

Final Degree Project

**PLURIPOTENT STEM CELL BANK FOR  
FREQUENT HAPLOTYPES OF THE  
SPANISH POPULATION**

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**Biotechnology Degree**

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# Resum

**Títol:** Banc de cèl·lules mare pluripotents per haplotips freqüents a la població espanyola.

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**Paraules clau:** Cèl·lules mare pluripotents induïdes, reprogramació d'iPSCs, caracterització d'iPSCs, haplotips, rebuig immunitari.

Els continguts principals d'aquest TFG es basen en la generació de cèl·lules mare pluripotents induïdes en el marc del projecte iPS-PANIA que està realitzant el grup *Teràpia amb cèl·lules mare pluripotents induïdes* liderat per la Dra. Anna Veiga.

iPS-PANIA té l'objectiu de seleccionar mostres de sang de cordó umbilical (CBUs) que cobreixin el 25% dels haplotips més comuns de la població espanyola. El procés comença amb la extracció de les cèl·lules CD34 per la seva posterior reprogramació a cèl·lules mare pluripotents induïdes i la seva posterior caracterització.

Aquestes cèl·lules seran generades sota condicions lliures de components animals i les Bones Pràctiques de Manufactura (GMP) per la seva posterior utilització en aplicacions clíniques. Aquestes línies cal que siguin generades amb els corresponents requeriments ètics i legals.

Aquest TFG està enfocat en seguir el procediment de generació d'una de les línies i elaborar una guia extensa que conté els passos necessaris per ampliar el percentatge d'haplotips disponibles de la població espanyola.

Ampliar el número de línies dels diferents haplotips de la població espanyola ajudarà en un futur a apropar-nos a la medicina personalitzada i poder utilitzar aquestes línies amb l'haplotip específic de cada pacient, per eliminar la probabilitat del rebuig immunitari i a la vegada, per acurtar el temps necessari de generació d'un òrgan per a un transplantament.

# Abstract

**Title:** : Pluripotent stem cell bank for frequent haplotypes in Spanish population

**Author:** Laia Valor Marcelino

**Supervisor:** Dr. Bernd Kuebler ( IDIBELL) and Dolors Antón Solà (UVic)

**Date:** June 2022

**Keywords:** Induced pluripotent stem cell, iPSCs reprogramming, iPSCs characterization, haplotypes, immune rejection.

The main content of this final grade project is the generation of induced pluripotent stem cells (iPSCs) in the frame of the iPS-PANIA project of the group *Therapy with induced pluripotent stem cells* lead by Dra. Anna Veiga.

iPS-PANIA has the objective to select cord blood units (CBUs) covering 25% of the most common haplotypes from the Spanish population and to isolate CD34 cells from these CBUs for their reprogramming and the characterization of generated iPSC clones.

iPSCs lines are generated under xeno-free and Good Manufacturing Practices (GMP) conditions for their future use in clinical applications. These lines must also be generated with all ethical and legal requirements.

The TFG is focused on the procedures necessary for the generation of one of the iPSCs lines and, to write an extense detailed protocol that contains all the steps to give enough information to, in a future, continue generating more haplotypes iPSCs lines from the Spanish population in the future.

The increase of the number of iPSCs lines for different haplotypes will help in a near future to be closer to personalized medicine and to be able to use these iPSCs lines to match the haplotype of a specific patient, to eliminate immune rejection and to shorten the time necessary to generate patient derived organ for a transplantation.

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# 1. Introduction

Regenerative Medicine is one of the most innovative fields that is potentially expanding and improving day by day. It is one of the medicine branches that develop methods to regrow, repair or replace damaged or diseased cells, organs, or tissues to establish normal function.

Nowadays regenerative medicine includes a wide group of biomedical approaches to clinical therapies that may involve the use of stem cells. Some examples are the generation and use of therapeutic stem cells, tissue engineering and the production of artificial organs.

The main problem in medicine is to achieve a wide number of required organs needed for transplantations and try to avoid immune rejection. Regenerative Medicine is trying to be closer to personalized medicine to solve this kind of problems faster and easily using stem cells. [8]

There're three types of stem cells; Embryonic stem cells (ESC), adult stem cells and pluripotent stem cells. Regenerative medicine focus on reprogramming somatic cells in a pluripotent state.

Cells from inner cells mass (ICM) of the human embryo are pluripotent in their nature which means they have self-renewal capacity and they can differentiate into cells representing three germ layers. This is possible due to the expression of functional pluripotent factors.

Two Japanese biomedical scientists called Kazutoshi Takahashi and Shinya Yamanaka showed that four human transcription factors; octamer-binding protein 3/4 (OCT3/4), SOX2, Krüpel-like factor 4 (KLF4) and MYC, collectively referred to as OSKM, are enough to induce somatic cells into pluripotent stem cells. [1]

iPSCs are one type of stem cells that can differentiate into any kind of cell and have autorenewal capacities. To achieve this type of stem cells two types of reprogramming can be done: Integrating and non-integrating methods. Integrating methods are based on vectors that get integrated into the genome of host cells, potentially causing disruption or aberrant activation of neighbouring genes with a high risk of reactivation of the exogenous reprogramming factors. Methods as nucleofection with episomal vectors, transduction with Sendai viruses and transfection of synthetic mRNA are integration-free methods which mean there's no integration of the vectors into the host genome. [10,21,23]



Generation of iPSCs from patient cells will be useful to eliminate the risk of immune rejection as patient specific cells may be used for transplantation. Nowadays the main problems with iPSCs generation for specific patient cases are their elevated prices, it's high workload and the time needed to complete all the steps to use iPSCs for a transplantation which is between 9 and 12 months.

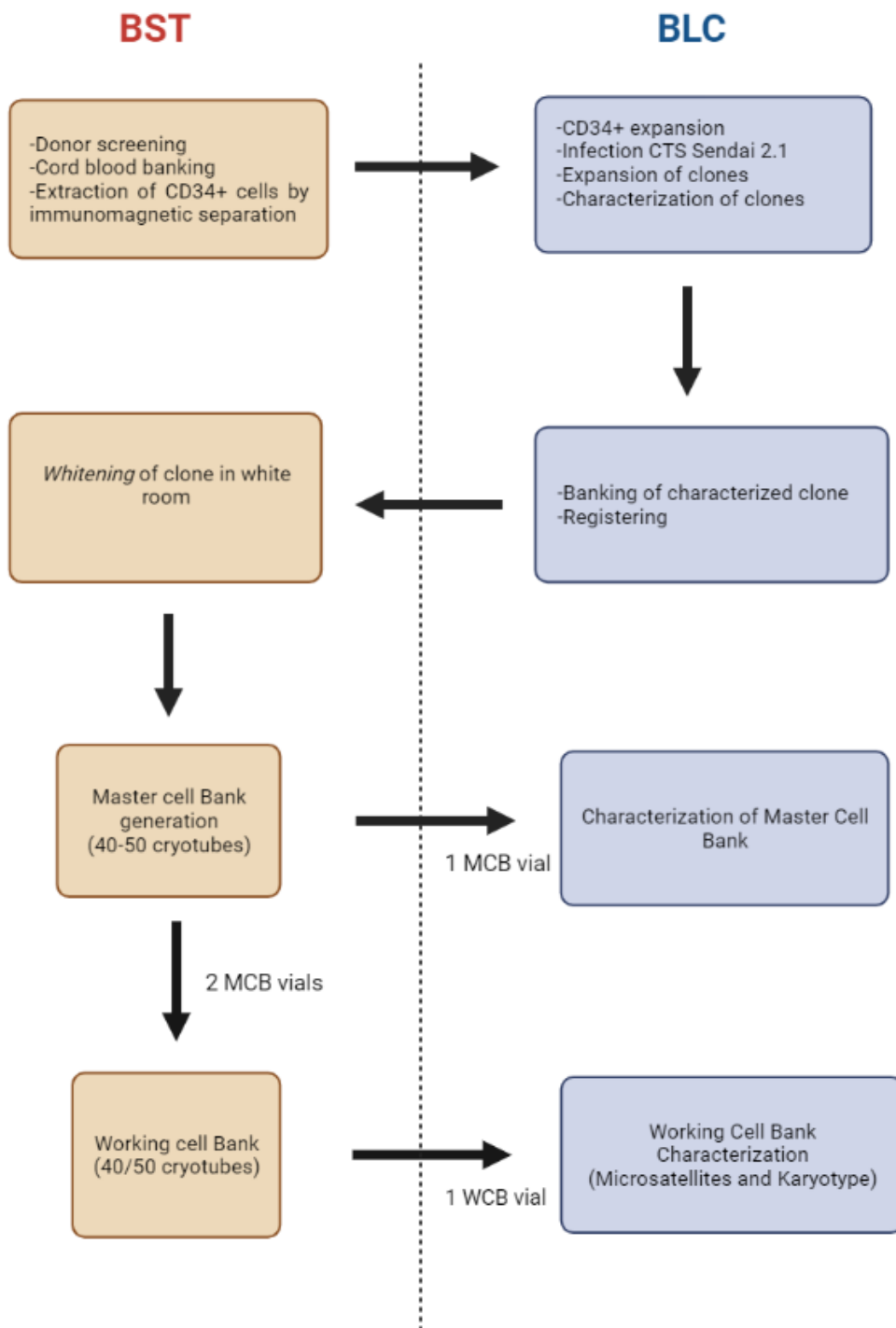
Personalized medicine is still a slow process that needs to be optimized to be useful in the near future as a solution for most of the diseases. To get closer to this objective, the leader investigator Dra Veiga from the group *Therapy with induced pluripotent stem cells* has started a project to generate a bank of hiPSCs (human induced pluripotent stem cells) for frequent haplotypes of the Spanish population. [24]

Haplotype is defined as the inheritance of a cluster of single nucleotide polymorphisms (SNPs), which are variations at single positions in the DNA sequence among individuals. Nowadays, the variability of haplotype between patients is still a barrier for the transplantation of tissues and organs. [30,31]

The project called IPS-PANIA has his objective on the generation of 7 different iPSCs lines under clinical grade condition corresponding to different haplotypes trying to cover around 25% of the spanish population haplotypes. [22, 28]

The starting material, CD34+ somatic cells, were isolated at Blood and Tissue Bank (BST). iPSCs lines were generated and characterized at the Bellvitge Biomedical Research Institute (IDIBELL), specifically at the National Stem Cell Bank (BST), under not clinical grade conditions. The generated iPSCs got back to the BST to be cultured under GMP conditions in a white room and then, a Master Cell Bank and a working Cell Bank (MCB and WCB, respectively) was generated. [4]

The generation of the MCB includes cell expansion of cells and crypreservation under clinical grade conditions in 40-50 cryovials containing at least one million viable cells. To produce the WCB, two MCB vials were thawed, expanded and frozen. iPSCs from MCB and WCB were characterized again at the BLC.



**Figure 1.** IPS-PANIA project methodology to generate 7 iPSCs lines under clinical grade conditions showing the specifics tasks of BST (Blood and Tissue bank) and BLC (National Stem Cell Bank)

The starting material is homozygous umbilical cord blood where HLAs from donors with different haplotypes were used to be reprogrammed and expanded. Most specifically, CD34 hematopoietic stem cells were isolated from umbilical cord blood samples and collected until the reprogramming process starts.

Umbilical cord blood samples are used because compared with other adult tissue they are less prone to somatic mutations and their HLA information is available through registries in each country. Another advantage to use HLA haplotype from homozygote donors lies in the ability to HLA match a significant number of patients using a relatively small number of well selected donors. [2]

CD34 cells are reprogrammed by their transduction with Sendai Virus. Sendai Virus transduction is a non-integrating method by a mouse and rat respiratory RNA virus which is non-pathogen to humans. For the reprogramming, a commercially available kit that contains three different virus constructs was used.

Generating a human iPSC-bank includes not only the generation of iPSCs, but also, required by Spanish law, the characterization of the resulting clones. The characterization is done to confirm the identity, the self-renewal capacity and the differentiation capacity of the cells.

