

# **Characterization of Human Induced Pluripotent Stem Cells**

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Bachelor's Thesis  
December 2013  
Degree Programme in  
Laboratory Sciences

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## ABSTRACT

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Tampere University of Applied Sciences  
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LAMPUOTI, JARKKO:

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Bachelor's thesis 57 pages, appendices 6 pages  
December 2013

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Induced pluripotent stem cells (iPSC) are cells reprogrammed from somatic cells with a set of defined factors. Induced pluripotent stem cells have promising applications in cell and tissue modeling, drug screening, and possibly also in future regenerative therapies. IPS cells exhibit characteristics and potency similar to embryonic stem cells and similar methods are employed to characterize and differentiate them. The objective of this study was to confirm the quality of the iPS cell lines for use in research. The purpose of the study was to characterize qualitatively six iPS cell lines with respect to pluripotency using immunocytochemistry, polymerase chain reaction, and microscopy.

The expression of viral transgenes, endogenous pluripotency markers, and germ layer related genes was studied with PCR after reverse transcription. The expression of pluripotency marker proteins was analyzed with indirect immunofluorescence staining. Cell colony morphology was assessed with visual microscopy.

Each of the six cell lines were successfully characterized. All lines exhibited all or nearly all of the pluripotency markers and at least one marker for each germ layer. No transgene activity was observed. Colony morphology was characteristic for human pluripotent stem cells.

The results indicated that all cell lines studied were pluripotent stem cell lines and may thus be applied in research protocols. The origin of problems encountered with individual samples in PCR remains unknown but may imply the need for additional examination. To further study the possible differences in the gene expression results with different concentrations, a high-yielding RNA extraction protocol must be devised.

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Key words: iPS, characterization, stem cells, immunofluorescence, PCR

## TIIVISTELMÄ

Tampereen ammattikorkeakoulu  
Laboratorioalan koulutusohjelma

JARKKO LAMPUOTI:

Ihmisen uudelleenohjelmoitujen pluripotenttien kantasolujen karakterisointi

Opinnäytetyö 57 sivua, joista liitteitä 6 sivua  
Joulukuu 2013

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Indusoidut pluripotentit kantasolut (iPS-solut) ovat uudelleenohjelmoituja soluja, jotka on tuotettu kuljettamalla tietyt transkriptiotekijät erilaistuneeseen soluun. iPS-soluilla on lukuisia lupaavia käyttökohteita, kuten solu- ja kudsmallinnus, lääkeainetutkimukset sekä tulevaisuudessa mahdollisesti myös kliiniset hoidot. Indusoidut pluripotentit kantasolut ja alkion kantasolut ovat piirteiltään samankaltaisia ja niiden karakterisoinnissa ja erilaistamisessa hyödynnetään samoja menetelmiä. Tämän tutkimuksen tavoitteena oli varmistaa tutkimuksessa käytettävien iPS-solulinjojen laatu. Työn tarkoitus oli kvalitatiivisesti karakterisoida kuusi iPS-solulinjaa pluripotenssitekijöiden osalta käyttäen polymeraasiketjureaktiota, immunofluoresenssiväryäystä ja mikroskopointia.

Työssä tutkittiin virusperäisten siirtogeenien, solujen omien pluripotenssigeenien sekä alkiokerrosten geenien ilmentymistä PCR-tekniikalla. Pluripotenssitekijöiden ilmentymistä proteiinitasolla tutkittiin immunofluoresenssiväryäyksellä. Solukolonioiden muoto tutkittiin mikroskoopilla.

Kaikki karakterisoidut solulinjat ilmensivät lähes kaikkia tutkittuja pluripotenssitekijöitä. Virusperäiset siirtogeenit eivät olleet aktiivisia. Kolonioiden morfologia oli pluripotenteille kantasoluille tyypillinen.

Saatujen tulosten perusteella kaikki tutkitut solulinjat ovat pluripotenteja kantasolulinjoja ja niitä voidaan käyttää tutkimuksessa. PCR-analyysiin liittyneiden ongelmien syitä ei tiedetä, mutta menetelmää voidaan tarvittaessa pyrkiä kehittämään. Geeniekspression erojen vertailemiseksi eri RNA-konsentraatioilla tulee kehittää paremman saannon tuottava eristysmenetelmä.

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## 1 INTRODUCTION

Induced pluripotency refers to a condition where normally non-pluripotent somatic cells are forced to regress into a pluripotent state. First demonstrated by professor Yamanaka and his team with mouse fibroblasts and subsequently with human fibroblasts, induced pluripotency has become a prominent tool in biomedical research. (Takahashi & Yamanaka 2006; Takahashi et al. 2007.)

This thesis was done at the Heart research group in the Institute of Biomedical Technology at the University of Tampere. The group exploits and differentiates patient-specific induced pluripotent stem cells (iPSC) in the study of genetic heart diseases.

The objective of this study was to confirm the quality of the iPS cell lines used in the Heart group for research purposes. The purpose of the study was to characterize six iPS cell lines qualitatively using immunocytochemistry, polymerase chain reaction, and microscopy. In addition to valid research, correct characterization aids the use and development of protocols. The cell lines characterized in this thesis are analyzed with respect to cell pluripotency and transgene expression. Other specifics, such as disease-related mutations, are not assessed herein.

## **2 LITERATURE REVIEW**

### **2.1 Induced pluripotent stem cells**

#### **2.1.1 Stem cell potency**

Stem cells are cells that are capable of proliferating extensively and differentiating towards several other cell types (Kolios & Moodley 2013). Depending on the differentiation capacity, stem cells are classified into different categories: totipotent, pluripotent, multipotent, and oligopotent stem cells (Mitalipov & Wolf 2009; Kolios & Moodley 2013). Zygotes are considered to be totipotent cells, giving rise to all cells, including pluripotent embryonic stem cells and extraembryonic tissues (Lovell-Badge 2007; Mitalipov & Wolf 2009). Pluripotent stem cells are defined as cells capable of differentiating into any of the three germ lines and thus all adult cell types (Thomson et al. 1998; Mitalipov & Wolf 2009). Multipotent cells are derived from pluripotent cells and capable of differentiating into some cell types derived from a single germ layer, such as mesenchymal stem cells which form adipose tissue and bone, among others (Pittenger et al. 1999; Mitalipov 2009; Kolios & Moodley 2013). Oligopotent cells are considered tissue-resident progenitor cells with the potency to form a limited number of terminally differentiated cells (Kolios & Moodley 2010). Examples of oligopotent cells are lymphoid stem cells, forming B and T cells (Janeway et al. 2007, 15; Alberts et al. 2008, 1457). It may be noted that oligopotent cells are not always defined as stem cells if a stem cell is considered a cell able to proliferate indefinitely (Seaberg & van der Kooy 2003).

### 2.1.2 Induction

Cells resembling pluripotent embryonic cells (ESC) have previously been generated from somatic cells with two notable methods, nuclear transfer to oocytes, and fusion with ES cells (Yamanaka 2007). A breakthrough in reprogramming somatic cells came about when several studies displayed that fibroblasts from mouse and human are reprogrammable with defined factors, utilizing a viral vector (Maherali et al. 2007; Okita, Ichisaka & Yamanaka 2007; Takahashi et al. 2007; Wernig et al. 2007; Yu et al. 2007; Park et al. 2008). Subsequently, reprogramming has succeeded with multiple other cell types (Aoi et al. 2008; Hanna et al. 2008; Stadtfeld & Hochedlinger 2010).

The induction of somatic cells into iPS cells is frequently carried out using four transcription factors, OCT3/4, SOX2, Klf4, and c-Myc, also referred to as the Yamanaka factors. Other combinations of factors have also been employed, including 1-4 factors in most cases. (Stadtfeld & Hochedlinger 2010.) All factors are not always required, but OCT3/4 and SOX2 are generally employed (Eminli et al. 2008; Rizzino 2009; Stadtfeld & Hochedlinger 2010).

c-Myc is a factor occasionally replaced or left unused, as it is a known proto-oncogene and thus problematic with possible clinical applications when transduced with an integrating virus (Okita et al. 2007; Stadtfeld & Hochedlinger 2010). However, as it is shown that transgene integration into cell genome is not necessary for induced pluripotency, non-integrating methods have been devised to eliminate insertional mutagenesis (Okita et al. 2008; Varas et al. 2009). One of these methods exploits the use of non-integrating *Sendai* RNA virus (Fusaki et al. 2009). Other methods include the use of plasmids, episomes, reprogramming proteins, small molecules, and miRNA, among others (Huangfu et al. 2008; Yu et al. 2009; Grskovic et al. 2011; Miyazaki et al. 2012). Sometimes incomplete silencing or re-activation of transgenes occurs with retrovirally induced cells and may cause dependence on the transgene expression, tumorigenesis, and interference with development and differentiation (Stadtfeld & Hochedlinger 2010; Toivonen et al. 2013). Cell lines in this thesis have been produced with either retroviral or Sendai-viral induction (section 3.1.1). A schematic illustration of the procedure is presented in figure 1.

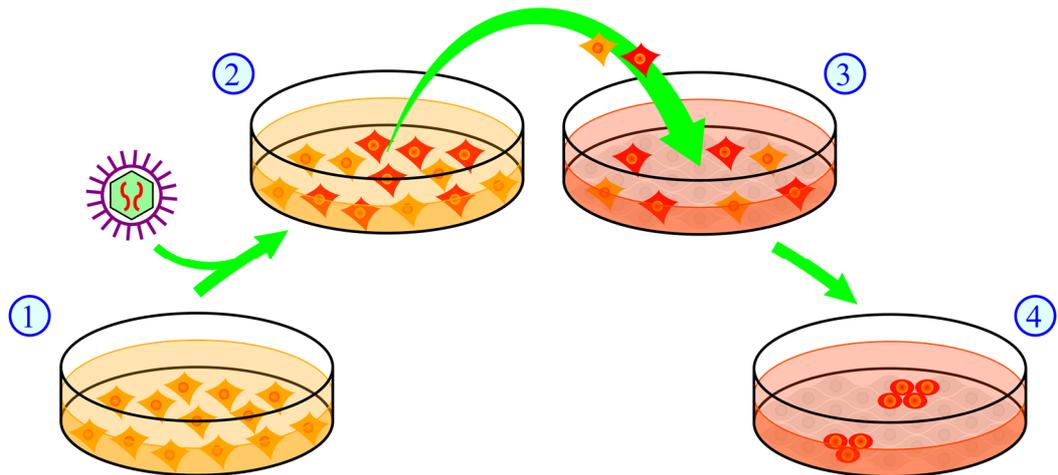


FIGURE 1. Induction protocol. Phases: (1) The isolation and culture of somatic cells. (2) Transferring of reprogramming factors. (3) Harvesting the cells and culturing in pluripotent cell conditions (on a feeder layer). (4) The formation of iPS cell colonies. (Image: Y. Tambe 2007)

When expressed in cells, the factors affect transcription, activating an expression network of several pluripotency-associated factors and series of events proceeding towards pluripotency (Kim et al. 2008; Wei et al. 2009). OCT3/4 and SOX2 are considered as core factors working synergistically, while c-Myc and Klf4 are thought to modify the chromatin structure for OCT3/4 and SOX2 binding (Takahashi 2007). In addition, c-Myc is proposed to universally amplify active gene expression, thereby enhancing the induction process (Nie et al. 2012). Obstacles that somatic cells must overcome in the course of reprogramming include the inhibition of somatic regulators, senescence and apoptotic pathways, the induction of proliferation, the activation of pluripotency loci, and independence from exogenous factor expression (Stadtfield & Hochedlinger 2010). Endogenous factors expressed after induction are briefly addressed in section 2.2.2.

### 2.1.3 Applications

iPS cells have prominent applications in cell and tissue modeling, drug discovery, gene repairing, and disease pathogenesis studies *in vitro*. In contrast to embryonic stem cells, iPS cells have the feature of being patient and disease-specific enabling the study of inherited conditions with iPS-cell derived cells and tissues without ethical concerns and limited availability surrounding embryonic cells and oocytes. Also, regenerative therapeutical applications could be conducted without the issue of tissue rejection. (Takahashi 2007; Yamanaka 2007; Stadtfelt & Hochedlinger 2010; Robinton & Daley 2012.) Figure 2 shows a scheme describing the applications of iPSC.

Human ES cells and iPS cells do not seem to exhibit significant differences in global gene expression patterns (Guenther et al. 2010). It is shown, however, that some iPS cells may retain a transient epigenetic memory especially in early passages (Kim et al. 2010). These differences with other concerns such as tumorigenesis need to be conclusively addressed before medical applications (Hyun et al. 2007).

