Dreams

DREAMS - Drug REpurposing with Artificial intelligence for Muscular disorderS

[D1.1] Characterization and Quality control of iPSC lines and myogenicderivatives.

Project Founded by the European Commission		
DISSEMINATION LEVEL		
PU	Public	☒
SEN	Sensitive	☐

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List of Abbreviations

TERM	DEFINITION
CPP	Committee for the Protection of Persons
CNM	Centronuclear Myopathy
DMD	Duchenne Muscular Dystrophy
DNM2	Dynamin 2
EDMD	Emery-Dreifuss muscular dystrophy
GSDII	Glycogen-Storage Disease Type II
hiPSC	human induced Pluripotent Stem Cell
NMD	Neuromuscular Dystrophy
PBMC	Peripheral blood mononuclear cell
qPCR	quantitative Polymerase Chain Reaction
SOP	Standard Operation Protocol
WT	Wild-Type

Content of the report

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Table 1. Standard Operation Procedures (SOPs)

Cell type	Method	
hiPSC	Reprogramming of PBMC in hiPSC (**Annex A** - PBMC Reprogramming using Sendaï Virus)	
	Culture of hiPSC (**Annex B** - hiPSC Culture)	
	Quality Control	Karyotyping & SNPs Genotyping (**Annex C** - hiPSC Karyotyping & SNPs Genotyping)
		qPCR sendaï non-integration (**Annex D** - hiPSC/Myoblast/Myotube qPCR)
		Immunofluorescence (**Annex E** - hiPSC Immunofluorescence)
		Cytometry (Annex F - hiPSC Cytometry)
Myoblast derived from hiPSC	Differentiation of hiPSC into Myoblasts (**Annex G** - Myoblast Differentiation)	
	Culture of Myoblasts derived from hiPSC (**Annex H** - Myoblast Culture)	
	Quality Control	Immunofluorescence (**Annex I** - Myoblast/Myotube Immunofluorescence)
		qPCR (**Annex D** - hiPSC/Myoblast/Myotube qPCR)
		Cytometry (**Annex J** - Myoblast Cytometry)
Myotubes derived from hiPSC	Differentiation of Myoblasts derived from hiPSC Into Myotubes (**Annex K** - Myotube Differentiation)	
	Quality Control	Immunofluorescence (**Annex I** - Myoblast/Myotube Immunofluorescence)
		qPCR (**Annex D** - hiPSC/Myoblast/Myotube qPCR)

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1 Introduction

This report provides a detailed molecular characterisation and quality control assessment of induced pluripotent stem cell (iPSC) lines and their myogenic derivatives derived from healthy controls, Cas9-inducible lines, and patients with five different neuromuscular dystrophies (NMDs): Dynamin 2 Centronuclear Myopathy (CNM), Duchenne Muscular Dystrophy (DMD), Emery-Dreifuss Muscular Dystrophy (EDMD), Glycogen-Storage Disease Type II (Pompe), and Danon Disease. This characterisation includes the expression of pluripotency markers, karyotypic stability, and a detailed molecular analysis of their myogenic derivatives, including banking reports, expression of myogenic markers, myoblast proliferation, and terminal differentiation of myotubes. Along with the production of iPSC lines and their myogenic derivates, a key aspect of our work has been the development and implementation of Standard Operating Procedures (SOPs). These SOPs were crafted by our team to ensure the rigorous, reproducible generation and differentiation of iPSC lines. The SOPs cover each step from cell line initiation to the final validation of myogenic derivatives, providing a structured framework that strengthens the entire characterisation process. By standardising these procedures, we aim to guarantee the consistency and reliability of our cellular models, which are essential for downstream applications such as drug screening and disease modelling.

1.1 Acquisition of Cellular Materials

Due to delays in obtaining the necessary ethical approval from the Committee for the Protection of Persons (CPP), which was originally anticipated for January 2024 but finally only granted in May, our project faced significant challenges in initiating the direct collection of cells from patients. To adapt to this setback and maintain progress in our research timeline, we initiated an alternative approach. Instead of waiting to collect patient samples directly, we began by reprogramming peripheral blood mononuclear cells (PBMCs) previously sampled by APHP or AFM Telethon and, when available, to use previously generated human induced pluripotent stem cells (iPSCs). This strategic pivot allowed us to continue our experimental workflows and data generation, essential for the project's advancement. To meet the deadlines and provide to the partners myogenic cells for at least one cell line per disease in the first 6 months, priority was given to the muscular dystrophies for which blood samples had been sampled by APHP (EDMD, DMD and CNM). Utilising these sources ensured that our research could proceed without further delay for three diseases, thus minimising the impact on our overall project timeline and objectives. For DMD we have initiated a collaboration with the INSERM unit led by Onnik Agbulut who provided us three IPS cell line for DMD.
For Pompe disease, we've been able to move forward thanks to the clinician Edoardo Malfatti (APHP) who sent us blood samples from two patients. In the meantime, to start our research on this disease we have used immortalized cells and iPSC from Coriell repository. Now that the ethical committee has approved our project, a third patient for Pompe disease will be sampled this summer. In the same way we will also sample three patients suffering from Danone disease in July. Finally, all the WT iPS cell lines, including the inducible cas9 cell line were generated before DREAMS and their full QC will be provided in the next deliverable D1.2.

1.2 Cell reprogramming strategy

Our strategy for reprogramming the cells focuses on developing cell lines for diseases where none or few cell lines were available, ensuring that at least one cell line is available for each disease of the DREAMS consortium. Although the DREAMS project was originally designed to use a single reprogramming method (Sendaï method), some of the external hiPSC were reprogrammed by other methods as detailed in Table 2. Accordingly, to maintain robust controls within our study, we established two control groups, each using both reprogramming techniques. This approach ensures a balanced experimental framework, allowing us to compare the outcomes of these methodologies directly. In an additional strategic move, we capitalised on

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the availability of both PBMC and hiPSC from the same DMD patients to compare the effects of the two different reprogramming methods on identical genetic backgrounds, enhancing the depth and applicability of our findings.

Table 2. hiPSC reprogramming.

Disease	Cell Line	Mutation	Reprogrammation		Availability			
			Done by	Method	hiPSC		Myogenic derivatives	
					Produced	QC*	Produced	QC**
Wild-type (WT)	WT-1		CECS	Episome	🕓	🕓	✓	✓
	WT-2		CECS	Sendaï	🕓	🕓	✗	✗
	WT-3		CECS	Sendaï	🕓	🕓	✗	✗
	WT-4		CECS	Episome	🕓	🕓	✗	✗
Emery-Dreifuss Muscular Disease (EDMD-LMNA)	EDMD-LMNA-1	c.745C>T	CECS	Episome	✓	✓	✓	✓
	EDMD-LMNA-2	c.976T>A	CECS	Episome	✓	✓	🕓	🕓
	EDMD-LMNA-3	c.340_342del	CECS	Episome	✓	✓	✓	✓
Centronuclear myopathy (CNM-DNM2)	CNM-DNM2-1	c.1106 G>A	CECS	Sendaï	✓	✓	🕓	🕓
	CNM-DNM2-2	c.1393C>T	CECS	Sendaï	🕓	🕓	✗	✗
	CNM-DNM2-3	c.1565G>A	CECS	Sendaï	🕓	🕓	✗	✗
Glycogenose type 2 (GSDII)	GSDII-1	c.-32-13T >G c.1888+1G>A	CECS	Sendaï	🕓	🕓	✗	✗
	GSDII-2	c.-32-13T >G c.1888+1G>A	CECS	Sendaï	🕓	🕓	✗	✗
	GSDII-3				✗	✗	✗	✗
Duchenne myopathy (DMD)	DMD-1.2	c.1990C>T	External source	Episome	✓	✓	✓	✓
	DMD-2.2	c.4870C>T	External source	Episome	🕓	🕓	✗	✗
	DMD-3.2	c.6439-1G>A	External source	Episome	🕓	🕓	✗	✗
Danon disease (DN)	DN-1				✗	✗	✗	✗
	DN-2				✗	✗	✗	✗
	DN-3				✗	✗	✗	✗

** QC: Quality control. Includes Immunofluorescence (OCT4 and NANOG), Cytometry (SSEA4 and TRA-1-81), SNPs and Karyotyping.** QC Quality control. Includes immunofluorescence in both Myoblast and Myotubes (CD56, DESMIN, MF20 MYOD, MYOG and PAX7), Cytometry (CD56 and CD82) and qPCR (DP427M, DESMIN, MYOD, MYOG, NANOG, OCT4). ✓: Available; 🕓: In Progress; ✗: To do.*

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2 hiPSC Production and Quality Control tests

Production reports of 6 hiPSC lines: 1) EDMD-LMNA-1, 2) EDMD-LMNA-2, 3) EDMD-LMNA-3, 4) CNM-DNM2-1 and 5) DMD-1.2

2.1 Production report of EDMD-LMNA-1 hiPSC

Immunofluorescence

The immunofluorescence images in Figure 1 show a strong expression of the pluripotency markers OCT4 and NANOG in EDMD-LMNA-1 hiPSC, indicating that these cells remain undifferentiated and pluripotent.

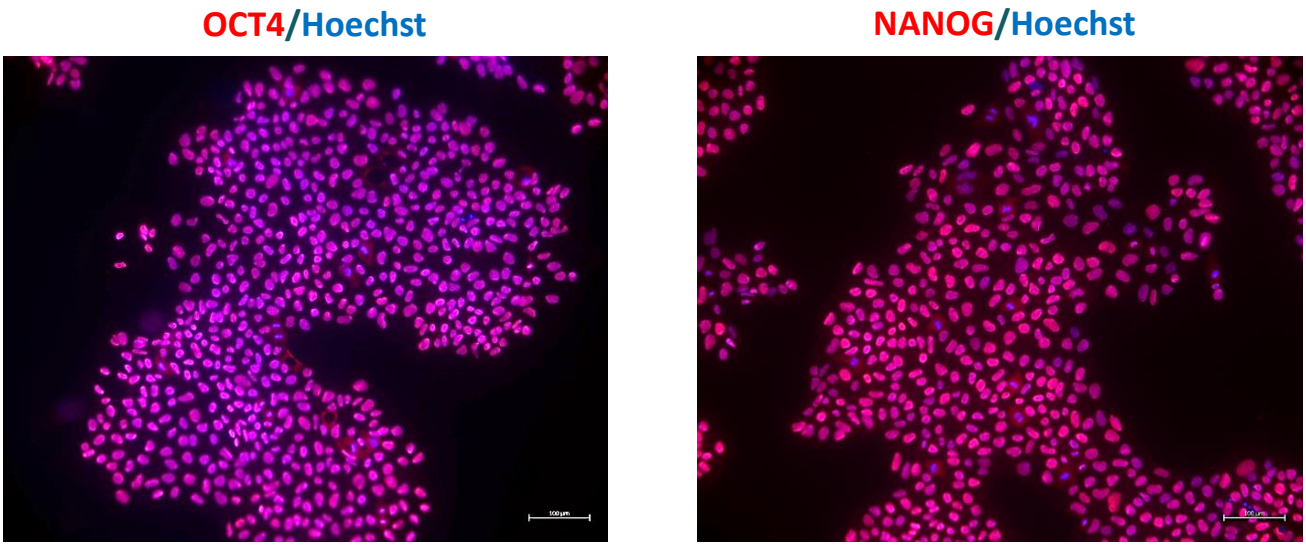

Figure 1. Immunofluorescence Quality Control Images of EDMD-LMNA-1 hiPSC.

Flow Cytometry

The flow cytometry analysis illustrated in **Figure 2** shows that 99.1% of EDMD-LMNA-1 hiPSC are double marked for SSEA4 and TRA-1-81, confirming that most of these cells are pluripotent.

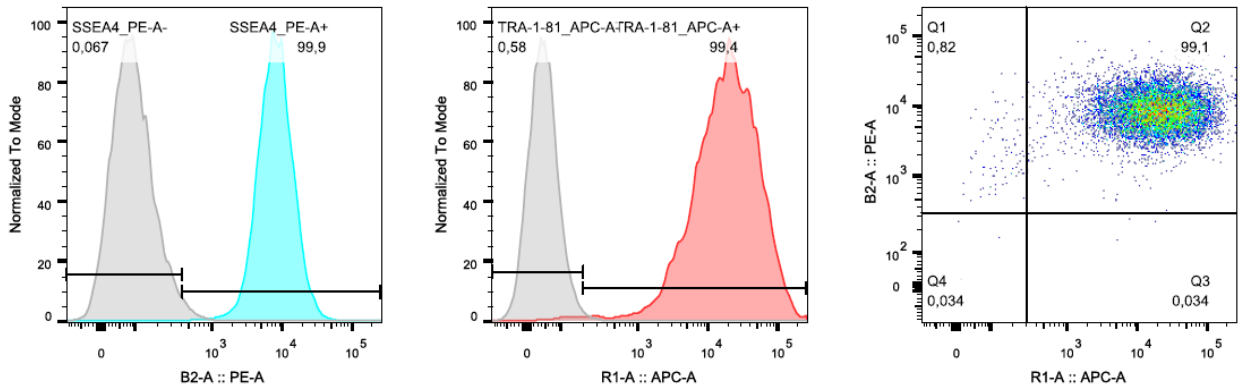

Figure 2. Flow Cytometry Analysis of SSEA4 and TRA-1-81 Markers in EDMD-LMNA-1 hiPSC.

