Assessing the Cross-Reactivity of Mumps Antibody Between the Vaccine Genotype A and the Circulating Genotype G

By

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Abstract:

Twenty-four mumps outbreaks were reported in nine provinces across Canada between January 1, 2016, and July 31, 2018. The outbreaks predominantly affected vaccinated young adults and were due to mumps virus (MuV) genotype G, whereas the current vaccine (Jeryl-Lynn) is a mixture of two genotype A strains. The observed outbreaks might result from waning vaccine immunity or antigenic differences between the vaccine and circulating strain, resulting in a population susceptible to MuV of genotype G.

The mumps virus encodes seven proteins, including two glycoproteins, HN and F, responsible for the attachment and fusion of the viral envelope to the target cell membrane. These glycoproteins are important targets for the immune system to produce neutralizing antibodies, which are believed to be protective against infection. We hypothesize that antigenic differences in the MuV glycoproteins between the vaccine and circulating strains result in reduced recognition by neutralizing antibodies, leaving individuals susceptible to mumps.

To study cross-reactivity between genotype A and genotype G, we established stable cell lines expressing the F and the HN proteins. Western Blot and immunofluorescence assay (IFA) were used to verify protein expression. Cross-reactivity of antibodies between genotypes was assessed by IFA analysis using patient sera from vaccinated individuals without genotype G infection (vaccinated-only), unvaccinated individuals with a history of genotype G infection (infected-only), and vaccinated individuals with genotype G infection (vaccinated-infected). A limiting dilution assay was used to determine antibody titer, defined as the reciprocal of the last

serum dilution at which fluorescence could be detected, and the geometric mean titer (GMT) was calculated. Titer ratios were calculated by dividing the genotype A titer by the genotype G titer and the arithmetic mean was then calculated.

Titer ratios showed that the vaccinated-only individuals had a higher titer for genotype A than for genotype G. In contrast, the infected-only individuals had a lower titer for genotype A compared to genotype G. Notably, no difference in titer ratio was observed for individuals who had been vaccinated and subsequently infected with mumps. Additionally, a comparison of GMT in different groups showed that vaccinated-only individuals had lower titers for genotype G than infected-only and vaccinated-infected individuals. The confidence interval was calculated for all the groups for both the HN and F protein, and a significant decrease in genotype A titer compared to genotype G was observed in the infected-only individuals for the F protein. These results show a differential antibody response to vaccine vs. wild-type strains of MuV, which may explain the increased susceptibility of mumps in vaccinated young adults. However, there is no correlate of protection for mumps infection, and therefore, it is difficult to conclude whether these titer differences make individuals susceptible to MuV. Nevertheless, contrary to the current assumption that the immune response to MuV genotypes is monotypic, these results show differences in antibody reactivity between MuV genotype A and genotype G.

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List of Abbreviations:

AA – Amino Acid
DAPI – 4',6-diamidino-2-phenylindole
DMEM – Dulbecco's Modified Eagle Medium
DNA – Deoxyribonucleic Acid
EBV – Epstein Barr Virus
EIA – Enzyme Immunoassay
ELISA – Enzyme-Linked Immunosorbent Assay
F – Fusion Protein
GAG – Lentiviral protein encoding the structural and polymerase gene
GFP – Green Fluorescent Protein
GMT – Geometric Mean Titer
HN – Hemagglutinin Protein
IFA – Immunofluorescence Assay
IgA – Immunoglobulin A
IgG – Immunoglobulin G
IgM – Immunoglobulin M
JL – Jeryl Lynn
JL2 – Jeryl Lynn Strain 2
JL5 – Jeryl Lynn Strain 5
Kb – Kilobase
L – Large Protein
M – Matrix Protein
mAbs – Monoclonal Antibodies
MCS – Multiple Cloning Sites
MMR – Measles, Mumps, and Rubella
MMRV – Measles, Mumps, Rubella, and Varicella
MuV – Mumps Virus
NGS – Next Generation Sequencing
NP – Nucleocapsid Protein
NDV – Newcastle Disease Virus
P – Phosphoprotein
PBS – Phosphate-buffered Saline
FBS – Fetal Bovine Serum
MEM – Minimal Essential Medium
PCR – Polymerase Chain Reaction
Pol – HIV Polymerase
Rev – Lentiviral Protein Encoding Regulatory Gene Required for Replication
RnP – Ribonuclear Protein
RNA—Ribonucleic Acid
RT-PCR – Reverse Transcription Polymerase Chain Reaction

SH – Small Hydrophobic Protein
SNV – Single Nucleotide Variant
TBS – Tris-buffered Saline
TBS-T – Tris-buffered Saline with 0.1% Tween – 20
TNF-β – Tumor Necrosis Factor B
VSV-G – Vesicular Stomatitis Virus G
WGS – Whole Genome Sequence

1. Introduction

1.1 Mumps Virus

Mumps virus (MuV) belongs to the Paramyxoviridae family, a group of viruses that infect humans and include the measles, canine distemper, parainfluenza, Newcastle disease viruses, and respiratory syncytial virus (1). Mumps virus (MuV) is a species in the genus Rubalavirus, with a 15.4 kb genome (2). The viral infectious nature of MuV was first demonstrated by Johnson and Goodpasture when they infected rhesus macaques with bacteria-free, filter-sterilized preparation of saliva obtained from infected individuals (3).

Transmission of MuV is via respiratory droplets, particularly in settings with prolonged close contact, such as closed communities, sports teams, and university campuses (2). The incubation period following exposure to MuV is 16-18 days (2). 30-50% of mumps infections are known to be asymptomatic or result in mild symptoms such as fever, headaches, muscle aches, and swelling of the salivary glands, especially the parotid glands (1). These relatively mild symptoms are observed in young children infected with mumps and usually last a week (4). In contrast, adults infected with mumps may experience more severe complications, such as inflammation of the testis (orchitis), ovaries (oophoritis), pancreas (pancreatitis), and brain and spinal cord (meningitis), as well as deafness (4, 5). Mumps-infected individuals, whether symptomatic or asymptomatic, have been shown to shed the virus in their saliva one week

before symptom onset and one week after recovery, resulting in outbreaks in areas of close contact (5).

Before vaccination programs were established, mumps accounted for 10% of viral meningitis with an incidence rate of 1/1000 (6). The introduction of the MMR vaccine in 1971 decreased the observed cases by 99% (6). The mumps vaccine is a live attenuated vaccine with different virus strains used worldwide (5-6). These include the Leningrad-Zagreb vaccine, the Rubini vaccine, the Urabe AM9 vaccine, and the Jeryl Lynn Vaccine (5, 6-11).

The Leningrad-3 has been used in the Soviet Union, while the Lenigrad-3-Zagreb strain is used in Croatia and India and resulted in 9 cases of aseptic meningitis per 10,000 vaccine doses (7). The Rubini vaccine strain was used in Switzerland and was proven to be ineffective, resulting in children being susceptible to MuV infections (8-9). The Urabe Am9 strain was used in Belgium, Canada, France, Italy, Japan, and many other countries but was eventually discontinued due to an unacceptable incidence of aseptic meningitis (10). Japan has removed the mumps vaccine from the vaccination program, resulting in high rates of mumps cases, with over a million cases reported annually (10-12).

North America and several other countries have shifted primarily to the Jeryl Lynn (JL) vaccine, a mixture of JL5 and JL2 strains derived from a genotype A isolate (26). Seroconversion rates following two doses of the Jeryl Lynn vaccine range from 80% to 94%, resulting in high mumps immunity level (13-14). The mumps vaccine is currently available as a trivalent vaccine MMR (Measles, Mumps, and Rubella) or a quadrivalent vaccine MMRV (Measles, Mumps,

Rubella, and Varicella) (14). Two doses of the vaccine are given, the first at 12-15 months and the second at 4-6 years of age (14).

MuV is considered a monotypic virus, and so far, 12 genotypes from A to N (excluding E and M) have been defined based on the RNA sequence of the small hydrophobic (SH) gene (15). Genotype A was the basis for Jeryl Lynn and other vaccine strains and it has been eliminated, along with genotypes B, I, J, L, and N (15-16). Genotypes C, D, F, G, H, and K are currently circulating, but in Europe, The United States of America, and Canada, genotype G is circulating almost exclusively, resulting in recurrent outbreaks (15-16).

The diagnosis of mumps typically involves the detection of viral RNA or serological confirmation (5). In mumps, high levels of IgM antibodies are observed during the primary viral infection (17). IgM antibodies are detected by ELISA, but may result in false negative results if samples are collected too early during infection or if the individual has been recently vaccinated (5, 17-18). Additionally, in vaccinated individuals, IgM antibodies may not be elevated during MuV re-infection due to the presence of an anamnestic IgG response (17). This poses a challenge in primarily using IgM serology to diagnose MuV infection (17).

For unvaccinated individuals, diagnosis of mumps usually involves detection of IgM antibodies or the monitoring of IgG antibodies (5, 19). If a four-fold rise in IgG titers is observed between sera taken in acute (2-3 days after onset of symptoms) and convalescent phase (8 to 12 weeks later), individuals are deemed positive for mumps (5, 19). The increase in IgG titer is observed in cases of primary mumps infections, but it is usually not observed in cases of re-infection of vaccinated individuals due to the presence of an early robust secondary IgG

response (5, 19). Antibody maturation occurs through the process of somatic mutations, resulting in increased sensitivity and higher affinities following secondary exposure to the antigen (20-22). Maturation of IgG antibodies from low to high affinity could be used to distinguish recent from remote primary infections of measles and rubella (20-21). However, in mumps, IgG antibodies do not mature to high affinity, and therefore, this method is not clinically useful (22).

Notably, no immunological correlate of protection for mumps has been identified, and no serological titer differences by EIA based methods are observed between vaccinated individuals or individuals infected with MuV (23). A study by Rota et al. looking at the 2009 New York mumps outbreak due to genotype G reported 296 confirmed MuV cases, and of those, only 51% were positive by IgM detection using the CDC EIA IgM capture kit (23). IgG antibodies were also studied using the CDC EIA IgG capture kit, and a negative result was obtained for 3% of the cases, and 0.7% were indeterminate (23). These results show that IgM and IgG testing may sometimes be unreliable for MuV diagnosis in a vaccinated population, and instead, it would be better to use RT-qPCR assays (23). RT-qPCR testing is the preferred method as it will detect the presence of the virus and allow for genotyping (24-25). Virus nucleic acid detection can be done from saliva, CSF, and urine, within the first week of infection, for maximum sensitivity (24-26). RT-qPCR is performed on nucleic acid isolated to amplify either the SH gene, the N gene, or the F gene for viral RNA detection (24-25). Whole genome sequencing (WGS) of MuV or complete sequencing of the SH gene are essential to identify the circulating genotype for epidemiological and surveillance reasons (26).

The MuV genome contains seven linked transcription units, including the four core proteins: the virion/phosphoprotein (V/P), the large protein (L), the nucleoprotein (NP), and the matrix protein (M); two glycoproteins, the hemagglutinin (HN) and the fusion (F) protein; and a membrane-associated small hydrophobic protein (SH) (Figure 1) (2, 27-28). The P and the L protein form the ribonuclear protein complex (RnP), which replicates the negative sense genome strand into a positive sense strand, facilitating viral transcription and replication (28). The NP packages the 3' to 5' viral RNA strand into the nucleocapsid, used by the RnP complex during transcription and genome replication (28). The M protein plays an essential role in localizing the RnP complex to the host cell membranes expressing the F and HN glycoproteins, facilitating the budding of the newly formed virions (2, 27). Both the V and the SH proteins play an important role in immune evasion by inhibiting INF-β and TNF-α signaling, resulting in decreased anti-viral gene transcription and host defense response (28-29).

Furthermore, the two glycoproteins, HN and F, play an essential role in viral attachment and entry into the host cell and in the spread of newly formed virions. They are also important targets for the host immune system (30-31). Specifically, the HN protein binds to sialic acid receptors triggering a conformational change in the F protein, resulting in syncytium formation and membrane fusion of the virus and the host cells (30-32). The F protein induces pH-dependent virus fusion with the host plasma membrane (32). The virus initially infects the upper respiratory epithelial cells and replicates in the glandular epithelium cells, resulting in virus shedding into saliva (32). MuV then infects mononuclear cells, leading to systemic viremia, infecting epithelial cells in the parotid gland, testis, pancreas, kidneys, and the central nervous system (32). The humoral antibody response inhibits viremia by producing neutralizing IgA and

IgM antibodies upon infection, followed by an IgG response (1). IgA plays an essential role in restricting the replication of the mumps virus, and the level of IgA in the saliva is inversely correlated with the load of MuV found in the saliva (34). Non-neutralizing antibodies and T-cells have been shown to play an important role in protecting against SARS-CoV-2, HIV, and cytomegalovirus by activating antibody dependent cytotoxicity and the complement system, resulting in viral clearance and inhibition, but the role of T-cells and non-neutralizing antibody during MuV infection is poorly understood (33, 35). Studies have shown that the virus can replicate effectively in T-cells, and migrating of MuV-infected T-cells leads to virus dissemination to different organs, resulting in systemic viremia (33, 35). The ability of MuV to infect epithelial cells and spread through the lymphatic system leads to systemic viremia, resulting in a wide range of symptoms observed (31).

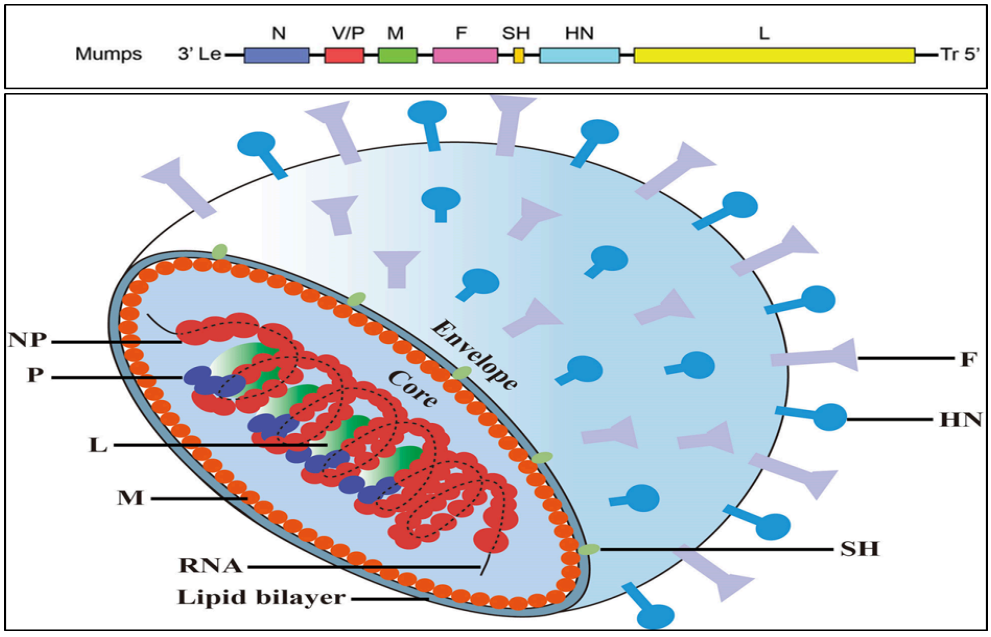


Figure 1. The MuV Genome. The HN and F glycoproteins play a role in the viral entry into the host cell, and are essential targets for the immune system. The figure was modified from Wu 2021 (5)—copyright Frontiers Microbiology.

1.2 Mumps outbreaks in Canada

Since 2006, several sporadic mumps outbreaks have been observed in highly vaccinated populations caused by MuV strains belonging to genotype G (36). From 2016-2018, there was an increase in mumps incidence which resulted in 24 outbreaks across nine Canadian provinces (36-38). The largest outbreak occurred in Manitoba from September 2016 to December 2018, resulting in 2,223 cases (Table 1) (37). The outbreak occurred mainly in northern communities and university campuses, with most cases reported in young adults aged 18 to 29 (37). 70% of the cases were in fully vaccinated individuals, and 24% were in partially vaccinated individuals, meaning they had two doses of MMRV or MMR vaccine or one dose of the vaccine, respectively (37). The disease severity was lower in vaccinated individuals than compared to non-vaccinated individuals. Risk of hospitalization, orchitis, and meningitis were reduced by 46%, 28%, and 72% in partially vaccinated individuals and 55%, 36%, and 83% for fully vaccinated individuals (37). The MMRV and the MMR vaccine used in Canada contain the Jeryl Lynn vaccine, which is a mixture of two genotype A strains, JL5 and JL2, whereas the recent outbreaks observed are caused by genotype G strains (37). It is possible that the circulating genotype G strains are antigenically different from the vaccine genotype A resulting in a possibility for immune escape (36). Additionally, mumps outbreaks in Canada and elsewhere could also be due to waning vaccine immunity, which would be particularly pronounced for genotype G (38-39).

Table 1. Characteristics of the Manitoba 2016-2018 Mumps Outbreak.

	Regional Health Authority					
Characteristics	**Northern**	**Winnipeg**	**Prairie Mountain**	**Southern Health -Sante Sud**	**Interlake - Eastern**	**Total**
Cases, N (%)	1566 (70.4 %)	370 (16.6%)	104 (4.7%)	98 (4.4%)	85 (3.8%)	2223 (100%)

Median age (years)	26	24	26	24	27	25
Unvaccinated (%)[a]	51 (5.9%)	20 (11.4%)	3 (6.4%)	5 (10.9%)	4 (8.3%)	20 (7.0%)
Partially Vaccinated (%)[b]	200 (23.1%)	42 (24.0%)	10 (21.3%)	9 (19.6%)	17 (35.4%)	278 (23.5%)
Fully Vaccinated (%)[c]	616 (71.0%)	113 (64.6%)	34 (72.3%)	32 (69.6%)	27 (56.3%)	822 (69.5%)

[a]No prior vaccination history with MMR or MMRV. [b]One dose of MMR or MMRV vaccine. [c]Two or more doses of MMR or MMRV vaccine. The table was modified from Wei et al, 2022 (37). Copyright Canada Communicable Disease Report: Public Health Service 2018-2019.

Waning vaccine immunity is one of the several potential factors causing mumps outbreaks. In the pre-vaccination era, cases were primarily observed in children, and mumps infection in childhood resulted in lifelong protection (40). Natural infection has decreased since the introduction of the mumps vaccination, and immunity is now primarily due to vaccination (40). Studies have shown that vaccine immunity for mumps wanes with time and the risk of reinfection increases by 10% per year post-vaccination (35-39). If ten years have passed since the second dose of the vaccine, the chance of infection increases significantly (35-39). Recent outbreaks have been observed in young adults who have acquired immunity to mumps by vaccination rather than natural immunity (37). Waning immunity could be one of the factors resulting in sporadic outbreaks because enough time has passed since vaccination, and vaccine-induced antibodies have decreased, rendering individuals susceptible to mumps (36-37). A third booster dose has been recommended in outbreak settings to control mumps outbreaks and has been shown to decrease the incidence rate in communities who have received it (40). However, studies have shown that after one year of receiving the third booster dose, antibody titers decrease and return to levels similar to those before the booster dose (40-41). Therefore, the longevity of protection of the third booster dose to prevent mumps

outbreaks is uncertain (40-41). Adverse events, such as headaches, joint problems, diarrhea, and lymphadenopathy, have been observed in individuals receiving a third booster dose of the mumps vaccine in a non-outbreak setting (42). If used, mumps vaccine booster doses should be used to control outbreaks, and more studies are needed to understand the effectiveness of booster doses in a non-outbreak setting (38, 40-41).

1.3 MuV Glycoproteins

1.3.1 HN Protein

The HN protein is a 582aa glycoprotein and a major envelope protein known to elicit an antibody response (43). It is essential for membrane fusion and viral entry into the host cells (43). The HN protein preferentially binds to trisaccharide with α2,3 linked sialic acid, glycan motifs sialyl Lewis^{x} (SLe^{x}), and GM2 ganglioside (GM2-glycan) receptors for viral entry into the host cells (43-45). These glycan structures are found in different tissues throughout the body, resulting in viral tropism (46). Upon binding of the HN protein to the sialic-glycan systems, the HN protein induces a structural change of the F protein, promoting viral fusion between the viral envelope and host cell membrane (45-47). The neuraminidase activity of the HN protein cleaves the sialic acid residues from the progeny virus to prevent viral tethering on the cell surface, viral self-agglutination, and allows viral particle release from the host cells (43, 45).

The structure and function of the HN protein has been described in detail in a recent review by Jasmine Frost and me (48). Briefly, HN protein is a type II membrane protein with the N terminus oriented toward the cytoplasm and the C terminus in the extracellular domain (47, 49). The HN protein's predominant secondary structure is a β-sheet, with the head domain

composed of a 6-bladed β-propeller fold consisting of 4-strands of β-sheets along with four α-helices (47, 49). Two monomers forming a homodimer are tilted approximately 90° to each other, and two homodimers form a tetramer which plays an essential role in triggering the F protein for fusion (45, 47). HN motifs include the leucine zipper motif for protein dimerization, the hemagglutinin receptor binding site (residues 405-410), and the neuraminidase motif (residues 240-245) (46, 49). The membrane anchorage domain site is from residues 35-93 and the protein contains nine potential glycosylation sites (47-53). Surface expressed residues 265-288, 329-340, and 352-360 are known B cell epitopes and important in antibody-mediated virus neutralization (54). The residues 113-130, 375-403, and 440-443 have been experimentally validated and described as potential epitopes that escape neutralization by mAbs (56-57). Computational analysis to predict B-cell and T-cell epitope have indicated several B-cell (199-207, 220-240, 261-266, 269-272, 284-296, 327-331, and 334-363) and T-Cell (74-82, 88-96, 157-165, 326-334, 503-512) epitopes (55-60).

Three aa differences are observed for the HN protein between the JL5 genotype A strain and the JL2 genotype strain (39, 58). Thirty-one aa differences have been reported in the literature when comparing genotype A vaccine strains (JL2 and JL5) vaccine strain and the genotype G circulating strains (Table 2). Four aa variations result in the loss of N-glycosylation sites, and one aa variation results in the addition of a N-glycosylation site (26 ,60-62). Six aa variations are found in the membrane anchorage domain, eleven aa variations are in B-cell and T-cell epitopes, and five aa variations have been studied in escape neutralization, while three aa variations were similar between the JL2, and genotype G strains, but not JL5. (58, 62, 61). Variation at positions 279 and 287 result in a mismatch of CD4+ and CD8+ responses and results

in loss of T-helper epitope, preventing a B cell response (58). Amino acid at positions 336, 354, and 356 have been associated with neurovirulence in rat models and have been found in samples sequenced from individuals with neurological symptoms (26, 39). These variations result in HN protein structure and bond changes in the HN changes, which could affect antibody binding and immune response (63). Amino acid variations in the antigenic regions and glycosylation sites in the HN protein between the vaccine strains (genotype A) and the circulating strain (genotype G) could result in MuV virus immune escape resulting in re-emergence of mumps in the vaccinated population (59-63).

Table 2. Amino Acid Variation in MuV HN protein between genotype A and genotype G circulating strains.

Position	Amino Acid Variations	Strain/Genotype	Potential Effect	Reference
6	L6F	G	N/A	60
9	M9I	G	N/A	60
12	N12S	G	Loss of N glycosylation	62
14	F14V	G	Loss of N glycosylation	62
21	V21S	G	N/A	60
37	A37G	G	Change in Membrane Anchorage Domain	60
56	I56V	G	Change in Membrane Anchorage Domain	60
63	S63G	G	Change in Membrane Anchorage Domain	60
77	E77K* E77A	JL2, G	Change in Membrane Anchorage Domain and Change in T-cell Epitope	58
80	A80T	G	Change in Membrane Anchorage Domain and Change in T-cell Epitope	58
81	V81M* V81I	JL2, G	Change in Membrane Anchorage Domain and Change in T-cell Epitope	58
94	H94Y	G	Change in T-cell Epitope	60
97	T97M	G	N/A	60
121	N121S	G	Neutralization Escape	60
122	R122S	G	Neutralization Escape	60

123	N123K	G	Neutralization Escape	60
129	T129S	G	Loss of N glycosylation	60
153	A153S	G	N/A	60
203	K203N	G	Change in B-cell Epitope	62
279	I279T	G	Change in B-cell and T-cell Epitope	62, 63
287	I287V	G	Change in B-cell and T-cell Epitope	62, 63
317	K317R	G	N/A	60
330	S330G	G	Loss of N glycosylation and Change in B-cell Epitope	60
336	S336L	G	Change in B-cell Epitope	39, 63
354	Q354P	JL2, G	Change in B-cell Epitope	39
356	E356D	G	Change in B-cell Epitope	39, 63
372	S372N	G	N/A	60
375	V375I	G	Neutralization Escape	60
399	N399S	G	Neutralization Escape	60
464	K464N	G	Addition of N glycosylation	60
577	A577T	G	N/A	60

The table was modified from Frost et al. 2022 (48)

1.3.2 F protein

The F protein is a 538aa surface glycoprotein and an essential target for the immune system along with the HN protein (64). The F protein is a class one fusion glycoprotein and depends on the HN protein's co-expression (65). The binding of the HN protein to the host cell receptors results in the cleavage of the receptor and activation of the F protein (65). Conformational changes in the F protein at neutral pH mediate the fusion of lipid and viral membranes, allowing the RnP complex to enter the host cells (29, 65). The exact mechanism that controls viral fusion and viral entry into the host cells is unknown (29).

The structure and function of the F protein has also been described in detail in the above-mentioned review article by Jasmine Frost and me (48). The F protein contains three hydrophobic regions, including the amino-terminal signal peptide (aa1-19), an amino terminal part of F1 (aa103-128), and the carboxyl-terminal membrane domain (aa483-512) (63). The F

protein is synthesized as a precursor protein molecule (F0) which is then cleaved into the active form by the host protease Furin at residues 98-103 (66). The active forms contain two disulfide-linked polypeptides, F1, the transmembrane subunit, and F2, the distal subunit (67). The F protein has two discontinuous heptad repeat domains at the ectodomain, forming a six-helix bundle, and a 3-4-4-4-3 residue spacing, conserved in RSV F protein structure (64). The F protein has six glycosylation sites (66). Monoclonal antibodies (mAbs) against the F protein have shown that inhibition of the F protein can block the spread of virus release and prevent the spread of virus to adjacent cells (67). Amino acid positions 221, 323, and 373 have been determined to be in conformation-neutralizing epitopes, and glycosylation at position 373 results in immune escape from anti-F mAbs (67).

As for the HN protein, amino acid differences between genotype A and genotype G could result in structural changes in the F protein resulting in immune escape (65). Twenty-six amino acid variations have been reported in the literature when comparing the genotype A and genotype G strains (Table 3). Six aa variations resulted in changes in the signal peptide regions, two aa variations are in the protease cleavage site, one aa variation is found close to the cleavage site, and one aa changes the amino-terminal part of F1 (58, 68). Variations in the cleavage site are hypothesized to increase cleavage and MuV fusion (65). Studies have shown that amino acid variation S195F is known to increase MuV neurovirulence and increase fusogenicity of the F protein (39, 58). Amino acid variations in the F protein could result in immune escape of the MuV by vaccine-induced neutralizing antibodies, resulting in the observed mumps outbreaks.

Table 3. Amino Acid Variation in MuV F protein between genotype A strains and genotype G strains.

Position	Amino Acid Variation	Genotype	Potential Effect	Reference
2	K2R	G	Change in Signal Peptide Region	60
3	V3A	G	Change in Signal Peptide Region	60
4	S4F	G	Change in Signal Peptide Region	60
7	I7T	G	Change in Signal Peptide Region	60
14	F14V	G	Change in Signal Peptide Region	60
16	S16F	G	Change in Signal Peptide Region	60
96	G96R	G	N/A	60
97	S97L	G	Close to Protease Cleavage Site	60
100	H100Y	G	Change in Protease Cleavage Site	60
101	K101R	G	Change in Protease Cleavage Site	60
115	V115F	G	Change in the amino-terminal region of F1	60
141	K141R	G	N/A	60
195	S195F	G	Increased Fusogenecity and Neurovirulence	39, 60
209	P209T	G	N/A	60
273	I273L	G	N/A	60
274	N274D	G	N/A	60
280	T280A	G	N/A	60
350	A350T	G	Change in Protease Cleavage Site	60
389	S389N	G	N/A	60
409	S409R	G	N/A	60
413	L413V	G	N/A	60
425	L425I	G	N/A	60
439	S439F	G	N/A	60
454	I454S	G	N/A	60
432	A492V	G	N/A	60
530	S530G	G	N/A	60

The table was modified from Frost et al. 2022 (48)

1.4 MuV Serology

The MuV genotype G strains have caused recent outbreaks in highly vaccinated populations, whereas the vaccine strain is genotype A (2-6, 43-46). Therefore, it is possible that antigenic differences between genotype A and genotype G result in reduced recognition of

vaccine-induced antibodies, leaving individuals susceptible to mumps (43-46). The waning of vaccine-induced immunity against genotype G may be more pronounced than for genotype A (44). Enzyme immunoassays (EIA) and plaque reduction neutralization test (PRNT) based assays have been used to study the role of waning immunity and antigenic variations by measuring differences in total and neutralizing antibodies between genotype A and genotype G (69-75). EIA assays have been used to study waning immunity and population immunity, but no credible correlation in titer was observed between the two genotypes for individuals who had been infected with mumps or individuals immune to mumps in outbreak settings (72-75). Studies have shown that individuals who receive one dose of vaccine are more susceptible to mumps than individuals who have received two doses, but no cutoff in IgG titer has been determined (69-75). EIA assays to measure IgG subclasses have identified that IgG2 and IgG4 subclasses levels remain low for both the genotype A and genotype G strains, while IgG1 and IgG3 were significantly high in infected individuals, but the significance of these findings is unclear (74-76).

PRNT is the gold standard for detecting neutralizing antibodies against viruses, and studies looking at GMT observed a 2-6-fold reduction in titers against genotype G compared to vaccinated (genotype A) individuals (74). Rubin et al. (2008) showed that the geometric mean neutralizing antibody titer for genotype G was approximately half of that for genotype A (70). Other studies have also shown that vaccine-induced antibodies can neutralize the genotype G strain in plaque reduction neutralization tests, but the level of neutralization in genotype G was lower than in genotype A (64, 69). On the contrary, results from other studies in our laboratory and in the literature have found no correlation between MuV genotype A and G PRNT titers (74, 76). Unpublished results from our laboratory have also failed to show a difference in IgG

neutralizing titer between genotype A and genotype G antigens. This could be due to the fact that neutralization efficacies depend on the evolution of MuV strains rather than a genotype specific response (51). In fact, studies have shown that the immune response against MuV is not genotype specific but rather it is strain specific, different strains have different neutralizing antibody titers depending on the antigenic variations in immunodominant regions (78-82). Differences in virus titer, virus strain, and non-specific neutralization such as viral self-agglutination, can also affect the results observed for PRNT, resulting in a lack of correlation between studies due to the use of different virus strains and cultures (77). Sero-epidemiology studies performed in our laboratory for measles show a good correlation between ELISA, Bioplex 2200, and PRNT titers; however, when applied to mumps, no correlation was observed (manuscript in preparation). Also, no serological test and correlate of protection of MuV have been determined to predict who is at risk and who is immune to mumps (44, 82-85). The role of antibody immune response to protect from mumps re-infection is unclear, and better methods are needed to study differences in antibody response between genotypes (82-85).

1.5 Rationale and Hypothesis

1.5.1 Rationale for Using IFA

EIA-based assays and PRNT are commonly used serological tests for mumps but have shown contradictory results (82-85). These could be due to the difference in assays, as EIA-based assays measure total antibodies, whereas PRNT specifically measures neutralizing antibodies (74). Also, for EIA-based assays, the amount of antigen and kits used may vary due to the lack of standards available, resulting in the observed variability in results (82-85).

Inconsistent PRNT results may stem from variability in viral strains, viral titer, and viral infectivity, resulting in artifactual differences in cross-reactivity (82).

To obviate these differences, I decided to use an Immunofluorescence assay (IFA) system using stable cell lines expressing the HN and F proteins of genotype A and genotype G. These cells present these proteins in their native conformation, without other interfering viral antigens.

IFA quantitation is independent of the concentration of the antigen, as long as these proteins are expressed in excess of their cognate antibodies, and therefore variation in expression between different cell line will not influence the observed titer of bound antibodies

In fact, an IFA assay could be considered a binding system where the antibodies (Ab) are the receptors and the antigenic proteins (Ag) are the ligands. The kinetics of this system at equilibrium binding will be:

$$Equation\ 1:\ [Ab{\cdot}Ag] = \frac{[Abt][Ag]}{Kd\ +[Ag]}$$

Where:

- [Ab·Ag] is the concentration of antibodies bound to the expressed antigen, measured by the fluorescence of the secondary Ab.
- $[Ab_t]$ is the total amount of antibody added to the system, the Bmax, i.e. the maximum binding capacity.
- [Ag] is the concentration of the antigen protein expressed on the cells and it is kept constant in the IFA system.

- K_d is the dissociation constant of [Ab.Ag], or the affinity of the ligand for its receptor. For Ab/Ag interactions, K_d is in the nM or pM range and therefore the system rapidly reaches the Bmax plateau at low concentration of ligand, the Ag in this case.

Equation 1 describes a square hyperbole similar to the Michaelis-Menten curve and, as serum is serially diluted, [Ag] would be in excess and the system will be at the plateau or Bmax, and all the molecule of Ab will be bound by the Ag on the cells. Therefore, the amount of bound Ab will be independent of the concentration of the Ag and dependent only on the amount of antibody.

This ensures that variations in the expression of different antigens on the surface of the cells will not impact on the amount of bound Ab as the serum approaches the limiting dilution.

1.5.2 Rationale for the Project

Since 2006, multiple mumps outbreaks caused by MuV genotype G have been observed in the Canadian population. Most cases have been in fully vaccinated individuals with a mean age of 25. These outbreaks could be caused by waning vaccine immunity as enough time has passed since vaccination and vaccine-induced antibodies have waned, resulting in the population becoming susceptible to MuV. Another reason could be waning immunity against genotype G is more pronounced due to amino acid variations resulting in genotype G strains that do not fully cross-react with vaccine-induced immunity. I decided to study the cross-reactivity of neutralizing antibodies between genotype A and genotype G using an in vitro IFA system. Stable cell lines were generated using a lentivirus system to examine antibody

binding differences in the HN and F glycoproteins as they are the primary target for the immune system. A transient protein expression system results in variable protein expression and nonspecific responses that could be avoided using stable lentivirus transduced cell lines. An immunofluorescence assay was used to assess antibody binding between genotype A and genotype G. Limiting dilution series were performed. The last dilution at which a signal was obtained was noted as the titer for serum, similar to the approach used for Epstein-Barr virus (EBV) diagnostic. Serum from vaccinated individuals with no history of MuV infection was stained to look at the cross-reactivity of vaccine antibodies (genotype A) against genotype A and genotype G glycoproteins. Serum from unvaccinated individuals infected with genotype G were stained to look at the cross-reactivity of genotype G antibodies against glycoproteins from genotype A and genotype G. Serum from vaccinated individuals with genotype G infection was obtained to look at the titer of genotype A and genotype G antibodies for MuV glycoproteins.

Hypothesis:

Amino acid variations in genotype G, the circulating strain, reduce the recognition of epitopes by neutralizing antibodies from vaccination by genotype A, making the population susceptible to MuV.

Objectives:

1) To create stable cell lines expression of the MuV glycoproteins (HN and F) for genotype A and genotype G.
2) Determine the cross-reactivity of MuV HN and F antibodies between genotype A and genotype G.

2. Methods

2.1 Lentivirus System

Lentiviruses are a genus of retroviruses, possessing an RNA genome that is converted to DNA by reverse transcriptase and subsequently integrated in the host DNA (88-90). Lentiviruses are widely used to transduce genes of interest or stable reporter constructs, both *in vitro* models within mammalian cell lines and *in vivo* animal models (88-90). The use of lentiviruses results in stable long-term protein expression, compared to transient transfections which lose expression over time (90).

The third-generation lentiviral vectors were used in my work as they are designed to ensure efficient gene delivery and maximize safety (91). Additionally, lentiviruses genes necessary for replication are present on a different packaging plasmid (gag plasmid, rev plasmid, and VSV-G plasmid), making the pseudovirus particle replication incompetent (92-95). The expression vector encodes a multi-cloning site for the gene of interest, a WPRE gene, and a selection marker gene to select lentivirus transduced cells along with the SV40 gene for stable propagation (95). The expression vector also includes a PuC promoter and an ampicillin resistance gene for replication and selection of plasmid expression in *E. coli* cells (93).

2.2 Cloning

2.1.1 Codon Optimization

The HN and F protein sequences for genotype A lab reference strain VE-12343 and genotype G CDC strain were available in the lab through previous WGS of mumps specimens submitted to the NMLB (Table 4) (Jasmine Frost, PhD thesis, University of Manitoba, 2023). HN and F gene sequences were codon optimized by Dr. Michael Carpenter's laboratory to increase protein expression using codon bias (96). Since most amino acids are encoded by multiple codons and different species favor different tRNA for protein translation, codon optimization targets the most abundant tRNAs in the species of interest to increase protein expression (96). A Kozak sequence was added to the 5' site of the codon-optimized DNA sequence to enhance protein translation from the correct initiation codon (Table 5) (97). The optimized DNA sequences were ordered commercially from Genscript for the HN protein and Thermofisher for the F protein.

Table 4. Amino Acid Sequences for MuV Glycoproteins Used for Lentivirus Transfection. The underlined sequences are the spacer and flag tag sequence at the end of proteins.

Protein	Genotype	Codon Optimized DNA Sequence 5' to 3'
HN	A	MEPSKLFIMSDNATFAPGPVVNAAGKKTFRTCFRILVLSVQAVILILVIVTLGELIRMINDQGLSNQLSSITDKIRESAAVIASAVGVMN QVIHGVTVSLPLQIEGNQNQLLSTLATICTNRNQVSNCSTNIPLINDLRFINGINKFIIEDYATHDFSIGHPLNMPSFIPTATSPNGCTRIP SFSLGKTHWCYTHNVINANCKDHTSSNQYVSMGILAQTASGYPMFKTLKIQYLSDGLNRKSCSIATVPDGCAMYCYVSTQLETDDYA GSSPPTQKLILLFYNDTITERTISPSGLEGNWATLVPGVGSGIYFENKLIFPAYGGVLPNSTLGVKLAREFFRPVNPYNPCSGPQQELDQ RALRSYFPSYFSSRRVQSAFLVCAWNQILVTNCELVVPSNNQTLMGAEGRVLLINNRLLYYQRSTSWWPYELLYEISFTFTNYGQSSV NMSWIPIYSFTRPGSGHCSGENVCPIVCVSGVYLDPWPLTPYRHQSGINRNFYFTGALLNSSTTRVNPTLYVSALNNLKVLAPYGTQ GLFASYTTTTCFQDTGDASVYCVYIMELASNIVGEFQILPVLARLTITGASDYKDDDDK
HN	G	MEPSKFFTISDSATFAPGPVSNAANKKTFRTCFRILALSVQAVTLILVIVTLGELVRMINDQGLSNQLSSITDKIRESATMIASAVGVMN QVIHGVTVSLPLQIEGNQNQLLATLATICTSQKQVSNCSTNIPLVNDLRFINGINKFIIEDYATHDFSIGHPLNMPSFIPTATSPNGCTRIP SFSLGKTHWCYTHNVINANCKDHTSSNQYVSMGILVQTASGYPMFKTLKIQYLSDGLNRKSCSIATVPDGCAMYCYVSTQLETDDYA GSSPPTQKLTLLFYNDTVTERTISPSGLEGNWATLVPGVGSGIYFENKLIFPAYGGVLPNSTLGVKLAREFFRPVNPYNPCSGPQQDLD QRALRSYFPSYFSNRRIQSAFLVCAWNQILVTNCELVVPSSNQTMMGAEGRVLLINNRLLYYQRSTSWWPYELLYEISFTFTNSGPSS VNMSWIPIYSFTRPGSGNCSGENVCPTTCVSGVYLDPWPLTPYSHQSGINRNFYFTGALLNSSTTRVNPTLYVSALNNLKVLAPYGT QGLFASYTTTTCFQDTGDASVYCVYIMELASNIVGEFQILPVLTRLTITGASDYKDDDDK

F	A	MNAFPVICLGYAIFSSSICVNINTLQQIGYIKQQVRQLSYYSQSSSSYVVVKLLPNIQPTDNSCEFKSVTQYNKTLSNLLLPIAENINNIAS PSLGSRRHKRFAGIAIGIAALGVATAAQVTAAVSLVQAQTNARAIAAMKNSIQATNRAVFEVKEGTQQLAIAVQAIQDHINTIMSTQL NNMSCQILDNQLATSLGLYLTELTTVFQPQLINPALSPISIQALRSLLGSMTPAVVQATLSTSISAAEILSAGLMEGQIVSVLLDEMQMI VKINIPTIVTQSNALVIDFYSISSFINNQESIIQLPDRILEIGNEQWRYPAKNCKLTRHHMFCQYNEAERLSLETKLCLAGNISACVFSPIA GSYMRRFVALDGTIVANCRSLTCLCKSPSYPIYQPDHHAVTTIDLTSCQTLSLDGLDFSIVSLSNITYTENLTISLSQTINTQPIDISTELSKV NASLQNAVKYIKESNHQLQSFSVGSKIGAIIVSALVLSILSIIISLLFCCWAYIATKEIRRINFKTNHINTISSSVDDLIRY<u>GASDYKDDDDK</u>
F	G	MKVSLVTCLGFAVFSFSICVNINILQQIGYIKQQVRQLSYYSQSSSSYIVVKLLPNIQPTDNSCEFKSVTQYNKTLSNLLLPIAENINNIASP SPGSRRHKRFAGIAIGIAALGVATAAQVTAAVSLVQAQTNARAIAAMKNSIQATNRAVFEVKEGTQQLAIAVQAIQNHINTIMNTQL NNMSCQILDNQLATSLGLYLTELTTVFQPQLINPALSPISIQALRSLLGSMTPAVVQATLSTSISAAEILSAGLMEGQIVSVLLDEMQVIV KINIPTIVTQSNALVIDFYSISSFINNQESIIQLPDRILEIGNEQWSYPAKNCKLTRHNIFCQYNEAERLSLESKLCLAGNISACVFSPIAGSY MRRFVALDGTIVANCRSLTCLCKSPSYPIYQPDHHAVTTIDLTACQTVSLDGLDFSIVSLSNITYAENLTISLSQTINTQPIDISTELIKVNAS LQNAVKYIKESNHQLQSVSINSKIGAIIIAALVLSILSMIISLLFCCWAYIATKEIRRINFKTNHINTISSSVDDLIRY<u>GASDYKDDDDK</u>

Table 5. Codon Optimized DNA Sequence. Kozak sequence and flag tag were added to the protein sequences and are underlined

Protein	Genotype	Amino Acid Sequence
HN	A	<u>gccacc</u>ATGGAACCGTCAAAGTTGTTCATCATGTCTGATAACGCCACCTTTGCCCCTGGCCCCGTGGTGAATGCCGCTGGCAAAAAGA CCTTCCGGACCTGCTTCAGAATCCTGGTGCTGAGTGTGCAGGCCGTGATTCTGATCCTGGTGATTGTGACACTGGGCGAGCTGATCA GAATGATCAACGACCAGGGCCTGAGCAATCAACTGAGCTCCATCACCGACAAGATCCGGGAAAGCGCAGCTGTCATCGCCAGCGCC GTGGGCGTGATGAACCAAGTGATCCACGGCGTTACAGTGAGCCTGCCACTGCAGATCGAGGGCAACCAGAACCAGCTGCTGAGCA CACTGGCCACAATCTGCACCAACCGGAATCAGGTGTCCAACTGCTCCACAAACATACCCCTCATCAACGACCTGAGATTCATCAACG GAATCAACAAGTTCATCATCGAAGATTACGCCACACACGACTTCAGCATCGGACACCCTCTGAACATGCCTTCTTTCATCCCCACAGC CACCAGCCCCAACGGCTGCACAAGAATCCCTAGCTTCAGtCTGGGAAAGACACATTGGTGCTACACCCACAACGTGATCAATGCTAA TTGCAAGGACCACACCAGCAGCAACCAATACGTGAGCATGGGAATTCTGGCCCAGACCGCCAGTGGCTACCCCATGTTCAAGACCC TGAAAATCCAGTACCTGAGCGACGGCCTGAATAGAAAGAGCTGCTCCATCGCCACCGTGCCTGATGGtTGTGCCATGTACTGCTACG TGTCCACCCAGCTGGAAACAGACGACTACGCCGGCTCTTCTCCACCTACCCAGAAACTGATCCTGCTCTTCTACAACGATACCATCAC CGAGAGAACCATCAGCCCTAGCGGCCTGGAAGGCAATTGGGCCACACTGGTGCCTGGAGTGGGATCTGGAATCTACTTCGAAAAC AAGCTGATCTTCCCTGCCTACGGCGGAGTCCTGCCGAATTCTACCCTGGGCGTGAAGCTAGCCAGAGAGTTCTTCCGGCCTGTGAAC CCCTATAACCCTTGTAGCGGCCCTCAGCAGGAGCTGGATCAGAGAGCCCTGCGGAGCTACTTTCCAAGCTATTTTAGCAGCAGAAG AGTGCAGTCTGCTTTTCTGGTGTGCGCCTGGAACCAGATCCTAGTTACCAACTGCGAGCTGGTCGTGCCCTCCAACAACCAGACCCT GATGGGCGCCGAGGGTAGaGTGCTGCTGATCAACAACAGACTGCTGTACTACCAGCGCAGCACCTCCTGGTGGCCCTACGAGCTGC TGTACGAGATCAGCTTCACCTTCACCAACTACGGCCAGtctAGCGTGAACATGAGCTGGATTCCAATCTACAGCTTCACAAGACCTGG ATCTGGCCACTGTAGCGGCGAGAACGTGTGTCCTATCGTGTGCGTGTCTGGAGTGTACCTGGACCCCTGGCCTCTGACCCCTTATAG ACACCAGAGCGGCATCAACCGGAACTTCTACTTCACCGGCGCTCTCCTGAACTCATCCACCACTCGGGTCAACCCTACACTCTACGTG TCTGCCCTGAATAATCTGAAGGTGCTGGCTCCTTACGGTACACAAGGCCTGTTCGCCTCTTATACAACCACCACCTGTTTTCAGGACA CCGGCGACGCCAGCGTGTACTGCGTGTACATCATGGAGCTGGCCAGCAACATCGTGGGCGAATTCCAGATCCTGCCCGTCCTGGCC AGACTGACAATCACC<u>GGCGCTTCTGATTACAAGGACGATGATGACAAGTGA</u>
HN	G	<u>gccacc</u>ATGGAACCTAGCAAGTTCTTCACCATTTCTGATAGCGCCACCTTTGCCCCCGGCCCTGTGTCCAATGCCGCCAACAAGAAAAC CTTCAGAACCTGTTTTAGAATCCTGGCCCTGAGCGTGCAAGCCGTGACACTGATCCTGGTCATCGTGACCCTGGGCGAGCTGGTGC GGATGATCAACGACCAGGGTCTGAGCAACCAGCTGTCTAGTATTACAGACAAGATCAGAGAGAGCGCTACCATGATCGCCAGCGCC GTGGGAGTCATGAACCAGGTGATaCACGGCGTAACCGTGTCCCTGCCTCTGCAGATCGAGGGCAACCAGAACCAGCTGCTGGCCAC CCTGGCaACAATCTGCACCAGCCAGAAGCAGGTGTCCAACTGCAGCACAAACATCCCTCTGGTGAATGACCTGAGATTCATCAACGG CATCAACAAATTCATCATCGAAGATTACGCCACACACGACTTCAGCATCGGCCACCCCCTGAATATGCCTAGCTTTATCCCTACAGCCA CAAGCCCAAATGGCTGCACCAGAATCCCTAGCTTCAGCCTGGGCAAGACCCACTGGTGCTACACCCATAATGTGATCAACGCTAATT GCAAGGACCACACCTCTTCTAACCAGTACGTGTCCATGGGCATCCTGGTCCAGACCGCCTCTGGCTACCCTATGTTCAAGACCCTCAA GATCCAGTACCTGAGCGACGGCCTCAATAGAAAGAGCTGCTCCATTGCCACCGTGCCTGACGGATGTGCCATGTACTGCTACGTGTC AACACAGCTGGAAACGGACGACTACGCCGGCTCAAGCCCACCTACCCAGAAACTGACACTGCTTTTTTACAACGACACCGTGACCG AGAGAACAATCAGCCCCTCAGGCCTGGAAGGCAATTGGGCCACACTGGTGCCCGGCGTTGGAAGCGGCATCTACTTCGAGAACAA GCTGATCTTCCCCGCCTACGGaGGCGTGCTGCCTAACTCGACCCTCGGCGTGAAGCTGGCTAGAGAGTTCTTCCGGCCCGTGAACCC TTACAACCCGTGCAGCGGCCCACAGCAGGATCTGGATCAGCGCGCCCTGCGGAGCTACTTCCCTTCTTATTTTAGCAACAGGCGGAT CCAATCCGCCTTCCTGGTTTGTGCTTGGAACCAGATCCTGGTGACCAATTGTGAACTGGTGGTGCCAAGCAGCAATCAAACGATGAT GGGCGCTGAGGGAAGAGTGCTGCTGATCAACAACAGACTGCTGTATTACCAGAGAAGCACCAGCTGGTGGCCCTACGAGCTGCTG TACGAGATCAGCTTTACCTTCACCAACAGCGGCCCTAGCAGCGTGAACATGAGCTGGATCCCCATCTACTCCTTCACCAGACCCGGC AGCGGCAACTGCAGCGGCGAAAACGTcTGTCCAACAACCTGCGTGTCTGGCGTGTATCTGGACCCTTGGCCTCTGACACCTTACAGC

		CACCAGAGCGGAATCAACCGGAACTTCTACTTCACCGGCGCTCTTCTGAACAGCTCTACAACCCGGGTGAACCCCACACTGTACGTa TCTGCTCTGAACAACCTGAAGGTGCTGGCACCTTATGGCACCCAGGGACTGTTCGCCAGCTACACTACAACCACATGCTTCCAGGAC ACCGGAGATGCCTCCGTGTACTGCGTGTACATCATGGAACTGGCCTCTAACATCGTGGGCGAGTTCCAAATCCTGCCTGTGCTGACCC GGCTGACCATCACAGGCGCTTCTGATTACAAGGACGATGATGACAAGTGA
F	A	gccaccATGAACGCTTTTCCAGTGATCTGCCTGGGATACGCCATCTTCAGCAGCTCAATCTGCGTGAACATCAACACCCTGCAGCAAAT CGGCTACATCAAGCAGCAGGTGCGGCAGCTGAGCTACTACTCTCAGAGCAGCTCCAGCTACGTGGTGGTGAAGCTGCTGCCTAACA TCCAGCCTACCGACAACAGCTGCGAATTCAAGAGCGTGACCCAGTACAACAAGACCCTGTCTAACCTGCTCCTGCCTATCGCCGAAA ACATCAACAACATCGCTAGTCCAAGCCTGGGCTCAAGACGGCATAAGCGGTTCGCCGGCATCGCCATCGGAATCGCCGCTCTTGGC GTGGCCACAGCCGCTCAGGTTACAGCGGCCGTGTCCCTGGTGCAGGCCCAGACCAACGCCAGAGCCATCGCCGCTATGAAAAACA GCATCCAGGCCACAAACAGAGCCGTCTTCGAGGTGAAAGAGGGCACACAACAACTGGCTATCGCCGTGCAAGCCATCCAGGACCA CATTAACACAATCATGAGCACCCAGCTGAACAACATGAGCTGCCAGATCCTGGATAATCAGCTCGCCACCAGTCTGGGACTGTACCT GACTGAACTGACAACCGTGTTTCAGCCCCAGCTGATCAATCCTGCTCTGTCCCCTATCAGCATCCAAGCCCTGCGCAGCCTGCTGGG CTCCATGACACCTGCTGTCGTGCAGGCCACCCTGAGTACAAGCATCAGCGCCGCCGAGATCCTGAGCGCCGGACTGATGGAAGGCC AGATCGTGTCTGTGCTGCTGGACGAGATGCAGATGATCGTGAAGATCAACATACCCACCATCGTTACCCAGTCTAACGCCCTCGTGAT CGATTTCTACAGCATCAGCAGCTTTATCAATAACCAGGAGTCCATTATCCAGCTGCCCGACAGAATCCTGGAAATCGGCAATGAGCAG TGGCGGTACCCTGCCAAGAACTGTAAACTGACCAGACACCACATGTTCTGCCAGTATAACGAGGCCGAGAGACTGAGCCTGGAAAC CAAGCTGTGTCTGGCCGGCAACATCTCCGCCTGCGTGTTCAGCCCCATCGCTGGCTCTTACATGAGAAGGTTCGTGGCCCTGGATGG CACCATCGTGGCCAATTGCAGAAGCCTGACCTGTCTGTGCAAGAGCCCTAGCTACCCCATCTACCAGCCTGATCACCACGCCGTGAC AACAATCGACCTGACCAGCTGTCAGACACTGAGCCTGGACGGCCTGGACTTCAGCATCGTGTCTCTGAGCAACATCACCTACACCGA GAACCTGACCATCTCCCTCAGCCAGACCATCAACACCCAGCCTATCGACATCAGCACCGAGCTGTCTAAAGTGAACGCCAGCCTCCA GAACGCCGTGAAGTACATCAAGGAGAGCAATCACCAGCTGCAAAGCTTCAGCGTGGGCTCCAAGATCGGCGCCATCATCGTTTCTG CCCTGGTGCTGAGCATCCTGAGCATTATCATCAGCCTGCTGTTCTGCTGCTGGGCTTATATCGCCACCAAGGAAATCCGGAGAATCAA CTTCAAGACAAACCACATTAATACCATTTCCAGCTCTGTGGACGACCTGATCAGATACGGCGCTTCTGATTACAAGGACGATGATGAC AAGTGA
F	G	gccaccATGAAGGTGTCTCTGGTCACATGTCTGGGCTTCGCCGTGTTTAGTTTCAGCATCTGCGTGAACATCAATATCTTGCAACAGATC GGCTACATCAAGCAGCAGGTGAGACAGCTGAGCTACTACAGCCAAAGCAGCTCATCTTACATCGTGGTGAAGCTGCTGCCTAACATC CAGCCTACAGACAACAGCTGCGAGTTCAAGTCCGTGACCCAGTACAACAAGACCCTGTCTAACCTGCTGCTTCCTATCGCAGAAAAC ATCAACAATATTGCCAGTCCCAGCCCCGGATCAAGACGGCACAAGAGATTCGCTGGCATCGCCATCGGAATCGCCGCCCTGGGCGT GGCCACCGCCGCCCAGGTAACCGCGGCCGTGAGCCTGGTGCAGGCTCAGACCAACGCCAGAGCCATCGCAGCCATGAAAAACAG CATCCAGGCCACAAACCGGGCCGTGTTCGAGGTGAAGGAAGGCACCCAGCAACTGGCCATCGCCGTGCAGGCTATCCAGAATCAC ATCAACACCATCATGAACACCCAGCTGAACAACATGAGCTGTCAGATCCTGGATAACCAGCTGGCCACATCTCTGGGCCTGTACCTGA CCGAGCTGACCACCGTGTTTCAGCCACAGCTCATCAACCCCGCCCTGAGCCCCATCAGCATCCAAGCCCTGAGATCCCTGCTGGGCA GCATGACCCCTGCTGTCGTGCAGGCCACCCTGTCCACAAGCATCTCTGCCGCCGAAATCCTCTCCGCTGGCCTGATGGAAGGCCAGA TCGTGTCTGTGCTGCTGGACGAGATGCAGGTCATCGTGAAGATCAATATTCCTACCATTGTTACACAGTCCAATGCCCTGGTGATCGAT TTTTACTCTATCTCCAGCTTCATCAACAATCAGGAGAGCATTATCCAGCTGCCTGATAGAATCTTGGAGATCGGCAACGAGCAGTGGA GCTACCCTGCCAAGAACTGCAAGCTGACCAGACACAACATCTTCTGCCAGTATAACGAGGCCGAACGGCTGTCCCTCGAGAGCAAG CTGTGCCTGGCCGGAAACATCAGCGCCTGTGTGTTCTCTCCTATCGCTGGCAGCTACATGAGAAGGTTCGTGGCCCTGGACGGCACC ATCGTTGCCAACTGCAGAAGCCTGACCTGCCTGTGTAAAAGCCCTTCTTATCCTATCTACCAGCCCGACCACCACGCCGTGACCACAA TTGATCTGACAGCCTGCCAGACAGTGAGCCTGGACGGCCTGGACTTCAGCATTGTTAGCCTGAGTAACATAACATACGCCGAGAACC TGACCATCAGCCTGAGCCAGACCATCAACACCCAACCAATCGACATCTCTACAGAGCTTATCAAAGTGAACGCTTCTCTGCAAAACG CCGTCAAATACATCAAGGAAAGCAACCATCAGCTGCAGAGCGTGTCCATCAACAGCAAGATCGGGGCTATTATTATCGCTGCCCTGG TGCTGTCCATCCTGAGCATGATTATCAGCCTGCTGTTCTGCTGCTGGGCCTACATCGCTACTAAGGAAATCCGGCGGATCAATTTCAA GACCAATCACATCAATACAATCAGCAGCAGCGTGGACGACCTGATCAGATACGGCGCTTCTGATTACAAGGACGATGATGACAAGTG A

2.1.2 Polymerase Chain Reaction

Virus stocks were available in the laboratory for the genotype A vaccine strain VE-12343, and genotype G CDC strain; these were used for MuV RNA isolation. For the non-codon optimized clones, viral RNA was isolated using the Qiagen Viral isolation kit (Qiagen, Cat#52906). Superscript IV (ThermoFisher, Cat# 1890050) reverse transcriptase was used to

convert the RNA into cDNA using gene-specific primers (Table 6). Polymerase Chain Reaction (PCR) was used to amplify non-codon optimized sequences.

Primers were designed to amplify complete gene sequences, and additional nucleotides were added to include restriction digest sites Xba1 and NotI (Table 6). Xba1 and NotI restriction sites were used for cloning since they were absent from the HN and F gene sequence but present in the multiple cloning sites in the vector. Platinum SuperFii PCR master mix (Invitrogen, cat#12358010) was used to amplify gene sequence for the HN and F protein and verify the presence of HN and F gene sequence in lentivirus transduced cells. The PCR reaction mixture was prepared based on the SuperFii protocol guideline, as mentioned in Table 7. PCR was run on a Veriti thermal cycler (Thermofisher Scientific, cat# 4375786) using the thermal profile provided in Table 8.

Table 6. Primer sequence for PCR amplification. Extra Nucleotides and restriction sites are underlined.

Protein Target	Genotype	Primer	Restriction Enzyme	Primer Sequence (5' to 3')
Hemagglutinin	A	Forward	XbaI	cacaTCTAGAgccaccATGGAACCGTCAAAGTT
Hemagglutinin	G	Forward	XbaI	cacaTCTAGAgccaccATGGAACCATGGAACCTAGC
Fusion	A	Forward	XbaI	cacaTCTAGAgccaccATGAACGCTTTTCCAG
Fusion	G	Forward	XbaI	cacaTCTAGAgccaccATGAAGGTGTCTCTGG
Hemagglutinin and Fusion	A and G	Reverse	NotI	gtgtGCGGCCGCTCACTTGTCATCATCGTCCT

Table 7. Reagents for SuperFii PCR reaction.

Reagents	**Volume**	**Concentration**
2X Platinum SuperFi II Master Mix	25 µL	1X
Template DNA (2ng/µL)	1 µL	0.04 nM

10µM Forward Primer	2.5 µL	0.5 µM
10µM Reverse Primer	2.5 µL	0.5 µM
Water	Up to 50 µL	-

Table 8. SuperFii PCR reaction amplification parameters.

Step	Cycles	Parameter	Temp (°C)	Time (mm: ss)
1	1	Initial Denaturation	98	2:00
2	35	Denaturation Annealing Extension	98 60 72	0:10 0:10 1:00
3	1	Final Extension	72	5:00
4	1	Hold	4	Indefinitely

Cloned PCR products were visualized by running a 0.7% agarose gel in TAE buffer using Azure Biosystems gel imager C200 (VWR, C200). 75ng/µL of PCR product was mixed with NEB 6X DNA gel loading dye (NEB, cat#B7024S), and 10µL of samples were loaded into the gel. The agarose gel was allowed to run for 50 minutes at 100V and visualized using the Gel Doc auto-exposure function. The QIAxcel High-Resolution DNA kit (Qiagen, cat#929002) was used to verify the presence of the genes in lentivirus-transduced cells. If the correct band size was observed, PCR products were cleaned using QIAquick PCR Purification Kit (QIAGEN, cat#28104) as per manufacturer instructions.

2.1.3 Restriction Digestion and Ligation:

The pCDH-CMV-NEO vector for lentivirus generation was generously provided by Dr. Michael Carpenter's laboratory at the NMLB. The vector already contained the human CD81 gene inserted in the multiple cloning site (98). The human CD81 gene was therefore digested out of the vector using Xba1 and Not1. A pCDH-EF1α-GFP-Puro vector was purchased from system biosciences and similarly the vector was digested using Xba1 and Not1 to insert the MuV

HN and F amplicons. For the digestion, 1µg of PCR amplicons and the two vectors were digested with Xba1 and Not1 restriction enzymes in CutSmart buffer (NEB, B7204) for 1 hour at 37°C. Subsequently, the digested amplicons and vectors were run on a 0.7% agarose gel for 40 mins at 70V. The PCR bands were then excised from the gel and then extracted from the gel using the QIAEX II gel extraction kit (QIAGEN, cat#20021). DNA concentrations were measured using Nanodrop (Thermofisher, cat#ND-2000).

NEB T4DNA ligase (NEB, cat#M0202S) was used to ligate the vectors and inserts at a 3:1 insert-to-vector ratio for optimal ligation. The ligation reaction was carried out using the T4 DNA ligase in ligation buffer (NEB, catB0202S) overnight at 16°C. Vector maps for all the clones using the pCDH-CMV-NEO are provided in Figure 2.

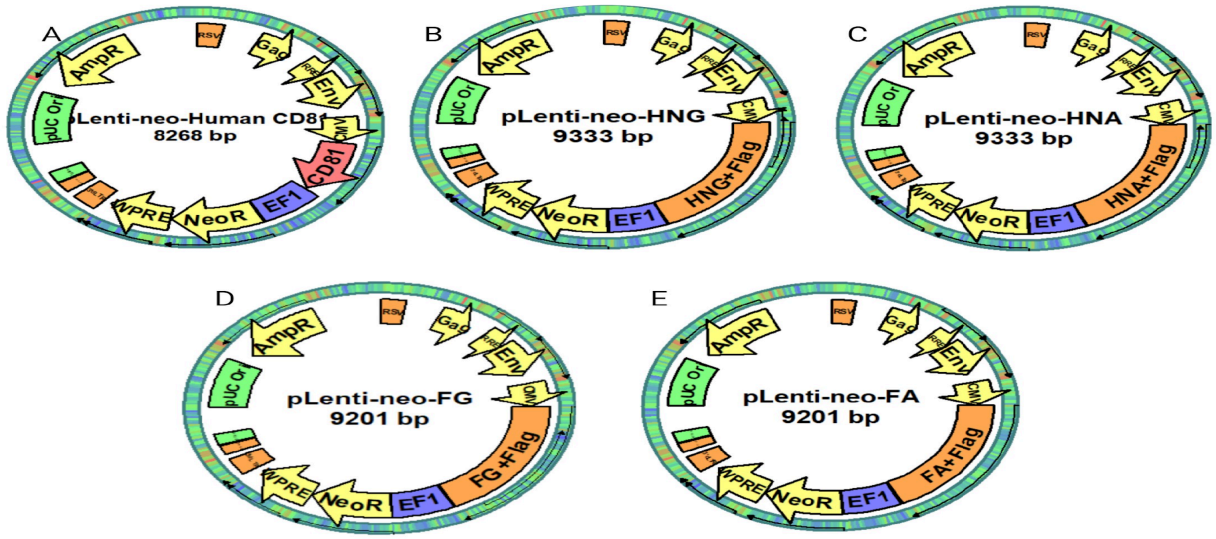


Figure 2. Plasmid maps of the vectors used for Lentivirus stable cell lines. A) pCDH-CMV-Neo (Lenti-neo-Human CD81) vector digested to remove the human CD81 gene and clone in the mumps glycoproteins fragments. B) CMV vector cloned with genotype G HN protein and flag tag (HNG). C) CMV vector cloned with genotype A HN protein and flag tag (HNA). D) CMV vector

cloned with genotype G F protein and flag tag (FG). E) CMV vector cloned with genotype A F protein and flag tag(FA).

2.1.4 Bacterial Cultures:

Following the manufacturer's protocol, the ligation products were transformed into One Shot TOP10 Escherichia coli chemically competent cells (Invitrogen, cat# C4040-3). The transformed cells were then plated on Luria-Bertani (LB) agar plates containing 50µg/mL Carbenicillin (National Microbiology Lab Media services) and incubated overnight at 37°C. Isolated single colonies were grown overnight in 5mL of LB broth containing 100µg/mL of Ampicillin. The QIAprep Spin Miniprep Kit (QIAGEN, cat# 271024) was used to isolate plasmids following the manufacturer's instructions.

Restriction digestions with clone-specific enzymes (Table 9) were performed, and the digested products were analyzed with 0.7% agarose gels. Once the correct band sizes were observed, clones were sent to DNA Core for Illumina NGS sequencing, to ensure that no SNV or frameshift mutations occurred in the cloned insert or in the vector. Single colonies containing the clones were grown in 50 mL of LB broth containing 100µg/mL of Ampicillin. The QIAGEN Plasmid Plus Midi Kit (QIAGEN, cat# 12943) was used to harvest and purify plasmids following the manufacturer's instructions for transient transfection and lentivirus generation.

Table 9. Restriction digest enzymes are used to verify cloning success.

Clone Name	Restriction Enzyme	Expected bands	Expected band size (Kb)
HNA (no codon optimization)	EcorI	3	8175, 1166, 492
HNG (no codon optimization)	EcorI	3	8175, 1166, 492

HNA – Codon Optimized	MfeI	3	6136, 2219, 978
HNG – Codon Optimized	MfeI	4	6136, 2219, 705, 273
FA – Codon Optimized	MfeI	3	6136, 2456, 609
FG – Codon Optimized	MfeI	3	6136, 2553, 532

2.1.5 Cell Culture Techniques

293TN cells

Dr. Michael Carpenter's laboratory at the NMLB kindly provided the 293TN cells. These cells are derived from the human embryonic kidney 293 cell line (HEK293) and they are highly susceptible to transfection by lentiviruses. The cells have been transformed with the SV40 large T antigen plasmid, which contains a neomycin resistance marker. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium containing 4mM L-glutamine, 4.5g/L glucose (DMEM Gibco, cat# 10566-016) supplemented with 10% fetal bovine serum (FBS) (Gibco, cat# 12484-028), 100units/mL of Penicillin, and 100µg/mL of streptomycin (Gibco, cat#15140-122). Cells were incubated in a humidified incubator at 37°C with 5% CO_2. Cells were passaged every 2-3 days by removing the media and washing the cells with phosphate-buffered saline (PBS) at pH 7.4 (National Microbiology Lab Media services). TrypleE trypsin (Gibco, cat# 12605010) was added for 5 minutes to dissociate the cells from the flask. DMEM complete medium was used to inactivate the TrypleE, and the cells were seeded at a dilution of 1:20 in 25 mL of fresh media.

Vero Cells

Vero cells (ATCC, cat#CCL-81) were derived from the kidney of adult African green monkeys. Researchers have previously used this cell line for transient transfection of mumps polypeptides and mumps virus isolation (99-101). Cells were maintained in Minimal Essential Medium (MEM) (Gibco, cat# 11095-080) with 5% FBS in a humidified incubator at 37°C with 5% CO_2. The cell line was used for lentivirus transduction to express the mumps HN and F glycoproteins cloned in the pCDH-CMV-NEO vector which contains the neomycin resistance marker. 500µg/mL of Geneticin (Gibco, Cat# 10131-027) an analog of neomycin was added to the media to select and maintain expression of the mumps glycoprotein. Cells were passaged every 3 to 4 days by removing the media and washing the cells with phosphate-buffered saline (PBS) at pH 7.4; TrypleE (Gibco, cat# 12605010) was then used to dissociate the cells from the flask for approximately 5 minutes at 37°C with 5% CO_2. MEM complete medium was added to neutralize the TrypleE, and the cells were seeded at a ratio of 1:6 in 25 mL of fresh media.

2.1.6 Lentiviral Production

293TN cells were used for transient transfection of the purified clones to confirm protein expression. $2.5x10^5$ cells/mL were seeded in 6-well plates for 24 hours until 70% confluence for transient transfections. 2µg of plasmid with 200µL of opti-MEM (Gibco, cat# 31985070) and 6µL of Roche X-tremeGene HP (Roche, cat#6366244001) were used for transfection of cells. The DNA: lipid transfection mixture was incubated at room temperature for 15 minutes, and then the mixture was added dropwise to the cells. Fresh complete media was added to the cells 24 hours post-transfection. Cells were fixed and stained for immunofluorescence, and protein lysates were collected for Western Blots 48 hours post-transfection.

Once protein expression was confirmed, 293TN cells were used for lentivirus production. $2x10^6$ cells/mL were seeded in 10cm plates for 24 hours to achieve a 70-80% cell confluency. 1.7µg of lentivirus mumps protein expression vector, 1mL of opti-MEM, and 16µL of packaging mix containing 2.8µg of gag plasmid and rev plasmid, and 2.7µg of VSV-G plasmid (provided by Dr. Michael Carpenter) was added to a sterile Eppendorf tube. 30µL of Roche X-tremeGene HP was added to the tube, and the mixture was incubated for 15 minutes and then added dropwise to the cells. Transfected cells were returned to the incubator, and fresh media was added to the cells 24 hours post-transfection. 48 hours post-transfection, lentivirus cells were harvested from the supernatant. Cells were checked under the microscope to see if they adhered to the plate. If cells were floating, the supernatant was transferred to a 15 mL centrifuge tube. Cells were centrifuged at 2000xg for 10 minutes at 4°C. The supernatant was then filtered through a 45µM cellulose acetate filter (VWR, Cat# 28145-481) with a syringe in a 15 mL falcon tube. 2.5mL of PEG-IT (polyethylene glycol) (System Biosciences cat#LV810A-1) was added to the tube, gently mixed, and stored at 4°C overnight to concentrate the viral particles. The following day, the virus particles in the PEG-IT mixture was centrifuged at 1500xg for 40 minutes at 4°C. The supernatant was removed, and the virus pellet was resuspended in 1mL of cold PBS and stored as 200µL aliquots at -80°C.

2.1.7 Vero Cells Transduction

Vero cells at a concentration of 50,000 cells/mL were seeded in a 24-well plate to achieve a 50% confluency. The following day, 100µL of lentivirus, 97µL of complete media, and 3.2µL of 0.5mg/ml of polybrene (Sigma, cat# H9268-5G) were added to transduce the Vero cells (Figure 3). 197µL of complete medium with 3.2µL of 0.5mg/ml of polybrene was added to

non-infected control cells. The cells/virus mixture was rocked continuously to ensure the cells remained in contact with the virus mixture. After 7 hours, the virus mixture was removed, and replaced with 1mL of complete medium, and incubated for 24 hours. Cells were washed once with media, and fresh new medium was added to the cells after 24 hours. 72 hours post-transduction, cells were checked under the microscope for adherence and to ensure no cell death was observed. Fresh medium containing 700µg/mL of Geneticin (G418) sulfate (Gibco, Cat# 10131-027) was added to the cells. Cells were split in the complete medium with antibiotic to select for lentivirus-transduced cells. The selection was continued until all the cells in the cell selection control well were dead (approximately 2 weeks). Once cells expressing the lentivirus were obtained, the antibiotic concentration was reduced to 500µg/mL. Cells were maintained in 5% FBS containing 500µg/mL of G418 sulfate.

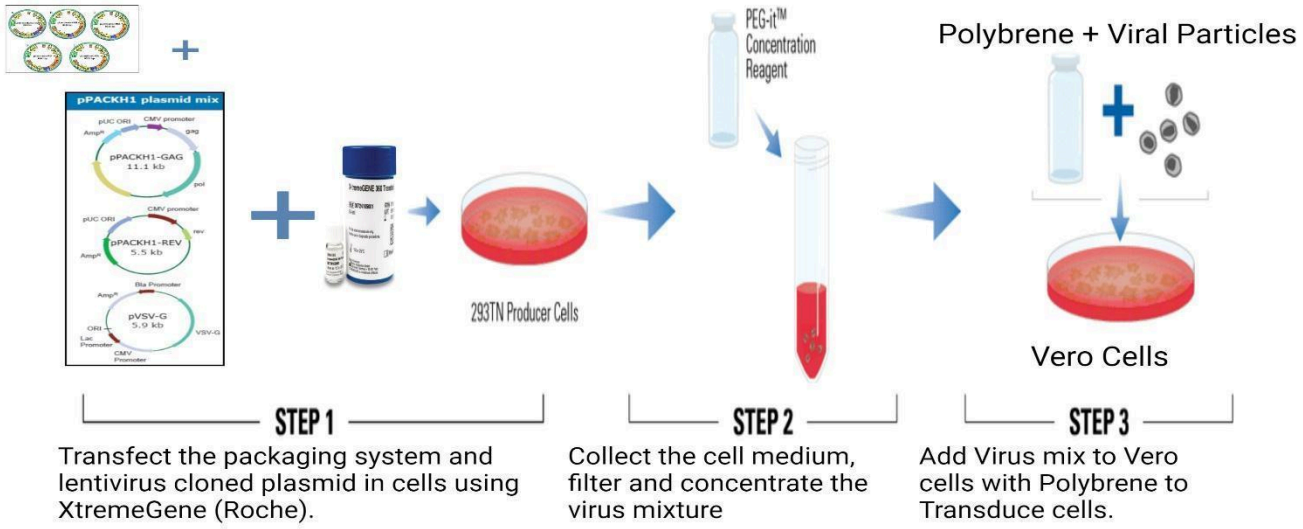


Figure 3. Methods for Stable cell line generation using lentivirus. The figure is modified from Lentivector Expression Systems: System Biosciences.

2.2 Protein Expression

2.2.1 Western Blot

Western Blot was performed on cell lysates collected from transiently transfected cells 48 hours post-transfection to ensure protein expression. Transiently transfected 293TN cells and lentivirus stable cell lines were grown in 6 well plates by adding $2x10^6$ cells/mL and harvesting protein lysate at 80% confluency. 100µL of 2% SDS lysis buffer (National Microbiology Lab Media Services) was added to the wells to lyse the cells and cell lysates were harvested. The lysate was collected in a QIA shredder (Qiagen, Cat# 79656) and centrifuged for 1 minute at 13000rpm to homogenize the cell lysate. 10µL of cell lysate was mixed with 20uL of Laemmli loading buffer(4x) with 2uL of 40mM DTT and then heated at 75°C for 10 minutes, cooled to 4°C, and stored at -80°C.

30µL of cell lysate was added to each well of a 4-20% SDS gradient polyacrylamide gels (Genscript, Cat#M42010), and 10µL of Chameleon Duo Pre-stained Protein Ladder (LI-COR, Cat#928-60000) was used as size control. The gel was run at 200V for 35 minutes with TRIS-MOPS-SDS running buffer (Genscript, Cat#M00138). A PVDF membrane was activated in methanol and soaked in PVDF equilibration buffer (Genscript, Cat#L00727), and proteins were transferred to the membrane using the Genscript E blot system. The membrane was then blocked for 1 hour in 1:1 dilution of Intercept blocking buffer (LI-COR, Cat#927-60001) in TBS. The membrane was incubated in 10mL of primary antibody solution, including 10µL of M2 flag mouse primary antibody (Sigma Aldrich, Cat#F1804) in 5mL of Intercept blocking buffer and 5mL of TBS-T. The membrane was stored overnight at 4°C and then washed 3 times for 5 minutes with TBS-T. The membrane was then incubated for 1 hour at room temperature in the

dark with 1:10000 dilution of Goat anti-mouse IRDye 680 (LI-COR, Cat#926-68070) in 1:1 of Intercept blocking buffer with TBS-T and washed 3 times for 5 minutes with TBS-T and 1 time with TBS before imaging. Membranes were blotted dry and imaged with the 680nm and 800nm wavelength channels for 2 minutes on the Odyssey FC imager (LI-COR, Cat# LIC-9201-00).

2.2.2 Immunofluorescence

Immunofluorescence assay (IFA) was performed to confirm protein expression in stable cell lines as well as to look at antibody binding differences between mumps genotype A and genotype G. 30,000 transduced Vero cells were seeded in 400µL in eight well chamber slides (Thermofisher, Cat#177402) in complete cell medium (MEM with 5% FBS and geneticin) and incubated in a humidified chamber at 37°C in 5% CO_2. The following day, the medium was removed, and the cells were washed once with PBS. Cells were fixed with 400µL of 4% paraformaldehyde (Thermofisher, cat# J61899.AK) for 10 minutes at room temperature, and then washed 3 times with 400µL PBS. PBS was removed, and cells were permeabilized with 400µL 0.5% Triton X (Fisher Scientific, Cat# M1122981001) in PBS for 5 minutes at room temperature. After 3 washes with 400µL PBS, cells were blocked for 1 hour with 400µL 1% BSA in TBS (Thermofisher, Cat# 37520).

The C terminus of the proteins contained a flag tag to determine protein localization and expression. The tag was necessary as there are no monoclonal antibodies available for the HN and F proteins of MuV. Positive mumps IgG serum, as determined by PRNT and Bioplex 2200, was used as a positive control. Negative controls included secondary antibody only, ‘strip serum’ (serum stripped from IgG antibodies), and a mumps negative serum obtained from a donor that

had not been infected or vaccinated for mumps. Test sera samples were kindly provided by Cadham Provincial Laboratory and included: patients only vaccinated for mumps (genotype A only), patients both vaccinated for mumps and infected with mumps (both genotype A and genotype G), and patients who were not vaccinated for mumps and only had a mumps infection (genotype G only), as described in detail in Results.

Secondary goat anti-mouse antibody conjugated with Alexa Fluor 488 (Thermofisher, cat# A55058) was used at a dilution of 1:1500 to bind to the primary flag antibody. Secondary goat anti-human mouse antibody conjugated with Alexa Fluor 555 (Thermofisher, cat#A-21433) was used at a dilution of 1:2000 to detect antibody binding from patient sera. Cells were incubated with 400µL of secondary antibodies diluted in 1% BSA with TBS-T for 1 hour at room temperature in the dark and then washed 3 times with PBS-T to remove any unbound antibodies. 400µL DAPI (Thermofisher, cat# D21490) diluted at 1:37000 in PBS was added to the cells for 5 minutes at room temperature in the dark to stain the nucleus, after which cells were washed once with PBS. 400µL of PBS was added to the cells, and the slides were then stored at 4°C until imaging.

Slides were imaged using the EVOS FL Color Imaging System (Life Technologies, cat# AMF4300). Images were captured at 345nm absorbance/455nm emission for DAPI (blue), 496nm absorbance /518nm emission for flag tag (green), and 555nm absorbance /565nm emission (red). Each image was acquired separately and saved individually, and the channels were overlaid together and saved as one file. The patient serum antibody binding (red channel) images were all collected on PowerPoint. The last dilution in which a signal was observed was recorded as the titer of the test serum as done for EBV diagnostics (102-103).

3. Results: MuV Genotype A and Genotype G Stable Cell Lines

3.1 Lentivirus Vector Cloning

3.1.1 MuV genotype A and genotype G Strains

MuV genotype A VE-12343 and the genotype G CDC strain were available in the lab and used for cloning in the lentiviral vectors. Strain VE-12343 is closely related to the MuV JL5 strain. Whole genome sequencing to confirm virus sequences was performed using the Illumina platform by Dr. Jasmine Frost.

The amino acid sequences of the HN and F proteins of the genotype A strain (VE-12343) and genotype G strain (CDC strain) were compared. MEGA amino acid alignment was used to identify differences between the two genotypes. For the MuV HN protein, thirty-one amino acid (aa) variations were observed between the two genotypes. Table 10 shows the amino acid differences in genotype G when compared to genotype A. Two aa variations resulted in changes to the N-glycosylation sites; the N12S variation resulted in the loss of N-glycosylation, while H464N created an additional potential N-glycosylation sites. Five aa variations led to a change in the membrane anchorage domain, five aa variations affected the predicted B and T cell epitope, and six aa potentially led to escape neutralization, as previously suggested (see Literature references in Table 10). Sixteen aa differences between genotype A and genotype G are unique, and have not been previously identified in the literature. Some of these variations could potentially affect antibody binding and reduce antibody cross-reactivity between the two genotypes.

Table 10. Amino Acid Variation in MuV HN protein between genotype A and genotype G strains.

Position	Amino Acid Variation	Potential Effect	Literature Reference or Unique
6	L6F	N/A	60
8	I8T	N/A	Unique
9	M9I	N/A	60
12	N12S	Loss of N-Glycosylation	61
21	V21S	N/A	60
25	G25N	N/A	Unique
37	V37A	Change in Membrane Anchorage Domain	Unique
44	I44T	Change in Membrane Anchorage Domain	Unique
56	I56V	Change in Membrane Anchorage Domain	60
80	A80T	Change in Membrane Anchorage Domain and T cell Epitope	56
81	V81M	Change in Membrane Anchorage Domain and T cell Epitope	56
113	S113A	N/A	Unique
121	N121S	Neutralization Escape*	Unique
122	R122Q	Neutralization Escape*	Unique
123	N123K	Neutralization Escape*	Unique
135	I135V	N/A	Unique
218	A218V	N/A	Unique
279	I279T	Change in B-cell and T-cell Epitope	26, 61
287	I287V	Change in B-cell and T-cell Epitope	26, 61
356	E356D	Change in B-cell Epitope	26, 61
372	S372N	N/A	26, 60
375	V375I	Neutralization Escape*	60
399	N399S	Neutralization Escape*	60
403	L403M	N/A	Unique
442	Y442S	Neutralization Escape*	Unique
444	Q444P	N/A	Unique
464	H464N	Addition of N-glycosylation	60
473	I473T	N/A	Unique
474	V474T	N/A	Unique
490	R490S	N/A	Unique
577	A577T	N/A	60

*Variations at these sites have resulted in neutralization escape in some genotype A strains and may cause neutralization escape in genotype G strain.

For the MuV F protein, twenty-eight amino acid variations were observed between the genotype A vaccine strain and the genotype G CDC strains. Table 11 shows the amino acid difference in genotype G compared to genotype A. Eight aa variations in the signal peptide region, and three aa variations in the carboxy terminal region were observed. Twenty differences between genotype A and genotype G are unique, and have not been previously identified in the literature. The potential effects of these differences have yet to be determined and some could result in changes in protein conformation and binding sites and reduced recognition from the immune system.

Table 11. Amino Acid Variation in MuV F protein between genotype A and genotype G strains.

Position	Amino Acid Variation	Potential Effect	Literature Reference or Unique
2	N2K	Change in Signal Peptide Region*	60
3	A3V	Change in Signal Peptide Region*	60
4	F4S	Change in Signal Peptide Region*	60
5	P5L	Change in Signal Peptide Region*	Unique
7	I7T	Change in Signal Peptide Region*	60
11	Y11F	Change in Signal Peptide Region*	Unique
13	I13V	Change in Signal Peptide Region*	60
16	S16F	Change in Signal Peptide Region*	60
24	T24I	N/A	Unique
49	V49I	N/A	Unique
95	L95P	N/A	Unique
170	D170N	N/A	Unique
177	S177N	N/A	Unique
269	M269V	N/A	Unique
318	R318S	N/A	Unique
330	H330N	N/A	Unique
331	M331I	N/A	Unique
345	S345T	N/A	Unique
409	S409A	N/A	Unique
413	L413V	N/A	60
431	T431A	N/A	Unique
454	S454I	N/A	60

477	F477V	N/A	Unique
479	V479I	N/A	Unique
480	G480N	N/A	Unique
488	V488I	Change in Carboxy Terminal*	Unique
489	S489A	Change in Carboxy Terminal*	Unique
498	I498M	Change in Carboxy Terminal*	Unique

*These mutations are present in the carboxy terminal and the signal peptide region and may cause changes in protein signaling.

3.1.2 MuV HN Gene Amplification

RNA was isolated from genotype A (VE-12343) and genotype G (CDC) viral culture strains available in the lab using the Qiagen viral RNA extraction kit. The RNA was converted into cDNA using superscript IV, and the cDNA was PCR amplified using SuperFii polymerase using gene-specific primers listed in Table 6. Superscript IV was chosen for cDNA synthesis as it is known to be highly efficient and has increased sensitivity and higher thermostability (101). SuperFii was chosen as it has high fidelity, resulting in low mutation rates and high accuracy during PCR amplification (102). The HN gene is 1.7kb long, and agarose gel analysis of the PCR product observed a band at 1.7kb (Figure 4). This shows that the HN gene was amplified using SuperFii PCR amplification.

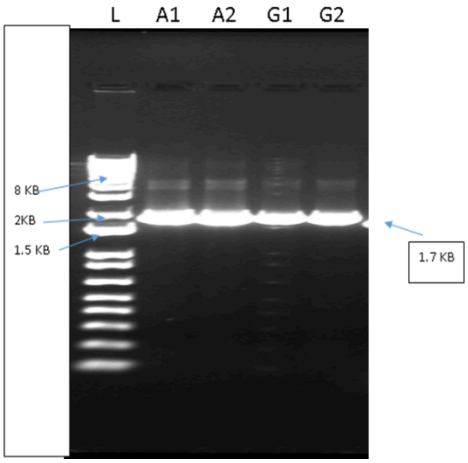


Figure 4. PCR amplification of MuV HN Gene. MuV genotype A and genotype G HN genes were reverse transcribed using the SSIV system and PCR amplified using the SuperFii polymerase. Two PCR replicates were confirmed by agarose gel analysis PCR 1 and 2 for genotype A (A1 and A2) and for genotype G (G1 and G2).

3.1.3 Vector and PCR Enzyme Digest

The CMV and EF1α promoters are known to have varying transduction efficacy in different cell lines (106). Hence, a vector with the CMV promoter (CMV vector) and a vector with the EF1α promoter (EF1α vector) were used to study which promoter was more efficient in expressing the MuV HN protein in 293TN and Vero Cells (Figure 5). The EF1α vector contains a multi-cloning site (MCS), a GFP, and a puromycin selection marker (Figure 5A). The CMV vector includes a neomycin selection marker and the human CD81 gene instead of a MCS (Figure 5B). The human CD81 gene was digested out using restriction enzymes and the MuV HN or the F genes were cloned into the vector.

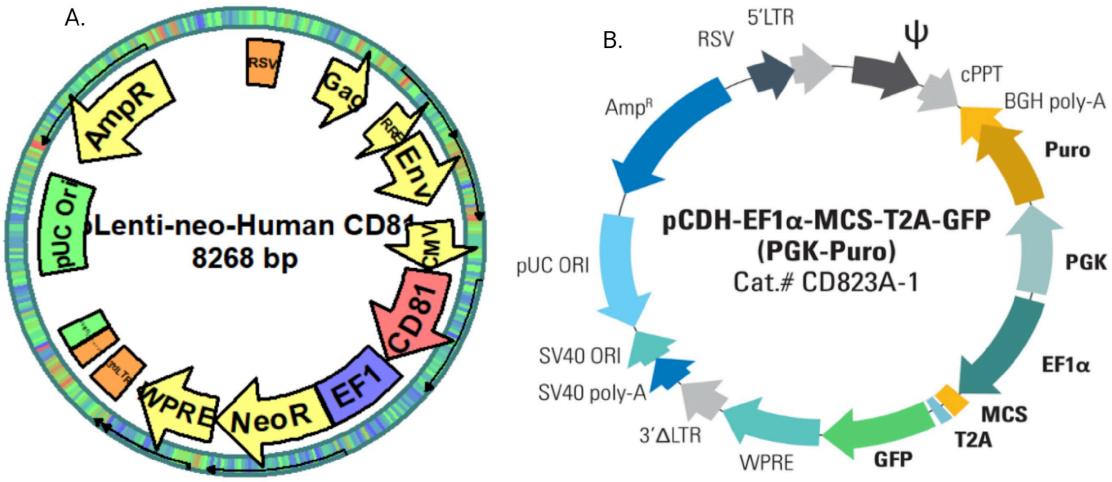


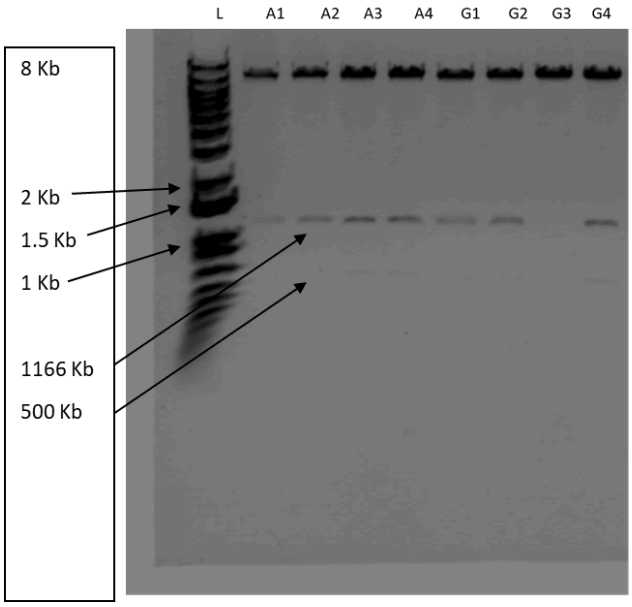
Figure 5. Lentivirus vector used for MuV stable cell lines. Vector A is the CMV vector which contains the CMV promoter and a CD81 region which is removed by restriction digestion. Vector B contains the EF1α promoter site and an MCS to clone the MuV HN and the F gene.

Briefly, the vectors and the PCR-amplified products were digested using the XbaI and NotI restriction enzymes and an agarose gel was run to purify the digested vector and PCR bands for ligation. The expected band size of 1.7Kb was observed for the PCR bands and cut

under UV light. Vector digestion was also performed for both the EF1α vector and the CMV vector. The restriction vector and the PCR fragments were ligated using the T4 DNA ligase. The resulting ligation products were transformed into *E.coli* cells.

3.1.4 Plasmid Preps and Restriction Digestion

The ligated plasmid were transformed into *E.coli* cells, and a single *E.coli* colony was picked and cultured for screening for the correct clone, by restriction digestion. For each of the four clones (Genotype A and Genotype G, in the CMV, or EF1α Vector), two colonies were digested with EcorI. The expected results included two bands, one at 8 Kb and one at 1.16 kB. The correct band sizes were observed for both colonies for genotype A and genotype G CMV vector and Genotype A EF1α vector but only one colony for genotype G EF1α vector (Figure 6). To ensure the absence of SNV or frameshift mutations in the cloned plasmid resulting from cloning or during bacterial culture the clones were submitted for NGS sequencing to the DNA Core Facility.

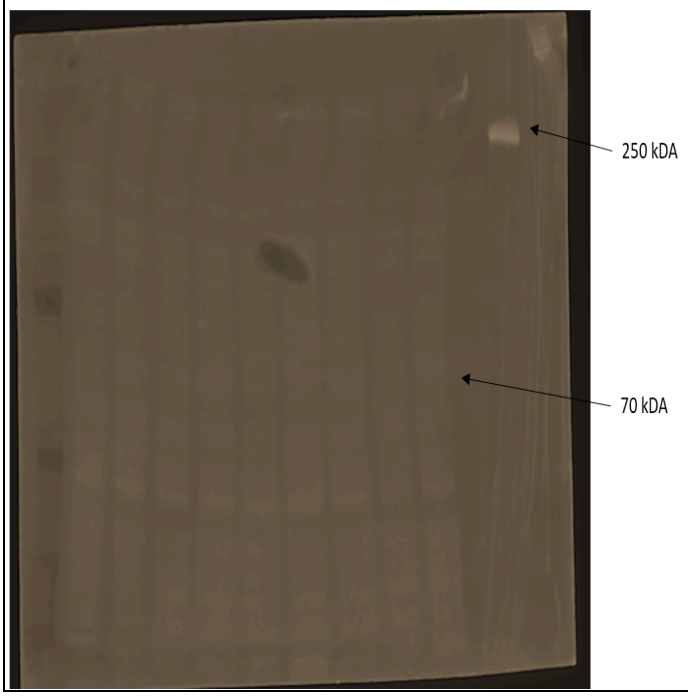


Labels	Samples	Clone Successful
L	Ladder	N/A
A1	Genotype A CMV Vector (1)	Yes
A2	Genotype A CMV Vector (2)	Yes
A3	Genotype A EF1α Vector (1)	Yes
A4	Genotype A EF1α Vector (2)	Yes
G1	Genotype G CMV Vector (1)	Yes
G2	Genotype G CMV Vector (2)	Yes
G3	Genotype G EF1α Vector (1)	No
G4	Genotype G EF1α Vector (2)	Yes

Figure 6. Restriction Digestion of Lentiviral Vectors with EcorI. CMV Vector and EF1α were digested with ECORI to confirm successful plasmid cloning. The expected band sizes were 8175 Kb, 1166 Kb, and 500 Kb. Two clones of each of the 4 clones were selected for analysis, and all the clones were correct and showed the expected band's size except Clone G3 (Genotype G EF1α vector).

3.2 Transient Transfections to Confirm Protein Expression

Once the plasmids sequences were verified by NGS, Midiprep was performed to obtain sterile and purified plasmids for transformation. 293TN cells were used for transformation as these cell lines have a high transfection efficiency (107). Transient transfection was performed, and protein lysate was isolated for Western Blot, 48 hours post-transfection. Western Blot was performed using the flag antibody as no monoclonal antibody is available for the MuV HN protein. No protein expression was observed in the Western Blot (Figure 7). SARS-CoV2 spike protein with the flag tag added at the C terminus was provided by Dr. Michael Carpenter as a positive control, which showed a positive band at 250kb. These results show that the Western Blot was run successfully, but no flag tag signal for the HN protein was detected, as the protein was not expressed or expressed in sufficient amounts for detection by the Western Blot.

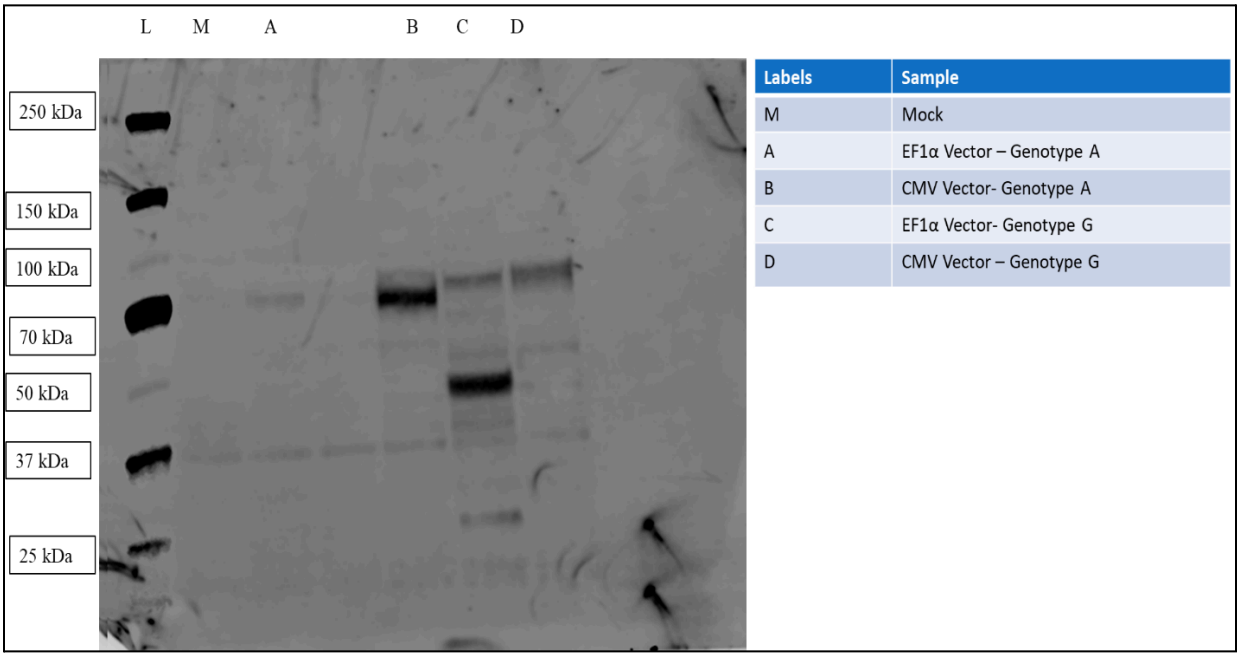


Labels	Sample	Lysate
L	Ladder	
M	Mock	SDS Lysis
A1	Genotype A CMV Vector	SDS Lysis
A2	Genotype A CMV Vector	SDS Lysis
A3	Genotype A EF1α Vector	SDS Lysis
A4	Genotype A EF1α Vector	SDS Lysis
G1	Genotype G CMV Vector	NP40 Lysis
G2	Genotype G CMV Vector	NP40 Lysis
G3	Genotype G EF1α Vector	NP40 Lysis
G4	Genotype G EF1α Vector	NP40 Lysis
S	Spike Protein Flag Control	SDS Lysis

Figure 7. Western Blot analysis of HN protein with flag tag. Transfected cells were extracted by SDS lysis or NP40 lysis to detect the flag tag on the C-terminus of the HN protein. The SARS-Cov-2 spike protein was used as a positive control, and a band was observed at 250 kDa. No flag tag was detected for the MuV HN protein clones at the expected 70kDa position**.**

Since the clone obtained by direct PCR of the MuV virus failed to express a detectable amount of NH protein, codon optimization was used with the aim to increase protein expression and translational efficiency (96). Codon optimization uses codon usage bias by favoring the most abundant tRNAs to increase protein expression. Codon optimization clones were generated using the same cloning strategy as non-codon optimized sequences. Transient transfections were performed and the Western Blot results showed a band at 70kDa, as expected for the HN protein expression for both the EF1α vector and the CMV vector for genotype A and genotype G (Figure 8). Two bands were observed for genotype G HN protein with the EF1α vector, the first band at 70kDa and a second smaller band at 50kDa. The observed 50kDa band could be due to non-specific cleavage of the HN protein or protein degradation.

Due to the additional band observed for genotype G HN protein with the EF1α vector, we decided not to use the vector for lentivirus preparation. The CMV vector was selected to make lentivirus as it showed good protein expression and no protein cleavage.



Labels	Sample
M	Mock
A	EF1α Vector – Genotype A
B	CMV Vector- Genotype A
C	EF1α Vector- Genotype G
D	CMV Vector – Genotype G

Figure 8. Western Blot analysis of codon-optimized HN protein with flag tag using an anti-flag antibody. Cells were lysed and proteins were run on Western Blot to detect the HN protein's flag tag on the C-terminus. Licor Chameleon duo pre-stained ladder was used, the dark bands pre-stained with AF680 and the lighter bands were stained AF800. For genotype, A CMV and EF1α clones and genotype G CMV, the expected HN protein band was observed at 70 kDa. For the genotype G clone with the EF1α vector, the correct band was observed at 70 kDa, but a cleaved band at 50 kDa was also observed.

IFA was performed for native and codon optimized clones to detect the HN protein in transfected 293TN cells, as it is more sensitive than Western Blot to confirm protein expression. For the CMV vector, two primary antibodies were tested: anti-flag antibody as the primary antibody with goat anti-mouse Alexa Fluor 488 (green channel) as the secondary antibody and an archival human anti-mumps serum (186-89 and) as the primary antibody, with goat anti-human Alexa Fluor 555 as the secondary antibody (red channel). Non-transfected 293TN

cells were used as cell negative control, and 'strip serum' (serum stripped of IgG antibodies) was tested with Alexa Fluor 555 (red channel), as serum background control. For the native codon clones no signal was observed in the cell control, confirming the absence of non-specific antibody binding (Figure 9). Additionally, for the non-codon optimized, the anti-flag and the anti-mumps serum to detect the HN protein expression showed a weak fluorescence signal, and flag and anti-mumps serum localized in the cell (Figure 9). These results show that the HN protein was expressed in low amounts for the CMV vector for genotype A and genotype G in non-codon optimized clones.

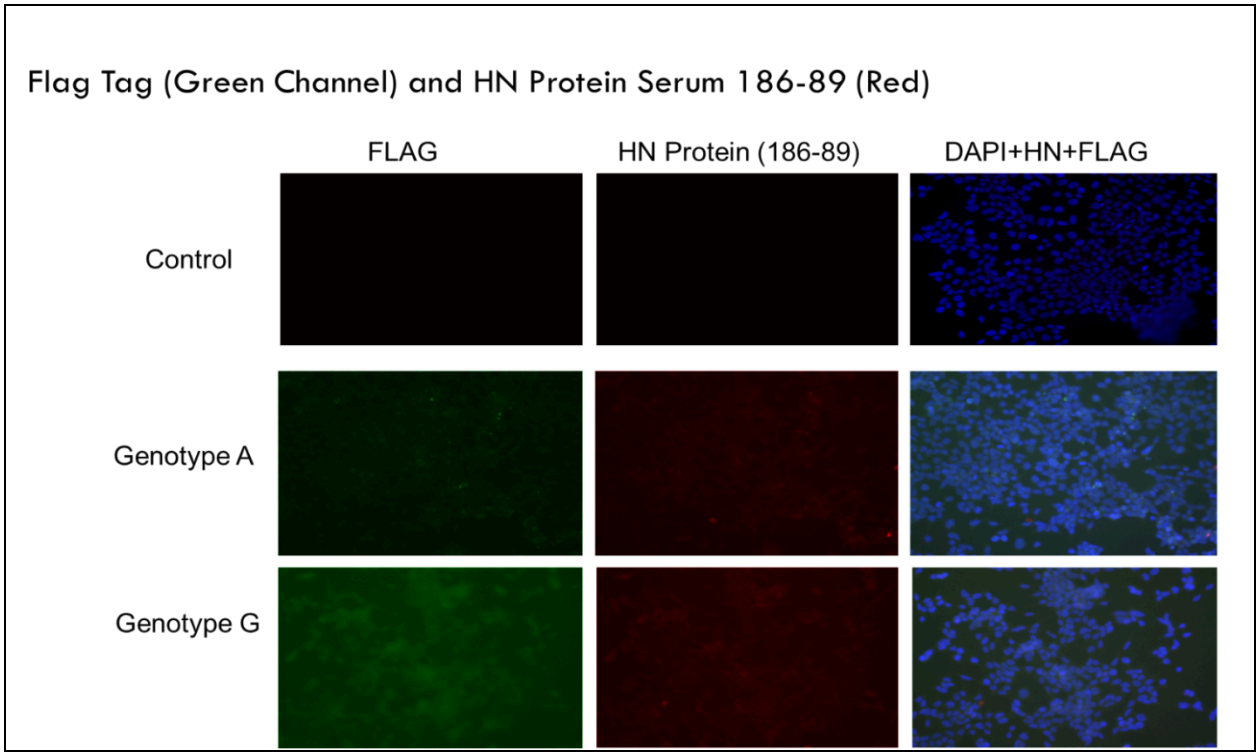


Figure 9. IFA with transiently transfected cells with genotype A and genotype G with CMV Vector. Control cells were non-transfected 293TN cells. 293TN cells were transfected with HN protein, clones for genotype A and genotype G with CMV vector. Human anti-mumps serum 186-89 was used to assess HN protein presence. Flag tag was used to confirm HN protein expression. Weak HN protein expression was observed.

IFA was also performed for the non-codon optimized GFP expressing EF1α vector to verify if the EF1α promoter was better at expressing the MuV HN protein. Anti-flag with goat anti-mouse 555 and anti-mumps serum (186-89) with goat anti-human Alexa Fluor 555 were used to see HN protein expression. 293TN non-transfected cells were used for negative control. Transient transfection showed high levels of GFP for genotype A, but low levels of GFP were observed for genotype G (Figures 10A and 10B). Weak signals were observed for the HN protein (Figure 10A and 10B). These results show that the HN protein was expressed in low amounts also for the EF1α vector, although strong GFP expression was observed for genotype A. For genotype G, weak GFP expression was observed.

In summary, weak protein expression was observed by Western Blot and IFA for all non-codon optimized clones. Hence, the clones were not used for lentivirus generation and IFA analysis on codon optimized clones was performed.

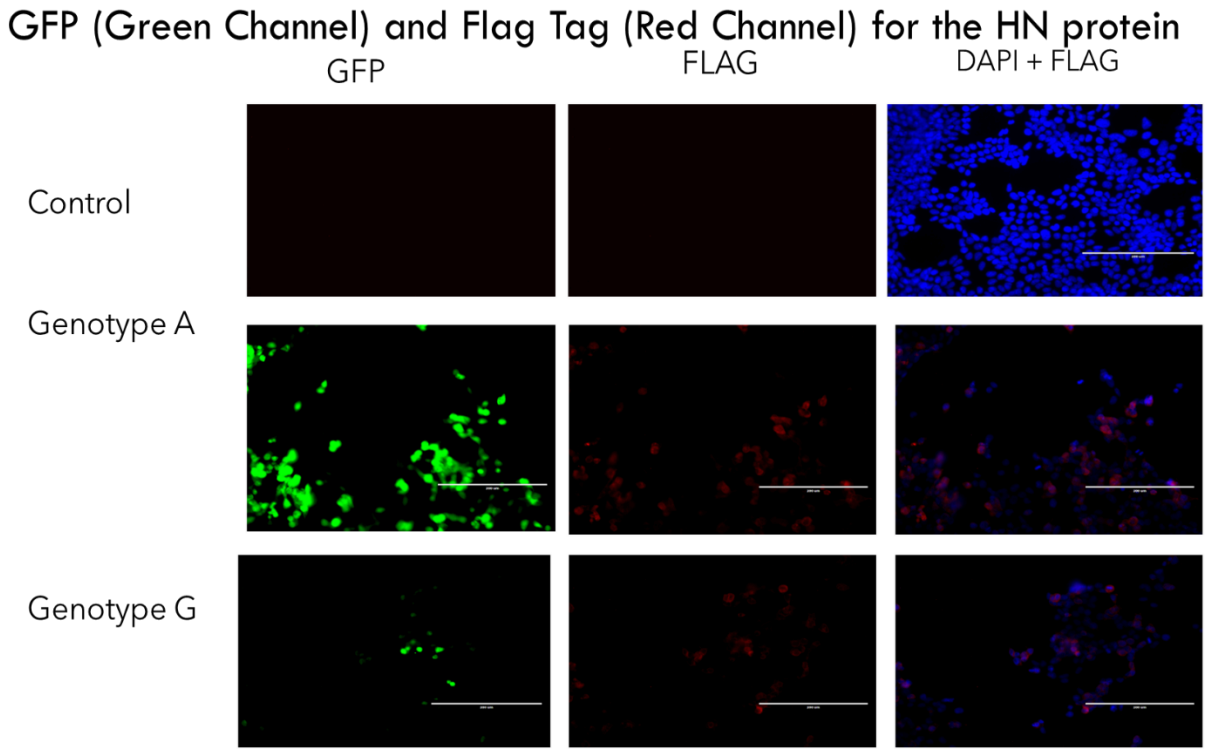


Figure 10A. IFA with transiently transfected cells with genotype A and genotype G with EF1α Vector. Control cells were non-transfected 293TN cells. 293TN cells were transfected with HN protein, clones for genotype A and genotype G with EF1α vector. Anti-flag was used assess HN protein expression. GFP was used to confirm HN protein expression and transfection efficiency. Weaker antibody binding to the HN protein and low levels of GFP was observed for genotype G clone.

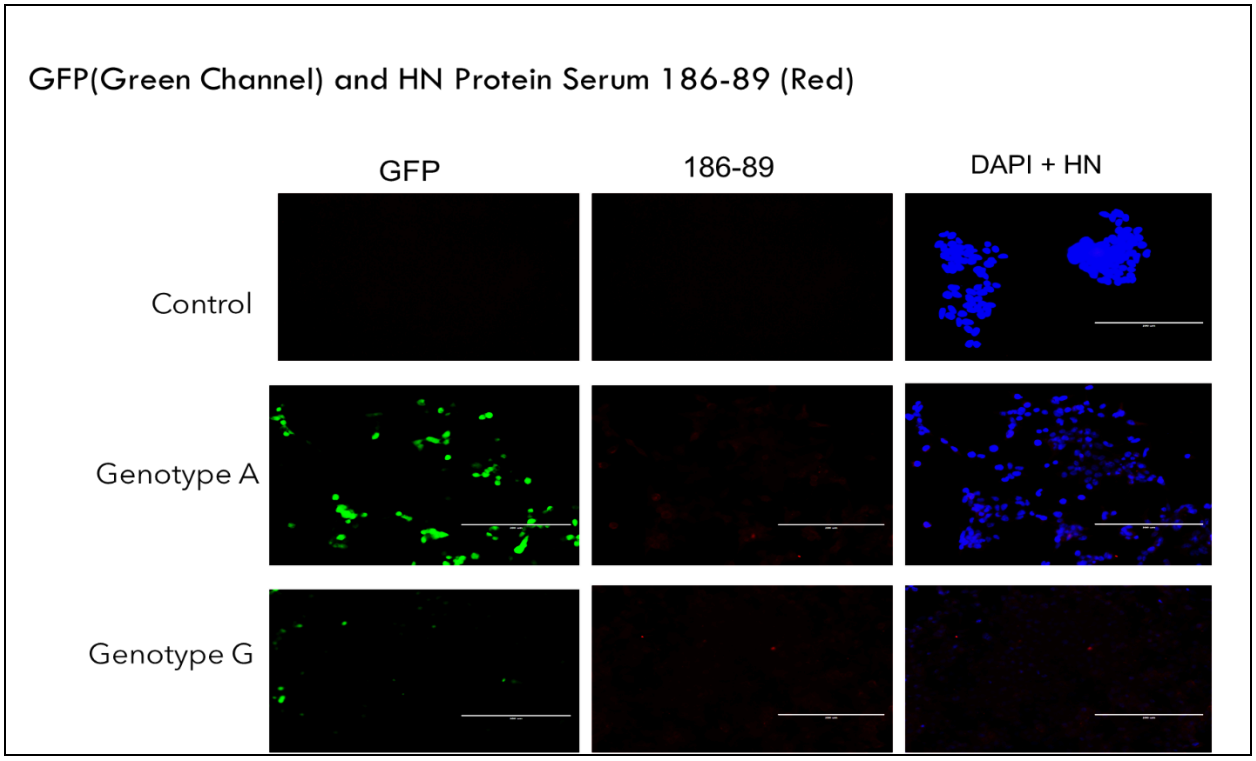


Figure 10B. IFA with transiently transfected cells with genotype A and genotype G with EF1α Vector. Control cells were non-transfected 293TN cells. 293TN cells were transfected with HN protein, clones for genotype A and genotype G with EF1α vector. Anti-mumps serum 186-89 assessed HN protein expression and binding. GFP was used to confirm HN protein expression and transfection efficiency. Weaker antibody binding to the HN protein and low levels of GFP was observed for genotype G clone.

IFA analysis was then performed on transiently transfected cells with the CMV vector plasmid containing codon-optimized HN and F protein genes, for both genotype A and genotype G. IFA results showed high levels of HN protein expression based on anti-mumps serum and anti-flag antibodies (Figure 11). Also, the HN protein colocalized with the flag tag added at the C-terminus of the HN protein. These results show strong HN protein expression after codon optimization. Hence, the F protein was codon-optimized for genotype A, and genotype G, and expressed in the CMV vector. Clones containing the genotype A and genotype G F genes in the CMV vector were also generated and IFA was performed to see F protein expression. Similar to

the HN protein, IFA results showed high levels of F protein expression in the CMV vector for genotype A and genotype G (Figure 12).

In conclusion, the CMV vector with the codon optimized HN and F genes for genotype A and genotype G showed good protein expression by IFA. These CMV vector clones were therefore used to generate lentiviruses for stable transmission.

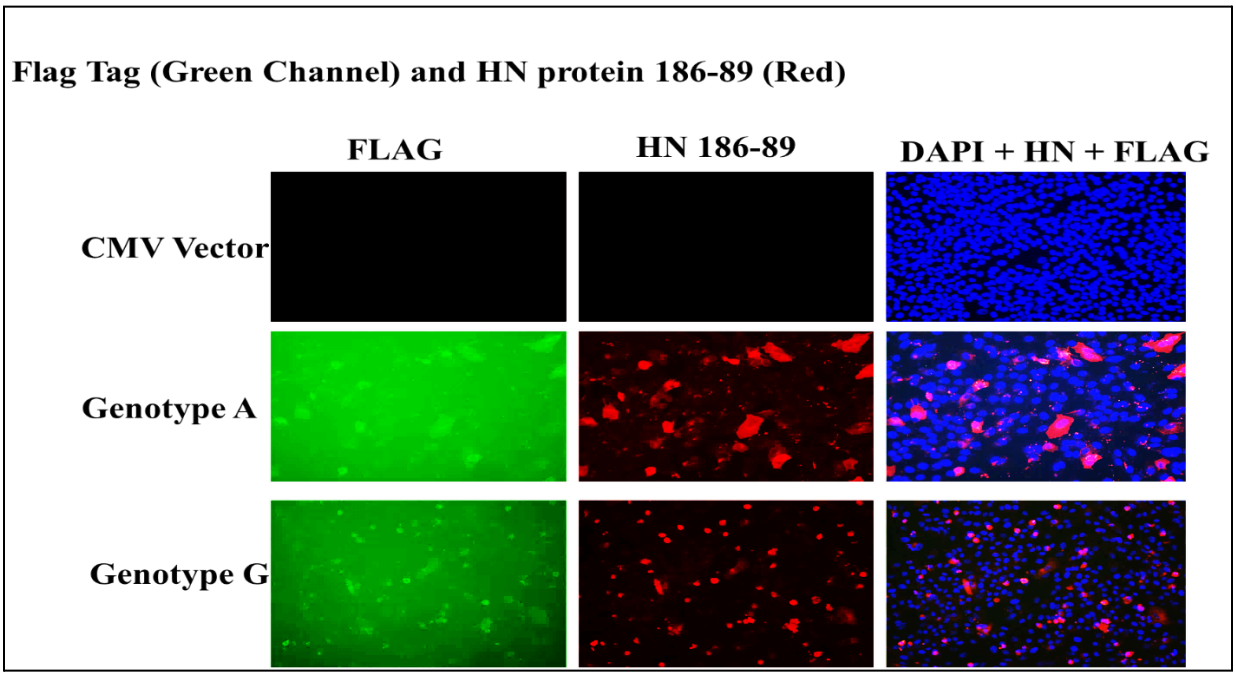


Figure 11. IFA with transiently transfected cells codon-optimized HN protein genotype A and genotype G with CMV Vector. Control 293TN cells were transfected with the CMV vector expressing the CD81. 293TN cells were transfected with CMV vector expressing the HN protein for genotype A and genotype G with CMV vector. Anti-mumps serum 186-89 was used to assess HN protein binding. Flag tag antibodies were used to confirm HN protein expression. Good HN protein expression was observed and flag tag and HN protein co-localized.

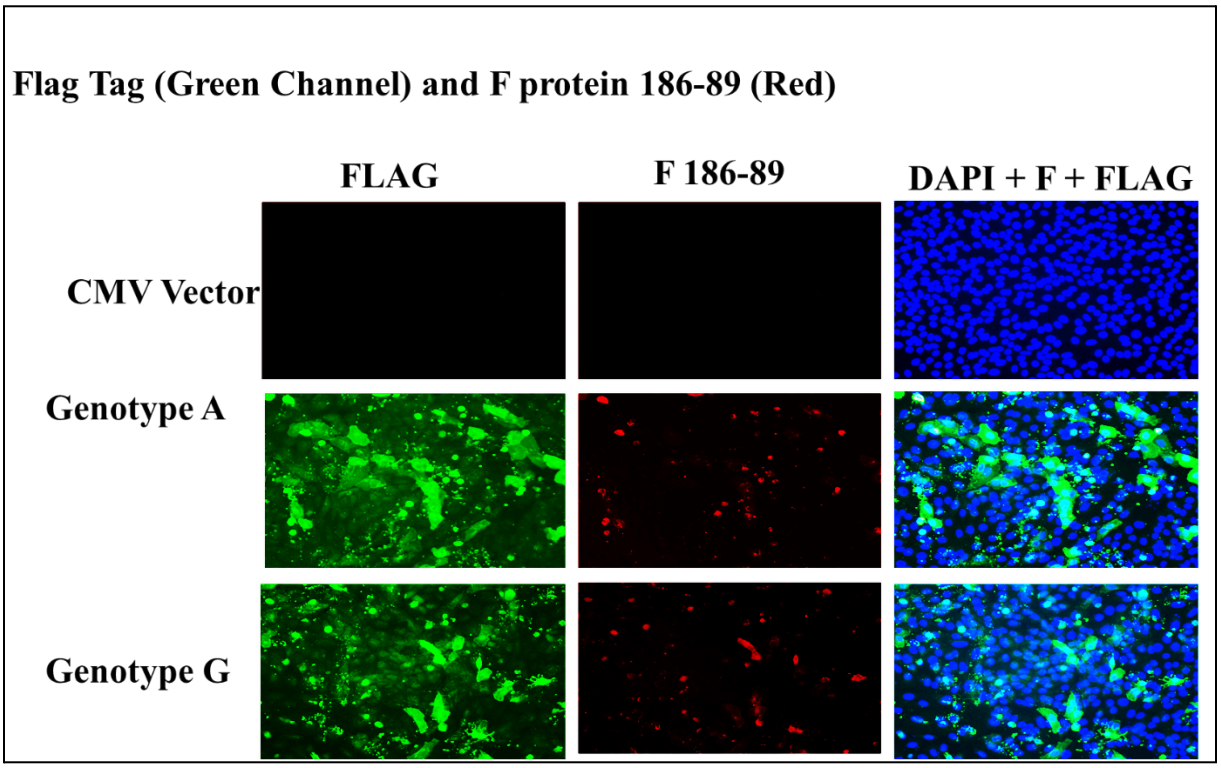
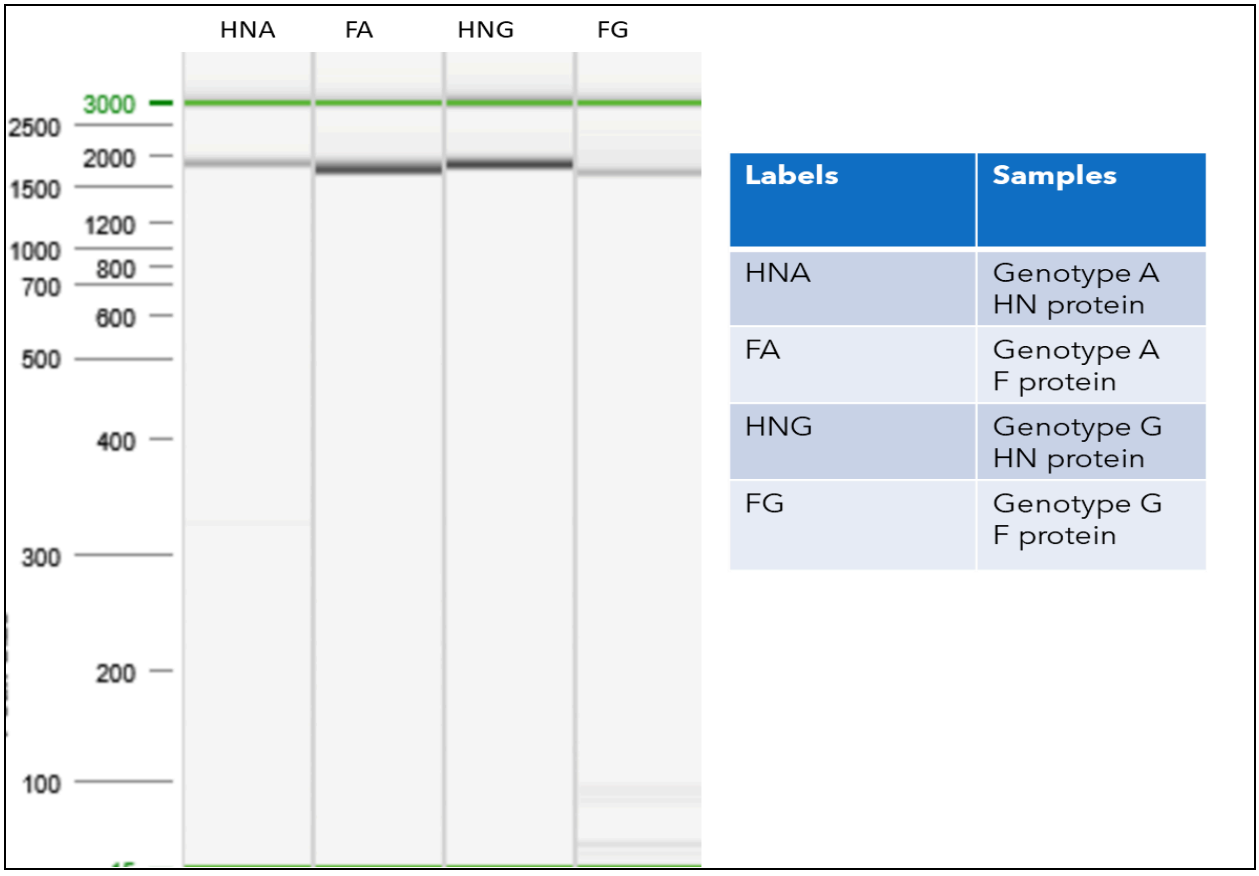


Figure 12. IFA with Transiently transfected cells codon-optimized F protein genotype A and genotype G with CMV Vector. Control cells were CMV vector expressing the CD81 gene in 293TN cells. 293TN cells were transfected with CMV vector expressing F protein for genotype A and genotype G with CMV vector. Anti-mumps serum 186-89 was used to assess F protein binding. Flag Tag was used to confirm HN protein expression. Strong F protein expression was observed and flag Tag and F protein co-localized.

3.3 Stable Cell Lines for HN and F Glycoprotein

Lentivirus particles were produced in the 293TN cell line using the CMV vector HN and F codon-optimized clones for genotype A and genotype G (Figure 2). Vero cells were then transduced with lentivirus particles at a 1:2 ratio, and transduced cells were selected by Geneticin antibiotic selection. Transduced cells were treated with Geneticin antibiotic to kill non-transduced cells for 14 days. In non-transduced Vero cells treated with Geneticin, complete cell death was observed at 14 days.

To confirm that the HN and F genes were expressed from the integrated lentivirus, transduced cells were seeded in 6 well plates, and RNA isolation was performed by adding DNaseI to remove DNA. Isolated RNA was used to make cDNA using Superscript IV and was PCR amplified using SuperFii polymerase. PCR products were analyzed on the QIAxcel Advanced gel electrophoresis instrument and correct band sizes were observed for the HN and F transcripts for genotype A and genotype G (Figure 13). These results show that the lentivirus transduction was successful and the HN and F MuV glycoproteins gene transcripts could be detected in the stable cell lines.



Labels	Samples
HNA	Genotype A HN protein
FA	Genotype A F protein
HNG	Genotype G HN protein
FG	Genotype G F protein

Figure 13.RT-PCR gel analysis MuV stable cell lines expressing the HN and F protein. RNA was extracted using the Qiagen RNA Kit and reverse transcribed using Superscript IV. PCR was performed using gene-specific primers to verify the presence of MuV F and HN transcripts.

Expected band sizes of 1.7kb for HNA and HNG and 1.6kb for FA and FG was observed for all the lentivirus-transfected cell lines.

IFA was then performed to confirm HN and F protein expression in lentivirus generated stable cell lines. DAPI was used to stain the nuclei, while the flag antibody was used to confirm the protein expression. Serum F, a mumps negative serum was obtained from an individual with no history of vaccination or infection and confirmed negative for mumps antibodies by ELISA and Bioplex 2200, served as a negative control to test non-specific IgG binding. The serum 186-89 which exhibited a high antibody titer by ELISA and Bioplex 2200, was used as a positive control to ensure the successful staining. 'Strip serum' (serum stripped of IgG antibodies) was used as serum negative control; flag antibody only was used as a positive control to verify HN and F protein expression and that no bleed-through was observed between the green and the red channels. A secondary antibody only condition (secondary only) was used to assess non-specific binding.

No background and bleed-through was observed for both the HN and the F protein for genotype A and genotype G for strip serum, flag only and secondary only antibodies (Figure 14A and Figure 15A). The F serum showed no binding to the HN and F proteins, indicating that there is no non-specific IgG binding (Figure 14B and Figure 15B). Serial dilution series of the positive serum 186-89 showed a decrease in signal with dilution, as expected (Figure 14C and Figure 15C). All the cells were expressing the HN and F protein based on the flag tag binding, proving that the stable cell line selection was complete, and the cells were expressing the glycoprotein.

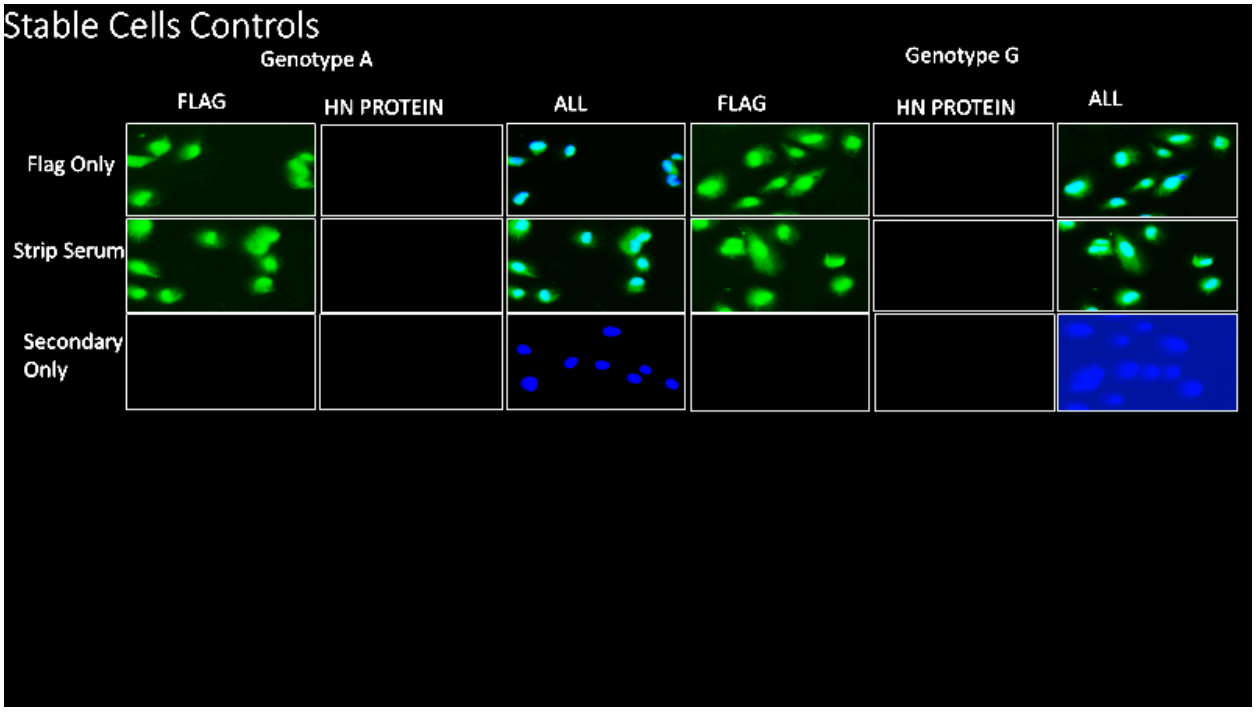


Figure 14A. IFA Lentivirus transduced stable cell lines for genotype A and G HN protein. Flag antibody (flag only) was used to control for bleed-through between channels. Strip serum was used at a 1:32 dilution; secondary antibody only was used as serum control. No non-specific binding was observed for the HN protein. 'All' contains DAPI, green channel (flag) and the red channel (HN Protein) all merged together.

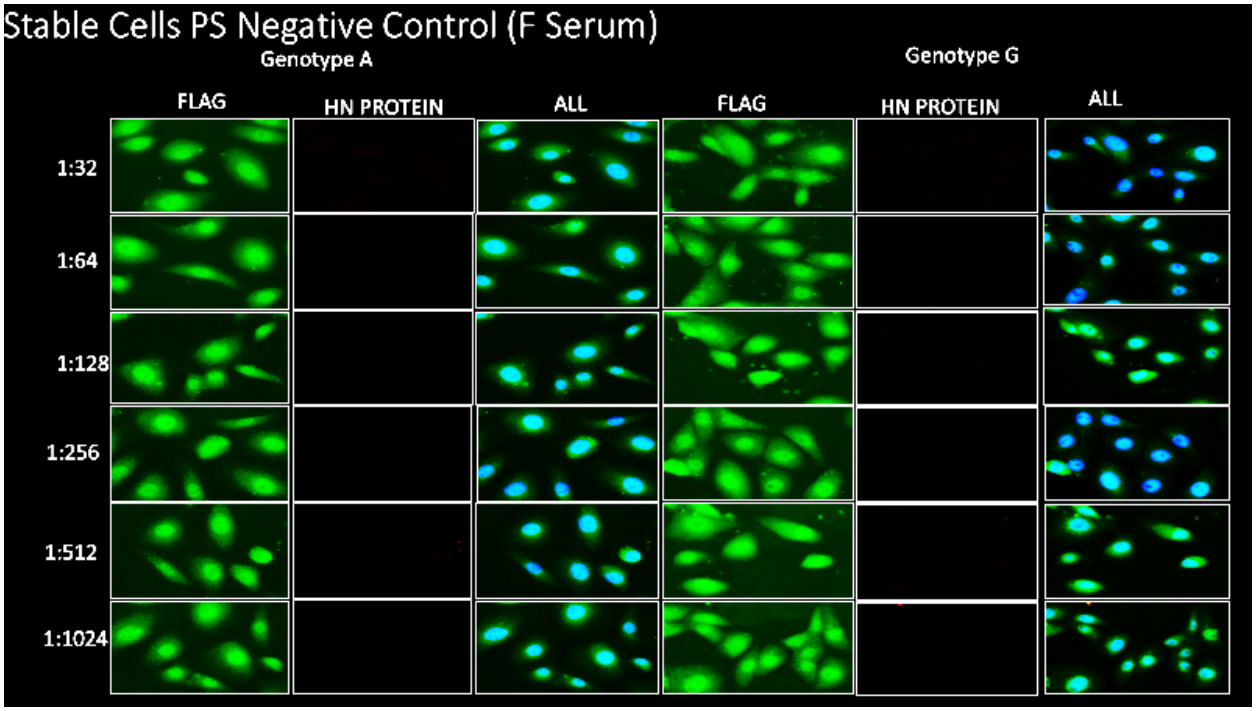


Figure 14B. IFA Lentivirus transduced stable cell lines for Patient Serum (PS) Negative Control (F serum) genotype A and G HN protein. Flag antibody (flag only) was used without anti-mumps serum to ensure HN protein expression. Serum F was used as negative control as it was negative for MuV antibody by ELISA and BioPlex 2200. 'All' contains DAPI, green channel (flag) and the red channel (HN Protein) all merged together.

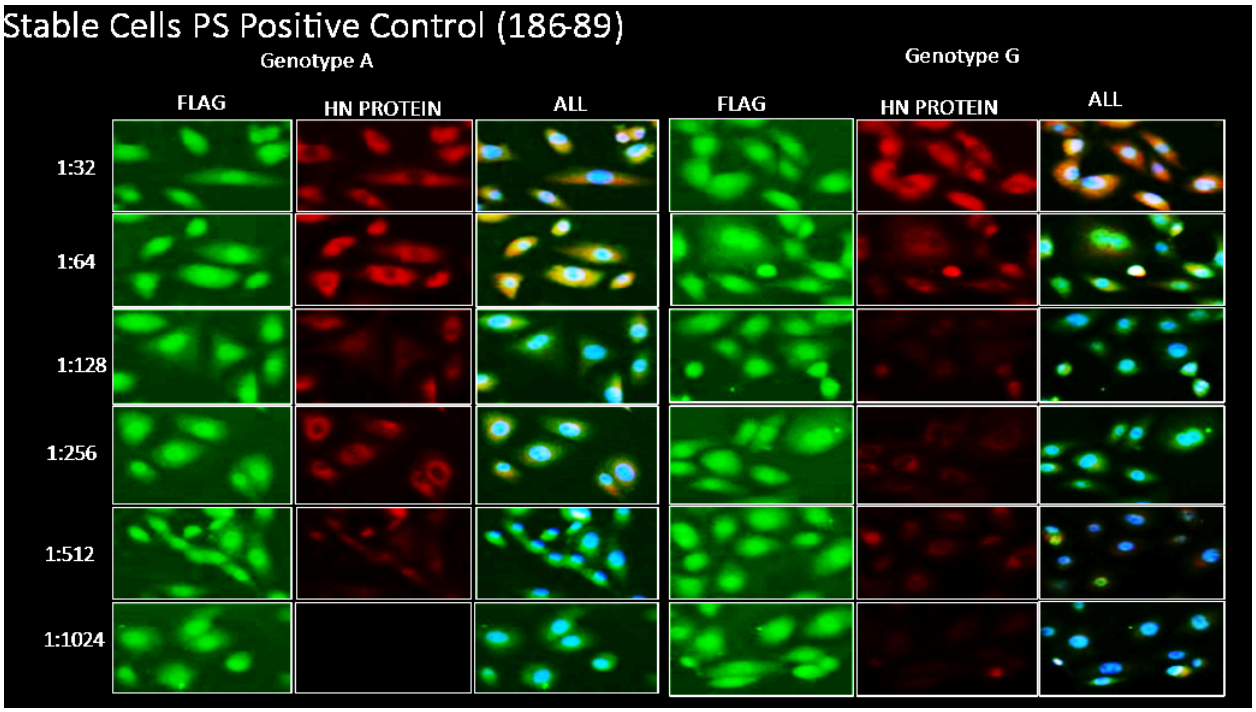


Figure 14C. IFA Lentivirus transduced stable cell lines for Patient Serum (PS) positive control 186-89) genotype A and G HN protein. Flag antibody (flag only) was used without anti-mumps serum to ensure F protein expression. Serum 186-89 was used as a positive control to test IFA staining as it shows good binding to the HN protein. 'All' contains DAPI, green channel (flag) and the red channel (HN Protein) all merged together.

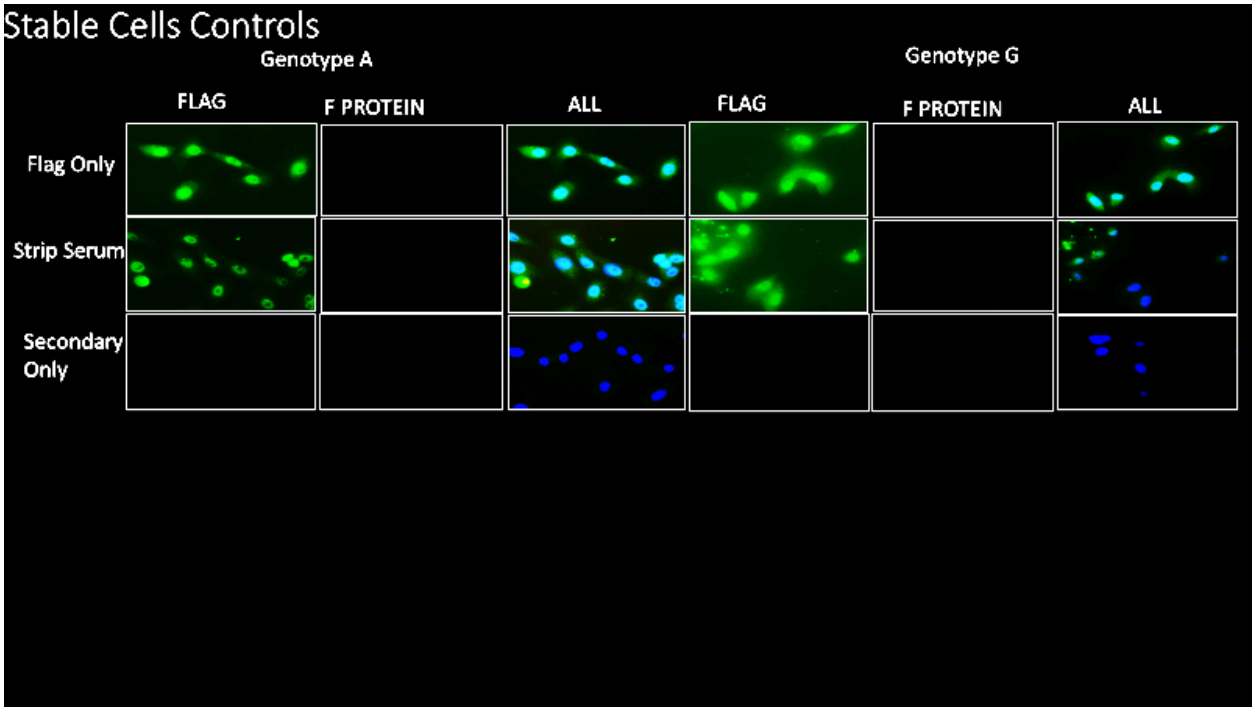


Figure 15A. IFA of Lentivirus transduced stable cell line for genotype A and genotype G F protein. Flag only was used to control for bleeding between channels. Strip serum was used at a 1:32 dilution; secondary only was used as serum control. No non-specific binding was observed for the F protein. 'All' contains DAPI, green channel (flag) and the red channel (HN Protein) all merged together.

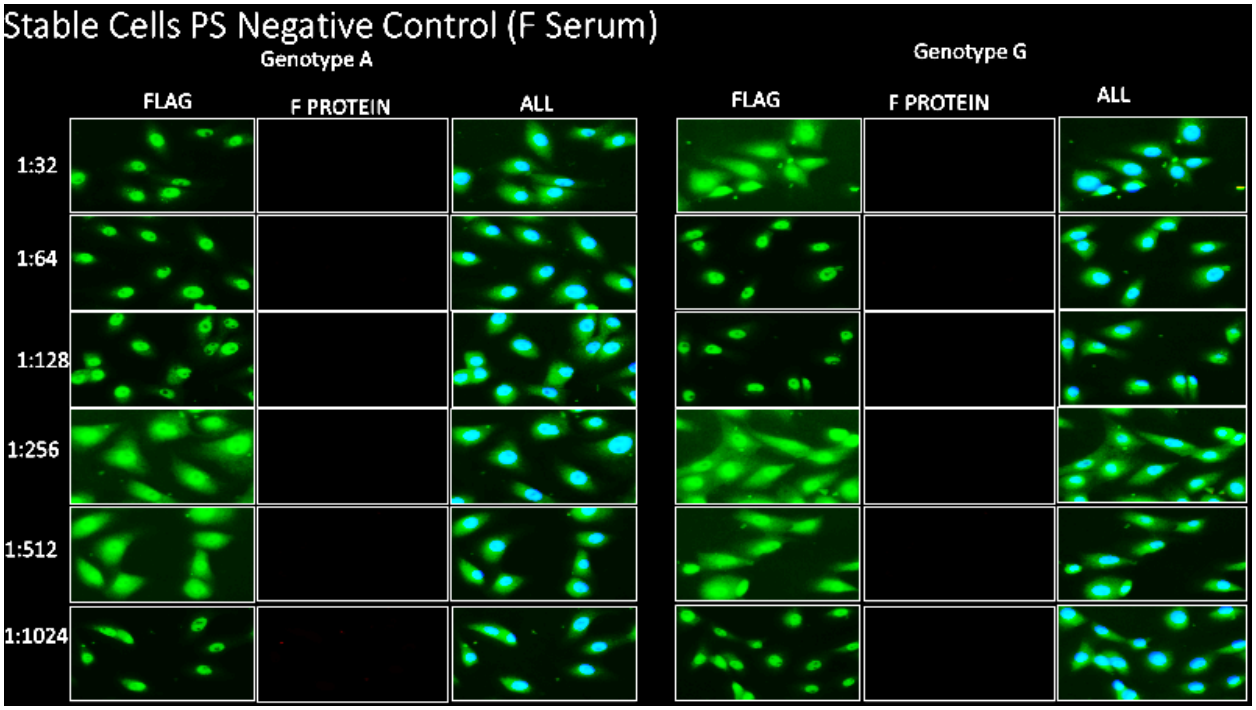


Figure 15B. IFA of Lentivirus transduced stable cell line for Patient Serum (PS) negative control F serum) genotype A and genotype G F protein. Flag antibody (flag only) was used without anti-mumps serum to ensure F protein expression. Serum F was used as a serum negative control as it was negative for MuV antibody by ELISA and BioPlex 2200. 'All' contains DAPI, green channel (flag) and the red channel (HN Protein) all merged together.

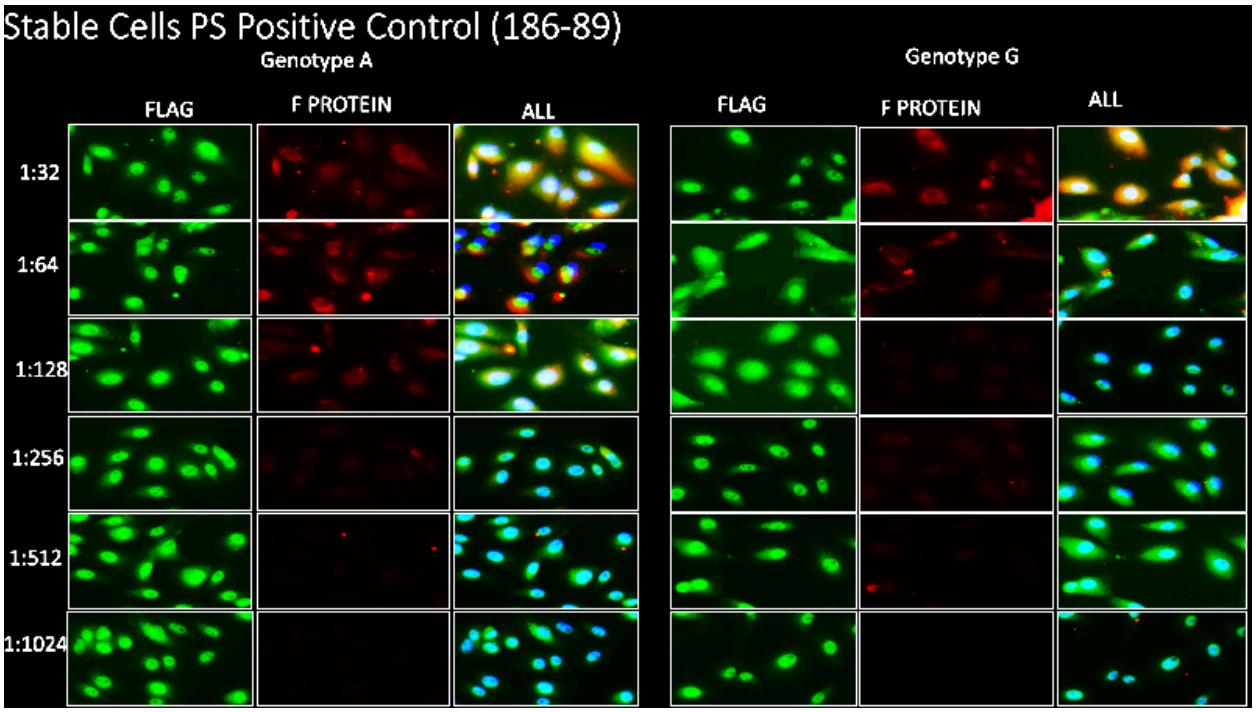


Figure 15C. IFA Lentivirus transduced stable cell line for Patient Serum (PS) positive control

186-89) genotype A and genotype G F protein. Flag antibody (flag only) was used without anti-mumps serum to ensure F protein expression. Serum 186-89 was used as a positive control to test IFA staining as it shows good binding to the F protein. 'All' contains DAPI, green channel (flag) and the red channel (HN Protein) all merged together.

In conclusion, these results show that the lentivirus transduced cell lines express the MuV HN and F glycoproteins, at a level that will allow the detection of anti-HN and anti-F antibodies using this IFA technique. The transfected cell lines cultured in the presence of the selection antibiotic Geneticin appear to stably express the HN and F proteins for at least 56 passages.

The first objective to create stable cell lines expressing the MuV glycoproteins for genotype A and genotype G was achieved.

4. Results: Antibody Cross Reactivity Between MuV Genotype A and Genotype G Using Stable Cell Lines

4.1 IFA assay to determine antibody cross-reactivity in MuV genotype in Patient Serum

IFA is being used to detect the presence of antibodies and their ability to react with different antigens, form immune complexes, and it allows determination of the localization of the proteins within the cell (107). It enables us to conserve the natural structure of the protein, and proteins with low expression can be detected (107). For example, IFA using limiting dilution series has been used as gold standard for EBV diagnostic serology, to obtain quantitative results of the antibody titers against specific viral antigens (108-109). IFA is a low throughput assay, but it is very sensitive and independent of variability of antigen expression, as detailed above (Section 1.5).

Table 12. List of Primary Antibody and Patient Sera for Immunofluorescence Microscopy.

Antibody/Patient Serum	Use	Number of Samples	Dilution Series
Flag*	Positive Control	N/A	1:300
Strip Serum	Negative Control	N/A	1:32
F Serum	Negative Control	N/A	1:32-1:4012
186-89	Positive Control	N/A	1:32-1:4012
Mumps Vaccinated-Only (V)**	Test Serum	19	1:32-4012
Mumps Vaccinated-Infected (CB)**	Test Serum	10	1:32-4012
Mumps Infected-Only (I)**	Test Serum	9	1:32-4012

*Flag tag was tested with every serum and was imaged in the green channel, whereas the protein of interest (HN or F) was in the red channel. **Test Cadham Provincial Laboratory provided Test Patient serum antibody and was used with 2-fold dilutions from 1:32 to 1:4012.

IFA was used to determine antibody binding differences between MuV genotype A and genotype G glycoproteins. Limiting dilution series was used to determine the serum titer endpoint. Two-fold serial dilutions of the patient sera were performed from 1:32 to 1: 4012. The reciprocal of the last dilution at which a signal was observed was considered the serum IgG titer. DAPI was used to stain the nuclei, and flag antibody was used to confirm the protein expression. Serum F was used as a negative control for background, and serum 186-89 was used as a positive control to ensure the staining was successful. The test serum results were always compared to the negative and positive control, and flag tag images were taken to prove the protein expression (Appendix). 19 samples were available for the vaccinated-only group, 9 for the infected-only group, and 10 for the vaccinated-infected groups (Table 12). Mumps vaccinated-only included individuals who had been vaccinated with the mumps vaccine and had no history of mumps infection. Mumps vaccinated-infected only group included individuals who had been vaccinated for mumps and infected with MuV genotype G strains. The mumps infected-only group included individuals who had been infected with MuV genotype G strains and had not received the mumps vaccine. A representative image for each of the group's

Vaccine (V) (Figure 16), Infected (I) (Figure 17), and vaccinated-infected (CB) (Figure 18) for the stable cells expressing the F protein is shown. The negative control showed no background, and positive control confirmed that the staining procedure worked well. Limiting dilutions series showed a progressive decrease in signal in all groups, and the last dilution at which a signal was observed was noted as the titer (Table 13 and 14). For the vaccine group representative image, the serum titer for genotype A titer was 2048, and the genotype G titer was 512. For the infected group representative image, the serum titer genotype A titer was 256, and the genotype G titer was 2048. The vaccinated-infected group representative image (CB) the serum titer was 2048 for both genotype A and genotype G. The complete set of IFA images for cells expressing the F and the HN proteins is shown in the appendix.

Titer was scored for all the samples by 3 independent evaluators. The geometric mean titer was calculated to examine antibody binding between genotype A and genotype G.

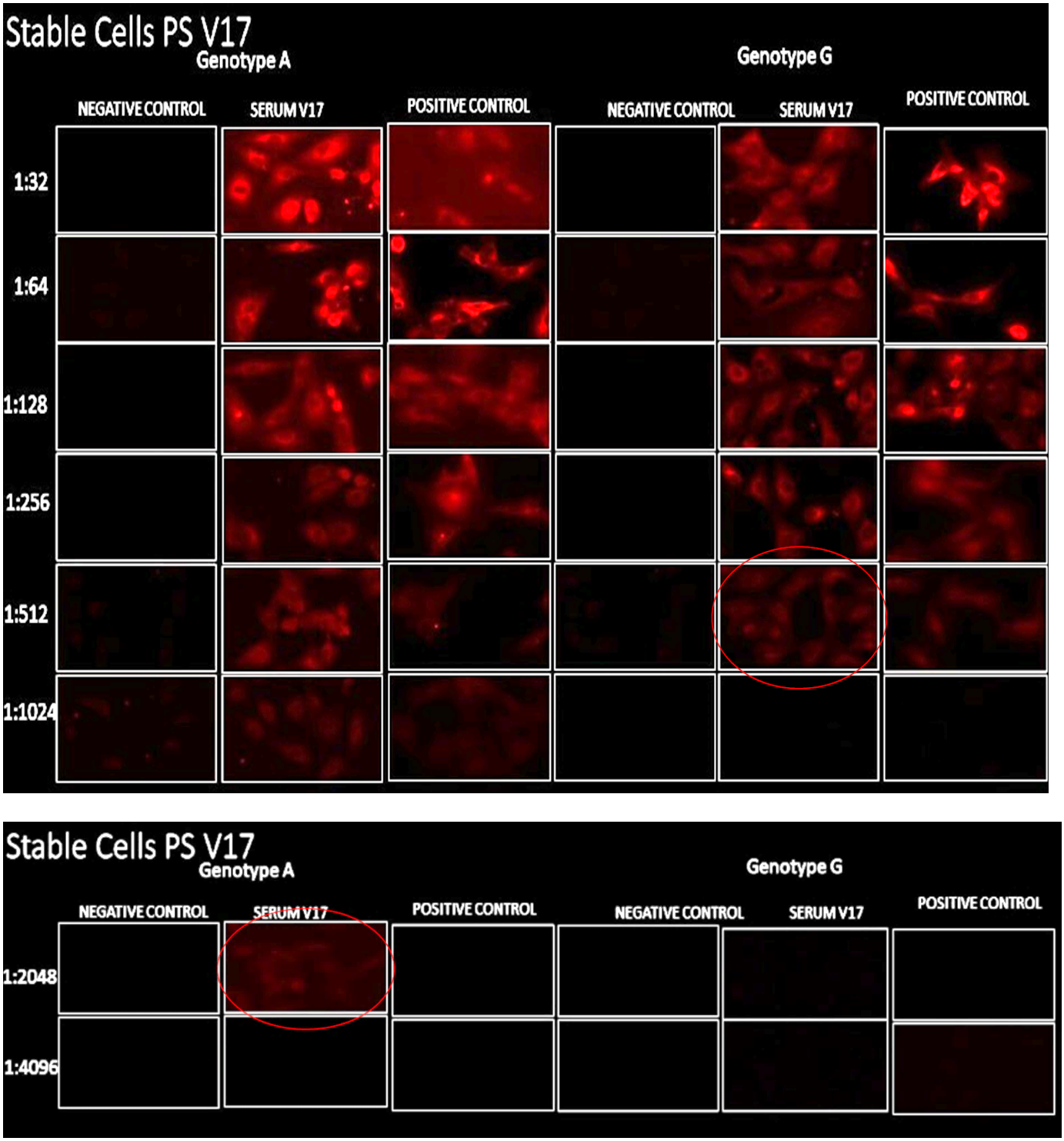
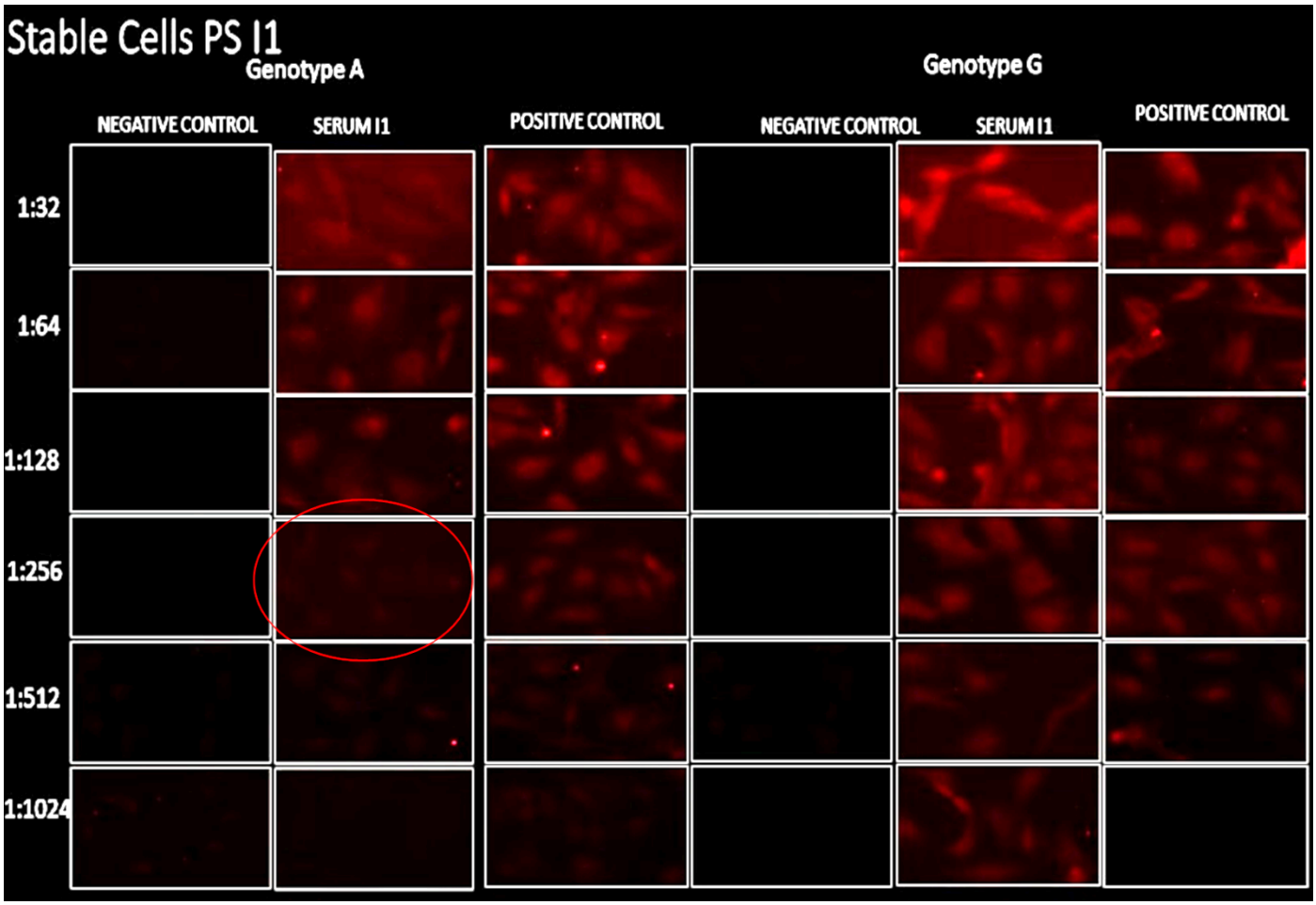


Figure 16.Limiting dilution of vaccine serum sample#17 in F protein expressing cells. The MuV negative F serum was used as a negative control for the background. Serum 186-89 was used as a positive control to show that the staining procedure worked well. The serum titer for genotype A was 2048, and for genotype G was 512 (red circles).


Stable Cells PS I1
Genotype A
Genotype G
NEGATIVE CONTROL
SERUM I1
POSITIVE CONTROL
NEGATIVE CONTROL
SERUM I1
POSITIVE CONTROL
1:32
1:64
1:128
1:256
1:512
1:1024

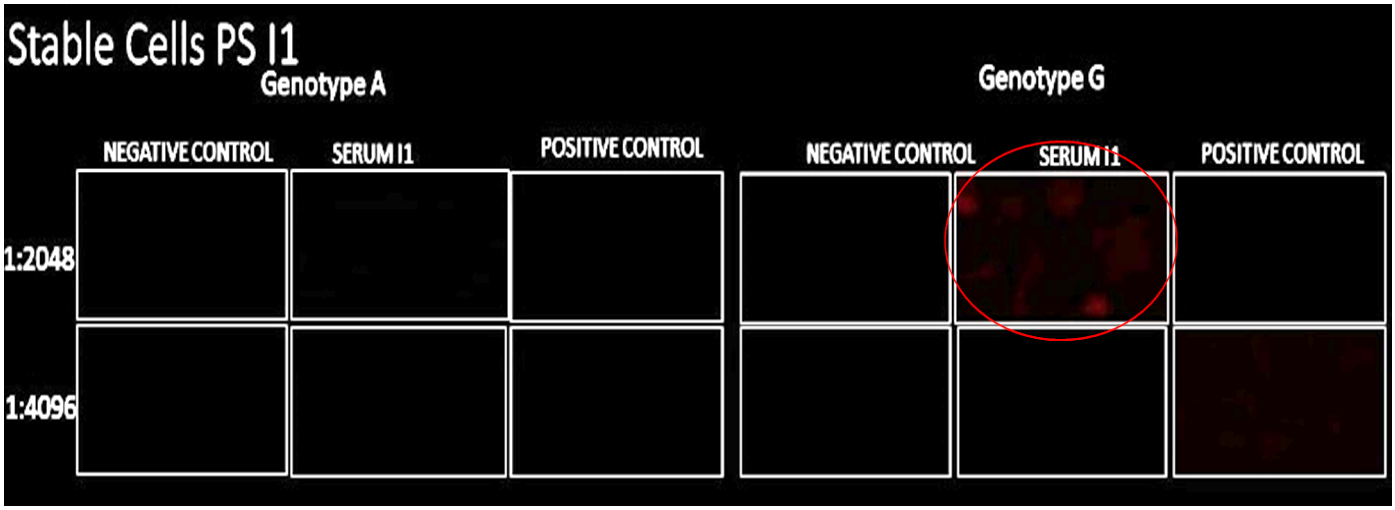


Figure 17. Limiting dilution of Infected serum sample#1 in F protein expressing cells. The MuV negative F serum was used as a negative control for the background. Serum 186-89 was used as a positive control to show that the staining procedure worked well. The serum titer for genotype A was 256, and for genotype G was 2048 (red circles).

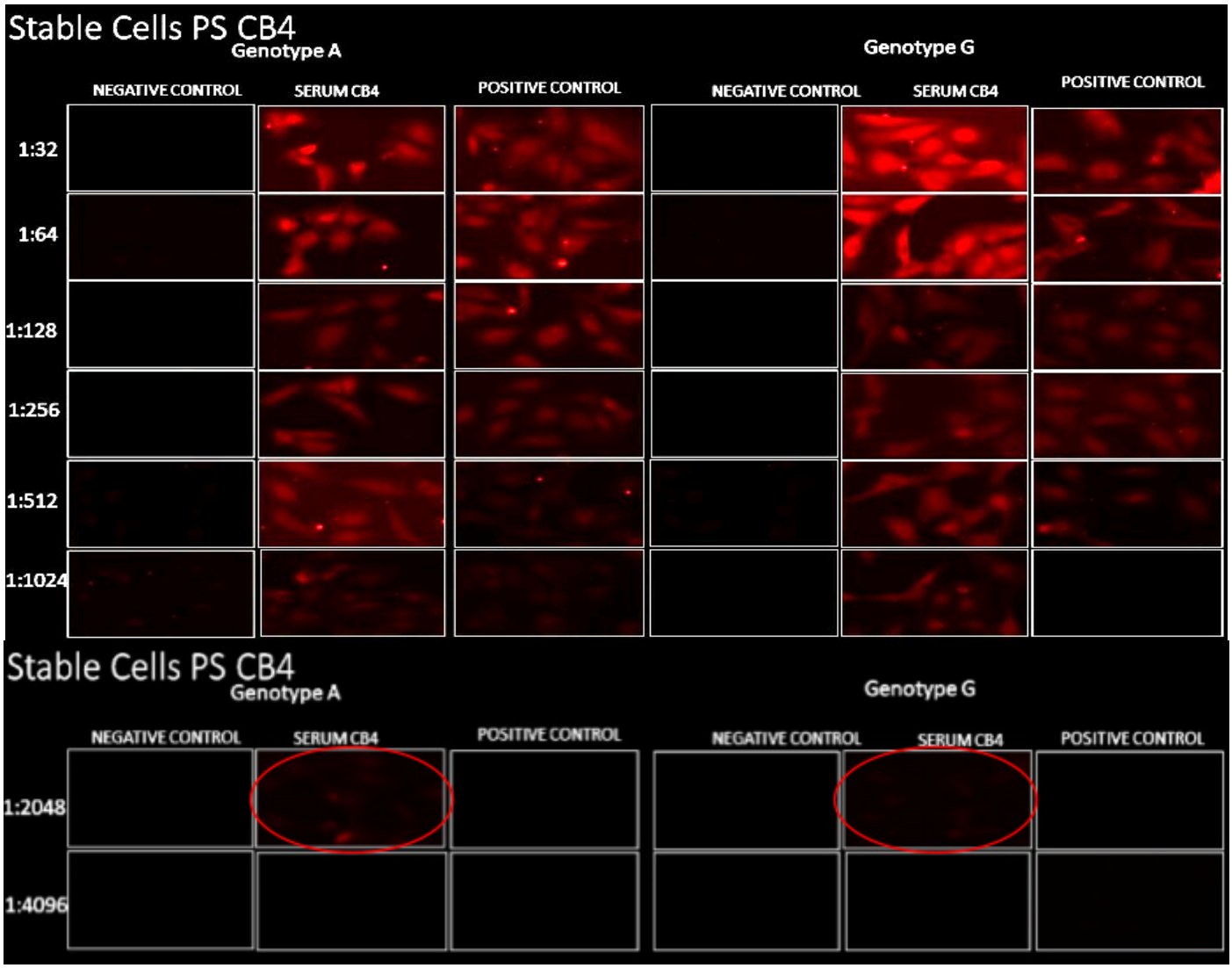


Figure 18. Limiting dilution of Vaccinated-Infected (Both CB) serum sample#4 in F protein expressing cells. The MuV negative F serum was used as a negative control for the background. Serum 186-89 was used as a positive control to show that the staining procedure worked well. The serum titer for genotype A and genotype G was 2048 (red circles).

The serum titer for all the groups vaccinated-only, infected-only, and vaccinated-infected can be found in the HN and F protein tables 13 and 14.

Table 13. Serum titer levels for MuV HN protein.

Sample Number	Vaccinated		Infected		Vaccinated-Infected	
	A	G	A	G	A	G
1	2048	512	512	1024	2048	2048
2	2048	1024	512	2048	2048	2048
3	512	128	256	512	2048	2048
4	2048	1024	1024	2048	2048	2048
5	2048	256	2048	2048	2048	2048
6	512	256	512	2048	2048	2048
7	512	256	1024	2048	2048	2048
8	512	128	1024	2048	2048	2048
9	2048	256	512	1024	2048	2048
10	512	512			2048	2048
11	512	256				
12	512	128				
13	2048	512				
14	256	128				
15	256	128				
16	64	32				
17	256	128				
18	256	32				
19	256	256				

Table 14. Serum titer levels for MuV F protein.

Sample Number	Vaccinated		Infected		Vaccinated-Infected	
	A	G	A	G	A	G
1	512	128	256	2048	2048	2048
2	256	256	256	2048	2048	2048
3	256	256	512	512	2048	2048
4	1024	512	64	1024	2048	2048
5	64	64	512	2048	512	2048
6	512	128	512	1024	2048	2048
7	256	128	512	2048	2048	2048
8	512	256	128	2048	2048	2048
9	1024	512	1024	2048	512	2048
10	2048	512			512	1024
11	1024	512				
12	2048	1024				
13	2048	1024				

14	1024	512				
15	1024	1024				
16	1024	1024				
17	2048	512				
18	2048	512				
19	1024	512				

In conclusion, the IFA with limiting dilution series did not show non-specific antibody binding in the negative controls (serum F, strip serum and secondary only). The positive control (serum 186-89) always showed a strong signal and a linear decrease in signal along the dilution series. Flag tag and the HN or F protein seemed to co-localize as expected.

4.2 Geometric Mean Titers and Titer Ratios:

Statistical analysis was performed by calculating the geometric mean titer (GMT) for the different sample groups to determine if there are significant differences between anti-A and anti-G antibodies. Different individuals will have a varying response to mumps; some individuals will exhibit a low immune response, while others will show a high immune response. Hence, titer data are not normally distributed, and calculating the arithmetical mean will be positively skewed towards higher titer values (110). Therefore, the geometric mean titer was calculated as it uses a logarithmic scale resulting in a bell-shaped distribution to control for high-end titer values (111). The GMT is the n^{th} root of the product of all the sample values (n) (111).

Equation 2: Geometric Mean = GMT = $(x_1 \cdot x_2 \cdot \ldots \cdot x_n)^{1/n}$

The GMT was calculated for all the 6 groups by using individual antibody titers for the HN and F proteins shown in Table 13 and 14. For the HN protein antibodies, the GMT for genotype A was 592, four times as that of genotype G, which was 213 for vaccinated-only

individuals (Figure 19). For infected-only individuals, the genotype A titer was 645, half as that of genotype G, which was 1505(Figure 19). In vaccinated-infected individuals, the GMT was 2048 for genotype A and 2048 for genotype G, with no significant difference in titer was observed (Figure 19). The GMT for genotype G in the vaccinated-only group was 213, seven times lower than that of the infected group, which was 1505, and nine times lower for the vaccinated-infected groups, which was 2048(Figure 19).

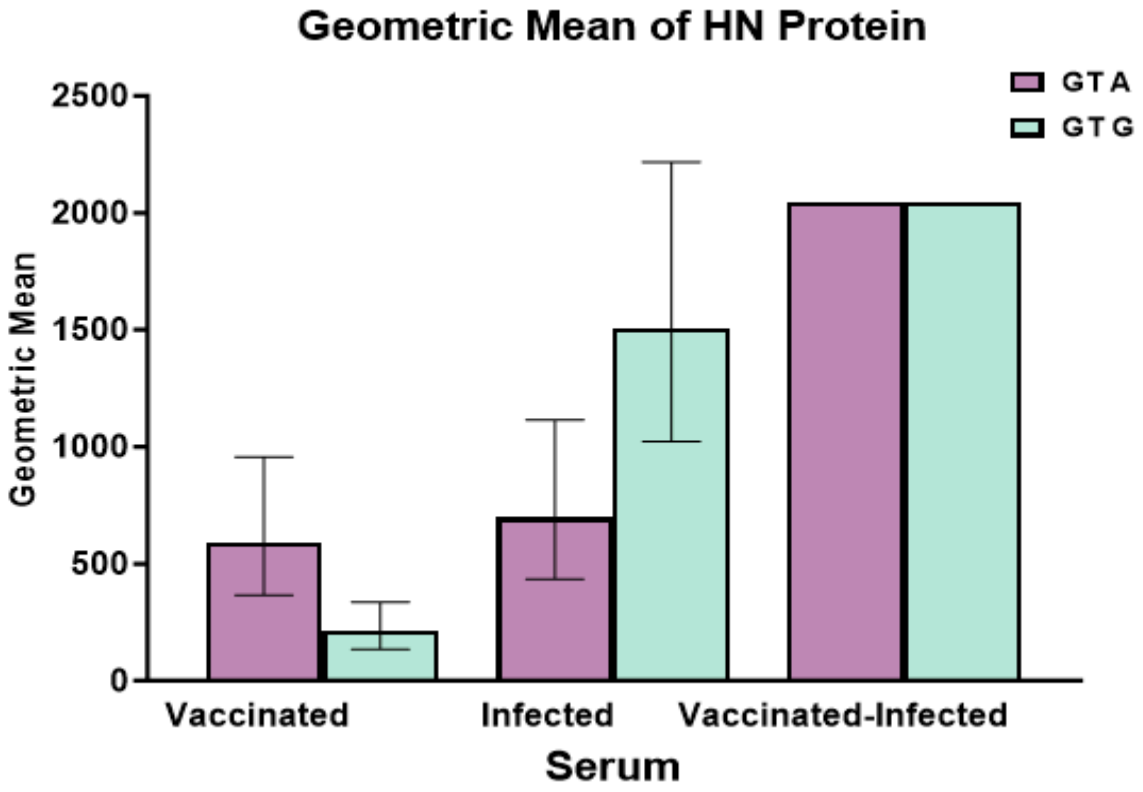


Figure 19. Geometric Mean Titer of HN protein. The vaccine group includes individuals vaccinated for mumps. The infected group includes individuals who did not receive the vaccine for mumps and had been infected with genotype G. Vaccinated-infected group includes individuals who had been vaccinated and infected with mumps. The GMT for genotype G in the vaccine group was significantly lower than compared to genotype G in the infected and both groups. The error bars represent the 95% confidence interval.

Similar results were obtained for the F protein. The GMT for genotype A was 765, twice that of genotype G, which was 369, for vaccinated-only individuals (Figure 20). For the infected-only individuals, the genotype type A titer was 348, a quarter as that of genotype G, which was 1505. In vaccinated-infected individuals, the GMT was 1351 for genotype A and 1910 for genotype G, with no significant difference in titer was observed (Figure 20). The only significant difference in titer, based on 95% confidence interval, was observed in the F protein among infected-only individuals as antibody titer against genotype A was a quarter as that of genotype G (Figure 20). In the vaccinated group, the GMT for genotype G was four times lower than that of the infected group, and six times lower than vaccinated-infected groups (Figure 20). One of the study's limitations is that the sample number tested for each serum group was low. An increased number of samples will allow for a better assessment of the results and the assay.

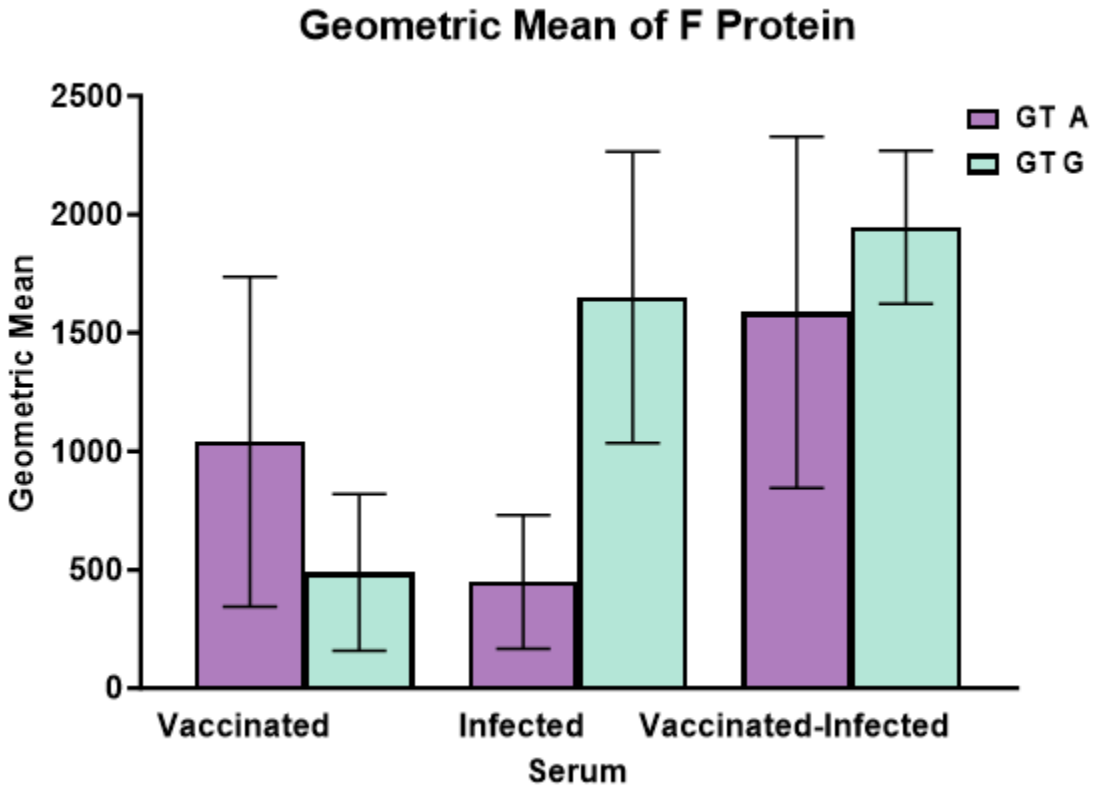


Figure 20. Geometric Mean Titer of F protein. The vaccine group includes individuals vaccinated for mumps. The infected group includes individuals who did not receive the vaccine for mumps and had been infected with genotype G. Vaccinated-infected group includes individuals who had been vaccinated and infected with mumps. The infected group, genotype A, had a significantly lower GMT than genotype G. The GMT for genotype G in the vaccine group was significantly lower than compared to genotype G in the infected and both groups**.** The error bars represent the 95% confidence interval.

Since variations in absolute titer among individuals and between groups increase the variance of these samples, the ratios between titers against genotype A and genotype G, the was calculated for each serum. The arithmetic mean within each group of patients was then calculated. The arithmetic mean was chosen as the titer ratios follow a normal distribution, and there is no bias towards higher end values. Calculating the A/G titer ratio for each serum normalizes the results for the considerable individual differences in antibody titer. A titer ratio

of one indicates no difference between the two genotypes, while a titer ratio of more than one implies genotype A titer is higher than genotype G, and titer ratio less than one implies genotype A titer is lower than that of genotype G. For the HN protein, the vaccinated-infected group had a mean titer ratio of one meaning no differences between genotype A and genotype G (Figure 21). In contrast, for the vaccinated-only group, the mean titer ratio was 3.68, and for the infected-only group, the mean titer ratio was 0.43 (Figure 21). 95% confidence intervals were calculated for the 3 groups, and statistical differences were observed.

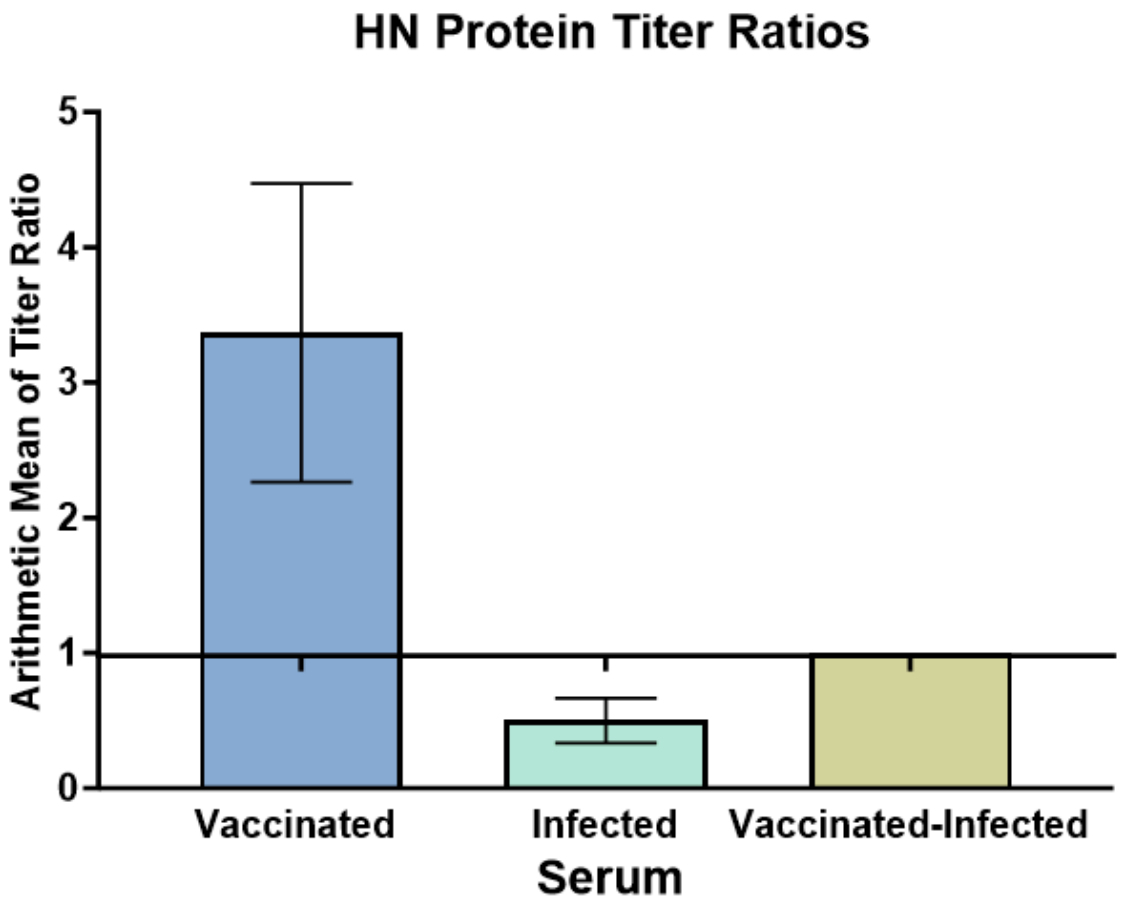


Figure 21. Arithmetic Mean Titer of HN Protein Titer Ratios. Titer ratios were calculated by dividing the genotype A titer by genotype G (genotype A/genotype G). The vaccinated group includes individuals vaccinated for mumps. The infected group includes individuals who did not

receive the vaccine for mumps and had been infected with genotype G. Vaccinated-infected group includes individuals who had been vaccinated and infected with mumps. The error bars indicate the 95% confidence interval.

Similarly, titer ratios and the arithmetic mean were calculated for the F protein. The vaccinated-infected group had a titer ratio of 0.80. In contrast, the vaccinated-only group had a mean titer ratio of 2.3, while the infected-only group had a titer of 0.33 (Figure 22). Significant statistical difference from a ratio of 1 was observed for the vaccinated and the infected groups, while there was no significant difference for the vaccinate-infected group. Significant differences were also observed between the vaccinated group against the vaccinated-infected group). No significant differences were observed between the infected group against the vaccinated-infected group (Figure 22). These results show that there are some differences in antibody cross-reactivity between vaccination and infection.

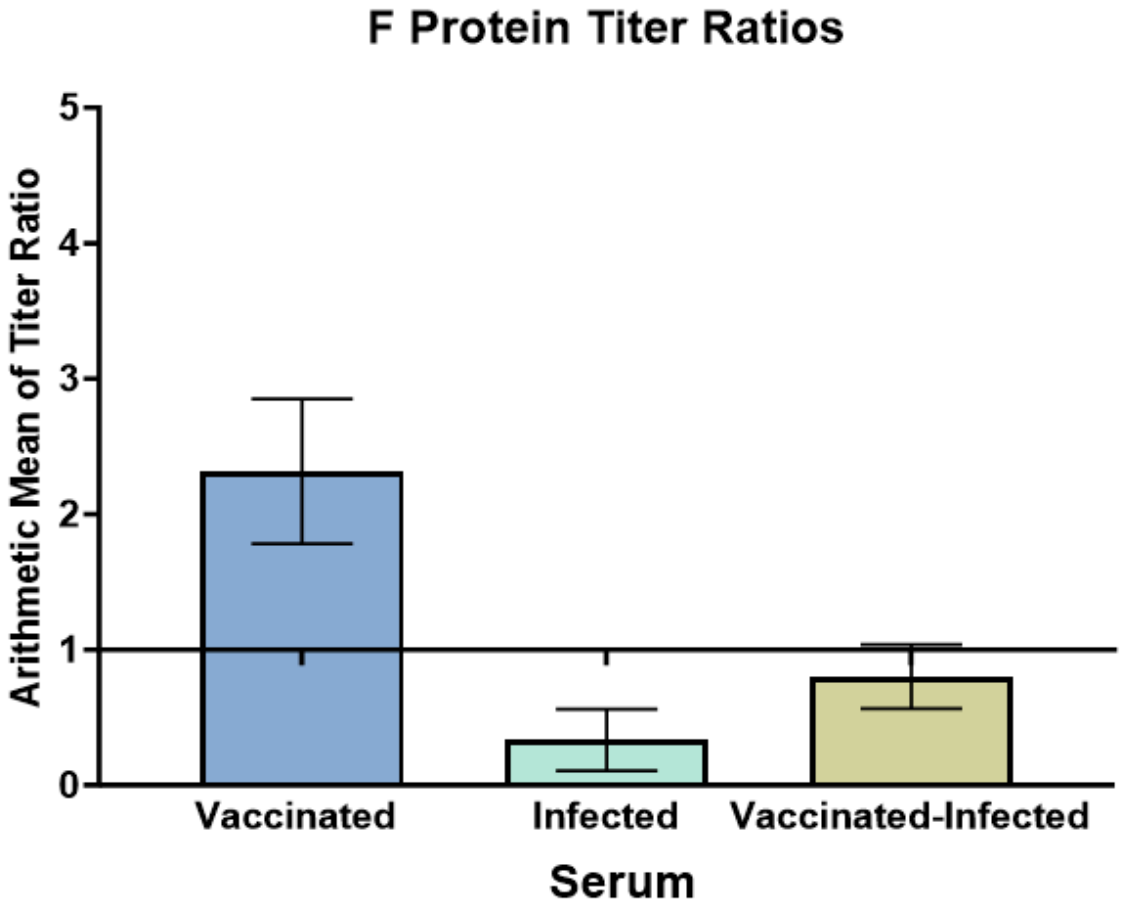


Figure 22. Geometric Mean Titer of F protein Titer Ratios. Titer ratios were calculated by dividing the genotype A titer by genotype G (genotype A/genotype G). The vaccinated group includes individuals vaccinated for mumps. The infected group includes individuals who did not receive the vaccine for mumps and had been infected with genotype G. Vaccinated-infected group includes individuals who had been vaccinated and infected with mumps. The error bars indicate the 95% confidence interval.

5. Discussion

5.1 Amino Acid Variations in HN and F Glycoproteins

Two doses of the MuV genotype A vaccine have reduced the incidence rate of mumps by 99% (6). However, several outbreaks of MuV genotype G, the circulating strain, have been observed in Canada since 2006 (39). Amino acid variations in genotype G, compared to genotype A, combined with waning vaccine immunity, could result in reduced recognition of mumps antigens by neutralizing antibodies, resulting in the population being susceptible to MuV. Amino acid difference between genotype A VE-12343 strain and MuV genotype G CDC strain was compared for the HN and F proteins, the MuV envelope glycoproteins. Thirty-one amino acid differences were observed between genotype A and genotype G in the HN protein. Fifteen of these aa variations have been reported in the literature for genotype G and sixteen aa variations are unique, meaning they have not been reported in the literature for genotype G. Among the sixteen unique amino acids, five aa variations (aa25, aa121, aa122, aa123, and aa442) have been reported in genotypes F, H and I (75, 112). Genotypes F, H and I are known to be circulating in Asia and South America (39, 75). Eleven aa variations are unique and have not been reported in other genotypes. The thirty-one amino acid variations found in genotype A and genotype G resulted in changes in N-glycosylation sites (aa12, aa464), membrane anchorage domain (aa37, aa44, aa56, aa80, aa81), and predicted B and T cell epitopes (aa20, aa81, aa279, aa287, aa356), and few variations have been found in predicted neutralization escape sites (aa121, aa122, aa123, aa375, aa399, aa442) (40, 48, 61). T-cell epitopes prediction identified several peptides which were expressed on antigen-presenting cells and used to identify HLA binding by monoclonal antibodies (62). It was observed that amino acid variations

aa80, aa81, aa279 and aa287 have been shown to evade neutralization, reduce HLA binding, and result in a mismatch of CD4 and CD8 T-cell response (26, 60). These variations are present in the genotype G strains and hence, could result in a reduced T-cell response compared to genotype A. The amino acid variations aa356 and aa464 present in genotype G strains have been associated with neurovirulence, but the exact cause of neurovirulence remains to be elucidated (48, 60). Variations at I279T, I287V, and E356D, resulted in changes in B cell epitope, and variation at H464N added a glycosylation site resulted in structural changes from large and uncharged amino acids being replaced by small and charged amino acids (48). These changes result in structural changes, increased hydrogen bonds, and the addition of charged amino acids that can potentially increase virus infectivity and reduce recognition of neutralizing antibodies (26, 48, 62).

Twenty-eight amino acid differences were observed in the F protein between genotype A and genotype G. Eight aa have been reported in literature and were found in genotype G strains. Twenty aa variations are unique, and of these four aa (aa49, aa318, aa345 and aa409) have been found in genotypes A, (SBL-1, Enders, and Kilham strains), C, and D strains (112-113). These genotype A and genotypes C and D strains are mostly found circulating in Europe and the Middle East. For the F protein, eight aa variations between genotype A and genotype G results in changes in the signal peptide region (aa2, aa3, aa4, aa5, aa7, aa11, aa13, and aa16) and 3 aa variations occurred in the carboxy-terminal (aa488, aa489, and aa49840 (37, 48, 112-113). Variations in the signal peptide region and carboxy-terminal could affect protein binding, cleavage and dimerization, but further studies are needed to understand the effect of variations on F protein.

Hence, antigenic variations and differences in viral epitopes between the vaccine strain and circulating strain may play an essential role in the resurgence of mumps due to reduced recognition of neutralizing antibodies and/or T cell response.

5.2 Lentiviral Vector Cloning

To assess the feasibility of producing cell lines expressing the MuV glycoproteins, HN and F genes from genotype A and genotype G were cloned into lentivirus vectors under the control of the CMV or the EF1α promoters, and transient transfections in 293TN cells were performed A limitations of the study was the unavailability of monoclonal antibody for mumps HN and F proteins; hence a flag tag was added to the C-terminus and anti-flag antibody was used for protein detection. Anti-flag antibodies showed no protein expression for both the CMV and the EF1α vectors. The lack of HN protein expression may have been due to low transfection efficiency or a reduced cellular viability, although 293TN cells were used and are known for their high transfection efficiency (114). Viability results show no decrease in cellular viability and transfection efficiency was good based on GFP expression for the EF1α vector, hence, the lack of protein detection could be due to low protein expression. IFA was also used to confirm protein expression in transiently transfected cells as it is more sensitive than a Western Blot. For HN protein detection (positive control), an in-house MuV positive serum (186-89) control was used as it demonstrated high levels of neutralizing antibody by PRNT. The positive serum (186-89) showed very low protein expression for both the CMV and the EF1α vectors. It was hypothesized that low protein expression could be due to codon usage bias that the MuV virus prefers specific tRNA, which may be limited in 293TN cells, affecting protein translation;

therefore, codon optimization was performed for the HN gene and cloned into lentiviral vectors (115).

Transient transfection with the codon-optimised transcripts was then performed. Western Blot results showed good protein expression for the HN protein using the CMV vector for genotype A and genotype G. For the EF1α vector, expressing the genotype A HN protein showed good protein expression but for genotype G a second band was observed at 50 kDA. This could be due to non-specific cleavage of the HN protein or could be due to genotype G effects. Literature review showed no HN protein size difference between the HN protein for genotype A and genotype G so non-specific or genotype G effect is unlikely. Another possible reason for a nonspecific band or low GFP expression for genotype G could be an EF1α vector effect. The CMV vector does not show any nonspecific protein cleavage; this is only seen in the EF1α vector. The EF1α vector has a T2A sequence (EGRGSLLTCGDVEE**NPGP**), which is a self-cleaving peptide to separate the protein of interest (MuV HN protein) from GFP (116). The T2A sequence induces ribosomal skipping during the translation of the protein, resulting in the generation of polyproteins from a single mRNA strand (116-117). The ribosome skipping occurs between the proline(P) and glycine(G), resulting in a lack of the peptide bond between the two acid acids (116-117). The HN protein for genotype G has an NSGP (aa440-444) sequence in the middle of the protein, which is absent in genotype A NYGQ (aa440-444). The presence of charged amino acids (serine) before the glycine (G) and proline (P) could result in ribosomal skipping, which is absent in genotype A. It was then hypothesized that the presence of a 50 kDa band could be due to the presence of T2A peptide in the EF1α vector and is only observed in genotype G because of the presence of charged amino acids before a GP sequence. Whatever

the reasons for the non-specific HN protein bands on the Western Blot, it was decided to use the CMV vector was used for the final lentivirus transformation, for both genotype A and genotype G.

IFA was performed on cells transfected with the CMV vectors to confirm protein expression for genotype A and genotype G. The mumps positive serum (serum 186-89) and flag tag was used to detect HN protein. IFA results showed a strong signal for the mumps serum and the flag antibodies used to detect HN protein and flag tag and they co-localized well, without any non-specific binding in negative controls. Since codon optimized CMV vector HN clones (genotype A and genotype G) worked well, CMV vector F protein clones were also generated. Western Blot and IFA were performed and they showed good protein expression for the F protein.

5.3 Stable Cell Lines

Lentivirus particles were produced and transduced in Vero cells to express the HN and F protein. One of the limitations of lentivirus stable cell line generation is random insertion within the genome, which could lead to disruption of normal cell proliferation and regulation (116). Hence, the cells were passaged for a few weeks for complete selection, and cellular morphology and growth properties were compared to non-transduced cells to ensure no changes in cell proliferation rate. IFA was performed to confirm protein expression in stable cell lines. A limiting dilution series was performed to obtain titer, which is the reciprocal last dilution at which a signal is observed. For positive control, MuV 186-89, an in-house MuV serum, was used, and the HN and F protein signal seemed to co-localize well with the flag tag. Limiting dilution series

showed a decrease in signal, proving that the IFA protocol was working and could be used to test patient serum.

5.4 Antibody Cross – Reactivity Using Stable cells lines.

IFA was used to study antibody binding differences between genotype A and genotype G. A Limiting dilution series was used to determine the titer endpoint, and titer was recorded for vaccinated individuals, infected individuals, and vaccinated-infected individuals. Geometric mean titer was used to compare titer differences between the different groups, as immune response to vaccination and infection varies between individuals depending on their health and environment (118-119). The antibody response to mumps is expected to vary considerably between individuals and therefore the titers will not be normally distributed, with higher titers skewing the distribution. Therefore, the geometric mean was used as it used a logarithmic scale to normalize positively skewed results and allow for titer comparison (110).

The results showed that in vaccinated individuals titers against genotype A were more than double that for genotype G (ratio of about 3.3 for the HN protein and 2.3 for the F protein), whereas in unvaccinated individuals that were infected with mumps the ratio was reversed (0.4 for the HN protein and 0.3 for the F protein). Vaccinated individuals who were also infected with mumps had higher IFA titers for both genotype A and genotype G antibodies, with a ratio not significantly different from 1.

These results strongly support the hypothesis that there is not a full cross-reactivity between vaccine (genotype A) and wild type genotype G infection and that vaccinated

individuals may not be fully protected against mumps reinfection with genotype G strains, especially when vaccine immunity wanes in young adults.

In previous literature, Rubin et al, (2008) compared serum obtained from children post-vaccination for viral neutralization by PRNT (70). They observed that the neutralization value (ND50) was twice as high for genotype A vaccine strains compared to genotype G outbreak strains (70). Santak et al, (2006) obtained sera from mice immunized with genotype A vaccine strains and genotype G Dutch strains, performed PRNT, and variations from the vaccine strains resulted in decreased neutralization capacity (61). The development and efficacy testing of the mumps genotype G recombinant genotype G vaccine found that in mice models, an increase in neutralizing titer for genotype G strains is observed for up to one-year post-vaccination (123). The study also showed the genotype G vaccine was effective in mice who had been immunized with two doses of MMR and MMRV vaccine, as they were able to develop an anti-G antibody response (123). The study shows that a lack of cross-reactivity exists between mumps genotype A and genotype G in mouse models, and a new vaccine is needed.

These studies are in agreement with these IFA results that there is a decrease in antibody titer for genotype G in vaccinated individuals. Antigenic variations between the MuV genotype A and genotype G glycoproteins could be the cause of a decrease in neutralizing titer observed in genotype G. There is no correlate of protection of MuV known. Therefore, it is difficult to conclude that the low titer observed for genotype in vaccinated individuals is still protective against MuV or leads individuals to becoming susceptible to MuV.

EIA-based assays are used to measure the total levels of antibodies, and they are commonly used to study vaccine immunogenicity, whereas PRNT measures the levels of neutralizing antibodies (43-44, 120-122). Studies looking at protective titers by PRNT and EIA-based assays have shown no correlation between neutralizing and total antibody titers. Unpublished results from our laboratory show that individuals with a high antibody titer for mumps by ELISA have shown low antibody titer by Bioplex 2200 or neutralization levels by PRNT. The lack of correlation in results could be because the different ELISA, Bioplex 2200, and PRNT use different virus strains, and mumps IgG response could be strain-specific rather than genotype specific. As the correlate of protection in assays and lack of correlation between assays, it makes it more challenging to conclude that the lower titer observed for vaccinated-only individuals for the HN and F protein genotype G by IFA leaves individuals susceptible to MuV.

Statistical significance was measured by calculating a 95% confidence interval for all the groups. Significant differences between genotype A and genotype G were only observed for the F protein-infected group. The F protein also had a higher GMT for all the groups compared to the HN protein. Although the GMT was higher for the F protein, some individuals had a higher titer against the HN protein than the F protein. Therefore, it can be concluded that the immune response varies from individual to individual, as some might have a higher antibody response against the HN protein, whereas others may have a higher response against the F protein. No literature exists on HN and F protein titer differences between individuals or if a higher immune response is generated for the MuV F protein than the HN protein.

Individuals who had not been vaccinated and infected (infected-only) had an GMT of 645 for genotype A, a similar titer to vaccinated-only individuals who had an GMT of 592 for genotype A for the HN protein. For the F protein, the infected-only group had a GMT of 348 for genotype A, whereas the vaccinated-only group had a titer of 765 for genotype G. Vaccinated individuals had a significantly lower genotype G titer than compared to infected individuals for both the HN and the F protein. A high mean titer was observed for the vaccinated-infected group, but no significant titer differences were observed in the group between genotype A and genotype G for the HN and the F protein.

A stronger immune response is generated during natural infection than compared to vaccination (120). Hence, a strong immune response is observed in individuals who have been infected, and an even higher response is observed in individuals who have been vaccinated-infected compared to individuals who have been vaccinated. Also, if serum samples for the infected group and vaccinated-infected group are collected during an active mumps infection, high levels of anamnestic antibody titer will be observed.

Also, the high titer observed in genotype A for the infected group and individuals vaccinated-infected could be due to the presence of common epitopes between the two genotypes and some degree of cross-reactivity. Infected individuals generate a strong antibody response against the common epitopes in the vaccine, resulting in an increased titer (122). In future studies, it would be important to test the pre-absorption and blocking of common epitopes to see if there are any antibody-binding differences between unique epitopes.

In conclusion, significant titer differences are observed between genotype A and genotype G for the HN and the F protein; however, more data is needed to determine if this leads to individual susceptibility to MuV. A limitation of the study is a small sample size was available for all the different groups. A larger sample size would allow us to conclude if the observed results are applicable to a population and if there indeed are differences between genotype A and genotype G. A larger sample size would increase the statistical power of the observed results and would provide validation of the results, that antigenic differences between the MuV glycoproteins result in insufficient cross-reactivity and thus a new vaccine may be needed.

6. Conclusion and Future Work

6.1 Conclusion

The goal of the project was to study if amino acid variations in genotype G, the circulating strain, result in reduced recognition of epitopes by neutralizing antibodies from vaccination, which is genotype A, making the population susceptible to circulating MuV strians.

The first objective was to establish stable cells expressing the MuV HN and F glycoproteins to study antibody cross-reactivity between genotype A and genotype G. Genotype A and genotype G CDC strain was used to create stable cell lines. Thirty-one aa variations for the HN protein and twenty-eight aa variations were observed between the two genotypes. During the project, I was able to create stable cell lines expressing the MuV HN and F glycoproteins using codon-optimized DNA fragments and a lentivirus system. This is the first time MuV HN and F protein stable cell lines have been created in Vero cells.

The second objective of the project was to determine antibody cross-reactivity between HN and F proteins for genotype A and genotype G. I was able to establish an IFA assay to study antibody binding using MuV serum and limiting dilution series. To study cross-reactivity antibody titer in vaccinated, infected, and vaccinated-infected individuals was determined by IFA to study titer differences between the group in genotype A and genotype G. Titer differences were observed between the different groups, but the only significant titer difference was found in genotype between the vaccine and the infected group, for the F protein significant titer difference was found between genotype A and genotype G in the infected group. There is no correlation of protection for MuV; hence, it is challenging to conclude whether these titer differences make individuals susceptible to MuV. However, titer ratio results show that differences in cross-reactivity exist between genotype A and genotype G vaccinated, infected, and vaccinated-infected groups for the HN and F protein, and a new genotype G mumps vaccine may be needed.

6.2 Future Work

The next step would be to confirm the result using a larger sample set for each of the groups to see if there are significant and established differences between genotype A and genotype G cross-reactivity. This will allow for better statistical power and better representation of the differences in the population level. It would also be informative to see if the addition of the pre-absorption step to block common epitopes results in differences in antibody binding between the genotypes. This will require further optimization of the IFA assay.

Another important study would be to assess if the difference in cross-reactivity is specific to genotype G CDC strain or observed in other genotype G circulating strains. Also, mutants expressing specific aa variations must be sought to see which variations are responsible for the selectivity of the immune response. It would be instructive to study if the cross-reactivity is genotype G specific or common to all non-A genotypes, such as the F genotype circulating in China. It would be interesting to study if this selectivity observed between genotype A and genotype G, also occurs in genotype F.

In addition, it would be ideal to have serum pre-vaccination and serum from one, two, and three doses post-vaccination to study if there is a rise in antibody titer after vaccination. Studies have reported similar titer levels in individuals who have had two doses versus individuals who have had three doses of vaccination. In many cases, adverse reactions have been reported to three doses of vaccination. Hence, it would be important to study if there are titer differences after different doses of vaccination. Waning immunity studies can also be designed for the established IFA by obtaining serum at various time points after vaccination and studying the statistical differences between the different age groups.

Animal models for MuV have major limitations; hence, it is a challenge to study the correlation of protection of MuV. It would be important to obtain serum pre and post-MuV outbreak to study titer differences against the neutralizing proteins and the total levels of antibody by IFA to see if there are significant differences between the titer and determine what levels of antibody titer are protective of MuV. Further studies would give us information to

determine if reduced levels of neutralizing antibodies leave individuals susceptible to MuV and if a genotype G vaccine is needed.

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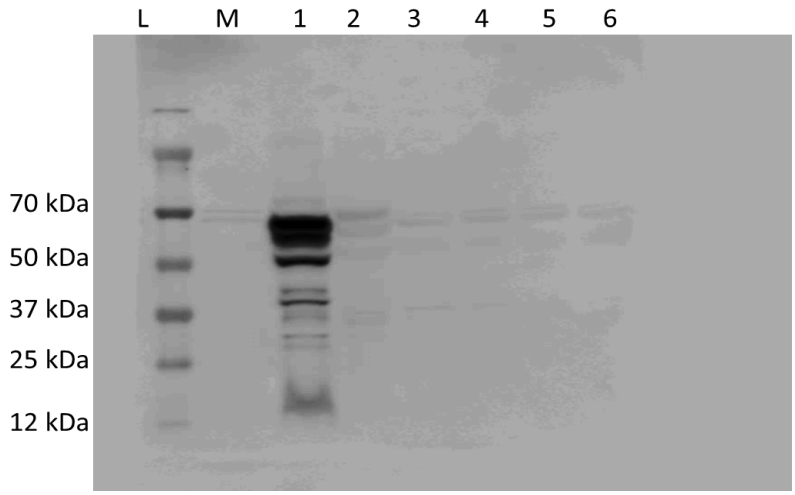
Appendix:

Western Blot results:



Labels	Samples
L	Ladder
M	Mock
1	Genotype A F Protein Transfected
2	Genotype G F Protein Transfected
3	Genotype A HN Protein Stable Cell Line
4	Genotype G HN Protein Stable Cell Line
5	Genotype A F Protein Stable Cell Line
6	Genotype G F Protein Stable Cell Line

Figure 1. Western Blot of mumps transfected with F protein clones and HN and F stable cell lines. M was non-transfected Vero cells. 293TN cells transiently transfected with genotype A and genotype G F protein clones showed strong protein expression for F protein transfected cells (1 and 2). Stable cell lines for HN and F proteins showed weak protein expression (3-6). Non-specific binding was in the blot as the flag antibody has lots of non-specific binding.

Appendix:

IFA Results of HN Protein

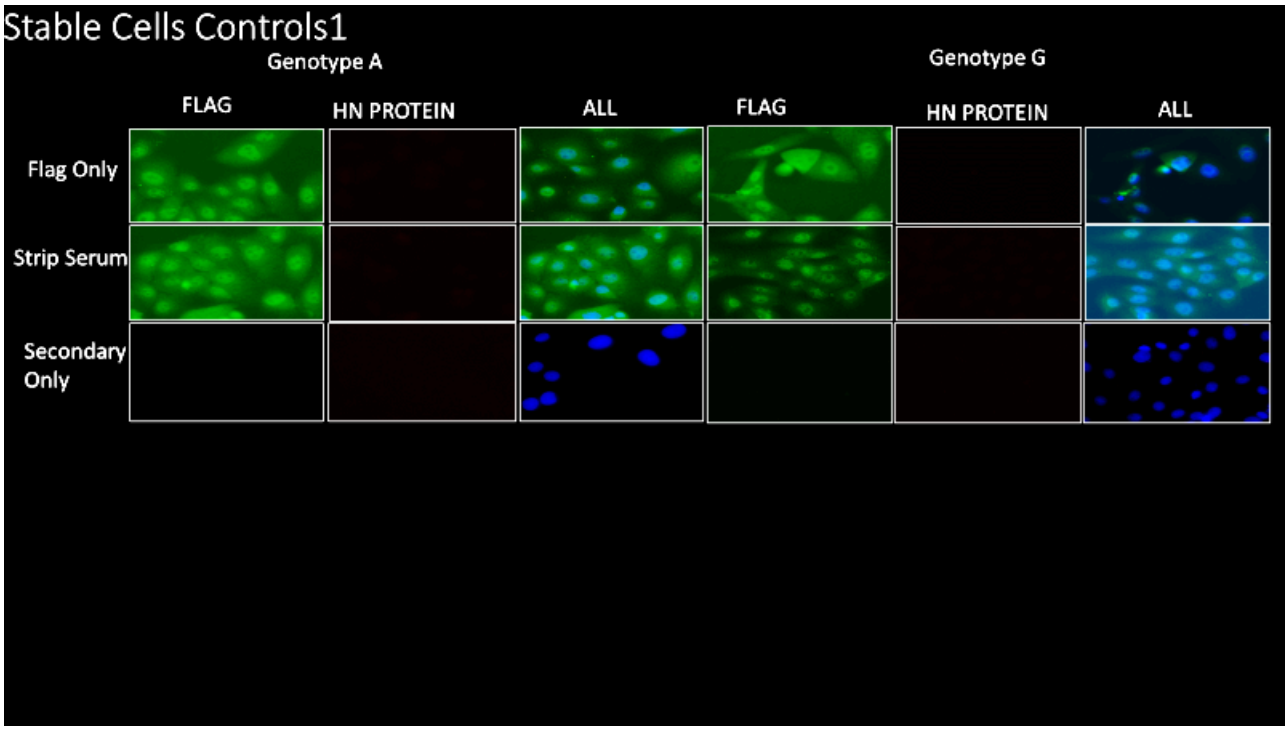


Figure A-1a. Stable cells ControlBatch#1.

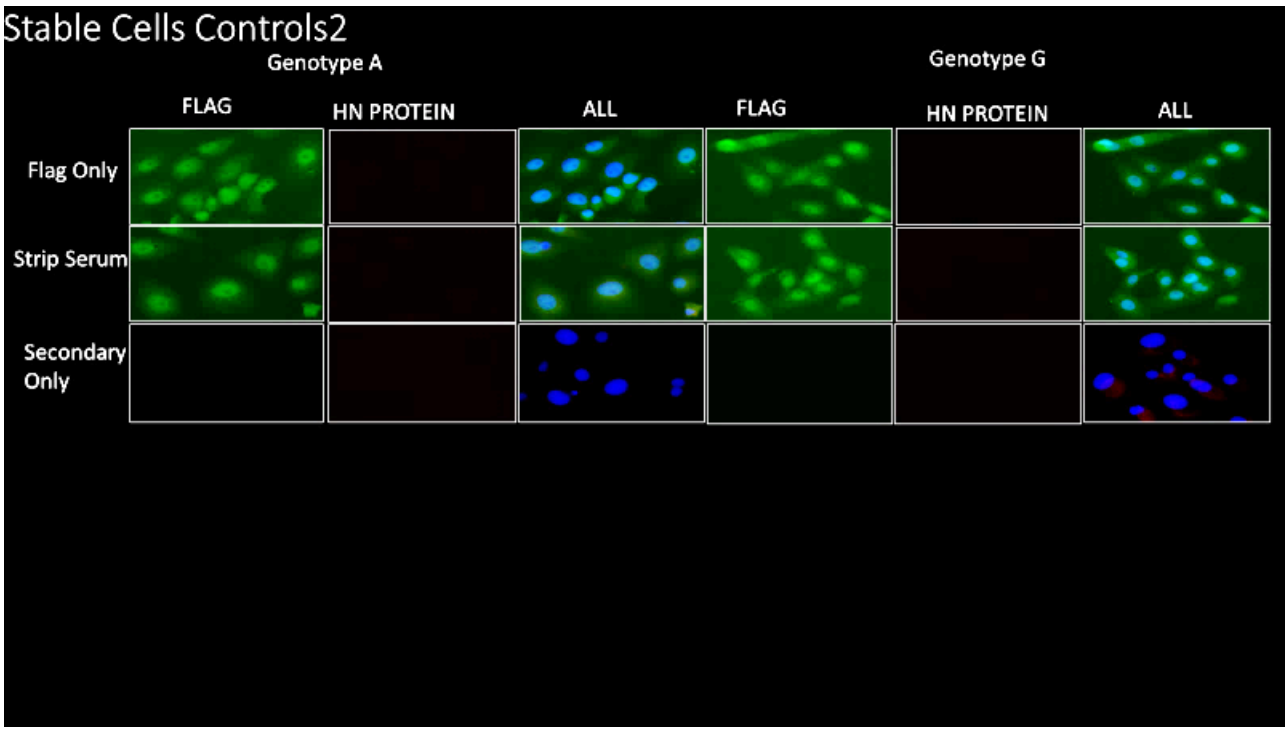


Figure A-1b. Stable cells ControlBatch#2.

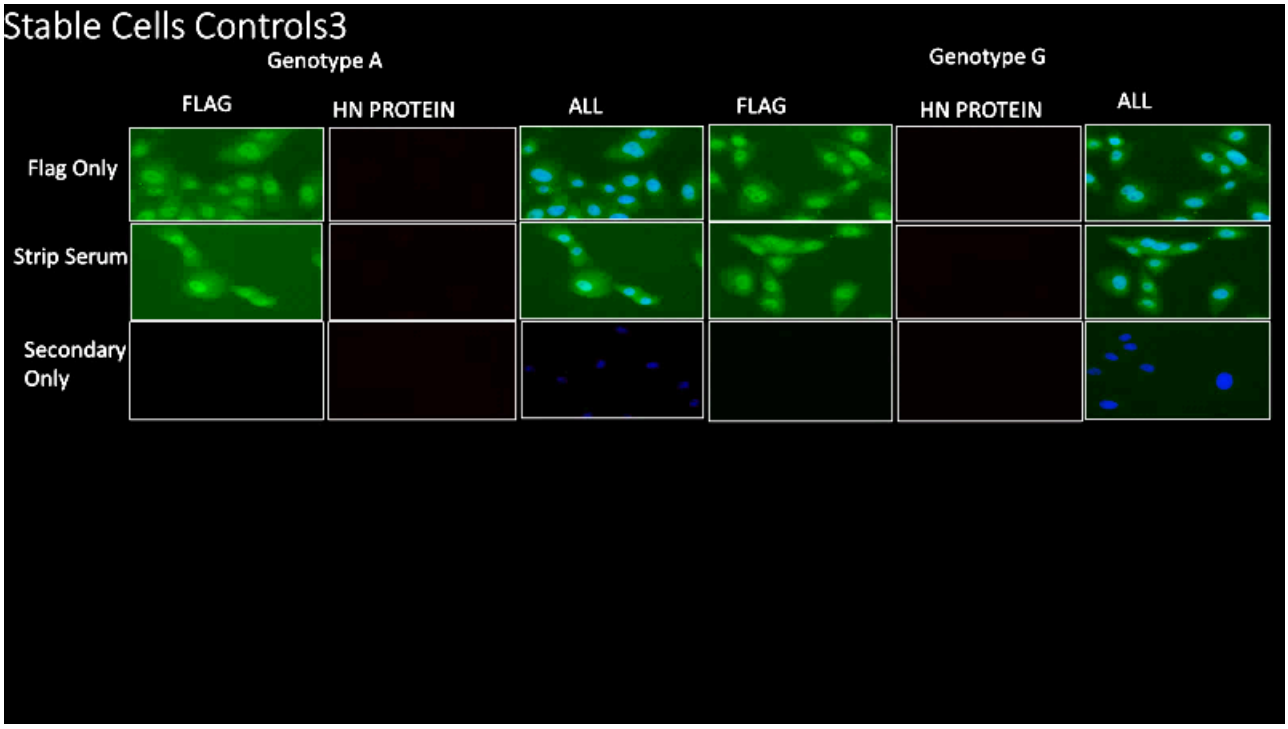


Figure A-1c. Stable cells ControlBatch#3.

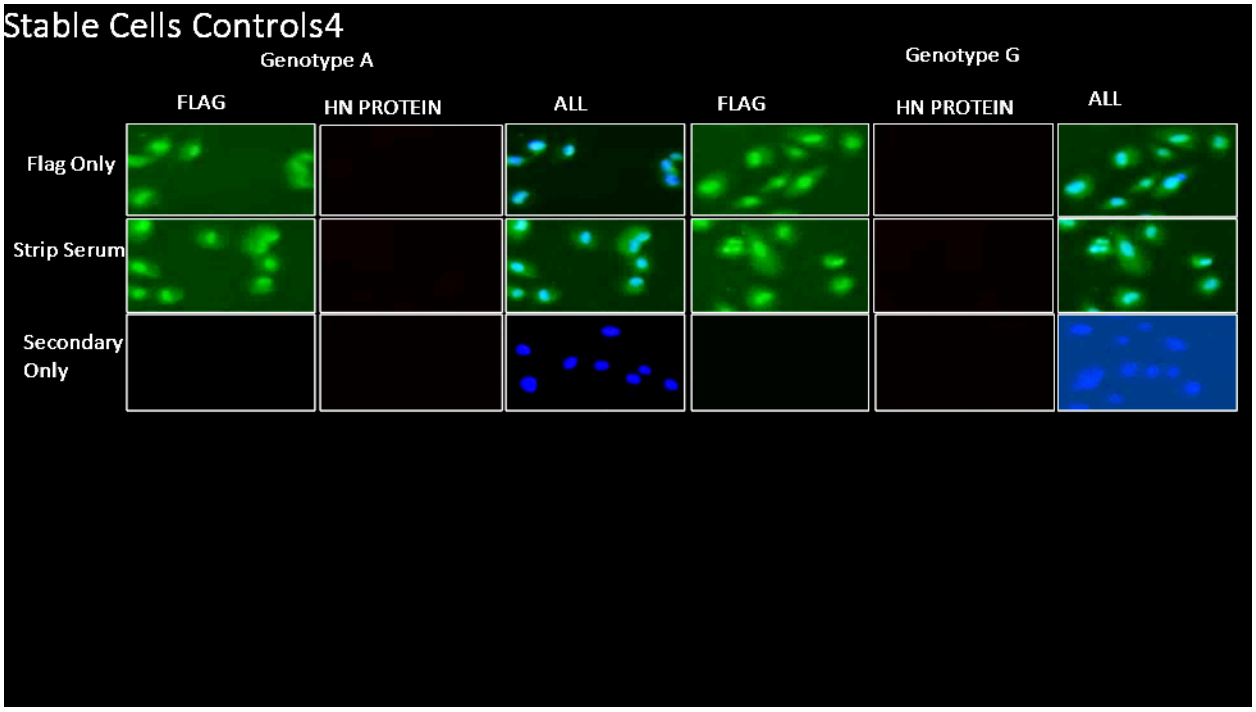


Figure A-1d. Stable cells ControlBatch#4.

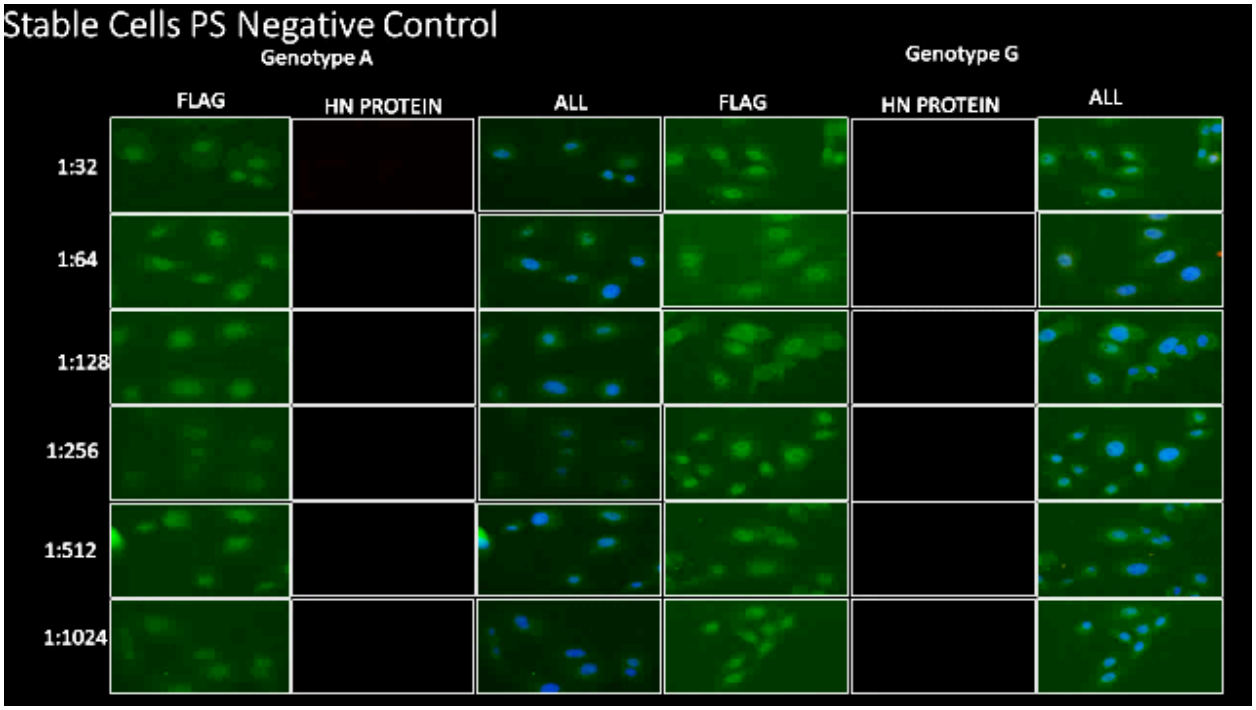


Figure A-2a. Stable cells Negative ControlBatch#1.

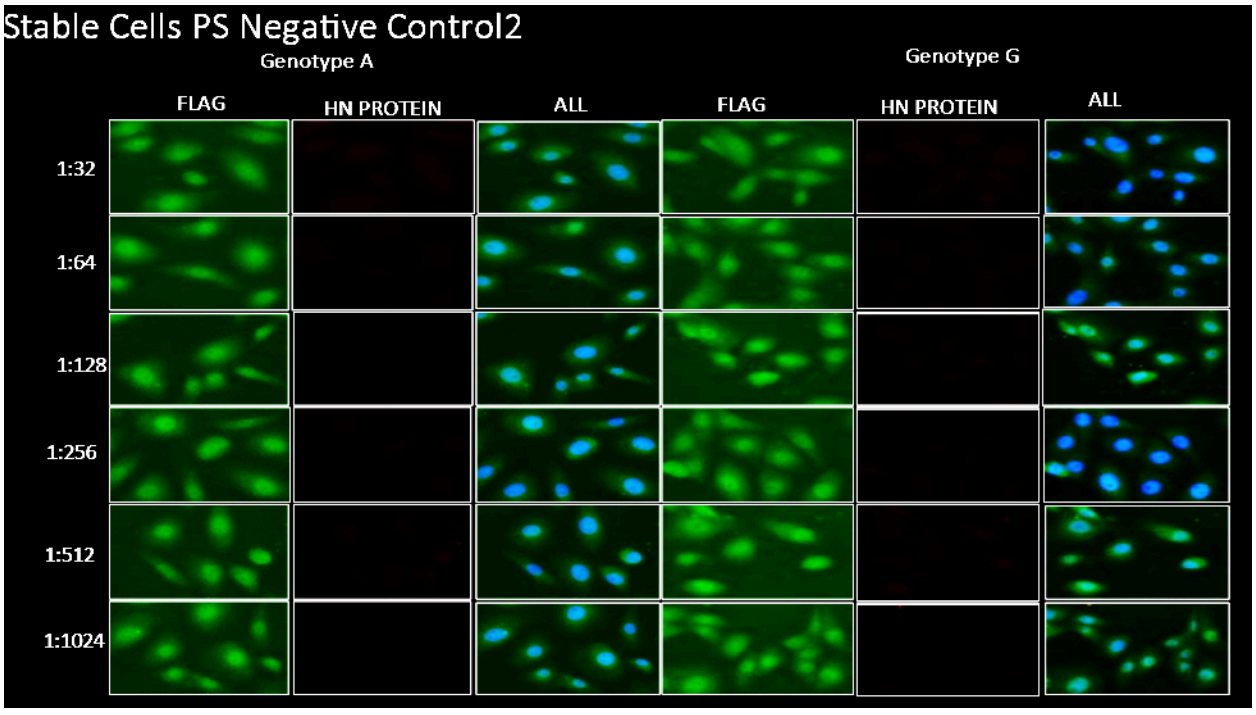


Figure A-2b. Stable cells Negative ControlBatch#2.

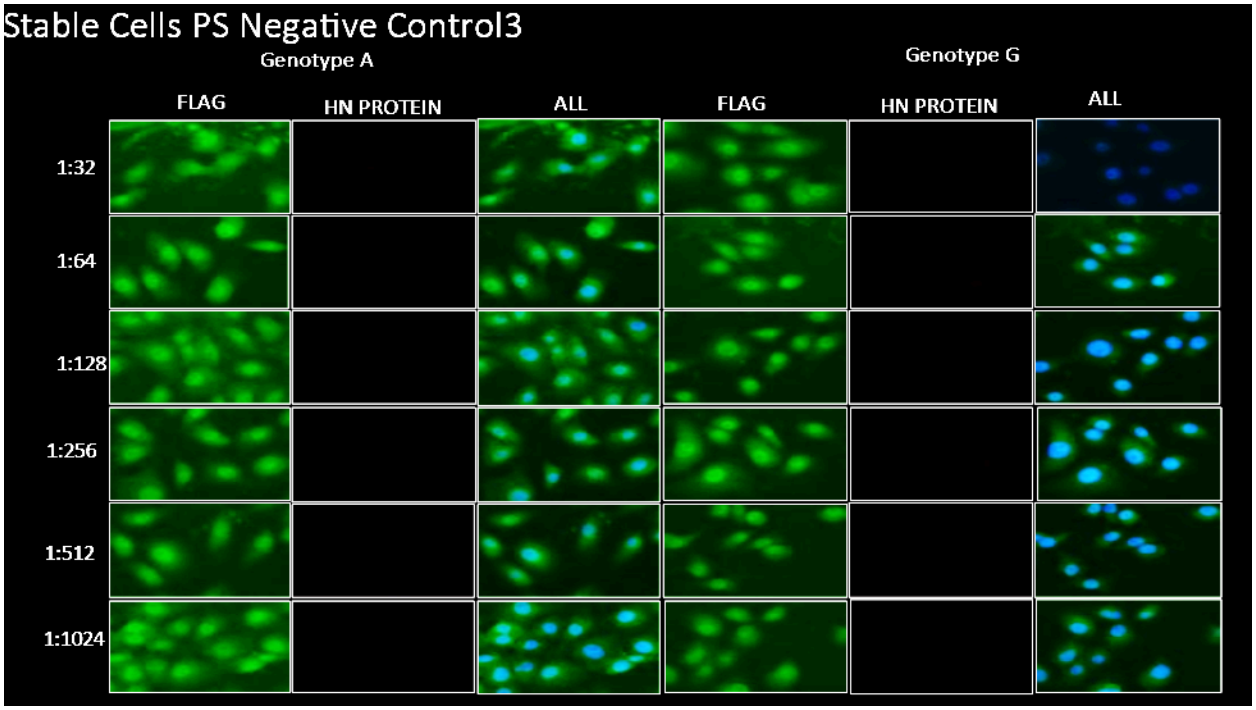


Figure A-2c. Stable cells Negative ControlBatch#3.

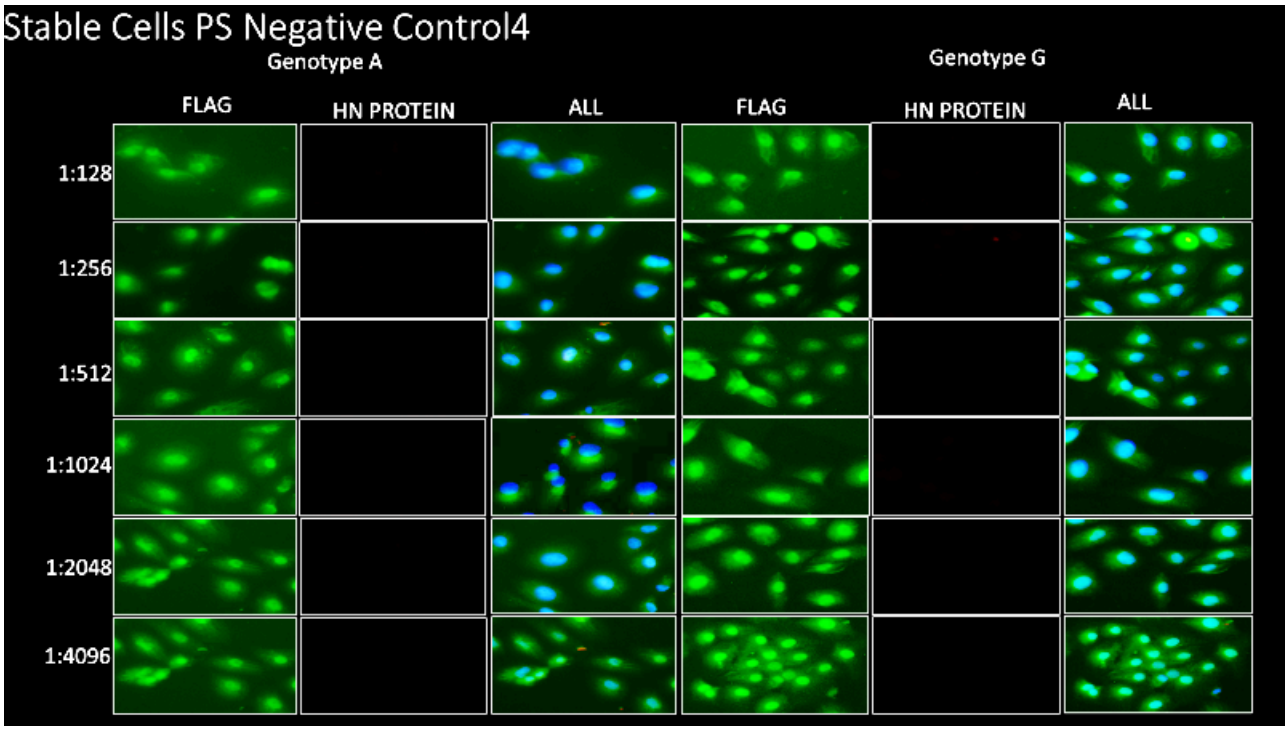


Figure A-2d. Stable cells Negative ControlBatch#4.

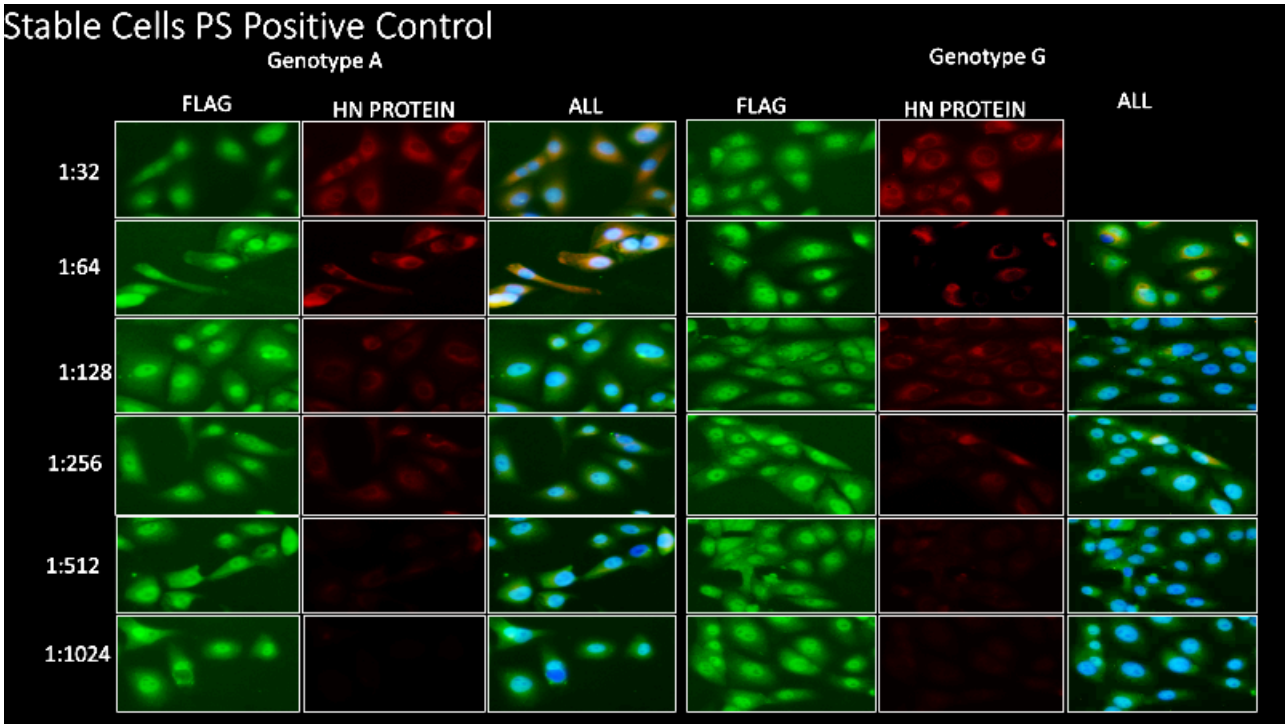


Figure A-3a. Stable cells Positive ControlBatch#1.

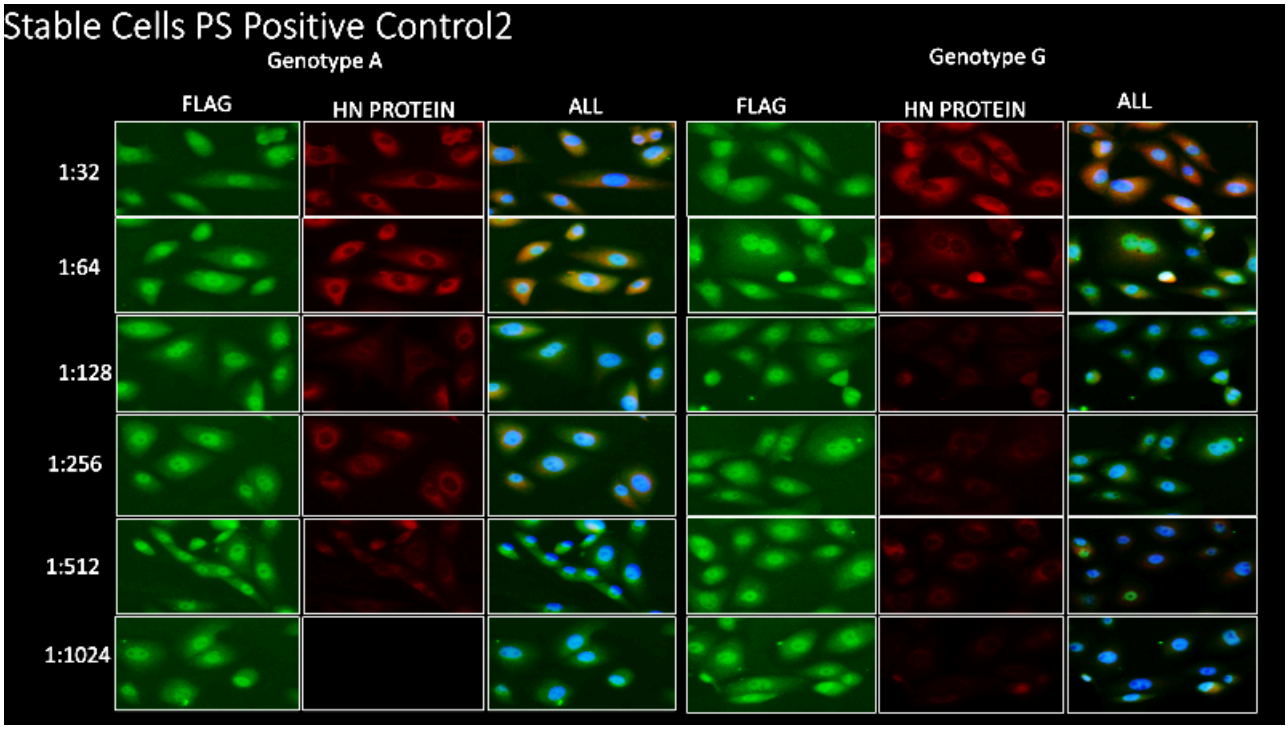


Figure A-3b. Stable cells Positive ControlBatch#2.

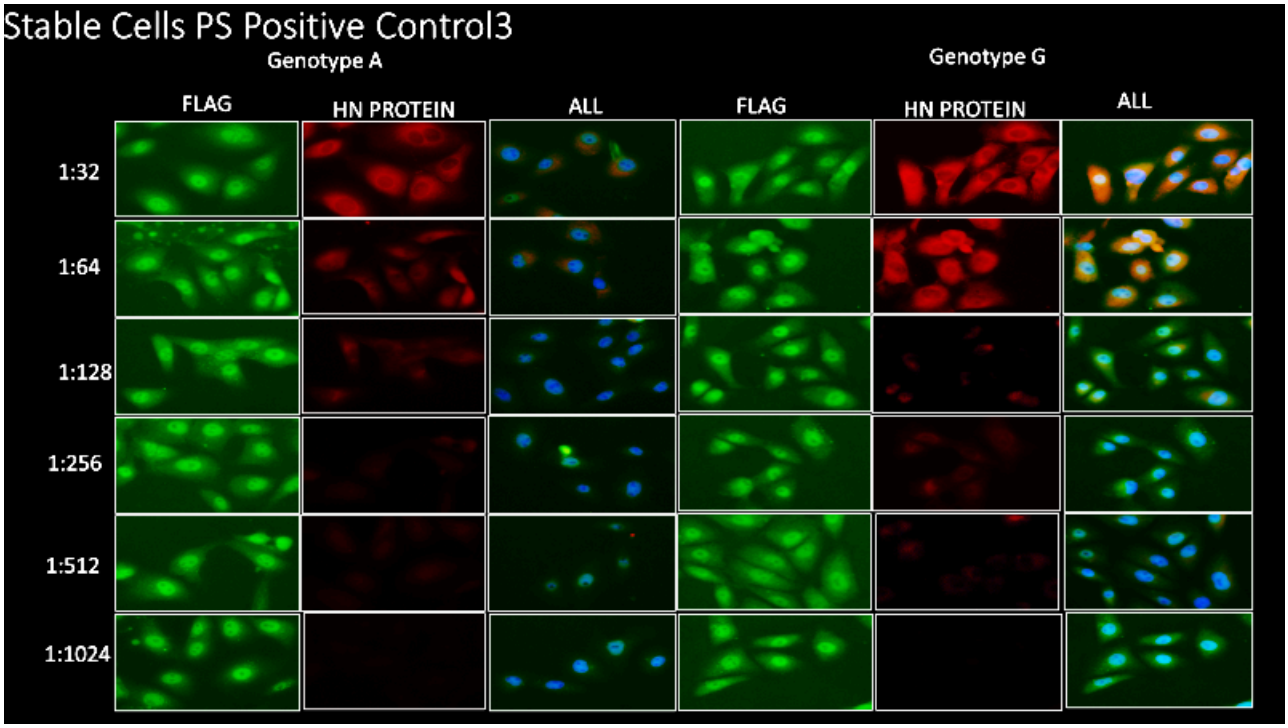


Figure A-3c. Stable cells Positive ControlBatch#3.

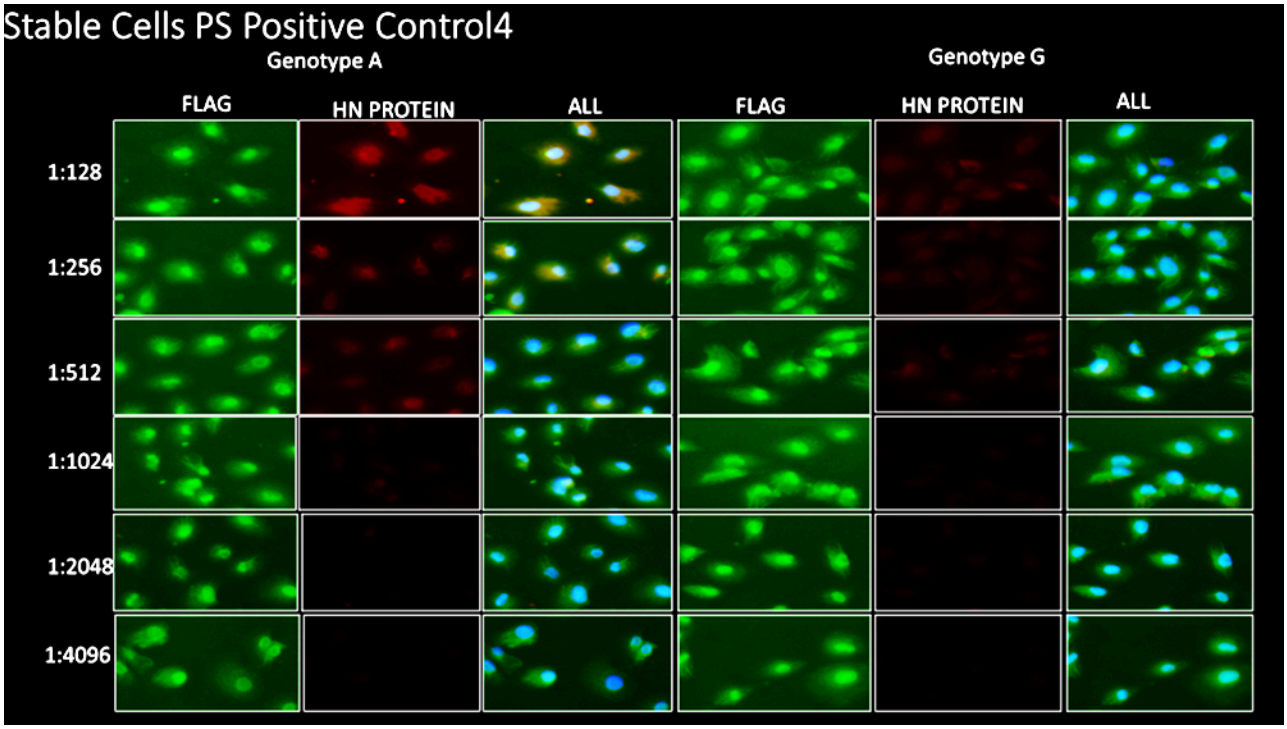


Figure A-3d. Stable cells Positive ControlBatch#4.

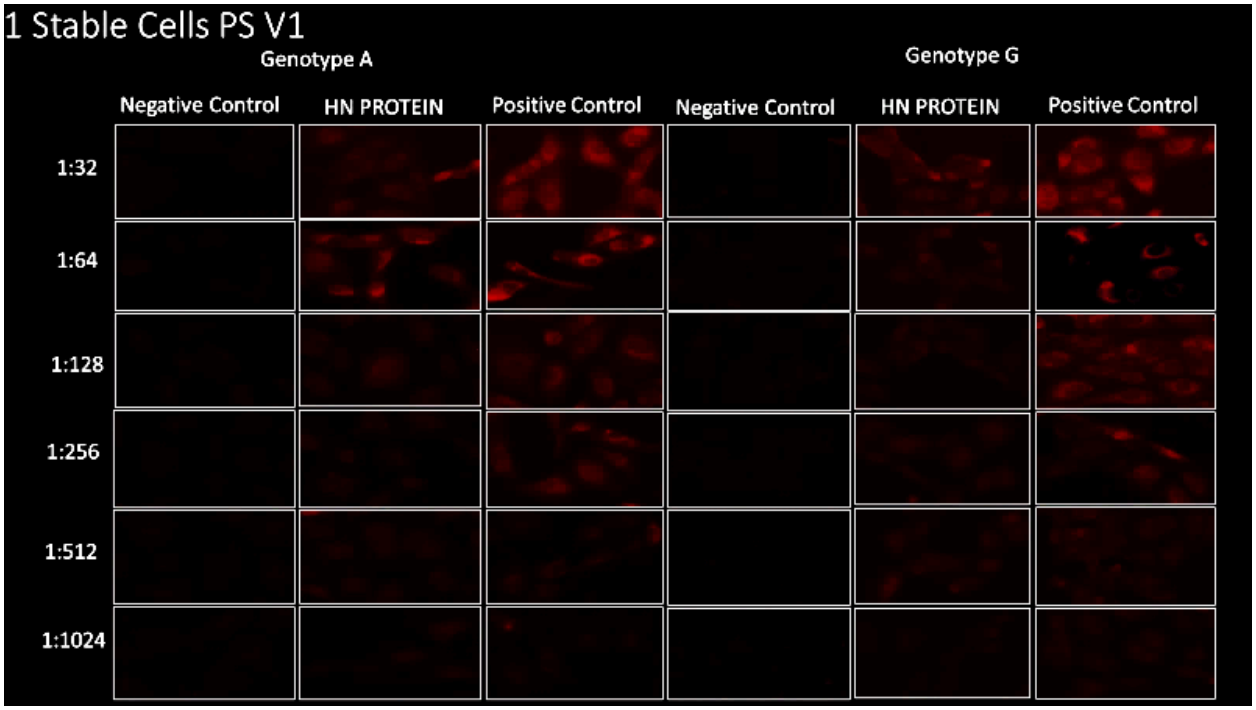


Figure A-4a. Stable cells Vaccine Sample#1.

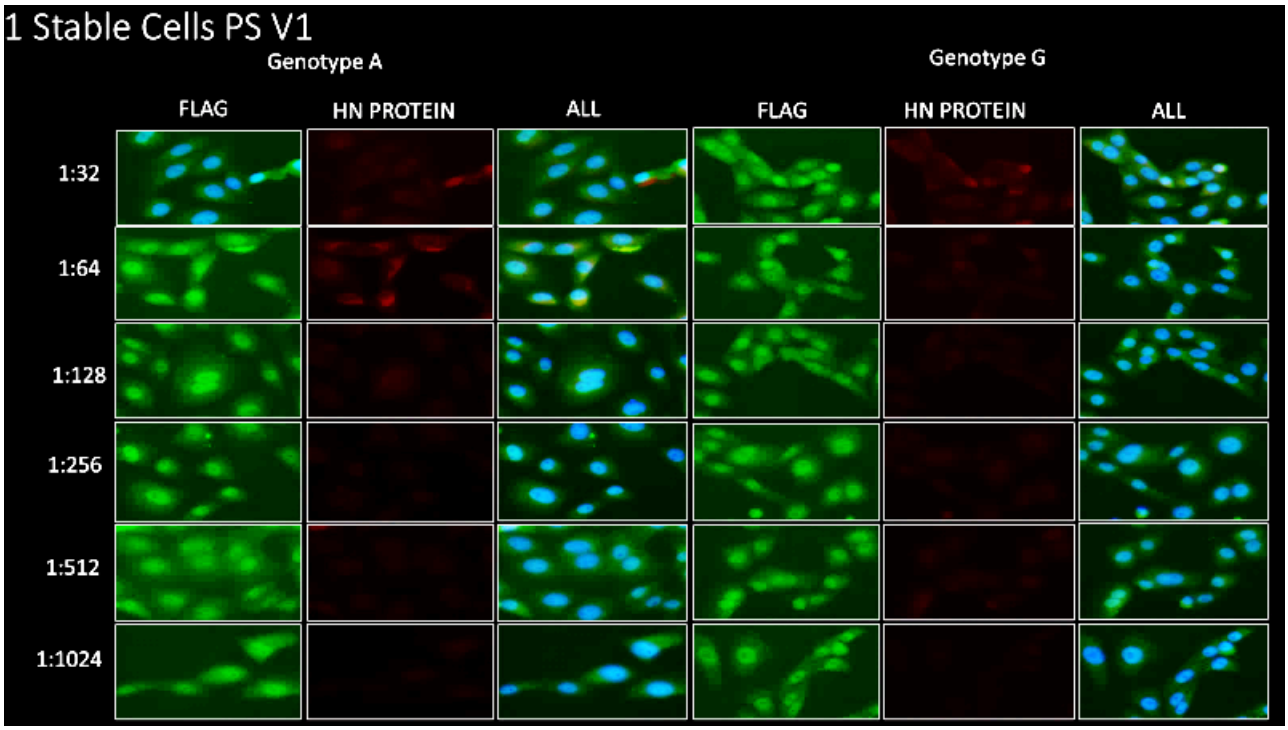


Figure A-4b. Stable cells Vaccine Sample #1.

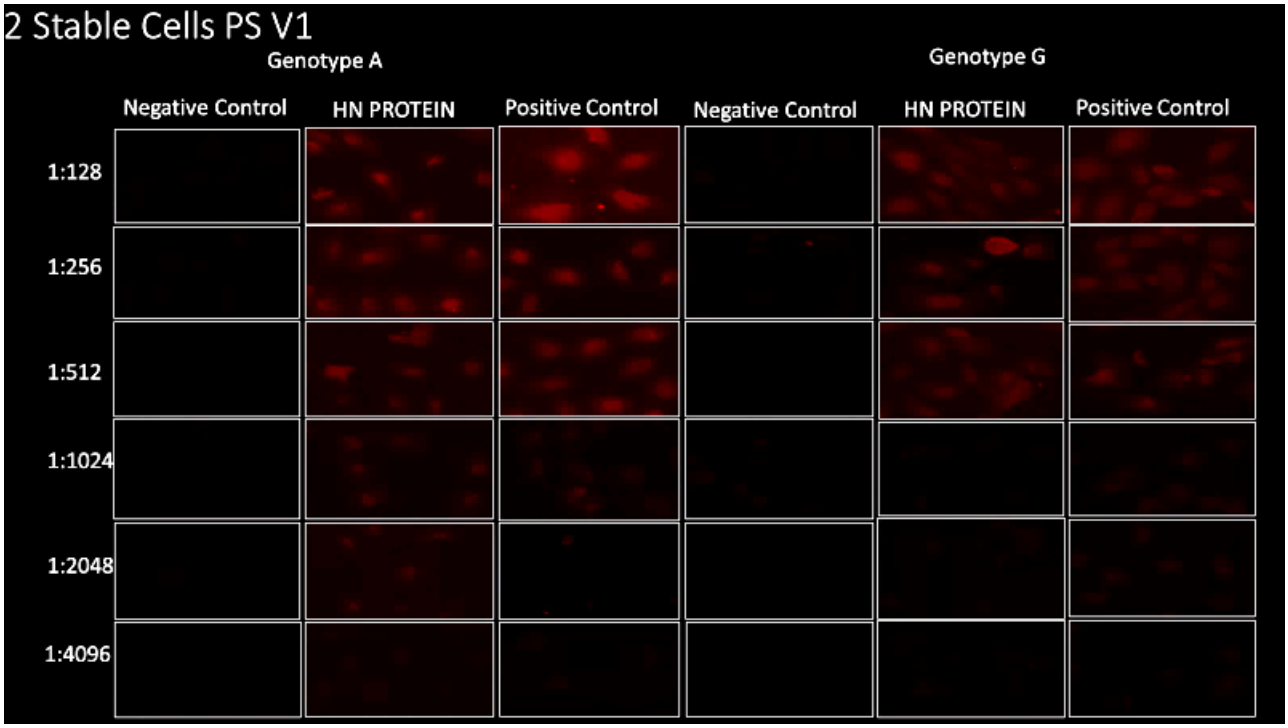


Figure A-4c. Stable cells Vaccine Sample #1.

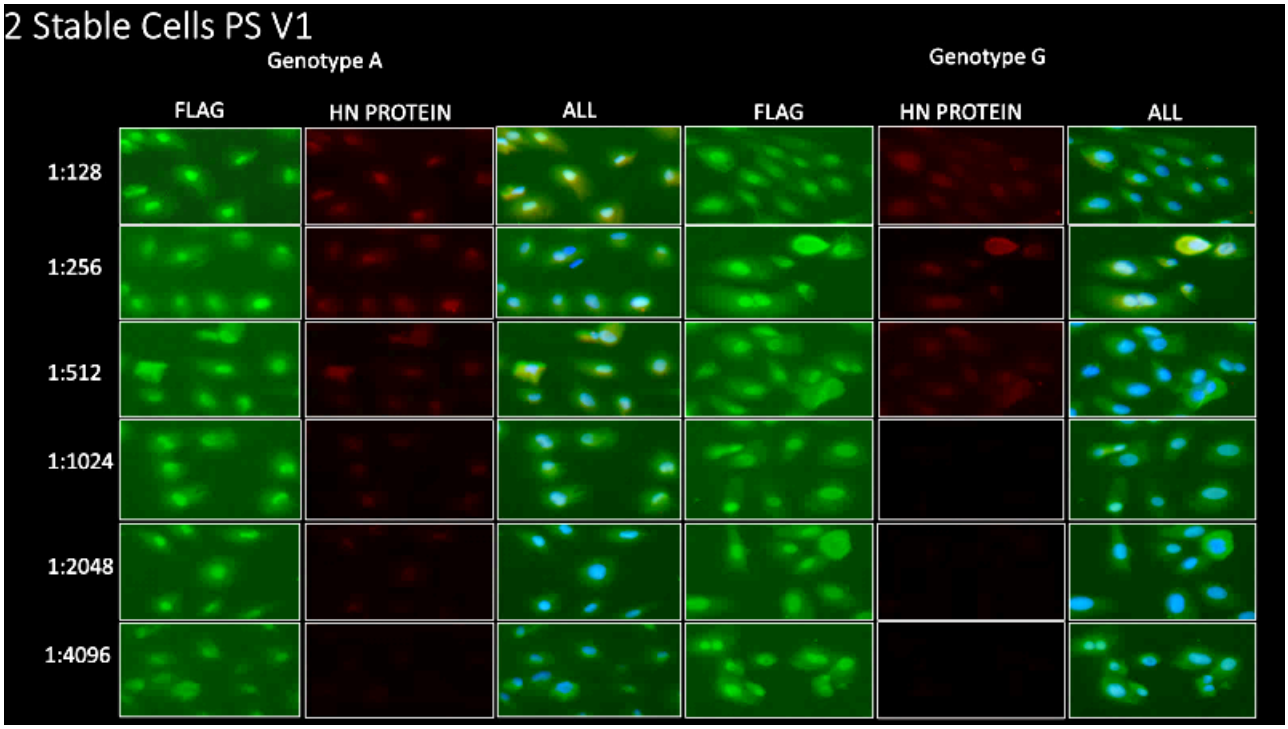


Figure A-4d. Stable cells Vaccine Sample #1.

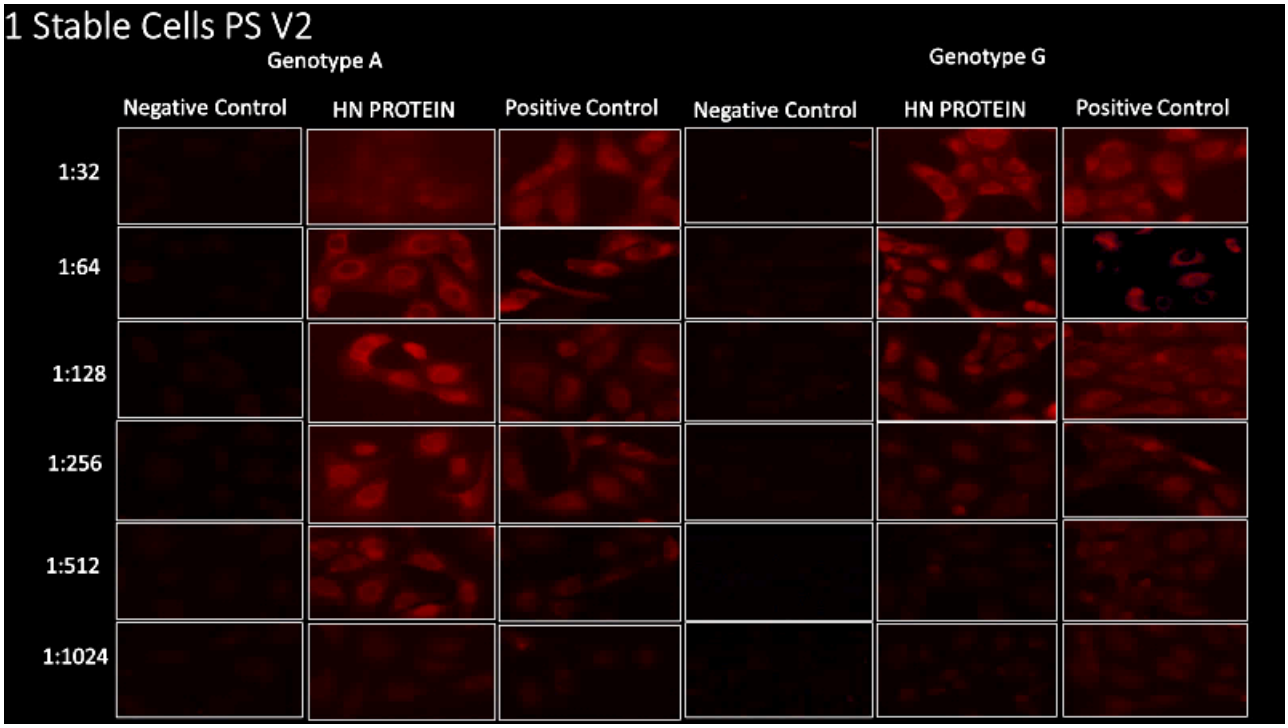


Figure A-5a. Stable cells Vaccine Sample#2.

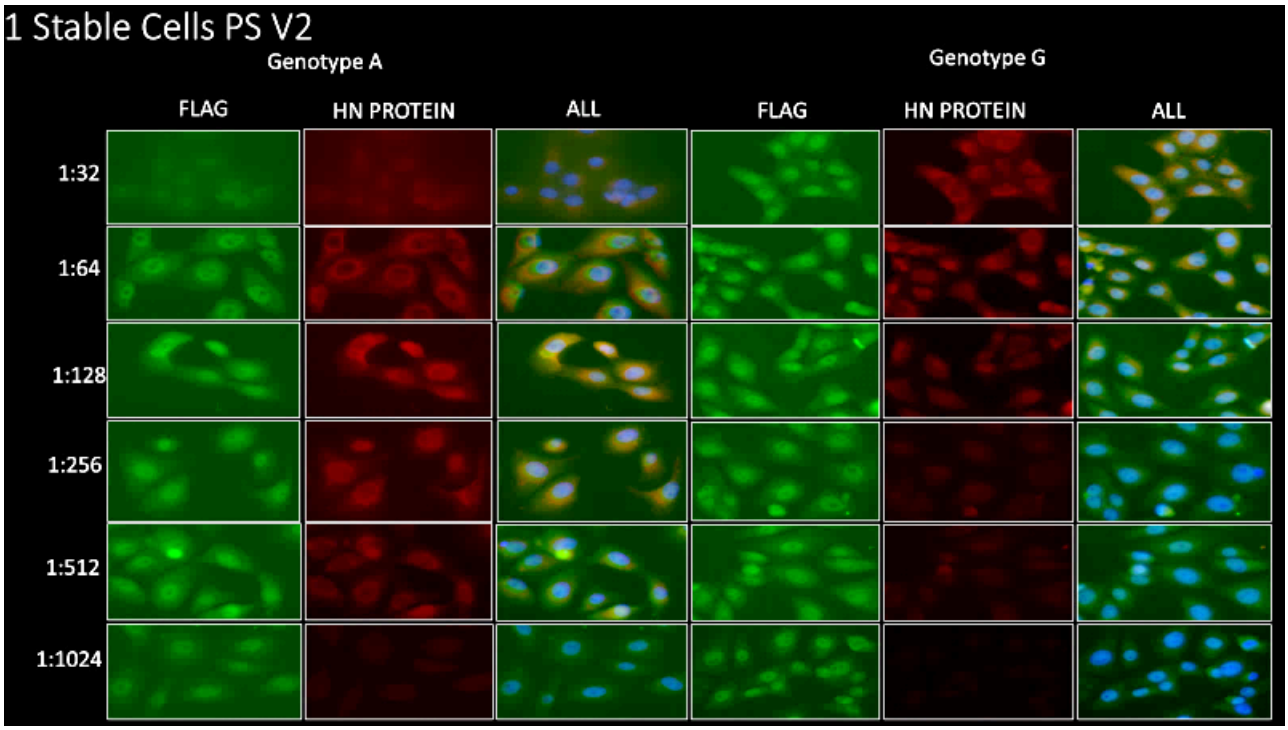


Figure A-5b. Stable cells Vaccine Sample #2.

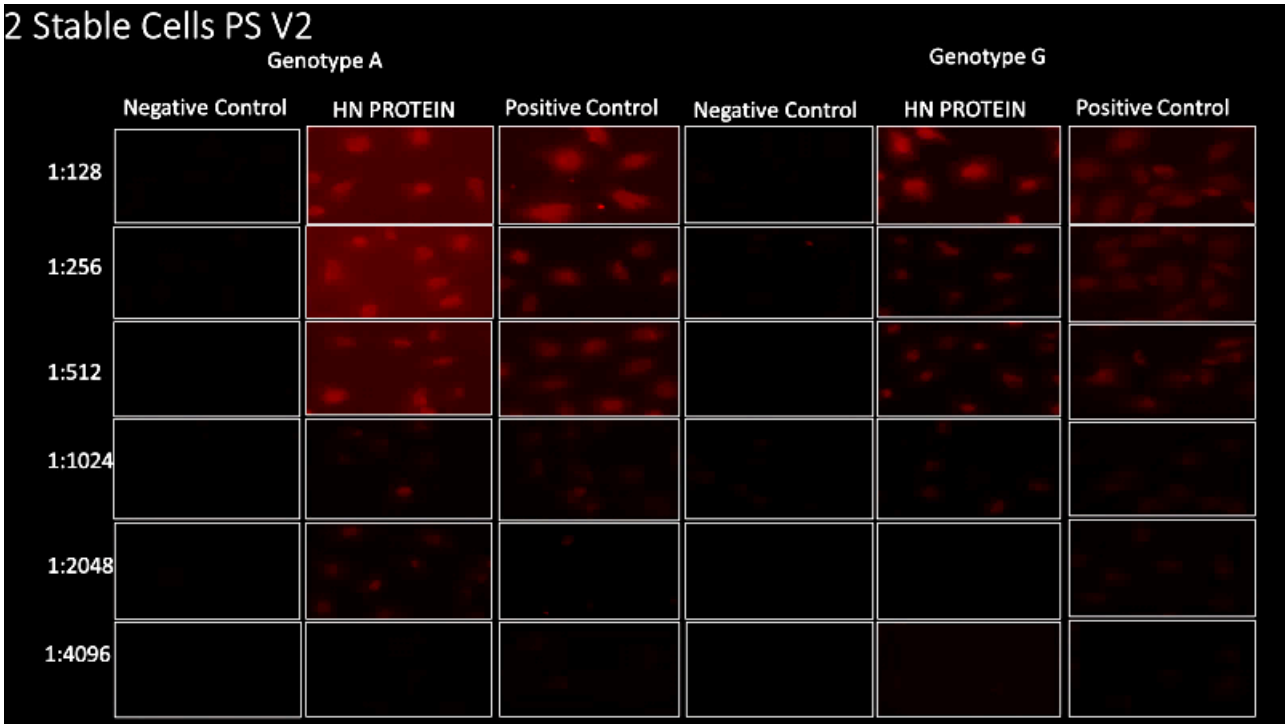


Figure A-5c. Stable cells Vaccine Sample #2.

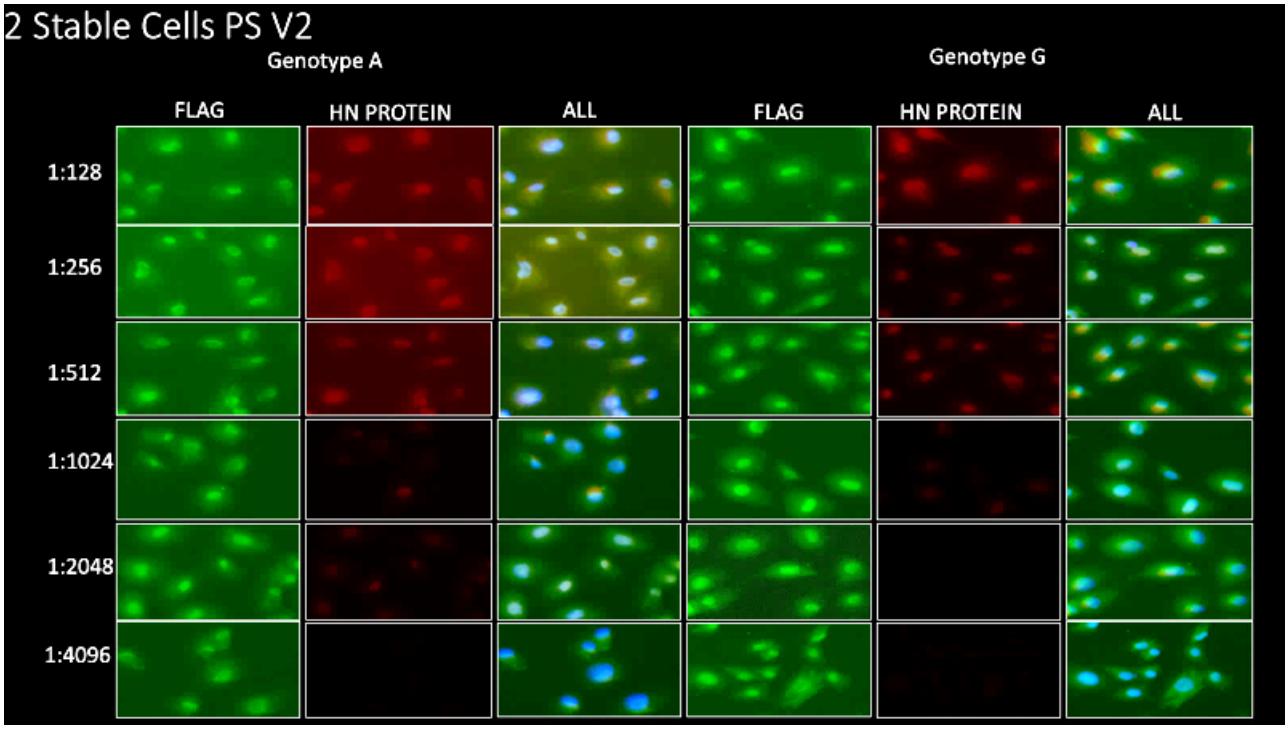


Figure A-5d. Stable cells Vaccine Sample #2.

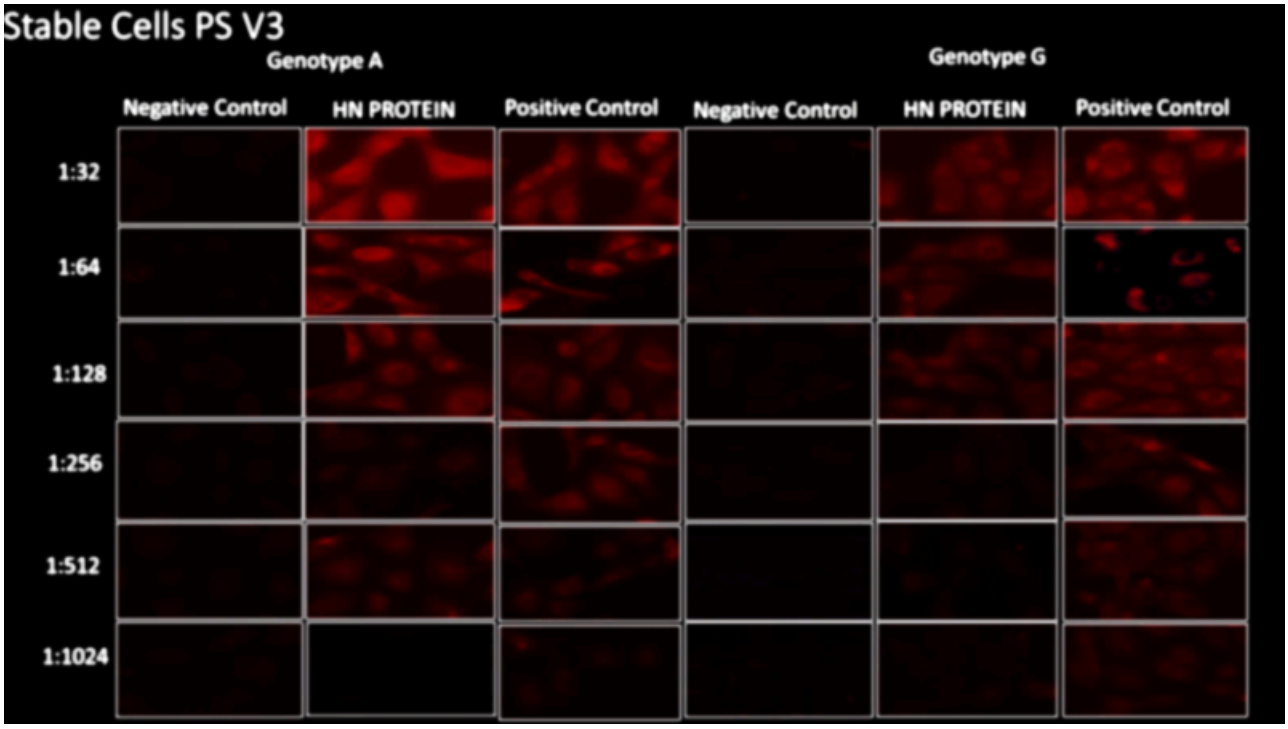


Figure A-6a. Stable cells Vaccine Sample#3.

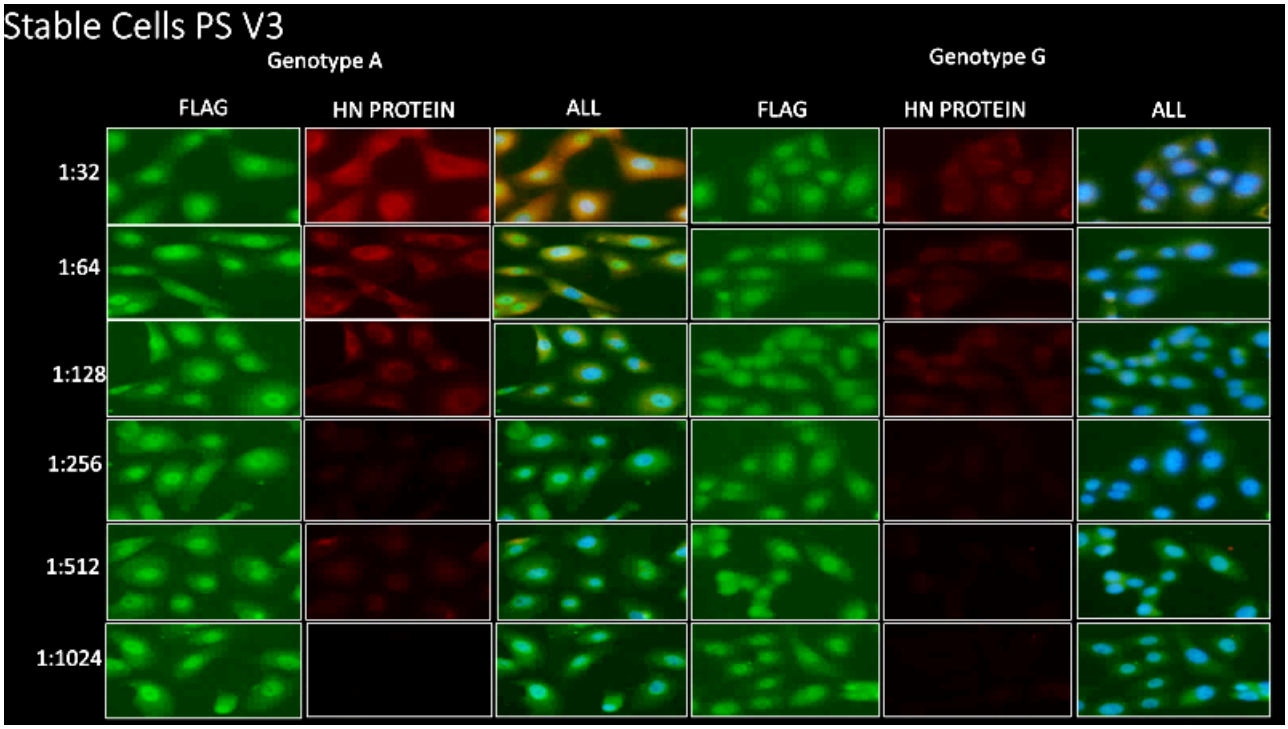


Figure A-6b. Stable cells Vaccine Sample #3.

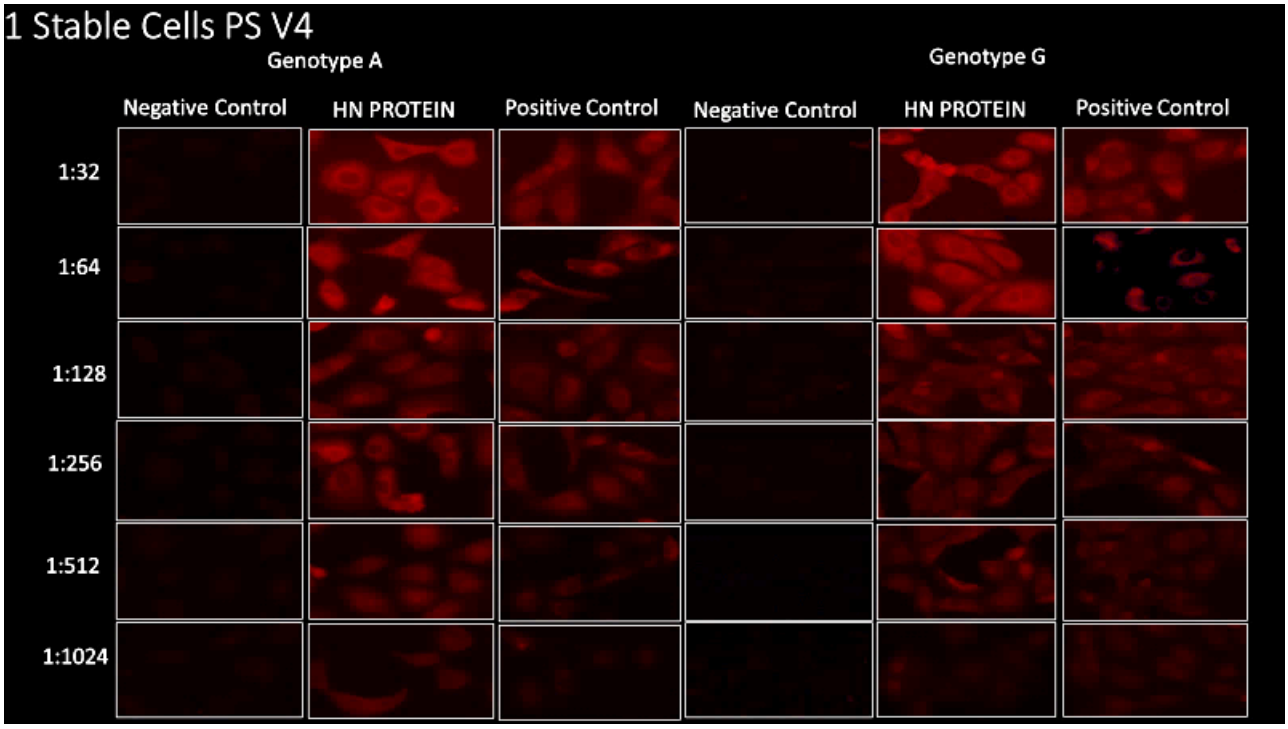


Figure A-7a. Stable cells Vaccine Sample#4.

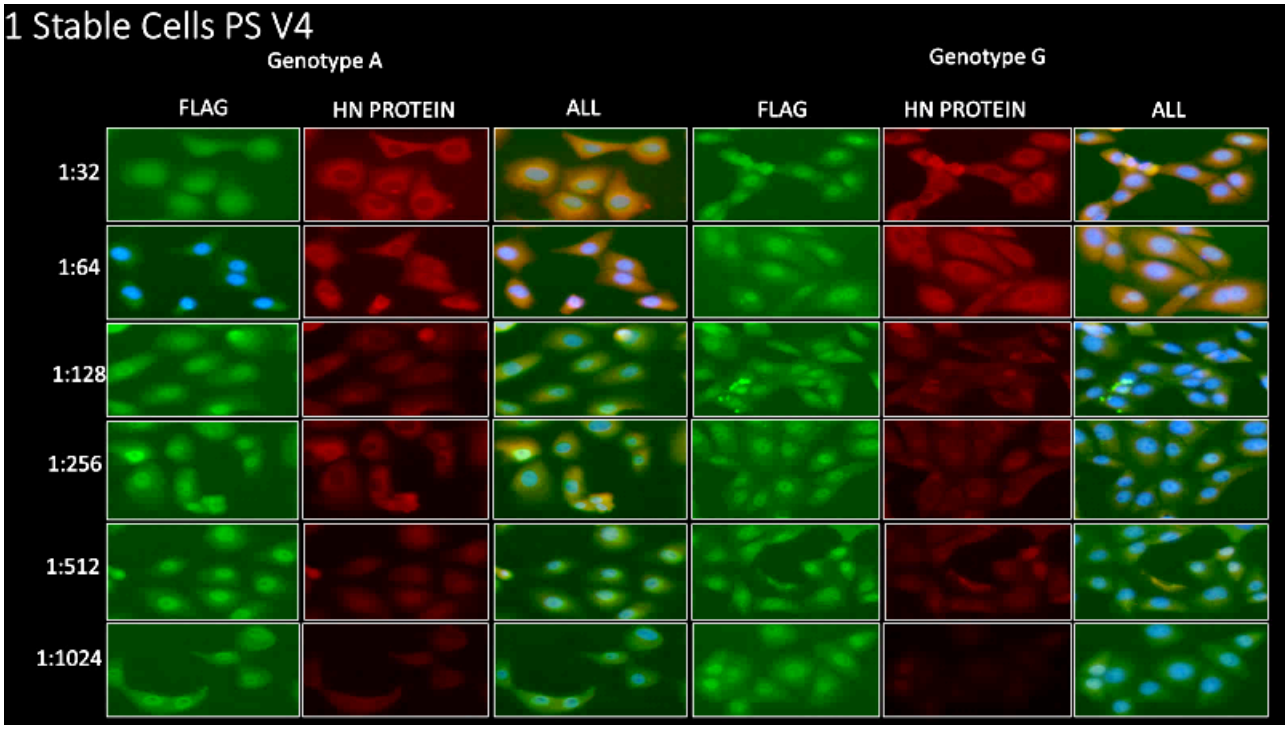


Figure A-7b. Stable cells Vaccine Sample #4.

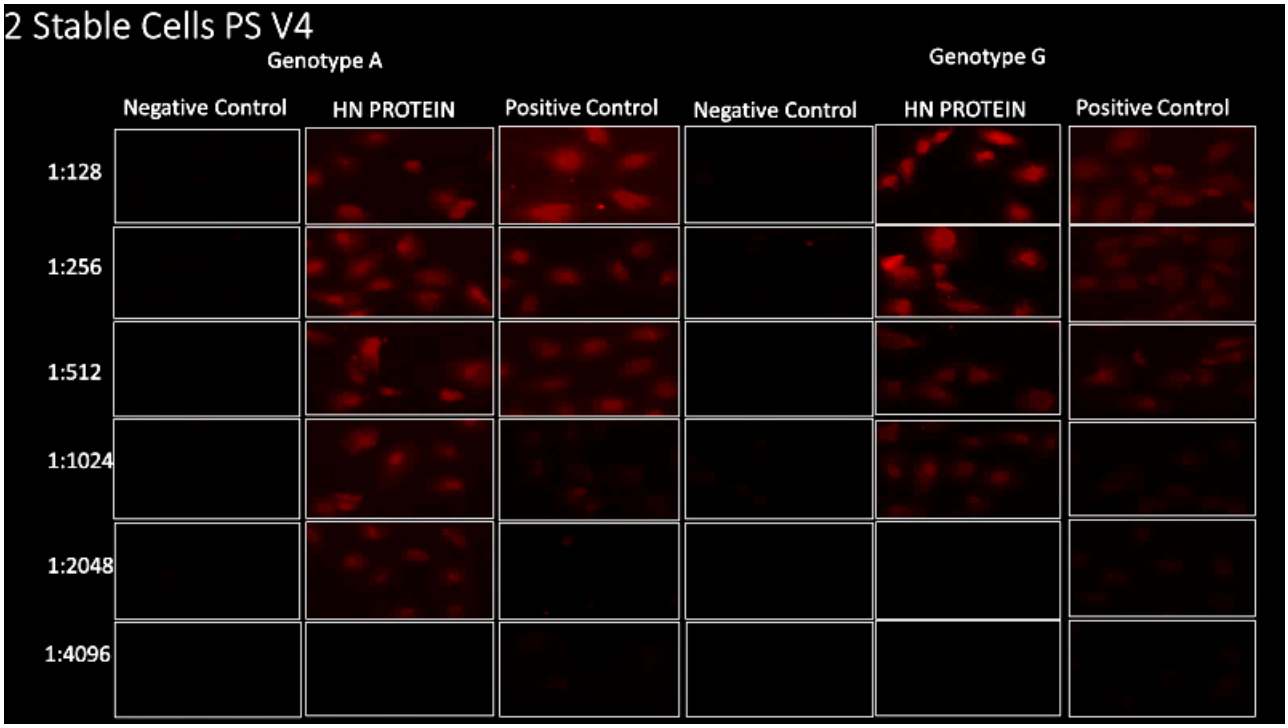


Figure A-7c. Stable cells Vaccine Sample #4.

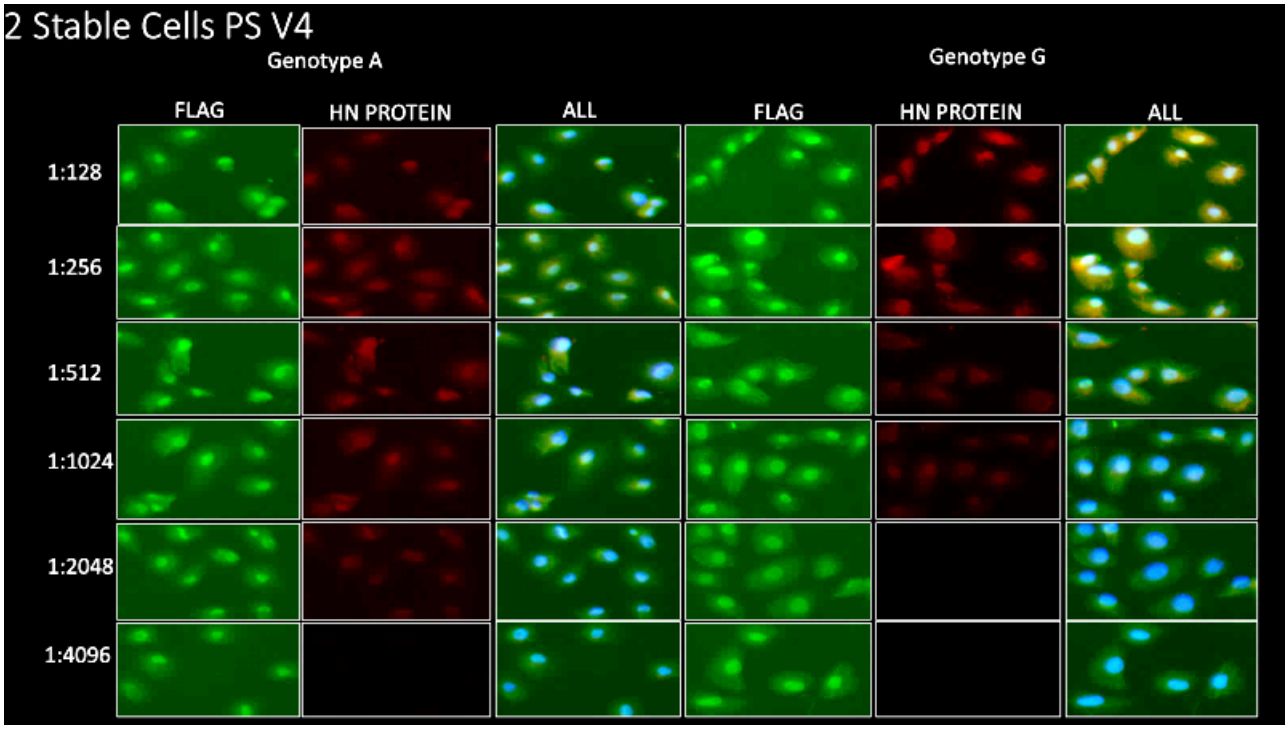


Figure A-7d. Stable cells Vaccine Sample #4.

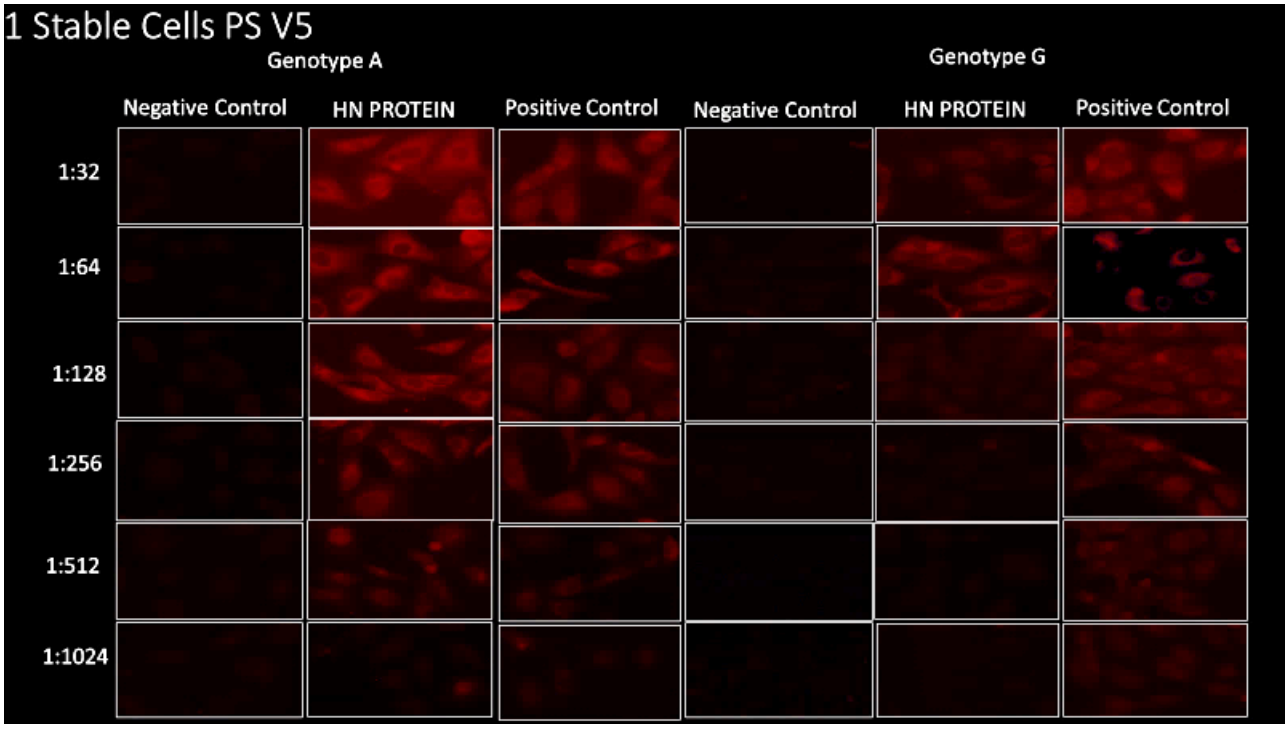


Figure A-8a. Stable cells Vaccine Sample#5.

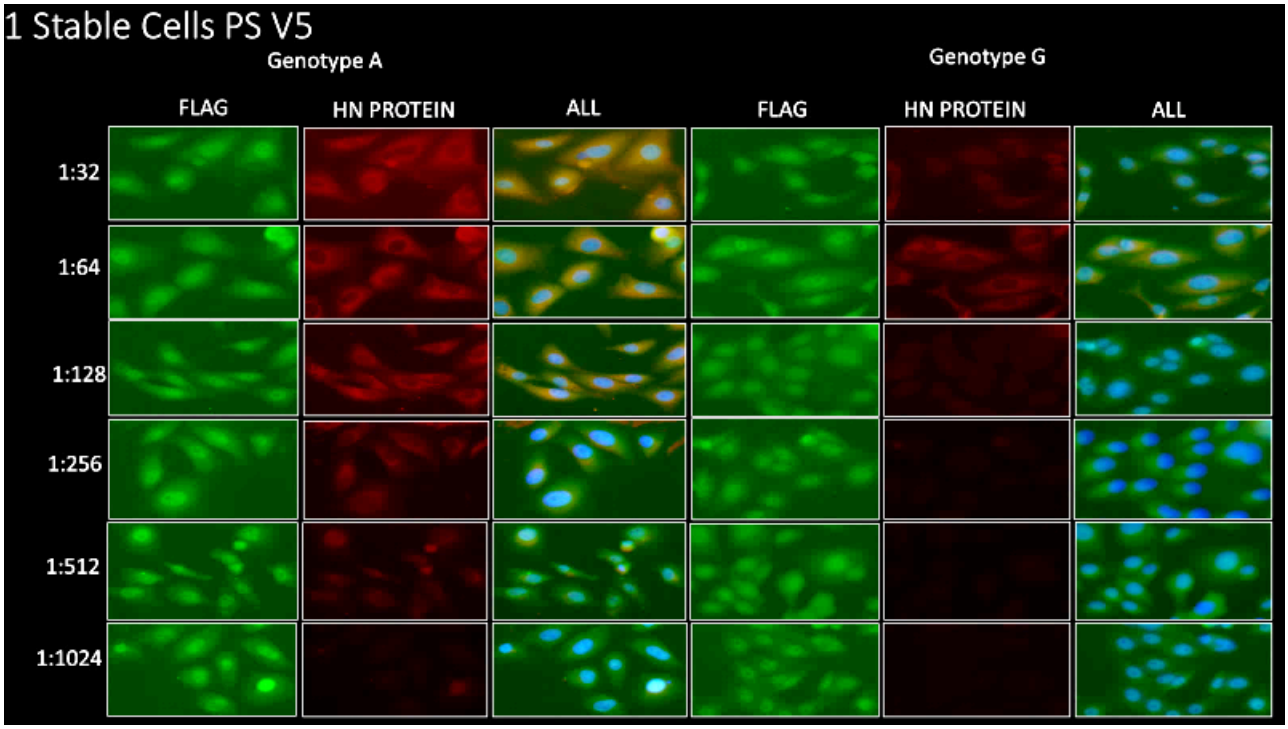


Figure A-8b. Stable cells Vaccine Sample #5.

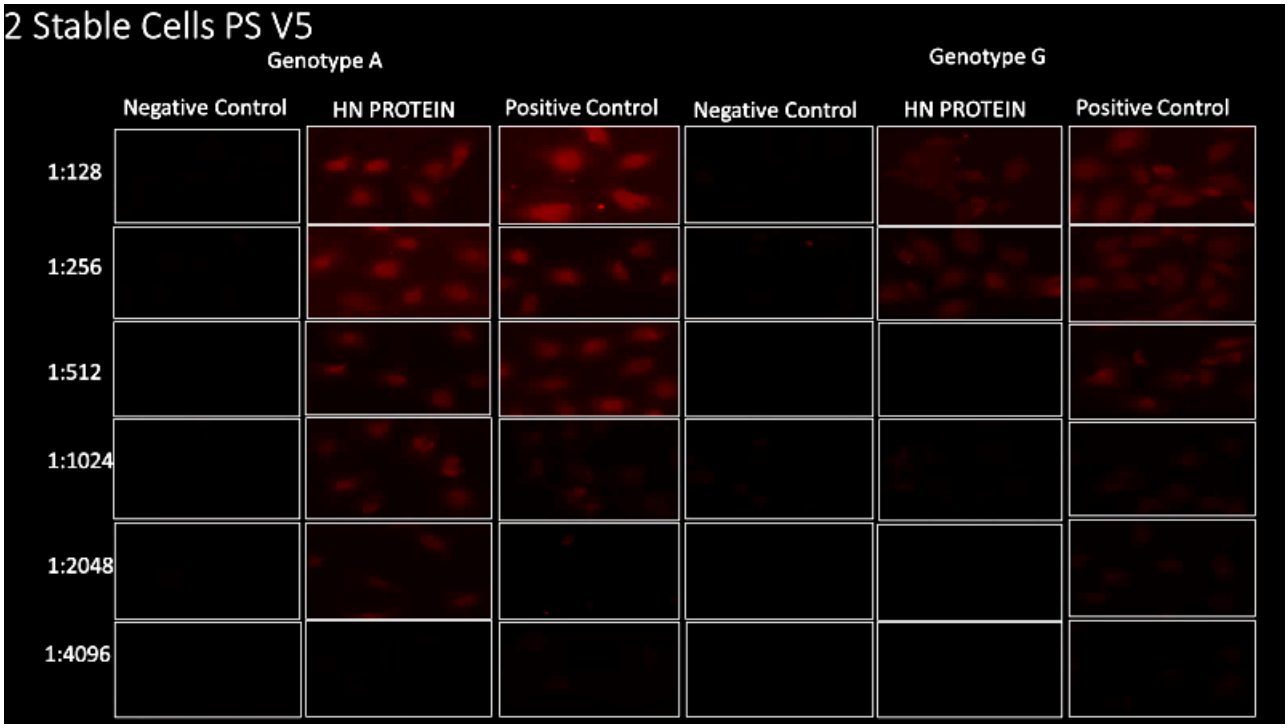


Figure A-8c. Stable cells Vaccine Sample #5.

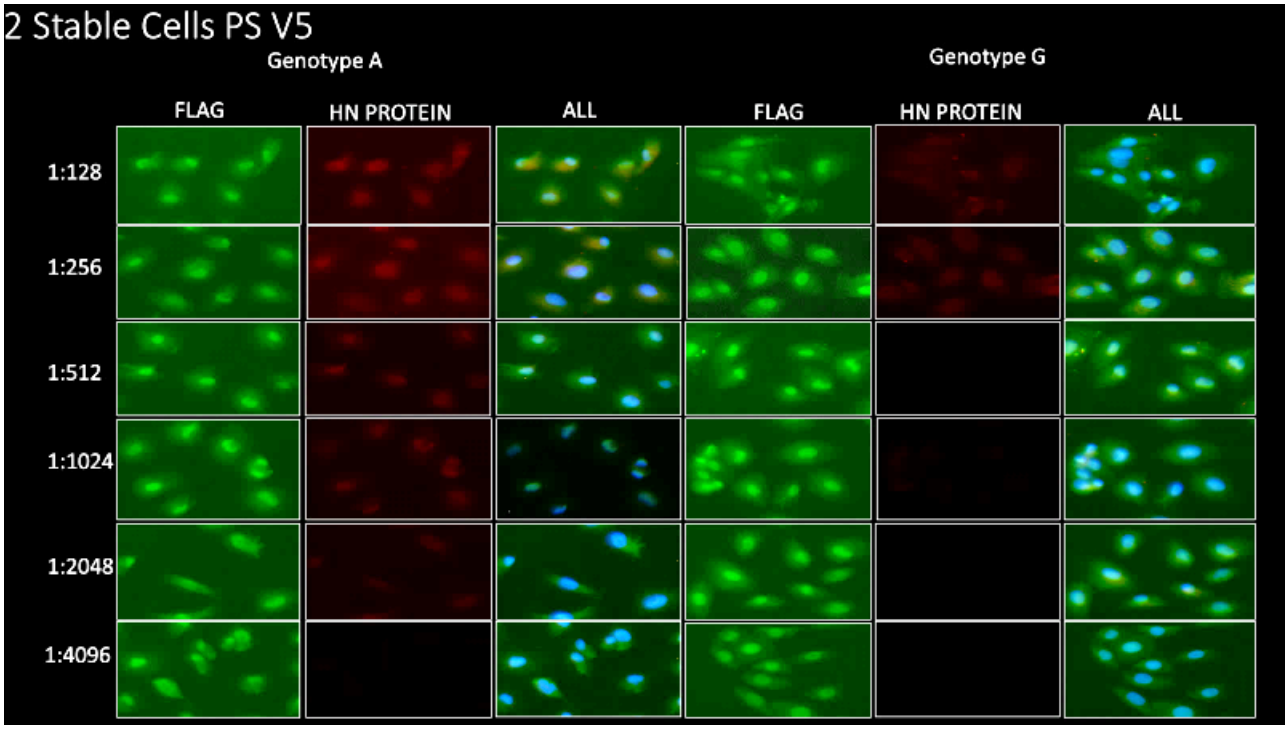


Figure A-8d. Stable cells Vaccine Sample #5.

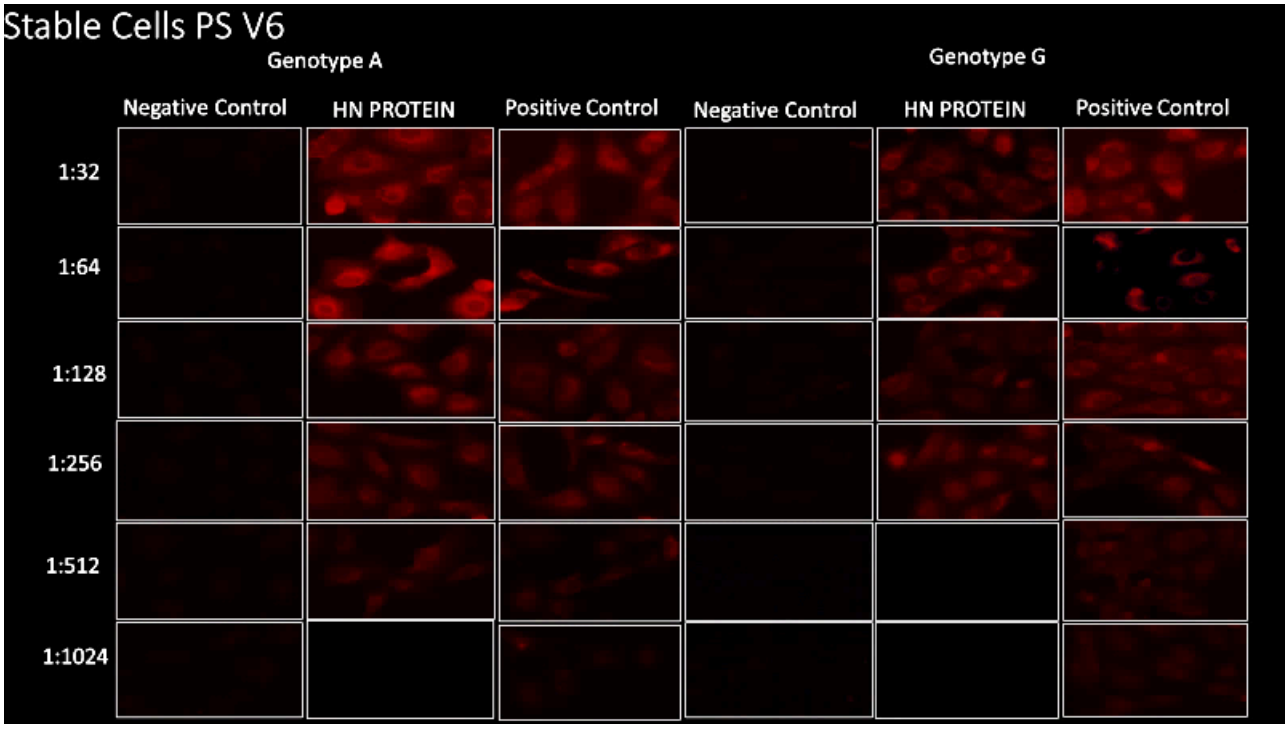


Figure A-9a. Stable cells Vaccine Sample#6.

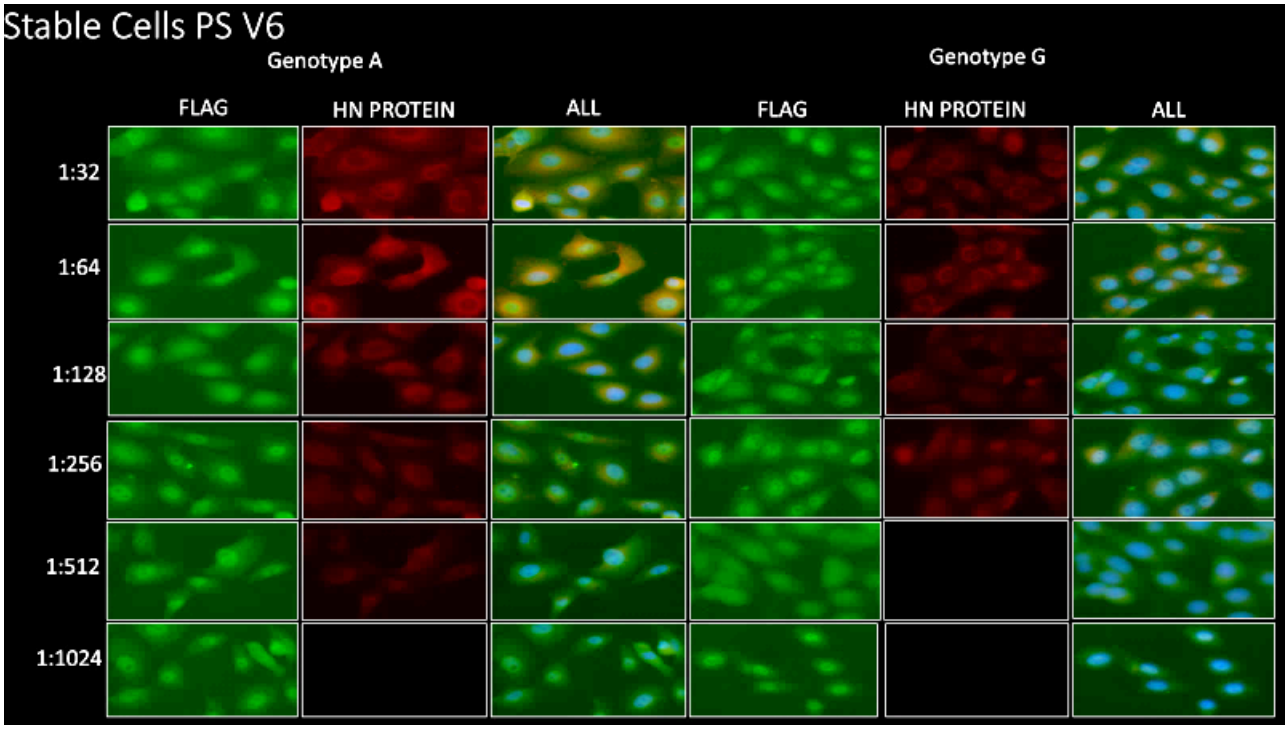


Figure A-9b. Stable cells Vaccine Sample #6.

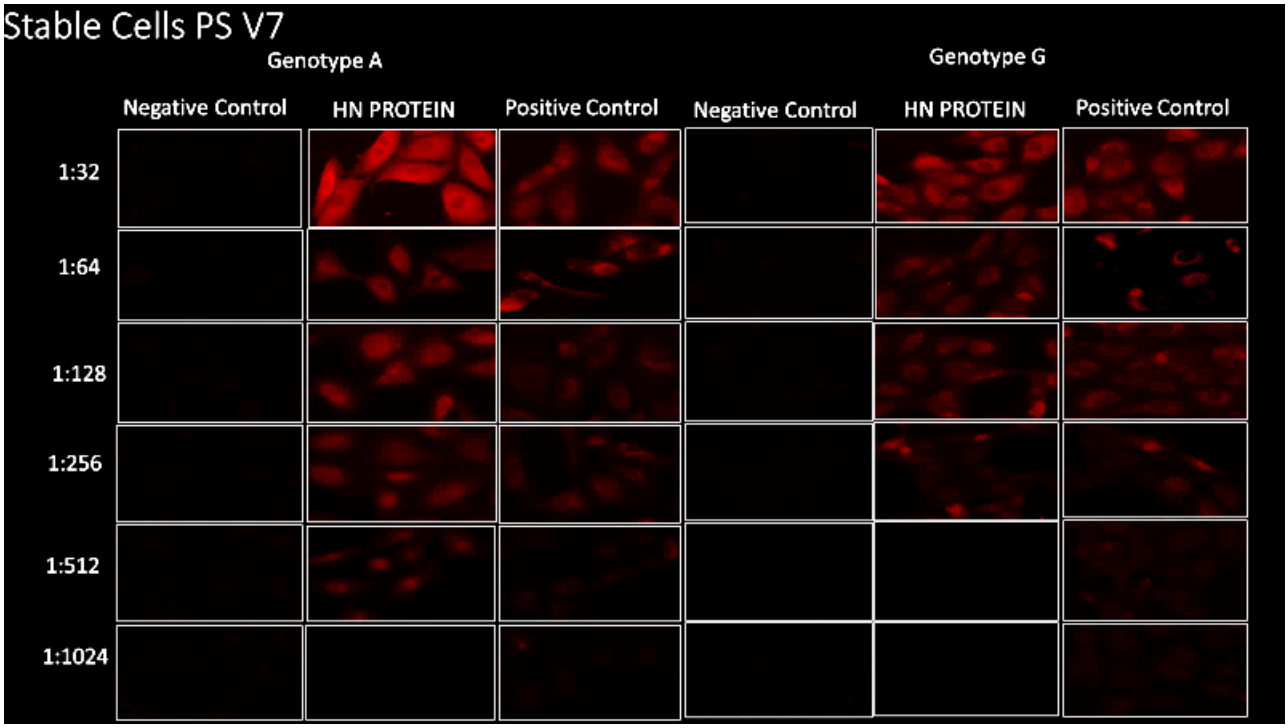


Figure A-10a. Stable cells Vaccine Sample#7.

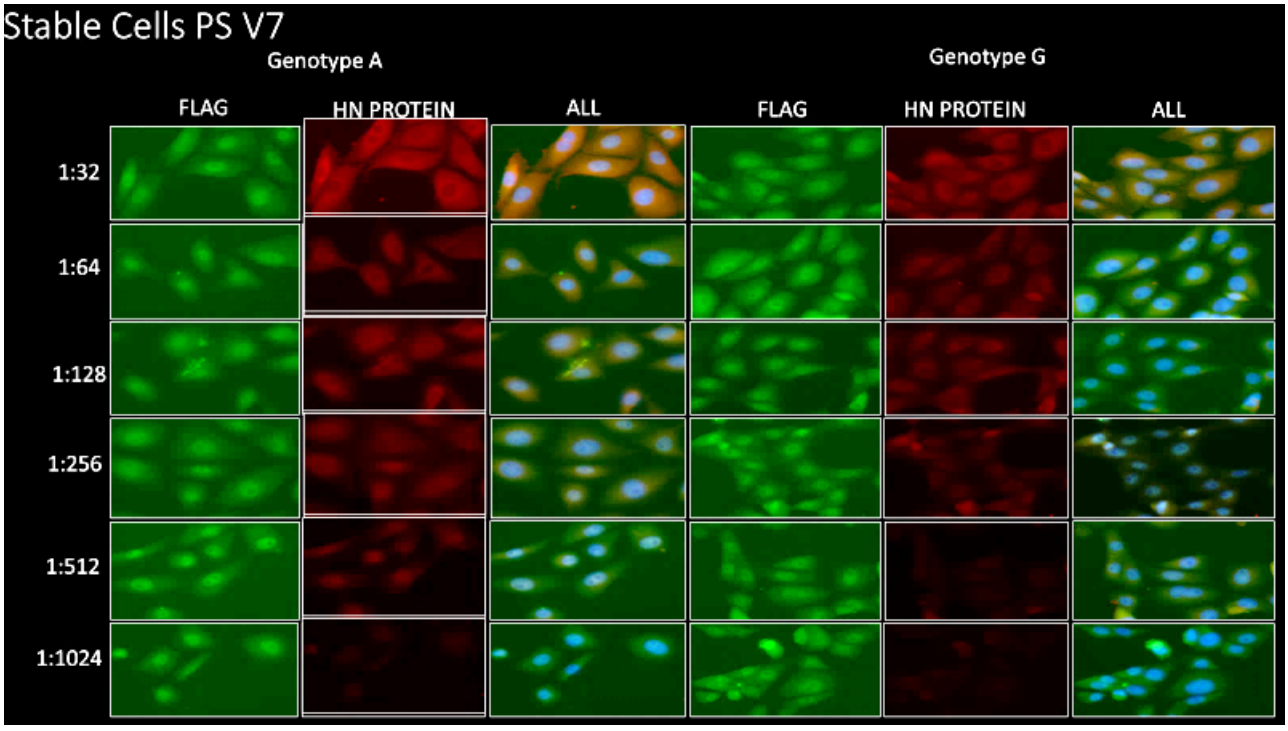


Figure A-10b. Stable cells Vaccine Sample #7.

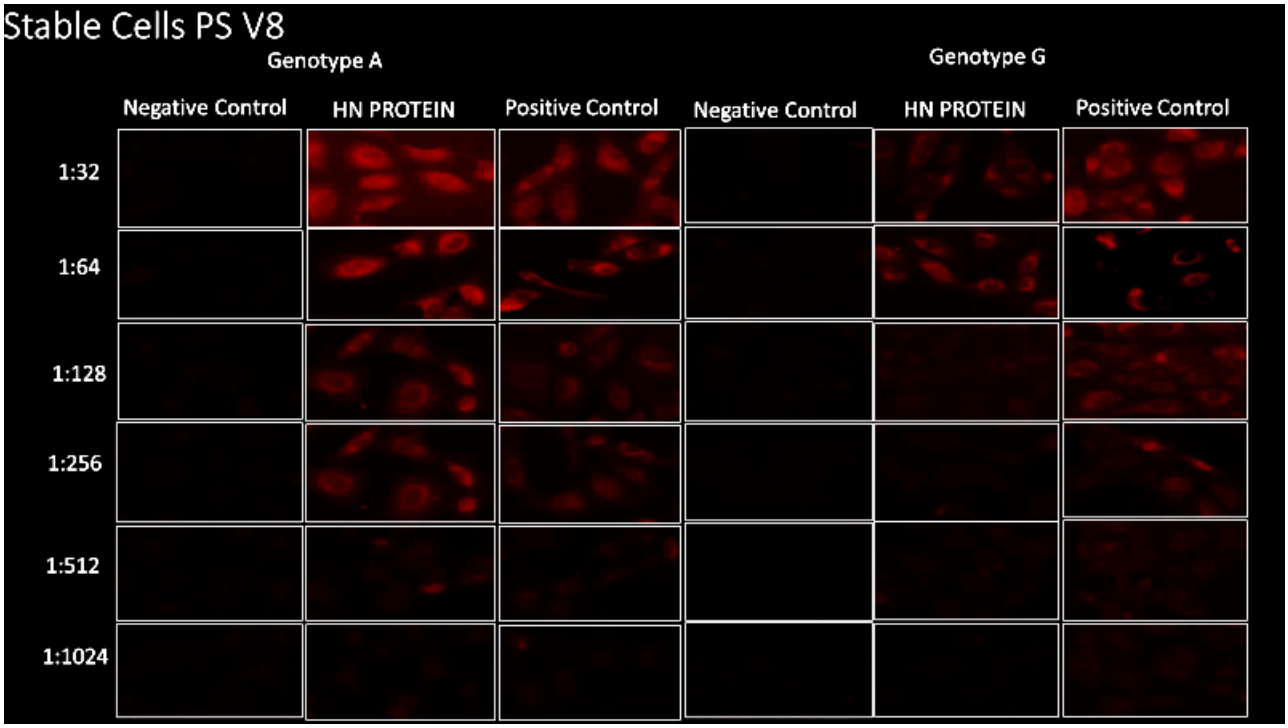


Figure A-11a. Stable cells Vaccine Sample#8.

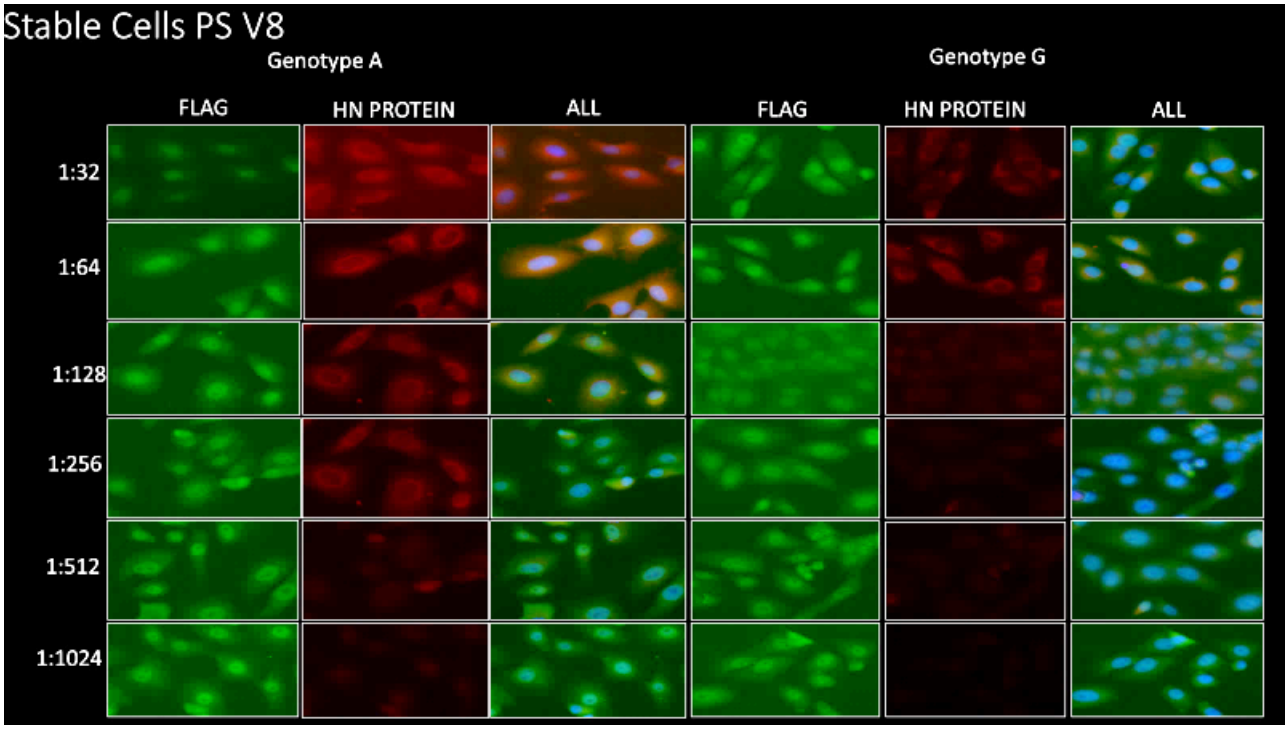


Figure A-11b. Stable cells Vaccine Sample #8.

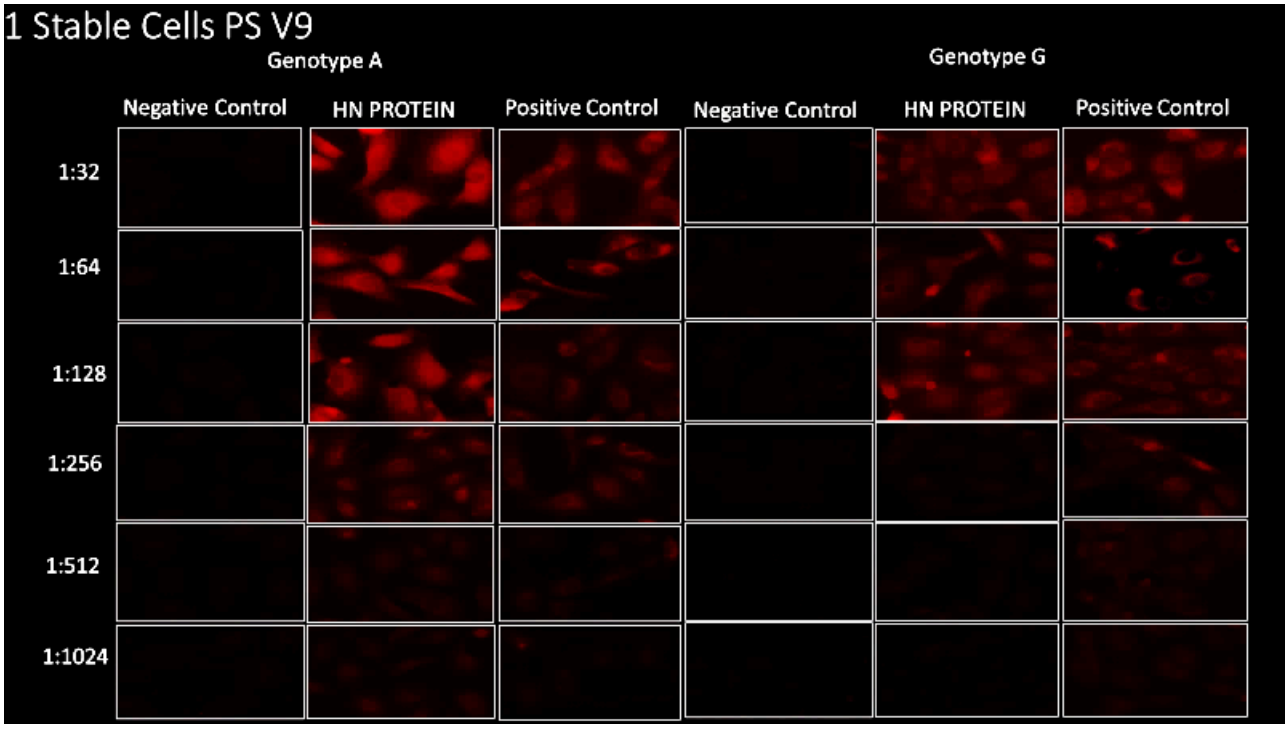


Figure A-12a. Stable cells Vaccine Sample#9.

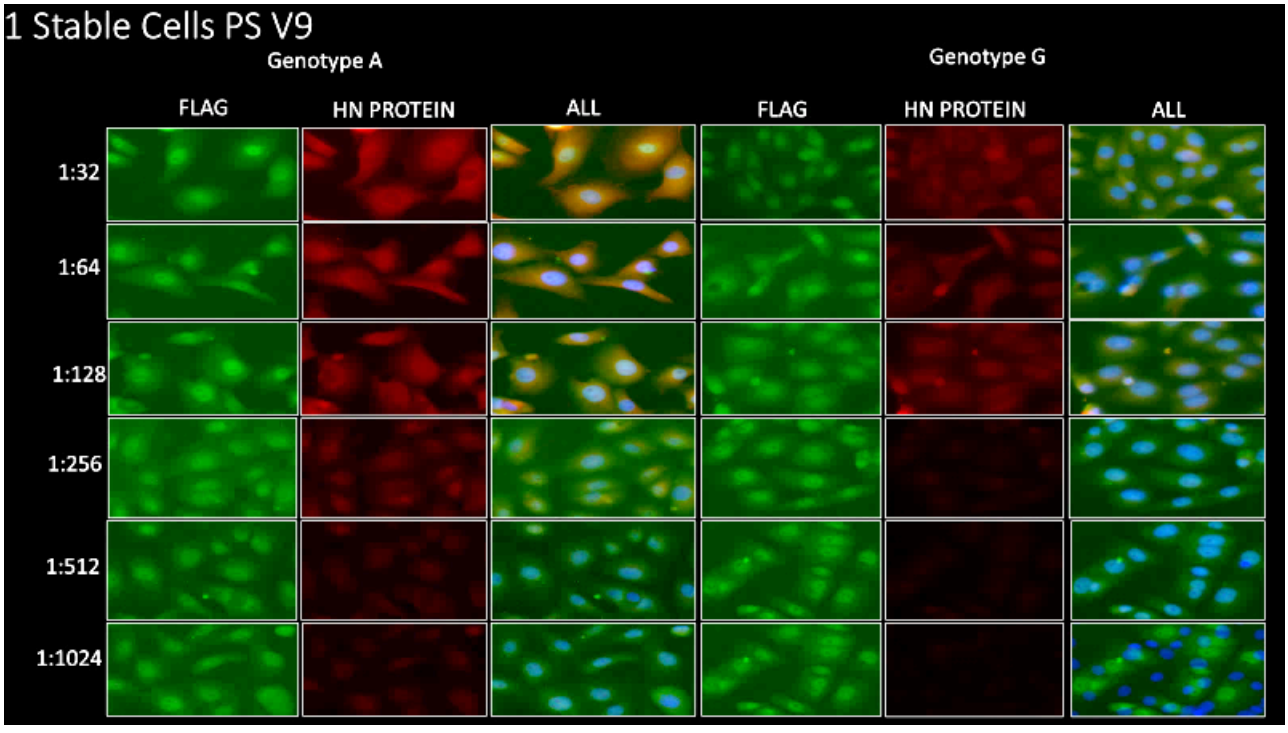


Figure A-12b. Stable cells Vaccine Sample #9.

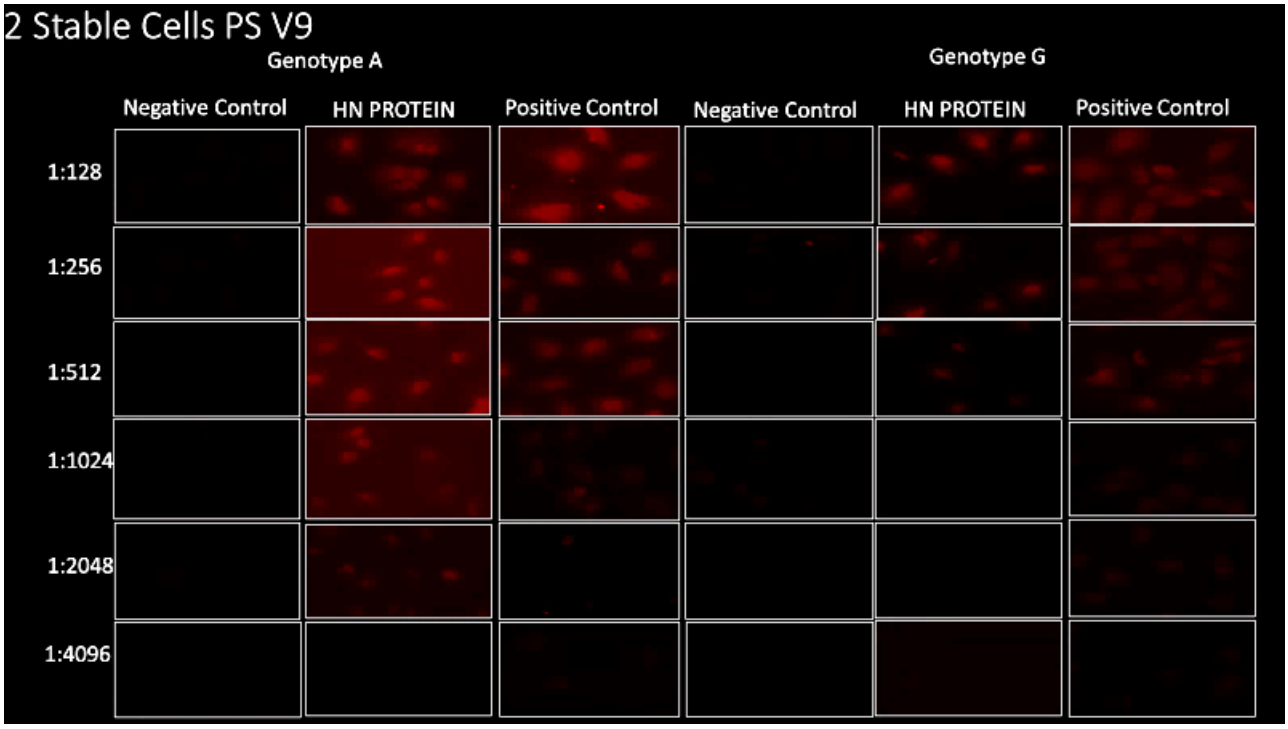


Figure A-12c. Stable cells Vaccine Sample #9.

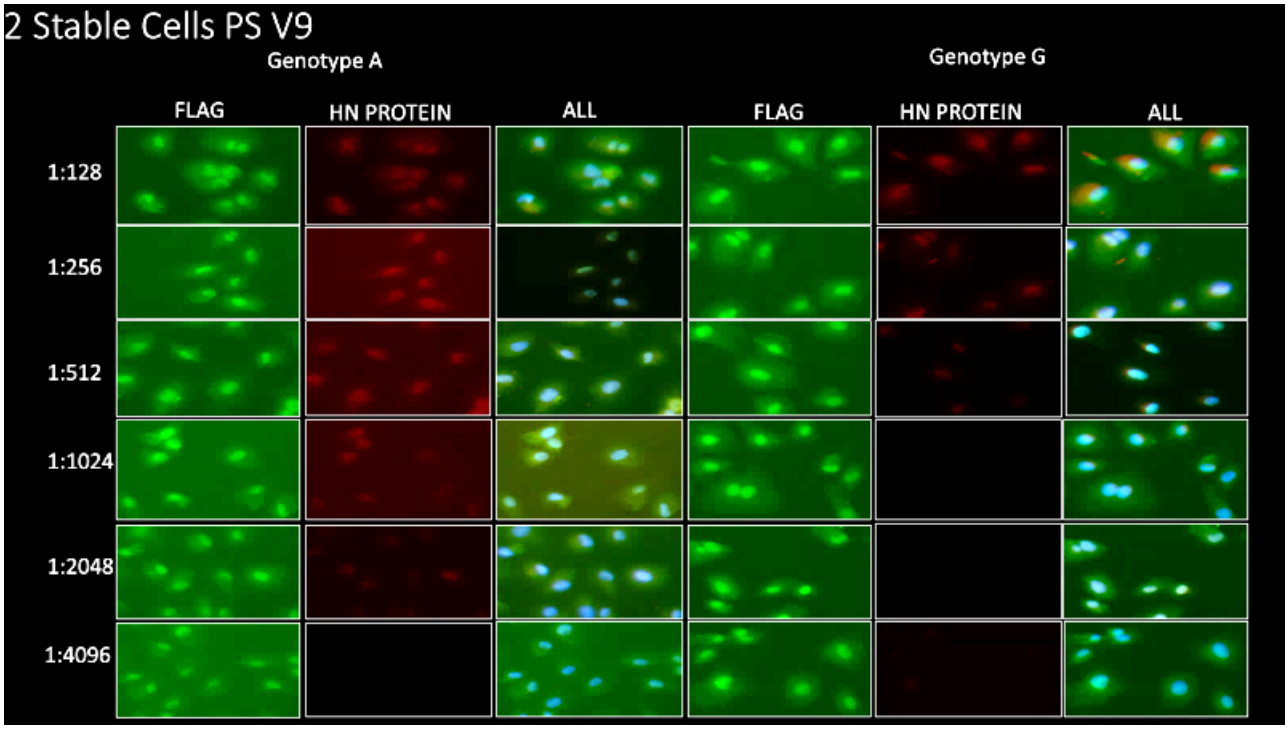


Figure A-12d. Stable cells Vaccine Sample #9.

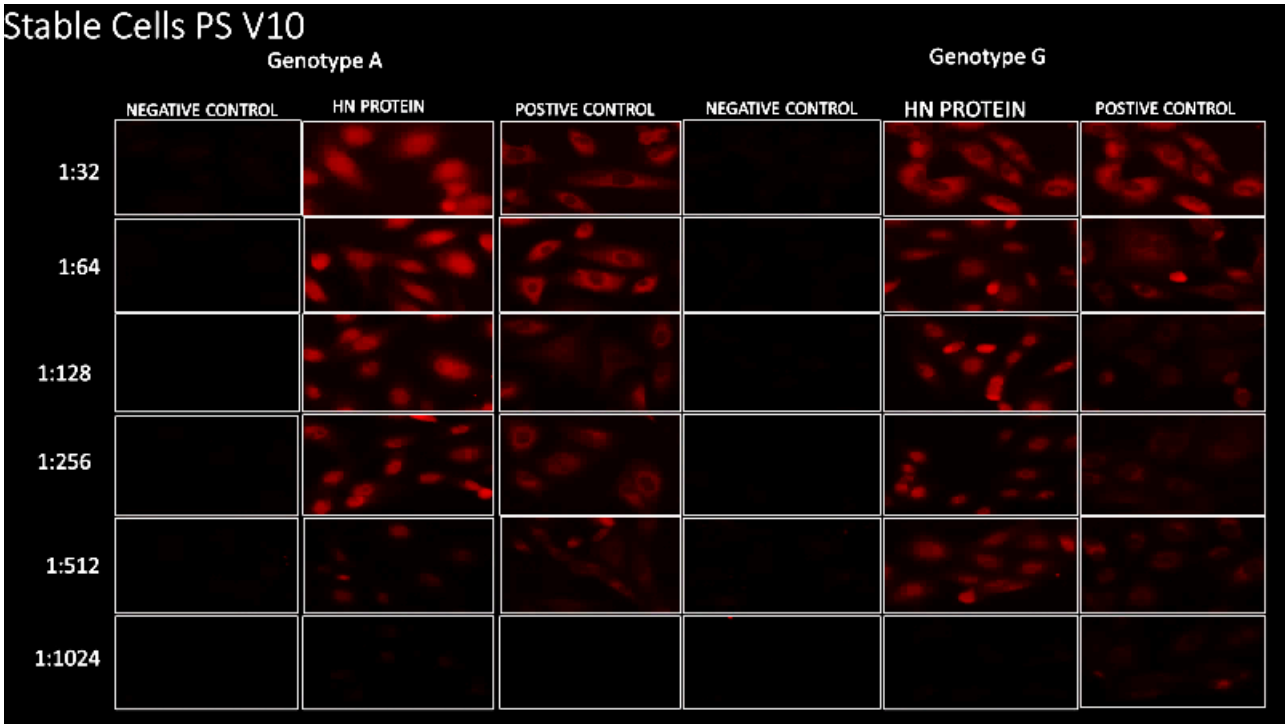


Figure A-13a. Stable cells Vaccine Sample#10.

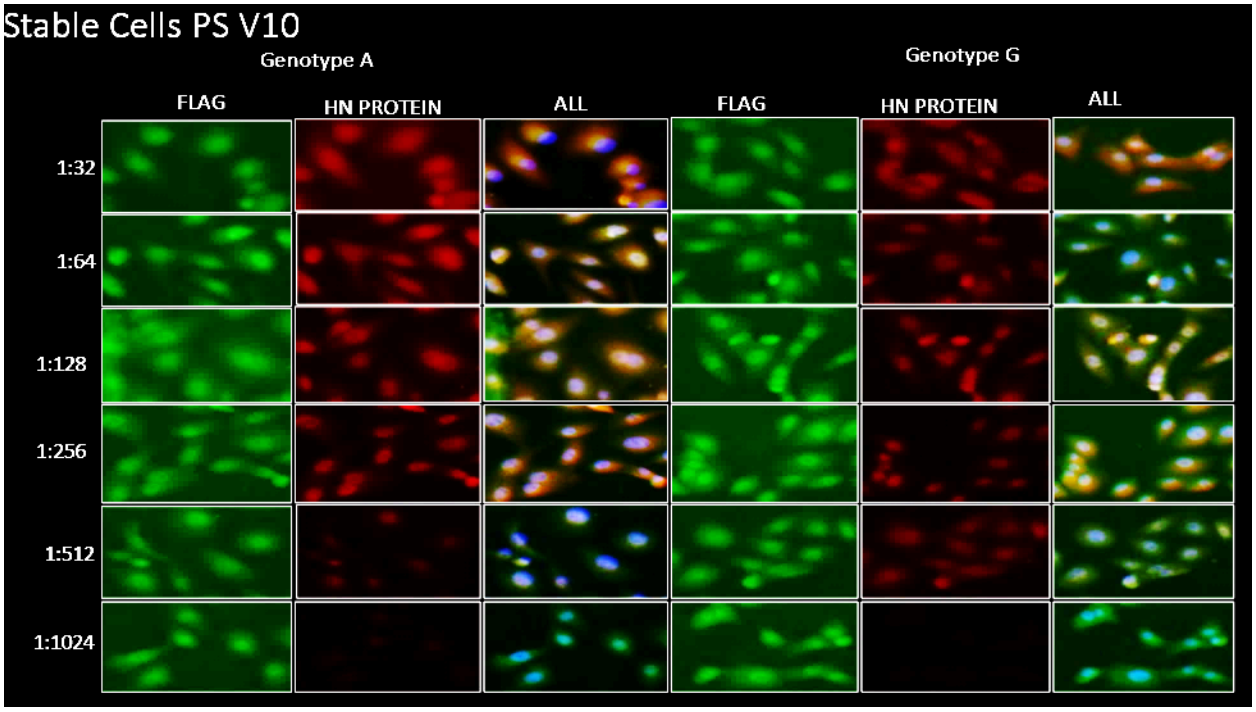


Figure A-13b. Stable cells Vaccine Sample #10.

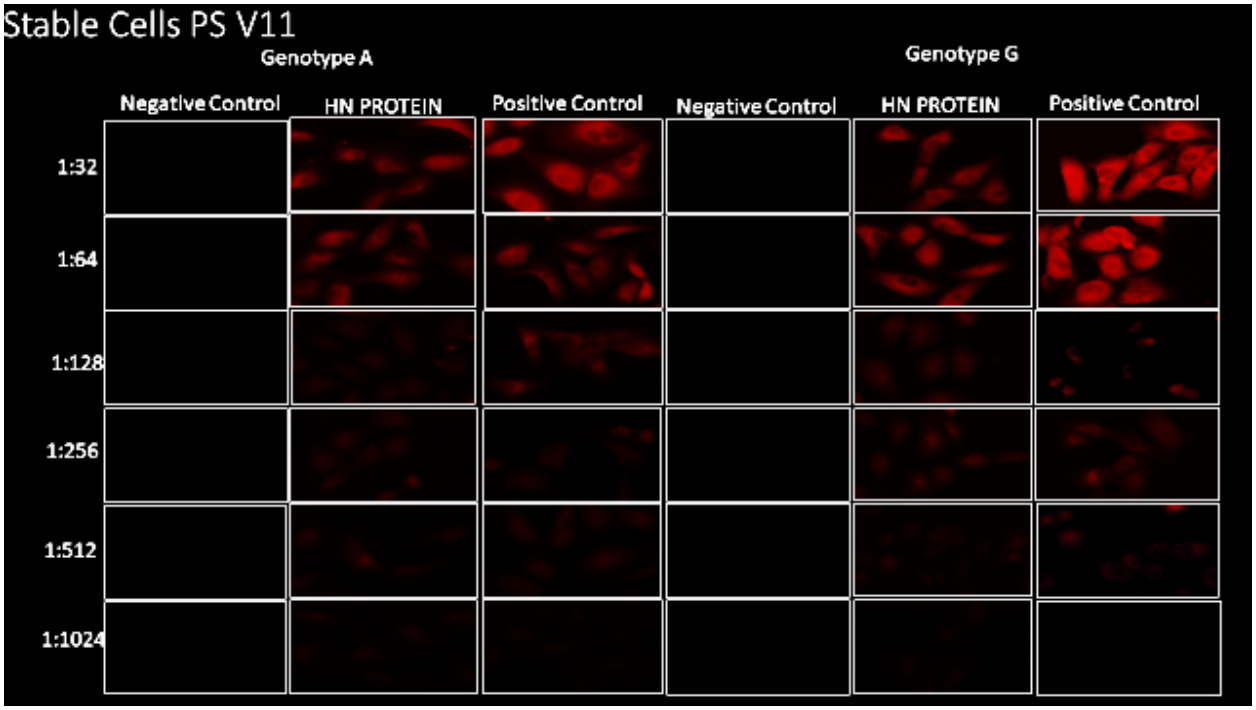


Figure A-14a. Stable cells Vaccine Sample#11.

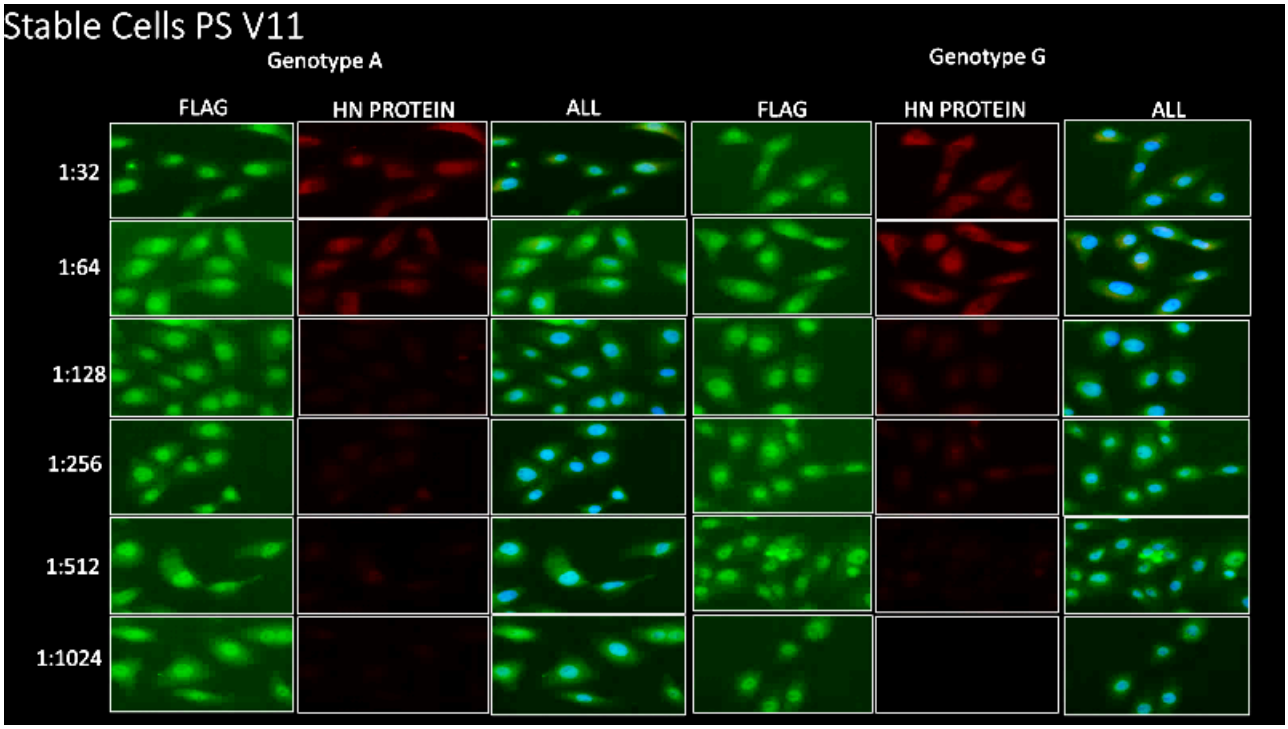


Figure A-14b. Stable cells Vaccine Sample #11.

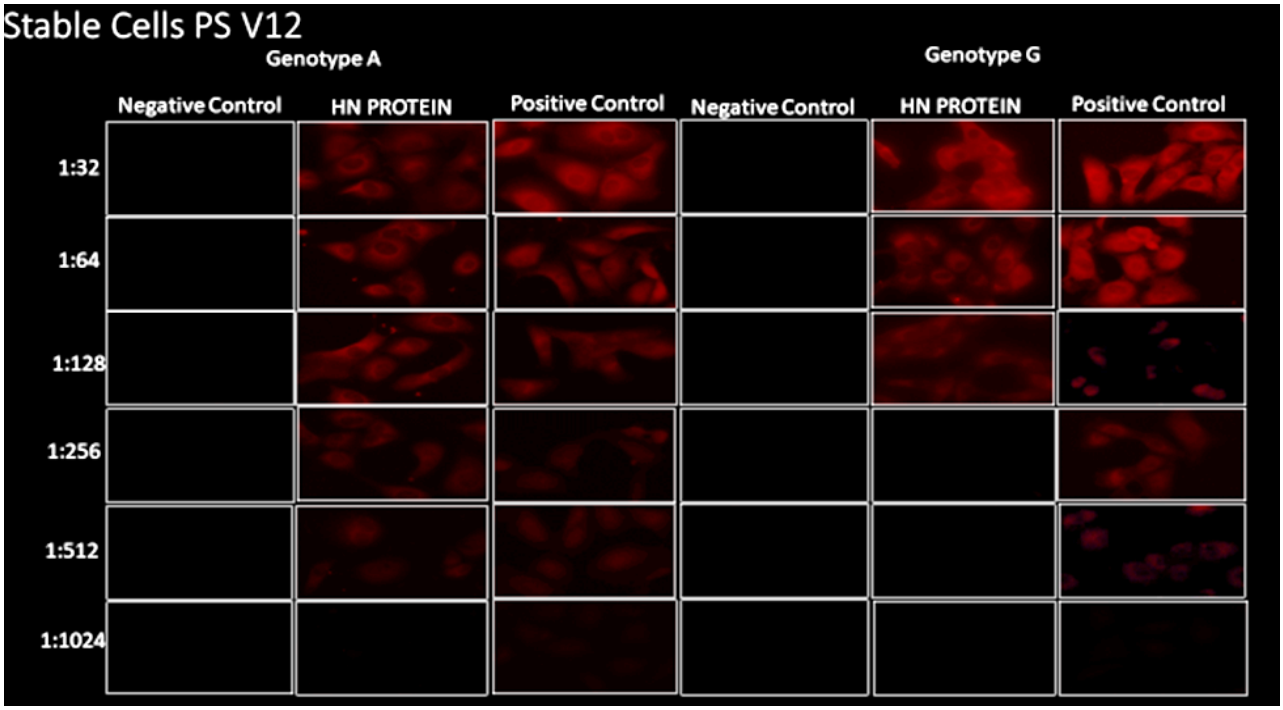


Figure A-15a. Stable cells Vaccine Sample#12.

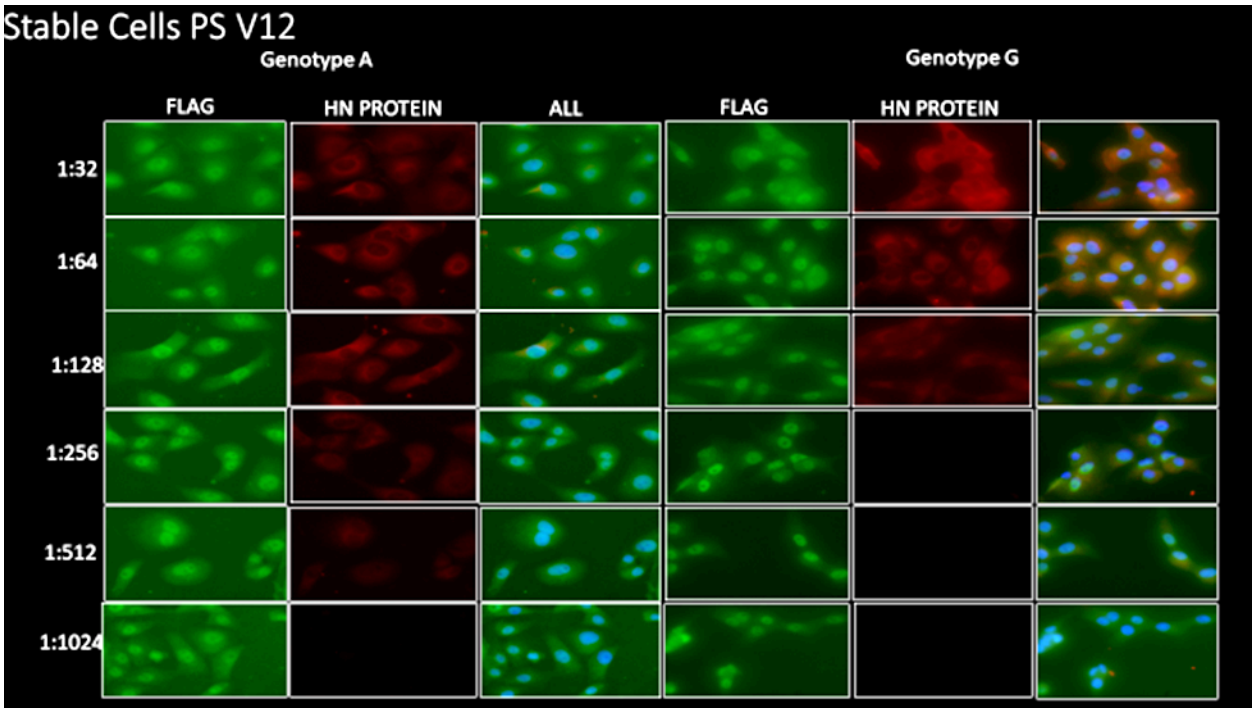


Figure A-15b. Stable cells Vaccine Sample #12.

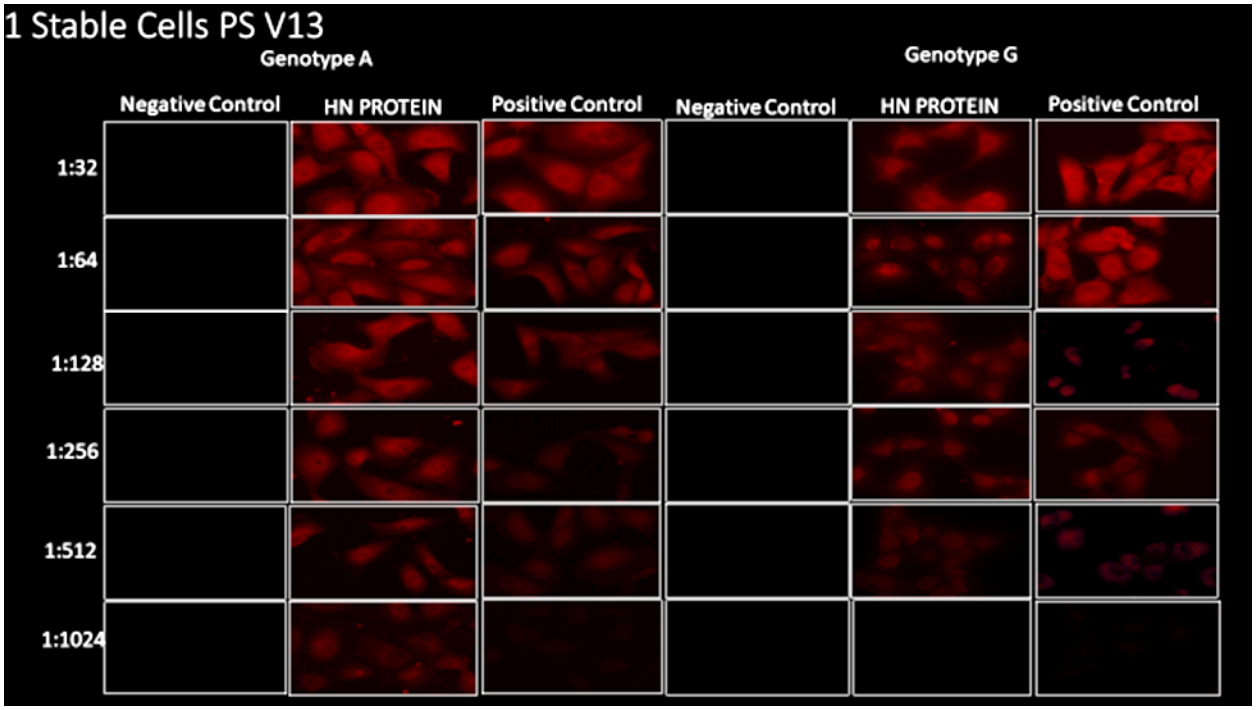


Figure A-16a. Stable cells Vaccine Sample#13.

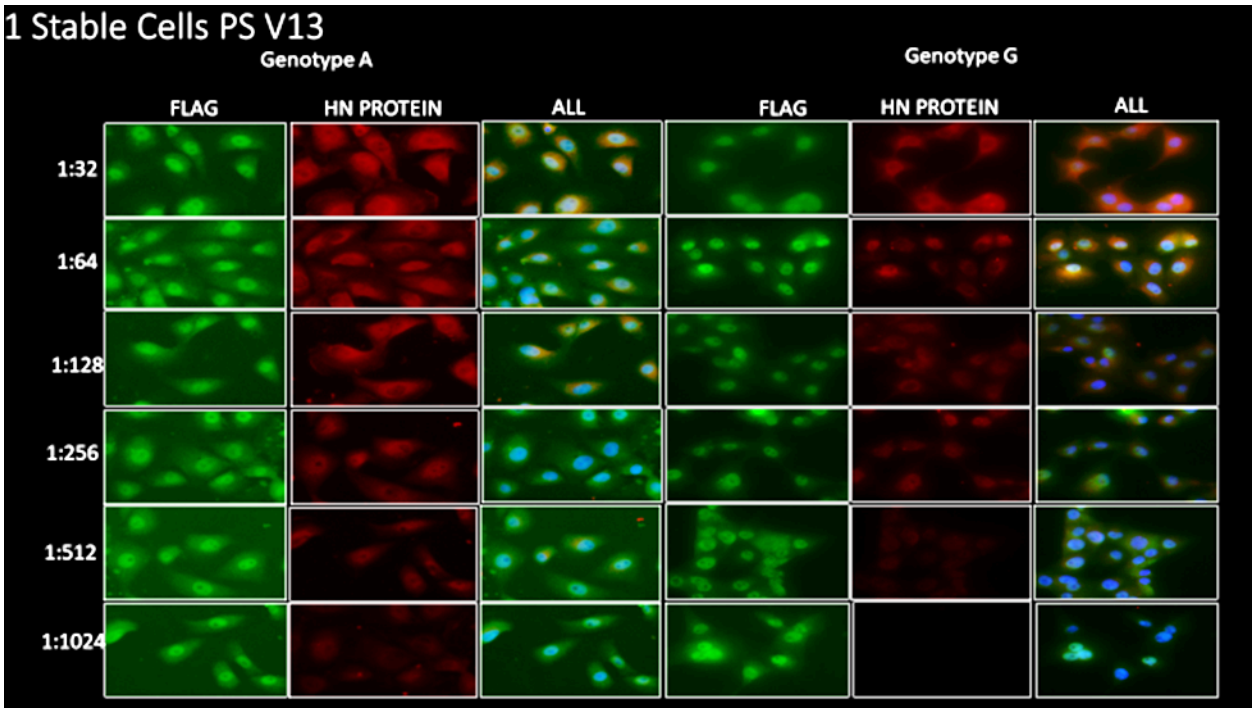


Figure A-16b. Stable cells Vaccine Sample #13.

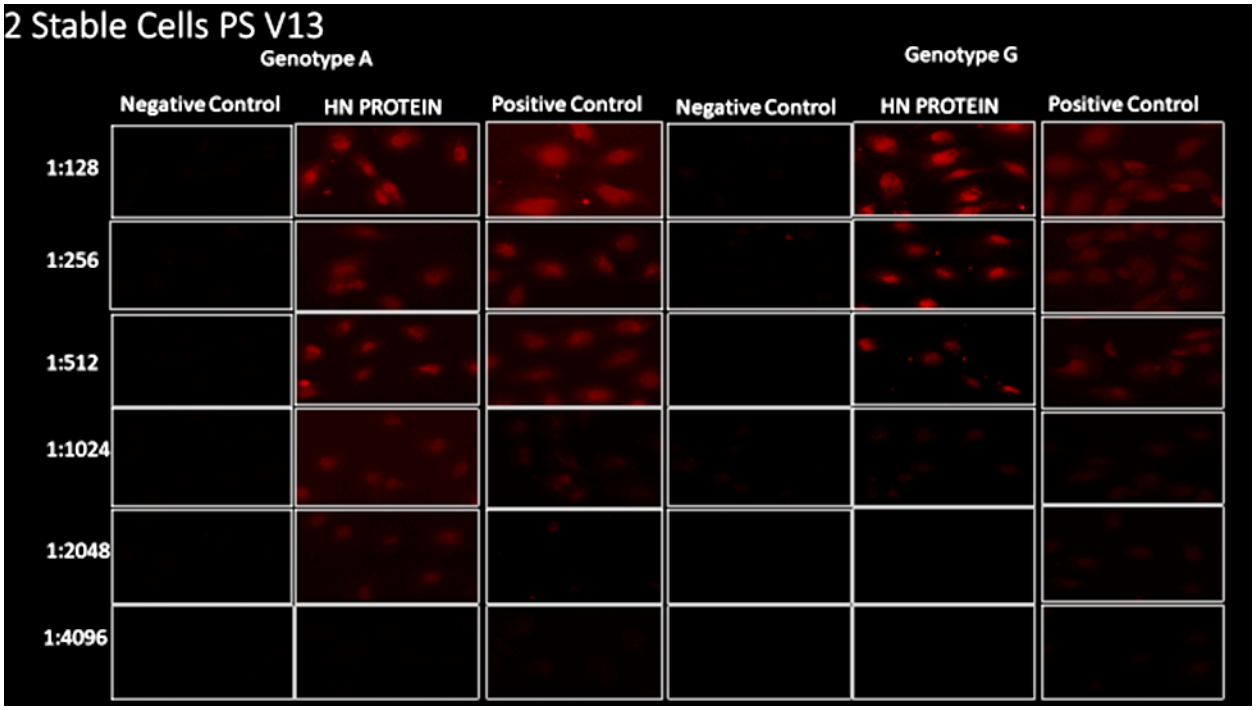


Figure A-16c. Stable cells Vaccine Sample #13.

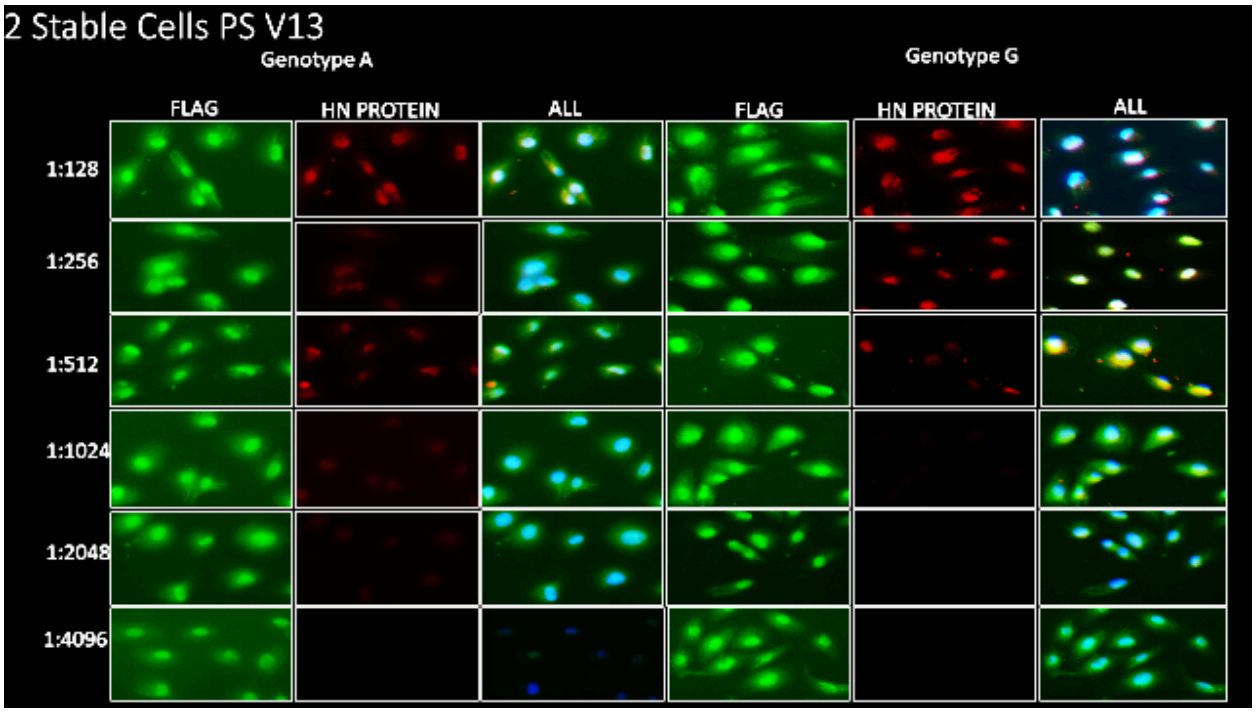


Figure A-16d. Stable cells Vaccine Sample #13.

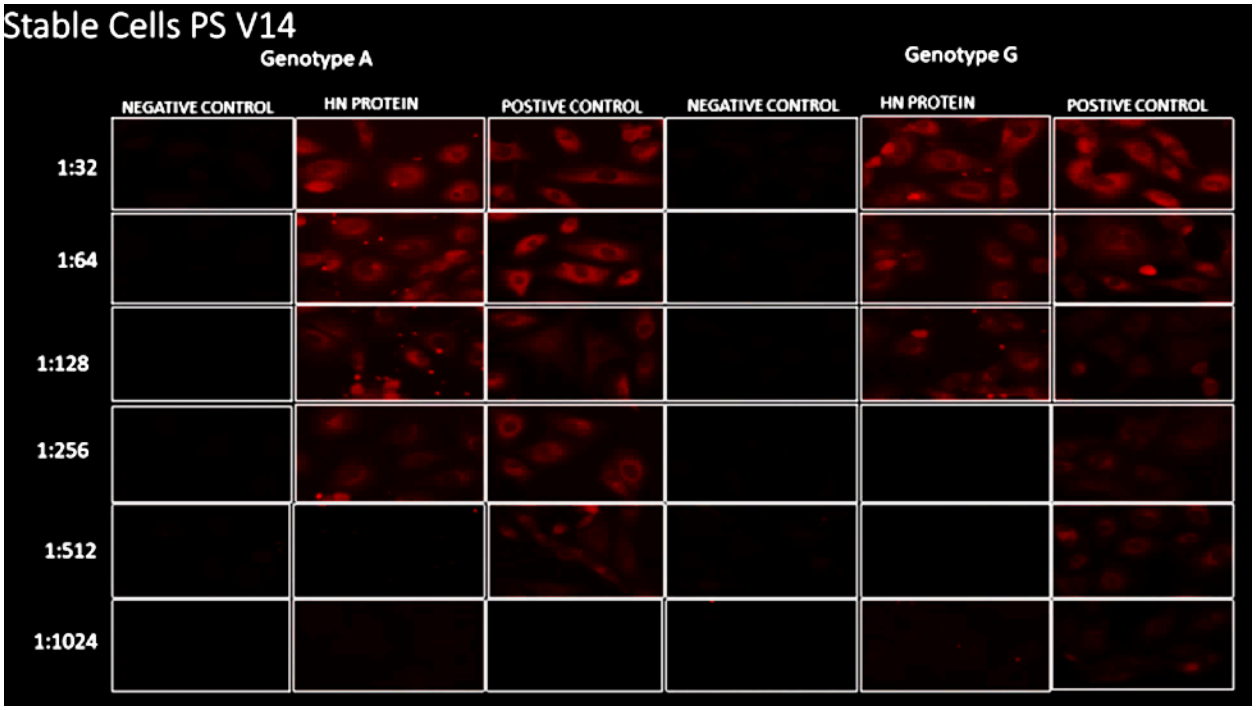


Figure A-17a. Stable cells Vaccine Sample#14.

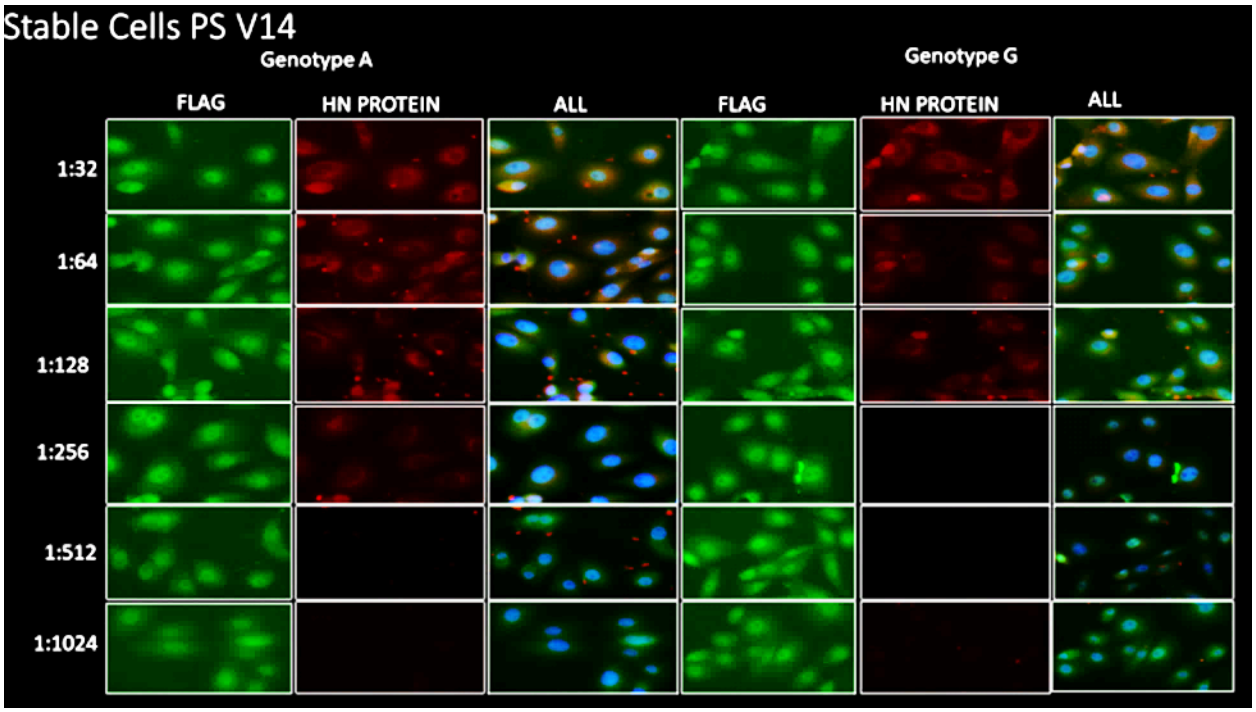


Figure A-17b. Stable cells Vaccine Sample #14.

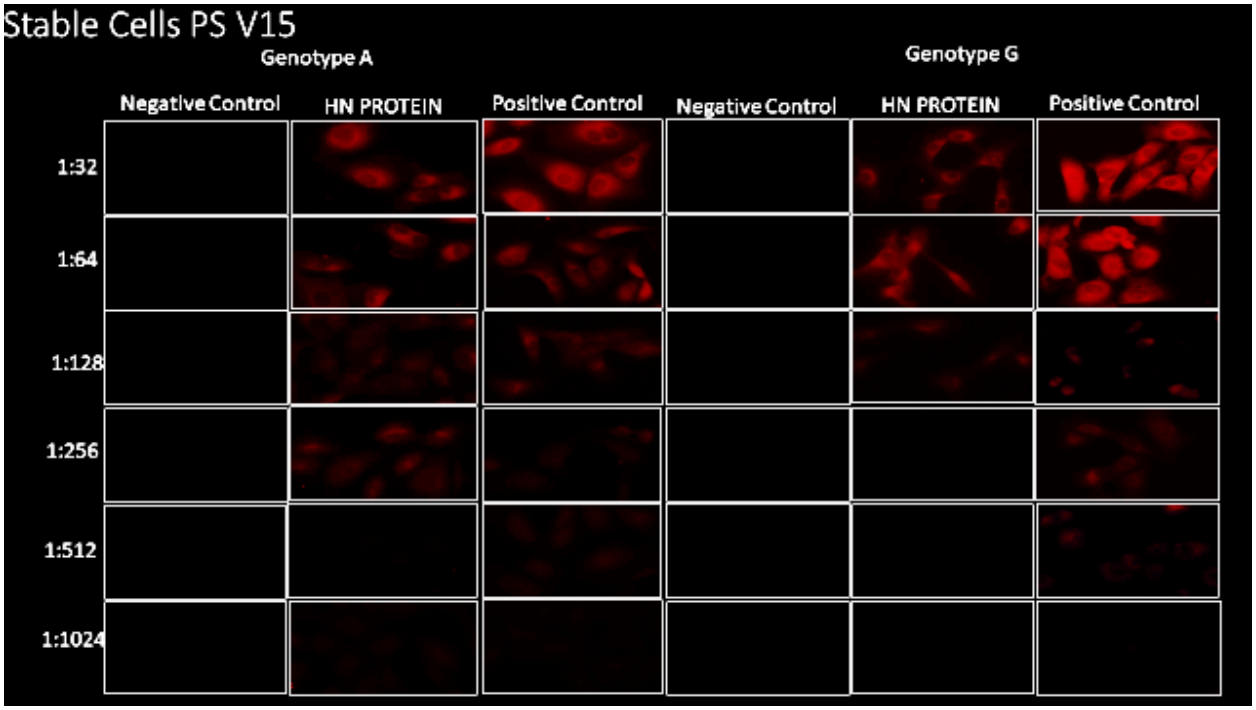


Figure A-18a. Stable cells Vaccine Sample#15.

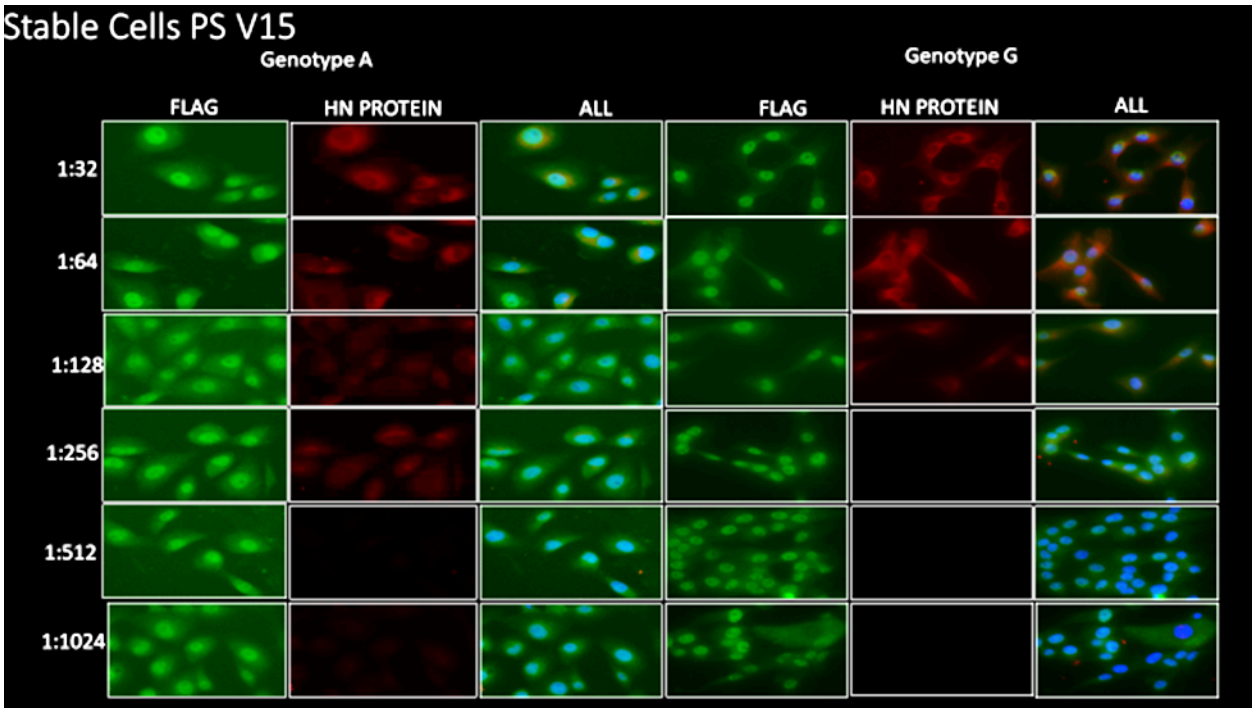


Figure A-18b. Stable cells Vaccine Sample #15.

