Zinc transporter SLC30A2 genetic variations and health implications

By

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department Human Nutritional Sciences

University of Manitoba

Winnipeg

1 Abstract

The SLC30A2 zinc transporter has been investigated due to its important role in zinc secretion into human milk. SLC30A2 is expressed in mammary epithelial cells, and the presence of genetic variations in this transporter could cause low zinc transport into the milk, leading to Transient Neonatal Zinc Deficiency (TNZD) in newborns. Through bioinformatics analysis 22 SNPs were identified. Therefore, we aim to identify the functional changes caused by these 22 SNPs. By subcloning the SLC30A2 open reading frames into the Gateway expression plasmid tagged with red and green fluorescent proteins (mCherry, tGFP). Four SNPs were introduced by mutagenesis and tagged with mCherry. We transfected individual plasmids into mammary epithelial cells (HC11) and observed cellular targeting using epifluorescent imaging. The most common variants located to secreting endosomes and membrane in HC11 cells. Incorrect targeting of SLC30A2 causes mislocalization. Based on our results, it may be possible to identify mothers carrying risk genotypes for infant zinc deficiency.

2 Acknowledgment

I would like to express my sincere gratitude to my advisor Dr. James K. Friel for the continuous support through this project. Thank you Dr. Friel for your knowledge, guidance and unconditional support, I am very thankful to have the opportunity to work with you.

To my laboratory group Chenxi Cai, Wafaa Qaasem, Sarah Jorgensen and Zakir Hossain, thank you all for your support.

I would like to thank my Co-supervisor Dr. Peter Eck, for his invaluable knowledge and guidance through the research project. To my committee member Dr. William L. Diehl-Jones who supported me through the project.

I would like to thank Graduate Studies and the Human Nutritional Science department for the financial support.

To my strength, my beloved parents Ramon and Rebeca thank you for your unconditional love and support in every step I decide to take. To my dear big brother, Hugo, whom I deeply love, he is always behind me in every step I take and fully support me in all my decisions, thank you for letting me grow by your side. To my dearest cousins Humberto and Carmina who have been there always, for making our friendship grow throughout the years, always together no matter what.

To my best friends back home in Mexico: Rosa Elia, Arlyn, Nora, Patricia, Daniel, Mercedes, Claudia, Yendi, Alberto, Sebastian, Ana Cristina and Karimme. You have taught me that no matter the distance we will be always be together.

To my dearest friend Alejandra Serrano Leon, you made me cherish even more every moment of our “Masters journey”, without you, this experience would not have been the

same. I cannot thank you enough for all your support and unconditional friendship. To Mrs. Elvia Cloutier, thank you for receiving me in your home and make me feel like part of your family.

To my dear friends, Yanan, Chenxi, Sarah, Jyoti, Miriam, Wafaa, Heather, Mohammad and David, thank you for your lovely friendship.

This is one of the most valuable experiences that I have had, I could not have done it without all the people that surround me, I am deeply grateful for all the blessings in my life.

3 Dedication

Without my family I would not be able to follow my dreams.

This thesis is dedicated to my parents Ramon and Rebeca and my brother Hugo for their belief and support through this journey.

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6 Literature review

6.1 Zinc

Zinc is one of the most important intracellular trace elements (Tapiero & Tew, 2003), and is involved in numerous cell processes. It is found in the cytosol, in cellular organelles, and the nucleus. It is also required for the correct functioning of more than 300 enzymes (McCall, Huang, & Fierke, 2000). This micronutrient can be found in animal and plant dietary sources.

6.2 Zinc metabolism

6.2.1 Zinc requirements

In Canada, average zinc consumption is estimated to be between 15.2 and 19.9 mg per day, and food contributes over 99% of the intake (Government of Canada, 1997).

The recommended dietary allowance (RDA) for adult males is 11 mg per day, and adolescent women between 13 and 18 years old require between 8 mg and 9 mg per day. It is also known that in several physiological stages, requirements may increase; for example the demand for zinc increases by 18.5 % in women between the ages of 14 and 18 years old. In women of more than 19 years of age the requirements increase 17.2% (http://ods.od.nih.gov/factsheets/Zinc-HealthProfessional/#h6).

Another important physiologic stage is lactation. During lactation women between 14 and 18 years have to increase the consumption of this micronutrient to 13 mg per day, women older than 19 years of age need to consume 12 mg per day.

Adequate consumption of zinc during lactation is of great importance, due to the requirements of the newborn for healthy growth and development.

The requirements for children increase by age. For male children up to 12 years of age the requirements are between 3 to 7 mg/day. As for females children the requirements are 9 mg/day (Government of Canada, 1997; Roohani, Hurrell, Kelishadi, & Schulin, 2013)

6.2.2 Zinc absorption and excretion

Zinc homeostasis is maintained by several physiological mechanisms. Primarily it is regulated via the gastrointestinal system through the process of absorption of dietary zinc, secretion and excretion of endogenous zinc (Nancy F. Krebs, 2000).

Zinc absorption occurs mostly in the small intestine. It is secreted in the intestinal and pancreatic juices, which enter the lumen of the intestine (Smolin, Grosvenor, & Gurfinkel, 2012). The equilibrium of this micronutrient in the human body is maintained through consumption (Government of Canada, 1997; Nancy F. Krebs, 2000). If zinc levels in the body are low, endogenous dietary zinc will be reabsorbed, but if the level is too high, less zinc will be reabsorbed and more will be eliminated in the feces. This is the reason why zinc-deprived adults show increased efficiency, whereas adults with elevated consumption show reduced efficiency in absorption. Due to this mechanism it is difficult to determine the amount of zinc absorbed in the human body. In general 33% of zinc is absorbed. This process is due to a carrier-mediated mechanism(Government of Canada, 1997; Hempe & Cousins, 1991).

It is well-known that zinc from animal sources is better absorbed than the plant sources.

There are several micronutrients that interact with absorption of zinc (Moran et al., 2012). Foods, like cereals, contain phytates which bind to zinc and impair absorption of this micronutrient in composite meals (Smolin et al., 2012). On the other hand, protein contained in a meal facilitates zinc absorption. The high availability of zinc in animal

sources is due to the presence of glycine, histidine, lysine, methionine and cysteine which also facilitate zinc absorption (Lönnerdal, 2000).

Zinc contained in animal sources such as red meat, eggs, dairy products and liver is more bioavailable than zinc from plant sources (Lönnerdal, 2000). According to the Canada Food Guide the best source of zinc is beef and crab (Smolin et al., 2012).

For exclusively breastfed infants the only source of this micronutrient is human milk.

Infants are born with reserves of zinc, iron and copper in the liver which are sufficient during breast feeding, however zinc concentration in milk is higher in order to the infant zinc requirements (Dórea, 2002).

6.2.3 Zinc nutrient interaction

Zinc can re-bind with organic molecules once it enters into the small intestine, which can affect its ability to be absorbed. Zinc is transported as an organic ion that is dissociated from organic molecules (Smolin et al., 2012).

Some organic molecules such as phytate bind zinc strongly, and thus can deplete or block zinc absorption by blocking the function of zinc transporters. Phytate, the salt of phytic acid, is responsible for the storage of phosphorus in plant tissues. Phytate binding can cause zinc deficiency and in some cases iron deficiency (Thompson & Manore, 2011).

Vegetarians have high risk of developing zinc deficiency due to the high content of phytic acid in seeds, bran and nuts. Even though the content of phosphorus in these foods is not bioavailable for humans, it can inhibit the absorption of several micronutrients (zinc, calcium, magnesium, iron), due to chelation. Phytic acid is also known to bond with niacin causing pellagra (H. W. Lopez et al., 2001) (http://www.westonaprice.org/food-features/living-with-phytic-acid)

Zinc interacts with other micronutrients; it has been shown that supplementing about 5 mg/day of zinc will decrease the absorption of copper. Therefore high zinc concentration in the intestine decreases the concentration of copper in the mucosal cell cytosol affecting the portal effluent. The high concentrations of zinc or copper inhibit the absorption.

Calcium is one of the micronutrients that have shown to impair the absorption of zinc in animals but no linkage has been found between calcium consumption and zinc absorption in humans. It appears that calcium itself has no negative effect on zinc absorption. As an example, 750 mg/L of calcium in a soy formula, which was 57.6% more than the established content that, was added to a milk formula. Zinc absorption was increased due to the larger portion of calcium bonded with phytate making zinc more bioavailable (Nancy F. Krebs, 2000; Lönnerdal, 2000).

However, in a study performed in postmenopausal women supplemented with calcium, showed depleted zinc absorption (Wood & Zheng, 1997). Therefore, it appears that when the ratio between these micronutrients is high a negative interaction could occur (Sandström, 2001).

There is not only a tight relationship between calcium, phytate and zinc.

Iron works in synergy with these micronutrients. With high concentration of iron, zinc absorption can be depleted if it is given in water solution. Whereas in solid food, specifically with protein containing histidine, zinc becomes more bioavailable, and the absorption is increased. It was shown that the supplementation of iron drops (30 mg/d) to infants for six months did not affect zinc absorption, according to plasma zinc concentrations (Sandström, 2001).

6.3 Milk zinc concentration

The primary function of breastfeeding is the transmission of nutrients from the mother to the infant for proper early development. Nutritional status during the first months of life in exclusively breast feed infants is dependent on the nutrient composition of human milk. Therefore, lactation is an important factor in maternal zinc homeostasis, particularly in populations at risk for low dietary zinc intake (Dorea, 2000; N F Krebs, 1999). The amount of zinc transferred daily through the human milk (HM) is around 1-3 mg. Maternal zinc requirements during pregnancy are lower than those during the lactation period.

Zinc concentration in human milk decreases 50% in the first 7 days of lactation. The average concentration of zinc in human milk after birth is high, approximately 30- 45 μmol (2 to 3mg/day), and after early weeks of postpartum normally decline after 2-3 month postpartum to approximately 15 μmol (1 mg/day) (N F Krebs, 1998).

It decreases continuously by the sixth month until the concentration of zinc falls 20%. On the other hand, milk volume increases from 553 mL to 1673 mL, while zinc concentration decreases around 60% , zinc does not increase within milk volume (Dorea, 2000). Even though zinc concentration is decreased, the infant receive the adequate amount, in addition to that zinc absorption in the infant gut is increased significantly (N F Krebs, 1998)

Nutrients concentration in human milk depend on physiological factors such as maternal nutritional status, nutrient interaction (other micronutrients and macronutrients), dietary intake and general health conditions of the mother (Food and Nutrition Board, 2013).

6.4 Zinc deficiency in infants

Adequate zinc intake is of great importance for infants for neurological development, immune function and growth. Adequate zinc intake primarily depends on breast milk or milk formula; therefore, premature infants are more vulnerable to zinc deficiency due to insufficient body storage and low absorption capacity (Kuramoto, Igarashi, & Tagami, 1991). Premature infants will utilize hepatic zinc storage (36.7 mg) faster than full term infants. The hepatic zinc is retained in the last three months of gestation. The quantity of zinc that the infant could receive during the first three months of lactation is around 108 mg (Dorea, 2000; Lasry et al., 2012). Adequate intake (AI) of zinc for infants is 2 mg/day female and males. This could be met by the quantity of zinc that is transferred by the mammary glands (3 mg/dl) (Qian, Wang, Tang, Zhang, & Cai, 2012).

Even though, zinc concentration in breast milk during the first three months of lactation is high, sometimes absorption is not ideal. This could be caused by different factors: inadequate intake, a decrease in absorption or the increase of zinc demand caused by special health conditions (malaria, pneumonia or diarrhea the demand of zinc is increased) or genetics (Dauncey, Shaw, & Urman, 1977). Zinc deficiency has been described in breast fed infants since 1979. Severe zinc deficiency is considered rare especially if those infants that are born from healthy women but may occur in cases of prematurity, malnutrition, total parenteral nutrition and burns where the demand of zinc is high (Dorea, 2002). In cases where zinc deficiency was reported in healthy term infants, the deficiency has been attributed to a decrease in zinc secretion in maternal breast milk (Dorea, 2000; Murthy *et al.*, 2010).

Many studies have been done on infants who present with low zinc concentration in plasma (Friel et al., 1993; Gulani, Bhatnagar, & Sachdev, 2011). Some of them have been supplemented with zinc and their condition has been improved. Commonly the first diagnosis of low zinc plasma concentration is *Acrodermatitis Enteropathica* (AE). This condition is treated by lifelong supplementation of zinc. It has been found that after zinc supplementation is withdrawn in some infants, AE does not reappear (Glover & Atherton, 1988). This leads to speculation that low plasma zinc concentration could be associated with another genetic disorder.

Transient neonatal zinc deficiency (TNZD) is another acute health condition which can be developed in infants between 0 and 6 months of age. This condition is diagnosed when zinc levels in the blood are less than 60 μg/ 100 mL. The normal concentration range for an infant is 100-160 μg/ 100 mL (El Fékih et al., 2011). This condition has been linked with genetic variations in the breast feeding mother that lead to low zinc milk concentration.

6.5 Low zinc plasma concentration and genetics

Zinc has gained special interest recently, especially in the field of genetics. It has been reported that zinc deficiency places infants at risk of growth impairment, neural development and immune function (Aggett & Harries, 1979), (Shah & Sachdev, 2001). According to the World Health Organization (WHO) and the United Nations Children's Fund (UNICEF), it was estimated that about one third of the world's population is living in regions where there is a high risk of zinc deficiency but the exact prevalence of pregnant and lactating women with zinc deficiency is unknown (de Benoist *et al.*, 2007). Zinc has three general functions: catalytic, structural and regulatory. Each of these

functions can be linked with gene expression. The catalytic functions are related to RNA synthesis; zinc ions are necessary for the catalytic reaction of RNA polymerase, the enzyme that produces RNA. The structural role of zinc is demonstrated in zinc-finger motif proteins, which are zinc-bonding domains responsible for binding DNA sequences in transcription factors (Cousins, 1998). There are several zinc-fingers which are characterized by the nature and gap of their amino acids residues, involving four cysteine and/or histidine residues in several arrangements (Dreosti, 2001). The regulatory function of zinc is directly related to gene expression. Zinc regulates the transcription of a gene in synaptic signalling and the metallothionein gene. This is recognized by the interaction with a Zn–binding transcription factor that recognizes the MRE (metal response elements) in the promoter region of the metallothionein gene (Cousins, 1998; Vallee, 1983).

In infants low plasma zinc concentration is diagnosed as either Transient Neonatal Zinc Deficiency (TNZD) or, as mentioned before, *Acrodermatitis Enteropathica* (AE).

There have been genetic studies done both in mothers that present with low zinc milk concentrations and infants who present with symptomatic zinc deficiency. Three genes have been related to low zinc milk concentration. SLC30A4/ZnT4 is expressed in several tissues such as testis, uterus, kidneys, ovaries and mammary glands. The presence of a mutation in this gene in mice is lethal for the pups. It causes low zinc levels in breast milk which can be corrected by maternal zinc supplementation. This mutation has not been found in humans (Murgia, Vespignani, Rami, & Perozzi, 2006)

Zinc transporter,SLC30A2/ZnT2, is expressed in several tissues: kidney, prostate, placenta and mammary epithelial cells. Recently, this transporter has been linked with

zinc deficiency in healthy newborns. The presence of genetic variations in humans lead to transient neonatal zinc deficiency which is not correctable by maternal zinc supplementation (Chowanadisai, Lönnerdal, & Kelleher, 2006; Lasry et al., 2012). Therefore the function and importance of this transporter must be elucidated in order to obtain knowledge to help prevent zinc deficiency in the newborn.

6.6 Zn transporters in mammary epithelial cells

In mammalian cells, zinc is found in three sites: bonded with proteins, methallotheins, and in cytoplasmic vesicles and organelles (Falcón-Pérez & Dell'Angelica, 2007).

Zinc is transported through the plasma membrane and organelle membranes by two integral membrane proteins; Zrt (ZIP), known as solute-linked carrier 39A (SLC39A), and SLC30 (ZnT) family/cation diffusion facilitator (Falcón-Pérez & Dell'Angelica, 2007). In human cells there are ten ZnT and fifteen ZIP transporters have unique tissue expression (Liuzzi & Cousins, 2004).

These transporters show opposite roles in cellular zinc transport. The family ZnT (SLC30) acts reducing the intracellular zinc levels carrying zinc from the cytoplasm to organelles (McMahon & Cousins, 1998). These transporters have been found in intracellular compartments, endosomes, Golgi or endoplasmic reticulum. There are more than 100 members of this family but only ten (SLC30) have been recognized in mammals (ZnT1, 2, 3 ,4…10) (Palmiter & Huang, 2004). ZnT family is subdivided into three subfamilies, all the members of this family are have six transmembrane domains (TMD) in the N- and C- termini on the cytoplasmatic side of the membrane (Palmiter & Huang, 2004).

ZIP (SLC39) transporters promote zinc influx into the cell or vesicular zinc release into cytoplasm. It transport zinc from the organelles or extracellular space to the cytoplasm. ZIPs have eight transmembrane domains (TMD) (Falcón-Pérez & Dell'Angelica, 2007; Kambe, 2011). There are fourteen members of this family found in the human genome. This family has the opposite function of the SLC30 transporters, meaning that its main role is to increase the zinc content of the cells (Kambe, 2011). The expression of the zinc transporters are tissue specific and have subcellular specific localization especially in secretory tissues.

Zinc is transported from the maternal blood to the Mammary Epithelial Cells (MECs) for secretion into the alveolar lumen (McCormick, Velasquez, Finney, Vogt, & Kelleher, 2010). This process involves several zinc transporters.

Four zinc transporters have been identified previously in mammary epithelial cells (ZnT1, ZnT2, ZnT4, and Zip3). These proteins regulate zinc homeostasis in the MEC's and play a major role in zinc concentration in human milk (Kelleher, Seo, & Lopez, 2009).

Recently other zinc transporters have been identified in MEC's reveals the presence of more than four zinc transporters. Additionally six other zinc transporters (ZnT5,Zip1,Zip8,Zip10,Zip11 and Zip13) were identified in the secreting phenotype MEC's (Kelleher et al., 2012). The expression of these transporters is controlled by hormonal stimuli during lactation which affect their subcellular localization.

Zip transporters are localized in MECs, and they were identified as being localized in the membrane to transport zinc into the cell which is then further mobilized into endoplasmic reticulum (ER) and Golgi apparatus or into secretory vesicles (Kambe, 2011). The Golgi

apparatus is the sub-cellular compartment for zinc storage in MEC's during lactation. ZIP11 and ZIP7 may be involved in zinc efflux to the milk from the Golgi apparatus (Kelleher et al., 2012). Zip5, Zip8, Zip10 and Zip3 are localized in the basal membrane of the MEC's, suggesting that these may play an important role in zinc uptake (Kelleher et al., 2012). Zip3 is implicated in zinc uptake to supply zinc into milk (Kelleher & Lönnerdal, 2005). The presence of Zip8 and Zip 10 expression suggest that these could have regulatory elements during lactation (Kelleher et al., 2012).

