A Library Screen for Yeast Mutants Defective in Transformation by the Lithium Acetate / Single Stranded DNA / Polyethylene Glycol Method

By

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A Thesis
Submitted to the Faculty of Graduate Studies
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for the degree of Master of Science

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MASTER OF SCIENCE

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ABSTRACT

In molecular biology, the term "transformation" is used to describe the genetic change which occurs when foreign DNA is taken up and stably expressed in a cell. Transformation has offered a great promise in the genetic manipulation of organisms; however the mechanisms behind the process are poorly understood. Current transformation methods in yeast result in less than 5% of cells which successfully express foreign DNA. A transformation screen was performed on a deletion library of yeast mutants derived from parent strain BY4742 to elucidate genes that could affect the transformation process. A total of 4827 mutants were screened, resulting in 115 mutants identified that displayed reduced transformation efficiencies when compared to the parent strain. The gene ontogeny of each mutant was referenced from the Saccharomyces Genome Database and mutants were categorized into groups based on biological function. The largest group consisted of mutants with genes of unknown function, while the remaining groups had a near equal distribution of mutants with genes that functioned in transcription, translation, transport, and vacuole formation. Immunofluorescence of several yeast strains showed DIG-labeled plasmid DNA bound to less than 5% of cells following transformation by LiAc/ssDNA/PEG method. Identifying the genes involved in yeast transformation will offer clues to this process in higher eukaryotic system which will allow for development of more efficient methods for both systems.

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1. INTRODUCTION

1.1 Transformation as a molecular tool

Frederick Griffith first coined the term "transformation" in 1928 while studying the pneumonia causing bacteria *Streptococcus pneumoniae* (Griffith 1928). A series of experiments lead to the conclusion that some "transforming principle" was responsible for converting an avirluent strain into virulent one. In the early 1940s, the team of Avery, MacLeod, and McCarty continued Griffith's work and eventually identified that DNA was the "transforming principle" Griffith described. Today, the process of introducing foreign DNA into a cell producing a genetic alteration is termed transformation. In the last couple of decades, genetic transformation process has become a powerful tool for the study of gene function and structure. The discovery of restriction endonucleases and the ability to clone eukaryotic DNA has lead to great growth in the field of molecular biology. A wealth of information can be learned by isolating individual genes from complex organisms and studying them in simpler organisms such as bacteria or yeast. Transformation is no longer limited to bacteria; it can be performed on yeast, plants, and mammalian cells. Although the methods are all different, the underlying principle is the same; the genetic transfer and expression of foreign DNA into a cell.

1.2 Yeast transformation techniques

1.2.1 Transformation by spheroplasting

Spheroplasting was the method used for the first successful transformation of yeast in 1978 (Hinnen *et al.* 1978). The yeast cell wall was removed by enzymatic digestion and the spheroplasts were osmotically stabilized in a solution of sorbitol/Tris/CaCl before and incubation with plasmid DNA and polyethylene glycol (PEG). The treated cells were resuspended in regeneration top agar and poured onto selection plates. Hinnen's original spheroplasting method yielded approximately 81 transformants/μg plasmid DNA. The method was further refined with the addition of carrier DNA into the transformation mix resulting in efficiencies to $5x10^6$ transformants/μg plasmid DNA (Burgers *et al.* 1987). The preparation of spheroplasts and their regeneration in solid media remains laborious, time consuming, and transformation efficiencies can be diminished by poor spheroplast re-growth in regeneration agar (Becker *et al.* 1991).

1.2.2 Metal Alkali Transformation

An alternative transformation method using intact whole yeast cells was developed in 1983 (Ito *et al.* 1983). This method did not require the removal of the yeast cell wall but rather the incubation of whole yeast cell with alkali metal ions and PEG. Ito investigated the effects of various metal ion combinations on yeast transformation. It was

found that Ca^{2+} ions, which were used to induce competence in *Escherichia coli* (Mandel *et al.* 1992), showed no effect on yeast transformation. Monovalent cations were shown to increase transformation up to 800 fold versus divalent cations. Metal ions including Li^+, Na^+, K^+, Cs^+, and Rb^+ were shown to induce competence in *S. cerevisiae.* Ito reported that lithium ions in the form of lithium acetate were the most effective metal ion to induce transformation in yeast. Lithium acetate resulted in an approximate 7 and 14 fold increase over the next best (Cs^+) and worst (K^+) monovalent ion respectively. Ito noted the absence of PEG in the transformation mix greatly diminished the transformation efficiency. Although Ito's method of transformation was simpler and less time consuming than Hinnen's spheroplasting method, it only achieved a maximum of 400 transformants /μg plasmid DNA.

1.2.3 Lithium acetate / Single stranded carrier DNA / Polyethylene glycol Transformation

The transformation method developed by Ito *et al.* has since been refined and improved into a high efficiency protocol (Schiestl *et al.* 1989). More than $1x10^6$ transformants/μg plasmid DNA has been reported when yeast cells are treated with a mixture of LiAc/ssDNA/PEG along with a 20 minute heat shock at 42°C (Gietz *et al.* 1992). The addition of single stranded carrier DNA is thought to bind to yeast cell surface and occupy DNA binding sites (Gietz *et al.* 1995). The LiAc/PEG/ssDNA method calls for a 500 fold excess single-stranded carrier DNA to the transformation mix which binds non-specifically and with greater affinity to the yeast cell wall leaving double stranded plasmid DNA more available for uptake. PEG was found to be essential to yeast

transformation having a strong effect on DNA binding to cell surfaces (Gietz *et al.* 1995; Zheng *et al.* 2005).

1.2.4 Transformation by Electroporation

Transformation by electroporation of mammalian cells had previously been accomplished (Potter *et al.* 1984). In 1989, Delorme published a protocol for transforming yeast cells with foreign DNA quickly and efficiently without removing or pre-treating the cell wall (Delorme 1989). Yeast cells were grown to log phase and received no chemical treatment prior to electroporation with plasmid DNA. Delorme achieved a maximum transformation efficiency of 4500 transformants/μg plasmid DNA. The method was further improved upon by osmotically stabilizing cells in 1 M sorbitol following electroporation (Becker *et al.* 1991). This method increased transformation efficiency to $3x10^5$ transformants/μg plasmid DNA. Transformation by electroporation was further refined by incubating cells in a mixture of lithium acetate and dithiothreitol prior to electroporation followed by stabilization in sorbitol (Thompson *et al.* 1998). A 15-300 fold improvement in transformation efficiency (up to 1.4×10^6 tranformants/μg plasmid DNA) was achieved in yeast strains that transformed poorly by other means (Thompson *et al.* 1998).

1.3 Factors Influencing Transformation

The majority of the yeast cell wall is composed of mannoproteins and glucans which provide structure and rigidity (Aguilar-Uscanga *et al.* 2003). Proteinase K and β-mercaptoethanol have previously been reported to remove mannoproteins and glucans resulting in increased porosity of the wall (Orlean *et al.* 1986). It was later reported that yeast cells treated with proteinase K, β-mercaptoethanol, and LiCl resulted in a leaky cell membrane as detected by endogenous RNA in the medium (Brzobohaty *et al.* 1986). Brozobohaty (1986) hypothesized the increased cell permeability facilitated the passage of transforming DNA across the cell wall.

Lithium treatment of cells has become essential in both electroporation and LiAc/ssDNA/PEG transformation methods. Pretreatment of cells with lithium acetate consistently results in several fold increased transformation over no pretreatment (Zheng *et al.* 2005). The exact effect of lithium treatment on cells is unknown but several hypotheses exist. The addition of the monovalent cation may be needed to reduce the electrostatic repulsion between the negatively charged transforming DNA and the partially negatively charged yeast cell surface (Eddy *et al.* 1958). Thompson *et al.* suggested lithium acetate enhanced pore formation (Thompson *et al.* 1998), while Gietz *et al.* suggested it increased the cell's capacity to bind DNA by exposing DNA binding sites (Gietz *et al.* 1995). Atomic force microscope (AFM) imaging has revealed that cells treated with lithium show rougher surfaces than those left untreated indicating increased cell permeability is a result of changes to cell surface structure (Zheng *et al.* 2005).

PEG is an essential component in both the spheroplasting and LiAC/ssDNA/PEG transformation methods. PEG is a water soluble polymer that may affect both conformational changes and aggregation of cells (Ito *et al.* 1983). Gietz and Schiestl (1995) have suggested PEG facilitated DNA precipitation onto the surface of a cell which was later confirmed by Zheng *et al.* (Zheng *et al.* 2005). They concluded PEG had a strong effect on inducing DNA binding to yeast cell surfaces. In the presence of PEG, the percentage of cells with bound DNA increased at three different DNA concentrations (Zheng *et al.* 2005).

The use of both lithium acetate and PEG to induce binding of DNA to the yeast cell surface is more effective than using either lithium acetate or PEG alone. Together the two components show a synergistic effect. Zheng *et al* demonstrated that lithium by itself would cause binding of DNA to 23.1% of cells, whereas PEG had a percentage of 61.7. However, when used together, lithium and PEG enhanced binding of DNA to cells at 98.2% (Zheng *et al.* 2005).

Heat shock is an important step in the LiAc/ssDNA/PEG yeast transformation method. Heat shock is commonly used in bacterial transformation and has been previously shown that both the temperature and duration of heat shock affects transformation efficiencies (Cosloy *et al.* 1973). It induces transcription of special proteins that function as molecular chaperones that protect important structural proteins and enzymes from stress induced degradation (Ellis *et al.* 1991). Gietz *et al* (1995) hypothesized that cellular changes as a result of heat shock could affect the levels or activities of specific proteins the might affect the transport of plasmid DNA to the nucleus or eliminate components that would degrade naked plasmid DNA.

Carrier DNA has become an essential component of spheroplasting, electroporation, lithium acetate transformation methods. Addition of denatured salmon sperm carrier DNA was shown to increase transformation efficiency at least 20 fold (Schiestl *et al.* 1989). Schiestl and Gietz (1989) were able to show that single-stranded carrier DNA could increase transformation efficiency by almost 100 fold over double stranded carrier DNA. They suggested that single stranded carrier DNA was more efficient at binding to non-specific binding sites on the yeast cell surface leaving plasmid DNA available for successful uptake by the cell (Gietz *et al.* 1995). The percentage of cells with bound plasmid DNA was shown to decrease from 95% to 28% with the addition of excess single stranded carrier DNA (Zheng *et al.* 2005).

Any of the methods previously described could be used to introduce foreign DNA into yeast to be stably expressed. The different transformation procedures have been refined and optimized to give maximum transformants; however, very little is known about the biological process that occurs during transformation. Transformation could be described as a multi-step process in which any one or a combination of the steps outlined below could lead to reduced transformation efficiencies.

1) Presentation of foreign DNA to a cell
2) Binding of exogenous DNA to the cell
3) Uptake of foreign DNA into the cell
4) Protection of naked DNA from host nucleases
5) Transport of foreign DNA to the nucleus
6) Uptake of foreign DNA into the nucleus
7) Expression of foreign DNA via the host transcription machinery

Several groups have looked at different components of the process to try to elucidate the factors involved. In bacteria, several proteins have been identified that are responsible for binding and internalization of exogenous DNA. The naturally

transforming bacterium, *Bacillus subtilis,* is able to bind exogenous double stranded DNA through the use of a ComEA membrane protein and internalize it through a ComEC protein (Inamine *et al.* 1995). ComEA was found to bind DNA on its C-terminus end and deletion of the protein severely affected the transformation efficiency (Provvedi *et al.* 1999). The proteins show conservation in other bacterial species including *Neisseria gonorrhoeae* and *Streptococcus pneumoniae* and are a requirement for genetic transformation (Chen *et al.* 2001; Berge *et al.* 2002).

There is likely no conserved transformation pathway in eukaryotes; however, cells when made competent, can be induced to transform. Wong *et al.* (2007) showed that liposomes complexed with DNA can enter the cell via two distinct routes; caveolae-mediated internalization or clathrin mediated endocytosis (Wong *et al.* 2007). His group was able to demonstrate the delivery of complexed DNA from the late endocytic pathway to the nucleus.

The *Saccharomyces cerevisiae* organism offers powerful models for investigating the molecular mechanisms involved in eukaryotic cell cycle. *S. cerevisiae* cells offer several advantages over other cells that make them suitable for biological study including rapid growth, dispersed cells, and a well defined genetic system (Oliver, 2007). They are also non-pathogenic and can be handled with little precautions. The complete *S. cerevisiae* genome was successfully sequenced in 1996 and found to be 12 Mb in length, spanning 16 chromosomes (Goffeau *et al.* 1996). One of the best resources that has arisen from the completed genome is the creation of deletion mutants covering 96% of the open reading frames (ORFs) in *S. cerevisiae*. Based on the completed sequence, one mutant was created for every predicted protein encoding gene (Giaever *et al.* 2002).

To date, 6,609 ORFs have been identified in *S. cerevisiae,* of which 70.49% (4,659 ORFs) have been identified to code for a gene product, 17.17% (1,135 ORFs) are uncharacterized, and 12.33% (815 ORFs) are dubious (http://www.yeastgenome.org/cache/genomeSnapshot.html).

1.4 Thesis objectives

Our laboratory is interested in uncovering the genes that affect the transformation process. Current transformation methods result in less than 5% of yeast cells successfully incorporating and expressing foreign DNA (R.D.Gietz personal communication). Gene disruption is a powerful molecular tool which allows for the better understanding of gene function. A *S. cerevisiae* deletion library was obtained from Open Biosystems in which the phenotype displayed by each mutant reflects a complete loss of function of a gene. The advantage of the deletion library is the identity of the gene is known, so we can use a reverse genetics approach to look for phenotypes resulting from the loss of the gene. In this project the phenotypic change we want to identify is each mutant's transformation ability in an attempt to identify genes involved in this process.

An effective method to investigate the transformation process is to study mutants which are defective in the process. The objective of this project was to use a LiAc/ssDNA/PEG microtitre plate transformation method developed in our lab to screen a library consisting of 4827 yeast deletion mutants to identify genes involved in the transformation process. Direct immunofluorescence was used to probe DIG labeled DNA transformed into yeast to observe a moment in the transformation process. Several poorly

transforming yeast strains with defects in different biological functions were characterized to visualize differences between them. Understanding the genes involved in yeast cell transformation will offer clues to this process in higher eukaryotic systems. Better methods could then be devised to increase transformation efficiencies in both yeast and mammalian systems.

2. MATERIALS AND METHODS

2.1 Yeast strains

Table 1. List of *S. cerevisiae* strains used and their genotype

Strain	Genotype
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*
KGY37	*MATa ade2 gal4 gal80 his3-Δ200 trp1-Δ901, leu2::pUC18 ura3::GAL1-lacZ, lys2::UASG 17 MERS(x3) GAL1-HIS3*
DGY233	*MAT a, ade2-1, lys2-1, ura3-52, leu2-3,112, his3-Δ200, trp1-Δ1, bar1-Δ1*

A yeast knock-out library was purchased from Open Biosystems derived from *Saccharomyces cerevisiae* strain BY4742. The Saccharomyces Genome Deletion Project is a collection gene-disruption mutants was created covering 96% of the yeast genome. Knockout strains were created through a two-step PCR-based approach. Each ORF was replaced with a KanMX antibiotic resistance marker along with a unique 20 base pair nucleotide barcode sequence (Giaever *et al.* 2002). The yeast knock-out library was formatted in 96-well microtitre plates as frozen glycerol stocks (YPD media and glycerol). Plates were maintained in a freezer at -80°C.

The yeast strains KGY37 and DGY233 were obtained from frozen stock collections of Dr. R.D. Gietz.

2.2 Bacterial Strain

Escherichia coli strain Mg7α was provided by Dr. R.D. Gietz and contains the following genotype: F-, *hsdR17*(r_K^- m_K^+), *glnV44, thi-1, deoR, recA1, relA1, supE44, Δ(lacZYA-argF) U169, λ-, (Φ80dlacΔ(lacZ)M15), pyrFΔ168, leuBΔ211, hisBΔ277,*

trpCΔ137, endAΔ605 (Griffith *et al.* 2003). This strain was used to produce plasmid DNA used in this project.

2.3 Plasmids

Table 2. List of plasmids used for this project

Plasmid Name	Vector Size (bp)	*E. coli* Selection	*S. cerevisiae* selection	GenBank Accession #	Source
YEPlac 181	5741	Amp	leu2	X75460	(Gietz *et al.* 1988)
YEPlac 112	4889	Amp	trp1	X75458	(Gietz *et al.* 1988)
pRKO-pBRori	10047	Amp	trp1		Pfizer

The plasmids YEplac 181, YCplac 33, YEplac 112, and pRKO-pBRori (RKO IV) were obtained from Dr. R. D. Gietz, Department of Biochemistry and Medical Genetics, University of Manitoba. All plasmids are shuttle vectors with the ability to replicate in both *Escherichia coli* and *Saccharomyces cerevisiae*.

Plasmids YEplac 181 and YEplac 112 contain the 2 μm origin of replication (2 μm circle) for multi copy replication in yeast (Gietz *et al.* 1988). Plasmid YEplac 181 contains the LEU2 gene which will complement yeast strains containing a leu2 mutation. Plasmid YEplac 112 contains the yeast TRP1 gene which can be selected for in yeast strains that contain a trp1 mutation.

Plasmid RKO IV is an autonomously replicating plasmid containing ARS1, CEN4, and is stably maintained but present in low copy numbers, typically found at 1-2 copies per cell (Tschumper *et al.* 1987). RKO IV contains the yeast TRP1 which can be selected for in strains containing a *trp1* mutation.

2.4 DNA precipitation by Ethanol

The aqueous phase solutions containing plasmid DNA was precipitated by addition of 2.5 volumes of ice cold ethanol and 1/10 volume of 3 M sodium acetate (pH 6.0). The samples were mixed and incubated at -20°C for at least 1 hr. DNA was harvested by centrifugation at 13000 x *g* for 15 min at 4°C. The supernatant was removed and pellet washed with room temperature 70% ethanol then DNA collected again by centrifugation. The pellet was air dried at room temperature and dissolved in an appropriate volume of TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA).

2.5 DNA precipitation by Polyethylene Glycol (PEG)

DNA samples contaminated with RNA oligonucleotides were precipitated with polyethylene glycol as recommended by (Paithankar *et al.* 1991). A 33 µl solution of 20% PEG_{Mr8000} / 5 M NaCl was added to 50 µl DNA sample and incubated in ice water for at least 1 hour. DNA was collected by centrifugation at 13000 x *g* at 4°C for 15 min. The pellet was rinsed with 150 µl of room temperature 70% ethanol, air dried, then dissolved in 25 µl of TE.

2.6 DNA Labeling with Digoxigenin (DIG)

Non-radioactive random primed DNA probes were created using a Dig High Prime DNA Labeling Kit obtained from Roche Applied Science (Cat # 11585614910).

Plasmid RKO IV was digested with restriction enzyme PvuII (see Section 2.11) to produce a linear plasmid with a COS site on each end. Linearized plasmid was collected by ethanol precipitation (see Section 2.4) in a microcentrifuge tube and 1 μg of digested template DNA was added to sterile ddH_20 to a final volume of 16 μl. The tube was placed in a boiling water bath for 10 min to denature the DNA then chilled in an ice water bath. Four microliters of DIG-High Prime mix was added and the contents gently mixed with a pipette. The reaction was allowed to proceed for 20 hr at 37°C and stopped by incubation in a water bath at 65°C for 15 min. The DNA was collected by PEG precipitation as described above.

2.7 DIG DNA labeling efficiency

The efficiency of the labeling reaction was determined by direct detection. A dilution series of DIG-labeled DNA along with defined DIG-labeled control standard were spotted onto a piece of nylon membrane. The membrane was placed under a UV lamp for 10 min for DNA cross linking to membrane to occur. The nylon membrane was transferred to a Petri dish containing 20 ml maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH to 7.5) and incubated for 2 min at room temperature. The membrane was transferred to a new dish containing 10 ml of blocking solution (Roche cat# 11096176001) and incubated for 30 min with gentle shaking. Binding of antibody to DIG labeled probe was accomplished by transferring membrane to a new dish containing 10 ml antibody solution and incubated with shaking for another 30 min. Washing of membrane was accomplished with two 15 min incubations in washing buffer

(0.1 M maleic acid, 0.15 NaCl, 0.3% (v/v) Tween20, pH to 7.5). The membrane was then equilibrated to pH 9.5 with 2 minute incubation in 10 ml detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH to 9.5). The membrane was placed DNA side up onto a piece of saran wrap and 1 ml of chemiluminescent substrate for alkaline phosphatase (Roche cat # 11755633001) was spotted before membrane was wrapped in plastic wrap. The membrane was placed into a development fold along with a piece of Kodak imaging film (cat# 1438795) and exposed for 15-25 min. The intensity of spots of DNA-labeled probe was compared to the control and efficiency determined.

2.8 Isolation of plasmid DNA from bacteria using a Qiagen™ column

Table 3. List of solutions used for plasmid purification

Solutions	Contents
Resuspension buffer	50 mM Tris-Cl, pH 8.0; 10 mM EDTAl 100 µg/ml RNase A
Lysis buffer	200 mM NaOH, 1% SDS
Neutralization buffer	3.0M potassium acetate, pH 5.5
Equilibrium buffer	750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol; 0.15% Triton X-100
Wash buffer	1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol
Elution buffer	1.25 M NaCl; 50 mM Tris-Cl, pH 8.5; 15% isopropanol

Plasmid DNA was purified using a Qiagen™ column and the reagents used listed in Table 3. A single colony from transformed *Escherichia coli* Mg7α cells was inoculated into a volume of 2 ml LB media containing ampicillin (100 µg/ml). The starter culture was incubated for 8 hours at 37°C on a rotary shaker at 300 RPM then transferred to a volume of 250 ml LB media containing ampicillin (100 µg/µl) and allowed to grow overnight at 37°C with shaking.

The cells were harvested by centrifugation at 5000 RPM for 5 min. The bacterial pellet was resuspended in 10 ml cold resuspension buffer kept at 4°C. Ten milliliters of lysis buffer was added and mixed, and the contents incubated at room temperature for 5 min. Twenty ml of cold neutralization buffer was added and contents mixed by inverting the tube several times. The mixture was then poured into a QIAfilter cartridge and incubated for 10 min at room temperature. QIAGEN-tip 500 columns were equilibrated with 10 ml equilibrium buffer and the flow through discarded. The cell lysate was then filtered through the QIAfilter into the barrel of the QIAGEN-tips. Eluent from the QIAGEN-tips were collected and discarded. Two 30 ml volumes of wash buffer was allowed to flow through the column and subsequently discarded. The DNA was eluted by addition of 15 ml elution buffer into a new collection tube and precipitated with ethanol as described in Section 2.4.

2.9 Gel Electrophoresis

A 0.7% (w/v) to 2.0% (w/v) agarose gel was cast depending on the requirement by adding the appropriate amount of electrophoresis grade agarose (Sigma Chemical Co. Ltd) to 100 ml of 1 x TAE buffer (40 mM Tris-acetate, 2 mM EDTA) and dissolved in a microwave until no granules remained. Agarose solution was cooled to ~50°C and 4 µl of 10 mg/ml ethidium bromide was added and gently mixed into solution. The mixture was poured into a casting mould with the appropriate sized combs for the loading wells. After the gel had solidified it was transferred to a horizontal electrophoresis chamber. The comb was removed and the chamber filled with 1 x TAE buffer (see Section 6.3).

Samples were loaded in wells along with DNA ladder (10 µg of 1 kb DNA ladder solution (10 µl of 1kb DNA ladder New England Biolabs, 100 µl of 10 x loading buffer, 890 µl ddH_20)). Samples were electrophoresed at 100V for 60-80 min and fragments were visualized under long wave UV light.

2.10 Determination of DNA concentration

Double stranded plasmid DNA concentration was determined using the formula:

DNA concentration (µg/ml) = (50 µg/ml)(A_{260})(dilution factor)

where A is the measured absorbance of the sample at 260 nm measured with a spectrophotometer.

2.11 Restriction enzyme digestion

Restriction enzymes were obtained from Promega or New England Biolabs, and digestions were carried out according to manufacturers' directions. One to 10 µg of plasmid RKO IV was digested in a microcentrifuge tube. The components were added in the following order:

Table 4. Components of restriction enzyme digestion

Sterile ddH_20	16.3 µl
RE 10X buffer	2.0 µl
Acetylated BSA (10 µg/µl)	0.2 µl
Plasmid DNA	1.0 µl
Restriction enzyme (10 u/µl)	0.5 µl
Final Volume	**20 µl**

The contents were mixed gently by pipetting and incubated at the manufacturers' specified optimal temperature for 1 to 4 hours. Enzyme inactivation was accomplished by incubation for 15 min in a 65°C water bath.

2.12 Preparation of electrocompetent *Escherichia coli*

E. coli strain Mg7α was made electrocompetent by the methods of Dower (Dower *et al.* 1988). One liter of LB medium (see Appendix 6.2.1) was inoculated with the *E. coli* strain Mg7α and incubated at 37°C on a New Brunswick Gyratory Shaker Model G2 rotary shaker for 4 to 6 hrs at 200 RPM until an OD_{600} between 0.5 and 1 was reached. The culture was rapidly chilled in an ice water bath and cells collected by centrifugation at 5000 x *g* for 15 min at 4°C. The cell pellet was washed in 1 L cold sterile ddH_20, collected by centrifugation, resuspended in 500 ml cold sterile ddH_20, collected again and resuspended in 20 ml cold sterile 10% (v/v) glycerol. Cells were collected once more and resuspended in 2 ml cold sterile 10% (v/v) glycerol then aliquoted into 25 µl samples. The samples were frozen with liquid nitrogen and stored at -80°C.