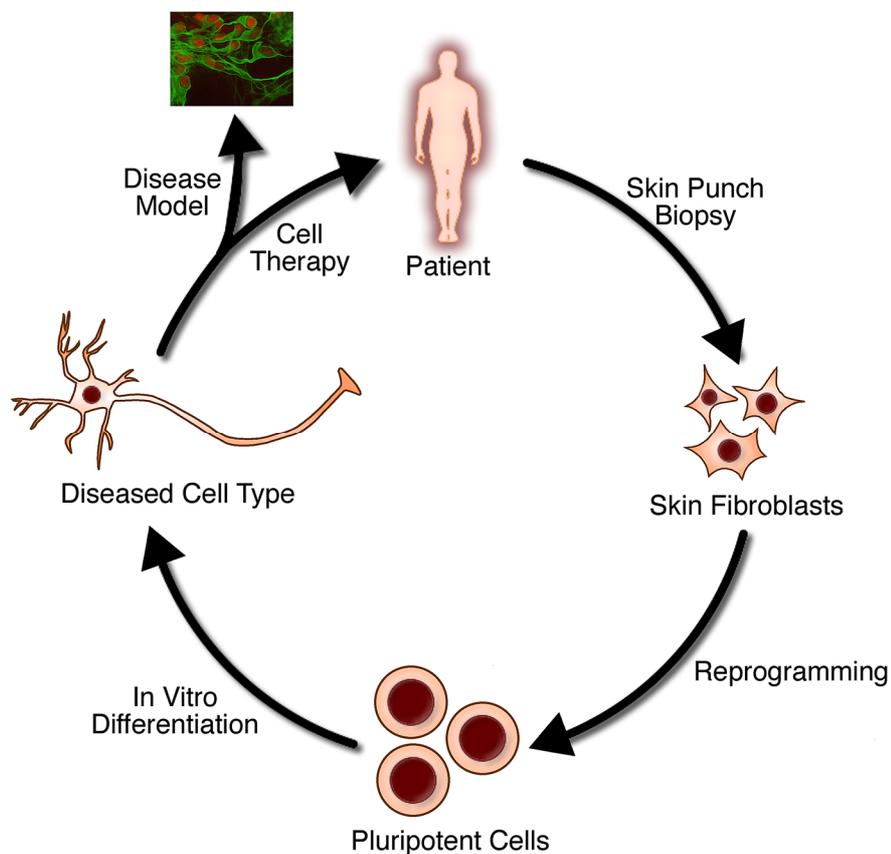


FIGURE 2. Applications for induced pluripotent stem cells. (Rodolfa 2008, modified)

## 2.2 Cell line characterization and pluripotency

### 2.2.1 Profile of pluripotency

The characterization of cells includes a set of defined tests to reach certainty on the cell type. iPS cells are frequently characterized with the criteria applied to ES cells (Ng & Choo 2010). As previously mentioned, there are some reported differences between iPS and ES cell characteristics considering expression patterns, but slight differences exist also between different iPSC and ESC lines. In the course of extended culture, however, iPS cells come to resemble ES cells probably due to the completion of reprogramming. (The International Stem Cell Initiative 2007; Ng & Choo 2010.) Features assessed by characterization include cell and colony morphology, growth rate, the expression of surface markers and other antigens, the expression of pluripotency marker genes, methylation statuses, enzyme levels, and differentiation into somatic cell types in vitro and in vivo (table 1).

TABLE 1. Pluripotency indicating conditions frequently studied in cell line characterization protocols. (Takahashi et al. 2007; Ng & Choo 2010; Tiscornia et al. 2011)

| Feature   | Status/marker/function  |
|---|---|
| Morphology  | Cells: high nucleus/cytoplasm ratio<br>Colonies: round, tight, smooth borders |
| Growth rate   | Comparable to ESC, $T_d \approx 40-50$ h.                                     |
| Surface marker proteins                                 | SSEA3, SSEA4, TRA 1-60, TRA 1-81, et al                                       |
| Undifferentiated ES markers                             | Nanog, OCT3/4, REX1, SOX2, GDF3, FGF4, ESG1, DPPA2, hTERT, et al              |
| Unmethylated promoters of pluripotency-associated genes | Nanog, OCT3/4, REX1, et al  |
| Enzyme levels   | Elevated telomerase levels<br>Elevated alkaline phosphatase levels            |
| Embryoid bodies (in vitro diff.)                        | Differentiation into the three germ layers                                    |
| Directed differentiation (in vitro)                     | Differentiation into specified cell types                                     |
| Teratoma formation (in vivo diff.)                      | Differentiation into the three germ layers                                    |

Among pluripotency markers, Nanog and OCT3/4 are regarded as archetypal pluripotent stem cell markers (The International Stem Cell Initiative 2007). In addition, it is noted that OCT3/4 and SOX2 work together through feedback loops regulating both their own transcription and other pluripotency-associated genes (Rizzino 2009). In this thesis, six antigens and five endogenous pluripotency marker genes are determined (table 2). The analysis of in vitro formed embryoid bodies is also carried out. Embryoid bodies are in vitro cell aggregates consisting of spontaneously differentiating cells (Kurosawa 2007). The target is to obtain cells from all three embryonic layers.

TABLE 2. Gene and antigen markers analyzed. (Ben-Shushan et al. 1998; Shi et al. 2006; Schopperle & DeWolf 2007; The International Stem Cell Initiative 2007; Rizzino 2009; Theunissen & Silva 2011; Nie et al. 2012.)

| Marker   | Analyzed expression | Role/type  |
|----------|---------------------|--|
| c-Myc    | Gene                | Expression amplifier, reinforcing the pluripotent state                        |
| Nanog    | Gene, antigen       | Establishment of induced pluripotency, resisting differentiation               |
| OCT3/4   | Gene, antigen       | Pluripotency maintenance and induction   |
| Rex1     | Gene                | Transcription factor (zinc finger protein), regulated by OCT3/4, SOX2, & Nanog |
| SOX2     | Gene, antigen       | Pluripotency maintenance and induction   |
| SSEA4    | Antigen             | Stage-specific embryonic antigen, cell surface glycolipid                      |
| TRA 1-60 | Antigen             | Stem cell surface keratan sulfate antigen epitope                              |
| TRA 1-81 | Antigen             | Stem cell surface keratan sulfate antigen epitope                              |

### 2.2.2 Factors maintaining pluripotency in cell culture

Culturing conditions including culturing wells, media, incubator environment, and supportive matrices affect strongly the condition of the cell culture. Feeder cells are used frequently in cell culture. Feeders are cells secreting substances that support the cultured cells and provide a matrix to attach. They are rendered incapable of proliferation with chemical or physical means.

Mouse embryonic fibroblast (MEF) cells are commonly used in pluripotent stem cell cultures, where MEF feeder layer aids in the undifferentiated propagation of cells. Fibroblast feeders secrete a mixture of growth factors including transforming growth factor  $\beta$  (TGF $\beta$ ), Activin A, Wnt proteins, BMP signaling antagonists, and some cell types also fibroblast growth factors (FGFs). Activin A is shown to induce OCT3/4 and Nanog, among others. FGF is essential for human embryonic stem cell self-renewal and has been shown to act in concert with pluripotency-maintaining signaling networks. FGF may be added into media as a supplement for maintaining stem cell pluripotency. (Vallier et al. 2005; Levenstein et al. 2006; Eiselleova et al. 2008; Abraham et al. 2010, Rajala et al. 2010; Pekkanen-Mattila et al. 2012.)

To gain reproducible and reliable results and to maintain a stable cell culture over extended periods, several defined media and feeder-free culture systems have been suggested and experimented with. For instance, fetal bovine serum (FBS) has been in many cases replaced with serum replacements and feeder layers with gel and protein-based systems. (Abraham et al. 2010, Rajala et al. 2010.)

## **2.3 Methods for characterization**

### **2.3.1 RNA extraction**

Intact, high-quality, and high-yield RNA is required for many purposes in molecular biology. (Muyal et al. 2009; Vomelová et al. 2009). In this thesis, total RNA is isolated to study the expression of genes via the polymerase chain reaction of transcribed cDNA. There are three principal methods for RNA isolation: organic solvent (usually phenol-chloroform) extraction, adsorption method, and isopycnic gradient separation (Muyal et al. 2009; Vomelová et al. 2009).

Compared to large molecule DNA extraction, shorter RNA molecules tolerate more mechanical stress and can be isolated from cells with disruptive methods. However, RNA is susceptible to degradation by omnipresent ribonucleases and factors such as heat, elevated pH, divalent cations, and extended storage periods. (Muyal et al. 2009; Wilson & Walker 2010, 165.)

Guanidinium isothiocyanate is a cationic detergent and a chaotropic salt which is used to lyse the sample and to denature endogenous ribonucleases. In the organic solvent method, the lysate is mixed usually with phenol-chloroform solution and centrifuged. Proteins precipitate between the phases. Upper phenolic phase containing nucleic acids is collected and RNA is precipitated with isopropanol, centrifuged and subsequently washed with ethanol. Deproteinisation can be carried out several times to ensure the purity of the RNA. (Muyal et al. 2009; Vomelová et al. 2009; Wilson & Walker 2010, 184-165.)

The adsorption method is based on the interactions of RNA and an adsorptive matrix in the presence of a chaotropic salt. The adsorptive surface may be in the form of beads, coating, or a solid matrix. Materials used in these systems are i.a. silica, polystyrene-latex materials, cellulose, and glass fibres. Depending on the specific system used, the contaminants are washed or centrifuged away. (Vomelová et al. 2009.) This method is employed for extracting RNA in this thesis using a commercial silica-based spin column kit (Macherey-Nagel 2011). See figure 3 for illustration.

In isopycnic gradient method, the sample is lysed as with other methods. The method is based on density differences between cellular components in a cesium salt solution. The solution is ultracentrifuged and the layer containing RNA is collected and precipitated. This method can be applied to isolate high purity RNA free of cellular components. (Vomelová et al. 2009; Wilson & Walker 2010, 164.)

Previously described methods are used for total RNA extraction but methods exist to isolate e.g. only mRNA (Vomelová et al. 2009; Wilson & Walker 2010, 167). Today, the most widely used techniques are the phenol-chloroform extraction and the silica-gel column based method. The silica-gel column method has been demonstrated to isolate longer RNA fragments intact, although the total RNA yield may be higher with the phenol-chloroform extraction. (Muyal et al. 2009.)

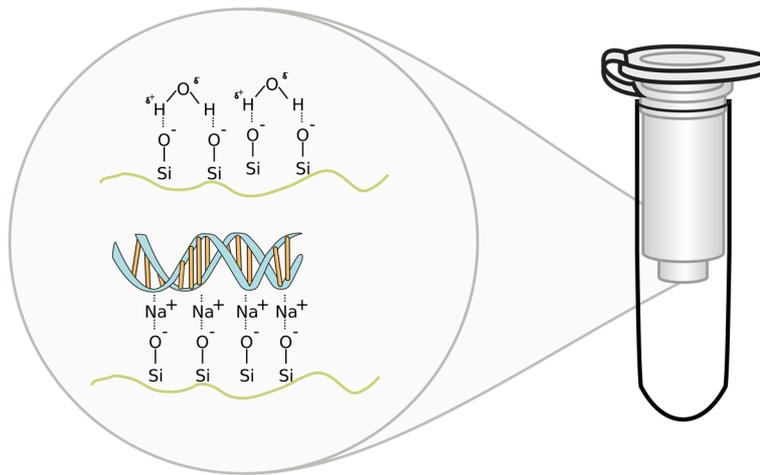


FIGURE 3. Silica-based spin column. (Image: Wikimedia Commons 2008)

### 2.3.2 cDNA transcription

To produce starting material for a polymerase chain reaction, isolated RNA has to be transcribed into complementary DNA (cDNA) (Alberts et al. 2008, 545). The transcription is carried out with reverse transcriptase (RT) enzyme in a solution containing random primers and deoxynucleotides (Wilson & Walker 2010, 201). Reverse transcriptase produces single-stranded DNA molecules on an RNA template (Alberts et al. 2008, 542).

### 2.3.3 Polymerase chain reaction

Polymerase chain reaction (PCR) is a widely and commonly used method in molecular biology. It employs a thermostable DNA polymerase enzyme, which amplifies any desired DNA sequence in a chain reaction where the amount of the sequence doubles in each cycle. PCR is an extremely sensitive method, detecting minuscule amounts of target DNA. (Alberts et al. 2008, 545; Campbell & Farrell 2012, 377.)

For the DNA polymerase to attach and begin synthesizing new DNA strands from deoxynucleotides (dNTPs), oligonucleotide primers are required. Primers are designed to complementary bind to both strands of DNA, one primer for each strand, together bracketing the segment to be amplified. To design the primers, knowledge of the original sequence is required. (Alberts et al. 2008, 545; Wilson & Walker 2010, 179.) Figure 4 shows an illustration of primers with DNA strands.

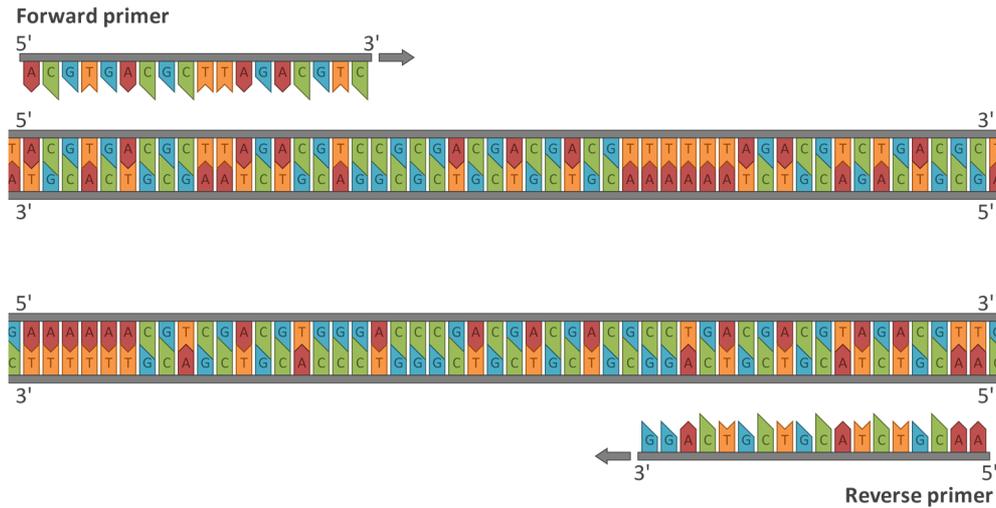


FIGURE 4. Primers showing complementarity with DNA strands. (Image: Wheeler 2013)

PCR includes three main phases. First, the reaction mixture is heated to **denature** the DNA and separate the two strands. Second, the temperature is lowered sufficiently for the primers to **anneal** to the strands. Third, the temperature is raised to optimize the activity of the DNA polymerase and begin the **extension**, or elongation, of strands. These three phases are repeated for multiple cycles, e.g. 20-30. (Alberts et al. 2008, 544; Wilson & Walker 2010, 181; Campbell & Farrell 2012, 377.) The phases are depicted in figure 5. PCR reactions are carried out in tubes inside a programmable thermocycler. Annealing temperature is calculated for each primer-pair according to its properties (Campbell & Farrell 2012, 377).