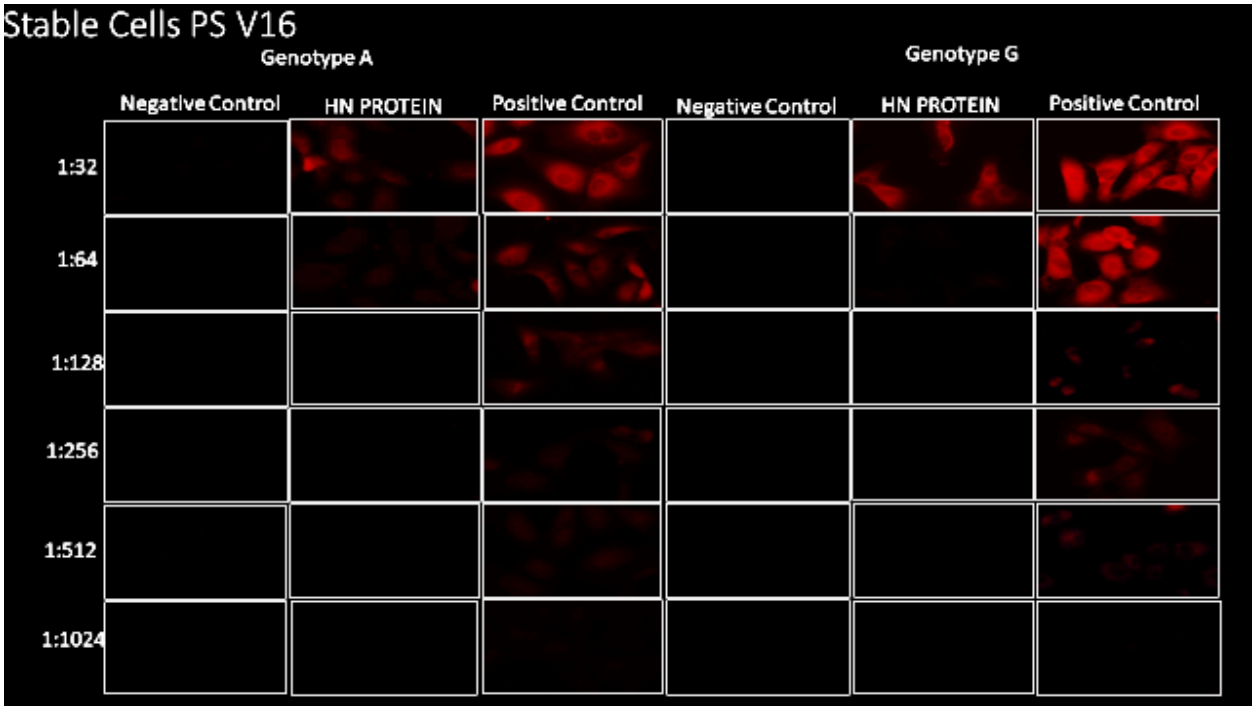


Figure A-19a. Stable cells Vaccine Sample#16.

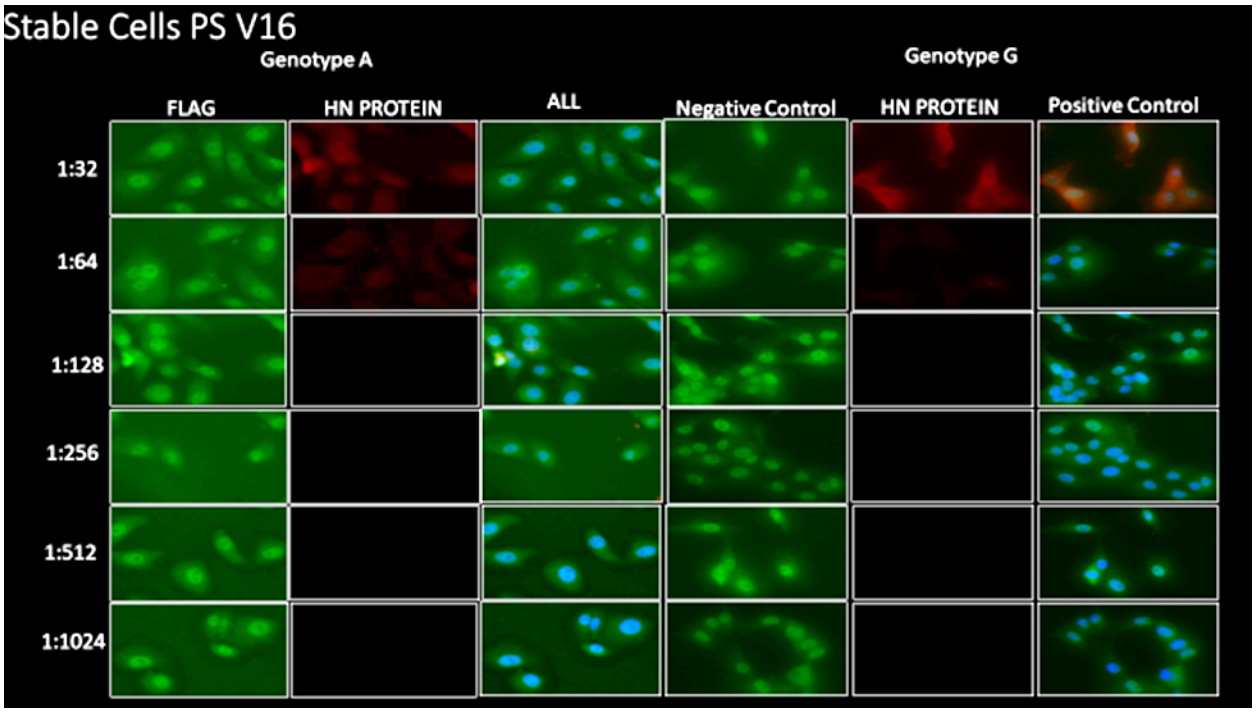


Figure A-19b. Stable cells Vaccine Sample #16.

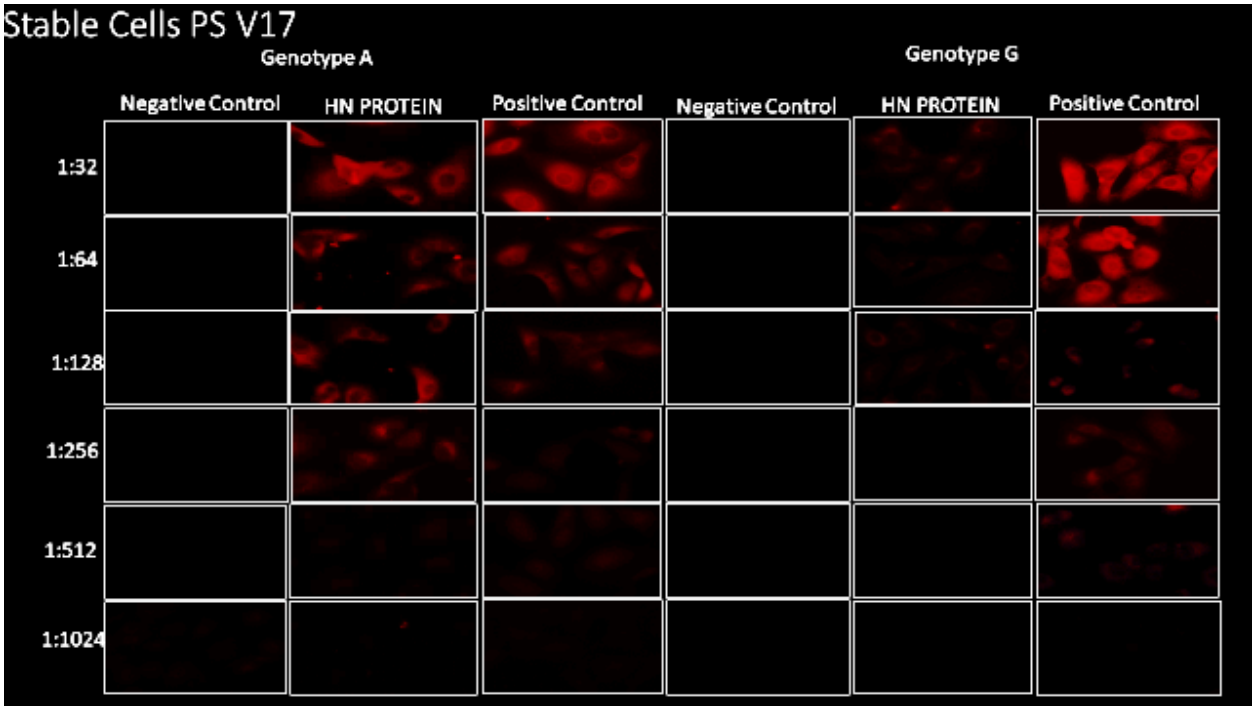


Figure A-20a. Stable cells Vaccine Sample#17.

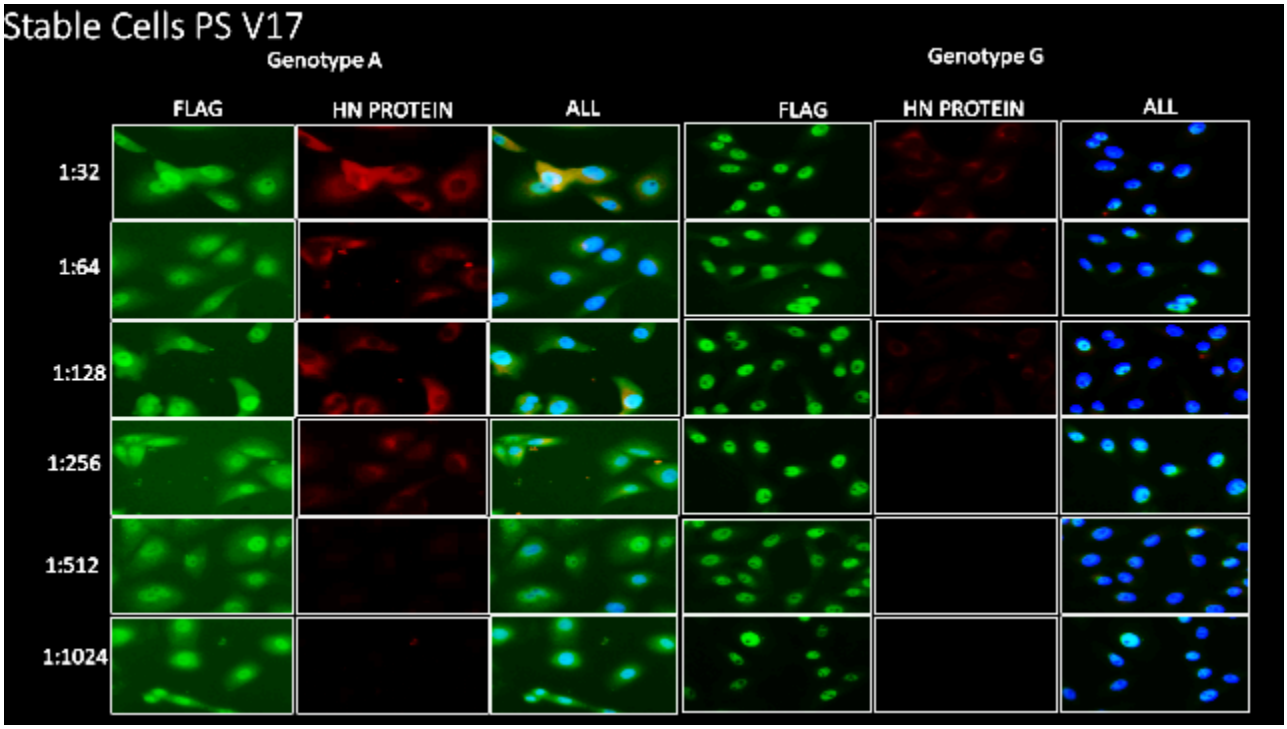


Figure A-20a. Stable cells Vaccine Sample#17.

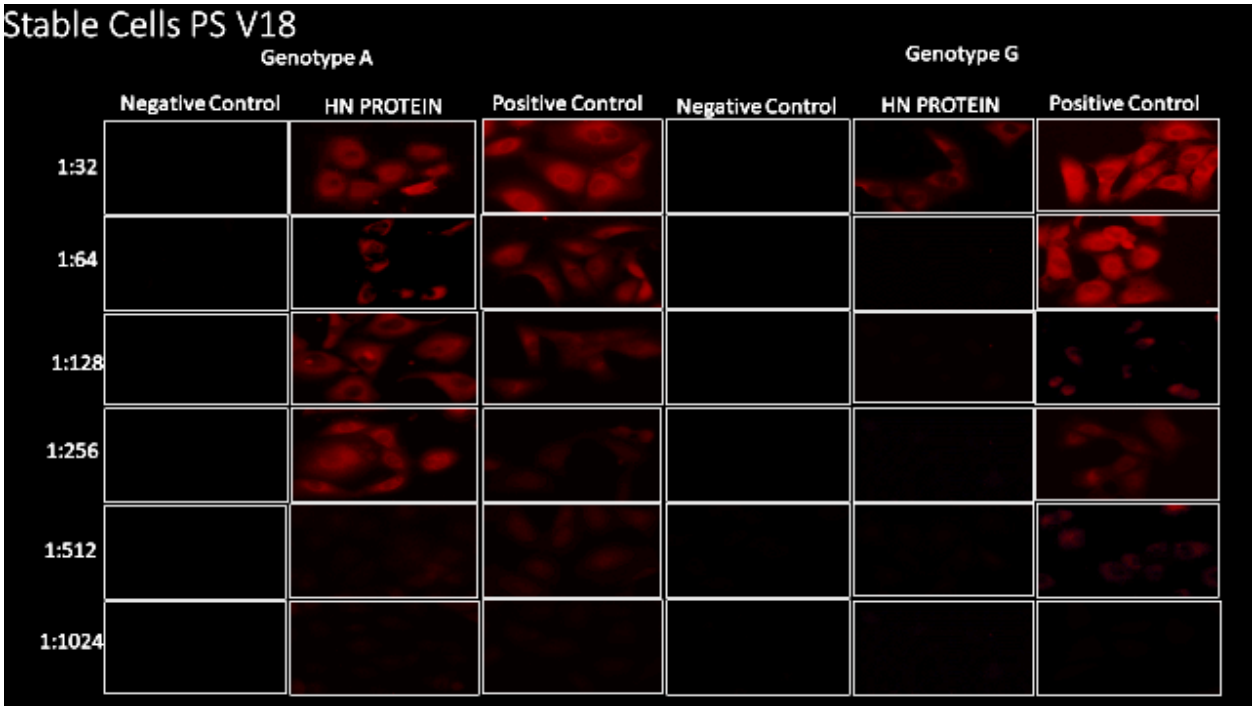


Figure A-21a. Stable cells Vaccine Sample#18.

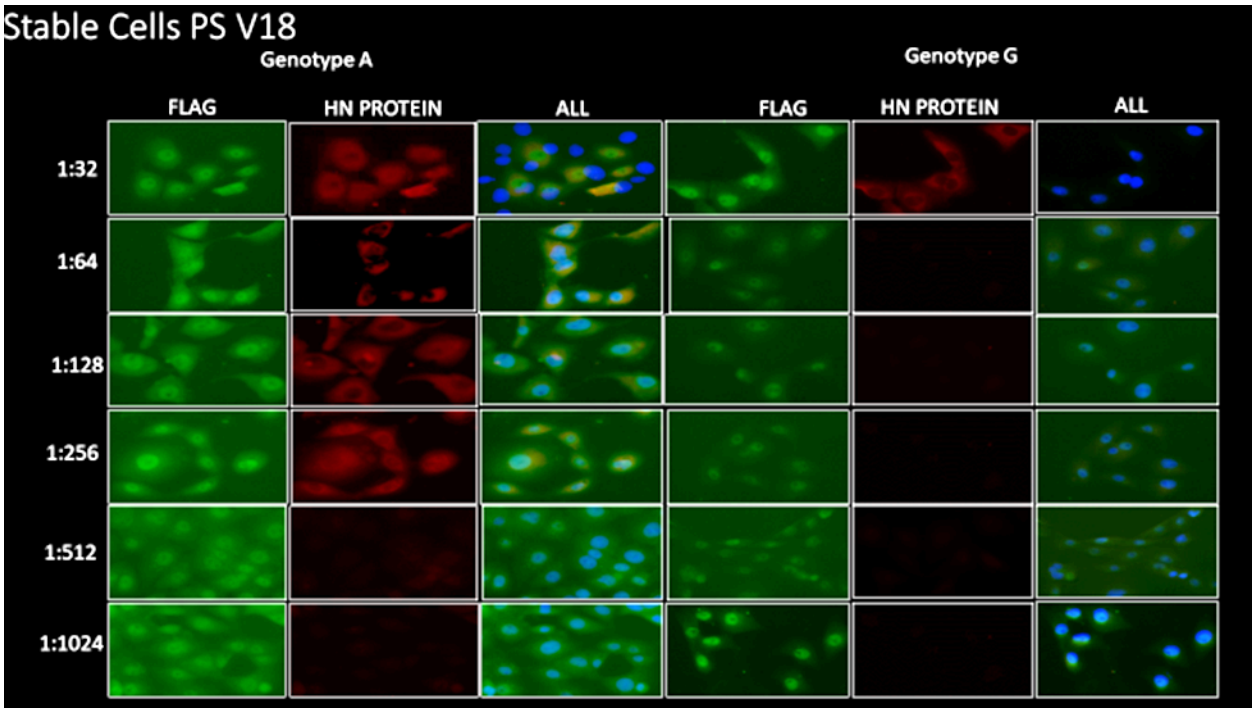


Figure A-21b. Stable cells Vaccine Sample #18.

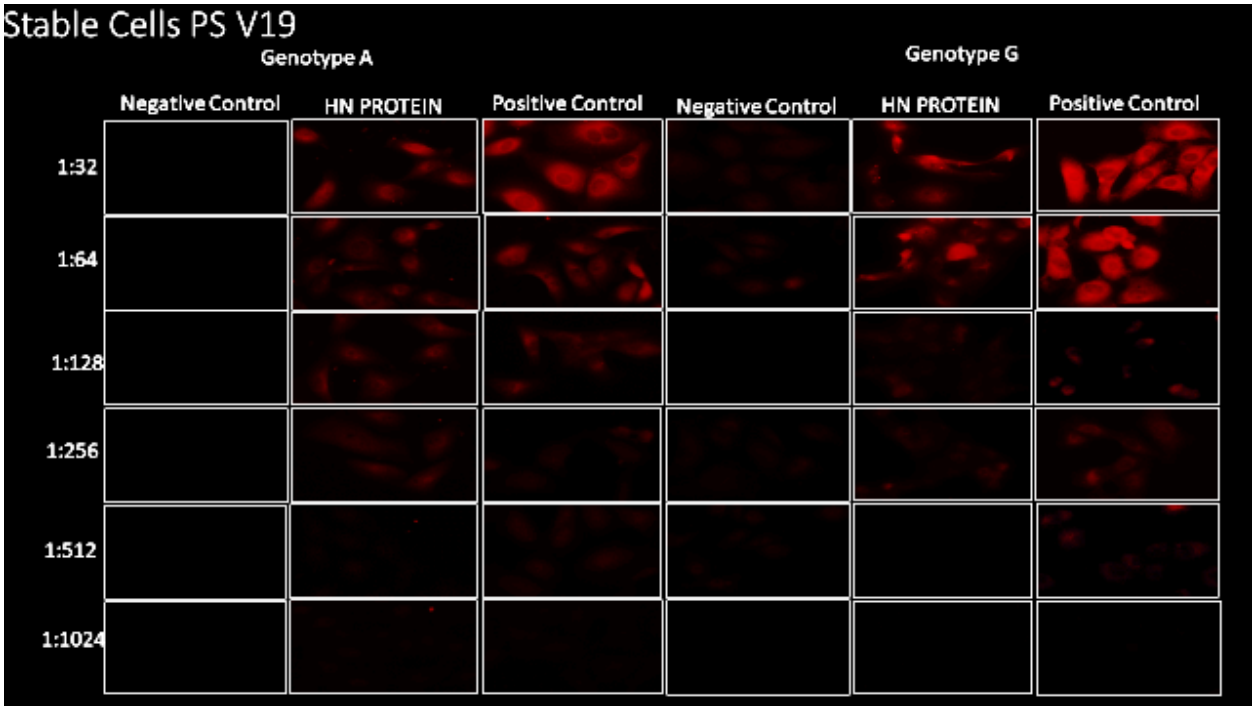


Figure A-22a. Stable cells Vaccine Sample#19.

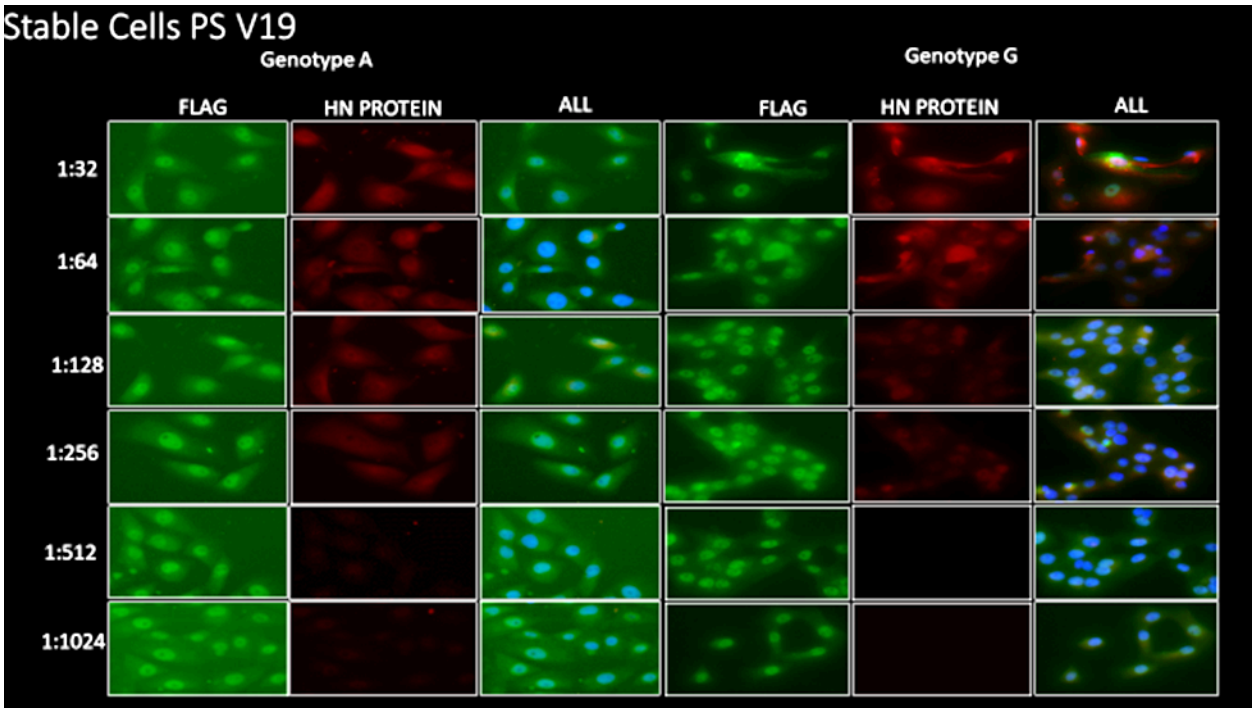


Figure A-22b. Stable cells Vaccine Sample #19.

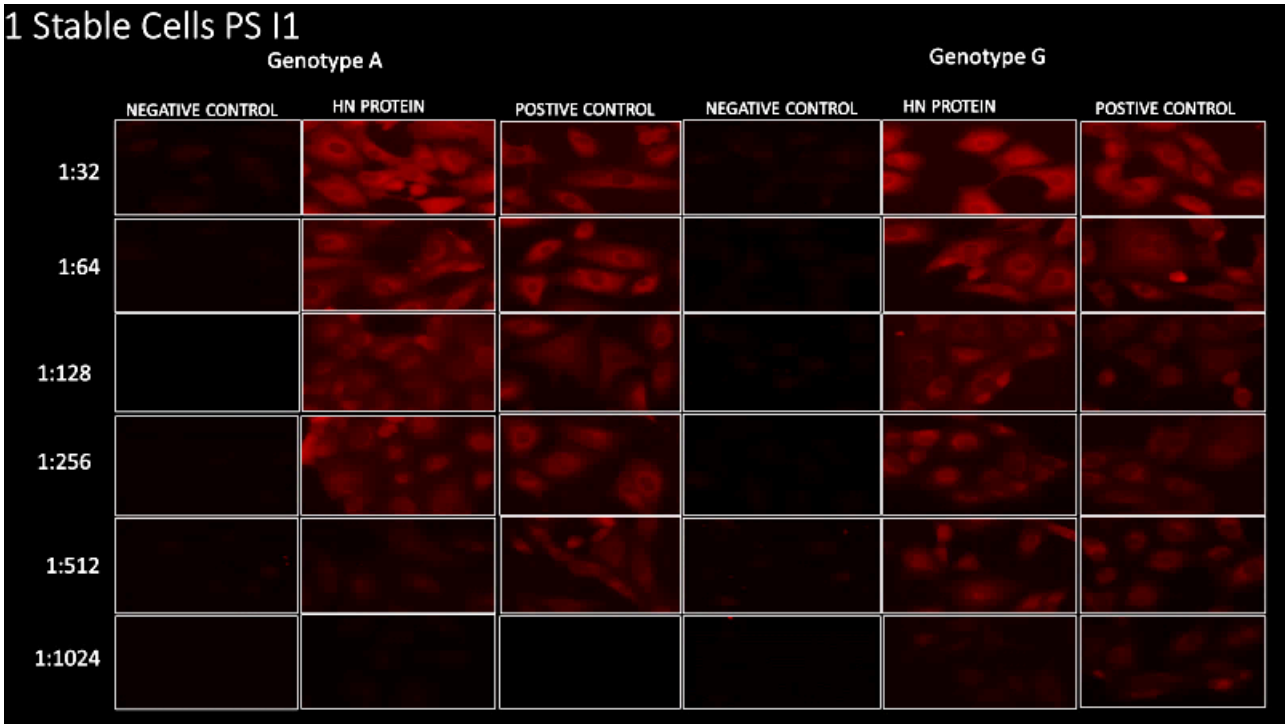


Figure A-23a. Stable cells Infected Sample#1.

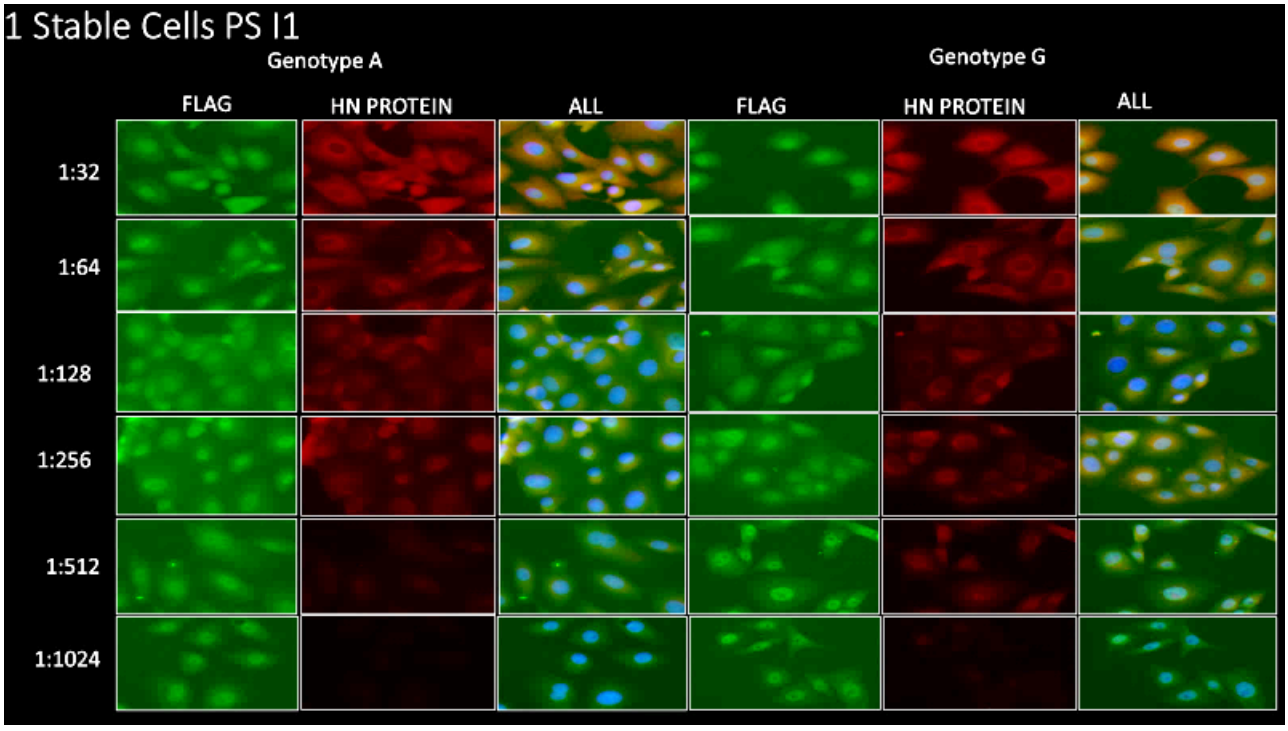


Figure A-23b. Stable cells Infected Sample #1.

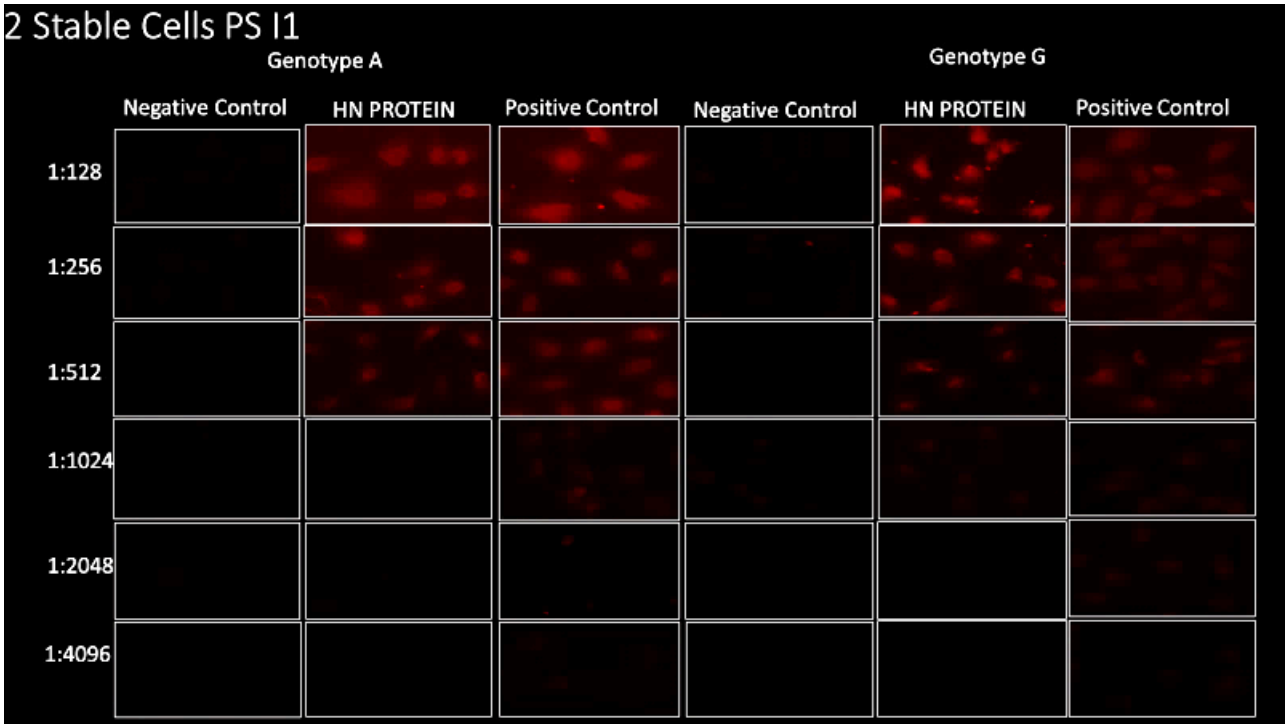


Figure A-23c. Stable cells Infected Sample #1.

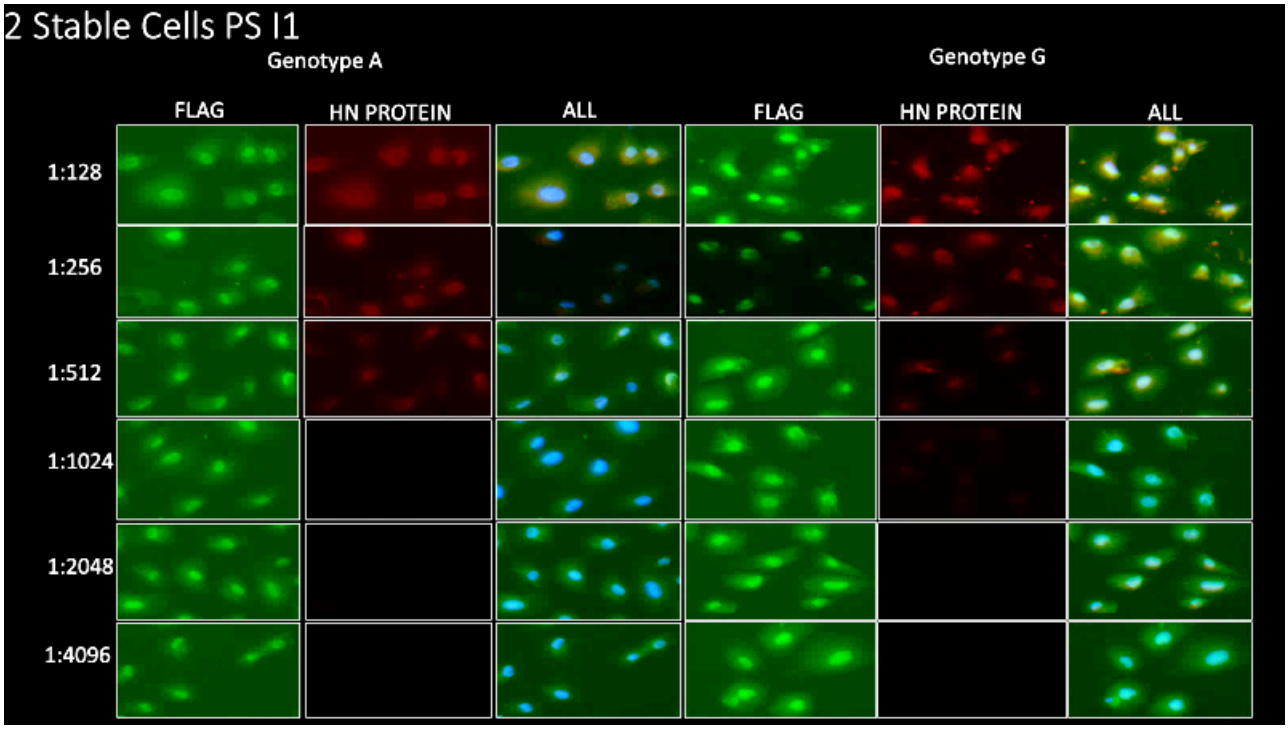


Figure A-23d. Stable cells Infected Sample #1.

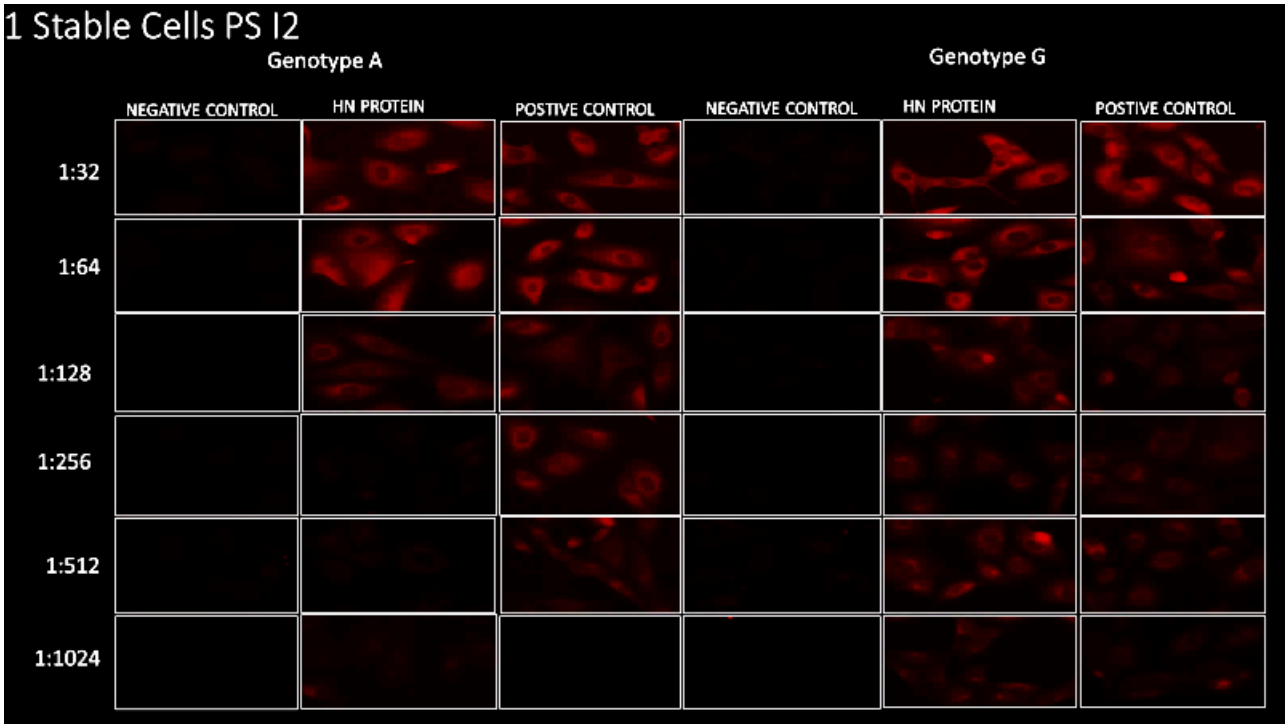


Figure A-24a. Stable cells Infected Sample#2.

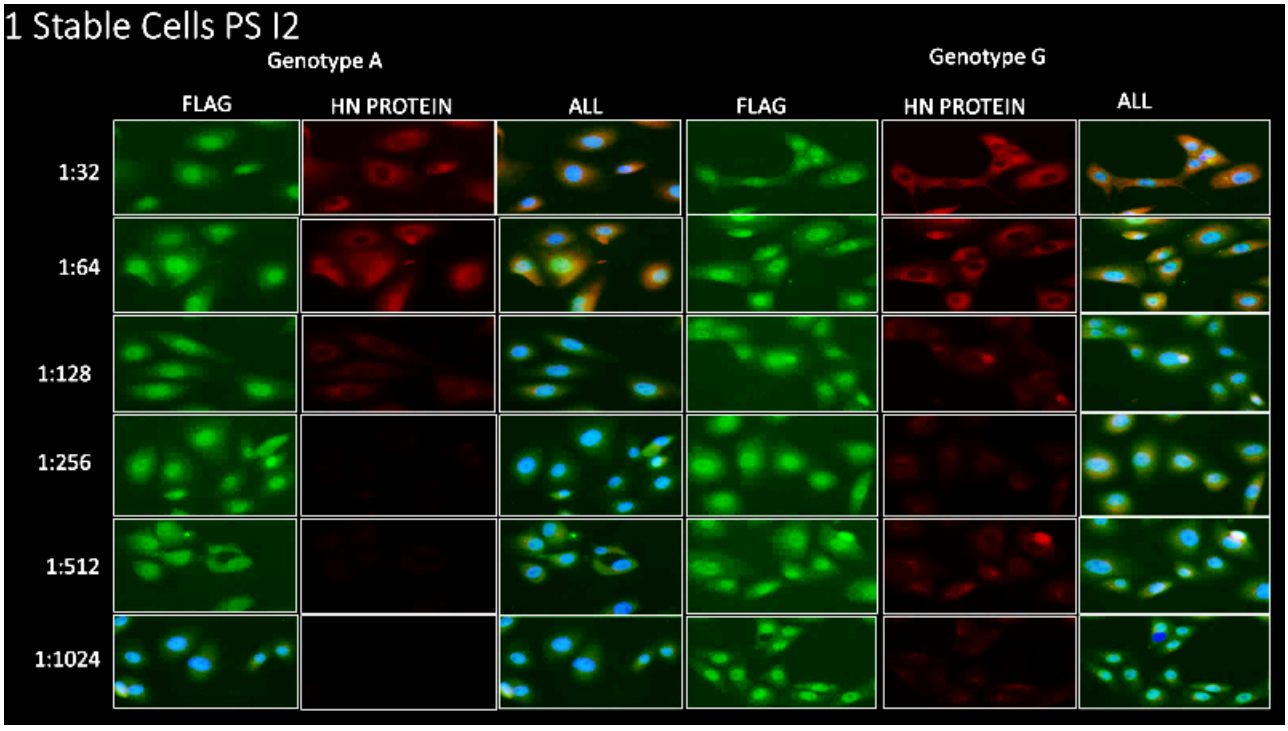


Figure A-24b. Stable cells Infected Sample #2.

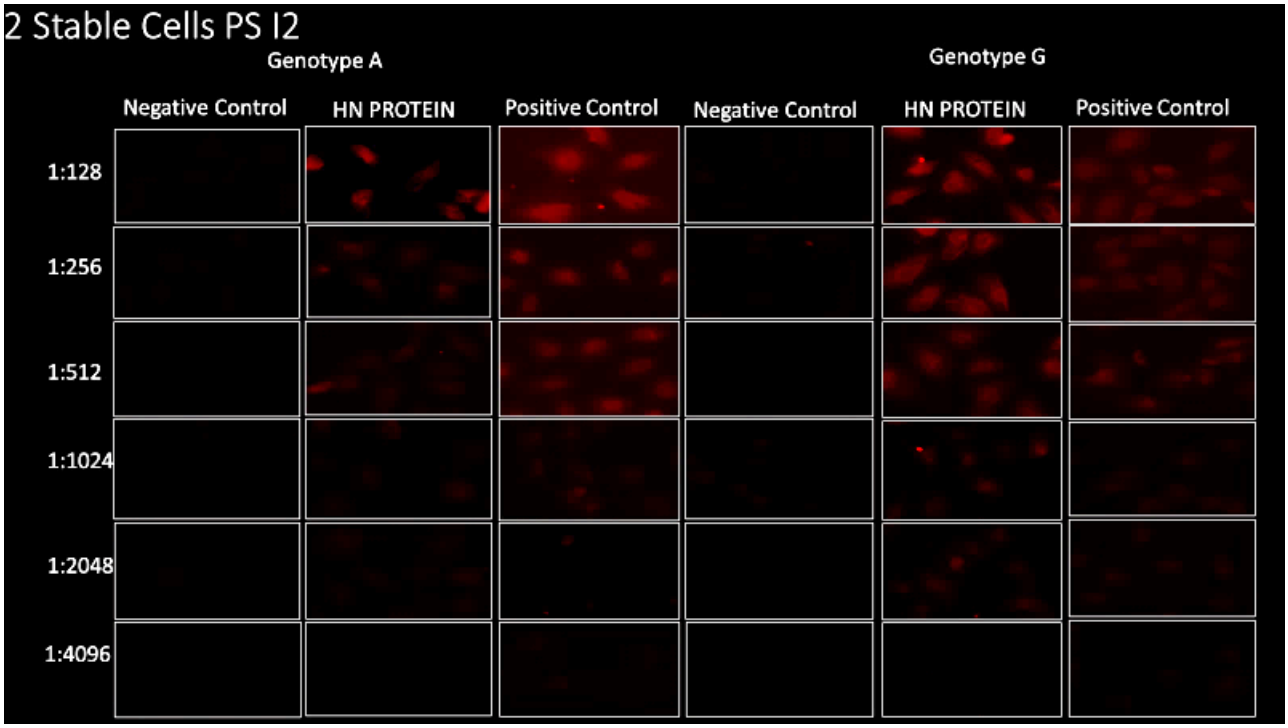


Figure A-24c. Stable cells Infected Sample #2.

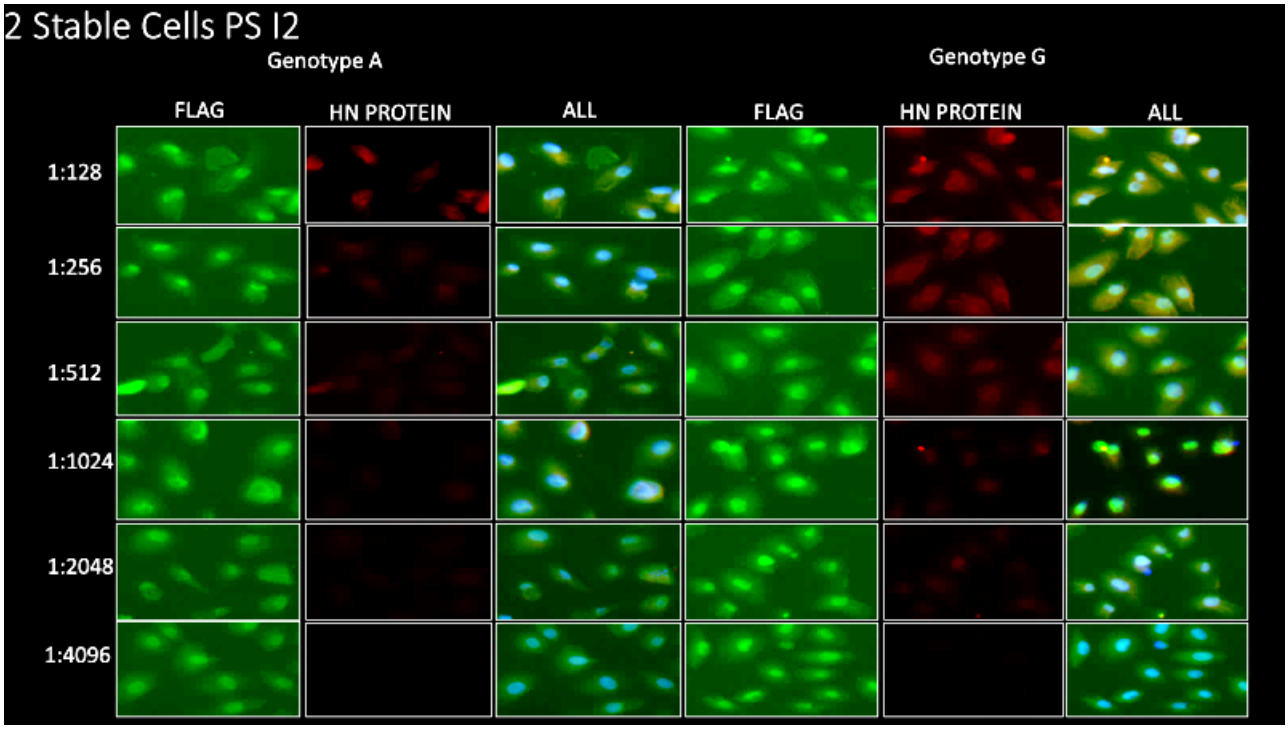


Figure A-24d. Stable cells Infected Sample #2.

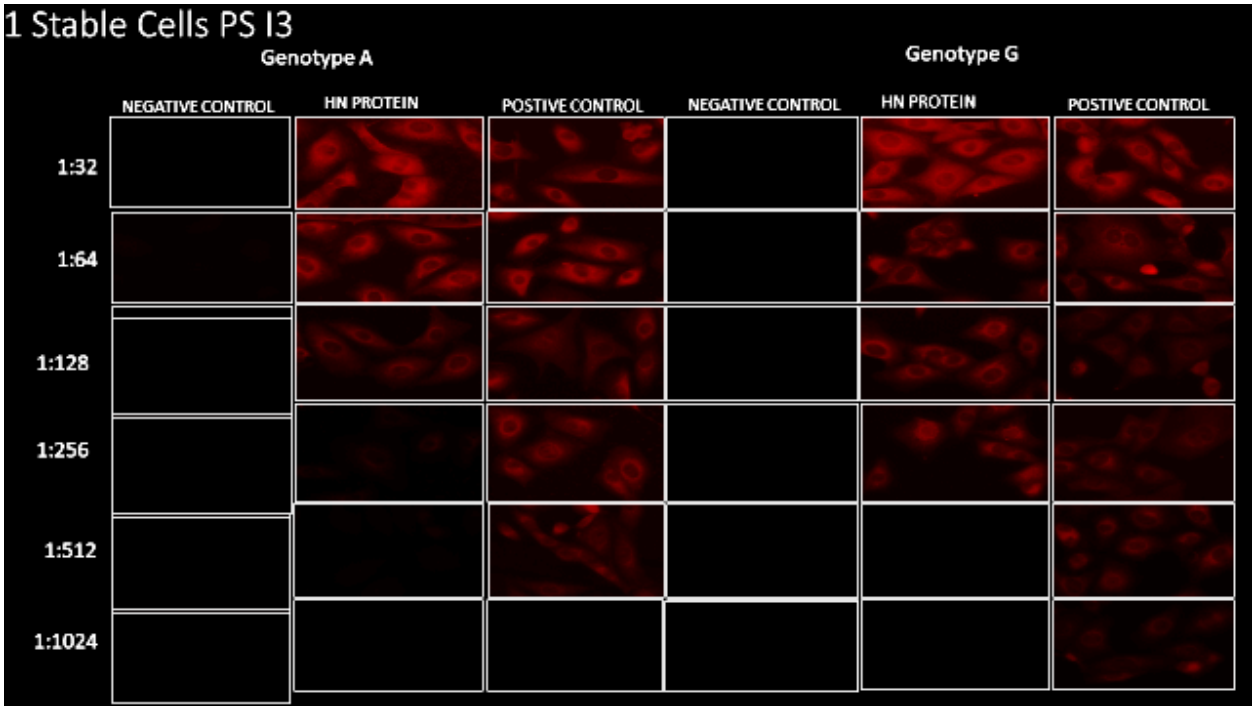


Figure A-25a. Stable cells Infected Sample#3.

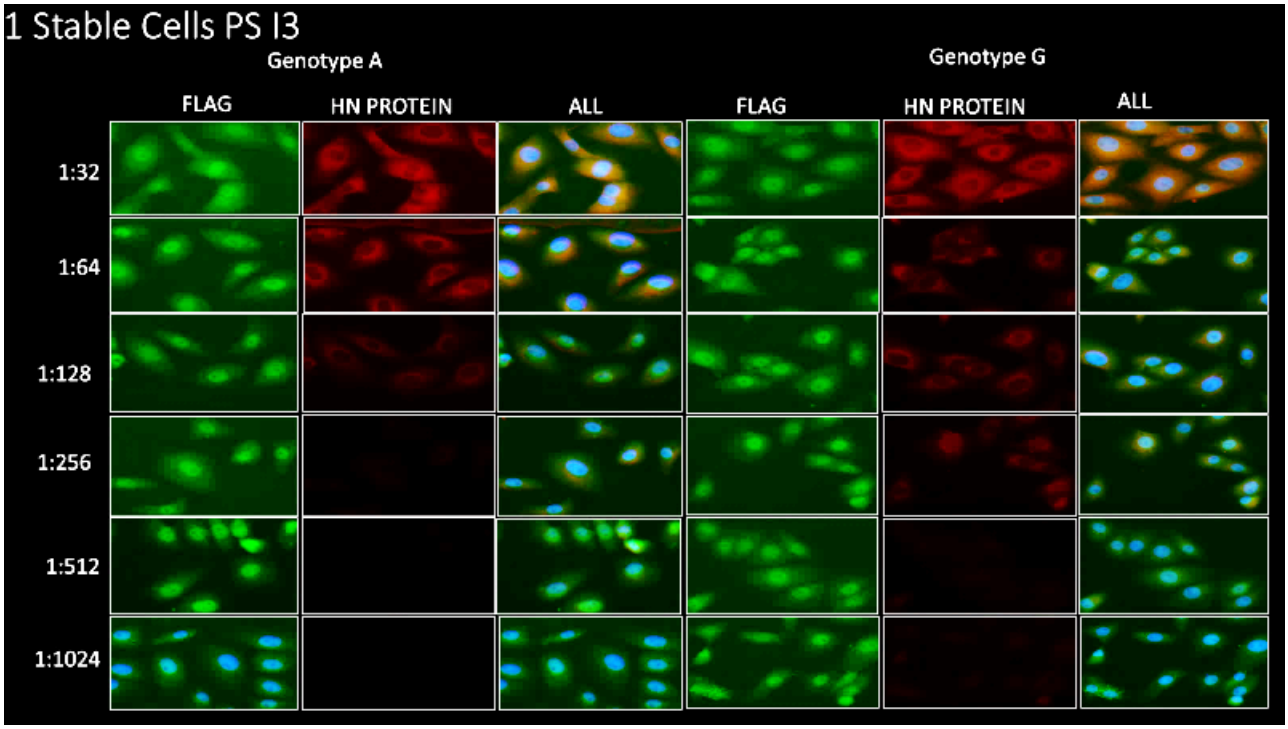


Figure A-25b. Stable cells Infected Sample #3.

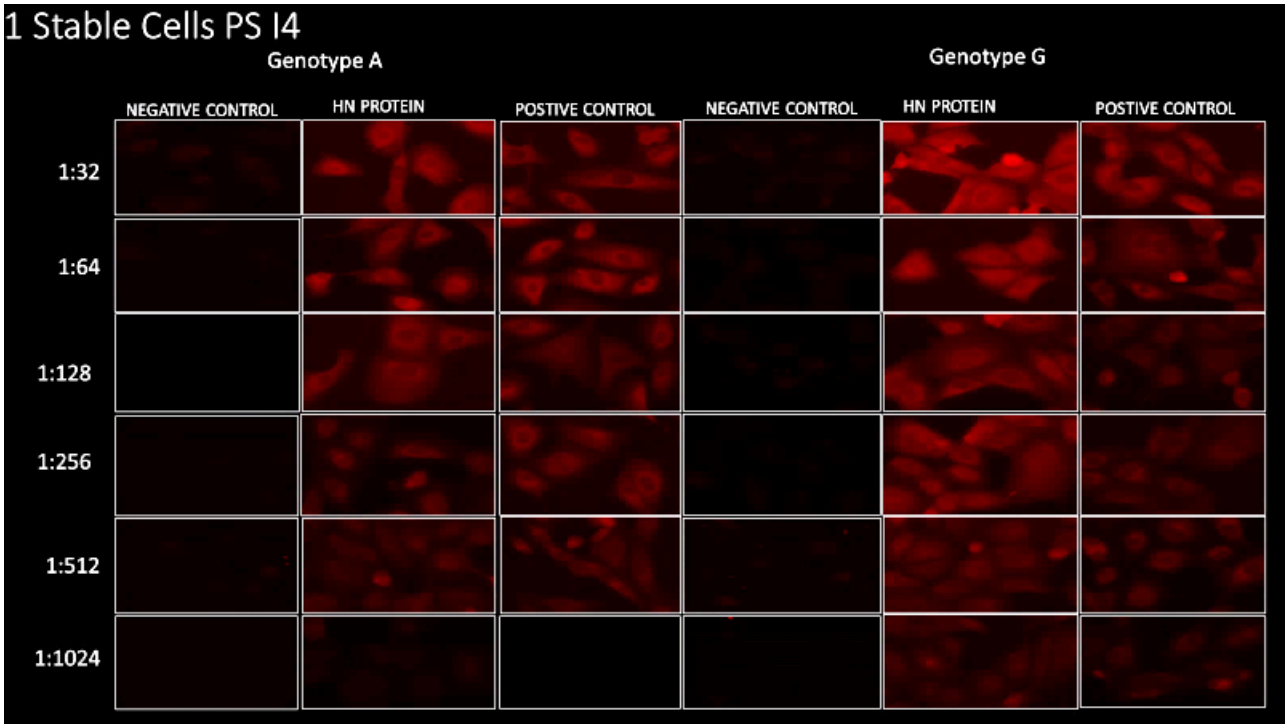


Figure A-26a. Stable cells Infected Sample#4.

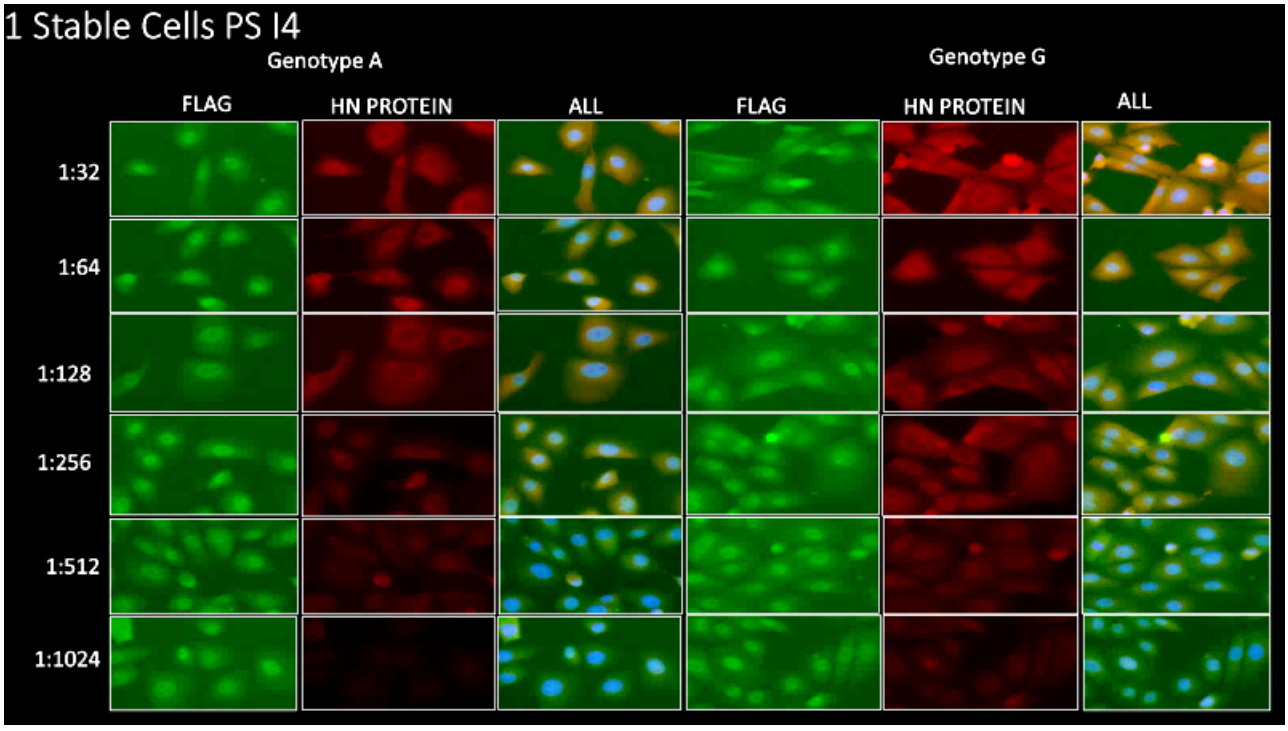


Figure A-26b. Stable cells Infected Sample #4.

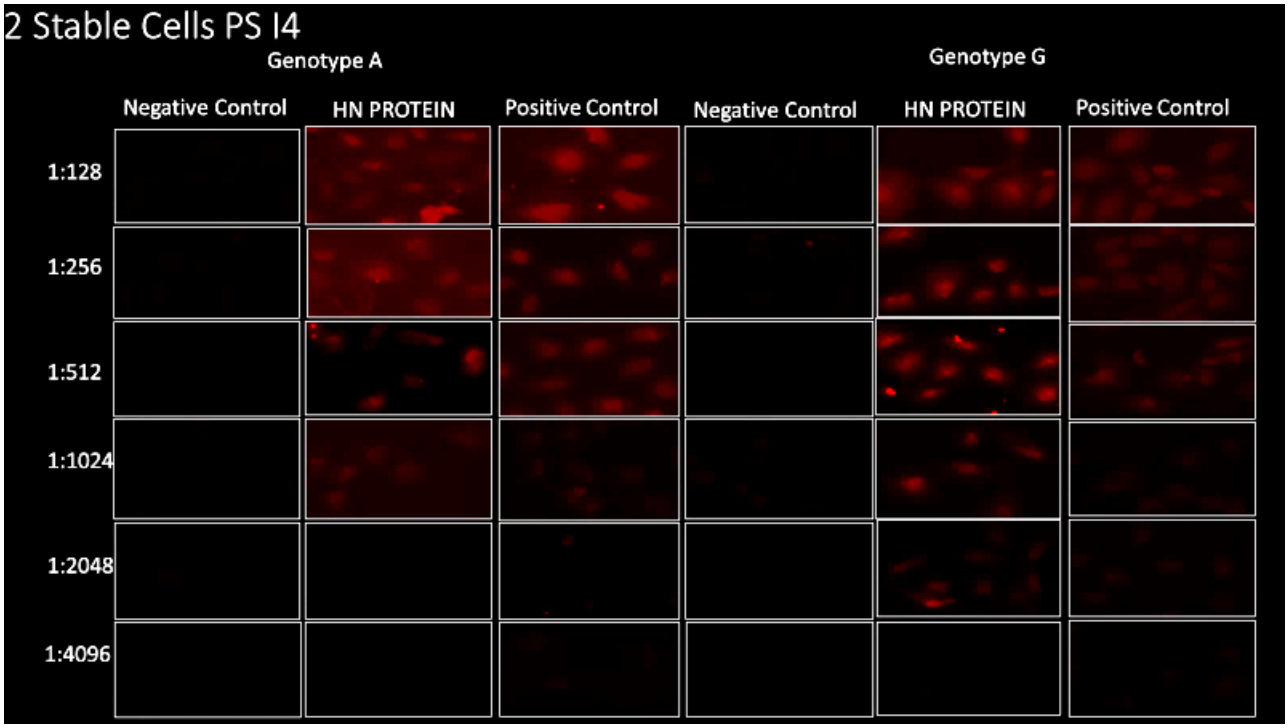


Figure A-26c. Stable cells Infected Sample #4.

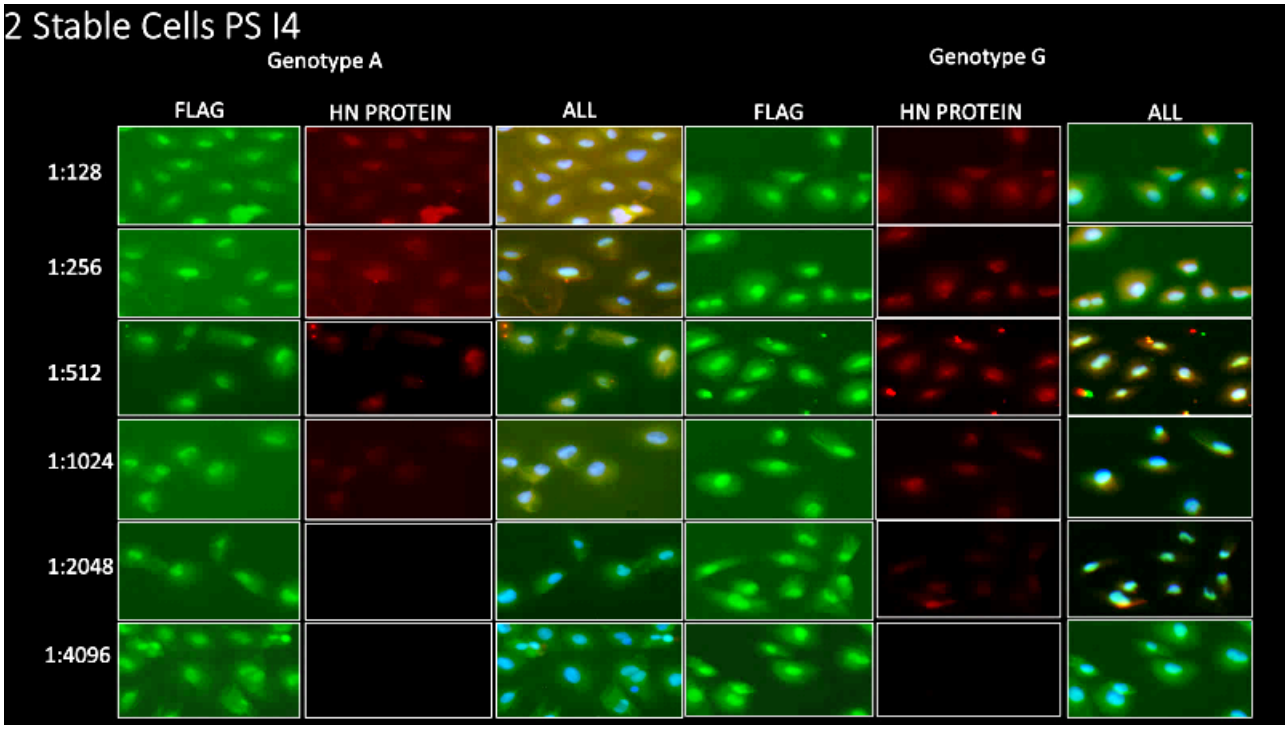


Figure A-26d. Stable cells Infected Sample #4.

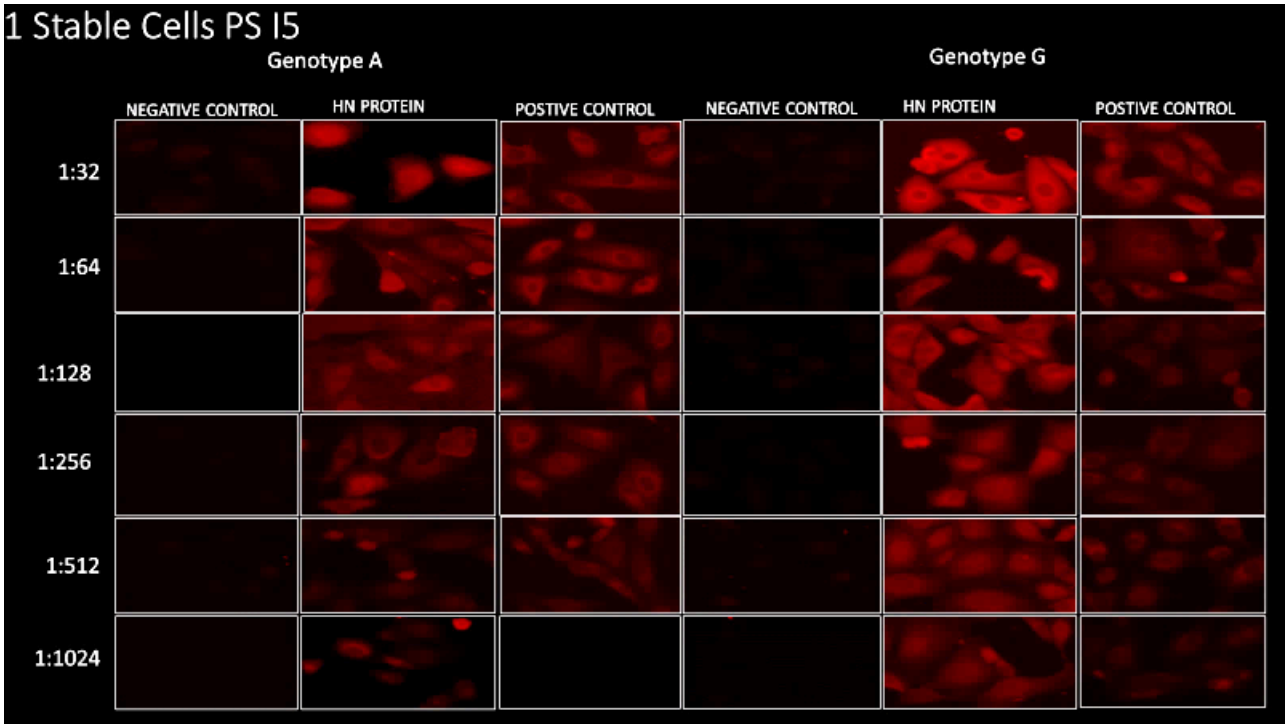


Figure A-27a. Stable cells Infected Sample#5.

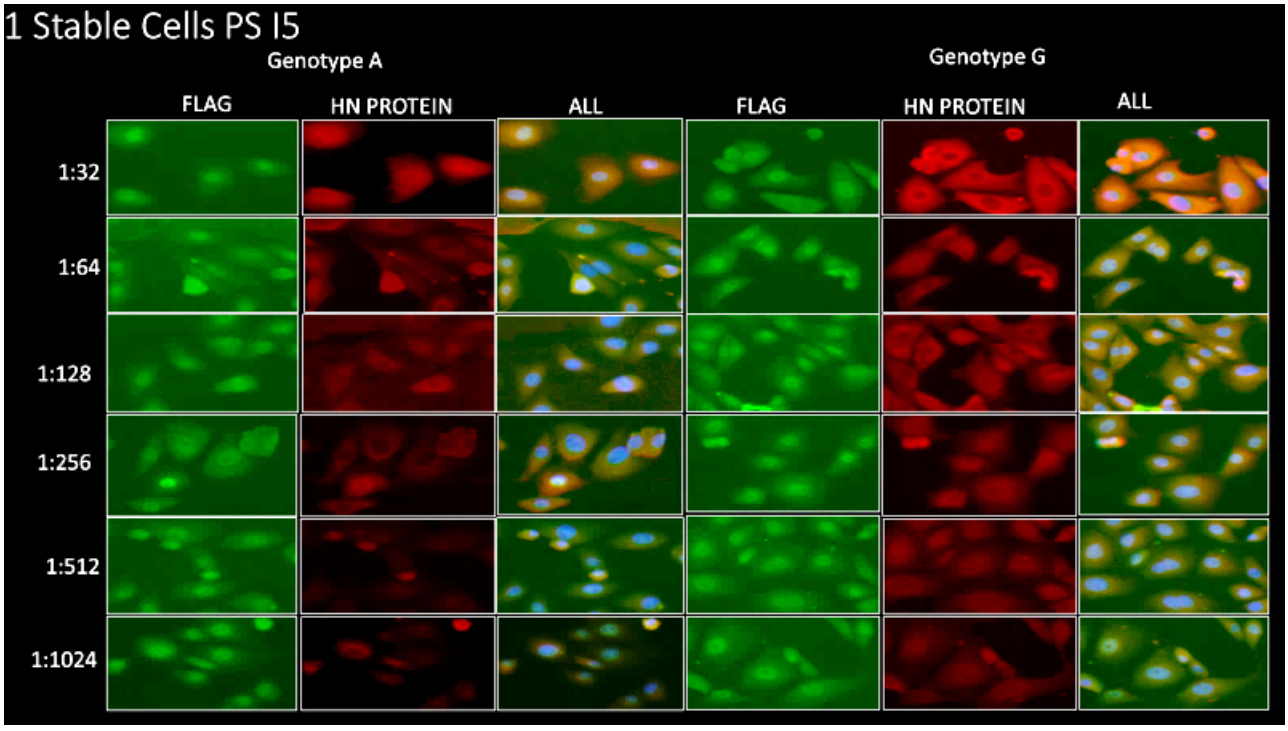


Figure A-27b. Stable cells Infected Sample #5.

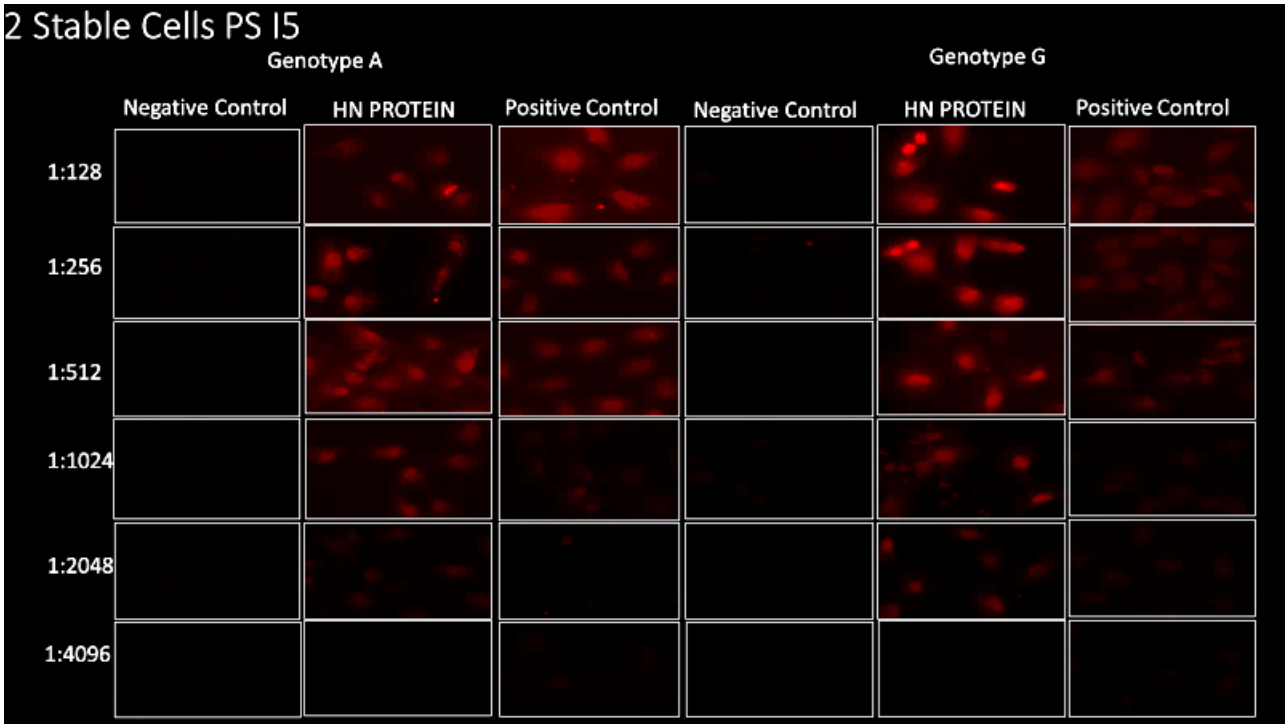


Figure A-27c. Stable cells Infected Sample #5.

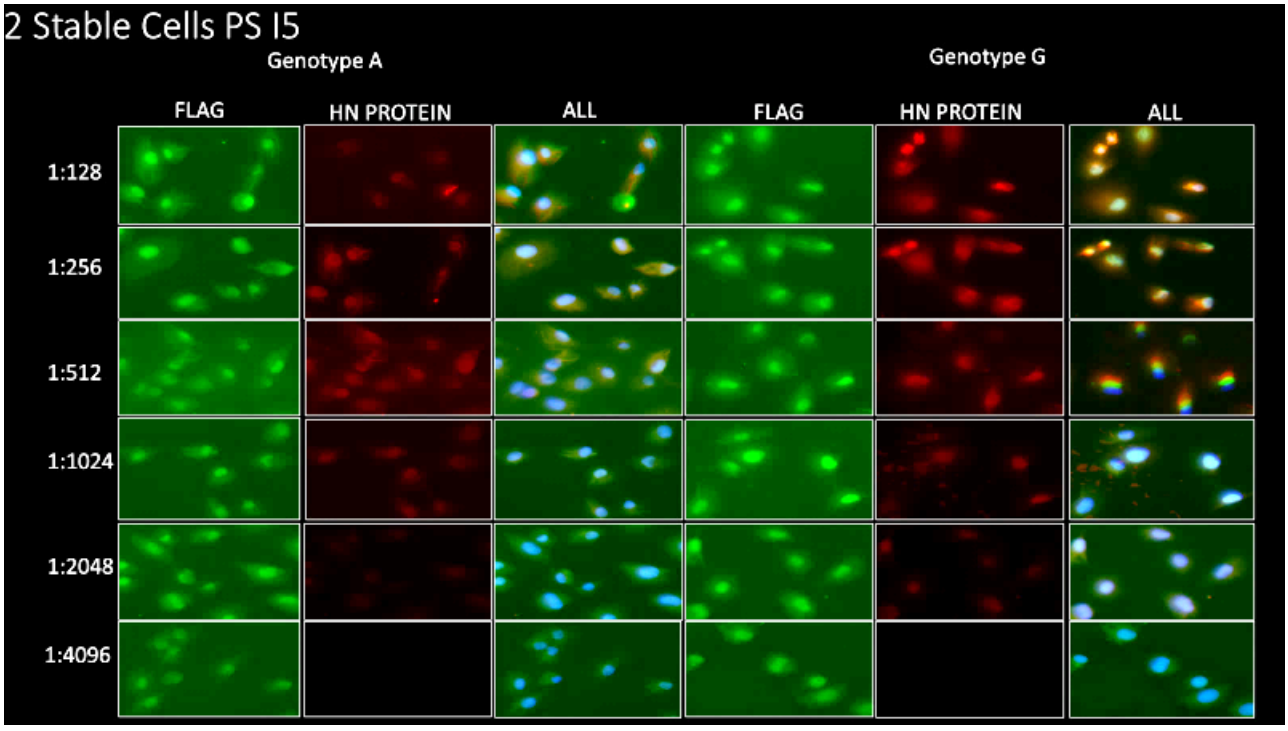


Figure A-27d. Stable cells Infected Sample #5.

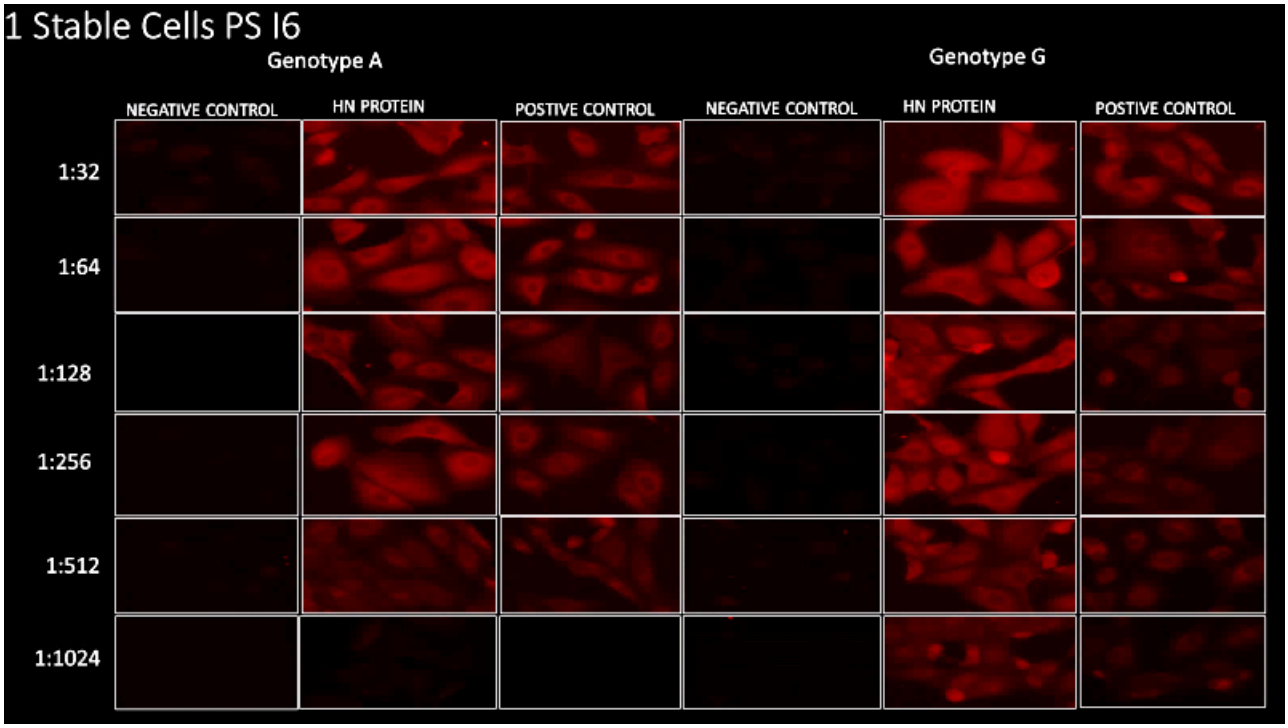


Figure A-28a. Stable cells Infected Sample#6.

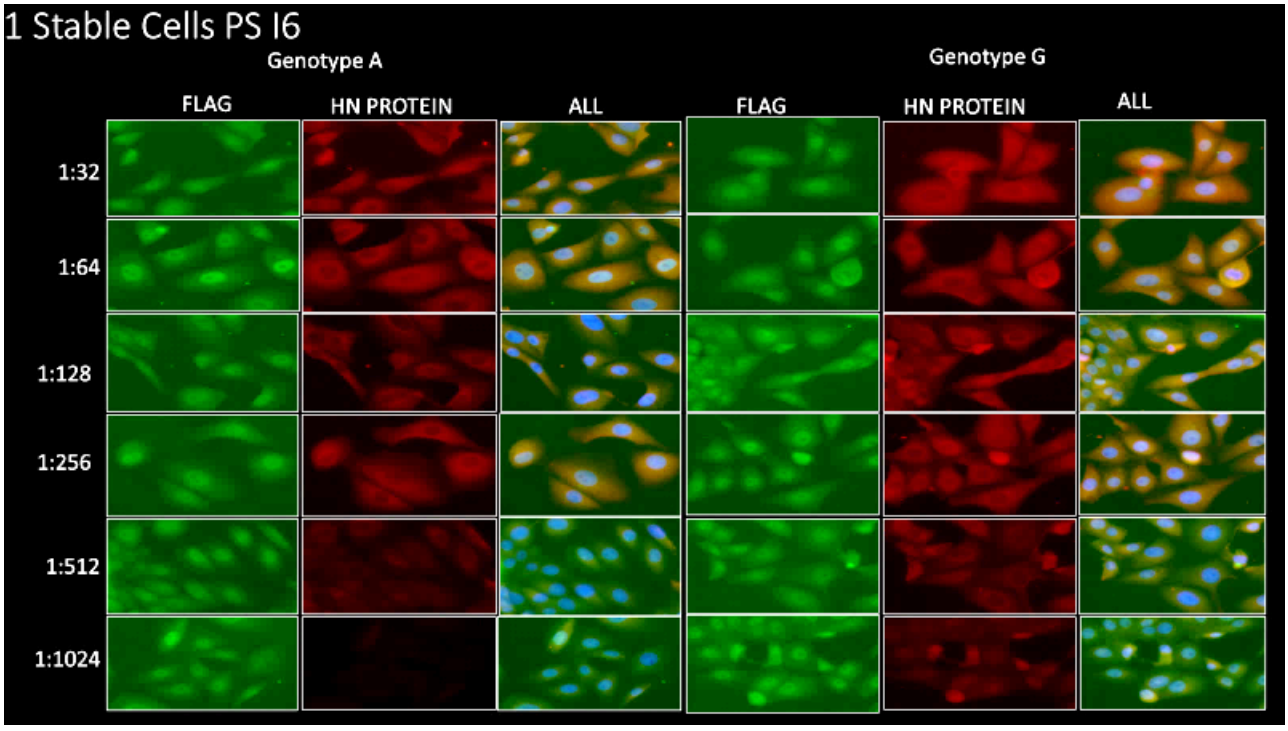


Figure A-28b. Stable cells Infected Sample #6.

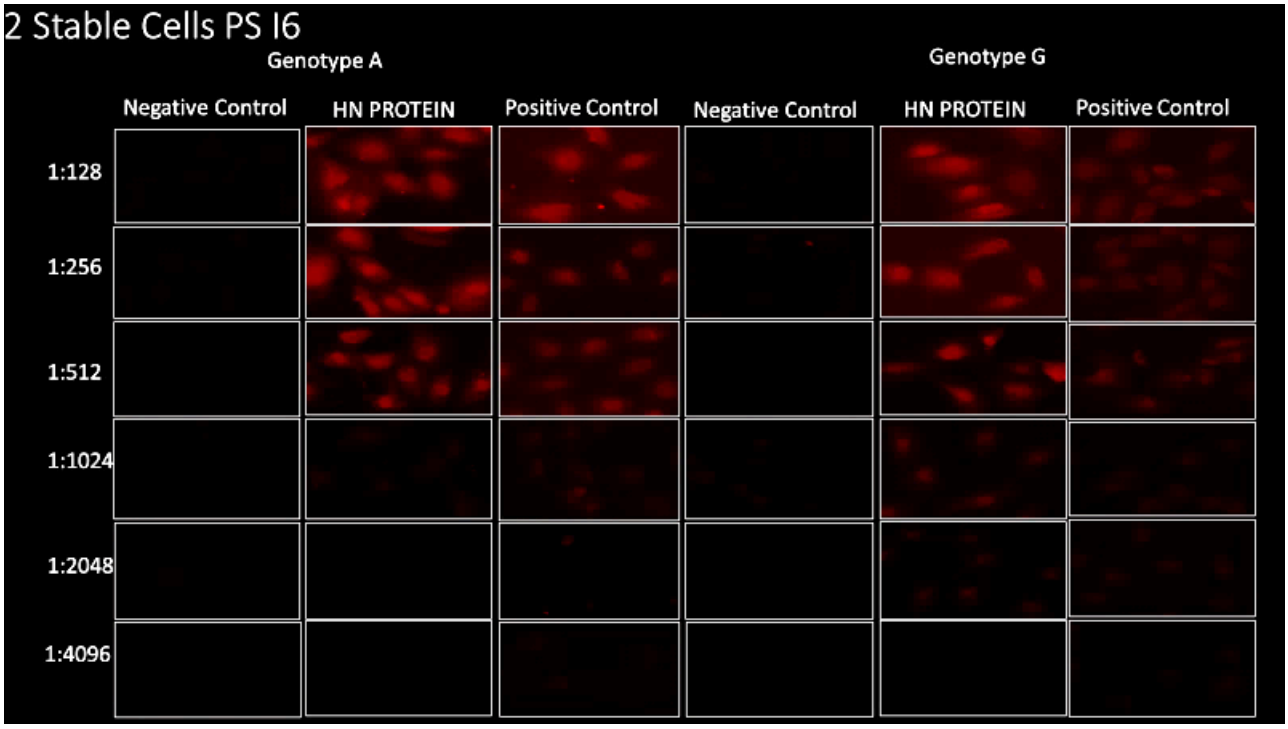


Figure A-28c. Stable cells Infected Sample #6.

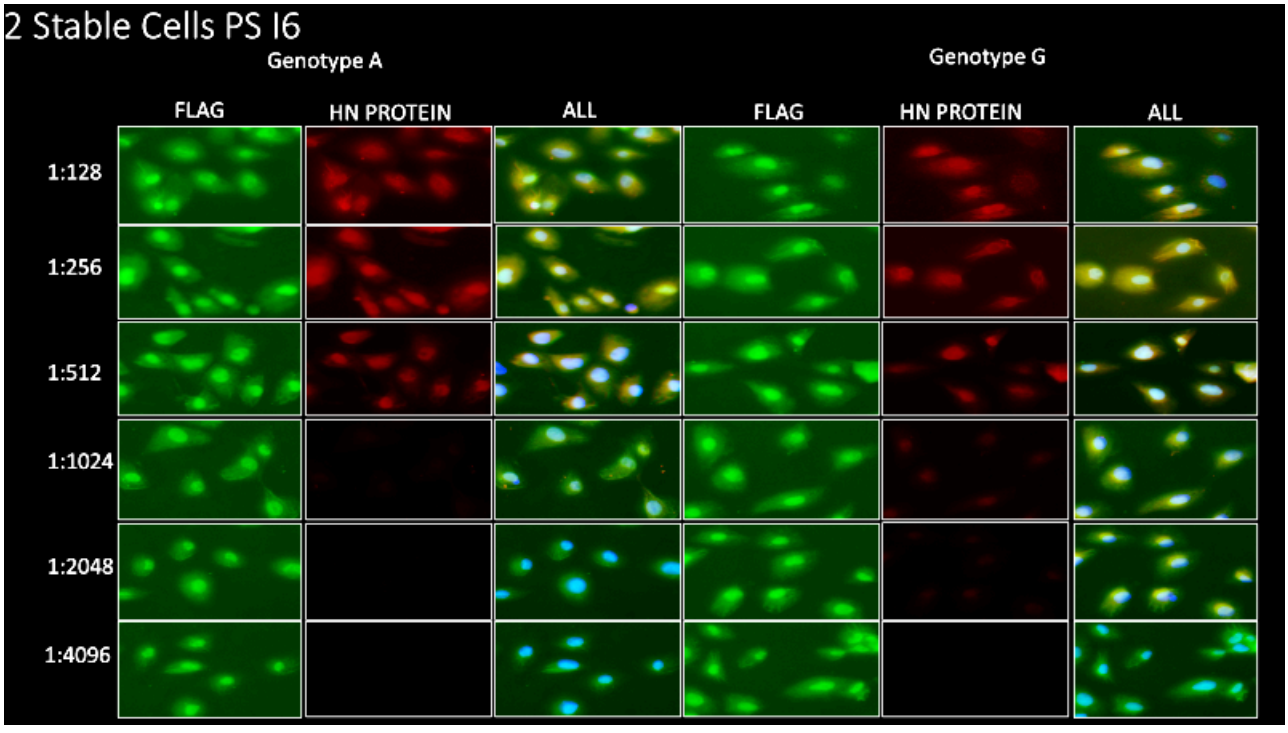


Figure A-28d. Stable cells Infected Sample #6.

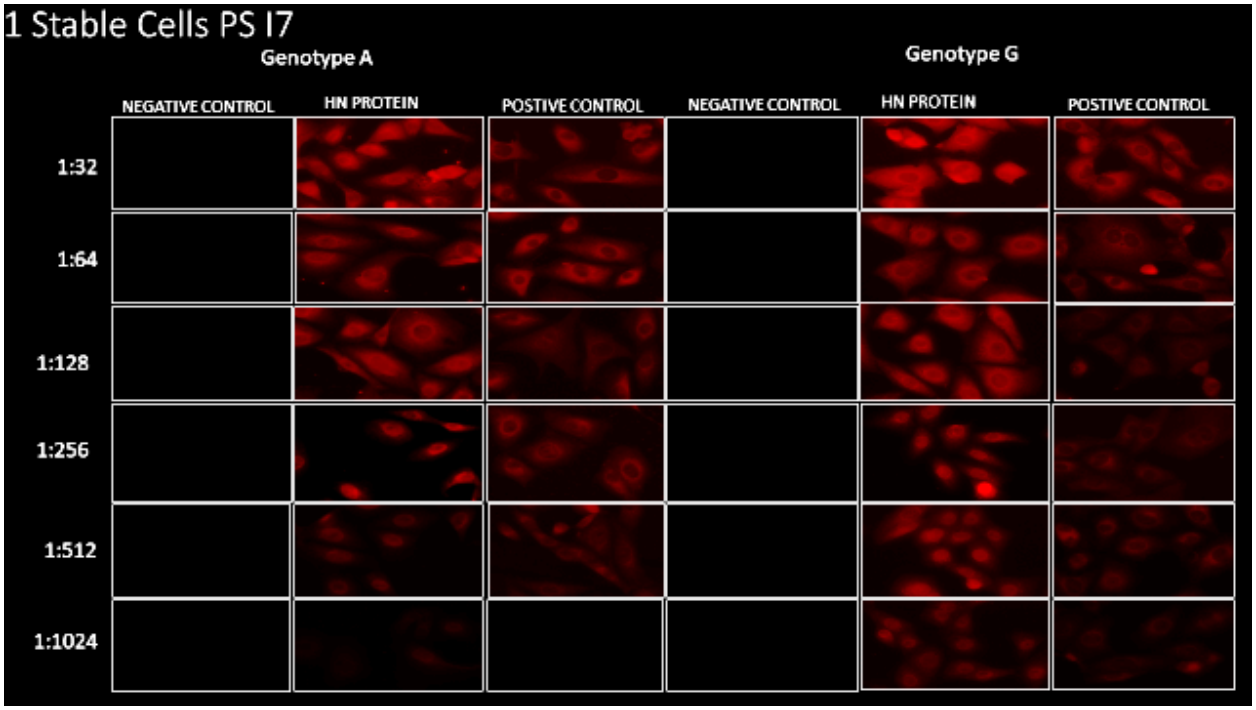


Figure A-29a. Stable cells Infected Sample#7.

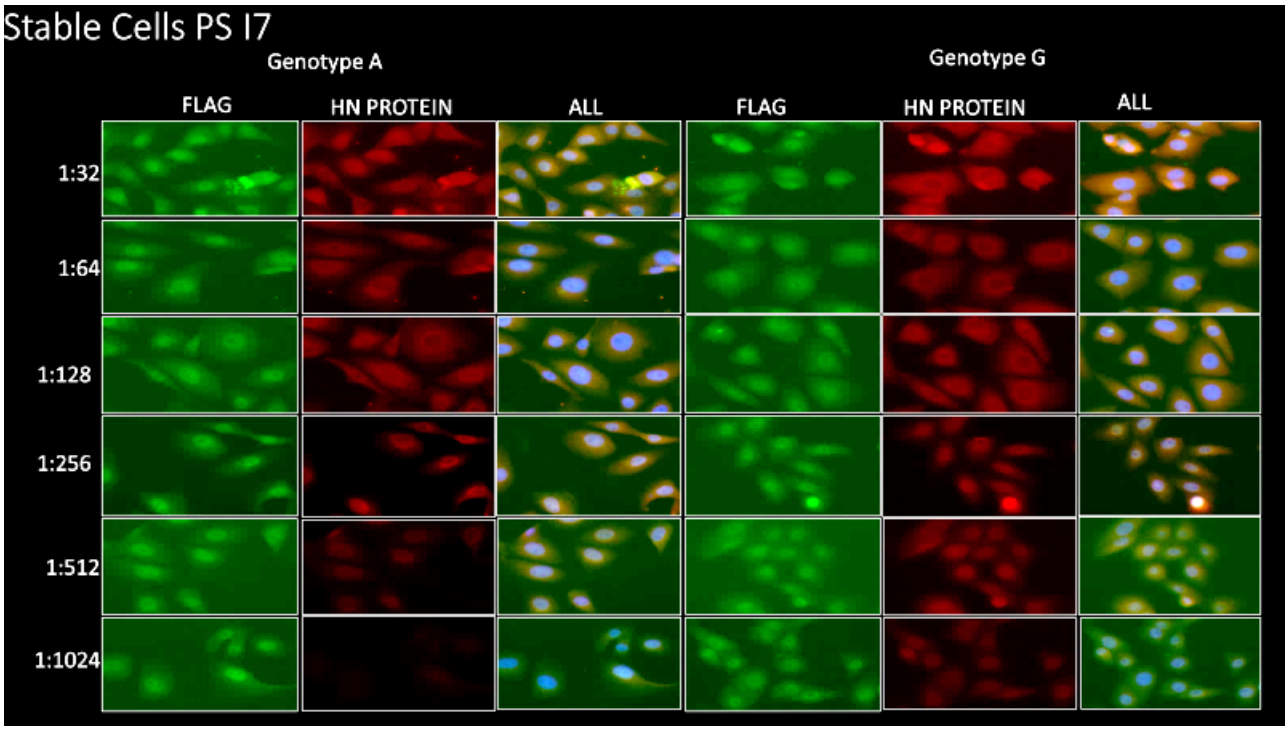


Figure A-29b. Stable cells Infected Sample #7.

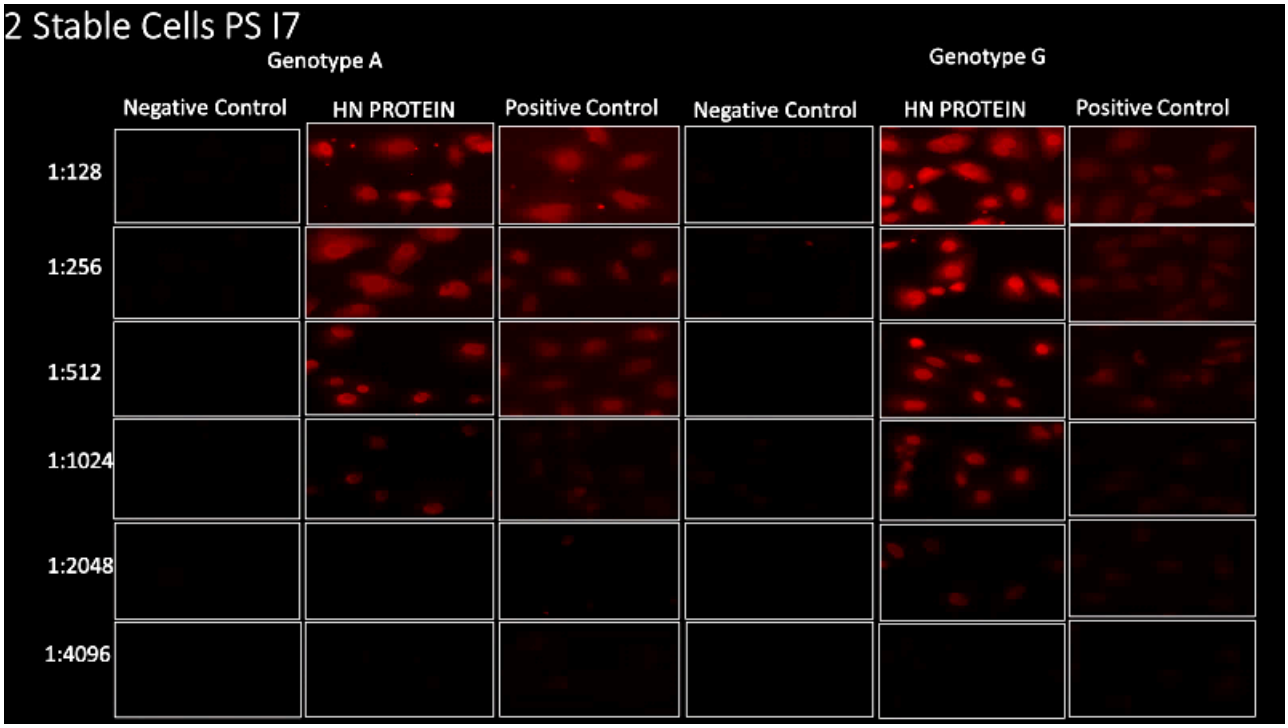


Figure A-29c. Stable cells Infected Sample #7.

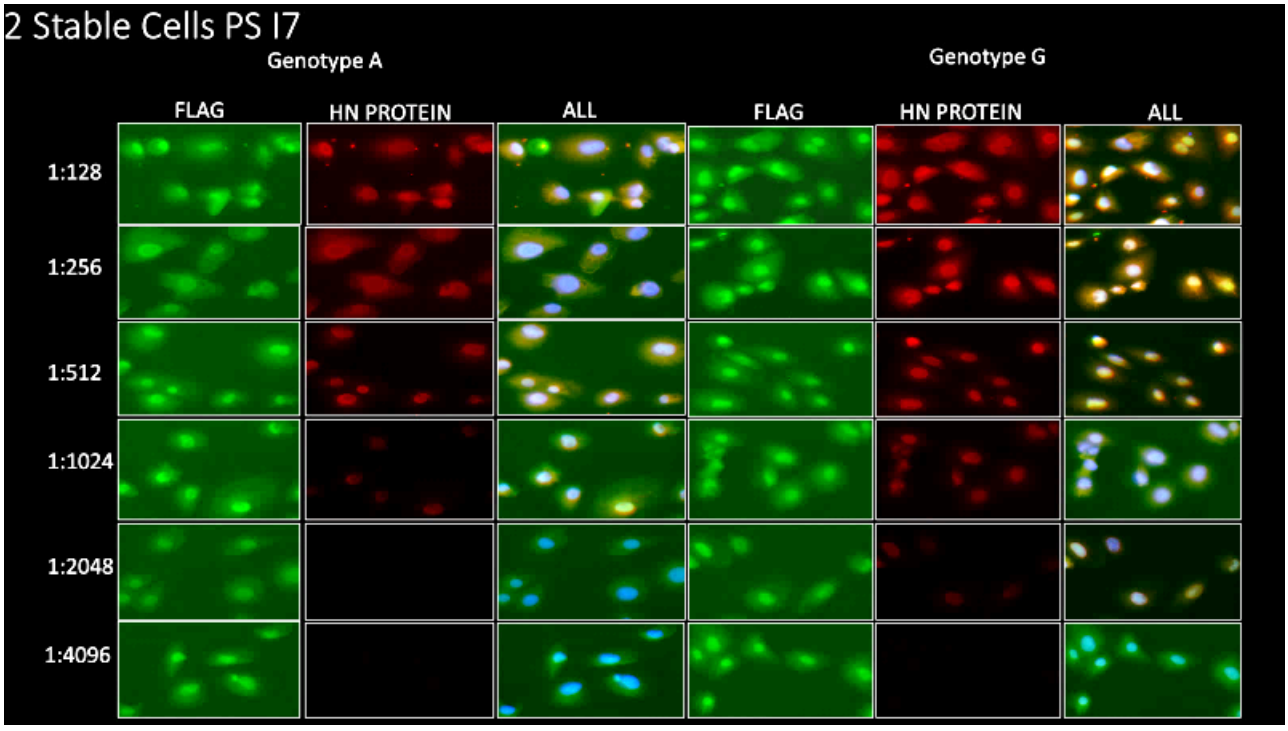


Figure A-29d. Stable cells Infected Sample #7.

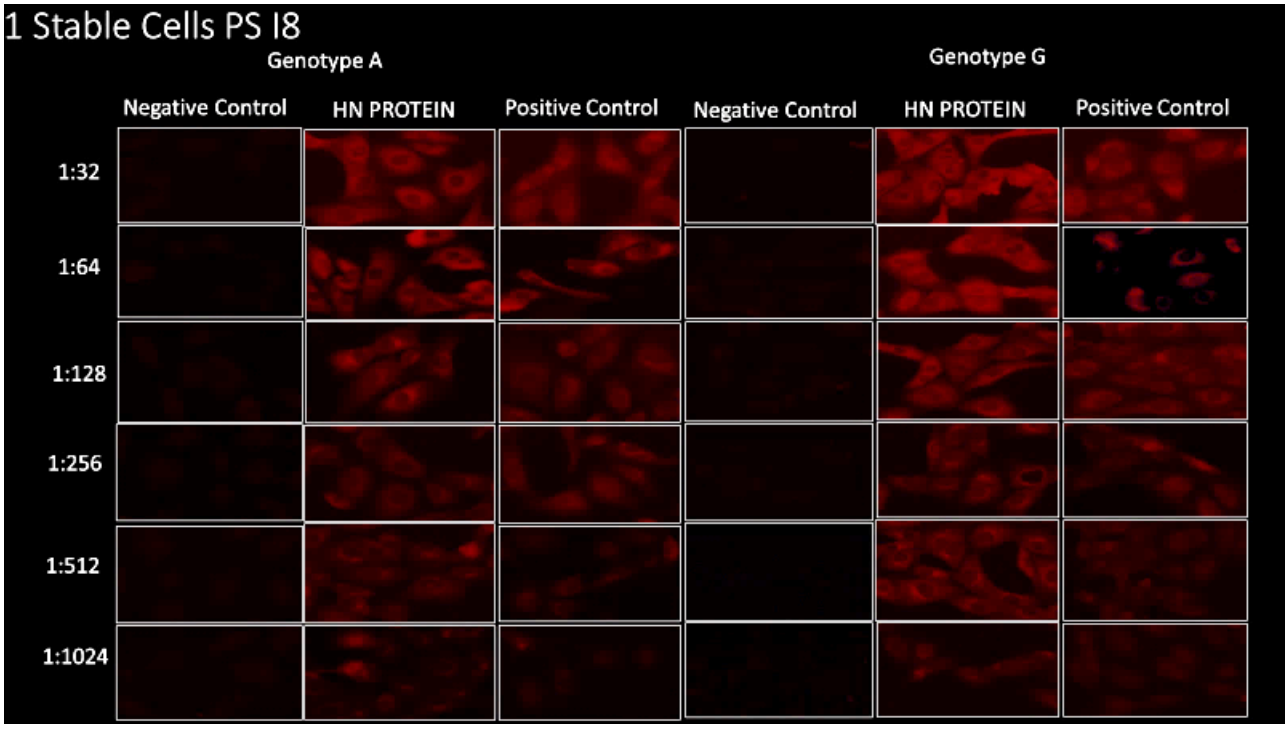


Figure A-30a. Stable cells Infected Sample#8.

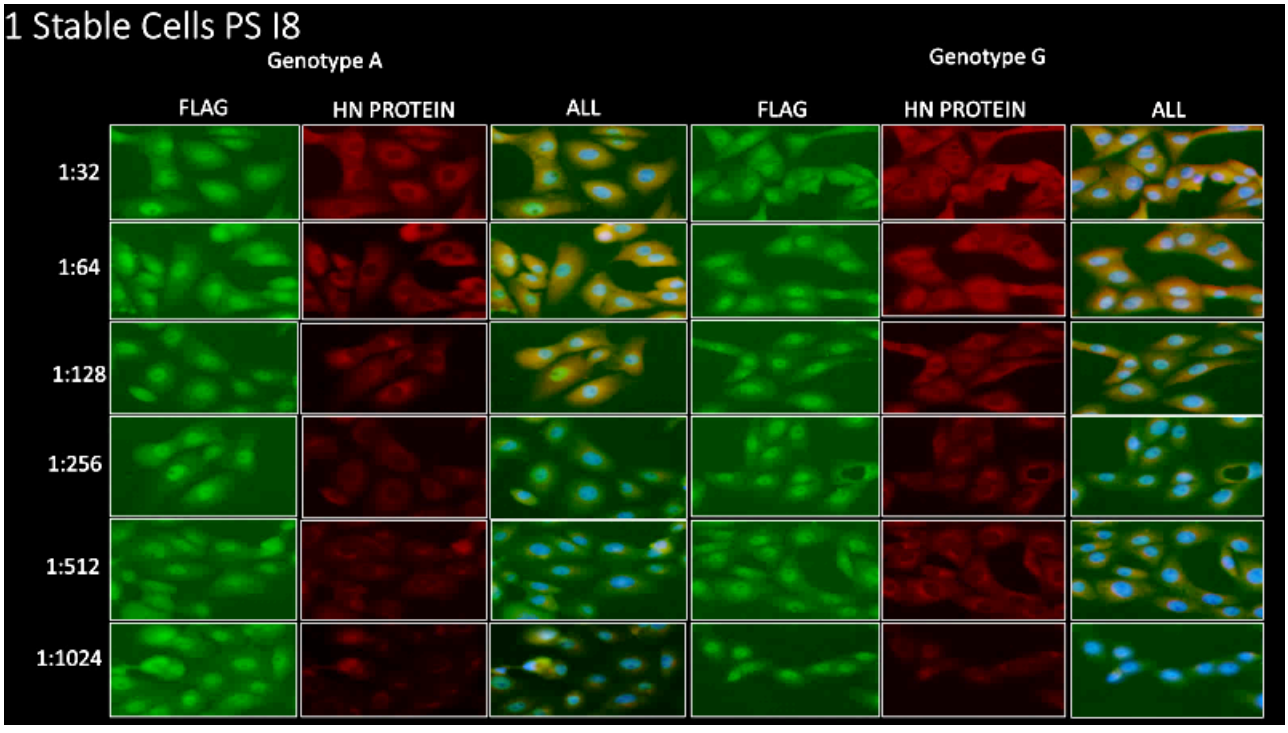


Figure A-30b. Stable cells Infected Sample #8.

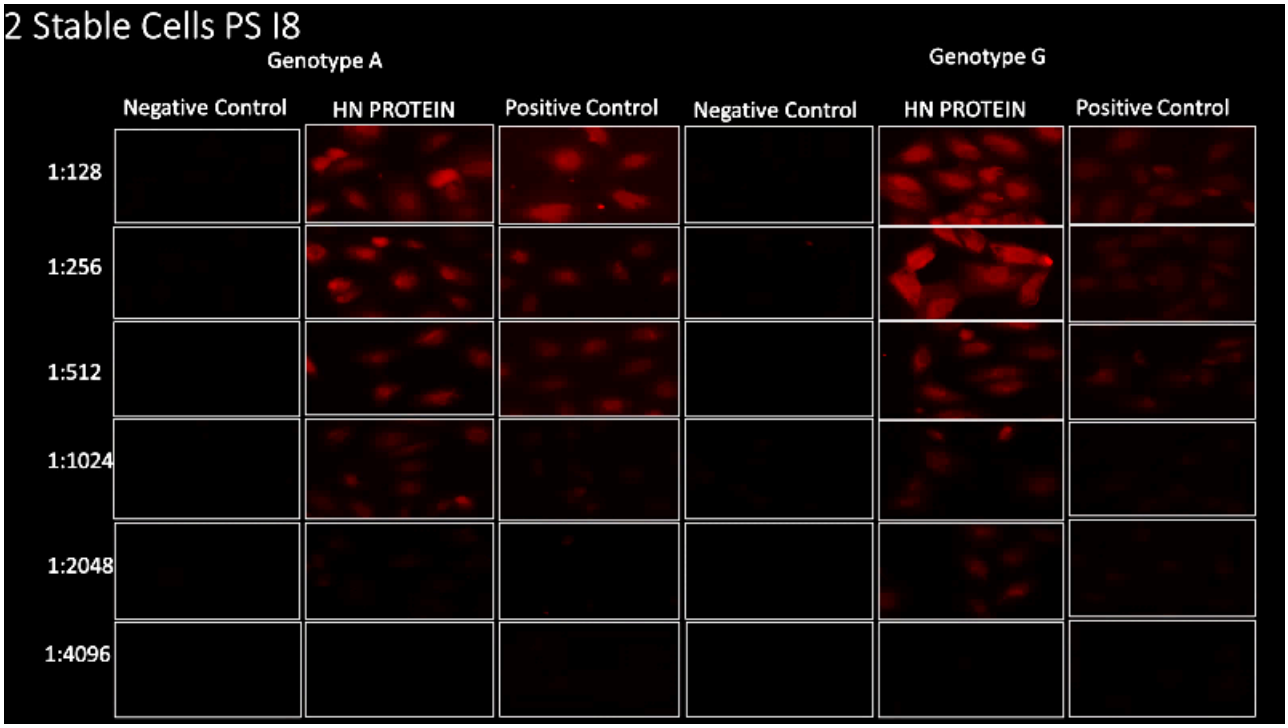


Figure A-30c. Stable cells Infected Sample #8.

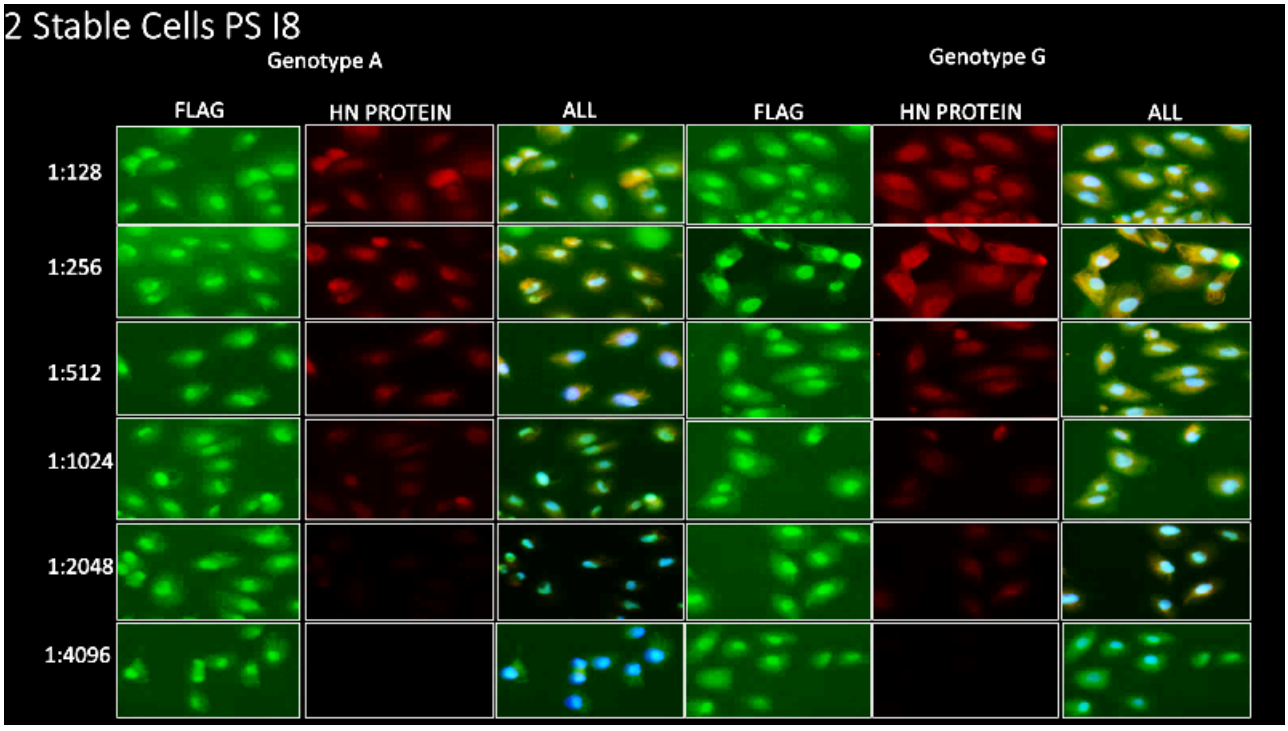


Figure A-30d. Stable cells Infected Sample #8.

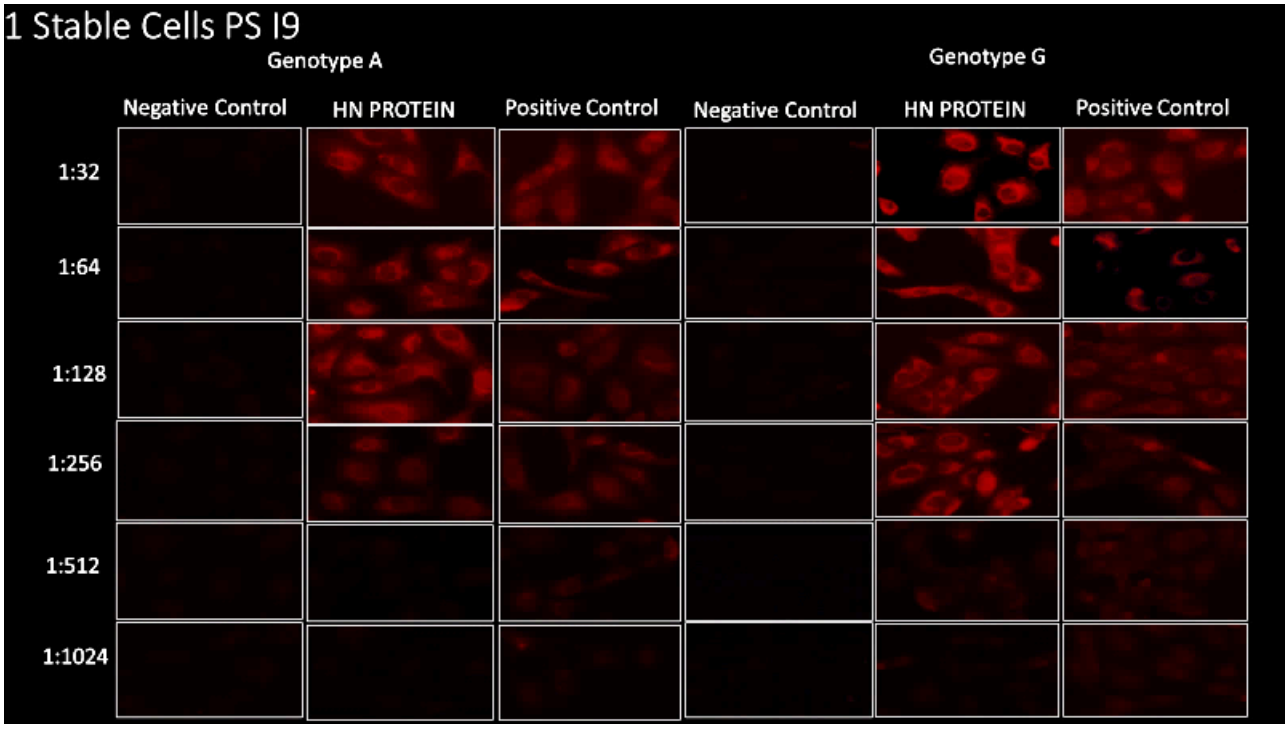


Figure A-31a. Stable cells Infected Sample#9.

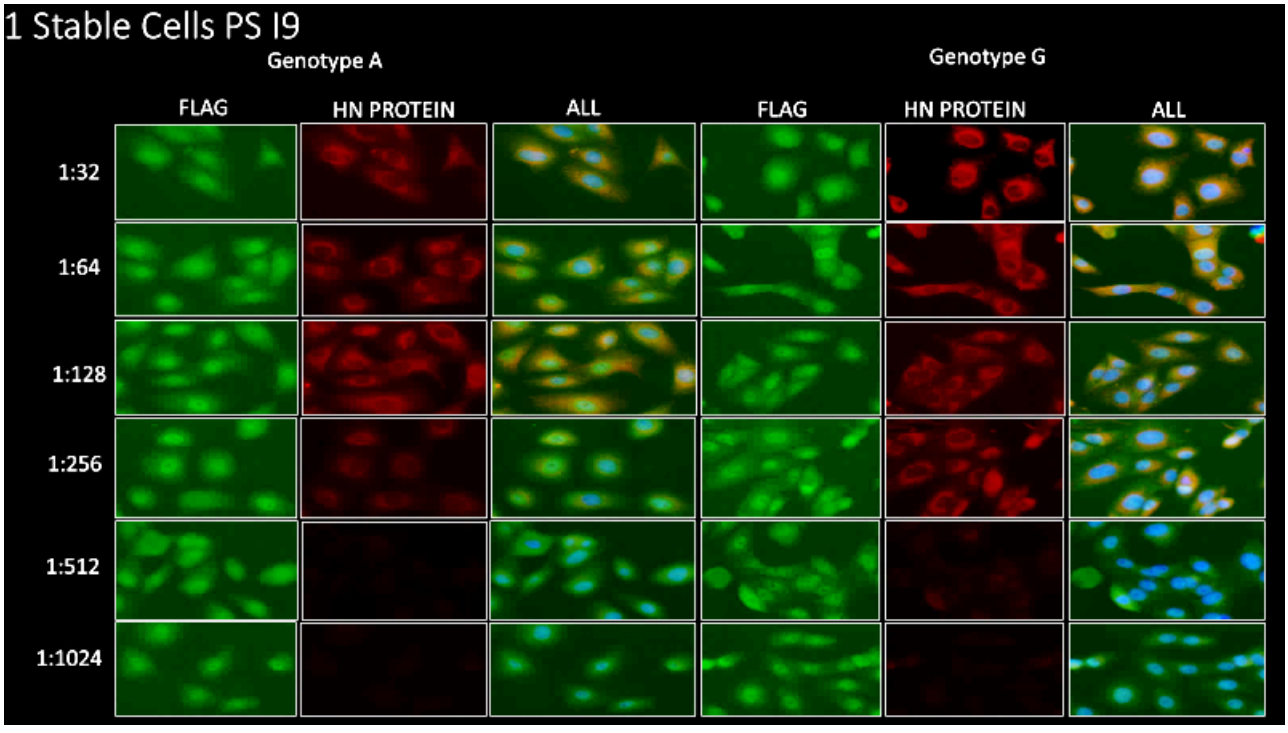


Figure A-31b. Stable cells Infected Sample #9.

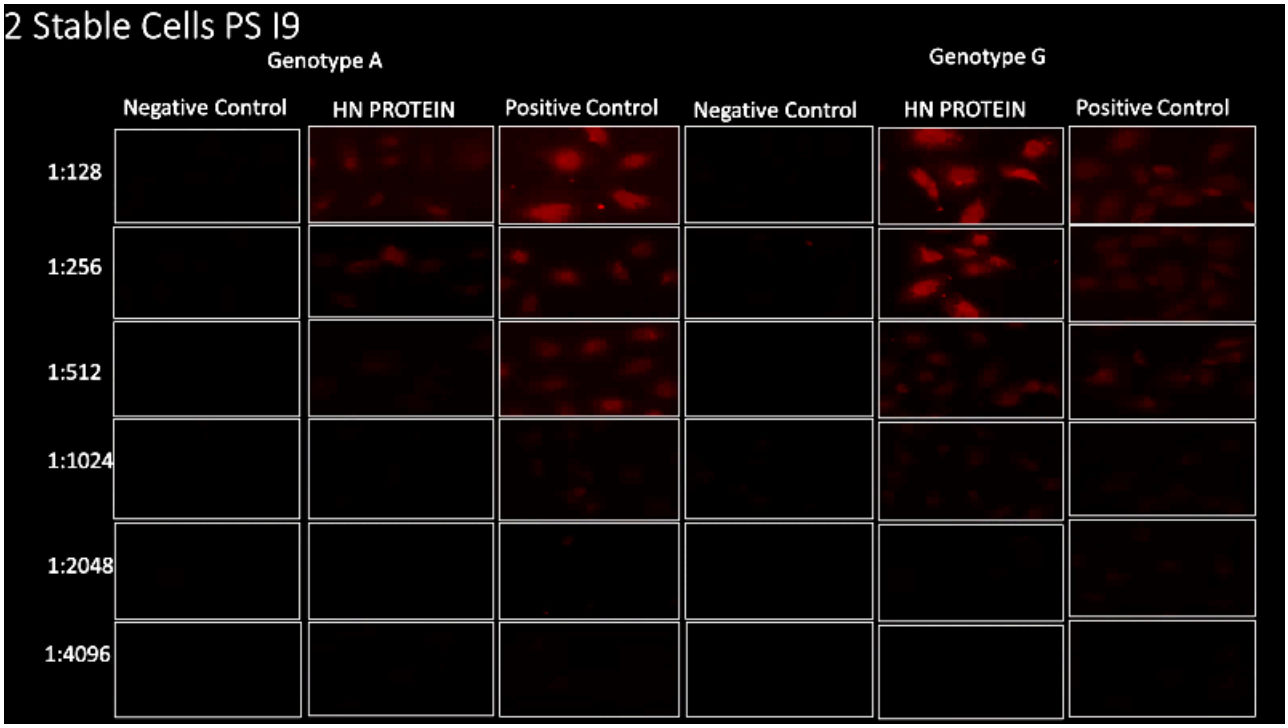


Figure A-31c. Stable cells Infected Sample #9.

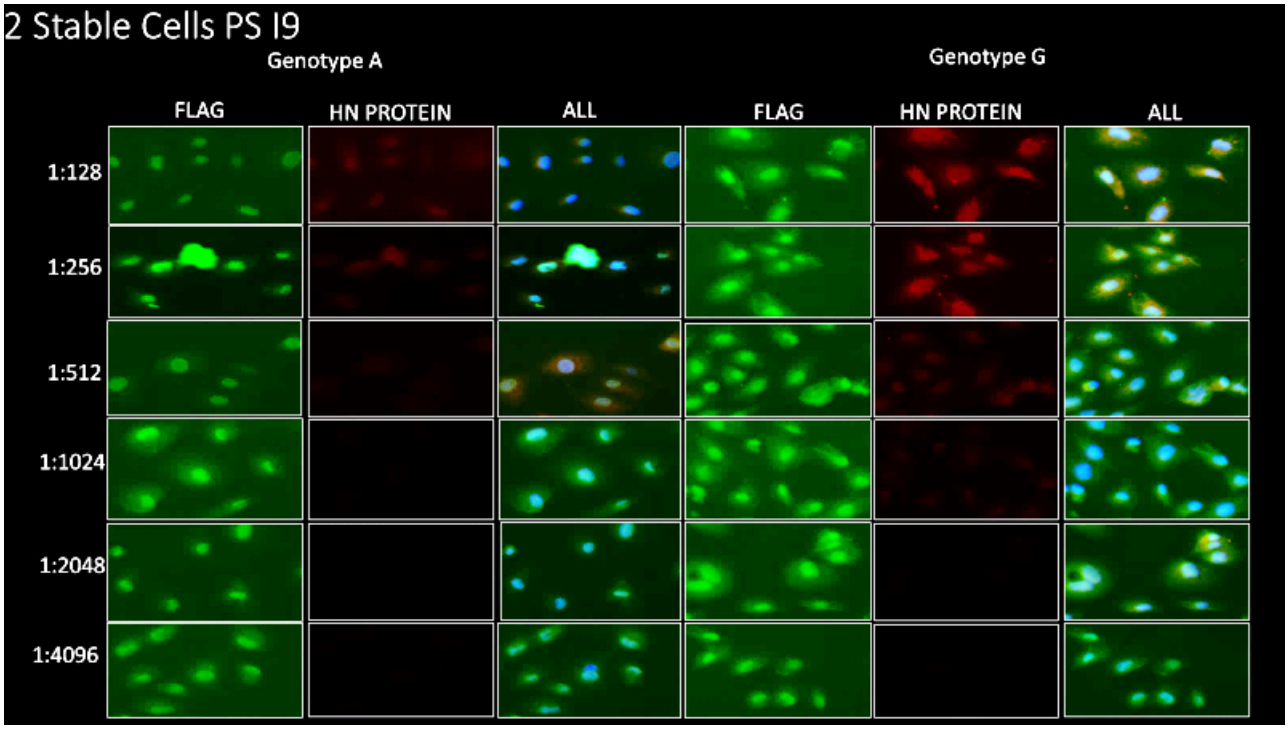


Figure A-31d. Stable cells Infected Sample #9.

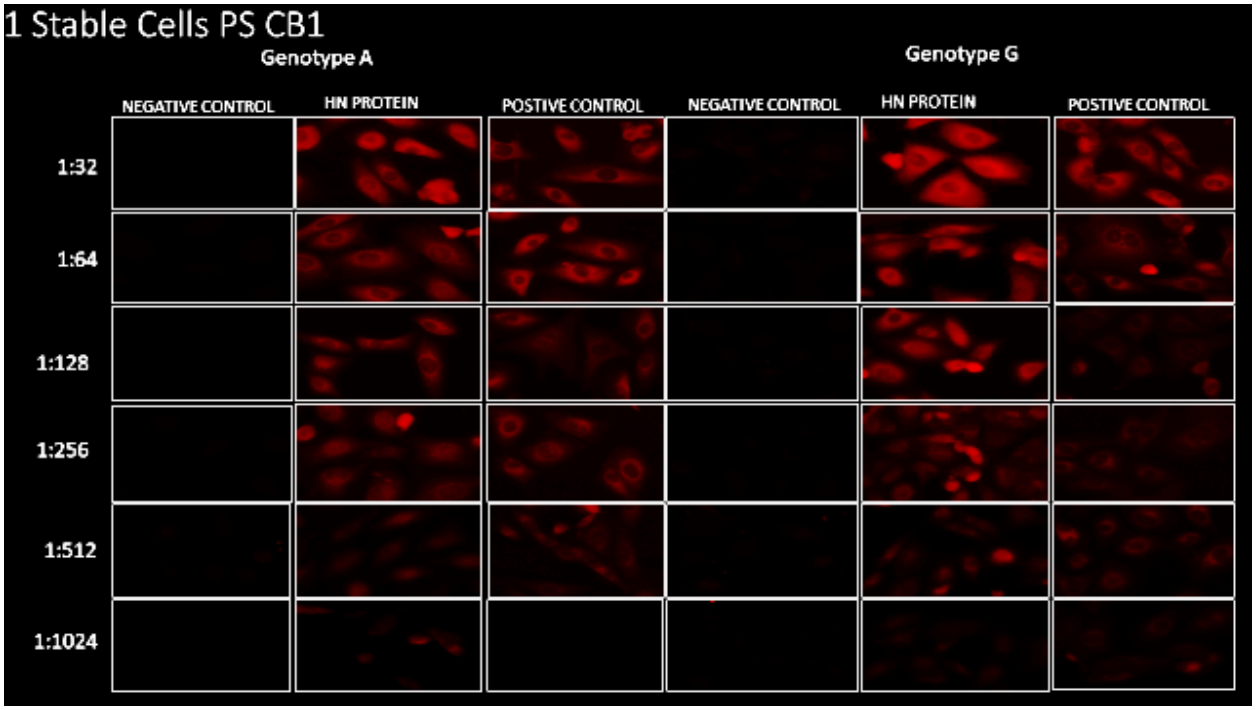


Figure A-32a. Stable cells Vaccine-Infected (CB) Sample #1.

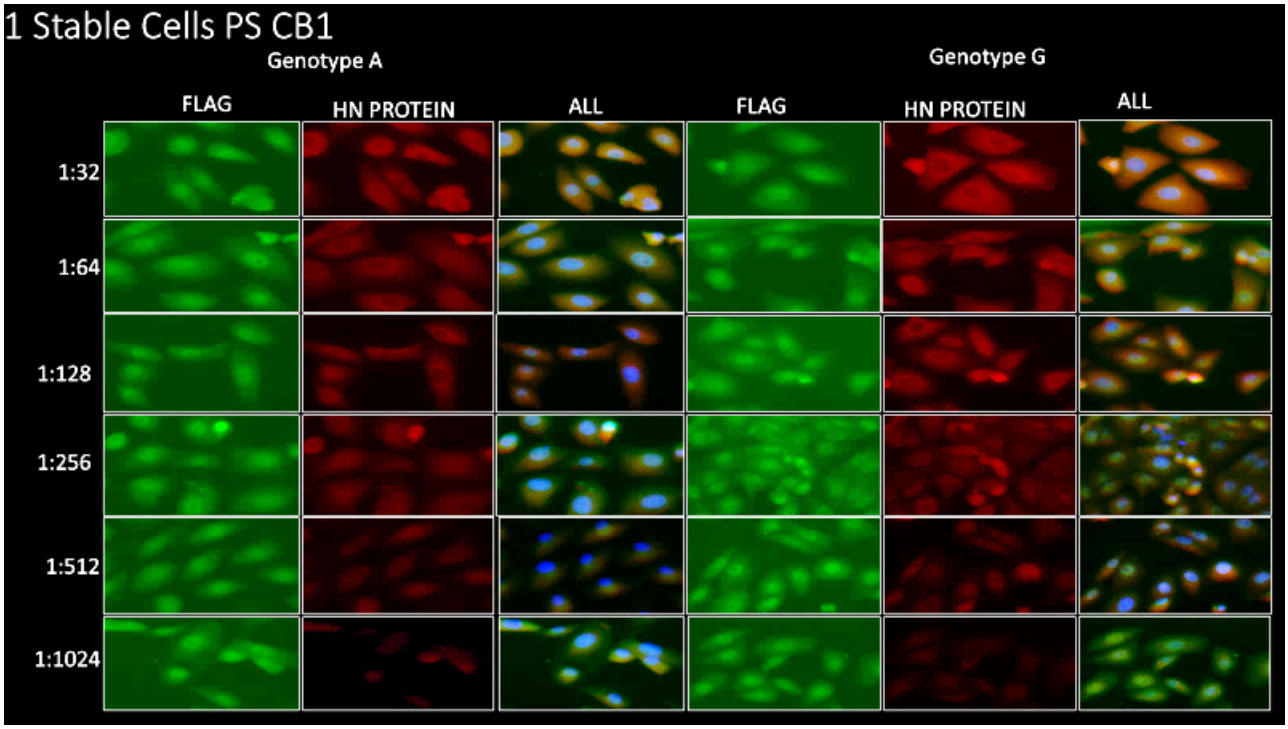


Figure A-32b. Stable cells Vaccine-Infected (CB) Sample #1.

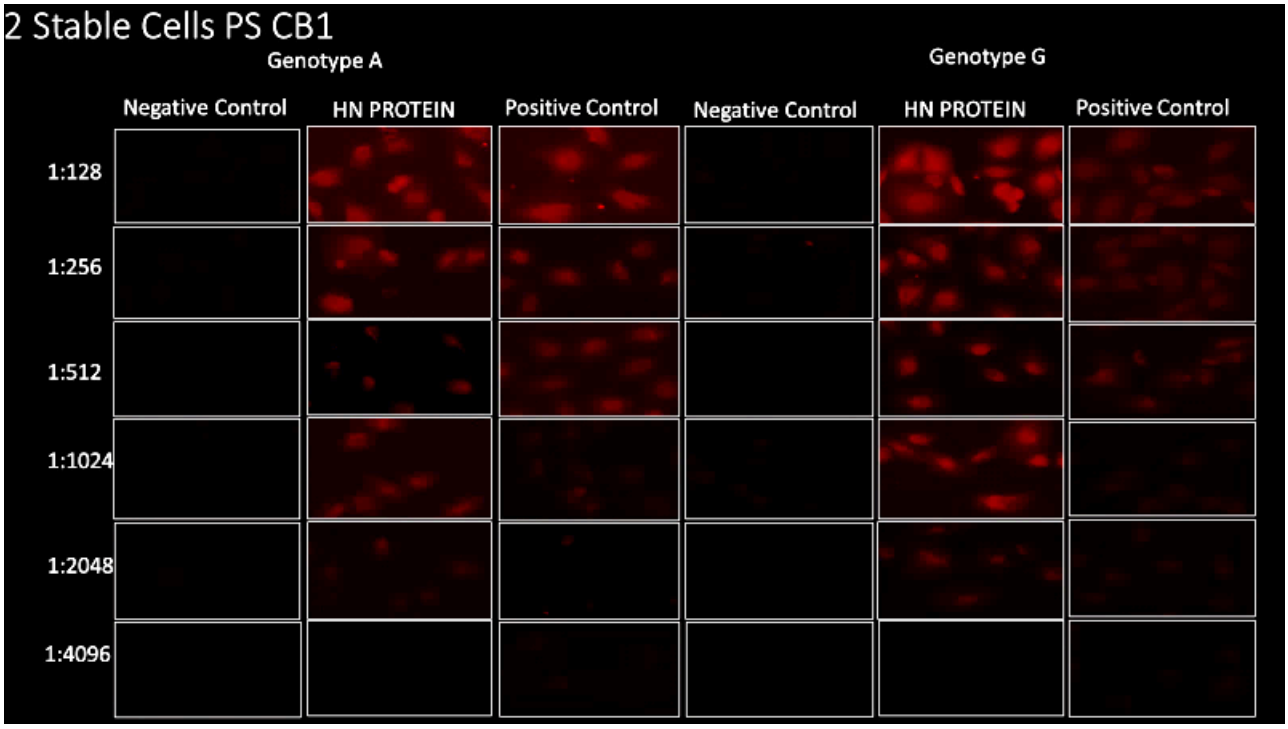


Figure A-32c. Stable cells Vaccine-Infected (CB) Sample #1.

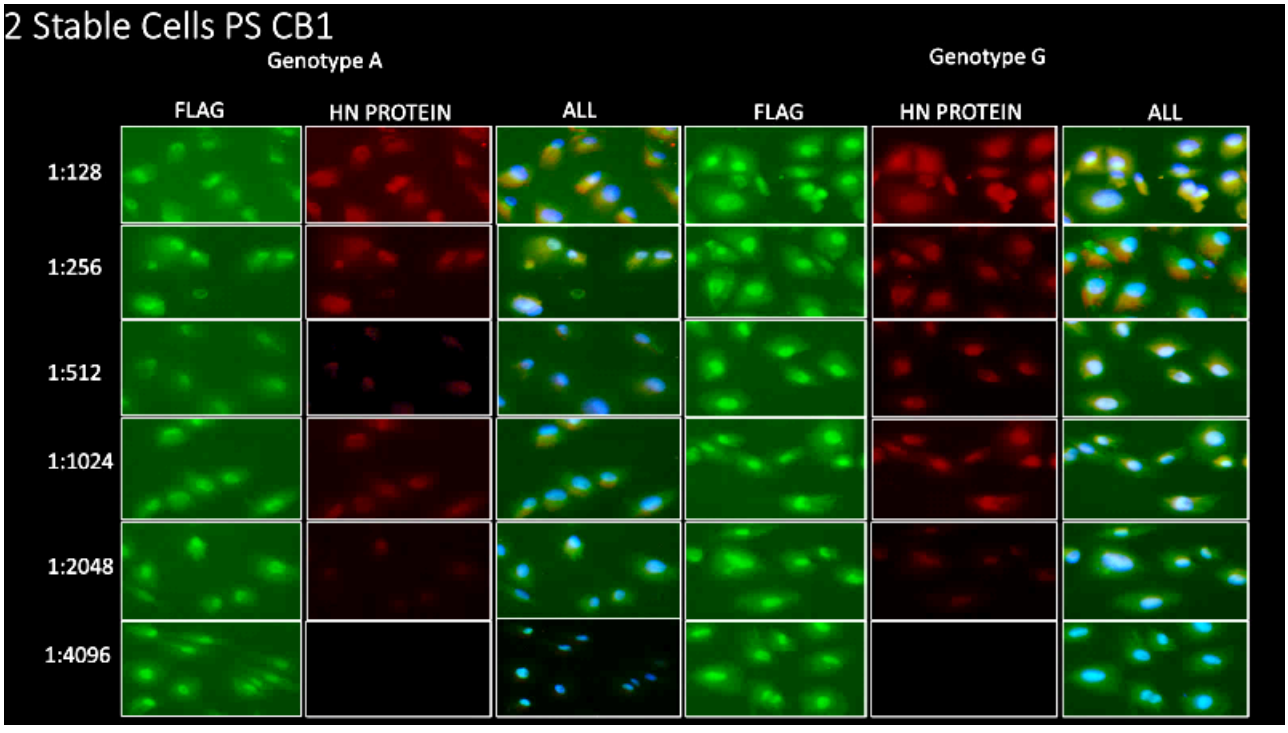


Figure A-32d. Stable cells Vaccine-Infected (CB) Sample #1.

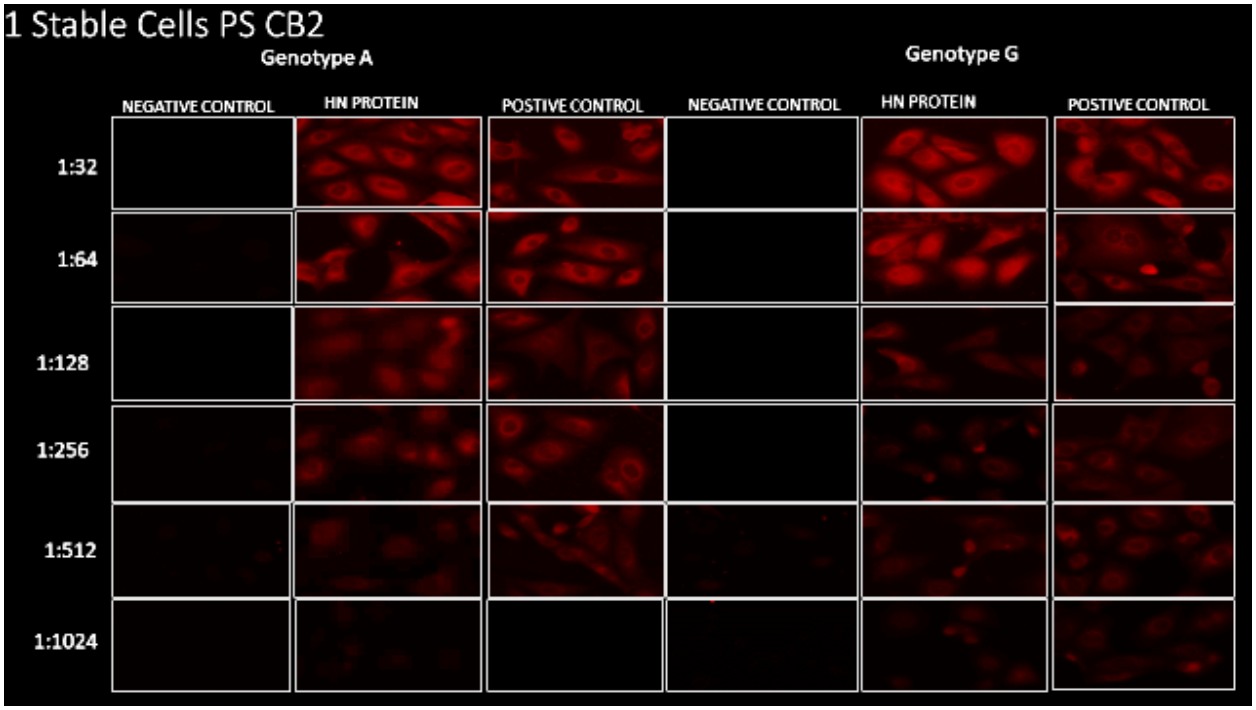


Figure A-33a. Stable cells Vaccine-Infected (CB) Sample#2.

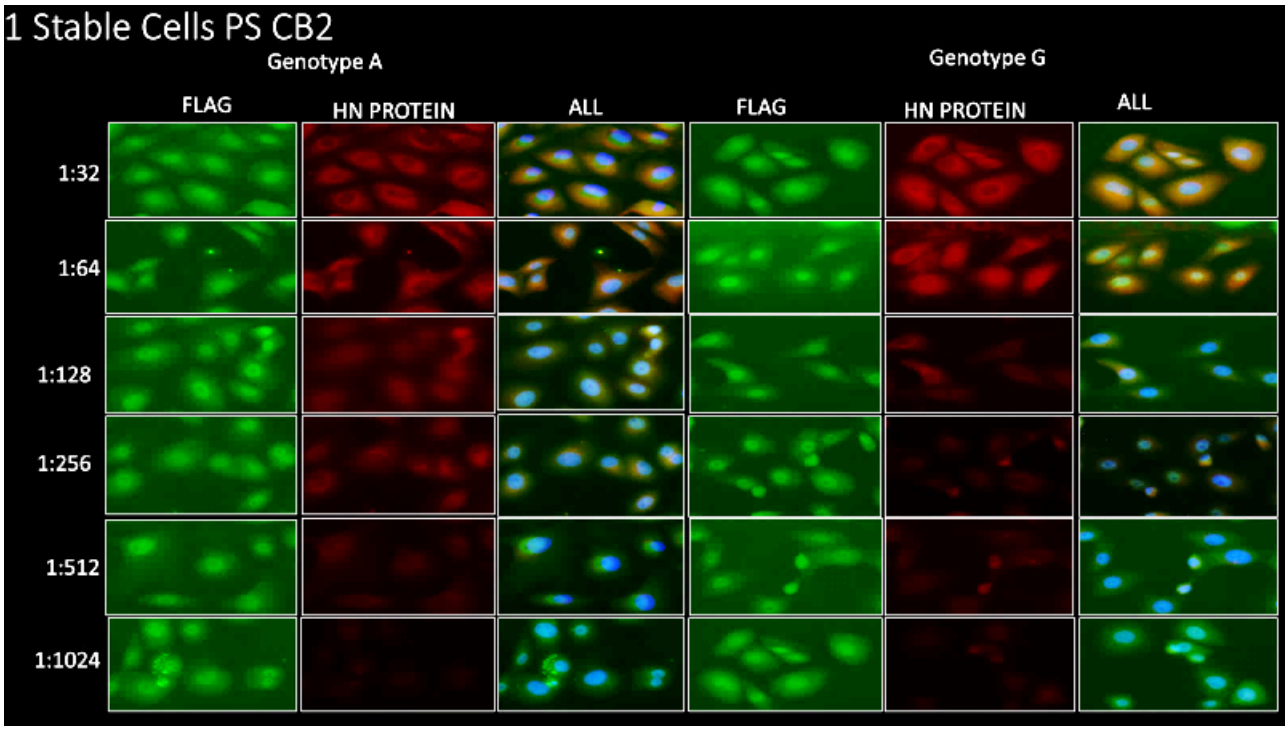


Figure A-33b. Stable cells Vaccine-Infected (CB) Sample #2.

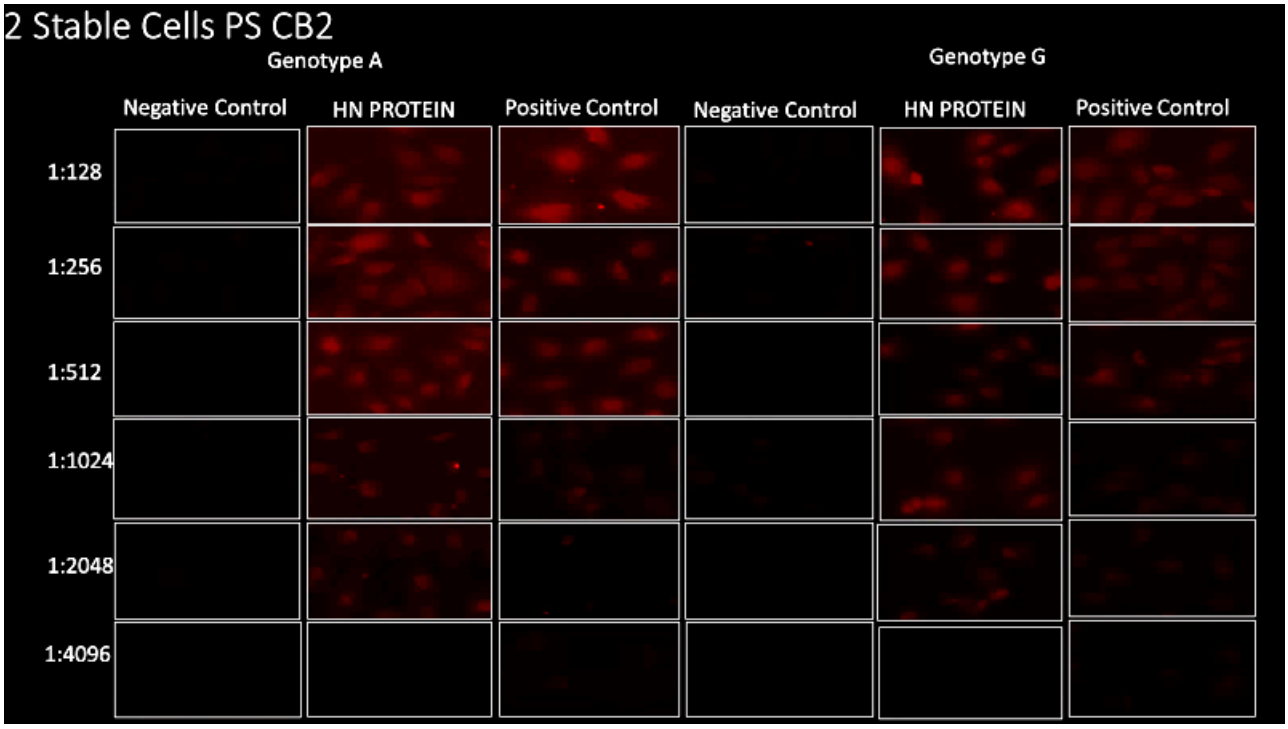


Figure A-33c. Stable cells Vaccine-Infected (CB) Sample #2.

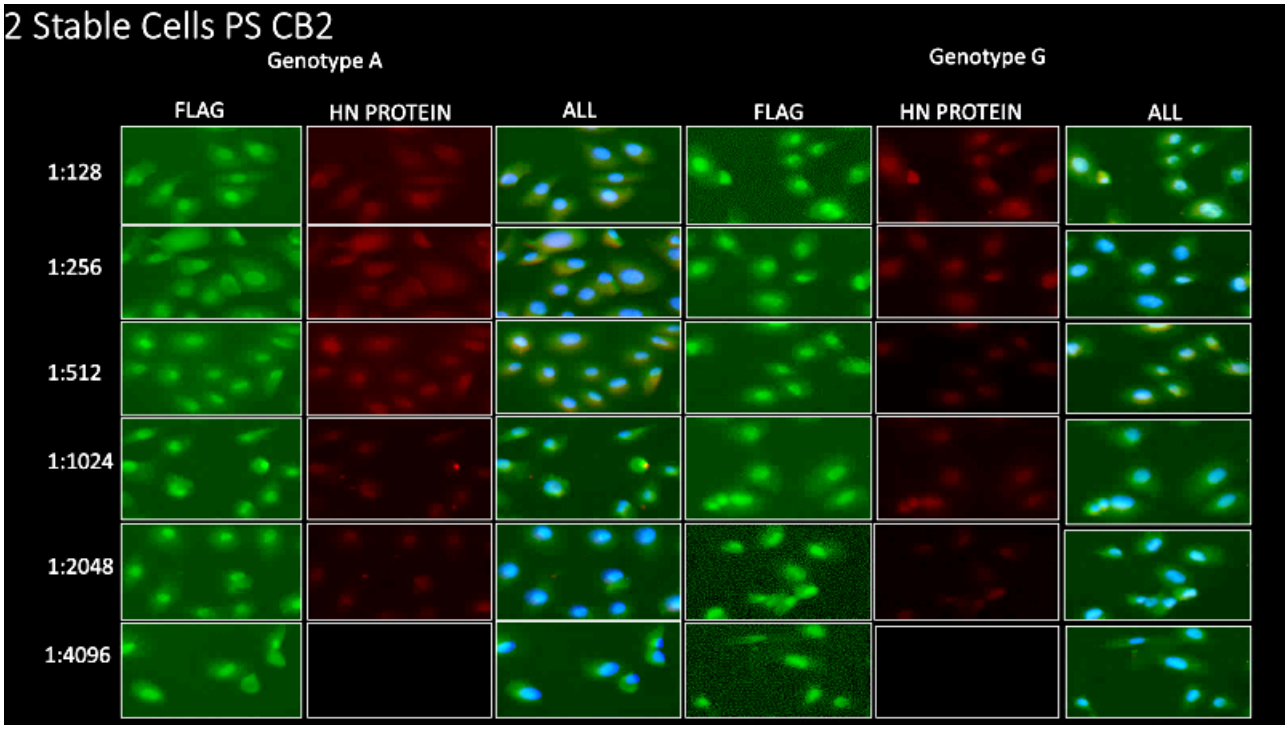


Figure A-33d. Stable cells Vaccine-Infected (CB) Sample #2.

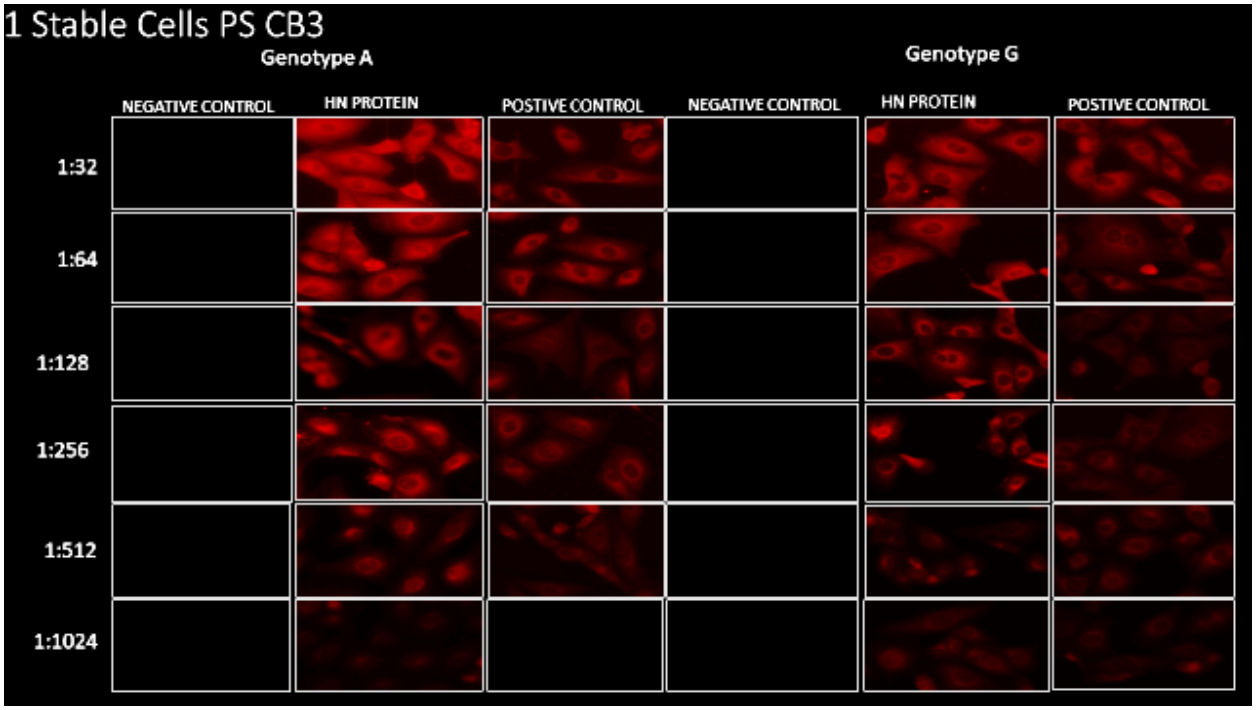


Figure A-34a. Stable cells Vaccine-Infected (CB) Sample#3.

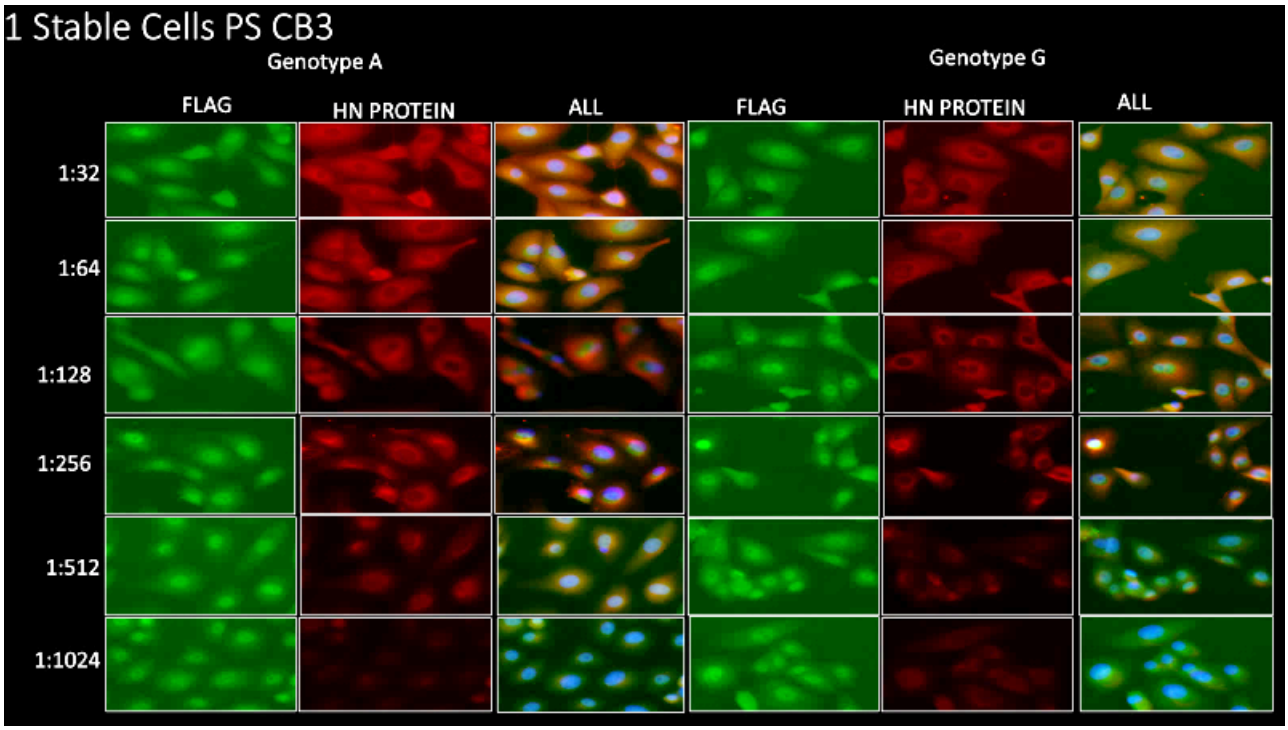


Figure A-34b. Stable cells Vaccine-Infected (CB) Sample #3.

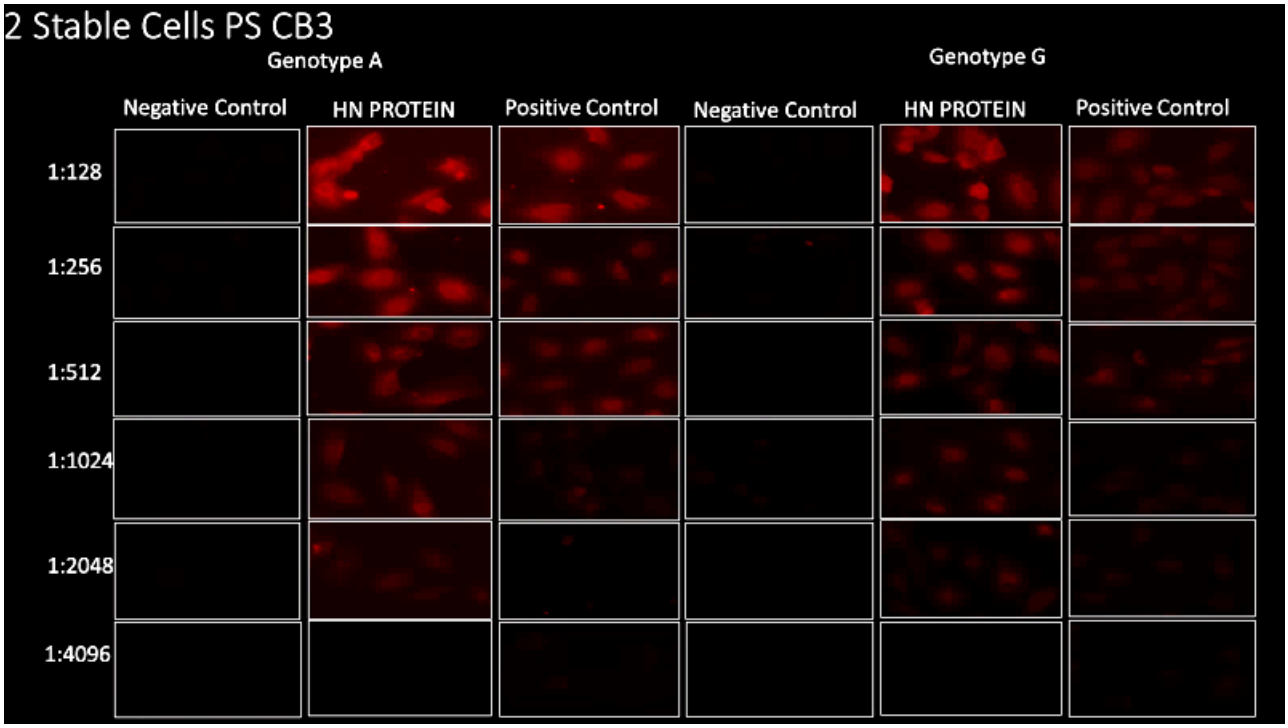


Figure A-34c. Stable cells Vaccine-Infected (CB) Sample #3.

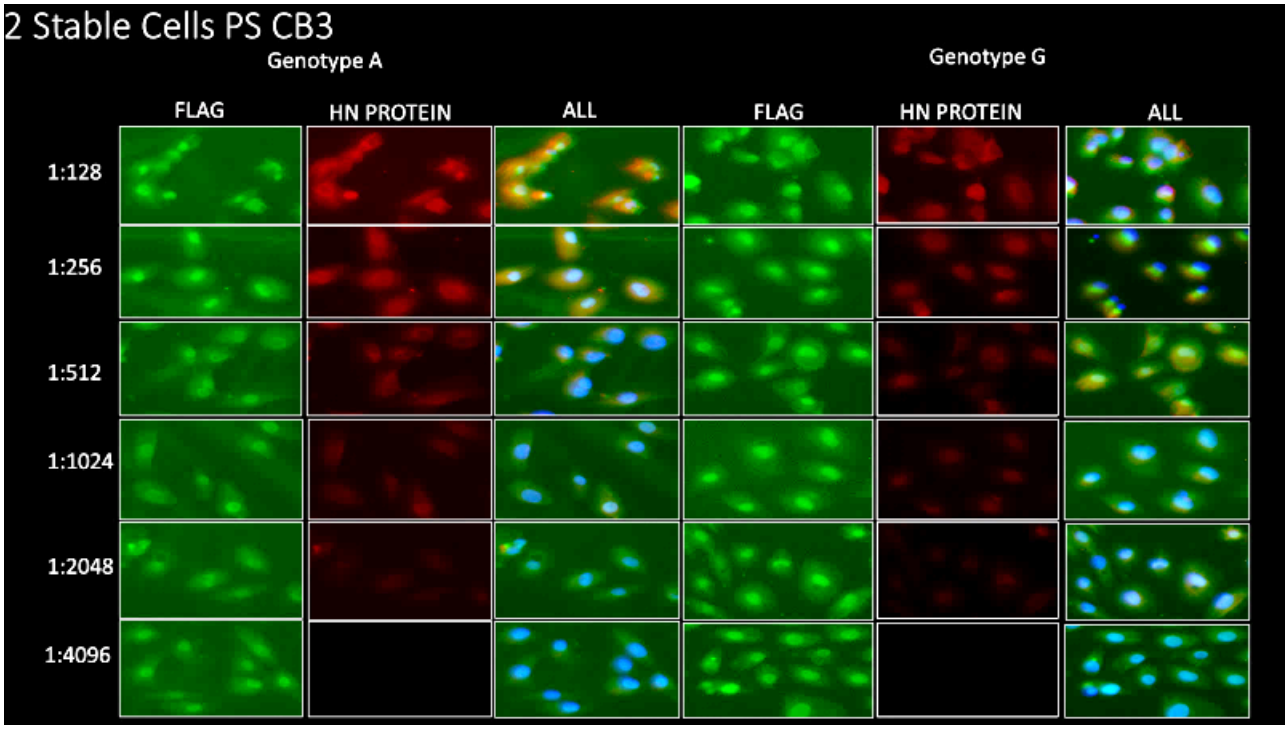


Figure A-34d. Stable cells Vaccine-Infected (CB) Sample #3.

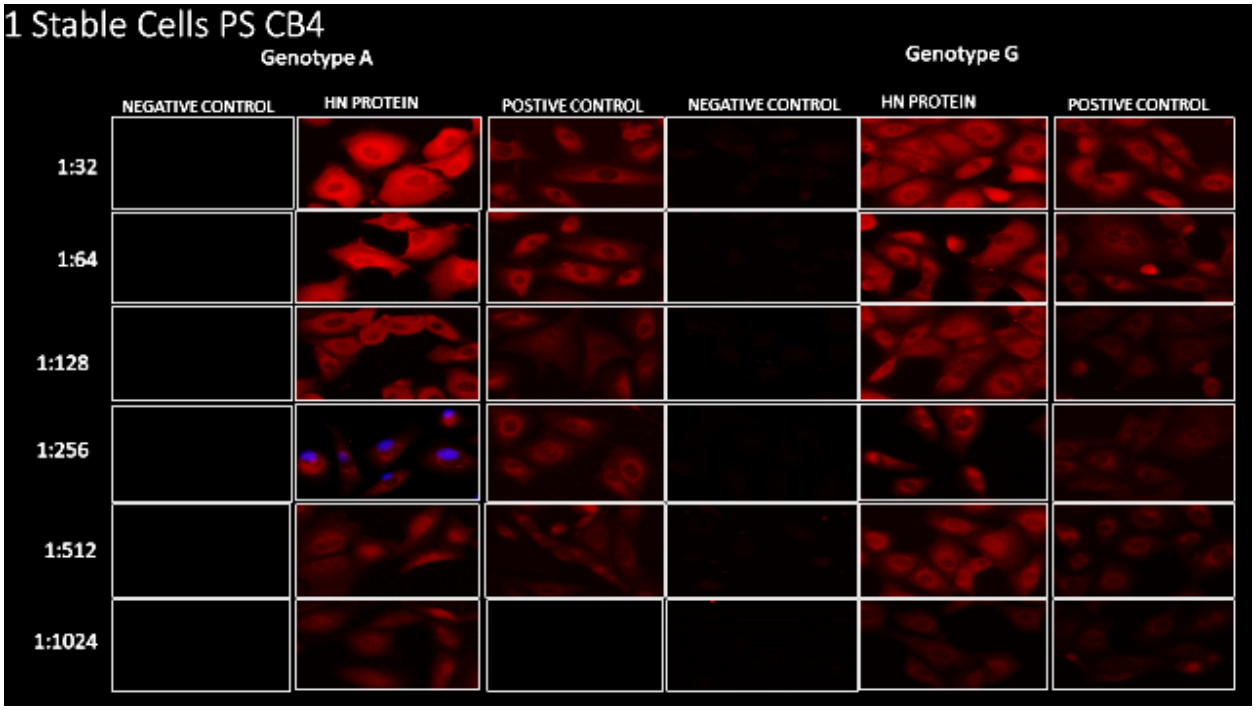


Figure A-35a. Stable cells Vaccine-Infected (CB) Sample#4.

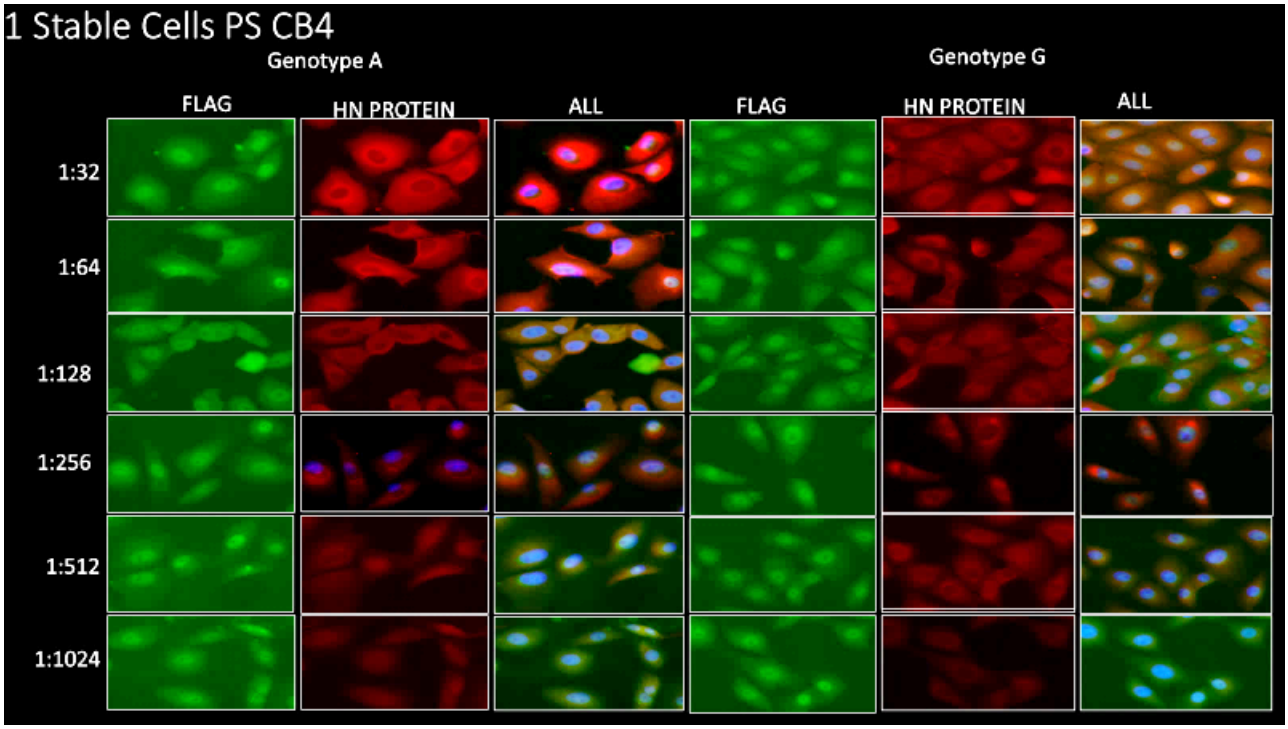


Figure A-35b. Stable cells Vaccine-Infected (CB) Sample #4. Note: Forgot to save red channel Alone

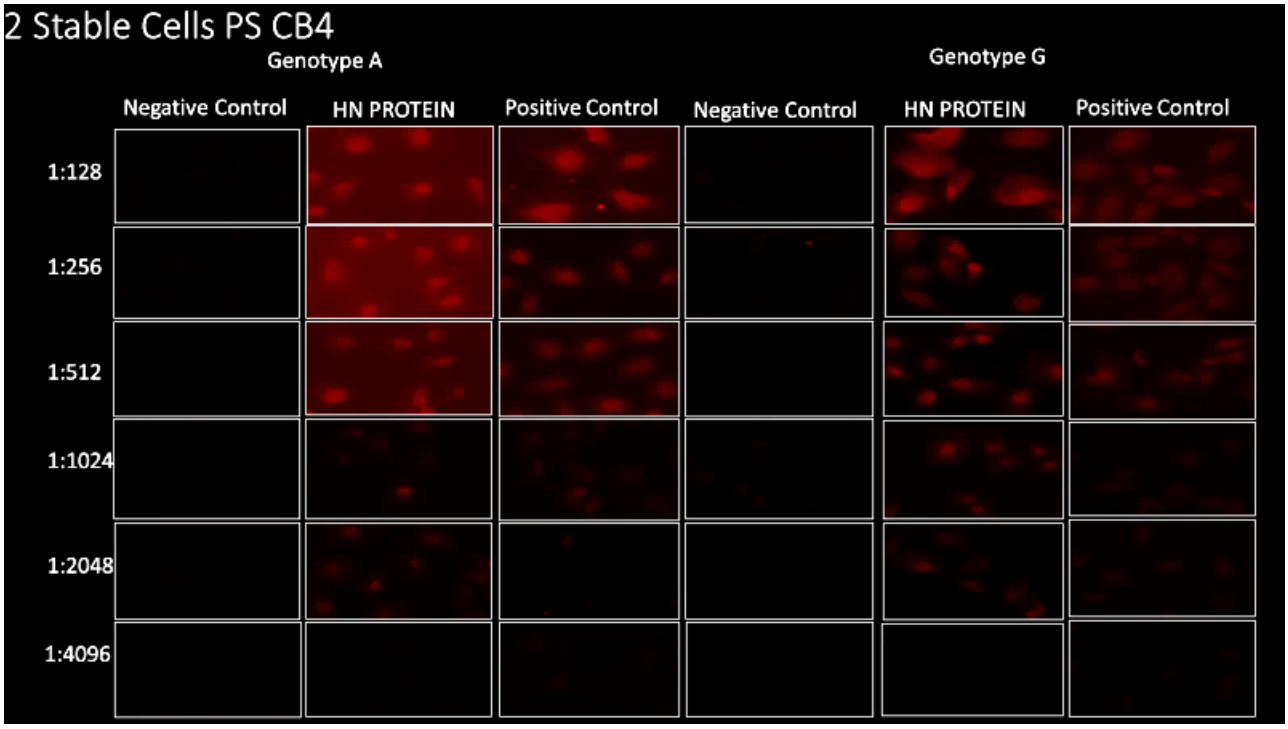


Figure A-35c. Stable cells Vaccine-Infected (CB) Sample #4.

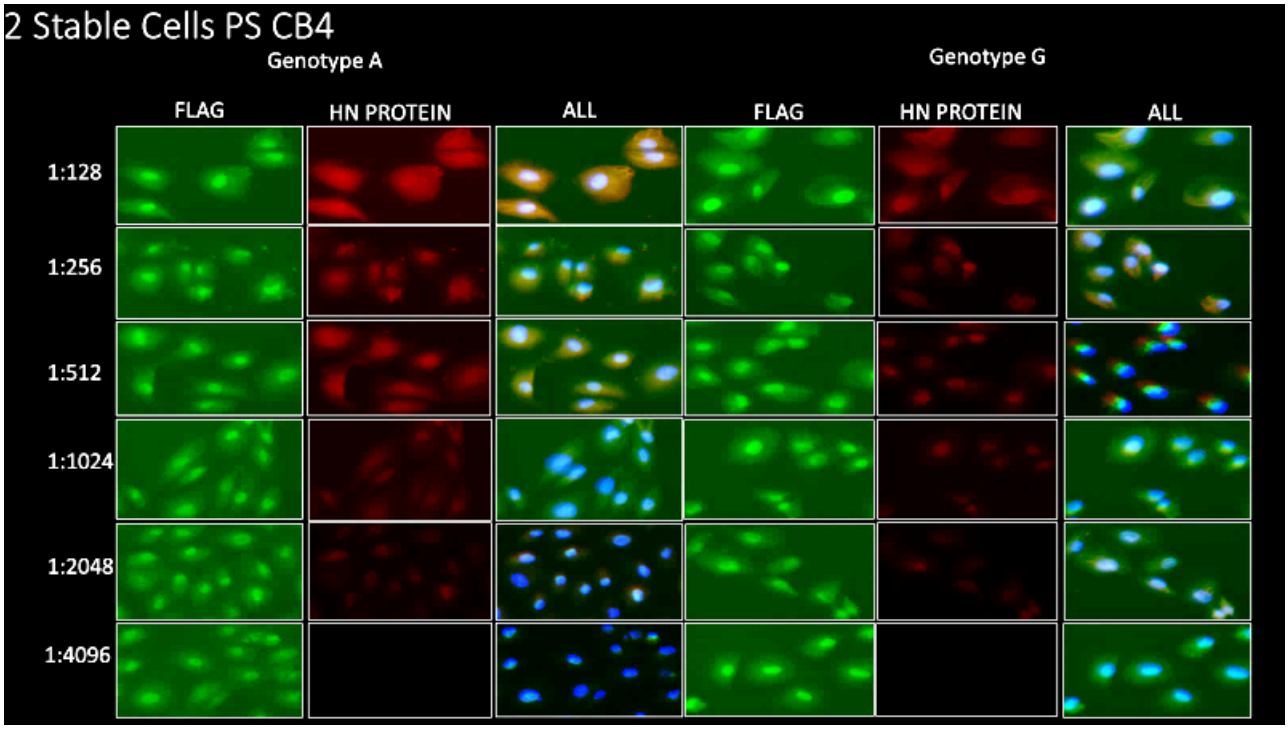


Figure A-35d. Stable cells Vaccine-Infected (CB) Sample #4.

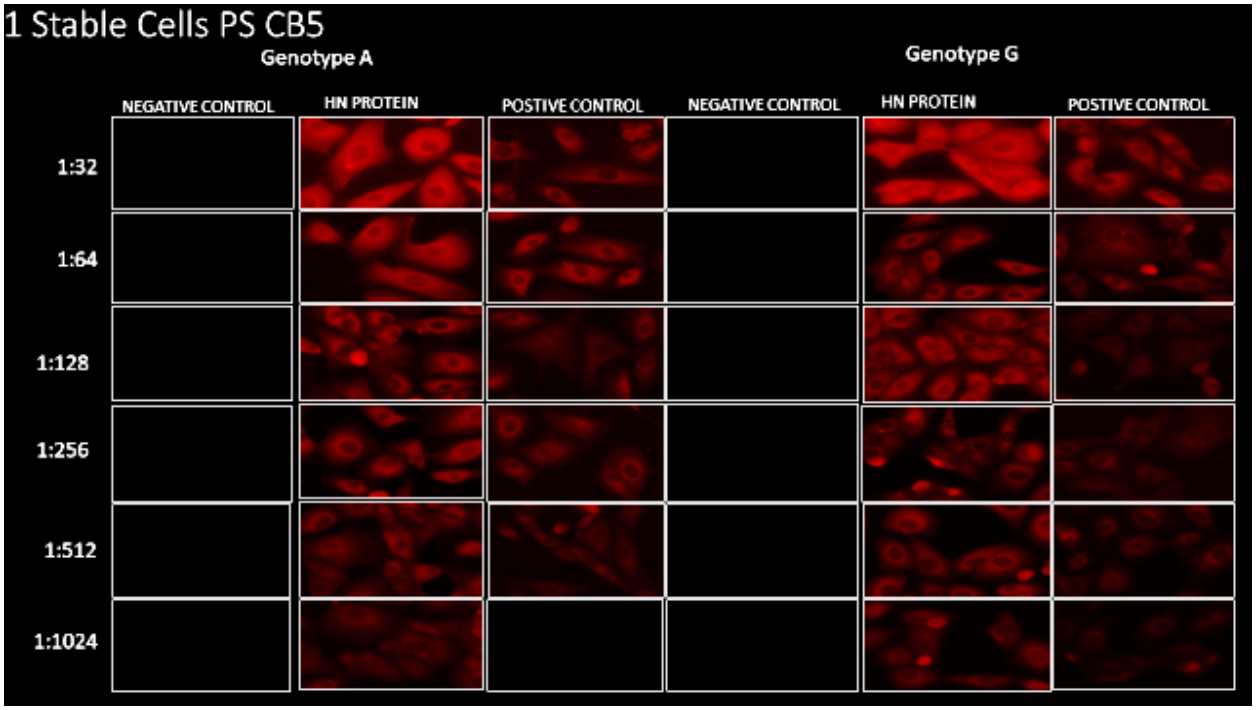


Figure A-36a. Stable cells Vaccine-Infected (CB) Sample#5.

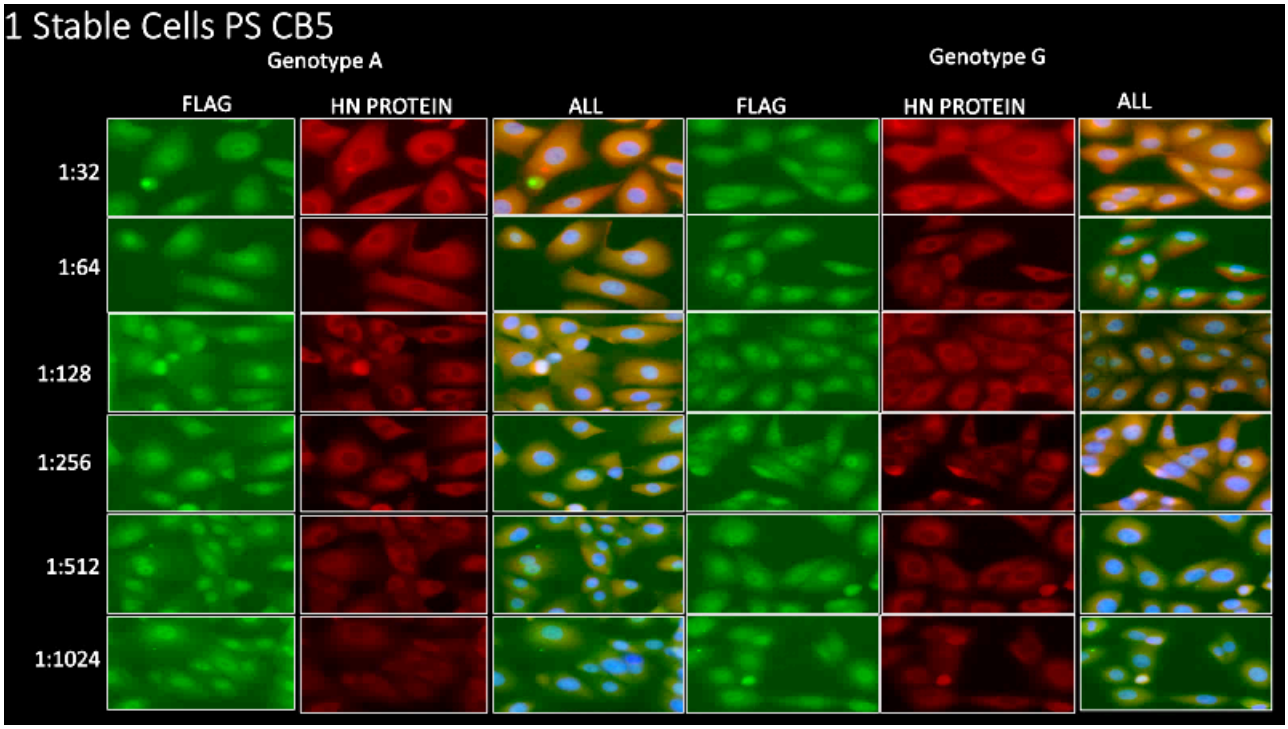


Figure A-36b. Stable cells Vaccine-Infected (CB) Sample #5.

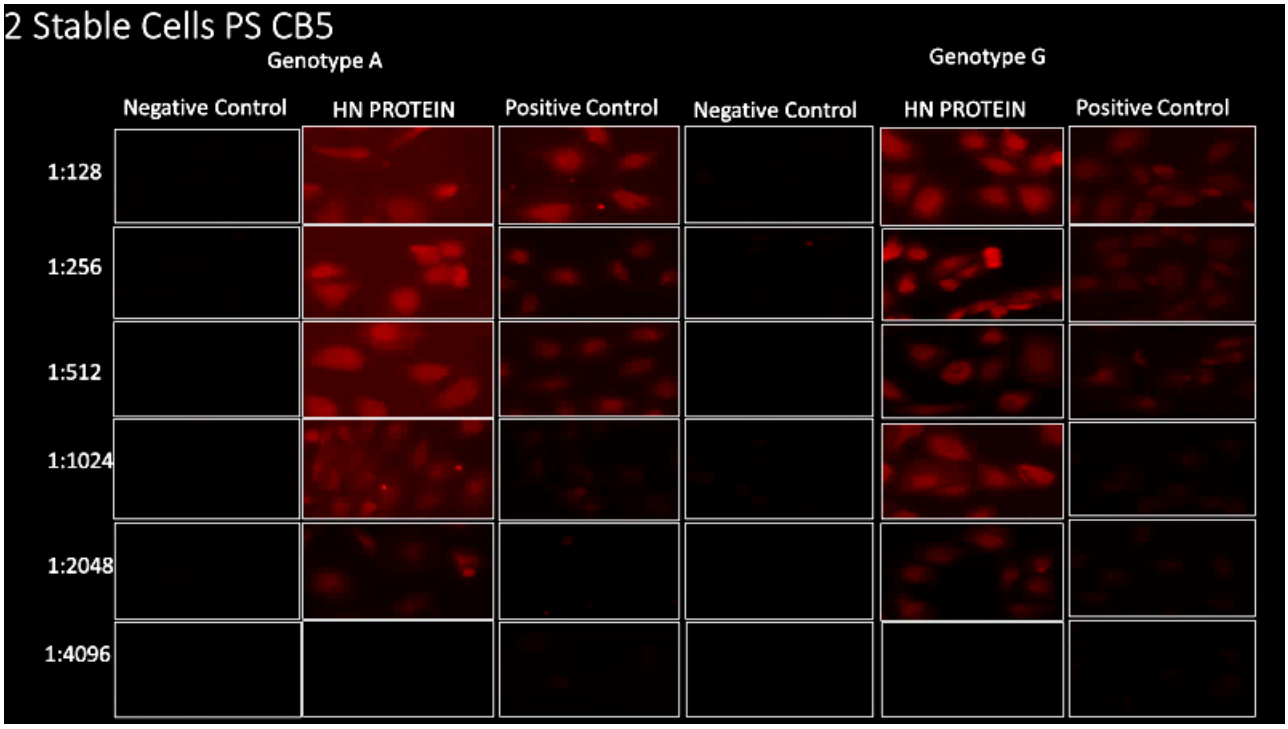


Figure A-36c. Stable cells Vaccine-Infected (CB) Sample #5.

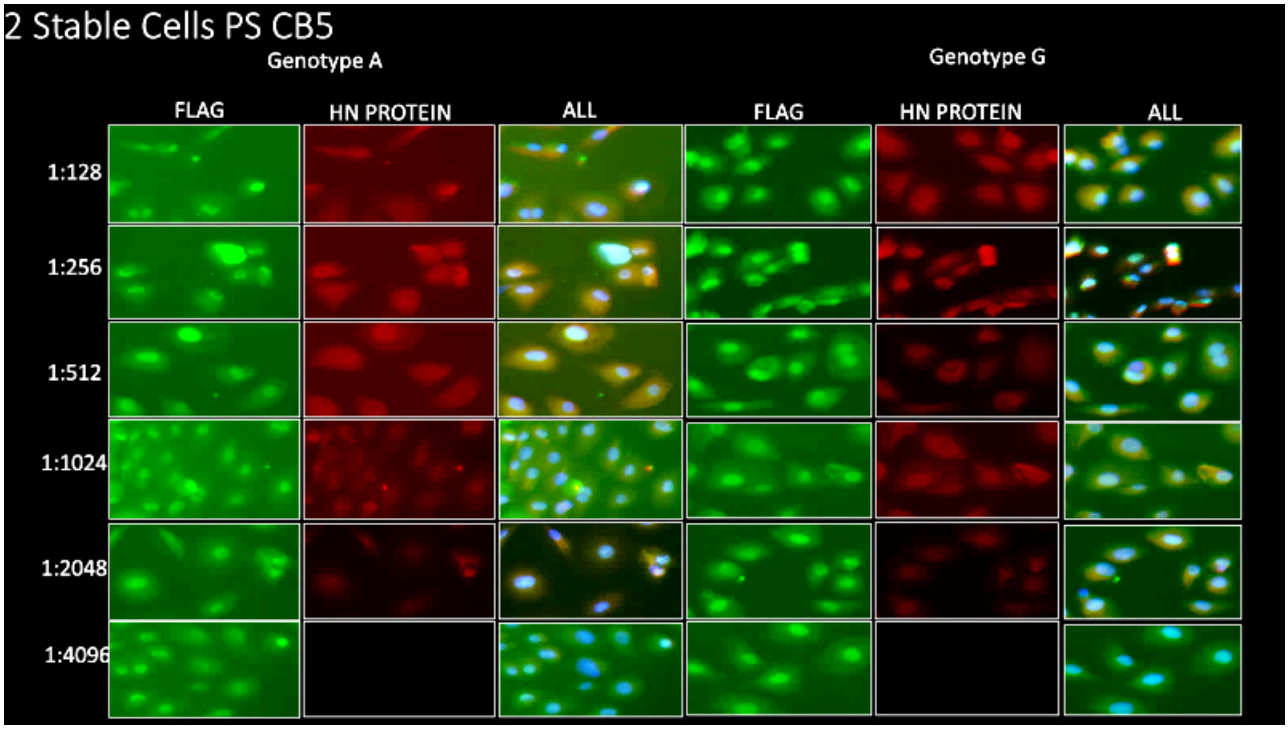


Figure A-36d. Stable cells Vaccine-Infected (CB) Sample #5.

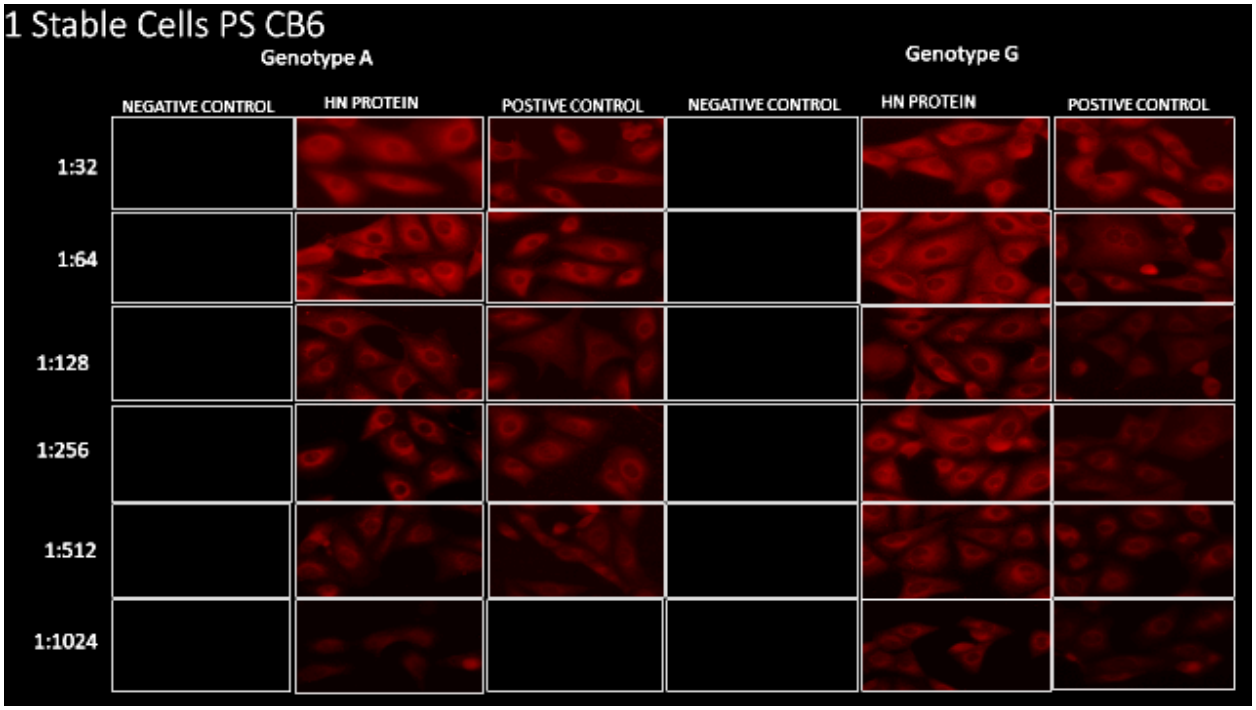


Figure A-37a. Stable cells Vaccine-Infected (CB) Sample#6.

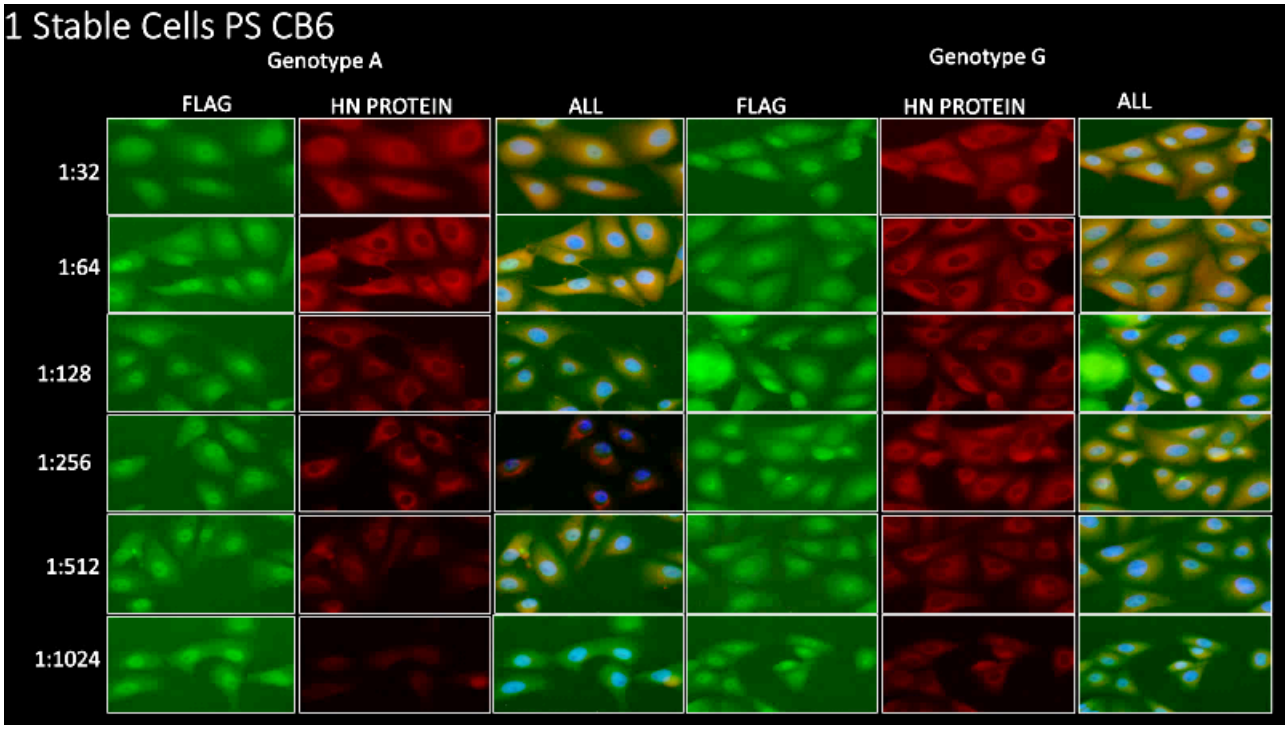


Figure A-37b. Stable cells Vaccine-Infected (CB) Sample #6.

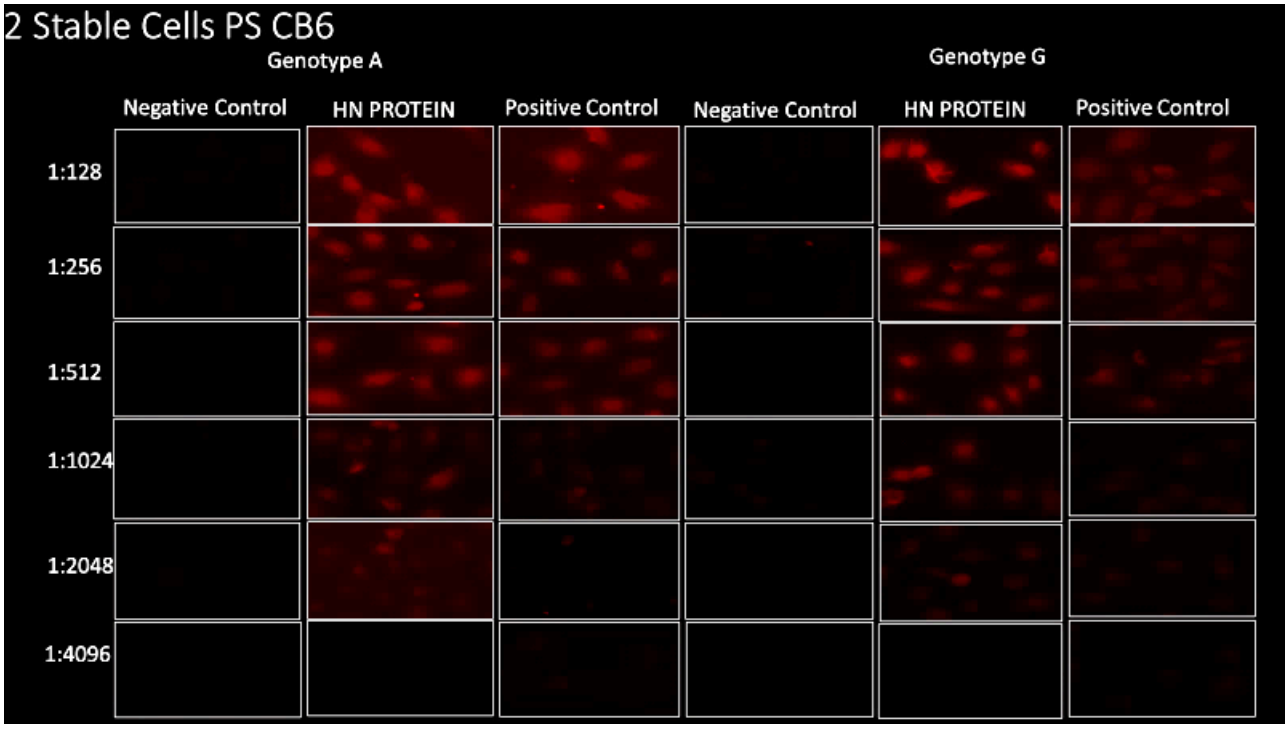


Figure A-37c. Stable cells Vaccine-Infected (CB) Sample #6.

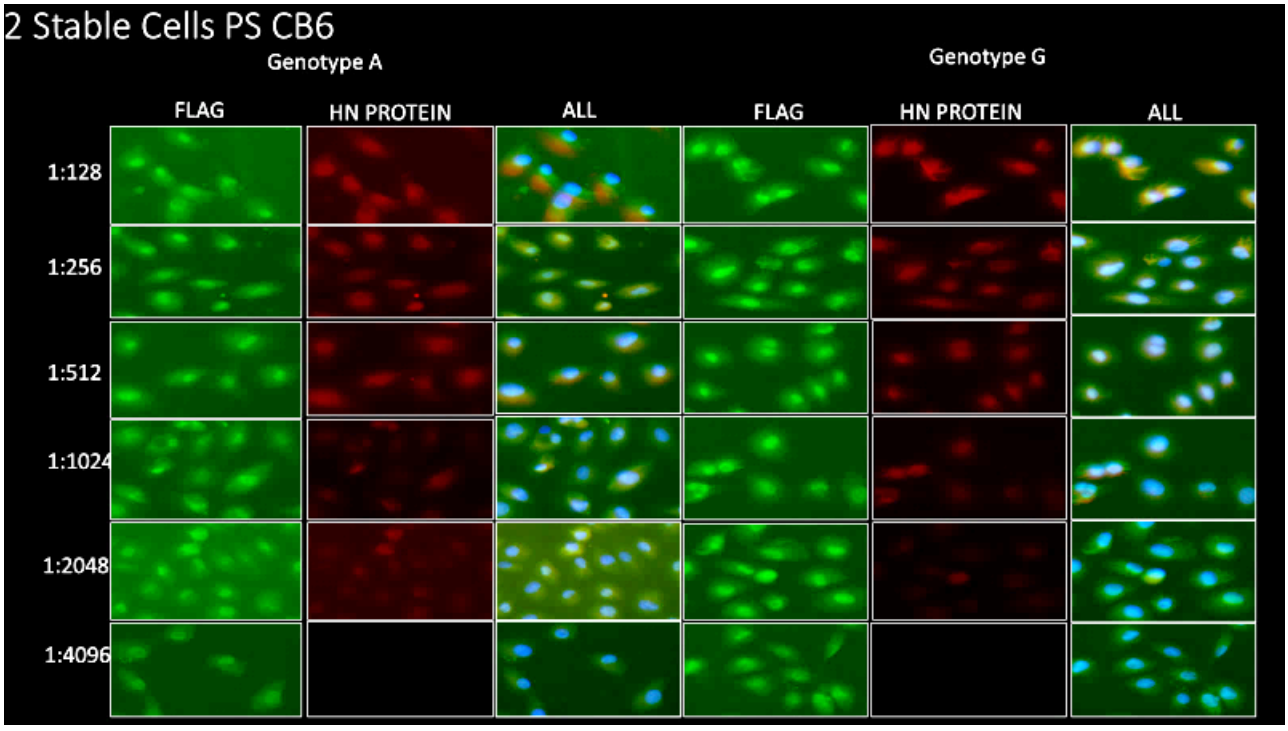


Figure A-37d. Stable cells Vaccine-Infected (CB) Sample #6.

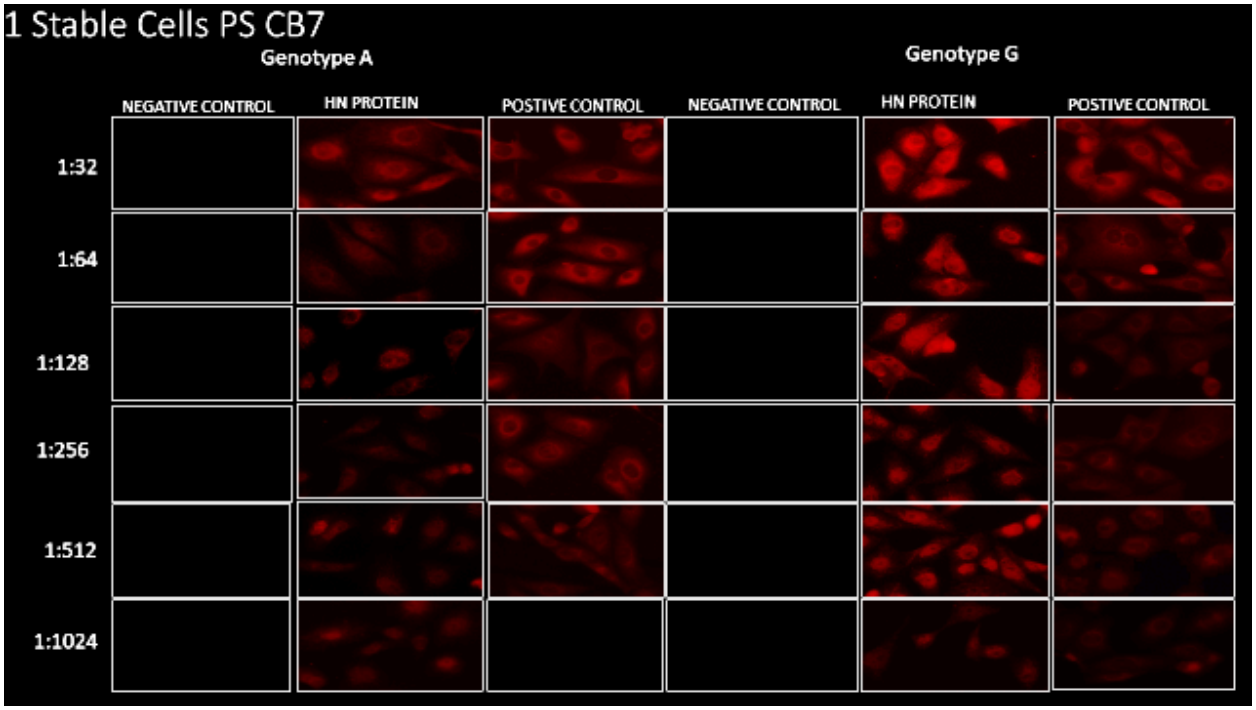


Figure A-38a. Stable cells Vaccine-Infected (CB) Sample#7.

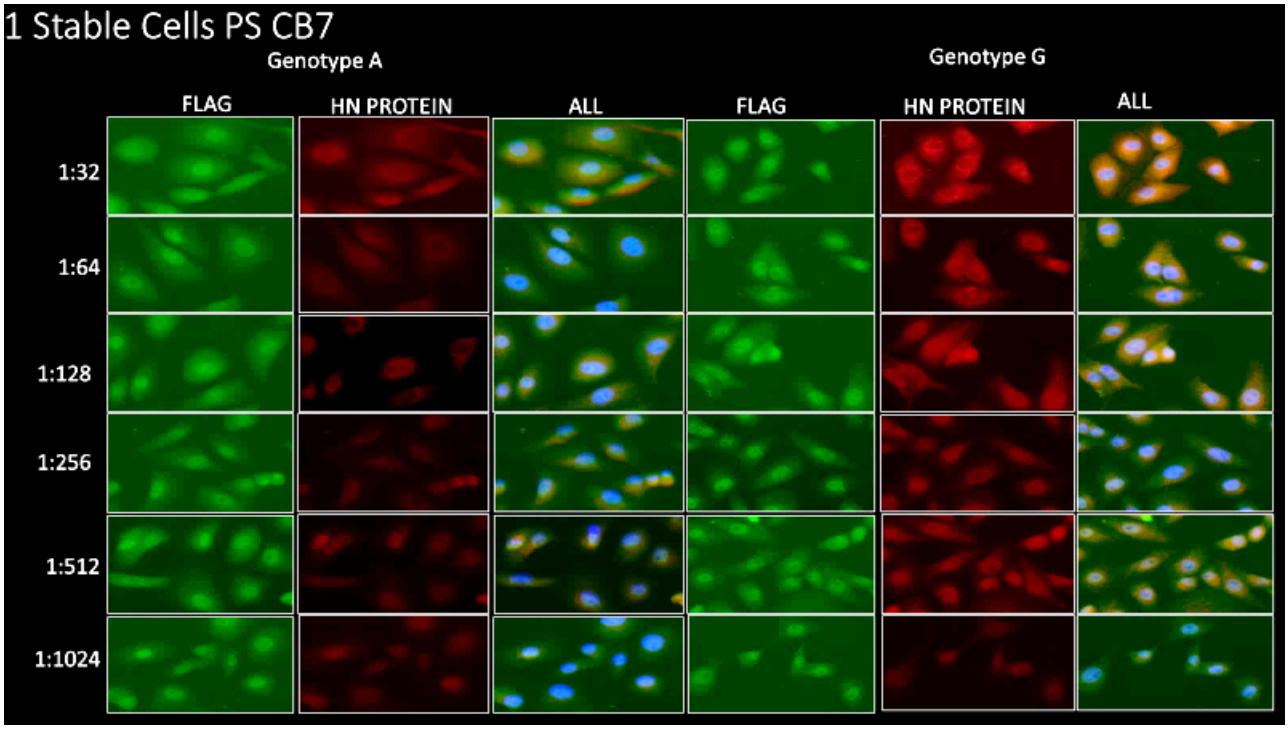


Figure A-38b. Stable cells Vaccine-Infected (CB) Sample #7.

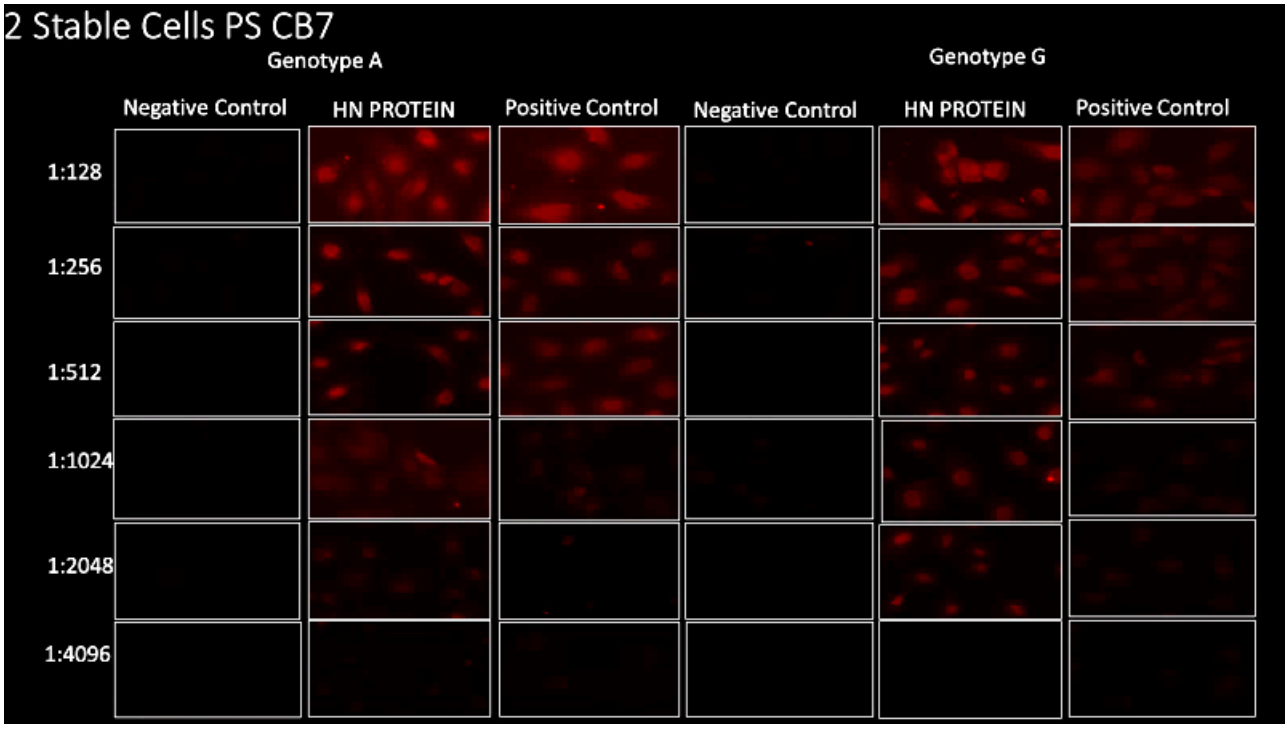


Figure A-38c. Stable cells Vaccine-Infected (CB) Sample #7.

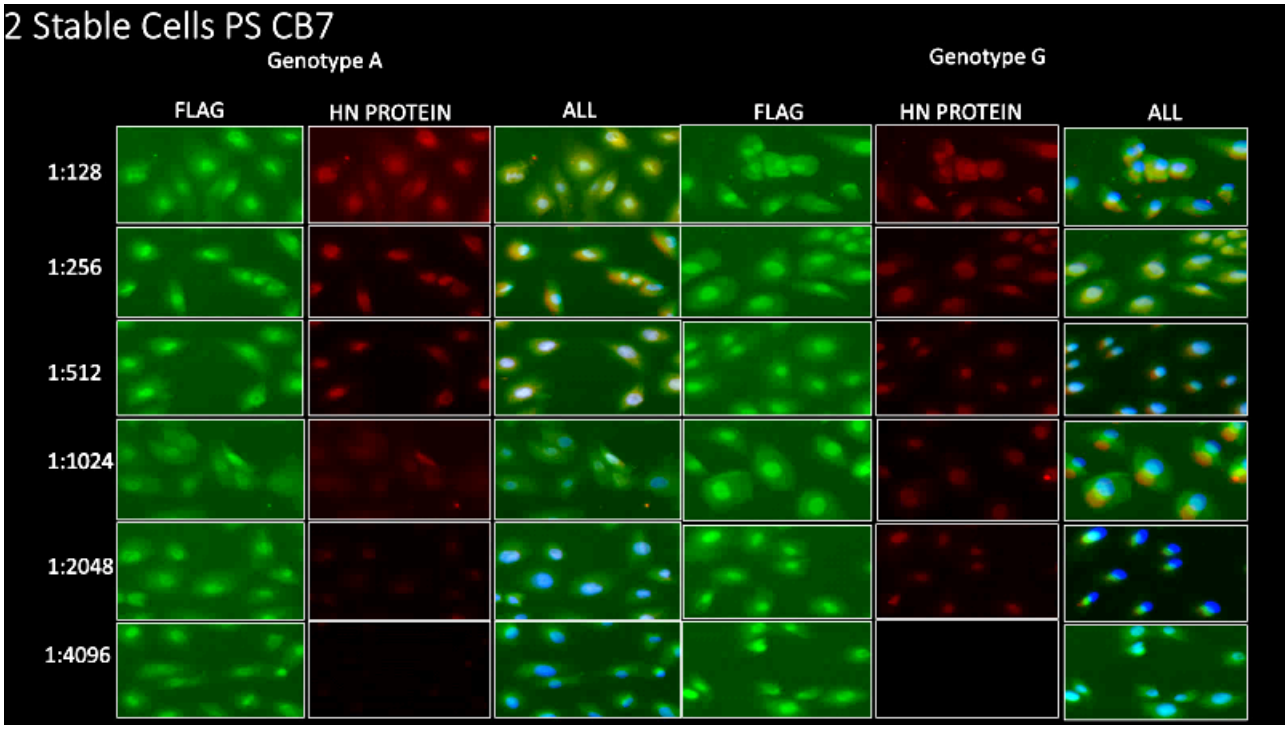


Figure A-38d. Stable cells Vaccine-Infected (CB) Sample #7.

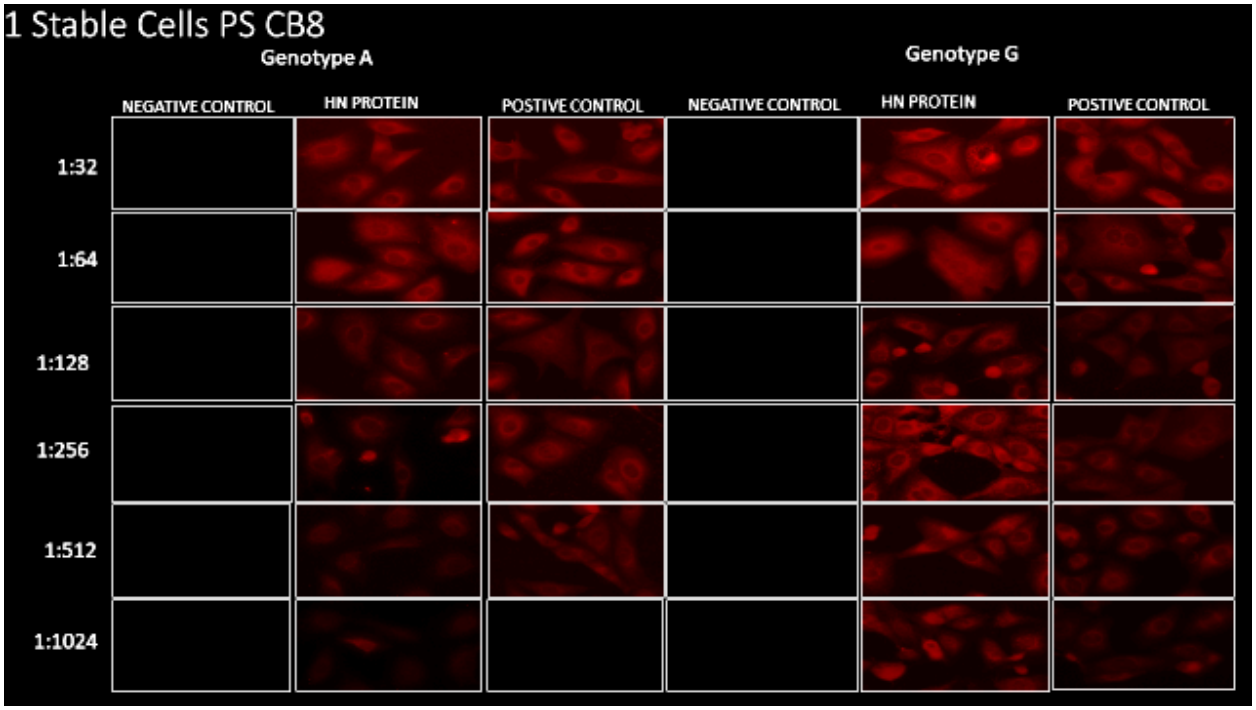


Figure A-39a. Stable cells Vaccine-Infected (CB) Sample#8.

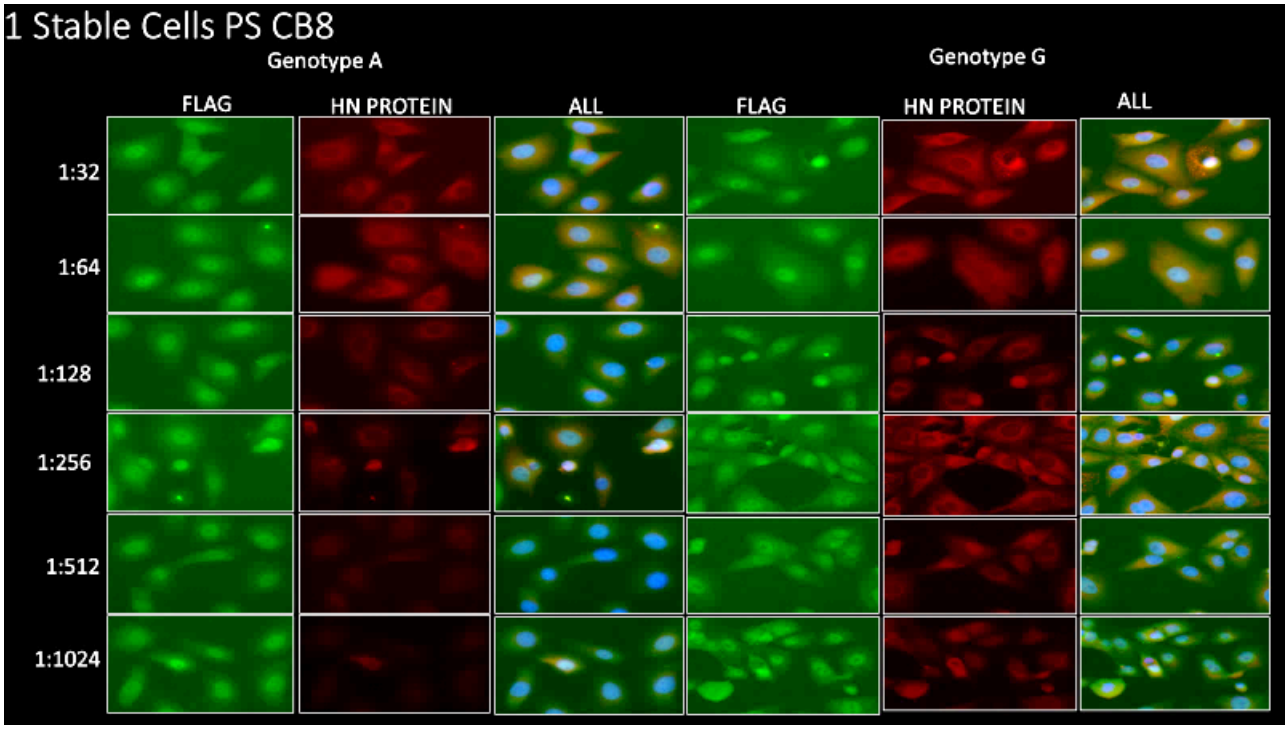


Figure A-39b. Stable cells Vaccine-Infected (CB) Sample #8.

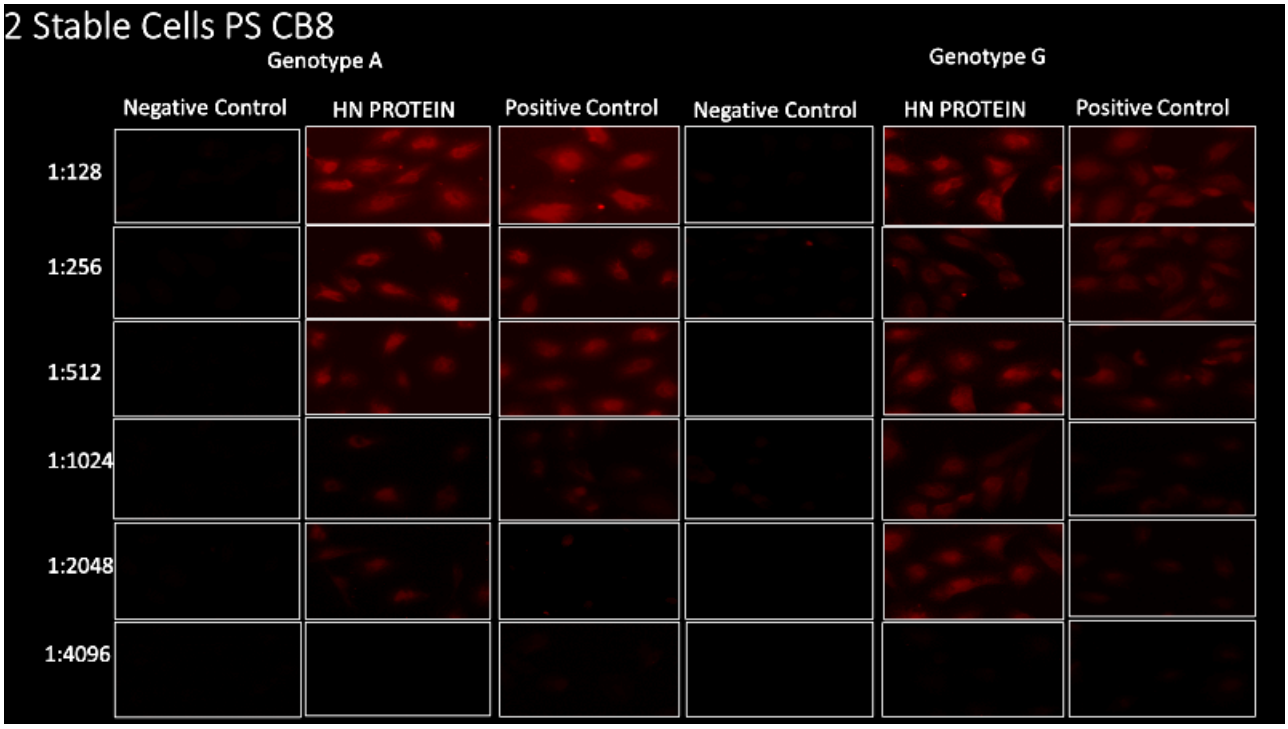


Figure A-39c. Stable cells Vaccine-Infected (CB) Sample #8.

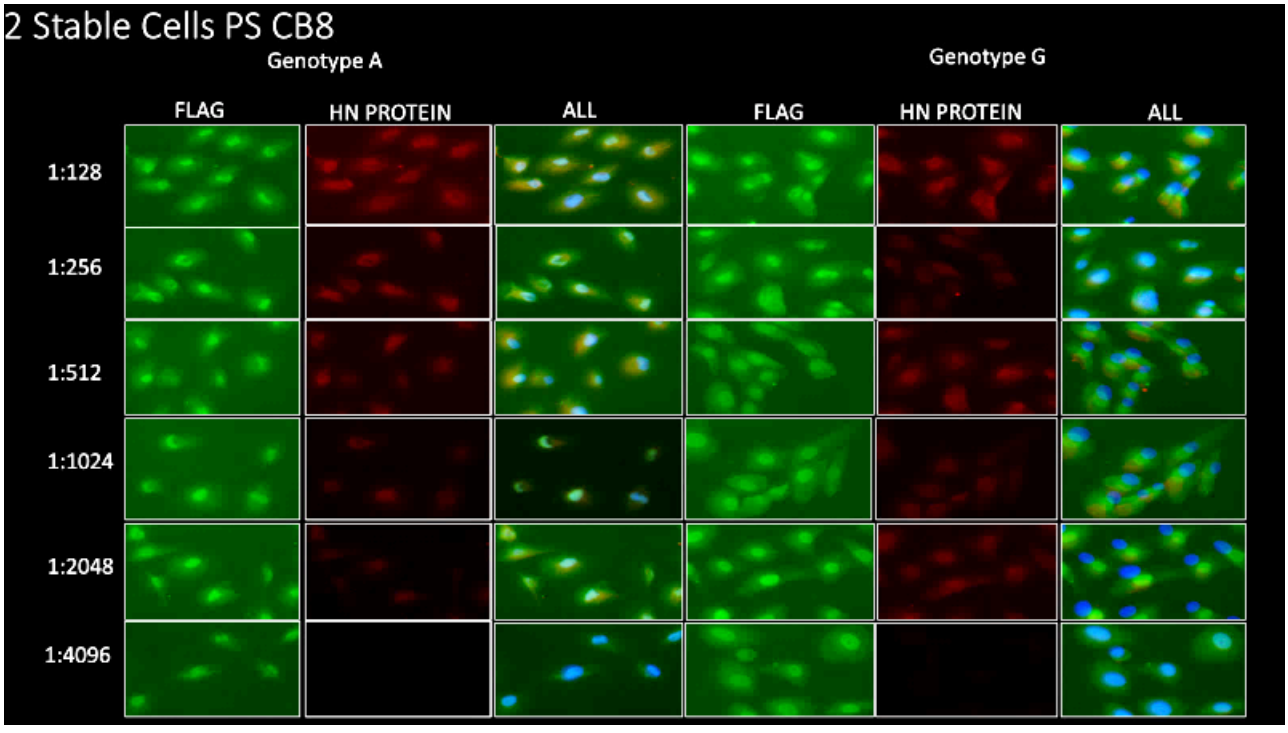


Figure A-39d. Stable cells Vaccine-Infected (CB) Sample #8.

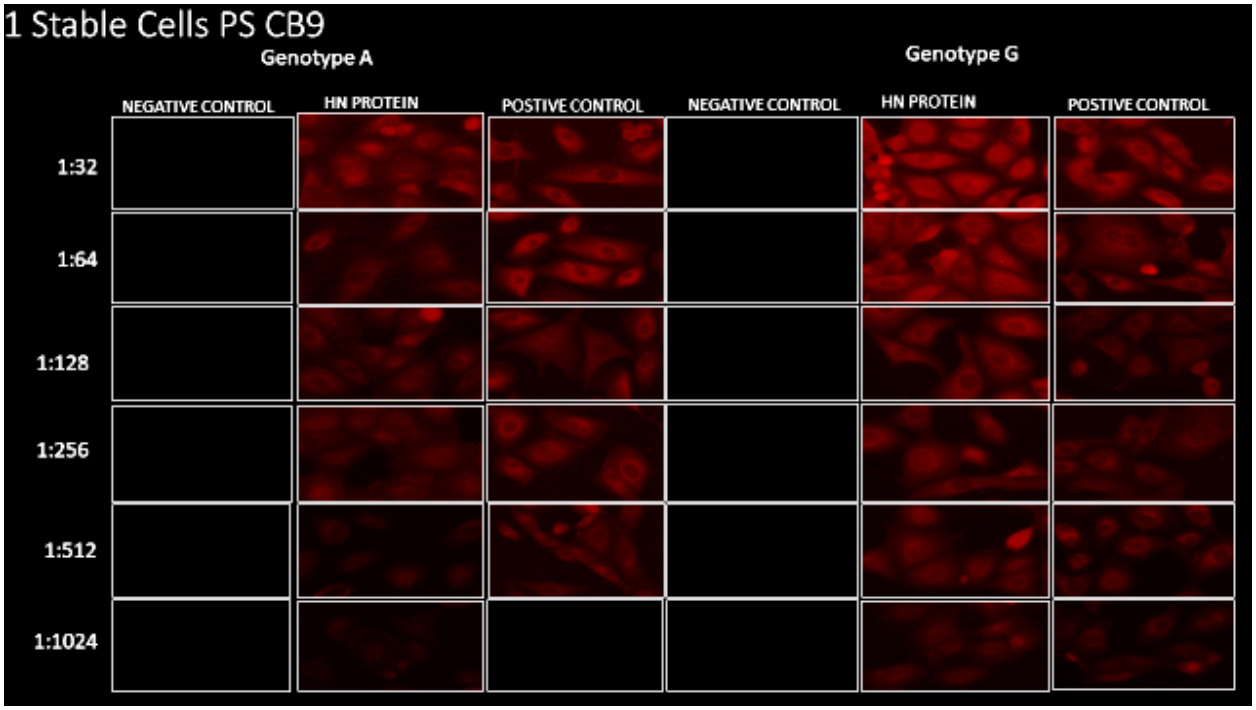


Figure A-40a. Stable cells Vaccine-Infected (CB) Sample#9.

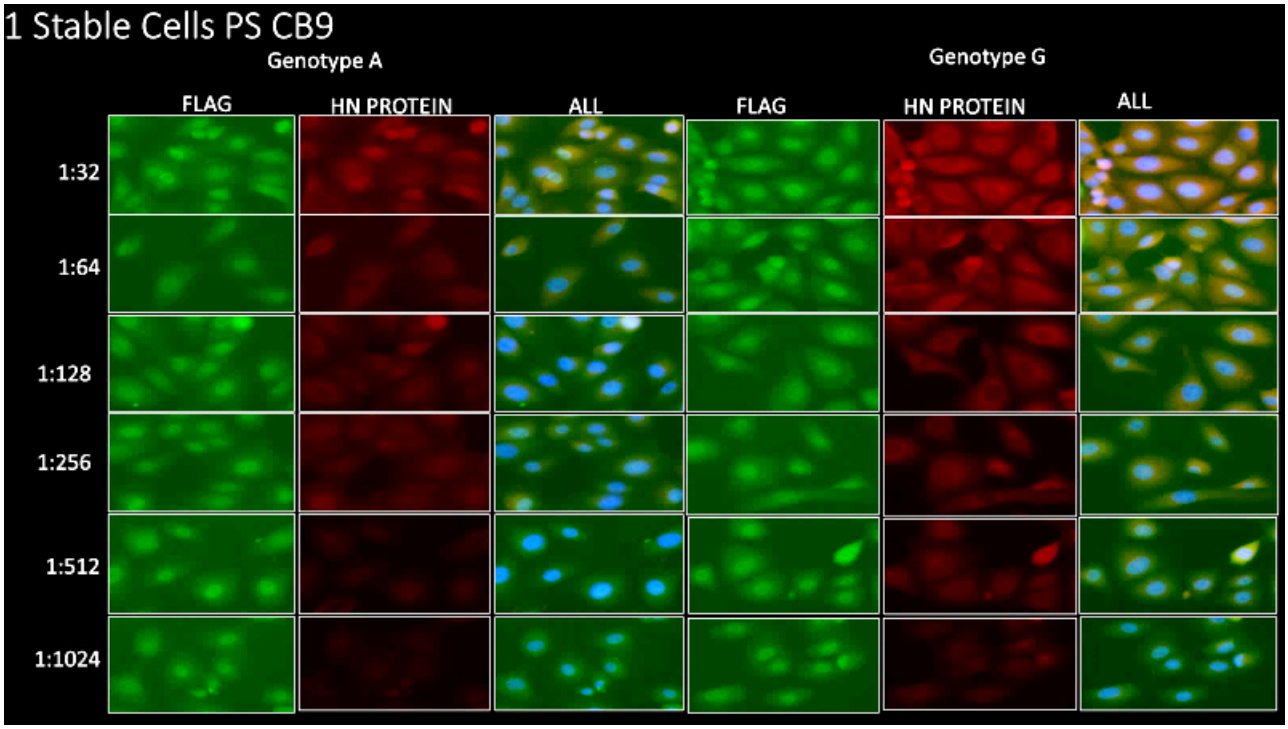


Figure A-40b. Stable cells Vaccine-Infected (CB) Sample #9.

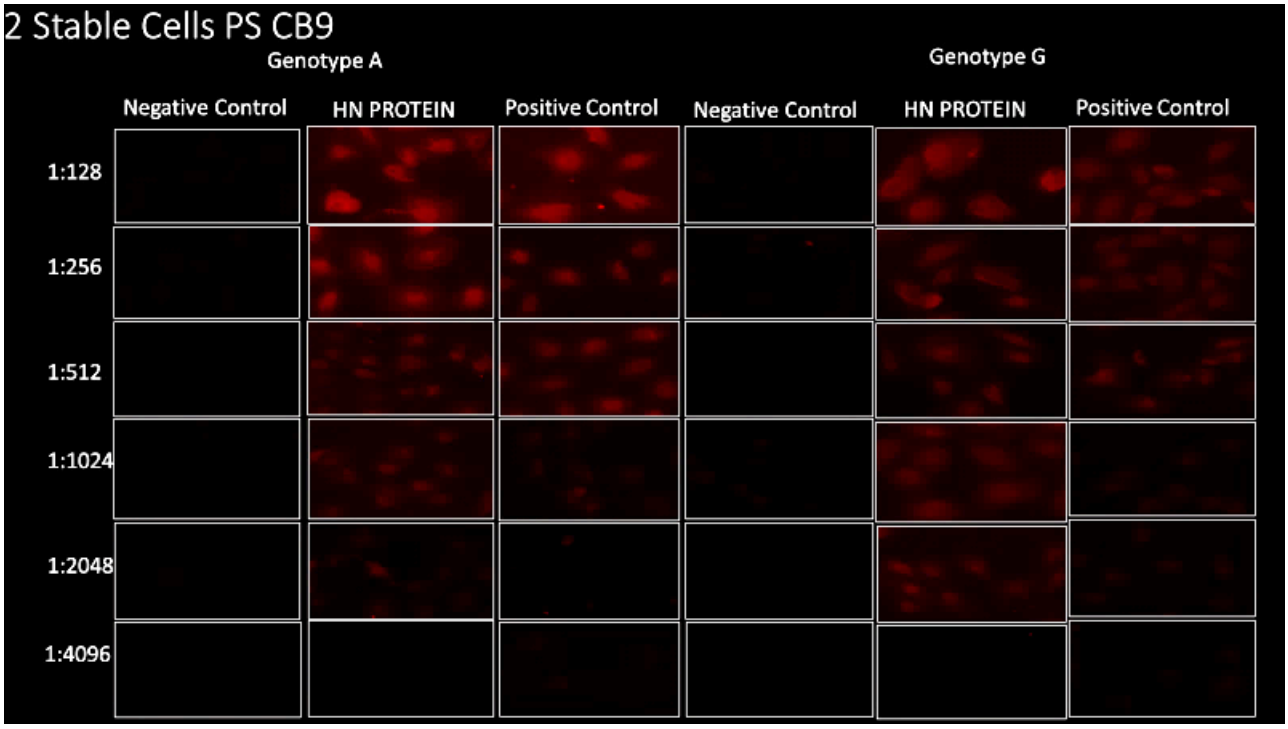


Figure A-40c. Stable cells Vaccine-Infected (CB) Sample #9.

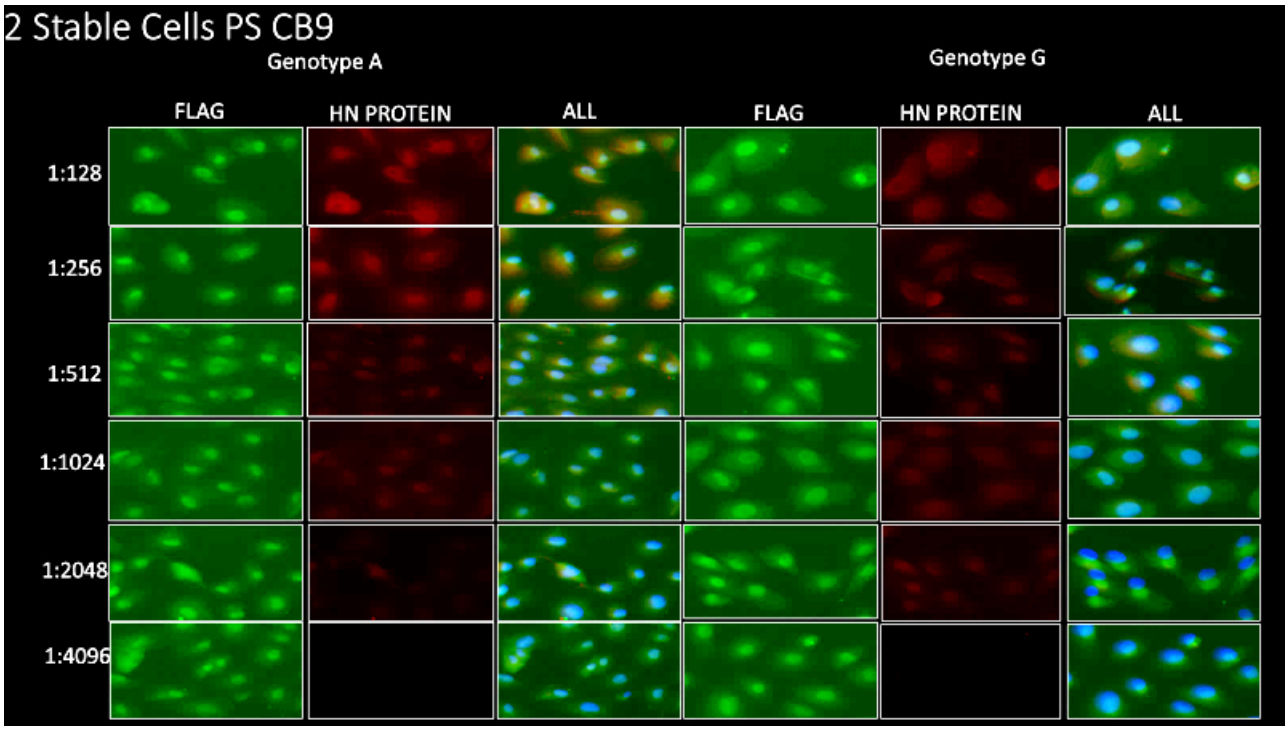


Figure A-40d. Stable cells Vaccine-Infected (CB) Sample #9.

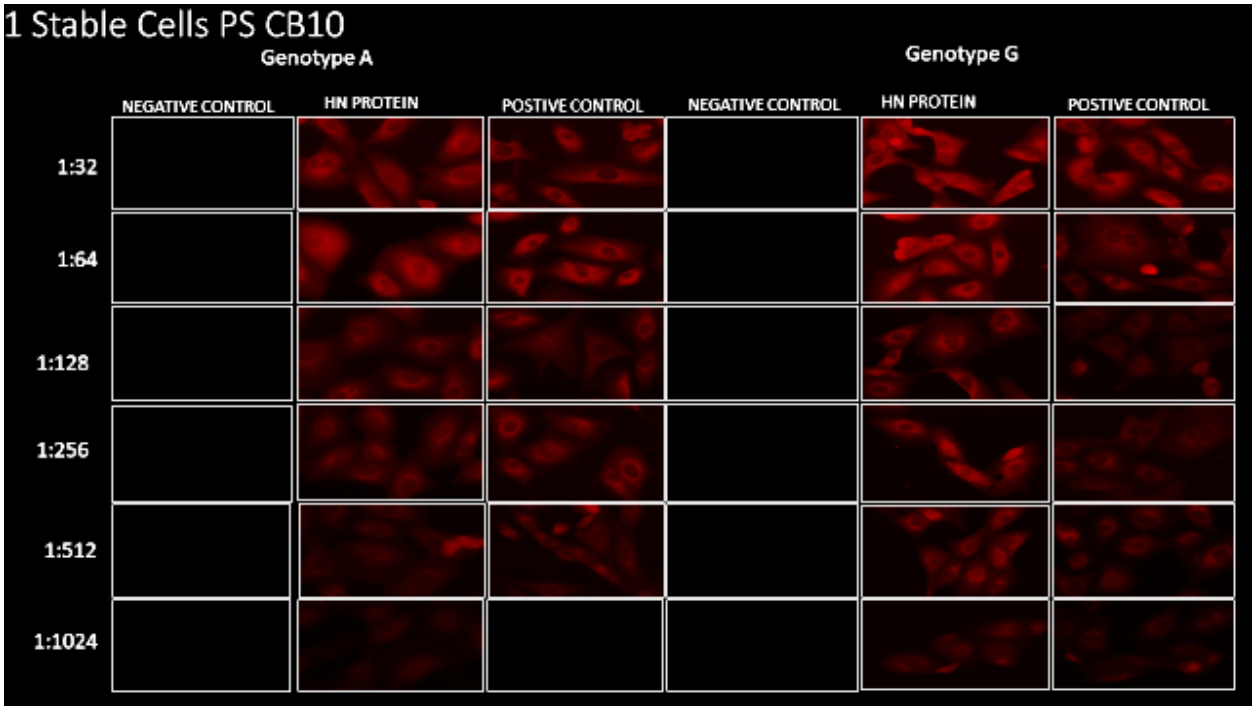


Figure A-41a. Stable cells Vaccine-Infected (CB) Sample#10.

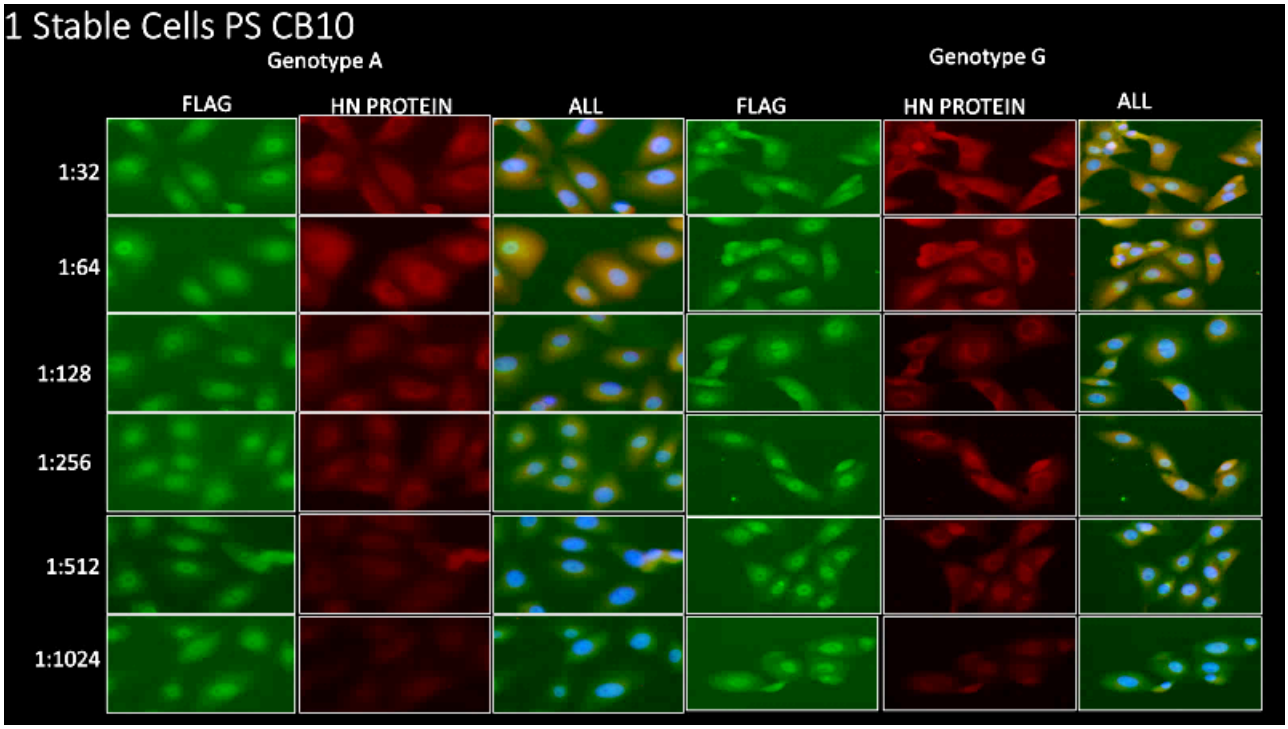


Figure A-41b. Stable cells Vaccine-Infected (CB) Sample #10.

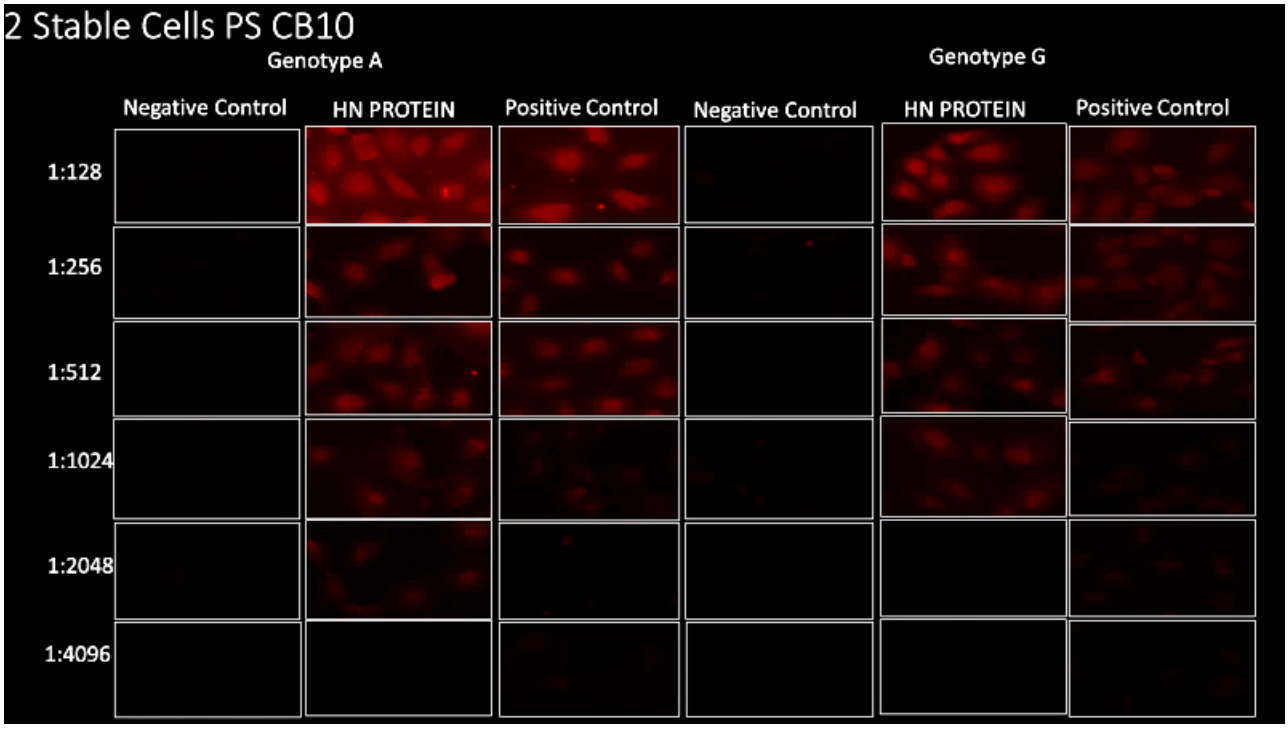


Figure A-41c. Stable cells Vaccine-Infected (CB) Sample #10.

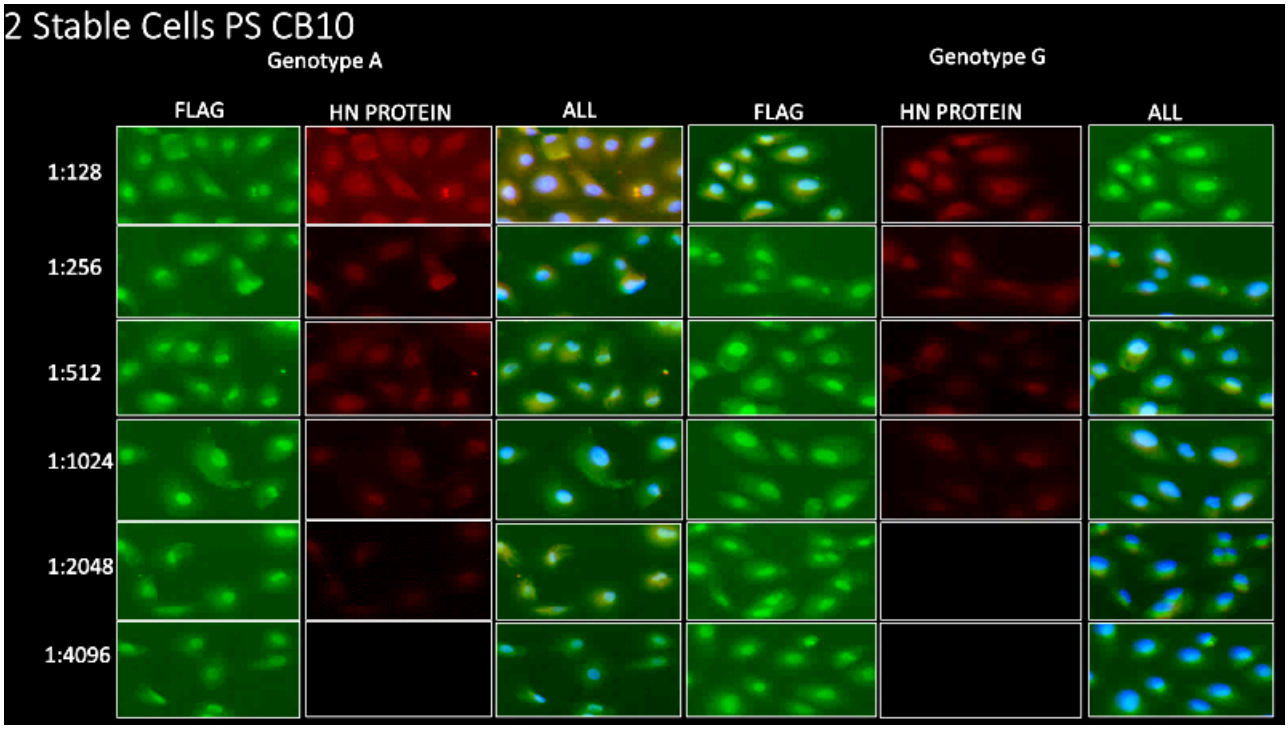


Figure A-41d. Stable cells Vaccine-Infected (CB) Sample #10.

IFA Results of F Protein

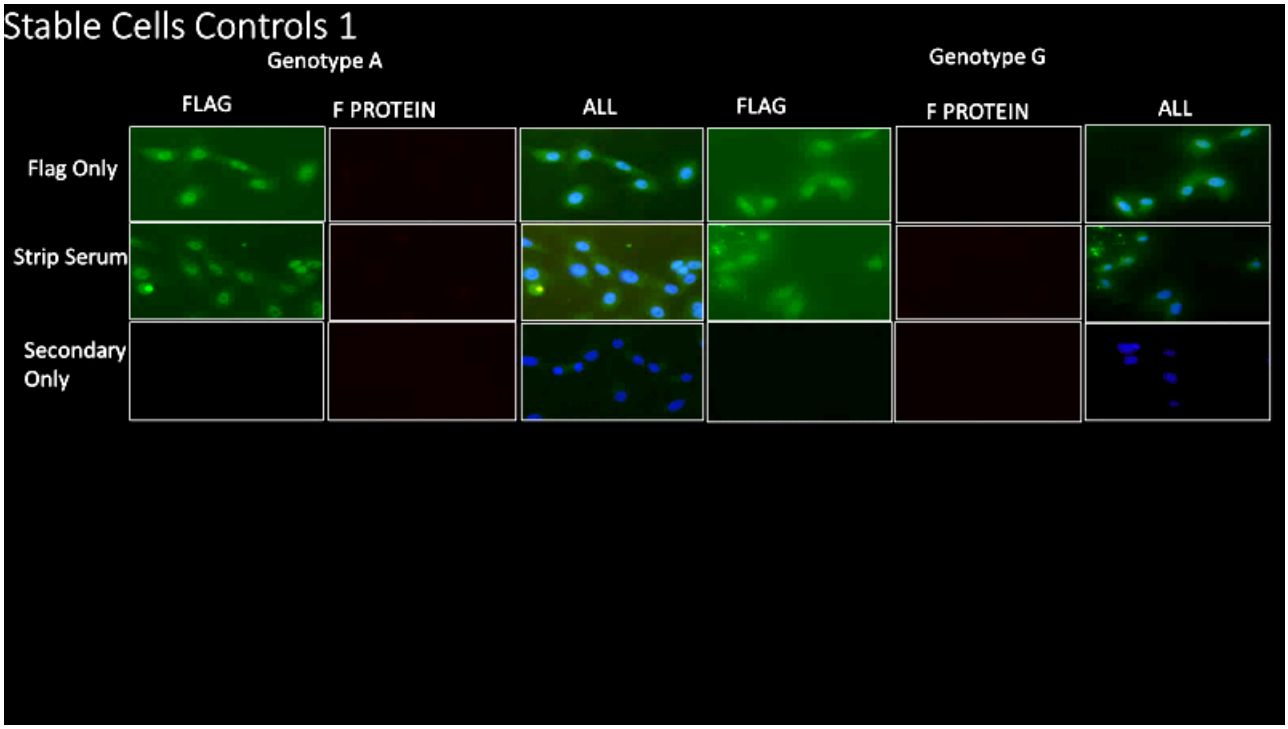


Figure A-42a. Stable cells Control Batch#1.

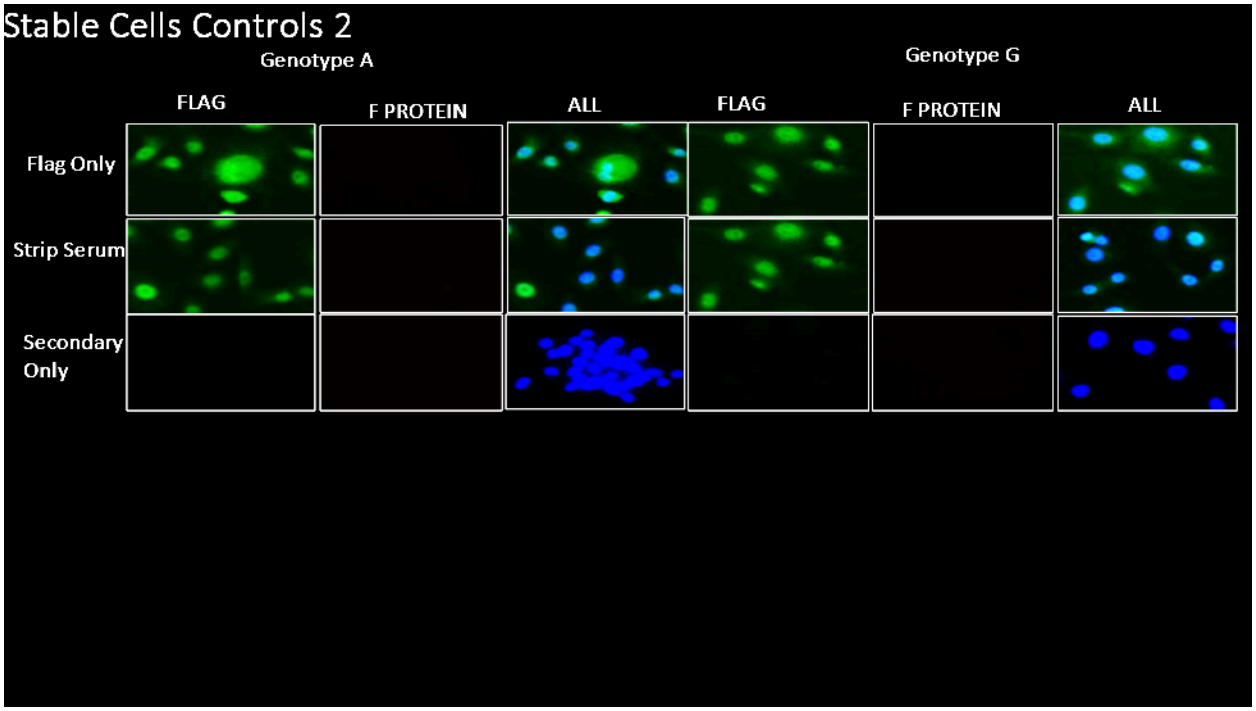


Figure A-42b. Stable cells Control Batch#2.

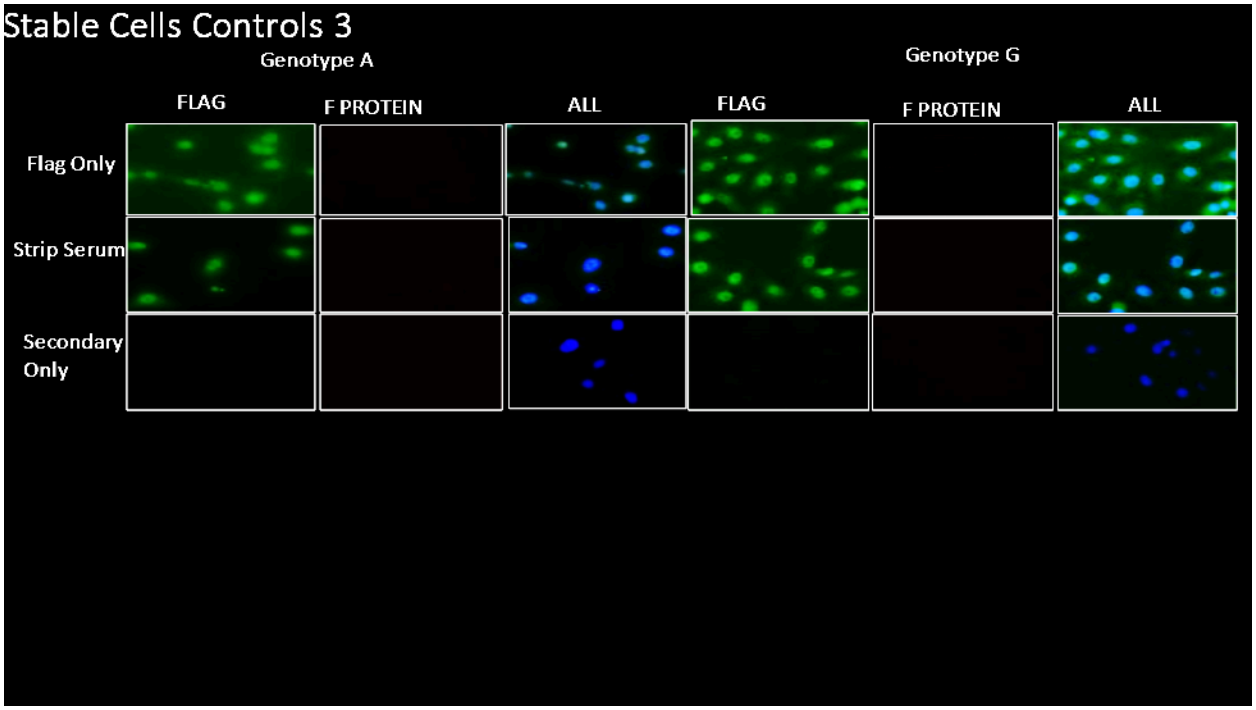


Figure A-42c. Stable cells Control Batch#3.

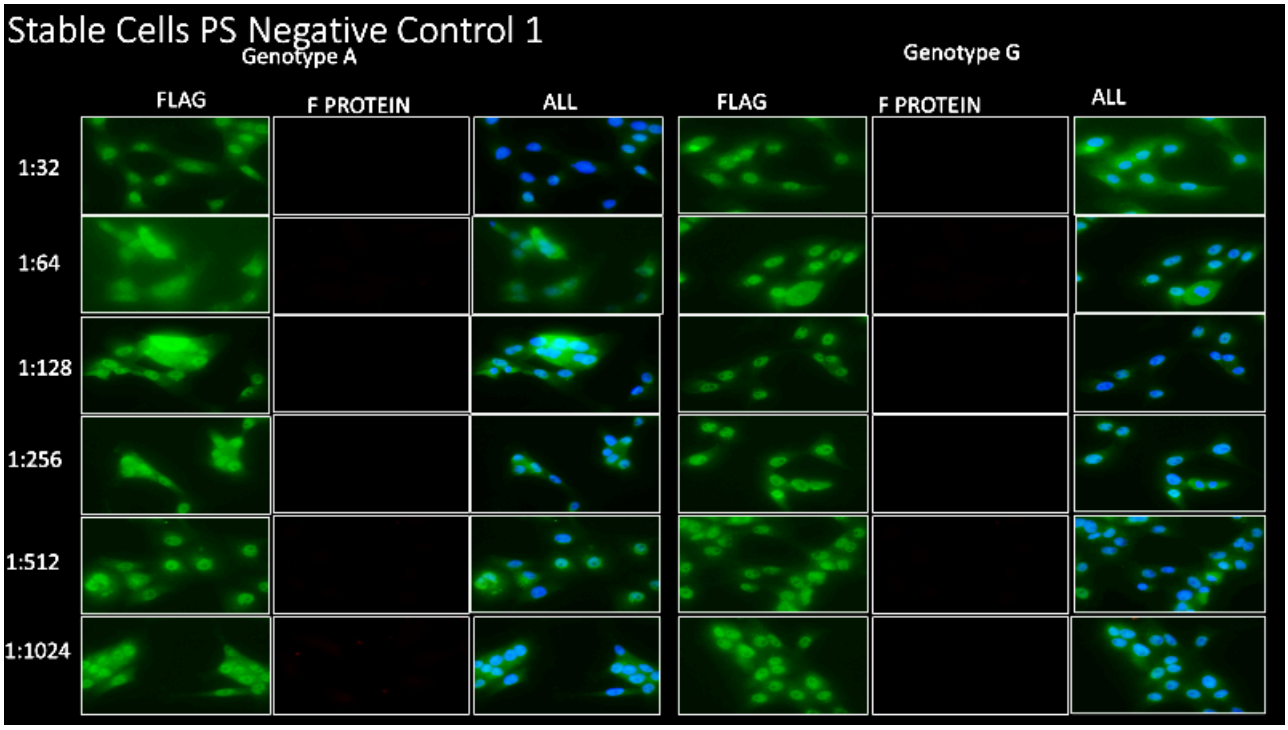


Figure A-43a. Stable cells Negative Control Batch#1.

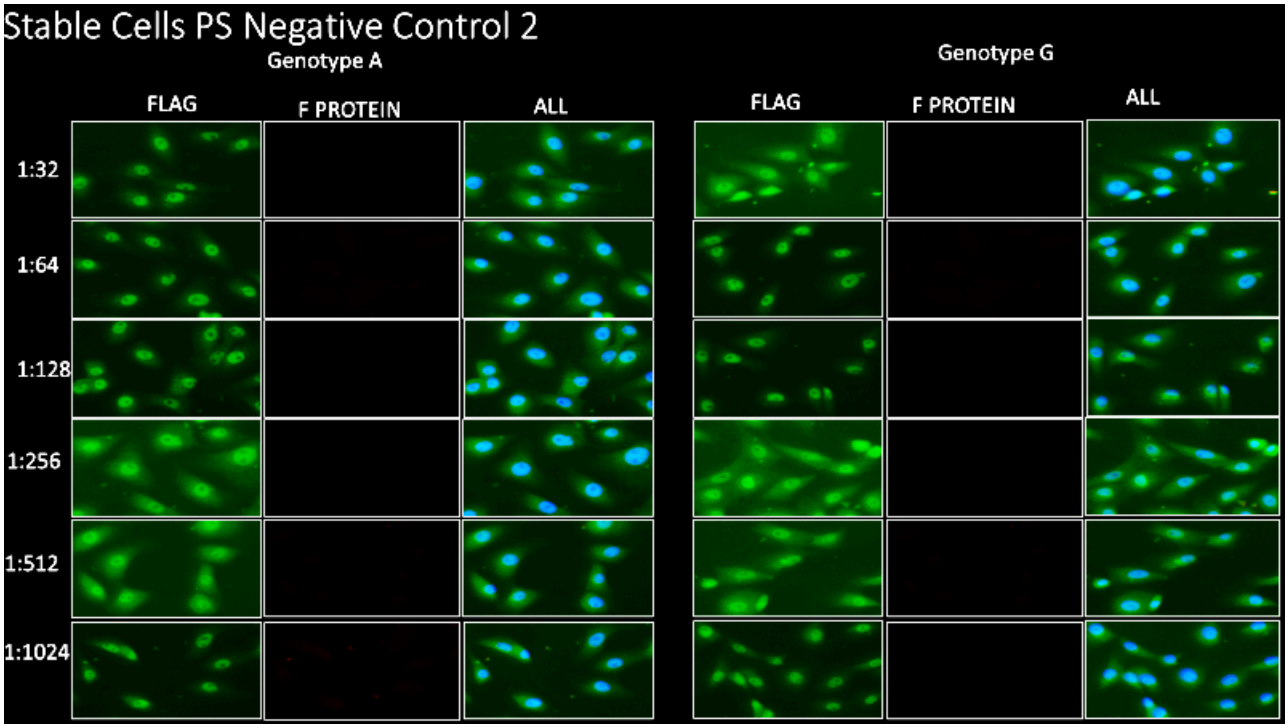


Figure A-43b. Stable cells Negative Control Batch#2.

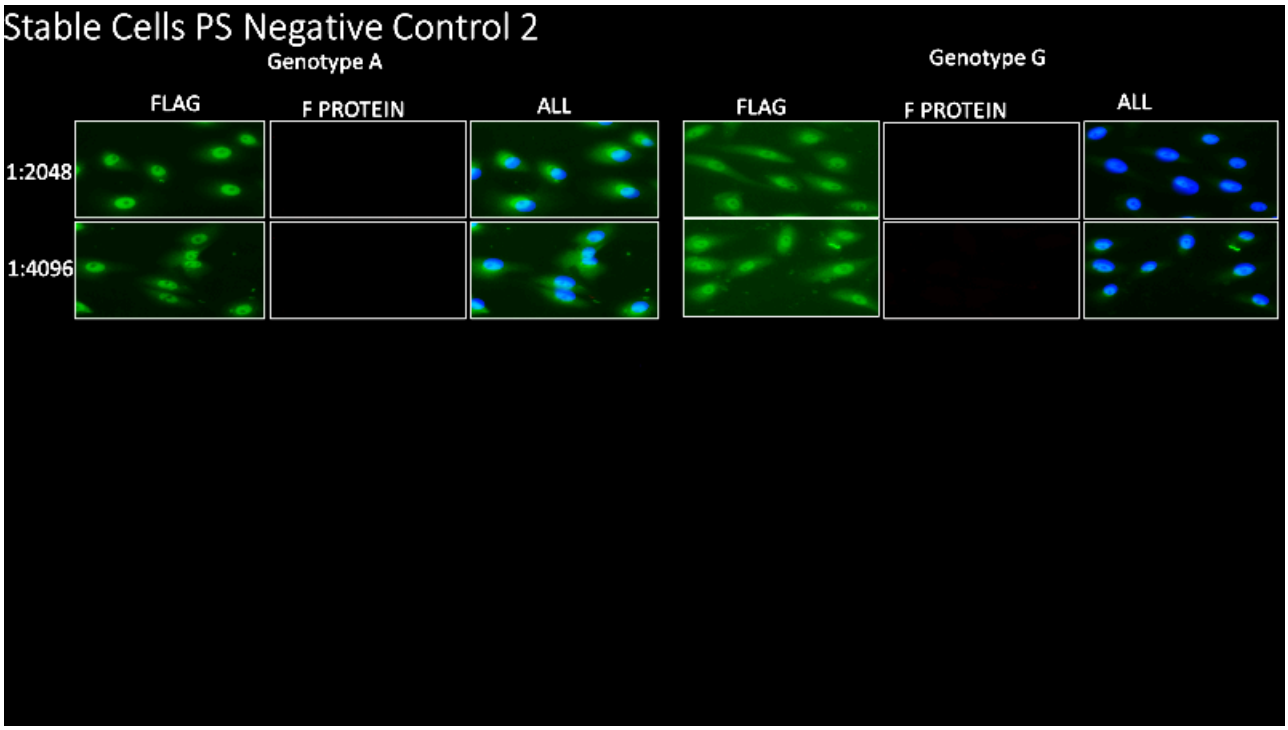


Figure A-43c. Stable cells Negative Control Batch#2.

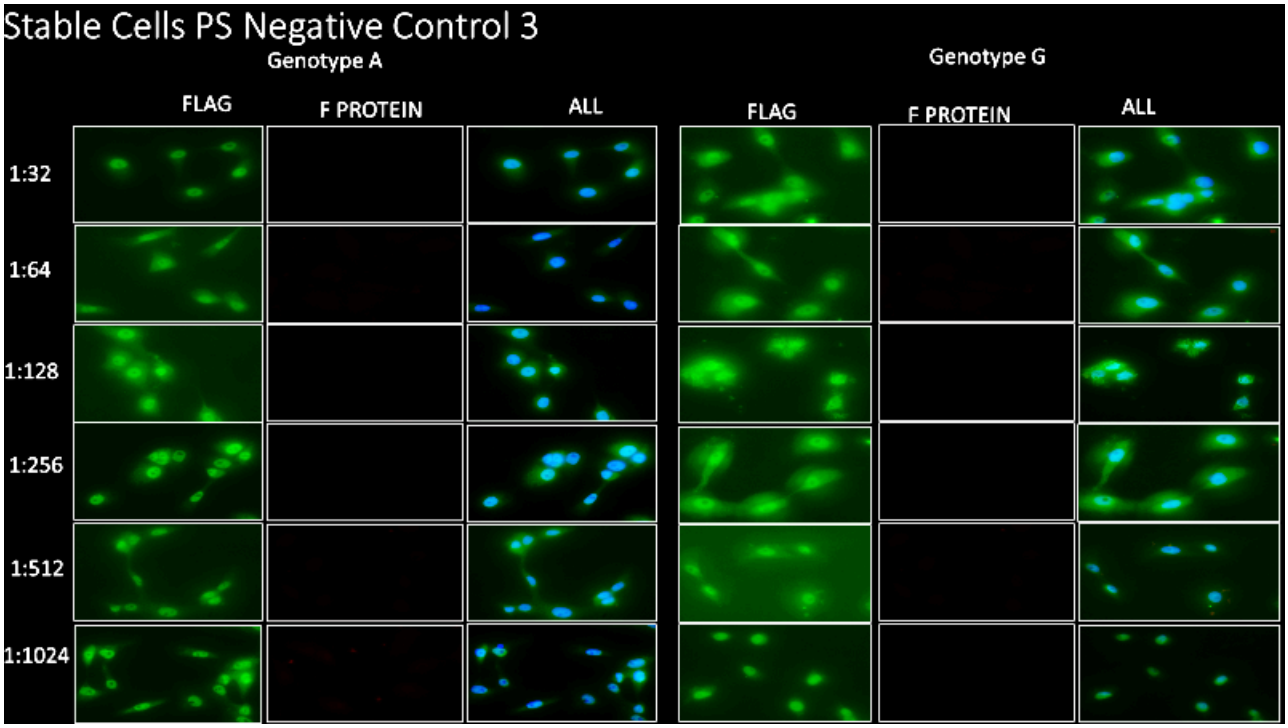


Figure A-43d. Stable cells Negative Control Batch#3.

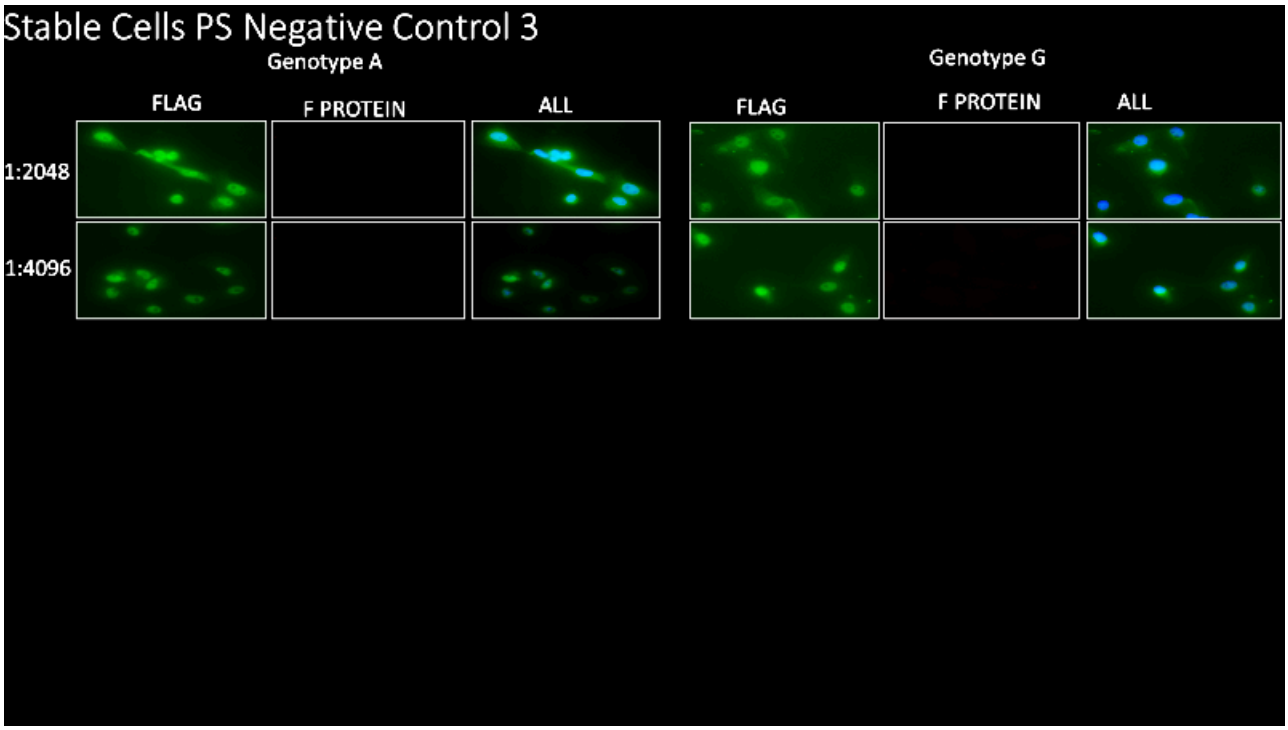


Figure A-43e. Stable cells Negative Control Batch#3.

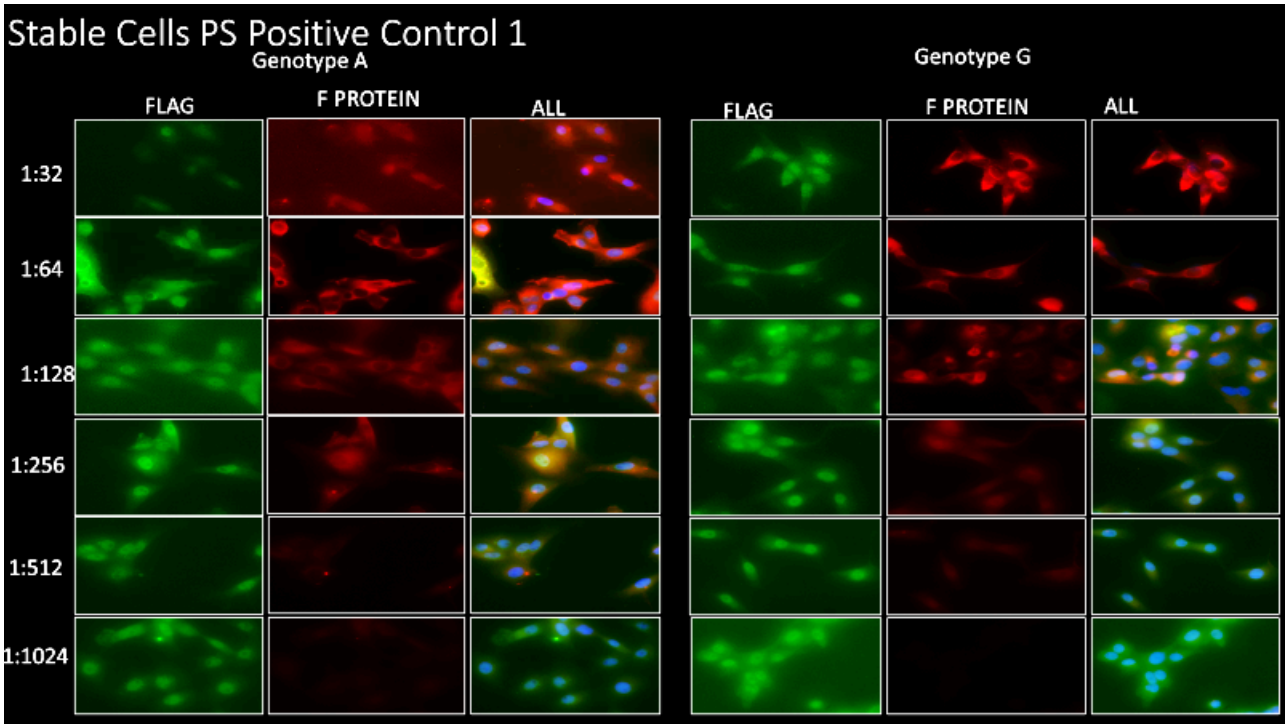


Figure A-44a. Stable cells Positive Control Batch#1.

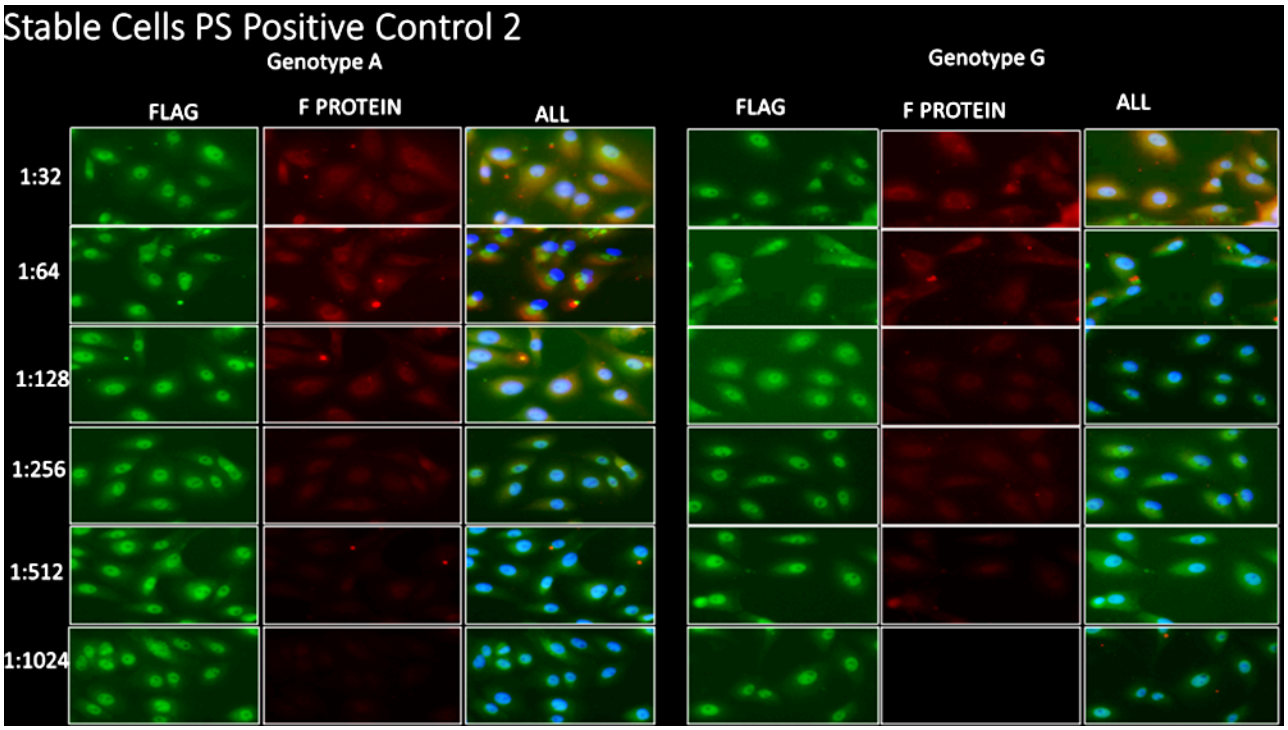


Figure A-44b. Stable cells Positive Control Batch#2.

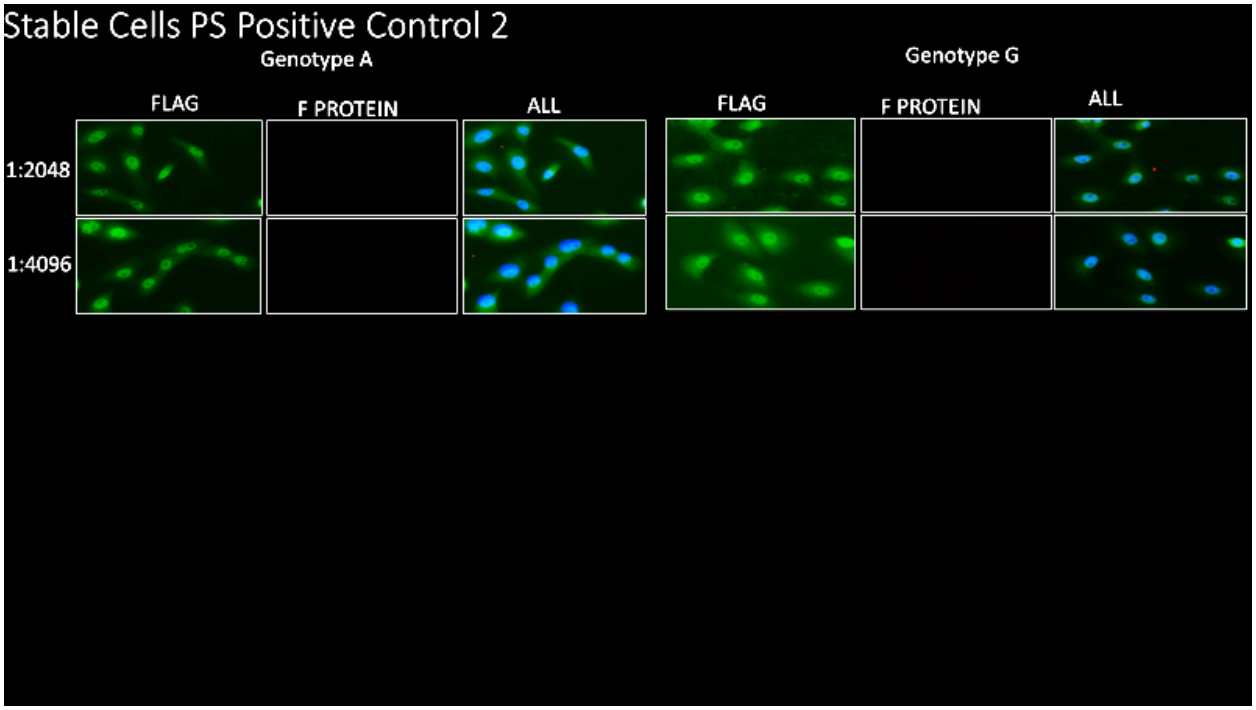


Figure A-44c. Stable cells Positive Control Batch#2.

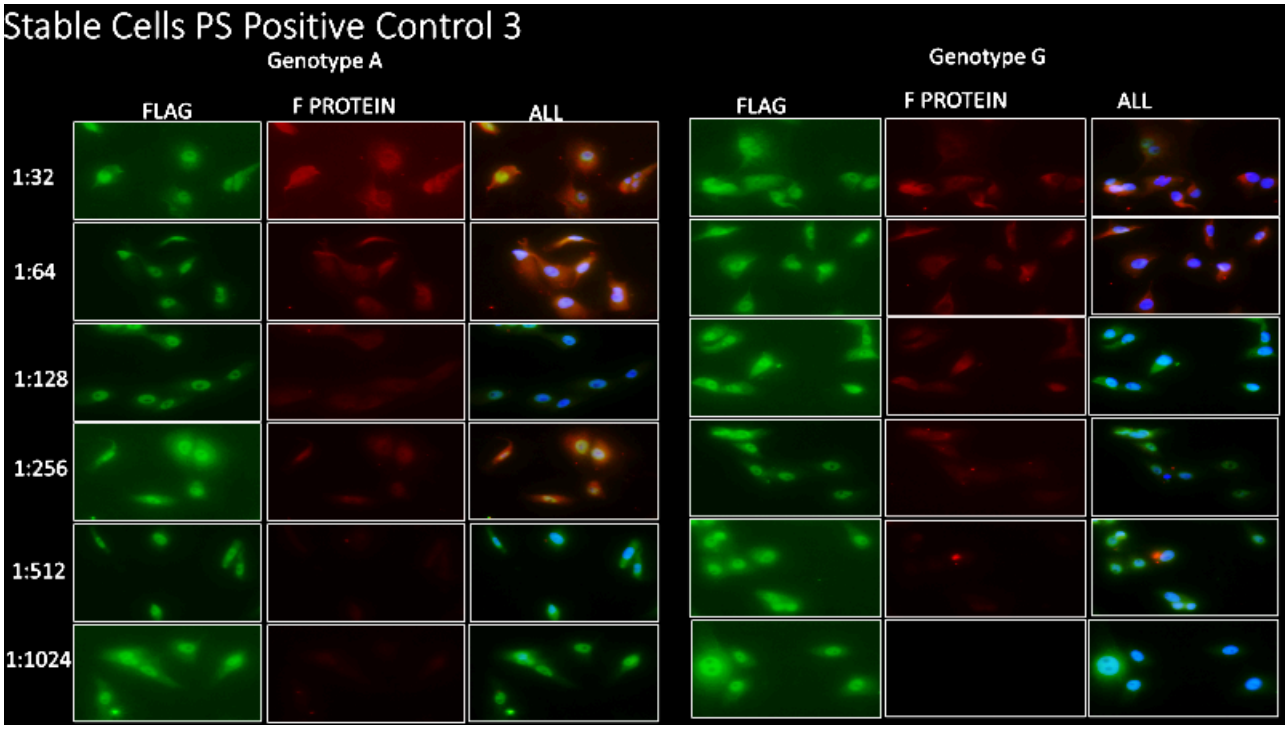


Figure A-44d. Stable cells Positive Control Batch#3.

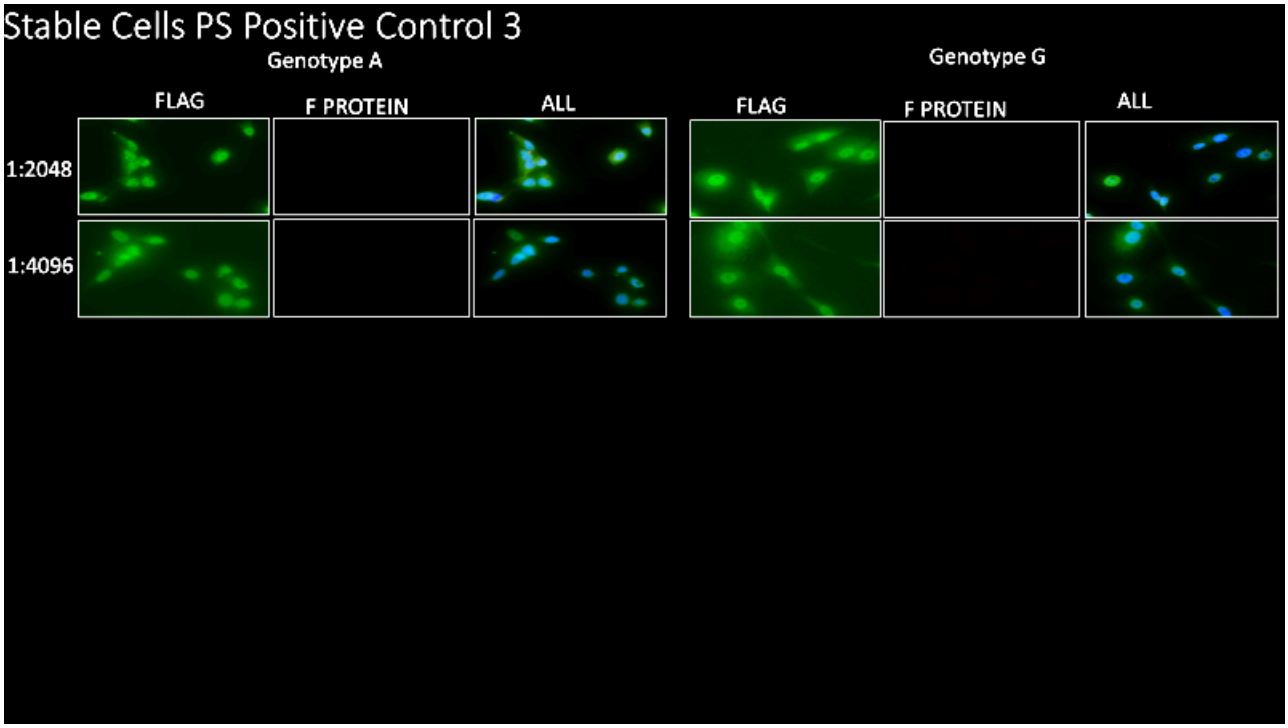


Figure A-44e. Stable cells Positive Control Batch#3.

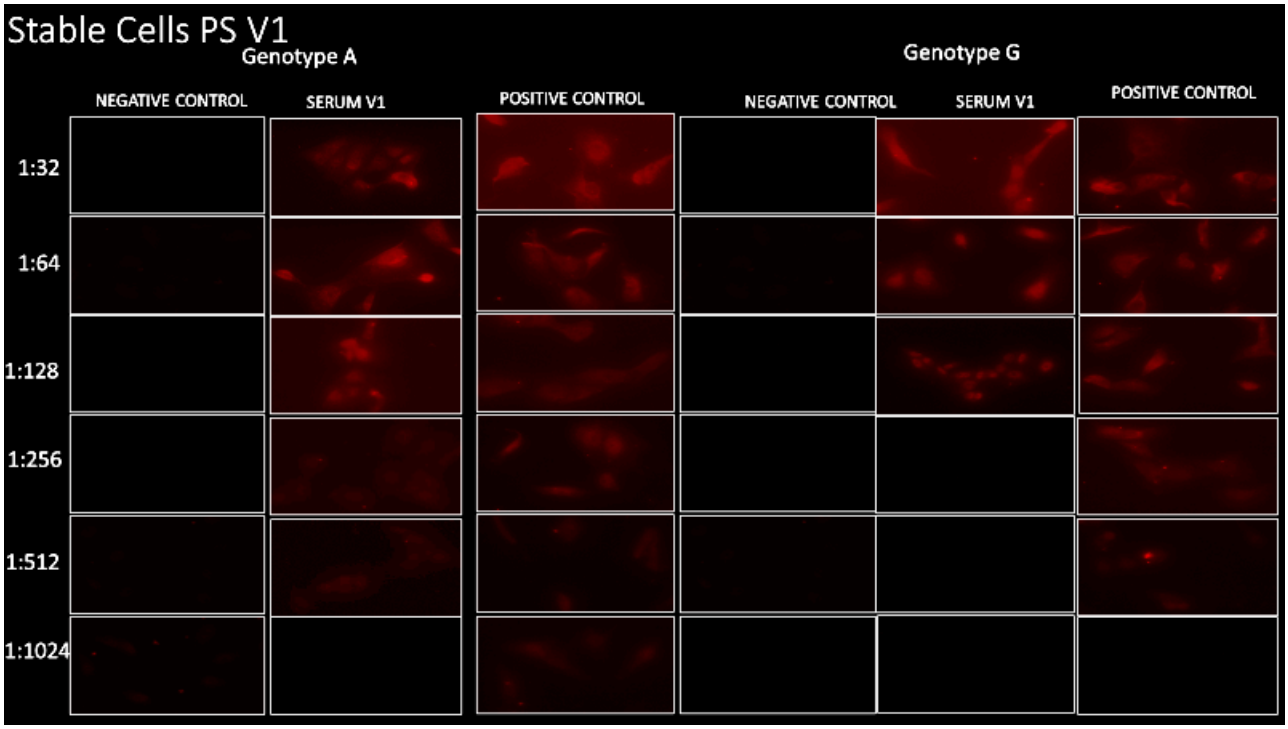


Figure A-45a. Stable cells Vaccine Sample#1.

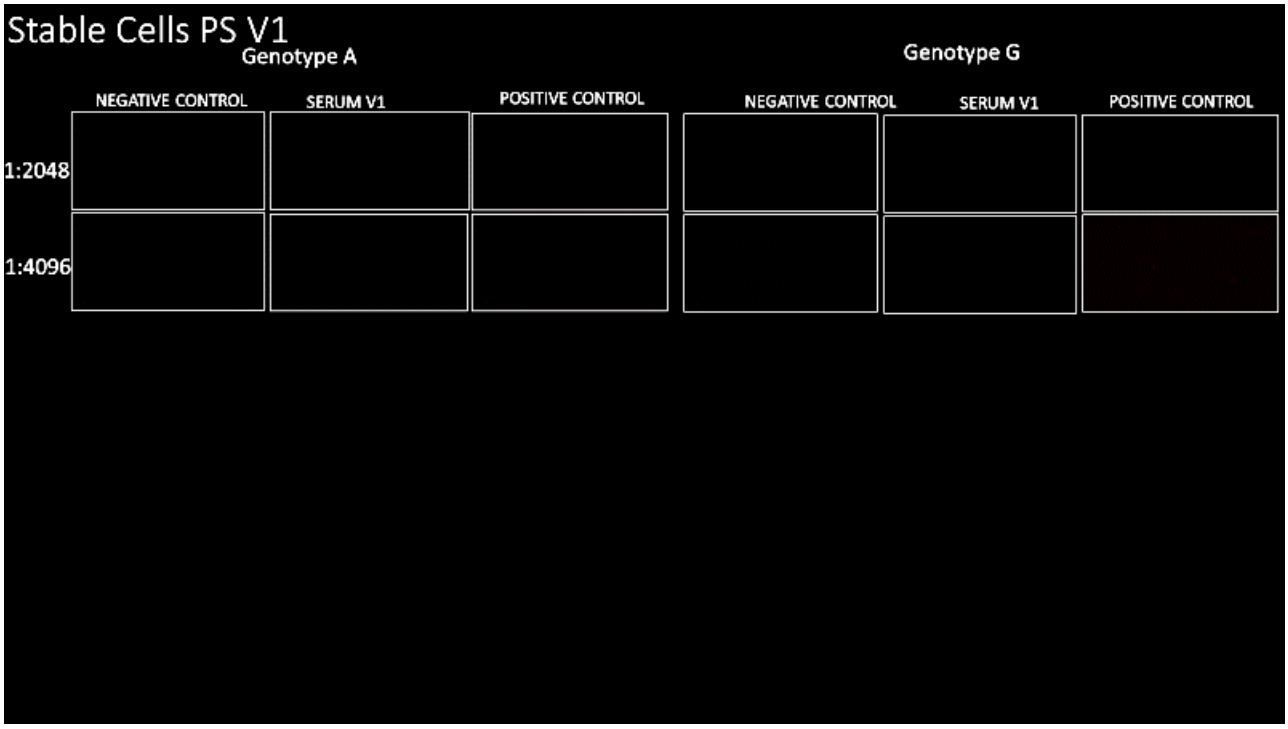


Figure A-45b. Stable cells Vaccine Sample#1.

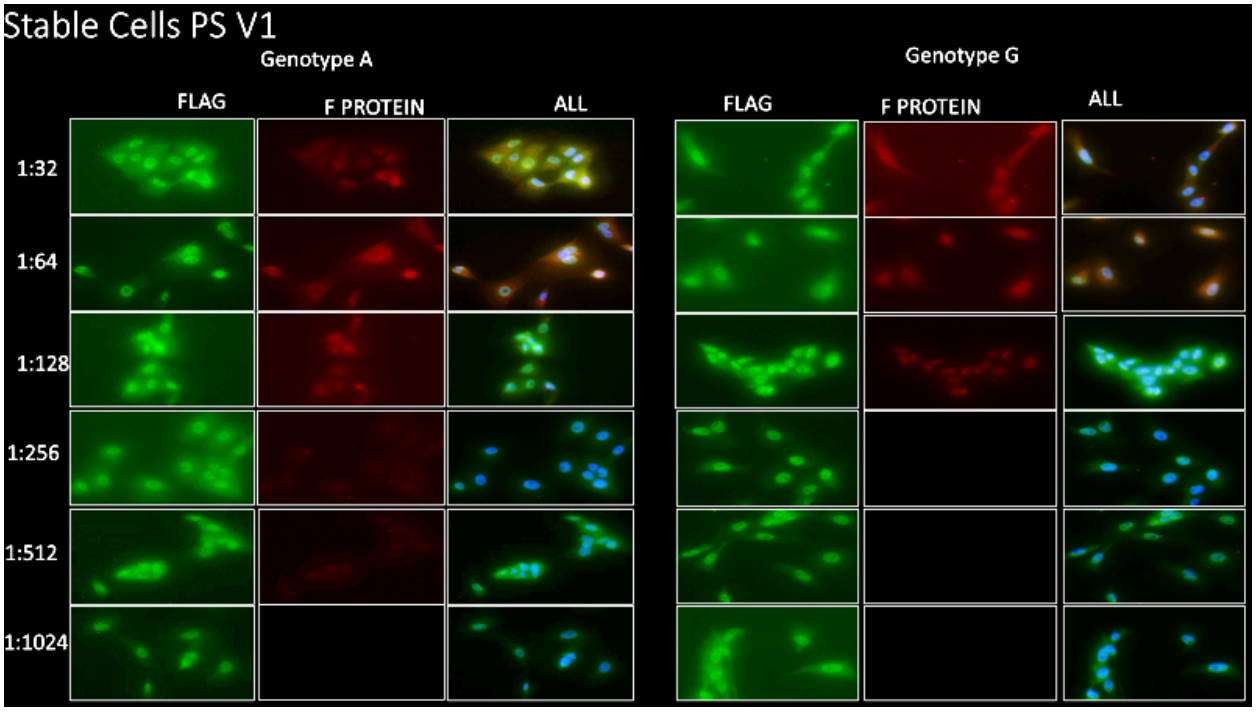


Figure A-45c. Stable cells Vaccine Sample#1.

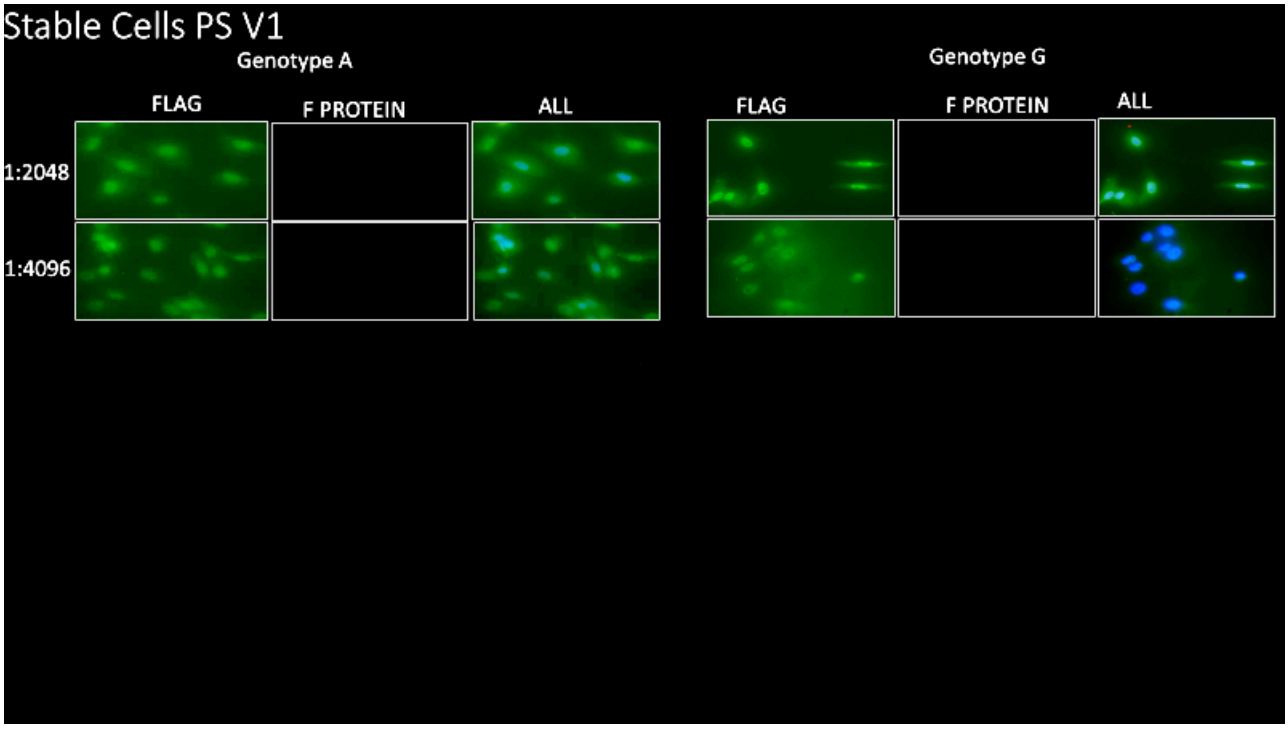


Figure A-45d. Stable cells Vaccine Sample#1.

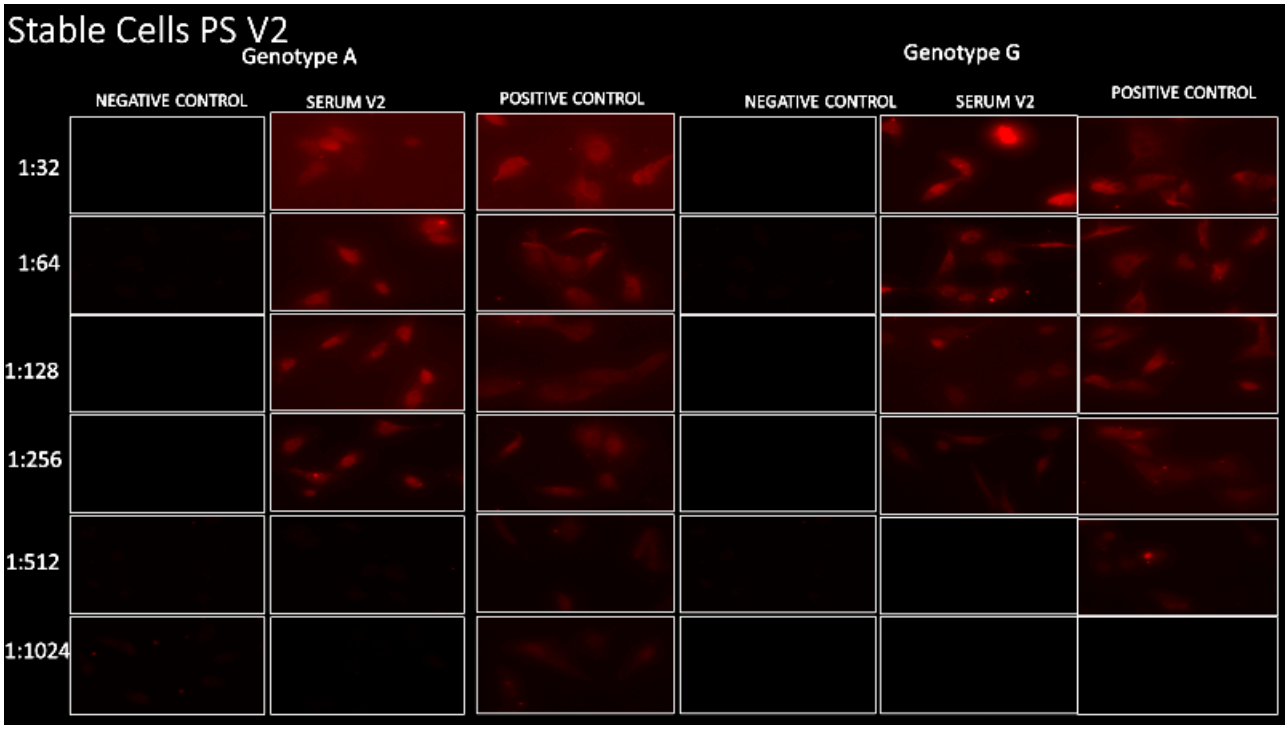


Figure A-46a. Stable cells Vaccine Sample#2.

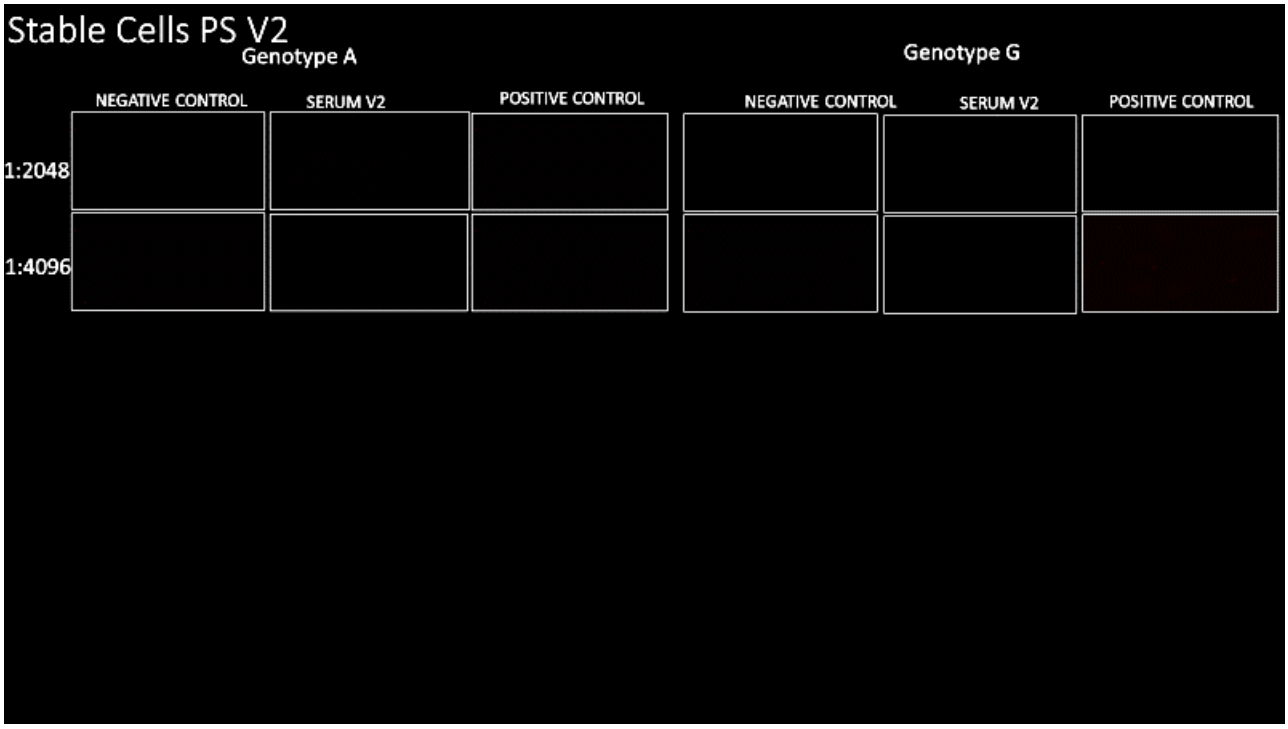


Figure A-46b. Stable cells Vaccine Sample#2.

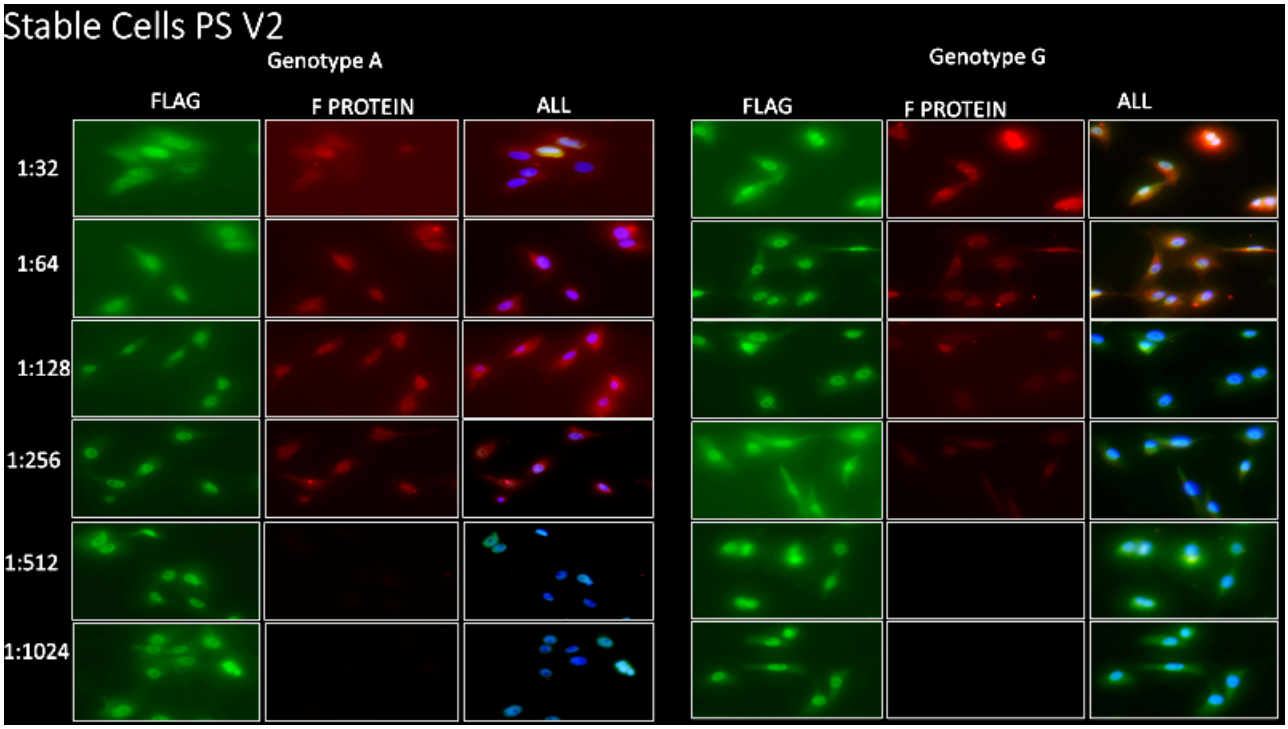


Figure A-46c. Stable cells Vaccine Sample#2.

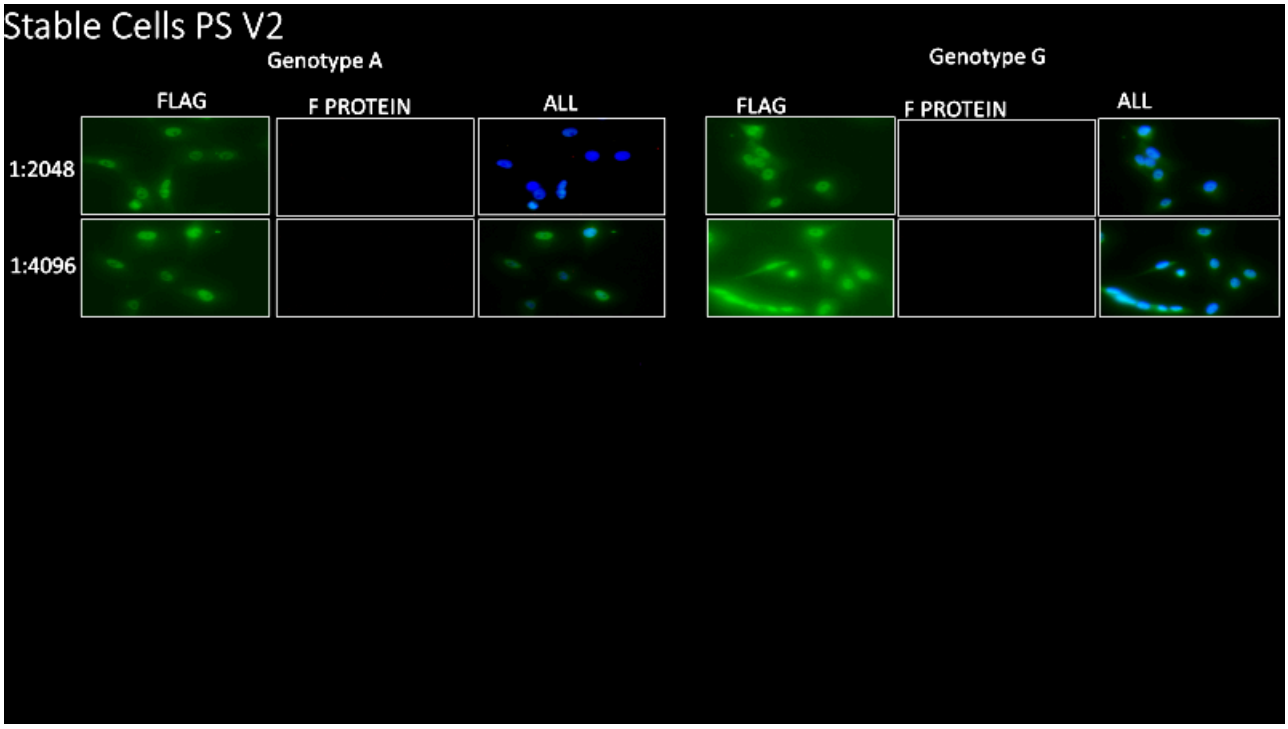


Figure A-46d. Stable cells Vaccine Sample#2.

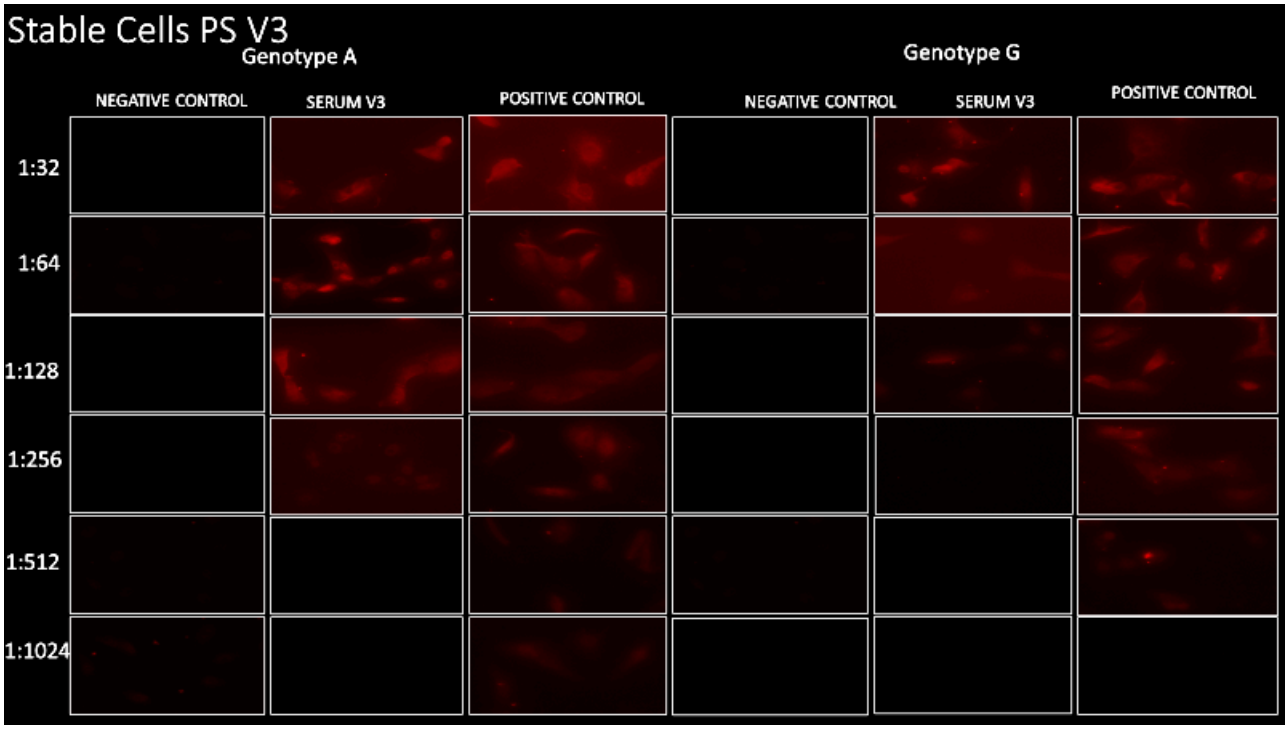


Figure A-47a. Stable cells Vaccine Sample#3.

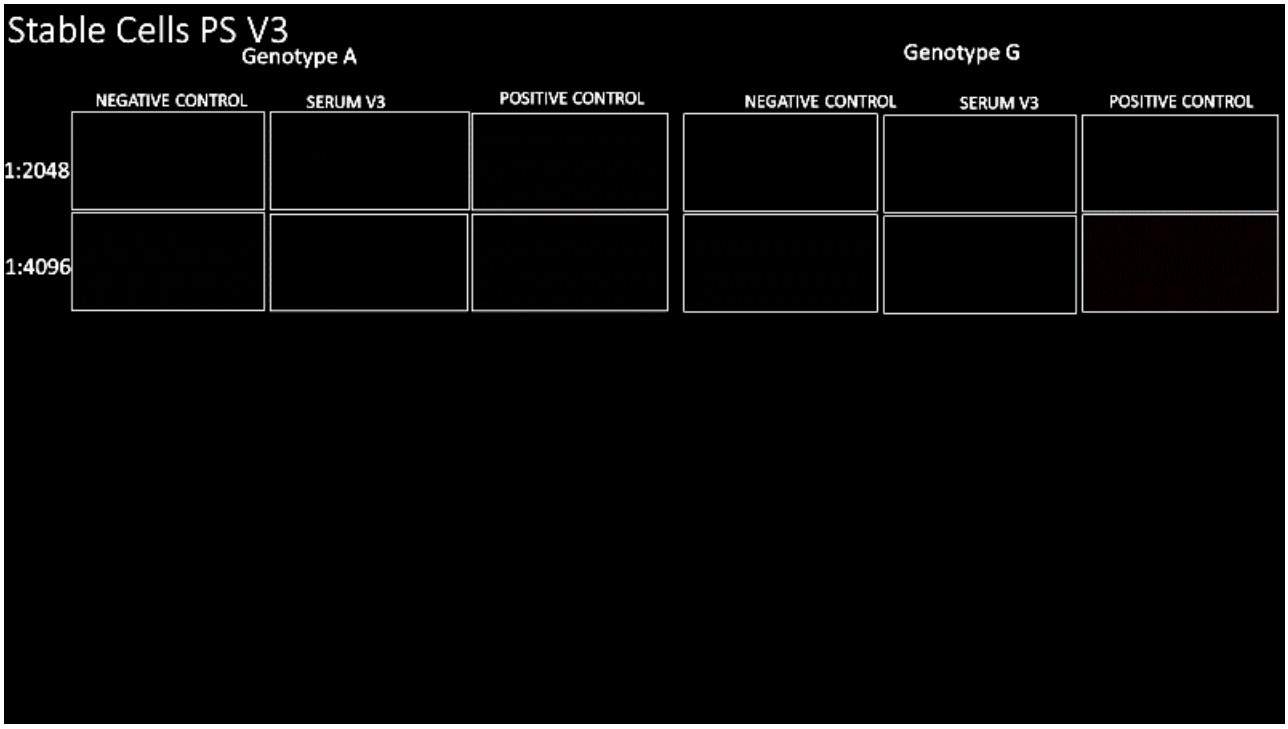


Figure A-47b. Stable cells Vaccine Sample#3.

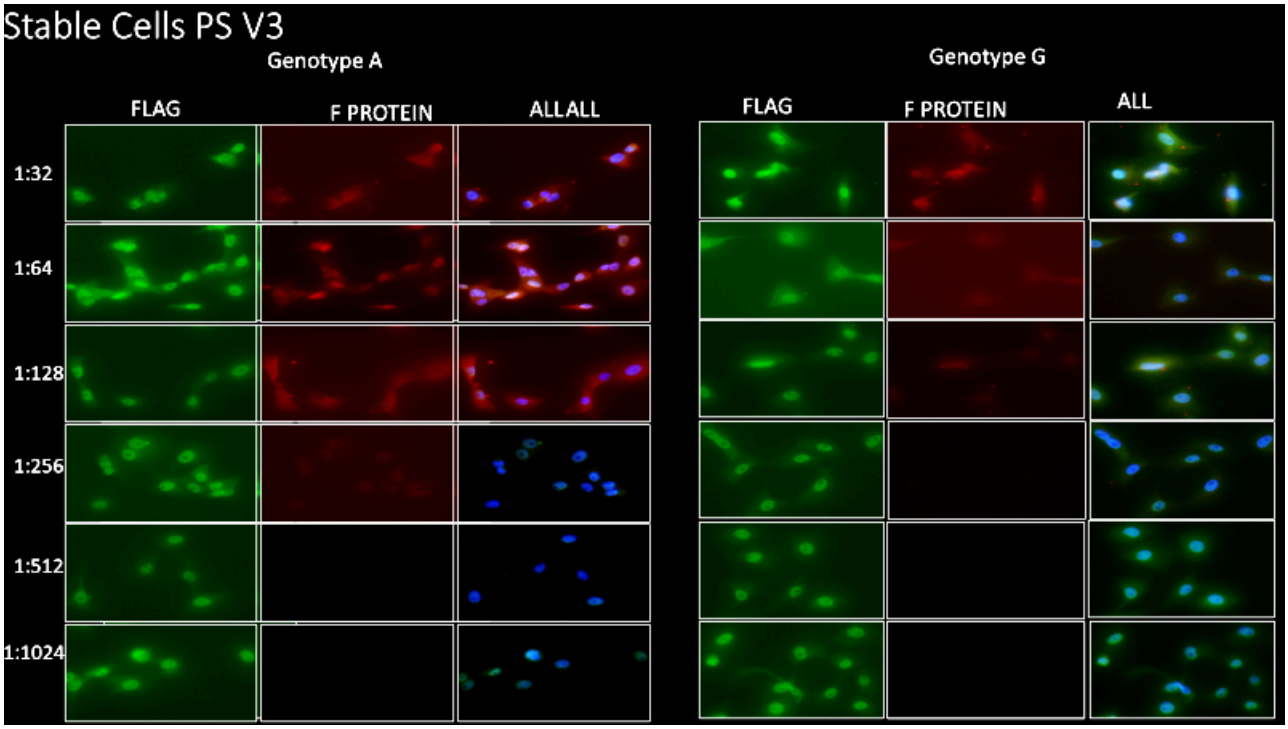


Figure A-47c. Stable cells Vaccine Sample#3.

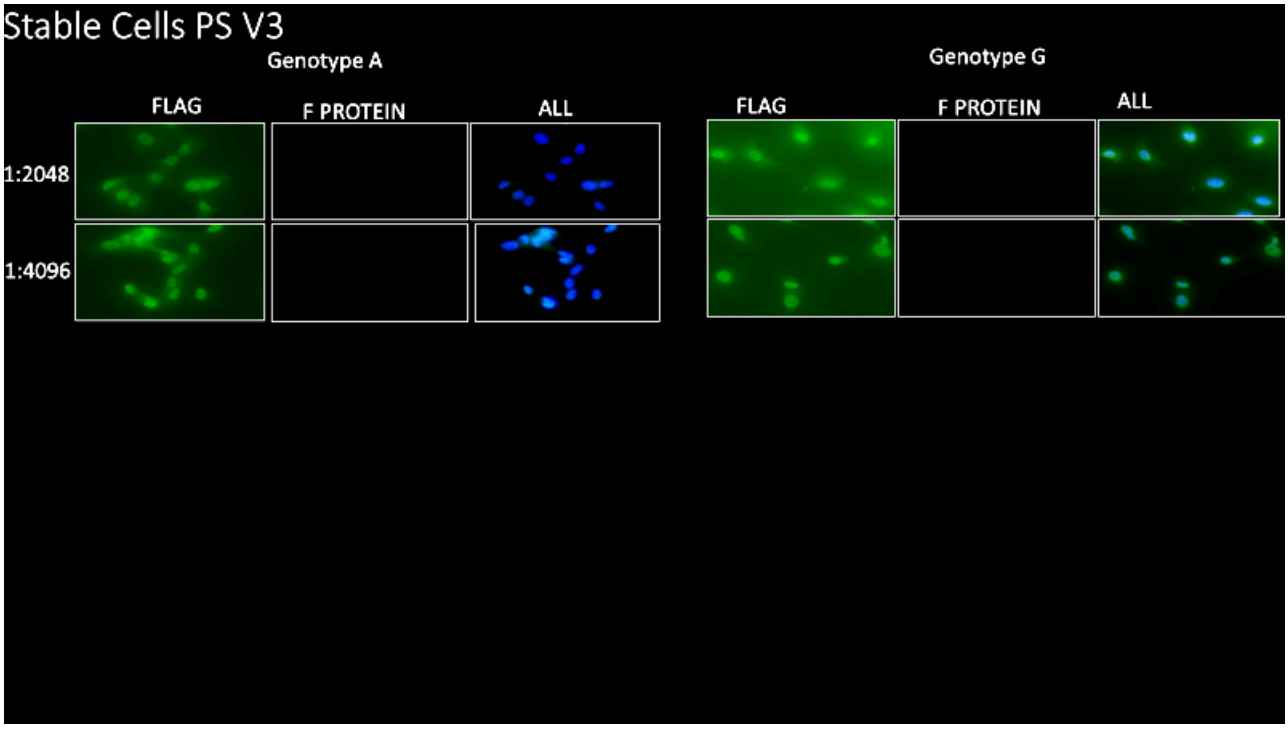


Figure A-47d. Stable cells Vaccine Sample#3.

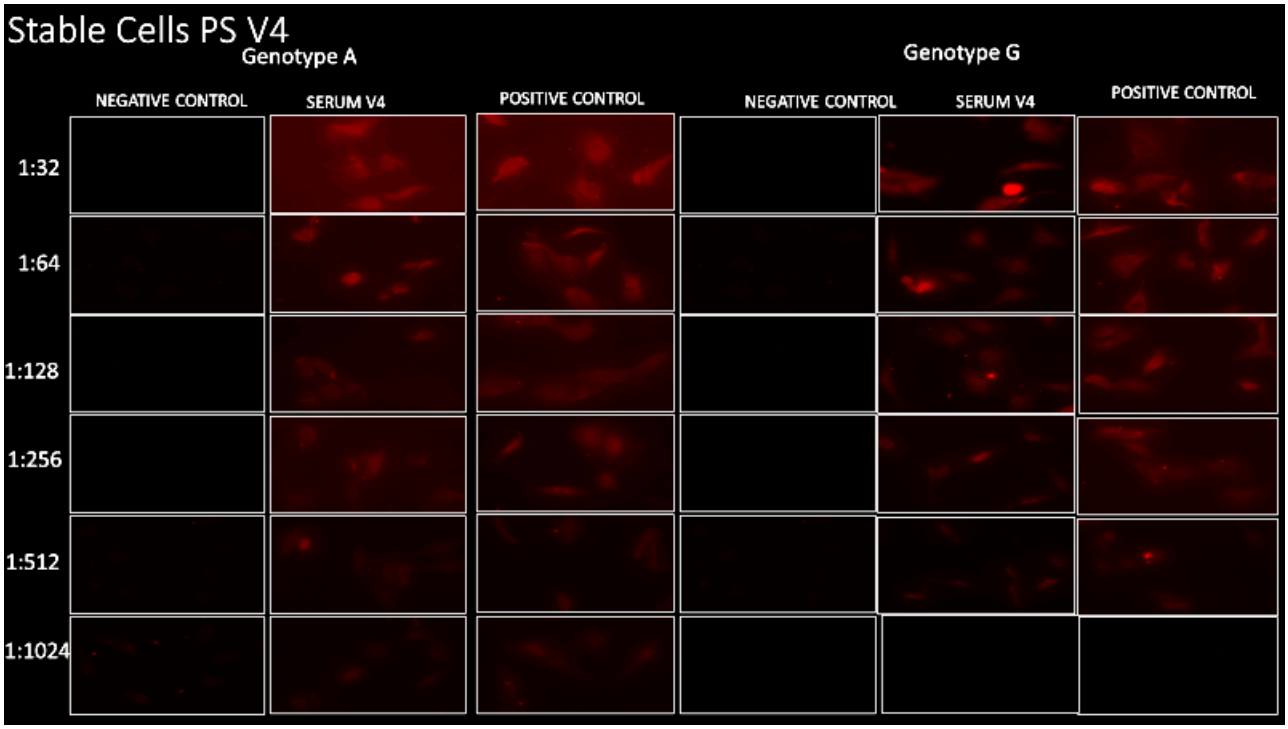


Figure A-48a. Stable cells Vaccine Sample#4.

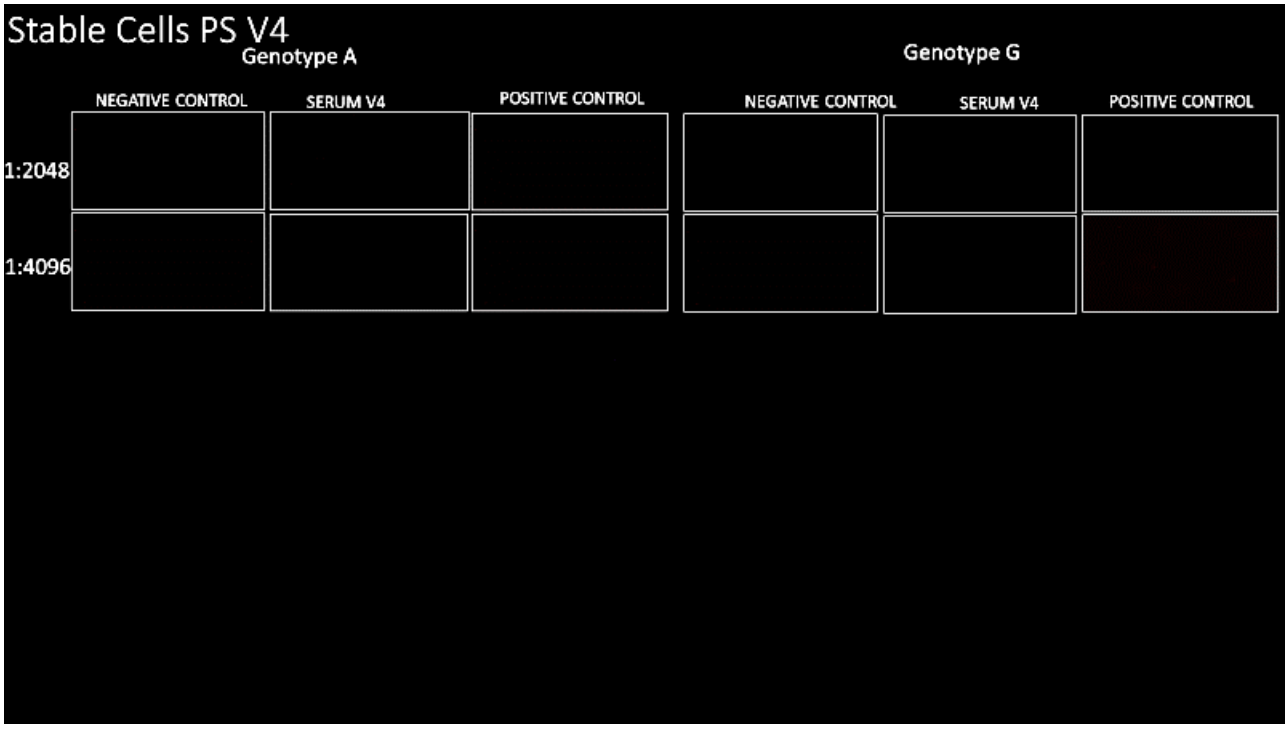


Figure A-48b. Stable cells Vaccine Sample#4.

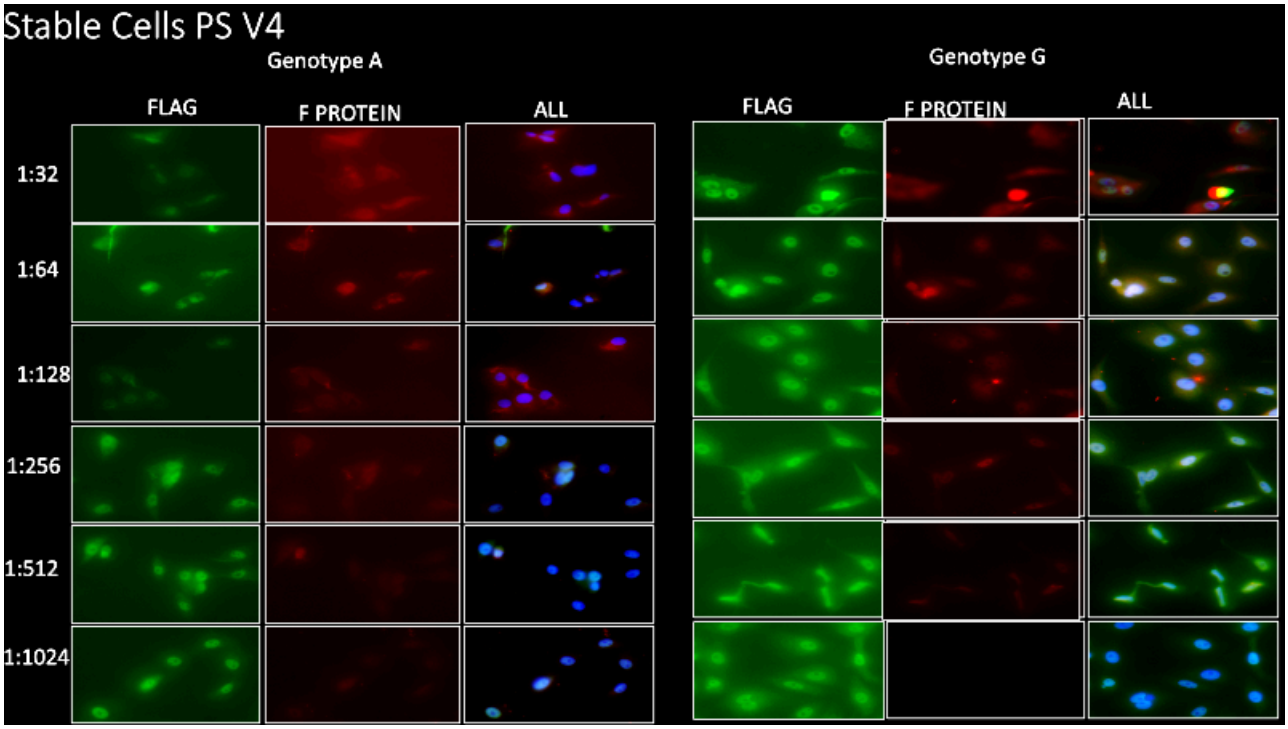


Figure A-48c. Stable cells Vaccine Sample#4.

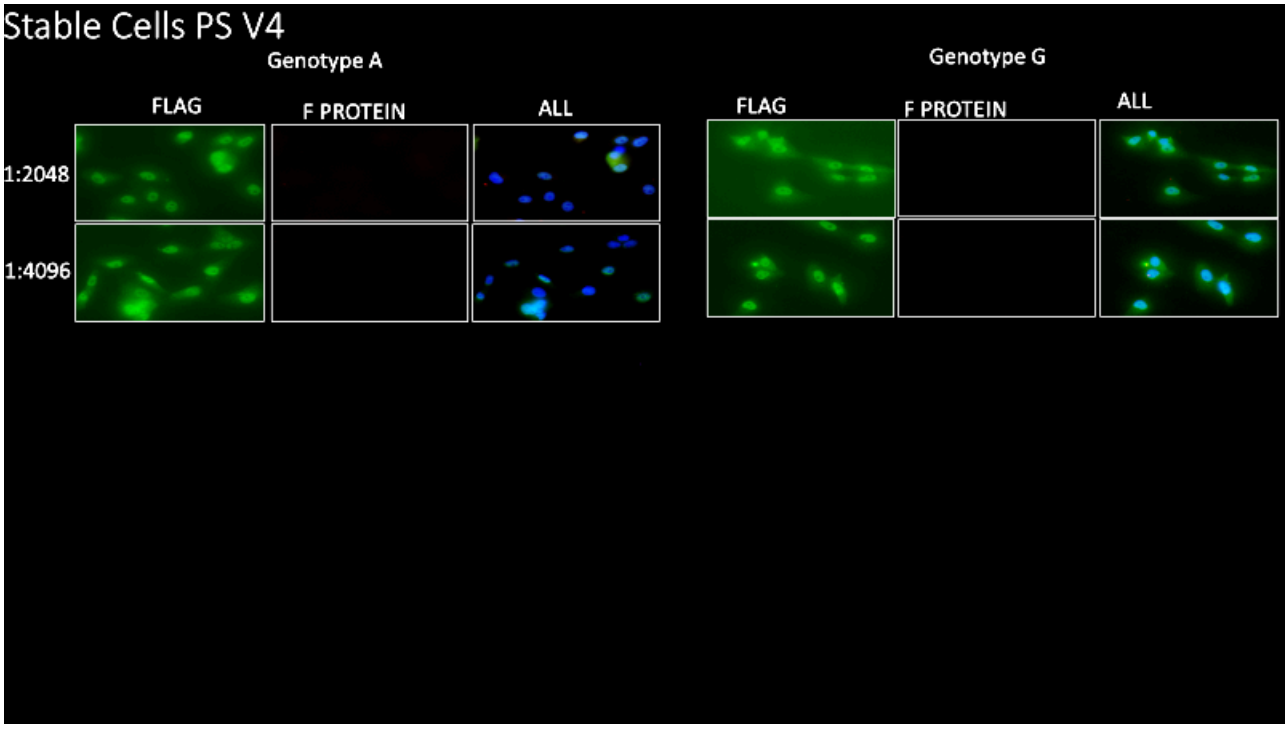


Figure A-48d. Stable cells Vaccine Sample#4.

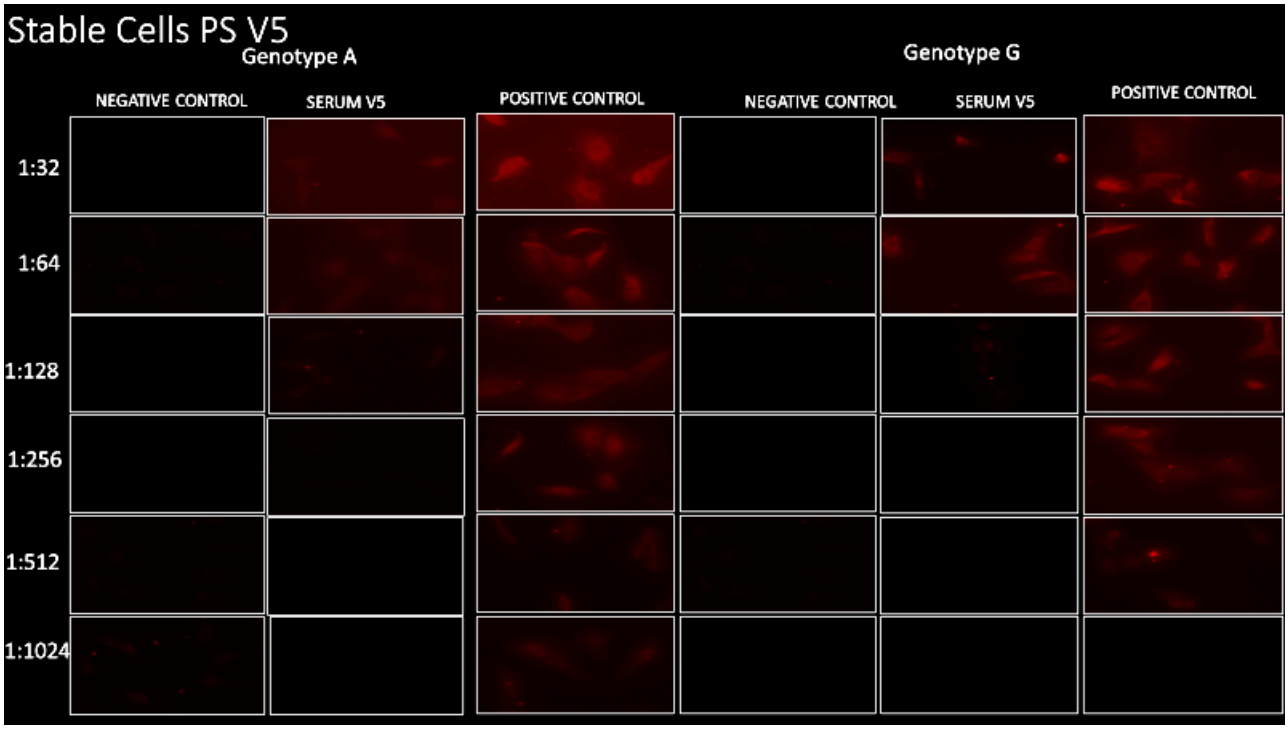


Figure A-49a. Stable cells Vaccine Sample#5.

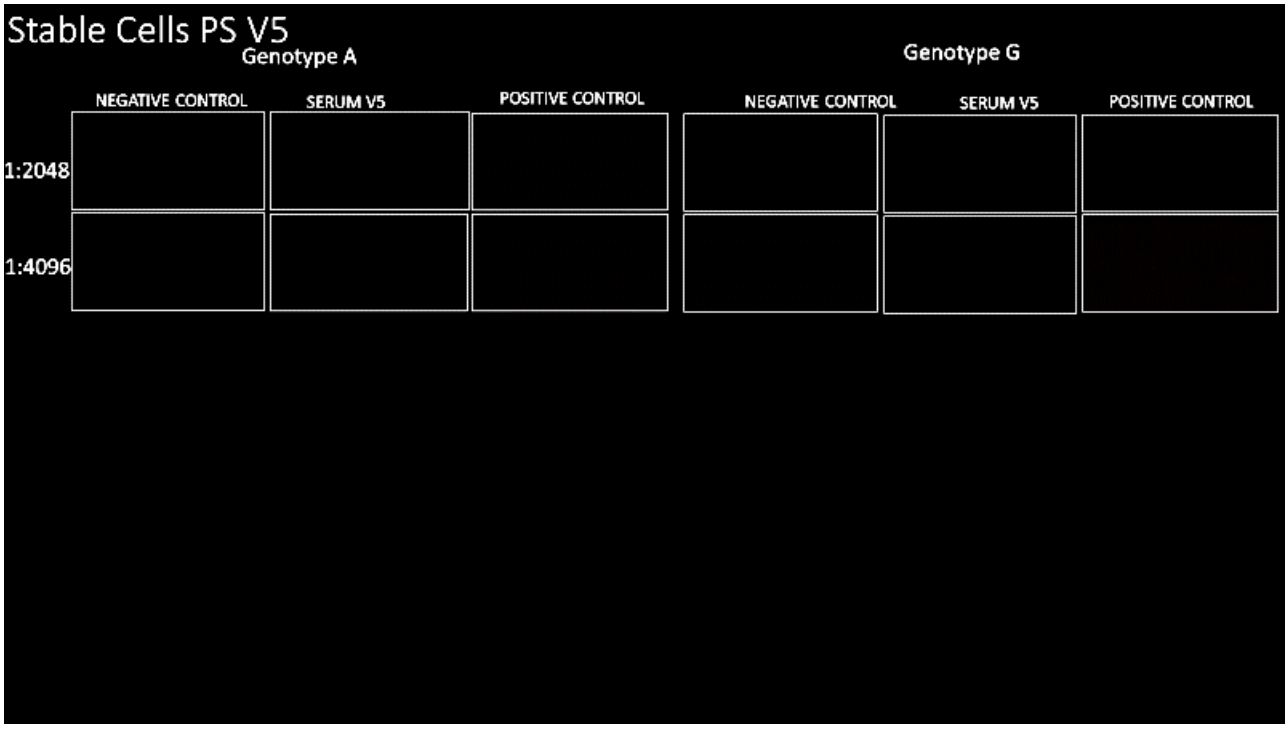


Figure A-49b. Stable cells Vaccine Sample#5.

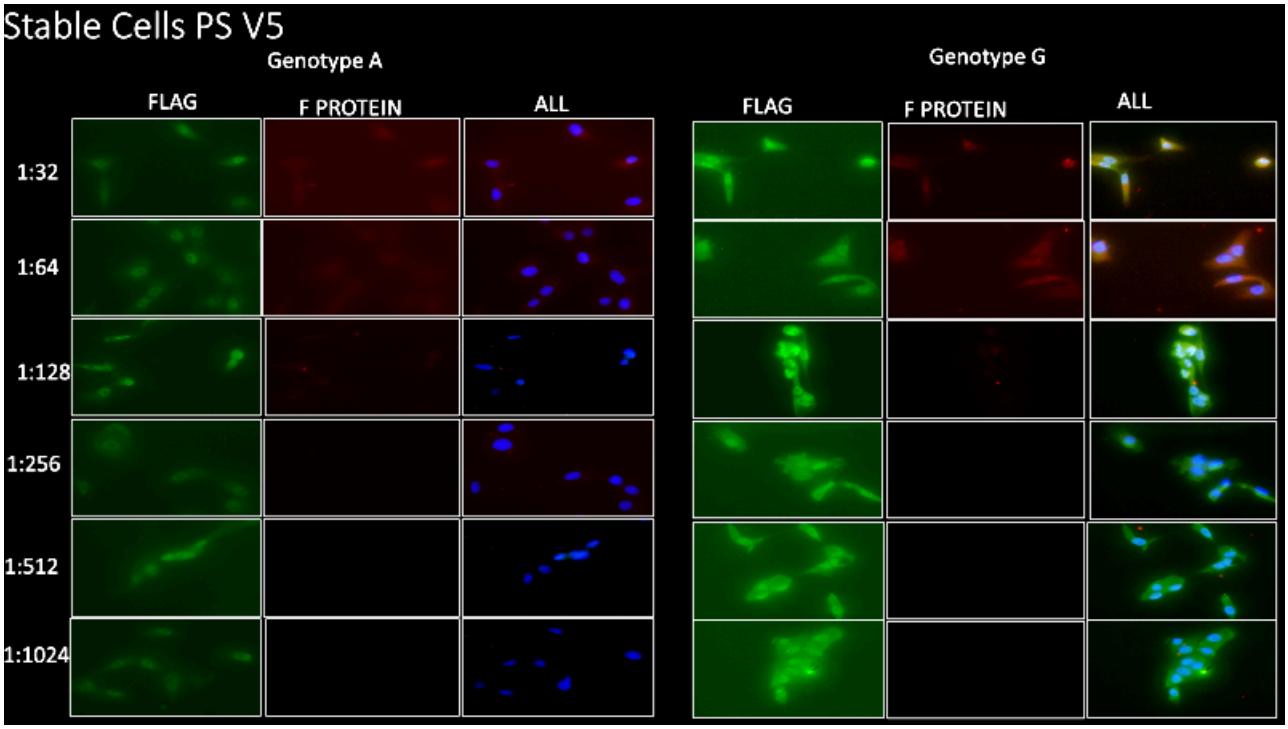


Figure A-49c. Stable cells Vaccine Sample#5.

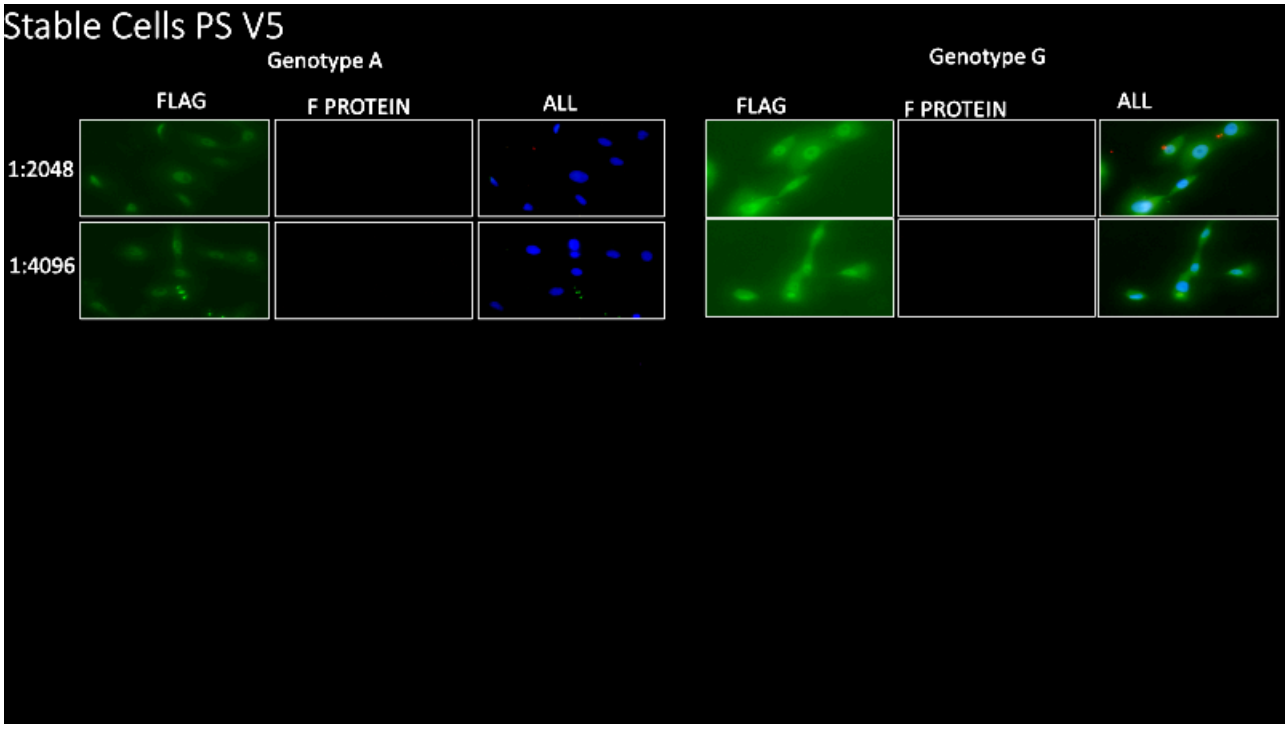


Figure A-49d. Stable cells Vaccine Sample#5.

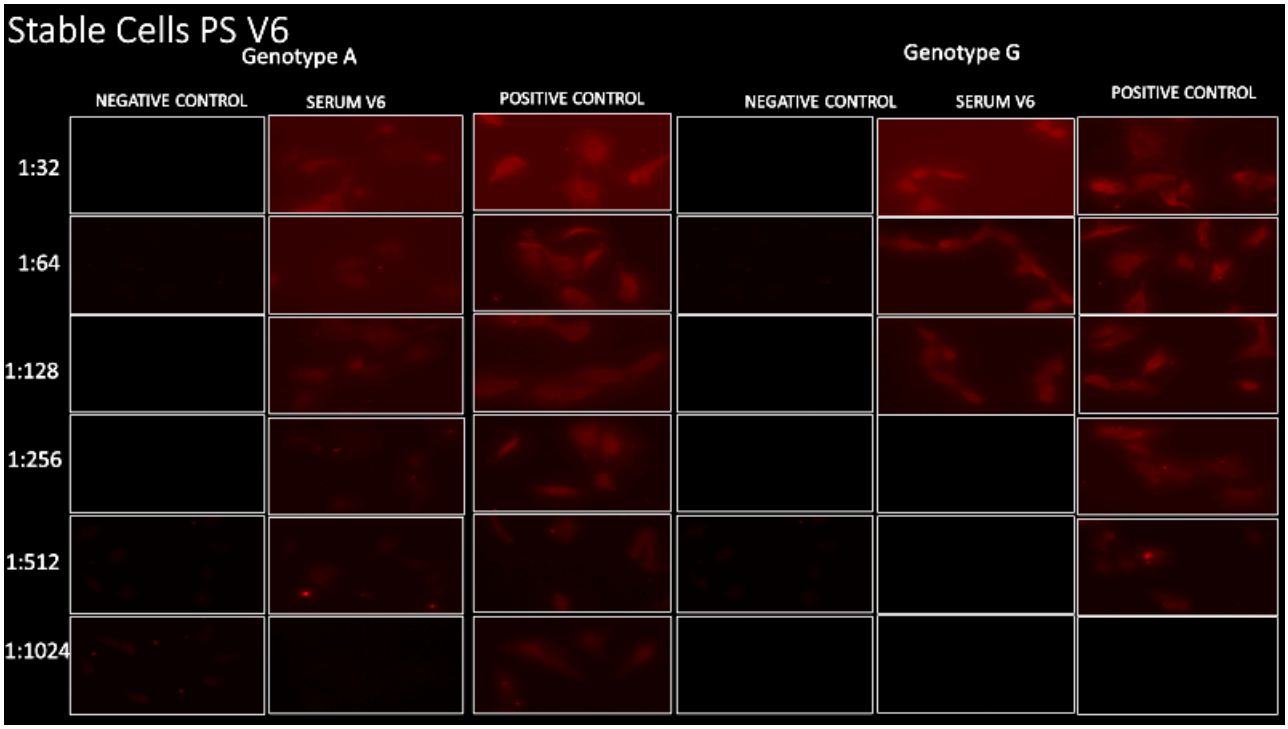


Figure A-50a. Stable cells Vaccine Sample#6.

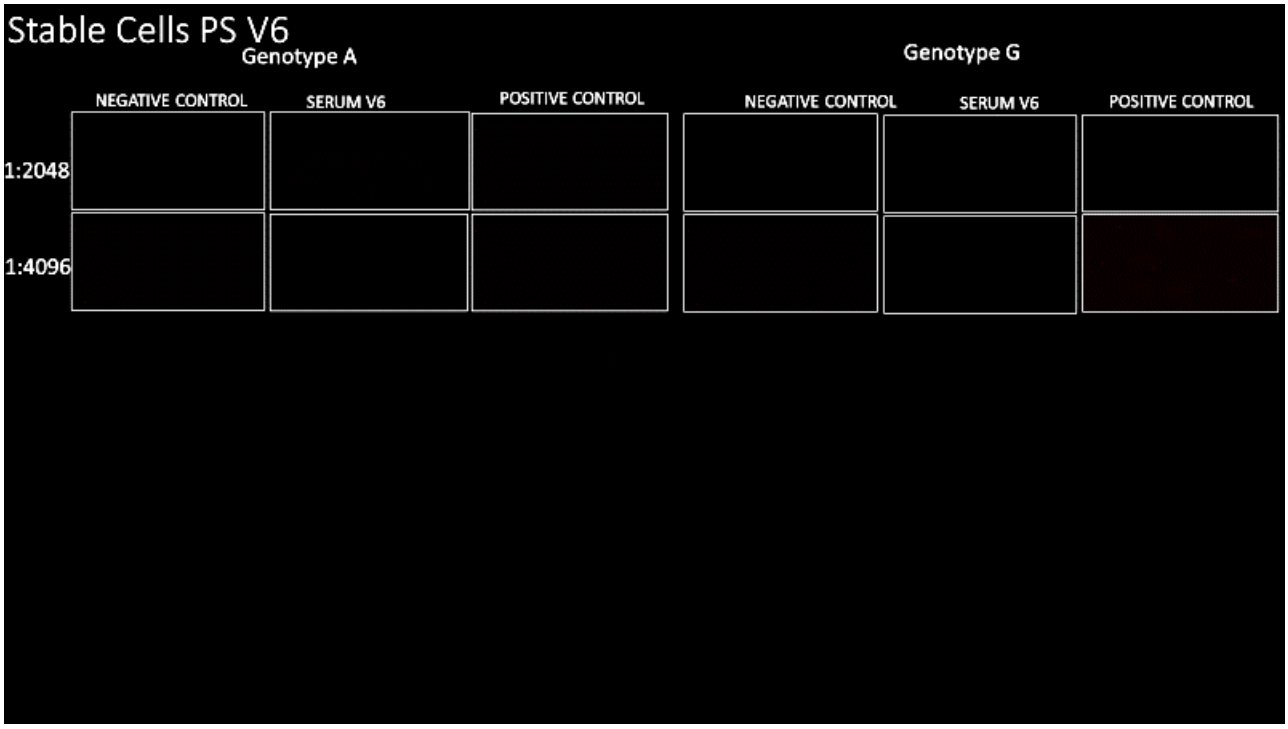


Figure A-50b. Stable cells Vaccine Sample#6.

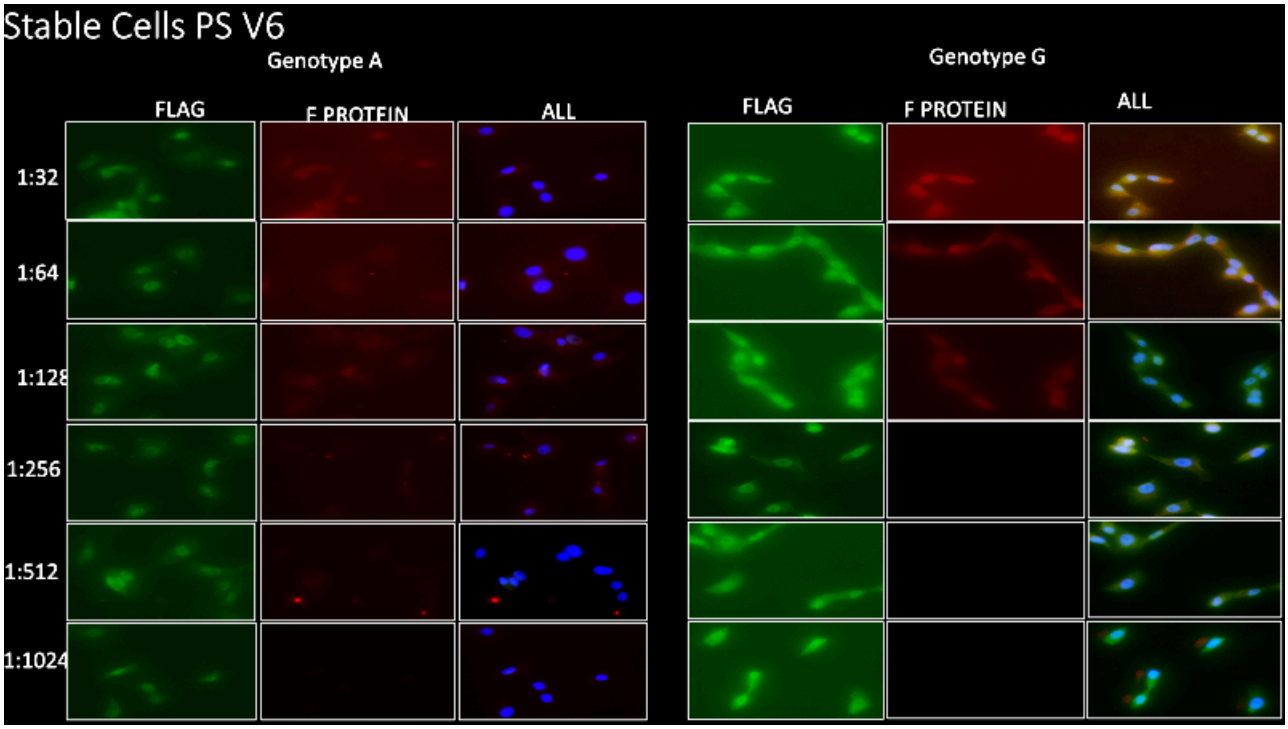


Figure A-50c. Stable cells Vaccine Sample#6.

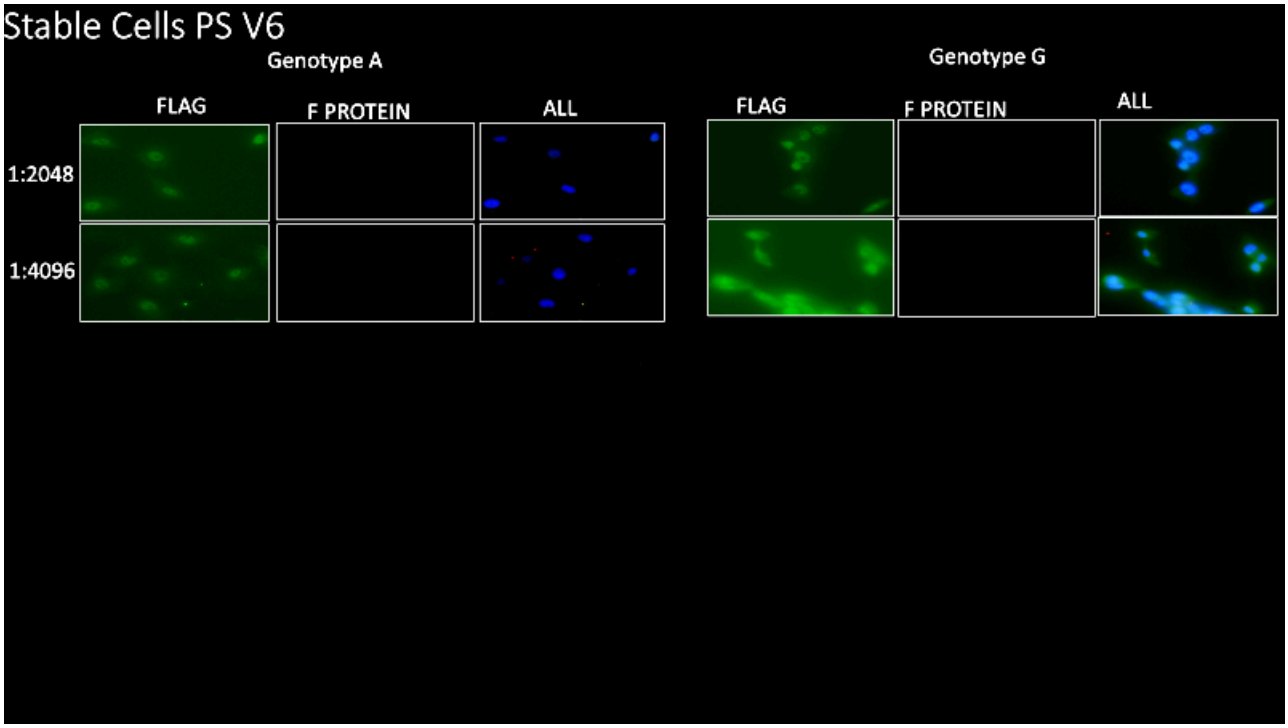


Figure A-50d. Stable cells Vaccine Sample#6.