2.13 Transformation of electrocompetent *Escherichia coli*

A frozen 25 µl aliquot of electrocompetent Mg7α cells was thawed on ice. A micropipette was used to add 200 ng of plasmid of interest to cells. The mixture was mixed then transferred to an ice chilled 0.1 cm gap BioRad electroporation cuvette. The cuvette was immediately pulsed at 1.25 kV, 25 µF, with a 400 Ω resistor in a BioRad

Gene Pulser. One ml of cold SOC medium (see Appendix I 6.2.2) was immediately added to cuvette and contents mixed with a micropipette. The cells were transferred to a 1.5 ml microcentrifuge tube and placed in a 37°C incubator for 30 min. Samples of 2, 20, and 200 µls were plated onto LB plates containing ampicillin (100 µg/ml) and incubated overnight at 37°C.

2.14 Transformation of *Saccharomyces cerevisiae*

2.14.1 Reagents and solutions

Lithium Acetate 1.0M (LiAc)

A 1.0M stock solution was prepared by dissolving 102 g Lithium acetate (Sigma Chemical Co LTD., L-6883) in 800 ml sterile nanopure water. Volume was brought up to 1000 ml and the solution was sterilized by autoclaving for 20 min at 121°C. This stock solution was stored at room temperature.

Polyethylene Glycerol MW 3350 (PEG 50% w/v)

A 50% w/v solution was prepared by dissolving 500 g of PEG molecular weight 3350 (Sigma Chemical Co. Ltd., 93640) in 300 ml sterile nanopure water. The volume was brought up to 1000 ml and solution transferred to a glass bottle and sterilized by autoclaving for 20 min at 121°C. Stock solution was kept at room temperature tightly capped to prevent evaporation.

Single-stranded Carrier DNA (ssDNA 2.0 mg/ml)

A 2 mg/µl stock solution was made by dissolving 200 mg of single-stranded salmon sperm DNA (Sigma Chemical Co. Ltd., D1626) in 100 ml of TE on a stirring plate. Samples were stored frozen at -20°C in 1 ml aliquots. Prior to use, the carrier DNA was denatured in a boiling water bath for 5 min and immediately placed in an ice water bath to prevent re-annealing. Boiled samples were re-used no more than 3 times before new a sample was used to reduce loss of activity.

2.15 Growth of *Saccharomyces cerevisiae*

Yeast strains were grown in liquid YPAD medium (YPD supplemented with adenine hemisulphate (Sherman 2002). Starter cultures of yeast were grown to stationary phase in a 2 or 5 ml YPAD (see Appendix I 6.1.1) culture inoculated from a single colony streaked from a solid YPAD plate. Cultures were incubated overnight at 30°C on a rotary shaker at 200 RPM. Cell concentration was determined from 1/10 dilution spotted on a haemocytometer. The overnight culture was inoculated to $5x10^6$ cells/ml in fresh 2X YPAD (see Appendix I 6.1.2) and allowed to grow for at least 2 divisions (cell density $2x10^7$ cells/ml) before being harvested for use.

2.16 Storage *of Saccharomyces cerevisiae* stock

Long term storage of yeast stock was accomplished by the method described by (Sherman 1986). Yeast strains were streaked on solid YPAD plates and incubated at 30°C for 2 days. The rounded end of a toothpick was used to scrape cells off the plate.

The blob of cells were then transferred to a cryo-vial containing 1 ml of sterile 20% (w/v) glycerol solution and resuspended by vortex mixing. Vials were stored frozen at -80°C

2.17 Transformation of *Saccharomyces cerevisiae* by Electroporation

Transformation by electroporation was carried out by the methods of Thompson *et al.* (1998). A yeast culture was grown to log phase as described in section 2.15. Cell culture was transferred to 50 ml polypropylene tubes and harvested by centrifugation at 3500 x *g* for 5 min in an IEC Centra 4 centrifuge. The supernatant was decanted the cells washed by resuspension in 25 ml sterile ddH_2O. Cells were harvested again by centrifugation and treated with 25 ml of electroporation solution (0.1 M lithium acetate, 10 mM dithiothreitol (DDT), 1mM EDTA, 10 mM Tris-Cl). The mixture was incubated at room temperature for 1 hr. The cells were collected by centrifugation for 5 min and resuspended in 25 ml ice cold sterile ddH_2O. The cells were collected and washed again in 25 ml ice cold sterile ddH_2O and resuspended in 10 ml ice cold 1 M sorbitol. Following another round of centrifugation the cell pellet was resuspended in 1 M sorbitol to a reach a final volume of 500 µl cell suspension. Aliquots of 50 µl of cell suspension were transferred to microcentrifuge tubes. Fifty µl was used per transformation which corresponded to $1x10^8$ cells. One µl (100 ng) plasmid DNA was mixed into cell suspension and incubated on ice for 5 min. The content of the microcentrifuge tube was transferred to a chilled 0.2 cm electroporation cuvette. Using a BioRad Gene Pulser, the cells were electroporated at 1.5 kV, 25 µF, and 200 Ω. Immediately following electroporation, 1 ml of cold 1 M SOC (see Appendix I 6.2.2) was added to the cuvette

and the contents gently mixed with a pipette. Cells were then transferred to a new microcentrifuge tube. Samples volumes of 2, 20, and 200 µl were plated onto SC- plates and incubated at 30°C for 2-4 days.

2.18 Transformation of *Saccharomyces cerevisiae* by Spheroplasting

A single yeast colony was inoculated into a 5 ml volume of YPAD and grown to log phase as described in Section 2.15. Yeast cells were harvested by centrifugation at 3500 x *g* for 5 min. The supernatant was discarded and cell pellet washed once by re-suspension in 20 ml sterile ddH_20 and collected by centrifugation followed by another wash in 20 ml sterile 1 M sorbitol. Cells were collected again and supernatant discarded. Cells were resuspended carefully in the following mix: (20 ml 1.0 M SCE, 200 µl Dithiothreitol, and 50 µl Zymolyase 20T (Seikagaku Cat# 120493) (10 mg/ml)).

Cells were incubated in a water bath at 30°C and digestion of cell wall allowed to occur for 20 min. Spheroplasts were collected by gentle centrifugation at 100 x *g* for 5 min and followed by washing once in 20 ml 1 M sorbitol and once in 20 ml STC (see Appendix I 6.3.4). Spheroplasts were harvested by centrifugation and resuspended again in STC to a final volume of 500 µl. An aliquot of 50 µl, corresponding to $1x10^8$ cells, was transferred to a micro centrifuge tube and 1 µl (100 ng) of plasmid DNA was added and mixed gently. The mixture was incubated for 10 min at room temperature before 1 ml of 20% (w/v) PEG8000 was mixed in followed by another 10 min incubation at room temperature. Spheroplasts were collected by centrifugation and the supernatant was discarded. Cells were resuspended in 150 µl SOS (see Appendix I 6.3.4) solution and

incubated for 30 min at 30°C. Cells were mixed with 8 ml of pre-warmed 48°C TOP agar (see appendix) and poured onto pre-warmed (30°C) SC-LEU plates containing sorbitol. Plates were allowed to dry before incubation for 3-4 days at 30°C to allow for colony formation.

2.19 Transformation of *S. cerevisiae* by microtitre plate assay

Microtitre plate transformation in 96-well plates was used to transform a single plasmid into many yeast strains. A 96-prong replicator (custom made) was sterilized by flaming with 95% ethanol. Frozen yeast knock-out strains were thawed at 30°C and inoculation was performed by lowering the replicator into the thawed microtitre plate and spotting a small amount of liquid (approximately 10 µl) onto 150 mm YPAD plates (see Appendix I 6.1.3). The spots were allowed to dry and plates were incubated at 30°C for 2-3 days to allow the yeast patches sufficient time for growth.

An 8 channel repeating multi-well micropipettor (Eppendorf) was used to add 150 µl of sterile dd$H_2$0 to the wells of a sterile microtitre plate. The replicator was sterilized as above and cooled by dipping in sterile dd$H_2$0 with excess water removed by shaking. The replicator was aligned and placed into the yeast patches of the inoculation plate and moved in a circular motion to transfer yeast cells to the prongs. Cells were then transferred to microtitre plates by submersing the prong tips into the water and cells suspended by gentle agitation. Re-growth in liquid culture was accomplished using a sterile replicator to transfer a small amount of the resuspended cells into a new microtitre plate containing 100 µl of 2X YPAD medium. Plates were incubated for 5 hours at 30°C with shaking on a rotary shaker at 300 RPM.

Cells were collected by centrifugation for 5 min at 1400 x *g* in an IEC Centra IEC4 centrifuge with an IEC-244 rotor designed for microtitre plates. The supernatant was removed by holding the plate and flicking supernatant into a sink. The cells were washed by addition of 100 µl of sterile ddH_20 and resuspended by vigorous shaking with the rotary shaker at 300 RPM for 5 min. Cells were collected again and supernatant discarded. A transformation mix without PEG was prepared as shown below:

Table 5. Microtitre plate transformation mix without PEG for 100 wells

Component	**Volume (µl)**
LiAc 1.0 M	1.5 ml
Single Stranded Carrier DNA 2mg/mL	2.0 ml
Plasmid DNA (100 ng/µl) + ddH_20	1.5 ml
Total Volume	**5.0 ml**

Fifty µl of the mix was added to each well and cells were resuspended by shaking at 300 RPM for 5 min. 100 µl of PEG (50% w/v) was pipetted into each well and cells were resuspended again by shaking at 300 RPM for 5 min. Plates were placed in a plastic bag to reduce evaporation during heat shock and incubated at 42°C for 1 hr.

Heat shocked cells were collected by centrifugation at 1400 x *g* for 5 min and the supernatant discarded. Fifty µl of sterile water was added to each well and cells resuspended by shaking at 300 RPM. The supernatant was removed and the previous step repeated. A sterile replicator was lowered into the resuspended cells and used to transfer approximately 10 µl of cell suspension to 150 mm SC-LEU (see Appendix 6.1.4) and YPAD plates. Plates were allowed to dry at room temperature before incubation at 30°C for 3-4 days to allow colonies to form.

2.20 Transformation of *S. cerevisiae* by LiAc / ssDNA / PEG method (non-microtitre)

S. cerevisiae were transformed by the modified method of (Gietz *et al.* 1995). Cells were grown to log phase as described in Section 2.15. Cell culture was transferred to a 50 ml polypropylene centrifuge tubes and centrifuged at 3500 x *g* for 5 min in an IEC centrifuge with an IEC 801 rotor. The supernatant was decanted and cell pellet washed by resuspension in 25 ml sterile ddH_20. Cells were collected again by centrifugation and water discarded. The cell pellet was then resuspended in 1 ml of 100 mM LiAc and transferred to a 1.5 ml microcentrifuge tube. Treated cells were incubated for 10 min at 30°C before centrifugation at 10,000 x *g* for 30 seconds in a Thermo IEC microcentrifuge. The supernatant was discarded and pellet resuspended again in 100 mM LiAc to reach a final volume of 500 µl. This volume corresponds to $1x10^9$ cells and allows for 10 transformation reactions ($1x10^8$ cells/transformation). For each transformation

50 µl of cell suspension was transferred to a new microcentrifuge tube and collected by centrifugation at 10,000 x *g* for 15 seconds. The supernatant was removed and cells were resuspended in 351 µl transformations mix which is optimized for 1 x 10^8 cells (see Table 6).

Table 6. Components of LiAc/ssDNA/PEG transformation mix

Component	**Volume (µl)**
PEG 3350 50% (w/v)	240
LiAc 1.0 M	36
ssDNA 2mg/mL	50
Plasmid DNA (100 ng/µl)	1
Sterile ddH_20	24
Total Volume	**351**

Treated cells were incubated at 30°C for 10 min then heat shocked for 20 min in a 42°C water bath. Heat shocked cells were collected by centrifugation and transformation mix discarded. Cell pellet was then resuspended in 1 ml of sterile ddH_2O by gentle mixing with a micropipette. Samples of sizes 2, 20, and 200 µl were plated onto SC-plates and incubated at 30°C for 2-4 days.

2.21 Immunofluorescence of *Saccharomyces cerevisiae*

Immunofluorescence was performed on yeast strains transformed by the LiAc/ssDNA/PEG method (Section 2.20) with DIG-labeled plasmid DNA (Section 2.6). Following centrifugation and removal of the transformation mix, the treated cells were gently washed in 500 µl ddH_2O. Cells were centrifuged at 400 x *g* for 3 min and the water was discarded. The pellet was then resuspended in 500 µl of a solution of (40 mM KPO_4 (pH 6.5) / 500 µM $MgCl_2$ / 37% formaldehyde) and incubated at room temperature for 1 hr on a test tube rocker. Following fixation, cells were spun down and gently washed twice in 500 µl KPO_4 (pH 6.5) / 500 µM $MgCl_2$. Cells were then washed once more in 500 µl of 1.2 M sorbitol, resuspended in 500 µl of the same, and stored overnight at 4°C or placed on ice for immediate use. Microscope cover slips were prepared in advance by coating each of them in 1 ml of 0.1% polylysine solution (>400,000MW) for 10 min. Cover slips were washed 4 times with sterile ddH_2O and allowed to dry in a dust free environment. For each cover slip, 100 µl of cell suspension was spotted and allowed to settle at room temperature for 20 min. Using aspirator, the remaining liquid was removed and cells were blocked by addition of 500 µl (PBS (pH 7.4) / 0.5% BSA / 0.5%

ovalbumin / 0.5% Tween20) for 30 min at room temperature. The blocking solution was then aspirated off and cells were incubated in blocking solution containing antibody of either Anti-DIG labeled with either FITC or rhodamine and incubated at room temperature for at least 2 hours. A dilution series of 1:100 to 1:5000 was performed with antibody with a 1:100 antibody dilution determined to give the best staining when viewed under the confocal microscope.

Blocking solution containing antibody was aspirated off and cells were then washed 4 times in blocking solution for 5 min each time. Nucleus staining was achieved by spotting 500 µl of 4'-6-Diamidino-2-phenylindole (DAPI) (10mg/ml) and incubation for 1 minute before removal by aspiration followed by 2 times washing in blocking solution. Twenty µl of mounting solution (Vectashield Cat #H-1000) was spotted onto microscope slides. Remaining blocking solution was aspirated off and cells were mounted immediately cell side down onto microscope slides. Slides were sealed with clear nail polish (Sally Hansen's "Hard as Nails") and stored in the dark at -20°C until ready to be viewed.

3. RESULTS

Our laboratory is interested in identifying the genes that affect the transformation process. Current yeast transformation methods result in less than 5% of yeast cells successfully incorporating and expressing foreign DNA (R.D.Gietz personal communication). The fact that all cells undergo the same treatment but less than 5% of cells become transformation competent is a mystery. By screening our library of mutants we hope to identify the genes that affect the process which may give us clues to developing an understanding of the mechanisms of transformation.

Frequency of transformation has been shown to be affected by strain background (Johnston *et al.* 1981) therefore prior to the library screen, the parent strain BY4742 was characterized. Several variables were tested to develop the optimal transformation conditions. These conditions were then applied in the development of the LiAc/ssDNA/PEG microtitre plate assay used to screen the mutant library derived from BY4742. Following the microtitre plate screen, identified mutants were individually re-screened in liquid culture under more controlled conditions to determine individual transformation efficiencies.

Additional transformation methods, including electroporation and spheroplasting, were performed on several identified mutants and the transformation efficiencies compared. Differences between methods could possibly give clues to the transformation process. Fluorescence imaging was then used to visualize the transformation process using a plasmid DNA labeled with DIG. Several mutant strains were transformed and

characterized using fluorescence microscopy to identify differences or patterns between strains.

3.1 BY4742 Parent strain characterization

BY4742 is the parent strain from which the library of mutants was derived, therefore aside from the specific gene deletion are isogenic to the parent strain. Having no previous experience with this strain, several characteristics were identified to devise and efficient transformation protocol that would be applied to the rest of the knock-outs. Growth time was measured to determine the amount of time needed to reach the required number of cells for transformation. The transformation efficiency during re-growth in 2X YPAD was measured to determine the time point at which cell competency was best. In addition, use of DMSO in the LiAc/ssDNA/PEG transformation mix was tested to determine if transformation efficiency could be further increased as suggested by previous reports ((Hill *et al.* 1991).

3.1.1 Determination of BY4742 growth curve

The growth curve of BY4742 was determined to identify the doubling time of this strain. The lithium acetate transformation protocol (Gietz *et al.* 2002) calls for yeast cells to be re-grown for two divisions from an initial inoculum of $5x10^6$ to a final titer of $2x10^7$ cells/ml. An over night culture of BY4742 was inoculated to $5x10^6$ cells/ml in 50 ml of 2X YPAD in a 250 ml flask and placed on shaker at 200 RPM. Samples were taken every 30 min and cell titer determined by using a haemocytometer. Figure 1 shows the growth curve of BY4742 over a 5 hour time period. The graph shows BY4742 cells in

the 2X YPAD medium initially double at 2 hr time point and again at just under the 4 hr time point.

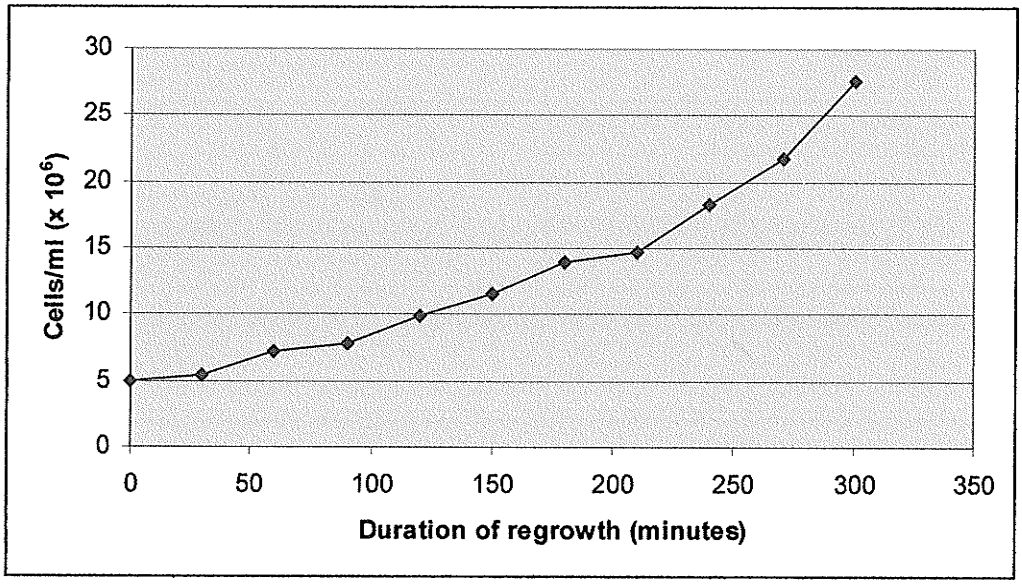


Figure 1. *S. cerevisiae* strain BY4742 growth curve in 2X YPAD

A 250 ml flask was inoculated to $5x10^6$ cells/ml with 50 ml 2X YPAD and grown at 30°C with shaking at 200 RPM. Cell titers were determined every half hour using a haemocytometer.

3.1.2 Transformation versus growth phase

The transformation efficiency of BY4742 cells during re-growth in 2X YPAD was investigated to determine an optimal time to culture cells for transformation. BY4742 cells were cultured as described in Section 2.15 and samples removed every 30 min for transformation. Volumes were taken so that $1x10^8$ cells were transformed each time with 100 ng of plasmid YEplac 181 by the LiAc/ssDNA/PEG method (see Section 2.20). Treated yeast cells were plated on SC-LEU plates and colonies counted following 3-4 days growth. Figure 2 shows the results from an average of two experiments. At the

90 min time point, the number of transformants remained below 2500. However, at the 120 min time point that number almost quadruple to 9600 transformants. Following the 150 min and 180 min time points, the number of transformants increased approximately to $2.8x10^4$ and $4.6x10^4$ respectively. The number of transformants decreased slightly to $4.0x10^4$ at the 210 min time point but recovers to $6.5x10^4$ at the 240 min time point. There was one more increase in transformation to 98000 transformants at the 270 min time point with no further increases at the 300 min time point.

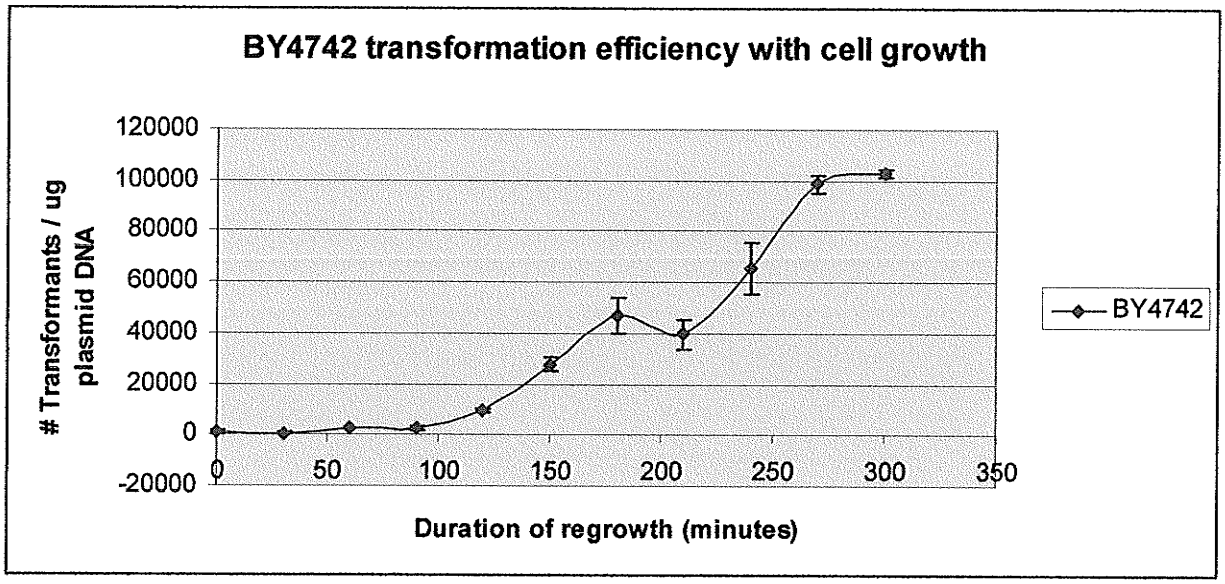


Figure 2. BY4742 transformation frequency with cell growth

BY4742 was inoculated at a titer of $5x10^6$ cells/ml in 200 ml of 2X YPAD and incubated at 30°C with shaking. $1x10^8$ cells were sampled every 30 min from the cell culture and transformed with 100 ng plasmid YEplac 181. Treated cells were plated on SC-LEU plates and incubated at 30°C for 3 days after which the number of transformants was determined. Data points were plotted as the mean of two experiments with error bars representing the standard deviation.

3.1.3 Synchronous versus asynchronous transformation

The effect of growth phase on transformation efficiency was investigated to determine if a particular stage in the cell cycle would result in more transformants. A synchronous and asynchronous culture of yeast strain DGY233 was transformed and the resulting number of transformant compared. Yeast strain DGY233 is of mating type MATa and contains a mutation in the BAR1 gene, which causes cells to arrest at G1 phase in cell division when treated with α-factor (Moore 1984; Jenness *et al.* 1987).

An overnight culture of DGY233 was used to start two new cultures in fresh 2X YPAD at a cell titer of $5x10^6$ cells/ml. One culture was incubated for two cell divisions before being harvested and transformed as described in Section 2.20. The other culture was arrested with α-factor (Sigma) at a concentration of 20 ng/ml to synchronize cells at G1 phase. Cells were incubated until >80% showed "shmoo" formation (approximately 3 hrs). Cells were collected and washed to remove α-factor then inoculated in fresh 2X YPAD until two divisions were reached before being transformed. The growth curve of both synchronous and asynchronous cultures was determined by sampling cells every 30 min as described in Section 3.1.1 and the results shown in Figure 3. The asynchronous culture initially doubles at the 2 hr time point and again sometime just after the 3½ hr time point. The synchronous culture required a longer incubation before its first doubling at approximately the 2½ hr time point followed by a second doubling at the 4 hr time point.

Synchronous and asynchronous cultures were transformed as described in Section 2.20. Following transformation, cells were plated on SC-LEU plates incubated for 3 days

at 30°C. Figure 4 shows approximately 26000 transformants/μg plasmid DNA were produced in both synchronous and asynchronous cultures. There were no significant differences in the transformation numbers of either culture.