ZnT transporters are known to be mainly localized on endosomes/lysosomes on MECs secretory vesicles allowing accumulation of zinc in these vesicles which traffic through the membrane to secrete zinc into the lumen by exocytosis (Kambe, 2011). ZnT5 may play a major role in zinc accumulation in Golgi apparatus by incorporating it into specific proteins that increase during the lactation period, which are part of the secretory mechanism of the mammary cell (Kelleher et al., 2012). Zinc deficiency has been linked with a mutation in ZnT2. This transporter was previously identified in secretory vesicles in mammary epithelial cells. It is up-regulated by the lactogenic hormone, prolactin. The ZnT4 has been identified in the Golgi apparatus and also is associated with the apical membrane, therefore ZnT4 could export zinc into milk from the Golgi apparatus to the cell membrane (Michalczyk, Varigos, Catto-Smith, Blomeley, & Ackland, 2003). The presence of genetic variations in the ZnT4 is associated with low zinc concentration in milk in mice, causing death of the pups (Qian, Lopez, Seo, & Kelleher, 2009).

Zinc secretion into milk is complex, influenced by several factors and conditions. The presence of lactogenic hormones is necessary for zinc transport into the milk (Kelleher et al., 2012). Also it has been shown that BMI can influence zinc milk concentration in

humans, according to zinc concentration measurements on 750 Chinese breast feeding women on the 42nd postpartum day (Qian et al., 2012). This was associated with three genetic variations in the MEC's. In 2006, two genetic variations were identified in the SLC30A2/ZnT2 that affect zinc concentration in human milk (HM) leading to Transient Neonatal Zinc Deficiency (TNZD) (Chowanadisai et al., 2006).

6.7 The SLC30A2 gene

The SLC30A2 gene is also known as ZnT2 gene and has an official full name which is the solute carrier family 30 (zinc transporter) member 2. The highest tissue expression is found in placenta followed by eye, kidney, ovaries, and breast and lowest in head, neck, lung, and uterus (Seve *et al.*, 2004).

The tissue expression of this gene is unique in reproductive and secretory tissues such as placenta, ovary, prostate gland, and mammary gland. The protein ZnT2 is encoded by the SLC30A2 gene. The gene protein products may function as active transmembrane zinc transporters within or out of the cell, or as sequestrant of zinc (Lopez & Kelleher, 2009).

ZnT2 transporter is in some specialized cells, like β cells, lateral prostate, pigment epithelial cells in the retina, mammary cells among other. These are known to have vesicles/granules (Kambe, 2011). This transporter is widely expressed in pancreas, due to its significant role in endogenous zinc loss by regulating the excretion into the intestinal track influencing dietary zinc requirement.

In baby hamster kidney cells (BHK) the SLC30A2 was localized in late endosomes, not being the normal intracellular location due to its dependence on chaperones. Zinc is stored by the ZnT2 in intracellular organelles like late endosomes, releasing zinc slowly,

after zinc accumulation through these vesicles throughout the cell membrane into the cytoplasm (Palmiter & Huang, 2004).

In rat acinar AR42J cells, ZnT2 was localized in zymogen granules. The expression of ZnT2 was stimulated and increased by oral zinc and by inducing into a secretory phenotype (Qian et al., 2009) This protein has been reported to be expressed in prostate cells, where zinc transport its mediated by a binding protein metallotheionein which acts as a chaperone for zinc uptake (Costello, Liu, Zou, & Franklin, 1999).

Also it has been detected in non-lactating and non-secreting mammary epithelial cells (MEC's). Therefore ZnT2 plays a major role in zinc metabolism in the non-secreting and secreting phenotype of MEC's (Seo, Lopez, & Kelleher, 2011).

Also it has been shown that mammary cells zinc production, storage and secretion is in response to prolactin in mammary cells. Moreover the presence of prolactin increases the expression of ZnT2 in mammary cells (Qian et al., 2009).

This gene has two splice variants; the two variants are protein coding (V. Lopez & Kelleher, 2009).

According to the NCBI Gene bank database (November 2013), there are 2 isoforms for SLC30A2: isoform1 (long isoform) results from transcript variant 1 which encodes the longer isoform, isoform 2 (short isoform) results from transcript variant 2 as a result of the splicing from exon number 3a from isoform 1(Liuzzi & Cousins, 2004; NCBI, 2012).

Both isoforms have a long 3' untranslated region, isoform I of 1,118 and isoform II 972 bp.

These two isoforms are localized in different organelles in mammary epithelial cells. Each has a different role (V. Lopez & Kelleher, 2009).

Isoform I was detected in vesicular compartments, endosomes and exocytotic vesicles. This isoform facilitates zinc accumulation in the vesicular compartments allowing zinc secretion from the cell through intracellular vesicular movement trafficking to the plasma membrane. Stimulation with lactogenic hormones (prolactin) increases it expression and zinc secretion as well (V. Lopez & Kelleher, 2009)

The second isoform was detected in the cell membrane. The expression of this isoform is not affected by hormonal stimuli. The main function of the short isoform consists in zinc export via the cell directly out of the membrane (V. Lopez & Kelleher, 2009). The over expression of isoform I decreases expression of isoform II, leading to the hypothesis that the shorter isoform does not play a major role in zinc secretion into the milk.

Furthermore, the over expression of the long isoform leads to a greater accumulation of zinc, therefore the sequestration of zinc in secretory vesicles is increased protecting the cells against too high zinc concentration. In mammary cells this mechanism allows for the accumulation and release of great quantities of zinc into the milk (V. Lopez & Kelleher, 2009).

6.7.1 SLC30A2 single nucleotide polymorphisms (SNPs)

There are seven genetic variations reported in humans which have been linked with incorrect function of SLC30A2. The missense polymorphisms could result in amino acid changes and subsequently may alter protein function. Seven have been identified in lactating women who present with either low milk zinc concentration or low plasma concentration. The first SNP is rs35235055, leucine to proline (Leu23Pro); rs35623192 is the second arginine to cysteine (Arg340Cys). In one study, ZnT2 gene with the SNPs (rs35235055) and (rs35623192) were ectopically expressed and further examined for

their localization and function in the MECs. Analysis revealed mislocalization of the protein product carrying the SNP (rs35235055) genotype (Leu23Pro) to lysosomes and the protein product carrying the SNP (rs35623192) genotype (Arg340Cys) to Golgi apparatus which subsequently resulted in zinc accumulation and increased oxidative stress, respectively (Seo & Kelleher, 2010).

In 2006, TNZD was reported in two exclusively breastfed infants. The infants were genotyped to verify if the origin of the condition was due to a mutation in the SLC39A4 which causes Acrodematitis Enteropathica. The result was negative. Later on the mothers were genotyped, showing two missense mutations in the *SLC30A2* gene. This was causing decreased zinc secretion into the human milk therefore the infants developed TNZD (Chowanadisai et al., 2006).

A Chinese study on lactating women who presented with low milk concentration was conducted. This *SLC30A2* gene of these women was sequenced, the authors found five different SNPs localized in different regions of the long isoform of the SLC30A2. The milk zinc content was correlated with non-genetic factors such as BMI, birth, infant gender, age, birth weight, vitamin D supplementation and caesarean delivery preterm delivery. Other non-genetic factors were not significantly related to the zinc concentration in milk.

Rs120245468 was identified to occur in the promoter region. This SNP did not show any association between low zinc milk concentrations and the genetic variation. Rs117153535 and 1031A>G was associated with low milk zinc in breastfeeding women (Qian et al., 2012)

To date a total of 5 missense mutations in humans in the isoform one of the *SLC30A2* have been described (Figure 1). Only two have been tested *in vitro* (rs35235055 and rs35623192).

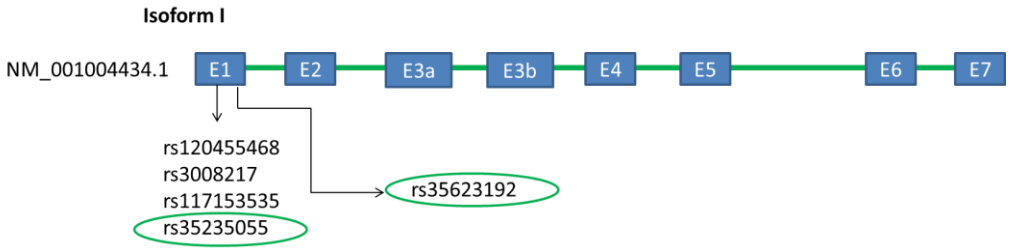


Figure 1 Six genetic variations present in humans

These two genetic variations were tested *in vitro* and they show miss localization of the SLC30A2/ZnT2 which reduces up to 75% milk zinc content in HM. Therefore it is important to know what changes occur if this missense mutations are present in the SLC30A2. Genetic variations could be a potential marker to diagnose TNZD before birth.

6.8 HC11 Mammary epithelial cells from mice

The HC11 cell line is a clonal derived from COMMA-1D mouse mammary epithelial cell line (Chammas, Taverna, Cella, Santos, & Hynes, 1994). This cell line was isolated from a midpregnant BALB/c mice mammary gland.

The HC11 cell line requires growth factors, such as Epidermal Growth Factor (EGF) and Insulin. It can be differentiated by prolactin exposure (Chammas et al., 1994).

HC11 show normal specific properties for a normal mammary gland function and has been used to study hormonal regulation of MEC's (Humphreys & Rosen, 1997).

HC11 cells have been used to examine uptake and secretion of distinct compounds in milk (Danielsson, 2007). Recently, interest in zinc transport has increased; therefore this

cell line has been used to express zinc transporters and to explore their interactions with other mediators.

7 Methods

7.1 Bioinformatics search of gene structure SLC30A2

Information about the SLC30A2 gene is contained in the National Center for Biotechnology Informations (NCBI) databases including PubMed, UniGene and dbSNP. Under NCBI we narrowed the search under "Gene", using the key words SLC30A2 and ZnT2.

7.2 Single Nucleotide Polymorphisms of SLC30A2

Genetic variations of the SLC30A2 gene were searched in the NCBI databases in dbSNP. Under Gene View we searched for missense Single Nucleotide Polymorphisms (SNPs). The human genome sequence genetic variations of SLC30A2 were downloaded from Gene View for 22 missense SNPs.

7.3 Tissue Expression

Tissue expression was searched in the UniGene data base Hs.143545 from the EST (Expressed sequence tags) profile (http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.143545).

7.4 Primer design

Sequencer 5.0 software was used for primer design of both splice variants and 22 missense genetic variations. Sequences were downloaded from RefSeq of NCBI and SNP (refSNP) cluster, respectively, into sequencer 5.0 (Table1 and 2).

7.5 Clinical findings

More genomic related information and clinical linkages were obtained by searching in distinct genomic databases, such as NCBI, Pubmed, GeneCards®, Ensembl and Catalogue of Genome-Wide Association Studies (GWAS). Key words used were: SLC30A2, ZnT2, zinc deficiency, breast milk, zinc, and zinc transporting system, mammary gland, and ZnT2 genetic variations. Primer design Sequencer 5.0 software was used for both splice variants of the SLC30A2 and the genetic variations of Isoform I. The human genome sequence of the SLC30A2 gene was downloaded from the NCBI data base

(http://www.ncbi.nlm.nih.gov/nuccore/?term=SLC30A2+AND+srcdb_refseq[PROP])

under FASTA files from RefSeq. The mRNA transcripts from Isoforms I and II were downloaded from the NCBI reference sequence with accession number NM_032513.3 and NM_001004434.1, respectively. As for genetic variations, sequences were imported from Reference SNP (refSNP) Cluster.

7.6 Sub-cloning of SLC30A2 Isoform I and Isoform II

Human SLC30A2 Isoform I was subcloned from placenta cDNA. As for Isoform II, the Open Reading Frame (CCSB Human ORFeome SLC30A2 Clone without stop codon Accession#: BC006251) was purchased from Fisher. They were fused with N-terminal tGFP fluorescent protein and mCherry, respectively. The Open Reading Frame (ORF) of Isoform I (long isoform) was amplified with the sense primer 5'-ATGGAGGCCAAGG AGAAGCAG-3' and the antisense primer 5'-TCAGTCTGAGGGGCCCTGGCA-3'. Isoform II (short isoform) was amplified with the sense primer 5'-ATGGAGGCCAAGG AGAAGCAG-3' and the antisense primer 5'-TCAGTCTGAGGGGCCCTGGCA-3'. The

PCR conditions for amplification of Isoform I was performed as follows: initial denaturation at 98℃ for 30s, followed by 35 cycles of denaturation at 98℃ for 5s, annealing at 65℃ for 10s, extension at 72℃ for 1:30 min and final extension at 72℃ for 5 min. The reaction contained a total volume of 20 µl, composed of 1µl of forward primer (10 µmol) and 1µl of reverse primer (10 µmol), 4 µl of Taq 5X Master Mix (New England BioLabs), 2 µl of cDNA (69.5 ng/µl) and 12 µl of dH_2O. The amplicons (5 µl) were separated on a 1% agarose gel (UltraPure™ Agarose, Invitrogen) conducted with Tris-Acetate-EDTA buffer (50x TAE, ThermoScientific) diluted 1X, identified by staining in Ethidium Bromide and visualized using Quantity One Software (Bio-Rad Laboratories Inc®). Transcripts were confirmed by electrophoresis. Verified transcripts were ligated into a TOPO TA Cloning® vector and transfected into *E.coli* (TOPO cloning Kit, Invitrogen) (Figure 2). Competent cells were transformed (Table 4) and seeded in kanamacin plates.

Positive clones were selected and grown in 3 ml kanamacin containing LB Broth (Invitrogen) for 16h (Table 5) with shaking. The plasmid DNA extraction was performed using the QIAprep Spin Miniprep Kit following the manufacturer's protocol (QIAgen, Venlo, Limburg). Isoform I plasmid was sequenced by The Center for Applied Genomics (TCAG), Sick Kids (Toronto, Ontario). All sequencing was performed by The Center for Applied Genomics (TCAG), Sick Kids (Toronto, Ontario).

As mentioned before, Isoform II was amplified with the sense primer 5'-ATGGAGGCCAAGGAGAAGCAG-3' and the antisense primer 5'-TCAGTCTGAGGGGCCCTGGCA-3'. The PCR conditions for amplification of Isoform II were performed as follows: initial denaturation at 98℃ for 30s, followed by 35 cycles

of denaturation at 98℃ for 5s, annealing at 65℃ for 10s, extension at 72℃ for 1:30 min and final extension at 72℃ for 5 min.

The reaction contained a total volume of 20µl, composed of 2µl of forward primer (10µmol) and 2µl of SLC30A2 reverse primer (10µmol), 0.2 µl of Phusion High Fidelity enzime (New England BioLabs), 0.4 µl of dNTPs, 4 µl 5XHF Buffer(New England BioLabs), 2µl of Human ORF SLC30A2 Isoform II and 9.4 µl of dH_2O. Then the amplicons were separated on a 2% gel (UltraPure™ Agarose, Invitrogen) and identified by electrophoresis. The positive amplicons were separated and purified by QIAquick PCR Purification Kit (QIAgen) according to the manufacturer's protocol. Concentration was measured by Nanodrop 2000 (ThermoFisher, 2009) for every extraction (Figure 3).

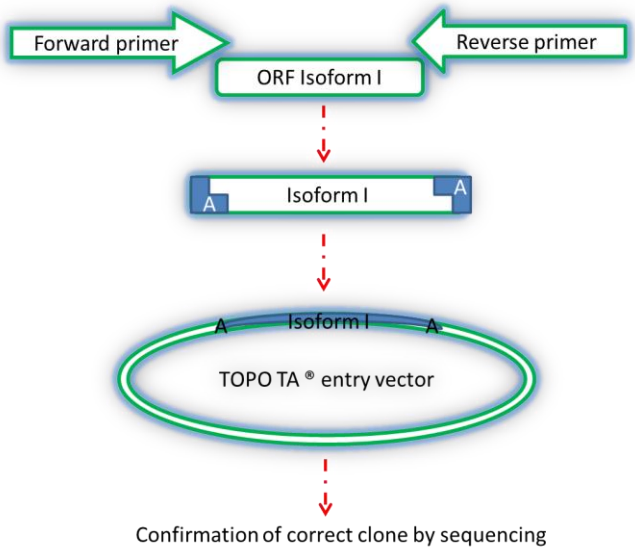


Figure 2 Cloning of WT Isoform I from placenta cDNA

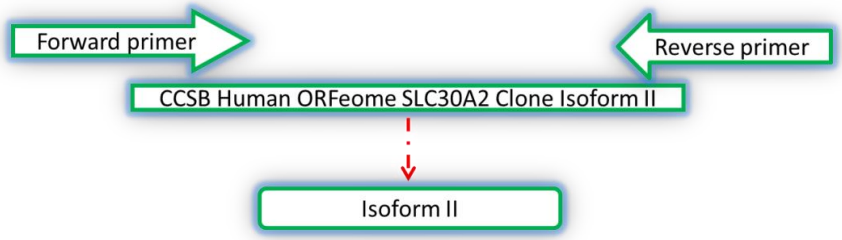


Figure 3 Cloning of Isoform II using CCSB Human ORFeome SLC30A2 Clone

7.7 Generation of human SLC30A2 construct Isoform I and II

We created an expression construct using Invitrogen Gateway® Destination vector pcDNA/V5-DEST for tGFP-SLC30A2 Isoform I and pcDNA/V5-DEST for mCherry-SLC30A2 for Isoform II with N-terminal expression. We created an SLC30A2 Isoform I PCR fragment overlapping with tGFP and an SLC30A2 Isoform II PCR fragment overlapping with mCherry to be further fused in a second round of PCR.

Isoform I was fused with tGFP green fluorescent protein using the sense primer (tGFP R1 nostop-SLC30A2F1)5’GCAGATGCCGGTGAAGAAATGGAGGCCAAGGAGAAGC AG-3’ and the antisense primer (SLC30A2 R1stop) 5'-TCAGTCTGAGGGGCCCTGG CA-3'. The PCR conditions for overlapping tGFP and Isoform I were as follows: initial denaturation at 98℃ for 30s, followed by 35 cycles of denaturation at 98° C for 5s, annealing at 65° C for 10s, extension at 72℃ for 1:30 min and final extension at 72℃ for 5 min. The reaction contained a total volume of 20µl, composed of 1µl of tGFP R1 nostop-SLC30A2 forward primer (10µmol) and 1µl of SLC30A2 reverse primer R1Stop (10µmol), 0.2 µl of Phusion High Fidelity enzyme (New England BioLabs), 4 µl 5XHF Buffer (New England BioLabs), 2µl of TOPO plasmid dilution Isoform I cDNA (101.8 ng/µl), 0.8 µl of dNTPs and 11.4µl of dH_2O. The amplicons (5µl) were separated on a 1%

agarose gel (UltraPure™ Agarose, Invitrogen) conducted with Tris-Acetate-EDTA buffer (50x TAE, ThermoScientific) diluted 1X, Ethidium Bromide contained (Invitrogen, 10 mg/ml) and visualized using Quantity One Software (Bio-Rad Laboratories Inc®). The positive amplicons were purified by QIAquick PCR Purification Kit (QIAgen) according to the manufacturer's protocol. Concentration was measured (Figure 4).