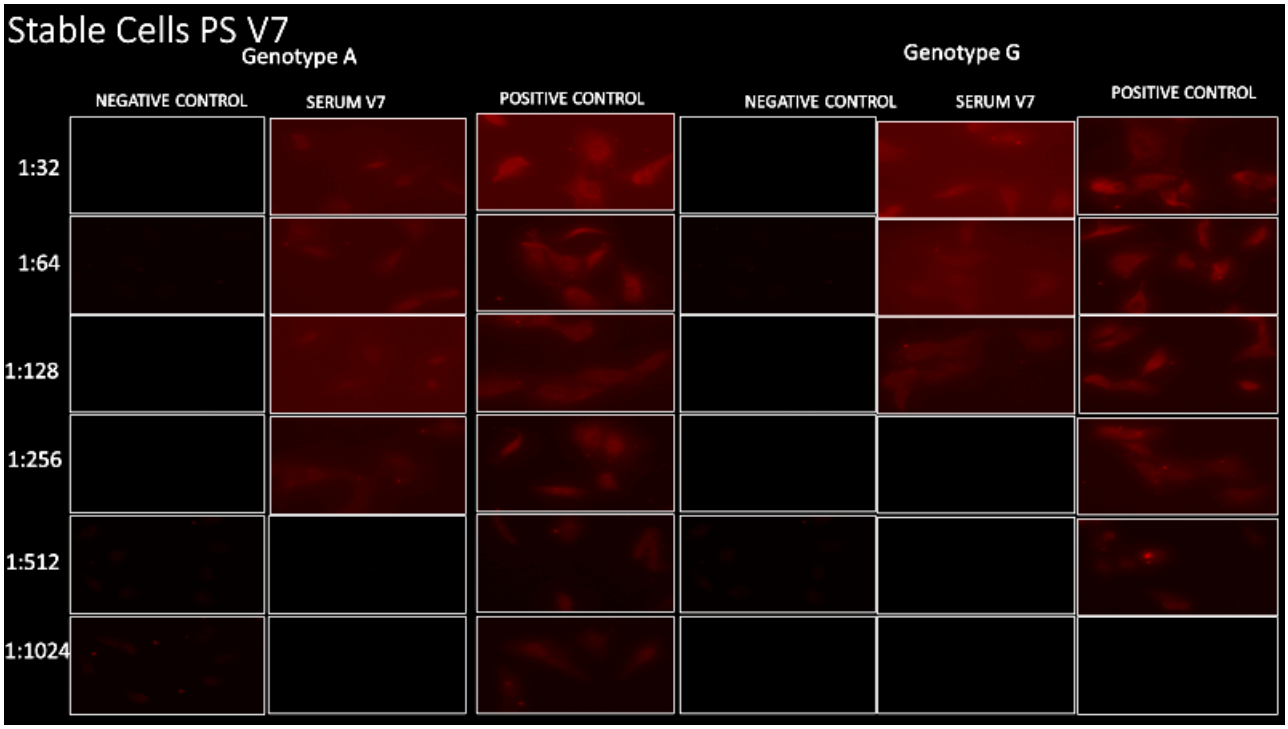


Figure A-51a. Stable cells Vaccine Sample#7.

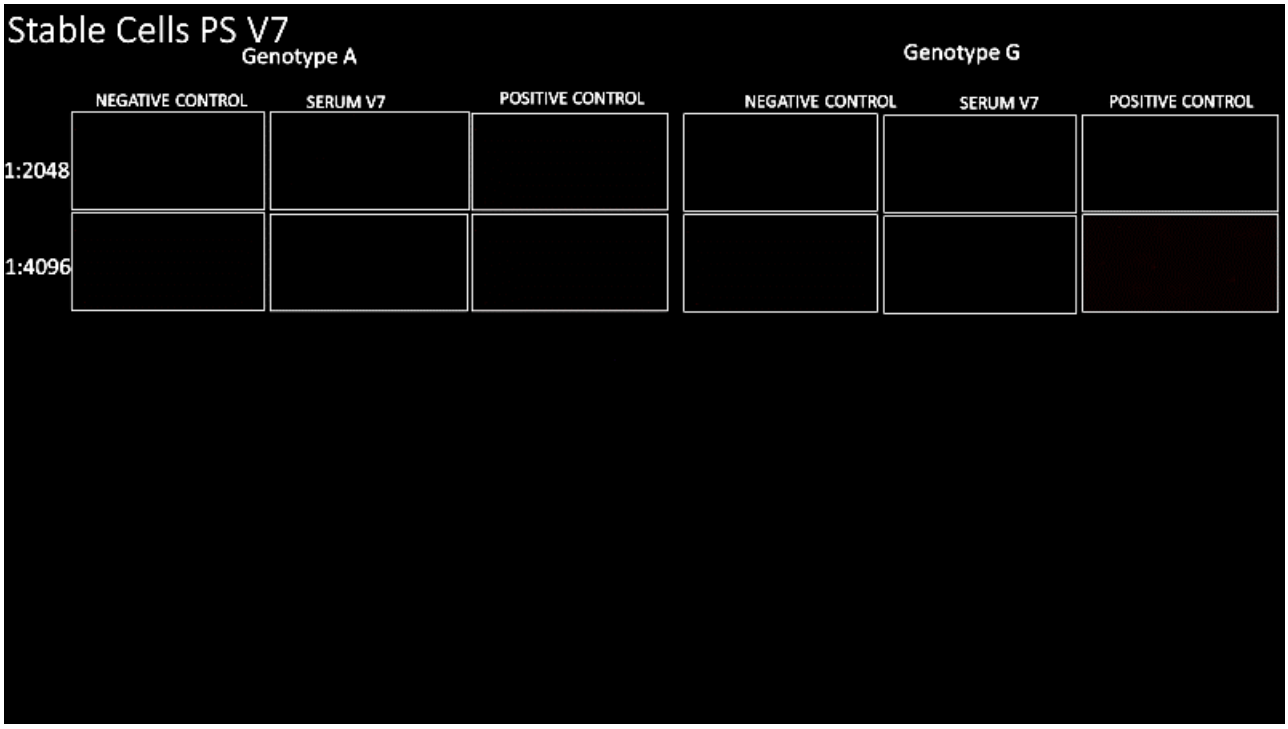


Figure A-51b. Stable cells Vaccine Sample#7.

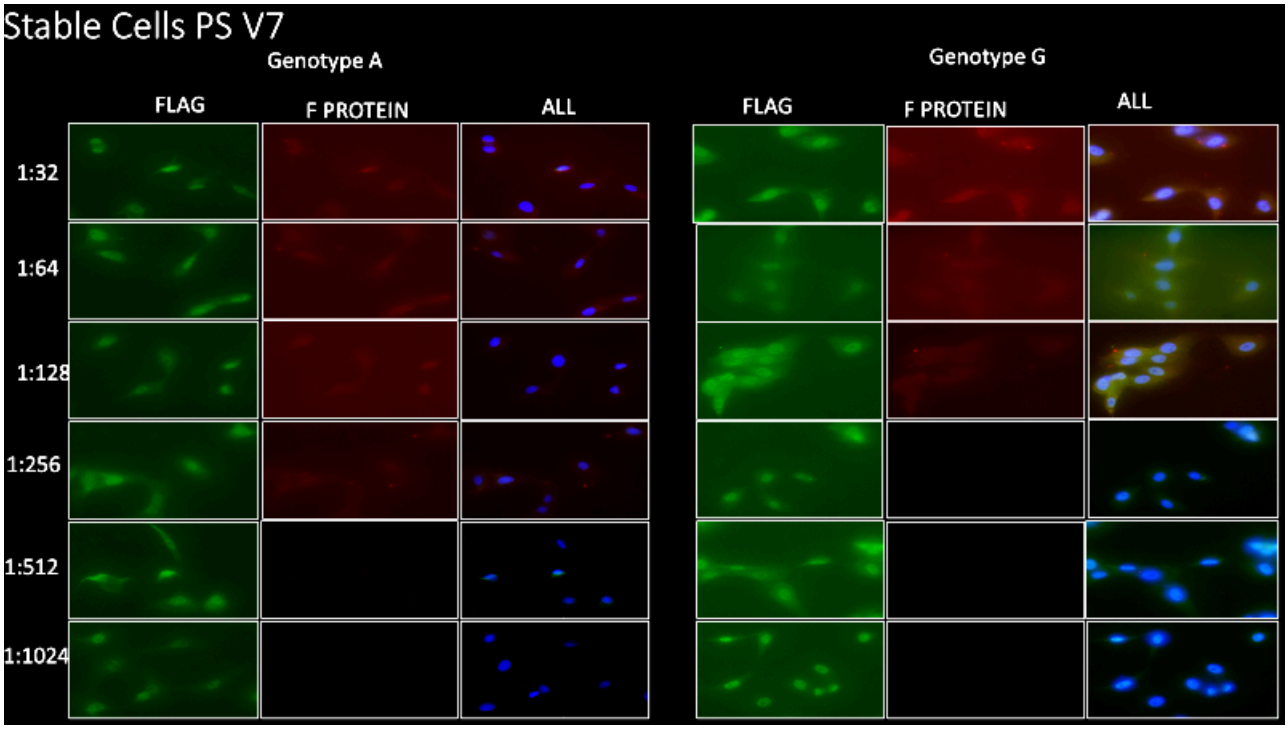


Figure A-51c. Stable cells Vaccine Sample#7.

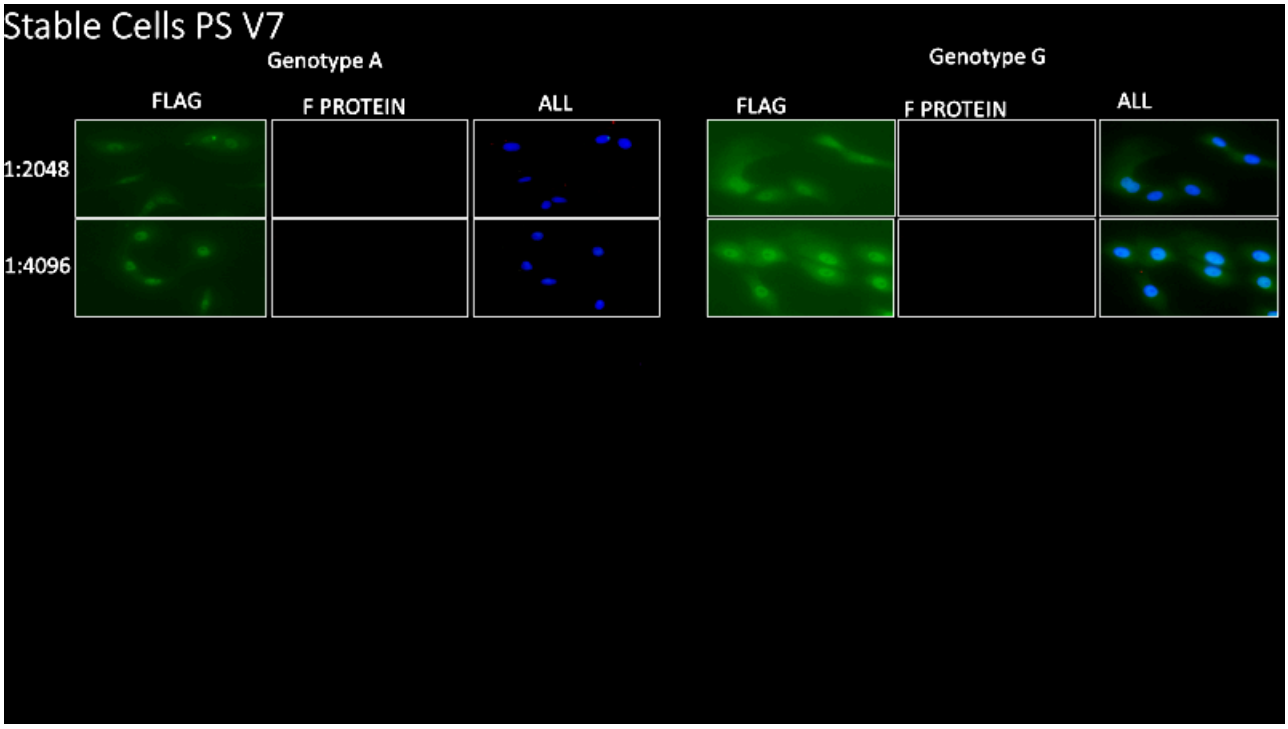


Figure A-51d. Stable cells Vaccine Sample#7.

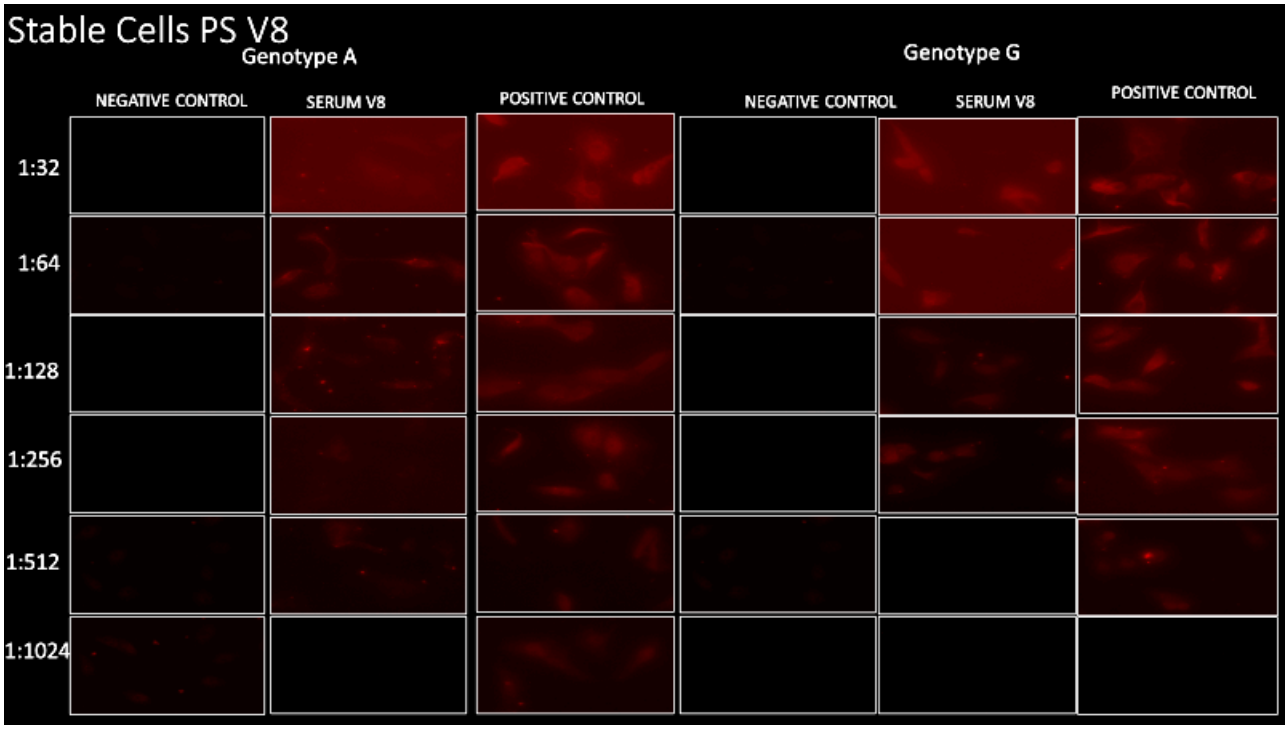


Figure A-52a. Stable cells Vaccine Sample#8.

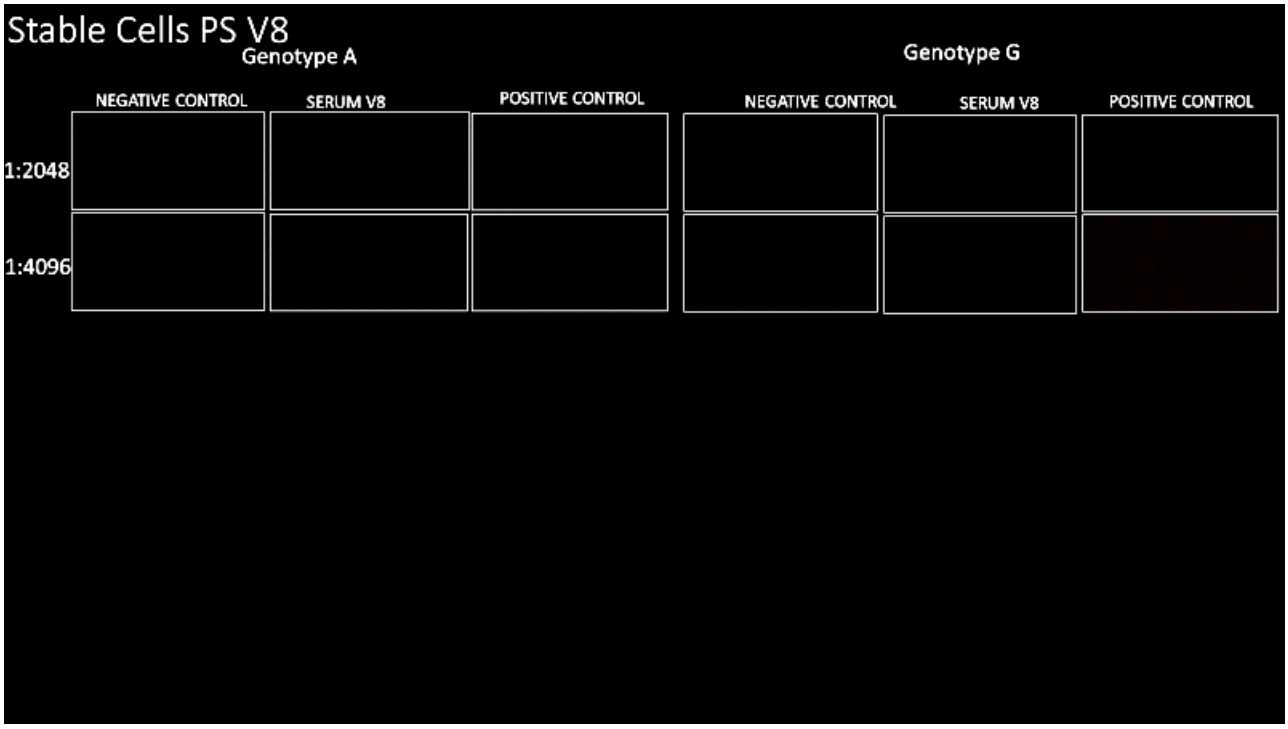


Figure A-52b. Stable cells Vaccine Sample#8.

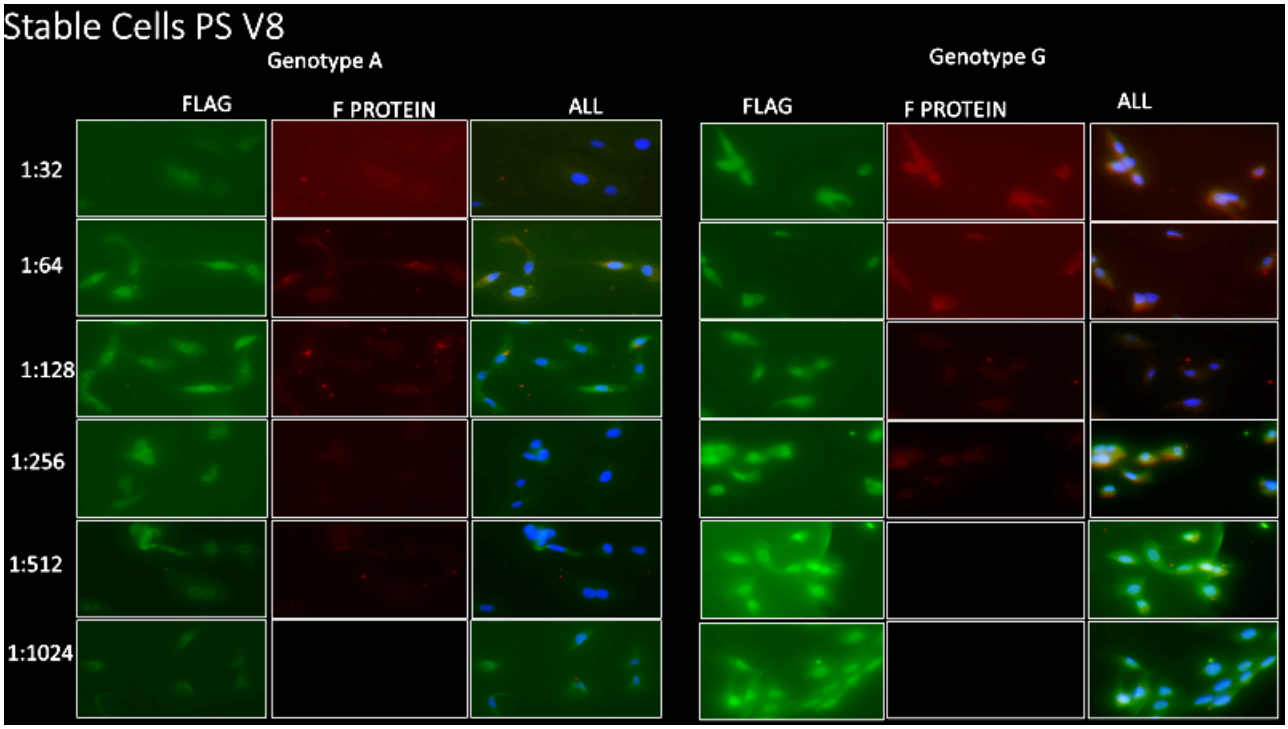


Figure A-52c. Stable cells Vaccine Sample#8.

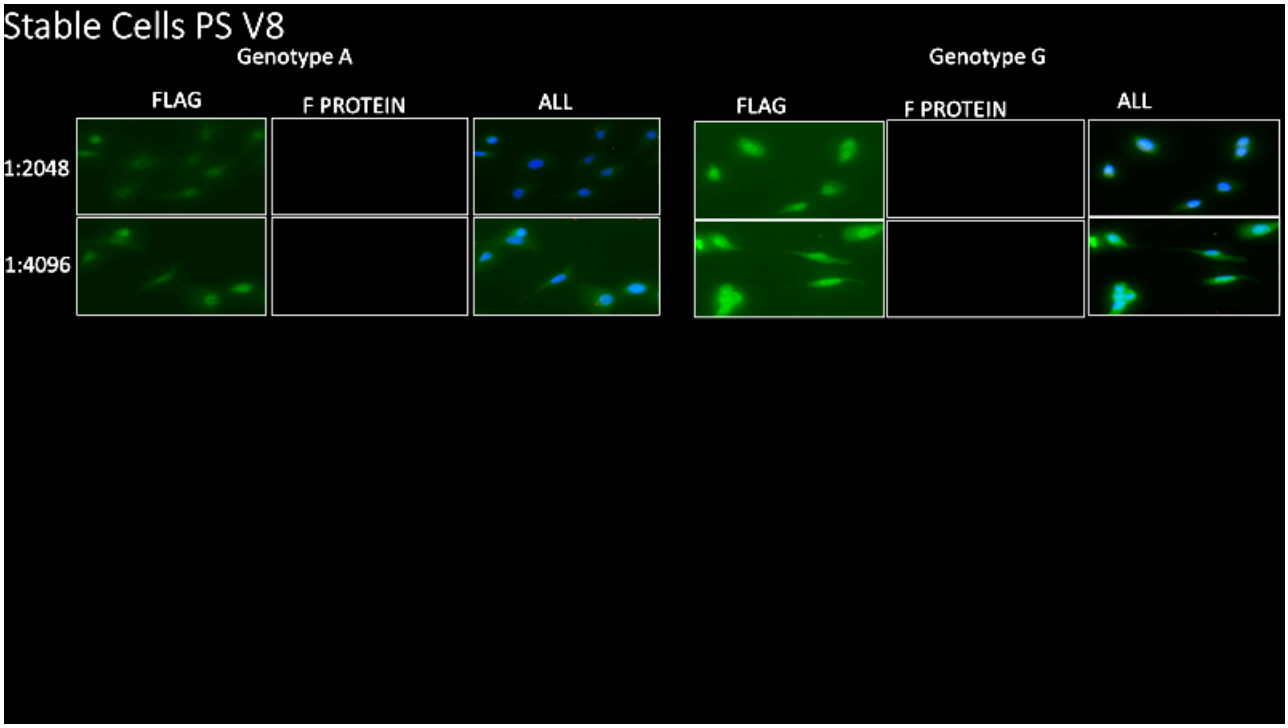


Figure A-52d. Stable cells Vaccine Sample#8.

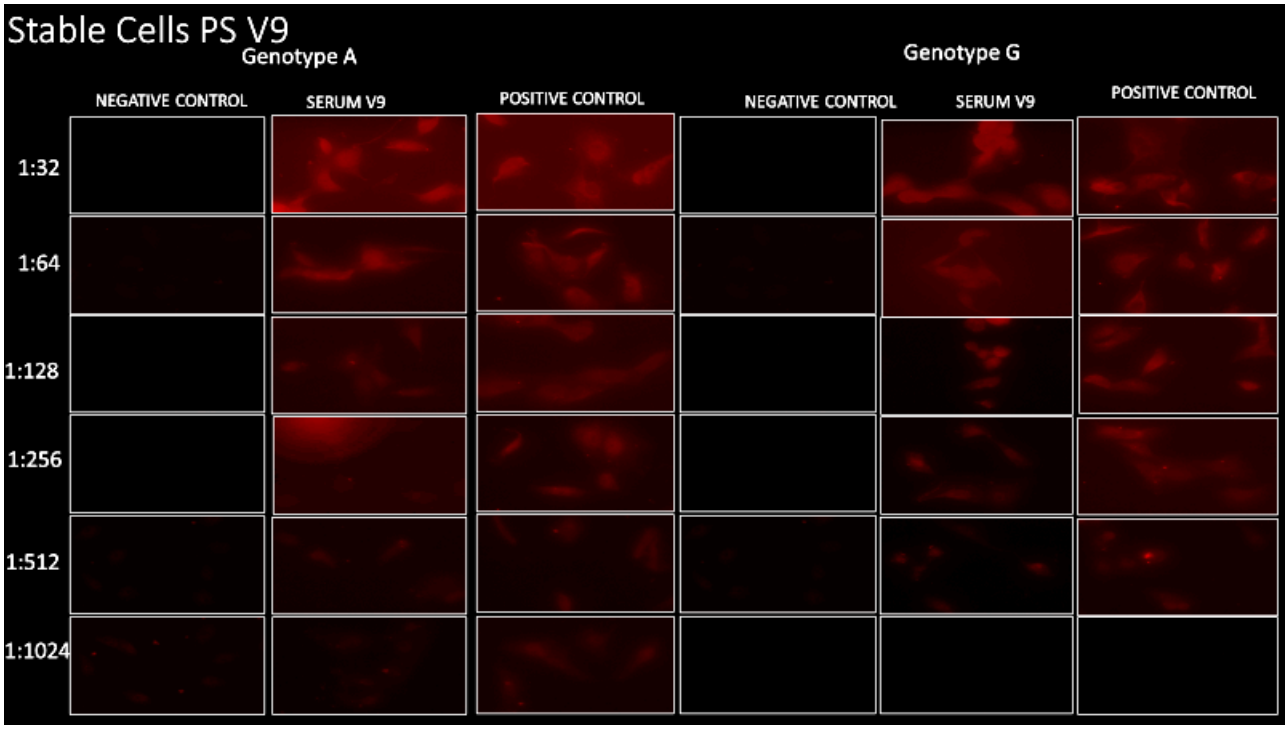


Figure A-53a. Stable cells Vaccine Sample#9.

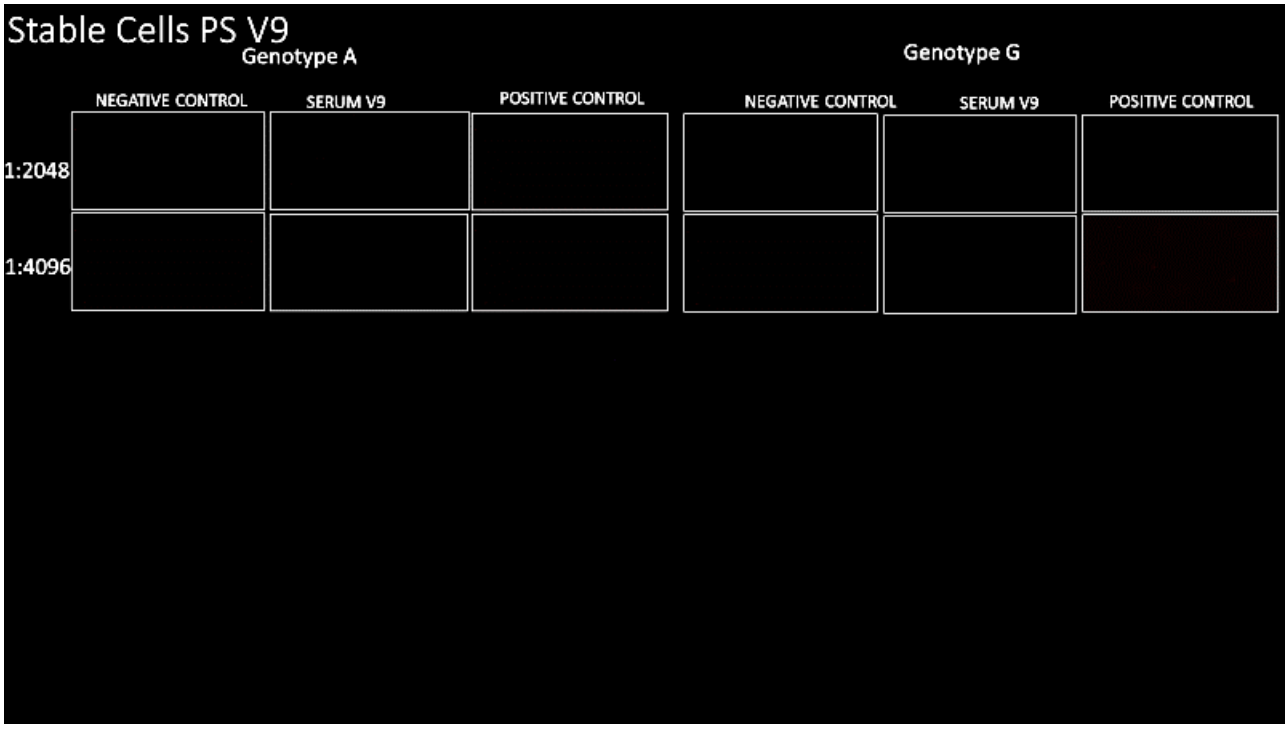


Figure A-53b. Stable cells Vaccine Sample#9.

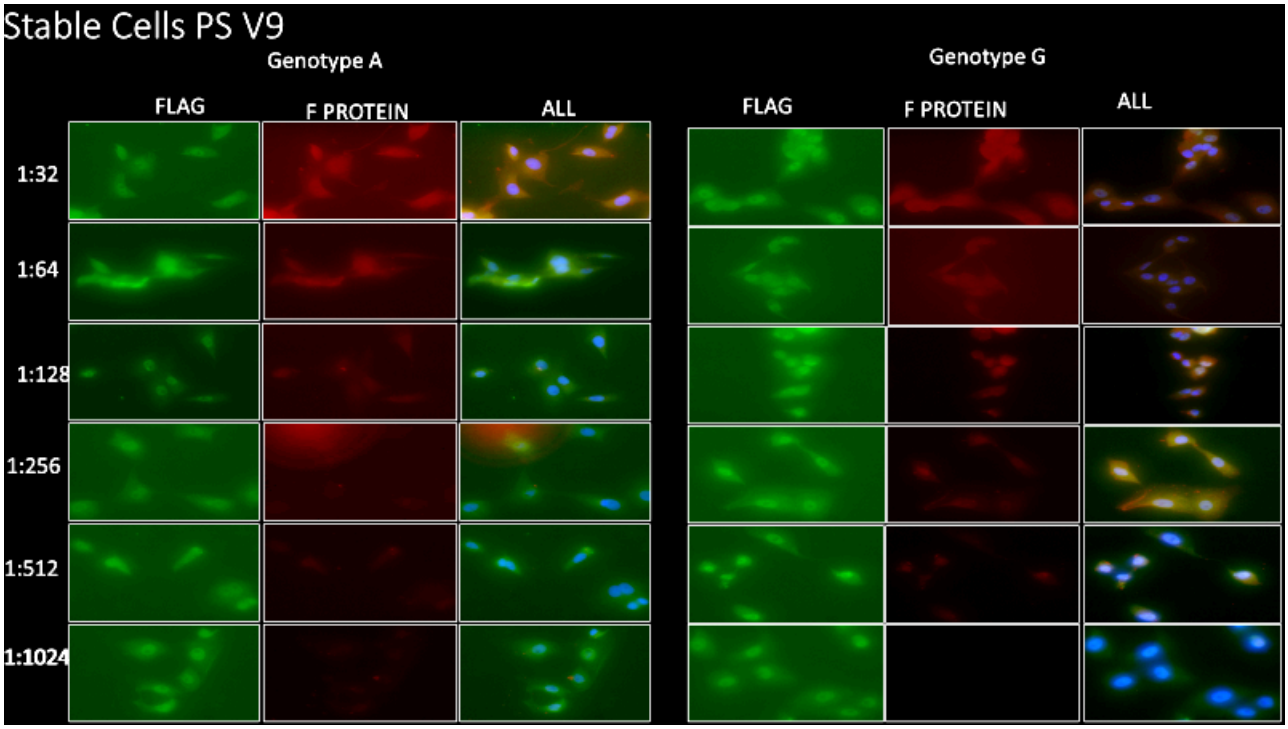


Figure A-53c. Stable cells Vaccine Sample#9.

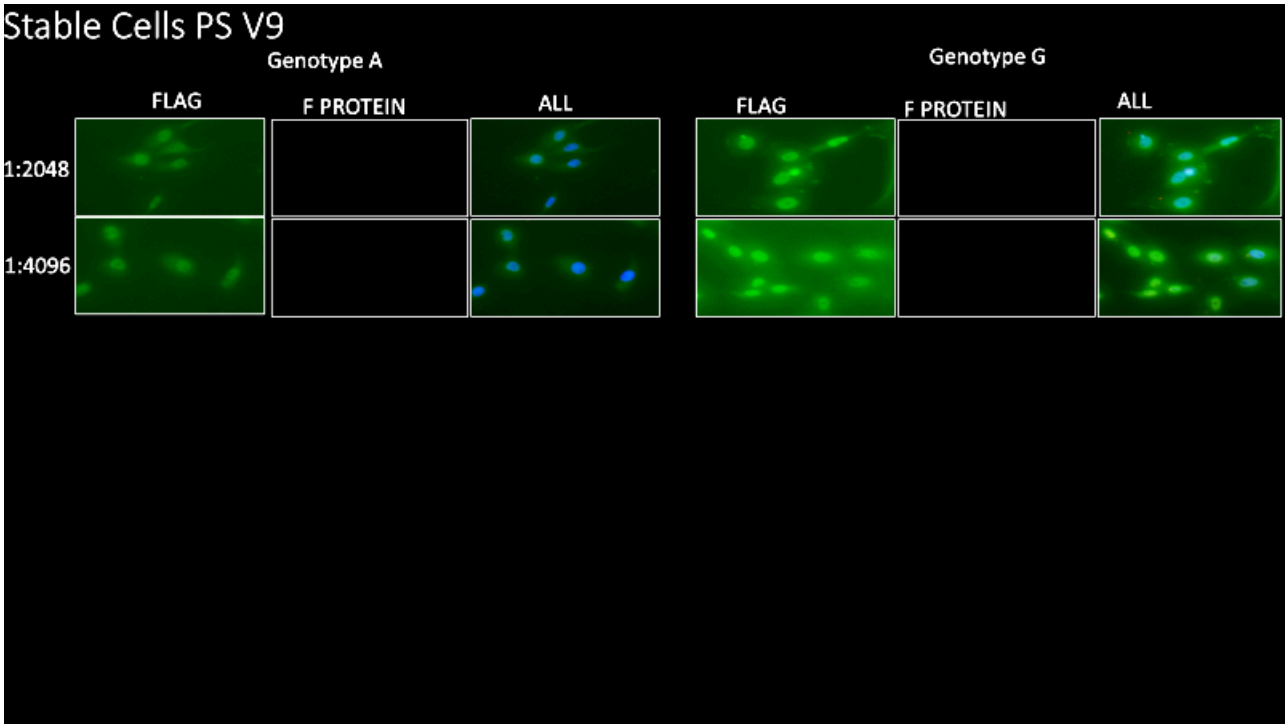


Figure A-53d. Stable cells Vaccine Sample#9.

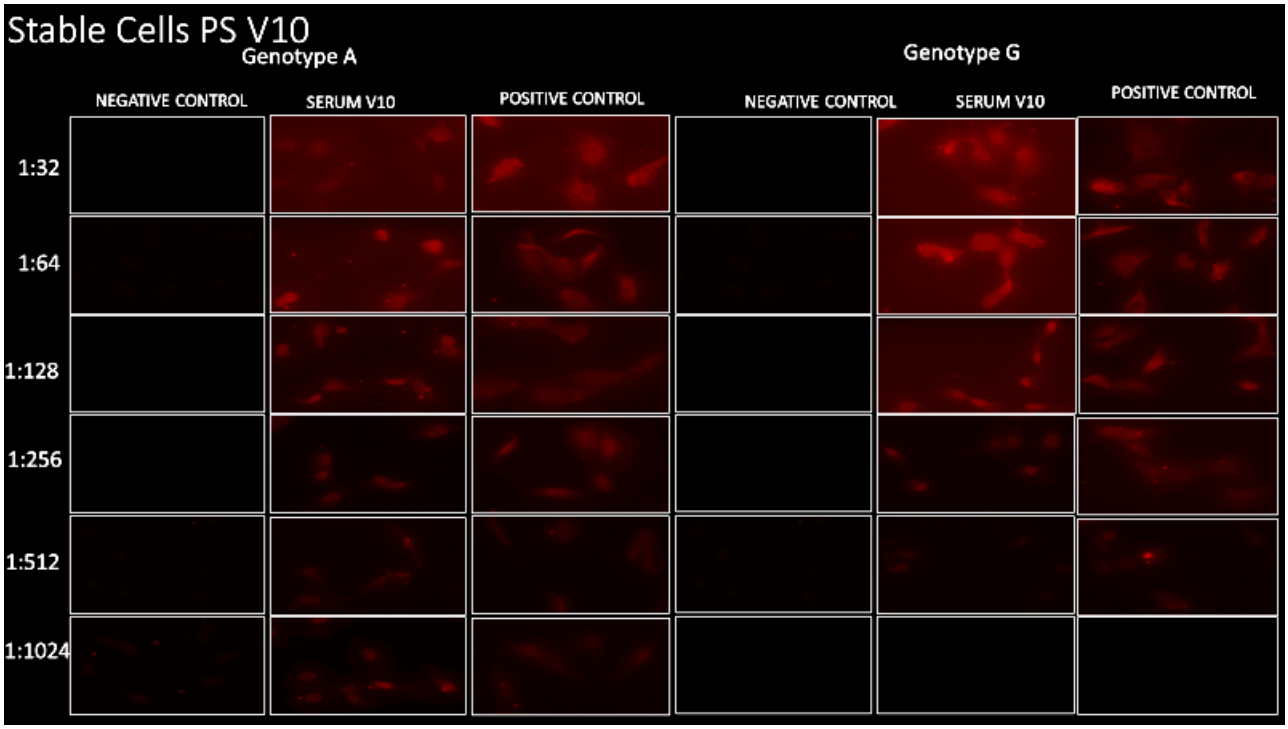


Figure A-54a. Stable cells Vaccine Sample#10.

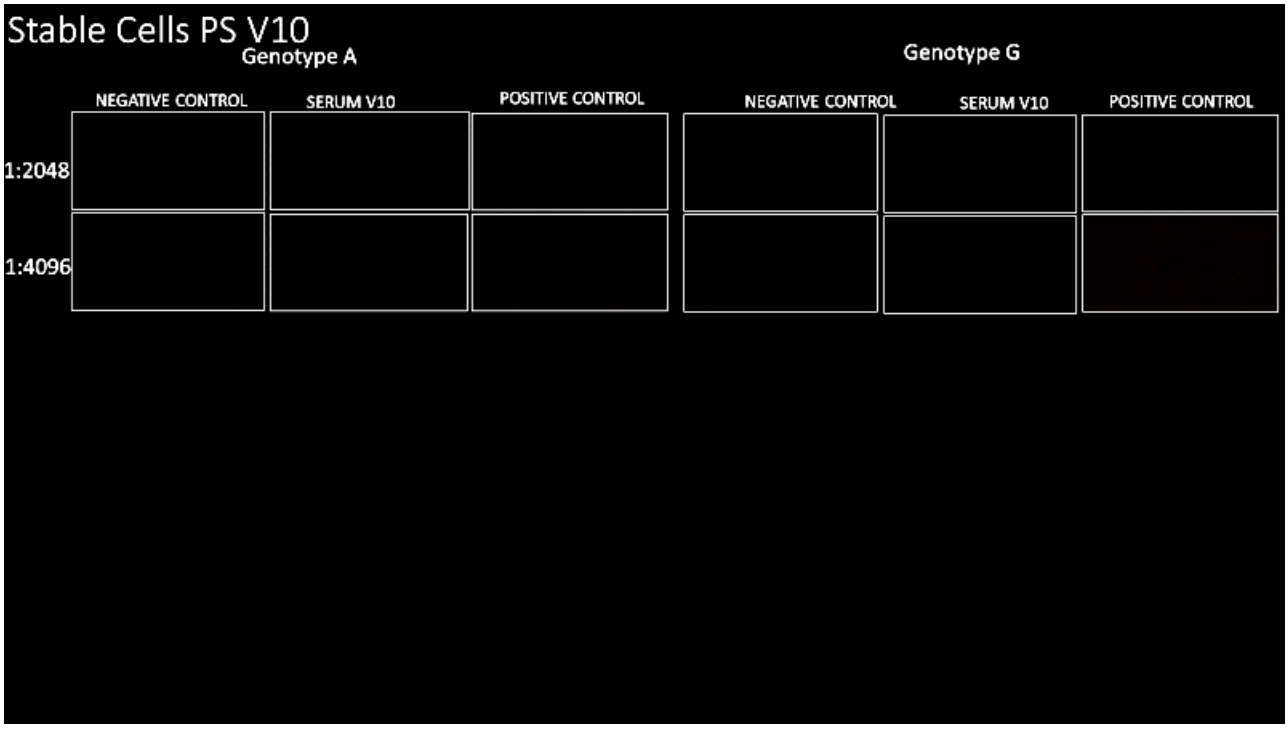


Figure A-54b. Stable cells Vaccine Sample#10.

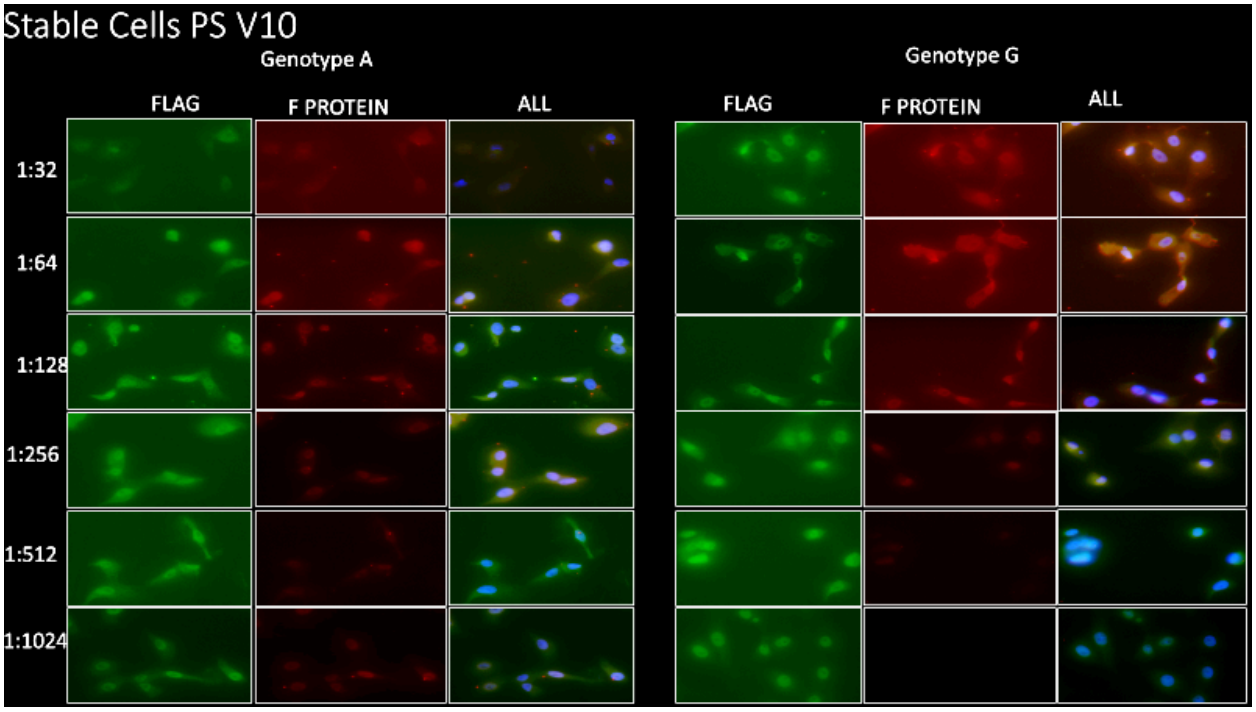


Figure A-54c. Stable cells Vaccine Sample#10.

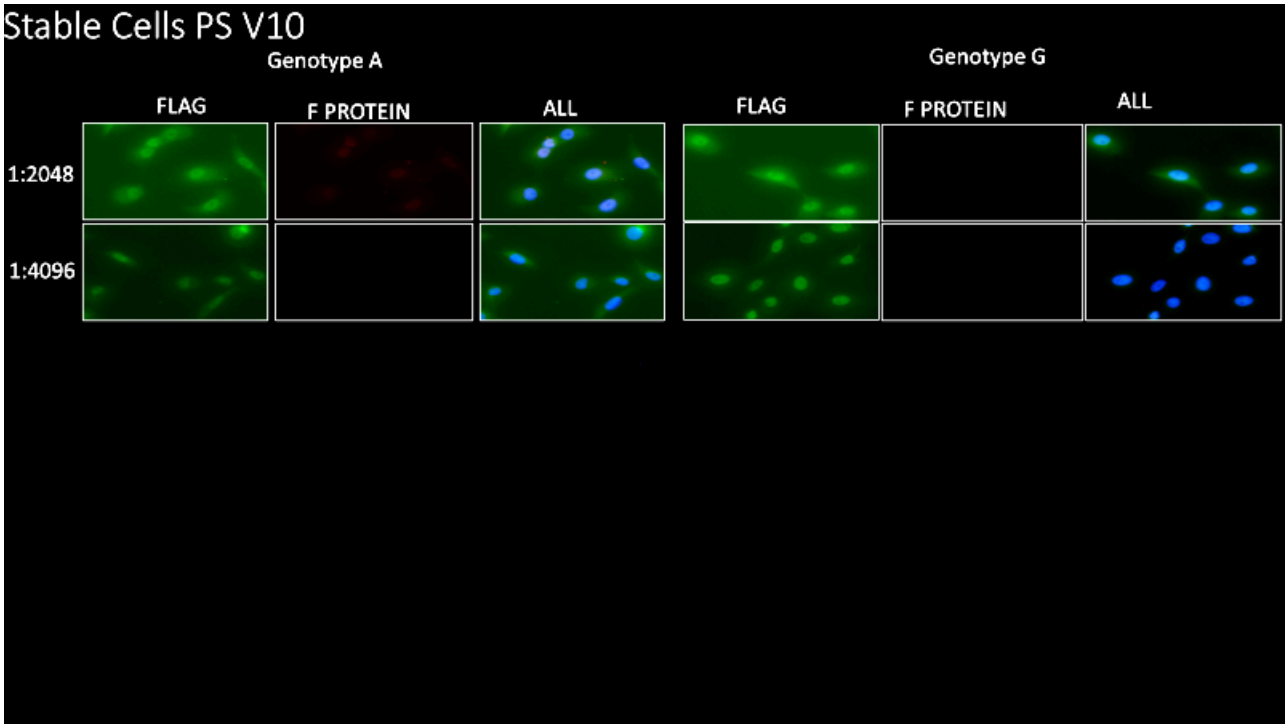


Figure A-54d. Stable cells Vaccine Sample#10.

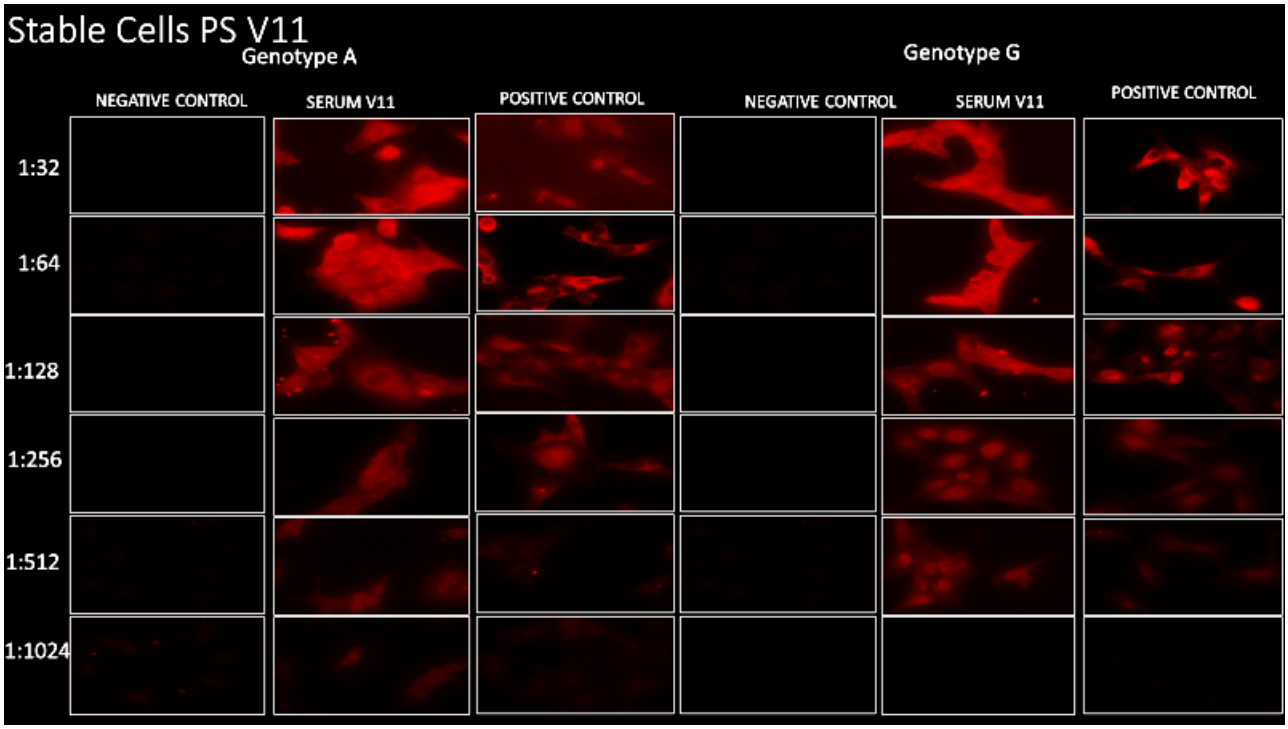


Figure A-55a. Stable cells Vaccine Sample#11.

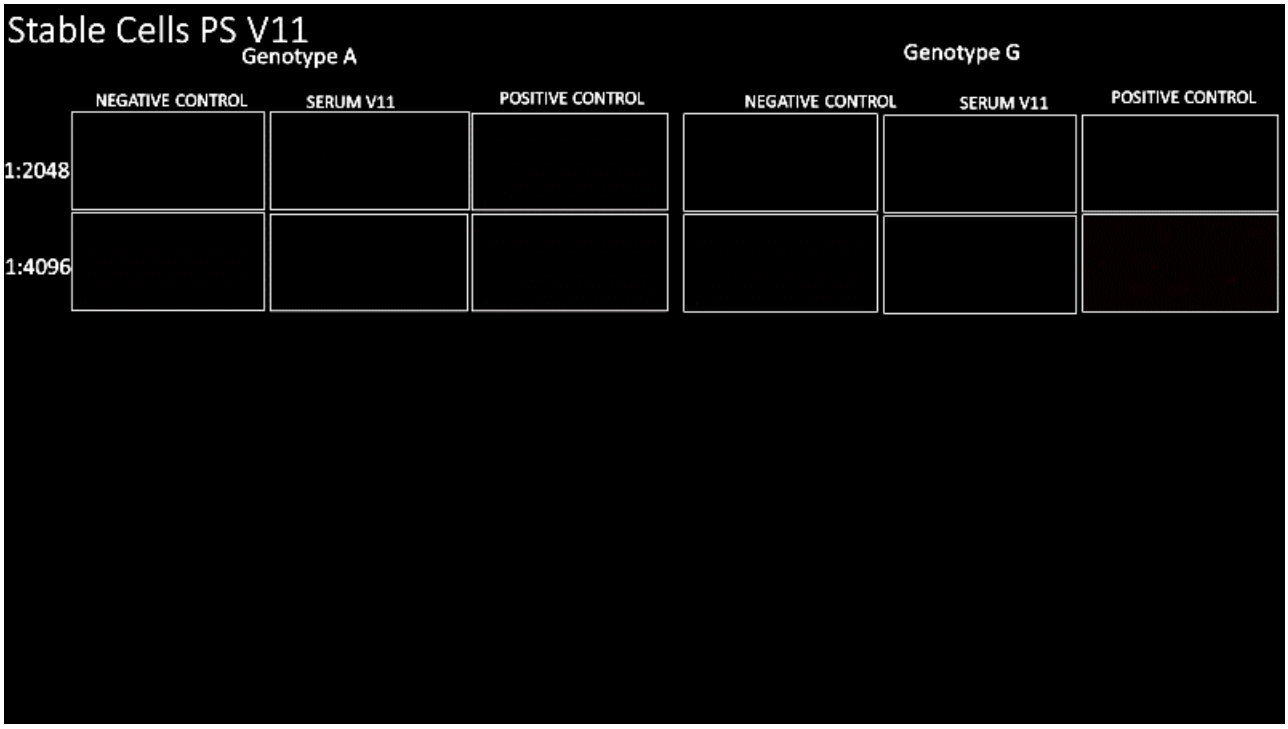


Figure A-55b. Stable cells Vaccine Sample#11.

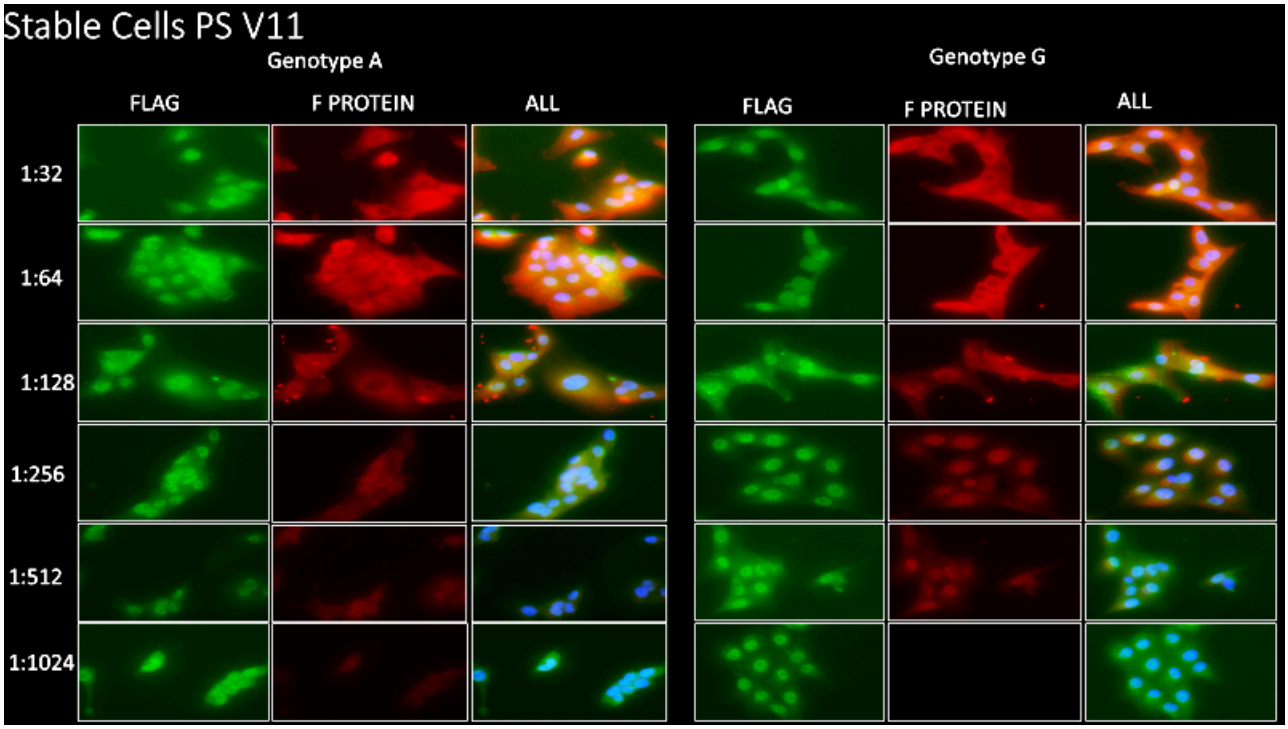


Figure A-55c. Stable cells Vaccine Sample#11.

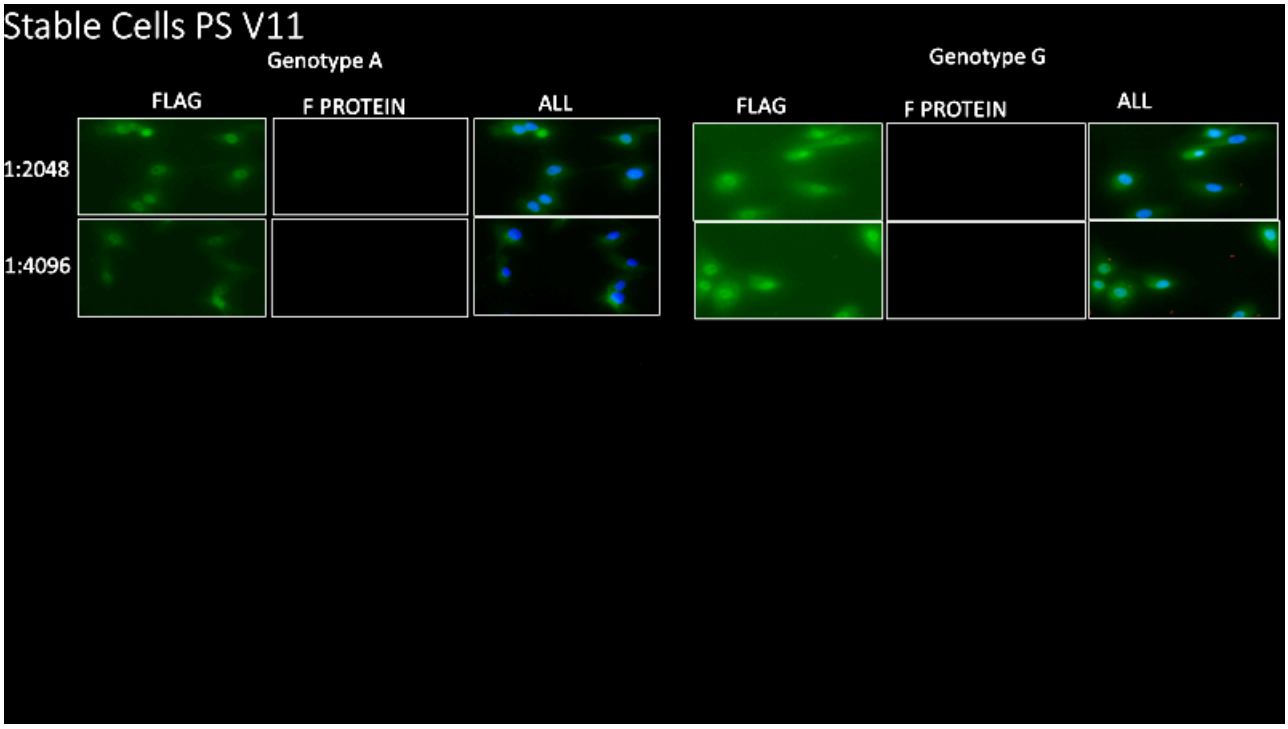


Figure A-55d. Stable cells Vaccine Sample#11.

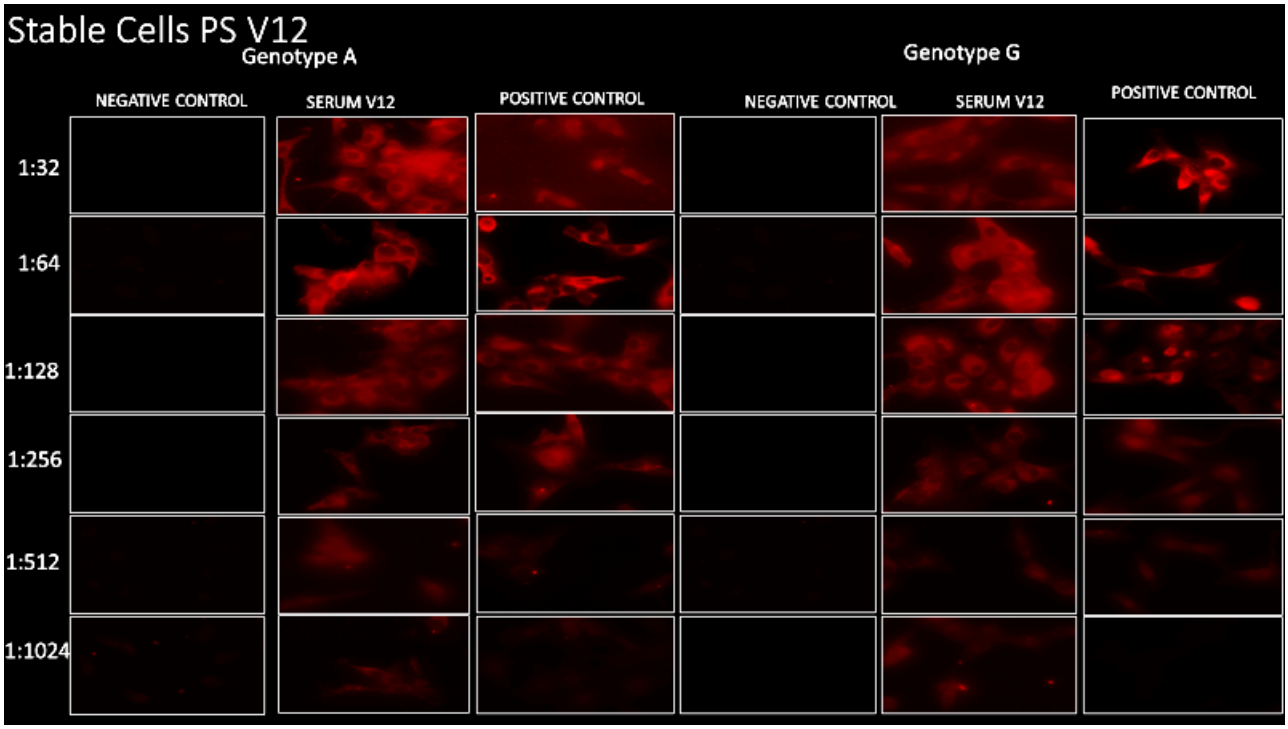


Figure A-56a. Stable cells Vaccine Sample#12.

Figure A-56b. Stable cells Vaccine Sample#12.

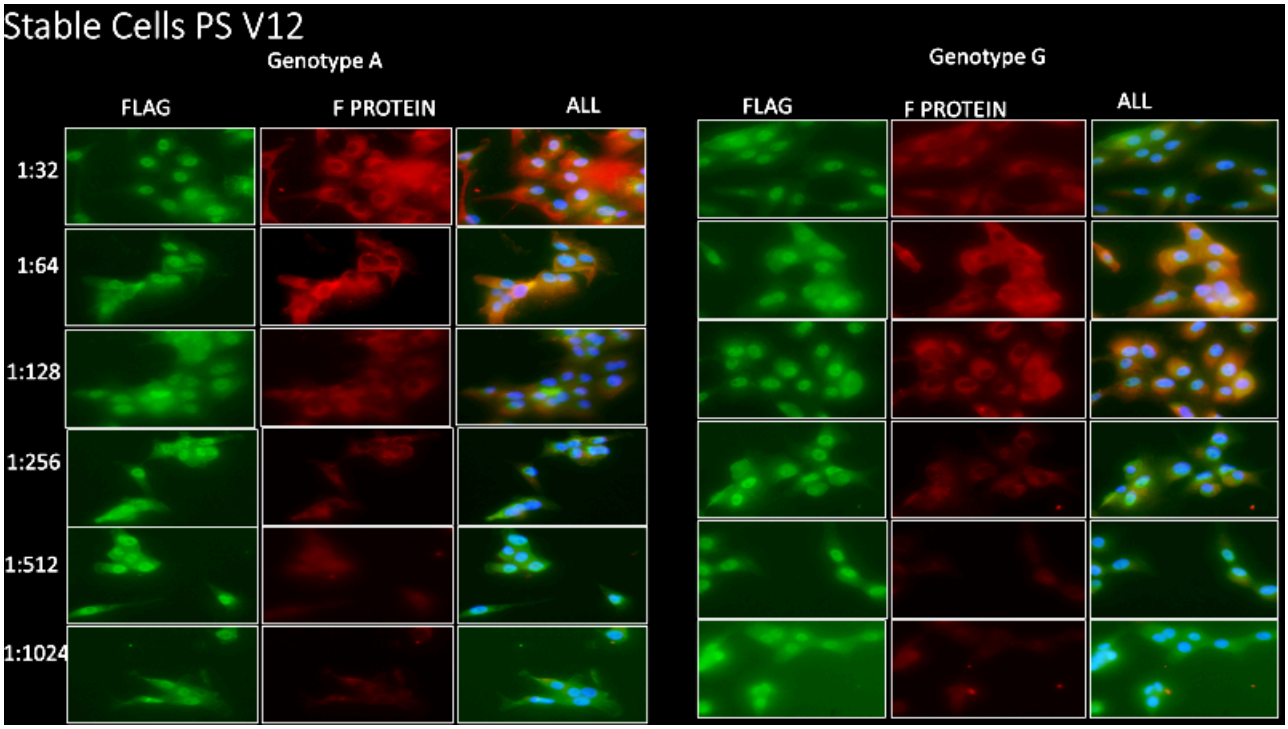


Figure A-56c. Stable cells Vaccine Sample#12.

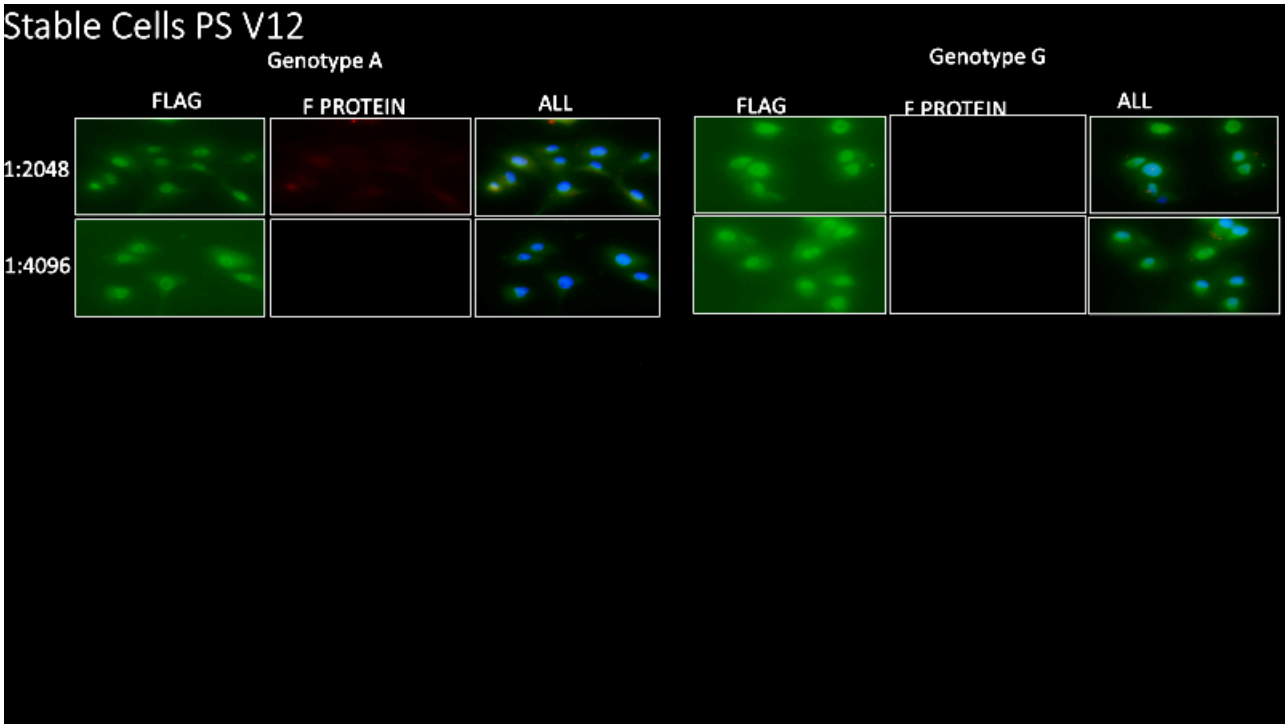


Figure A-56d. Stable cells Vaccine Sample#12.

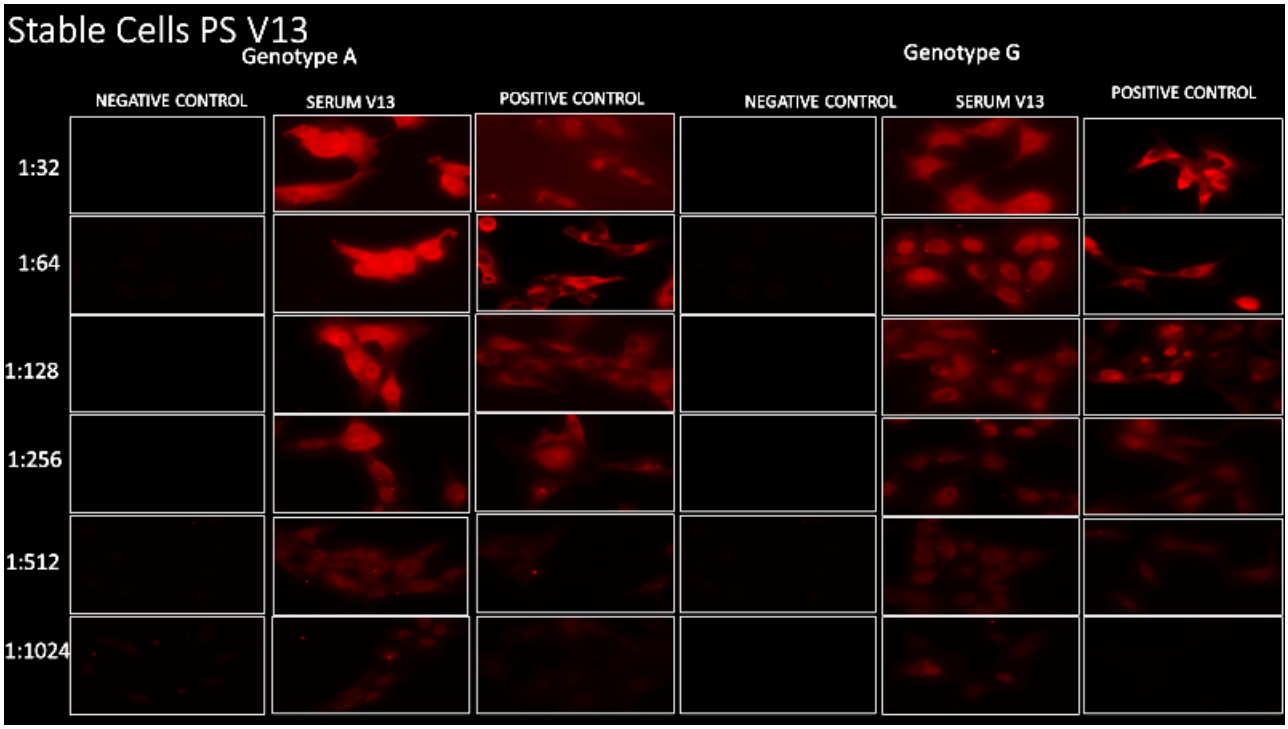


Figure A-57a. Stable cells Vaccine Sample#13.

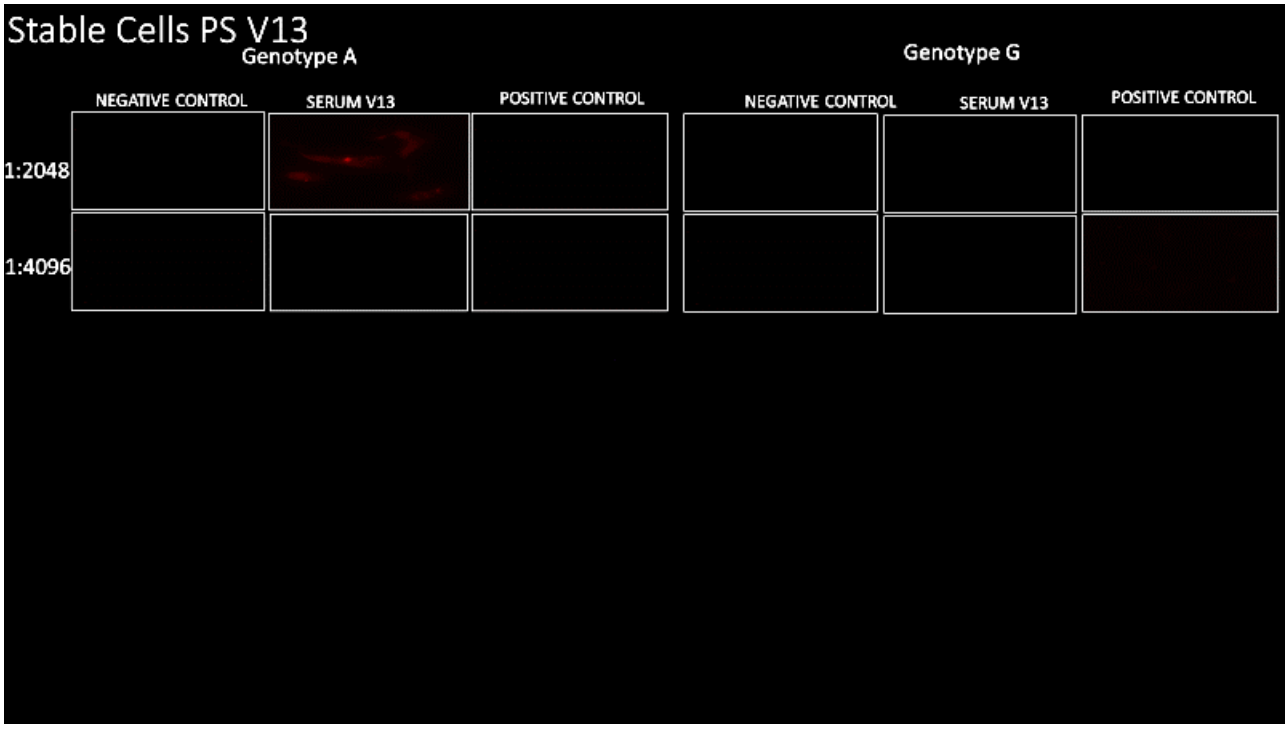


Figure A-57b. Stable cells Vaccine Sample#13.

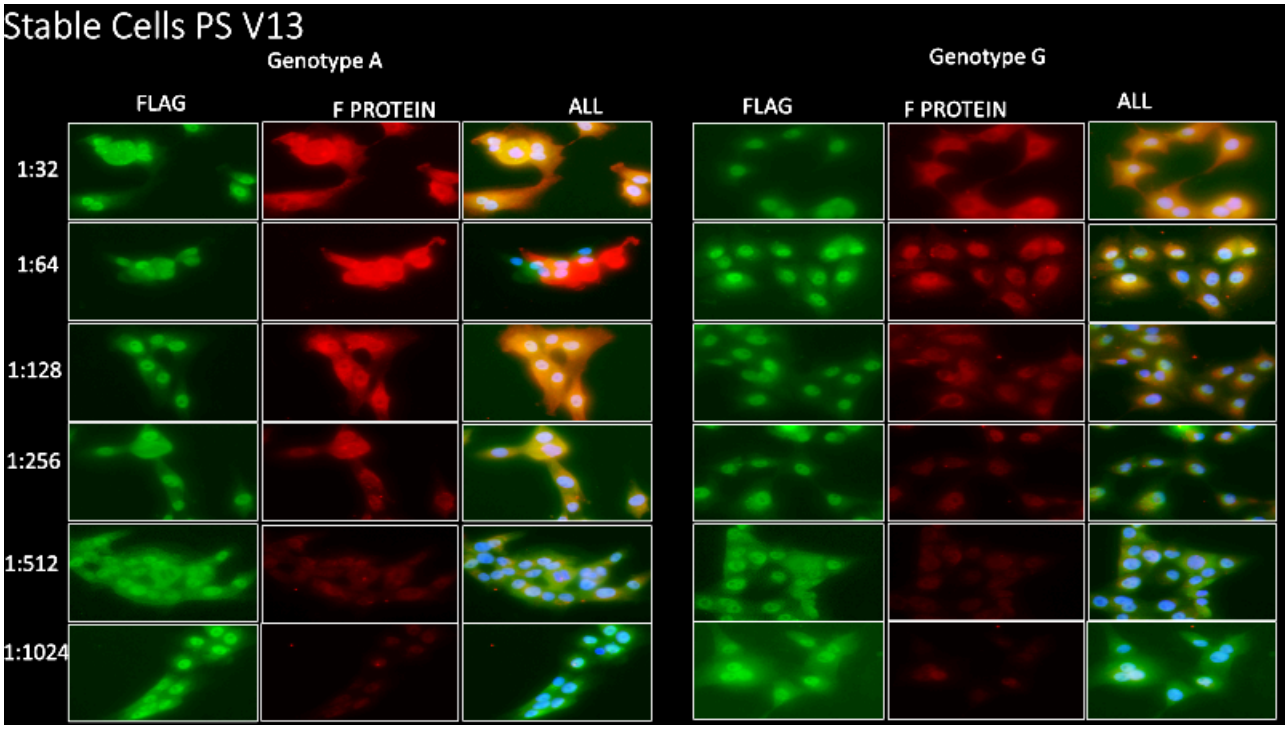


Figure A-57c. Stable cells Vaccine Sample#13.

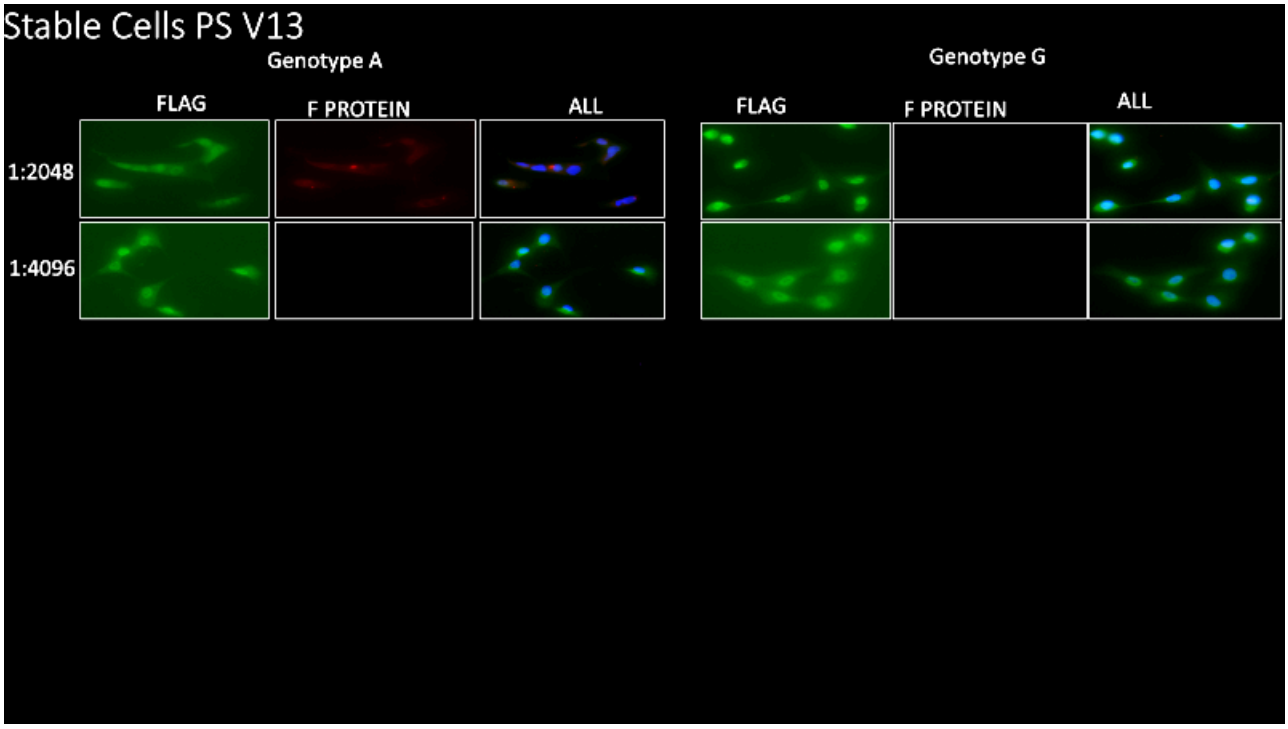


Figure A-57d. Stable cells Vaccine Sample#13.

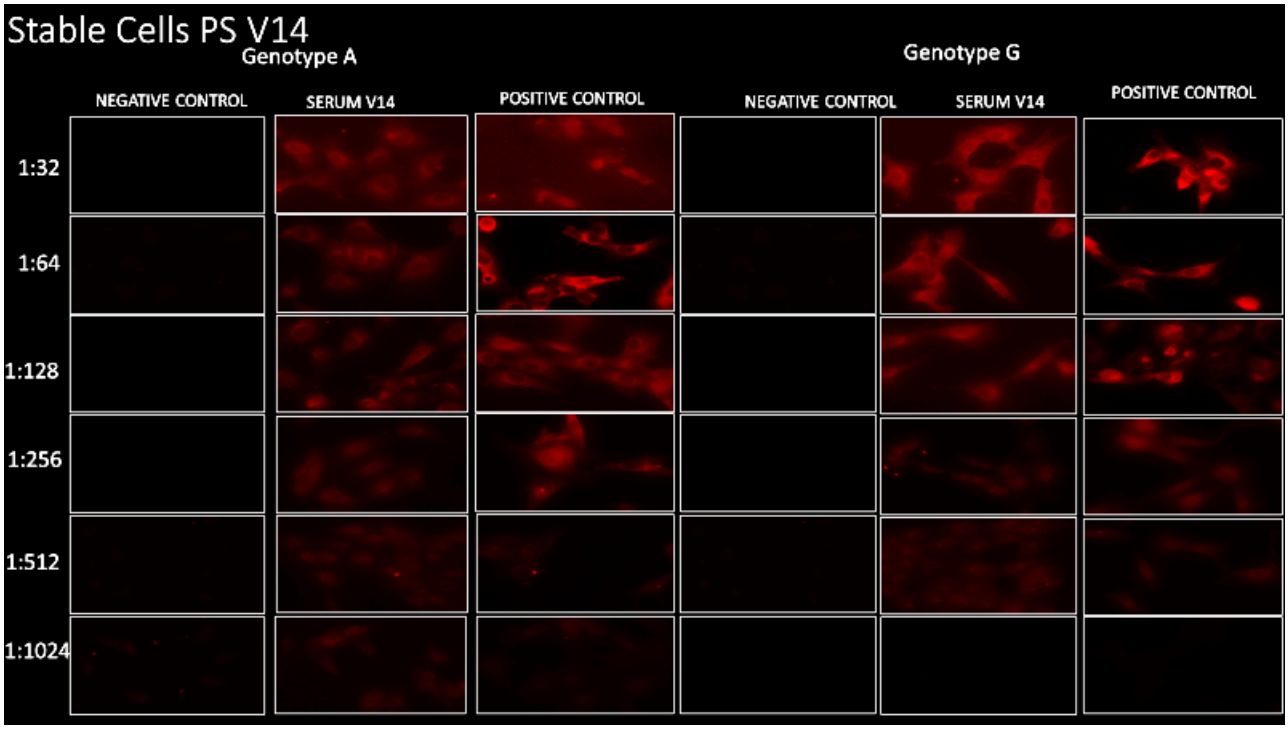


Figure A-58a. Stable cells Vaccine Sample#14.

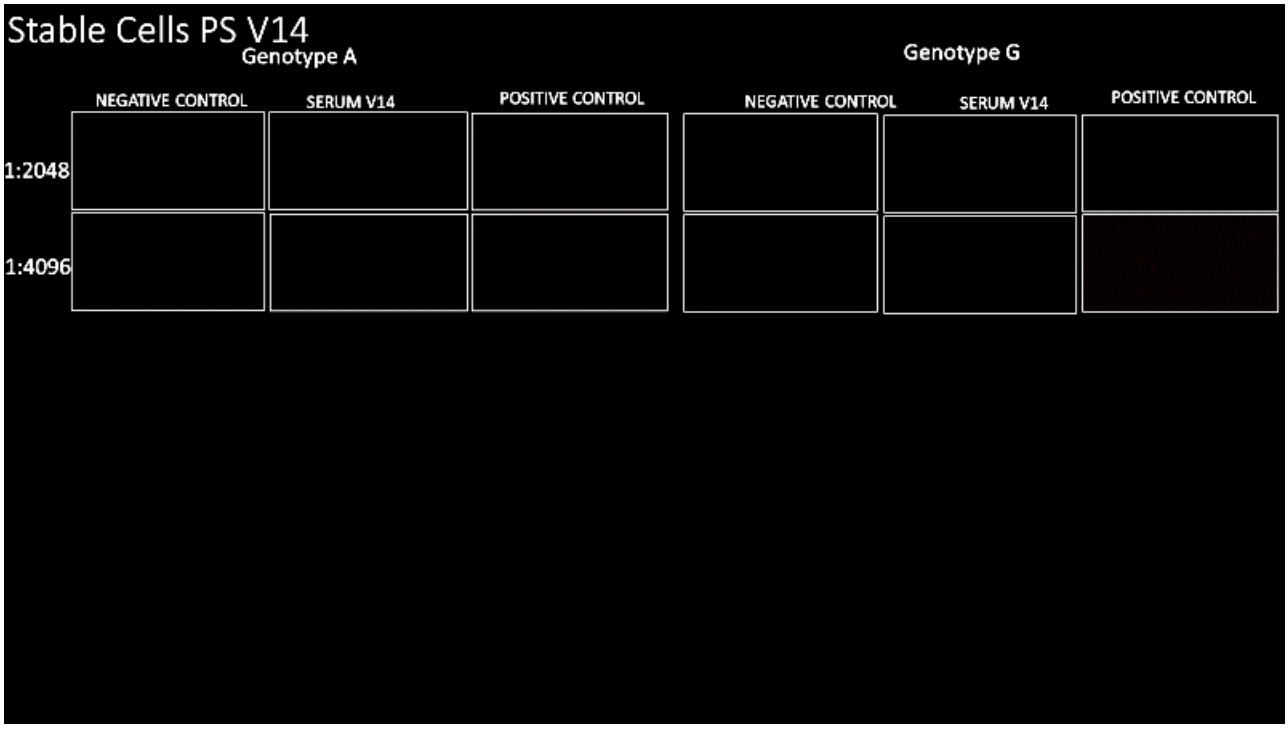


Figure A-58b. Stable cells Vaccine Sample#14.

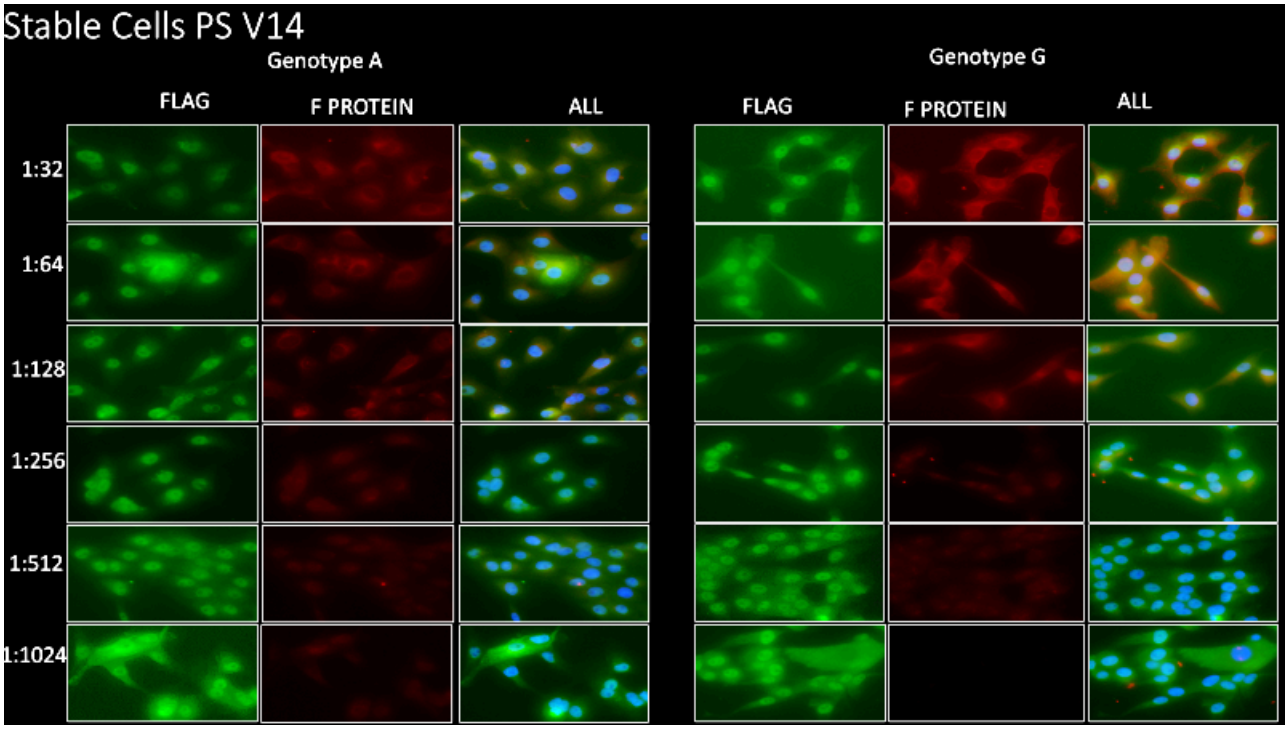


Figure A-58c. Stable cells Vaccine Sample#14.

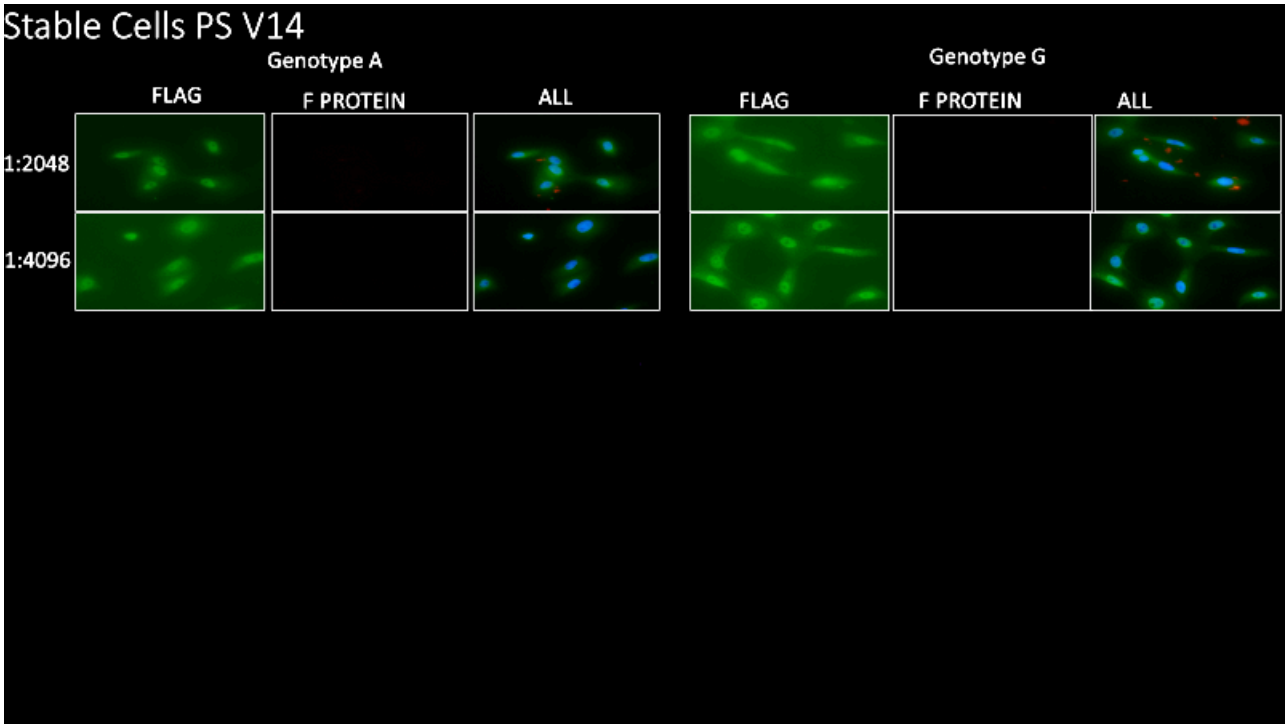


Figure A-58d. Stable cells Vaccine Sample#14.

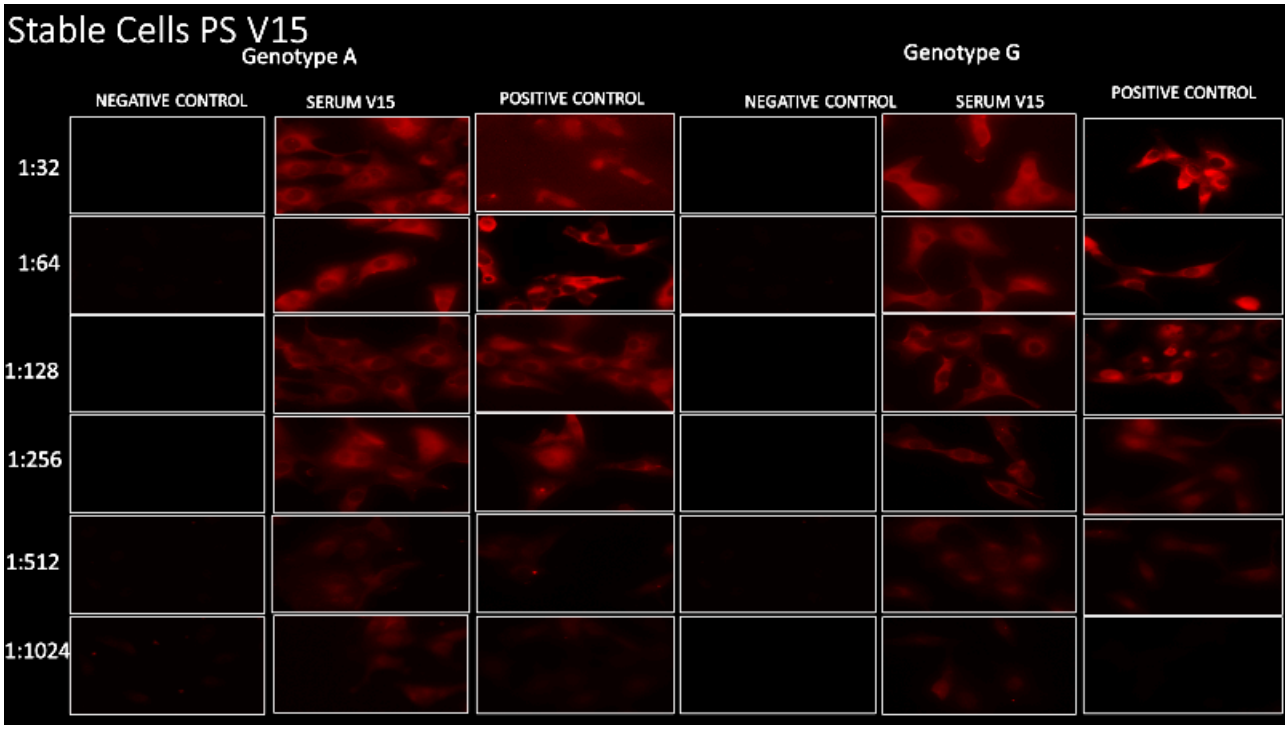


Figure A-59a. Stable cells Vaccine Sample#15.

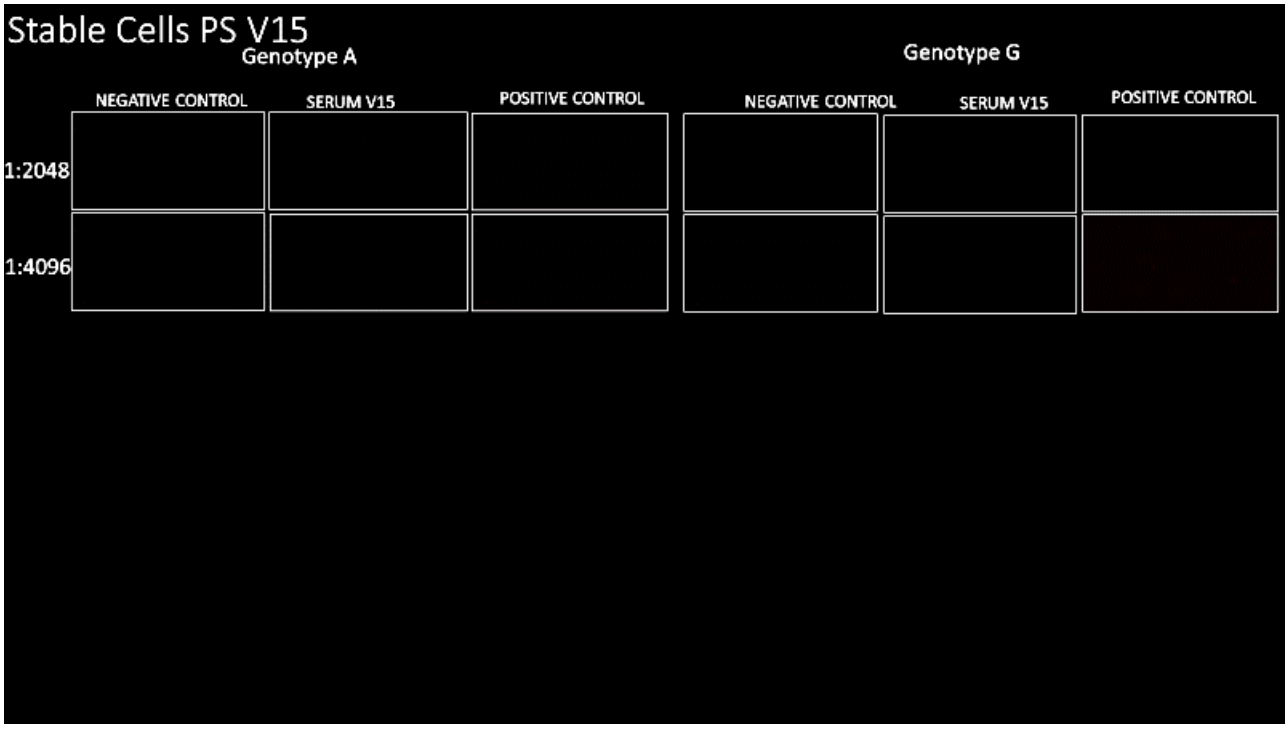


Figure A-59b. Stable cells Vaccine Sample#15.

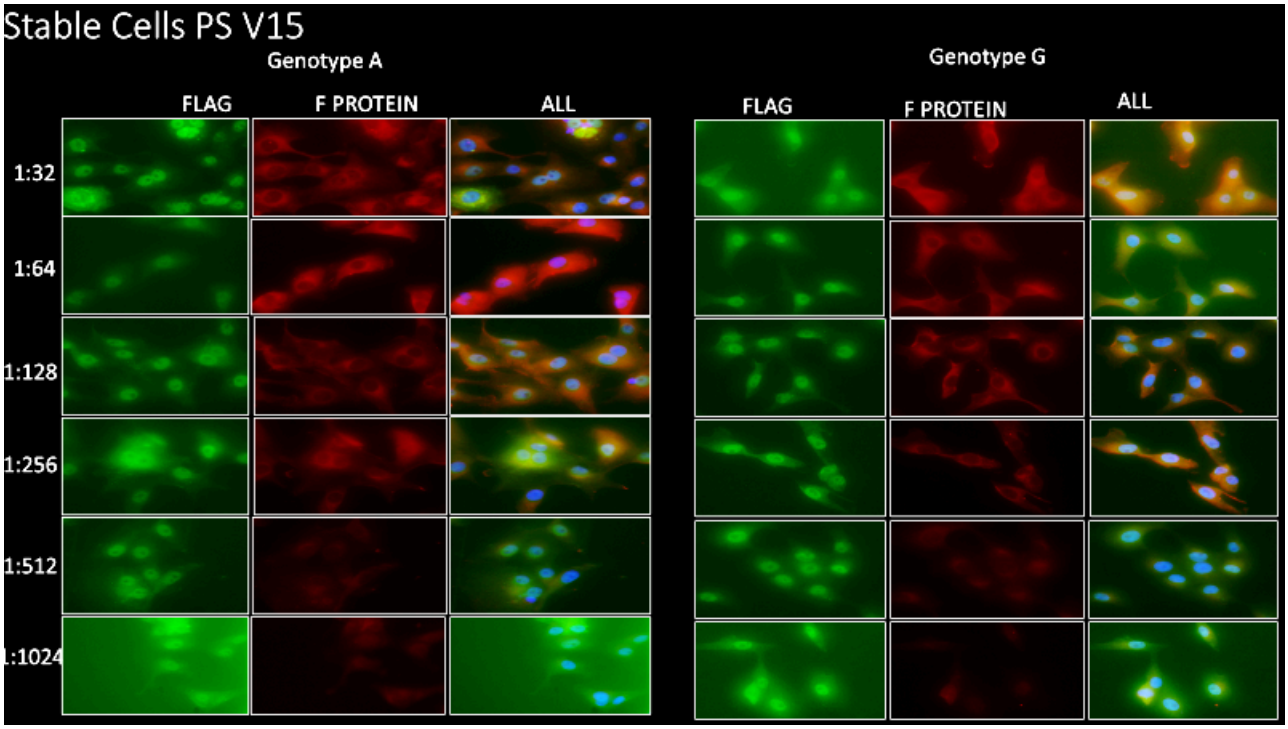


Figure A-59c. Stable cells Vaccine Sample#15.

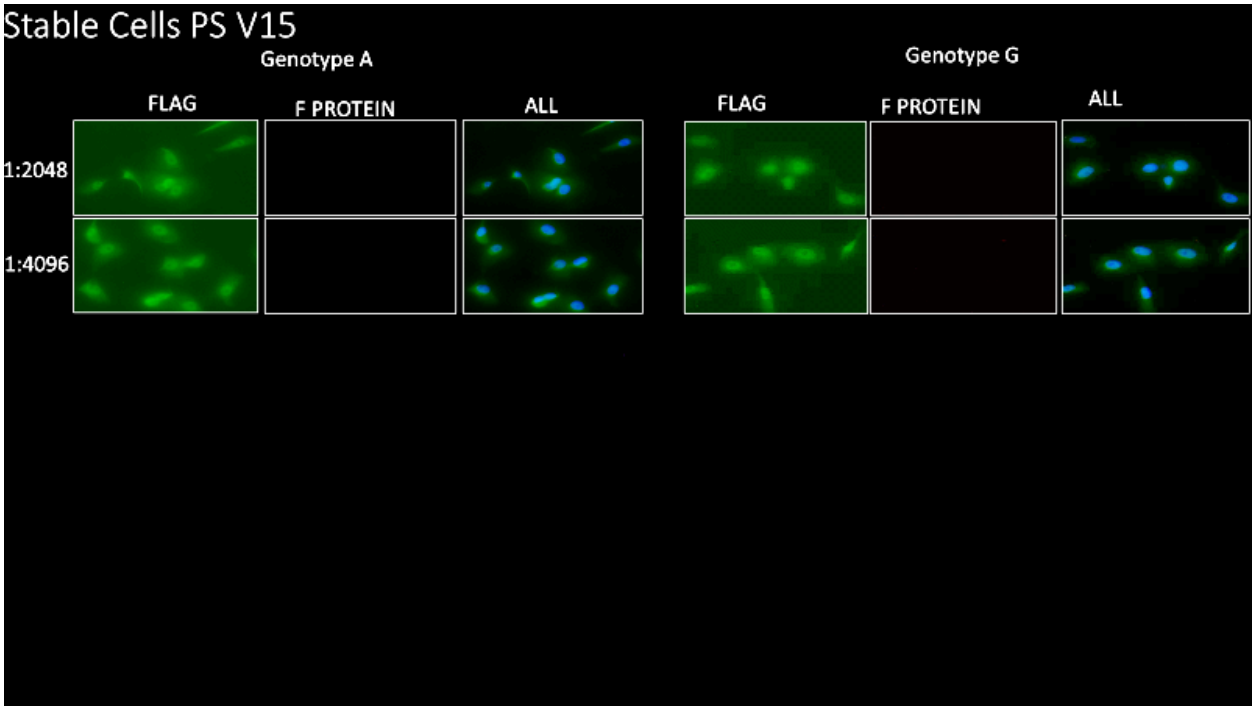


Figure A-59d. Stable cells Vaccine Sample#15.

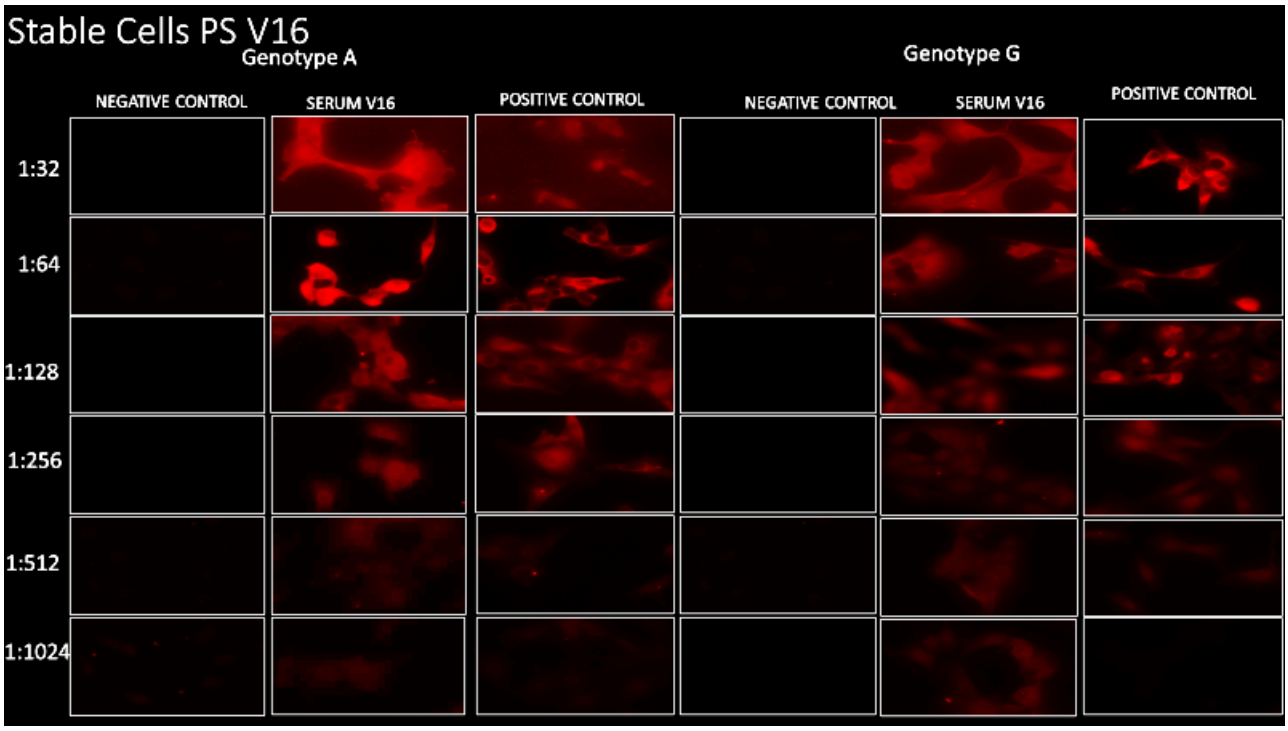


Figure A-60a. Stable cells Vaccine Sample#16.

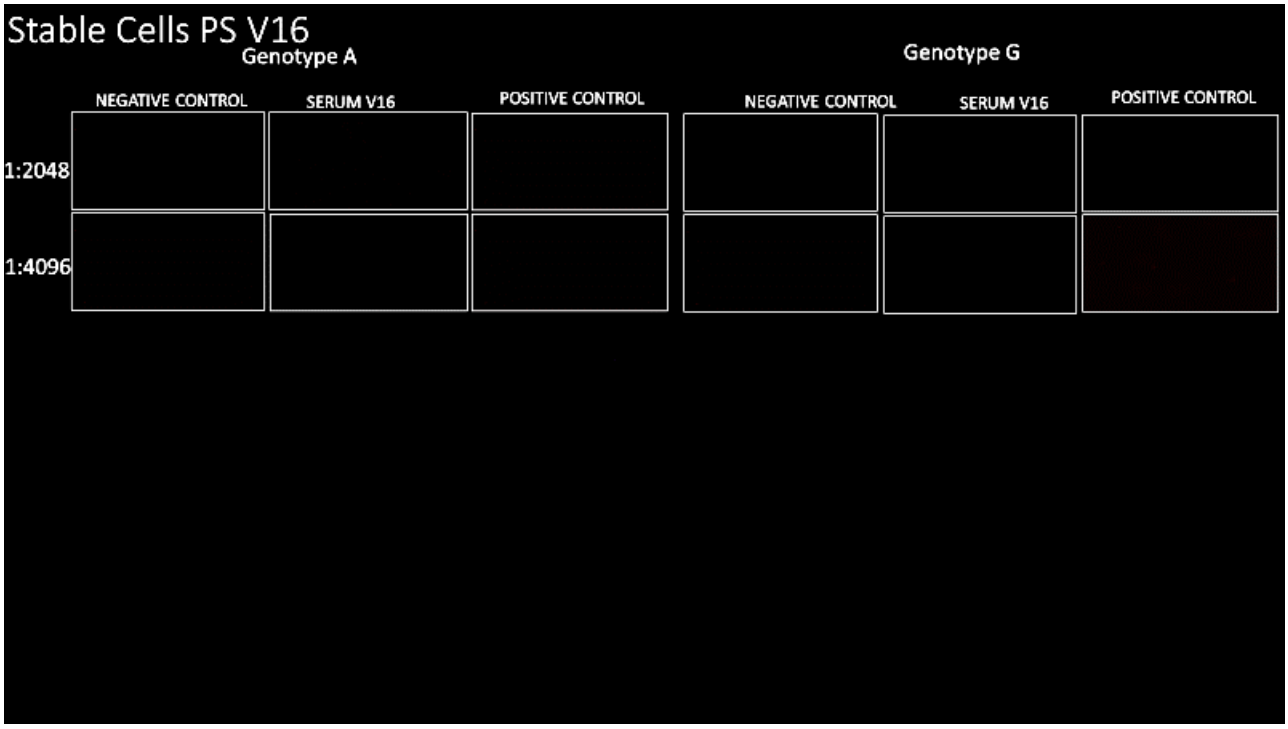


Figure A-60b. Stable cells Vaccine Sample#16.

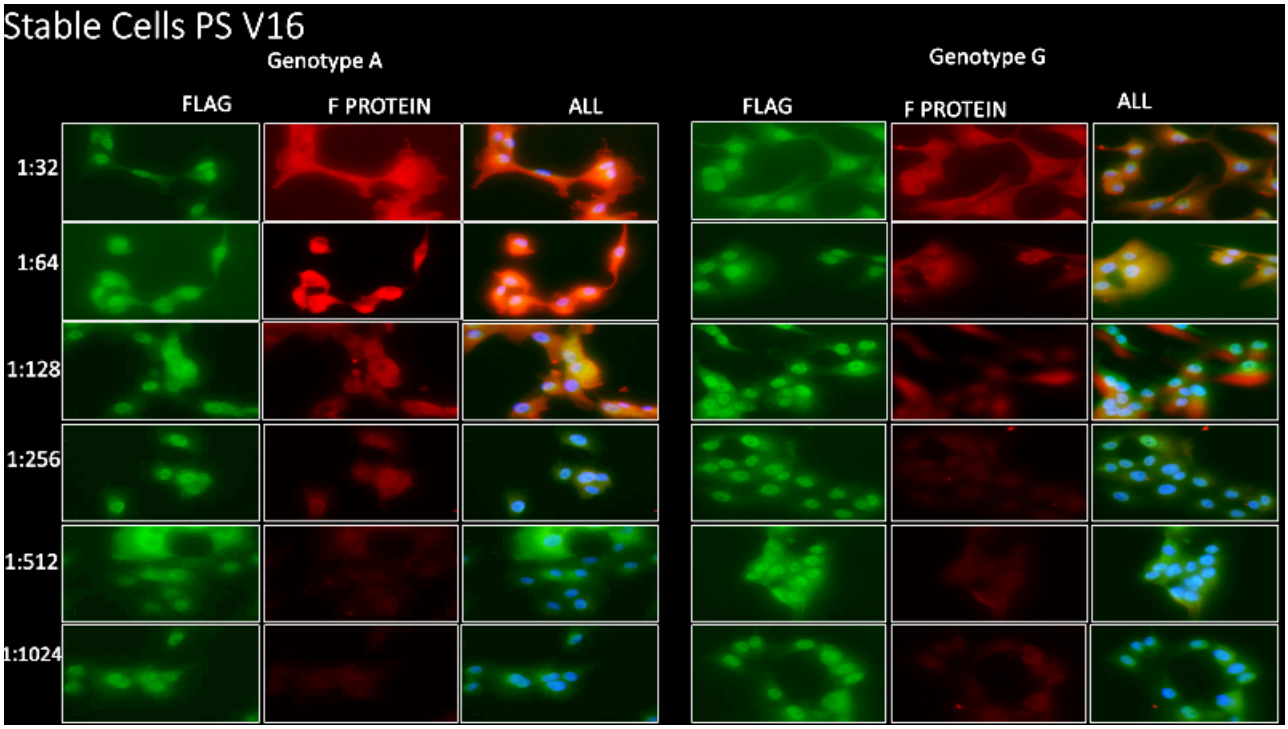


Figure A-60c. Stable cells Vaccine Sample#16.

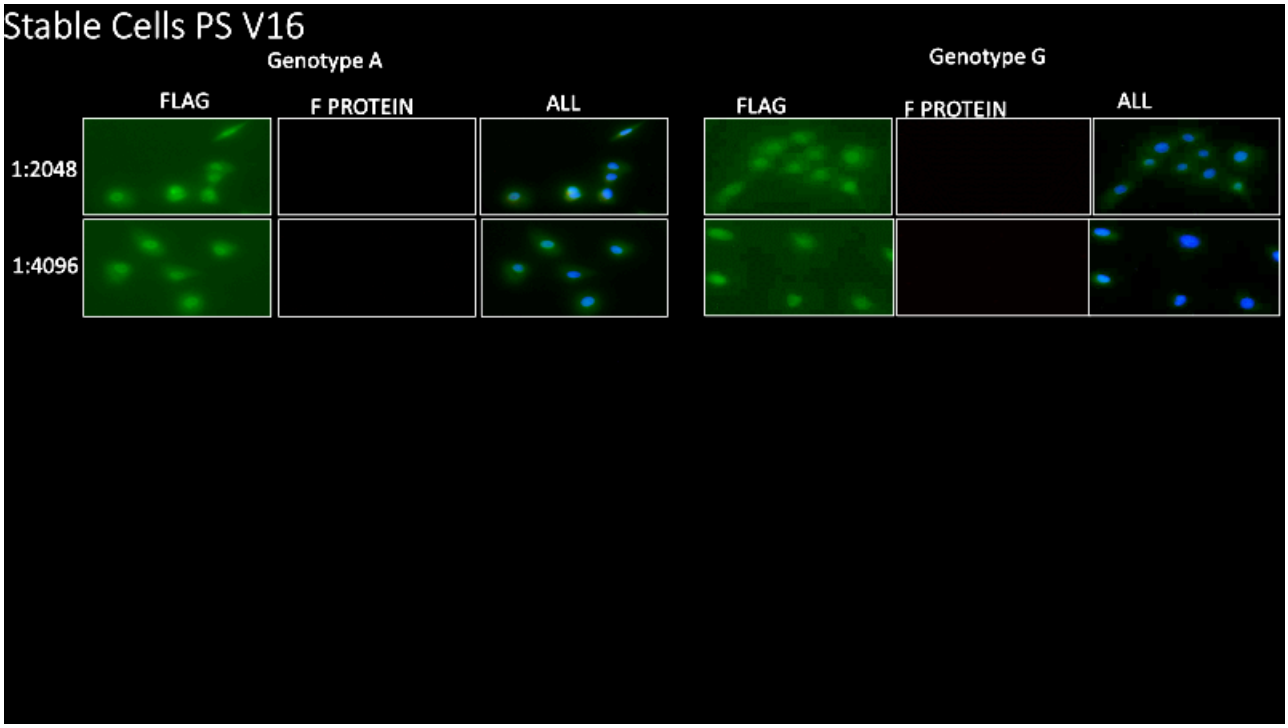


Figure A-60d. Stable cells Vaccine Sample#16.

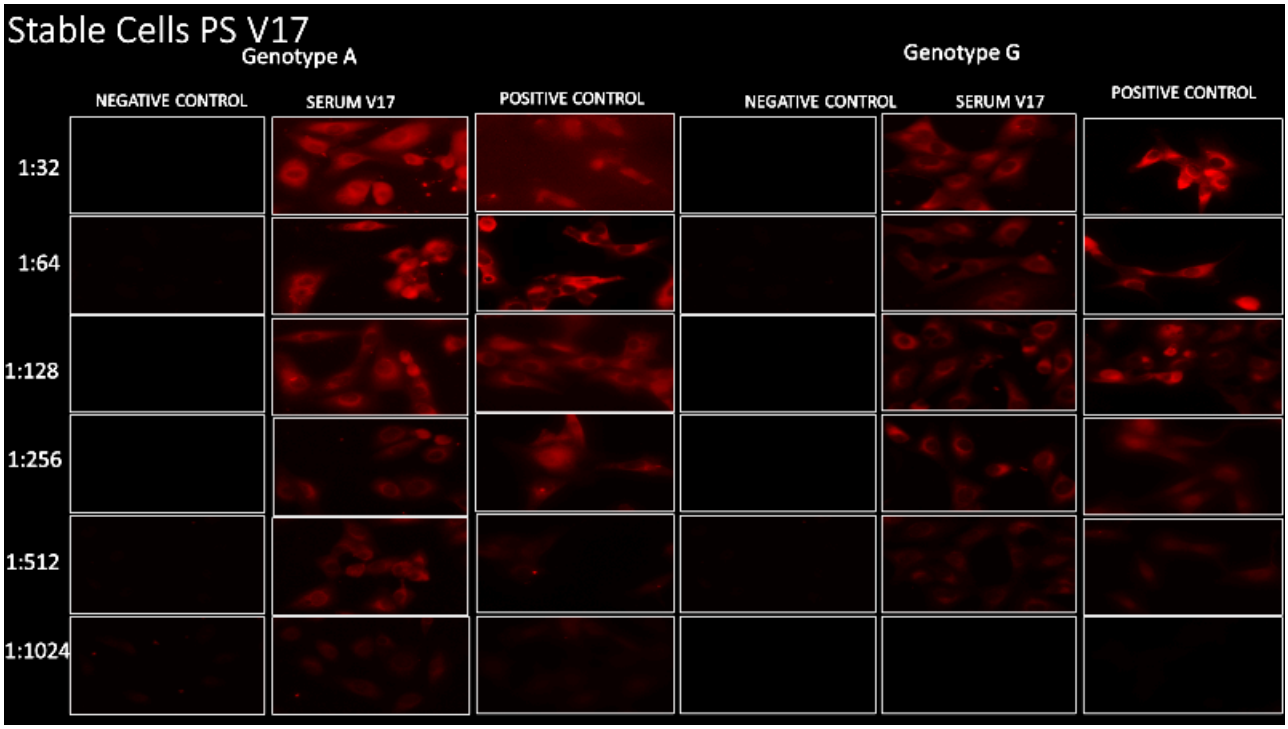


Figure A-61a. Stable cells Vaccine Sample#17.

Figure A-61b. Stable cells Vaccine Sample#17.

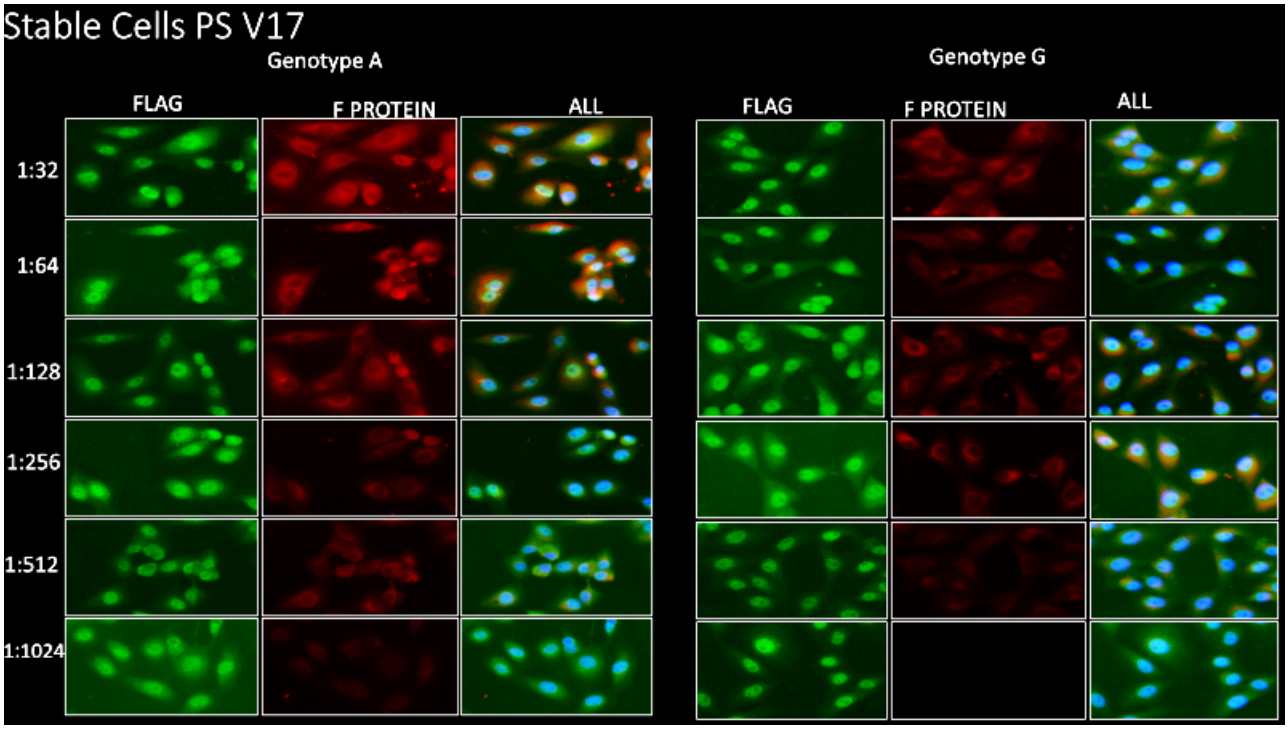


Figure A-61c. Stable cells Vaccine Sample#17.

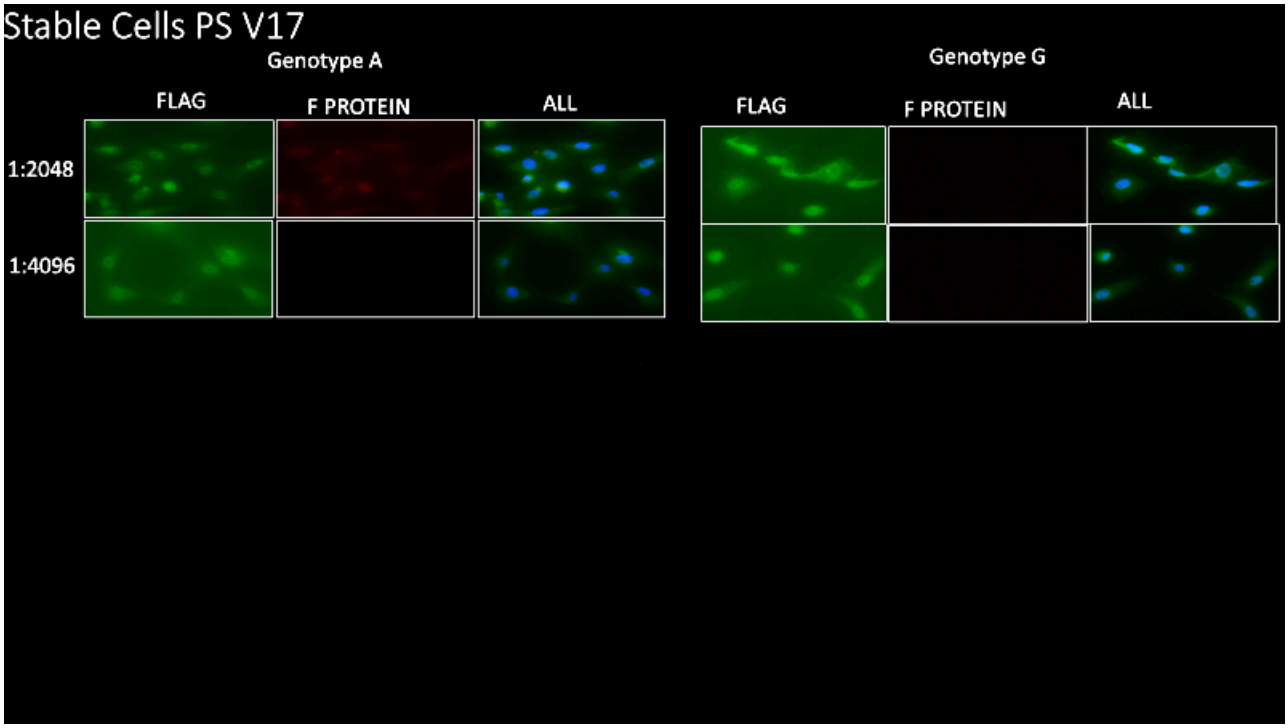


Figure A-61d. Stable cells Vaccine Sample#17.

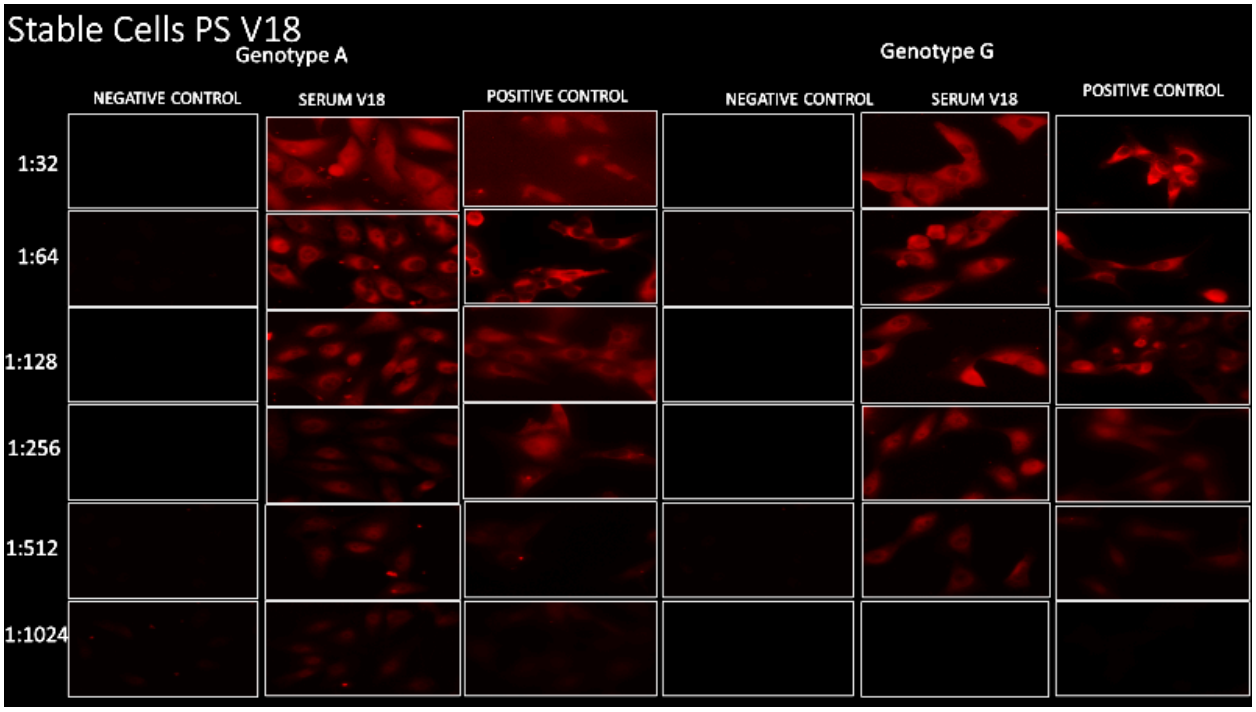


Figure A-62a. Stable cells Vaccine Sample#18.

Figure A-62b. Stable cells Vaccine Sample#18.

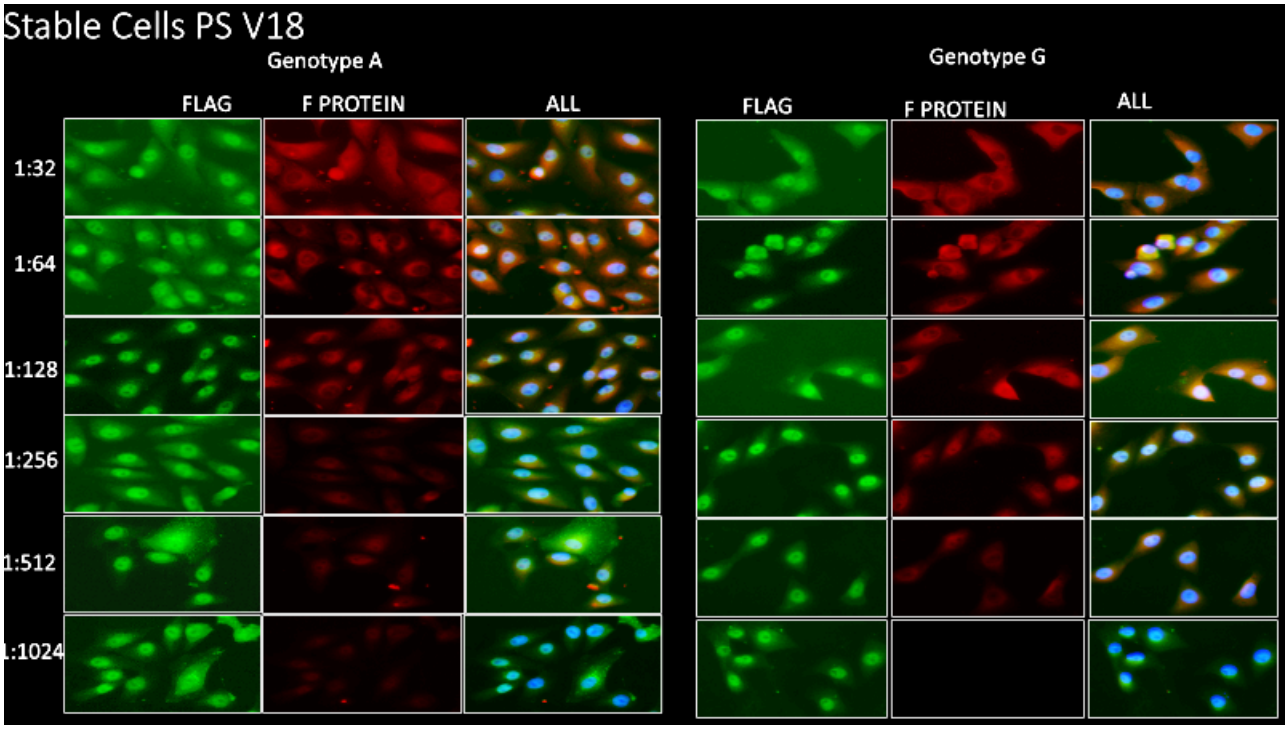


Figure A-62c. Stable cells Vaccine Sample#18.

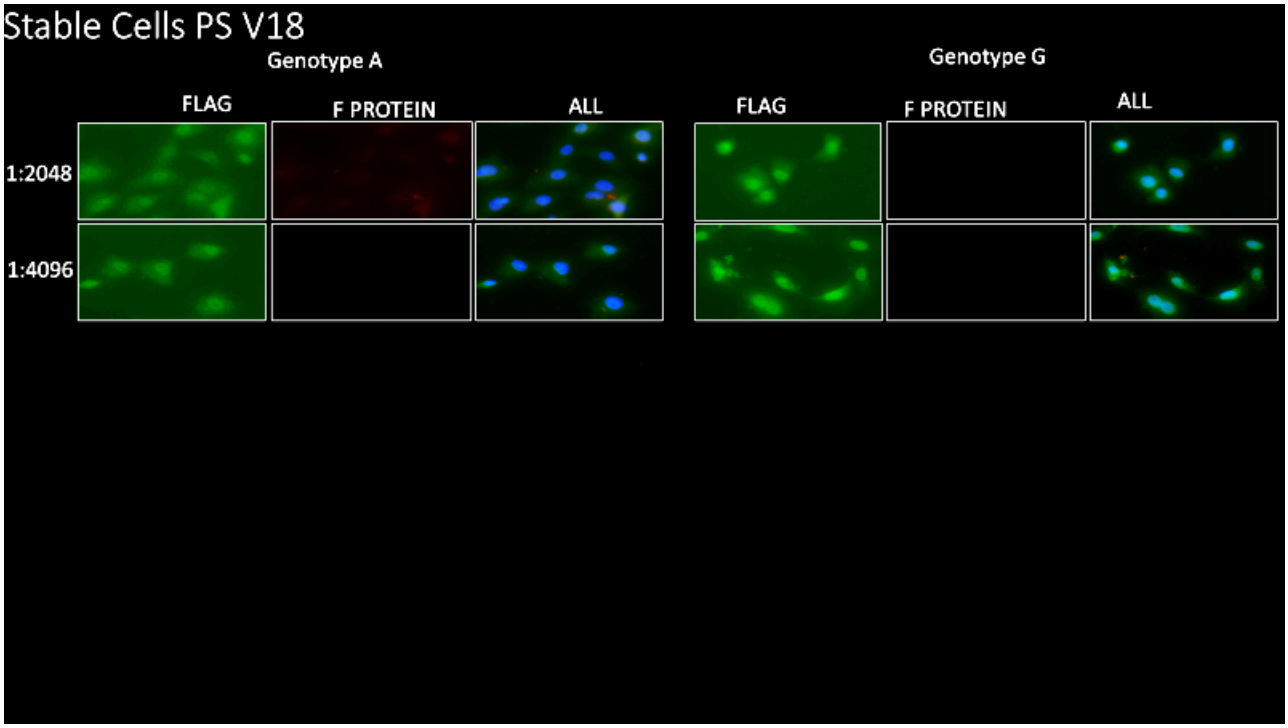


Figure A-62d. Stable cells Vaccine Sample#18.

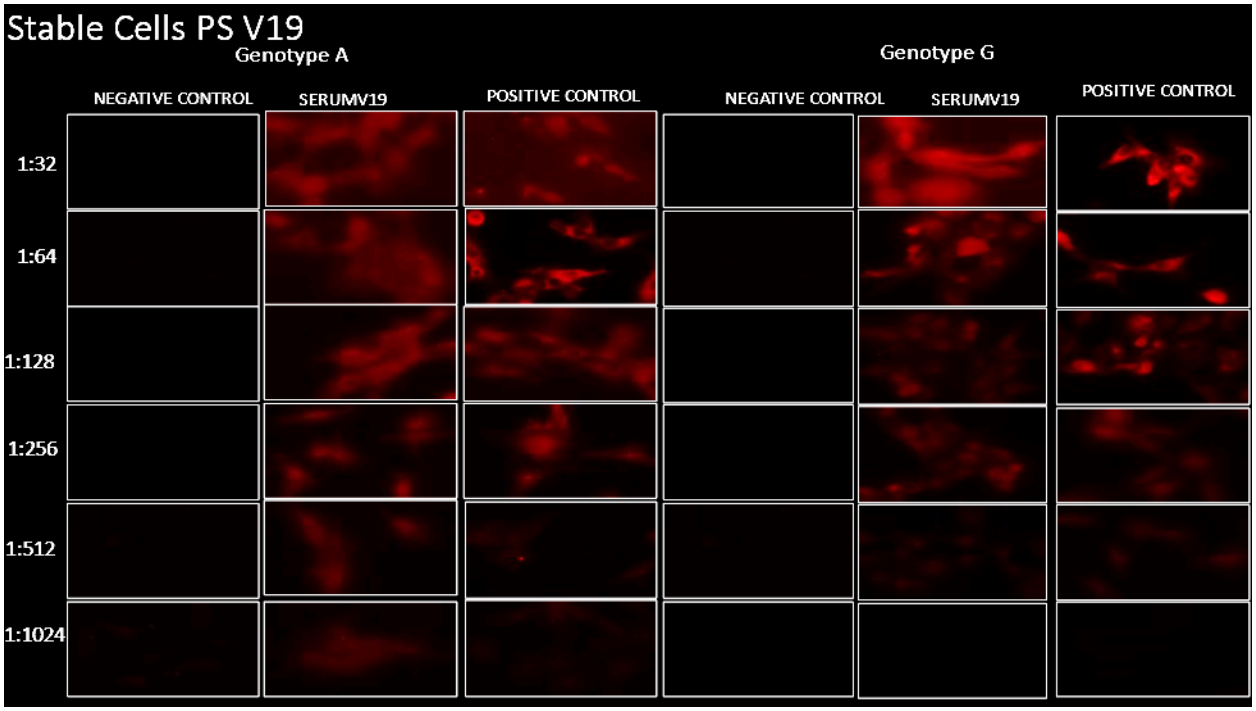


Figure A-63a. Stable cells Vaccine Sample#19.

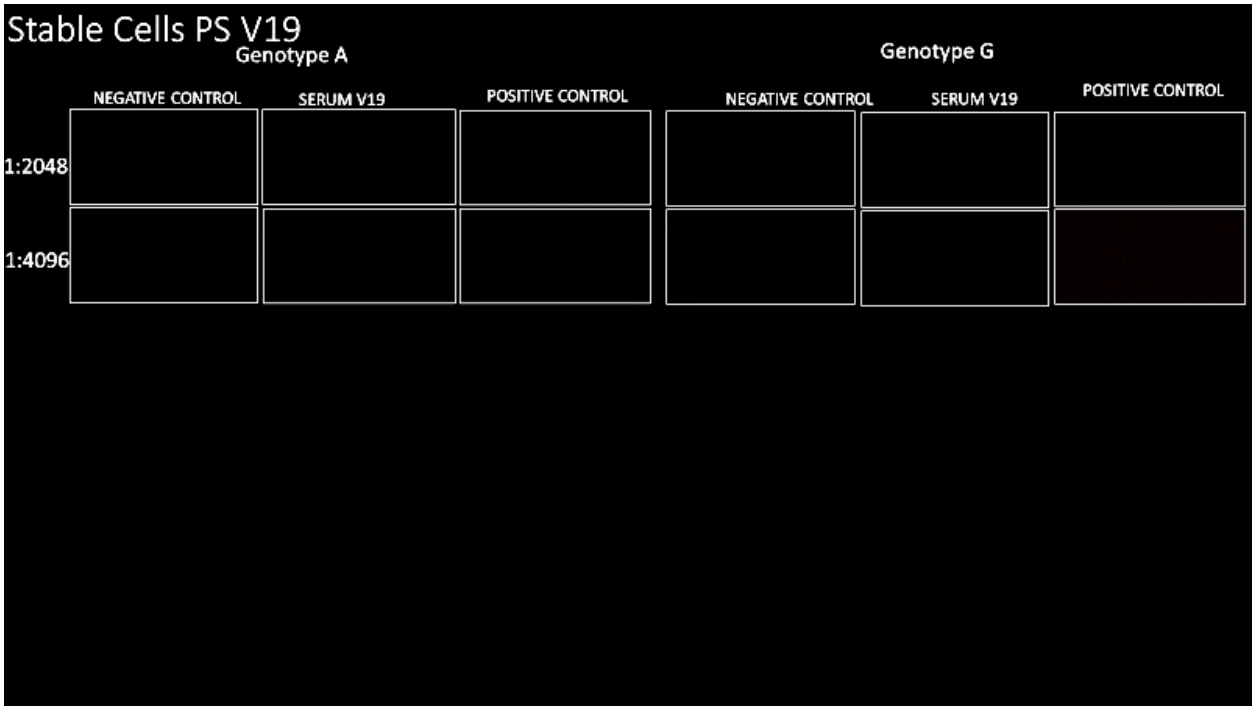


Figure A-63b. Stable cells Vaccine Sample#19.

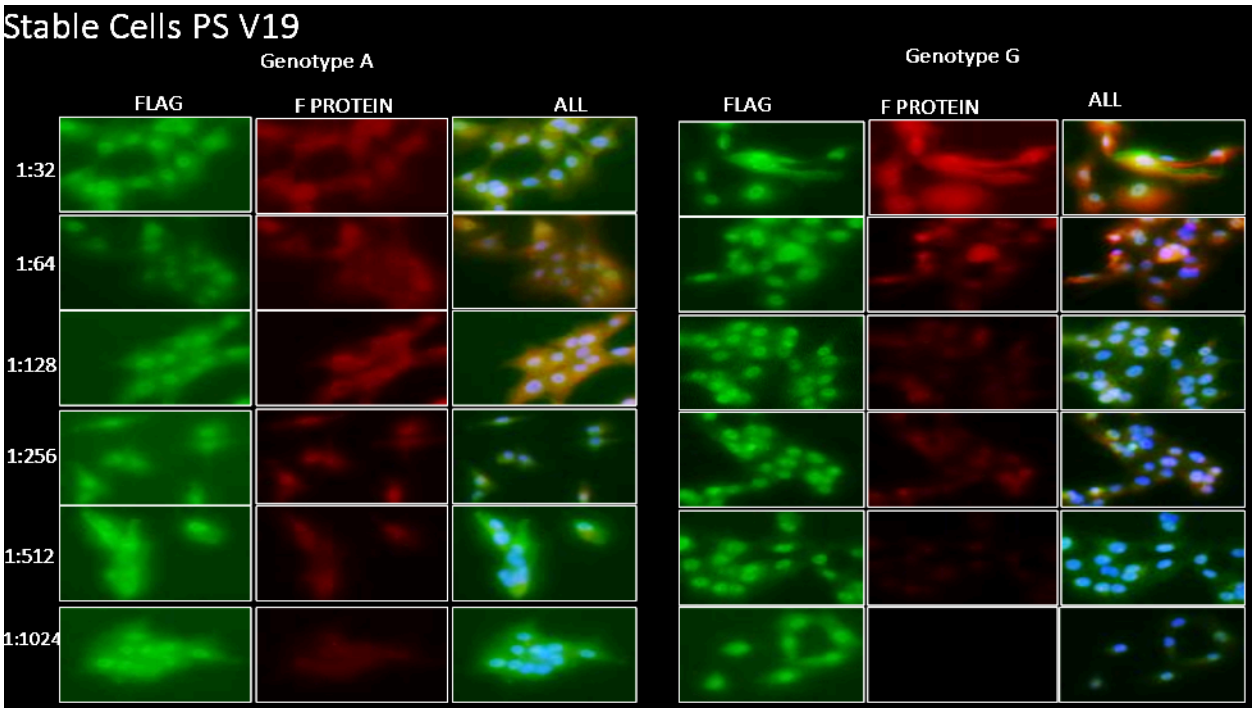


Figure A-63c. Stable cells Vaccine Sample#19.

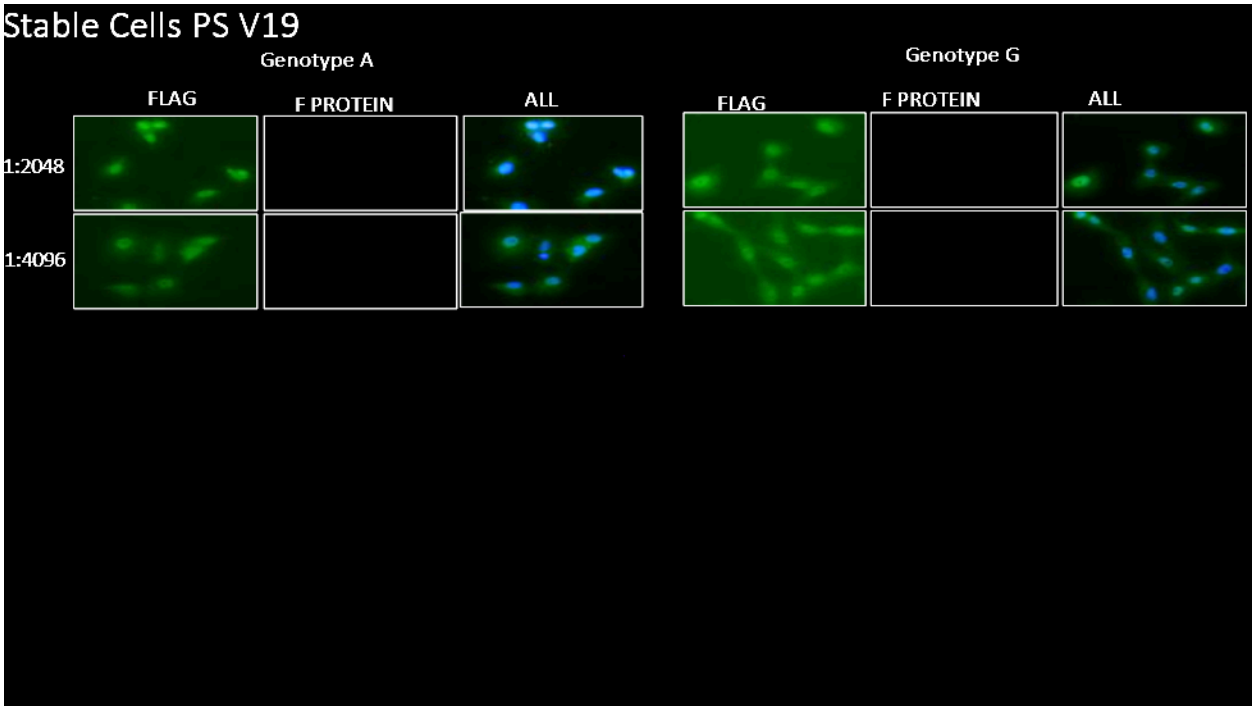


Figure A-63d. Stable cells Vaccine Sample#19.

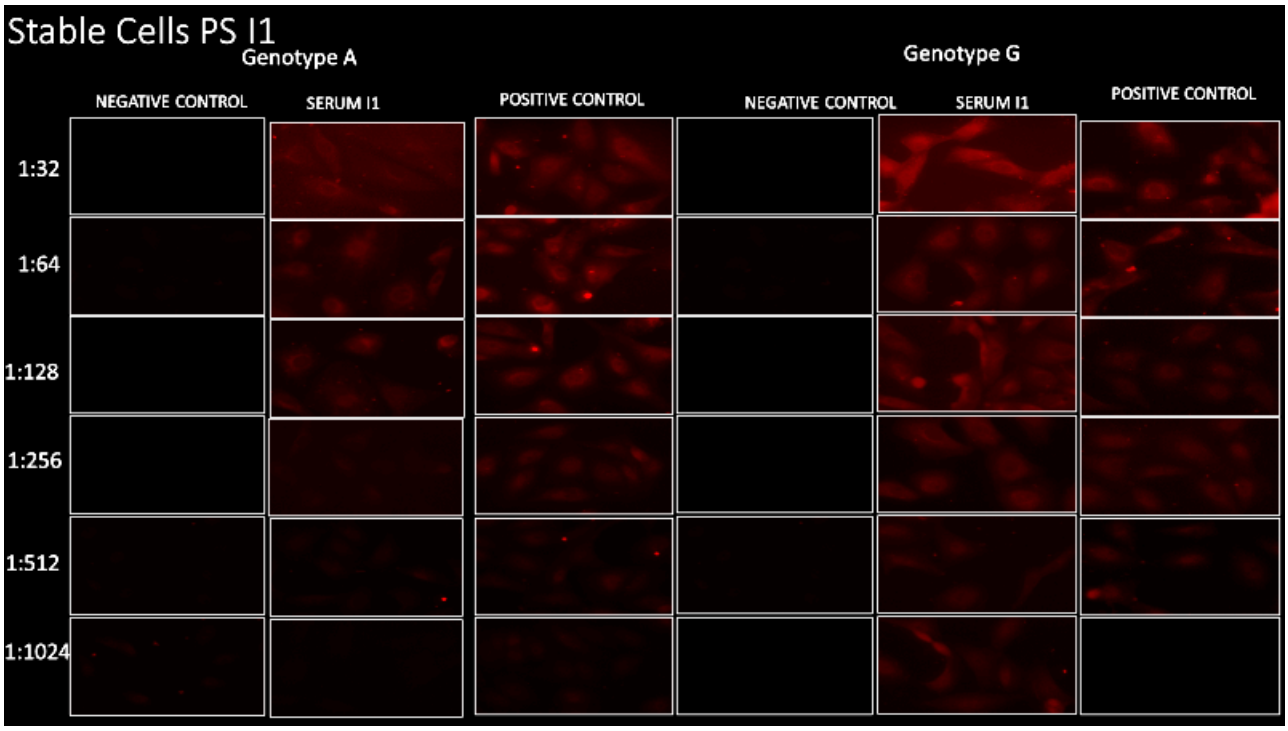


Figure A-64a. Stable cells Infected Sample#1.

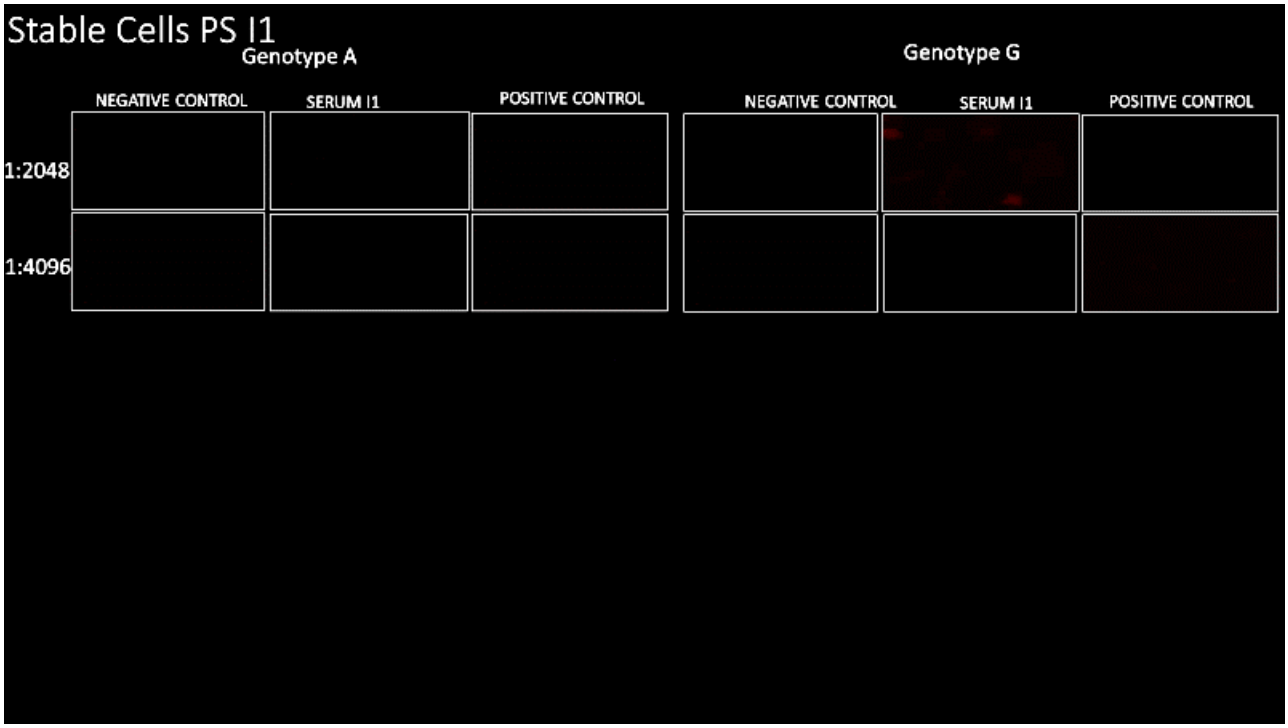


Figure A-64b. Stable cells Infected Sample#1.

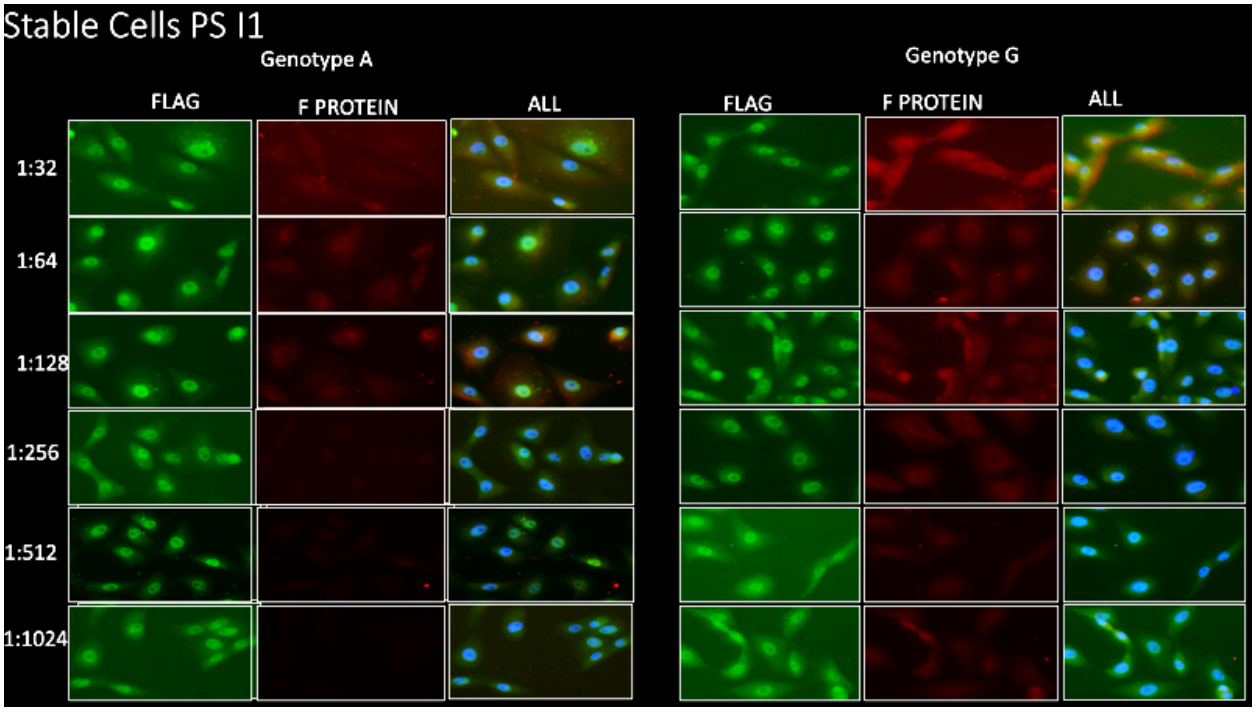


Figure A-64c. Stable cells Infected Sample#1.

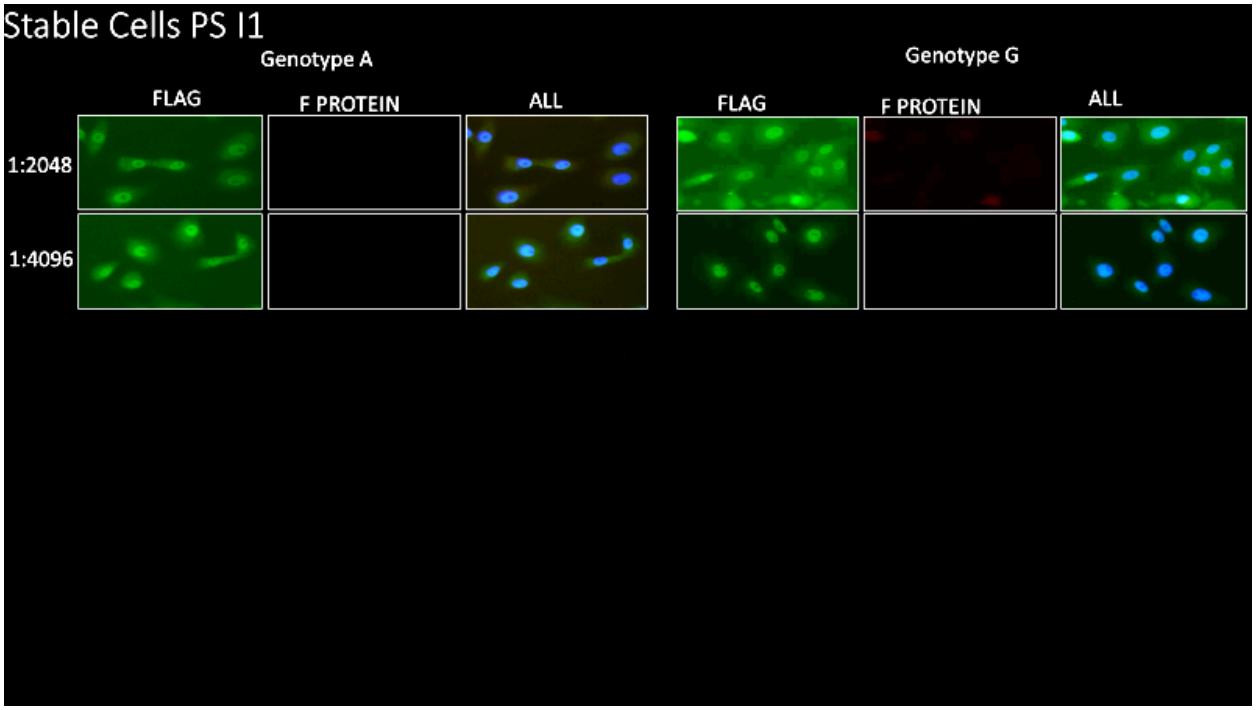


Figure A-64d. Stable cells Infected Sample#1.

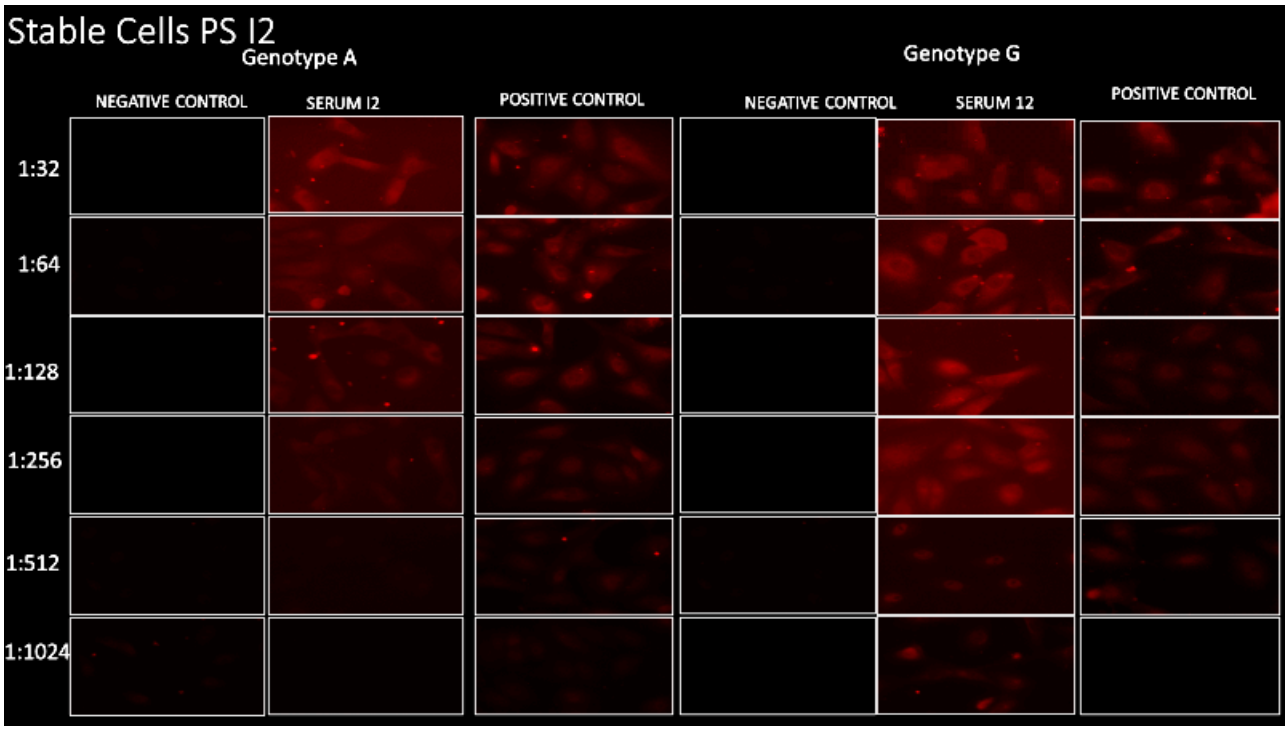


Figure A-65a. Stable cells Infected Sample#2.

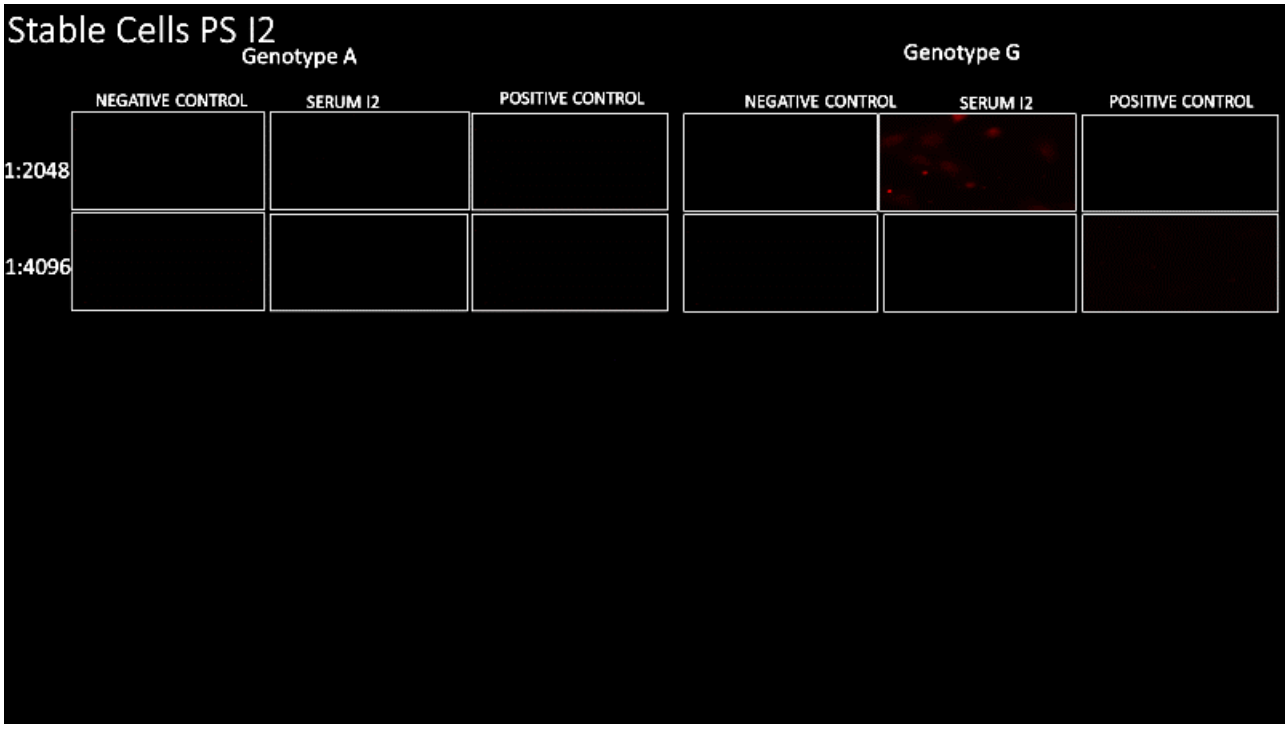


Figure A-65b. Stable cells Infected Sample#2.

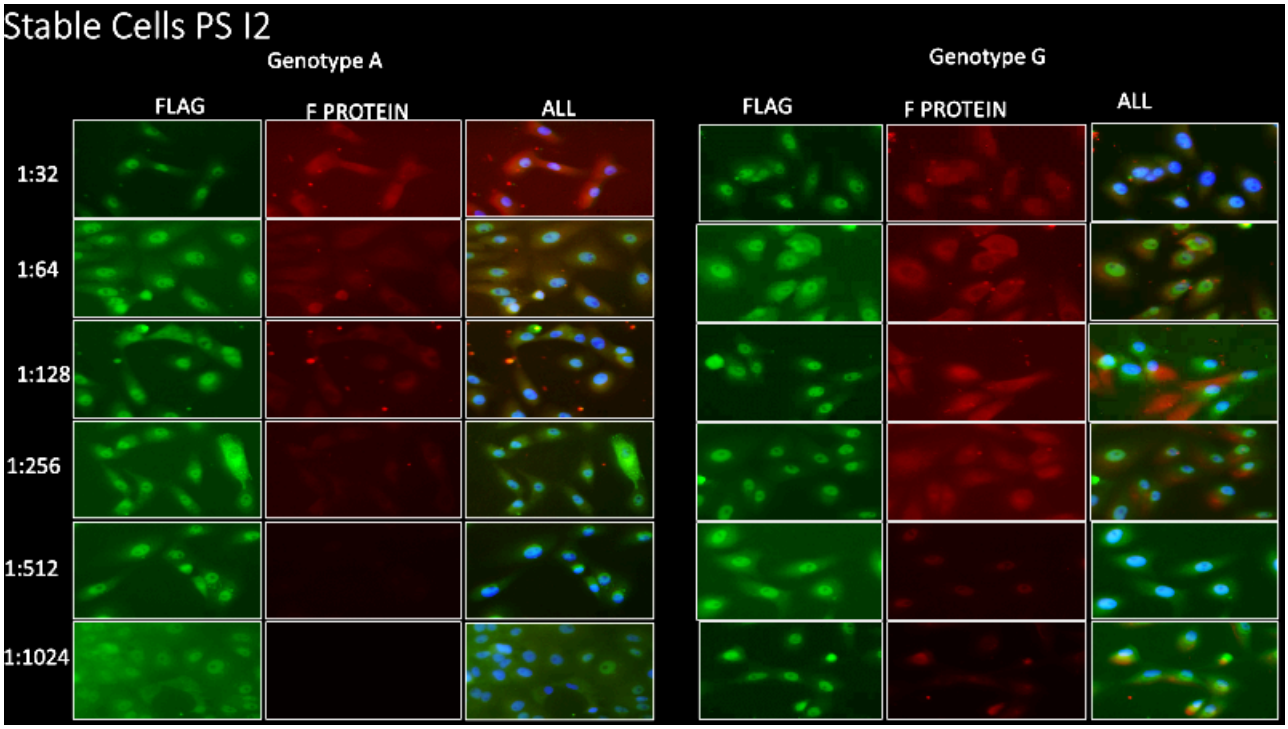


Figure A-65c. Stable cells Infected Sample#2.

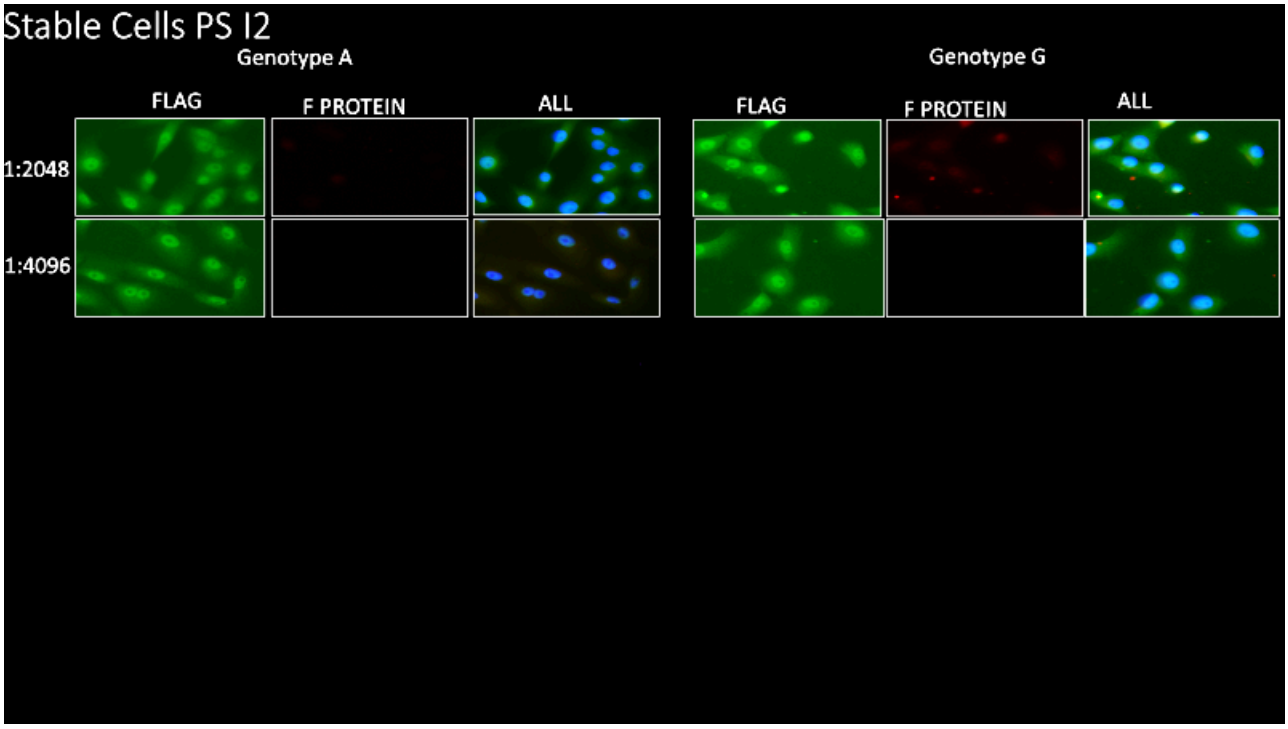


Figure A-65d. Stable cells Infected Sample#2.

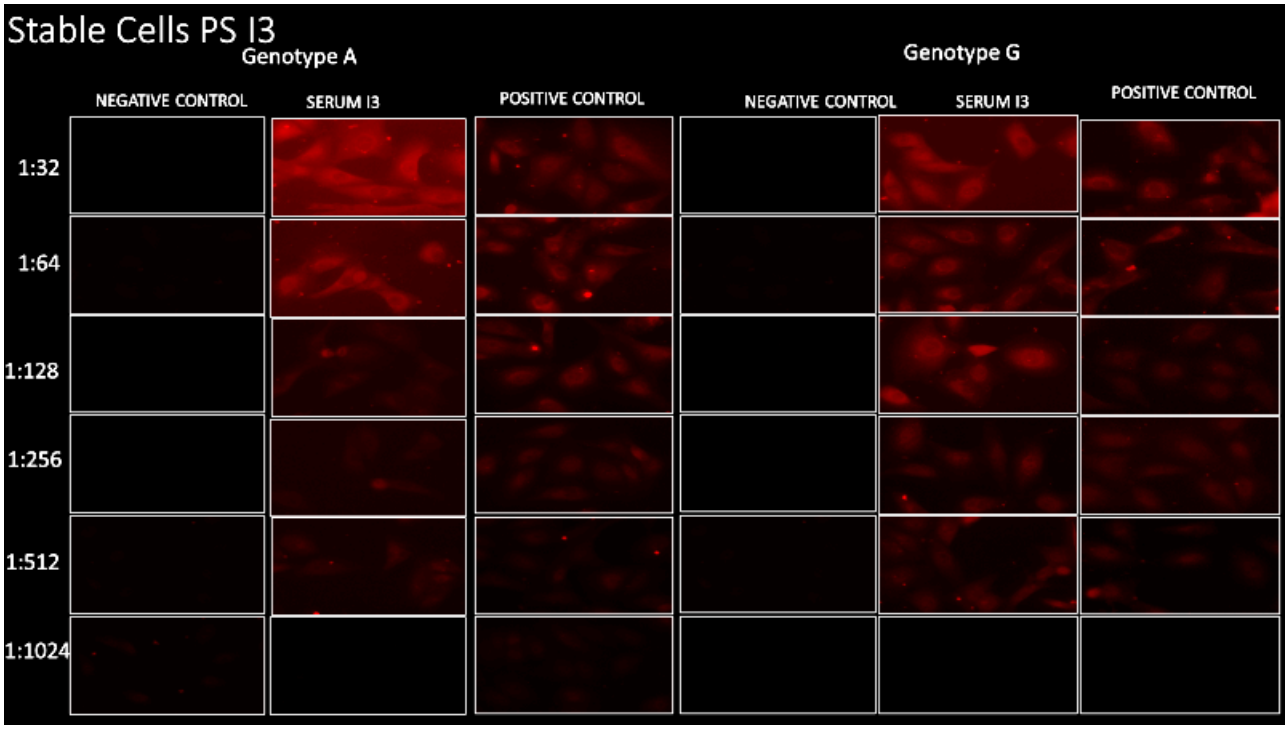


Figure A-66a. Stable cells Infected Sample#3.

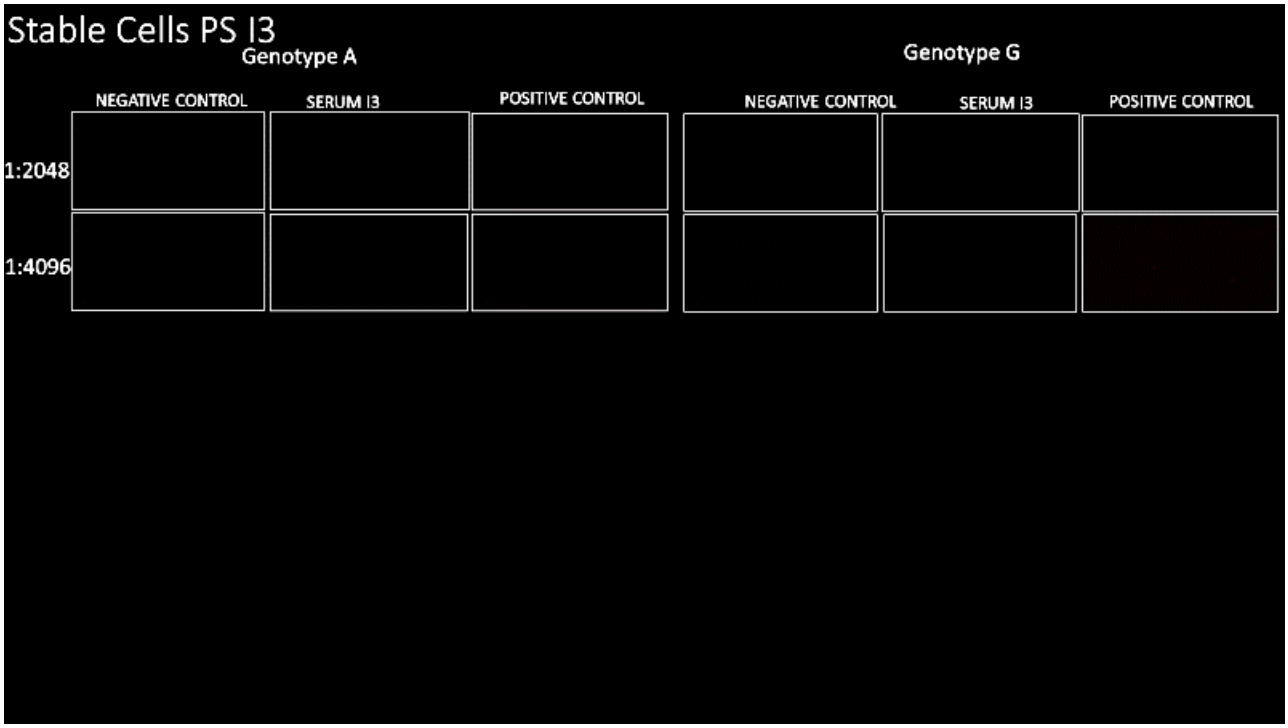


Figure A-66b. Stable cells Infected Sample#3.

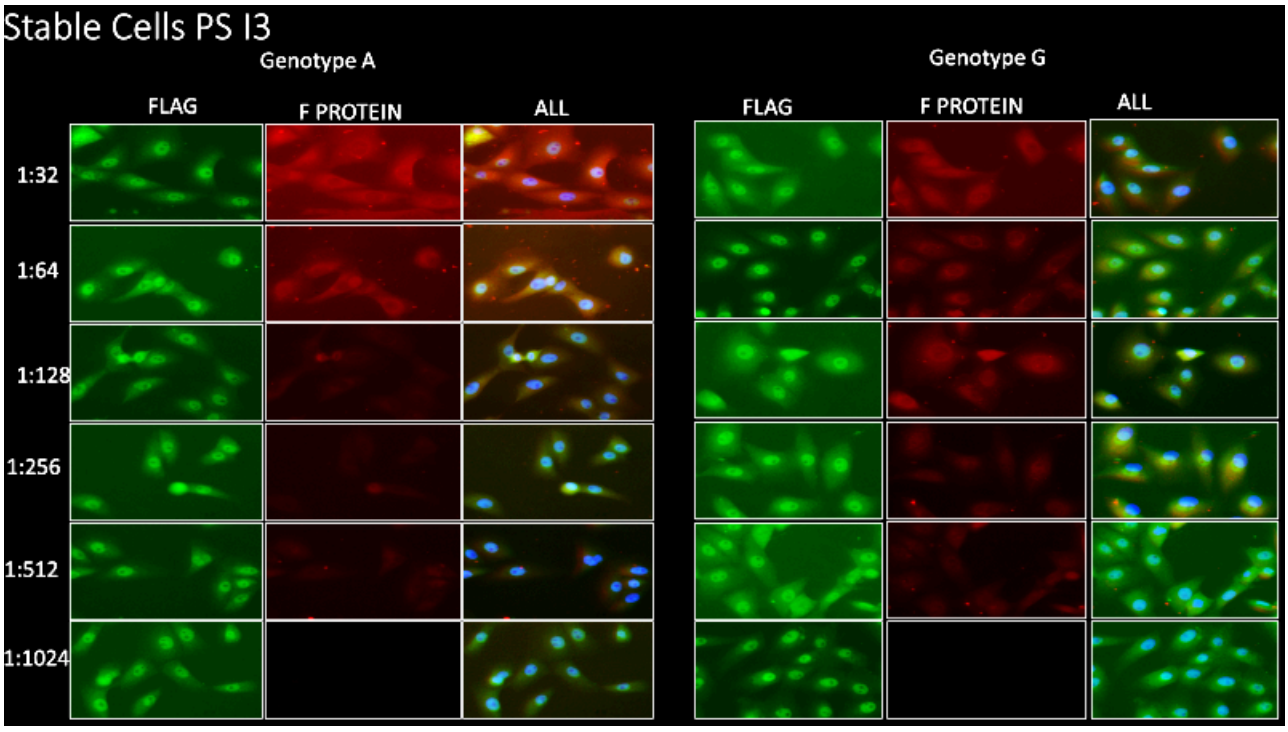


Figure A-66c. Stable cells Infected Sample#3.

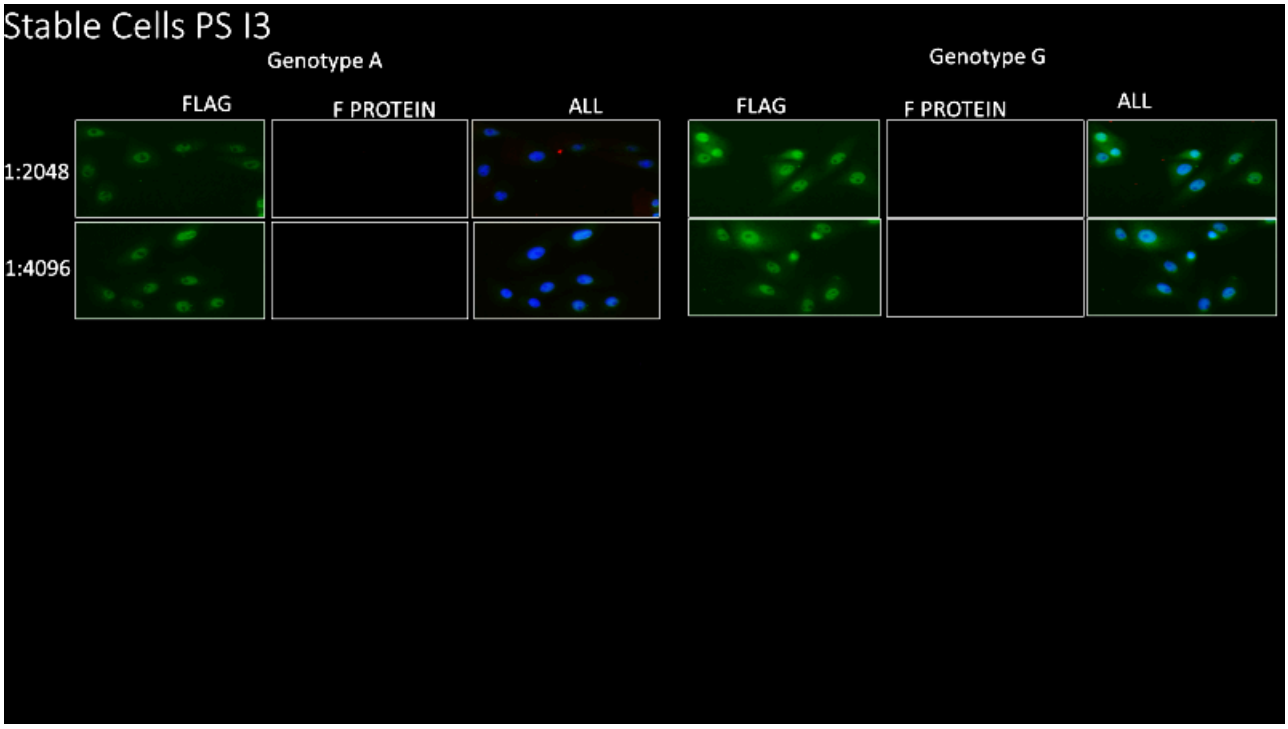


Figure A-66d. Stable cells Infected Sample#3.

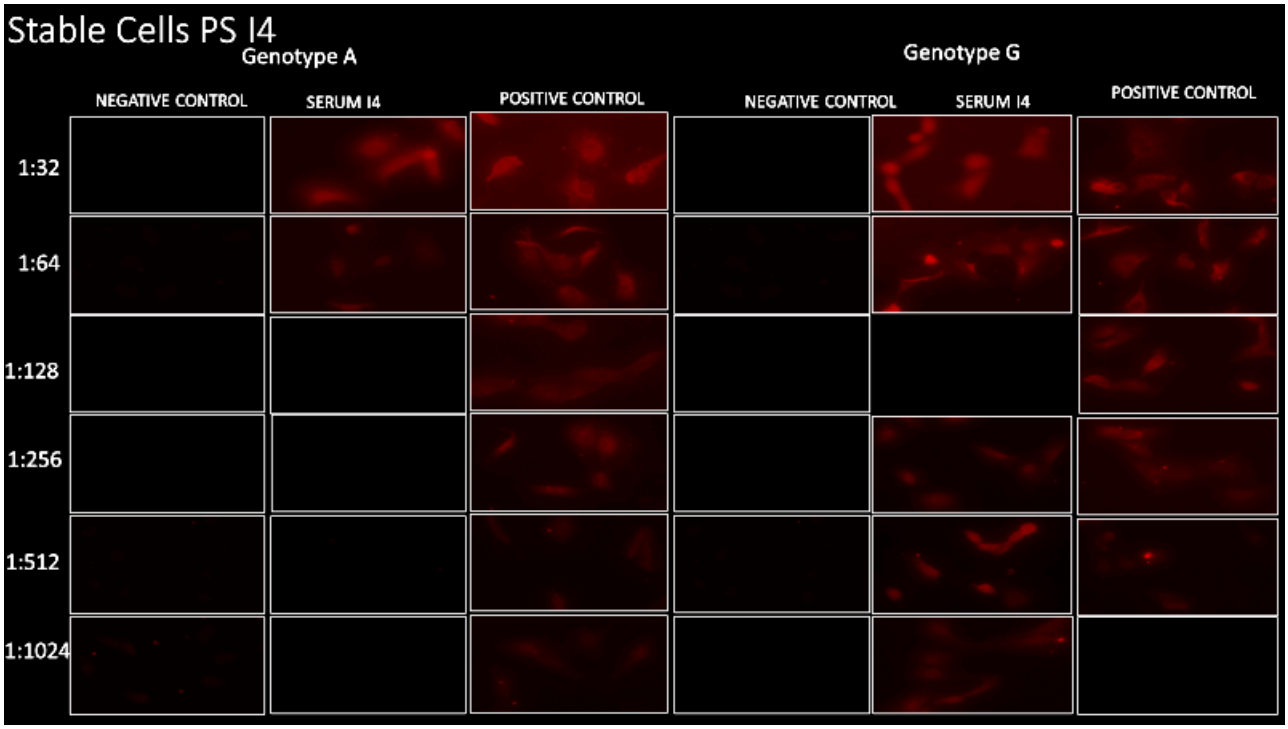


Figure A-67a. Stable cells Infected Sample#4.

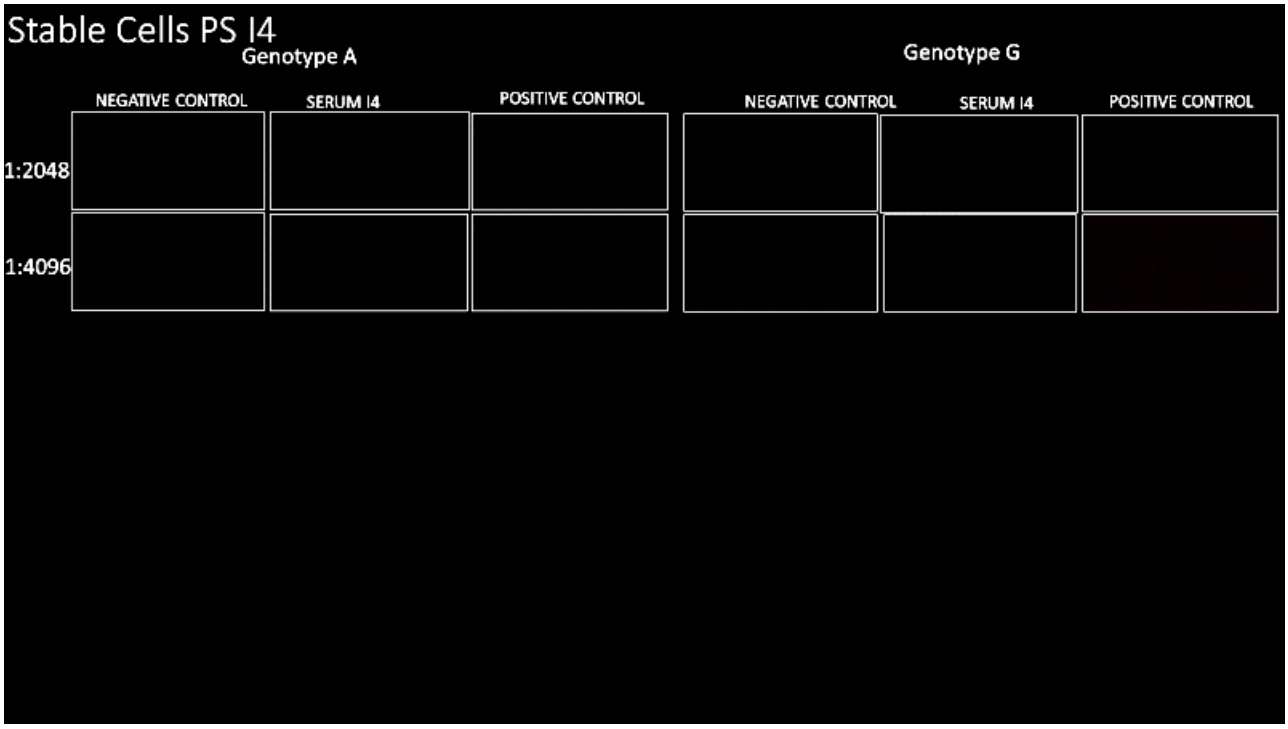


Figure A-67b. Stable cells Infected Sample#4.

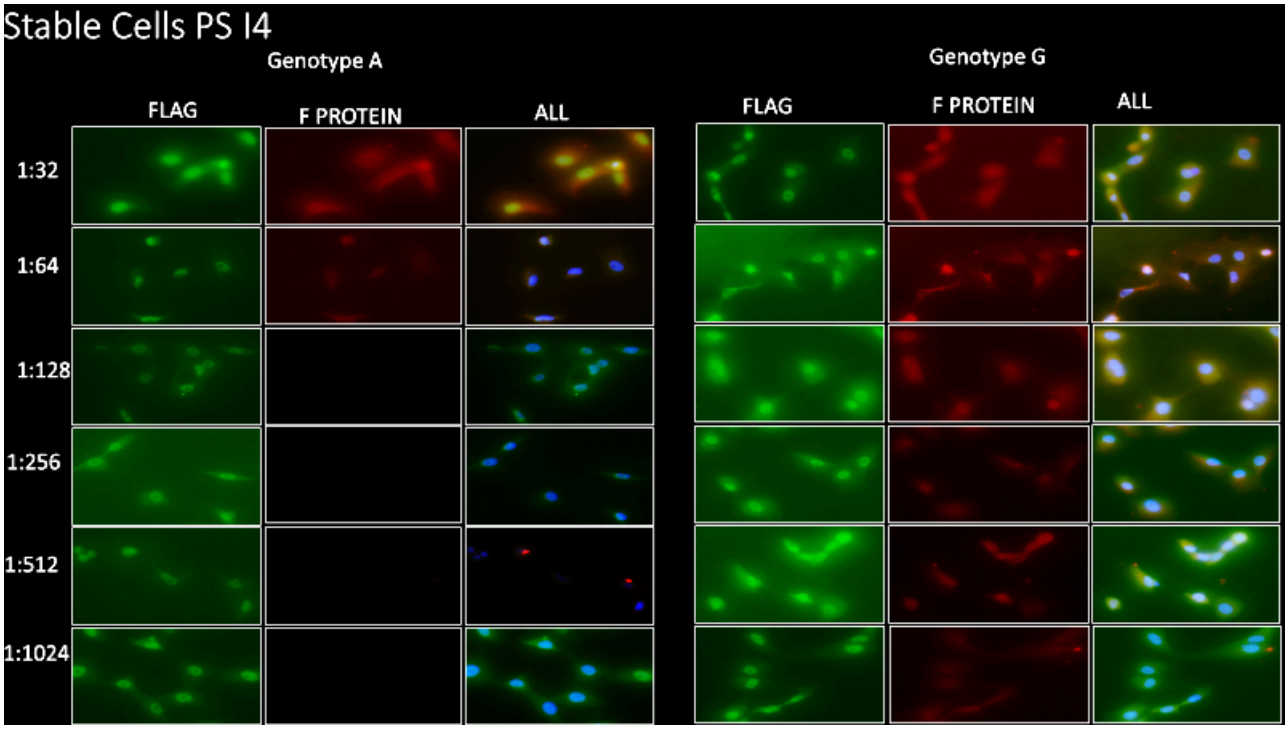


Figure A-67c. Stable cells Infected Sample#4.

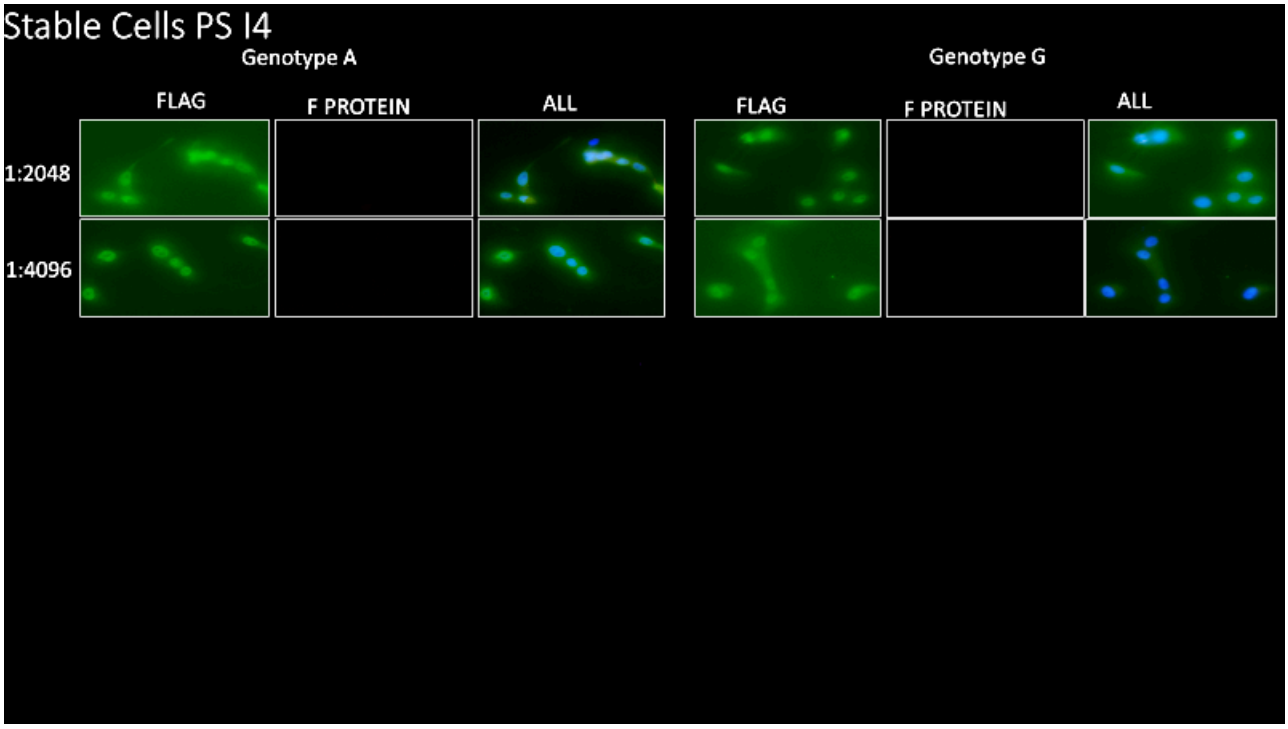


Figure A-67d. Stable cells Infected Sample#4.

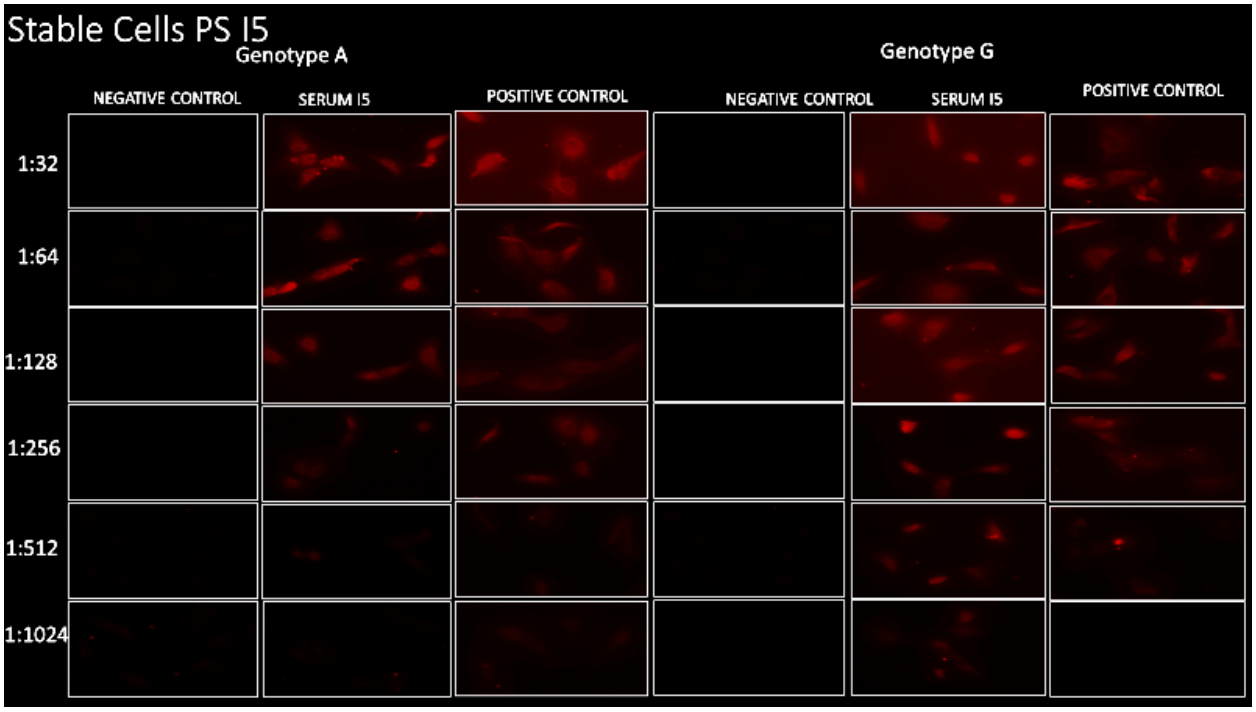


Figure A-68a. Stable cells Infected Sample#5.

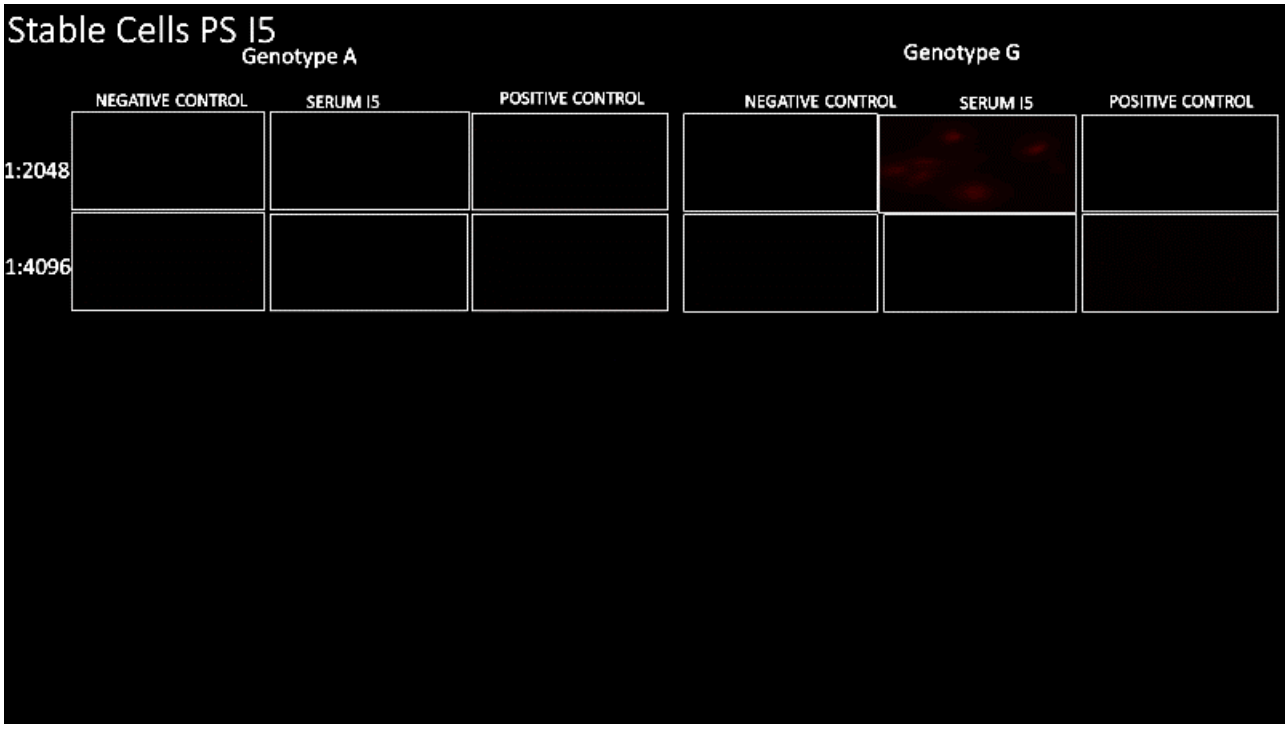


Figure A-68b. Stable cells Infected Sample#5.

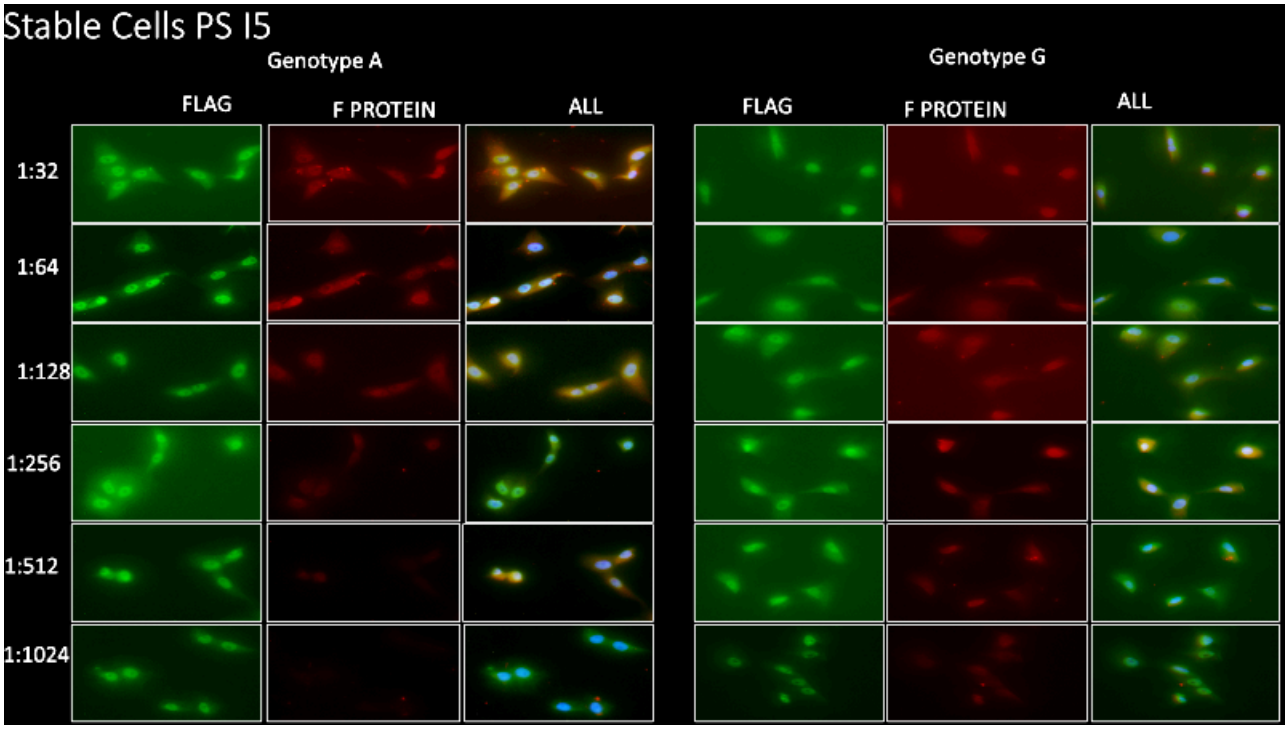


Figure A-68c. Stable cells Infected Sample#5.

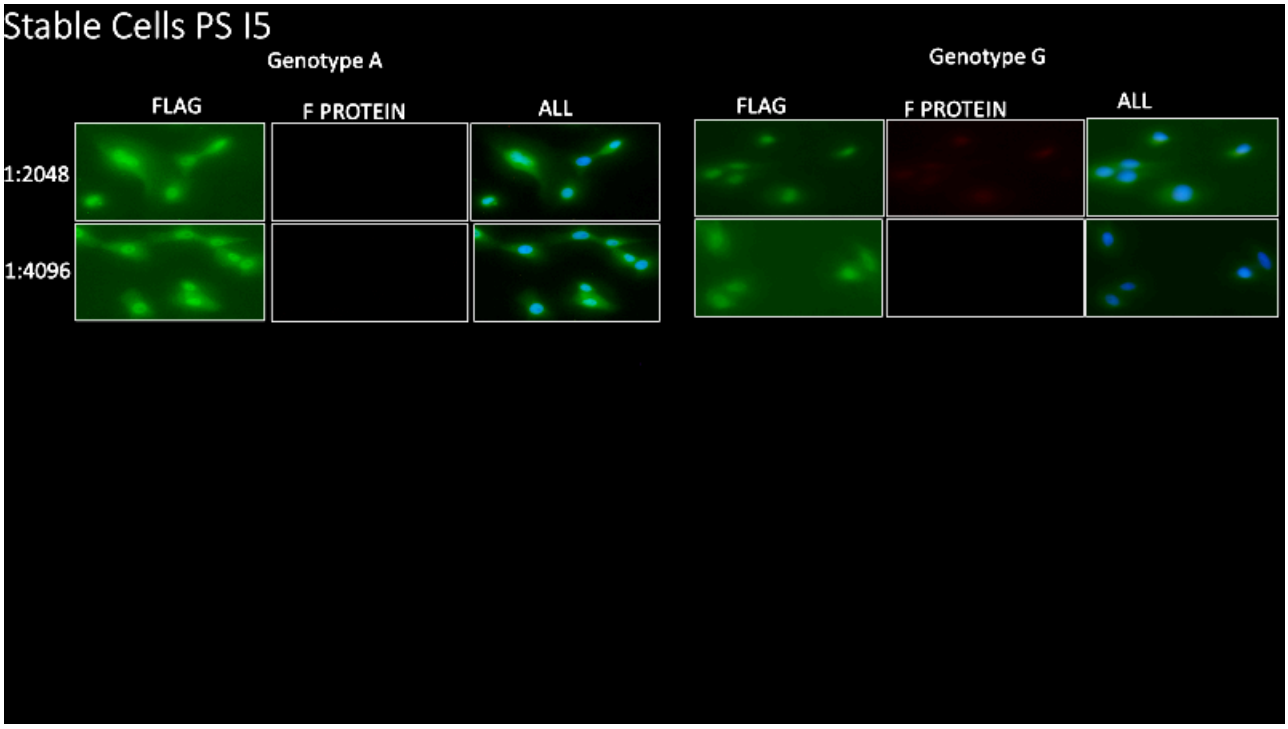


Figure A-68d. Stable cells Infected Sample#5.

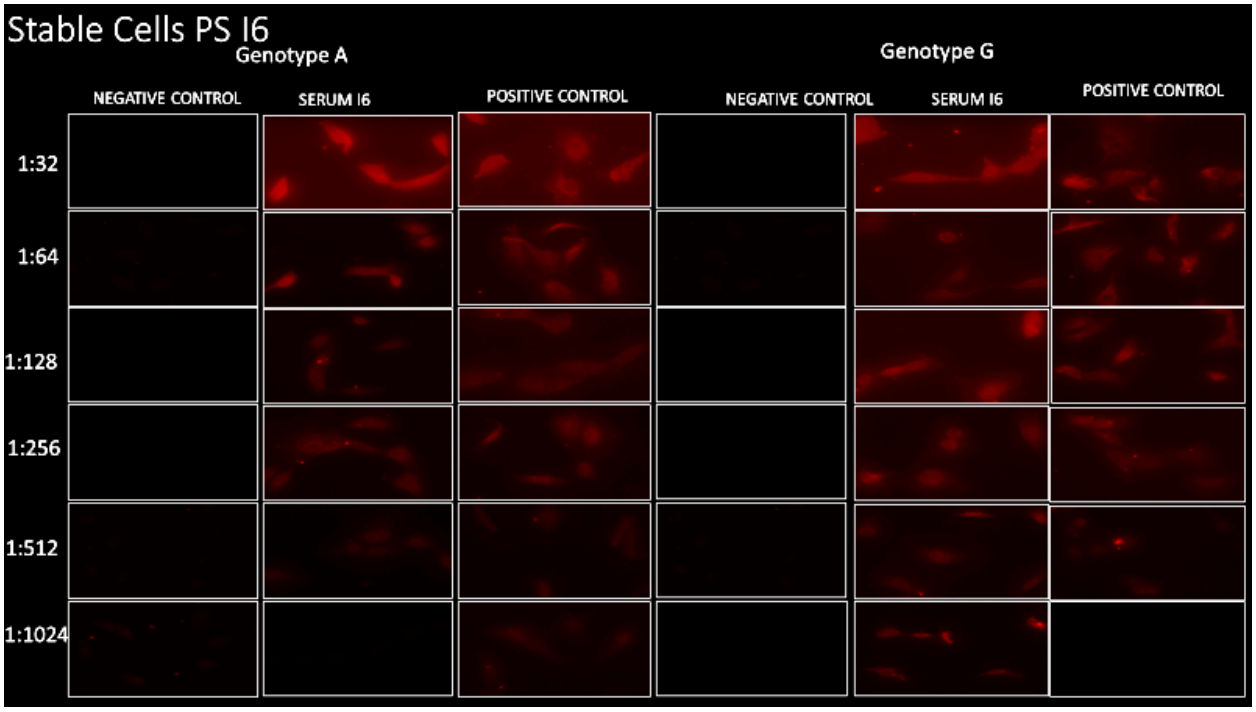


Figure A-69a. Stable cells Infected Sample#6.

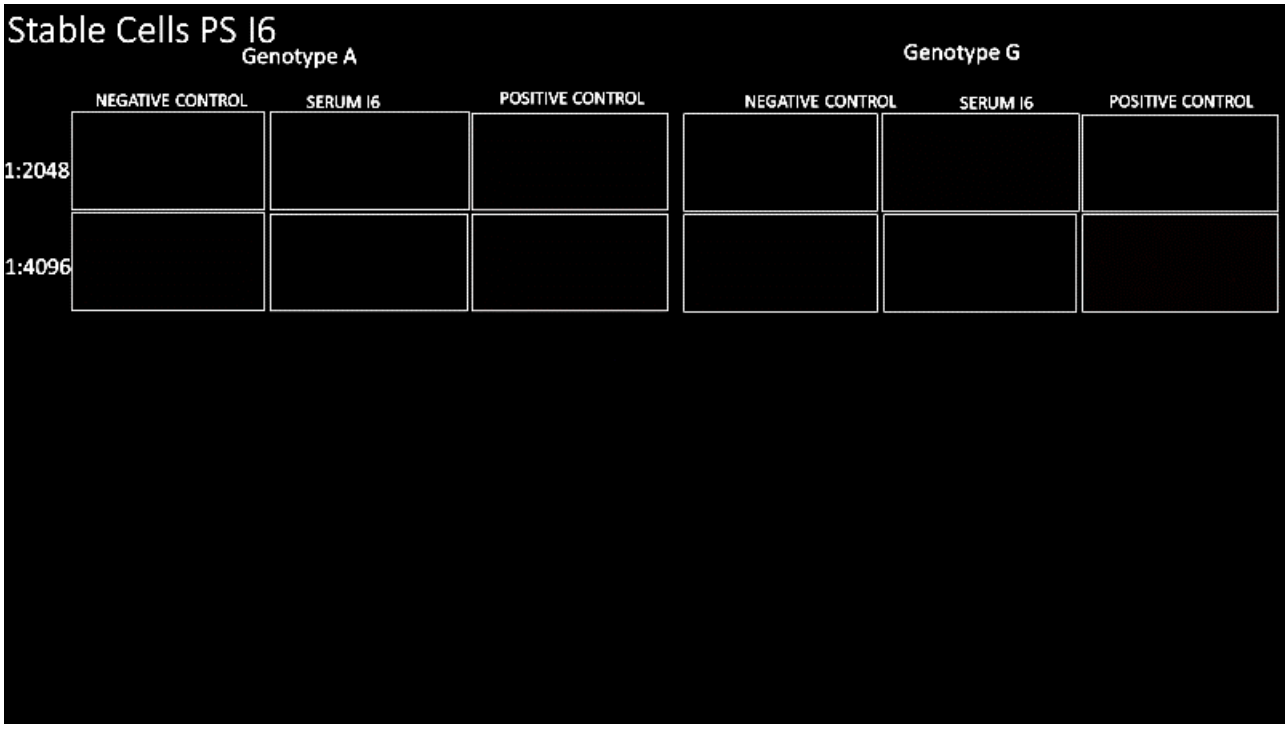


Figure A-69b. Stable cells Infected Sample#6.

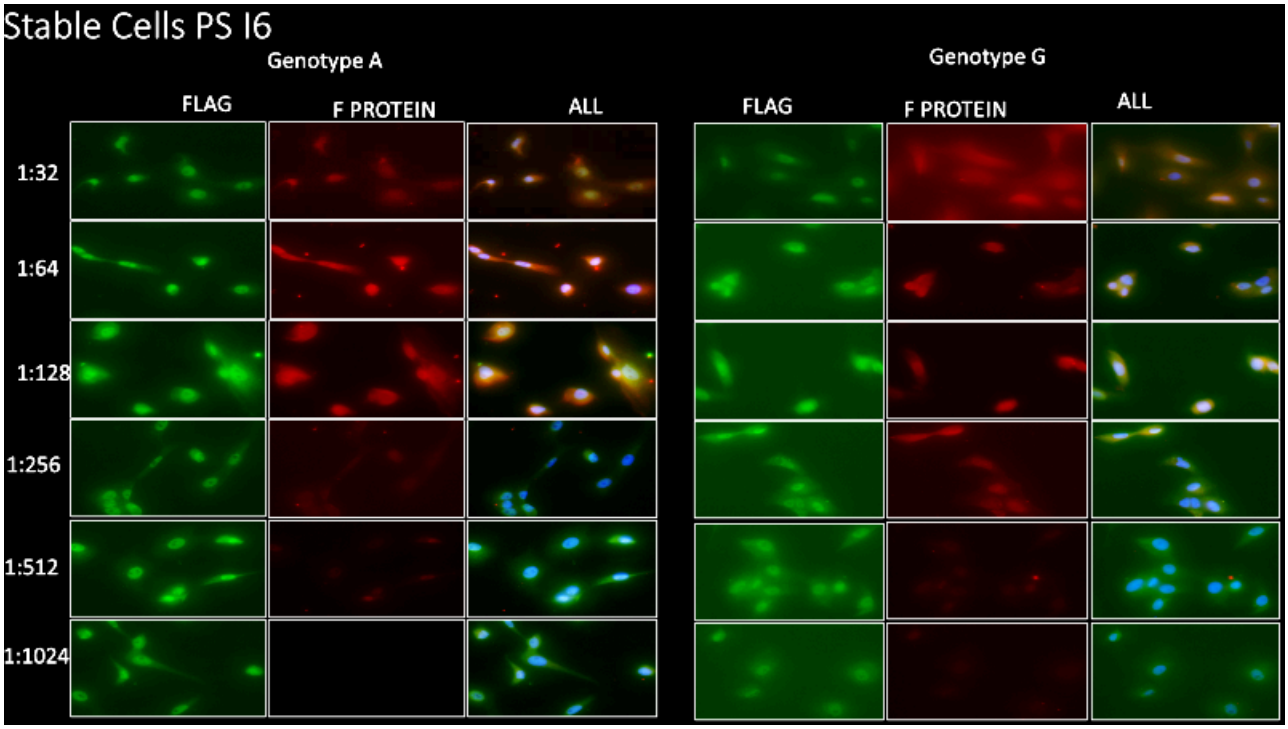


Figure A-69c. Stable cells Infected Sample#6.

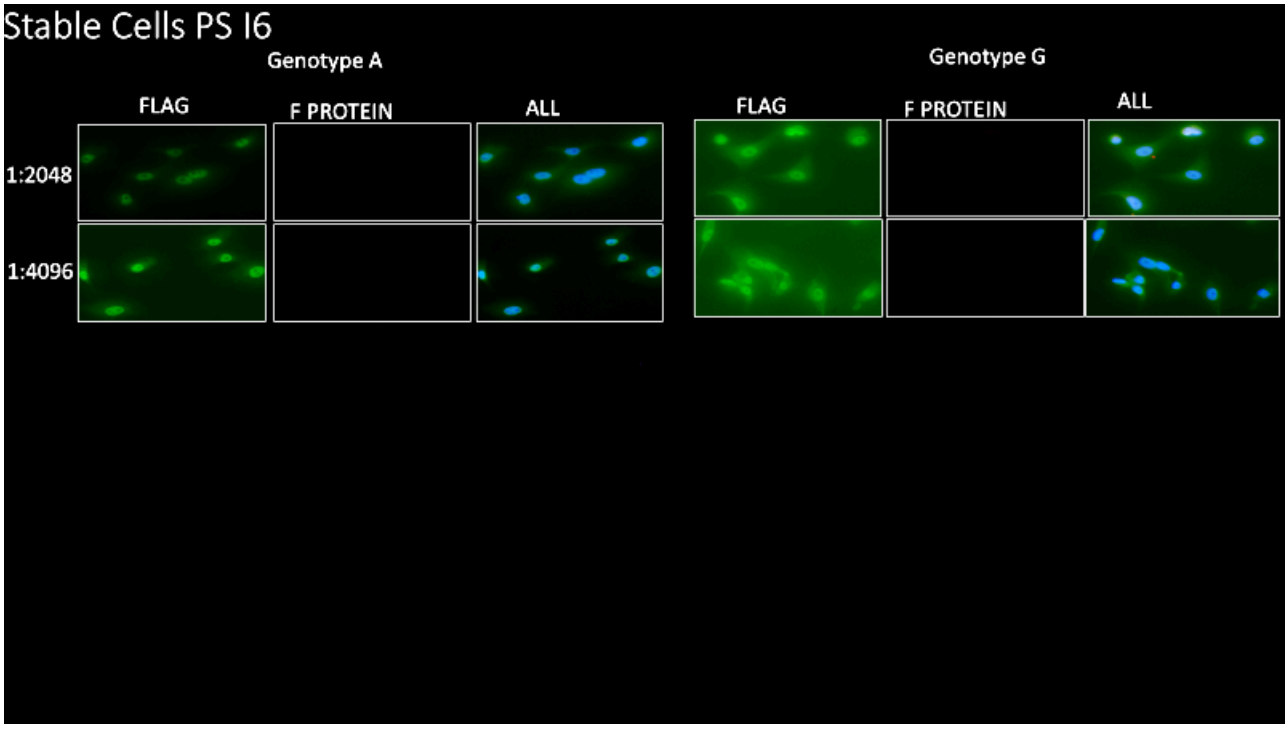


Figure A-69d. Stable cells Infected Sample#6.

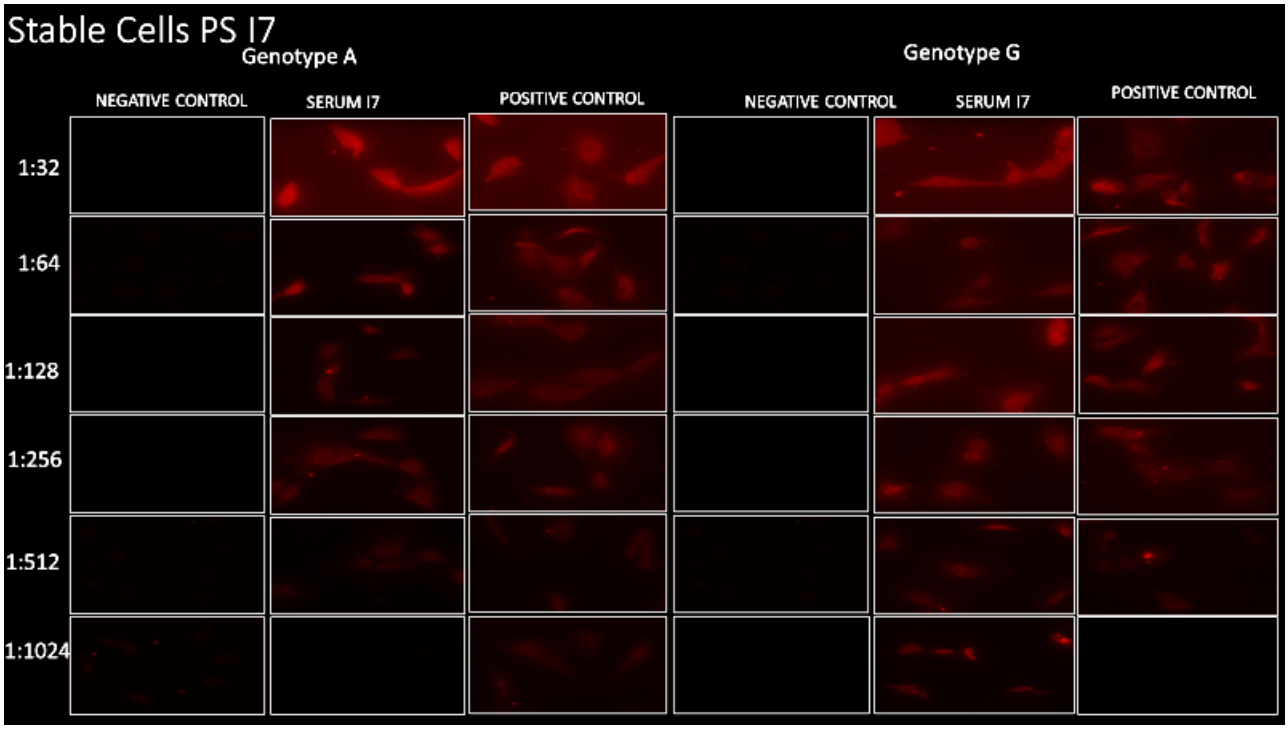


Figure A-70a. Stable cells Infected Sample#7.

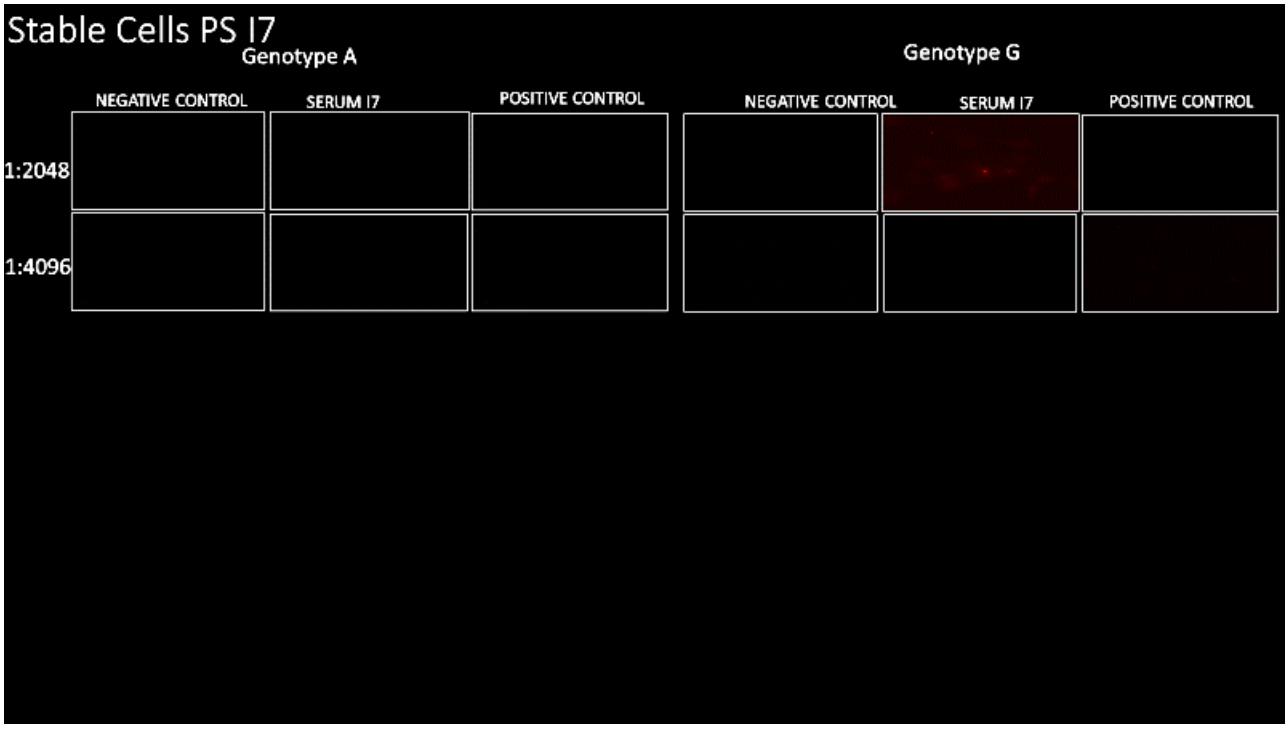


Figure A-70b. Stable cells Infected Sample#7.

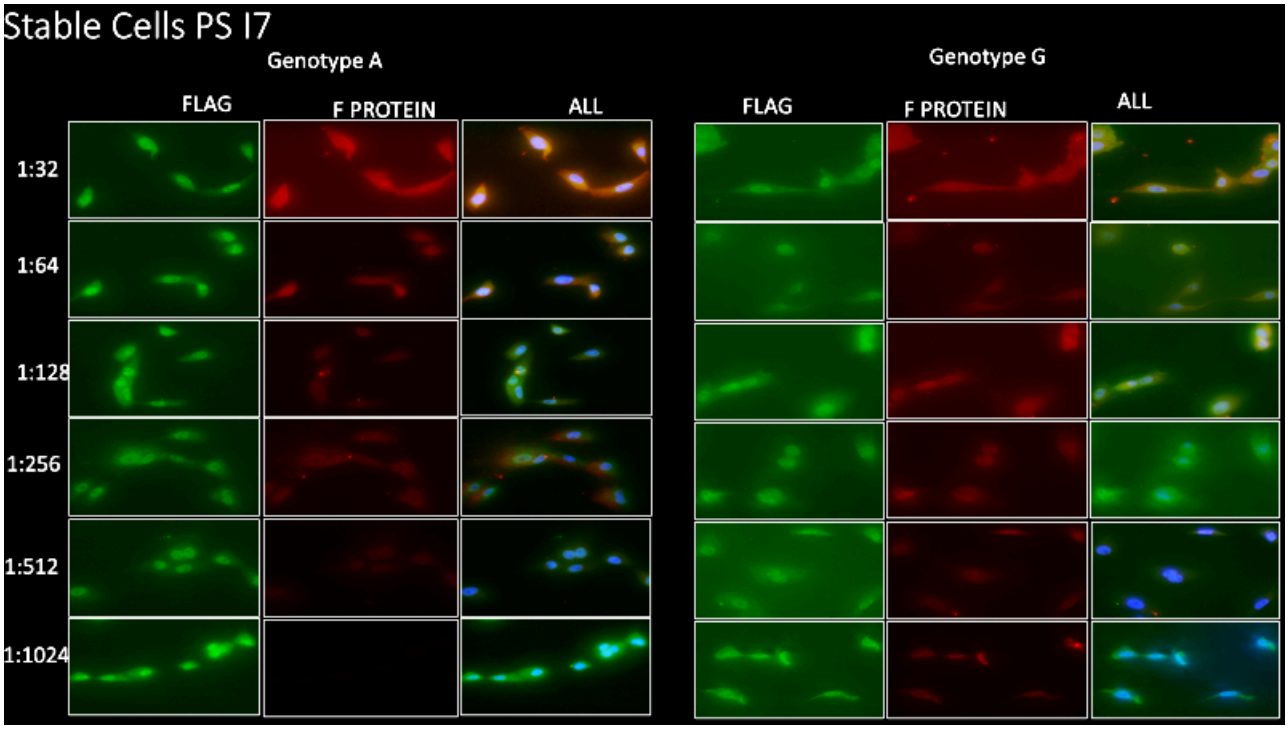


Figure A-70c. Stable cells Infected Sample#7.

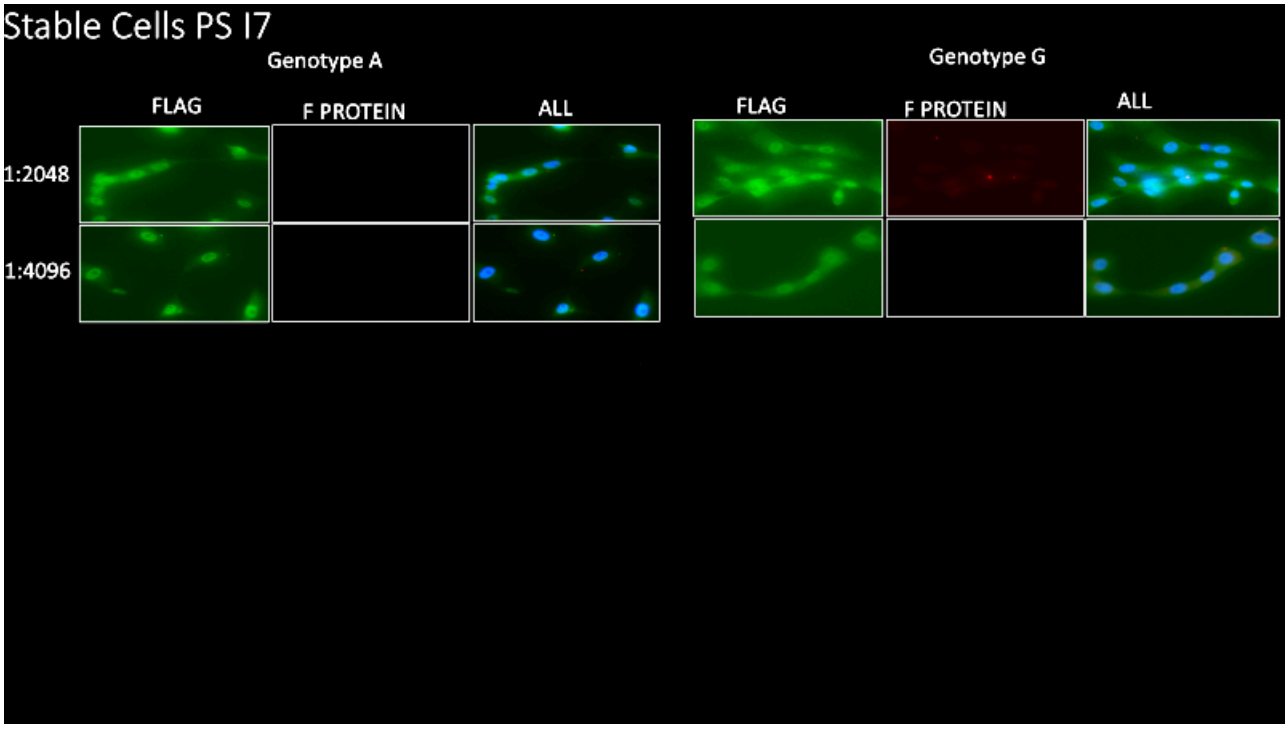


Figure A-70d. Stable cells Infected Sample#7.

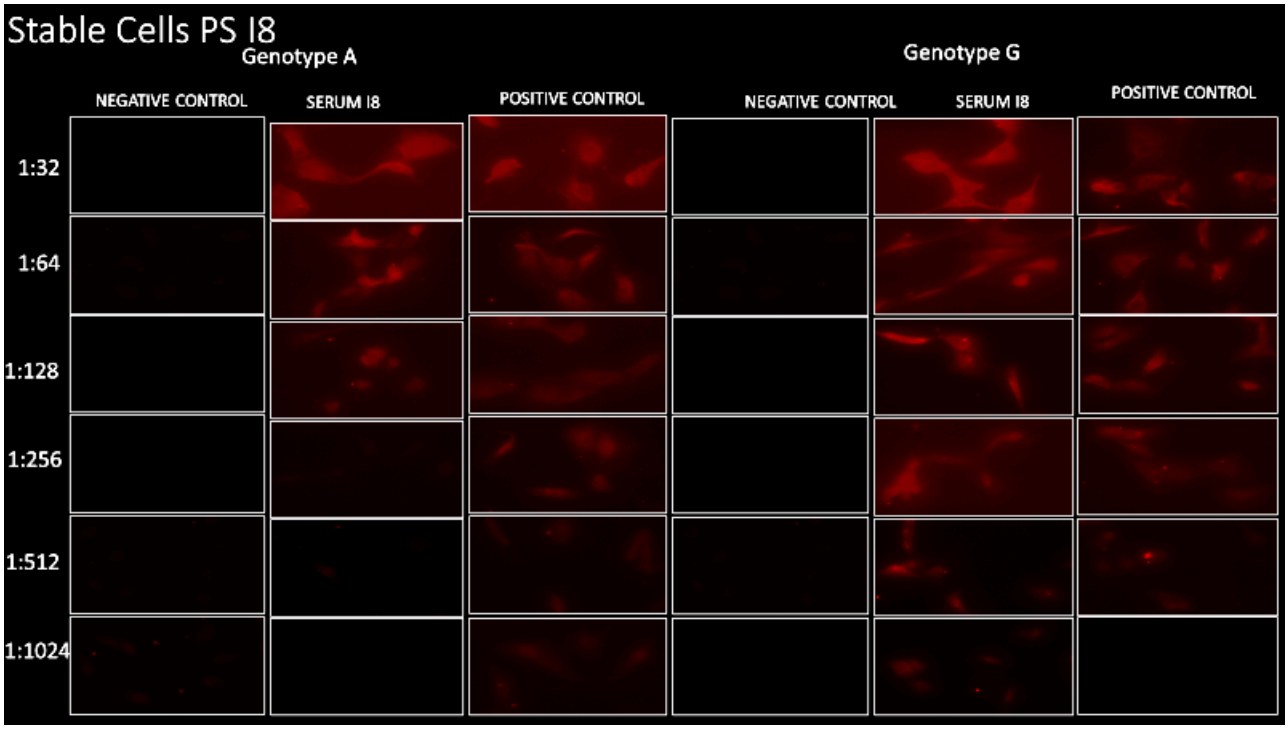


Figure A-71a. Stable cells Infected Sample#8.

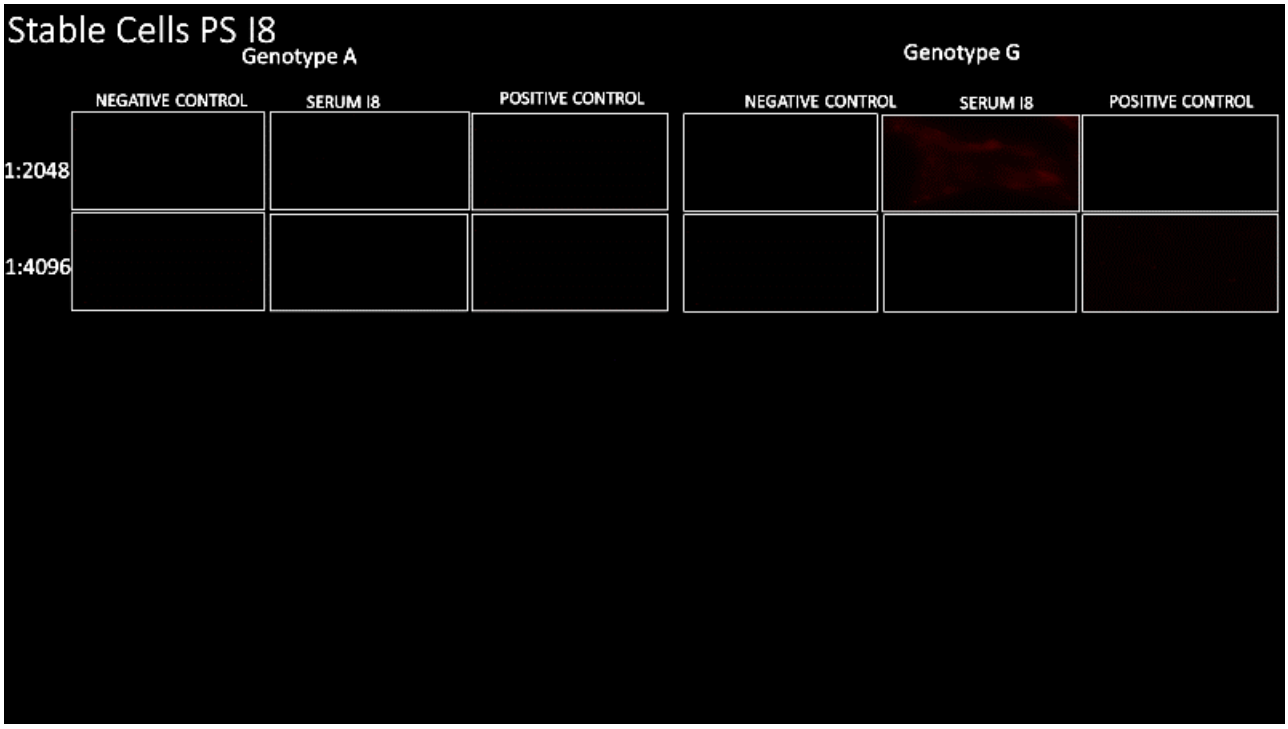


Figure A-71b. Stable cells Infected Sample#8.

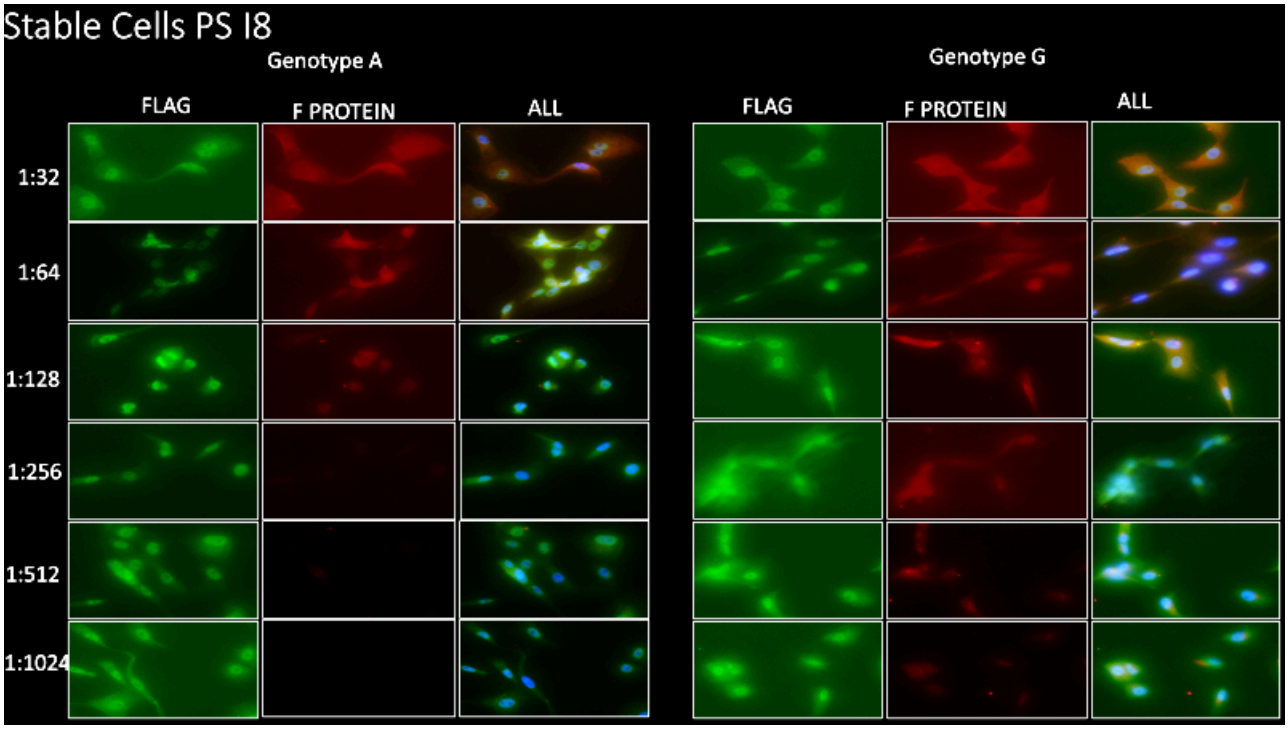


Figure A-71c. Stable cells Infected Sample#8.

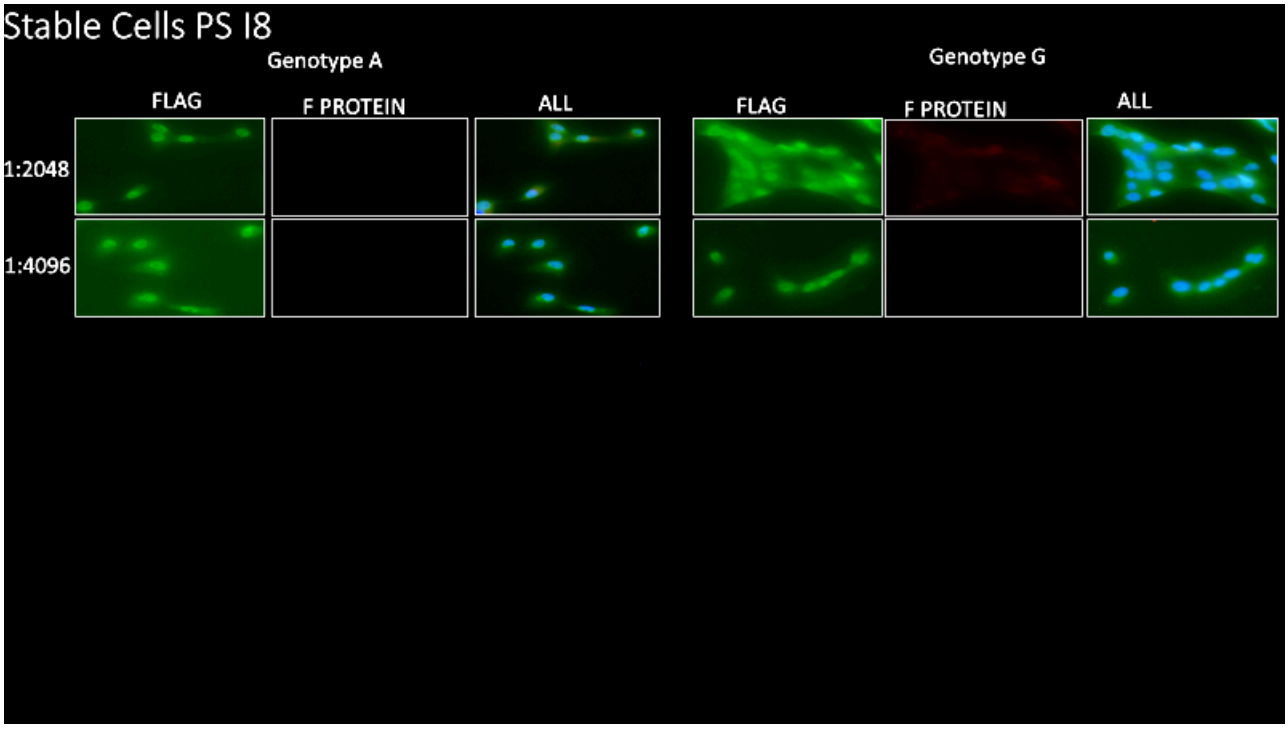


Figure A-71d. Stable cells Infected Sample#8.

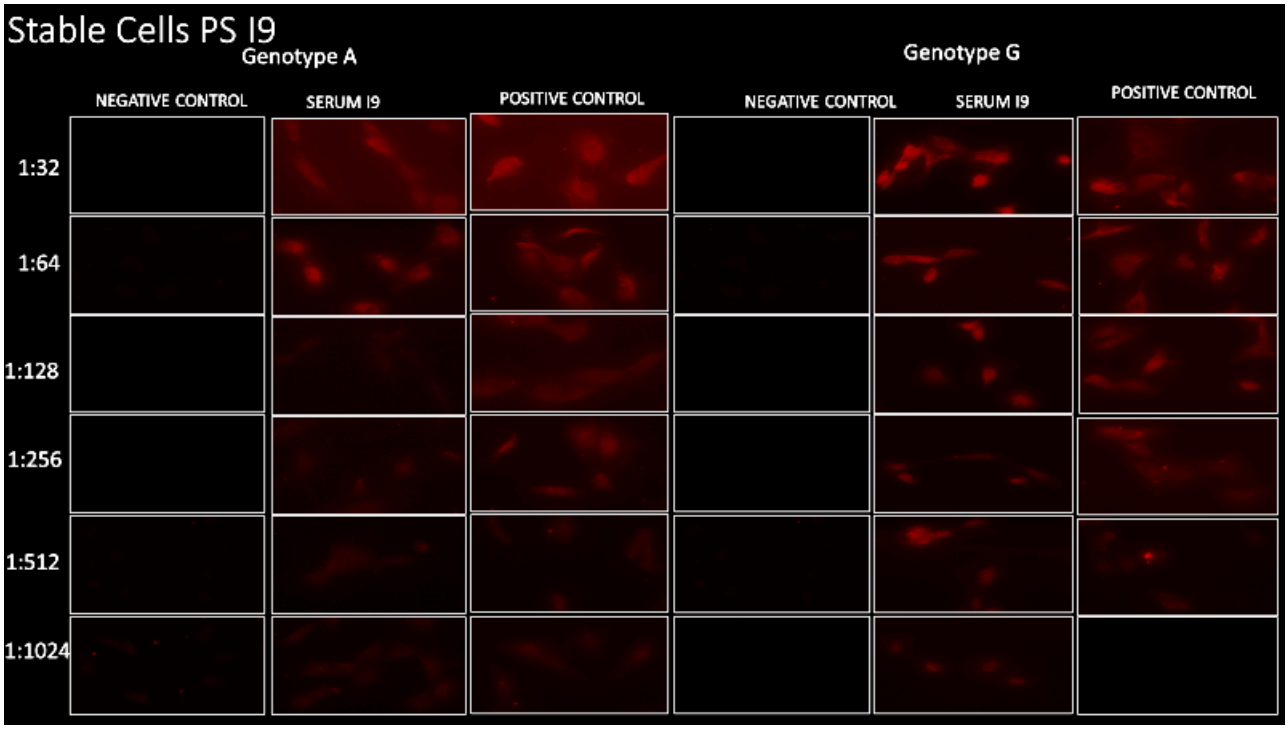


Figure A-72a. Stable cells Infected Sample#9.

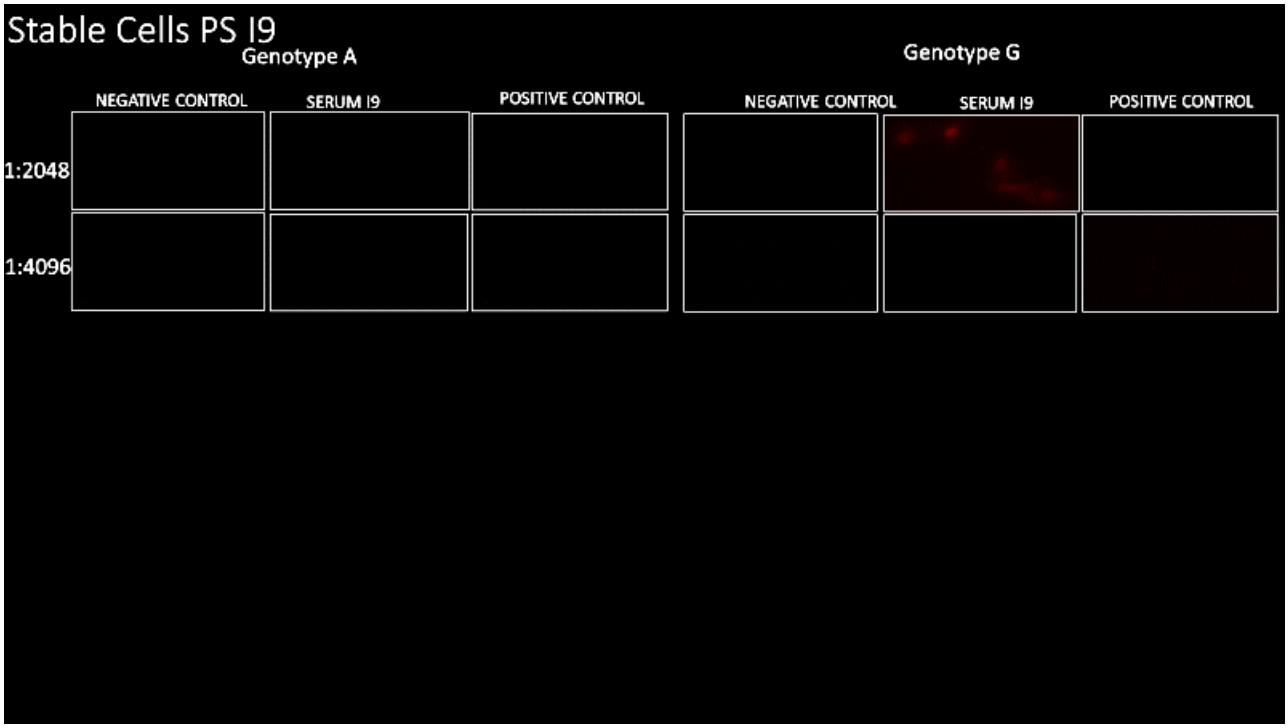


Figure A-72b. Stable cells Infected Sample#9.

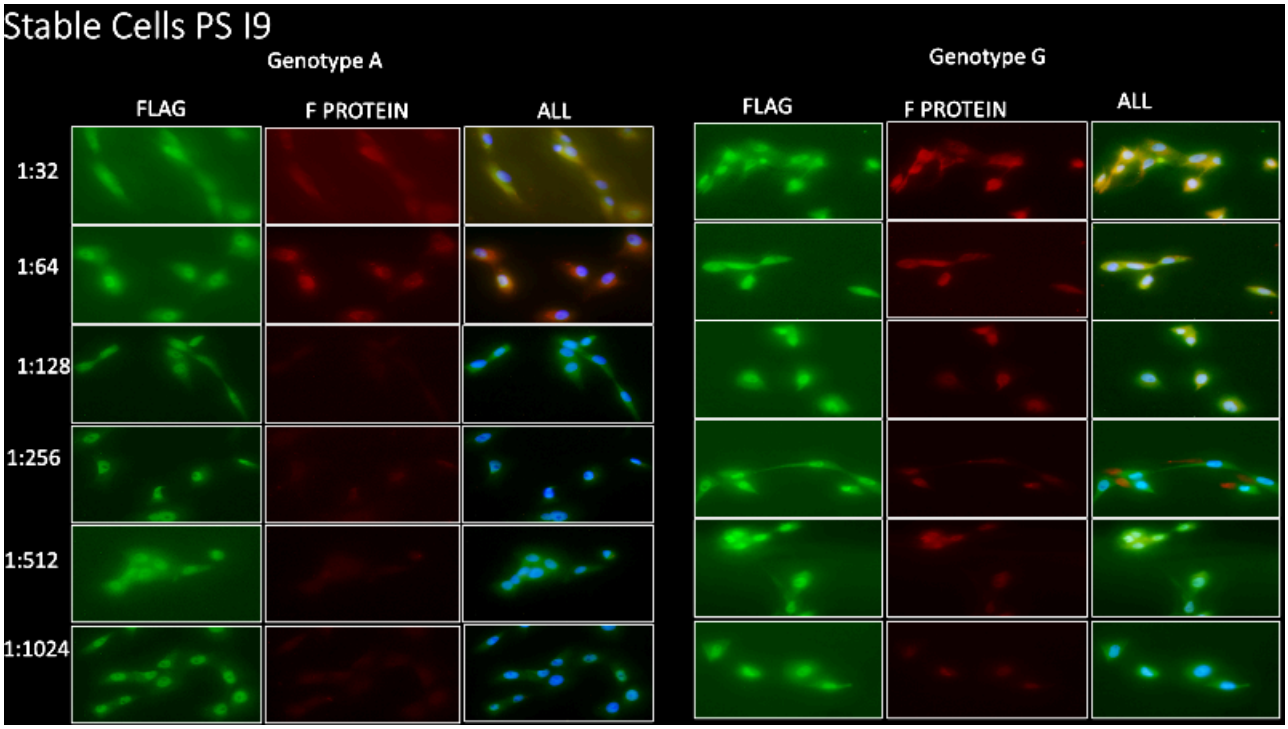


Figure A-72c. Stable cells Infected Sample#9.

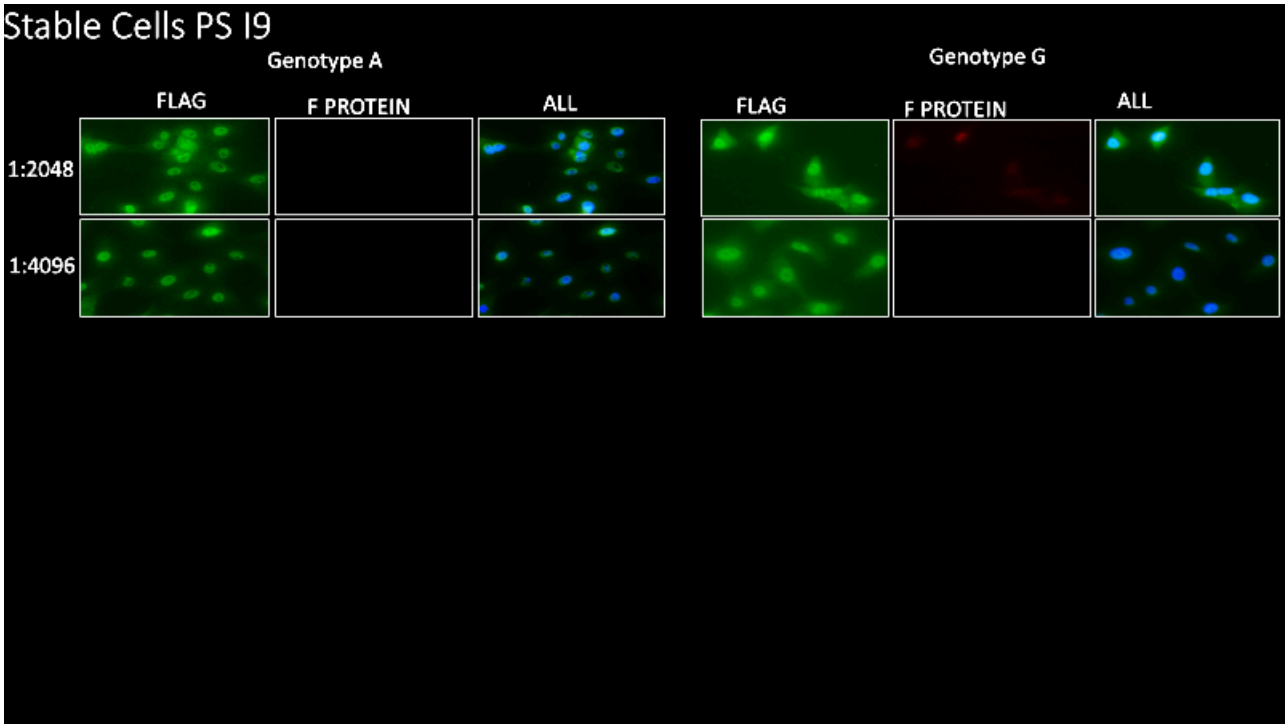


Figure A-72d. Stable cells Infected Sample#9.

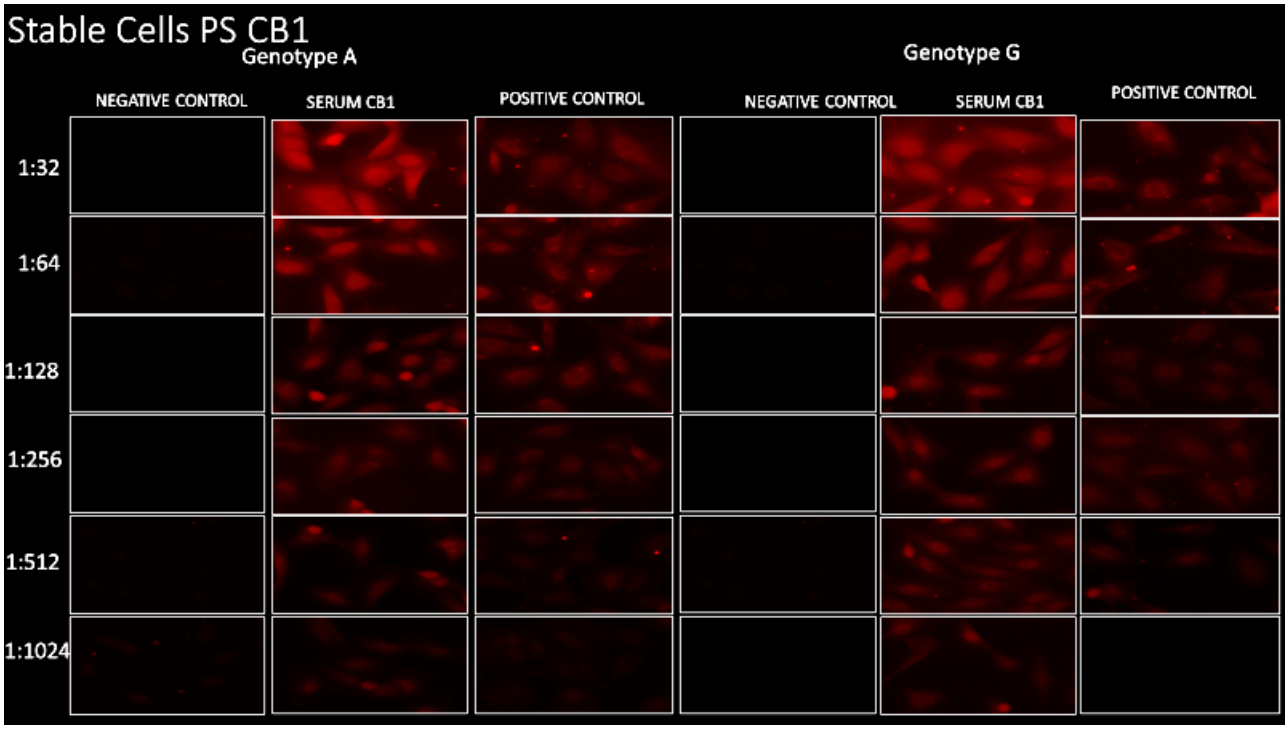


Figure A-73a. Stable cells Vaccine-Infected (CB) Sample#1.

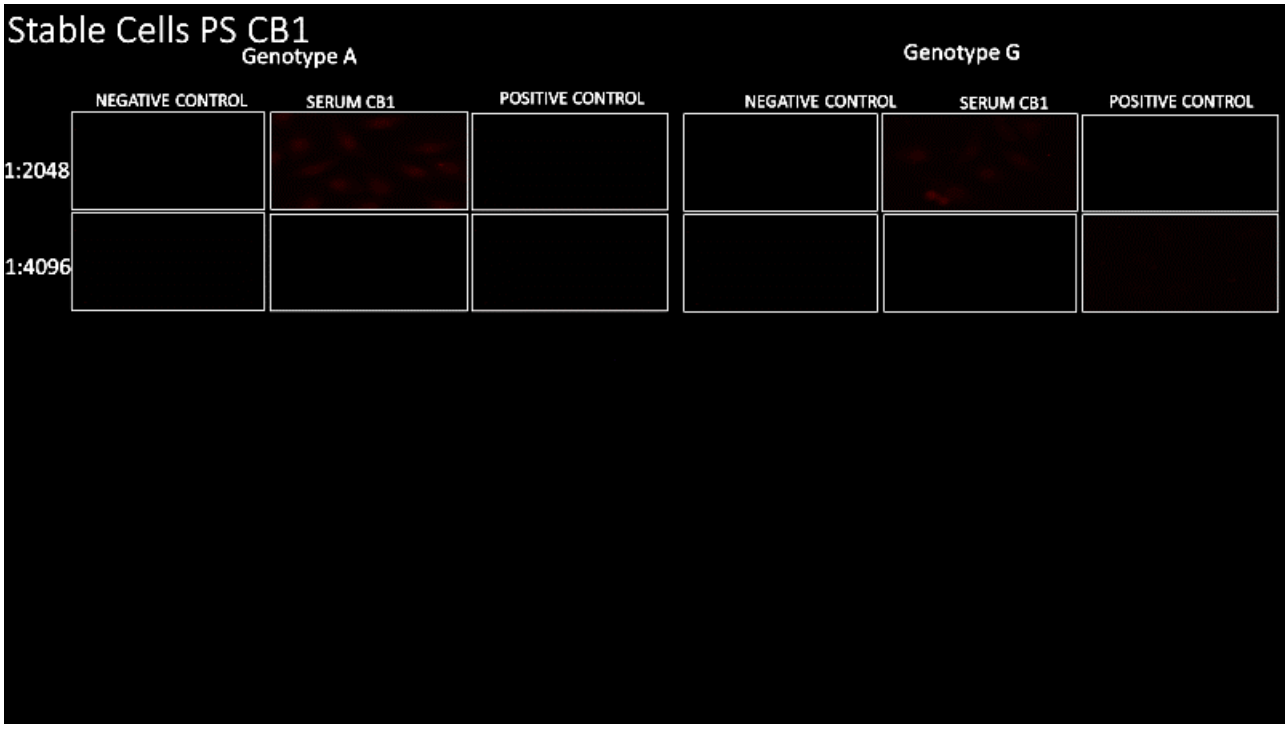


Figure A-73b. Stable cells Vaccine-Infected (CB) Sample#1.

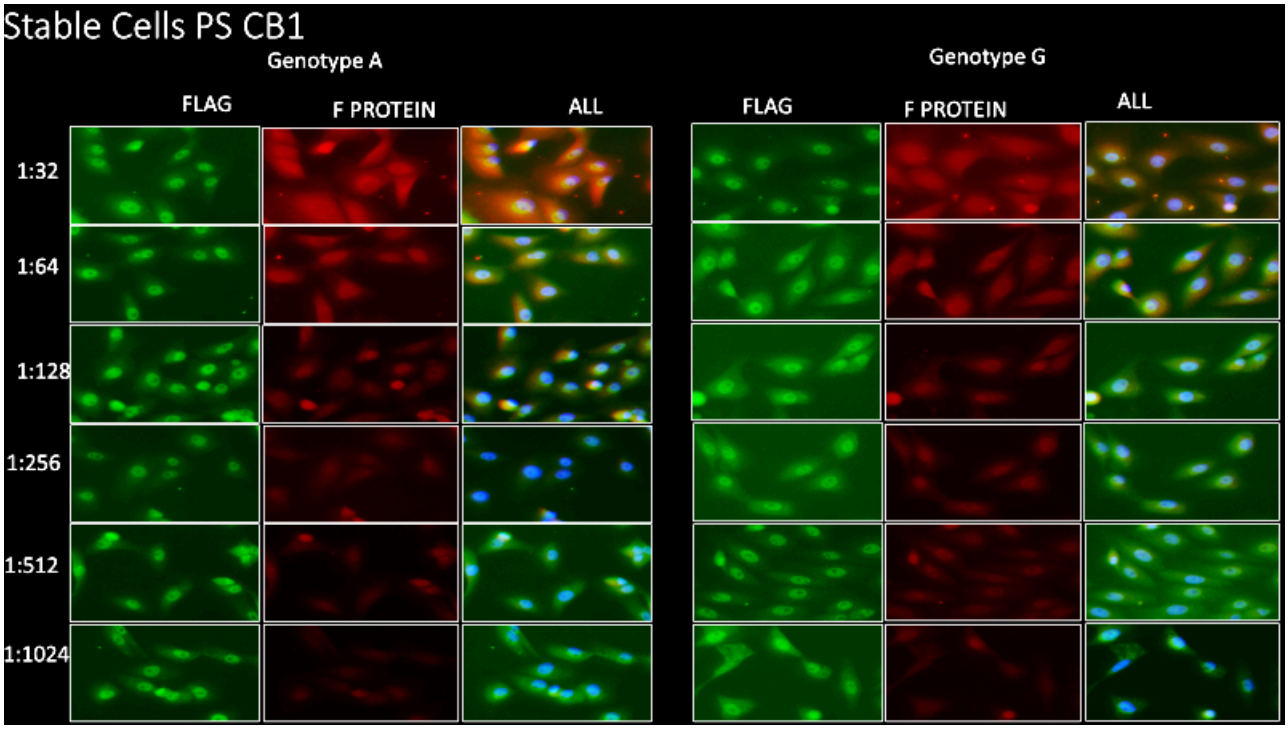


Figure A-73c. Stable cells Vaccine-Infected (CB) Sample#1.

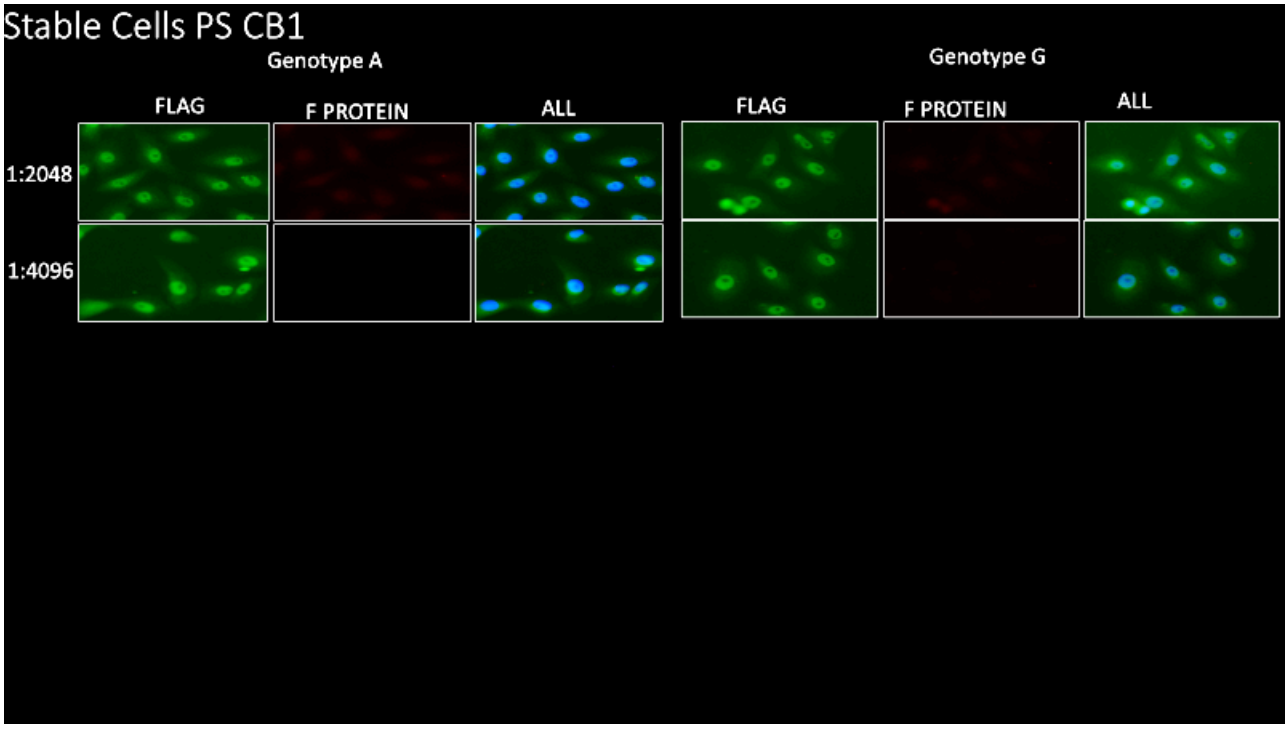


Figure A-73d. Stable cells Vaccine-Infected (CB) Sample#1.

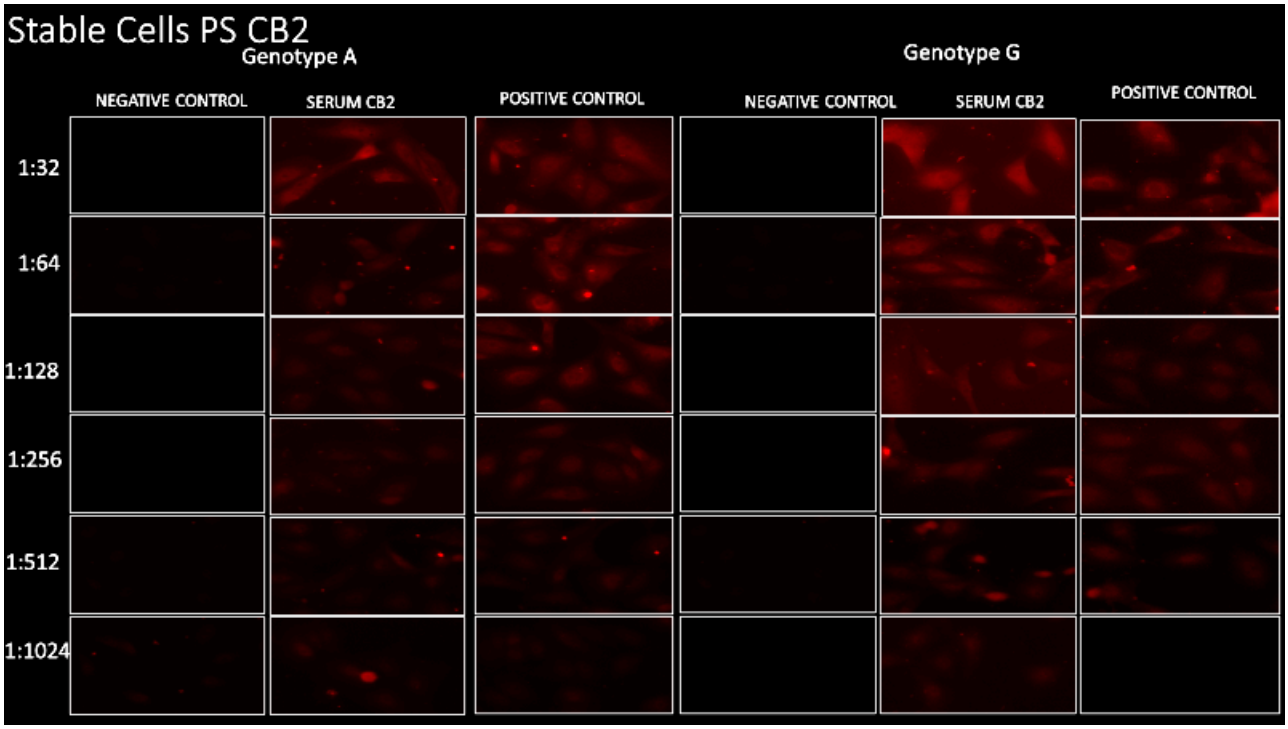


Figure A-74a. Stable cells Vaccine-Infected (CB) Sample#2.

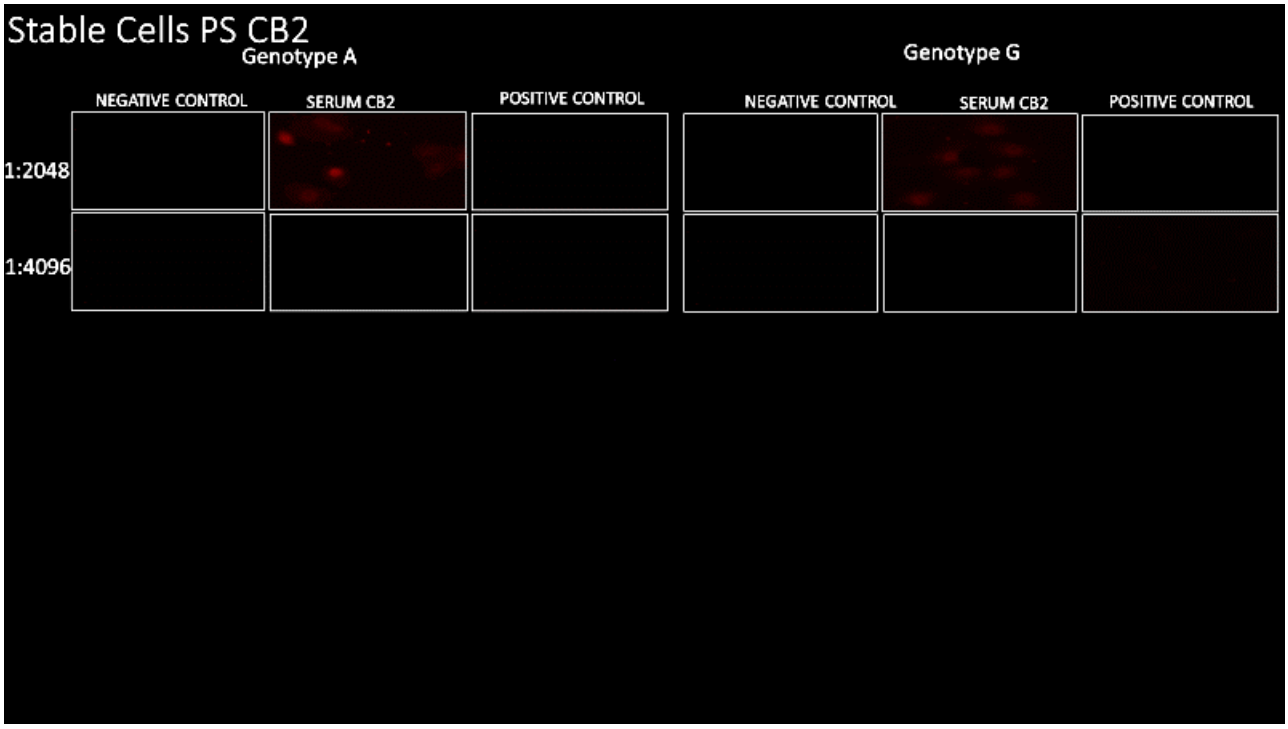


Figure A-74b. Stable cells Vaccine-Infected (CB) Sample#2.

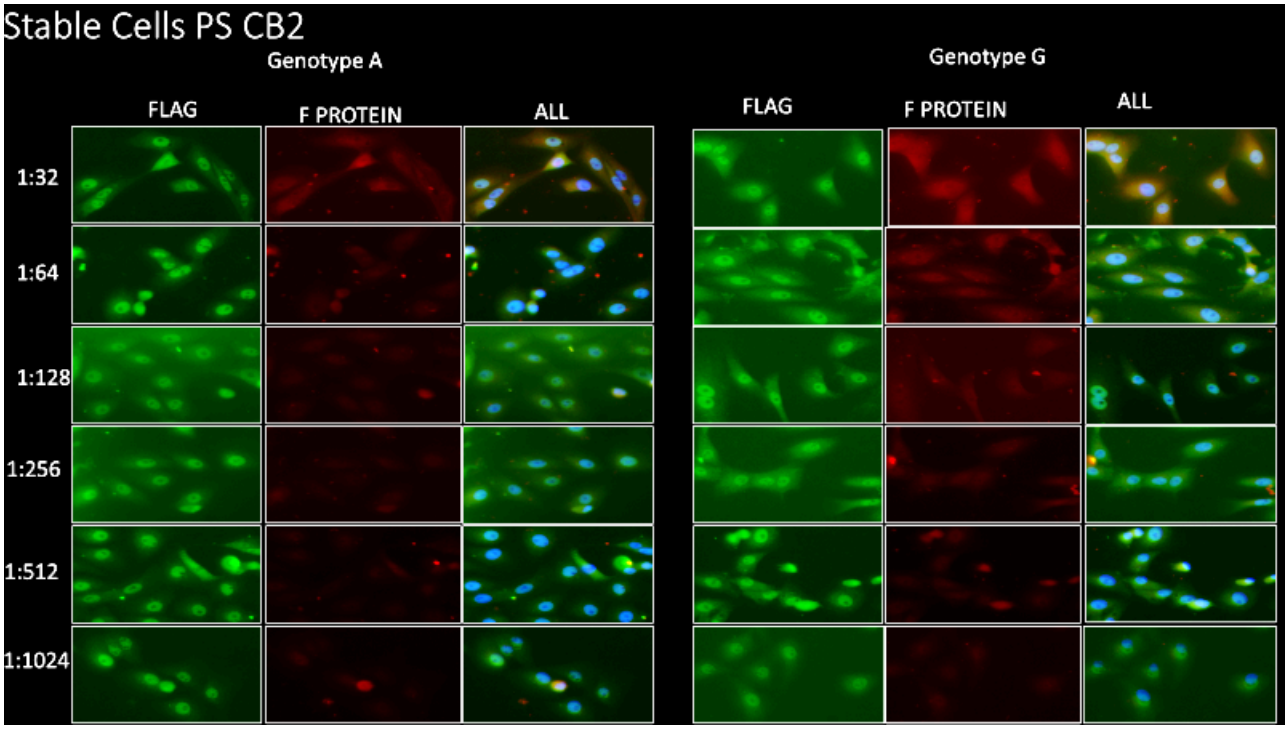


Figure A-74c. Stable cells Vaccine-Infected (CB) Sample#2.

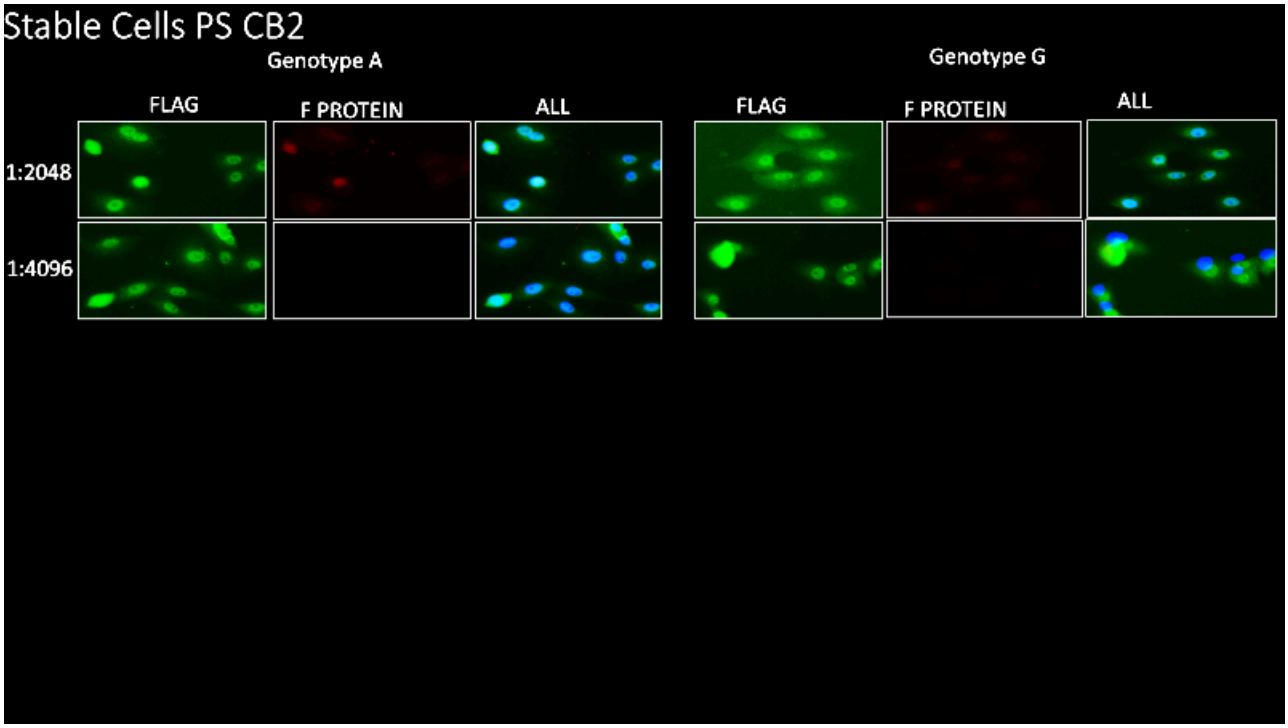


Figure A-74d. Stable cells Vaccine-Infected (CB) Sample#2.

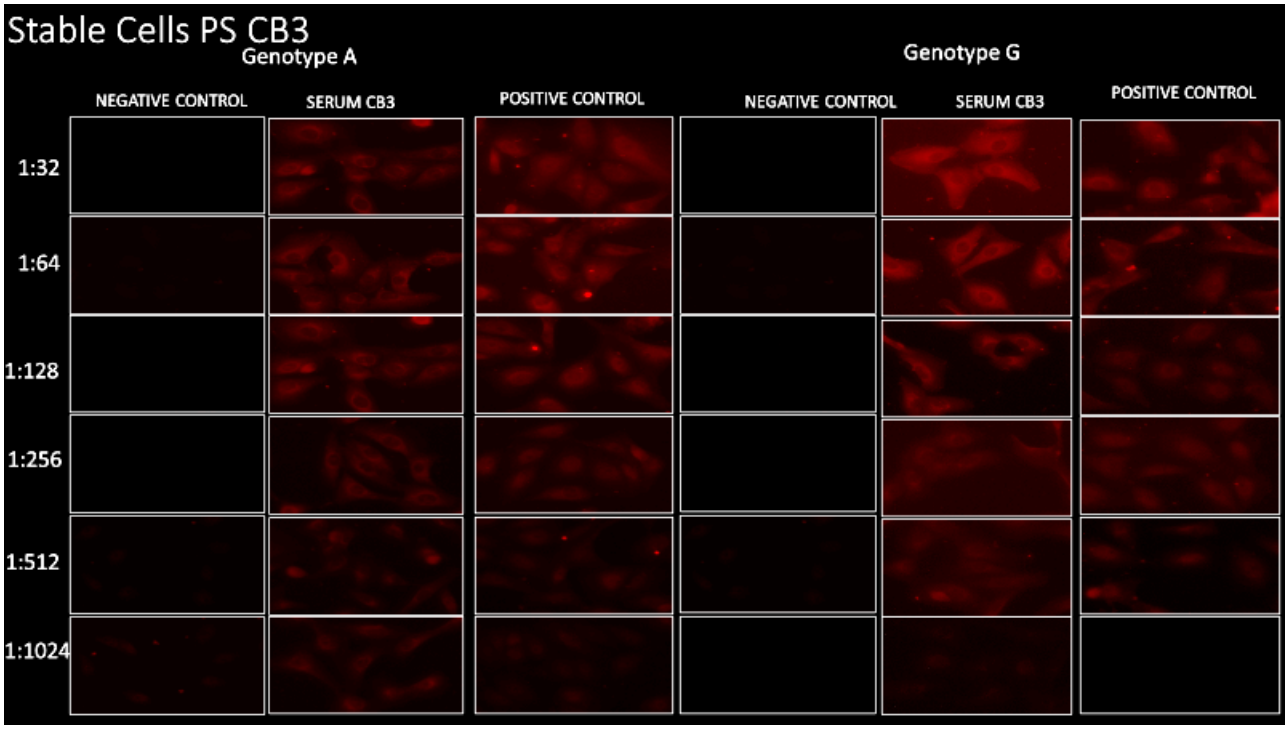


Figure A-75a. Stable cells Vaccine-Infected (CB) Sample#3.

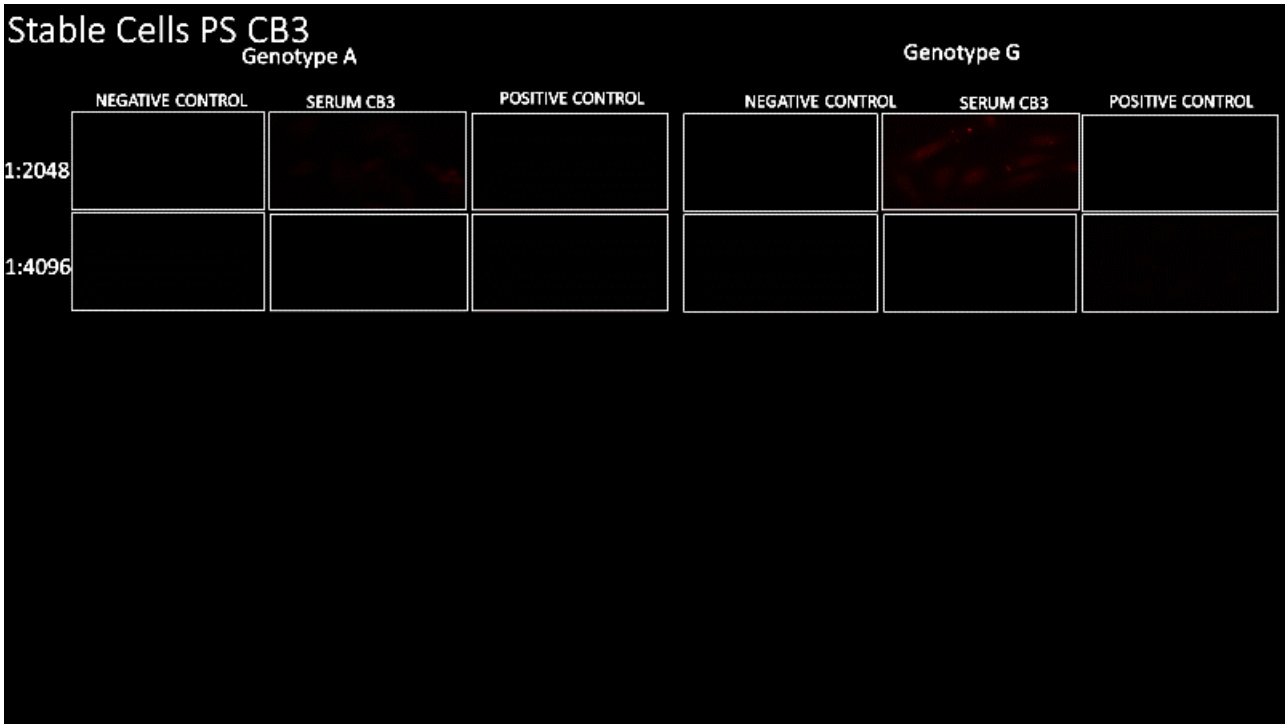


Figure A-75b. Stable cells Vaccine-Infected (CB) Sample#3.

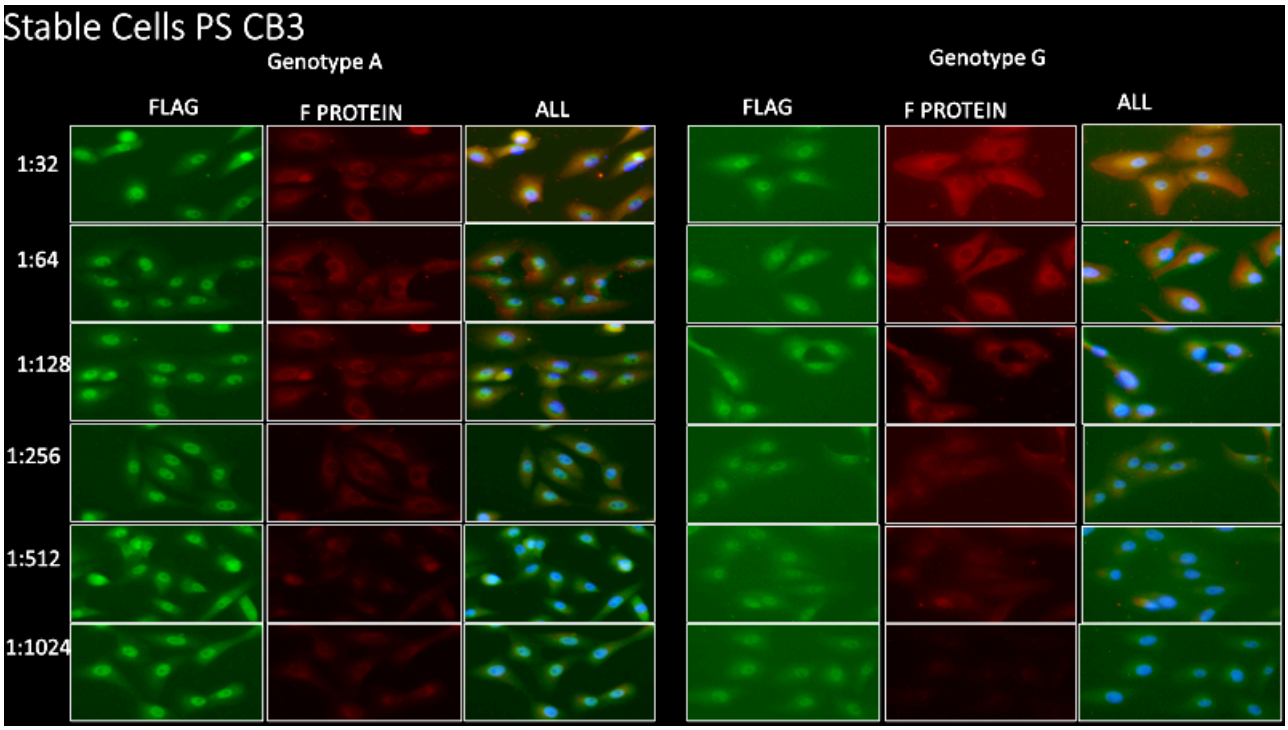


Figure A-75c. Stable cells Vaccine-Infected (CB) Sample#3.

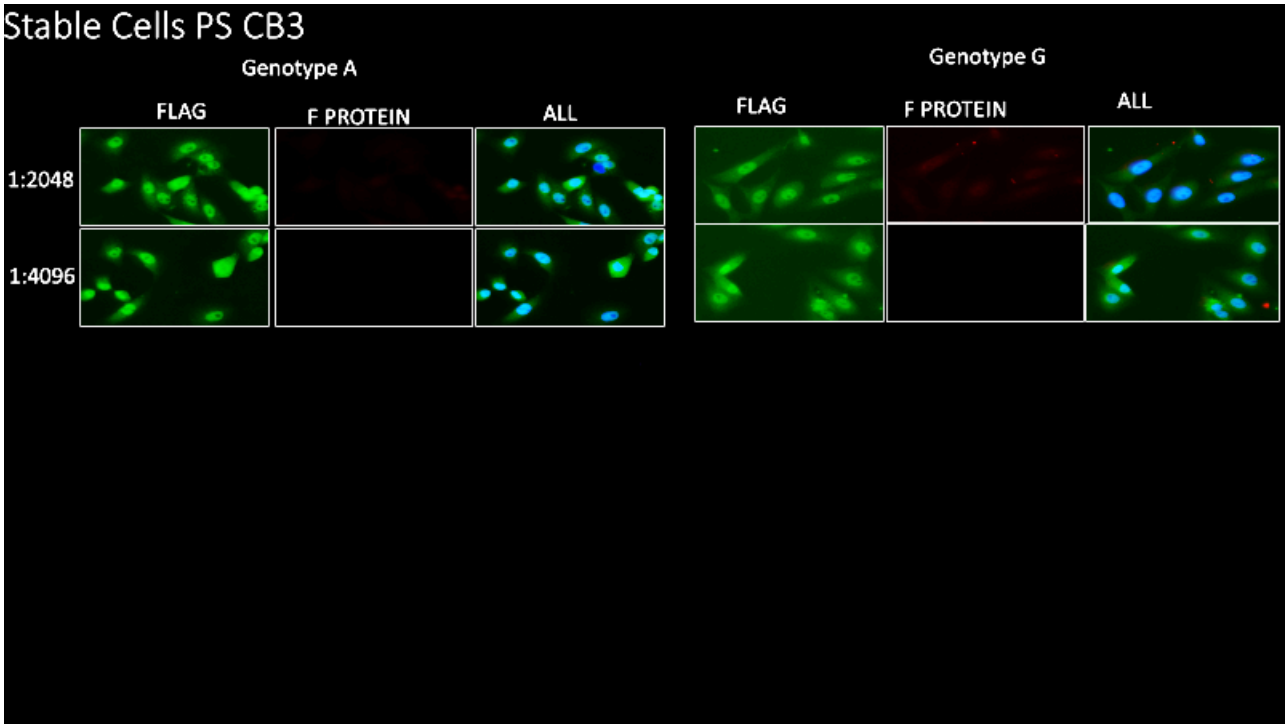


Figure A-75d. Stable cells Vaccine-Infected (CB) Sample#3.

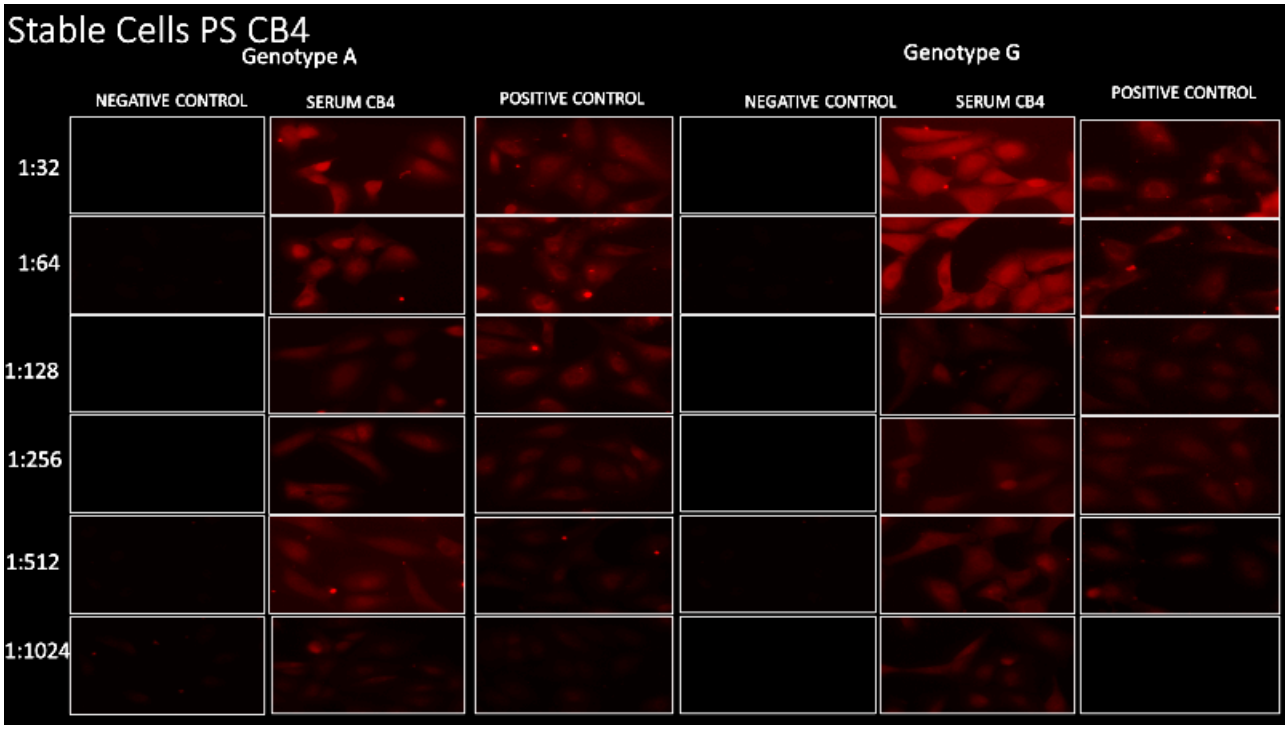


Figure A-76a. Stable cells Vaccine-Infected (CB) Sample#4.

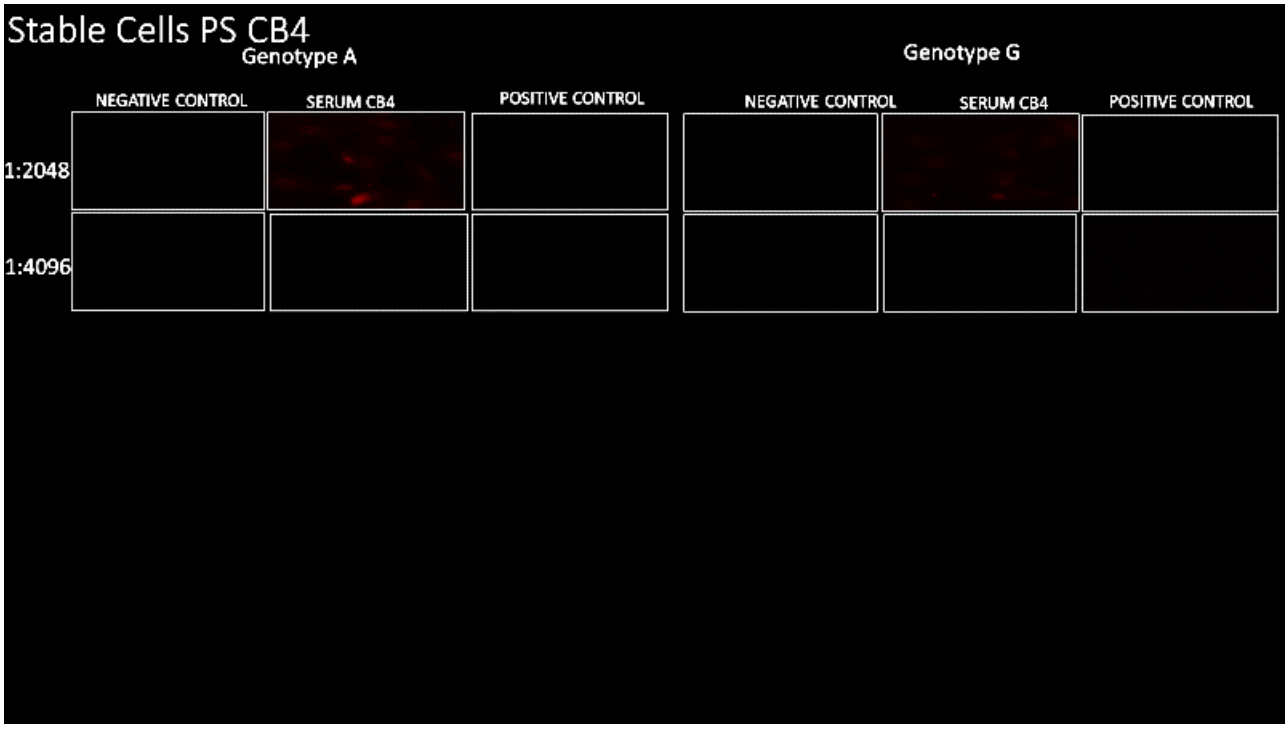


Figure A-76b. Stable cells Vaccine-Infected (CB) Sample#4.

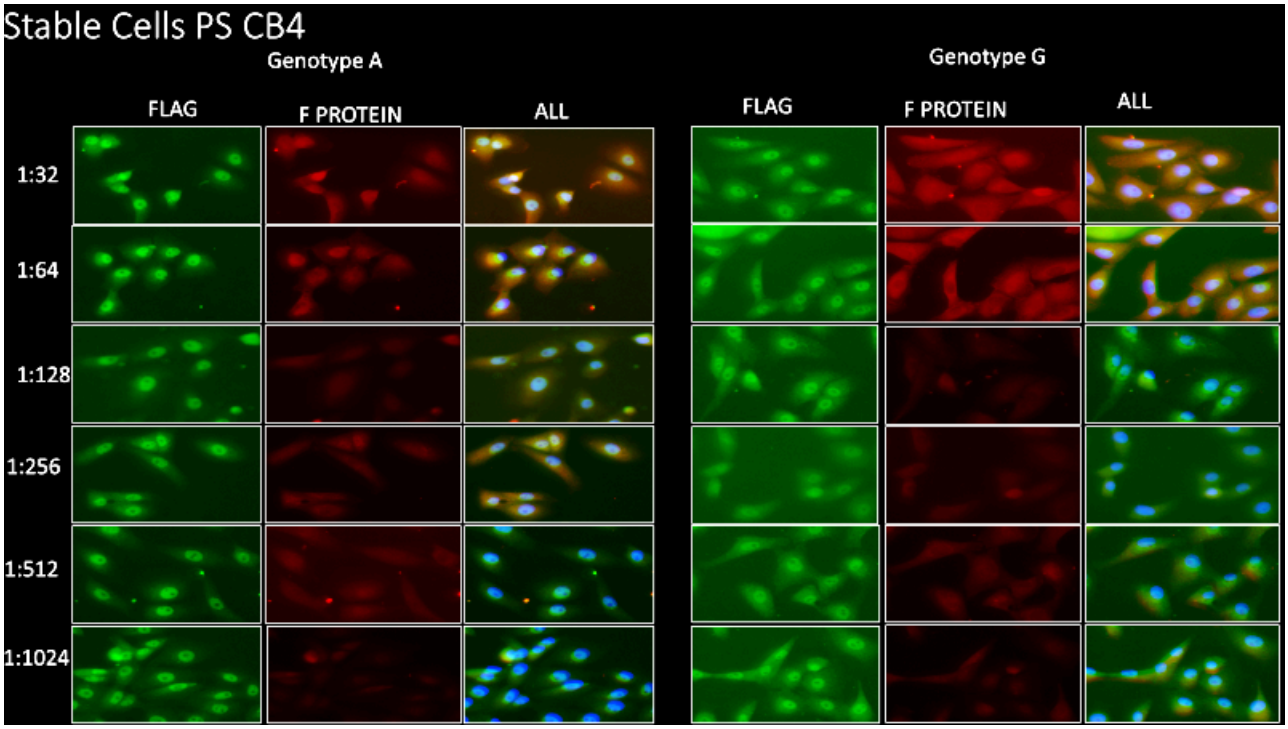


Figure A-76c. Stable cells Vaccine-Infected (CB) Sample#4.

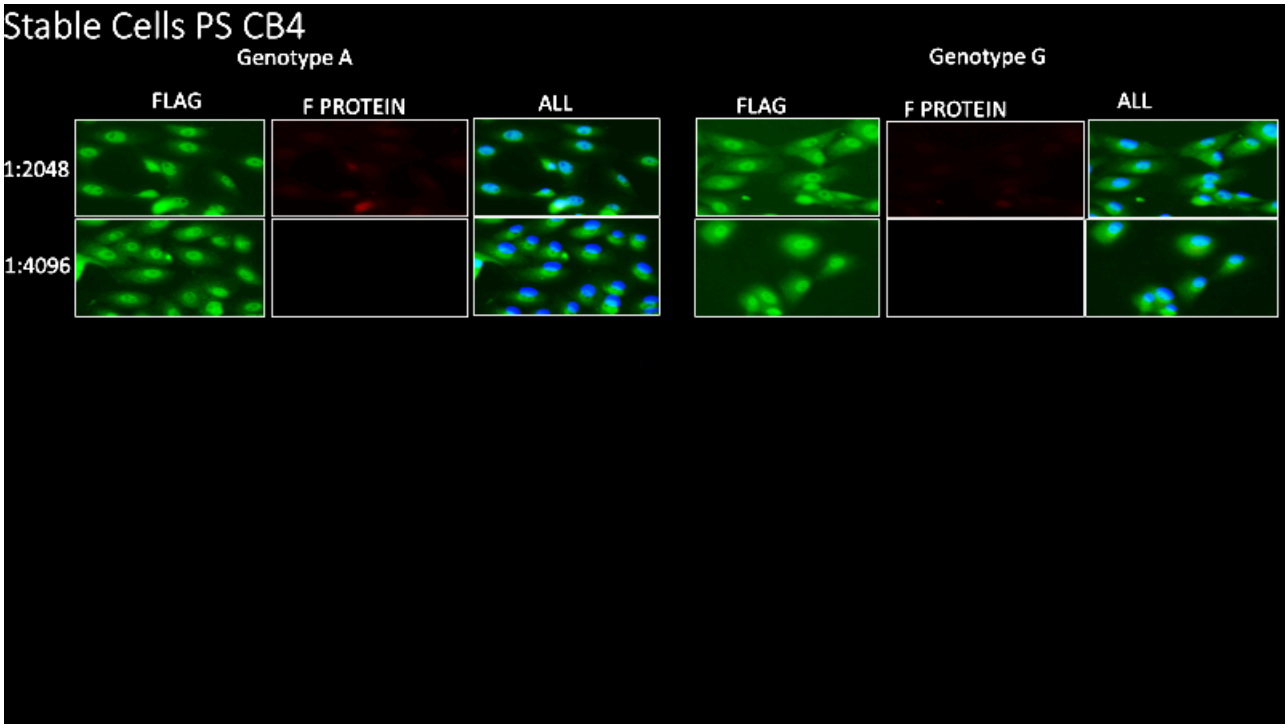


Figure A-76d. Stable cells Vaccine-Infected (CB) Sample#4.

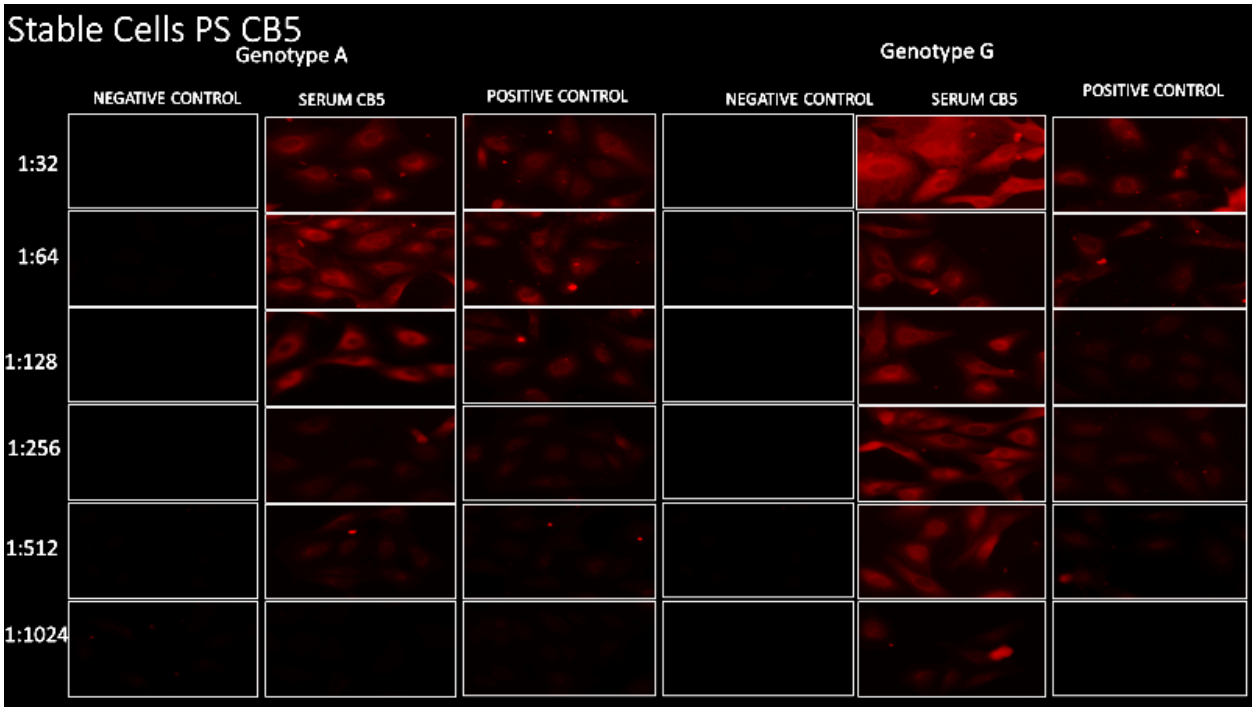


Figure A-77a. Stable cells Vaccine-Infected (CB) Sample#5.

Figure A-77b. Stable cells Vaccine-Infected (CB) Sample#5.

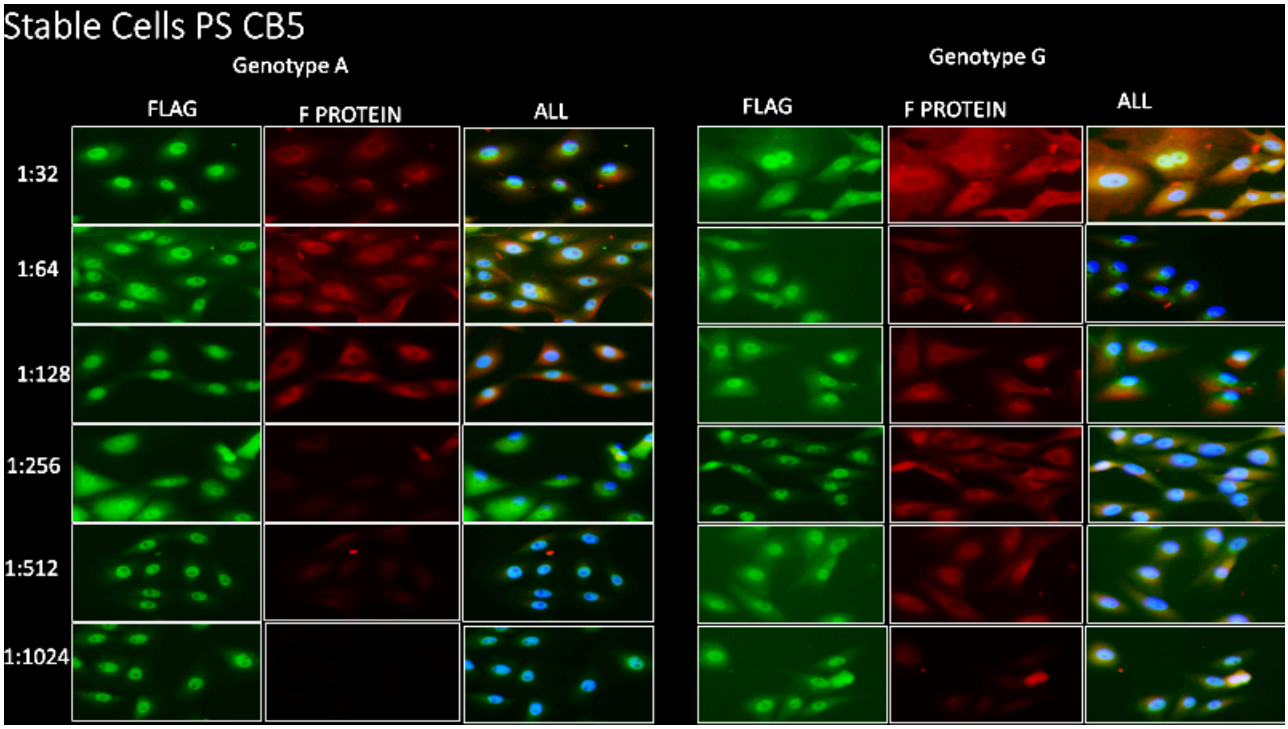


Figure A-77c. Stable cells Vaccine-Infected (CB) Sample#5.

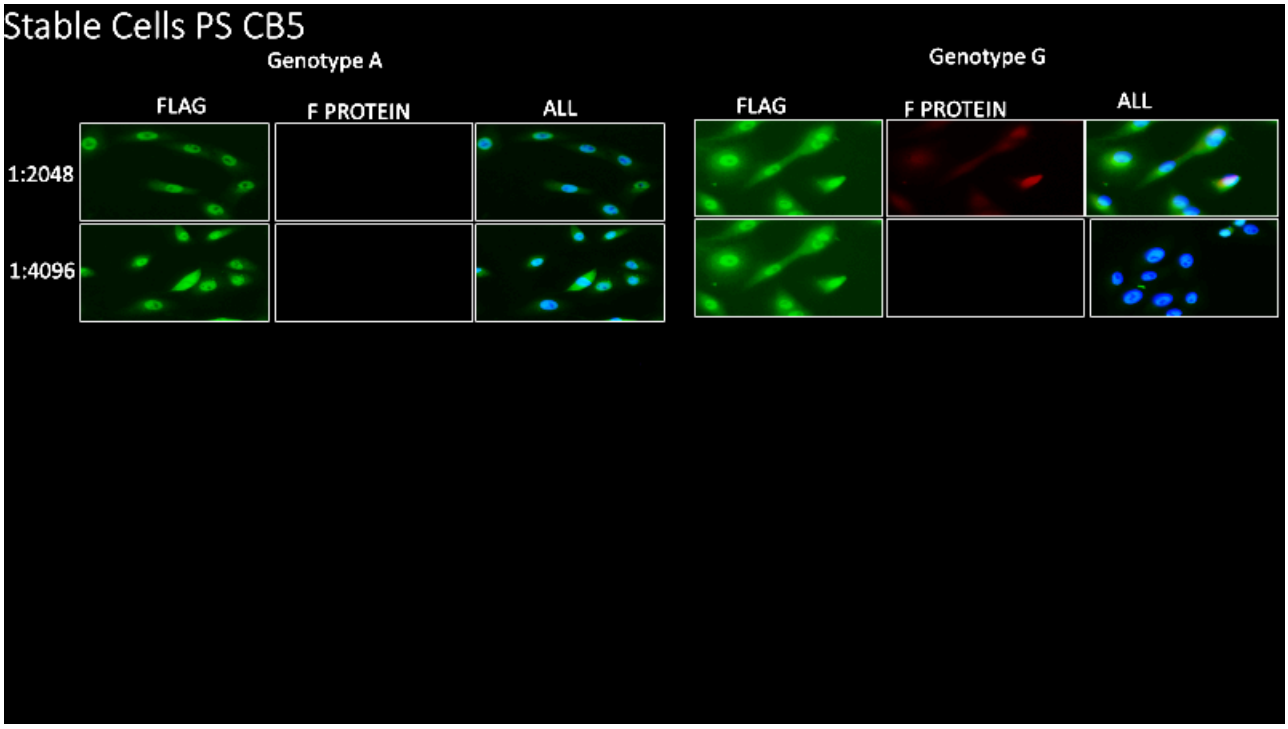


Figure A-77d. Stable cells Vaccine-Infected (CB) Sample#5.

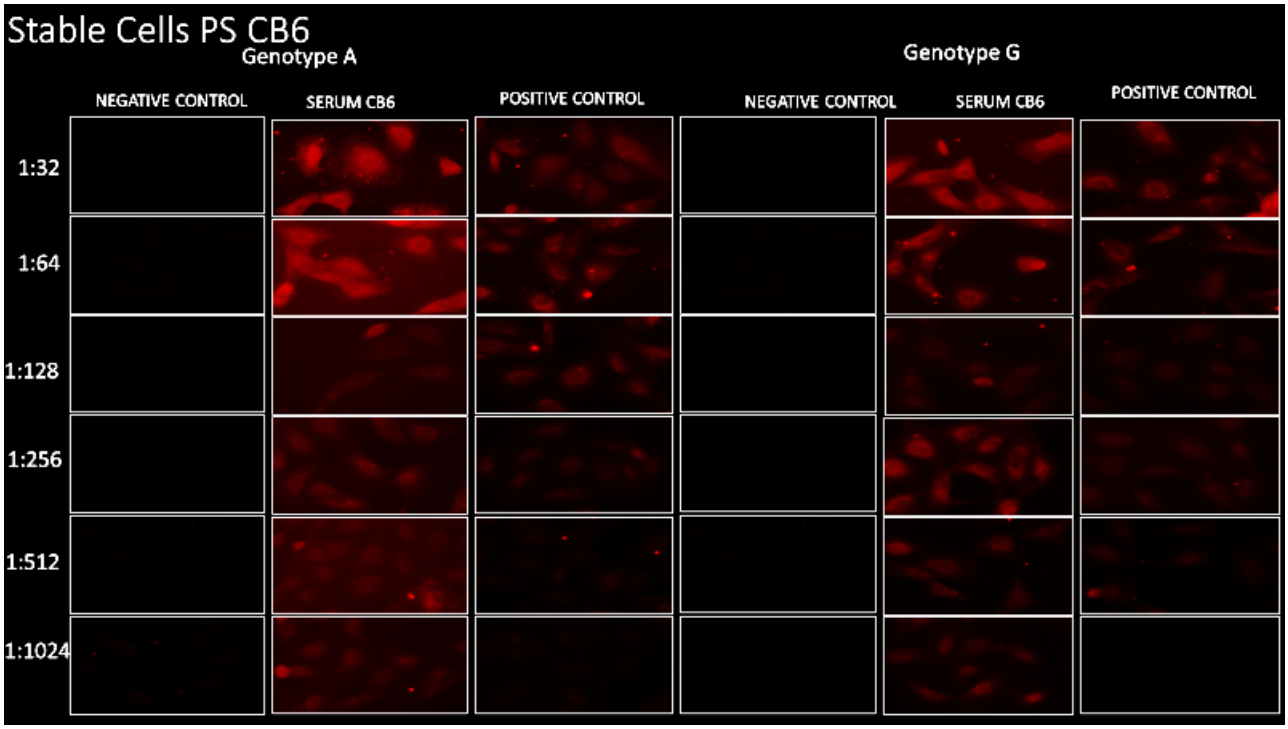


Figure A-78a. Stable cells Vaccine-Infected (CB) Sample#6.

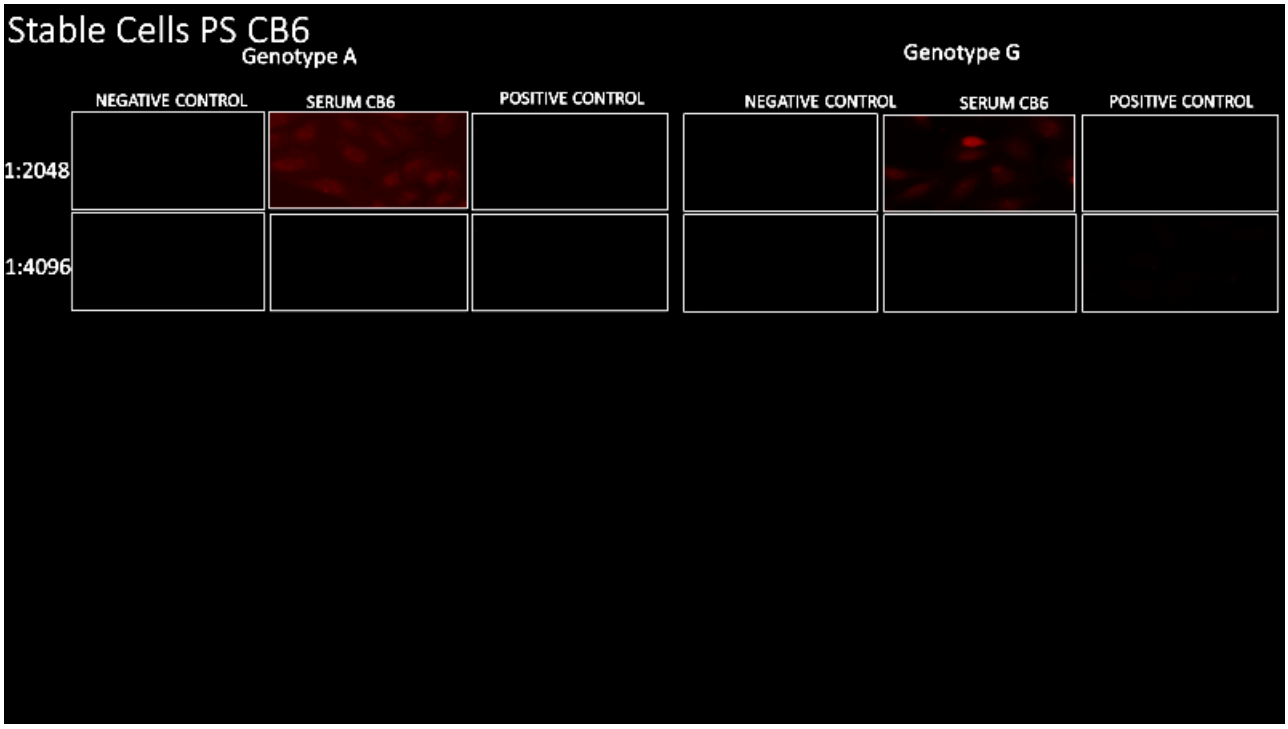


Figure A-78b. Stable cells Vaccine-Infected (CB) Sample#6.

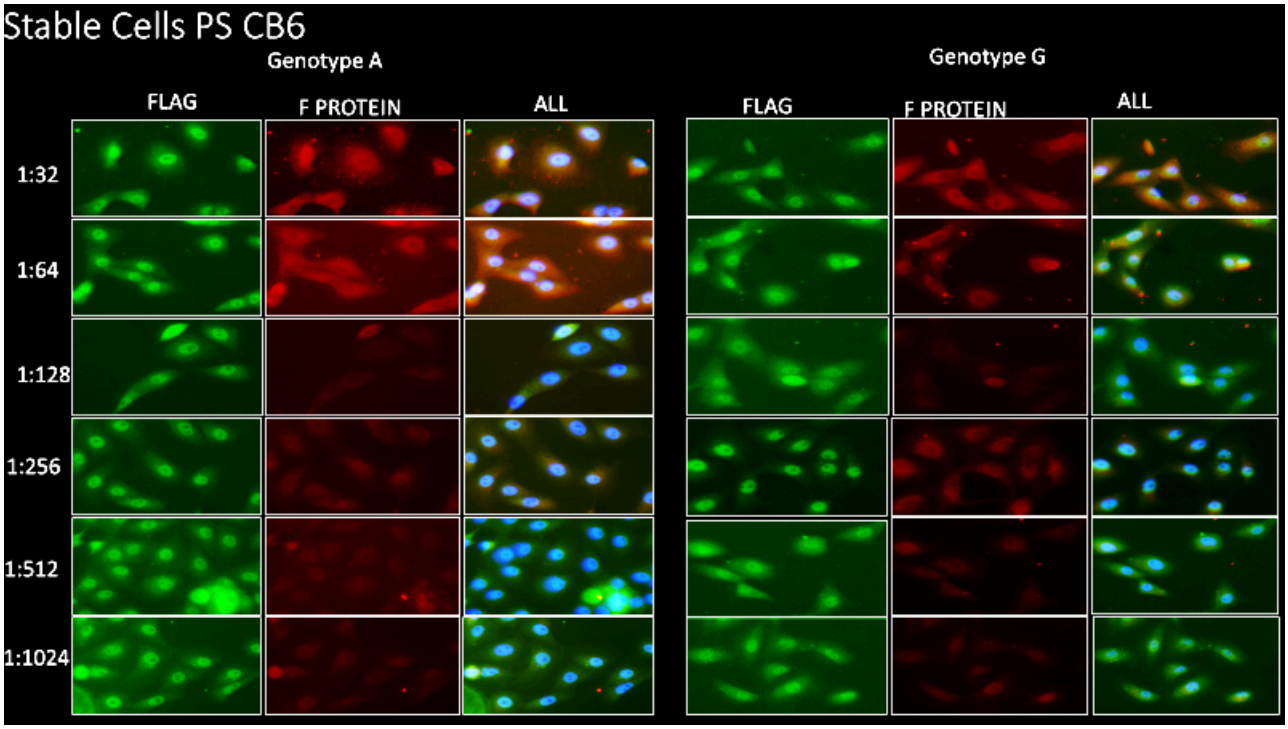


Figure A-78c. Stable cells Vaccine-Infected (CB) Sample#6.

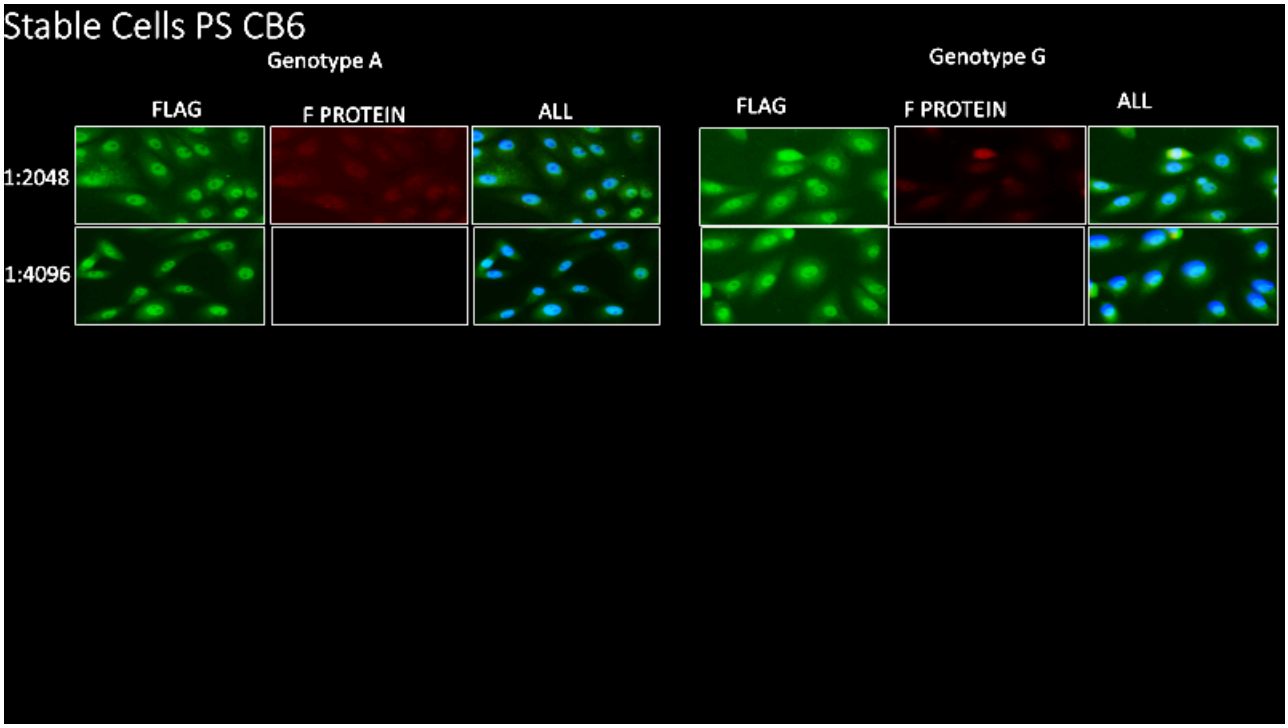


Figure A-78d. Stable cells Vaccine-Infected (CB) Sample#6.

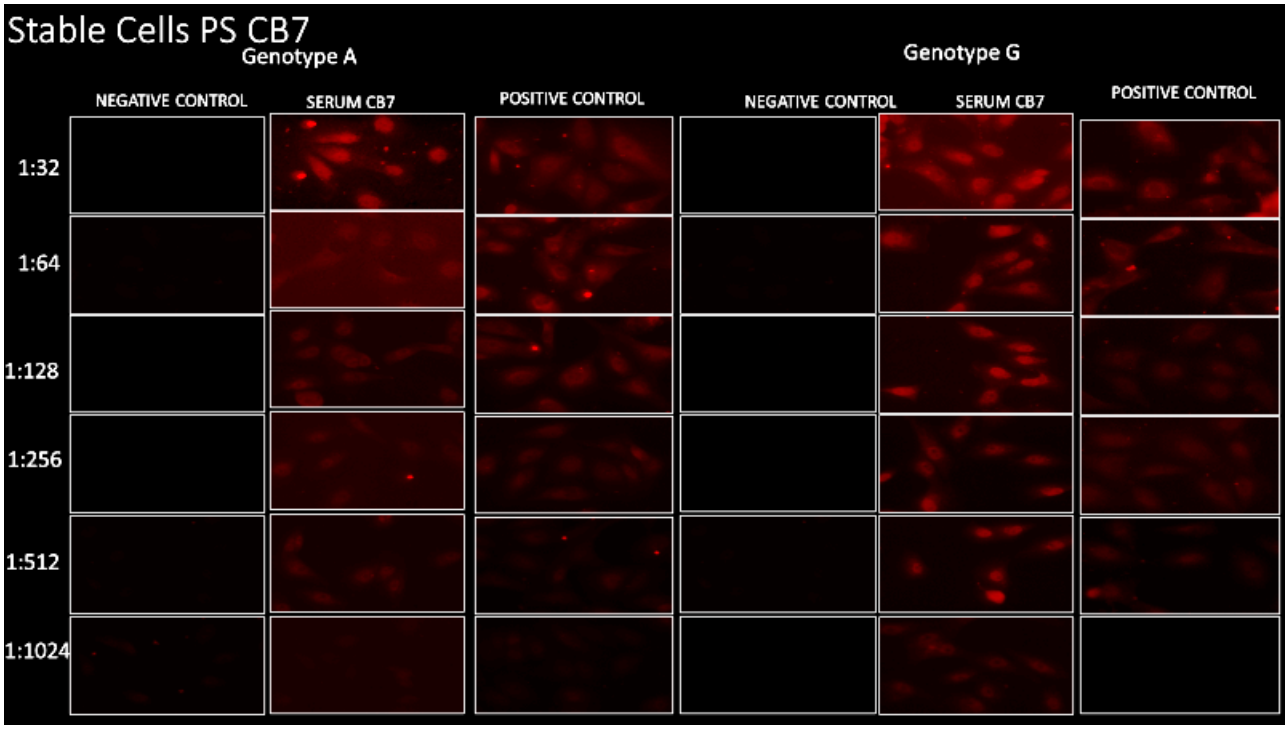


Figure A-79a. Stable cells Vaccine-Infected (CB) Sample#7.

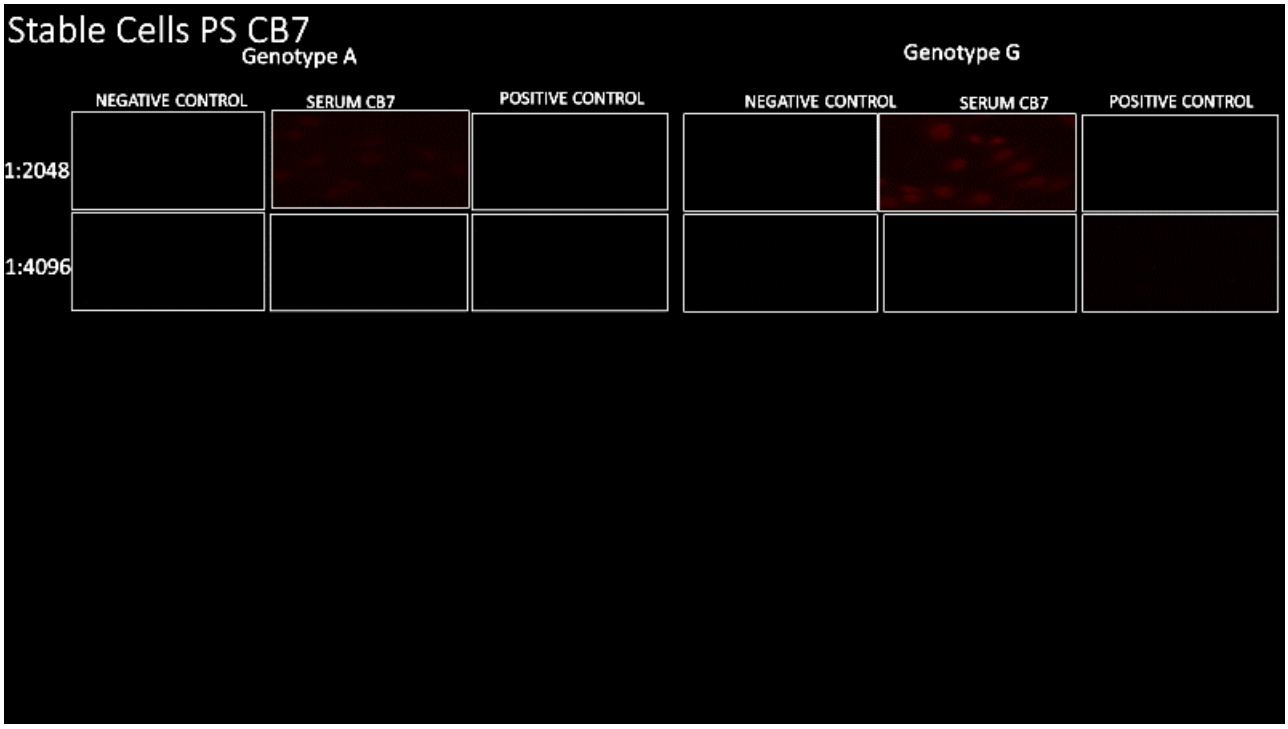


Figure A-79b. Stable cells Vaccine-Infected (CB) Sample#7.

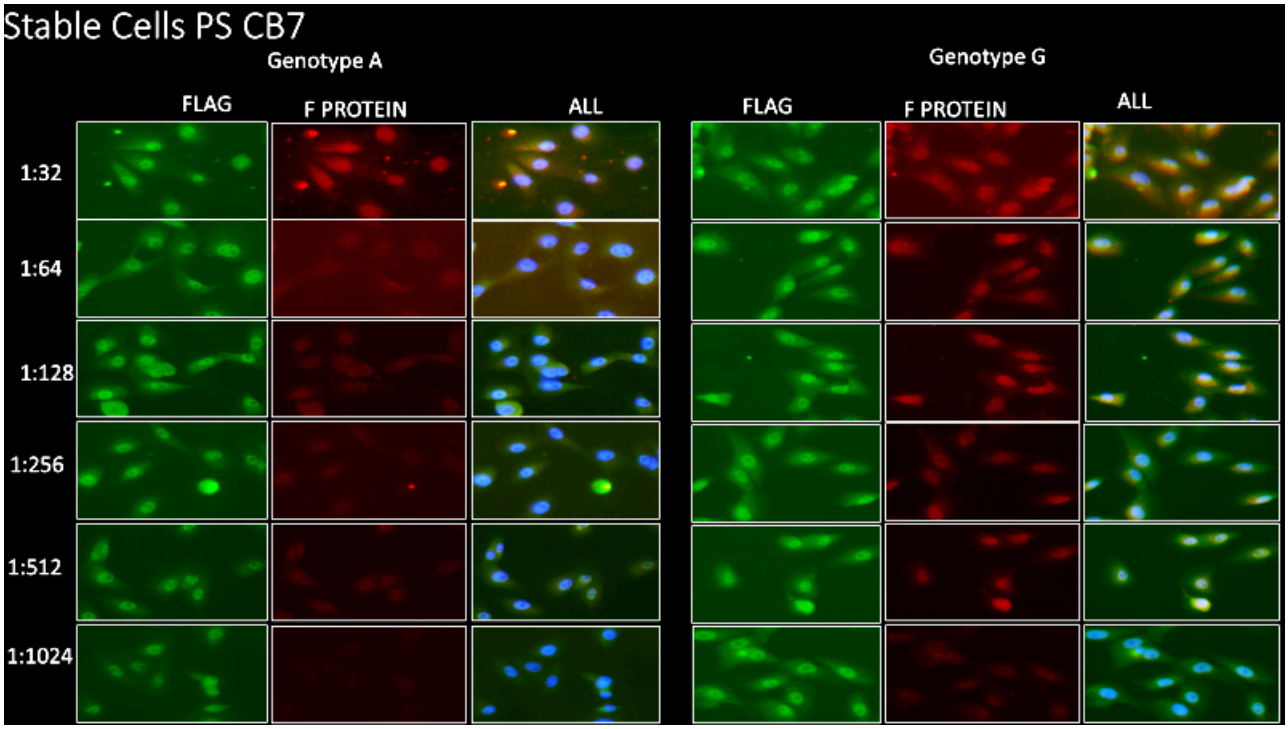


Figure A-79c. Stable cells Vaccine-Infected (CB) Sample#7.

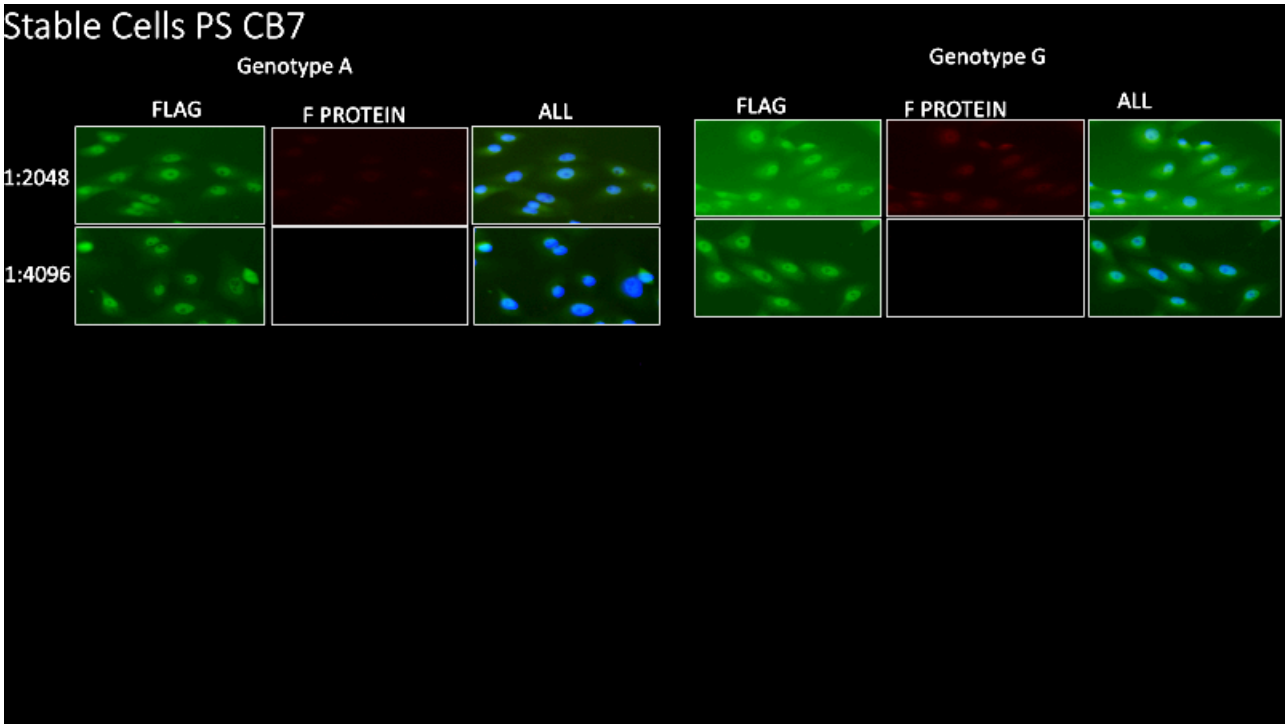


Figure A-79d. Stable cells Vaccine-Infected (CB) Sample#7.

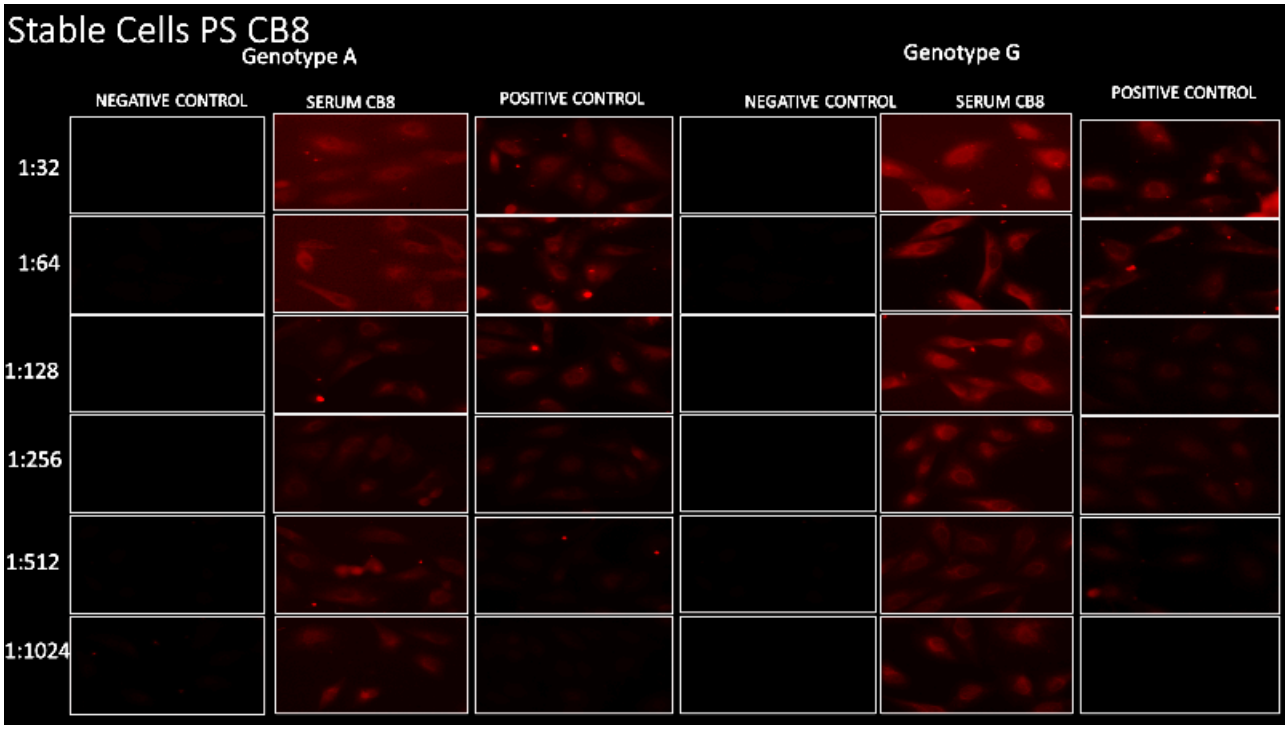


Figure A-80a. Stable cells Vaccine-Infected (CB) Sample#8.

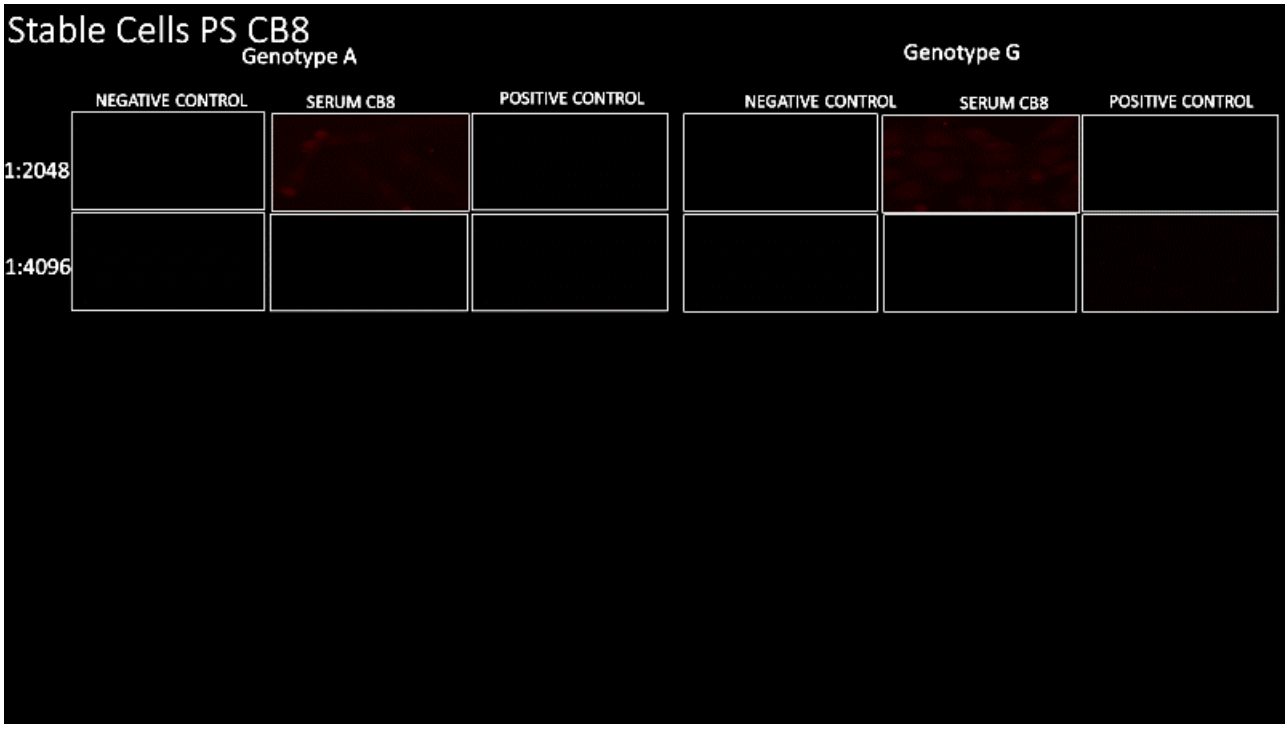


Figure A-80b. Stable cells Vaccine-Infected (CB) Sample#8.

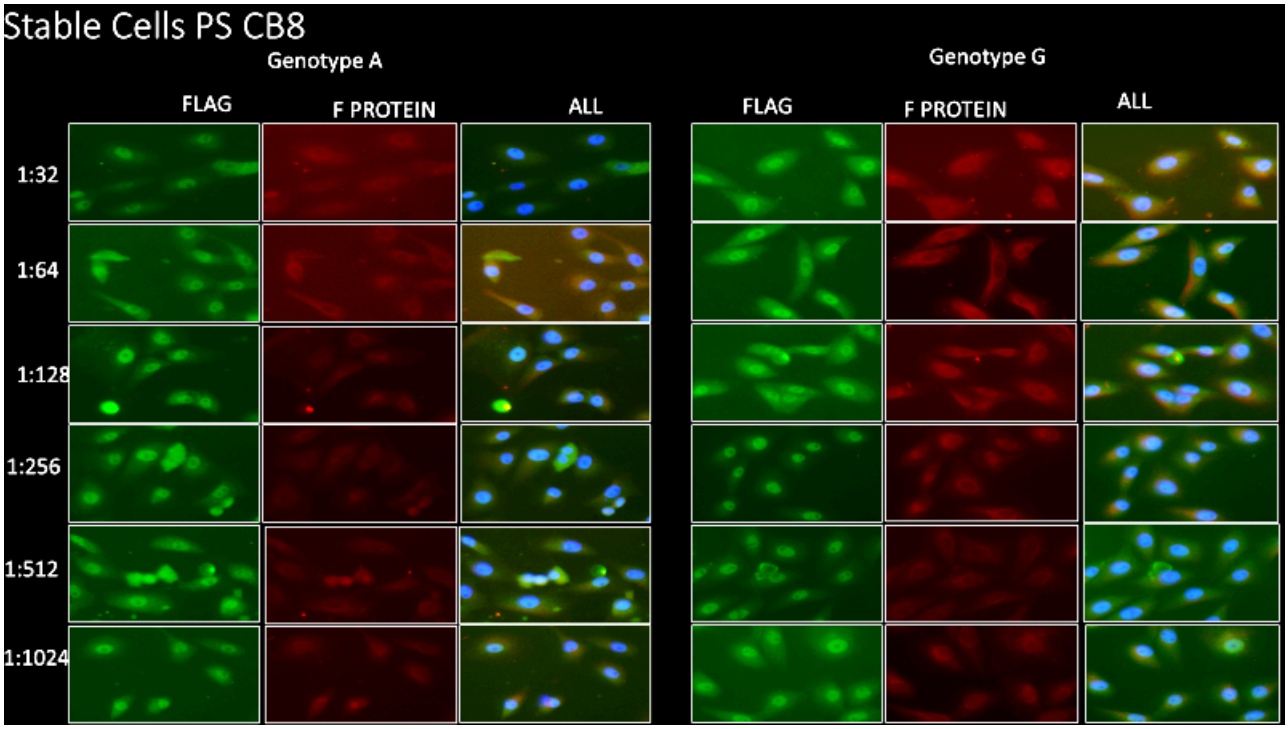


Figure A-80c. Stable cells Vaccine-Infected (CB) Sample#8.

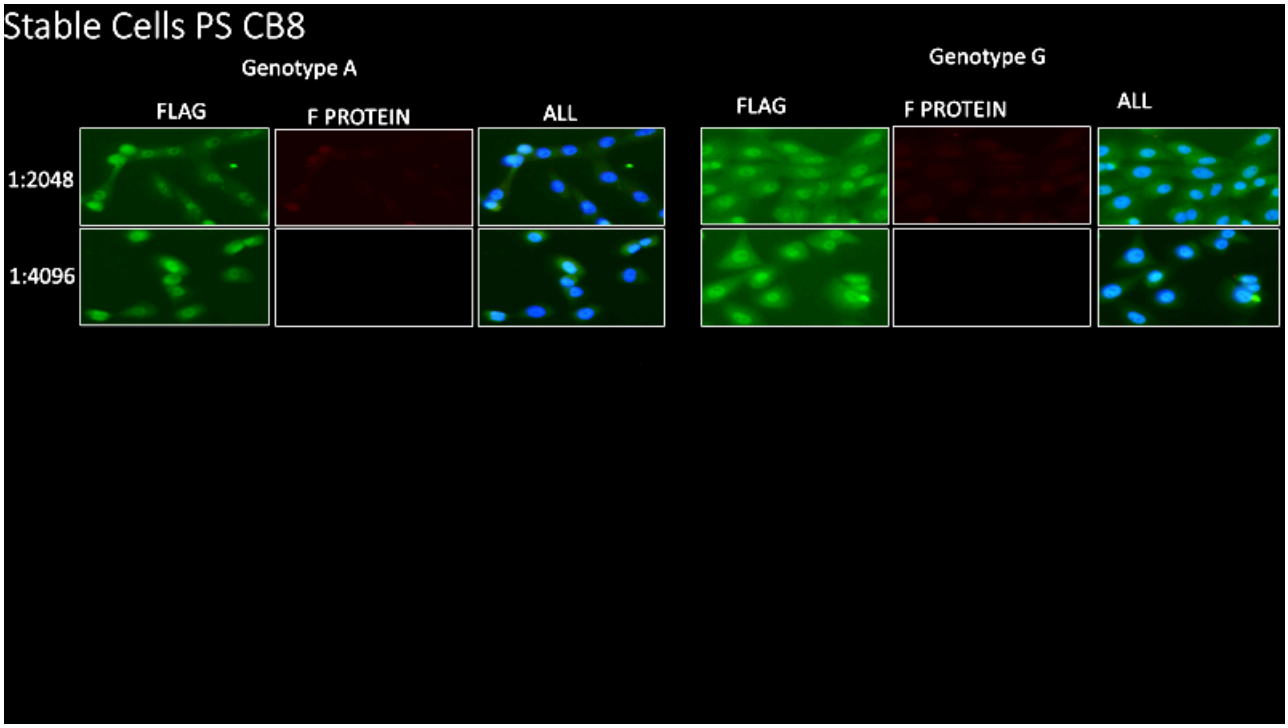


Figure A-80d. Stable cells Vaccine-Infected (CB) Sample#8.

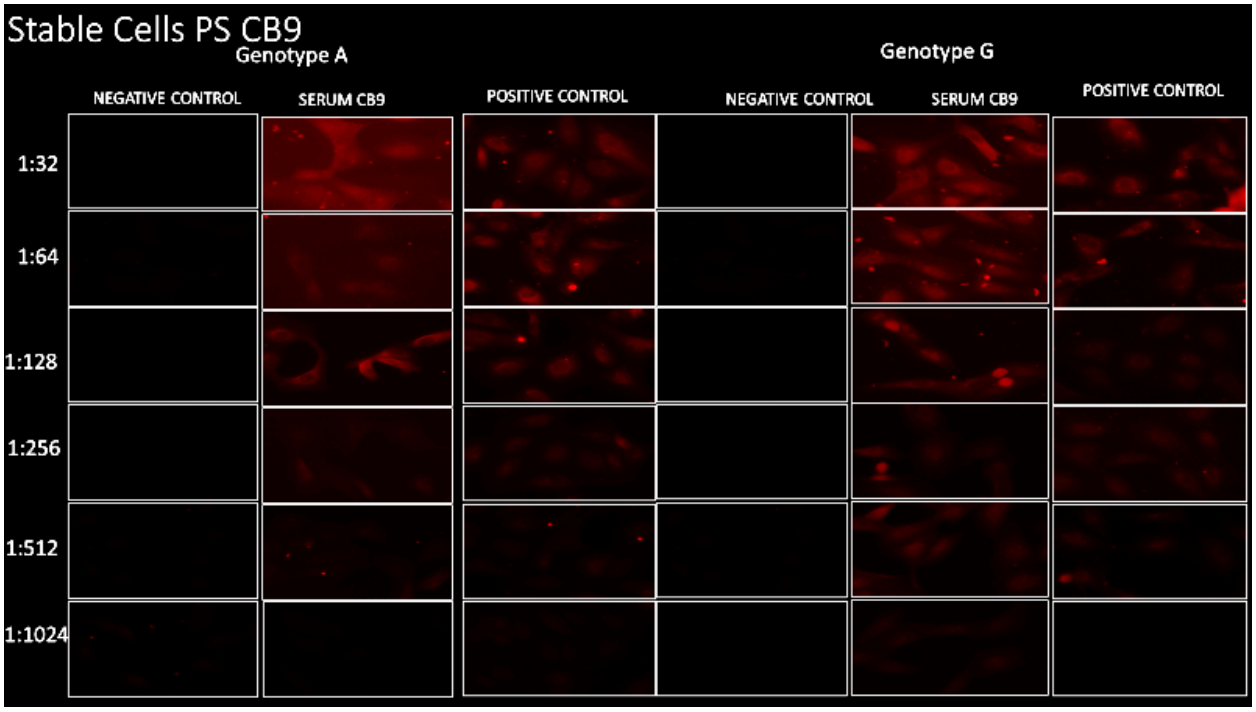


Figure A-81a. Stable cells Vaccine-Infected (CB) Sample#9.

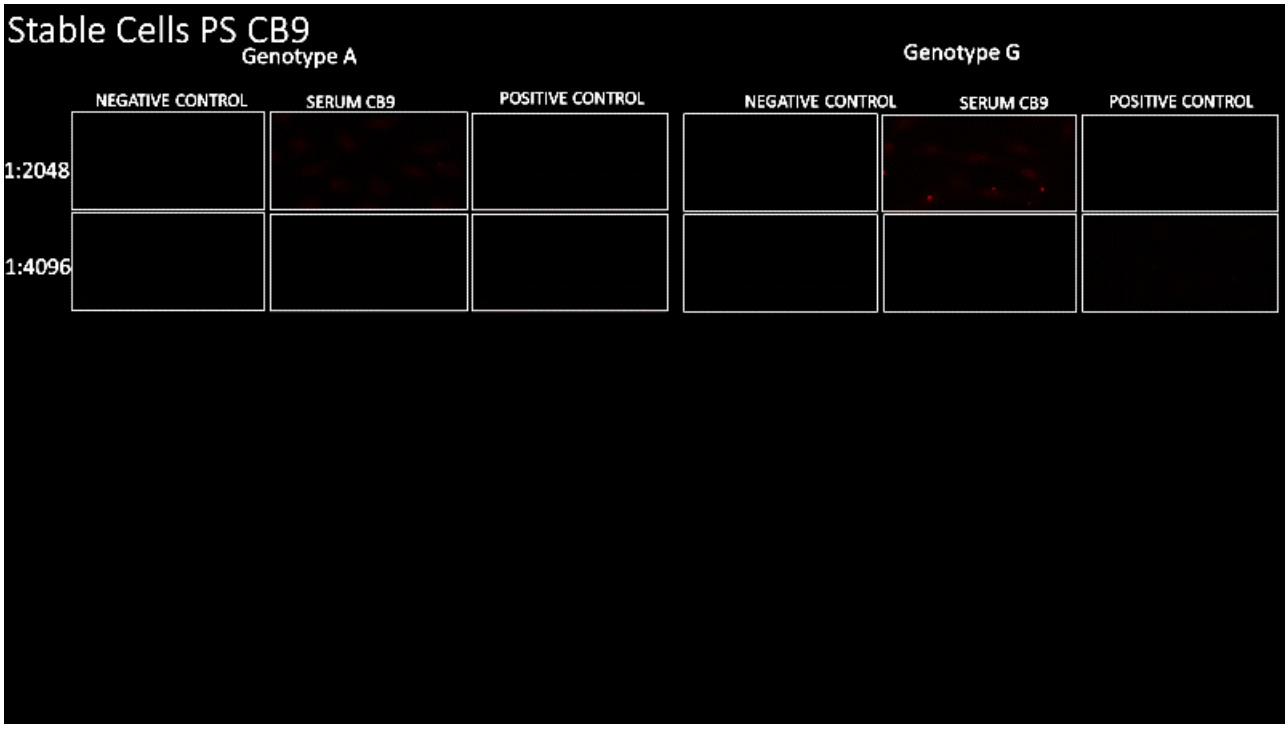


Figure A-81b. Stable cells Vaccine-Infected (CB) Sample#9.

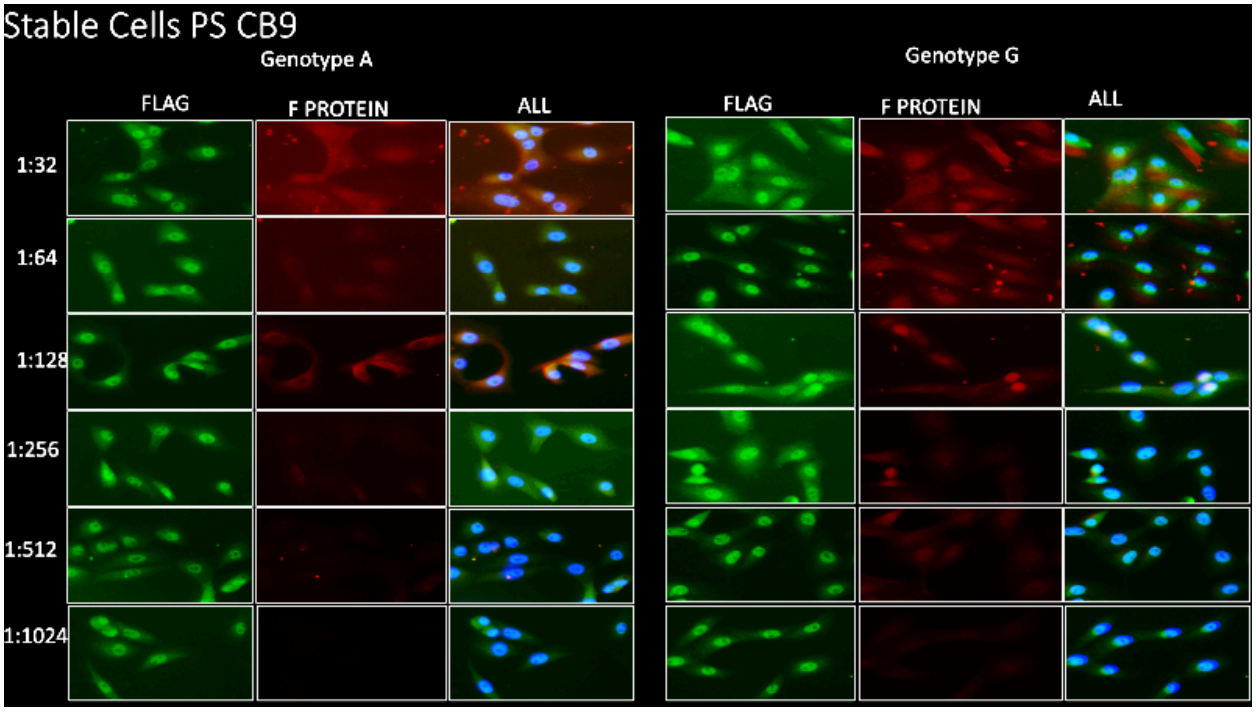


Figure A-81c. Stable cells Vaccine-Infected (CB) Sample#9.

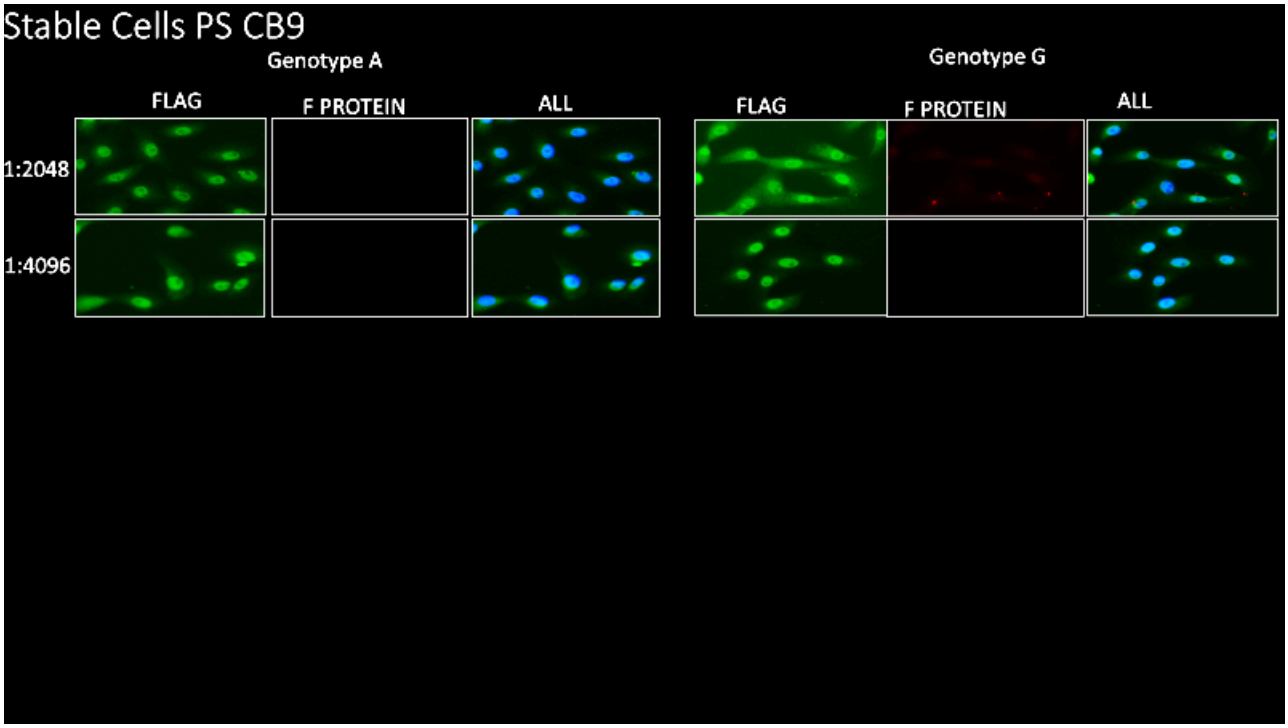


Figure A-81d. Stable cells Vaccine-Infected (CB) Sample#9.