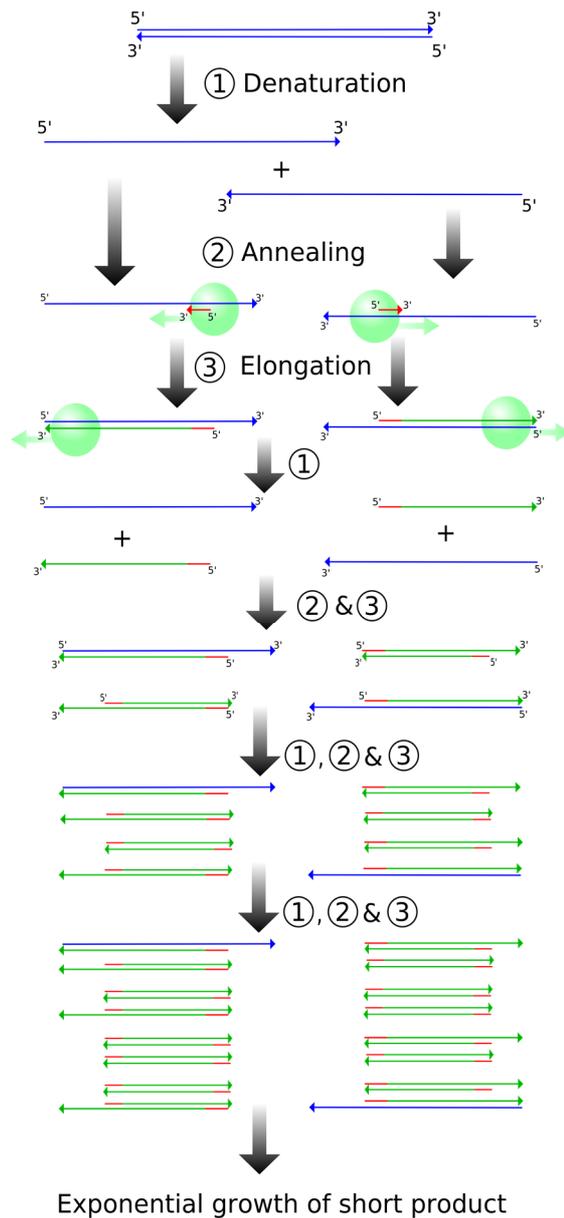


FIGURE 5. PCR phases. (Image: Ball 2007)

Reaction products can be visualized after the separation with gel electrophoresis. Running the DNA sample into an agarose gel using direct electric current separates DNA fragments, as larger fragments take more time to migrate through the pores in the gel matrix. DNA is frequently visualized using ethidium bromide as a component in the gel. Ethidium bromide intercalates between the stacked nucleotide base pairs and fluoresces when illuminated with UV light. (Watson et al. 2004, 648; Wilson & Walker 2010, 168.)

### 2.3.4 Immunofluorescence staining

Antibodies, also known as immunoglobulins, are proteins produced by plasma cells that bind specifically to particular substances referred to as antigens (Eales 2003, 41; Janeway et al. 2007, 824). Antibodies share the same fundamental structure (figure 6) (Wilson & Walker 2010, 270). Antibodies are utilized in biosciences to identify specific molecules in biological matrices.

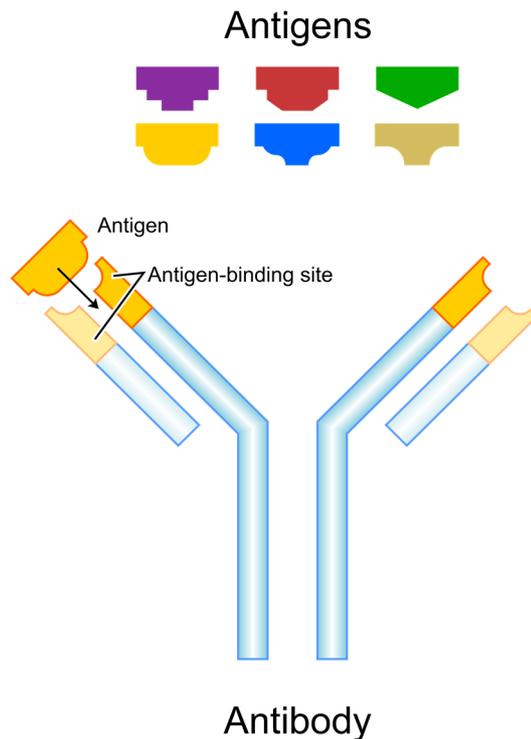


FIGURE 6. Simplified presentation of a Y-shaped antibody. (Image: Wikimedia Commons 2007)

Immunofluorescence staining refers to immunochemical methods based on the use of fluorescent dyes attached to antibodies. The location of the antigens in a sample is ascertained when the sample is illuminated with a dye-specific excitatory wavelength. Fluorescent label may be directly attached to the antibody or indirectly to a secondary antibody which, in turn, binds to the primary antibody. (Janeway et al. 2007, 735-737.) An indirect method is elucidated in figure 7.

A matter that is taken into account when using fluorescent dyes is a phenomenon called photobleaching, denoting damage to the dye when overexcited with light (Bernas et al. 2004; Wilson & Walker 2010, 116). Photobleaching is prevented with adequate staining practices, such as a dim environment and foil shielding. Anti-fading agents are also frequently included in mounting media for stained specimens (Longin et al. 1993; Ono et al. 2001).

In addition to staining the target molecules, common cell structures are frequently counterstained to obtain a more comprehensive view and vivid photographs. A common counterstain for cells is DAPI (4'-6-diamidine-2-phenyl indole), which stains DNA by insertion inside the helix (Larsen et al. 1989).

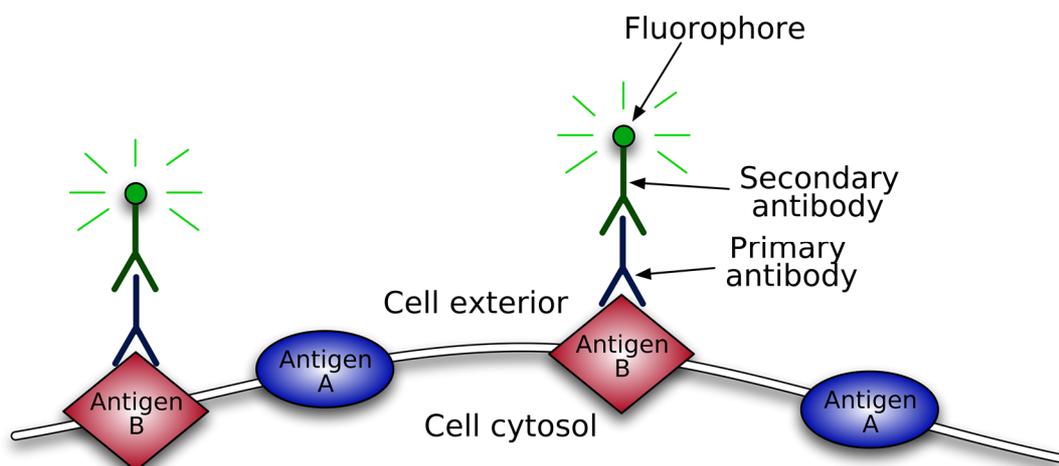


FIGURE 7. Indirect immunofluorescence staining. (Image: Munch 2007)

### 3 MATERIALS AND METHODS

#### 3.1 Cell culture

##### 3.1.1 Origin of the cells

The five human iPS cell lines characterized in this thesis had been produced from fibroblast cells obtained from skin biopsies. Skin biopsies had been taken from cardiac patients carrying a certain type of abnormality in their genome predisposing them to heart disease. A control cell line (wildtype) used had been produced from blood cells of a healthy individual. Cell lines are listed in table 3 with the induction method used. Abbreviations used in the names of the cell lines are explained in table 4.

TABLE 3. Cell lines.

| Cell line       | Induction method    |
|-----------------|---------------------|
| UTA.07001.CPVT  | Retrovirus          |
| UTA.05303.CPVT  | Retrovirus          |
| UTA.05404.CPVT  | Retrovirus          |
| UTA.01402.HCMTs | <i>Sendai virus</i> |
| UTA.02912.HCMTs | <i>Sendai virus</i> |
| UTA.11505.WTsb  | <i>Sendai virus</i> |

TABLE 4. Abbreviations in cell line designations. Latter capitalized letters indicate the disease-related genotype and lowercase letters technicalities.

| Abbreviation | Explanation / disease genotype                        |
|--------------|---|
| UTA          | University of Tampere                                 |
| CPVT         | Catecholaminergic polymorphic ventricular tachycardia |
| HCM          | Hypertrophic cardiomyopathy                           |
| WT           | Wildtype  |
| s            | Sendai-viral induction                                |
| b            | Blood cell-derived                                    |

### 3.1.2 Culture conditions

The cell cultures were maintained in a cell culture laboratory under aseptic conditions. The culturing plates were kept in an incubator set to 37 °C temperature. The temperature was monitored on a daily basis. The carbon dioxide levels in the incubator were set to 5.0% for controlling the medium pH (Chakrabarti & Chakrabarti 2001). The incubator was humidified by keeping sterile water on a tray inside the incubator.

The cells were cultured on regular plastic 6-well plates with gelatin coating and mouse embryonic fibroblast (MEF) feeder layer (see section 3.1.3). KSR medium was used to support cell growth. KSR medium is a serum free, KnockOut™ Serum Replacement (KO-SR) based cell culture medium (contents in appendix 1). Before use, KSR medium was supplemented with fibroblast growth factor (bFGF). The morphology of the cell colonies was assessed visually and colonies were photographed with an inverted phase contrast microscope system (see figures 8 & 9, section 4.1).

### 3.1.3 Passaging

The cell lines were enzymatically passaged once a week to prevent the colonies from overgrowing. For the passaging, a MEF feeder layer was prepared on the wells. Passage numbers were used to follow the approximate age of the cultures, one unit standing for approximately one week.

First, the wells were coated with gelatin. The coating was prepared by applying 1 ml of autoclave sterilized 0.1% gelatin (porcine, type A) in dH<sub>2</sub>O onto the wells and letting gelatin bind for an hour at room temperature. After binding, excess gelatin was aspirated. Mitotically inactivated MEF cells were stored in gas phase liquid nitrogen storage (Millipore™ EmbryoMax® PMEF P3 Strain CF-1 MitoC treated). The MEF cell vials containing 5 million cells per vial were thawed in 37 °C water bath. Pre-warmed MEF medium was carefully added into the thawed ampoule to reduce osmotic shock (for MEF medium contents, see appendix 1). The suspension was then transferred into a 50 ml tube of pre-warmed MEF medium and carefully mixed. Cell suspension was transferred into the gelatin-coated wells adding 2.5 ml of suspension per well. The plates were kept in an incubator overnight to let the MEF cells attach.

On the following day, the MEF medium was aspirated from the wells and replaced with pre-warmed KSR+FGF medium, 1.5 ml/well. From the iPSC wells to be passaged, the MEF layer and inferior colonies were detached using sterile pipette tips. The medium with detached material was aspirated from the wells. 1 ml of 1 mg/ml collagenase IV solution was added onto the wells and incubated for 5 minutes at room temperature. The collagenase solution was then aspirated and a sufficient amount of KSR+FGF medium was added. Cell colonies were carefully detached by pipetting and transferred within the medium into new wells.

Some of the cells were occasionally frozen for storage purposes. Before freezing, the feeder layer was removed and aspirated, the cell colonies were scraped off with a sterile instrument and cell colony suspension was centrifuged at 300 G for 5 minutes. Supernatant was aspirated and freezing medium (appendix 1) was added, 0.5 ml/vial. Cell suspension was transferred into cryovials. 0.5  $\mu$ l of ROCK-inhibitor was added into each vial to aid cell survival (Claassen et al. 2009). Subsequently, the vials were transferred inside a cooling container into a -80 °C freezer and next day into gas phase liquid nitrogen storage. The thawing of the cells was carried out in the same fashion as with the MEF cells.

#### **3.1.4 Embryoid body culture**

For embryoid body culture, the cells were passaged from regular 6-well plates into low-attachment 12-well plates with no feeder layer or gelatin coating. 2 wells from 6-well plate were split to 4 wells on 12-well plates. The culturing medium was embryoid body medium with no FGF and a higher concentration of KO-SR compared to KSR medium (appendix 1). The medium was changed completely once a week for the 5-week growing period. The cells were incubated in the same environment as the iPS cells.