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Karyotyping

The karyotype analysis in Figure 3 confirmed the genomic stability of the EDMD-LMNA-1 iPSC line, showing no chromosomal abnormalities.

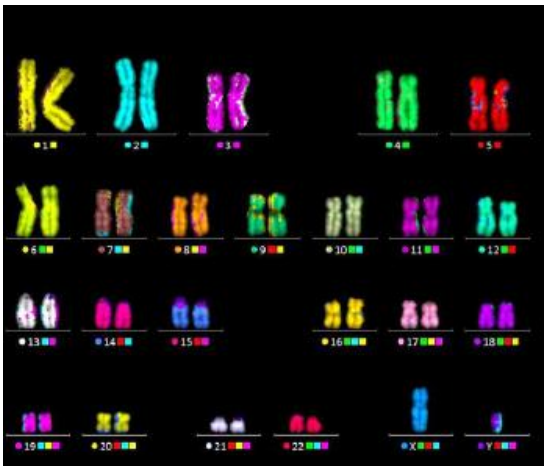

Figure 3: Karyotype Analysis of EDMD-LMNA-1 hiPSC.

SNPs

SNP karyotyping and genotyping in Figure 4 were performed to further ensure the genetic integrity of the EDMD-LMNA-1 iPSC line, confirming the absence of major genetic alterations.

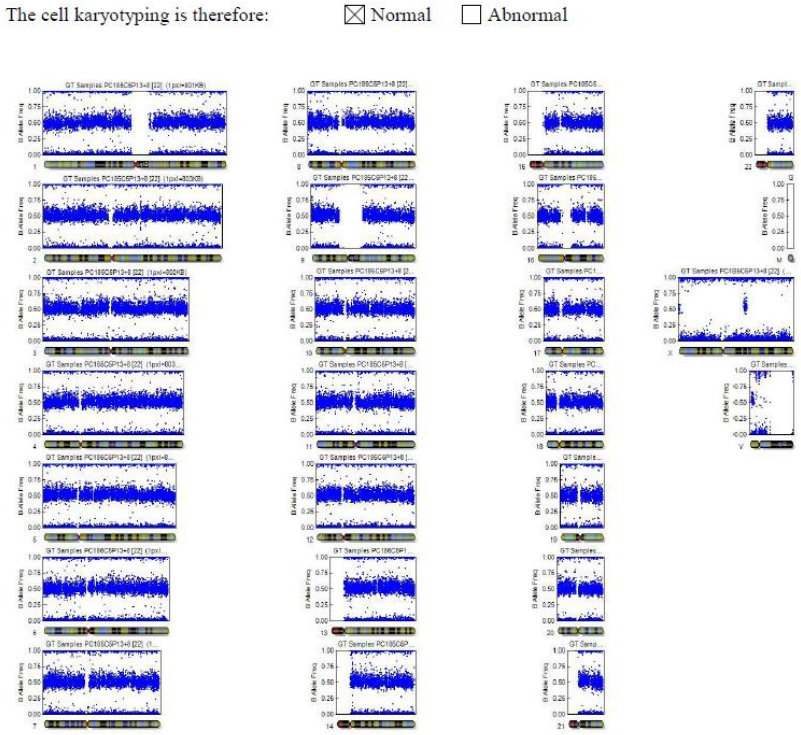

Figure 4: SNP karyotyping and genotyping analysis of EDMD-LMNA-1 hiPSC.

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2.2 Production report of EDMD-LMNA-2 hiPSC

Immunofluorescence

The immunofluorescence images in Figure 5 show a strong expression of the pluripotency markers OCT4 and NANOG in EDMD-LMNA-2 hiPSCs, indicating that these cells remain undifferentiated and pluripotent.

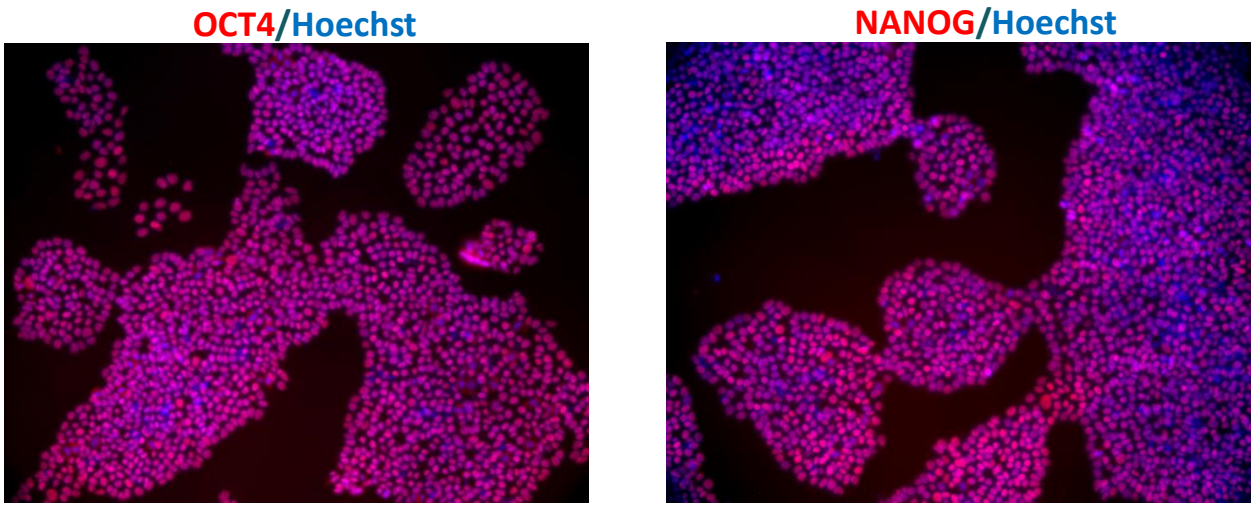

Figure 5: Immunofluorescence Quality Control Images of EDMD-LMNA-2 hiPSC.

Flow Cytometry

The flow cytometry analysis illustrated in Figure 6 shows that 99.4% of EDMD-LMNA-2 hiPSCs are double-marked for SSEA4 and TRA-1-81, confirming that most of these cells are pluripotent.

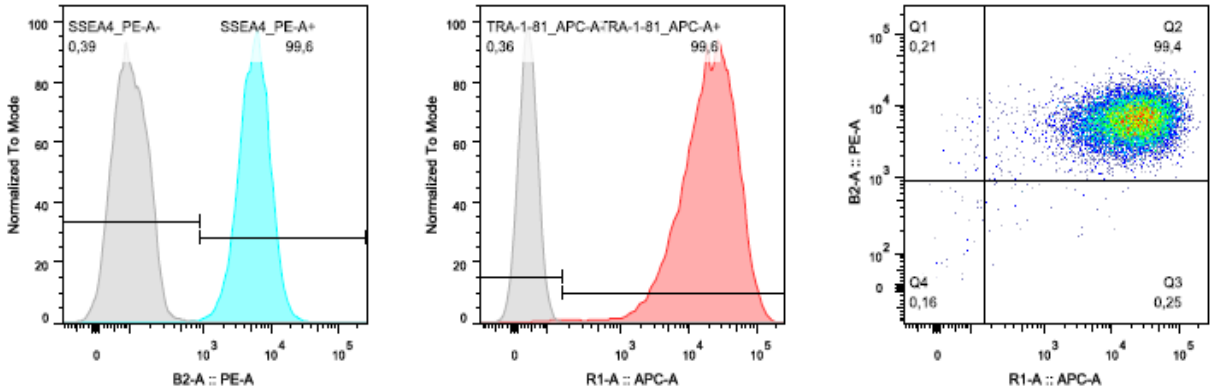

Figure 6: Flow Cytometry Analysis of SSEA-3, SSEA-4 and TRA-1-81 Markers in EDMD-LMNA-2 hiPSC.

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Karyotyping

The karyotype analysis in Figure 7 confirmed the genomic stability of the EDMD-LMNA-2 hiPSC line, showing no chromosomal abnormalities.

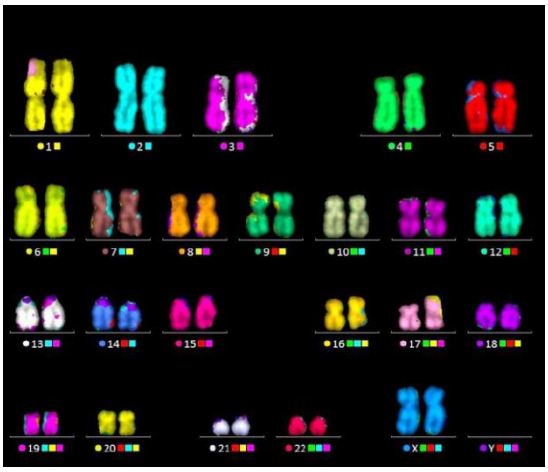

Figure 7: Karyotype Analysis of EDMD-LMNA-2 hiPSC.

SNPs

SNP karyotyping and genotyping in Figure 8 were performed to further ensure the genetic integrity of the EDMD-LMNA-2 iPSC line, confirming the absence of major genetic alterations.

Production

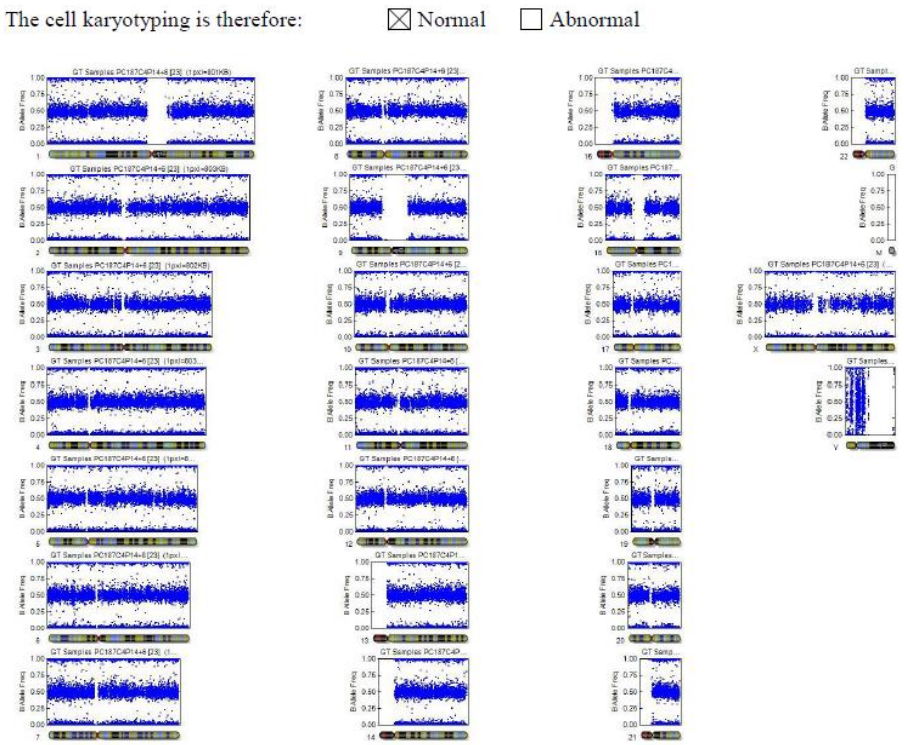

Figure 8: SNP karyotyping and genotyping analysis of EDMD-LMNA-2 hiPSC.

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2.3 Production report of EDMD-LMNA-3 hiPSC

Immunofluorescence

The immunofluorescence images in Figure 9 show a strong expression of the pluripotency markers OCT4 and NANOG in EDMD-LMNA-3 hiPSCs, indicating that these cells remain undifferentiated and pluripotent.

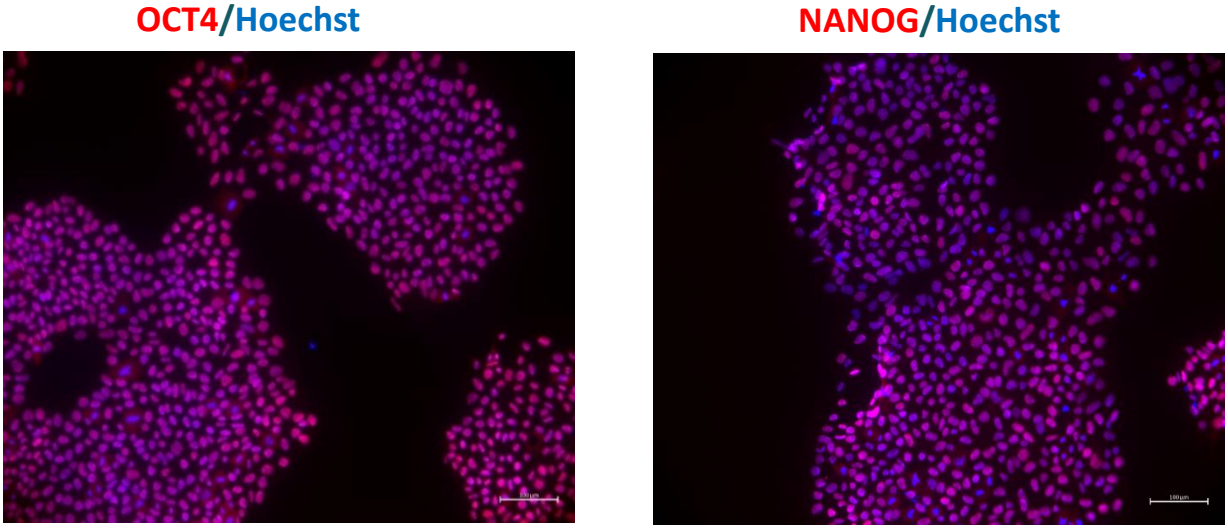

Figure 9: Immunofluorescence Quality Control Images of EDMD-LMNA-3 hiPSC.