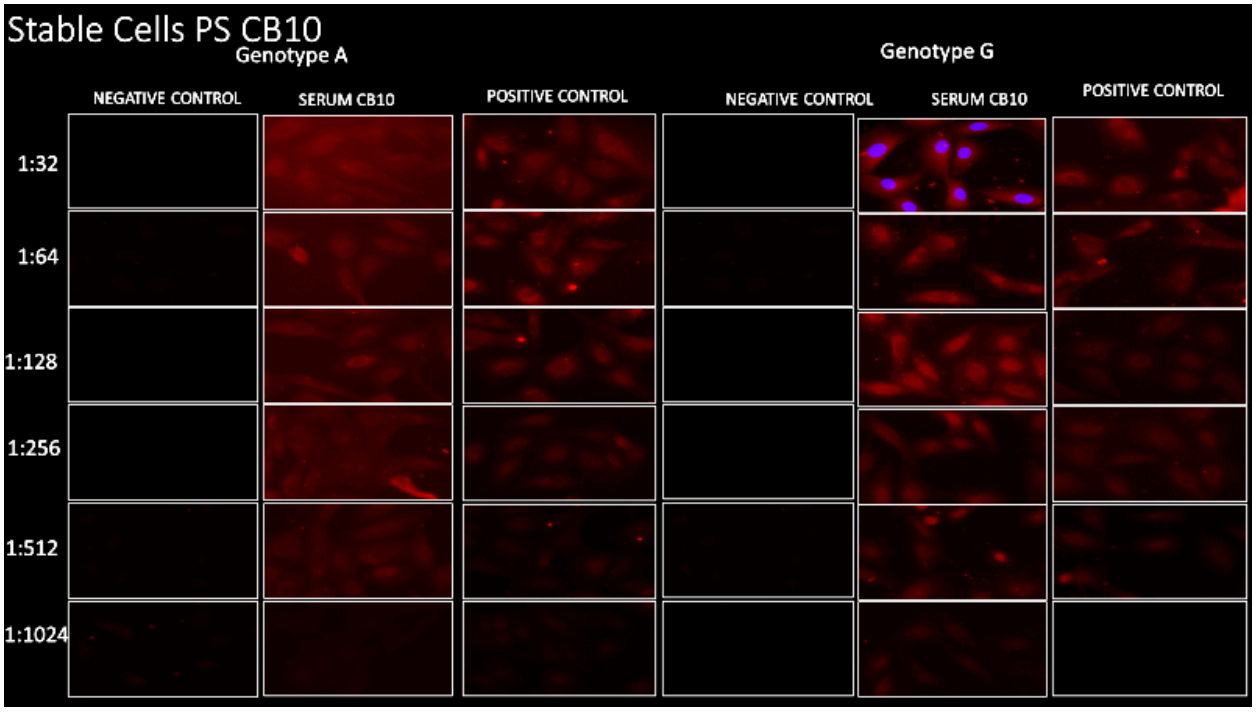


Figure A-82a. Stable cells Vaccine-Infected (CB) Sample#10.

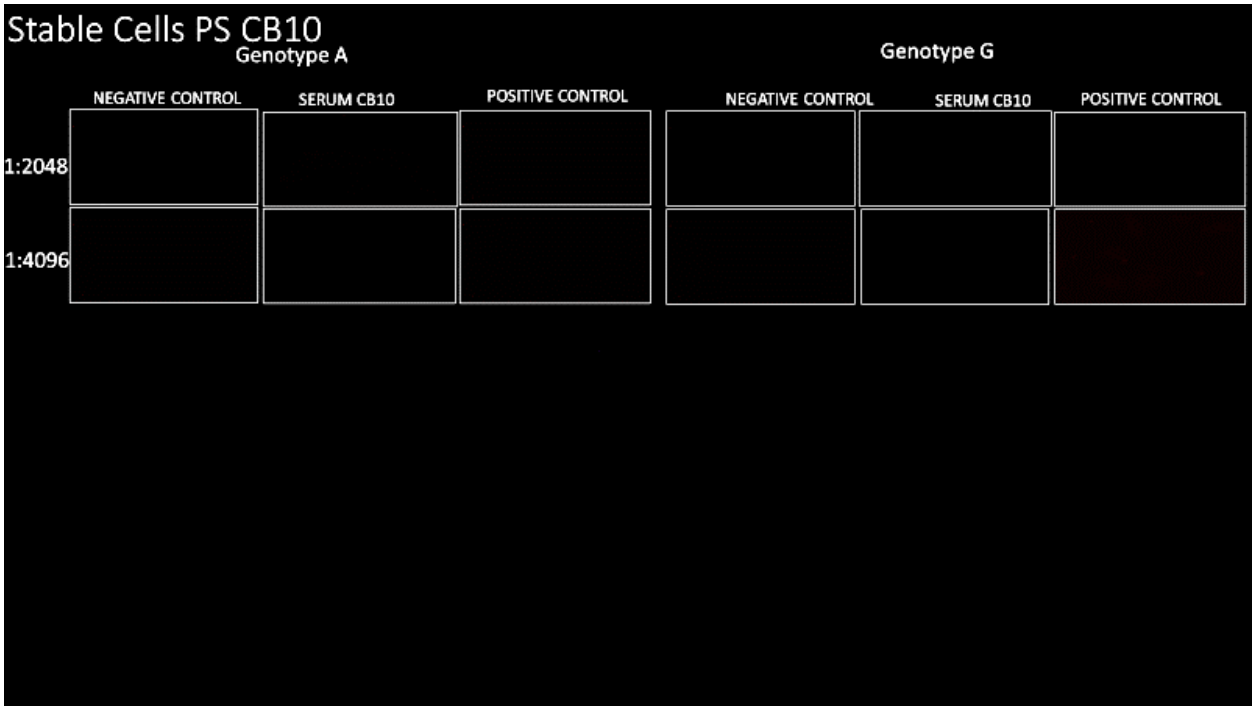


Figure A-82b. Stable cells Vaccine-Infected (CB) Sample#10.

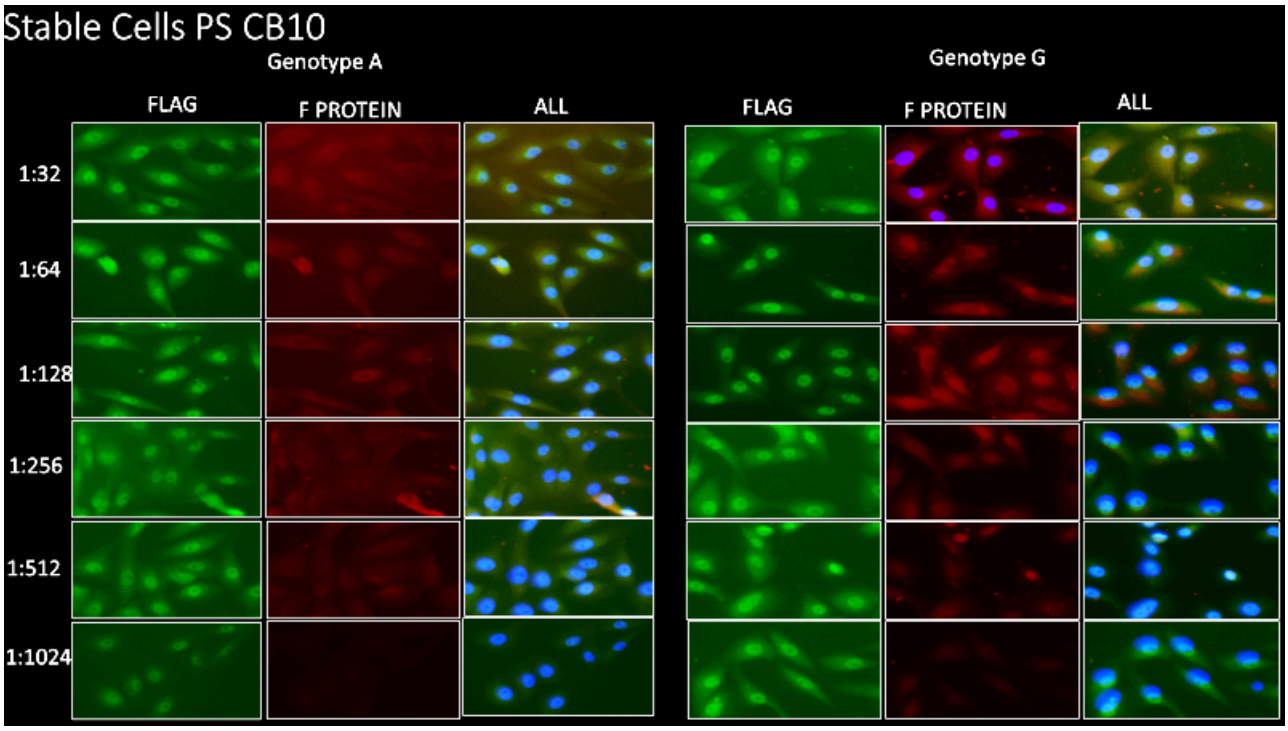


Figure A-82c. Stable cells Vaccine-Infected (CB) Sample#10.

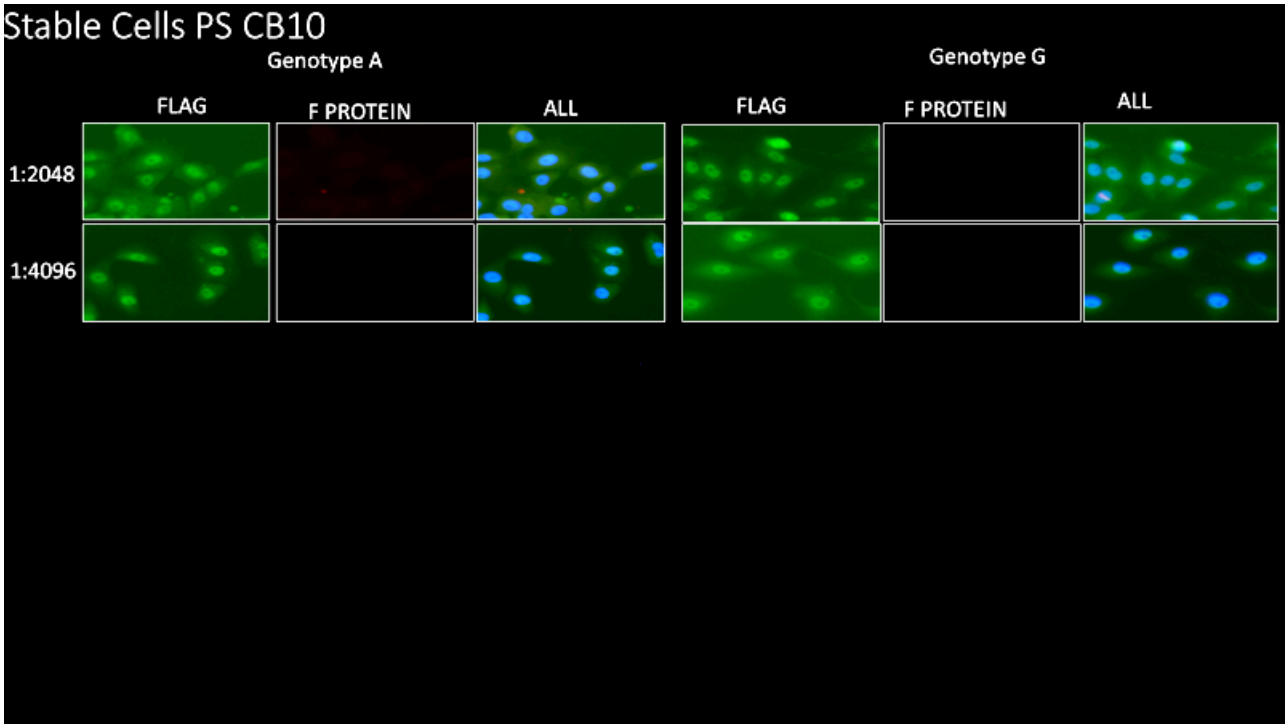


Figure A-82d. Stable cells Vaccine-Infected (CB) Sample#10.

Appendix:

IFA Results of HN Protein

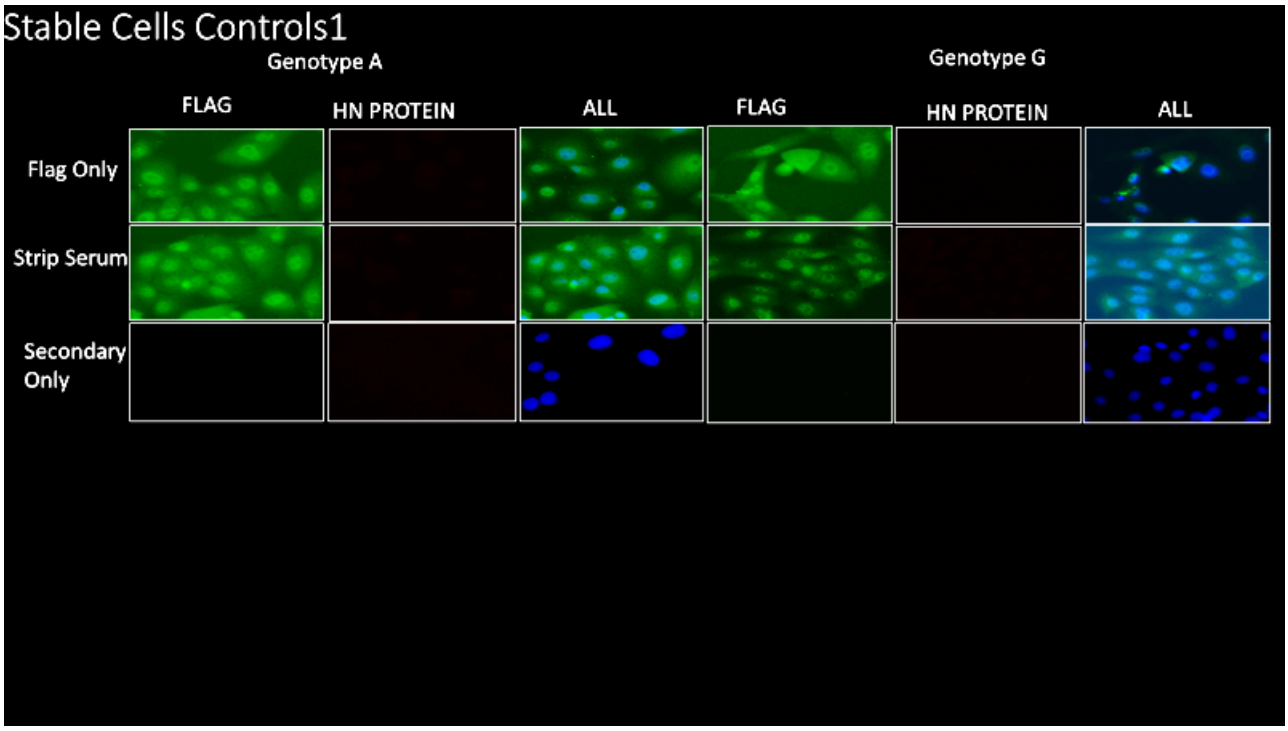


Figure A-1a. Stable cells ControlBatch#1.

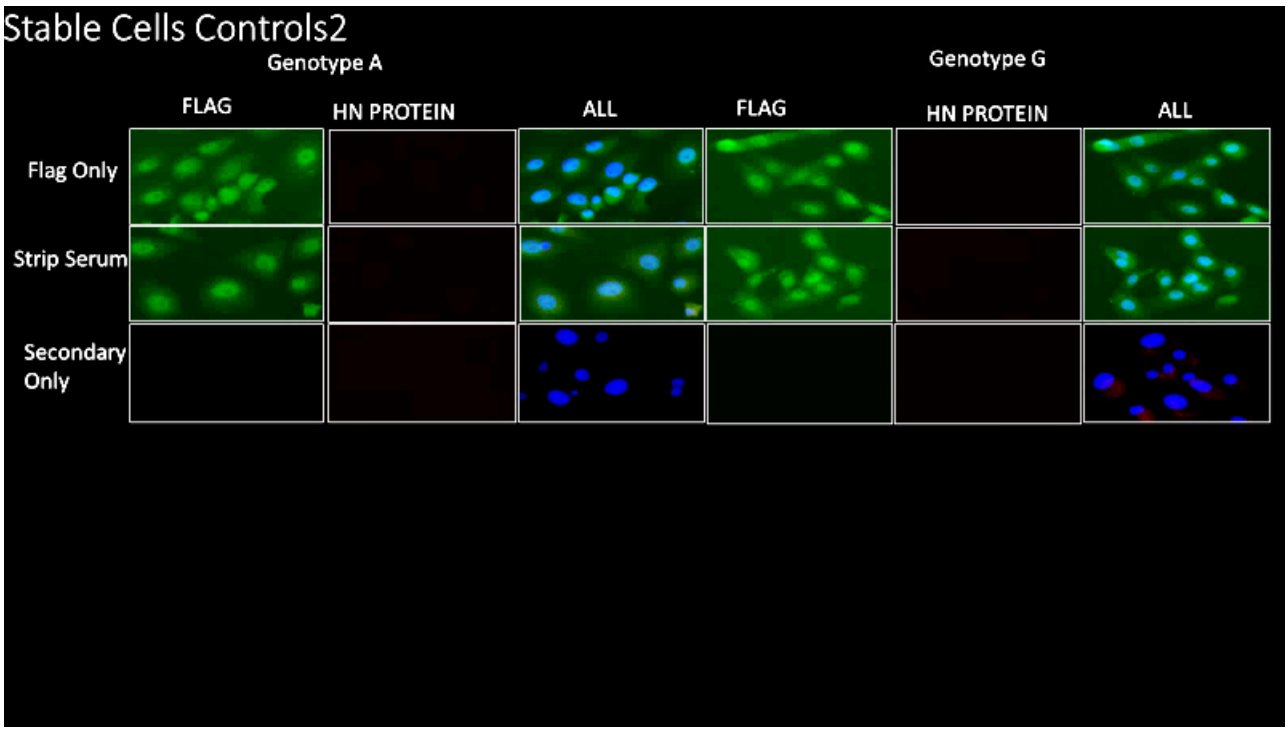


Figure A-1b. Stable cells ControlBatch#2.

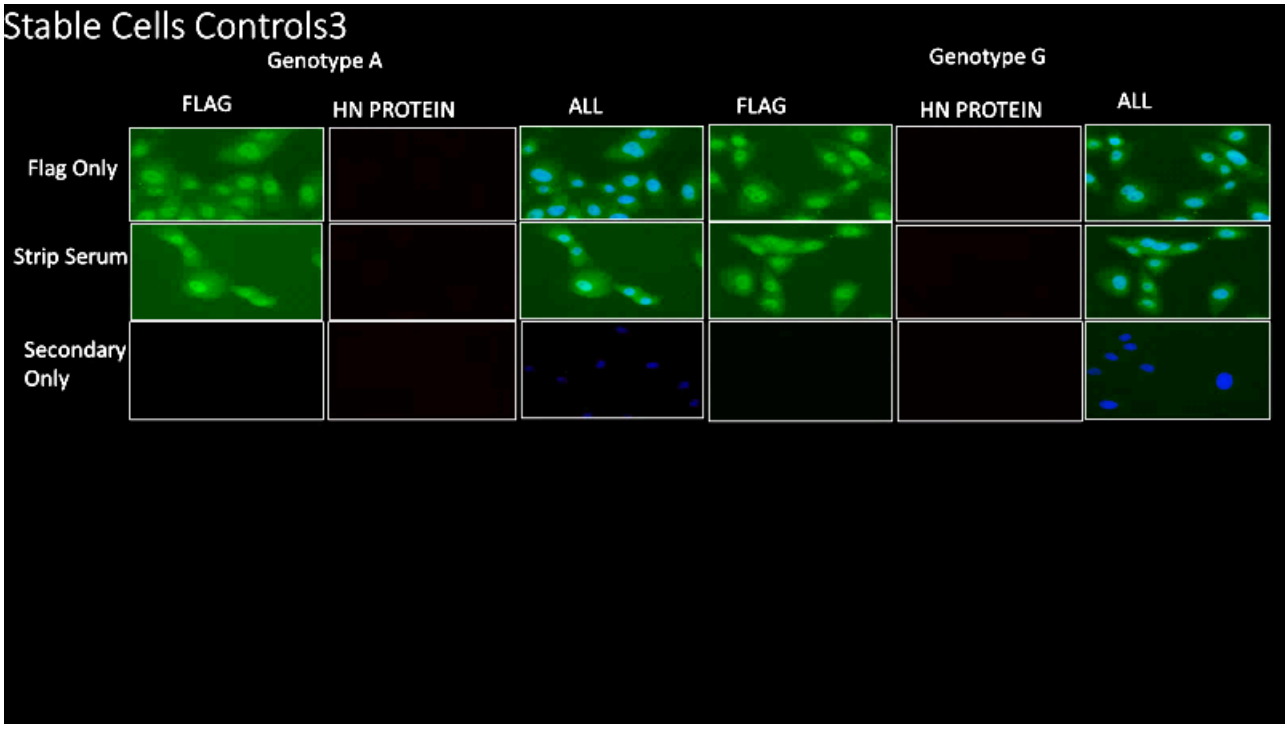


Figure A-1c. Stable cells ControlBatch#3.

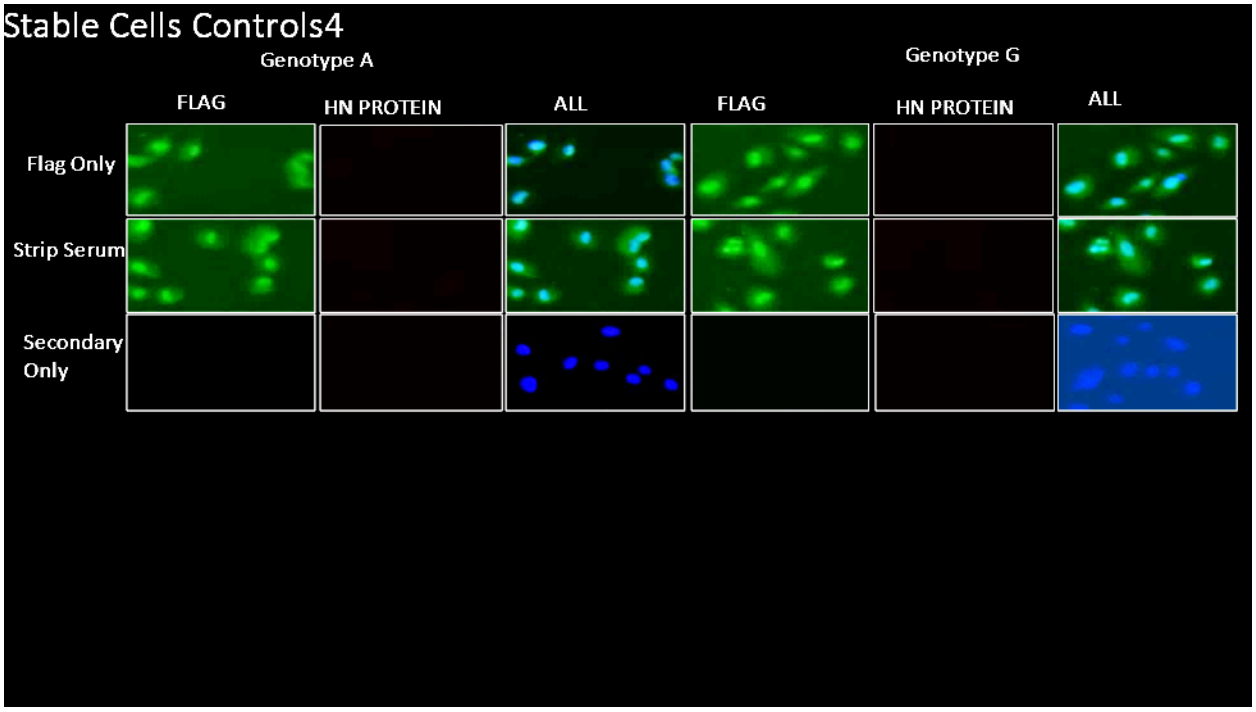


Figure A-1d. Stable cells ControlBatch#4.

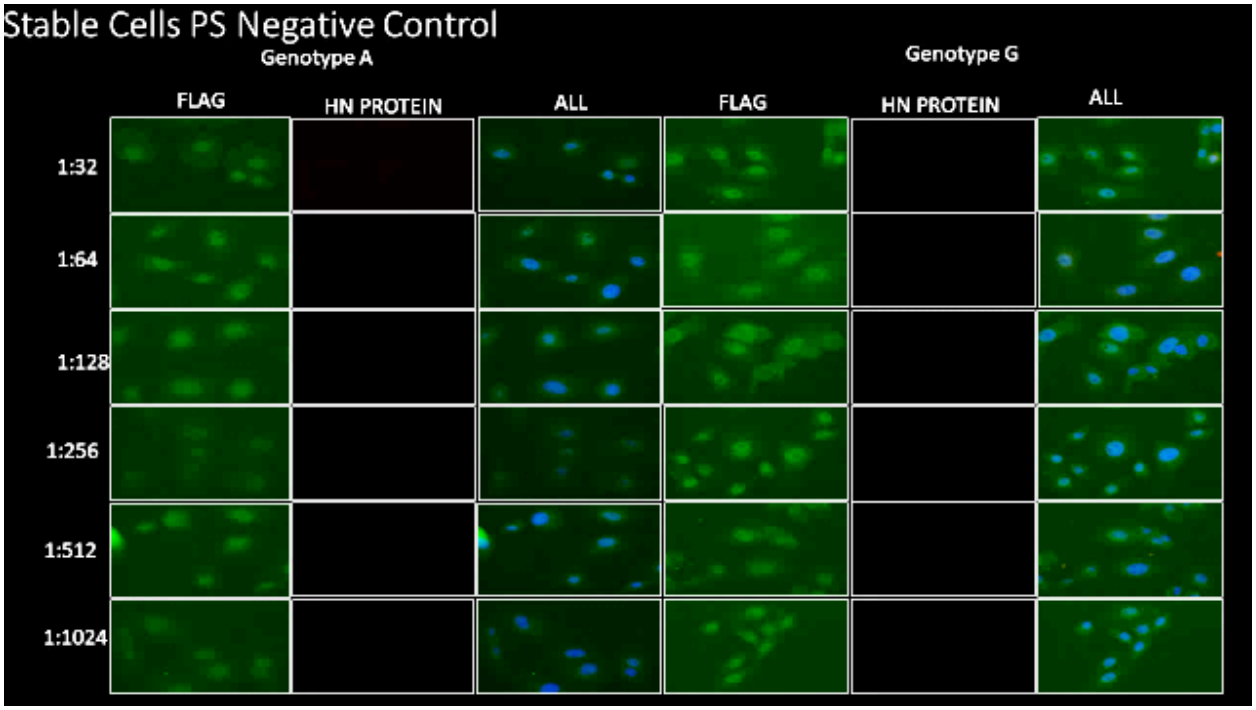


Figure A-2a. Stable cells Negative ControlBatch#1.

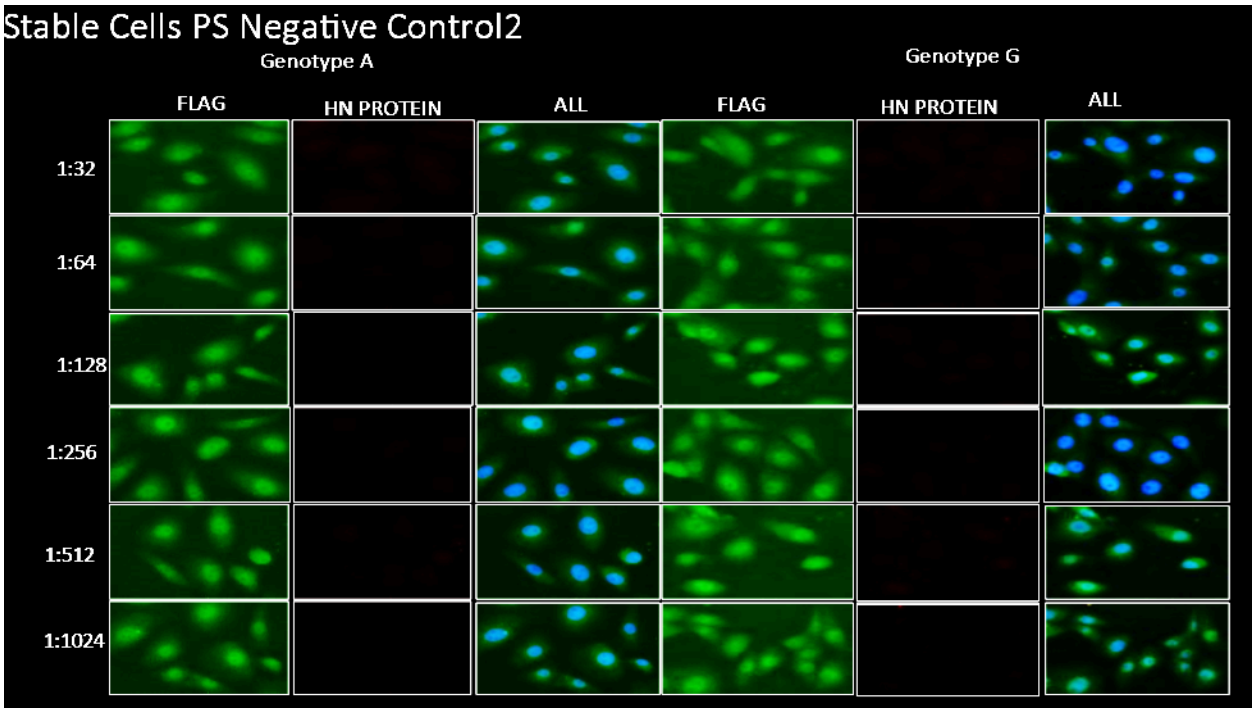


Figure A-2b. Stable cells Negative ControlBatch#2.

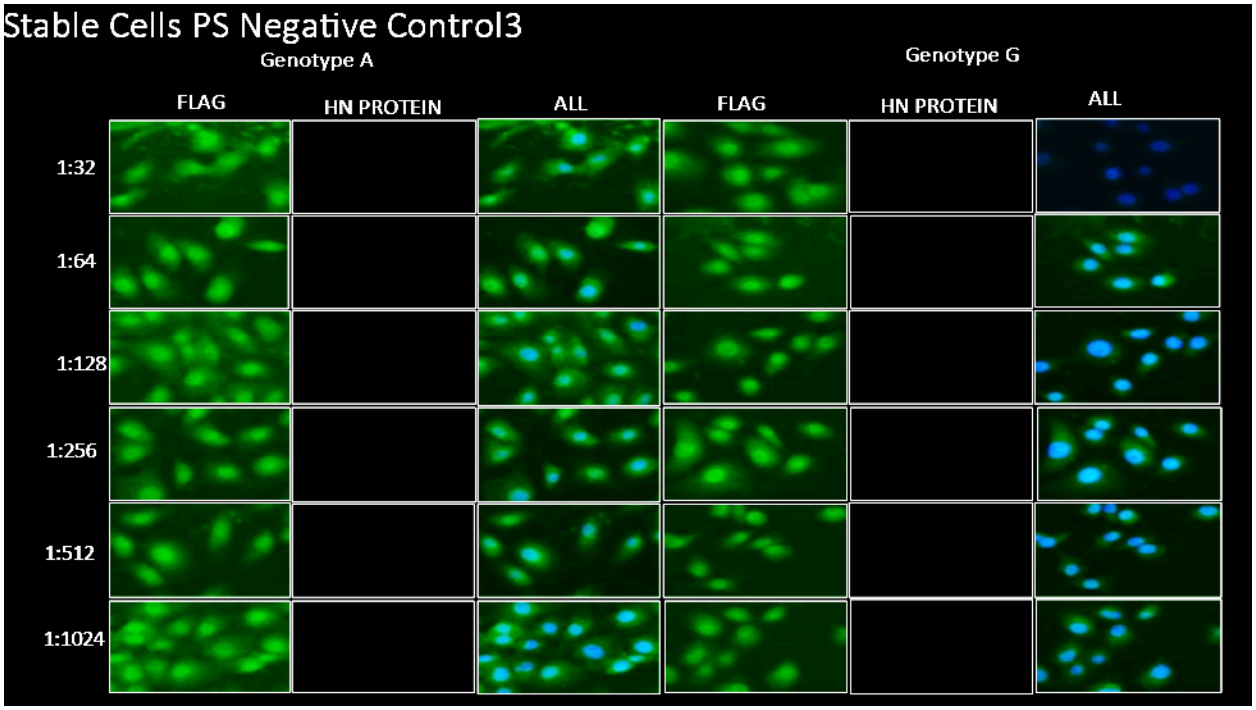


Figure A-2c. Stable cells Negative ControlBatch#3.

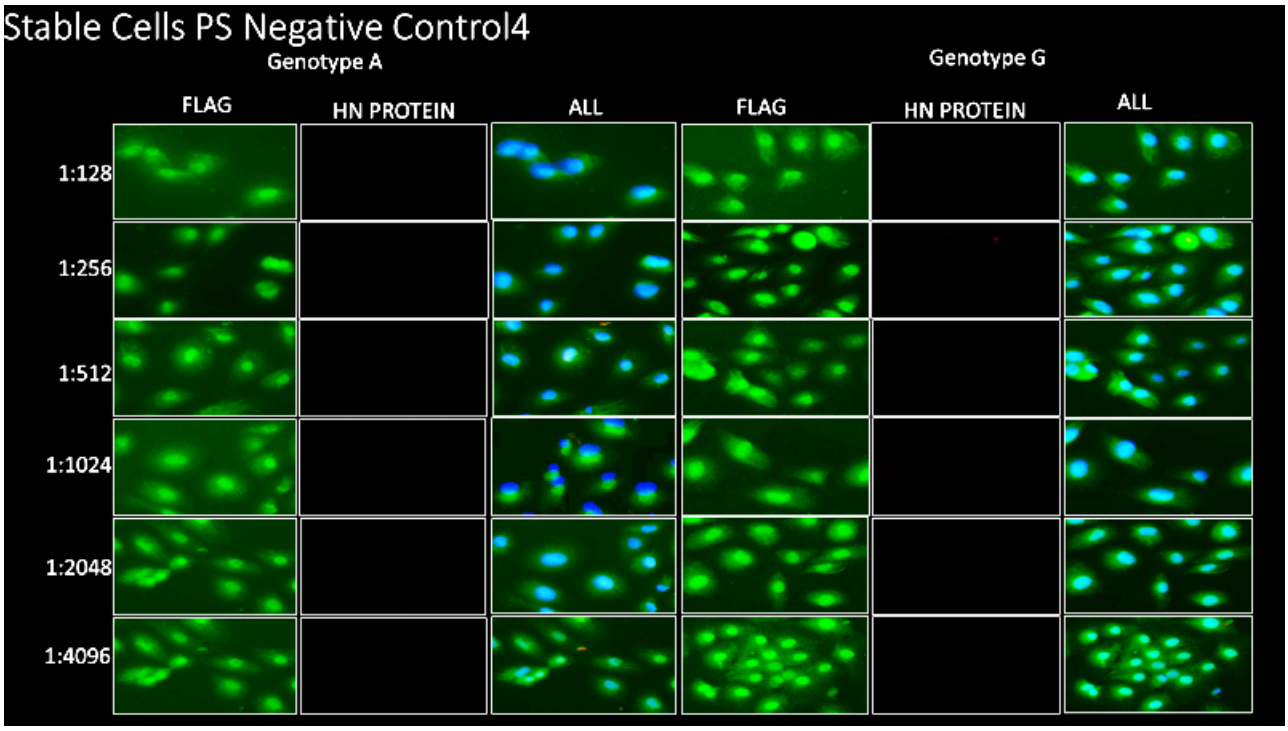


Figure A-2d. Stable cells Negative ControlBatch#4.

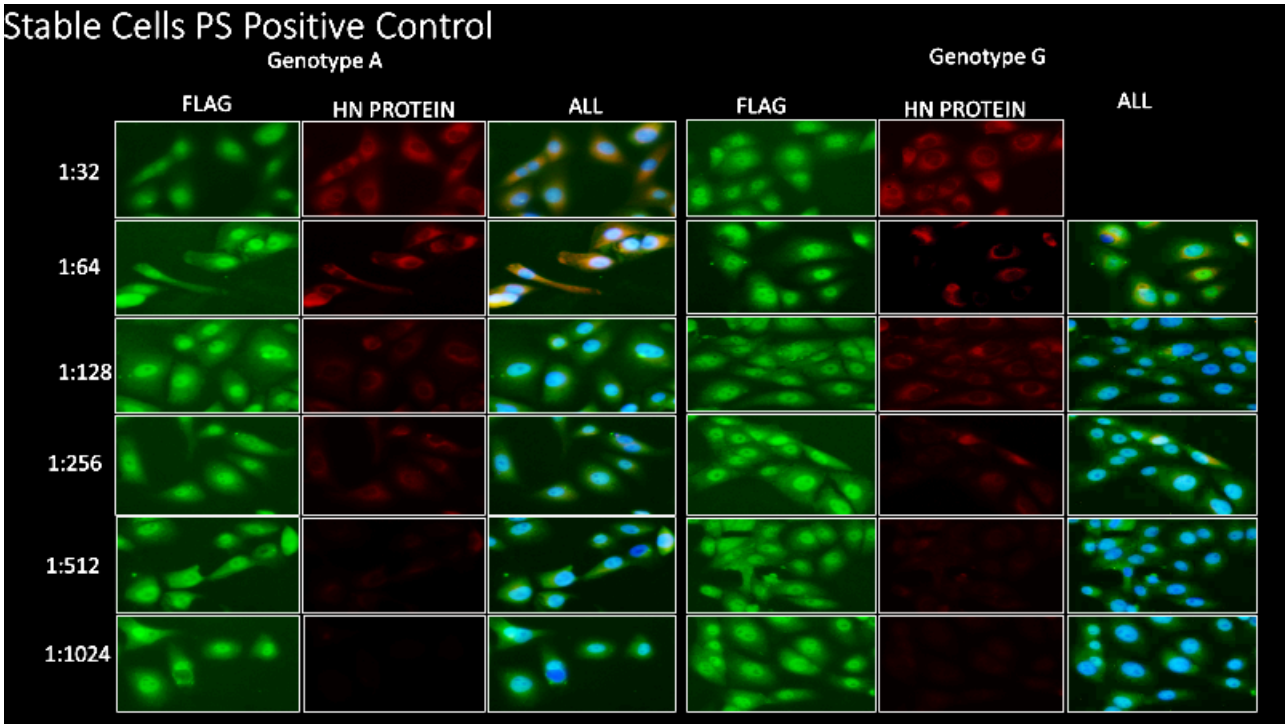


Figure A-3a. Stable cells Positive ControlBatch#1.

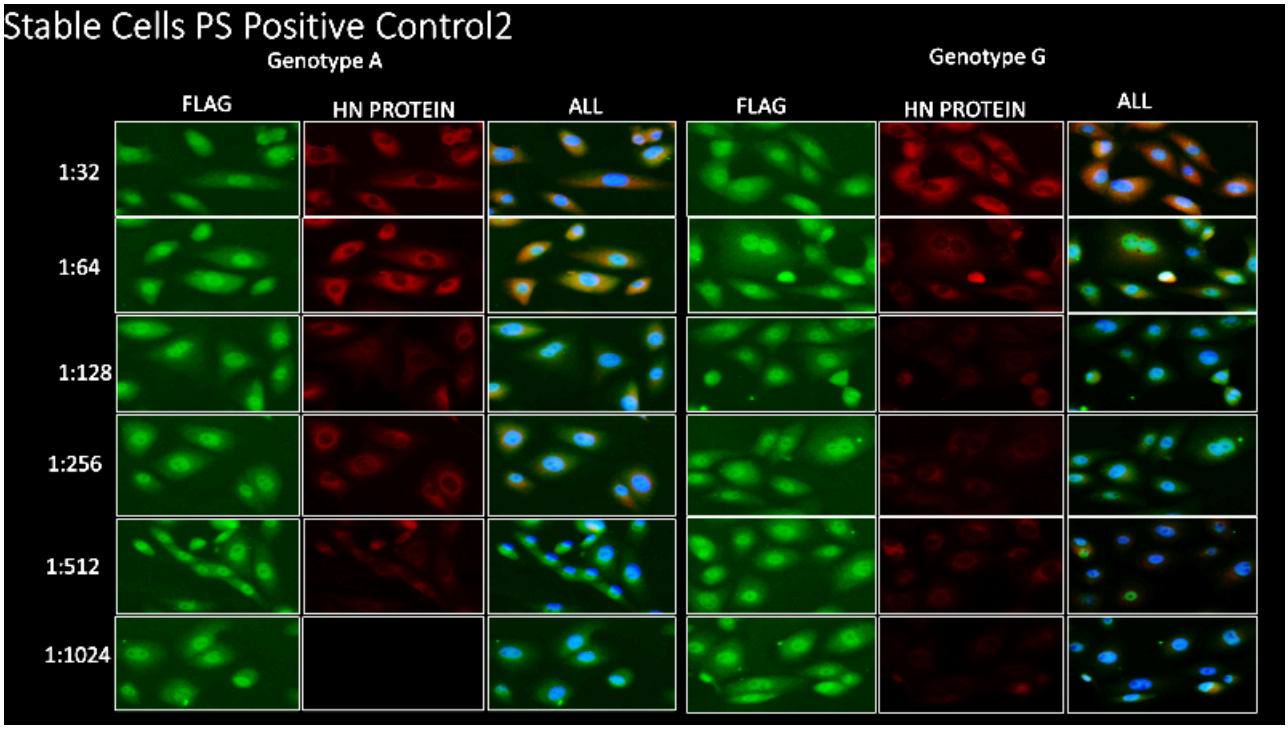


Figure A-3b. Stable cells Positive ControlBatch#2.

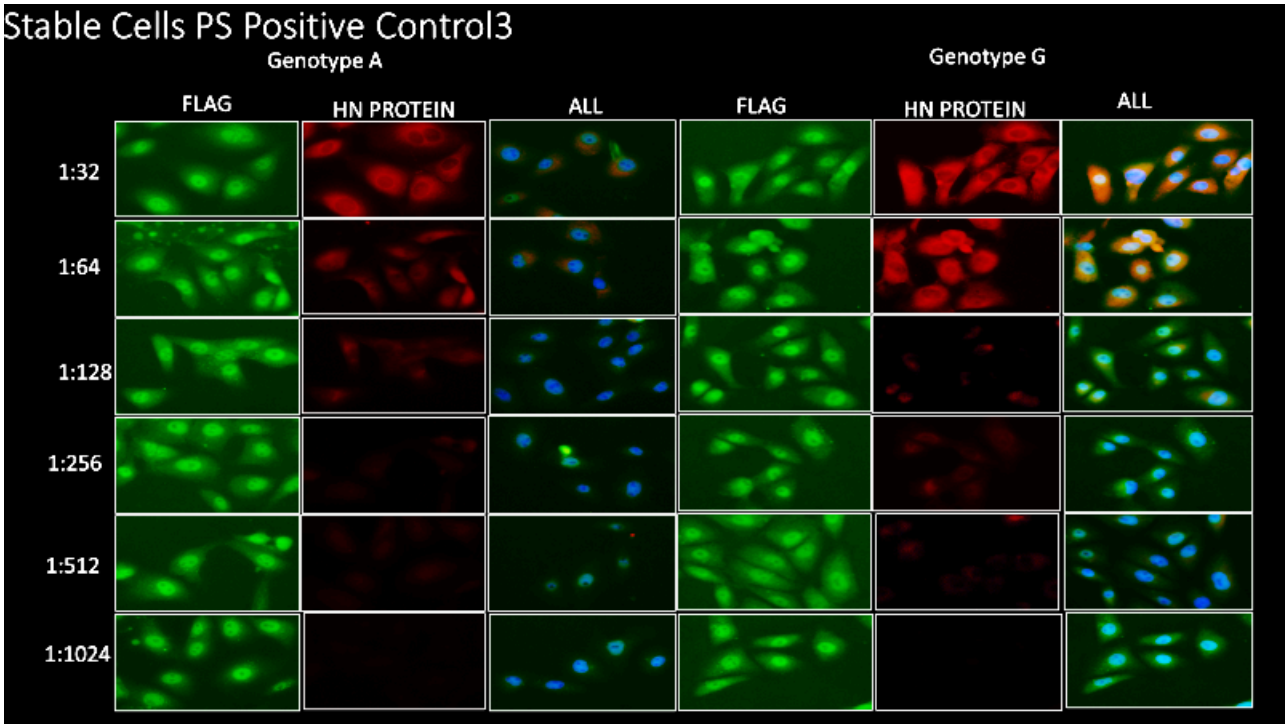


Figure A-3c. Stable cells Positive ControlBatch#3.

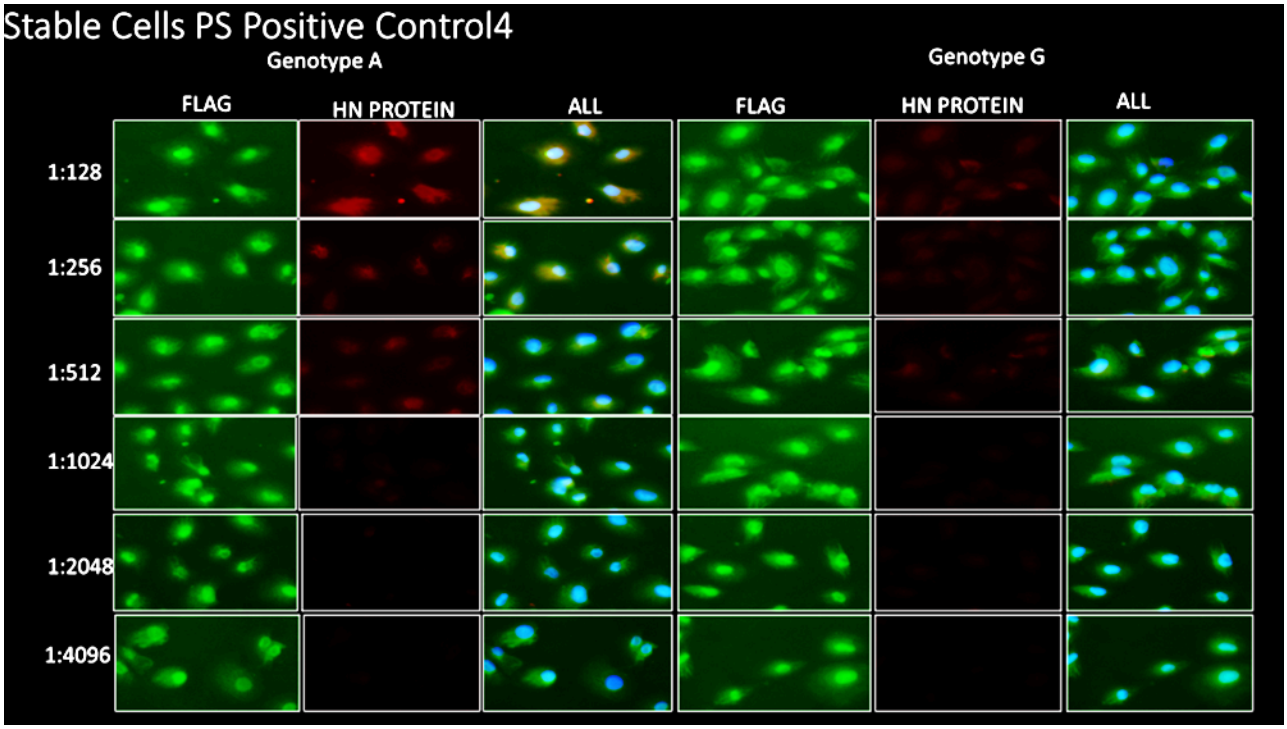


Figure A-3d. Stable cells Positive ControlBatch#4.

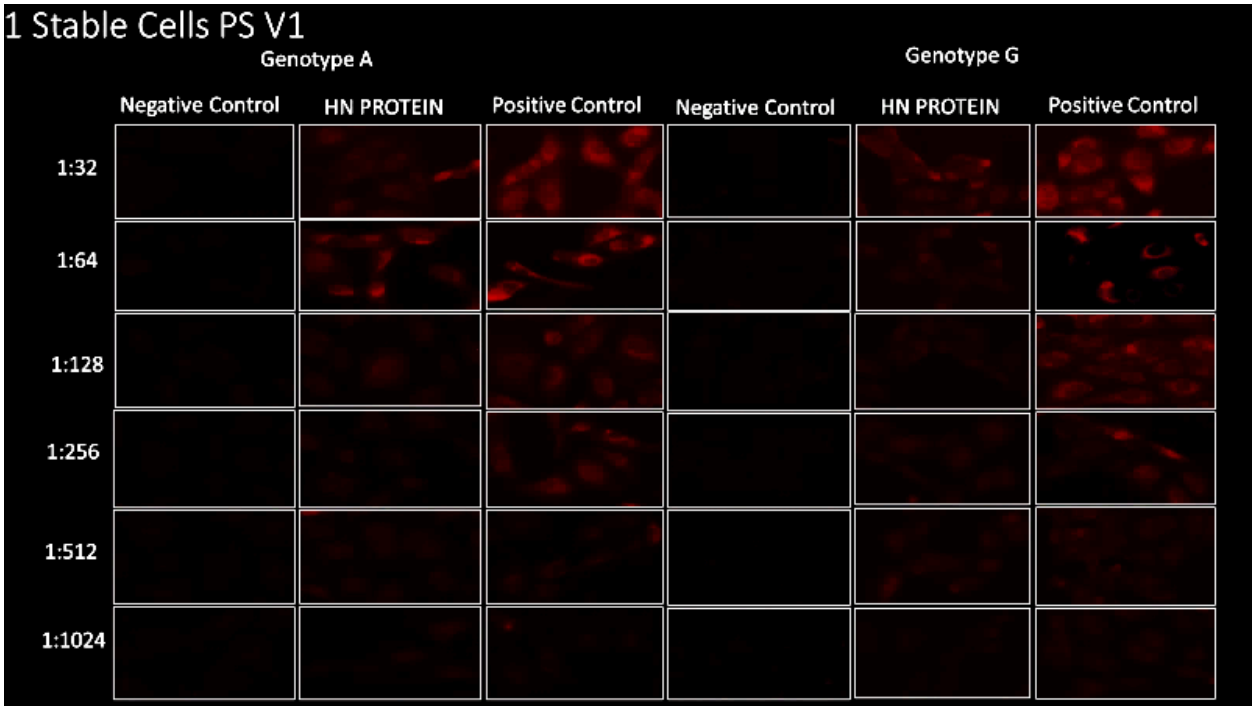


Figure A-4a. Stable cells Vaccine Sample#1.

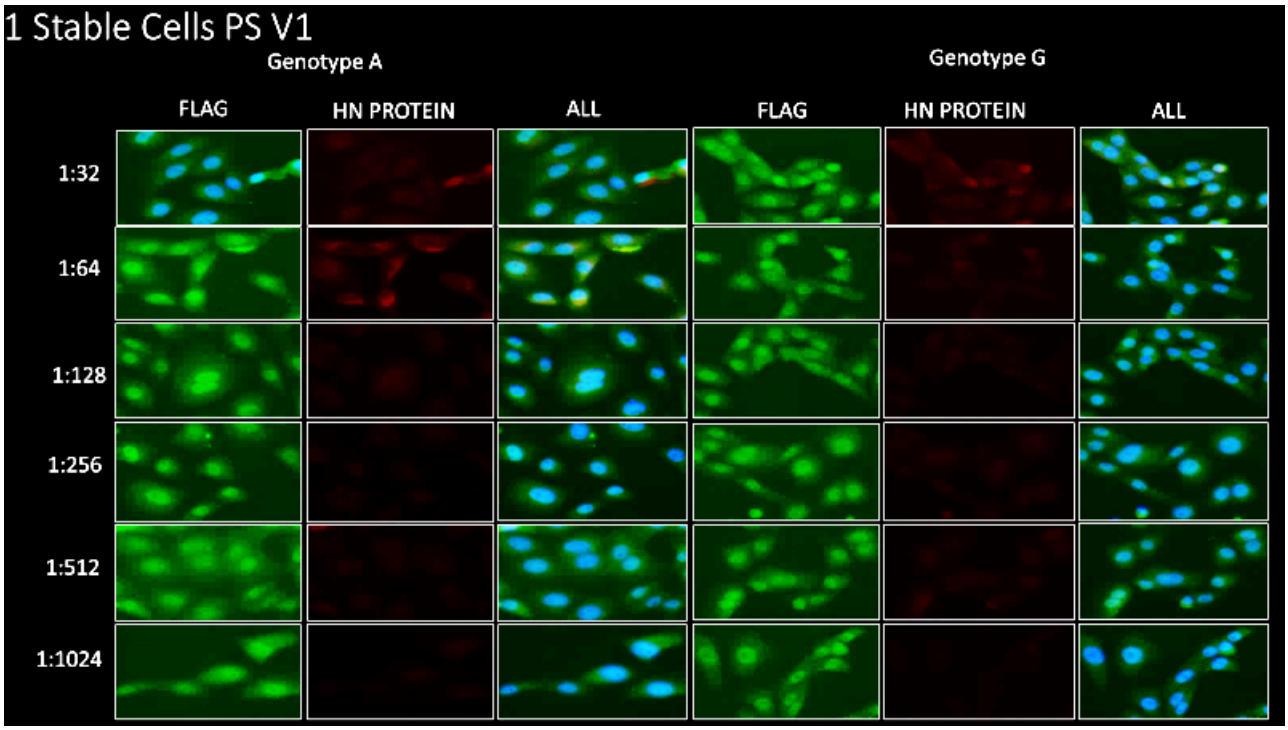


Figure A-4b. Stable cells Vaccine Sample #1.

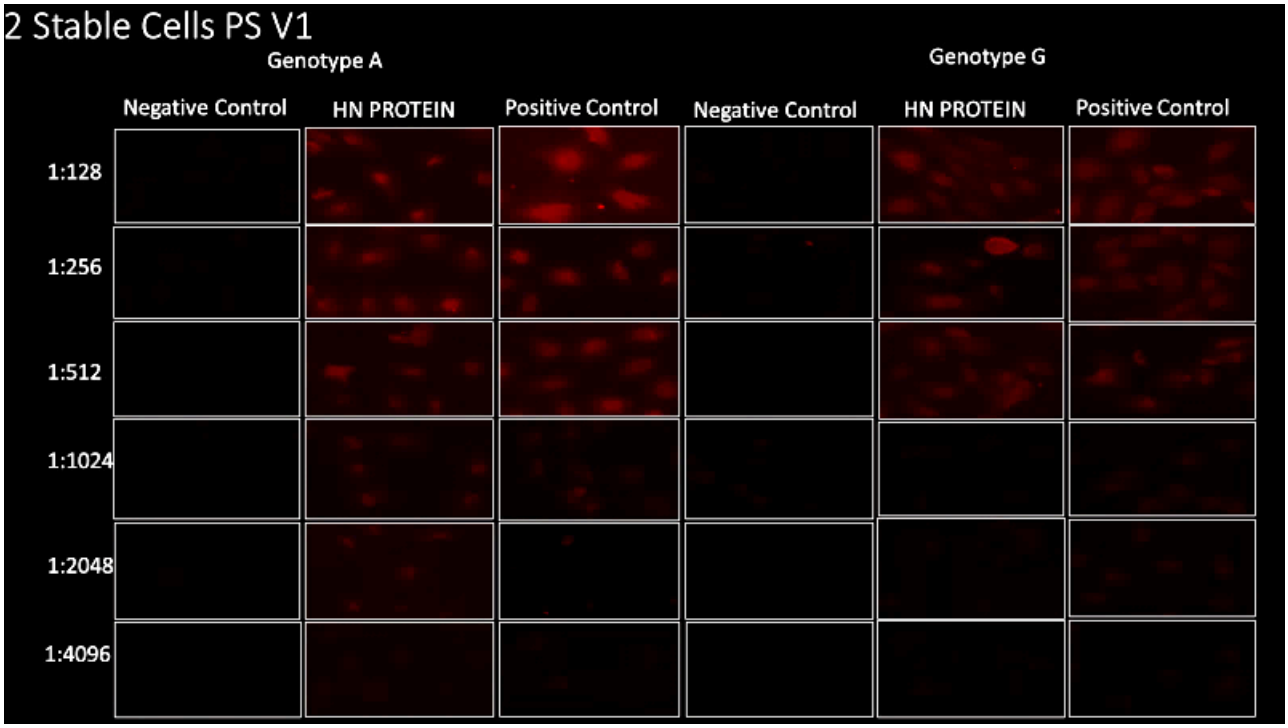


Figure A-4c. Stable cells Vaccine Sample #1.

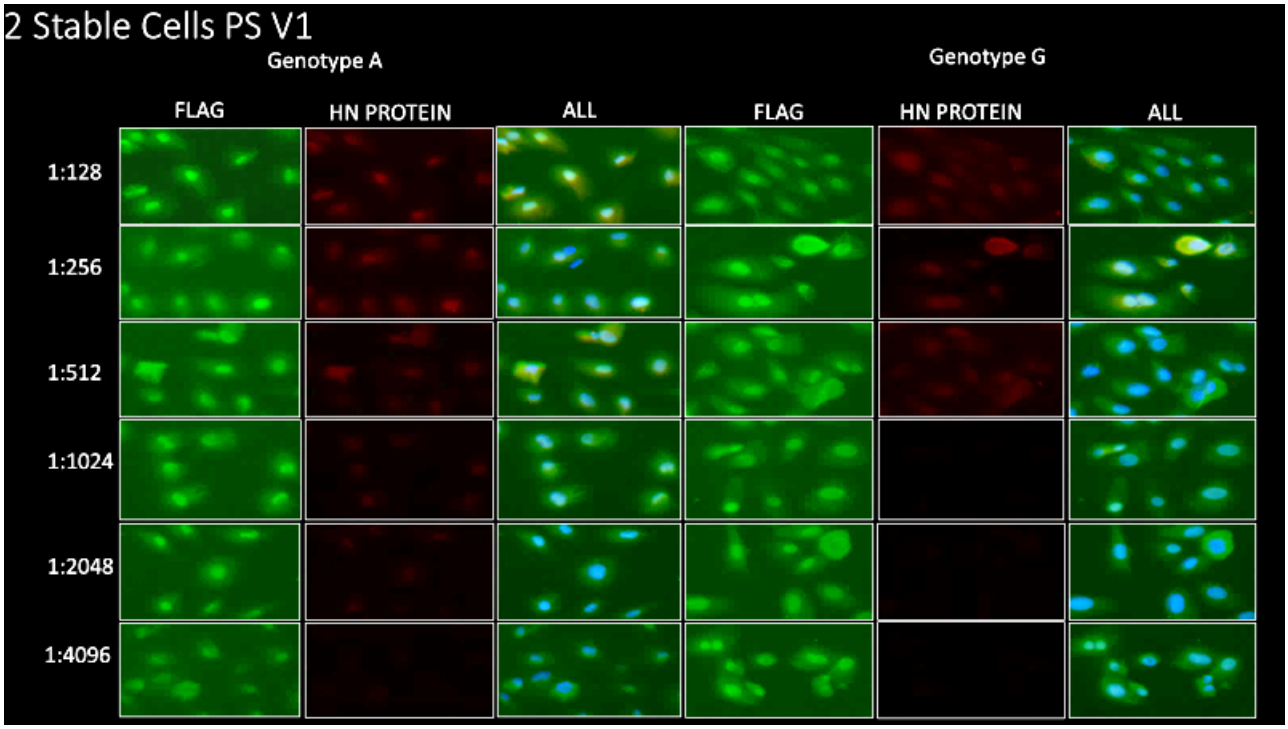


Figure A-4d. Stable cells Vaccine Sample #1.

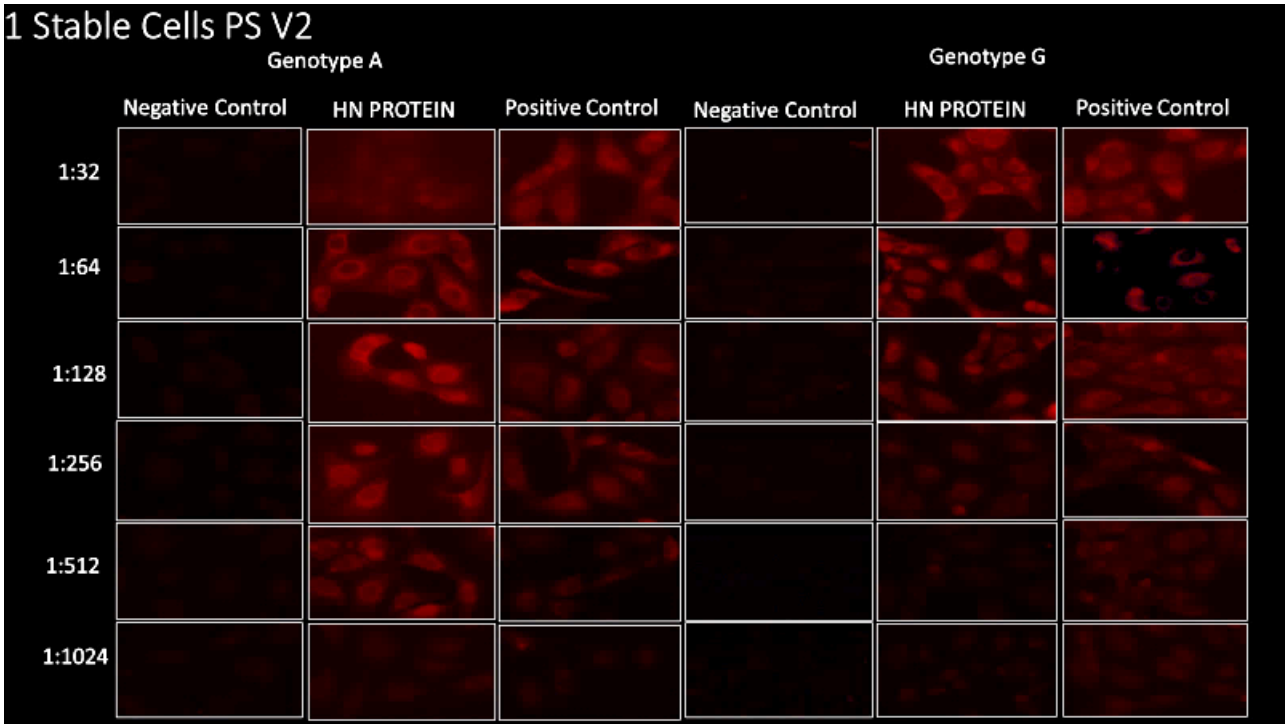


Figure A-5a. Stable cells Vaccine Sample#2.

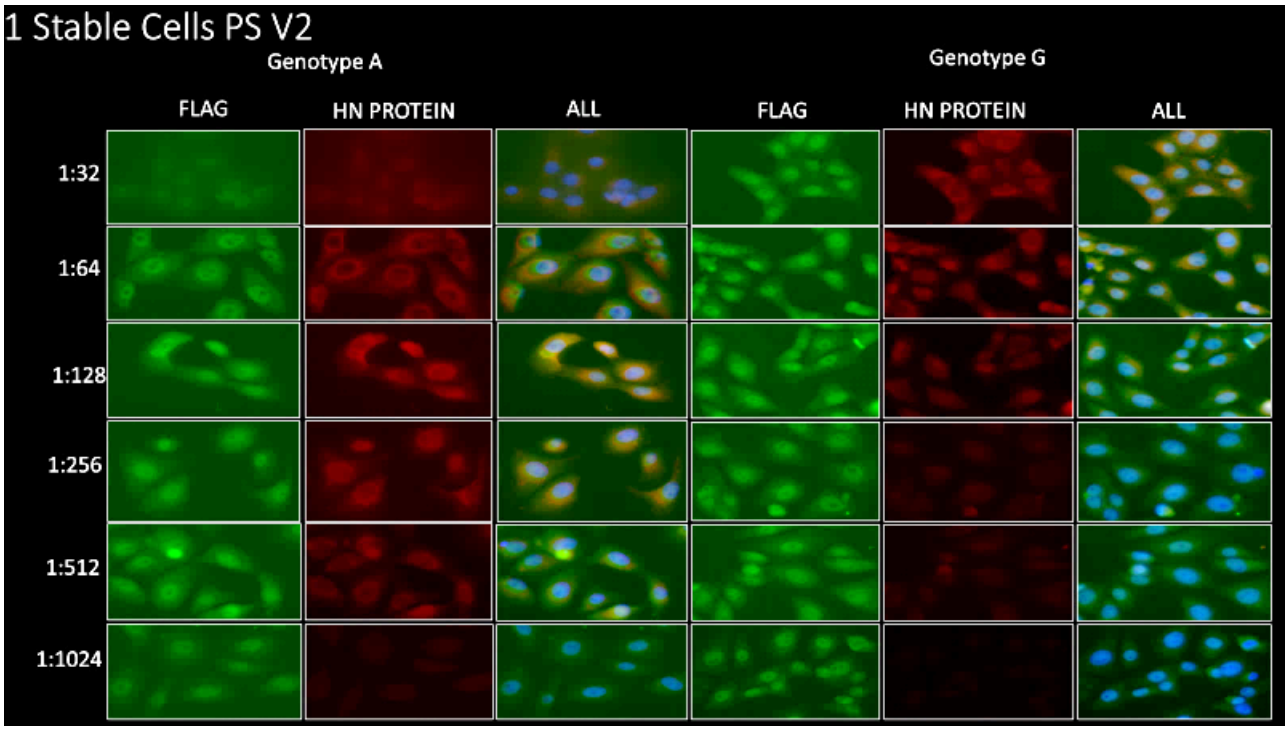


Figure A-5b. Stable cells Vaccine Sample #2.

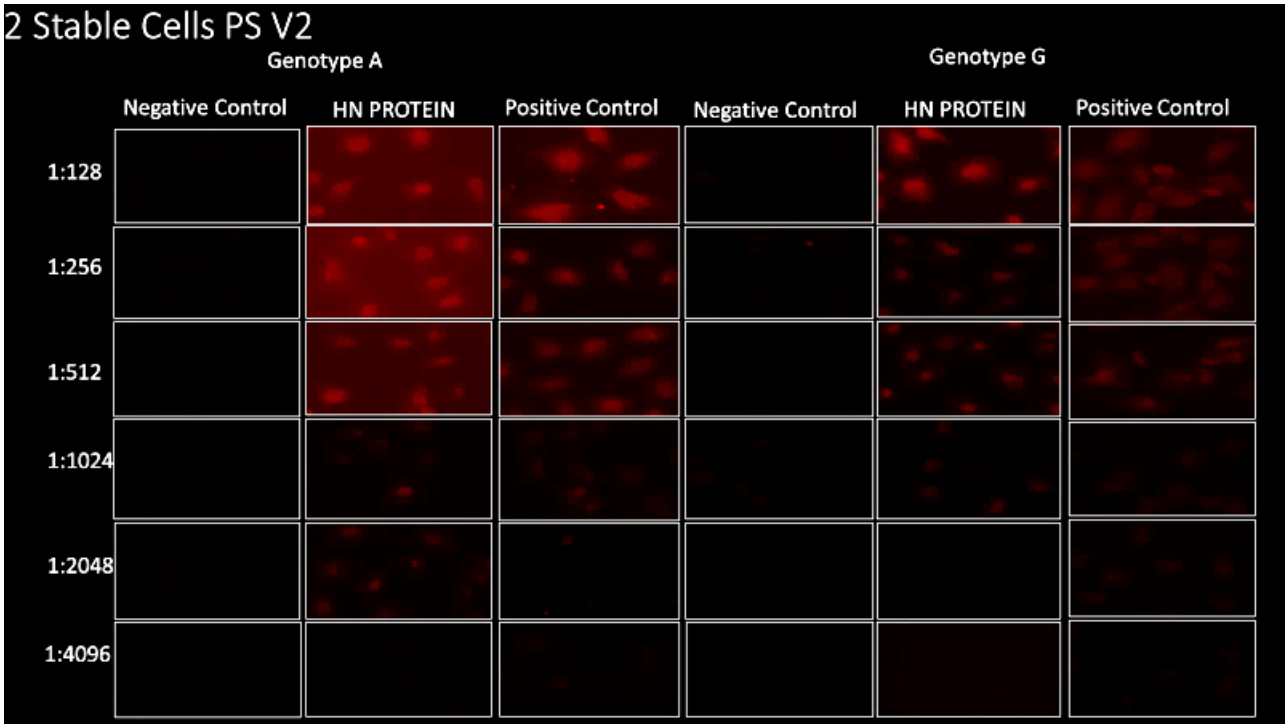


Figure A-5c. Stable cells Vaccine Sample #2.

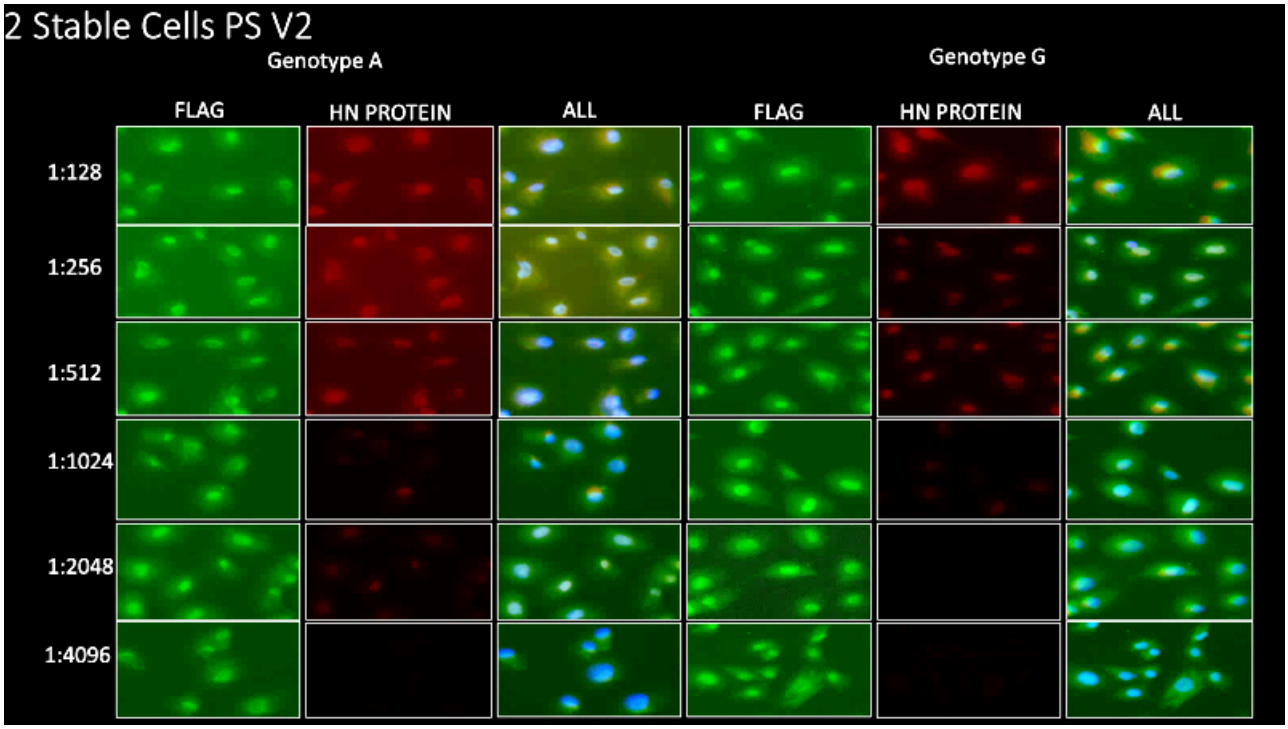


Figure A-5d. Stable cells Vaccine Sample #2.

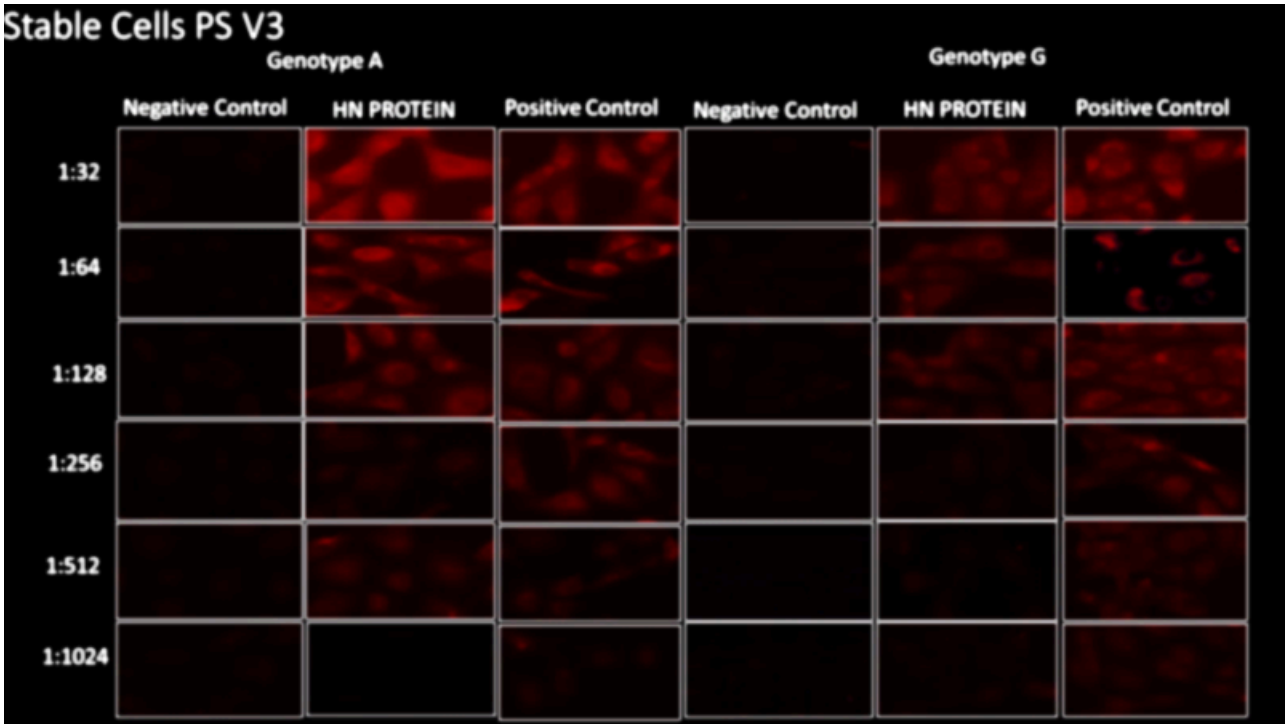


Figure A-6a. Stable cells Vaccine Sample#3.

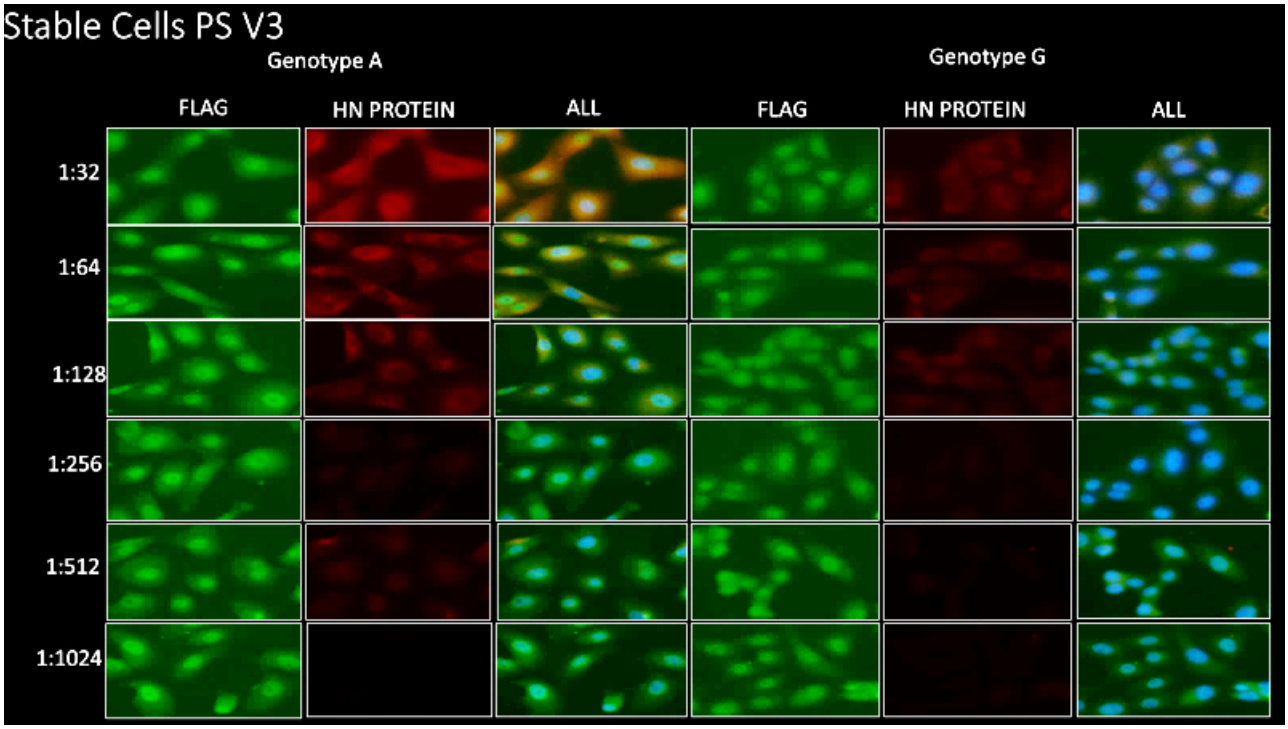


Figure A-6b. Stable cells Vaccine Sample #3.

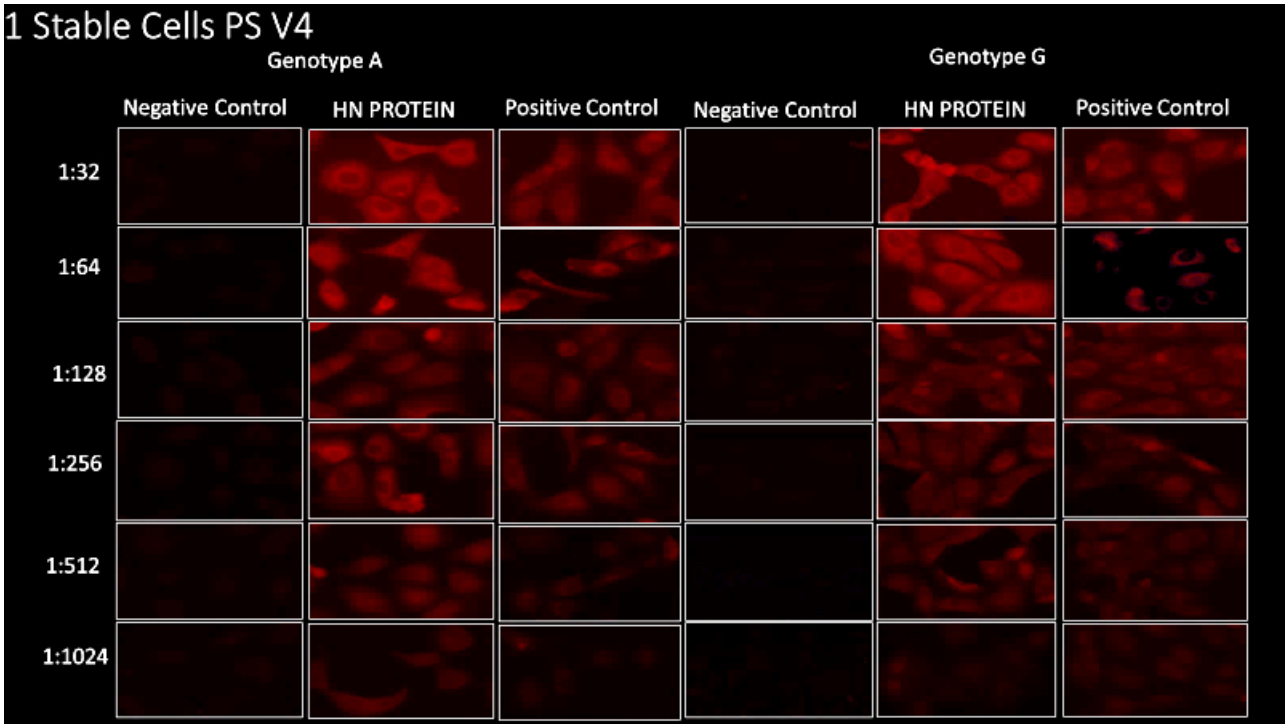


Figure A-7a. Stable cells Vaccine Sample#4.

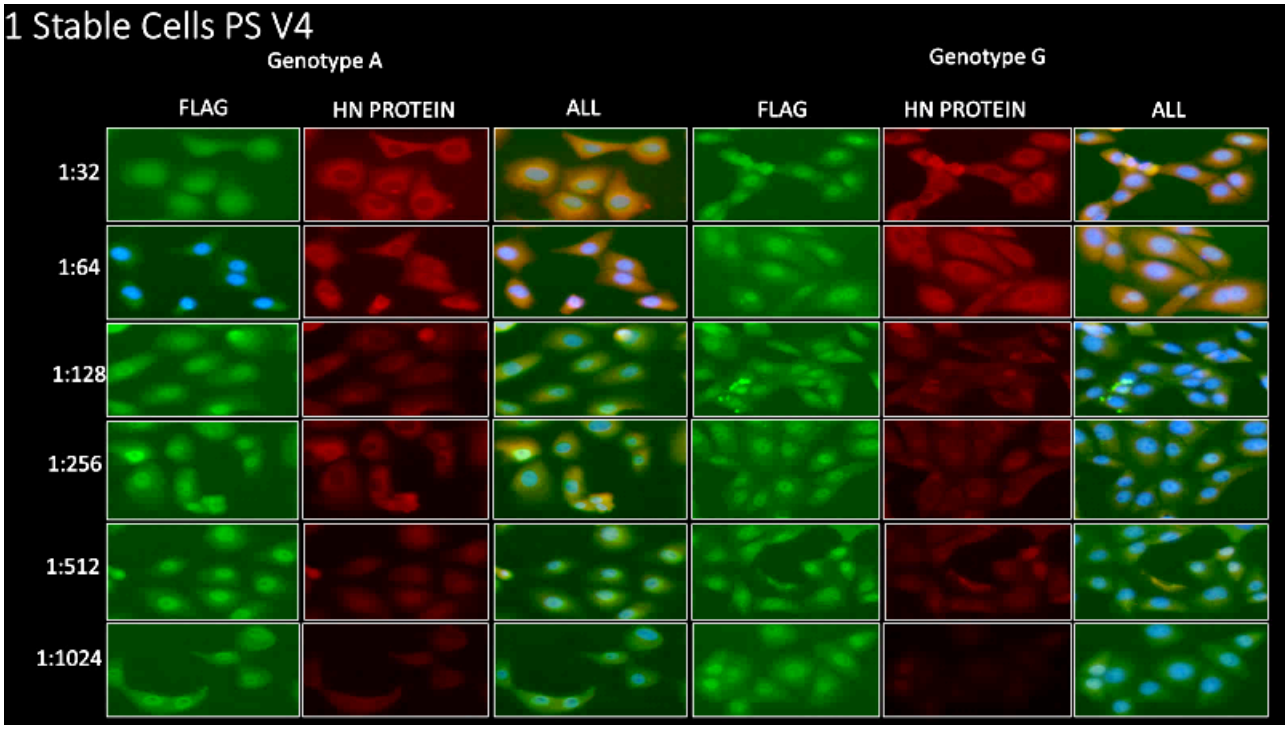


Figure A-7b. Stable cells Vaccine Sample #4.

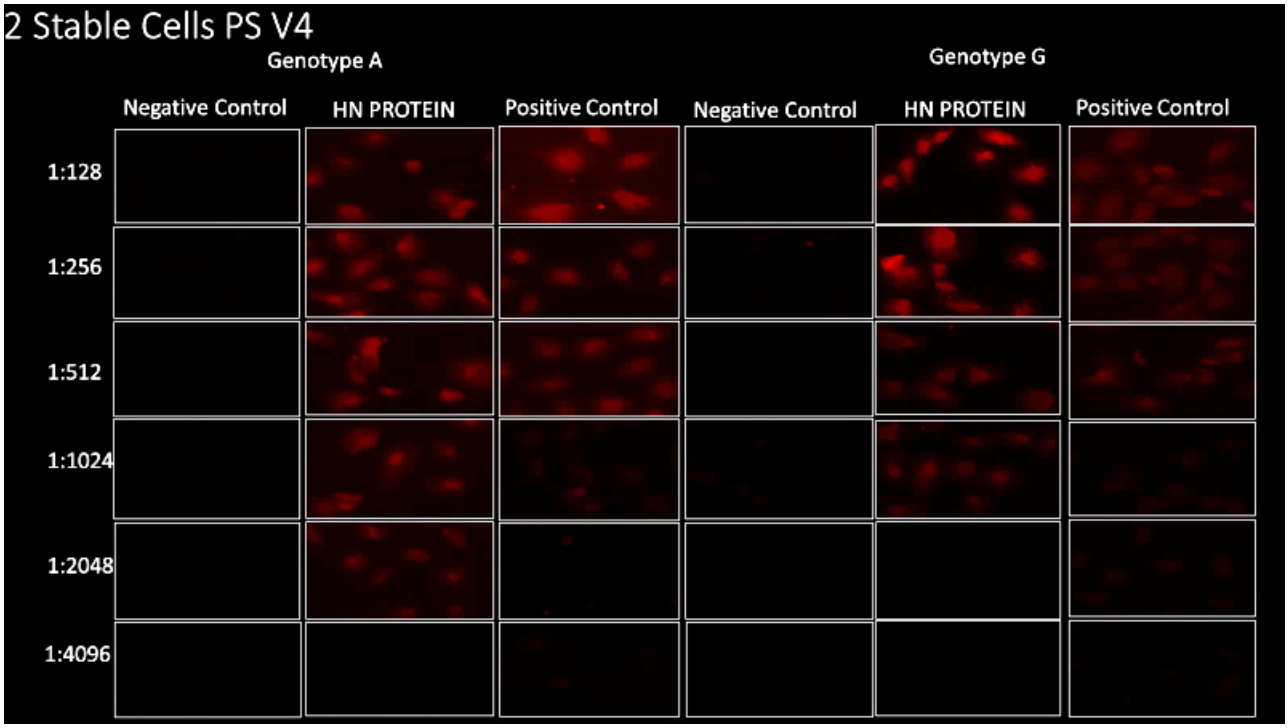


Figure A-7c. Stable cells Vaccine Sample #4.

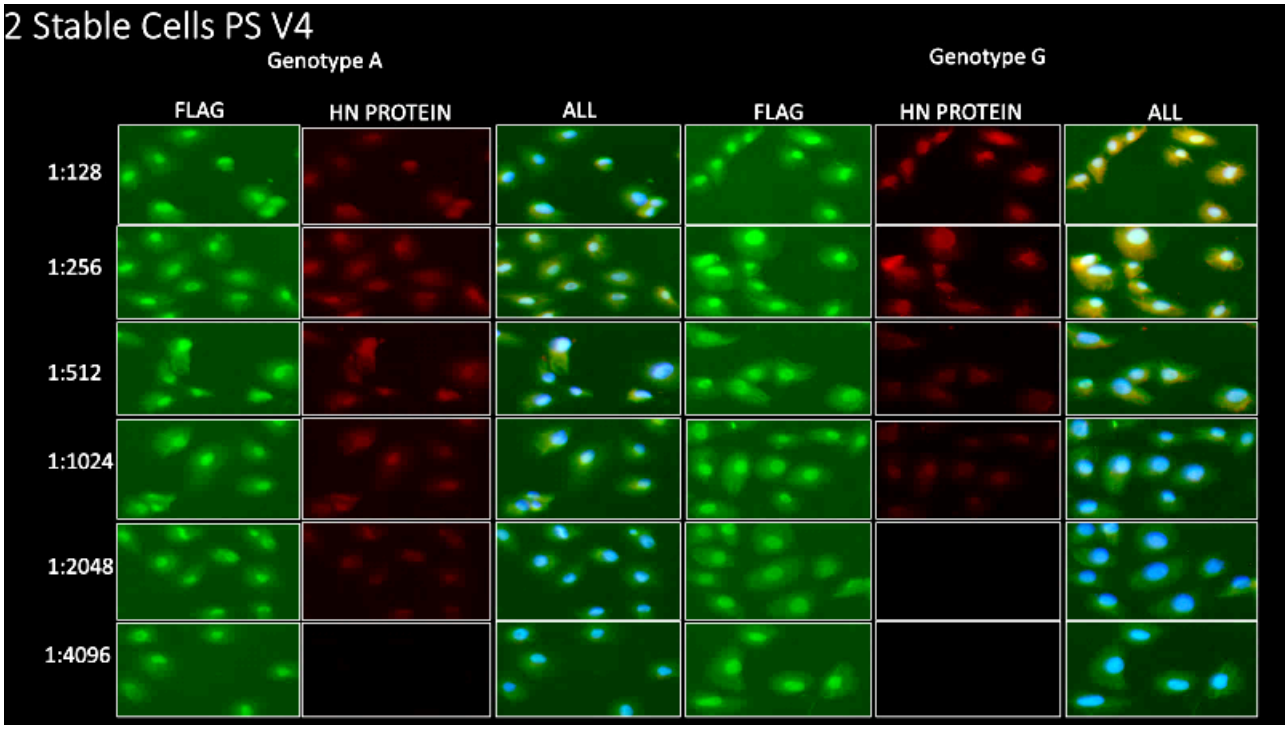


Figure A-7d. Stable cells Vaccine Sample #4.

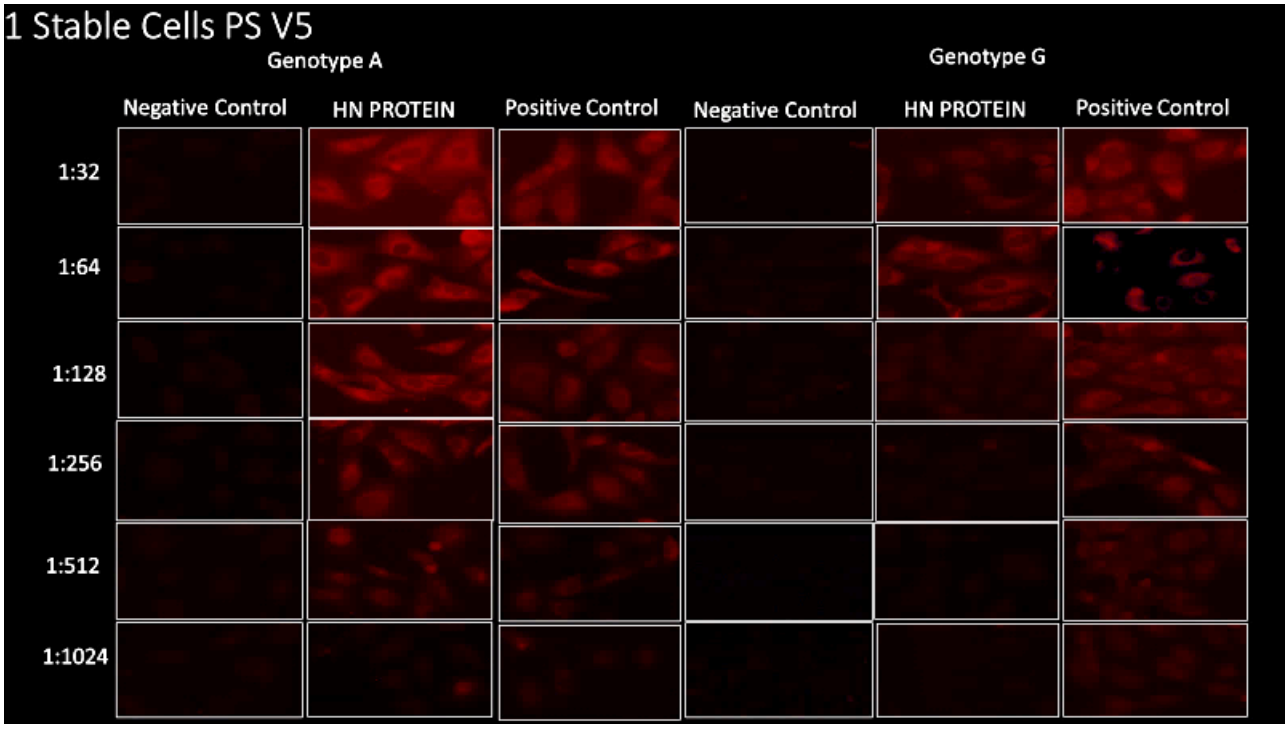


Figure A-8a. Stable cells Vaccine Sample#5.

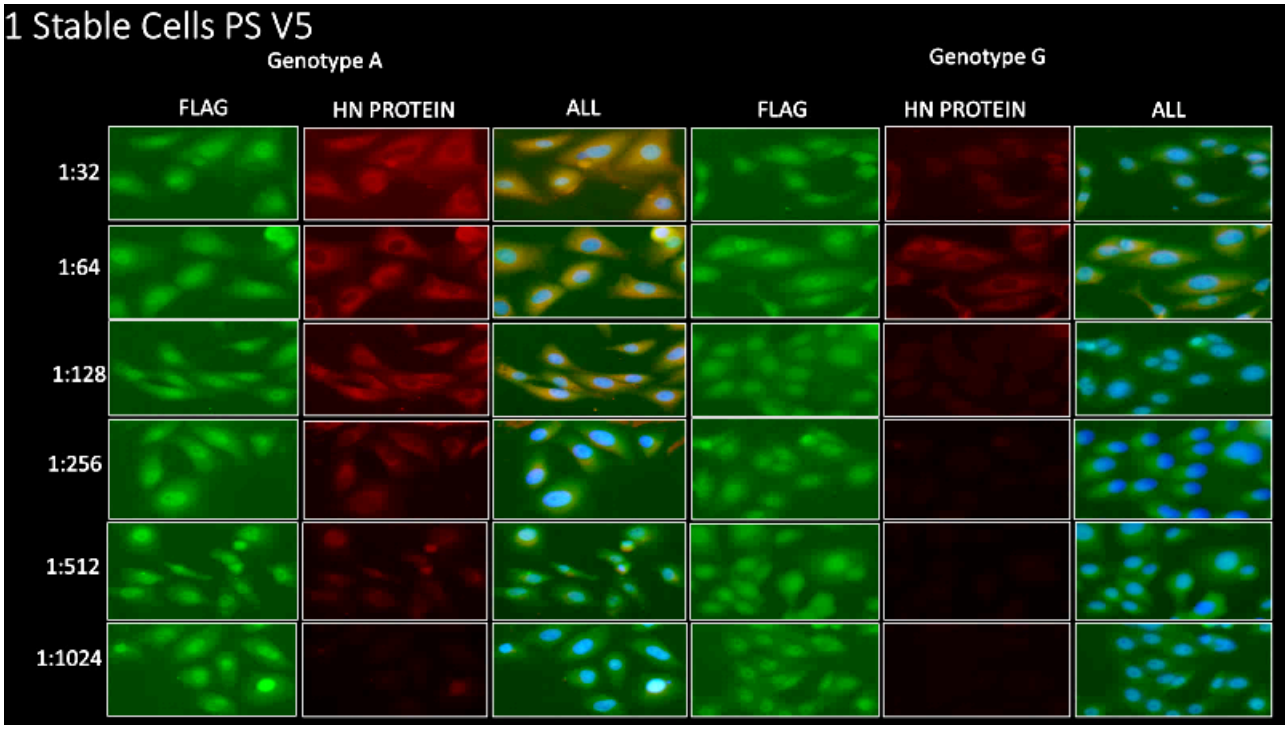


Figure A-8b. Stable cells Vaccine Sample #5.

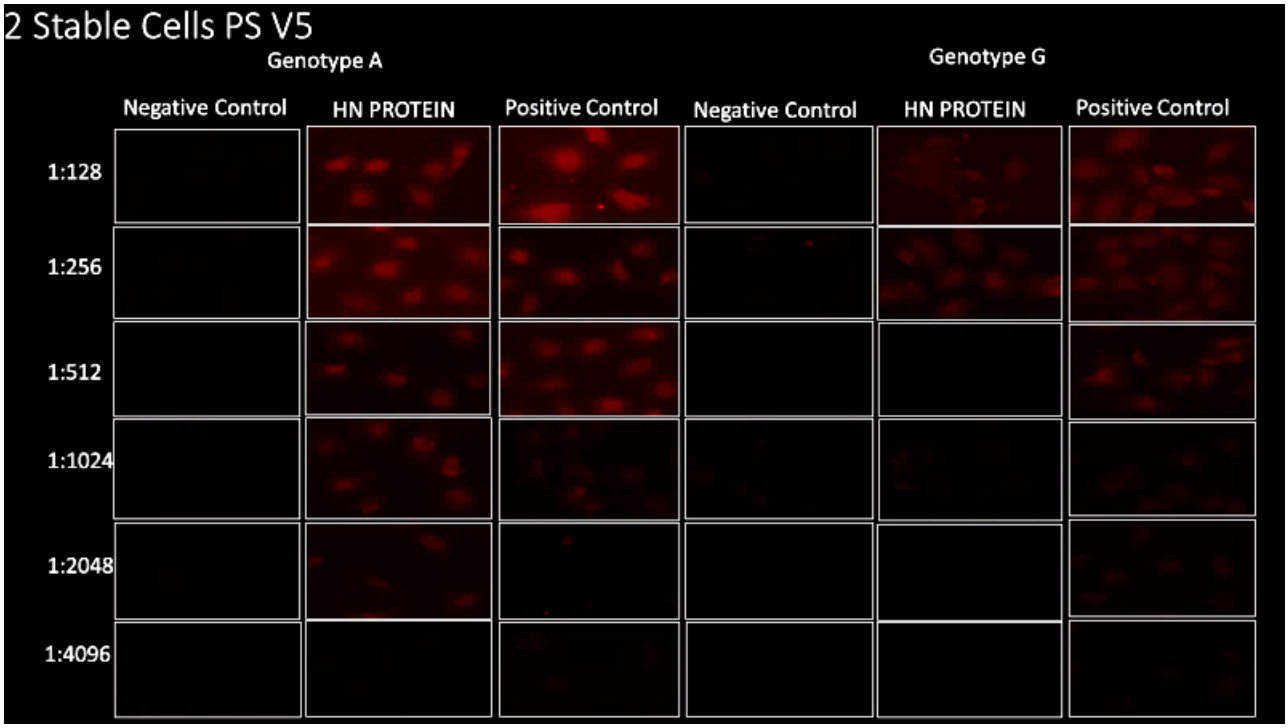


Figure A-8c. Stable cells Vaccine Sample #5.

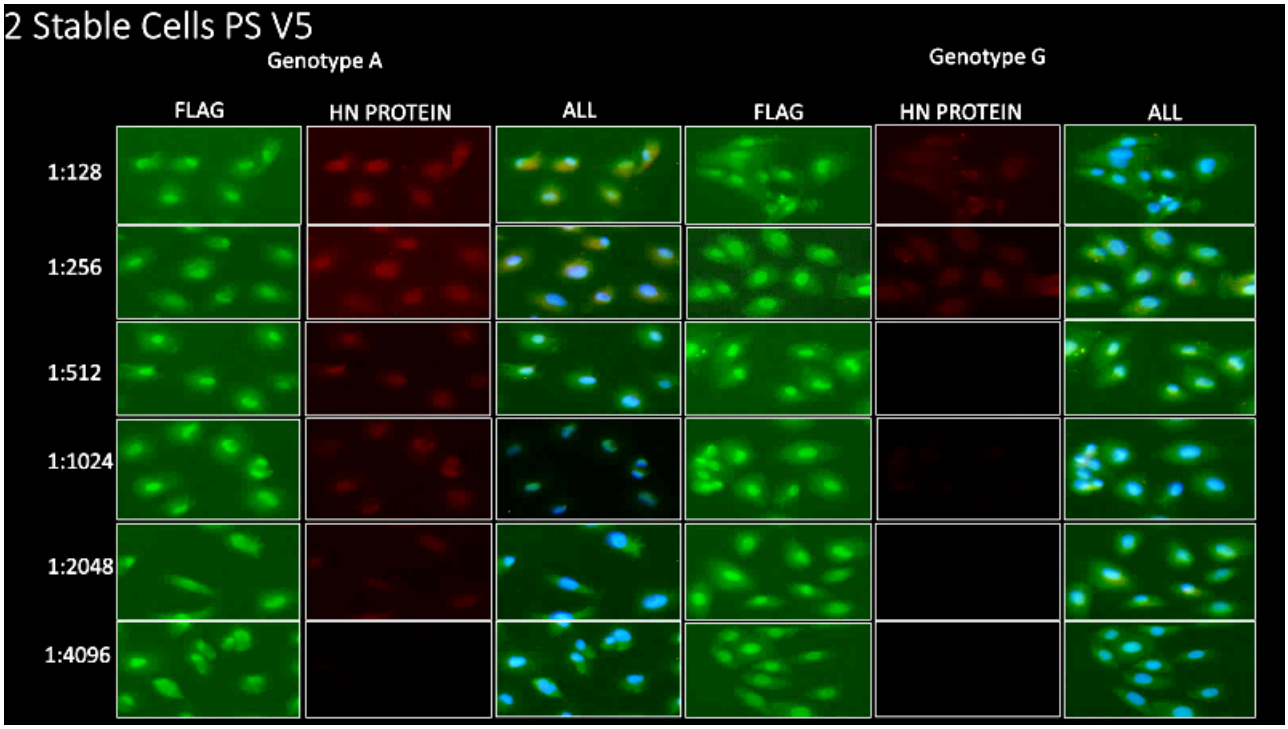


Figure A-8d. Stable cells Vaccine Sample #5.

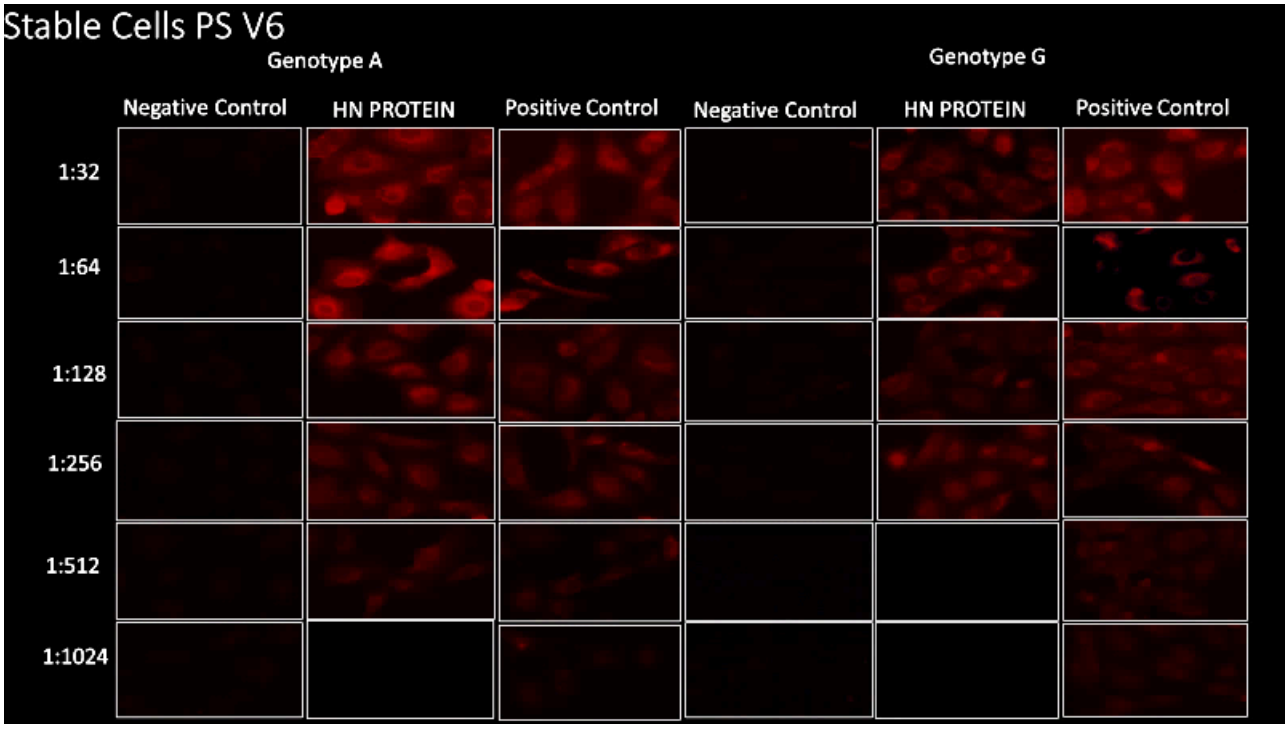


Figure A-9a. Stable cells Vaccine Sample#6.

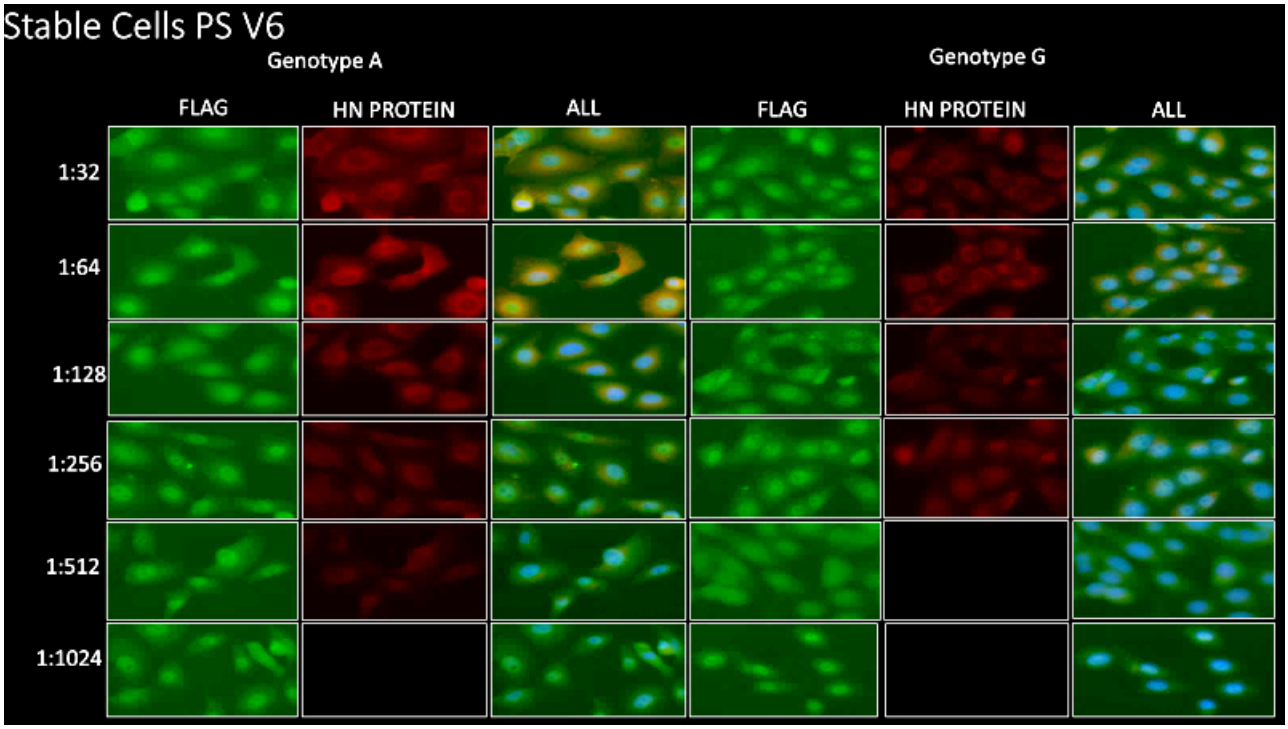


Figure A-9b. Stable cells Vaccine Sample #6.

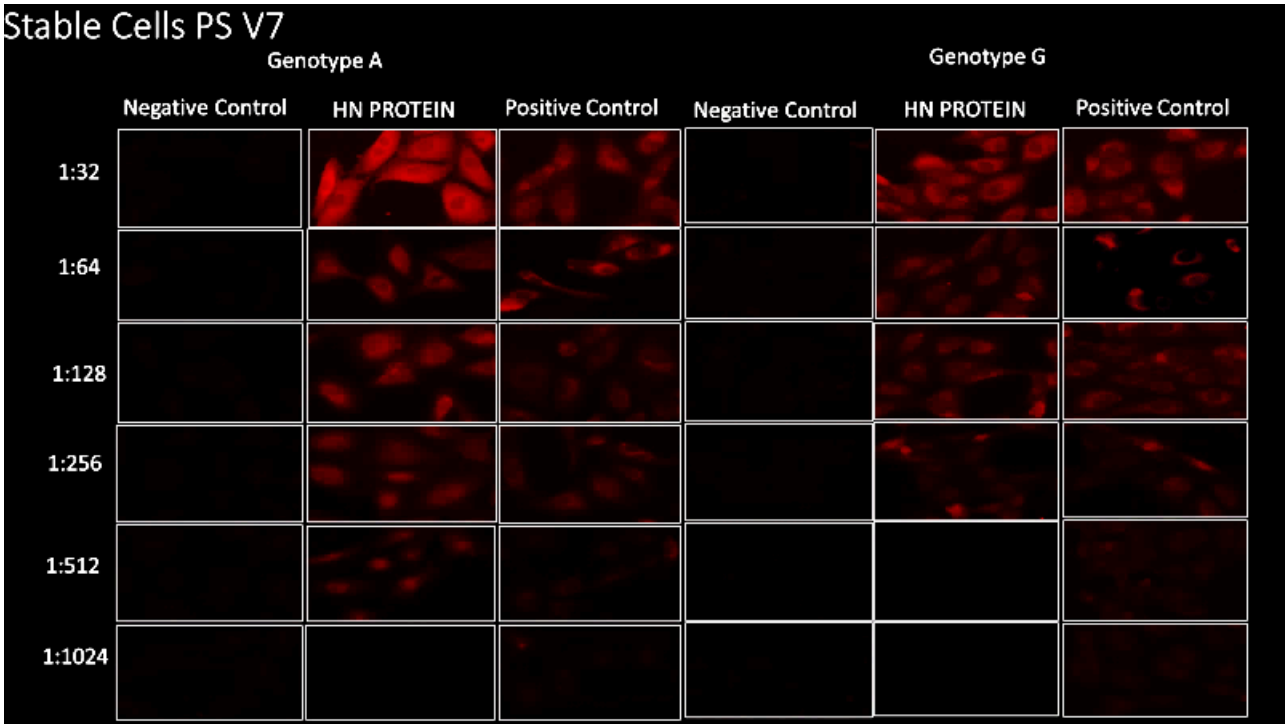


Figure A-10a. Stable cells Vaccine Sample#7.

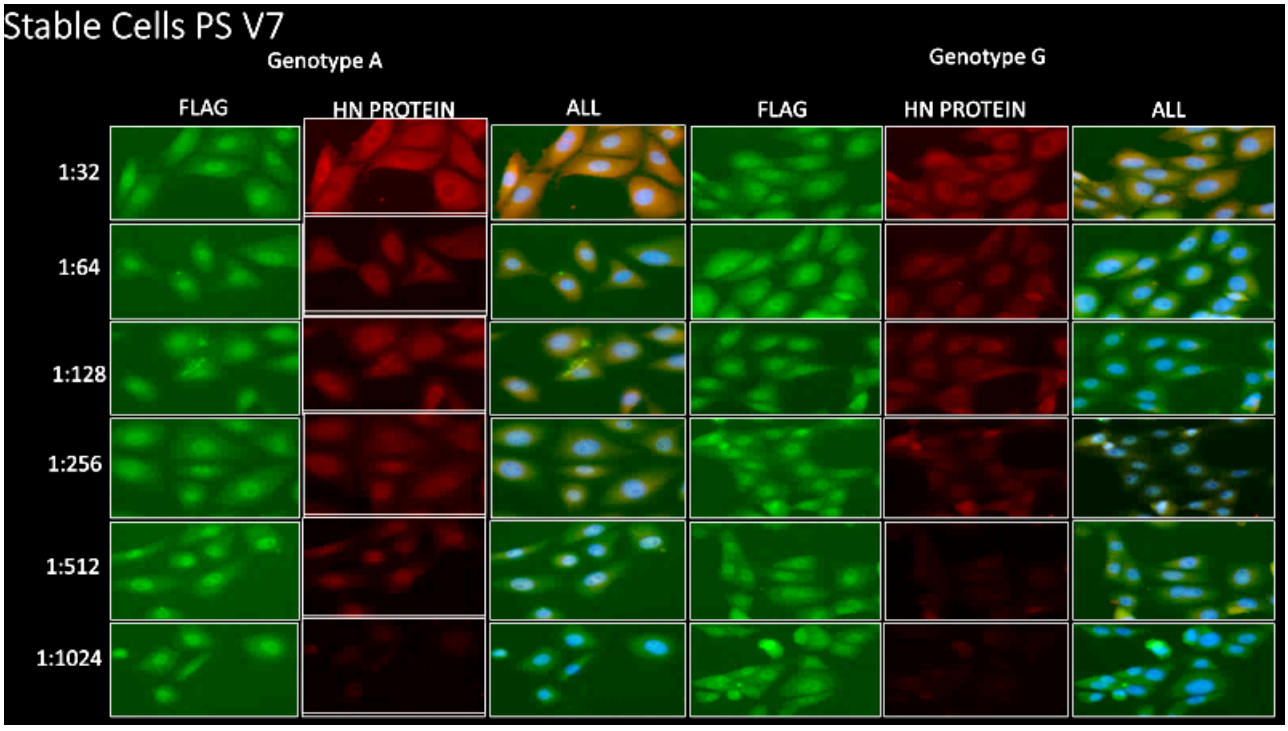


Figure A-10b. Stable cells Vaccine Sample #7.

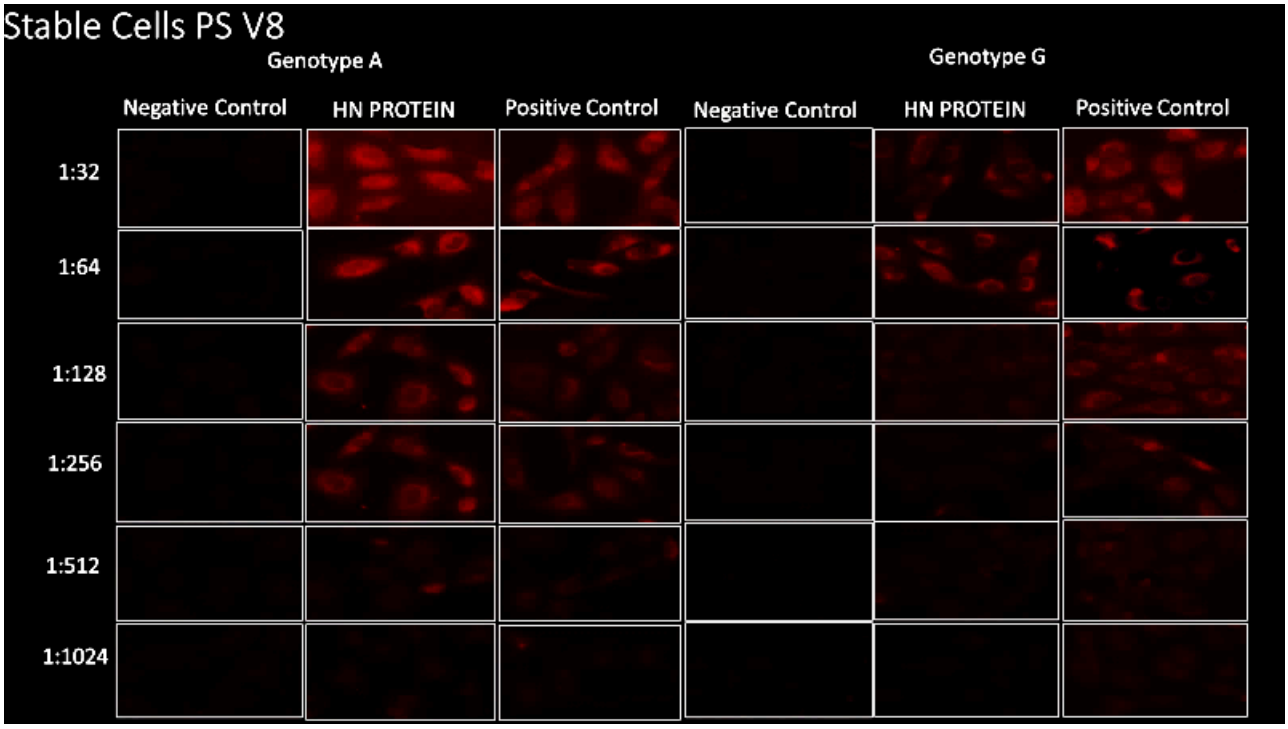


Figure A-11a. Stable cells Vaccine Sample#8.

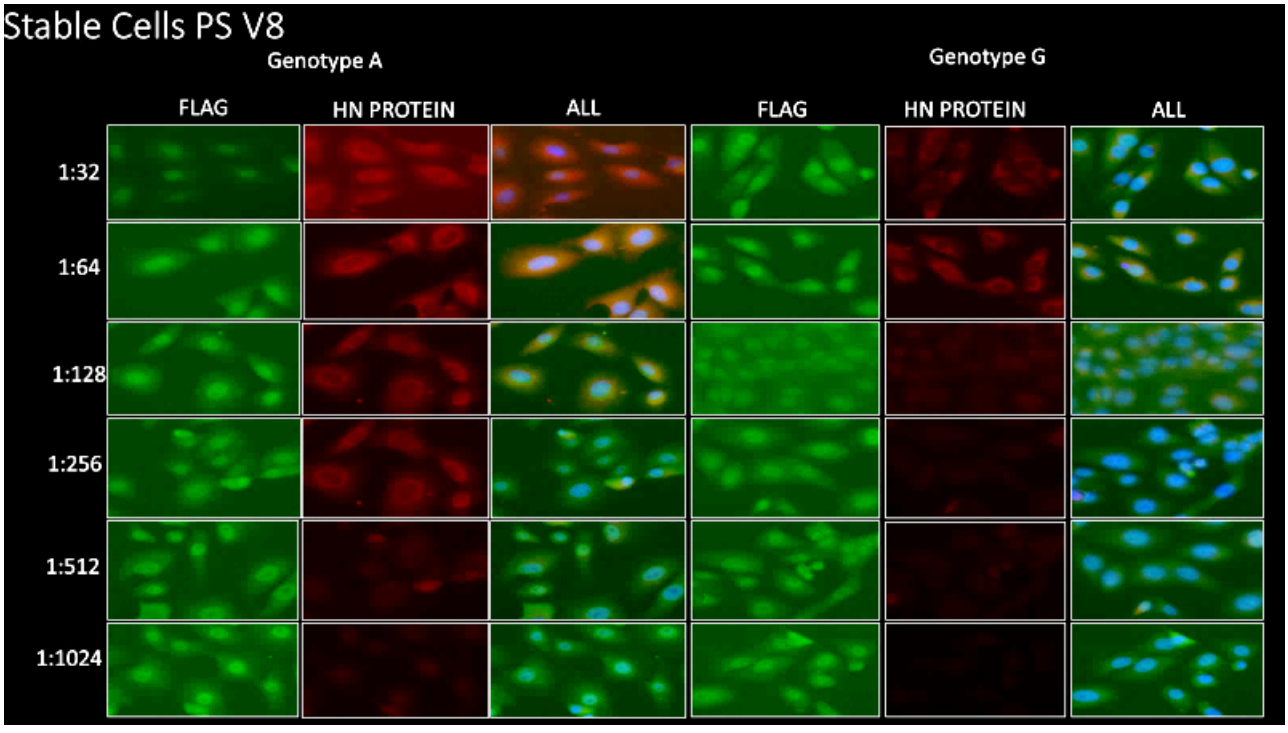


Figure A-11b. Stable cells Vaccine Sample #8.

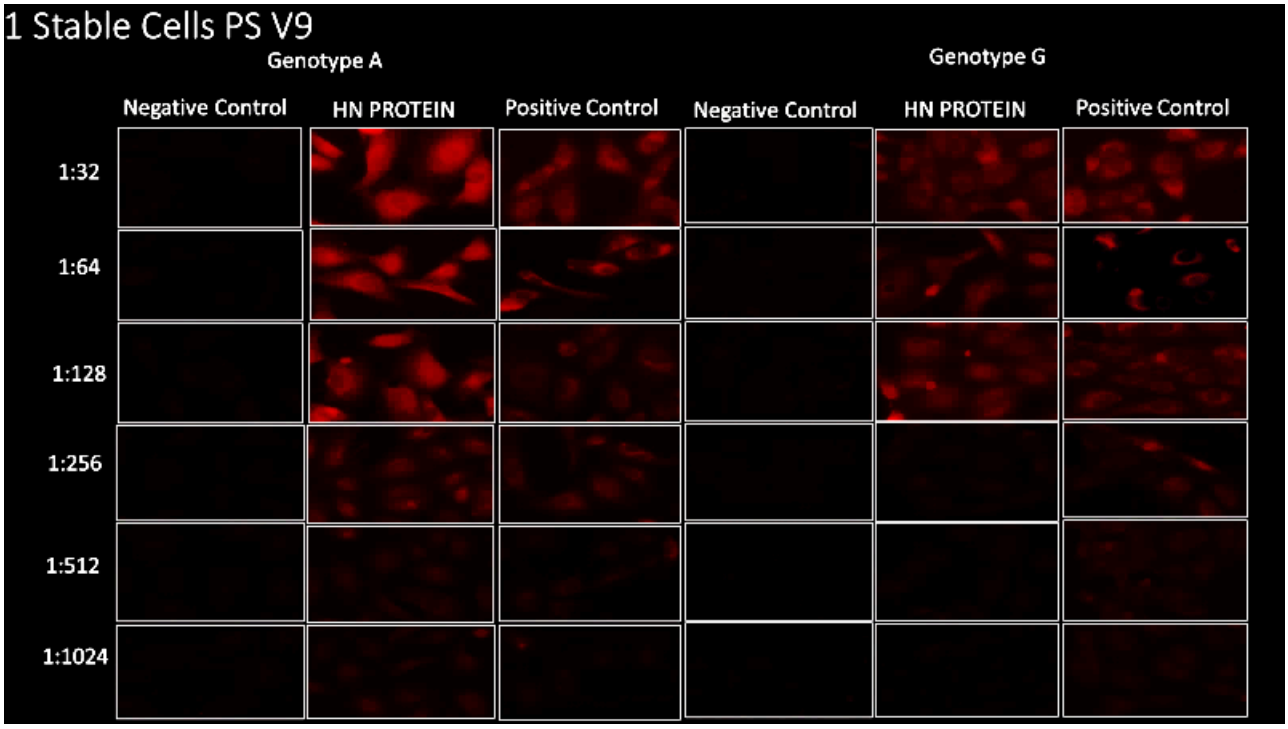


Figure A-12a. Stable cells Vaccine Sample#9.

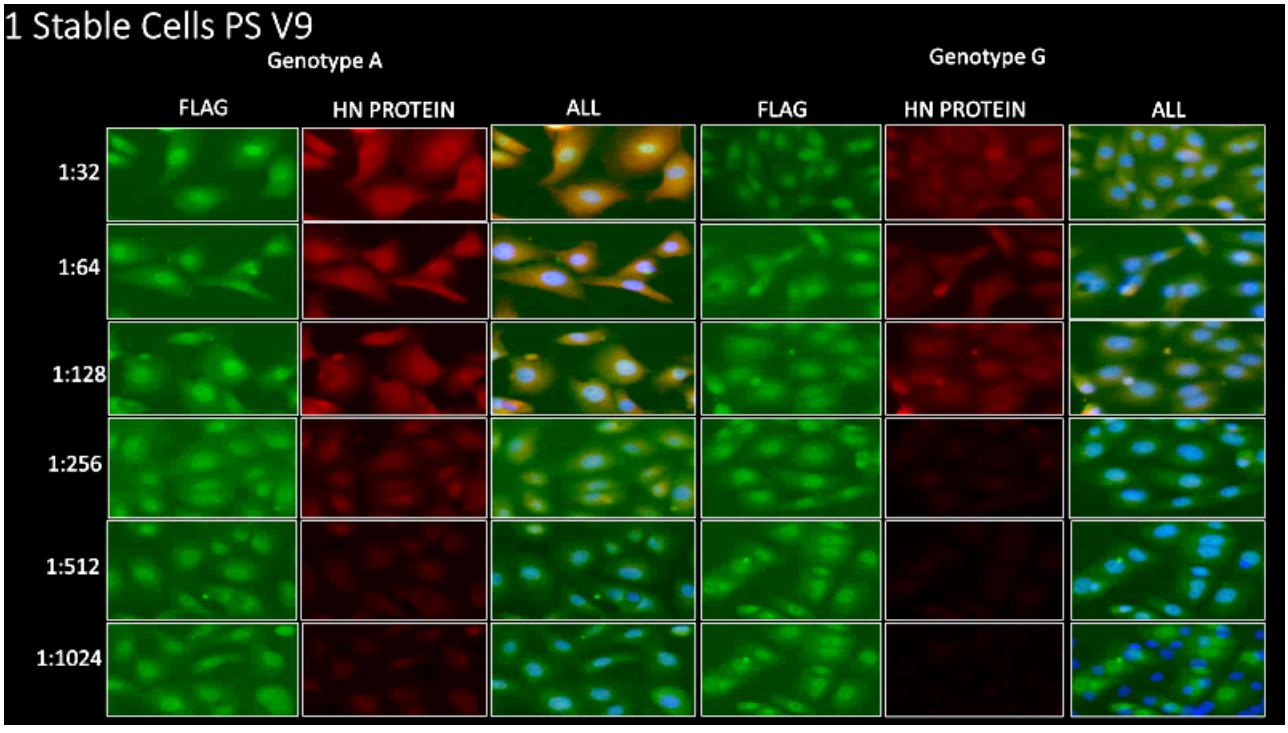


Figure A-12b. Stable cells Vaccine Sample #9.

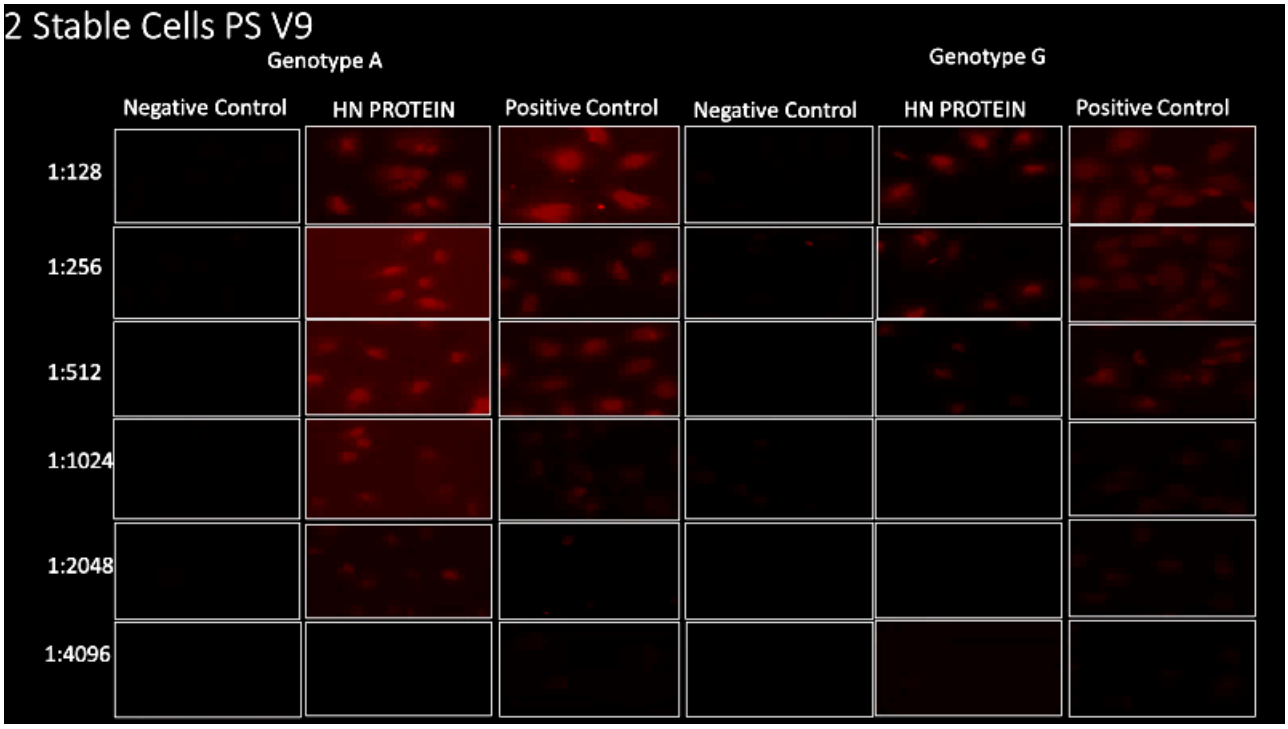


Figure A-12c. Stable cells Vaccine Sample #9.

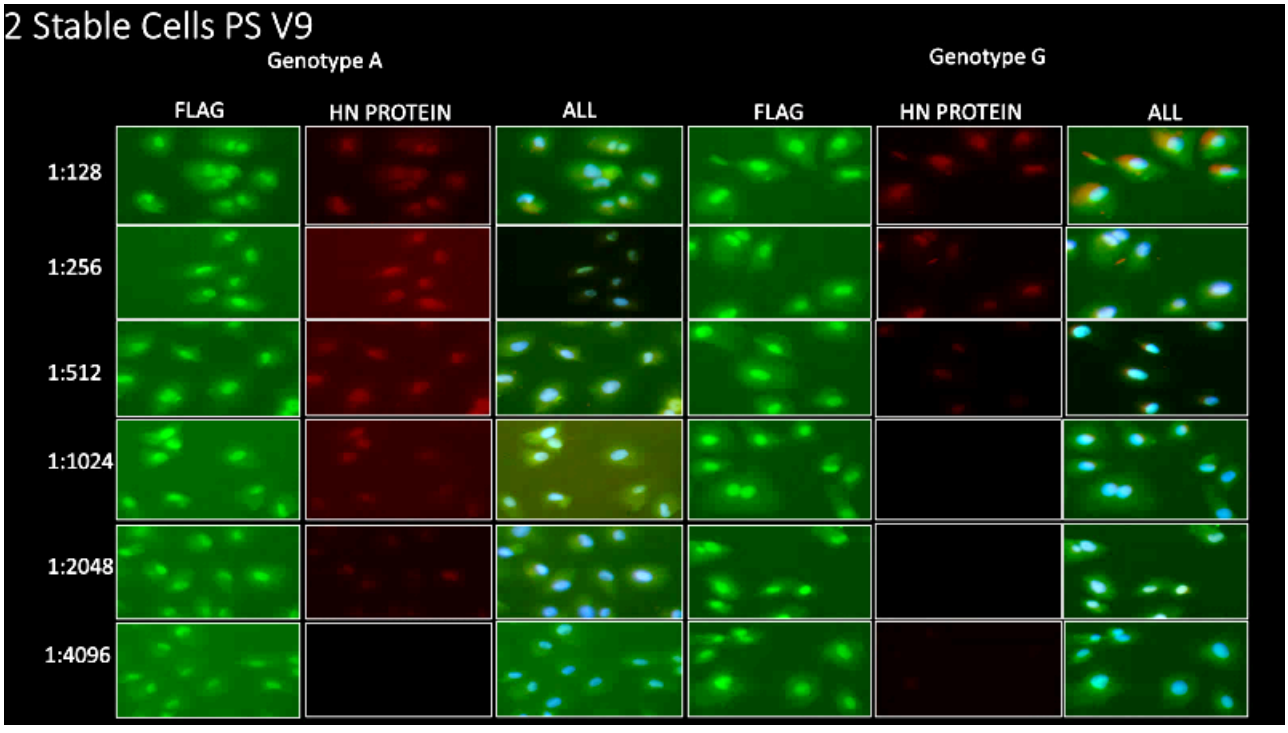


Figure A-12d. Stable cells Vaccine Sample #9.

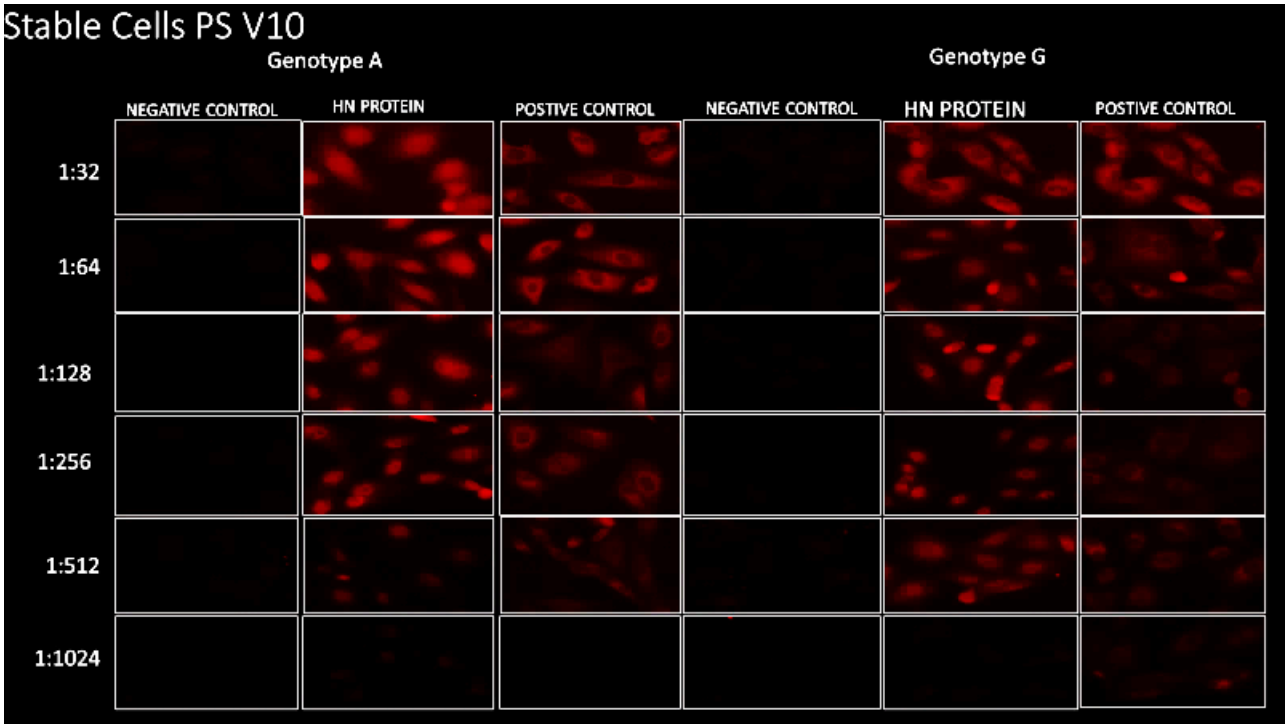


Figure A-13a. Stable cells Vaccine Sample#10.

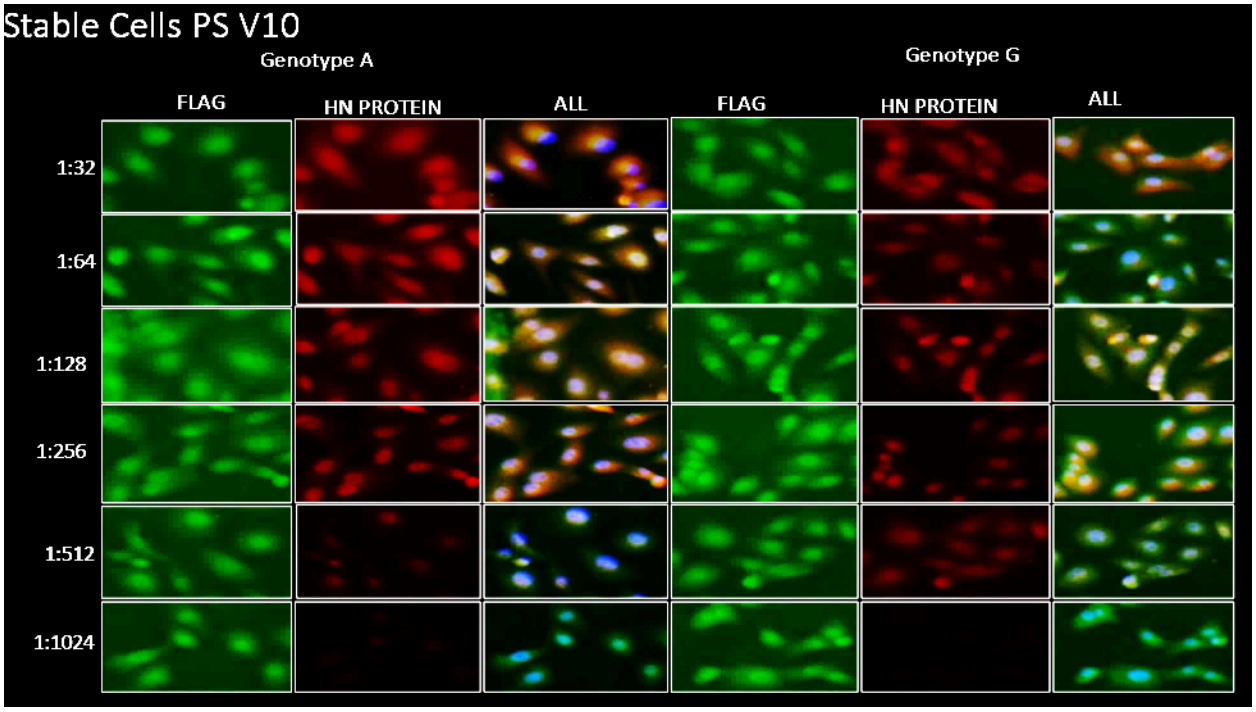


Figure A-13b. Stable cells Vaccine Sample #10.

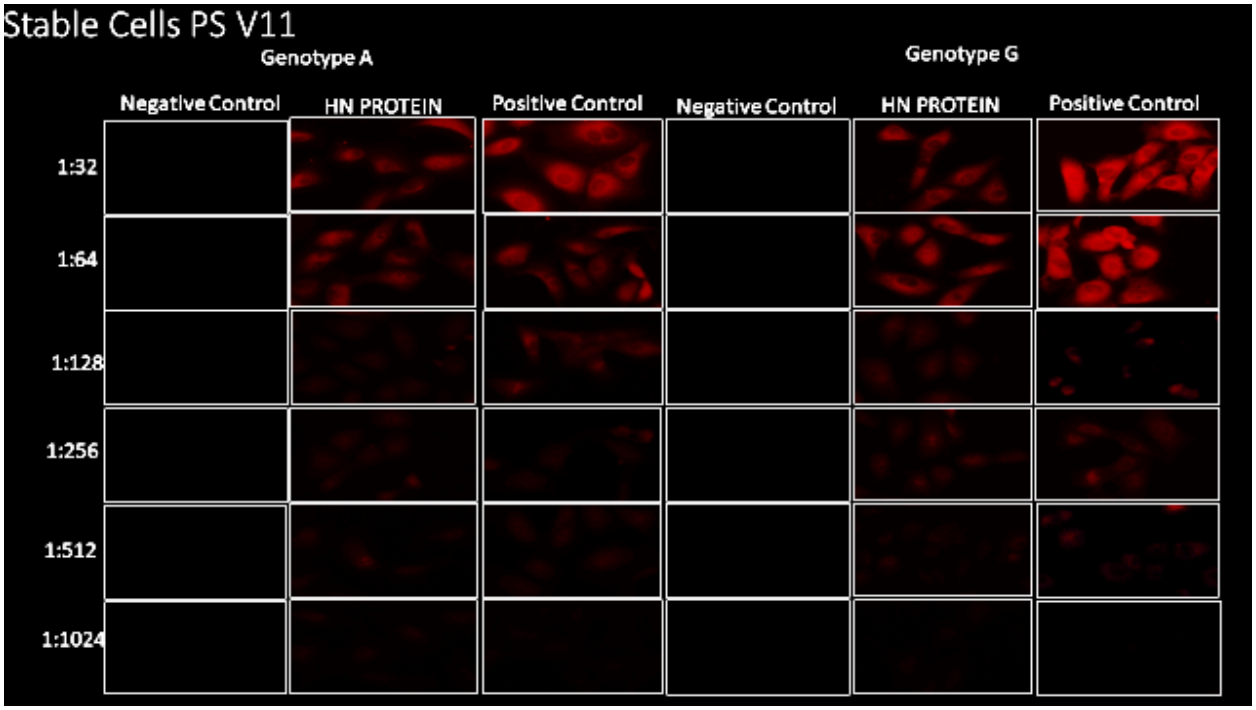


Figure A-14a. Stable cells Vaccine Sample#11.

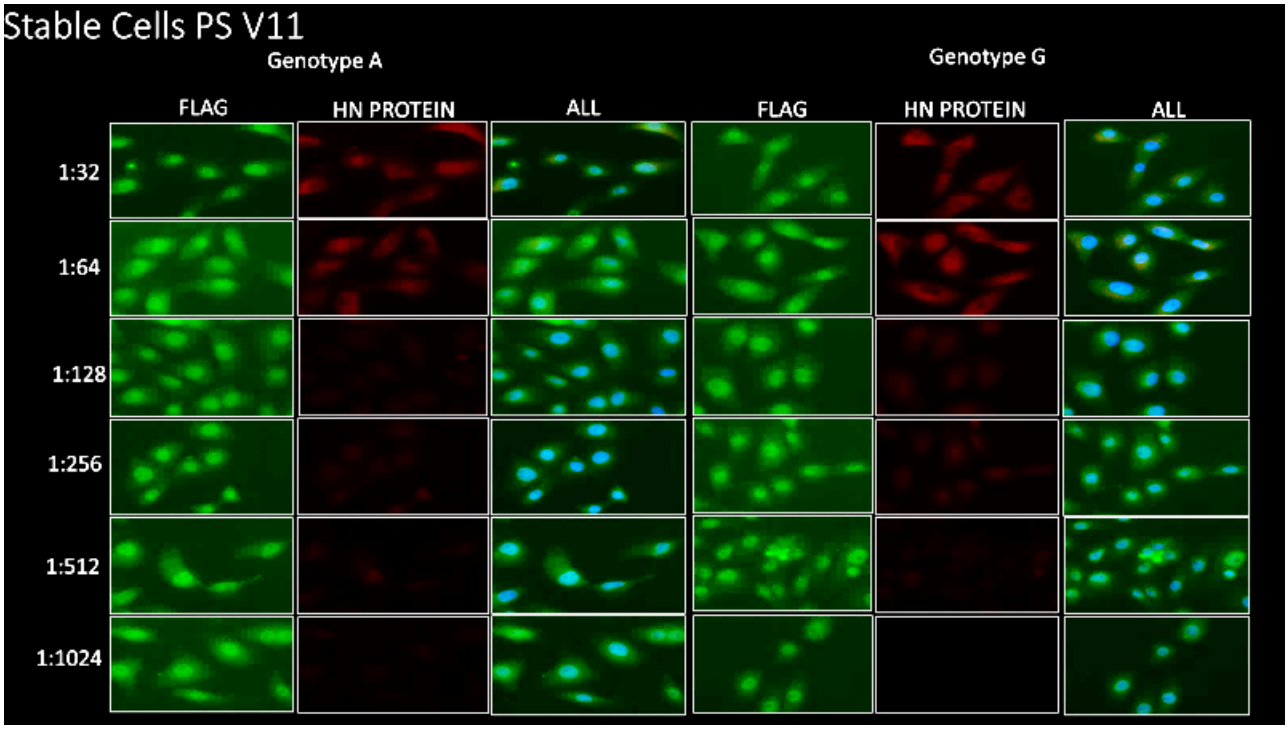


Figure A-14b. Stable cells Vaccine Sample #11.

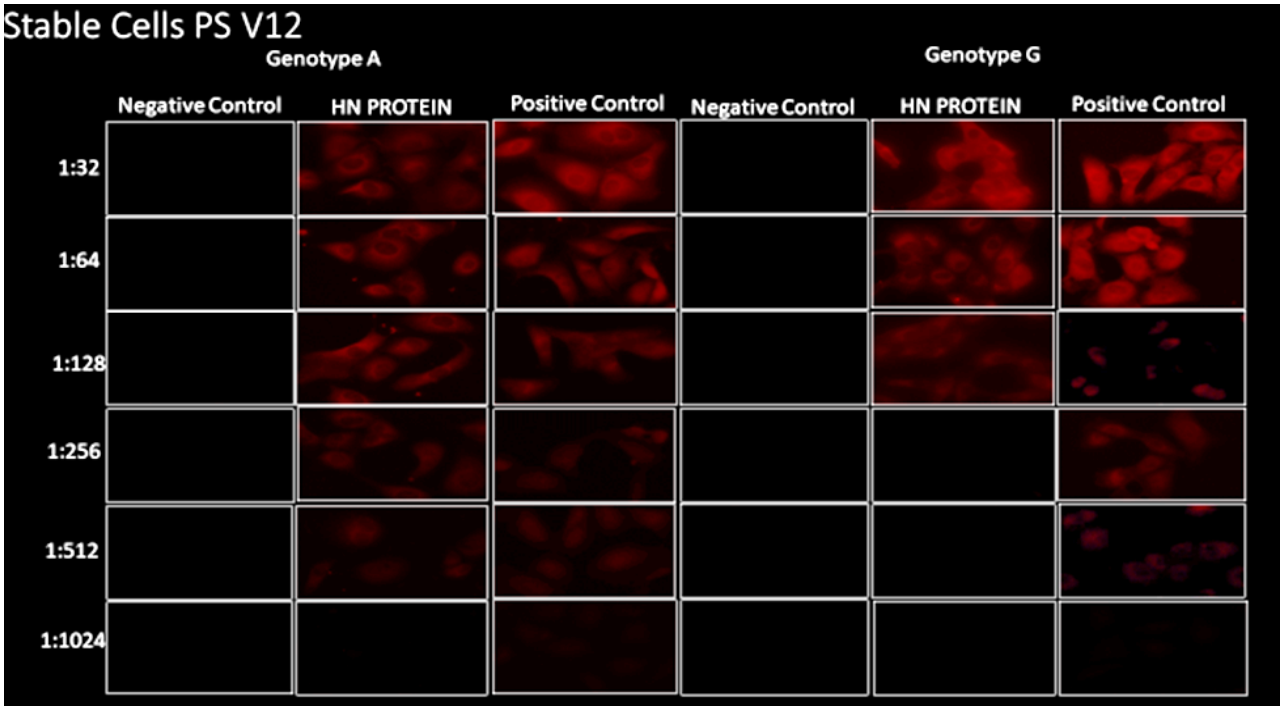


Figure A-15a. Stable cells Vaccine Sample#12.

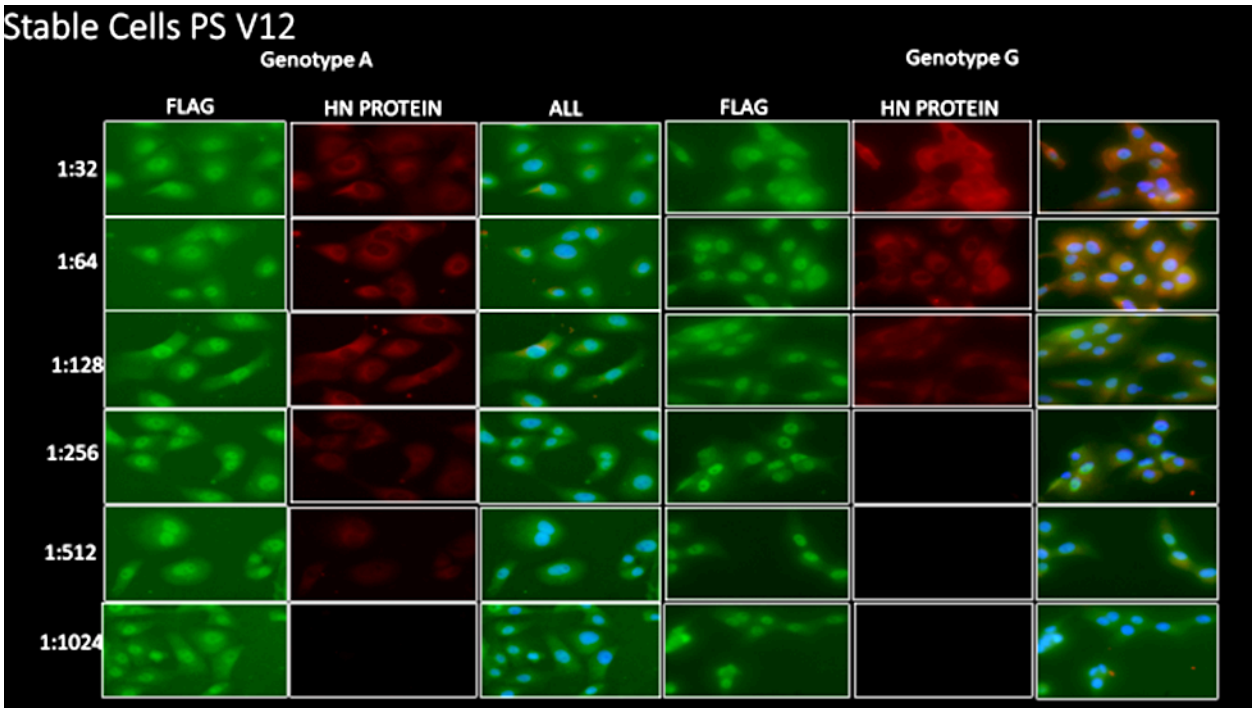


Figure A-15b. Stable cells Vaccine Sample #12.

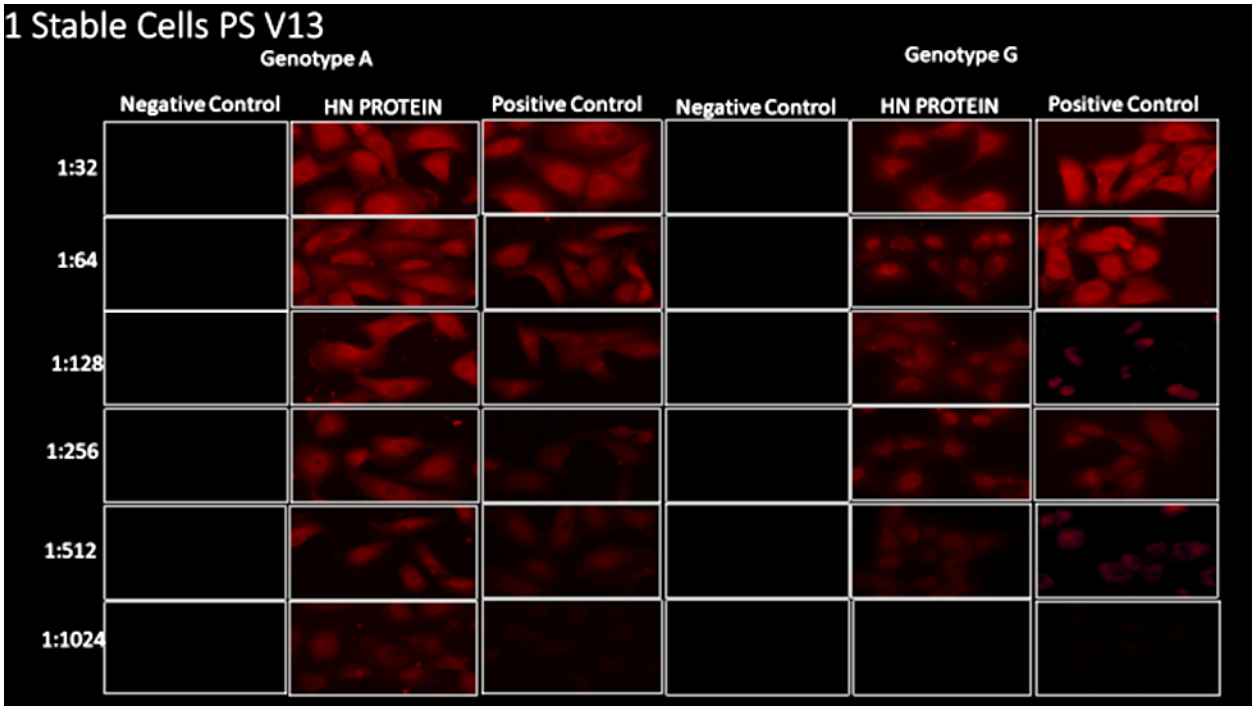


Figure A-16a. Stable cells Vaccine Sample#13.

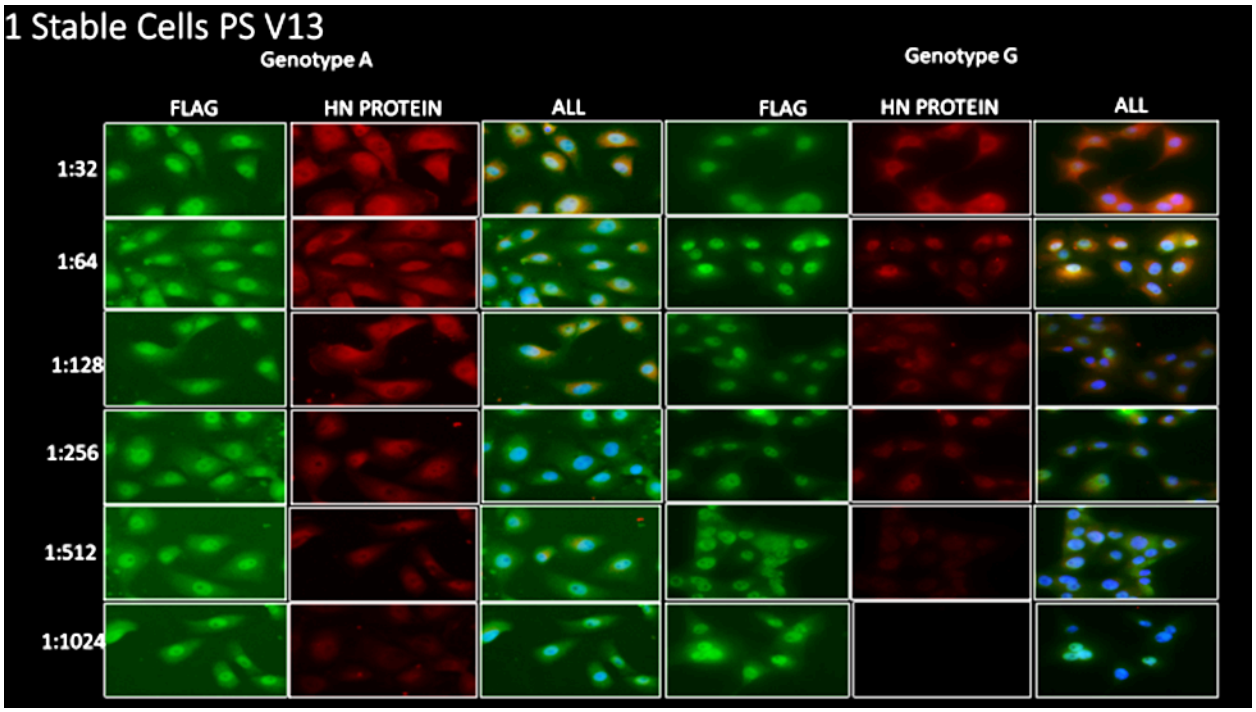


Figure A-16b. Stable cells Vaccine Sample #13.

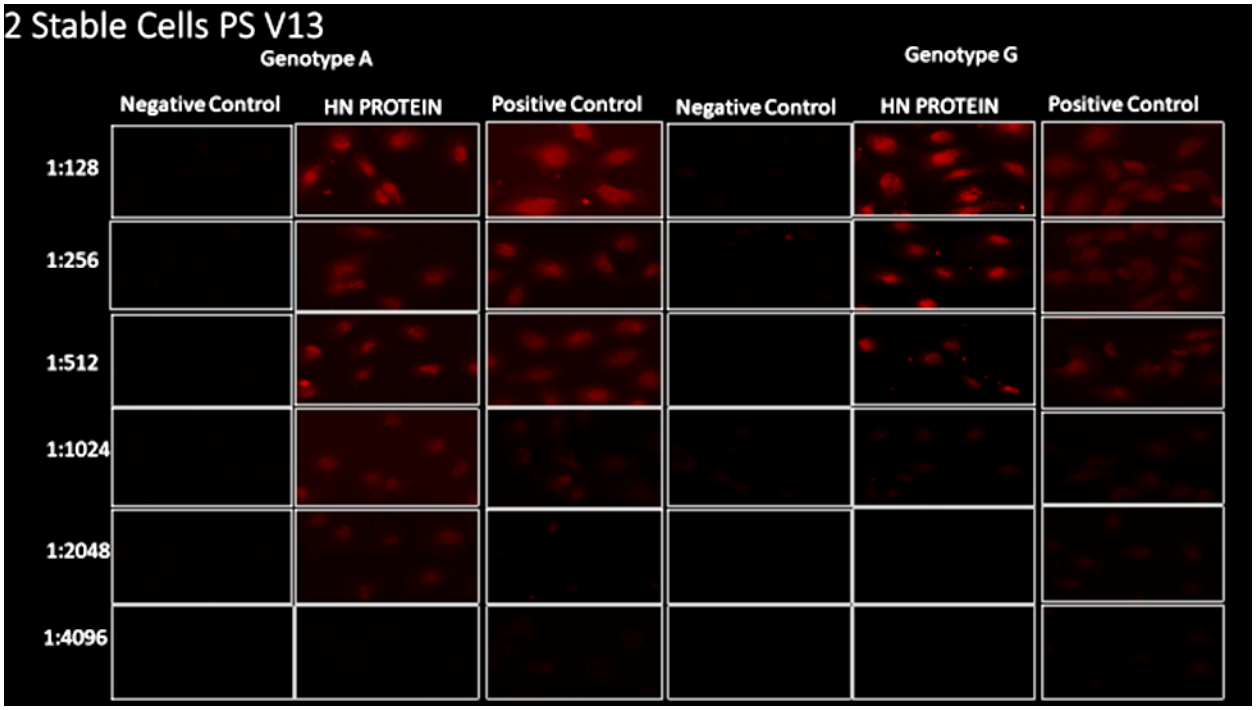


Figure A-16c. Stable cells Vaccine Sample #13.

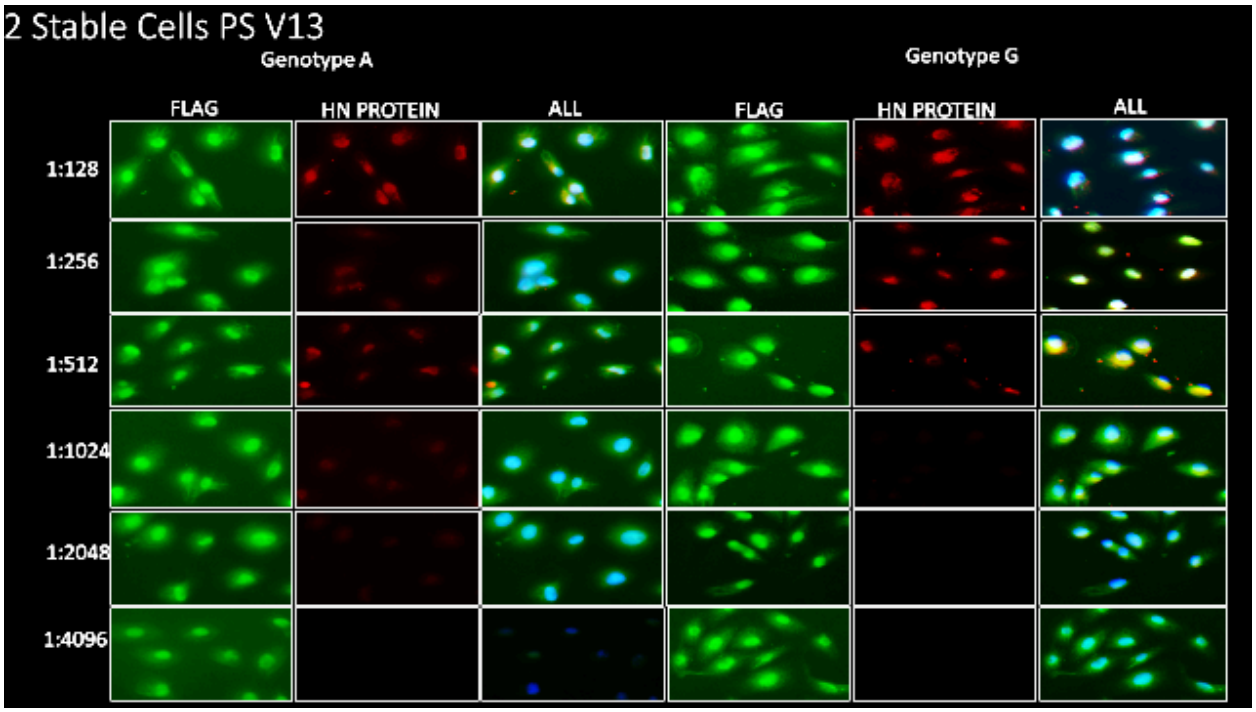


Figure A-16d. Stable cells Vaccine Sample #13.

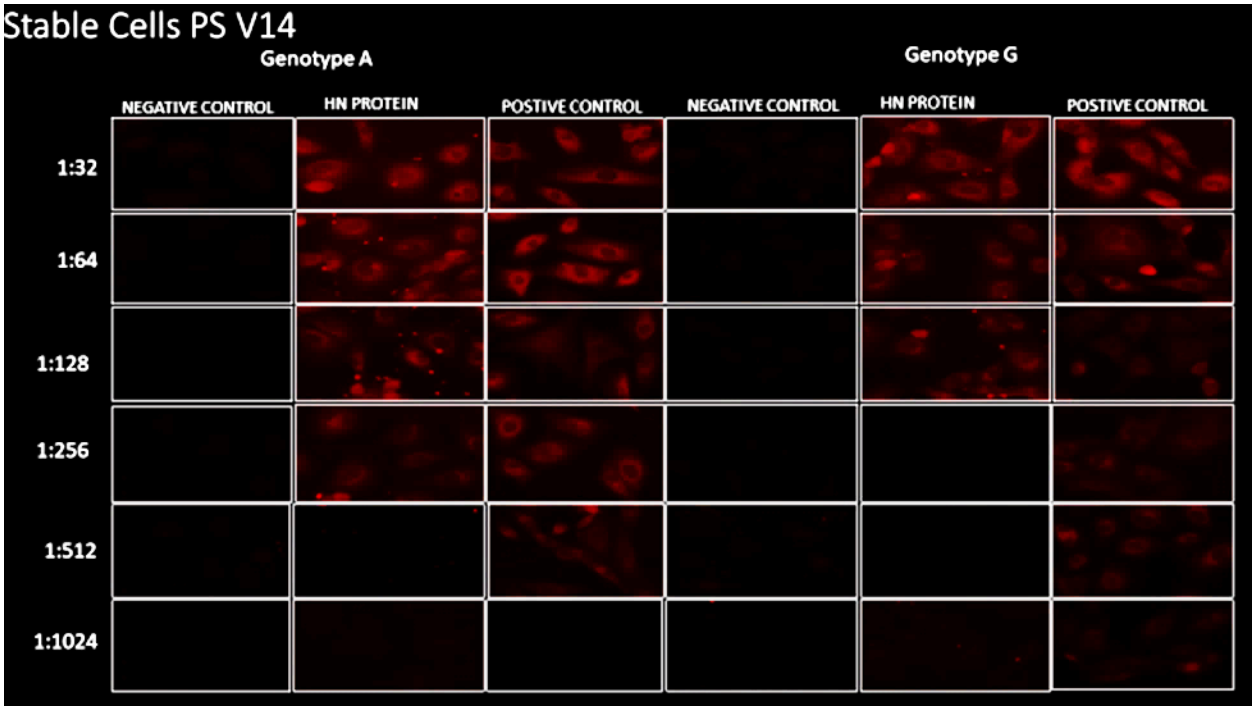


Figure A-17a. Stable cells Vaccine Sample#14.

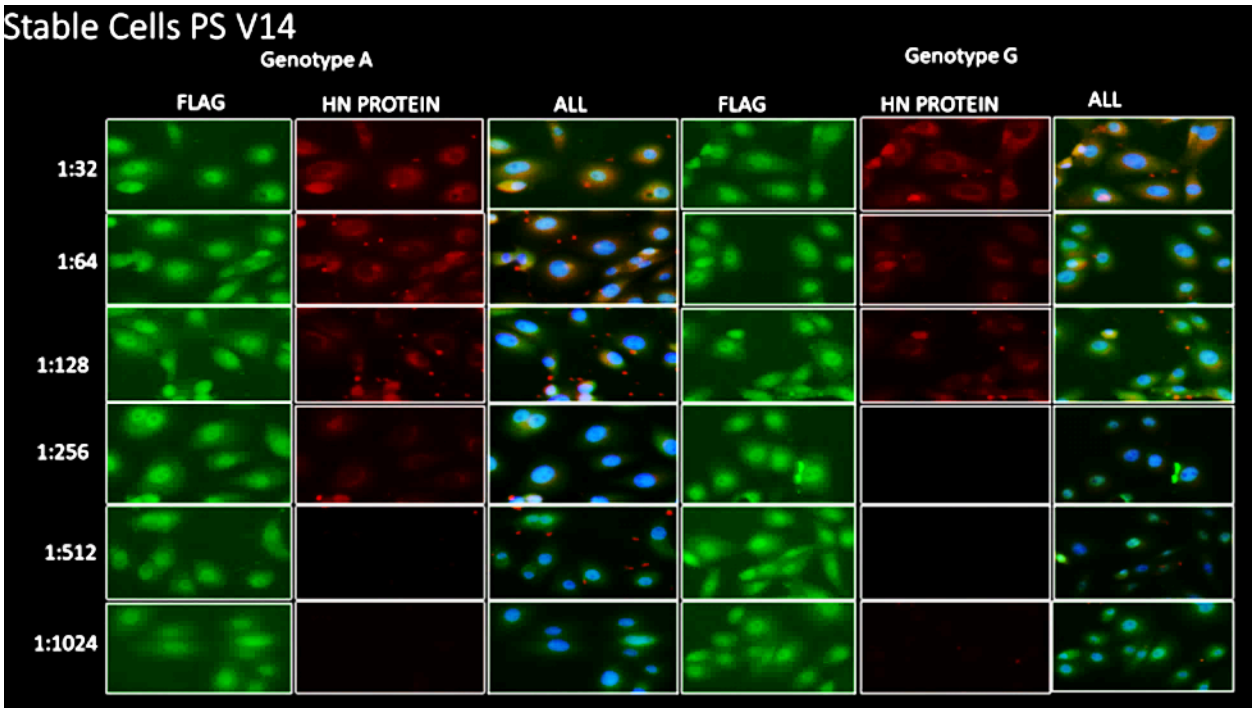


Figure A-17b. Stable cells Vaccine Sample #14.

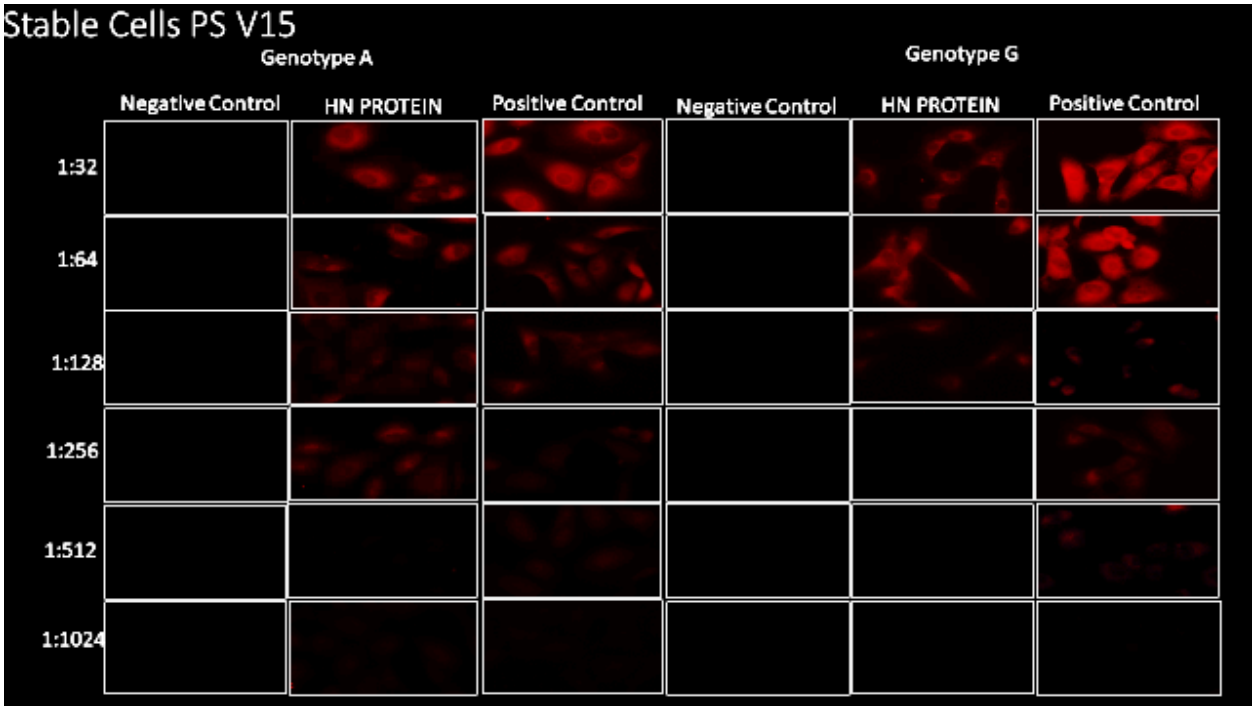


Figure A-18a. Stable cells Vaccine Sample#15.

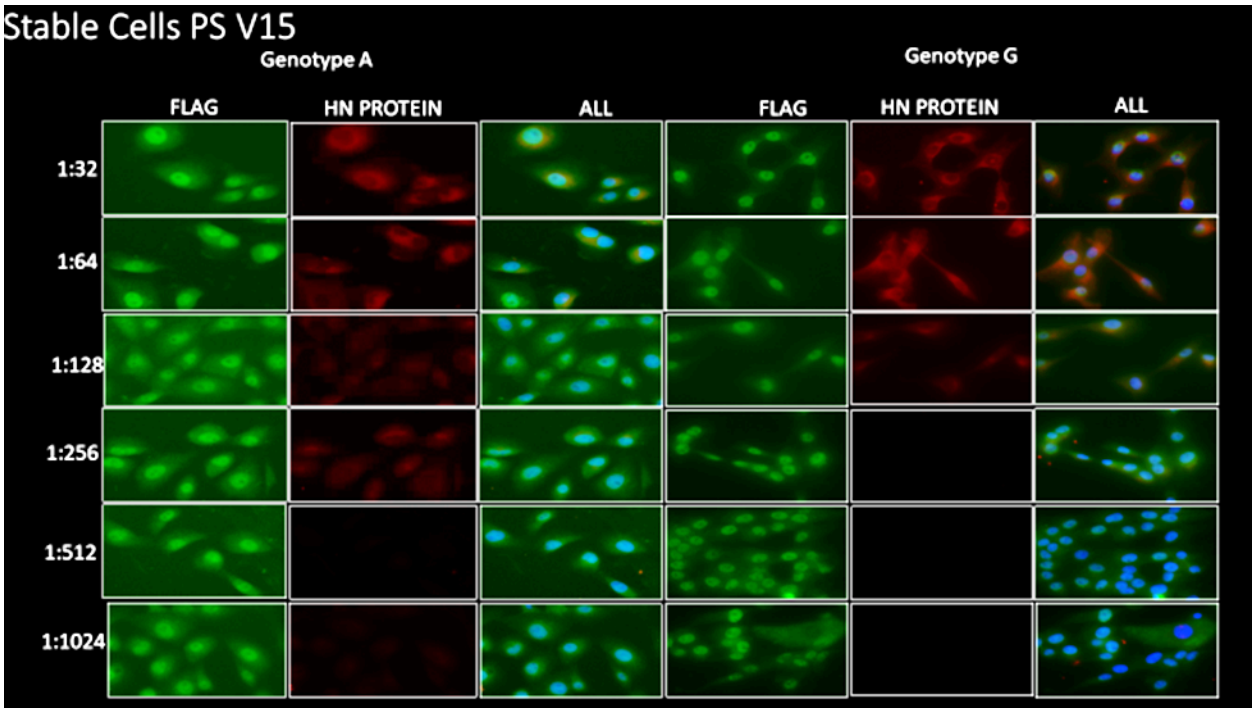


Figure A-18b. Stable cells Vaccine Sample #15.

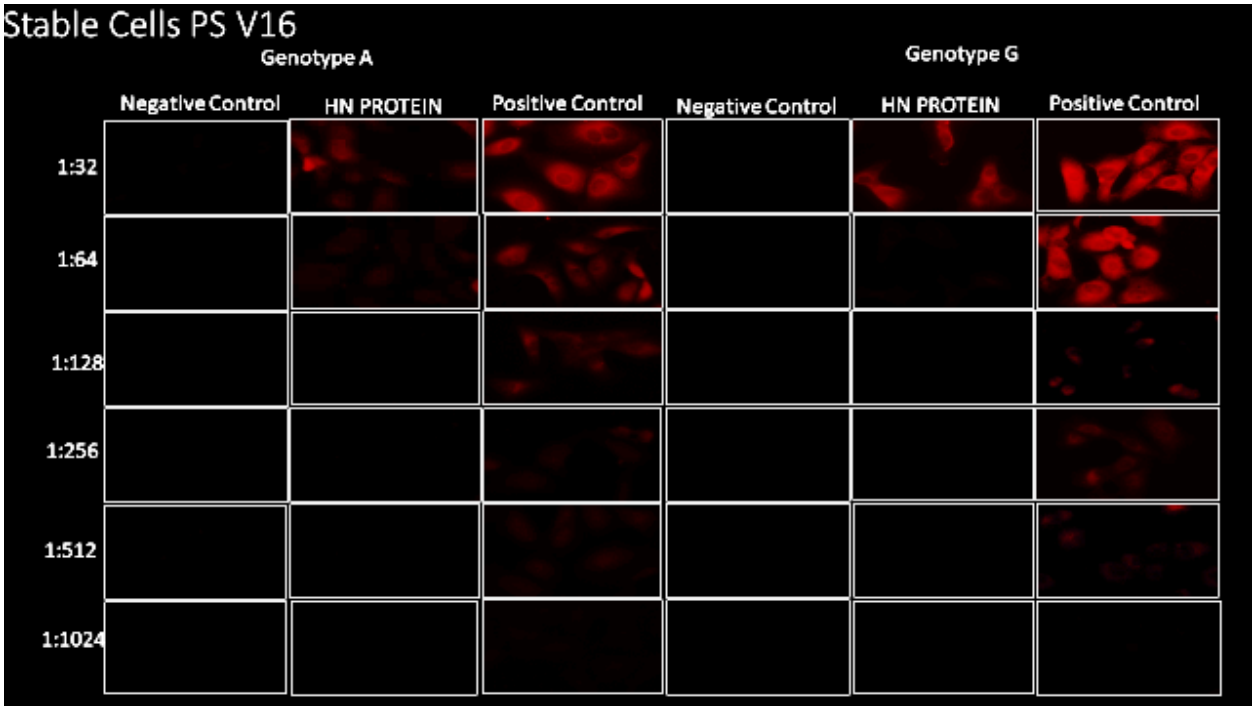


Figure A-19a. Stable cells Vaccine Sample#16.

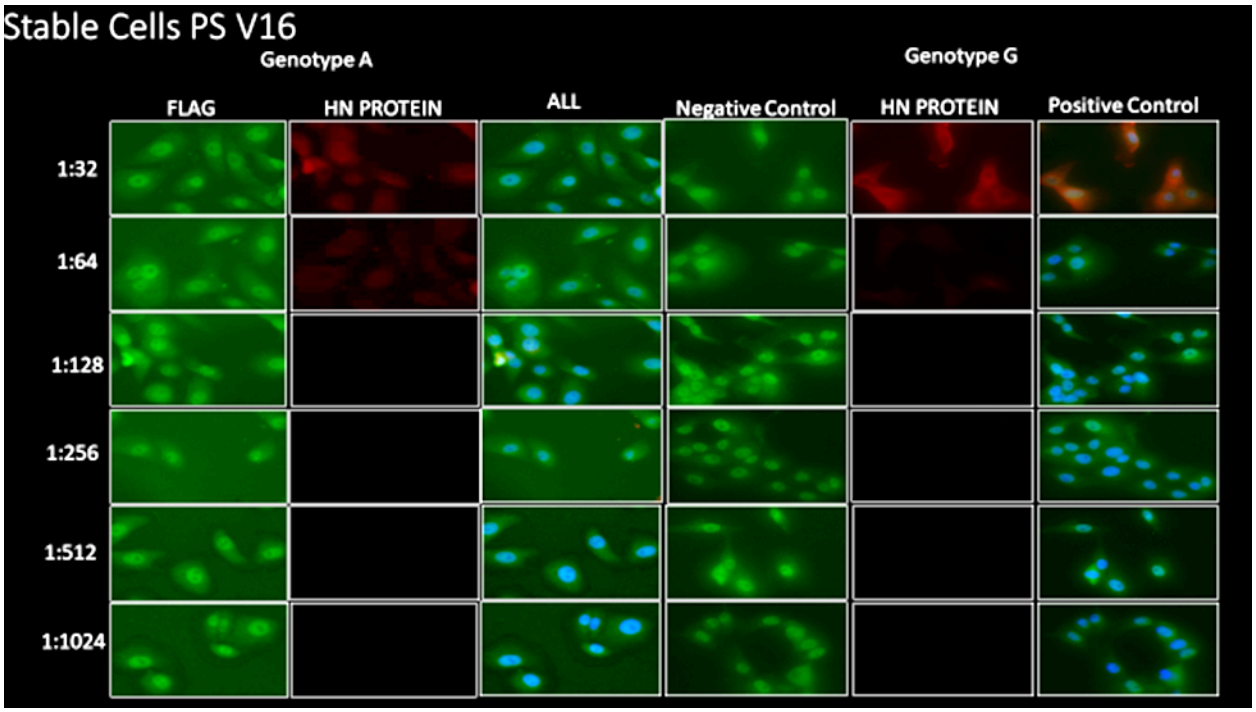


Figure A-19b. Stable cells Vaccine Sample #16.

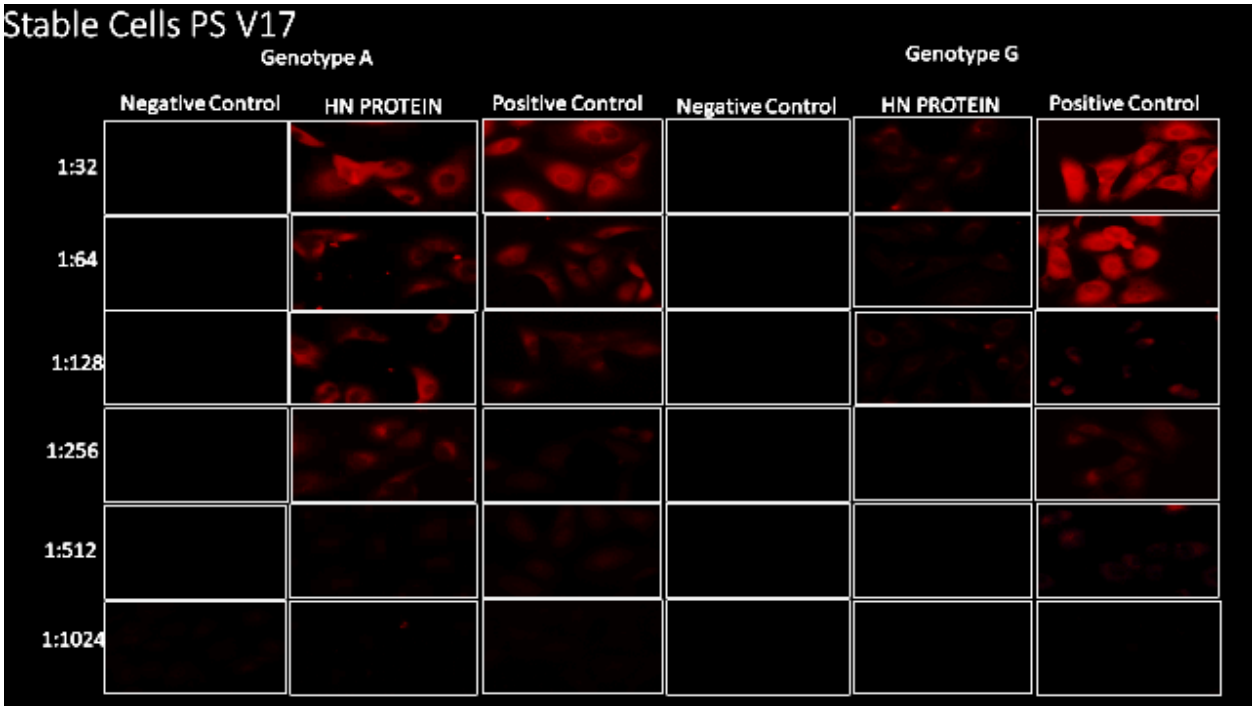


Figure A-20a. Stable cells Vaccine Sample#17.

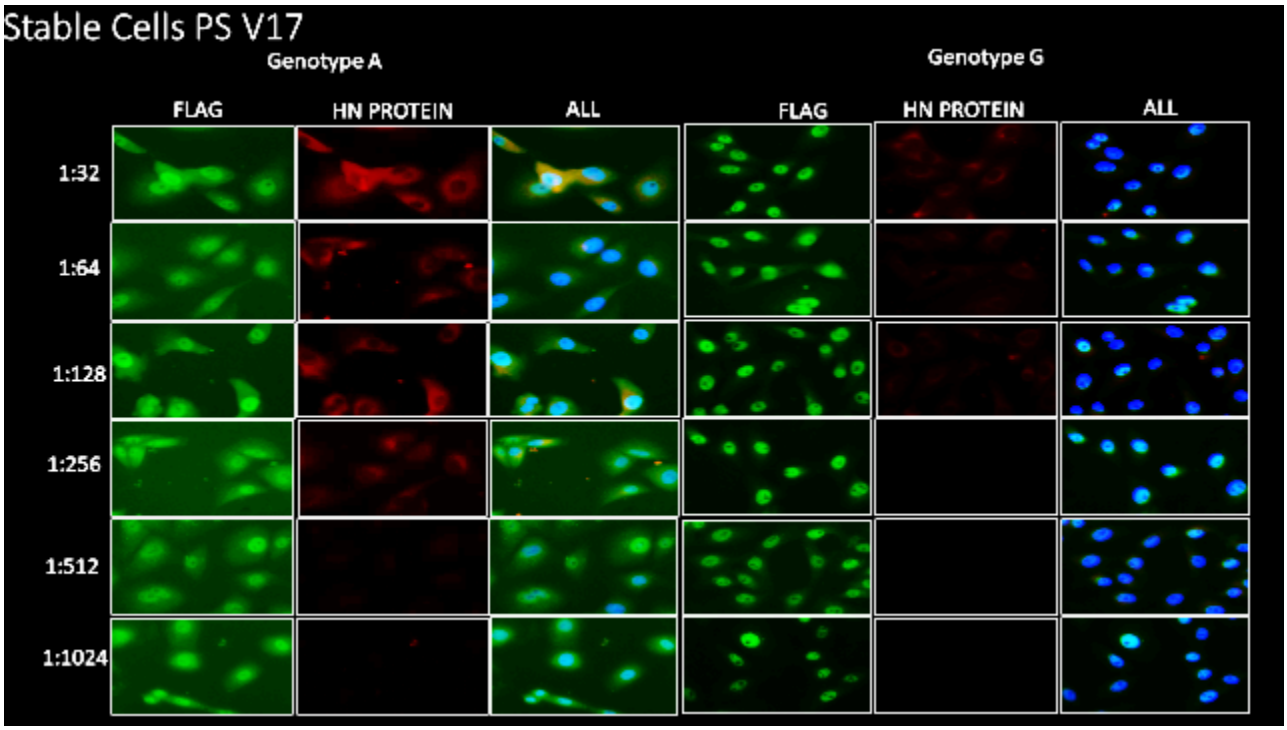


Figure A-20a. Stable cells Vaccine Sample#17.

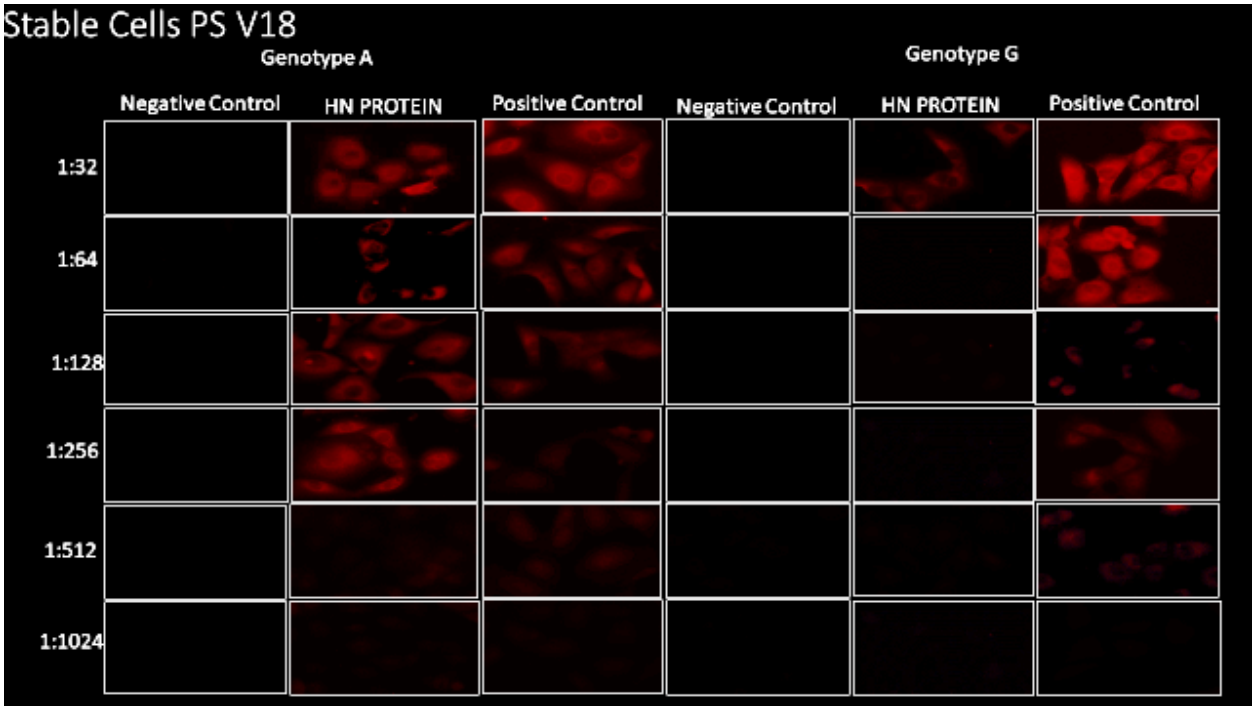


Figure A-21a. Stable cells Vaccine Sample#18.

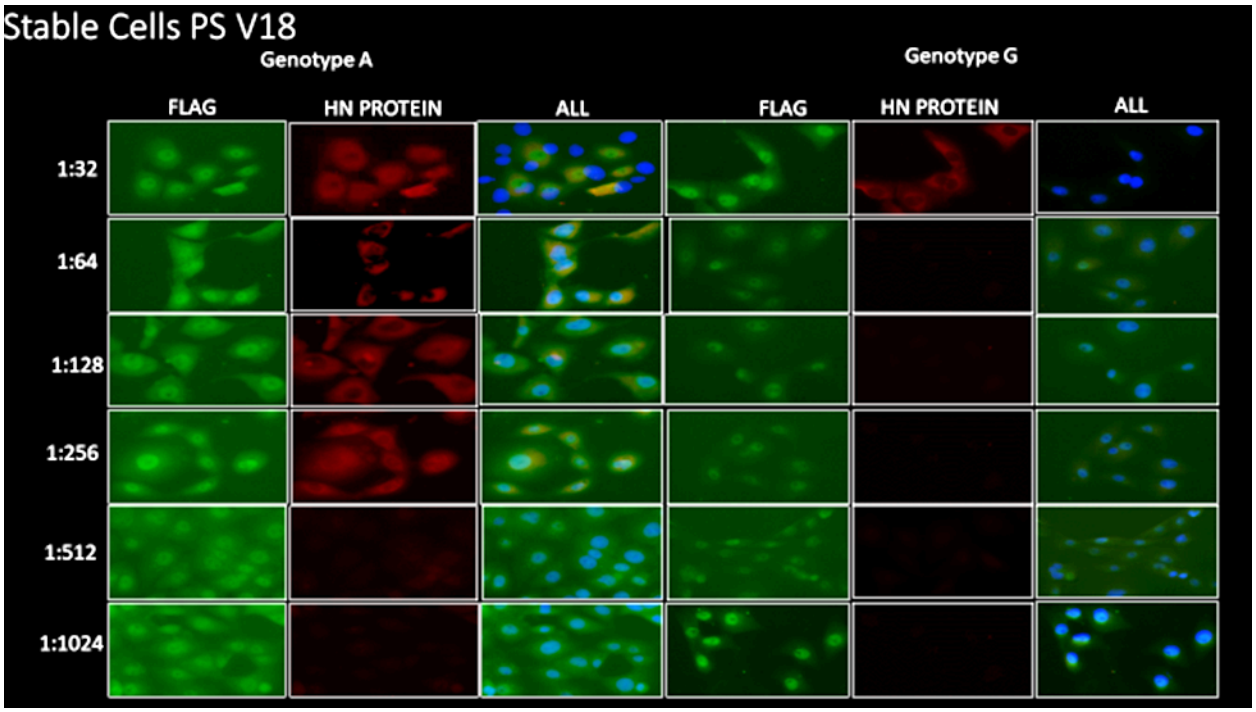


Figure A-21b. Stable cells Vaccine Sample #18.

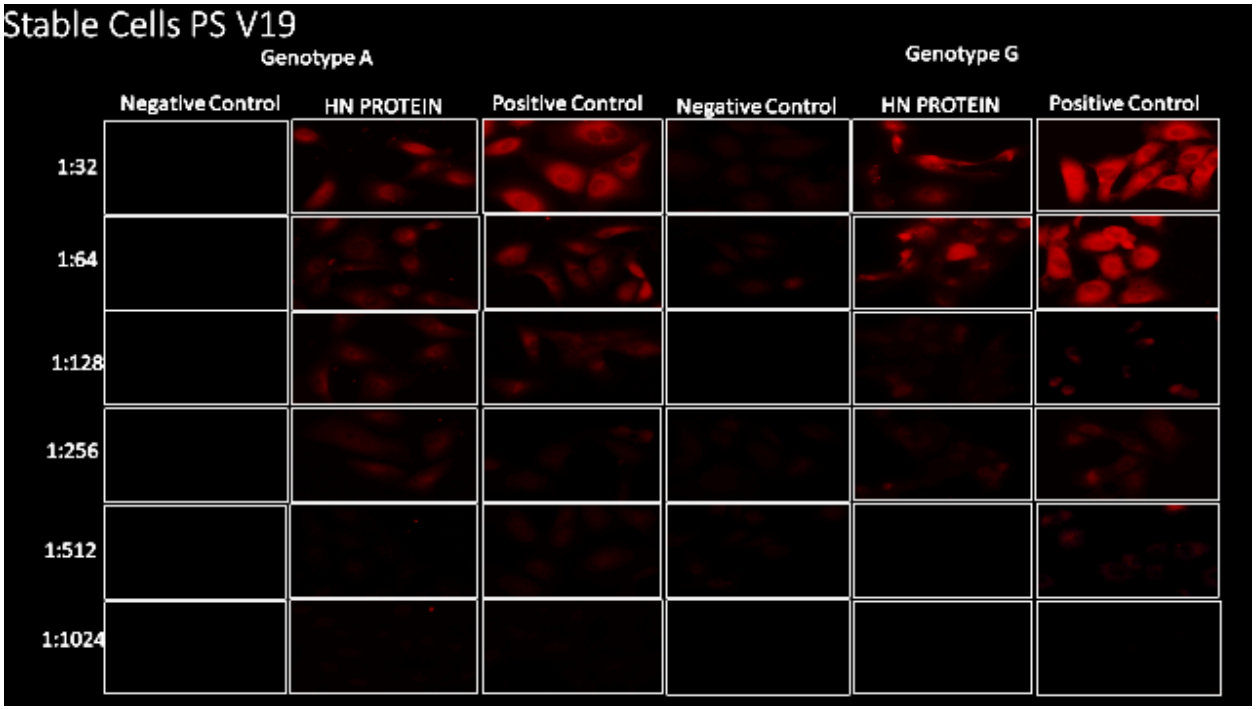


Figure A-22a. Stable cells Vaccine Sample#19.

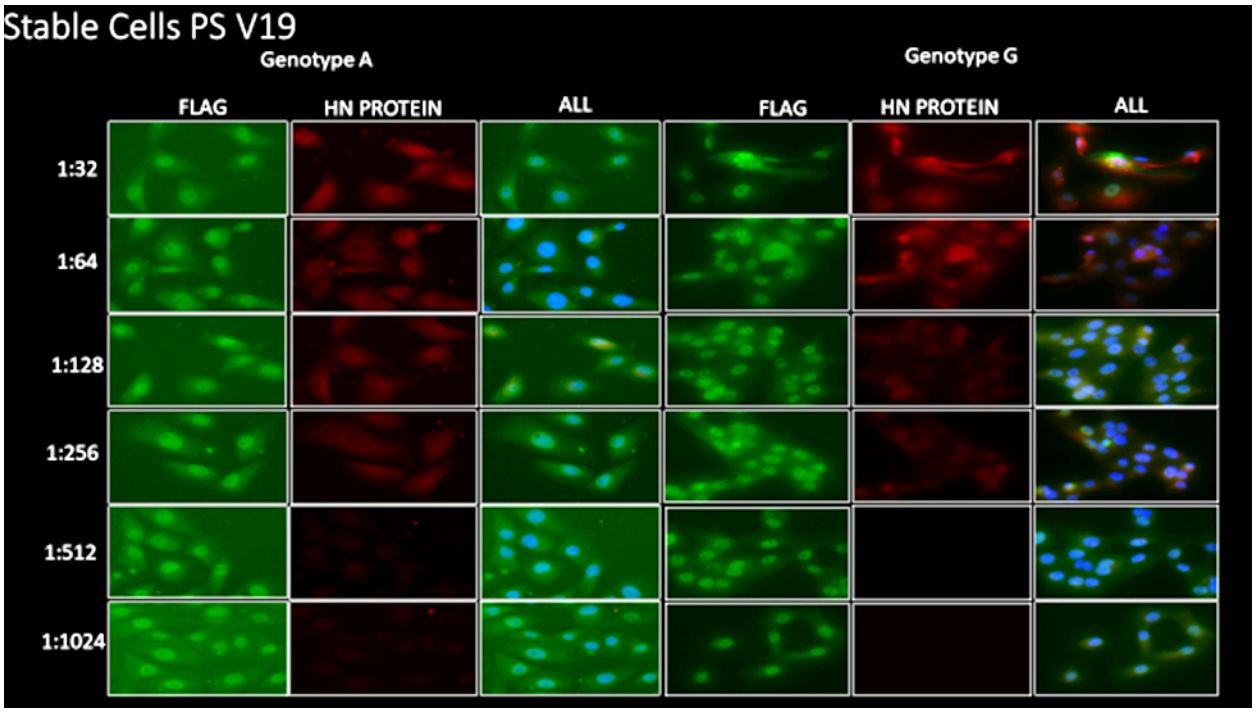


Figure A-22b. Stable cells Vaccine Sample #19.

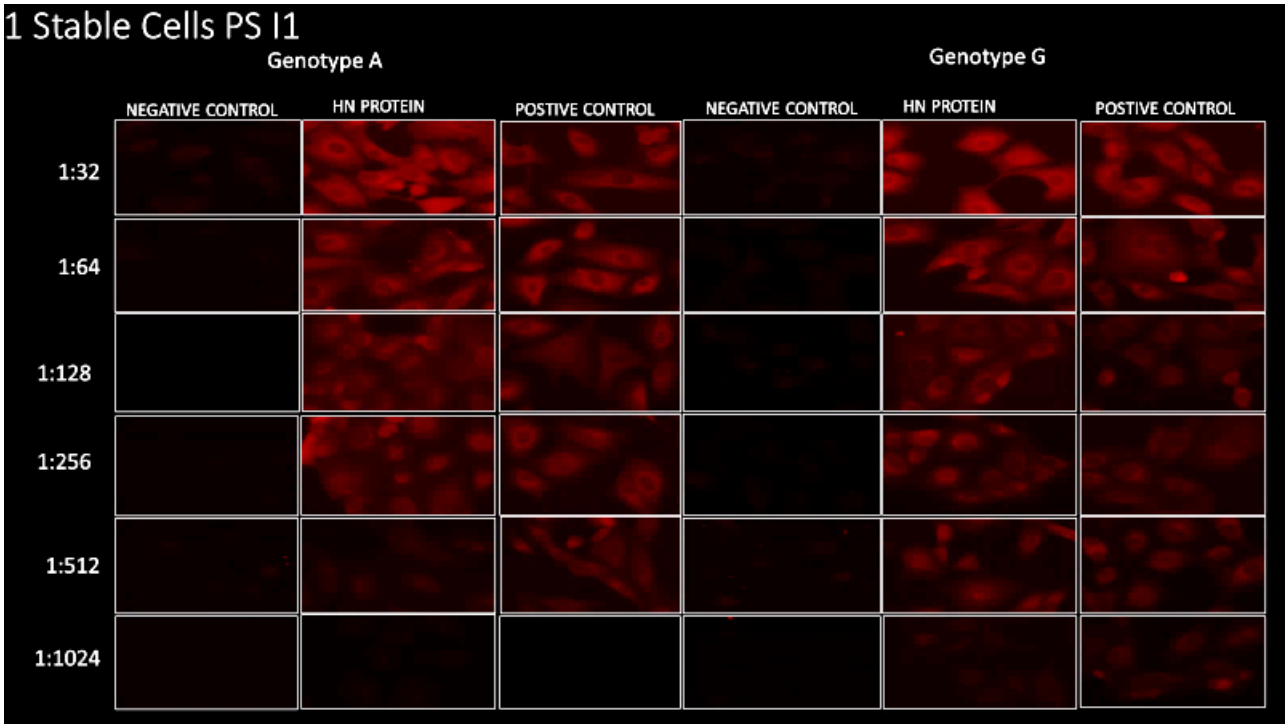


Figure A-23a. Stable cells Infected Sample#1.

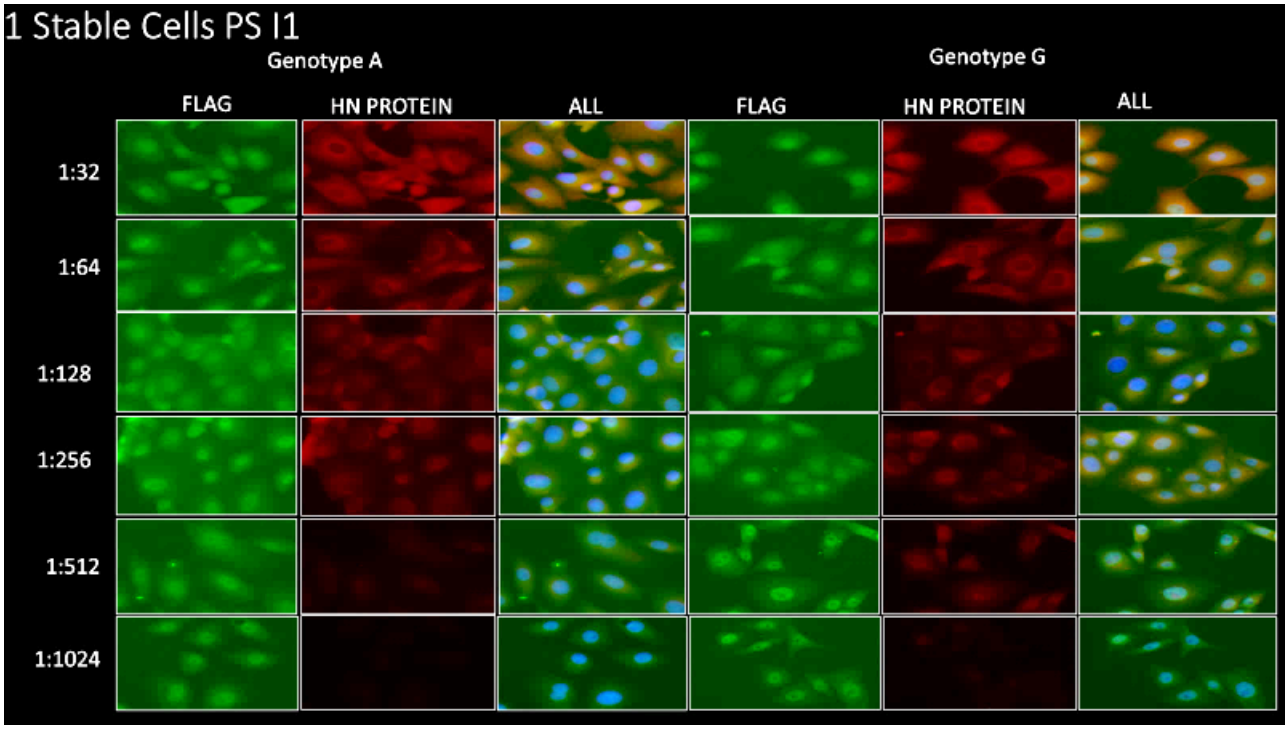


Figure A-23b. Stable cells Infected Sample #1.

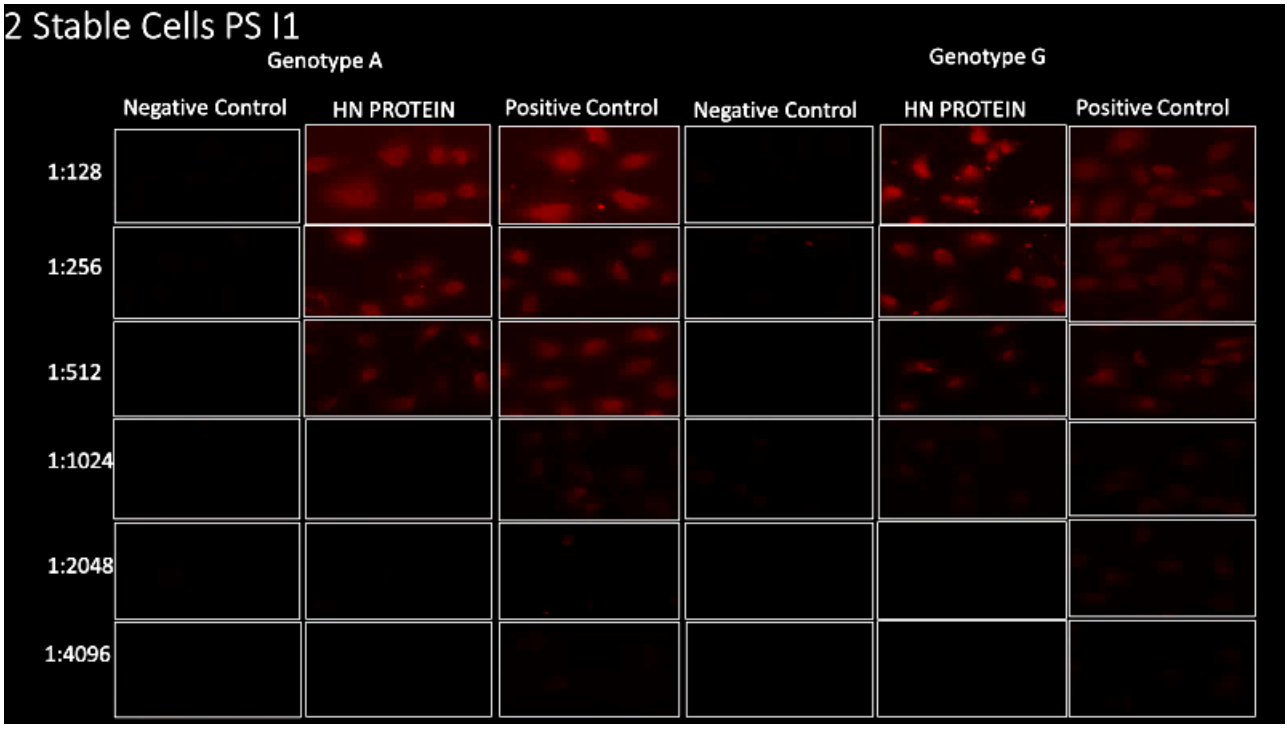


Figure A-23c. Stable cells Infected Sample #1.

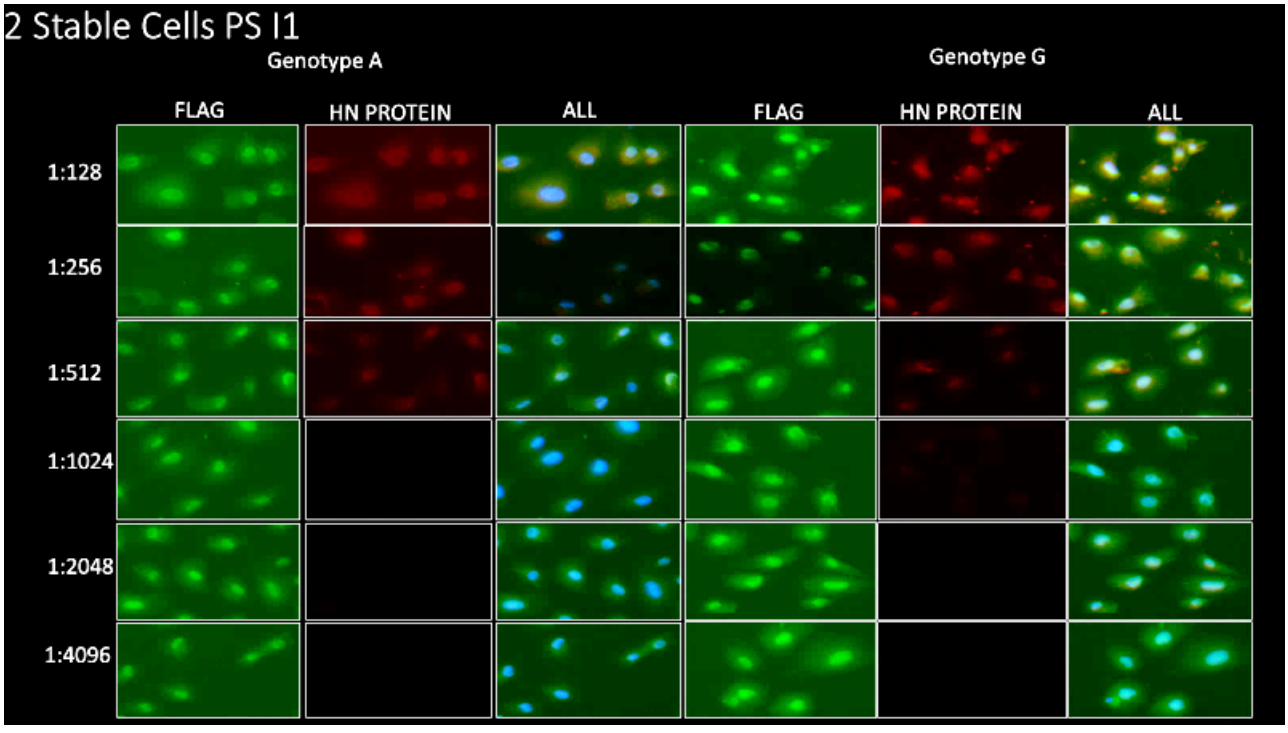


Figure A-23d. Stable cells Infected Sample #1.

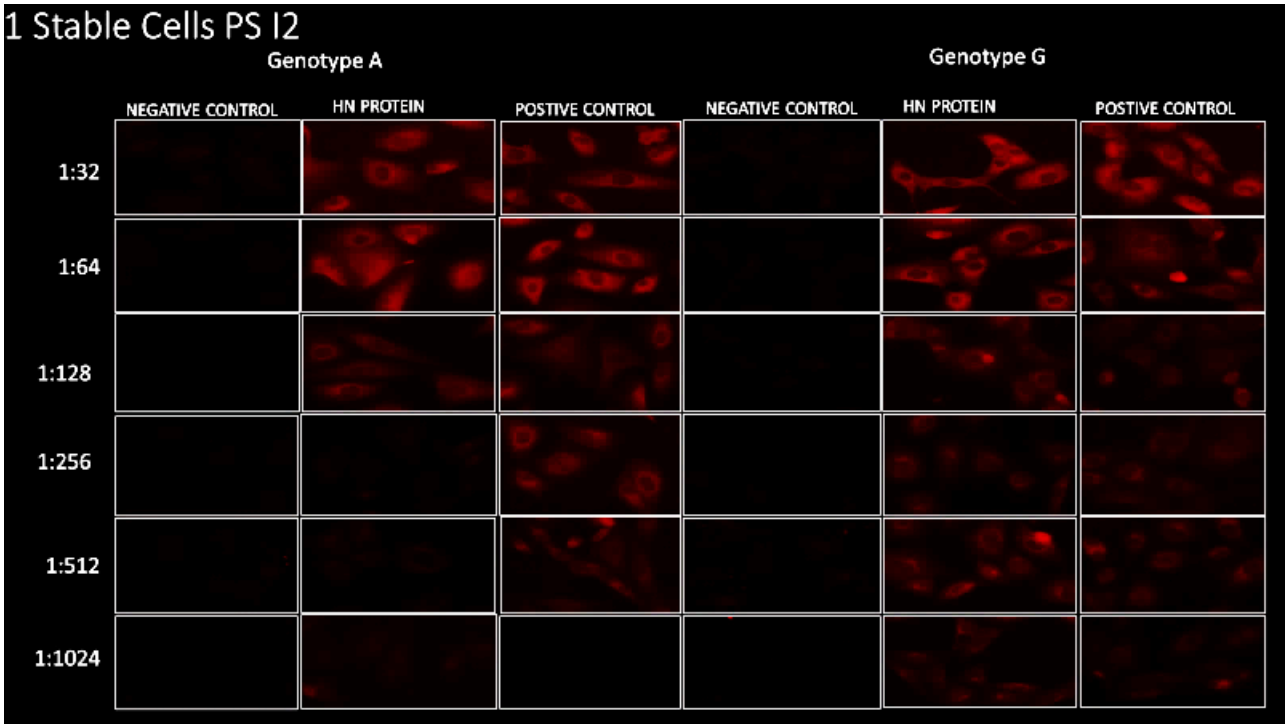


Figure A-24a. Stable cells Infected Sample#2.

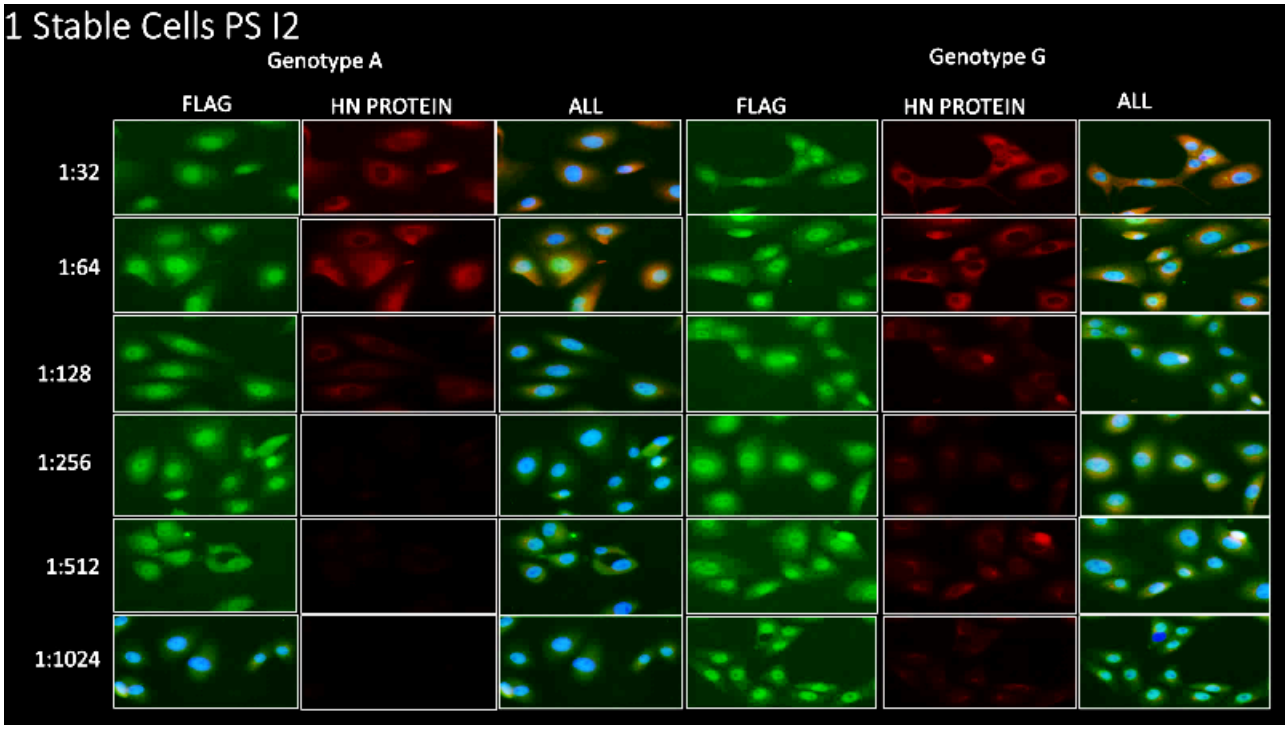


Figure A-24b. Stable cells Infected Sample #2.

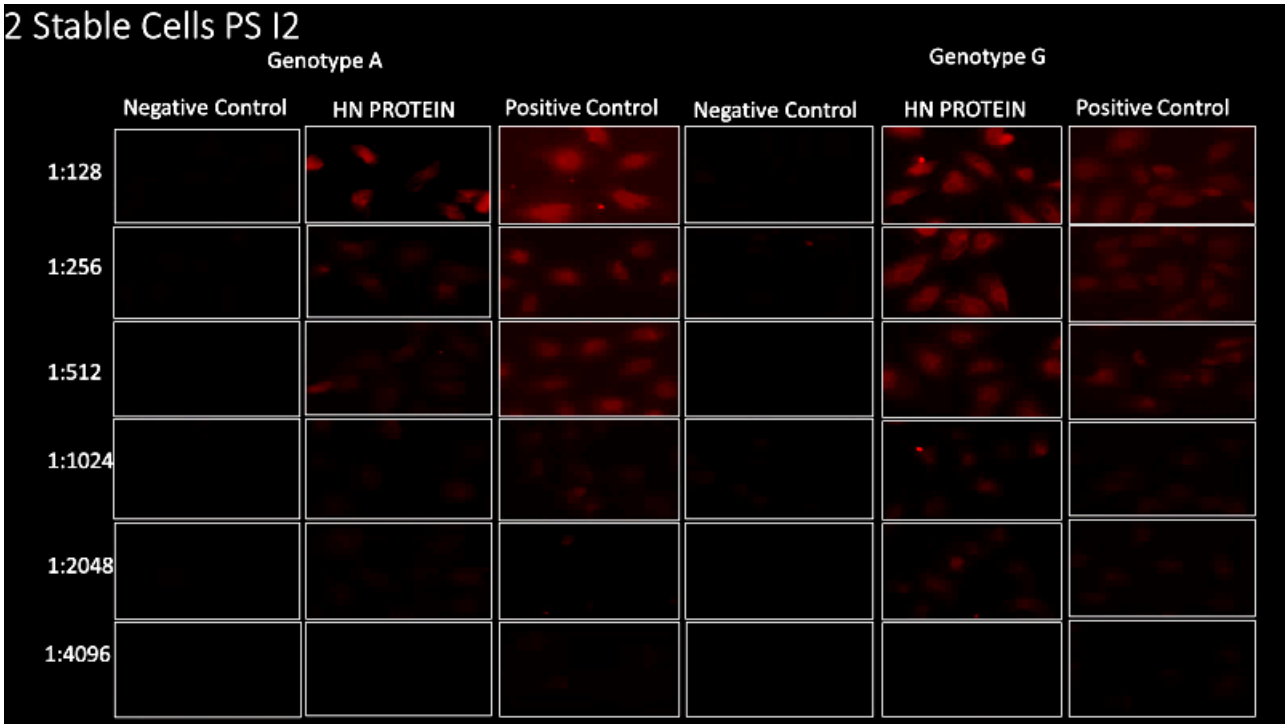


Figure A-24c. Stable cells Infected Sample #2.

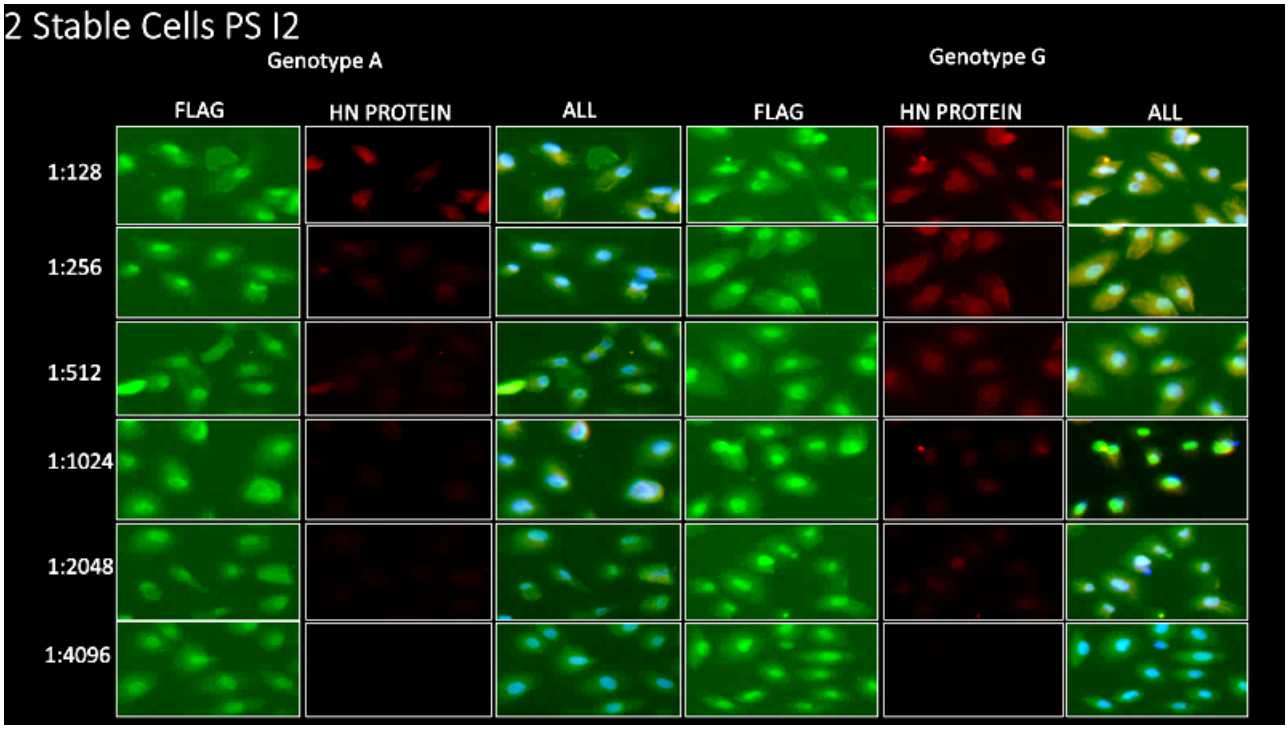


Figure A-24d. Stable cells Infected Sample #2.

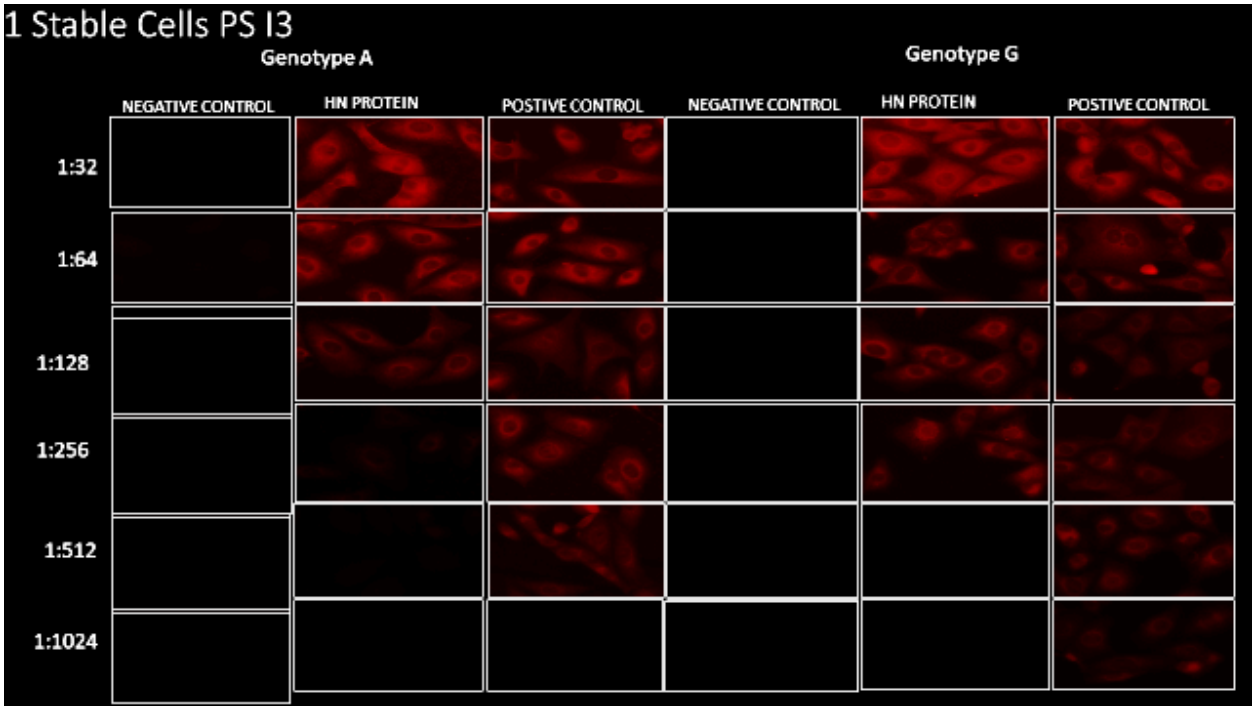


Figure A-25a. Stable cells Infected Sample#3.

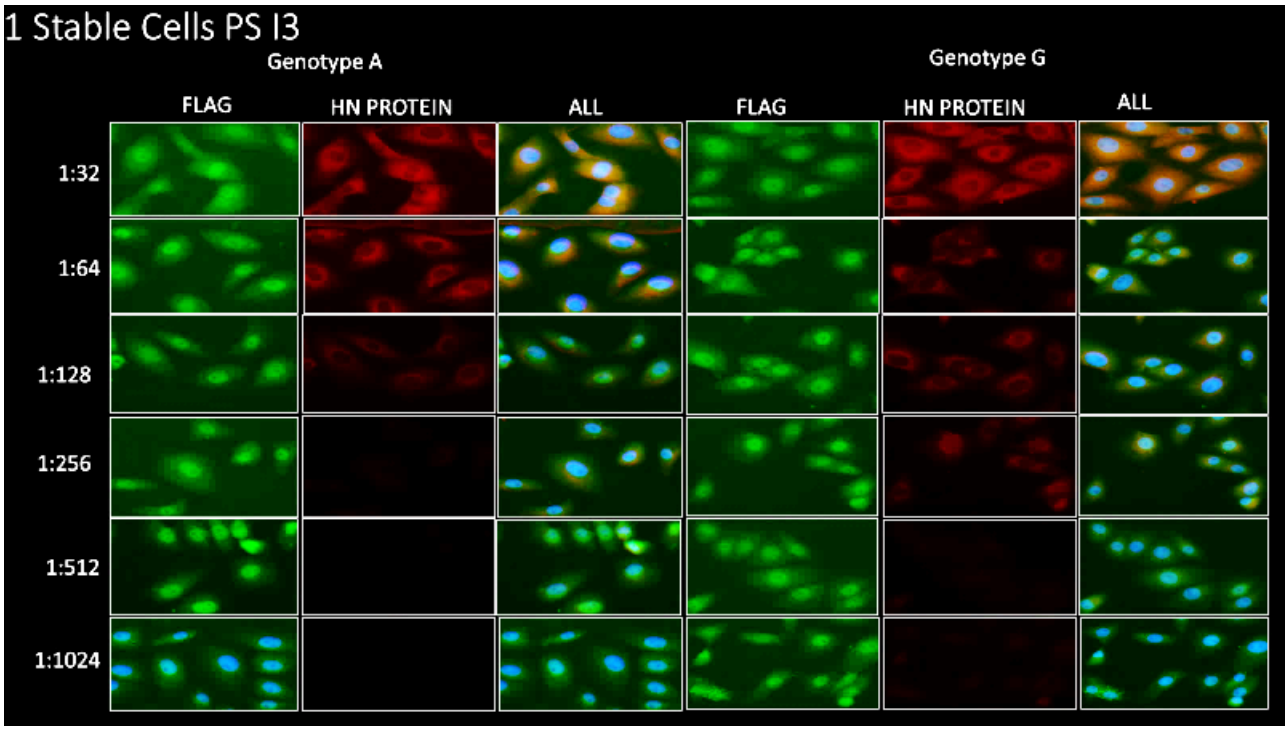


Figure A-25b. Stable cells Infected Sample #3.

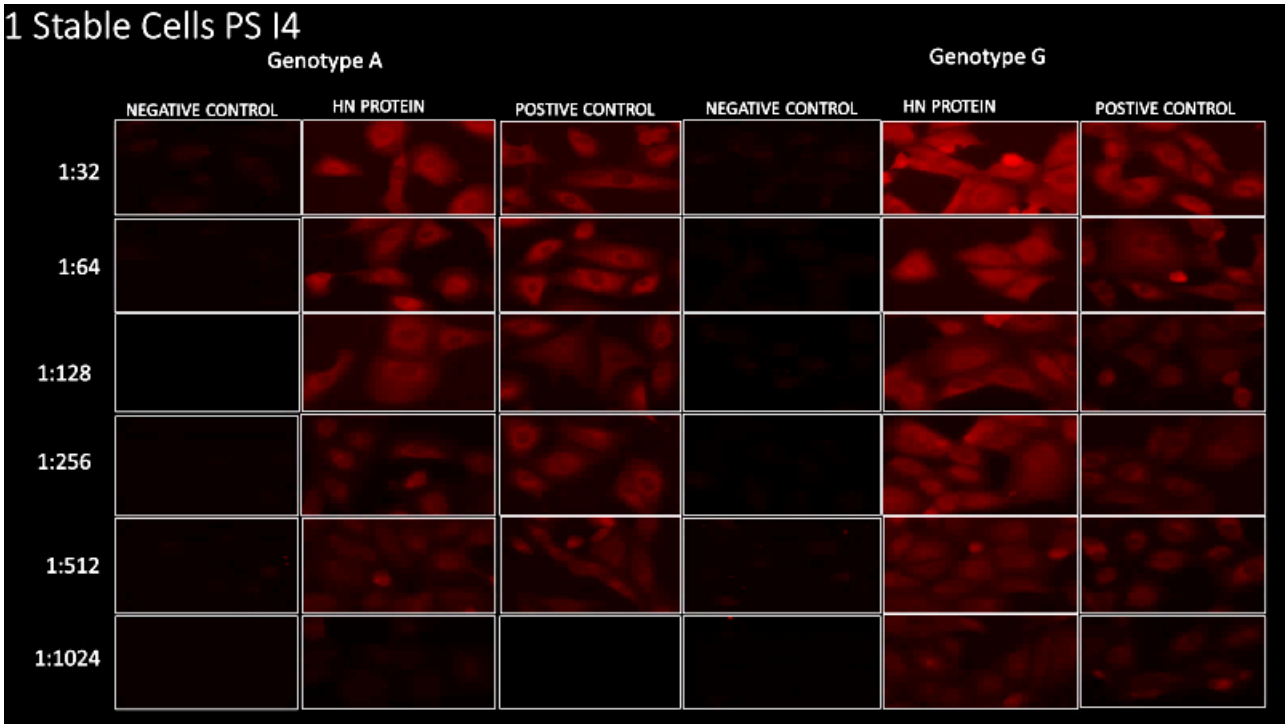


Figure A-26a. Stable cells Infected Sample#4.

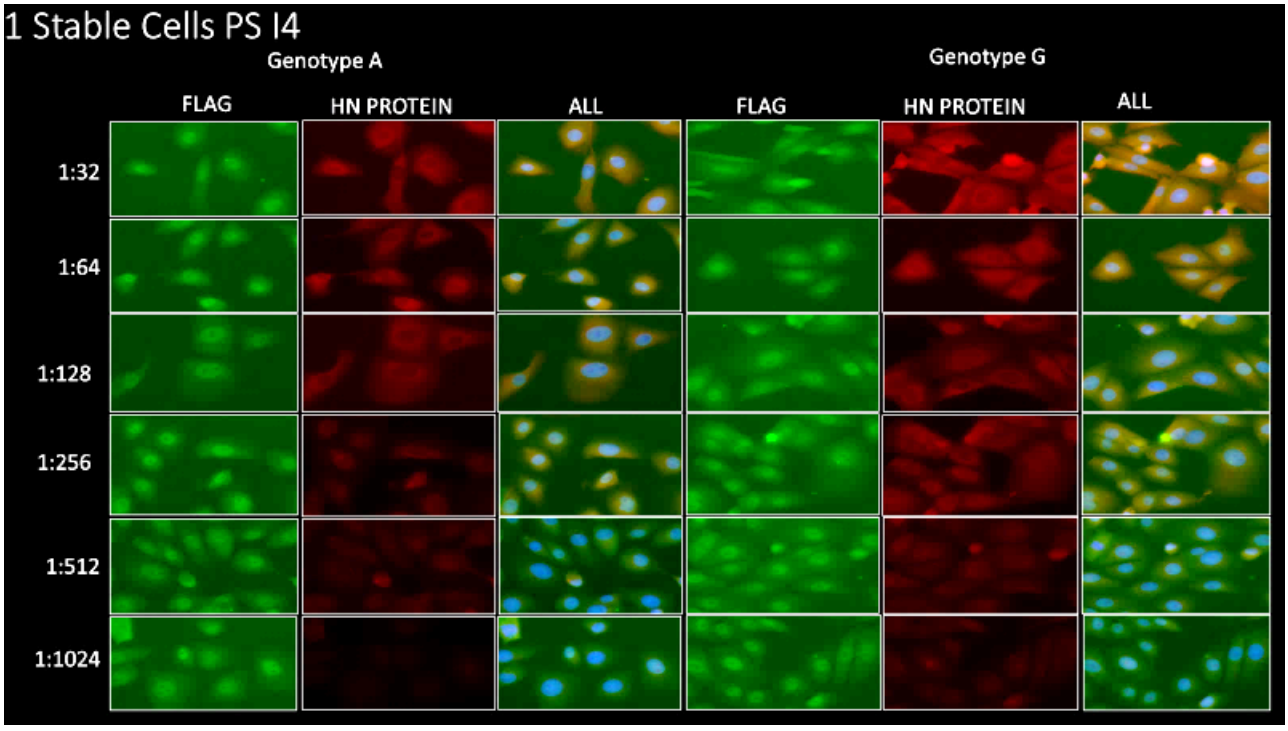


Figure A-26b. Stable cells Infected Sample #4.

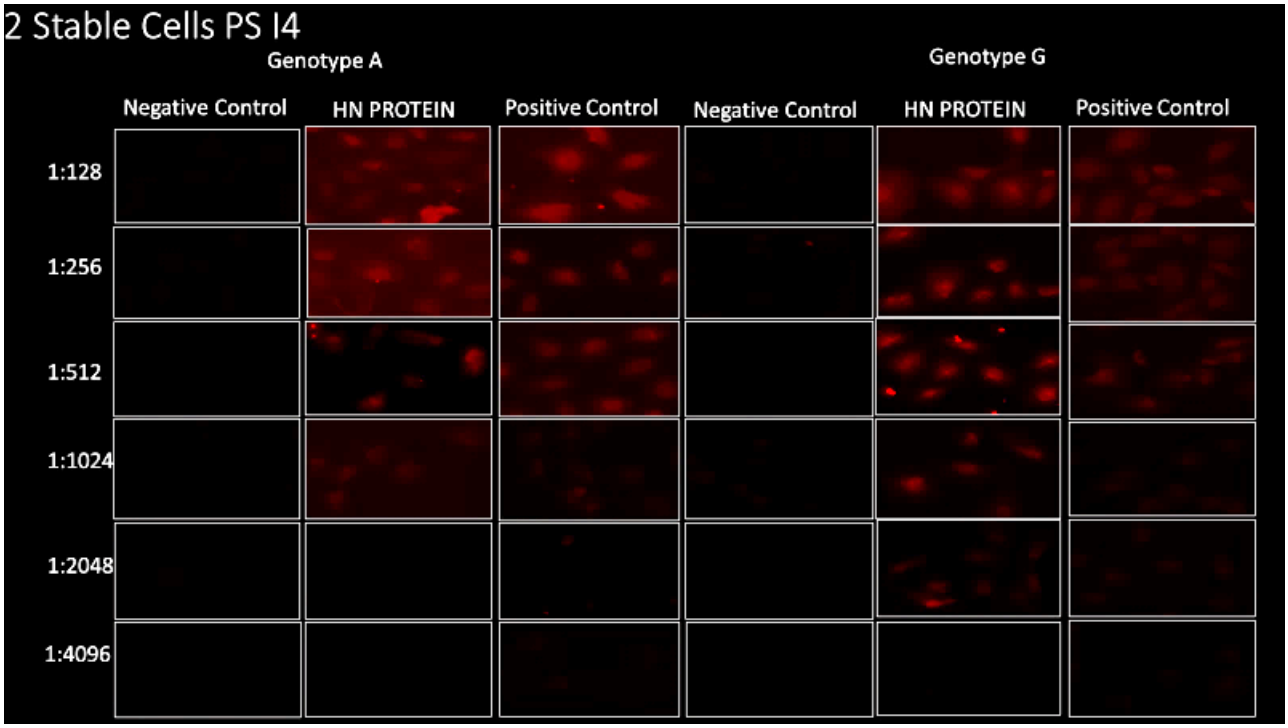


Figure A-26c. Stable cells Infected Sample #4.

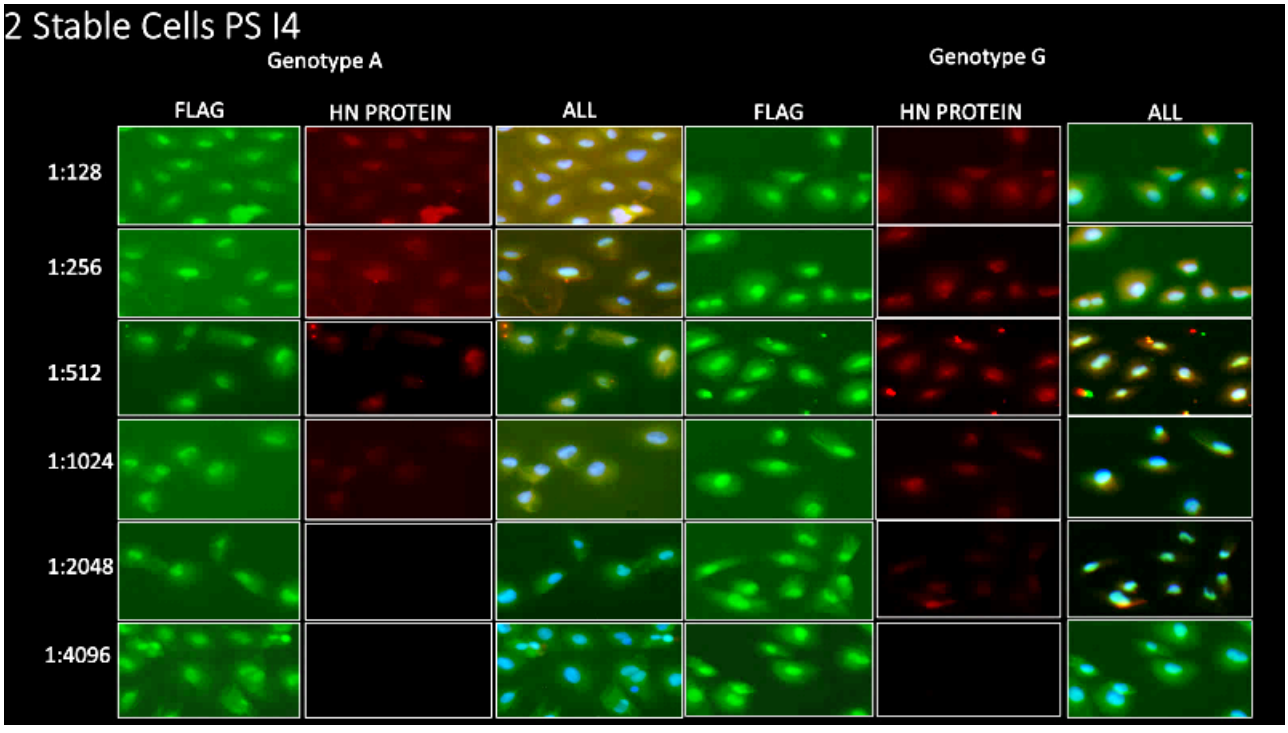


Figure A-26d. Stable cells Infected Sample #4.

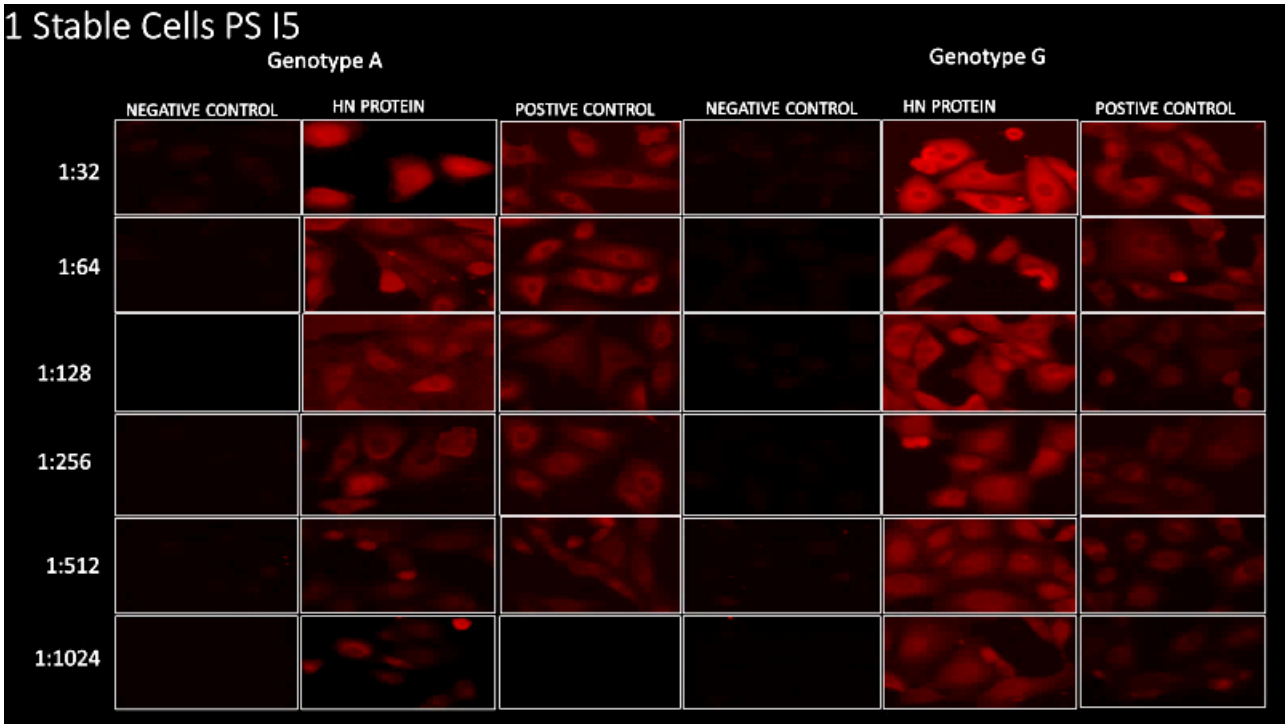


Figure A-27a. Stable cells Infected Sample#5.

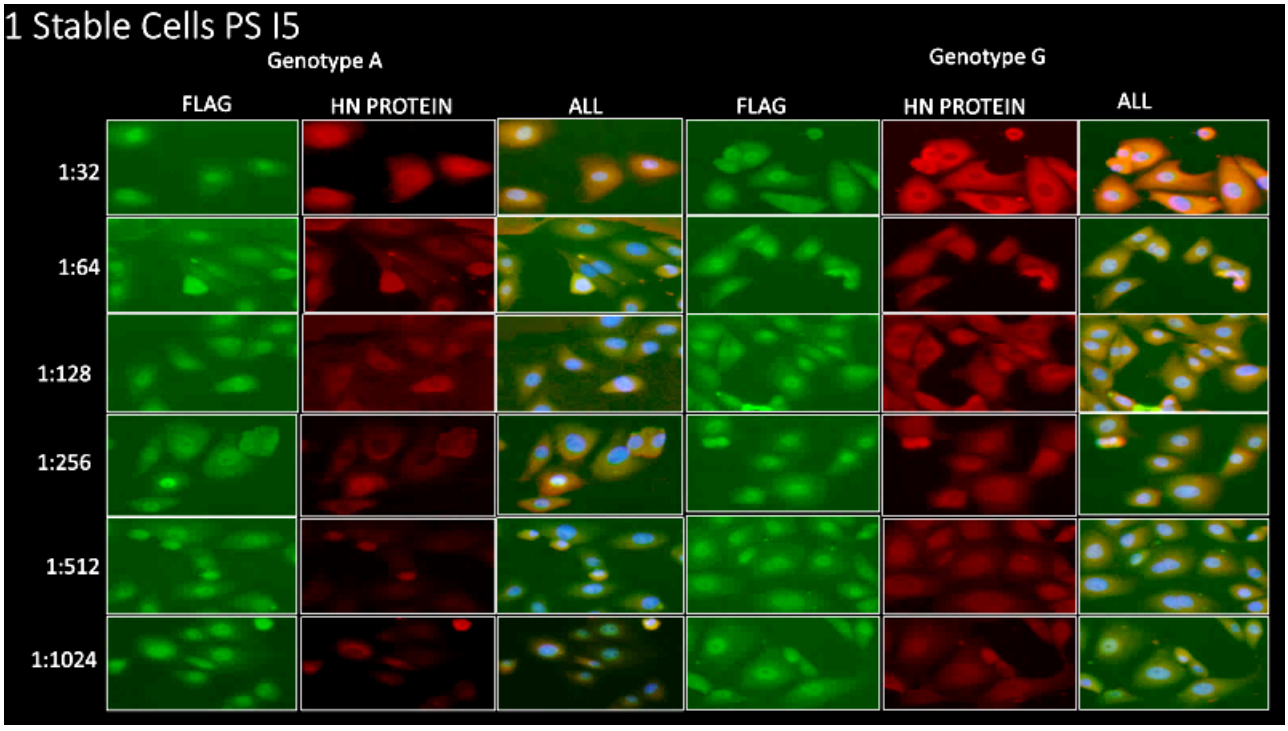


Figure A-27b. Stable cells Infected Sample #5.

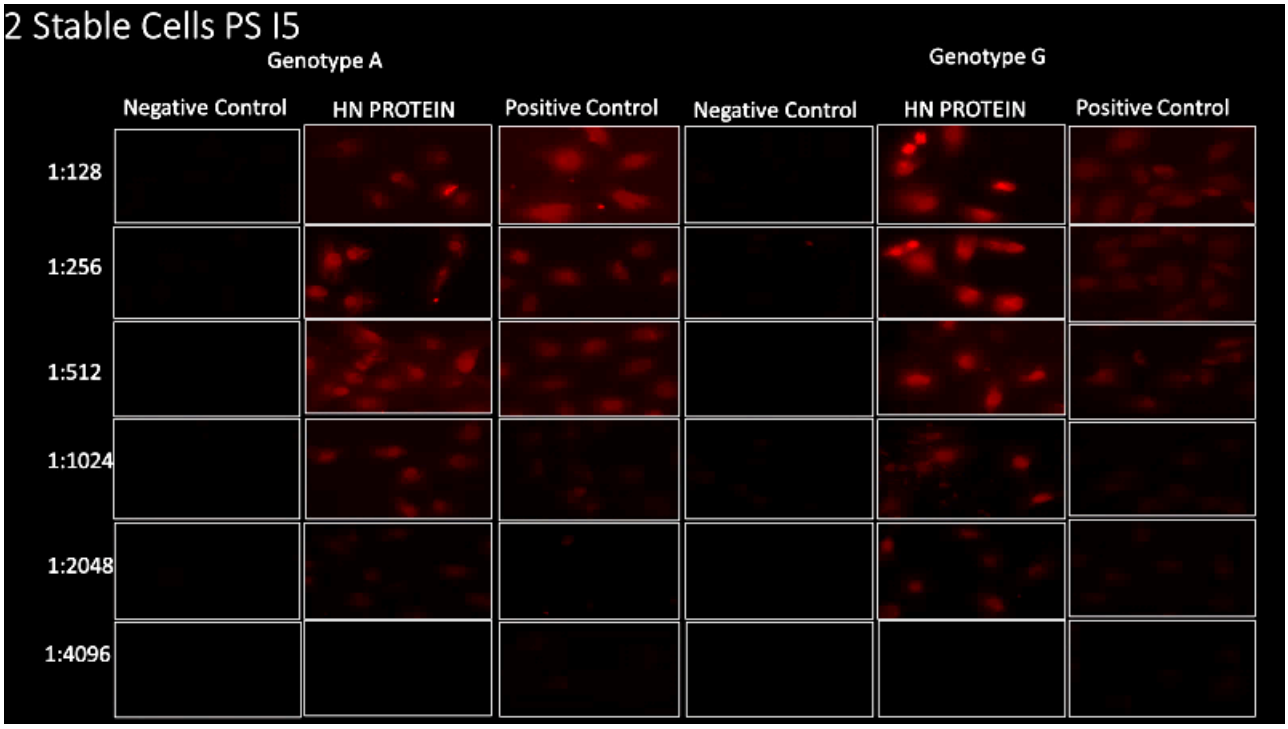


Figure A-27c. Stable cells Infected Sample #5.

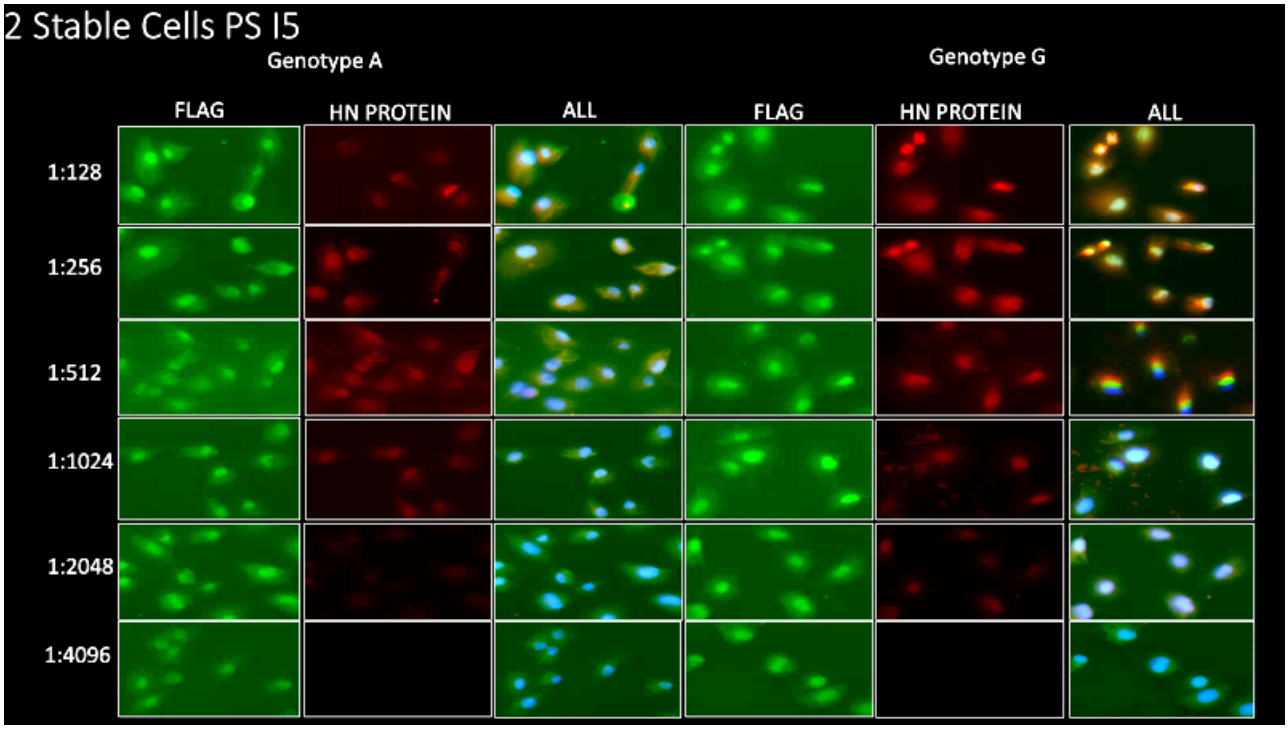


Figure A-27d. Stable cells Infected Sample #5.

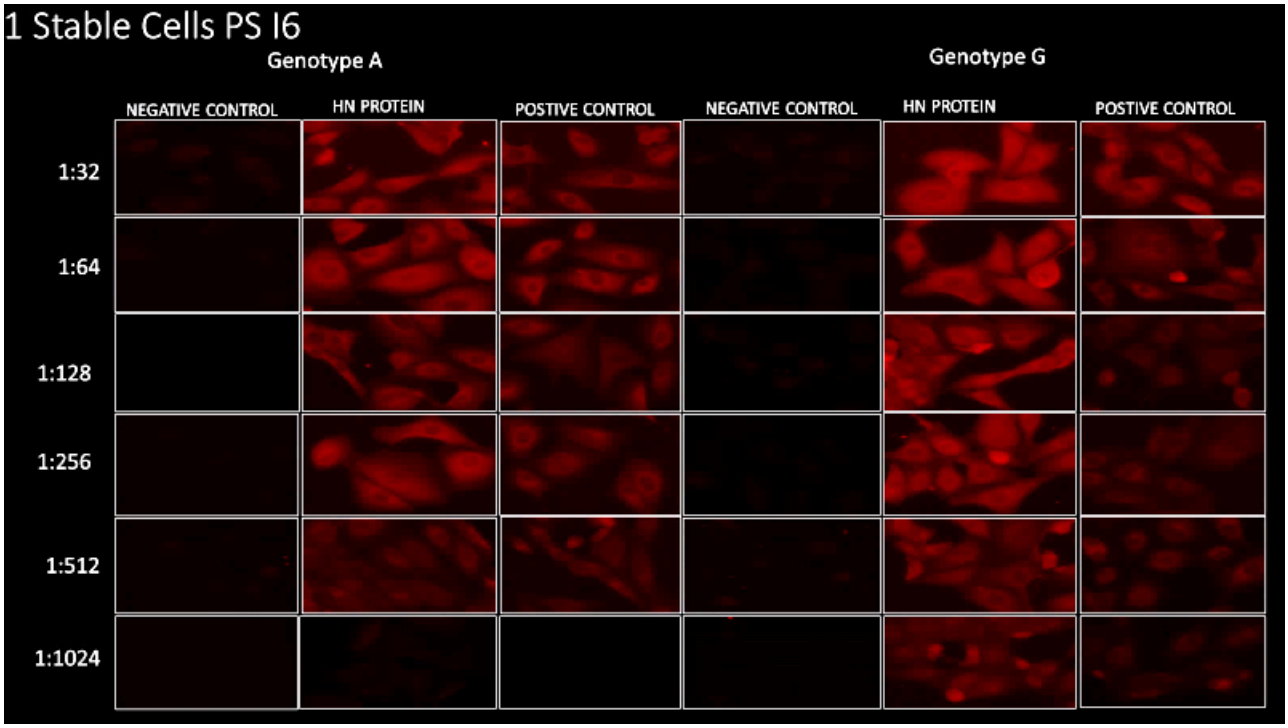


Figure A-28a. Stable cells Infected Sample#6.

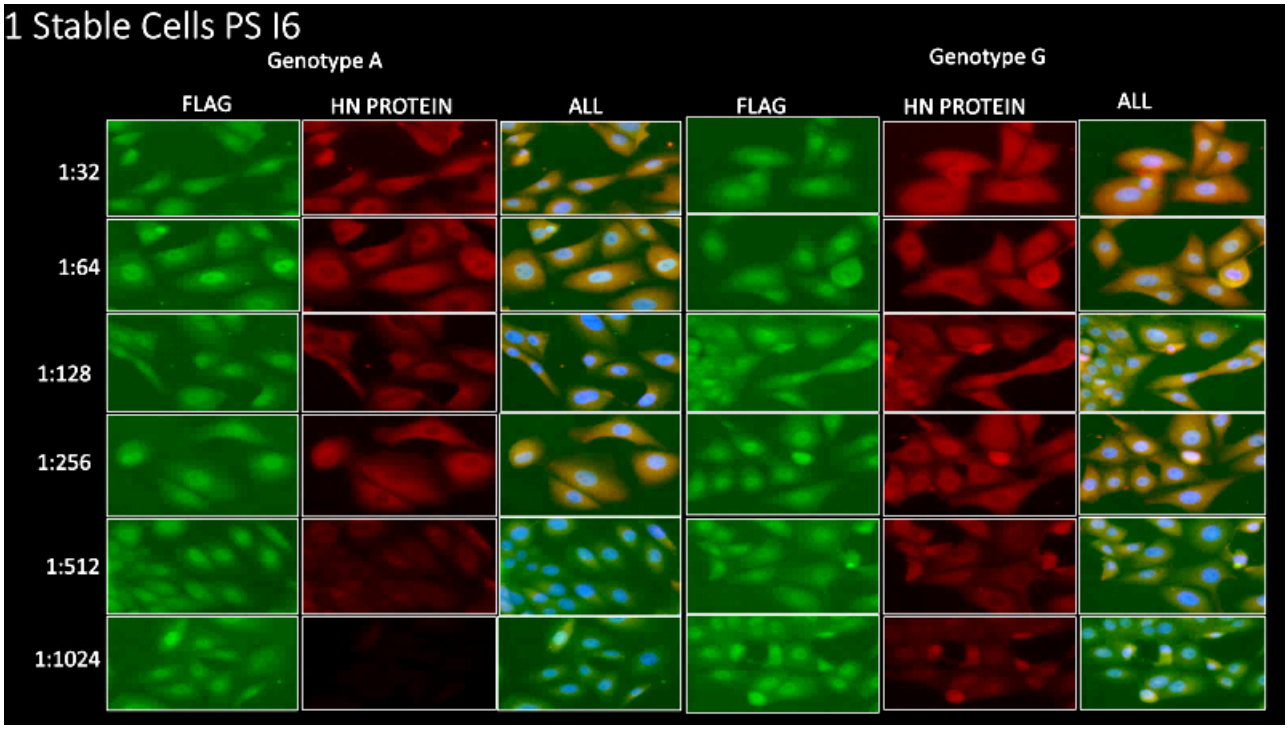


Figure A-28b. Stable cells Infected Sample #6.

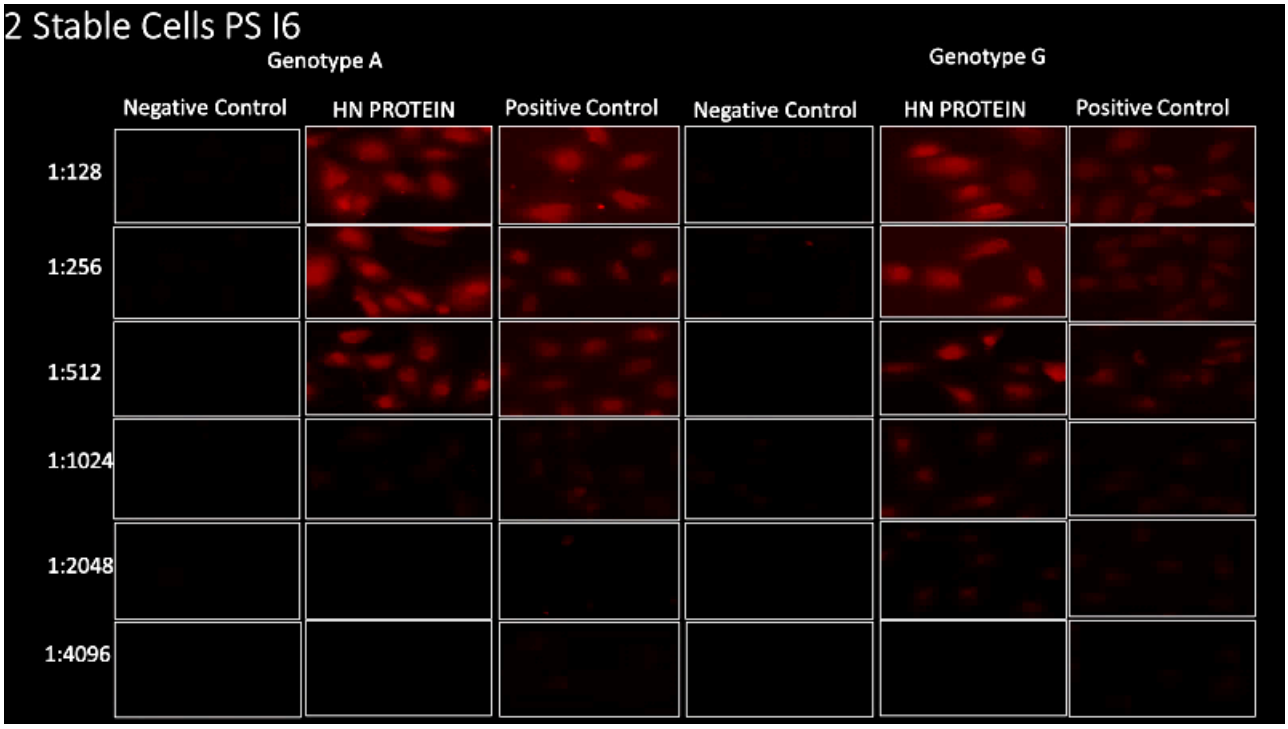


Figure A-28c. Stable cells Infected Sample #6.

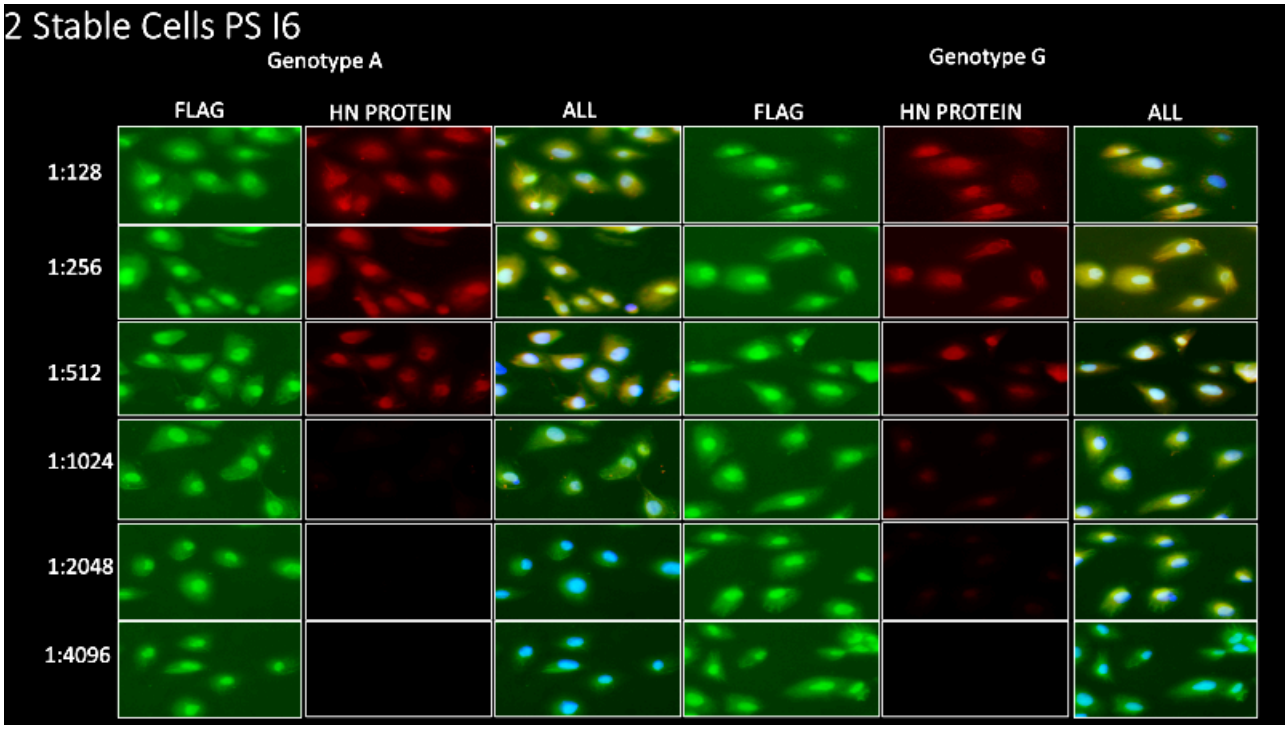


Figure A-28d. Stable cells Infected Sample #6.

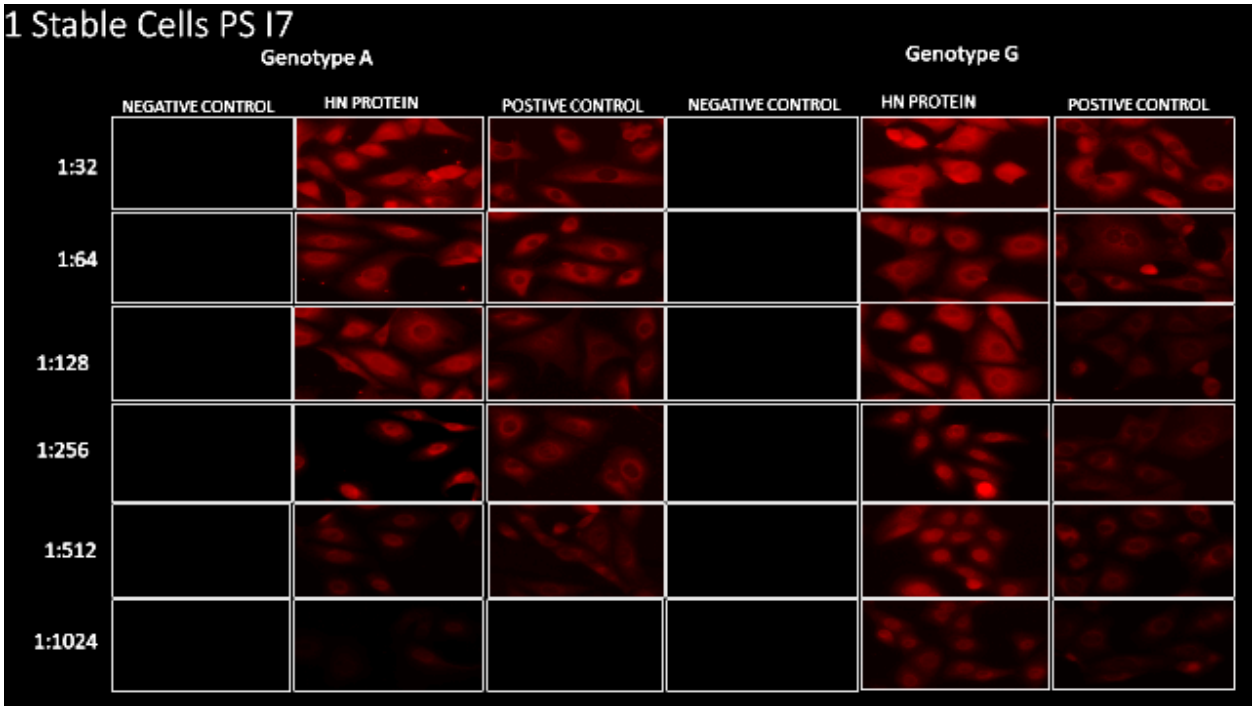


Figure A-29a. Stable cells Infected Sample#7.

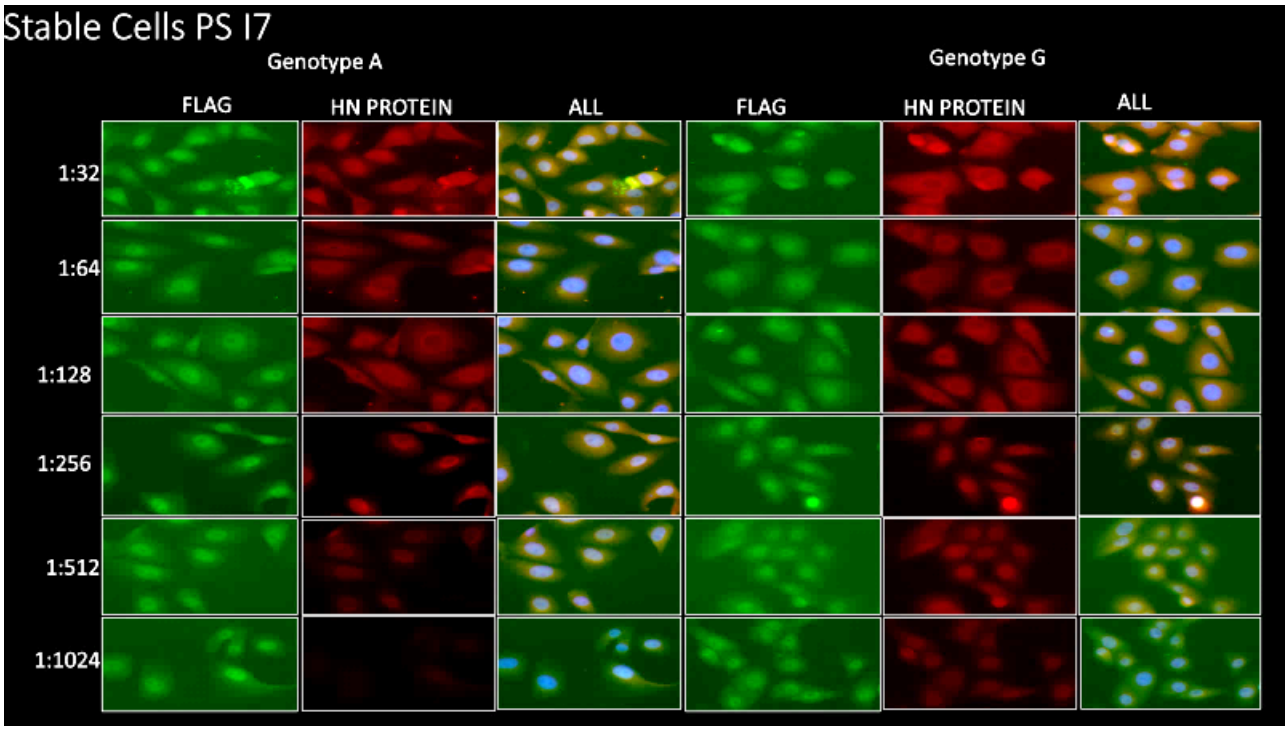


Figure A-29b. Stable cells Infected Sample #7.

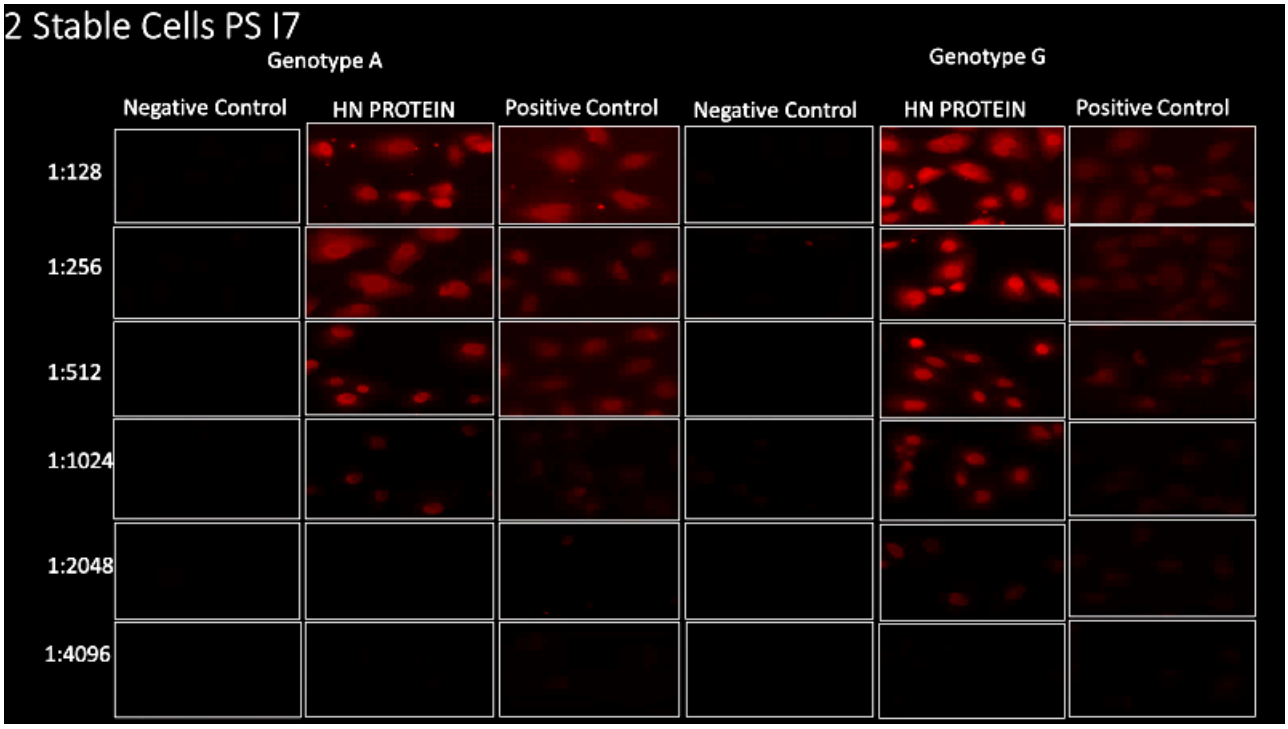


Figure A-29c. Stable cells Infected Sample #7.

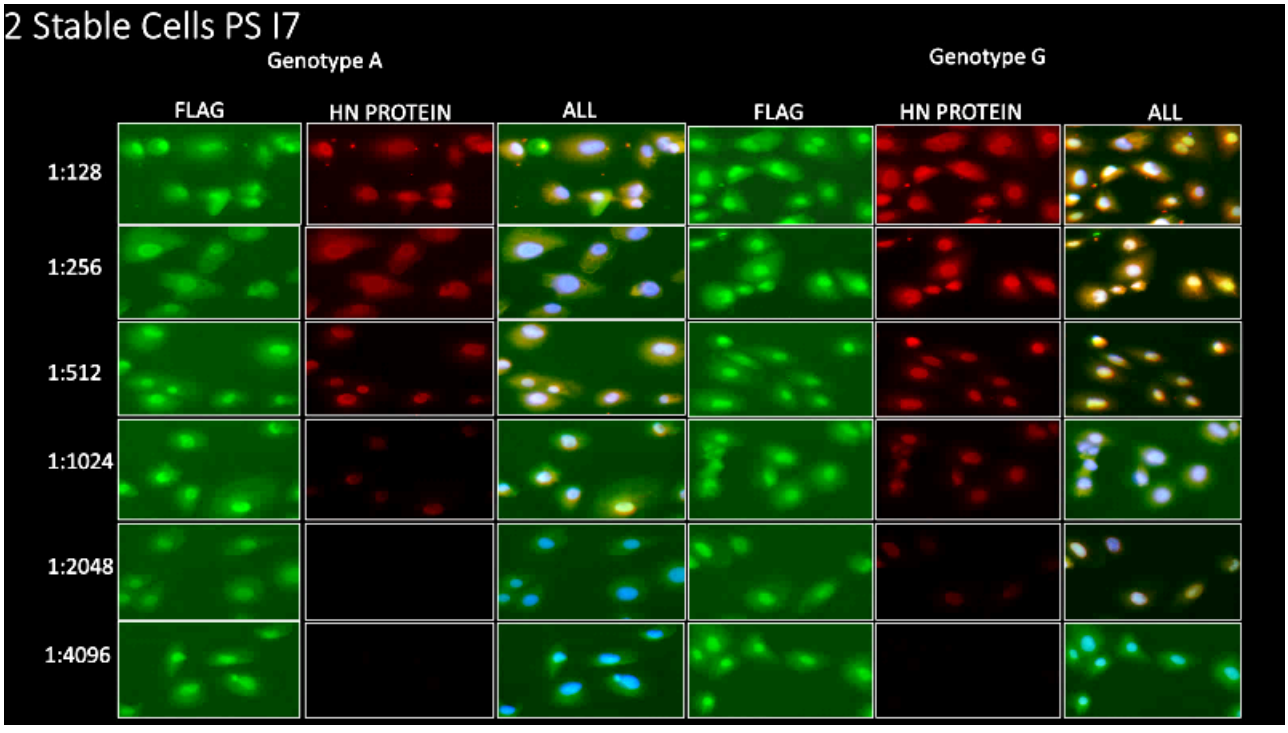


Figure A-29d. Stable cells Infected Sample #7.

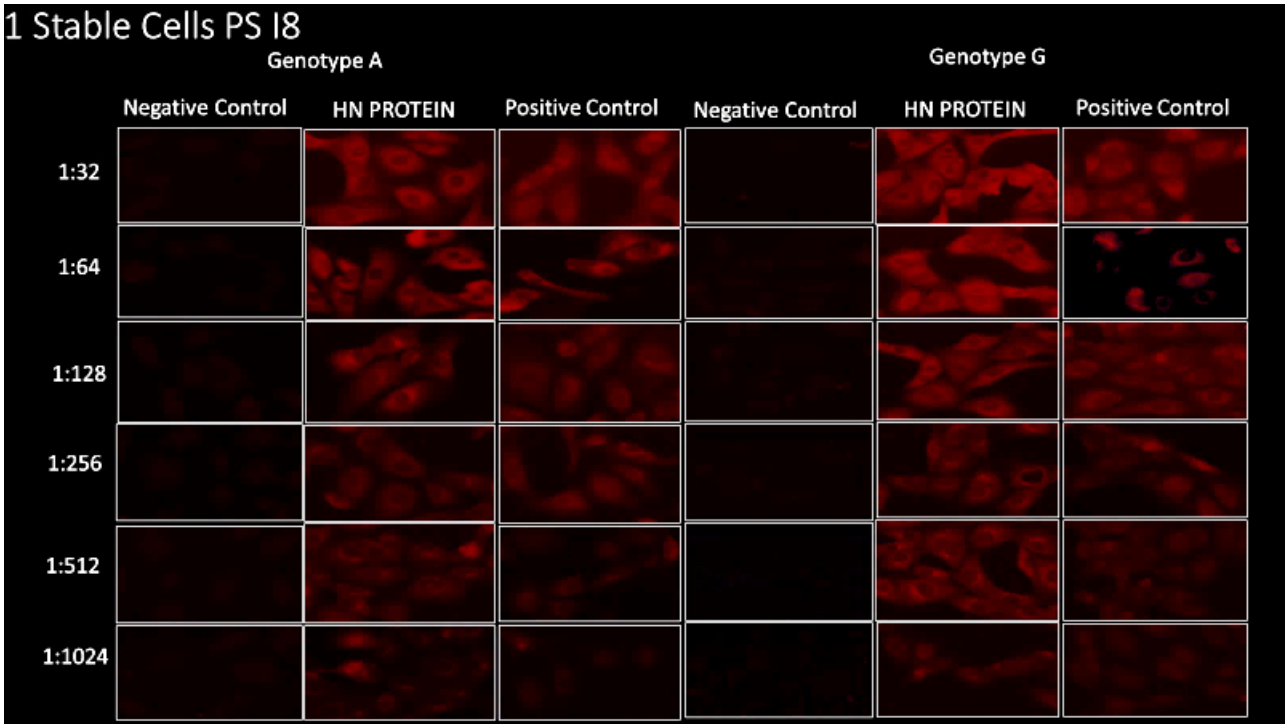


Figure A-30a. Stable cells Infected Sample#8.

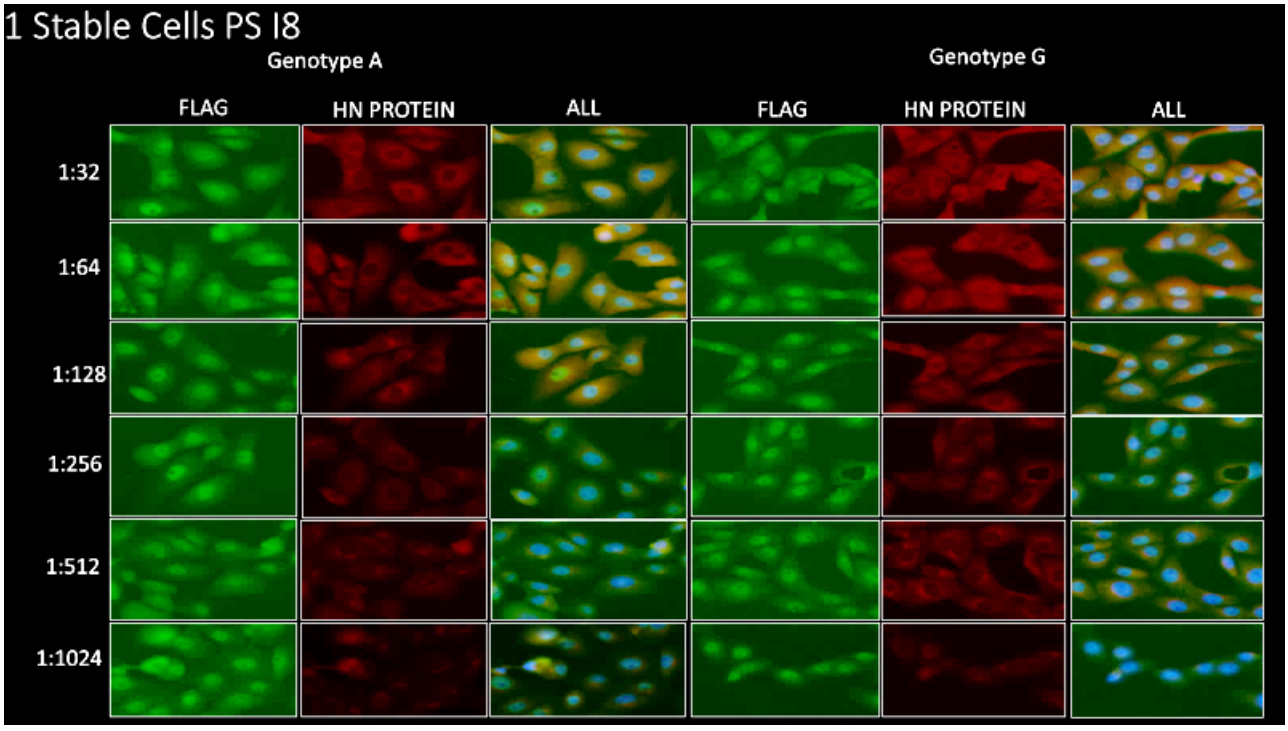


Figure A-30b. Stable cells Infected Sample #8.

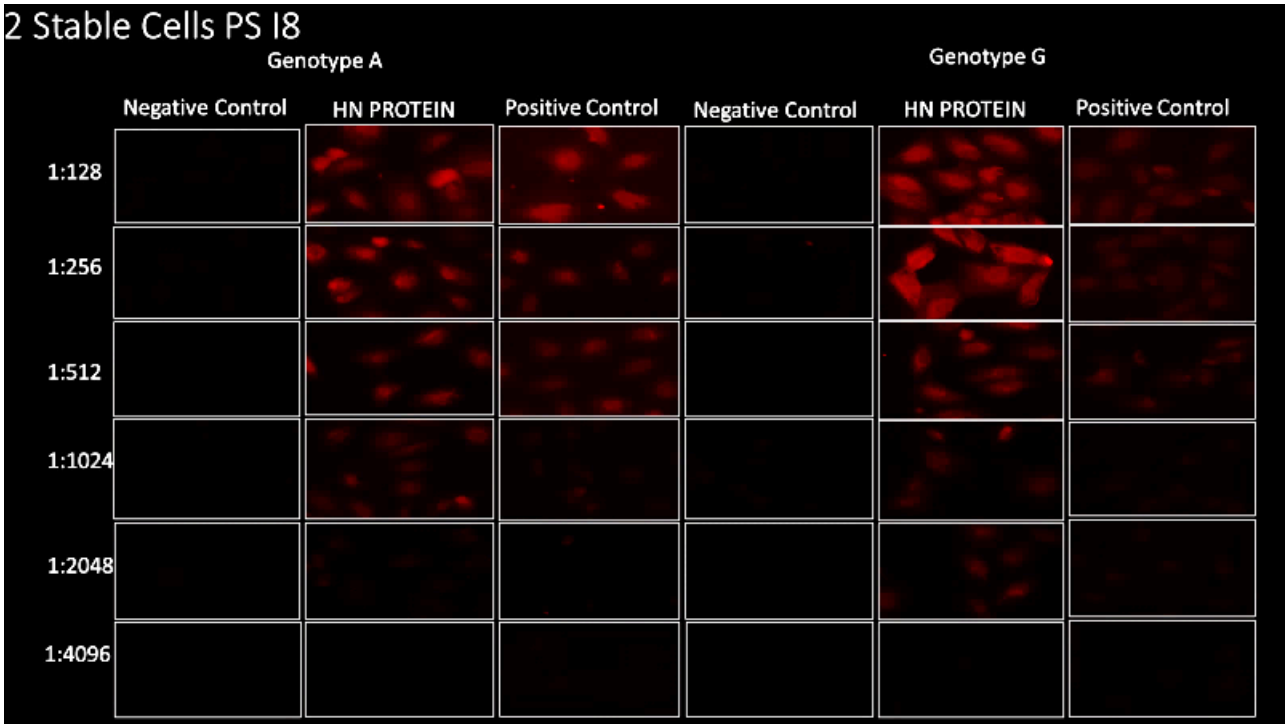


Figure A-30c. Stable cells Infected Sample #8.

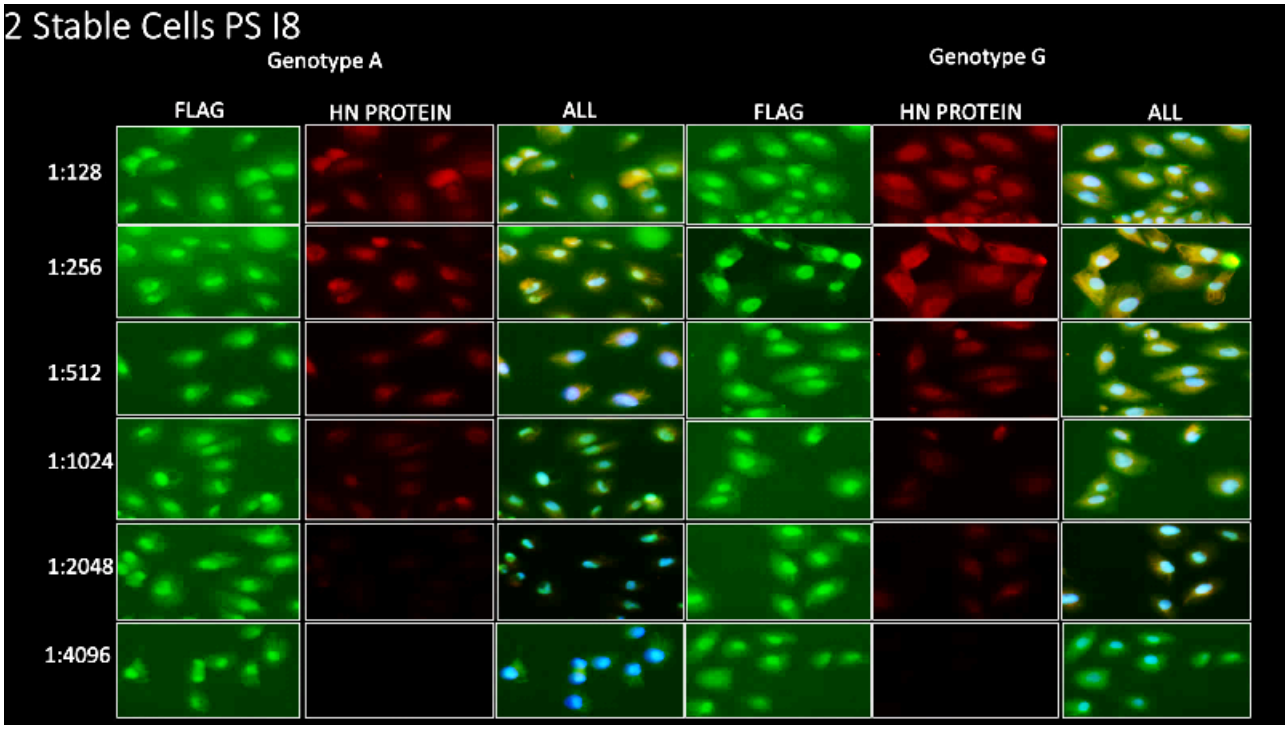


Figure A-30d. Stable cells Infected Sample #8.

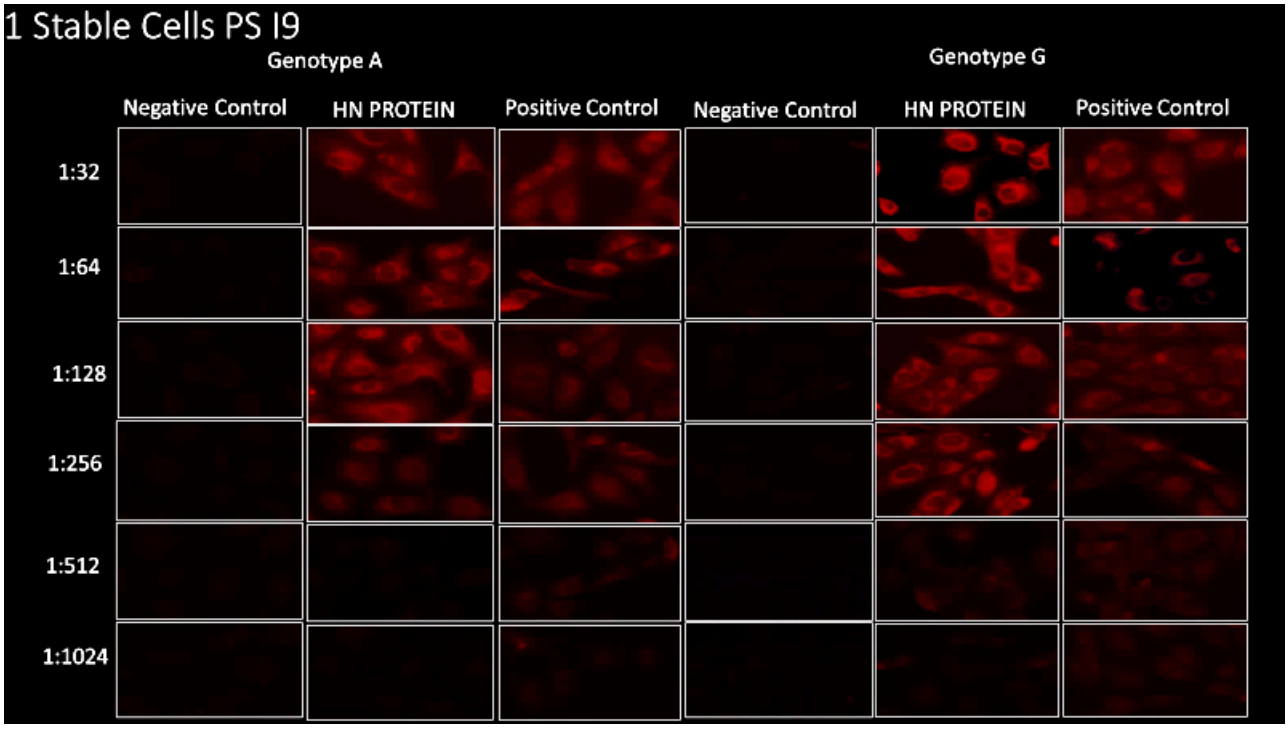


Figure A-31a. Stable cells Infected Sample#9.

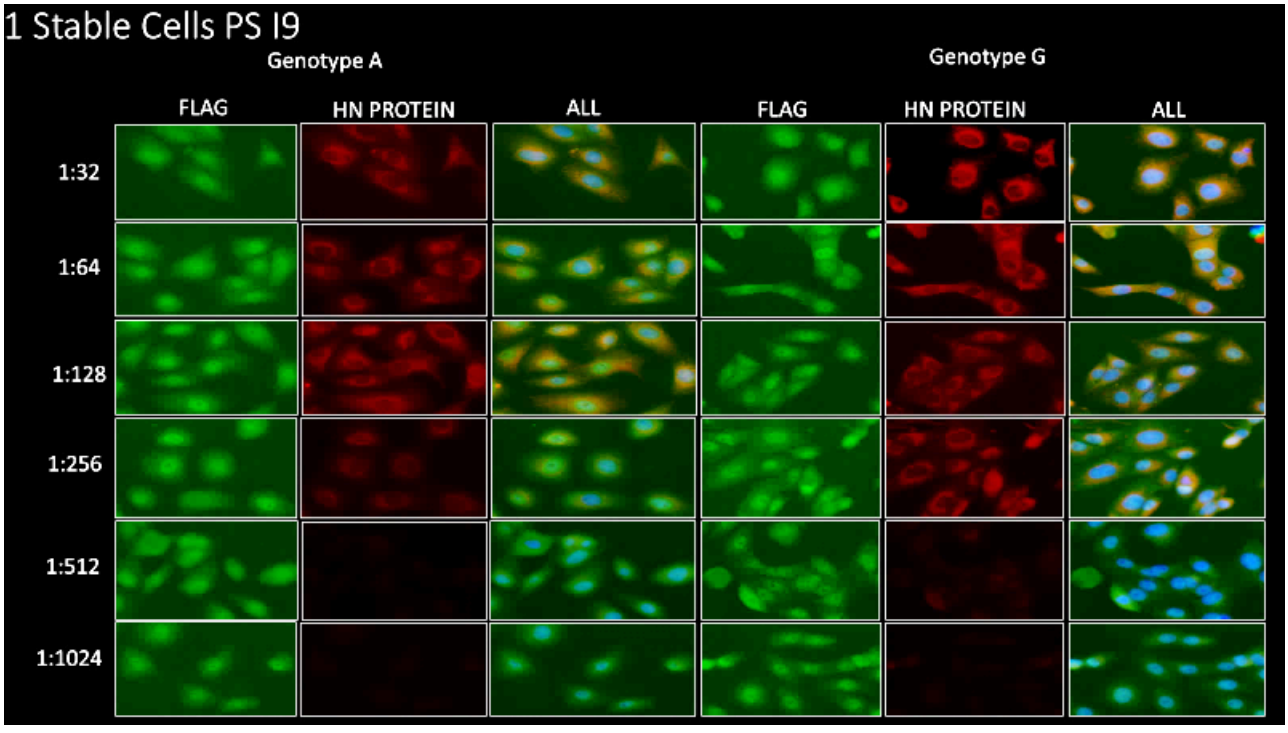


Figure A-31b. Stable cells Infected Sample #9.

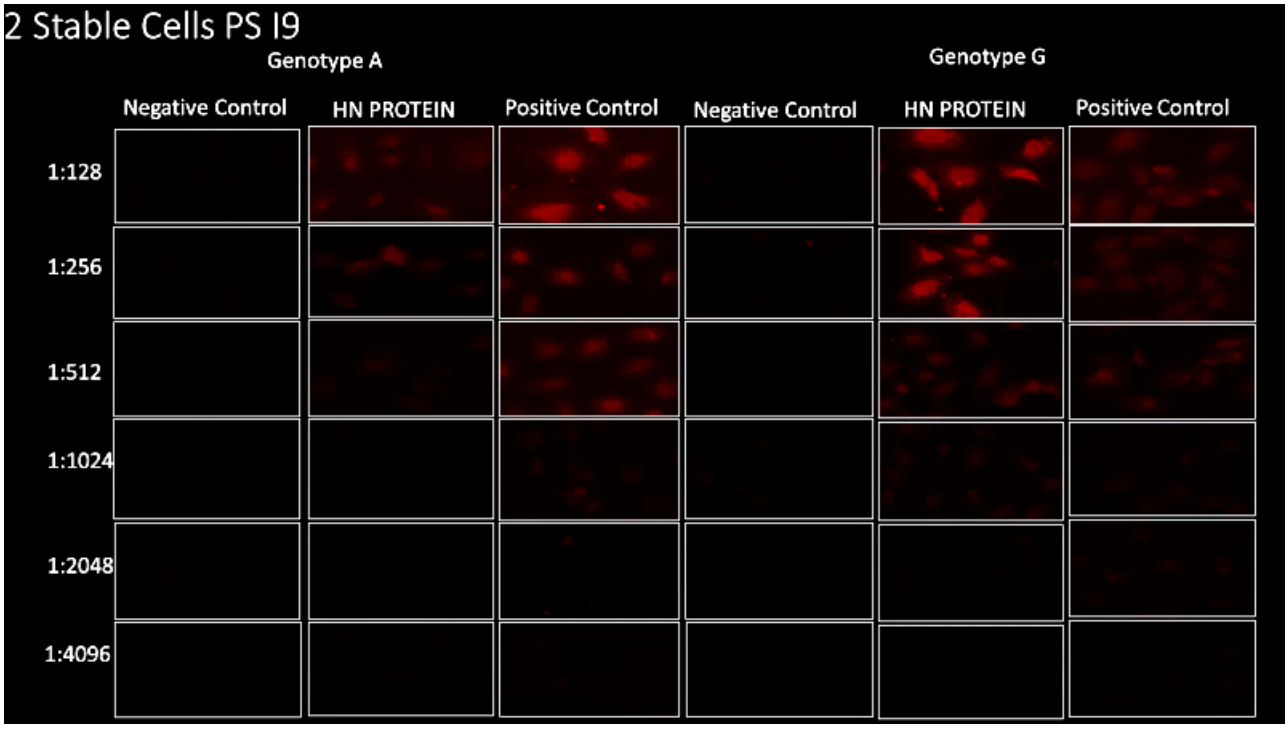


Figure A-31c. Stable cells Infected Sample #9.

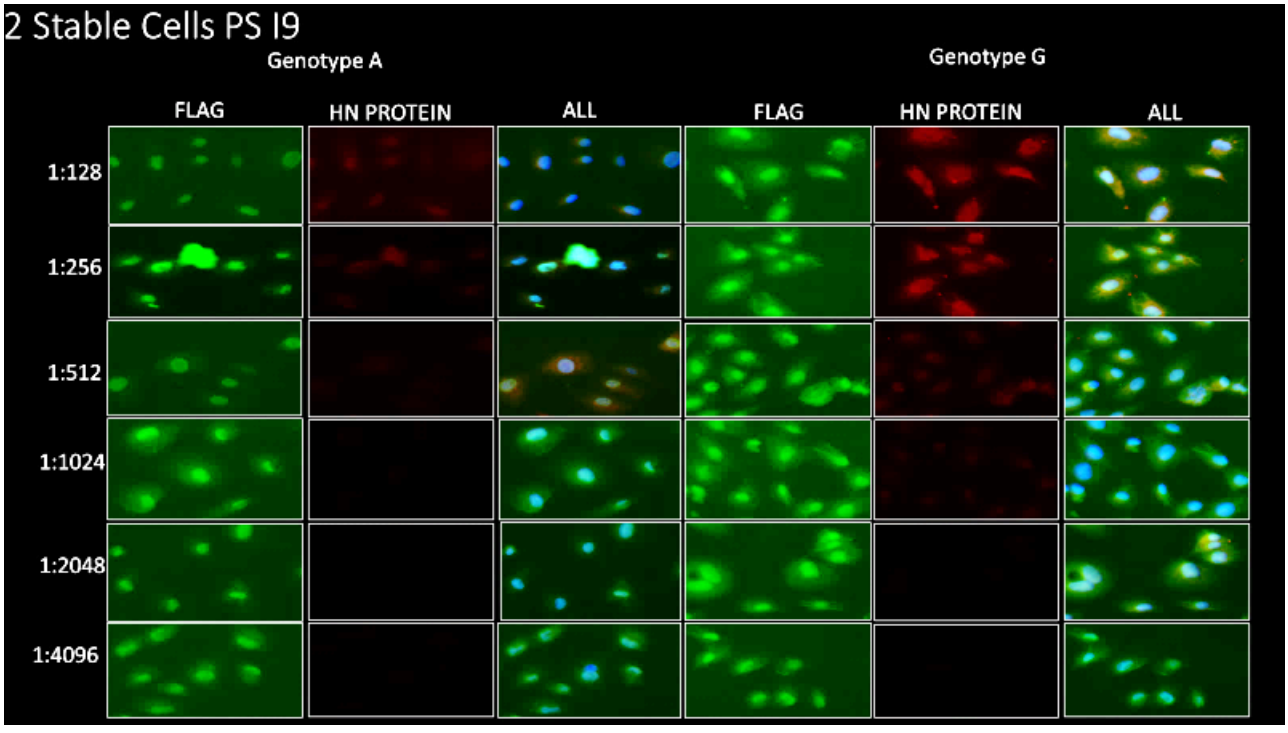


Figure A-31d. Stable cells Infected Sample #9.

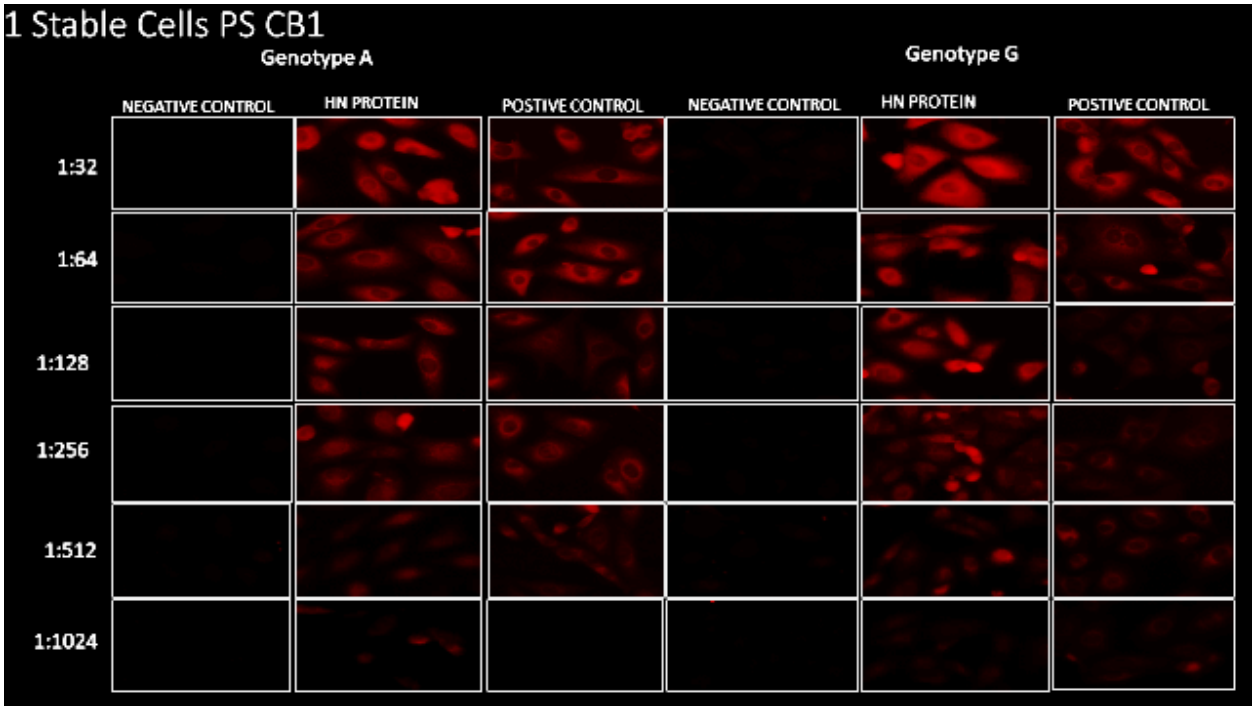


Figure A-32a. Stable cells Vaccine-Infected (CB) Sample #1.

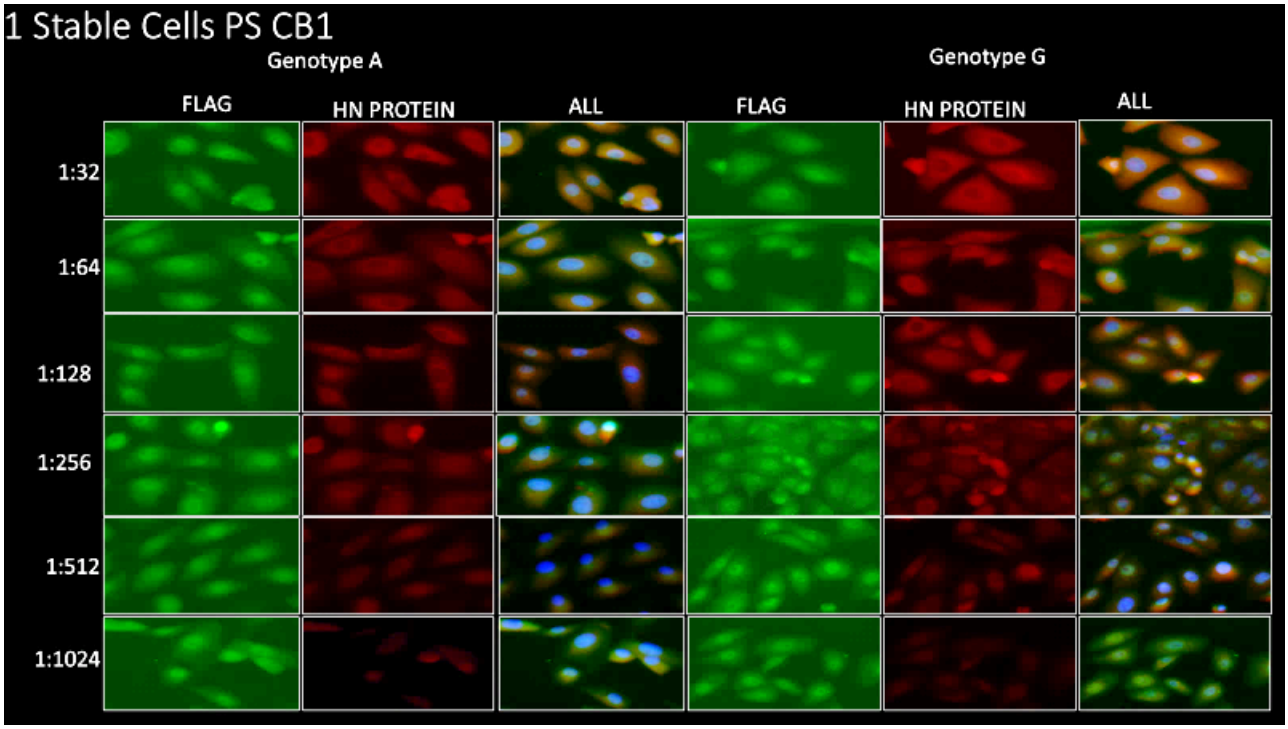


Figure A-32b. Stable cells Vaccine-Infected (CB) Sample #1.

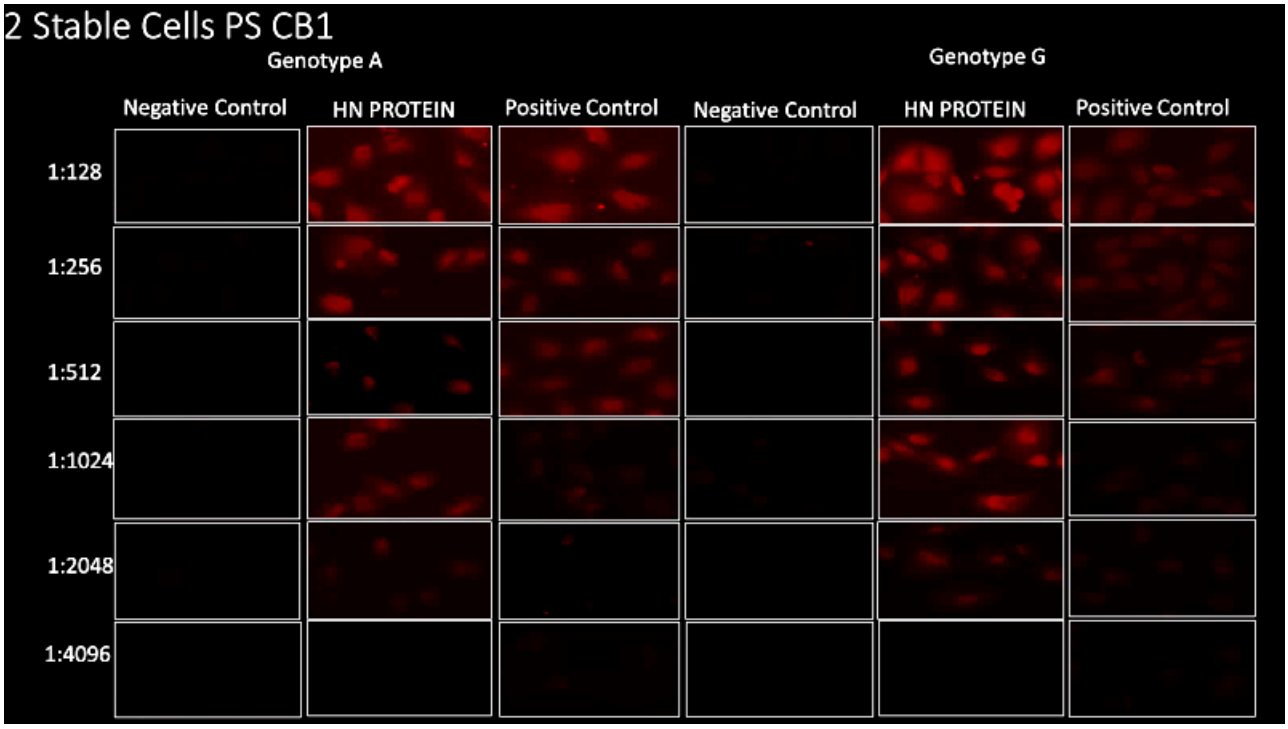


Figure A-32c. Stable cells Vaccine-Infected (CB) Sample #1.

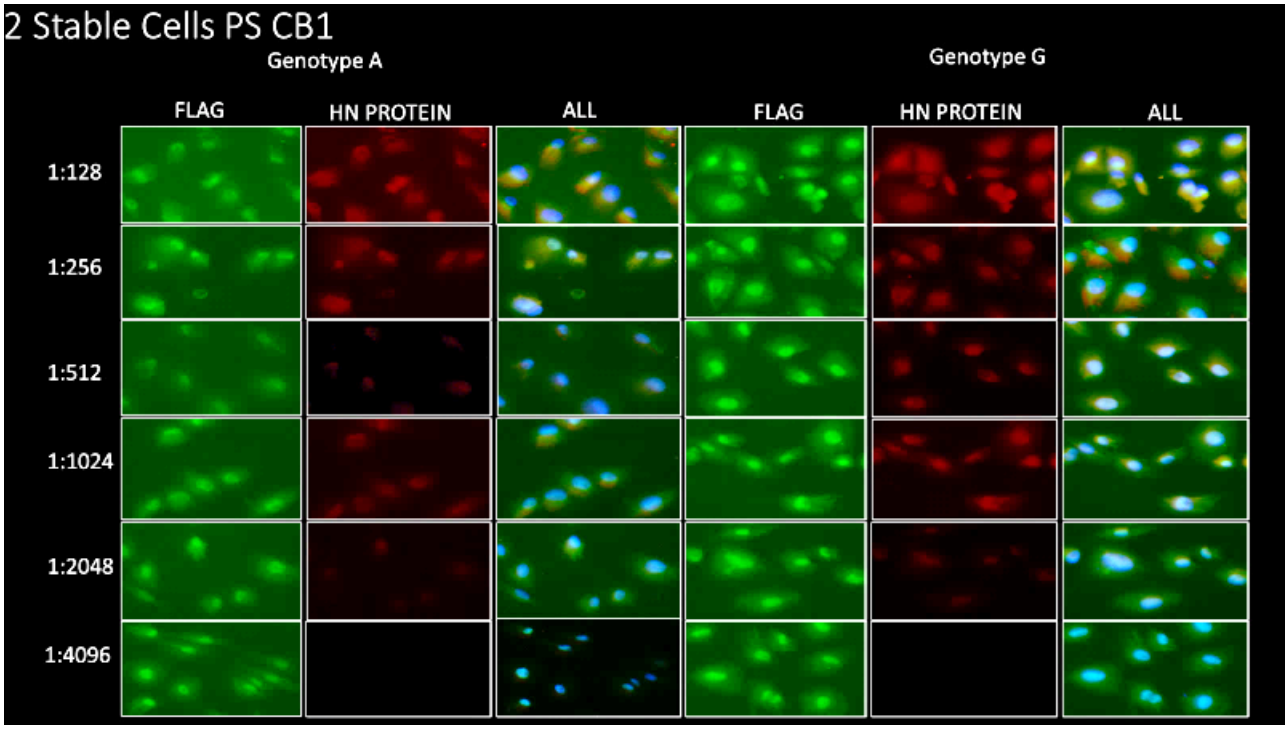


Figure A-32d. Stable cells Vaccine-Infected (CB) Sample #1.

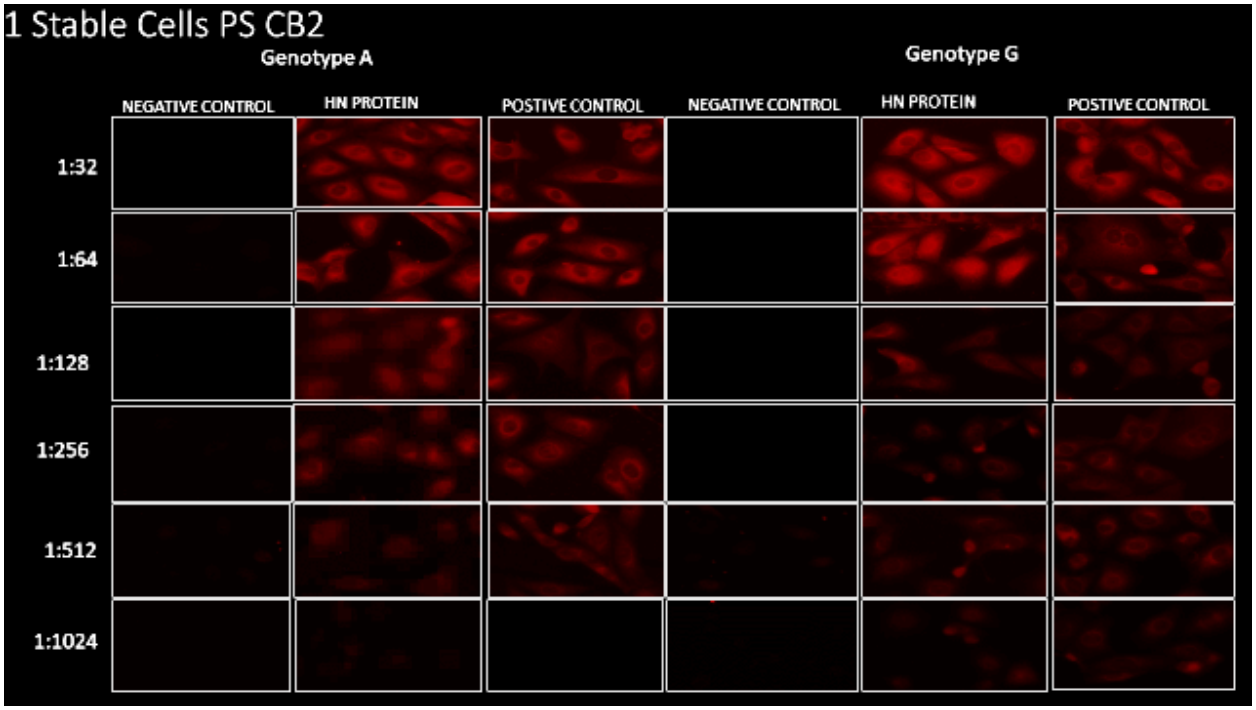


Figure A-33a. Stable cells Vaccine-Infected (CB) Sample#2.

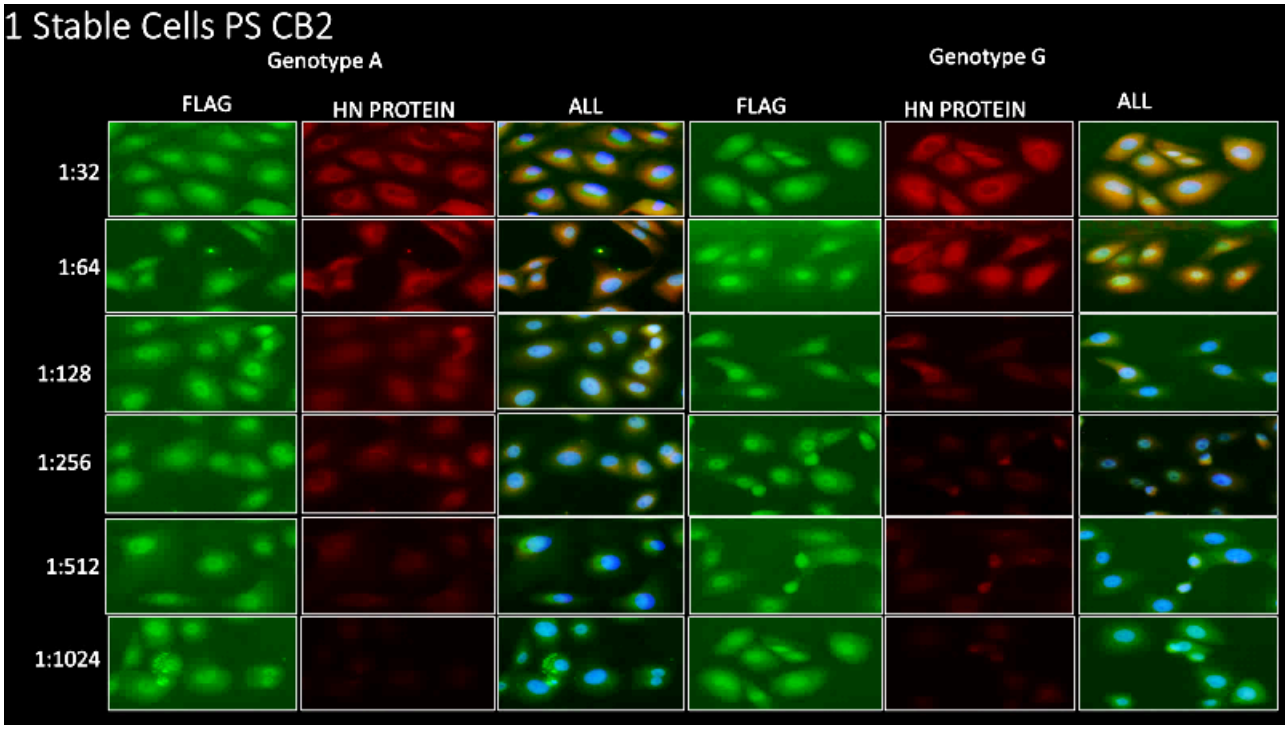


Figure A-33b. Stable cells Vaccine-Infected (CB) Sample #2.

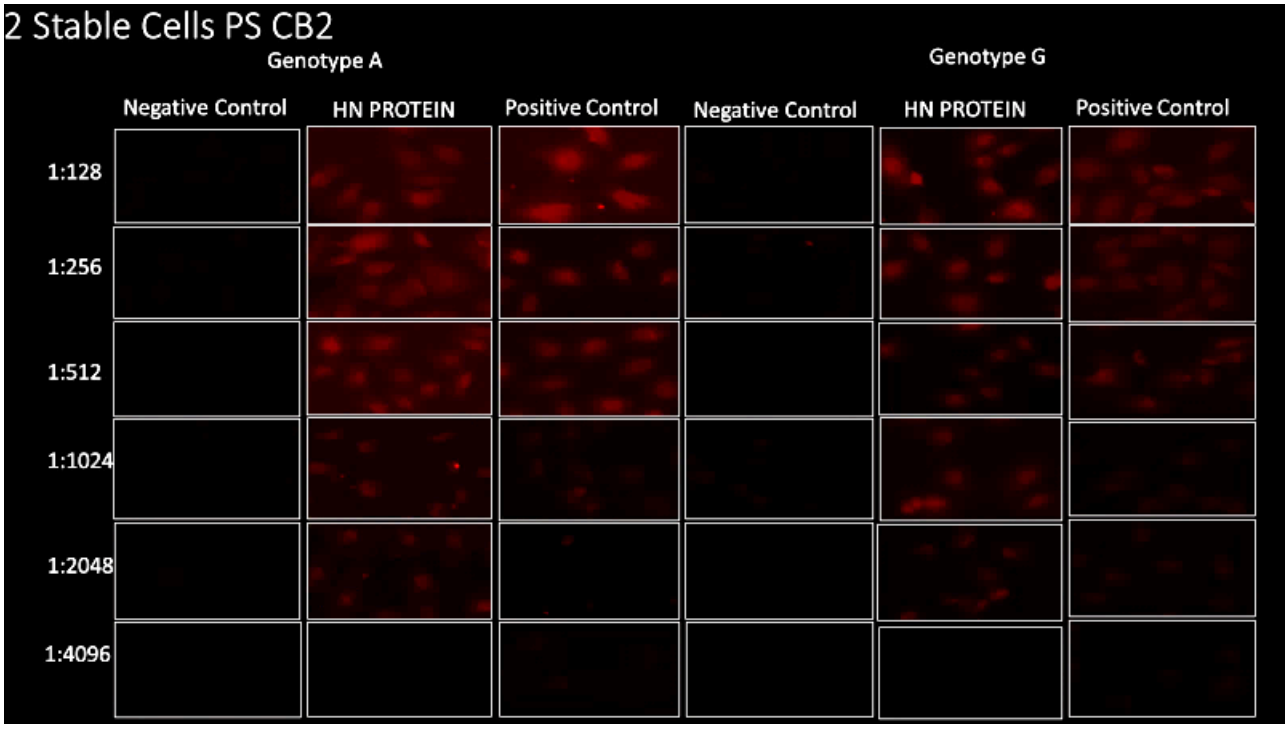


Figure A-33c. Stable cells Vaccine-Infected (CB) Sample #2.

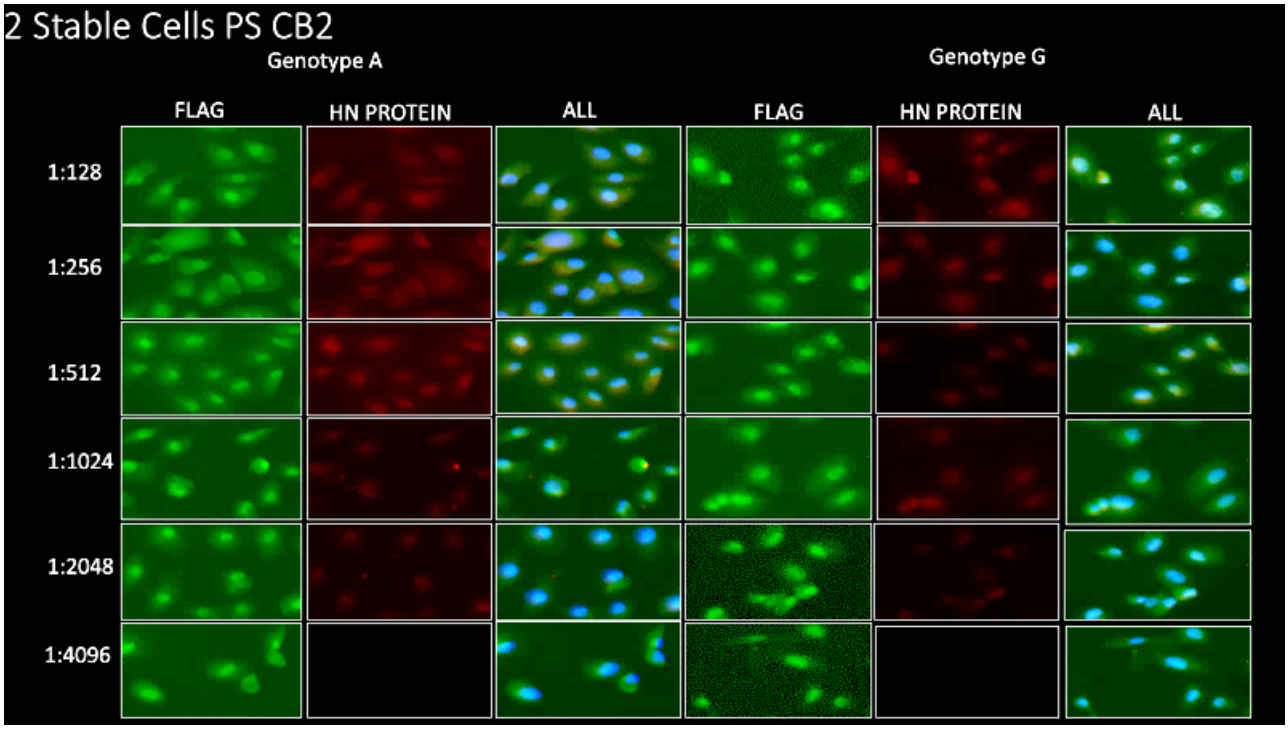


Figure A-33d. Stable cells Vaccine-Infected (CB) Sample #2.

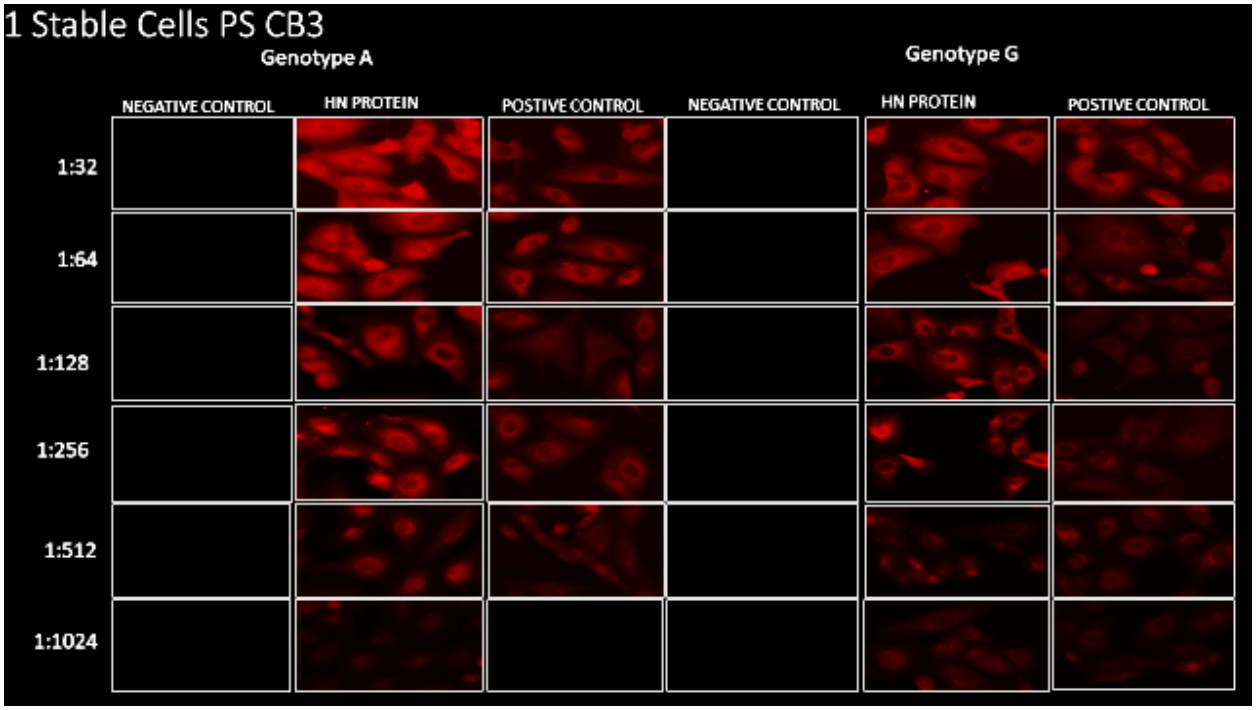


Figure A-34a. Stable cells Vaccine-Infected (CB) Sample#3.

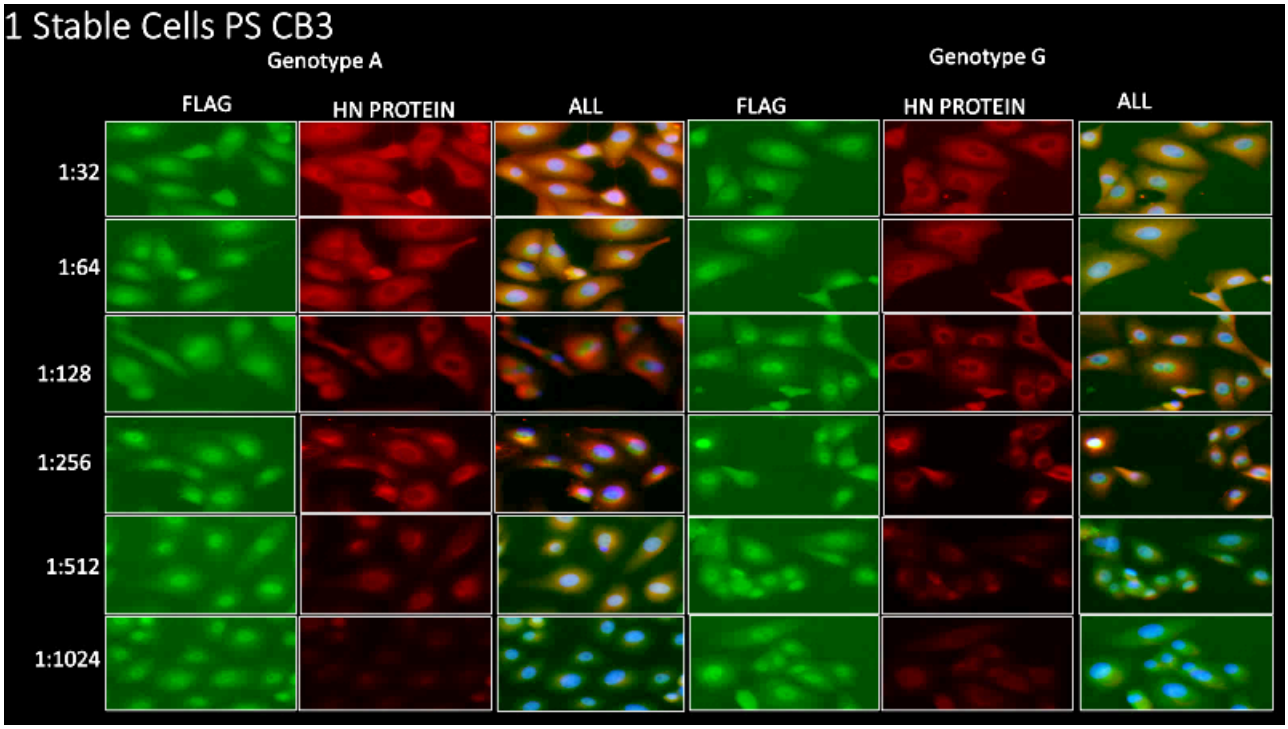


Figure A-34b. Stable cells Vaccine-Infected (CB) Sample #3.

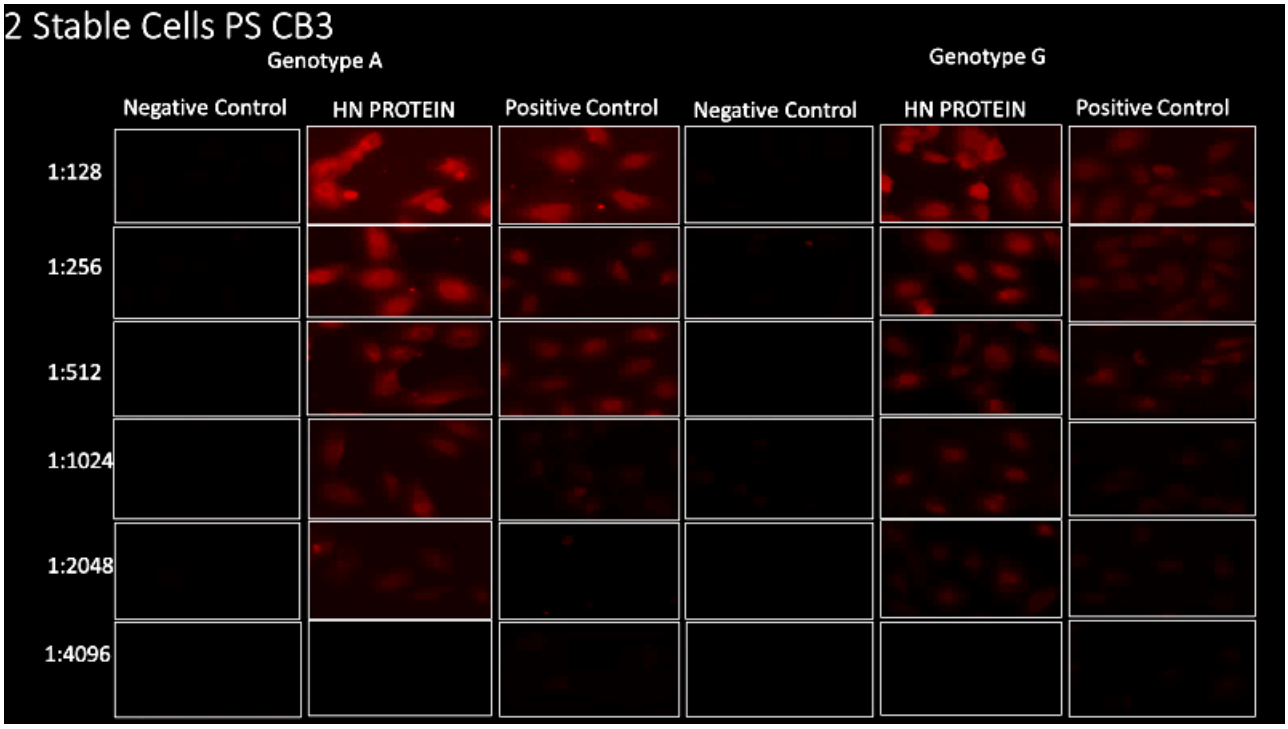


Figure A-34c. Stable cells Vaccine-Infected (CB) Sample #3.

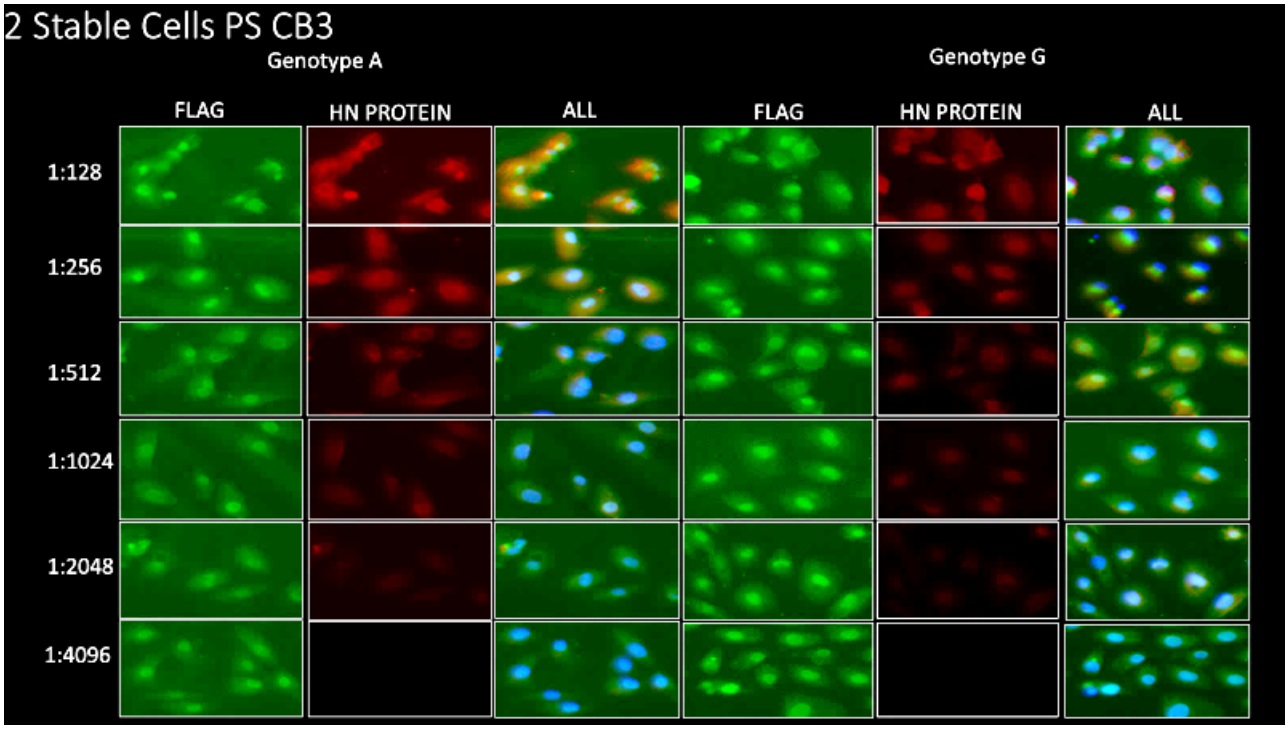


Figure A-34d. Stable cells Vaccine-Infected (CB) Sample #3.

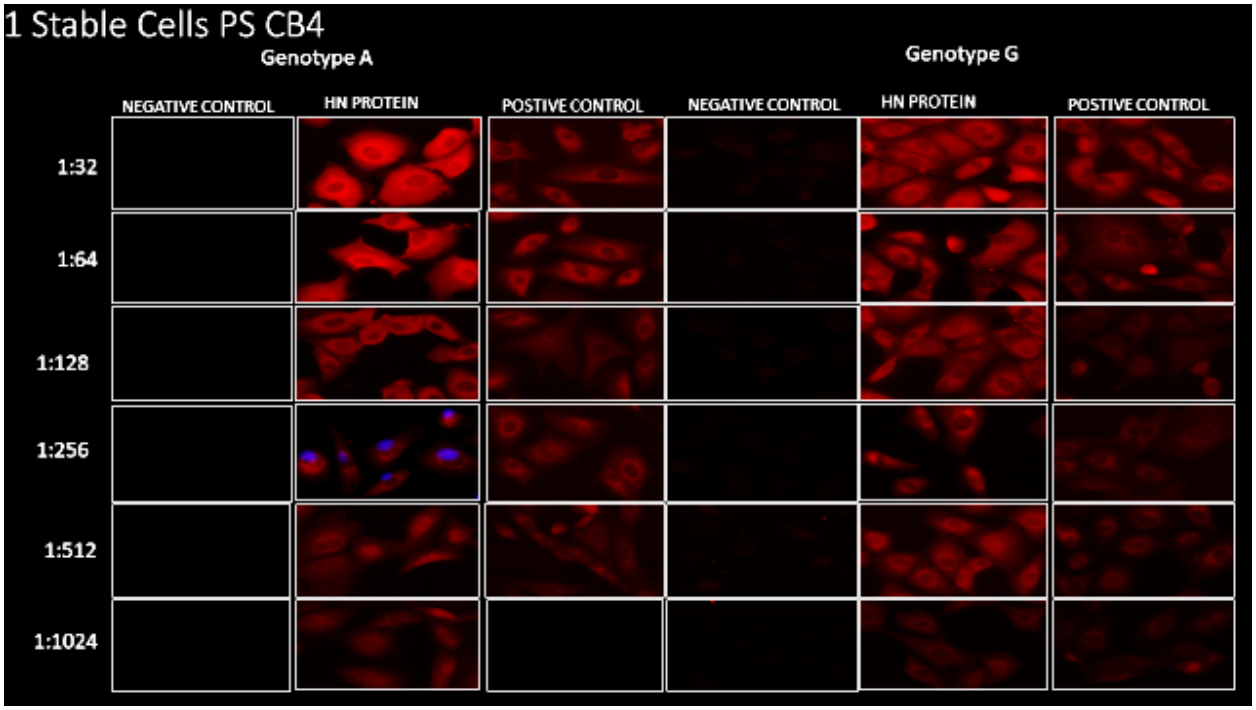


Figure A-35a. Stable cells Vaccine-Infected (CB) Sample#4.

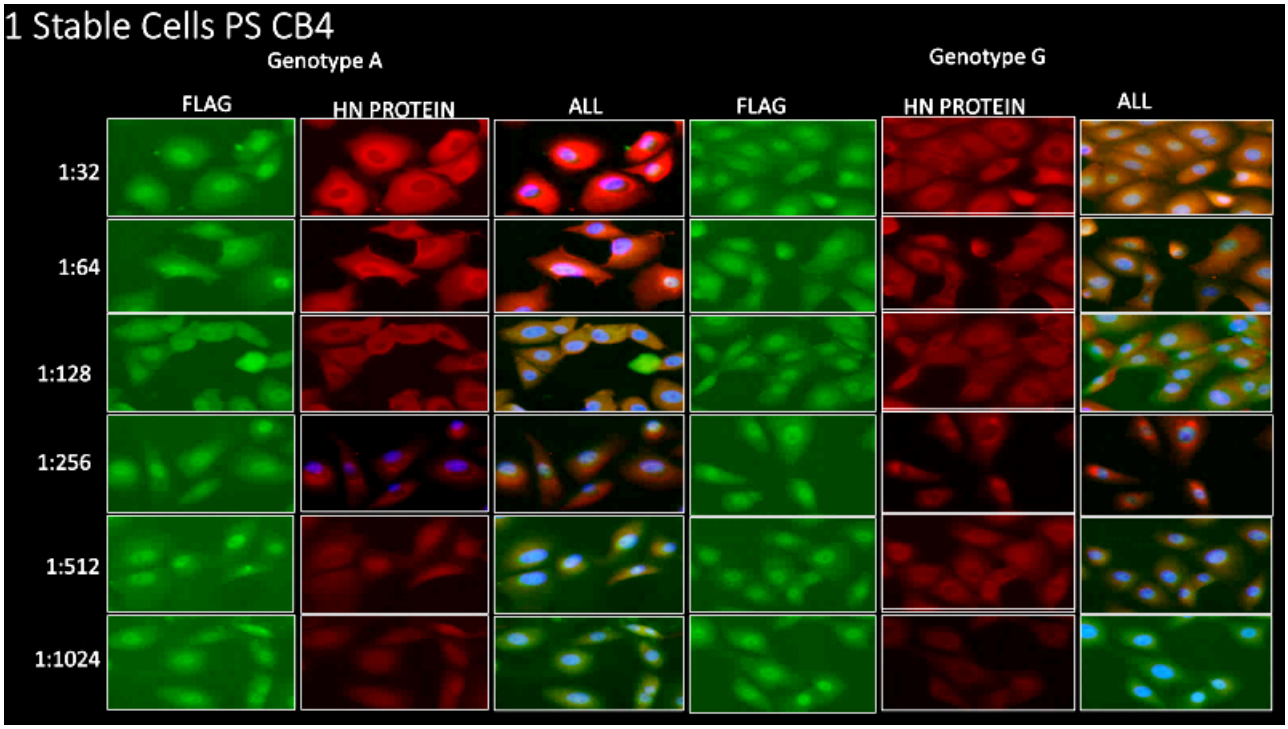


Figure A-35b. Stable cells Vaccine-Infected (CB) Sample #4. Note: Forgot to save red channel Alone

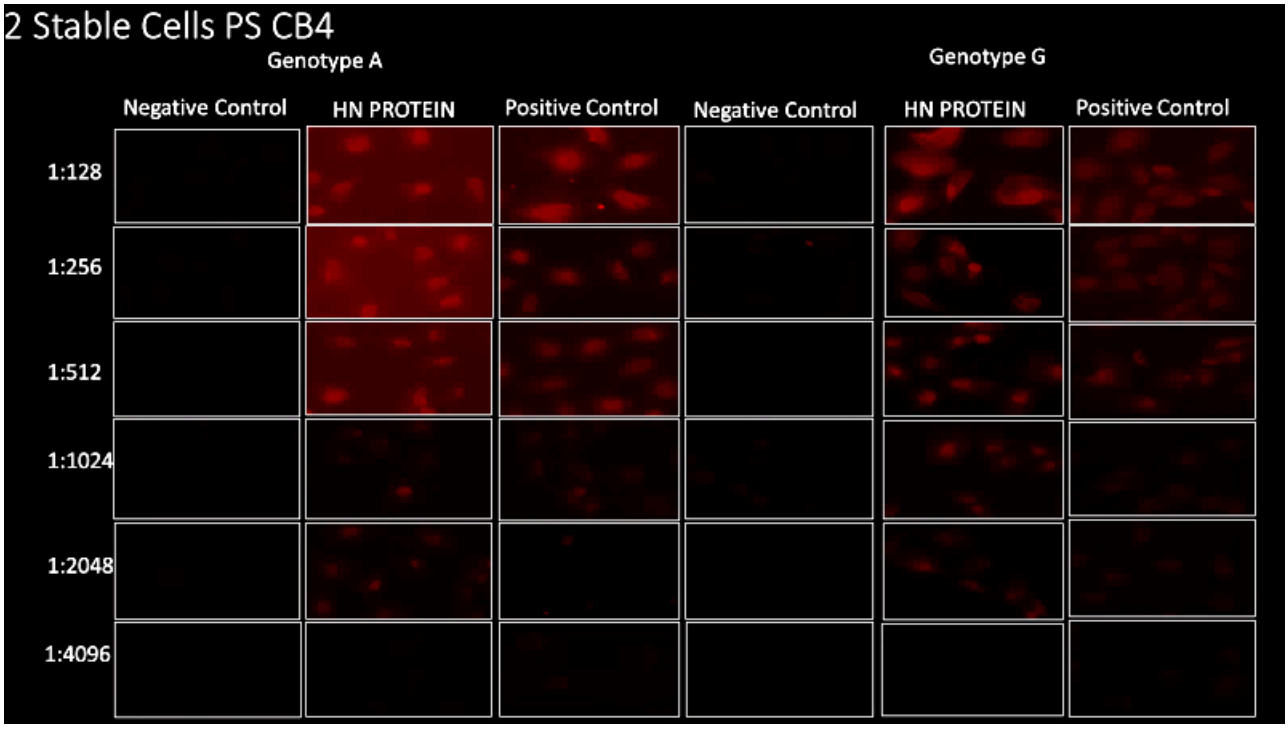


Figure A-35c. Stable cells Vaccine-Infected (CB) Sample #4.

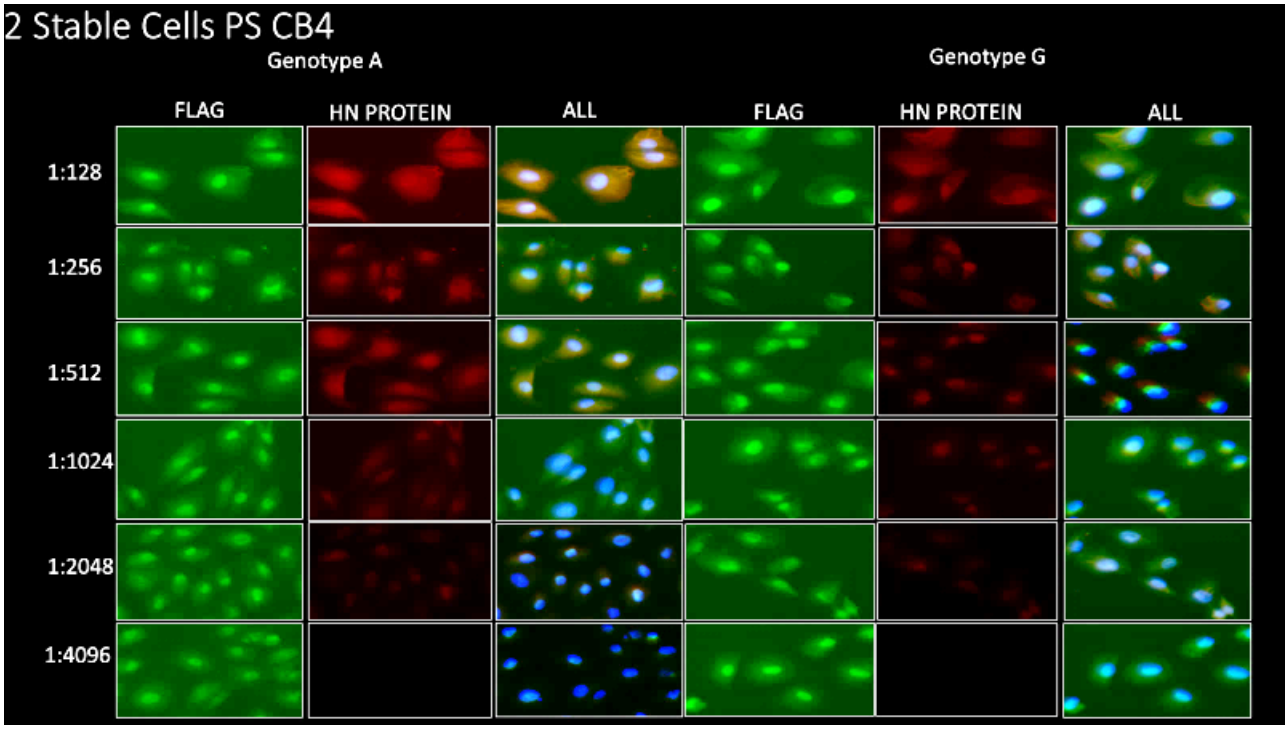


Figure A-35d. Stable cells Vaccine-Infected (CB) Sample #4.

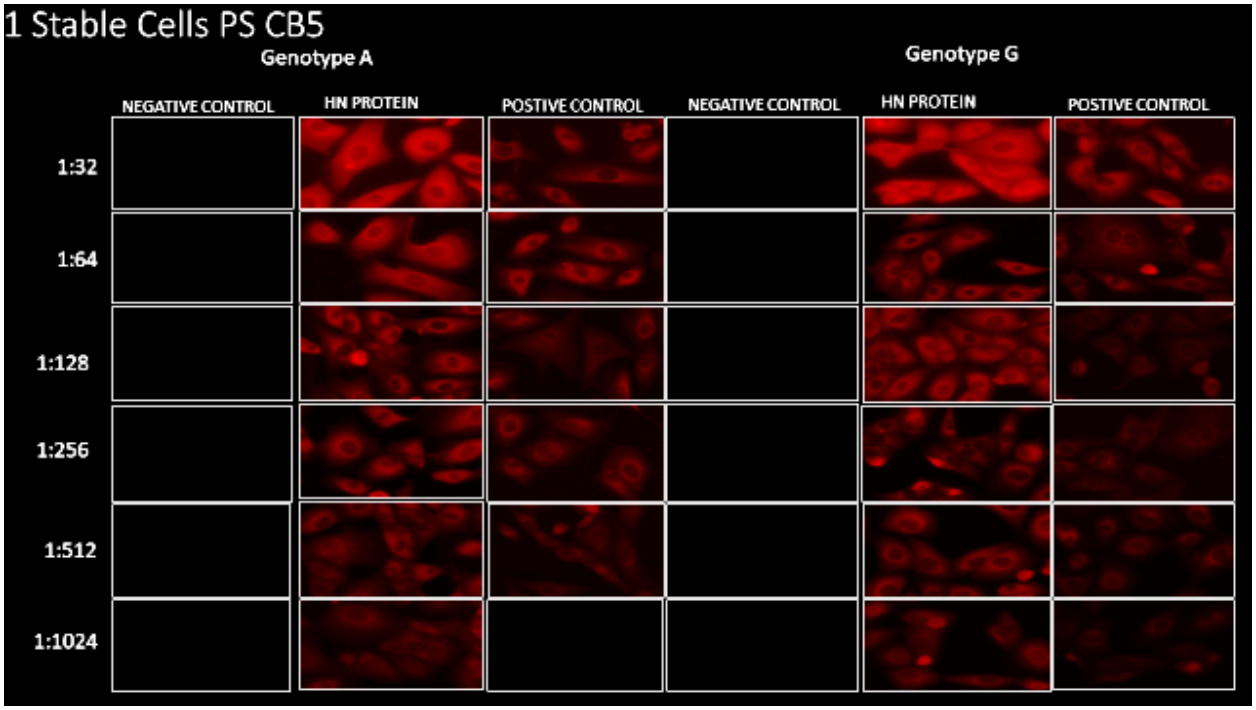


Figure A-36a. Stable cells Vaccine-Infected (CB) Sample#5.

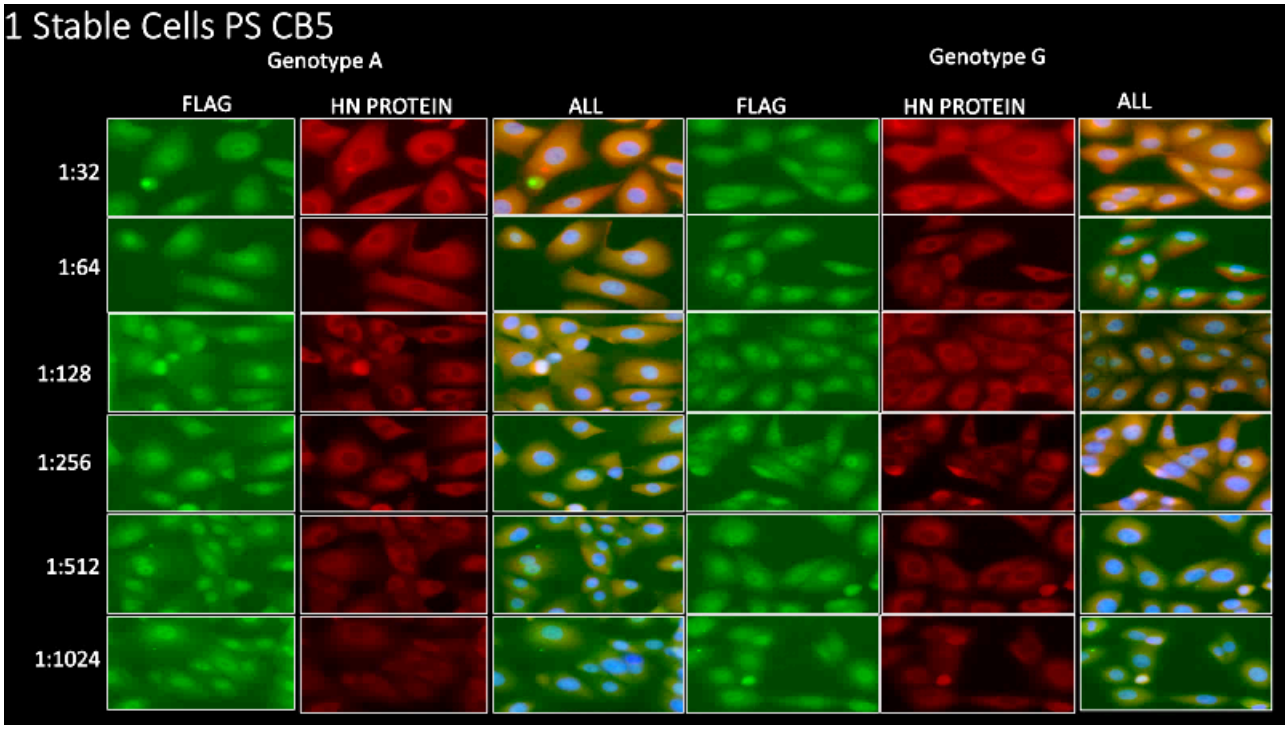


Figure A-36b. Stable cells Vaccine-Infected (CB) Sample #5.

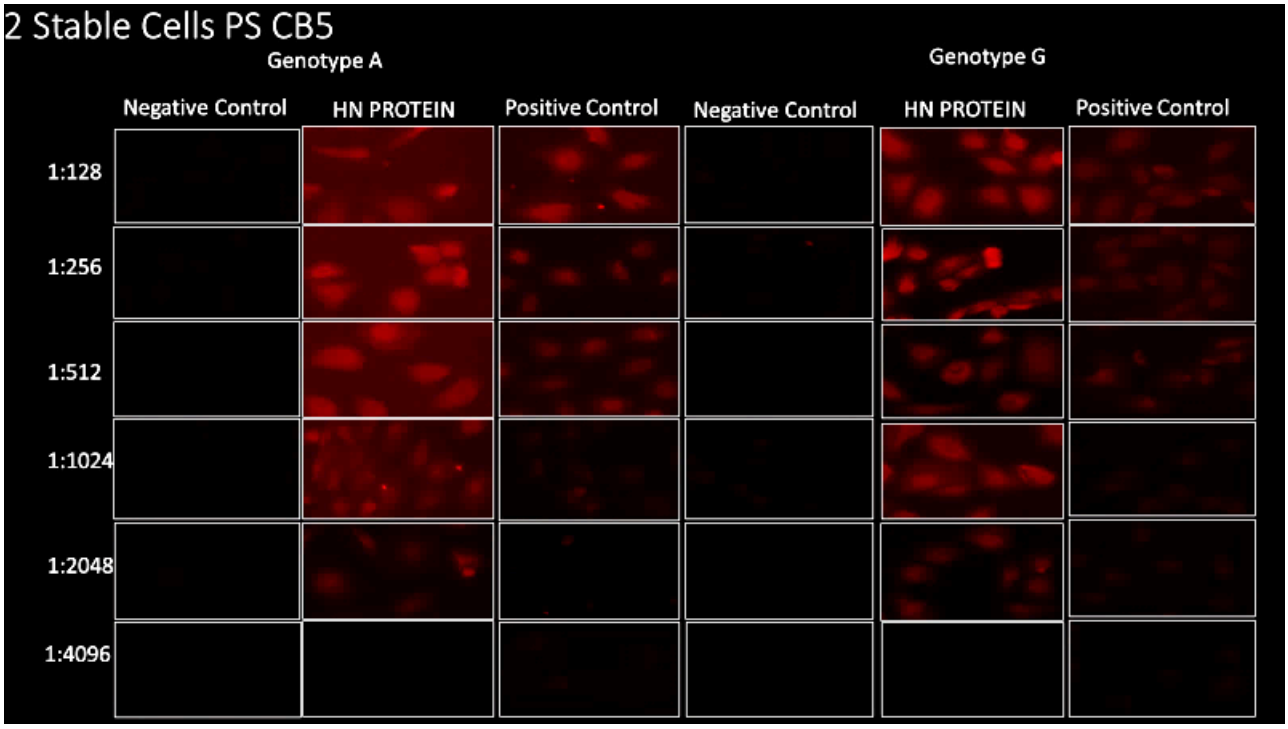


Figure A-36c. Stable cells Vaccine-Infected (CB) Sample #5.

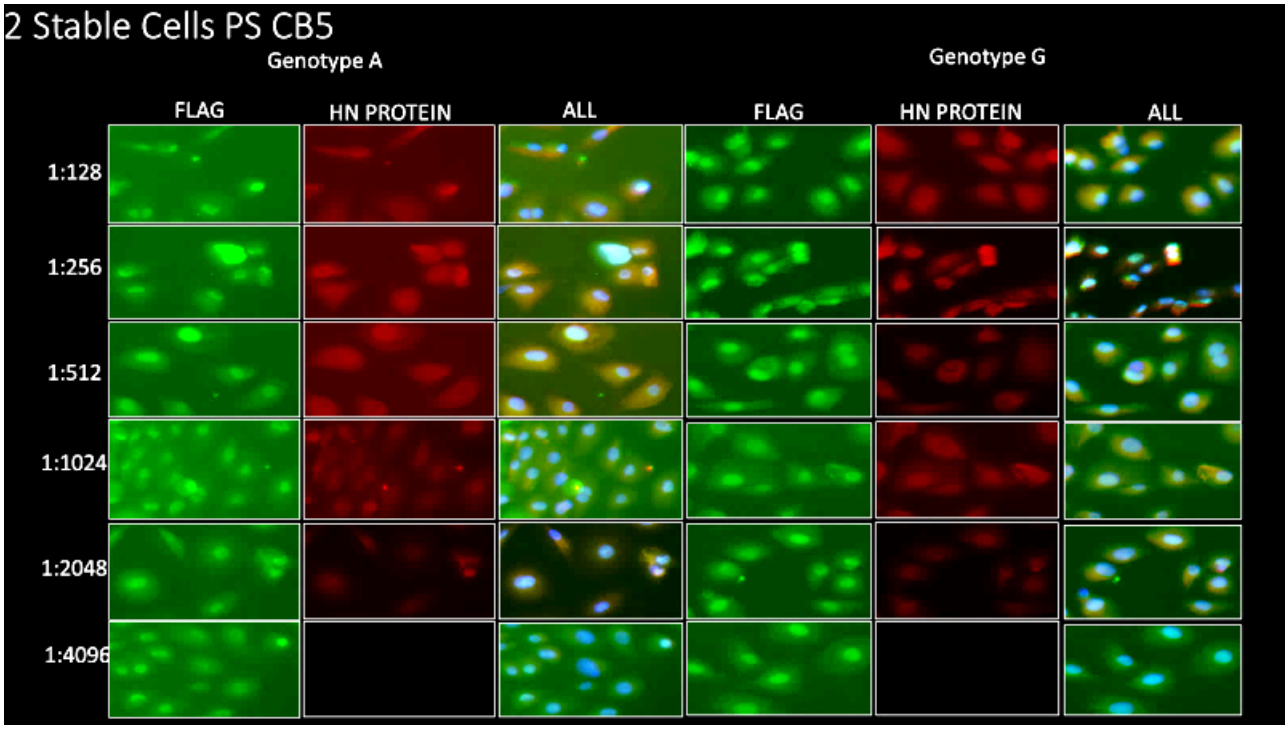


Figure A-36d. Stable cells Vaccine-Infected (CB) Sample #5.