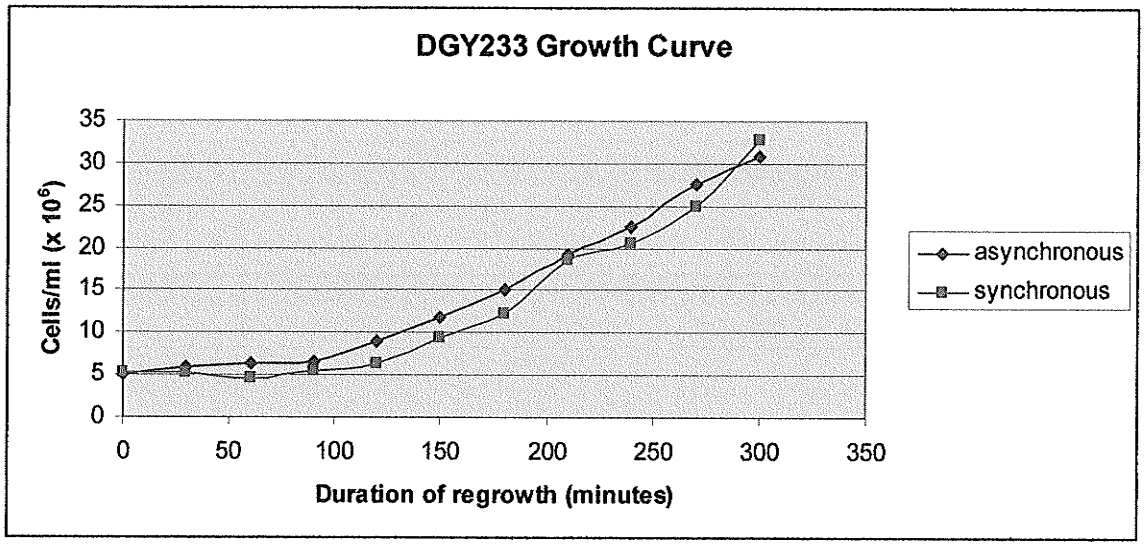


Figure 3. Growth curve of a synchronous and asynchronous culture of *S. cerevisiae* strain DGY233 in 2X YPAD

DGY233 was inoculated into two new cultures in 250 ml flasks to $5x10^6$ cells/ml. One culture was allowed to grow without interference. The other culture was arrested at G1 as described in Section 3.1.3 then allowed to proceed to cell division. Cell titers were determined every half hour using a haemocytometer.

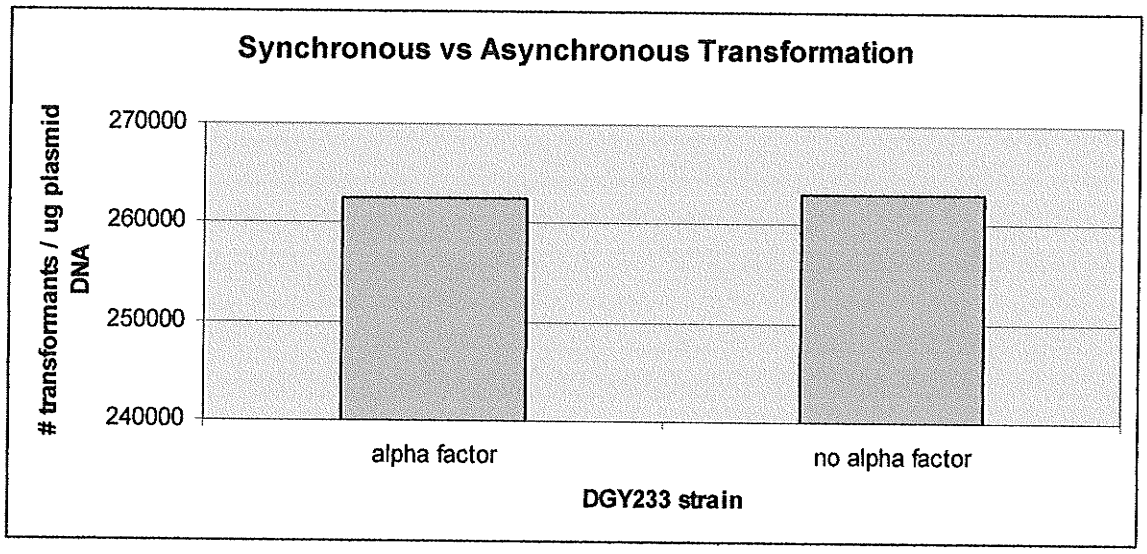


Figure 4. Comparison of transformation ability in synchronous and asynchronous DGY233 cells

Two cultures of DGY233 were started with one culture allowed to grow asynchronously to 2 divisions, while the other was arrested in G1 with α-factor then grown synchronously to 2 divisions before being transformed by LiAc/ssDNA/PEG method (see Section 2.20).

3.1.4 The effect of cell cycle synchronization on transformation ability

The effect of growth phase on transformation efficiency was investigated to determine if any particular stage in the cell cycle would result in higher transformation efficiencies. An overnight culture of DGY233 was inoculated to $5x10^6$ cells/ml in a total volume of 200 ml 2X YPAD and arrested with α-factor as described in Section 3.1.3. Arrested cells were collected, washed, transferred to new 2X YPAD without α-factor, and allowed to proceed through the cell cycle. Samples were taken so that an identical number of cells were collected every 30 min and transformed as described in Section 3.1.2. Figure 5 shows the transformation results over a 6 hr period. The transformation efficiency at the initial three time points remains below 2500 transformants/μg plasmid

DNA. At the 90 min time point the efficiency had risen to $9.8x10^4$ transformants/μg plasmid DNA and continued to rise steadily to $2.2x10^5$ transformants/μg plasmid DNA by the 180 min time point. There was a reduction to $1.7x10^5$ at the 210 min time point but recovered and plateaued to $2.5x10^5$ transformants/μg plasmid DNA over the final three time points.

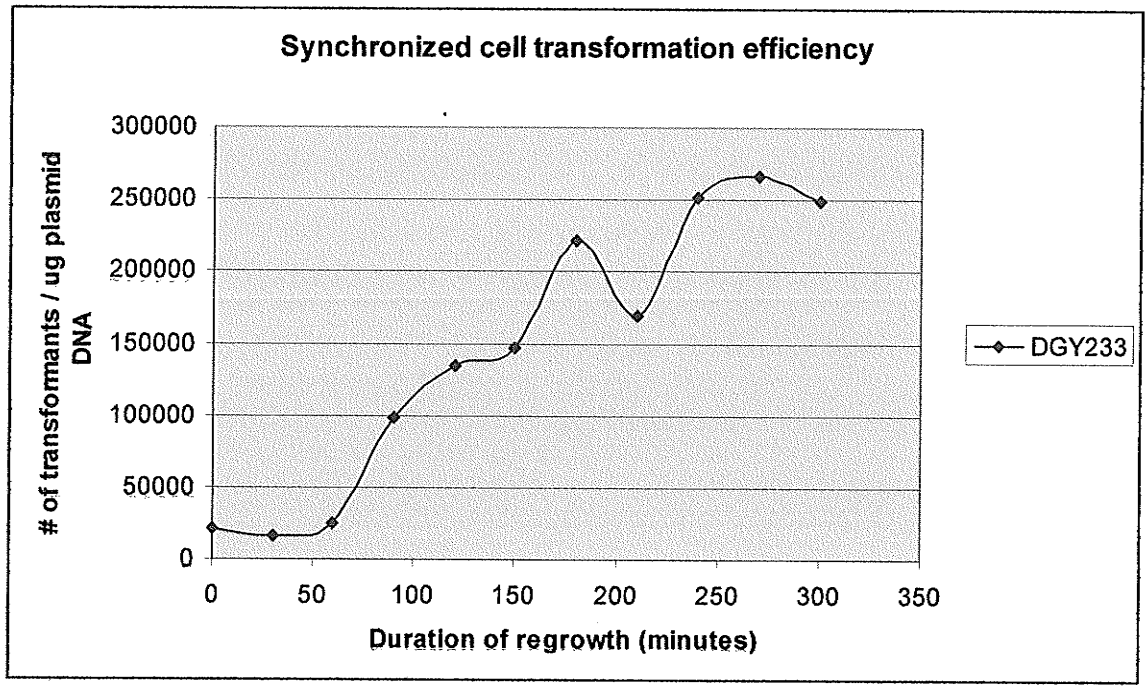


Figure 5. Transformation frequency from a synchronized yeast culture

A culture of DGY233 was synchronized at G1 with α-factor for 3 hours before being re-grown in α-factor free 2X YPAD. Transformations by LiAc/ssDNA/PEG with plasmid YEplac 181 were carried out every 30 minutes with $1x10^8$ cells used in each transformation (see Section 2.20).

3.1.5 Dimethyl Sulfoxide (DMSO) modified transformation

The addition of DMSO to the LiAc/ssDNA/PEG transformation mix was investigated to determine the effects on transformation efficiency. A 10% (v/v) DMSO solution had previously been demonstrated to significantly increase transformation efficiency (Hill *et al.* 1991). A 5% (v/v) and 10% (v/v) DMSO (Sigma D-8418) transformation mix was used to determine if significant increases would also be exhibited by our BY4742 strain. Transformation was performed as described in Section 2.20 with a 5% (v/v) or 10% (v/v) DMSO transformation mix (see Table 7). Treated cells were plated on SC-LEU plates and the number of transformants determined after incubation for 3 days at 30°C.

Table 7. Five and ten percent DMSO transformation mix

Components	**5 % DMSO**	**10% DMSO**
PEG 3350 50% (w/v)	240.0 µl	240.0 µl
LiAc 1.0 M	36.0 µl	36.0 µl
ssDNA 2mg/mL	50.0 µl	50.0 µl
Plasmid DNA (100 ng/µl)	1.0 µl	1.0 µl
Sterile ddH_20	6.4 µl	- µl
DMSO	17.6 µl	35.0 µl
Total Volume	**351 µl**	**362 µl**

Table 8 shows the average of two experiments along with the standard deviation. The addition of DMSO to the transformation mix did not increase the transformation efficiency for *S. cerevisiae* BY4742. Transformation of BY4742 without addition of DMSO resulted in an efficiency of 75600 transformants/µg plasmid DNA. At 5% (v/v) DMSO, the transformation efficiency was similar to that without DMSO. At 10% (v/v)

DMSO there was a greater than 3 fold decrease resulting in 19625 transformants/μg plasmid DNA.

Table 8. Comparison of DMSO transformation methods on *S. cerevisiae* BY4742

Method	# transformants/ μg DNA	STD. Deviation
No DMSO	75600	± 3020
5% DMSO (v/v)	73650	±11075
10% DMSO (v/v)	19625	± 4082

3.2 Library screen for mutants defective in transformation ability

3.2.1 High efficiency microtitre plate transformation library screen

A transformation protocol for yeast in 96-well microtitre plates (Gietz *et al.* 2007) was adapted to screen a library of knock-out mutants. The optimal conditions for growth, heat shock, and chemical treatment were tested for maximum transformation efficiency and a protocol was developed and described in Section 2.19. The microtitre plate assay was used to introduce a single plasmid into many different yeast strains simultaneously.

A knock-out library was purchased from Open Biosystems consisting of 4827 gene disruption mutants each derived from BY4742 (Giaever *et al.* 2002). The knock-out library was transformed using the shuttle plasmid YEplac 181 and screened for mutants displaying above or below normal transformation efficiencies. The phenotype displayed by each mutant reflects a complete loss gene function. Therefore, identifying mutants deficient in transformation could suggest a possible involvement in the transformation process. Mutants were transformed as described in Section 2.19 and treated cells were

spotted using a 96 prong replicator to place approximately 10 µl of cells in a grid like pattern onto both YPAD and SC-LEU plates.

Putative transformation mutants were identified by visual inspection of plates. Mutants were identified by plate number and the plate coordinates which were referenced from a Microsoft™ excel file supplied with the library. Mutant growth was compared between YPAD and SC-LEU to identify mutants of interest. A mutant was deemed deficient in transformation ability if treated cells grew well on the YPAD plate but fewer than 5 colonies were identified on the SC-LEU plate. Figure 6 displays the growth of treated cells on YPAD and SC-LEU plates of microtitre plate 132. Five mutants of interest were identified from plate 132 in which three where identified as transformation deficient (mutants 132E3, 132F7, and 132H8 circled in yellow, Figure 6A and 6B). The remaining 2 mutants were identified as possibly sensitive to the treatment (mutants 132C6 and 132E11, circled in red, Figure 6A and 6B). Strains which showed very good growth on SC-LEU plates and YPAD plates were identified as potential mutants with increased transformation ability (see mutants 149A6, 149H1, and 149H12) circled blue in Figure 6C and 6D).

In total, 4827 yeast knock out strains were screened by microtitre plate transformation resulting in 177 mutants identified and categorized as having either increased/decreased transformation ability. Of the 177 mutants identified, 34 showed a possible sensitivity to the treatment, 6 showed a potential increased transformation efficiency, and remaining 137 showed a potential decreased in transformation efficiency. Identified mutants were isolated from the initial inoculation plate and streaked for single colonies on YPAD plates. Strains were stored frozen as described in Section 2.16.

Using the Microsoft™ excel file supplied by OpenBiosystems, the specific gene deletion in each mutant was referenced and then identified from the *Saccharomyces Genome Database* (http://www.yeastgenome.org/). Table 17 (see Appendix II) displays the 177 mutants identified from the microtitre plate screen along with the corresponding knocked-out gene.

Figure 6

(A)

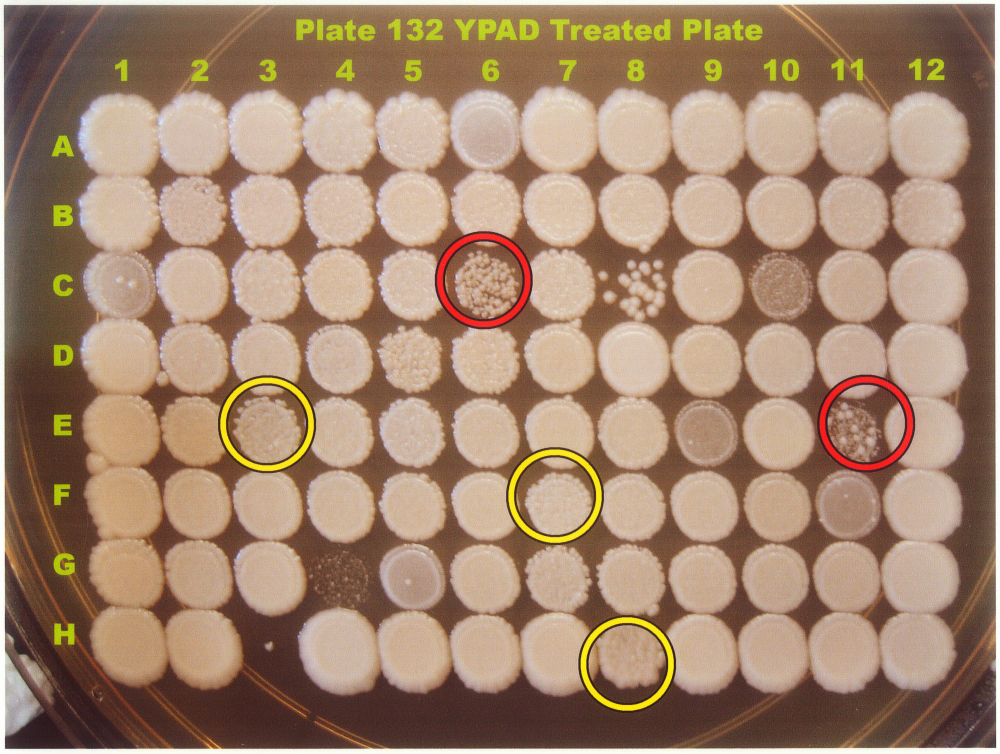


Plate 132 YPAD Treated Plate
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

(B)

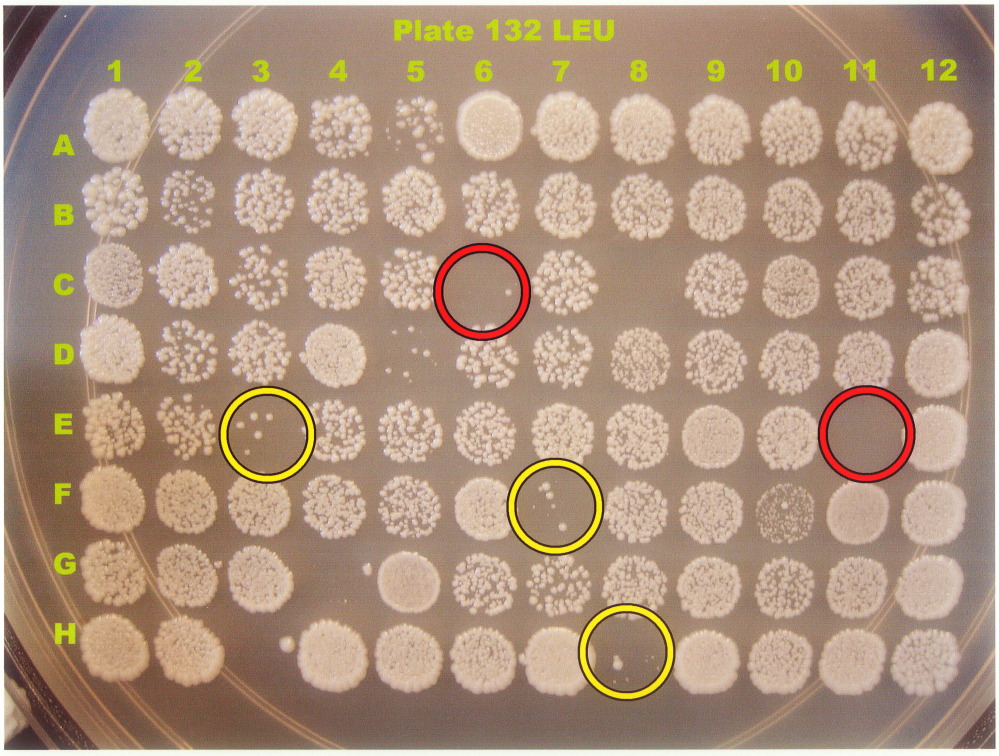


Plate 132 LEU
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

Figure 6

(C)

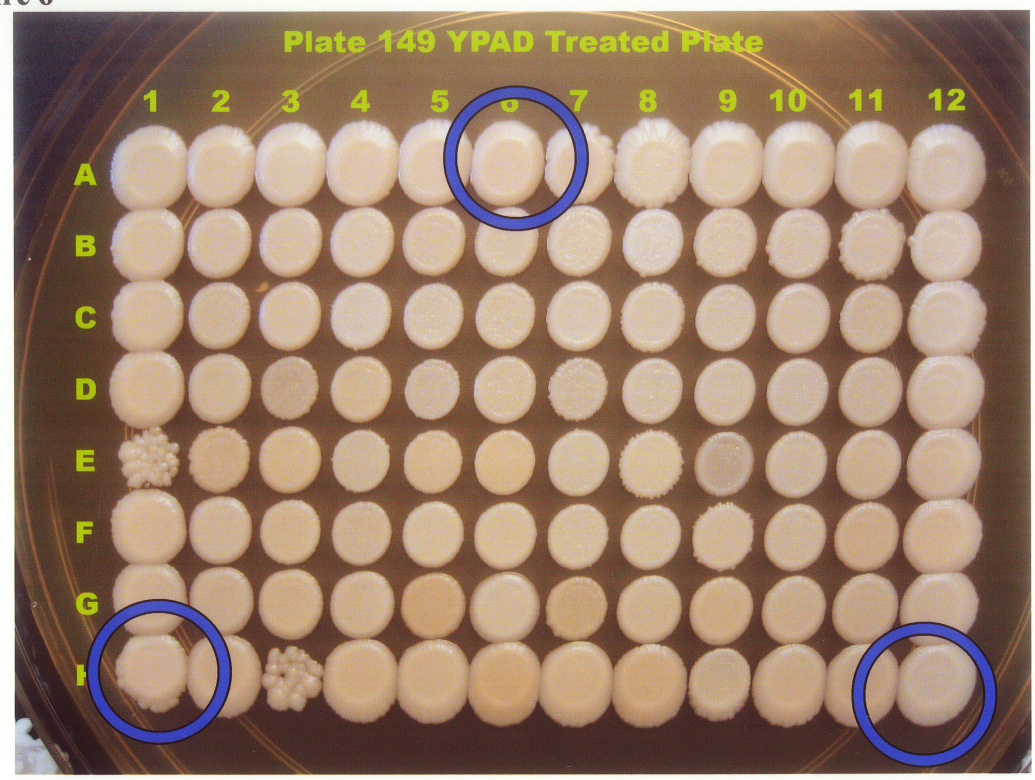


Plate 149 YPAD Treated Plate
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

(D)

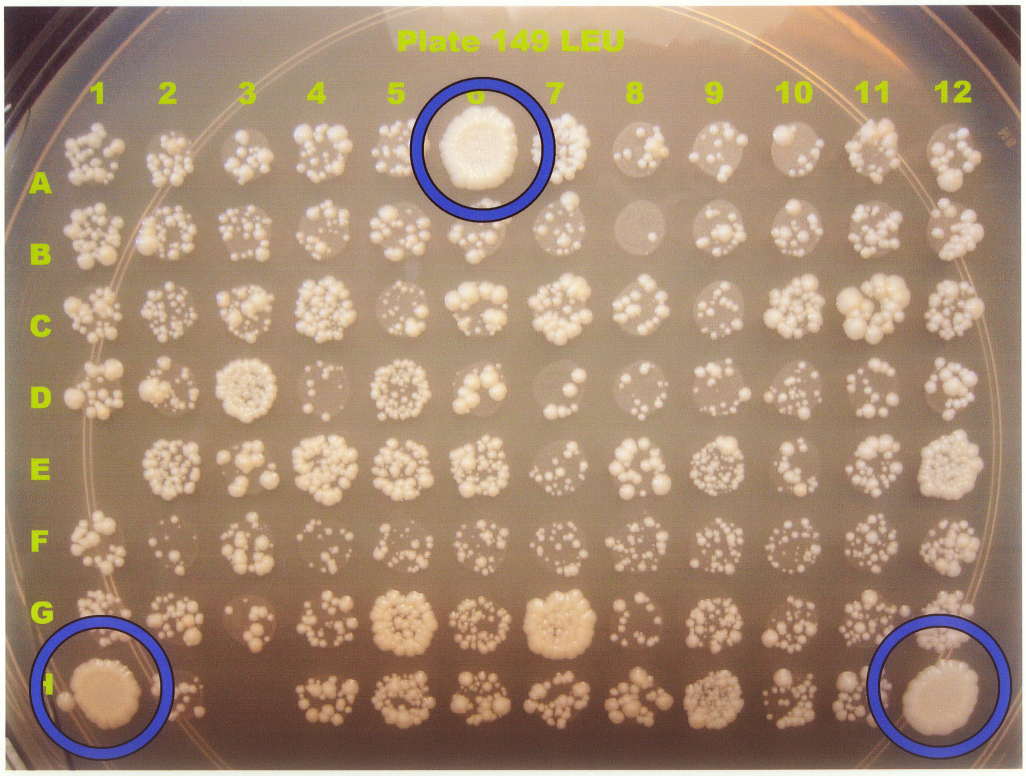


Plate 149 LEU
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

Figure 6. Microtitre plate transformation

Yeast deletion library was transformed with plasmid YEplac 181 and plated on YPAD and SC-LEU plates as described in Section 2.19. Results of two microtitre plates are shown. Plate 132 shown in 6A and 6B where 6A is YPAD and 6B is SC-LEU. Plate 149 shown in 6C and 6D where 6C is YPAD and 6D is SC-LEU. The yellow circles identify mutants considered transformation defective. The red circles identify mutants that showed sensitivity to the treatment. The blue circles identify mutants that showed increased transformation rates.

3.2.2 Individual Liquid Culture Re-screen

A number of the mutants identified were then re-screened individually in liquid culture to determine the individual transformation efficiencies. Individual colonies were inoculated into a starter culture of YPAD and grown as described in Section 2.15. Mutants were grown for two divisions and transformed by the LiAc/ssDNA/PEG method as described in Section 2.20. Treated cells were plated on YPAD and SC-LEU plates and incubated for 3-5 days. Table 9 displays the results of individual screens of the parental strain, BY4742, and *erg6* (103D10) mutant.

Table 9. Comparison of # colonies formed after transformation

Yeast Strain	**# of colonies when specified dilution spotted**		**Calculated # of transformants/µg plasmid DNA**
	20 µl	**200 µl**	
BY4742	204	TNTC	102000
103D10 (*erg6*)	0	2	200

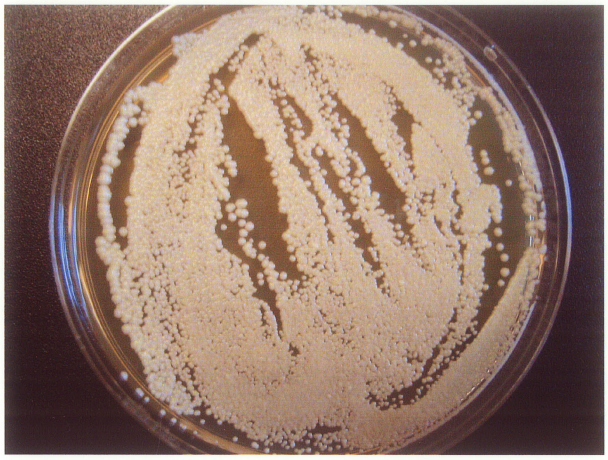
Figure 7A and 7C shows growth of both strains when 2 µl of cells are spotted on YPAD plates after treatment. When 20 µl of cells are spotted on SC-LEU plates, 204 colonies were seen for BY4742 (Figure 7B) and none for *erg6* (103D10) mutant (Figure 7D). Increasing the volume of spotted cells to 200 µl resulted in too many to count for BY4742 and only 2 colonies for *erg6* (103D10) mutant (Figure 6E).