Characterization of the lines includes several steps:

1. Molecular characterization
  - 1.1 qRT-PCR
2. In vitro pluripotency test
  - 2.1 Immunocytochemistry
  - 2.2 AP staining
3. In vitro differentiation test
  - 3.1 Embryoid bodies formation
  - 3.2 Immunocytochemistry
4. Karyotyping
5. DNA fingerprinting
  - 5.1 Microsatellites from somatic cells and from iPSC clones.

After the generation and characterization of the lines, they have to be registered and banked at the Spanish National Institute of Health.

This project will help with the problems mentioned before, making it possible to use iPSCs from the bank to shorten the process needed for future transplantations for specific patients. [24,29]

Related to this project, South Korea is one of the countries that have generated iPSCs lines for the top 10 haplotypes in Korea. It is shown that these haplotypes are at relatively high frequency in most Asian populations, but in non-Asian populations at a low

frequency. These results suggest that some of the HLA-homozygous iPSC lines generated in Korea will be useful not only to cover the Korean population but to cover other populations with high or low frequency of HLA haplotypes.

It is estimated that in a country with limited HLA variability, such as Japan, around 140 lines could provide coverage for 90% of the population and around 80 lines to cover more than 50% of the population. [2]

Working with Anna Veiga's investigation group makes me able to follow and help with the process to generate this iPSCs lines and to establish an extensive guide that contains the methodology for the generation of more haplotype matched lines of the Spanish population to increase the coverage of the lines. This will serve for basic investigation, future clinical trials and to improve personalized medicine.

## 2. Objectives

Generation of iPSCs under Xenofree and GMP conditions for the most common haplotypes of the spanish population will provide to the society the chance to be closer to the use of personalized medicine and to make progress on medicine investigation and clinical trials.

The objectives are:

- To learn iPSCs culture techniques in aseptic conditions and the methodology to characterize iPSCs clones.
- To make an extensive protocol describing the isolation, generation and characterization of iPSCs from one of the 7 lines from a cord blood unit with the haplotype 11-27-1

## 3. Methodology

### 3.1 Study area

An extensive protocol was set up and improved to reprogram CD34+ cells isolated from human cord blood. Main goal was to achieve an iPSC-bank generated from 7 different donors covering 25% of the most frequent haplotypes of the Spanish population.

To set up the methodology, CD34+ cells were isolated from cord blood units (CBU) which were not used to generate the iPSC bank. The prior investigation and information about how to generate this haplotype has been obtained by an extensive literature search of articles related with the generation of iPSCs lines and about the methods needed to achieve it.

This guide will help researchers from the area of Regenerative Medicine to generate more iPSC lines in order to achieve the highest possible coverage of Spanish haplotypes, or, on the other hand, encourage researchers from other countries to start to generate iPSC banks from the main haplotypes of their countries.

### 3.2 Study design

Before writing this guide, I learnt how to generate iPSCs from somatic cells like human fibroblasts and peripheral blood mononuclear cells (PBMCs). In this step, my prior study plan was as follows:

1. The Sample collection from the cord blood donors and extraction of CD34+ cells with immunomagnetic beads was done at the Blood and tissues bank (BST) and extracted CD34+ cells were moved to the Barcelona Stem Cell Bank (BLC). Due to that, this step will be explained by bibliographic information from papers published by the BST and others. [4]
2. Reprogram CD34+ cells to iPSCs following and helping during all the processes of the generation of the last haplotype *H2-11-27-1 CBiPS8 Sv4F I1* and complete the background information with bibliographic sources. [22]
3. Follow and help with the characterization of the above mentioned haplotype and complete the technical information with bibliographic tools. [11]

### 3.3 Population studied

For this investigation, donors with the most frequent haplotypes of Spanish population were chosen. In total, cord blood units from 7 different haplotypes were used covering 25% of the most frequent haplotypes of Spain:

**Table 1.** *iPSCs from 7 most frequent haplotypes of Spanish population*

| 7 most frequent haplotypes of the Spanish population used to generate the iPSC bank |
|---|
| 29-44-7   |
| 30-18-3   |
| 3-7-15  |
| 1-8-3   |
| 33-14-1   |
| 24-7-15   |
| 11-27-1   |

In this protocol, the methodology of generation and characterization of iPSCs is explained using CD34 cells with the haplotype *H<sub>Z</sub>-11-27-1* as an example.

All cord blood donors were informed about the project and the interest of their homozygous donated samples. They must answer a health questionnaire to be sure they don't have genetic diseases or other incompatibilities and finally they have to sign an informed consent for the donation.

109 cord blood units were genotyped in high resolution and 43 different haplotypes were identified. Finally, cord blood units (CBUs) were chosen with a content of at least  $2 \times 10^6$  cells and verifications on frozen segments were performed for 25 units with the goal to generate seven haplotypes that represents the top number of haplotypes to give the widest coverage of the Spanish population.

### 3.4 Place where the study is developed

The project has been carried out at the Barcelona Stem Cell Bank located in Bellvitge Biomedical Investigation Institute (IDIBELL), with the investigation group *Therapy with pluripotent stem cells* lead by Dra. Anna Veiga. The project has been started in 2018 with the generation of the seven haplotypes and will be finished this year (2022).

CD34+ cells coming from cord blood samples were collected and isolated at the BST and were moved to the IDIBELL for their reprogramming and characterization. Some steps during characterization of the lines were done at different IDIBELL platforms, such as histology, molecular and microscopy platforms. I could follow all the processes done at the IDIBELL performed with the last haplotype.

## 3.5 Interventions

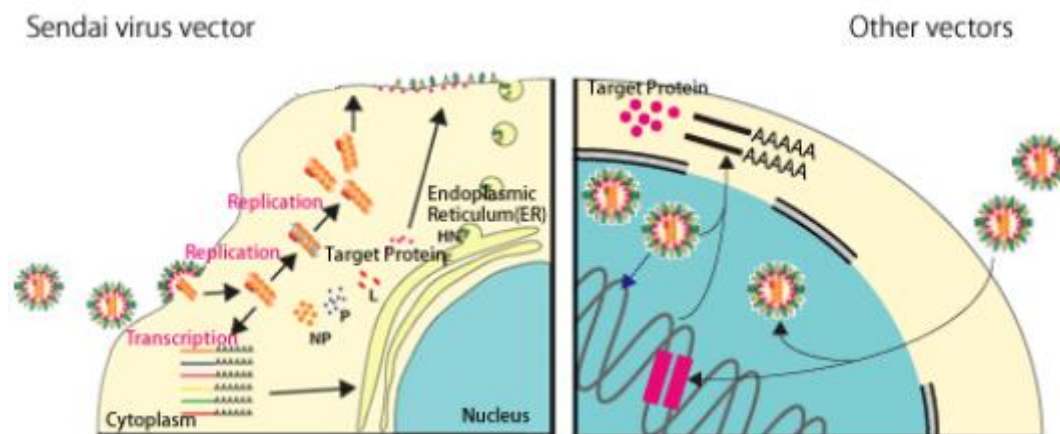
### 3.5.1 Infection with Sendai Virus 2.1

Sendai Virus is a respiratory virus of mouse and rat, belonging to the Paramyxoviridae family. It is an enveloped virus of 150-250 nm in diameter with a single chain RNA genome in the minus sense, containing 15,384 bases.

Sendai Virus is formed by six genes coding for viral proteins in the following order starting from the 3' end:

1. Nucleocapsid protein (NP): Makes the core nucleocapsid complex with the genome RNA.
2. Phosphoprotein (P): Small subunit of the RNA polymerase.
3. Matrix protein (M): Supports the envelope structure from the inside.
4. Fusion protein (F): Fuses the viral envelope with the cell membrane when the virus enters the cell.
5. Hemagglutinin-Neuraminidase (HN): Recognises the cell surface receptor called sialic acid.
6. Large Protein (L): Large subunit of RNA polymerase.

Sendai virus naturally infects cells by attaching itself to sialic acid receptor, that is present on the surface of different cells. As it's a non-integrating virus, it doesn't integrate into the genome of the cell. It infects the cell and goes through genome replication and protein synthesis and finally the new virus particles are assembled and released.



**Figure 2.** Comparison of the lifecycles of non-integrating SeV vectors and other integrating vectors

Infection with Sendai Virus to reprogram a somatic cell to iPSCs, is done by use of the commercially available Sendai reprogramming kit called *CTS Cytotune-iPS 2.1 Sendai Reprogramming kit* [33]. This kit uses vectors based on a modified, non-transmissible form of Sendai Virus to deliver and express key genetic factors necessary for reprogramming somatic cells into iPSCs. Specifically, this kit uses vectors that are non-integrating into the genome of the host cell and have been engineered to increase



biological and environmental safety. The kit contains three Sendai Virus constructs containing four reprogramming factors that are the OSKM factors; hKOS (hKlf4, Oct4 and Sox2), hL-Myc and hKlf4. [32]

Table 2 shows the titer of each three constructs (units of viruses capable to infect cells in each ml) and depending on the lot, the titer can be slightly different.

Important information for this step is the number of cells needed for reprogramming. In general, 500 000 cells from peripheral blood (Peripheral blood mononuclear cells, PBMCs) or 50 000 purified CD34+ cells are enough to use for the infection. After determining the number of cells, the Multiplicity of Infection (MOI) must be calculated. MOI means the amount of virus particles for each cell you want to infect.

Table 2 shows the information from the kit that have been used to reprogram the last haploline clone with their corresponding titer.

**Table 2.** *Composition of Cytotune 2.1 kit*

| Component           | Titer (CIU/mL)       | pH   | Osmolarity |
|---------------------|----------------------|------|------------|
| Cytotune 2.1 hKOS   | 0.85x10 <sup>8</sup> | 6.22 | 621 mOsm   |
| Cytotune 2.1 hL-Myc | 0,98x10 <sup>8</sup> | 6.32 | 618 mOsm   |
| Cytotune 2.1 hKlf4  | 0,60x10 <sup>8</sup> | 6.16 | 622 mOsm   |

The number of cells to infect is 50.000 with a MOI of 5:2,5:3. To calculate the volume of each virus construct the following formula has to be used:

$$\text{Volume of virus } (\mu\text{l}) = \frac{\text{MOI (CIU/cell)} \times (\text{number of cells})}{\text{titer of virus (CIU/mL)} \times 10^{-3} (\text{mL}/\mu\text{L})}$$

**Figure 3.** *Formula to calculate the volume of virus (μl)*

50 000 cells, MOI 5, hKOs:  $(5 \times 50\,000) / (0.85 \times 10^8) \times 10^{-3} = 2,94 \mu\text{l}$

50 000 cells, MOI 2,5, hL-Myc:  $(2,5 \times 50\,000) / (0.98 \times 10^8) \times 10^{-3} = 1,28 \mu\text{l}$

50 000 cells, MOI 3, hKlf4:  $(3 \times 50\,000) / (0.60 \times 10^8) \times 10^{-3} = 2,5 \mu\text{l}$

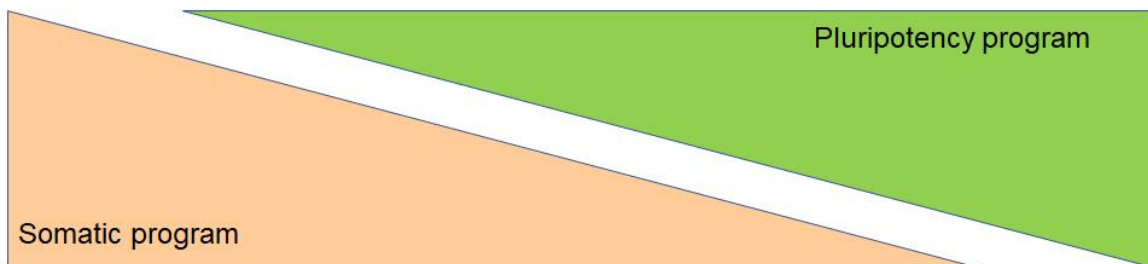
Transduction protocol:

1. Add the calculated volumes of the three Sendai virus constructs to 1 ml of Stem Pro 34 (SP34 complete medium) supplemented with 4 μg/ml of polybrene and cytokines (100ng/ml SCF, 20ng/ml IL-3, 20ng/ml TPO and 100ng/ml FH3-L). Add 50000 CD34+ cells.