Flow Cytometry

The flow cytometry analysis illustrated in Figure 10 shows that 99.0% of EDMD-LMNA-3 hiPSCs are double-marked for SSEA3 and TRA-1-81, confirming that most of these cells are pluripotent.

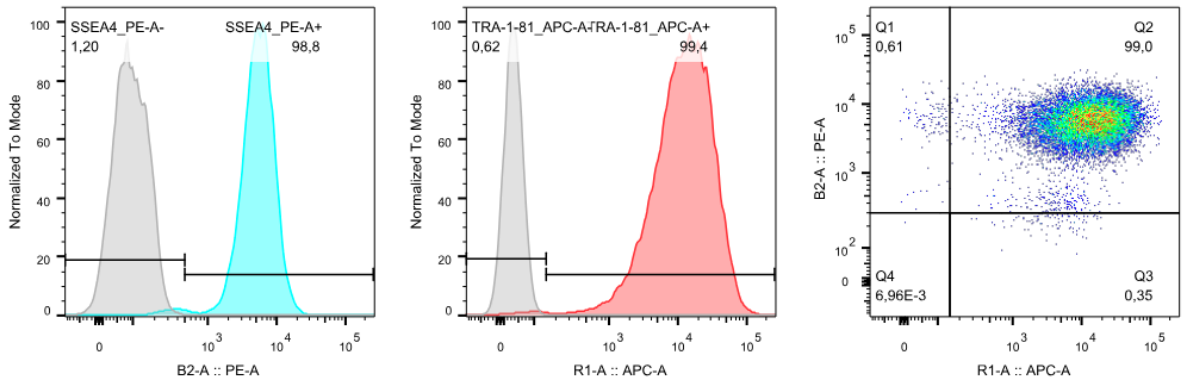

Figure 10: Flow Cytometry Analysis of SSEA-3, SSEA-4 and TRA-1-81 Markers in EDMD-LMNA-3 hiPSC.

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Karyotyping

The karyotype analysis in Figure 11 confirmed the genomic stability of the EDMD-LMNA-3 iPSC line, showing no chromosomal abnormalities.

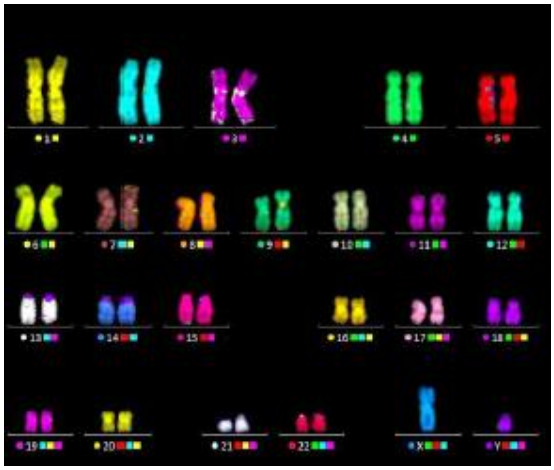

Figure 11: Karyotype Analysis of EDMD-LMNA-3 hiPSC.

SNPs

SNP karyotyping and genotyping analysis in Figure 12 were performed to further ensure the genetic integrity of the EDMD-LMNA-3 iPSC line, confirming the absence of major genetic alterations.

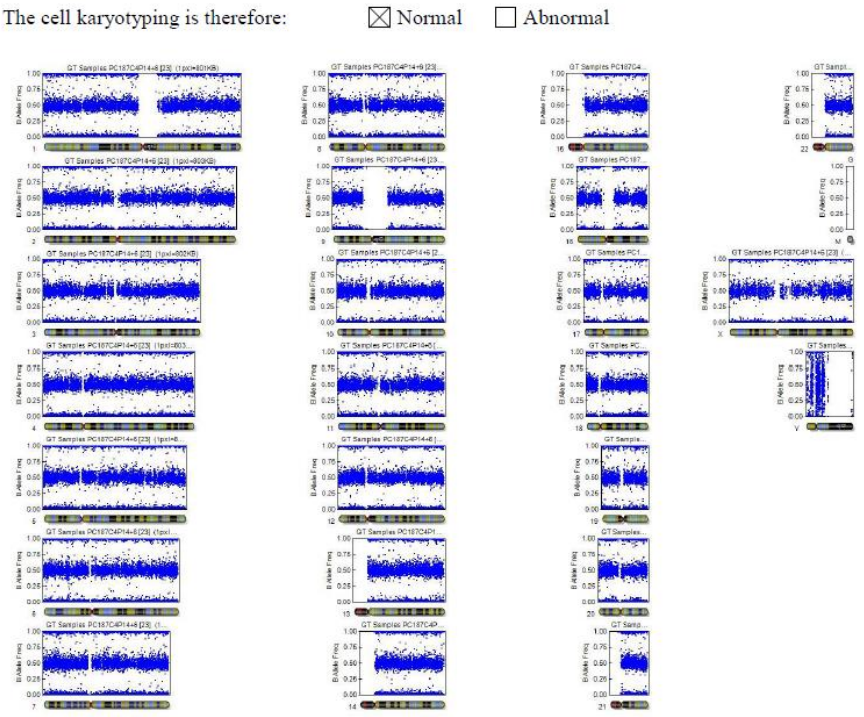

Figure 12: SNP karyotyping and genotyping analysis of EDMD-LMNA-3 hiPSC.

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2.4 Production report of CNM-DNM2-1 hiPSC

Immunofluorescence

The immunofluorescence images in Figure 13 show strong expression of the pluripotency markers OCT4 and NANOG in CNM-DNM2-1 hiPSC, indicating that these cells remain undifferentiated and pluripotent.

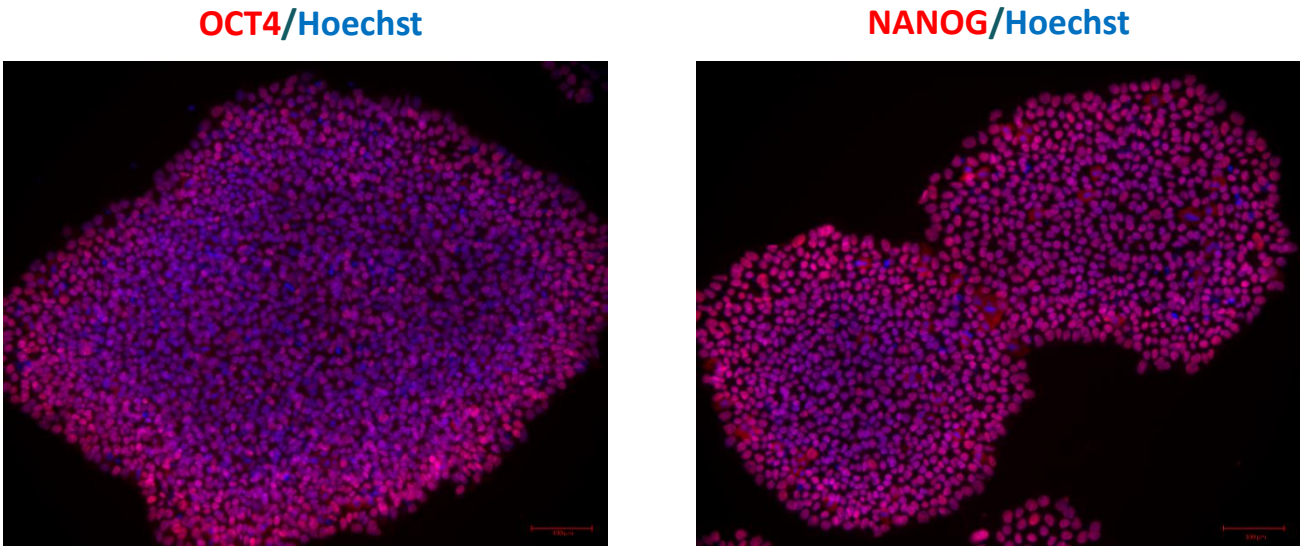

Figure 13: Immunofluorescence Quality Control Images of CNM-DNM2-1 hiPSC.

Flow Cytometry

The flow cytometry analysis illustrated in Figure 14 shows that 98.4% of CNM-DNM2-1 hiPSCs are double marked for SSEA3 and TRA-1-81, confirming that most of these cells are pluripotent.

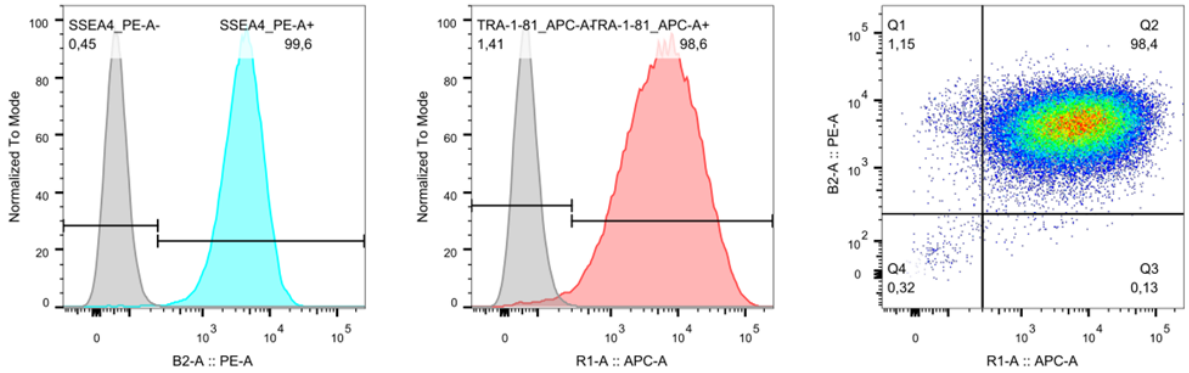

Figure 14: Flow Cytometry results of SSEA4 and TRA-1-81 markers on CNM-DNM2-1 hiPSC.

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Karyotyping

The karyotype analysis in Figure 15 confirmed the genomic stability of the CNM-DNM2-1 iPSC line, showing no chromosomal abnormalities.

Figure 15: Karyotyping analysis of CNM-DNM2-1 hiPSC.

SNP

The SNP karyotyping and genotyping analysis of CNM-DNM2 hiPSCs illustrated in Figure 16 indicates a duplication on chromosome 7 (q11.23) and a deletion on chromosome 11 (p14.3). These results highlight potential genomic variations in the CNM-DNM2 hiPSC line but does not indicate that it is due to the reprogramming since we did not evaluate the karyotype in PBMC. While no genes of importance are present in these regions, further investigation is required to determine if these variations were initially present in the patient.

chromosome	default	zone	start	end	default size
7	duplication	q11.23	76.137.375	76.720.582	583Kb
11	deletion	p14.3	23.437.553	23.915.409	479Kb

Figure 16: SNP karyotyping and genotyping analysis of CNM-DNM2 hiPSC.

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2.5 Production report of DMD-1.2 hiPSC

Immunofluorescence

The immunofluorescence images in Figure 17: Immunofluorescence Quality Control Images of DMD1.2 hiPSC. show strong expression of the pluripotency markers OCT4 and NANOG in DMD-1.2 hiPSC, indicating that

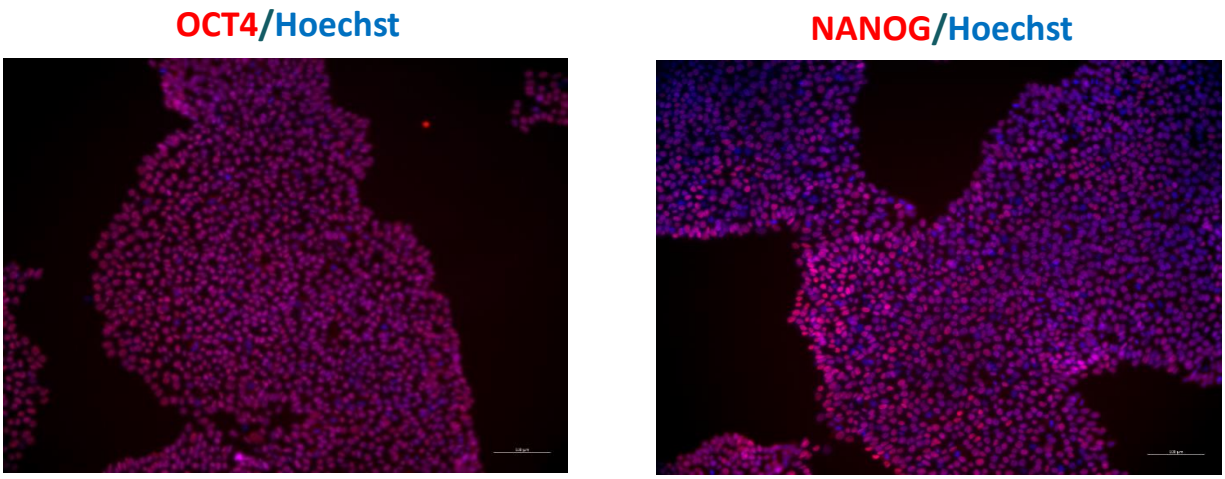

Figure 17: Immunofluorescence Quality Control Images of DMD1.2 hiPSC.

these cells remain undifferentiated and pluripotent.