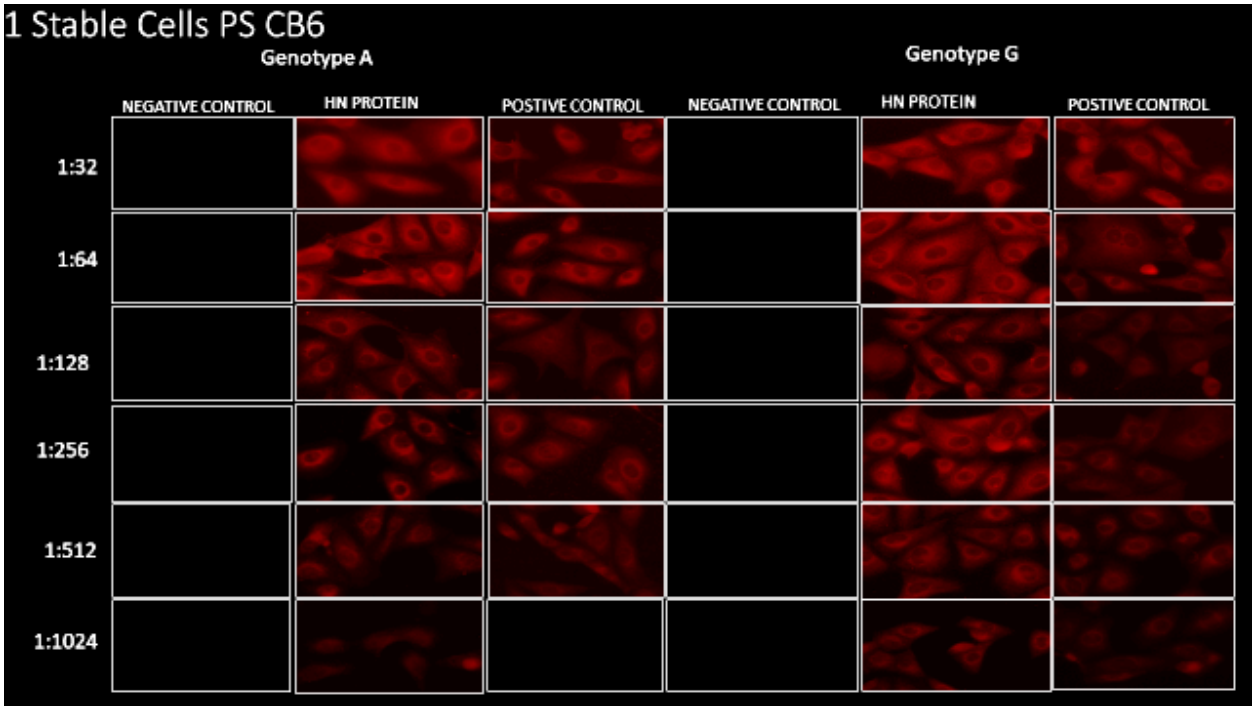


Figure A-37a. Stable cells Vaccine-Infected (CB) Sample#6.

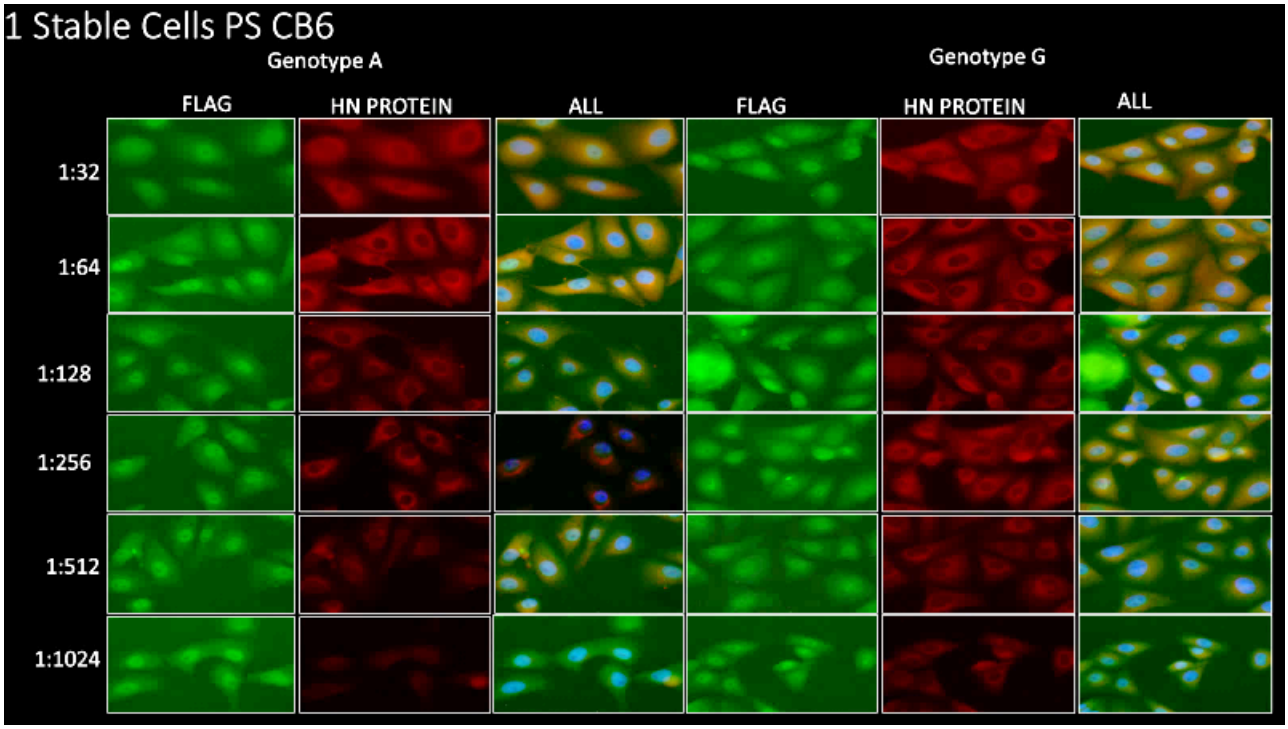


Figure A-37b. Stable cells Vaccine-Infected (CB) Sample #6.

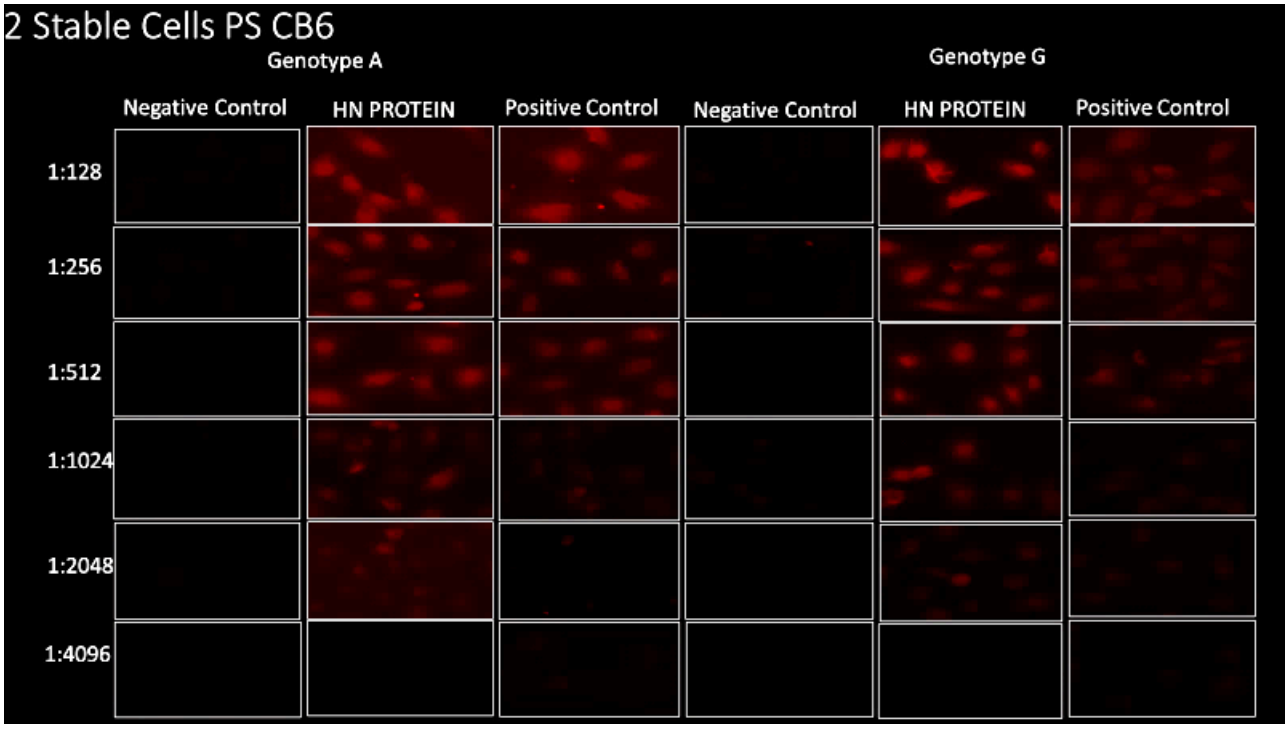


Figure A-37c. Stable cells Vaccine-Infected (CB) Sample #6.

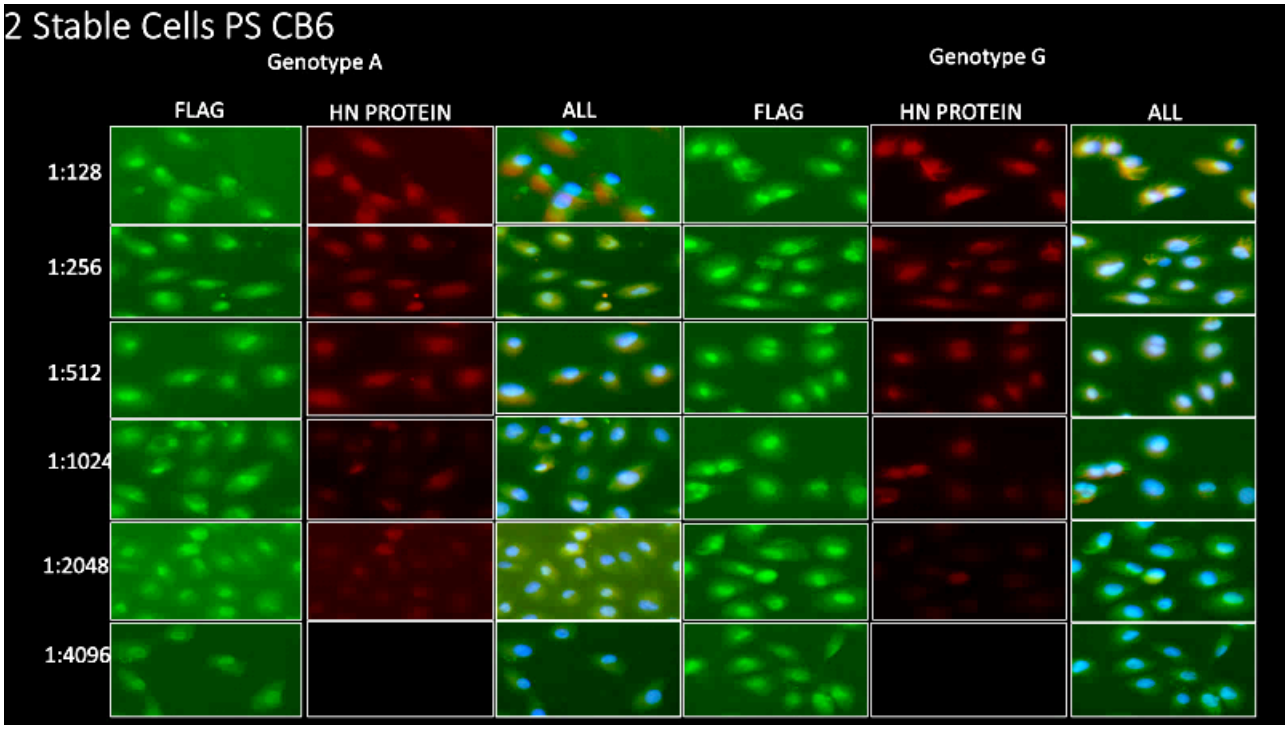


Figure A-37d. Stable cells Vaccine-Infected (CB) Sample #6.

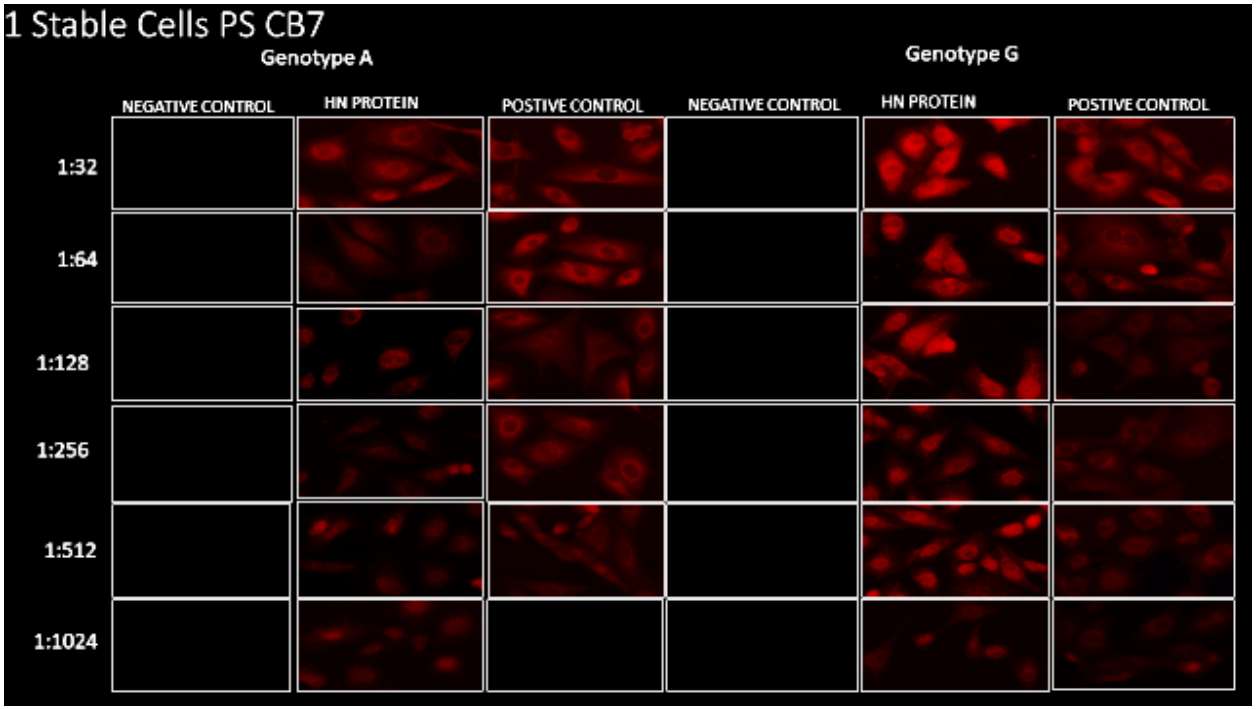


Figure A-38a. Stable cells Vaccine-Infected (CB) Sample#7.

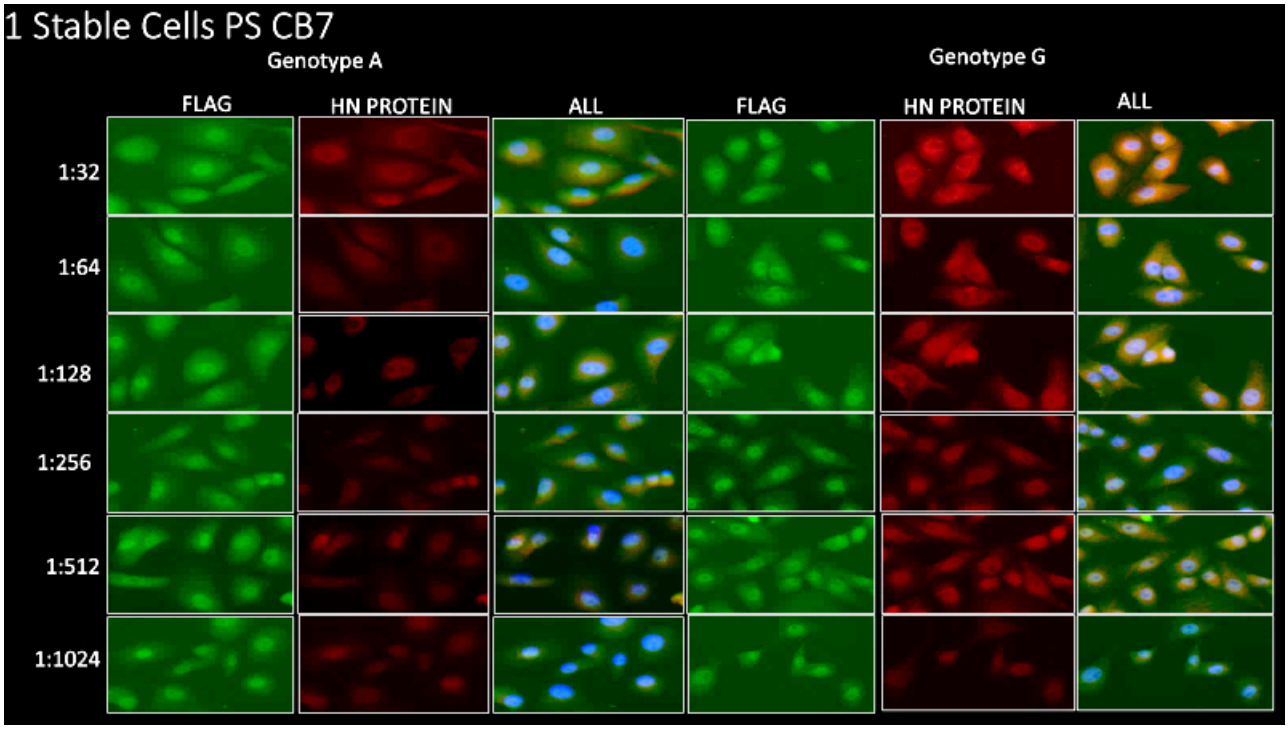


Figure A-38b. Stable cells Vaccine-Infected (CB) Sample #7.

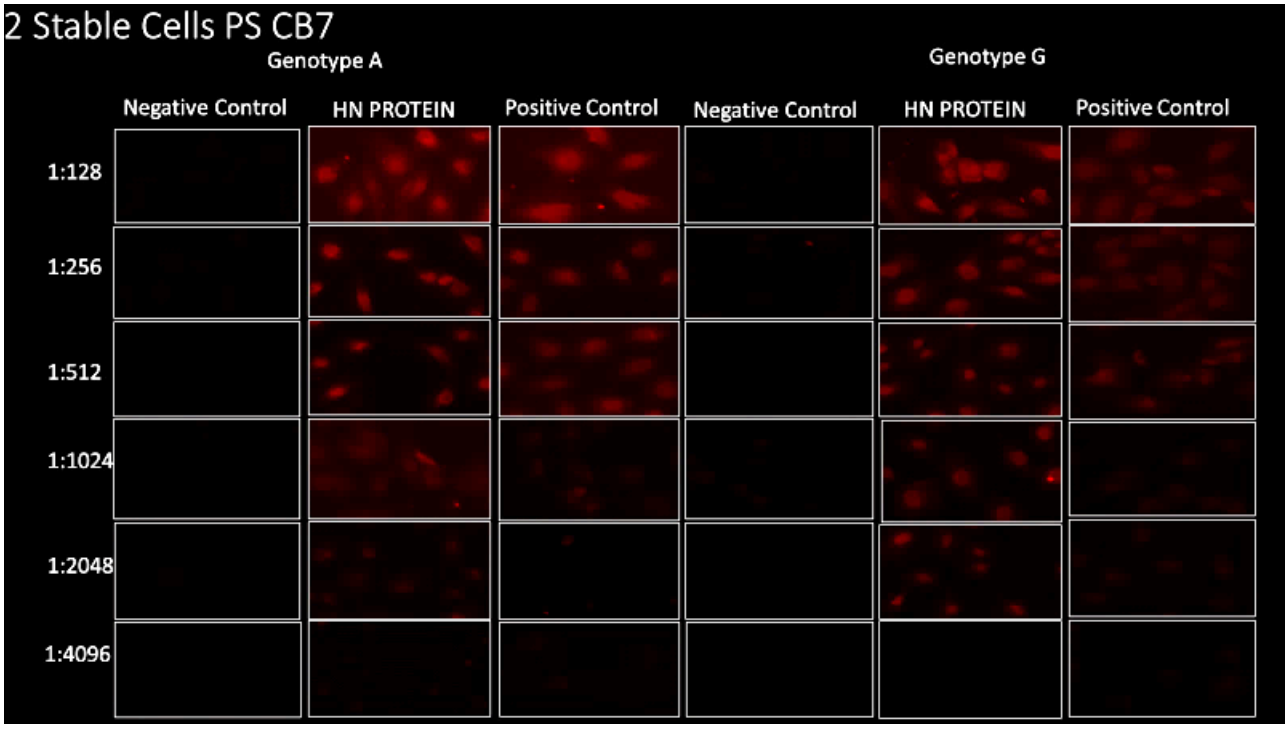


Figure A-38c. Stable cells Vaccine-Infected (CB) Sample #7.

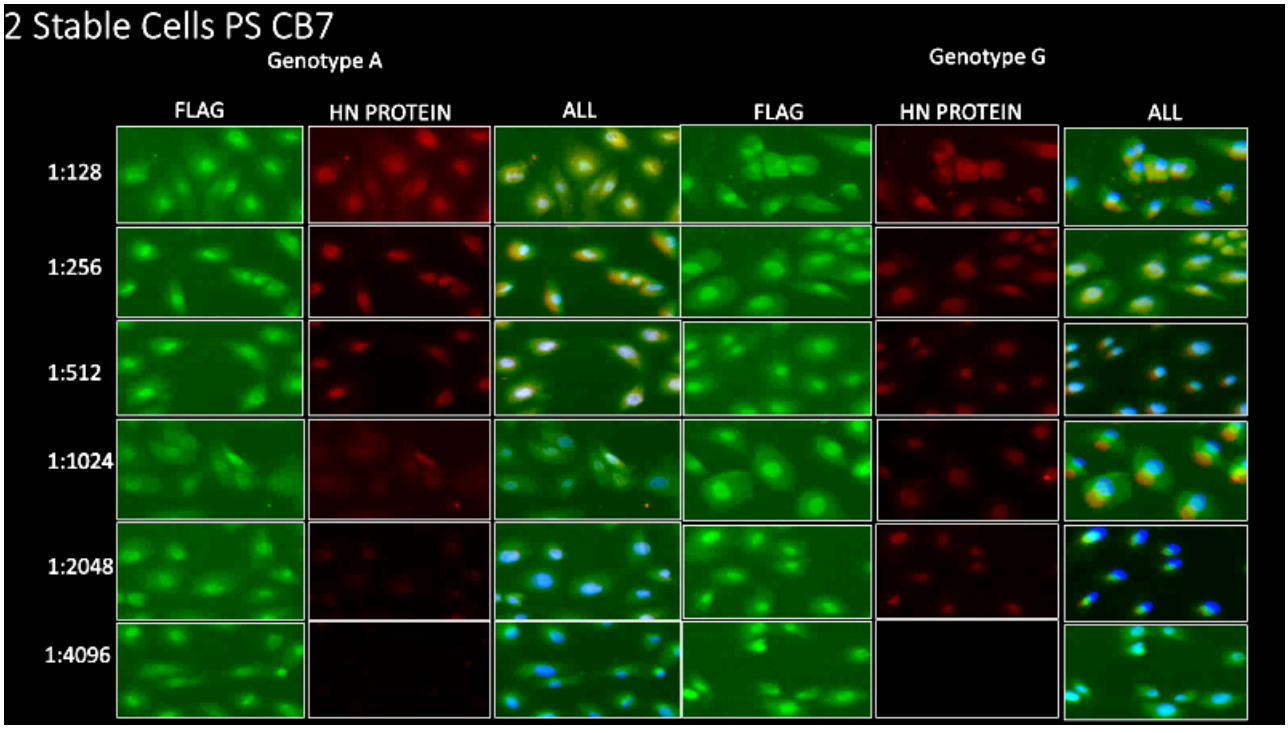


Figure A-38d. Stable cells Vaccine-Infected (CB) Sample #7.

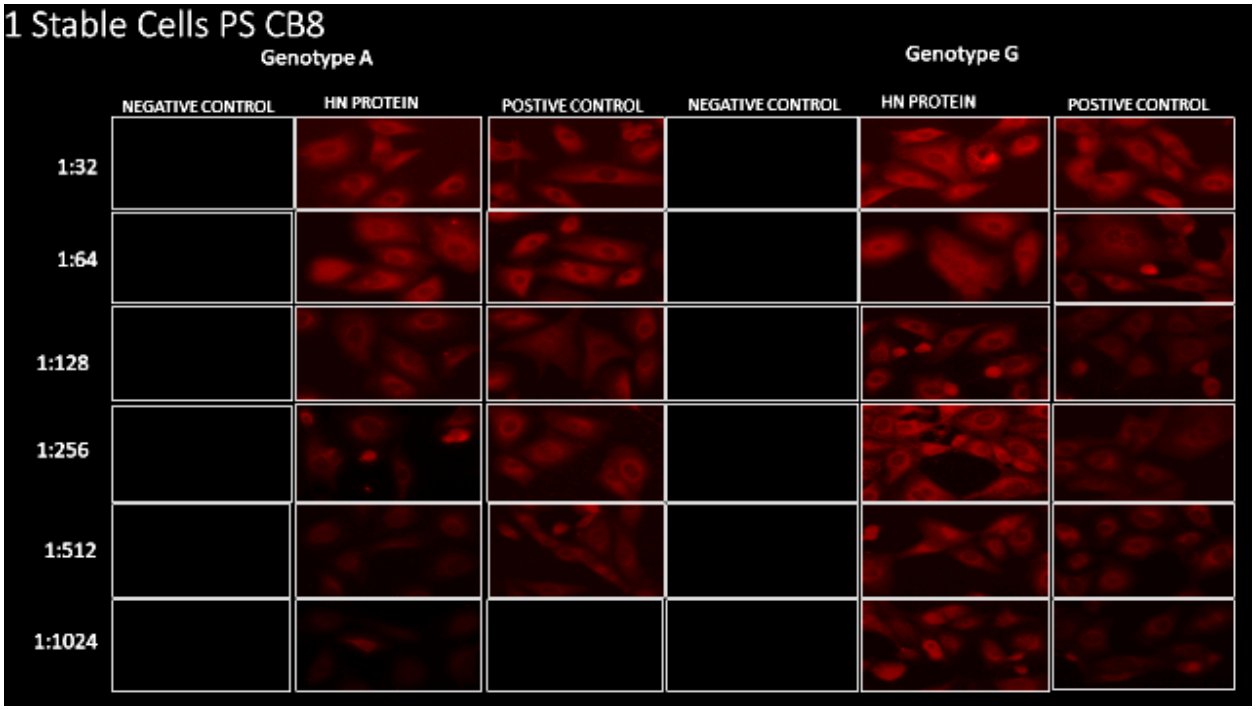


Figure A-39a. Stable cells Vaccine-Infected (CB) Sample#8.

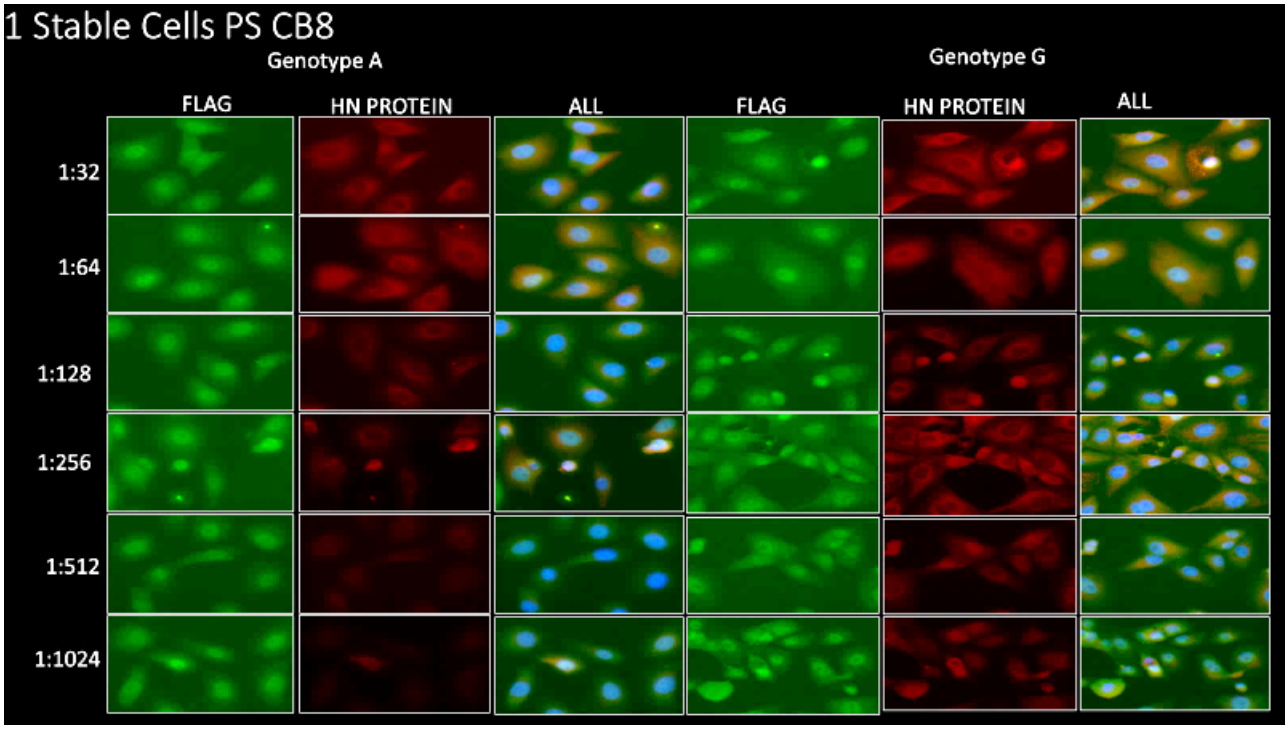


Figure A-39b. Stable cells Vaccine-Infected (CB) Sample #8.

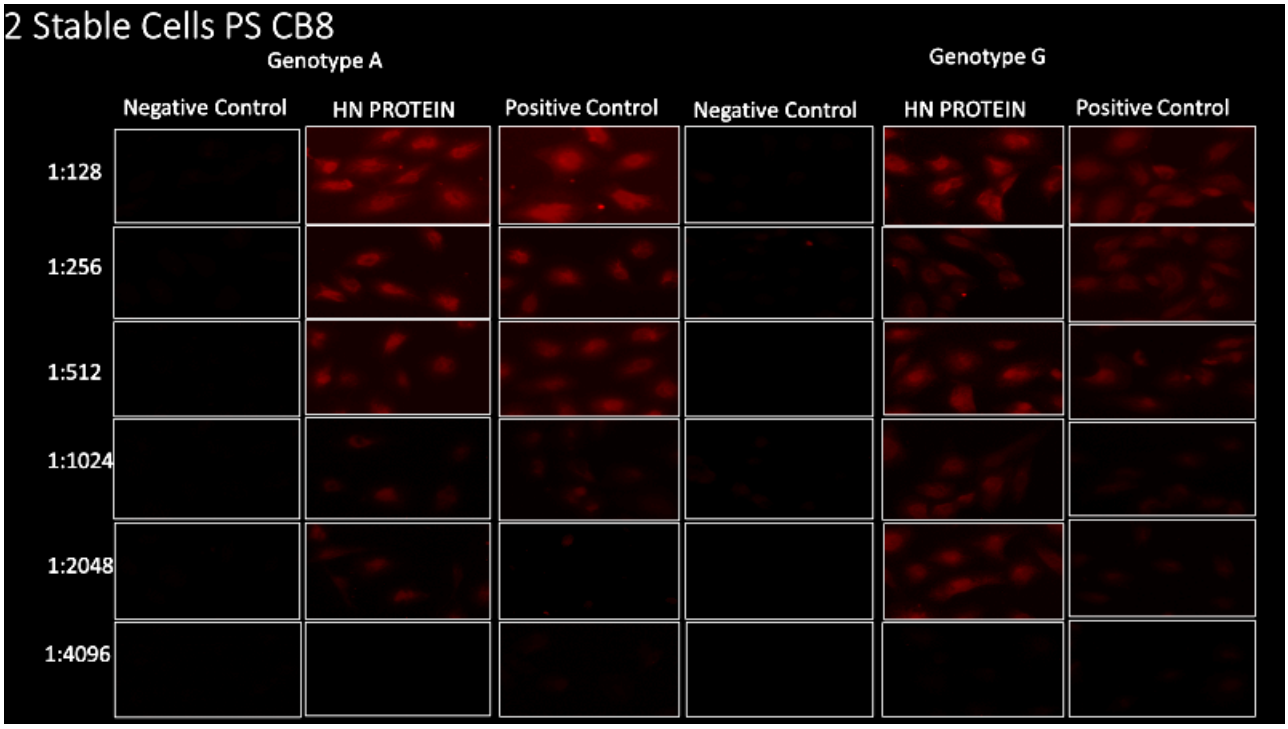


Figure A-39c. Stable cells Vaccine-Infected (CB) Sample #8.

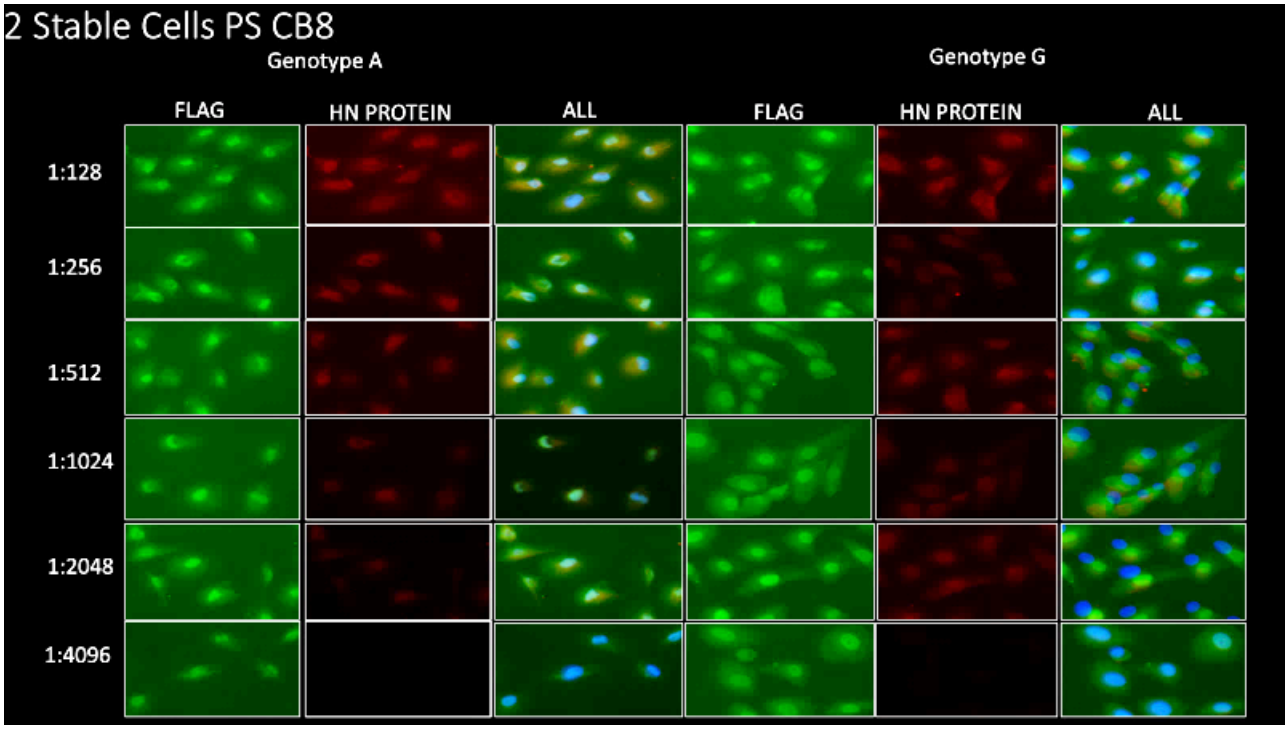


Figure A-39d. Stable cells Vaccine-Infected (CB) Sample #8.

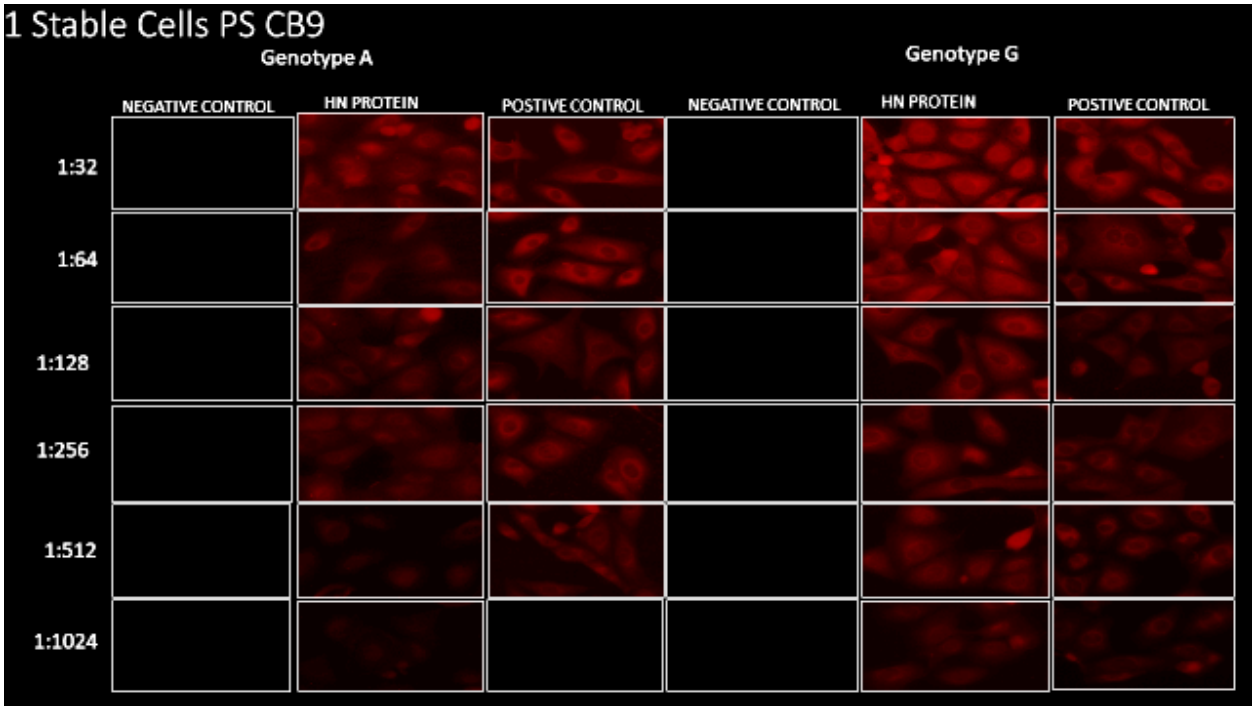


Figure A-40a. Stable cells Vaccine-Infected (CB) Sample#9.

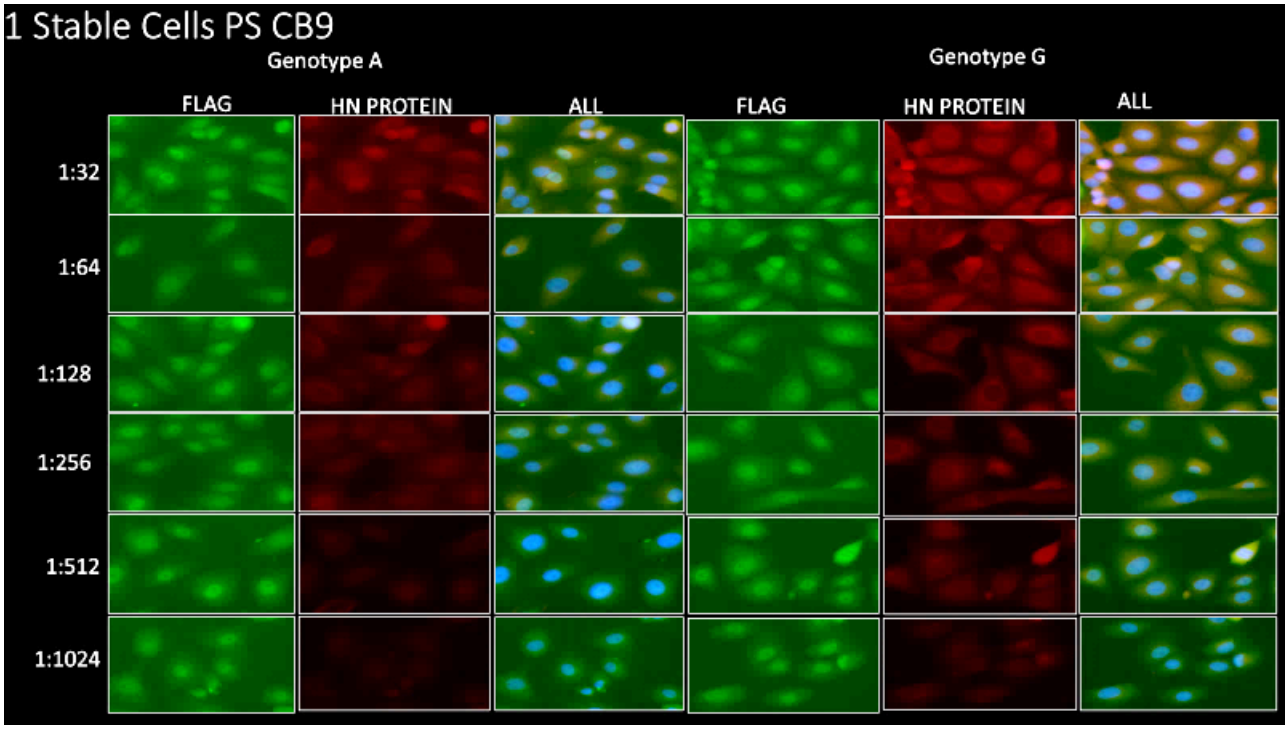


Figure A-40b. Stable cells Vaccine-Infected (CB) Sample #9.

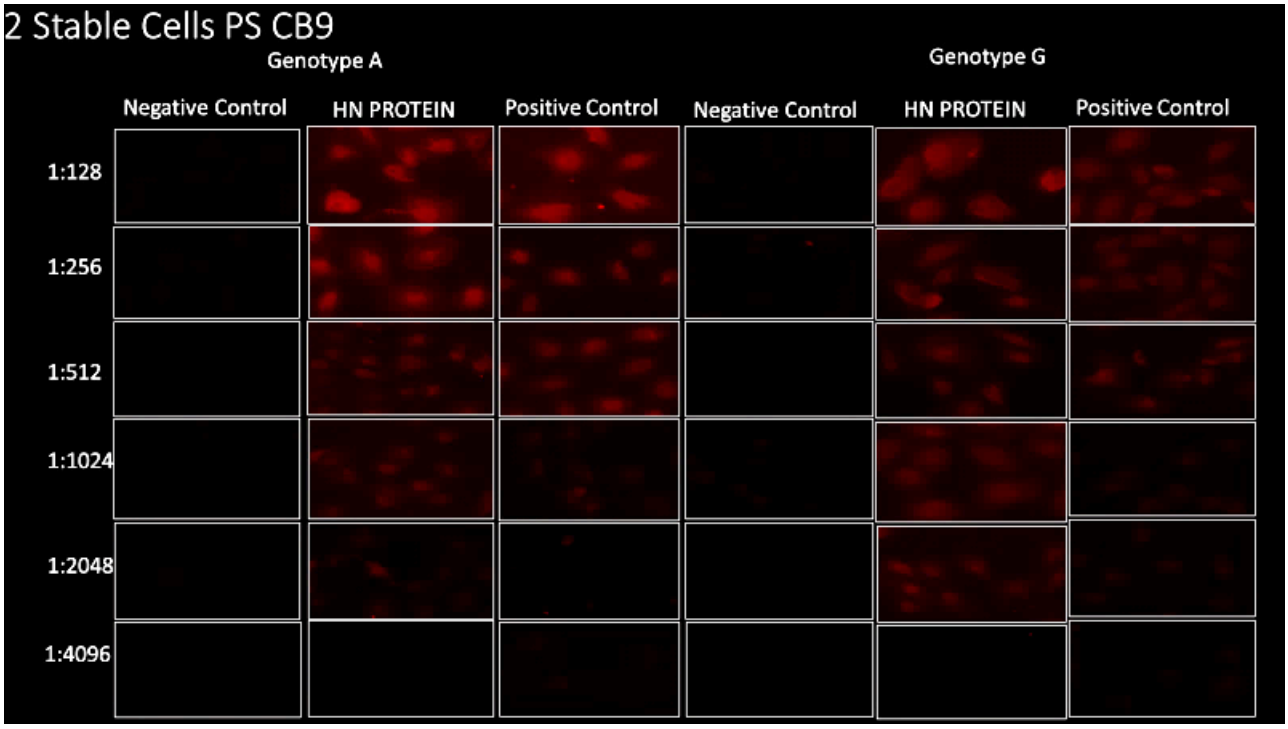


Figure A-40c. Stable cells Vaccine-Infected (CB) Sample #9.

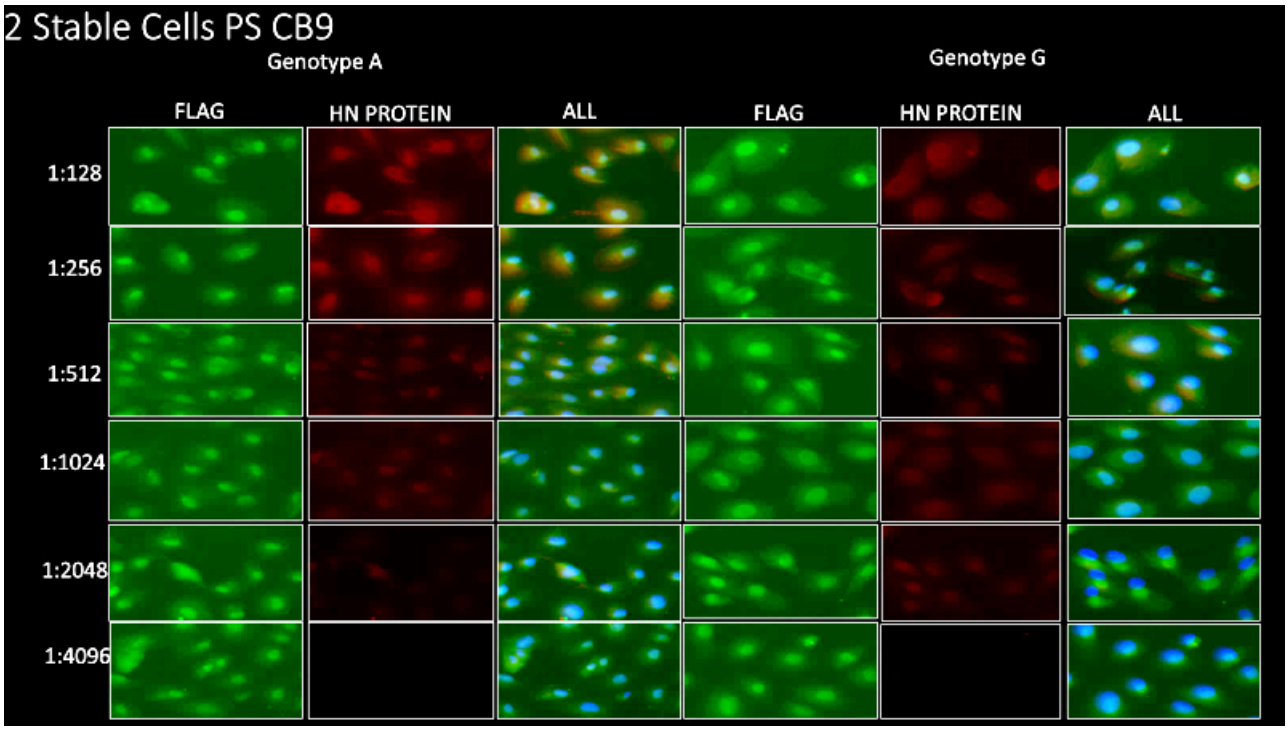


Figure A-40d. Stable cells Vaccine-Infected (CB) Sample #9.

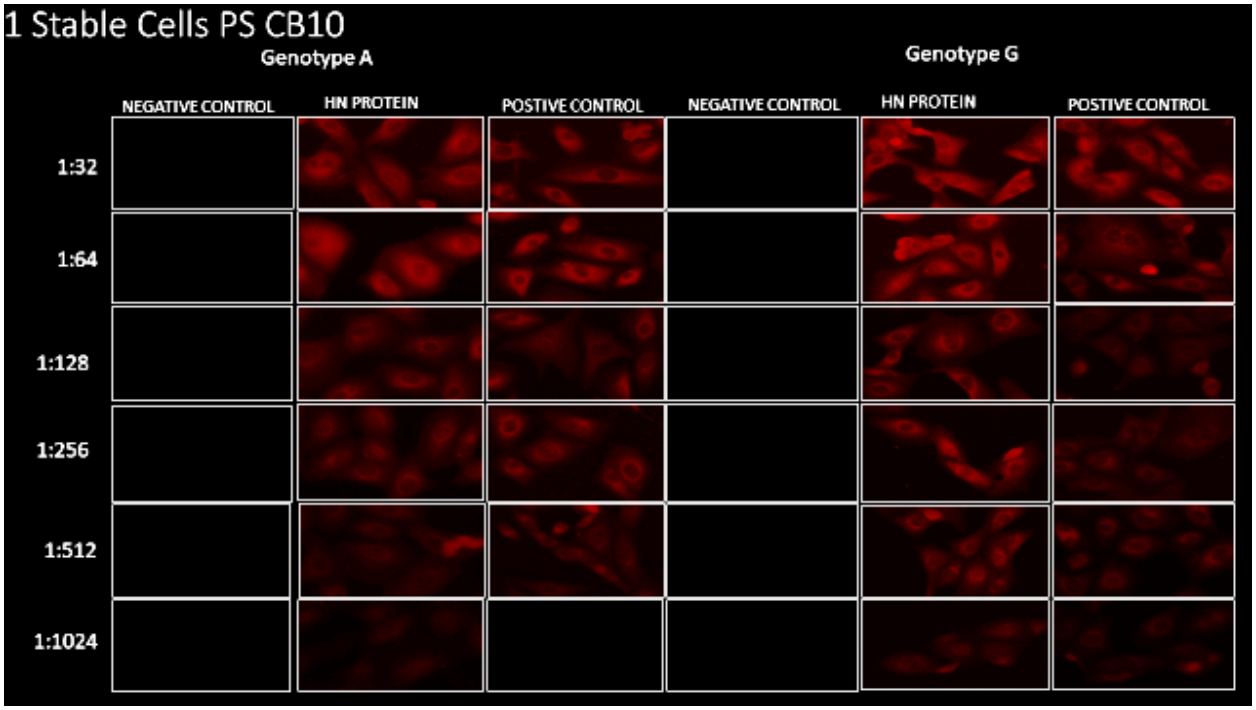


Figure A-41a. Stable cells Vaccine-Infected (CB) Sample#10.

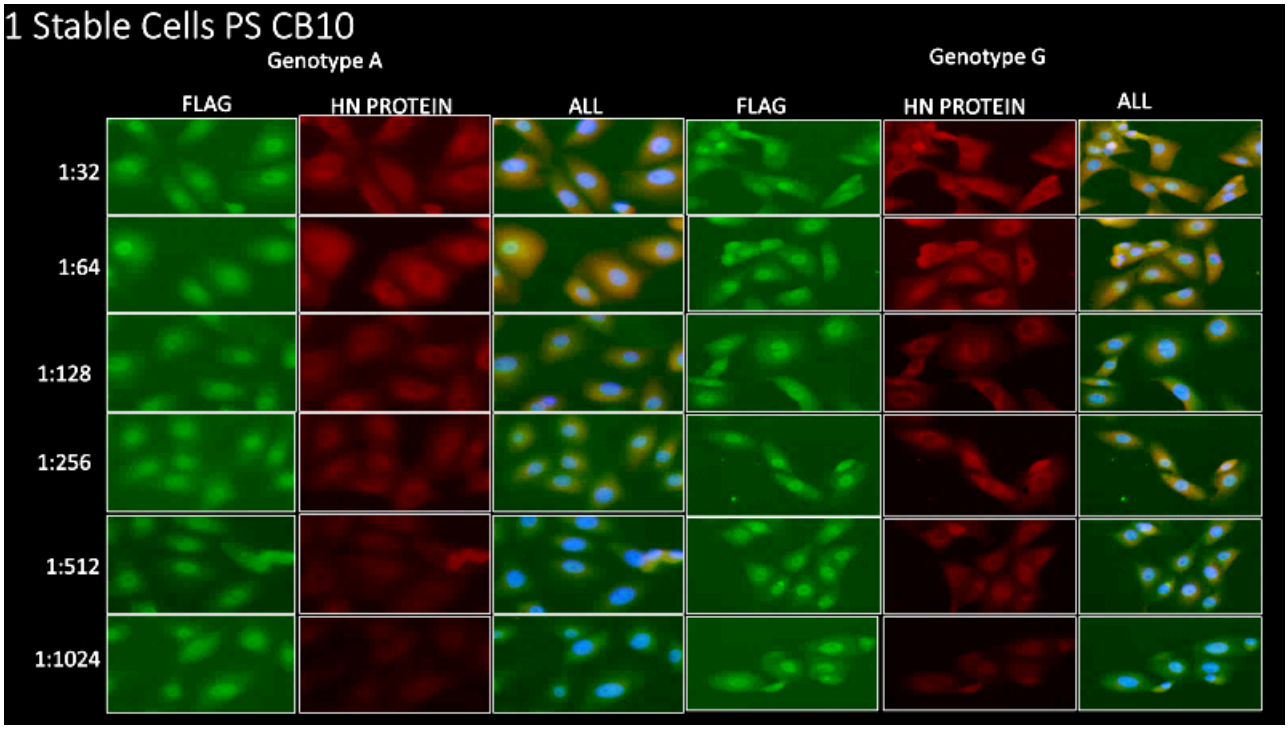


Figure A-41b. Stable cells Vaccine-Infected (CB) Sample #10.

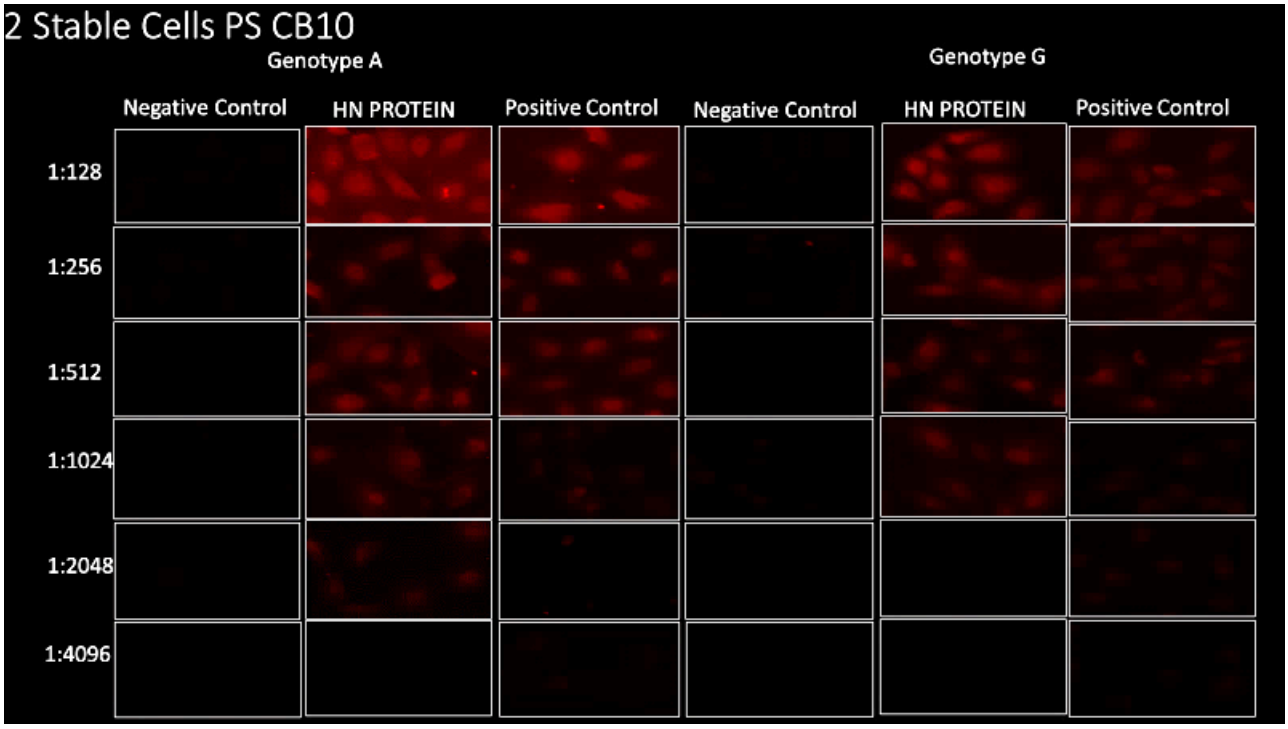


Figure A-41c. Stable cells Vaccine-Infected (CB) Sample #10.

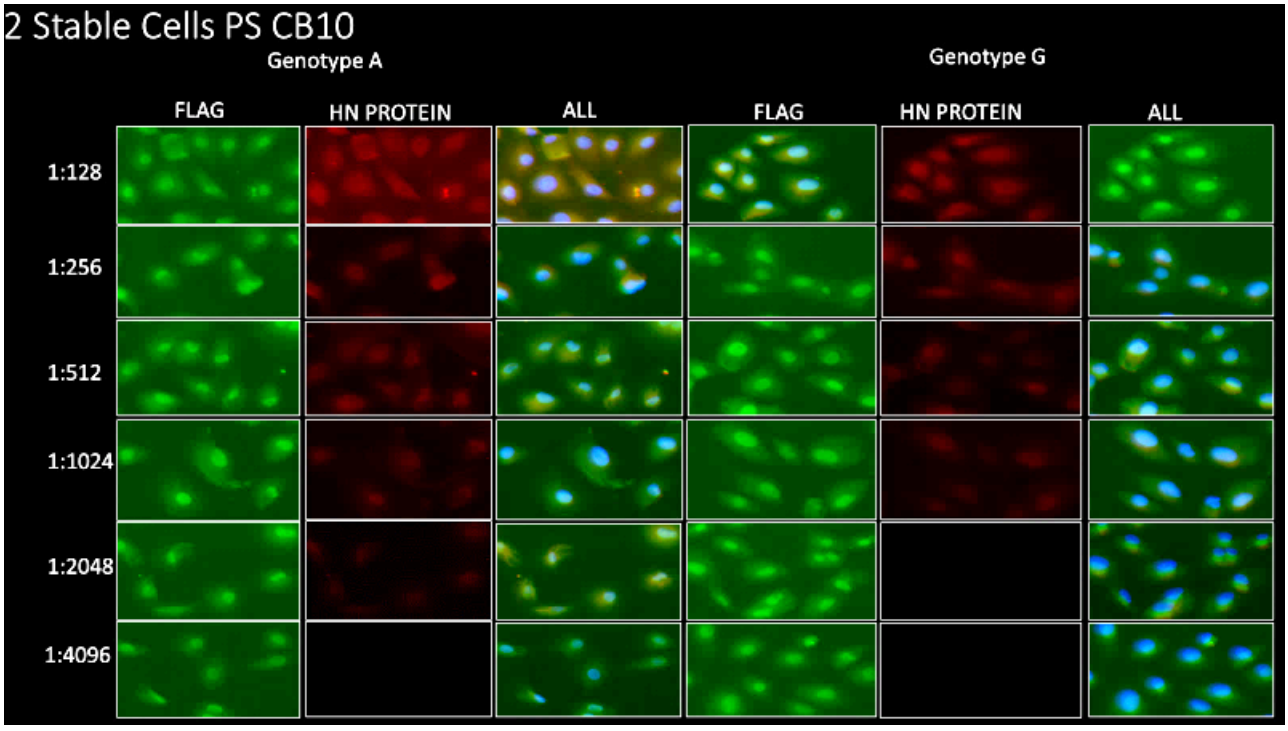


Figure A-41d. Stable cells Vaccine-Infected (CB) Sample #10.

IFA Results of F Protein

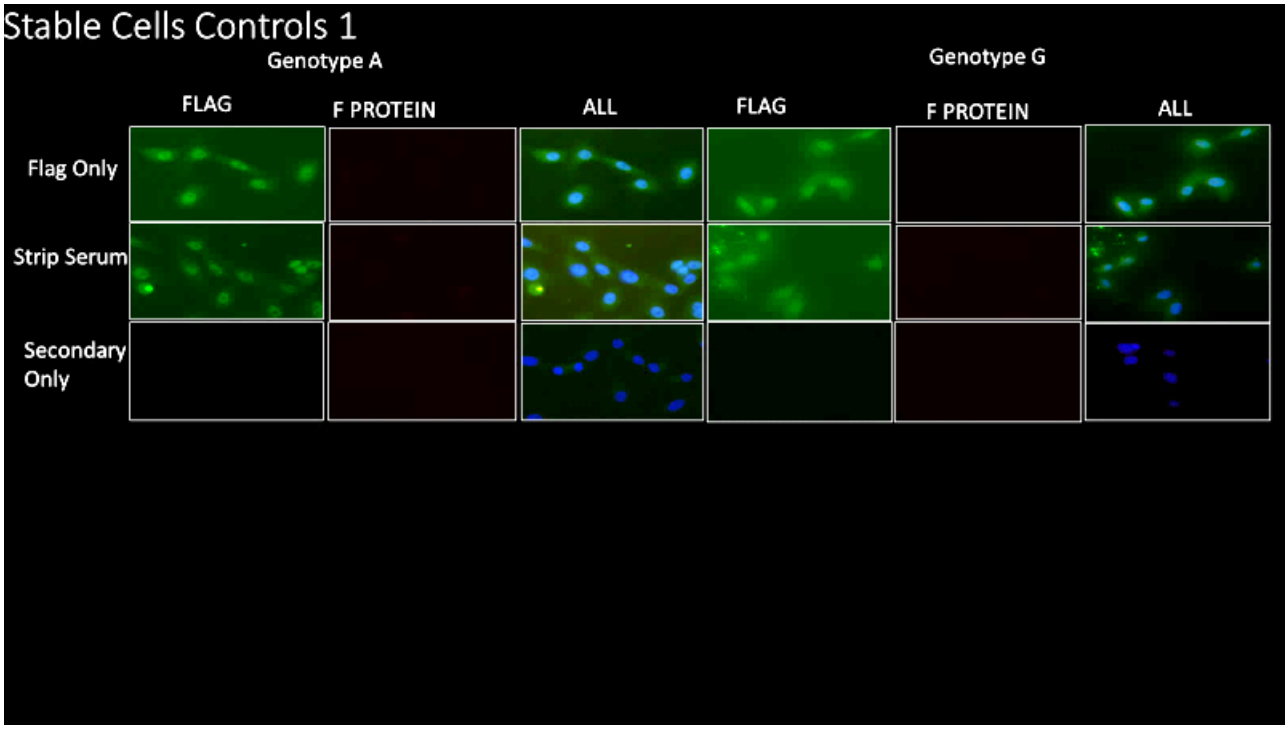


Figure A-42a. Stable cells Control Batch#1.

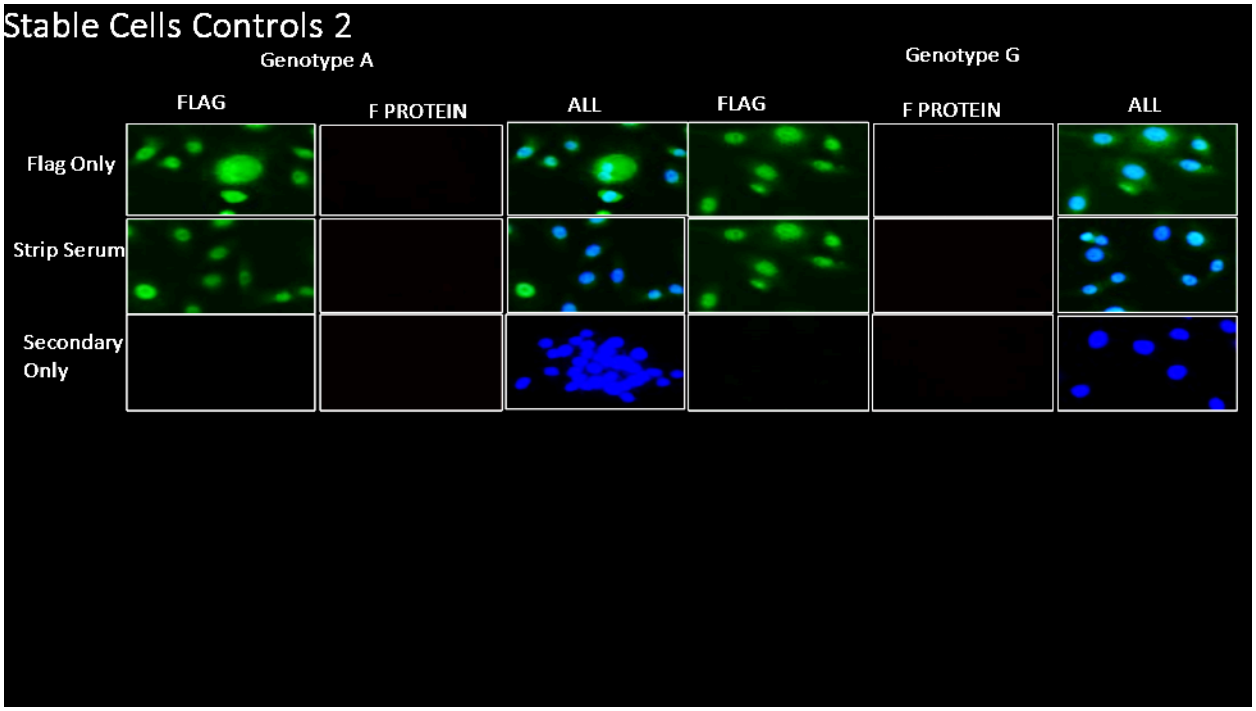


Figure A-42b. Stable cells Control Batch#2.

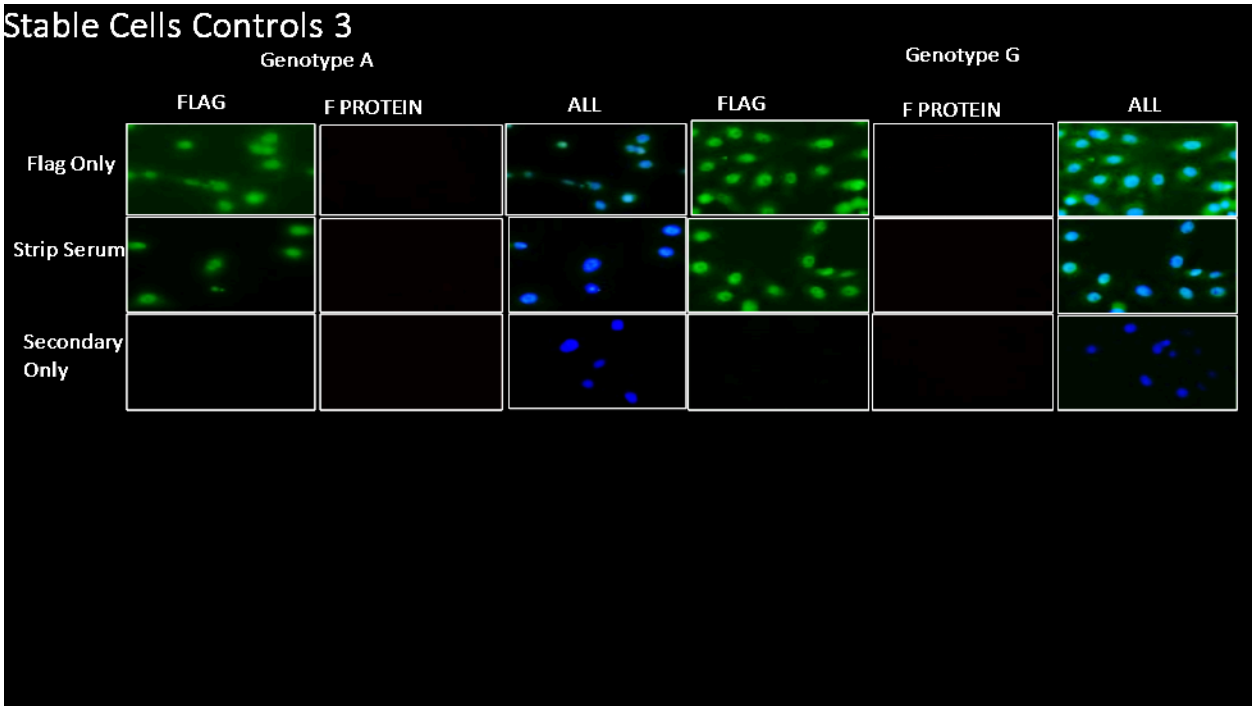


Figure A-42c. Stable cells Control Batch#3.

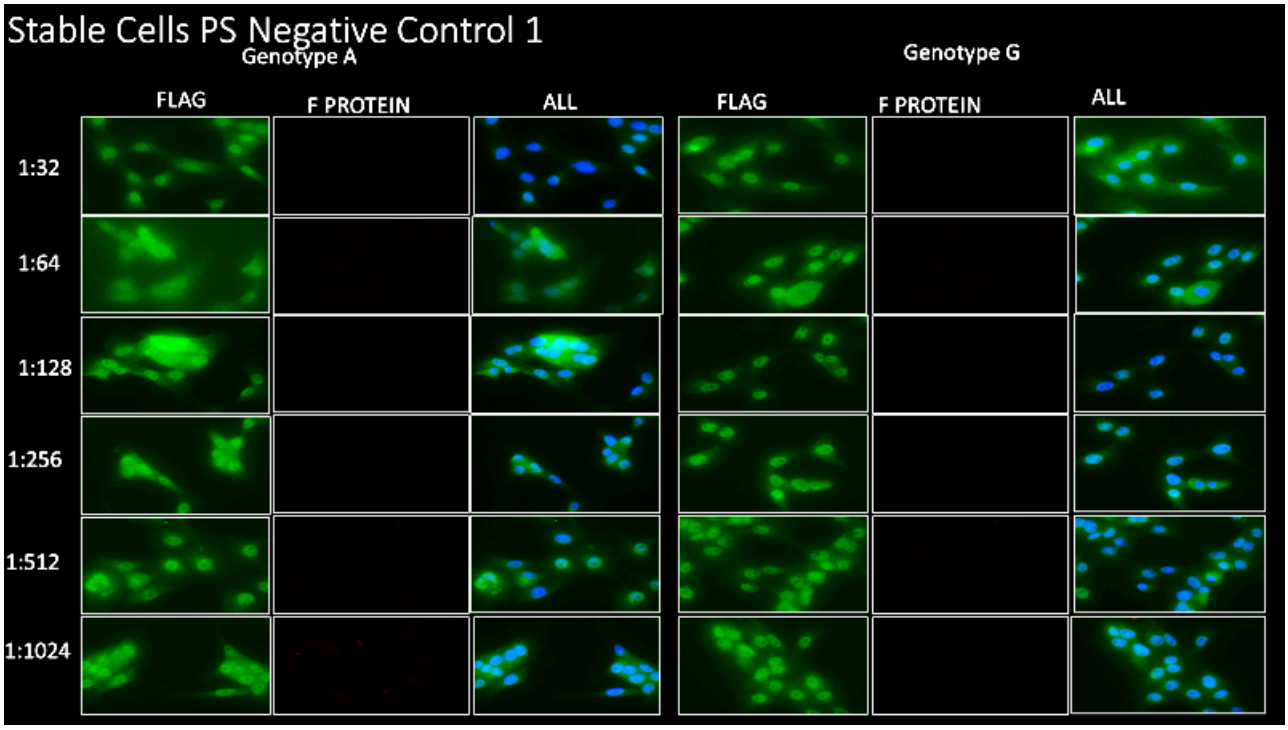


Figure A-43a. Stable cells Negative Control Batch#1.

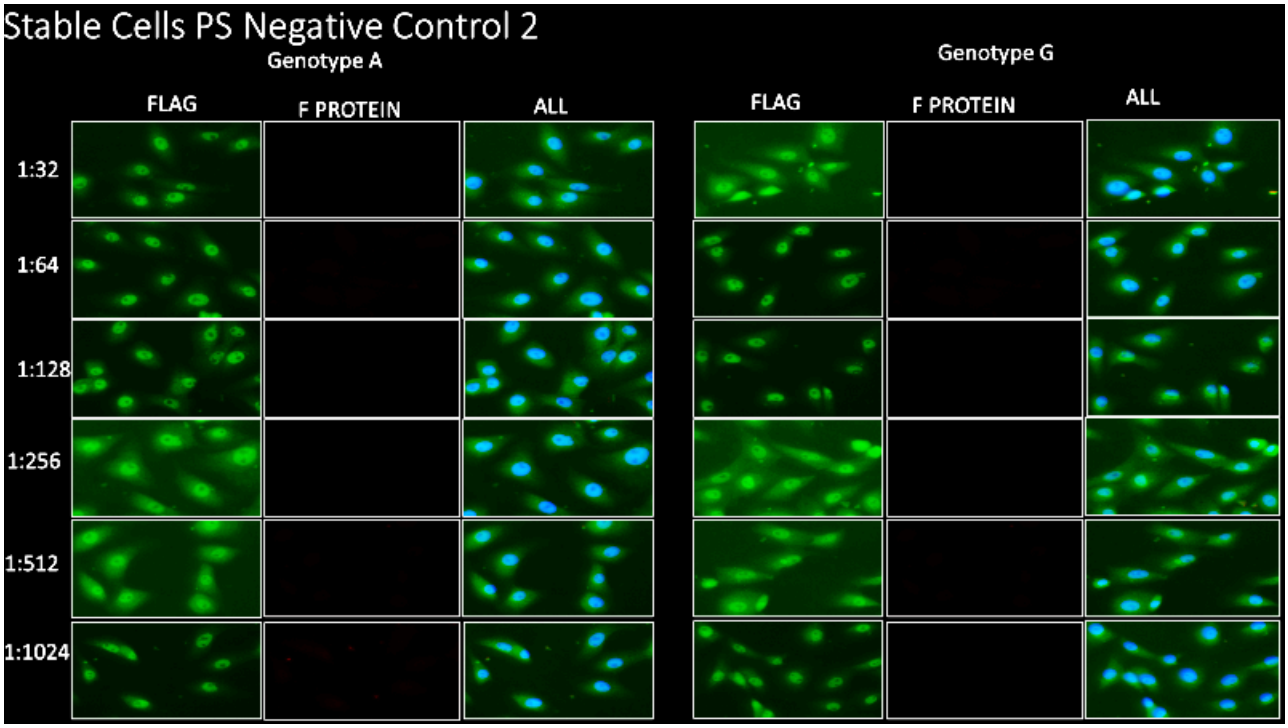


Figure A-43b. Stable cells Negative Control Batch#2.

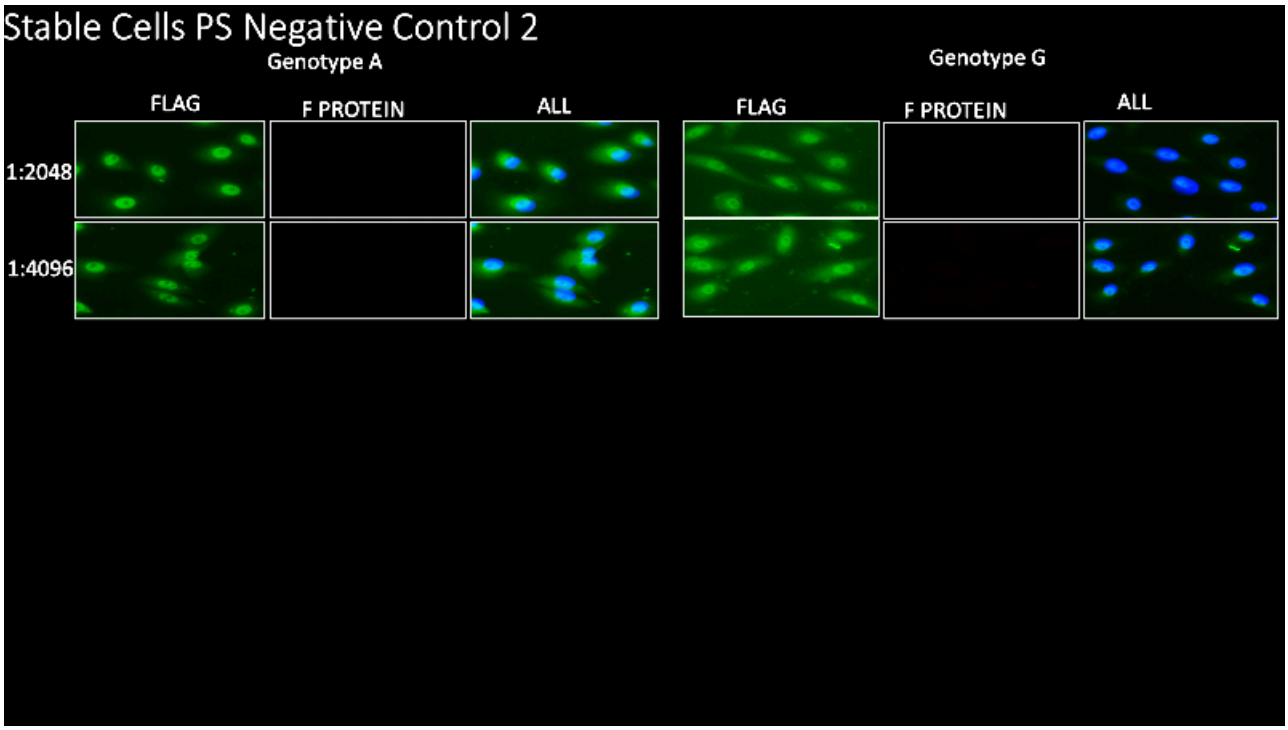


Figure A-43c. Stable cells Negative Control Batch#2.

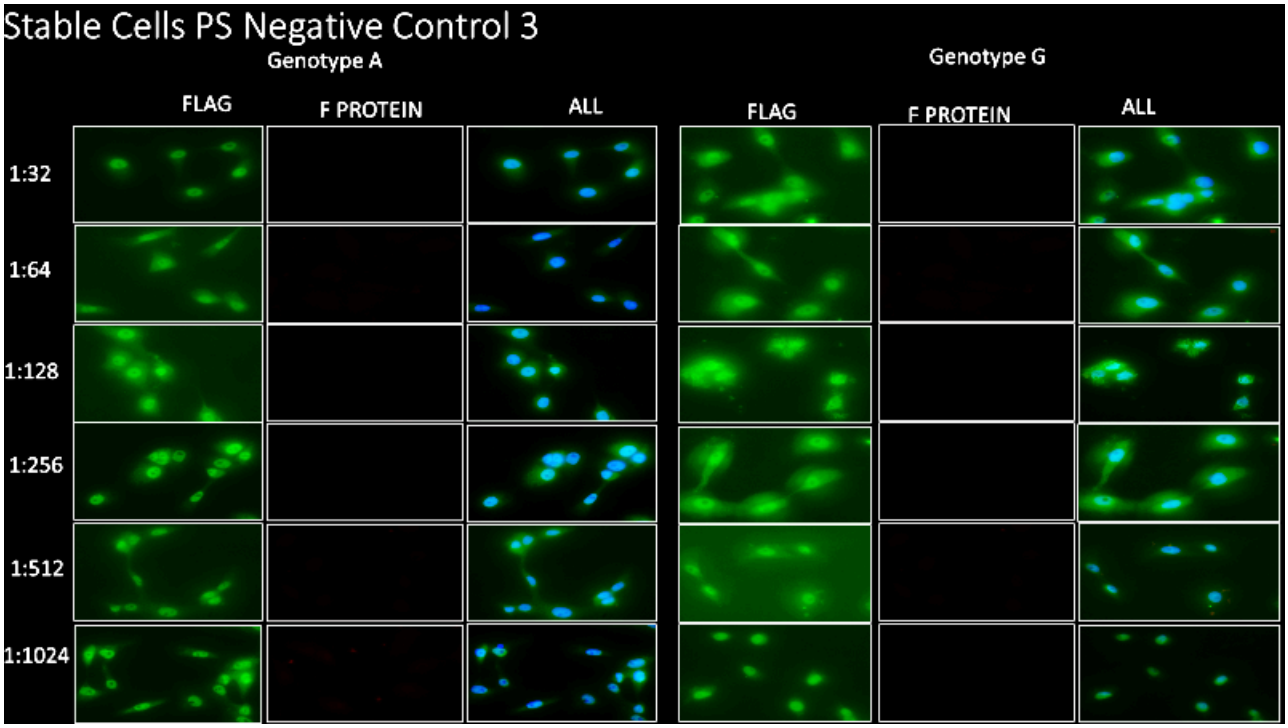


Figure A-43d. Stable cells Negative Control Batch#3.

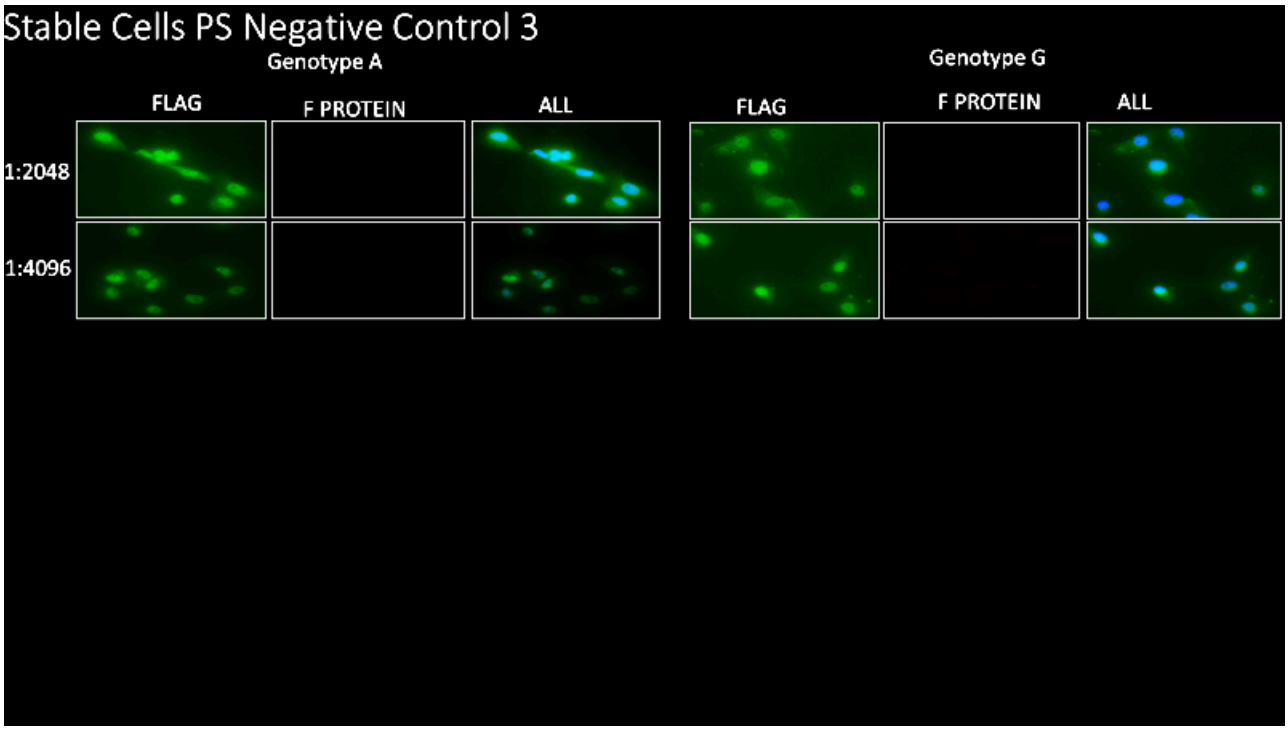


Figure A-43e. Stable cells Negative Control Batch#3.

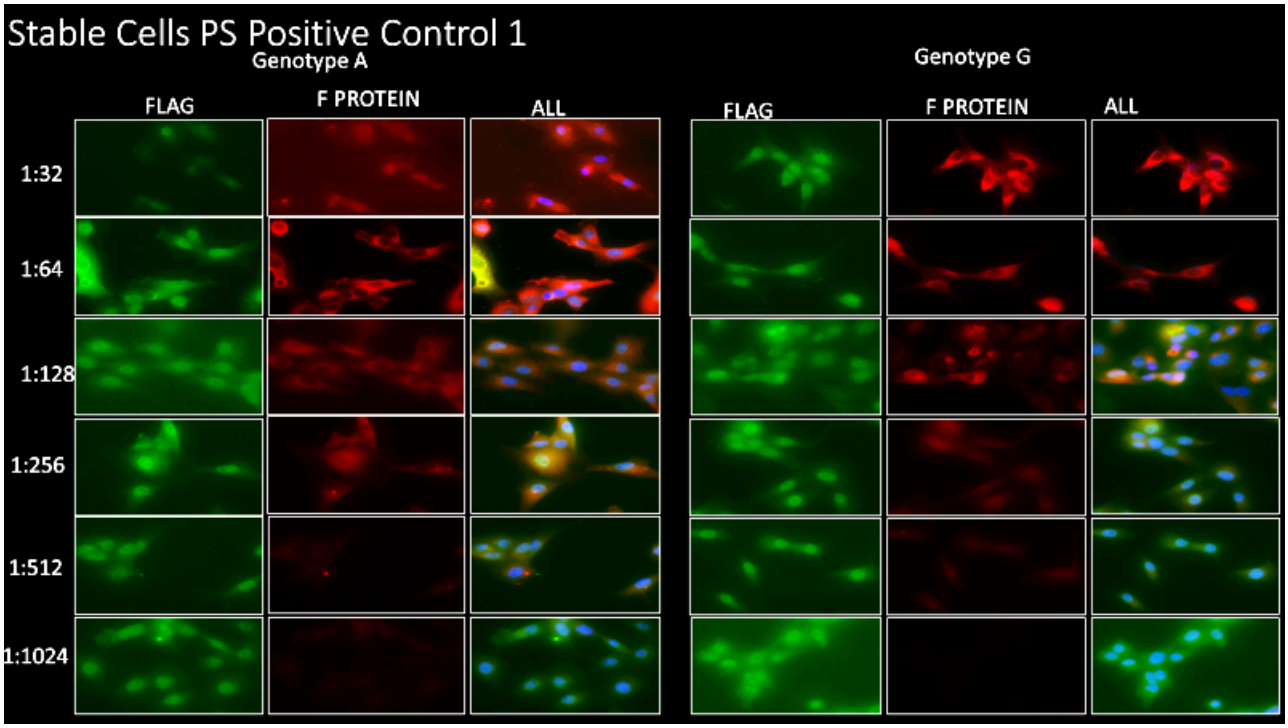


Figure A-44a. Stable cells Positive Control Batch#1.

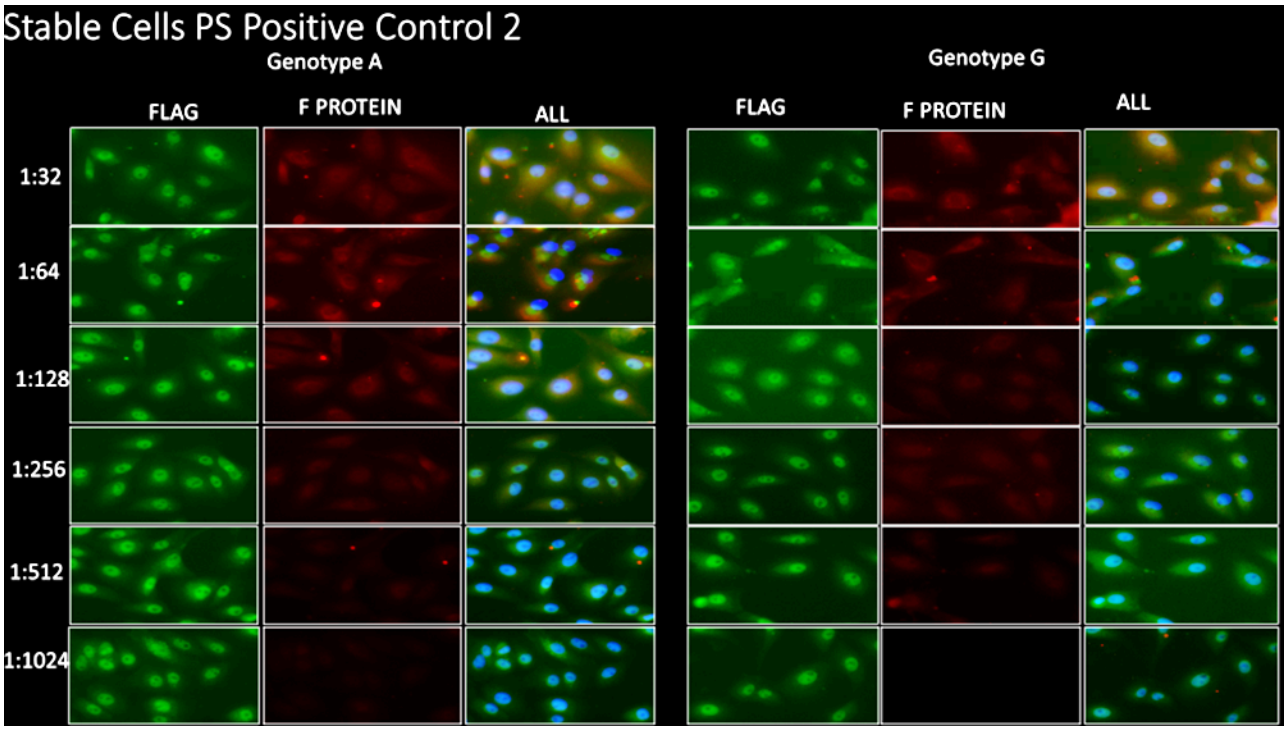


Figure A-44b. Stable cells Positive Control Batch#2.

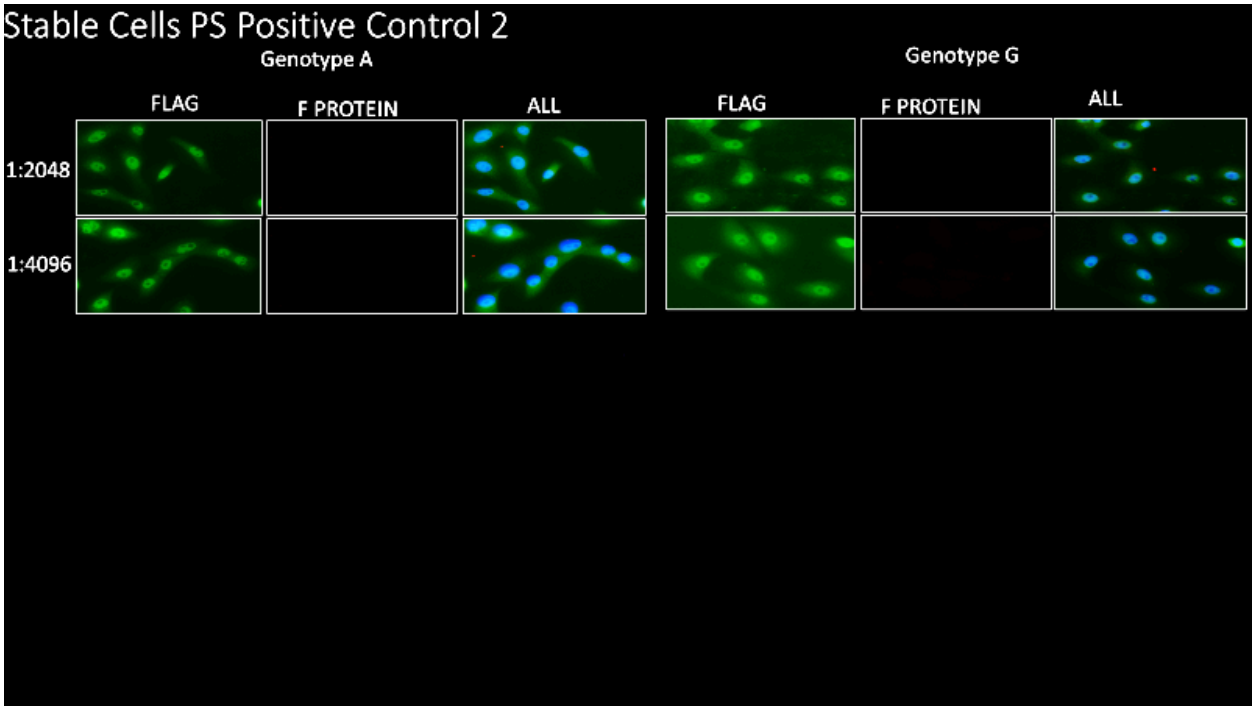


Figure A-44c. Stable cells Positive Control Batch#2.

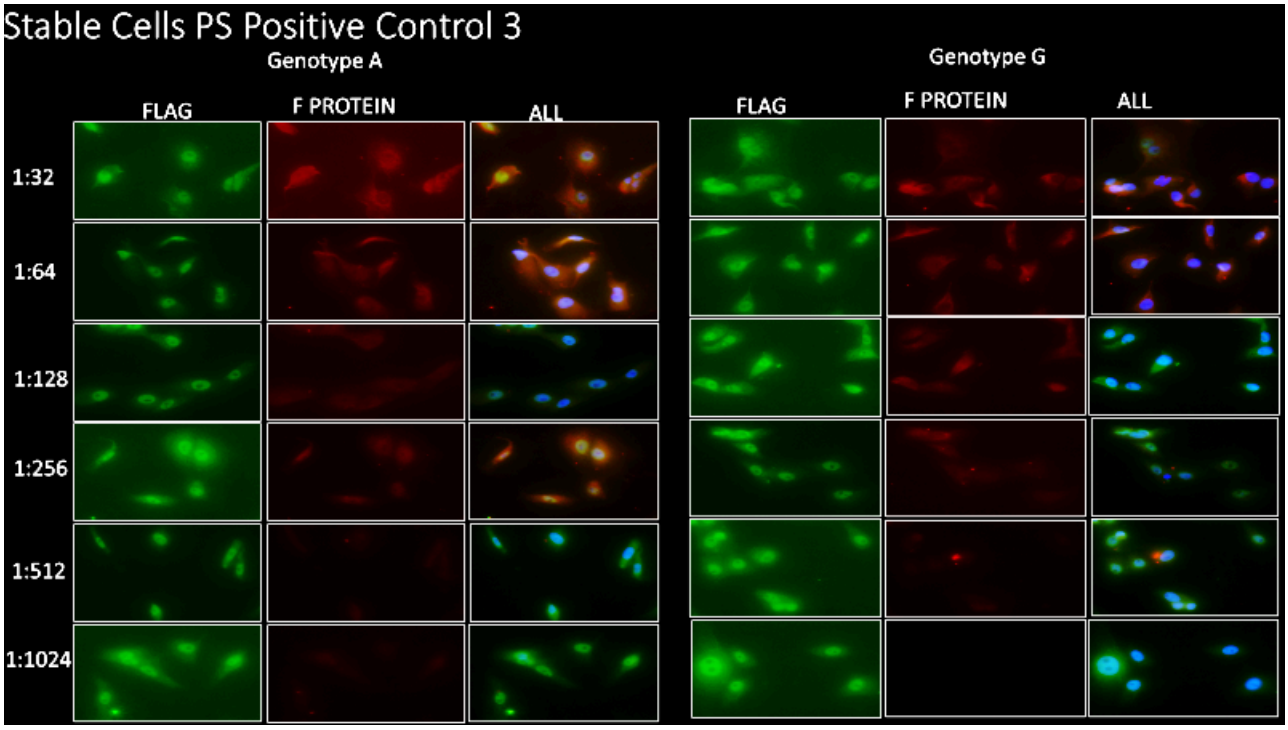


Figure A-44d. Stable cells Positive Control Batch#3.

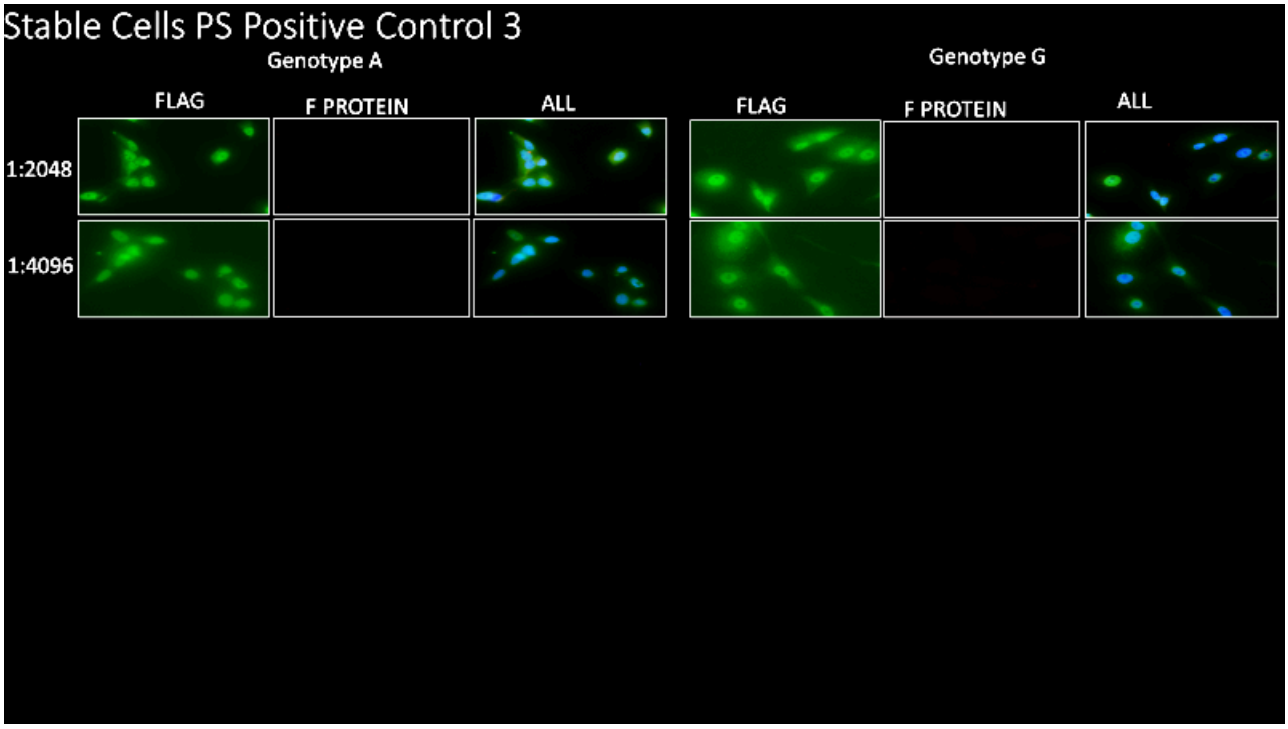


Figure A-44e. Stable cells Positive Control Batch#3.

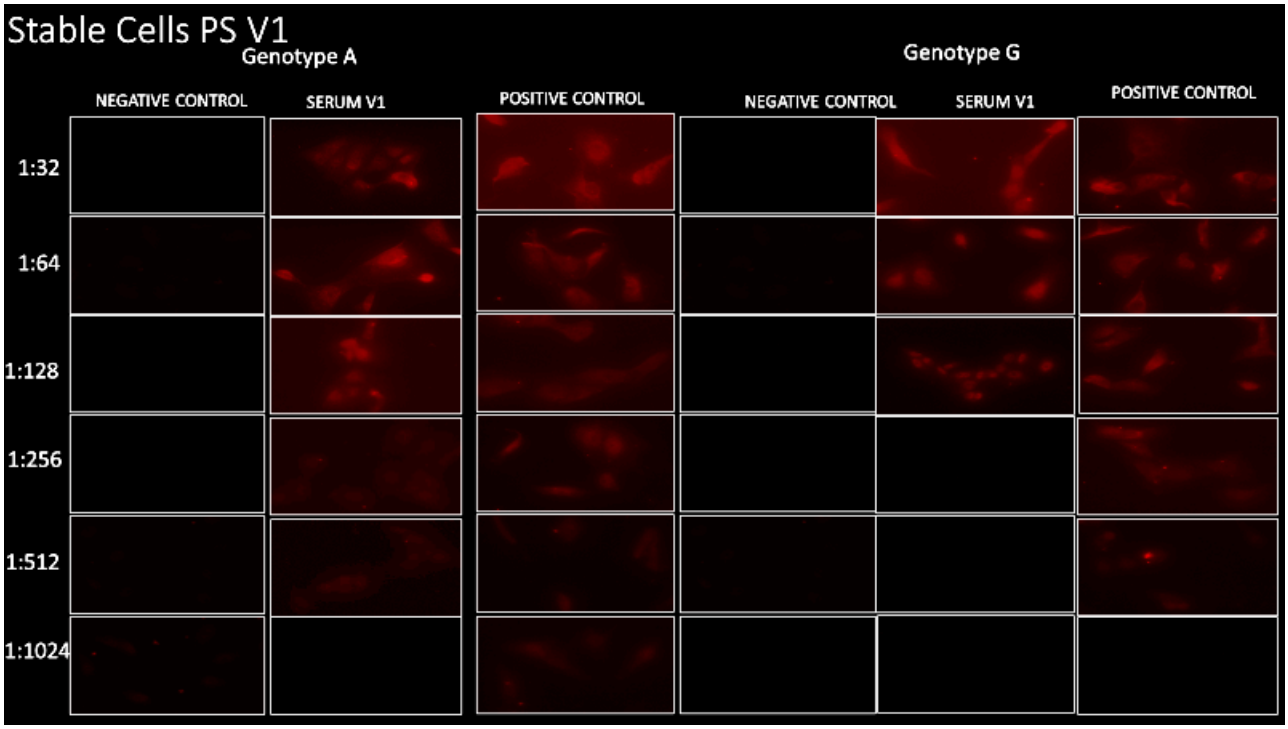


Figure A-45a. Stable cells Vaccine Sample#1.

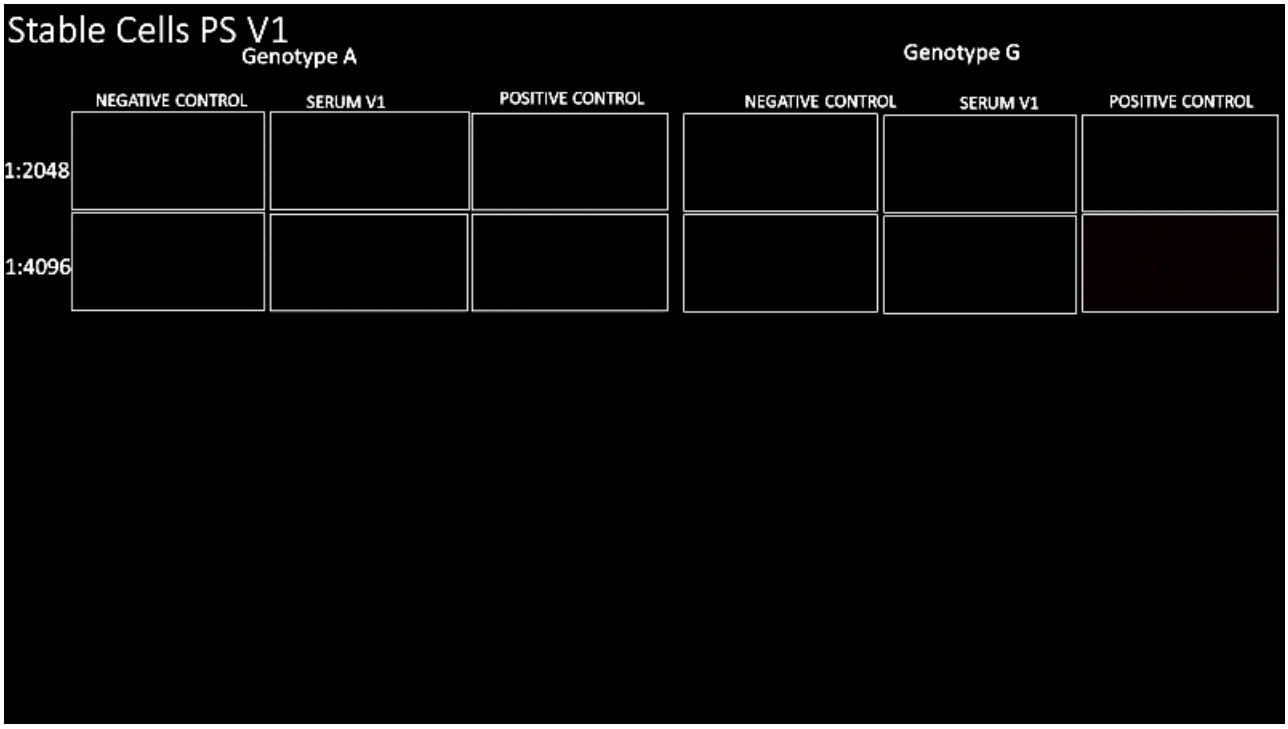


Figure A-45b. Stable cells Vaccine Sample#1.

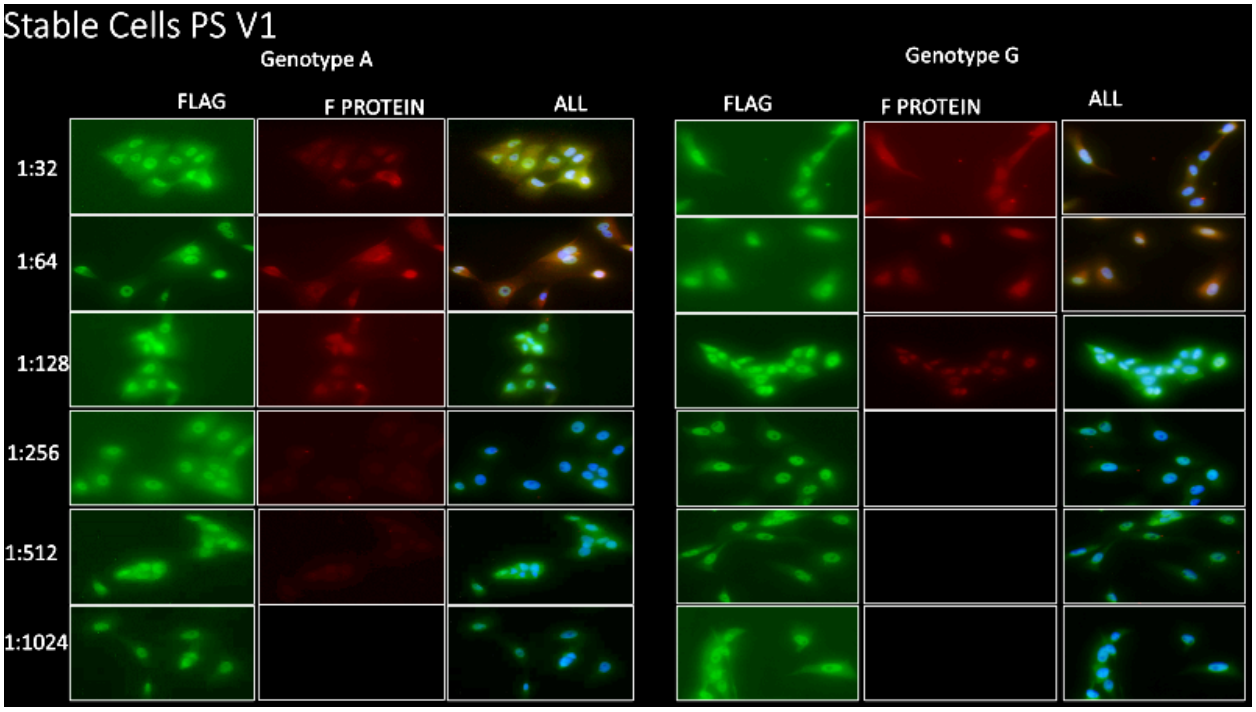


Figure A-45c. Stable cells Vaccine Sample#1.

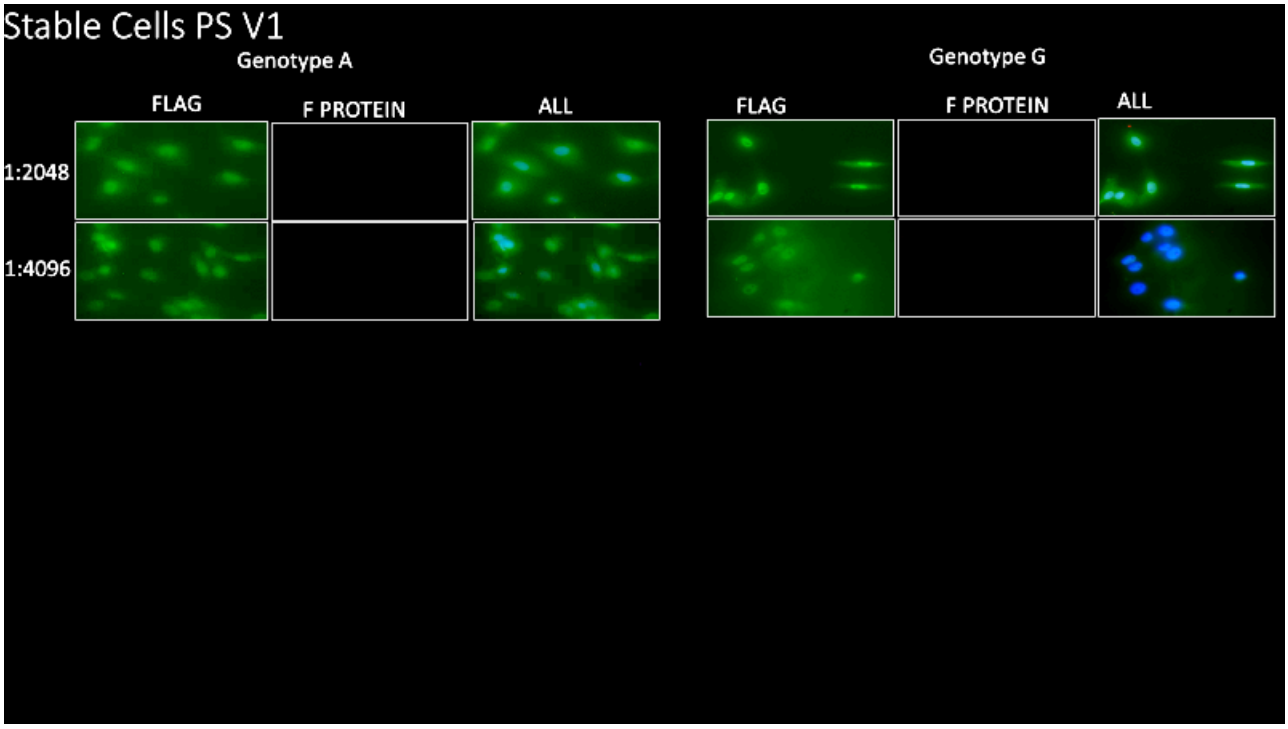


Figure A-45d. Stable cells Vaccine Sample#1.

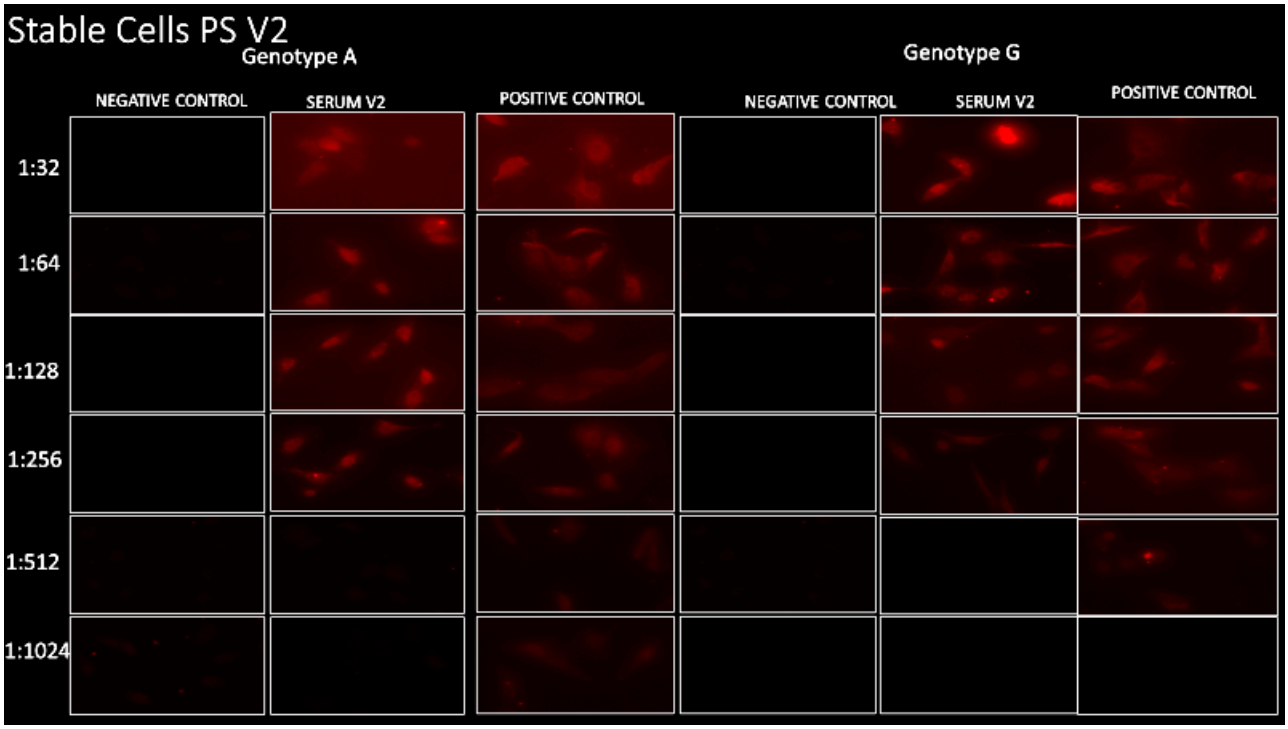


Figure A-46a. Stable cells Vaccine Sample#2.

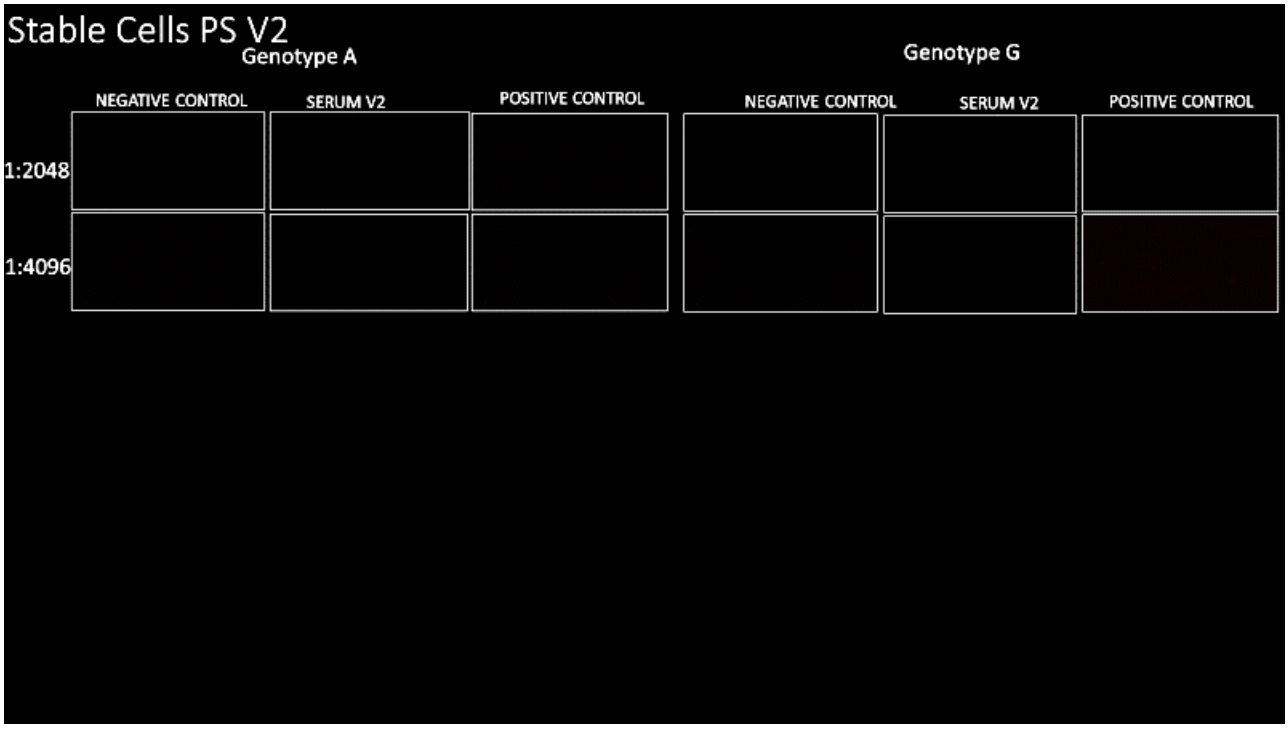


Figure A-46b. Stable cells Vaccine Sample#2.

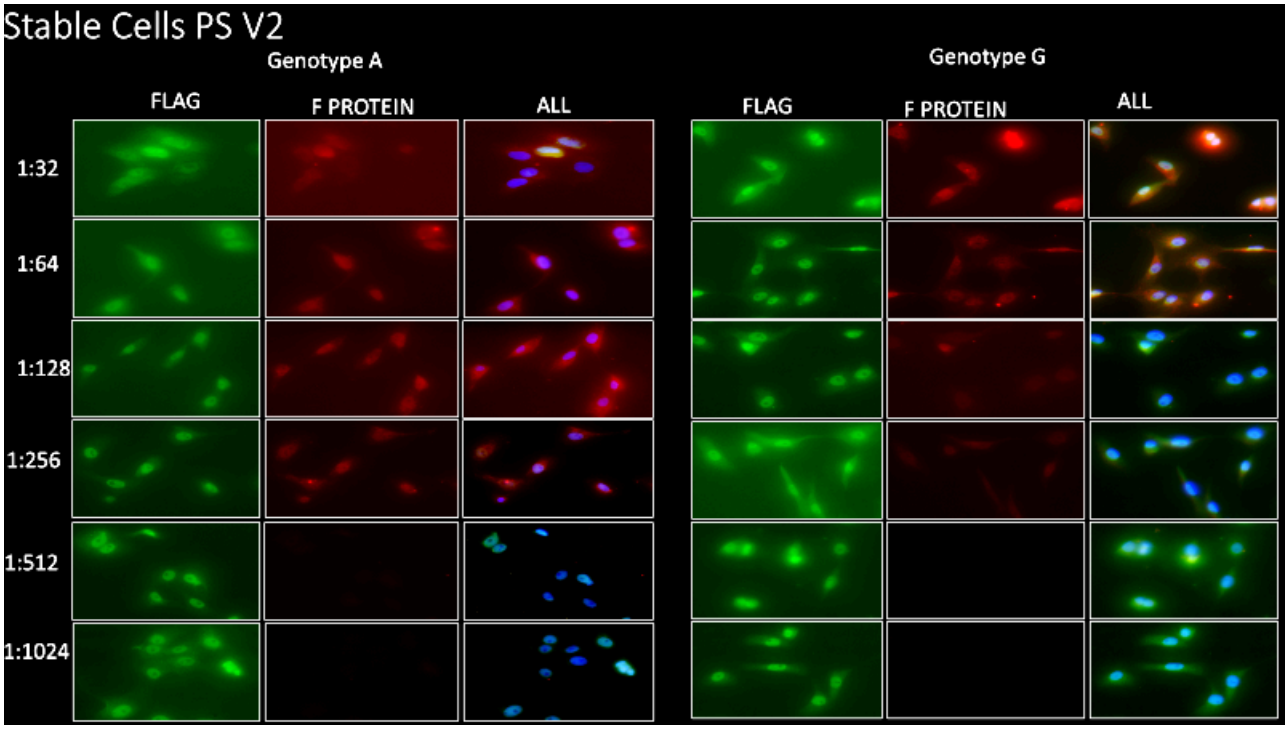


Figure A-46c. Stable cells Vaccine Sample#2.

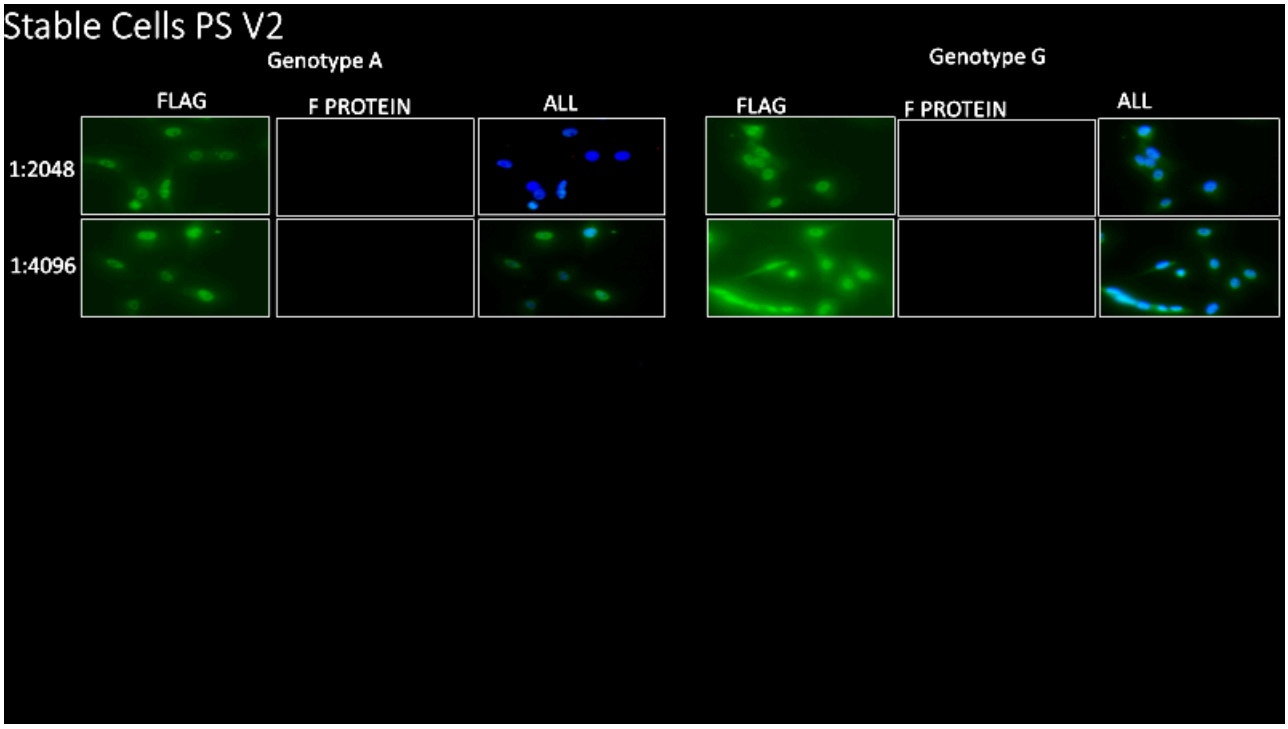


Figure A-46d. Stable cells Vaccine Sample#2.

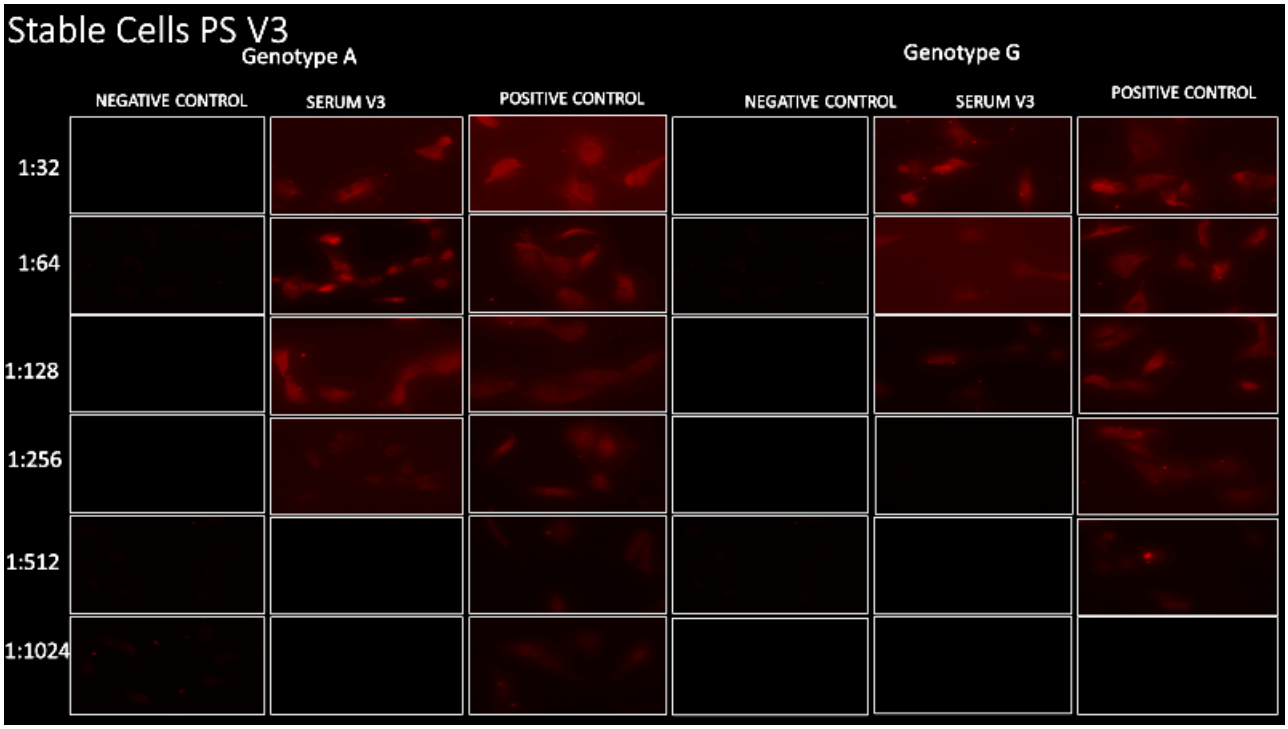


Figure A-47a. Stable cells Vaccine Sample#3.

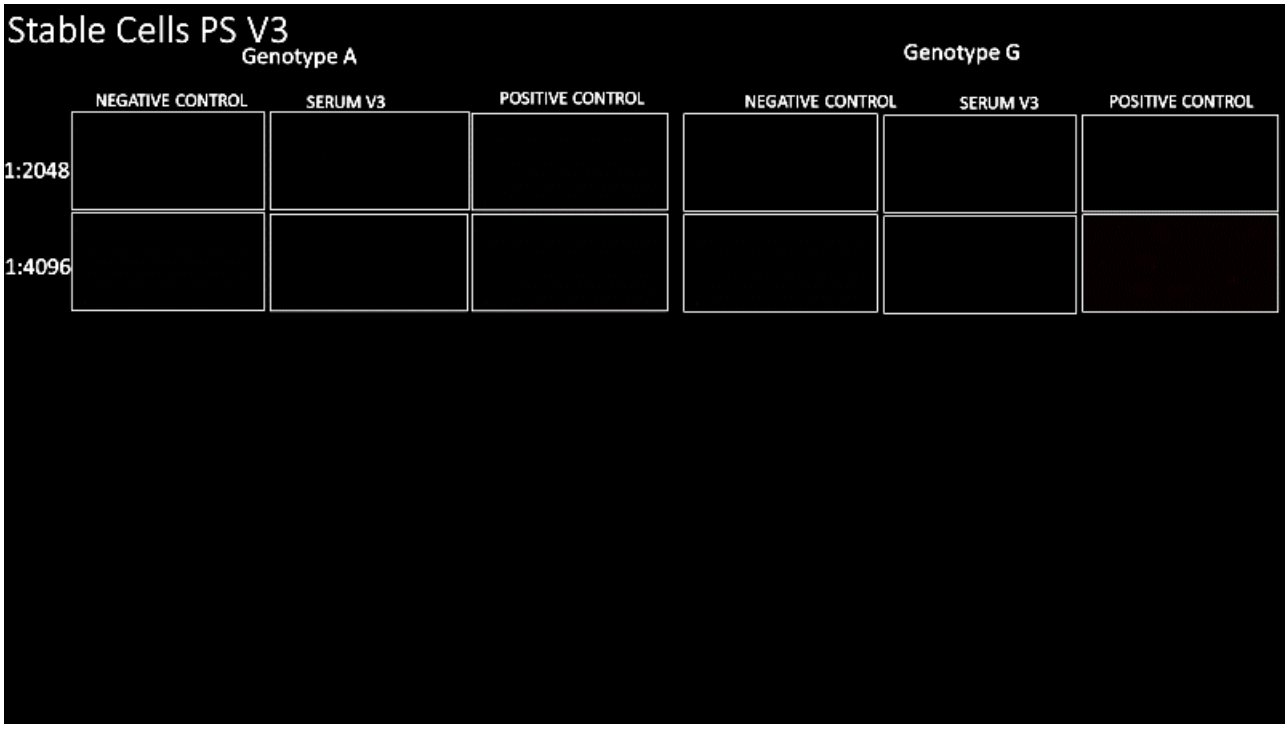


Figure A-47b. Stable cells Vaccine Sample#3.

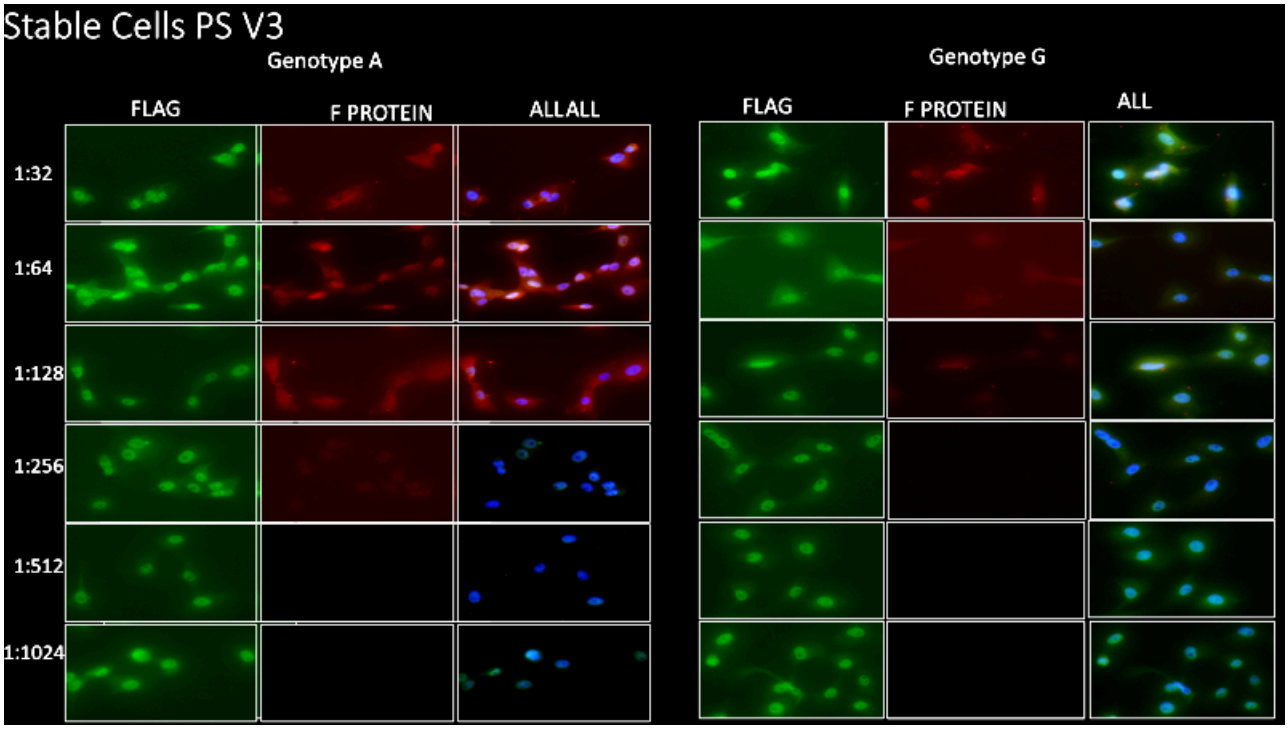


Figure A-47c. Stable cells Vaccine Sample#3.

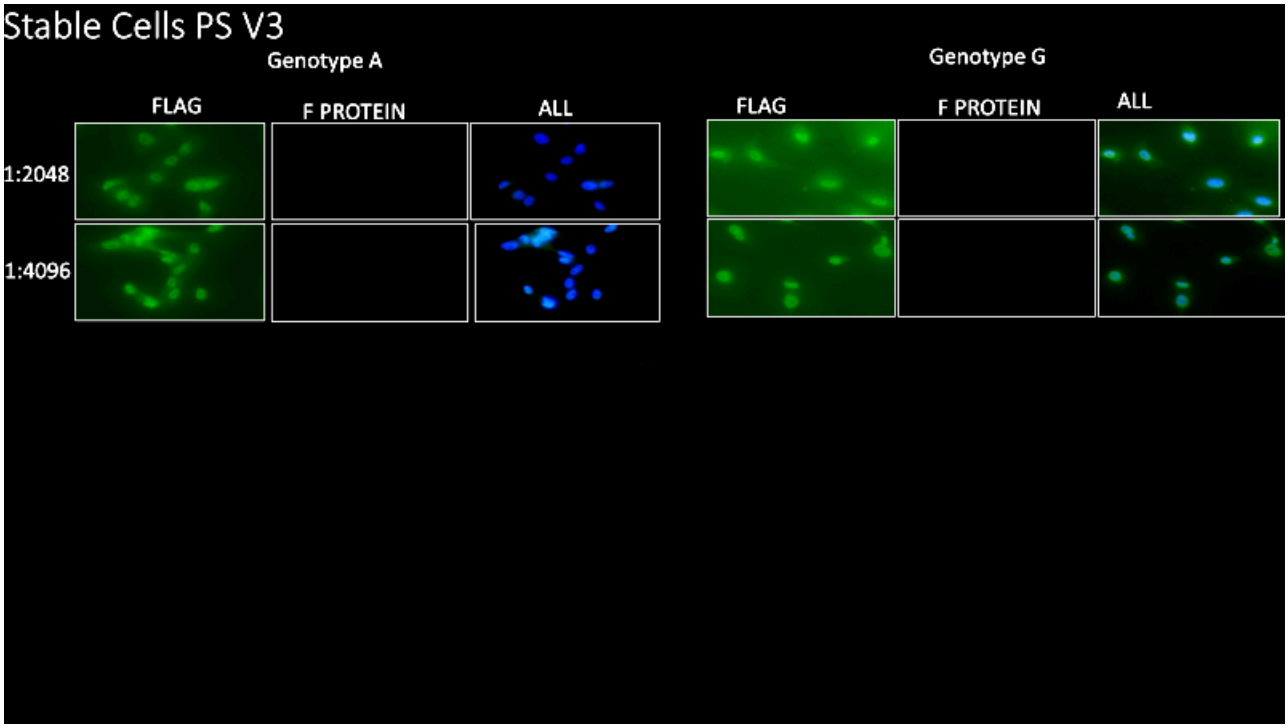


Figure A-47d. Stable cells Vaccine Sample#3.

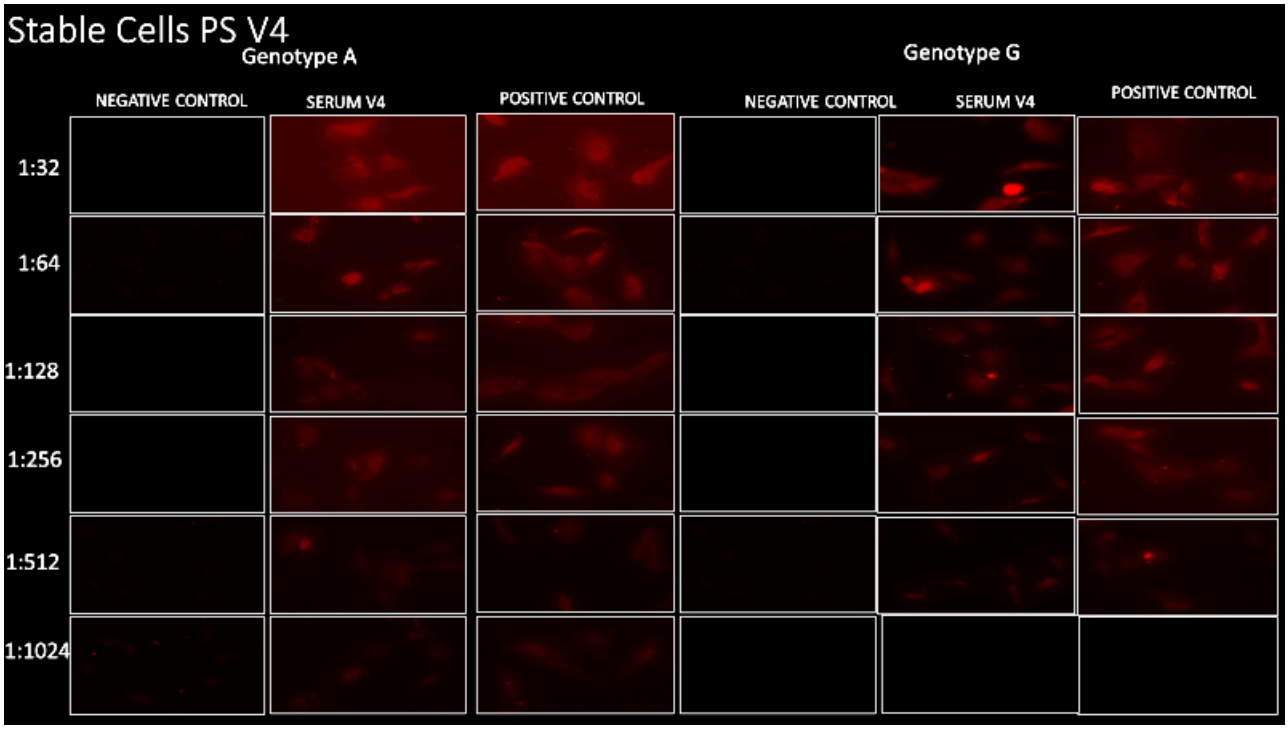


Figure A-48a. Stable cells Vaccine Sample#4.

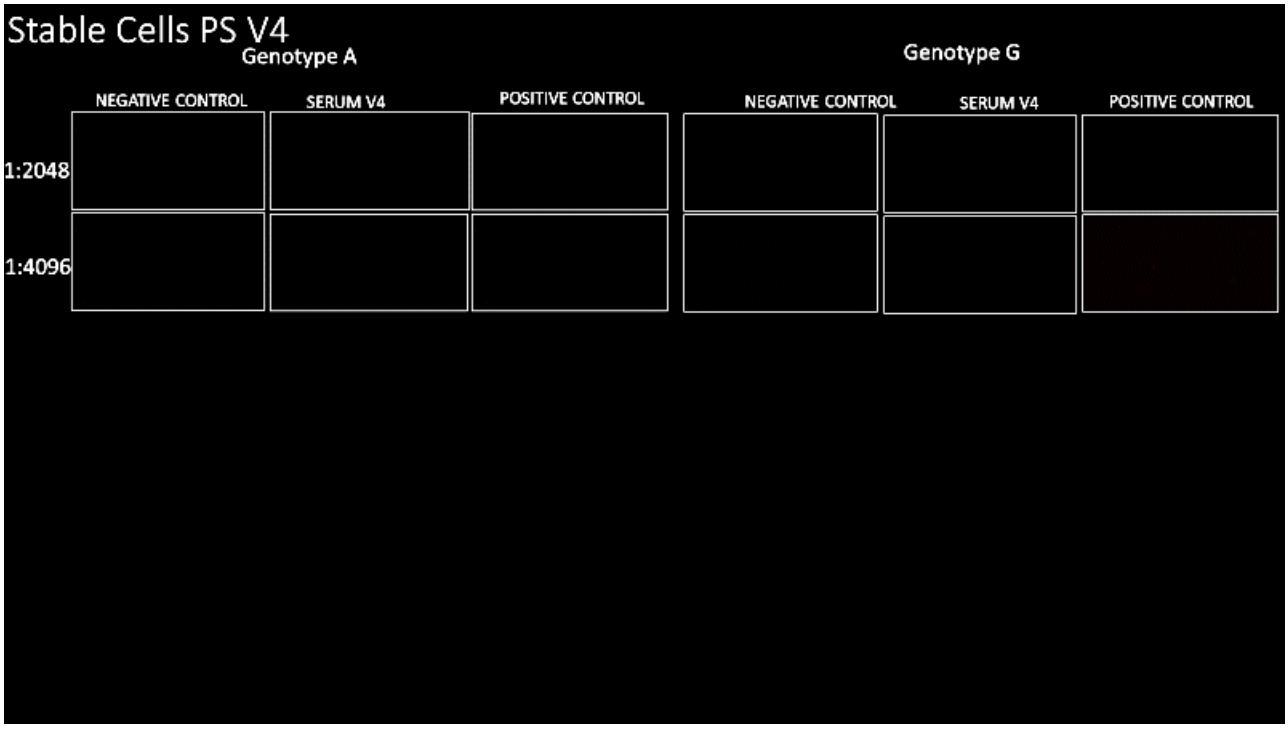


Figure A-48b. Stable cells Vaccine Sample#4.

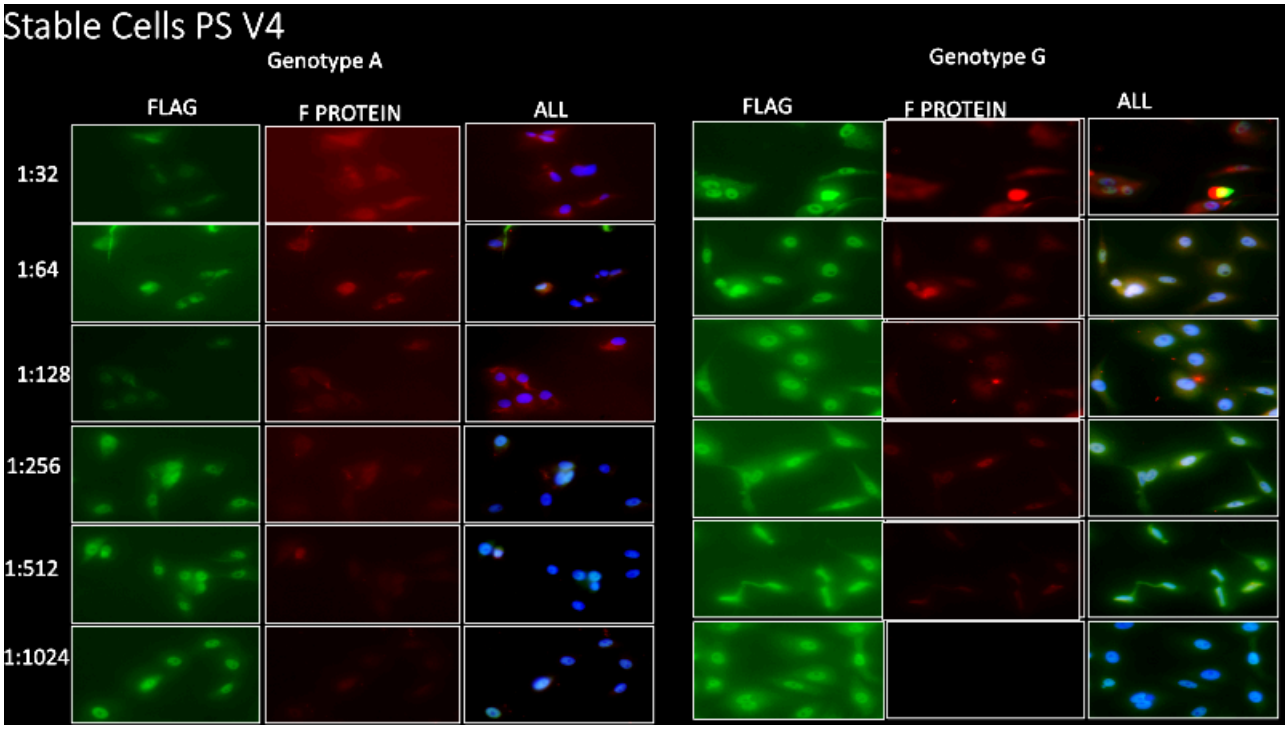


Figure A-48c. Stable cells Vaccine Sample#4.

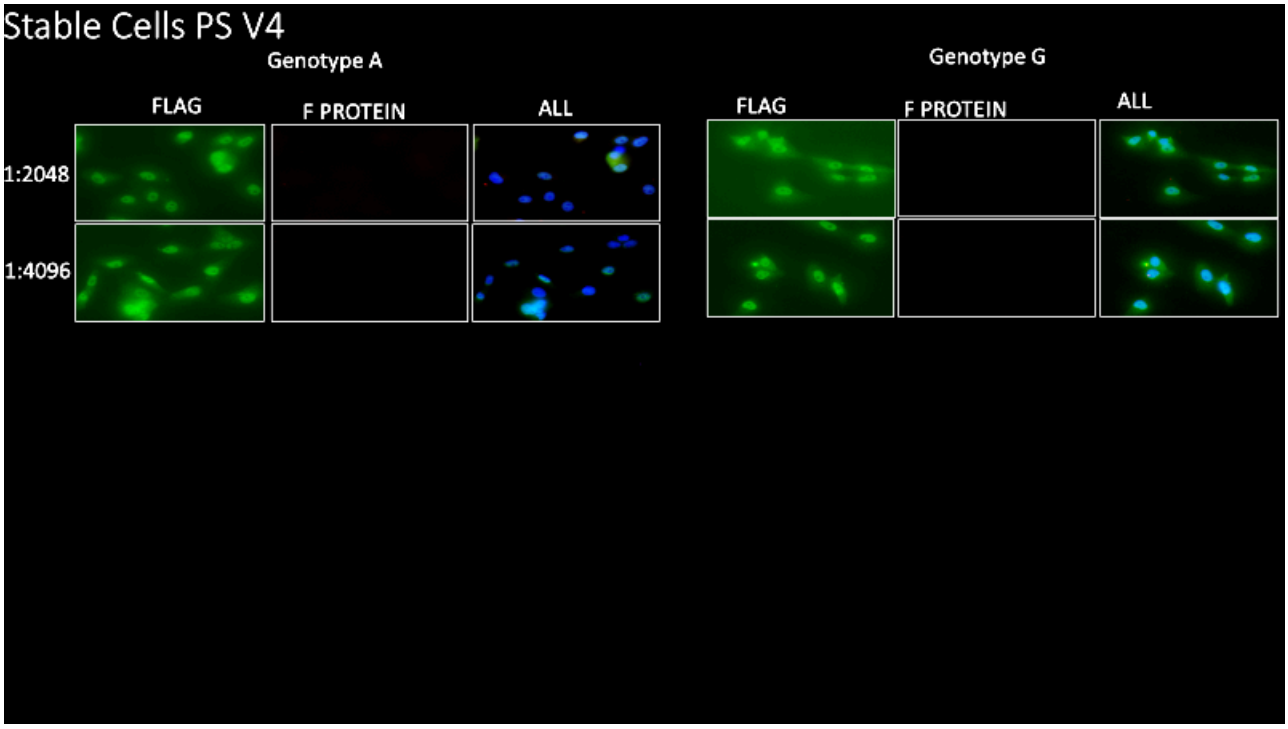


Figure A-48d. Stable cells Vaccine Sample#4.

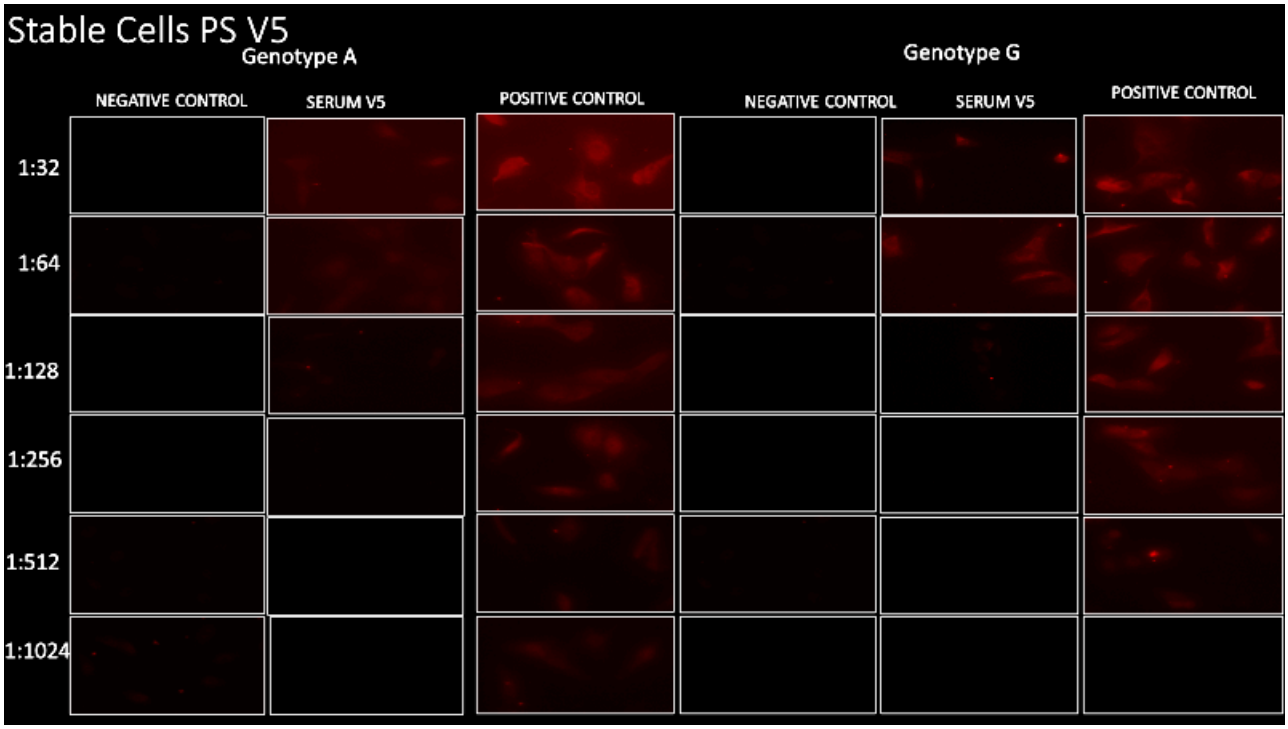


Figure A-49a. Stable cells Vaccine Sample#5.

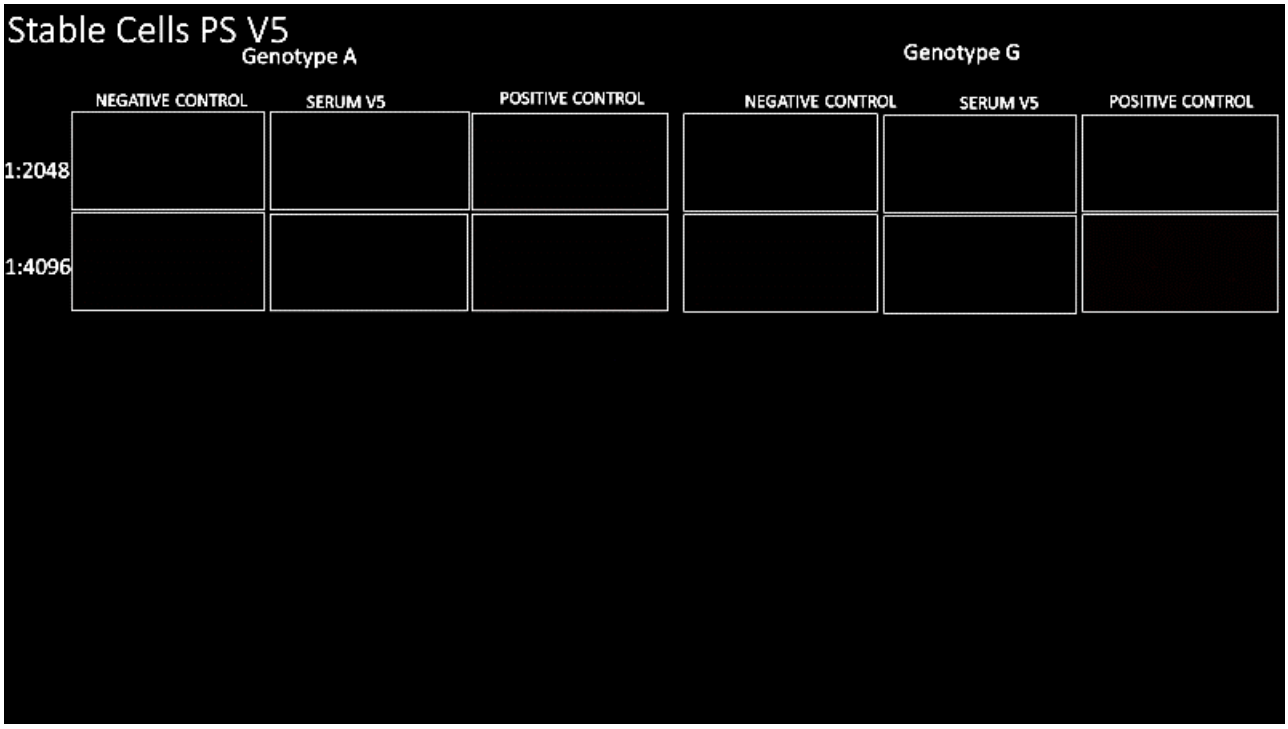


Figure A-49b. Stable cells Vaccine Sample#5.

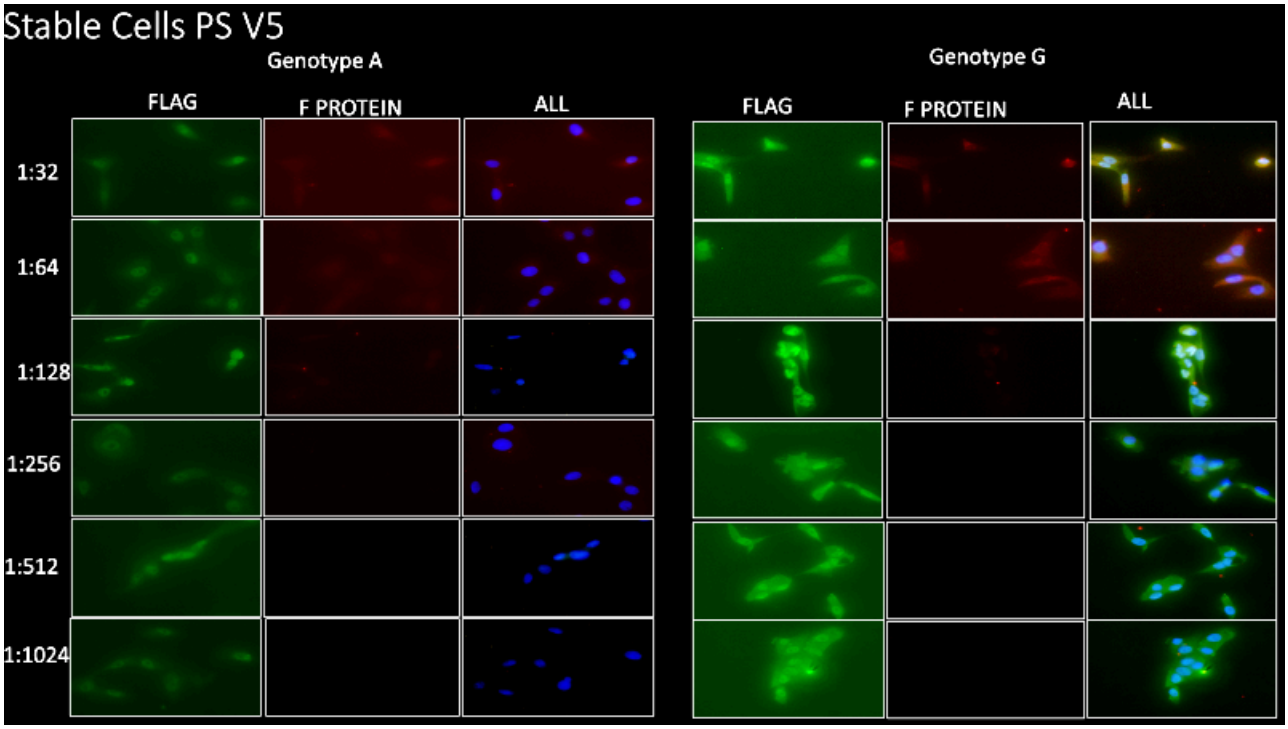


Figure A-49c. Stable cells Vaccine Sample#5.

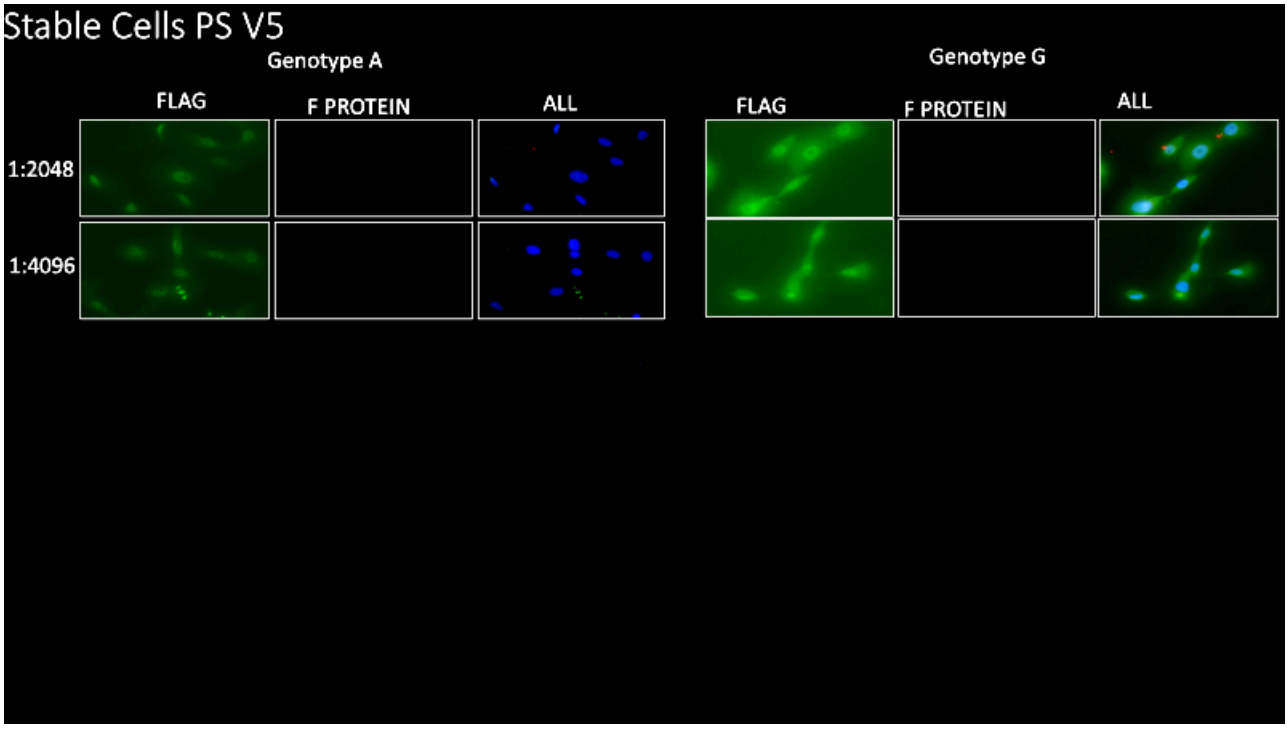


Figure A-49d. Stable cells Vaccine Sample#5.

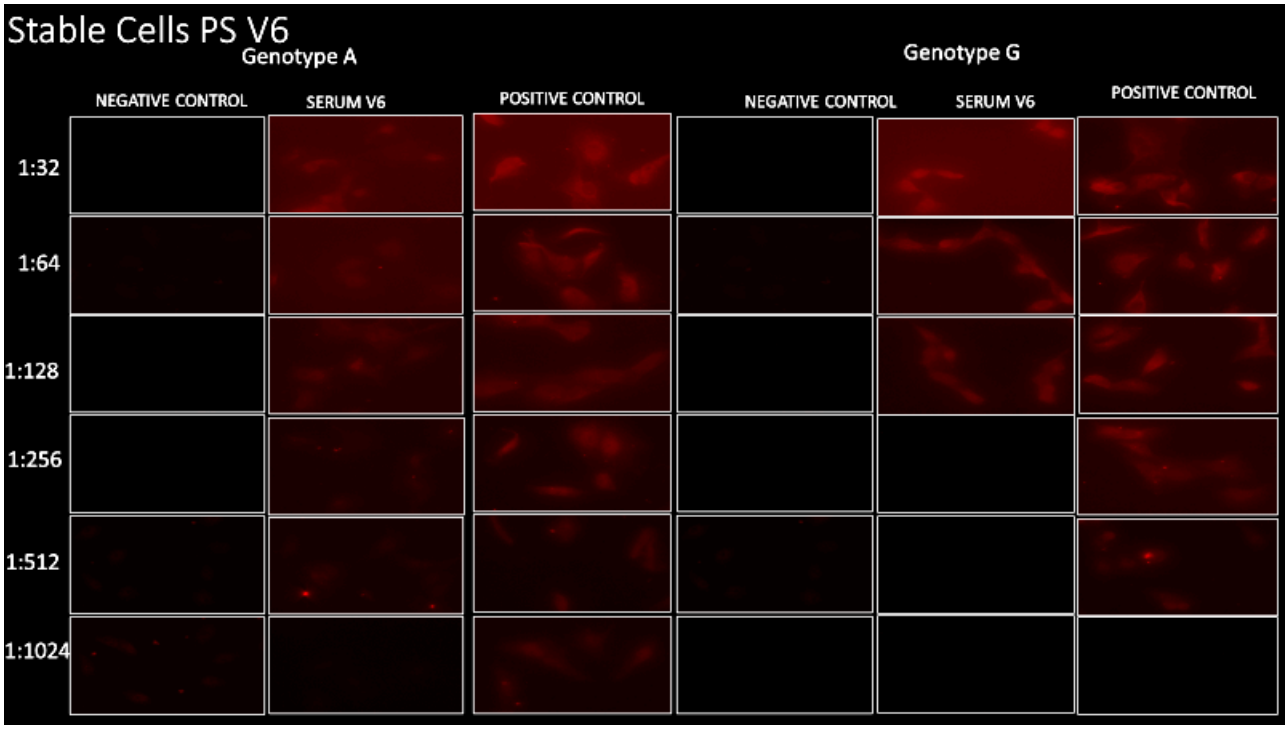


Figure A-50a. Stable cells Vaccine Sample#6.

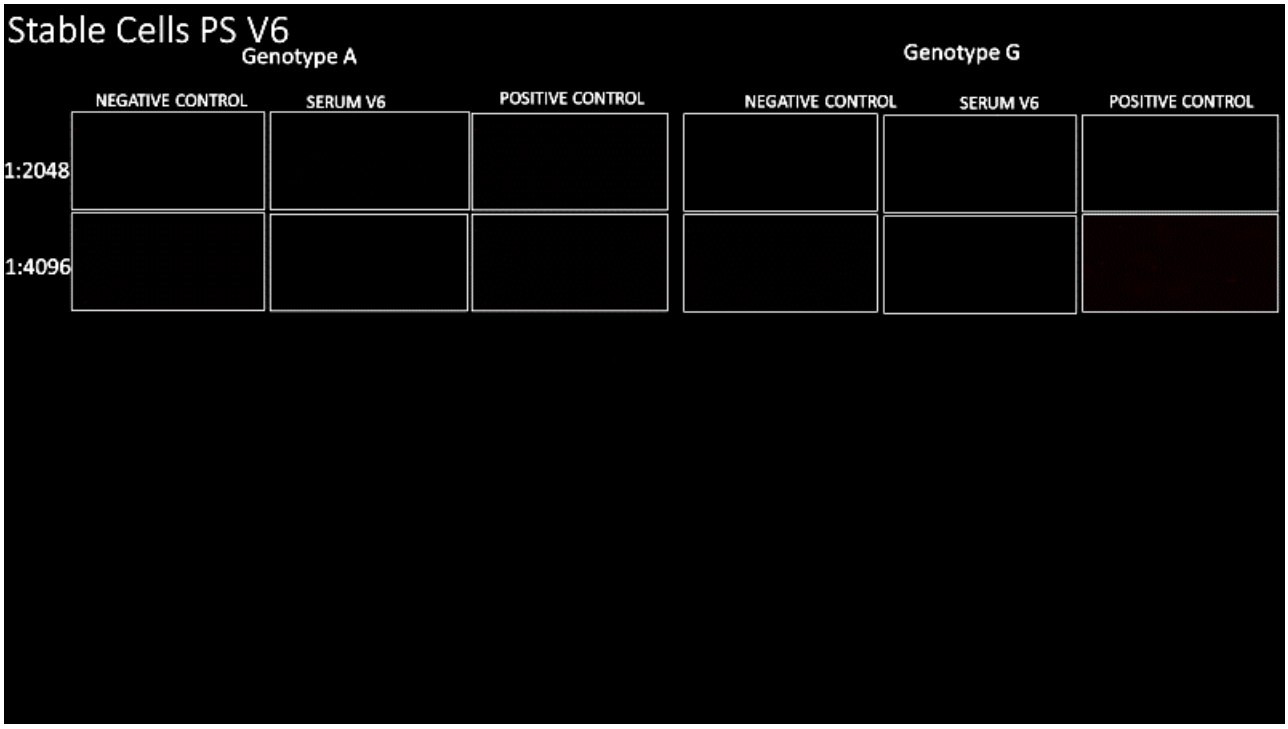


Figure A-50b. Stable cells Vaccine Sample#6.

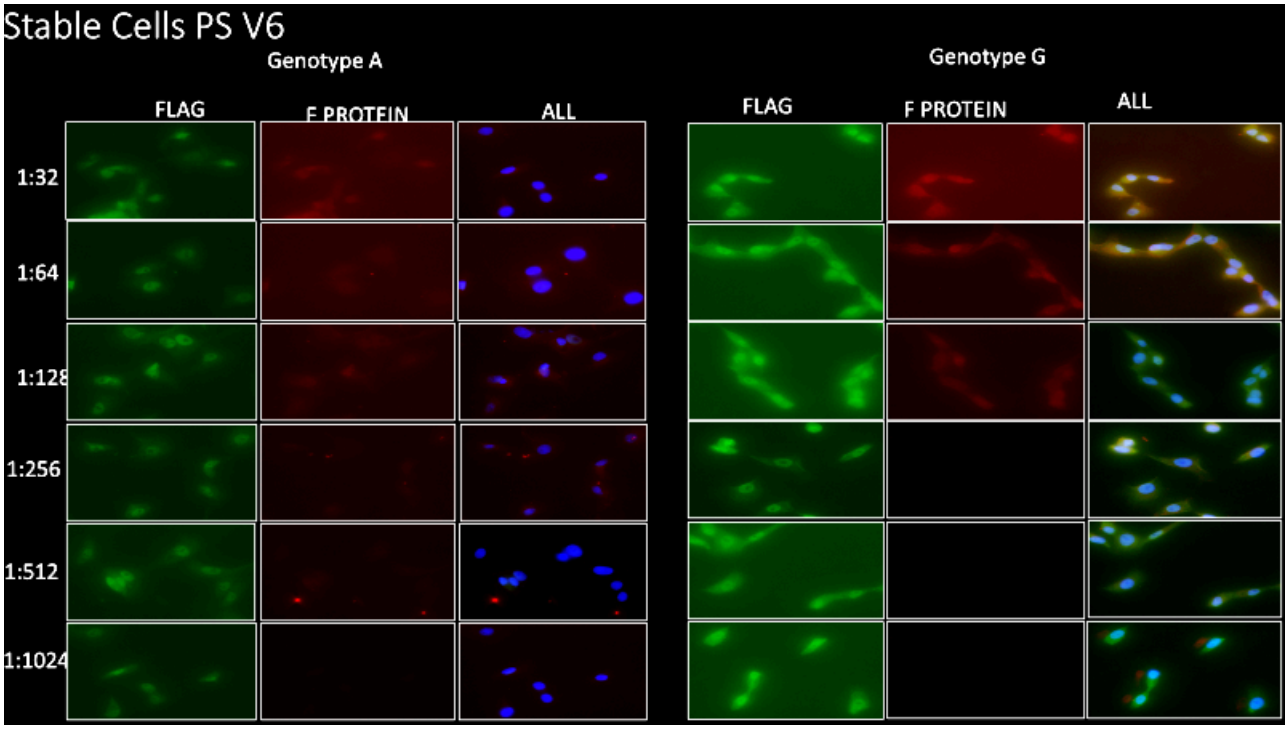


Figure A-50c. Stable cells Vaccine Sample#6.

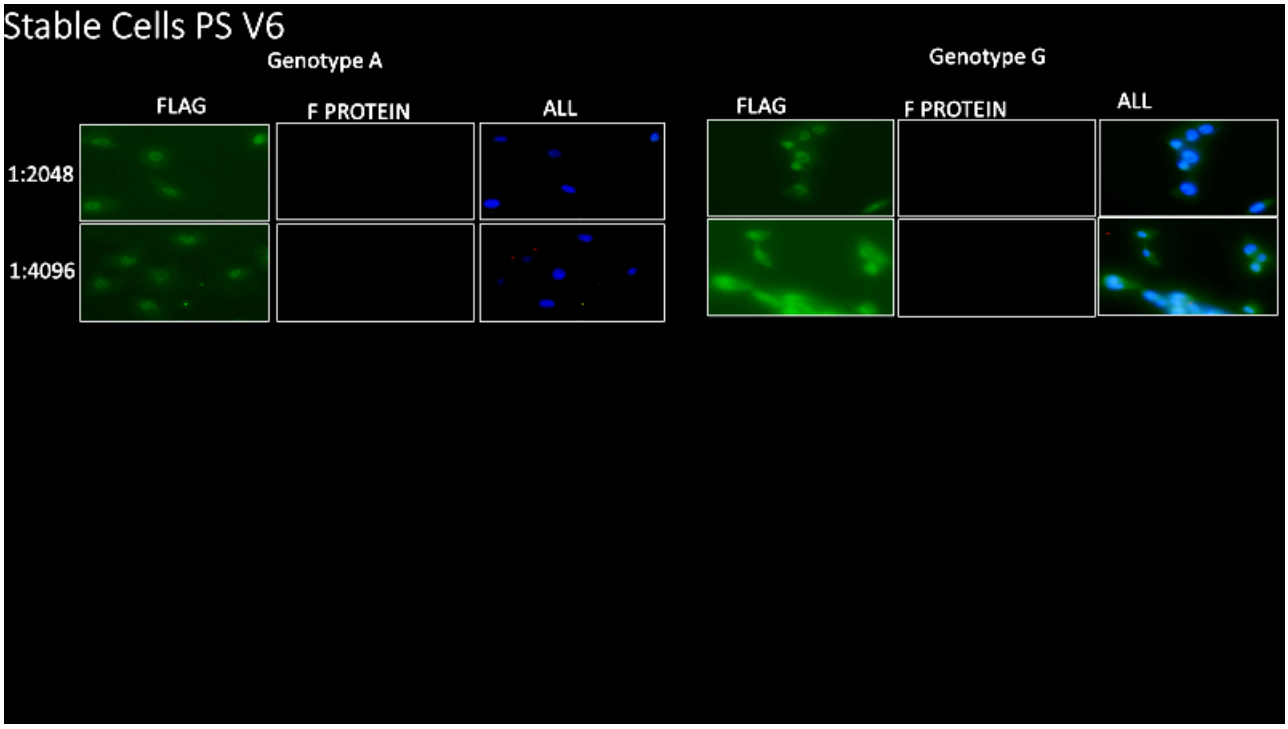


Figure A-50d. Stable cells Vaccine Sample#6.

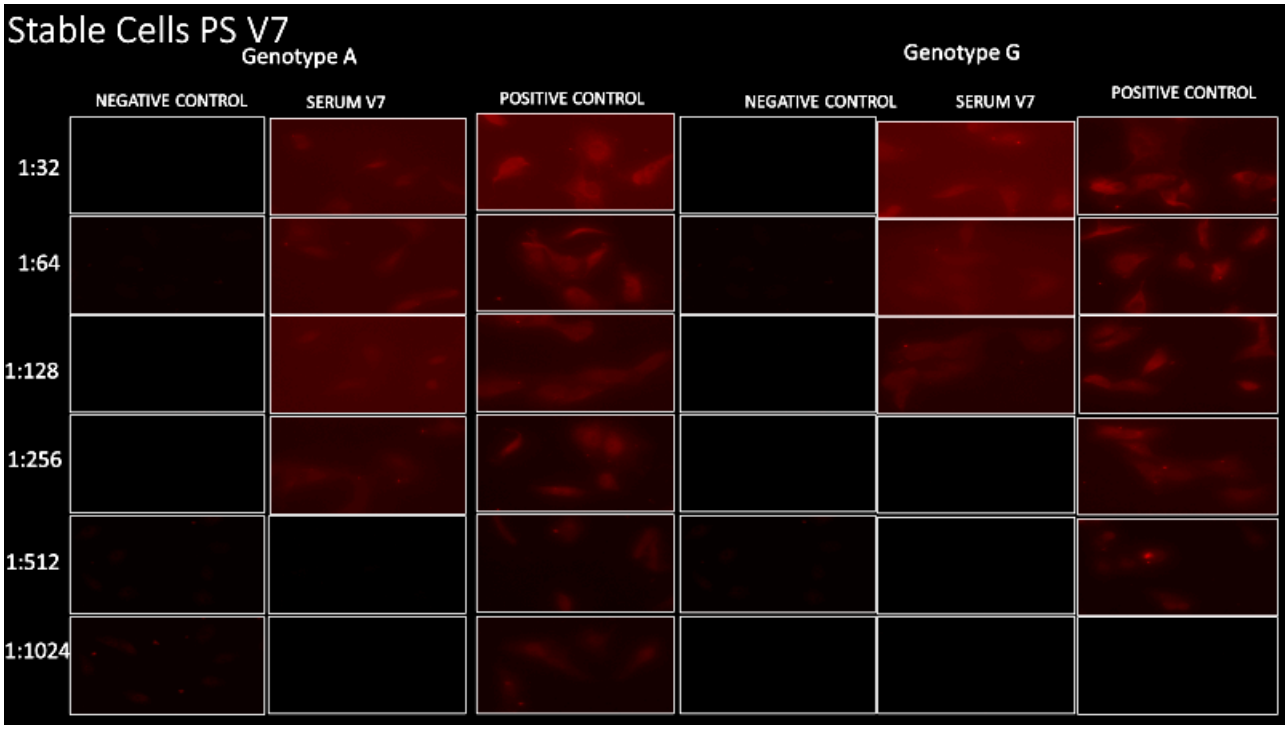


Figure A-51a. Stable cells Vaccine Sample#7.

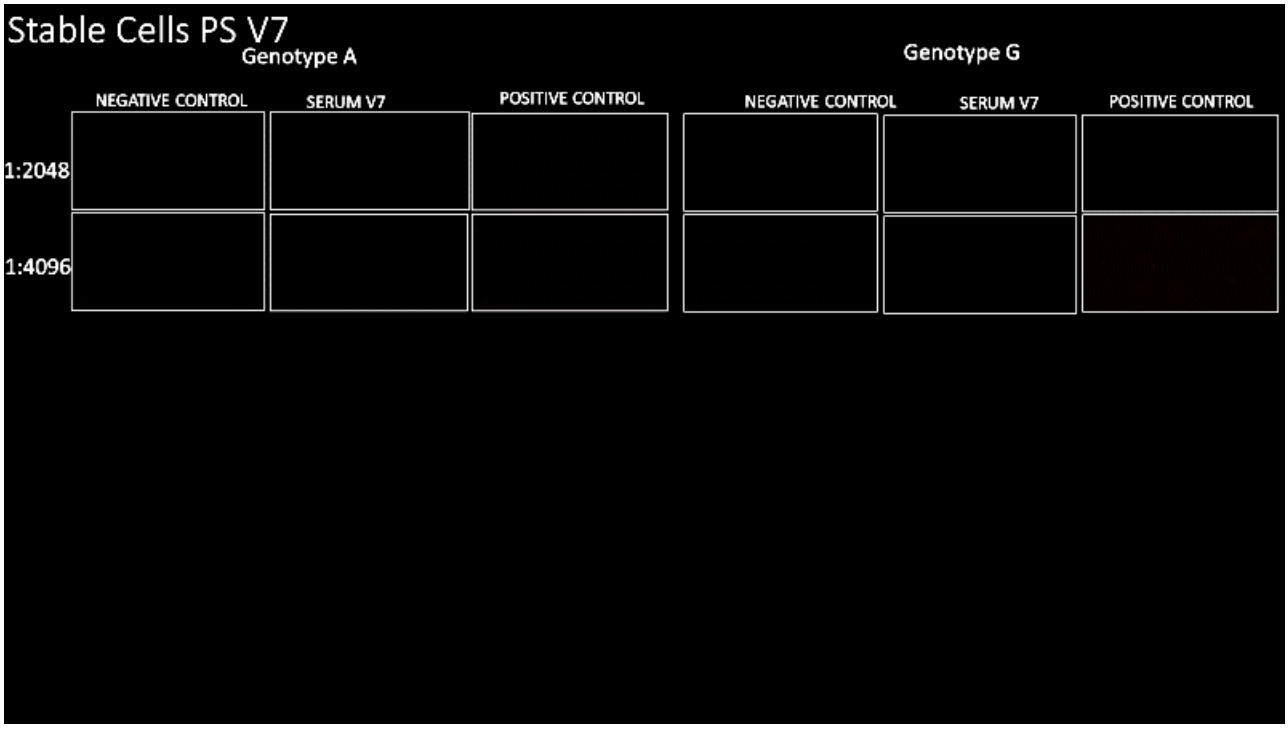


Figure A-51b. Stable cells Vaccine Sample#7.

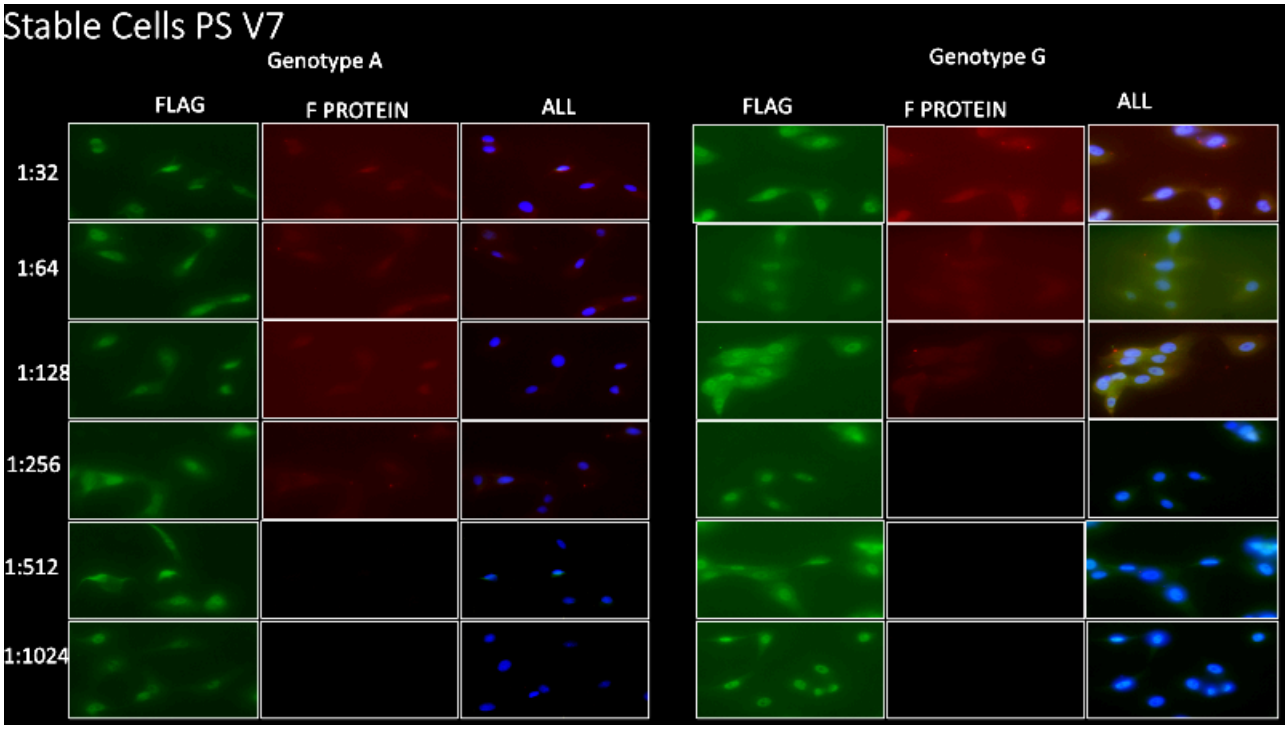


Figure A-51c. Stable cells Vaccine Sample#7.

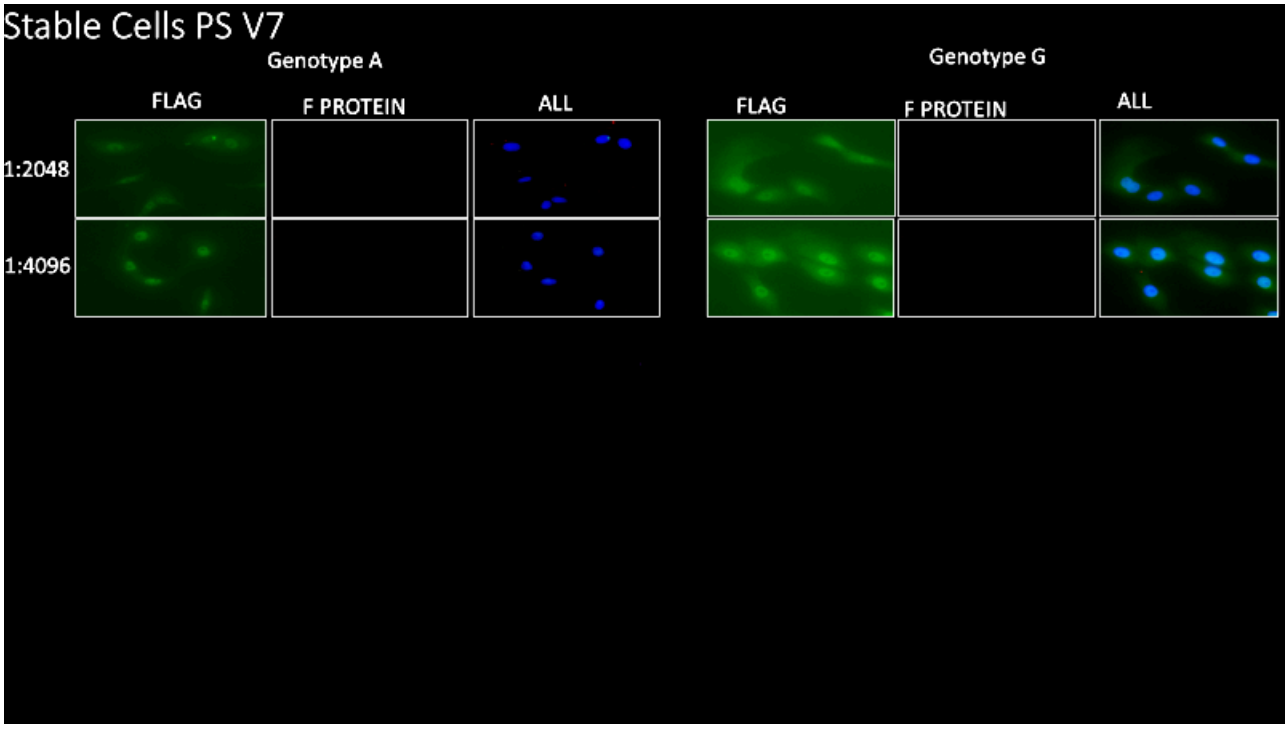


Figure A-51d. Stable cells Vaccine Sample#7.

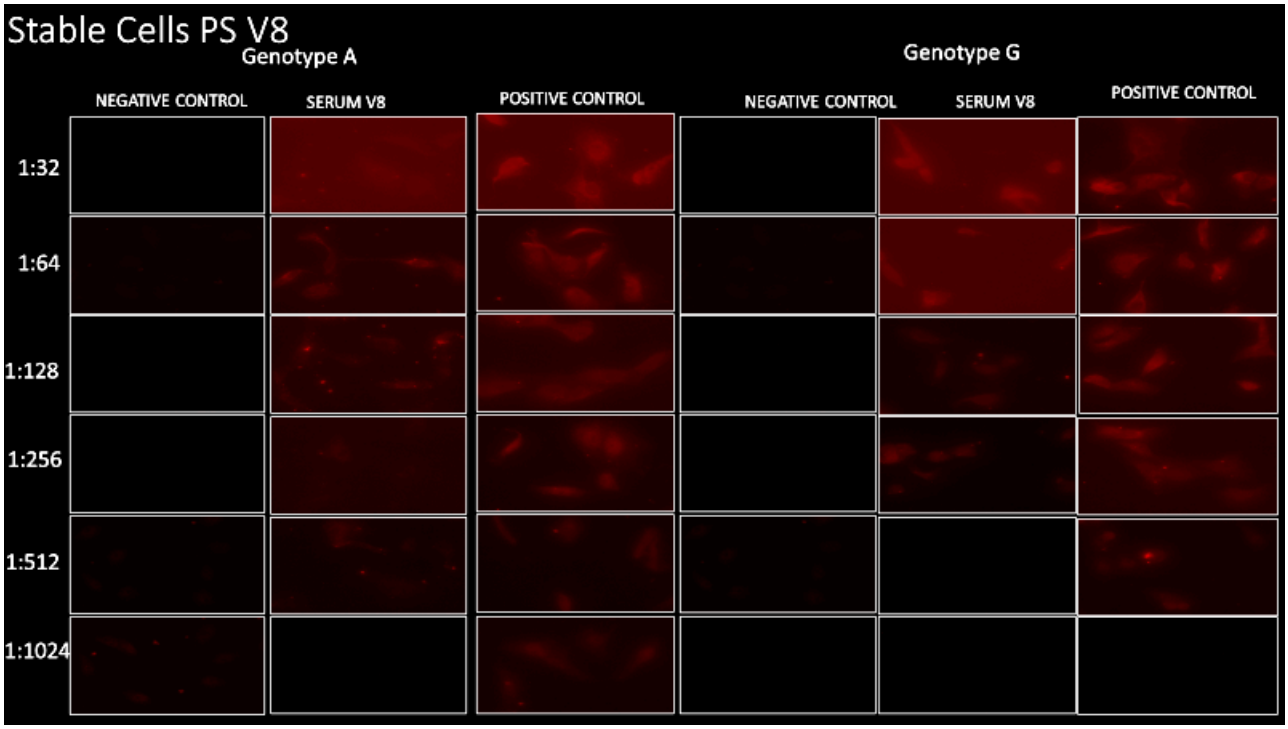


Figure A-52a. Stable cells Vaccine Sample#8.

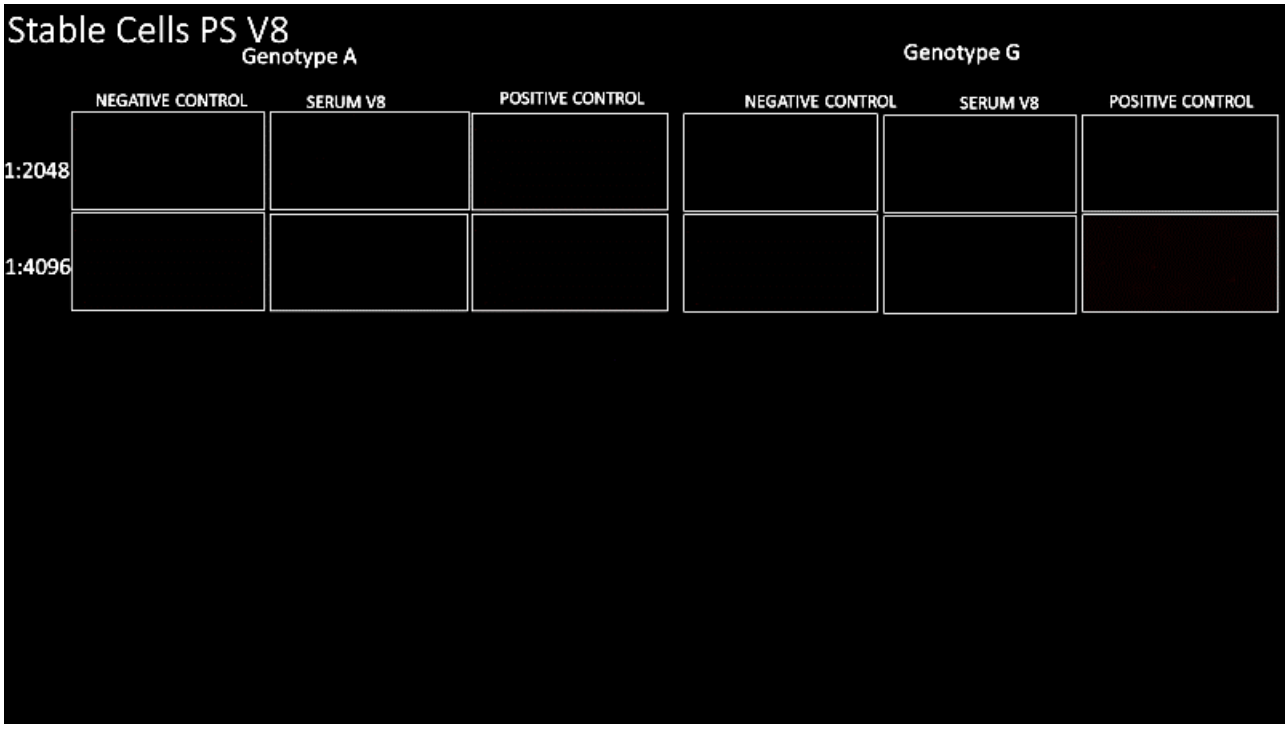


Figure A-52b. Stable cells Vaccine Sample#8.

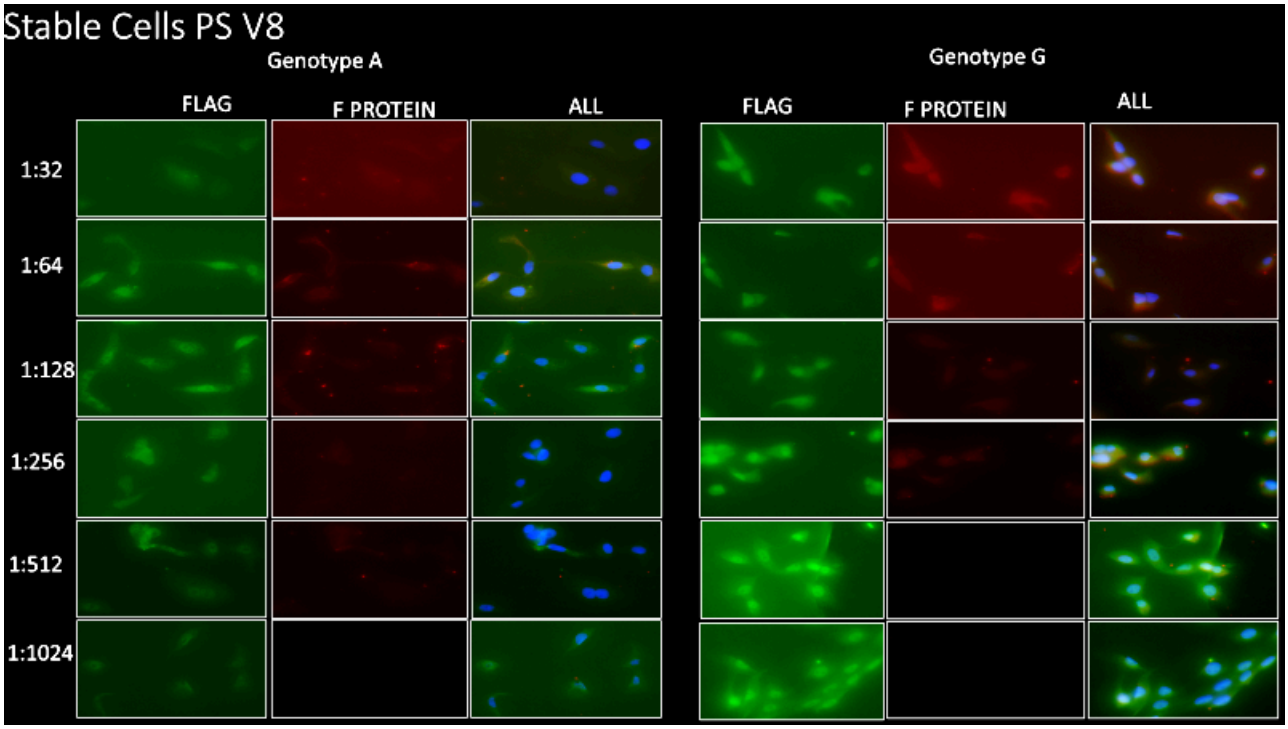


Figure A-52c. Stable cells Vaccine Sample#8.

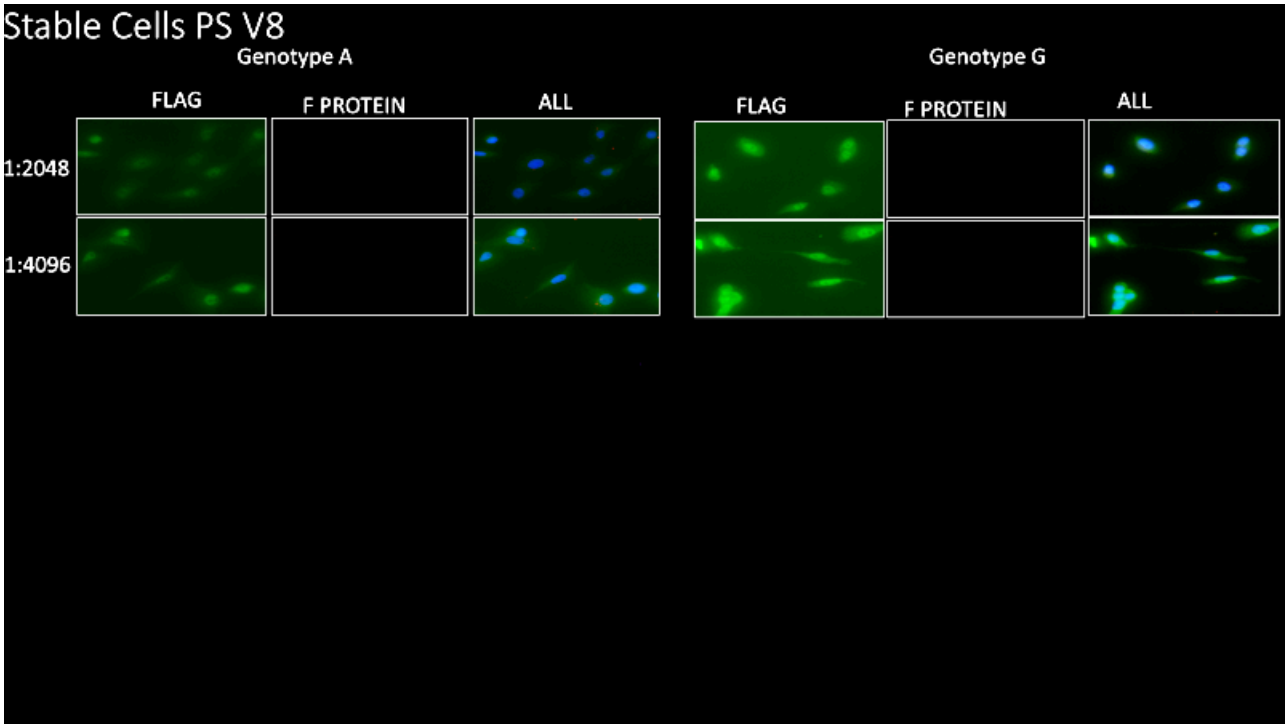


Figure A-52d. Stable cells Vaccine Sample#8.

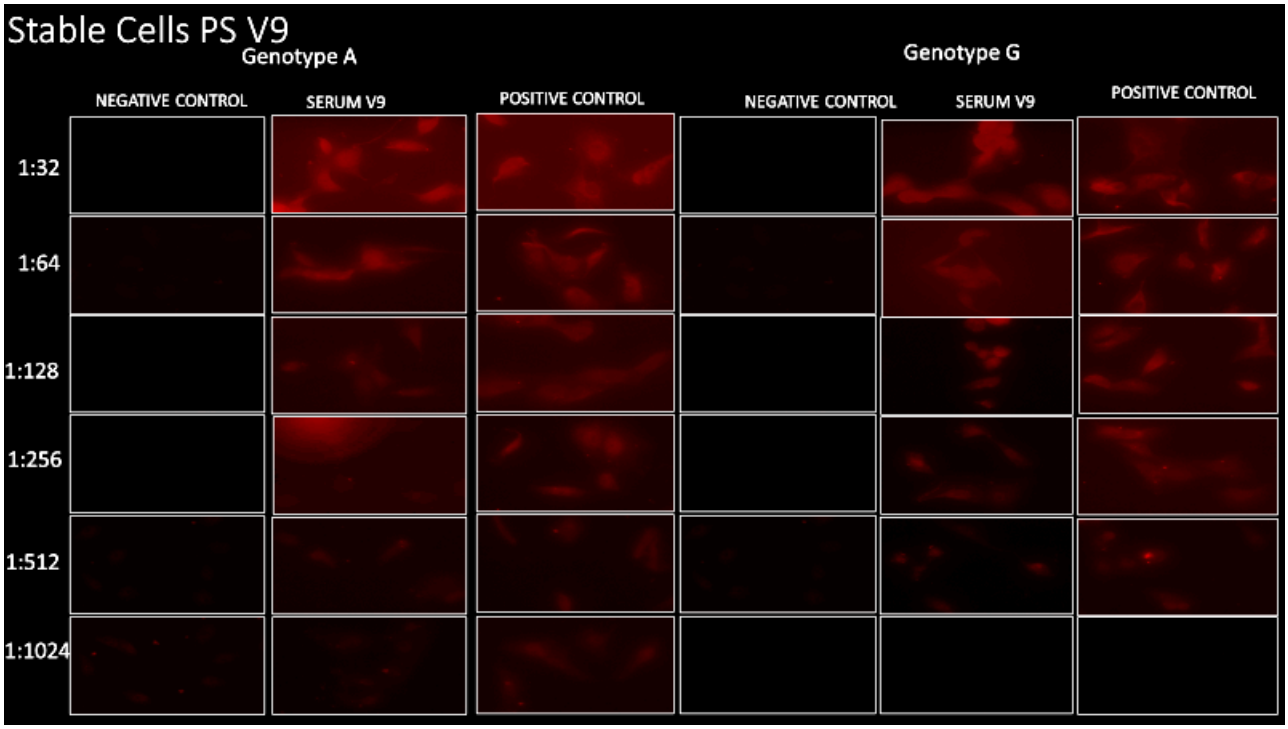


Figure A-53a. Stable cells Vaccine Sample#9.

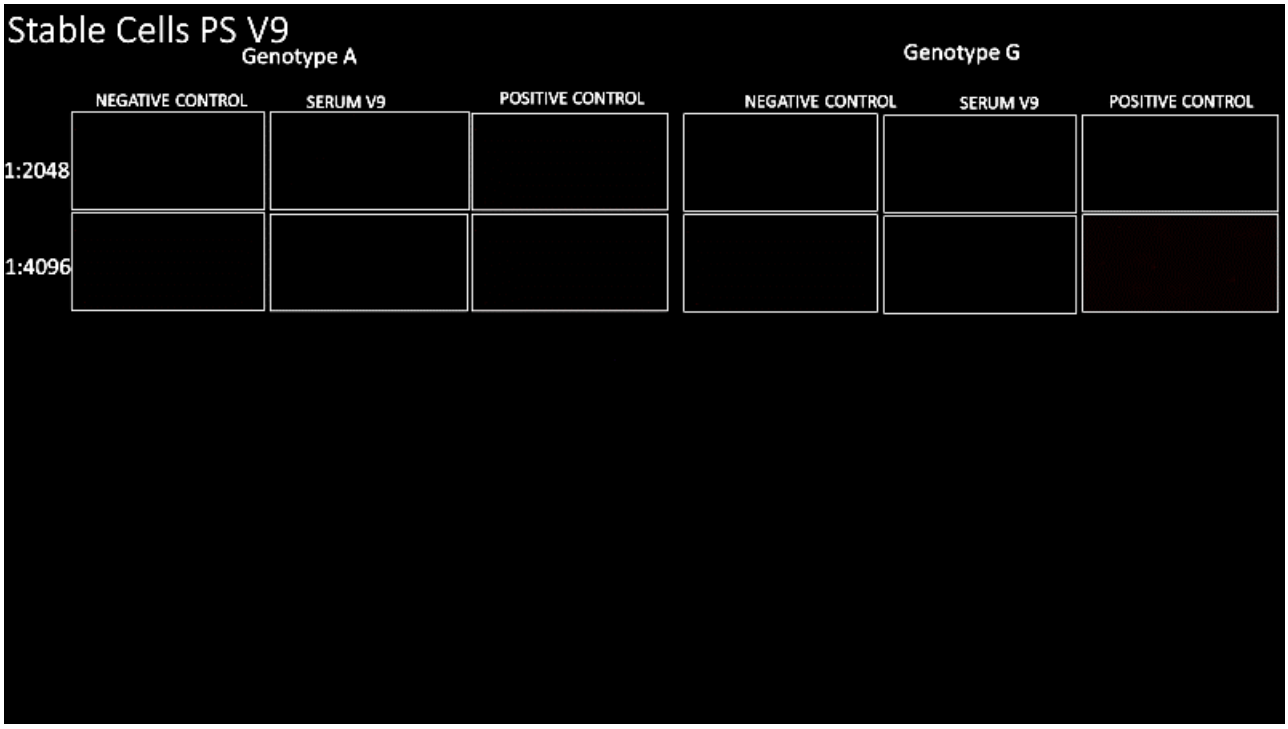


Figure A-53b. Stable cells Vaccine Sample#9.

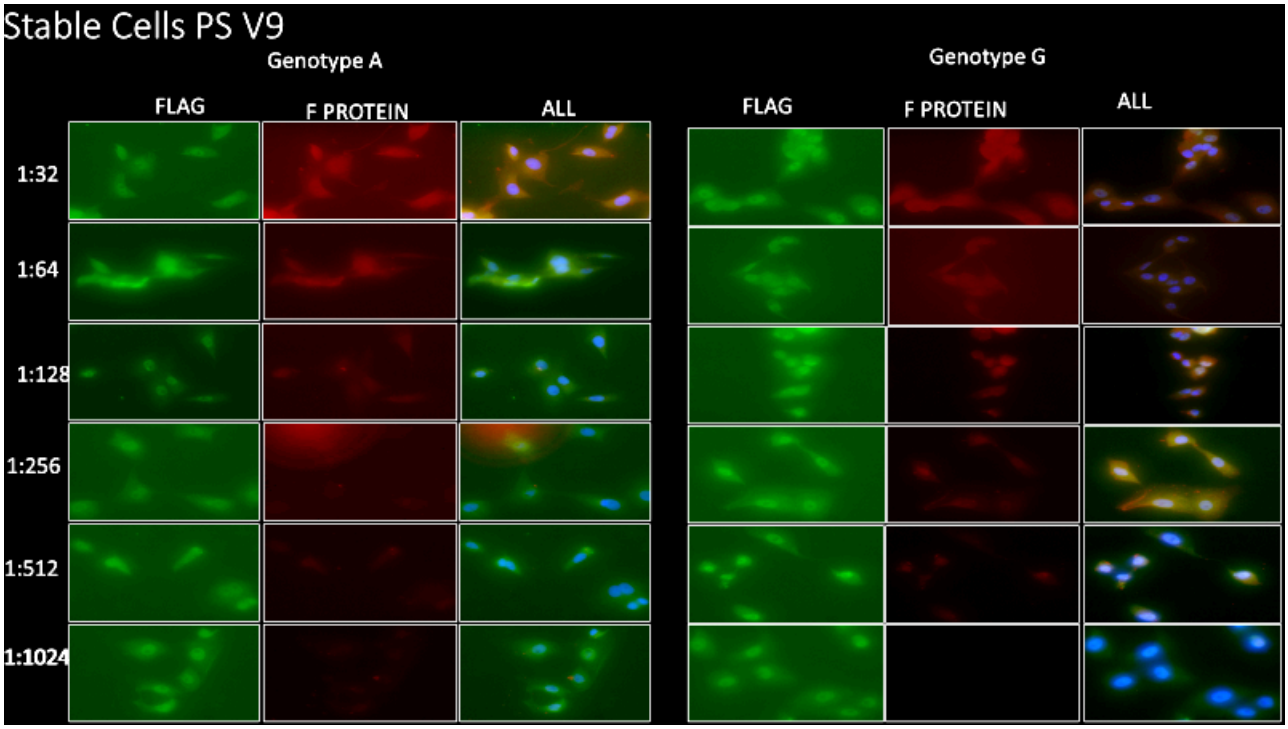


Figure A-53c. Stable cells Vaccine Sample#9.

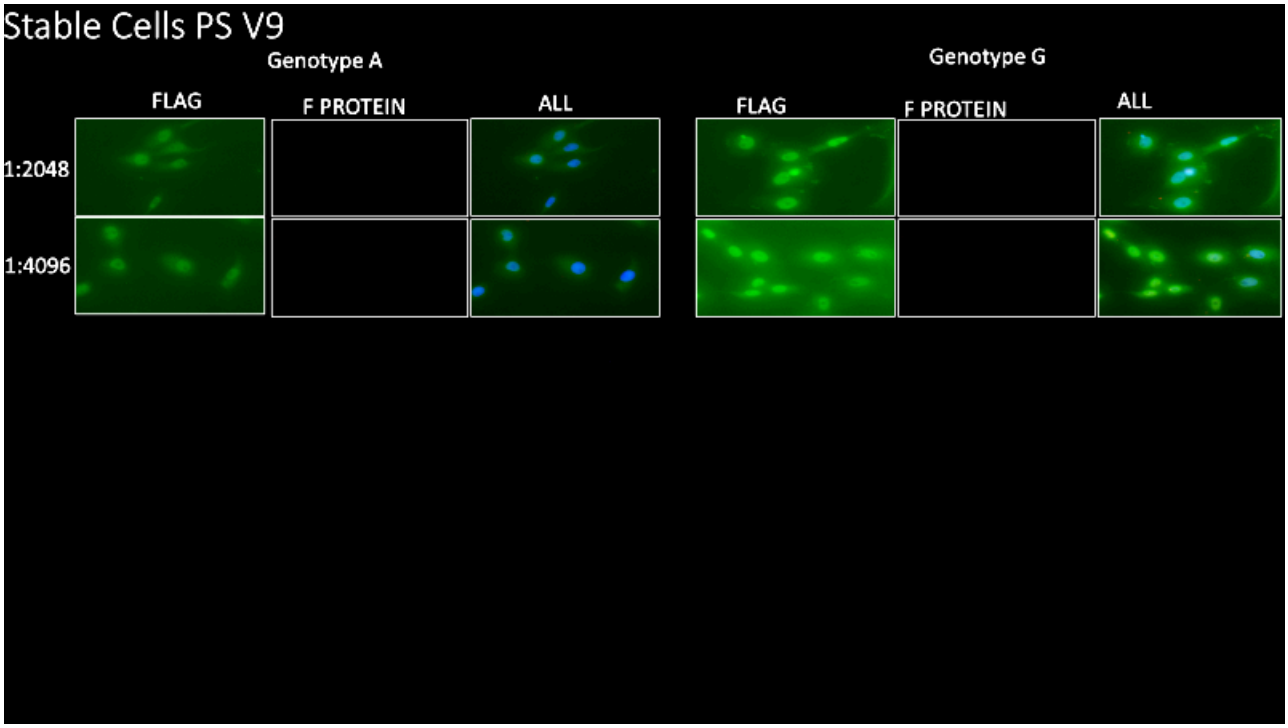


Figure A-53d. Stable cells Vaccine Sample#9.

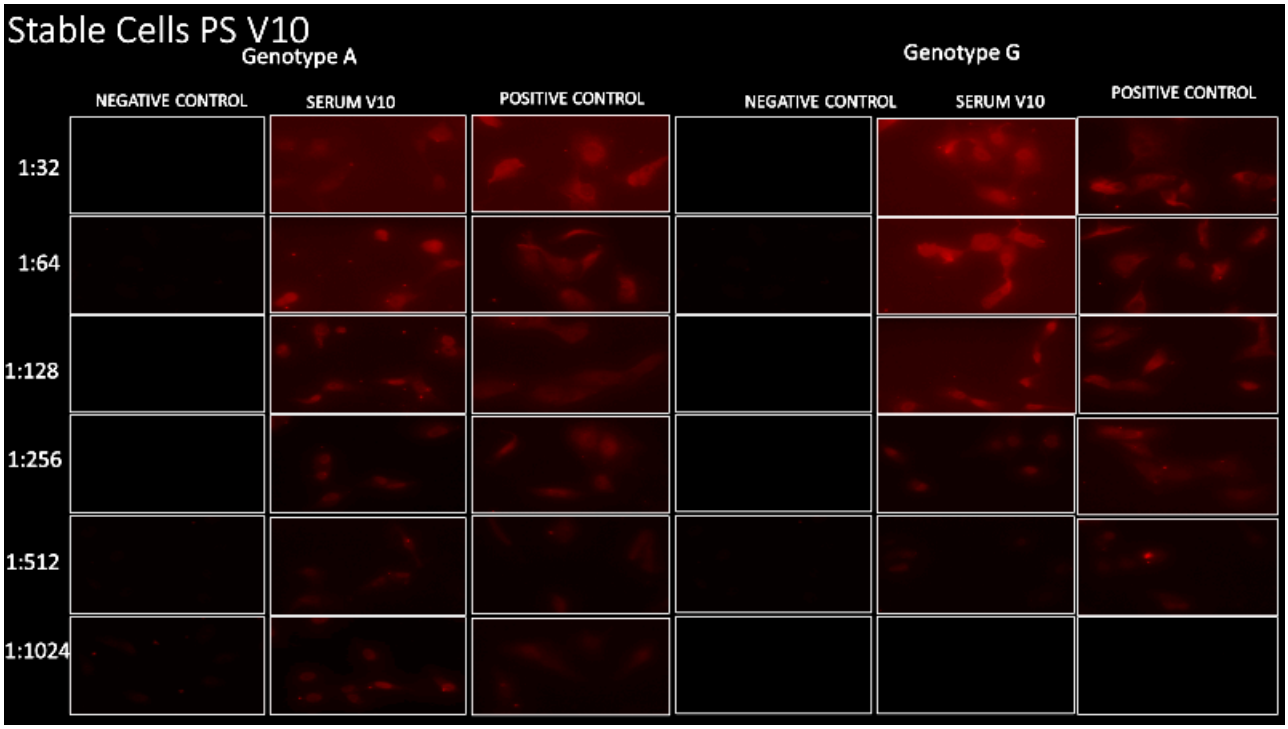


Figure A-54a. Stable cells Vaccine Sample#10.

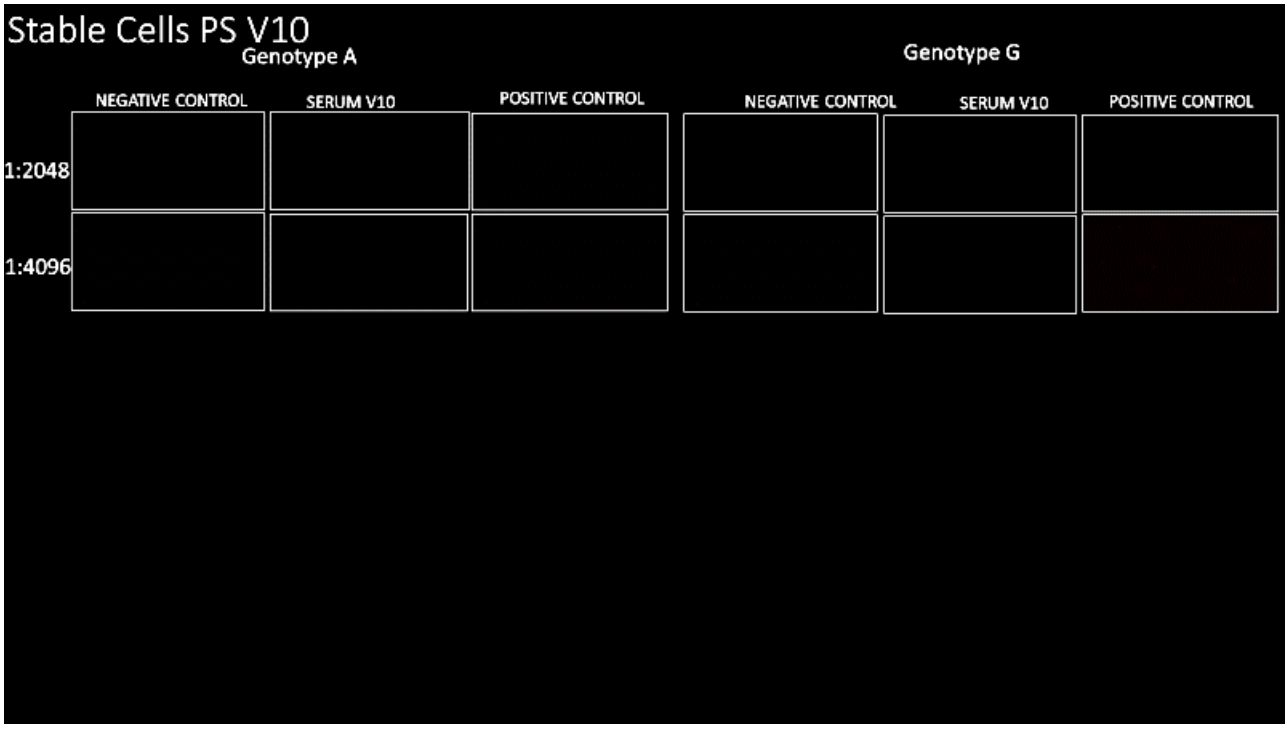


Figure A-54b. Stable cells Vaccine Sample#10.

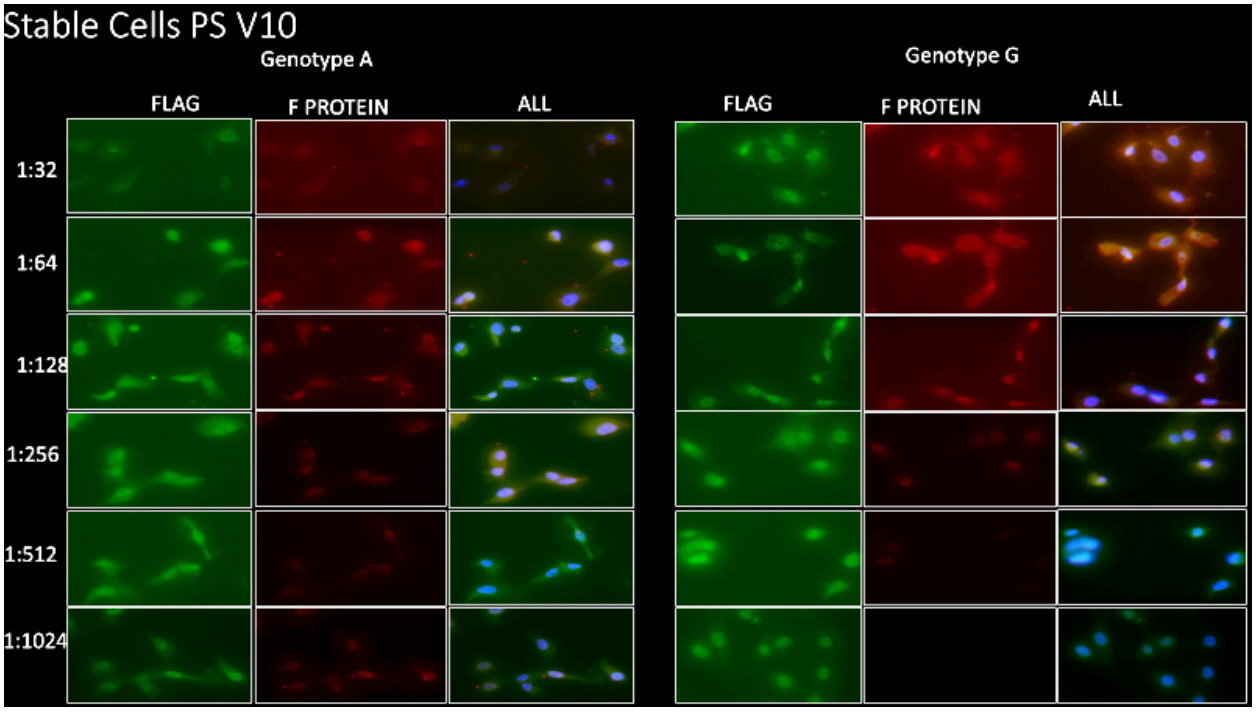


Figure A-54c. Stable cells Vaccine Sample#10.

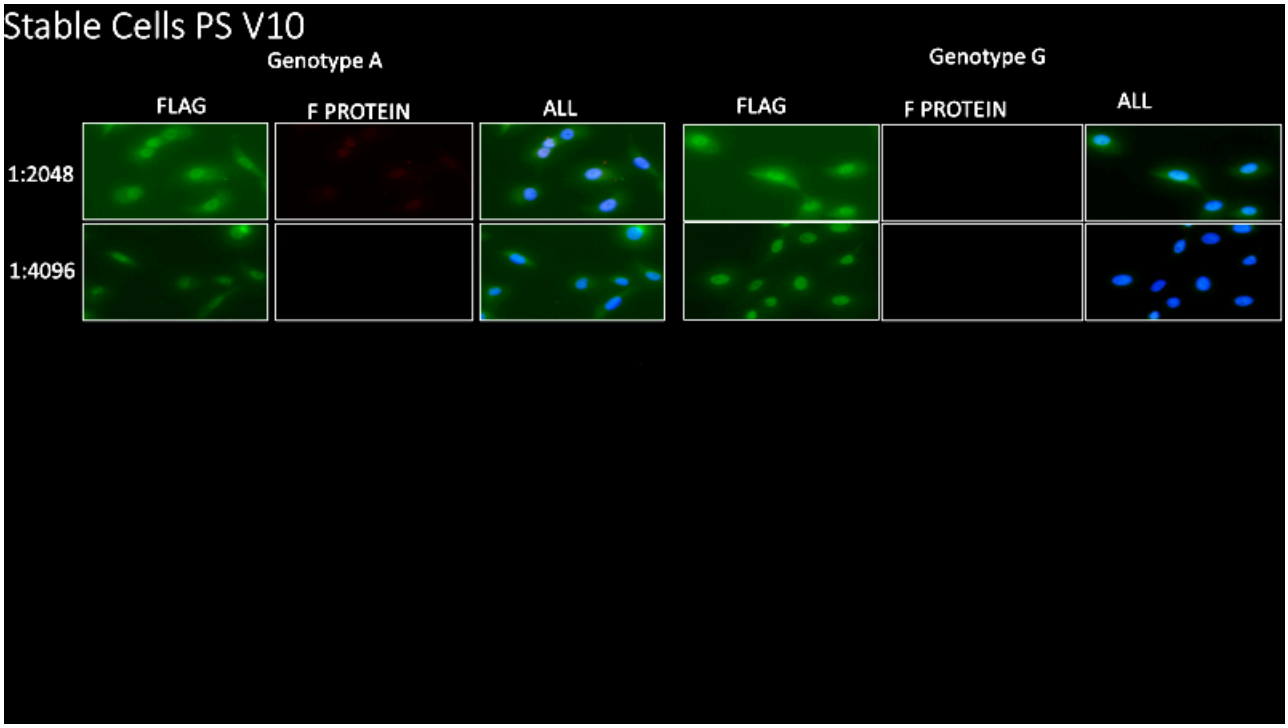


Figure A-54d. Stable cells Vaccine Sample#10.

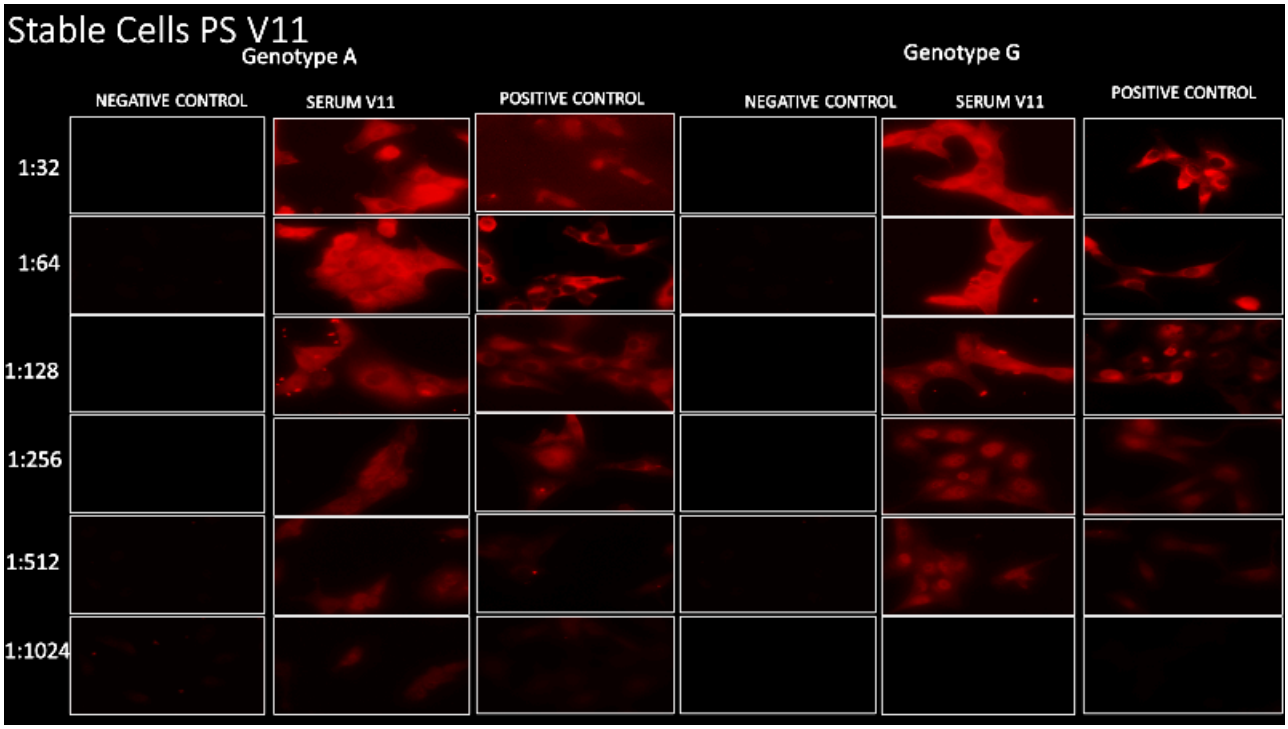


Figure A-55a. Stable cells Vaccine Sample#11.

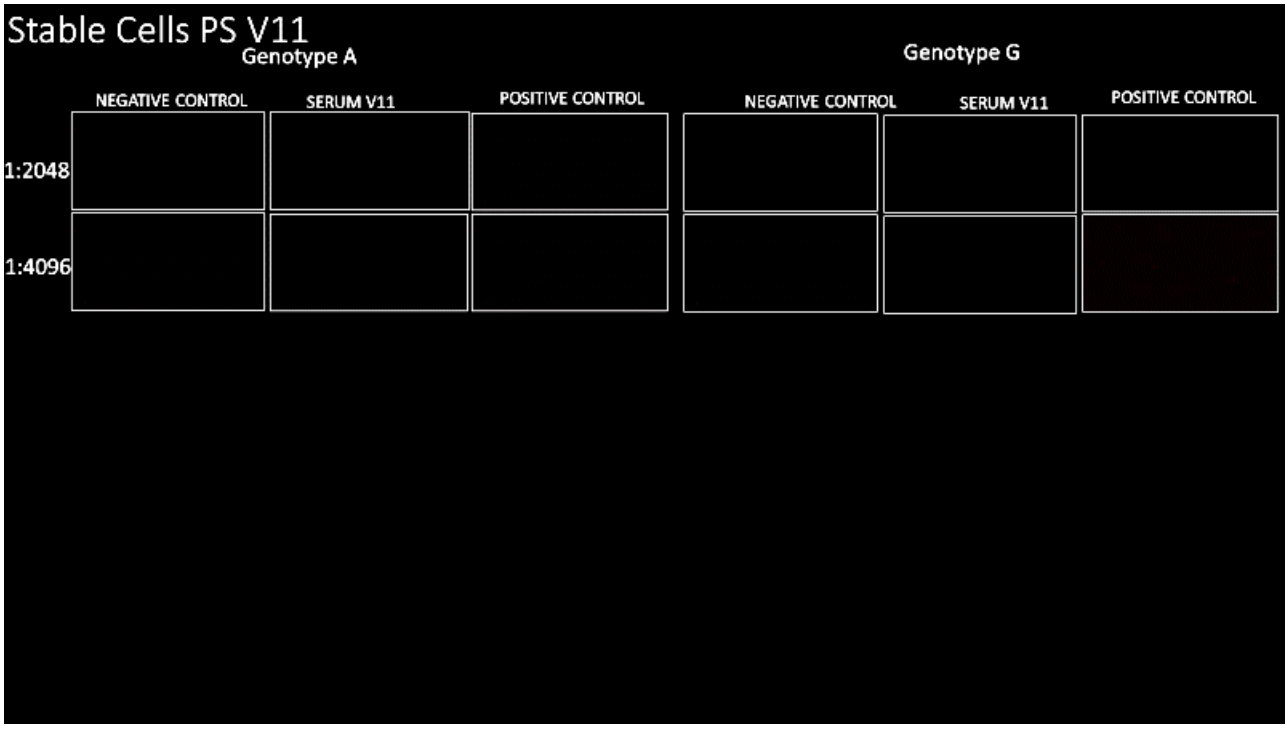


Figure A-55b. Stable cells Vaccine Sample#11.

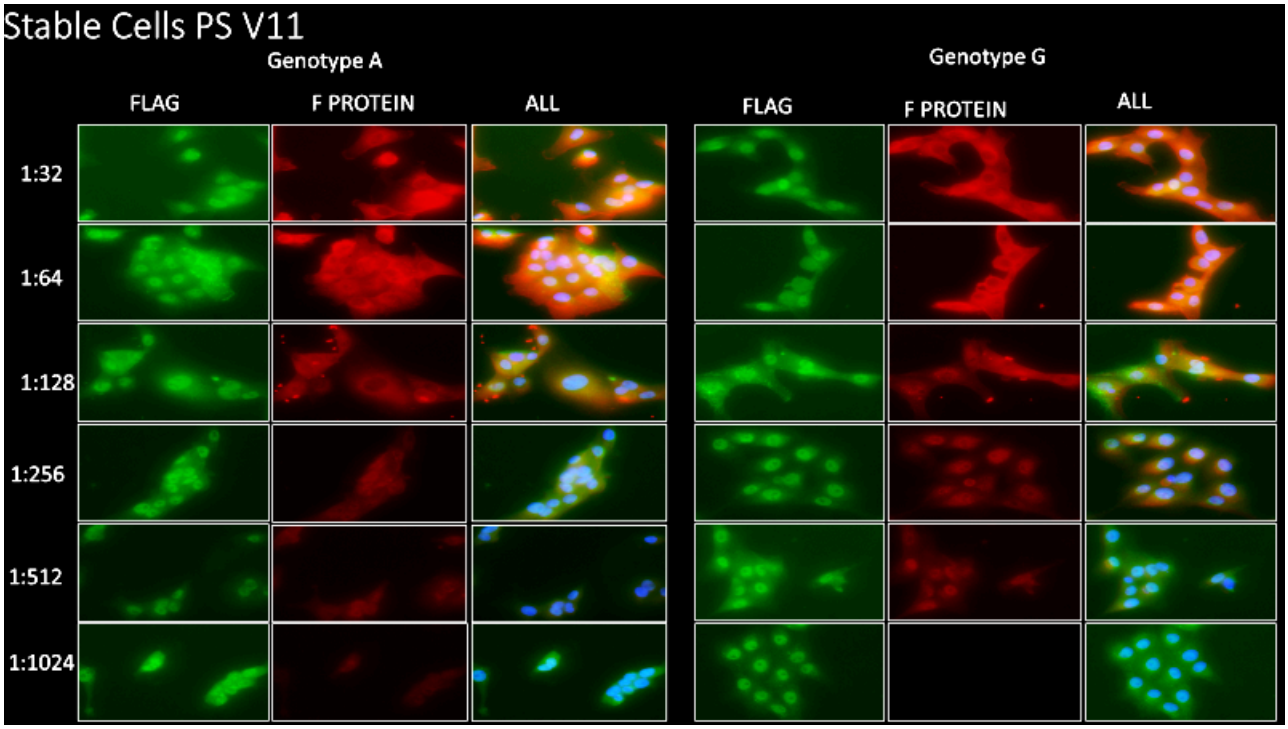


Figure A-55c. Stable cells Vaccine Sample#11.

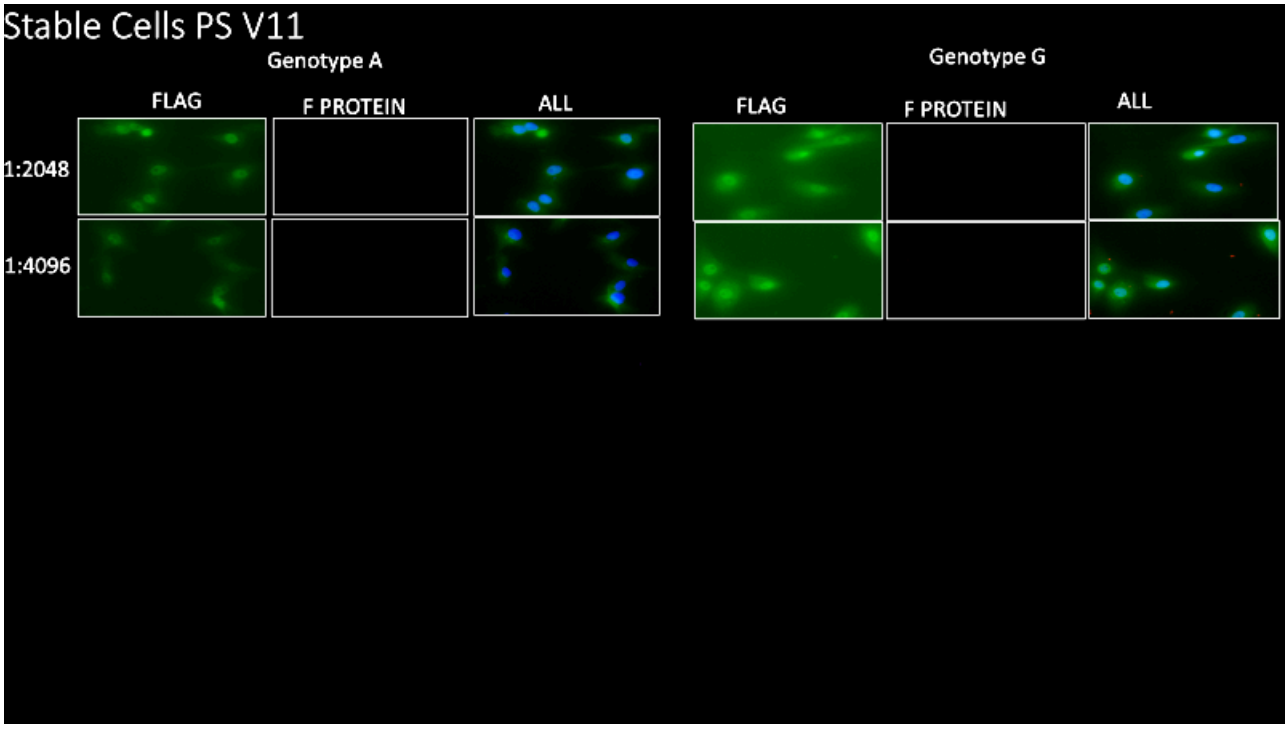


Figure A-55d. Stable cells Vaccine Sample#11.

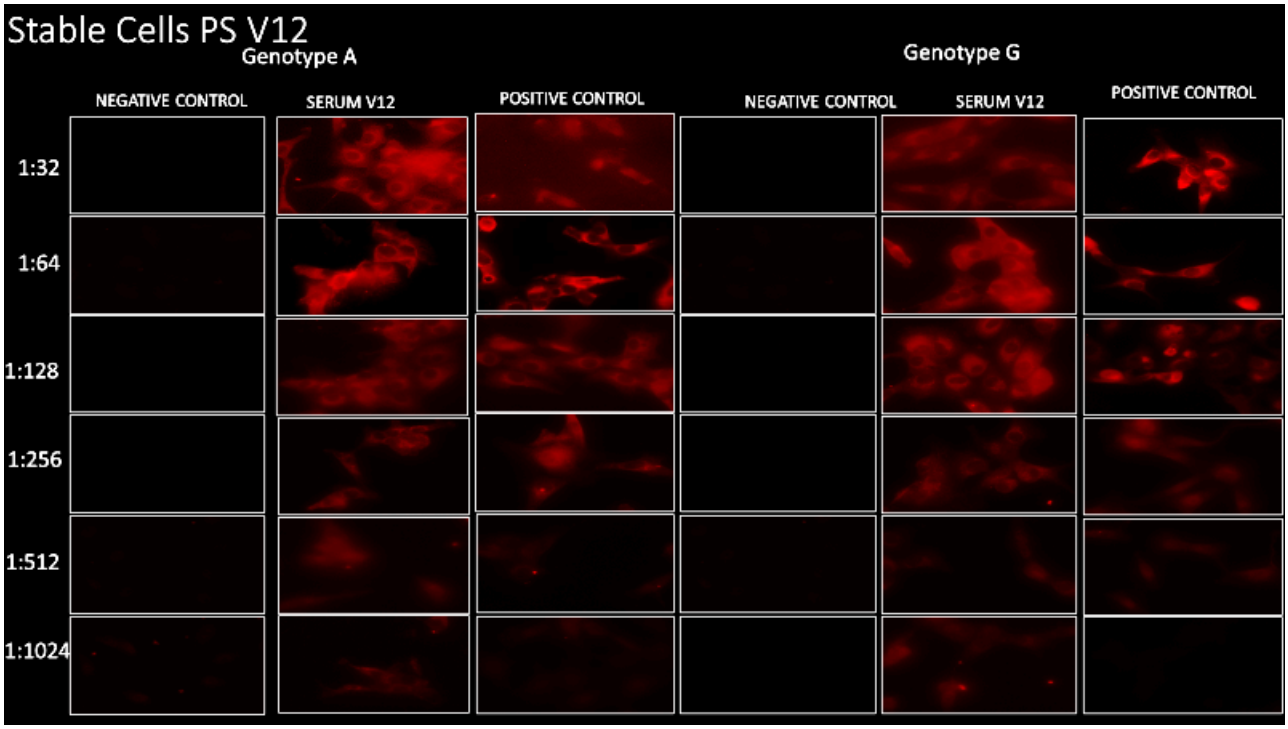


Figure A-56a. Stable cells Vaccine Sample#12.

Figure A-56b. Stable cells Vaccine Sample#12.

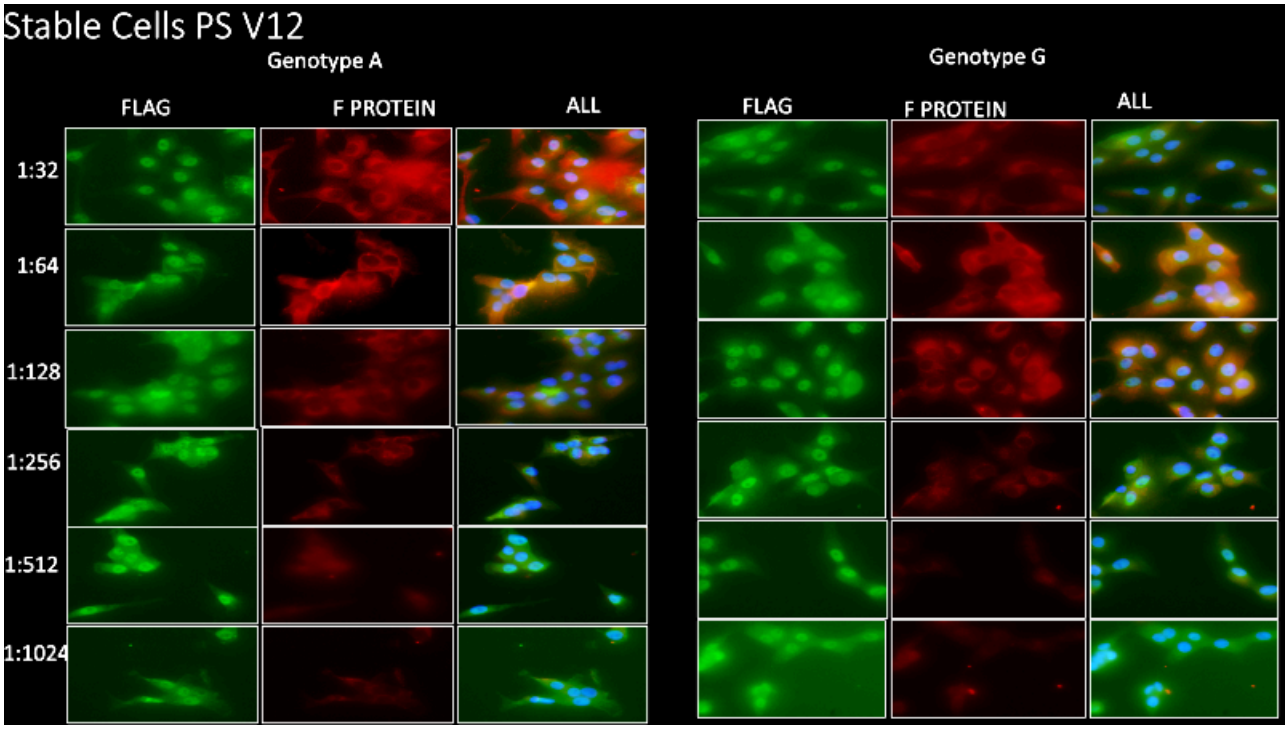


Figure A-56c. Stable cells Vaccine Sample#12.

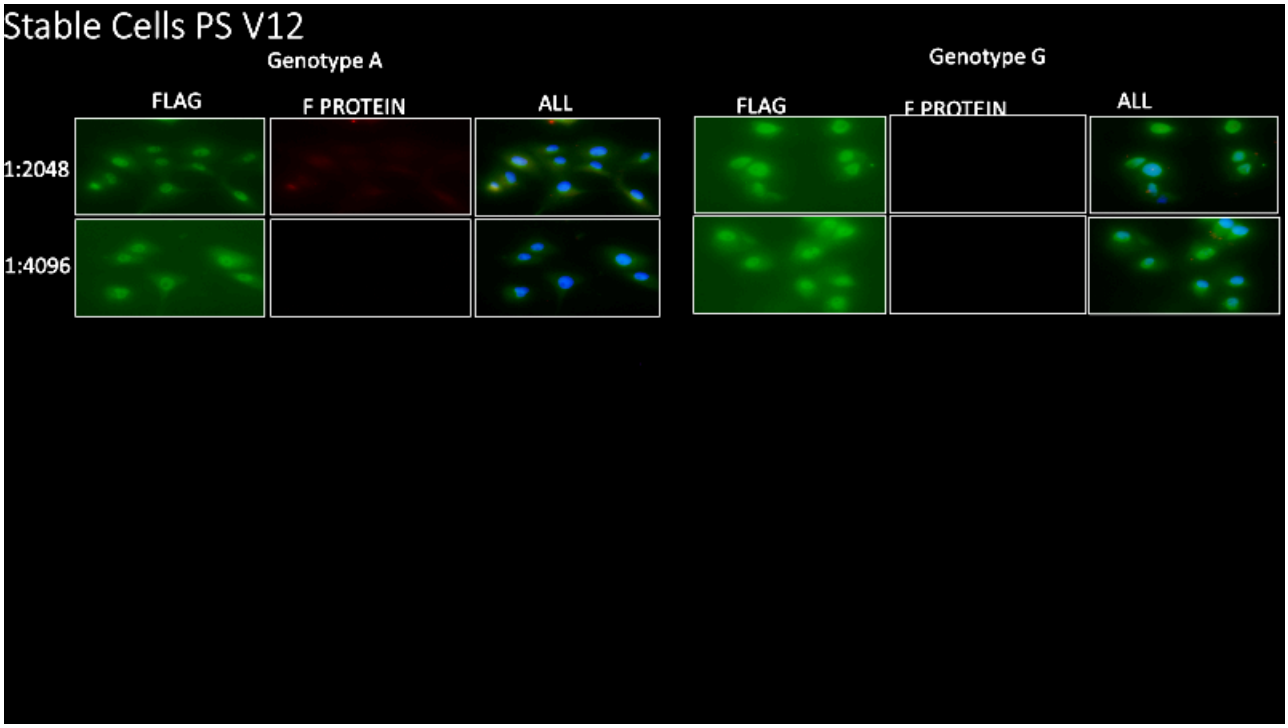


Figure A-56d. Stable cells Vaccine Sample#12.

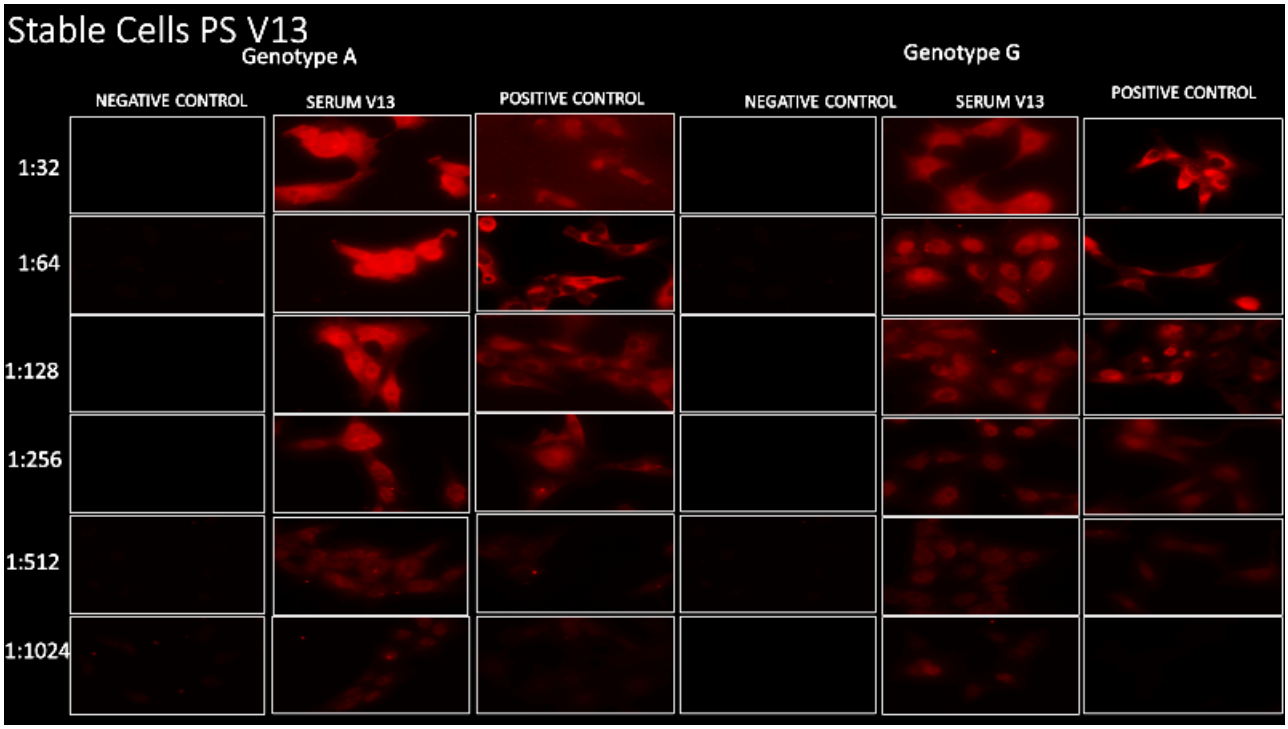


Figure A-57a. Stable cells Vaccine Sample#13.

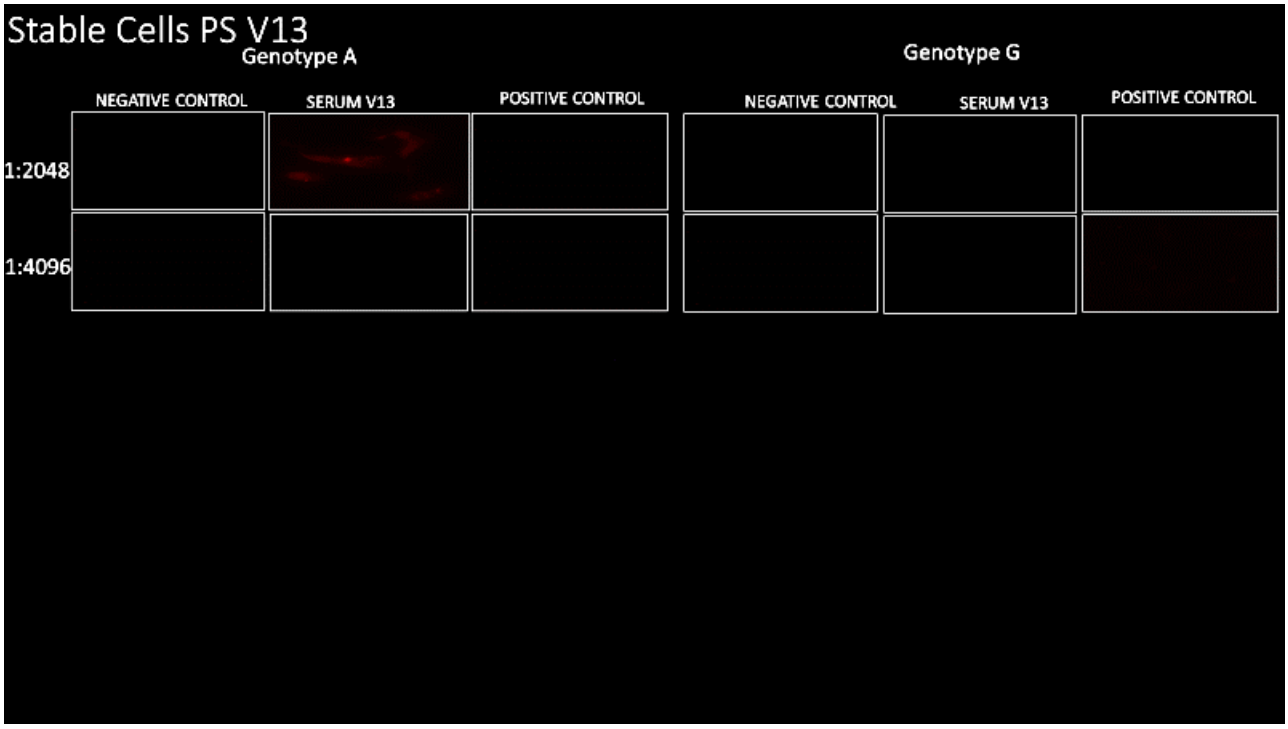


Figure A-57b. Stable cells Vaccine Sample#13.

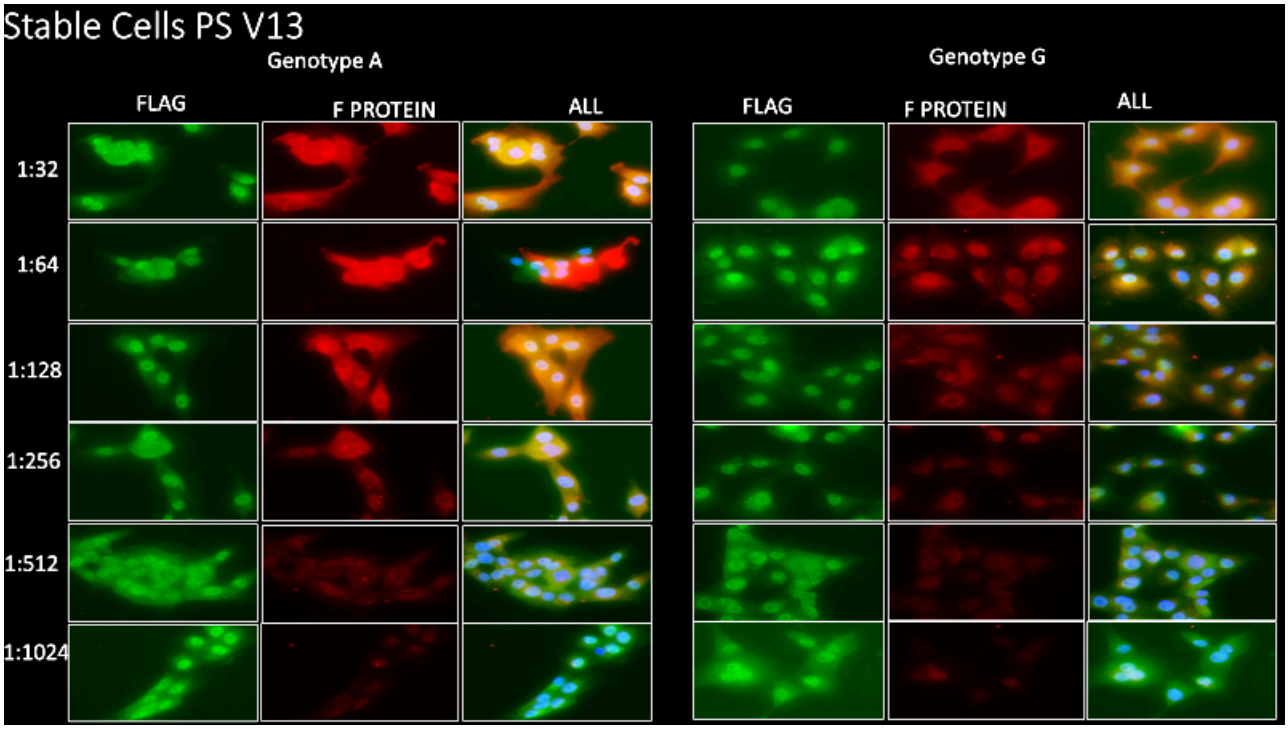


Figure A-57c. Stable cells Vaccine Sample#13.

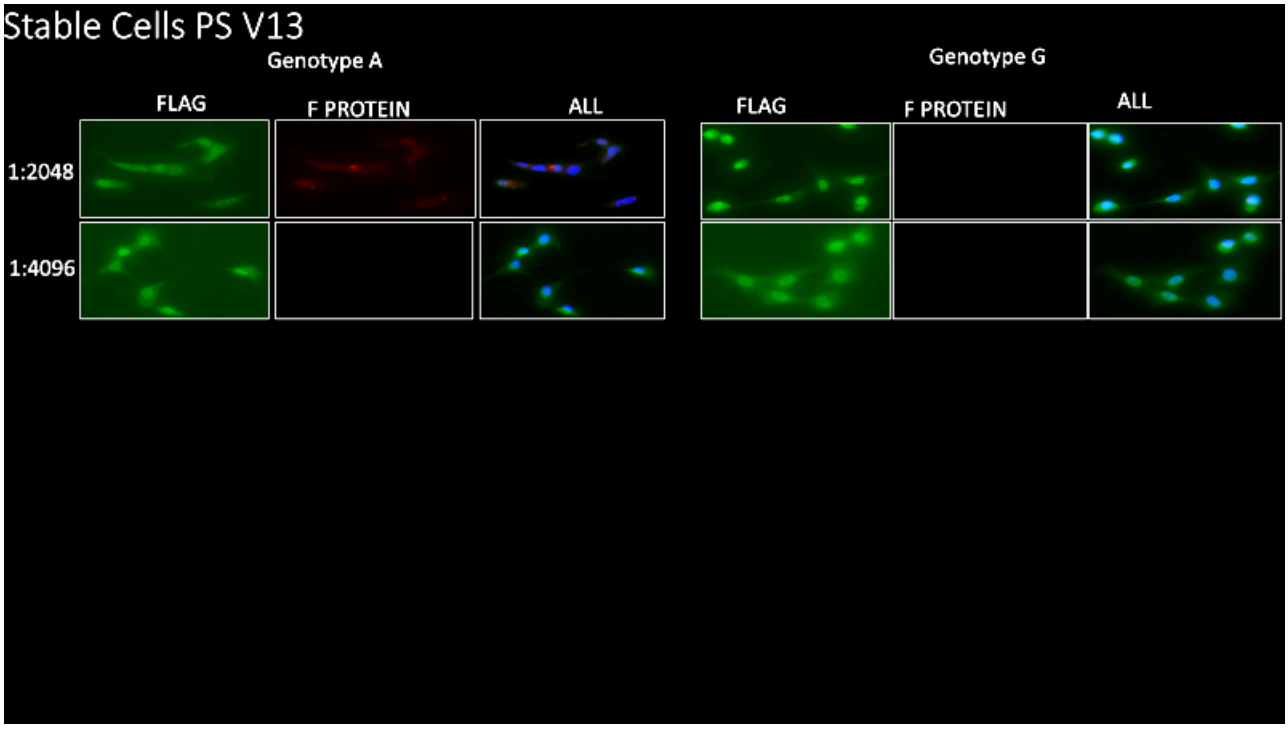


Figure A-57d. Stable cells Vaccine Sample#13.

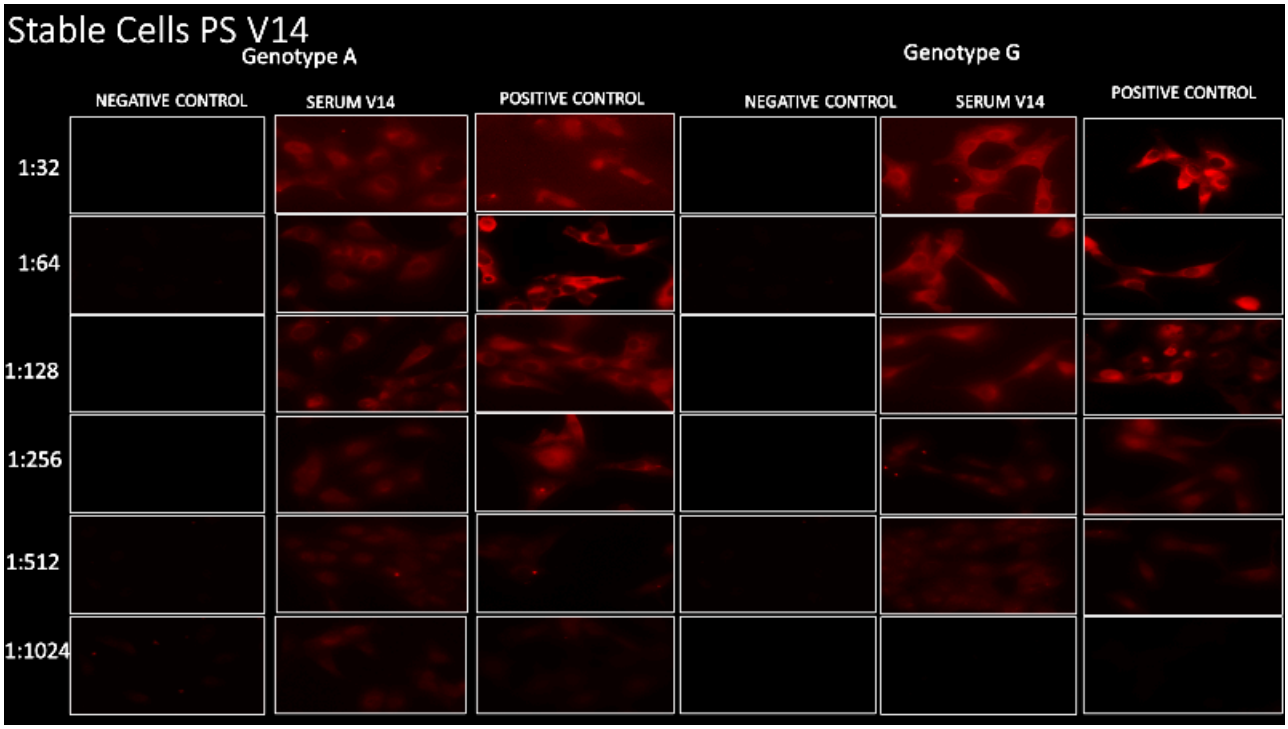


Figure A-58a. Stable cells Vaccine Sample#14.

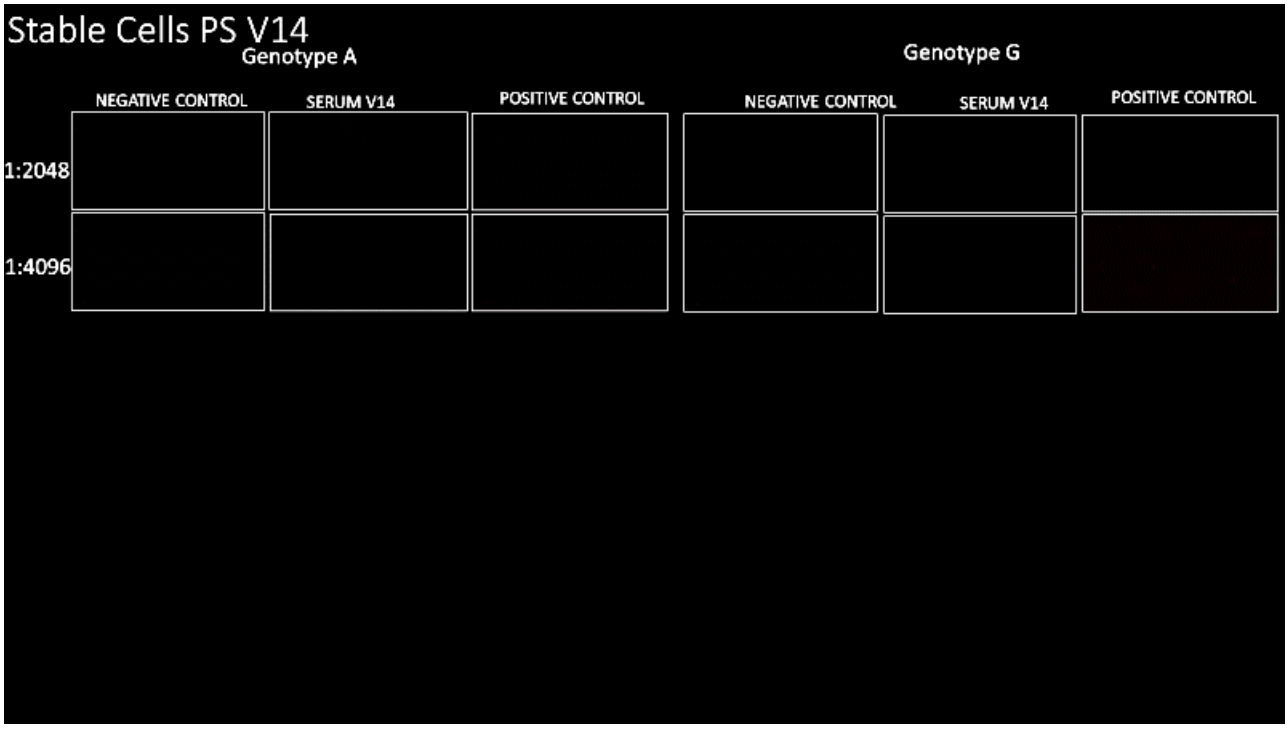


Figure A-58b. Stable cells Vaccine Sample#14.

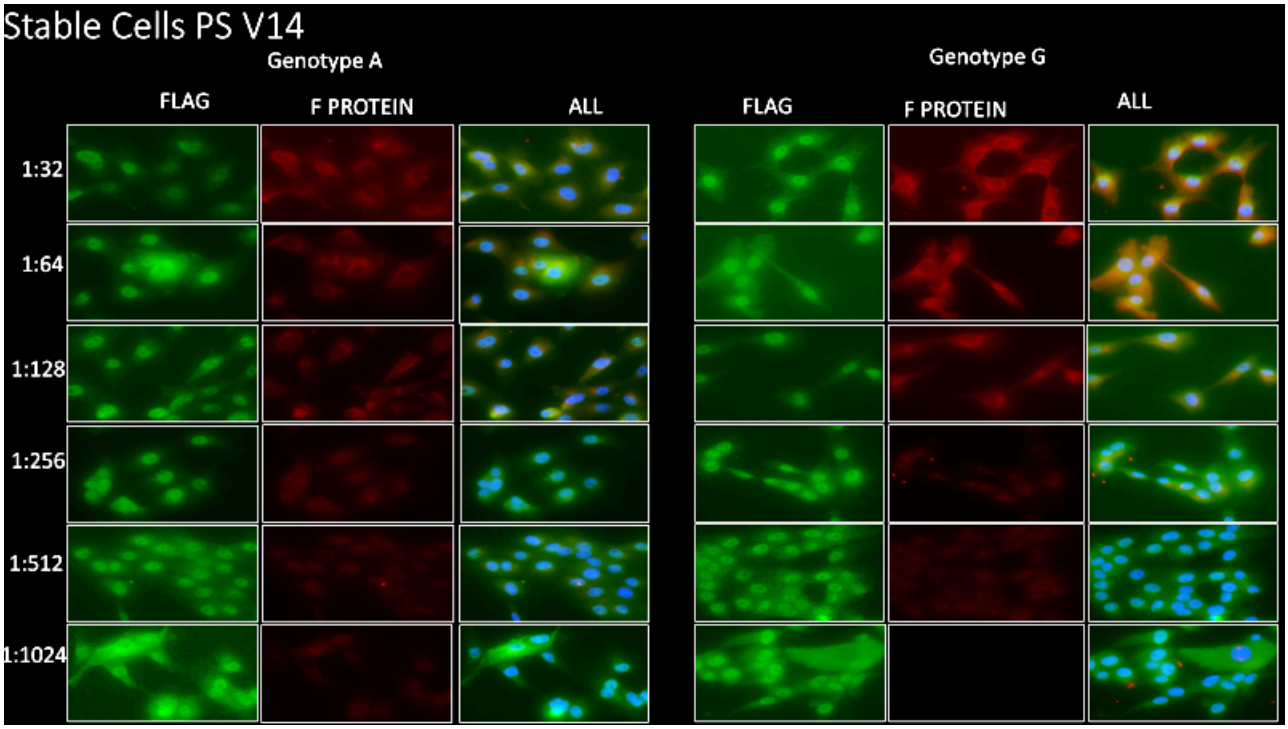


Figure A-58c. Stable cells Vaccine Sample#14.

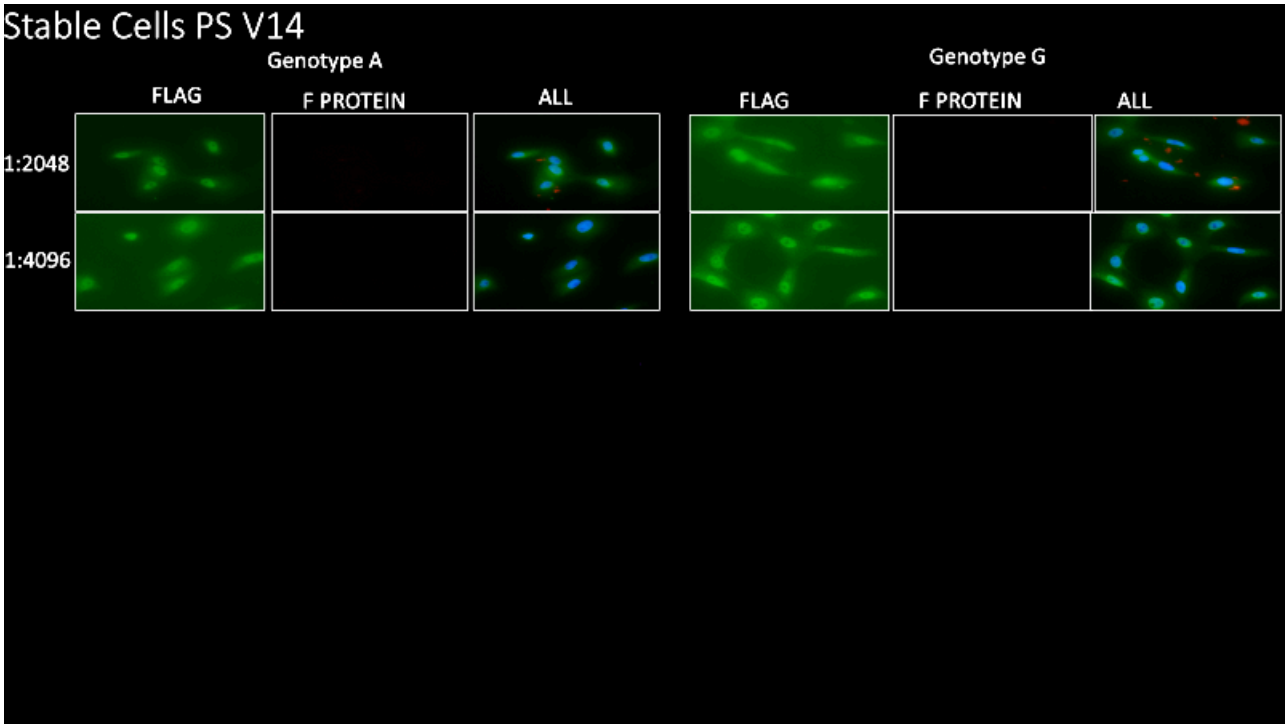


Figure A-58d. Stable cells Vaccine Sample#14.

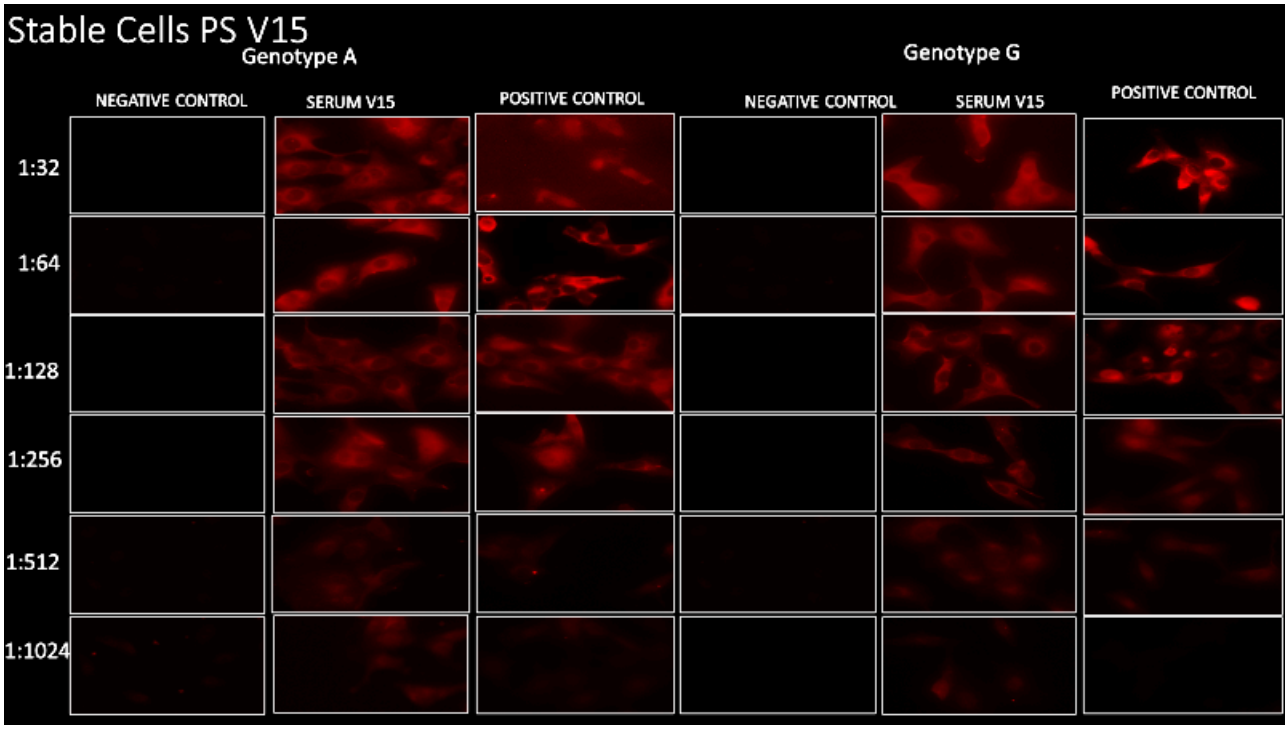


Figure A-59a. Stable cells Vaccine Sample#15.

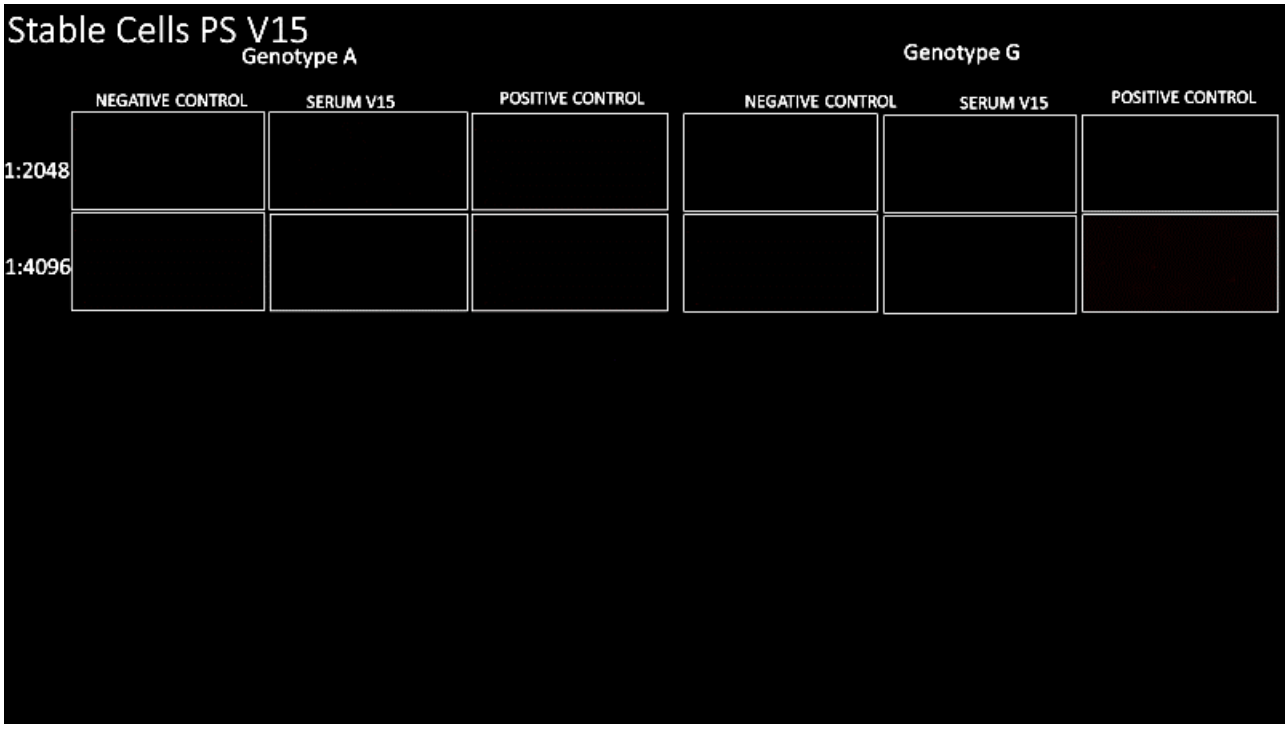


Figure A-59b. Stable cells Vaccine Sample#15.

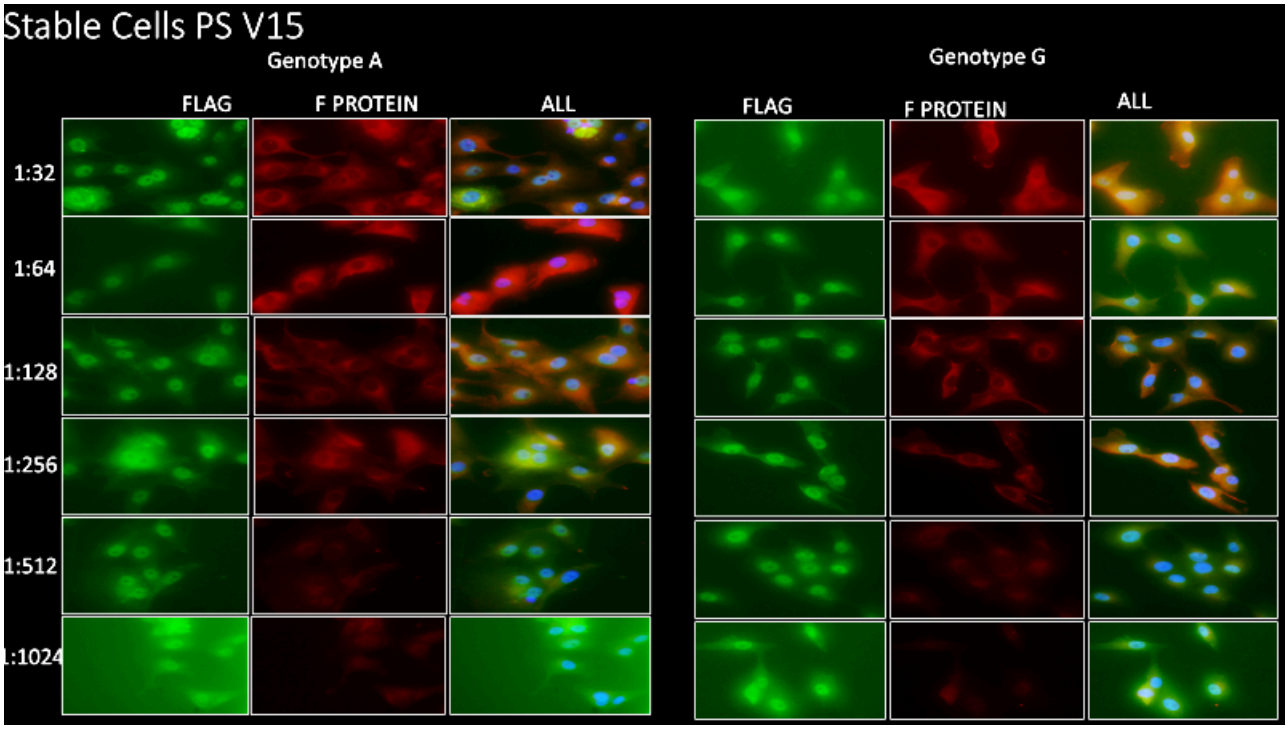


Figure A-59c. Stable cells Vaccine Sample#15.

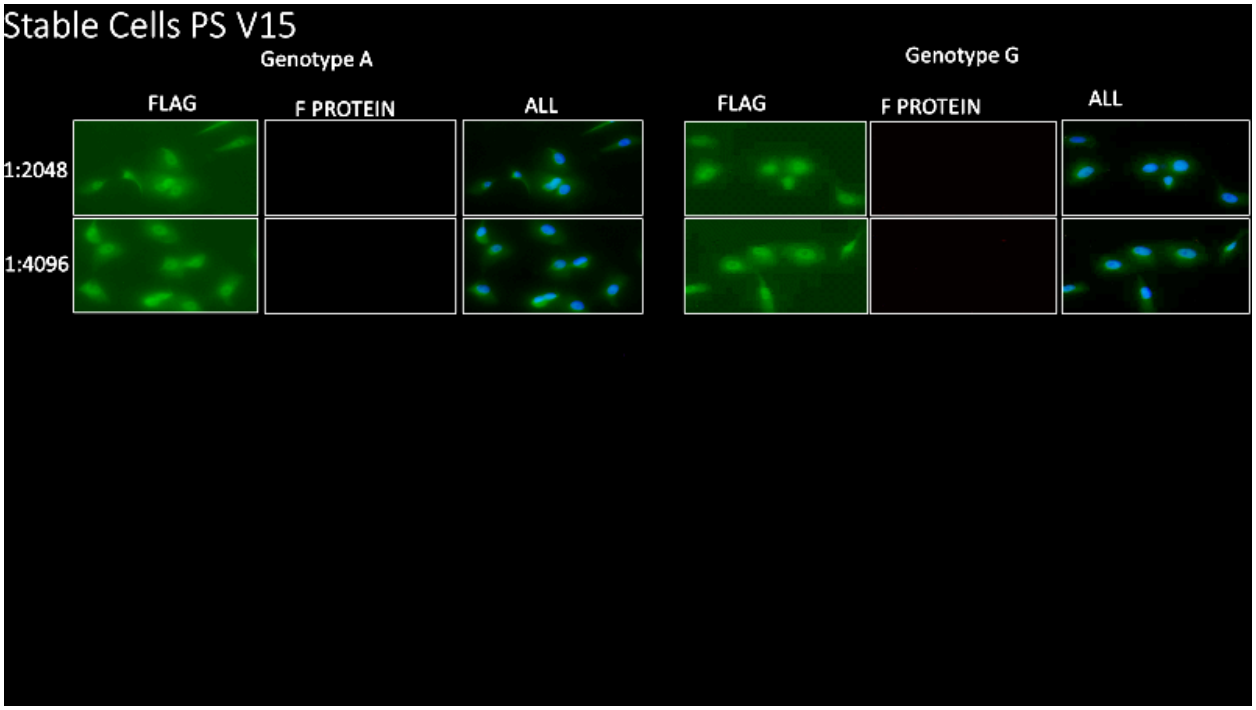


Figure A-59d. Stable cells Vaccine Sample#15.

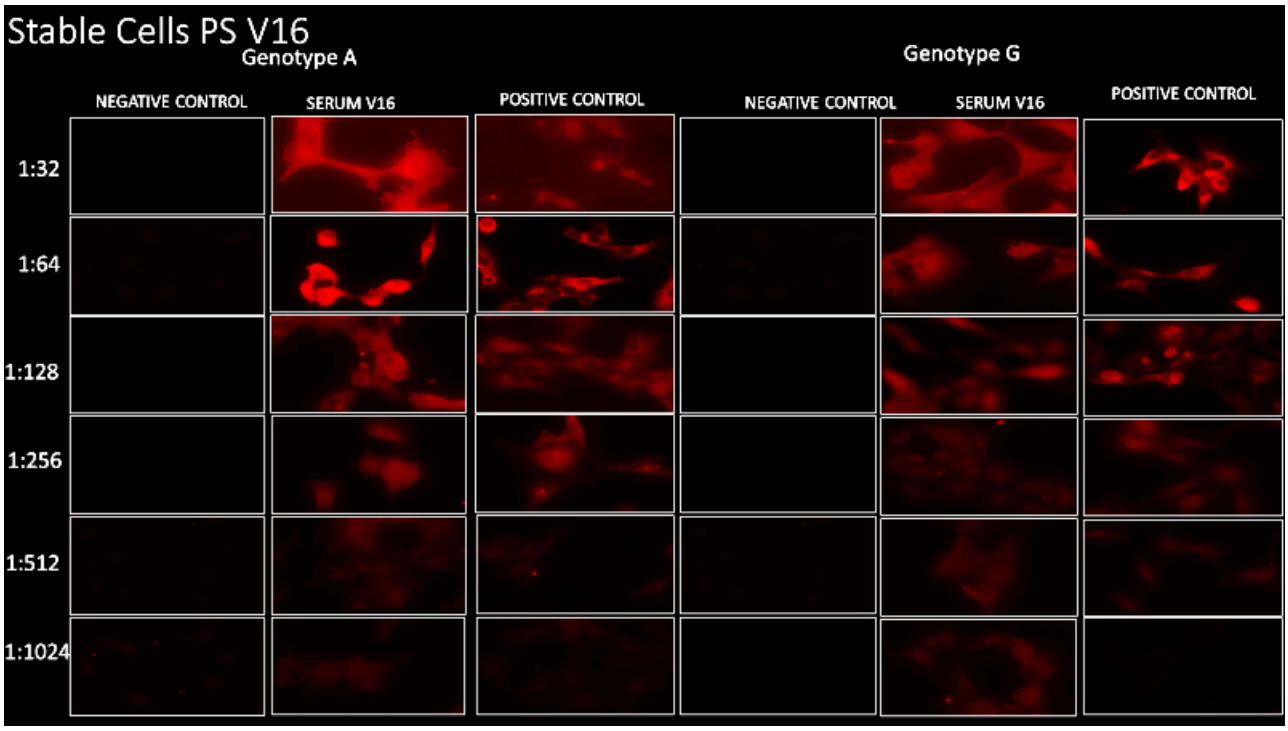


Figure A-60a. Stable cells Vaccine Sample#16.

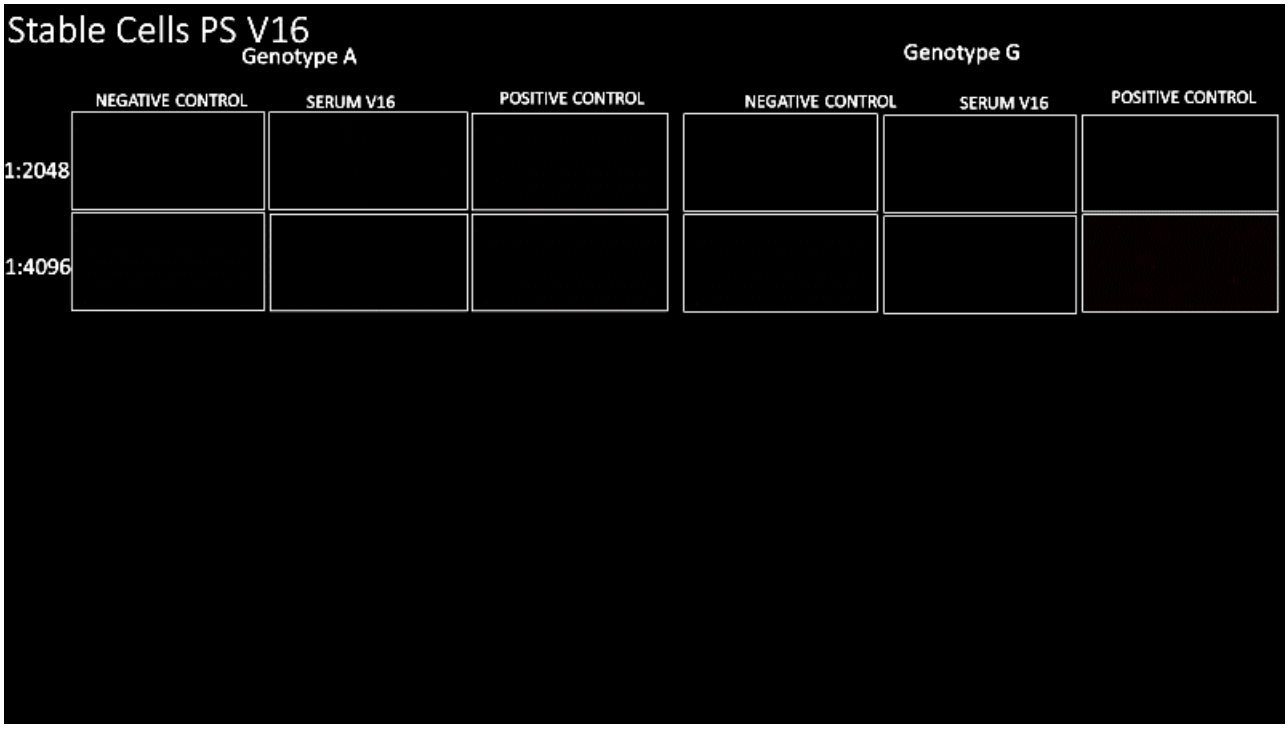


Figure A-60b. Stable cells Vaccine Sample#16.

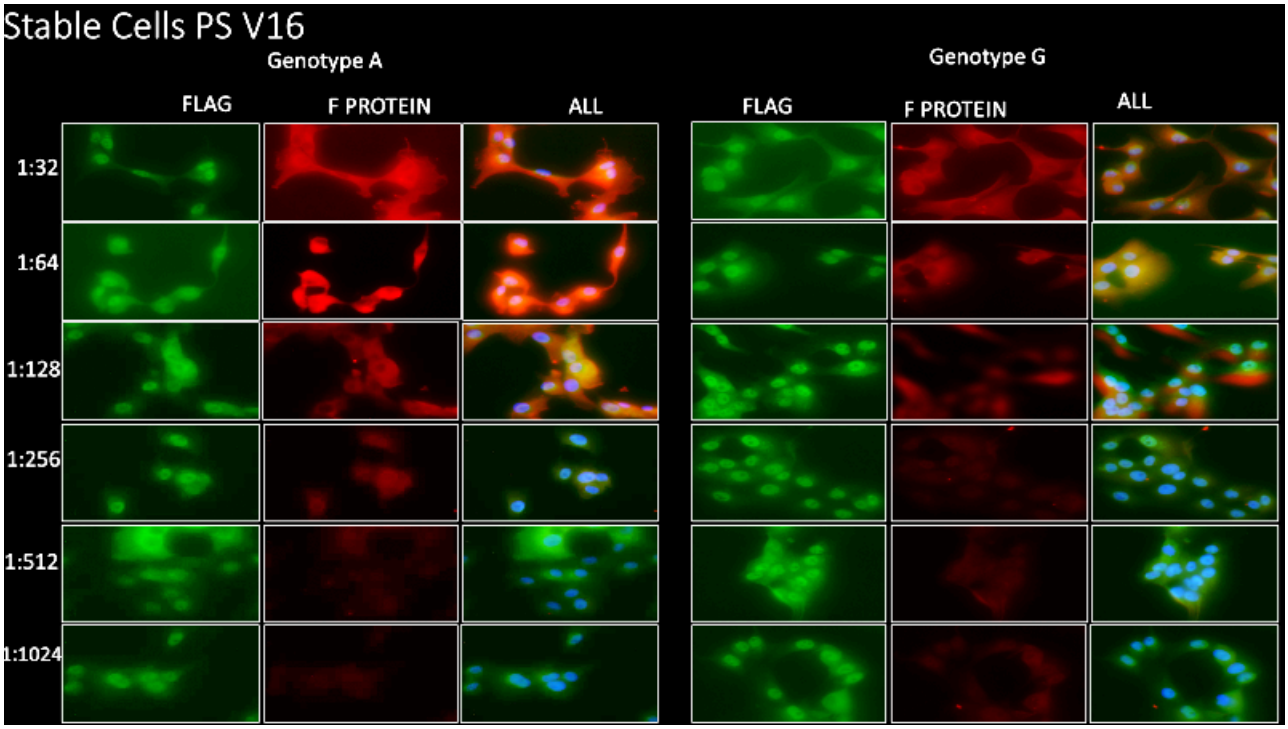


Figure A-60c. Stable cells Vaccine Sample#16.

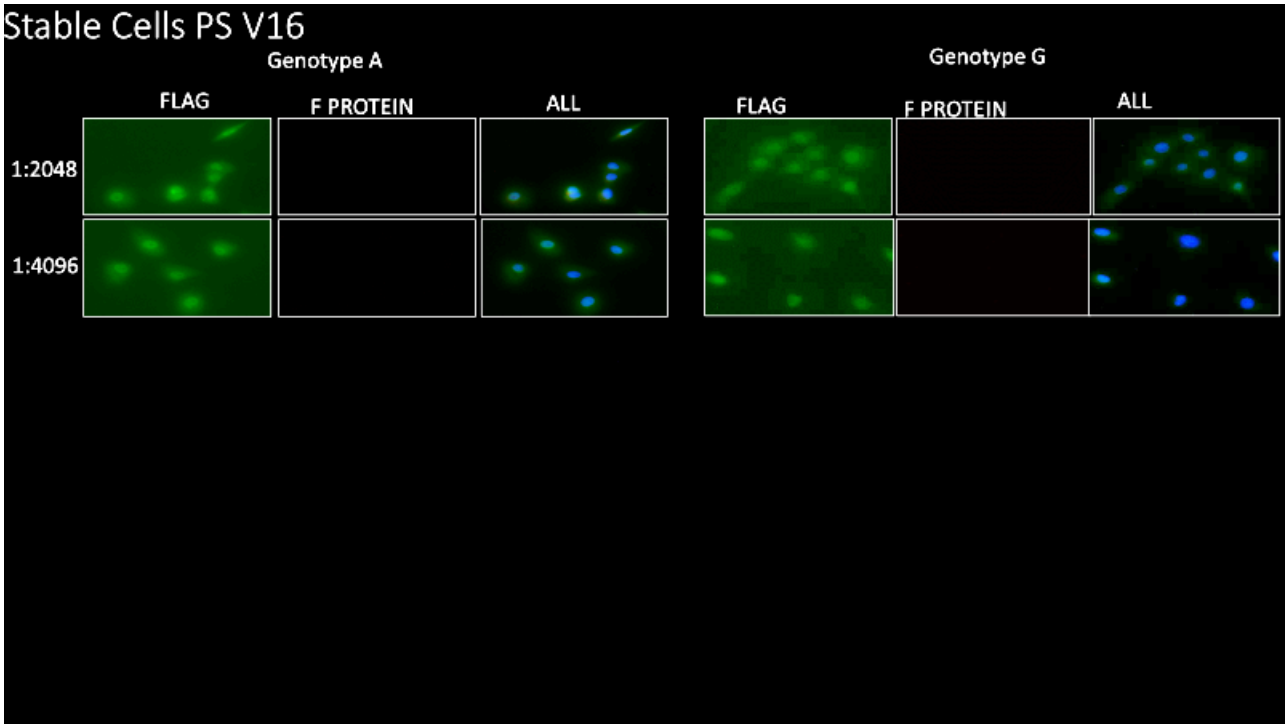


Figure A-60d. Stable cells Vaccine Sample#16.

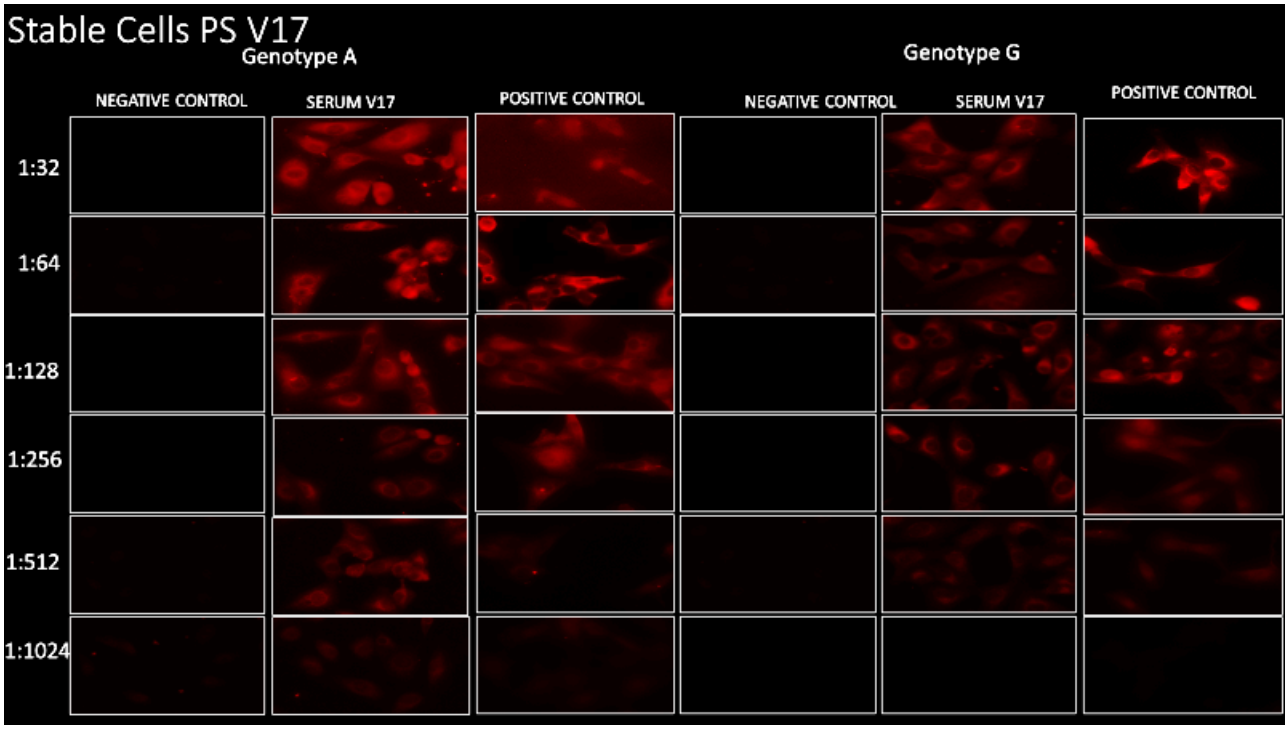


Figure A-61a. Stable cells Vaccine Sample#17.

Figure A-61b. Stable cells Vaccine Sample#17.

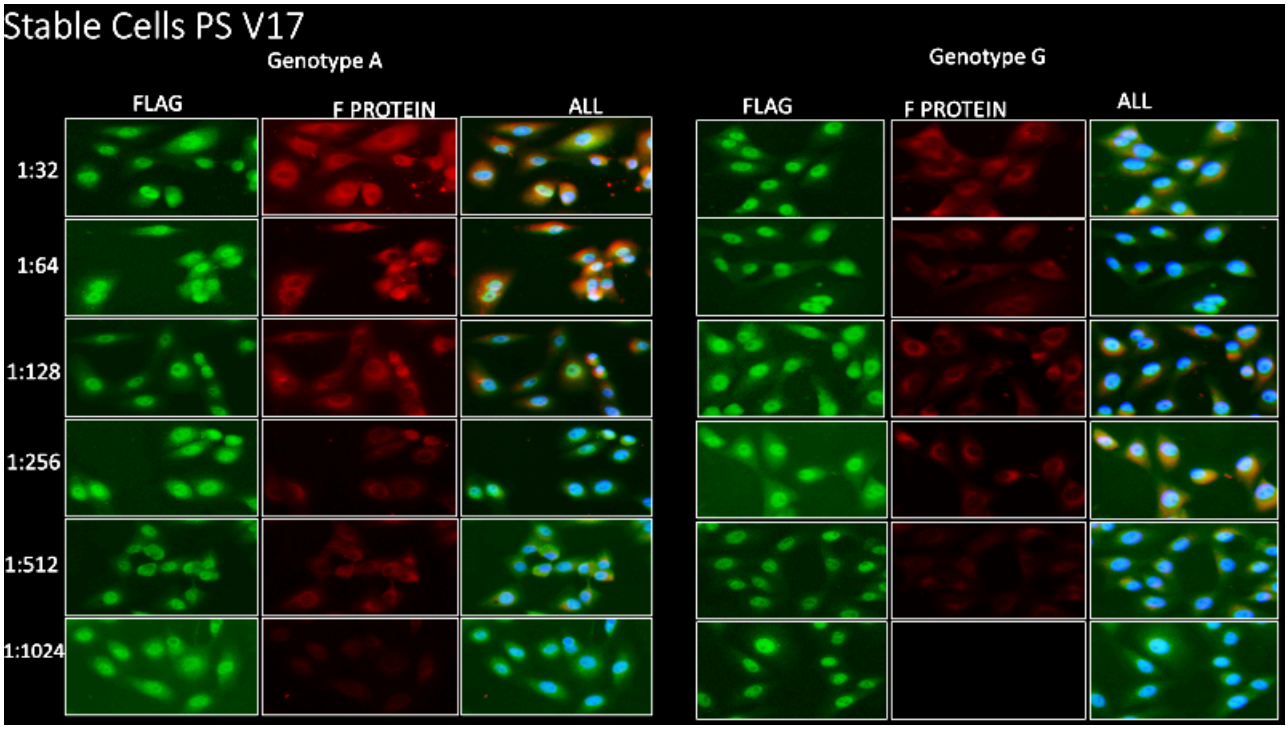


Figure A-61c. Stable cells Vaccine Sample#17.

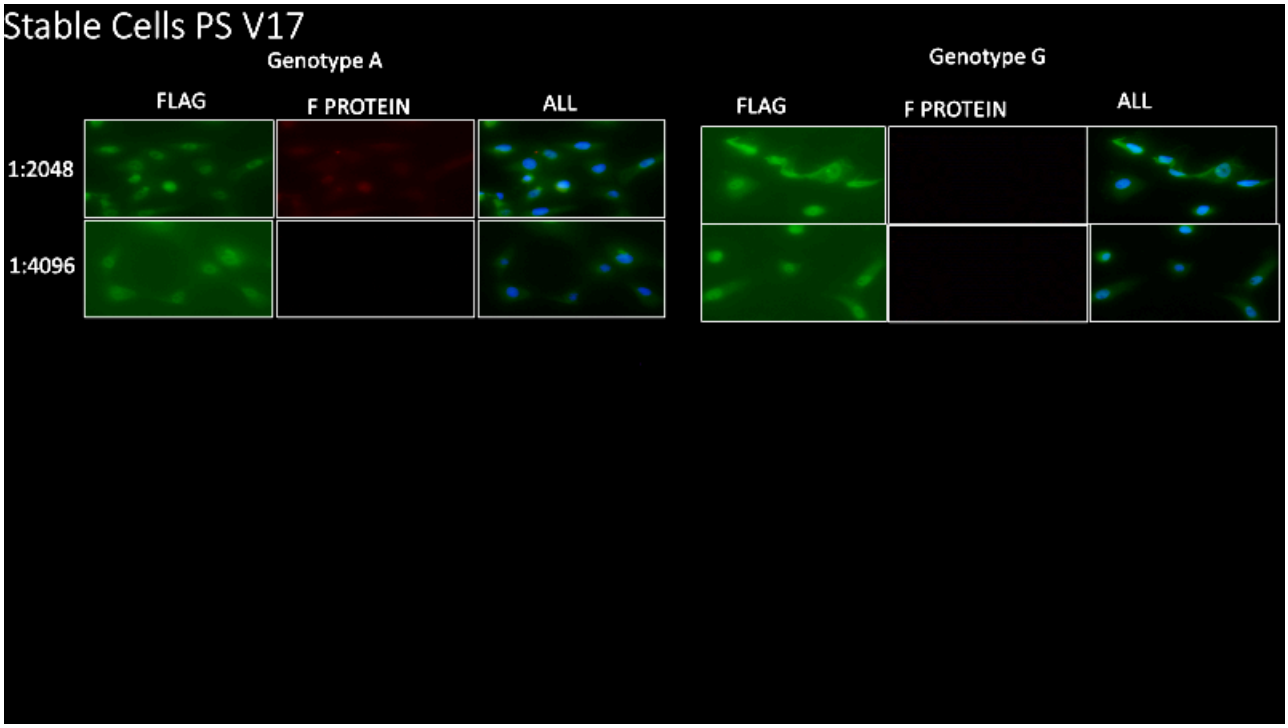


Figure A-61d. Stable cells Vaccine Sample#17.

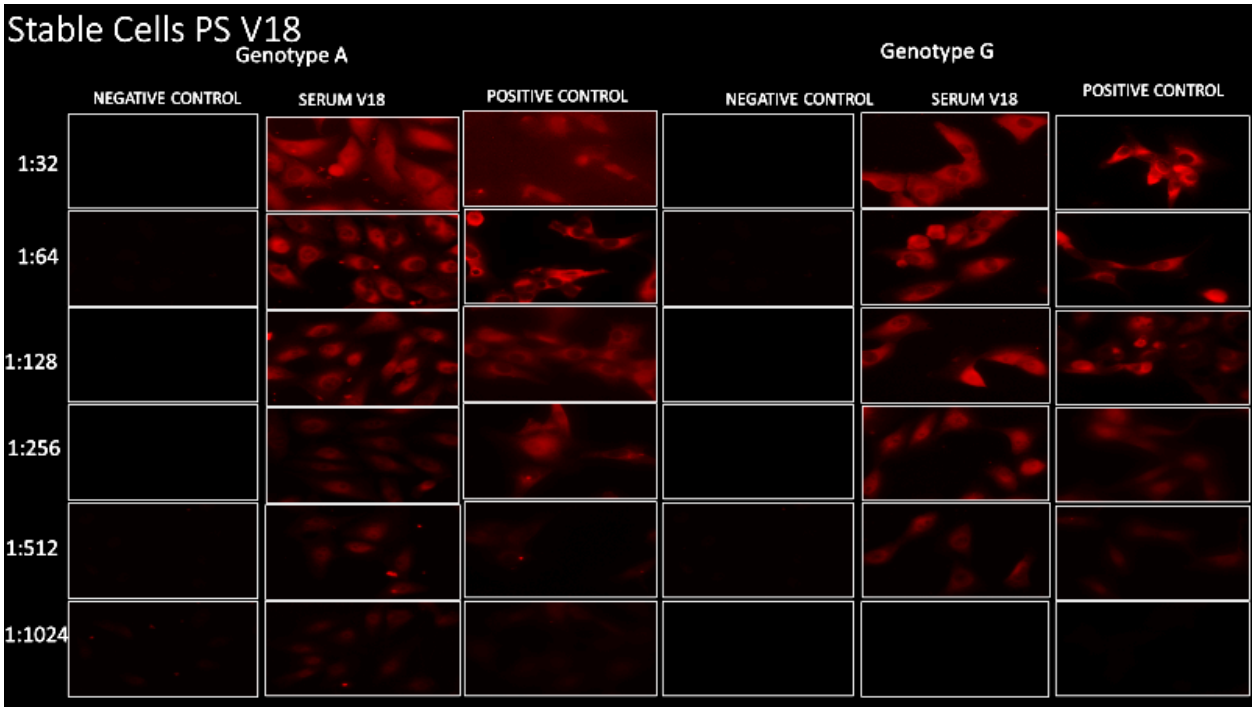


Figure A-62a. Stable cells Vaccine Sample#18.

Figure A-62b. Stable cells Vaccine Sample#18.

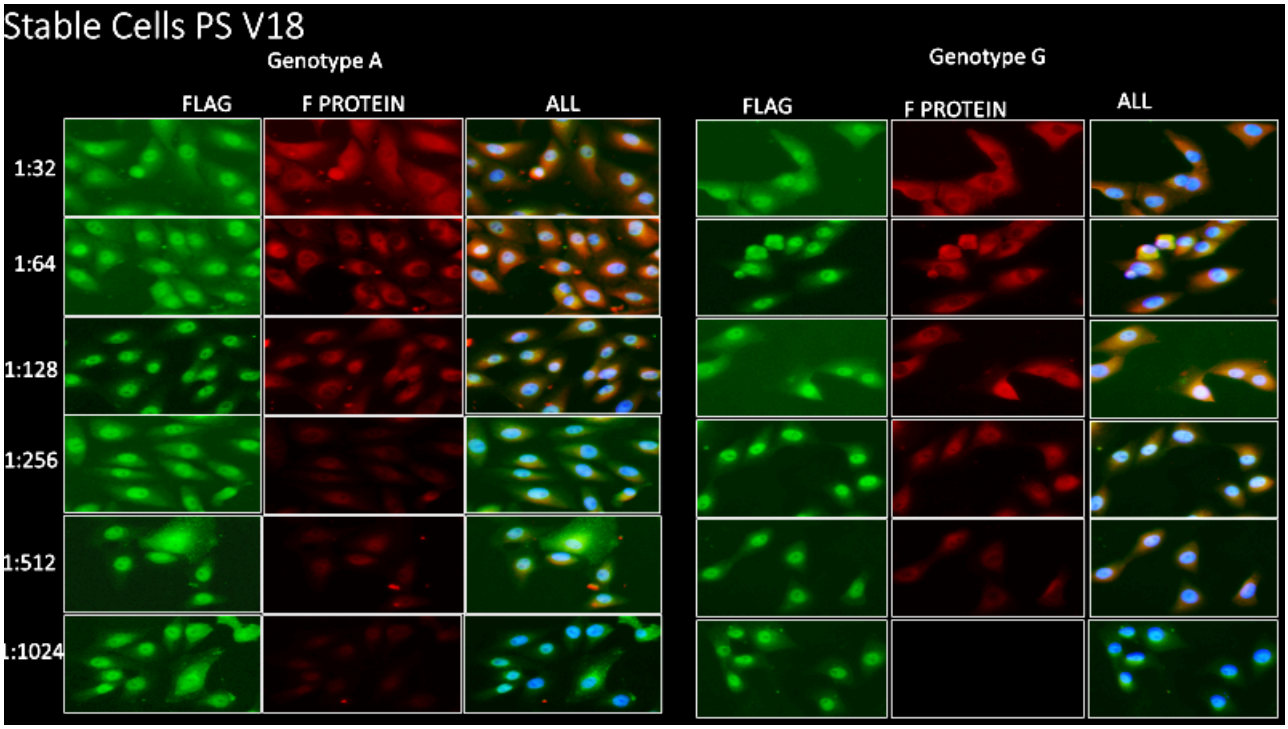


Figure A-62c. Stable cells Vaccine Sample#18.

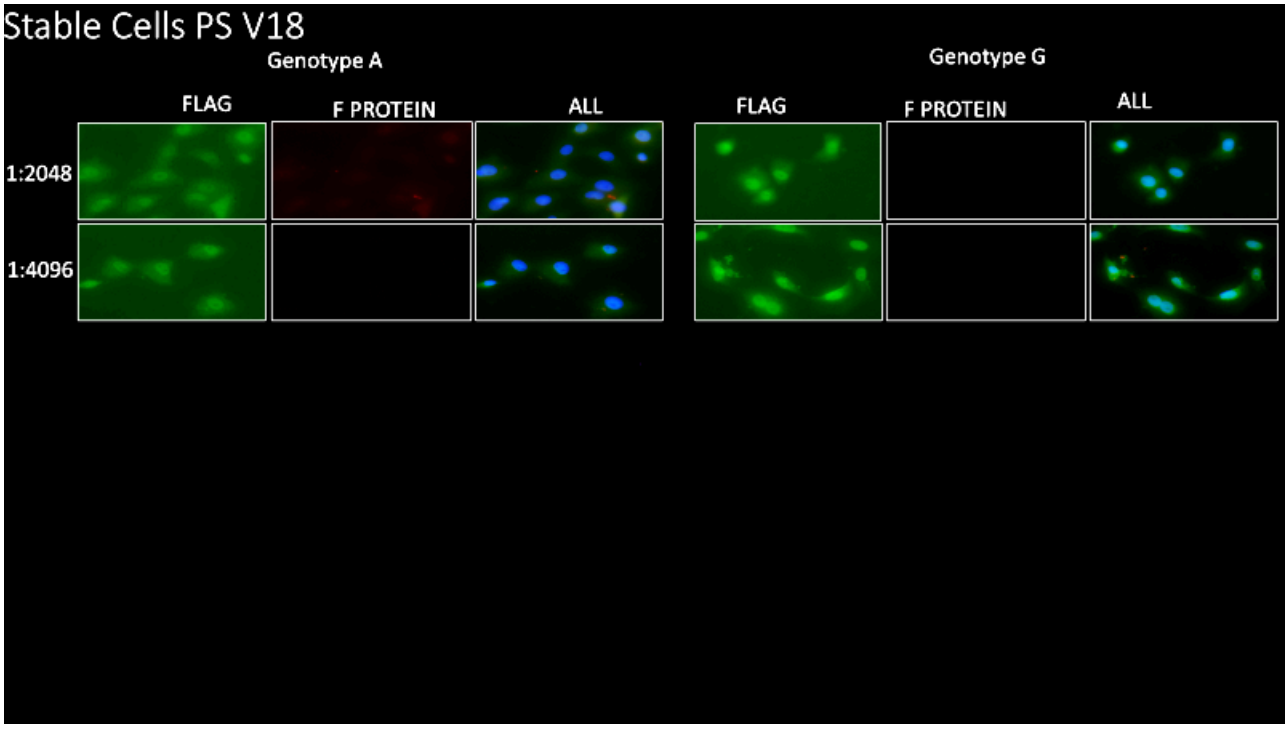


Figure A-62d. Stable cells Vaccine Sample#18.

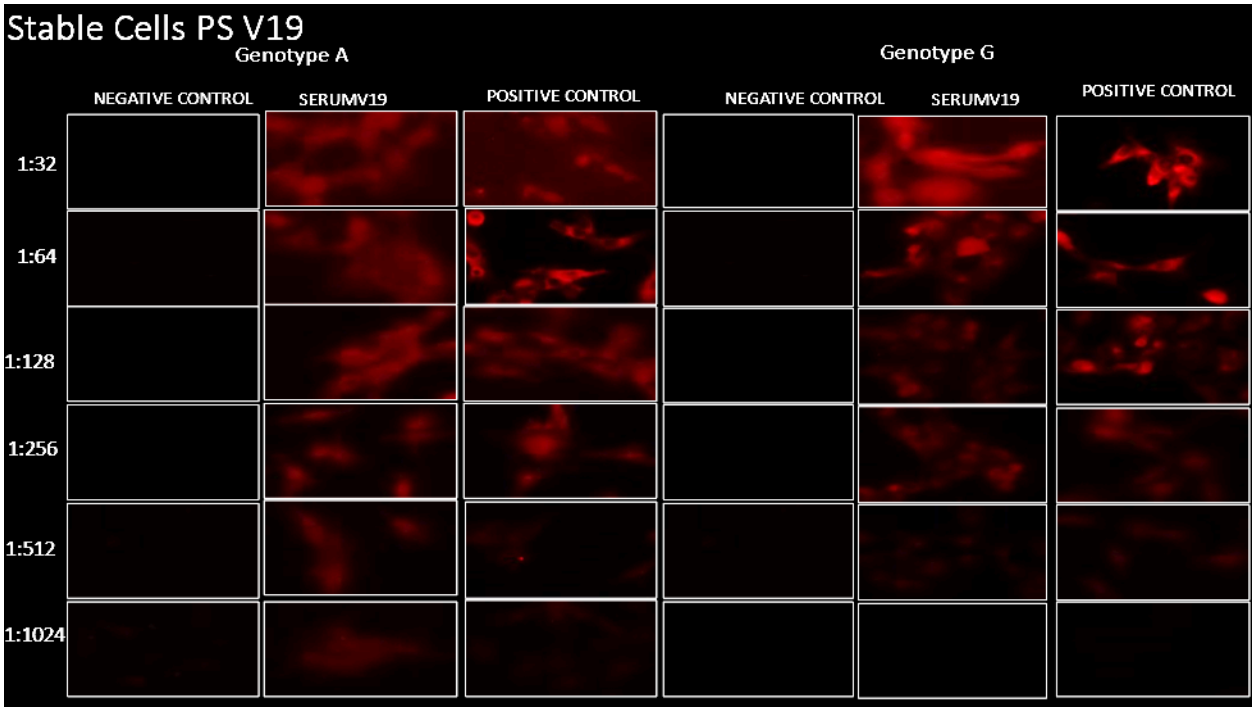


Figure A-63a. Stable cells Vaccine Sample#19.

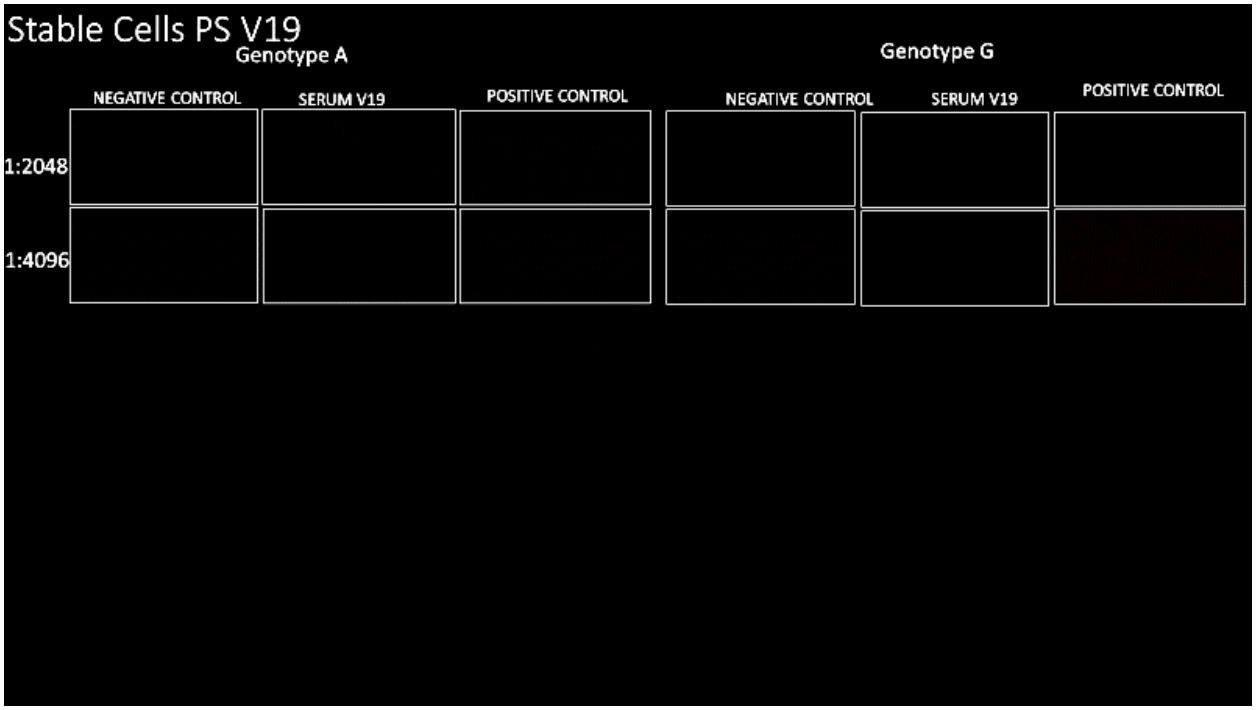


Figure A-63b. Stable cells Vaccine Sample#19.

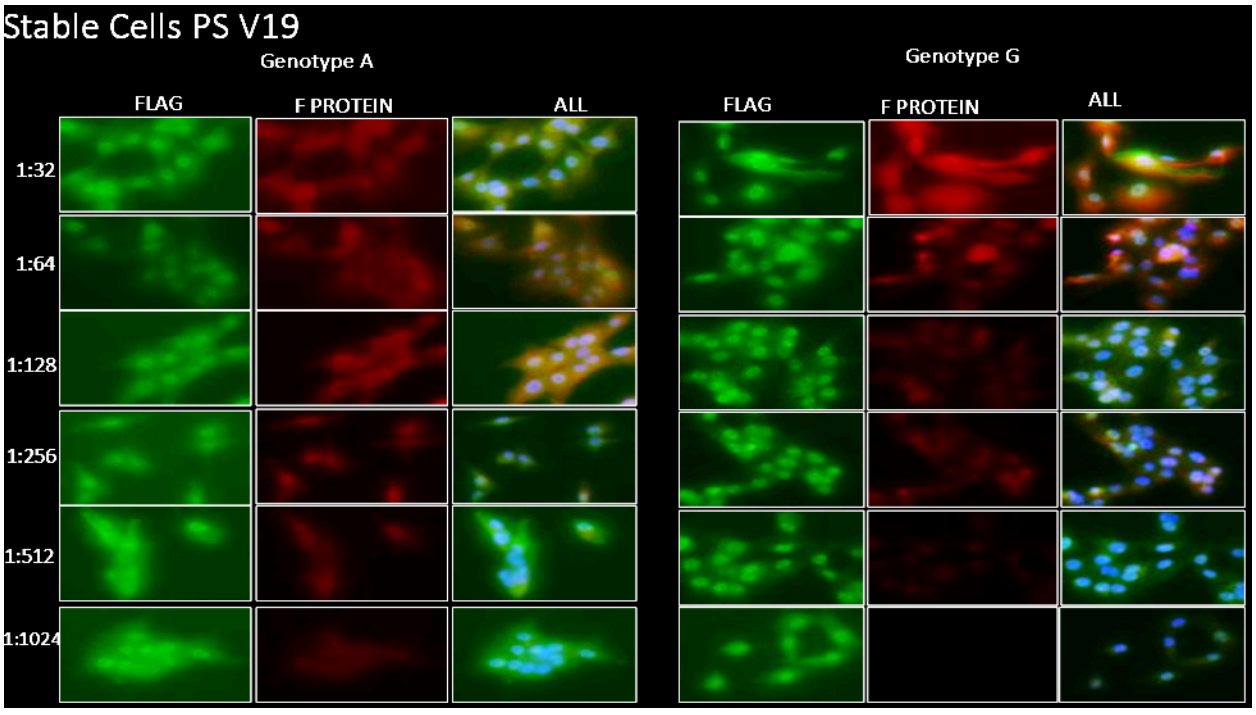


Figure A-63c. Stable cells Vaccine Sample#19.

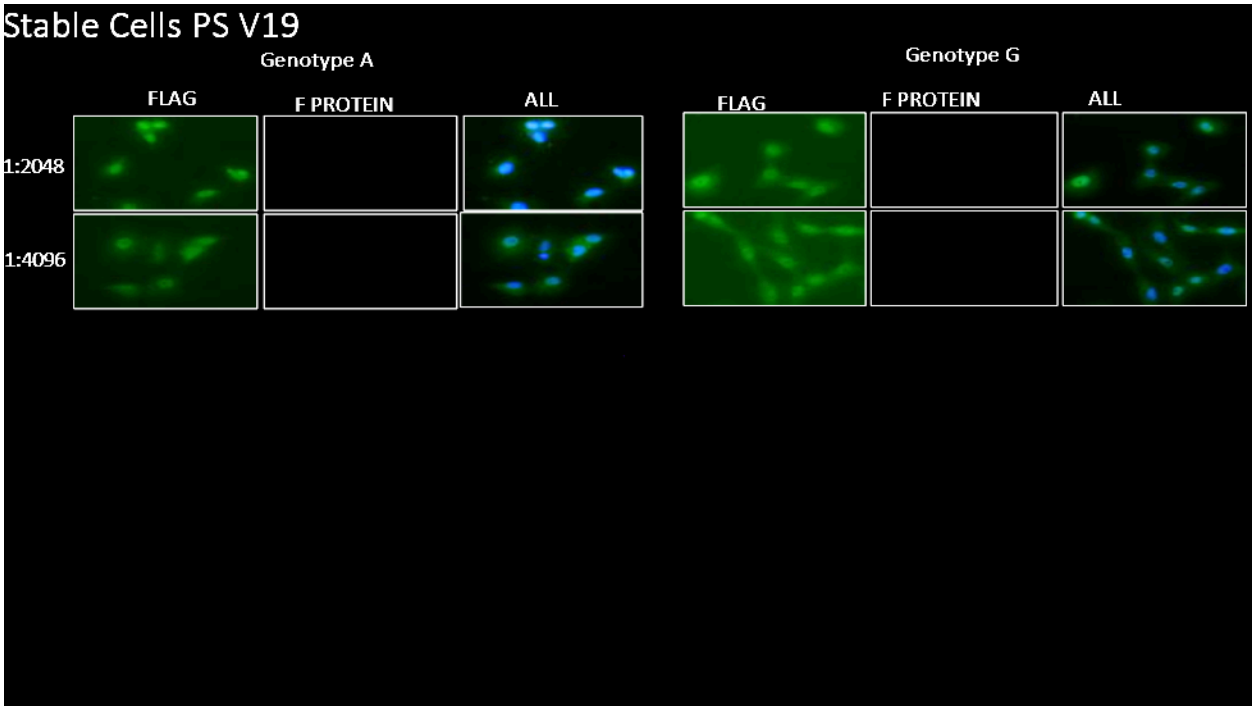


Figure A-63d. Stable cells Vaccine Sample#19.

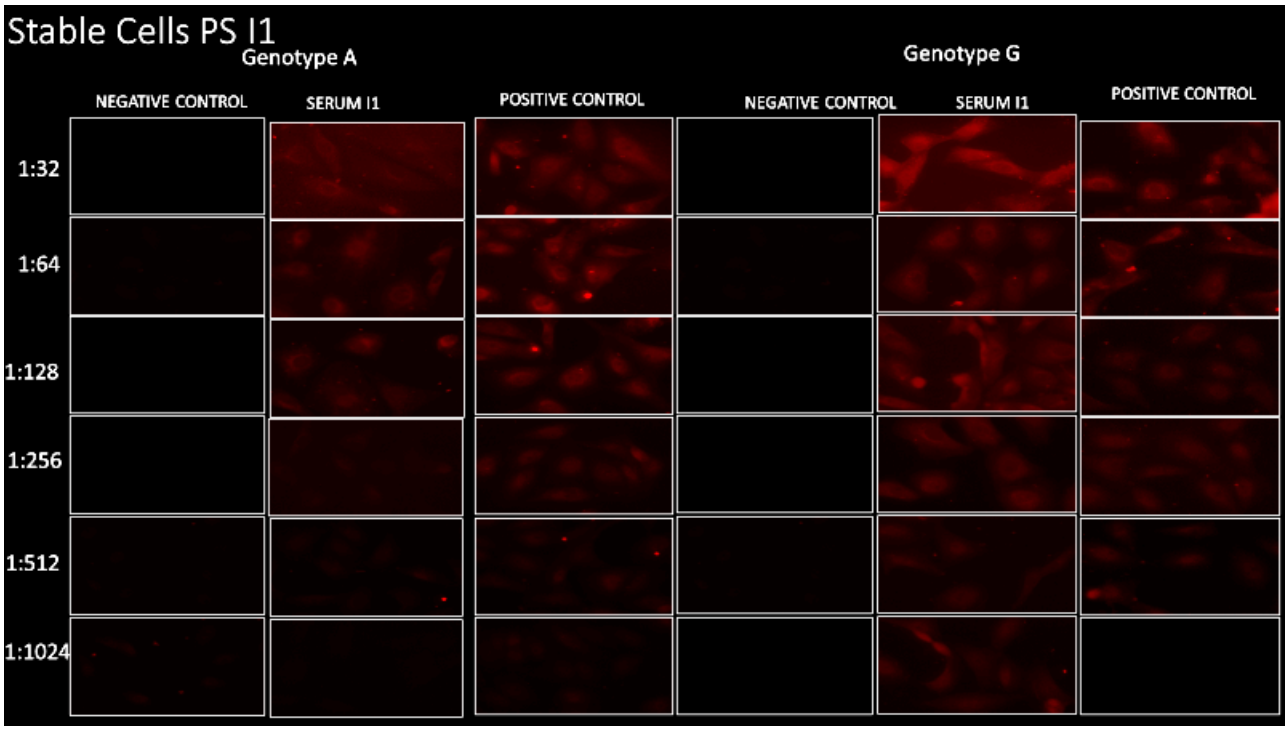


Figure A-64a. Stable cells Infected Sample#1.

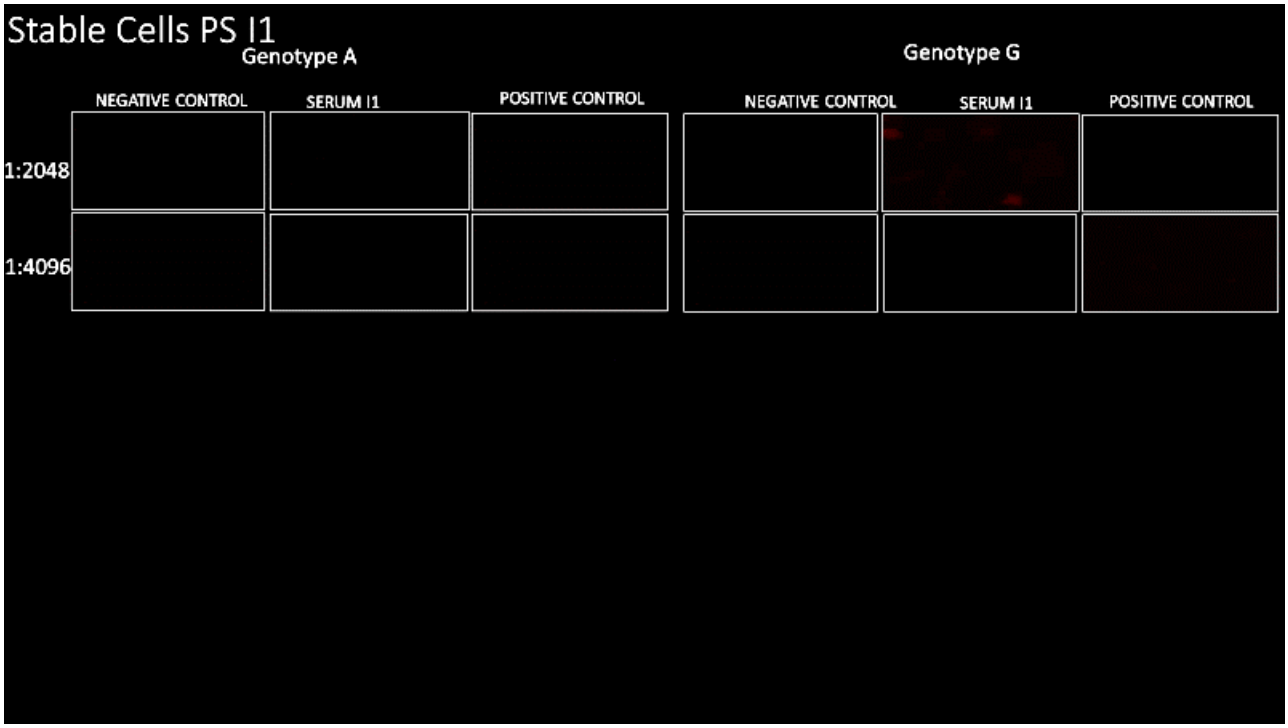


Figure A-64b. Stable cells Infected Sample#1.

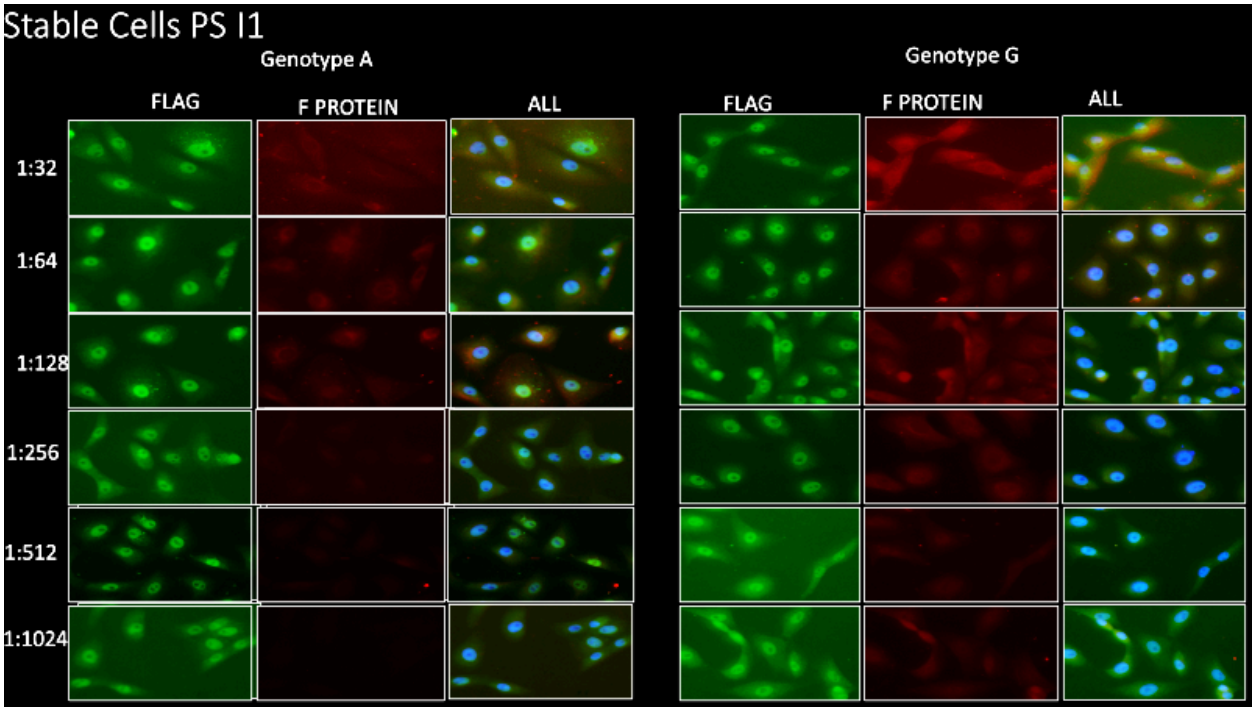


Figure A-64c. Stable cells Infected Sample#1.

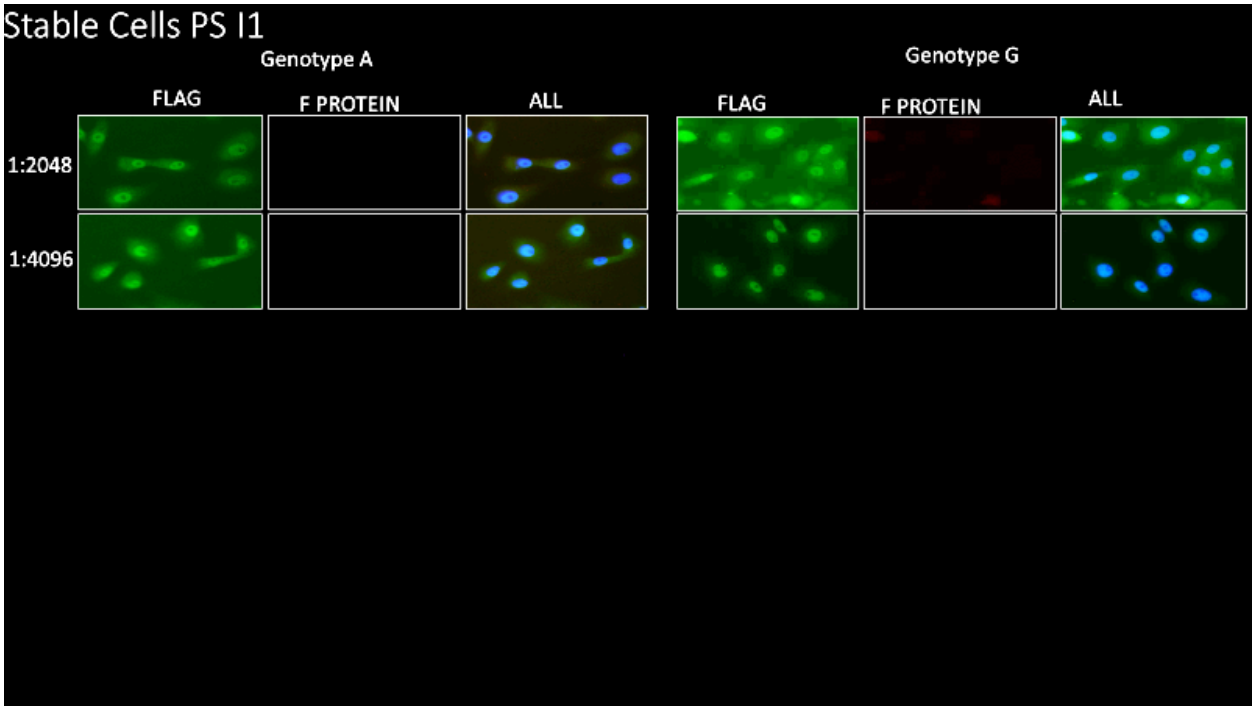


Figure A-64d. Stable cells Infected Sample#1.

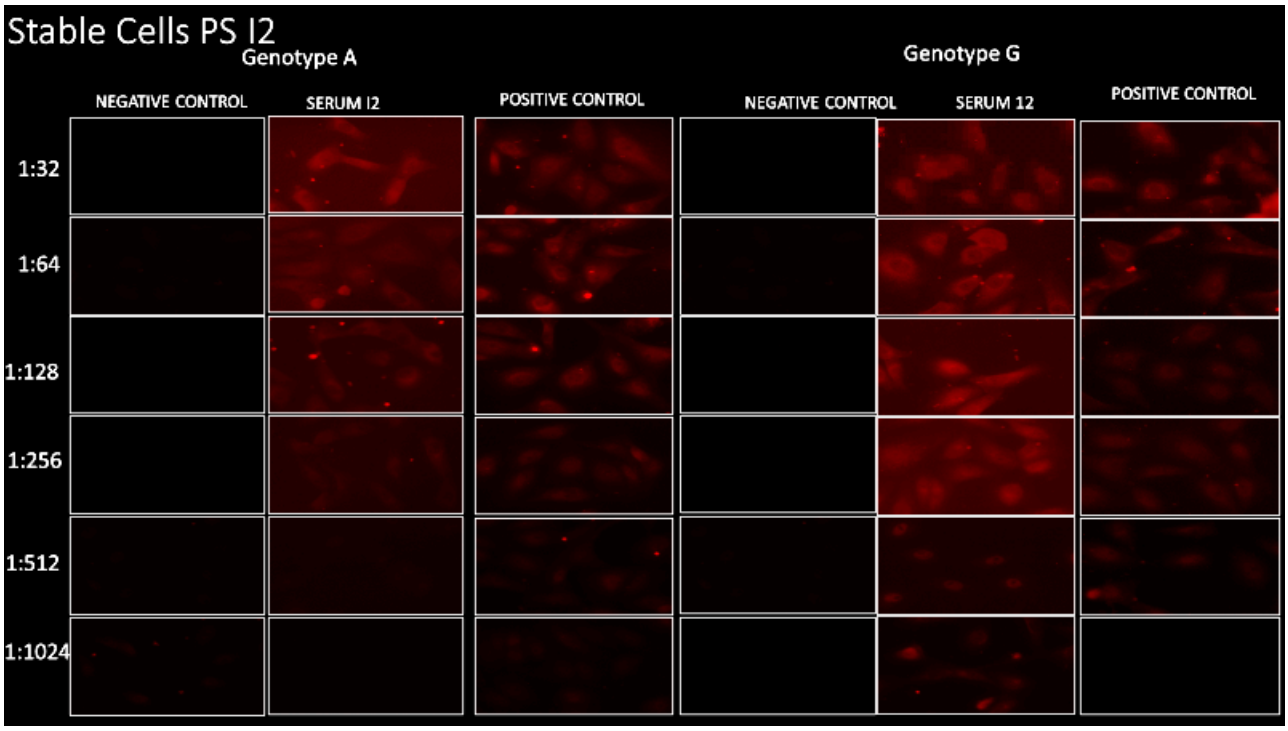


Figure A-65a. Stable cells Infected Sample#2.

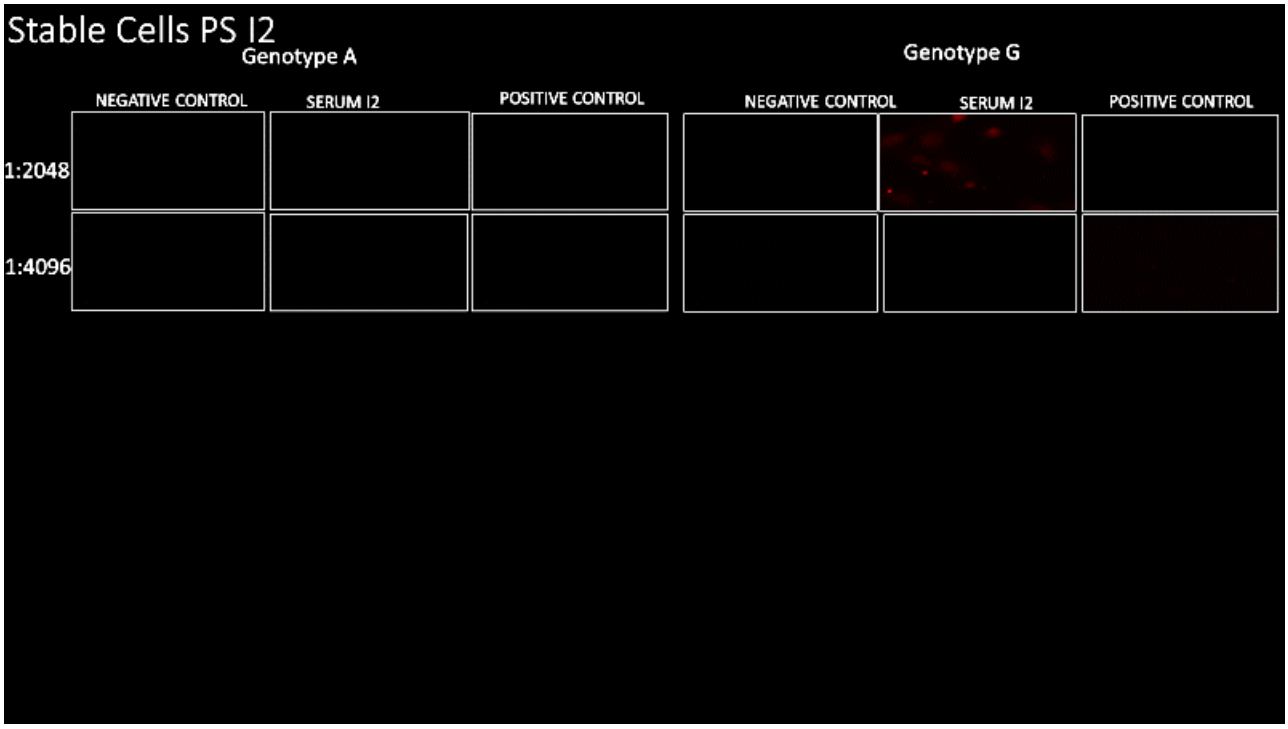


Figure A-65b. Stable cells Infected Sample#2.

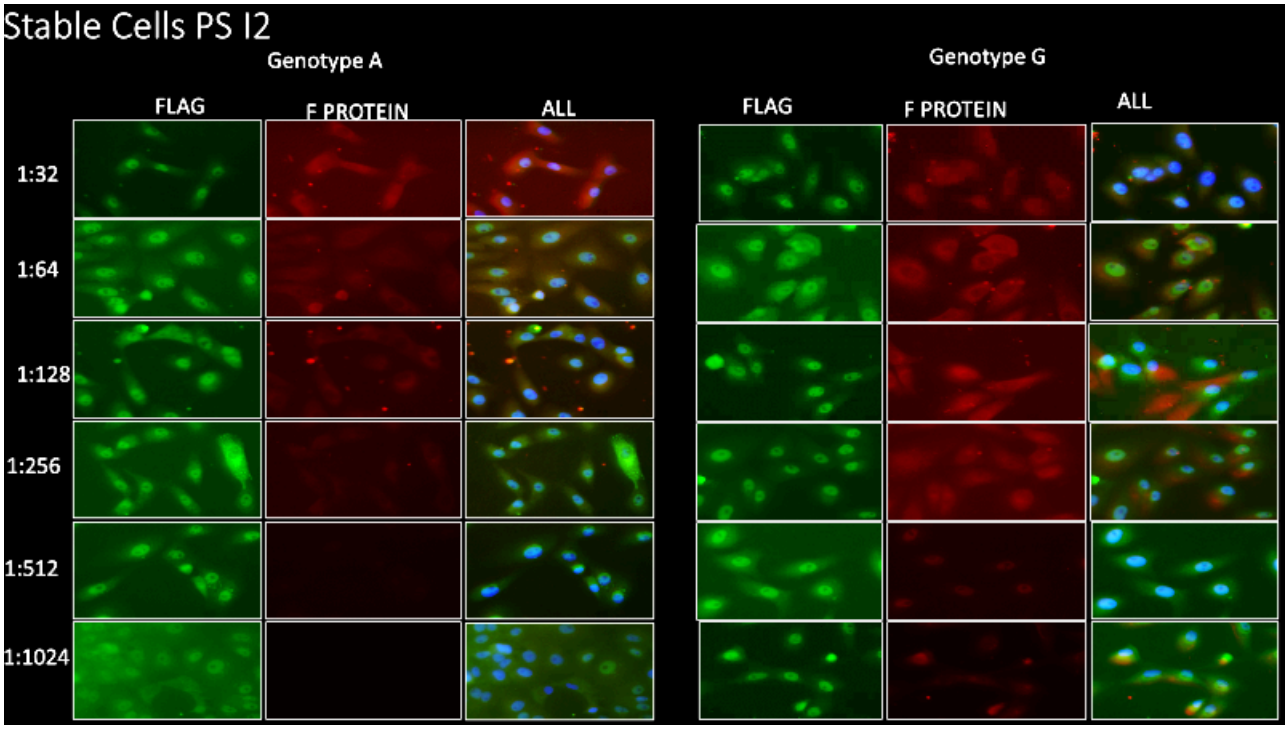


Figure A-65c. Stable cells Infected Sample#2.

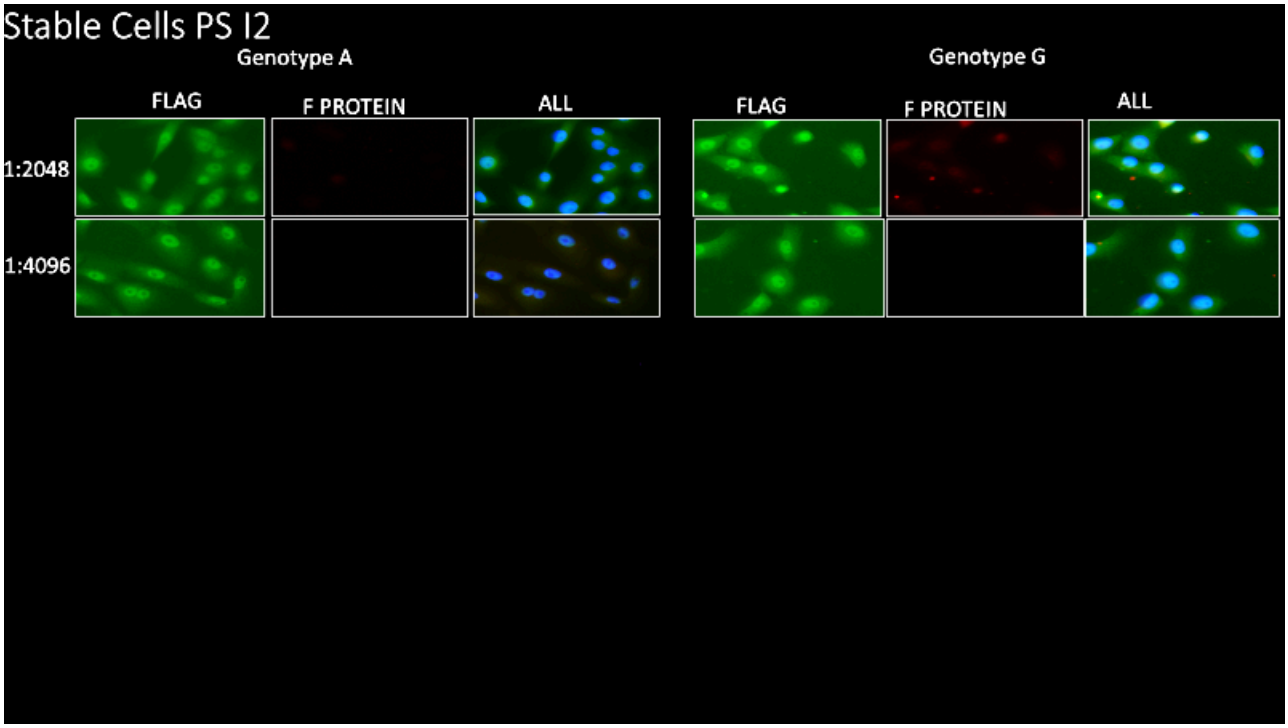


Figure A-65d. Stable cells Infected Sample#2.

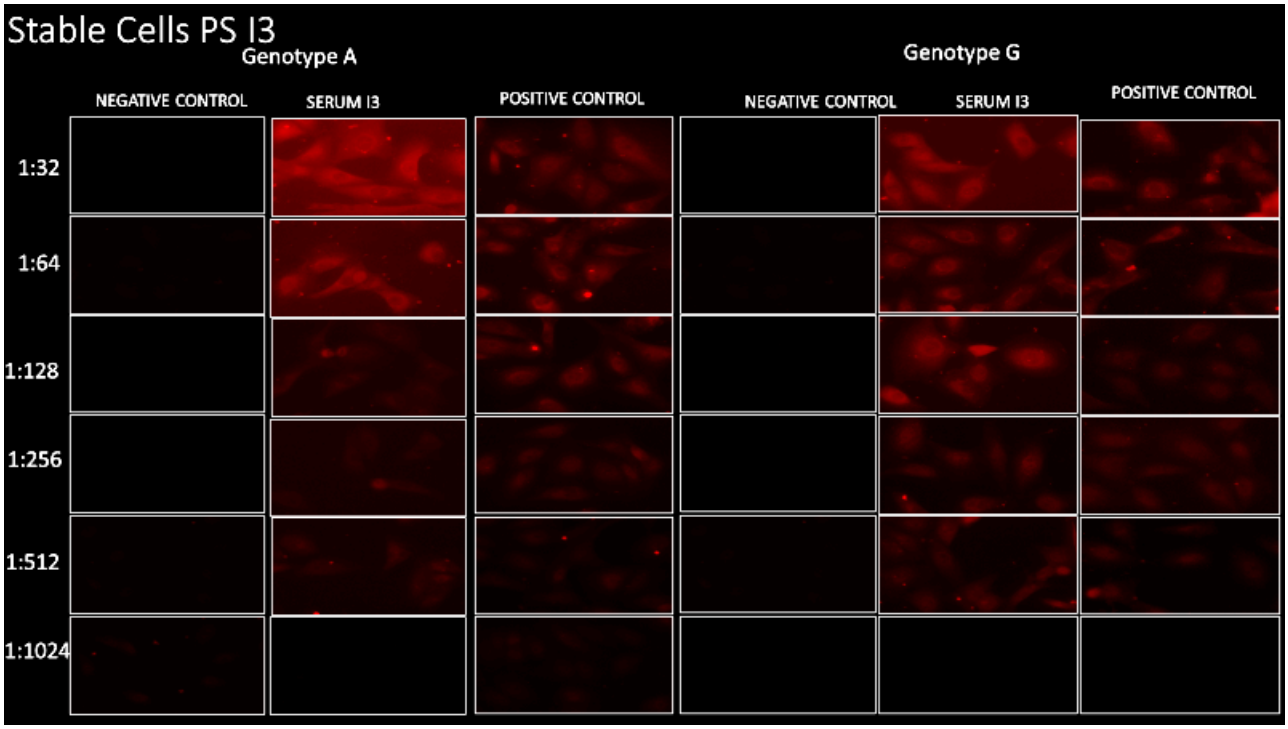


Figure A-66a. Stable cells Infected Sample#3.

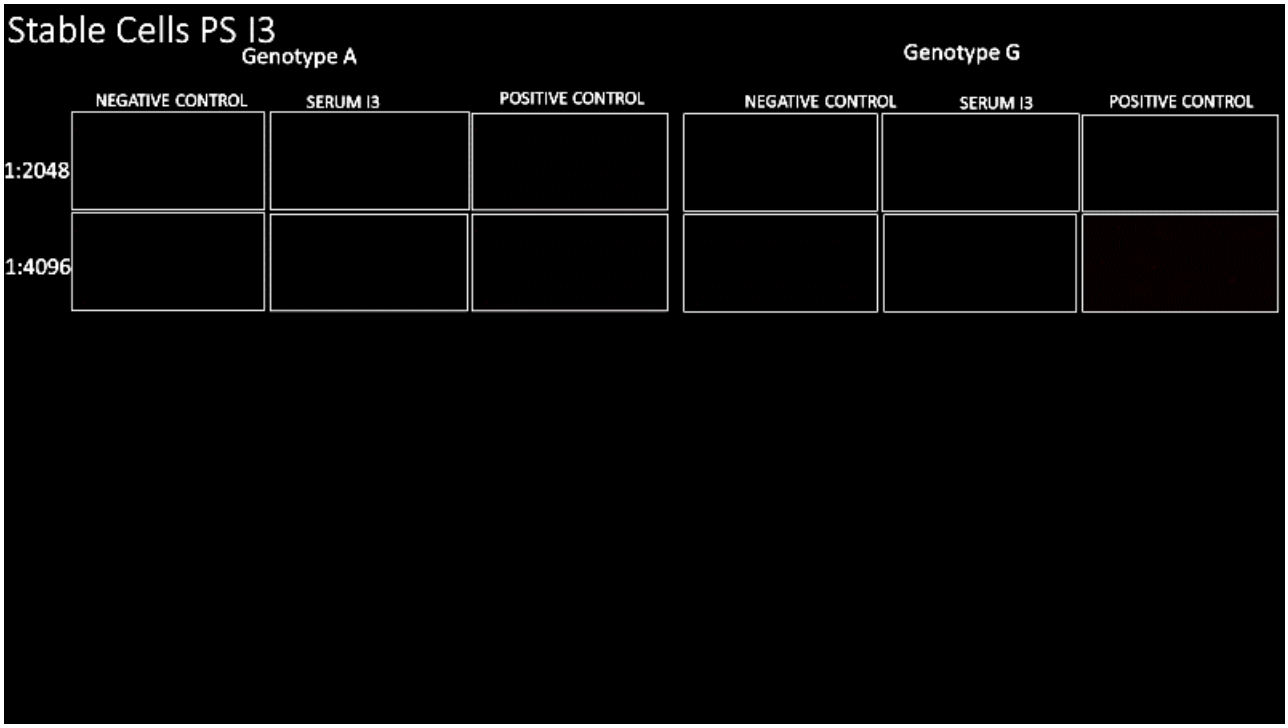


Figure A-66b. Stable cells Infected Sample#3.

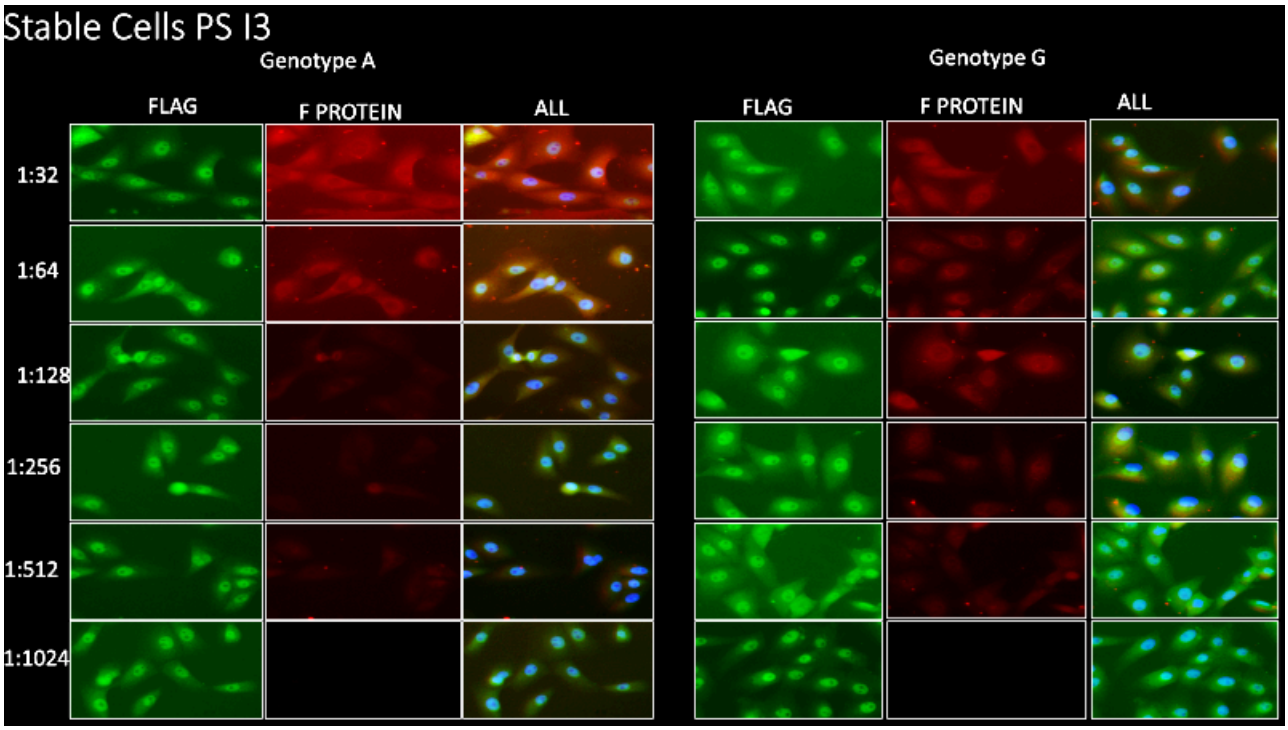


Figure A-66c. Stable cells Infected Sample#3.

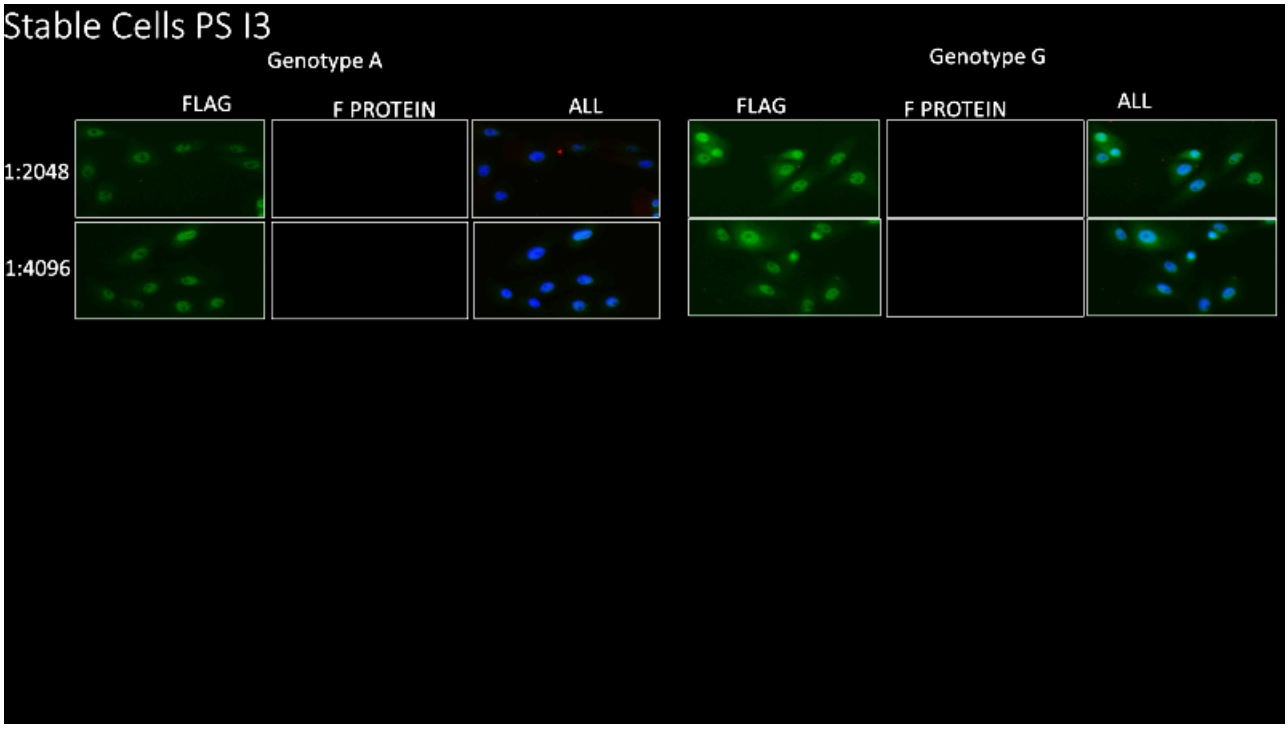


Figure A-66d. Stable cells Infected Sample#3.

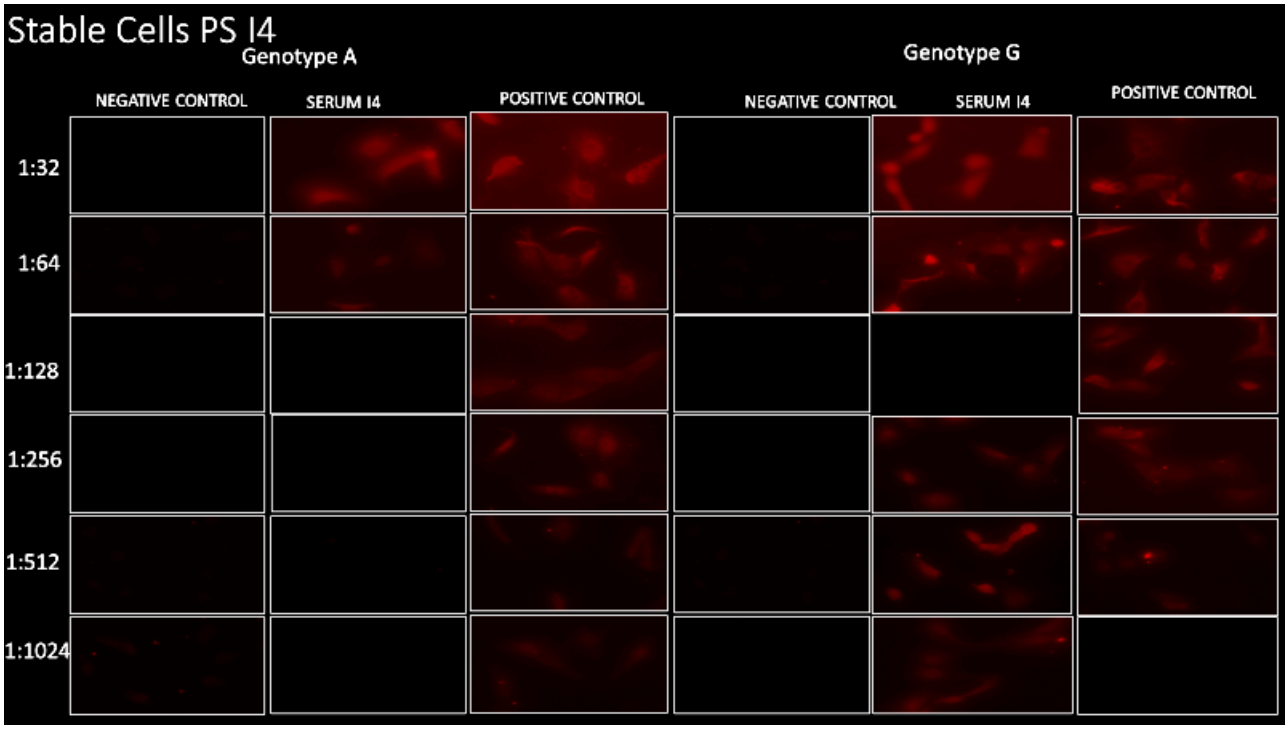


Figure A-67a. Stable cells Infected Sample#4.