2. Centrifuge the cells with the virus at 1000g for 30 minutes.
3. Resuspend the cells without aspirating the medium with the virus and transfer to an ultra-low attachment plate.
4. Incubate the cells over night at 37°C and 5% CO<sub>2</sub>.

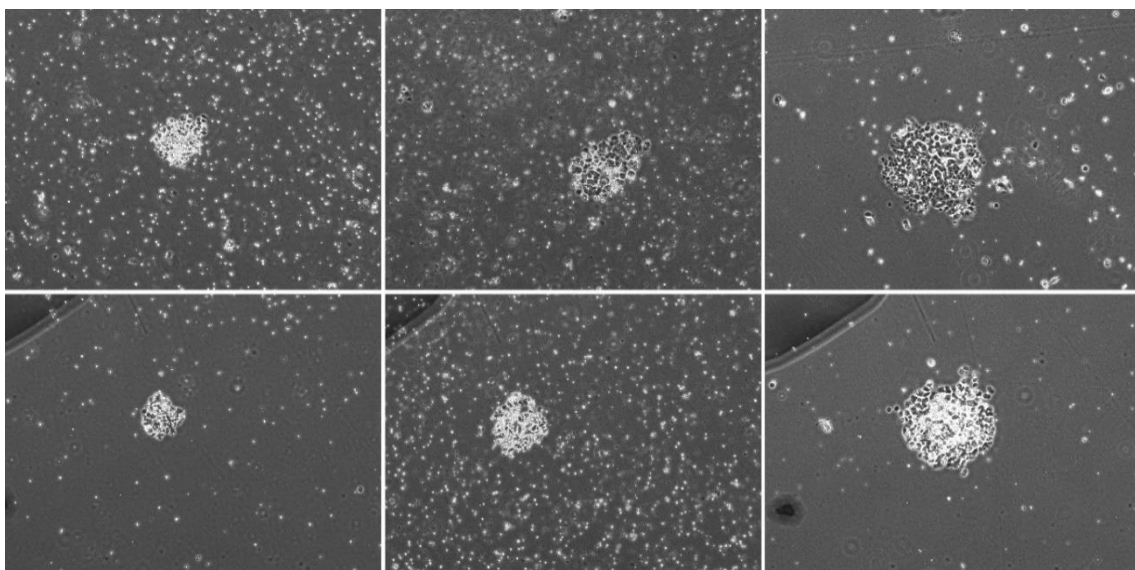
### 3.5.2 Reprogramming of CD34+ cells to iPSCs

Once the cells have been infected by Sendai Virus, the OSKM transcription factors are activating genes related with pluripotency and are repressing other genes determining somatic cells. [1]



**Figure 4.** Schematic way to explain how iPSCs repress somatic genes and activate pluripotency genes during reprogramming.

When somatic cells start to express those genes related with pluripotency, they start to change their morphology. To recognise emerging colonies of iPSCs that will finally get reprogrammed, it's important to control the plates every day. Characteristic morphology for iPSCs are the formation of colonies with well-defined borders of proliferating cells with a big nucleus and a small cytosol.



**Figure 5.** Rising of iPSC colonies from CD34+ cells transduced with OSKM factors. Each row shows an iPSC clone growing up from a single CD34+ cell at day 7,8 and 9 after transduction

A total of 50.000 cells were infected but just a small portion of these cells will be reprogrammed to iPSCs. From the total cells, just 5/10% will be infected by the virus and only 0,1% of the infected cells will develop to iPSCs. That means, from 50.000 cells, just 5.000 will be infected and of these, only 5 cells will be reprogrammed to iPSCs.

Approximately 6-8 days after infection, first colonies of iPSC clones emerge on the plates and after reaching an appropriate size, single colonies were manually picked and seeded in new plates previously coated with LN521 in Essential 8 Flex medium. During the next 8 weeks, colonies that are candidates to develop to iPSC should grow doing splits from the colonies selected. [33]

In this project the conditions of iPSCs generation must be CTS quality; it means use xeno-free products (doesn't contain non-human derived products). For that reason, iPSCs generation will be done under feeder-free conditions and laminin coated (LN521) dishes.

The specific protocols are the next ones:

**DAY 1 after transduction:** Replace the medium with fresh StemPro-34 medium containing cytokines by spinning to remove the Sendai Vectors.

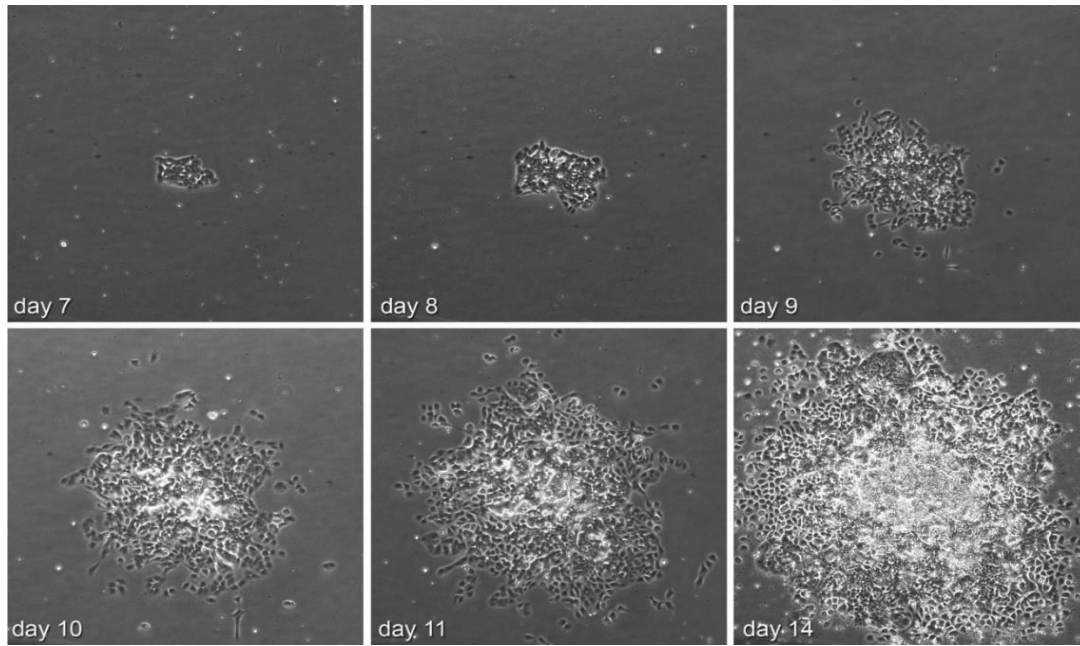
**DAY 3:** Plate the transduced cells on laminin-coated culture dishes in complete StemPro-34 medium without cytokines.

**DAY 4-6:** Replace half of the spent medium with fresh complete StemPro-34 medium without cytokines.

**DAY 7:** Start transitioning into Essential 8 Medium by replacing half of the StemPro-34 medium without cytokines with complete iPSCs medium.

**DAY 8:** Replace the entire medium with Essential 8 medium to conclude the transitioning and continue culturing cells on laminin-coated culture dishes.

**DAY 9-28:** Replace medium with fresh Essential 8 Medium every day and monitor the culture vessels for the emergence of iPSC colonies. When iPSC colonies are ready to transfer, pick single colonies and transfer onto fresh laminin-coated culture dishes for expansion. Each colony was formed by one infected CD34 cell and is called one clone. From all cord blood sample one clone is selected for full characterization.



**Figure 6.** *Feeder-free colony formation growing after 7, 8, 9, 10, 11 and 14 days after infection*

### 3.5.3 Characterization

#### Molecular characterization:

To select a clone for full characterization, it's important to choose a clone that doesn't contain Sendai virus transcripts or genes. Therefore, from at least 4 clones of each line a molecular characterization has to be performed. [2,11]

#### 🚦 qRT-PCR :

Preparation for molecular characterization requires the expansion of clones to freeze criotubes from early passages and prepare cell pellets and criotubes at passage 7.

First step of the characterization of a clone is a molecular characterization by a qRT-PCR. qRT-PCR is a technique where a specific enzyme called retrotranscriptase synthesizes cDNA from RNA.

qRT-PCR has three main parts:

-RNA extraction:

From cell pellets, RNA is extracted by the Maxwell technology using magnetic beads. While working with RNA it's important to use micropipettes cleaned with RNAase and use tips specifically for RNA.



**Figure 7.** Maxwell machinery

Maxwell machinery showed in figure 7 has several advantages:

- Avoid sample contamination by our DNA
- Avoid working with toxics as used by other RNA extraction techniques
- 16 samples can be processed at the same time.
- It's economic compared to other techniques.
- Required DNase treatment included inside the machine.

Protocol for RNA extraction:

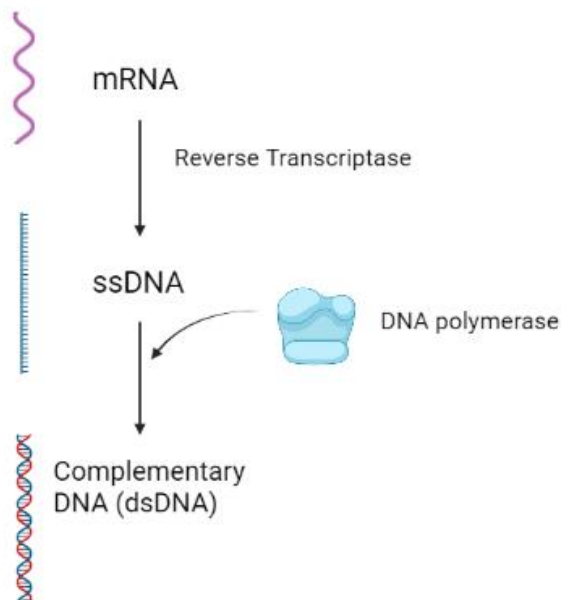
1. Expose Maxwell machinery for 5 minutes to ultraviolet light
2. Sample preparation: Dissolve RNA by adding 200  $\mu\text{L}$  of a buffer composed by a homogenization solution and triglycerol. Add 200  $\mu\text{L}$  of lysis solution.
3. Mix 15 sec and spin down sample.
4. Add a total volume of 400  $\mu\text{L}$  into the dishes of the Maxwell machinery
5. Add 5  $\mu\text{L}$  of DNAase to the yellow well of the machine, wait till the solution turns to green.
6. Add 50  $\mu\text{L}$  of elution buffer (nuclease free water) to the wells in front of the plaque
7. Incubate for 50 minutes at room temperature.
8. Freeze RNA samples at  $-80^{\circ}\text{C}$  till retrotranscription process is started.



**Figure 8.** Wells inside Maxwell machinery

-Retrotranscription

Once RNA has been extracted, the complementary DNA must be synthesised. This step is performed in a block cycler by a specific enzyme (Reverse transcriptase). [\[36\]](#)



**Figure 9.** Steps to synthesise cDNA from RNA sample

Steps for the cDNA synthesis from RNA sample:

1. To prepare the template-primer mix, thaw the components listed in table 3 and place them on ice
2. Briefly centrifuge all the reagents before setting up the reactions
3. Set up the reaction components in a nuclease free microcentrifuge tube placed on ice.

**Table 3.** *Reaction components to microcentrifuge*

| Reagent  | Volume       | Final conc.                          |
|--|--------------|--------------------------------------|
| total RNA or poly (A)+mRNA                                   | variable     | 1µg total RNA *or 10 ng poly A)+mRNA |
| Primer: choose either  |              |                                      |
| Anchored-oligo(dT) <sub>18</sub> Primer, 50 pmol/µl (Vial 5) | 1 µl         | 2.5 µM                               |
| OR Random Hexamer Primer, 600 pmol/µl (Vial 6)               | 2 µl         | 60 µM                                |
| OR sequence-specific Primer                                  | variable     | 0.5 – 2.5 µM                         |
| Water, PCR Grade (Vials 7 or 9)                              | variable     | to make total volume = 13 µl         |
| <b>Total</b>   | <b>13 µl</b> |                                      |

4. Mix the reagents and centrifuge briefly to collect the sample at the bottom of the tube
5. Optional: Denature the template-primer mixture by heating the tube for 10 min at 65°C in a block cycler. This step ensures denaturation of secondary structures of the RNA.
6. Immediately cool the tube on ice
7. Add the components of the RT mix in the order listed in table 4 to the tube containing the template-primer mix.