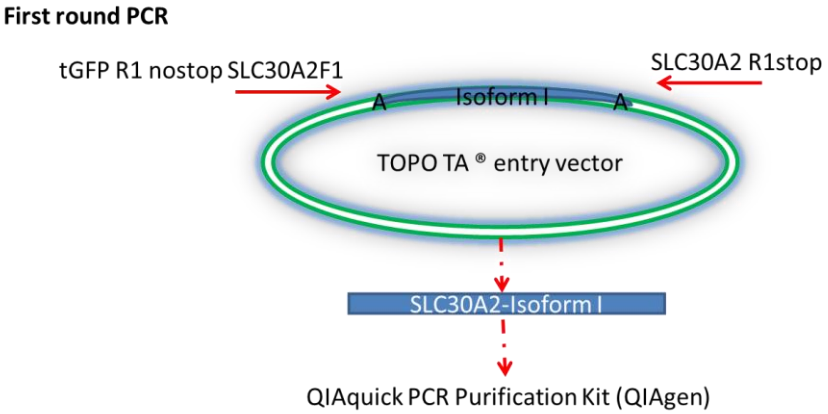


Figure 4 First round PCR Isoform I

The Second round of PCR was performed with AttB primers using the sense primer (tGFPATGKozakF1attB1)5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCATGGAGAGCGACGAGAGC-3' and the antisense primer (SLC30A2 R1stop attB2) 5'GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGTCTGAGGGGCCCTGGCA-3'. The PCR conditions were as already mentioned (page 22, second paragraph). The reaction contained a total volume of 40 µl, composed of 4 µl of tGFP -ATG-Kozak-F1-attB1 primer (10µmol) and 4µl of SLC30A2 R1stop attB2 (10µmol), 0.4 µl of Phusion High Fidelity enzime (New England BioLabs), 8µl 5XHF Buffer (New England BioLabs), 0.8µl dNTPs, 1.3µl of Phusion tGFP SLC30A2 purified product (31.7ng/µl), 0.8 µl tGFP PCR product (53.2 ng/µl) and 20.7 µl of dH2O. The amplicons were separated on a 2% gel (UltraPure™ Agarose, Invitrogen) and identified by

electrophoresis as mentioned before (page 21, first paragraph). The positive amplicons were extracted by QIAquick Gel Extraction Kit (QIAgen) according to the manufacturer's protocol. Concentration was measured (Figure 5).

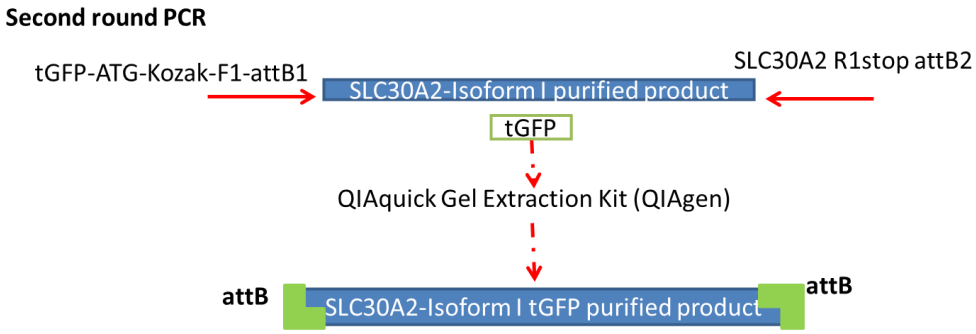


Figure 5 Second round PCR of Isoform I with aTTB primers

The second round construct purified was subcloned into a pDONR™221 Gateway entry vector by BP cloning reaction following the manufacturer's protocol (Invitrogen). The competent cells were then transformed (Table 4) and seeded in kanamacin plates.

Positive clones were selected and grown in 3ml in kanamacin containing LB Broth (Invitrogen) for 16h (Table 5) with shaking. The plasmid DNA extraction was performed using QIAprep Spin Miniprep Kit following the manufacturer's protocol (QIAgen). (Figure 6)

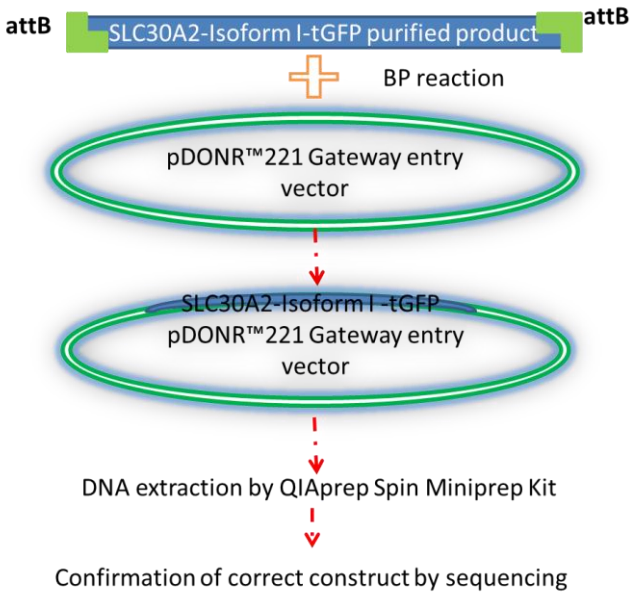


Figure 6 SLC30A2-Isoform I-tGFP introduced into pDONR vector

The correct construct (tGFP-SLC30A2 Isof I) was sequenced and confirmed by the ORF alignment using Sequencer 5.0. The tGFP-SLC30A2 Isof I was transferred into the pcDNA/V5-DEST Gateway destination vector by Gateway LR cloning reaction (Figure 7)

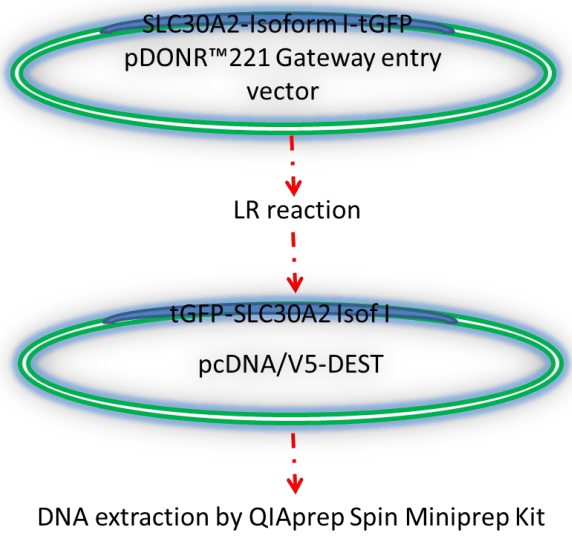


Figure 7 Recombination of entry clone pDONR and Destination Vector V5-DEST

Isoform II was fused with mCherry red fluorescent protein using the sense primer 5'-GACGAGCTGTACAAG ATGGAGGCCAAGG AGAAGCAG-3' (mCherry R1 nostop-SLC30A2 F1 phusion primer) and the antisense primer 5'-TCAGTCTGAGGGGCCCTGGCA-3' (SLC30A2 R1stop). The reaction contained a total volume of 40µl, composed of the sense primer mCherry R1 nonstop SLC30A2 F1 phusion 2µl, 2 µl of the antisense primer SLC30A2 R1stop, 8µl of QF buffer, 0.8µl of dNTPs, 0.4µl of Phusion High Fidelity enzyme, 1µl of SLC30A2 Isoform II (11.5 ng/µl) and 25.8µl of dH_2O. Then, the amplicons were separated on a 2% gel (UltraPure™ Agarose, Invitrogen) and identified as by electrophoresis. The positive amplicons were purified according with the QIquick PCR Purification Kit (Figure 8).

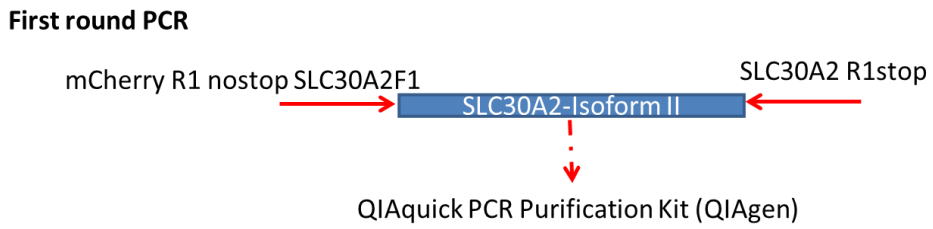


Figure 8 First round of Isoform II

The second round of Isoform II PCR was performed with AttB primers using the sense primermCherryATGKozakF1attB15'GGGGACAAGTTTGTACAAAAAAGCAGGCTT CGCCACCATGGTGAGCAAGGGCGAG-3' and the antisense SLC30A2 R1stop attB2 5'GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGTCTGAGGGGCCCTGGCA -3'. The PCR conditions were as described on page 21, third paragraph. The reaction contained a total volume of 40µl, composed of 4µl of mCherry -ATG-Kozak-F1-attB1 primer (10µmol) and 4µl of SLC30A2 R1stop attB2 (10µmol), 0.4 µl of Phusion High Fidelity enzime (New England BioLabs), 8µl 5XHF Buffer (New England BioLabs), 0.8µl dNTPs, 1.3µl of Phusion mCherry SLC30A2 purified product (49.7ng/µl), 0.8 µl mCherry PCR product (64.8 ng/µl) and 20.7µl of dH_2O. The amplicons were separated on a 2% gel (UltraPure™ Agarose, Invitrogen) and identified by electrophoresis. The positive amplicons were extracted by QIAquick Gel Extraction Kit (QIAgen) according to the manufacturer protocol. Concentration was measured. The amplicons were separated on a 2% gel (UltraPure™ Agarose, Invitrogen) and identified by electrophoresis. The positive amplicons were extracted by a QIAquick Gel Extraction Kit (QIAgen) according to the manufacturer's protocol. Concentration was measured by Nanodrop (ThermoFisher, 2009).

The purified second round construct was subcloned into pDONR™221 Gateway entry vector by a BP cloning reaction following the manufacturer's protocol (Invitrogen) (Figure 9).

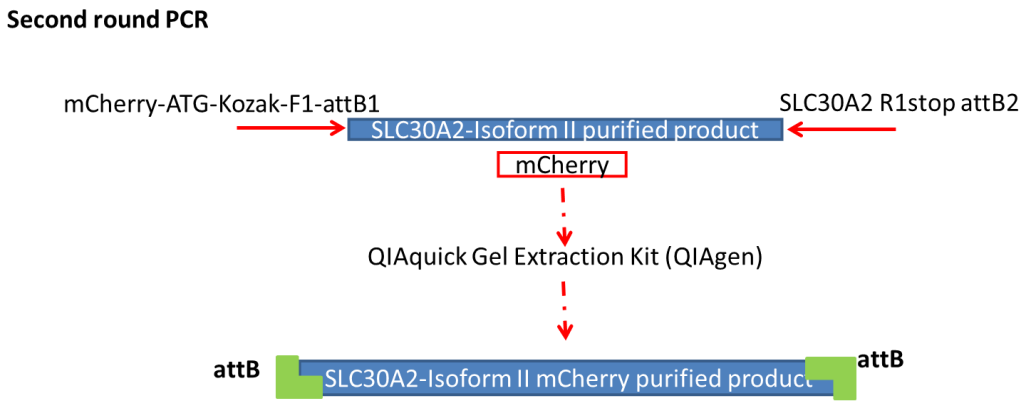


Figure 9 Second round PCR SLC30A2-Isoform II

After this, competent cells were transformed (Table 4) and seeded in kanamacin plates. Positive clones were selected and grown in 3ml kanamacin containing LB Broth (Invitrogen) for 16h (Table 5) with shaking. The plasmid DNA extraction was performed using a QIAprep Spin Miniprep Kit following the manufacturer's protocol (QIAgen). The correct construct (mCherry-SLC30A2 Isof II) was sequenced (Figure 10).

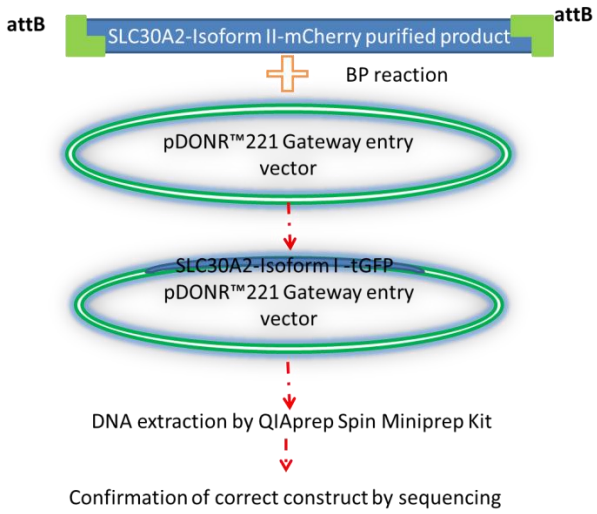


Figure 10 SLC30A2-Isoform II-mCherry introduce into pDONR vector

The mCherry-SLC30A2 Isof II was transferred into the pcDNA/V5-DEST Gateway destination vector by the Gateway LR cloning reaction (Figure 11)

Following this, both constructs tGFP-SLC30A2 Isof I and mCherry-SLC30A2 Isof II, were sequenced.

Later, the pcDNA™/V5-DEST expression vector was utilised to express SLC30A2 Isof I and SLC30A2 Isof II in HC11 cells.

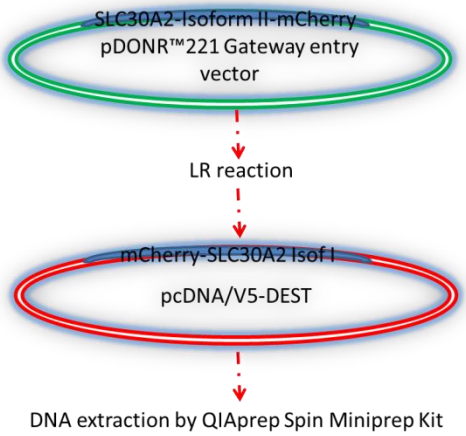


Figure 11 Recombination of entry vector pDONR and destination vector V5-DEST

7.8 Mutagenesis of Isoform I with 22 missense SNPs

The 22 missense single nucleotide polymorphisms were identified on NCBI under SNP GeneView. The sequences were retrieved from dbSNP and primer design was performed with Sequencer 4.8. The 22 SNPs were inserted by Single Site-Directed Mutagenesis (Hsieh & Vaisvila, 2013).

We followed two approaches for the same assay. **The first approach** was based on five PCR reactions. PCR I was performed using the sense primer of the WT Isoform I and the antisense primer of each SNP (Table 2) with the following PCR conditions: initial denaturation at 98℃ for 30s, followed by 35 cycles of denaturation at 98℃ for 5s, annealing at 65℃ for 10s, extension at 72℃ for 1:30 min and final extension at 72℃ for 5 min. The template used for this reaction was TOPO plasmid dilution Isoform I SLC30A2 cDNA (101.8 ng/µl). Total volume for each reaction was 10 µl, the reaction was composed by 1µl of forward primer (10µmol) and 1µl of reverse primer (10µmol), 0.1 µl of Phusion High Fidelity enzime (New England BioLabs), 0.2 µl of dNTPs, 2 µl 5XHF Buffer(New England BioLabs), 9.4µl of dH_2O and TOPO plasmid dilution Isoform I SLC30A2 cDNA (101.8 ng/µl). Following this, the amplicons (5µl) were separated on a 1% agarose gel (UltraPure™ Agarose, Invitrogen) conducted with Tris-Acetate-EDTA buffer (50x TAE, ThermoScientific) diluted 1X, identified by staining in Ethidium Bromide and visualized using Quantity One Software (Bio-Rad Laboratories Inc®). Positive amplicons were separated and purified by QIAquick PCR Purification Kit (QIAgen) according to the manufacturer's protocol. The concentration was measured.

Following this, PCR II was performed using the sense primer of each SNP (Table 2) and the antisense primer of Isoform I (Table 1) using the following PCR conditions: initial denaturation at 98℃ for 30s, followed by 35 cycles of denaturation at 98℃ for 5s, annealing at 65℃ for 10s, extension at 72℃ for 1:30 min and final extension at 72℃ for 5 min. The template used for this reaction was TOPO plasmid dilution Isoform I SLC30A2 cDNA (101.8 ng/µl). Total volume for each reaction was 10 µl, the reaction was composed by 1µl of forward primer (10µmol) and 1µl of reverse primer (10µmol), 0.1 µl of Phusion High Fidelity enzime (New England BioLabs), 0.2 µl of dNTPs, 2 µl 5XHF Buffer(New England BioLabs), 9.4µl of dH_2O and TOPO plasmid dilution Isoform I SLC30A2 cDNA (101.8 ng/µl). The amplicons (5µl) were separated on a 1% agarose gel (UltraPure™ Agarose, Invitrogen) conducted with Tris-Acetate-EDTA buffer (50x TAE, ThermoScientific) diluted 1X, identified by staining in Ethidium Bromide and visualized using Quantity One Software (Bio-Rad Laboratories Inc®). Positive amplicons were separated and purified by a QIAquick PCR Purification Kit (QIAgen) according to the manufacturer's protocol. The concentration was measured. Having the two purified PCR amplicons, PCR III was performed to ligate both linear fragments into the SLC30A2 Isoform I. To perform the reaction we used the sense primer 5'-ATGGAGGCCAAGG AGAAGCAG-3' and the antisense primer 5'-TCAGTCTGAGGGGCCCTGGCA-3'. Total volume for each reaction was 20 µl, the reaction was composed by 1µl of forward primer (10µmol) and 1µl of reverse primer (10µmol), 0.1 µl of Phusion High Fidelity enzime (New England BioLabs), 0.2 µl of dNTPs, 2 µl 5XHF Buffer(New England BioLabs), 9.4µl of dH_2O and 1µl of PCR I and PCR II purified products. Following this, the amplicons (5µl) were separated on a 1% agarose gel (UltraPure™ Agarose,

Invitrogen) conducted with Tris-Acetate-EDTA buffer (50x TAE, ThermoScientific) diluted 1X, identified by staining in Ethidium Bromide and visualized using Quantity One Software (Bio-Rad Laboratories Inc®). Positive amplicons were separated and purified by QIAquick PCR Purification Kit (QIAgen) according to the manufacturer's protocol. Further the concentration was measured. Isoform I was fused with mCherry red fluorescent protein using the sense primer 5'-GACGAGCTGTACAAG ATGGAGGCCAAGG AGAAGCAG-3' (mCherry R1 nostop- SLC30A2 F1 phusion primer) and the antisense primer 5'-TCAGTCTGAGGGGCCCTGGCA-3' (SLC30A2 R1stop). The reaction contained a total volume of 20µl, composed of the sense primer mCherry R1 nonstop SLC30A2 F1 phusion 1µl, 1µl of the antisense primer SLC30A2 R1stop, 2µl of QF buffer, 0.2µl of dNTPs, 0.1µl of Phusion High Fidelity enzyme, 1µl of SNP SLC30A2 mutagenesis Isoform I (for concentration, Table 7) and 9.4µl of dH_2O. Following this procedure the amplicons were separated on a 2% gel (UltraPure™ Agarose, Invitrogen) and identified by electrophoresis. Positive amplicons were extracted by QIAquick Gel Extraction Kit (QIAgen) according to the manufacturer's protocol. Concentration was measured. The Second round PCR was performed with AttB primers using the sense primer (mCherry-ATG-Kozak-F1-attB1) mCherry-ATG-Kozak-F1-attB1 5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCATGGTGAGCAAGG GCGAG-3' and the antisense primer (SLC30A2 R1stop attB2) 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTC TCAGTCTGAGGGGCCCTGGCA-3'. The PCR conditions were as mentioned. The reaction contained a total volume of 20µl, composed of the sense primer mCherry -ATG-Kozak-F1-attB1 primer (10µmol) 1µl and 1µl of the antisense primer SLC30A2 R1stop attB2, 2µl of QF buffer, 0.2µl of

dNTPs, 0.1µl of Phusion High Fidelity enzyme, 1µl of each SNP_n SLC30A2 mutagenesis (phusion) Isoform I (for concentration, Table 8) and 9.4µl of dH_2O. The amplicons were separated on a 1% gel (UltraPure™ Agarose, Invitrogen) and identified by electrophoresis (Figure 12).