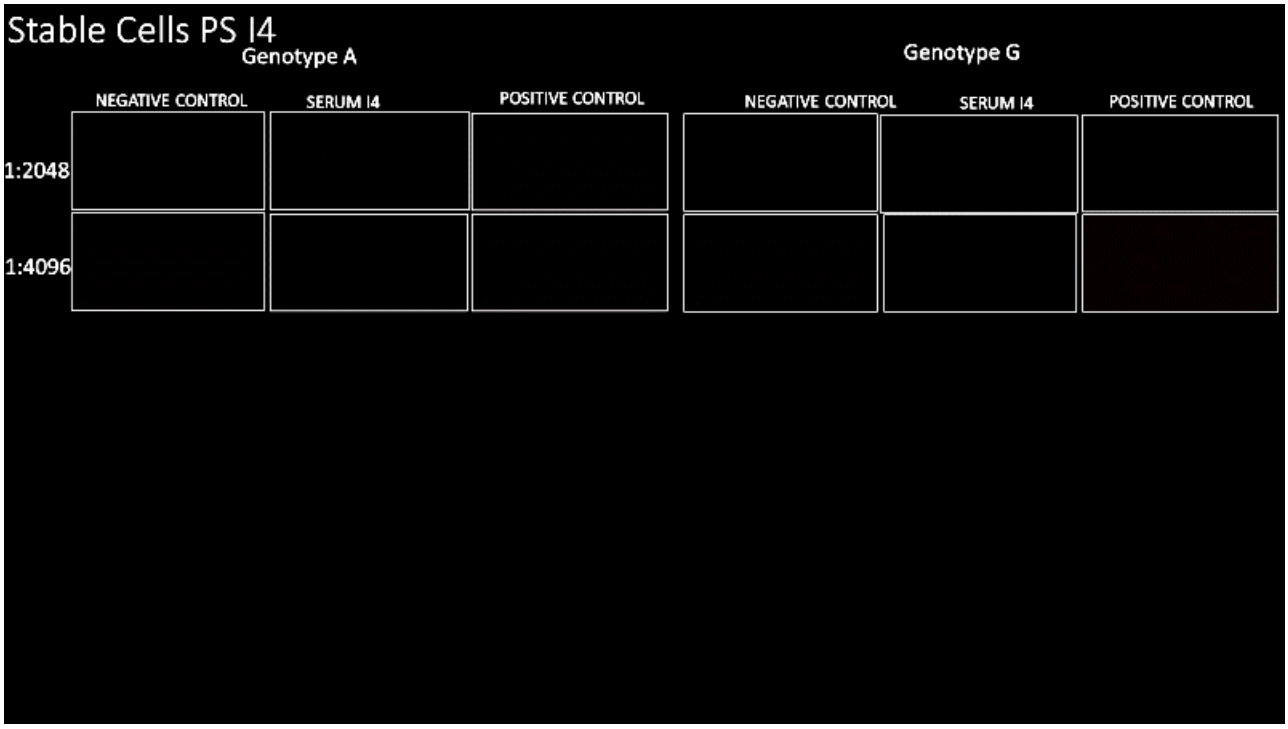


Figure A-67b. Stable cells Infected Sample#4.

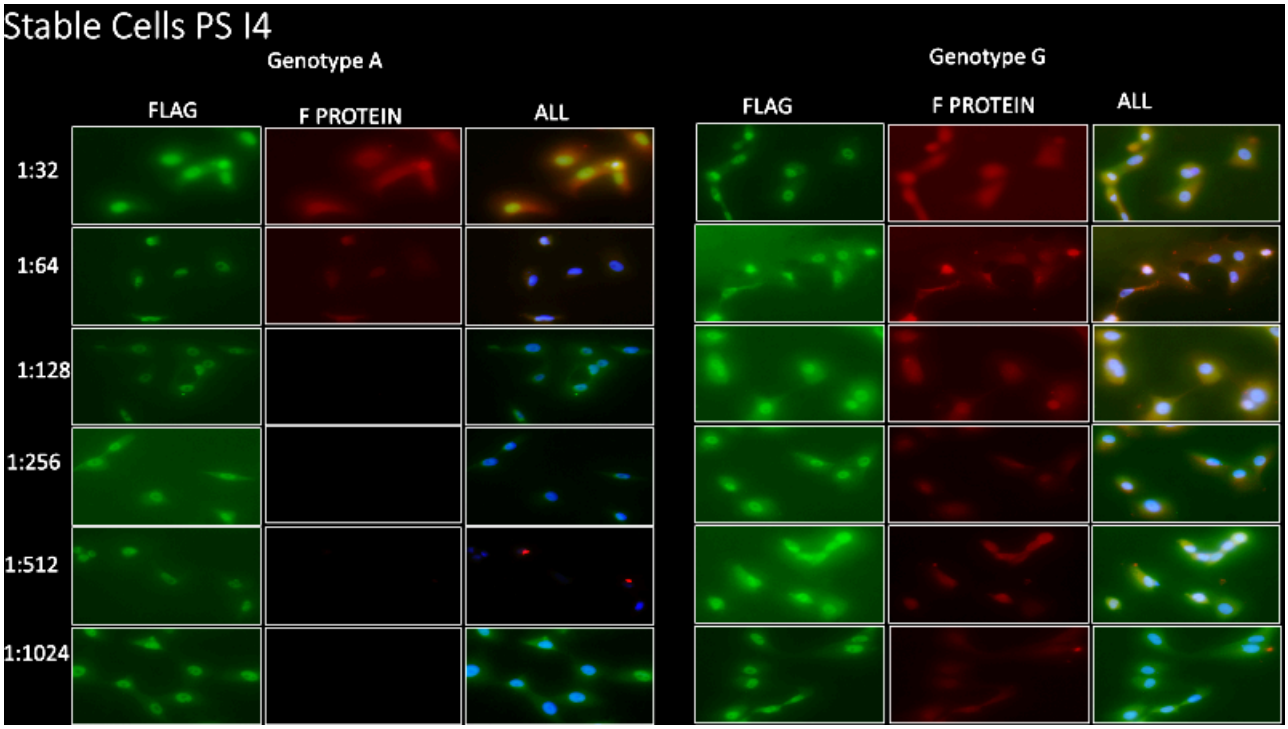


Figure A-67c. Stable cells Infected Sample#4.

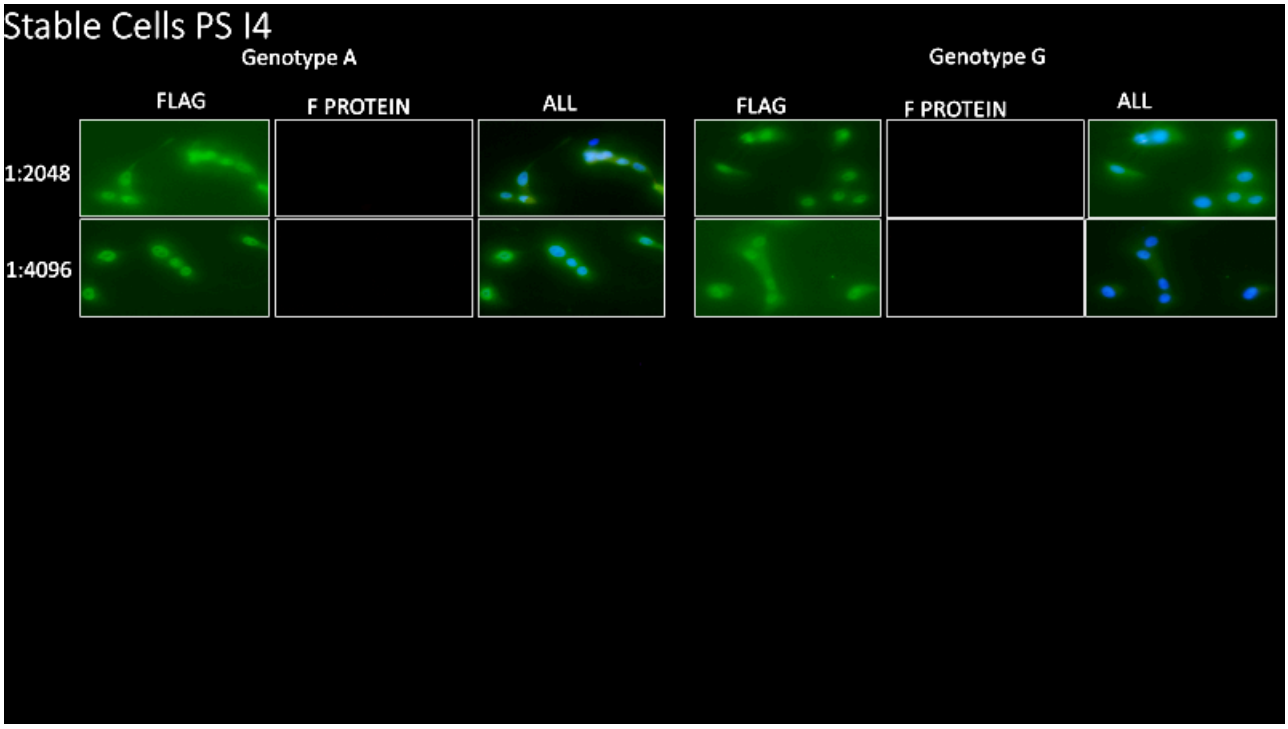


Figure A-67d. Stable cells Infected Sample#4.

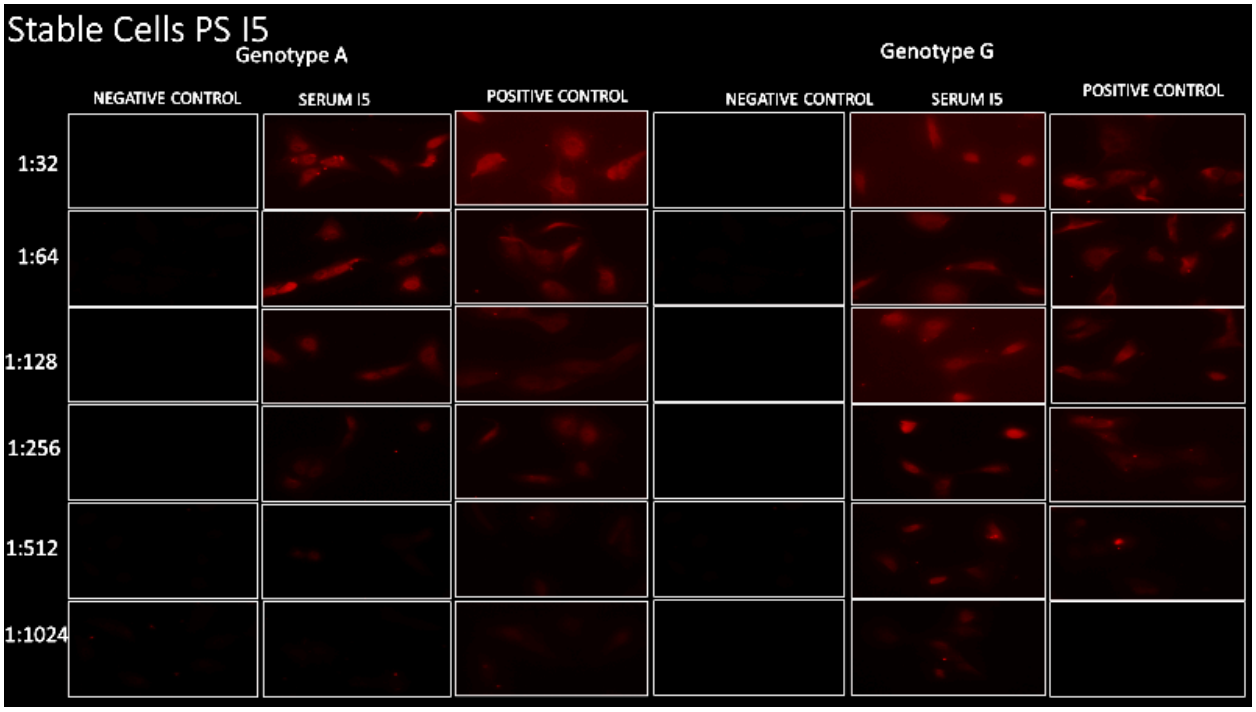


Figure A-68a. Stable cells Infected Sample#5.

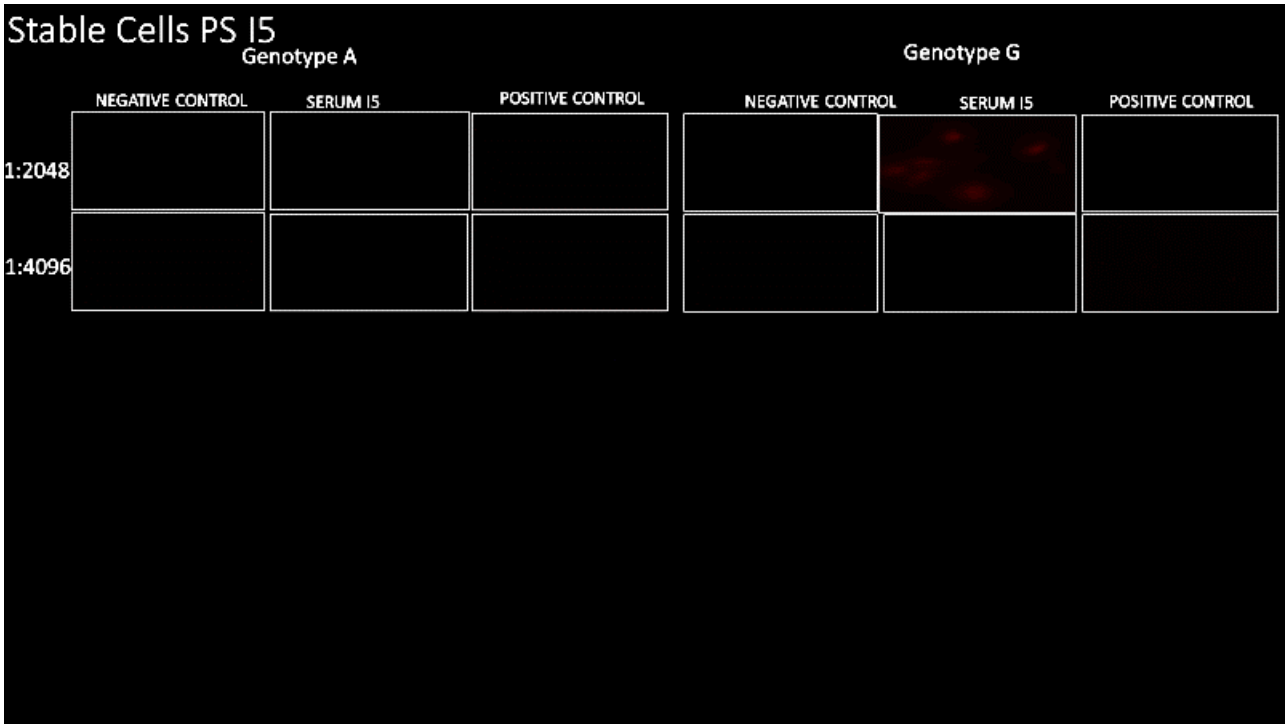


Figure A-68b. Stable cells Infected Sample#5.

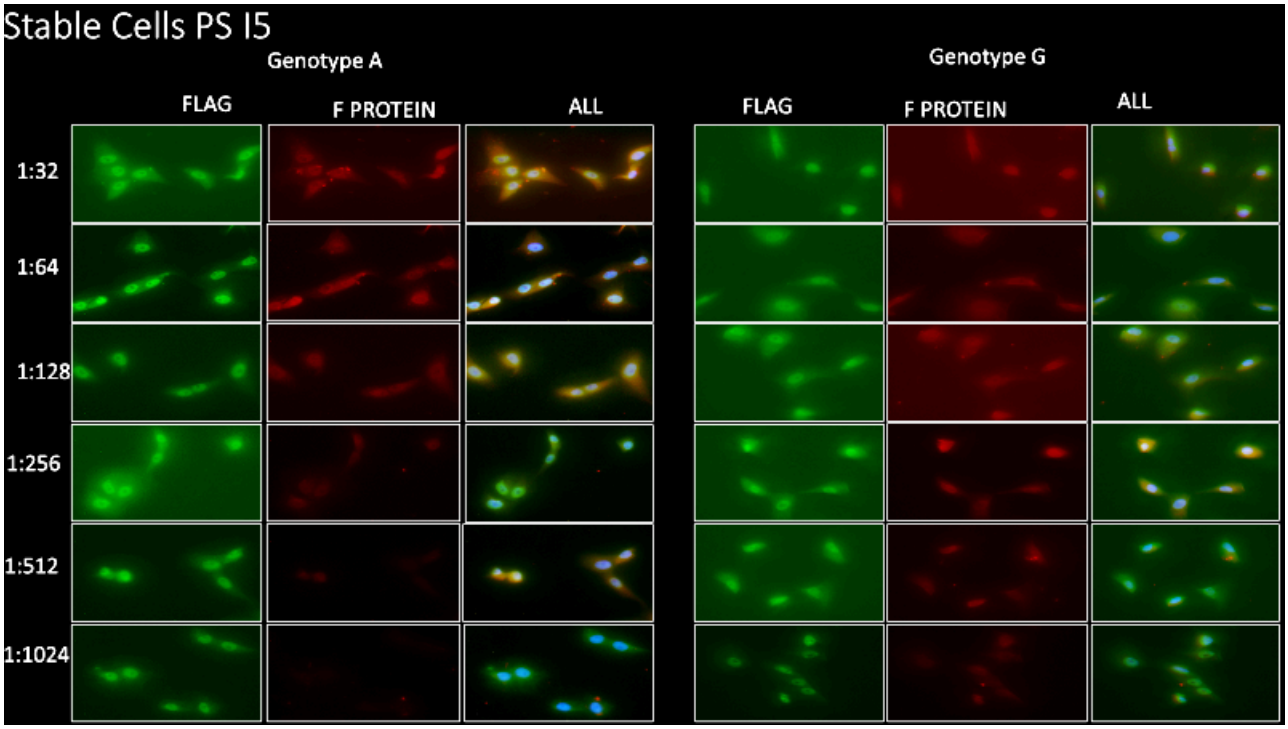


Figure A-68c. Stable cells Infected Sample#5.

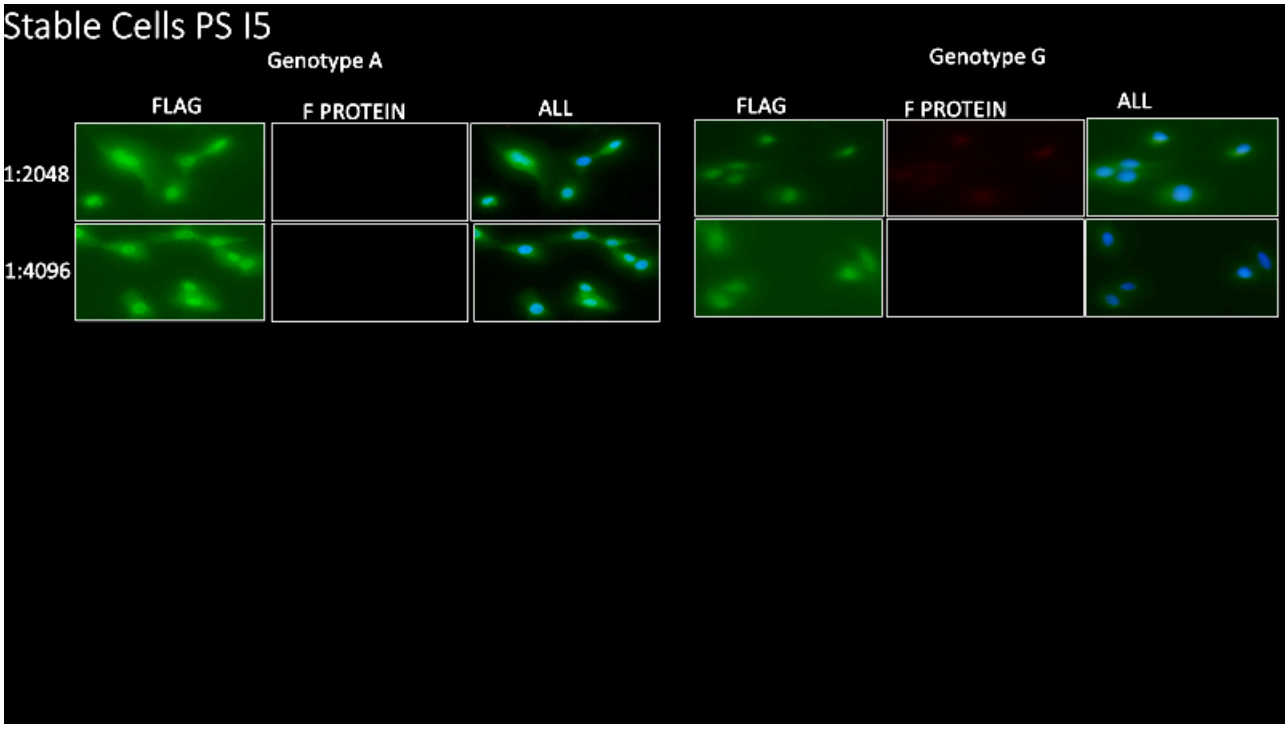


Figure A-68d. Stable cells Infected Sample#5.

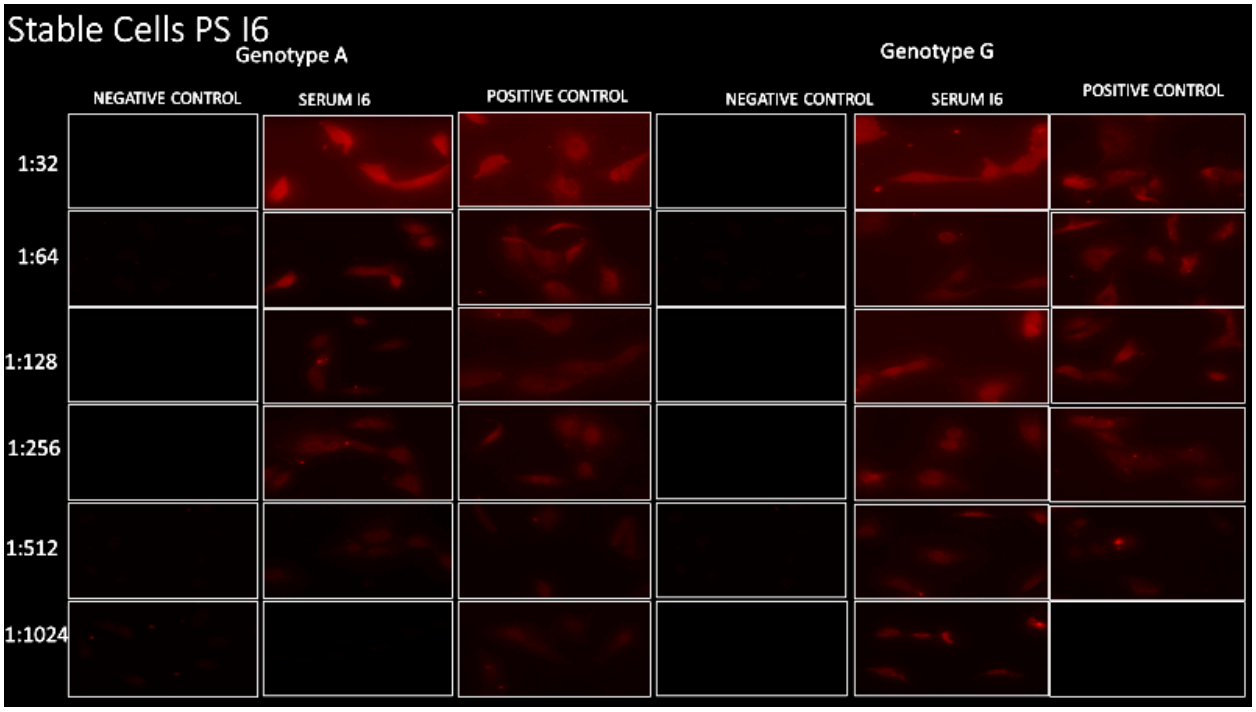


Figure A-69a. Stable cells Infected Sample#6.

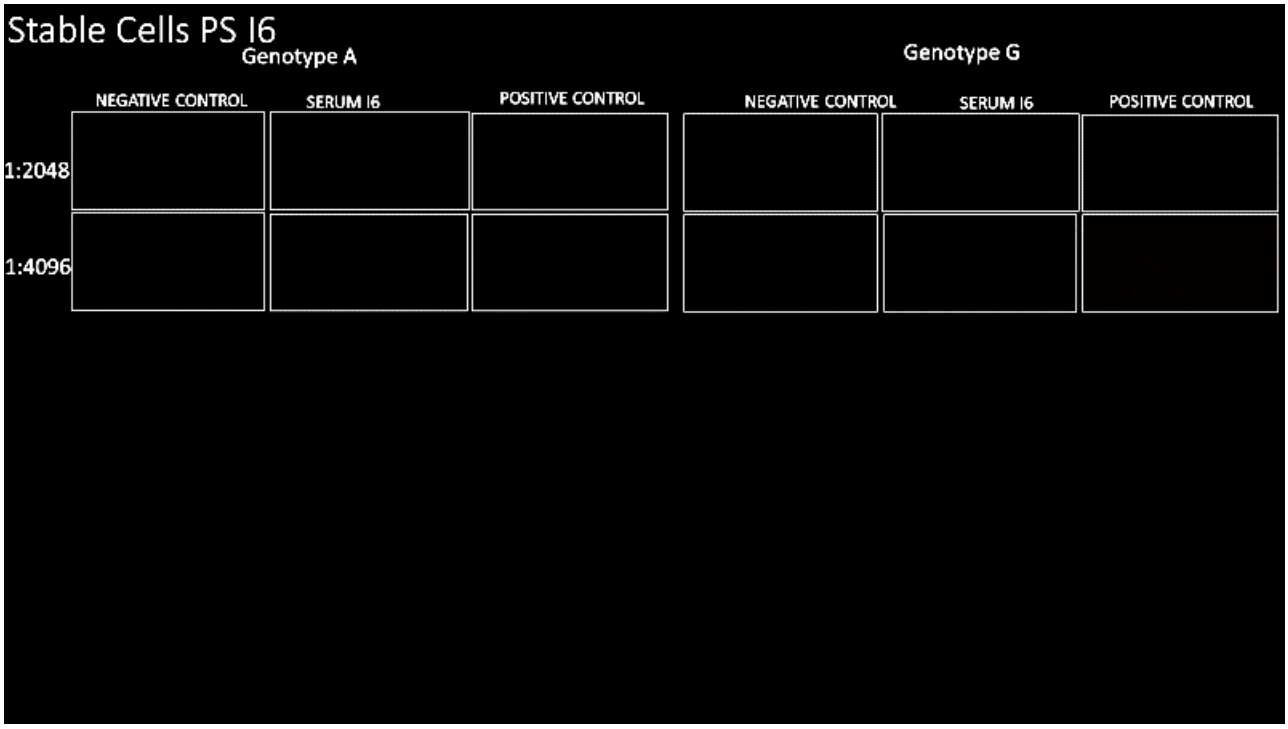


Figure A-69b. Stable cells Infected Sample#6.

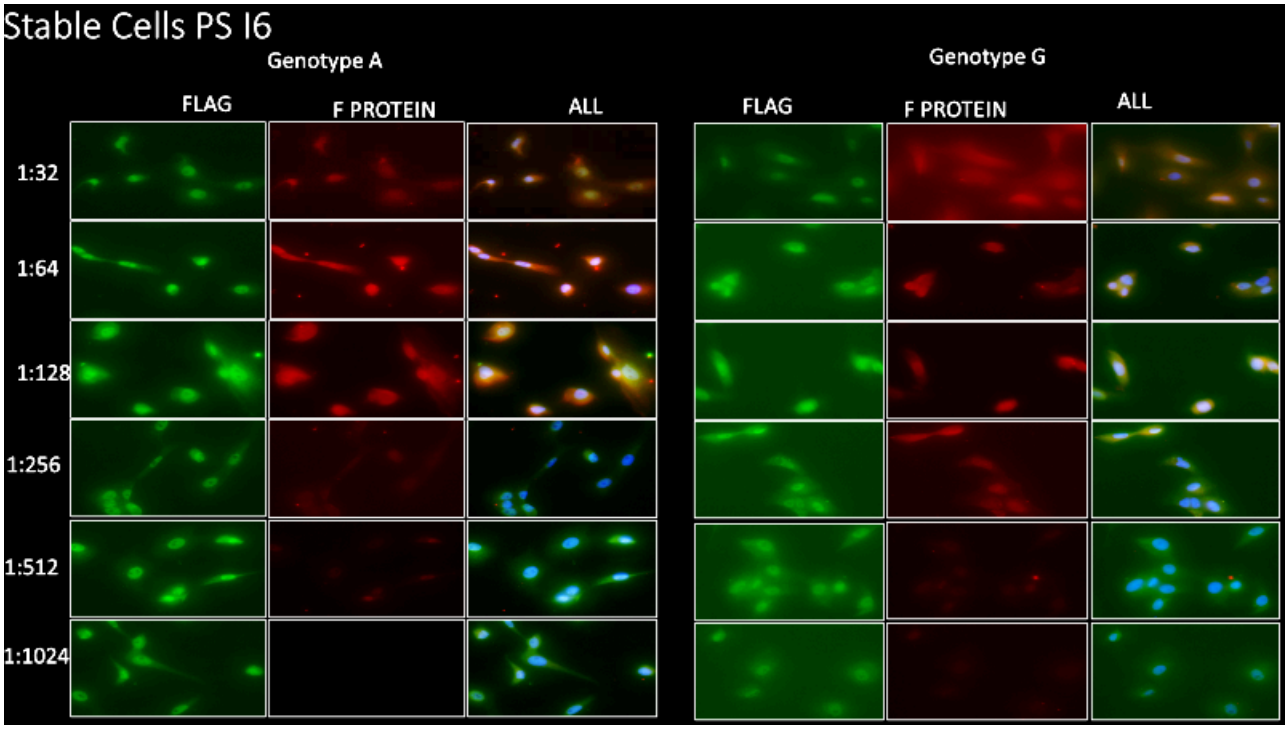


Figure A-69c. Stable cells Infected Sample#6.

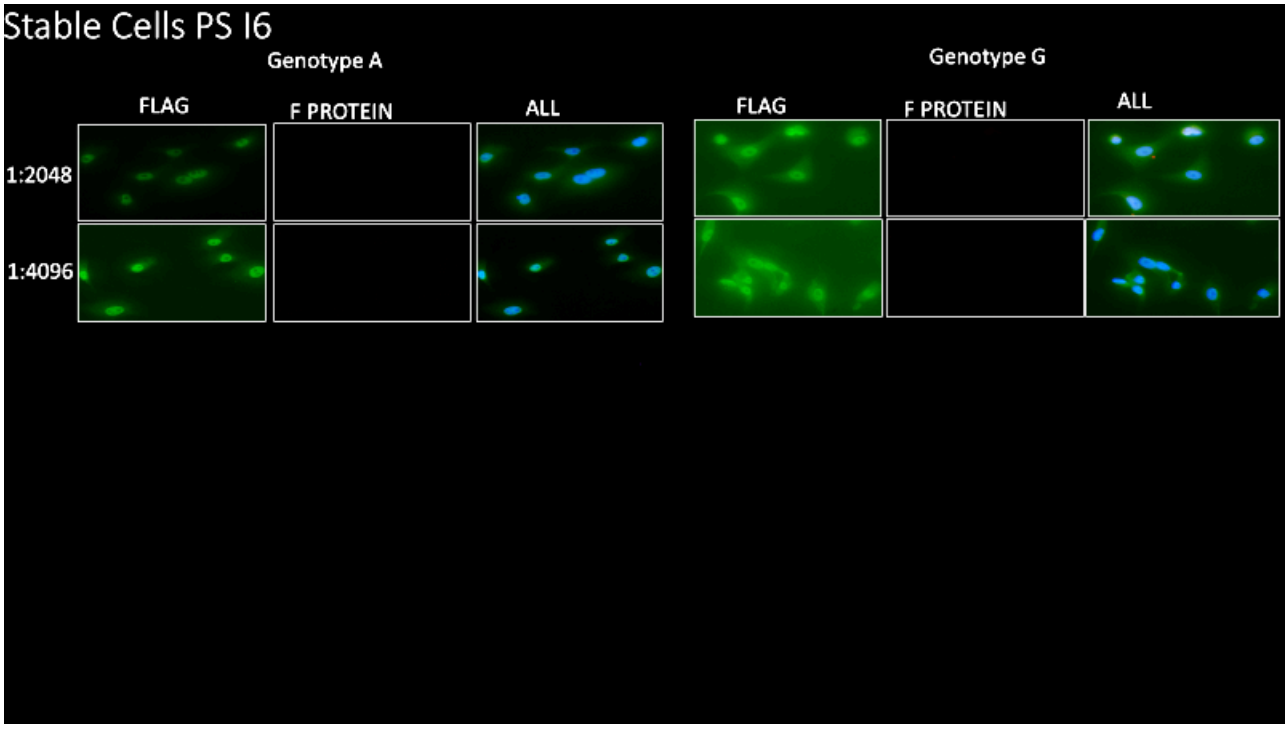


Figure A-69d. Stable cells Infected Sample#6.

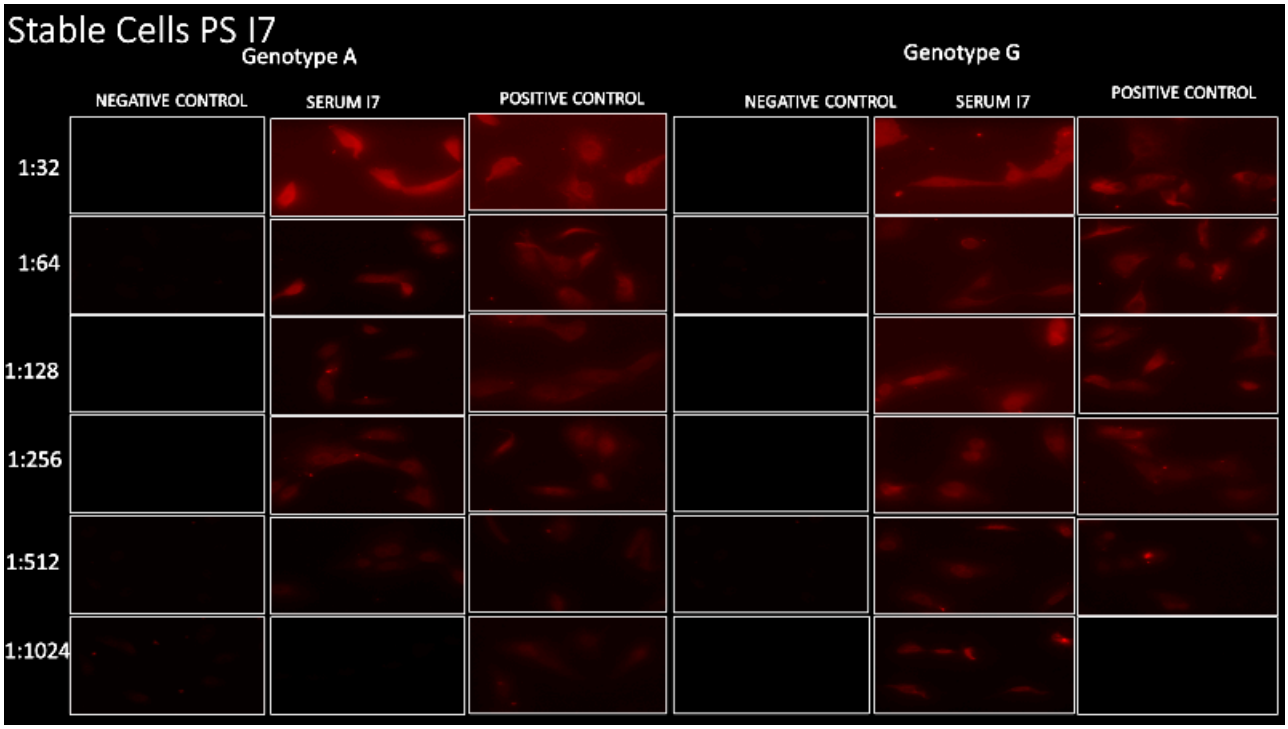


Figure A-70a. Stable cells Infected Sample#7.

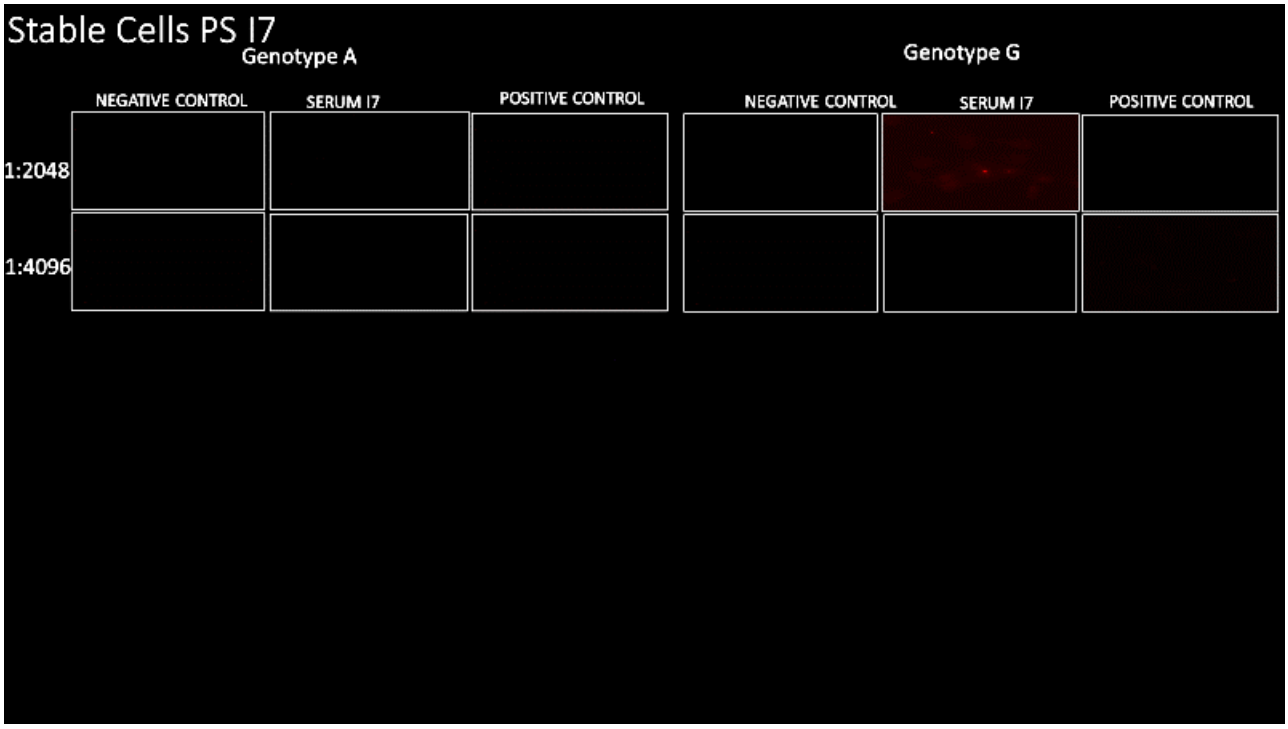


Figure A-70b. Stable cells Infected Sample#7.

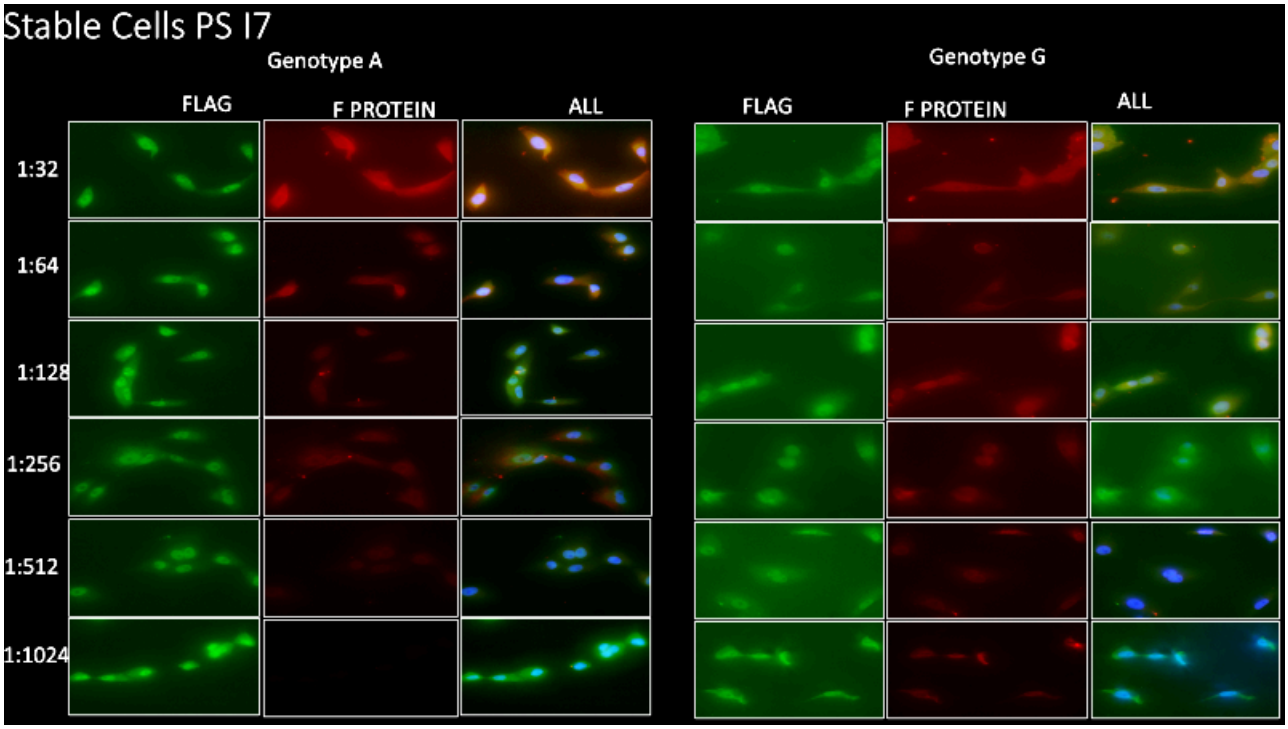


Figure A-70c. Stable cells Infected Sample#7.

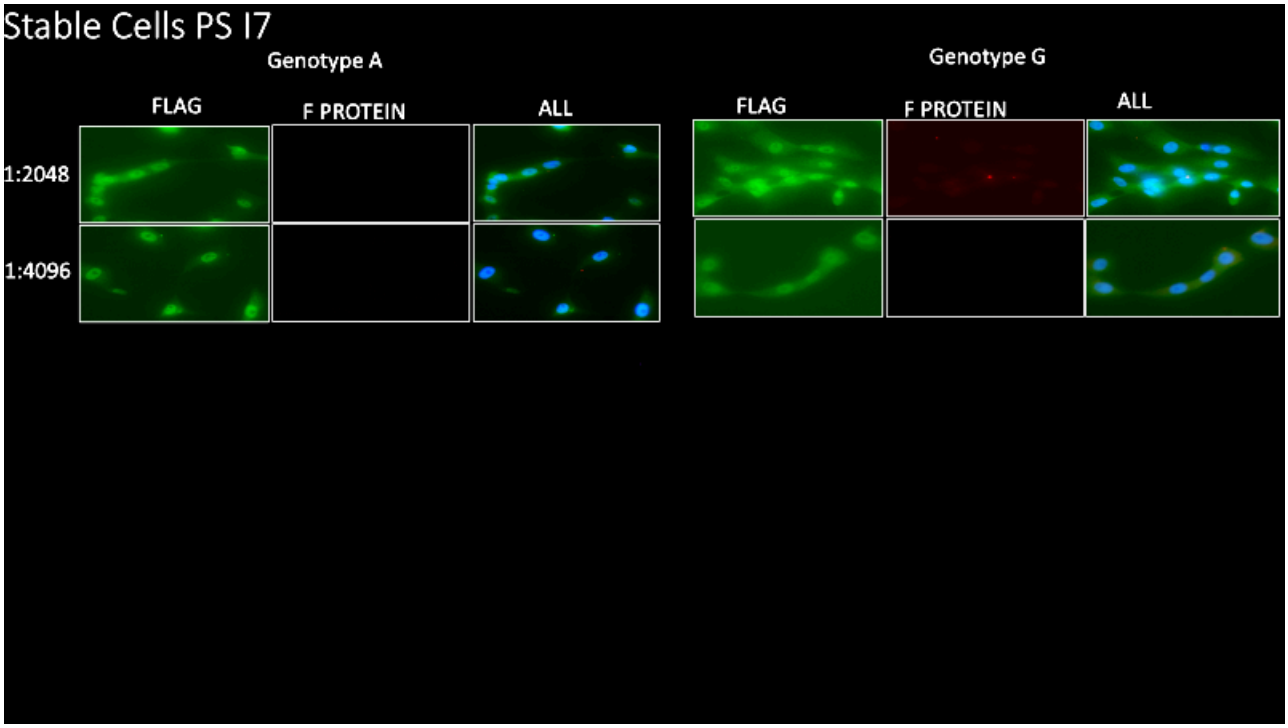


Figure A-70d. Stable cells Infected Sample#7.

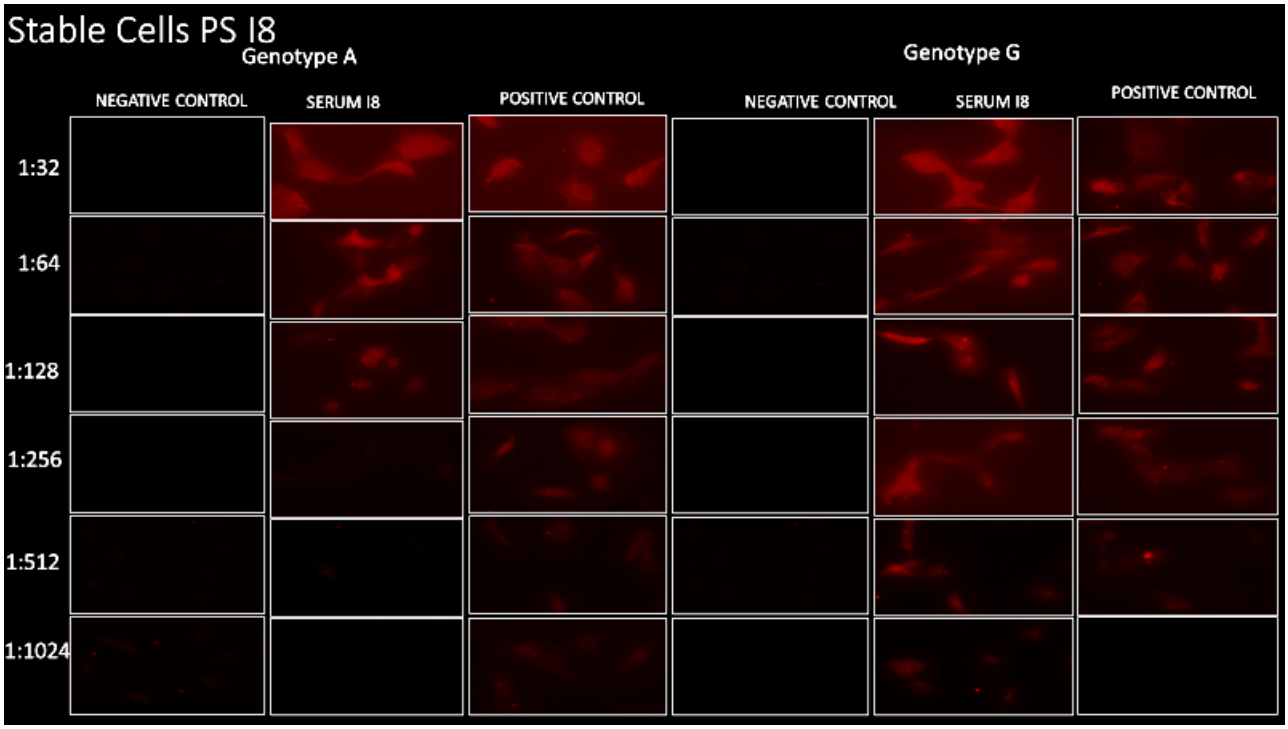


Figure A-71a. Stable cells Infected Sample#8.

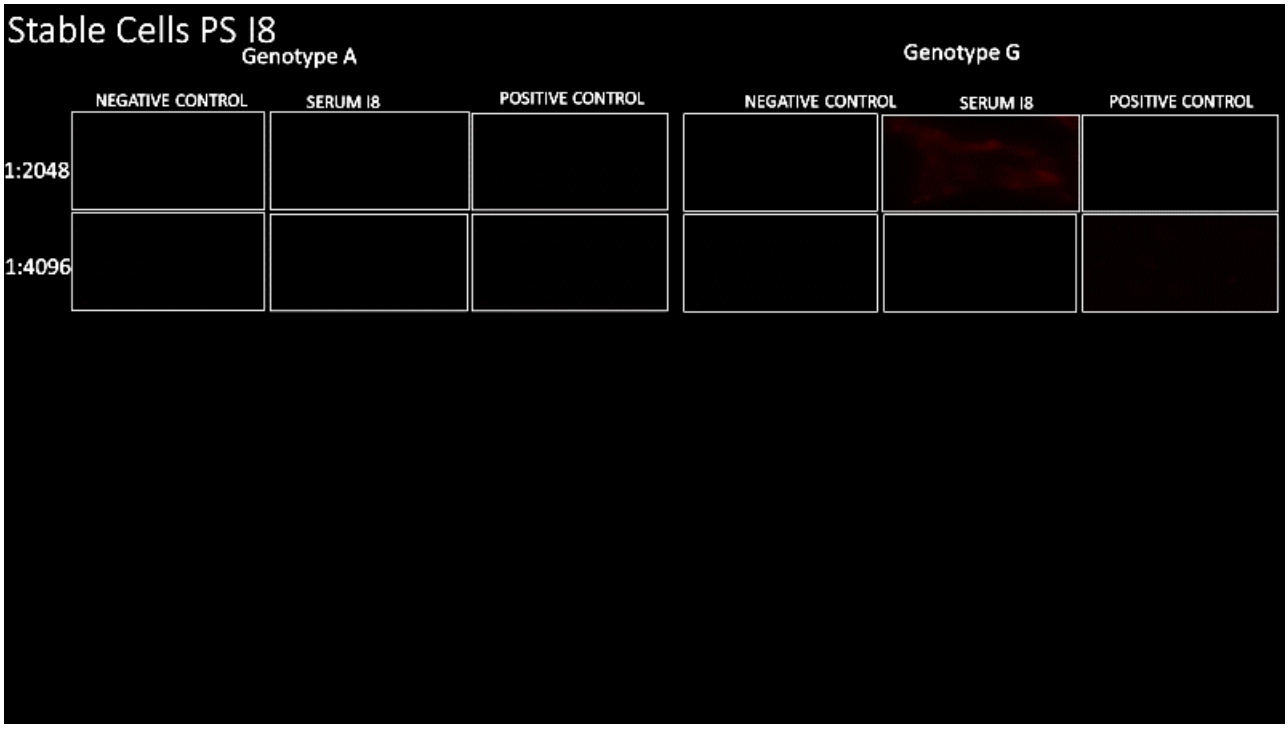


Figure A-71b. Stable cells Infected Sample#8.

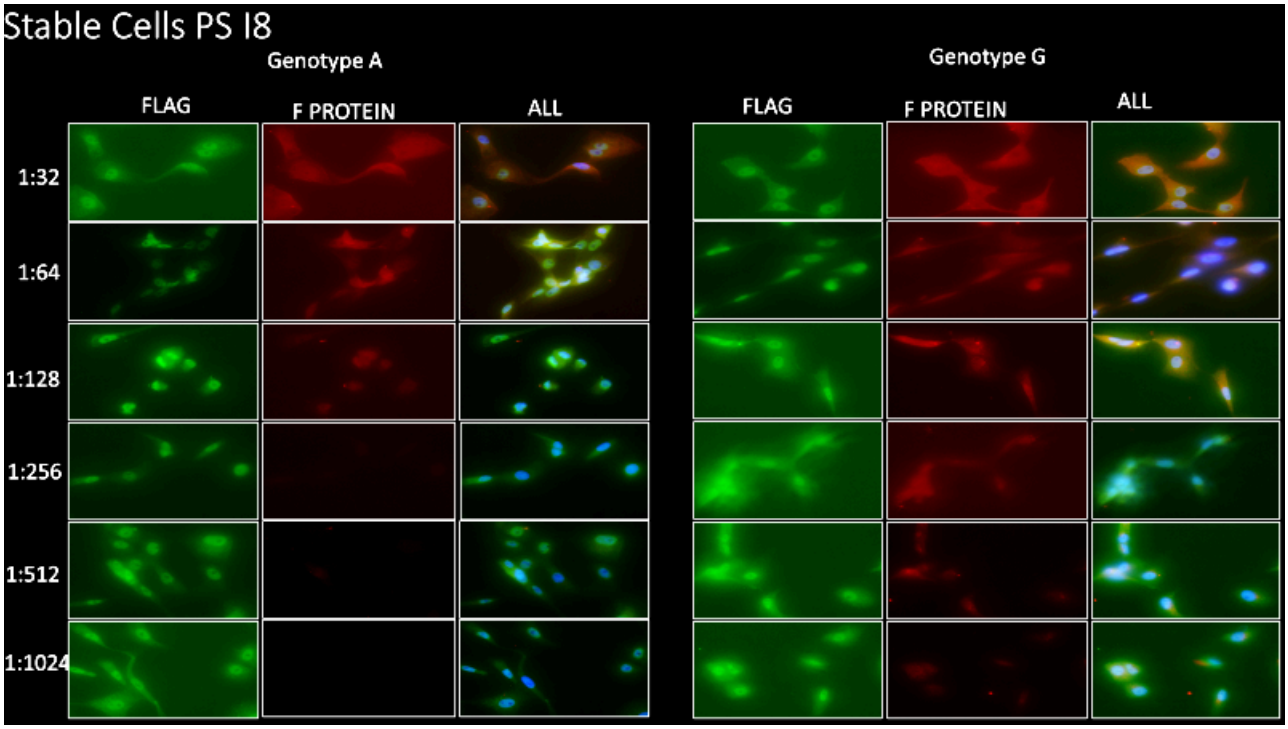


Figure A-71c. Stable cells Infected Sample#8.

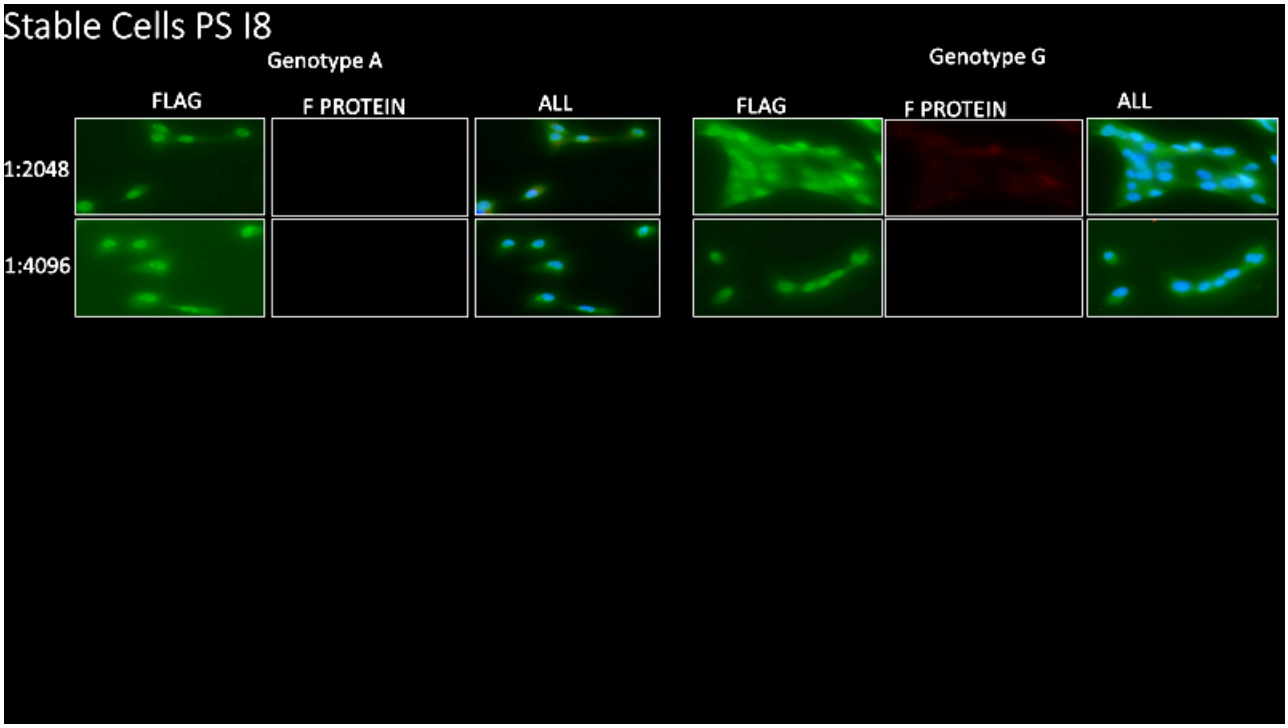


Figure A-71d. Stable cells Infected Sample#8.

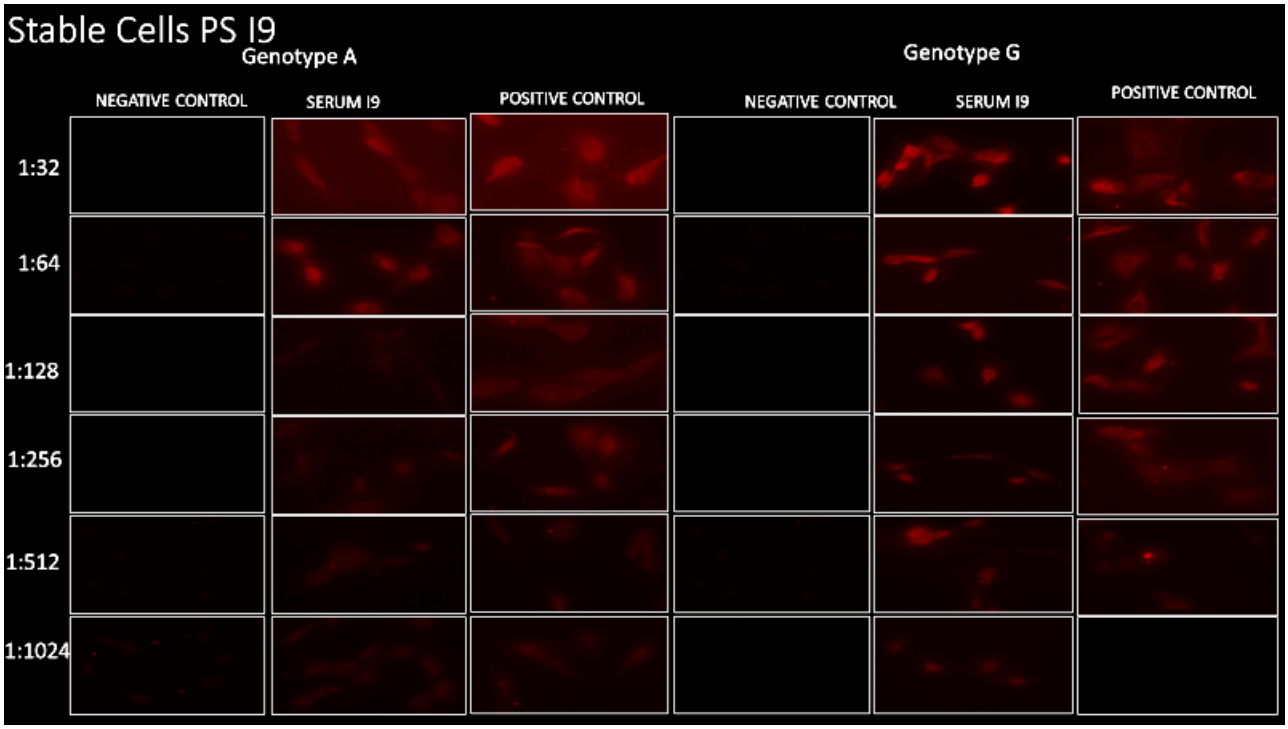


Figure A-72a. Stable cells Infected Sample#9.

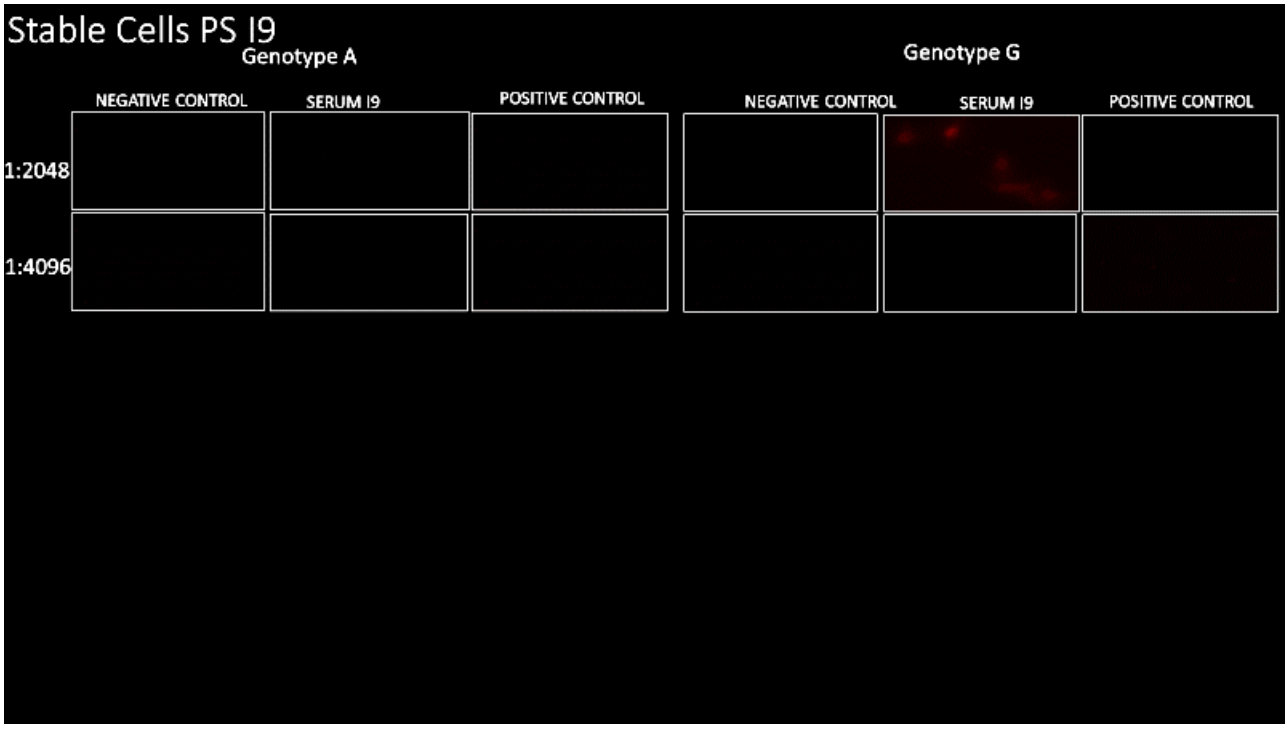


Figure A-72b. Stable cells Infected Sample#9.

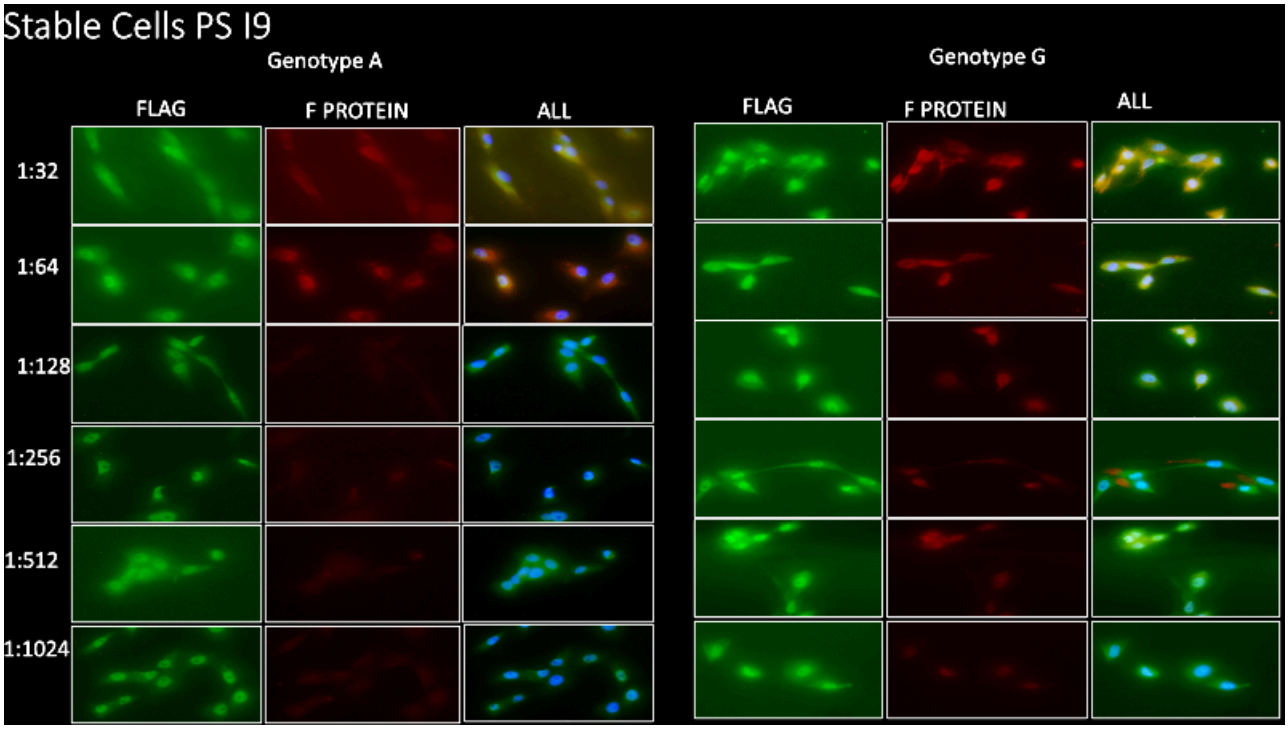


Figure A-72c. Stable cells Infected Sample#9.

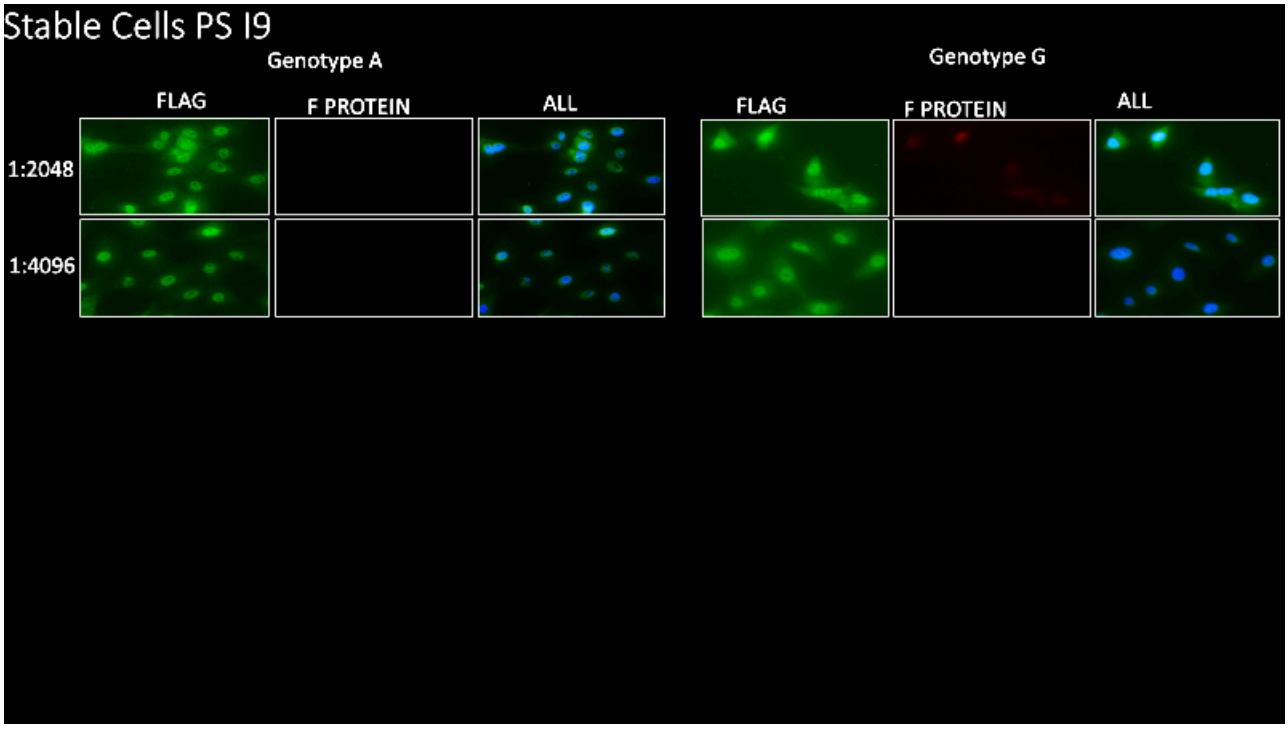


Figure A-72d. Stable cells Infected Sample#9.

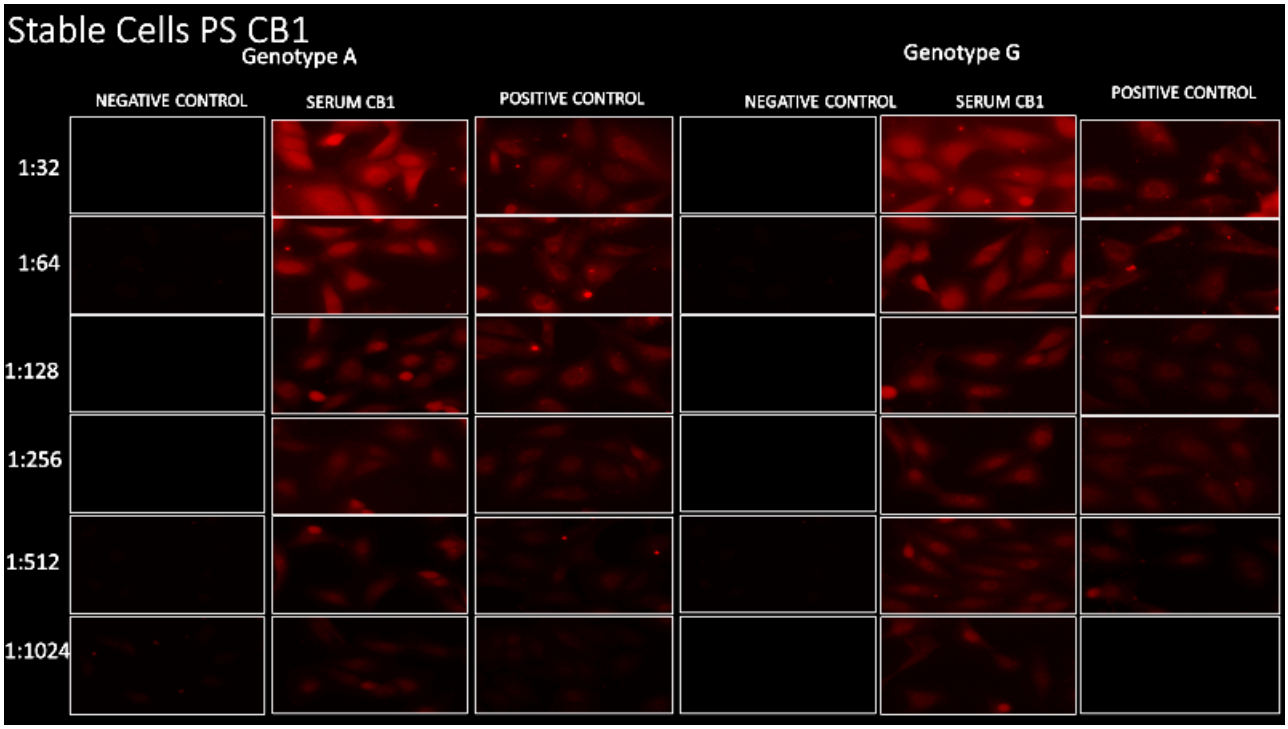


Figure A-73a. Stable cells Vaccine-Infected (CB) Sample#1.

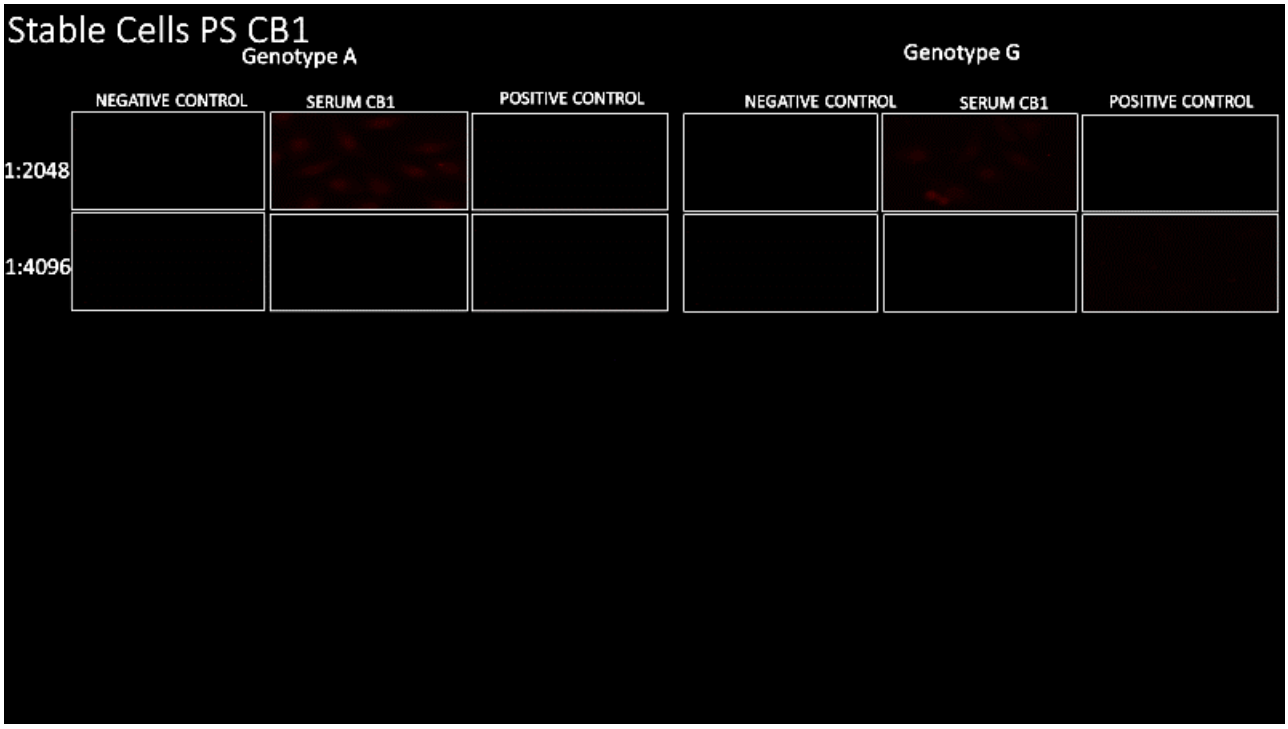


Figure A-73b. Stable cells Vaccine-Infected (CB) Sample#1.

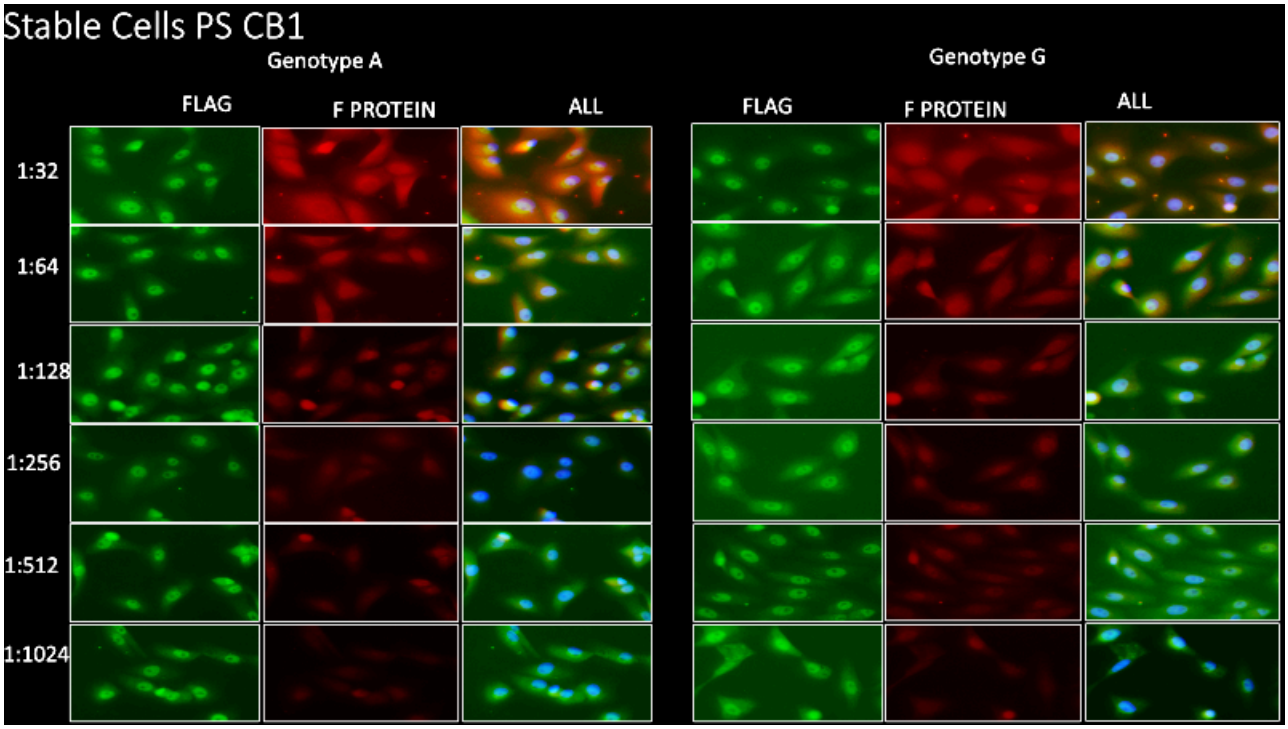


Figure A-73c. Stable cells Vaccine-Infected (CB) Sample#1.

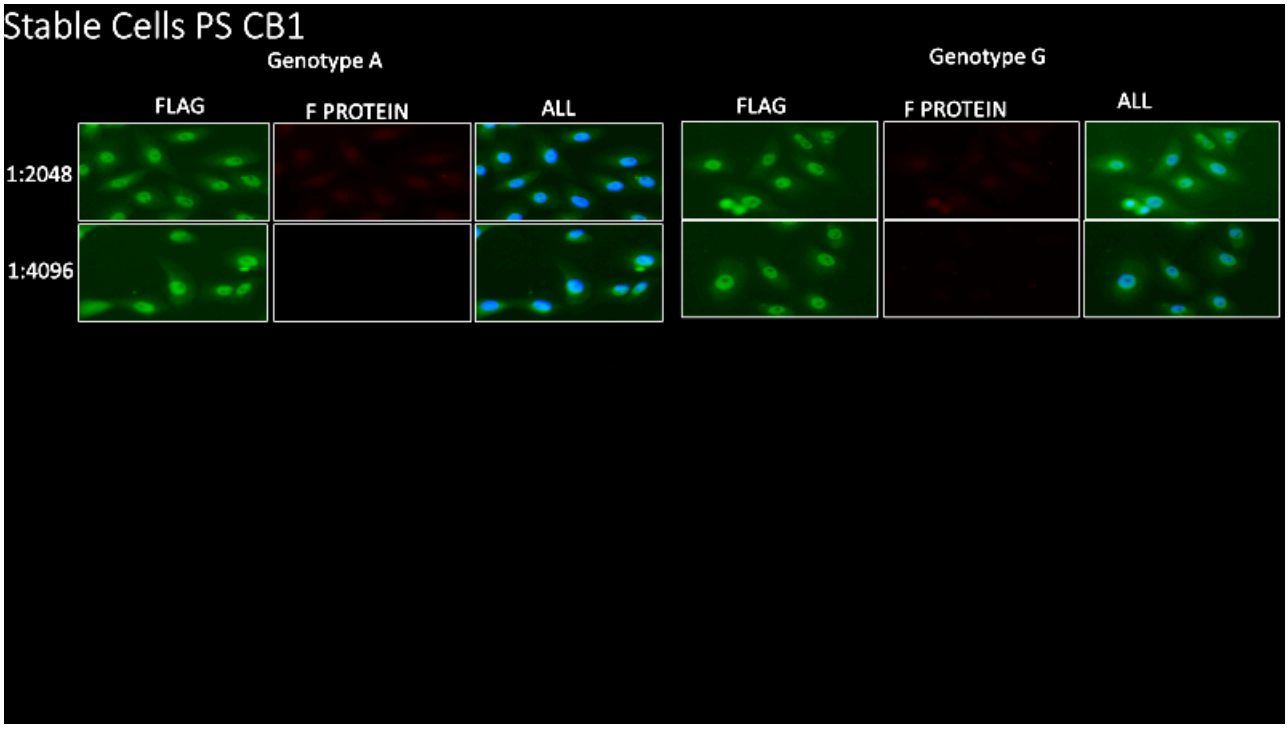


Figure A-73d. Stable cells Vaccine-Infected (CB) Sample#1.

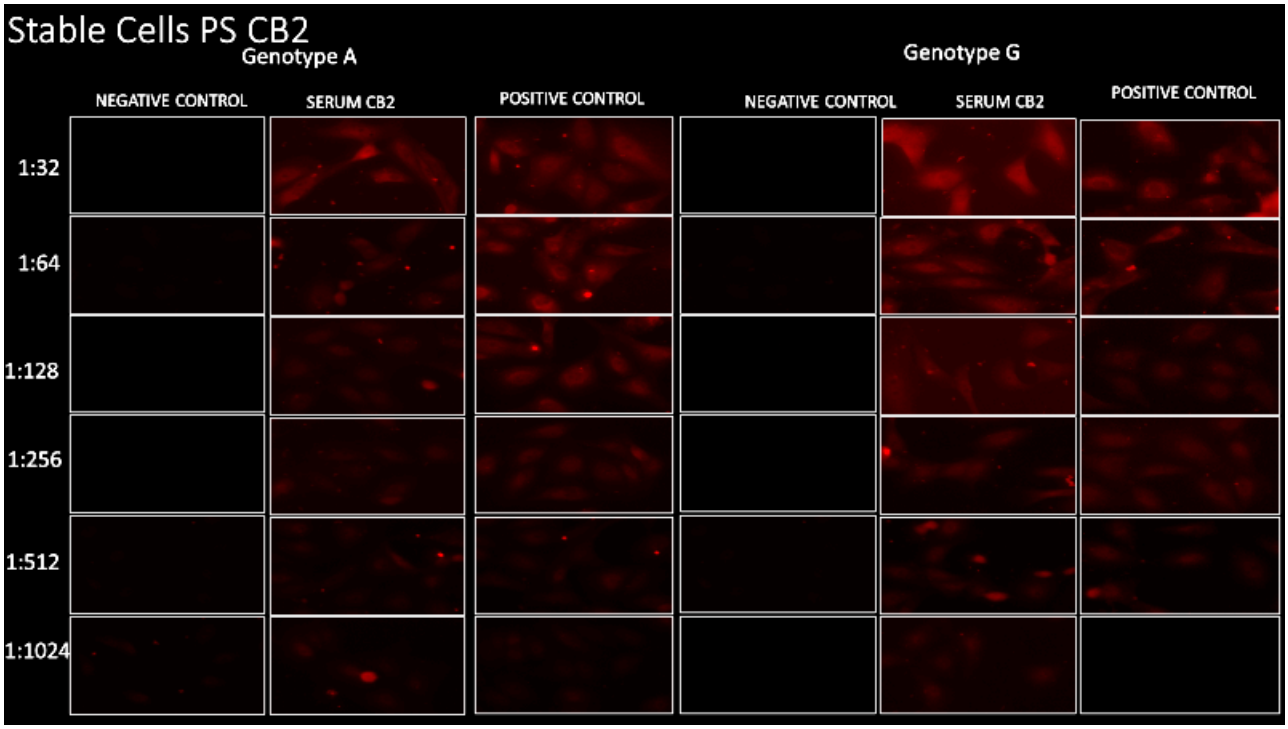


Figure A-74a. Stable cells Vaccine-Infected (CB) Sample#2.

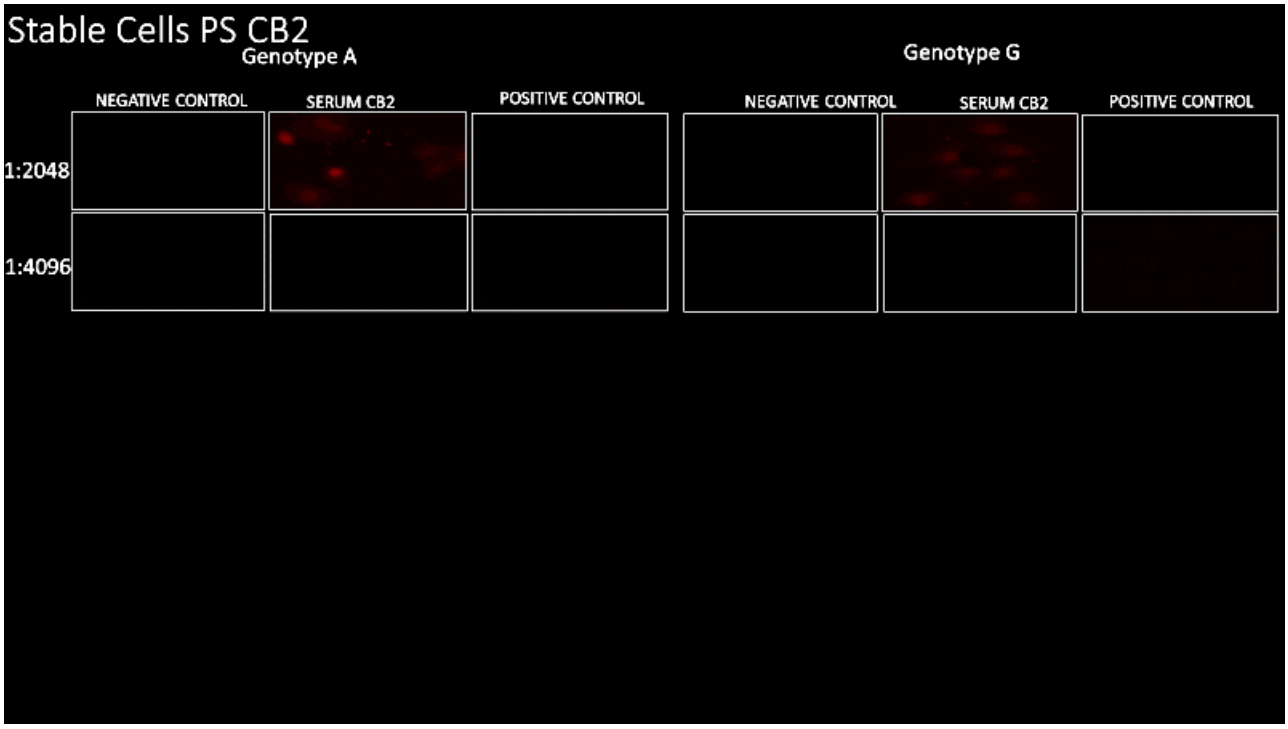


Figure A-74b. Stable cells Vaccine-Infected (CB) Sample#2.

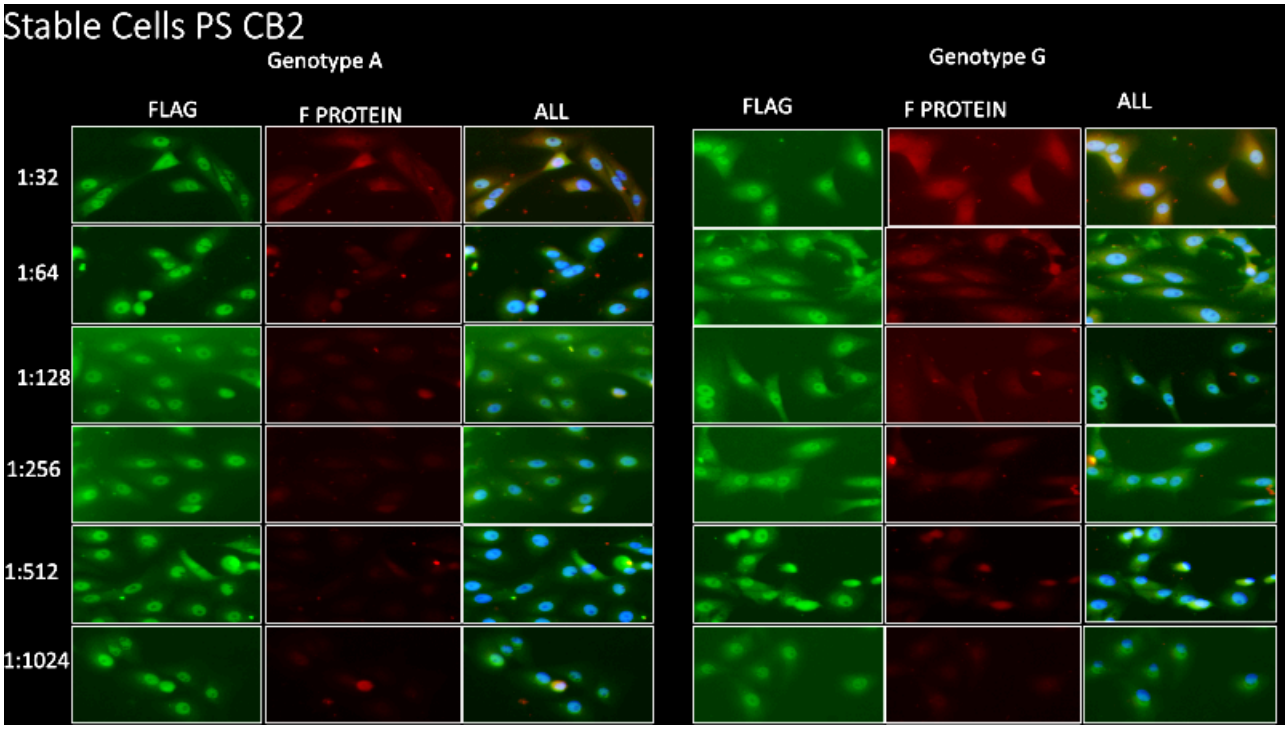


Figure A-74c. Stable cells Vaccine-Infected (CB) Sample#2.

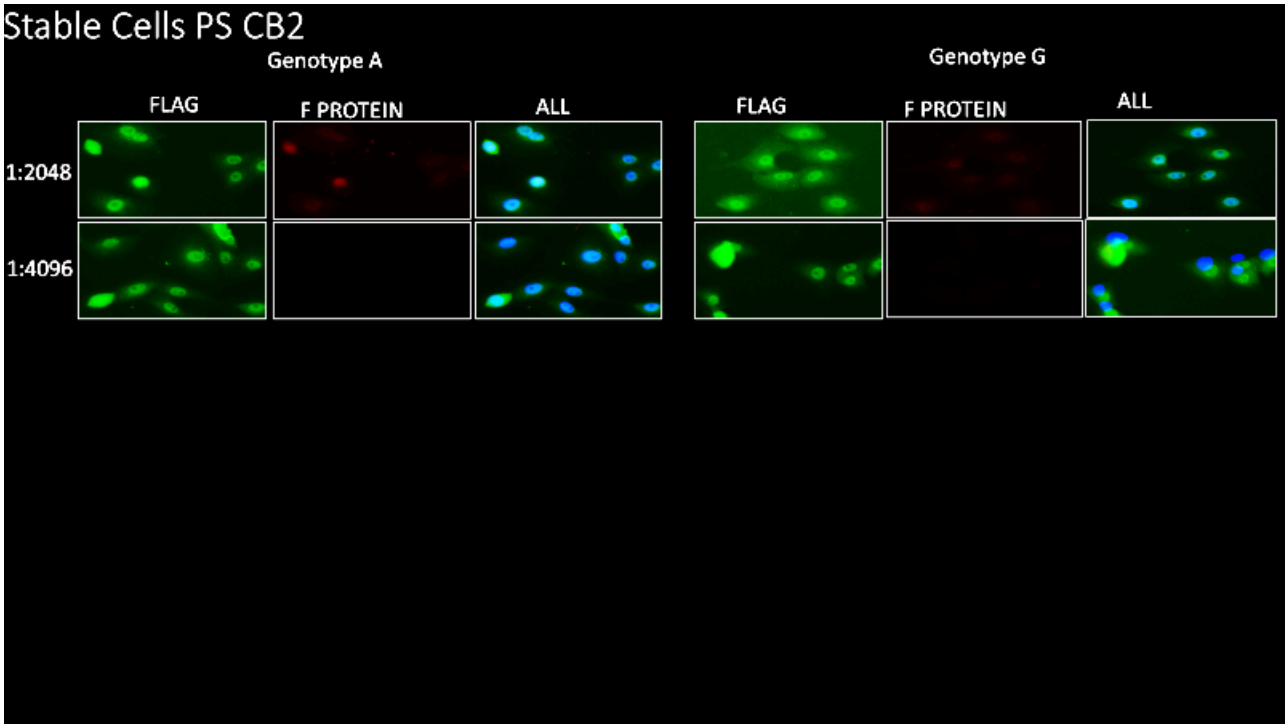


Figure A-74d. Stable cells Vaccine-Infected (CB) Sample#2.

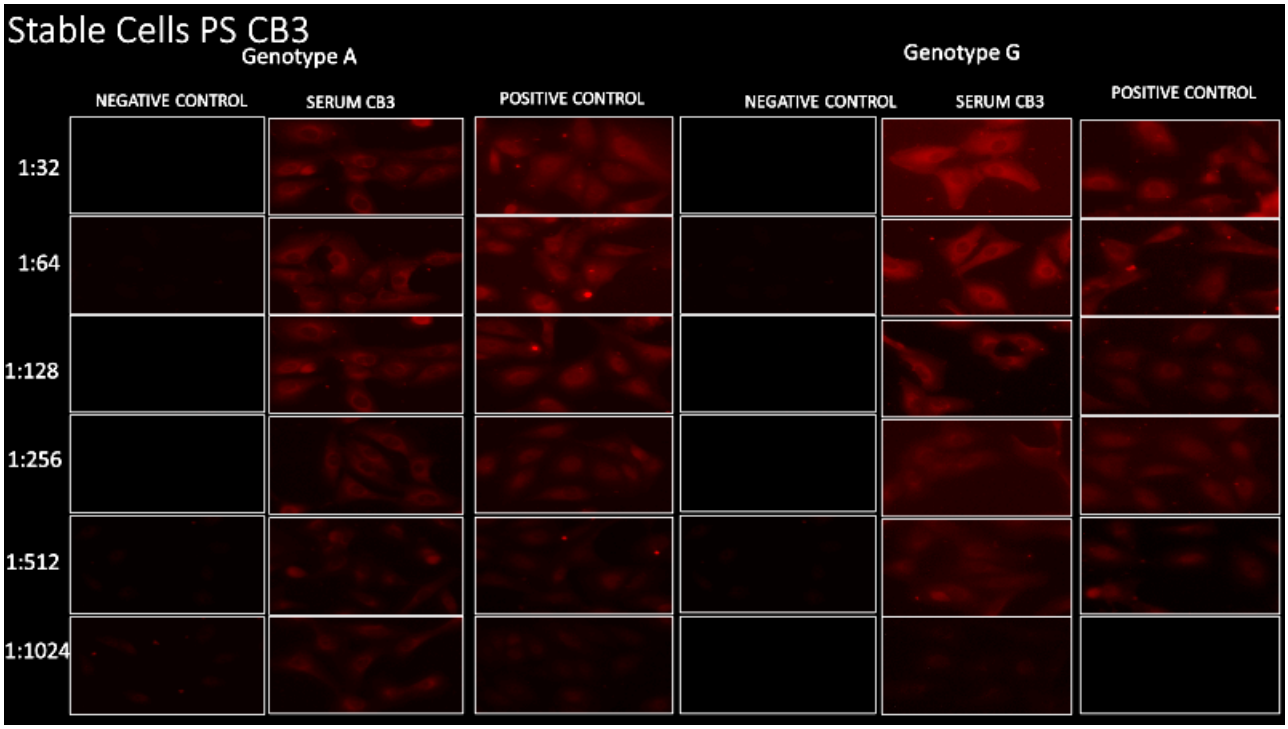


Figure A-75a. Stable cells Vaccine-Infected (CB) Sample#3.

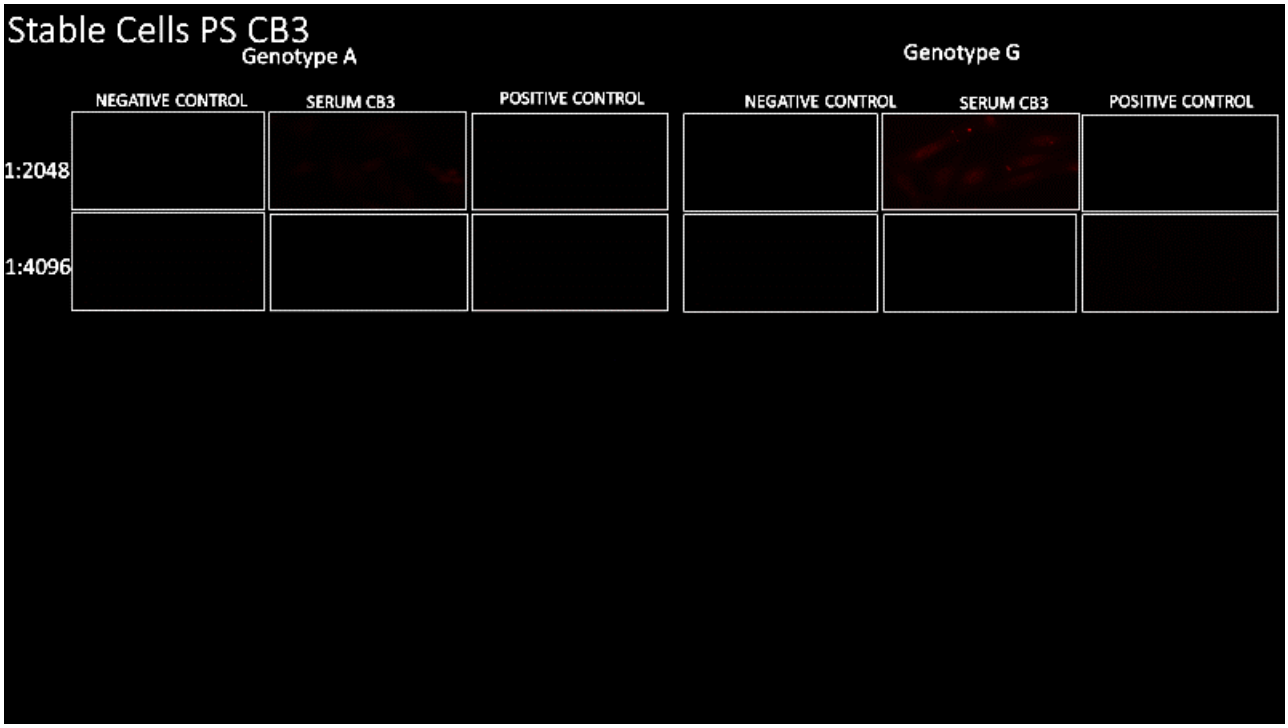


Figure A-75b. Stable cells Vaccine-Infected (CB) Sample#3.

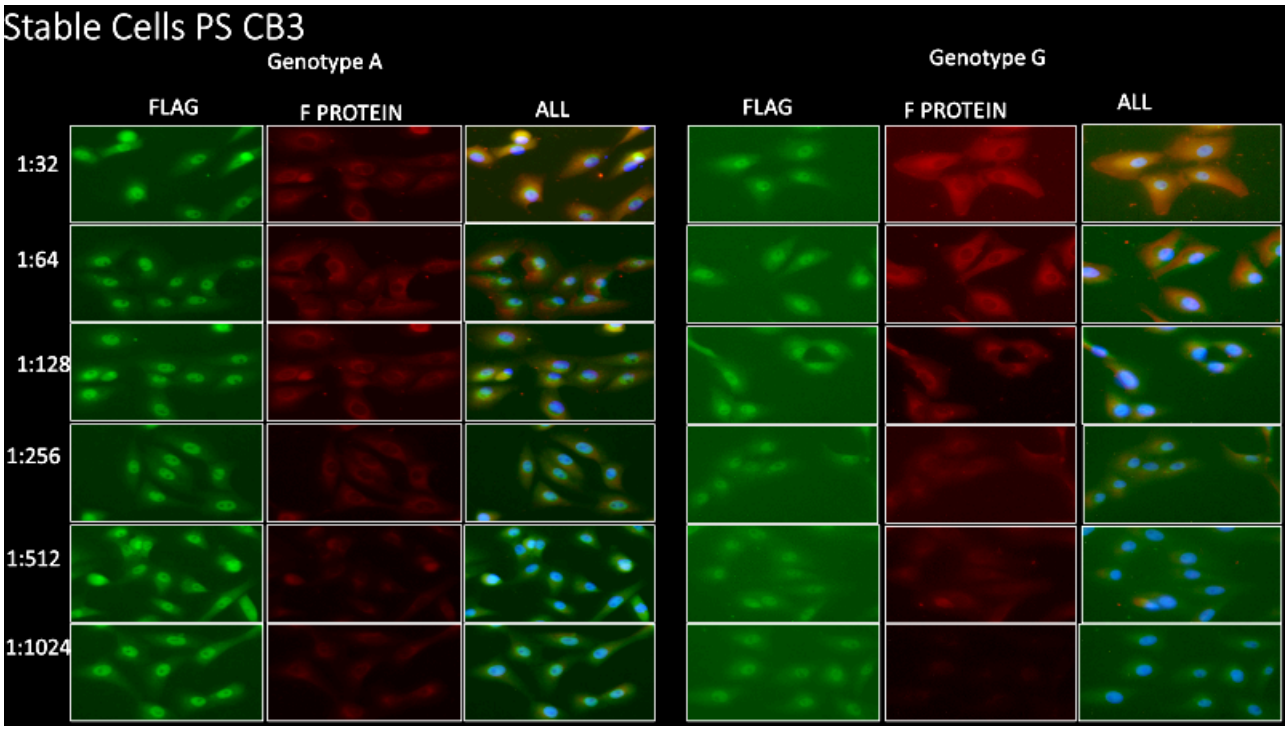


Figure A-75c. Stable cells Vaccine-Infected (CB) Sample#3.

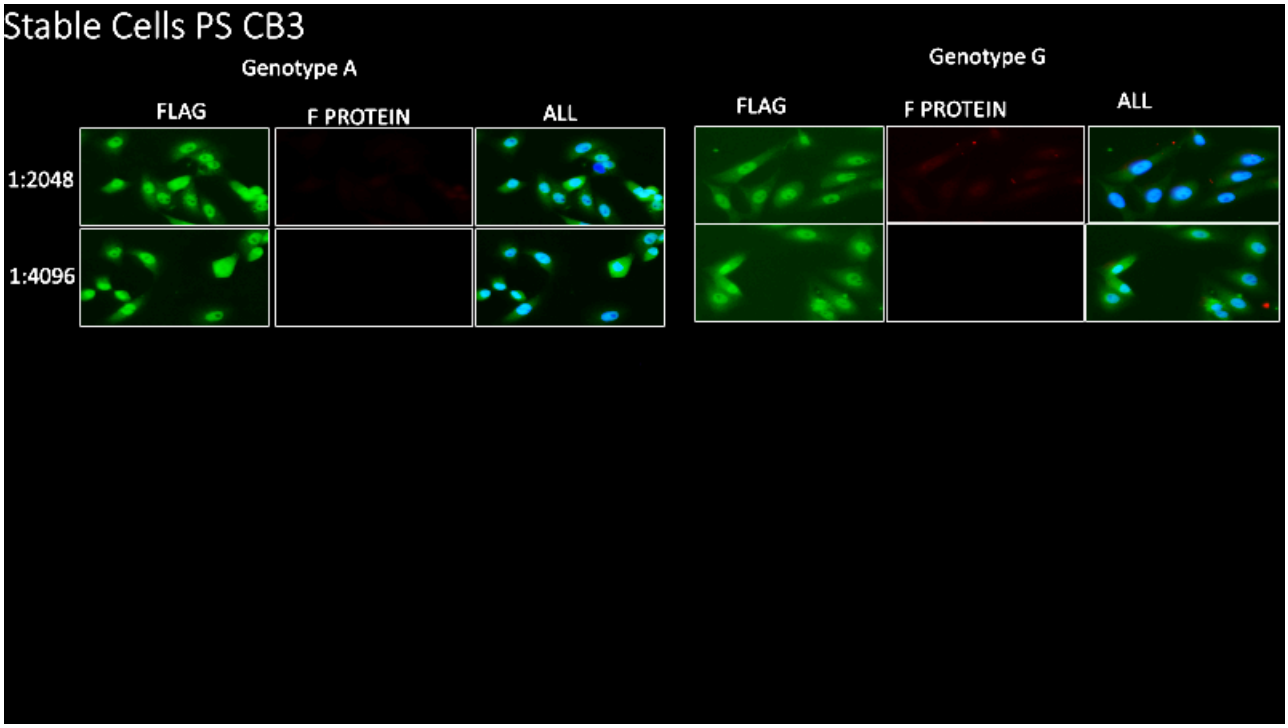


Figure A-75d. Stable cells Vaccine-Infected (CB) Sample#3.

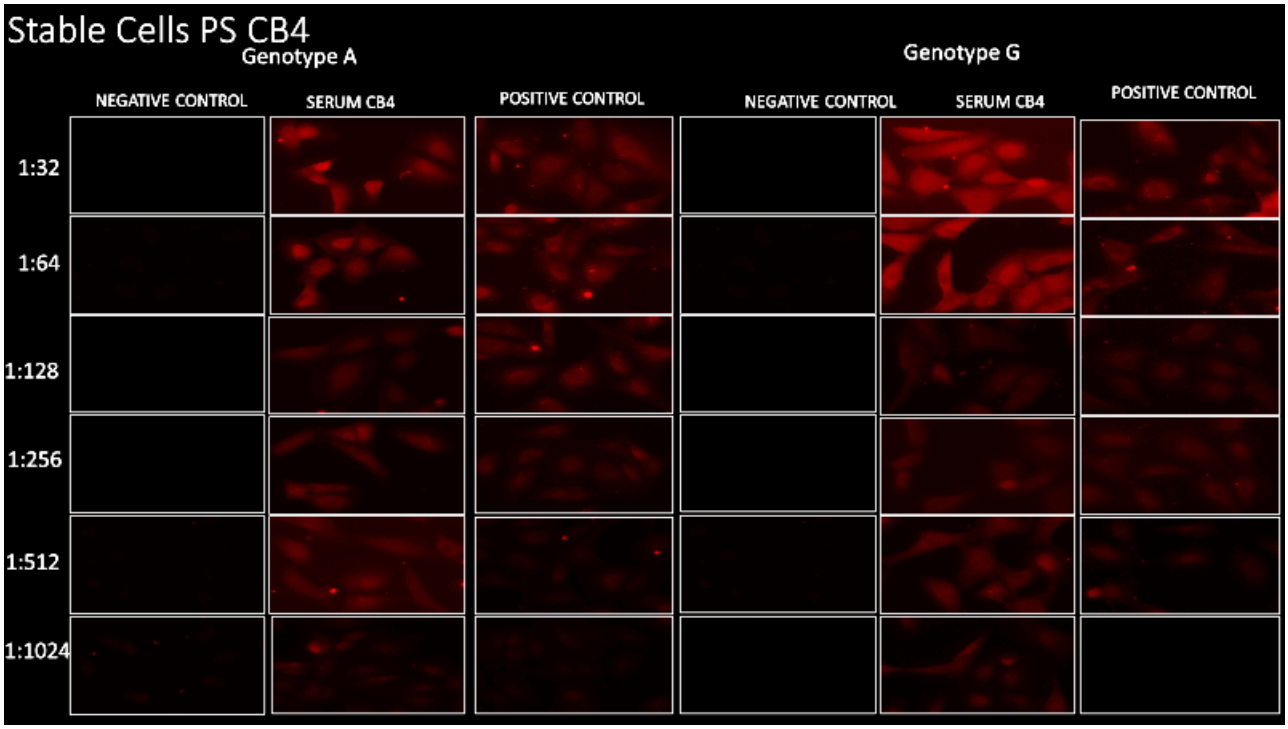


Figure A-76a. Stable cells Vaccine-Infected (CB) Sample#4.

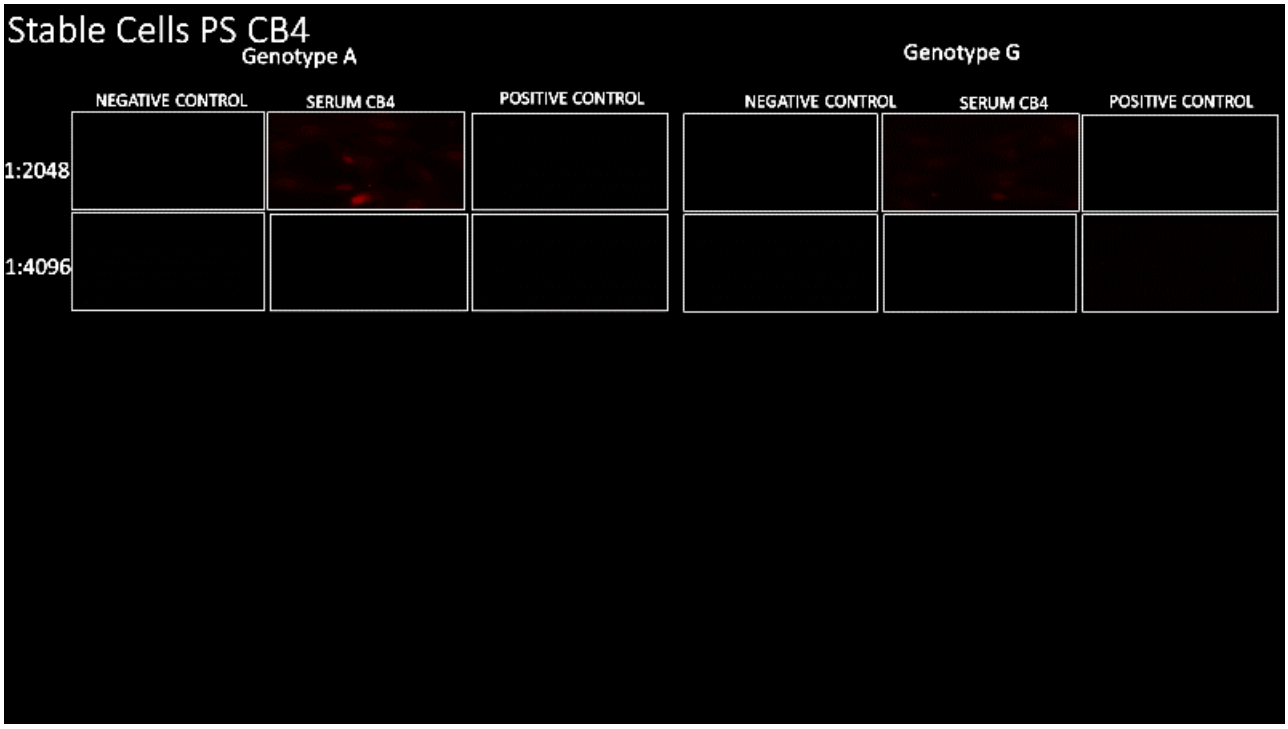


Figure A-76b. Stable cells Vaccine-Infected (CB) Sample#4.

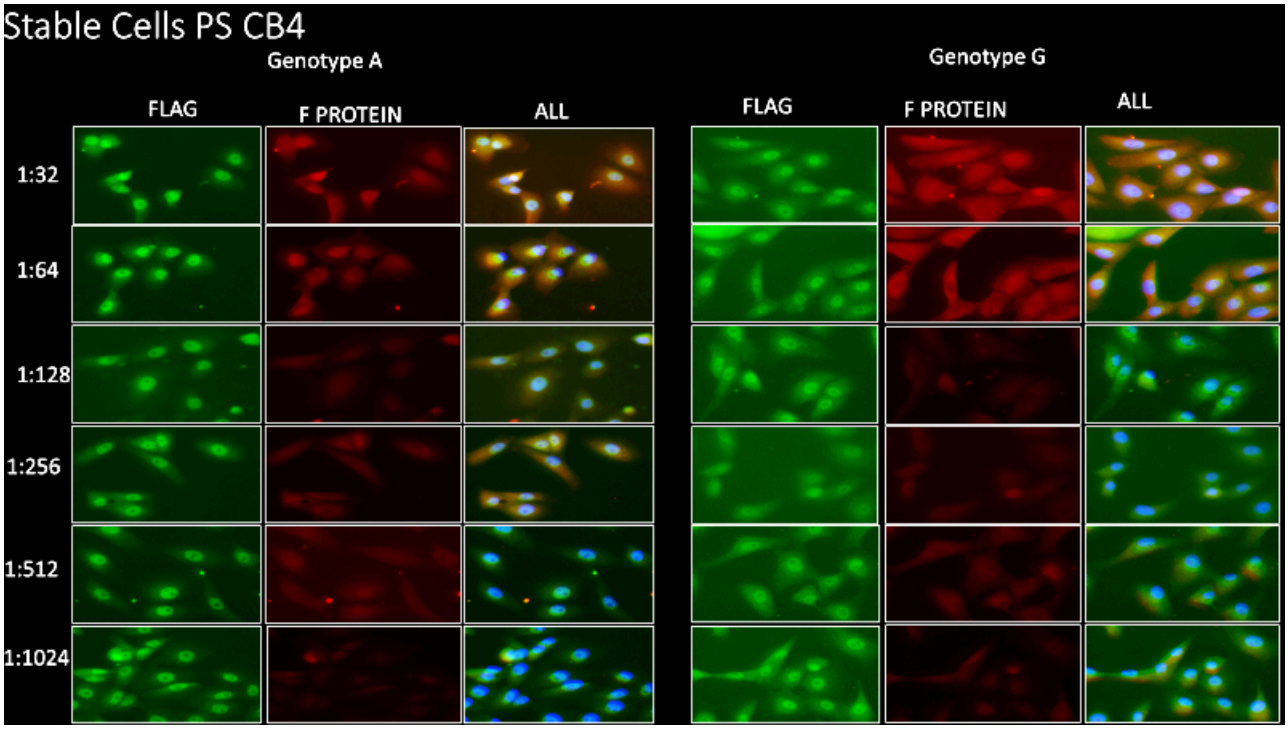


Figure A-76c. Stable cells Vaccine-Infected (CB) Sample#4.

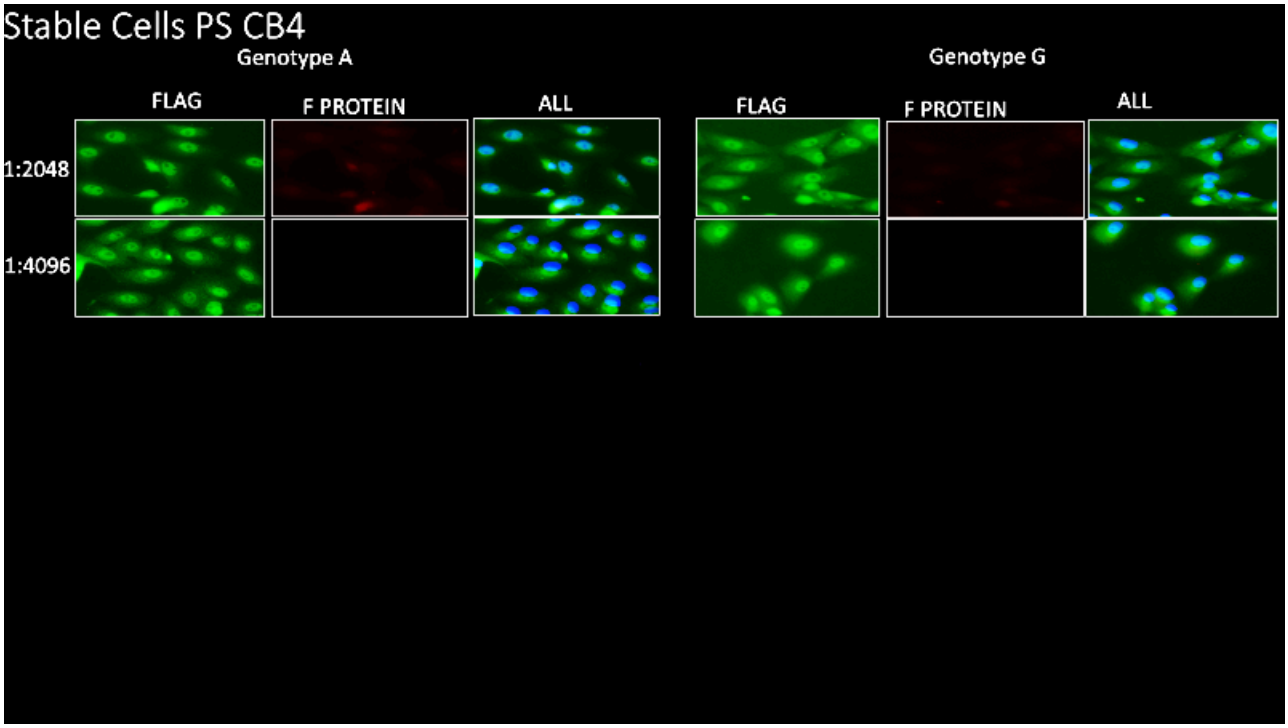


Figure A-76d. Stable cells Vaccine-Infected (CB) Sample#4.

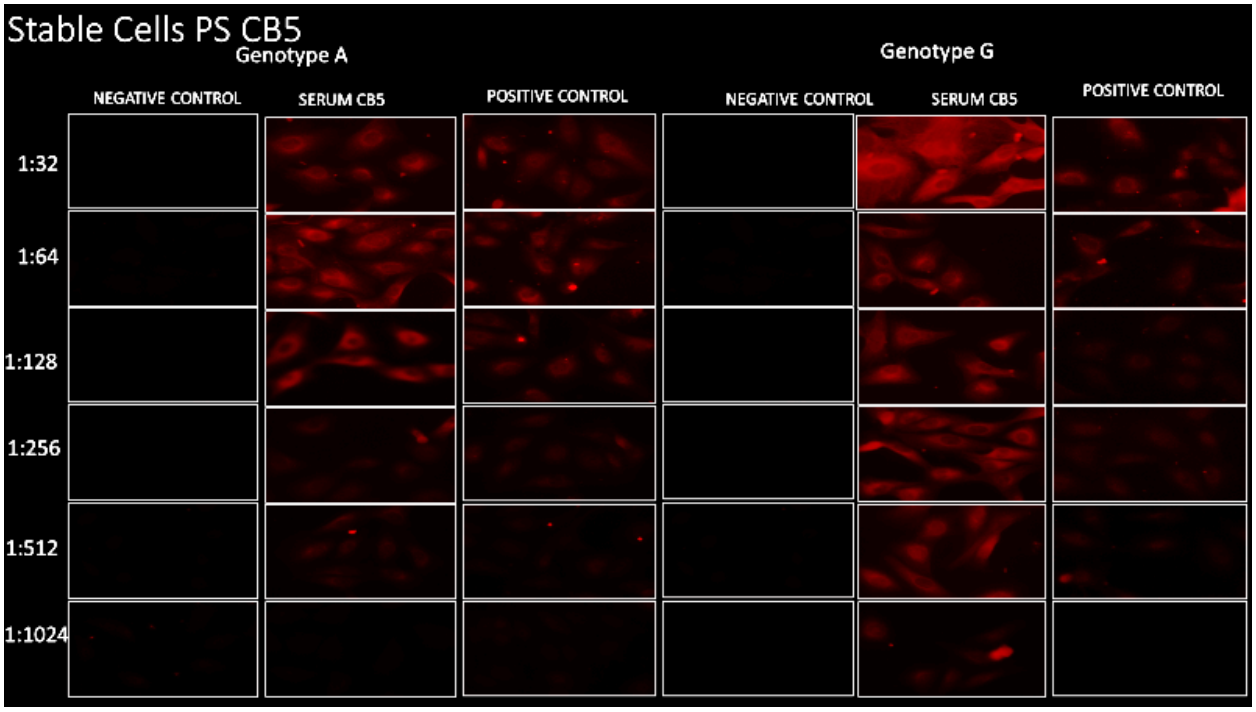


Figure A-77a. Stable cells Vaccine-Infected (CB) Sample#5.

Figure A-77b. Stable cells Vaccine-Infected (CB) Sample#5.

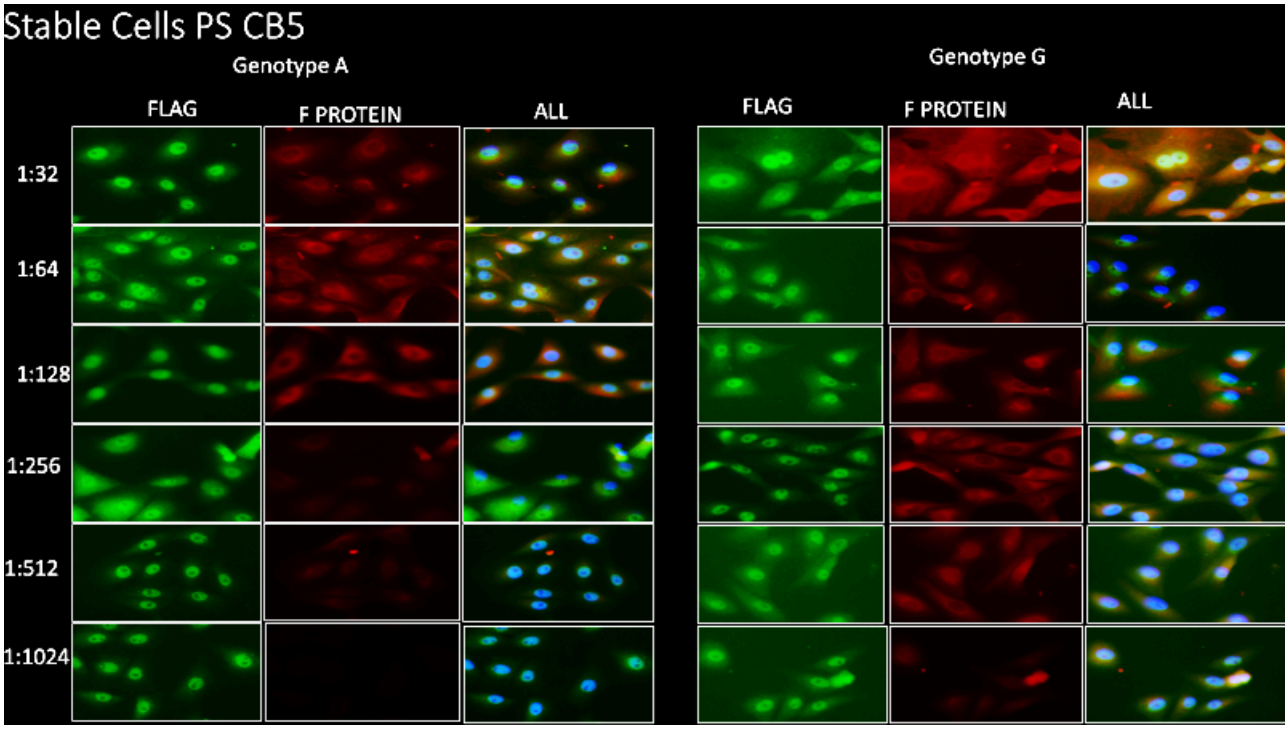


Figure A-77c. Stable cells Vaccine-Infected (CB) Sample#5.

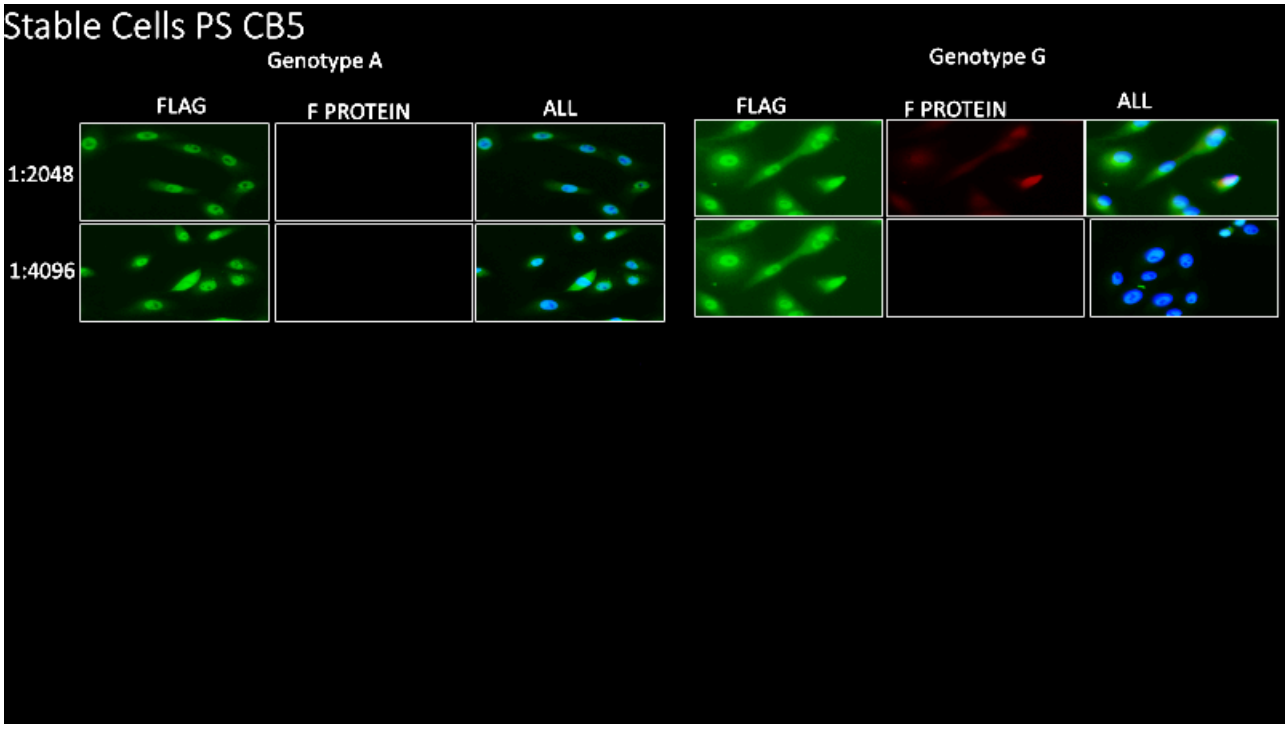


Figure A-77d. Stable cells Vaccine-Infected (CB) Sample#5.

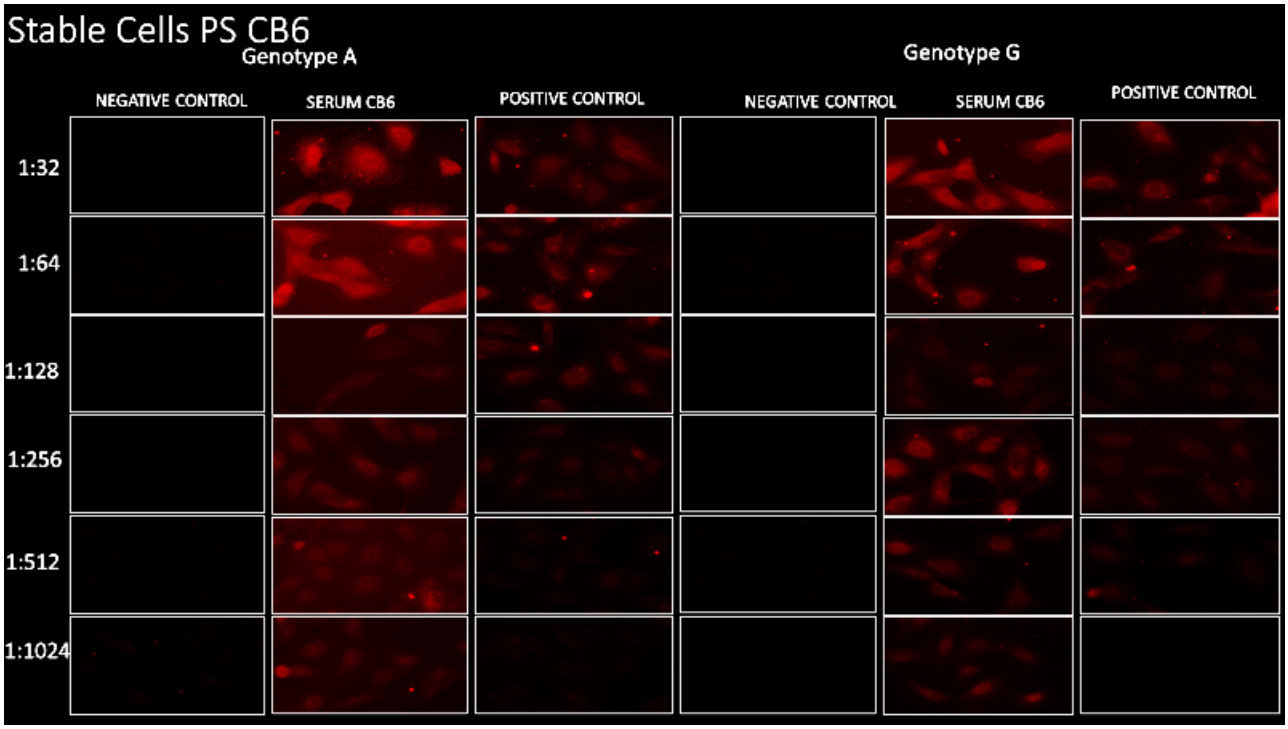


Figure A-78a. Stable cells Vaccine-Infected (CB) Sample#6.

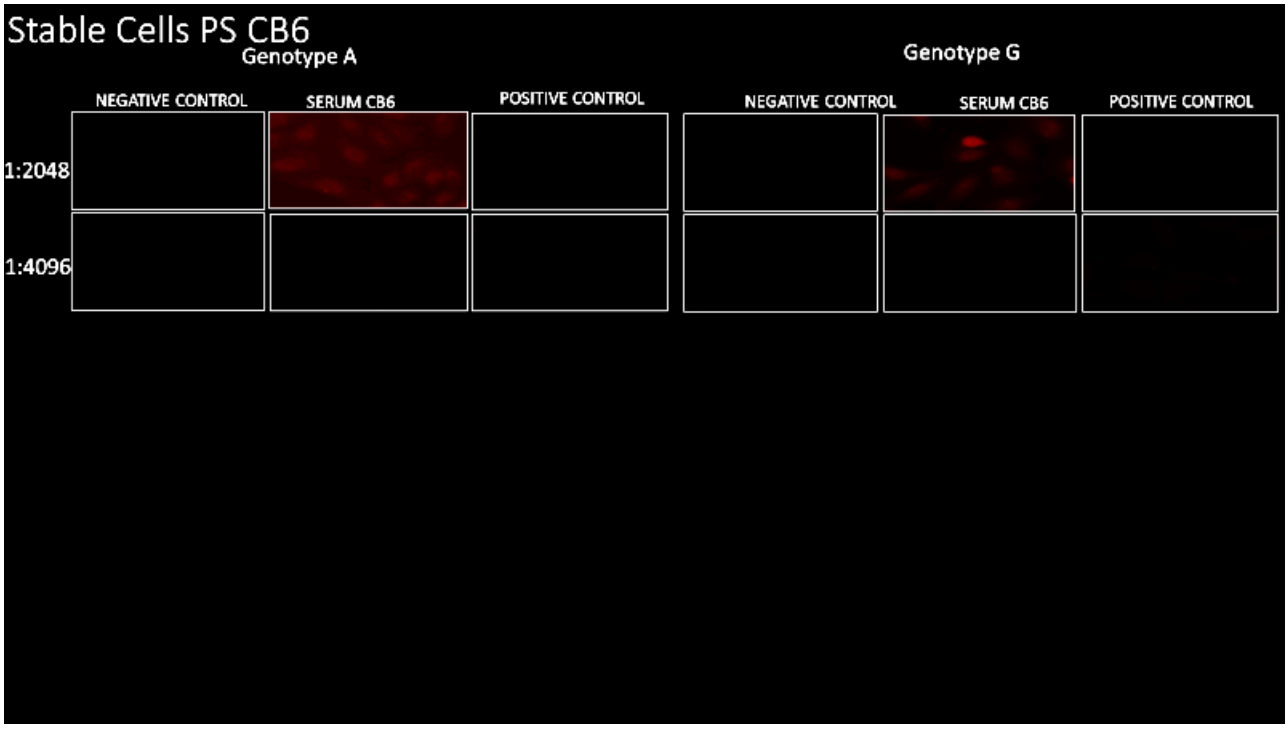


Figure A-78b. Stable cells Vaccine-Infected (CB) Sample#6.

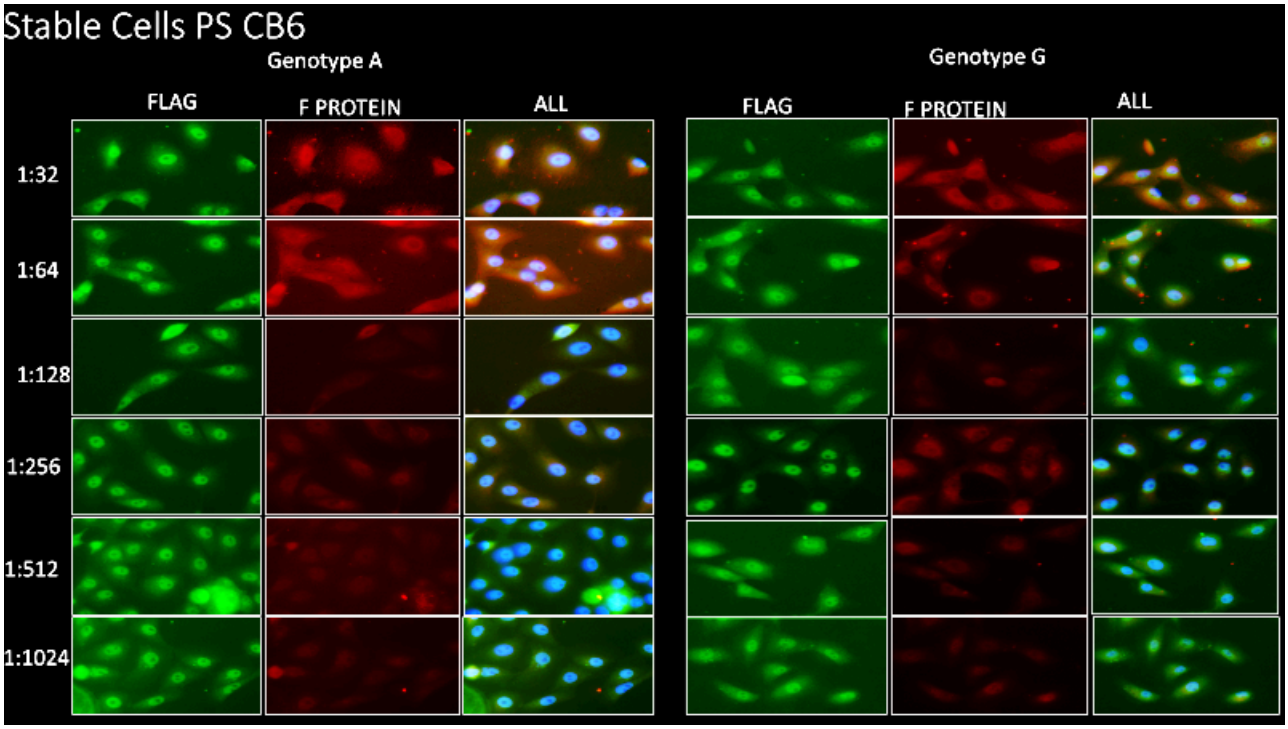


Figure A-78c. Stable cells Vaccine-Infected (CB) Sample#6.

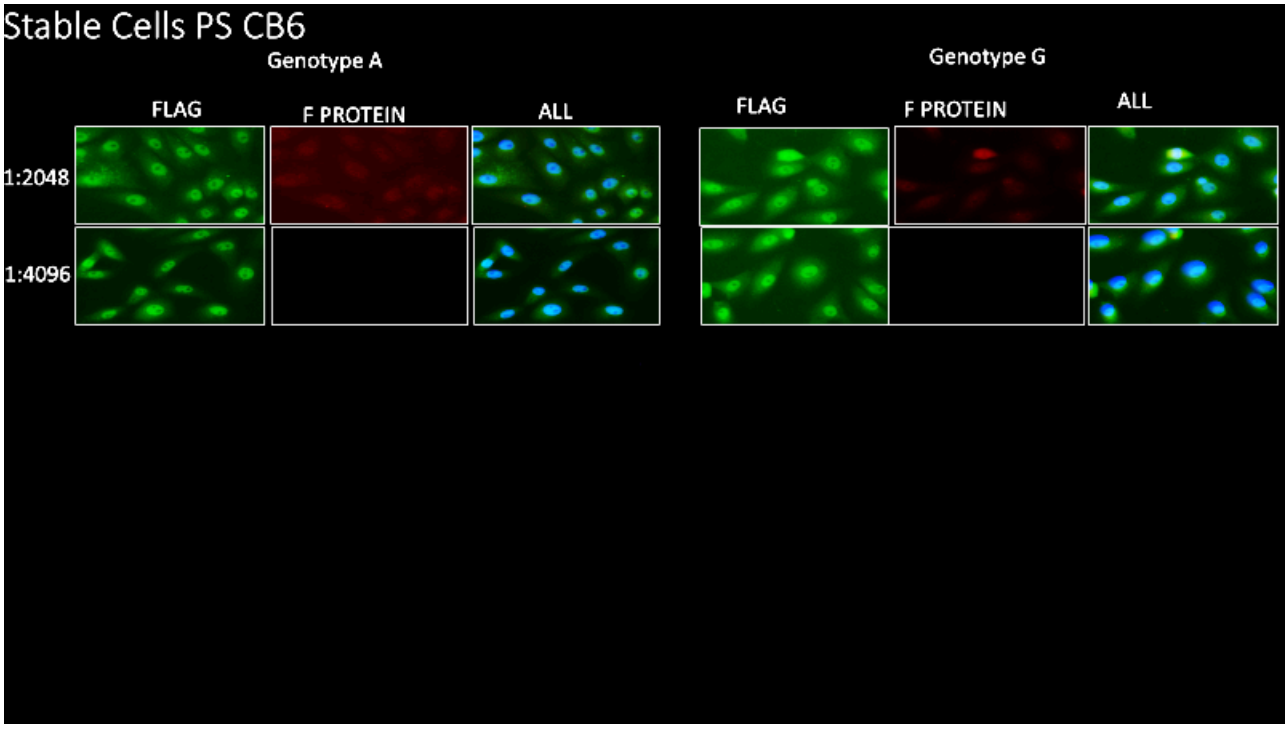


Figure A-78d. Stable cells Vaccine-Infected (CB) Sample#6.

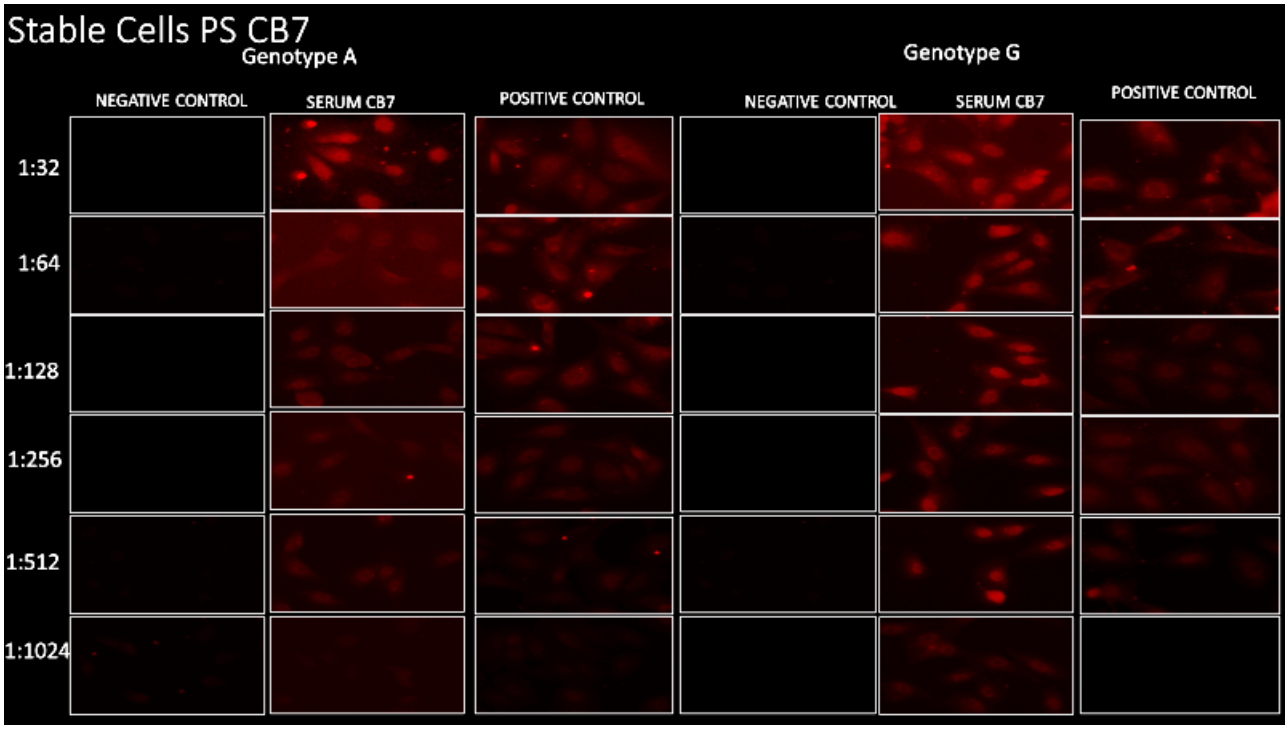


Figure A-79a. Stable cells Vaccine-Infected (CB) Sample#7.

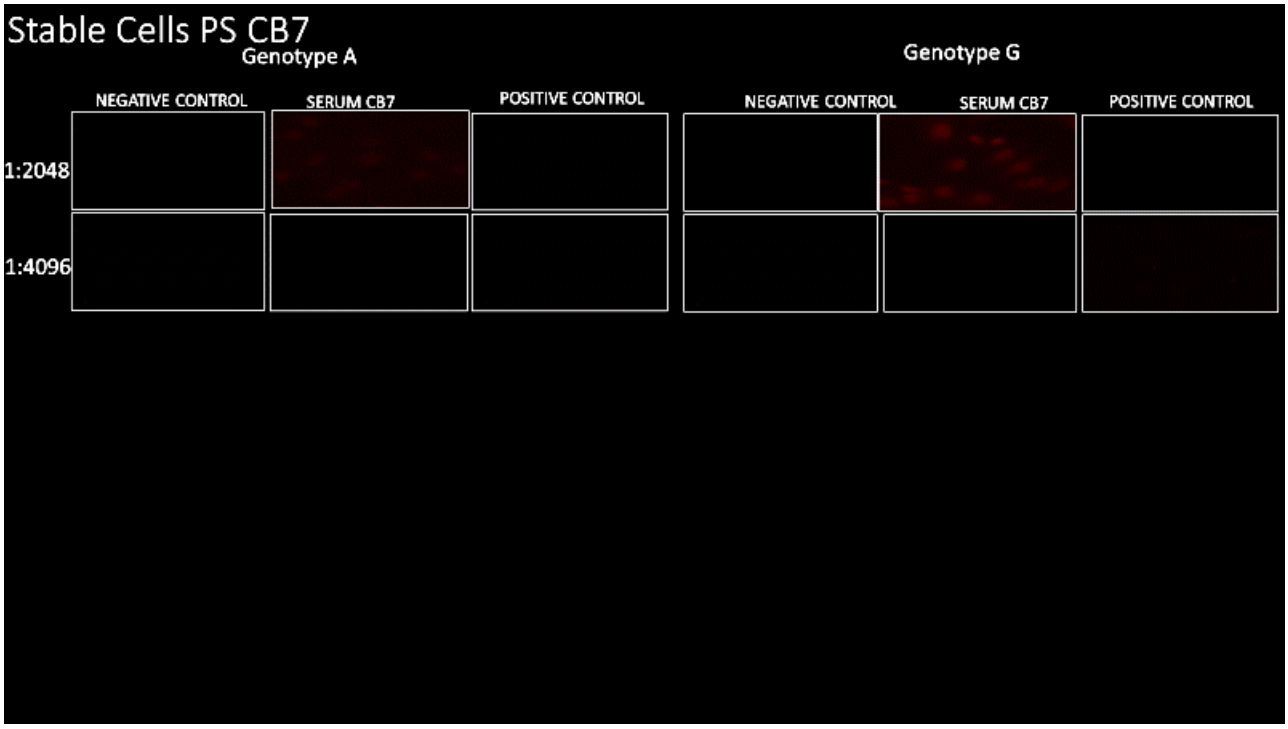


Figure A-79b. Stable cells Vaccine-Infected (CB) Sample#7.

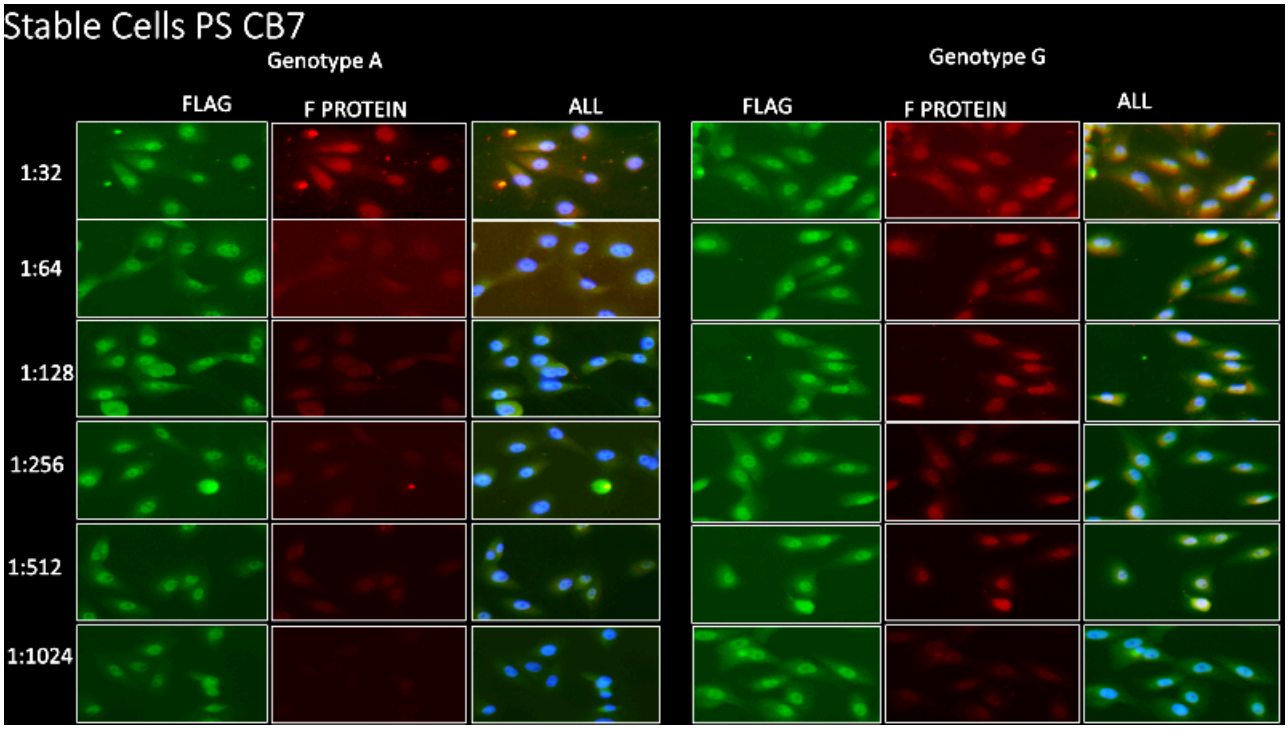


Figure A-79c. Stable cells Vaccine-Infected (CB) Sample#7.

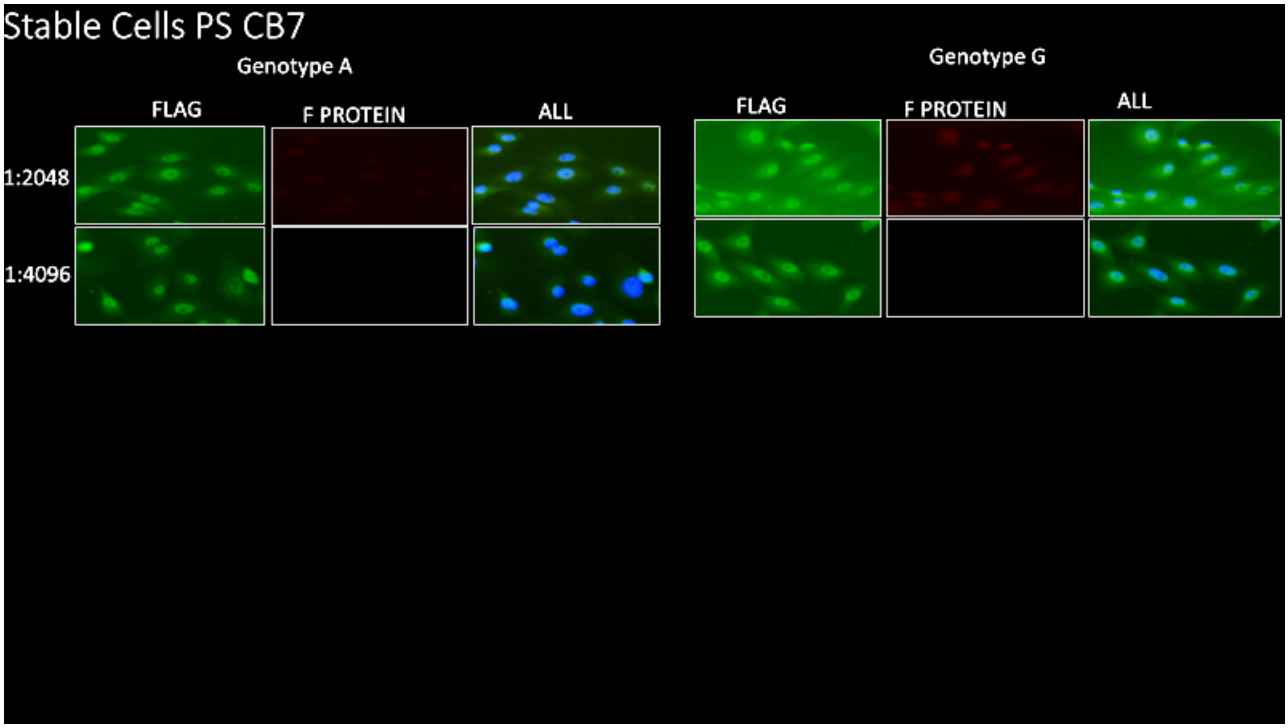


Figure A-79d. Stable cells Vaccine-Infected (CB) Sample#7.

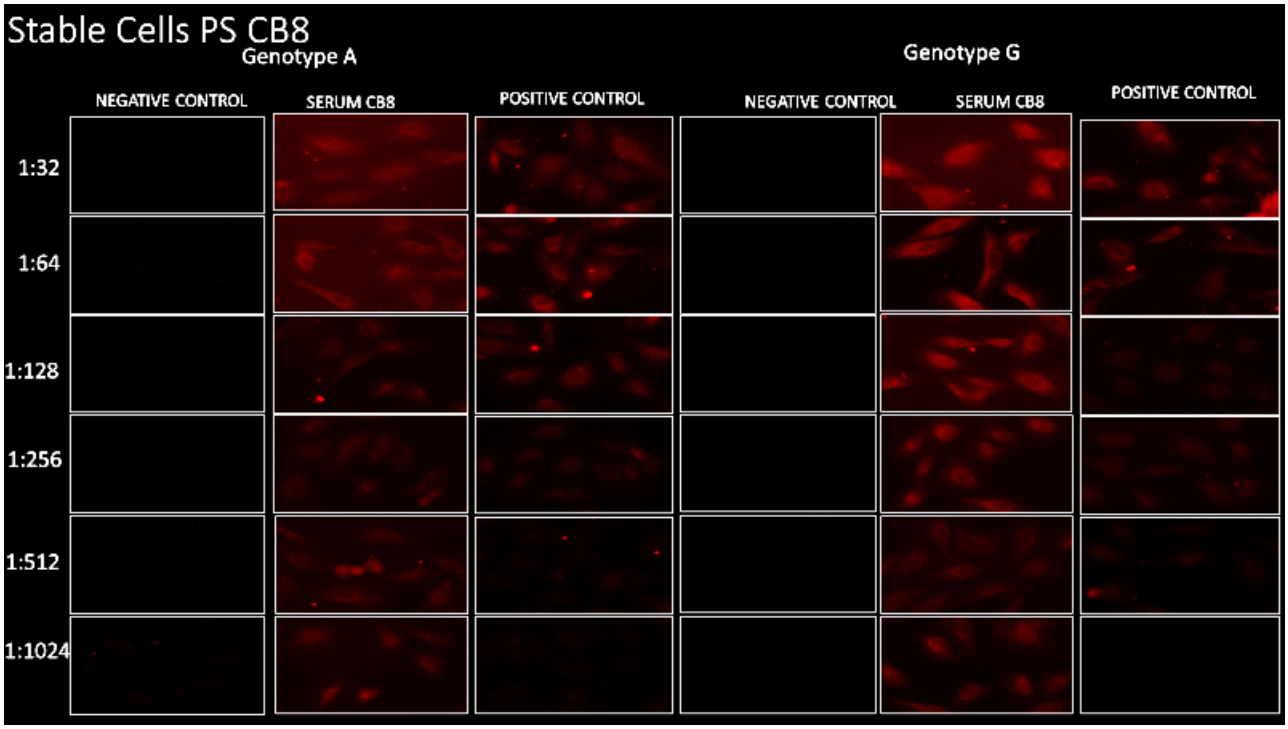


Figure A-80a. Stable cells Vaccine-Infected (CB) Sample#8.

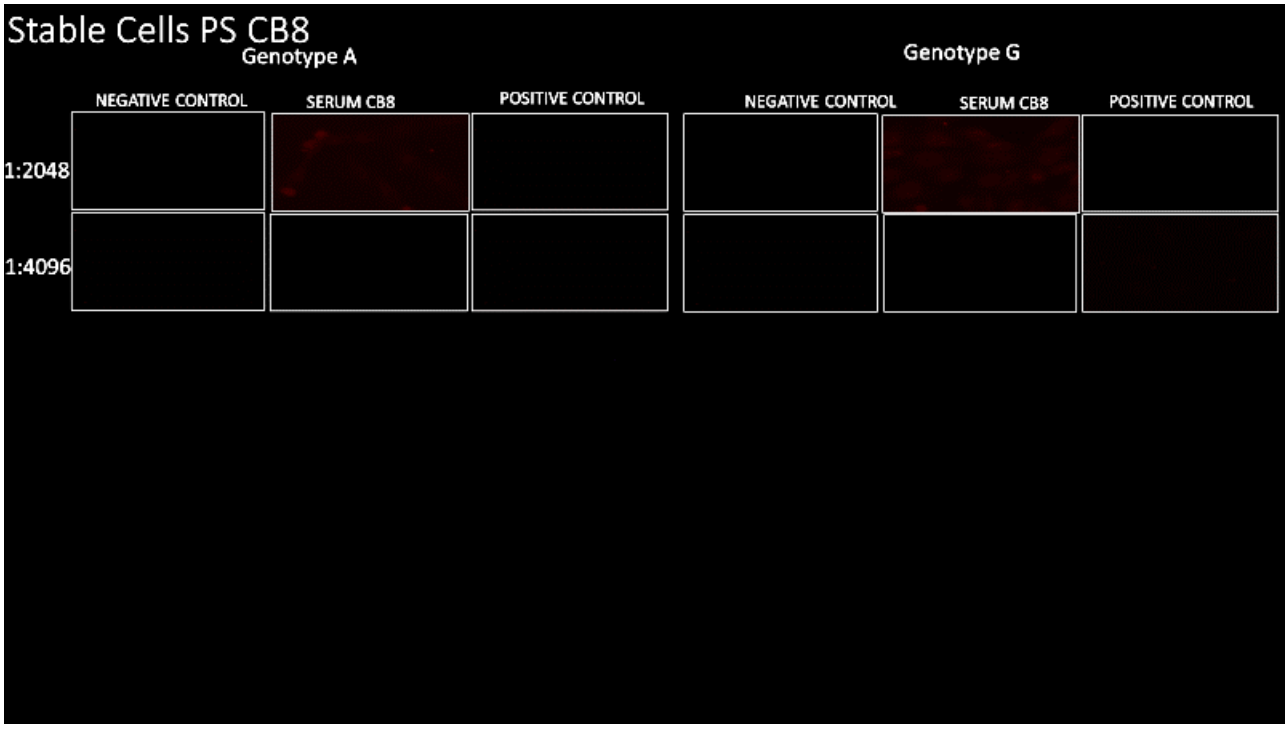


Figure A-80b. Stable cells Vaccine-Infected (CB) Sample#8.

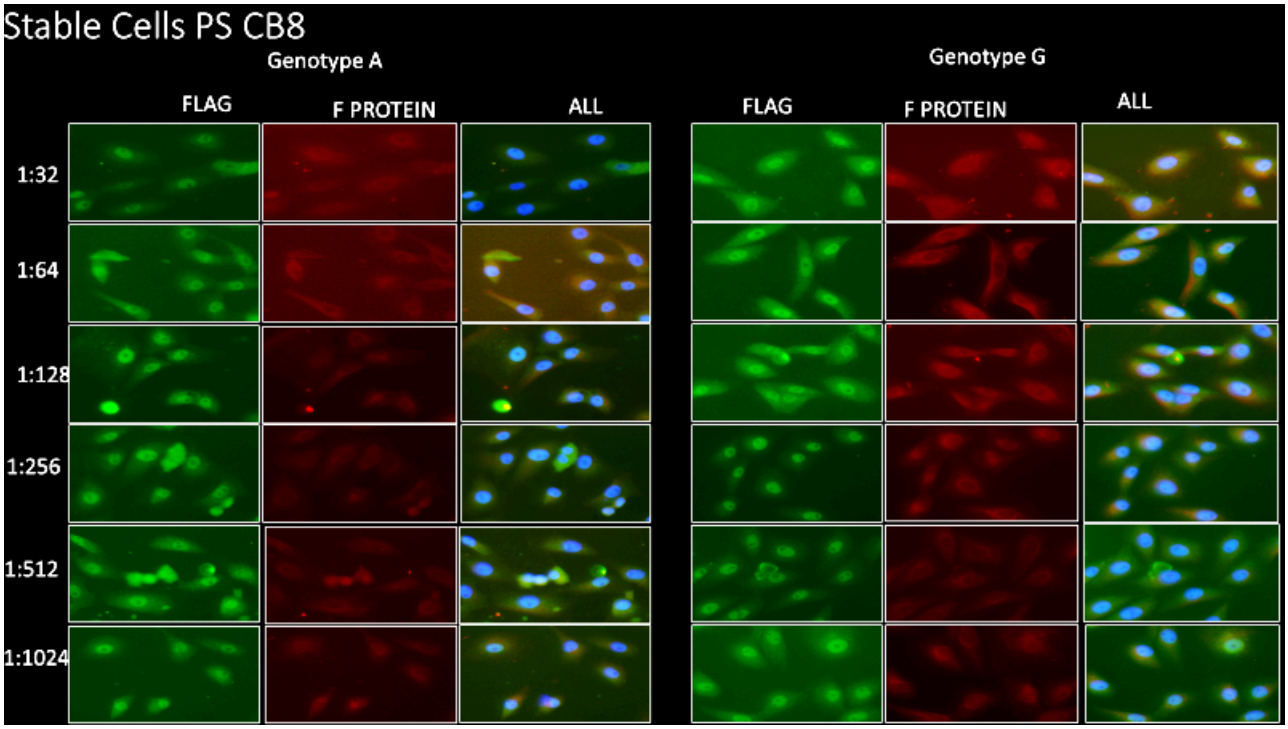


Figure A-80c. Stable cells Vaccine-Infected (CB) Sample#8.

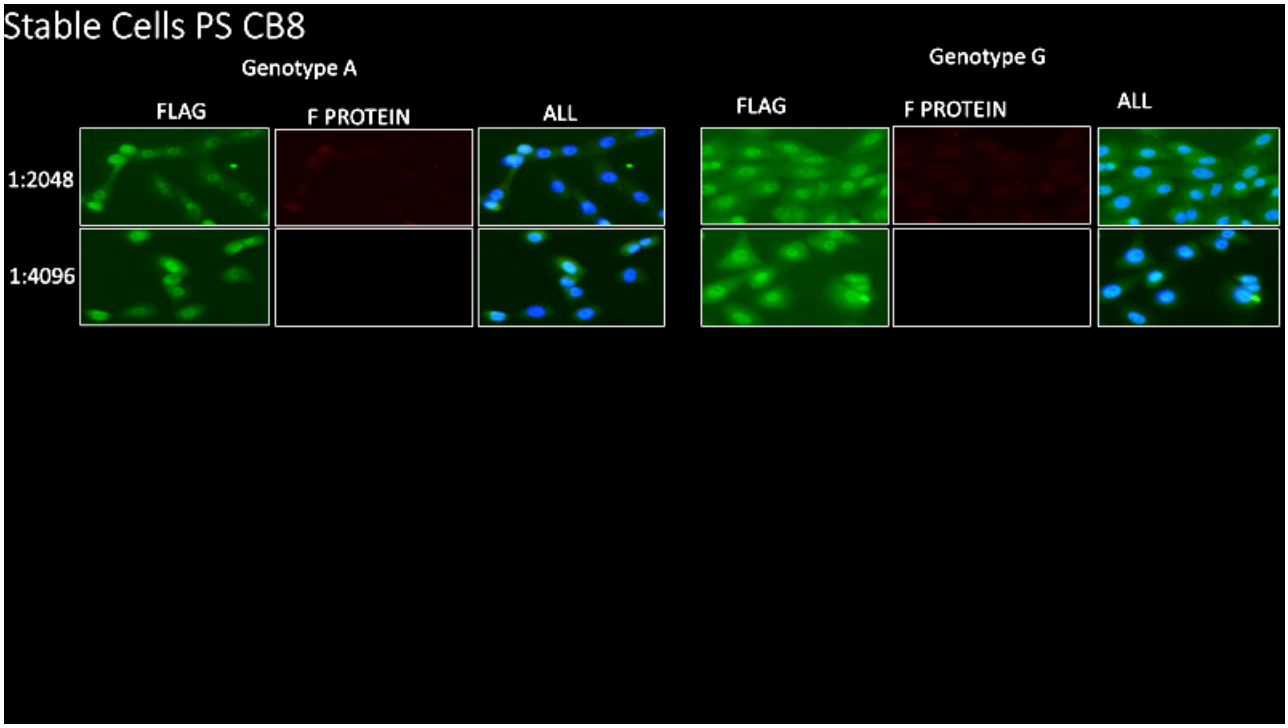


Figure A-80d. Stable cells Vaccine-Infected (CB) Sample#8.

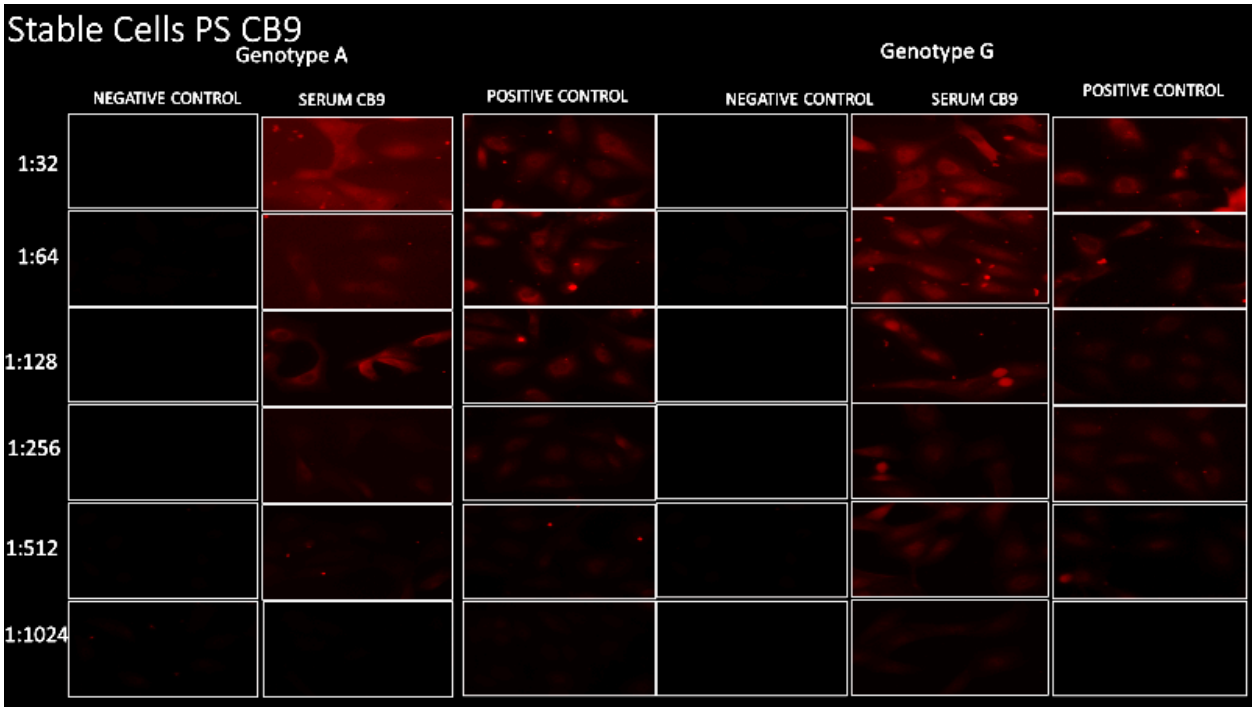


Figure A-81a. Stable cells Vaccine-Infected (CB) Sample#9.

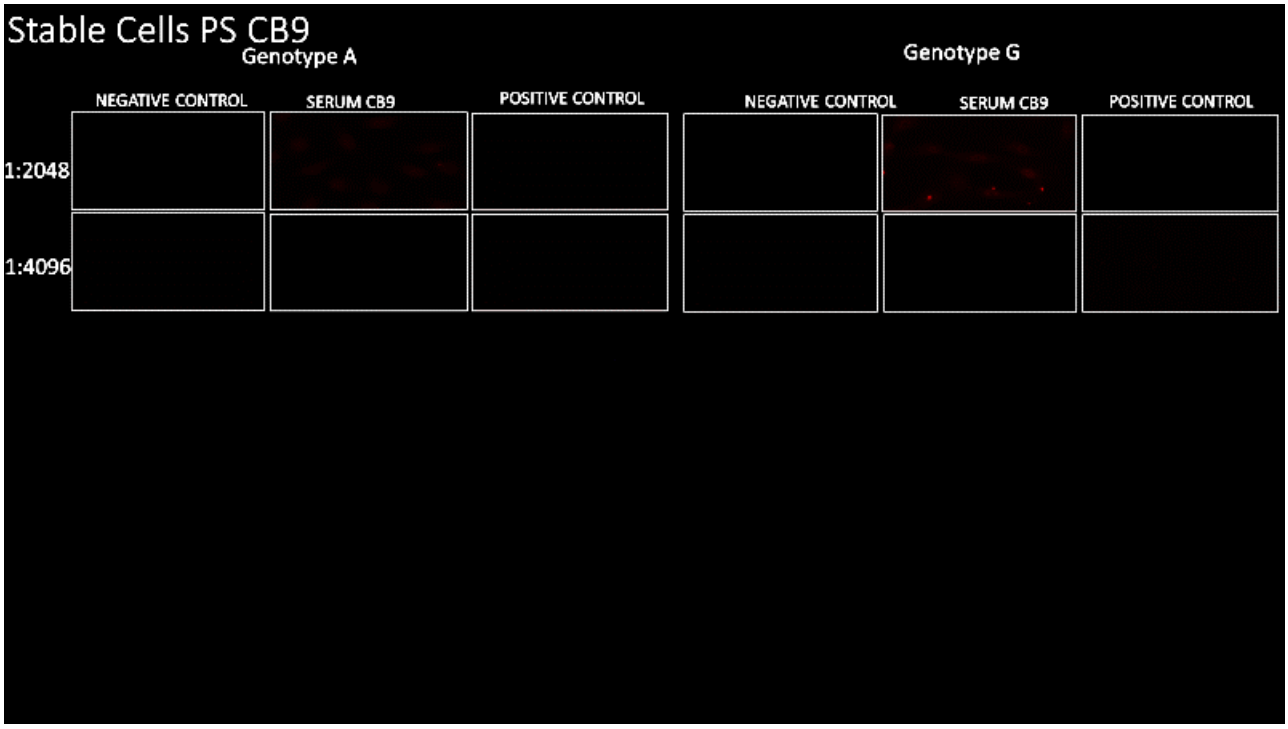


Figure A-81b. Stable cells Vaccine-Infected (CB) Sample#9.

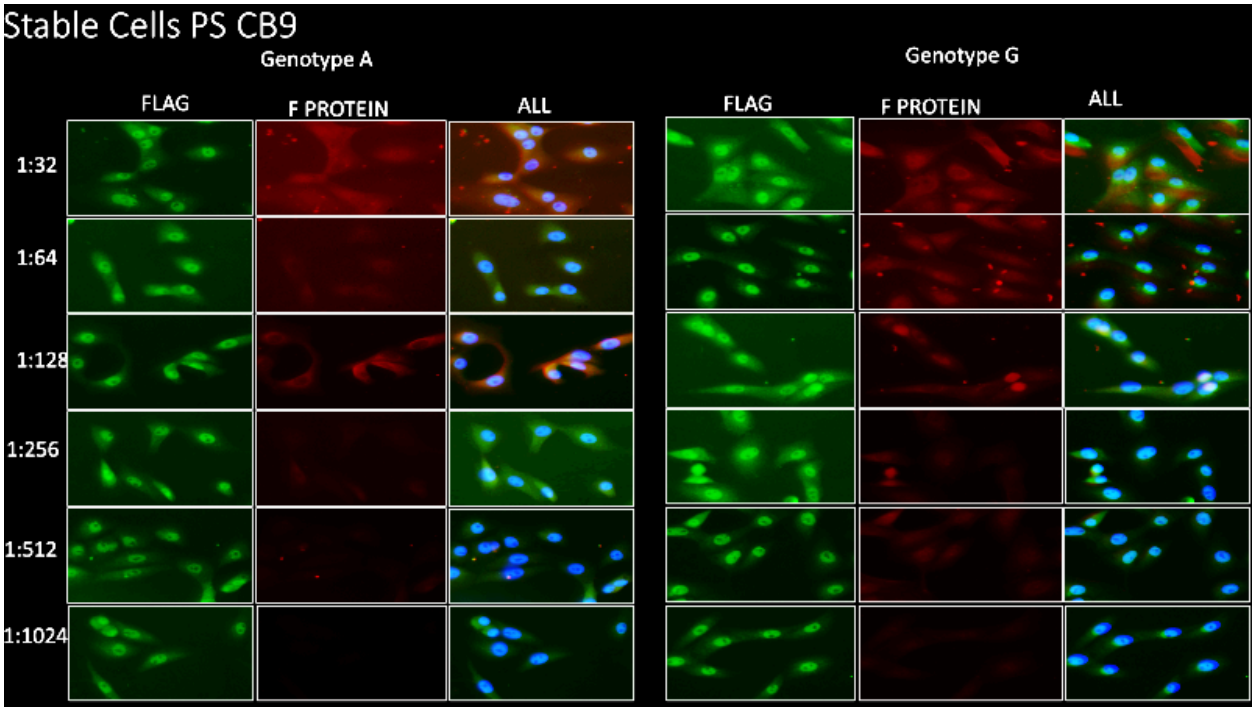


Figure A-81c. Stable cells Vaccine-Infected (CB) Sample#9.

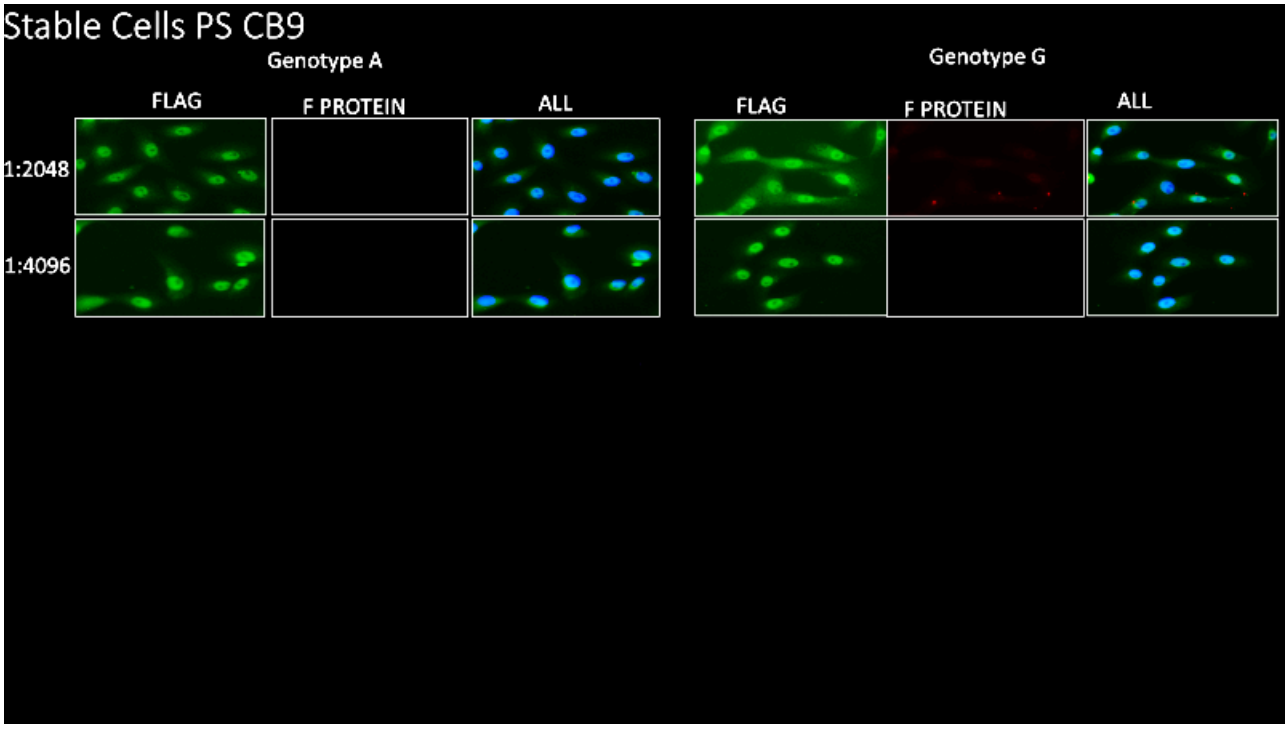


Figure A-81d. Stable cells Vaccine-Infected (CB) Sample#9.

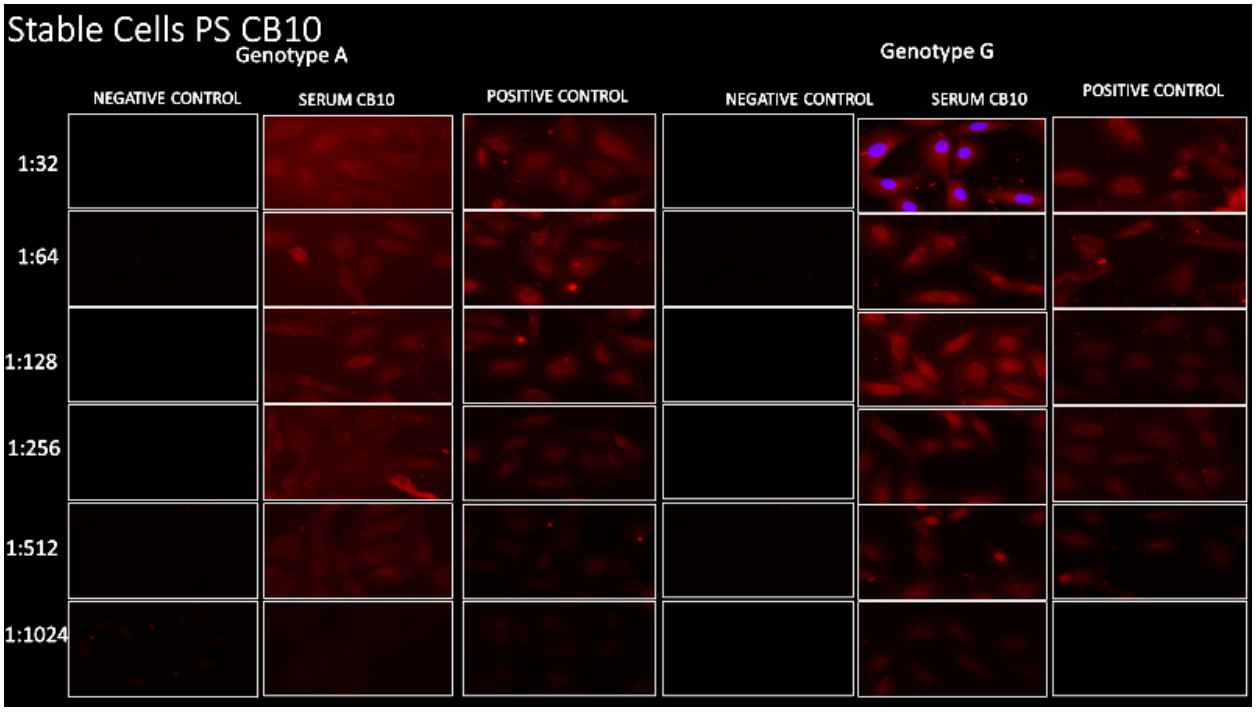


Figure A-82a. Stable cells Vaccine-Infected (CB) Sample#10.

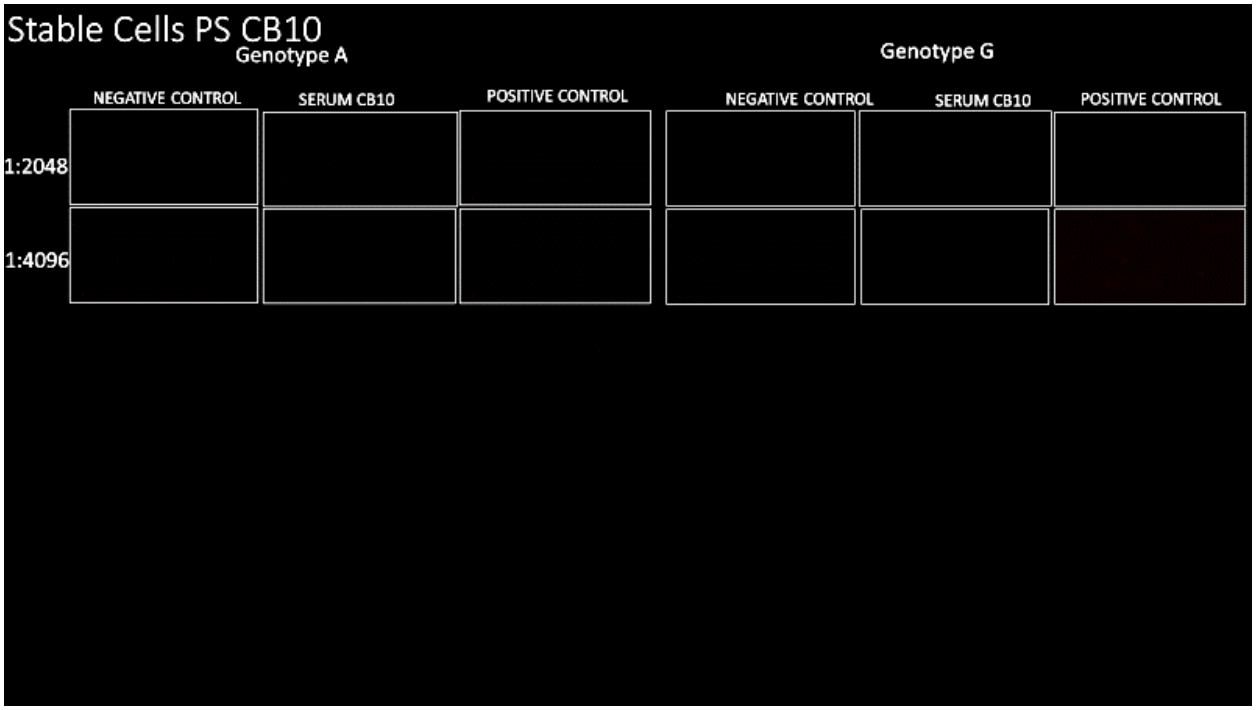


Figure A-82b. Stable cells Vaccine-Infected (CB) Sample#10.

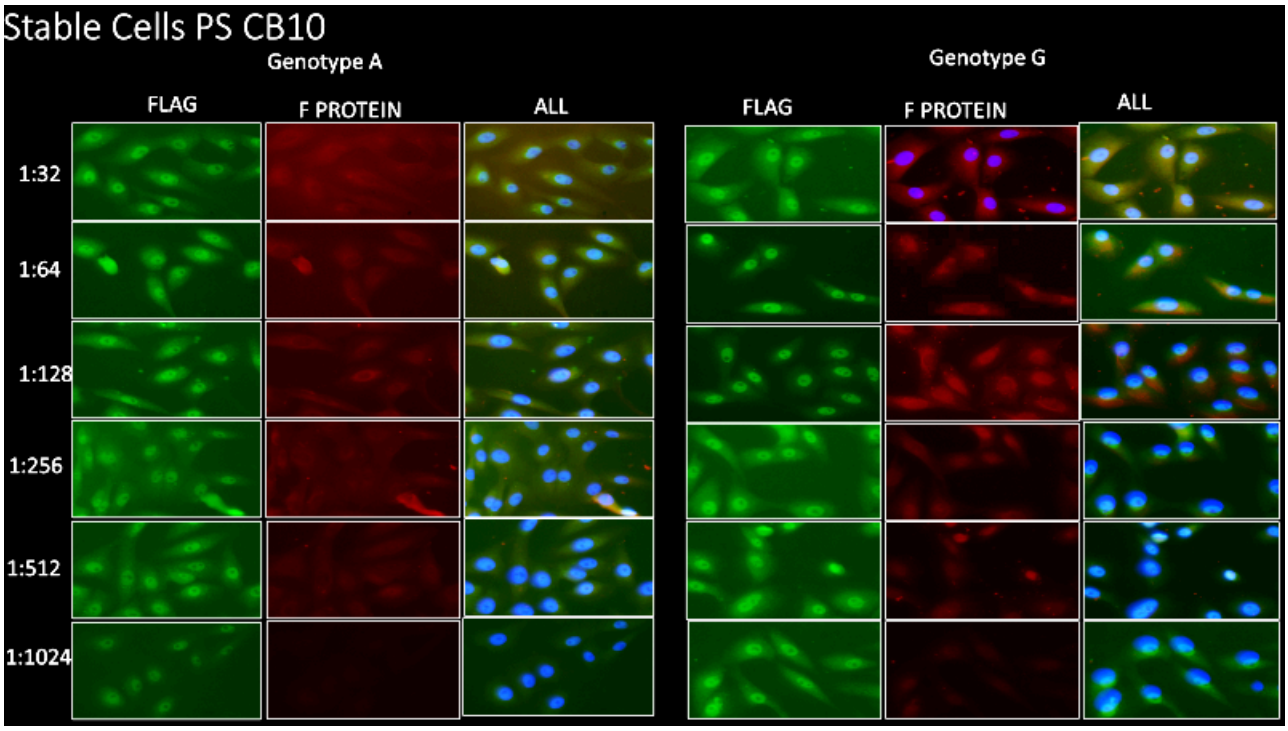


Figure A-82c. Stable cells Vaccine-Infected (CB) Sample#10.

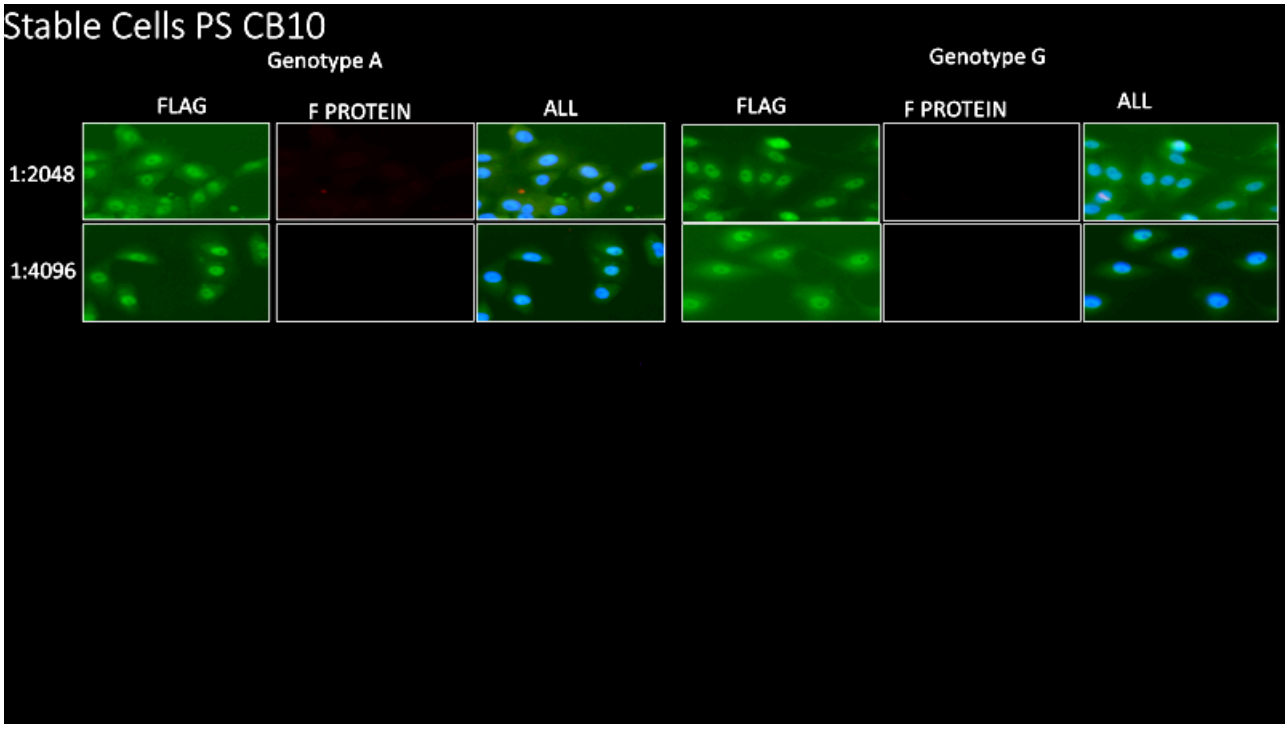


Figure A-82d. Stable cells Vaccine-Infected (CB) Sample#10.

Appendix:

IFA Results of HN Protein

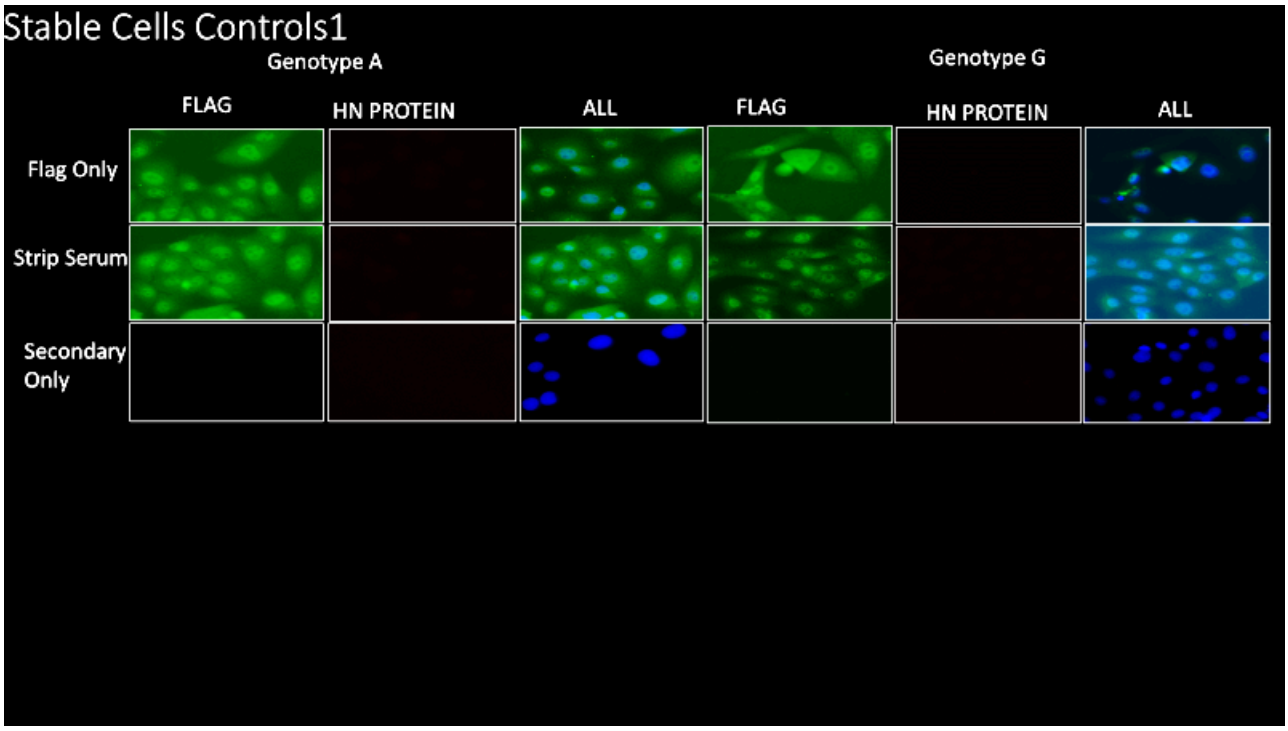


Figure A-1a. Stable cells ControlBatch#1.

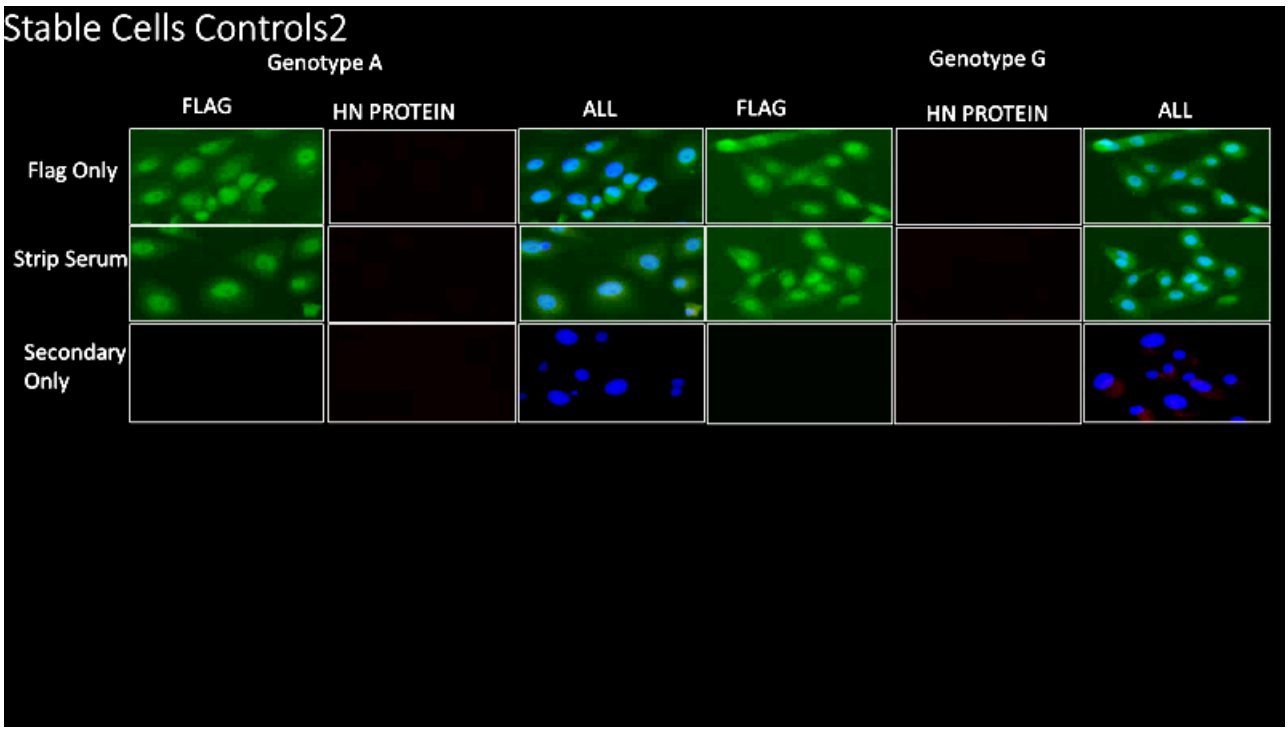


Figure A-1b. Stable cells ControlBatch#2.

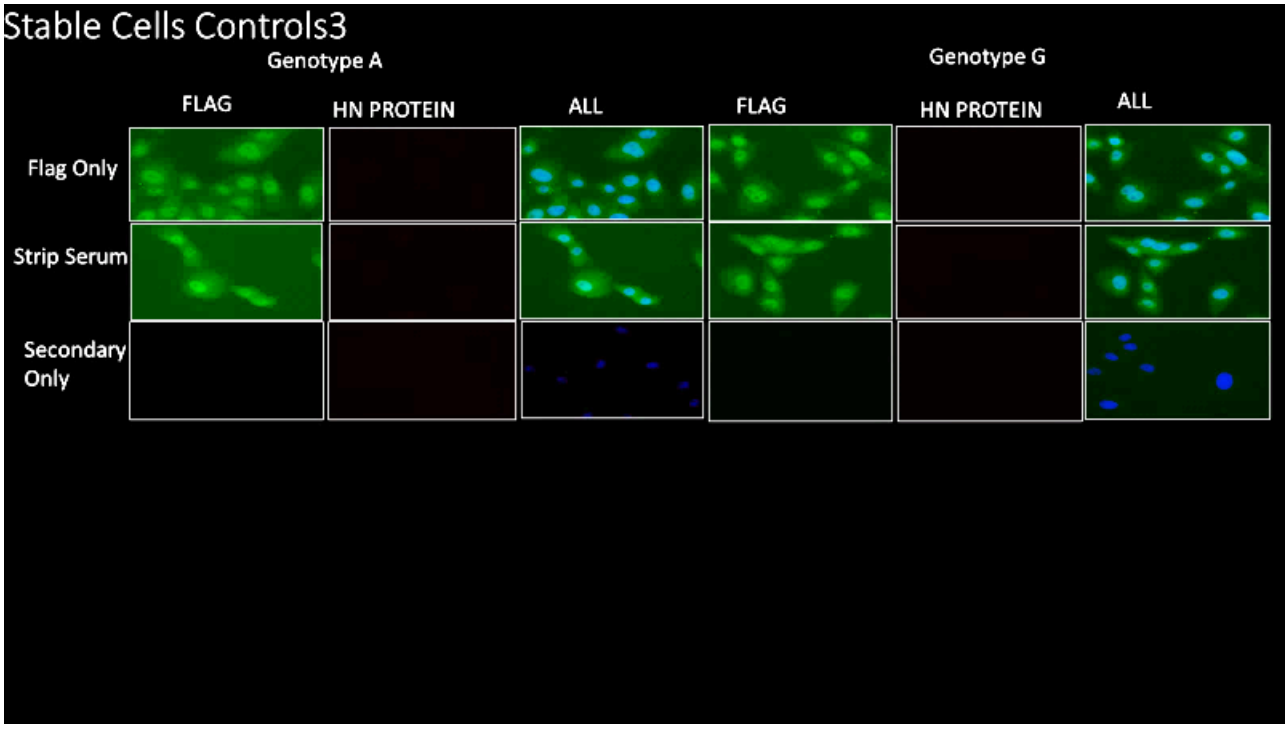


Figure A-1c. Stable cells ControlBatch#3.

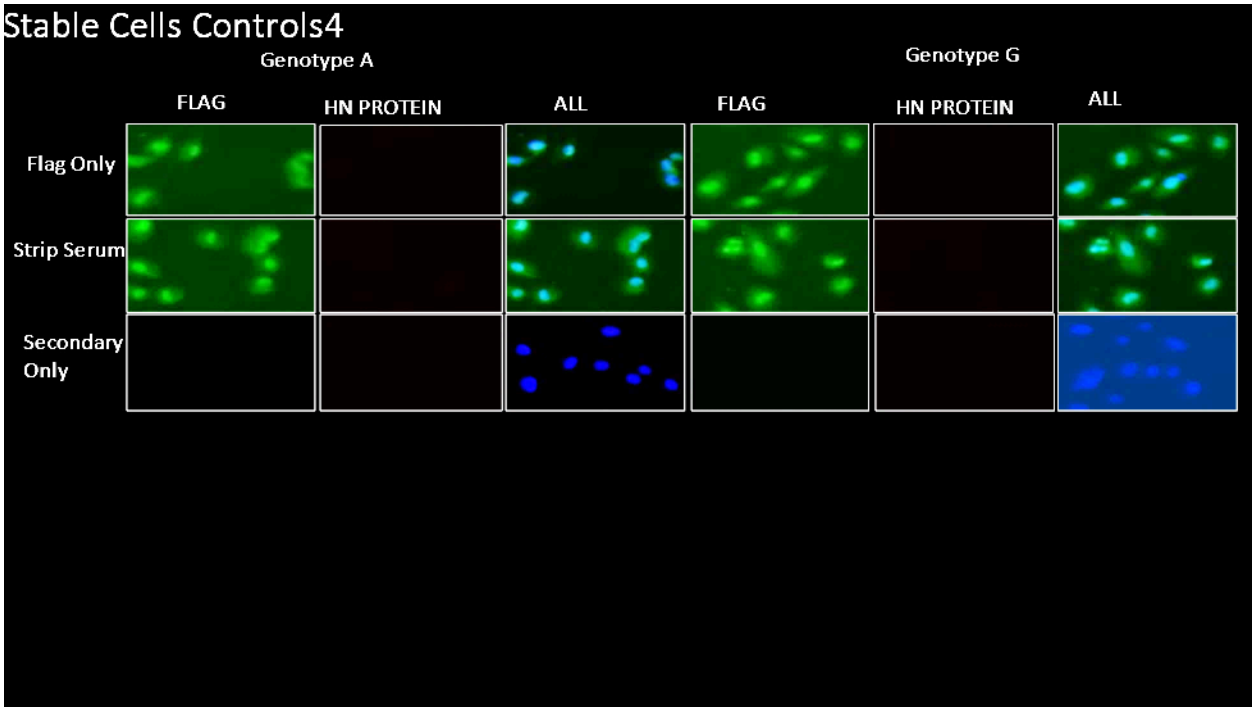


Figure A-1d. Stable cells ControlBatch#4.

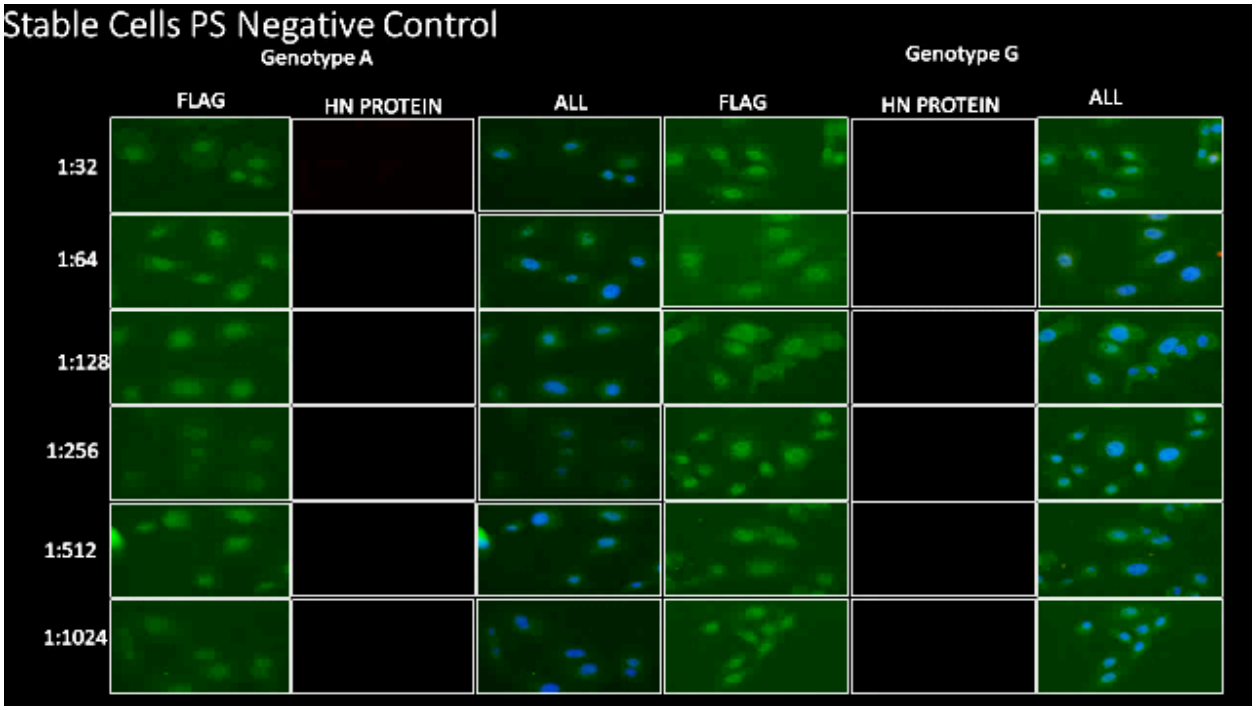


Figure A-2a. Stable cells Negative ControlBatch#1.

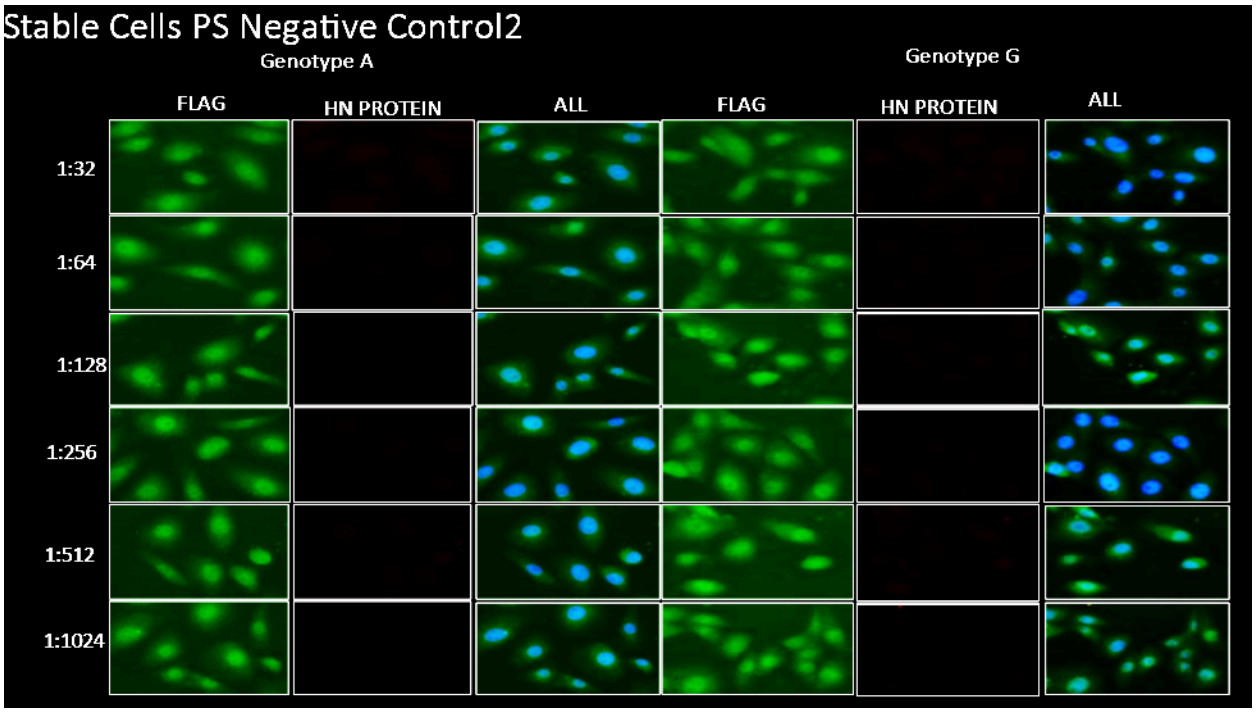


Figure A-2b. Stable cells Negative ControlBatch#2.

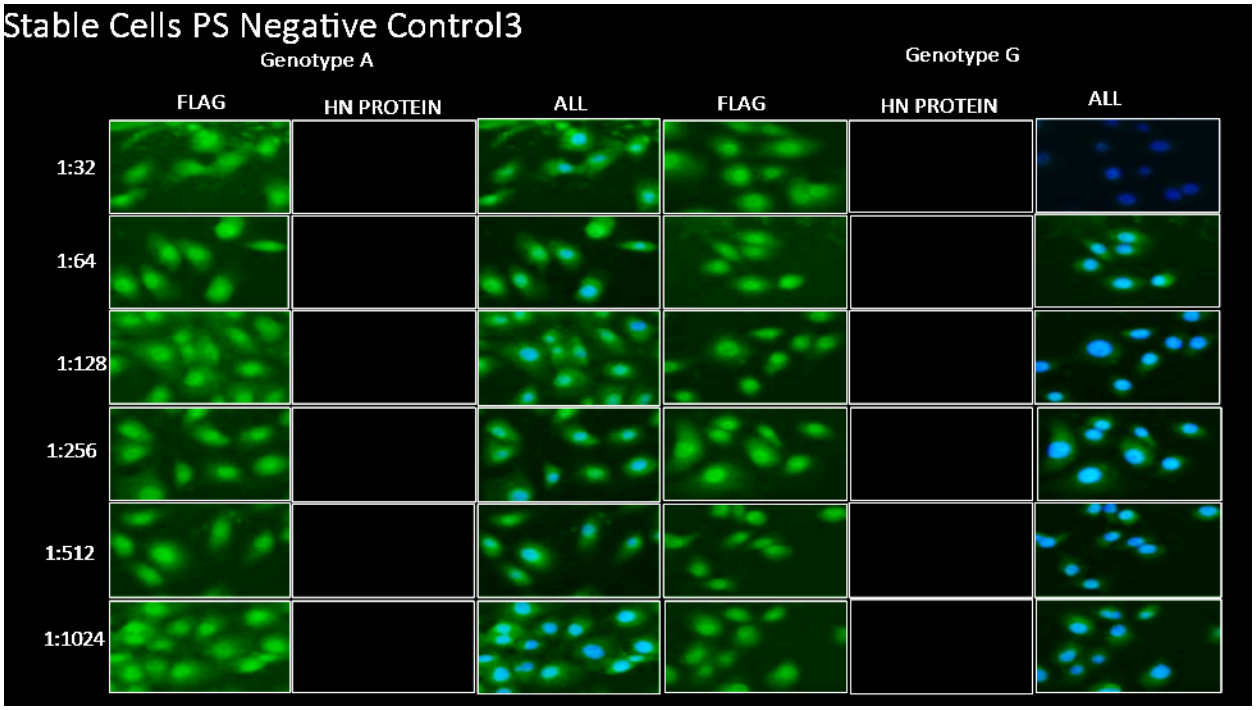


Figure A-2c. Stable cells Negative ControlBatch#3.

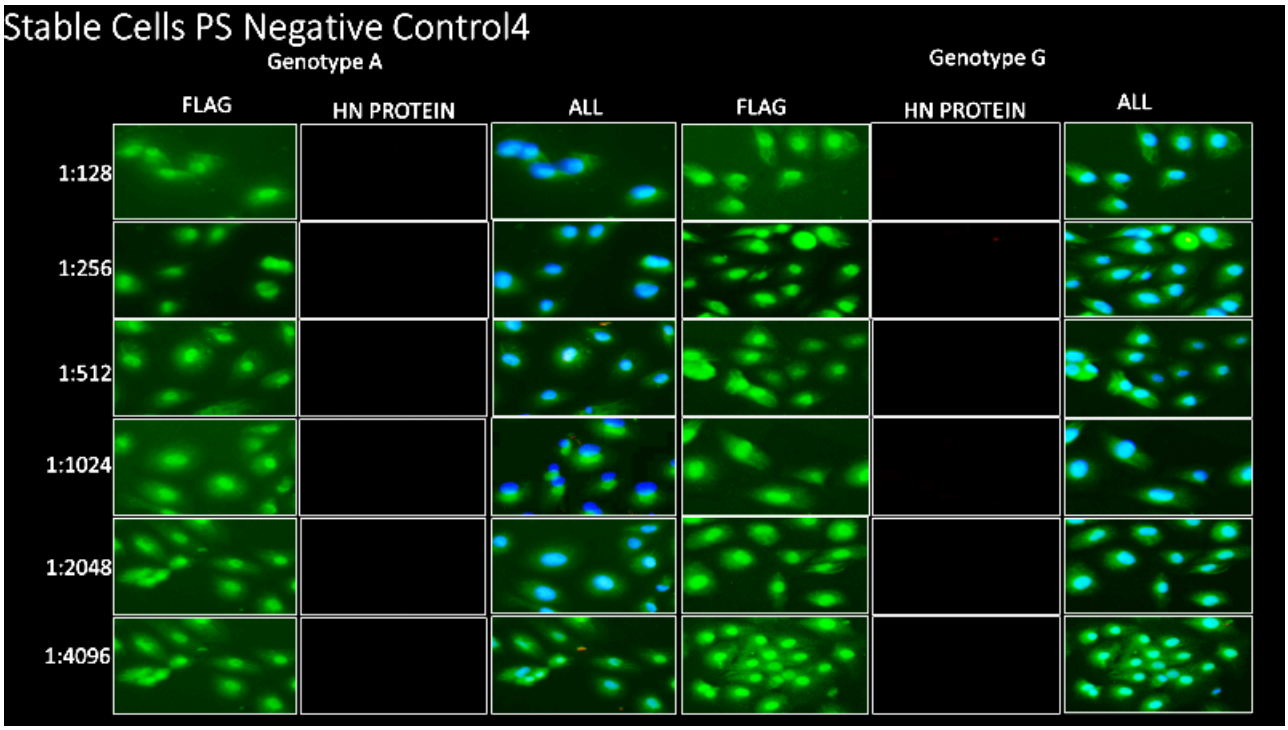


Figure A-2d. Stable cells Negative ControlBatch#4.

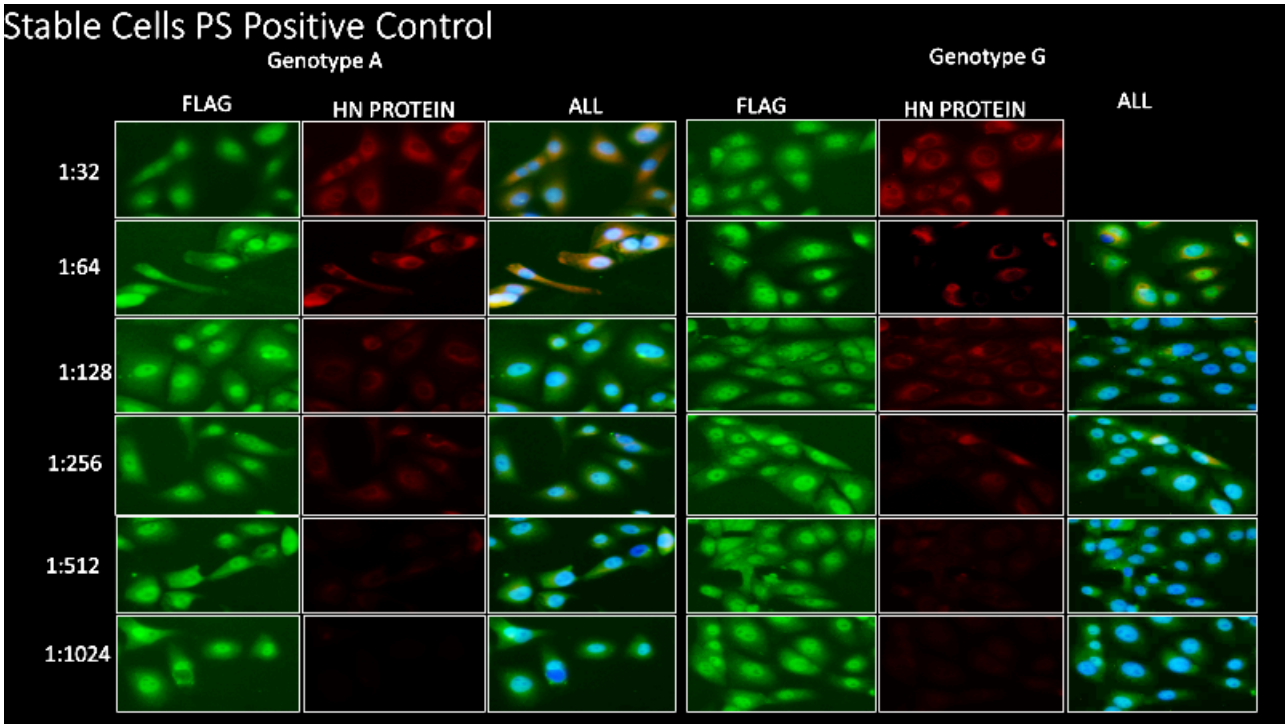


Figure A-3a. Stable cells Positive ControlBatch#1.

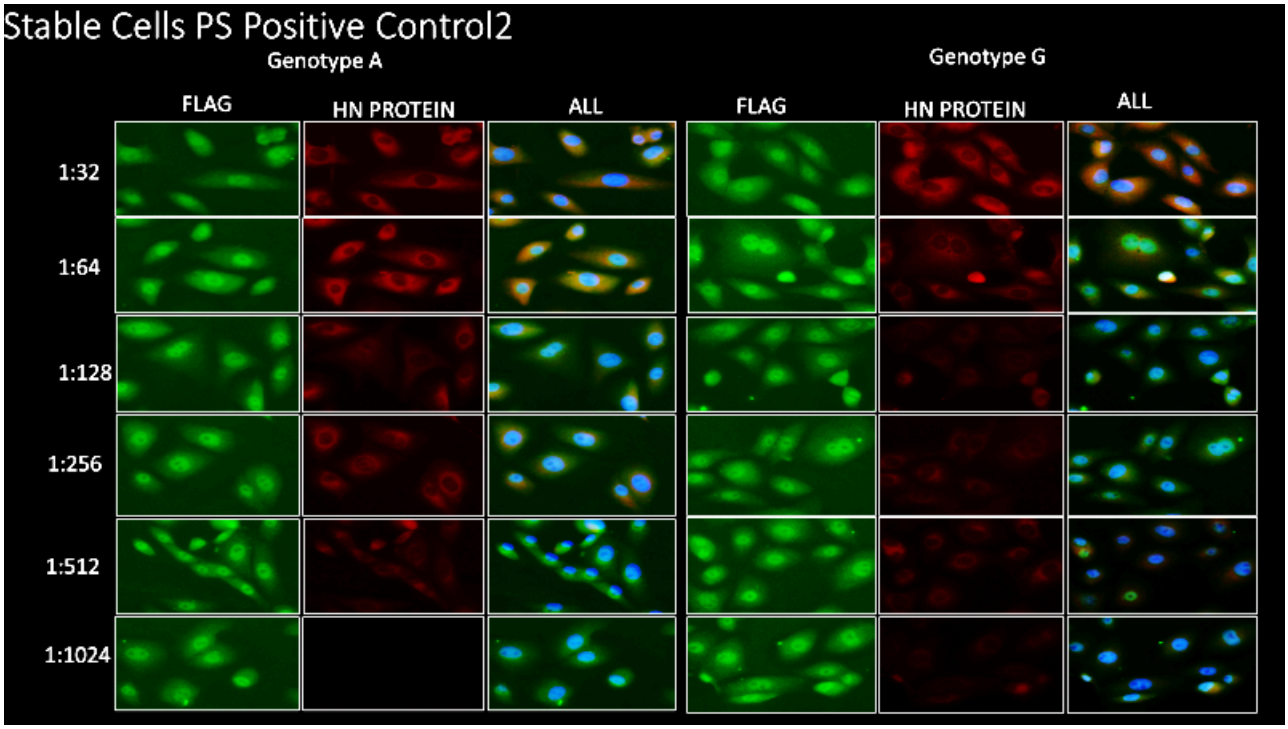


Figure A-3b. Stable cells Positive ControlBatch#2.

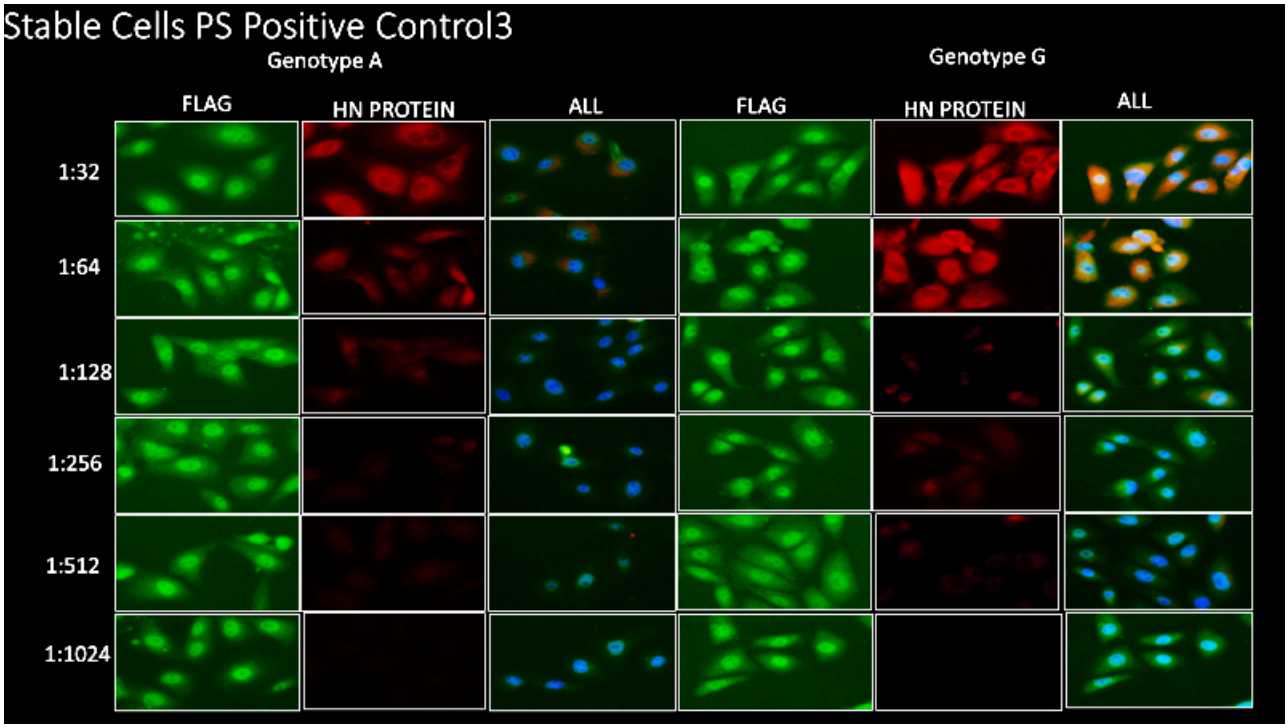


Figure A-3c. Stable cells Positive ControlBatch#3.

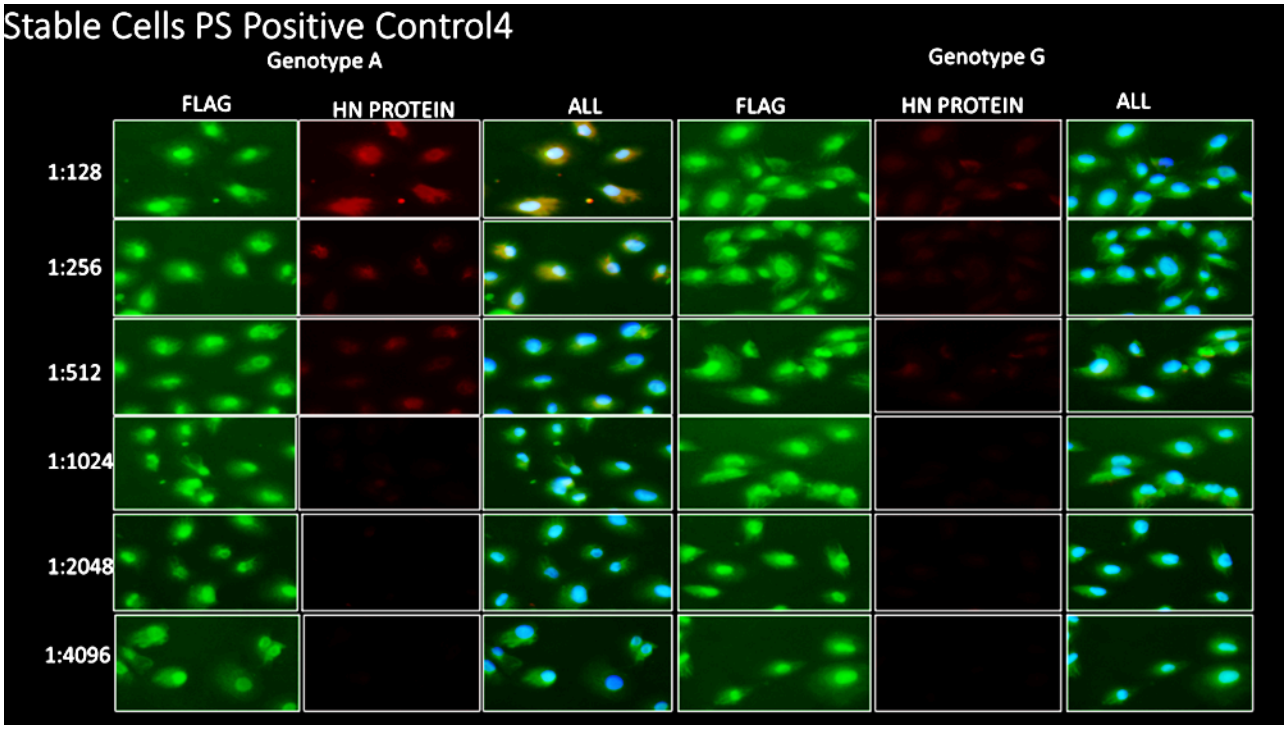


Figure A-3d. Stable cells Positive ControlBatch#4.

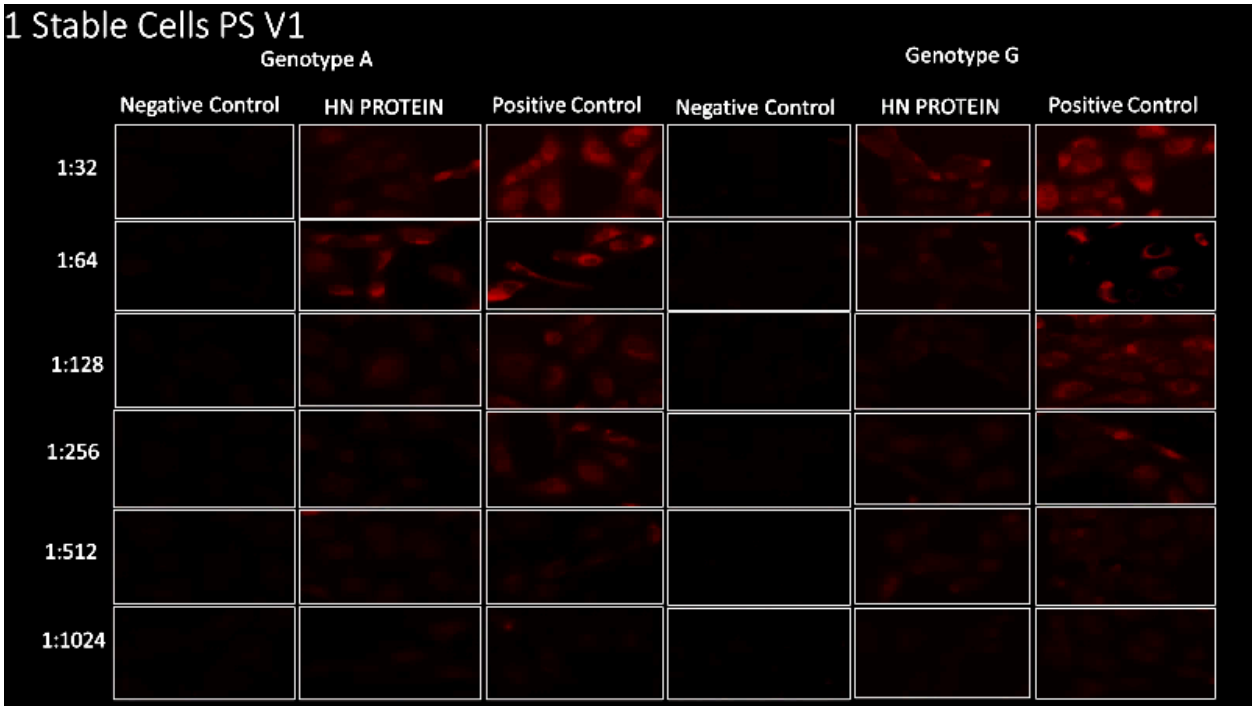


Figure A-4a. Stable cells Vaccine Sample#1.

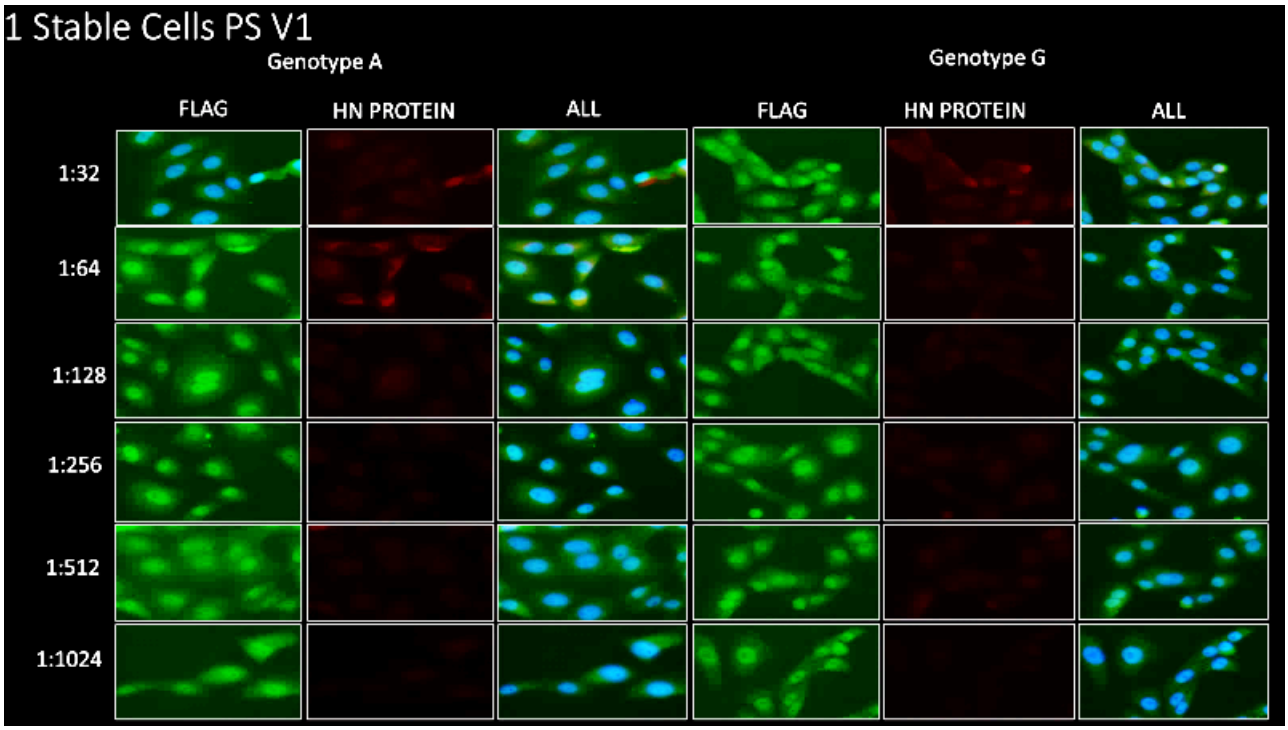


Figure A-4b. Stable cells Vaccine Sample #1.

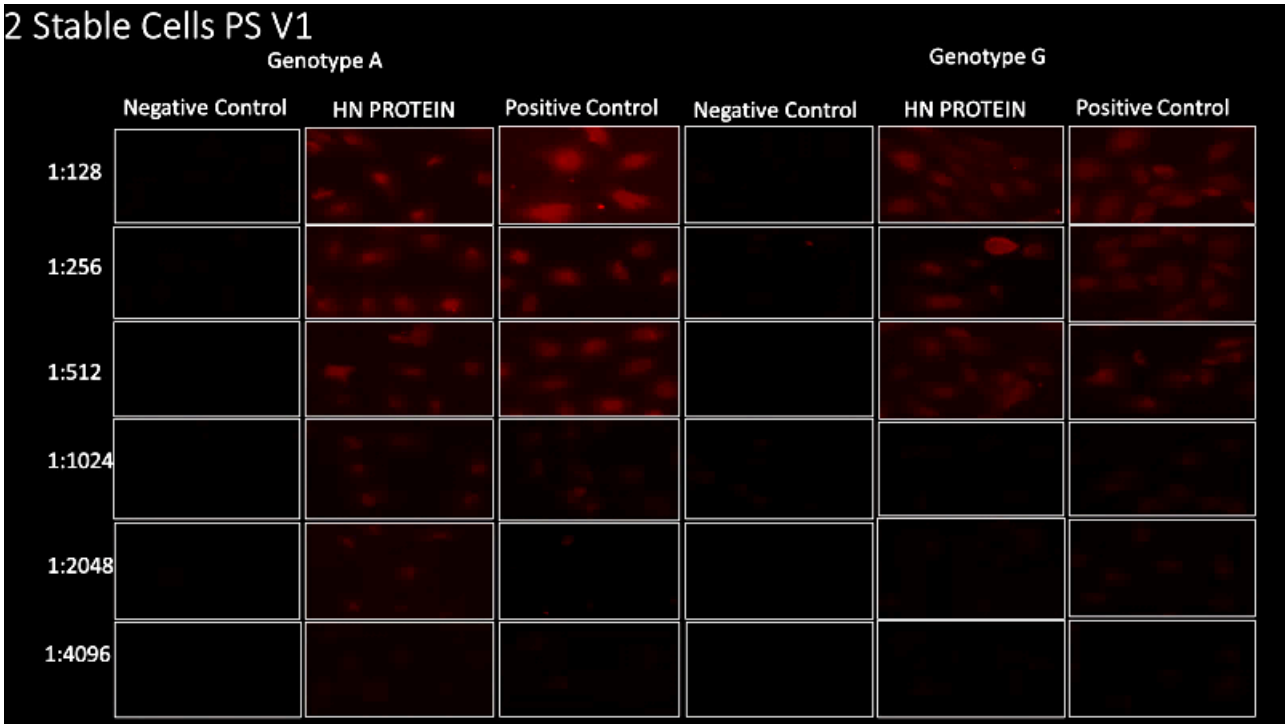


Figure A-4c. Stable cells Vaccine Sample #1.

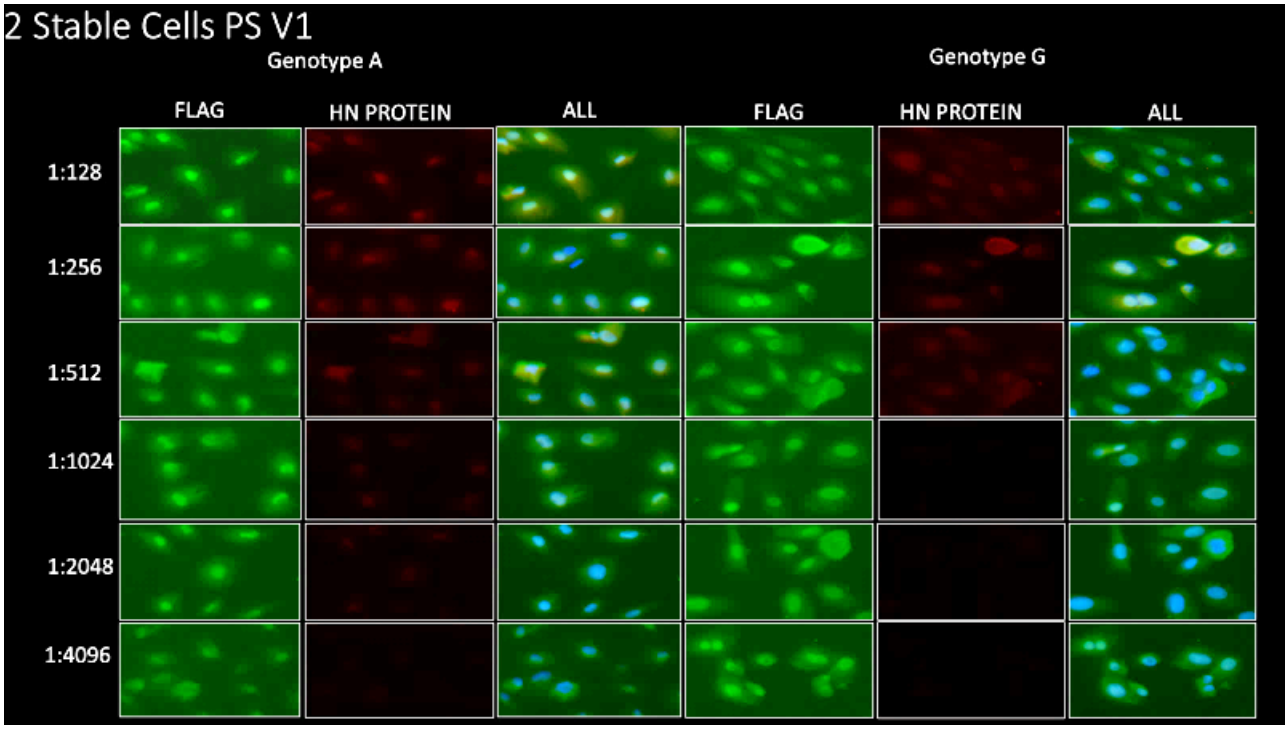


Figure A-4d. Stable cells Vaccine Sample #1.

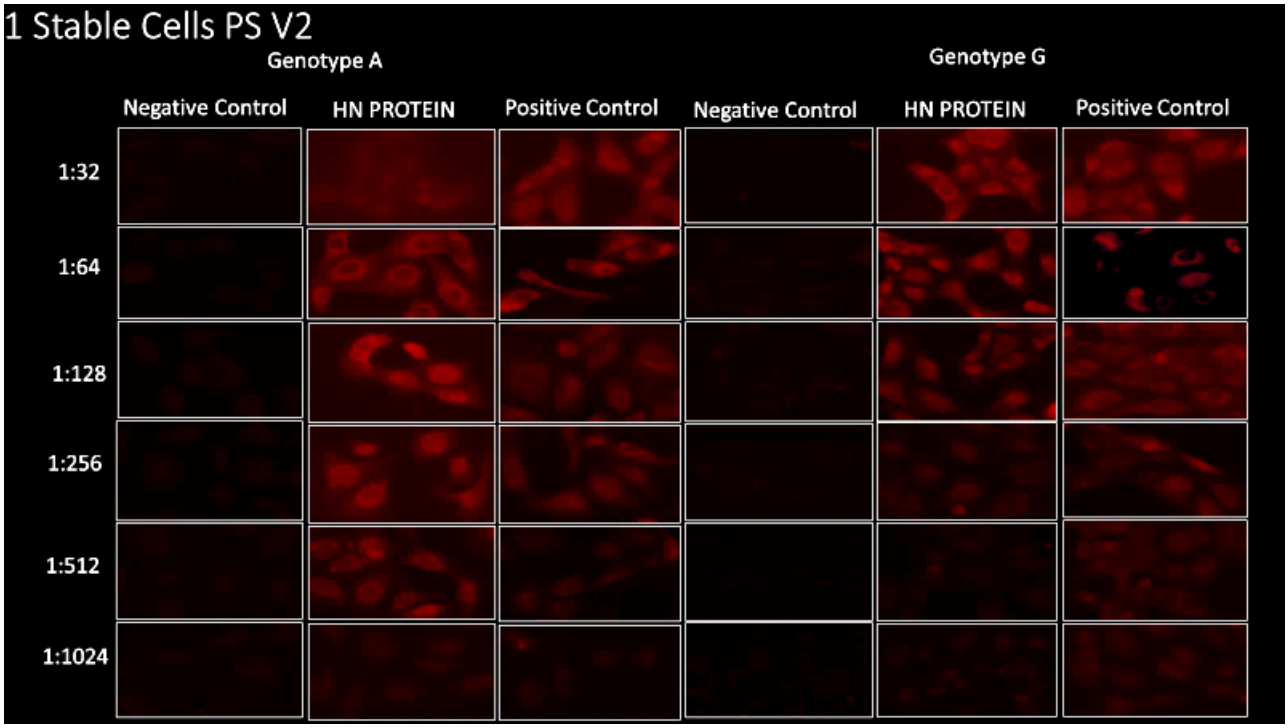


Figure A-5a. Stable cells Vaccine Sample#2.

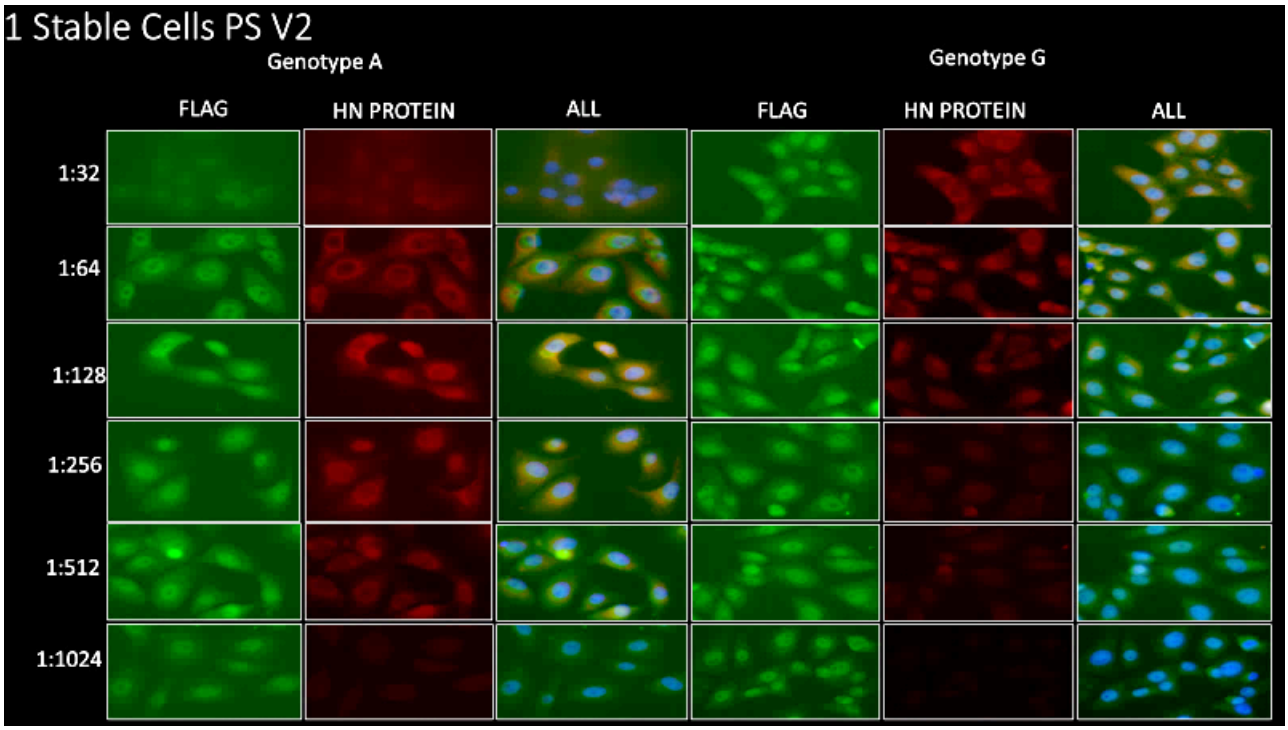


Figure A-5b. Stable cells Vaccine Sample #2.

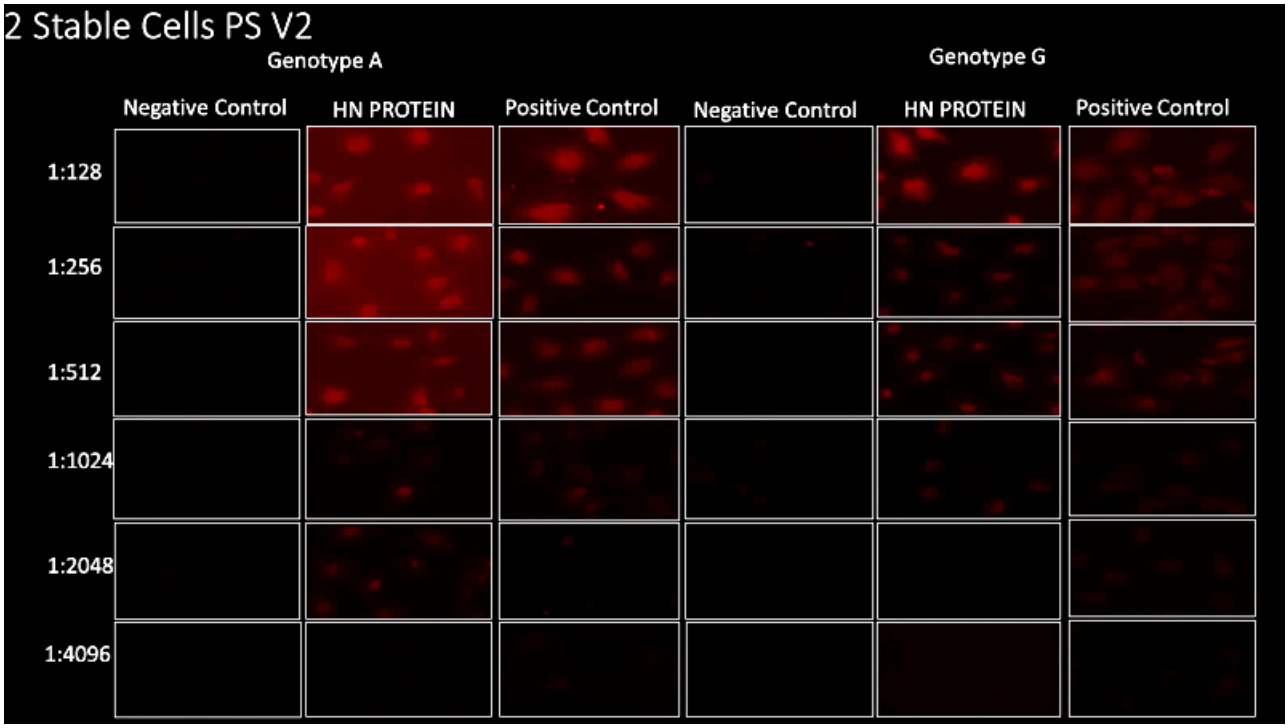


Figure A-5c. Stable cells Vaccine Sample #2.

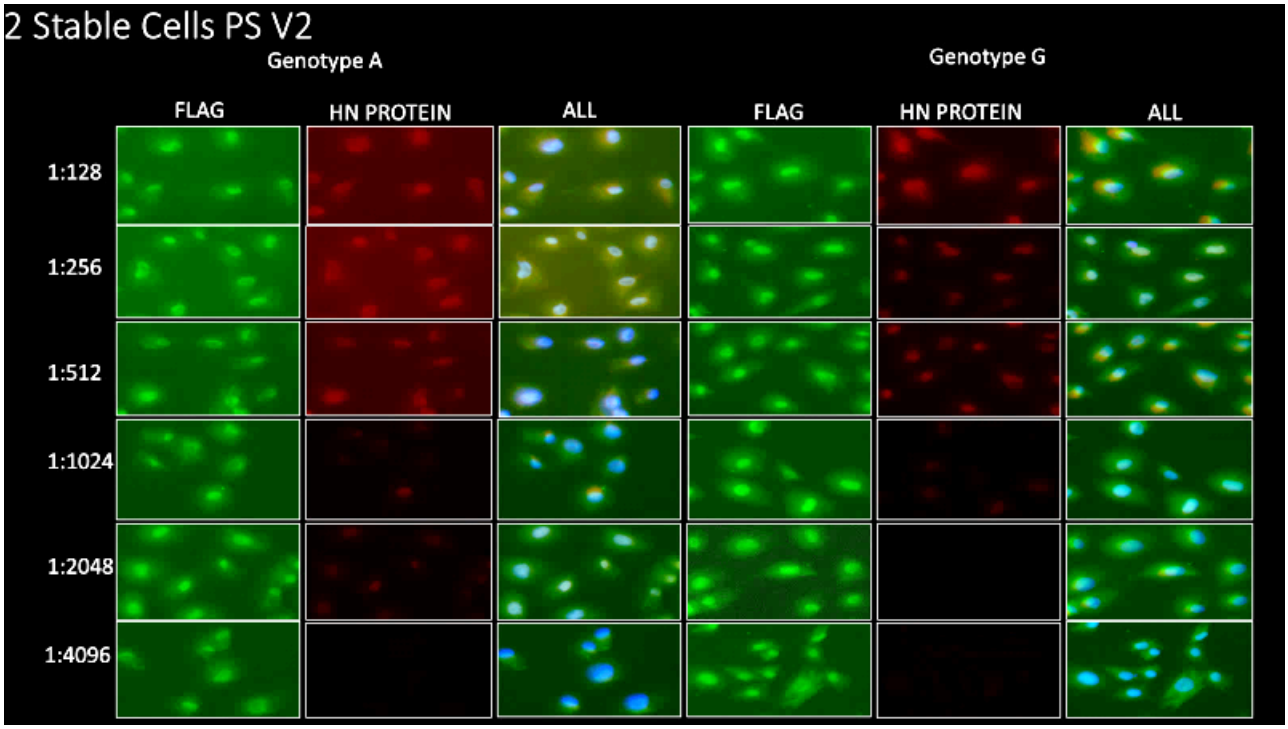


Figure A-5d. Stable cells Vaccine Sample #2.

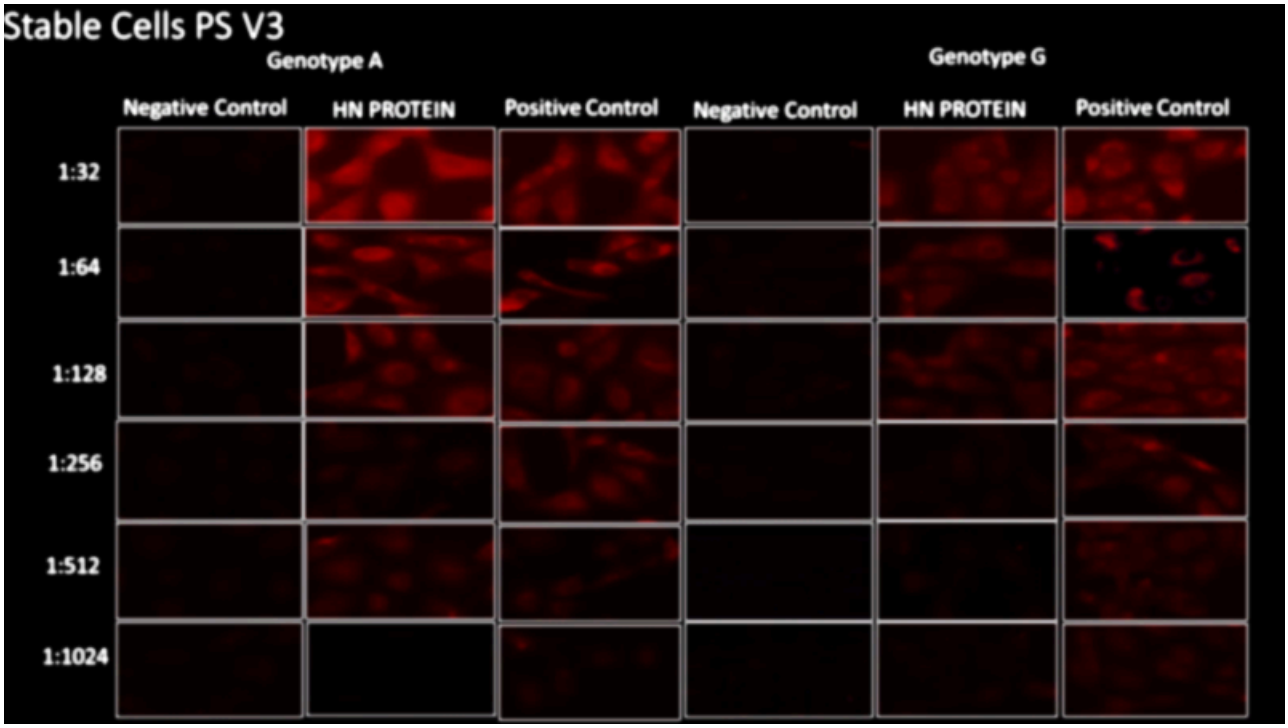


Figure A-6a. Stable cells Vaccine Sample#3.

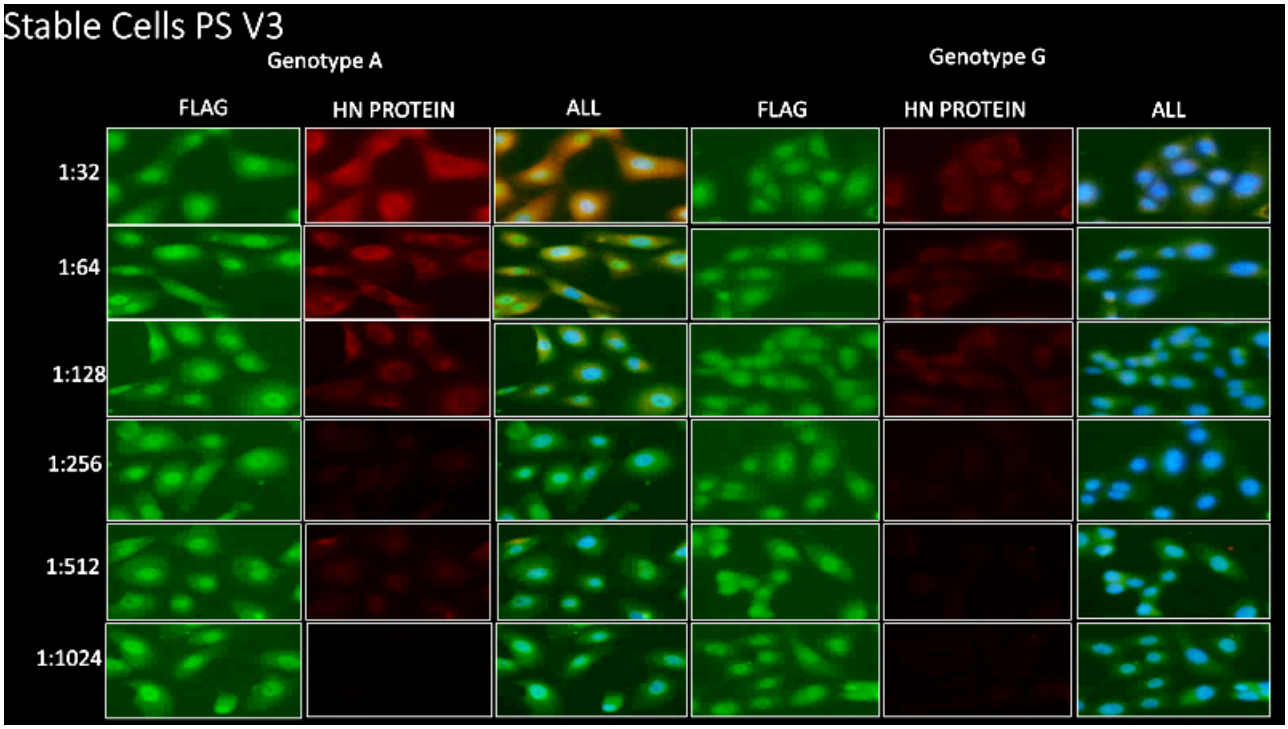


Figure A-6b. Stable cells Vaccine Sample #3.

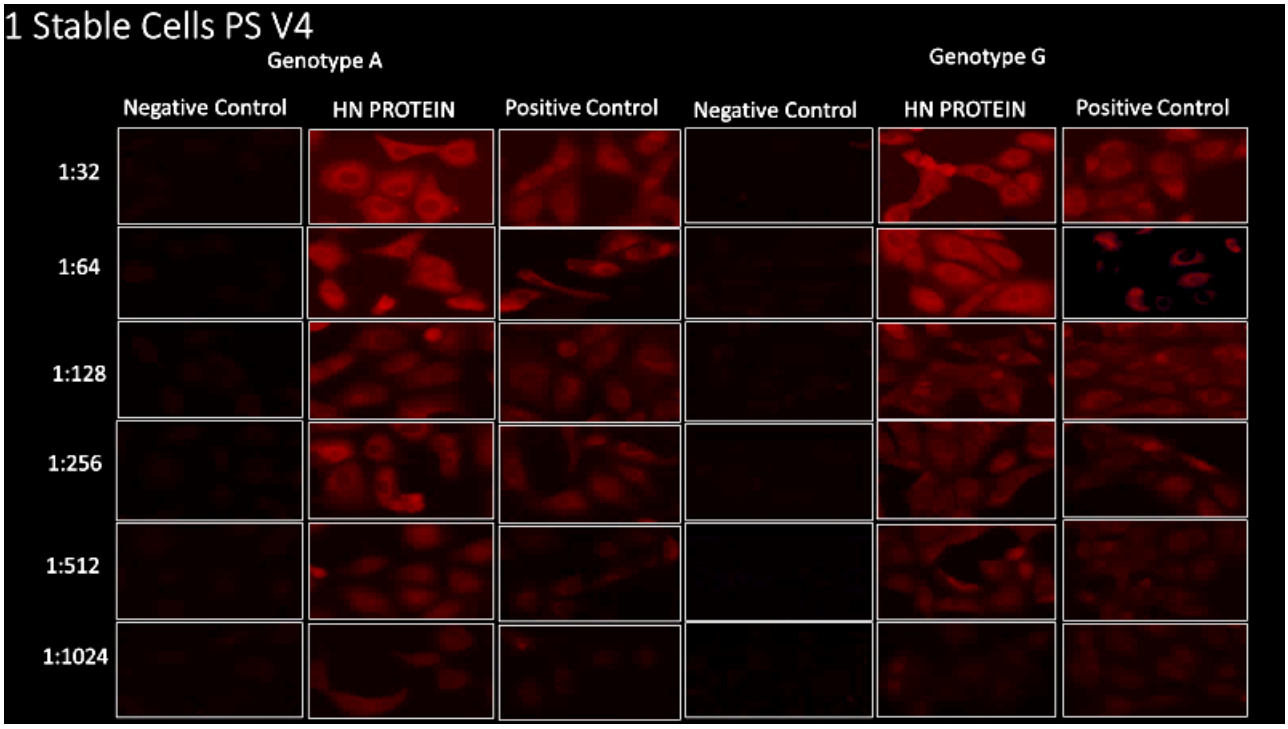


Figure A-7a. Stable cells Vaccine Sample#4.

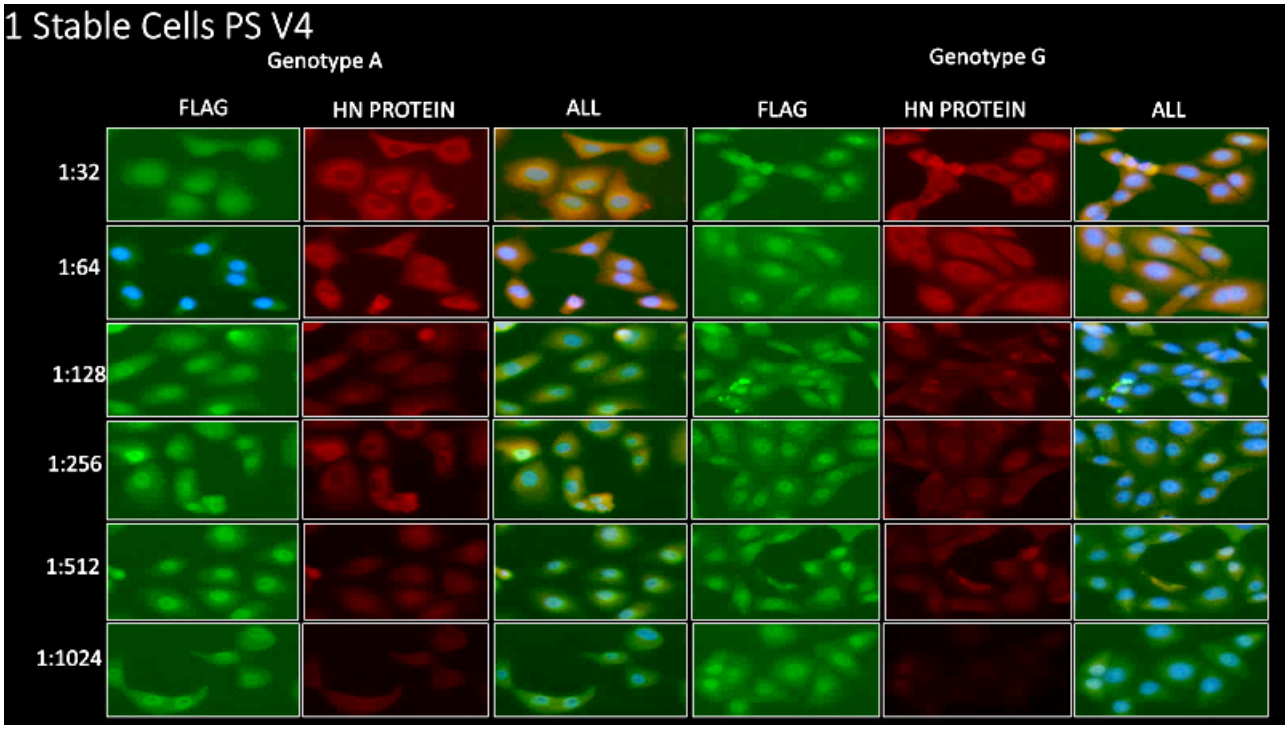


Figure A-7b. Stable cells Vaccine Sample #4.

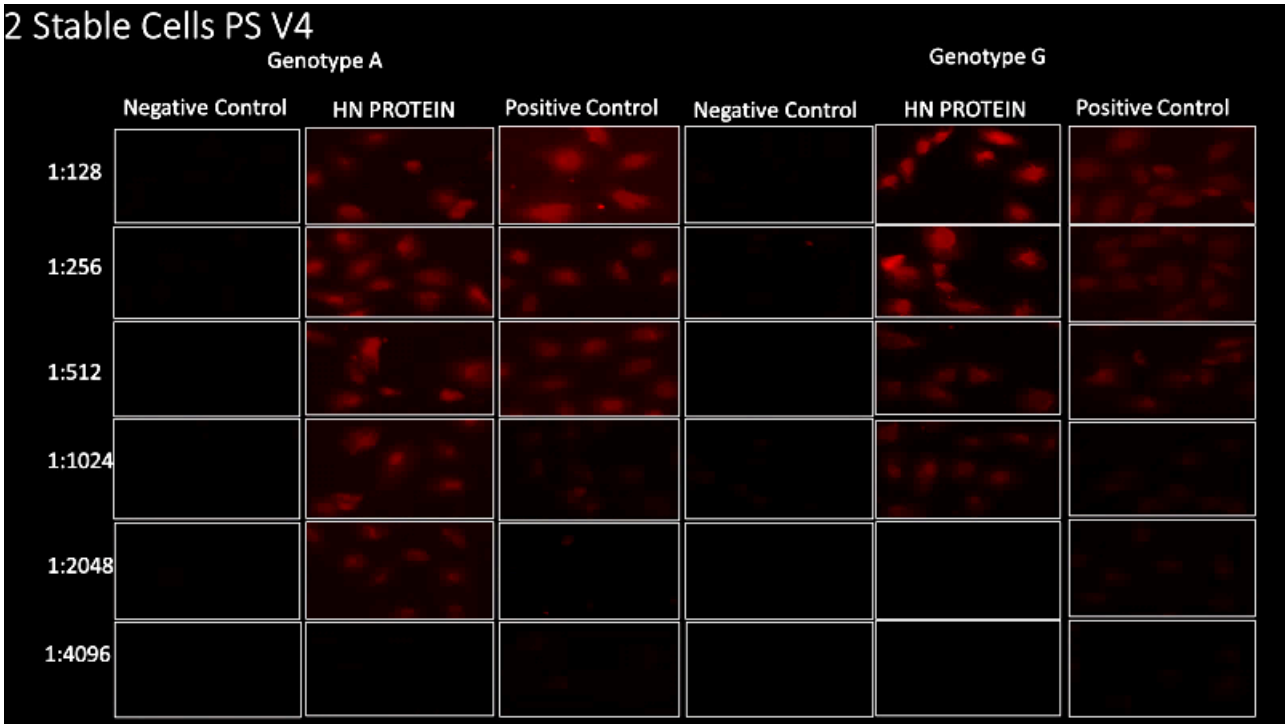


Figure A-7c. Stable cells Vaccine Sample #4.

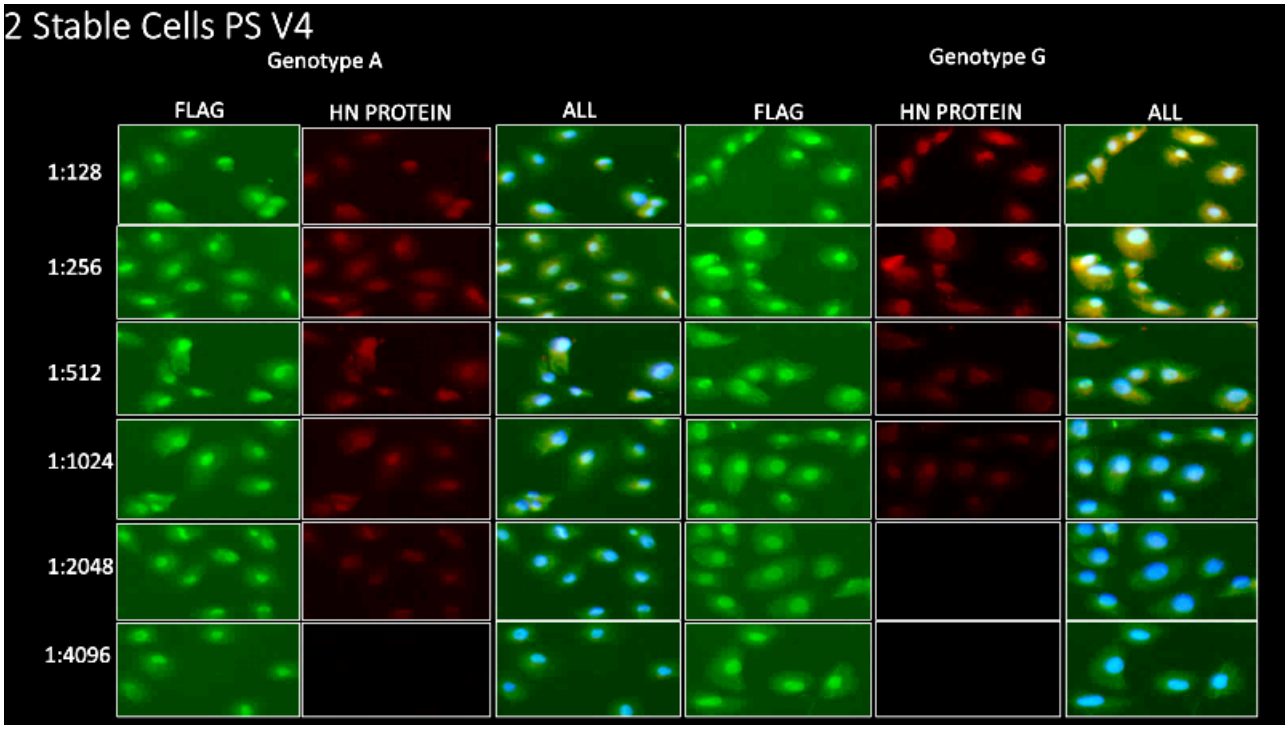


Figure A-7d. Stable cells Vaccine Sample #4.

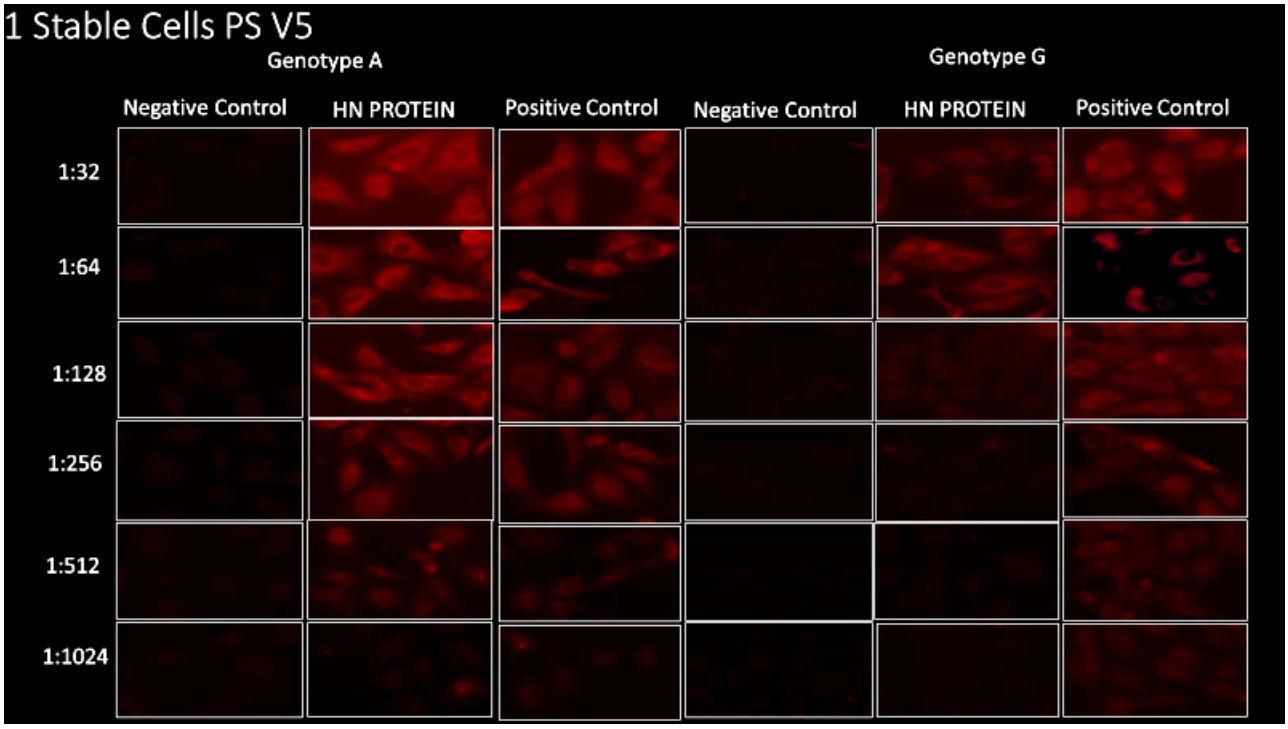


Figure A-8a. Stable cells Vaccine Sample#5.

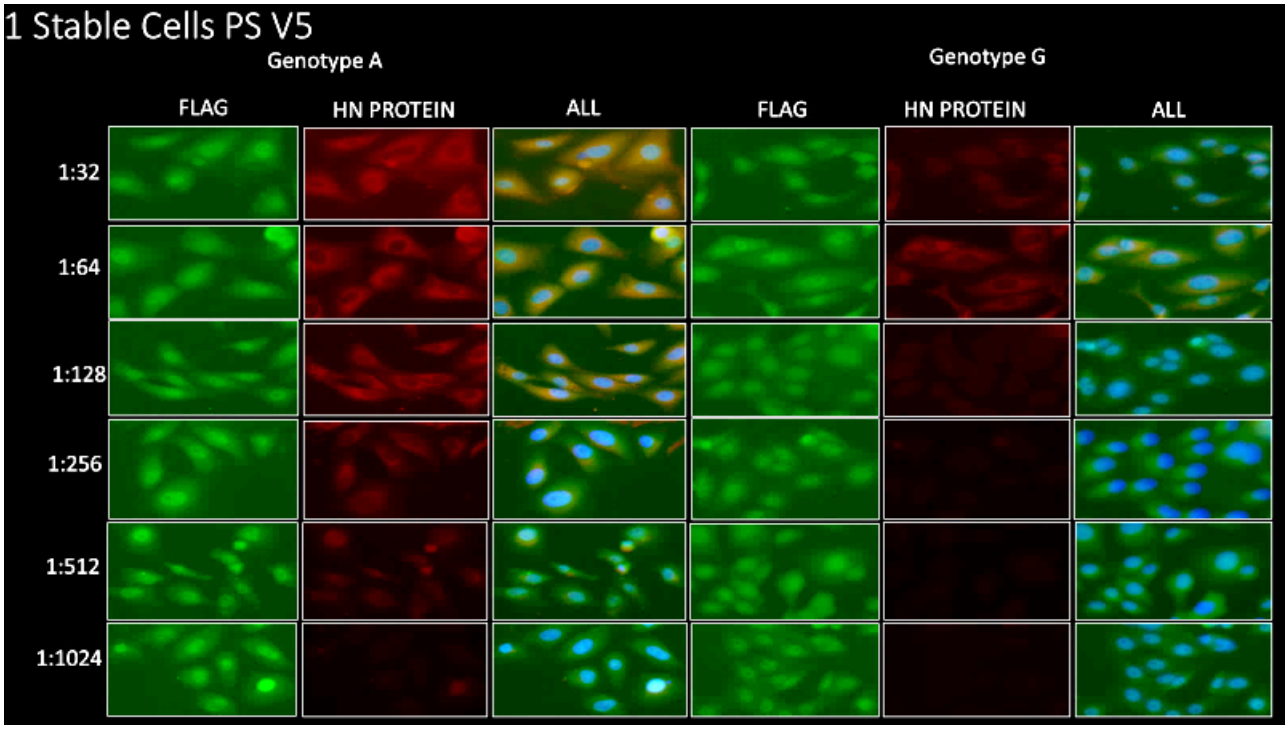


Figure A-8b. Stable cells Vaccine Sample #5.

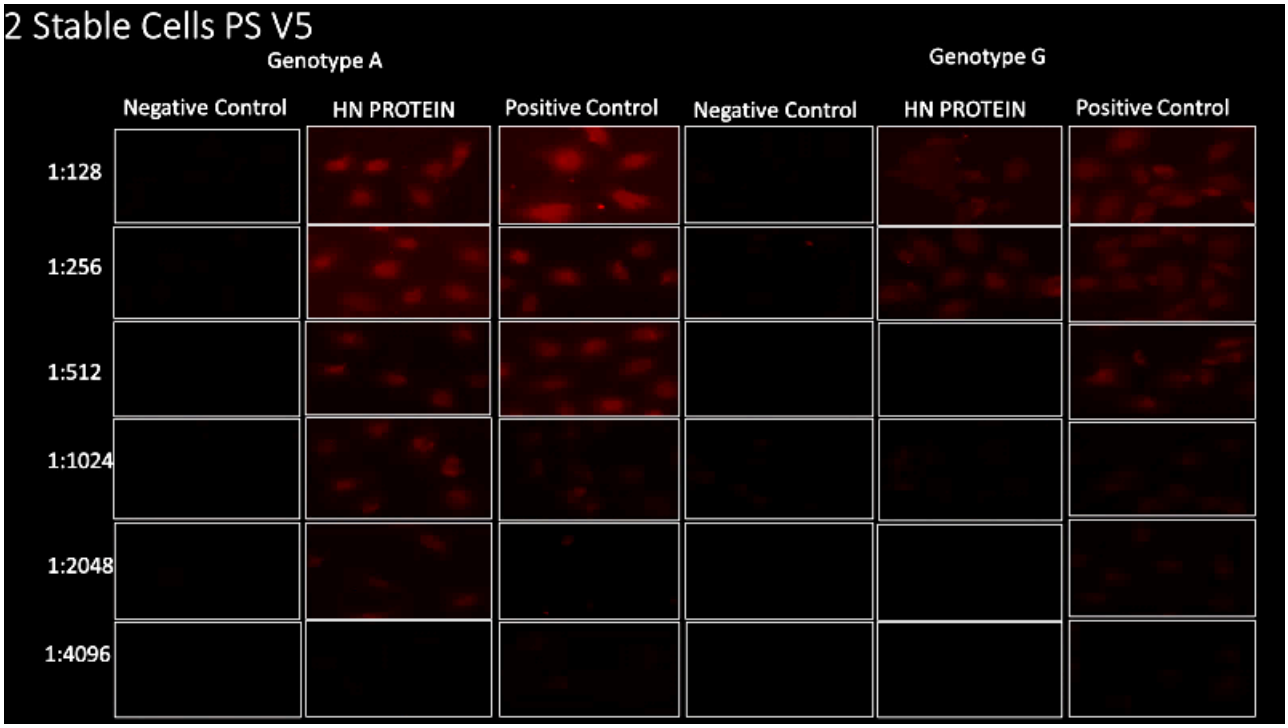


Figure A-8c. Stable cells Vaccine Sample #5.

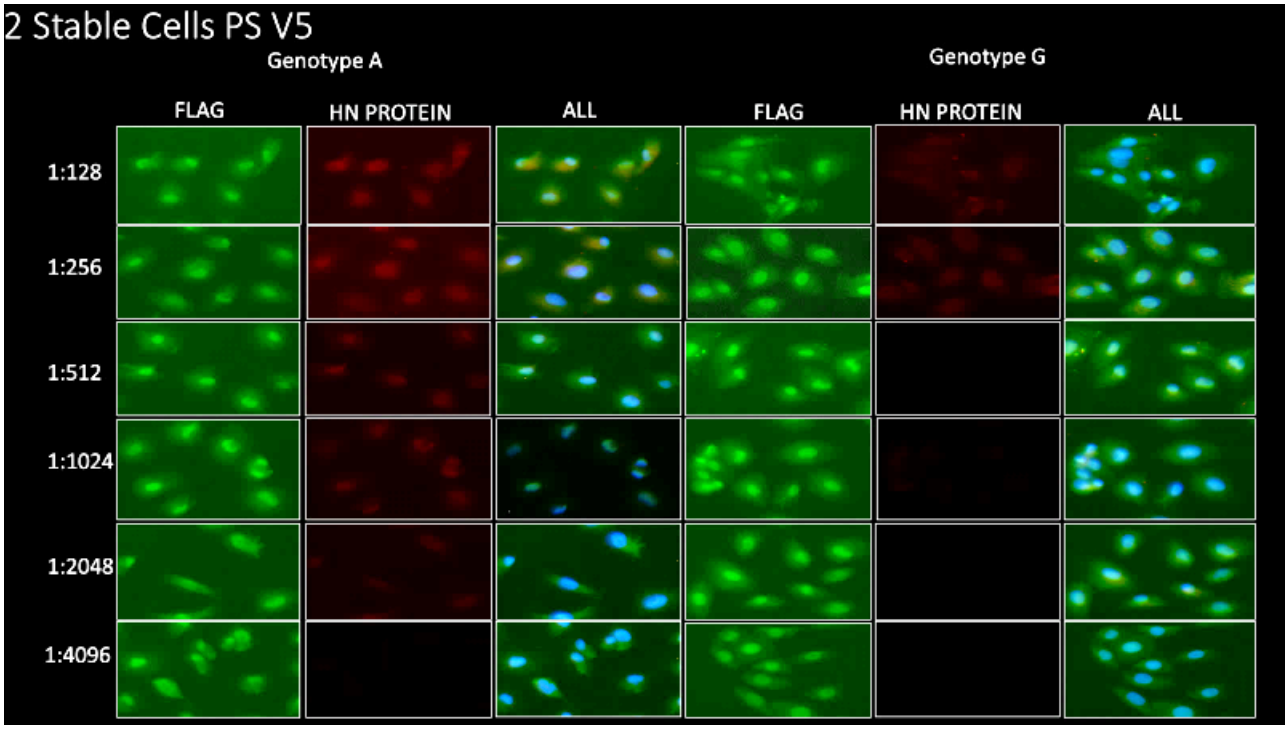


Figure A-8d. Stable cells Vaccine Sample #5.

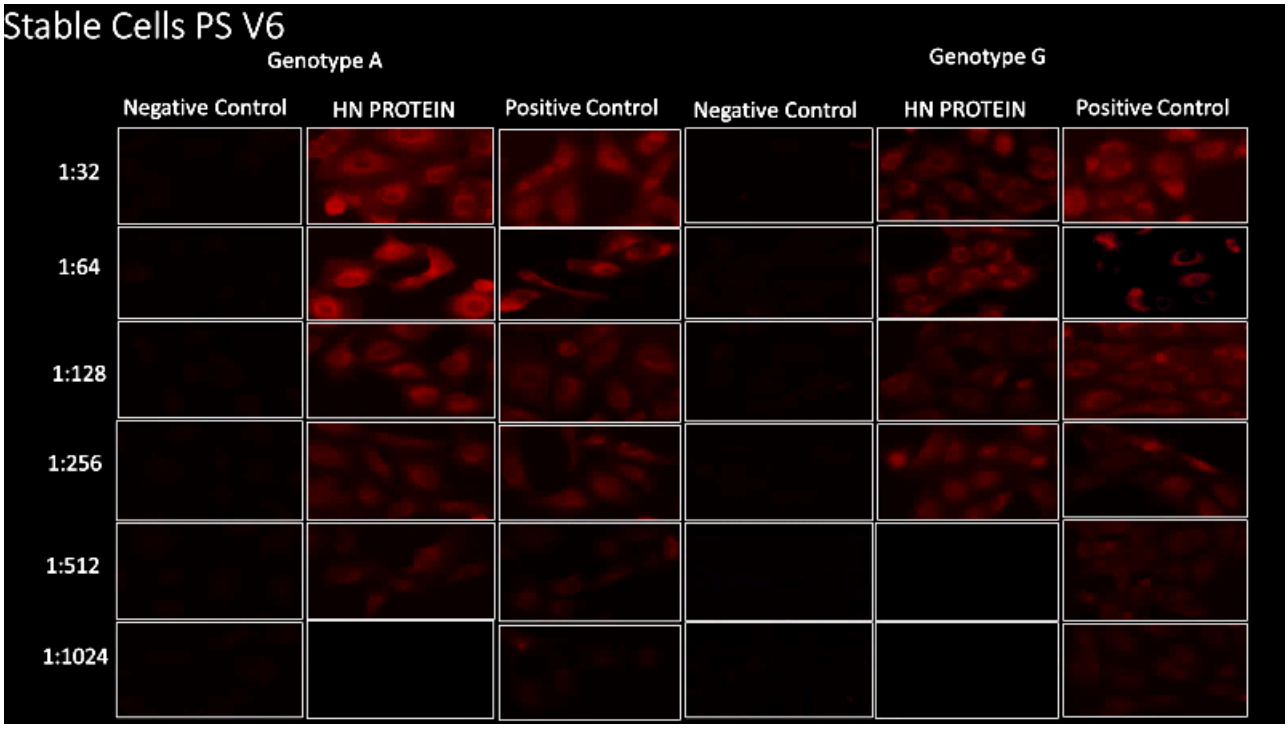


Figure A-9a. Stable cells Vaccine Sample#6.

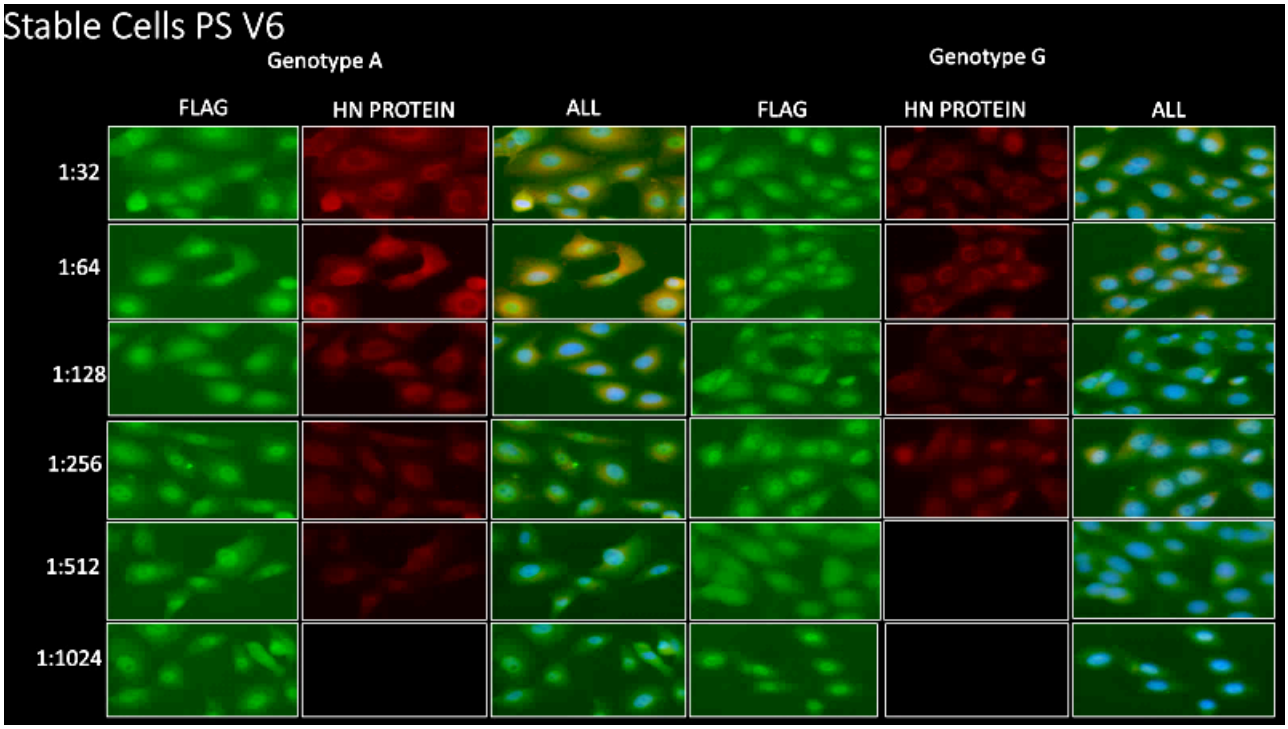


Figure A-9b. Stable cells Vaccine Sample #6.

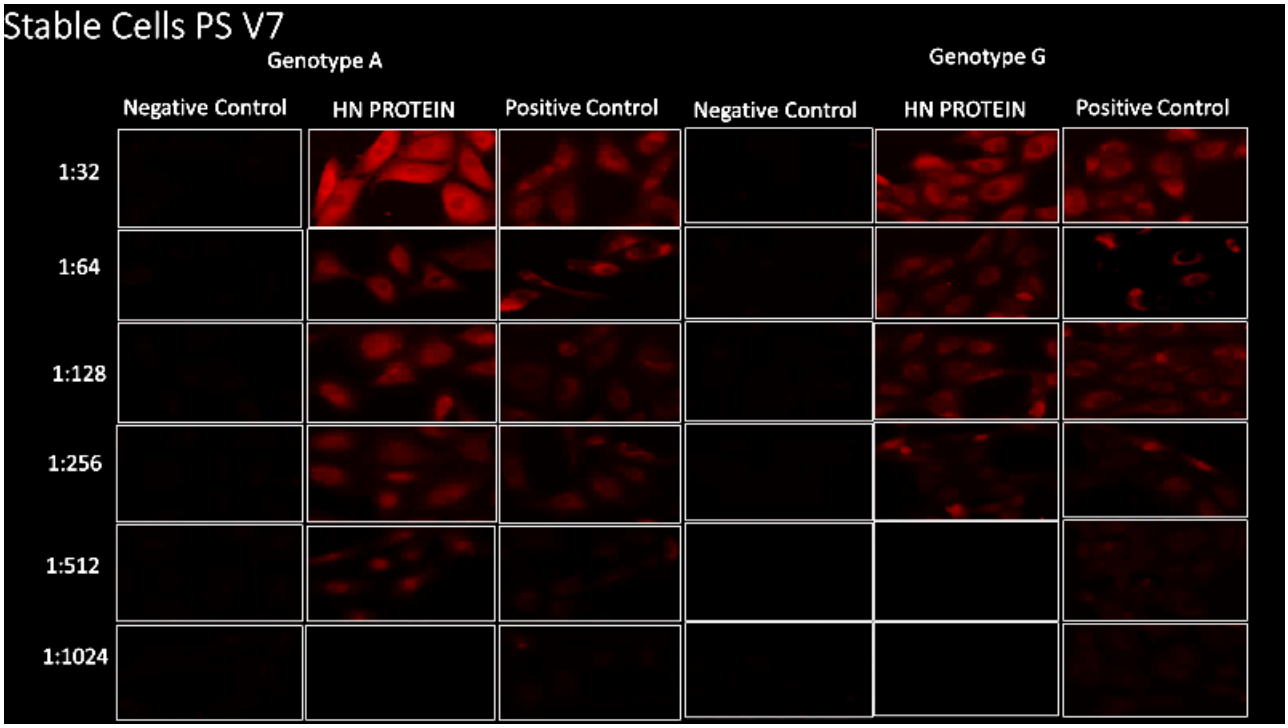


Figure A-10a. Stable cells Vaccine Sample#7.

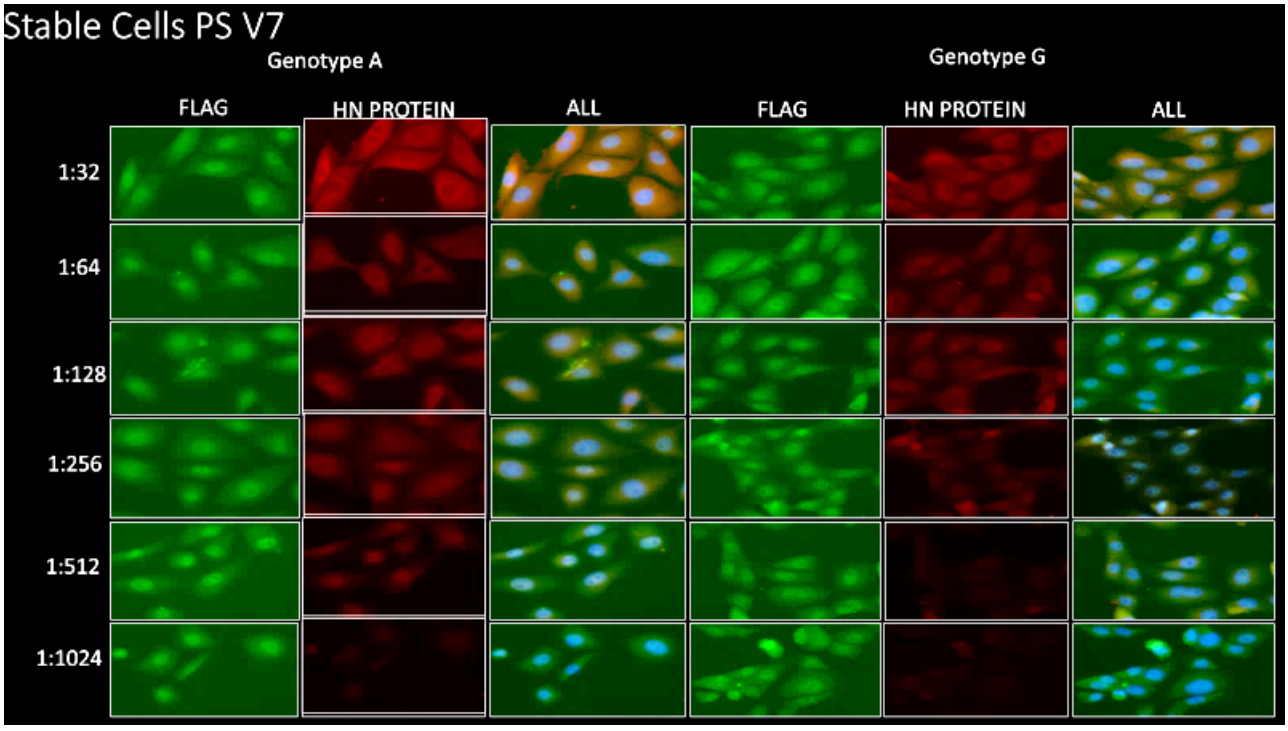


Figure A-10b. Stable cells Vaccine Sample #7.

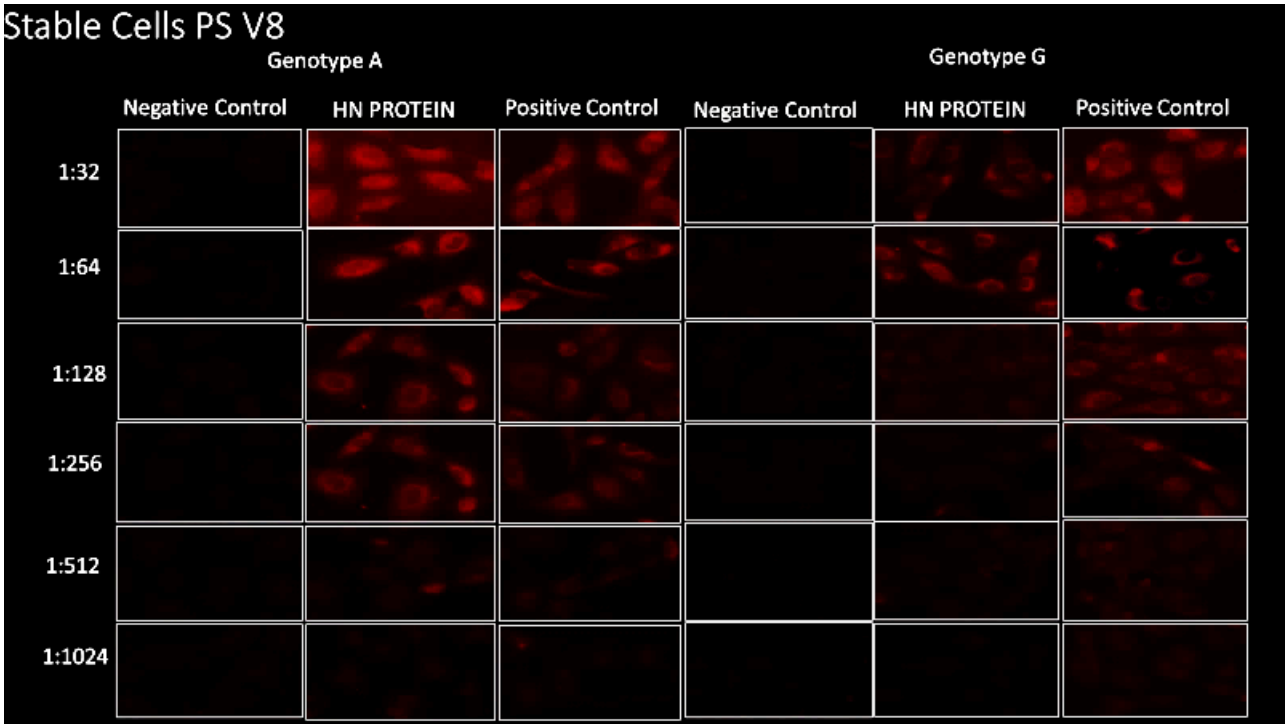


Figure A-11a. Stable cells Vaccine Sample#8.

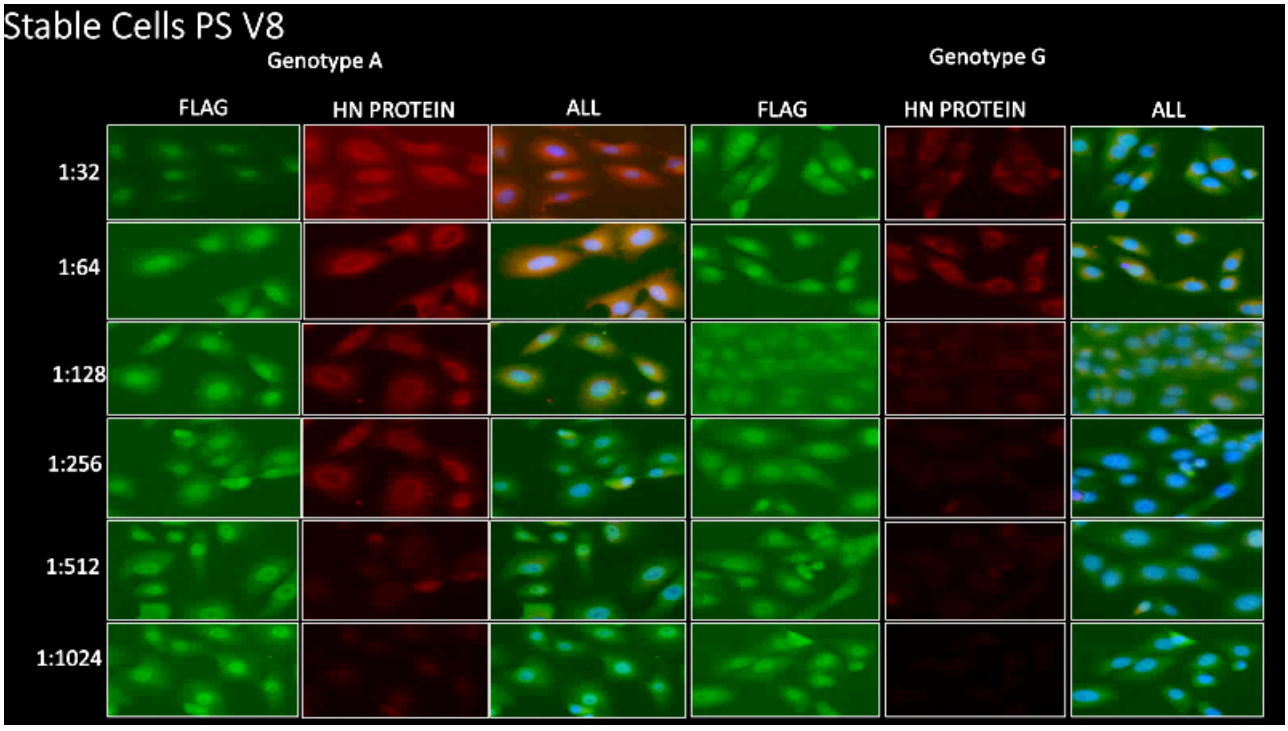


Figure A-11b. Stable cells Vaccine Sample #8.

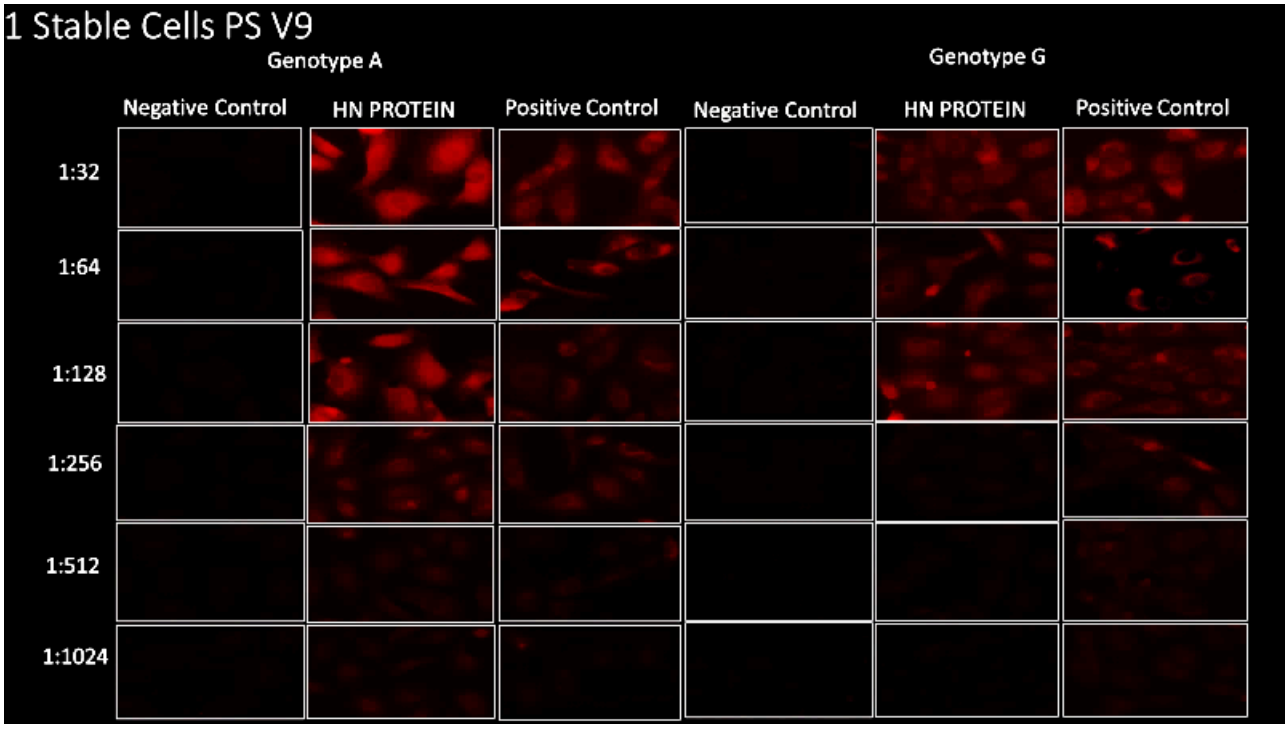


Figure A-12a. Stable cells Vaccine Sample#9.

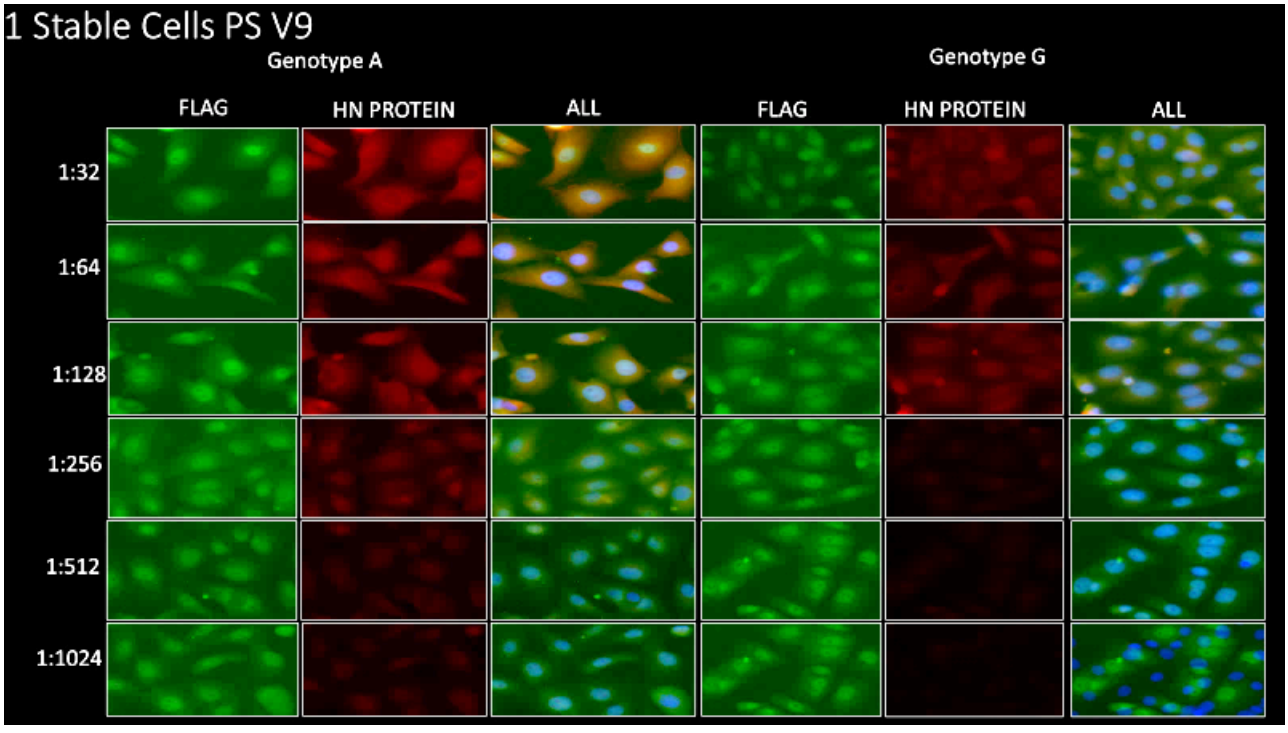


Figure A-12b. Stable cells Vaccine Sample #9.

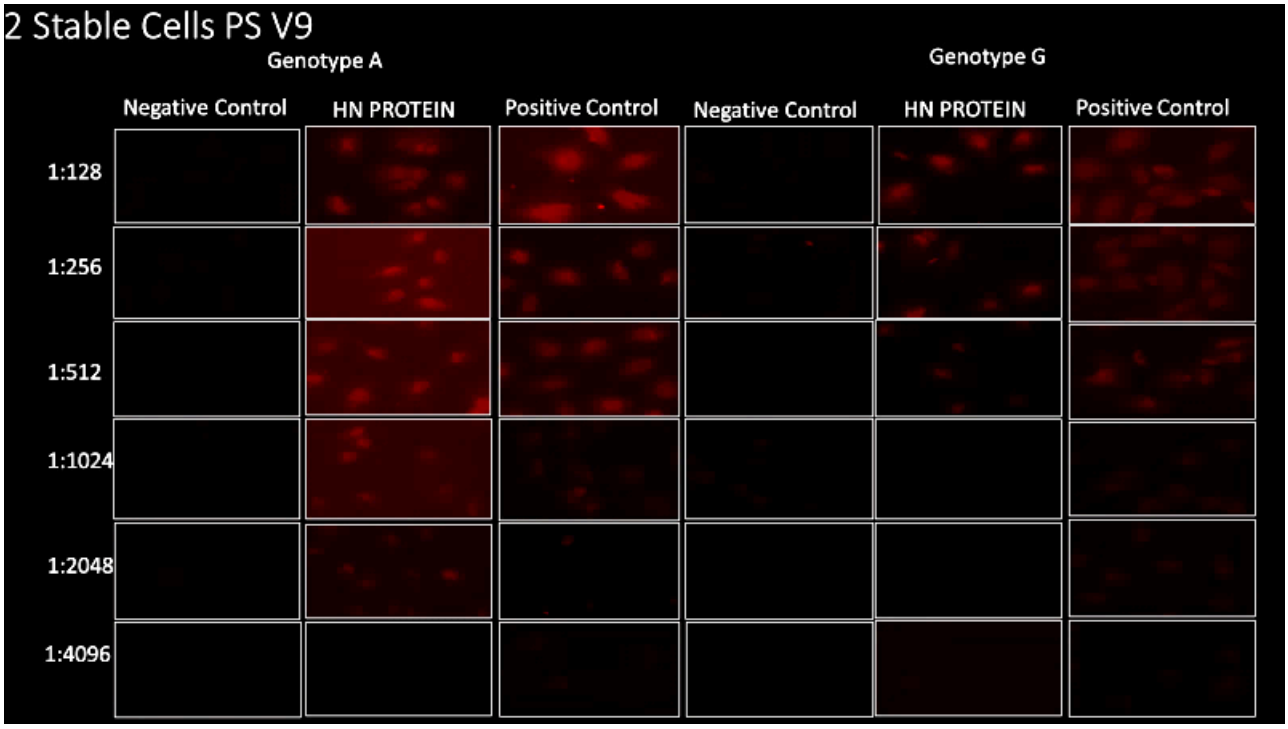


Figure A-12c. Stable cells Vaccine Sample #9.

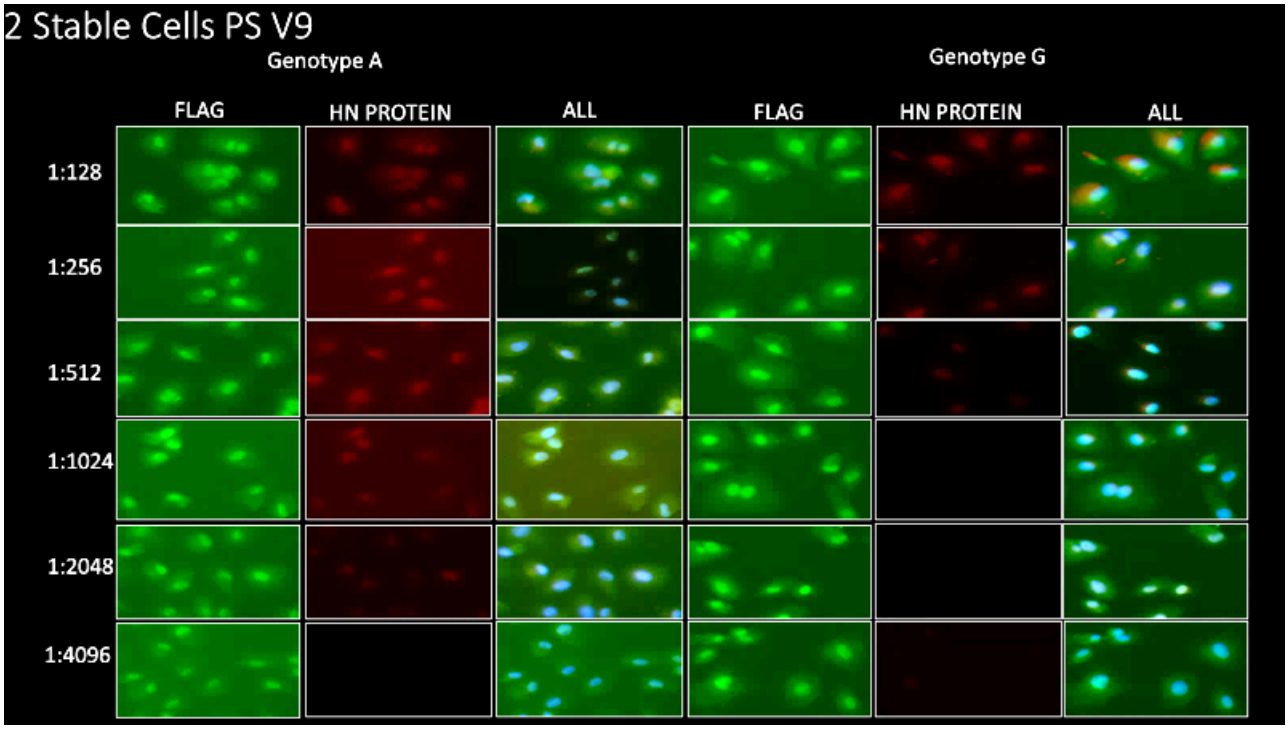


Figure A-12d. Stable cells Vaccine Sample #9.

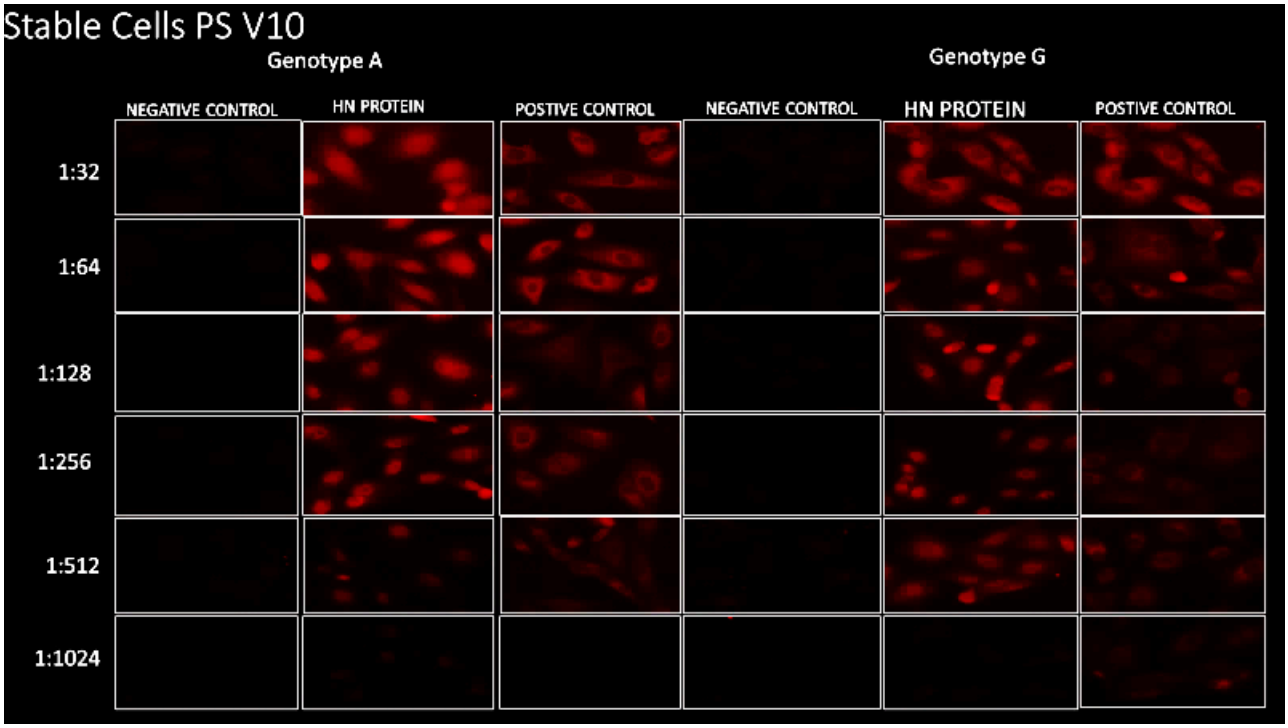


Figure A-13a. Stable cells Vaccine Sample#10.

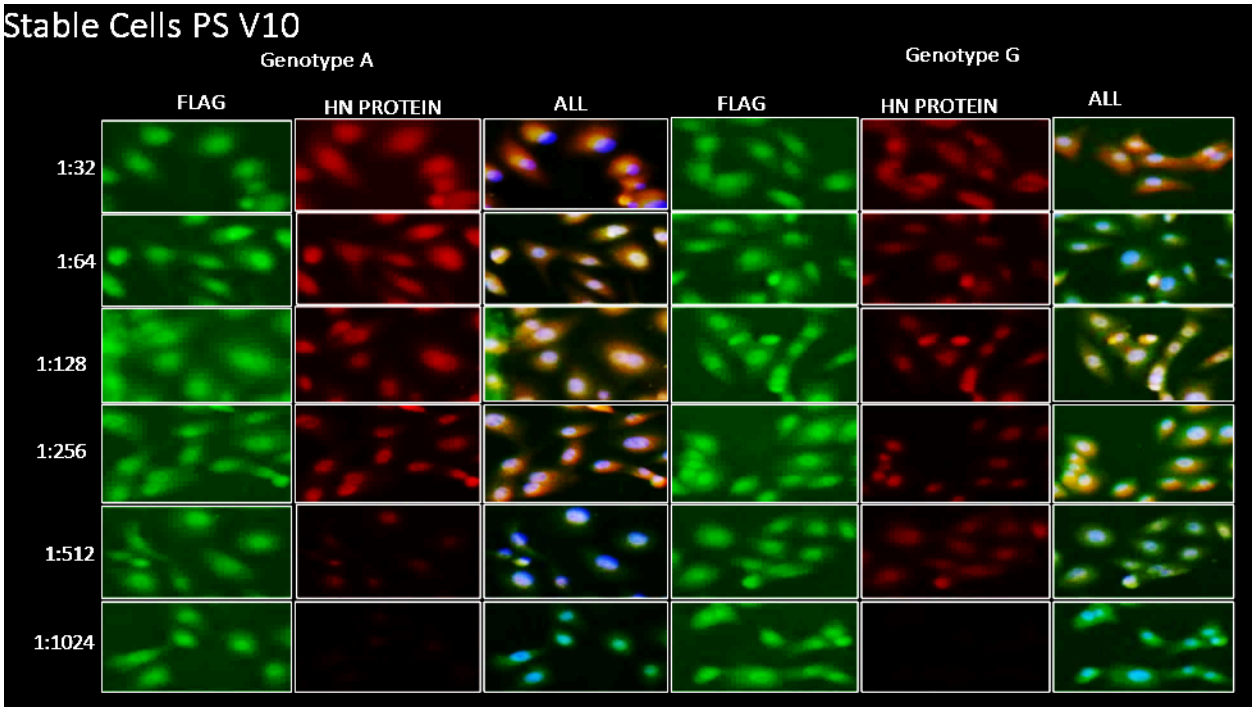


Figure A-13b. Stable cells Vaccine Sample #10.

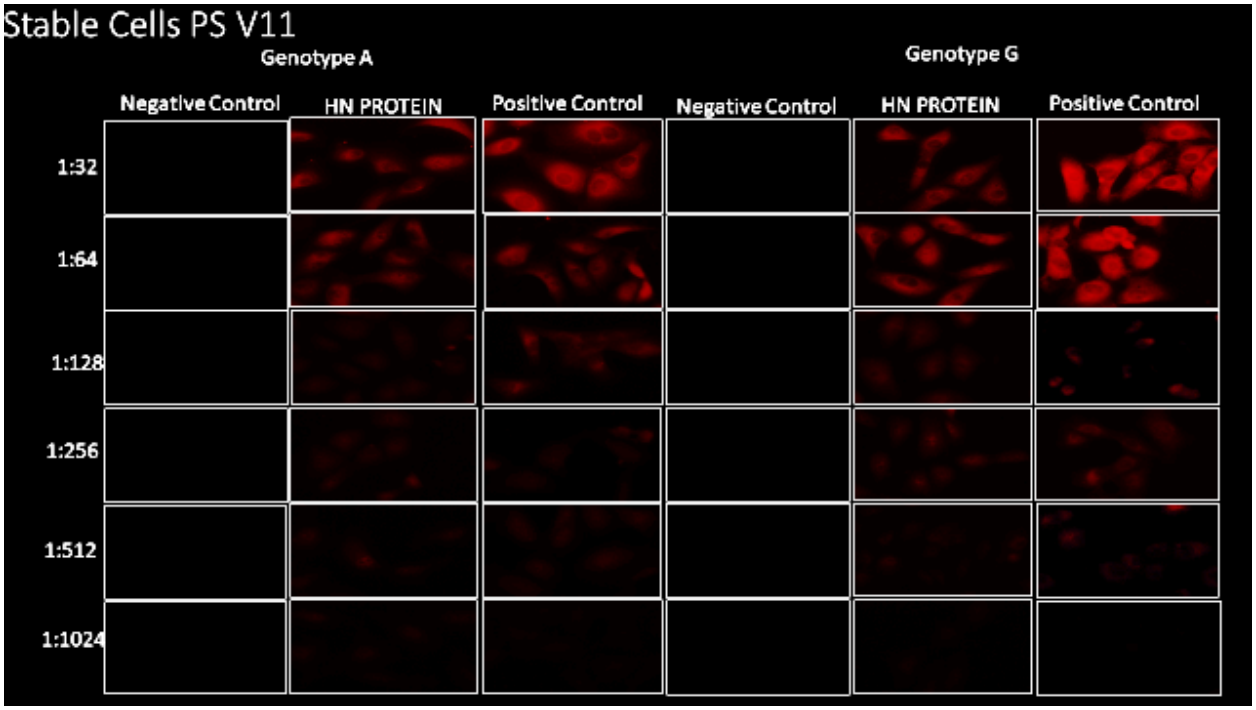


Figure A-14a. Stable cells Vaccine Sample#11.

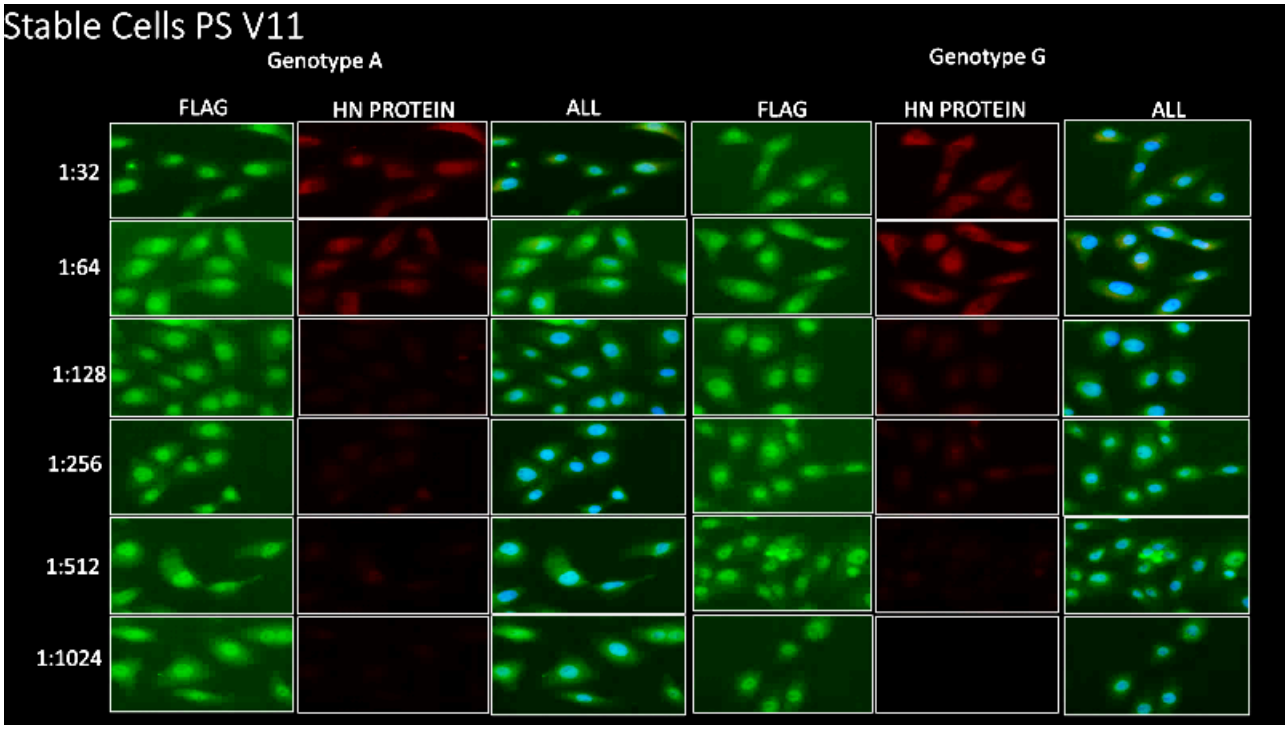


Figure A-14b. Stable cells Vaccine Sample #11.

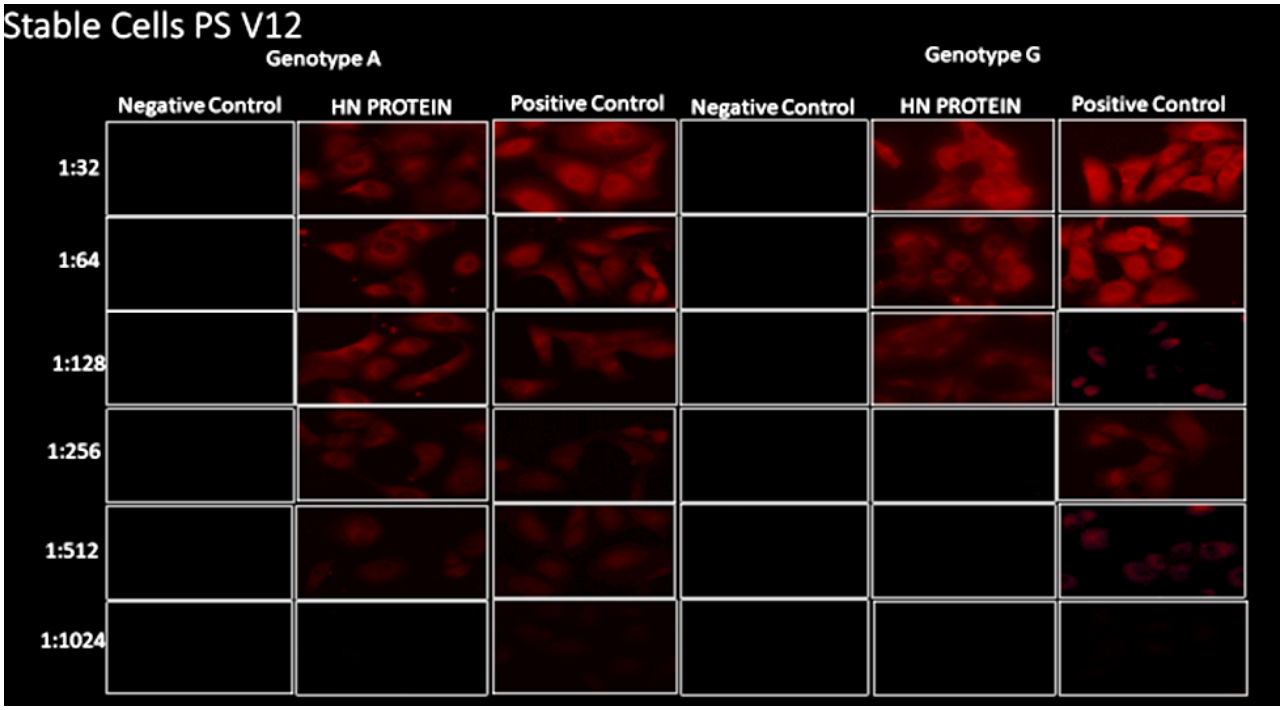


Figure A-15a. Stable cells Vaccine Sample#12.

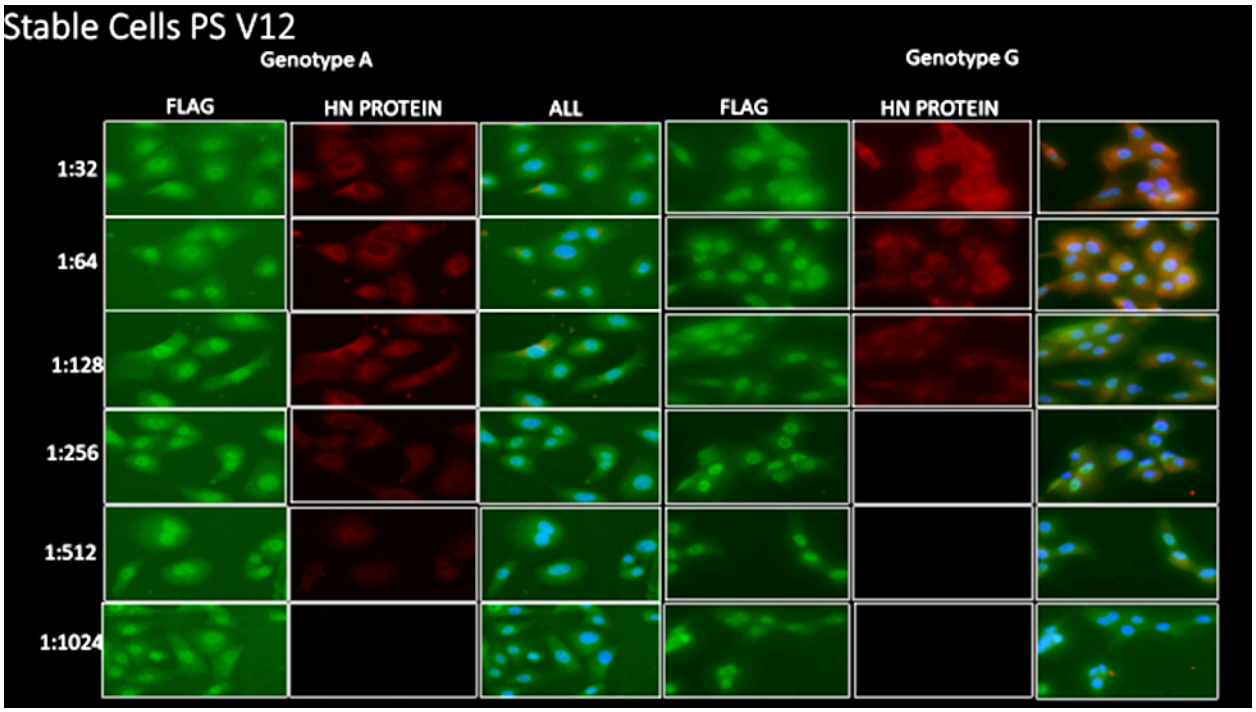


Figure A-15b. Stable cells Vaccine Sample #12.

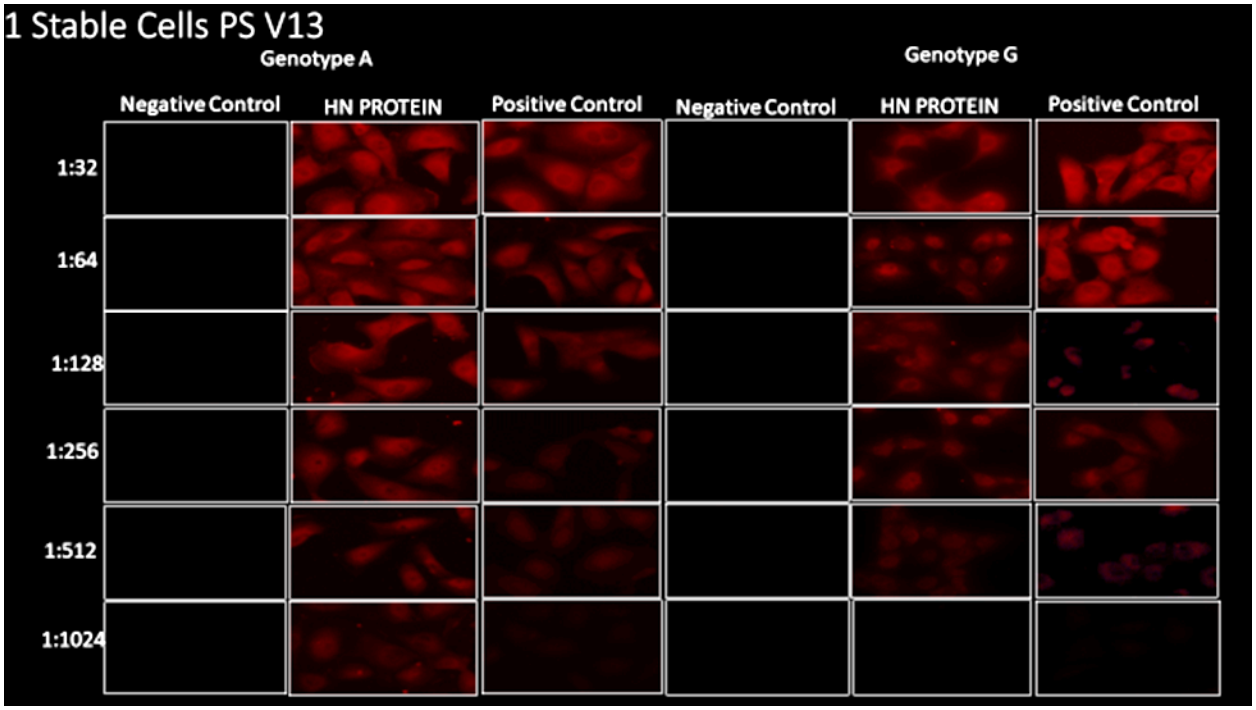


Figure A-16a. Stable cells Vaccine Sample#13.

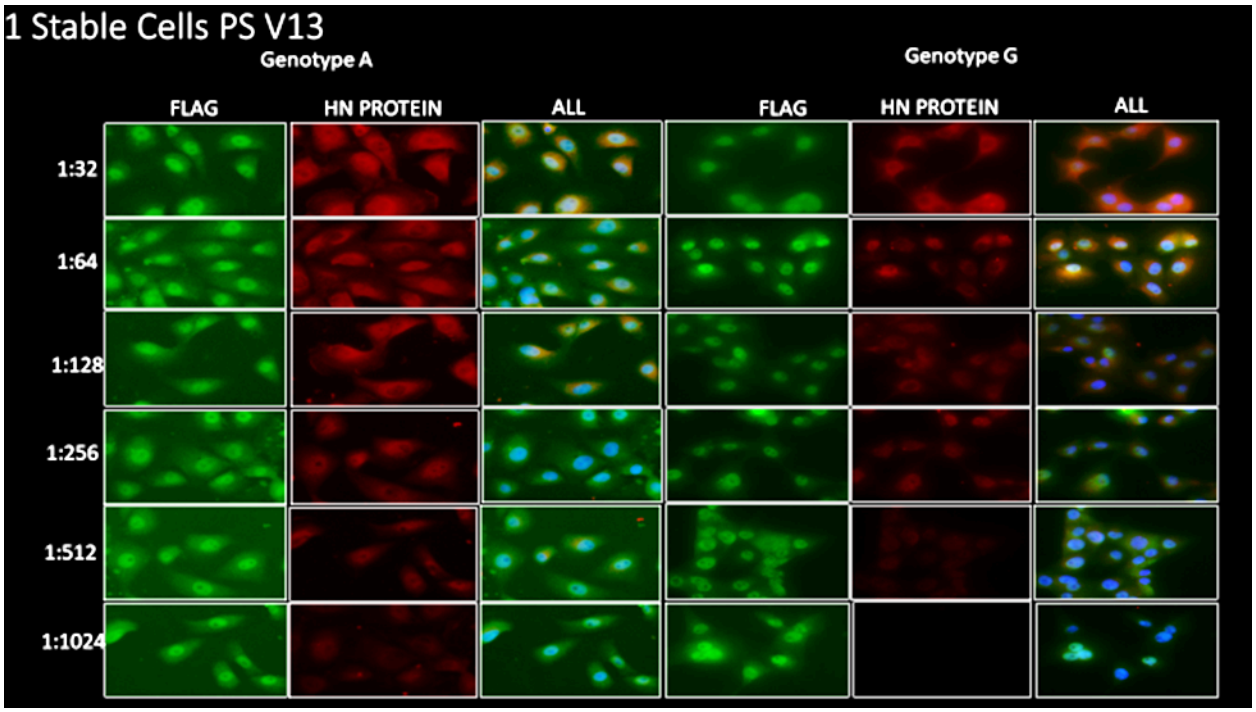


Figure A-16b. Stable cells Vaccine Sample #13.

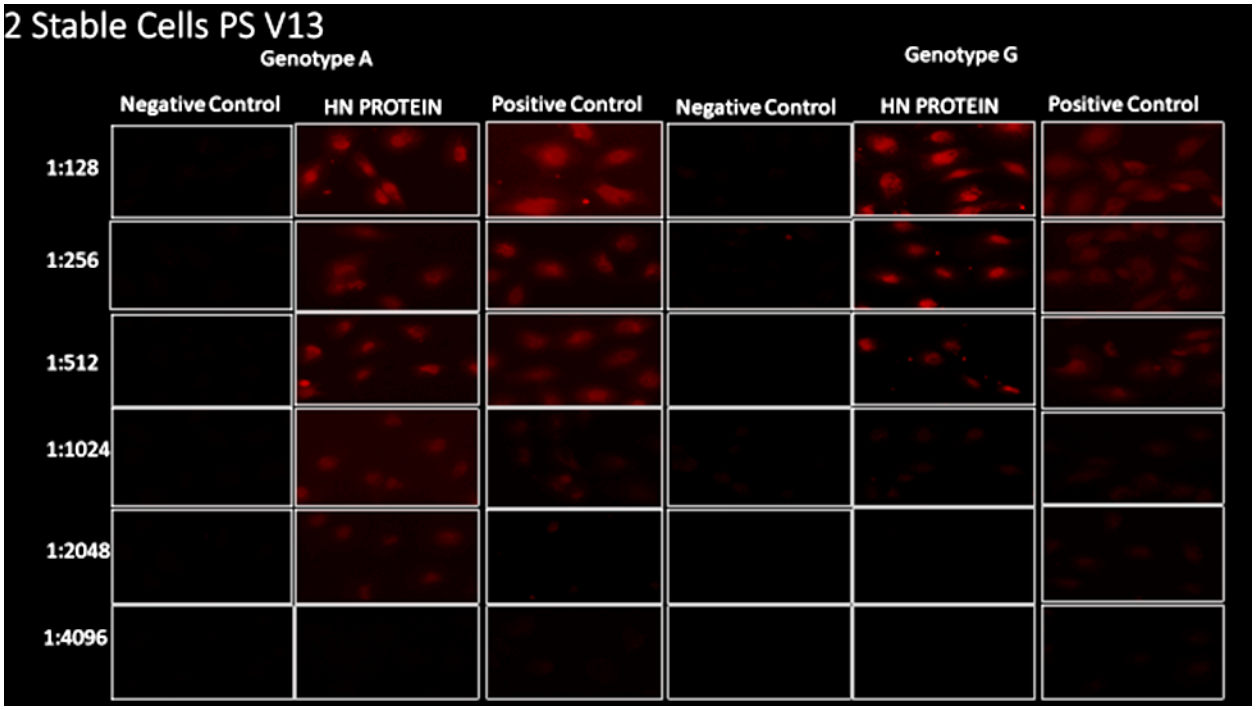


Figure A-16c. Stable cells Vaccine Sample #13.

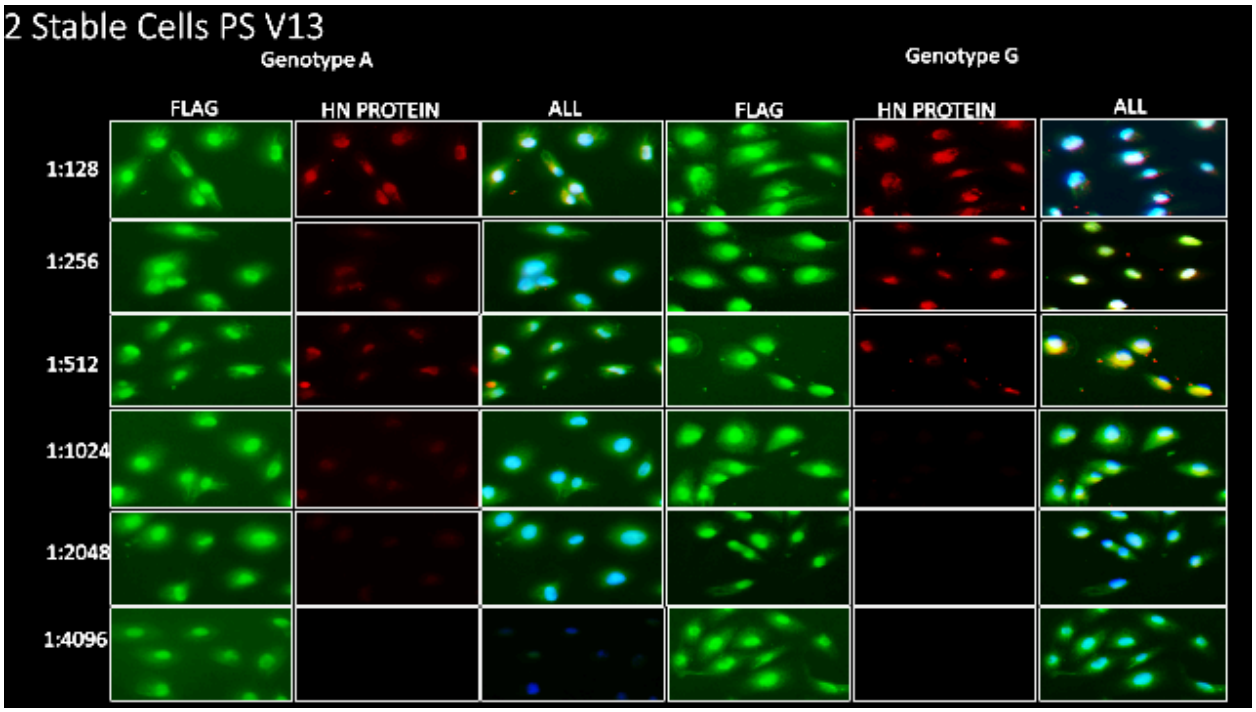


Figure A-16d. Stable cells Vaccine Sample #13.

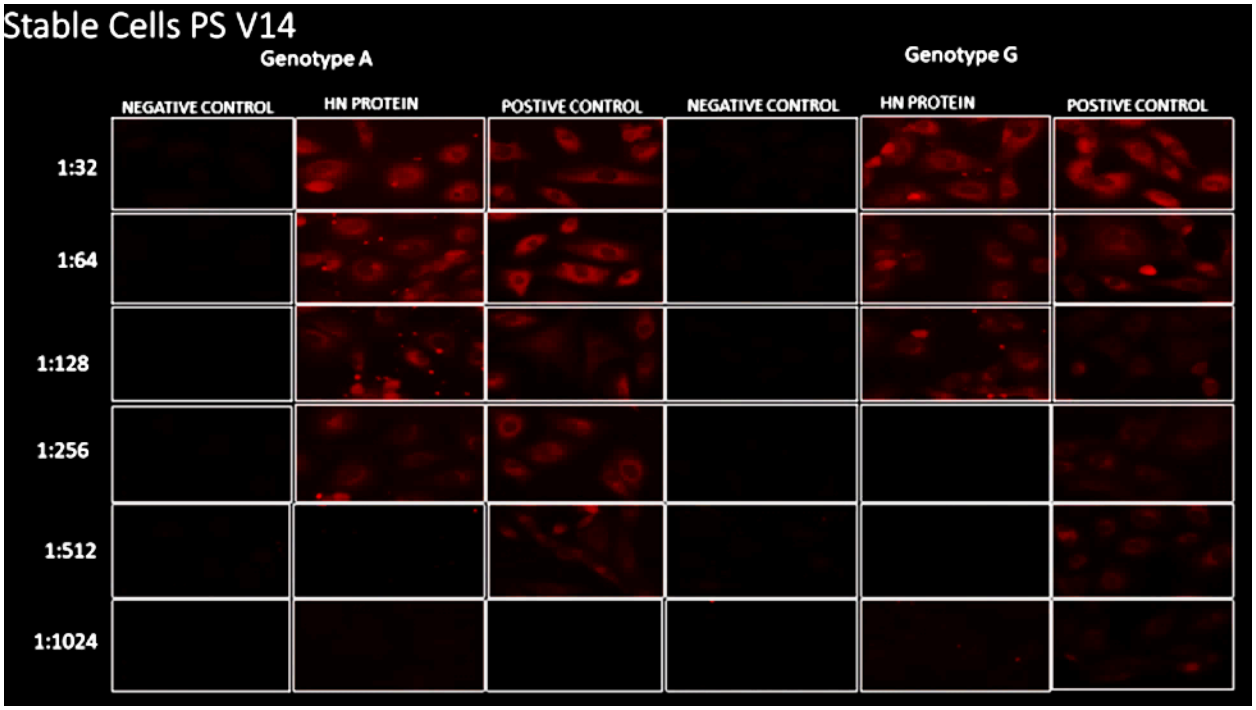


Figure A-17a. Stable cells Vaccine Sample#14.

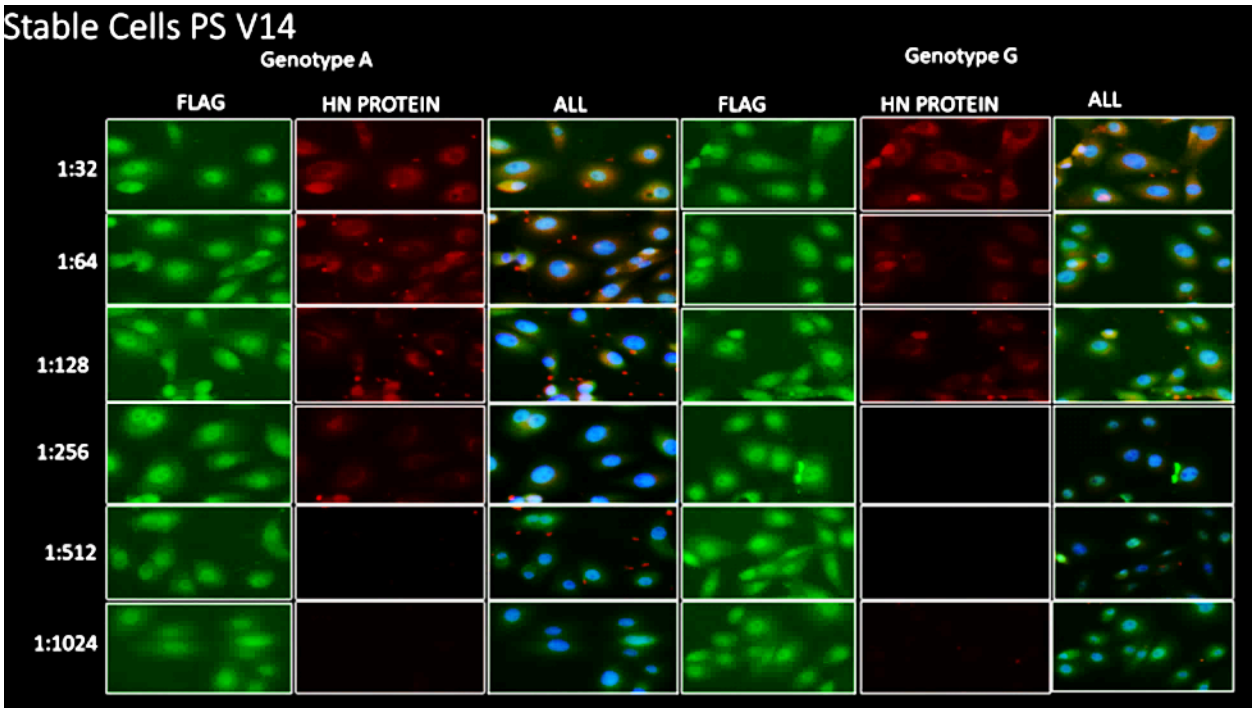


Figure A-17b. Stable cells Vaccine Sample #14.

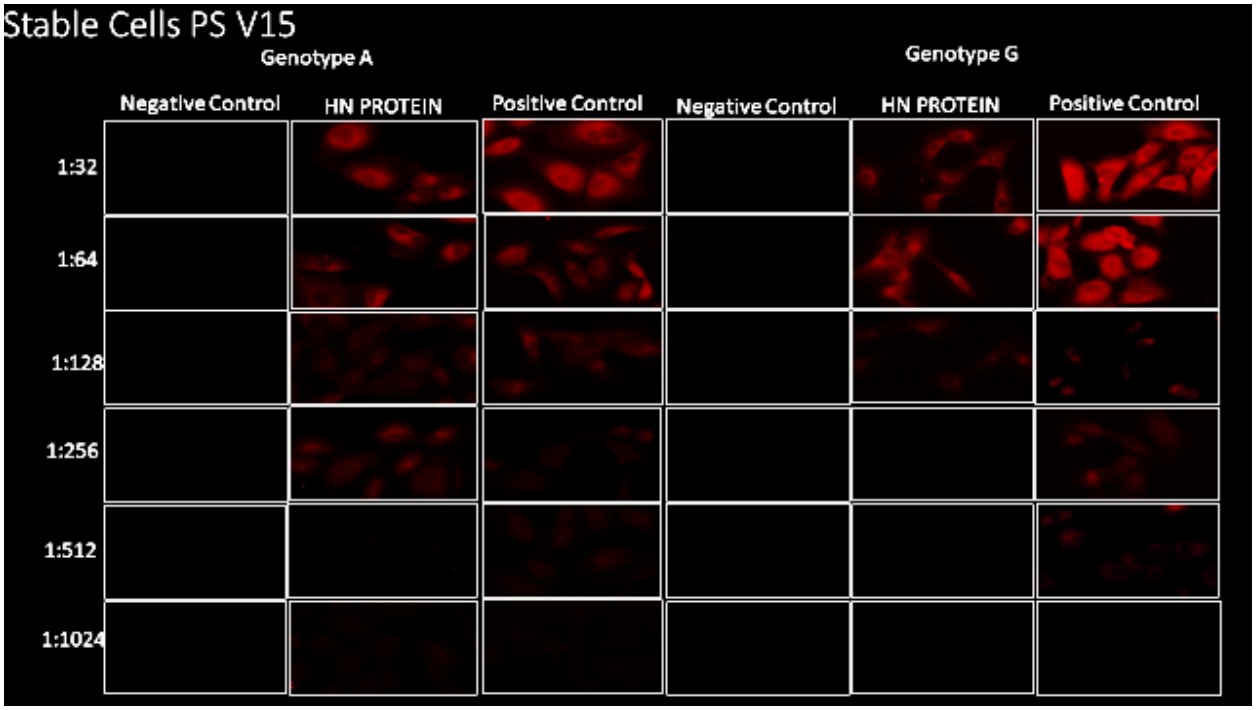


Figure A-18a. Stable cells Vaccine Sample#15.

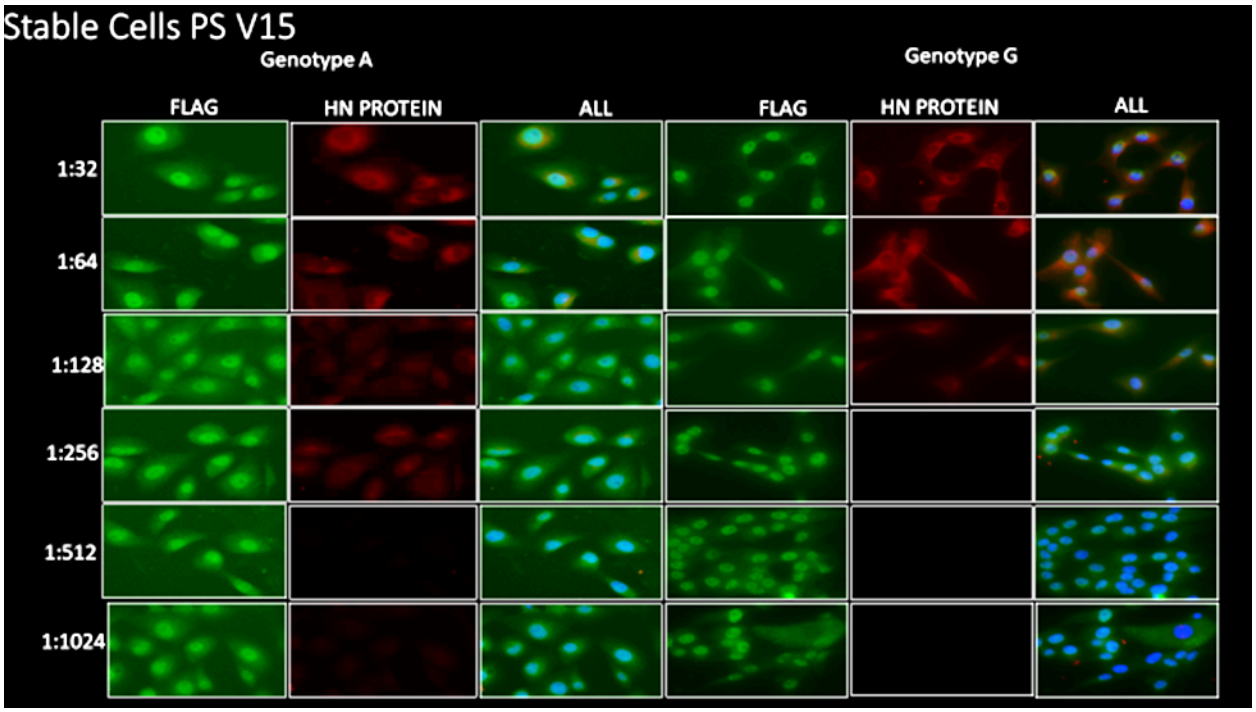


Figure A-18b. Stable cells Vaccine Sample #15.

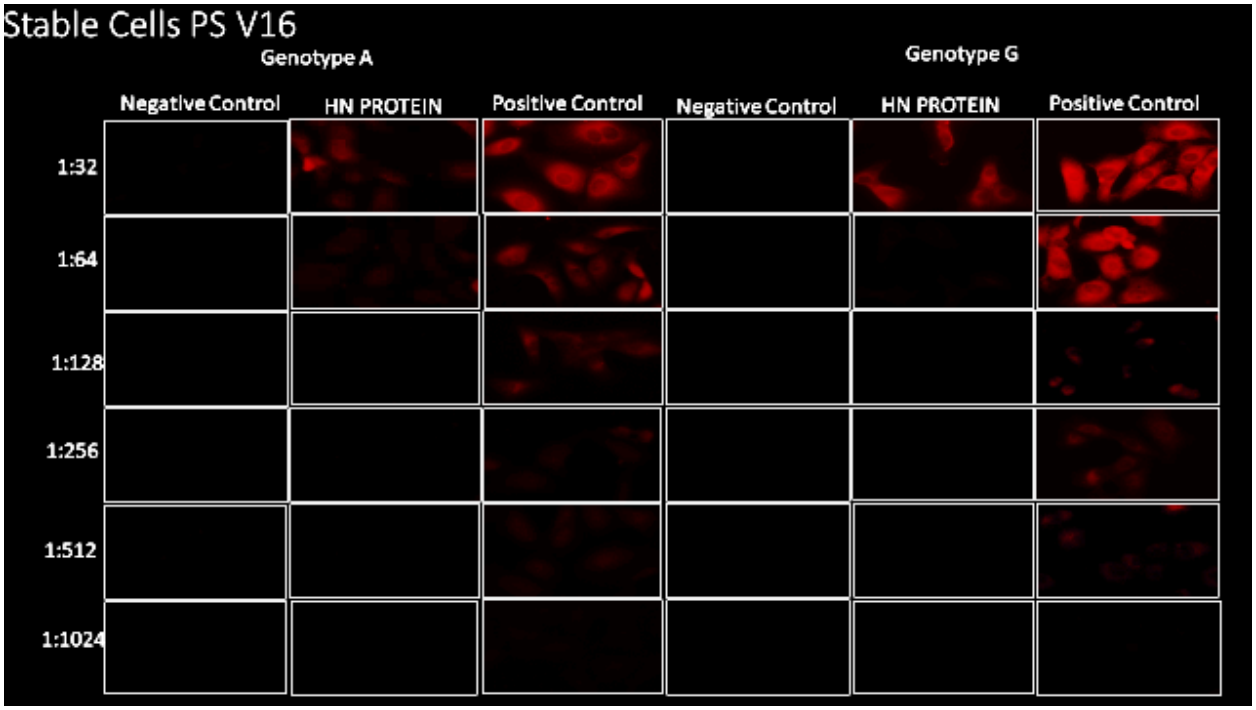


Figure A-19a. Stable cells Vaccine Sample#16.

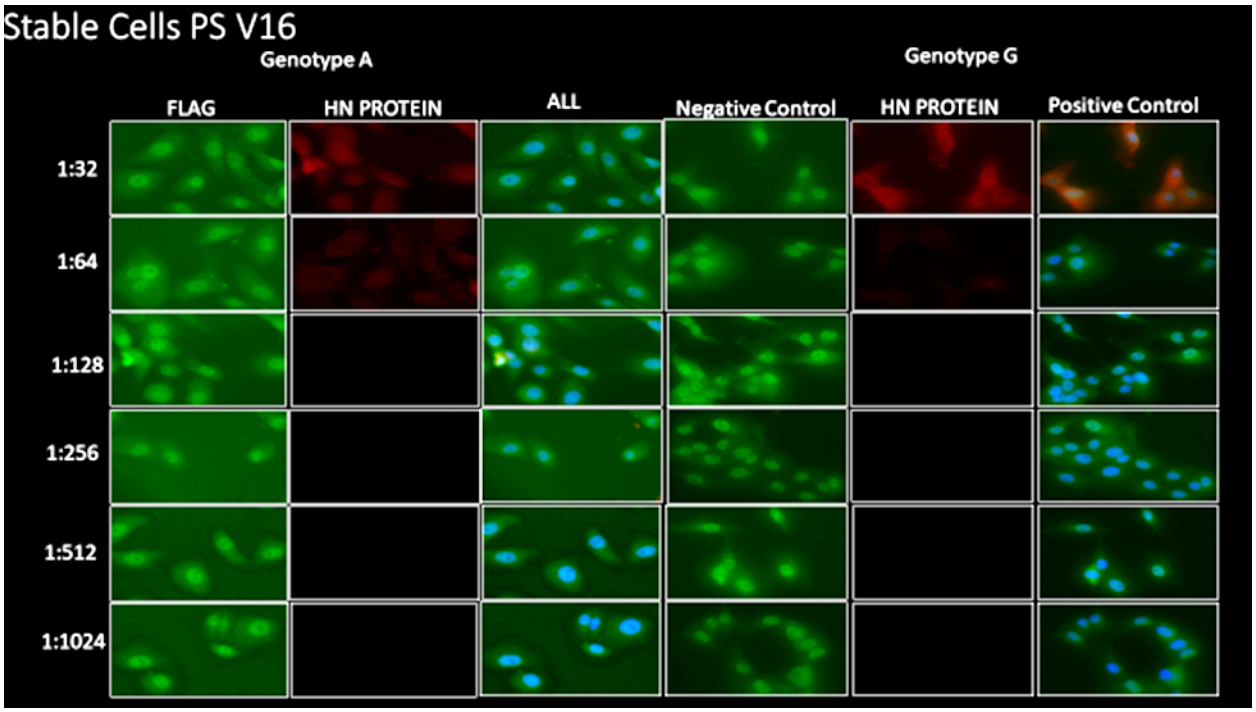


Figure A-19b. Stable cells Vaccine Sample #16.

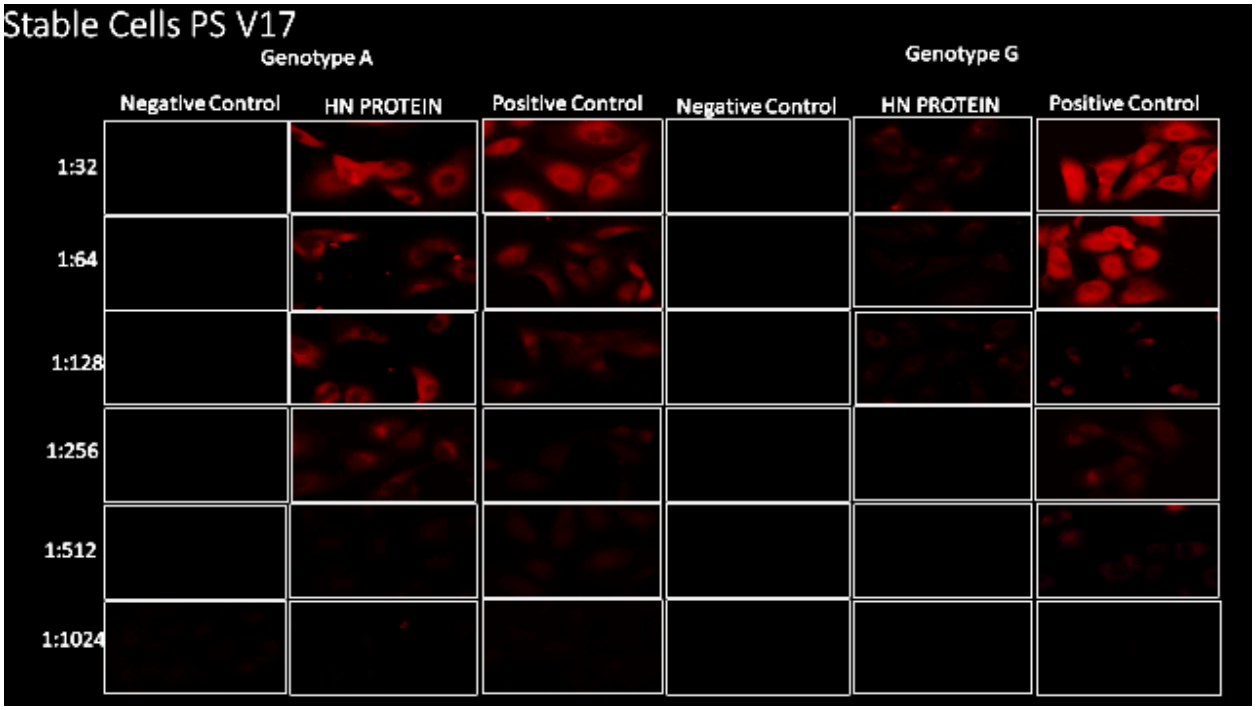


Figure A-20a. Stable cells Vaccine Sample#17.

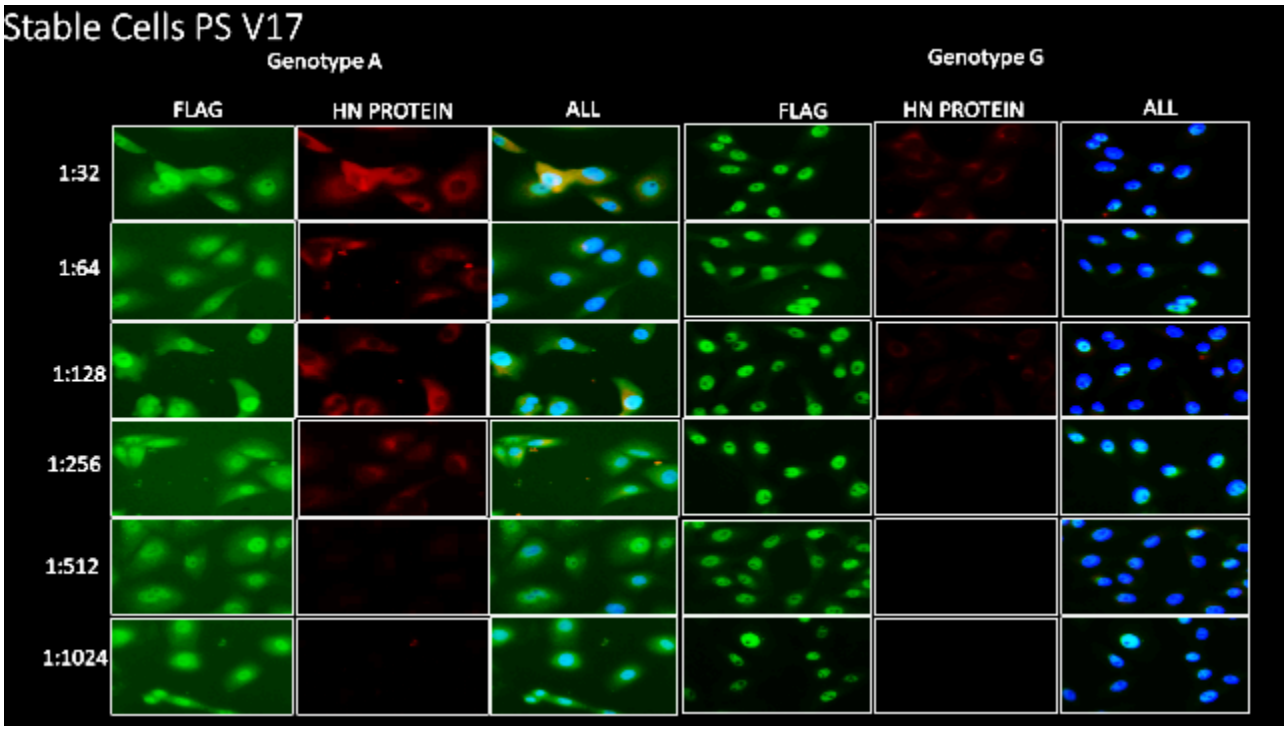


Figure A-20a. Stable cells Vaccine Sample#17.