Table 9 shows the transformation efficiency of parent strain, BY4742, to be 1.02×10^5 transformants/µg plasmid DNA for that single experiment. An average of several experiments was taken and a transformation efficiency of 8×10^5 transformants/µg DNA plasmid was used as the base to which all other mutants were compared. Transformation efficiencies of re-screened mutants were calculated and compared to the

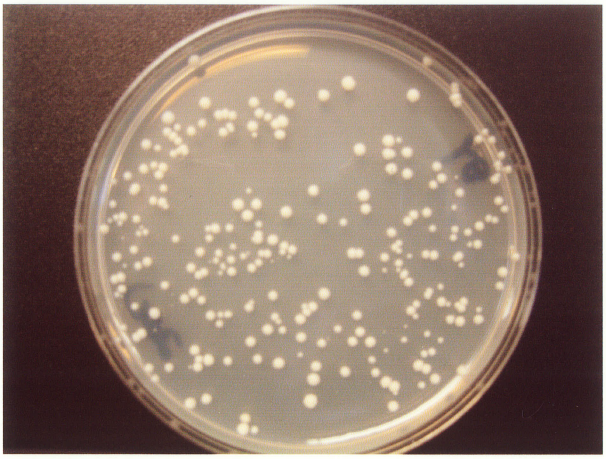
transformation efficiency of the parent strain and shown as a percentage (see Tables 18-22 in Appendix II).

BY4742

(A) YPAD 2 µl



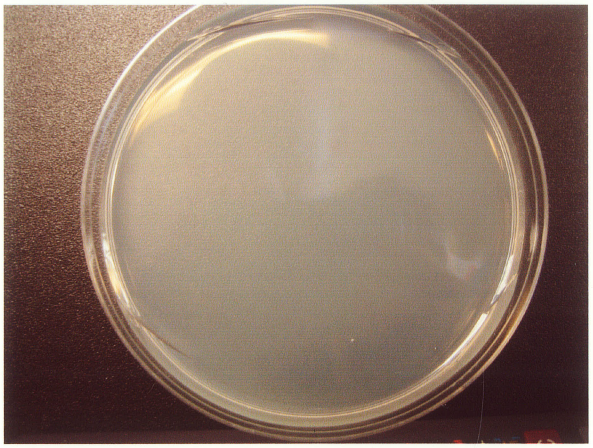
(B) SC-LEU 20 µl



103D10 (*erg6*)

(C) YPAD 2 µl

(D) SC-LEU 20 µl



(E) SC-LEU 200 µl

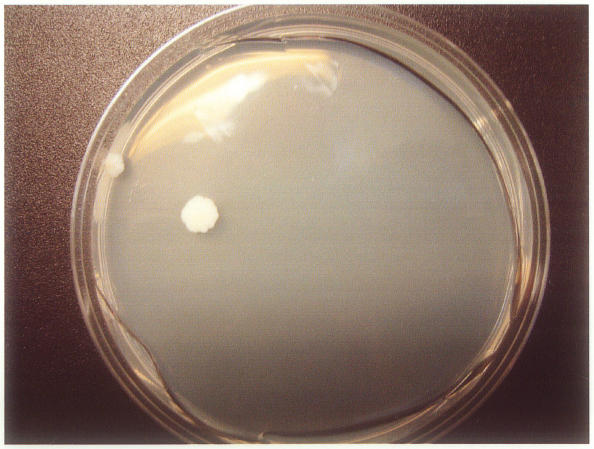


Figure 7. Transformation of *S. cerevisiae* BY4742 & 103D10 by LiAc/ssDNA/PEG method

Parent strain BY4742 and *erg6* mutant strain were transformed by chemical treatment and plated in various dilutions. Both strains were plated on YPAD plates, 7A and 7C, and SC-LEU plates, 7B, 7D, and 7E. Transformation efficiencies are shown in Table 9.

3.2.3 LiAc/ssDNA/PEG treatment sensitive mutants

A number of mutants from the initial microtitre plate screen were identified as treatment sensitive based their reduced growth after treatment on YPAD and SC-LEU plates when compared to the healthy grow on initial inoculation plate. This may be a result of lithium in the treatment since increased concentrations of lithium ions are toxic to yeast (Sychrova 2004). The poor growth displayed could also be from the strain's inherent poor growth which would not have provided adequate numbers for transformation. Several of these mutants were re-screened by the LiAc/ssDNA/PEG method along with two strains known for poor growth characteristics and the results shown in Table 10. Samples 1 through 7 were mutants identified as treatment sensitive and had transformation efficiencies ranging from 0.04% to 35.16% when compared to BY4742. The majority of the mutants plated on YPAD medium resulted in spotty growth except 132E11 mutant which displayed slightly better growth. Samples 8 and 9 (Table 10) were also identified from the screen but were known to have poor growth and long doubling times as noted in library information. A re-screen of those 2 mutants (150D10 and 170D11) resulted in poor growth on YPAD plates and consequently poor transformation efficiencies.

Table 10. Treatment sensitive mutants re-screened

Sample	Plate #	Position	Gene	%	YPAD
1	129	E5	BUD27	10.81	Poor Growth
2	132	C6	RPS7A	0.4	Poor Growth
3	132	E11	CTK3	35.16	Med. Growth
4	133	A3	STO1	6.47	Poor Growth
5	133	B12	VMA13	0.08	Poor Growth
6	133	F10	SNT309	15.75	Poor Growth
7	134	G5	PPA1	0.04	Poor Growth
8	150	D10	SPT20	24.19	Poor Growth
9	170	D11	MET7	0.94	Poor Growth

3.2.4 Re-screen of mutants with potentially increased transformation ability

Four of the six strains identified as increased transformation mutants were re-screened for transformation efficiencies and the results shown in Table 11. Only the *bmh1* mutant had a comparable transformation rate to that of the parent strain, while the remaining three mutants (*ram1, deg1, and ydr417c*) did not perform as well in the liquid culture re-screen.

Table 11. High TRAFO mutant re-screen

Plate #	Position	Gene	%	YPAD
149	H1	YDR417C	12.75	Med. Growth
147	A6	RAM1	45.60	Good Growth
149	H12	DEG1	55.58	Good Growth
149	A6	BMH1	97.76	Good Growth

3.2.5 Distribution of mutants based on biological function

The individual re-screening of mutants was performed to remove false positives that may have arisen from the microtitre plate screen. Removal of false positives and treatment sensitive mutants reduced the list to 115 transformation deficient mutants. Mutants in the Saccharomyces Genome Database have been classified by standardized gene ontology terms, which describe gene product attributes. The function of each gene was acquired from the *Saccharomyces Genome Database* (see Appendix II) and the distribution of mutants by biological function shown in Figure 8. Mutants with an unknown gene function created the largest class of transformation defective mutants with 28 followed by miscellaneous genes with 14 members. The translation and transport group had 12 members each and the remaining groups contained between 4 and 11 members involved in process ranging from DNA repair to cell wall synthesis.

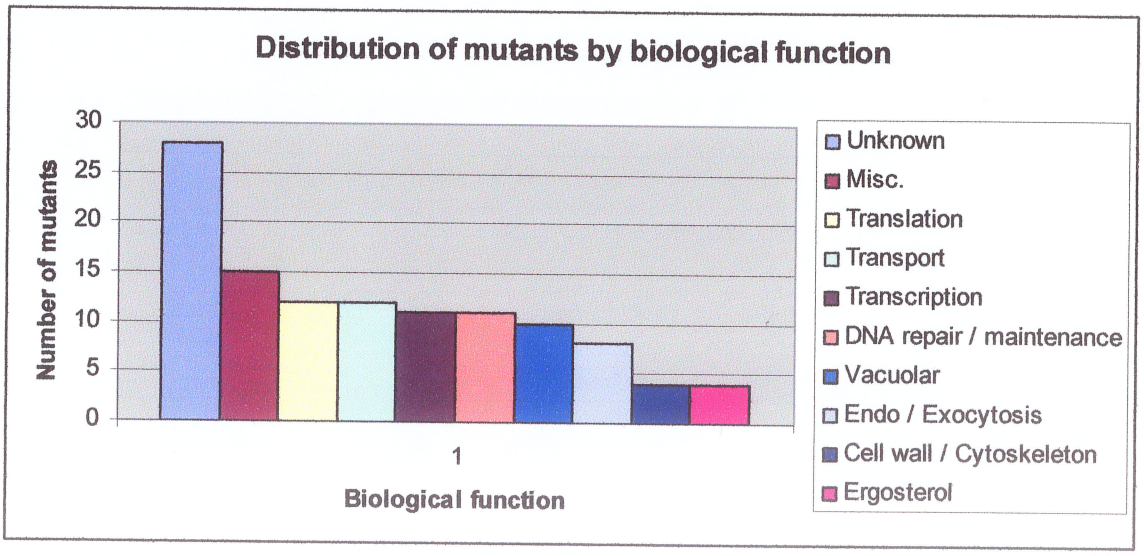


Figure 8. Distribution of mutants by biological function
The gene ontology of 115 mutants from LiAc/ssDNA/PEG transformation re-screen were referenced from the *Saccharomyces Genome Database* (www.yeastgenome.org) and categorized by function.

3.3 Transformation by other methods

A comparison of transformation efficiencies using different transformation methods was explored to determine if the observed transformation trends were a result of the procedure itself. Several transformation defective mutants were selected and transformed using both electroporation and the spheroplasting method (Section 2.17 and 2.18). A comparison of the differences between methods and the resulting transformation efficiencies could possibly give clues to the transformation process

3.3.1 Electroporation

Parent strain BY4742 and three mutants were selected and transformed by electroporation as described in Section 2.17 and the result are shown in Figure 9. The parent strain, BY4742, showed an average efficiency of $3.5x10^4$ transformants/μg plasmid DNA while the *erg28* (112G6) mutant had the next highest with an average of $3.0x10^4$ transformants/μg plasmid DNA followed by *erg2* (104D5) and *erg6* (112G6) at $1.8x10^4$ and $3.6x10^3$ transformants/μg plasmid DNA respectively.

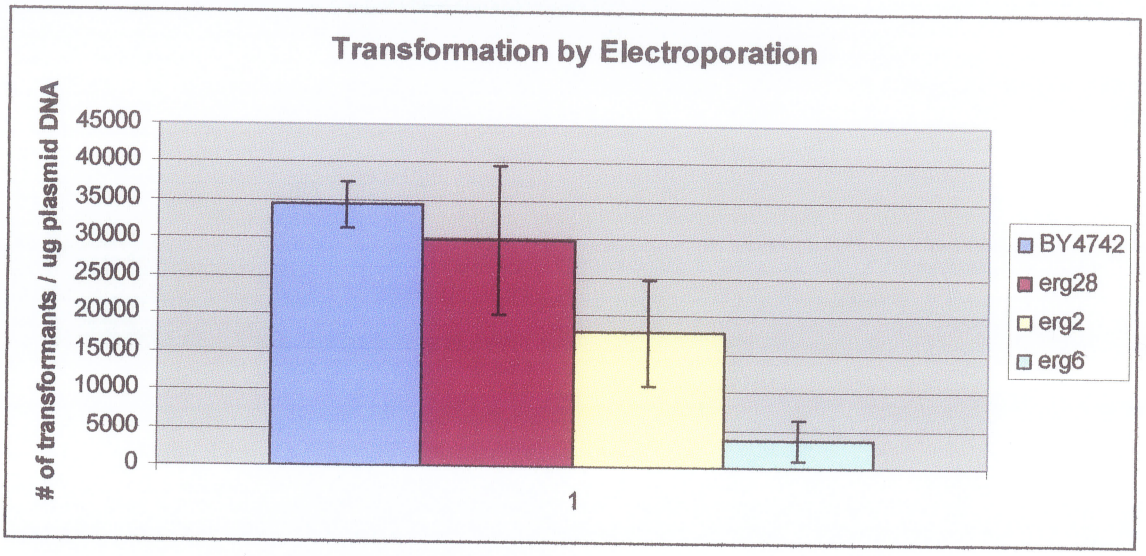


Figure 9. Electroporation transformation frequencies of several strains
Three ergosterol mutants and parent strain BY4742 were transformed by electroporation (Section 2.18). The transformation efficiencies were plotted as the average of two experiments with the standard deviation shown.

3.3.2 Transformation by spheroplasting

The same yeast strains used in the electroporation technique were also subjected to transformation by spheroplasting. Yeast strains were transformed as described in Section 2.18 and the result shown in Figure 10. Colonies were counted after 3-4 days incubation and results shown in Figure 9. Parent strain BY4742 showed an average of 8.4×10^4 transformants/µg plasmid DNA while *erg28* (112G6) mutant had the next highest number of transformants with an average of 1.0×10^4 transformants/µg plasmid DNA followed by *erg2* (104D5) and *erg6* (112G6) at 2200 and 900 transformants/µg plasmid DNA respectively.

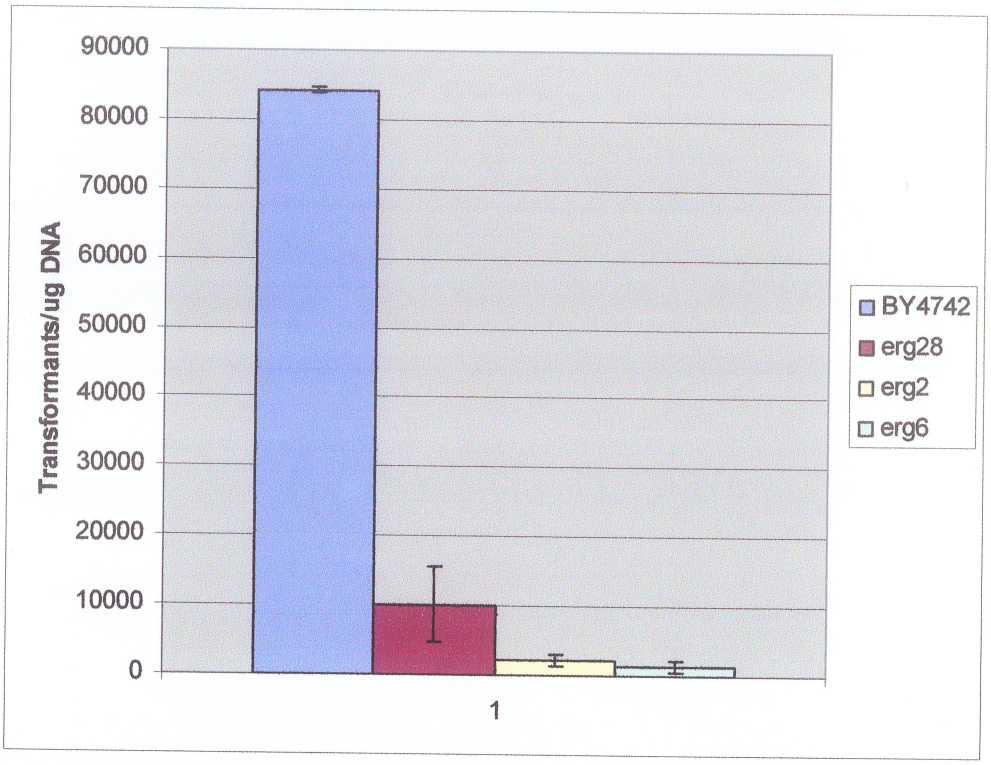


Figure 10. Spheroplast transformation frequencies of several strains
Three ergosterol mutants and parent strain BY4742 were transformed by the spheroplasting method (Section 2.18). The transformation efficiencies were plotted above as an average of two experiments with the standard deviation shown.

The number of transformants resulting from the three methods were compared to the parent strain and shown as a percentage in Table 12. None of the three mutants displayed transformant numbers higher than BY4742 with any of the methods. The lowest transformation efficiency belonged to the *erg6* mutant followed by *erg2* and *erg28* mutants across all three methods.

Table 12. Comparison of transformation efficiencies from different methods

Strain	LiAc/ssDNA/PEG	Electroporation	Spheroplasting
erg6 (103D10)	0.04 %	11.38 %	1.07 %
erg2 (104D5)	0.65 %	51.82 %	2.62 %
erg28 (112G6)	1.13 %	85.44 %	11.90 %

3.4 *S. cerevisiae* KGY37 transformation with DIG labeled plasmid

The growth curve of KGY37 was characterized as described in Section 3.11 to determine the optimal time to harvest cells for transformation. Figure 11 shows the growth curve of KGY37 over a 5 hour time period. KGY37 cells doubled at approximately the 2 hr time point and again at prior to the 4 hr time point.

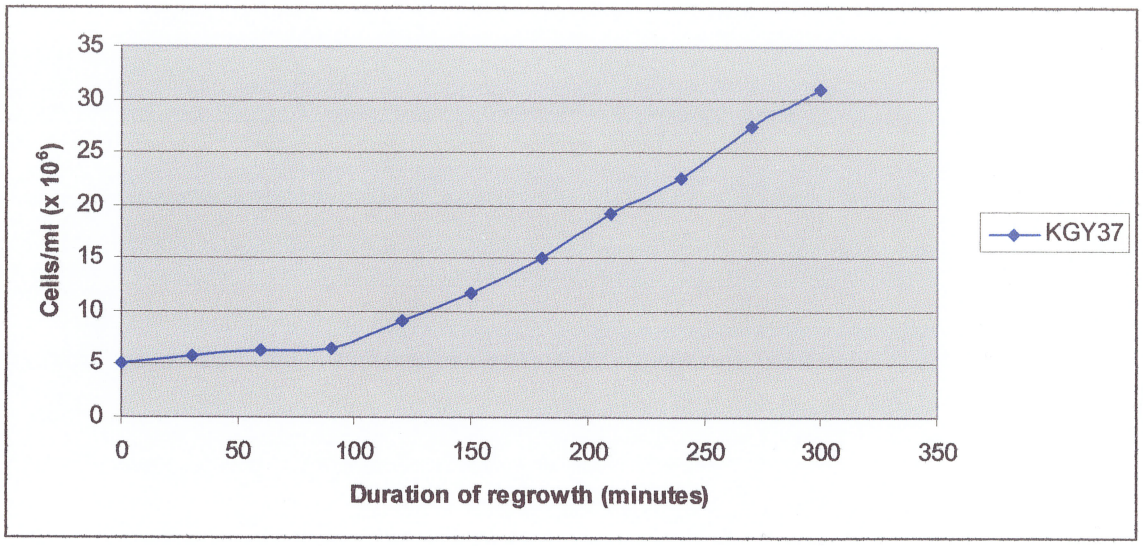


Figure 11. *S. cerevisiae* strain KGY37 growth curve in 2X YPAD

A 250 ml flask was inoculated to $5x10^6$ cells/ml with 50 ml 2X YPAD and grown at 30°C with shaking at 200 RPM. Cell titers were determined every half hour using a haemocytometer.

KGY37 cells were transformed by LiAc/ssDNA/PEG method (see Section 2.20) with plasmids of different sizes to determine their effect on transformation efficiency. Plasmid RKO IV was labeled with DIG as described in Section 2.6. Two circular plasmids, YEplac 112 and unlabeled RKO IV, along with linearized DIG labeled

RKO IV plasmid were transformed into KGY37 and the results shown in Table 13. Transformation efficiency was highest with YEplac 112 followed by RKO IV and DIG labeled RKO IV. Transformation with YEplac112 resulted in a 6 and 164 fold difference between unlabeled RKO IV and DIG labeled RKO IV plasmids respectively. Furthermore, transformation with DIG labeled RKO IV plasmid resulted in a 24 fold decrease in efficiency over unlabeled plasmid.

Table 13. KGY37 transformation efficiency with different plasmids

Plasmid	**Size**	**Transformants/ug DNA**
YEplac 112	4889bp	878000 ± 31113
RKO IV	10047bp	131700 ± 39640
DIG labeled RKO IV	-	5338 ± 1150

3.5 Immunofluorescence

Yeast immunofluorescence was used to determine if plasmid DNA labeled with DIG could be visualized during transformation by LiAc/ssDNA/PEG. Plasmid RKO IV was labeled with DIG as described in Section 2.6. The labeled plasmid was probed with an anti-DIG antibody conjugated to a fluorochrome and detected by fluorescence microscopy.

3.5.1 Immunofluorescence of KGY37 cells

Digoxigenin labeled RKO IV plasmid was transformed into KGY37 cells and immunofluorescence microscopy was used to investigate the transformation process. Cells were transformed as with DIG labeled plasmid as described in Section 2.20, then fixed, incubated with anti-DIG antibody, stained, and mounted as described in Section 2.21. Fluorescence microscopy was used to detected anti-DIG antibody conjugated with rhodamine bound to labeled plasmid using the appropriate filters.

3.5.2 Experimental Controls

Several sets of experimental controls were performed to determine factors that could affect the immunofluorescence results. KGY37 was examined for autofluorescence

properties before and after the transformation process. Anti-DIG antibody was examined for its specificity. Table 14 shows the experimental conditions used and the associated Figures.

Table 14. KGY37 experimental control conditions

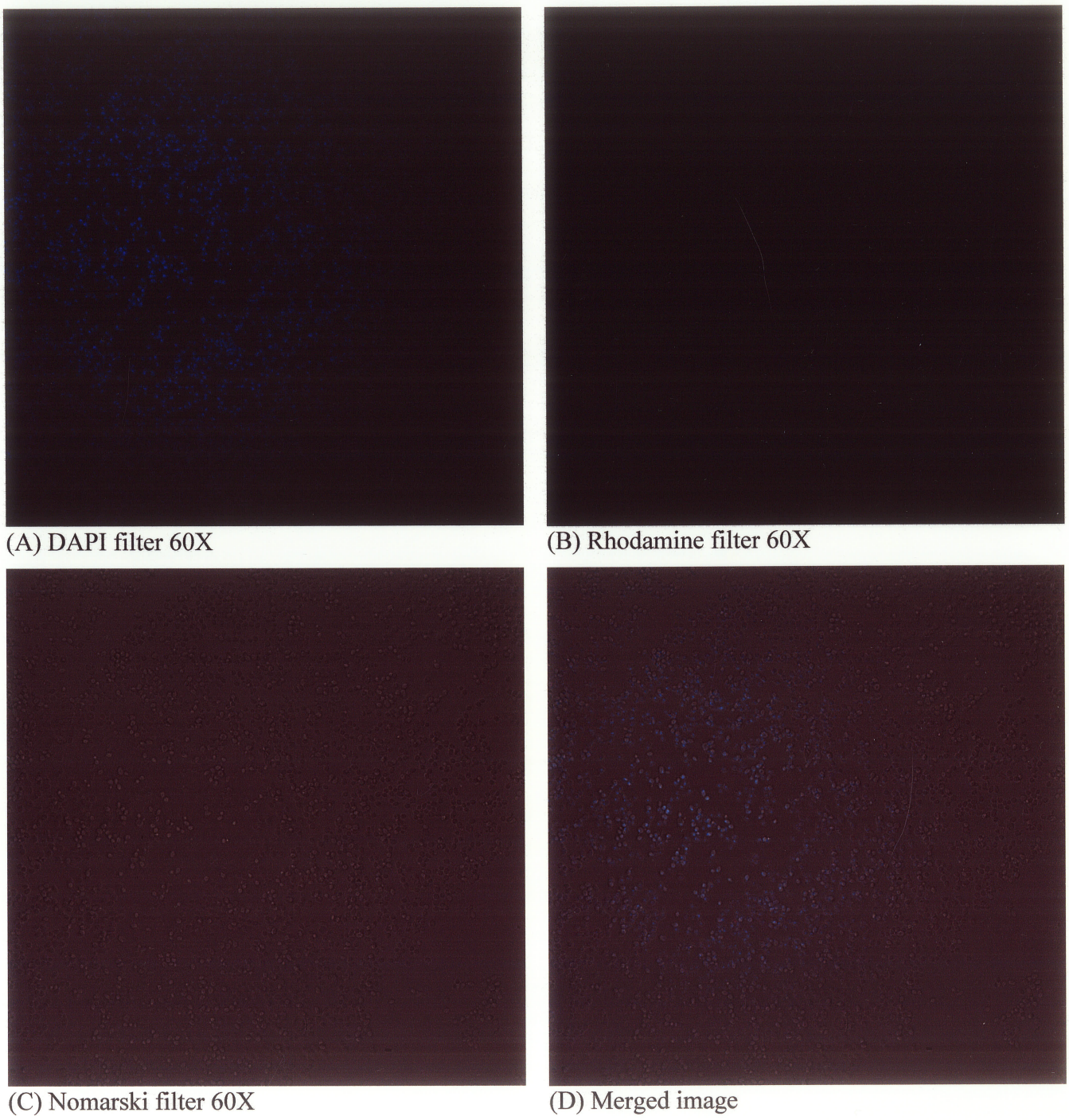
KGY37	LiAc/ssDNA/PEG Treatment	Plasmid	Antibody	Anti-DIG Signal
Figure 12	No	No	No	No
Figure 13	No	No	Yes	No
Figure 14	Yes	No	No	No
Figure 15	Yes	No	Yes	No
Figure 16	Yes	Yes	No	No

Two cultures of KGY37 cells were grown to log phase and harvested. One culture was fixed and stained with DAPI and the second culture with both DAPI and anti-DIG antibody before mounting to microscope slides (see Section 2.21) with the results shown in Figures 12 and 13 respectively. Images in Figures 12 and 13 were taken with an Olympus FV500 confocal microscope at 600X and 1800X magnification respectively. No anti-DIG antibody signal is detected in Figure 12 from the untreated KGY37 cells fixed with DAPI only. Figure 12D shows the merged images of Figure 12A (DAPI filter), Figure 12B (rhodamine filter), and Figure 12C (Nomarski filter). Similarly, there no signal was detected in Figure 13B of KGY37 cells that were stained with anti-DIG antibody.