Flow Cytometry

The flow cytometry analysis in Figure 18 shows that 95.4% of DMD-1.2 hiPSCs are double-marked for SSEA4 and TRA-1-81. This high level of double marking confirms that most DMD-1.2 cells express these pluripotency markers, indicating their undifferentiated state.

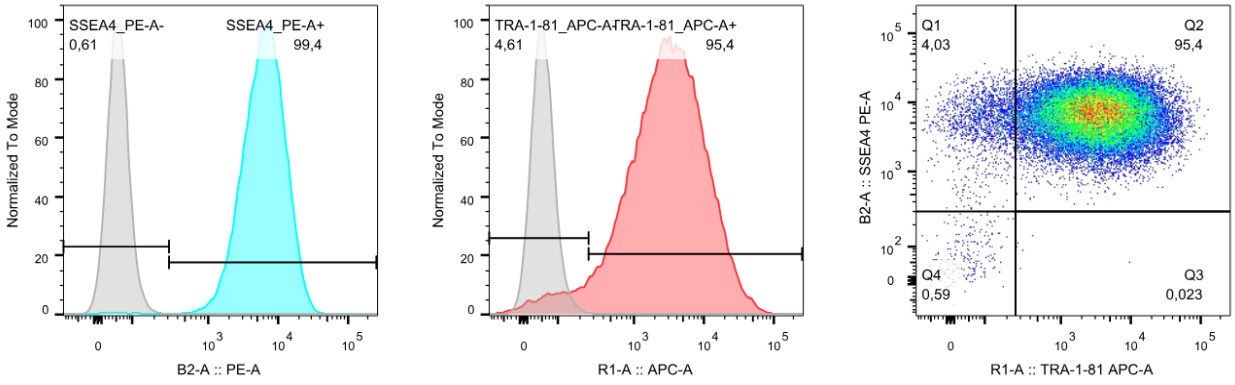

Figure 18: Flow Cytometry results of SSEA4 and TRA-1-81 markers on DMD-1.2 hiPSC.

This DMD iPS cell lines has been provided by INSERM. Karyotypes and SNP analysis will be done later in the project.

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3 Production reports of hiPSC-derived myoblasts/ myotubes

Four production reports of hiPSC-derived myoblasts/myotubes: 1) WT-1, 2) EDMD-LMNA-1, 3) EDMD-LMNA-3 and 4) DMD-1.2.

3.1 Report of WT-1 hiPSC-derived myoblasts/myotubes

Table 3. Quality control and production overview for WT-1 hiPSC-derived myoblasts.

Cell lines	Myoblast produced		
	Differentiation n°	Production	Delivery
WT-1	N°1	60,000,000 (60 tubes)	20 tubes Samsara
			20 tubes Inserm
	N°2	200,000,000 (200 tubes)	20 tubes Technion

Immunofluorescence

WT-1 hiPSC-derived myotubes and WT-1 hiPSC-derived myoblasts showed a high expression of Desmin, MF20 and CD56 and myotube cells expressed more MYOD and MYOG than myoblast cells (Figure 19).

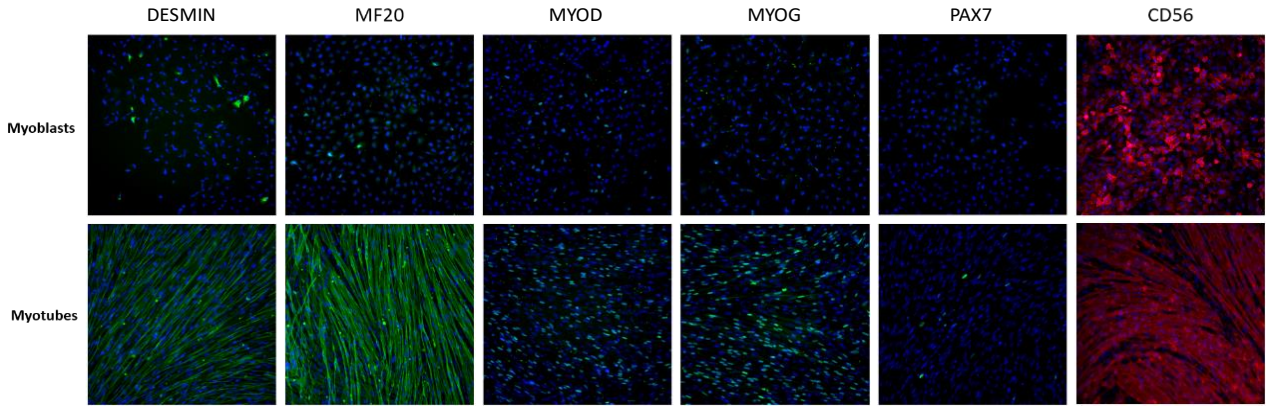

Figure 19: Immunofluorescence Quality Control Images of WT-1 hiPSC-derived myoblasts and myotubes.

Cytometry

As shown in Figure 20, the flow cytometry analysis shows that 99.0% of WT-1 hiPSC-derived myoblasts express CD56 and 100% express CD82, with 99.3% co-expressing both markers, confirming their myogenic identity.

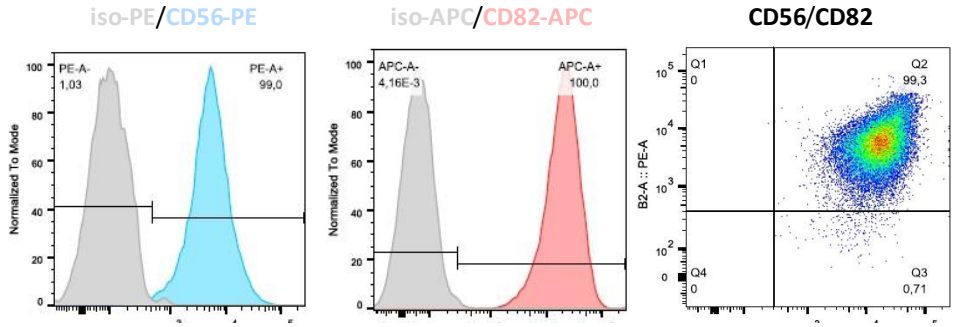

Figure 20: Flow Cytometry Analysis of CD56 and CD82 markers in WT-1 hiPSC-derived myoblasts (Iso-… = Control antibody for the fluorochrome of interest).

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Quantitative PCR

Based on the qPCR data shown in Figure 21, our WT-1 hiPSC-derived myoblasts and WT-1 hiPSC-derived myotubes do not express pluripotency biomarkers (NANOG, OCT4), indicating they are well differentiated. The high expression levels of DESMIN, MYOD, MYOG, and DP427M confirm that these cells are fully differentiated into myotubes at the final stage.

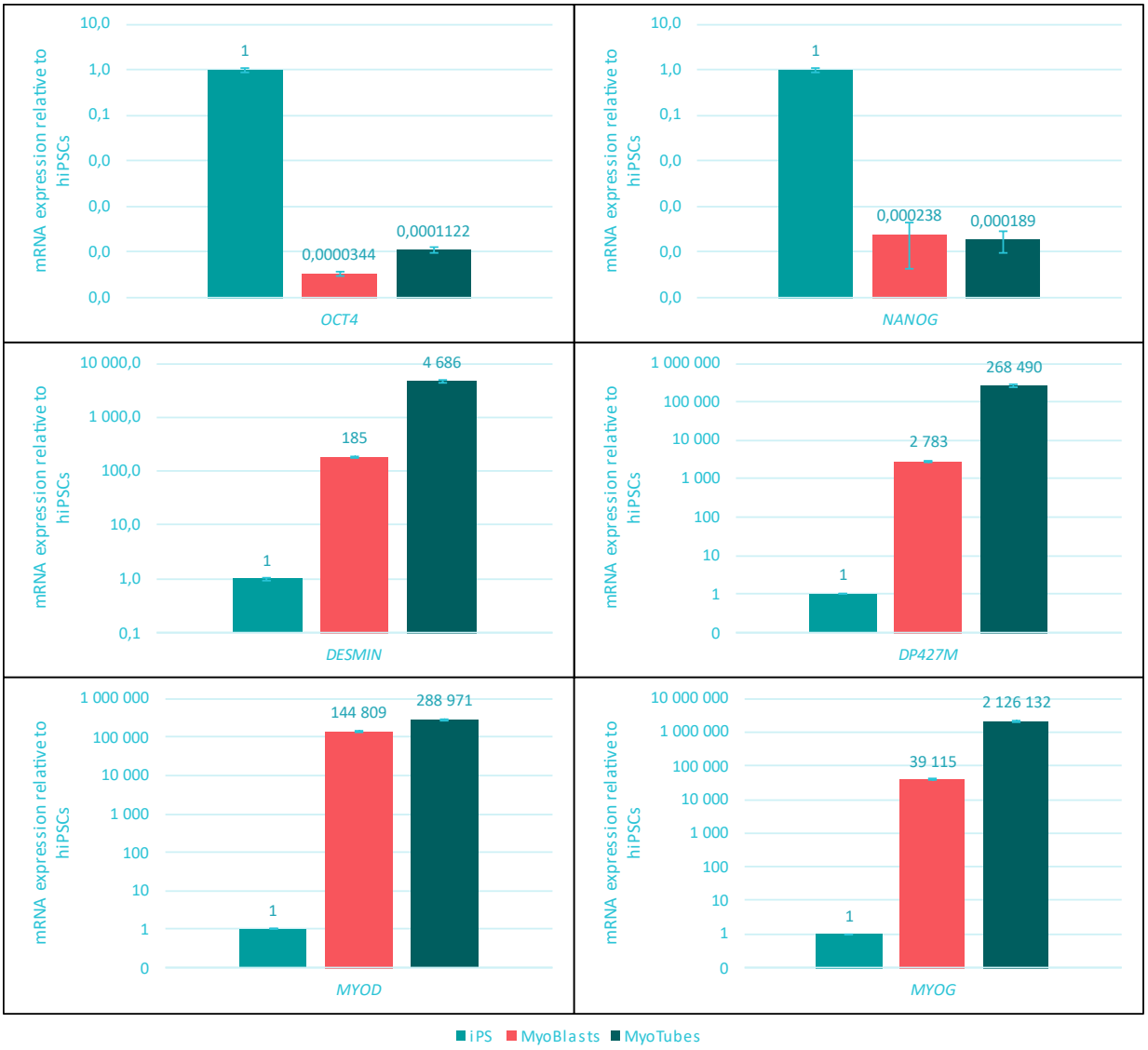

Figure 21: Quantitative PCR Results for WT-1 hiPSC-derived myoblasts and myotubes.

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3.2 Report of EDMD-LMNA-1 hiPSC-derived myoblasts/myotubes

Table 4. Quality control and production overview for EDMD-LMNA hiPSC-derived myoblasts.

Cell lines	Myoblast Differentiation n°	Myoblast Production	Myoblast Delivery
EDMD-LMNA-1	N°1	96 000 000	20 tubes Samsara
			20 tubes Inserm
			20 tubes Technion
EDMD-LMNA-2	N°1	130 000 000	
EDMD-LMNA-3	N°1	110 000 000	20 tubes Samsara
			20 tubes Inserm
			20 tubes Technion

Immunofluorescence

EDMD-LMNA-1 hiPSC-derived myoblasts and EDMD-LMNA-1 hiPSC-derived myotubes showed a high expression of Desmin, MF20 and CD56. EDMD-LMNA-1 Myotube cells express more MYOD and MYOG than EDMD-LMNA-1 Myoblast cells, as shown in Figure 22.