**Table 4.** *Reaction components to synthesise cDNA from RNA sample*

| Reagent   | Volume       | Final conc.               |
|---|--------------|---------------------------|
| Transcriptor Reverse Transcriptase Reaction Buffer, 5x conc. (Vial 2) | 4 µl         | 1x 8 mM MgCl <sub>2</sub> |
| Protector RNase Inhibitor, 40 U/µl (Vial 3)                           | 0.5 µl       | 20 U                      |
| Deoxynucleotide Mix, 10 mM each (Vial 4)                              | 2 µl         | 1 mM each                 |
| Transcriptor Reverse Transcriptase, 20U/µl (Vial 1)                   | 0.5 µl       | 10 U                      |
| <b>Total</b>  | <b>20 µl</b> |                           |

8. Mix the reagents
9. Place the tube in a thermal block cycler.
10. Depending on the primer used and the length of the target mRNA incubate the RT reaction as described below.
11. Inactivate the reverse transcriptase by incubation at 85°C for 5 min.



-cDNA amplification (PCR)

After the mRNA extraction and cDNA synthesis, a qPCR to proof the absence of expression of Sendai virus derived pluripotency factors has to be performed. Generated DNA fragments will be separated in an agarose gel.

DNA amplification:

- 1- Prepare the PCR mix showed in table 5:

**Table 5.** *PCR components*

| PCR mix for one reaction   |         |
|----------------------------|---------|
| Sterile milliQ water       | 10,1 µl |
| 5X Green Buffer            | 4 µl    |
| Primer F 10 µM             | 1,5 µl  |
| Primer R 10 µM             | 1,5 µl  |
| dNTPs 10 mM                | 0,5 µl  |
| MgCl <sub>2</sub> 25 mM    | 1,2 µl  |
| Go Taq Polymerase (5 U/µl) | 0,2 µl  |

- 2- Pipette 19 µl of the PCR mix per reaction tube

- 3- Add:

**Table 6.** *Samples analysed by PCR*

|                         |   |
|-------------------------|---|
| <b>Negative control</b> | 1 µl of steril water<br>1 µl of No RT sample<br>1 µl of negative control (sample of episomal) |
| <b>Sample</b>           | 1 µl of sample (or 5ng of cDNA)   |
| <b>Positive control</b> | 1 µl of positive sample   |

- 4- PCR program

The PCR is performed with 5 different primers pairs to detect Sendai virus backbone (SEV), virus derived KOS, Klf4, L-Myc and the housekeeping gene GAPDH.

-For SEV, L-MYC and GAPDH:

**Table 7.** *PCR cycles for SEV, L-MYC and GAPDH reprogramming factors*

| 1 cycle        | 35 cycles  |
|----------------|--|
| 95°C for 5 min | 95°C for 30 sec<br>55°C for 30 sec<br>72°C for 30 sec<br>72°C for 10 min |



-For KOS and Klf4:

**Table 8.** PCR cycles for KOS and Klf4 reprogramming factors

| 1 cycle        | 35 cycles  |
|----------------|--|
| 95°C for 5 min | 95°C for 30 sec<br>60°C for 30 sec<br>72°C for 30 sec<br>72°C for 10 min |

5- Run the samples on a 2% agarose gel

## In vitro pluripotency test

### Immunocytochemistry:

Immunocytochemistry is used to analyse the expression of certain proteins in a sample of cells using specific antibodies. Once the first antibody binds to the antigen of interest, the second antibody is added and binds to the first antibody. The second antibody carries a fluorescent dye and can be detected with a fluorescence microscope. [38]

**Table 9.** In vitro pluripotency markers that confirm pluripotent and stem cell characteristics.

| PLURIPOTENCY MARKERS   |
|--|
| <b>OCT4 (Octamer-binding transcription factor 4):</b> Transcription factor involved in the self-renewal of undifferentiated embryonic stem cells.  |
| <b>Nanog:</b> Transcription factor that plays a central role in maintaining pluripotency by suppressing cell determination factors.  |
| <b>TRA1-81:</b> Epitope expressed on podocalyxin, a type I transmembrane glycoprotein found in embryonic stem cells, embryonal carcinoma cells and induced pluripotent stem cells (iPS).   |
| <b>SOX2 (sex determining region Y-box 2):</b> Transcription factor that play an essential role in maintaining pluripotency of stem cells. It recruits other nuclear factors to activate pluripotent gene expression and repress genes involved in differentiation.                         |
| <b>SSEA-4 (Stage-specific embryonic antigen-4):</b> Glycosphingolipid express in the surface of embryonic stem cells, embryonal carcinoma cells and induced pluripotent stem cells (iPS) and is used as a marker.  |
| <b>TRA1-60:</b> Cell surface antigen in human embryonic stem cells, embryonal carcinoma cells and induced pluripotent stem cells (iPS).  |
| <b>SSEA-3 (Stage-specific embryonic antigen-3):</b> Glycosphingolipid, specifically, an oligosaccharide composed of five carbohydrate units connected to sphingolipid which plays a role in identifying many types of mammalian cells with pluripotent and stem cell-like characteristics. |

Immunocytochemistry of pluripotency factors is develop starting from undifferentiated iPSC in a slideflask.

The steps for immunocytochemistry protocol are:

1. Fixation with Paraformaldehyde (PFA) 4% for 20 minutes at room temperature (2ml/slide): fixation is required for preserving cellular and subcellular structure and to immobilize antigens. Once the sample is fixed, immunocytochemistry must be started before 10 days.  
1.1 - 3x washes with 1x PBS or 1x TBS for 10 minutes at room temperature.



**Figure 10.** iPSCs samples washed with 1x PBS or 1x TBS in room temperature

2. Add antigen retrieval for 1 hour at 60°C and cool down to RT for 15 minutes. Antigen retrieval enables an antibody to access the target protein
3. Add block buffer (TBS 1x / 0,5% triton / 6% Donkey serum) and incubate 1 hour at RT. Block buffer avoids unspecific binding of secondary antibodies.
4. Incubate samples with primary antibody for 48-72h at 4°C in TBS++ (TBS 1X, 0,1% triton, 6% donkey serum). Before adding the antibodies is important to dry the slides and mark them with PAP PEN to ensure antibodies get fix to the sample.

**Table 10.** Primary antibodies for endoderm, ectoderm and mesoderm markers for pluripotency assay

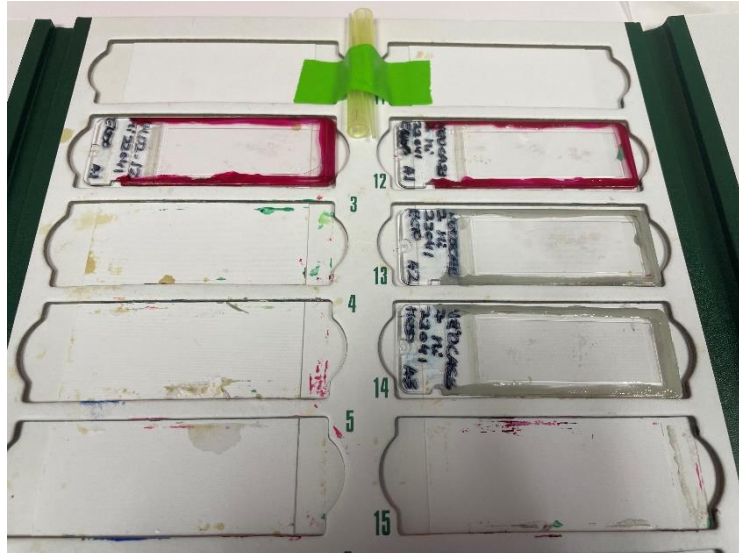
| ENDODERM            | ECTODERM                 | MESODERM           |
|---------------------|--------------------------|--------------------|
| OCT4 mouse igG 1:2  | SOX2 rabbit igG 1:100    | NanoG goat igG 1:5 |
| SSEA3 rat igM 1:200 | SSEA4 mouse igG 1:200    | Tra-1-81 niyse UgN |
|                     | Tra-1-60 mouse igM 1:100 | 1:100              |

5. Washes 3 times with TBS++ to remove unbound antibodies that didn't fix to the antigen of interest.
6. Incubate samples with secondary antibodies in TBS++ for 2 hours at 37°C.

**Table 11.** Secondary antibodies for endoderm, ectoderm and mesoderm markers for pluripotency assay

| ENDODERM                 | ECTODERM                 | MESODERM                 |
|--------------------------|--------------------------|--------------------------|
| Anti-mouse igG Cy2 1:200 | Anti-rabbit igG 1:200    | Anti-goat igG Cy2 1:200  |
| Anti-rat igM Cy3 1:200   | Anti-mouse igG Cy3 1:200 | Anti-mouse igM Cy3 1:200 |
|                          | Anti-mouse igG Cy5 1:200 |                          |

7. Wash 3 times with TBS. During the washing steps it's important to avoid bright light on the samples because the secondary antibodies are light sensitive.
8. Add DAPI 1:10000 for 10' at RT. DAPI is a dye that get intercalated in the nuclear DNA and emits fluorescence in the blue spectrum. In general, 500  $\mu$ L /slide are added.
9. Add mounting medium (add 3 drops of home-made fluorescent) to fix the coverslip at the slide and add nail polish avoiding the formation of bubbles.



**Figure 11.** Slides containing mounting medium and nail polish fixing the coverslip.

#### ✚ Alkaline Phosphatase staining (AP staining)

Alkaline Phosphatase test shows the activity of alkaline phosphatase (ALP). Pluripotent stem cells typically have high activity of Alkaline Phosphatase, and it is used as a biomarker that provides easier identification of pluripotency without compromising cell

integrity. To characterize in vitro pluripotency this assay is done using a kit where alkaline phosphatase acts on a fluorescent substrate changing the colour of the substrate from yellow to blue. [37]

The kit contains two different solutions, A and B, from which equal volumes were mixed. The solutions should not be exposed to light, because they are light sensitive.

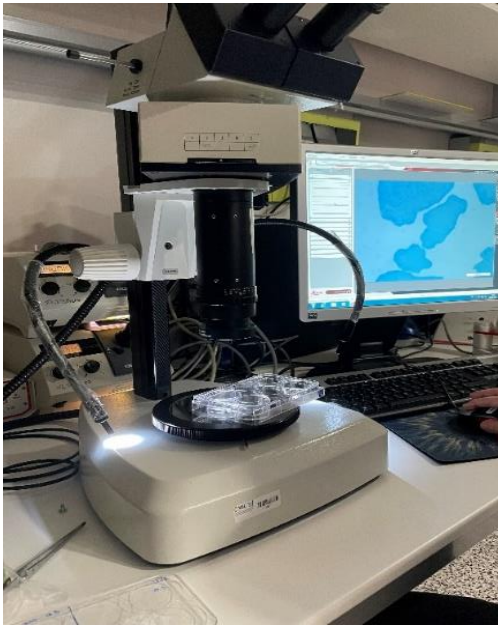
Protocol for AP-staining:

1. Take the plate with the iPSC cells and aspirate the medium.
2. Wash the well with 2ml of PBS.
3. Fix the cells with 1,5ml 4% PFA for 1 minute at room temperature.
4. Wash the well with 2ml of PBS
5. Add 1ml of the mixture A+B to the well to wash. Aspirate the solution and add again 1ml to the well. Incubate the plate for 10 min at room temperature in the dark.
6. After the incubation wash the well with 2ml of PBS and wait for 2 minutes.
7. If the cells in the plate are iPS cells, the colour from the substrate solution A+B will turn from yellow to blue colour.