Two additional cultures of KGY37 were grown to log phase and harvested. Both cultures were subjected to the LiAc/ssDNA/PEG treatment (see Section 2.20) however without the inclusion of plasmid DNA. One culture was then fixed and stained with DAPI while the other with both DAPI and anti-DIG antibody before mounting to microscope slides (see Section 2.21) with the results shown in Figures 14 and 15

respectively. Images in Figures 14 and 15 were taken with an Olympus FV500 confocal microscope at 1800X and 600X magnification respectively. Figure 14 shows that no signal is detected in cells subjected to the chemical treatment of the LiAc/ssDNA/PEG method (see Section 2.20). Similarly, no signal is detected in treated cells that were stained with anti-DIG antibody (see Figure 15B).

A negative control was performed and the results displayed in Figure 16. A culture KGY37 cells was grown to two divisions, harvested, and transformed with DIG labeled plasmid by the LiAc/ssDNA/PEG method (see Section 2.20). Treated cells were fixed and stained with DAPI only before mounting to microscope slides. Figures 16A (DAPI filter) and 16B (rhodamine filter) were taken with a confocal microscope at 600X magnification. In the absence of antibody staining no signal is detected in transformed cells (see Figure 16B).



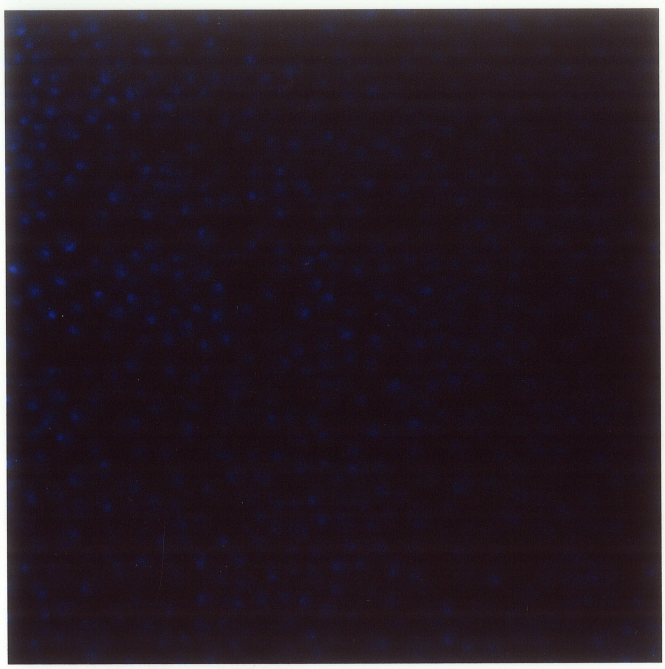
(A) DAPI filter 60X

(B) Rhodamine filter 60X

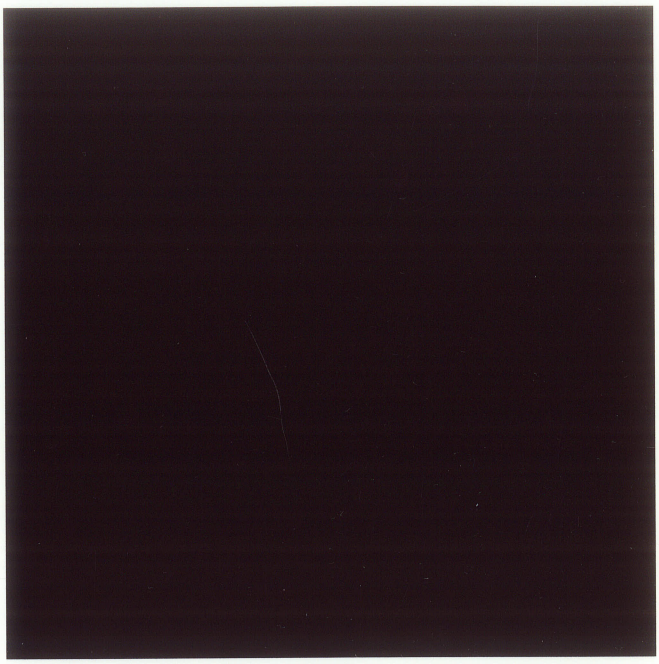
(C) Nomarski filter 60X

(D) Merged image

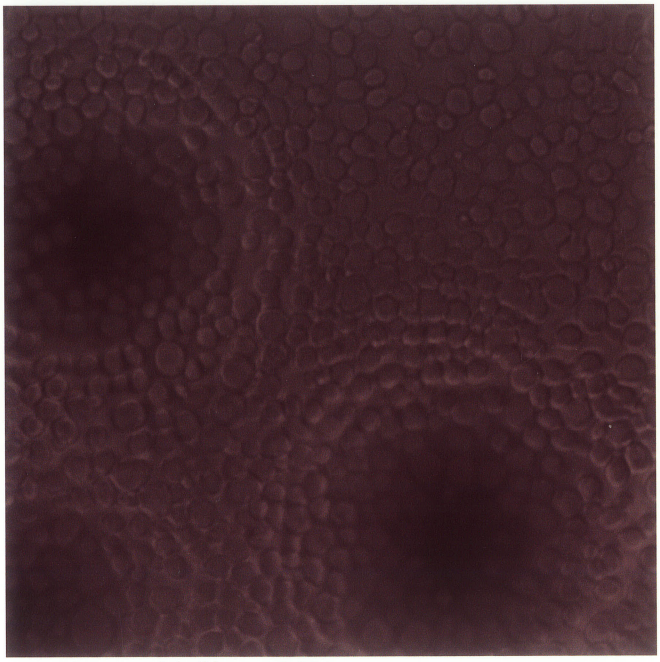
Figure 12. Untreated cells KGY37 cells stained with DAPI
Cells were grown to log phase, fixed, stained with DAPI, and mounted to slides as described in Section 2.21



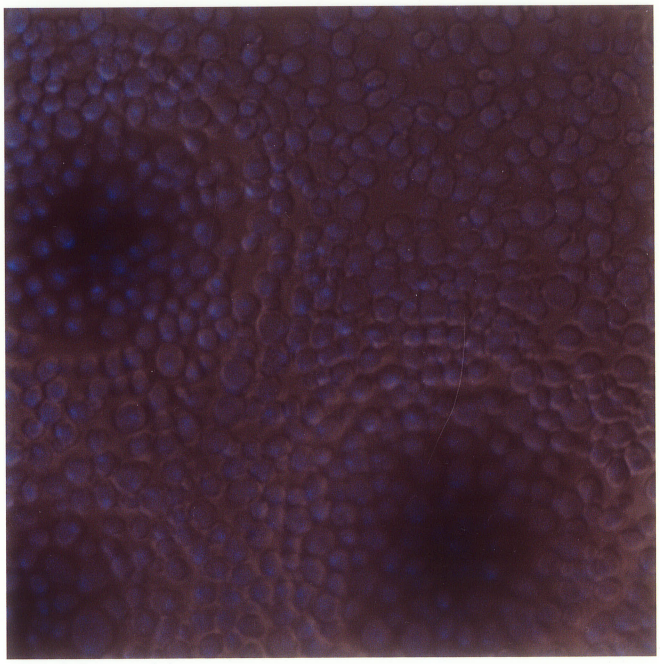
(A) DAPI filter 180X



(B) Rhodamine filter 180X

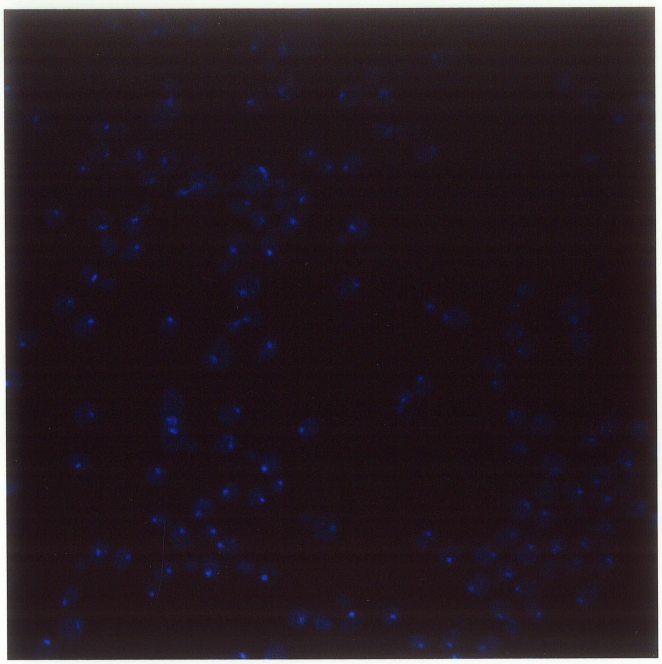


(C) Nomarski filter 180X

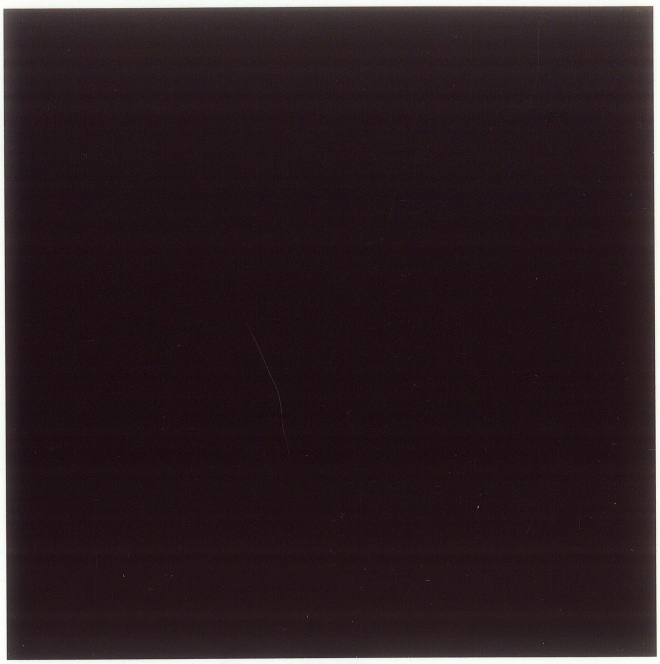


(D) Merged image

Figure 13. Untreated cells KGY37 cells stained with DAPI and anti-DIG antibody Cells were grown to log phase, fixed, stained with DAPI and anti-DIG antibody, and mounted to slides described in Section 2.21.

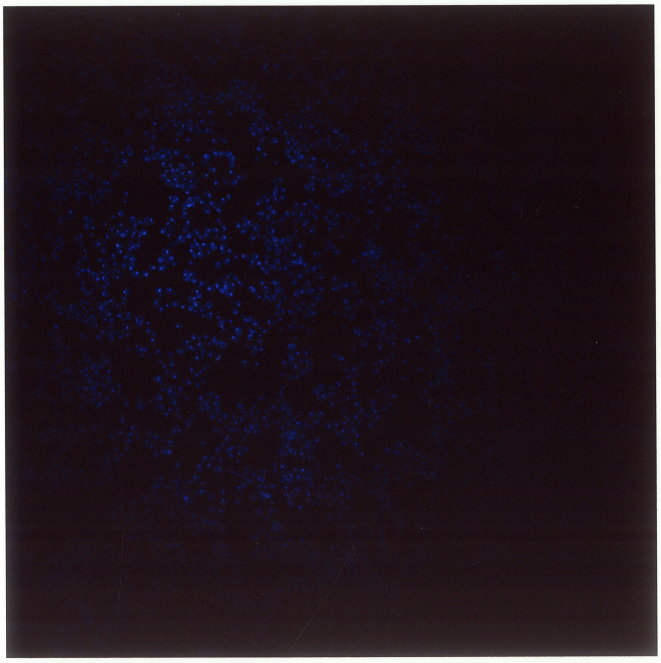


(A) DAPI filter 1800X



(B) Rhodamine filter 1800X

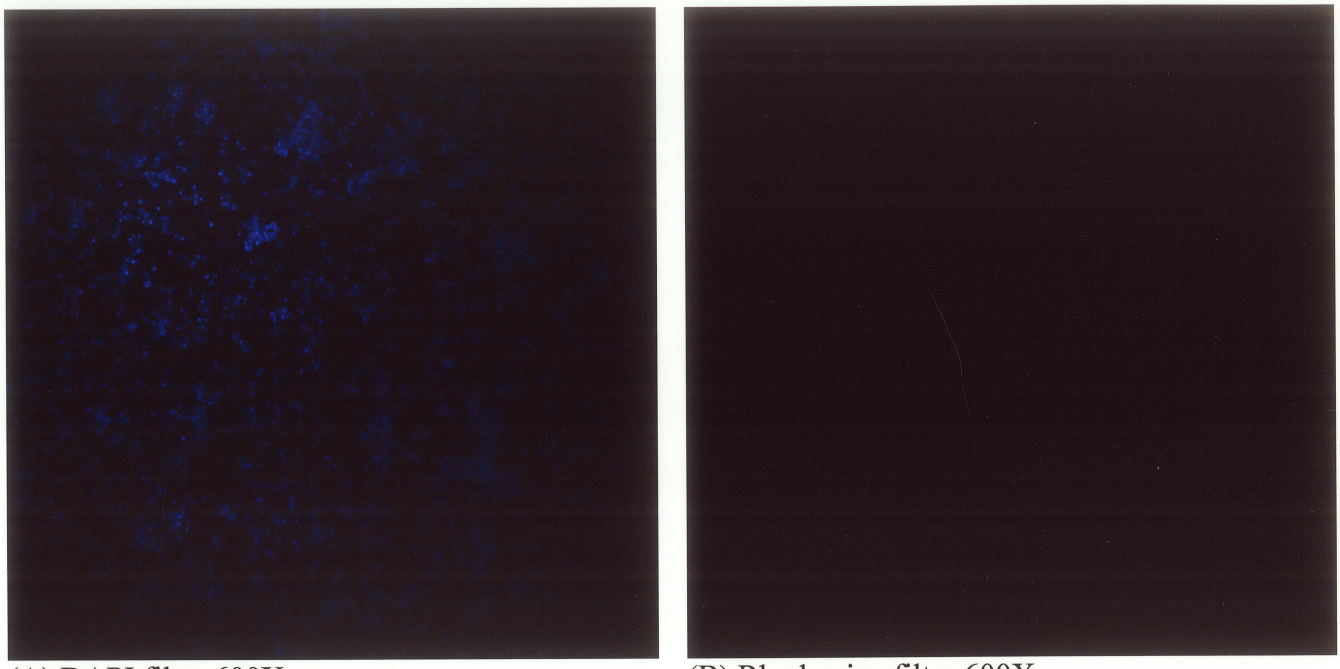
Figure 14. Treated cells KGY37 stained with DAPI
Cells were transformed as described in Section 2.20 without DIG-labeled plasmid, fixed, stained with DAPI, and then mounted to slides as described in Section 2.21.



(A) DAPI filter 600X

(B) Rhodamine filter 600X

Figure 15. Treated KGY37 cells stained with DAPI and anti-DIG antibody
Cells were transformed as described in Section 2.20 without DIG-labeled plasmid, fixed, stained, and mounted to slides as described in Section 2.21



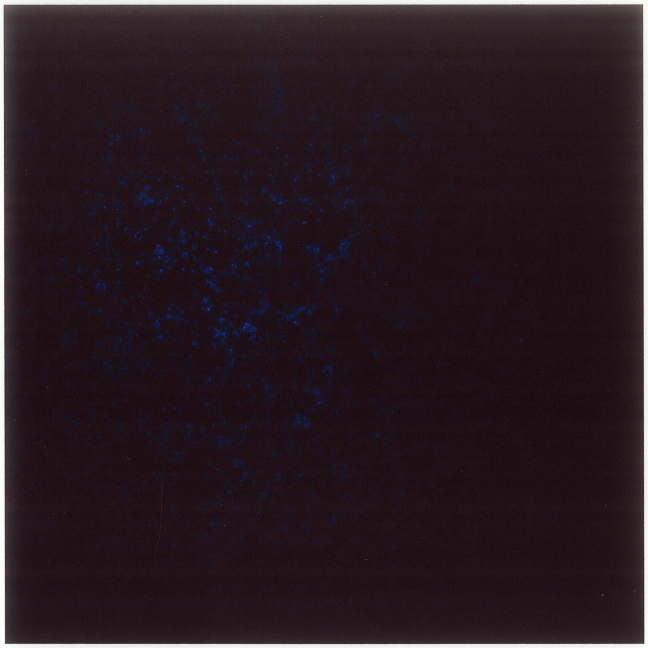
(A) DAPI filter 600X (B) Rhodamine filter 600X

Figure 16. KGY37 cells transformed with DIG labeled plasmid stained with DAPI. Cells were transformed as described in Section 2.20 with DIG-labeled plasmid, fixed, stained with DAPI, and then mounted to slides as described in Section 2.21.

3.5.3 Detection of DIG-labeled plasmid DNA in KGY37 cells

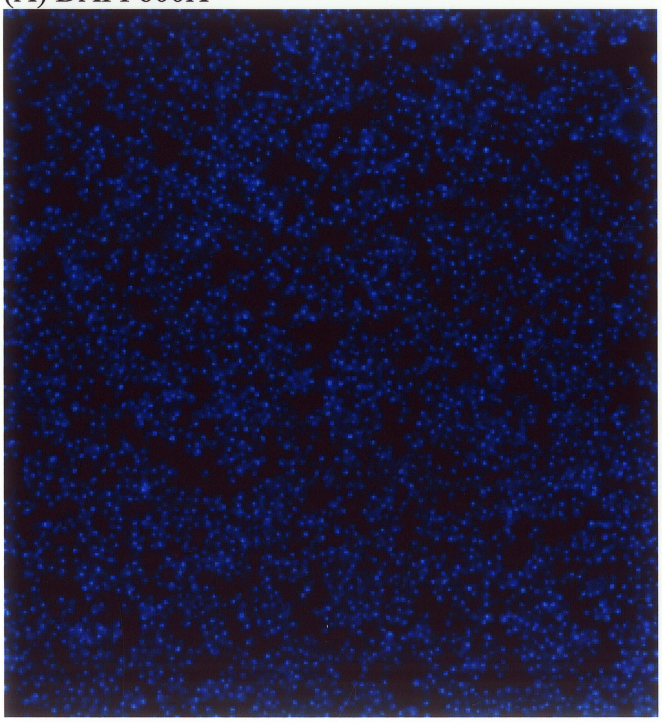
KGY37 cells were transformed by LiAc/ssDNA/PEG method (see Section 2.20) with DIG labeled RKO plasmid, fixed, stained with DAPI and anti-DIG antibody, and then mounted to microscope slides as described in Section 2.21. Figure 17 shows the resulting images in which probe was detected in transformed KGY37 cells. Figures 17A and 17B were taken on an Olympus FV500 confocal microscope while Figures 17C and 17D were taken with a Zeiss Axioimager Z1 microscope both of which were at 600X magnification. Figures 17A and 17C are images of DAPI stained cells while Figures 17B and 17D are images of cells displaying detected rhodamine signal in red. A potential transformation rate of 3.76% was determined from Figures 17C and 17D by comparing the number of cells with detected signal to the total number of cells.

The experiment was repeated and the result shown in Figure 18 at an increased magnification. Figures 18A through 18C were taken with the confocal microscope at 180X magnification. Figure 18C shows cells with detected signal circled in yellow. Figure 18D shows a 3X digital zoom of Figure 18C in which a single cell is observed to be coated in anti-DIG antibody. From Figure 18C, a potential transformation rate of 2.15% was determined by comparing the number of cells with signal to the total number of cells in the image.

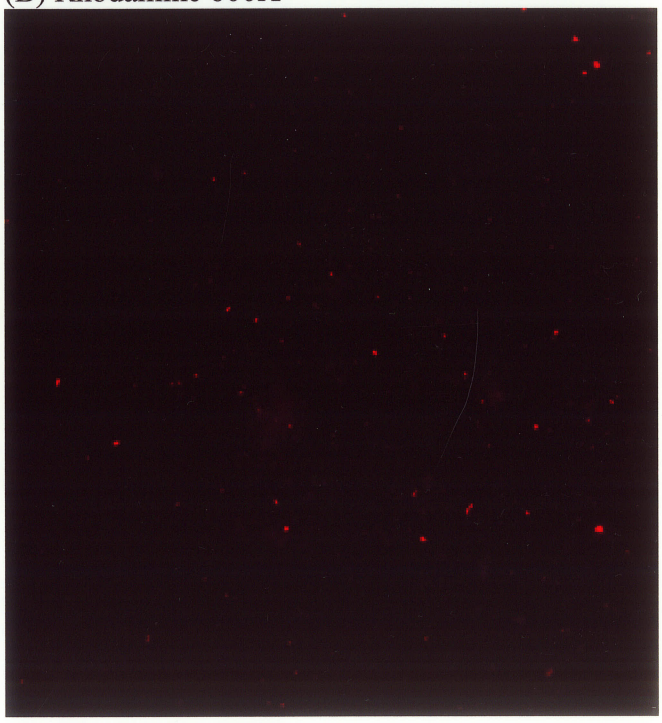


(A) DAPI 600X

(B) Rhodamine 600X

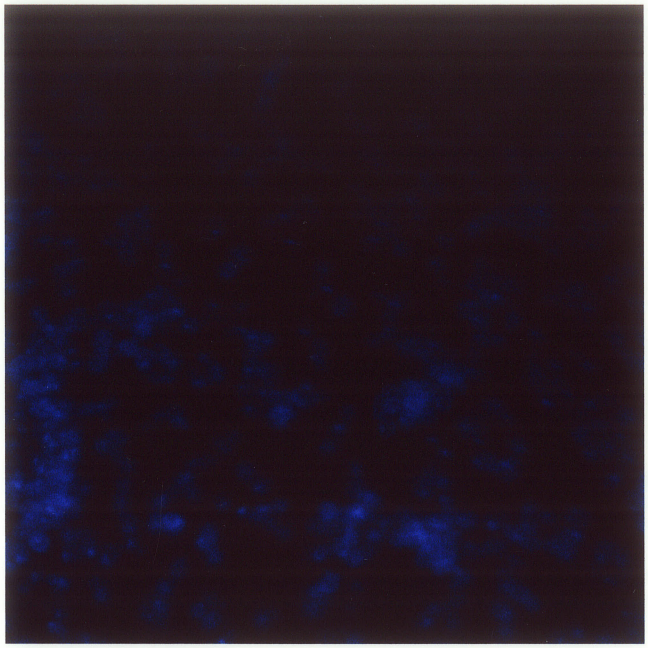


(C) DAPI 600X



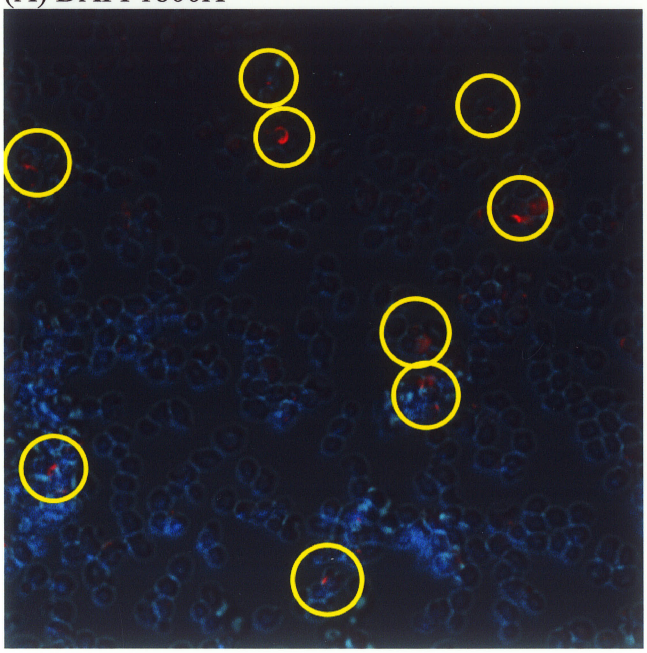
(D) Rhodamine 600X

Figure 17. KGY37 transformed with DIG labeled plasmid detected with anti-DIG antibody. KGY37 cells were transformed with DIG labeled DNA by LiAc/ssDNA/PEG method (Section 2.20), fixed, stained with DAPI and antibody, before mounting to slides.

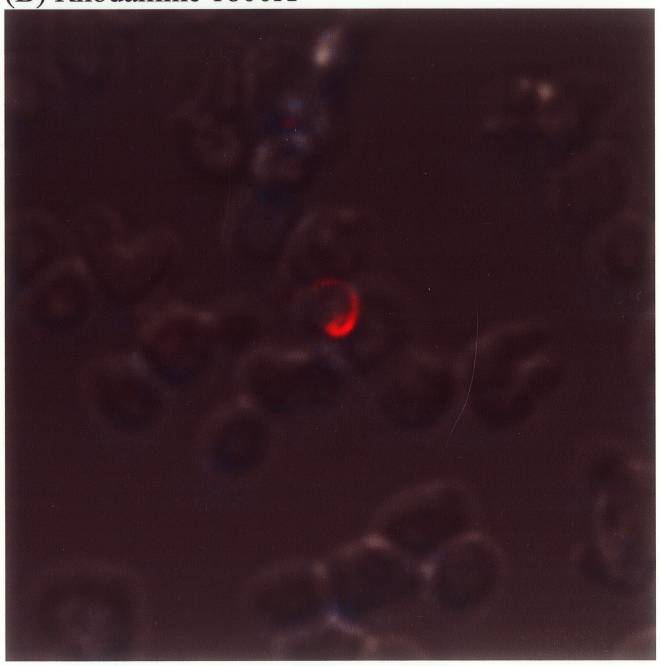


(A) DAPI 1800X

(B) Rhodamine 1800X



(C) Merged 1800X



(D) 3X digital zoom of Figure 18C

Figure 18. Increased magnification of KGY37 cells transformed with DIG labeled plasmid detected with anti-DIG antibody.
KGY37 cells were transformed with DIG labeled DNA by LiAc/ssDNA/PEG method (Section 2.20) fixed, stained with DAPI and antibody, before mounting to slides. Images were taken with a confocal microscope with cells showing bound probe circled in yellow.