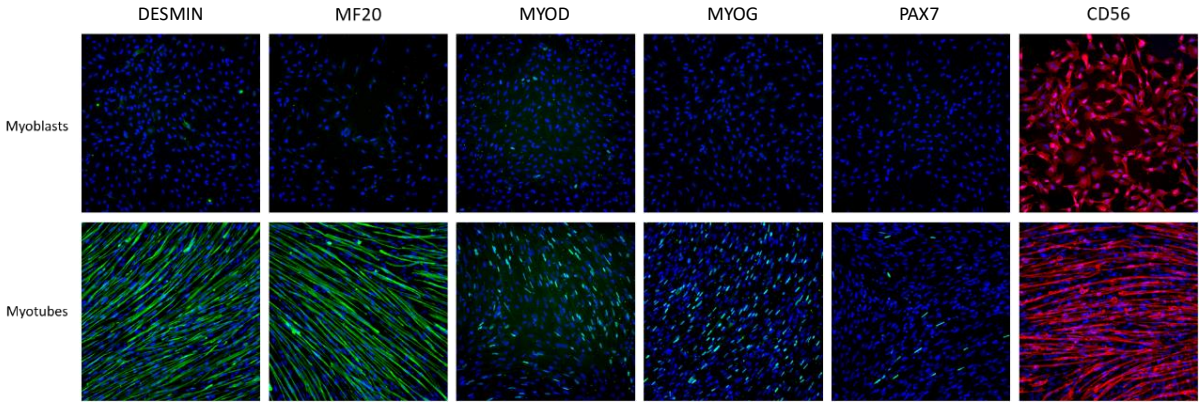

Figure 22: Immunofluorescence Quality Control Images of EDMD-LMNA-1 hiPSC-derived myoblasts and myotubes.

Flow Cytometry

As shown in Figure 23, the flow cytometry analysis shows that 95.4% of EDMD-LMNA-1 hiPSC-derived myoblasts express CD56 and 99.9% express CD82, with 95.5% co-expressing both markers, confirming their myogenic identity.

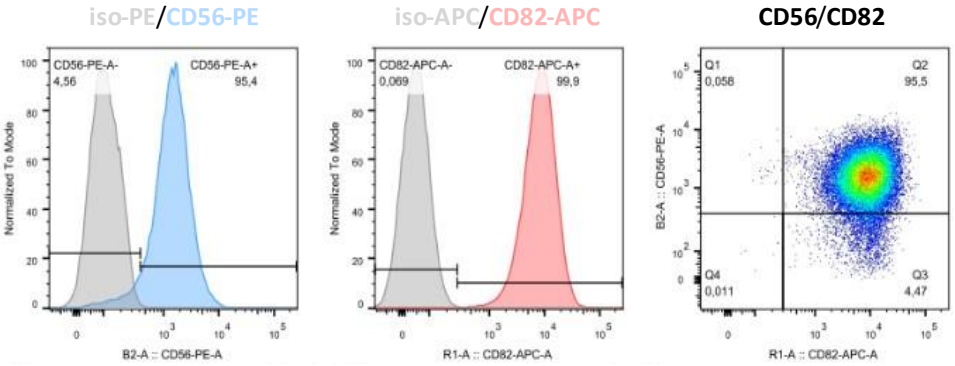

Figure 23. Flow Cytometry Analysis of CD56 and CD82 markers in EDMD-LMNA-1 hiPSC-derived myoblasts (Iso-… = Control antibody for the fluorochrome of interest).

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Quantitative PCR

Based on the qPCR data shown in Figure 24, our EDMD-LMNA-1 hiPSC-derived myoblasts and myotubes do not express pluripotency biomarkers (NANOG, OCT4), indicating they are well differentiated. The high expression levels of DESMIN, MYOD, MYOG, and DP427M confirm that these cells are fully differentiated into myotubes at the final stage.

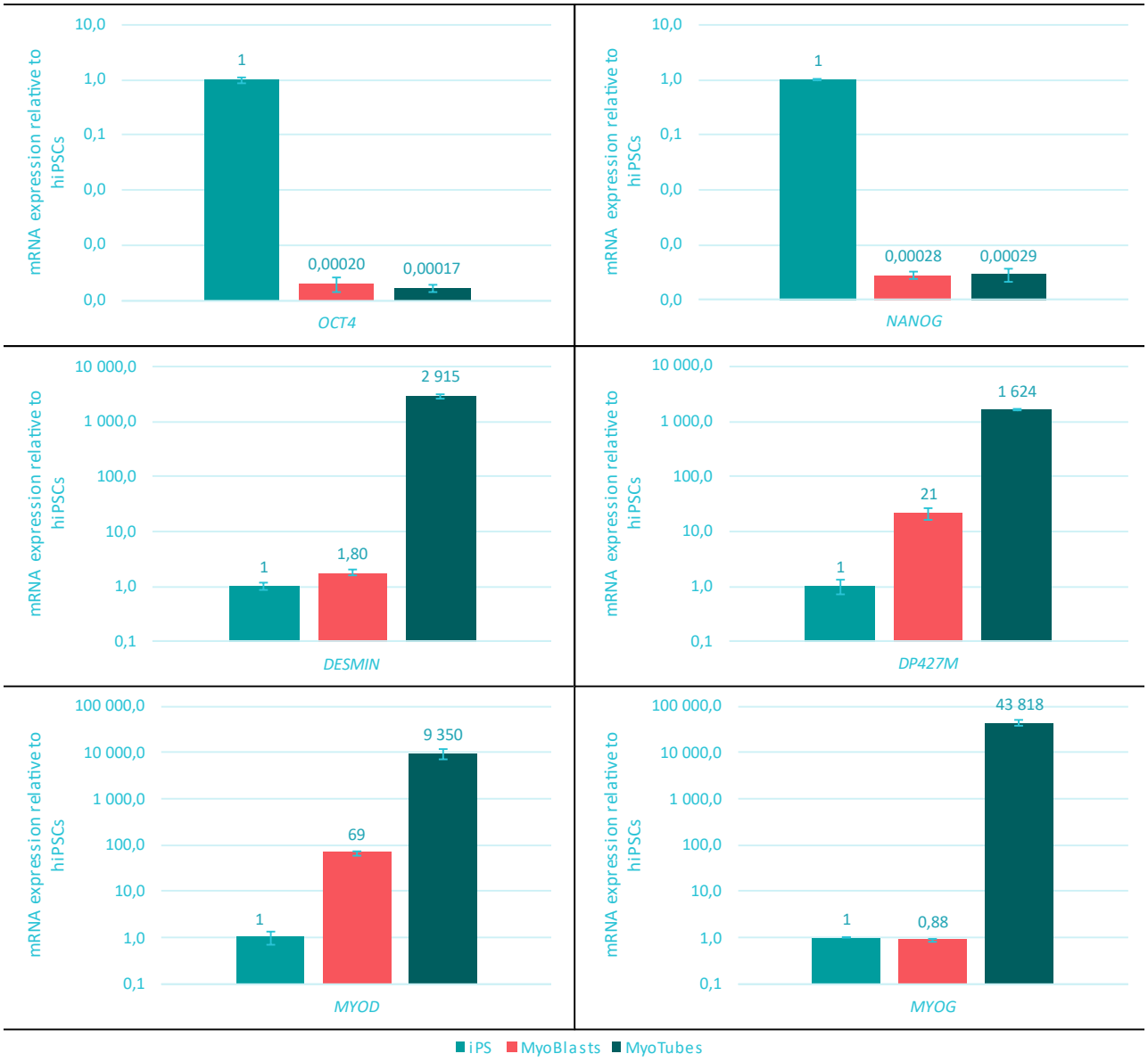

Figure 24. Quantitative PCR Results for EDMD-LMNA-1 hiPSC-derived myoblasts and myotubes.

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3.3 Report of EDMD-LMNA-3 hiPSC-derived myoblasts/myotubes

Immunofluorescence

EDMD-LMNA-3 hiPSC-derived myoblasts and EDMD-LMNA-3 hiPSC-derived myotubes showed a high expression of Desmin, MF20 and CD56, as shown in Figure 25. hiPSC-derived myotubes express more MYOD and MYOG than hiPSC-derived myoblasts.

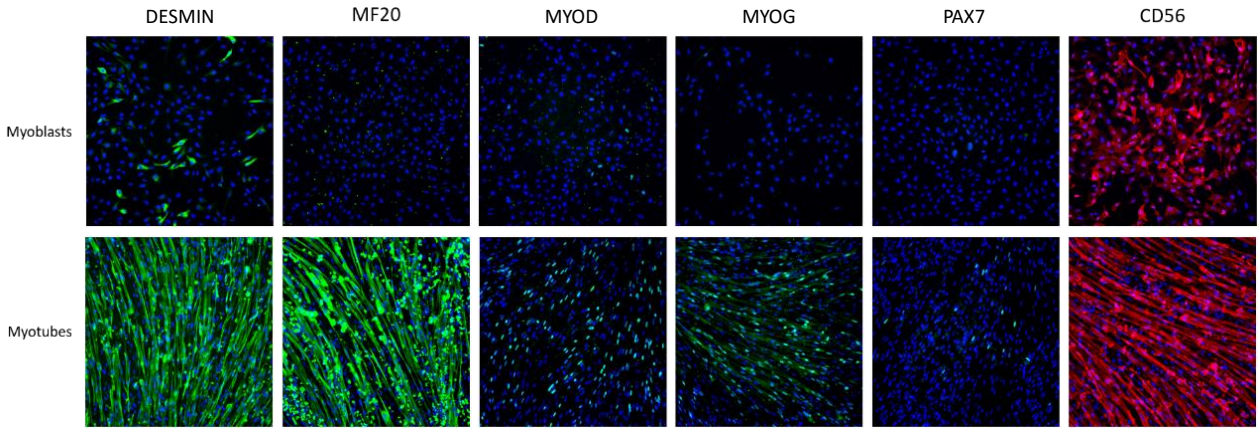

Figure 25: Immunofluorescence Quality Control Images of EDMD-LMNA-3 hiPSC-derived myoblasts and myotubes.

Flow Cytometry

The flow cytometry analysis in Figure 26 shows that 86.1% of EDMD-LMNA-3 hiPSC-derived myoblasts express CD56 and 100% express CD82, with 85.5% co-expressing both markers, confirming their myogenic identity.

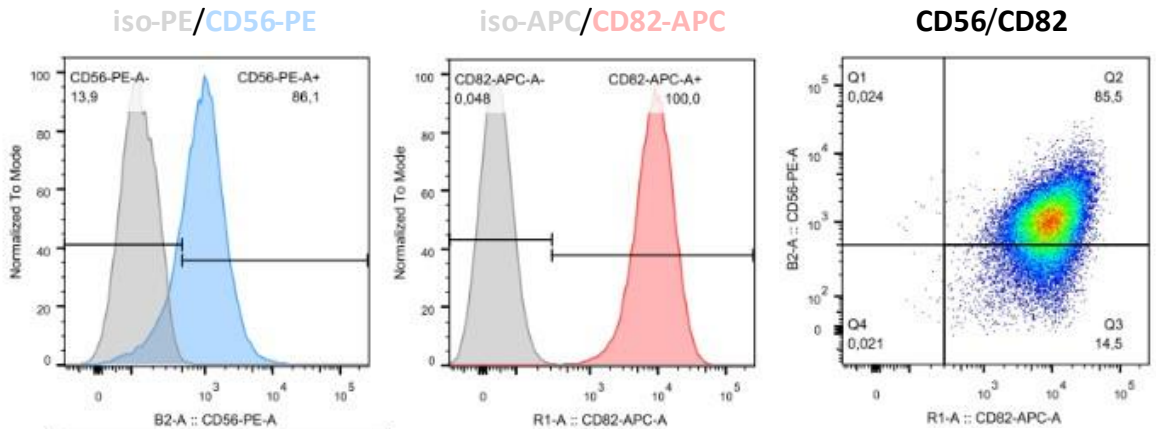

Figure 26: Cytometry Analysis of CD56 and CD82 markers in EDMD-LMNA-3 hiPSC-derived myoblasts (Iso-… = Control antibody for the fluorochrome of interest)

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Quantitative PCR

Based on the qPCR data shown in Figure 27, our EDMD-LMNA-3 hiPSC-derived myoblasts and myotubes do not express pluripotency biomarkers (NANOG, OCT4), indicating they are well differentiated. The high expression levels of DESMIN, MYOD, MYOG, and DP427M confirm that these cells are fully differentiated into myotubes at the final stage.