## 3.2 Characterization of cell lines

### 3.2.1 Obtaining cDNA

#### **RNA extraction with spin column method**

RNA extraction from the cells was carried out using Macherey-Nagel NucleoSpin® RNA II kit. The kit is a spin column system based on a silica matrix and extraction in the presence of guanidinium thiocyanate-containing buffer (Macherey-Nagel 2011). Cell samples were obtained from a single 6-well plate culturing well each. Prior to taking the sample, the cells were washed two times with PBS. Detached cell suspension was centrifuged, PBS was aspirated and 350 µl of RA1 buffer including 3.5 µl of β-mercaptoethanol was added. Applying the buffer lysed the cells and the samples were stored at -80 °C before the extraction.

The thawed sample was first centrifuged through NucleoSpin filter to clear the lysate. Ethanol was then added to the filtrated lysate to adjust RNA binding conditions. The mix was then loaded to NucleoSpin RNA II column and centrifuged. Membrane desalting buffer was added onto the filter and centrifuged to enhance the following rDNase digestion. RDNase solution was applied onto the filter for a 15 minute incubation at room temperature. RA2 buffer was added to the column and centrifuged to inactivate the rDNase. The column was then washed two times with RA3 buffer and centrifuged after the washes. Finally, RNA was eluted into a sterile collection tube with RNase-free water. (Macherey-Nagel 2011.) The procedure was carried out for each sample. The protocol is summarised in table 5.

TABLE 5. Total RNA isolation protocol for NucleoSpin® RNA II kit. (Macherey-Nagel 2011)

| Phase                         | Reagent  | Centrifugation at<br>11 000 g |
|-------------------------------|--|-------------------------------|
| Lyse cells                    | 350 µL RA1 + 3.5 µL β-mercaptoethanol                    |                               |
| Filtrate lysate               | NucleoSpin filter  | 1 min                         |
| Adjust RNA binding conditions | 350 µl 70 % ethanol                                      |                               |
| Bind RNA                      | NucleoSpin RNA II column                                 | 30 s                          |
| Desalt silica membrane        | 350 µl MDB   | 1 min                         |
| Digest DNA                    | 95 µl rDNase mix (10 % rDNase stock in H <sub>2</sub> O) | incubation at RT for 15 min   |
| Wash and dry silica membrane  | 1st wash: 200 µl RA2                                     | 30 s                          |
|                               | 2nd wash: 600 µl RA3                                     | 30 s                          |
|                               | 3rd wash: 250 µl RA3                                     | 2 min                         |
| Elute RNA                     | RNase-free H <sub>2</sub> O (supplied)                   | 1 min                         |

NanoDrop spectrophotometer was used to measure the concentration and purity of the RNA extracts. The results of the measurements are listed in table 6. Prior to the usage, RNA samples were stored at -80 °C. New cultured samples were taken from lines UTA.07001.CPVT and UTA.05404.CPVT due to the problems later encountered with PCR.

TABLE 6. RNA concentrations, purity and volume for cDNA transcription.

| Sample cell line     | Passage | RNA concentration | RNA purity (A <sub>260</sub> /A <sub>280</sub> ) | Volume for transcription |
|----------------------|---------|-------------------|--|--------------------------|
| UTA.07001.CPVT       | 15      | 13.3 ng/µl        | 2.26   | 10 µl                    |
| UTA.05303.CPVT       | 13      | 133.4 ng/µl       | 2.13   | 7.50 µl                  |
| UTA.05404.CPVT       | 19      | 74.41 ng/µl       | 1.91   | 10 µl                    |
| UTA.01402.HCMTs      | 10      | 53.28 ng/µl       | 2.20   | 10 µl                    |
| UTA.02912.HCMTs      | 14      | 109.6 ng/µl       | 2.17   | 9.12                     |
| UTA.11505.WTsb       | 29      | 98.33 ng/µl       | 2.15   | 10 µl                    |
| UTA.07001.CPVT (new) | 27      | 42.18 ng/µl       | 2.18   | 10 µl                    |
| UTA.05404.CPVT (new) | 31      | 119.9 ng/µl       | 2.16   | 8.34 µl                  |

### **cDNA transcription**

cDNA transcription was carried out using Applied Biosystems' High Capacity cDNA Reverse Transcription kit. The master mix was prepared for the reverse transcription reactions according to table 7. 10  $\mu$ l of the master mix was added to PCR tubes for each reaction. The amount of RNA for each reaction was determined to be the maximum from each cell line but  $\leq$  1000 ng, since all samples did not yield to 100 ng/ $\mu$ l concentration required for 1000 ng amount. The volume of each extract was calculated accordingly (table 6). The total reaction volume was 20  $\mu$ l. A control sample was prepared with reverse transcriptase replaced with H<sub>2</sub>O. The temperature program for the thermocycler is in table 8.

TABLE 7. Master mix components for reverse transcription in one reaction.

| Reagent                            | $\mu$ l |
|------------------------------------|---------|
| 10x RT buffer                      | 2.0     |
| 25x dNTP mix (100 $\mu$ M)         | 0.8     |
| 10x RT random primers              | 2.0     |
| MultiScribe™ Reverse Transcriptase | 1.0     |
| RNase inhibitor                    | 0.5     |
| Nuclease-free H <sub>2</sub> O     | 3.7     |
| Total volume                       | 10      |

TABLE 8. Temperature program for cDNA transcription.

| Step | Temperature | Time    |
|------|-------------|---------|
| #1   | 25 °C       | 10 min  |
| #2   | 37 °C       | 120 min |
| #3   | 85 °C       | 5 min   |
| #4   | 4 °C        | hold    |

### 3.2.2 Analysis of exogenous genes

To study the expression status of viral exogenes, previously transcribed cDNA was used for the PCR protocol. Master mixes were aseptically prepared for the reactions into Eppendorf tubes according to table 9. The DNA polymerase was from Thermo Scientific. In PCR, dimethyl sulfoxide (DMSO) disrupts base pairing and strand re-annealing and is used with some primers to prevent the formation of secondary structures especially in GC rich areas (Baskaran et al. 1996; Mammedov et al. 2008; Jensen et al. 2010). Primers used were designed to attach to the viral transgene *OCT3/4*, *SOX2*, *Klf4*, and *c-Myc* cDNA. A housekeeping gene, *GAPDH*, was used as a control. *GAPDH*, producing glyceraldehyde-3-phosphate dehydrogenase, is a maintenance gene and known to be perpetually expressed in cells to a degree (Warrington et al. 2000; Barber et al. 2005).

TABLE 9. Master mix components for one reaction in the analysis of exogenous genes.

| Reagent                                | µl         |
|--|------------|
| 10x buffer (DyNAzyme™)                 | 2,5        |
| dNTP mix (10 mM)                       | 0,5        |
| primer F (5 uM)                        | 2,5        |
| primer R (5 uM)                        | 2,5        |
| DNA Polymerase (DyNAzyme™ II)          | 0,25       |
| MgCl (25 mM)                           | 0,5        |
| DMSO (not for <i>GAPDH</i> master mix) | 1,25       |
| H <sub>2</sub> O                       | 14 (15,25) |
| Total volume                           | 24         |

The total volume of 24 µl of the master mix was used for each reaction. The master mixes were pipetted into PCR tube strips and 1 µl of the sample cDNA solutions were added. Transgene plasmid solutions were used as positive control samples. Three blank samples were used to check possible contamination: water from cDNA transcription and PCR master mixes and –RT sample from cDNA transcription. Reaction tubes were mixed by tapping and spun in a microcentrifuge. A standard temperature program was used for the reactions with 40 cycles and the annealing temperature of 55 °C (table 10).

TABLE 10. Temperature program for PCR cyclers in the analysis of exogenous genes.

| Step | Temperature | Time  |
|------|-------------|-------|
| #1   | 94 °C       | 2 min |
| #2   | 94 °C       | 30 s  |
| #3   | 55 °C       | 30 s  |
| #4   | 72 °C       | 1 min |
| #5   | 72 °C       | 5 min |
| #6   | 4 °C        | hold  |

} 40 cycles

A 2% w/v agarose gel was used to analyze the results. Ethidium bromide was used as a fluorescent DNA label. The gel was prepared by weighing 3 g of agarose and mixing it with 150 ml of TRIS-borate-EDTA (TBE) buffer solution. The suspension was brought to a boil and re-heated in a microwave oven so that the agarose was completely dissolved. 3.75  $\mu$ l of 10 mg/ml ethidium bromide was added and mixed into the sufficiently cooled solution. Gel was poured into a tray to polymerize for 45 min at room temperature. The samples were mixed with 4.2  $\mu$ l of 6x DNA loading dye (Thermo Scientific). 50 bp DNA ladder was used as a size standard. The gel was immersed in TBE and the samples were loaded into the wells. Electrophoresis was ran for 1 hour with a voltage of 80 V. The gel was photographed in UV light. For the results, see section 4.2.

### 3.2.3 Analysis of endogenous pluripotency genes

To study the expression of endogenous pluripotency marker transcription factors, a PCR protocol was carried out using the previously transcribed cDNA. Five analyzed factors were *Nanog*, *Rex1*, *OCT3/4*, *SOX2*, and *c-Myc*. *GAPDH* was used as a control as previously. The protocol was conducted as with transgene analyses, with the exception of using temperature gradient to perform several reactions with differing annealing temperatures concurrently. The master mix components for the reactions are listed in table 11 and thermocycler temperature program in table 12. The gel photographs can be found in section 4.3.

TABLE 11. Master mix components for one reaction in the analysis of endogenous genes.

| Reagent                            | $\mu\text{l}$ |
|------------------------------------|---------------|
| 10x buffer (DyNAzyme™)             | 2,5           |
| dNTP mix (10 mM)                   | 0,5           |
| primer F (5 $\mu\text{M}$ )        | 2,5           |
| primer R (5 $\mu\text{M}$ )        | 2,5           |
| DNA Polymerase (DyNAzyme™ II)      | 0,25          |
| MgCl (25 mM)                       | 0,5           |
| DMSO (for <i>Nanog</i> master mix) | 1,25          |
| H <sub>2</sub> O                   | 15,25 (14)    |
| Total volume                       | 24            |

TABLE 12. Temperature program for PCR cyclers in the analysis of endogenous genes.

| Step | Temperature | Primers                    | Time  |
|------|-------------|----------------------------|-------|
| #1   | 94 °C       |                            | 2 min |
| #2   | 94 °C       |                            | 30 s  |
| #3   | 45 °C       | <i>Nanog</i>               | 30 s  |
|      | 55 °C       | <i>GAPDH, Rex1</i>         |       |
|      | 60 °C       | <i>OCT3/4, SOX2, c-Myc</i> |       |
| #4   | 72 °C       |                            | 1 min |
| #5   | 72 °C       |                            | 5 min |
| #6   | 4 °C        |                            | hold  |

} 40 cycles

### 3.2.4 Analysis of embryoid bodies

After 5 weeks of culture, embryoid body samples were collected, RNA was extracted and cDNA transcribed as previously described with the iPS cells. RNA properties and volumes for cDNA transcription are listed in table 13. Six genes, two from each layer, were chosen for the subsequent PCR analysis (table 14). The master mix composition and temperature program were equivalent with the analyses of exogenous genes (tables 9 and 10). Agarose gel electrophoresis was carried out for the products, the results are in section 4.4.

TABLE 13. RNA concentrations and purity in embryoid body samples.

| Sample cell line | Passage | RNA concentration,<br>ng/ $\mu$ l | RNA purity<br>( $A_{260}/A_{280}$ ) | Volume for<br>transcription, $\mu$ l |
|------------------|---------|-----------------------------------|-------------------------------------|--------------------------------------|
| UTA.07001.CPVT   | 15      | 51.27                             | 2.21                                | 10                                   |
| UTA.05303.CPVT   | 11      | 117.1                             | 2.21                                | 8.54                                 |
| UTA.05404.CPVT   | 18      | 34.67                             | 2.15                                | 10                                   |
| UTA.02912.HCMTs  | 13      | 100.8                             | 2.19                                | 9.92                                 |
| UTA.01402.HCMTs  | 10      | 296.0                             | 2.16                                | 3.38                                 |
| UTA.11505.WTsb   | 33      | 102.6                             | 2.22                                | 9.75                                 |

TABLE 14. Analyzed embryoid body related genes and respective germ layers.

| Gene                                       | Related germ layer                                   |
|--|--|
| <i>SOX17</i>                               | Endoderm (Niakan et al. 2010; Wang et al. 2011)      |
| <i>AFP</i>                                 | Endoderm (Schroeder et al. 2012; Rajala et al. 2010) |
| <i>Nestin</i>                              | Ectoderm (Jagtap et al. 2011)                        |
| <i>PAX6</i>                                | Ectoderm (Surmacz et al. 2012)                       |
| <i><math>\alpha</math>-cardiac actinin</i> | Mesoderm (Poudel et al. 2011)                        |
| <i>VEGFR2/KDR</i>                          | Mesoderm (Takenaga et al. 2007)                      |

### 3.2.5 Immunofluorescence staining

For immunofluorescence staining, the cell lines were split for culturing on 12-well plates, each plate containing one cell line. The cells were cultured on seven wells, one well for each of the six antibodies and a secondary antibody control well. Before splitting, the wells were gelatin coated and plated with mouse embryonic fibroblast feeder cells as previously described.