3.5.4 Immunofluorescence of other yeast strains

Parent strain BY4742 and along with five mutants were selected based on different molecular defects (see Table 15) and transformed with DIG labeled plasmid DNA to determine if differences could be visualized between strains. Strains were transformed with DIG labeled RKO plasmid, fixed, stained with DAPI and anti-DIG antibody, and then mounted to microscope slides as described in Section 2.20 and 2.21.

Table 15. Strains used for immunofluorescence

Strain	Defect
BY4742	-
146A1 (*vma2*)	defective in proper vacuole formation
103D10 (*erg6*)	in ergosterol biosynthesis required for plasma membrane
103D8 (YML009W-B)	dubious orf
103G6 (*hof1*)	defective in cytokinesis
110H7 (*sac6*)	defective in organization and maintenance of actin cytoskeleton

Figure 19 displays the confocal images from a transformation of BY4742 with DIG labeled plasmid showing (A) DAPI stained cells, (B) rhodamine stained cells and (C) Nomarski image at 600X magnification. Figure 19D is an 1800X magnification of a merged Nomarski/rhodamine image which shows signal detected as red spots found in or bound to the perimeter of cells. Table 16 shows the potential transformation rates determined by comparing the number of cells displaying signal (circled in yellow Figure 19D) to the total number of cells in the image.

Figure 20 shows the results of a transformation with DIG labeled plasmid of a *vma2* mutant (146A1) defective in proper vacuole formation. Cells were viewed under the DAPI filter (Fig. 20A), rhodamine filter (Fig. 20B), and the Nomarski filter at 1800X

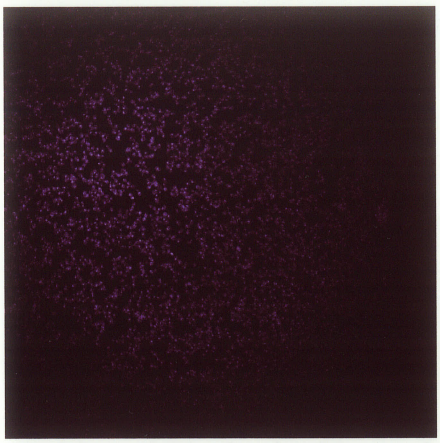
magnification on a confocal microscope. A DAPI/rhodamine merged image and DAPI/rhodamine/Nomarski are shown in Figures 20C and 20D respectively. Two cells circled in yellow (Figure18D) can be seen coated in anti-DIG antibody.

Figures 21A through 21D show the results of transformations of the remaining four mutants with DIG labeled plasmid and detection with anti-DIG antibody conjugated to rhodamine. Images were taken with a confocal microscope at 1800X magnification of (A) *erg6* (rhodamine/Nomarski filter), (B) YML009W-B, (rhodamine/Nomarski filter) (C) *hof1* (DAPI/rho/Nomarski), and (D) *sac6* (DAPI/rho/Nomarski) mutants transformed with DIG labeled DNA plasmid. Probe was detected in all four mutants either coating the cell or as distinct points within or on perimeter. The percentage of cells displaying signal were calculated and displayed in Table 16. The percentage of cells with detected probe ranged from 1.48% in YML009W-B (103D8) to 4.58% in *hof1* (103D8) mutant.

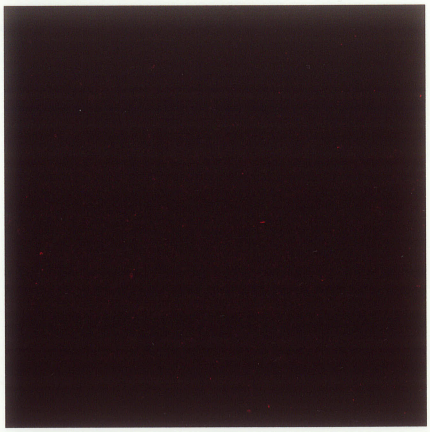
Table 16. Percentage of cells displaying fluorescence labeled plasmid DNA signal

Strain	# cells with probe	Total # of cells	% cells with signal
KGY37	9	418	2.15 %
BY4742	5	315	1.58 %
vma (146A1)	2	101	1.98 %
erg6 (103D10)	8	197	4.06 %
YML009W-B (103D8)	3	203	1.48 %
hof1 (103G6)	13	268	4.85 %
sac6 (110H7)	11	455	2.40 %

(A) DAPI 600X



(B)Rhodamine 600X



(C) Nomarski 600X

(D) Merged Rhodamine/Nomarski 1800X

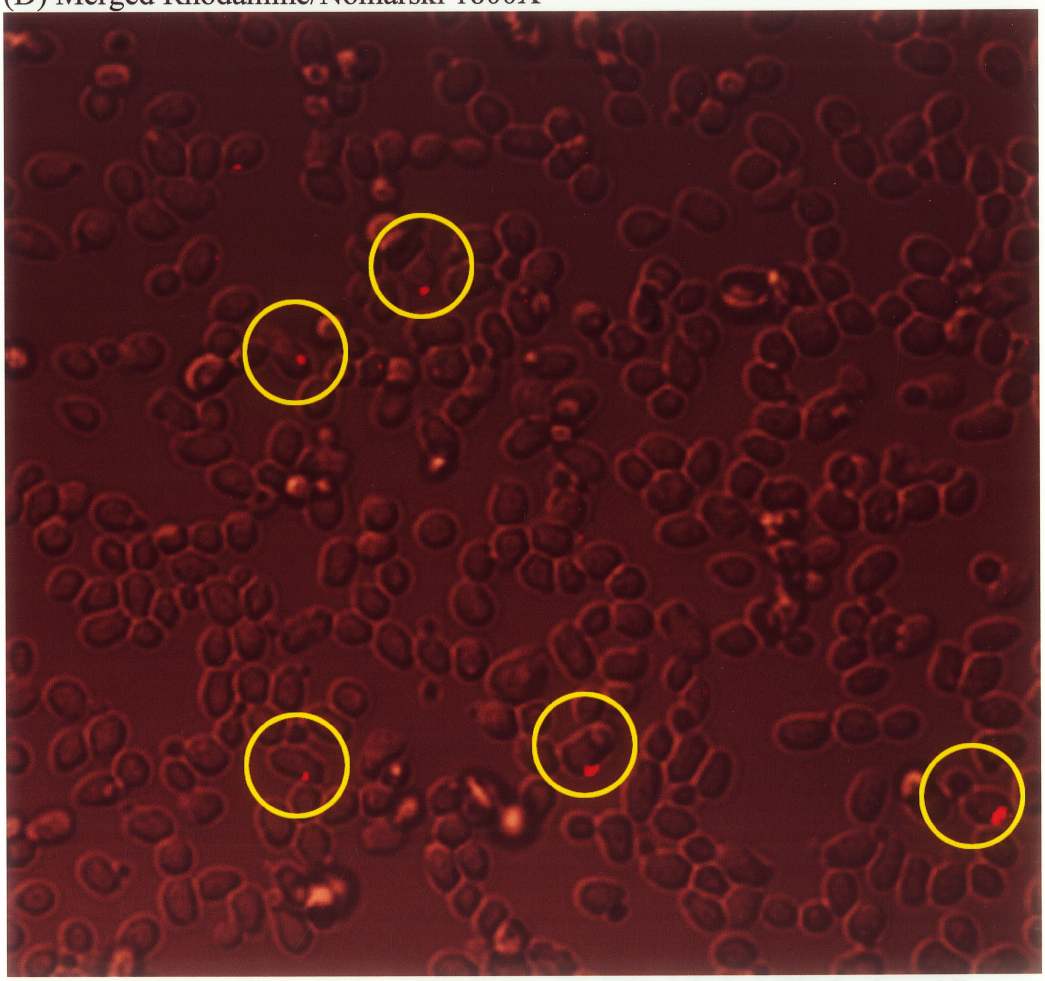
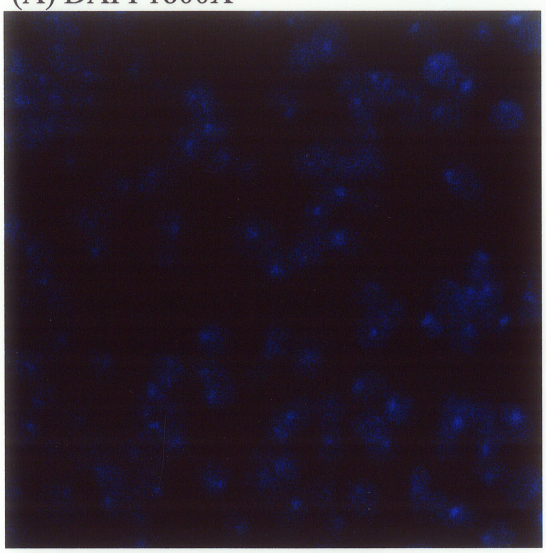
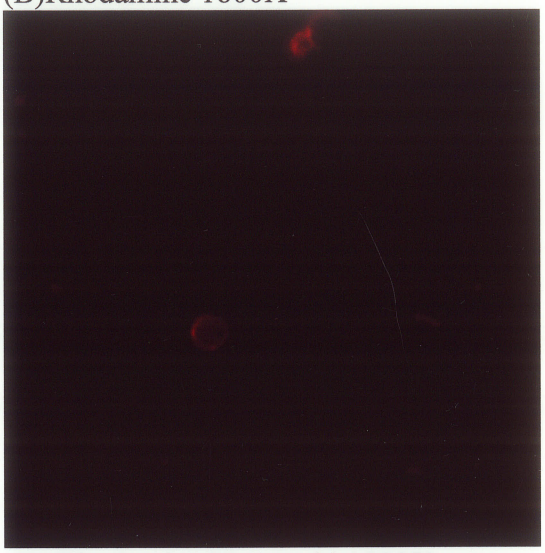


Figure 19. Transformation of BY4742 with DIG labeled RKO plasmid
Cells were transformed with labeled plasmid by LiAc/ssDNA/PEG method (see Section 2.20) then fixed, stained with DAPI and anti-DIG antibody, then mounted to slides as described in Section 2.21. Cells circled in yellow display presence of signal

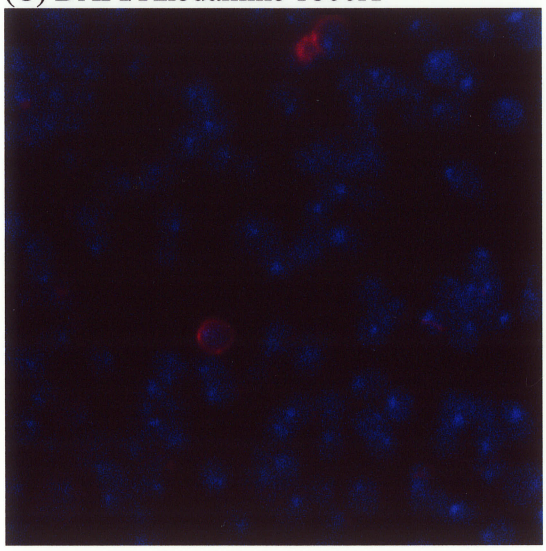
(A) DAPI 1800X



(B)Rhodamine 1800X



(C) DAPI/Rhodamine 1800X



(D) Rhodamine/Nomarski 1800X

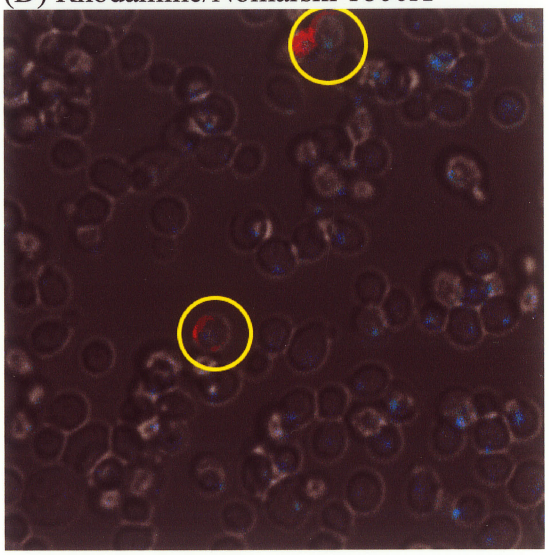
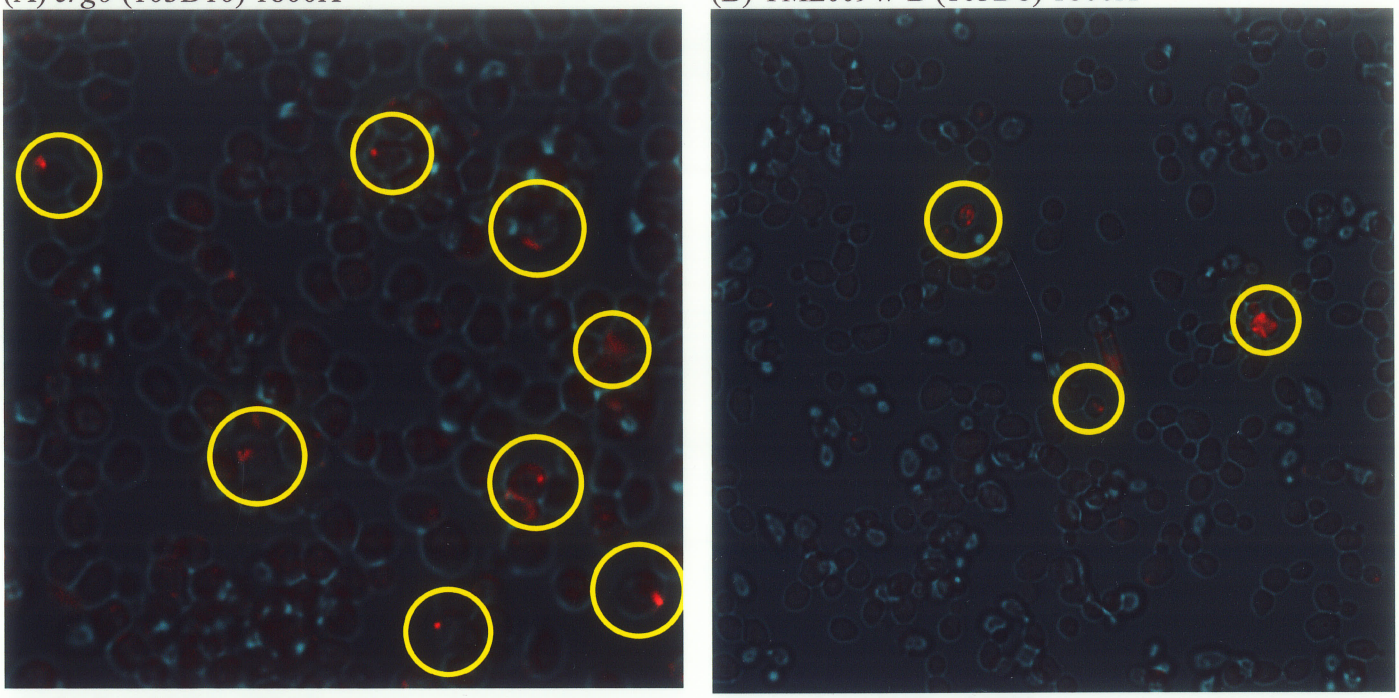


Figure 20. Transformation of *vma2* mutant (146A1) defective in proper vacuole formation. Cells were transformed with labeled plasmid by LiAc/ssDNA/PEG method (see Section 2.20) then fixed, stained with DAPI and Anti-DIG antibody, and mounted to slides as described in Section 2.21. Cells circled in yellow showed presence of signal.

(A) *erg6* (103D10) 1800X

(B) YML009W-B (103D8) 1800X



(C) *hof1* (103G6) 1800X

(D) *sac6* (110H7) 1800X

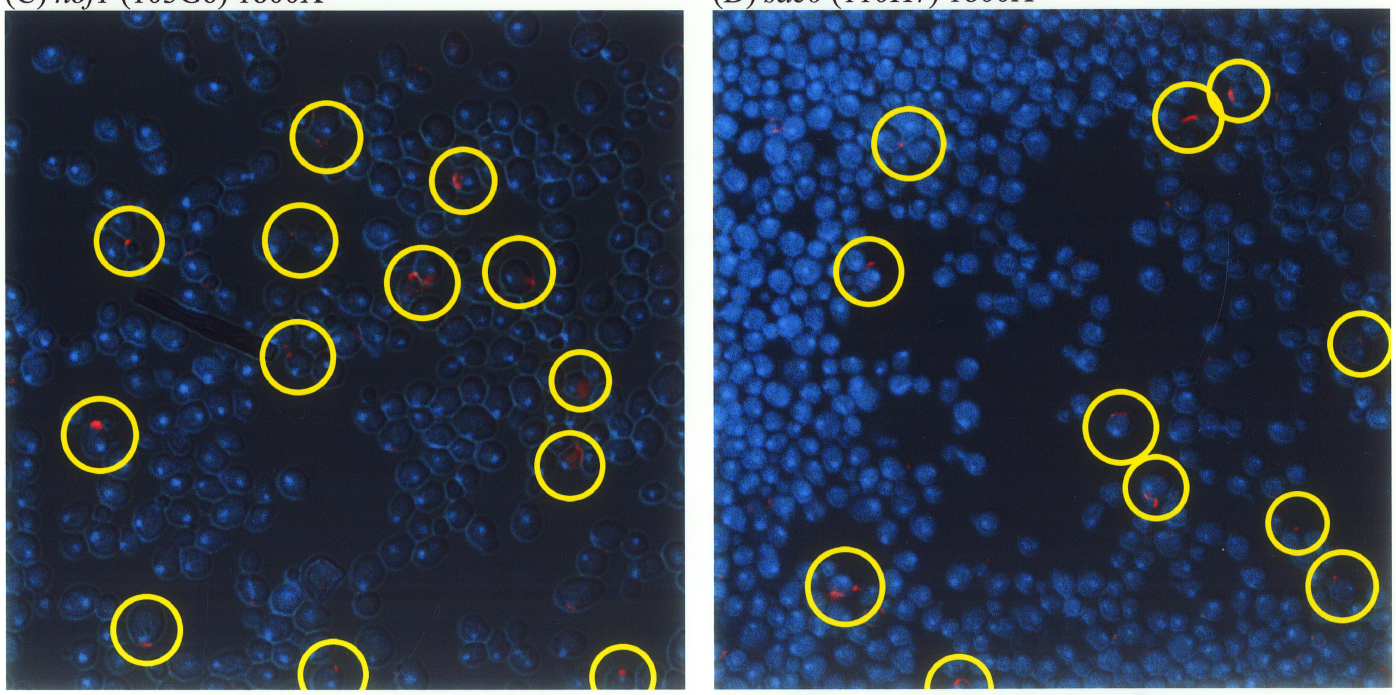


Figure 21. Detection of DNA labeled probe in four different mutant strains after transformation by LiAc/ssDNA/PEG. Strains were transformed with DIG labeled plasmid by LiAc/ssDNA/PEG method (see Section 2.20) then fixed, stained with DAPI and antibody, and then mounted to slides as described in Section 2.21. Transformation rates were calculated from cells with detected signal (circled in yellow) and displayed in Table 16.

4. DISCUSSION

4.1 BY4742 Parent strain characterization

A yeast deletion library derived from parent strain BY4742 was screened for mutants that displayed reduced transformation efficiencies. BY4742 was initially characterized prior to the library screen to develop transformation conditions that would be applied to the rest of the library.

Saccharomyces cerevisiae strain, BY4742, was derived from S288C strain to contain commonly used selectable markers and to reduce plasmid integration events which can interfere with a wider variety of molecular genetic applications (Brachmann *et al.* 1998). A yeast deletion library, consisting of 4827 knock-out mutants derived from BY4742 to contain single gene deletions, was purchased from Open Biosystems. The library was transformed by chemical treatment with LiAc/ssDNA/PEG and screened for mutants displaying irregular transformation efficiencies. Prior to library screening, the transformation ability of BY4742 was characterized to give a baseline for comparison to all the mutant strains.

4.2 Growth curve of *S. cerevisiae* strains used

Parent strain, BY4742, was determined to have an approximate doubling time of 2 hrs (see Figure 1) which was comparable the other strains used in this study. KGY37 and unsynchronized DGY233 cells were also determined to have a doubling time of

approximately 2 hrs (Figures 11, and 3 respectively). Cells inoculated into fresh 2X YPAD appeared to have an initial lag phase before cells could reach exponential phase. This was apparent in Figure 3 between synchronized and unsynchronized cells. Synchronized cells took an extra 30 min to double compared to the unsynchronized cells. The overnight culture used to prepare the unsynchronized culture was likely to contain some cells still actively dividing which when inoculated into fresh 2X YPAD for re-growth did not require an adjustment time. This could explain why the unsynchronized culture grew more quickly and would suggest the lag phase for DGY233 cells is approximately 30 min. It can then be reasoned the lag phase for BY4742 is also approximately 30 min because BY4742 and unsynchronized DGY233 cells had similar growth curves.

4.3 Transformation ability during re-growth in 2X YPAD

An optimal time to harvest BY4742 cells for transformation was determined by sampling cells every 30 min during re-growth in 2X YPAD for 5 hrs. The same number of cells ($1x10^8$) was transformed at each interval and the resulting number of transformants shown in Figure 2. There was a low basal rate of transformants until the 120 min interval at which time the number of transformants significantly increased (see Figure 2). Interestingly, this increase coincided with the first doubling of cells (see Figure 1) and could have been a result of plasmid that enters the nucleolus as it is dismantled and re-formed during mitosis. Following the first doubling, there was a continued increase in transformants until a plateau was reached at the 270 min time point with

approximately $1x10^5$ transformants/μg DNA. A similar trend was observed when the experiment was repeated with synchronized DGY233 cells (see Figure 5). Cells released from G1 phase and inoculated into fresh medium displayed a low basal rate of transformants. This was observed until the 90 min interval at which time the number of transformants increased significantly (see Figure 5). Transformant numbers continued to increase until a plateau was reached at the 240 min time point (see Figure 5) with approximately $2.5x10^5$ transformants/μg DNA.

The results of both experiments suggest that transformation ability is linked to cell growth and transformation competence is gained at some distinct point in the cell cycle. Cells in stationary or lag phase did not transform well as witnessed by the low transformant numbers from both BY4742 and DGY233 cells sampled at the 0 and 30 min time points (see Figure 2 and 5). For BY4742, the initial increase in transformants coincided with the first doubling of cells at the 120 min time point (see Figure 2) suggesting competency is gained before cell division. Furthermore, synchronized DGY233 cells released from G1 showed an approximate 5X increase in transformants between the 60 and 90 min time point which was well before cell doubling at the 150 min time point (see Figures 3 and 5). It is possible that an increase in transformant numbers could have been detected soon after the 60 min time point and perhaps for this strain, with a 2 hr doubling time, competency could have been gained in late G1 or early S phase.

4.4 Increasing transformation efficiency

Dimethylsulfoxide (DMSO) enhances penetration of chemicals and has been demonstrated to enhance the transformation efficiency in bacteria and yeast (Chung *et al.* 1988; Hill *et al.* 1991). Hill used a 10% (v/v) DMSO solution along with lithium acetate transformation to achieve a 25.8 fold increase in transformation in *S. cerevisiae* JRY188 (Hill *et al.* 1991). However, transformation of BY4742 with the addition of DMSO did not result in a similar increase. There was either no increase at 5% (v/v) or a 3 fold decrease at 10% (v/v) DMSO (see Table 8). The results found by Hill were likely a strain specific characteristic. DMSO at high concentrations (5 to 50%) has been shown to affect yeast ultrastructure unpredictably (Fassel *et al.* 1997) and may explain why *S. cerevisiae* BY4742 transformation efficiency does not improve.

4.5 Library screen by microtitre plate assay

A microtitre plate assay was used to screen the mutant knock-out library in a 96 well format. Two to four plates were screened at a time as described in Section 2.19. This method allowed the rapid screening of 4827 mutants with the results shown in Appendix II. From the screen, 177 mutants were identified as putative transformation mutants

Of the 177 mutants identified, 34 displayed poor growth on SC-LEU and YPAD plates after treatment which could have been the result of several factors. A strain's inherent slow growth would not have provided enough cells required for the

transformation. Neighbouring mutant strains could have out competed these strains for nutrients on solid medium resulting in smaller growth patches. Another likely possibility could be the strain's sensitivity to the microtitre plate assay itself. Seven of the mutants displaying poor growth and two known poor growers were individually re-screened (see Table 10) in liquid culture. Given proper doubling times, the mutants were still found to show poor growth and in turn poor transformation efficiencies. The lithium component in the transformation mix is likely to have caused cells to become sensitive. In glucose-grown cells lithium can reduces the steady state levels of UDP-glucose resulting in a defect on glycogen biosynthesis (Masuda *et al.* 2001). This could then explain the poor growth on solid medium and the resulting transformation efficiencies displayed by the mutants. All 34 of these lithium sensitive mutants were removed from the original list of 177. An alternate transformation method would have to be applied to determine if these mutants were truly deficient in transformation.