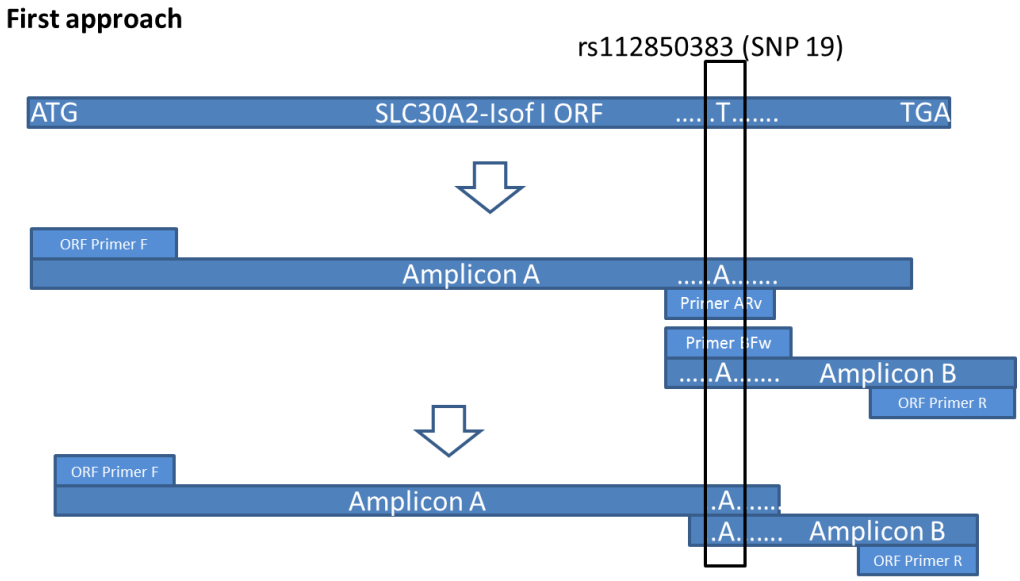


Figure 12 Site Directed Mutagenesis of SLC30A2 Isof I

7.8.1 Rapid ligation

The **second approach** followed was performed using the sense and antisense primer of each mutation, using the following PCR conditions: initial denaturation at 98℃ for 30s, followed by 35 cycles of denaturation at 98℃ for 5s, annealing at 65℃ for 10s, extension at 72℃ for 1:30 min and final extension at 72℃ for 5 min. The template used for this reaction was pcDNA™/ pDONR™221 Gateway entry vector (mCherry-SLC30A2 Isoform I) tagged with red fluorescent protein, sense and antisense primers that were used are presented in (table 2). Total volume for each reaction was 10 µl, the reaction was composed of 1µl of forward primer (10µmol) and 1µl of reverse primer (10µmol), 0.1 µl

of Phusion High Fidelity enzime (New England BioLabs), 0.2 µl of dNTPs, 2 µl 5XHF Buffer(New England BioLabs), 9.4µl of dH_2O and mCherry-SLC30A2 Isoform II (139.6 ng/µl). Following this, the amplicons (5µl) were separated on a 1% agarose gel (UltraPure™ Agarose, Invitrogen) conducted with Tris-Acetate-EDTA buffer (50x TAE, ThermoScientific) diluted 1X, identified by staining in Ethidium Bromide and visualized using Quantity One Software (Bio-Rad Laboratories Inc®). Positive amplicons were separated and purified by a QIAquick PCR Purification Kit (QIAgen) according to the manufacturer protocol. The concentration was measured.

The assembly of PCR fragments was performed by Gibson Assembly Reaction following the manufacturer protocol. Using 0.02- 0.5 pmols X (1µl) of PCR product, 4µl of deionized water, and 5µl of Gibson Assembly Master Mix to obtain a total volume of 10µl. Samples were incubated at 50℃ for 60 minutes, followed by storage on ice at -20℃ for subsequent transformation. One microliter of assembled product was transferred into competent cells and seeded in kanamacin plates. Positive clones were selected and grown in 3ml of kanamacin containing LB Broth (Invitrogen) tubes for 16h (Table 5) with shaking. The plasmid DNA extraction was performed using QIAprep Spin Miniprep Kit following the manufacturer's protocol (QIAgen). The concentration was measured.

After this, a PCR reaction was performed to confirm the presence of the mutated isoform and the fluorescent tag. Positive products were sequenced (Figure 13).

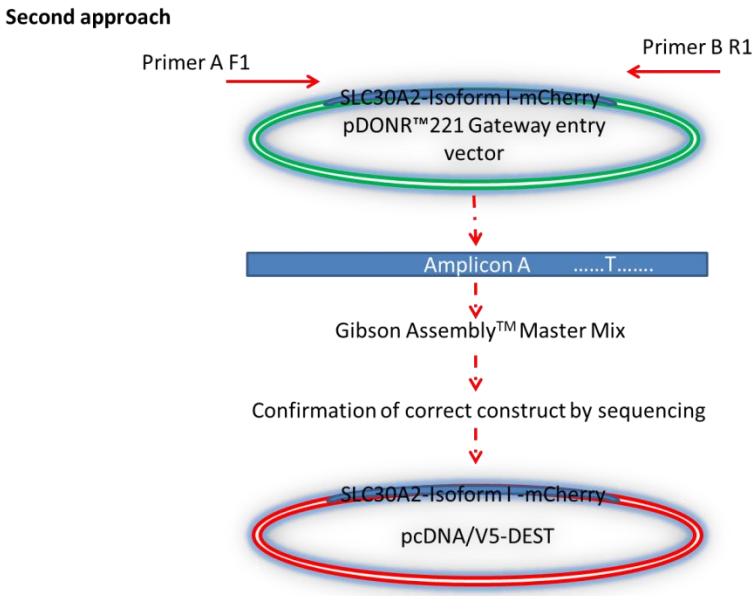


Figure 13 Second approach of mutagenesis of SLC30A2 Isof I, using rapid ligation

7.9 Cell culture and transient transfection of wild type SLC30A2 in HC11 mammary epithelial cells

Mouse mammary epithelial HC11 cells were a gift from Dr. Carrie Shemanko, University of Calgary (Calgary, Alberta).

HC11 cells were grown in Growth medium containing RMPI 1640 medium (Invitrogen) with Pen/Strep (Life Technology, 50µg/mL), EGF (10µg/ml; Sigma), insulin (5mg/ml; Sigma) and 10% Fetal Bovine Serum (FBS) at 37℃ in a humidified atmosphere of 5% CO_2 in a 75 mm flask (Thermo Fisher Scientific, Corning).

To differentiate HC11 cells into the secretory phenotype, HC11 cells were cultured in two different mediums, competence medium and differentiation medium. After attaining 98% confluence, cells were transferred into a competence medium containing RMPI 1640 (Invitrogen) with Pen/Strep (Life Technology, 50µg/mL), Epidermal Growth Factor (10µg/ml; Sigma), and 10% Fetal Bovine Serum at 37℃ in a humidified atmosphere of

5% CO_2 in a 75 mm flask (Thermo Fisher Scientific, Corning). After 4 days, cells were washed with 5ml sterile PBS and transferred to a Differentiation Medium containing RMPI 1640 (Invitrogen) with Pen/Strep (Life Technology 50µg/mL), Epidermal Growth Factor (10µg/ml; Sigma), insulin (5mg/ml; Sigma), prolactin (5µg/ml; Sigma) and 10% Fetal Bovine Serum at 37℃ in a humidified atmosphere of 5% CO_2 in a 75 mm flask (Thermo Fisher Scientific, Corning).

HC11 cells were seeded in 6-well tissue culture plates (Thermo Fisher Scientific, BioLite). After three days, cells were transfected with tGFP-SLC30A2 Isoform I and mCherry-SLC30A2 Isoform II pcDNA/V5-DEST in RPMI 1640 with 1% FBS, using Effectine transfection reagent with modification of the manufacturer's protocol (QIAGEN) Table 6.

7.10 Transient transfection of mutant SLC30A2 isoform I in HC11 mammary epithelial cells

After differentiation, HC11 cells were seeded in 6-well tissue culture plates (Thermo Fisher Scientific, BioLite). After three days, cells were transfected with tGFP-SLC30A2 Isoform I, mCherry Isoform II pcDNA/V5-DEST in RPMI 1640 with 1% FBS, using Effectene® Transfection Reagent with modification of the manufacturer's protocol (QIAGEN) Table 6.

To transfect HC11 cells with SNP 17, 5, 19 and 22 of SLC30A2 mCherry Isoform I pcDNA/V5-DEST. Cells were seeded in 6-well tissue culture plates (Thermo Fisher Scientific, BioLite) in RPMI 1640 with 1% FBS, using Effectene® Transfection Reagent with modification of the manufacturer's protocol (QIAGEN).

To track the secretory vesicles and visualize the localization, we used a Cyto-Tracer™ pCT-CD63-GFP (pCMV, Exosome/Secretory, CD63 Tetraspanin Tag), MJS BioLynx INC, cat no. SYCYTO120PA1, in both transfections.

7.11 Cellular Localization

72h post transfection HC11 cells were visualized by fluorescent microscopy (Anxiovert 200, ZEISS).

8 Results

8.1 Bioinformatics search of gene structure SLC30A2

SLC30A2 transporter is also known as ZNT2; ZnT-2; PP12488. It is located in chromosome 1 cytoband p35.3 starting at 26363299 base pair (bp) from the promoter region to 26373817 bp in the reverse strand. It is located in between the EXTL1 gene and TRIM63 gene. It is localized in the 10.52 kb region with a total of 10519 bp (Figure 14).

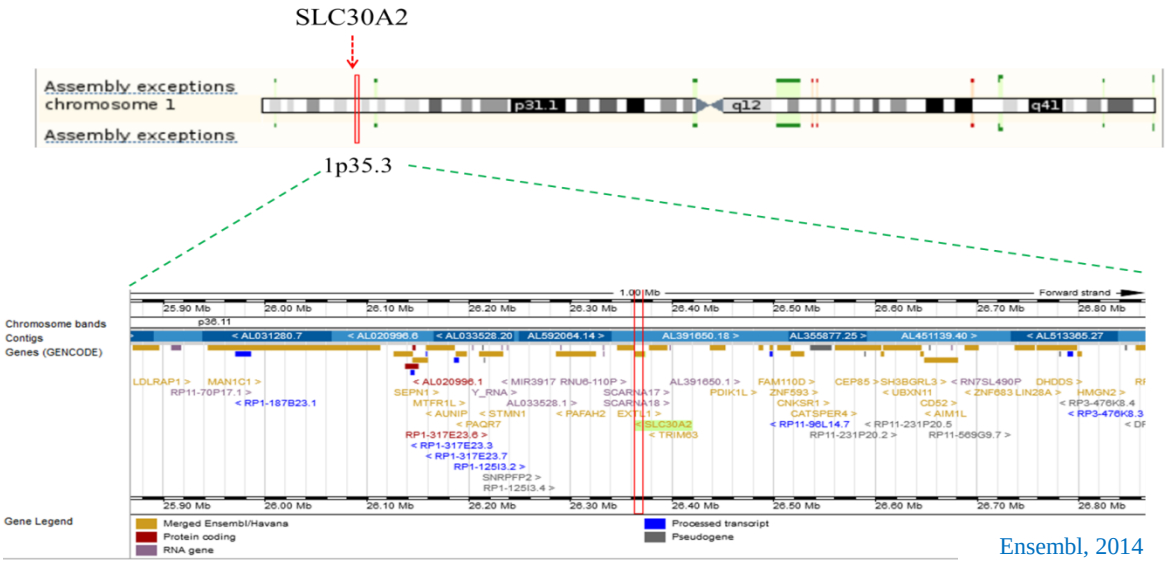


Figure 14 Overview of the SLC30A2 gene (Ensembl, 2014)

It encodes for two mRNA Isoforms according to NCBI databases. Both splice variants, long and short, encode for a functional protein. The first isoform, or long isoform, encodes for a protein containing 362 amino acids and is 1,186 bp long. This isoform consists of 8 exons and 9 introns, whereas Isoform II, the short isoform, has 7 exons and 8 introns (Figure 15)

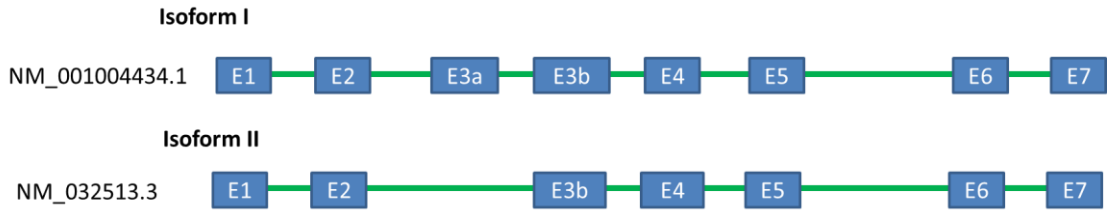


Figure 15 Structure of Isoform I and II

Isoform II is hypothesized to arise from the splice of exon number 3b of isoform I. This isoform has a deletion of exon number 3a. It encodes for a protein that consists of 32 amino acids, its length is 972 bp .

8.2 Single Nucleotide Polymorphisms of SLC30A2

In February 2013, there were reported 39 SNPs, of which 22 SNPs were missense, and to date a total of 56 single nucleotide polymorphisms (SNPs) are known, from which 35 are missense according to NCBI (February, 2014).

Table 10 Single Nucleotide Polymorphisms, reported on NCBI, February 2014

Chr. position	mRNA pos	dbSNP rs# cluster id	Hetero-zygosity	Function	dbSNP allele	Protein residue	Codon pos	Amino acid position
26365663	1324	rs35611719	N.D.	frame shift	-	Gln [Q]	3	371
				contig reference	C	Ser [S]	3	371
26365672	1315	rs374195497	N.D.	synonymous	G	Ala [A]	3	366
				contig reference	A	Ala [A]	3	366
26365707	1280	rs377192955	N.D.	missense	A	Lys [K]	1	355
				contig reference	G	Glu [E]	1	355
26365708	1279	rs147469106	0.001	synonymous	T	Ile [I]	3	354
				contig reference	C	Ile [I]	3	354
26365722	1265	rs144832711	0.001	missense	A	Met [M]	1	350
				contig reference	G	Val [V]	1	350
26365751	1236	rs145406127	0.001	missense	A	His [H]	2	340

Chr. position	mRNA pos	dbSNP rs# cluster id	Hetero-zygosity	Function	dbSNP allele	Protein residue	Codon pos	Amino acid position
				contig reference	G	Arg [R]	2	340
26365752	1235	rs35623192	0.001	missense	T	Cys [C]	1	340
				contig reference	C	Arg [R]	1	340
26365782	1205	rs149723161	0.001	missense	A	Thr [T]	1	330
				contig reference	G	Ala [A]	1	330
26366274	1184	rs372456339	N.D.	missense	A	Thr [T]	1	323
				contig reference	G	Ala [A]	1	323
26366275	1183	rs35020821	0.001	synonymous	T	Ile [I]	3	322
				contig reference	C	Ile [I]	3	322
26366320	1138	rs35925054	0.006	synonymous	C	His [H]	3	307
				contig reference	T	His [H]	3	307
26366346	1112	rs151175941	0.000	missense	A	Arg [R]	1	299
				contig reference	G	Gly [G]	1	299
26366353	1105	rs150251854	0.000	synonymous	A	Ser [S]	3	296
				contig reference	G	Ser [S]	3	296
26366364	1094	rs375429556	N.D.	synonymous	T	Leu [L]	1	293
				contig reference	C	Leu [L]	1	293
26366369	1089	rs149340896	0.004	missense	A	His [H]	2	291
				contig reference	G	Arg [R]	2	291
26366373	1085	rs369345160	N.D.	missense	A	Ile [I]	1	290
				contig reference	G	Val [V]	1	290
26366401	1057	rs200846211	0.002	synonymous	A	Gly [G]	3	280
				contig reference	G	Gly [G]	3	280
26367417		rs71004561	N.D.		-/CCC			
26367418		rs67103589	N.D.		(>6bp)			
				intron	(>6bp)			

Chr. position	mRNA pos	dbSNP rs# cluster id	Hetero-zygosity	Function	dbSNP allele	Protein residue	Codon pos	Amino acid position
26368230	1016	rs138246170	0.000	synonymous	C	Leu [L]	1	267
				contig reference	T	Leu [L]	1	267
26368249	997	rs202003978	0.002	synonymous	A	Ile [I]	3	260
				contig reference	C	Ile [I]	3	260
26368260	986	rs372442248	N.D.	missense	A	Ile [I]	1	257
				contig reference	G	Val [V]	1	257
26368261	985	rs1316852	0.139	synonymous	T	Phe [F]	3	256
				contig reference	C	Phe [F]	3	256
26369064	925	rs141247216	0.001	synonymous	A	Val [V]	3	236
				contig reference	G	Val [V]	3	236
26369074	915	rs201084300	0.001	missense	A	Asp [D]	2	233
				contig reference	G	Gly [G]	2	233
26369076	913	rs200039883	0.001	missense	A	Ile [I]	3	232
				contig reference	G	Met [M]	3	232
26369078	911	rs144129605	0.000	missense	G	Val [V]	1	232
				contig reference	A	Met [M]	1	232
26369080	909	rs369755786	N.D.	missense	C	Thr [T]	2	231
				contig reference	G	Ser [S]	2	231
26369097	892	rs201442115	0.002	synonymous	T	Ile [I]	3	225
				contig reference	C	Ile [I]	3	225
26369123	866	rs373747848	N.D.	missense	C	Leu [L]	1	217
				contig reference	G	Val [V]	1	217
26369159	830	rs377645638	N.D.	missense	G	Asp [D]	1	205
				contig reference	C	His [H]	1	205
26369173	816	rs201917367	N.D.	missense	A	Asp [D]	2	200
				contig	G	Gly [G]	2	200

Chr. position	mRNA pos	dbSNP rs# cluster id	Hetero-zygosity	Function	dbSNP allele	Protein residue	Codon pos	Amino acid position
				reference				
26369176	813	rs112127101	0.500	missense	G	Cys [C]	2	199
				contig reference	C	Ser [S]	2	199
26369894	784	rs200520278	0.001	missense	A	Lys [K]	3	189
				contig reference	C	Asn [N]	3	189
26369902	776	rs199807670	0.003	missense	T	Ser [S]	1	187
				contig reference	G	Ala [A]	1	187
26369915	763	rs373547628	N.D.	synonymous	A	Ser [S]	3	182
				contig reference	G	Ser [S]	3	182
26369919	759	rs148861822	0.000	missense	T	Met [M]	2	181
				contig reference	C	Thr [T]	2	181
26369971	707	rs199594277	0.001	missense	A	Lys [K]	1	164
				contig reference	G	Glu [E]	1	164
26369997	681	rs370485489	N.D.	missense	T	Met [M]	2	155
				contig reference	C	Thr [T]	2	155
26370002	676	rs147918642	0.000	synonymous	T	Val [V]	3	153
				contig reference	C	Val [V]	3	153
26370038	640	rs373563894	N.D.	synonymous	T	Ile [I]	3	141
				contig reference	C	Ile [I]	3	141
26370817	607	rs200234967	0.002	synonymous	A	Lys [K]	3	130
				contig reference	G	Lys [K]	3	130
26370907	517	rs374981775	N.D.	synonymous	A	Val [V]	3	100
				contig reference	C	Val [V]	3	100
26370927	497	rs369142908	N.D.	missense	G	Val [V]	1	94
				contig reference	C	Leu [L]	1	94
26371500	476	rs185398527	0.001	missense	A	Arg [R]	1	87

Chr. position	mRNA pos	dbSNP rs# cluster id	Hetero-zygosity	Function	dbSNP allele	Protein residue	Codon pos	Amino acid position
				contig reference	G	Gly [G]	1	87
26371530	446	rs200712540	0.001	missense	A	Thr [T]	1	77
				contig reference	G	Ala [A]	1	77
26371544	432	rs368255248	N.D.	missense	A	His [H]	2	72
				contig reference	G	Arg [R]	2	72
26371586	390	rs141752286	0.000	missense	A	Asp [D]	2	58
				contig reference	G	Gly [G]	2	58
26371669	307	rs199685973	0.001	missense	T	Cys [C]	3	30
				contig reference	G	Trp [W]	3	30
26371679	297	rs77193883	0.005	missense	A	Glu [E]	2	27
				contig reference	G	Gly [G]	2	27
26371689	287	rs112850383	0.500	missense	C	Arg [R]	1	24
				contig reference	T	Trp [W]	1	24
26371691	285	rs35235055	0.006	missense	C	Pro [P]	2	23
				contig reference	T	Leu [L]	2	23
26371699	277	rs372550969	N.D.	synonymous	A	Thr [T]	3	20
				contig reference	G	Thr [T]	3	20
26372347	258	rs377136186	N.D.	missense	T	Leu [L]	2	14
				contig reference	C	Pro [P]	2	14
26372367	238	rs144738392	0.000	missense	C	His [H]	3	7
				contig reference	G	Gln [Q]	3	7
26372371	234	rs142587047	0.001	missense	G	Arg [R]	2	6
				contig reference	A	Lys [K]	2	6

In table 10, are the genetic variations identified in the *SLC30A2*. The variations in color red are the missense SNPs, this color indicate the exons of the gene.