KSR+FGF medium was changed for the cells in 2-3 day intervals as described previously. After 5-7 days of culture, the culturing medium was removed and the cells were fixed (see table 15). Before fixing, the cells were washed two times with PBS for five minutes a wash. Fixing was carried out applying 350  $\mu$ l of 4 % w/v paraformaldehyde (PFA) solution into wells empty of liquid. The plates were incubated for 20 minutes at ambient temperature and subsequently washed two times with PBS as previously.

The fixed plates were stored at +4 °C in a cold storage room for varying periods of time, maximum time being two months. The passage data are listed in appendix 2.

Before treating the cells with primary antibodies, the wells were treated with blocking solution to reduce non-specific antibody binding (Kenna et al. 1985). Blocking was carried out applying normal donkey serum (NDS) based blocking solution to wells following an incubation of 45 minutes at ambient temperature (see table 15 & appendix 2). Blocking solution was aspirated and wells were washed once with primary+washing solution.

2 mg/ml primary antibody solutions were diluted with appropriate amounts of primary+washing solution to achieve desired dilutions. Onto each well, 750 µl of appropriate antibody solution was applied. Only primary+washing solution without the antibody was added to the control wells. The plates were incubated overnight at +4 °C. For the list of primary antibodies used, see table 16.

After incubation, the wells were washed three times with PBS for five minutes a wash. Secondary antibody solutions (2 mg/ml) were prepared by diluting antibody stock solutions with secondary+washing solution (appendix 2). Secondary antibody solutions were applied and incubated for one hour, protected from light. Secondary antibodies used were labeled with Alexa Fluor® 568, a sulfonated derivative of rhodamine (Panchuk-Voloshina et al. 1999) (table 17).

The wells were subsequently washed two times with PBS and then two times with PB (phosphate buffer), 5 minutes a wash. The wells were emptied of liquid. 1-2 drops of Vectashield® (Vector Laboratories) mounting solution with DAPI counterstain was added onto the wells. Finally, cover glasses were added. The stained plates were stored in a cold storage room at +4 °C, protected from light.

TABLE 15. Fluorescence staining protocol.

| Step                                    | Volume / well | Incubation at RT   |
|---|---------------|--------------------|
| Washing with PBS                        | 2 x 1 ml      | 5 min / wash       |
| Fixing with 4 % PFA                     | 350 $\mu$ l   | 20 min             |
| Washing with PBS                        | 2 x 1 ml      | 5 min / wash       |
| Blocking                                | 0.7 ml        | 45 min             |
| Washing with primary+washing solution   | 1 ml          | -                  |
| Applying primary antibodies             | 750 $\mu$ l   | overnight at +4 °C |
| Washing with secondary+washing solution | 3 x 1 ml      | 5 min / wash       |
| Applying secondary antibodies           | 750 $\mu$ l   | 60 min, dark       |
| Washing with PBS                        | 2 x 1 ml      | 5 min / wash, dark |
| Washing with PB                         | 2 x 1 ml      | 5 min / wash, dark |
| Mounting with Vectashield®              | 1-2 drops     | -                  |

TABLE 16. List of primary antibodies.

| Antibody                      | Dilution | Vendor       |
|-------------------------------|----------|--------------|
| Anti-human Nanog goat IgG     | 1:100    | R&D Systems® |
| Anti-human OCT 3/4 goat IgG   | 1:400    | R&D Systems® |
| Anti-human SSEA4 mouse IgG    | 1:100    | Santa Cruz   |
| Anti-human SOX2 goat IgG      | 1:200    | Santa Cruz   |
| Anti-human TRA-1-60 mouse IgM | 1:200    | Millipore™   |
| Anti-human TRA-1-81 mouse IgM | 1:200    | Millipore™   |

TABLE 17. List of secondary antibodies.

| Antibody  | Dilution | Vendor             |
|---|----------|--------------------|
| Alexa Fluor® 568 donkey anti-goat IgG (H+L)         | 1:800    | Life Technologies™ |
| Alexa Fluor® 568 goat anti-mouse IgG (H+L)          | 1:800    | Life Technologies™ |
| Alexa Fluor® 568 goat anti-mouse IgM ( $\mu$ chain) | 1:800    | Life Technologies™ |

The stained cells were photographed with a fluorescence microscope (Olympus IX51®) using 320 nm filter for DAPI and 560 nm filter for Alexa Fluor® 568 labels. Absorption maximum for Alexa Fluor® 568 dye is reported to be 578 nm and emission maximum 603 nm (Life Technologies 2013). For DAPI, these wavelengths are 347 nm and 448 nm, respectively (Kapuscinski 1990). Photographs were taken as grayscale and coloured with an image processing program. Red colour was used for the antibody and blue colour for DNA (DAPI). For the results, see section 4.5.

## 4 RESULTS

### 4.1 Morphology

The morphology of the iPS cell colonies was evaluated after days 2 and 6 after the previous passaging. Photographs are presented in figures 8 and 9. All of the photographs show a clear growth in colony sizes in four days indicating exponential proliferation. Doubling time was not determined for the cells. The cells exhibit small, round features in contrast to surrounding fibroblast feeder cells.

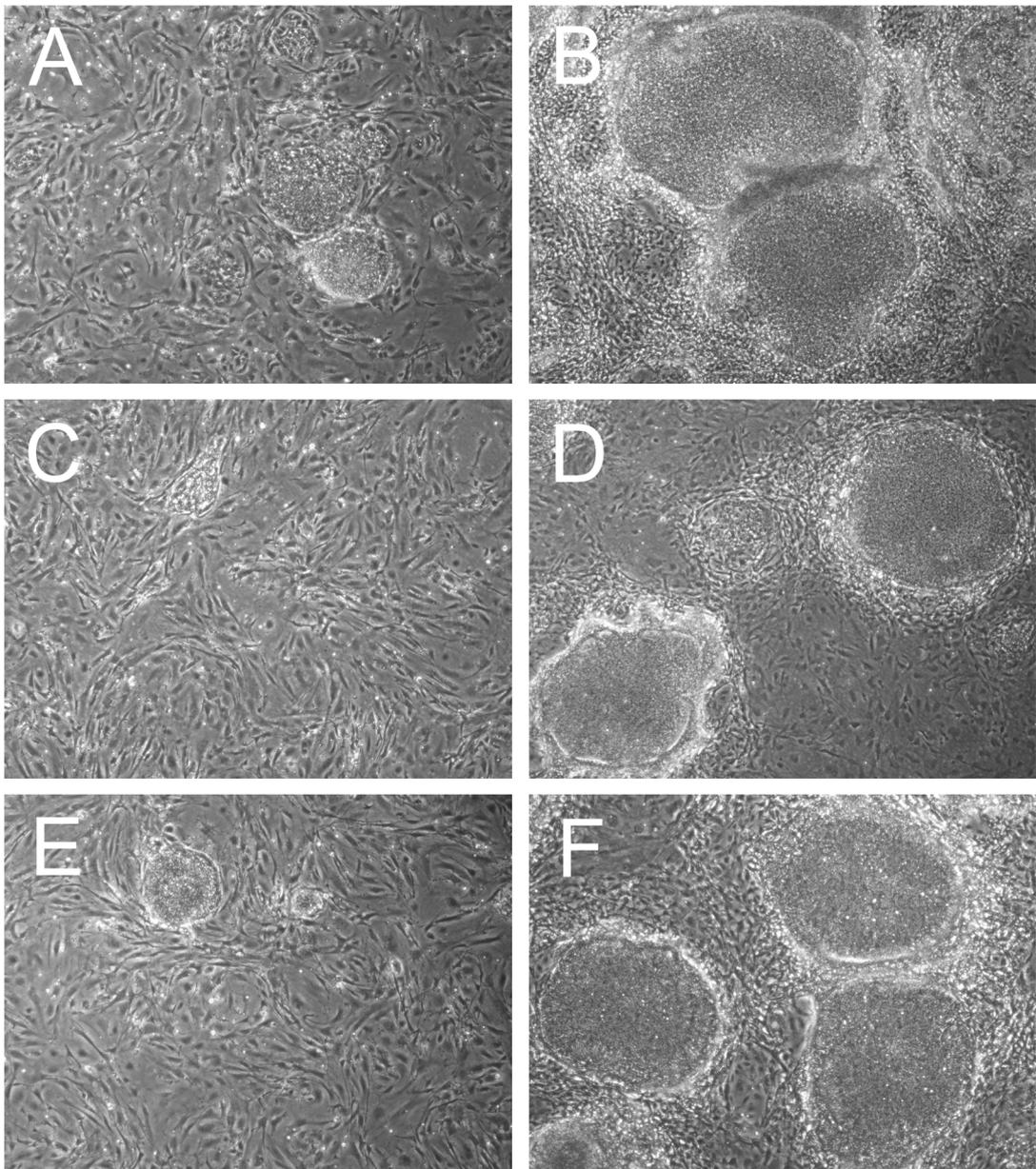


FIGURE 8. On the left: 2 days after the passaging, on the right: 6 days after the passaging. (A)&(B) UTA.07001.CPVT, (C)&(D) UTA.05303.CPVT, (E)&(F) UTA.05404.CPVT.

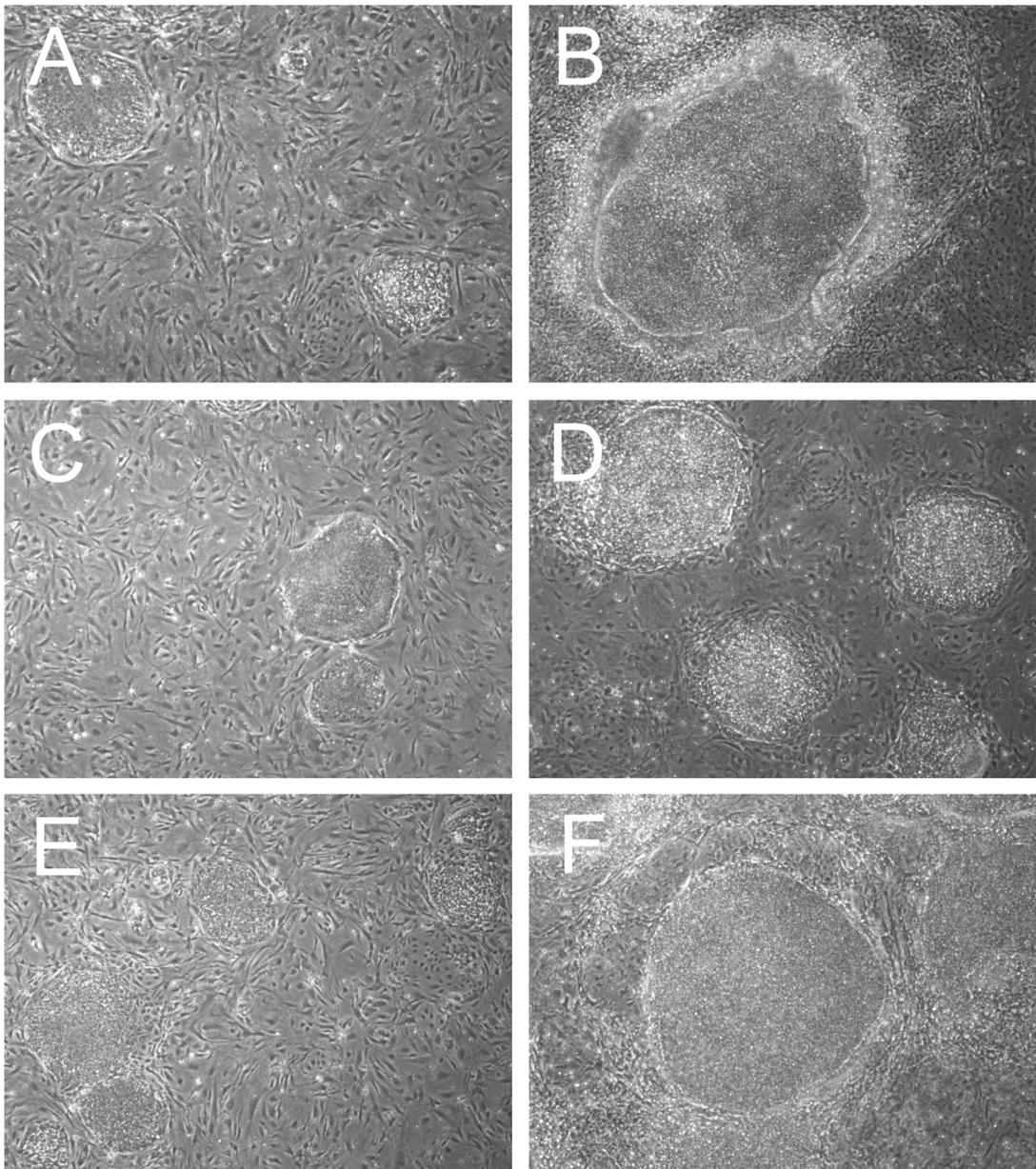


FIGURE 9. On the left: 2 days after the passaging, on the right: 6 days after the passaging.  
(A)&(B) UTA.02912.HCMTs, (C)&(D) UTA.01401.HCMTs, (E)&(F) UTA.11505.WTsb.