**Figure 12.** Alkaline phosphatase staining on iPSC to confirm pluripotency

8. Take a photo with the stereomicroscope.



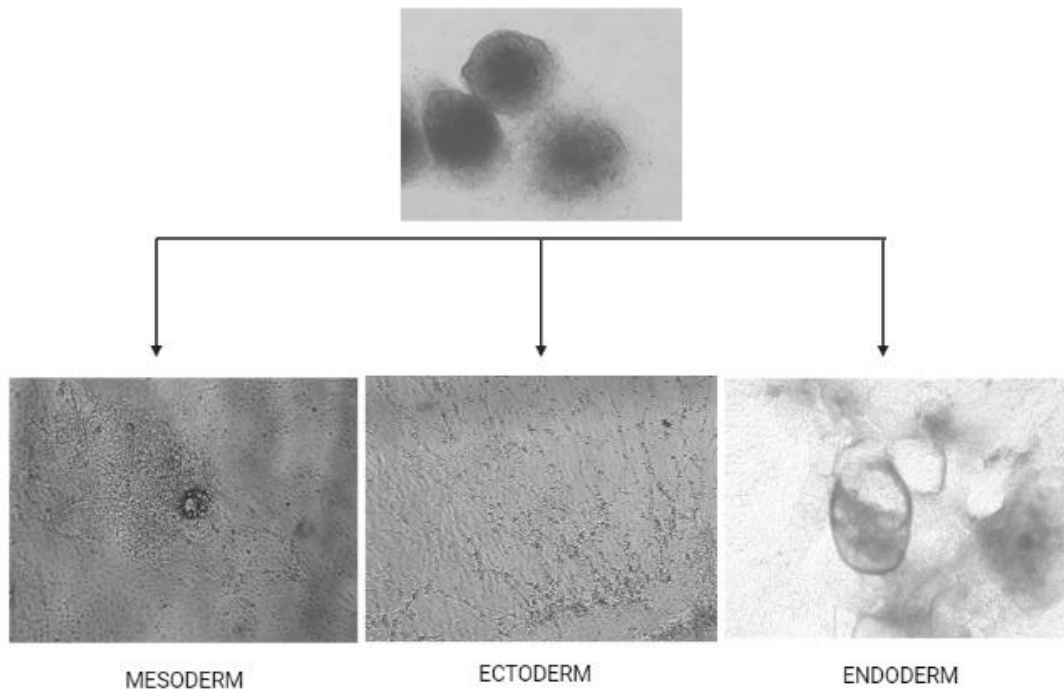
**Figure 13.** Stereomicroscope to analyse Alkaline phosphatase staining

### In vitro differentiation test

#### ✚ Embryoid bodies formation and in vitro differentiation:

Embryoid body formation and their differentiation is an assay to confirm the differentiation capacity of iPSCs towards the three germinal layers; ectoderm, endoderm, and mesoderm. [38]

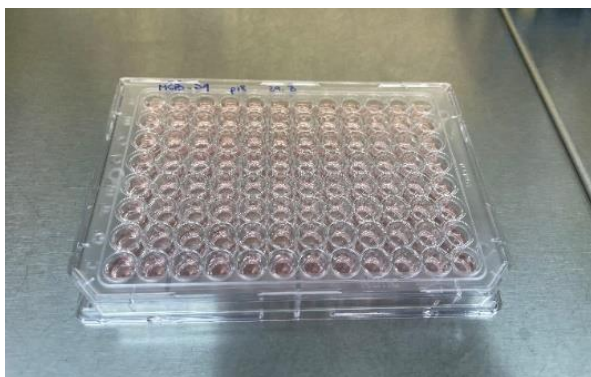
In differentiations towards ectoderm germ layer, structures that seems like neurons were observed. In endoderm germ layers globular structures were observed and in mesoderm germ layers muscular structures could be seen.



**Figure 14.** EB differentiation to the three germ layers; mesoderm, ectoderm, and endoderm, respectively.

The differentiation assay starts with iPSC cells in culture. The following steps will be performed:

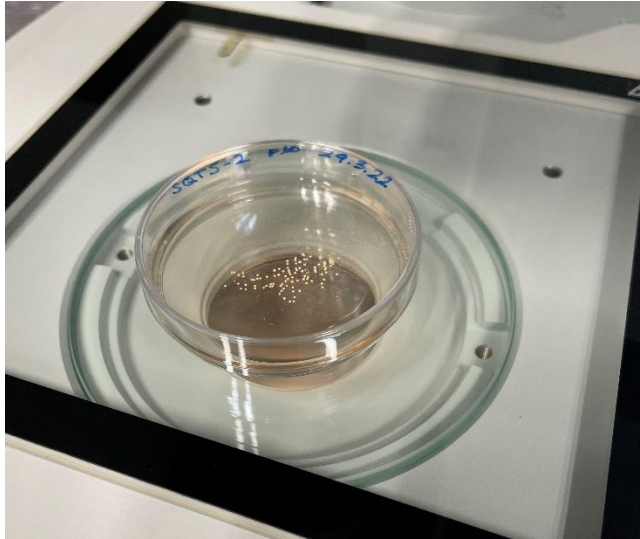
1. Trypsinize iPSCs with EDTA and transfer the cellular suspension to the wells of a 96-well plate.



**Figure 15.** iPSCs in 96-well plates after trypsinization

2. Centrifuge the cells at 800g for 10 minutes, to form a cell pellet at the bottom of the wells.
3. Take the cell pellets with a pipette and transfer them into an ultra-low attachment plate to avoid the attachment of the cells to the surface of the plate.
4. Concentrate the cells in the middle of the plate by moving the plate in circles. Take cells with a pipette and change them into another plate with fresh mTeSR.





**Figure 16.** Cells concentrated in ultra-low attachment plates

5. The cells will be incubated for 24 hours at 37°C. The next day, the cells are seeded in three slide flasks previously coated with matrigel in different media to force them to get differentiated into the three different germ layers. Take 30 cells per slide with a 1000 pippette and try to adhere them in the middle of the flask.
6. Incubate plates at 37°C overnight.

Composition of differentiation media:

**Table 12.** Composition of endoderm, ectoderm and mesoderm differentiation media

| ENDODERM               | ECTODERM               | MESODERM               |
|------------------------|------------------------|------------------------|
| KO-DMEM                | KO-DMEM                | 50% Neural basal media |
| Glutamax 1X            | Glutamax 1X            | 50% DMEM/F12 media     |
| NEEA 1X                | NEEA 1X                | N2 0.5%                |
| 2-Mercaptoethanol 0.1X | 2-Mercaptoethanol 0.1X | B27 1%                 |
| FBS-HyClone 10%        | FBS-HyClone 10%        | Glutamax 1X            |
| Pen-strepto 1X         | Pen-strepto 1X         | Pen-strepto 1X         |
|                        | 0.5 mM Ascorbic Acid   |                        |

EBs will be completely differentiated within 3 weeks to endoderm and mesoderm and within 4 weeks to ectoderm.

During those weeks growth will be observed, and the medium will be changed three times per week (Monday, Wednesday, and Friday).

To confirm the expression of specific markers for endo-, meso- and ectoderm, immunocytochemistry was performed.

 **Immunocytochemistry:**

Immunocytochemistry is used to analyse the expression of certain proteins in a sample of cells using specific antibodies. Once the first antibody binds to the antigen of interest, the second antibody is added and binds to the first antibody. The second antibody carries a fluorescent dye and can be detected with a fluorescence microscope. [38]

**Table 13.** *In vitro* differentiation markers that confirm the differentiation to mesoderm, ectoderm and endoderm, respectively.

| MESODERM   | ECTODERM   | ENDODERM  |
|--|--|---|
| <b>ASMA(Alpha-smooth muscle actin)</b> – Actin isoform that contributes to cell-generated mechanical tension observed only on vascular smooth muscle cells | <b>Tuj1 (beta-tubulin III)</b> – structural protein from the tubulin; also known as neuron-specific protein expressed primarily in neurons and used as a marker to distinguish it. | <b>AFP (alpha-fetoprotein)</b> – Most abundant serum protein in the developing embryo   |
| <b>GATA4</b> -Zing finger transcription factor related with myocardial differentiation and function.   | <b>GFAP (Glial fibrillary acid protein)</b> - fibrous protein that form the intermediate filaments of intracellular cytoskeleton, particularly of glial cells such as astrocytes.  | <b>FOXA2 (Forkhead Box A2)</b> – Transcription factor that plays an important role during development of multiple endoderm-derived organ systems. |

1. Fixation with Paraformaldehyde (PFA) 4% 20' (2ml/slide): Fixation is required for preserving cellular and subcellular structure and to immobilizes antigens. Once the sample is fixed, immunocytochemistry must be started before 10 days.
  - 1.1 3x washes 10' with PBS 1x or TBS 1x
2. Add block buffer (TBS 1x / 0,5% triton / 6% Donkey serum) and incubate 1 hour at RT. Block buffer tries to avoid unspecific unions of secondary antibodies.
3. Primary antibody incubation for 48-72h at 4°C diluting them in TBS++ (TBS 1X, 0,1% triton, 6% donkey serum). Before adding the antibodies is important to dry well the slides and mark it with PAP PEN to ensure antibodies get fix to the sample.



**Table 14.** Primary antibodies for endoderm, ectoderm and mesoderm markers for differentiation assay

| ENDODERM                             | ECTODERM                               | MESODERM                                  |
|--------------------------------------|--|---|
| Alpha-1-Fetoprotein rabbit igG 1:200 | Beta III- Tubulin TUJ1 mouse igG 1:500 | Alpha-Smooth Muscle Actin mouse igG 1:400 |
| FOXA2 goat igG 1:50                  | GFAP rabbit igG 1:500                  | GATA4 rabbit igG 1:25                     |

- 3x washes with TBS++ to clean those antibodies that didn't fix to the antigen of interest.
- Secondary antibody incubation diluting in TBS++ during 2h at 37°C.

**Table 15.** Secondary antibodies for endoderm, ectoderm and mesoderm markers for differentiation assay.

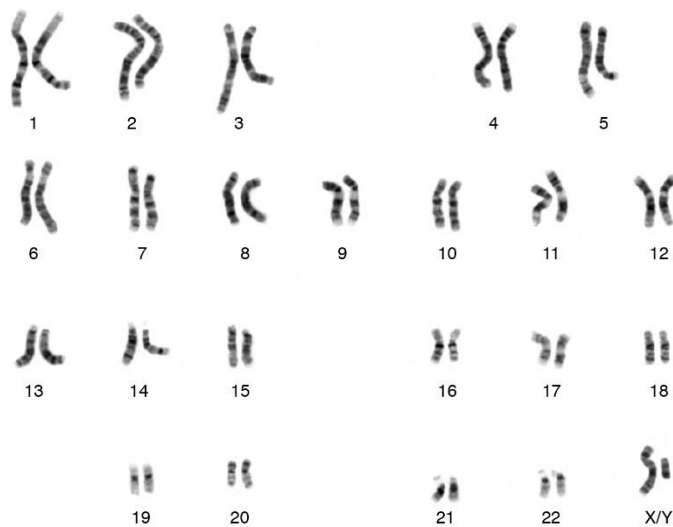
| ENDODERM                  | ECTODERM                  | MESODERM                  |
|---------------------------|---------------------------|---------------------------|
| Anti-rabbit igG Cy2 1:200 | Anti-mouse IgG Cy2 1:200  | Anti-mouse igG Cy2 1:200  |
| Anti-goat igG Cy3 1:200   | Anti-rabbit igG Cy3 1:200 | Anti-rabbit igG Cy3 1:200 |

- 3' washes with TBS. Between the washes it's important to avoid light focus on the sample because secondary antibodies are sensitive.
- Add DAPI 1:10000 for 10' at RT. DAPI is a sounding that get intercalated in the nuclear DNA and send out fluorescence in the blue of spectre. Normally, 500 microliters/slide is added.
- Add mounting medium (add 3 drops of home-made fluorescent medium in their case) to get fix the coverslip at slide and then add nail polish ensuring there's no bubbles before.

## Karyotyping

The Karyotype is the collection of all chromosomes of an individual organism. The technique that produces an image of individual chromosomes is called karyotyping. During the characterization of an iPSCs it's important to confirm that there are no abnormal numbers or structures of chromosomes in iPS cells.