Color blue is the 5', SNPs in color green, yellow and white are synonymous polymorphisms located in the introns of the SLC30A2.

From these genetic variations we chose to work with 22 mutations that were identified in February 2013, as missense mutations (Table 11).

Table 11 Single Nucleotide Polymorphisms used for mutagenesis of the SLC30A2

SNP number	SNP
1	rs144832711
2	rs145406127
3	rs35623192
4	rs149723161
5	rs151175941
6	rs201084300
7	rs200039883
8	rs144129605
9	rs201917367
10	rs112127101
11	rs200520278
12	rs199807670
13	rs148861822
14	rs185398527
15	rs200712540
16	rs141752286
17	rs199685973
18	rs77193883
19	rs112850383
20	rs35235055
21	rs144738392
22	rs142587047

8.3 Tissue expression

According to UniGene expression profile Hs.143545, the SLC30A2 gene has wide tissue expression. It is strongly expressed in placental and kidney tissues. It is also found in the

cervix, ovaries, thyroid, embryonic tissue, eyes and uterus. During disease, SLC30A2 was expressed in cervical, head and neck, lung and ovarian tumors (NCBI, 2013). Also, it has been linked with breast cancer and zinc accumulation in the mammary tissue (Kelleher et al., 2009).

Table 12 Tissue expression in the SLC30A2 (NCBI, February 2014)

		Hs.143545	
Cervix	20	1 /	48486
embryonic tissue	4	1 /	212896
eye	9	2 /	208840
kidney	61	13 /	210778
lung	2	1 /	334815
ovary	19	2 /	101488
placenta	105	30 /	283019
thyroid	21	1 /	46583
uterus	12	3 /	232093
cervical tumor	28	1 /	34484
head and neck tumor	7	1 /	133826
lung tumor	9	1 /	102765
normal	14	48 /	3328811
ovarian tumor	26	2 /	76185
embryoid body	14	1 /	69969
fetus	1	1 /	556978
adult	18	35 /	1921829

8.4 Sub-cloning and generation of SLC30A2 Isoform I and Isoform II

Isoform I was successfully sub-cloned from placenta cDNA. Five plasmids were isolated for Isoform I tag with tGFP the size expected was 1.8 kb, since Isoform I is 1.1 kb and the tGFP is 0.7 kb (Figure 16). After this the expression construct was created.

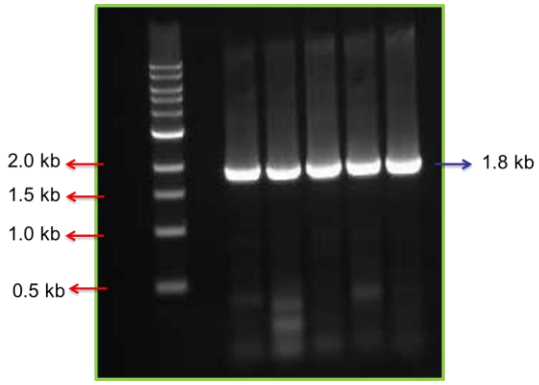


Figure 16 PCR visualization of the amplicon of Isoform I, fused with tGFP green fluorescent protein

Isoform II was successfully sub-cloned from CCSB Human ORFeome SLC30A2 clone. Five plasmids of Isoform II-mCherry were isolated the size expected was 1.67 kb, since Isoform II is 0.97 kb and the mCherry is 0.7 kb (Figure 16). After this the expression construct was created.

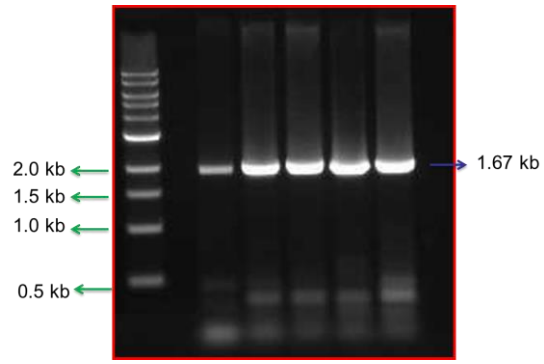


Figure 17 PCR visualization of the amplicon of Isoform II, fused with mCherry red fluorescent protein

Both constructs of the Wild Type (WT) SLC30A2 were confirmed by sequencing by The Center for Applied Genomics (TCAG), Sick Kids (Toronto, Ontario) (Figure 18 - 21). These products were further used for transient transfection in HC11 cells.

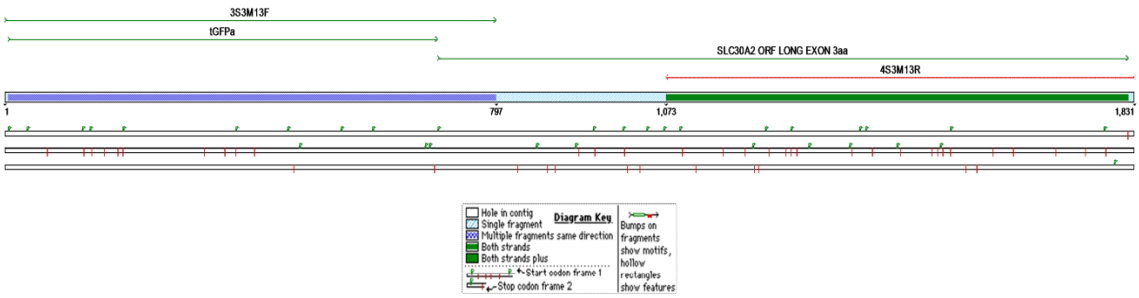


Figure 18 Alignment of the ORF of SLC30A2, fluorescent protein tGFP and the tGFP -SLC30A2-Isof I construct in the in the Sequencher 5.0

For sequencing the M13 forward and reverse primers were used, since the tGFP -SLC30A2-Isof I was inserted into pDONR vector. In figure 18, the upper green line labelled as **3SM13F** is the sequenced construct, the second green line on the left is the **tGFP** sequence. The mid line is the open reading frame of the **SLC30A2 Isoform I**.

The red line in the bottom, labelled as **4SM13R** is the reverse of the sequenced construct.

Figure 19 Alignment of the sequenced construct tGFP -SLC30A2-Isof I

As is observed in figure 18, the alignment was positive. When analyze the bases of the construct, ORF of SLC30A2 and the green fluorescent protein (figure 19) were perfectly aligned.

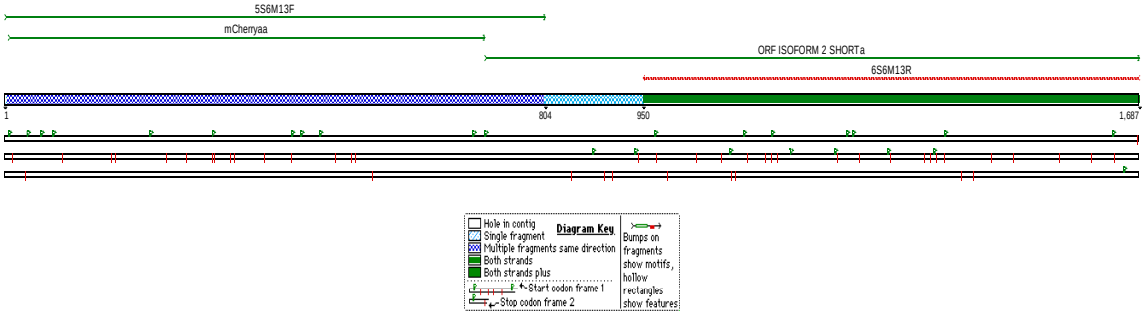


Figure 20 Alignment of the ORF of SLC30A2, fluorescent protein mCherry and the mCherry -SLC30A2-Isof I construct in the in the Sequencher 5.0

As for Isoform II, M13 forward and reverse primers were used to sequence mCherry - SLC30A2-Isof I inserted into pDONR vector. In figure 20, the upper green line **5S6M13F** is the sequenced construct; the second green line on the left is the **mCherry** sequence. The mid line is the open reading frame of the **SLC30A2 Isoform II**. The red line in the bottom, labelled as **6S6M13R** is the reverse of the sequenced construct.

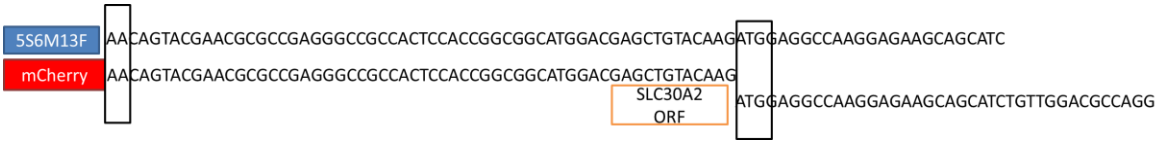


Figure 21Alingment of the sequenced construct mCherry -SLC30A2-Isof II

After alignment (figure 21), was confirmed the correct construct for Isoform II tagged with red fluorescent protein, mCherry.

8.5 Single Site-Directed Mutagenesis

Single Site-directed mutagenesis was performed with two distinct approaches.

The **first approach** was based on five PCR reactions. The 22 genetic variations were introduced individually into Isoform I.

The first PCR reaction was performed using the sense primer of WT Isoform I and the antisense primer of each SNP_n. Following this, PCR II was performed using the sense primer of each SNP_n and the antisense primer of WT Isoform I. There were nine positive amplicons (Figure 22- 24). Amplicons were separated and purified by a QIAquick PCR Purification Kit (QIAgen) according to the manufacturer's protocol. Concentration was measured.

Ten positive amplicons were separated and purified by a QIAquick PCR Purification Kit (QIAgen) according to the manufacturer's protocol for further concentration measurement.

Seven positive amplicons were separated for further purification. PCR II was performed for the SNPs, 13, 14, 15, 16, 17, 18, 19 and 21 (Figure 25). We separated eight positive amplicons for further purification. PCR III was performed to join PCRI and PCRII in order to introduce the mutation into Isoform I of the SLC30A2. This last PCR reaction was performed using sense and antisense primers of WT Isoform I SLC30A2 and all positive purified amplicons were utilised as a template in PCR III. For electrophoresis, a 1 kb DNA ladder was used, since the PCR product was not smaller than 0.5 kb.

Fourteen SNPs were successfully inserted into Isoform I SLC30A2 (Figure 26). After this, we successfully fused the mutated SLC30A2 with mCherry red fluorescent protein using the sense primer mCherry R1 nostop- SLC30A2 F1 phusion primer and the antisense primer SLC30A2 R1stop.

The eleven positive amplicons (Figure 27-28) were extracted and concentration was measured.

The attachment with the AttB primers was unsuccessful; therefore, the **second approach** was performed using the Gibson Assembly Reaction for the 22 genetic variations.

The PCR reaction contained the sense and antisense primer of each SNP. The template used for this reaction was pcDNA™/ pDONR™221 Gateway entry vector (mCherry-SLC30A2 Isoform I) tagged with red fluorescent protein. To confirm the correct size (6,580 bp) a 1% agarose gel was electrophoresed.

Nine positive amplicons were obtained: SNP 5 (rs151175941), SNP 9 (rs201917367) and 22 (rs142587047), SNP 12 (rs199807670), SNP 16(rs141752286), SNP 17(rs199685973) and SNP 20(rs35235055), SNP 8 (rs144129605) and SNP 19(rs112850383). Following this, concentration of each amplicon was measured.

Table 10 Concentration of positive PCR products

SNP	Concentration ng/µl
rs112850383	803.1
rs144129605	843.8
rs151175941	596.7
rs201917367	671.0
rs142587047	641.3
rs199807670	546.1
rs35235055	586.9
rs199685973	560.0
rs141752286	560.9

Based on the concentration of each PCR product, the Gibson Assembly™ Master Mix was prepared for each SNP.

Following this, competent *E.Coli* cells were transformed and grown in kanamycin plates. Then, plasmid DNA extraction was performed using QIAprep Spin Miniprep Kit following the manufacturer's protocol (QIAgen).

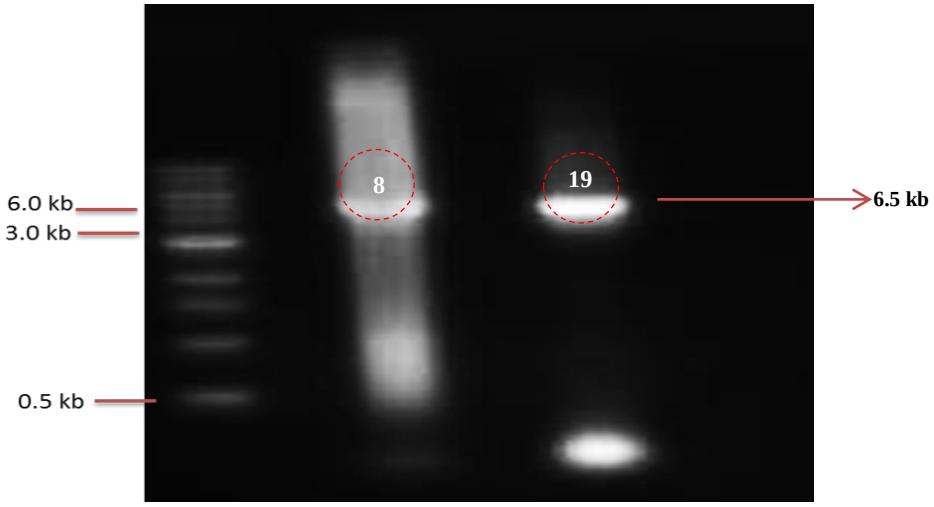


Figure 22 SNP 8 (rs144129605) and SNP 19(rs112850383) on pDONR™221 Vector, had the expected size, 6.5 kb

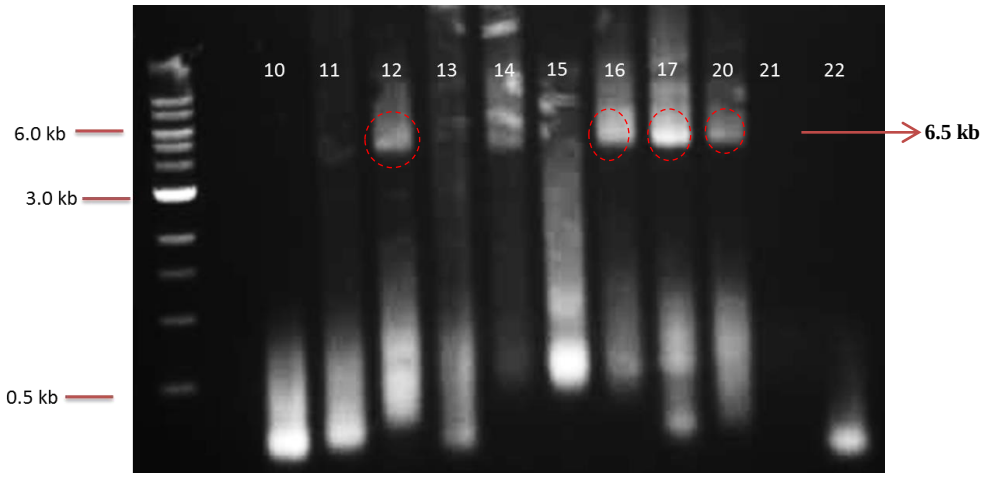


Figure 23SNP 12 (rs199807670), SNP 16(rs141752286), SNP 17(rs199685973) and SNP 20(rs35235055) pDONR™221 Vector, had the expected size, 6.5 kb

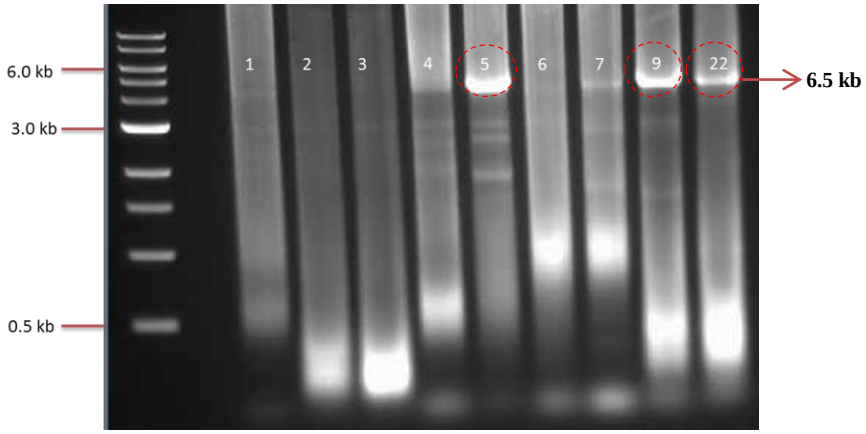


Figure 24 SNP 5 (rs151175941), SNP 9 (rs201917367) and 22 (rs142587047) pDONR™221 Vector, had the expected size, 6.5 kb

From figure 22-24, the expected size of the plasmid of the mutated SLC30A2 was 6.5 kb, since Isoform I is 1.1 kb, mCherry 0.7 kb and the pDONR™221 is 4.762 kb. The expected size was obtained as is observed in lane labelled as 8 and 19 in figure 22. In figure 23 also we obtained the expected size as observed in lane 12, 16, 17, 20 in lane 5, 9 and 22 in figure 23 as well. After obtaining the expected product it was sent for sequencing. The confirmation of the correct mutant SLC30A2 was performed by sequencing.