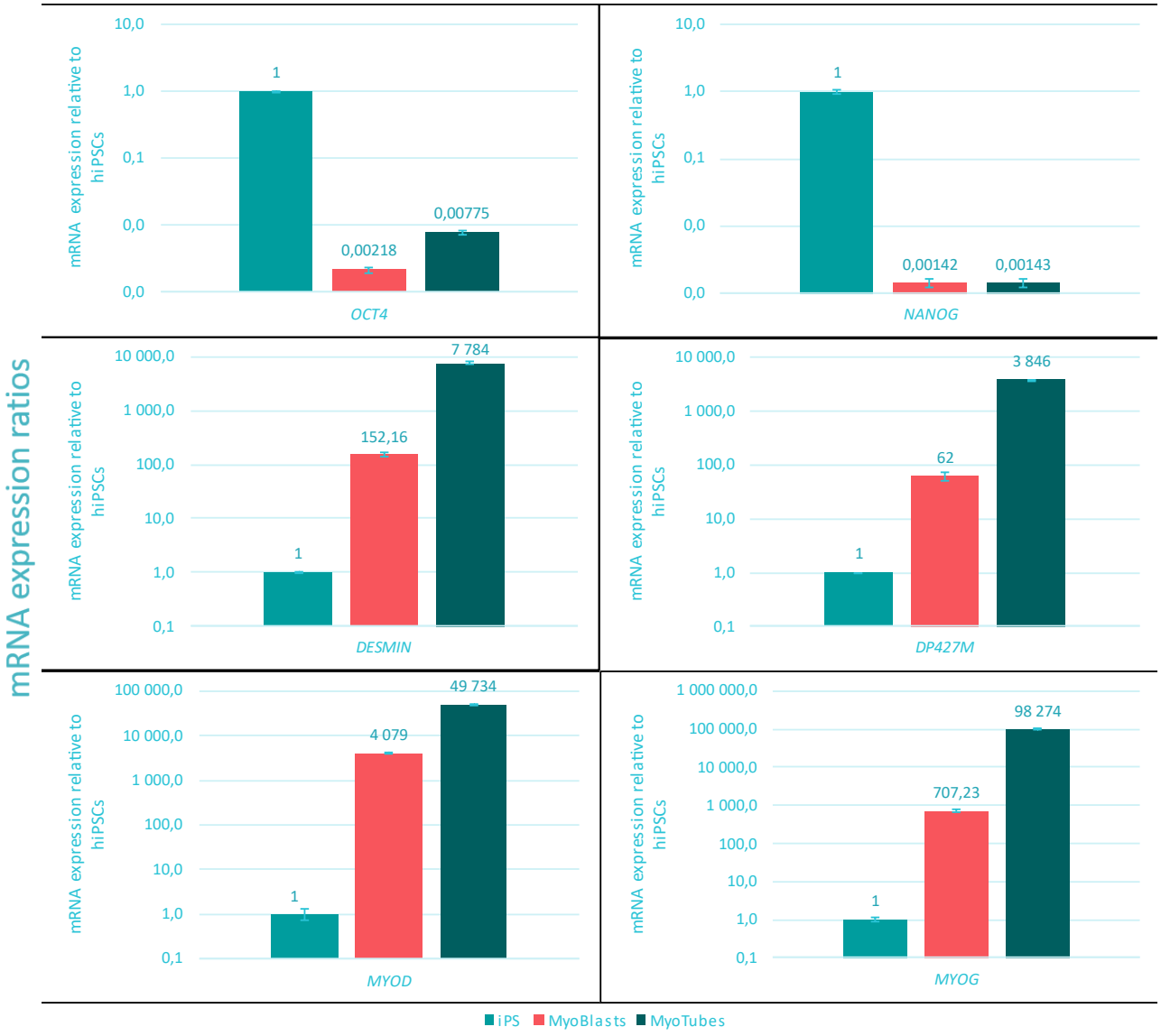

Figure 27: Quantitative PCR results for EDMD-LMNA-3 hiPSC-derived myoblasts and myotubes.

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3.4 Report of DMD1.2 hiPSC-derived myoblasts/myotubes

Table 5: Quality control and production overview for the DMD-hiPSC-derived myoblasts.

	Myoblast		
	Differentiation n°	Production	Delivery
DMD-1.2	N°1	87 000 000 (87 tubes)	20 tubes Inserm
			20 tubes Samsara
			20 tubes Technion
	N°2	130 000 000 (130 tubes)	

Immunofluorescence

DMD-1.2 hiPSC-derived myoblasts and DMD-1.2 hiPSC-derived myotubes show a high expression of Desmin, MF20 and CD56. DMD-1.2 myotube cells express more MYOD and MYOG than DMD-1.2 myoblast cells, as shown in Figure 28.

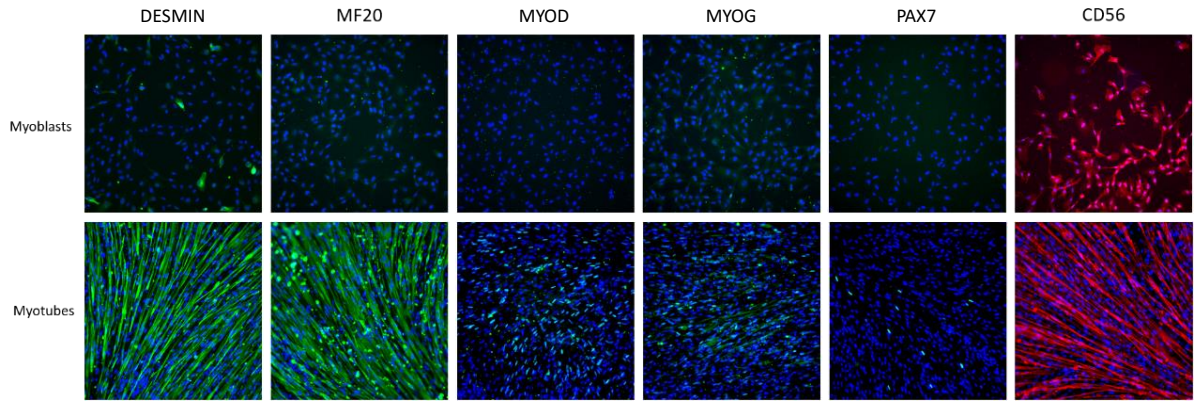

Figure 28: Immunofluorescence Quality Control Images of DMD-1.2 hiPSC-derived myoblast and myotubes.

Flow Cytometry

The flow cytometry analysis in Figure 29 shows that 91.3% of DMD-1.2 hiPSC-derived myoblasts express CD56 and 99.6% express CD82, with 91.0% co-expressing both markers, confirming their myogenic identity.

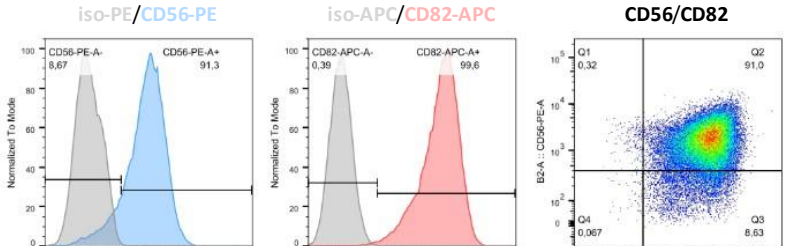

Figure 29: Flow Cytometry Analysis of CD56 and CD82 markers in DMD-1.2 hiPSC-derived (Iso-… = Control antibody for the fluorochrome of interest).

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qPCR Quality Control

Based on the qPCR data shown in Figure 30, our DMD-1.2 hiPSC-derived myoblasts and myotubes do not express pluripotency biomarkers (NANOG, OCT4), indicating they are well differentiated. The high expression levels of DESMIN, MYOD, MYOG, and DP427M confirm that these cells are fully differentiated into myotubes at the final stage.

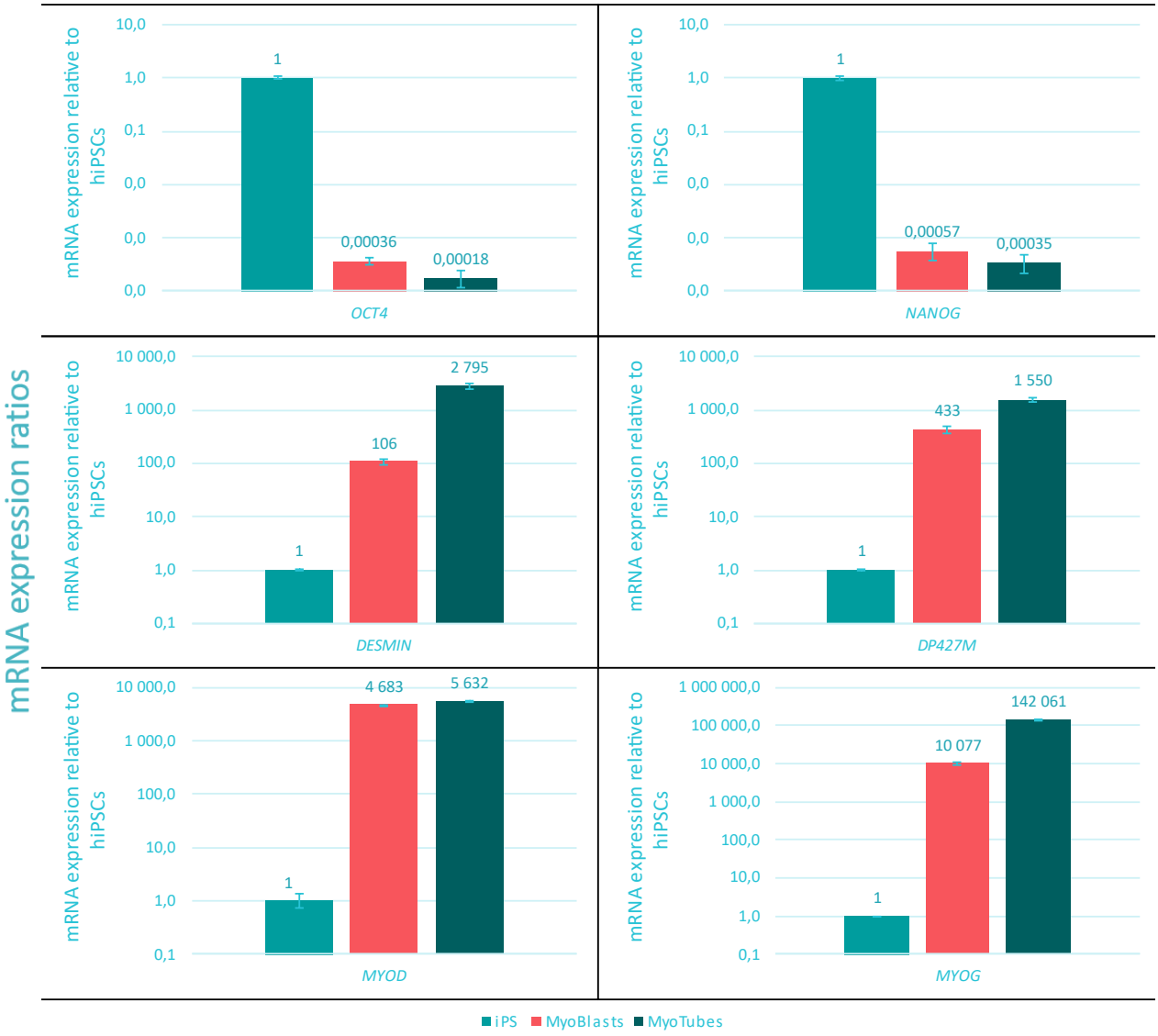

Figure 30: Quantitative PCR Results for DMD-1.2 hiPSC-derived myoblasts and myotubes.

4 SOP protocols

Multiple protocols were developed to ensure the standardisation of cell production, their characterisation and culture for all cell types of the DREAMS project, including hiPSC, Myoblast, and Myotubes. All these SOPs are referenced in Table 1 and included in Annex.

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5 Discussion

To date, most of the samples for the DREAMS consortium have been collected. Out of the 18 cell lines projected to be developed by the consortium, 9 are currently in production, in addition to 4 that are already produced in both hiPSC and myoblast cells. Moreover, at least one cell line for each disease is on its way to being established, except for the Danon disease. The delay in CPP approval and the insufficiency of access to both patients and laboratories specialising in this disease have contributed to this shortfall. To address the absence of Danon disease cell lines, we have organised a service provision that includes sampling the cells of all three patients in July 2024 and reprogramming them in late August, at the iPSC Nantes Platform, under the leadership of Laurent David, a leader in cell reprogramming.

Consequently, we have distributed at least one cell line corresponding to each disease speciality to our collaborators within the DREAMS consortium, enabling them to commence their respective work packages. Comprehensive SOPs for hiPSC culture, reprogramming, and quality control tests, as well as myoblast and myotube cell differentiation, culture, and quality control tests, have been developed by I-Stem and disseminated among all consortium partners. These essential protocols ensure that all collaborators can initiate their research activities and replicate the cell culture expertise that we have developed at I-Stem in their respective laboratories.

We are very optimistic about the progress made for the current deliverable, designated as 1.1. This deliverable lays the foundational groundwork for the subsequent deliverable, 1.2, planned for month 18. With 1.1 on track, we are confident that within one year, all remaining cell lines will be generated.

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Annex A - PBMC Reprogramming using Sendaï Virus

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1 References & prerequisites protocols

1.1 Product references

Table 6. References of different products needed to perform the protocol.