A normal karyotype shows 22 pairs of chromosomes called autosomes and the 23<sup>rd</sup> pair that determines the sex of an individual organism. In a normal karyotype a total of 23<sup>rd</sup> pairs of chromosomes should be observed. In the figure 17, the chromosomes are ordered from the largest chromosomes (number 1) to the smallest chromosome (number 22).



**Figure 17.** Normal karyotype with 22 pairs of autosome chromosomes and 1 pair determining the sex of the organism

There are different characteristics of karyotypes that are usually observed:

- Differences in the size of the chromosomes: could reflect different amounts of DNA duplications or segmental interchange of unequal lengths.
- Differences in the position of centromeres: could be referred to translocations in the DNA.
- Differences in basic number of chromosomes: could have resulted from successive unequal translocation which removed all the essential genetic material from a chromosome, permitting its loss without penalty to the organism or through fusion.
- Differences in number and position of satellites (small bodies attached to a chromosome by a thin thread)
- Differences in degree and distribution of heterochromatic regions (heterochromatic regions stain darker than euchromatin regions)

The Karyotype analysis is done by arresting the cell cycle in the metaphase. Metaphase is the phase where chromosomes are condensed and visible in the nucleus.

To stop the growth of the iPSCs in the metaphase phase of their cell cycle, a 70/80% confluent plate has to be incubated with Colcemid for three hours at 37°C. Colcemid is a generic name for N-methyl-N-deacetyl-colchicine, an antimitotic agent, which depolymerizes microtubules and thereby blocks mitosis and metaphase. [34]

After the incubation of the cells with colcemid:

1. Prepare Carnoy (to fix the cells): 1/4 acetic acid and 3/4 methanol. This step requires a gas extraction booth.
2. Take the plate with the cells and trypsinize them to get the cells in suspension.

3. Centrifuge at 130g for 5 minutes. Aspirate the medium.
4. Add drop by drop 5 ml of Potassium Chloride(KCl): KCl is an hypotonic solution which allows the cells to take in water and to let them swell; later on, the cells will explode more easily.
5. Incubate cells for 15 minutes at 37°C in the water bath.
6. Add 4 drops of Carnoy previously prepared.
7. Centrifuge at 130g for 5 minutes and aspirate the KCl.
8. Add drop by drop the rest of the Carnoy solution.
9. Store tube with cells at -20°C. Karyotypes will be analysed at the hospital Sant Joan de Deu, Barcelona.

## DNA fingerprinting

### Microsatellites from somatic cells and from iPSC clones

Microsatellites refers to a short segment of DNA, usually one to six or more base pairs in length, that is repeated multiple times in succession at a particular genomic location.

The number of repeated segments within a microsatellite varies among people, which makes them useful as markers. From the isolated CD34 cells, as well as from the iPSCs generated from them, gDNA is extracted and the microsatellites analysed by PCR. [35]

The microsatellites will be compared to make sure they are from the same donor.

## 4. Results

### 4.1 qRT-PCR

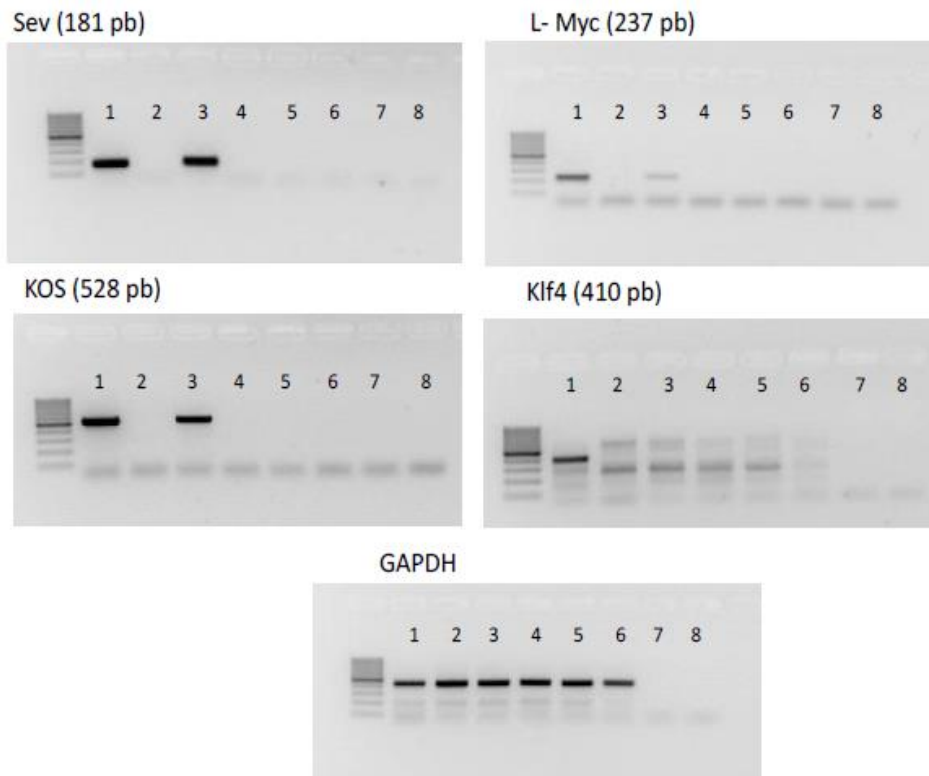
Figure 18 shows the absence of virus derived OSKM factors (Sev, L-Myc, KOS and Klf4) in different clones. The result is as expected because a non-integrating methodology was used to reprogram the CD34+ cells.

GADPH is used as a housekeeping gene; a gene that is expressed always in all the tissues. It serves as a positive control to confirm that equal amounts of cDNA were loaded and to ensure that the steps of qRT-PCR worked properly.

There are eight lanes in each gel to analyze different clones and controls to make sure that the PCR worked properly. For example, a positive control with a sample of a cell pellet prepared one week after infection (lane 1, these cells still contain the vectors with the OSKM factors) and 2 negative controls, where no amplification is expected.

Last lanes are for negative controls. Lane 6, showing cells not infected with sendai virus, to rule out unspecific amplification. Lane 7, showing infected cells without the enzyme reverse transcriptase and, lane 8, only water used for the PCR.

One of the iPSCs clones which shows no amplification of virus derived material (Figure 18, clones I1, I7 and I9) can be selected for full characterization. Clone I6 shows amplification of a fragment with the primers for SeV, L-Myc and KOS.



**Figure 18.** Gel electrophoresis results from qRT-PCR showing reprogramming factors *Kos*, *L-Myc*, *Klf4* and *Sev* and a housekeeping gene *GAPDH*

1. Control + SENDAI 2.1
2. Hz-11-27-1 CbiPS8 Sv4F-I1, P12
3. Hz-11-27-1 CbiPs8 Sv4F-I6, P13
4. Hz-11-27-1 CbiPS8 Sv4F-I7, P14
5. Hz-11-27-1 CbiPS8 Sv4F-I9, P12
6. Rb20234-C17 P12 (NEGATIVE CONTROL EPISOMAL SAMPLE)
7. SAMPLE NO RT
8. H2O

## 4.2 Immunocytochemistry for pluripotency test

Figure 19 shows expression of nuclear pluripotency factors Oct4, Nanog and Sox2 in green (from top to bottom) and pluripotency related membrane proteins SSEA3, TRA1-81, and SSEA4 in red and TRA1-60 in purple.

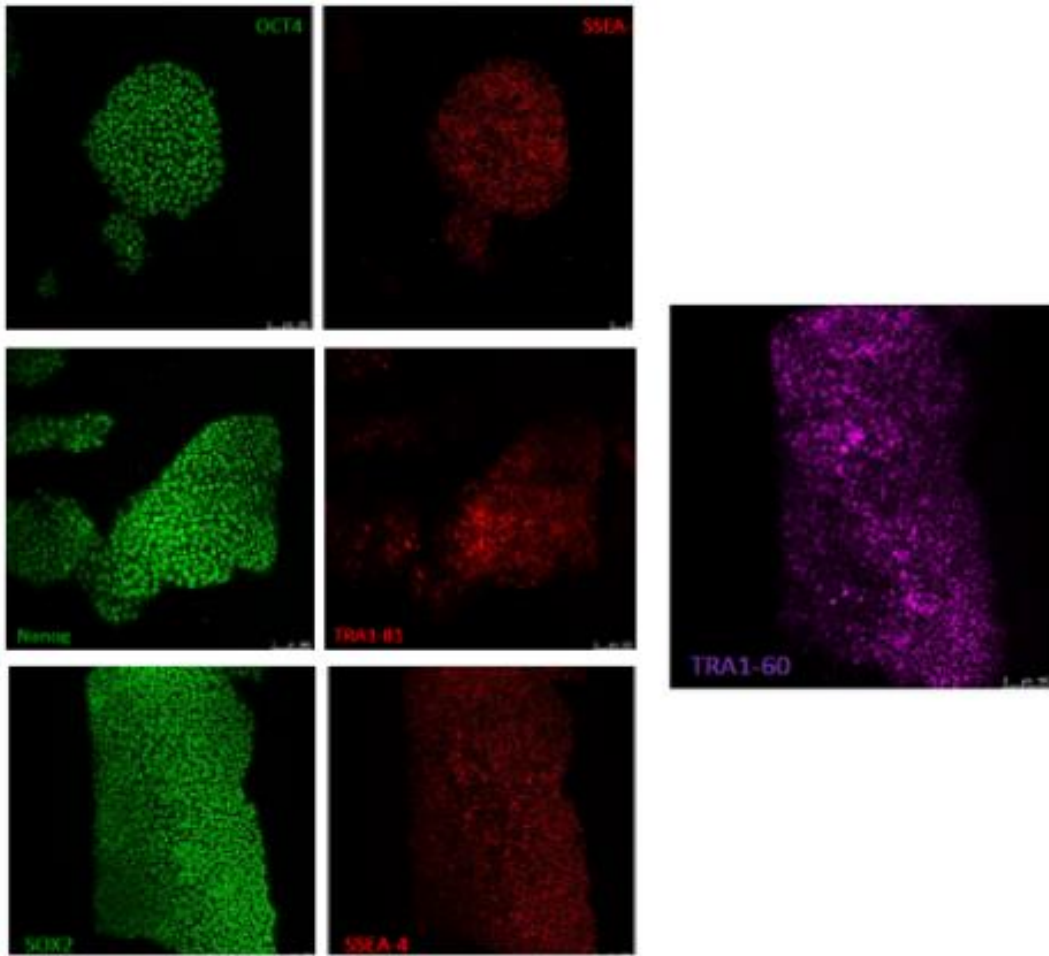
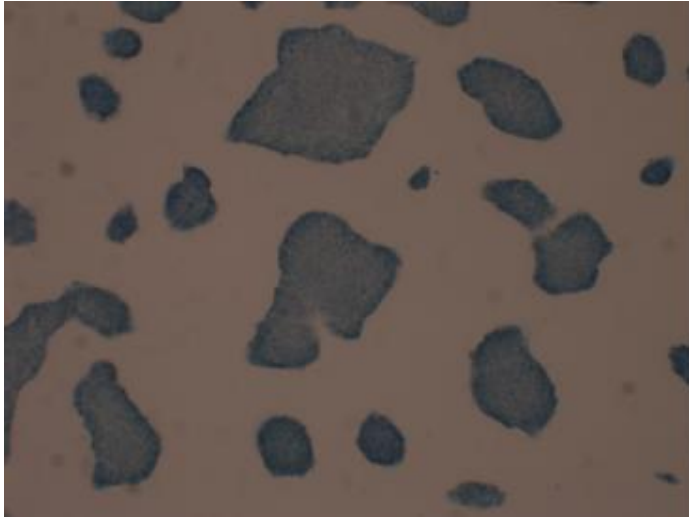


Figure 19. Immunocytochemistry results showing the presence of markers related with pluripotency

### 4.3 AP staining

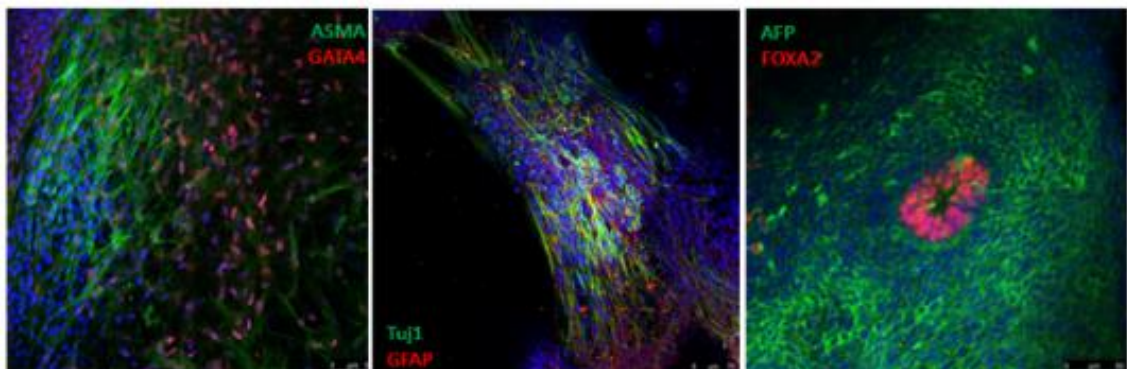
Figure 20 shows alkaline phosphatase staining. iPSC colonies turned the yellow substrate of the staining kit to blue, indication of high alkaline phosphatase activity.