After alignment in Sequencer 5.0, using ORF of Isoform I, mCherry red fluorescent protein and the sequence of the positive clones of rs199685973 (SNP 17), rs151175941 (SNP5) and rs112850383 (SNP19) and rs142587047 (SNP22) was confirmed that the bases of interest were inserted successfully. (Figure 25- 27)

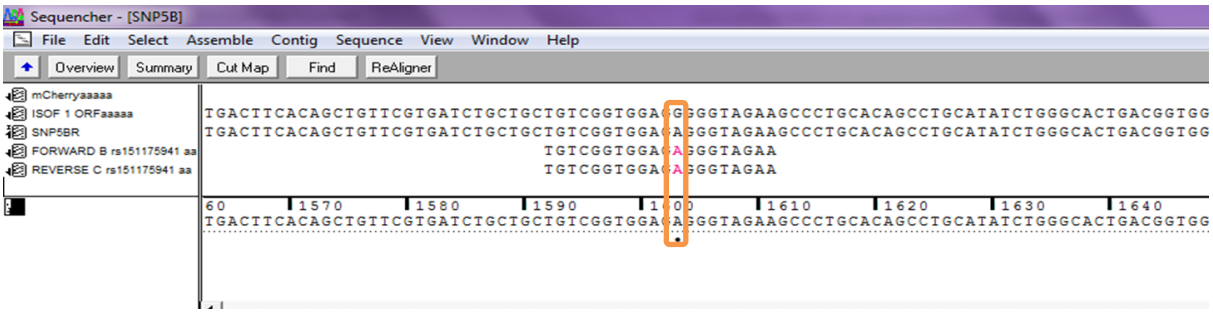


Figure 25 Sequence alignment of construct of the SLC30A2-rs151175941 (SNP 5) successfully inserted the change of base G/A

For rs151175941 (SNP5), the expected change of base in position number 895 (**G/A**) was inserted in the SLC30A2 Isoform I. This construct will be transferred to an expression system, for later utilization in transient transfection of HC11 cells.

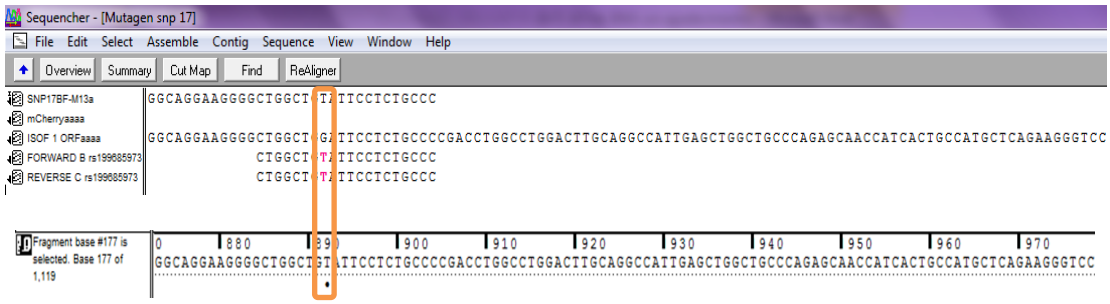


Figure 26 Alignment of sequence of construct of the SLC30A2-rs199685973 (SNP 17) successfully inserted the change of base in base number 90 (T)

In figure 26 the alignment of rs199685973 (SNP 17), showed the expected change of base **G/T** in position 90 confirming that the mutagenesis of the SLC30A2 Isoform I was succesfull. This construct will be transferred to an expression system in order to perform transient transfection.

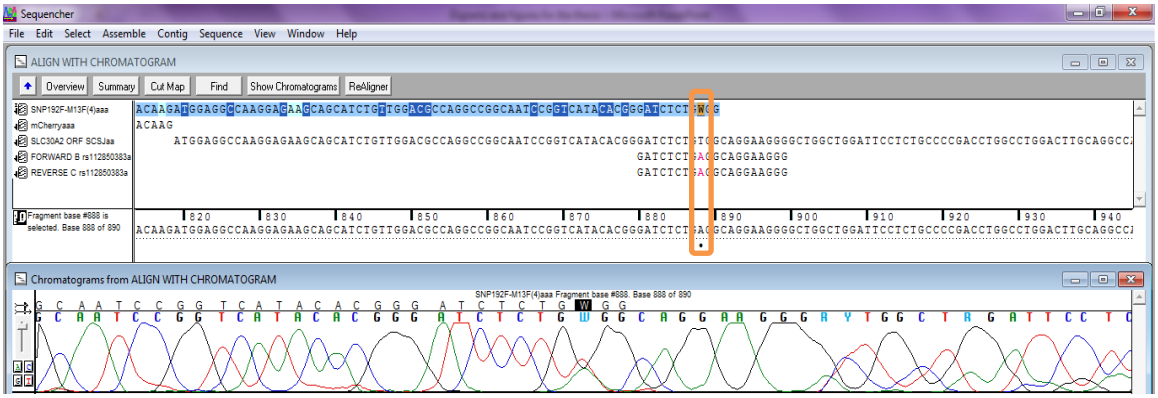


Figure 27 Alignment of sequences of construct of SLC30A2- rs112850383 (SNP 19)

After sequencing and alignment of the SLC30A2- rs112850383 (SNP 19) a weak signal (**W**) was identified on base number 70.

The weak signal shows that in the same plasmid we have a **T** and **A,** the base of interest is **A**. In order to isolate the clone of interest plasmid extraction was performed, but it was not successful. Therefore we did not use this clone for transient transfection.

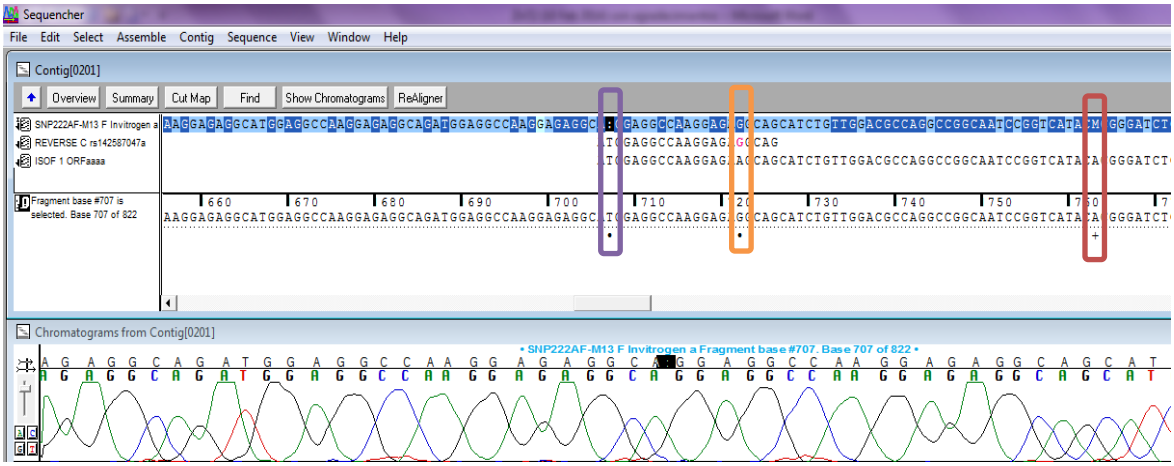


Figure 28 Sequence alignment of construct of the SLC30A2-rs142587047 (SNP 22)

For SNP 22 the change of base G/A in base number 18 was confirmed. In addition to that there was another mutation inserted in that clone in base number 2 **(A/T)** and an additional one in base number 58 **(A/C)**. This change of base causes change of amino acid Arginine/ Lysine in the protein, implying that the insertion of this mutation potentially could cause a change of function of the SLC30A2.

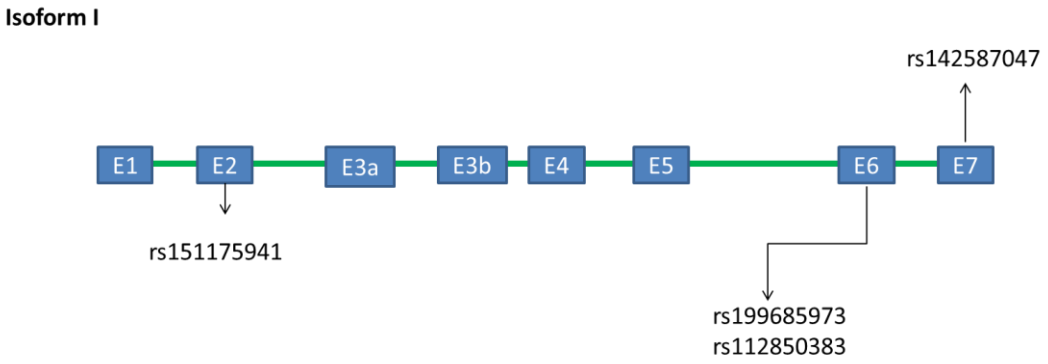


Figure 29 Localization of the SNPs introduce separately into the SLC30A2

The rs199685973 (SNP 17), rs151175941 (SNP5), rs112850383 (SNP19) and rs142587047 (SNP22) are localized in different regions in the SLC30A2 gene (Figure 28) Rs199685973 (SNP 17) and rs112850383 (SNP19), are localized in the exon number seven, as for rs151175941 (SNP5) and rs142587047 (SNP22) are localized in exon two and eight respectively. More detail is included in the following table:

Table 11 SNPs introduced successfully into the SLC30A2 (NCBI, February 2014)

SNP	Chr. position	Exon loc.	dbSNP rs# cluster id	Function	dbSNP allele	Protein residue	Codon pos	Amino acid pos
SNP 5	26366346	5	rs151175941	missense	A	Arg [R]	1	299
				contig reference	G	Gly [G]	1	299
SNP 17	26371669	7	rs199685973	missense	T	Cys [C]	3	30
				contig reference	G	Trp [W]	3	30
SNP 19	26371689	7	rs112850383	missense	C	Arg [R]	1	24
				contig reference	T	Trp [W]	1	24
SNP 22	26372371	8	rs142587047	missense	G	Arg [R]	2	6
				contig reference	A	Lys [K]	2	6

8.6 Transient transfection Wild Type SLC30A2 in HC11 cells

The transient transfection in HC11 cells was performed after cellular differentiation.

After 72h of transient transfection in HC11 cells, Isoform I and Isoform II tracking tGFP fluorescent protein and mCherry respectively (Figure 29).

Fluorescence was visualized by fluorescence microscope, and we found that Isoform I was over-expressed in some HC11 cells. We did not determine transfection efficiency.

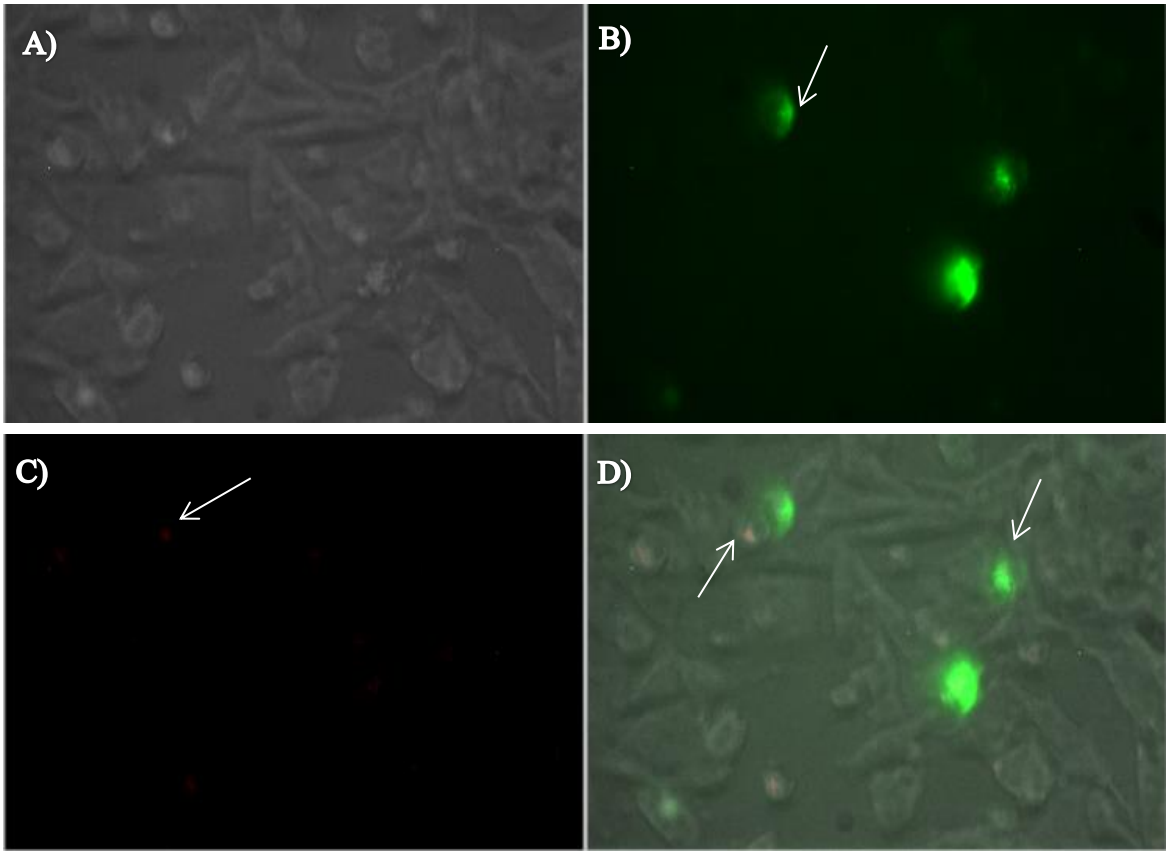


Figure 30 Fluorescent microscopy of HC11 cells SLC30A2 Isoform I and II after 72 hours of transfection

In panel **A**, figure 30, the HC11 cells were observed with bright light, on the panel **B**, the HC11 cell expressing the SLC30A2 Isoform I tagged with tGFP fluorescent protein, in this pool the secretory vesicular Cyto Tracer was added. The localization of Isoform I, as

we observed is on the secretory vesicles. In panel **C** we visualized the SLC30A2 Isoform II tagged with mCherry red fluorescent protein expressed in the cell. In the last panel, panel **D**, we observed the merge of SLC30A2 Isoform I and II with the secretory vesicular Cyto Tracer, localized in different sites of the HC11.

8.7 Transient transfection of mutant SLC30A2

The transient transfection in HC11 cells was performed after cellular differentiation.

After 4 days of transient transfection in HC11 cells, we visualized mutated Isoform I-mCherry-SNP22 (figure 31), SNP5 (figure 30) and 17(figure 32) and Cyto-tracer for secretory vesicles

HC11 transfected cells were visualized by fluorescence microscope, and we found that Isoform I is expressed in the HC11 cells.

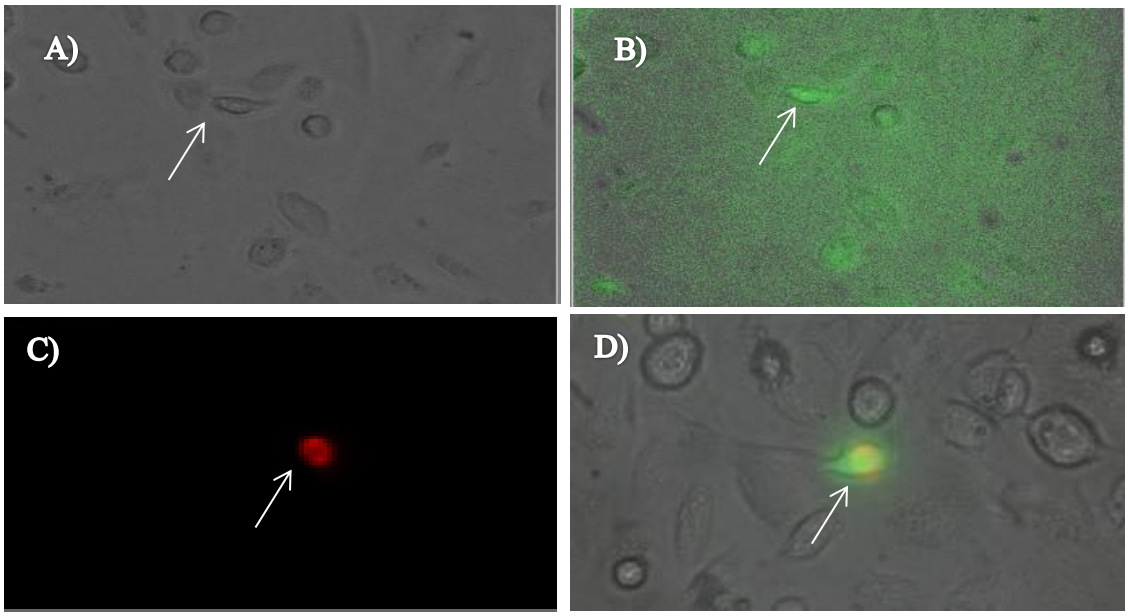


Figure 32 Fluorescent microscopy of HC11 cells transfected with SNP 22 (rs142587047) and green secretory vesicular Cyto Tracer after 72 hours of transfection; Panel A, bright light, panel B, GFP channel, panel C Alexa 546, panel D, merge of GFP and Alexa 546.

The first panel **A** (Figure 32)**,** we visualize the cell with bright light of the transfected HC11. In panel **B** we observe the Green secretory vesicular Cyto Tracer. On panel **C** the

SLC30A2 Isoform I- SNP5 is tagged with mCherry red fluorescent protein. The last panel, **D**, shows the merge of mutated SLC30A2 Isoform I- SNP5 tagged with mCherry red fluorescent protein and Green secretory vesicular Cyto Tracer.

As observed in the preliminary results in figure 31, panel **D** the presence of the SNP22 in the SLC30A2 Isoform I do not change the localization of the SLC30A2 in the HC11 cells.