Six mutants initially screened by the microtitre plate assay appeared to show increased transformation ability. However, of the four mutants re-screened in liquid culture, one was found to have similar transformation ability to the parent strain while the other three showed less than half the ability (see Table 11). This would suggest that results from the microtitre plate assay and the liquid assays are not always in agreement. False positives or false negatives could have arisen from the microtitre plate screen that would not give a true indication of a mutant's transformation ability. The discrepancies between assays used are likely a result of several factors. The number of cells transformed between assays was different. The number of cells transformed in liquid culture was kept constant for all strains while it varied between strains in the microtitre

plate assay. Mutants would have appeared to transform better in the microtitre plate assay than the liquid re-screen because of the difference in ratio of cells used. Growth conditions are also likely to have also caused differences between assays. In the liquid re-screen, growth times were tailored for each strain to allow for two cell divisions. Based on the doubling time of parent strain BY4742, a 4 hr growth time was used in microtitre plate assay to allow cells to reach two cell divisions. The use of a standardized time is likely to have resulted in mutants that did not reach two cell divisions or those that surpassed it. Mutants that surpassed two divisions could have then appeared to transform better in the microtitre plate assay than the liquid re-screen because of the increased number of cells used.

Competition between mutants grown on solid medium could also account for the false positives observed. From the microtitre screen, a unique phenomenon was observed (see Figure 6C) in which mutants spotted on the perimeter grid grew much better than those near the centre. Mutants spotted at the perimeter of YPAD plates tended to have bigger patches of growth that extended to the outside of the Petri dish and would most often come into contact with a neighbouring perimeter mutant (see Figure 6C). Mutants spotted inside the perimeter tended to be confined to the position in which they were spotted. These resulting circular patches of growth were smaller and mutants did not come into contact with neighbouring mutants (see Figure 6C). Having fewer neighbours to compete with for nutrients could explain the growth of the perimeter mutants. All six of the increased transformation mutants identified from the microtitre plate screen were located at positions on the perimeter or the grid in either rows A or H (see Figure 6 for grid pattern). Mutants 149H1 and 149H12 were found located at opposite corners where

competition would be the least (see Figure 6C). The observed growth phenomenon could then explain why these mutants were originally identified but later determined not be increased transformation mutants.

4.6 Individual mutant re-screen

Mutants identified from the microtitre assay were individually re-screened in liquid culture to verify the results of the initial plate screen. Transformation efficiencies were determined and compared to the parent strain and shown as a percentage in Tables 17-21 (see Appendix II). Following the re-screen, 23 mutants that initially displayed decreased transformation were found to have transformation efficiencies of 50% or more than the parent strain (see appendix II, Table 21). The results could be explained by the differences in assays mentioned above. The better performances could have resulted from a combination of better growth times or the increased aeration in a flask. The elimination of lithium sensitive mutants and false positives from the original list of identified mutants resulted in 115 mutants deemed to be transformation deficient.

4.7 Transformation by alternative methods

Several transformation defective mutants were selected and transformed using the electroporation and spheroplasting methods (see Section 2.17 and 2.18). The mechanisms involved in the two methods differ but the end result is the same: the introduction of foreign DNA into the cell. Transformation by electroporation involves the passage of a

transient electric pulse (Potter 1993) through two electrodes of an electroporation cuvette containing yeast cells. The underlying mechanism behind electroporation is not well understood; however, it is believed that electric pulses create instantaneous pores in the cell wall and membrane which DNA can use to diffuse into the cell (Sukharev *et al.* 1992). On the other hand, spheroplasting requires the enzymatic digestion of the cell wall followed by incubation of plasmid DNA with cells stabilized in sorbitol. The underlying idea here is that removal of the physical barrier, that is the cell wall, allows plasmid DNA to more easily gain entry into the cell. By comparing different transformation methods, a better understanding of the mechanism could be gained.

An *erg6* mutant was selected for transformation by electroporation and spheroplasting based on previously reported poor transformation efficiencies (Gaber *et al.* 1989; Veen *et al.* 2003). Another two *erg* mutants, *erg2* and *erg28,* identified from the microtitre plate screen were also selected because they contained defects in the same biosynthetic pathway as the *erg6* mutant.

Ergosterol is a sterol found in yeast that affects membrane fluidity and permeability much like cholesterol does in mammalian cells (Lees *et al.* 1995). It is a major component of the plasma membrane (Zinser *et al.* 1993) with the majority of the *ERG* genes in the ergosterol biosynthetic pathway being essential, except for five that function in the late steps (*erg2* to *erg6*) (Parks *et al.* 1995). Deletion in these mutants results in viable cells with an accumulation of different sets of sterols that are able to compensate for the lack of ergosterol.

4.8 Transformation of *erg* mutants by electroporation

Parent strain, BY4742, along with the three selected *erg* mutants was transformed by electroporation. Transformation efficiency was highest for BY4742 followed by *erg28*, *erg2* and *erg6* mutants. This is the same trend observed when the same strains were transformed by the LiAc/ssDNA/PEG method (see Table 12). When electroporation was used, the transformation ability of BY4742 was found to decrease while the *erg* mutant's increased. For BY4742 an average of $3.5x10^5$ transformants/μg DNA was obtained by electroporation compared to an average of $8x10^5$ transformants/μg DNA by LiAc treatment. The number oftransformants recovered from the *erg* mutants were significantly higher by electroporation. Less than $1x10^3$ transformants/μg DNA was observed by LiAc/ssDNA/PEG treatment for all *erg* mutants, whereas transformants ranged from $3x10^3$ to $3x10^5$ by electroporation (see Figure 8).

The instantaneous generation of pores by electroporation appears to increase transformation efficiency in *erg* mutants but have a negative effect on the parent strain. High levels of sterols are believed to be important for reducing the permeability of the outer membrane to protect cells against hostile environmental conditions (Eisenkolb *et al.* 2002). This could explain why the transformation efficiency of the parent strain decreased. The parent strain was able to synthesize wild-type membranes containing ergosterol that was able to protect cells from the affects of electroporation resulting in lower transformant numbers. By contrast, the altered sterol compositions in the *erg* mutants could have caused them to be more susceptible to the electroporation method.

4.9 Transformation of *erg* mutants by spheroplasting

Parent strain BY4742, along with the three selected *erg* mutants was transformed by spheroplasting. Transformation by spheroplasting resulted in the same transformation trend seen when electroporation and LiAc/ssDNA/PEG methods were used. Again, BY4742 showed the highest efficiency followed by *erg28*, *erg2* and *erg6* mutants (see Figure 9). The resulting transformation efficiencies were comparable to what was observed by LiAc/ssDNA/PEG transformation. Parent strain transformation efficiency was approximately $8.4x10^5$ transformants/μg plasmid DNA which was slightly higher than the $8x10^5$ transformants transformants/μg plasmid DNA observed by LiAc/ssDNA/PEG treatment. The *erg* mutants also showed a slight increase in transformants, however not as dramatic as transformation by electroporation. Transformation efficiencies for the mutants ranged between $9x10^2$ and $1x10^5$ transformants/μg plasmid DNA (see Figure 9).

The completion of three different transformation methods resulting in the same observed trend indicates a true deficiency in some part of the transformation process of the *erg* mutants. The individual transformation efficiencies may have differed across methods, but the same trend was observed when mutants were compared (see Table 12). The parent strain BY4742 always had the highest number of transformants followed by *erg28*, *erg2*, and *erg6*.

4.10 Differences in transformation methods

There appears to be a blockage in the transformation process of the *erg* mutants which the electroporation method was able to partially bypass but spheroplasting and LiAc/ssDNA/PEG treatment could not. Perhaps there exist multiple transformation pathways which are invoked by different transformation methods. The spheroplasting and LiAc/ssDNA/PEG methods produced similar results suggesting the same pathway was involved. Both methods are chemical treatments requiring an incubation time with plasmid DNA. It is possible that during the incubation time, plasmid DNA that comes into contact with the cell is absorbed through some cellular process such as endocytosis. The resulting endocytic vacuole could then be targeted to the nucleus.

An alternate transformation pathway could be involved when electroporation was used. Cells could bypass the endocytosis process because plasmid DNA could simply diffuse in when pores were generated by an electric pulse. From there it is possible that a protein could chaperone naked plasmid DNA to the nucleus. It was previously discovered that *erg2* and *erg6* mutants were defective in the formation of endocytic vesicles at the plasma membrane (Munn *et al.* 1999). Therefore, bypassing the endocytic process would remove a major barrier to DNA entry and may explain why the *erg* mutants showed high efficiencies by electroporation.

4.11 Immunofluorescence

4.11.1 Immunofluorescence of KGY37

Yeast strain KGY37 was initially selected for transformation with DIG labeled RKO plasmid because it possessed a higher transformation efficiency than parent strain BY4742 ($8.78x10^6$ versus $8x10^5$ transformants/μg DNA respectively, see Tables 8 and 13). KGY37 would give rise to more transformation events resulting in a better chance of visualizing DIG labeled plasmid during the process.

It was important to show that DIG labeled plasmid could gain entry into cells and be successfully incorporated. Transformation with the circular plasmids YEplac 112 and RKO IV resulted in a 6 fold difference in transformation efficiency when plasmid size was doubled from 5kb to 10kb (see Table 13). The decrease in efficiency with increase in plasmid size is not uncommon as it has also been found to occur in *E. coli* (Horáková 1998). It is possible that the increased size of the RKO IV plasmid causes difficulties for the cell machinery to transfer it across the cell membrane.

From Table 13 it was shown that DIG labeled RKO IV plasmid could be transformed into KGY37, however a lower transformation efficiency than unlabeled RKO IV plasmid was observed. The added size of the digoxigenin group to the plasmid could have reduced a cell's ability to take up the plasmid or it could be due to the fact that DIG labeled RKO IV is a linear plasmid. The ends of DIG labeled RKO IV plasmid, linearized for the purpose of labeling with DIG (see Section 2.6), contained two COS sites that required re-circularized by a host ligase in order to be properly expressed.

Failure to re-circularization is likely to have contributed to the lower transformation efficiency.

Immunofluorescence microscopy was used to detect DIG labeled DNA transformed into several yeast strains. Different yeast strains have been shown to have varying levels of autofluorescence (Billinton *et al.* 1998). Several controls (see Table 14) were performed to determine whether KGY37 showed autofluorescence along with the suitability of the anti-DIG antibody conjugated to rhodamine probe. No signal was detected when untreated KGY37 cells were viewed under the rhodamine filter in both the absence and presence of antibody (see Figures 12 and 13). This would suggest that untreated KGY37 cells do not autofluoresce and non-specific binding of antibody to untreated cells does not occur. Similarly, no signal was detected when KGY37 cells were subjected to the conditions of LiAc/ssDNA/PEG treatment (without DIG labeled plasmid) in both the absence and presence of antibody (see Figures 14 and 15). This would suggest that conditions of the treatment do not alter the autofluorescence properties of KGY37 and non-specific binding of antibody to treated cells does not occur. A final control suggested that DIG labeled plasmid DNA does not interfere with signal detection in KGY37 transformed with DIG labeled plasmid without antibody incubation (see Figure 16).

KGY37 cells transformed with DIG labeled plasmid and incubated with anti-DIG antibody conjugated to rhodamine showed presence of signal when viewed under a rhodamine filter (see Figures 17 and 18). Antibody probe was detected on a small minority of cells (2.15-3.76%) which is in contrast to what Zheng *et al.* (2005) discovered when pUC18 plasmid was stained with YOYO-1 was found bound to 69.9%

of cells. Several differences between experiments could account for the discrepancies. Two different yeast strains were used and differences their genetic background could explain the differences in binding properties (KGY37 was used in this experiment while yeast strain AY was used in Zheng's). The process of fixing, staining, and mounting of cells to slides resulted in additional cell washes that could have removed bound DNA, whereas Zheng's cells were dropped on glass slides for viewing immediately following the LiAc/ssDNA/PEG transformation process (Zheng *et al.* 2005).

Bound probe on KGY37 cells appeared as red spots at various locations in or on the surface of the cell. The majority of cells showed probe bound to the exterior of the cell. Some cells showed a distinct point of intensity while most were coated (see Figure 18). Cells were fixed immediately following transformation, in doing so the cells may not have had a chance to properly internalize the plasmid resulting in the majority of it bound to the cell surface. This is not surprising since the cell wall acts as a barrier to DNA and nonspecifically binds DNA (Gietz *et al.* 1995). Perhaps internalization of probe would be better seen if cells were given an incubation time in YPAD following transformation to allow cells to grow and internalize plasmid DNA.

4.11.2 Immunofluorescence of other yeast strains

Immunofluorescence microscopy of BY4742 and mutants revealed images similar to those found with KGY37 (see Figures 19–21). Probe was found bound to a minority of cells and typically on the exterior of the cell. The percentage of cells with bound probe ranged from 1.58 – 4.85%. Although mutants with different defects were used (see Table

15), no differences in the binding pattern of probe were observed. For all strains, probe was found either bound at distinct points inside/outside the cell or coating the exterior.

From these experiments, immediately following LiAc/ssDNA/PEG transformation only a small percentage (<5%) of cells will bind plasmid and the majority of which is appears bound to the exterior of the cell.

4.12 Gene Ontogeny

The Gene Ontology project provides a controlled vocabulary for the description of gene and gene product attributes. A common language is used to provide the description of the molecular function, biological process, and cellular component of gene products. The Gene Ontology terms for the biological process of the 115 mutants identified through the transformation screen was referenced from the *Saccharomyces Genome Database* and grouped into several categories shown in Figure 8.

Uncharacterized mutants

By far, the largest group identified from the transformation screen was mutants whose gene products were of unknown biological function (see Appendix II, Table 31). Transformation was reduced in 28 of these mutants by comparison the second largest group contained only 14 members. It is likely that with such a large group, one or more of these mutants directly affects the transformation process.

Cell wall / Cytoskeleton mutants

The four mutants shown in Table 23 were found to be involved in processes including budding and cytokinesis. The mutants, *bud16, pac1 and hof1*, were found to be defective in proper bud site selection, positioning of spindle apparatus at the bud site, and cytokinesis, respectively (Kamei *et al.* 1998; Ni *et al.* 2001; Lee *et al.* 2003). These defects could lead to an irregular budding pattern or unequal separation of chromosomes. The transformation deficiency observed in these mutants is likely the result of defects in growth caused by the respective gene deletion. The remaining mutant in this group (*tip1)* was found to be deficient in a mannoprotein found in the cell wall with possible lipase activity (Fujii *et al.* 1999). Perhaps the TIP1 gene product is needed for entry of plasmid DNA bound at the cell surface into cells through some action associated with its lipase activity.

DNA Repair / Maintenance mutants

The 11 mutants identified in Table 24 contained gene deletions that affected the repair and maintenance of DNA. The mutants, *lrp1, wss1, ddr48, and fyv6* were found to be unable to produce specific proteins in response to DNA damage (Maga *et al.* 1986; Erdemir *et al.* 2002; Wilson 2002; O'Neill *et al.* 2004). The MUM2 and ILM1 genes are required for meiotic DNA replication and mitochondrial DNA maintenance respectively (Entian *et al.* 1999; Davis *et al.* 2001). Three mutants (*snf6*, *snf12*, and *swi3*) identified from the transformation screen lacked the ability to form a fully functional SWI/SNF complex required for transcriptional activation through the modulation of chromatin

structure. Mutant *rtt106* was found to be unable to produce a protein which regulates TY1 transposition through chromatin silencing. If a cell was unable to repair or maintain its own DNA, it would be unlikely it to be able to do the same for foreign plasmid DNA. The transformation deficiency seen in these mutants is most likely attributed to the growth defects of these strains.

Endocytosis / Exocytosis mutants

Endocytosis/exocytosis mutants consisted of 8 members shown in Table 25 that were identified with poor transformation efficiencies. Only one mutant (*sac1*) was defective in exocytosis while the remaining seven in the endocytosis process. The ratio of endo/exocytosis mutants identified suggests that endocytosis is an important step in the transformation process. The single exocytosis gene product (*sac1*) is integral to the endoplasmic reticulum (ER) and is involved in protein sorting and trafficking (Whitters *et al.* 1993; Schorr *et al.* 2001). It is possible that foreign DNA that has entered the endosomal pathway could be targeted to the Golgi apparatus where it could be sorted through the help of Sac1p and re-targeted for the nucleus.

Endocytic mechanisms serve many important cellular functions including nutrient uptake, regulation of surface receptors, and maintaining cell polarity. Seven mutants were identified from the transformation screen with reduced transformation efficiencies and found to function in endocytosis. The gene products absent from mutants, *akr1*, *sla1*, *fen2*, *end3*, and *cup5* function at the cell membrane while the gene products from mutants *thr4* and *sac6* function at the nucleus and cytoplasm respectively (Adams *et al.* 1995;

Tang *et al.* 1997; Stolz *et al.* 1999; Roth *et al.* 2002; Warren *et al.* 2002) . Perhaps a part of the transformation process involves the entry of plasmid DNA into the cell is via vesicles endocytosed at the cell surface.

Ergosterol mutants

From the transformation screen, 4 mutants (see Table 26) were identified with defects in ergosterol biosynthesis. These mutants were unable to produce ergosterol required for normal membrane structure. Furthermore, several biochemical activities are disrupted in ergosterol mutants including amino acid and pyrimidine transport, resistance to antifungal agents and certain cations, and respiratory activity (Parks *et al.* 1995). Two of the ergosterol mutants (*erg2* and *erg6*) were previously shown to be defective in forming endocytic vesicles at the plasma membrane (Munn *et al.* 1999). The inability to form normal membrane structure along with the inability to compensate for the loss of ergosterol could explain why these mutants are transformation defective.

Transcription mutants

The 11 transcription mutants identified from the screen are shown in Table 28. The mutants, *ctk1, iwr1, not3, pgd1, ref2, rtg3, spt21*, and *srb2* were found to be defective in transcription from the RNA polymerase II promoter, while the mutants *rpa49* and *maf1* defective at the RNA polymerase I and III promoter respectively (Boguta *et al.*

1997; Lee *et al.* 1997). Gene disruption in these mutants would affect the cell's ability to produce the necessary transcripts for normal growth. The remaining mutant, *cha4*, had a defect in a transcription factor required for the utilization of serine/threonine as nitrogen sources (Holmberg *et al.* 1996). The defects in these mutants likely affect their growth and in turn their transformation efficiency.

Translation mutants

The twelve mutants (see Table 29) that make up this group contain genes that produced proteins that formed structural components of either the 60S or 40S ribosomal unit. It is likely these mutants do not directly affect the transformation process; however, their inability to form functional ribosomal units would affect overall growth which was shown to affect transformation efficiency (see Figure 2).

Transport mutants

Twelve mutants shown in Table 30 were found to function in transport/targeting of various biological pathways. Mutants *vps51, vps54,* and *snf8* were found to be defective in the targeting of proteins to vacuoles (Conibear *et al.* 2000; Teo *et al.* 2004) while *sec28* was defective in regulation of Golgi to ER protein traffic (Duden *et al.* 1998). Mutant g*tr1* was found to indirectly affect protein sorting because of its defect in phosphate transport (Bun-Ya *et al.* 1992). Perhaps defects in the above mutants could affect the transformation process through improper sorting and trafficking of plasmid

DNA into vacuoles targeted for the nucleus. Mutants *bap2* and *fat1* both code for proteins found at the plasma membrane, however *bap2* is defective in the uptake of extra cellular amino acids while *fat1* is defective in the uptake of exogenous fatty acids (Grauslund *et al.* 1995; Watkins *et al.* 1998). It is possible these proteins could inadvertently bind plasmid DNA on the surface and transport it into the cell. Mutants *air2, ist3,* and *nup188* were found to be defective in export of mRNA from the nucleus while *ltv1* and *kap120* were defective in proper export of a ribosomal subunit (Inoue *et al.* 2000; Shulga *et al.* 2000; Stage-Zimmermann *et al.* 2000; Gottschalk *et al.* 2001; Seiser *et al.* 2006). These mutants' inability to properly export mRNA and ribosomes could affect the translation of plasmid DNA and thus indirectly affect the transformation efficiency of these mutants.

Vacuolar mutants

From the transformation screen, 10 identified mutants had defects classified as vacuolar. All of these mutants except for *svl7* were either part of the vacuole proton pump or functioned in proton transport activity (see Table 32). The defect in vacuolar acidification rendered these mutants unable to maintain proper pH. The *svl7* mutant is known for defects in vacuolar sorting which results in improper trafficking of endocytic cargo (Shaw *et al.* 2003). Vacuolar acidification is important cellular process including endocytosis and membrane trafficking processes (Forgac 1999). It has previously been shown that DNA:liposome complexes could be internalized via caveolae which proceeded to the late endocytic pathway and was delivered to the nucleus (Wong *et al.* 2007). It is then possible the transformation process involves the endocytosis and

packaging of plasmid DNA from the cell surface into vacuoles. Cells unable to properly maintain and target these vacuoles would be unable to deliver plasmid DNA to the nucleus thus affecting the transformation efficiency.

Miscellaneous mutants

The mutants that did not fit into the other categories were placed in a miscellaneous category and their biological function shown in Table 27. Fifteen mutants were identified with gene deletions that affected biosynthetic processes ranging from amino acid to fatty acid synthesis. It is likely the transformation deficiency seen in these mutants is related to the growth defects resulting from the gene deletion. It is possible that some genes, which at first appear to have no easily identifiable affect on the transformation process, could contribute to the transformation defect through interactions with other proteins. Genes AAT2, PFK1, and SOD1 code for an aspartate amino transferase, phosphofructo kinase, and superoxide dismutase respectively, and have been found to associate with the gene product from ERG6, however the link between proteins is unclear (Ho *et al.* 2002; Hermjakob *et al.* 2004). The *erg6* mutant was previously shown to be transformation deficient (see Section 4.7 – 4.10). Perhaps these associations are required for successful transformation and deletion of these genes affects the function of Erg6p in a negative manner which leads to reduced transformation ability.

The genes identified from the screen could be placed into two categories; those that directly affected the transformation process and those that indirectly affected the

process. If entry of plasmid DNA was through endocytosis into vacuoles, then it is likely that the genes from the cell wall, ergosterol, endocytosis, transport, and vacuolar mutants would directly affect the process. Genes from the DNA repair, transcription, translation, and miscellaneous mutant groups could be seen to affect the transformation process indirectly through their respective defects that would affect overall growth. It is also possible these genes play roles in additional biological functions that affect the transformation process in ways not yet identified.

The distribution of mutants into several biological categories suggests that a successful transformation is the result of a multi-step event requiring the combined effects of many genes. Experimental evidence was found for associations between many of the genes identified. For example, Vma6p was found to interact with Vma2p and Sla1p (genes identified from the screen) and Vma21p (gene not identified from screen) (Uetz *et al.* 2000; Compton *et al.* 2006). The associations continued when Vma21p was found to interact Cup5p (gene identified from screen) and Erg25p (gene not identified from screen) (Miller *et al.* 2005). The layers of associations went further since Erg25p was found to interact with Erg28p, Erg2p, and Sac1p (Miller *et al.* 2005; Schuldiner *et al.* 2005) whose genes were all identified from the screen. It becomes evident that a defect in one gene could create a chain reaction disrupting the association of many gene products possibly involved in the transformation process resulting in reduced efficiency.

5. CONCLUSION

A yeast library containing 4827 deletion mutants generated from parent strain BY4742 was screened to identify genes that affect the transformation process. The library was screened by LiAc/ssDNA/PEG method in 96-well microtitre plates resulting in 177 mutants identified with abnormal transformation efficiencies. Mutants were individually re-screened in liquid culture and removal false positives and false negatives resulted in 115 mutants identified as transformation defective.

Several different transformation methods were performed on three poorly transforming *erg* strains. Different transformation efficiencies were observed from the different methods suggesting they affect the transformation process in unique ways. Although transformation efficiencies varied between methods, the same trend was observed in the order of those efficiencies. This is strong evidence that *erg* mutants are truly defective in transformation ability.

Immunofluorescence was used to observe a moment in the transformation process. Images showed DIG-labeled DNA bound to a small fraction of the total population of cells. The majority of signal detected appeared to be bound to the exterior of the cell and not in conjunction with the DAPI stained nucleus. Perhaps a longer incubation time following transformation is required for plasmid DNA to become internalized.

Further characterization of the genes identified from the library screen is required to elucidate the mechanisms behind transformation in yeast. Comparisons of transformation efficiencies of double mutants with their singular counterparts could help determine the magnitude of a gene defect on transformation. Understanding the genes

involved in the transformation process will hopefully lead to higher efficiency transformation methods in yeast and other mammalian systems.

6. APPENDIX I

6.1 *Saccharomyces cerevisiae* media

6.1.1 Liquid Yeast Peptone Adenine Dextrose Medium (YPAD)

Component	Mass / 600 ml	[Final]
Difco Bacto Yeast Extract	6 g	10 g/L
Difco Bacto Peptone	12 g	20 g/L
Dextrose	10 g	20 g/L
Adenine Hemisulphate	60 mg	100 mg/L

The ingredients were mixed into a 1 L Pyrex bottle and autoclaved for 20 min at 121°C. YPAD liquid medium was allowed to cool and stored at 30°C, the optimal yeast growth temperature.

6.1.2 2X Liquid Yeast Peptone Adenine Dextrose Medium (2X YPAD)

Component	Mass / 600 ml	[Final]
Difco Bacto Yeast Extract	12 g	20 g/L
Difco Bacto Peptone	24 g	40 g/L
Dextrose	20 g	40 g/L
Adenine Hemisulphate	60 mg	100 mg/L

2X YPAD medium used to re-grow yeast cultures to log phase. The ingredients were mixed into a 1 L Pyrex bottle, autoclaved for 20 min at 121°C, and stored at 30°C.