## 4.2 Exogenous genes

EtBr-labeled agarose gels were photographed using a UV-cabinet. Printed results are presented in figure 10. Photographs include 50-5000 bp standard DNA ladder with 50 bp intervals and brighter bands at 250 and 500 bp for approximate size determination. Expected nominal band sizes are listed in table 18. The results show without controversy that all transgenes were unexpressed. Positive controls and *GAPDH* are visible in each line (figure 10, A-D). In addition to the cell lines and genes labelled, a comprehensive chart including passage data is included in appendix 3. Cell line UTA.01401.HCMTs was an additional line not included in the thesis.

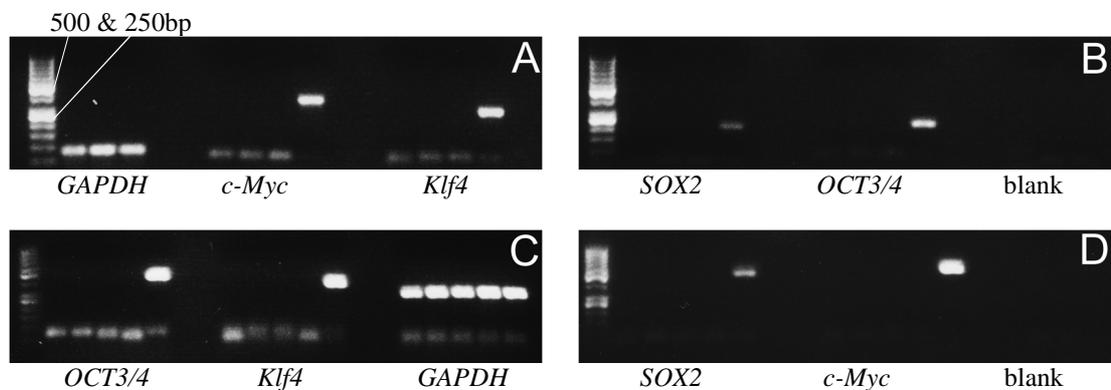


FIGURE 10. PCR results for exogenous genes. Samples are grouped by genes. On the left, 50-1000 bp ladder with 50 bp intervals with brighter bands at 250 and 500 bp.

(A) & (B) Band 1: UTA.07001.CPVT, band 2: UTA.05303.CPVT, band 3: UTA.05404.CPVT, band 4: positive control. (C) & (D) Band 1: UTA.02912.HCMTs, band 2: UTA.01401.HCMTs, band 3: UTA.01402.HCMTs, band 4: UTA.11505.WTsb, band 5: positive control.

TABLE 18. Transgene-related fragment sizes in base pairs.

|                          |         |
|--------------------------|---------|
| retro exo <i>OCT3/4</i>  | 200-250 |
| retro exo <i>SOX2</i>    | >200    |
| retro exo <i>Klf4</i>    | 250-300 |
| retro exo <i>c-Myc</i>   | <400    |
| Sendai exo <i>OCT3/4</i> | 483     |
| Sendai exo <i>SOX2</i>   | 451     |
| Sendai exo <i>Klf4</i>   | 410     |
| Sendai exo <i>c-Myc</i>  | 532     |
| <i>GAPDH</i>             | 302     |

### 4.3 Endogenous pluripotency genes

Gel photographs were obtained as previously described. Expected nominal band sizes are listed in table 19. The results presented here considering lines UTA.07001.CPVT and UTA.05404.CPVT have been obtained with the new samples (table 6). With the exception of *c-Myc*, all endogenous markers were expressed (figure 11). In two lines, *OCT3/4* expression was not observed (figure 11 (B)), so the analysis was repeated and expression then confirmed (figure 11 (D)). *c-Myc* exhibited weak expression in two lines. Table 20 summarizes the observed gene expression. In addition to cell lines and genes labelled, a comprehensive chart including the passage data is included in appendix 3.

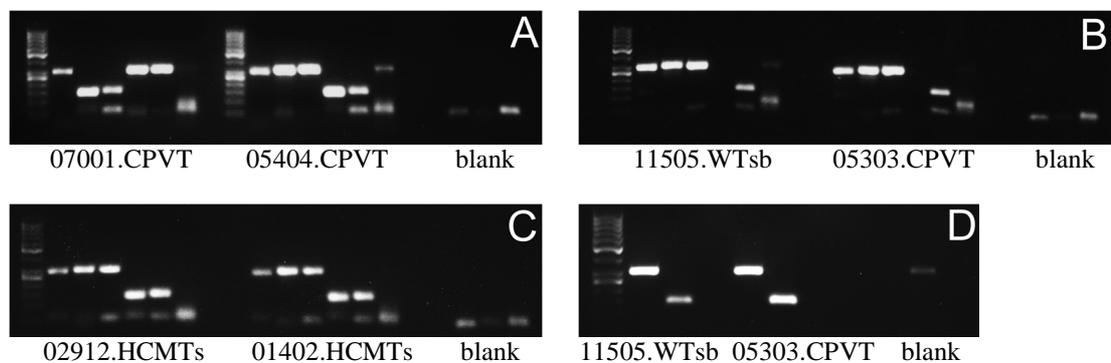


FIGURE 11. Endogenous pluripotency marker PCR results. Samples are grouped by cell lines. The order of genes in each series of bands from left to right: 1. *Nanog*, 2. *GAPDH*, 3. *Rex1*, 4. *OCT3/4*, 5. *SOX2*, 6. *c-Myc* (except UTA.07001.CPVT: 2. *OCT3/4*, 3. *SOX2*, 4. *GAPDH*, 5. *Rex1*). Supplementary bands in (D): 1.  *$\beta$ -actin*, 2. *OCT3/4*.

TABLE 19. Fragment sizes in base pairs.

|                    |     |
|--------------------|-----|
| Endo <i>Nanog</i>  | 287 |
| Endo <i>OCT3/4</i> | 144 |
| Endo <i>Rex1</i>   | 306 |
| Endo <i>SOX2</i>   | 151 |
| Endo <i>c-Myc</i>  | 328 |
| <i>GAPDH</i>       | 302 |

TABLE 20. The observed expression of endogenous pluripotency markers in cell lines.

Markings: + expression, - no expression, w: weak expression.

|                 | <i>Nanog</i> | <i>GAPDH</i> | <i>Rex1</i> | <i>OCT3/4</i> | <i>SOX2</i> | <i>c-Myc</i> |
|-----------------|--------------|--------------|-------------|---------------|-------------|--------------|
| UTA.07001.CPVT  | +            | +            | +           | +             | +           | -            |
| UTA.05303.CPVT  | +            | +            | +           | +             | +           | -            |
| UTA.05404.CPVT  | +            | +            | +           | +             | +           | w            |
| UTA.01402.HCMTs | +            | +            | +           | +             | +           | -            |
| UTA.02912.HCMTs | +            | +            | +           | +             | +           | -            |
| UTA.11505.WTsb  | +            | +            | +           | +             | +           | w            |

#### 4.4 Embryonic layer genes in embryoid bodies

Gel photographs were obtained as previously described. Expected nominal band sizes are listed in table 21. All lines exhibited the three embryonic layers with at least one gene per germ layer (figure 12, table 22). Two lines, UTA.07001.CPVT and UTA.01402.HCMTs expressed all studied genes including housekeeping gene *GAPDH*. In addition, two lines, UTA.05303.CPVT and UTA.11505.WTsb, failed to express *GAPDH*. *AFP* produced two different-sized bands, most evident in figure 12 (D). Table 22 summarizes the observed gene expression. In addition to the cell lines and genes labelled, a comprehensive chart including the passage data is included in appendix 3.

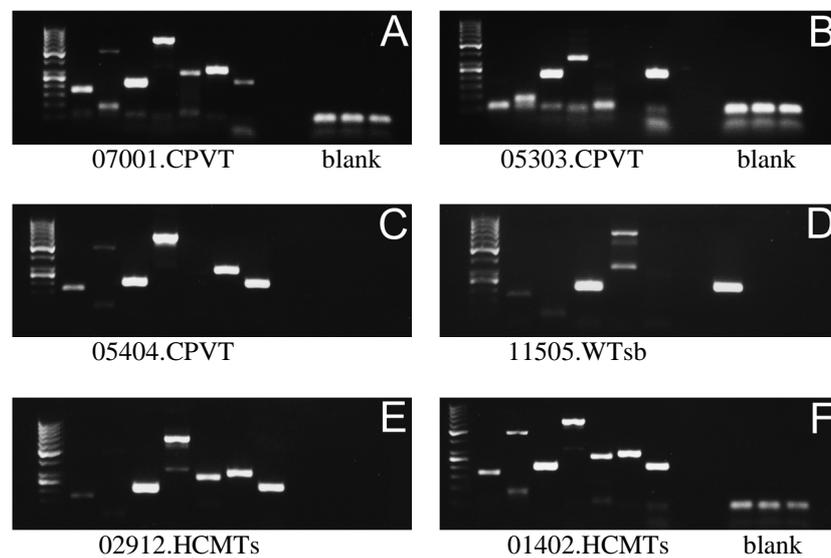


FIGURE 12. Embryonic germ layer marker PCR results. Samples are grouped by cell lines. The order of genes in each series of bands from left to right: 1. *SOX17*, 2.  *$\alpha$ -cardiac actinin*, 3. *Nestin*, 4. *AFP*, 5. *PAX6*, 6. *GAPDH*, 7. *VEGFR2/KDR*.

TABLE 21. EB-related PCR fragment sizes in base pairs.

|   |        |
|---|--------|
| EB <i>SOX17</i>                             | 120 bp |
| EB <i><math>\alpha</math>-cardiac actin</i> | 486 bp |
| EB <i>Nestin</i>                            | 208 bp |
| EB <i>AFP</i>                               | 672 bp |
| EB <i>PAX6</i>                              | 274 bp |
| EB <i>VEGFR2/KDR</i>                        | 218 bp |
| <i>GAPDH</i>                                | 302 bp |

TABLE 22. Observed gene expression in embryoid body samples. Markings: + expression, - no expression, w: weak expression.

|                            | <i>SOX17</i> | <i><math>\alpha</math>-actinin</i> | <i>Nestin</i> | <i>AFP</i> | <i>PAX6</i> | <i>GAPDH</i> | <i>VEGFR2</i> |
|----------------------------|--------------|------------------------------------|---------------|------------|-------------|--------------|---------------|
| Embryonic layer<br>(-derm) | endo         | meso                               | ecto          | endo       | ecto        | control      | meso          |
| UTA.07001.CPVT             | +            | +                                  | +             | +          | +           | +            | +             |
| UTA.05303.CPVT             | -            | -                                  | +             | +          | -           | -            | +             |
| UTA.05404.CPVT             | +            | w                                  | +             | +          | -           | +            | +             |
| UTA.01402.HCMTs            | +            | +                                  | +             | +          | +           | +            | +             |
| UTA.02912.HCMTs            | w            | -                                  | +             | +          | +           | +            | +             |
| UTA.11505.WTsb             | w            | -                                  | +             | +          | -           | -            | +             |

#### 4.5 Immunofluorescence staining

All of the six cell lines were successfully stained. Protein expression was observed with all six antibodies in all lines. None of the three fluorescent labeled secondary antibodies caused observable background staining or noise. There was no notable autofluorescence effects. Staining intensities differed both between cell lines and antibodies. Figure 13 shows one staining result of each antigen and figure 14 the three secondary antibody control stainings.

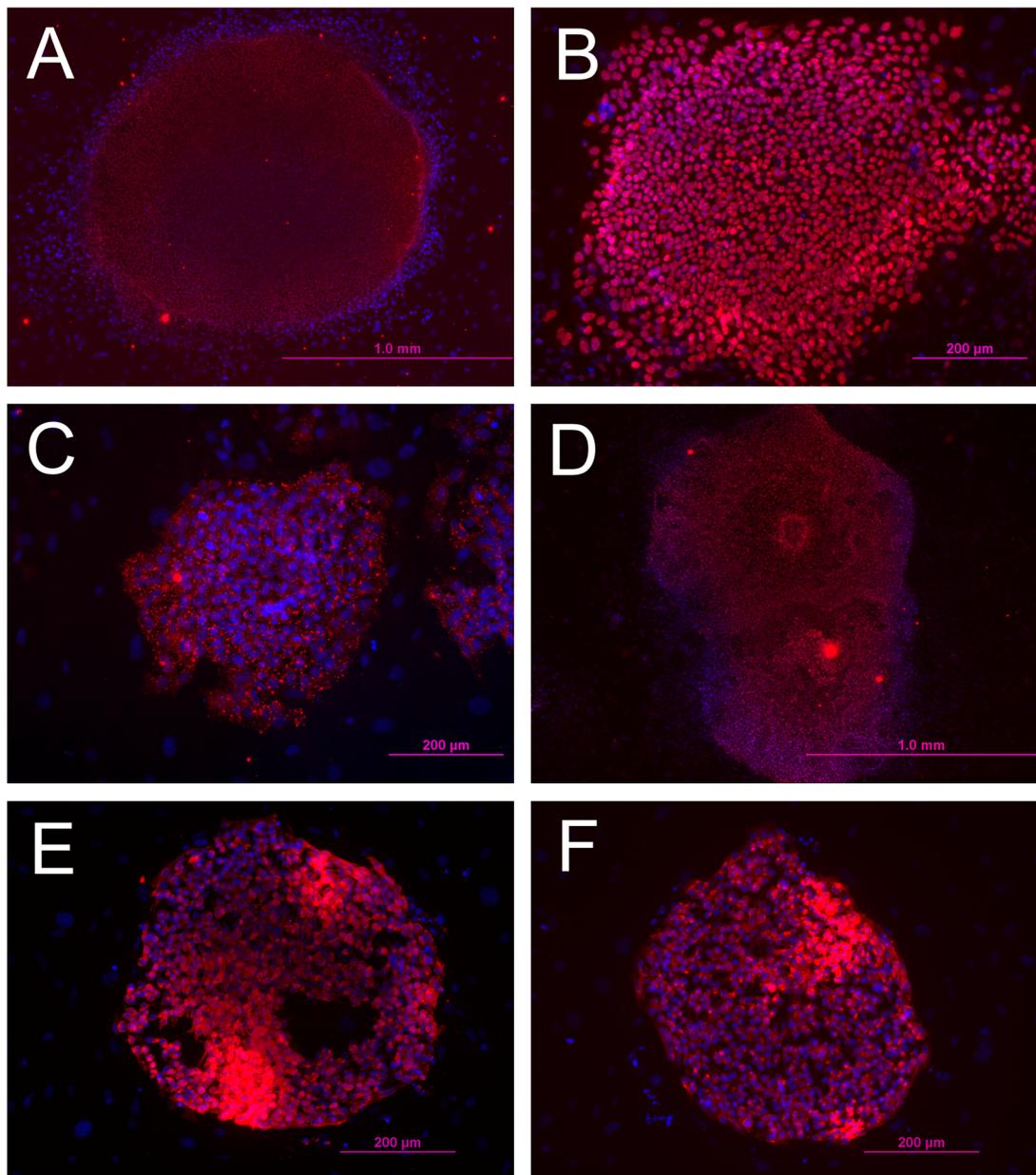


FIGURE 13. Immunofluorescence staining for pluripotency markers. Red color indicates antigen, blue DNA. (A) Nanog, (B) OCT3/4, (C) SSEA4, (D) SOX2, (E) TRA 1-60, (F) TRA 1-81.