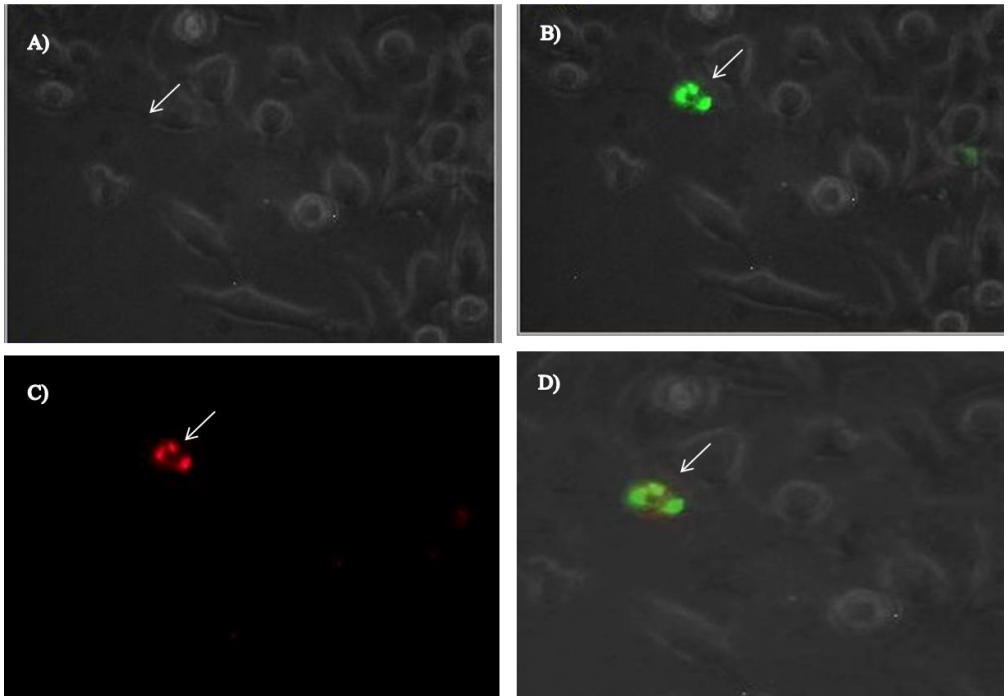


Figure 33 Fluorescent microscopy of HC11 cells transfected with SNP 17 (rs199685973) and green secretory vesicular Cyto-Tracer after 72 hours of transfection; Panel A, bright light, panel B, GFP channel, panel C Alexa 546, panel D, merge of GFP and Alexa 546.

In Figure 33, panel **A,** the transfected HC11 was visualized with bright light, panel **B,** we observe the HC11 cells transfected with green secretory vesicular Cyto Tracer. In panel **C** we visualize with bright light the transfected HC11 with SLC30A2 Isoform I –SNP17 tagged with red fluorescent protein with a green secretory vesicular Cyto Tracer. In panel **D**, the merge of transfected HC11 with mutated SLC30A2 Isoform I –SNP17 tagged with mCherry red fluorescent protein and green secretory vesicular Cyto Tracer.

In the preliminary results, as was observed in figure 32, panel **D,** the SLC30A2 Isoform I –SNP17 is localized in the same place as the secretory vesicular Cyto-Tracer; the presence of the SNP in the SLC30A2 does not affect the localization of the SLC30A2.

9 Discussion

Zinc depletion in human milk may occur due to mutations in zinc transporters in the mammary gland. Zinc concentrations in human milk decrease over the first months of lactation. This may be due to the increased volume of milk secreted per day or could involve genetic factors, including potential genetic variations in the mammary epithelial cells. One such factor would be a mutation in SLC30A2.

There are several mutations that have been identified in humans. They are hypothesized to change the functional protein leading to low zinc milk concentrations.

To date, there are more than 39 known genetic variations, 22 of which can potentially change the function and localization of SLC30A2 affecting zinc concentration in human milk. Therefore, the targeting of these 22 genetic variations to test localization of SLC30A2 is extremely important, due to the potential change of localization and possibly function of SLC30A2.

We aimed to insert a single genetic variation in SLC30A2 in order to track the localization of the mutated SLC30A2 transporter. Mislocalization could lead to misfunction of the transporter's ability to transfer zinc into human milk.

In order to know and confirm localization of Isoform I and II we cloned and sub-cloned the SLC30A2 transporter.

We followed a different approach for cloning and sub cloning from that used previously by Lopez & Kelleher (2009).

We amplified Isoform I from placental cDNA and Isoform II from a cDNA clone. Lopez & Kelleher (2009) amplified both isoforms from a cDNA clone. We genetically encoded the SLC30A2 labelling it with two different fluorescent proteins, tGFP and mCherry in order to visualize the two Isoforms.

Lopez & Kelleher (2009) and Chowanadisai et al. (2006) tagged Isoform I to a C terminus with two tandem hemagglutinin (HA) epitopes for later insertion into a pcDNA3.1/V5-His TOPO VECTOR. We used an N-terminal tGFP fluorescent protein (green fluorescent protein) to tag SLC30A2 Isoform I for later insertion into a pcDNA/V5-DEST.

Lopez and Kelleher (2009) identified Isoform I in distinct vesicular compartments, using two different vesicular marker antibodies: endosomal vesicle marker (mouse anti-human, M6PR) and exocytotic vesicle marker (anti-human vesicle-associated membrane protein-8). These two secondary antibodies are monoclonal; the results that were obtained were accurate. For Isoform II a polyclonal mouse anti-mouse pan-cadherin plasma antibody marker was used.

Immunoblot was used to verify the association with the cell membrane. The assay needs to be replicated to confirm that Isoform II is actually present in the cell membrane using a monoclonal antibody.

We confirmed the presence of tGFP-SLC30A2 Isoform I with a cellular green fluorescent Cyto-Tracer for exocytotic vesicles.

We confirmed the Lopez and Kelleher findings (2009) that Isoform I is localized in secretory vesicles. Isoform II was successfully transfected into the HC11 cells. We did not use any membrane Cyto-Tracer during transfection due to our interest in Isoform I

and the main role of zinc secretion into the human milk; therefore we cannot confirm that Isoform II is localized in the cell membrane.

To analyze the localization of the missense mutations we performed Site-Direct Mutagenesis.

Site-Directed Mutagenesis

We followed two approaches. In the **first approach,** we successfully fused the mutated SLC30A2 with mCherry red fluorescent protein using the sense primer mCherry R1 nostop- SLC30A2 F1 fusion primer and the antisense primer SLC30A2 R1stop. We then attempted attachment with the AttB primers, however the attempt was unsuccessful.

Methods used previously to mutate the SLC30A2 were based on the Phusion® Site-Directed Mutagenesis Kit (Qian et al., 2009; Seo & Kelleher, 2010), which is a three step procedure.

Based on the protocol of this kit we decided to follow a **second approach** for Site Directed Mutagenesis. Our second approach was based on two major steps. We obtained only seven positive amplicons for this PCR reaction. The second step was performed with the seven positive amplicons and a Gibson Assembly™ Master Mix.

We obtained four positive clones, tagged with mCherry red fluorescent protein. During analysis on the Sequencher 5.0 of rs112850383 (SNP 19) results showed a change of base in the expected base, number 70, to be either **A** or **T**. In order to isolate the correct clone with the change of base of interest (**A),** this base is the variation for this SNP; we transformed cells with this specific clone and extracted seven different plasmids. For rs199685973 (SNP 17) and rs151175941 (SNP5), we successfully inserted the change of base in base number 90 (**T/G**) and base number 895 (**A/G**) of the ORF respectively.

In the case of rs142587047 (SNP22), we were successful in inserting the desired mutation (**G/A**) in base number 17. In addition we found another variation inserted in base number 6. This caused an amino acid change (Arginine/ Lysine) in the expressed protein. We are not certain why an additional mutation was inserted. We checked and confirmed that the primers designed for SNP22 were correctly designed.

In 2006, Chowanadisai et al. reported on two exclusively breast-feed five month old infants with low serum zinc concentrations. They were delivered from mothers with normal zinc serum concentrations. After maternal sequence analysis a missense mutation was identified. This mutation is localized in **exon 2** and causes the substitution of base adenine to guanine, causing an amino acid change from histidine to arginine **(H54R)**. After transient transfection, immunobloting and a zinc uptake assay in HEK-293 cells Chowanadisai et al. (2006) determined that the presence of the H54R mutation in the SLC30A2 caused a decrease in zinc secretion due to protein misfolding, leading to TNZD in the infants. Similarly, another heterozygous mutation **G87R** was identified in two unrelated Ashkenazi Jewish mothers. The presence of this genetic variation lead to mislocalization in the Golgi apparatus and loss of zinc transport activity as reported in three different cell lines (HEK-293, HC11 and MCF-7). Further, two different SNP_s were identified in a Japanese woman by Itsumura et al (2013), which infants developed zinc deficiency.The presence of c.454T>C (exon 4) and c.887C>T (exon 7), result in low milk zinc concentrations, also causing TNZD in the infant. The effect of these two mutations has not yet been tested *in vitro*. Seo & Kelleher (2010) found that the presence of two new SNPs, rs35235055 and rs35623192, cause mislocalization of SLC30A2. SNP 1 (rs35235055) was found to be mislocalized into lysosomes, causing zinc accumulation.

The localization of SNP1 was visualized with rabbit anti-Lamp1 (polyclonal antibody). This genetic variation resides in the NH_2-Terminal, causing an amino acid change (Leu23Pro). The mislocalization of SLC30A2 causes a decrease in cytoplasmatic zinc pools, reducing zinc secretion. SNP 2 (rs35623192) was found to be mislocalized into the Golgi apparatus by a rabbit anti-P58 polyclonal marker. The presence of rs35623192 (T/C) eliminates the function of SLC30A2.

We used a red fluorescent protein (mCherry) to visualize rs199685973 (SNP 17), rs151175941 (SNP5) and rs142587047 (SNP22); to identify the localization of these genetic variations we used a green Cyto-Tracer for secretory vesicles. Our results showed that the presence of SNP 17, SNP 5 and SNP 22 do not affect the localization of SLC30A2 Isoform I. The amino acid change that is caused by SNP 22, (Arginine/ Lysine) can lead to a dominant negative effect in the function of SLC30A2 (NCBI, 2014).

We now know that the presence of SNP22 does not impact localization, but may cause zinc retention in other organelles or even protein misfolding leading to a negative effect on zinc transport. Also, either the presence of SNP 5, SNP 17 or SNP 22, could affect expression of SLC30A2.

SNPs 5, 17 and 22 could completely or partially affect the secretory function of SLC30A2 causing low milk zinc concentrations leading to TZN. During the lactation period, zinc transporters are regulated and stimulated by hormones (estrogen and prolactin) so that zinc homeostasis is maintained (Kelleher et al., 2009). In 2011 a study conducted in mice, (Kelleher et al, 2011), revealed that different Zip and ZnT transporters are involved in zinc transport from the mammary cells into the milk. It is possible that the

presence of these SNPs affects neither localization nor function on SLC30A2, because of the role of the other zinc transporters (ZnT4, 5; Zip 1, 3, 8, 10, 11, 13) that are expressed during the lactation period.

10 Conclusion

After transfection of WT SLC30A2 into HC11 cells, we confirm that Isoform I is localized in the secretory vesicles, due to the usage of vesicular Cyto-Tracer. Isoform II is localized in distinct cellular compartment, as the fluorescent images show.

After introduction of four missense SNPs, rs199685973 (SNP 17), rs151175941 (SNP5) and rs142587047 (SNP22), into SLC30A2 and transfection into HC11 cells, it was observed that these do not affect the localization of the SLC30A2 Isoform I in the HC11 cells. In order to explore the potential functional impacts on SLC30A2, further functional studies should be done.

11 Future directions

To explore the effect of SNPs 5, 22 and 17, we suggest future functional studies with HC11 cell line. For the effect of the SNPs in zinc secretion, we propose a zinc secretion assay with ^{65}Zn using a stable cell line with the WT- SLC30A2 Isoform I together with the SNP. This cell line should be grown in a zinc rich medium (100-200 μm). For later localization of labeled zinc, a fluorescent zinc-specific dye could be used in order to determine where zinc is accumulated or secreted.

12 Limitations

In this research the main focus was on the missense mutations, due to the potential to cause change of function of the functional protein for which the SLC30A2 encodes for. The polymorphisms introduced in the SCL30A2 were four missense mutations. Even though the nonsense polymorphisms could not change the function of the gene, the SNPs can be introduced and transfected in either mice or human mammary epithelial cells, to explore the possibility of mislocalization of the SLC30A2.

On the other hand, the cell model used to test the localization of the mutated SLC30A2 was mammary epithelial cell from mice, HC11, stimulated by prolactin, since we are focusing on the lactating period. The transient transfection in non-stimulated HC11 was not performed. It is important to know the difference in the localization of the SLC30A2 in the secreting phenotype and the non-secreting phenotype.

In addition, the cell line model could be changed or tested at the same time in a healthy human mammary epithelial cell. This would explore the similarities and differences in localization of this transporter in both cell lines.

13 Figures

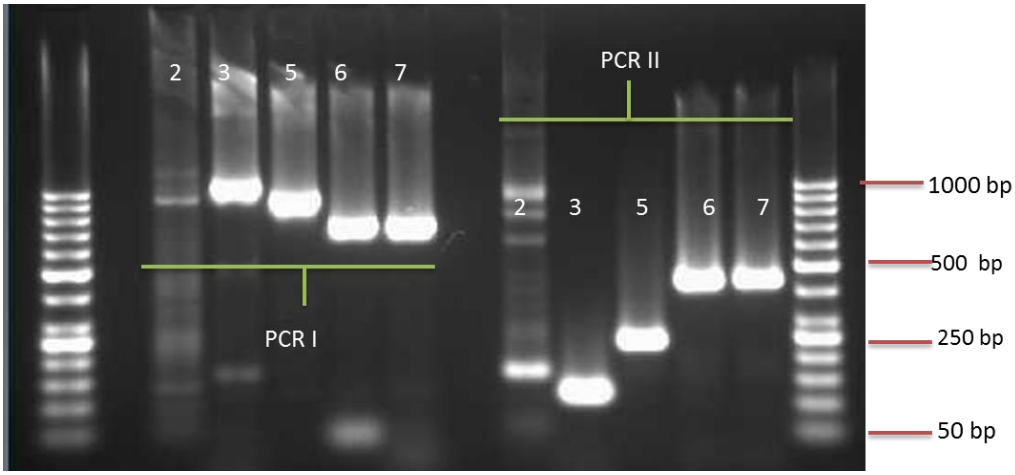


Figure 34 PCR I and PCRII of SNP 2, 3,5,6,7. The DNA Ladder used was GeneRulerTM 50bp

PCR I and II of mutagenesis first approach of SNP 2,3,5,6 and 7.

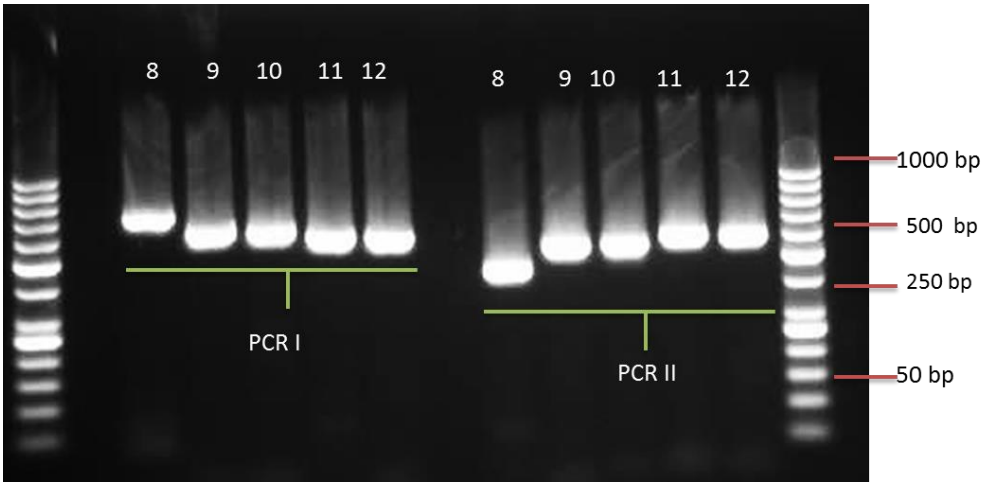


Figure 35 PCR I and PCR II of SNPs 8, 9,10,11,12. The DNA Ladder used was GeneRulerTM 50bp

PCR I and II of mutagenesis first approach of SNP 8, 9, 10, 11 and 12.

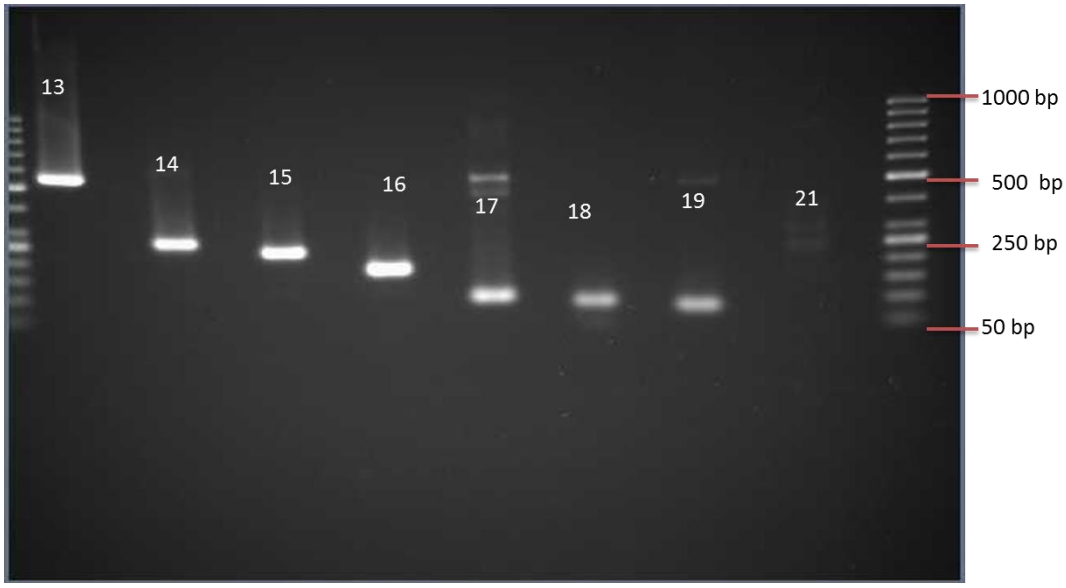


Figure 36 PCR I SNPs 13,14,15,16,17,18,19 and 21. The DNA Ladder used was GeneRulerTM 50bp

PCR I of mutagenesis first approach of SNP 13, 14, 15, 16, 17, 18, 19 and 21.

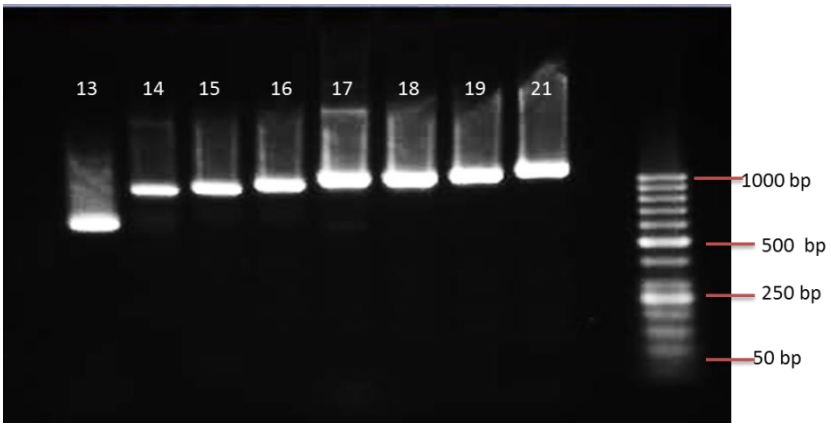


Figure 37 PCR II SNPs 13,14,15,16,17,18,19 and 21. The DNA Ladder used was GeneRulerTM 50bp

PCR II of mutagenesis first approach of SNPs 13, 14, 15, 16, 17, 18, 19 and 21.