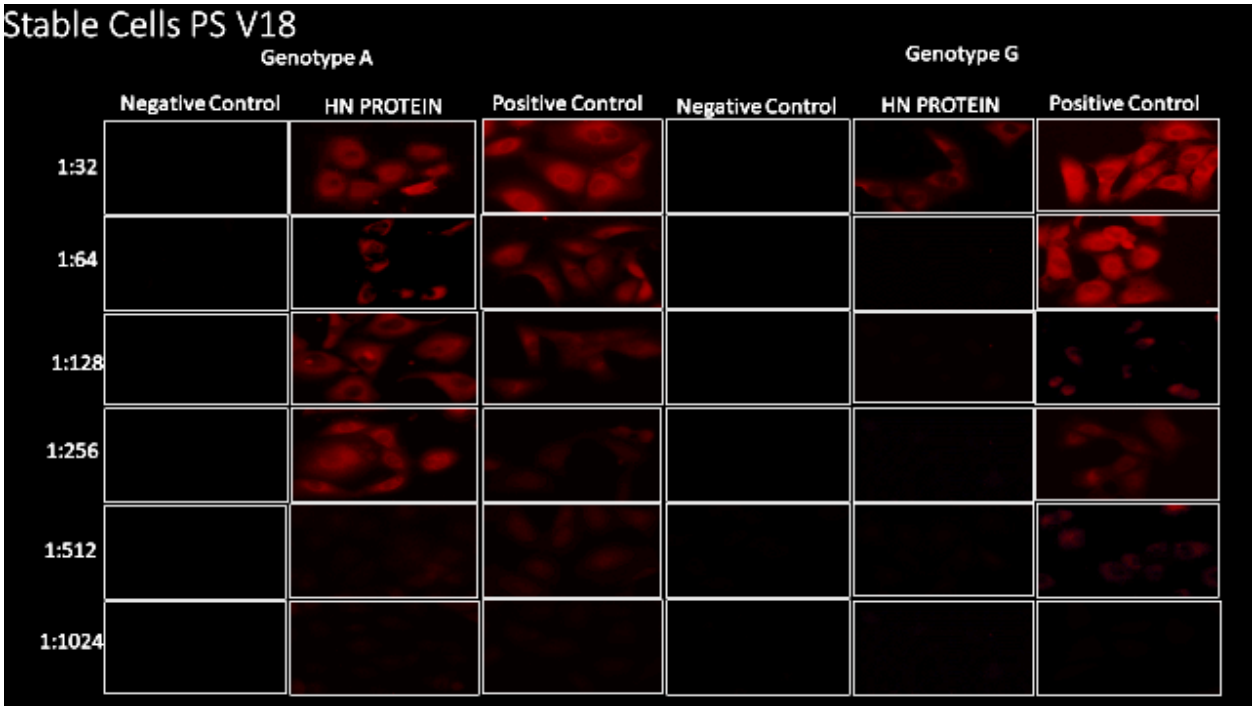


Figure A-21a. Stable cells Vaccine Sample#18.

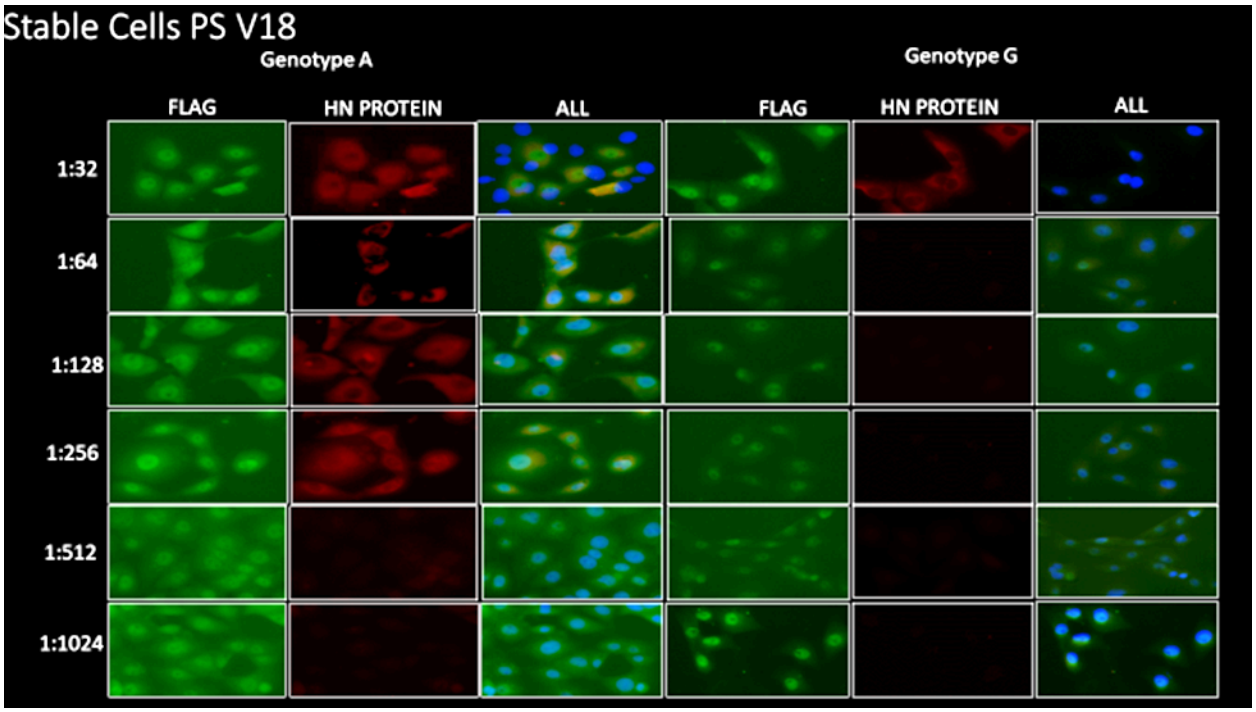


Figure A-21b. Stable cells Vaccine Sample #18.

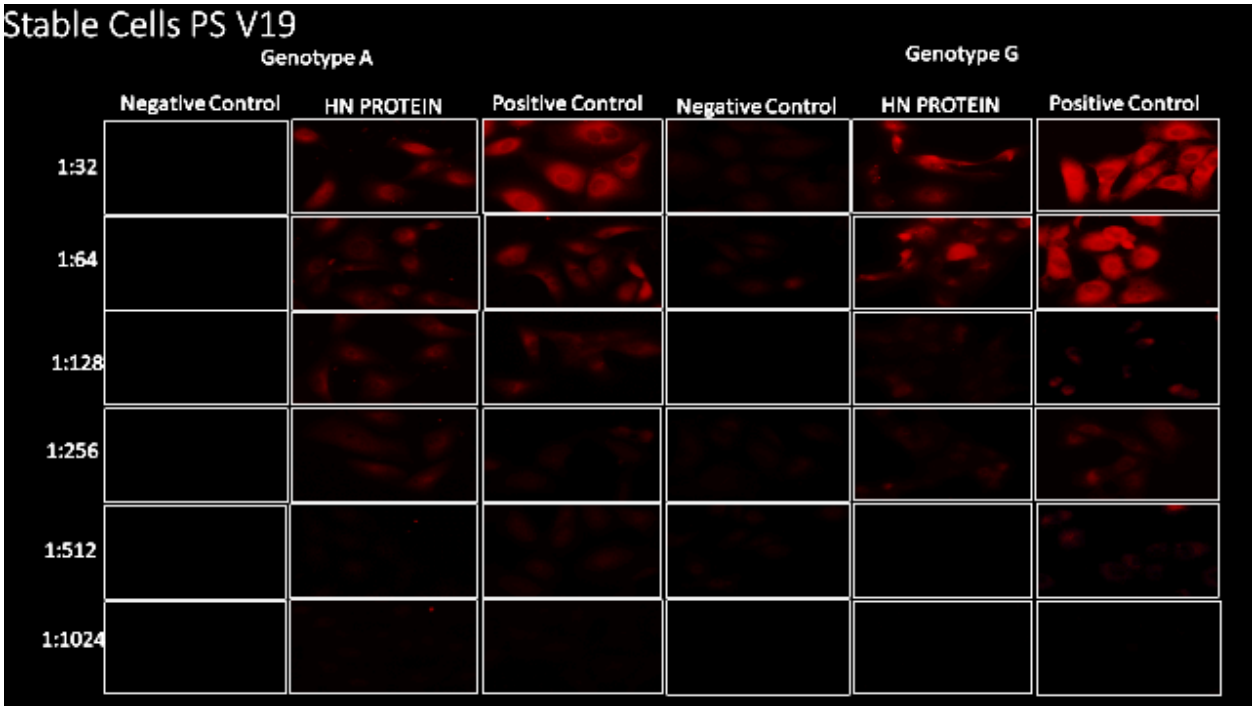


Figure A-22a. Stable cells Vaccine Sample#19.

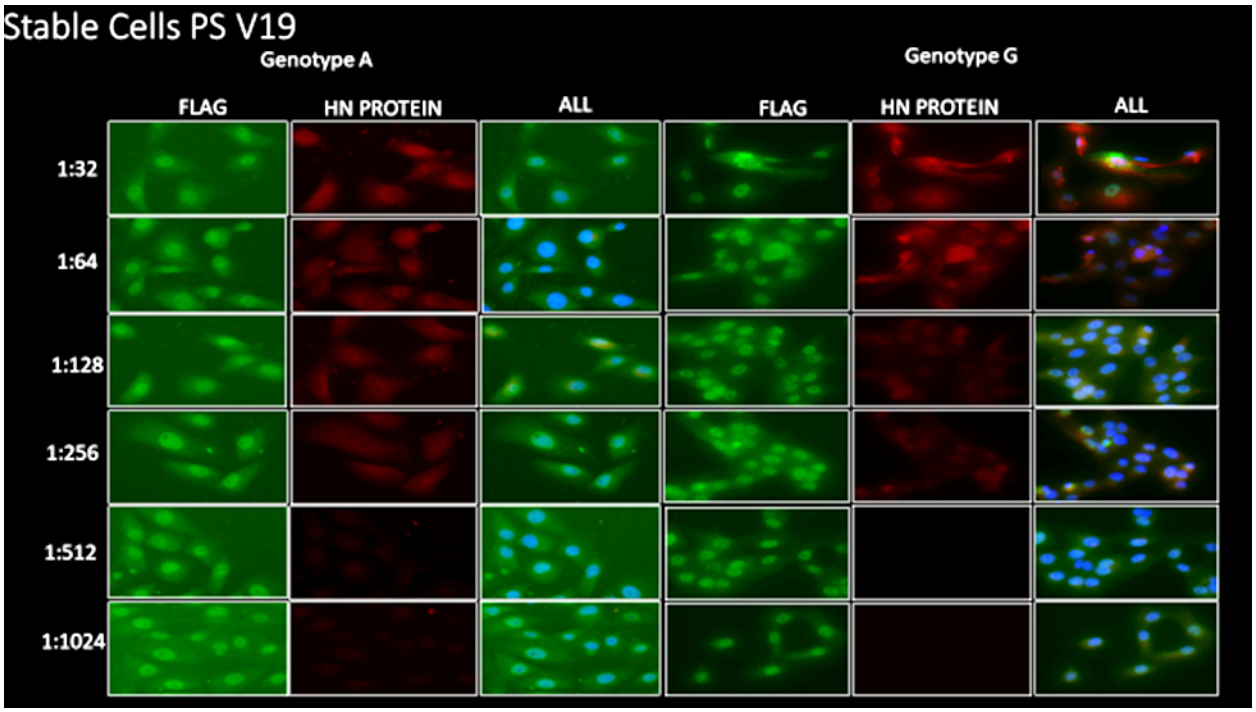


Figure A-22b. Stable cells Vaccine Sample #19.

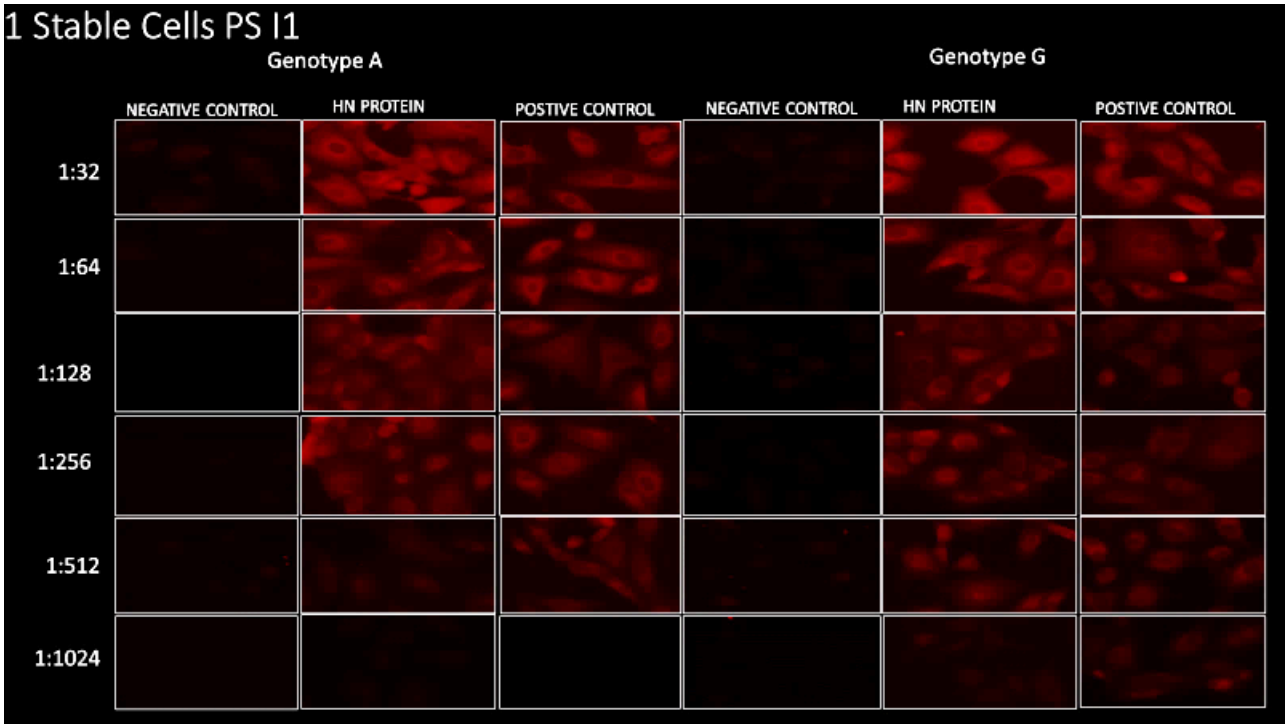


Figure A-23a. Stable cells Infected Sample#1.

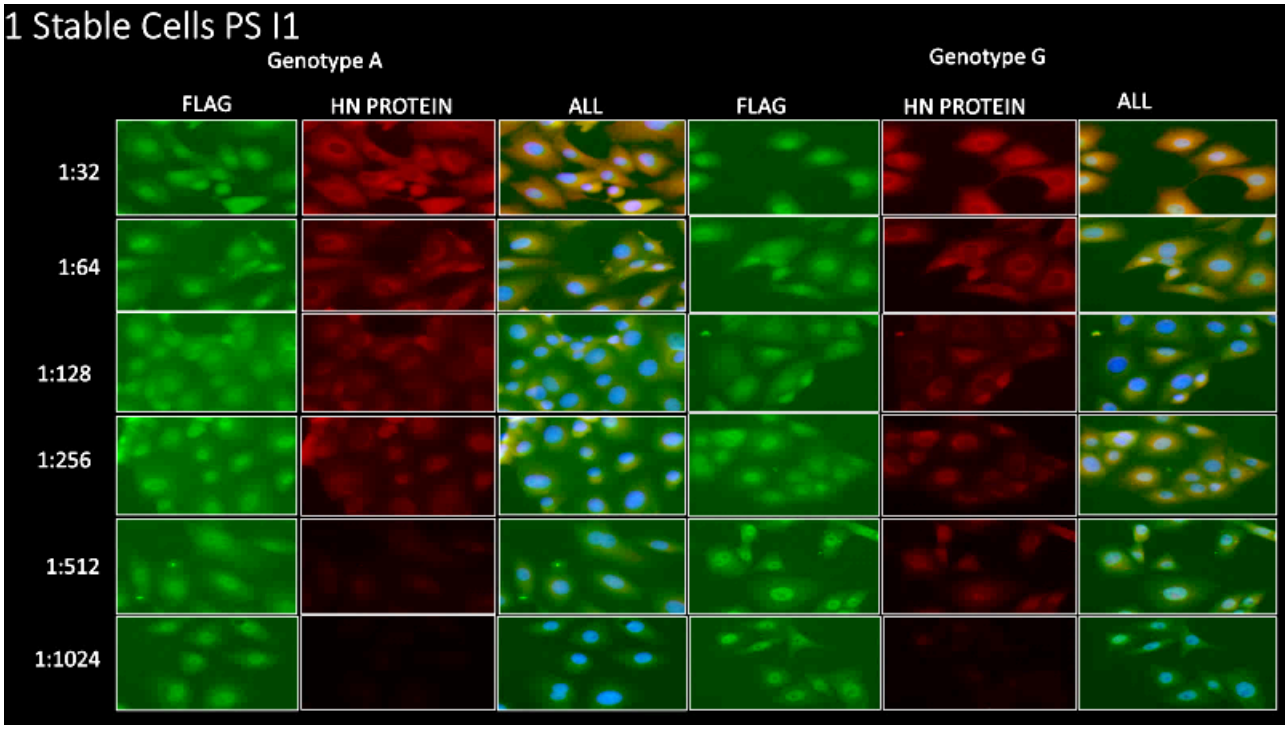


Figure A-23b. Stable cells Infected Sample #1.

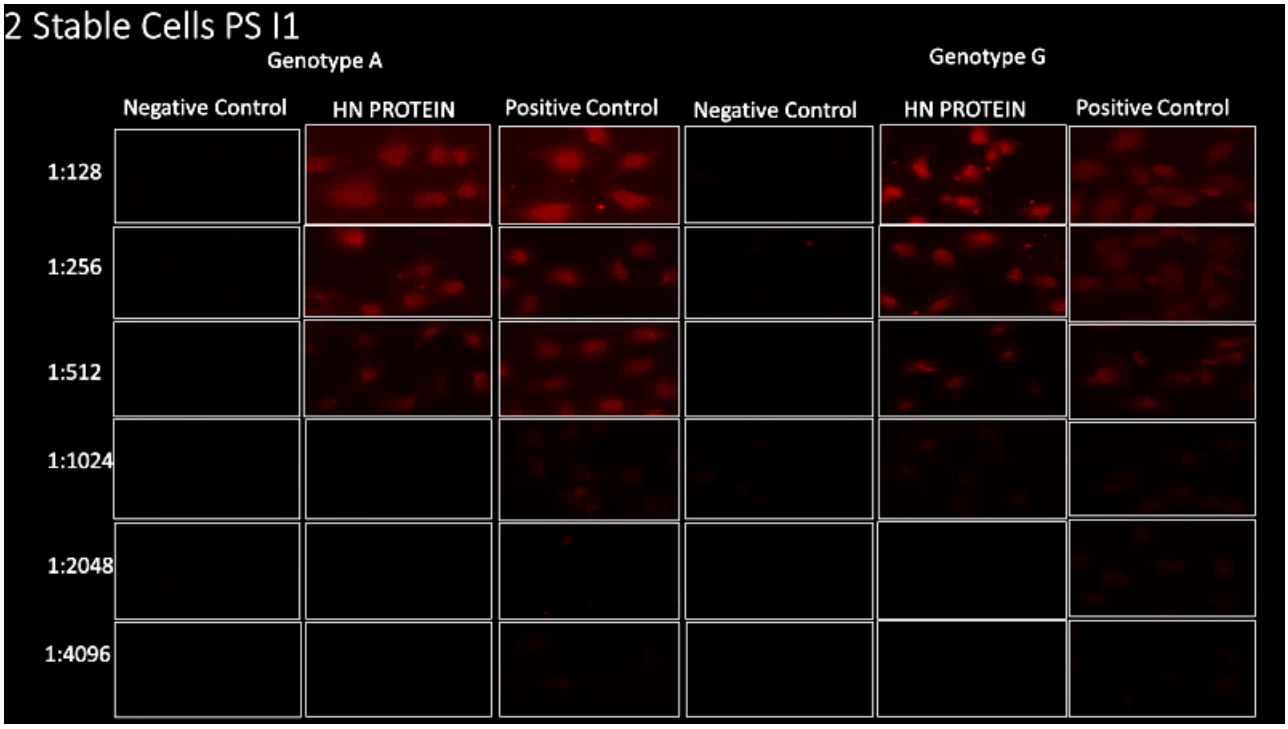


Figure A-23c. Stable cells Infected Sample #1.

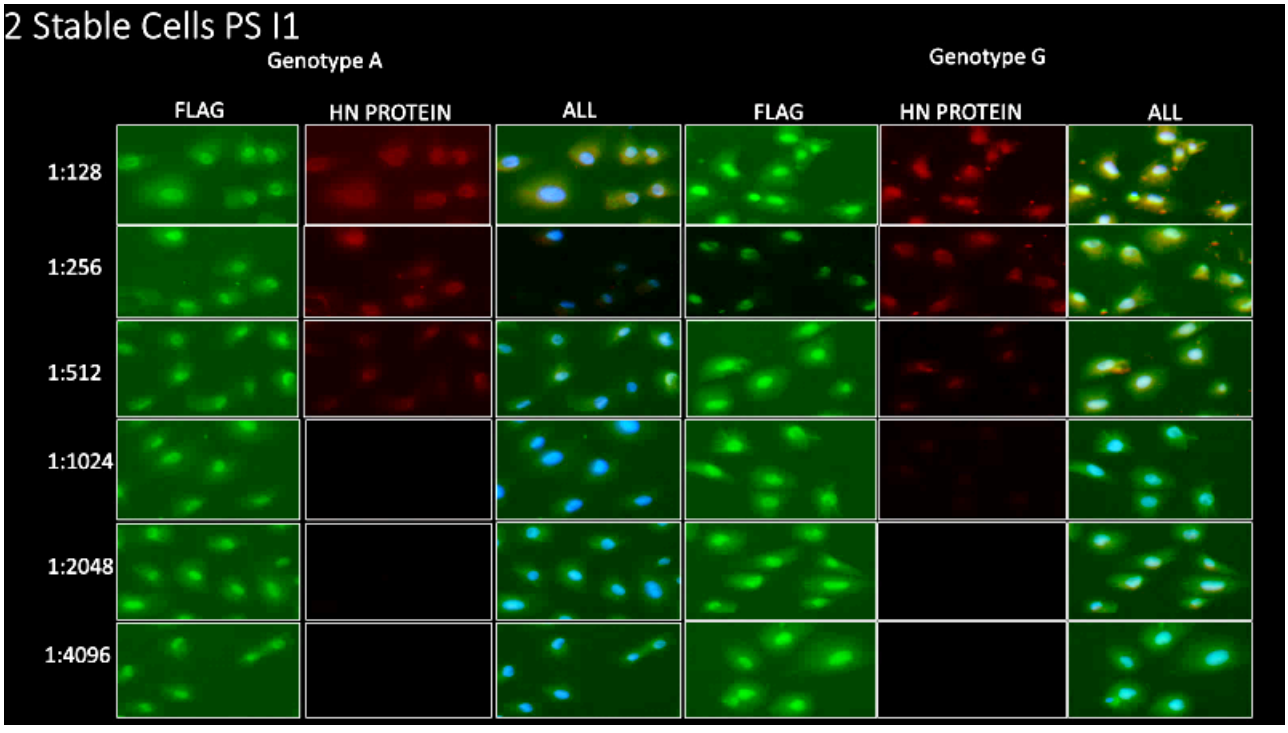


Figure A-23d. Stable cells Infected Sample #1.

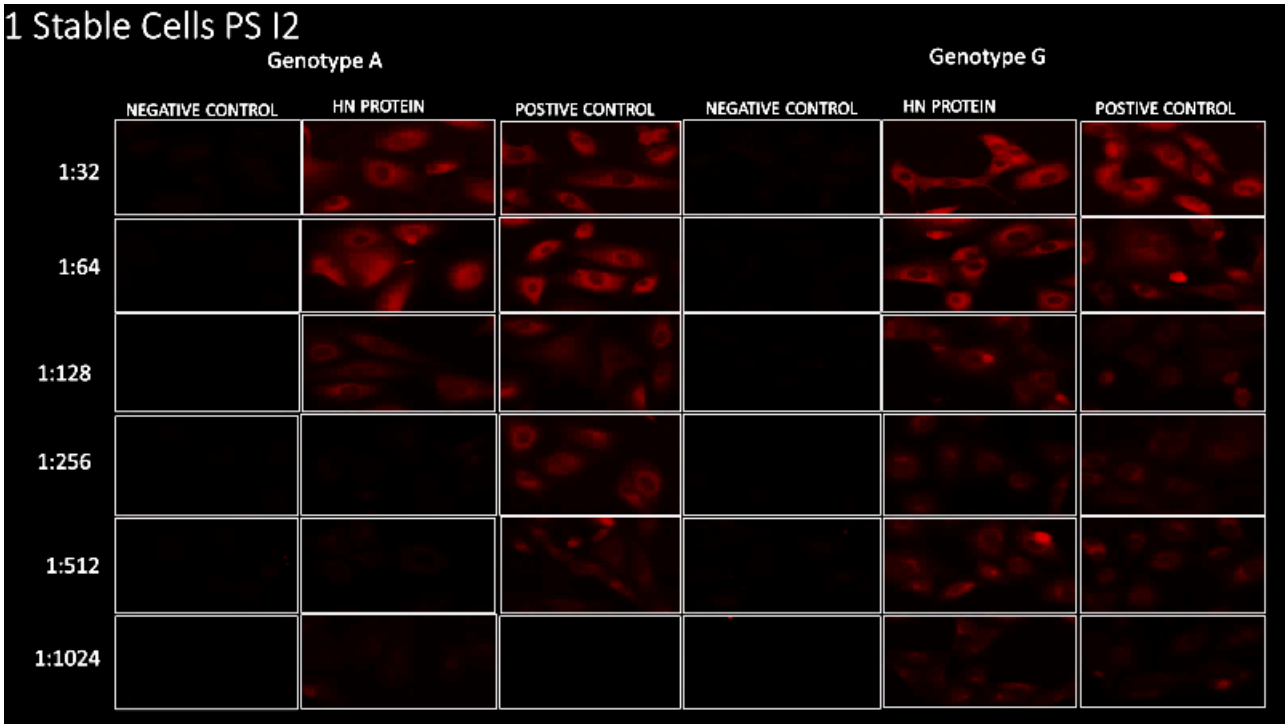


Figure A-24a. Stable cells Infected Sample#2.

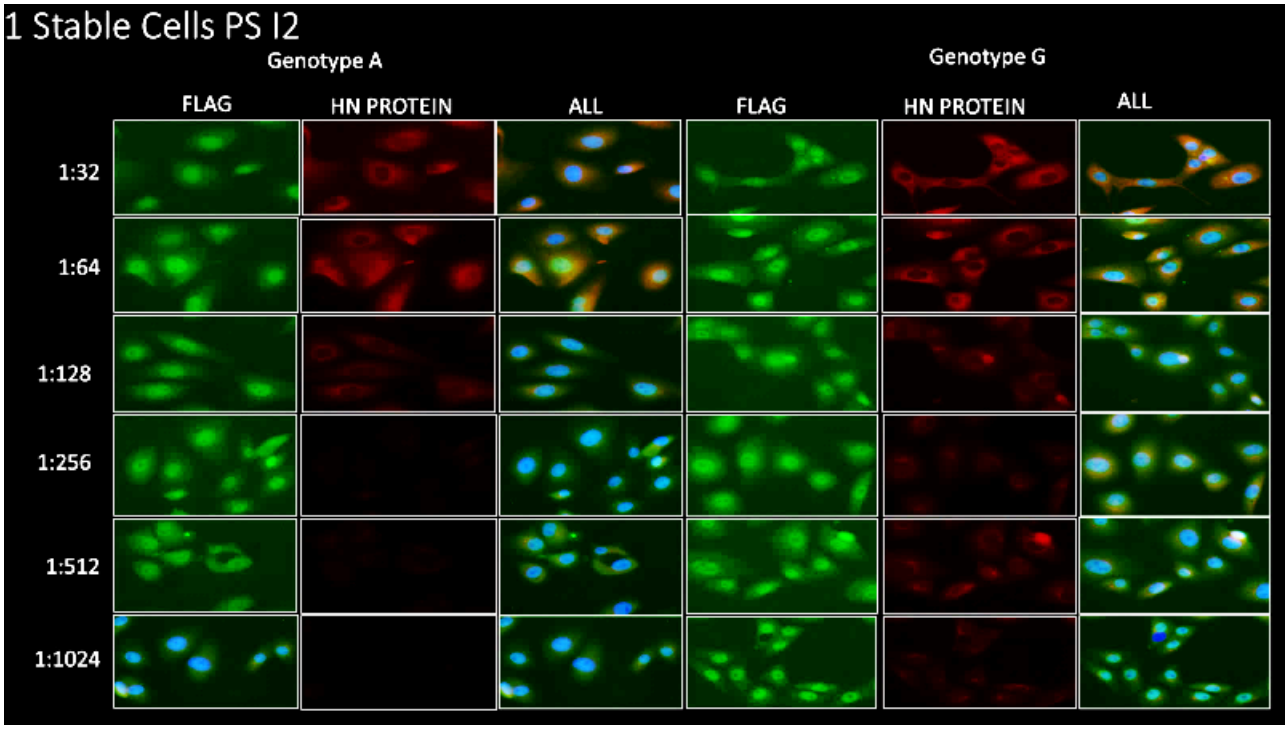


Figure A-24b. Stable cells Infected Sample #2.

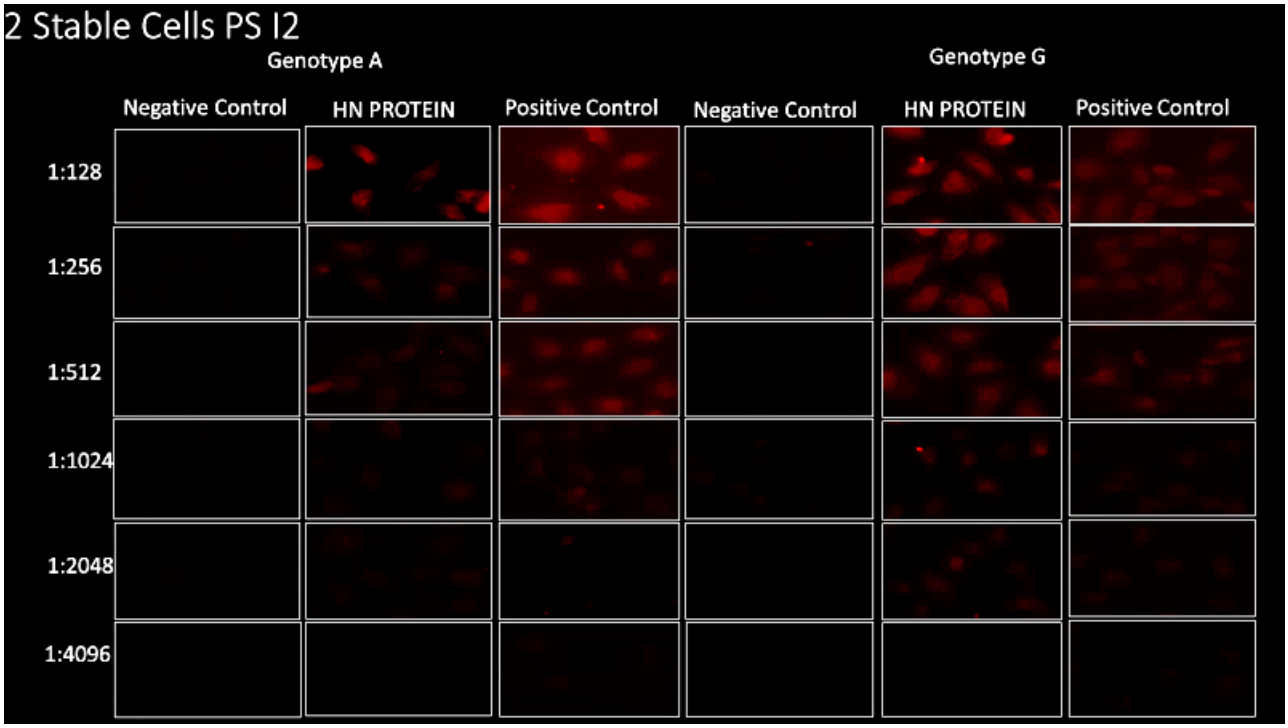


Figure A-24c. Stable cells Infected Sample #2.

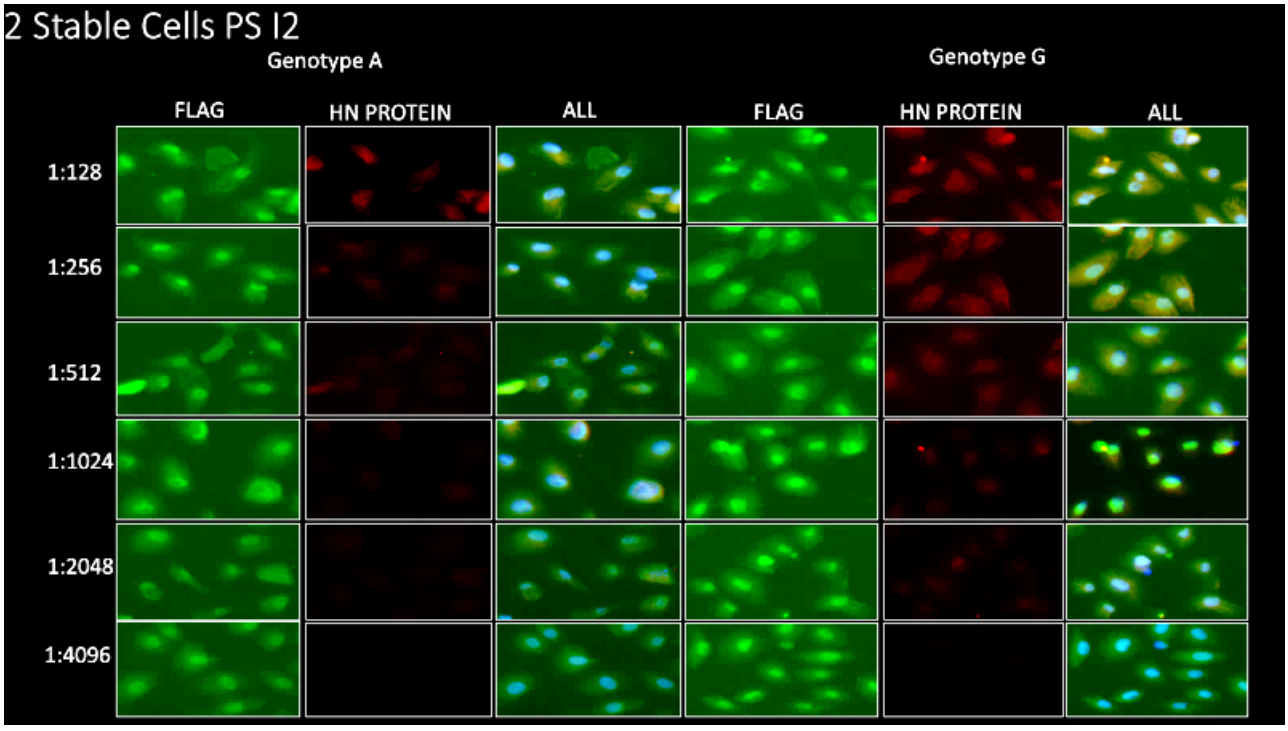


Figure A-24d. Stable cells Infected Sample #2.

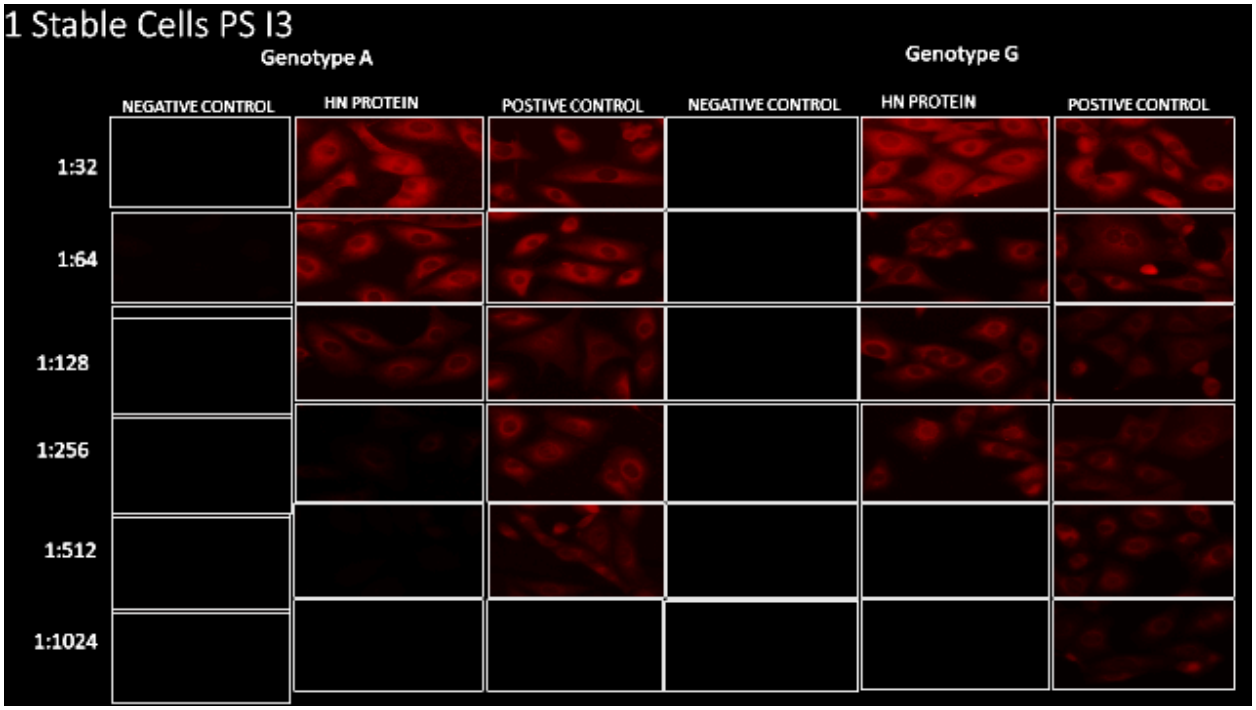


Figure A-25a. Stable cells Infected Sample#3.

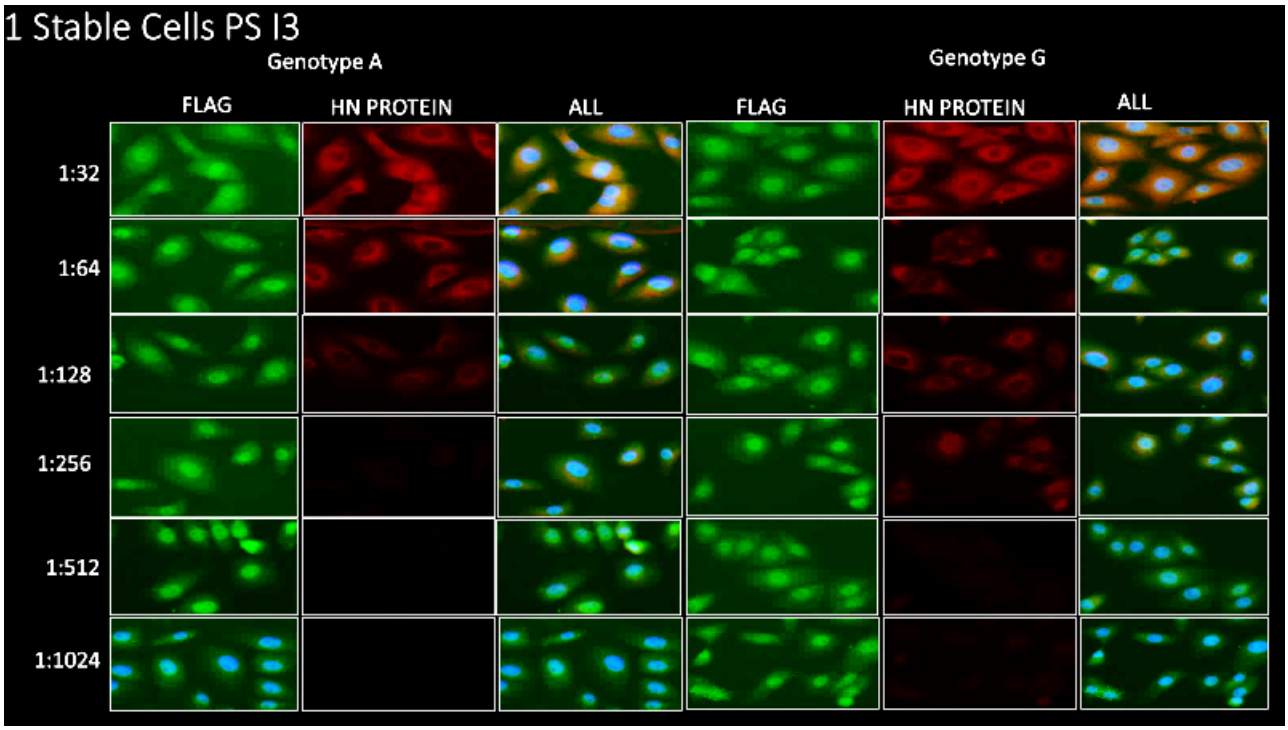


Figure A-25b. Stable cells Infected Sample #3.

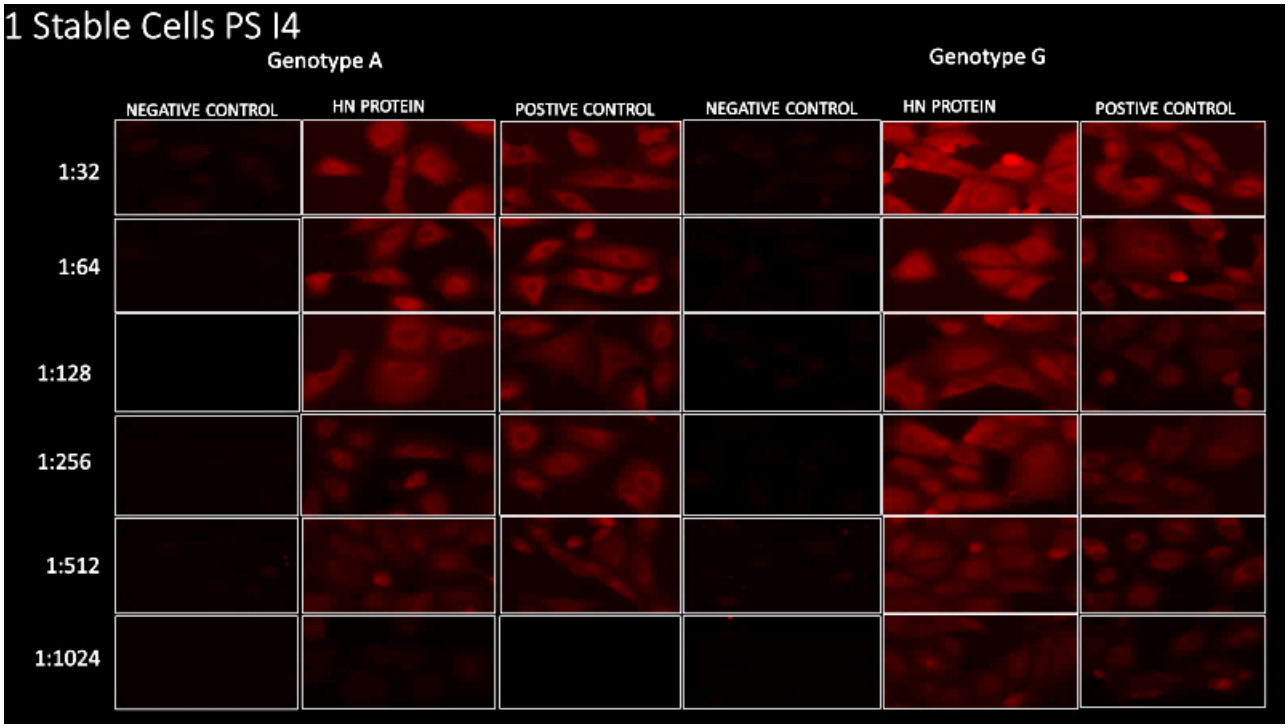


Figure A-26a. Stable cells Infected Sample#4.

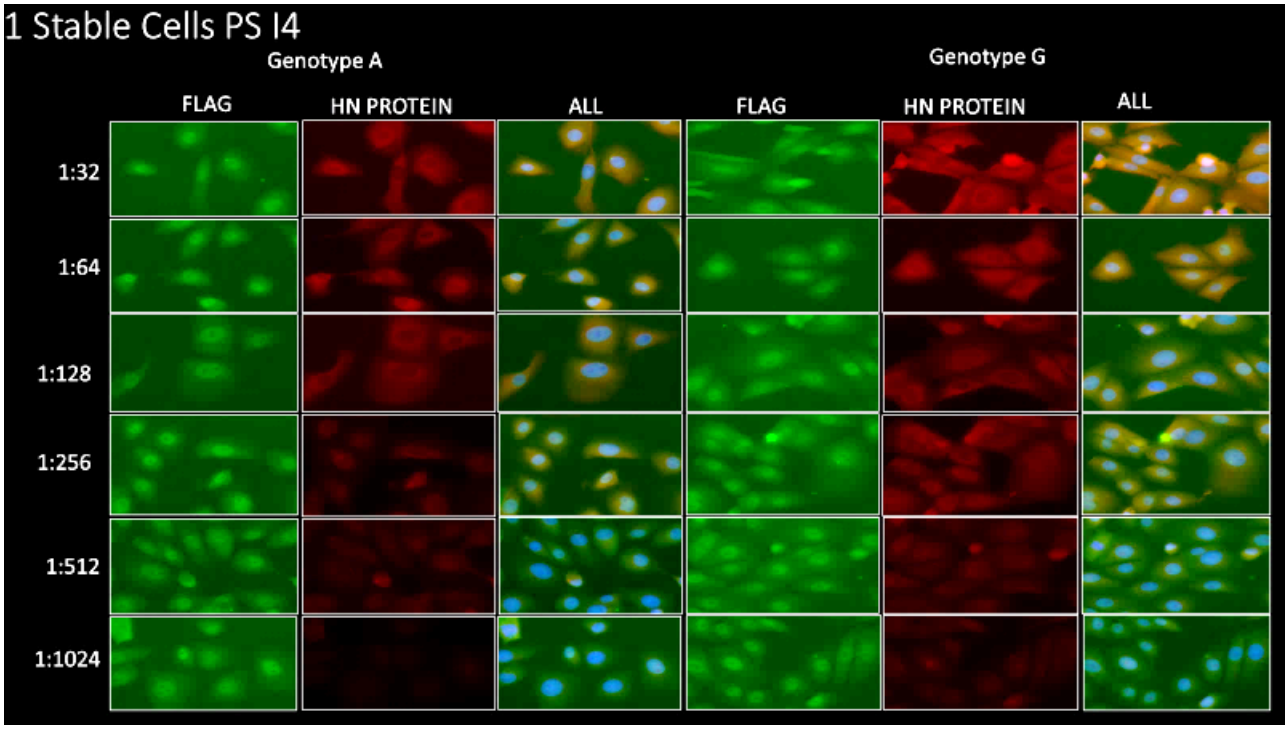


Figure A-26b. Stable cells Infected Sample #4.

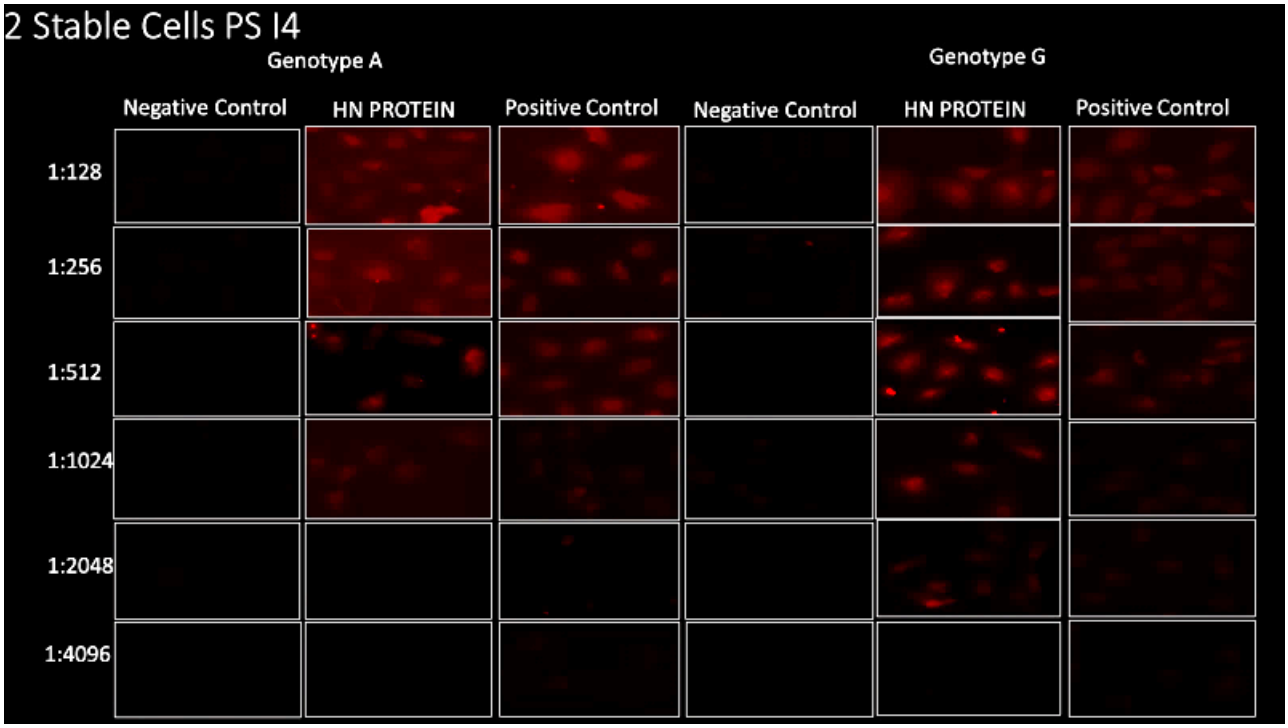


Figure A-26c. Stable cells Infected Sample #4.

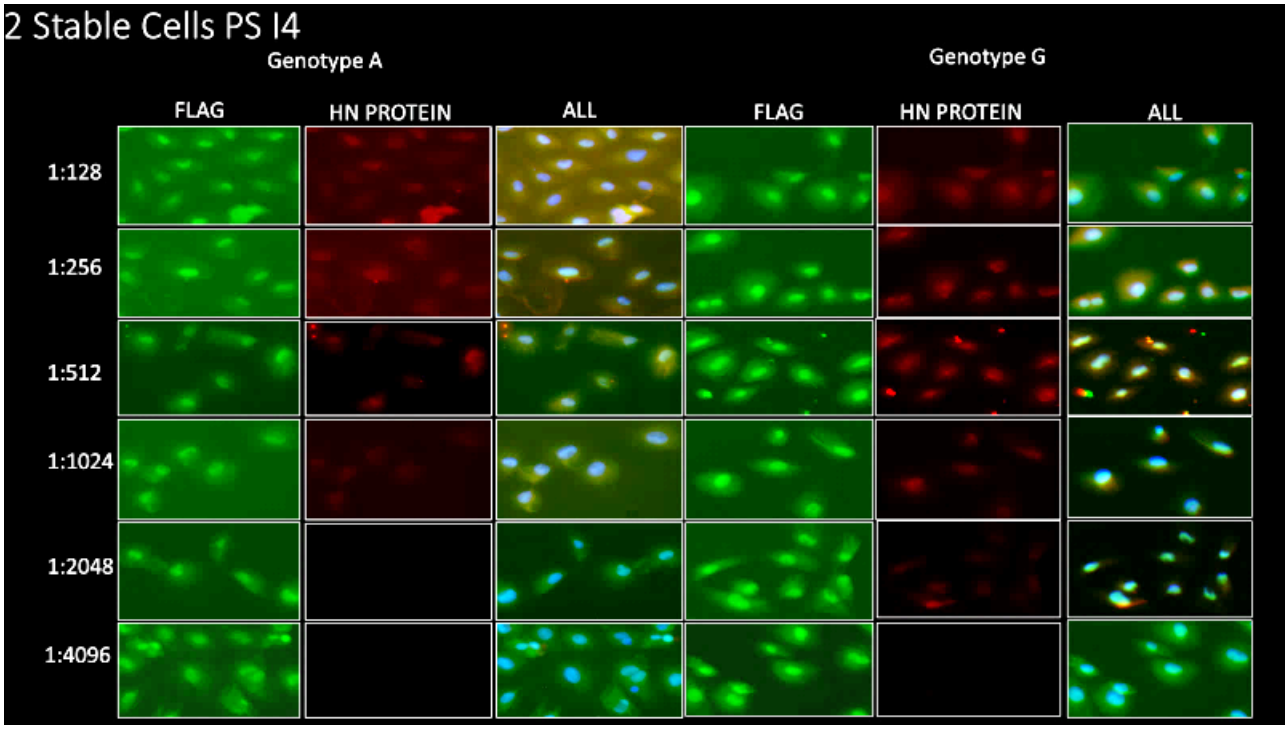


Figure A-26d. Stable cells Infected Sample #4.

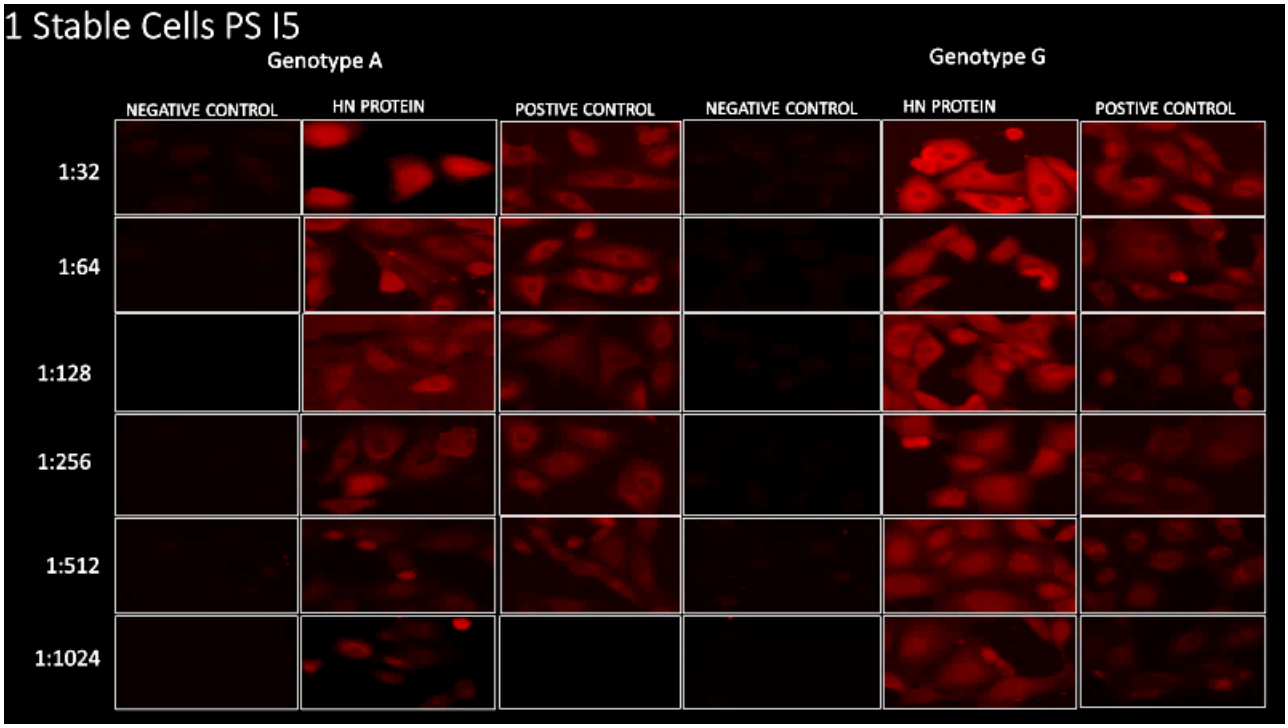


Figure A-27a. Stable cells Infected Sample#5.

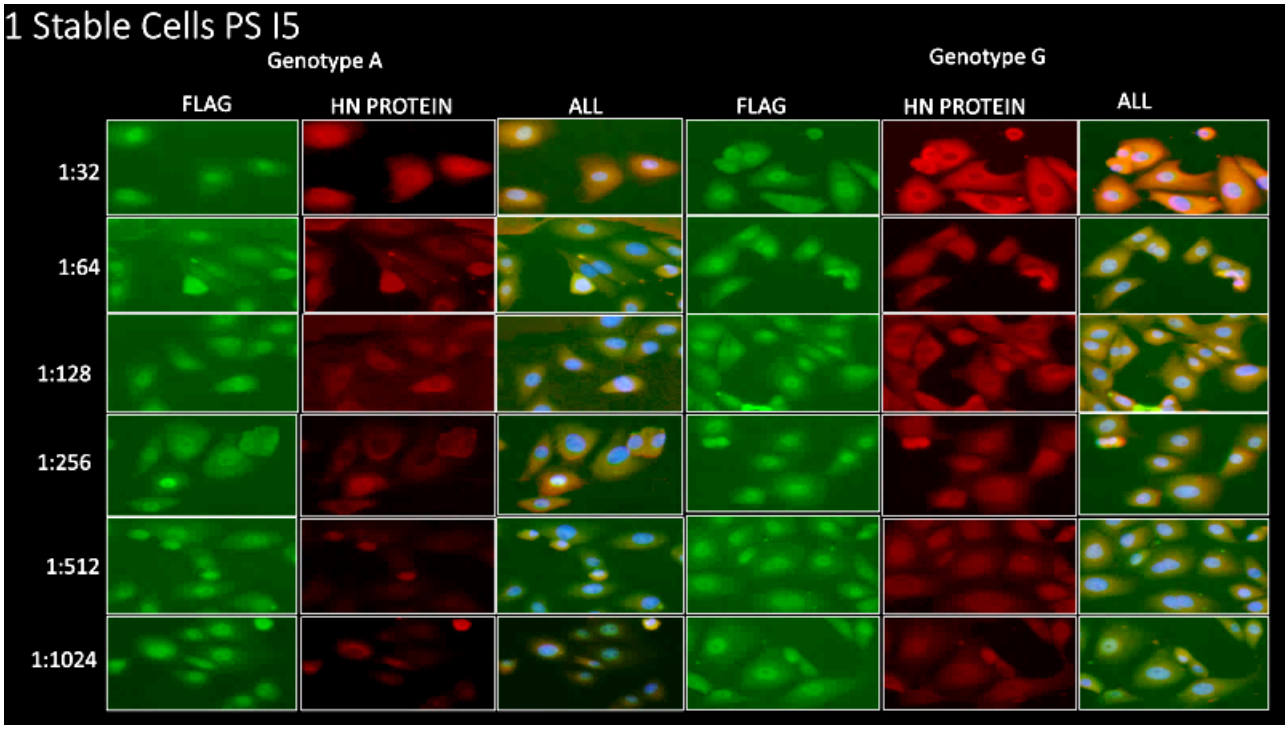


Figure A-27b. Stable cells Infected Sample #5.

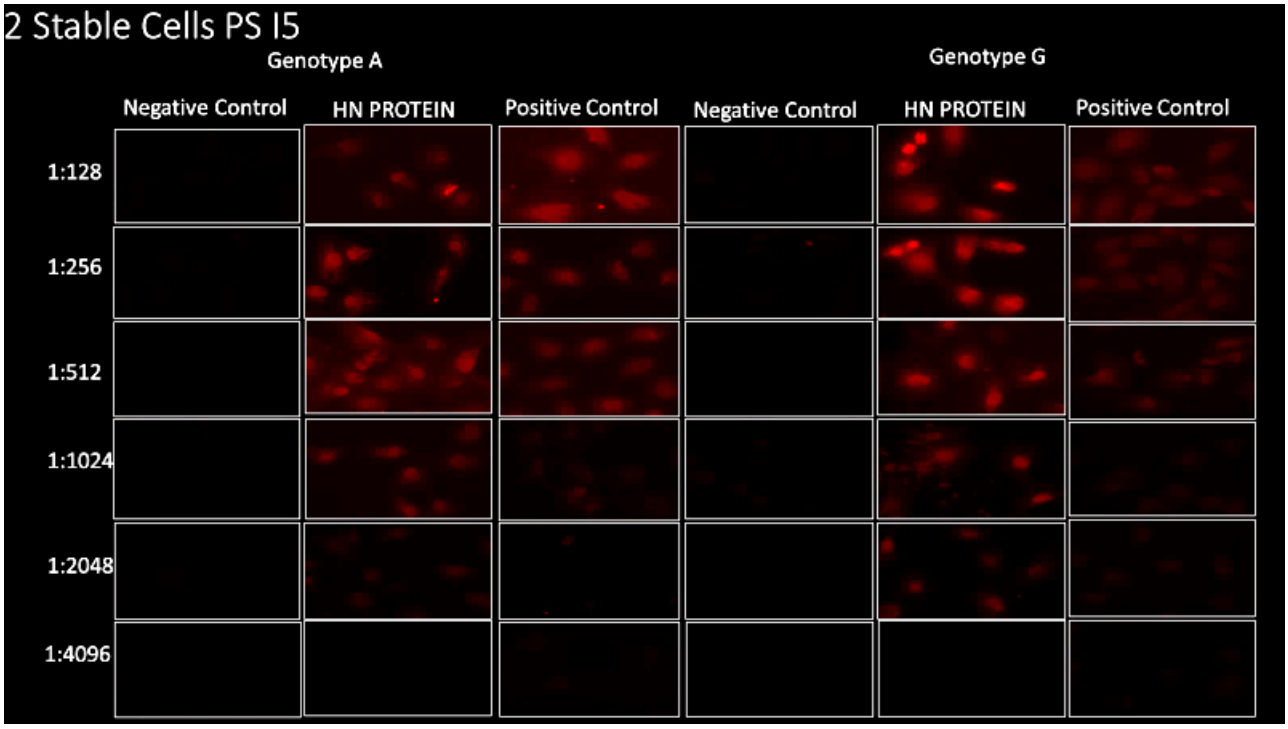


Figure A-27c. Stable cells Infected Sample #5.

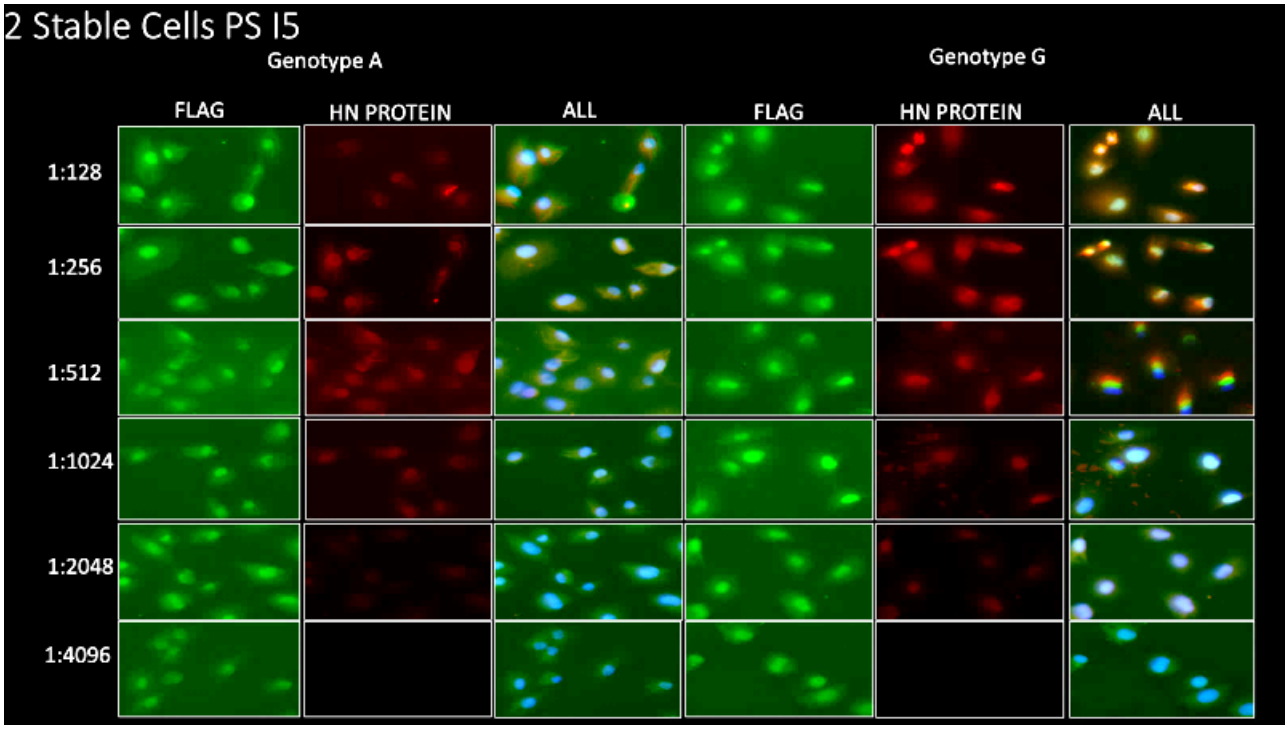


Figure A-27d. Stable cells Infected Sample #5.

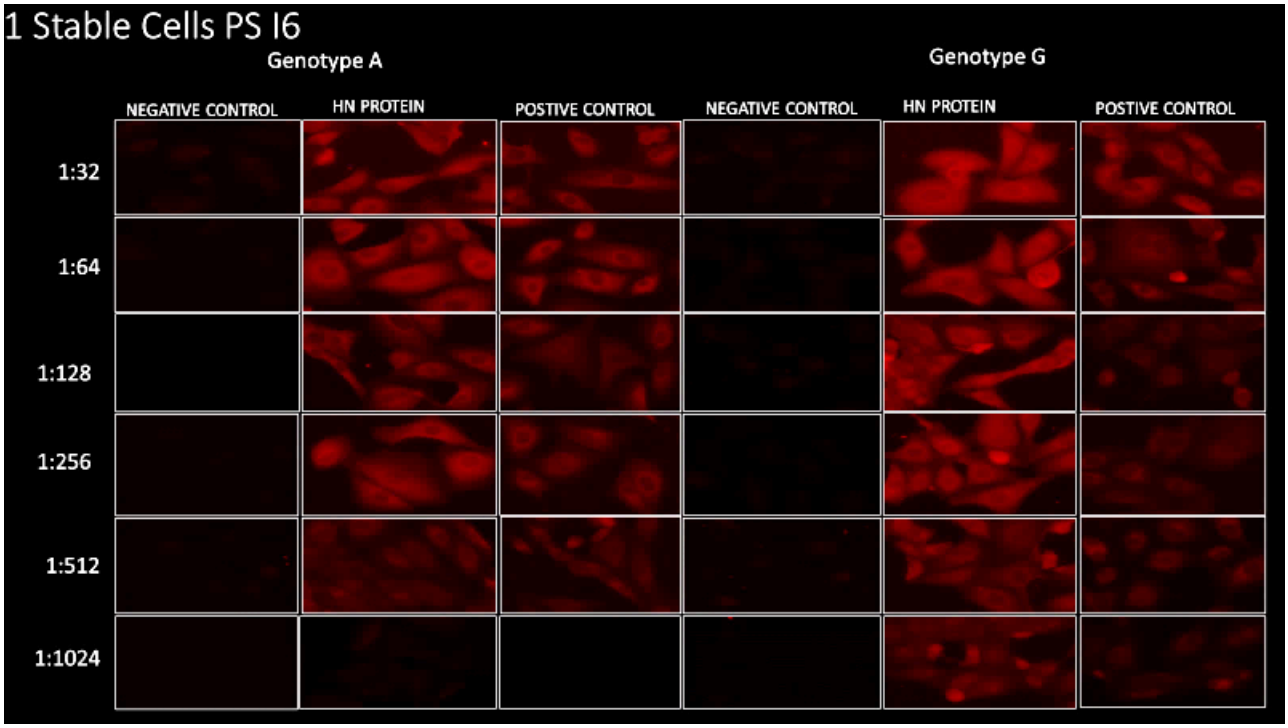


Figure A-28a. Stable cells Infected Sample#6.

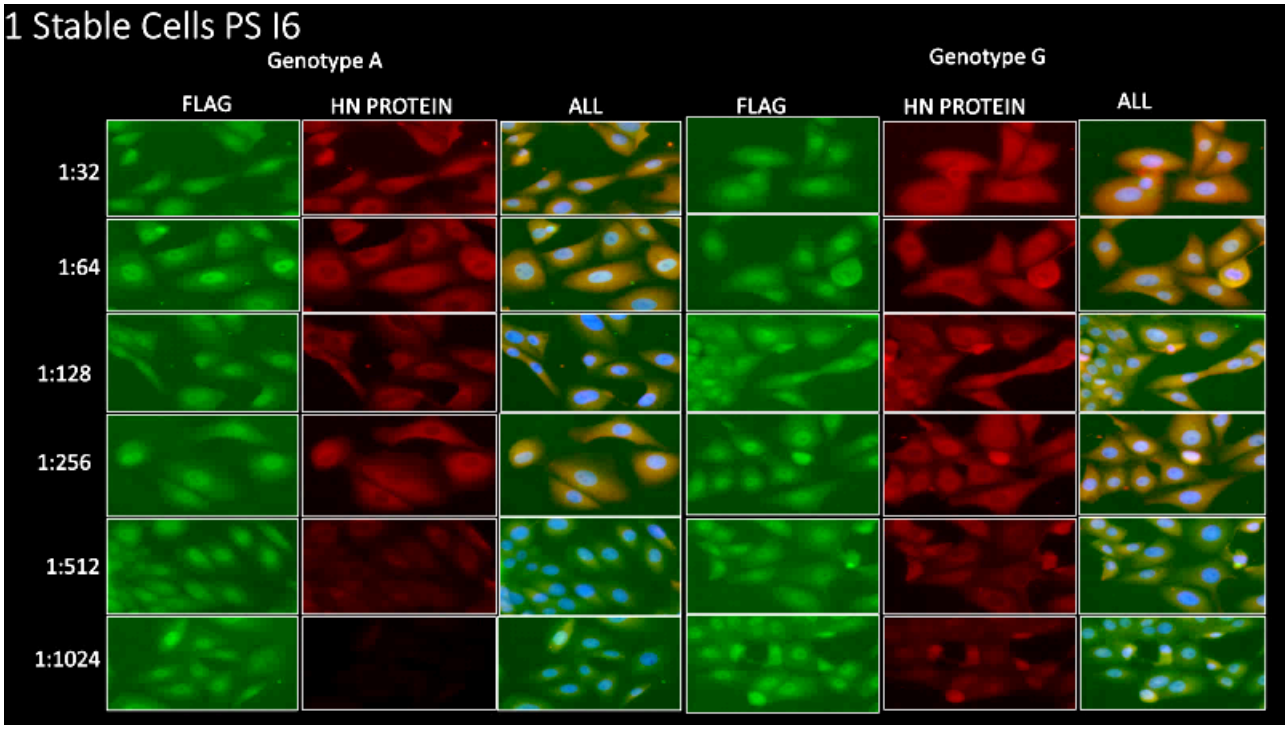


Figure A-28b. Stable cells Infected Sample #6.

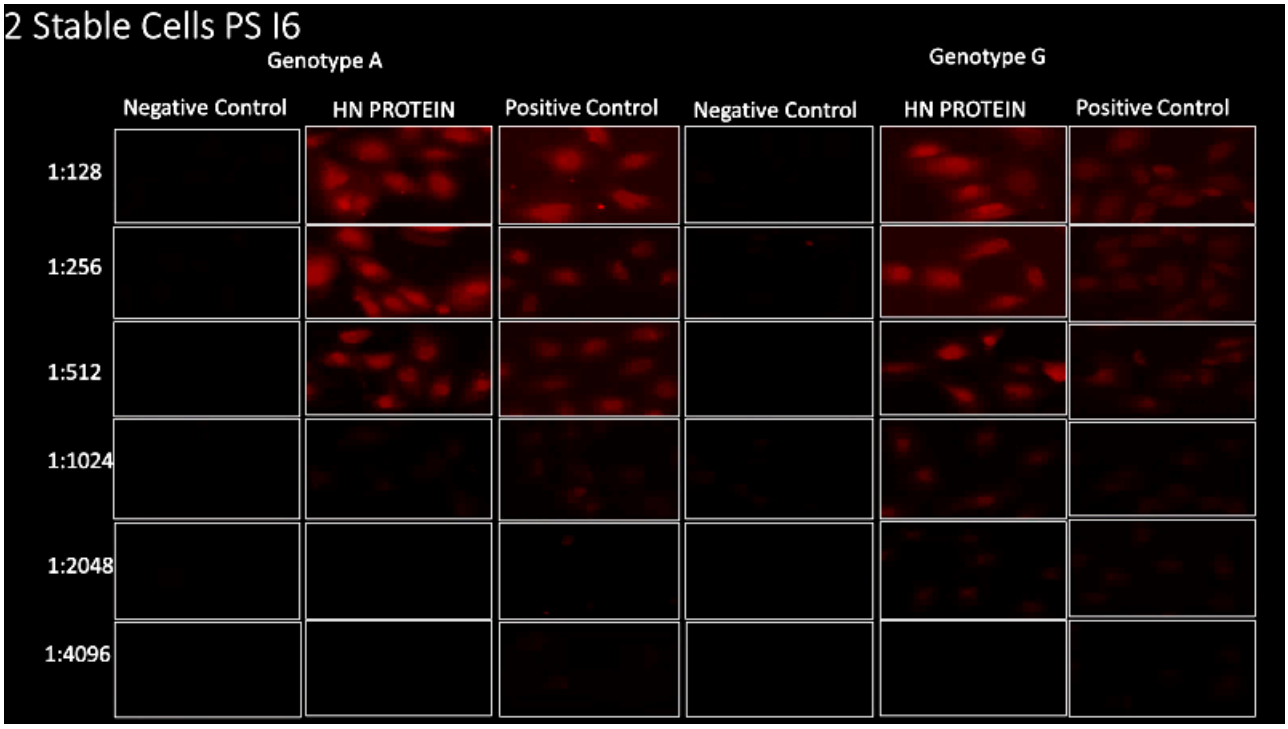


Figure A-28c. Stable cells Infected Sample #6.

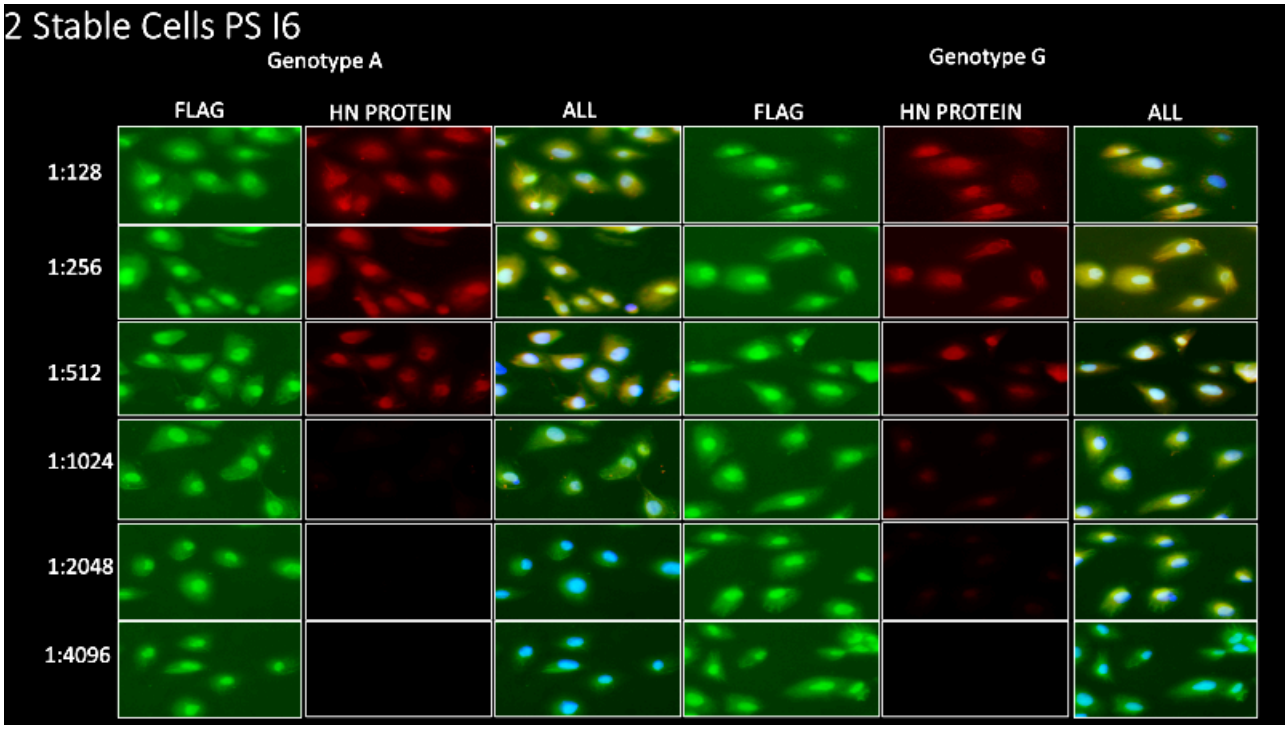


Figure A-28d. Stable cells Infected Sample #6.

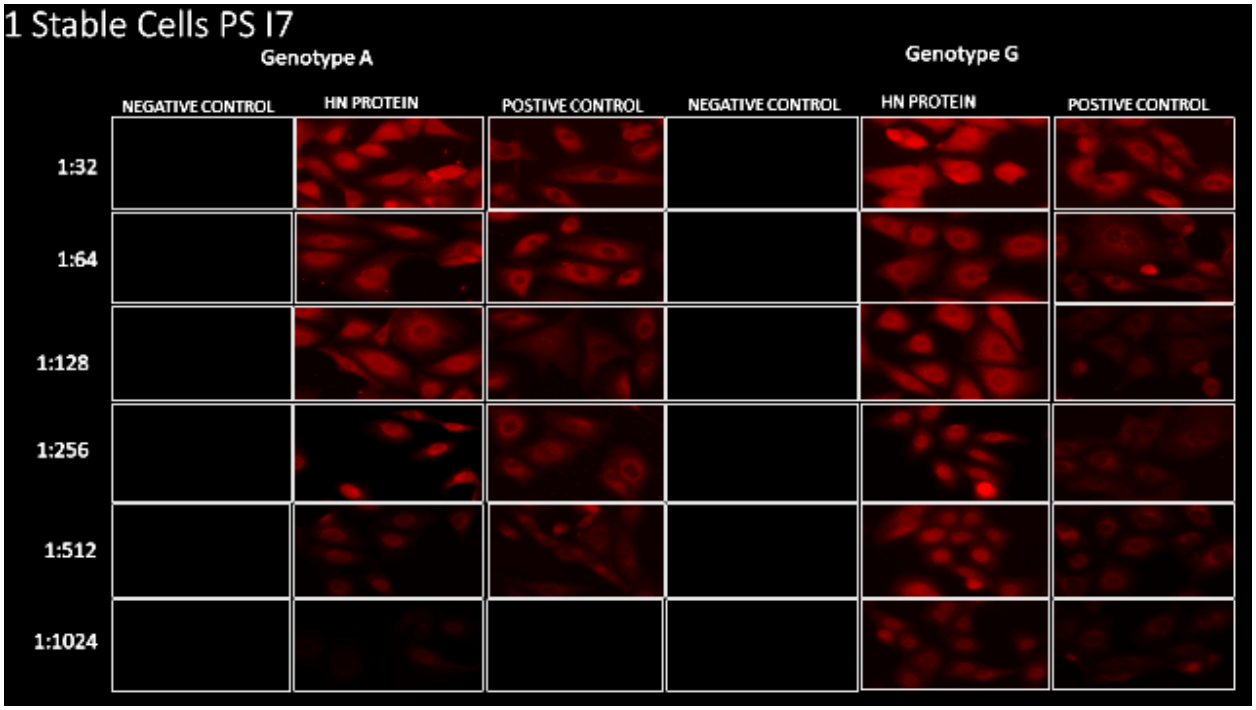


Figure A-29a. Stable cells Infected Sample#7.

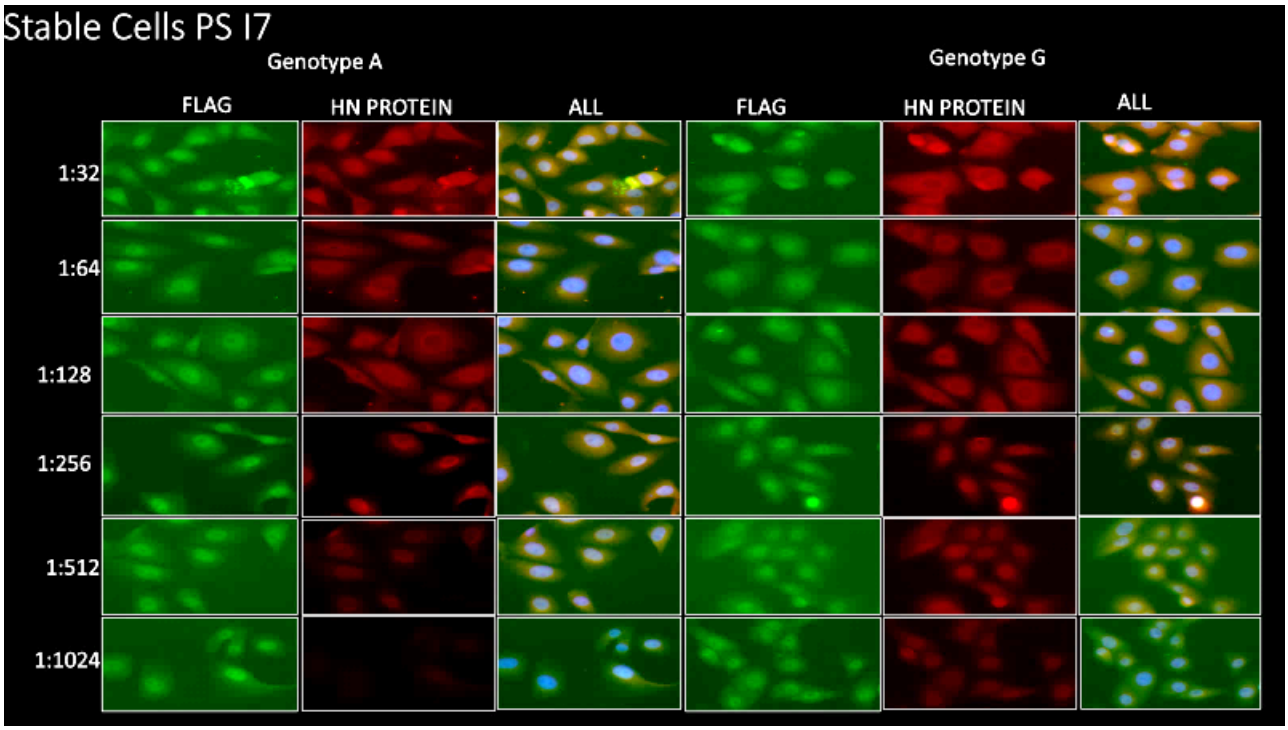


Figure A-29b. Stable cells Infected Sample #7.

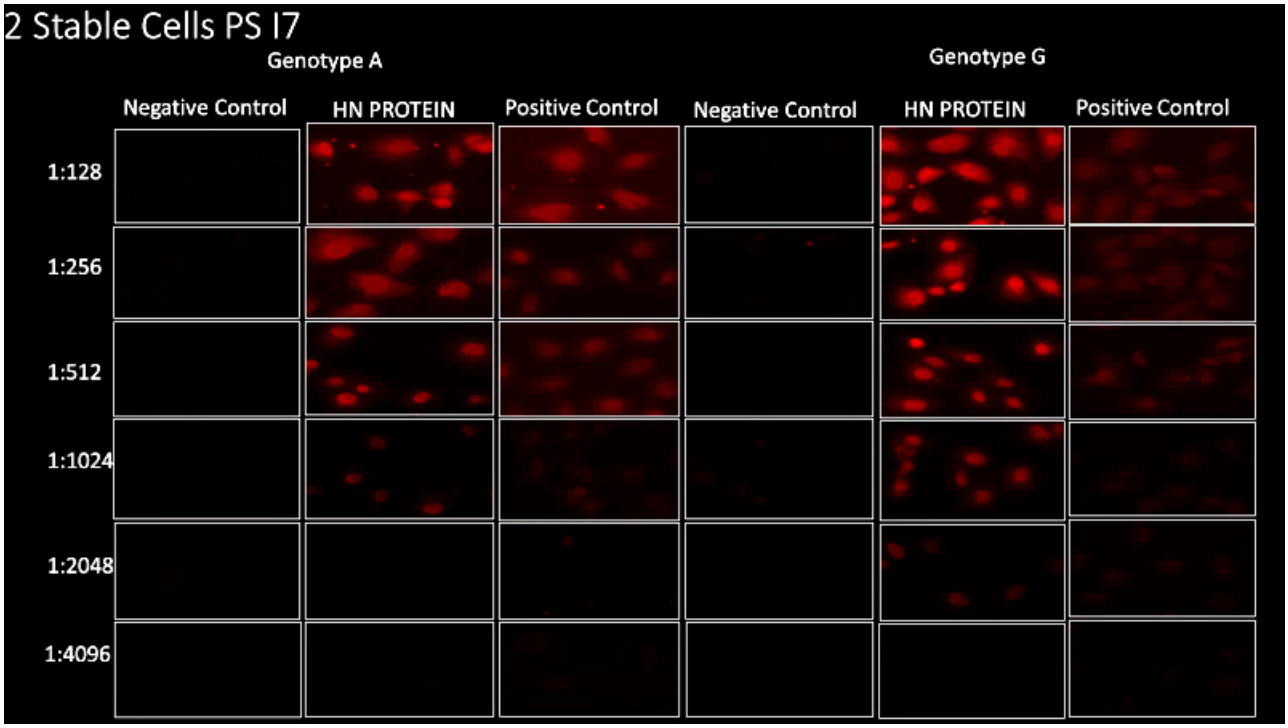


Figure A-29c. Stable cells Infected Sample #7.

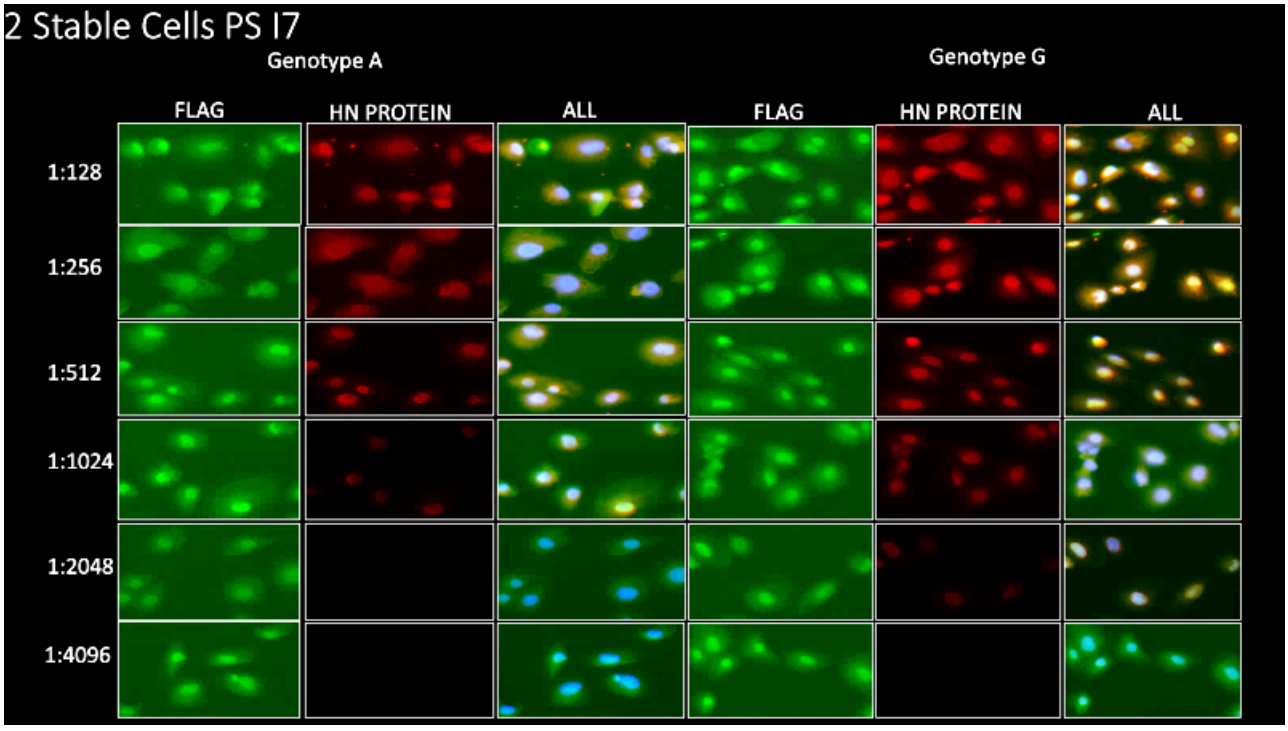


Figure A-29d. Stable cells Infected Sample #7.

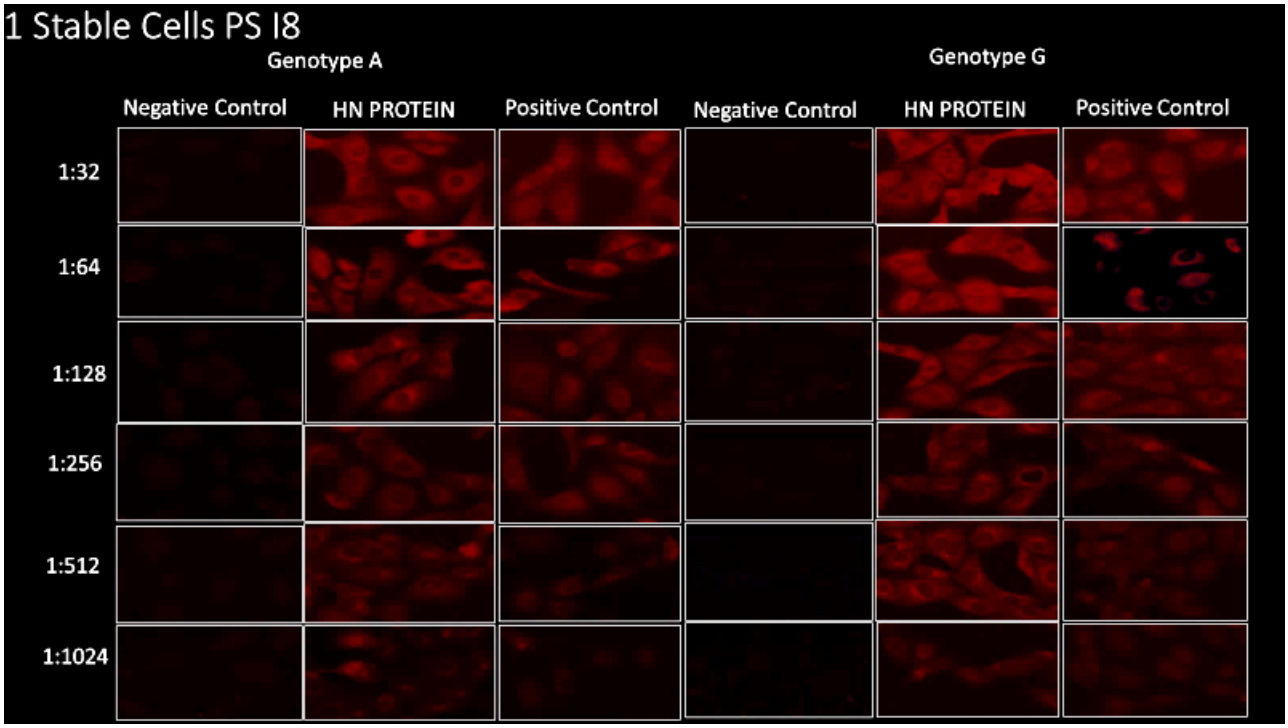


Figure A-30a. Stable cells Infected Sample#8.

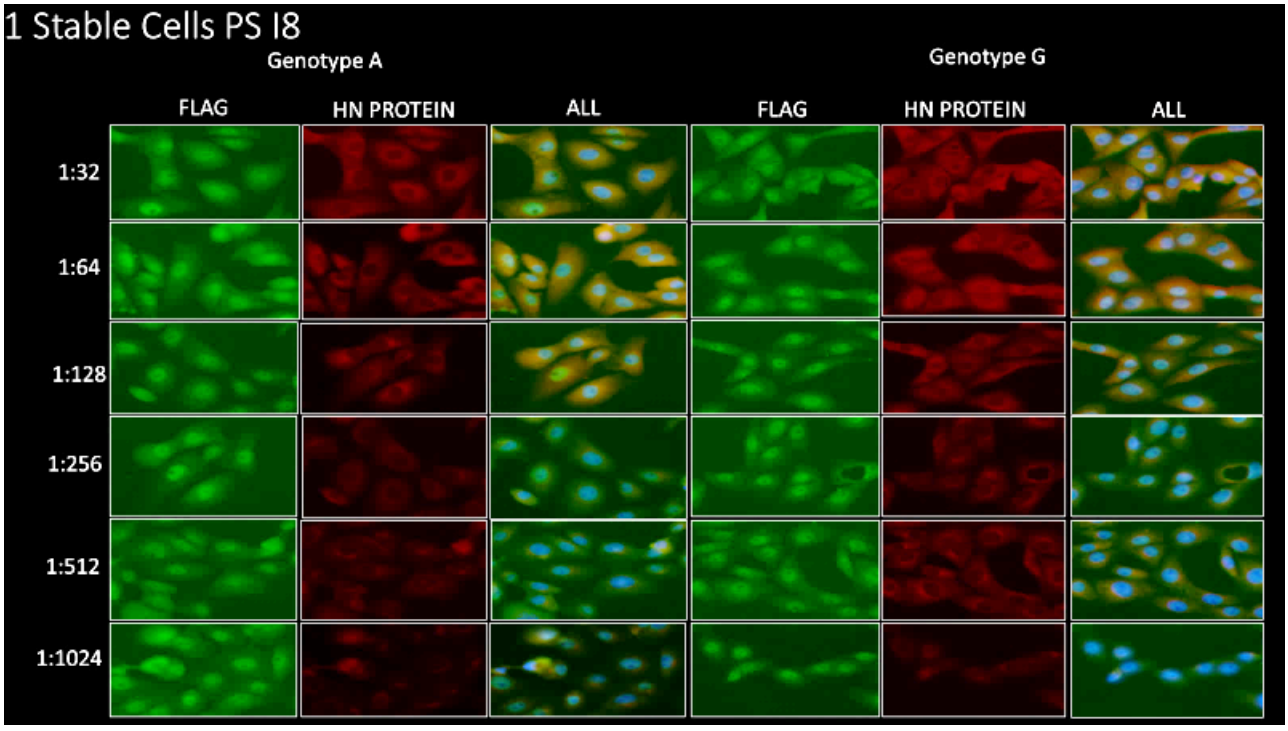


Figure A-30b. Stable cells Infected Sample #8.

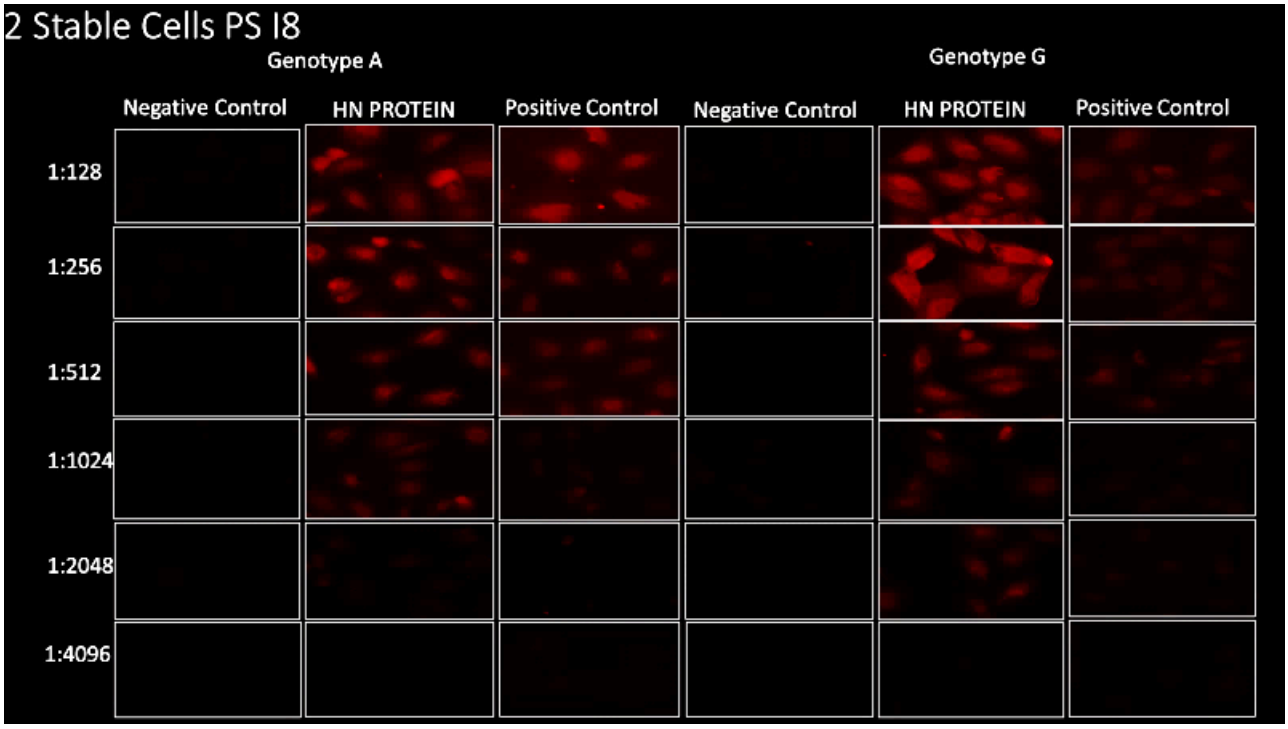


Figure A-30c. Stable cells Infected Sample #8.

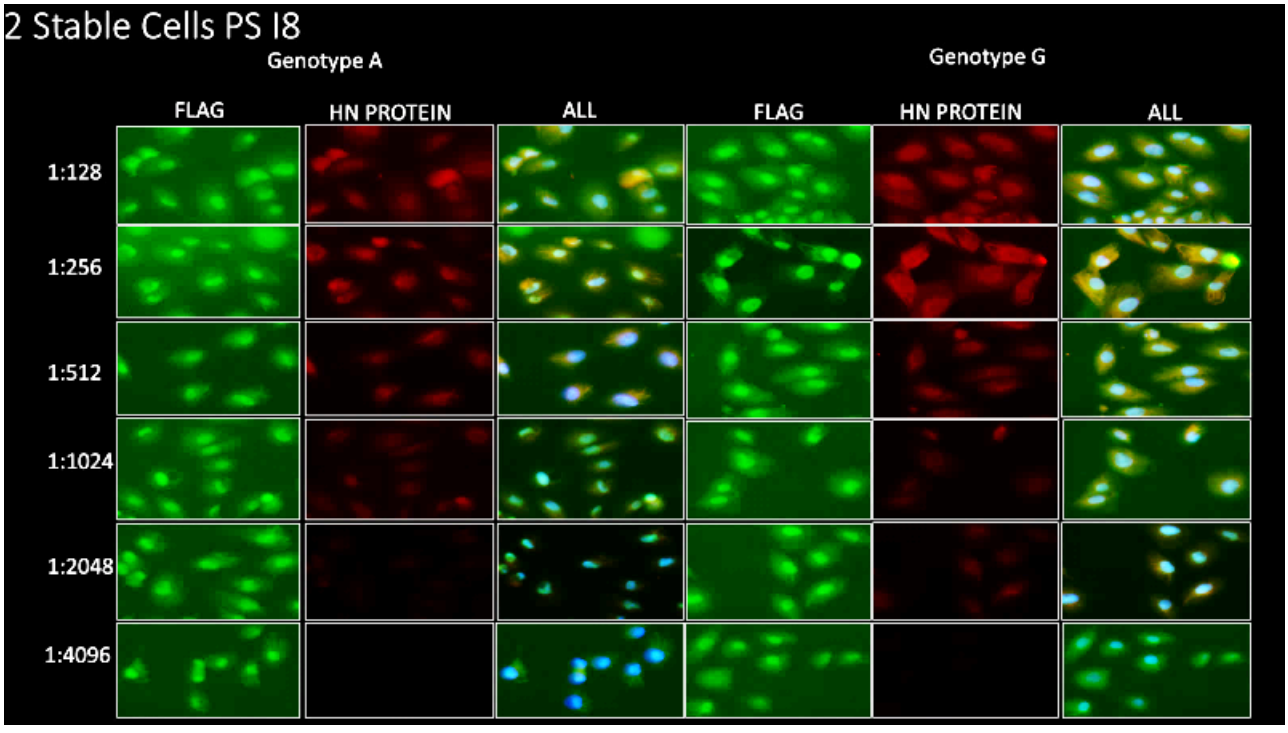


Figure A-30d. Stable cells Infected Sample #8.

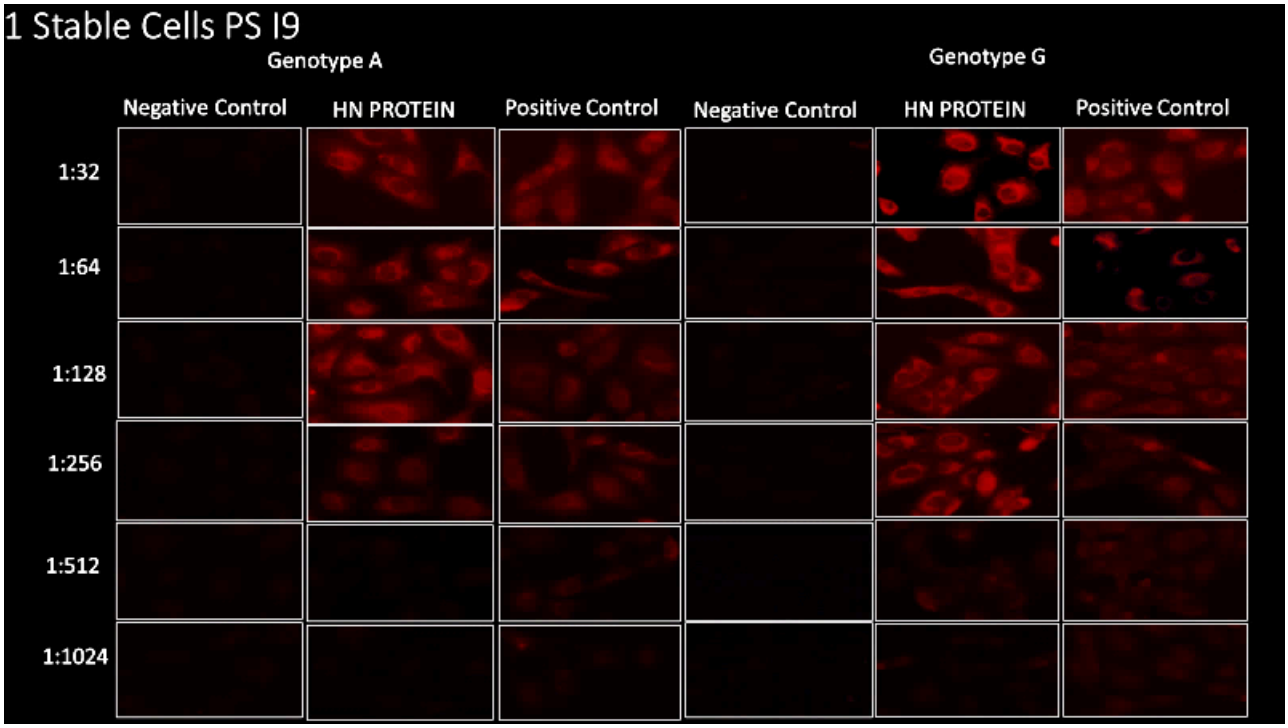


Figure A-31a. Stable cells Infected Sample#9.

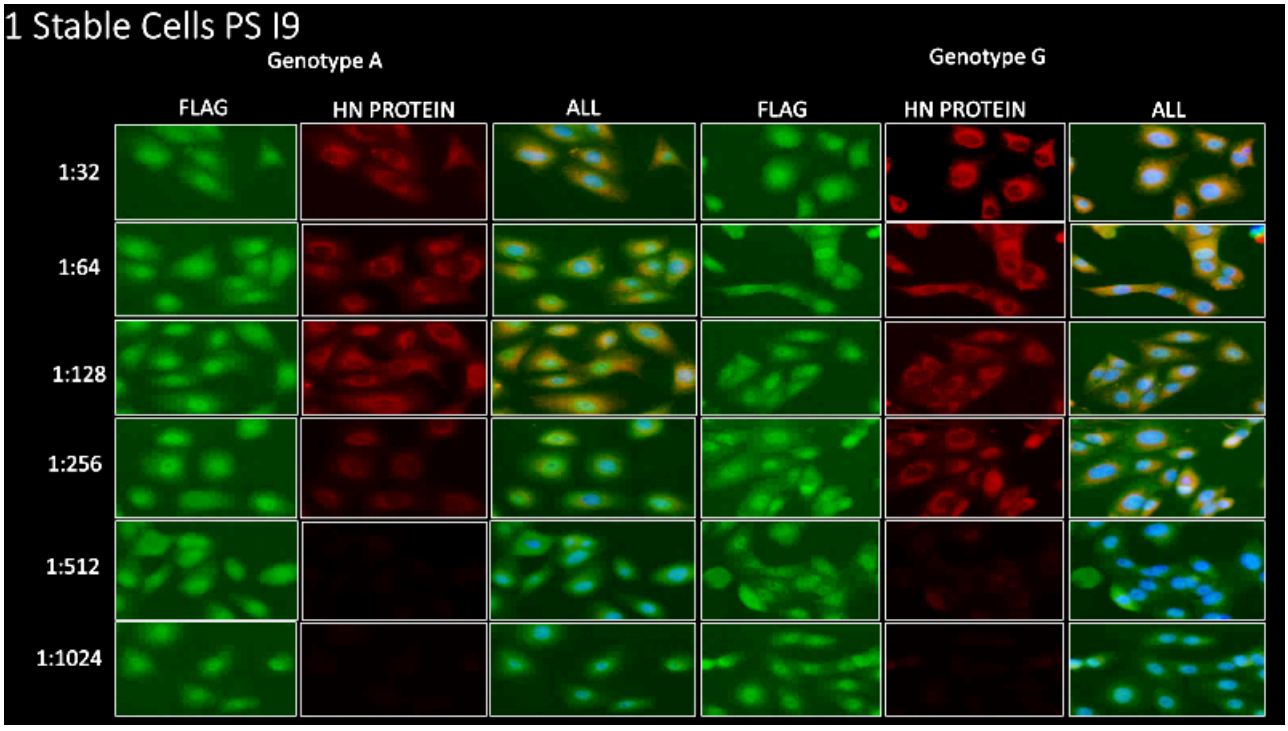


Figure A-31b. Stable cells Infected Sample #9.

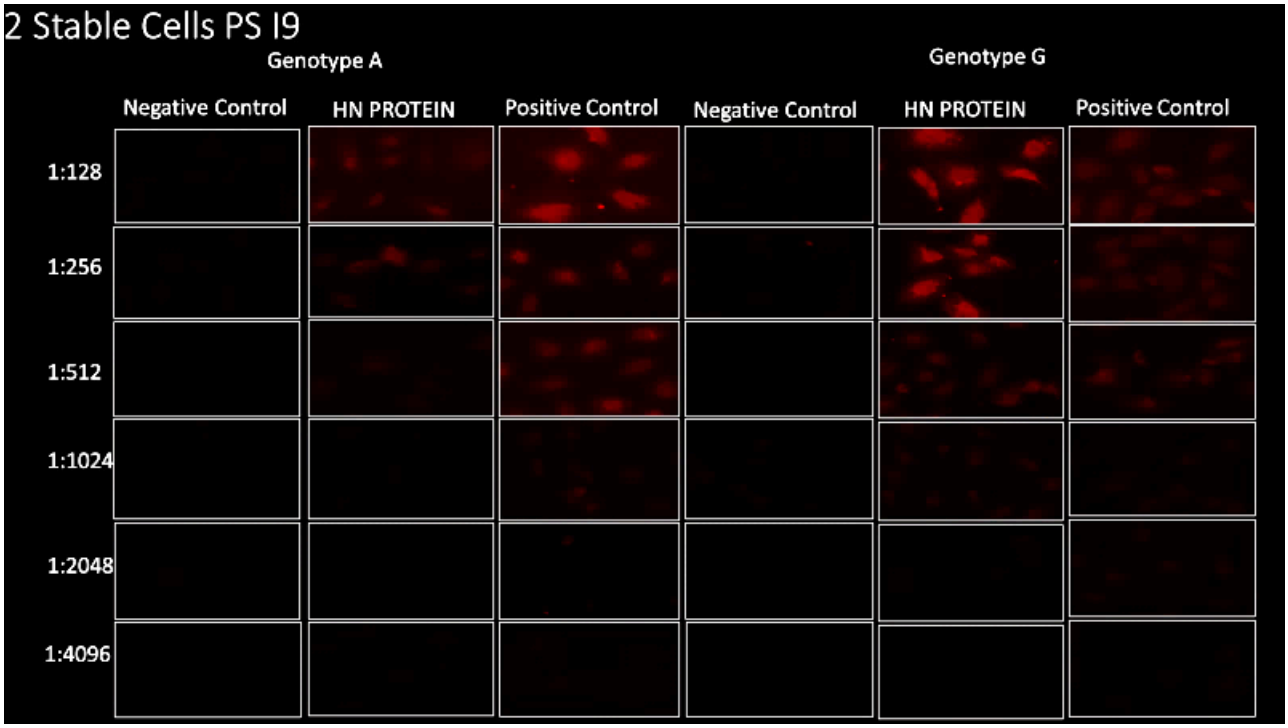


Figure A-31c. Stable cells Infected Sample #9.

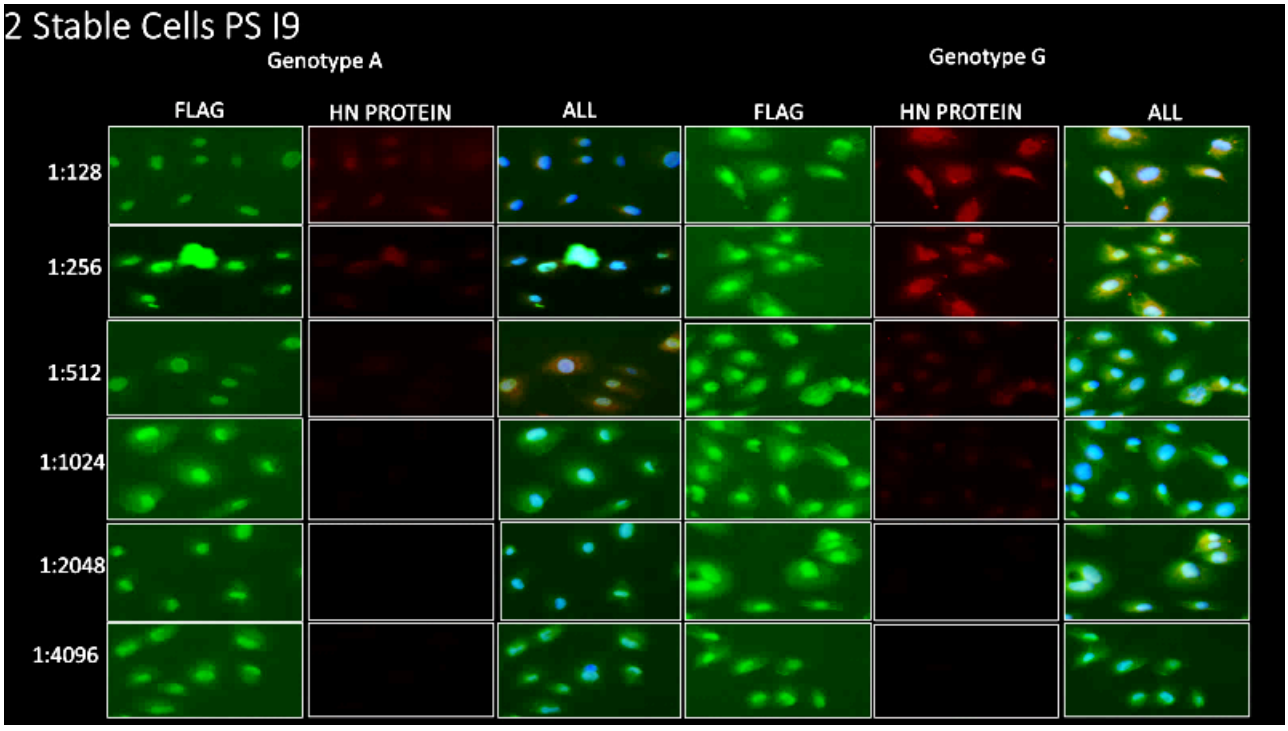


Figure A-31d. Stable cells Infected Sample #9.

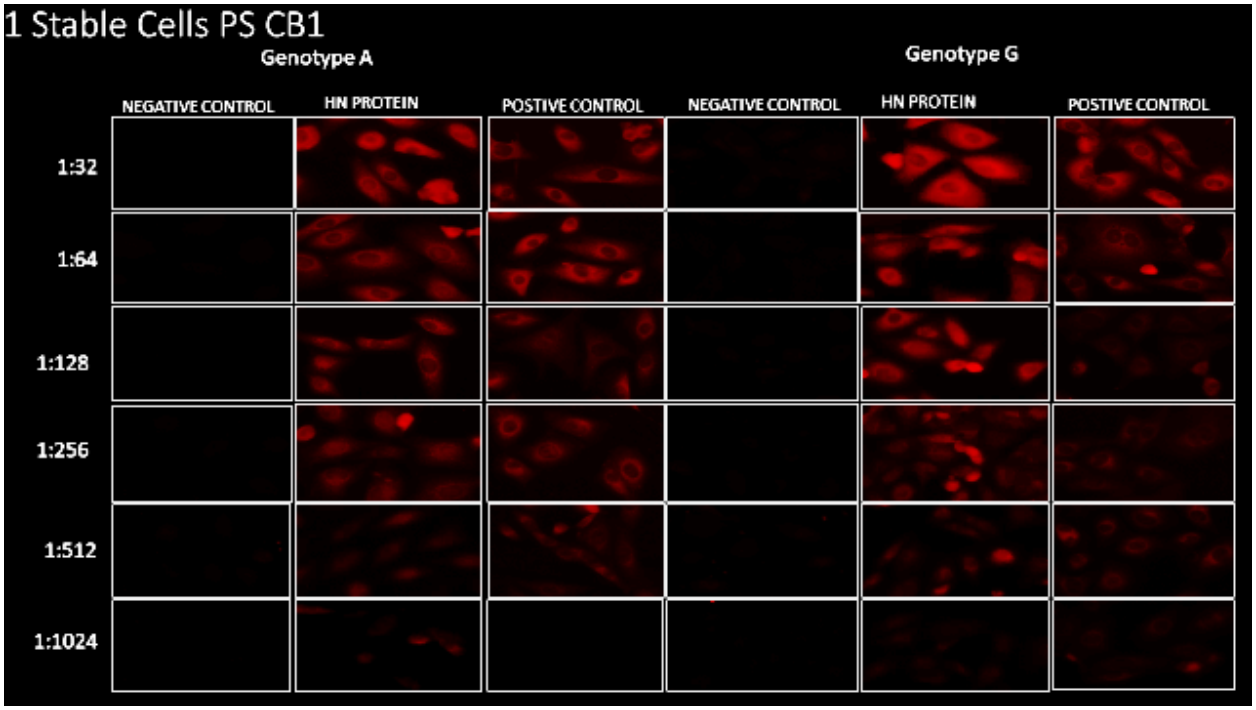


Figure A-32a. Stable cells Vaccine-Infected (CB) Sample #1.

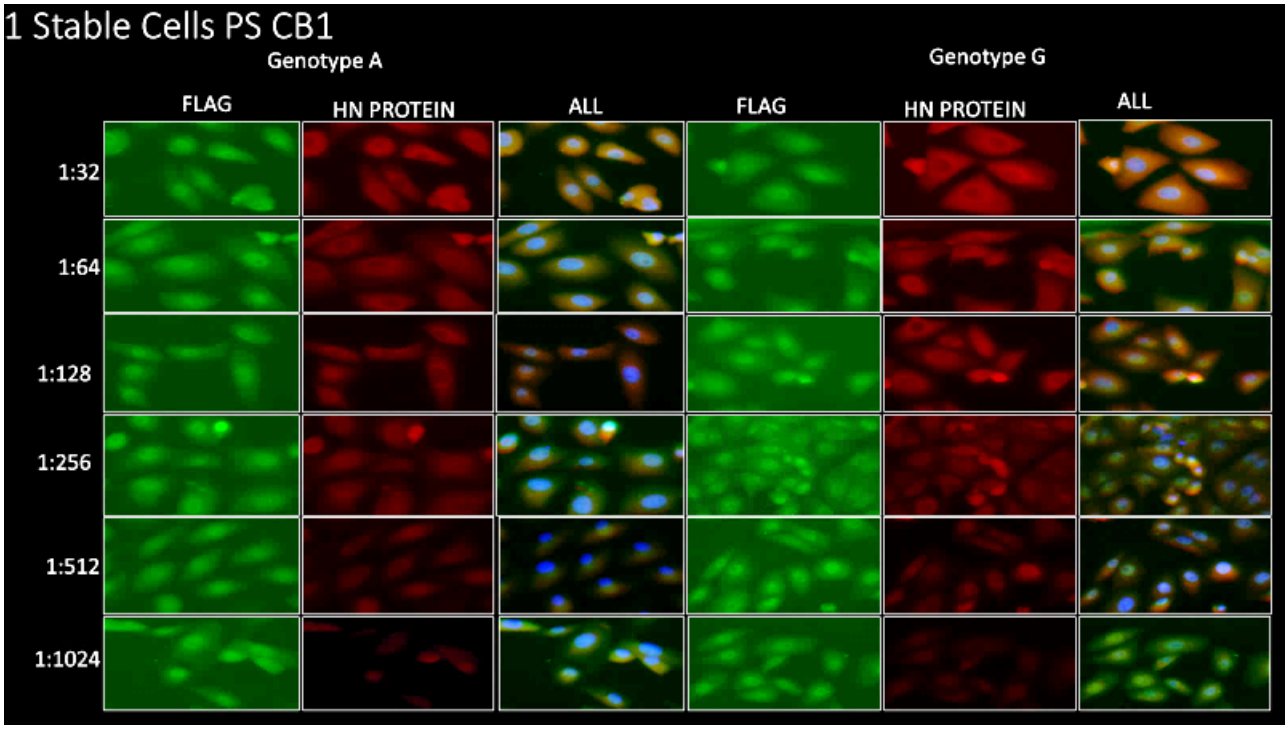


Figure A-32b. Stable cells Vaccine-Infected (CB) Sample #1.

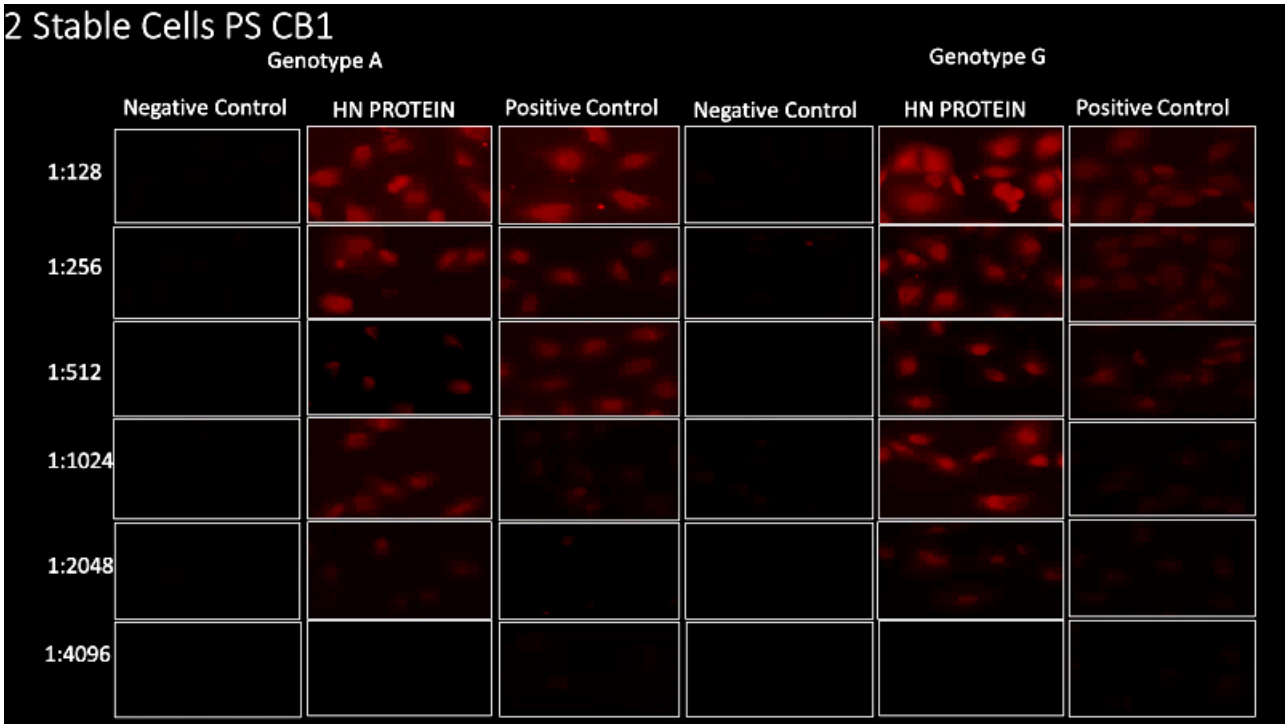


Figure A-32c. Stable cells Vaccine-Infected (CB) Sample #1.

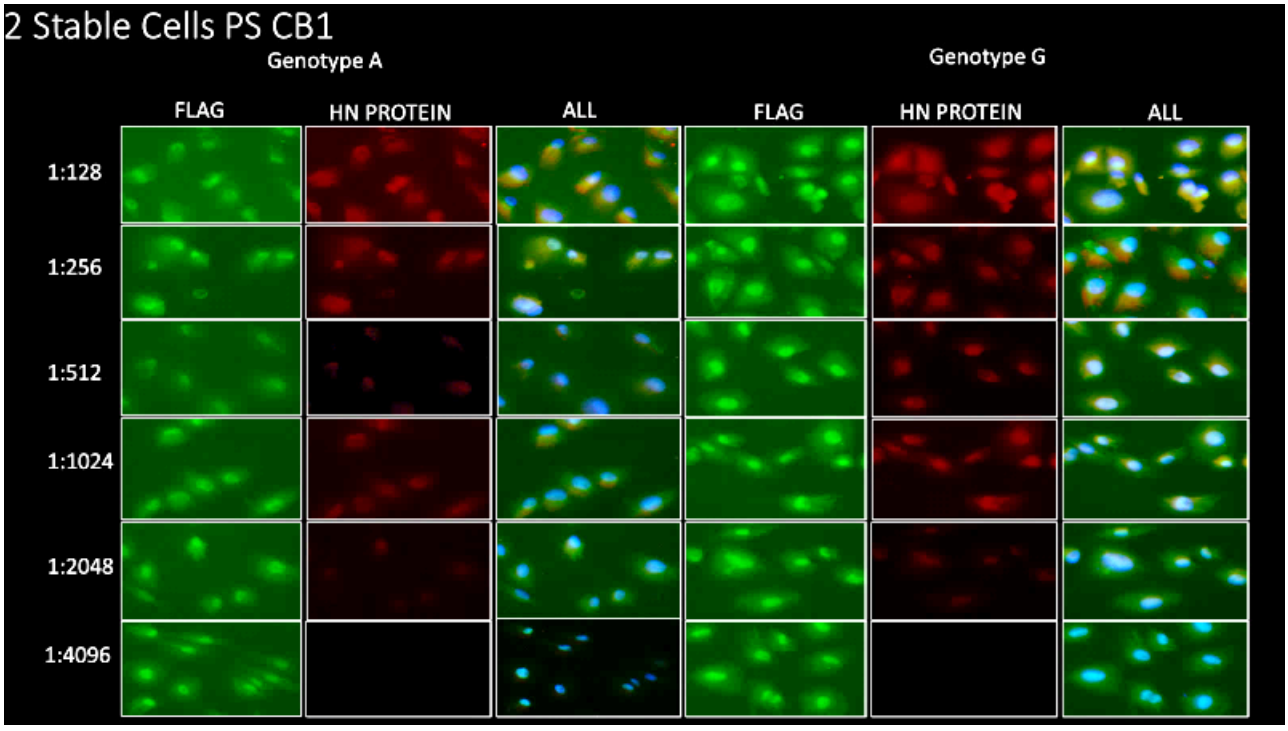


Figure A-32d. Stable cells Vaccine-Infected (CB) Sample #1.

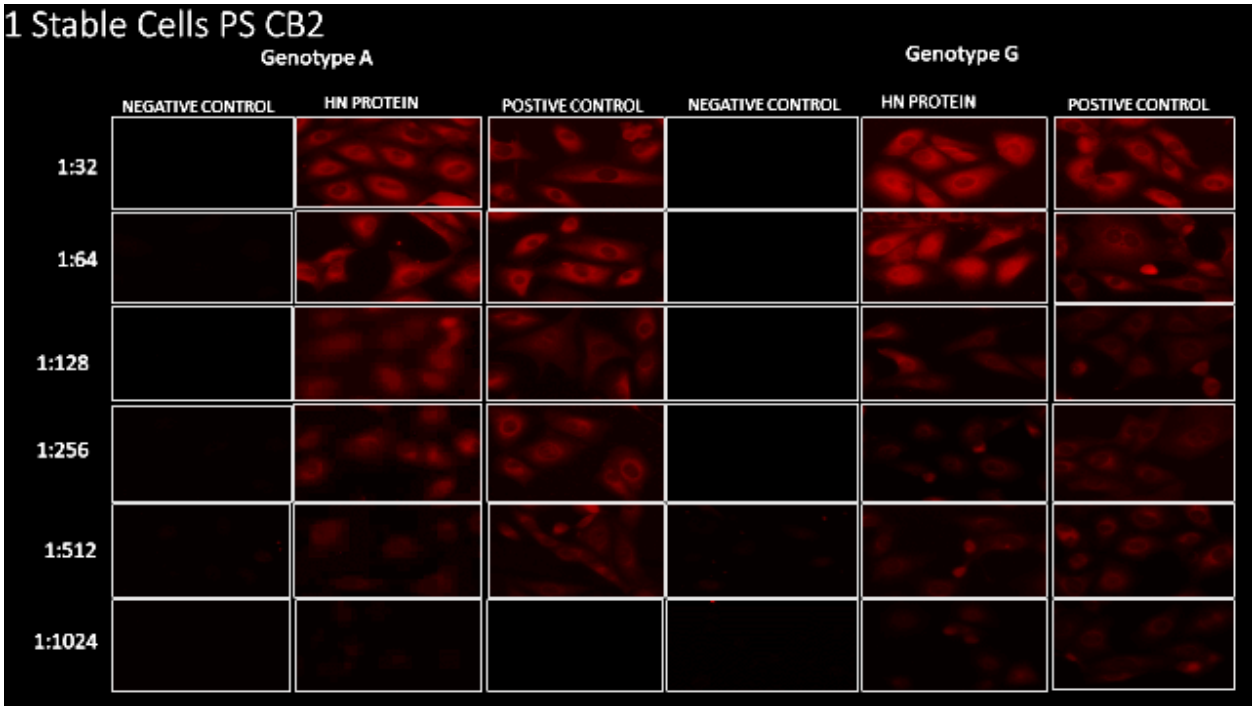


Figure A-33a. Stable cells Vaccine-Infected (CB) Sample#2.

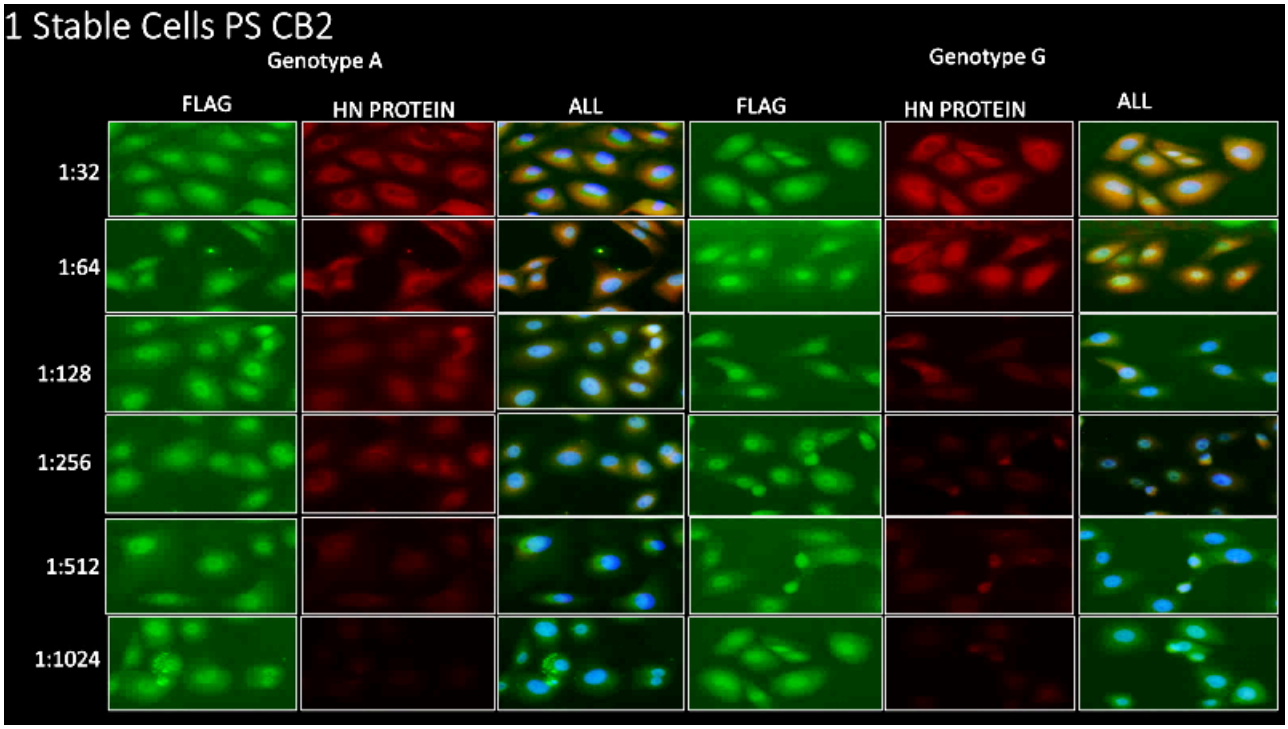


Figure A-33b. Stable cells Vaccine-Infected (CB) Sample #2.

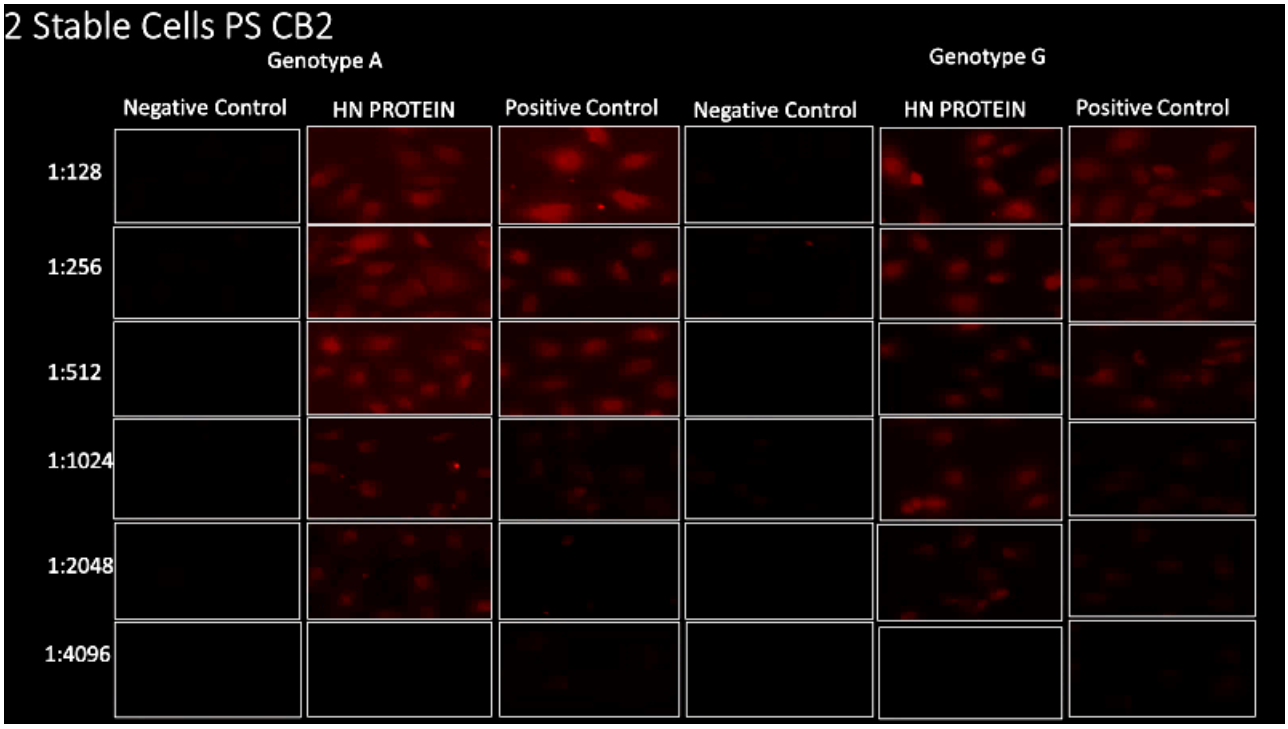


Figure A-33c. Stable cells Vaccine-Infected (CB) Sample #2.

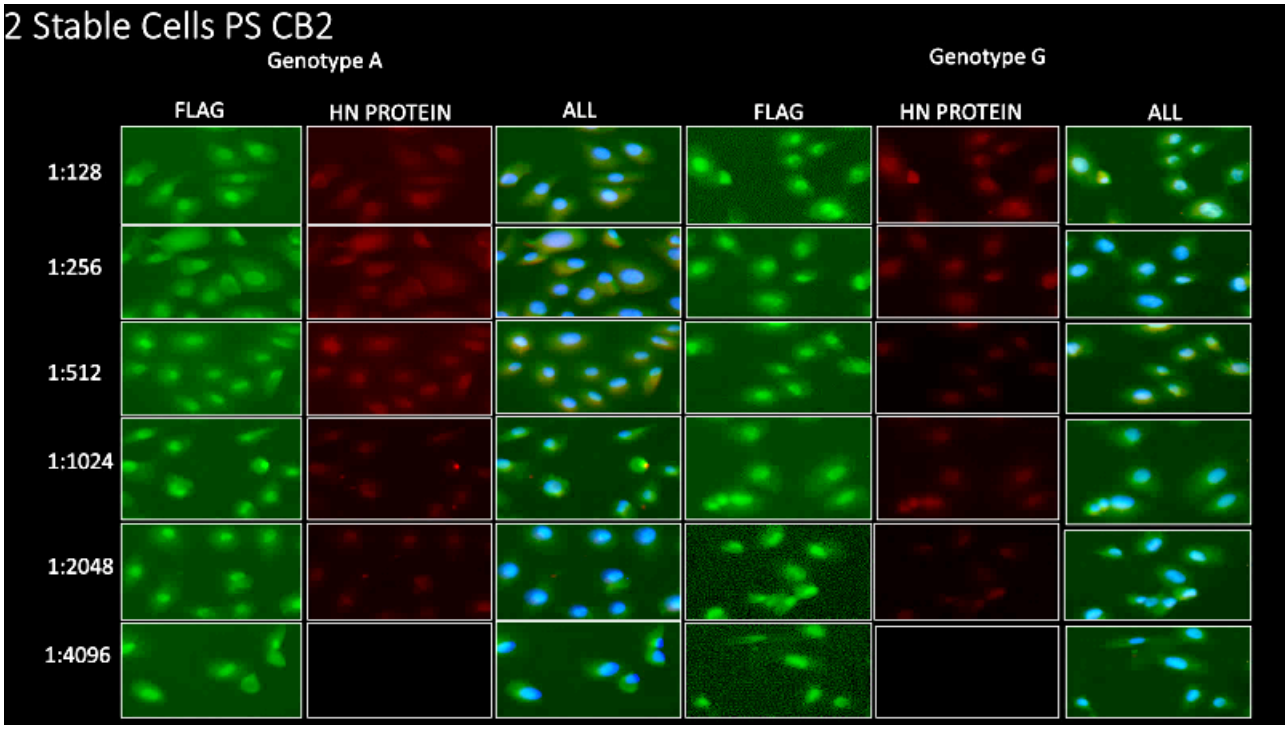


Figure A-33d. Stable cells Vaccine-Infected (CB) Sample #2.

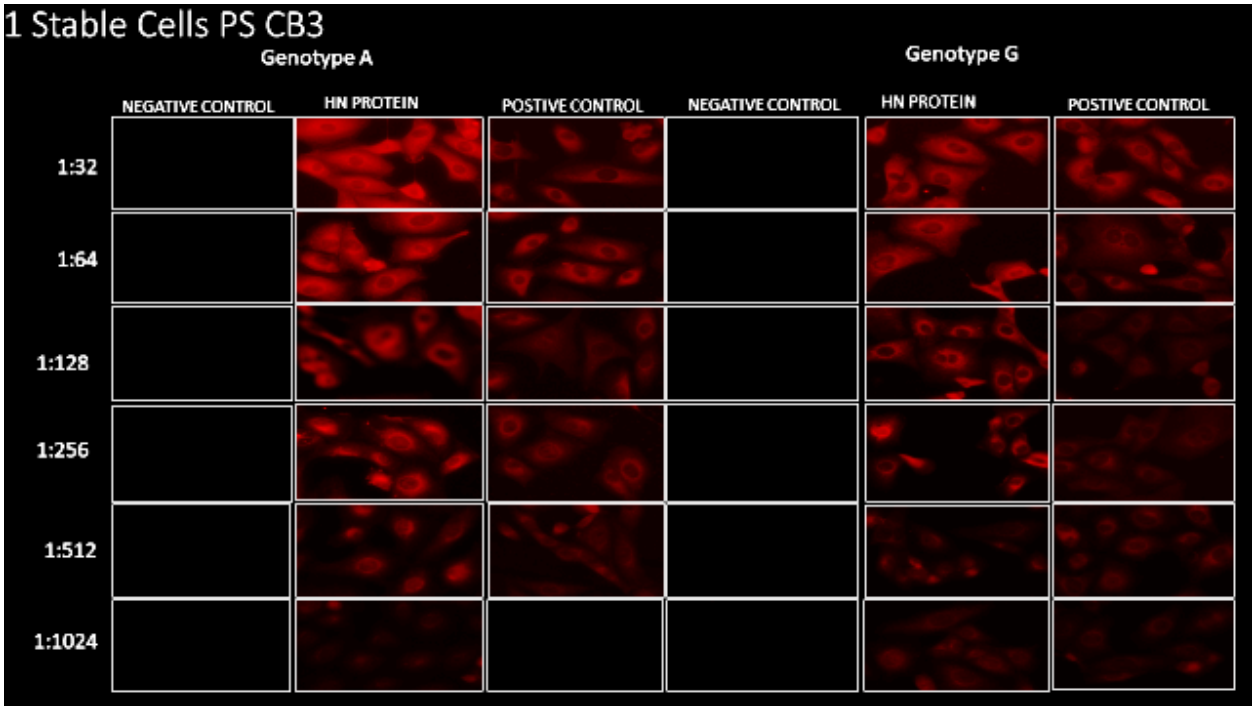


Figure A-34a. Stable cells Vaccine-Infected (CB) Sample#3.

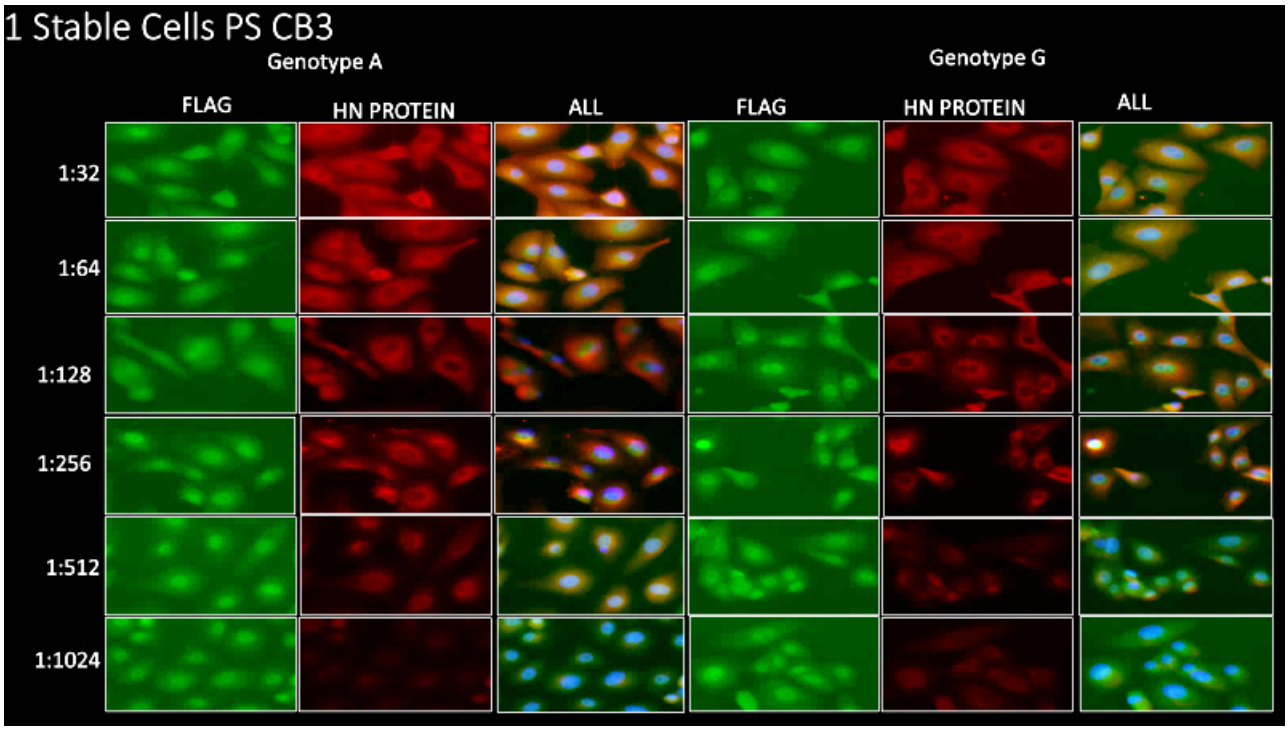


Figure A-34b. Stable cells Vaccine-Infected (CB) Sample #3.

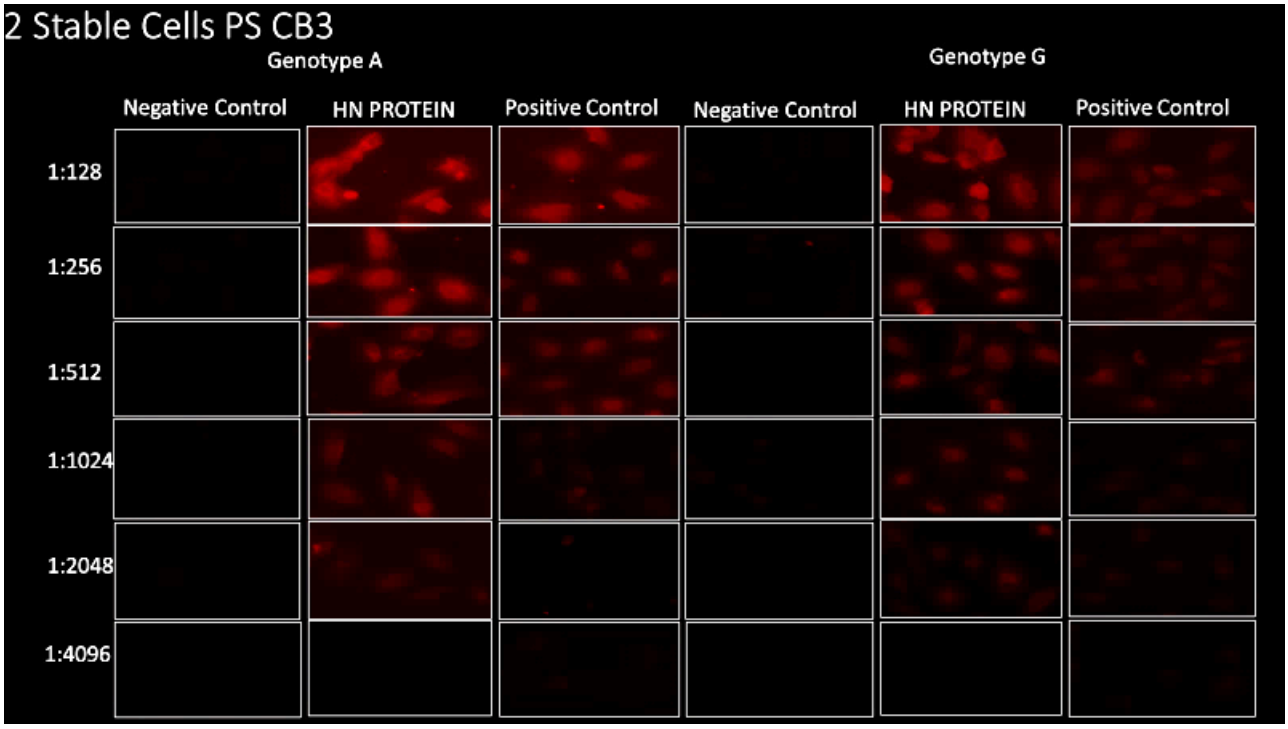


Figure A-34c. Stable cells Vaccine-Infected (CB) Sample #3.

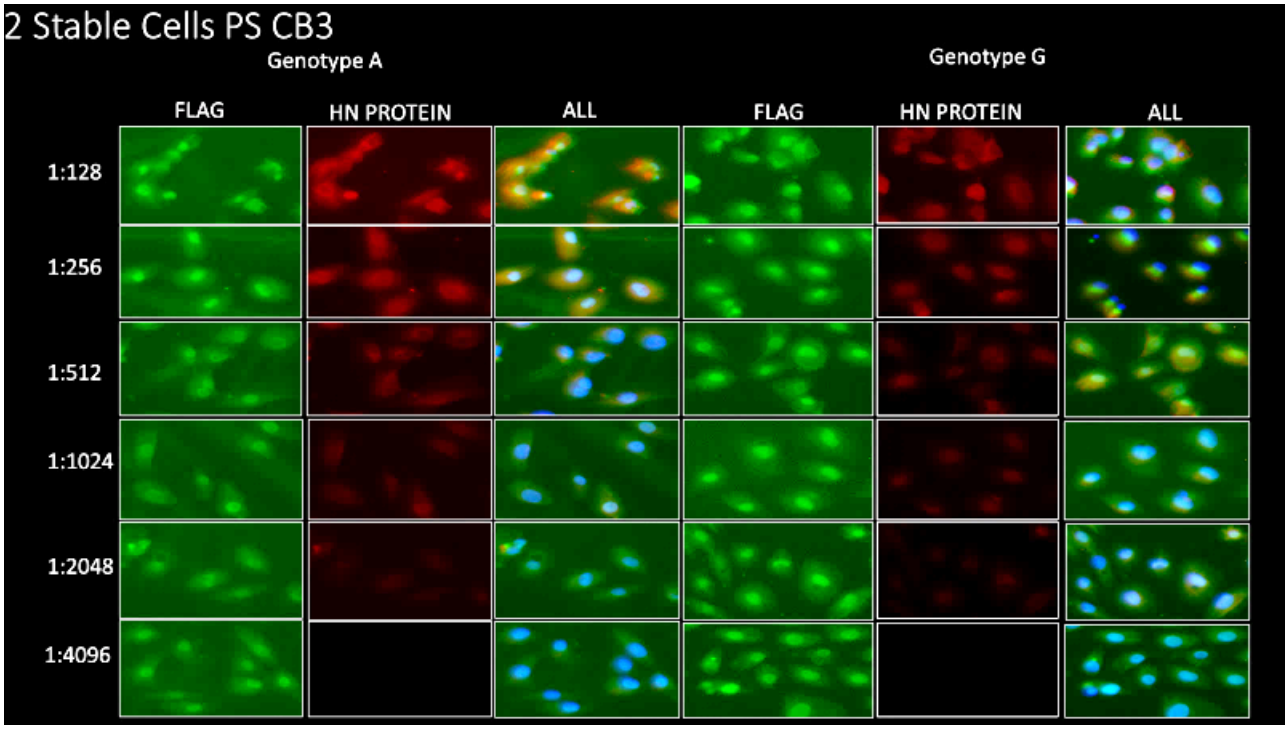


Figure A-34d. Stable cells Vaccine-Infected (CB) Sample #3.

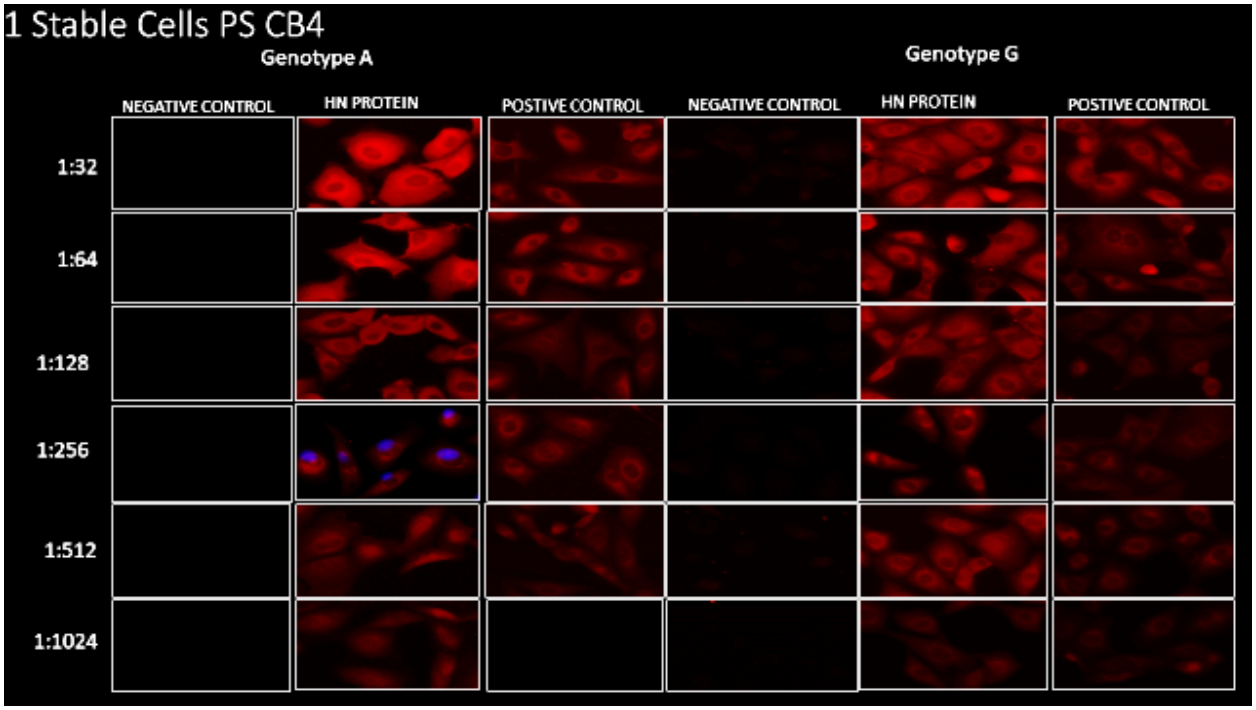


Figure A-35a. Stable cells Vaccine-Infected (CB) Sample#4.

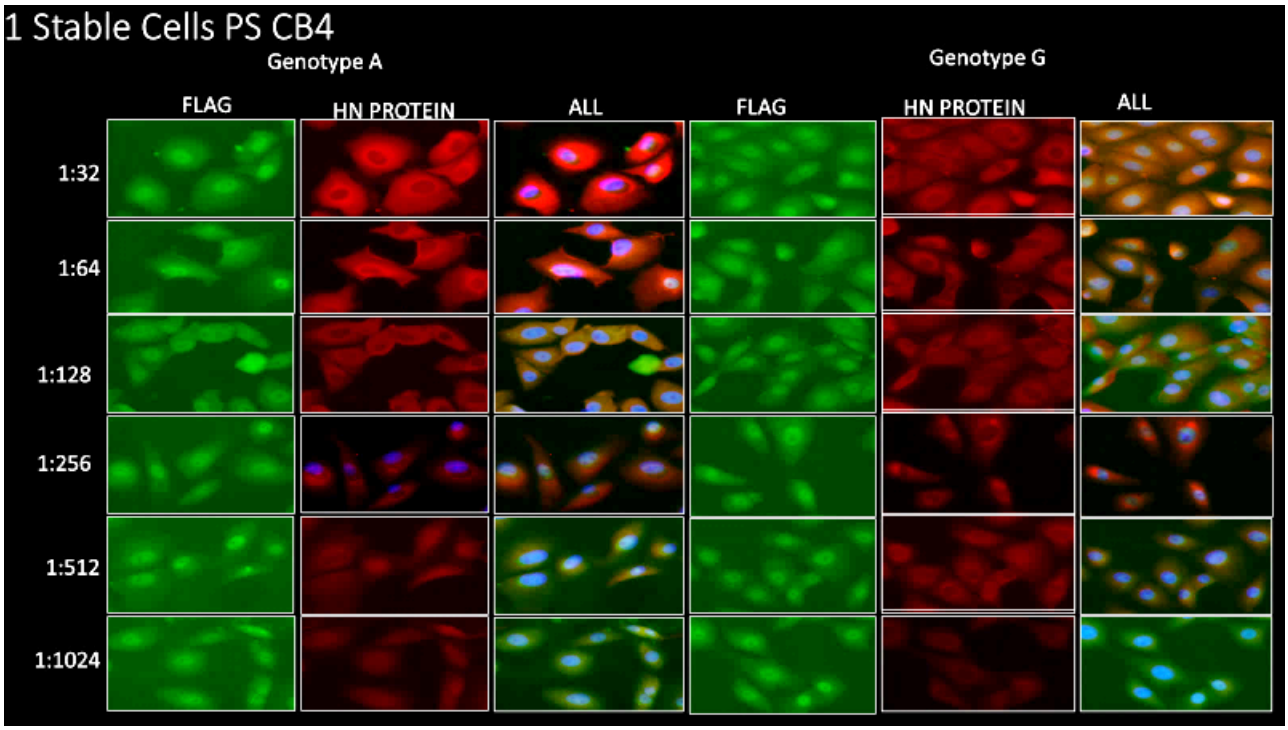


Figure A-35b. Stable cells Vaccine-Infected (CB) Sample #4. Note: Forgot to save red channel Alone

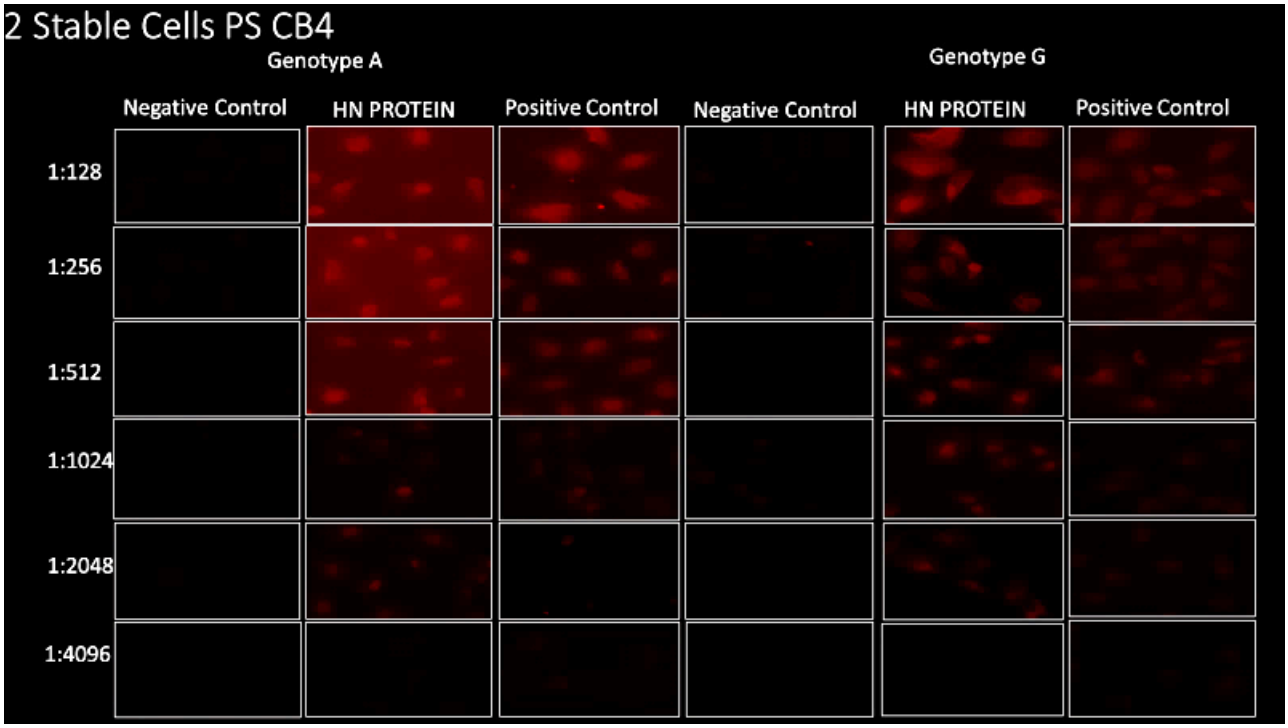


Figure A-35c. Stable cells Vaccine-Infected (CB) Sample #4.

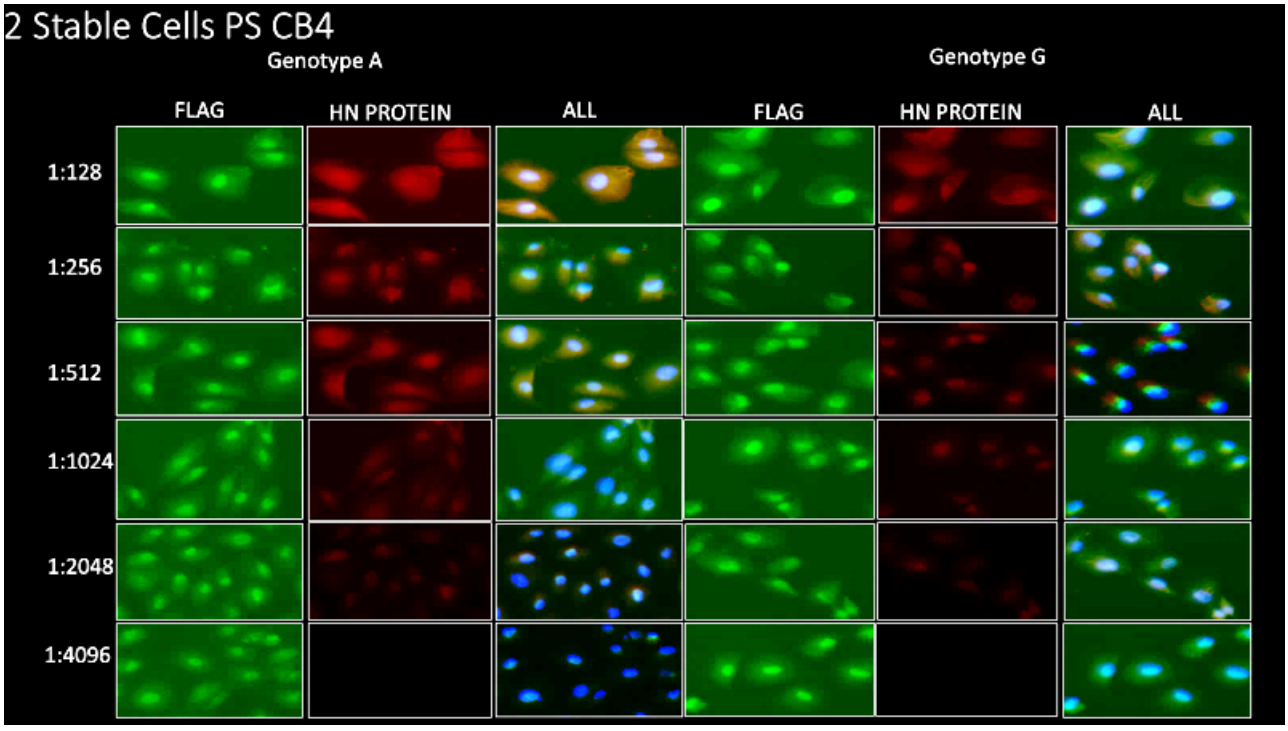


Figure A-35d. Stable cells Vaccine-Infected (CB) Sample #4.

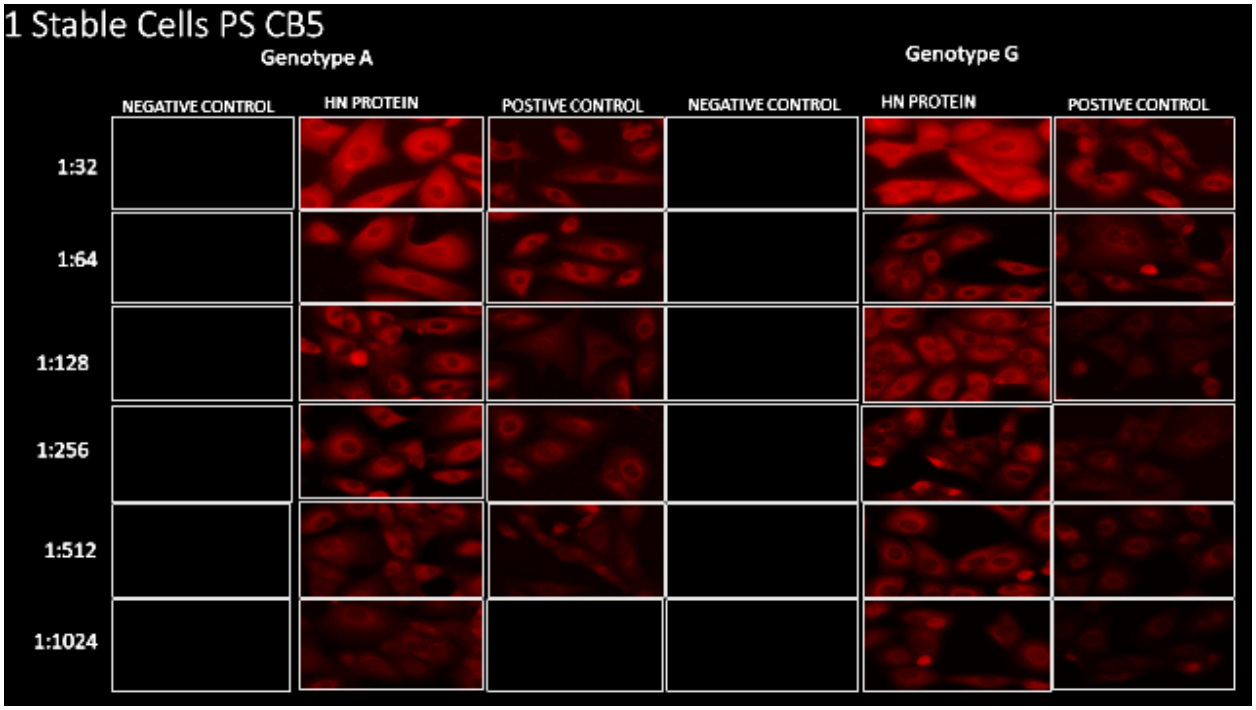


Figure A-36a. Stable cells Vaccine-Infected (CB) Sample#5.

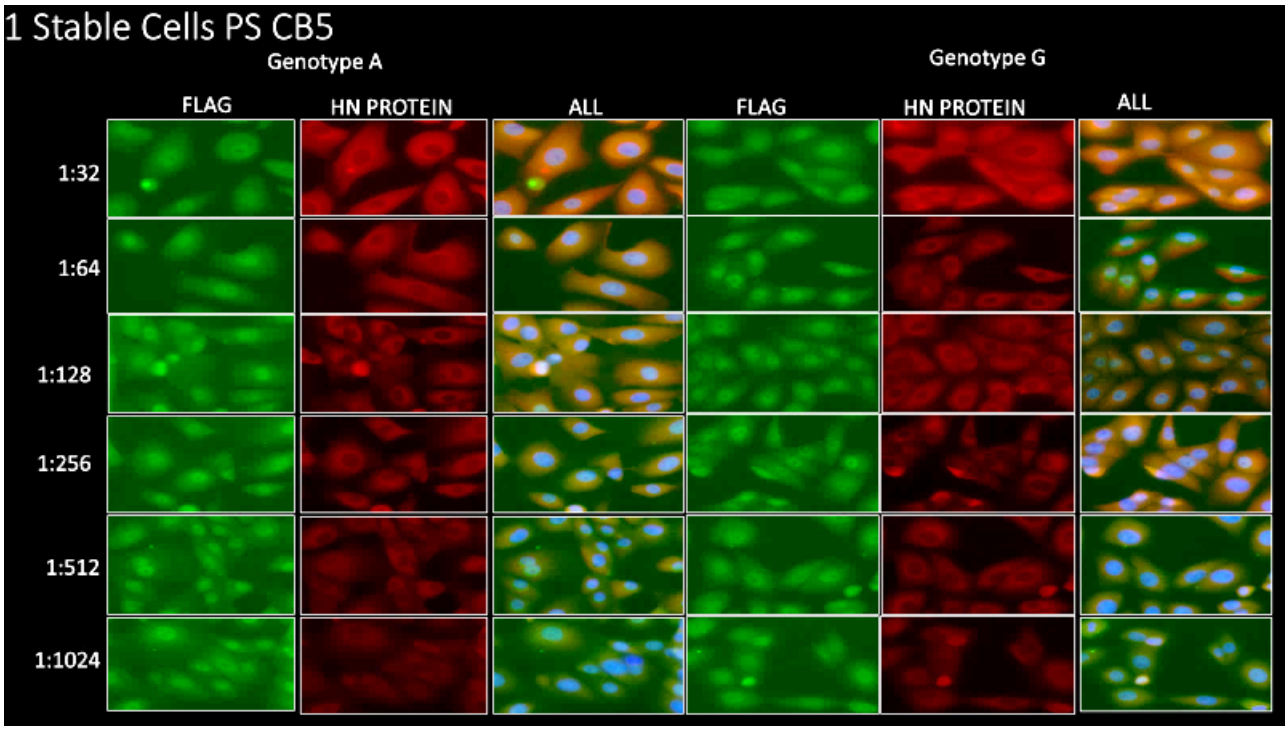


Figure A-36b. Stable cells Vaccine-Infected (CB) Sample #5.

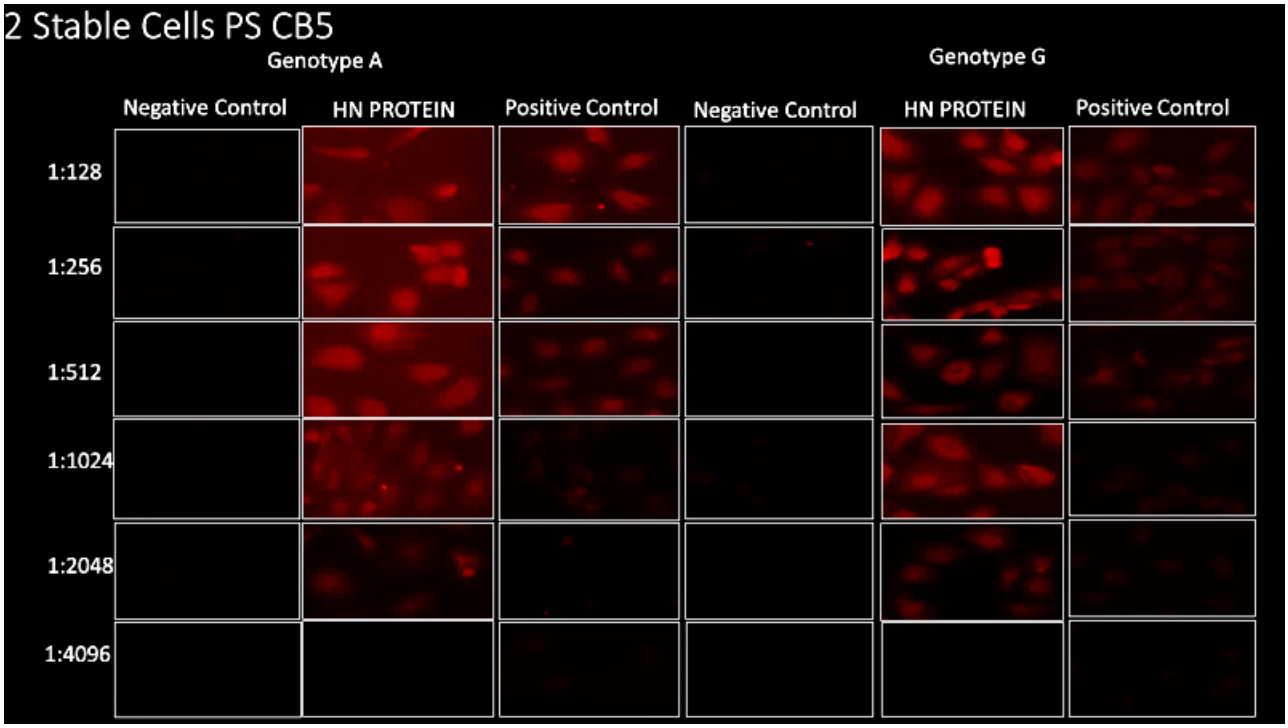


Figure A-36c. Stable cells Vaccine-Infected (CB) Sample #5.

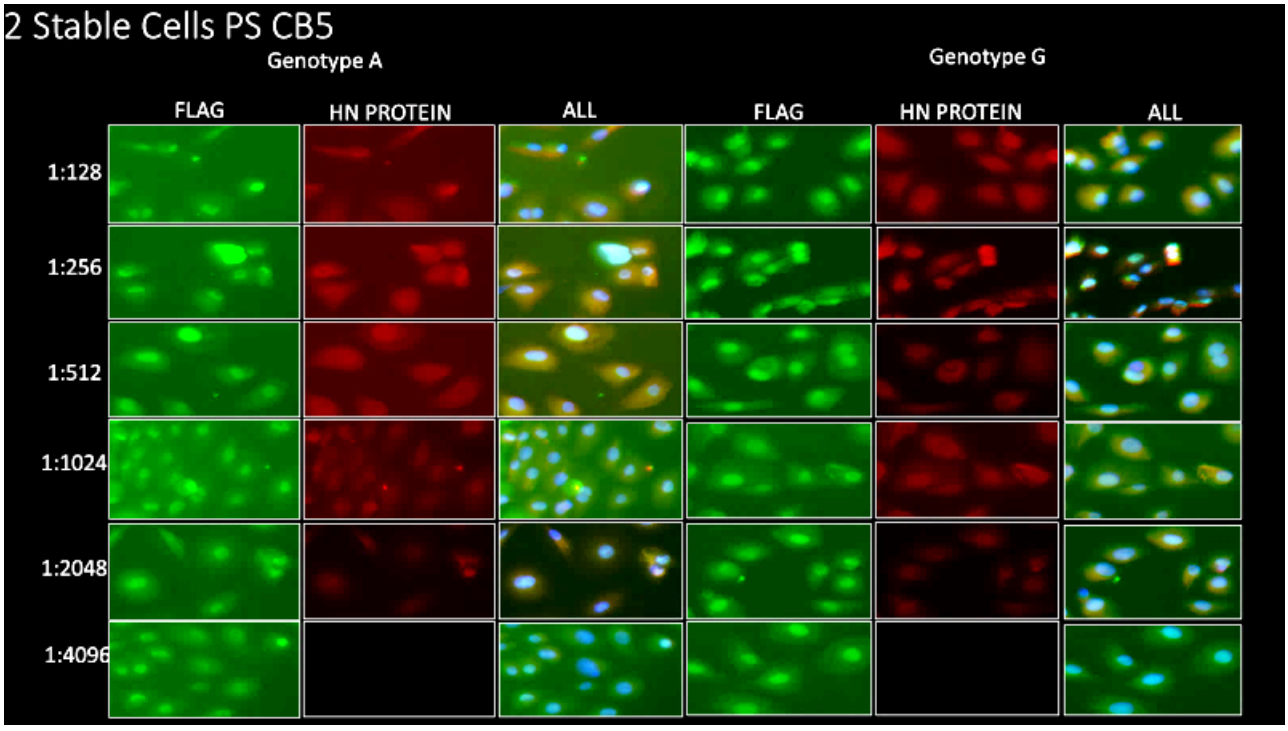


Figure A-36d. Stable cells Vaccine-Infected (CB) Sample #5.

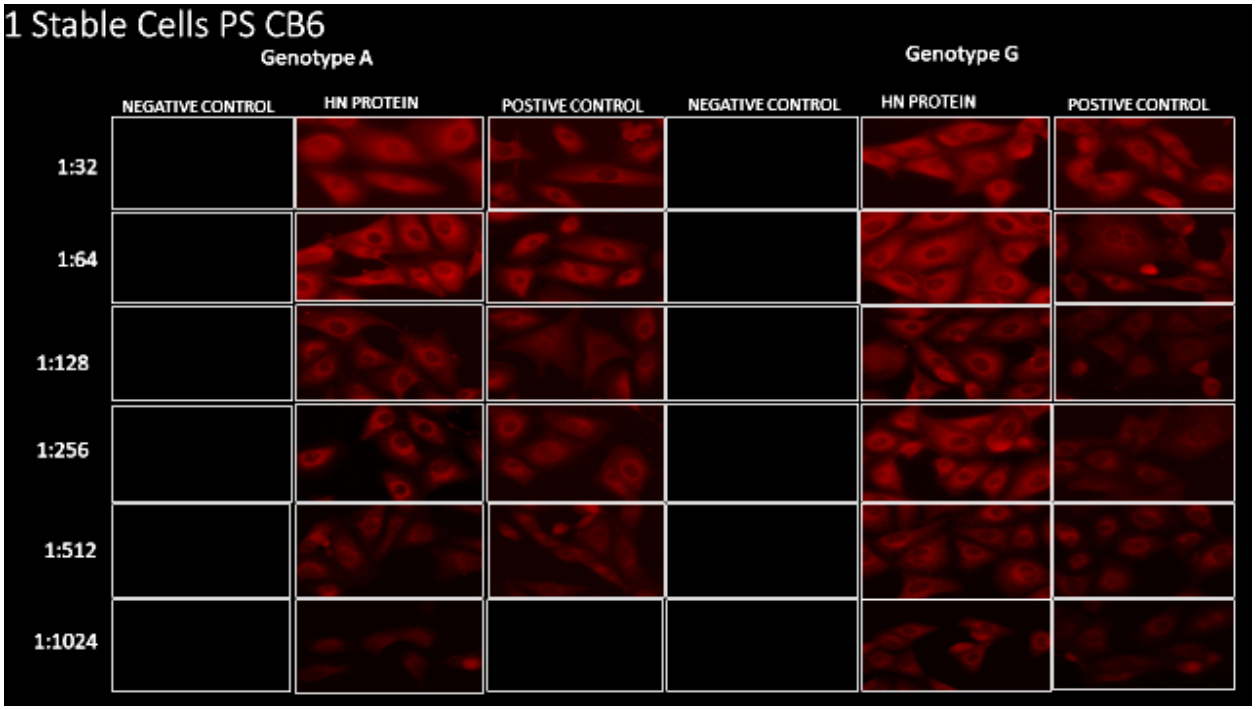


Figure A-37a. Stable cells Vaccine-Infected (CB) Sample#6.

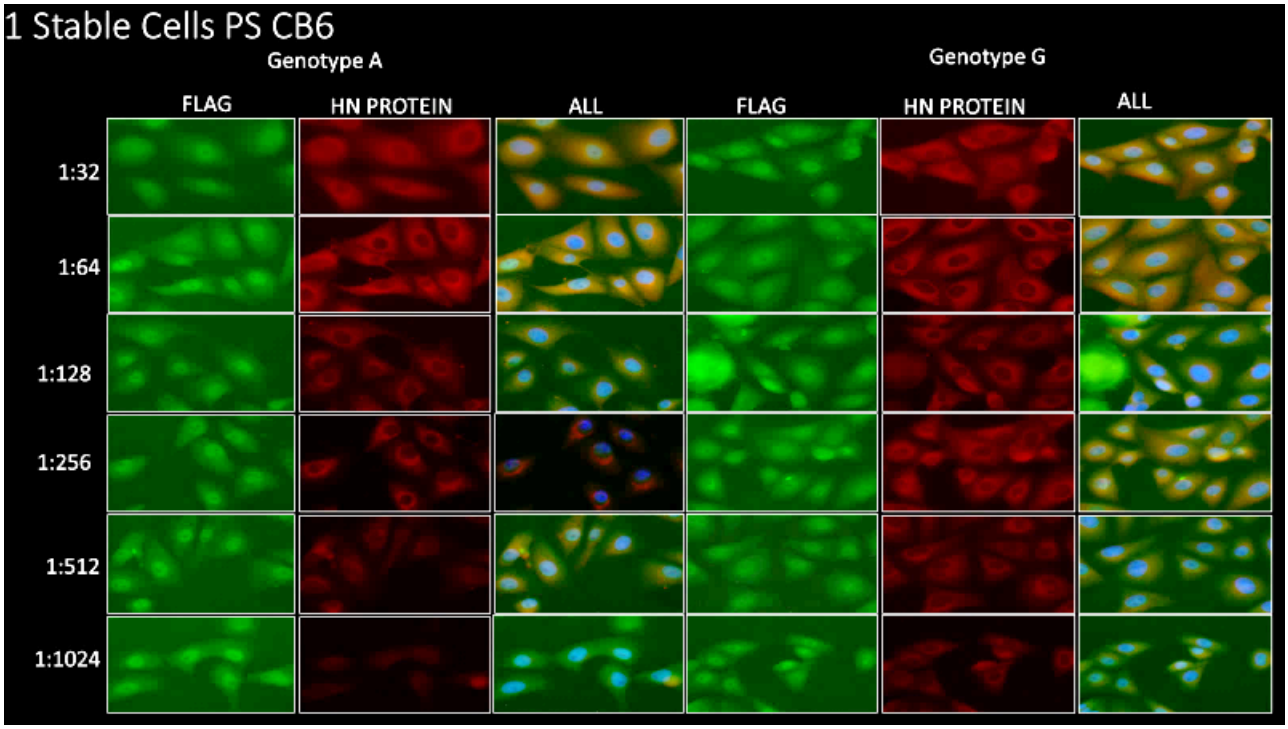


Figure A-37b. Stable cells Vaccine-Infected (CB) Sample #6.

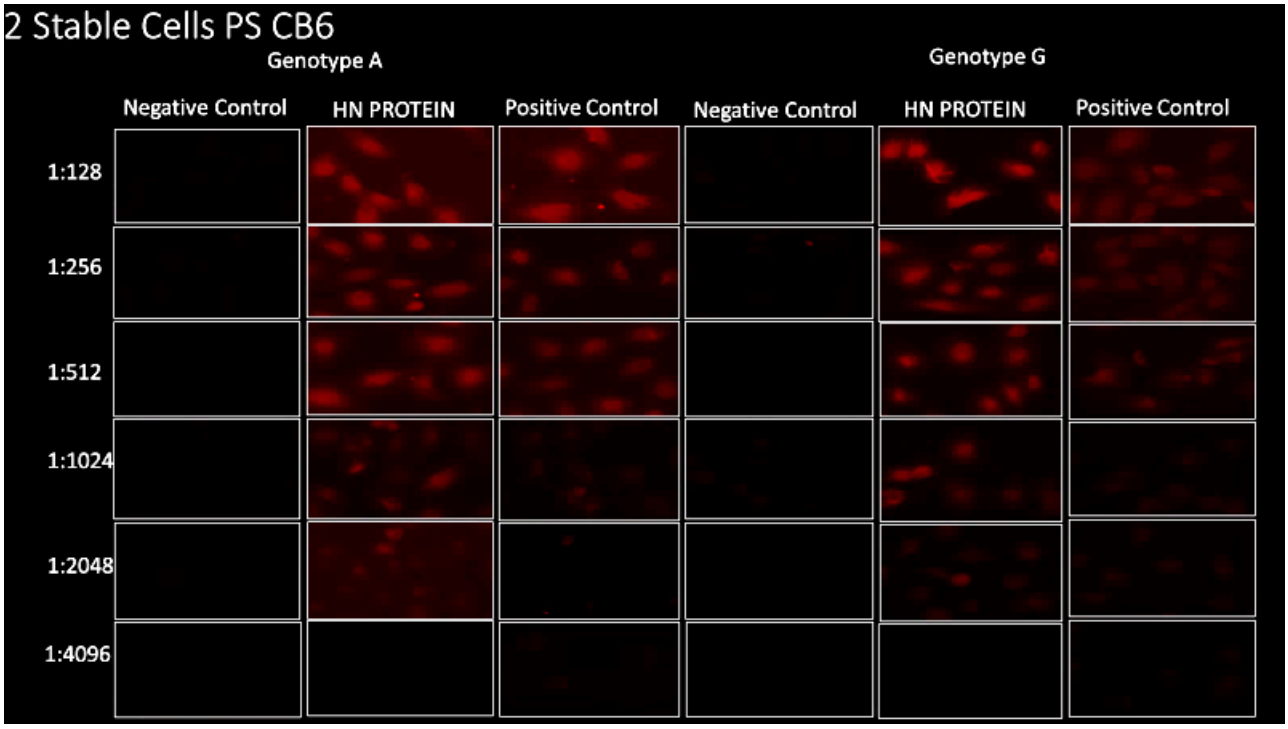


Figure A-37c. Stable cells Vaccine-Infected (CB) Sample #6.

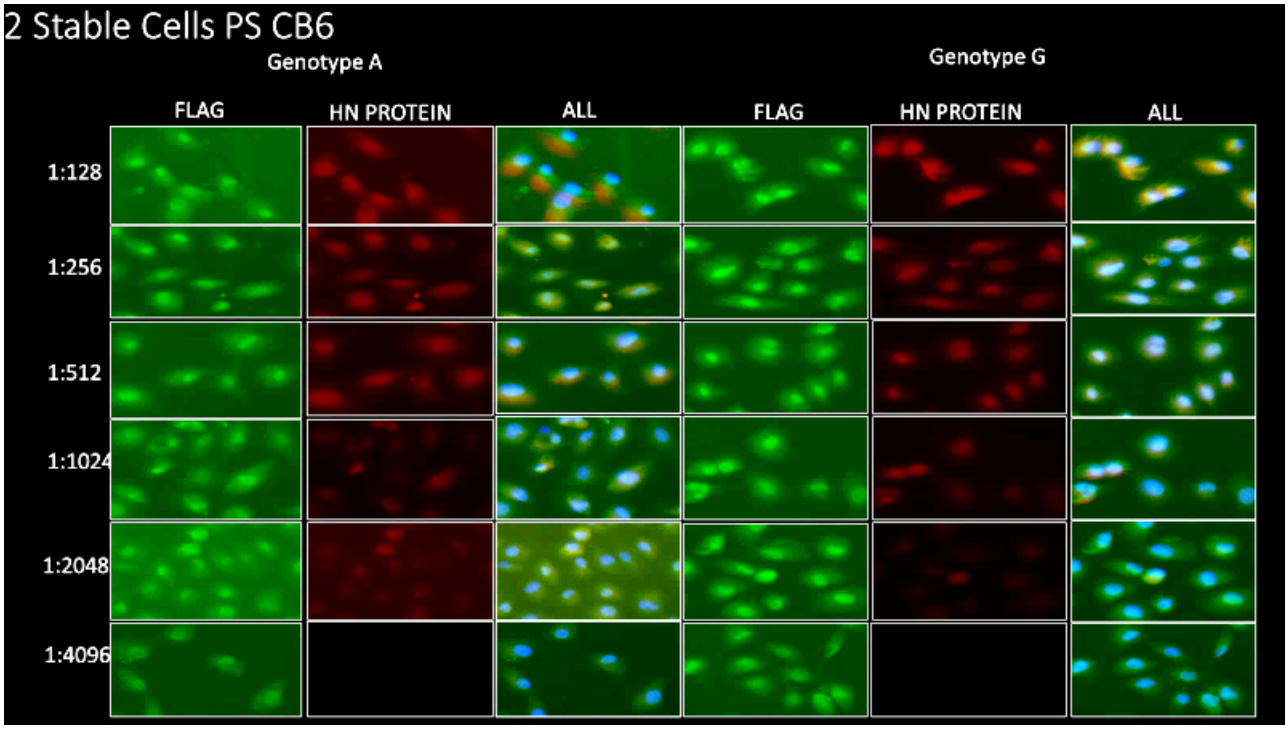


Figure A-37d. Stable cells Vaccine-Infected (CB) Sample #6.

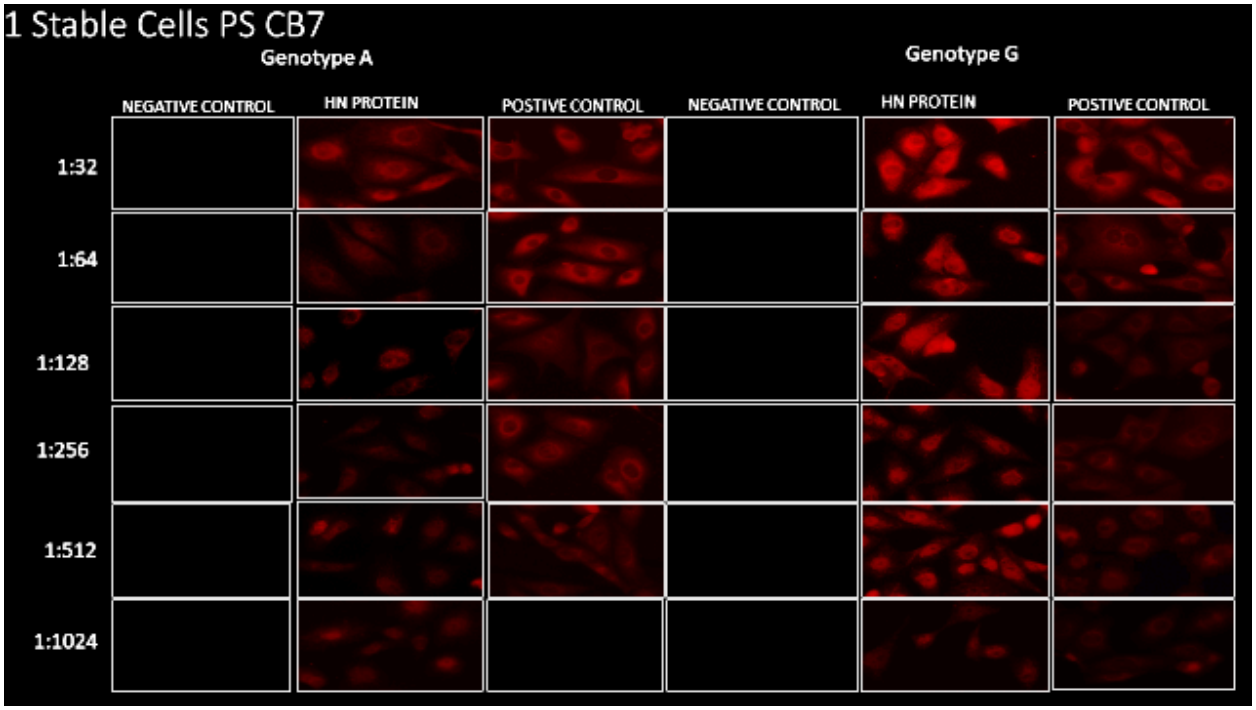


Figure A-38a. Stable cells Vaccine-Infected (CB) Sample#7.

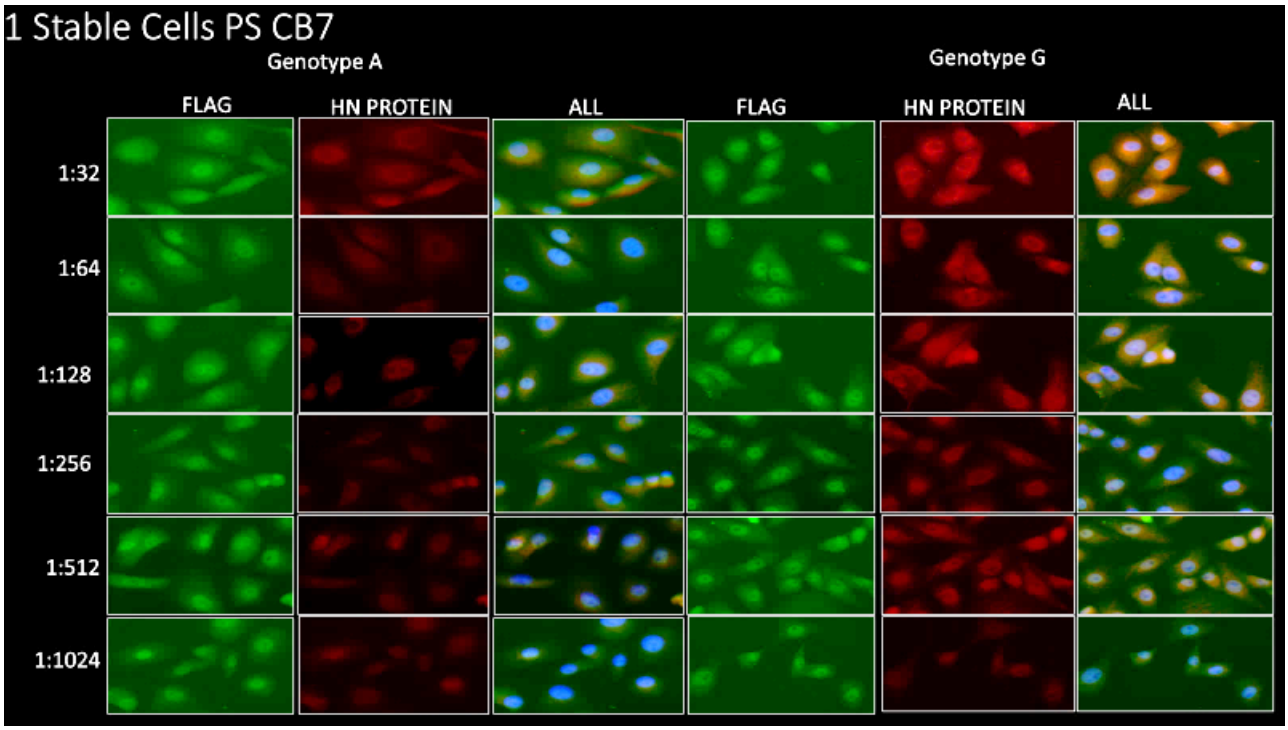


Figure A-38b. Stable cells Vaccine-Infected (CB) Sample #7.

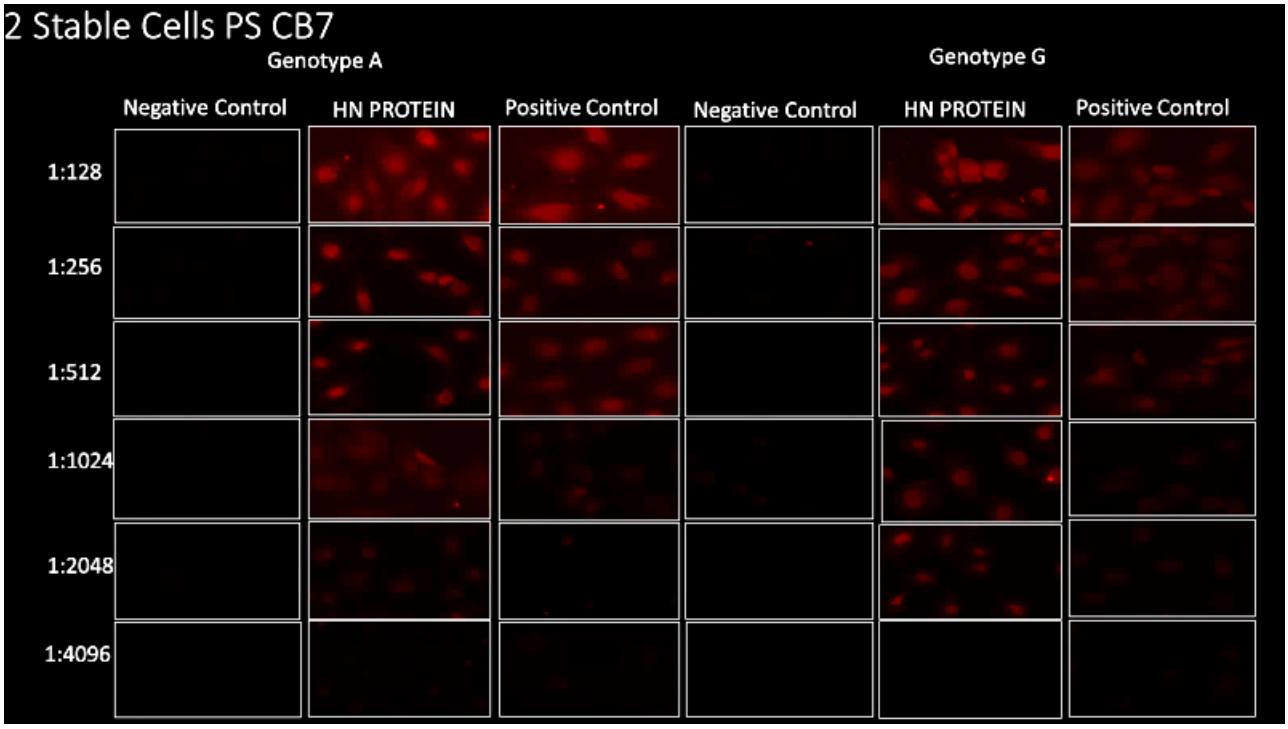


Figure A-38c. Stable cells Vaccine-Infected (CB) Sample #7.

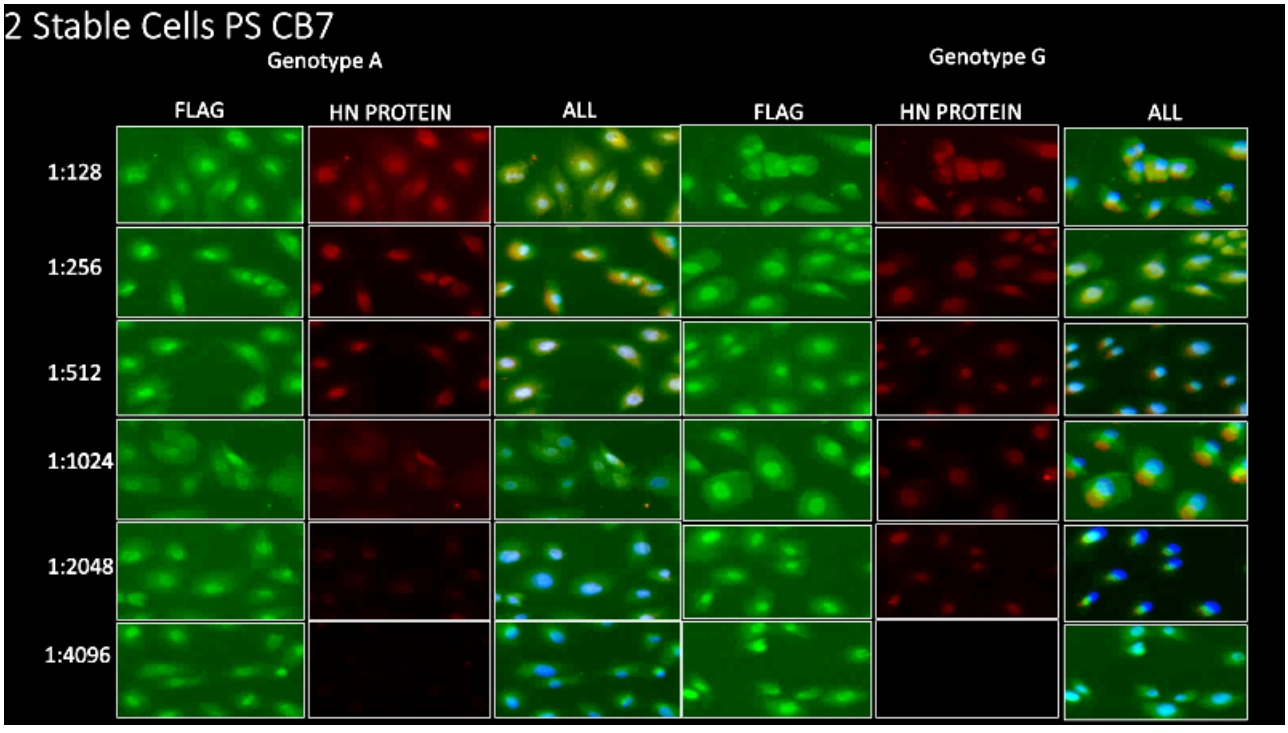


Figure A-38d. Stable cells Vaccine-Infected (CB) Sample #7.

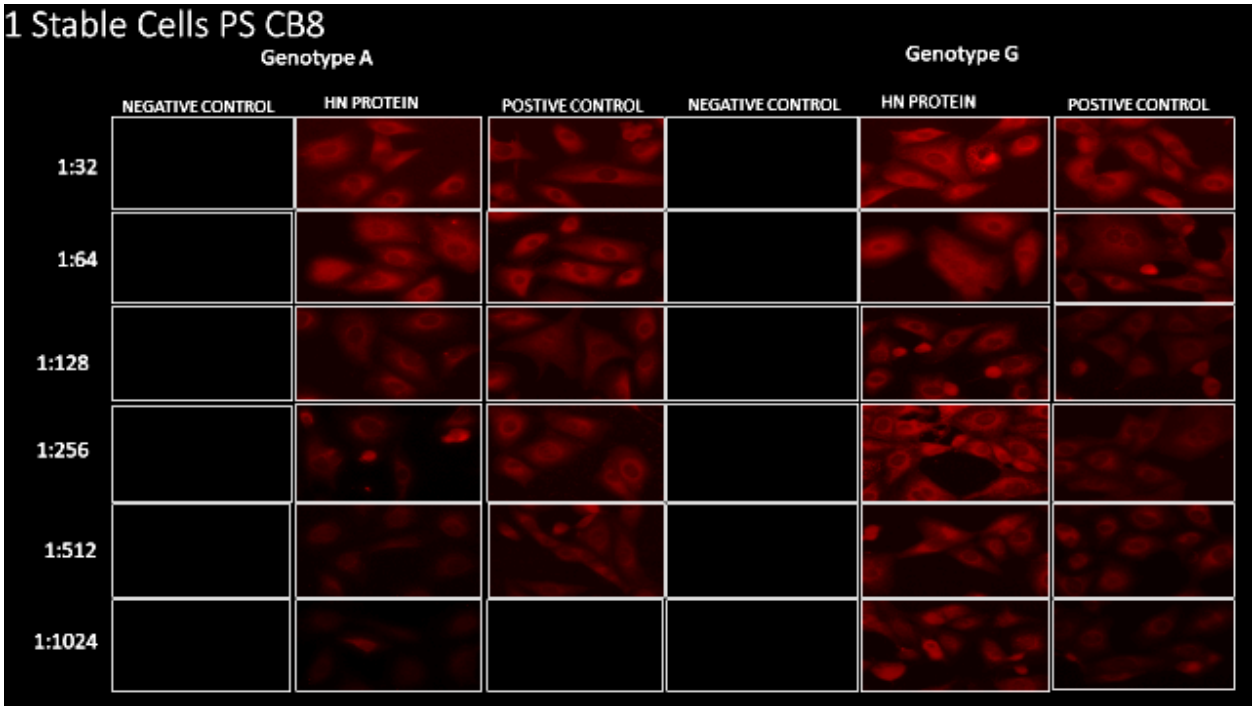


Figure A-39a. Stable cells Vaccine-Infected (CB) Sample#8.

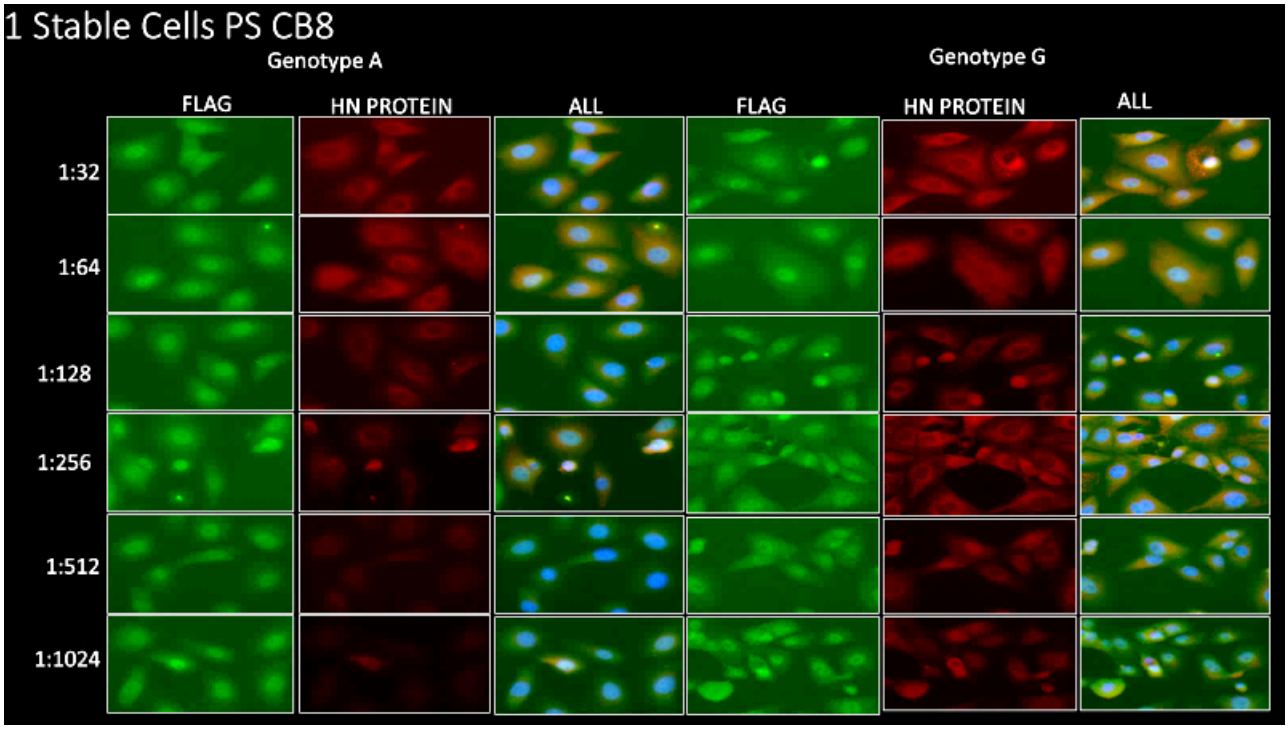


Figure A-39b. Stable cells Vaccine-Infected (CB) Sample #8.

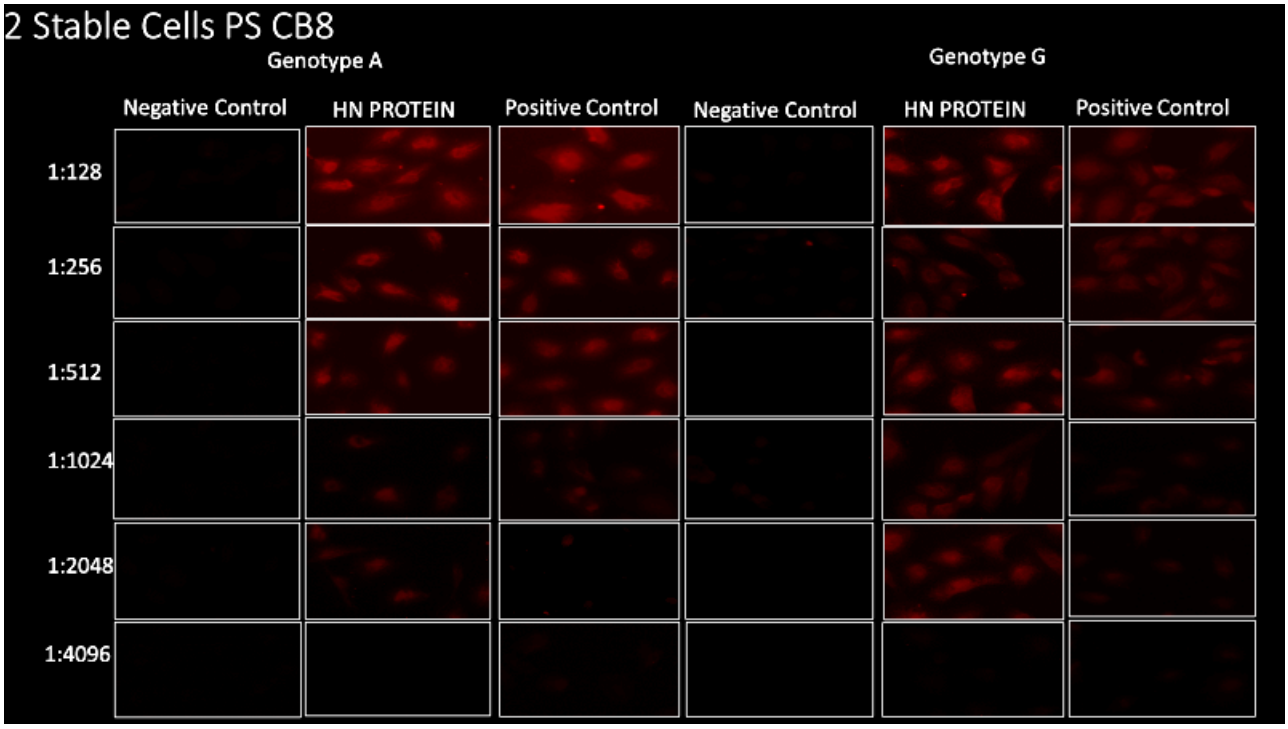


Figure A-39c. Stable cells Vaccine-Infected (CB) Sample #8.

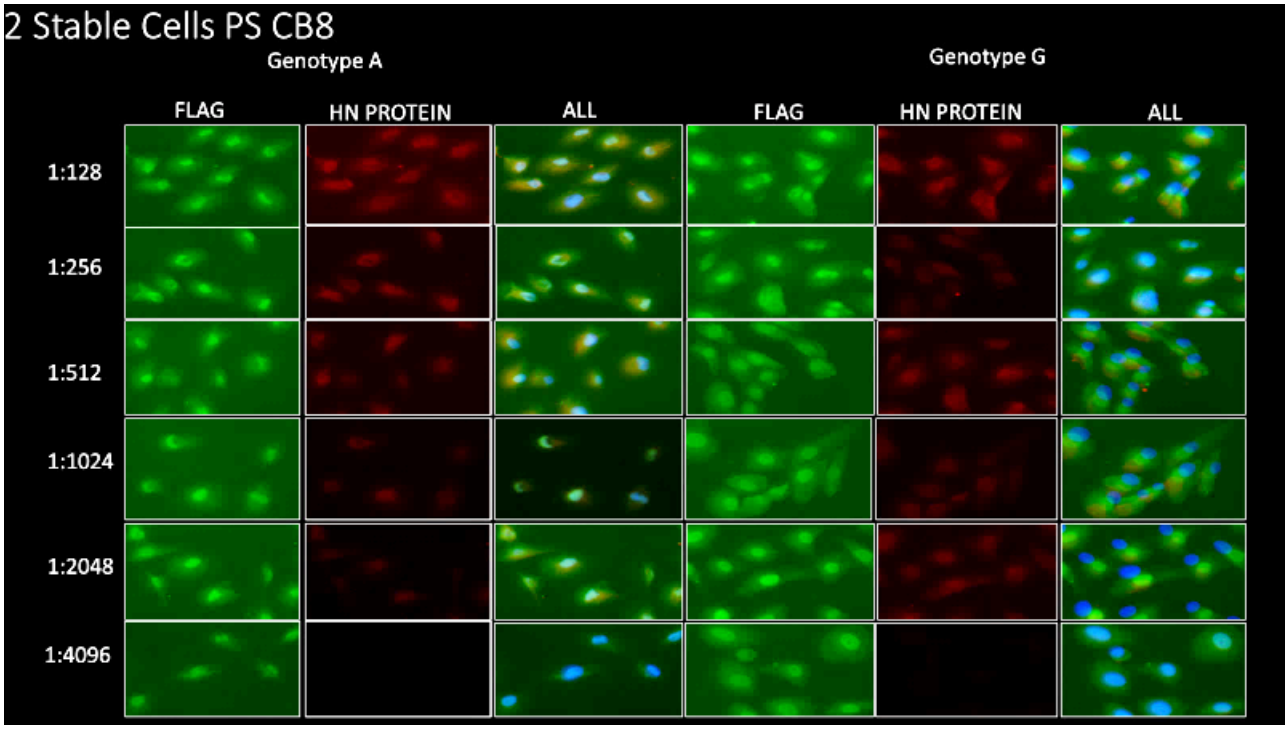


Figure A-39d. Stable cells Vaccine-Infected (CB) Sample #8.

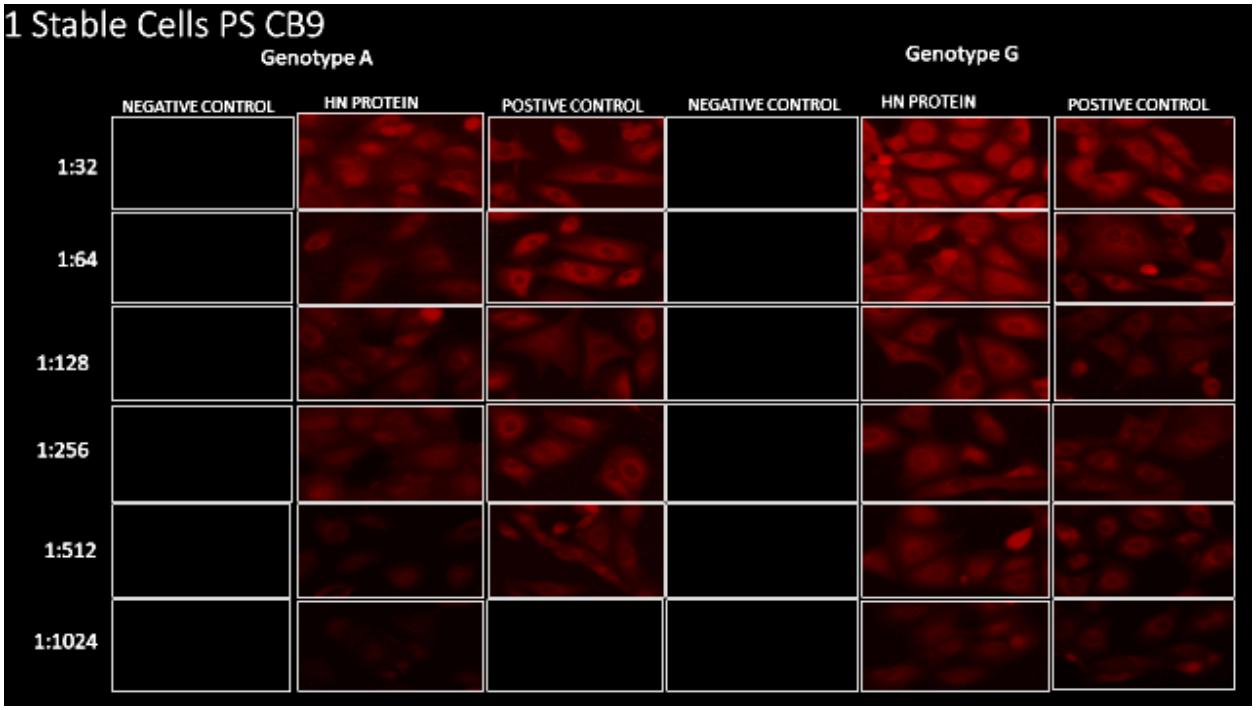


Figure A-40a. Stable cells Vaccine-Infected (CB) Sample#9.

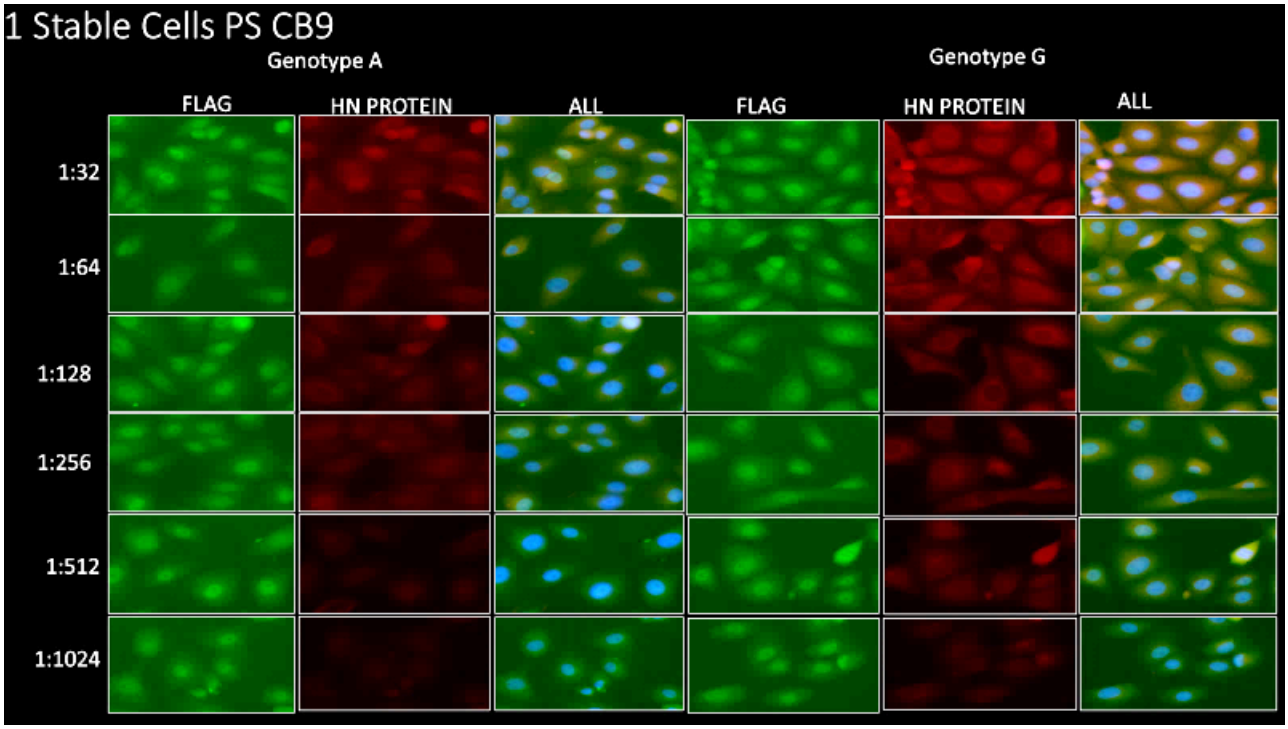


Figure A-40b. Stable cells Vaccine-Infected (CB) Sample #9.

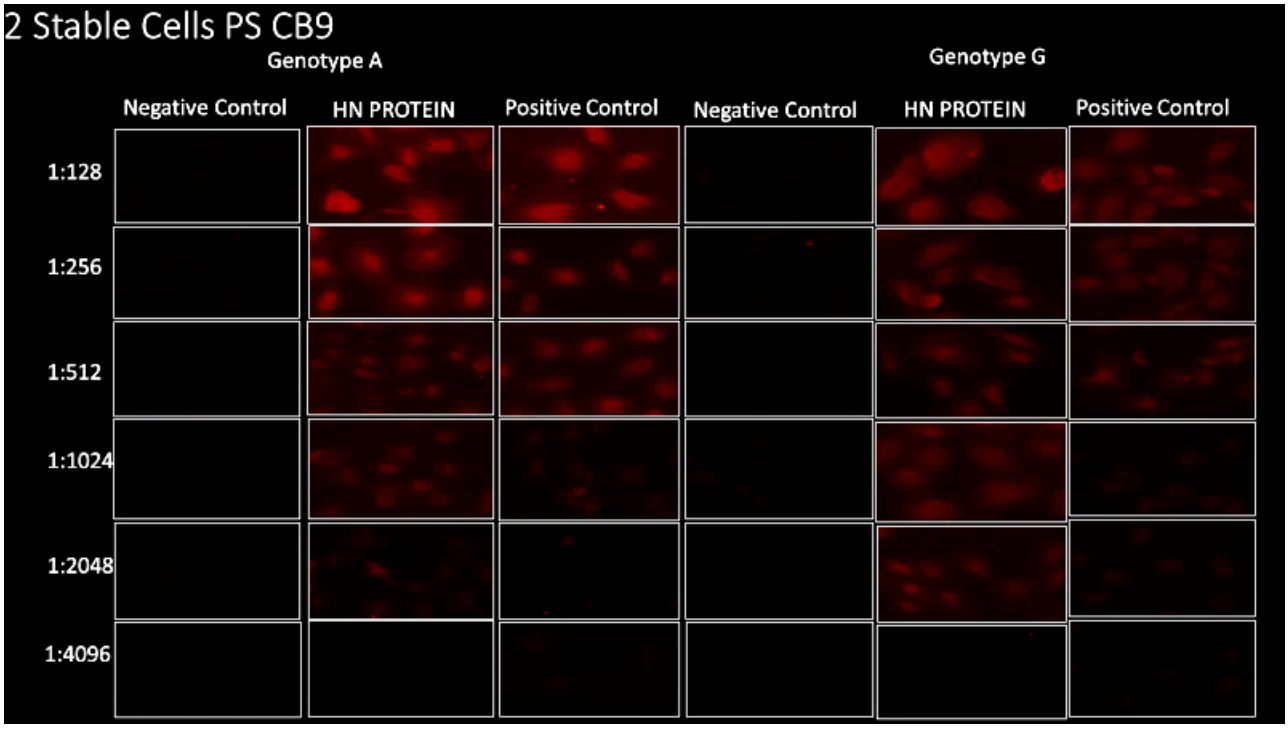


Figure A-40c. Stable cells Vaccine-Infected (CB) Sample #9.

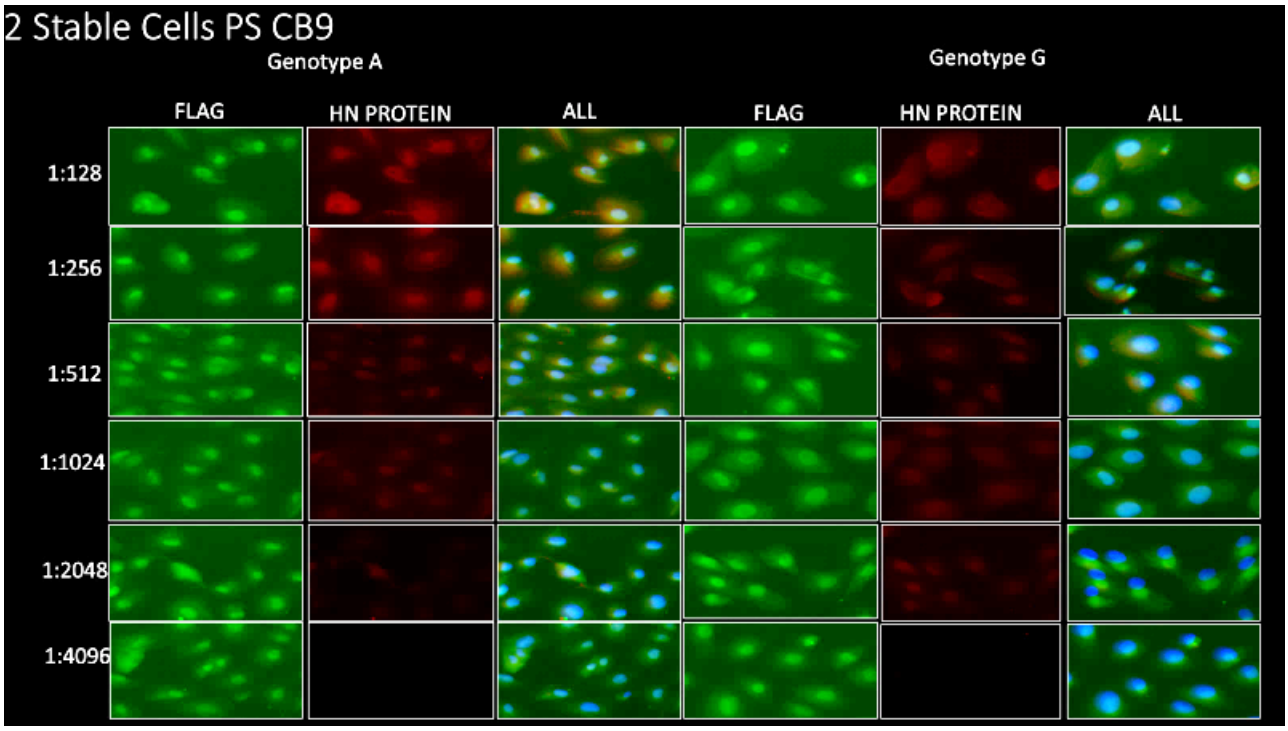


Figure A-40d. Stable cells Vaccine-Infected (CB) Sample #9.

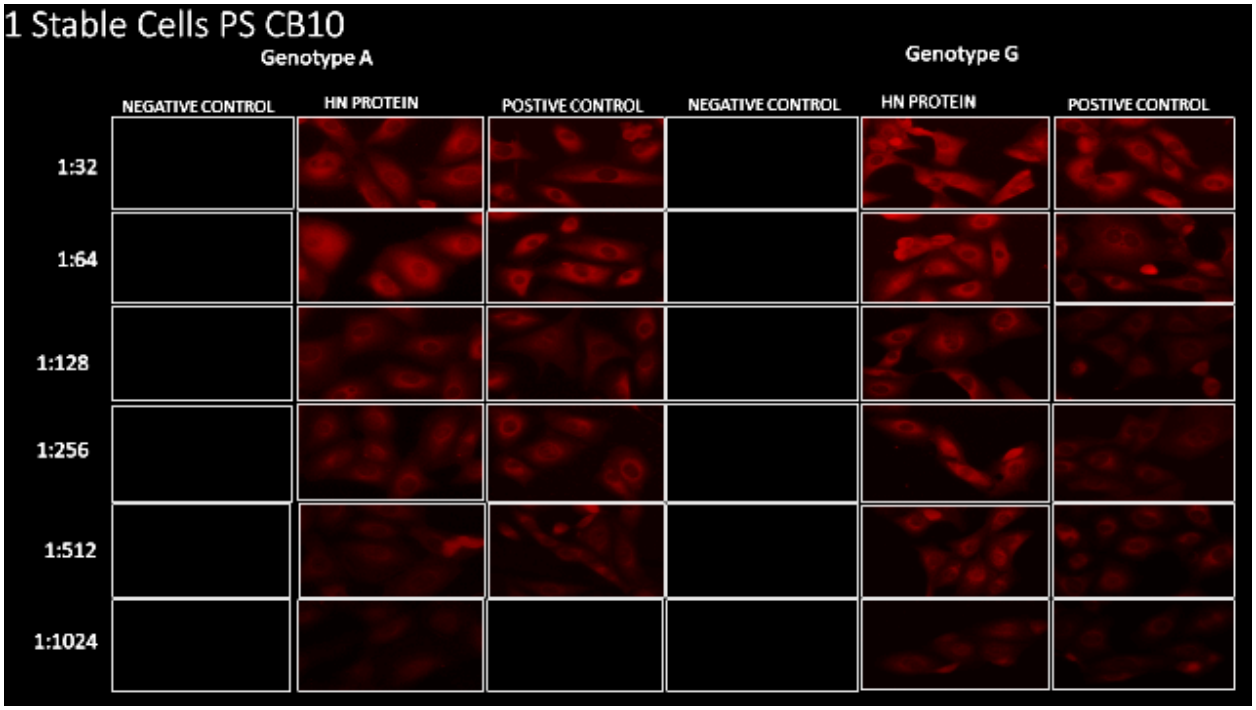


Figure A-41a. Stable cells Vaccine-Infected (CB) Sample#10.

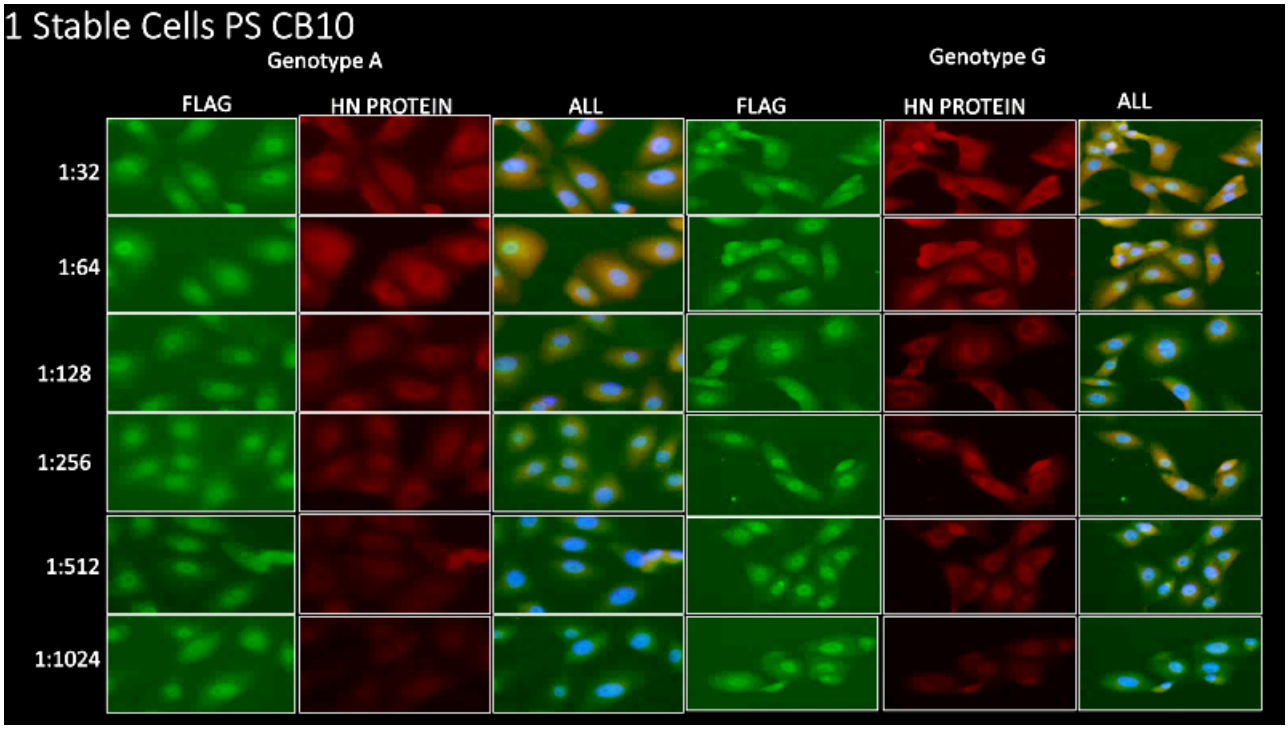


Figure A-41b. Stable cells Vaccine-Infected (CB) Sample #10.

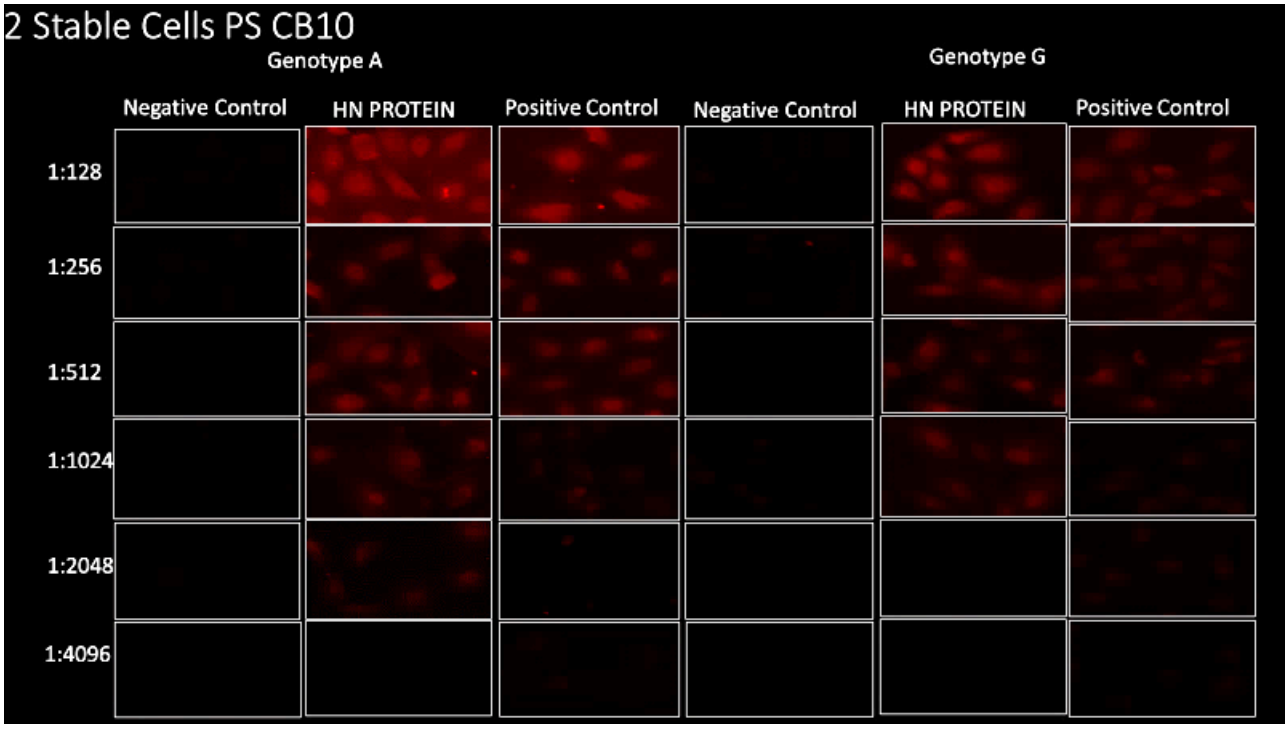


Figure A-41c. Stable cells Vaccine-Infected (CB) Sample #10.

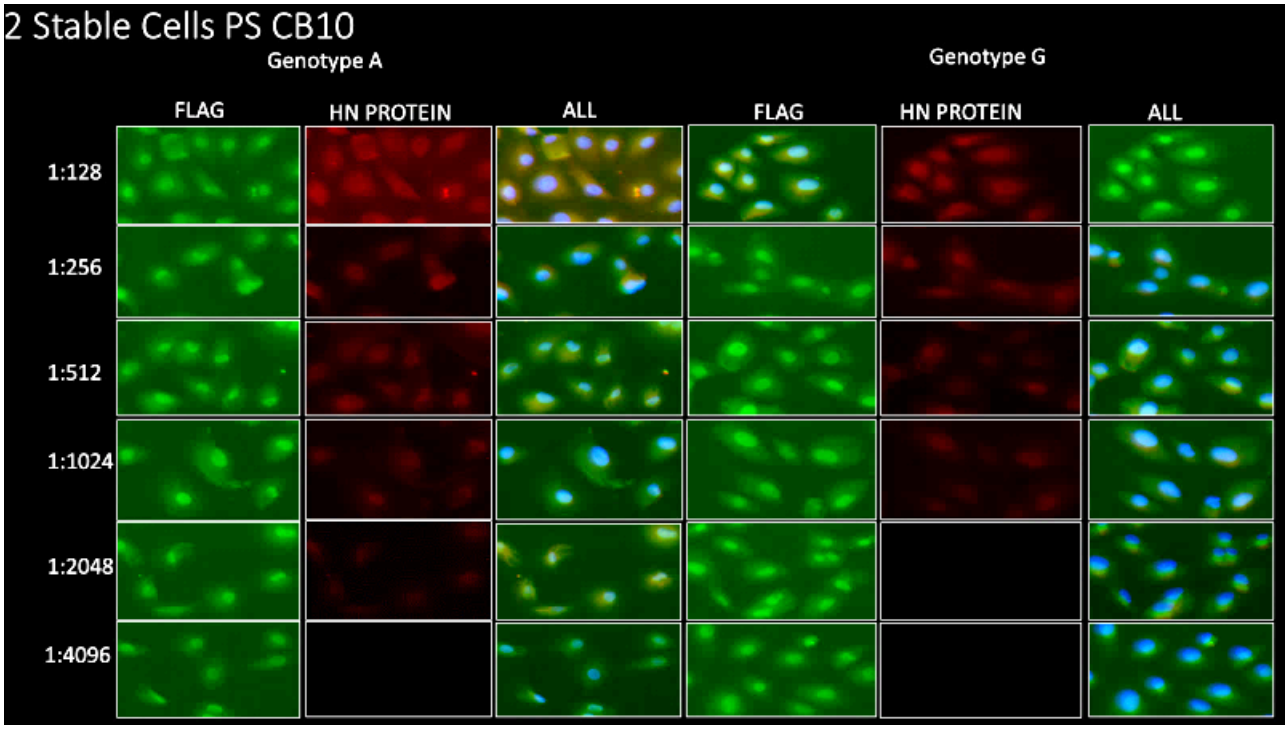


Figure A-41d. Stable cells Vaccine-Infected (CB) Sample #10.

IFA Results of F Protein

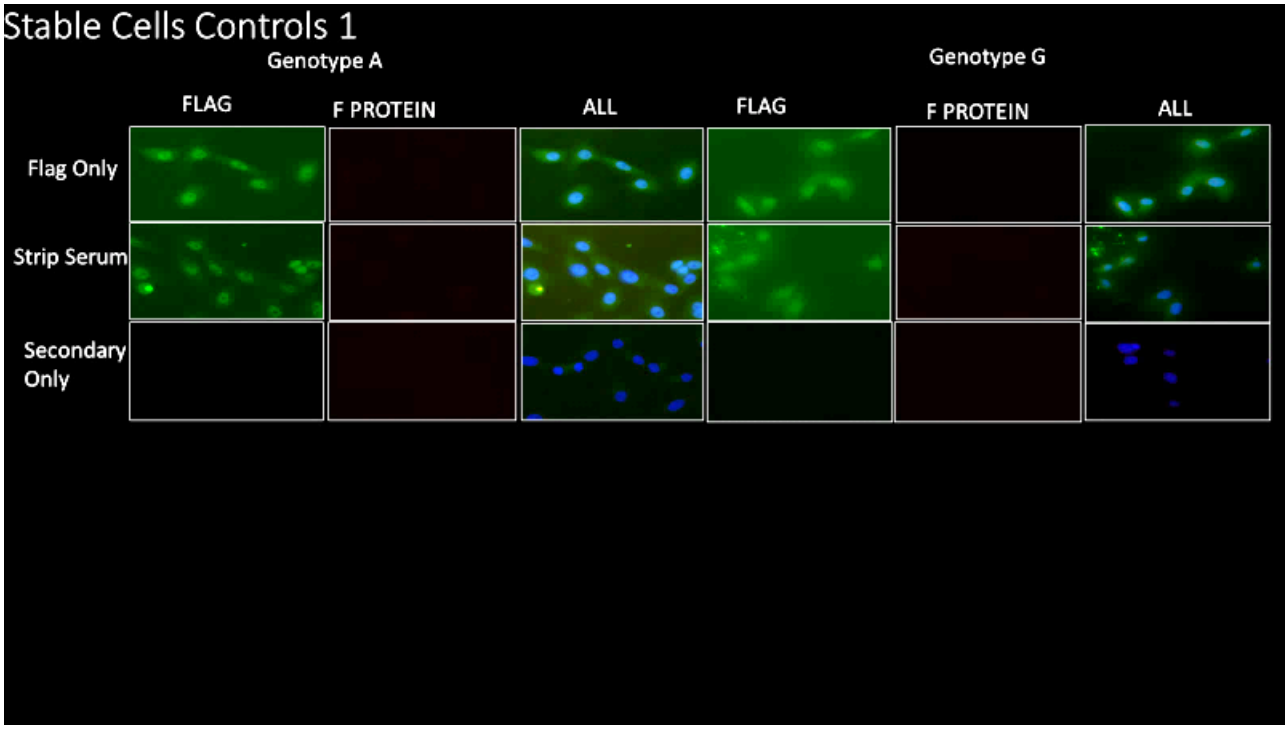


Figure A-42a. Stable cells Control Batch#1.

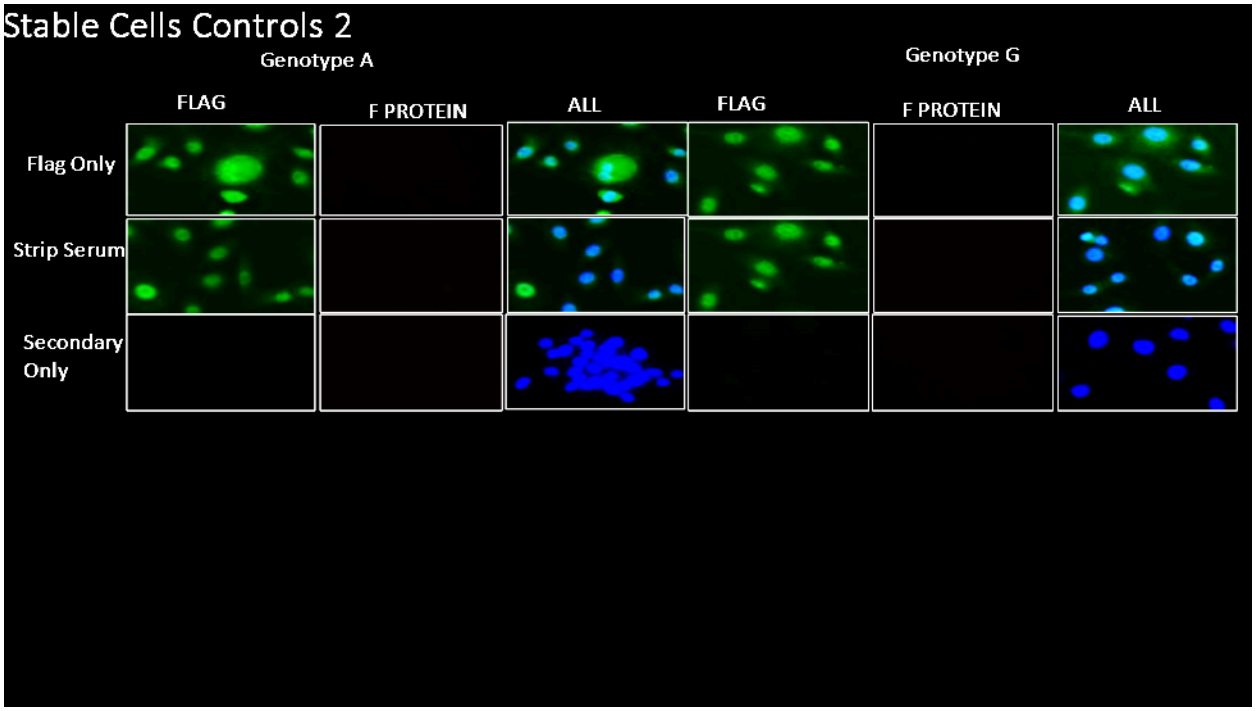


Figure A-42b. Stable cells Control Batch#2.

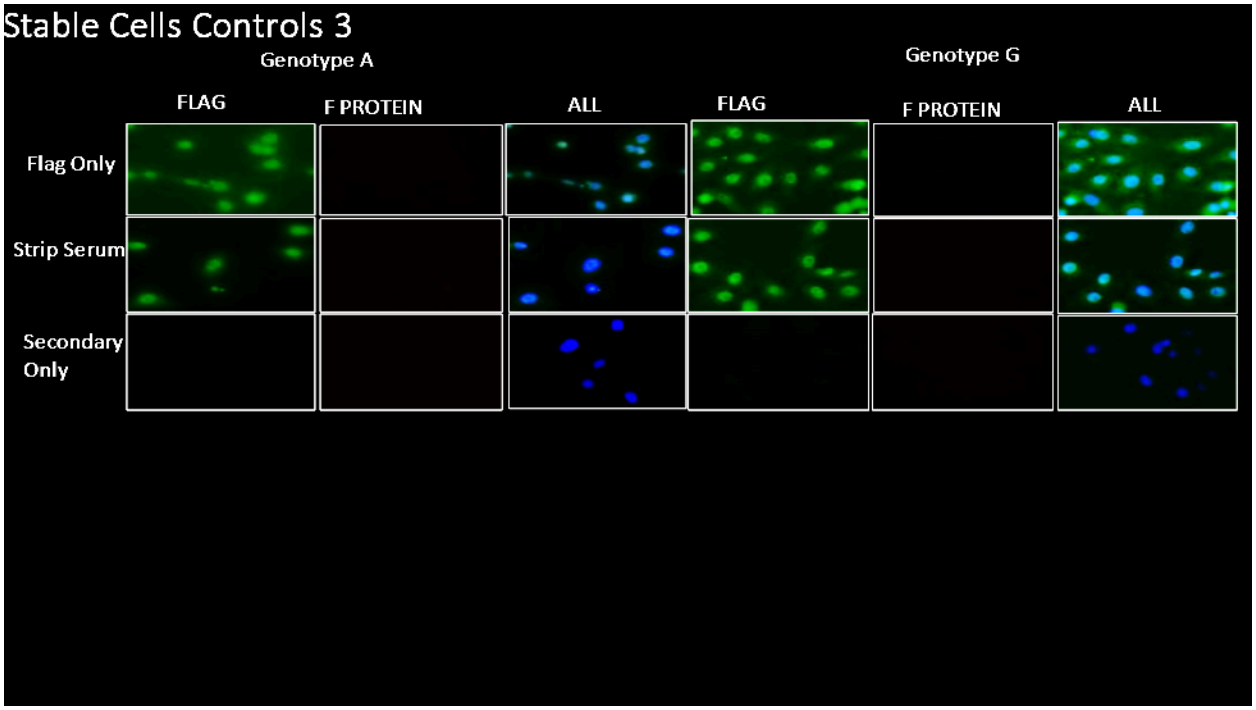


Figure A-42c. Stable cells Control Batch#3.

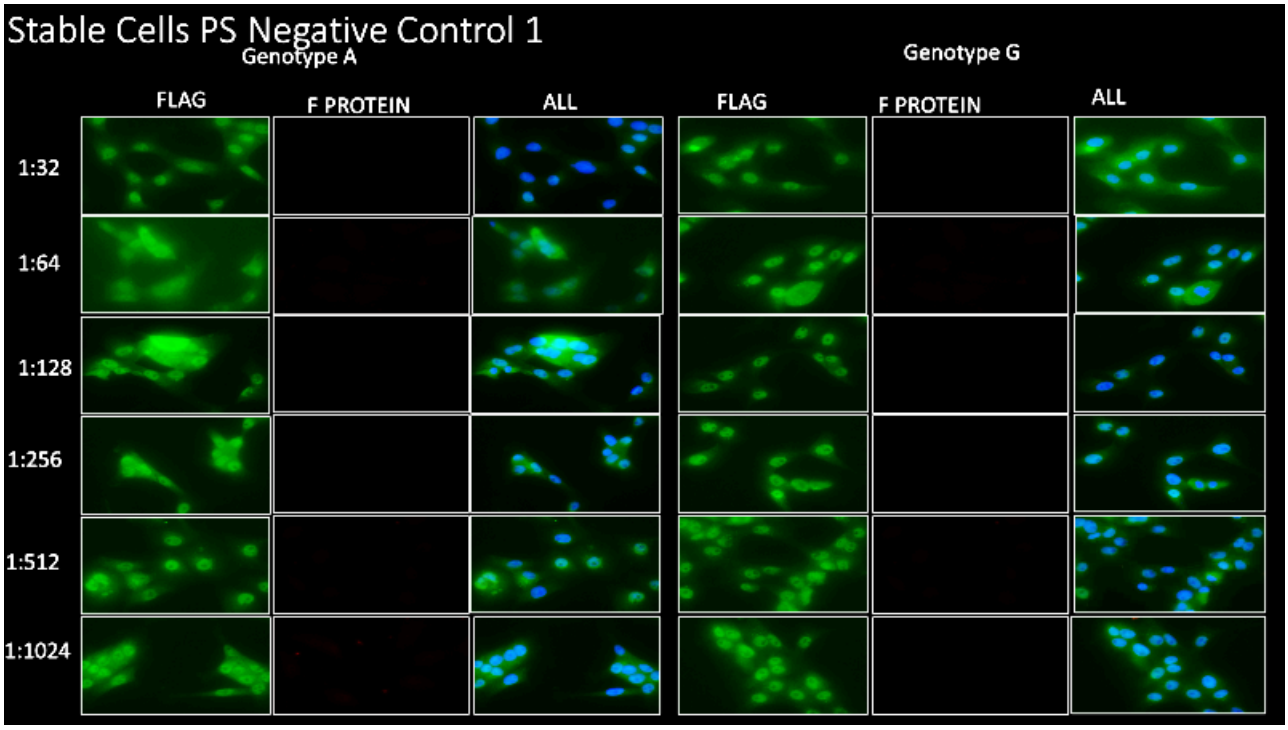


Figure A-43a. Stable cells Negative Control Batch#1.

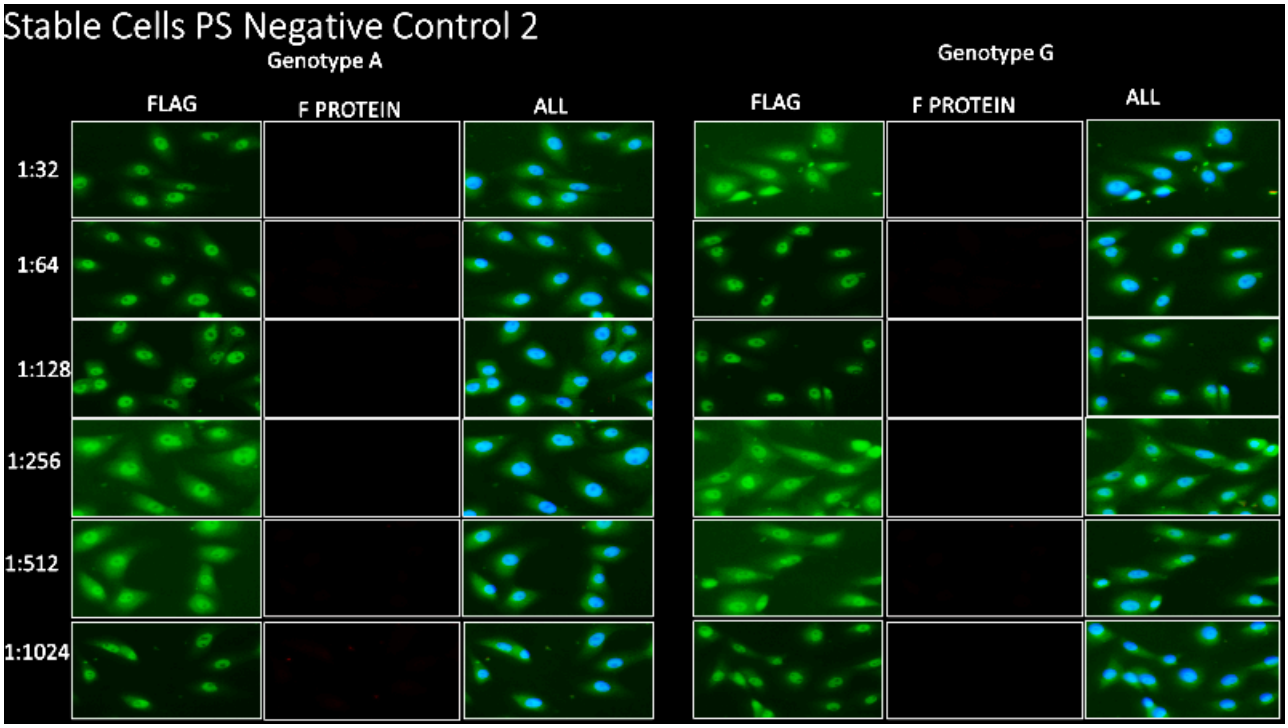


Figure A-43b. Stable cells Negative Control Batch#2.

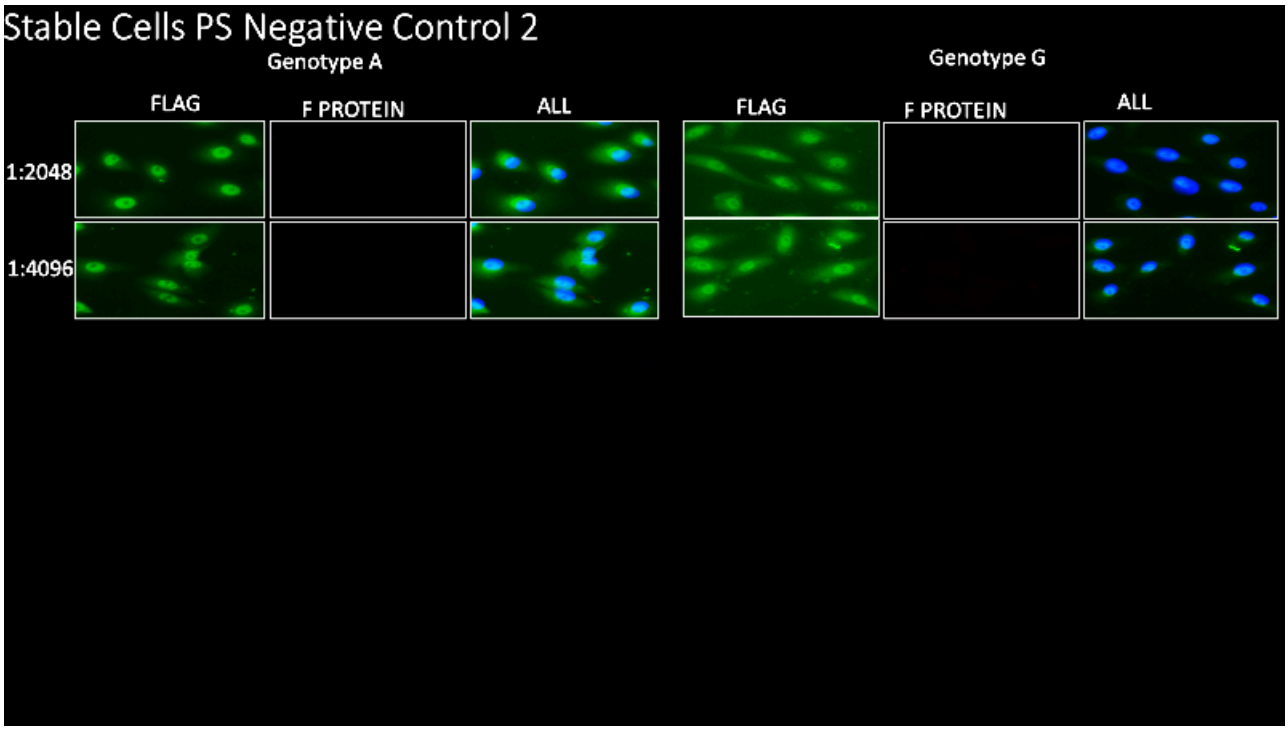


Figure A-43c. Stable cells Negative Control Batch#2.

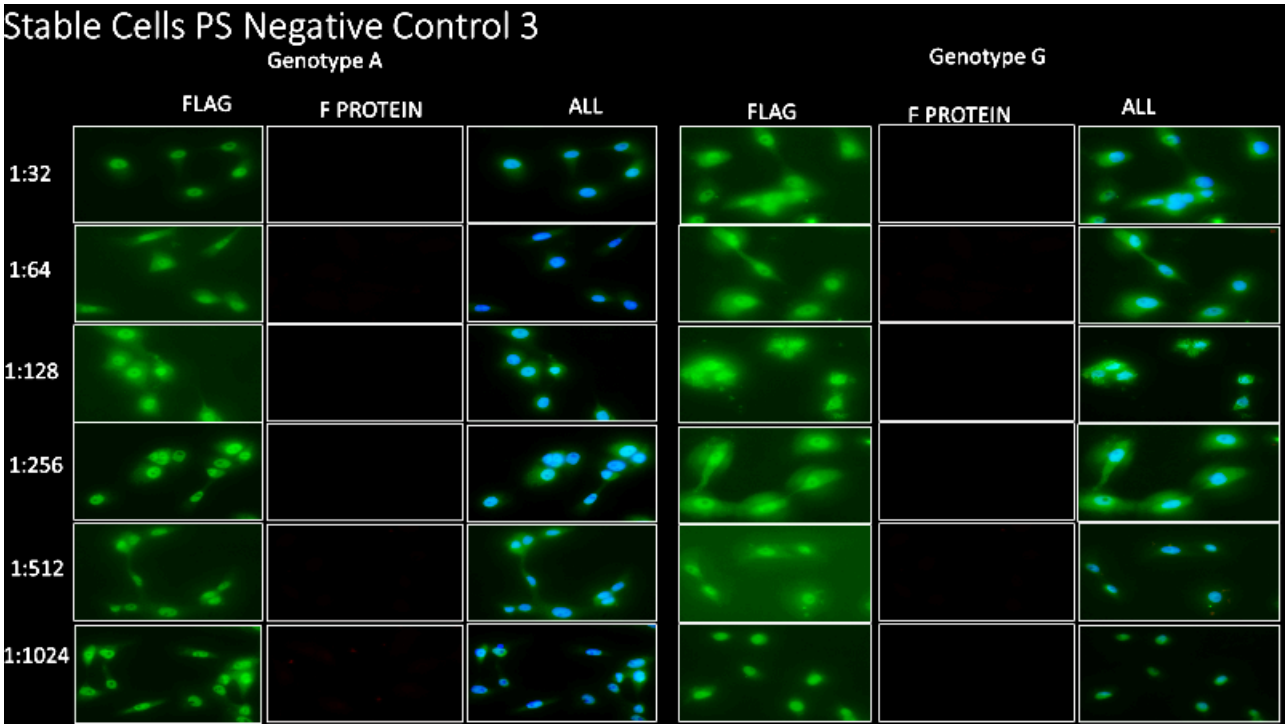


Figure A-43d. Stable cells Negative Control Batch#3.

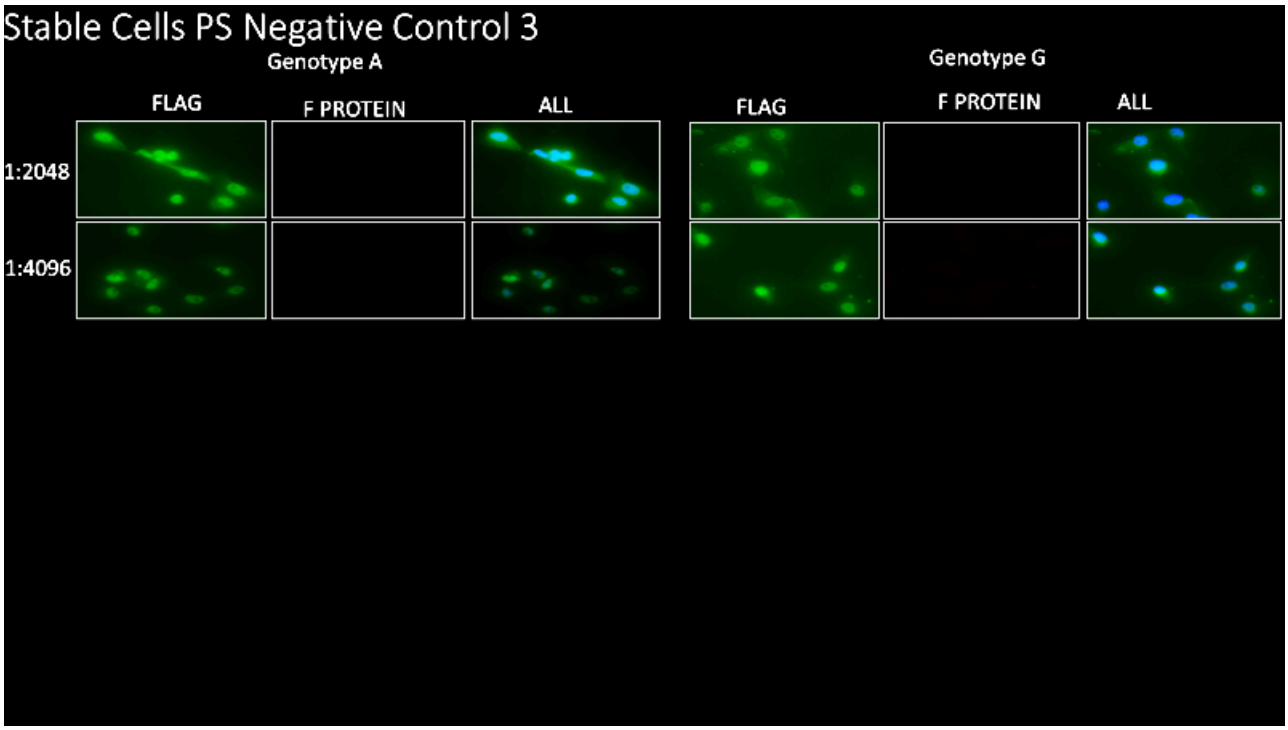


Figure A-43e. Stable cells Negative Control Batch#3.

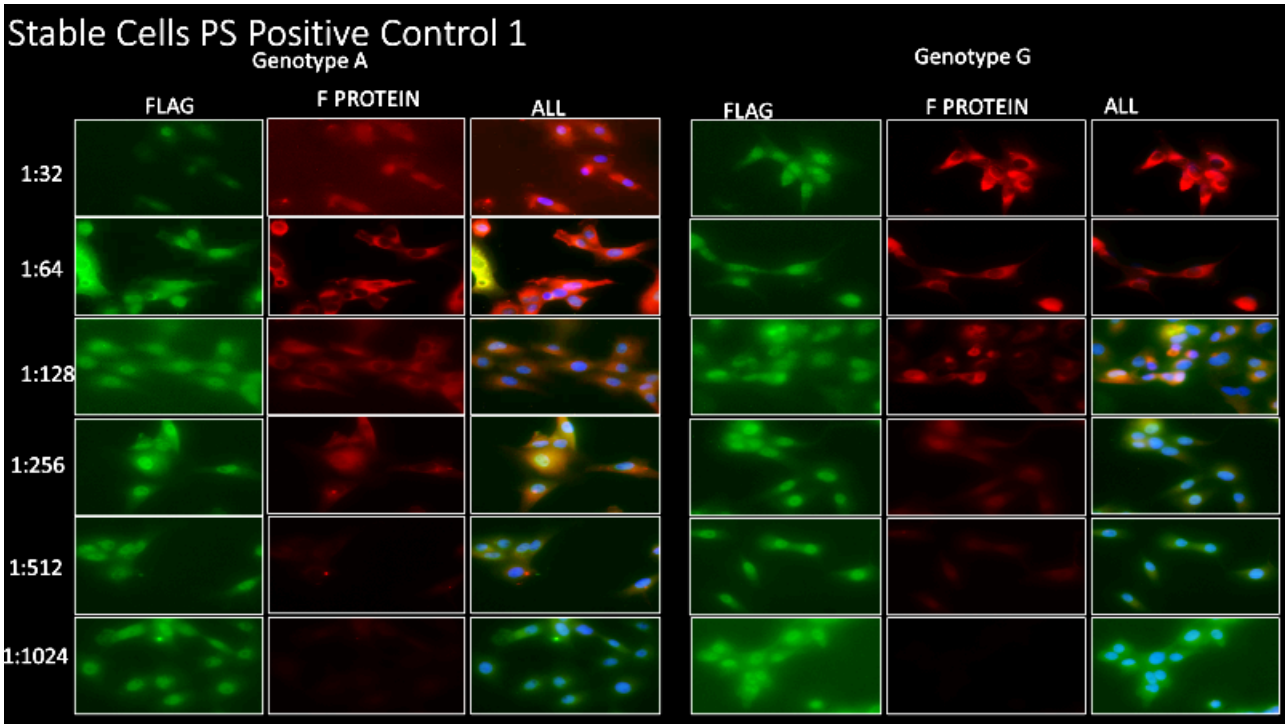


Figure A-44a. Stable cells Positive Control Batch#1.

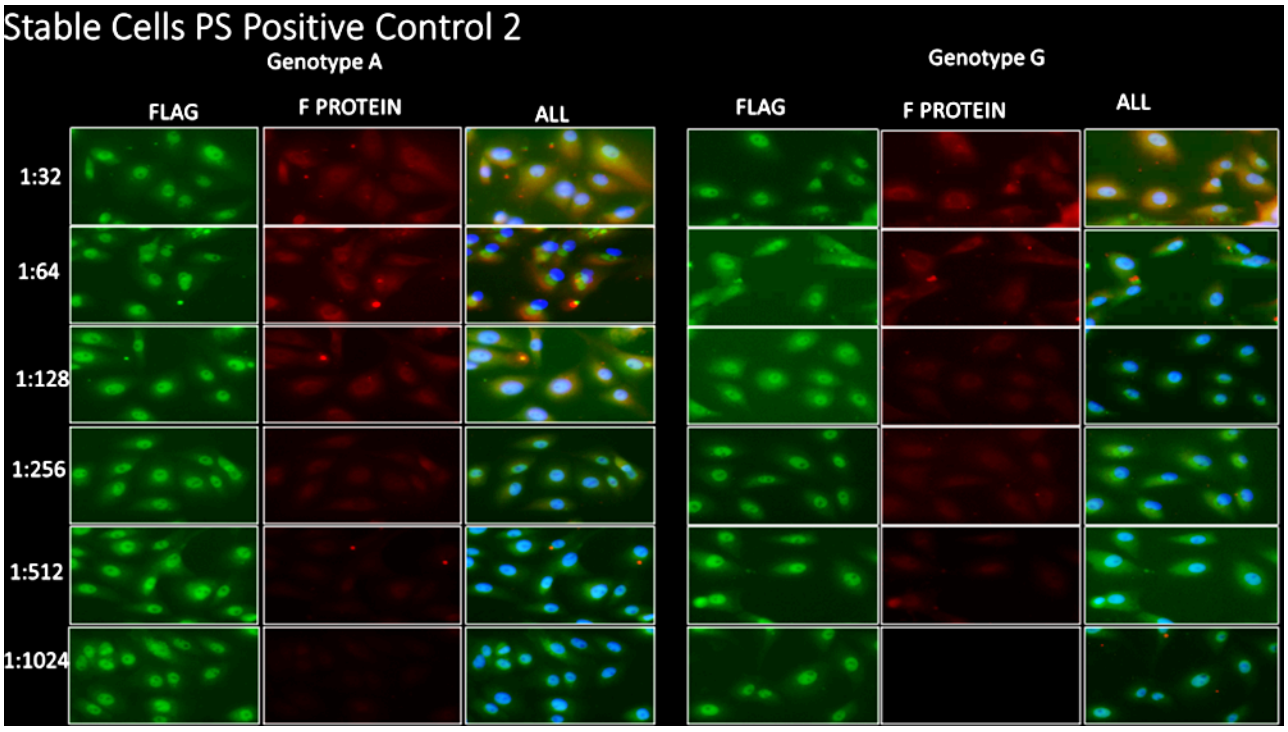


Figure A-44b. Stable cells Positive Control Batch#2.

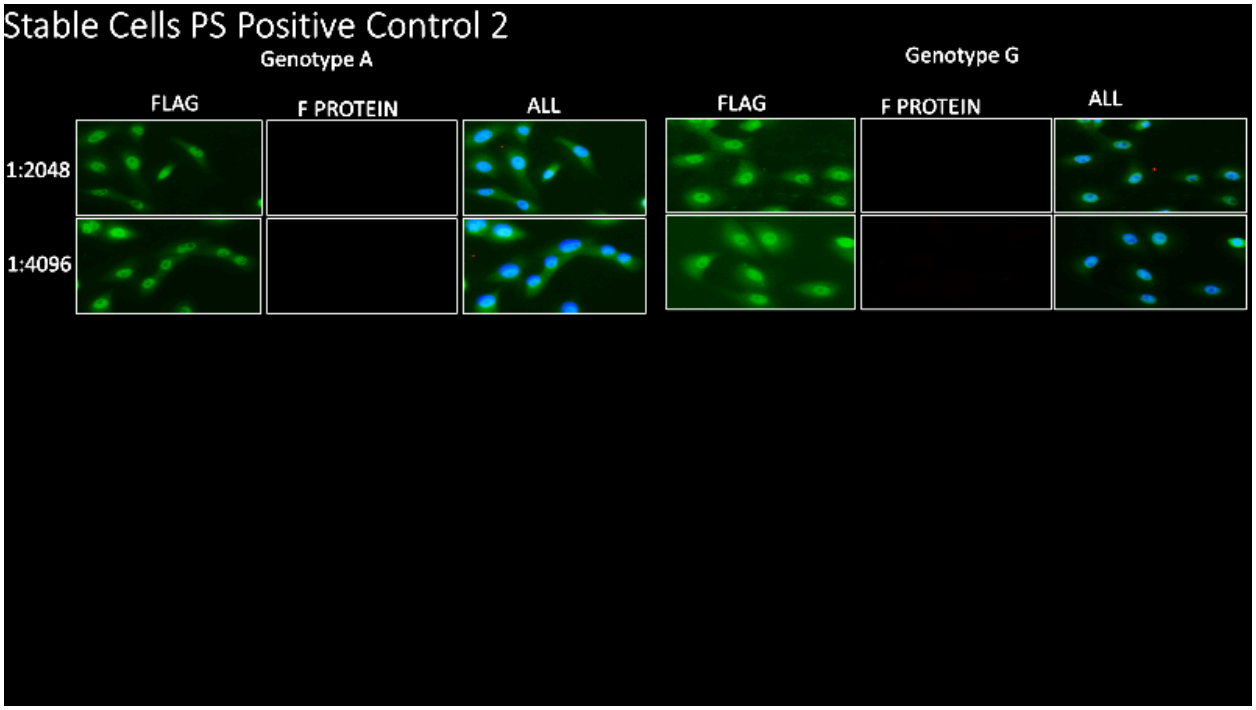


Figure A-44c. Stable cells Positive Control Batch#2.

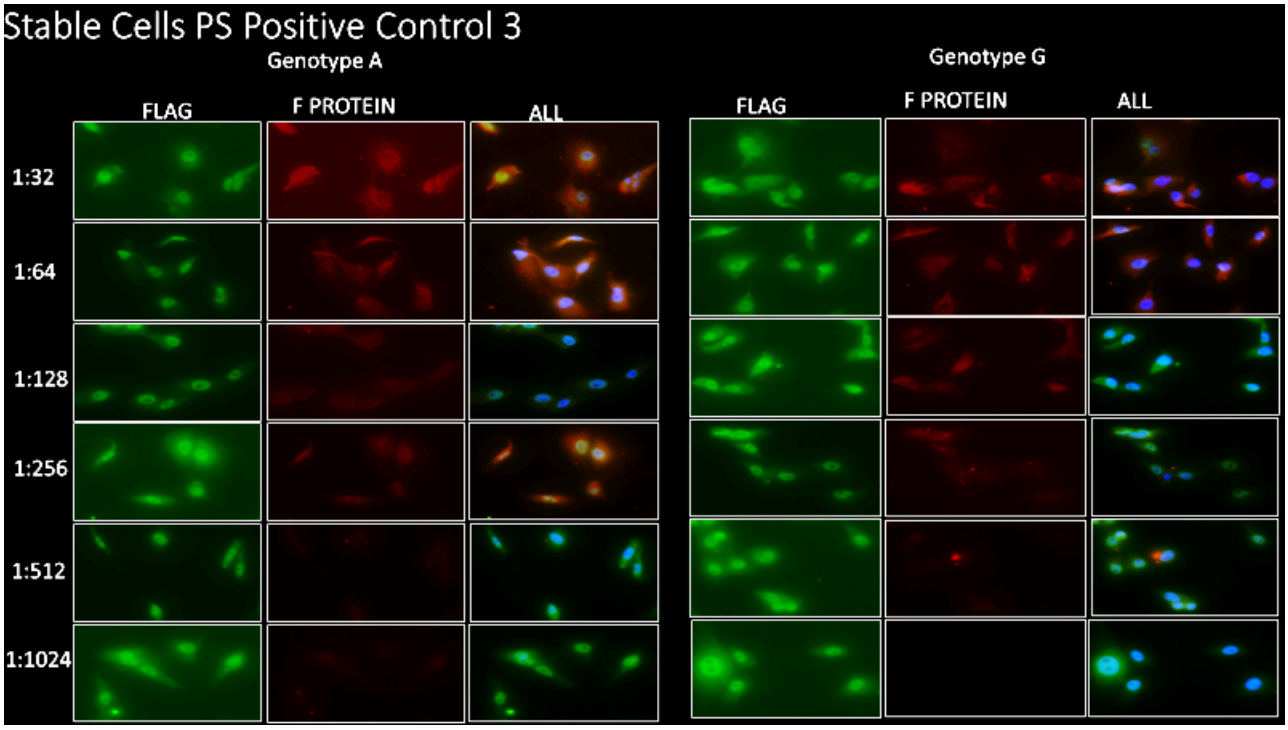


Figure A-44d. Stable cells Positive Control Batch#3.

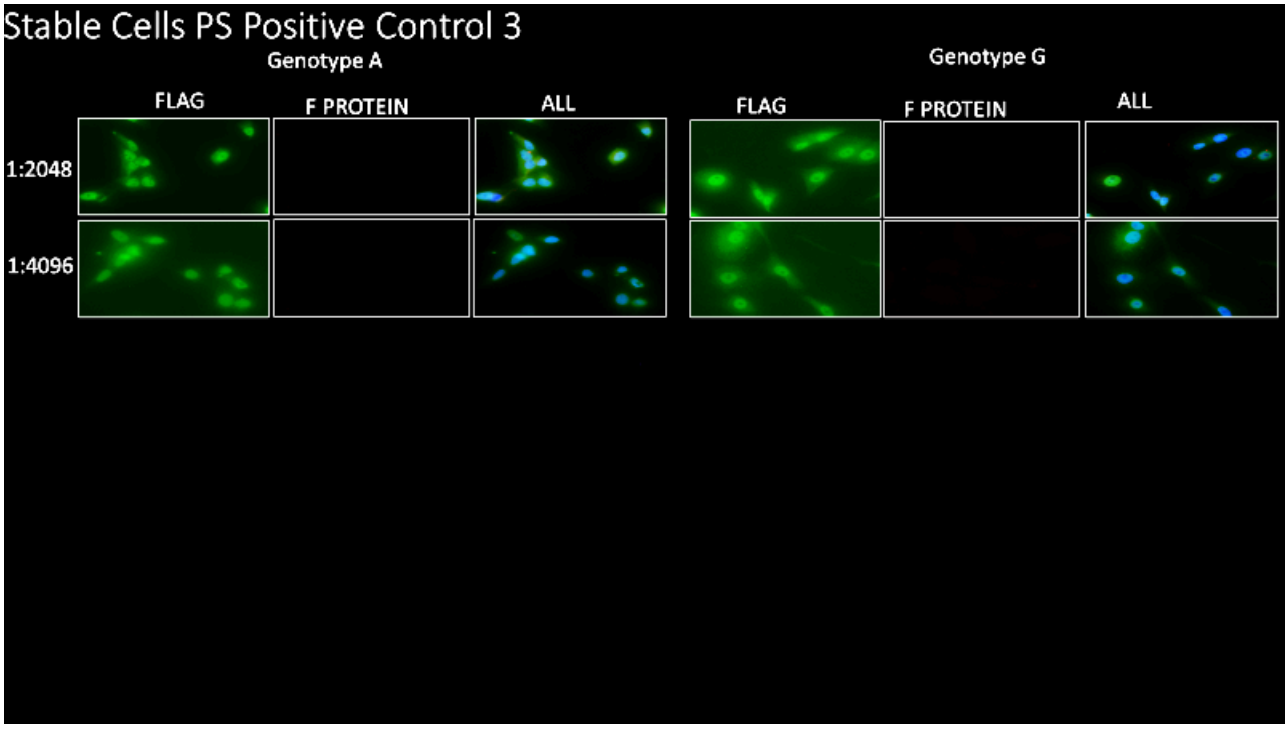


Figure A-44e. Stable cells Positive Control Batch#3.

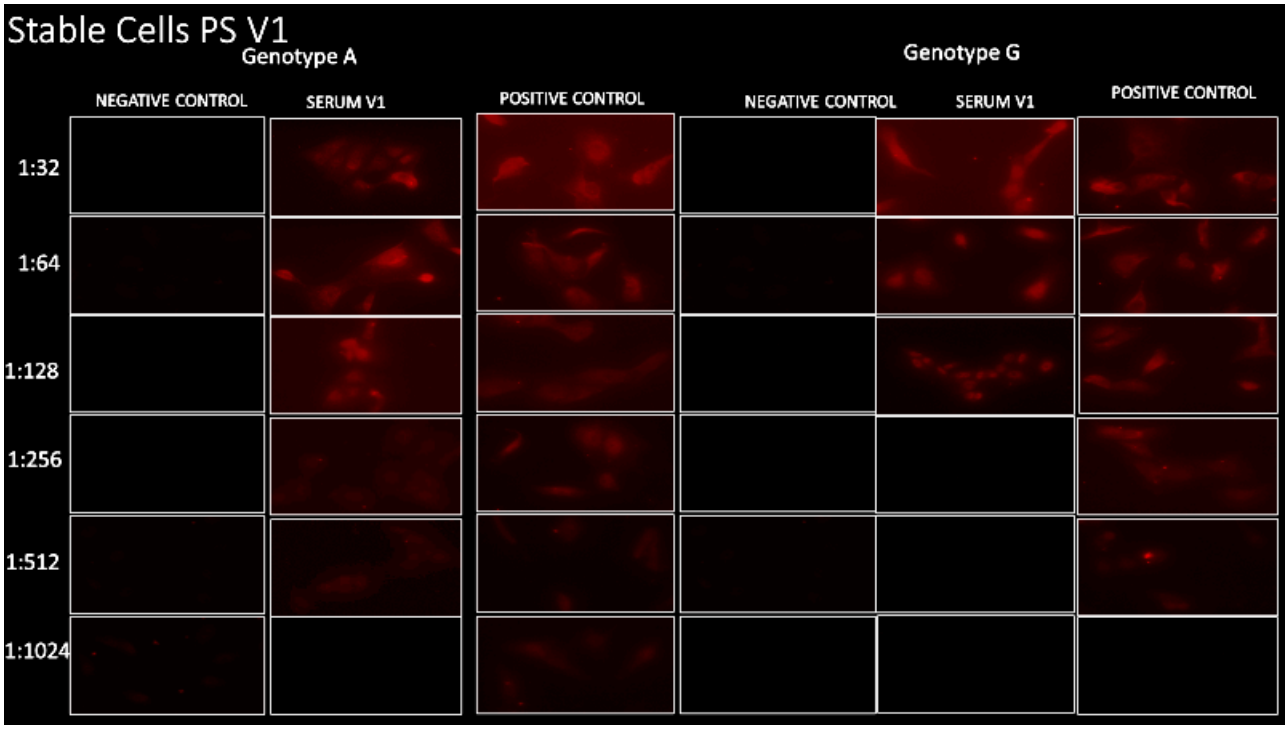


Figure A-45a. Stable cells Vaccine Sample#1.

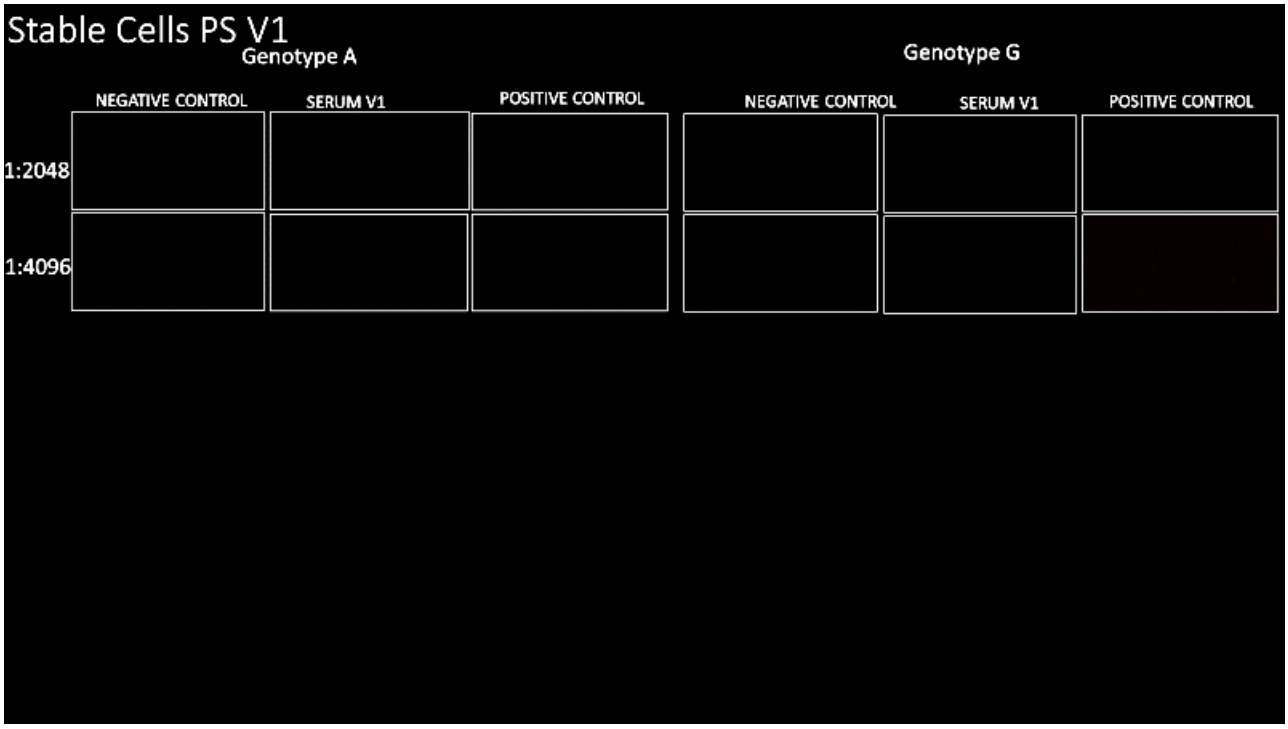


Figure A-45b. Stable cells Vaccine Sample#1.

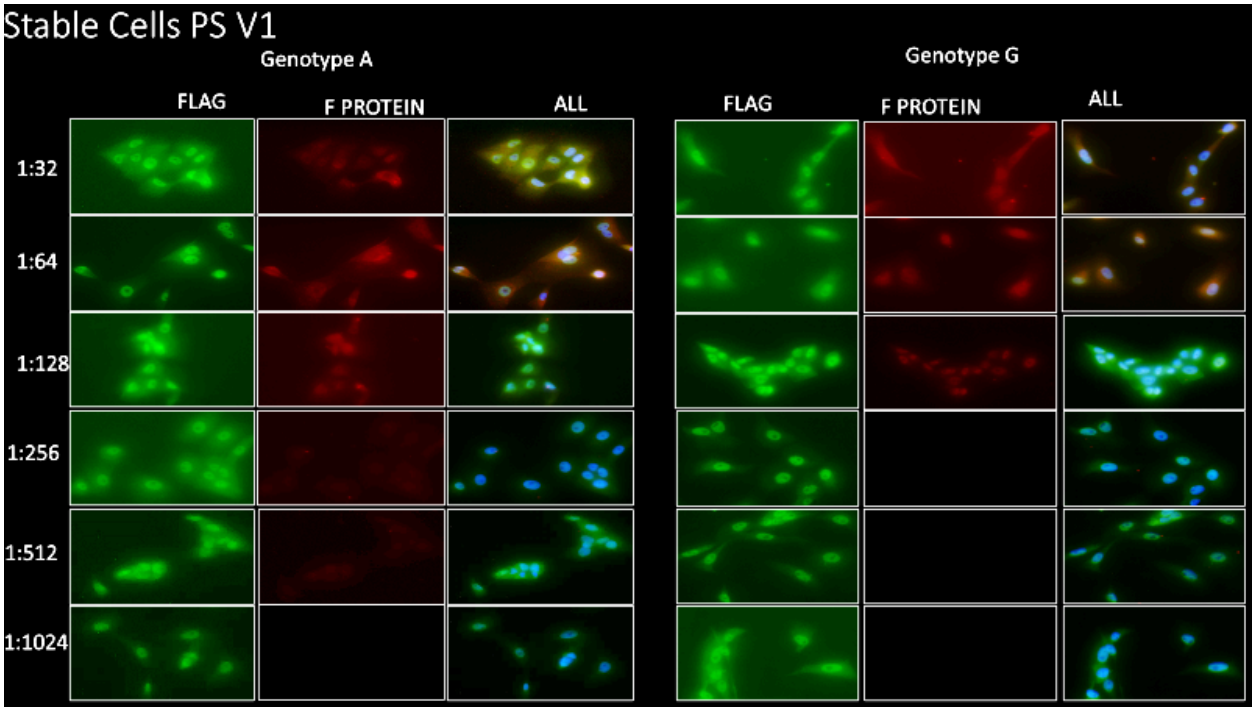


Figure A-45c. Stable cells Vaccine Sample#1.

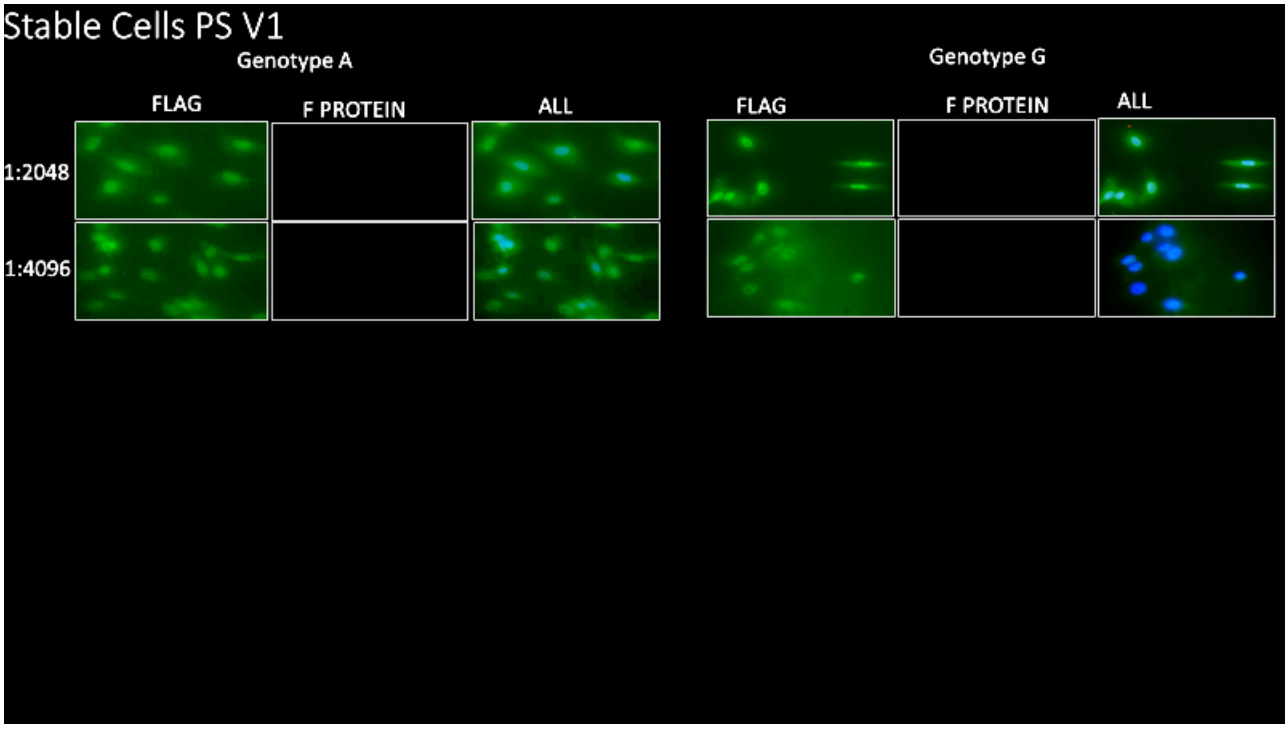


Figure A-45d. Stable cells Vaccine Sample#1.

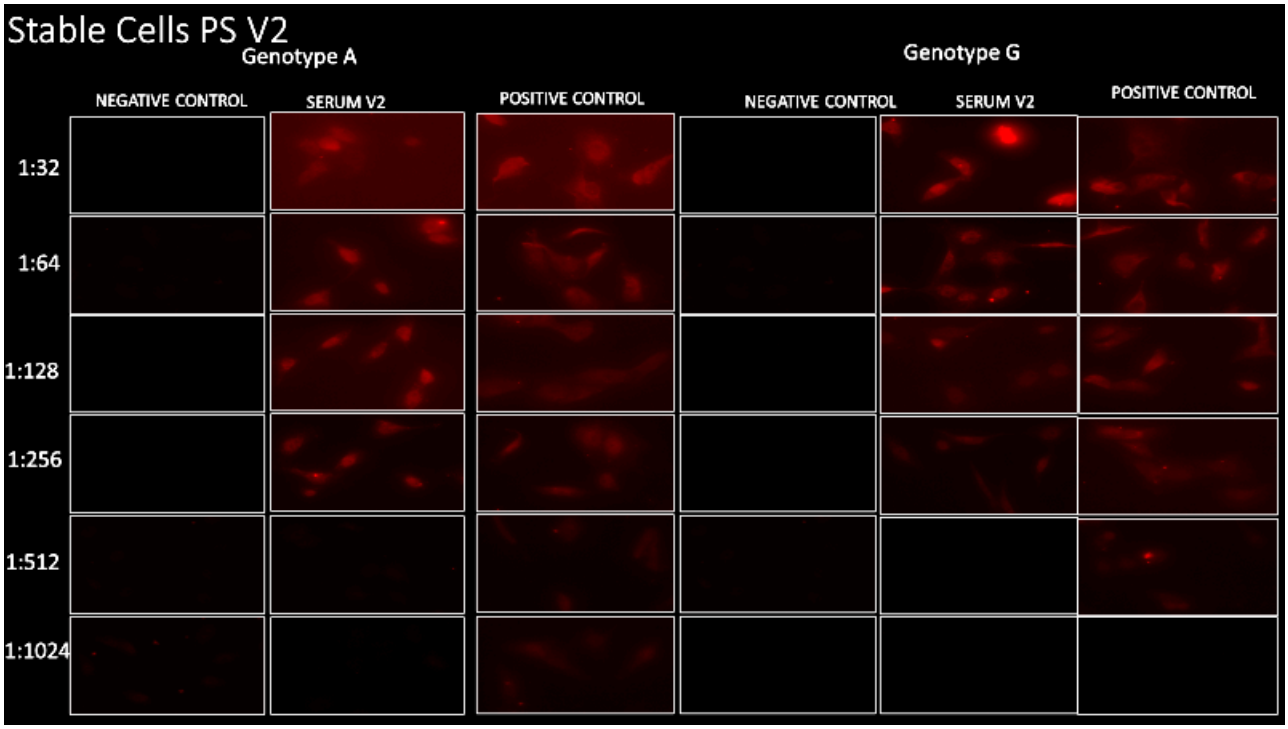


Figure A-46a. Stable cells Vaccine Sample#2.

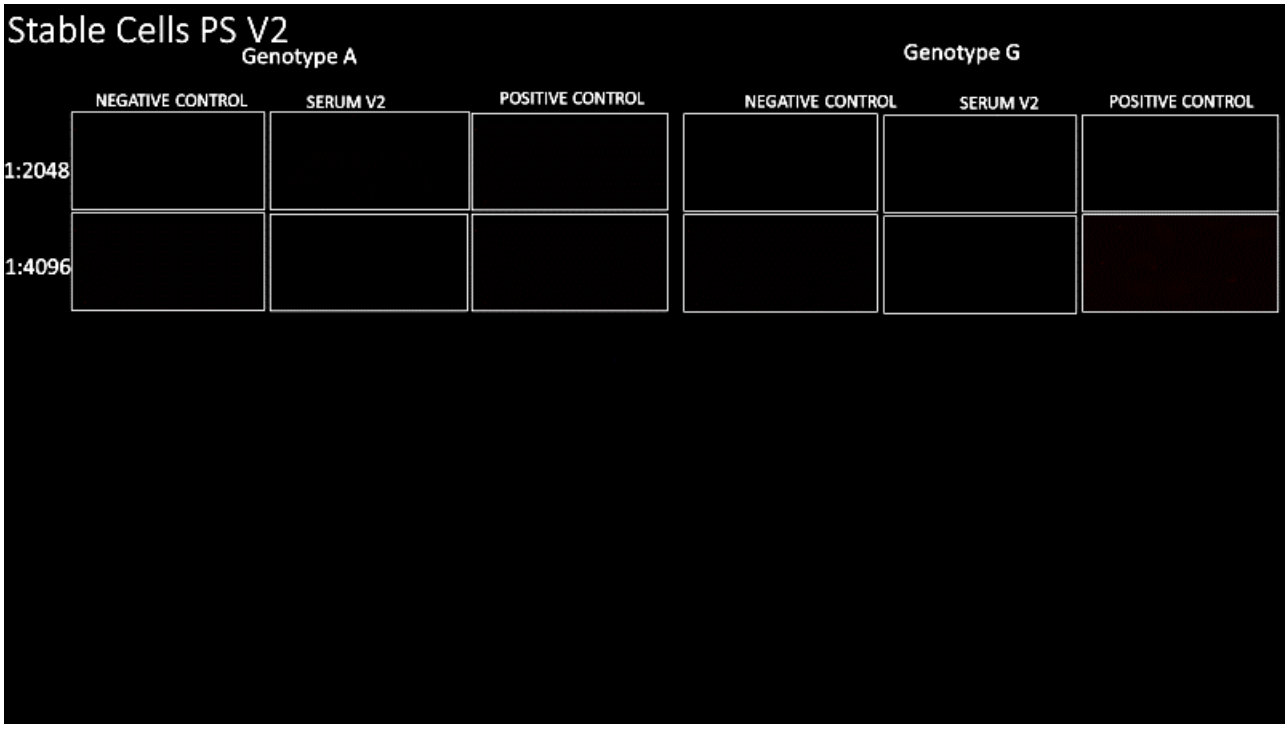


Figure A-46b. Stable cells Vaccine Sample#2.

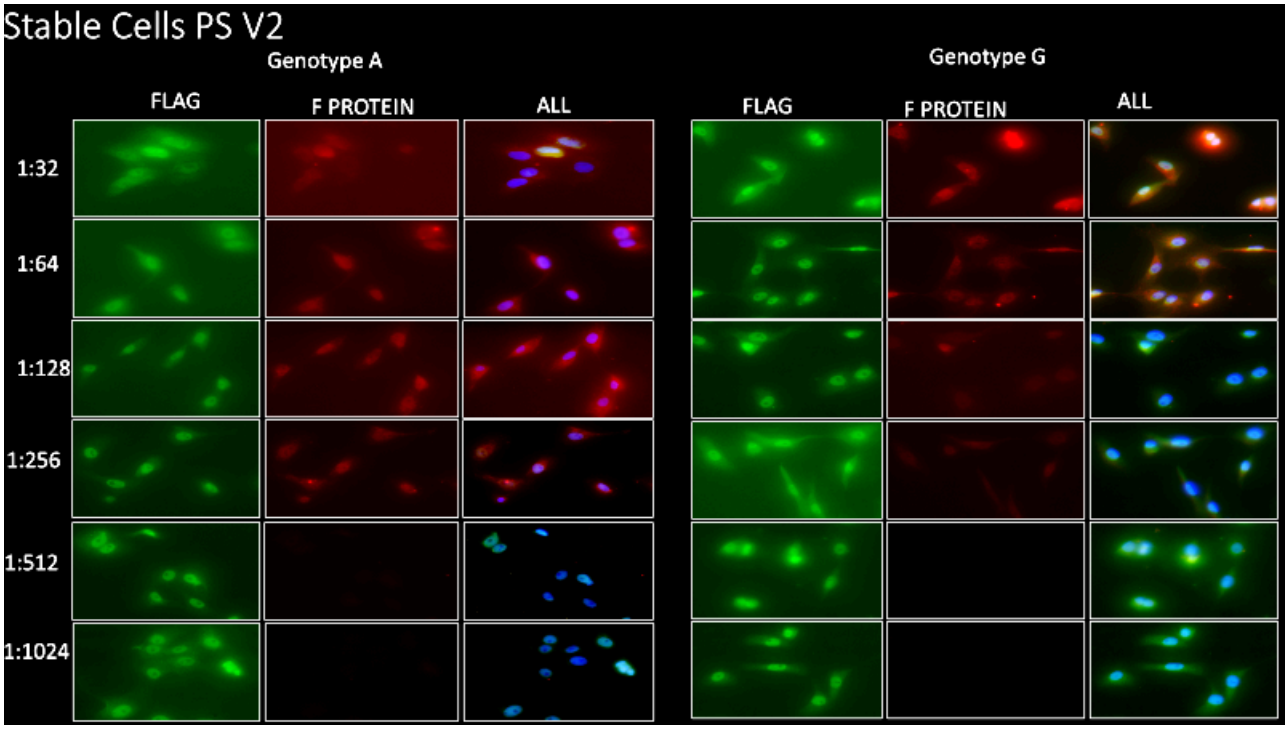


Figure A-46c. Stable cells Vaccine Sample#2.

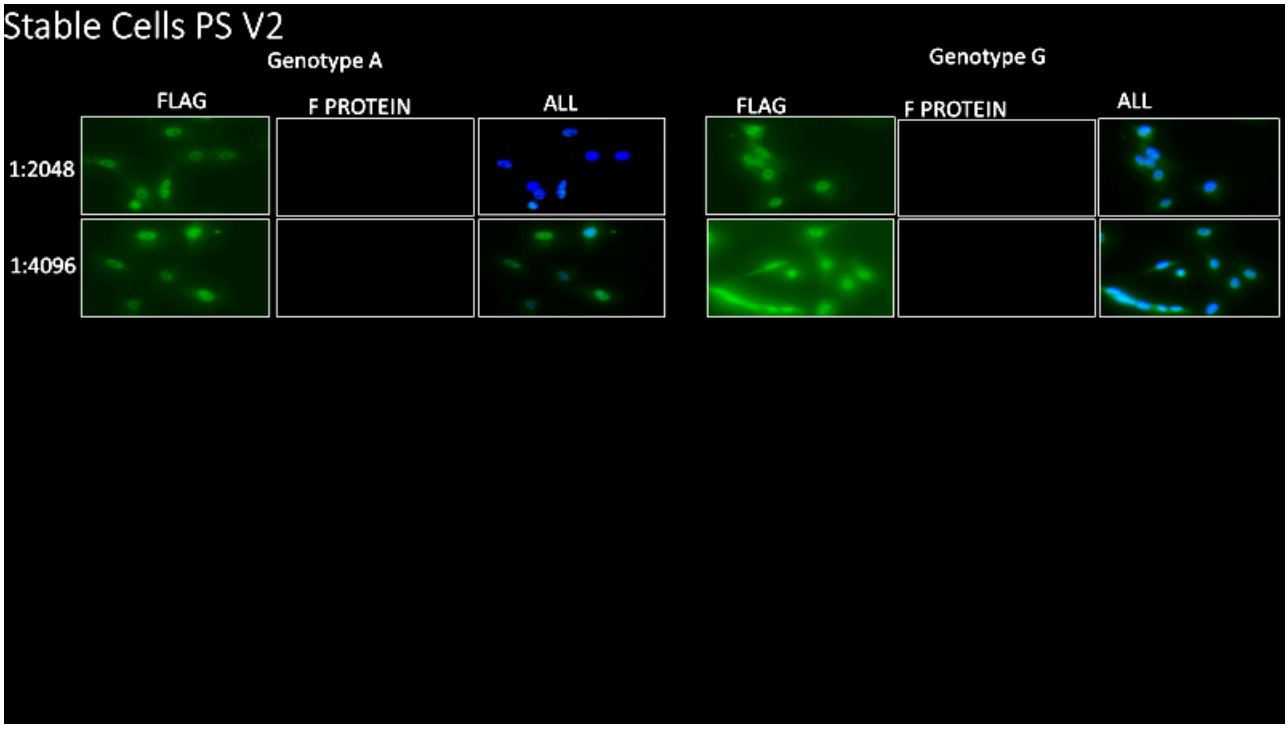


Figure A-46d. Stable cells Vaccine Sample#2.

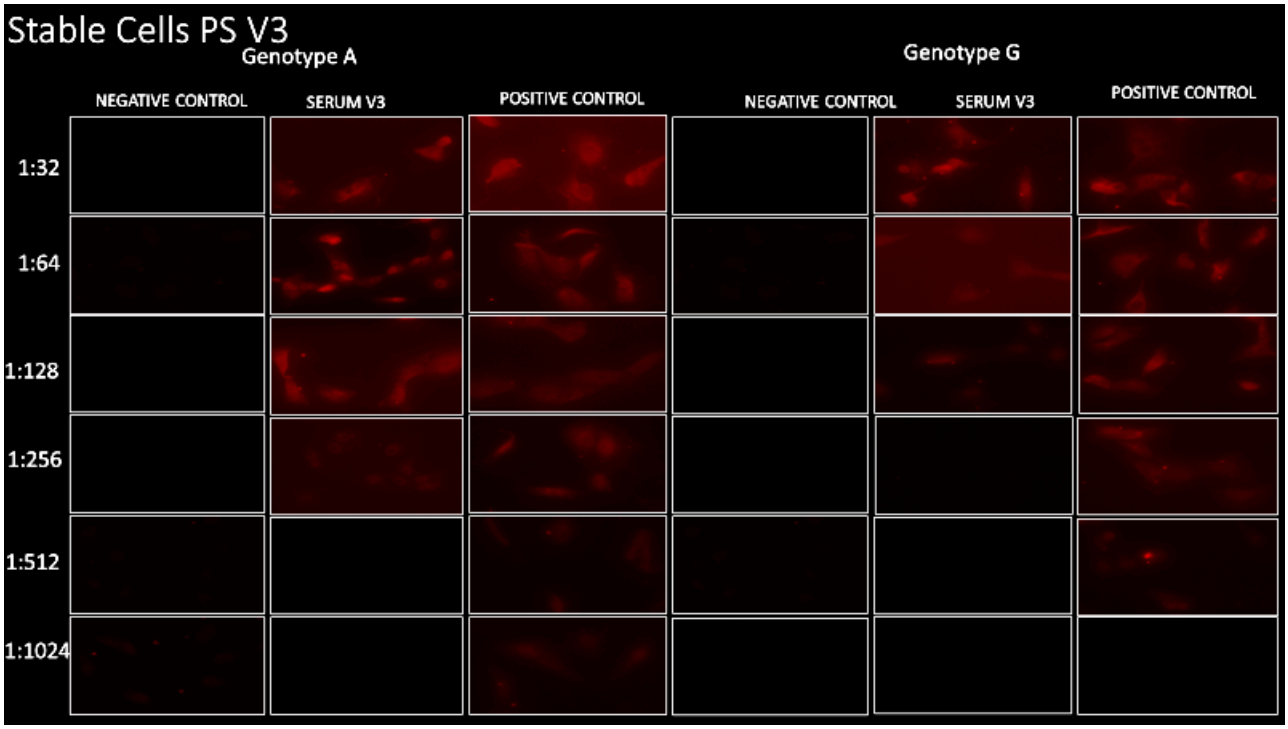


Figure A-47a. Stable cells Vaccine Sample#3.

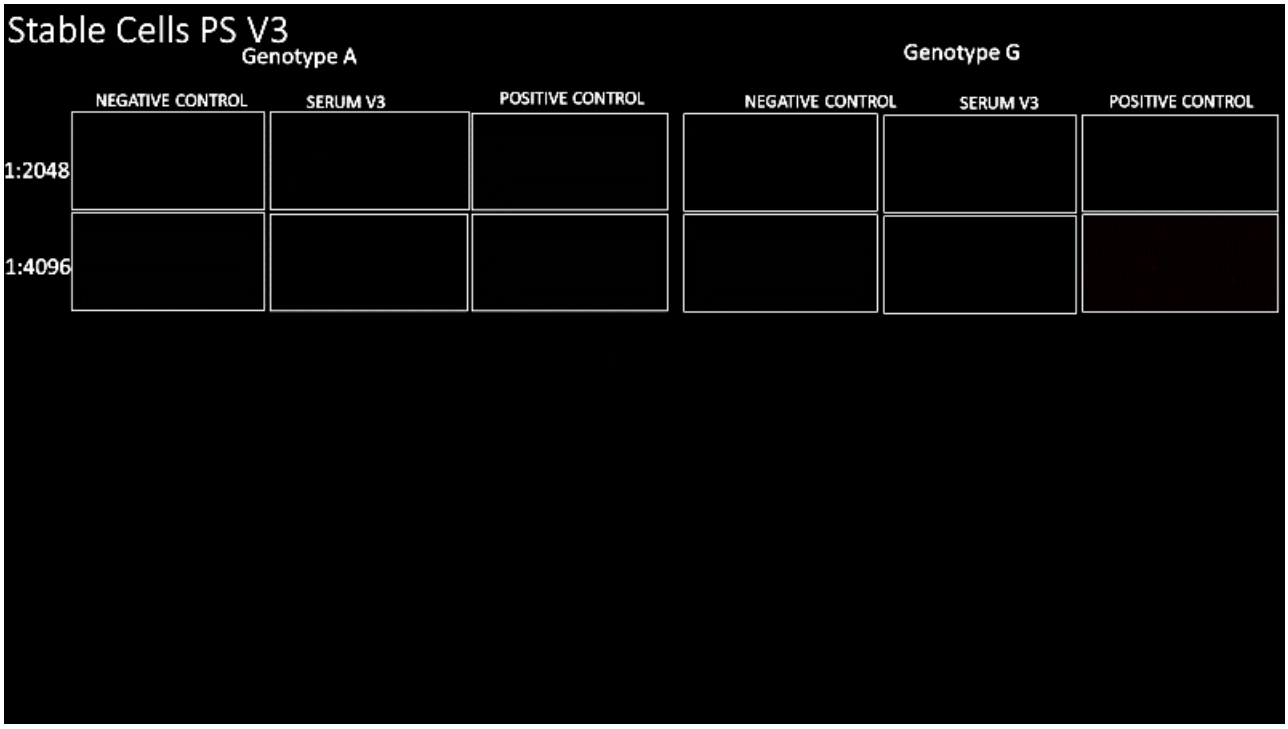


Figure A-47b. Stable cells Vaccine Sample#3.

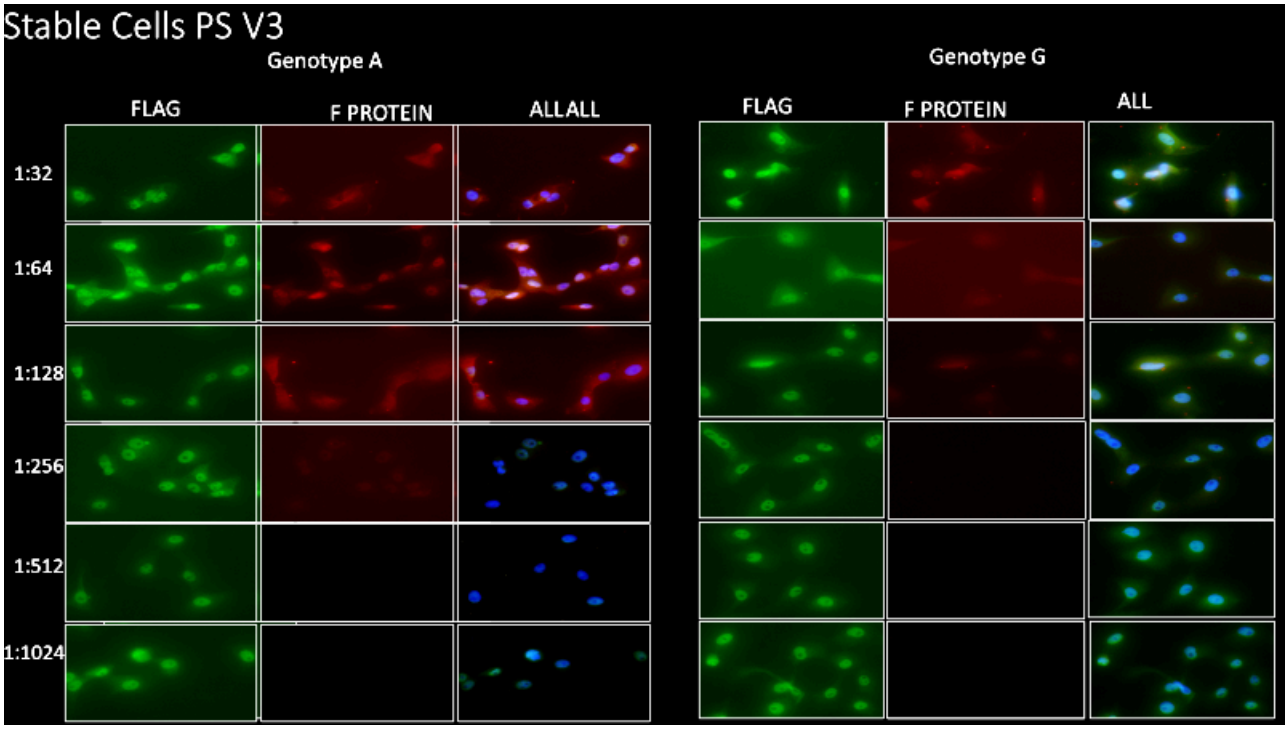


Figure A-47c. Stable cells Vaccine Sample#3.

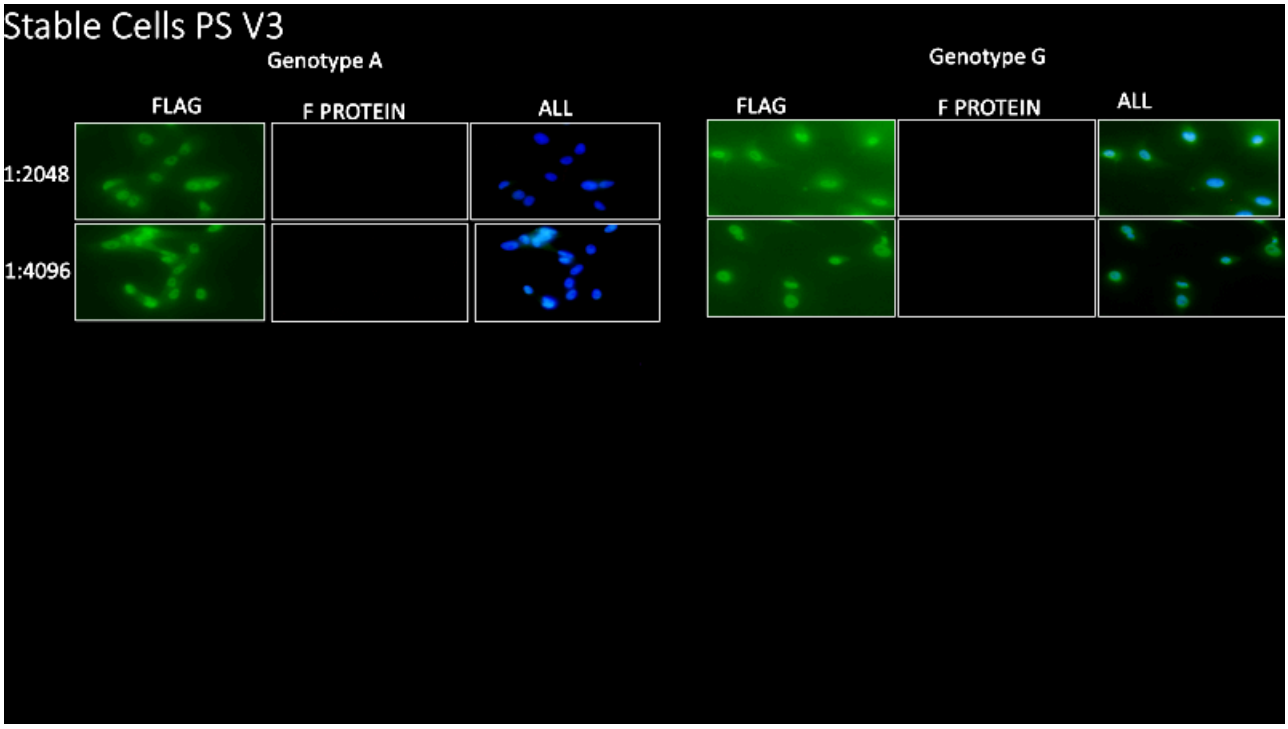


Figure A-47d. Stable cells Vaccine Sample#3.

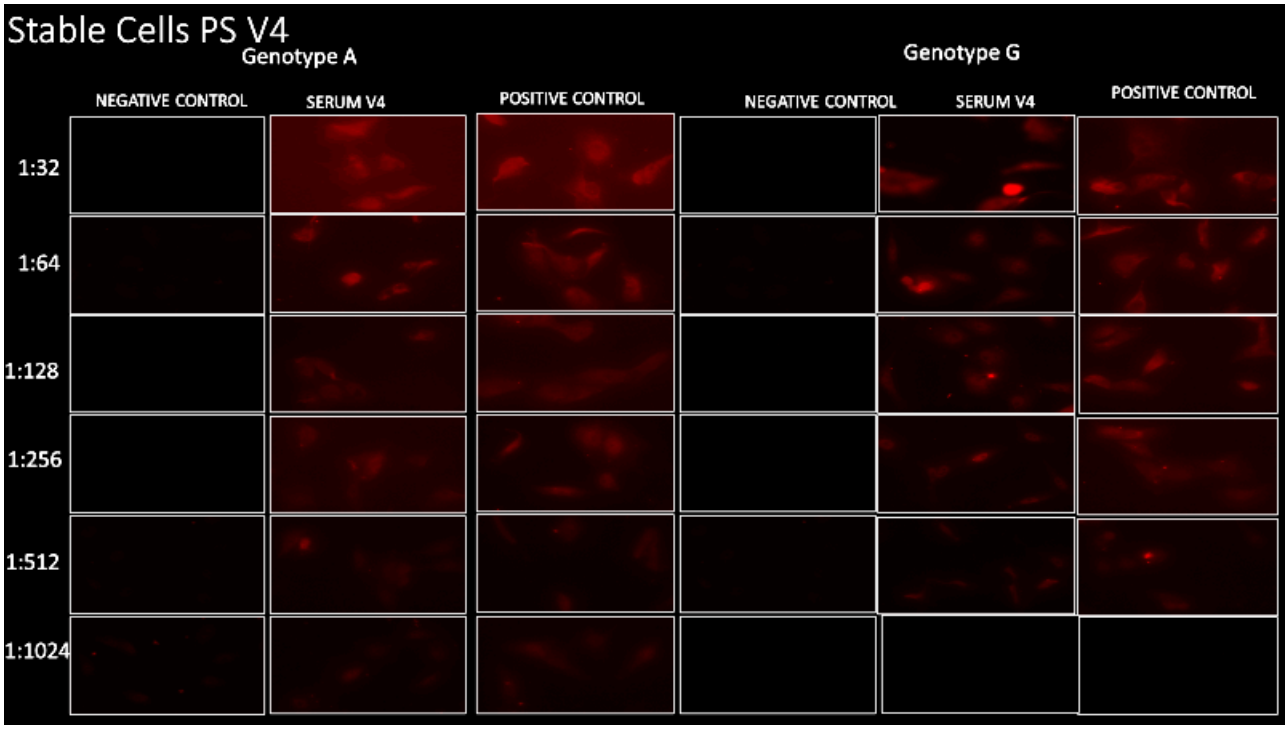


Figure A-48a. Stable cells Vaccine Sample#4.

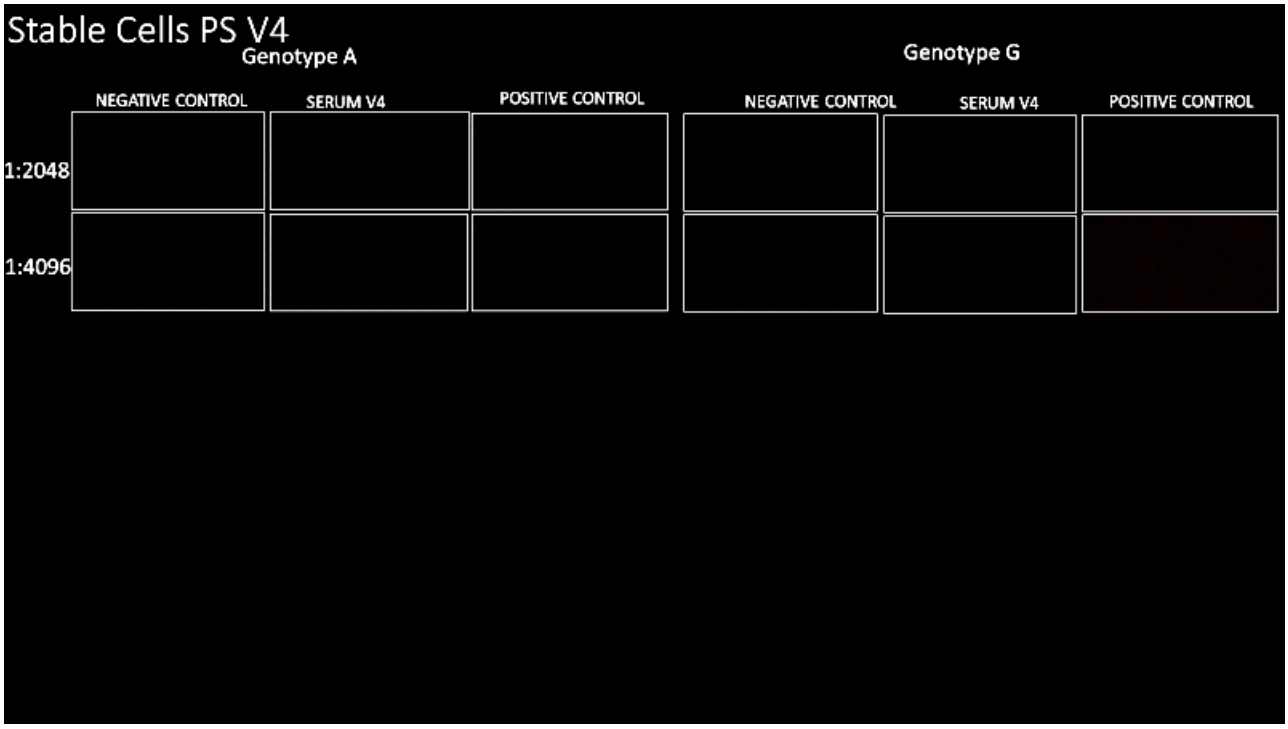


Figure A-48b. Stable cells Vaccine Sample#4.

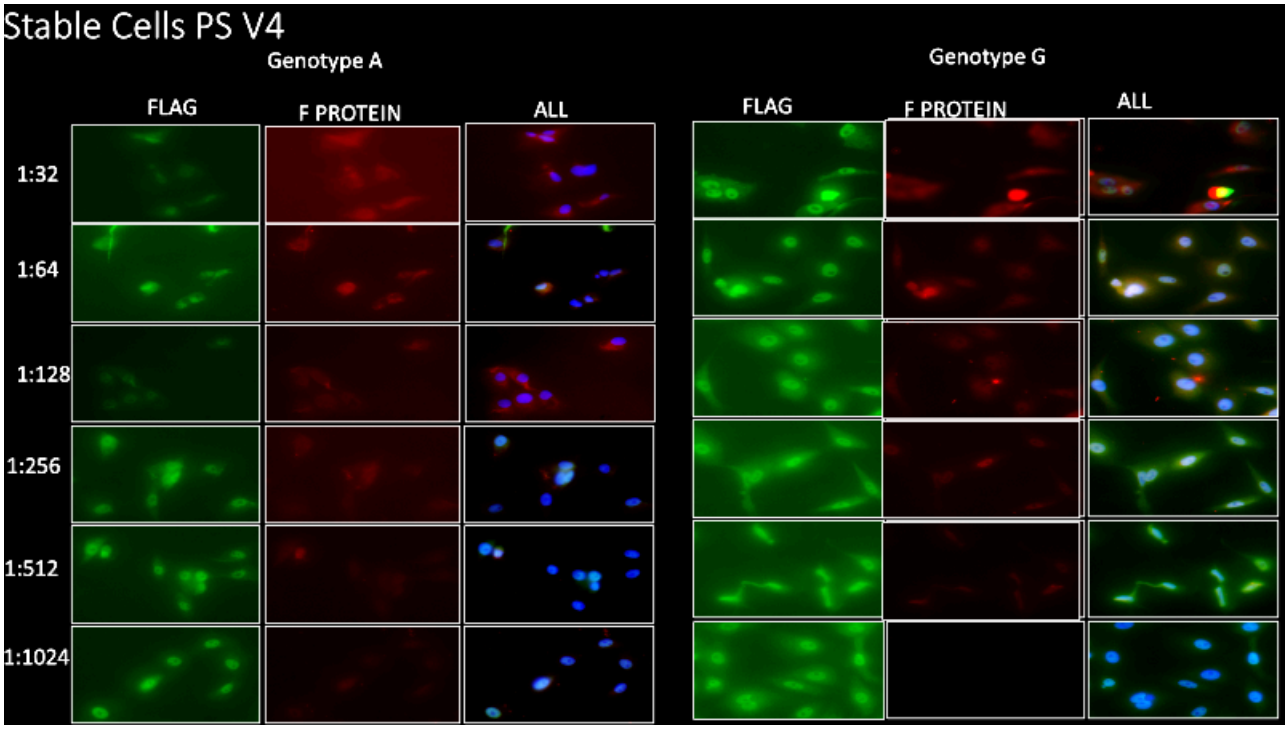


Figure A-48c. Stable cells Vaccine Sample#4.

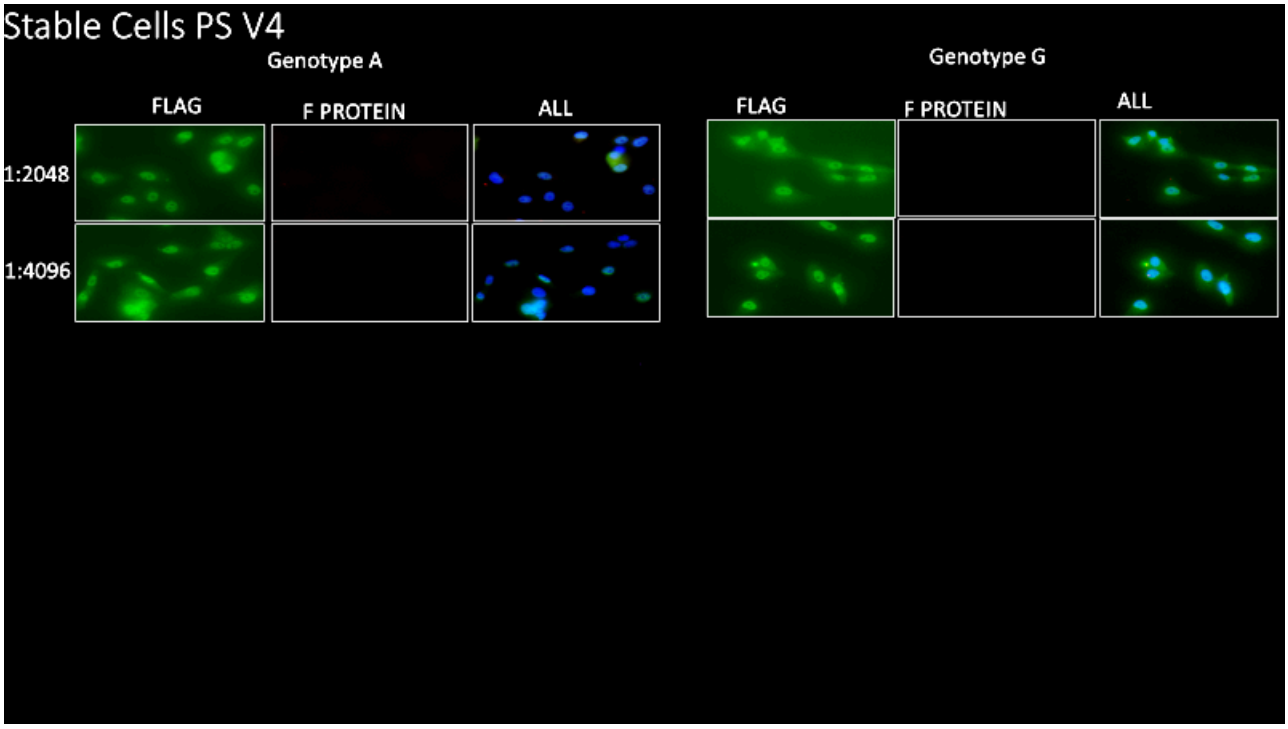


Figure A-48d. Stable cells Vaccine Sample#4.

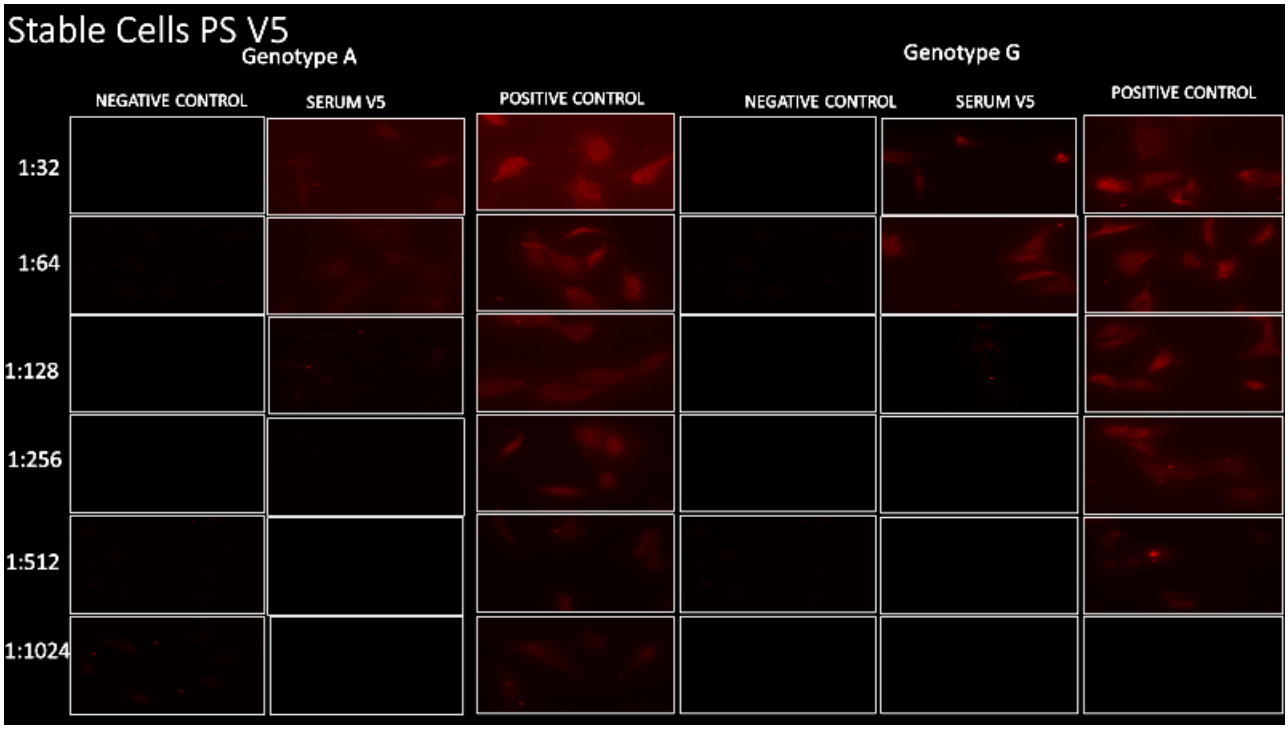


Figure A-49a. Stable cells Vaccine Sample#5.

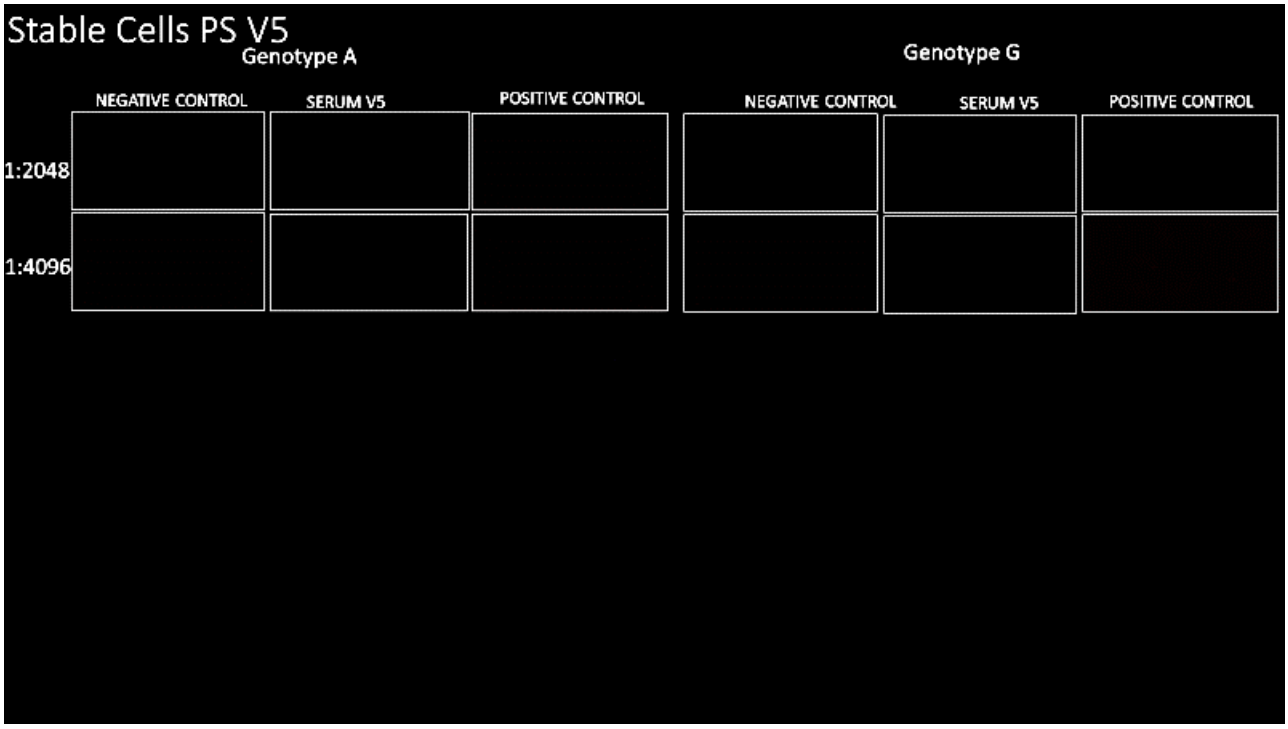


Figure A-49b. Stable cells Vaccine Sample#5.

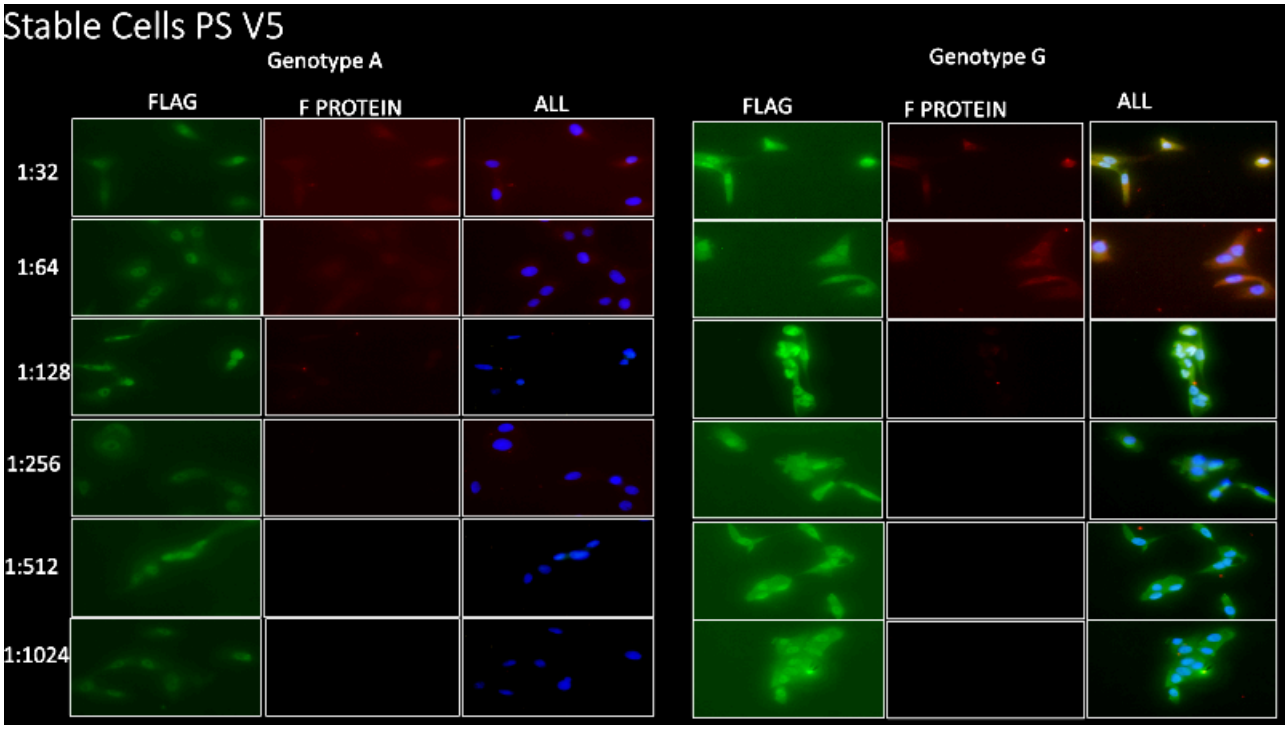


Figure A-49c. Stable cells Vaccine Sample#5.

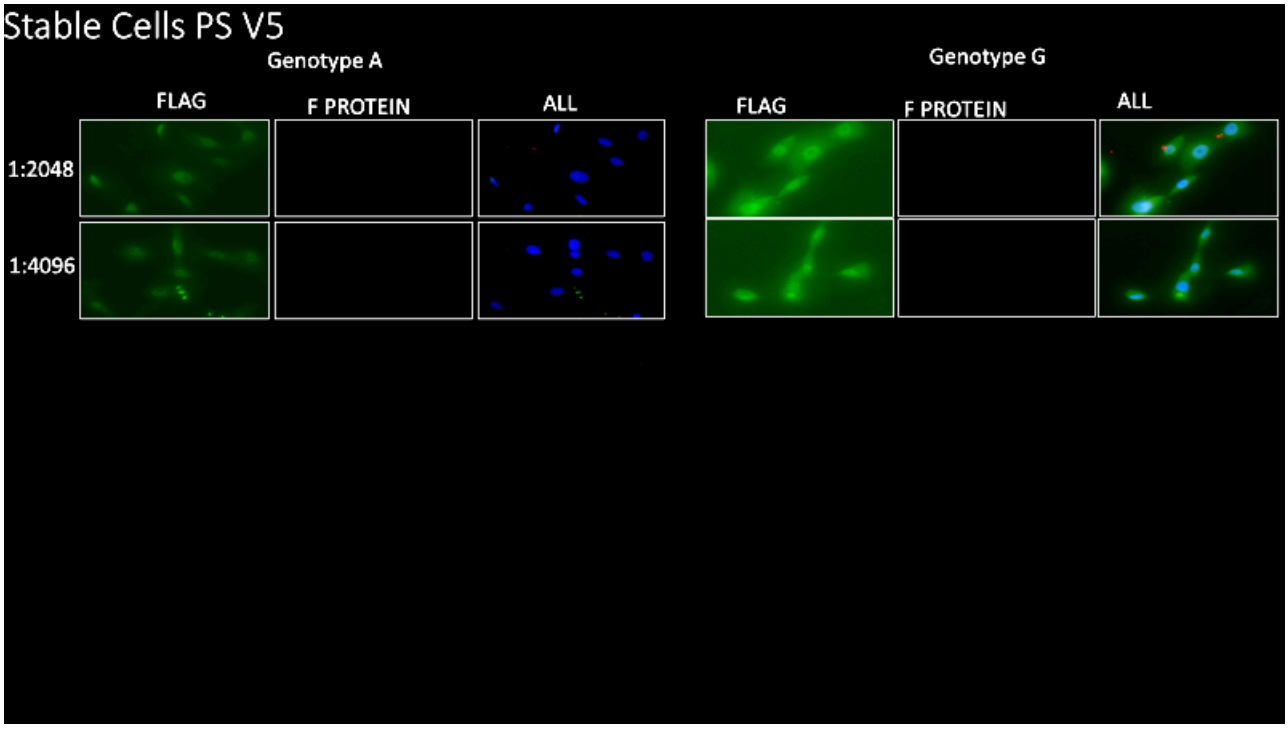


Figure A-49d. Stable cells Vaccine Sample#5.

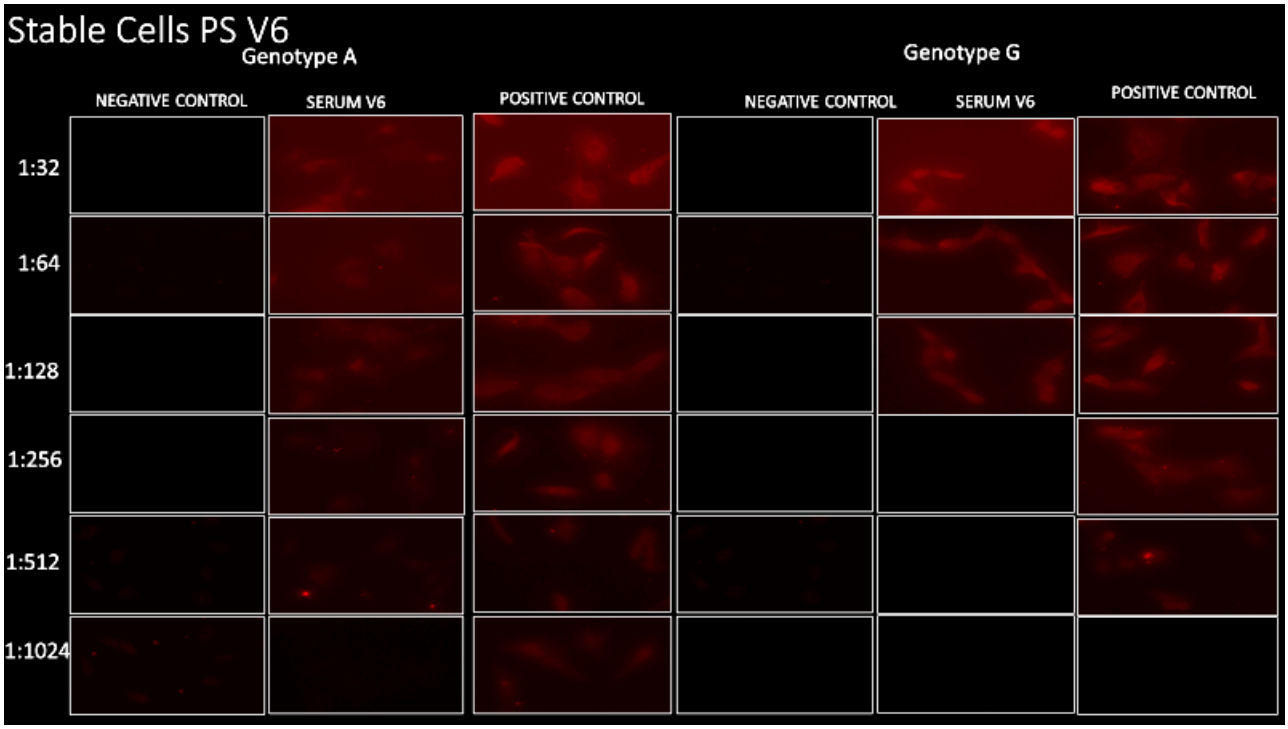


Figure A-50a. Stable cells Vaccine Sample#6.

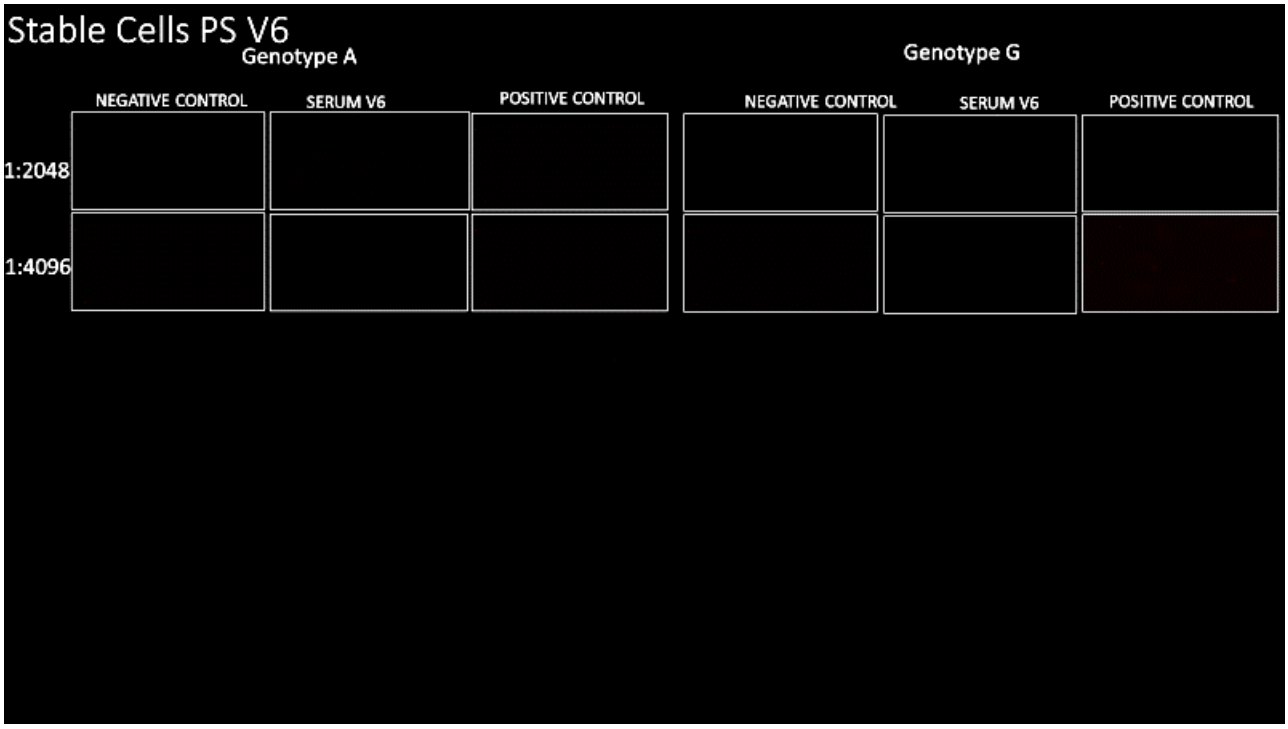


Figure A-50b. Stable cells Vaccine Sample#6.

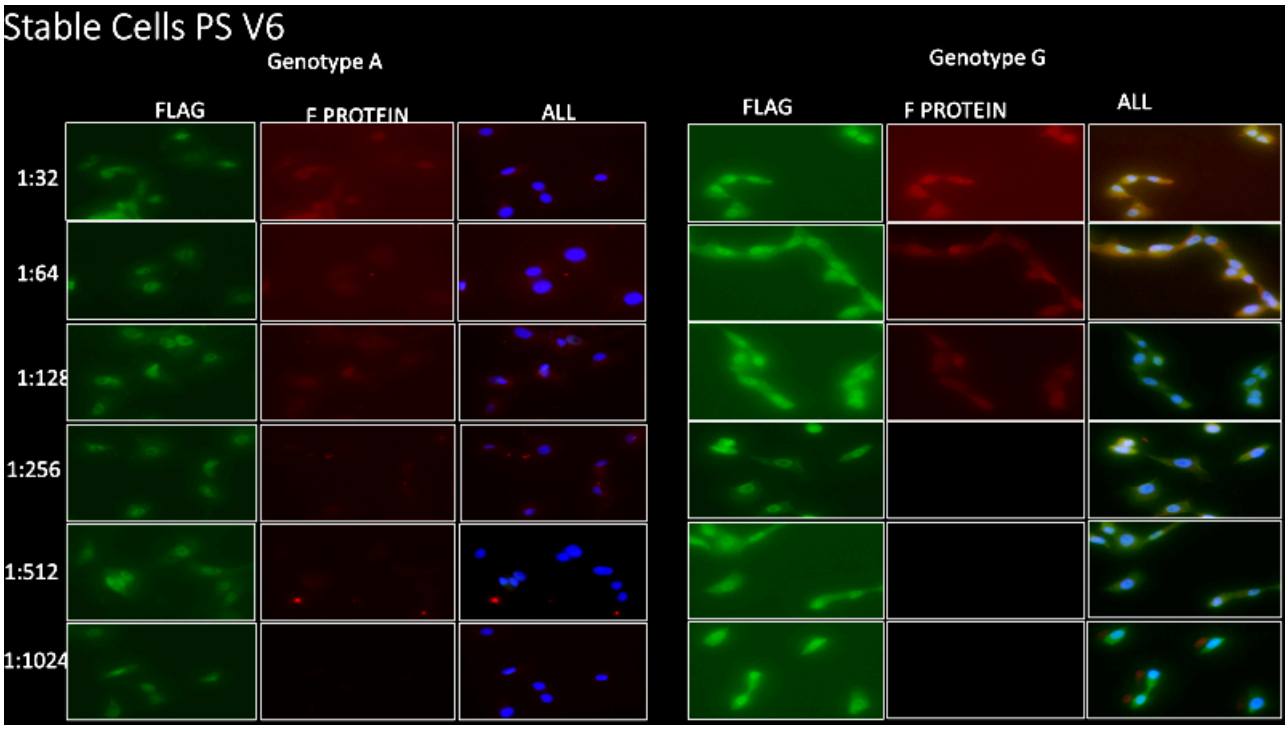


Figure A-50c. Stable cells Vaccine Sample#6.

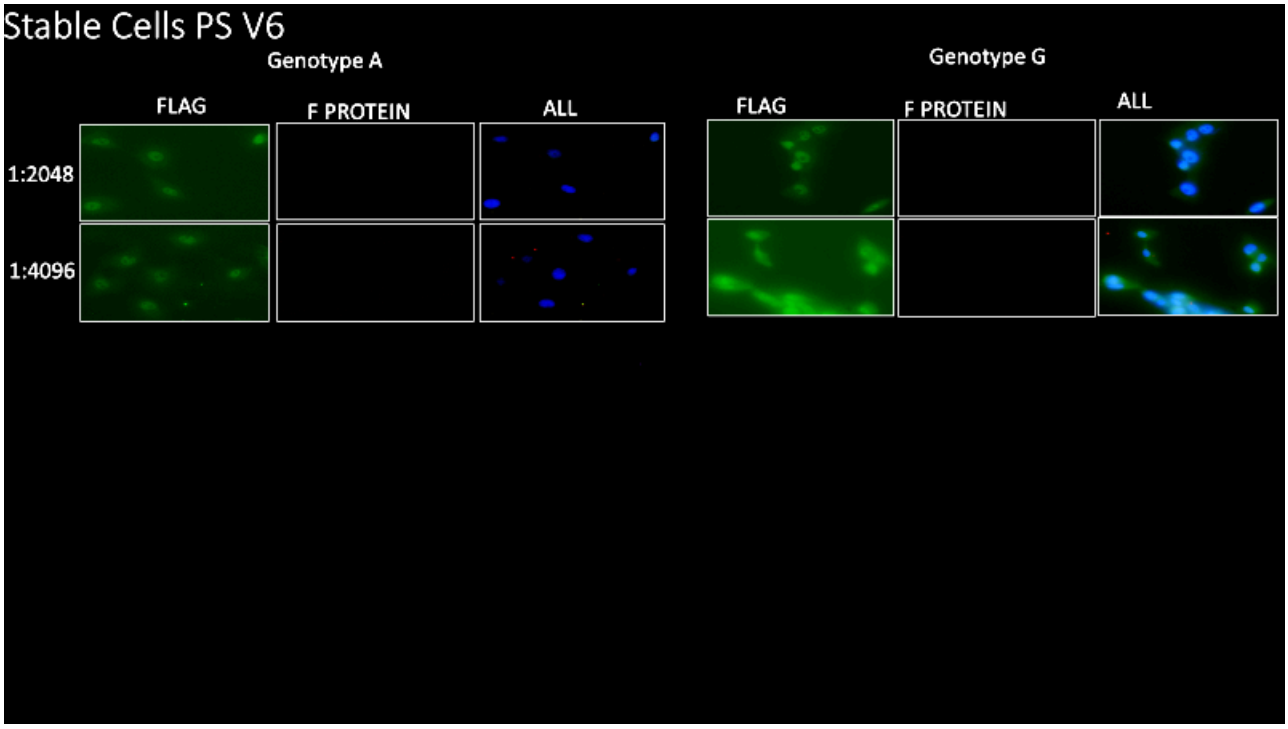


Figure A-50d. Stable cells Vaccine Sample#6.

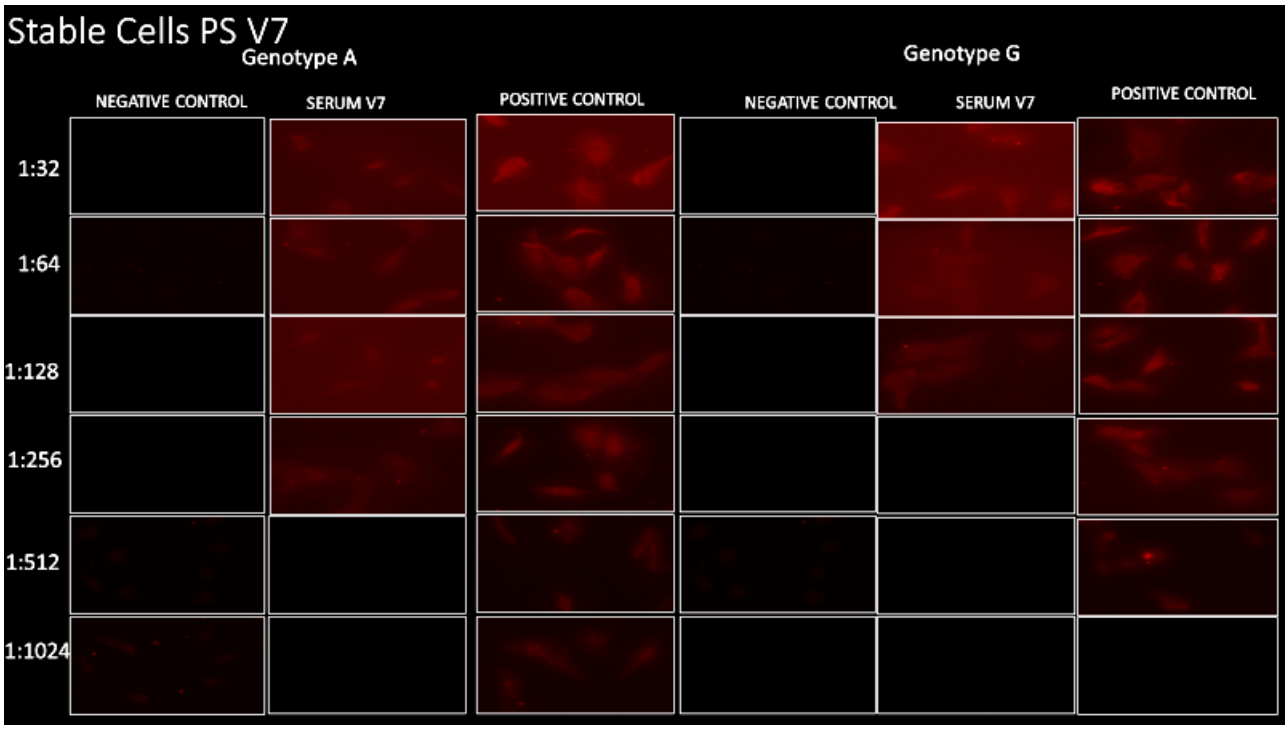


Figure A-51a. Stable cells Vaccine Sample#7.

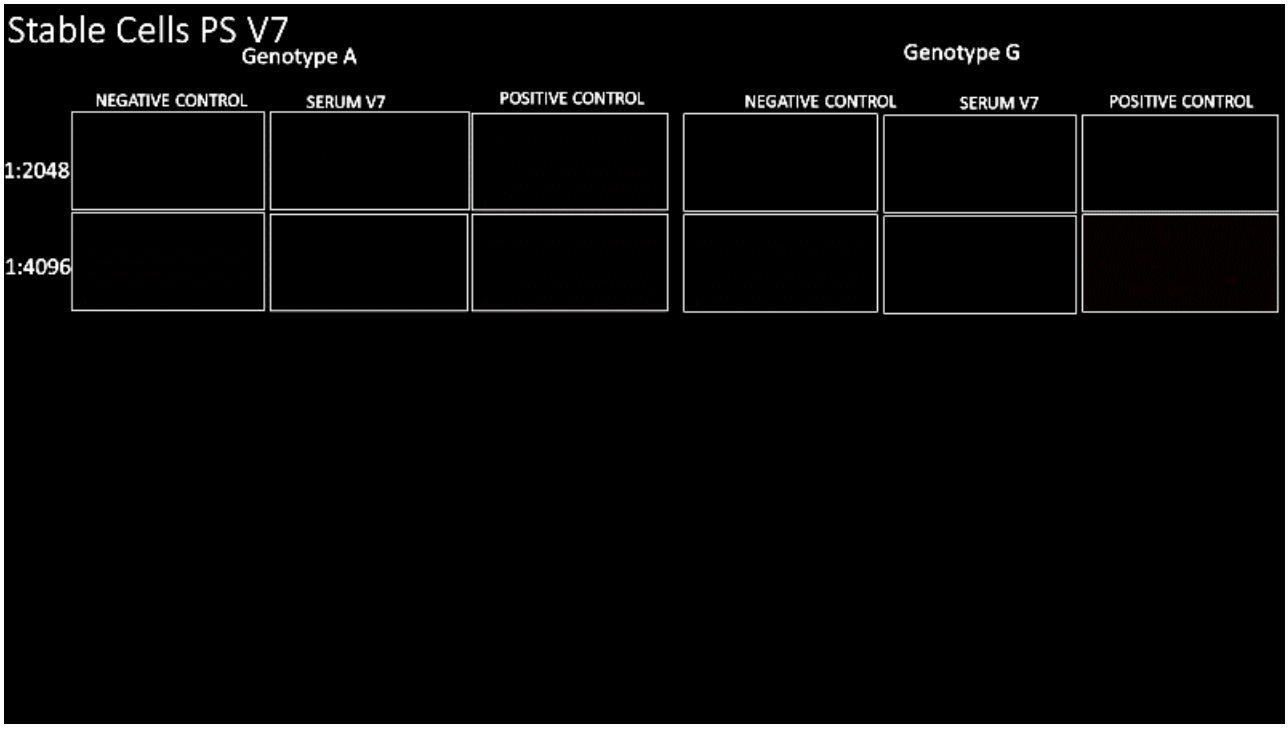


Figure A-51b. Stable cells Vaccine Sample#7.

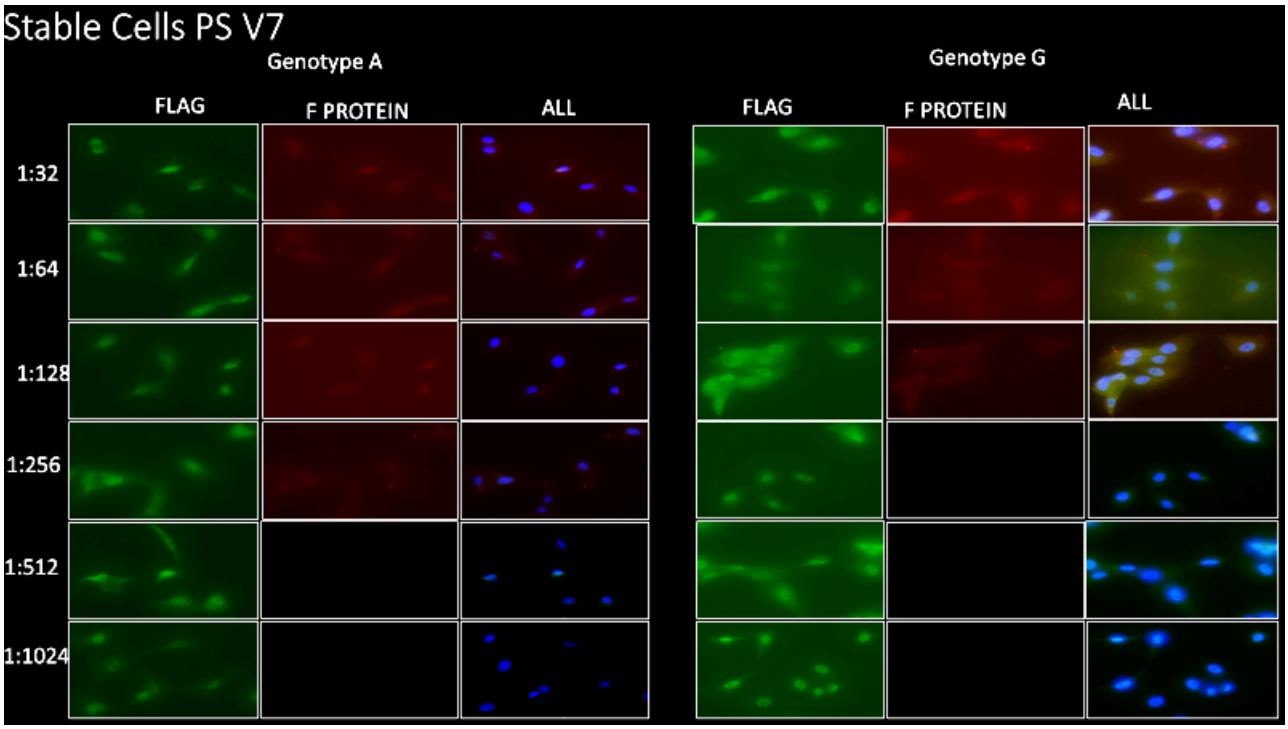


Figure A-51c. Stable cells Vaccine Sample#7.

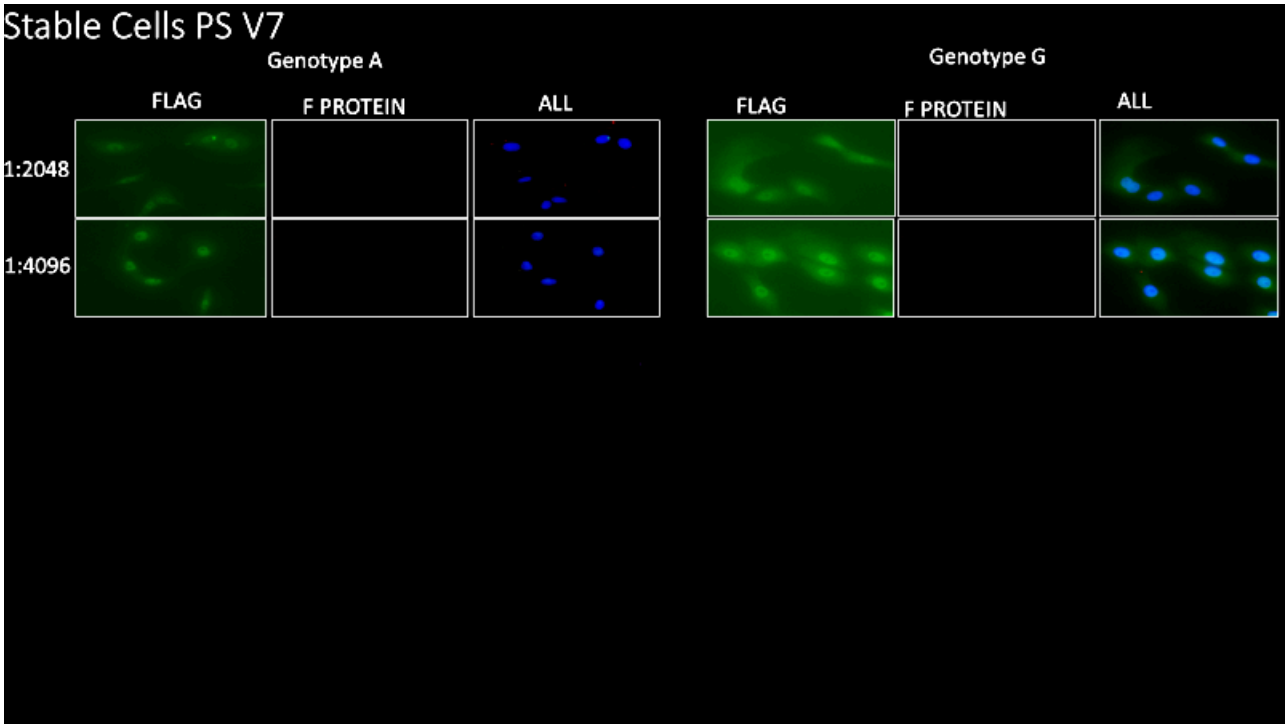


Figure A-51d. Stable cells Vaccine Sample#7.

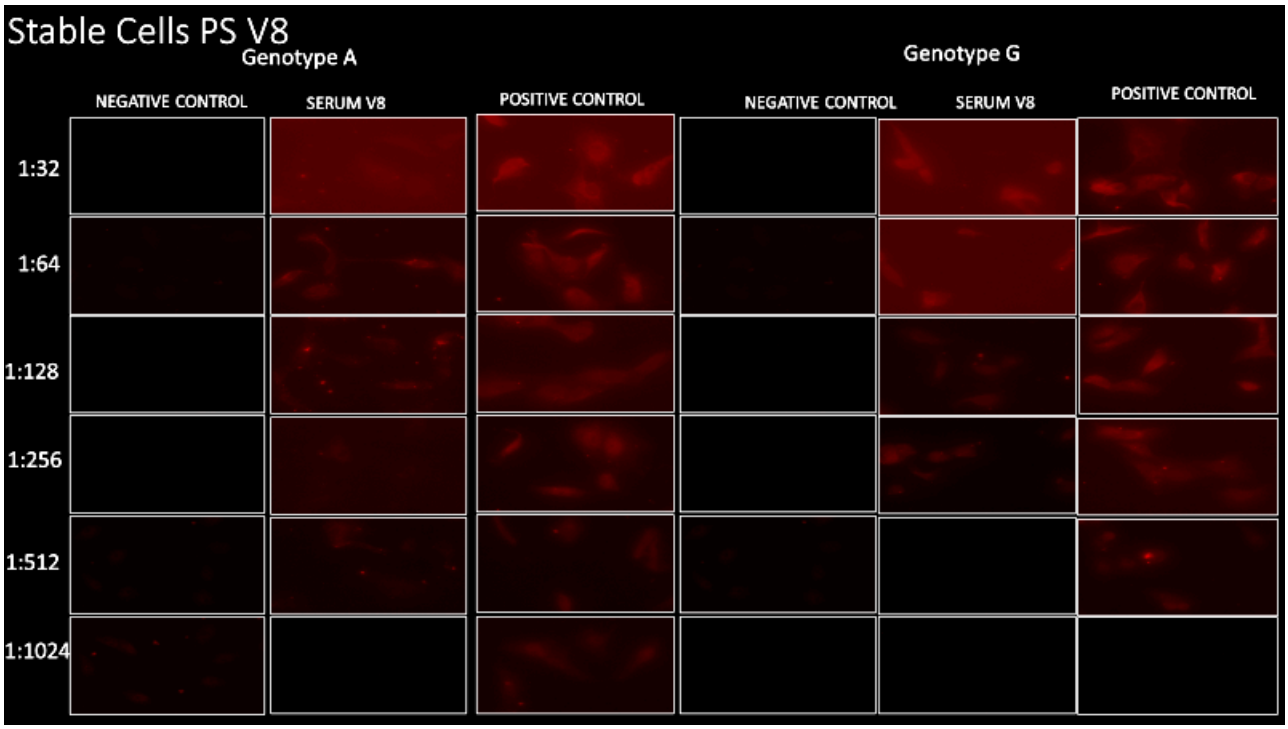


Figure A-52a. Stable cells Vaccine Sample#8.