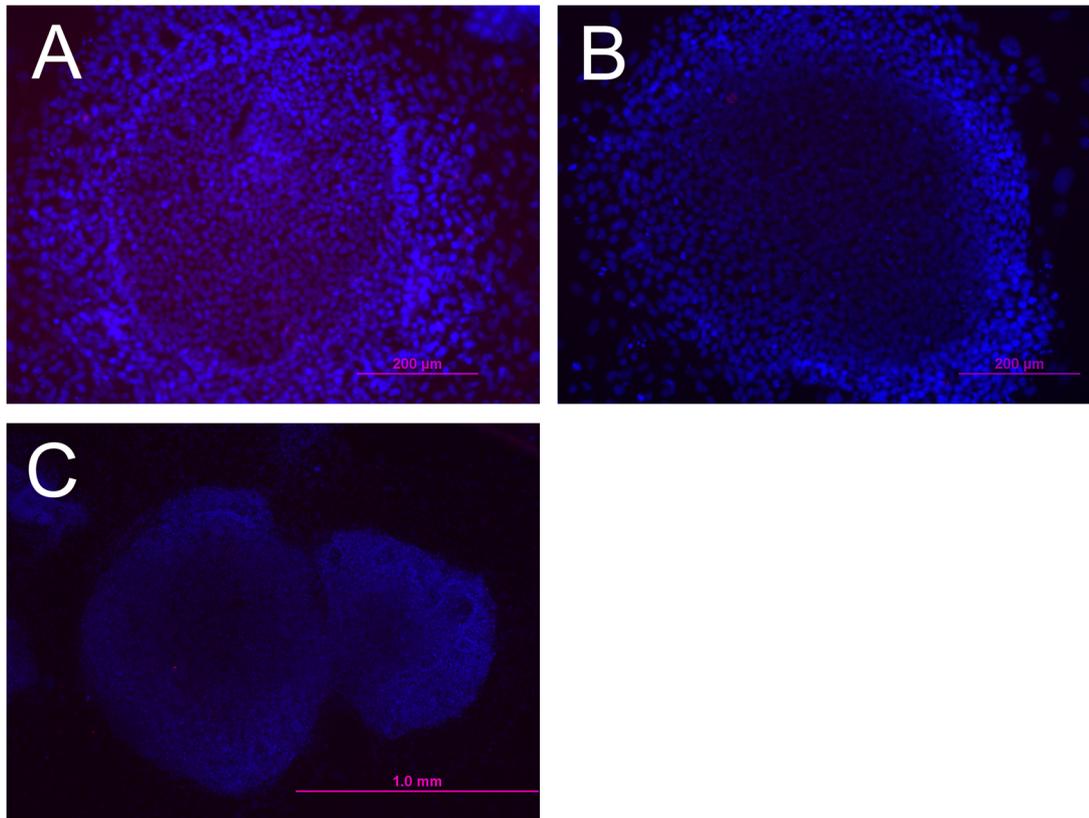


FIGURE 14. Secondary antibody control stainings. Red color indicates antigen, blue DNA. (A) Anti-goat IgG, (B) Anti-mouse IgG, (C) Anti-mouse IgM.

## 5 DISCUSSION

The morphology of iPS cell colonies was expected, exhibiting characteristics of human pluripotent stem cells, such as high nucleus-to-cytoplasm ratio, dense, flat, colonies and smooth colony borders. The characteristics differ notably from other cells, such as parental human dermal and mouse embryonic fibroblasts. Similar morphology has been described with other iPS cells in several studies. (Takahashi et al. 2007; Yu et al. 2009; Okita et al. 2011.)

The analyses of viral transgene expression were performed for cultures with passage number ranging from 10 to 29. The analyses exhibited no evidence of transgene expression, whether residual or due to reactivation. Transgenes are thus silenced in both the integrating retroviral-derived and non-integrating Sendai-viral-derived cell lines studied. Of the five endogenous factors analyzed, all but c-Myc were expressed, most importantly the key pluripotency factors OCT3/4 and Nanog. c-Myc is considered of value in reprogramming, but not necessarily as a pluripotency marker (Takahashi et al. 2007). All embryoid body samples analyzed included at least one marker for each of the three germ layers, thus presenting in vitro preliminary potential to differentiate into all adult cells. Embryoid bodies from cell line UTA.02912.HCMTs were accidentally cultured on regular plates rather than on low-attachment plates but it had apparently no effect on differentiation (figure 12, (E)). Passage numbers of lines used in endogenous marker studies ranged from 10 to 31 and in embryoid body culture from 10 to 33. Expression does not seem to have correlated with passage numbers. Passage range is not broad and the amount of data is insufficient for general implications.

The data from the immunofluorescence stainings support positive results for Nanog, OCT3/4 and SOX2. In addition, all other pluripotency markers were observed in all cell lines. The passage number of a line or date of fixation surprisingly did not correlate with the staining results in terms of colour intensity. Additionally, the colour intensity could not be reliably correlated with any specific practice or parameter and may be attributed to different amount of antigens in the samples. Antibody binding could also vary in samples due to differing pH values. Several washing steps should however have eliminated this possibility. Intensity differences may also arise from photobleaching.

RNA purity ratios ranged from 1,91 to 2,26 in iPS cell samples and from 2,15 to 2,22 in embryoid body samples. RNA is considered to be pure around ratio 2,0 and contaminated with protein or DNA below 1,8 (Vomelová et al. 2009; Wilson & Walker 2010, 167). The measured purities can be considered satisfactory. The originally contemplated comparison between low and high RNA concentration for the cDNA transcription and the subsequent PCR was discarded, since several samples yielded only low amounts of RNA. Advice from the kit manufacturer for better yields included pre-heating the elution water to 37-50 °C, incubating the elution water 5 minutes prior to the centrifugation, and repeating the elution with the once-through eluate. These notes should be taken into practice in the future while also pursuing to use samples with high cell count.

Double banding was shown with endoderm-related gene *AFP* and mesoderm-related  $\alpha$ -cardiac actinin in embryoid body PCR analyses (see figure 12). Two separate gel bands may have occurred if template DNA has formed secondary structures such as coiled DNA in addition to desired linear structure. Again, this might be caused by improper buffer conditions or the tendency of the specific DNA sequence to form secondary structures, which allows DNA polymerase to amplify partly only free uncoiled sequences. This might be resolved with use of additional reagents, such as DMSO and betaine. (Kang et al. 2005; Mammedov et al. 2008.)

*$\beta$ -actin* was used in place of *GAPDH* as a housekeeping gene control in one PCR reaction (figure 11, (D)) since *GAPDH* was for unknown reason not functioning properly. It has been argued over if *GAPDH* is a suitable housekeeping gene for different PCR analyses and noted that notable differences in expression levels exist between tissue types and some differences even between embryonic stem cells and iPS cells (Barber et al. 2005; Kim et al. 2012). However, in qualitative PCR even small amount ought to be sufficient for a positive result.

PCR analyses in general showed variation in the initial result reliability. Some of the reactions had to be repeated several times before succeeding. Reasons to these problems are not evident and they may sporadic as well as systematic. Sporadic errors are likely to arise in PCR due to the sensitivity of the method and susceptibility to contamination. Preparation of fresh primer dilutions before master mix preparation seemed to improve results, indicating contamination problems. Both types of errors can however signal that the method is not robust enough.

Even though this may be attributable to and inherent in the method, some modifications can be speculated with. These include the use of previously mentioned reagents, DMSO and betaine to improve PCR efficiency especially with sequences containing high amounts of GC (Mammedov et al. 2008). The successful use of BSA as an enhancer in combination with other reagents has also been reported (Farell & Alexandre 2012).

In agarose gels, some blanks and samples in general exhibited strong non-specific noise and primer-dimers (figure 12 (B), for instance). It was also noted that upper row samples in a two-row gel were systematically brighter than lower row samples (bright: figure 10 A & C; dim: figure 10 B & D). This may be due to optical properties of the gel and might pose a problem if also weak bands are to be sought for. While not deemed necessary, nonspecific noise could possibly be reduced in gels using a mRNA-specific RNA isolation method or mRNA-specific cDNA-transcription primers rather than random primers.

Although there were some slight differences between the Sendai-induced and retrovirally induced lines, such as the initial success with the PCR analysis, no difference can reliably be attributed to the difference in induction method on the basis of this data. As all required markers for pluripotency were identified and no transgene activity was observed, cells may be used as pluripotent stem cells in research protocols. It must however be noted that study of pluripotency does not sort out iPS cell lines with different disease-related genotypes and iPS cells and embryonic stem cells from each other. As contaminations may occur during cell culture and manipulation, these possibilities are to be searched with other methods.

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## APPENDICES

### Appendix 1. Media contents.

Contents of 50 ml aliquot of KSR medium.

|                                     |          |
|-------------------------------------|----------|
| KnockOut™ DMEM (KO-DMEM)            | 38,65 ml |
| KnockOut™ serum replacement (KO-SR) | 10 ml    |
| GlutaMax™                           | 0,5 ml   |
| Non-essential amino acids (NEAA)    | 0,5 ml   |
| Penicillin/Streptomycin             | 0,25 ml  |
| β-mercaptoethanol                   | 98 µl    |
| FGF (7,8 µg/ml)                     | 26 µl    |

Contents of 50 ml aliquot of MEF medium.

|                             |         |
|-----------------------------|---------|
| KnockOut™ DMEM (KO-DMEM)    | 44,5 ml |
| FBS (fetal bovine serum)    | 5 ml    |
| GlutaMax™                   | 0,5 ml  |
| Medium is sterile filtered. |         |

Freezing medium contents for 0,5 ml.

|  |              |
|--|--------------|
| FBS, fetal bovine serum                        | 90 %, 450 µl |
| DMSO, dimethyl sulfoxide                       | 10 %, 50 µl  |
| ROCK (rho-associated protein kinase) inhibitor | 0,5 µl       |

Contents of 50 ml aliquot of embryoid body medium.

|                                     |          |
|-------------------------------------|----------|
| KnockOut™ DMEM (KO-DMEM)            | 28,65 ml |
| KnockOut™ serum replacement (KO-SR) | 20 ml    |
| GlutaMax™ (Life Technologies)       | 0,5 ml   |
| Non-essential amino acids (NEAA)    | 0,5 ml   |
| Penicillin/Streptomycin             | 0,25 ml  |
| β-mercaptoethanol                   | 98 µl    |

## Appendix 2. Fluorescence staining additional data.

## Fluorescence staining sample passages.

| Cell line       | Passage |
|-----------------|---------|
| UTA.07001.CPVT  | 26      |
| UTA.05303.CPVT  | 14      |
| UTA.05404.CPVT  | 30      |
| UTA.02912.HCMTs | 10      |
| UTA.01402.HCMTs | 10      |
| UTA.11505.WTsb  | 32      |

## Fluorescence staining solutions.

| Solution                        | Contents   |
|---------------------------------|--|
| PBS                             | Phosphate buffered saline, $[\text{PO}_4^{3-}] = 0,01 \text{ M}$ , $\text{pH} = 7,4$ |
| 4 % PFA                         | 4 % w/v paraformaldehyde in purified water, filtered                                 |
| Blocking solution               | 10 % normal donkey serum, 0,1 % Triton X-100, 1 % bovine serum albumin in PBS        |
| Primary Ab + washing solution   | 1 % normal donkey serum, 0,1 % Triton X-100, 1 % bovine serum albumin in PBS         |
| Secondary Ab + washing solution | 1 % bovine serum albumin in PBS  |
| PB                              | Phosphate buffer, $[\text{PO}_4^{3-}] = 0,01 \text{ M}$ , $\text{pH} = 7,4$          |
| Mounting solution               | Vectashield® with DAPI (Vector Laboratories)   |