Products	Suppliers	References
Media preparation		
StemPro-34 SFM medium	Life Technologies	10639011
Human SCF Recombinant Protein	Life Technologies	PHC2111
Human Flt-3 Ligand (FLT3L) Recombinant Protein	Life Technologies	PHC9411
Human IL-3 Recombinant Protein	Life Technologies	PHC0035
Human IL-6 Recombinant Protein	Life Technologies	PHC0066
Penicillin-Streptomycin (P/S)	Life Technologies	15140122
L-glutamine (200 mM)	Life Technologies	25030081
Hexadimethrine bromide (Polybrene)	MedChemExpress	HY-112735
StemMACS iPS-Brew XF, human	Miltenyi Biotec	130-104-368
TeSR-E7 Medium for Reprogramming	STEMCELL Technologies	05914
DMEM, high glucose, GlutaMAX Supplement, pyruvate	Gibco	10569010
Fetal Bovine Serum	SIGMA	F4135
Dishes coating		
Gelatin solution	Sigma	G1393
Corning Matrigel hESC-Qualified Matrix, LDEV-free	Corning	354277
DMEM/F-12, GlutaMAX™ Supplement	Gibco	31331028
Reprogramming		
CytoTune-iPS 2.0 Sendai Reprogramming Kit	Invitrogen	A16517
C57BL/6 Mouse Embryonic Fibroblasts, irradiated	Gibco	A34961
D-PBS, no calcium, no magnesium	Life Technologies	14190094

1.2 Prerequisites

The following standard operating procedure concerns the reprogramming of Peripheral Blood Mononuclear Cells (PBMCs) using the *CytoTune-iPS 2.0 Sendai Reprogramming Kit*. As shown in Figure 31, this step takes place upstream of the differentiation process and enables the generation of induced pluripotent stem cells (hiPSC) from patients' PBMCs.

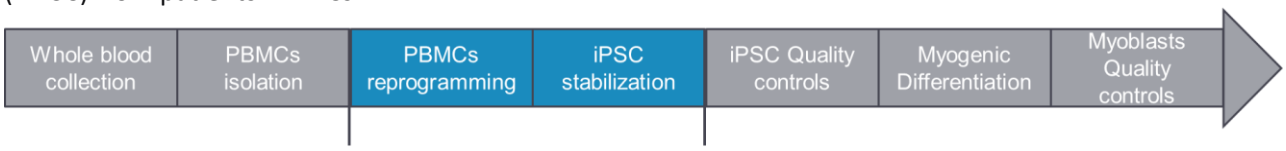

Figure 31. Chronological representation of main steps to generate myoblasts cells from induced pluripotent stem cells (hiPSC). The blue squares indicate the steps that will be described in this protocol.

Before carrying out the reprogramming protocol, PBMCs from fresh patients' whole blood samples must be isolated using the Ficoll-Paque protocol or other isolation techniques such as magnetic separation. It is possible to perform the reprogramming procedure using either fresh PBMCs, directly after their isolation, or frozen PBMCs. Therefore, it is possible to either directly plate de PBMCs on a 12-well plate (see part 3.1.1) or to freeze PBMCs on cryovials containing around 2.10^6 cells in FBS + 10%-DMSO (see part 3.1.2 to perform thawing).

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IMPORTANT: Ensure the use of sterile conditions and techniques at any step while performing this reprogramming process.

1.3 Media preparation

Several culture media are required at different steps of the reprogramming procedure. Make sure to prepare the required media before starting any step of the protocol. The following part will describe their preparation.

StemPro-34 SFM medium preparation

The StemPro-34 SFM medium (Life Technologies, ref: 10639011) is required in the early steps for PBMC growth.

- Thaw the frozen StemPro-34 Nutrient Supplement overnight at 4°C.
- Mix the thawed supplement well by gently inverting the vial several times. Aseptically transfer the entire contents of the vial (13 mL) to the bottle of StemPro-34 SFM. Swirl the bottle to mix and obtain a homogenous complete medium.
- Aseptically add L-Glutamine (Life Technologies, ref: 25030081) to a final concentration of 2 mM (5 mL of 200 mM L-Glutamine to 500 mL of medium).
- Complete StemPro-34 SFM medium (without cytokines) can be stored at 2–8°C for up to 4 weeks.

  NOTE: We recommend preparing aliquots of 45mL of complete StemPro-34 SFM medium and freezing them at -20°C to increase the conservation time. When needed, take out an aliquot from the freezer and store it at 2–8°C.

Cytokines reconstitution

For some steps, StemPro-34 SFM medium must be complemented with several cytokines: Human SCF Recombinant Protein (Life Technologies, ref: PHC2111); Human Flt-3 Ligand Recombinant Protein (Life Technologies, ref: PHC9411); Human IL-3 Recombinant Protein (Life Technologies, ref: PHC0033) and Human IL-6 Recombinant Protein (Life Technologies, ref: PHC0066). Those cytokines are supplied lyophilised and must be reconstituted and aliquoted as described below before use.

- Briefly centrifuge the vials to bring the contents to the bottom.
- Reconstitute the products with sterile demineralised water to a concentration of 100 µg/mL.
- Ensure that all the powder is well solubilised and homogenise the solution performing up and down pipetting.
- Prepare aliquots of 50 µL for SCF and FLT-3.
- Prepare aliquots of 10 µL for IL-3 and IL-6.
- Store at -80°C, thaw aliquot just before use and freeze it again at ≥-20°C.

Hexadimethrine bromide reconstitution

At a specific step, Hexadimethrine bromide (MedChemExpress, ref: HY-112735) (also known as polybrene) must be added to StemPro 34 SFM medium. This compound is supplied lyophilised and must be reconstituted and aliquoted as described below before use.

- Briefly centrifuge the vials to bring the contents to the bottom.

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- Reconstitute the products with sterile demineralised water to a concentration of 4 mg/mL to obtain a 1000X solution.
- Ensure that all the powder is well solubilised and homogenise the solution performing up and down pipetting.
- Prepare aliquots of 50 µL.
- Store at -80°C, thaw aliquot just before use and freeze it again at ≥ -20°C.

PBMC medium preparation

This medium consists of a complete StemPro-34 SFM medium containing the appropriate cytokines and Penicillin-Streptomycin (P/S). To know how to prepare the medium and the cytokines, see parts 1.3.1 and 1.3.2 respectively.

NOTE: This PBMC medium must be prepared on the day it will be used to avoid a decrease in cytokines activities. Do not reuse it the day(s) after.

- If complete StemPro-34 SFM is stored at -20°C (see part 1.3.1), warm it at room temperature.
- In a new 50 mL sterile tube, dispense the desired amount of complete StemPro-34 SFM medium.
- Thaw on ice and add the following cytokines and antibiotics to the complete StemPro-34 SFM medium:
    - **SCF at 0.1 µg/mL** final concentration (see part 1.3.2 to prepare stock tubes at 100 µg/mL).
    - **FTL3-L at 0.1 µg/mL** final concentration (see part 1.3.2 to prepare stock tubes at 100 µg/mL).
    - **IL-6 at 0.02 µg/mL** final concentration (see part 1.3.2 to prepare stock tubes at 100 µg/mL).
    - **IL-3 at 0.02 µg/mL** final concentration (see part 1.3.2 to prepare stock tubes at 100 µg/mL).
    - **Penicillin-Streptomycin** (Life Technologies, ref: 15140122) **at 0.5%** final concentration.
- Homogenise the medium.
- Store the cytokines at -20°C or -80°C.

This medium, supplemented with those cytokines and Penicillin-Streptomycin, is referred to later as "PBMC medium".

MEF medium

The following medium is required for the culture of Mouse Embryonic Fibroblasts (MEF) that are used as feeders for the growth of the hiPSC colonies.

- Transfer to a new 50 mL sterile tube the desired volume of "DMEM, high glucose, GlutaMAX Supplement, pyruvate" (Gibco, ref: 10569010).
- Add Fetal Bovine Serum (Sigma, ref: F4135) to have a final concentration of 10%.
- Mix well and store at 4°C if not used on the same day.
- Before use, it is possible to warm it at 37°C using a water bath.

This medium is referred to later as "MEF medium".

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TeSR-E7 medium preparation

Before the TeSR-E7 medium (STEMCELL Technologies, ref: 05914) can be used, the two kit components need to be mixed according to the following protocol to obtain the complete medium.

- Thaw 25X Supplement at room temperature (15 - 25°C) or at 2 - 8°C just prior to use. Mix thoroughly.
  NOTE: Once thawed, use immediately. Do not re-freeze.
- Add the totality (20 mL) of 25X Supplement to the 480 mL bottle of Basal Medium. Mix thoroughly.
- The medium is ready-to-use now. Use the complete medium within 2 weeks when stored at 2 - 8 °C.
- For longer storage, prepare 45 mL aliquots and store them at -20 °C for up to 1 month. Thaw aliquots of complete medium room temperature or overnight at 2 - 8°C. Once thawed, keep aliquots at 2 - 8 °C and use within 1 week.

iPS-Brew XF medium preparation

Before StemMACS iPS-Brew XF (Miltenyi Biotec, ref: 130-104-368) can be used, the two kit components need to be mixed according to the following protocol to obtain the complete medium.

- Thaw StemMACS iPS-Brew Supplement XF (50×) at 2 - 8 °C prior to use.
- To obtain the complete medium, add 10 mL StemMACS iPSBrew Supplement XF (50×) to 500 mL StemMACS iPS-Brew Basal Medium XF. Mix well.
- The medium is ready-to-use now. Use the complete medium within 2 weeks when stored at 2 - 8 °C.
- For longer storage, prepare 45 mL aliquots and store at -20 °C for up to 2 months. Thaw aliquots of complete medium room temperature or overnight at 2 - 8°C. Once thawed, keep aliquots at 2 - 8 °C and use within 2 weeks.

1.4 Dishes coating

Some dishes used for cell culture must be coated with either gelatin or Matrigel. Refer to Table 7 to see the amount of volume needed to cover the dishes depending on their type.

Table 7. Culture surfaces and cover volumes per cell culture dish.

Cell Culture Support	Culture Surface (cm^2)	Volume to add per dish
Center-Well Organ Cell (OC)	2.89	0.5 mL
35 mm dish (B3)	9.6	1 mL
60 mm dish (B6)	22.1	2 mL

Gelatin coating

GELATIN 0.1% PREPARATION

- Using a water bath set at 37°C, warm the bottle of gelatin solution (Sigma, ref: G1393) until it becomes liquid.
- Then, add 25 mL of this gelatin solution into a new 500 mL bottle of D-PBS (Life Technologies, ref: 14190094). Mix thoroughly to obtain a solution of D-PBS with 0.1% gelatin.
- Store this new solution and the gelatin bottle at 4°C.

COATING DISHES WITH GELATIN 0.1%

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- Take out the D-PBS-0.1% gelatin from the fridge (see part 1.4.1.1), and the dishes to coat.
- Dispense the appropriate volume of D-PBS-0.1% gelatin depending on the dish type (see Table 7) to cover all the surfaces.
- Incubate the dishes at room temperature for at least 15 minutes.
    - If used directly after 15 minutes, aspirate the remaining liquid just before adding the cell medium. Do not leave the gelatin-coated surface without liquid, it will dry!
    - If not used directly, seal the coated dishes with parafilm, and store them at 4°C for up to 10 days.

Matrigel coating

MATRIGEL ALIQUOTING

Warning: Matrigel sets very quickly if it is not cold. **Always work on ice!**

- Thaw the bottle of Corning Matrigel hESC-Qualified Matrix, LDEV-free, 5mL (Corning, ref: 354277) in a tray filled with ice at 4°C overnight.
- Place sterile tips and sterile Eppendorf for aliquoting at -20°C overnight.
- On Corning's website, click on ''quality certificate". Enter the product reference and batch number of your Matrigel's bottle and click on "Download certificate". In this certificate, it is indicated the "Dilution factor", indicates the volume of Matrigel aliquot.

    NOTE 1: With one aliquot, prepare 25 mL of Matrigel suspension in DMEM/F-12, GlutaMAX™ Supplement (Gibco, ref: 31331028).

    NOTE 2: Adapt the volume of the aliquot according to the volume of media to resuspend. We usually resuspend 1 Matrigel aliquot in 12.5 mL of DMEM/F-12, GlutaMax Supplement to avoid Matrigel wasting. In consequence, we aliquot half of the volume indicated on the ''Quality Certificate''.
- Before aliquoting, annotate sterile pre-cooled Eppendorf with ''*Matrigel*'' and the date of the day.
- Keep Matrigel on ice and place pre-annotated Eppendorf on ice. Add the appropriate volume of Matrigel per aliquot tube using cold tips.
- Immediately store aliquots of Matrigel at -20°C.

COATING DISHES WITH MATRIGEL

For the following steps, DMEM/F-12, GlutaMAX Supplement Medium (Gibco, ref: 31331028) and aliquoted Corning Matrigel hESC-Qualified Matrix, LDEV-free, 5 mL (Corning, ref: 354277) (See part 1.4.2.1) is required.

- Thaw the desired number of Matrigel aliquots on the ice depending on the number and type of culture support to coat (See Table 7).
- Prepare a sterile 50 mL tube, keep it on ice, and add the appropriate volume of cold DMEM/F-12, Glutamax Supplement depending on the number and size of Matrigel aliquots to use.
- With a 200 µL pipette take 100 µL of DMEM/F-12, GlutaMAX Supplement medium from the tube and practice up and down in the frozen aliquot of Matrigel to thaw it gently.
- Add the entire thawed aliquot of Matrigel to the tube with DMEM/F-12, GlutaMAX Supplement medium.
- Homogenise the solution.