**Figure 20.** *Alkaline Phosphatase results showing pluripotency activity in the iPSCs*

#### 4.4 EB formation and in vitro differentiation

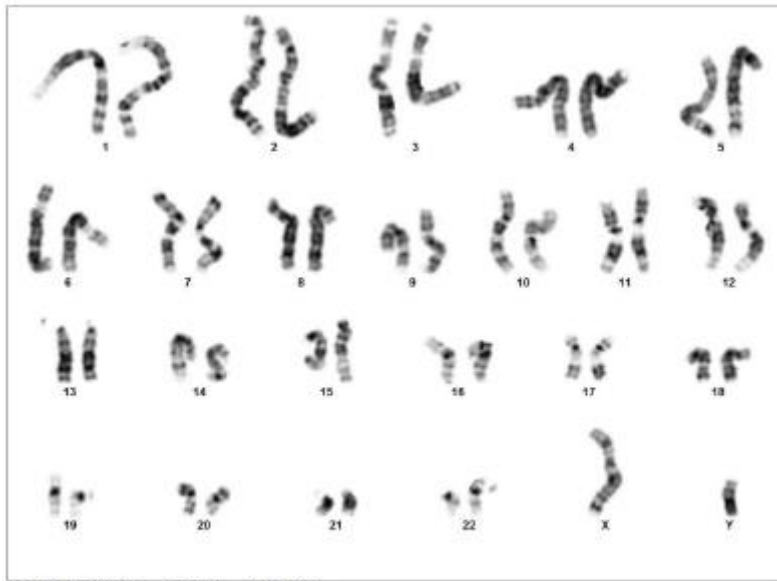
Once embryoid bodies were formed and differentiated to the three germinal layers, immunocytochemistry was performed. Figure 21 shows the expression of mesoderm marker ASMA and GATA4 (left panel, green and red, respectively), ectoderm marker Tuj1 and GFAP (middle panel, green and red, respectively), and endoderm marker AFP and FOXA2 (right panel, green and red, respectively). Nuclei were counterstained with DAPI (blue).



**Figure 21.** *Immunocytochemistry results showing the presence of markers related with differentiation*

#### 4.5 Karyotyping

Figure 22 shows the karyotype of the iPSC clone without any abnormal numbers or structures of chromosomes. A total of 46 chromosomes could be observed and the last two chromosomes determines the sex of the organism (XY).



Case: 13331150 Slide: 1 Cell: 13F

Result: 46,XY

**Figure 22.** Karyotype results showing 23rd pairs of chromosomes without anomalies

## 4.6 Microsatellites from somatic cells

**Table 16.** Microsatellites results from somatic cells sample

| Línea celular             | Loci STRs analizados |          |        |         |        |         |        |      |        |      |
|---------------------------|----------------------|----------|--------|---------|--------|---------|--------|------|--------|------|
|                           | TH01                 | D21S11   | D5S818 | D13S317 | D7S820 | D16S539 | CSP1PO | AMEL | vWA    | TPOX |
| CD34 24-7-15, I, 10.05.21 | 6; 9.3               | 28; 32.2 | 11; 13 | 11; 12  | 9; 12  | 8; 13   | 10; 11 | X; Y | 14; 16 | 8    |

## 4.7 Microsatellites from iPSC clones.

**Table 17.** Microsatellites results from iPSC clones samples

| Línea celular                  | Loci STRs analizados |          |        |         |        |         |        |      |        |      |
|--------------------------------|----------------------|----------|--------|---------|--------|---------|--------|------|--------|------|
|                                | TH01                 | D21S11   | D5S818 | D13S317 | D7S820 | D16S539 | CSP1PO | AMEL | vWA    | TPOX |
| Hz 24-7-15 CBiPS7 Sv4F-I12 p13 | 6; 9.3               | 28; 32.2 | 11; 13 | 11; 12  | 9; 12  | 8; 13   | 10; 11 | X; Y | 14; 16 | 8    |

Table 16 shows microsatellites from CD34 cells (the starting somatic cell) and table 17 shows microsatellites from the iPSC sample.

From 10 different loci, short terminal repeats were analyzed by PCR. Repeats from somatic CD34 positive cells (table 16) were compared with the number of the same loci from the generated iPSCs of clone I12 (table 17) indicating that both lines are from the same organism.



Overview of the characteristics and characterization results of all generated lines can be seen in Annex A and B (table 18 and table 19).

## 5. Discussion

The project iPS-PANIA has the objective to generate 7 iPSC lines with the highest possible covering of haplotypes of the Spanish population. 7 healthy cord blood units were selected covering 25% of the most frequent haplotypes of the Spanish population. [27]

Commercialized clinical-grade hiPSC lines are available from private companies (Fuji-CDI, in Wisconsin USA from the most 5 common HLA types matching to 35% of US population) and very few hiPSC lines are provided by public research organizations (RUCDR Infinite Biologics, Korea HLA-Typed hiPSC Banking (KHIB) and the Center for iPSC Cell Research and Application (CiRA)).

Selected haplotypes for the generation of the iPSC lines, described in the iPS-PANIA project, represent some of the most frequent HLA-A, B and DRB1 haplotypes in Spain. It is possible to share most of them with other populations in Europe, North and South America. [5]

In transplants, a perfect donor match occurs when the two alleles from loci HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1 are identical. Even if a donor is considered a perfect match, peptides derived from cellular proteins (minor histocompatibility antigens) can cause an immune response due to polymorphisms. This causes the need for immunosuppression, which is what is trying to be avoided via genetic modification techniques. [13]

Tissue donors criteria follows the national regulatory frameworks. Medical information of the donors and their families such as cancer, drugs abuse and inherited diseases, are required. Screening for risk factors associated with infection diseases; such as HIV, hepatitis B and C virus, malaria, Chagas disease and human T-cell lymphotropic virus I and II, are also needed.

The donation should be free and voluntary and the donor should be notified of the possible use in cellular therapies, genetic modification and international sharing and commercial uses of hiPSC lines. [4]

Food and Drug Administration (FDA) in US or the European Medicines Agency (EMA) in Europe, classifies hiPSC derivatives for cell therapy as Advanced Therapy Medicinal Products (ATMPs). This means, among other requirements, that iPSC must be produced under Good Manufacturing Practice (GMP) conditions and generate them under strict quality assurance. In fact, most of clinical trials are using hiPSCs generated in non-GMP conditions and then qualified for GMP by conversion in GMP culture conditions and additional testing. [9, 4]

During iPSCs generation and characterization, growth media, reagents and freezing solutions should be commercially animal serum-free and produced under GMP conditions.

hiPSC cryopreservation is essential for using them for clinical application. Specifically, freezing and banking must be also performed under good manufacturing practices (GMP). The cells must be transported in LN2 as it is the most secure way to transport cryopreserved material. [25]

## 6. Conclusion

The iPS-PANIA project gets patients closer to personalized cell therapy by generating an iPSCs bank that provides the starting material to differentiate them to any specialized cell, without the need of searching for compatible donors and reducing the need for immunosuppression after cell therapy.

The generation of 7 hiPSCs of HLA-matched from more frequent haplotypes of the spanish population will avoid the need for the generation of specific hiPSC for each patient reducing time and costs. Also, fewer medical complications, hospitalizations, lower mortality rates and better quality of life will be achieved after cell therapies.

In conclusion, learn and describe the methodology to generate an iPSCs haplobank for my Final Grade Project (TFG) will contribute not only to scientific divulgation, but also to an international effort to create national banks that can share iPSCs haplotypes increasing the possibilities to cover most of the population.

### 6.1 Limitations and improvements in future projects

One limitation according to the iPSCs generation protocol is that all the processes are very extensive and includes the participation of different platforms. During the preparation of my TFG I wanted to follow all the processes of the generation and characterization of one iPSCs line but some steps were performed by different IDIBELL scientific support platforms being very difficult to coordinate when to go. Although, I tried to generate this protocol as accurate as it is possible with the help from the different platforms from IDIBELL and at the same time, trying to explain the processes as well with bibliographic tools, to ensure everyone who wants to generate more iPSCs haplotypes can be guided by this TFG.

The 7 haplotypes cover around 25% of haplotypes of the spanish population and it's necessary to increase the percentage to offer to more patients these new treatments in the future. It is a difficult objective because wide number of haplotypes have to be generated to cover most part of the spanish population as it is a laborious and large process.

Moreover, in other countries the most frequent haplotypes can be different from spanish ones, so even if there are more scientists generating new haplotypes, there's the possibility they couldn't be useful for our country.

If I could follow with the project, I probably will try to increase the amount of haplotypes for covering more percentage of the population. After studying the possibility, if it is not viable because too many iPSCs lines have to be generated to cover a small portion more of the patients, I would try to focus on another objective.

Probably it could be useful to try to shorten the time necessary to use these cells for cell therapy. One idea is to differentiate these iPSCs lines from the bank to the most needed cell types to shorten the process and reduce the time ill patients have to wait until they can be operated.

## 7. Bibliography

- [1] Takahashi K. and Yamanaka S. (2016). A decade of transcription factor-mediated reprogramming to pluripotency. *Nature reviews*, vol.17, pp 183-191.
- [2] Lee S, Huh JY, Turner DM, et al. (2018). Repurposing the Cord Blood Bank for haplobanking of HLA-homozygous iPSCs and their usefulness to multiple populations. *Stem cells*. Vol 36 (10), pp 1552-1566.
- [3] Morishima Y, Azuma F, Kashiwase K, et al. (2017). Risk of HLA homozygous cord blood transplantation: implications for induced Pluripotent Stem Cell banking and transplantation. *Stem cells Translational Medicine*. Vol 7. pp 173-179.
- [4] Alvarez B, Vives J, Casaroli RP, et al. (2019). Adapting cord blood collection and banking standard operating procedures for HLA-homozygous induced Pluripotent Stem Cells production and banking for clinical application. *Journal of Clinical Medicine*. Pp 1-15.
- [5] Alvarez B, García I, Gayoso J, et al. (2021). Evaluation of the Spanish population coverage of a prospective HLA haplobank of induced pluripotent stem cell. *Stem Cell Research & Therapy*. pp 1-8.
- [6] Kobold S, Guhr A, Mah N, et al. (2020). A manually curated database on clinical studies involving cell products derived from Human Pluripotent Stem Cells. *Stem Cell Reports*. Vol 15. Pp 546-555.
- [7] Takahashi K, Tanabe K, Ohnuki M, et al. (2007). Induction of pluripotent stem cells from Adult Human Fibroblasts by defined factors. *Cell*. pp 861-872.
- [8] Andrades J, Becerra J, Muñoz R, et al (2014). Stem Cells Therapy for Regenerative Medicine: Principles of Present and Future Practice. *Journal of Biomedical Science and Engineering*. Vol 7. Pp 49-57
- [9] Ohmine S, Dietz AB, Deeds MC, et al. (2011). Induced Pluripotent Stem Cells From GMP-grade Hematopoietic Progenitor Cells and Mononuclear Myeloid Cells. *Stem Cell Research and Therapy*. Vol 2(6). pp 1-12.
- [10] Okita K, Matsumura Y, Sato Y, et al. (2011). A More Efficient Method to Generate Integration-free Human iPSC Cells. *Nat Methods*. Vol 8. pp 409-412.
- [11] Martí M, Mulero L, Pardo C, et al. (2013). Characterization of pluripotent stem cells. *Nat. Protoc*. Vol 8 (2). pp 223-253.
- [12] Petrus-Reurer S, Winblad N, Kumar P, et al. (2020). Generation of retinal pigment epithelial cells derived from human embryonic stem cells lacking human leukocyte antigen class I and II. *Stem cell Reports*. Vol 14 (4). pp 648-662.