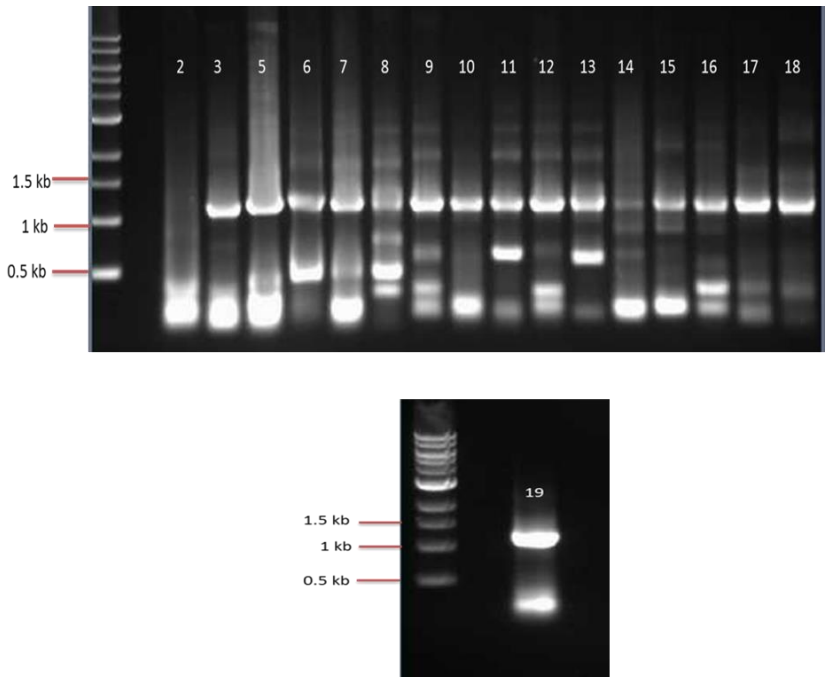
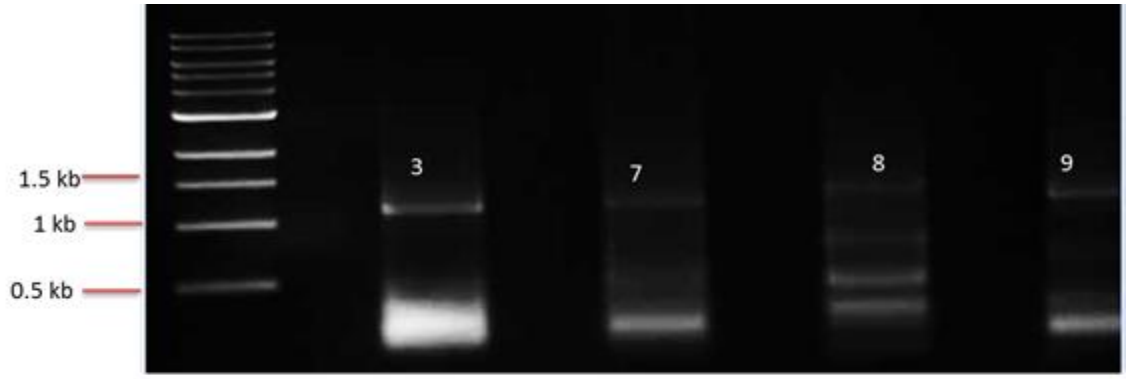
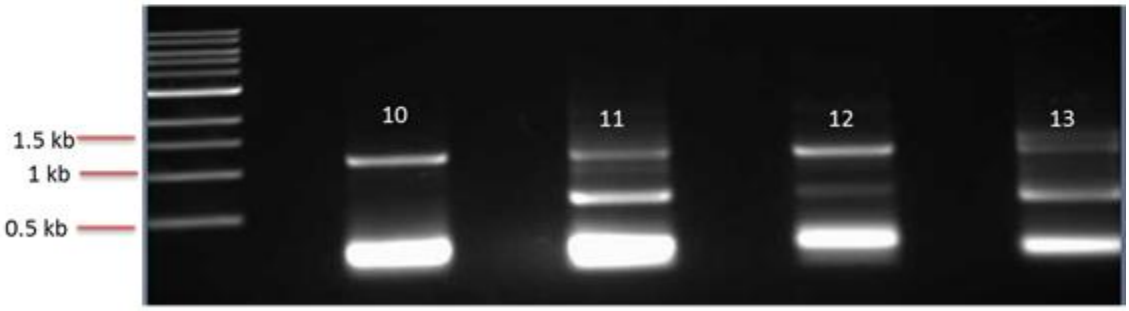


Figure 38 Fusion of PCR I and PCR II was successfully for fourteen SNPs, we obtained the correct size, 1.118 kb

PCR III (fusion of PCR I and PCR II) of mutagenesis first approach of positive amplicons of PCR I and PCR II SNP 13, 14, 15, 16, 17, 18, 19 and 21.



3) rs35623192, 7)rs200039883, 8) rs144129605, 9) rs201917367



10) rs112127101,, 11) 200520278, 12) rs199807670, 13) rs148861822

Figure 39 Positive amplicons after using fusion primers of SNP 3, 7,8,9, 10, 11, 12 and 13.

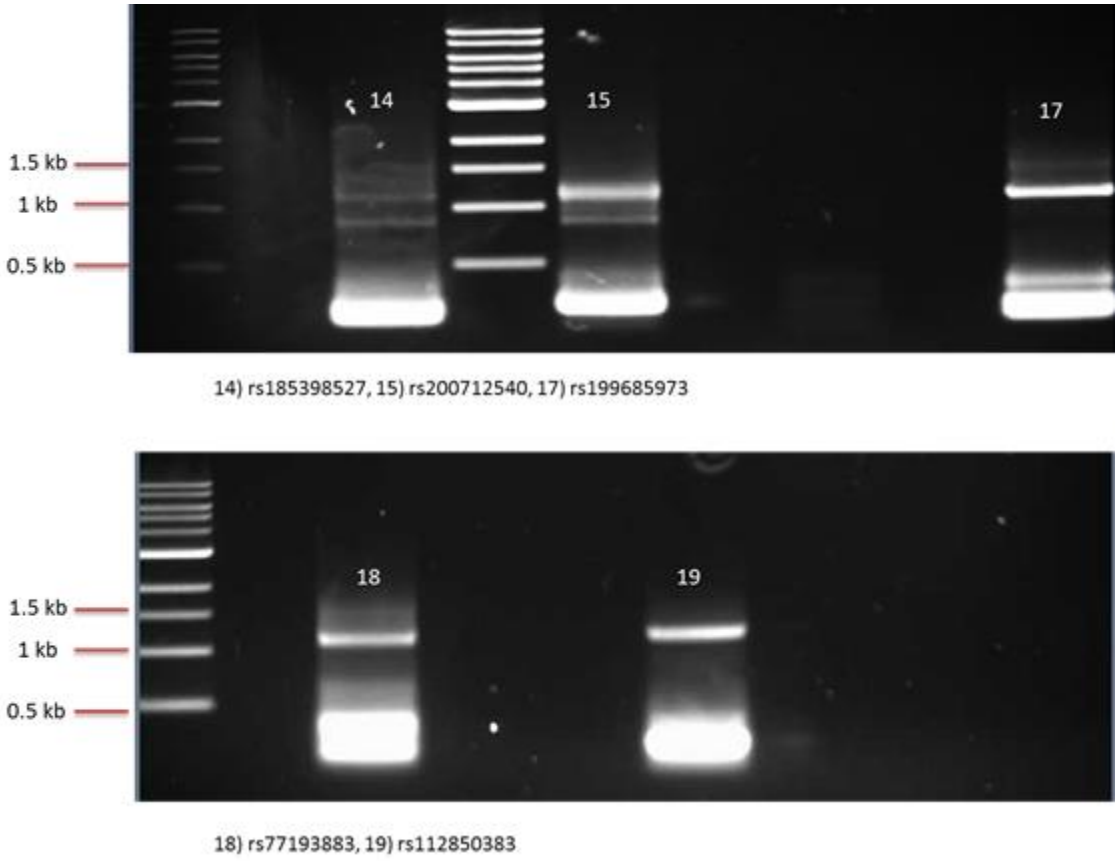


Figure 40 Positive amplicons after using fusion primers of SNPs 14, 15, 17, 18 and 19.

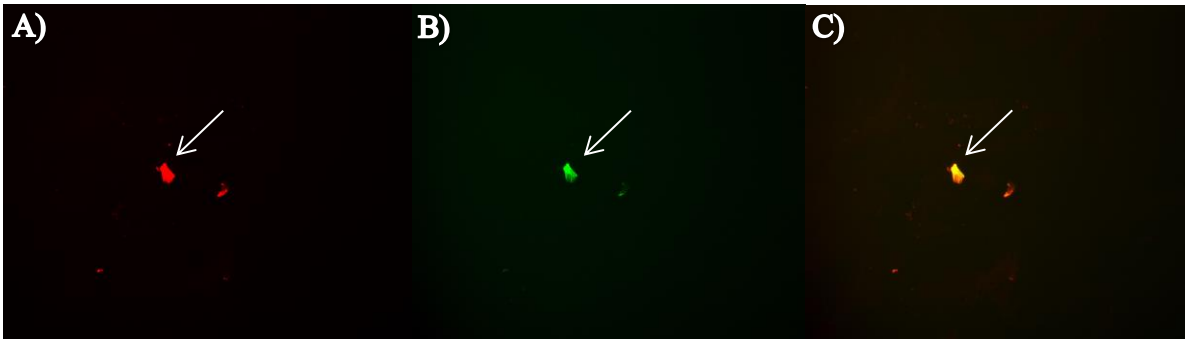


Figure 41 Fluorescent microscopy of HC11 cells transfected with SNP5 (rs151175941) and green secretory vesicular Cyto Tracer after 72 hours of transfection

In panel **A** (figure 31) the transfected HC11 with mutated SLC30A2 Isoform I- SNP5 tagged with mCherry red fluorescent protein, panel **B** Green secretory vesicular Cyto Tracer.

On the last panel **C**, the merge of mutated SLC30A2 Isoform I- SNP5 tagged with mCherry red fluorescent protein and Green secretory vesicular Cyto Tracer.

These are preliminary data for colocalization of SNP5, there is no resolution and we are not having the bright light in the HC11 cell, therefore the transfection and imaging should be performed.

14 Appendix

14.1 Table 1 Primer Sequences for isoform I and II with its respective fluorescent protein

Primer name	Sequence
mCherry/SLC30A2 Isoform 1	
SLC 30A2 F1	5'-ATGGAGGCCAAGG AGAAGCAG-3'
SLC 30A2 R1stop	5'-TCAGTCTGAGGGGCCCTGGCA-3'
SLC30A2 A R1stop attB2	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTC TCAGTCTGAGGGGCCCTGGCA-3'*
mCherry-ATG-Kozak-F1	5'-GCCACCATGGTGAGCAAGGGCGAG-3'
mCherry-ATG-Kozak-F1-attB1	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTC GCCACCATGGTGAGCAAGGGCGAG-3'
mCherry R1 nostop	5'-CTTGTACAGCTCGTCCAT-3'
mCherry R1 nostop-SLC30A2 F1 phusion primer	5'-GACGAGCTGTACAAGATGGAGGCCAAGGAGAAGC AG-3'*
tGFP/SLC30A2 Isoform 2 (exon 3 skpd)	
SLC 30A2 F1	5'-ATGGAGGCCAAGG AGAAGCAG-3'*
SLC30A2 R1stop	5'-TCAGTCTGAGGGGCCCTGGCA-3'*
SLC 30A2 R1stop attB2	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTC TCAGTCTGAGGGGCCCTGGCA-3'*
tGFP-ATG-Kozak-F1	5'-GCCACCATGGAGAGCGACGAGAGC-3'
tGFP -ATG-Kozak-F1-attB1	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTC GCCACCATGGAGAGCGACGAGAGC-3'
tGFP R1 nostop	5'-TTC TTC ACC GGC ATC TGC-3'
tGFP-ATG-Kozak-F1	5'-GCCACCATGGAGAGCGACGAGAGC-3'
tGFP R1 nostop-SLC30A2 F1	5'-GCAGATGCCGGTGAAGAAATGGAGGCCAAGGAGA AGAG-3'
tGFP -ATG-Kozak-F1-attB1	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTC GCCACCATGGAGAGCGACGAGAGC-3'

*these primers can be used for isoform I and Isoform II

14.2 Table 2 SNPs primer sequences Isoform I

SNP	PRIMER FORWARD B	PRIMER REVERSE C
rs144832711	TTCCACACCATGACCATCCA	TGGATGGTCATGGTGTGGAA
rs145406127	CTTGGAGGTGGCTGCTGGCT	AGCCAGCAGCCACCTCCAAG
rs35623192	ACAGCCAGCAGCTGCCTCCAA	TTGGAGGCAGCTGCTGGCTGT
rs149723161	AGAATACAGACACCCAGGCT	AGCCTGGGTGTCTGTATTCT
rs151175941	TGTCGGTGGAGAGGGTAGAA	TTCTACCCTCTCCACCGACA
rs149340896	TCACAGCTGTTCATGATCTG	CAGATCATGAACAGCTGTGA
rs201084300	AGCATGGATGTCCTAGTGGC	GCCACTAGGACATCCATGCT
rs200039883	CAGAGCATAGGTGTCCTAGT	ACTAGGACACCTATGCTCTG
rs144129605	TGCAGAGCGTGGGTGTCCTA	TAGGACACCCACGCTCTGCA
rs201917367	AGTCTGACCATGGGCACAGC	GCTGTGCCCATGGTCAGACT
rs112127101	CTTCACCAGTGTGGCCATGG	CCATGGCCACACTGGTGAAG
rs200520278	GCTGCGCTGTGGCTGTGAAAATCAT	ATGATTTTCACAGCCACAGCGCAGC
rs199807670	CGCTGTGTCTGTGAACATCA	TGATGTTCACAGACACAGCG
rs148861822	GCTGATCATGTCGGGCTGCG	CGCAGCCCGACATGATCAGC
rs185398527	GTTCATGATCAGAGAAGTCG	CGACTTCTCTGATCATGAAC
rs200712540	CTGTATGTAACCTCTGCCAT	ATGGCAGAGGTTACATACAG
rs141752286	CTCAGAAGGATCCTGACAGT	ACTGTCAGGATCCTTCTGAG
rs199685973	CTGGCTGTATTCCTCTGCCC	GGGCAGAGGAATACAGCCAG
rs77193883	CAGGAAGAGGCTGGCTGGAT	ATCCAGCCAGCCTCTTCCTG
rs112850383	GATCTCTGAGGCAGGAAGGG	CCCTTCCTGCCTCAGAGATC
rs35235055	CGGGATCTCCGTGGCAGGAA	TTCCTGCCACGGAGATCCCG
rs144738392	GGAGAAGCACCATCTGTTGG	CCAACAGATGGTGCTTCTCC
rs142587047	ATGGAGGCCAAGGAGAGGCAG	CTGCCTCTCCTTGGCCTCCAT

14.3 Table 3 SNPs sizes

SNP NUMBER	SNP	SIZE PCR 1 (bp)	SIZE PCR 2 (bp)
1	rs144832711	1058	81
2	rs145406127	112	1027
3	rs35623192	1026	114
4	rs149723161	996	143
5	rs151175941	903	236
6	rs201084300	710	429
7	rs200039883	707	432
8	rs144129605	705	432
9	rs201917367	612	527
10	rs112127101	605	534
11	rs200520278	572	572
12	rs199807670	571	568
13	rs148861822	553	586
14	rs185398527	268	871
15	rs200712540	239	900
16	rs141752286	183	956
17	rs199685973	102	1037
18	rs77193883	92	1047
19	rs112850383	81	1058
20	rs35235055	78	1061
21	rs144738392	31	1108
22	rs142587047	21	119

14.4 Table 4 Transformation of competent cells

1. Add Iµl of cloning sample into the competent cells (One Shot®Top10 Chemically Competent *E.Coli*, Invitrogen)
2. Incubate on ice for 30 min
3. Heath shock 30 sec/42°C
4. Remove vials from 42°C bath and place them on ice 2 min.
5. Add 250 µl of S.O.C medium
6. Shake vials horizontally at 37°C/1h at 225 rpm

7. Spread 50 µl and 250 µl o/n pre-warmed selective plates, incubate at 37°C

14.5 Table 5 Bacteria growth

1. Place LB Broth Media in 50 ml tubes (in a sterilized area), add the antibiotic needed (Kanamacin or Ampicilin)
2. Place 3ml of LB Broth media containing antibiotic into 15 ml tubes.
3. With a toothpick take one colony and put the stick into the tube with LB media.
4. Place all the tubes on the incubator at 37°C over night shaking at 225 RPM.

14.6 Table 6 Transfection protocol

HC11 (P24) –transfection Effectine	ng/ul	Control	Isoform I SLC30A2	Isoform II SLC30A2	Isoform I and Isoform II
EC		15	15	15	15
tGFP- ISOFORM 1 SLC30A2	420.4		0.48		0.2
mCherry - ISOFORM 2 SLC30A2	461.2			0.4	0.2
ISOFORM 1 AND 2 SLC30A2					
Enhencer		0.4	0.4	0.4	0.4
Effectine		1.25	1.25	1.25	1.25
RPMI 1%FBS, add to the complexes		350	350	350	350
RPMI 1%FBS, add to the cells		100/plate	100/plate	100/plate	100/plate
Total on each well about 1ml					

14.7 Table 7 PCR I and PCR II concentrations

#	PCR	SNP	CONCENTRATION
1	1	2	9.6
	2		13
2	1	3	27.7
	2		16.1
3	1	5	21.1
	2		18.1
4	1	6	25.1
	2		20.3
5	1	7	26.9
	2		15.6
6	1	8	24
	2		19.8
7	1	9	21.3
	2		21.9
8	1	10	19.2
	2		17.4
9	1	11	25.2
	2		22.5
10	1	12	22.8
	2		19.6
11	1	13	26
	2		34.3
12	1	14	15.8
	2		14.1
13	1	15	14.6
	2		16.3
14	1	16	16.5
	2		16.5
15	1	17	15.9
	2		25
16	1	18	13.1
	2		23.8
17	1	19	15.3
	2		25
18	1	21	
	2		20

14.8 Table 8 Positive SNP SLC30A2 mutagenesis Isoform I concentrations

#	SNP	CONCENTRATION
1	3	40.1
2	7	36.8
3	8	45.1
4	9	43.1
5	10	45.2
6	11	48.4
7	12	46.1
8	13	45.4
9	14	38.3
10	15	40.3
11	16	45.5
12	17	44.8
13	18	42
14	19	46.7

14.9 Table 9 Positive SNP SLC30A2 mutagenesis (fusion) Isoform I concentrations

#	SNP	CONCENTRATION
1	3	31.6
2	7	36.9
3	8	31.9
4	9	36.7
5	10	34.8
6	11	34.9
7	13	44.5
8	15	47.1
9	16	31.5
10	17	41.2
11	19	49.3

14.10 Gel preparation

1% Agarose gel

1. Weight 1 g of agarose.
2. Place it in a 400ml flask.
3. Dilute into 400 ml of TAE 50X (Tris-Acetate-EDTA).
4. Place it in the microwave for 3 minutes (until it melted completely).
5. Pour off into a casting tray and insert the comb.
6. Let it stand at room temperature for 5 minutes and store it at 4°C and let it stand at least for 20 minutes.

For the 2% agarose gel weight 2 g of Agarose and follow the above mention.

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