6.1.3 Solid YPAD Plates

Component	Mass / 600 ml		[Final]	
Difco Bacto Yeast Extract	6	g	10	g/L
Difco Bacto Peptone	12	g	20	g/L
Dextrose	10	g	20	g/L
Adenine Hemisulphate	60	mg	100	mg/L
Difco Bacto Agar	10	g	16.67	g/L

Yeast extract, peptone, dextrose, and adenine were mixed in a beaker and volume brought up to 600 ml with sterile nanopure water before being transferred into a 1 L flask containing agar. The medium was autoclaved for 25 min at 121°C and allowed to cool to 56°C before being poured into Petri dishes. Plates were left to dry overnight and stored upside down in the dark at room temperature.

6.1.4 Solid Synthetic Complete (SC) Selective Agar

Component	Mass / 600 ml		[Final]	
Difco Yeast Nitrogen Base w/o Amino Acids	1	g	1.67	g/L
Ammonium Sulphate	3	g	5	g/L
Dextrose	12	g	20	g/L
SC-Amino Acid Mix*	0.5	g	0.83	g/L
Difco Bacto Agar	10	g	16.67	g/L

Dextrose, yeast nitrogen base, and SC- amino acid mix were combined in a beaker and volume brought up to 600 ml with sterile nanopure water. The pH was titrated to 5.6 with 10 M NaOH and media was transferred to a 1 L flask containing agar. The medium was autoclaved for 25 min at 121°C and allowed to cool to 56°C before being poured into Petri dishes. Plates were left to dry overnight and stored upside down in the dark at room temperature.

***SC-Amino Acid Mix**

Stock Synthetic Complete dropout mix was made by adding powdered amino acids acquired from Sigma Chemical Co. Ltd into a plastic screw cap bottle along with 3 marbles (Rose 1987). SC-Leu, SC-Trp, and SC-Ura mix was made by omission of the appropriate amino acid (shown in bold).

Adenine hemisulfate	0.5g	Phenylalanine	2.0g
Arginine	2.0g	Serine	2.0g
Aspartic Acid	2.0g	Threonine	2.0g
Glutamic Acid	2.0g	**Tryptophan**	**2.0g**
Histidine HCL	2.0g	Tyrosine	2.0g
Inositol	2.0g	**Uracil**	**2.0g**
Leucine	**2.0g**	Valine	2.0g
Lysine HCL	2.0g	p-aminobenzoic acid	0.2g
Methionine	2.0g		

6.2 Bacterial Media

6.2.1 Luria-Bertani medium (LB)

Component	Mass / 600 ml	[Final]
Difco Bacto Tryptone	6 g	10 g/L
Difco Bacto Yeast Extract	3 g	5 g/L
Sodium Chloride (NaCl)	3 g	5 g/L
Difco Bacto Agar	10 g	16.67 g/L

The ingredients were mixed into a 1 L flask and autoclaved at 121°C for 20 min then cooled to 56°C before being poured into Petri plates. LB-amp plates were made by mixing 600 µl ampicillin (100mg/ml) into medium that was autoclaved and cooled to 56°C. Plates were stored in the dark at 4°C.

6.2.2 SOC

Ingredients	Mass (g) / 600 ml	Final Concentration g / L
Difco Bacto yeast extract	12 g	25 g
Difco Bacto tryptone	3 g	5 g
NaCl	0.36 g	0.6 g
KCl	0.108 g	0.18 g
Dextrose	12 g	20 g

E. coli cells were supplied with SOC media to support re-growth after electroporation. The ingredients were mixed with ddH_20 and brought to a final volume of 600 ml. The media was sterilized by autoclaving 121°C for 20 min then stored in a fridge at 4°C.

6.3 Buffers

6.3.1 DIG label Blocking 1X solution

Components	Volume
Blocking Solution (Roche cat# 11096176001)	3 ml
Maleic acid buffer	27 ml

A 1X working solution was prepared fresh for every reaction by diluting the 10X blocking solution with maleic acid buffer [0.1 M Maleic acid, 0.15 M NaCl, pH 7.5].

6.3.2 DIG label Antibody solution

Antibody solution was prepared by centrifuging a tube of anti-digoxigenin-AP (Roche cat# 11093274910) for 5 min at 15,000 x *g* then pipetting the necessary amount to a 1:10,000 dilution in DIG label blocking solution.

6.3.3 Tris-EDTA Buffer 10X (TE)

Components	Volume
Tris-HCl (pH 8.0)	50 ml
0.5M Na_2EDTA (pH 8.0)	10 ml
ddH_20	440 ml

The components were mixed in a 500mL glass bottle on a stir plate and pH was adjusted to 8. Working solutions were made by diluting to 1X with sterile ddH_20.

6.3.4 Tris-acetate EDTA Buffer 50X (TAE)

Components	Amount
Tris-base	242.0 g
0.5M Na_2EDTA (pH 8.0)	100.0 ml
Glacial acetic acid	57.1 ml
ddH_20	842.9 ml

The ingredients were mixed in a glass bottle on a stir plate and pH adjusted to 8.3. Working solutions were made by diluting to 1X with sterile ddH_20.

6.3.5 SCE Buffer

Component	Amount / 1000 ml	[Final]
Sorbitol	182 g	1 M
Trisodium citrate 1.0M (pH 5.8)	100 ml	100 mM
Na_2EDTA 0.5M (pH 8.0)	20 ml	10 mM

The ingredients were mixed together with sterile ddH_20 to a final volume of 1000 ml and sterile filtered.

6.3.6 STC Buffer

Component	Amount / 500 ml	[Final]
Sorbitol	91 g	1 M
Tris 1.0M (pH 7.5)	5 ml	10 mM
$CaCl_2$ 1.0M	5 ml	10 mM

The ingredients were mixed together with sterile ddH_20 to a final volume of 500 ml and sterile filtered.

6.3.7 Polyethylene Glycol 8000 20% (w/v)

Component	Amount / 100 ml
PEG8000	20 g
Tris 1.0M (pH 7.5)	1 ml
$CaCl_2$ 1.0M	1 ml

6.3.8 SOS

Component	Amount / 100 ml
YPAD	25 ml
Sorbitol	18.2 g
$CaCl_2$ 1.0M	700 µl
Uracil (1% solution)	270 µl

6.3.9 SORB TOP Agar

Component	Amount / 1000 ml	[Final]
Yeast Nitrogen Base w/o Amino Acids	1.7 g	1.7 g/L
Ammonium Sulphate	5 g	5 g/L
Dextrose	20 g	20 g/L
Sorbitol	182 g	182 g/L
SC-Amino Acid Mix*	0.83 g	0.83 g/L
Bacto agar	25 g	25 g/L

TOP agar was mixed with cell suspension and poured evenly across the top of an agar plate and allowed to solidify. The ingredients were mixed in a flask and sterilized by autoclaving for 20 min at 121°C.

6.3.10 SORB TOP Plates

Sorbitol top plates were used in recovery is transformed spheroplast. A 1 liter of media was prepared the same as SORB TOP agar except with the use of 20 g Bacto agar.

7. APPENDIX II

7.1 Mutants isolated from microtitre plate screen

Table 17. Isolated mutants from microtitre transformation plate screen

Plate #	Position	ORF	Gene Name	Transformation	Sensitivity to LiAc
102	D7	YLR056W	ERG2	down	no
102	G1	YLR098C	CHA4	down	no
102	G11	YLR114C	AVL9	down	no
103	A8	YML063W	RPS1B	down	no
103	D10	YML008C	ERG6	down	no
103	D8	YML009W-B	YML009W-B	down	no
103	F2	YMR014W	BUD22	down	yes
103	G6	YMR032W	HOF1	down	no
104	B10	YMR179W	SPT21	down	no
104	D5	YMR202W	ERG2	down	no
104	D7	YMR205C	PFK2	down	no
104	F2	YMR238W	DFG5	down	no
104	H1	YMR269W	YMR269W	down	no
105	D8	YNL302C	RPS19B	down	no
105	G5	YNL248C	RPA49	down	no
106	E2	YOR078W	BUD21	down	yes
106	H1	YOR312C	RPL20B	down	no
107	A2	YOR332W	VMA4	down	no
107	G8	YOL051W	GAL11	down	no
108	D8	YPL234C	TFP3	down	no
109	C2	YPL129W	TAF14	down	yes
109	C4	YPL125W	KAP120	down	no
109	E4	YPL097W	MSY1	down	yes
110	H7	YDR129C	SAC6	down	no
111	E6	YDR405W	MRP20	down	yes
112	A1	YEL027W	CUP5	down	no
112	B5	YEL046C	GLY1	down	no
112	B9	YEL050C	RML2	down	yes
112	G6	YER044C	ERG28	down	no
113	C4	YGR160W	YGR160W	down	yes
114	C12	YHR081W	LRP1	down	no
114	D10	YHR100C	YHR100C	down	yes
114	F10	YHR134W	WSS1	down	no
115	B7	YCL007C	YCL007C	down	yes
166	C4	YLR200W	YKE2	down	no
166	G7	YKL054C	DEF1	down	yes
117	C7	YKL118W	YKL118W	down	yes
117	C8	YKL119C	VPH2	down	yes
117	E1	YKL143W	LTV1	down	no

117	H1	YGR036C	CAX4	down	no
118	C7	YGR105W	VMA21	down	yes
118	H1	YOR167C	RPS28A	down	no
119	D5	YOR235W	YOR235W	down	no
119	E4	YOR251C	YOR251C	down	no
119	F5	YOR286W	FMP31	down	no
119	H9	YJL176C	SWI3	down	no
121	A3	YDR195W	REF2	down	no
121	C10	YLR027C	AAT2	down	no
121	D7	YEL029C	BUD16	down	no
123	B10	YGR020C	VMA7	down	yes
123	E5	YPL059W	GRX5	down	yes
123	G5	YPL031C	PHO85	down	yes
124	A2	YPL002C	SNF8	down	no
124	C10	YPR139C	VPS66	down	yes
124	C5	YPR131C	NAT3	down	yes
125	C2	YFR056C	YFR056C	down	yes
125	D11	YGR240C	PFK1	down	no
125	E11	YBL007C	SLA1	down	no
126	A3	YBL058W	SHP1	down	yes
126	D12	YGL024W	YGL024W	down	no
126	E1	YGL025C	PGD1	down	no
126	G11	YGL076C	RPL7A	down	no
126	G8	YGL070C	RPB9	down	yes
127	B4	YNL206C	RTT106	down	no
127	F2	YKL212W	SAC1	down	no
127	G12	YKR020W	VPS51	down	no
127	G2	YKR007W	MEH1	down	no
128	D2	YDR264C	AKR1	down	no
128	G11	YDR322W	MRPL35	down	no
128	H4	YDR332W	YDR332W	down	no
129	C11	YIL076W	SEC28	down	no
129	E5	YFL023W	BUD27	down	yes
129	G8	YFR001W	LOC1	down	yes
130	B3	YGR281W	YOR1	down	no
131	A9	YOL086C	ADH1	down	yes
131	E8	YER077C	YER077C	down	yes
131	F10	YHL025W	SNF6	down	no
132	C6	YOR096W	RPS7A	down	yes
132	E11	YML112W	CTK3	down	yes
132	E3	YML103C	NUP188	down	no
132	F7	YML121W	GTR1	down	no
132	H8	YMR116C	ASC1	down	no
133	A3	YMR125W	STO1	down	yes
133	B12	YPR036W	VMA13	down	yes
133	F10	YPR101W	SNT309	down	yes
134	A2	YJL077C	ICS3	down	no
134	F8	YHL011C	PRS3	down	yes
134	G5	YHR026W	PPA1	down	yes

134	G6	YHR041W	SRB2	down	no
134	G8	YHR067W	HTD2	down	no
135	B8	YLR423C	ATG17	down	no
135	C9	YLR441C	RPS1A	down	no
135	D3	YLR447C	VMA6	down	no
135	E2	YML010C-B	YML009C-A	down	no
135	G5	YAL047C	SPC72	down	yes
136	B7	YJR104C	SOD1	down	no
136	C2	YJR118C	ILM1	down	no
136	C6	YJR122W	CAF17	down	no
136	D2	YJR139C	HOM6	down	no
136	F6	YDL203C	ACK1	down	no
136	H2	YDL232W	OST4	down	no
137	A5	YDR005C	MAF1	down	no
137	A6	YDR006C	SOK1	down	no
137	B6	YDR024W	FYV1	down	no
137	B7	YDR025W	RPS11A	down	no
137	B9	YDR027C	VPS54	down	no
137	E12	YCR020W-B	HTL1	down	yes
137	F6	YCR028C	FEN2	down	no
138	F11	YDL115C	IWR1	down	no
138	F12	YDL116W	NUP84	down	no
138	G12	YDL130W	RPP1B	down	no
138	H12	YDL146W	LDB17	down	no
139	B8	YDL175C	AIR2	down	no
139	B9	YDL176W	YDL176W	down	no
139	D5	YDR271C	YDR271C	down	no
139	D6	YDR290W	YDR290W	down	no
139	E6	YGL235W	YGL235W	down	no
139	F11	YIL055C	YIL055C	down	no
139	F6	YIL038C	NOT3	down	no
139	H6	YBL103C	RTG3	down	no
140	A1	YBR006W	UGA2	down	no
140	A4	YBR009C	HHF1	down	no
140	A8	YBR014C	YBR014C	down	no
140	B7	YBR026C	ETR1	down	no
140	C7	YBR041C	FAT1	down	no
140	C8	YBR042C	YBR042C	down	no
140	D10	YBR057C	MUM2	down	no
140	D7	YBR53C	YBR53C	down	no
140	D9	YBR056W	YBR056W	down	no
140	E10	YBR072W	HSP26	down	no
140	E6	YBR067C	TIP1	down	no
140	E7	YBR068C	BAP2	down	no
140	G9	YNL023C	FAP1	down	no
141	C1	YNR023W	SNF12	down	no
141	E8	YNL133C	FYV6	down	no
141	F9	YNL084C	END3	down	no
142	D1	YIR005W	IST3	down	no

142	G5	YNL080C	YNL080C	down	no
142	H11	YNL119W	NCS2	down	no
142	H2	YNL097C	PHO23	down	no
143	A10	YDR202C	RAV2	down	no
143	A11	YDR205W	MSC2	down	no
143	D12	YJL175W	YJL175W	down	no
143	D8	YJL161W	FMP33	down	no
143	E10	YKL139W	CTK1	down	no
143	G3	YKR041W	YKR041W	down	no
144	A10	YCR046C	IMG1	down	no
144	B1	YCR053W	THR4	down	no
144	E11	YMR173W	DDR48	down	no
144	E12	YIL092W	YIL092W	down	no
144	F2	YOR364W	YOR364W	down	no
144	G10	YMR191W	SPG5	down	no
144	H11	YOR269W	PAC1	down	no
144	H12	YOR270C	VPH1	down	no
145	B9	YFR019W	SVL7	down	no
145	H10	YBR119W	MUD1	down	no
146	A1	YBR127W	VMA2	down	no
147	A6	YDL090C	RAM1	up	no
147	H4	YGL108C	YGL108C	down	no
147	H5	YGL109W	YGL109W	down	no
149	A6	YER177W	BMH1	up	no
149	B8	YMR054W	STV1	down	no
149	D7	YDR500C	RPL37B	down	no
149	H1	YDR417C	YDR417C	up	no
149	H12	YFL001W	DEG1	up	no
150	C5	YML073C	RPL6A	down	no
150	D10	YOL148C	SPT20	down	no
150	E10	YPR133W-A	TOM5	down	no
170	A4	YAL027W	YAL027W	up	no
170	C4	YLL027W	ISA1	down	no
170	D11	YOR241W	MET7	down	no
170	G5	YGR102C	YGR102C	down	no
171	A3	YCR094W	CDC50	up	no
171	C8	YPL268W	PLC1	down	no
171	D6	YBL093C	ROX3	down	no
171	D7	YPR072W	NOT5	down	no
171	E12	YER068W	MOT2	down	no

7.2 Transformation efficiencies of re-screened mutants

Table 18. Mutants with compared transformation efficiencies between 0-1%

Plate #	Position	Gene Name	%
110	H7	SAC6	0
112	B5	GLY1	0
121	C10	AAT2	0
125	D11	PFK1	0
136	C6	CAF17	0
143	G3	YKR041W	0
144	B1	THR4	0
103	D10	ERG6	0.04
134	G5	PPA1	0.04
135	D3	VMA6	0.04
133	B12	VMA13	0.08
136	D2	HOM6	0.19
144	F2	YOR364W	0.19
112	A1	CUP5	0.23
108	D8	TFP3	0.28
132	C6	RPS7A	0.4
128	H4	YDR332W	0.43
136	C2	ILM1	0.63
104	D5	ERG2	0.65
170	D11	MET7	0.94

Table 19. Mutants with compared transformation efficiencies between 1-10%

Plate #	Position	Gene Name	%
112	G6	ERG28	1.33
145	H10	MUD1	1.49
136	B7	SOD1	1.72
139	D6	YDR290W	1.77
145	B9	SVL7	1.96
121	A3	REF2	1.98
143	E10	CTK1	2.1
139	H6	RTG3	2.17
121	D7	BUD16	2.38
142	D1	IST3	2.57
132	H8	ASC1	2.65
144	H12	VPH1	2.75
142	G5	YNL080C	3.17
103	G6	HOF1	3.57
117	H1	CAX4	3.79
143	A10	RAV2	4.27
126	G11	RPL7A	4.38
137	B9	VPS54	4.56
138	G12	RPP1B	4.62
129	C11	SEC28	4.72
138	H12	LDB17	5.03
106	H1	RPL20B	5.38
133	A3	STO1	6.47
137	B6	FYV1	6.59
135	C9	RPS1A	7.01
136	H2	OST4	7.01
103	D8	YML009W-B	7.12
135	B8	ATG17	7.41
117	E1	LTV1	7.78
119	E4	YOR251C	7.94
141	E8	FYV6	8.4
105	D8	RPS19B	8.52
116	C4	YKE2	8.63
128	G11	MRPL35	8.63
125	E11	SLA1	8.65
127	G12	VPS51	8.86
134	A2	ICS3	9.17
102	G11	AVL9	9.2
144	A10	IMG1	9.7

Table 20. Mutants with compared transformation efficiencies between 10-25%

Plate #	Position	Gene Name	%
102	D7	ERG2	10.35
137	F6	FEN2	10.46
135	E2	YML009C-A	10.49
129	E5	BUD27	10.81
105	G5	RPA49	10.95
132	E3	NUP188	11.88
128	D2	AKR1	12.22
149	H1	YDR417C	12.75
104	B10	SPT21	12.78
138	F11	IWR1	13.29
132	F7	GTR1	13.43
142	H2	PHO23	14.66
137	B7	RPS11A	15.13
127	B4	RTT106	15.16
131	F10	SNF6	15.75
133	F10	SNT309	15.75
144	G10	SPG5	15.99
139	F6	NOT3	16.91
149	D7	RPL37B	20.00
127	F2	SAC1	20.03
147	H4	YGL108C	20.08
147	H5	YGL109W	21.35
127	G2	MEH1	21.76
134	G8	HTD2	22.21
139	E6	YGL235W	24.07
146	A1	VMA2	24.11
150	D10	SPT20	24.19
119	D5	YOR235W	24.41
140	E6	TIP1	24.49

Table 21. Mutants with compared transformation efficiencies between 25-50%

Plate #	Position	Gene Name	%
137	A5	MAF1	26.26
139	D5	YDR271C	27.50
134	G6	SRB2	29.36
140	E7	BAP2	29.43
140	G9	FAP1	30.74
144	H11	PAC1	30.87
143	D12	YJL175W	31.06
140	C7	FAT1	31.08
103	A8	RPS1B	31.25
143	D8	FMP33	33.35
132	E11	CTK3	35.16
104	H1	YMR269W	35.56
141	F9	END3	36.63
139	F11	YIL055C	41.05
119	H9	SWI3	45.58
147	A6	RAM1	45.60
144	E12	YIL092W	48.88

Table 22. Mutants with compared transformation efficiencies between 50-100+%

Plate #	Position	Gene Name	%
109	C4	KAP120	50.16
126	E1	PGD1	51.25
126	D12	YGL024W	54.14
149	H12	DEG1	55.58
140	B7	ETR1	59.08
114	F10	WSS1	68.83
137	A6	SOK1	75.17
102	G1	CHA4	77.00
143	A11	MSC2	78.14
144	E11	DDR48	80.97
119	F5	FMP31	89.71
140	A1	UGA2	89.82
149	A6	BMH1	97.76
139	B9	YDL176W	101.56
142	H11	NCS2	102.09
118	C7	RPS28A	112.30
139	B8	AIR2	140.91
124	A2	SNF8	144.62
140	D10	MUM2	205.19
114	C12	LRP1	217.53
140	D9	YBR056W	255.41
140	D7	YBR53C	324.76
141	C1	SNF12	588.73

7.3 Mutants categorized by biological function

Table 23. Cell wall / Cytoskeleton mutants

Cell wall / Cytoskeleton	
Gene	**Function**
BUD16	bud site selection
PAC1	nuclear migration, microtubule-mediated
HOF1	Cytokinesis
TIP1	cell wall organization and biogenesis

Table 24. DNA repair / maintenance mutants

DNA repair / maintenance	
Gene	**Function**
LRP1	DNA repair
WSS1	response to DNA damage stimulus
DDR48	DNA repair
MUM2	premeiotic DNA synthesis
ILM1	mitochondrial genome maintenance
SNF6	chromatin remodeling
SNF12	chromatin remodeling
SWI3	chromatin remodeling
RTT106	chromatin silencing at telomere
PHO23	chromatin modification
FYV6	double-strand break repair via nonhomologous end joining

Table 25. Endocytosis / Exocytosis mutants

Endocytosis / Exocytosis	
Gene	**Function**
AKR1	endocytosis
SLA1	endocytosis
FEN2	endocytosis
END3	endocytosis
THR4	endocytosis
SAC6	endocytosis, bipolar bud site selection
CUP5	endocytosis, vacuolar acidification, vacuole organization
SAC1	regulation of exocytosis

Table 26. Ergosterol mutants

Ergosterol	
Gene	**Function**
ERG2	ergosterol biosynthesis, endocytosis
ERG3	ergosterol biosynthesis
ERG6	ergosterol biosynthetic process
ERG28	ergosterol biosynthetic process

Table 27. Miscellaneous mutants

Miscellaneous	
Gene	**Function**
AAT2	aspartate biosynthetic process
CAX4	lipid biosynthetic process
ETR1	aerobic respiration
GLY1	glycine biosynthetic process
HOM6	homoserine biosynthetic process
HTD2	fatty acid biosynthetic process
MSC2	zinc ion homeostasis
NCS2	invasive growth
OST4	protein amino acid N-linked glycosylation
PFK1	Glycolysis
SOD1	age-dependent response to reactive oxygen species
SOK1	cAMP-mediated signaling
UGA2	glutamate catabolic process
YNL080C	protein amino acid N-linked glycosylation
YKE2	protein folding

Table 28. Transcription mutants

Transcription	
Gene	**Function**
CHA4	transcription factor activity
CTK1	regulation of transcription from RNA polymerase II promoter
IWR1	transcription from RNA polymerase II promoter
MAF1	regulation of transcription from RNA polymerase III promoter
NOT3	regulation of transcription from RNA polymerase II promoter
PGD1	regulation of transcription from RNA polymerase II promoter
REF2	transcription termination from Pol II promoter
RPA49	transcription from RNA polymerase I promoter
RTG3	transcription factor activity
SPT21	regulation of transcription from RNA polymerase II promoter
SRB2	regulation of transcription from RNA polymerase II promoter

Table 29. Translation mutants

Translation	
Gene	**Function**
ASC1	Translation
IMG1	Translation
MRPL35	Translation
RPS1A	Translation
RPL20B	ribosome biogenesis and assembly
RPL37B	Translation
RPL7A	Translation
RPP1B	Translation
RPS11A	Translation
RPS19B	Translation
RPS1B	Translation
RPS28A	Translation

Table 30. Transport mutants

Transport	
Gene	**Function**
AIR2	mRNA export from nucleus
BAP2	amino acid transport
FAT1	lipid transport
GTR1	phosphate transport
IST3	mRNA export from nucleus
KAP120	protein import into nucleus
LTV1	ribosomal small subunit export from nucleus
NUP188	protein export from nucleus
SEC28	ER to Golgi vesicle-mediated transport
SNF8	protein targeting to vacuole
VPS51	protein targeting to vacuole
VPS54	Golgi to vacuole transport

Table 31. Uncharacterized mutants

Uncharacterized			
Gene	**Function**	**Gene**	**Function**
AVL9	Unknown	YGL108C	Unknown
CAF17	Unknown	YGL109W	Unknown
FAP1	Unknown	YGL235W	Unknown
FMP31	Unknown	YIL055C	Unknown
FMP33	Unknown	YIL092W	Unknown
FYV1	Unknown	YJL175W	Unknown
ICS3	Unknown	YKR0411	Unknown
LBD17	Unknown	YML009C-A	Unknown
SPG5	Unknown	YML010W-A	Unknown
YBR056W	Unknown	YMR269W	Unknown
YDL176W	Unknown	YOR235W	Unknown
YDR271C	Unknown	YOR251C	Unknown
YDR290W	Unknown	YOR364W	Unknown
YDR332W	Unknown		
YGL024W	Unknown		

Table 32. Vacuolar mutants

Vacuolar	
Gene	**Function**
ATG17	autophagy
MEH1	vacuolar acidification
STV1	vacuolar acidification
VMA2	vacuolar acidification
VPH1	vacuolar acidification
RAV2	vacuolar acidification
VMA4	vacuolar acidification
TFP3	vacuolar acidification
VMA6	vacuolar acidification
SVL7	vacuole organization and biogenesis

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