- [13] Otsuka R, Wada H, Murata T, et al. (2020). Immune reaction and regulation in transplantation based on pluripotent stem cell technology. *Inflamm Regen*. Vol 40 (1). pp 12.
- [14] Yamanaka S. (2020) Pluripotent Stem Cell-based cell therapy-Promise and Challenges. *Cell Stem Cell*. Vol 27(4). pp 523-531.
- [21] Schlaeger TM, Daheron L, Brickler TR, et al. (2015). A comparison of non-integrated reprogramming methods. *Nat Biotechnol*. Vol 33(1). pp 58-63.
- [22] Hu K. (2014). All roads lead to Induced Pluripotent Stem Cells: The technologies of iPSC generation. *Stem Cells Dev*. Vol 23(12). Pp 1285-3000.
- [23] Haridhasapavalan KK, Borgohain MP, Dey C, et al. (2019). An insight into non-integrative gene delivery approaches to generate transgene-free induced pluripotent stem cells. *Gene*. Vol 686. pp 146-159.
- [24] Turner M, Leslie S, Martin NG, et al. (2013). Toward the development of a global induced pluripotent stem cell library. *Cell stem Cell*. Vol 13(4). pp 382-384.
- [25] Roh EY, Oh S, Yoon JH, et al. (2020). Umbilical cord blood units cryopreserved in the Public Cord Blood Bank; A breakthrough in iPSC haplobanking? *Cell Transplant*. Vol 29. pp 1-9.
- [26] Pappas DJ, Gourraud P, Le Gall C, et al. (2015). Human leukocyte antigen haplo-homozygous induced Pluripotent Stem Cell haplobank modeled after the California population: Evaluating matching in a multiethnic and admixed population. *Stem Cells Transl Med*. Vol 4(5). pp 413-418.
- [27] Taylor CJ, Peacock S, Chaundhry AN, et al. (2012). Generating an iPSC bank for HLA-matched tissue transplantation based on known donor and recipient HLA types. *Cell Stem Cell*. Vol 11(2). pp 147-152.
- [28] Huang CY, Liu CL, Ting CY, et al. (2019). Human iPSC banking: Barriers and opportunities. *J Biomed Sci*. Vol 26(1). pp 1-14.
- [29] Karanika S, Karantanos T, Li L, et al. (2015). Current challenges of iPSC-based disease modeling and therapeutic implications. *Cells*. Vol 34(22). pp 2815-2822.
- [30] Garg S. (2021). Computational methods for chromosome-scale haplotype reconstruction. *Genome Biology*. pp 1-24
- [31] Copley H, Elango M, Kosmoliaptis V, et al. (2018). Assessment of human leukocyte antigen immunogenicity: current methods, challenges and opportunities. *Current Opinion in Organ Transplantation*. Vol 23. pp 477-485.

- [32] Ping Chen I, Fukuda K, Fusaki N, et al. (2013). Induced Pluripotent Stem Cell reprogramming by Integration-free Sendai Virus vectors from peripheral blood of patients with Craniometaphyseal Dysplasia. *Cellular Reprogramming*. Vol 15. pp 503-513.
- [33] CTS™ CytoTune™ -iPS Sendai 2.1 Reprogramming Kit User Guide. *ThermoFisher Scientific*. pp 6-68
- [34] Campos B.P, Sartore C.R, Abdalia N.S, et al. (2009). Chromosomal spread preparation of Human Embryonic Stem Cells for Karyotyping. *Journal of Visualized Experiments*. Vol 31. pp 1-4.
- [35] The GenePrint® 10 System. *Promega Corporations*. pp 1-38.
- [36] Transcriptor First Strand cDNA Synthesis Kit. *Roche Diagnostics*. pp 1-27.
- [37] Stefková K, Procházková J and Pacherník J. (2015). Alkaline Phosphatase in Stem Cells. *Stem Cells int*. pp 1-11.
- [38] Ahmadian B, Syama A, Sivapatham R, et al. (2016). Detailed characterization of Human Induced Pluripotent Stem Cells manufactured for therapeutic Applications. *Stem Cell Rev*. Vol 12. pp 394-420



## Annex A

**Table 18.** Summary of the methodology and results for the 7 iPSCs lines generated and characterised.

|   |   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|---|
| Name of sample  | SPUC BAR<br>0048411   | SPUC VIZ<br>0074822   | SPUC MAL<br>0021923   | SPUC MAL<br>0040899   | SPUC MAD<br>0002340   | SPUC BAR<br>0077596   | SPUC BAR<br>0075408   |
| Name of iPSC clone                                      | Hz 29-44-7 CBiPS1<br>Sv4F-B8                                | Hz 30-18-3 CBiPS2<br>Sv4F-D10                               | Hz 3-7-15 CBiPS3<br>Sv4F-E9                                 | Hz 1-8-3 CBiPS4<br>Sv4F-F6                                  | Hz 33-14-1 CBiPS6<br>Sv4F-H6                                | Hz 24-7-15 CBiPS7<br>Sv4F-I12                               | Hz 11-27-1 CBiPS8<br>Sv4F-J1                                |
| Expansion of CD34+<br>cells, day 1<br>(Number of cells) | 0,23x10 <sup>6</sup>  | 1,14x10 <sup>6</sup>  | 0,66x10 <sup>6</sup>  | 2,2x10 <sup>6</sup>   | 0,63 x10 <sup>6</sup>                                       | 1,37 x10 <sup>6</sup>                                       | 0,61 x10 <sup>6</sup>                                       |
| Expansion of CD34+<br>cells, day 4<br>(Number of cells) | 0,87x10 <sup>6</sup>  | 1,65x10 <sup>6</sup>  | 1,17x10 <sup>6</sup>  | 3,46x10 <sup>6</sup>  | 1,57 x10 <sup>6</sup>                                       | 2,76 x10 <sup>6</sup>                                       | 1,056 x10 <sup>6</sup>                                      |
| Expansion factor  | 3,87  | 1,45  | 1,77  | 1,57  | 2,49  | 2,01  | 1,73  |
| Reprogramming<br>efficiency                             | 0,86%   | 1,8%  | 1,07%   | 1,29%   | 0,45%   | 0,47%   | 1,34%   |
| Karyotype (KT)  | 46, XY, P14   | 46, XY, P16   | 46, XY, P13   | 46, XX, P14   | 46, XX, P13   | 46, XY, P9  | 46, XX, P15   |
| AP-staining   | positive  | positive  | positive  | positive  | positive  | positive  | positive  |
| ICC pluripotency<br>marker                              | Oct4, Nanog, Sox2,<br>SSEA-3, SSEA-4,<br>TRA-1-60, TRA-1-81 | Oct4, Nanog, Sox2,<br>SSEA-3, SSEA-4,<br>TRA-1-60, TRA-1-81 | Oct4, Nanog, Sox2,<br>SSEA-3, SSEA-4,<br>TRA-1-60, TRA-1-81 | Oct4, Nanog, Sox2,<br>SSEA-3, SSEA-4,<br>TRA-1-60, TRA-1-81 | Oct4, Nanog, Sox2,<br>SSEA-3, SSEA-4,<br>TRA-1-60, TRA-1-81 | Oct4, Nanog, Sox2,<br>SSEA-3, SSEA-4,<br>TRA-1-60, TRA-1-81 | Oct4, Nanog, Sox2,<br>SSEA-3, SSEA-4,<br>TRA-1-60, TRA-1-81 |
| ICC differentiation<br>marker, Ectoderm                 | TUJ1, GFAP  | TUJ1, GFAP  | TUJ1, GFAP  | TUJ1, GFAP  | TUJ1, GFAP  | TUJ1, GFAP  | TUJ1, GFAP  |
| ICC differentiation<br>marker, Endoderm                 | AFP, FOXA2  | AFP, FOXA2  | AFP, FOXA2  | AFP, FOXA2  | AFP, FOXA2  | AFP, FOXA2  | AFP, FOXA2  |
| ICC differentiation<br>marker, Mesoderm                 | ASMA, ASA   | ASMA, GATA4   | ASMA, GATA4   | ASMA, GATA4   | ASMA, GATA4   | ASMA, GATA4   | ASMA, GATA4   |
| Microsatellites   | Matching with<br>somatic cells                              | Matching with<br>somatic cells                              | Matching with<br>somatic cells                              | Matching with<br>somatic cells                              | Matching with<br>somatic cells                              | Matching with<br>somatic cells                              | Matching with<br>somatic cells                              |
| KT post banking   | 46, XY, P16   | 46, XY, P17   | 46, XY, P13   | 46, XX, P16   | 46, XX, P15   | 46, XY, P14   | 46, XX, P15   |

## Annex B

**Table 19.** Summary of the Characterization Master Cell Bank and Working Cell Bank of the 7 iPSCs lines generated.

| Characterization Master Cell Bank    |   |   |   |   |   |   |   |
|--------------------------------------|---|---|---|---|---|---|---|
| Name of line                         | Hz 29-44-7 CBiPS1<br>Sv4F-B8                          | Hz 30-18-3 CBiPS2<br>Sv4F-D10                         | Hz 3-7-15 CBiPS3<br>Sv4F-E9                           | Hz 1-8-3 CBiPS4<br>Sv4F-F6                            | Hz 33-14-1 CBiPS6<br>Sv4F-H6                          | Hz 24-7-15 CBiPS7<br>Sv4F-I12                         | Hz 11-27-1 CBiPS8<br>Sv4F-J1                          |
| KT                                   | 46, XY, P22   | 46, XY, P22   | 46, XY, P20   | 46, XX, P17   | 46, XX, P18   | 46, XY, P17   | 46, XX, P18   |
| AP-staining                          | positive  | positive  | positive  | positive  | positive  | positive  | positive  |
| ICC pluripotency marker              | Oct4, Nanog, Sox2, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 | Oct4, Nanog, Sox2, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 | Oct4, Nanog, Sox2, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 | Oct4, Nanog, Sox2, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 | Oct4, Nanog, Sox2, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 | Oct4, Nanog, Sox2, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 | Oct4, Nanog, Sox2, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 |
| ICC differentiation marker, Ectoderm | TUJ1, GFAP  | TUJ1, GFAP  | TUJ1, GFAP  | TUJ1, GFAP  | TUJ1, GFAP  | TUJ1, GFAP  | TUJ1, GFAP  |
| ICC differentiation marker, Endoderm | AFP, FOXA2  | AFP, FOXA2  | AFP, FOXA2  | AFP, FOXA2  | AFP, FOXA2  | AFP, FOXA2  | AFP, FOXA2  |
| ICC differentiation marker, Mesoderm | ASMA, ASA   | ASMA, GATA4   | ASMA, GATA4   | ASMA, GATA4   | ASMA, GATA4   | ASMA, GATA4   | ASMA, GATA4   |
| Microsatellites                      | Matching with somatic cells                           | Matching with somatic cells                           | Matching with somatic cells                           | Matching with somatic cells                           | Matching with somatic cells                           | Matching with somatic cells                           | Matching with somatic cells                           |
| Characterization Working Cell Bank   |   |   |   |   |   |   |   |
| KT                                   | 46, XY, P23   | 46, XY, P23   | 46, XY, P21   | 46, XX, P21   | pending   | 46, XY, P20   | pending   |
| Microsatellites                      | Matching with somatic cells                           | Matching with somatic cells                           | pending   | Matching with somatic cells                           | pending   | pending   | pending   |