## Appendix 3. Pipetting charts for PCR.

1 (4)

**Pipetting chart for transgenes**

| well | upper row        |           | well | lower row        |            |
|------|------------------|-----------|------|------------------|------------|
| 1    | <b>standard</b>  |           | 1    | <b>standard</b>  |            |
| 2    | 07001.CPVT p. 15 | GAPDH     | 2    | 07001.CPVT p. 15 | exo SOX2   |
| 3    | 05303.CPVT p. 13 | GAPDH     | 3    | 05303.CPVT p. 13 | exo SOX2   |
| 4    | 05404.CPVT p. 19 | GAPDH     | 4    | 05404.CPVT p. 19 | exo SOX2   |
| 5    |                  |           | 5    | positive control | exo SOX2   |
| 6    |                  |           | 6    |                  |            |
| 7    | 07001.CPVT p. 15 | exo c-Myc | 7    |                  |            |
| 8    | 05303.CPVT p. 13 | exo c-Myc | 8    | 07001.CPVT p. 15 | exo OCT3/4 |
| 9    | 05404.CPVT p. 19 | exo c-Myc | 9    | 05303.CPVT p. 13 | exo OCT3/4 |
| 10   | positive control | exo c-Myc | 10   | 05404.CPVT p. 19 | exo OCT3/4 |
| 11   |                  |           | 11   | positive control | exo OCT3/4 |
| 12   |                  |           | 12   |                  |            |
| 13   | 07001.CPVT p. 15 | exo Klf4  | 13   |                  |            |
| 14   | 05303.CPVT p. 13 | exo Klf4  | 14   | H2O (cDNA)       | GAPDH      |
| 15   | 05404.CPVT p. 19 | exo Klf4  | 15   | no RT            | exo c-Myc  |
| 16   | positive control | exo Klf4  | 16   | H2O (PCR)        | exo c-Myc  |

| well | upper row         |            | well | lower row         |           |
|------|-------------------|------------|------|-------------------|-----------|
| 1    | <b>standard</b>   |            | 1    | <b>standard</b>   |           |
| 2    | 02912.HCMTs p. 14 | exo OCT3/4 | 2    | 02912.HCMTs p. 14 | exo SOX2  |
| 3    | 01401.HCMTs p. 9  | exo OCT3/4 | 3    | 01401.HCMTs p. 9  | exo SOX2  |
| 4    | 01402.HCMTs p. 10 | exo OCT3/4 | 4    | 01402.HCMTs p. 10 | exo SOX2  |
| 5    | 11505.WTsb p. 29  | exo OCT3/4 | 5    | 11505.WTsb p. 29  | exo SOX2  |
| 6    | positive control  | exo OCT3/4 | 6    | positive control  | exo SOX2  |
| 7    |                   |            | 7    |                   |           |
| 8    |                   |            | 8    |                   |           |
| 9    | 02912.HCMTs p. 14 | exo Klf4   | 9    | 02912.HCMTs p. 14 | exo c-Myc |
| 10   | 01401.HCMTs p. 9  | exo Klf4   | 10   | 01401.HCMTs p. 9  | exo c-Myc |
| 11   | 01402.HCMTs p. 10 | exo Klf4   | 11   | 01402.HCMTs p. 10 | exo c-Myc |
| 12   | 11505.WTsb p. 29  | exo Klf4   | 12   | 11505.WTsb p. 29  | exo c-Myc |
| 13   | positive control  | exo Klf4   | 13   | positive control  | exo c-Myc |
| 14   |                   |            | 14   |                   |           |
| 15   |                   |            | 15   |                   |           |
| 16   | 02912.HCMTs p. 14 | GAPDH      | 16   | H2O (cDNA)        | GAPDH     |
| 17   | 01401.HCMTs p. 9  | GAPDH      | 17   | no RT             | exo c-Myc |
| 18   | 01402.HCMTs p. 10 | GAPDH      | 18   | H2O (PCR)         | exo c-Myc |
| 19   | 11505.WTsb p. 29  | GAPDH      | 19   |                   |           |
| 20   | positive control  | GAPDH      | 20   |                   |           |

## Pipetting chart for endogenous pluripotency markers

2 (4)

| well | upper row        | primers       |
|------|------------------|---------------|
| 1    | <b>standard</b>  |               |
| 2    | 07001.CPVT p. 27 | Nanog         |
| 3    | 07001.CPVT p. 27 | <b>OCT3/4</b> |
| 4    | 07001.CPVT p. 27 | <b>SOX2</b>   |
| 5    | 07001.CPVT p. 27 | <b>GAPDH</b>  |
| 6    | 07001.CPVT p. 27 | <b>Rex1</b>   |
| 7    | 07001.CPVT p. 27 | c-Myc         |
| 8    |                  |               |
| 9    |                  |               |
| 10   | 05404.CPVT p. 31 | Nanog         |
| 11   | 05404.CPVT p. 31 | GAPDH         |
| 12   | 05404.CPVT p. 31 | Rex1          |
| 13   | 05404.CPVT p. 31 | OCT3/4        |
| 14   | 05404.CPVT p. 31 | SOX2          |
| 15   | 05404.CPVT p. 31 | c-Myc         |
| 16   |                  |               |
| 17   |                  |               |
| 18   | H2O (cDNA)       | Rex1          |
| 19   | no RT            | Nanog         |
| 20   | H2O (PCR)        | c-Myc         |

| well | upper row        | primers |
|------|------------------|---------|
| 1    | <b>standard</b>  |         |
| 2    | 11505.WTsb p. 29 | Nanog   |
| 3    | 11505.WTsb p. 29 | GAPDH   |
| 4    | 11505.WTsb p. 29 | Rex1    |
| 5    | 11505.WTsb p. 29 | OCT3/4  |
| 6    | 11505.WTsb p. 29 | SOX2    |
| 7    | 11505.WTsb p. 29 | c-Myc   |
| 8    |                  |         |
| 9    |                  |         |
| 10   | 05303.CPVT p. 13 | Nanog   |
| 11   | 05303.CPVT p. 13 | GAPDH   |
| 12   | 05303.CPVT p. 13 | Rex1    |
| 13   | 05303.CPVT p. 13 | OCT3/4  |
| 14   | 05303.CPVT p. 13 | SOX2    |
| 15   | 05303.CPVT p. 13 | c-Myc   |
| 16   |                  |         |
| 17   |                  |         |
| 18   | H2O (cDNA)       | Rex1    |
| 19   | no RT            | Nanog   |
| 20   | H2O (PCR)        | c-Myc   |

| well | lower row        | primers        |
|------|------------------|----------------|
| 1    | <b>standard</b>  |                |
| 2    | 11505.WTsb p. 29 | $\beta$ -actin |
| 3    | 11505.WTsb p. 29 | OCT3/4         |
| 4    |                  |                |
| 5    | 05303.CPVT p. 13 | $\beta$ -actin |
| 6    | 05303.CPVT p. 13 | OCT3/4         |
| 7    |                  |                |
| 8    |                  |                |
| 9    | H2O (cDNA)       | $\beta$ -actin |
| 10   | no RT            | $\beta$ -actin |
| 11   | H2O (PCR)        | $\beta$ -actin |

| well | upper row               | primers |
|------|-------------------------|---------|
| 1    | <b>standard</b>         |         |
| 2    | 02912.HCMTs p. 14       | Nanog   |
| 3    | 02912.HCMTs p. 14       | GAPDH   |
| 4    | 02912.HCMTs p. 14       | Rex1    |
| 5    | 02912.HCMTs p. 14       | OCT3/4  |
| 6    | 02912.HCMTs p. 14       | SOX2    |
| 7    | 02912.HCMTs p. 14       | c-Myc   |
| 8    |                         |         |
| 9    |                         |         |
| 10   | 01402.HCMTs p. 10       | Nanog   |
| 11   | 01402.HCMTs p. 10       | GAPDH   |
| 12   | 01402.HCMTs p. 10       | Rex1    |
| 13   | 01402.HCMTs p. 10       | OCT3/4  |
| 14   | 01402.HCMTs p. 10       | SOX2    |
| 15   | 01402.HCMTs p. 10       | c-Myc   |
| 16   |                         |         |
| 17   |                         |         |
| 18   | H <sub>2</sub> O (cDNA) | Rex1    |
| 19   | no RT                   | Nanog   |
| 20   | H <sub>2</sub> O (PCR)  | c-Myc   |

### Pipetting charts for embryoid body samples

| well | upper row               | primers                   | well | lower row        | primers                   |
|------|-------------------------|---------------------------|------|------------------|---------------------------|
| 1    | <b>standard</b>         |                           | 1    | <b>standard</b>  |                           |
| 2    | 07001.CPVT p. 15        | SOX17                     | 2    | 05404.CPVT p. 18 | SOX17                     |
| 3    | 07001.CPVT p. 15        | $\alpha$ -cardiac actinin | 3    | 05404.CPVT p. 18 | $\alpha$ -cardiac actinin |
| 4    | 07001.CPVT p. 15        | Nestin                    | 4    | 05404.CPVT p. 18 | Nestin                    |
| 5    | 07001.CPVT p. 15        | AFP                       | 5    | 05404.CPVT p. 18 | AFP                       |
| 6    | 07001.CPVT p. 15        | PAX6                      | 6    | 05404.CPVT p. 18 | PAX6                      |
| 7    | 07001.CPVT p. 15        | GAPDH                     | 7    | 05404.CPVT p. 18 | GAPDH                     |
| 8    | 07001.CPVT p. 15        | VEGFR2/KDR                | 8    | 05404.CPVT p. 18 | VEGFR2/KDR                |
| 9    |                         |                           | 9    |                  |                           |
| 10   |                         |                           | 10   |                  |                           |
| 11   | H <sub>2</sub> O (cDNA) | VEGFR2/KDR                | 11   |                  |                           |
| 12   | no RT                   | VEGFR2/KDR                | 12   |                  |                           |
| 13   | H <sub>2</sub> O (PCR)  | VEGFR2/KDR                | 13   |                  |                           |

4 (4)

| well | upper row               | primers                   | well | lower row        | primers                   |
|------|-------------------------|---------------------------|------|------------------|---------------------------|
| 1    | <b>standard</b>         |                           | 1    | <b>standard</b>  |                           |
| 2    | 05303.CPVT p. 11        | SOX17                     | 2    | 11505.WTsb p. 33 | SOX17                     |
| 3    | 05303.CPVT p. 11        | $\alpha$ -cardiac actinin | 3    | 11505.WTsb p. 33 | $\alpha$ -cardiac actinin |
| 4    | 05303.CPVT p. 11        | Nestin                    | 4    | 11505.WTsb p. 33 | Nestin                    |
| 5    | 05303.CPVT p. 11        | AFP                       | 5    | 11505.WTsb p. 33 | AFP                       |
| 6    | 05303.CPVT p. 11        | PAX6                      | 6    | 11505.WTsb p. 33 | PAX6                      |
| 7    | 05303.CPVT p. 11        | GAPDH                     | 7    | 11505.WTsb p. 33 | GAPDH                     |
| 8    | 05303.CPVT p. 11        | VEGFR2/KDR                | 8    | 11505.WTsb p. 33 | VEGFR2/KDR                |
| 9    |                         |                           | 9    |                  |                           |
| 10   |                         |                           | 10   |                  |                           |
| 11   | H <sub>2</sub> O (cDNA) | VEGFR2/KDR                | 11   |                  |                           |
| 12   | no RT                   | VEGFR2/KDR                | 12   |                  |                           |
| 13   | H <sub>2</sub> O (PCR)  | VEGFR2/KDR                | 13   |                  |                           |

| well | upper row               | primers                   | well | lower row         | primers                   |
|------|-------------------------|---------------------------|------|-------------------|---------------------------|
| 1    | <b>standard</b>         |                           | 1    | <b>standard</b>   |                           |
| 2    | 01402.HCMTs p. 10       | SOX17                     | 2    | 02912.HCMTs p. 13 | SOX17                     |
| 3    | 01402.HCMTs p. 10       | $\alpha$ -cardiac actinin | 3    | 02912.HCMTs p. 13 | $\alpha$ -cardiac actinin |
| 4    | 01402.HCMTs p. 10       | Nestin                    | 4    | 02912.HCMTs p. 13 | Nestin                    |
| 5    | 01402.HCMTs p. 10       | AFP                       | 5    | 02912.HCMTs p. 13 | AFP                       |
| 6    | 01402.HCMTs p. 10       | PAX6                      | 6    | 02912.HCMTs p. 13 | PAX6                      |
| 7    | 01402.HCMTs p. 10       | GAPDH                     | 7    | 02912.HCMTs p. 13 | GAPDH                     |
| 8    | 01402.HCMTs p. 10       | VEGFR2/KDR                | 8    | 02912.HCMTs p. 13 | VEGFR2/KDR                |
| 9    |                         |                           | 9    |                   |                           |
| 10   |                         |                           | 10   |                   |                           |
| 11   | H <sub>2</sub> O (cDNA) | VEGFR2/KDR                | 11   |                   |                           |
| 12   | no RT                   | VEGFR2/KDR                | 12   |                   |                           |
| 13   | H <sub>2</sub> O (PCR)  | VEGFR2/KDR                | 13   |                   |                           |