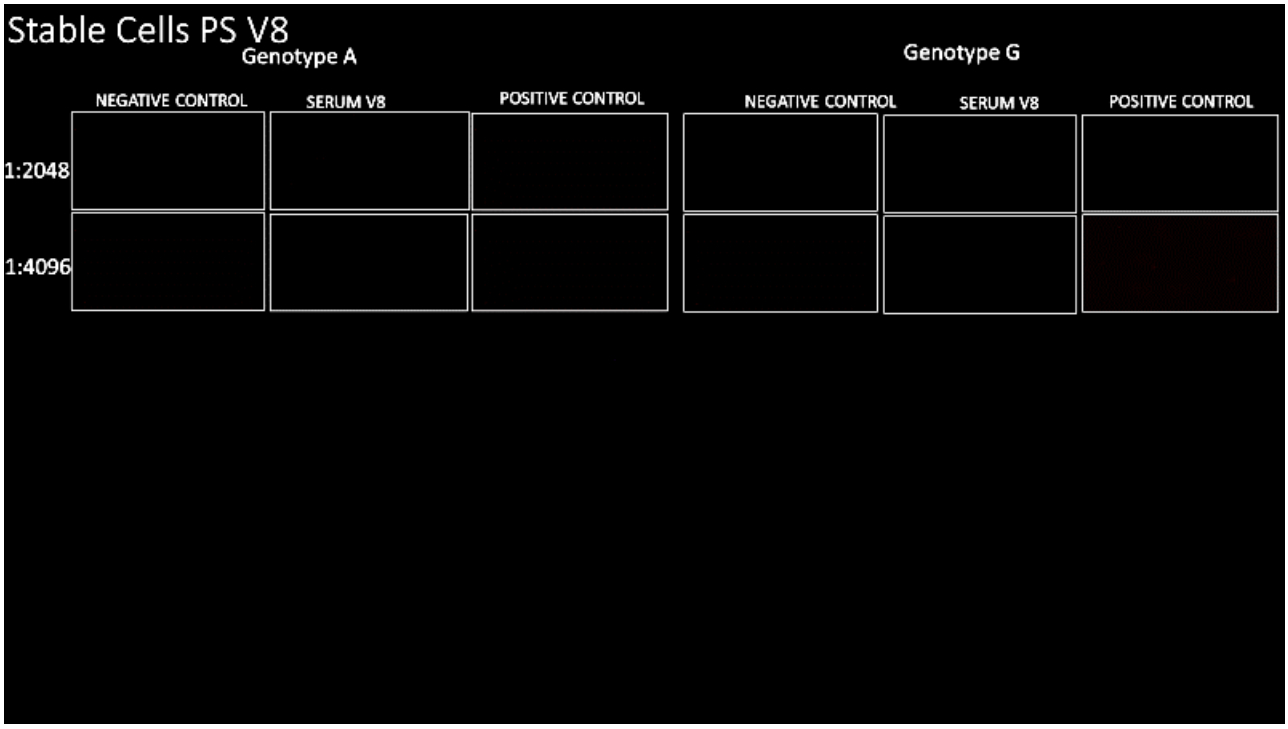


Figure A-52b. Stable cells Vaccine Sample#8.

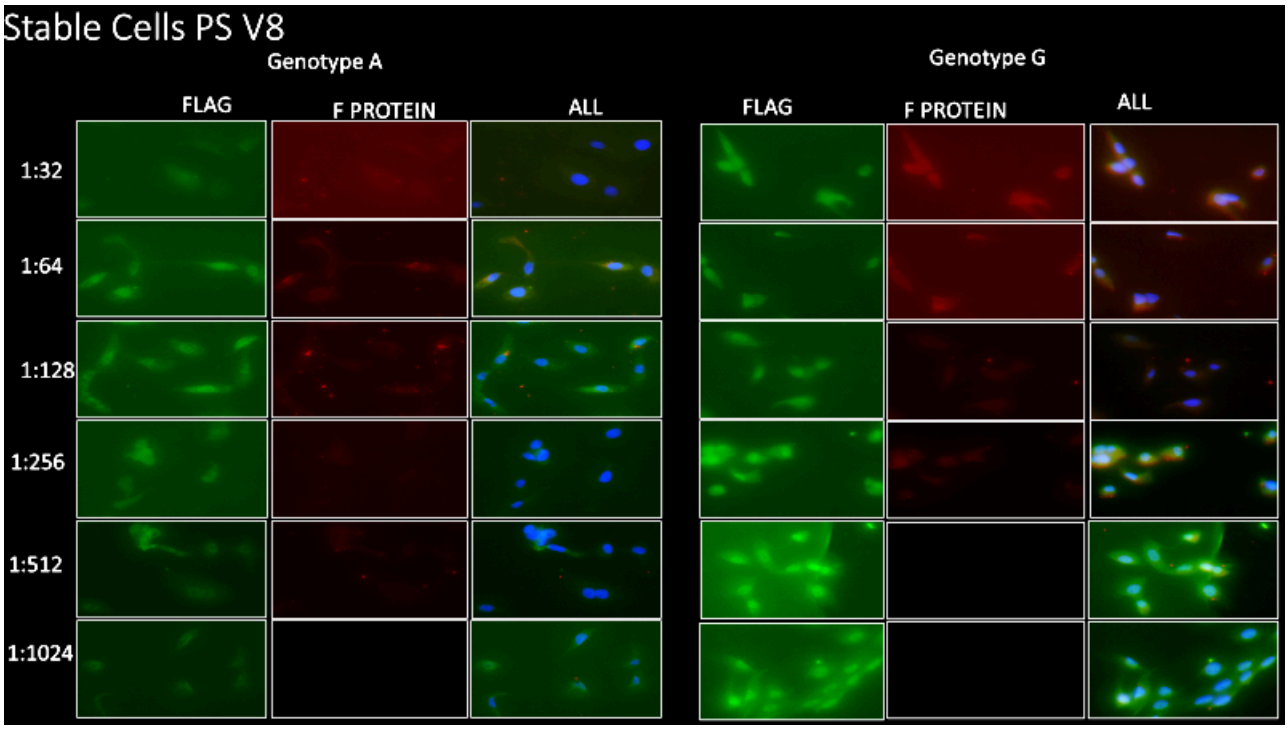


Figure A-52c. Stable cells Vaccine Sample#8.

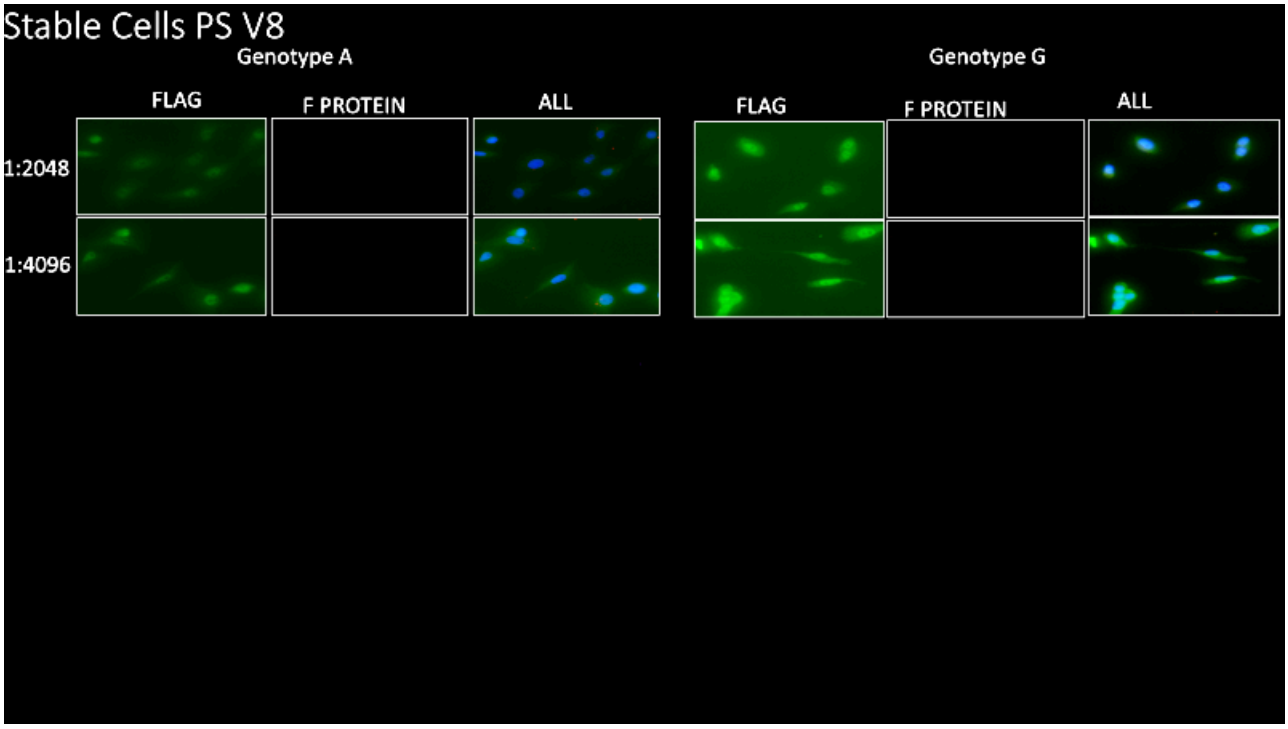


Figure A-52d. Stable cells Vaccine Sample#8.

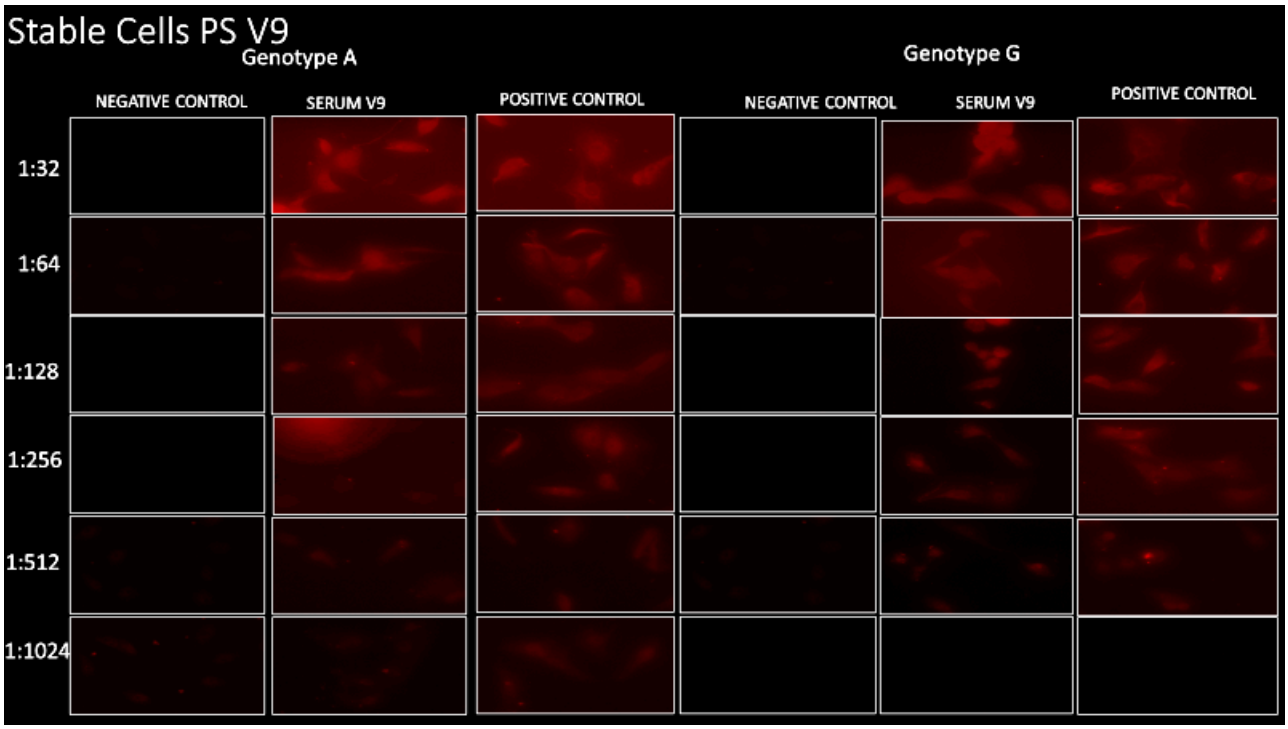


Figure A-53a. Stable cells Vaccine Sample#9.

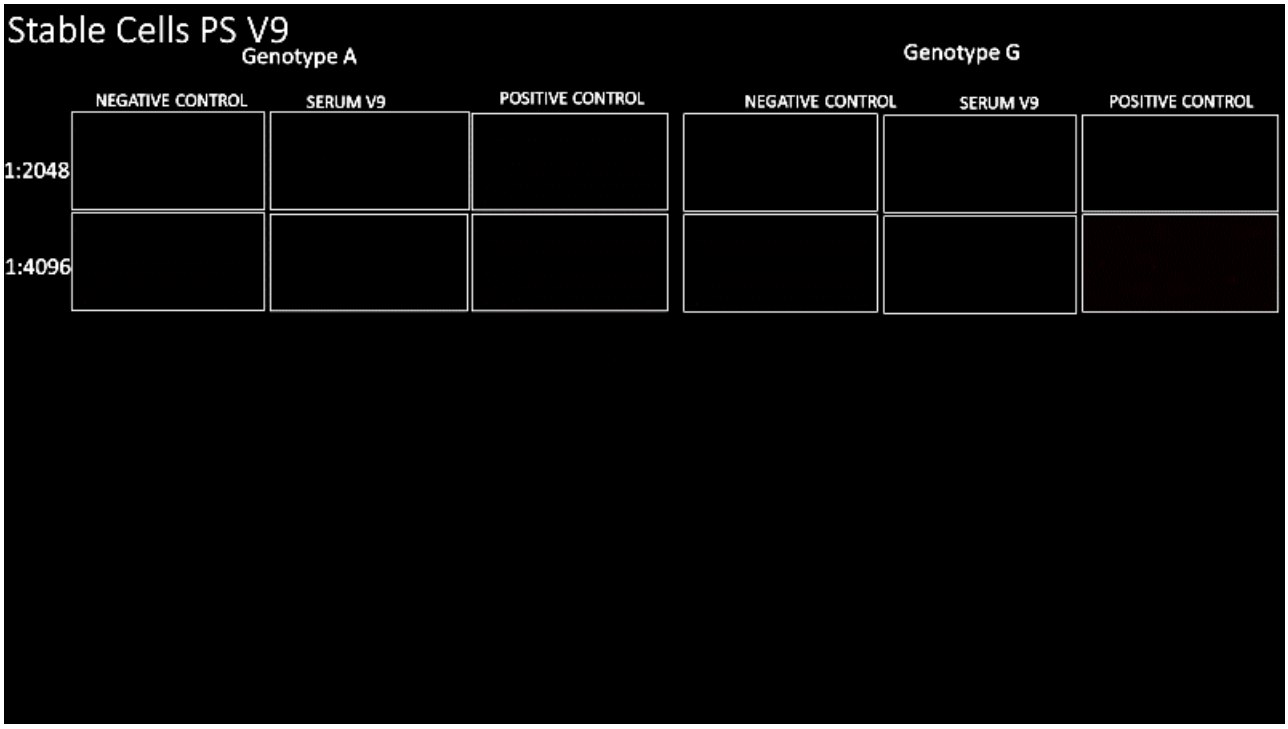


Figure A-53b. Stable cells Vaccine Sample#9.

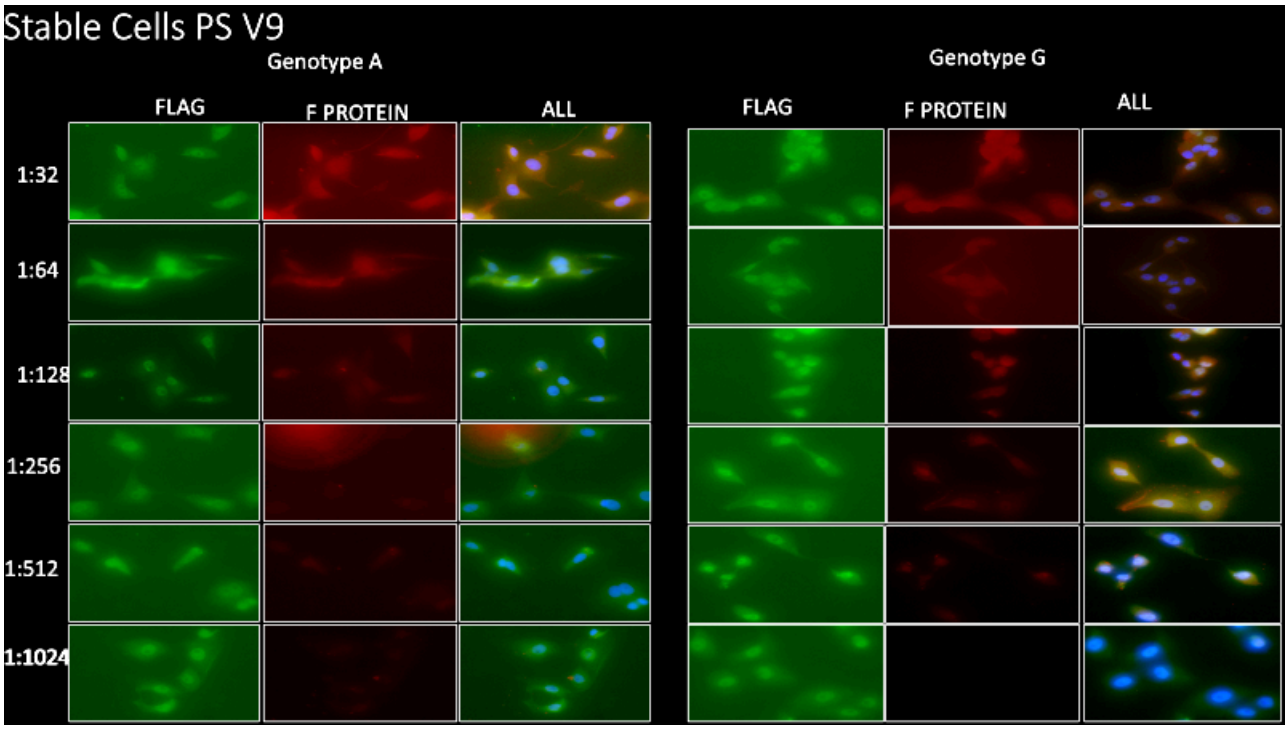


Figure A-53c. Stable cells Vaccine Sample#9.

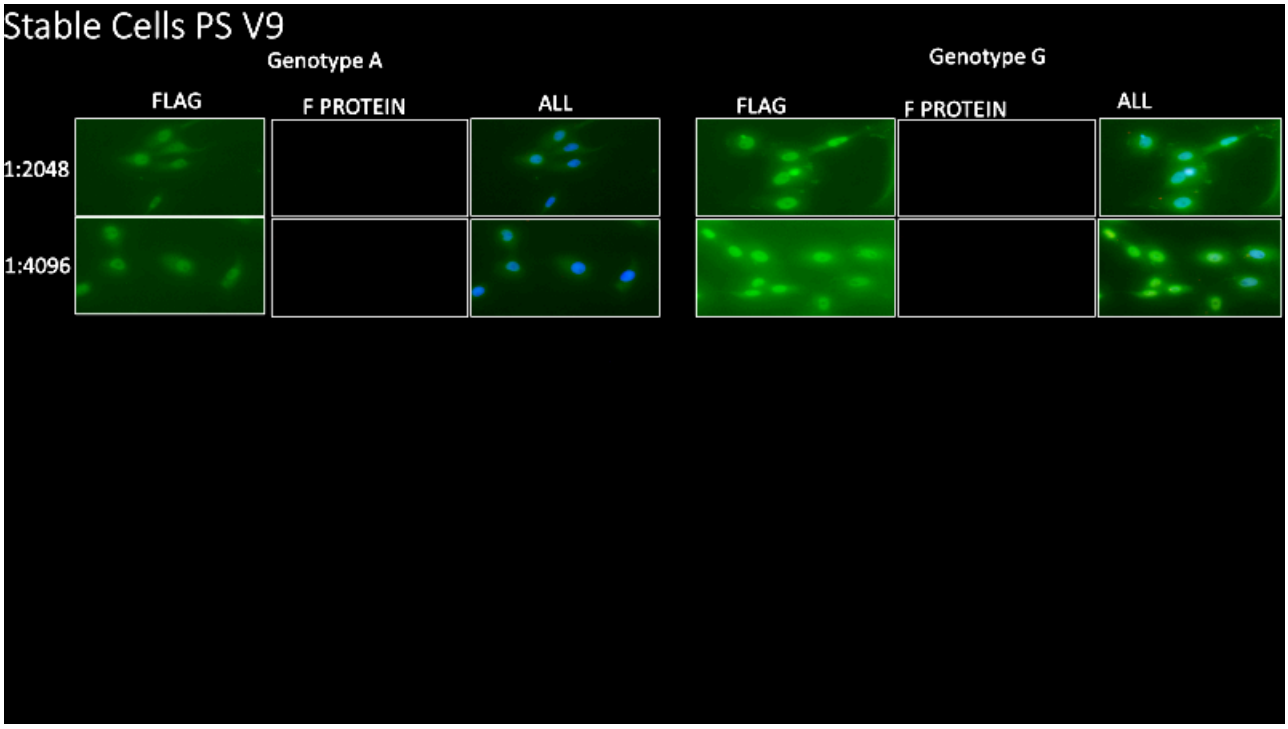


Figure A-53d. Stable cells Vaccine Sample#9.

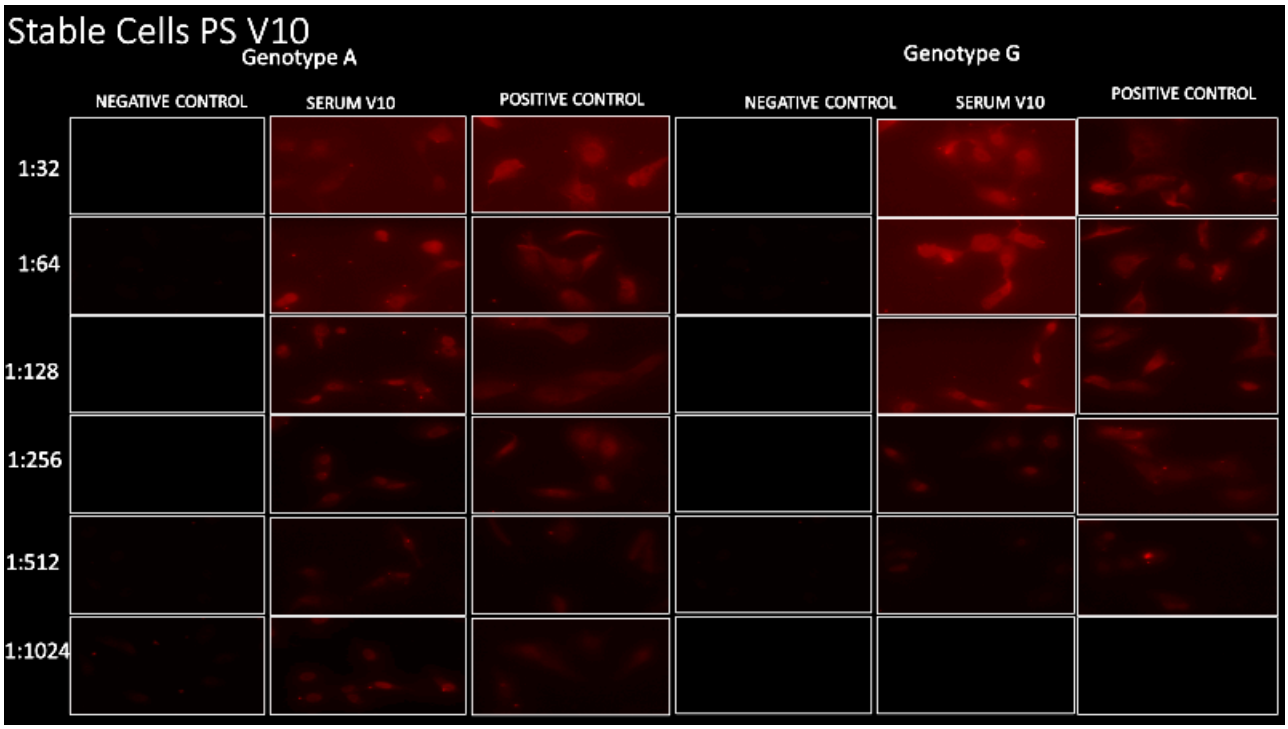


Figure A-54a. Stable cells Vaccine Sample#10.

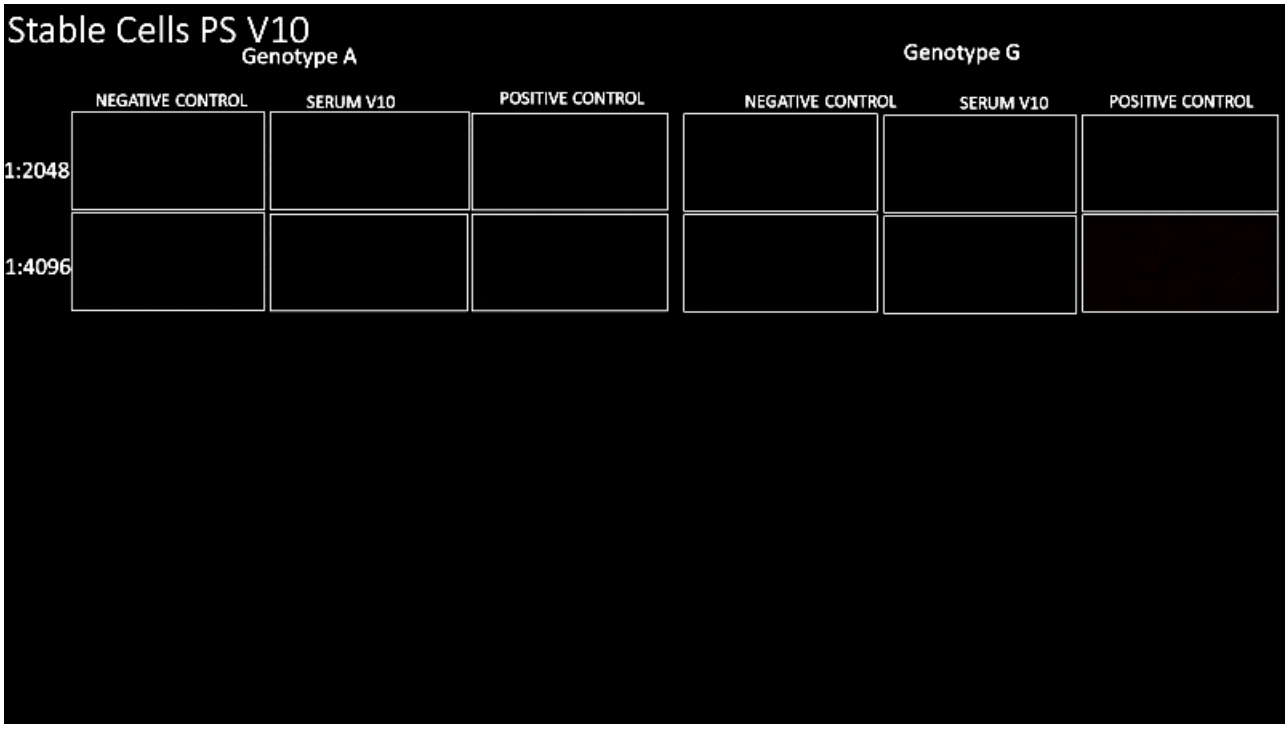


Figure A-54b. Stable cells Vaccine Sample#10.

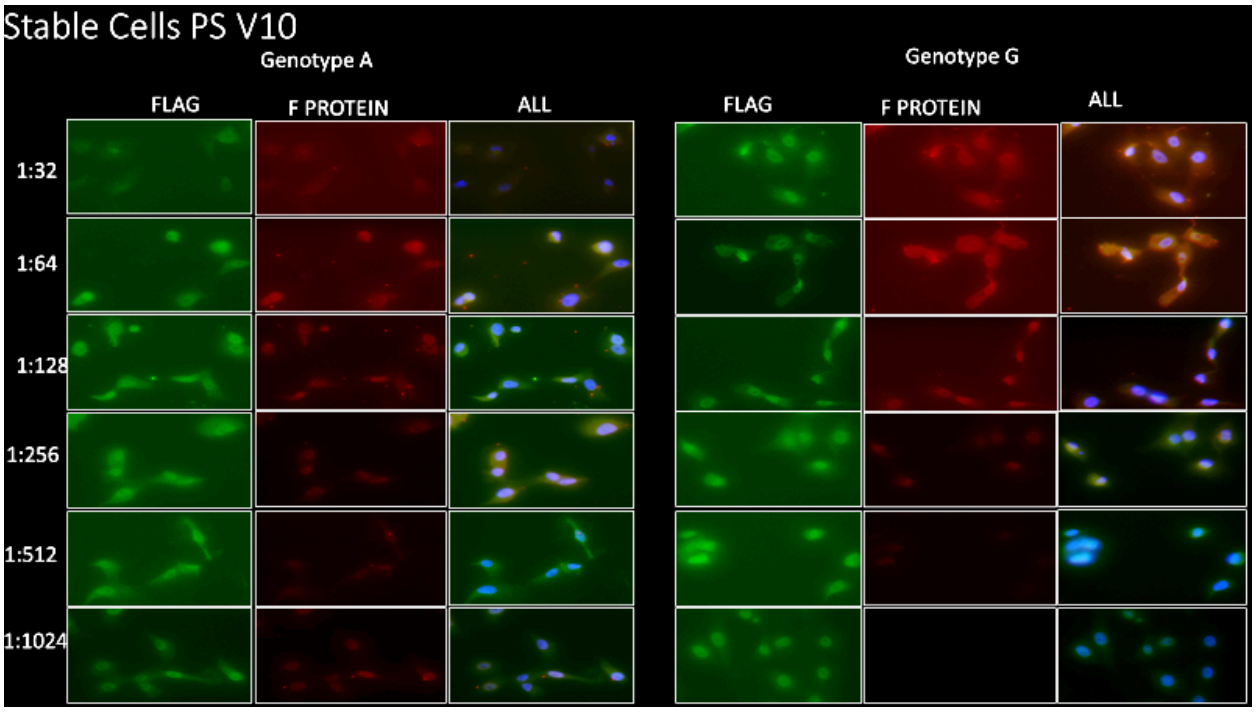


Figure A-54c. Stable cells Vaccine Sample#10.

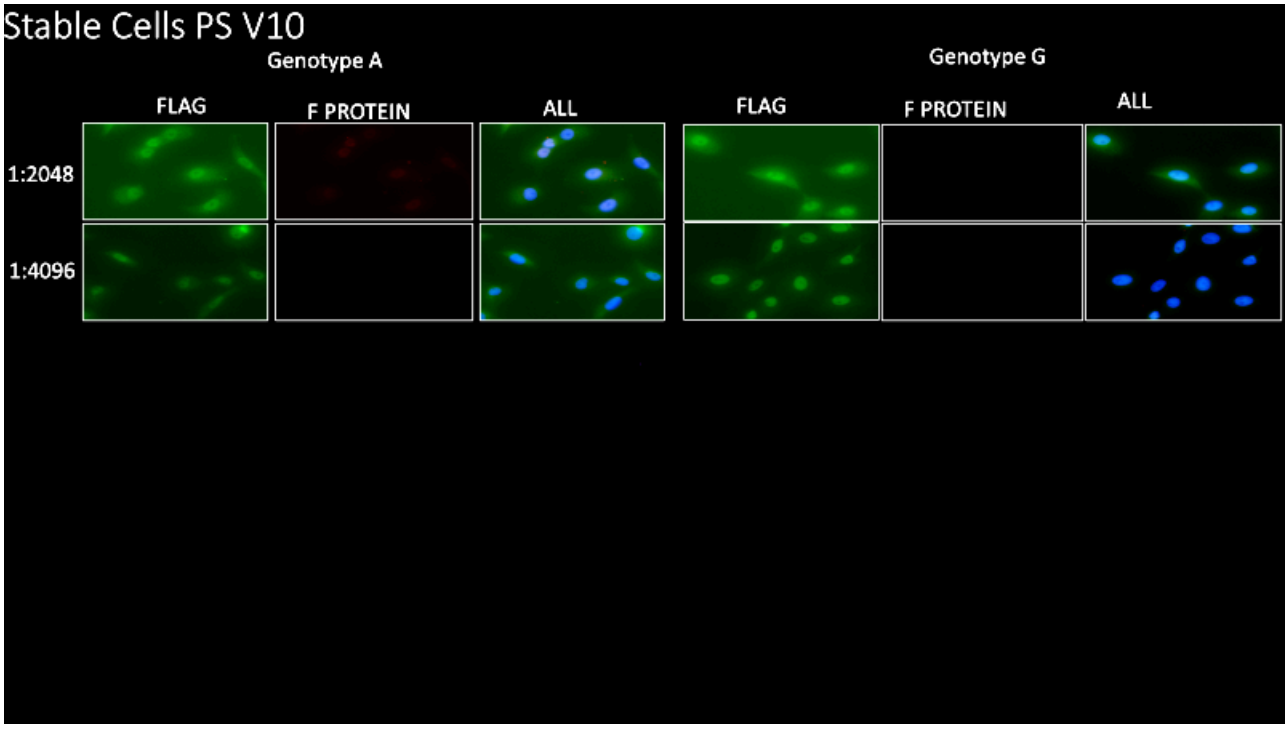


Figure A-54d. Stable cells Vaccine Sample#10.

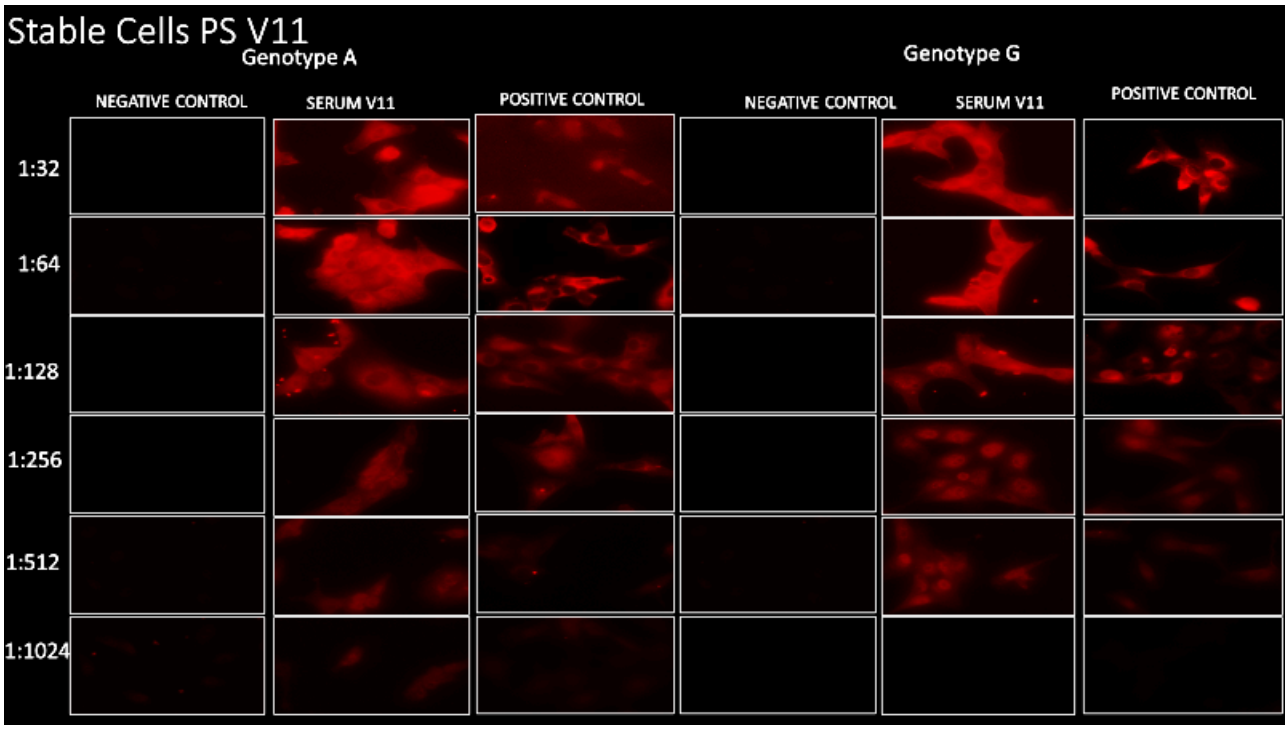


Figure A-55a. Stable cells Vaccine Sample#11.

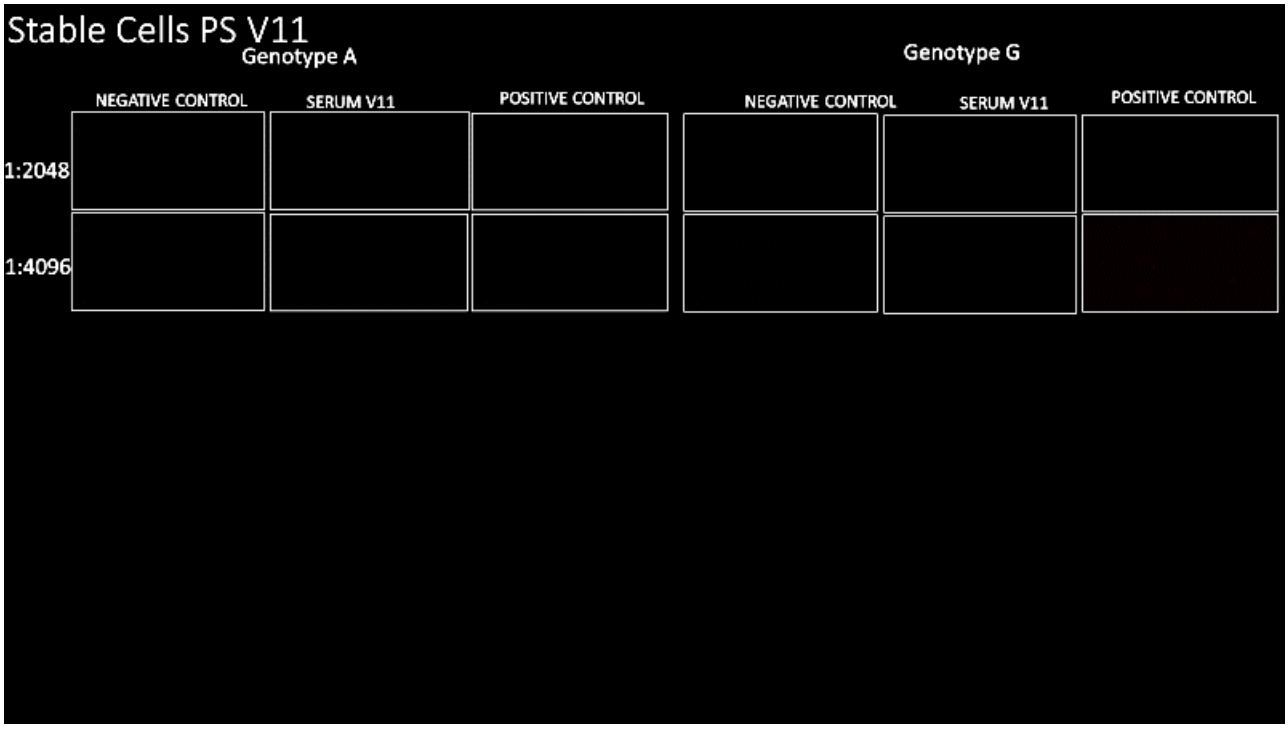


Figure A-55b. Stable cells Vaccine Sample#11.

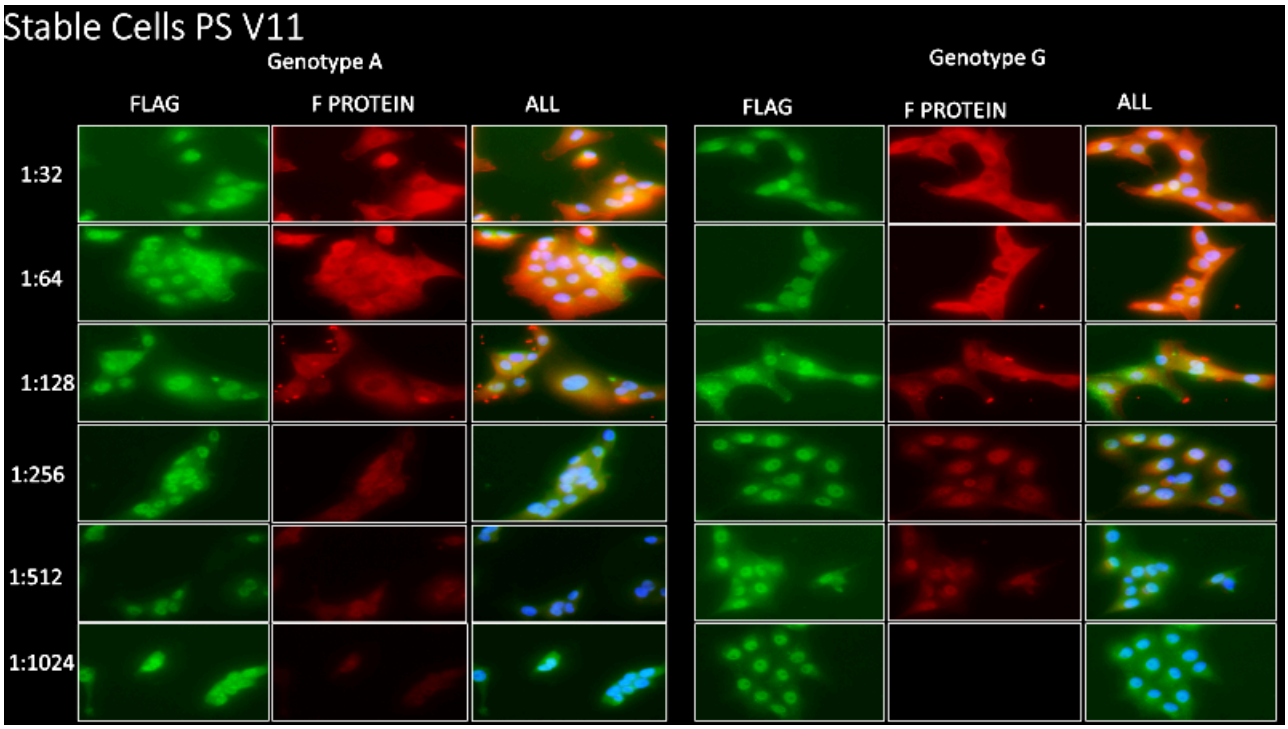


Figure A-55c. Stable cells Vaccine Sample#11.

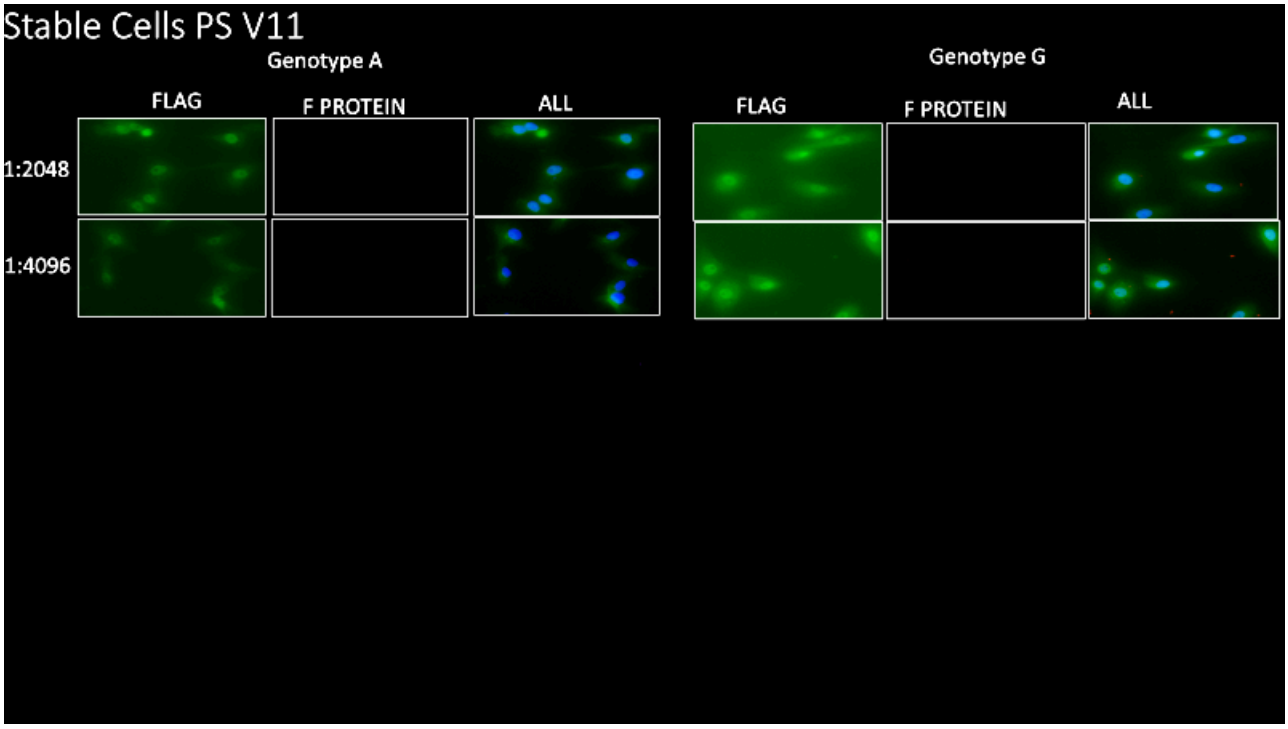


Figure A-55d. Stable cells Vaccine Sample#11.

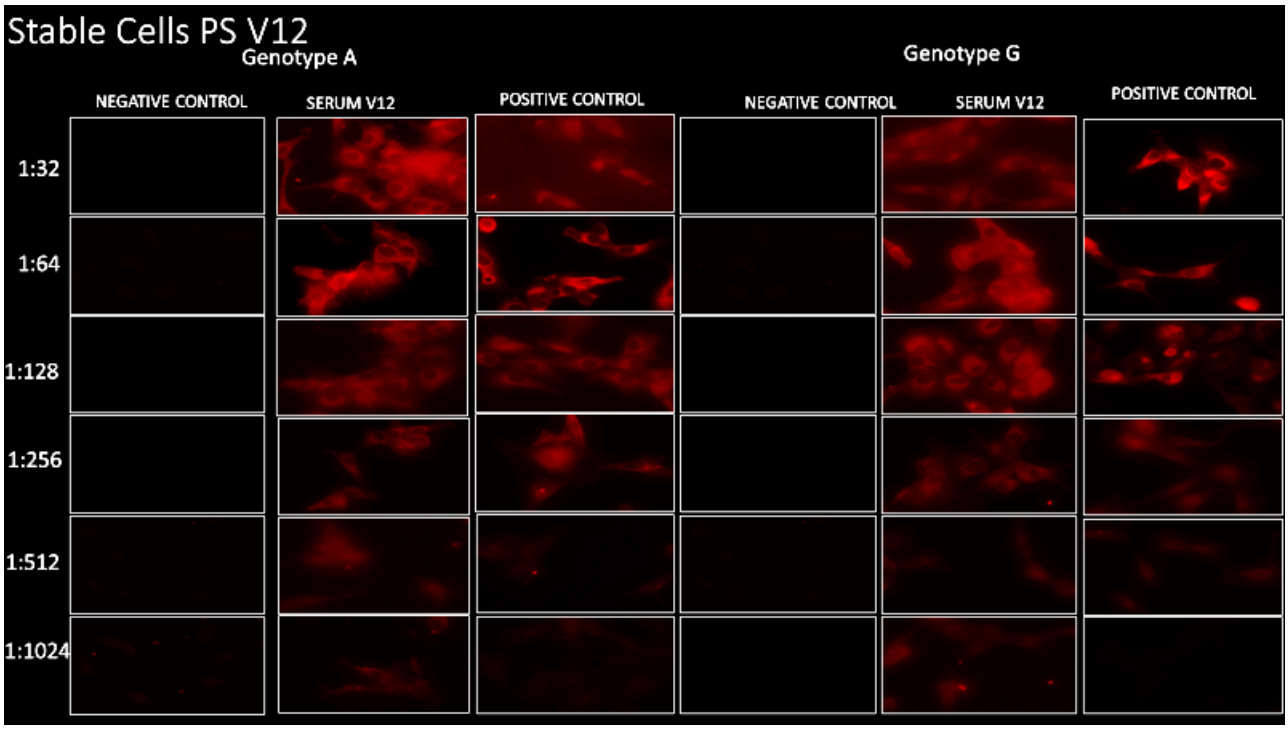


Figure A-56a. Stable cells Vaccine Sample#12.

Figure A-56b. Stable cells Vaccine Sample#12.

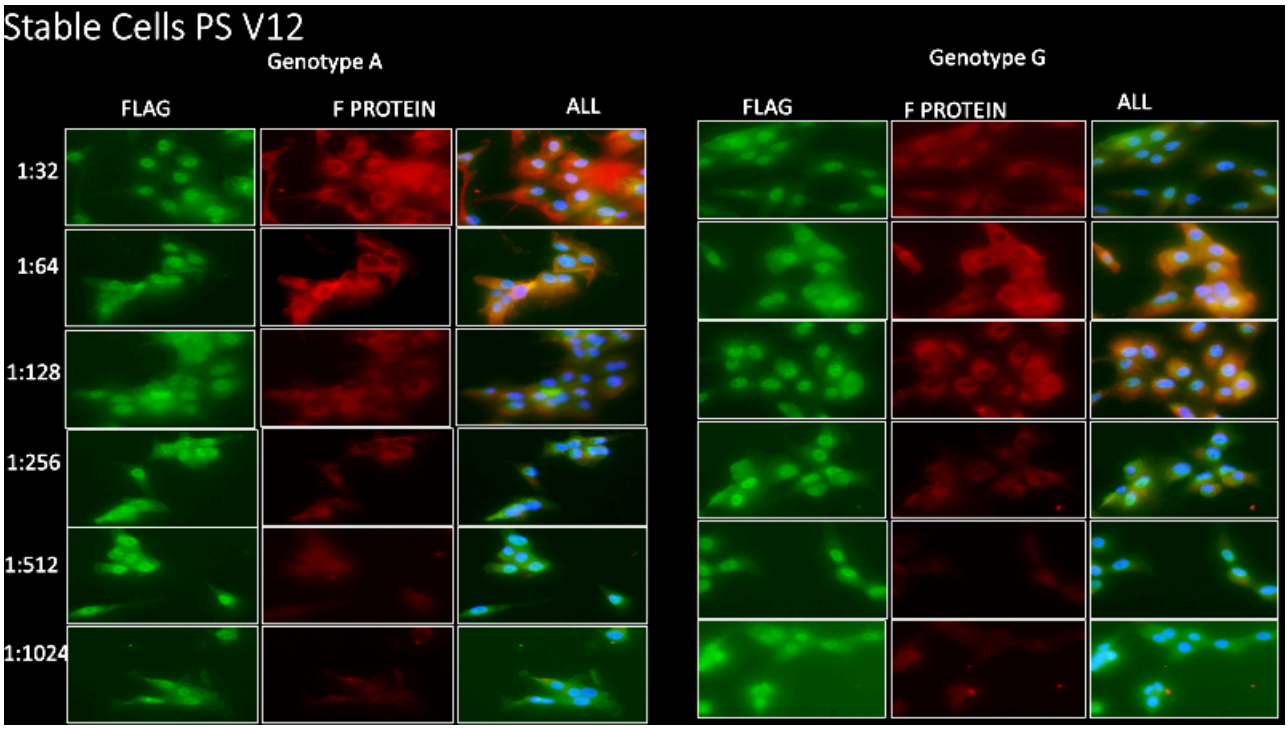


Figure A-56c. Stable cells Vaccine Sample#12.

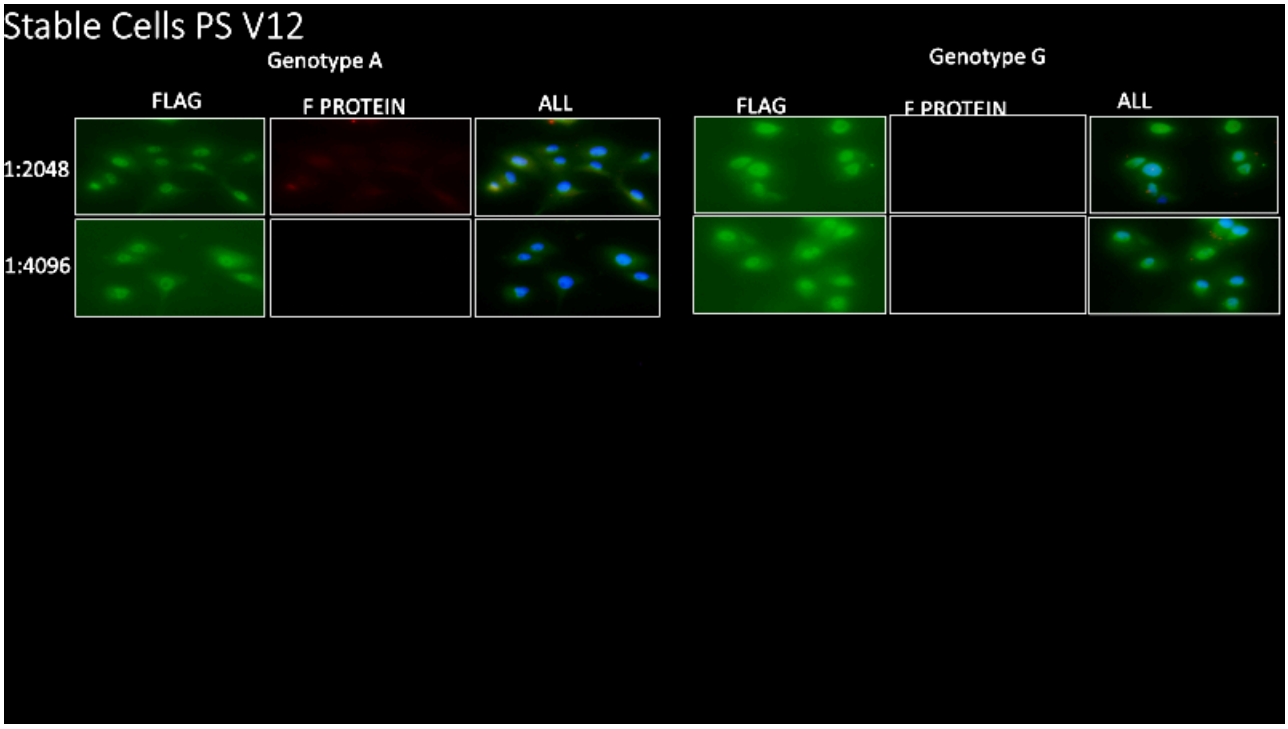


Figure A-56d. Stable cells Vaccine Sample#12.

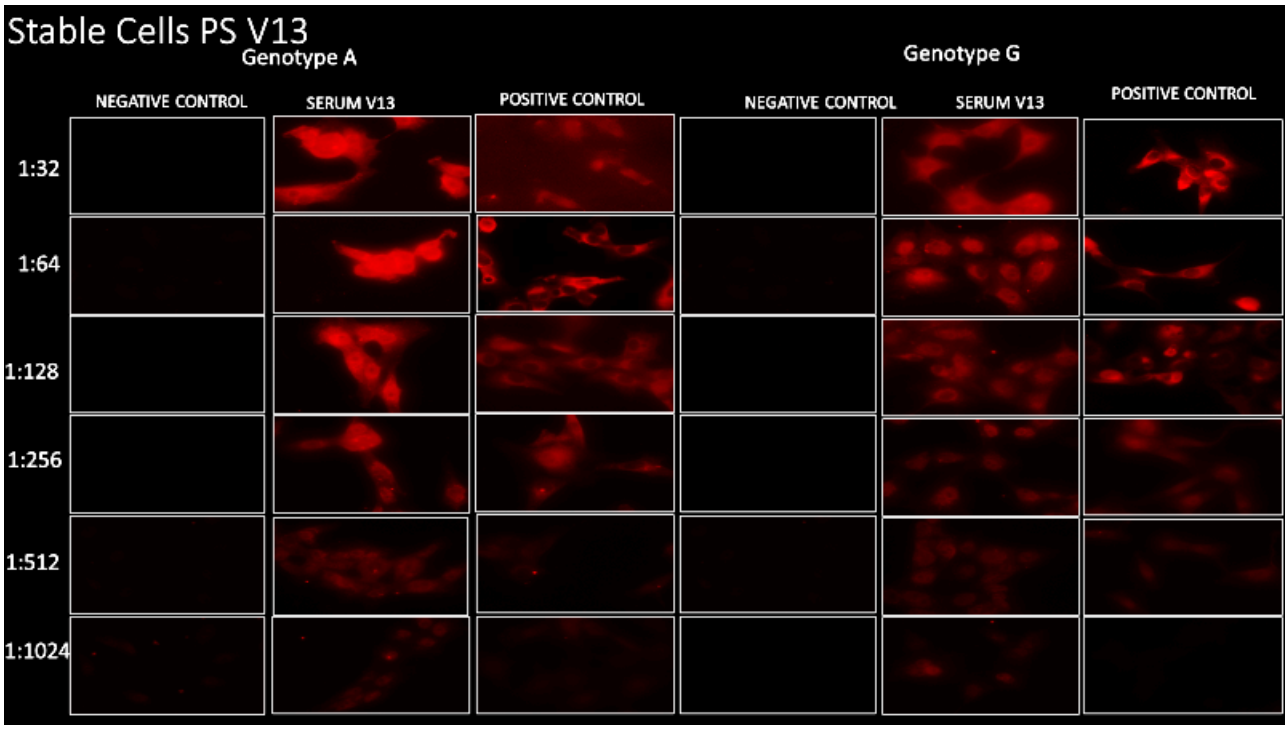


Figure A-57a. Stable cells Vaccine Sample#13.

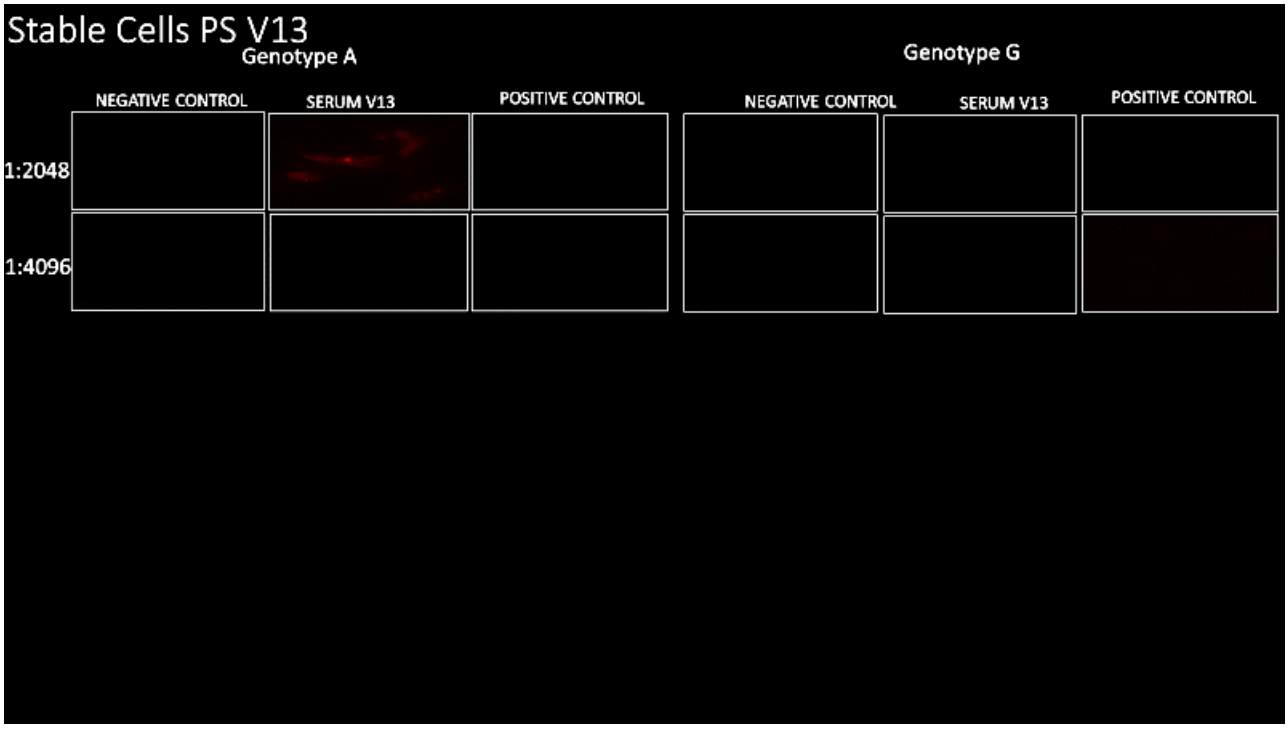


Figure A-57b. Stable cells Vaccine Sample#13.

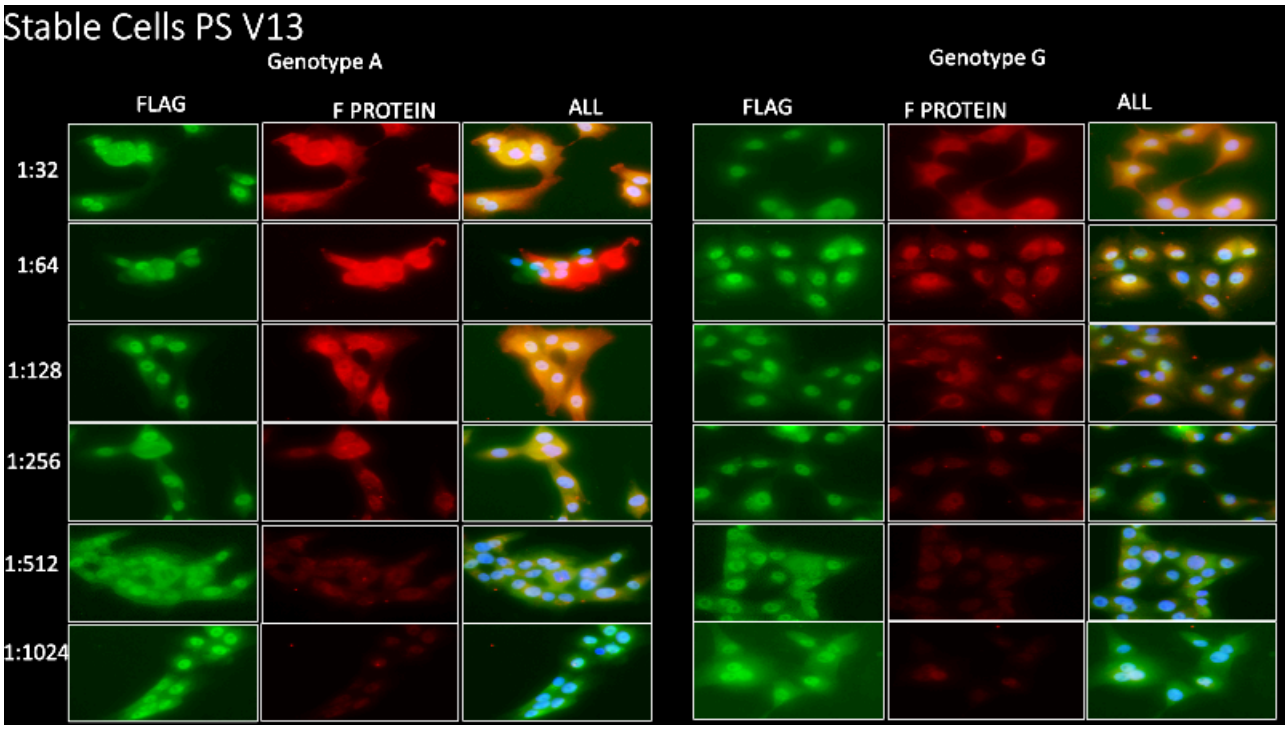


Figure A-57c. Stable cells Vaccine Sample#13.

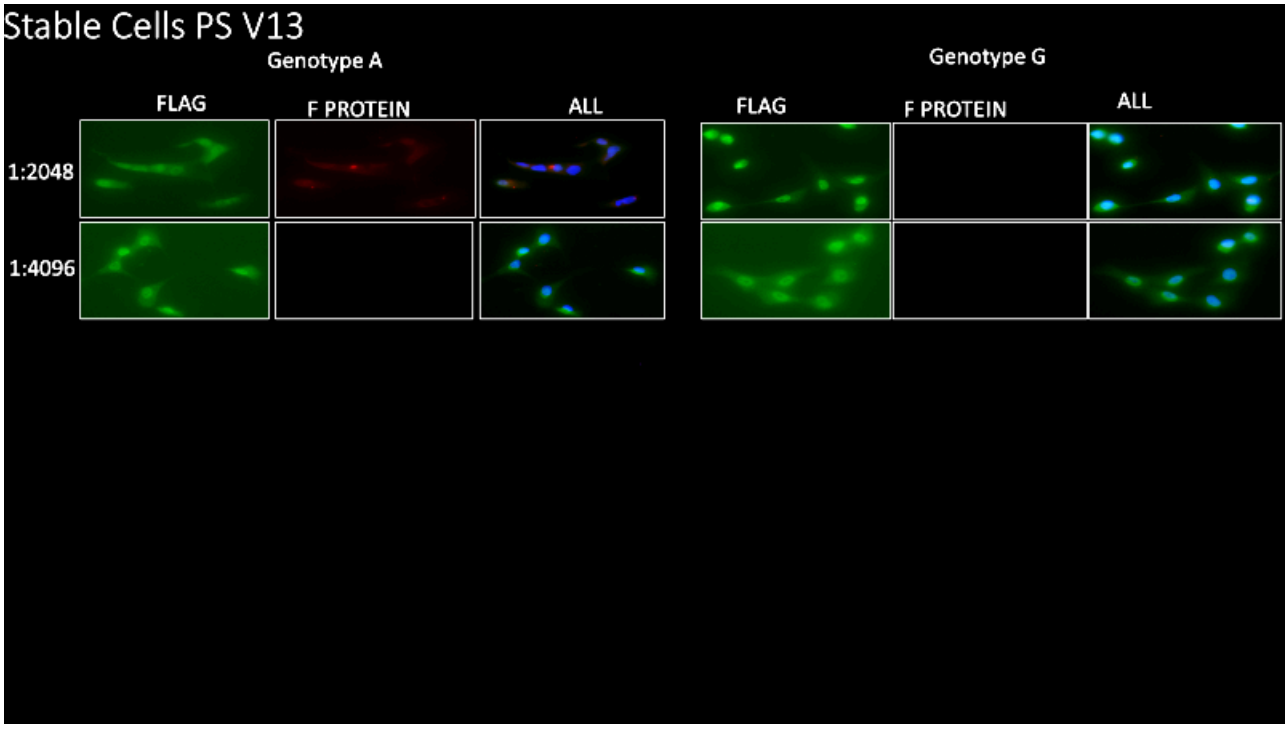


Figure A-57d. Stable cells Vaccine Sample#13.

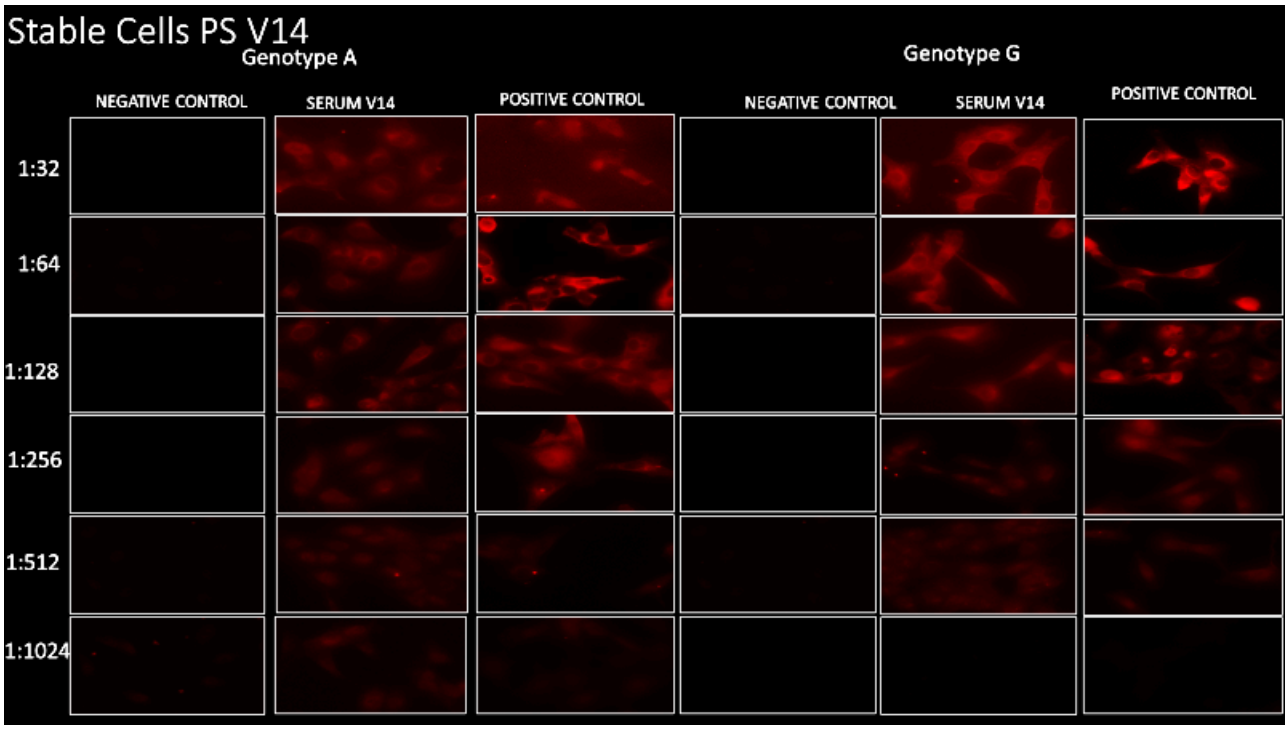


Figure A-58a. Stable cells Vaccine Sample#14.

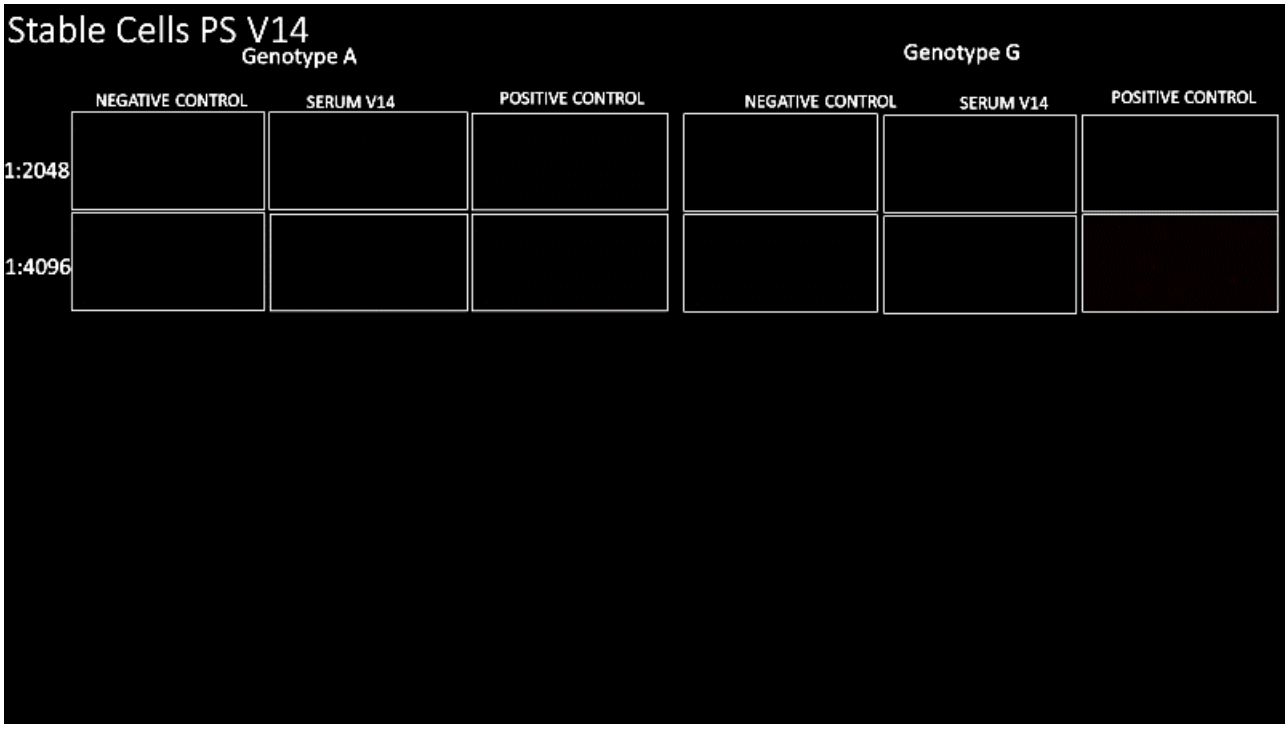


Figure A-58b. Stable cells Vaccine Sample#14.

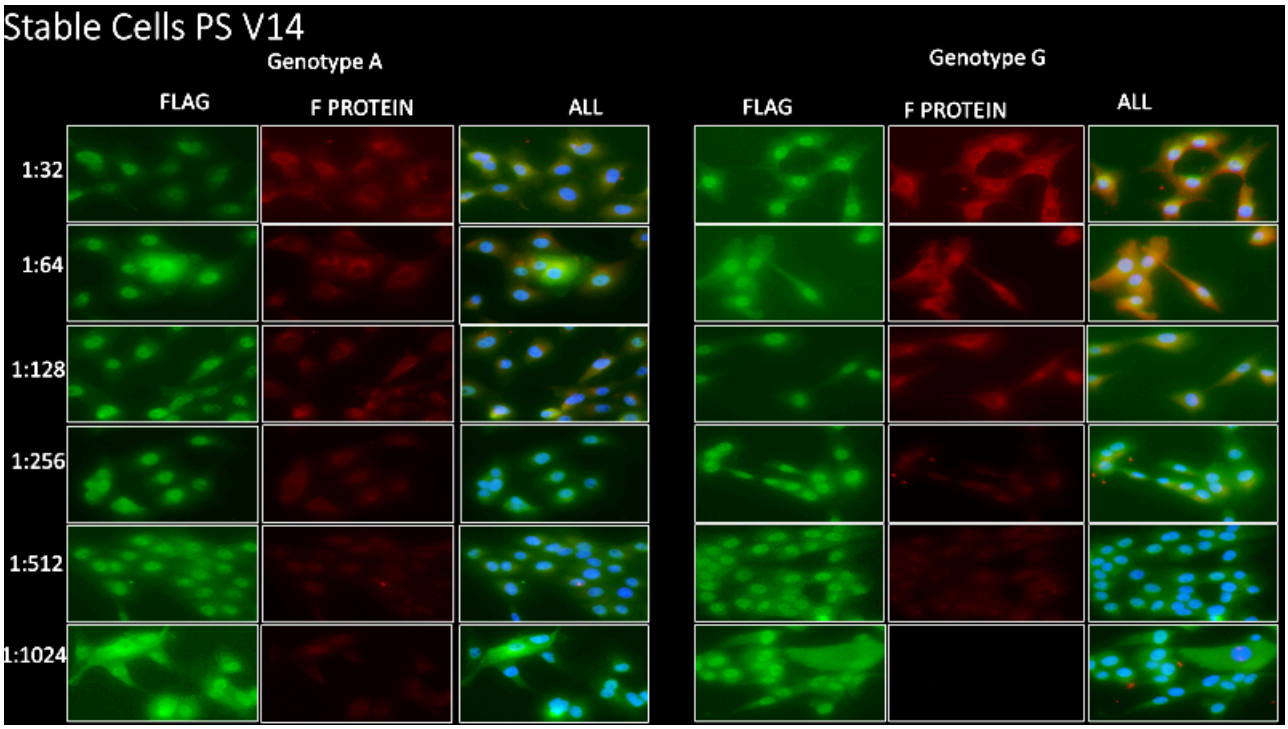


Figure A-58c. Stable cells Vaccine Sample#14.

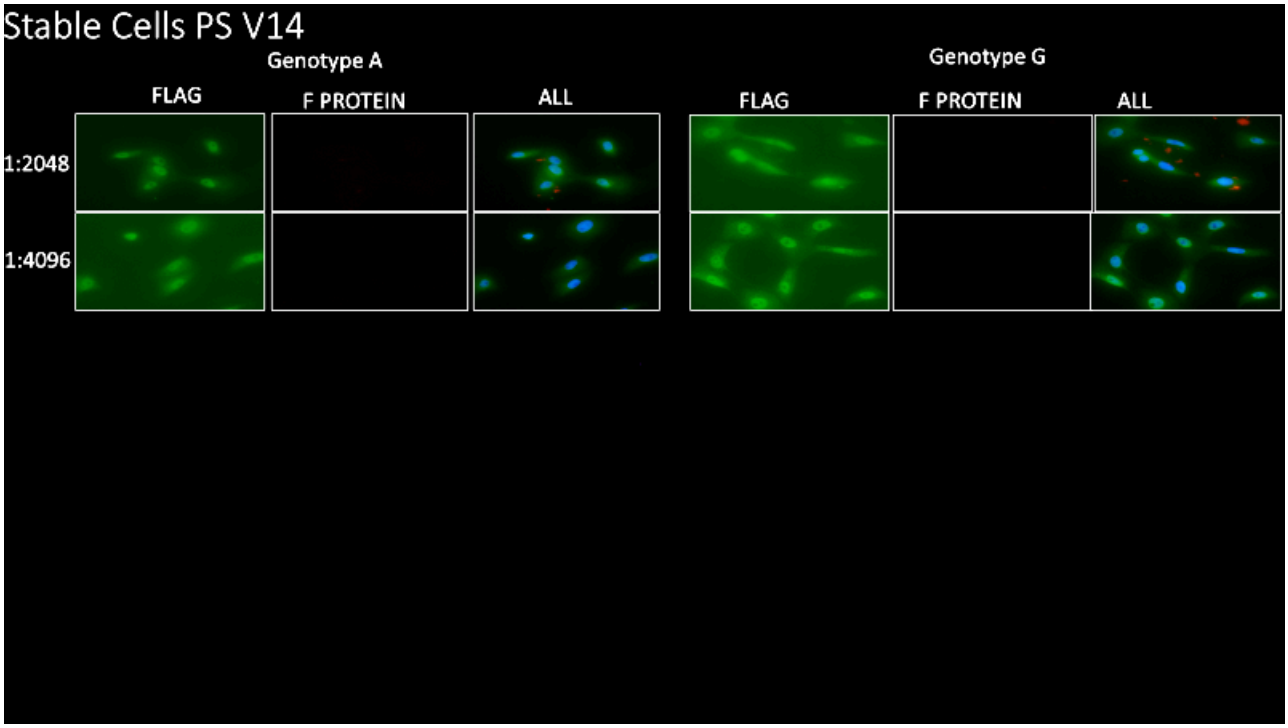


Figure A-58d. Stable cells Vaccine Sample#14.

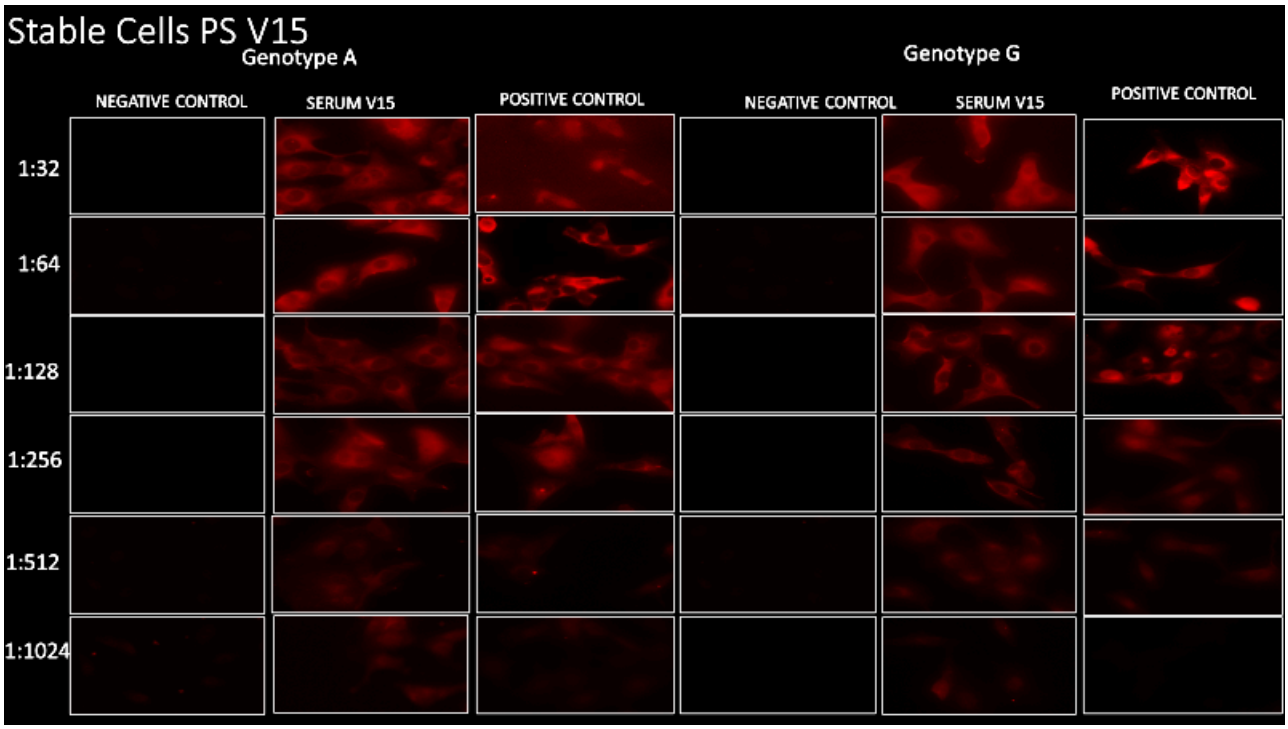


Figure A-59a. Stable cells Vaccine Sample#15.

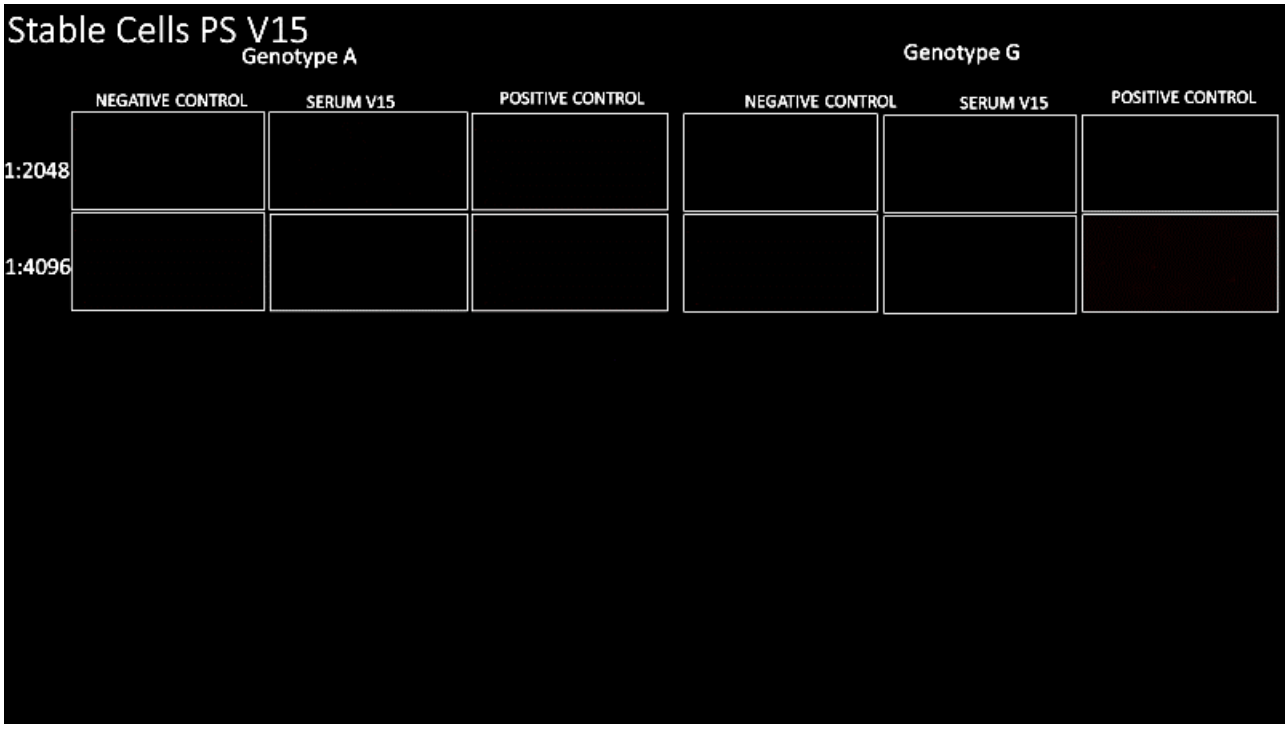


Figure A-59b. Stable cells Vaccine Sample#15.

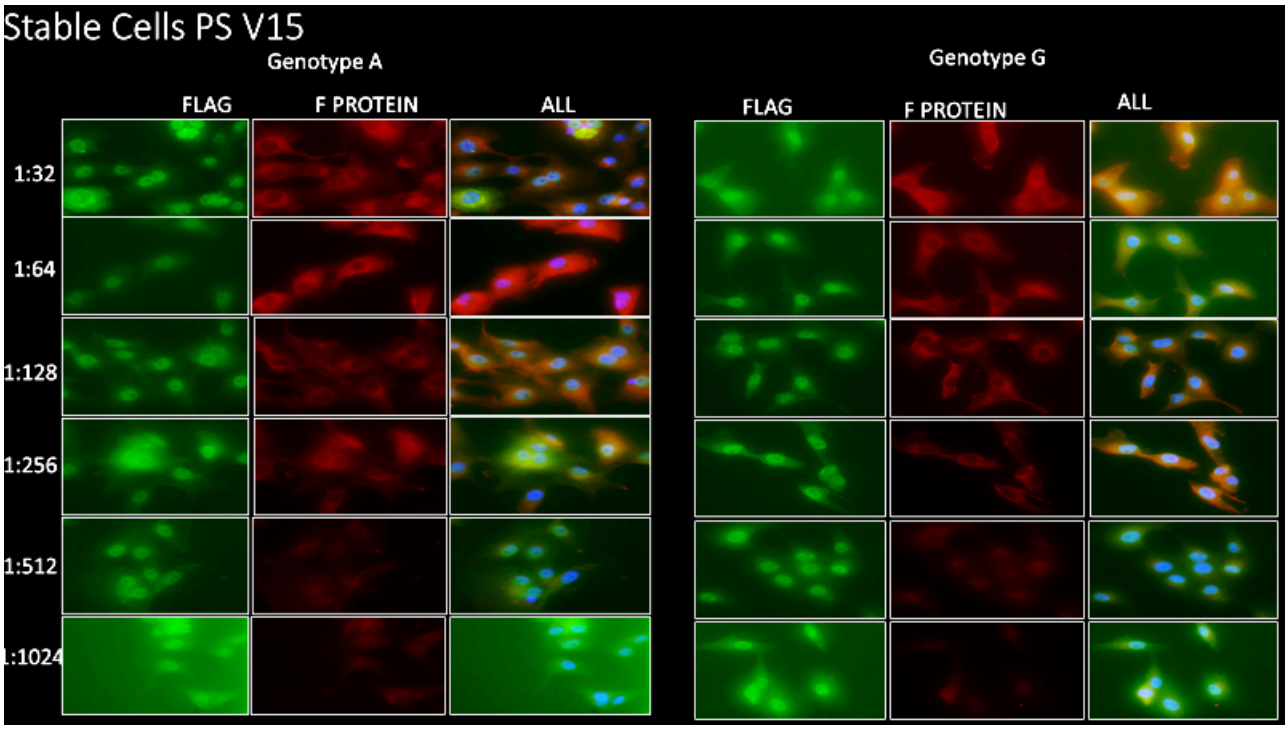


Figure A-59c. Stable cells Vaccine Sample#15.

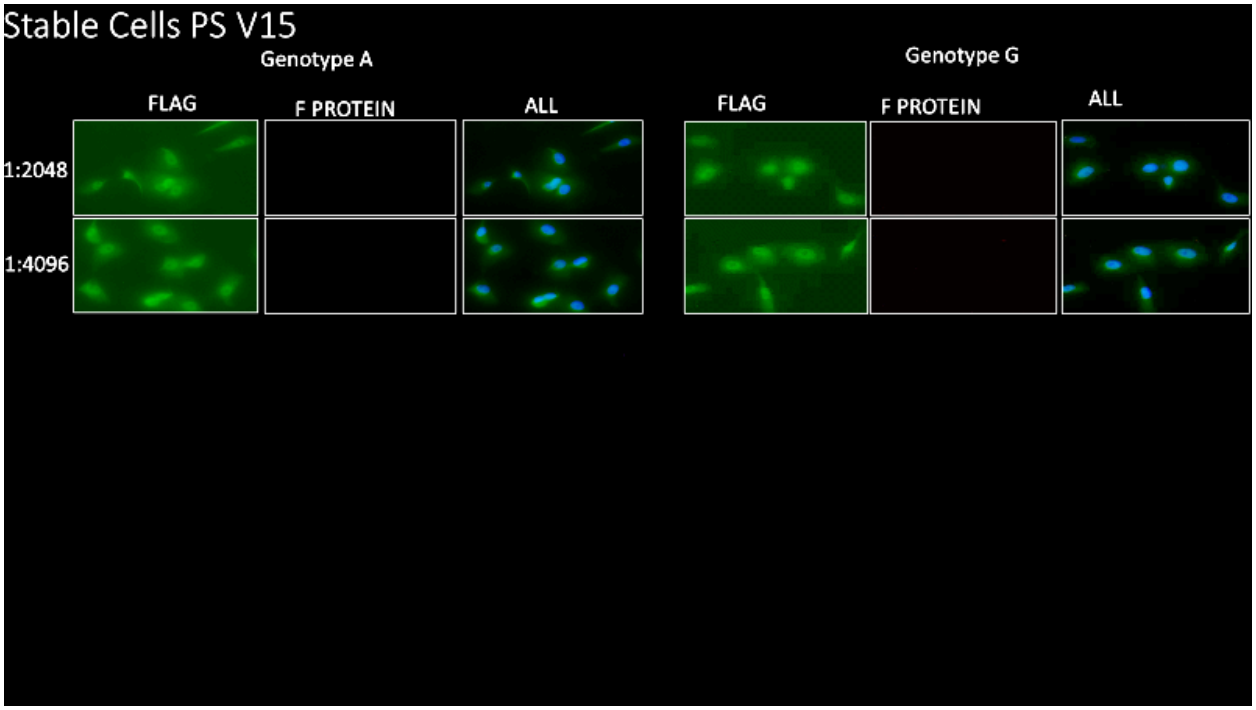


Figure A-59d. Stable cells Vaccine Sample#15.

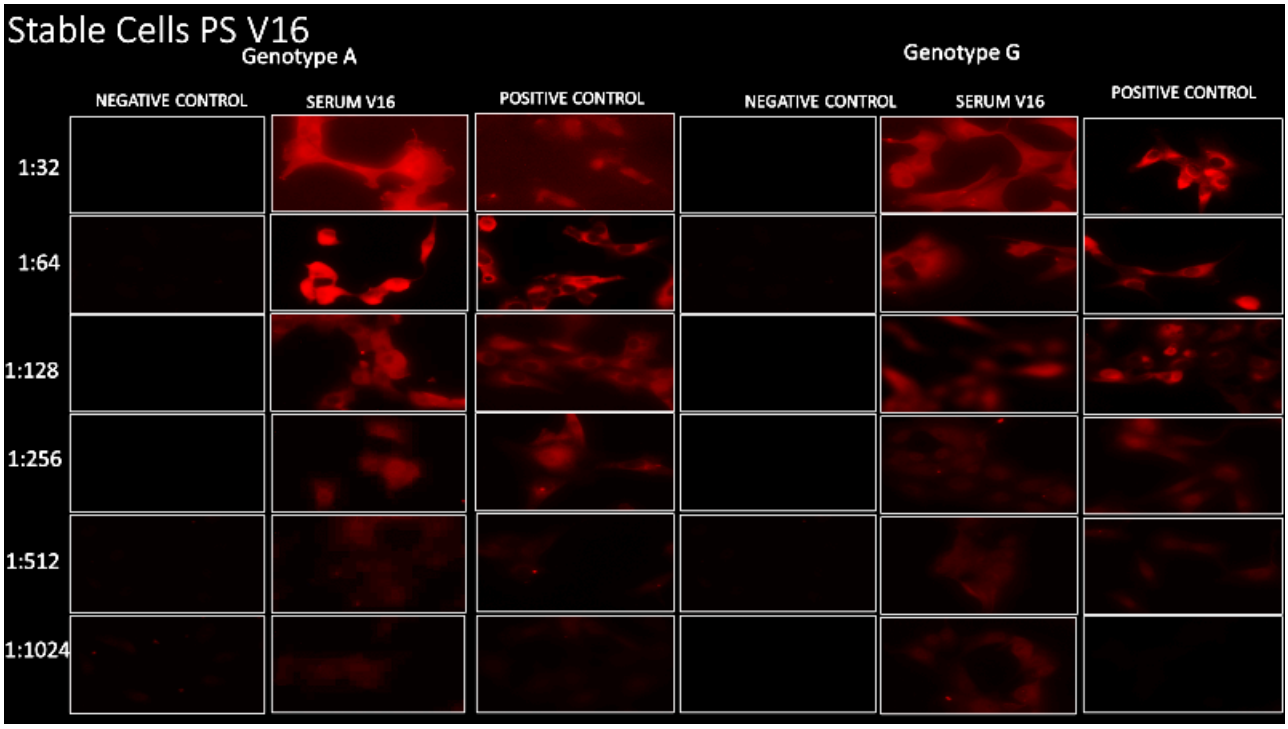


Figure A-60a. Stable cells Vaccine Sample#16.

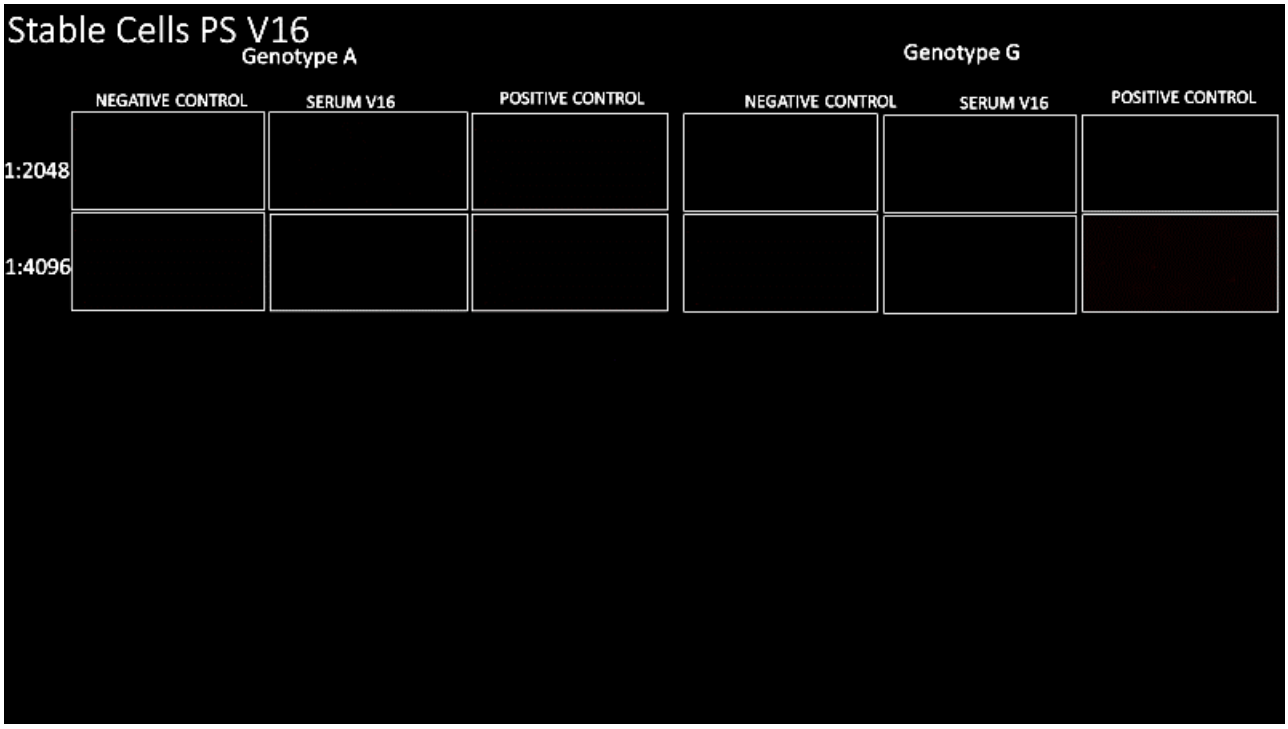


Figure A-60b. Stable cells Vaccine Sample#16.

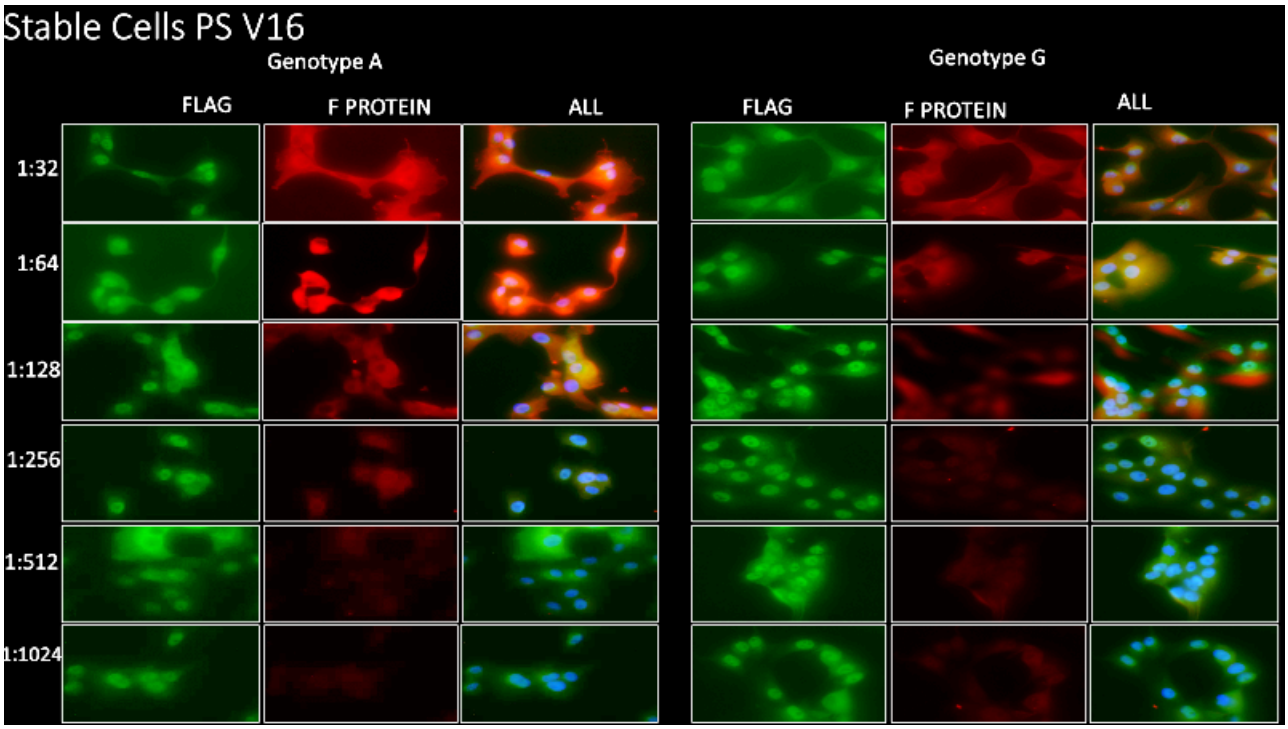


Figure A-60c. Stable cells Vaccine Sample#16.

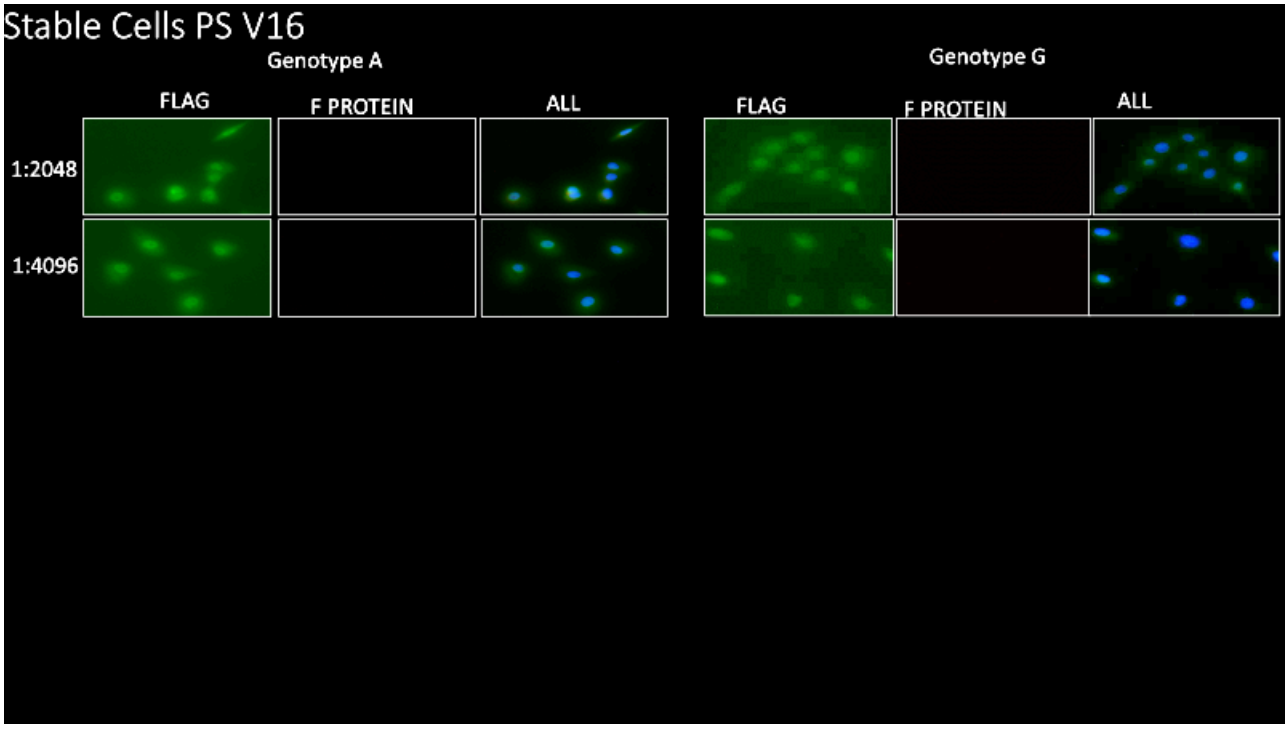


Figure A-60d. Stable cells Vaccine Sample#16.

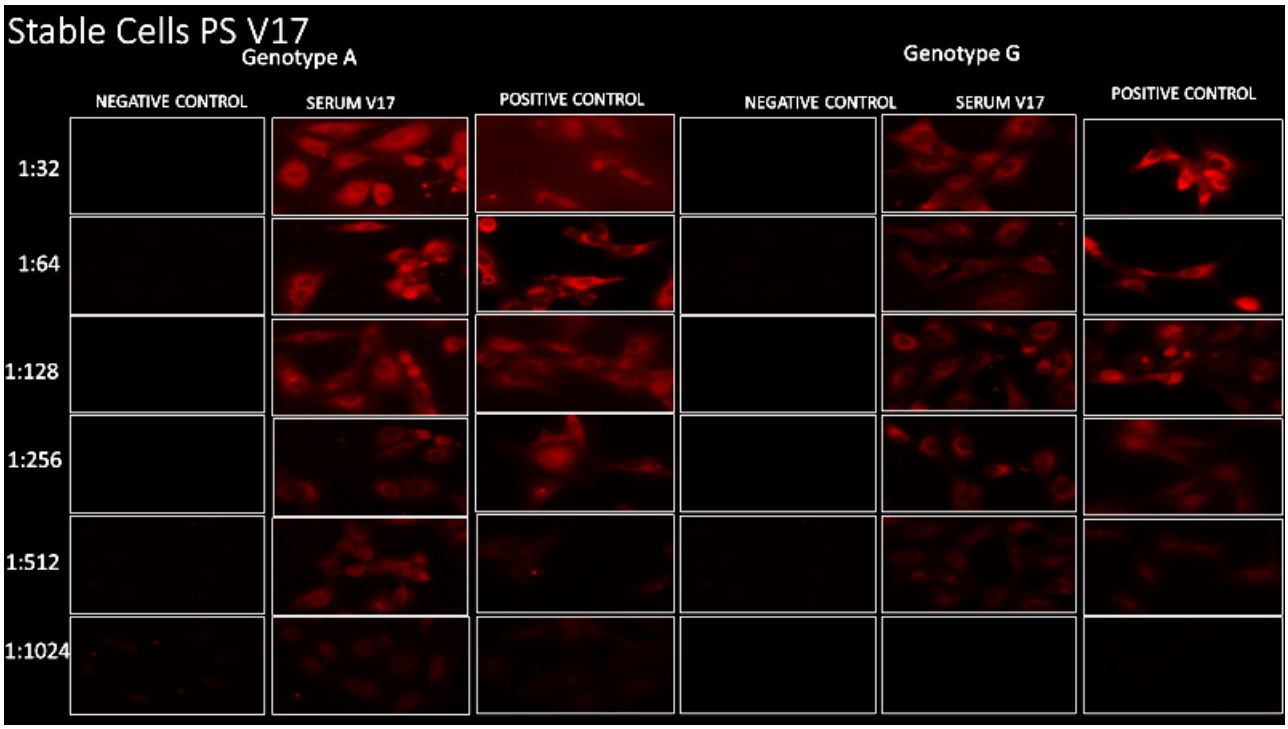


Figure A-61a. Stable cells Vaccine Sample#17.

Figure A-61b. Stable cells Vaccine Sample#17.

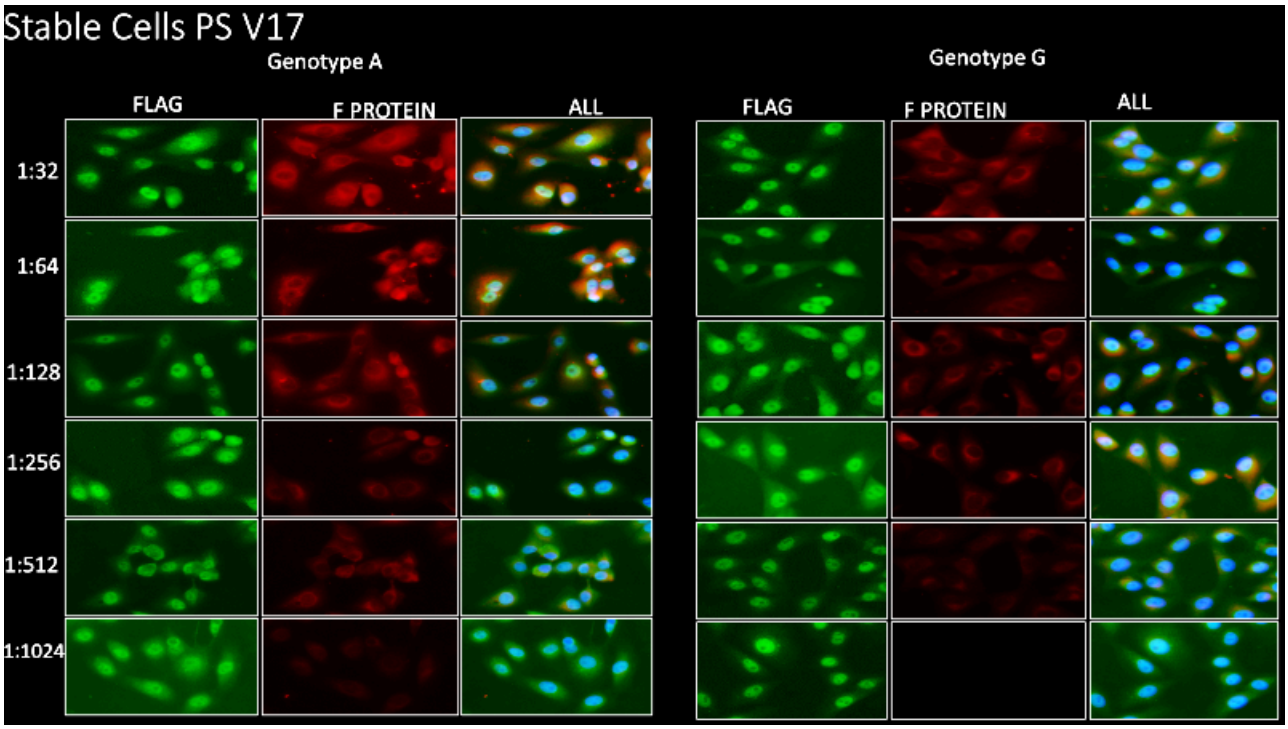


Figure A-61c. Stable cells Vaccine Sample#17.

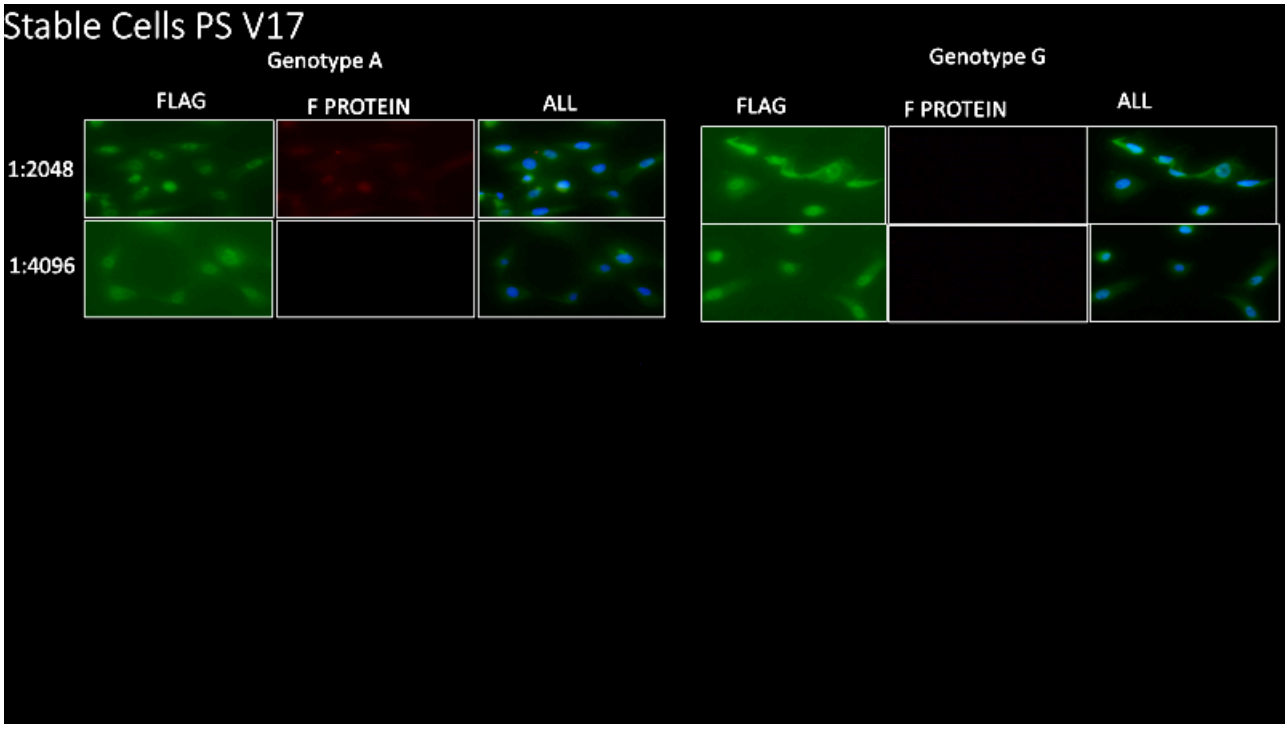


Figure A-61d. Stable cells Vaccine Sample#17.

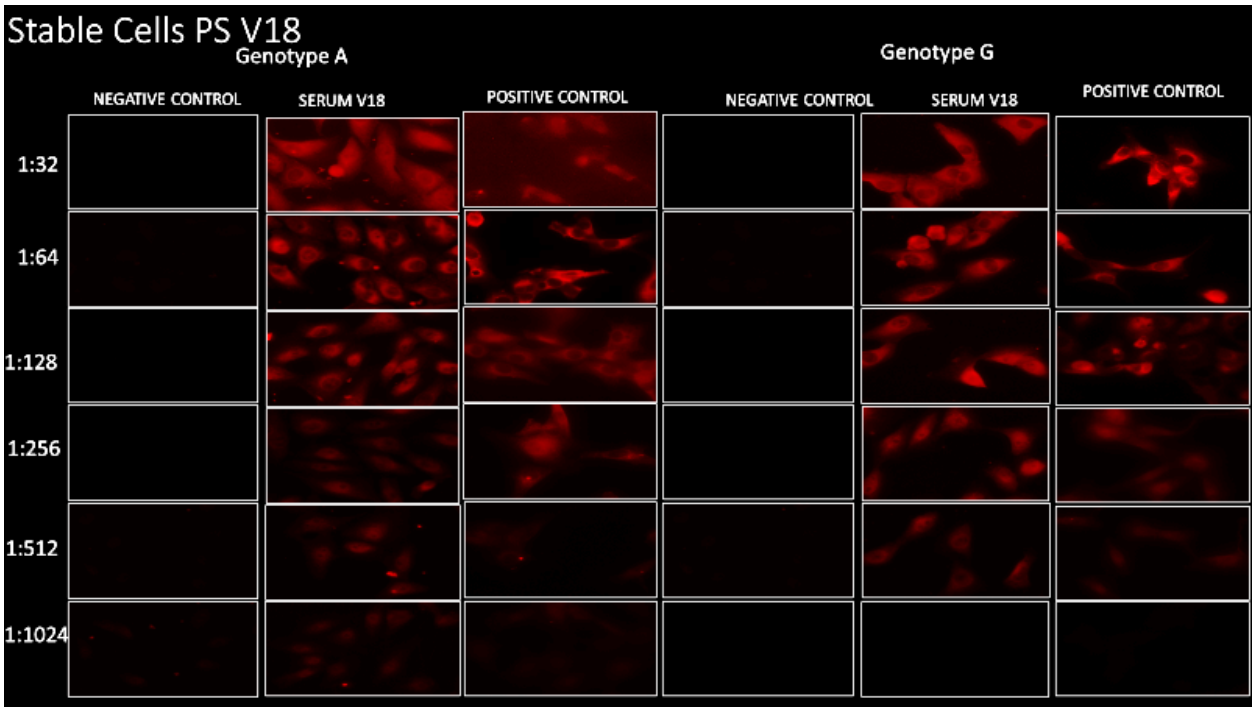


Figure A-62a. Stable cells Vaccine Sample#18.

Figure A-62b. Stable cells Vaccine Sample#18.

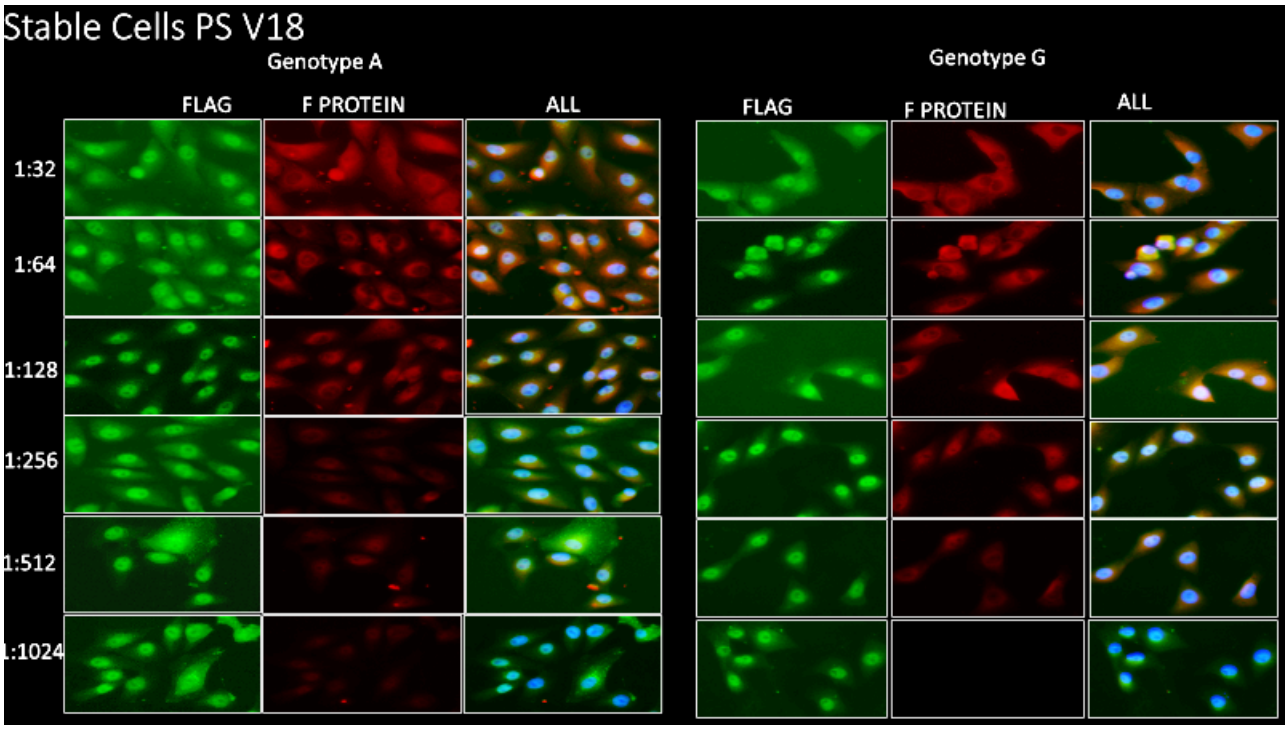


Figure A-62c. Stable cells Vaccine Sample#18.

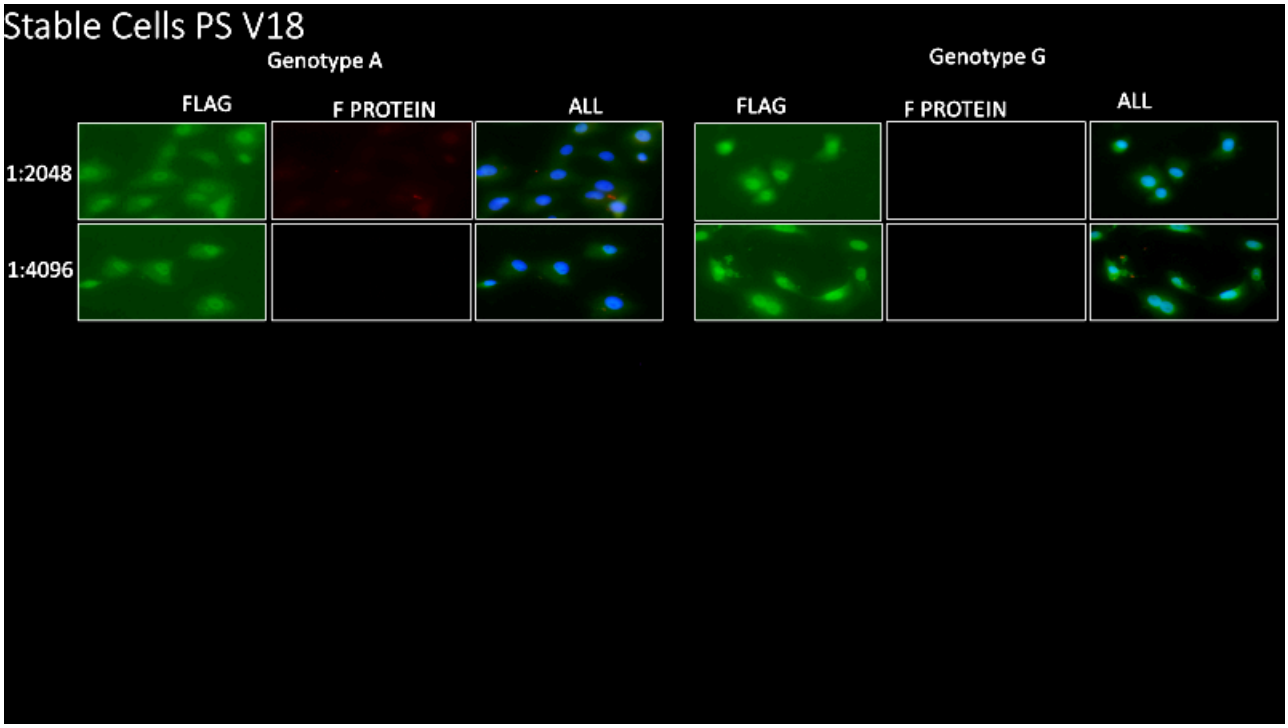


Figure A-62d. Stable cells Vaccine Sample#18.

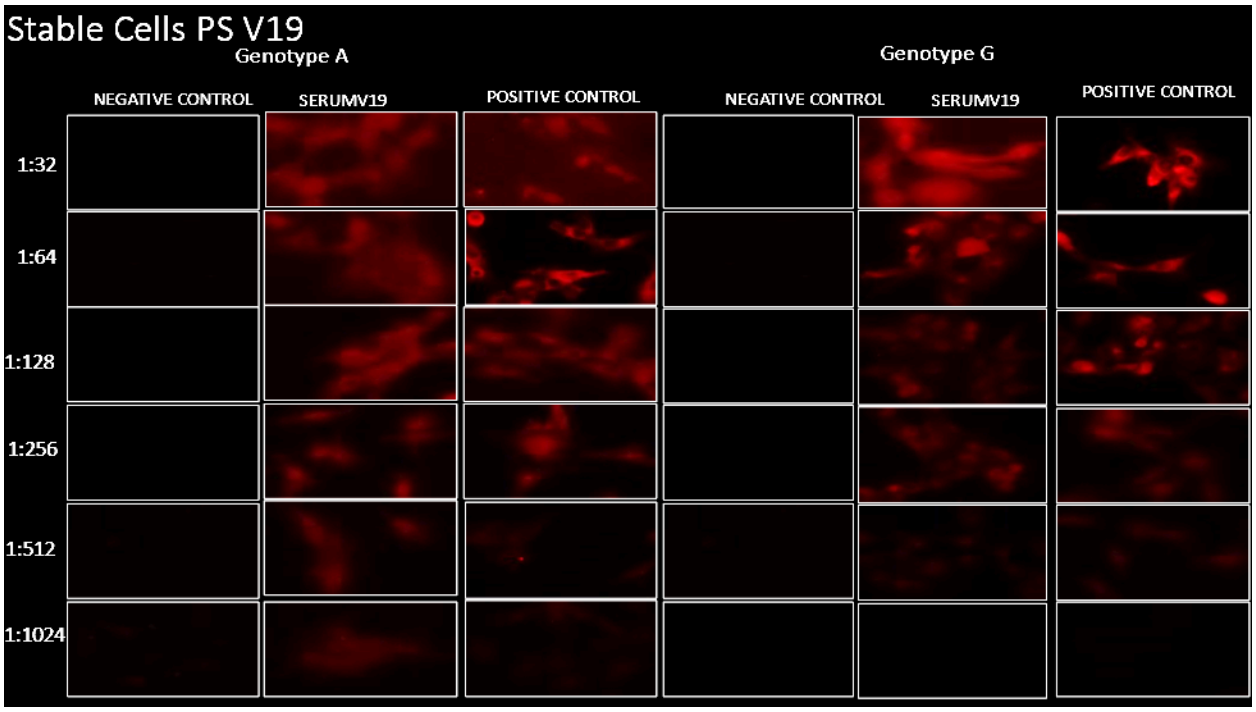


Figure A-63a. Stable cells Vaccine Sample#19.

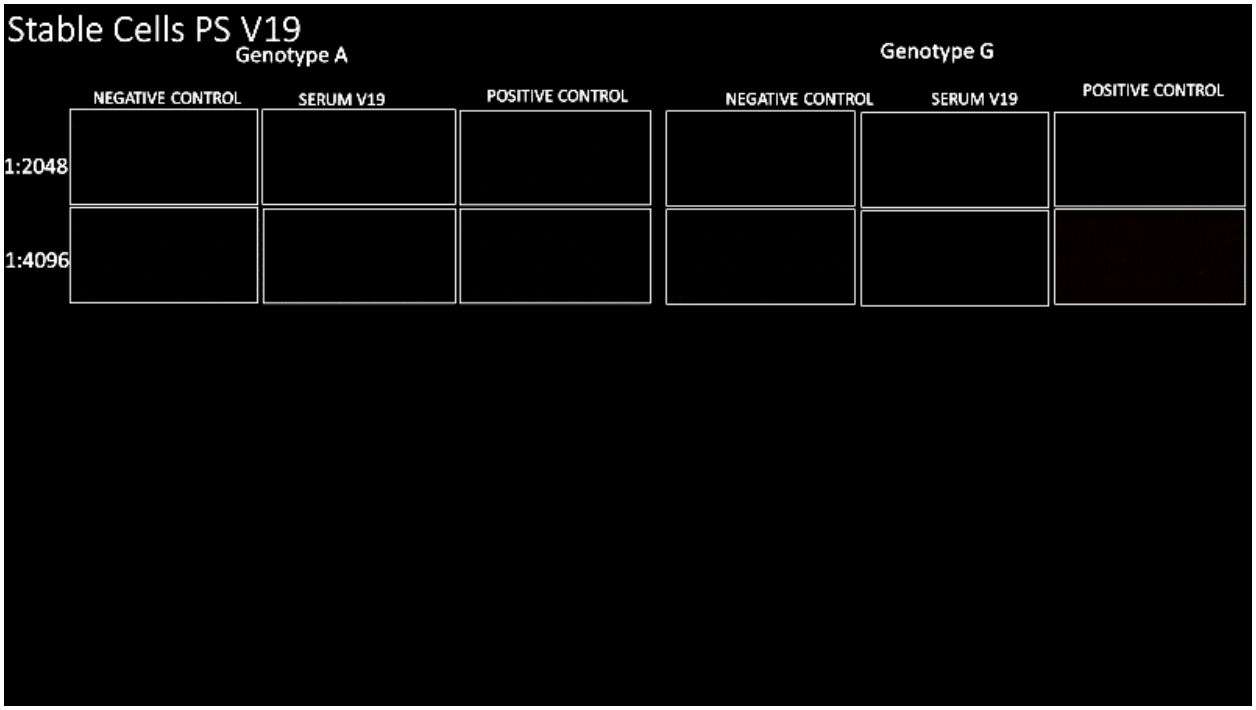


Figure A-63b. Stable cells Vaccine Sample#19.

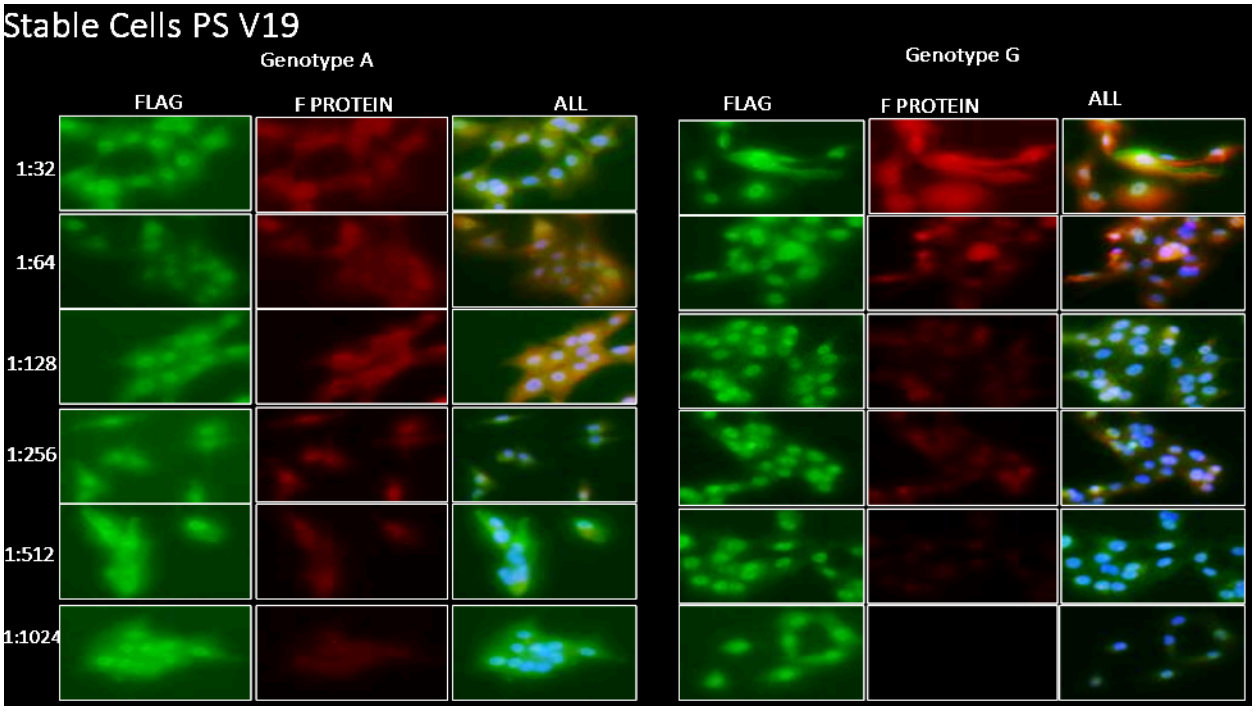


Figure A-63c. Stable cells Vaccine Sample#19.

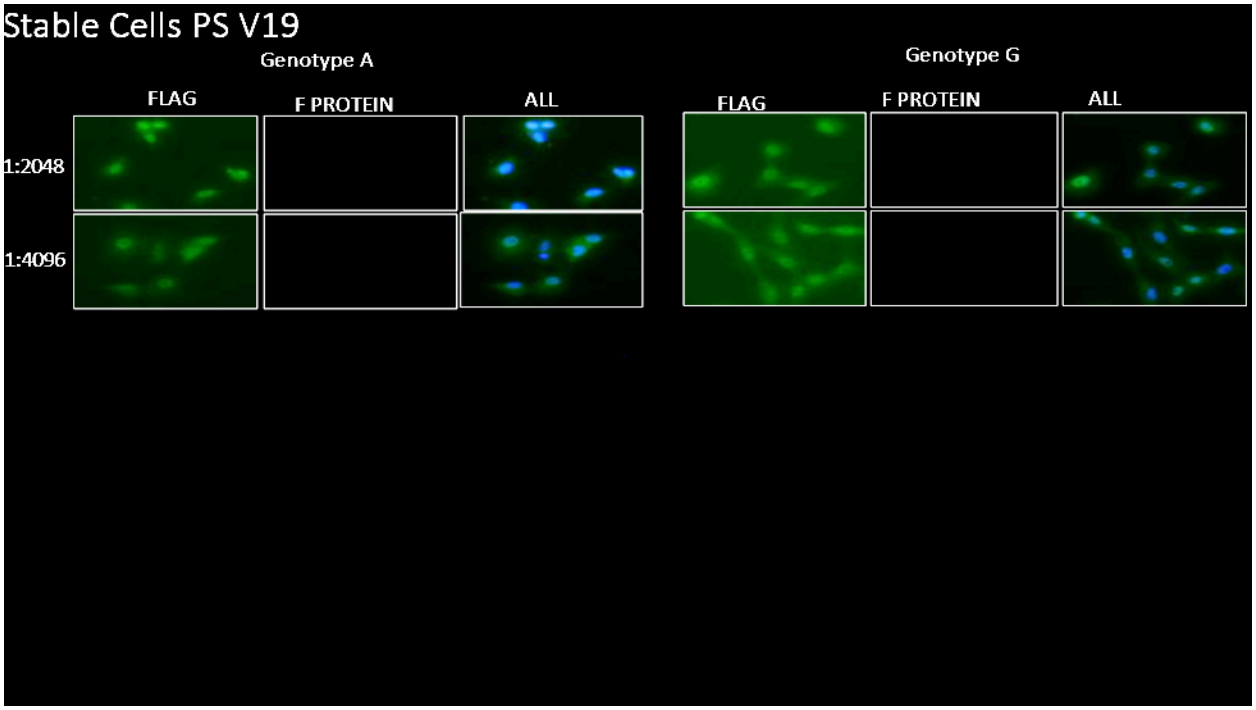


Figure A-63d. Stable cells Vaccine Sample#19.

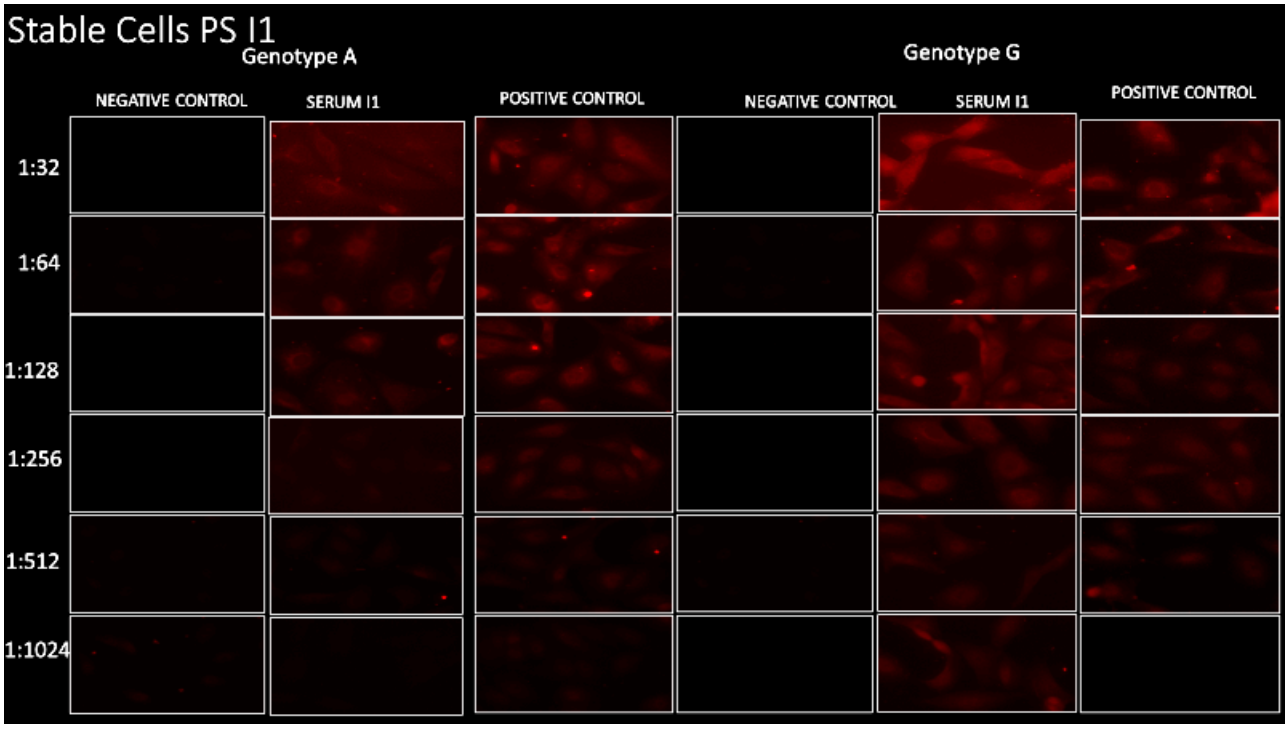


Figure A-64a. Stable cells Infected Sample#1.

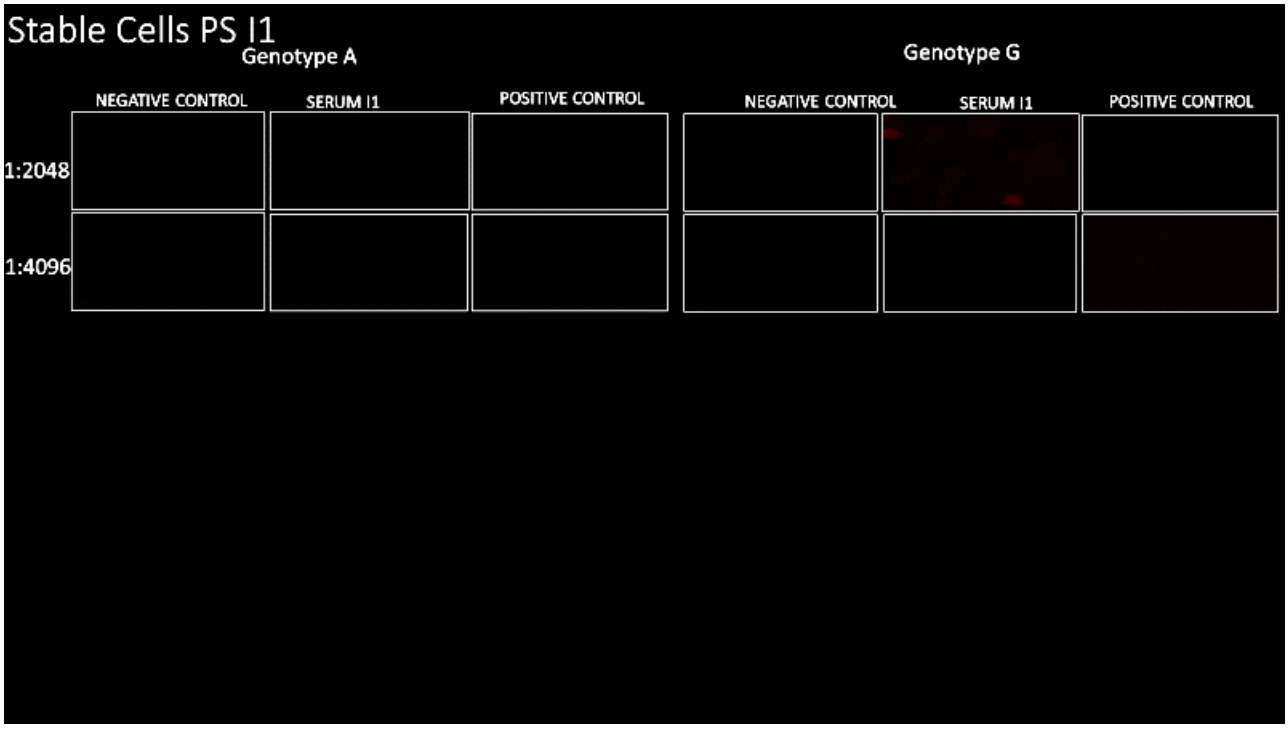


Figure A-64b. Stable cells Infected Sample#1.

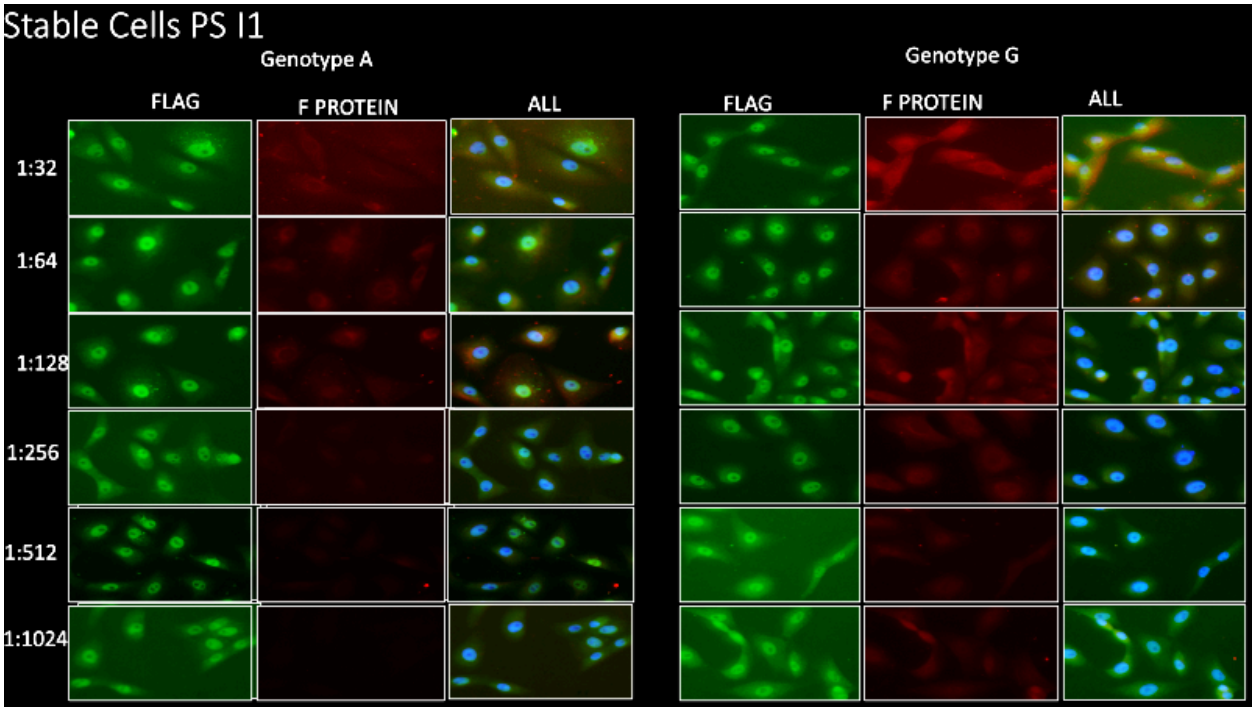


Figure A-64c. Stable cells Infected Sample#1.

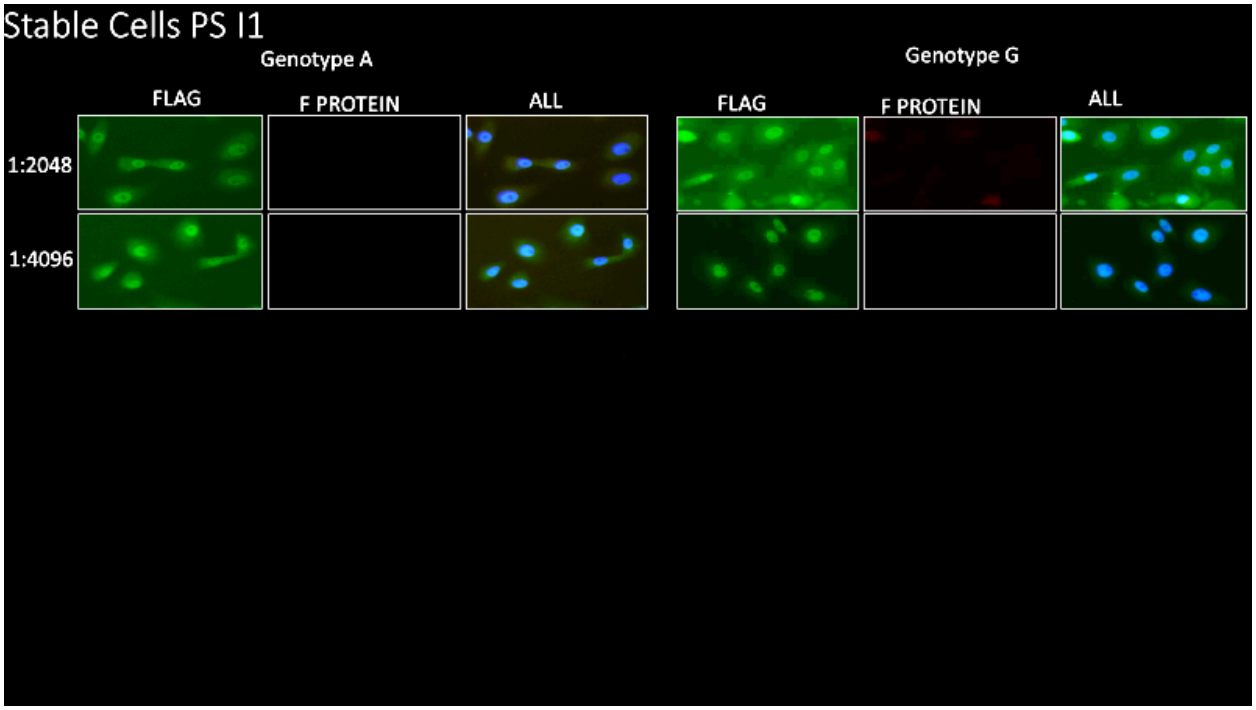


Figure A-64d. Stable cells Infected Sample#1.

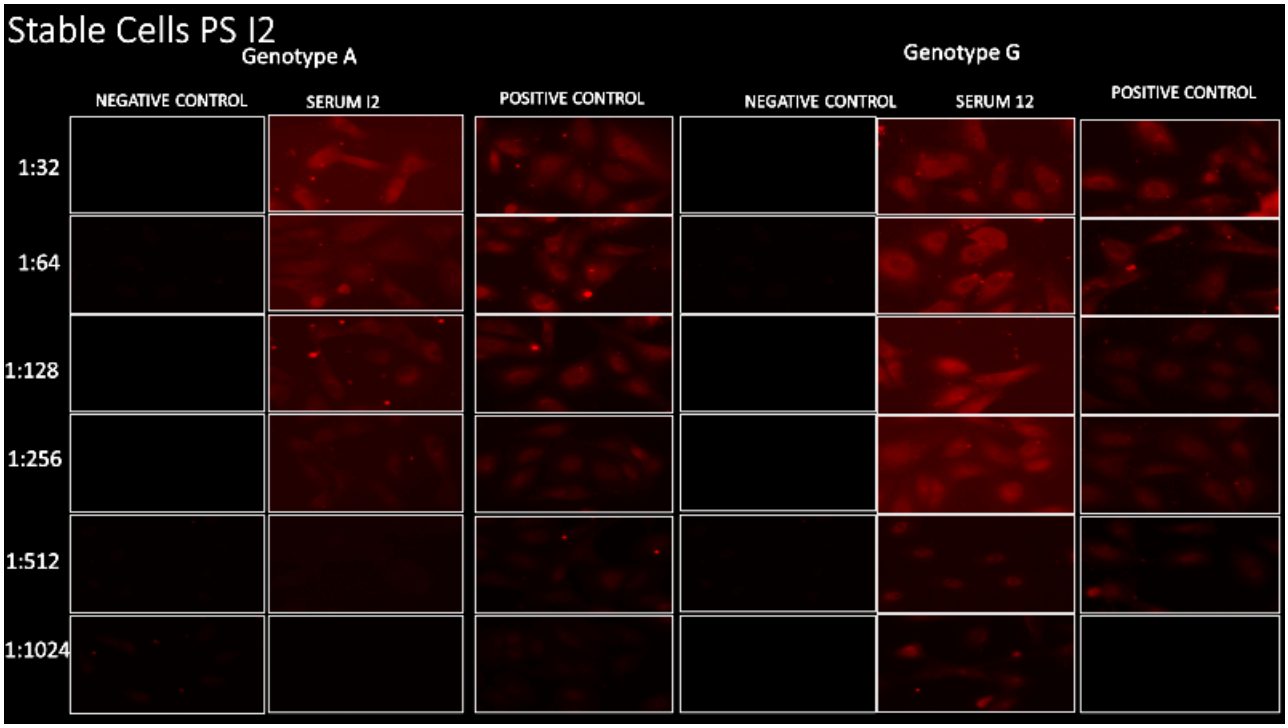


Figure A-65a. Stable cells Infected Sample#2.

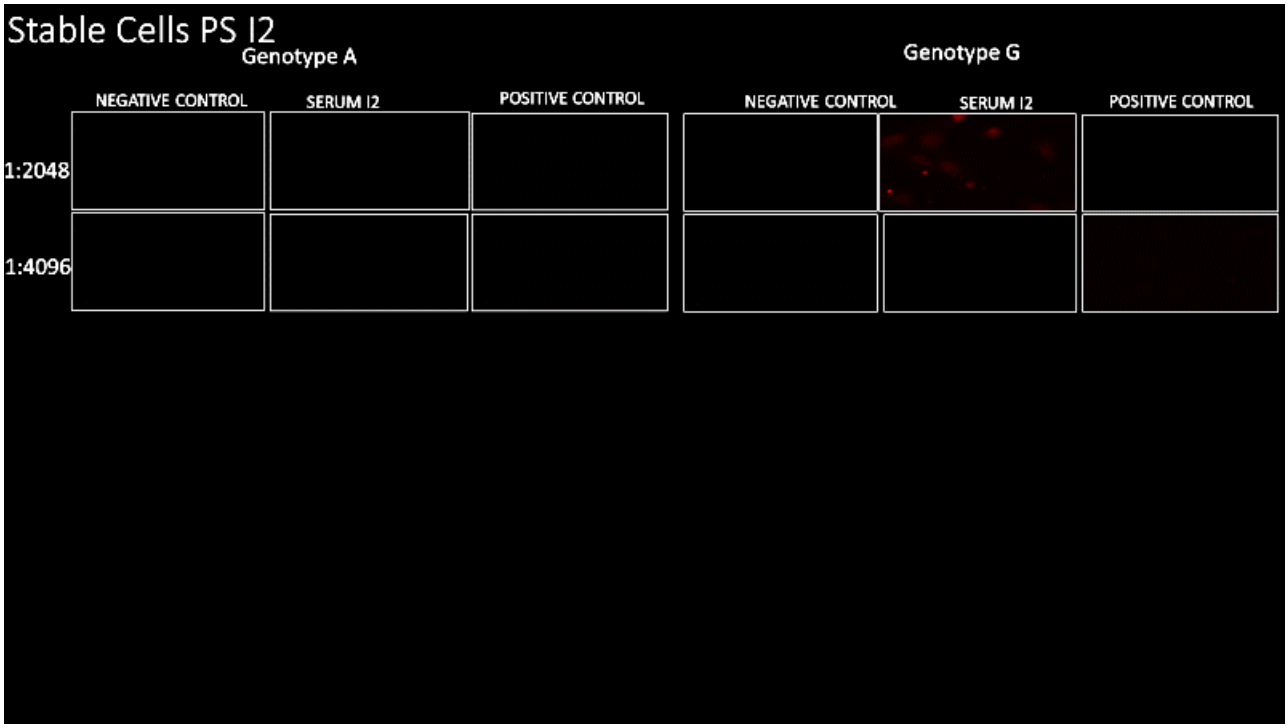


Figure A-65b. Stable cells Infected Sample#2.

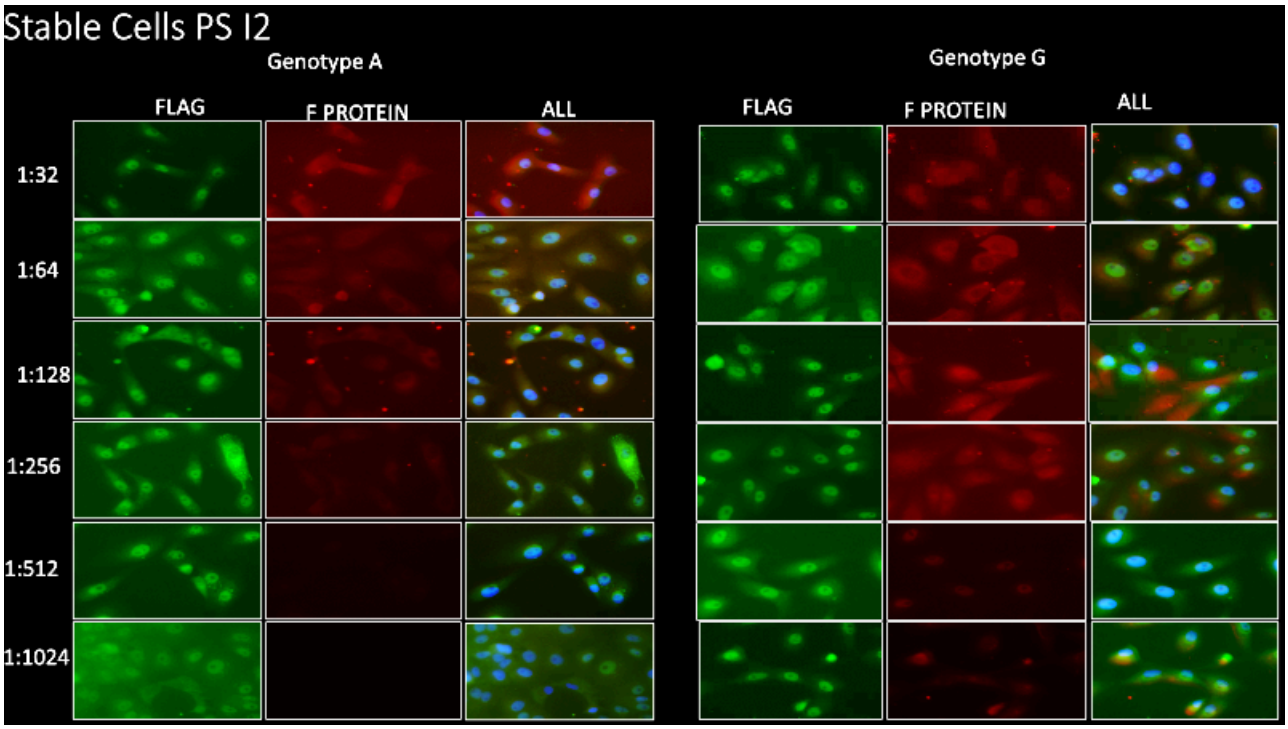


Figure A-65c. Stable cells Infected Sample#2.

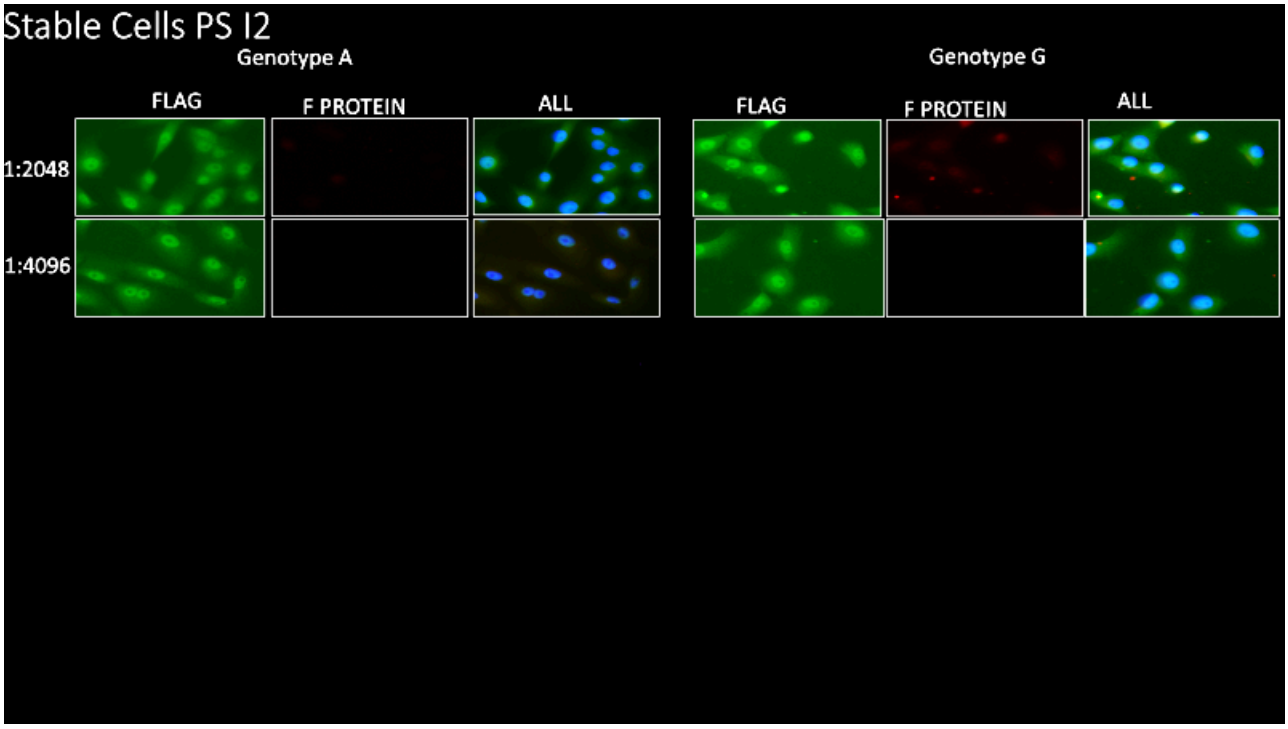


Figure A-65d. Stable cells Infected Sample#2.

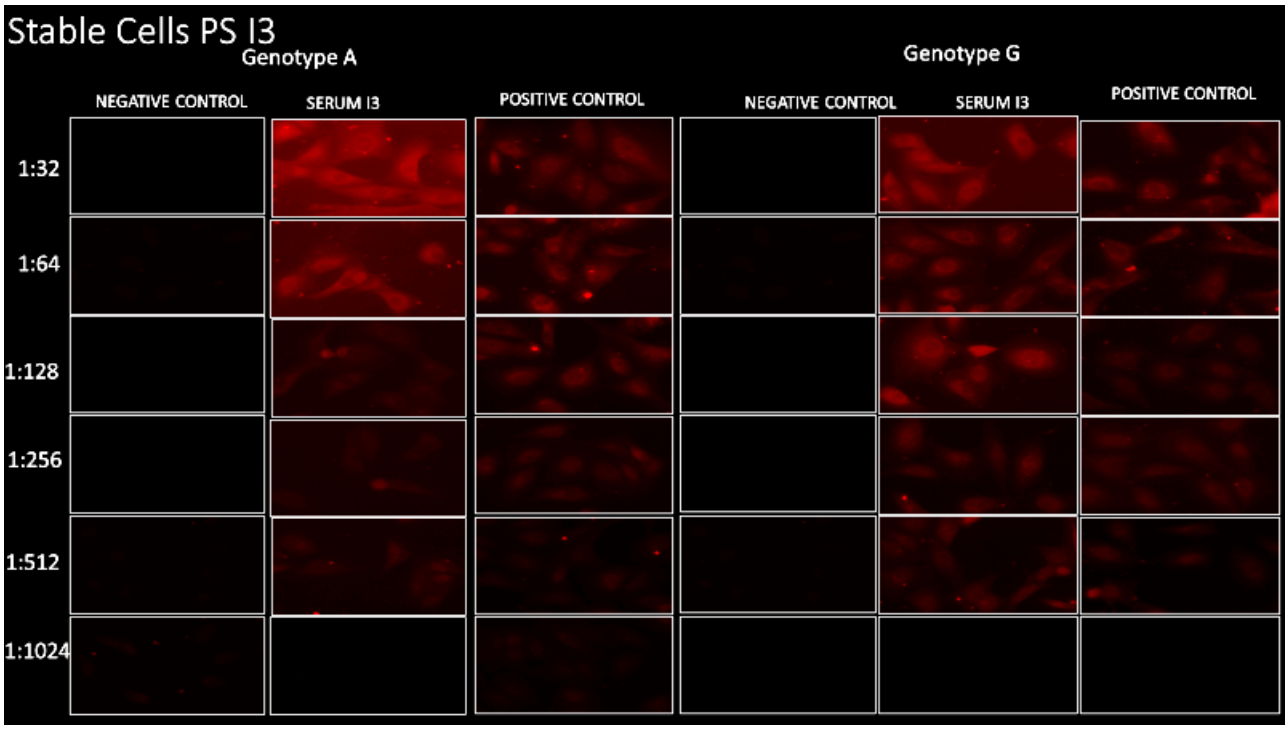


Figure A-66a. Stable cells Infected Sample#3.

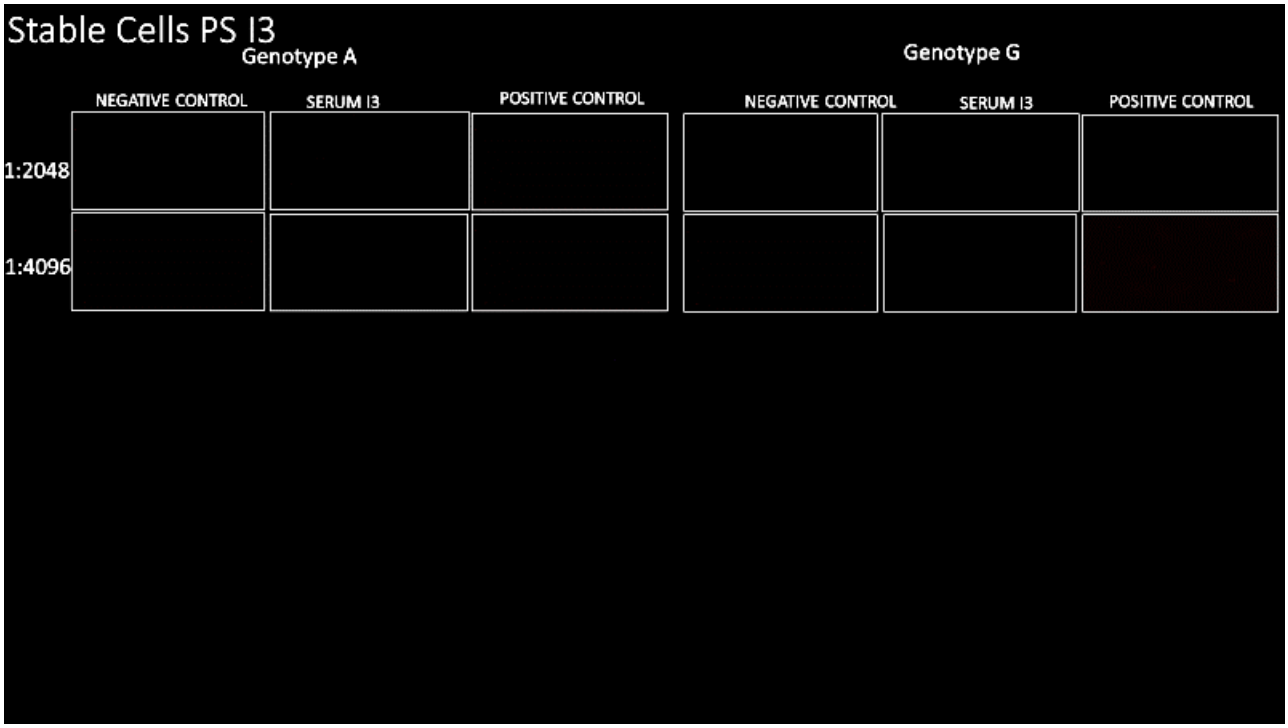


Figure A-66b. Stable cells Infected Sample#3.

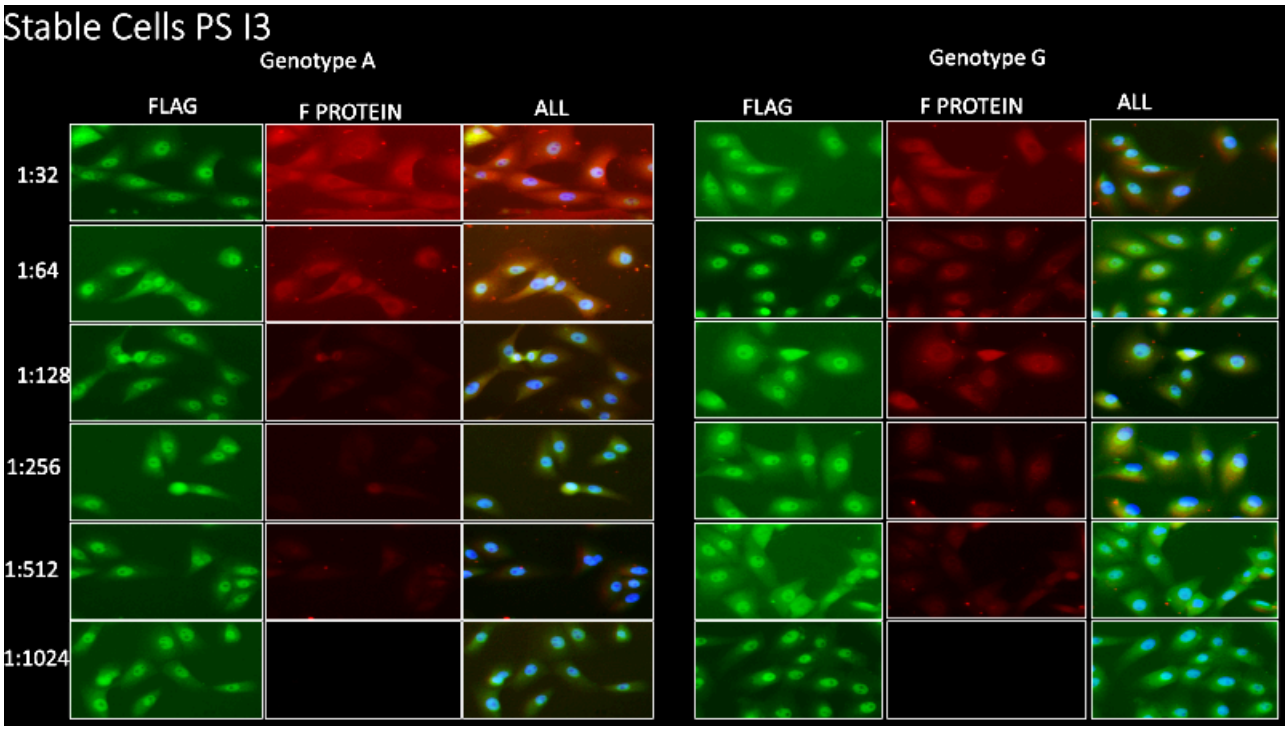


Figure A-66c. Stable cells Infected Sample#3.

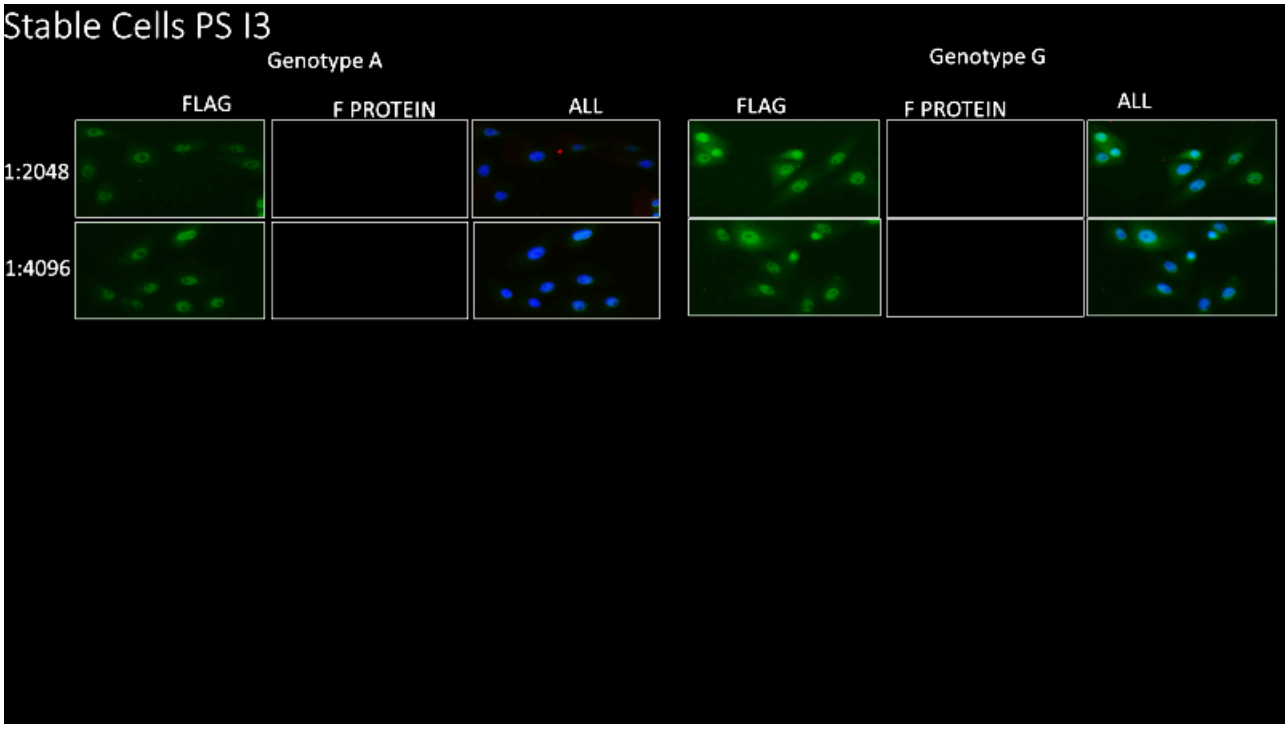


Figure A-66d. Stable cells Infected Sample#3.

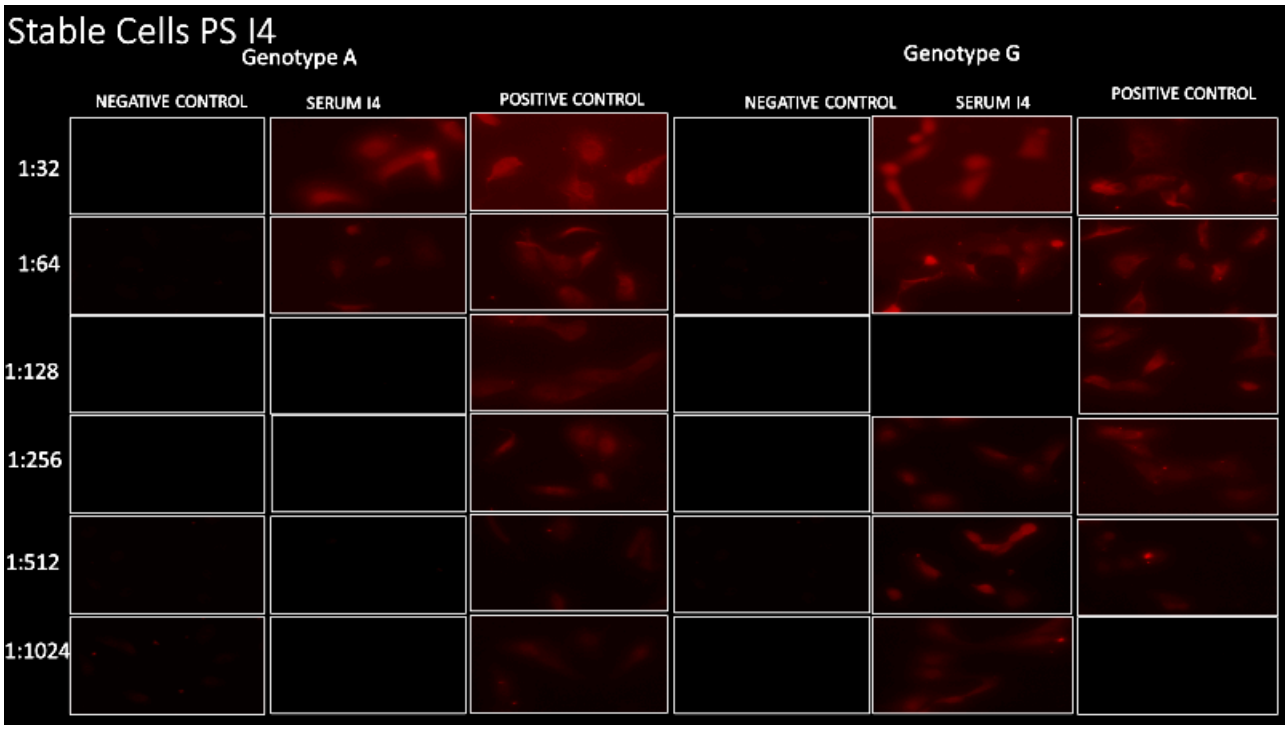


Figure A-67a. Stable cells Infected Sample#4.

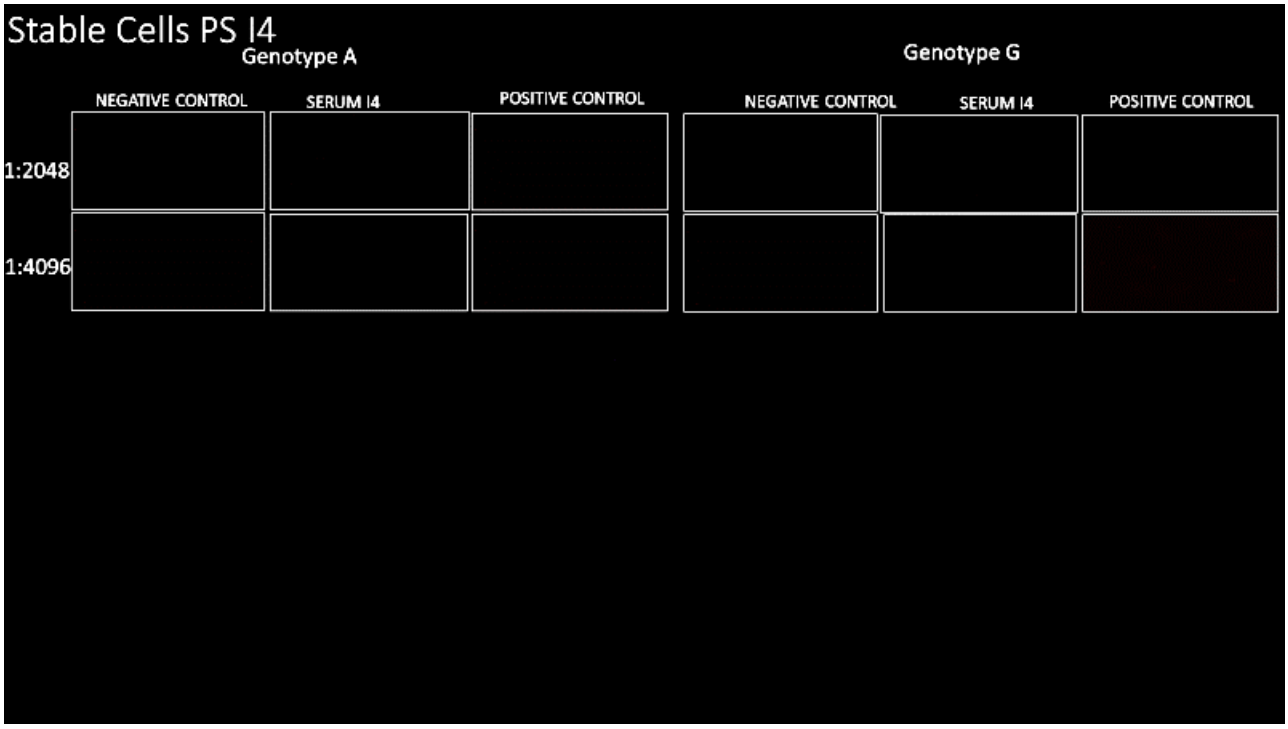


Figure A-67b. Stable cells Infected Sample#4.

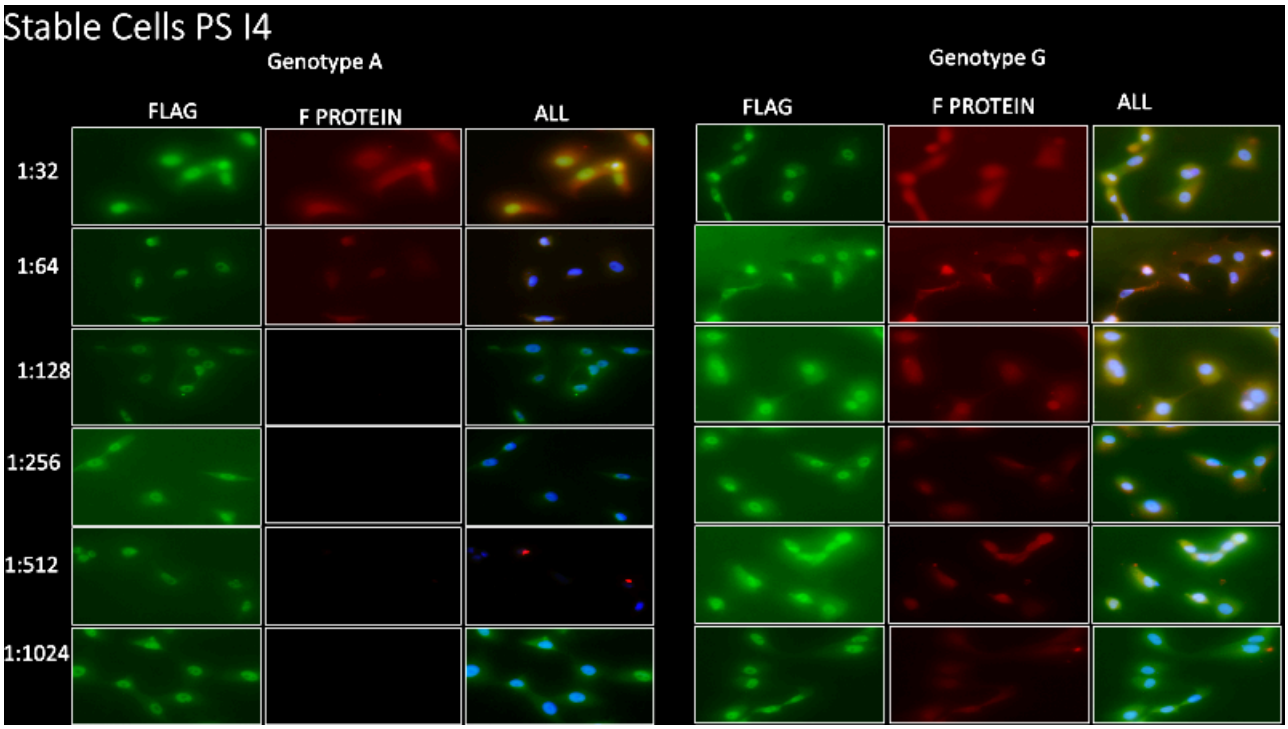


Figure A-67c. Stable cells Infected Sample#4.

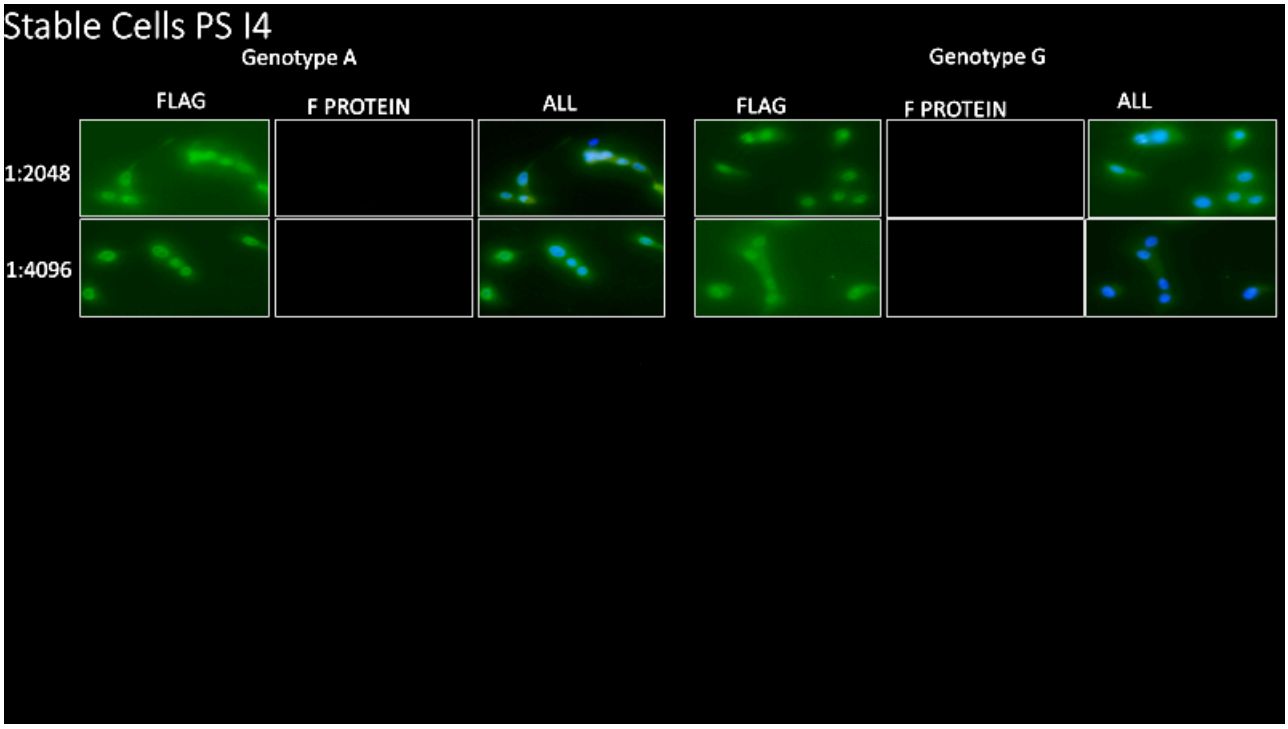


Figure A-67d. Stable cells Infected Sample#4.

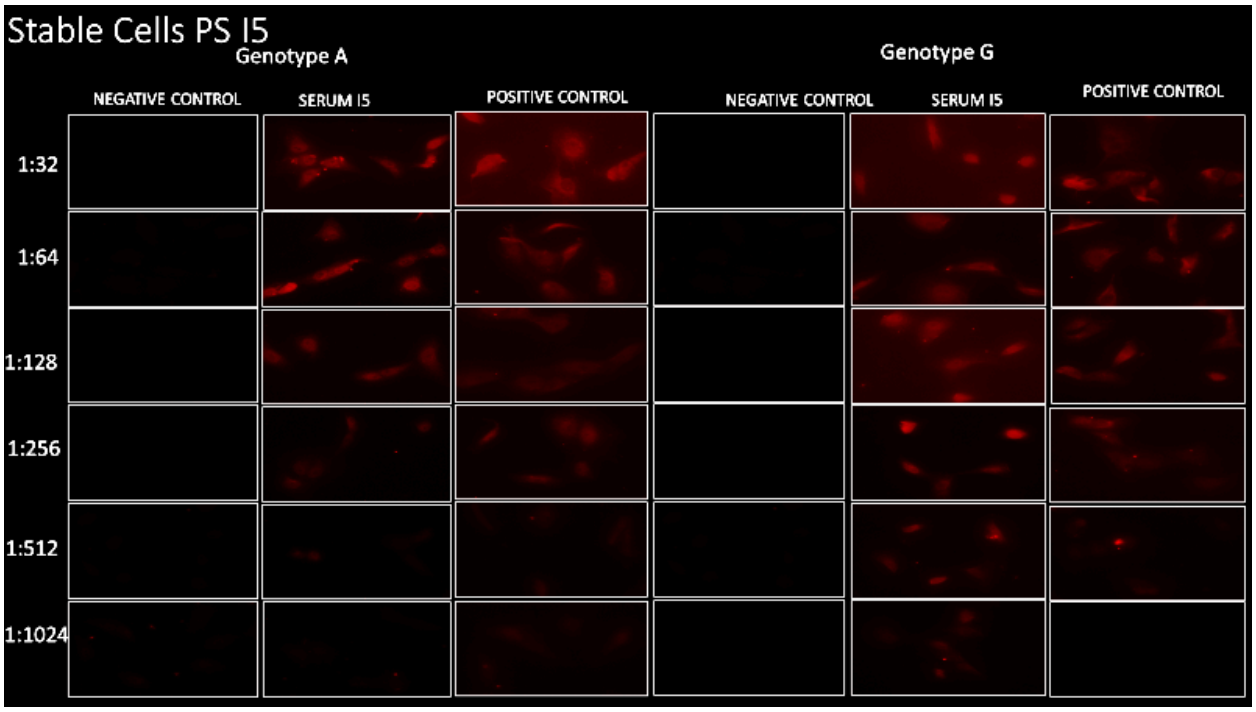


Figure A-68a. Stable cells Infected Sample#5.

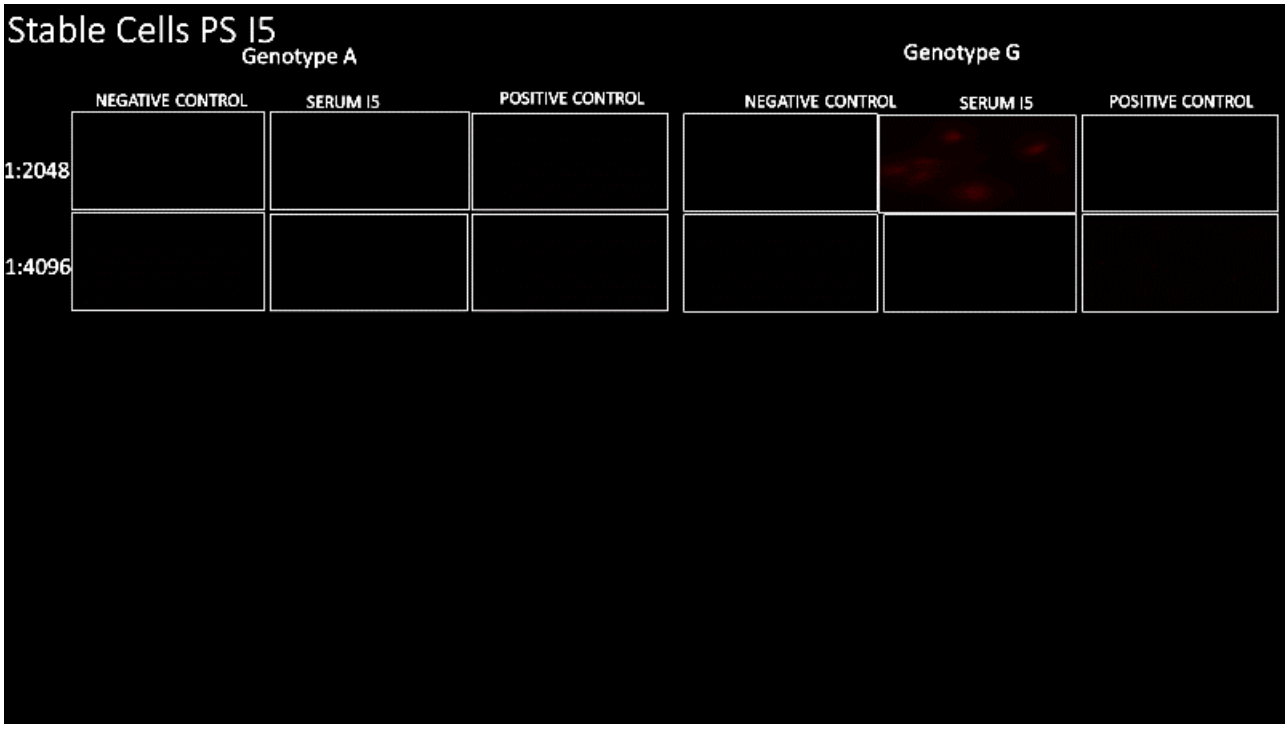


Figure A-68b. Stable cells Infected Sample#5.

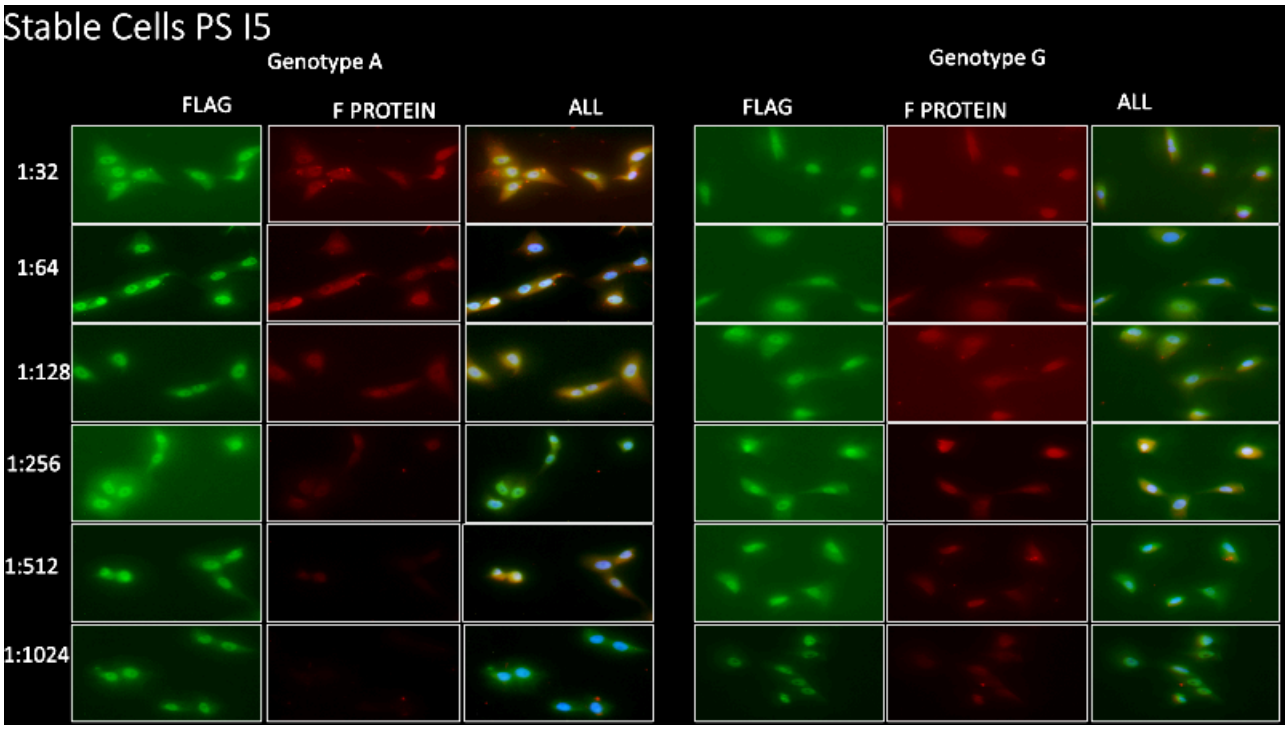


Figure A-68c. Stable cells Infected Sample#5.

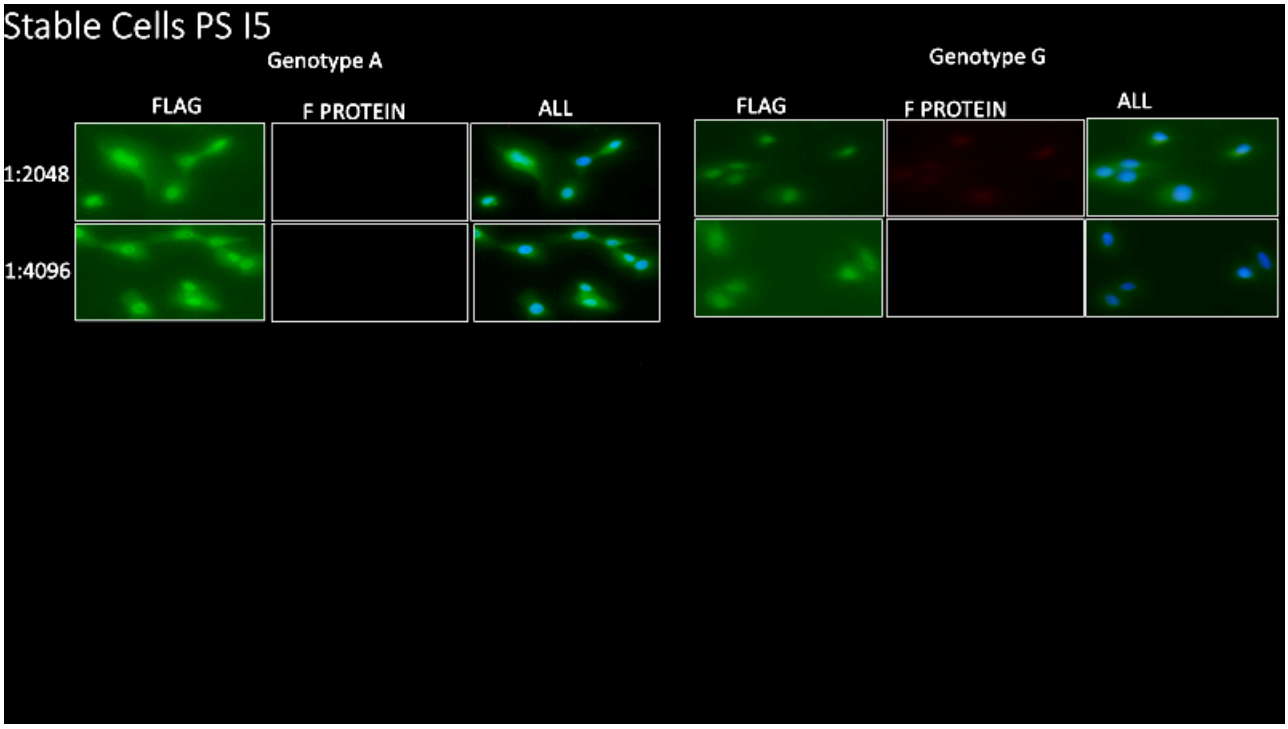


Figure A-68d. Stable cells Infected Sample#5.

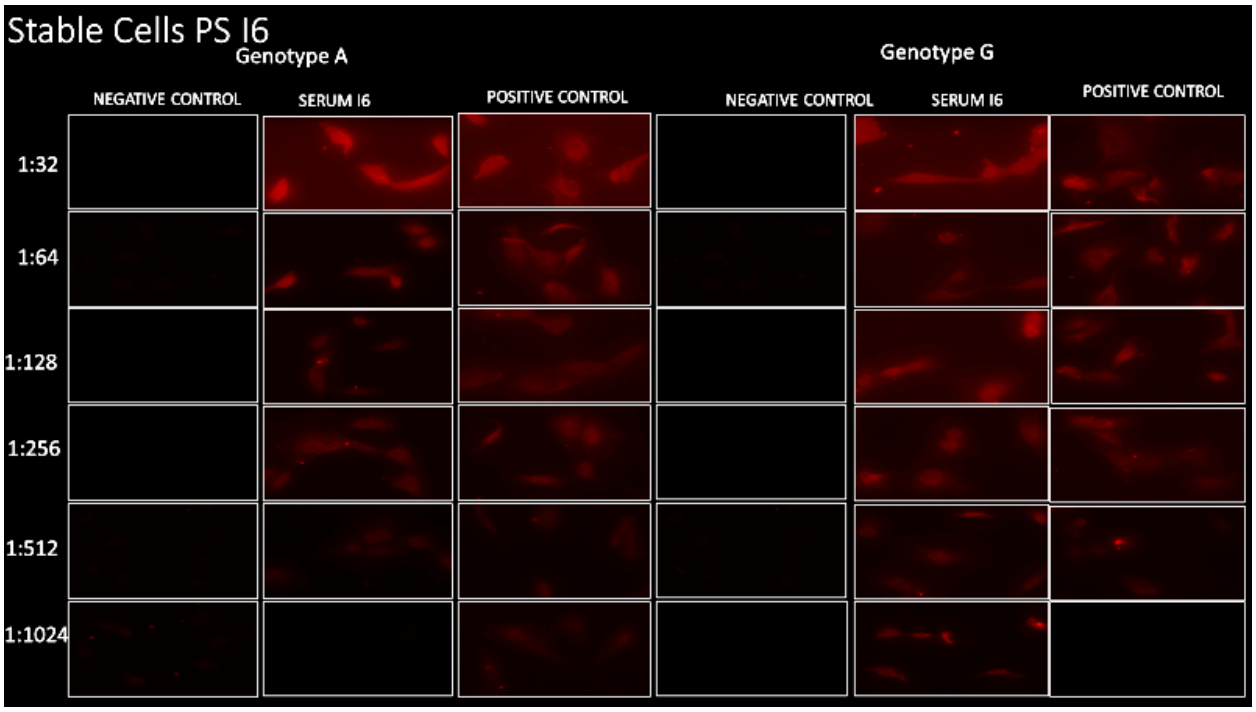


Figure A-69a. Stable cells Infected Sample#6.

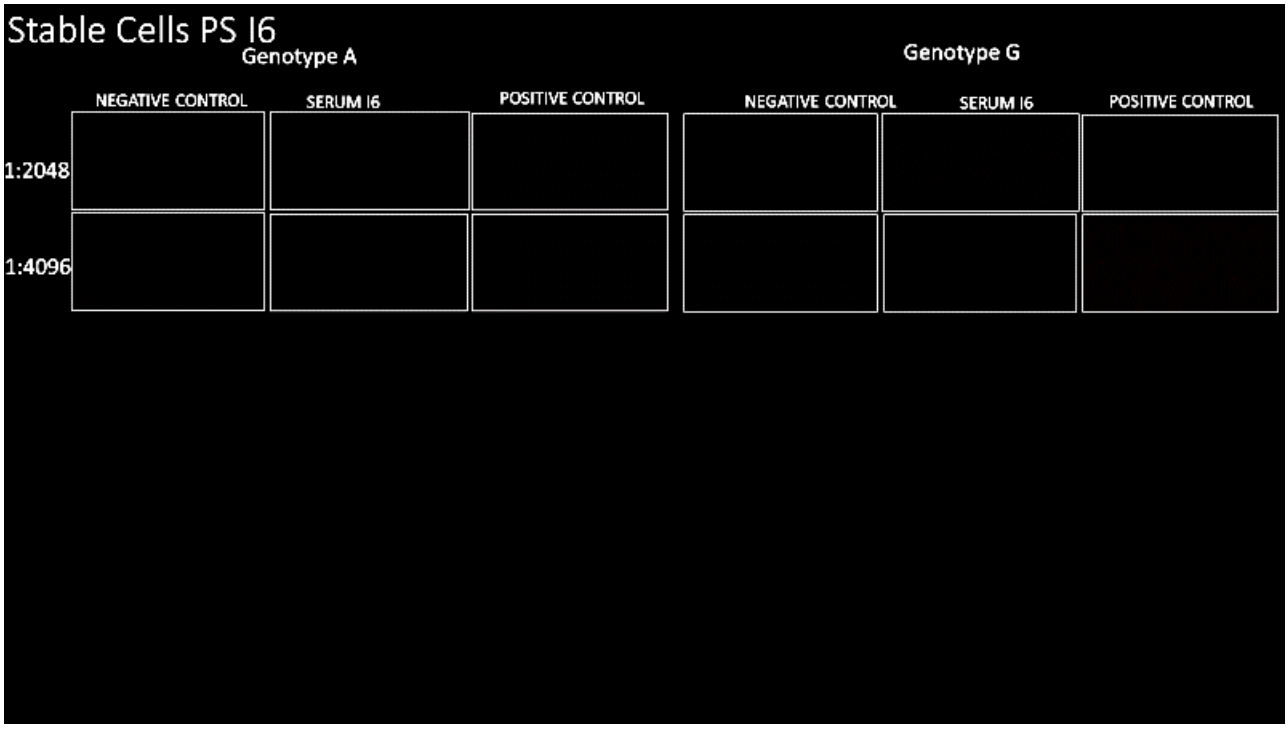


Figure A-69b. Stable cells Infected Sample#6.

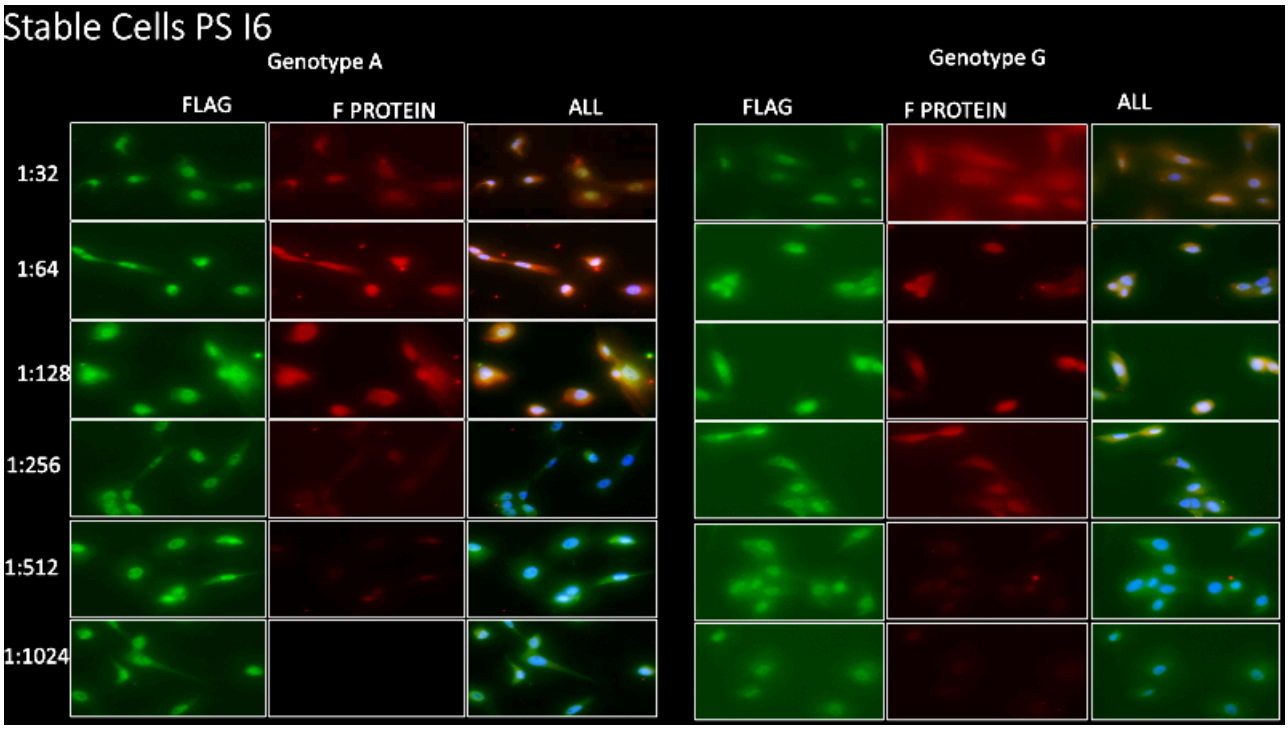


Figure A-69c. Stable cells Infected Sample#6.

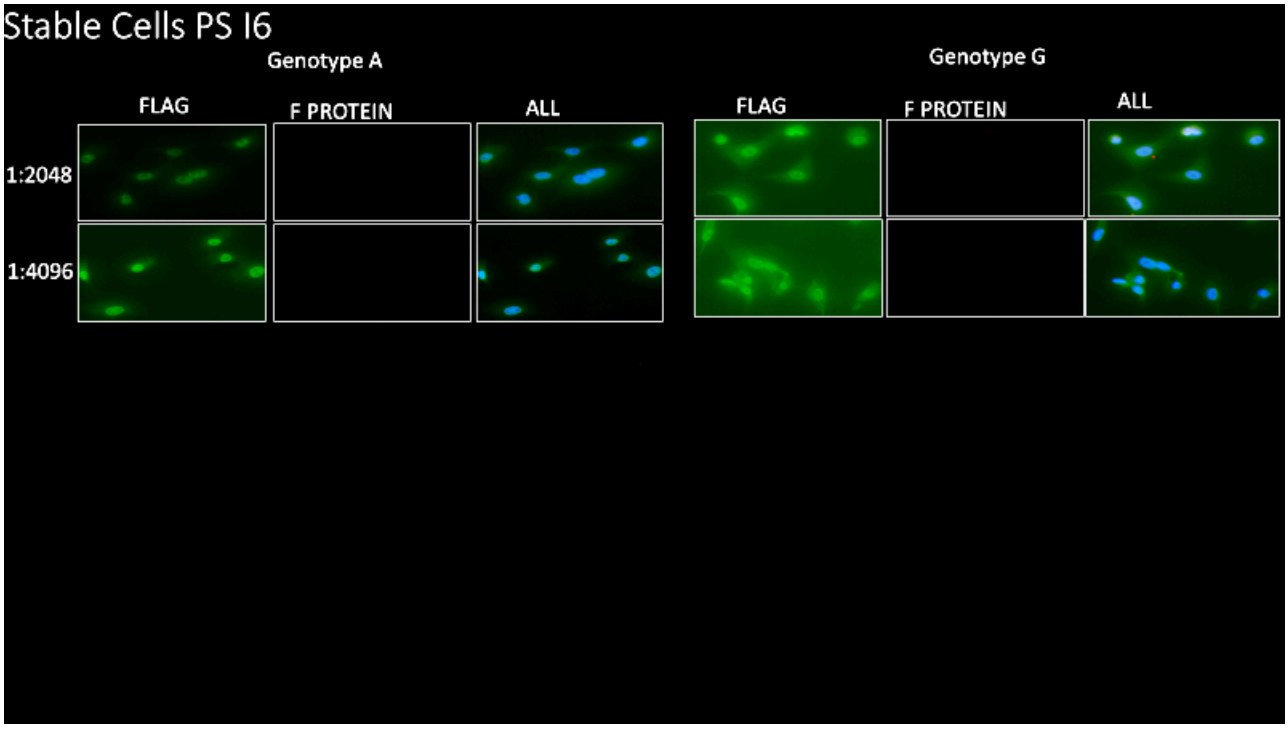


Figure A-69d. Stable cells Infected Sample#6.

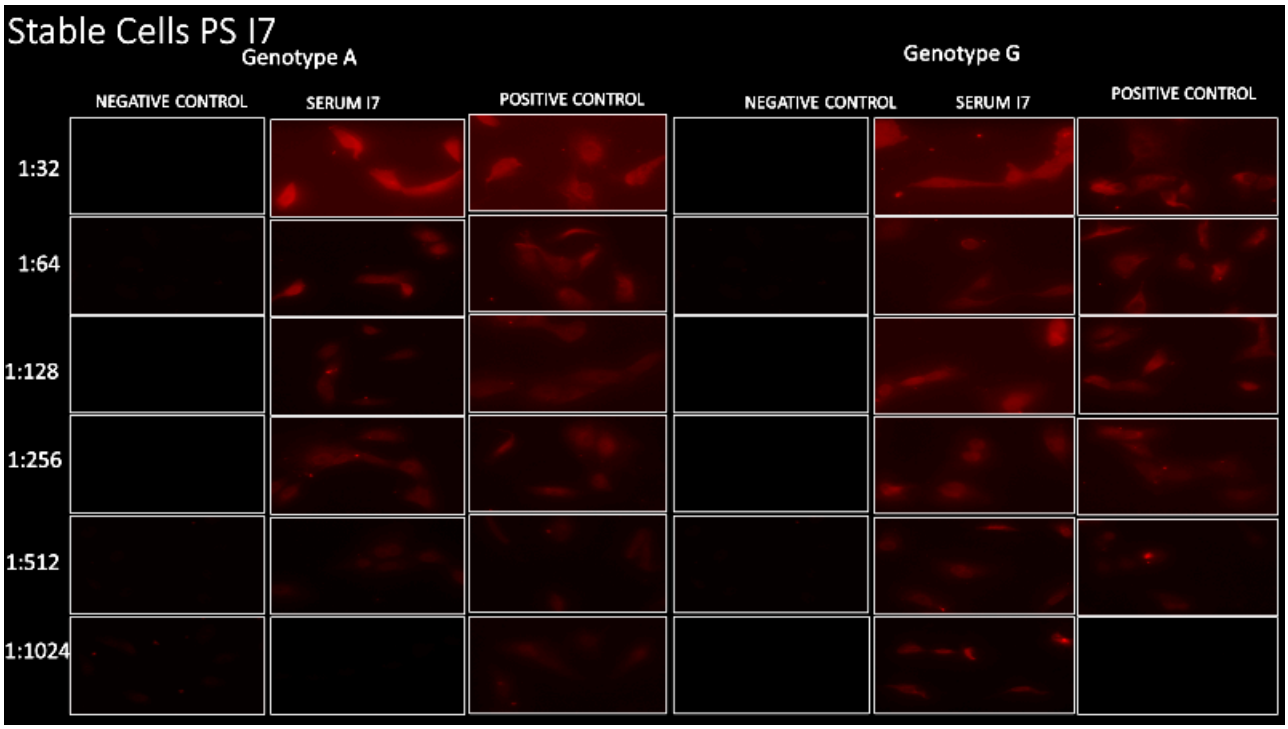


Figure A-70a. Stable cells Infected Sample#7.

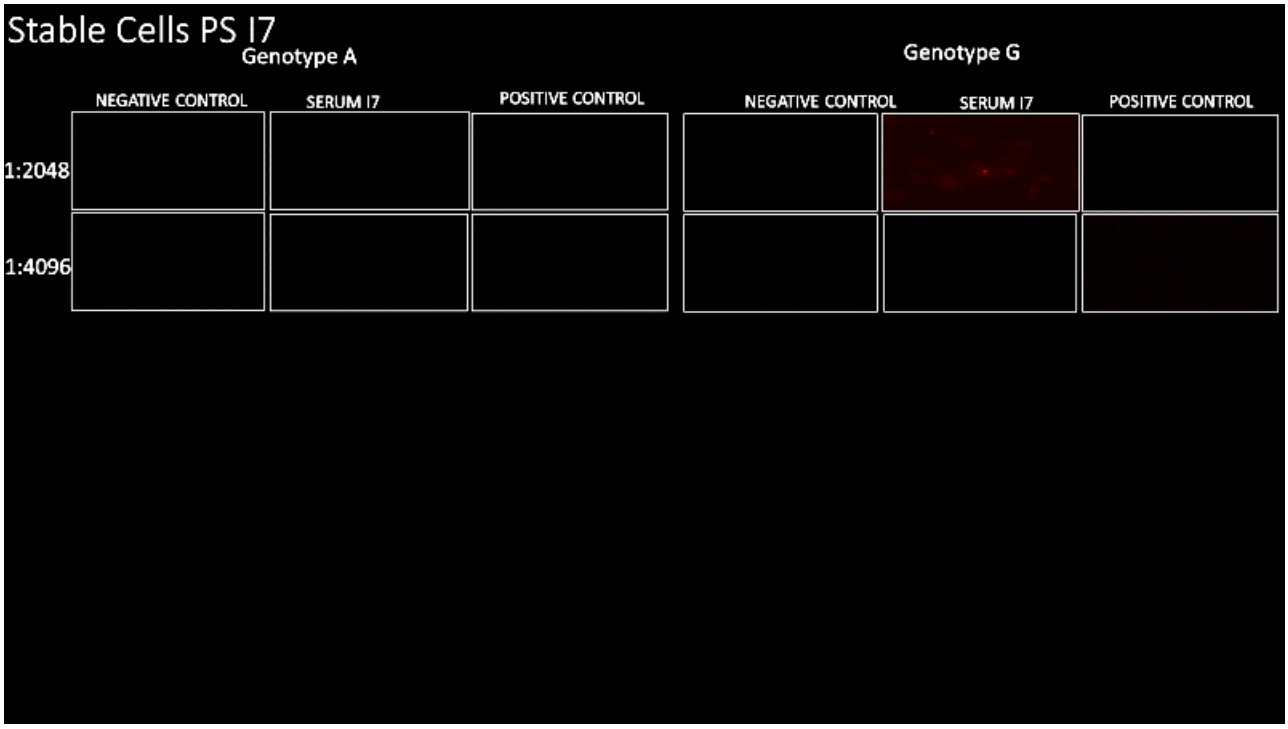


Figure A-70b. Stable cells Infected Sample#7.

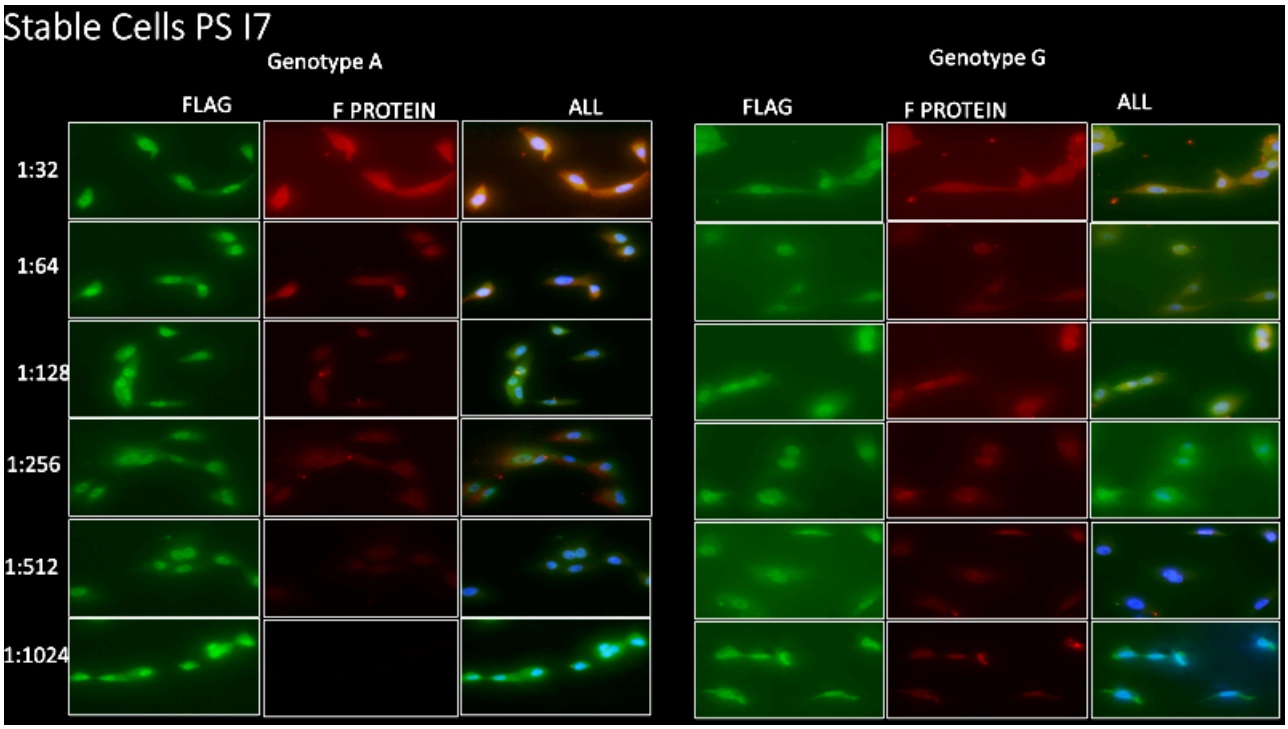


Figure A-70c. Stable cells Infected Sample#7.

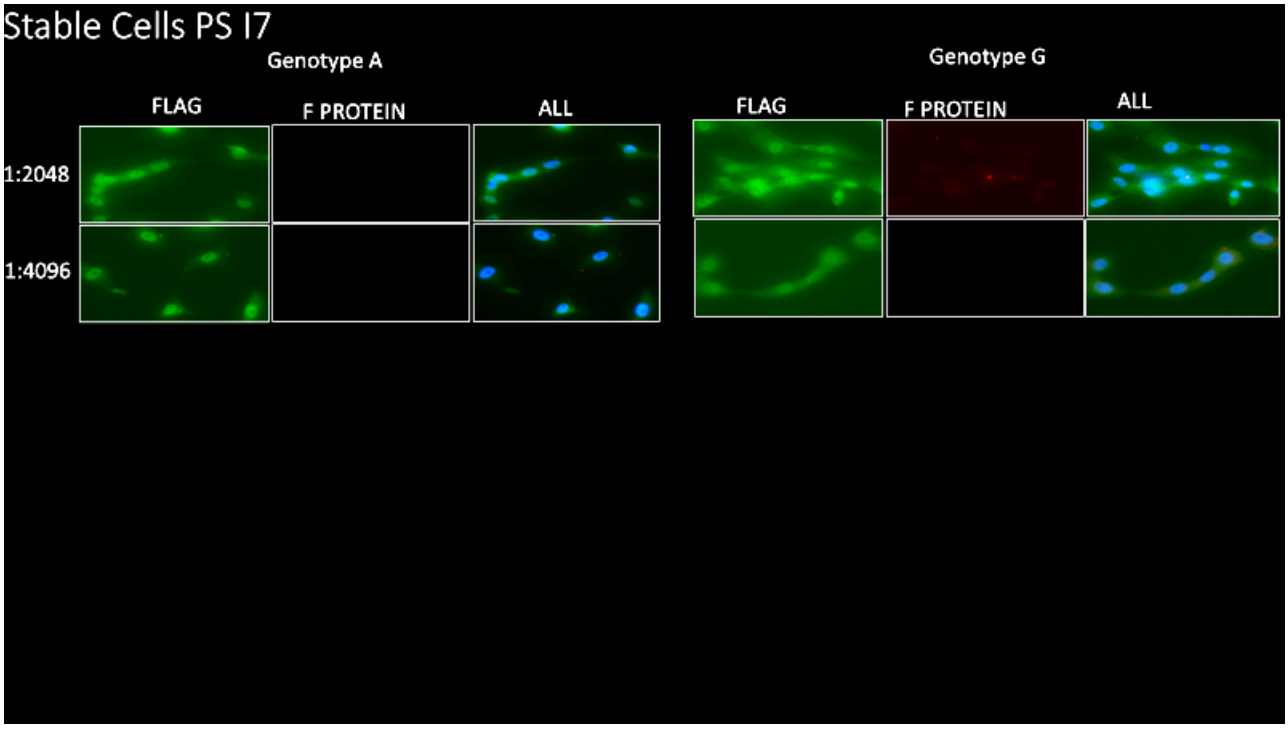


Figure A-70d. Stable cells Infected Sample#7.

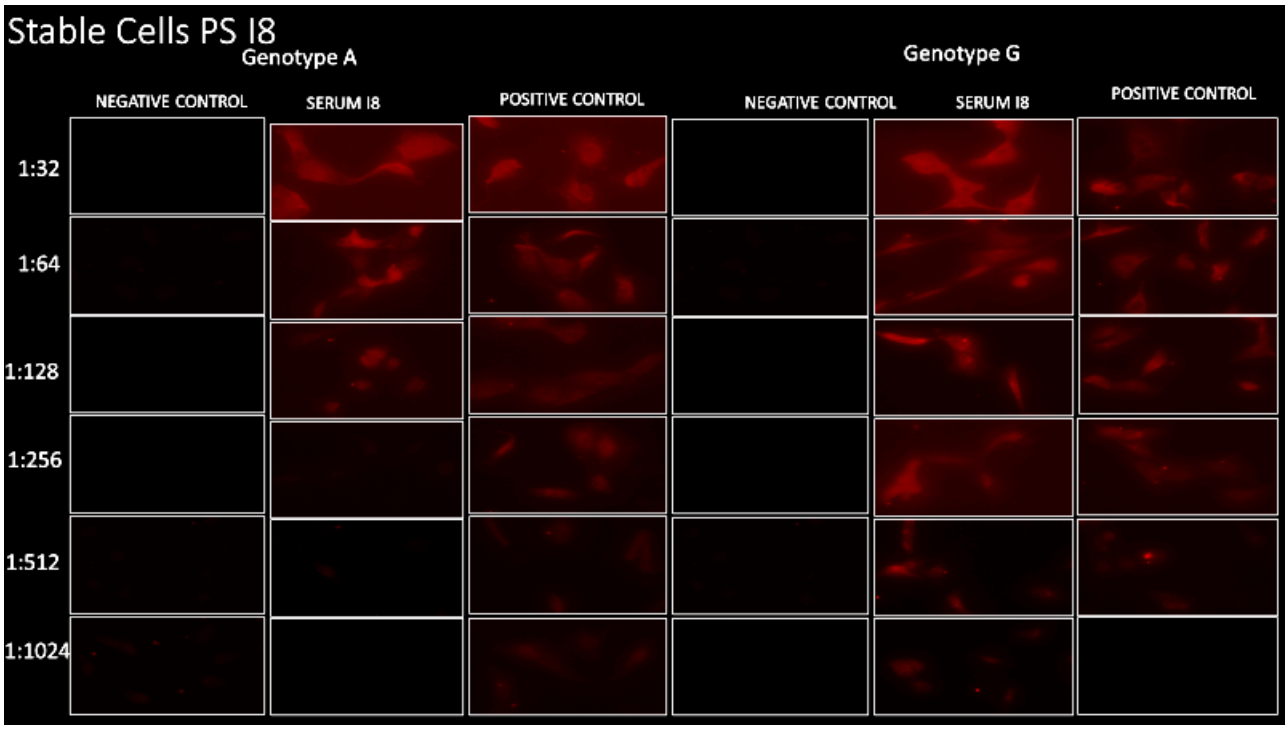


Figure A-71a. Stable cells Infected Sample#8.

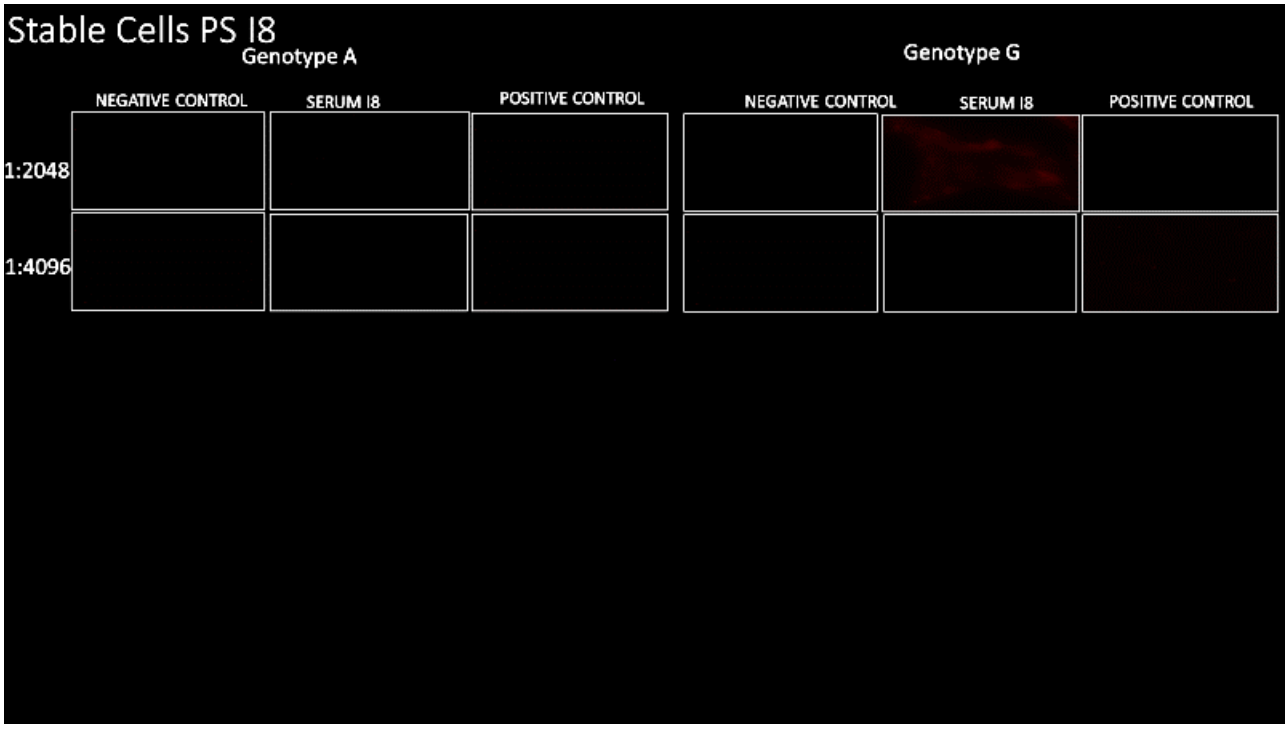


Figure A-71b. Stable cells Infected Sample#8.

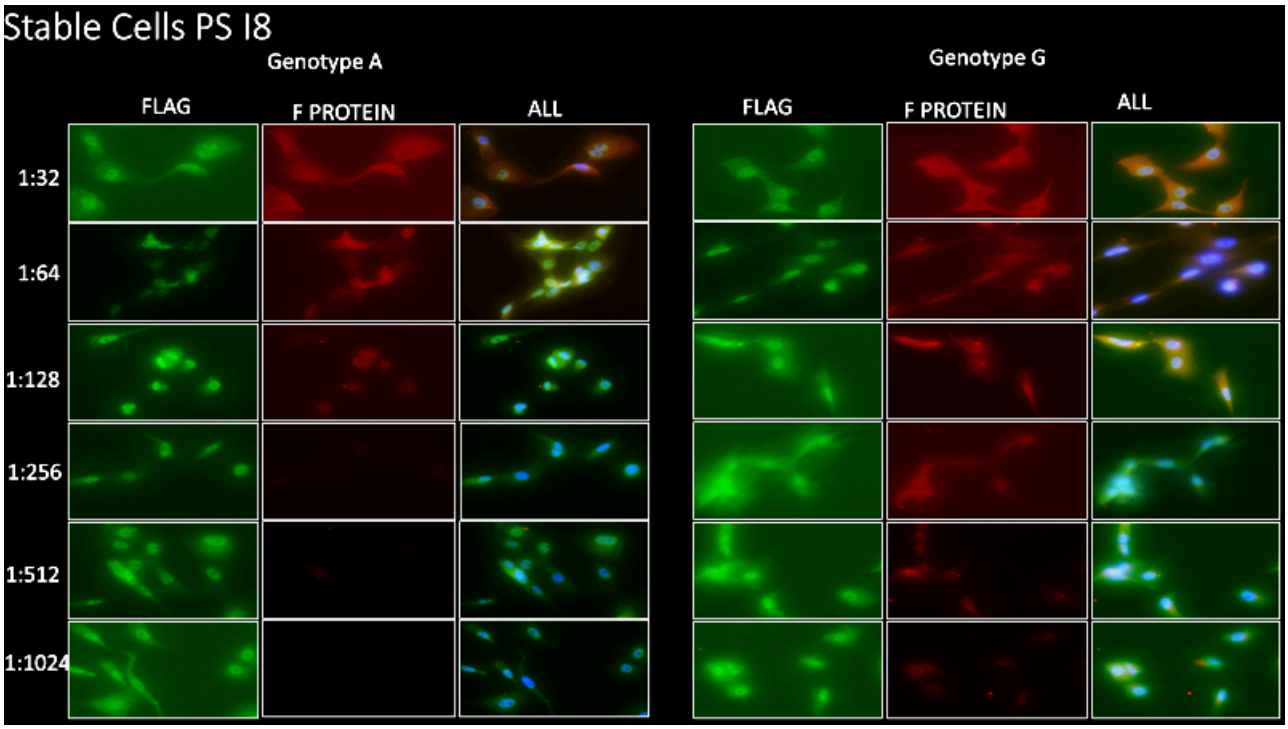


Figure A-71c. Stable cells Infected Sample#8.

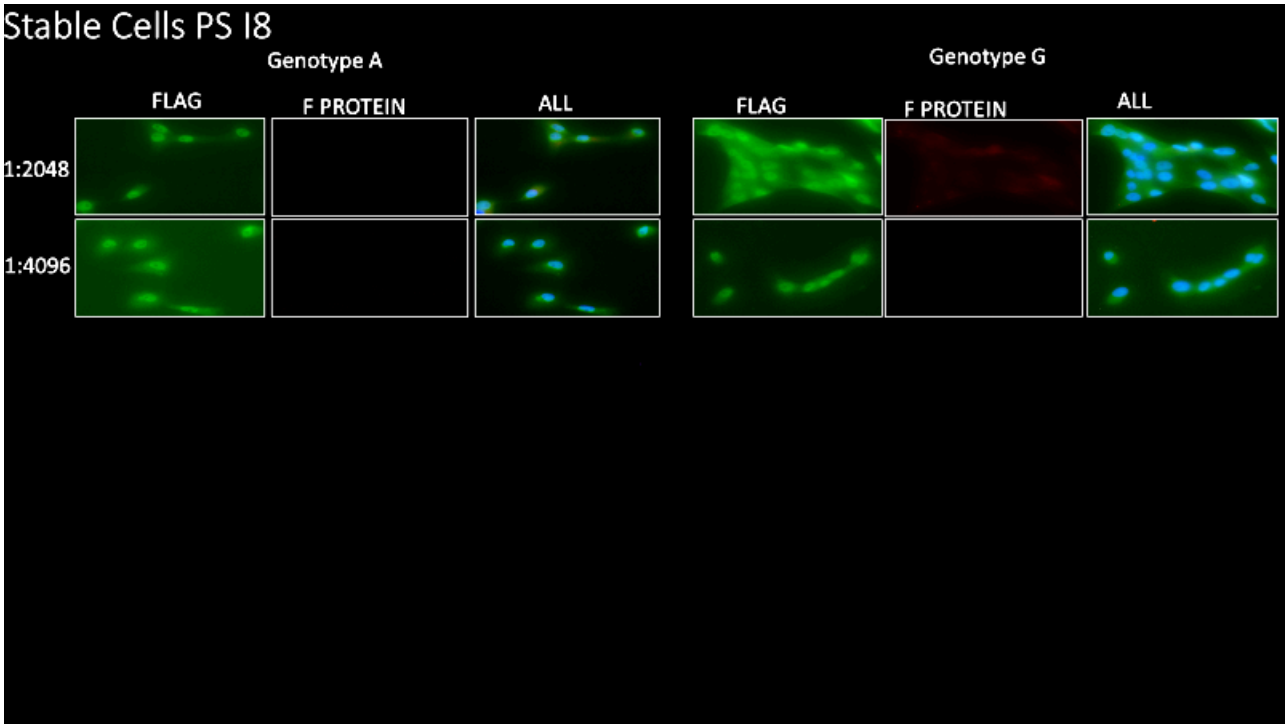


Figure A-71d. Stable cells Infected Sample#8.

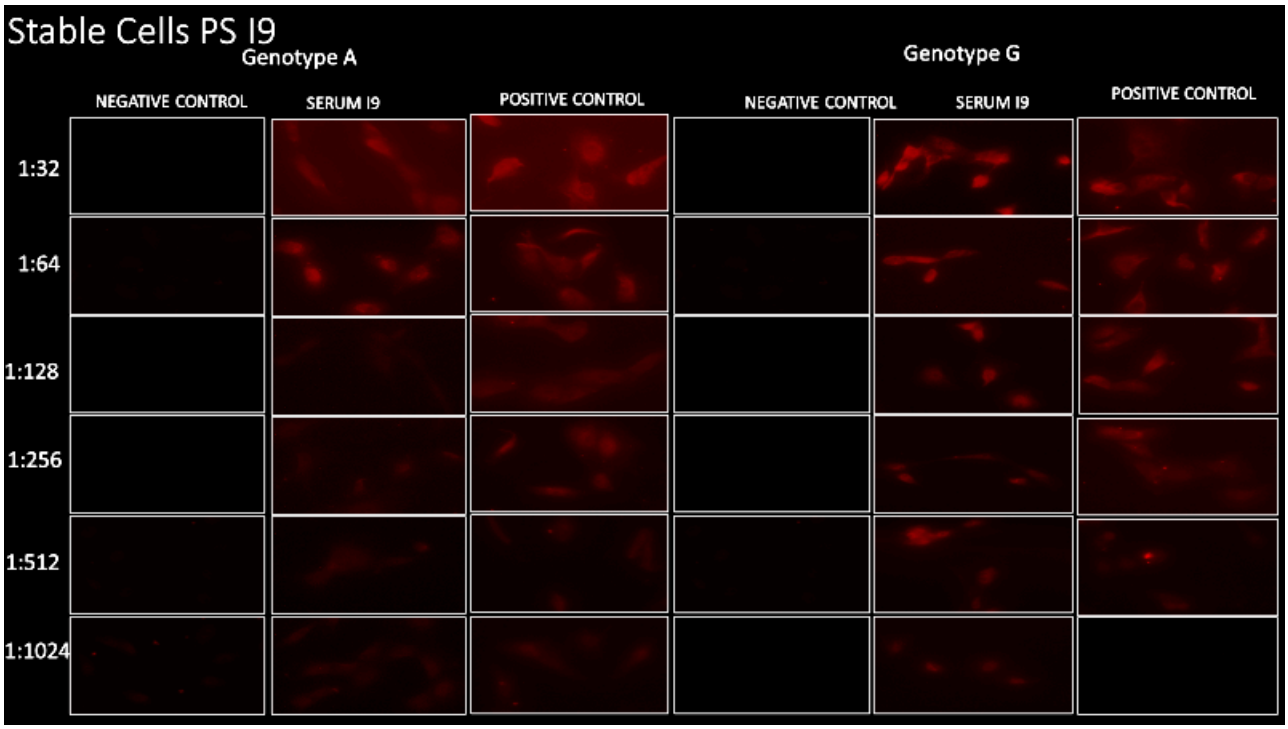


Figure A-72a. Stable cells Infected Sample#9.

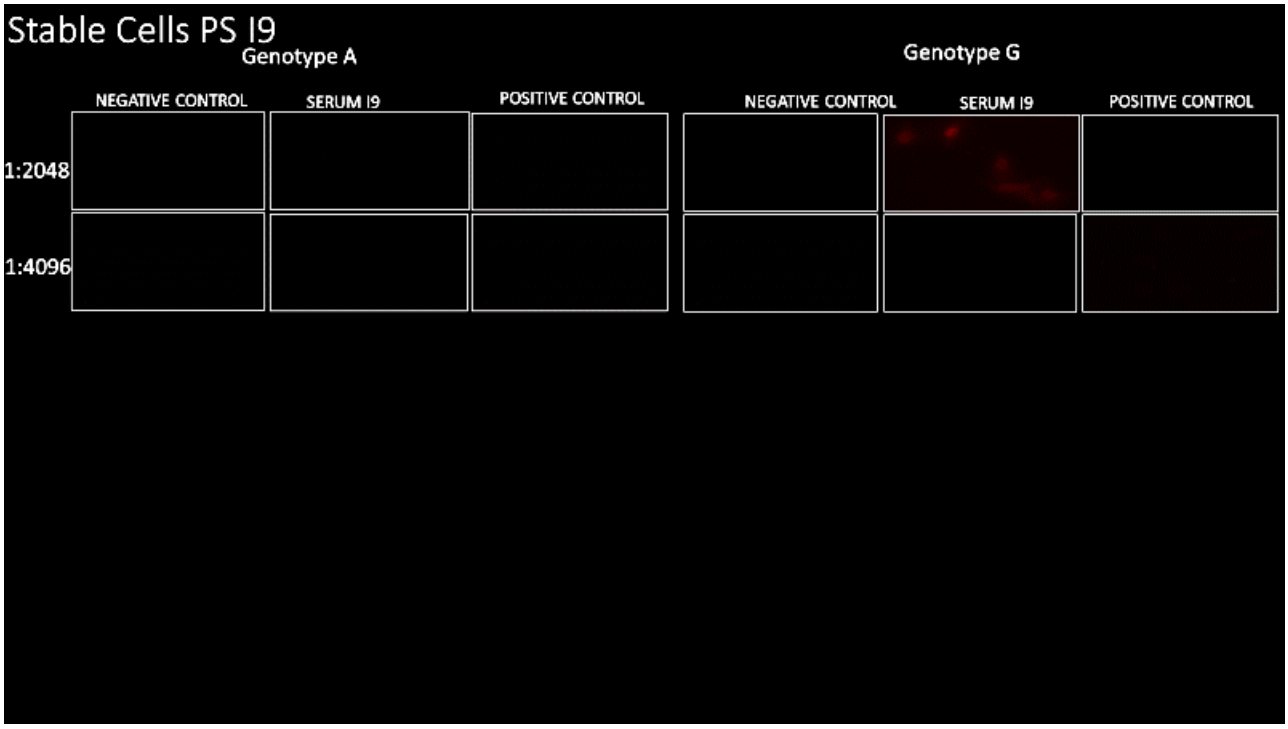


Figure A-72b. Stable cells Infected Sample#9.

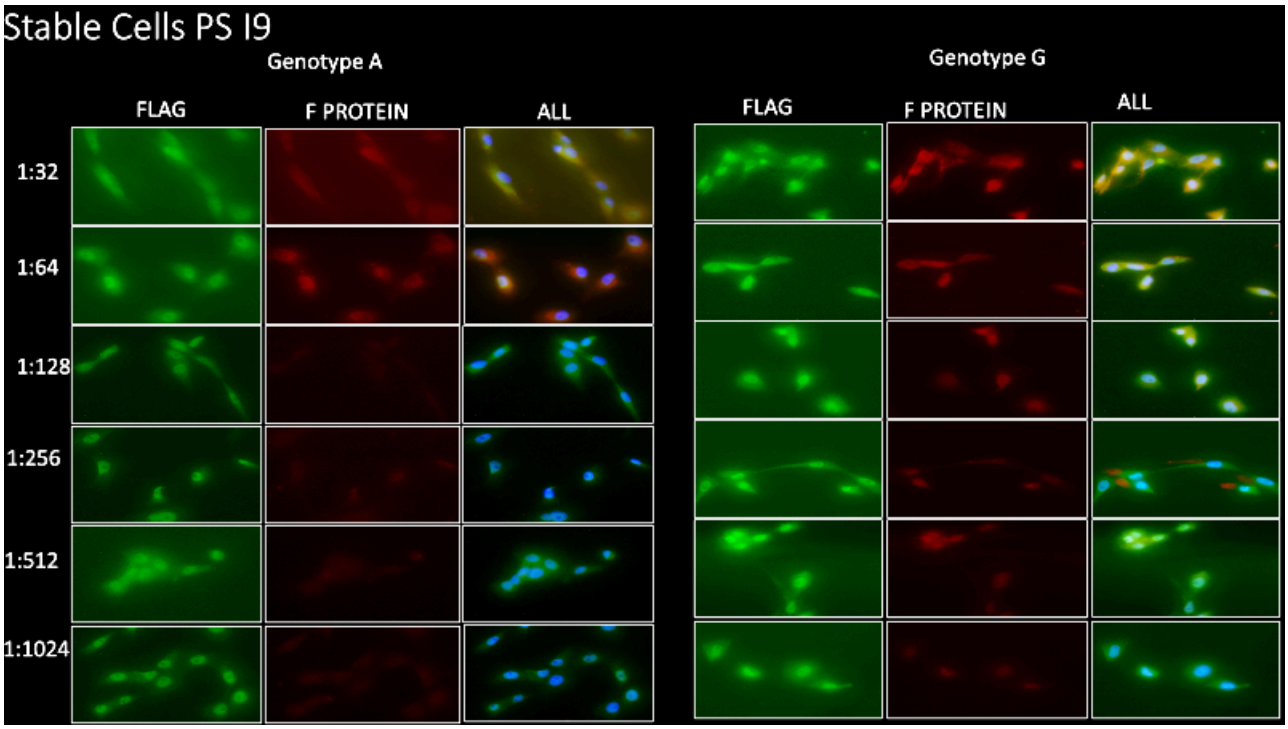


Figure A-72c. Stable cells Infected Sample#9.

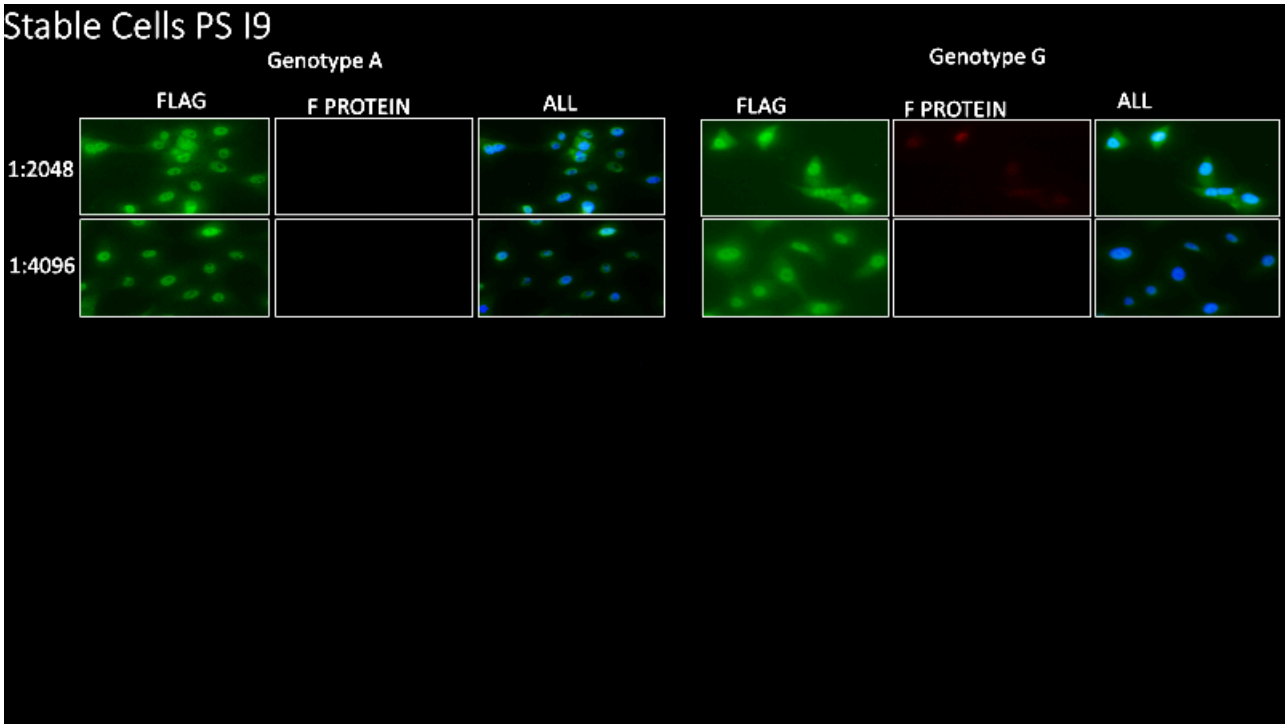


Figure A-72d. Stable cells Infected Sample#9.

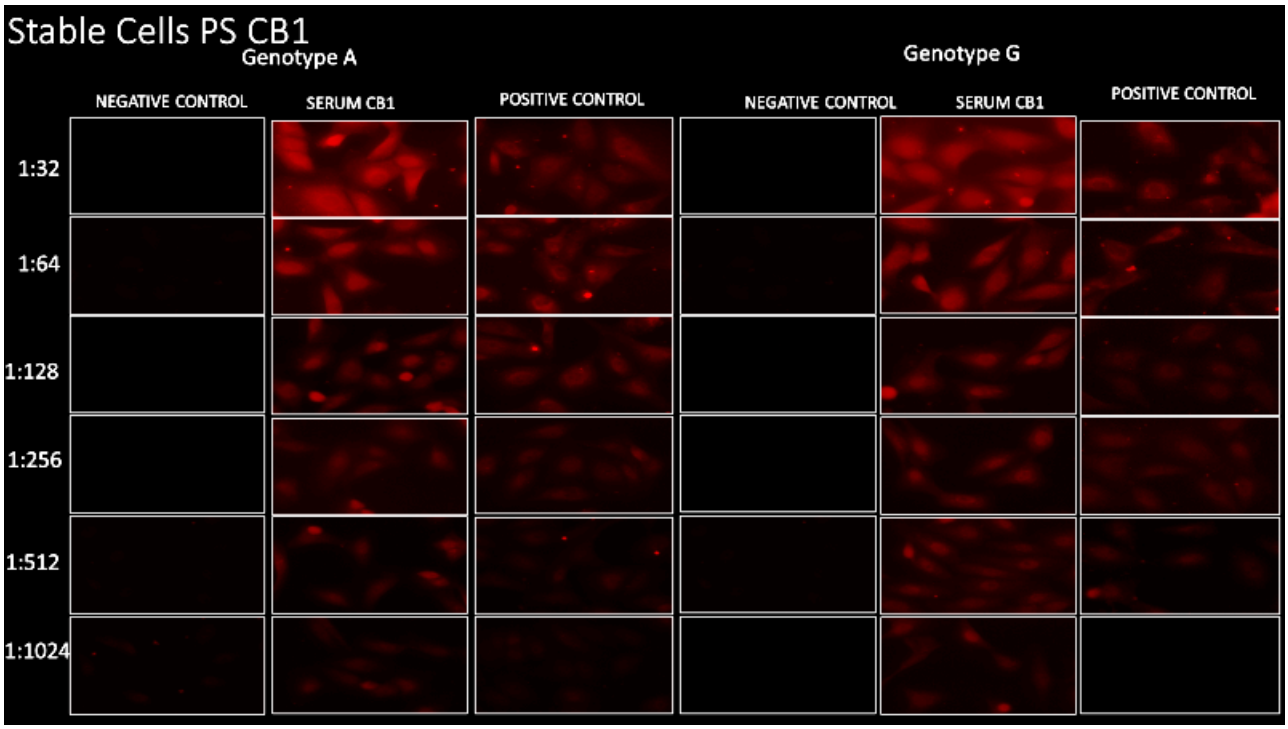


Figure A-73a. Stable cells Vaccine-Infected (CB) Sample#1.

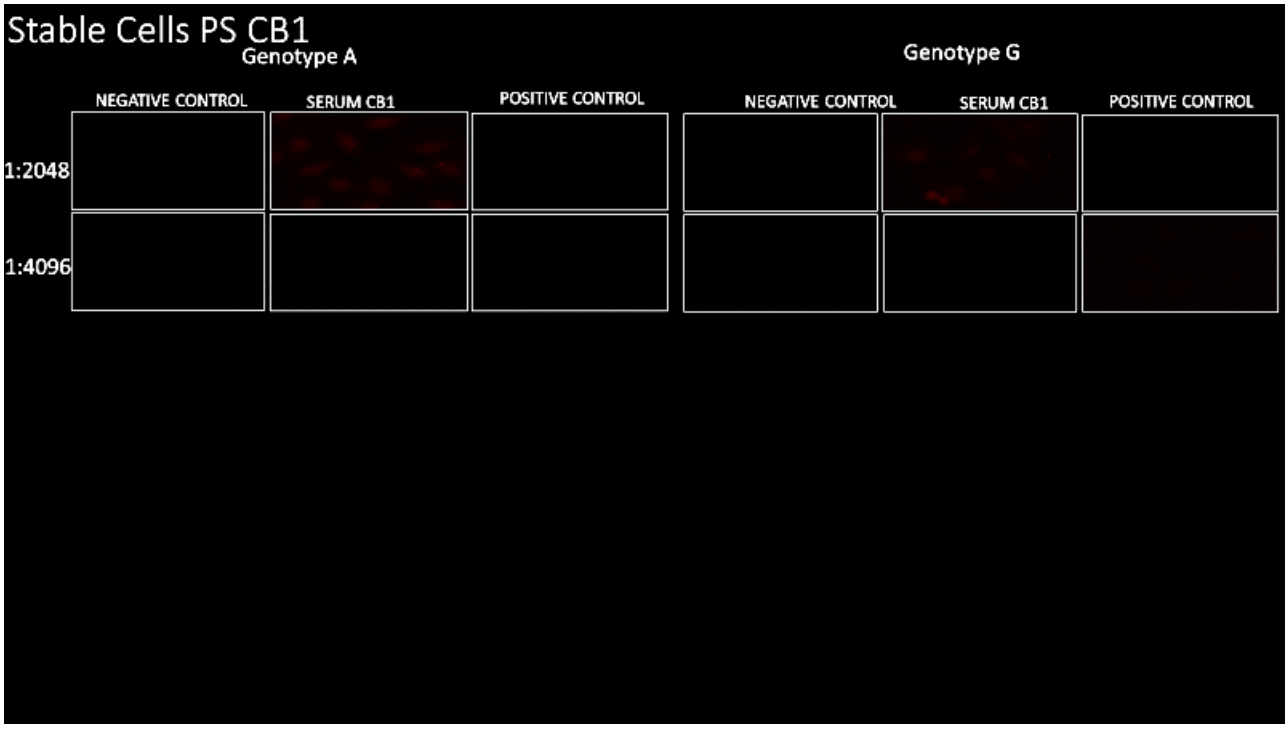


Figure A-73b. Stable cells Vaccine-Infected (CB) Sample#1.

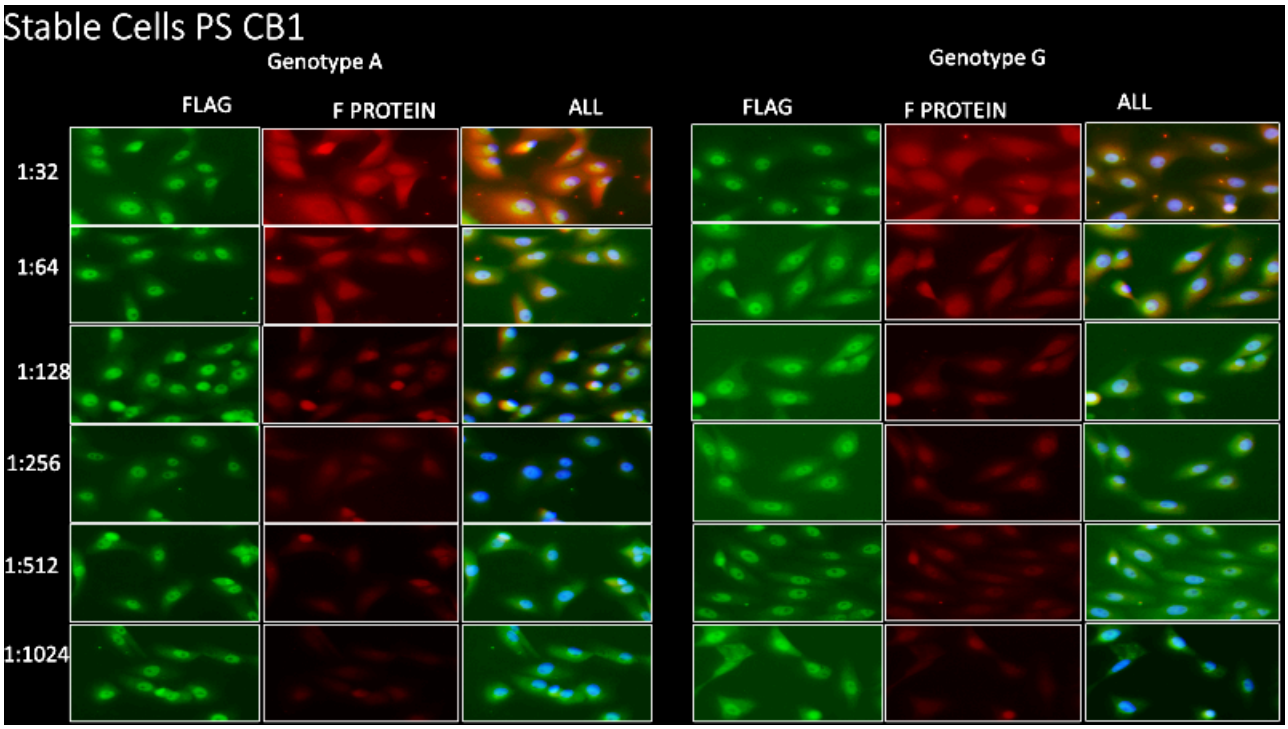


Figure A-73c. Stable cells Vaccine-Infected (CB) Sample#1.

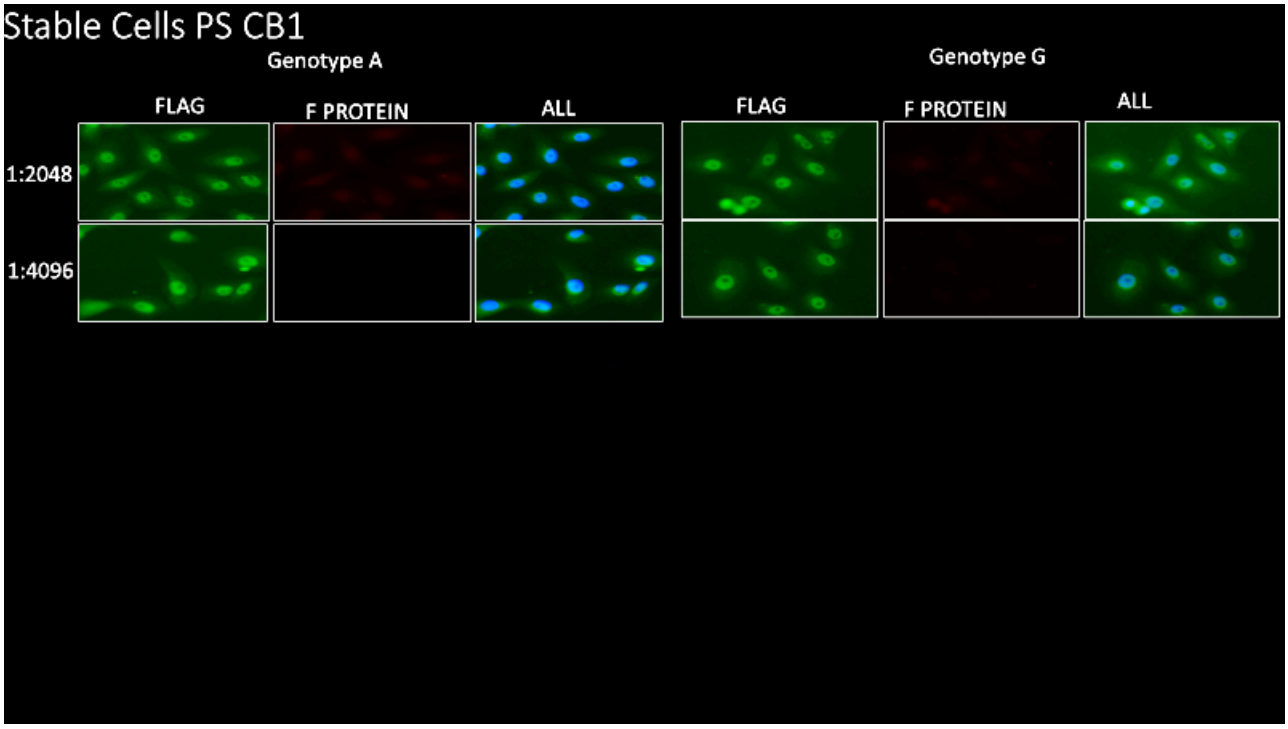


Figure A-73d. Stable cells Vaccine-Infected (CB) Sample#1.

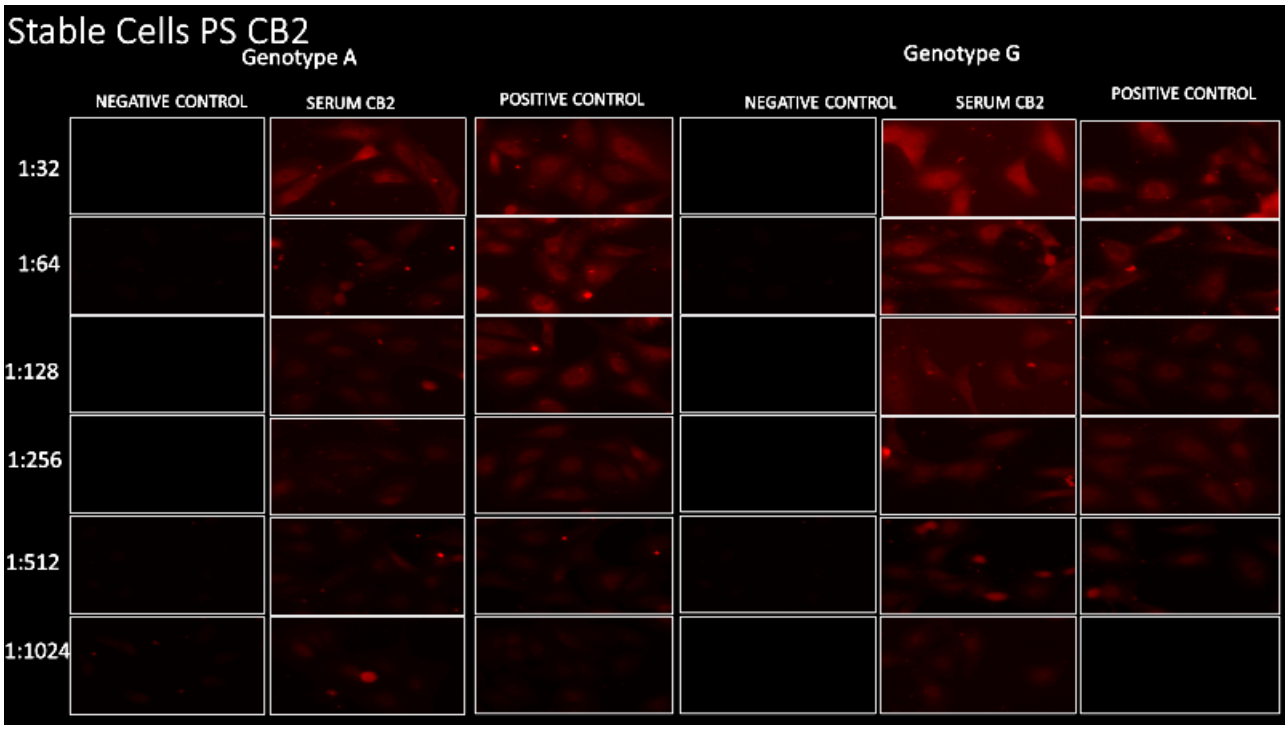


Figure A-74a. Stable cells Vaccine-Infected (CB) Sample#2.

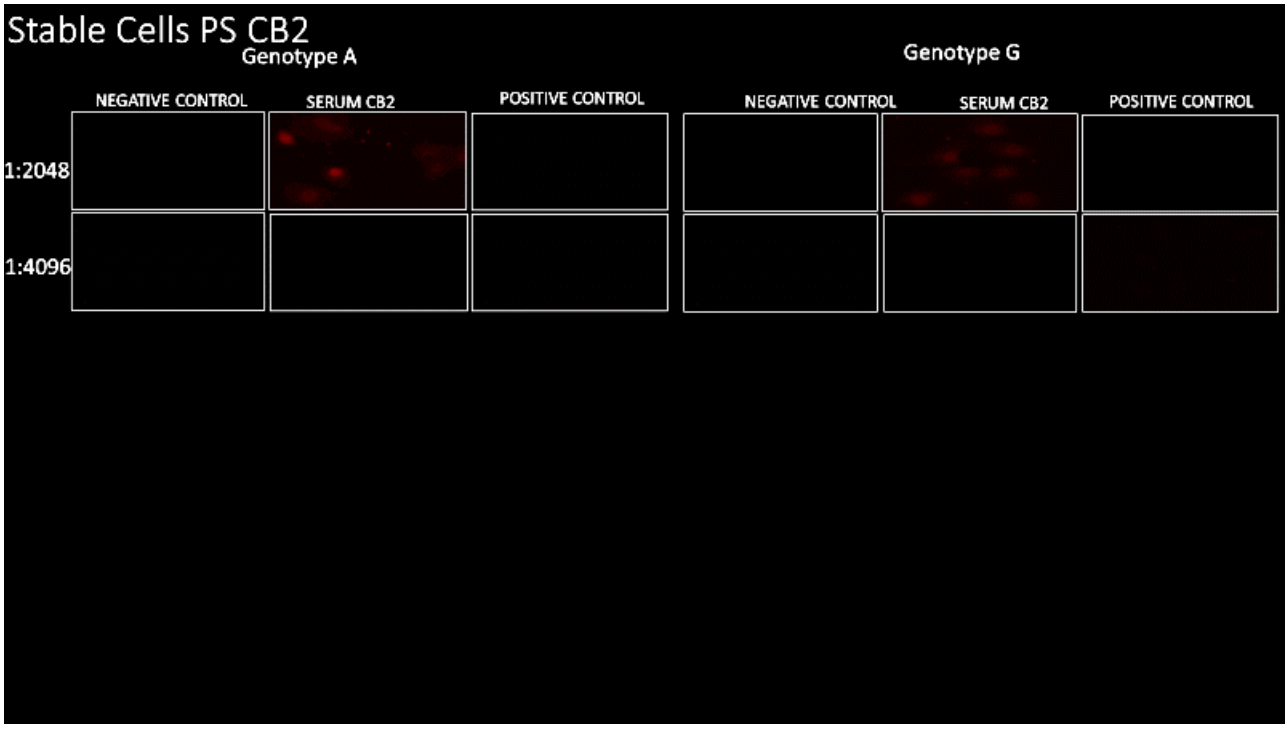


Figure A-74b. Stable cells Vaccine-Infected (CB) Sample#2.

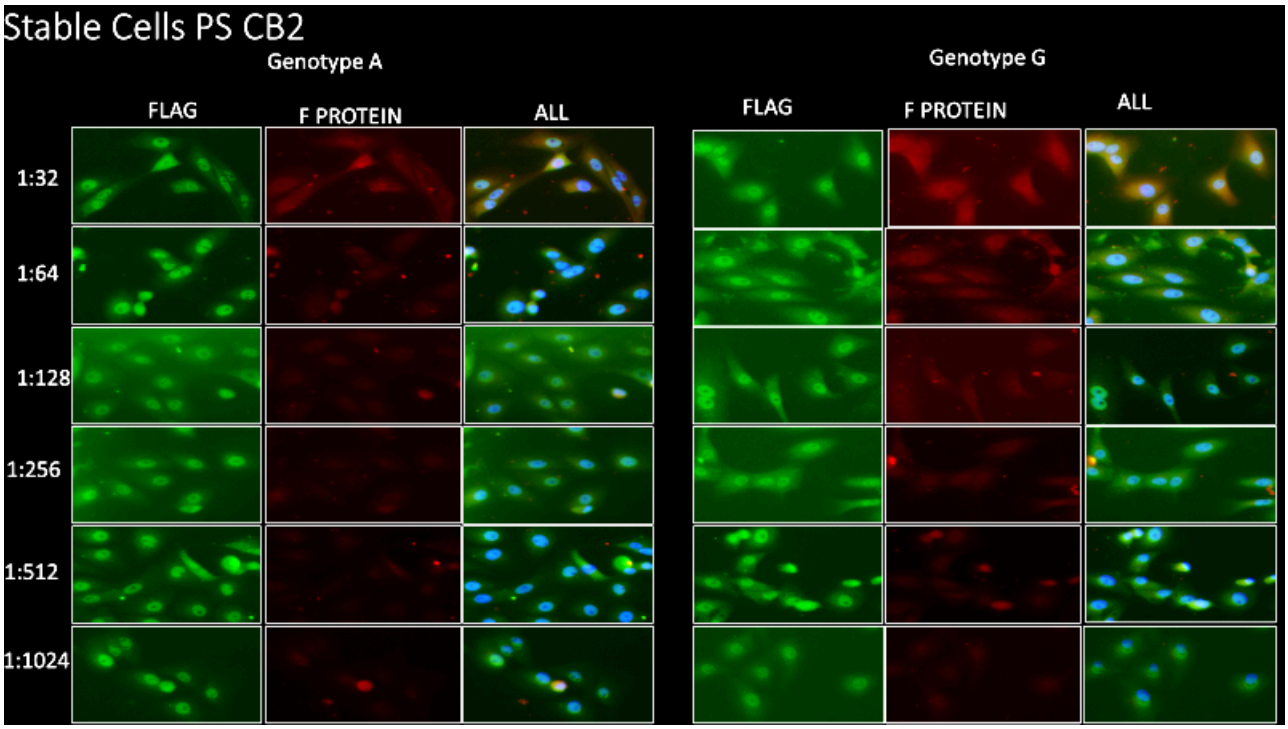


Figure A-74c. Stable cells Vaccine-Infected (CB) Sample#2.

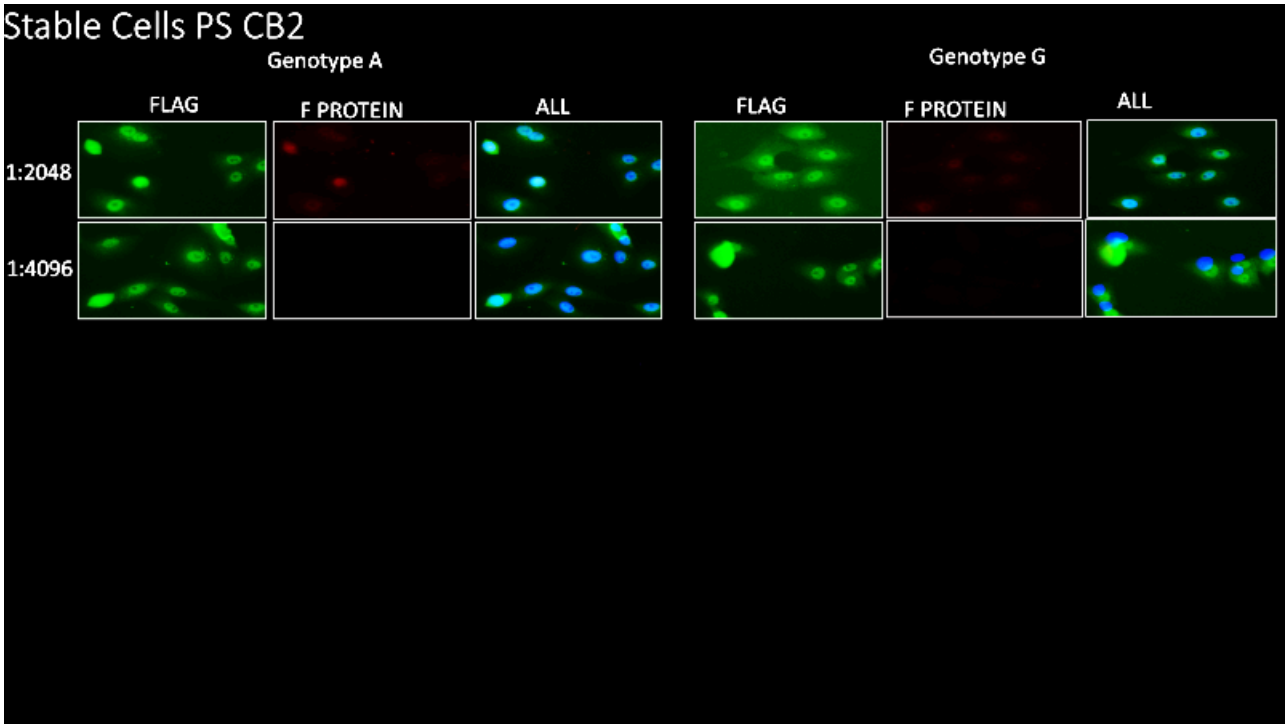


Figure A-74d. Stable cells Vaccine-Infected (CB) Sample#2.

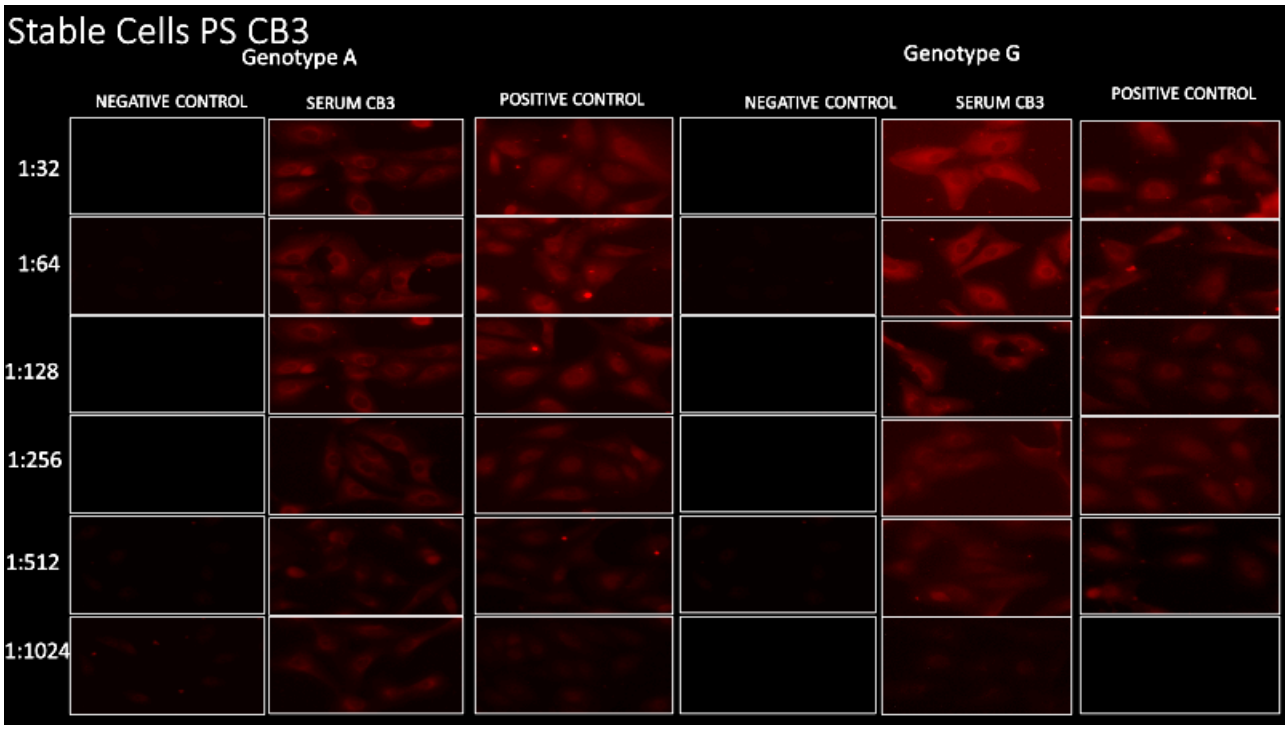


Figure A-75a. Stable cells Vaccine-Infected (CB) Sample#3.

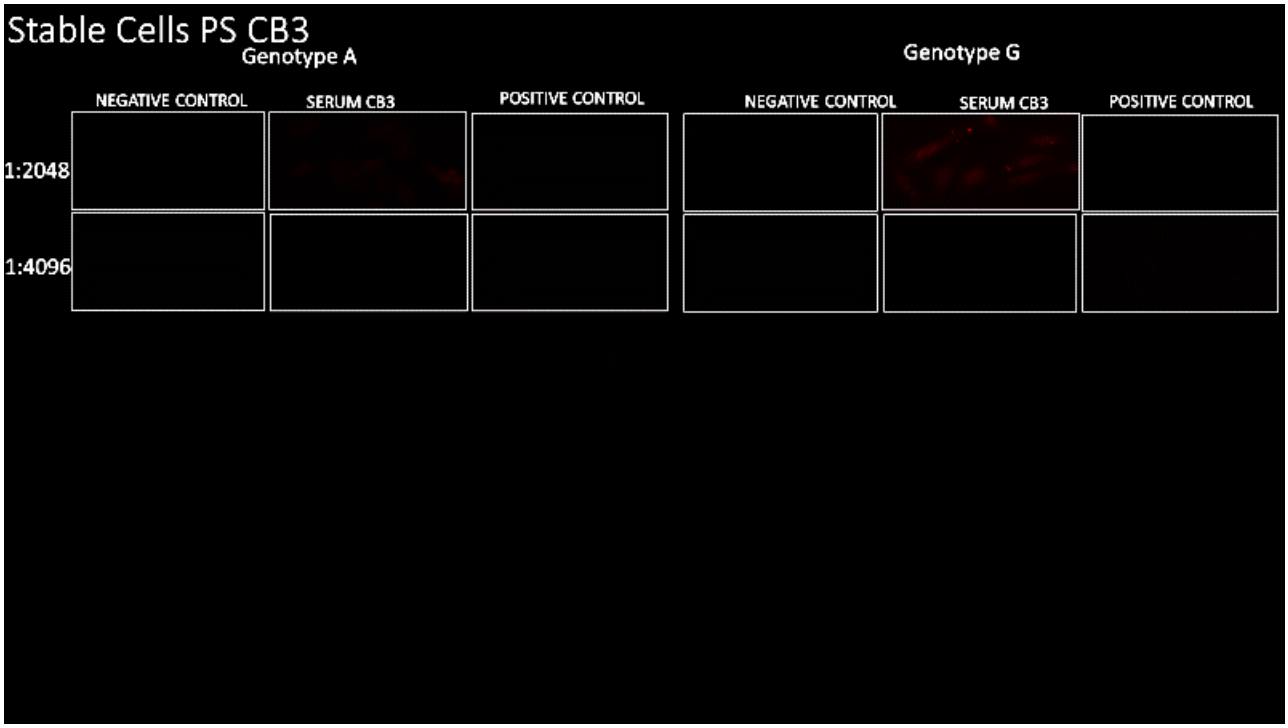


Figure A-75b. Stable cells Vaccine-Infected (CB) Sample#3.

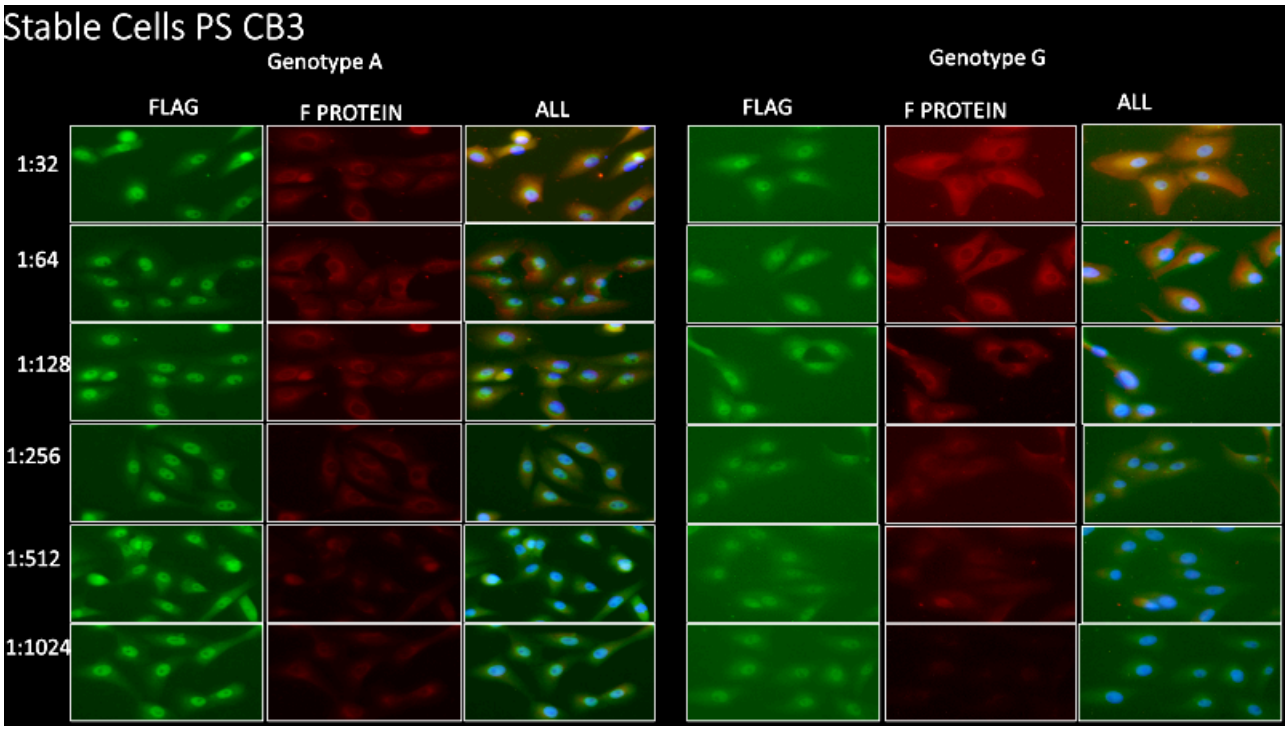


Figure A-75c. Stable cells Vaccine-Infected (CB) Sample#3.

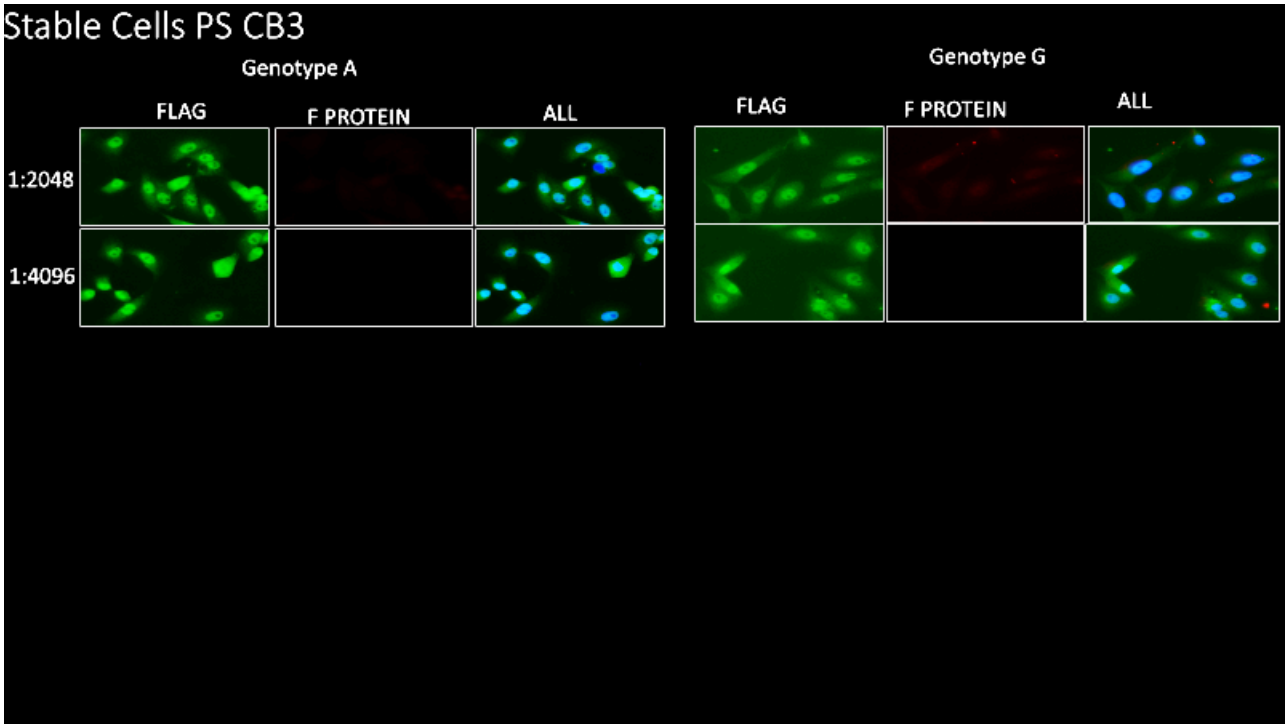


Figure A-75d. Stable cells Vaccine-Infected (CB) Sample#3.

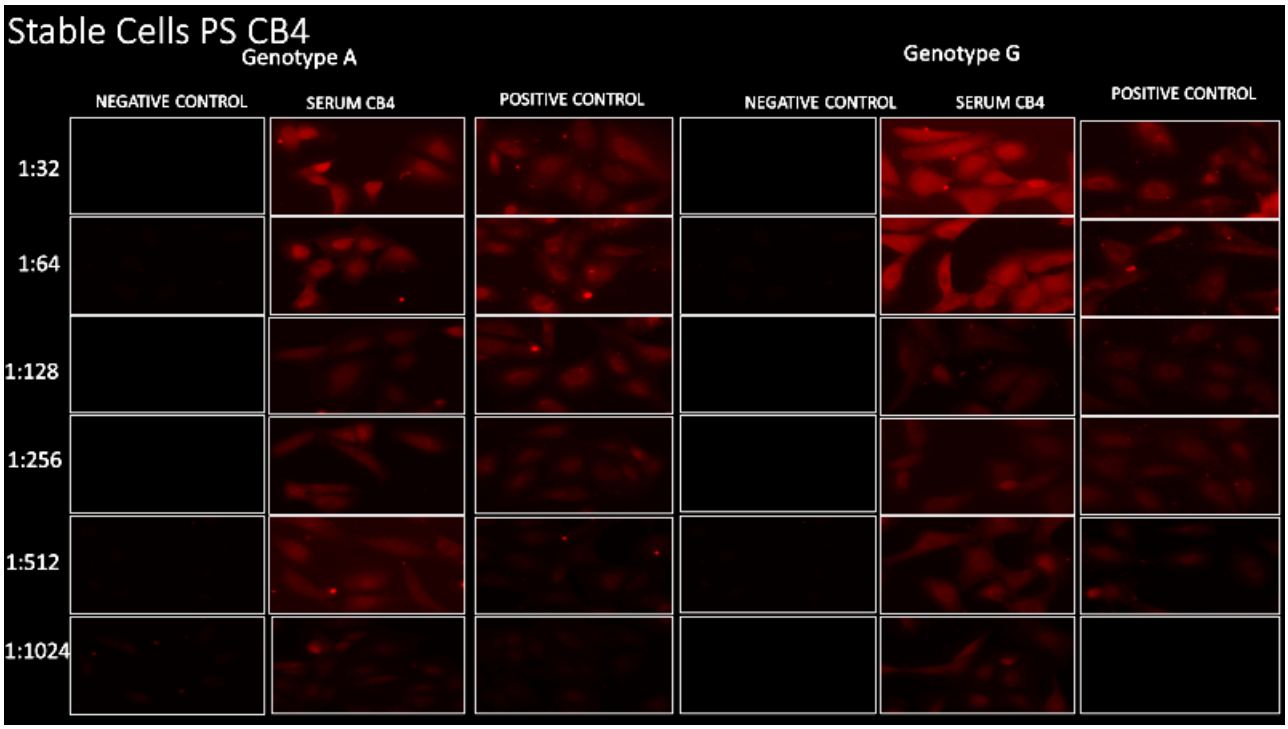


Figure A-76a. Stable cells Vaccine-Infected (CB) Sample#4.

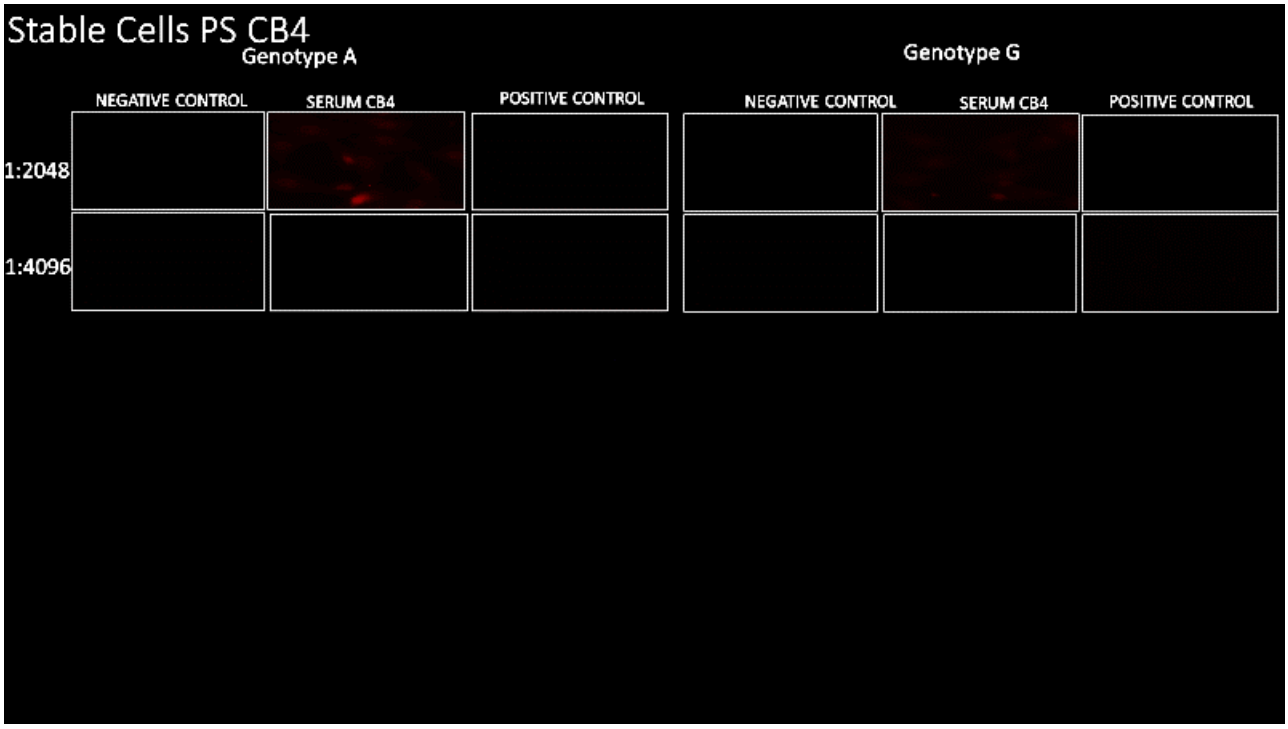


Figure A-76b. Stable cells Vaccine-Infected (CB) Sample#4.

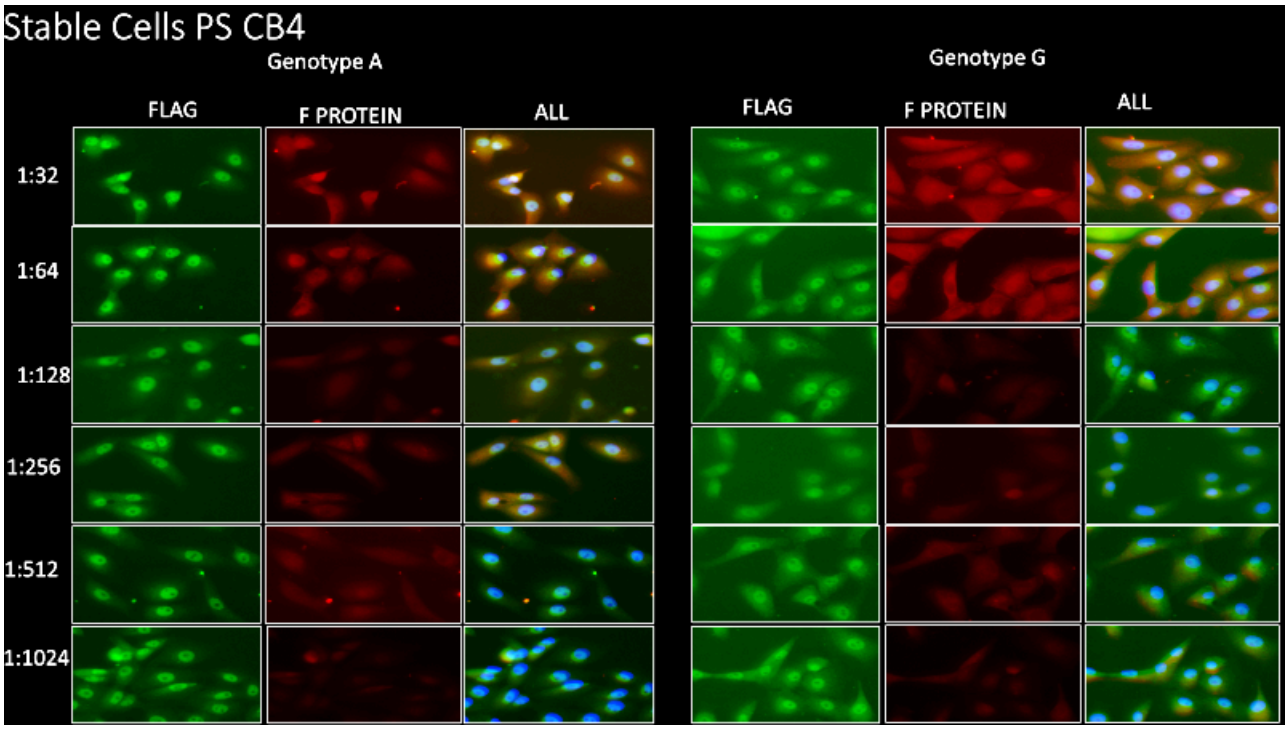


Figure A-76c. Stable cells Vaccine-Infected (CB) Sample#4.

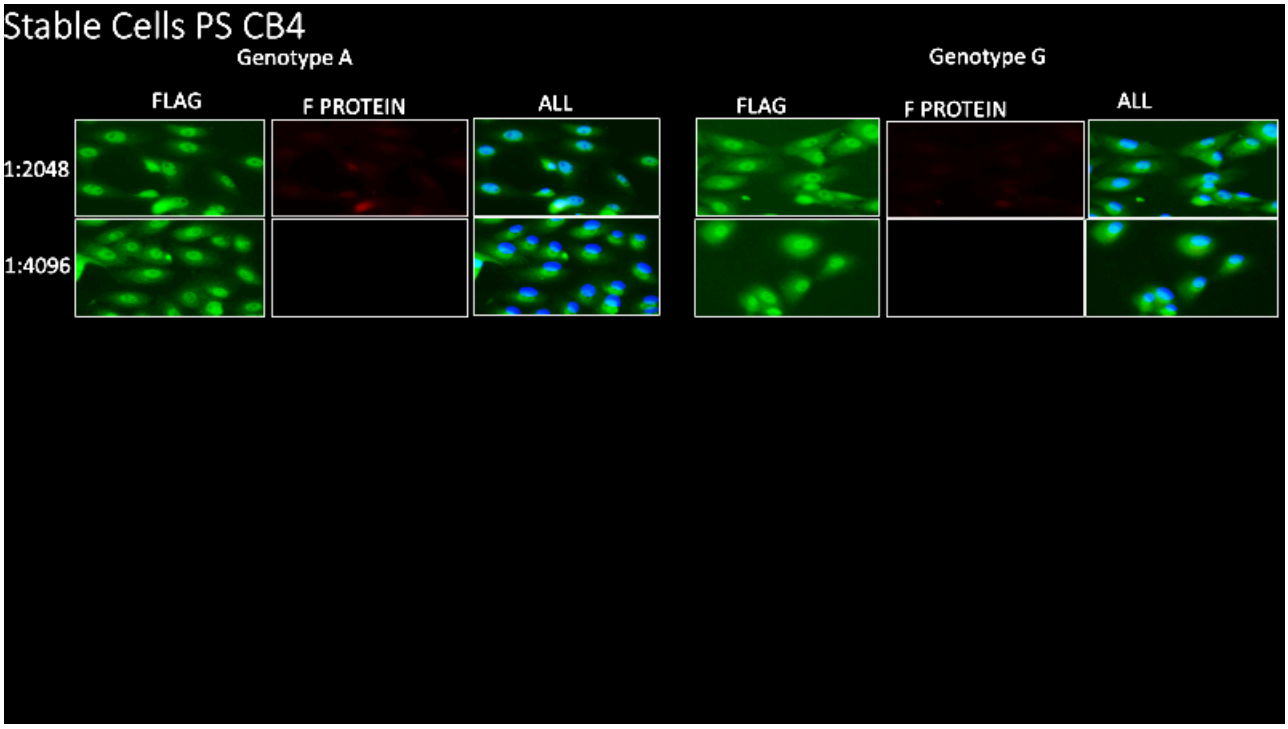


Figure A-76d. Stable cells Vaccine-Infected (CB) Sample#4.

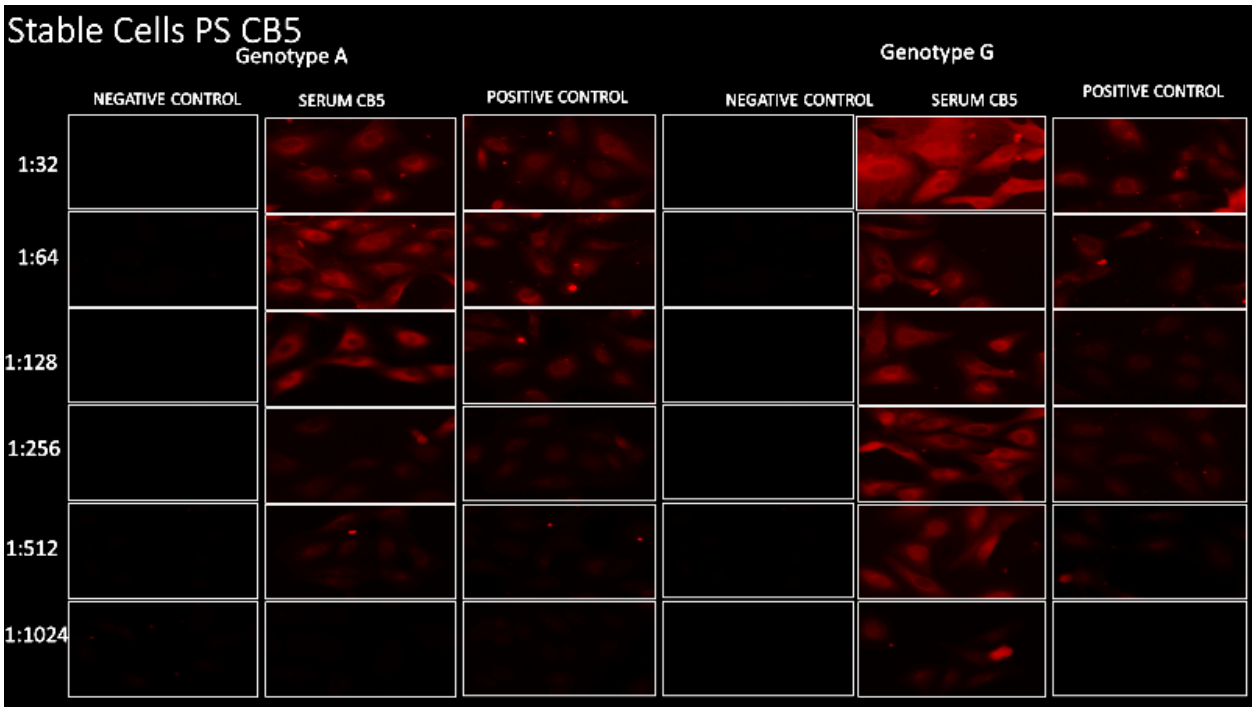


Figure A-77a. Stable cells Vaccine-Infected (CB) Sample#5.

Figure A-77b. Stable cells Vaccine-Infected (CB) Sample#5.

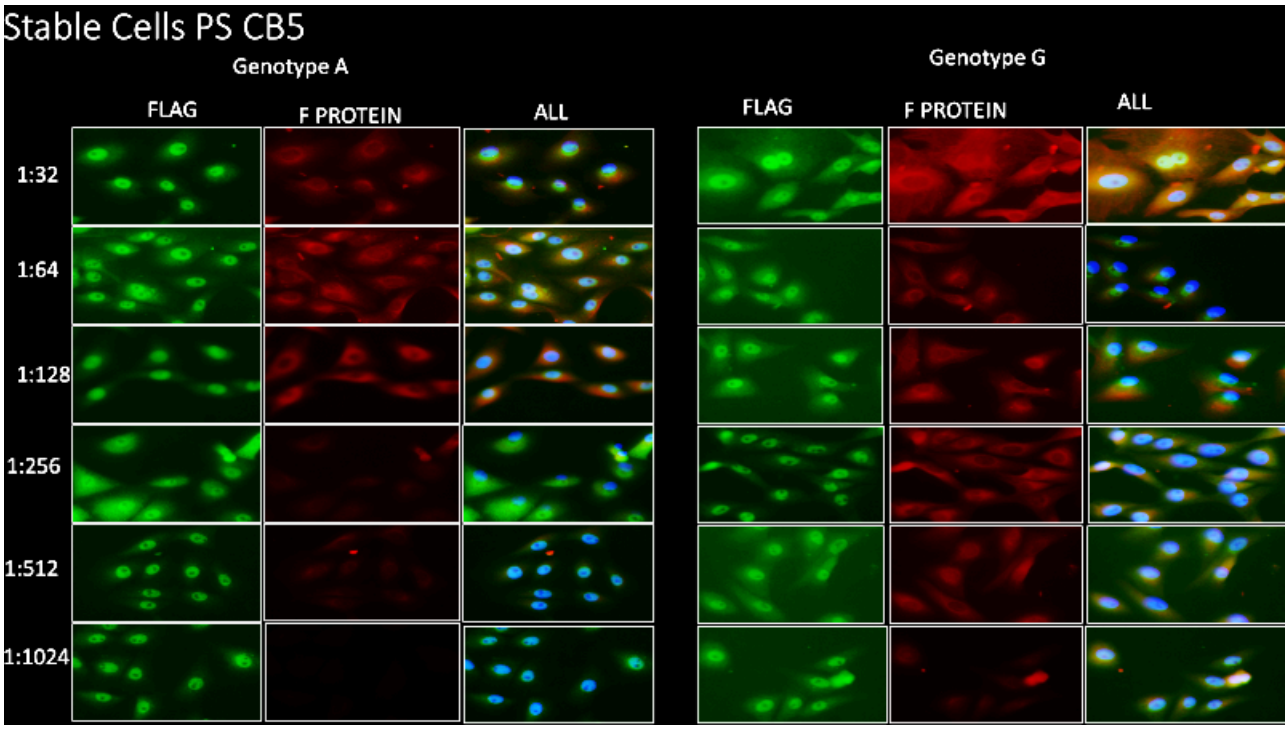


Figure A-77c. Stable cells Vaccine-Infected (CB) Sample#5.

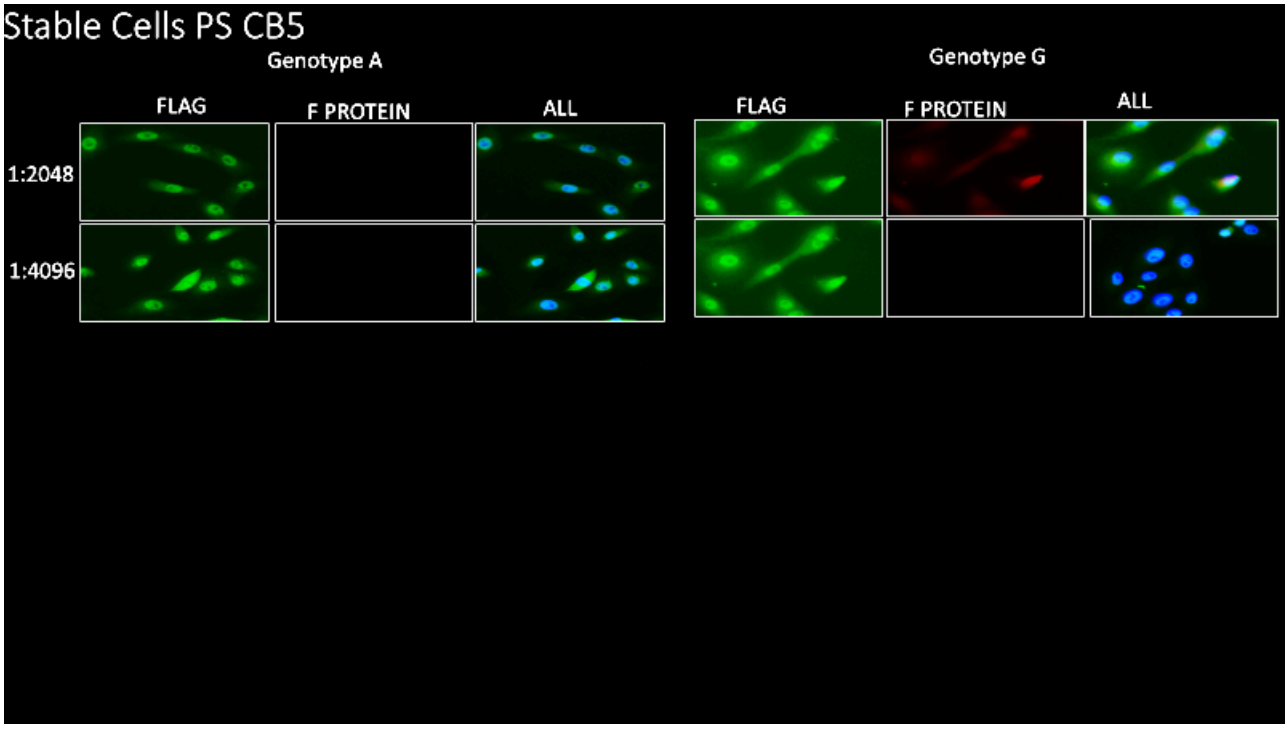


Figure A-77d. Stable cells Vaccine-Infected (CB) Sample#5.

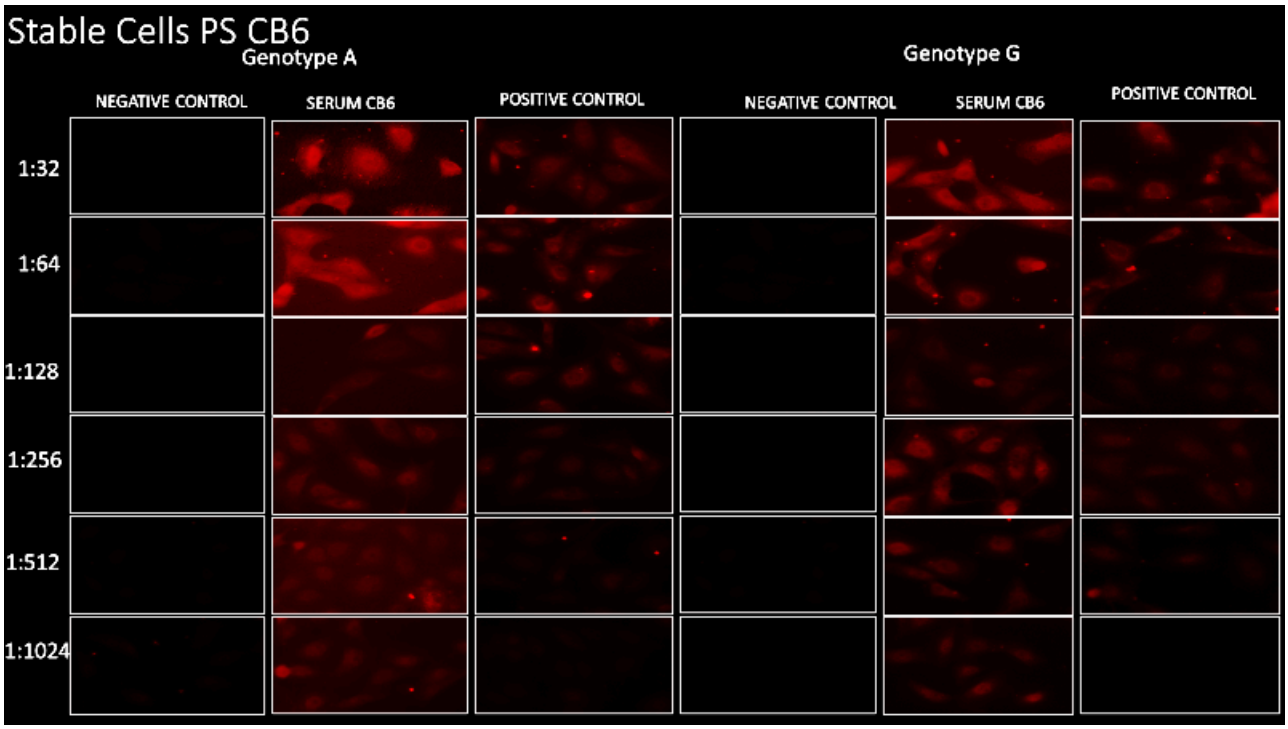


Figure A-78a. Stable cells Vaccine-Infected (CB) Sample#6.

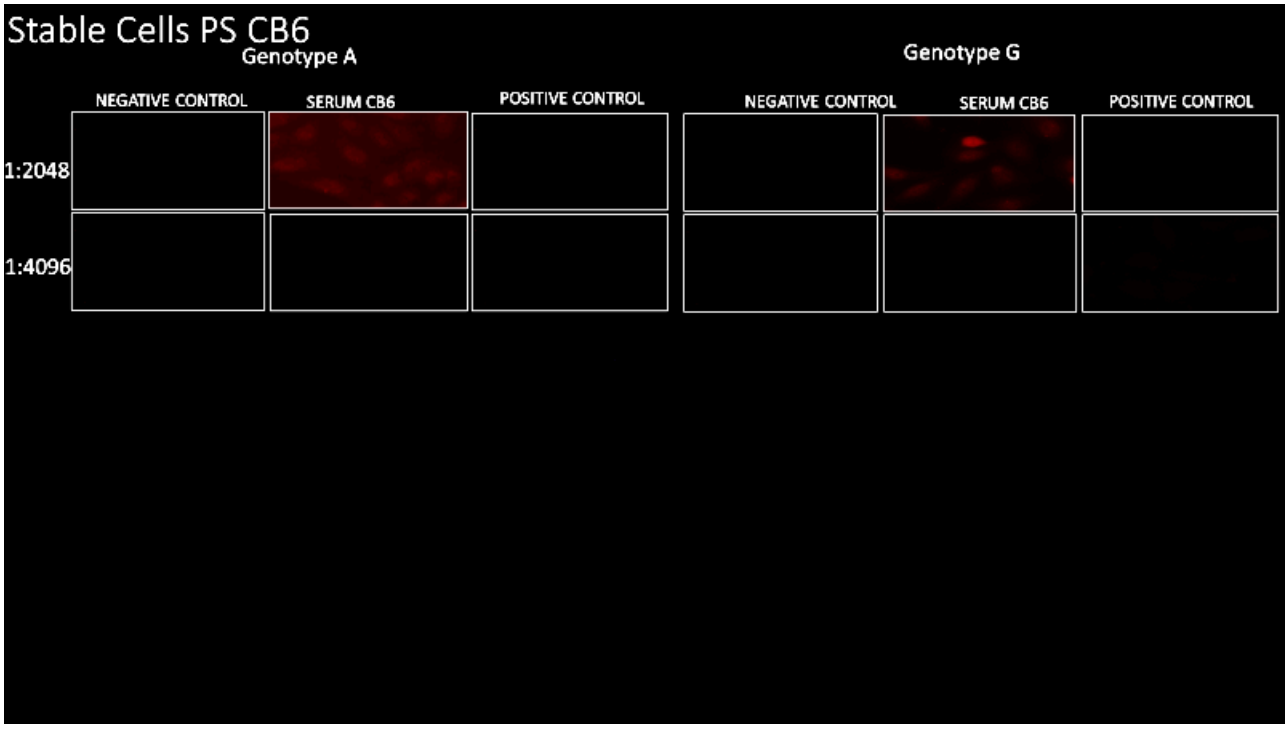


Figure A-78b. Stable cells Vaccine-Infected (CB) Sample#6.

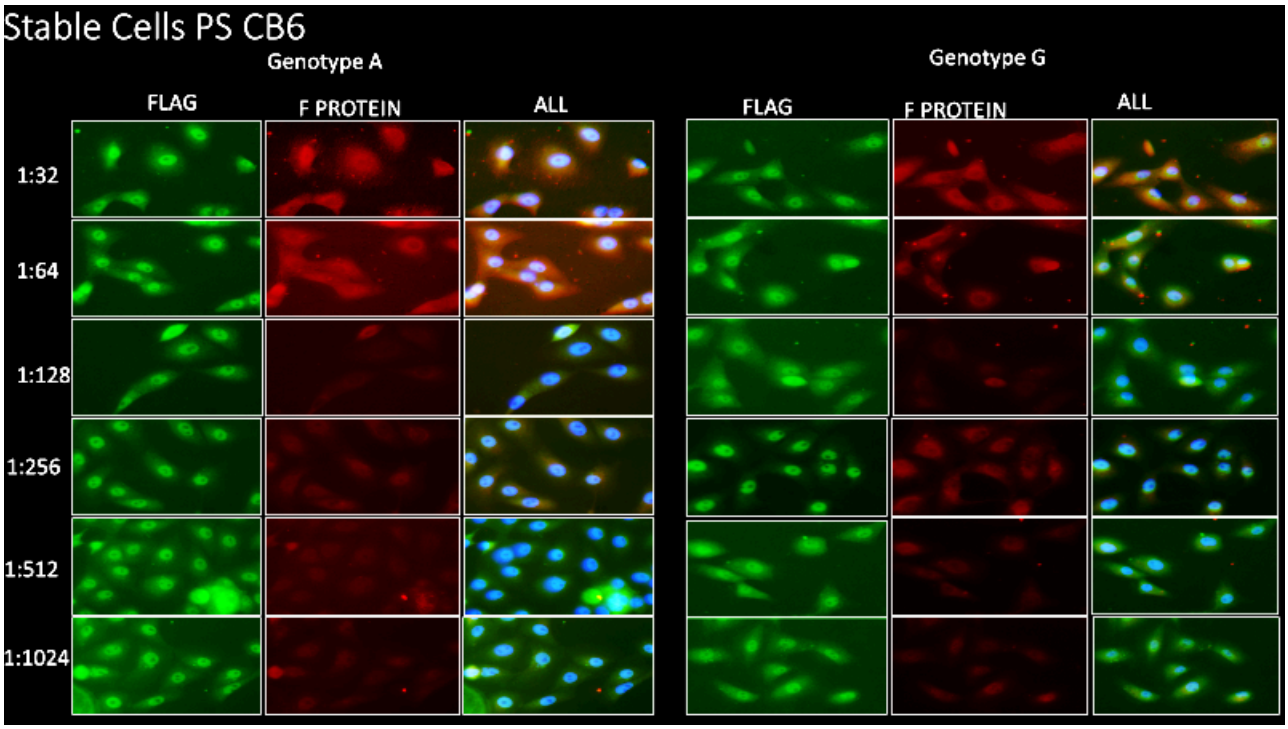


Figure A-78c. Stable cells Vaccine-Infected (CB) Sample#6.

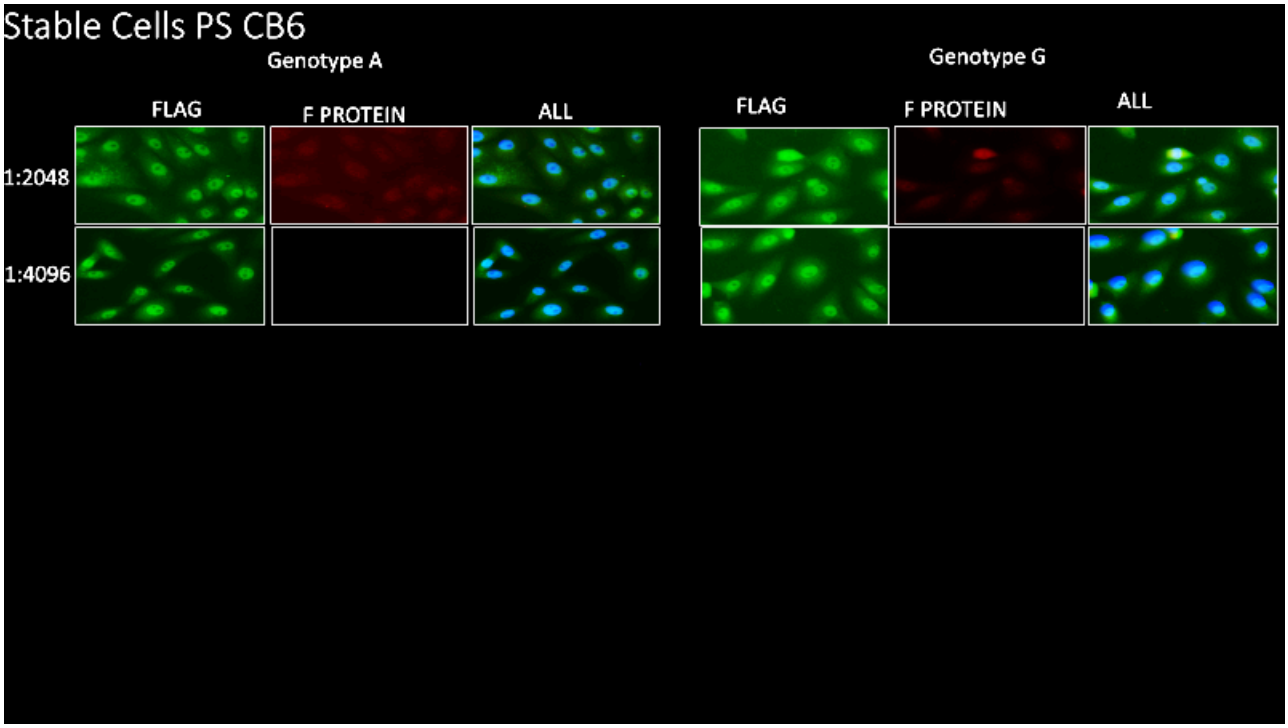


Figure A-78d. Stable cells Vaccine-Infected (CB) Sample#6.

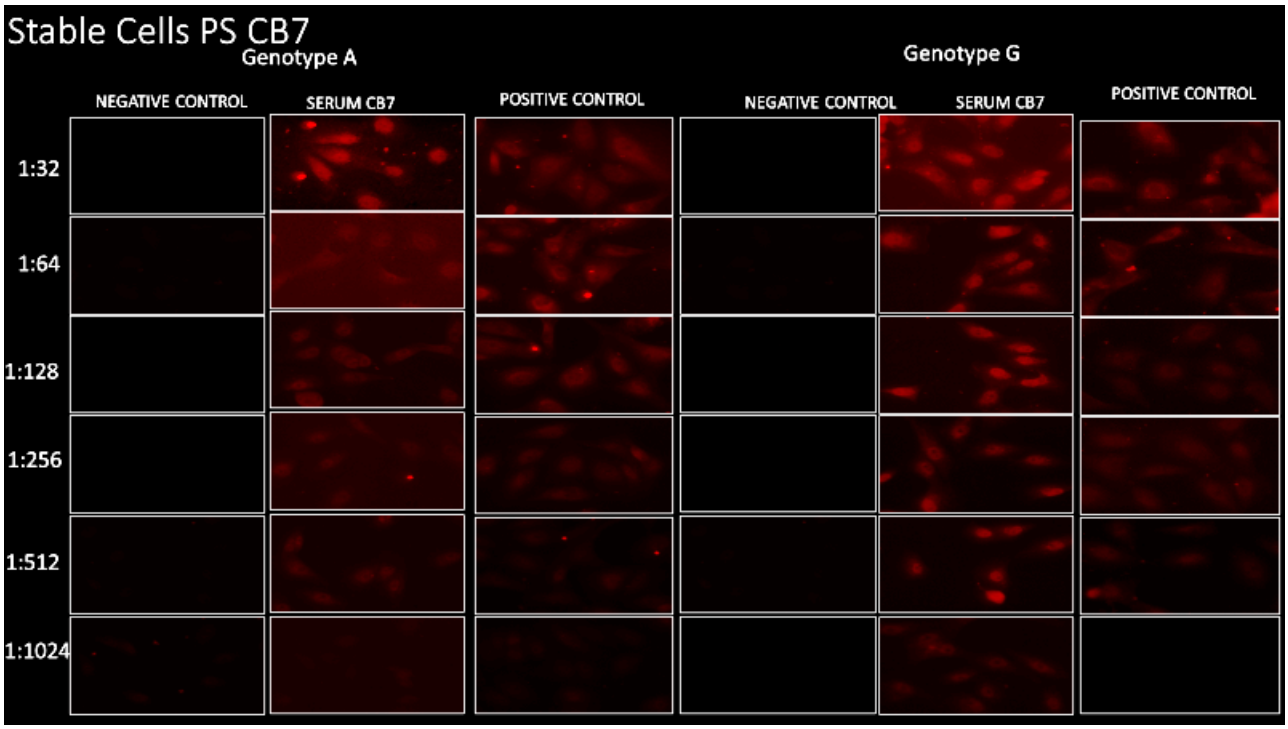


Figure A-79a. Stable cells Vaccine-Infected (CB) Sample#7.

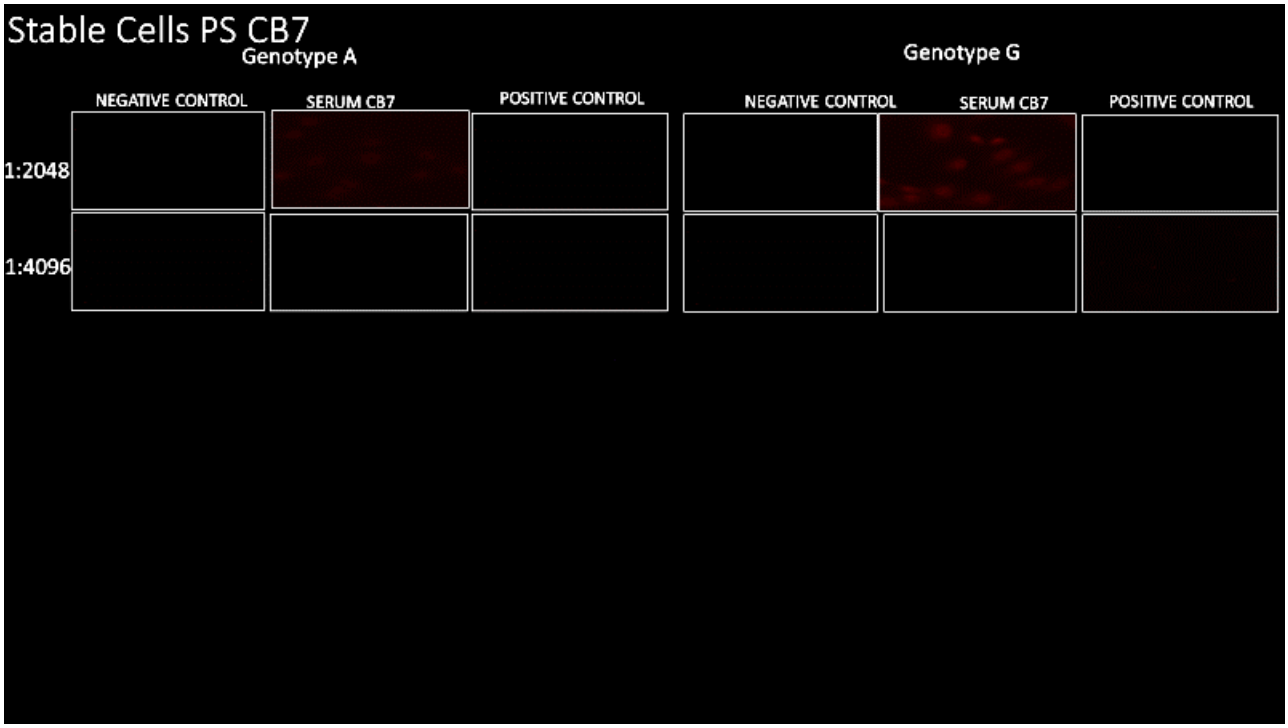


Figure A-79b. Stable cells Vaccine-Infected (CB) Sample#7.

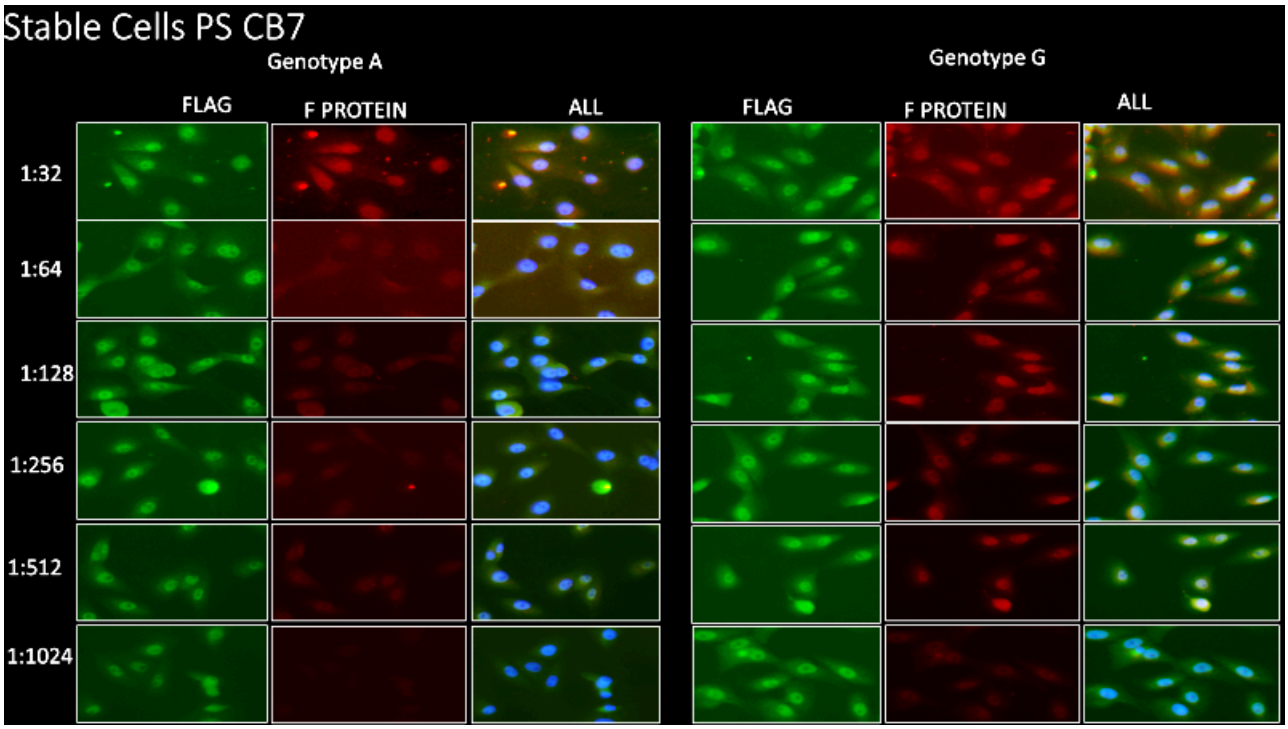


Figure A-79c. Stable cells Vaccine-Infected (CB) Sample#7.

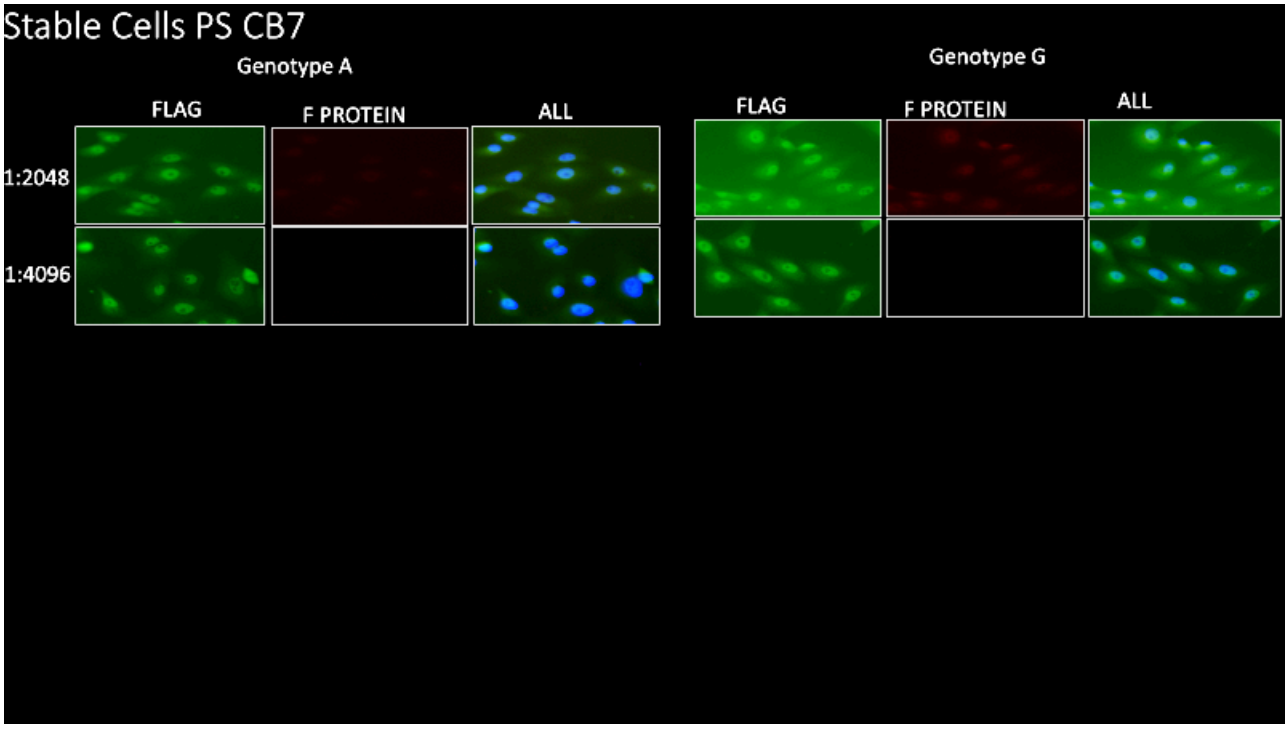


Figure A-79d. Stable cells Vaccine-Infected (CB) Sample#7.

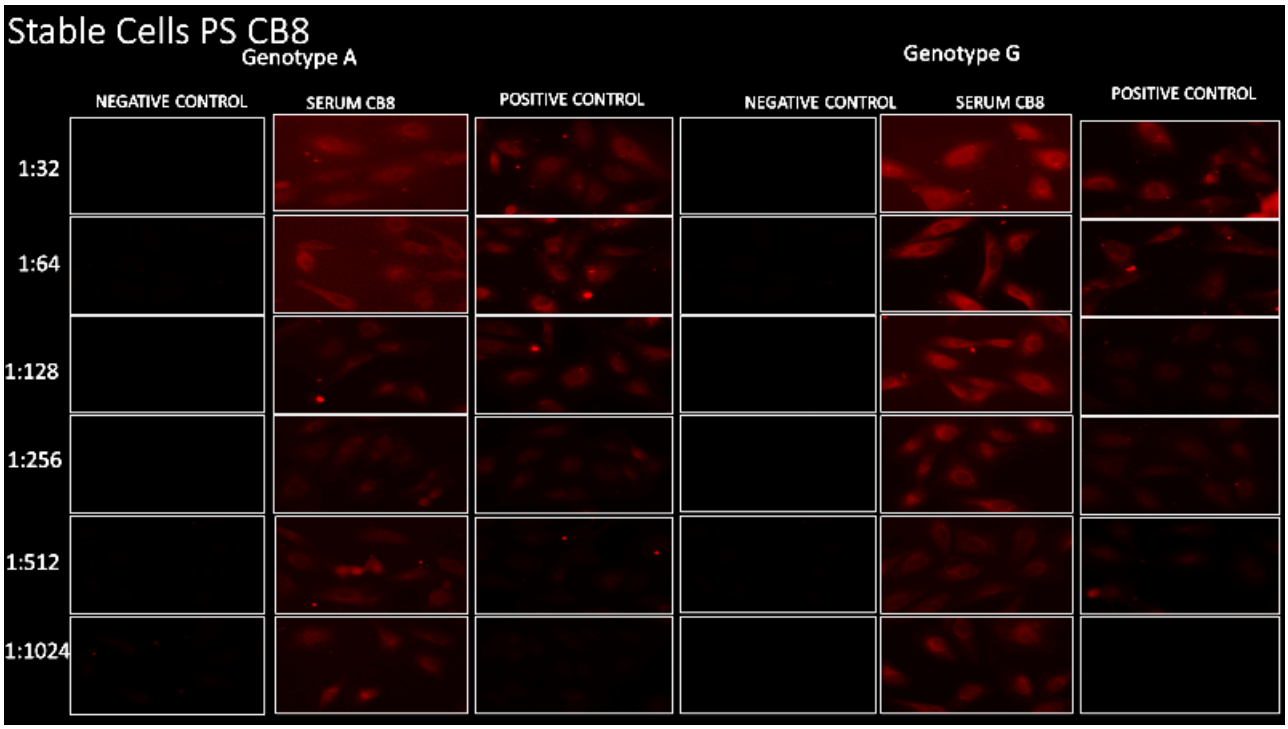


Figure A-80a. Stable cells Vaccine-Infected (CB) Sample#8.

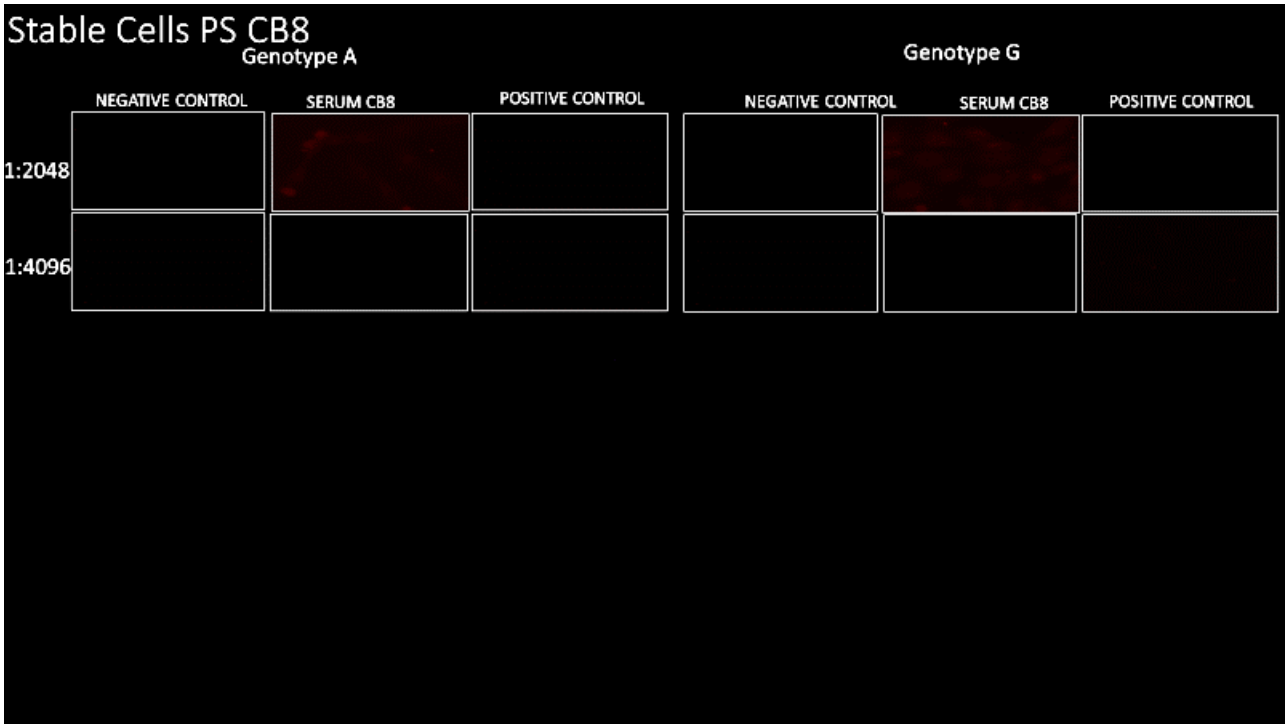


Figure A-80b. Stable cells Vaccine-Infected (CB) Sample#8.

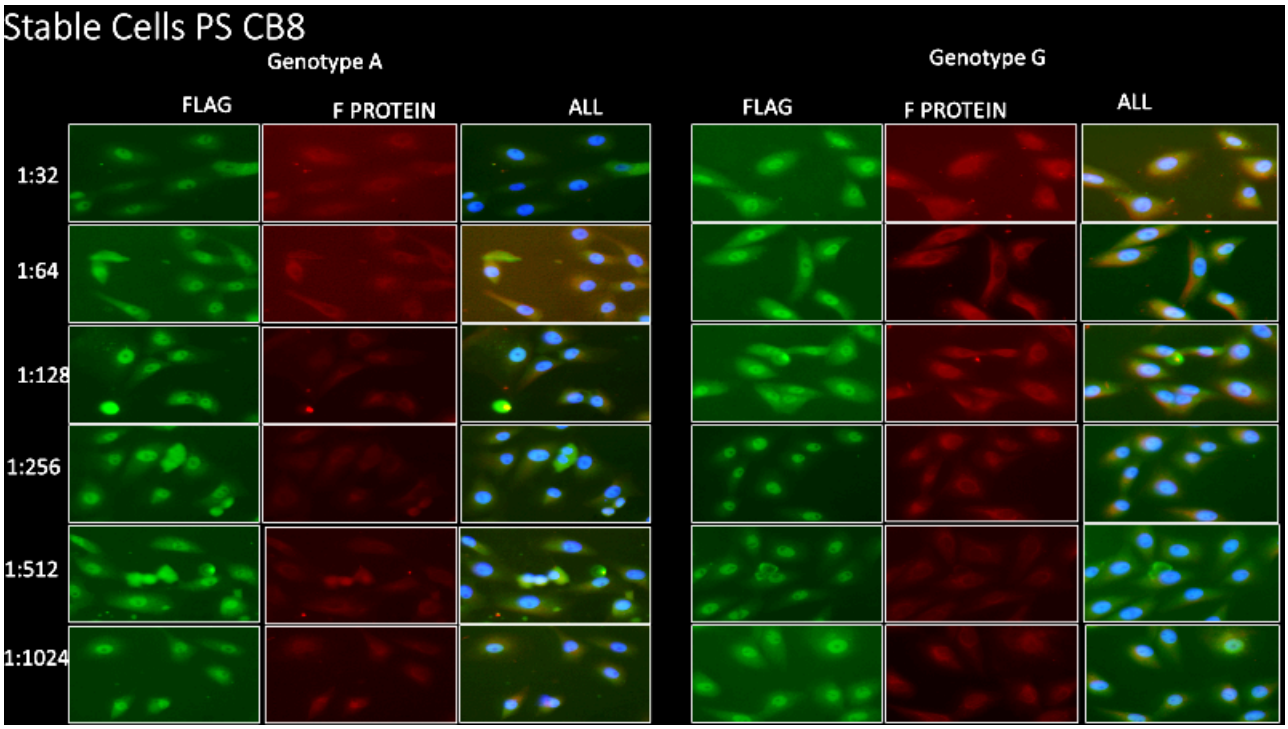


Figure A-80c. Stable cells Vaccine-Infected (CB) Sample#8.

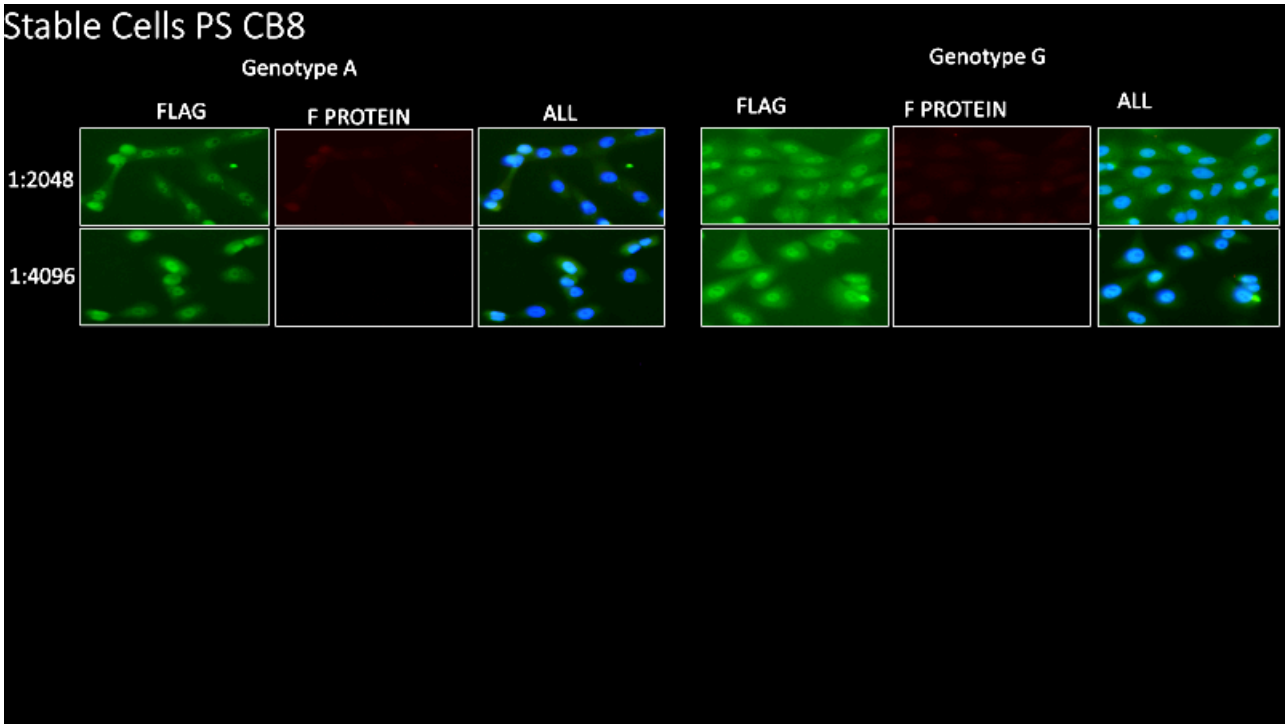


Figure A-80d. Stable cells Vaccine-Infected (CB) Sample#8.

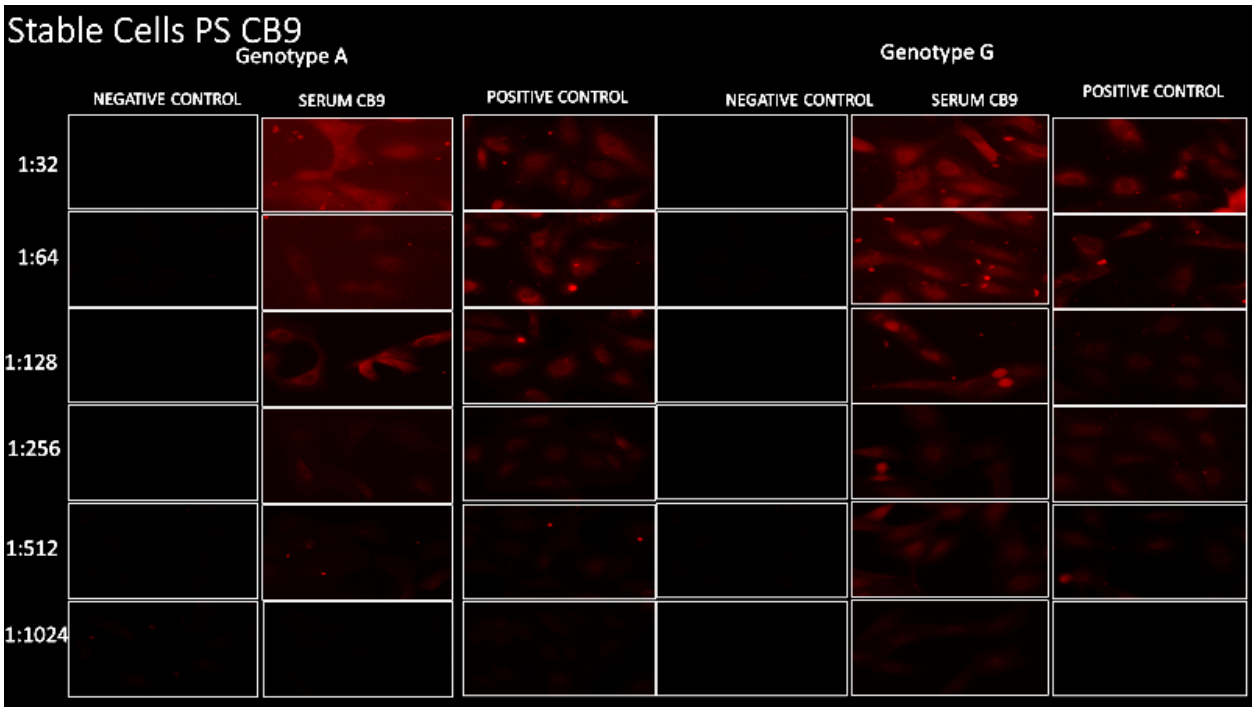


Figure A-81a. Stable cells Vaccine-Infected (CB) Sample#9.

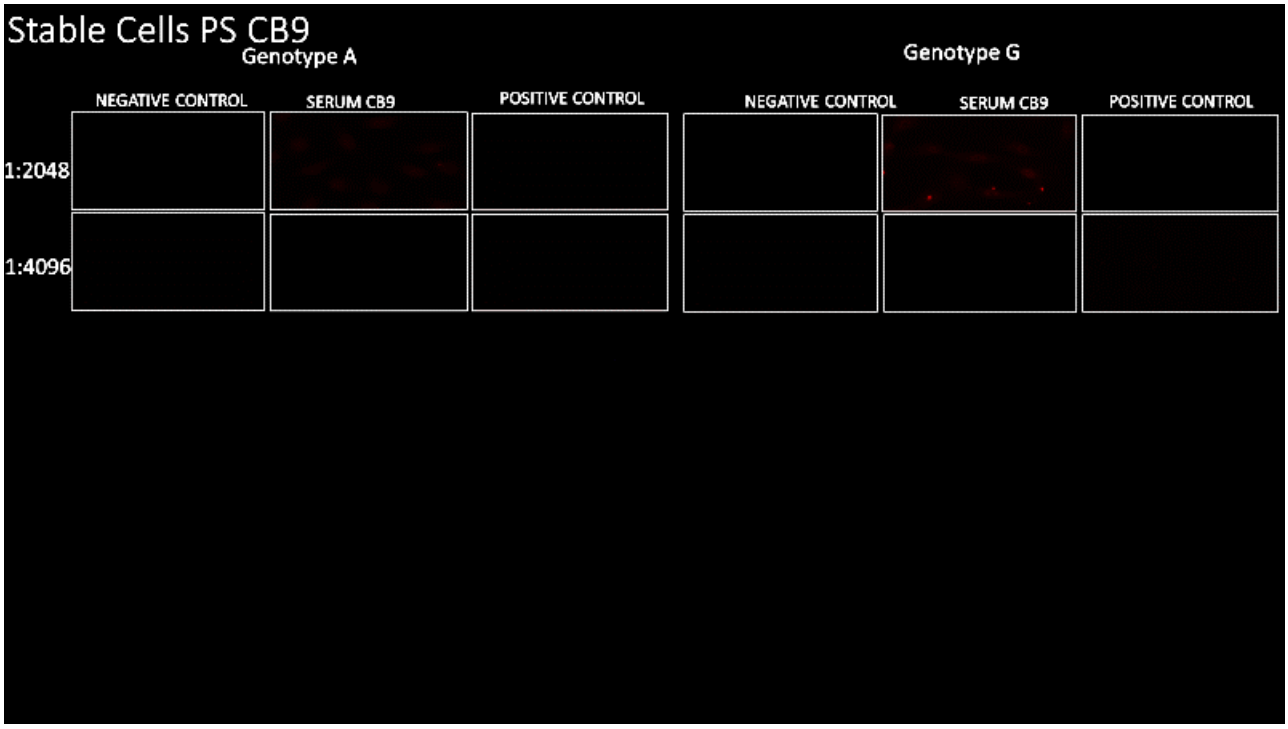


Figure A-81b. Stable cells Vaccine-Infected (CB) Sample#9.

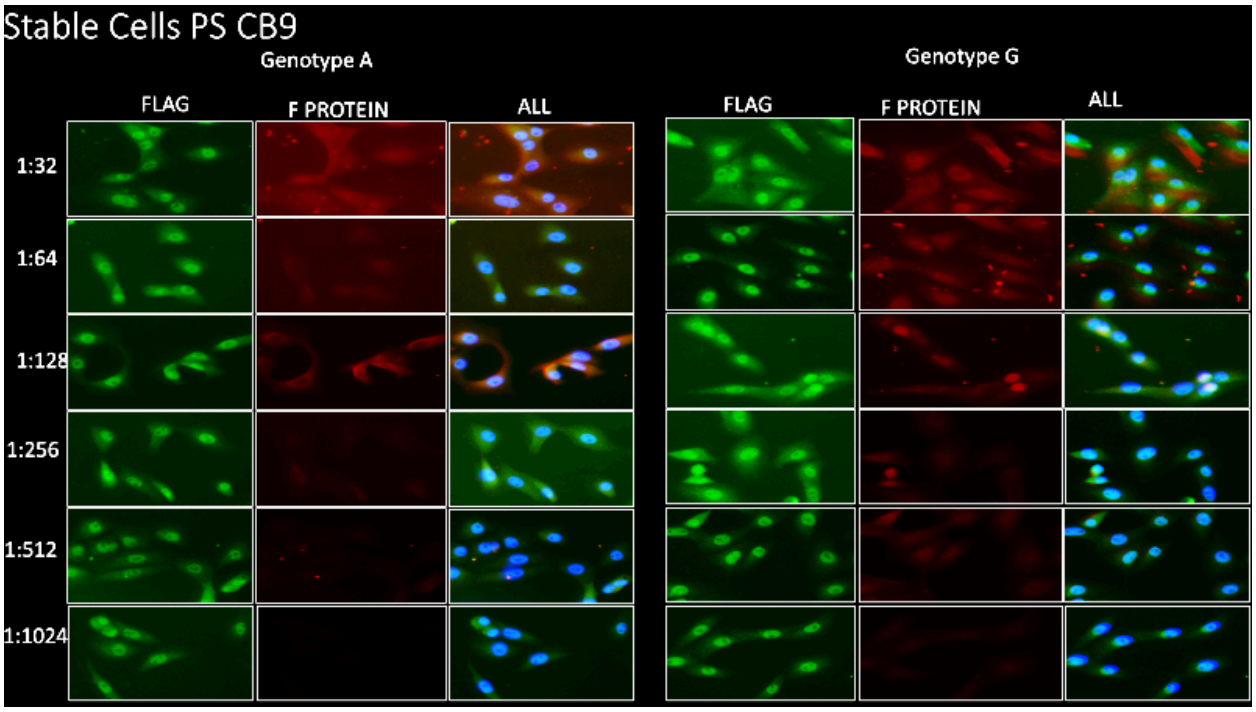


Figure A-81c. Stable cells Vaccine-Infected (CB) Sample#9.

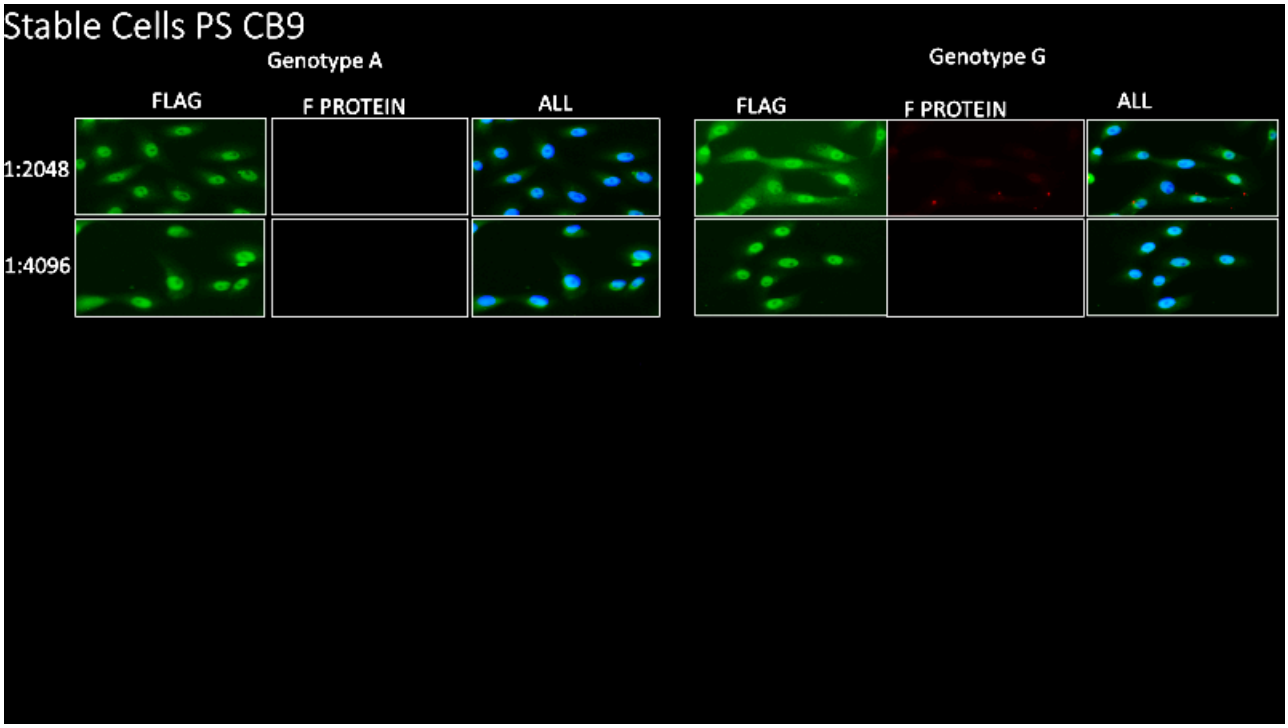


Figure A-81d. Stable cells Vaccine-Infected (CB) Sample#9.

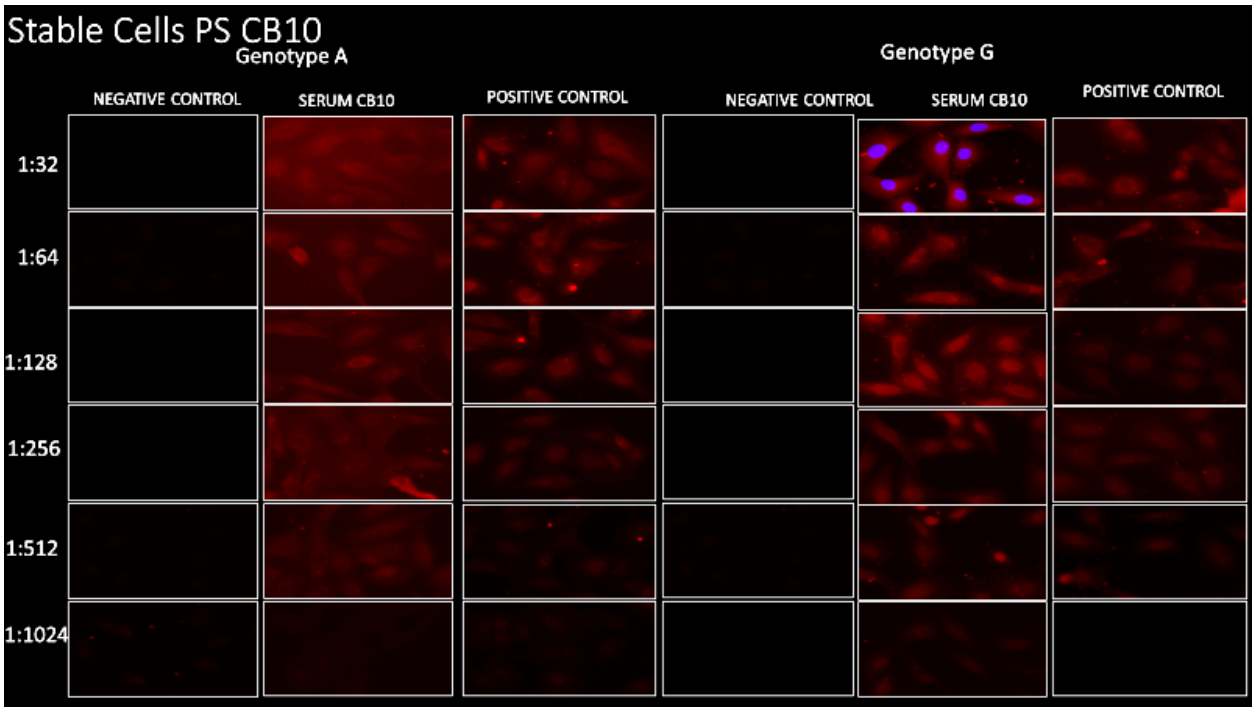


Figure A-82a. Stable cells Vaccine-Infected (CB) Sample#10.

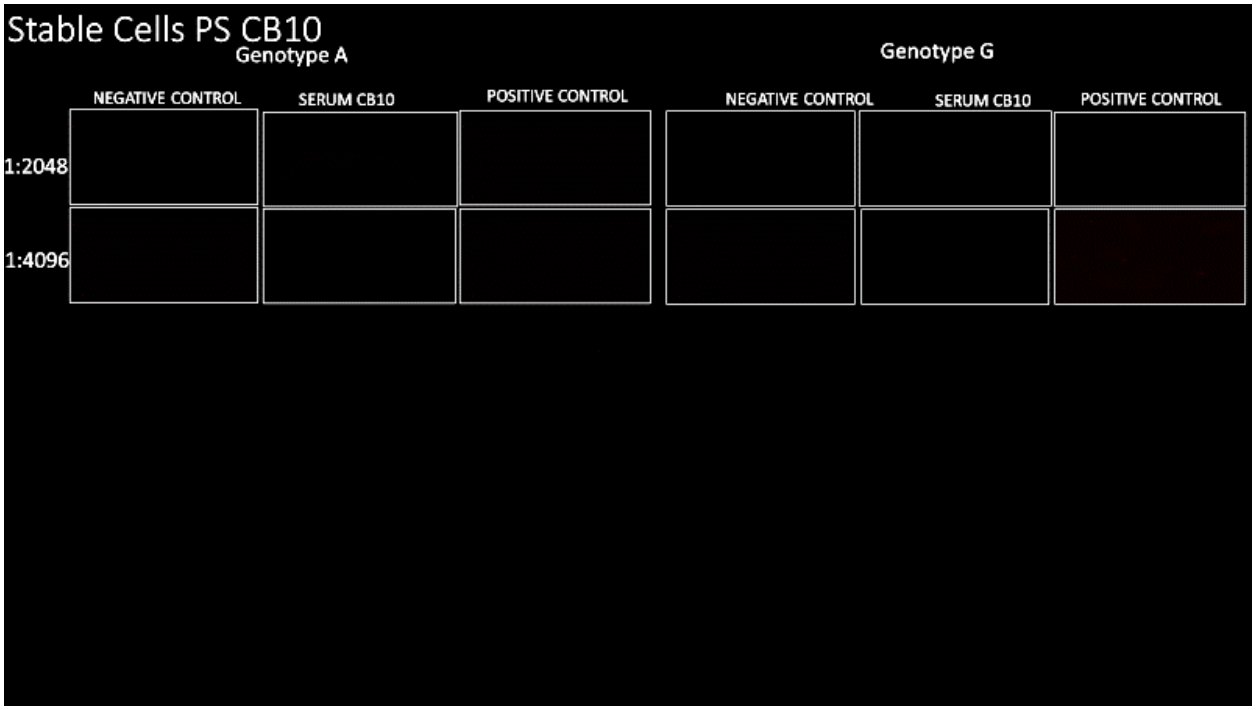


Figure A-82b. Stable cells Vaccine-Infected (CB) Sample#10.

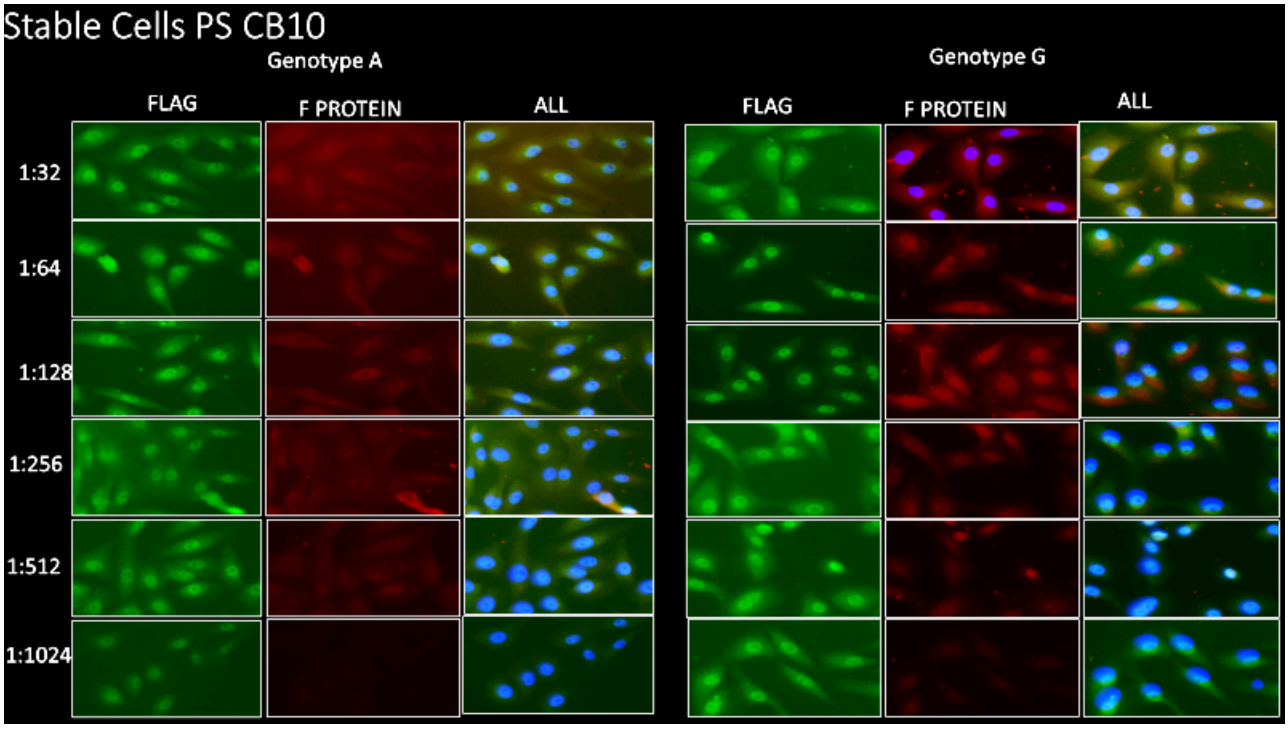


Figure A-82c. Stable cells Vaccine-Infected (CB) Sample#10.

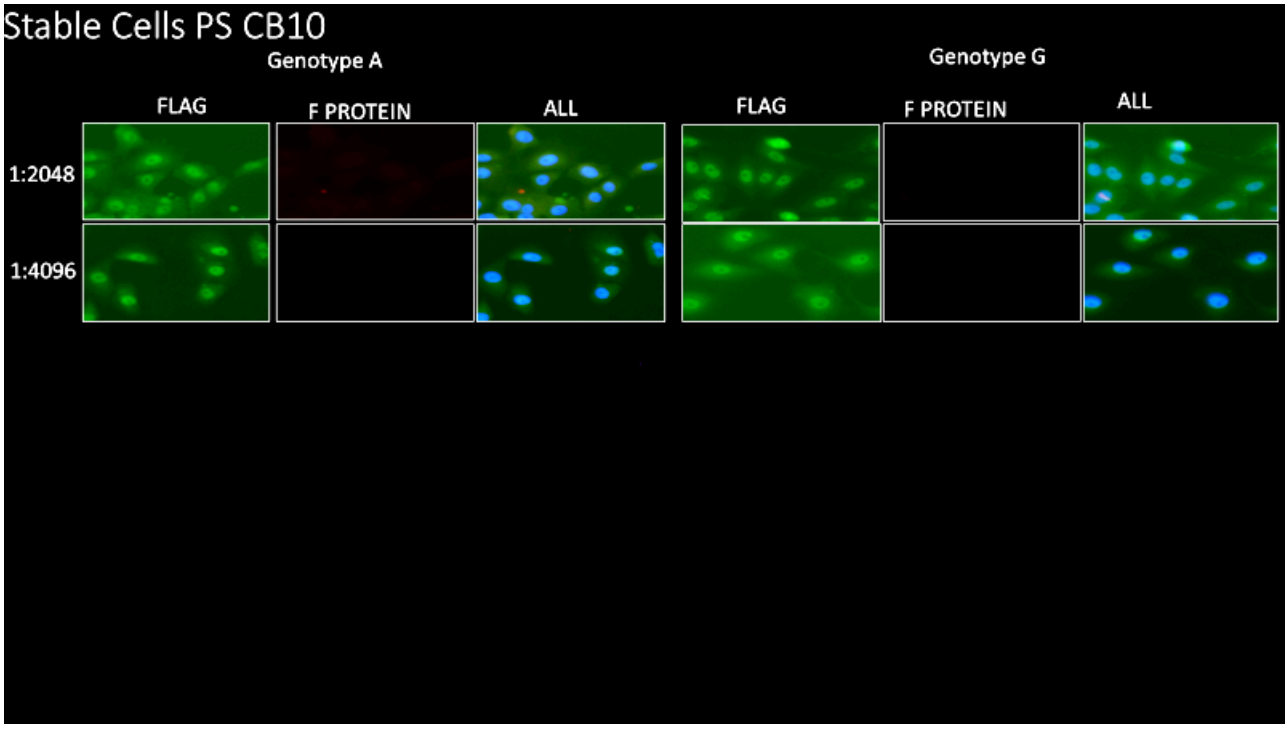


Figure A-82d. Stable cells Vaccine-Infected (CB) Sample#10.