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- Dispense the desired volume of DMEM/F-12 + Matrigel solution on the cell culture support according to volumes presented in Table 7.
- Ensure that the DMEM/F-12 + Matrigel solution is covering the entire surface of the cell culture support.
- Write "Matrigel" and the date of coating on the support.
- Incubate the dishes at room temperature for at least 1 hour to allow the Matrigel to polymerise:
    - If used directly after the polymerisation hour, aspirate the remaining liquid right before adding the cell medium. Do not leave the Matrigel-coated surface without liquid, it will dry!
    - If not used directly after the hour of polymerisation, seal the coated supports with parafilm, and store them at 4°C for up to 10 days.

1.5 CytoTune-iPS 2.0 Sendai Reprogramming Kit Preparation

The Cytotune-iPS 2.0 Sendai Reprogramming kit (Invitrogen, ref: A16517) contains three different tubes for three different reprogramming vectors (KOS, hc-Myc and hKlf4) of 100 µL each. For successful reprogramming, cells must be transduced using all three reprogramming vectors to provide the four Yamanaka factors to the cells (Oct4, Sox2, Klf4 and c-Myc). Thus, vectors must be mixed altogether following a MOI = 5-5-3 (KOS MOI=5, hc-Myc MOI=5, hKlf4 MOI=3), and aliquoted:
The titer of each CytoTune-2.0 Sendai reprogramming vector is lot-dependent. For the specific titer of your vectors, refer to the Certificate of Analysis (CoA) available on the Invitrogen website. Go to *thermofisher.com/cytotune* and search for the CoA by product lot number, which is printed on the vial. Avoid re-freezing and thawing of the reprogramming vectors since viral titers can decrease dramatically with each freeze/thaw cycle.

Use the specific titer of the vectors to calculate the volume of each vector needed to reach the target MOI (KOS MOI=5, hc-Myc MOI=5, hKlf4 MOI=3), considering several 200,000 cells to transduce. To do so, use the following formula:

$$\frac{MOI\ (CIU/cell) \times number\ of\ cells}{Titer\ of\ virus\ (CIU/cell)\ \times 10^{-3}}$$

Once the calculation is done (see example below), prepare an aliquot of Sendai virus containing all three vectors each, in sufficient quantity to transduce 200 000 cells:

- Using sterile technics, remove CytoTune-2.0 Sendai tubes from the -80°C storage.
- Thaw each tube one at a time by first immersing the bottom of the tube in a 37°C water bath for 5–10 seconds, and then removing the tube from the water bath and allowing it to thaw at room temperature. Once thawed, briefly centrifuge the tube and place it immediately on ice.
- Knowing that each tube contains 100 µL of vectors, dispense the calculated volumes of each of the three CytoTune-2.0 Sendai tubes into new sterile 0.5 mL tubes to obtain the maximum number of aliquots containing the appropriate volume of each vector.
- Store the Sendai virus aliquot (and what remains in the kit tubes) at -80°C.

<u>Example:</u> If the CoA corresponding to your CytoTune-2.0 Sendai reprogramming vector batch indicates the viral titers shown in Table 8, here is how to proceed to prepare aliquots with sufficient volume to transduce 200 000 cells respecting the target MOI5.5.3 (KOS MOI=5, hc-Myc MOI=5, hKlf4 MOI=3):

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Table 8. Example of viral titer of each CytoTune-2.0 Sendai reprogramming vector shown in the CoA of the Cytotune-iPS 2.0 Sendai Reprogramming kit. This information is required to calculate the volume of each vector to use to efficiently transduce cells.

Component	Titer* (CIU/mL)
CytoTune™ 2.0 hKOS	$1.5x10^8$ CIU/mL
CytoTune™ 2.0 hc-Myc	$1.5x10^8$ CIU/mL
CytoTune™ 2.0 hKlf4	$1.5x10^8$ CIU/mL

- *Volume to add for MOI=5 (For CytoTune 2.0 KOS and hc-Myc vectors) :*

$$\frac{MOI\ (CIU/cell) \times number\ of\ cells}{Titer\ of\ virus\ (CIU/cell)\ \times 10^{-3}} = \frac{5\ \times 200\ 000}{1.5 \times 10^8 \times 10^{-3}} = \frac{1\ 000\ 000}{150\ 000} = 6.67\ \mu L$$

- *Volume to add for MOI=3 (For CytoTune 2.0 hKlf4 vector) :*

$$\frac{MOI\ (CIU/cell) \times number\ of\ cells}{Titer\ of\ virus\ (CIU/cell)\ \times 10^{-3}} = \frac{3\ \times 200\ 000}{1.5 \times 10^8 \times 10^{-3}} = \frac{600\ 000}{150\ 000} = 4\ \mu L$$

- *Aliquots preparation:*

  Regarding the previous calculations, each aliquot must contain 6.67 µL of ***CytoTune-2.0 hKOS*** *and* ***CytoTune-2.0 hc-Myc*** *vectors and 4 µL of* ***CytoTune-2.0 hKlf4*** *vector. One aliquot can be used to transduce 200,000 cells. As the volume of vector per tube from the kit is 100 µL, prepare 15 aliquots (100 µL/6.67 µL = 15).*

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2 Protocol overview and principle

2.1 Protocol Overview

Reprogramming timeline

The major steps required for reprogramming peripheral blood mononuclear cells (PBMCs) using the CytoTune-iPS 2.0 Sendai Reprogramming Kit to generate hiPSCs cultured on MEF feeder-cells are shown below in Table 9. Note that the timeline is provided as a guideline for experimental planning; the actual timeline can vary based on the cell type and experimental conditions.

Table 9. Timeline representing major steps required for reprogramming PBMC using the Cytotune 2.0 Sendai Reprogramming Kit.

Temporality	Action
Day -4	PBMC seeding (either fresh or frozen)
Day -3	Add fresh medium
Day -2 and Day -1	Renew half of the medium
Day 0	Cells transduction with Sendaï virus (MOI = 5-5-3)
Day +1	Replace the whole medium and virus elimination
Day +2	Feeder cells preparation
Day +3	PBMCs transfer on feeders
Day +4	Renew half of the medium
Day +6	Renew half of the medium
Day +7	Transition from StemPro to TeSR E7 medium
Days +8, +10, +12	Replace the whole medium (E7), emergence of colonies, then transition to iPS Brew medium
Day -1 before P1	Feeder cells preparation
P1	Passage of emerging colonies
P1 to at least P10	hiPSC expansion and stabilisation

Principle of Cytotune-iPS 2.0 Sendai Reprogramming Kit

The goal of this system is to reprogram somatic cells, to obtain induced pluripotent stem cells which exhibit the same properties as embryonic stem cells. To do so, it uses vectors based on a modified, non-transmissible form of Sendai virus (SeV) to deliver and express key genetic factors necessary for reprogramming somatic cells into hiPSCs. Sendai virus is an enveloped virus of 150–250 nm in diameter whose genome is a single chain RNA in the minus sense. It infects cells by attaching itself to the sialic acid receptors. Interestingly, this reprogramming system uses vectors that are non-integrating and remain in the cytoplasm. The system uses a combination of three Sendai vectors which include the four Yamanaka factors: *OCT3/4*, *SOX2*, *KLF4*, and *C-MYC* (see Table 10).

Table 10. Presentation of the three Sendai viruses used in the CytoTune 2.0 Sendai Reprogramming kit, and their included Yamanaka factors.

CytoTune Sendai vector	Factors
CytoTune™ 2.0 *KOS*	Human *KLF4* Human *OCT3/4* Human *SOX2*
CytoTune™ 2.0 h*C-MYC*	Human *C-MYC*
CytoTune™ 2.0 h*KLF4*	Human *KLF4*

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3 Procedure for reprogramming PBMCs using the CytoTune-iPS 2.0 Sendai Reprogramming Kit

3.1 Day -4: PBMC Seeding

It is possible to perform the reprogramming procedure using either fresh PBMCs, directly after their isolation, or frozen PBMCs in FBS + 10%-DMSO.
For the following steps, freshly prepare PBMC medium (see part 1.3.4). This medium must be prepared the day it will be used to avoid a decrease in cytokines activities.
See Figure 32 to check PBMCs morphology in the well, after seeding.

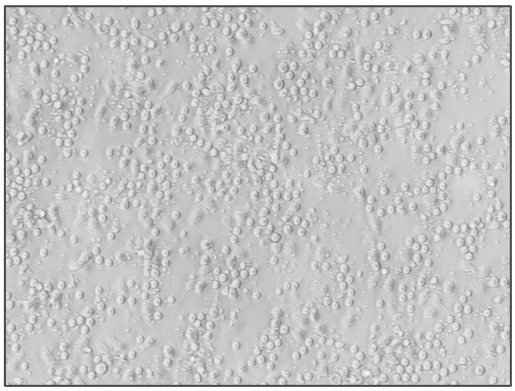

Figure 32. Morphology of PBMCs just after seeding in a 12-well plate at 1,000,000 cells/mL, corresponding to day -4. Magnification x200 - Image modification : Contrast +40%.

Seeding of fresh PBMCs

If working with fresh PBMCs, plate them just after their isolation using Ficoll-Paque or other isolation methods.

- Make sure to prepare enough quantity (allow 2 mL/well) of freshly prepared PBMC medium (see part 1.3.4 for recipe) and warm it at 37°C.
- Determine the viable cell count of your freshly isolated PBMCs using your method of choice (e.g., Countess Automated Cell Counter).
- Calculate the volume of cell suspension needed to collect 2 000 000 PBMCs:

$$\left(\frac{cell\ count\ (cells/mL)}{2\ 000\ 000\ (cells)} = volume\ to\ collect\ to\ have\ 2\ 000\ 000\ PBMCs\ (mL)\right)$$

- Dispense this volume in a new 15mL sterile tube.
- Centrifuge the tube at 200 x g for 5 min.
- Aspirate the supernatant without disturbing the pellet.
- Resuspend the cell pellet with 2 mL of pre-warmed at 37°C PBMC medium.
- Transfer that 2 mL of PBMC suspension to one well of a new 12-well plate.
- Move the plates in several quick, short, back-and-forth and side-to-side motions to ensure uniform distribution of cells.
- Incubate the cells at 37°C, 5% CO_2.

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Seeding of frozen PBMCs

- Make sure to prepare enough quantity (allow at least 10 mL for thawing and 2 mL/well) of freshly prepared PBMC medium (see part 1.3.4 for recipe) and warm it at 37°C.
- In a new 15 mL sterile tube, dispense 5 mL of Complete StemPro-34 SFM medium (see part 1.3.1) and warm it at 37°C.
- Remove the vial of PBMCs from liquid nitrogen storage. Thaw the vial quickly in a 37°C water bath. When only a small ice cube remains in the vial, remove it from the water bath.
- Gently transfer the PBMCs into the 15 mL sterile tube containing pre-warmed Complete StemPro-34 SFM medium.
- Centrifuge the cells at 200 x g for 10 minutes.
- Aspirate the supernatant without disturbing the pellet.
- Resuspend the pellet with 1 to 5 mL of PBMC medium depending on the pellet size.
- Determine the viable cell count using your method of choice (e.g., Countess Automated Cell Counter).
- Adjust the volume by adding PBMC medium to have 1 000 000 cells/mL.
    - If the concentration is inferior to 1 000 000 cells/mL, then centrifuge at 200 x g for 5 min, aspirate the supernatant and resuspend the pellet with the appropriate volume of PBMC medium to have 1 000 000 cells/mL.
- Dispense 2 mL of cell suspension per well of a new 12-well plate (=2 000 000 cells/well).
- Move the plates in several quick, short, back-and-forth and side-to-side motions to ensure uniform distribution of cells.
- Incubate the cells at 37°C, 5% CO_2.

3.2 Day -3: Add 1 mL of fresh medium

- Make sure to prepare enough quantity (allow at least 1 mL/well) of freshly prepared PBMC medium (see part 1.3.4 for the recipe). Warm it at room temperature.
- Observe the cells under a microscope and optionally take a picture to follow the cell's evolution (See Figure 33).
- Gently add 1 mL of PBMC medium per well.
- Incubate the cells at 37°C, 5% CO_2.

NOTE: Some cell death is generally observed the first day after the thaw. Some cells may adhere to the surface of the culture plate. Proceed with the cells in suspension and leave behind any attached cells. Cells will not proliferate but should maintain stable cell numbers for the first few days.

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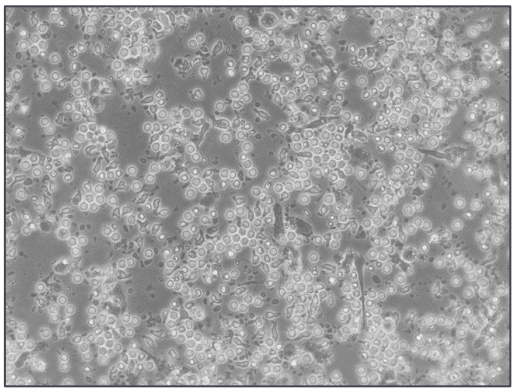

Figure 33. Morphology of PBMCs at day -3. Magnification x200 - Image modification : Contrast : +40%

3.3 Day -2: Renew half of the medium

⚠ As PBMCs are non-adherent cells, DO NOT use an aspiration system. ⚠

- Make sure to prepare enough quantity (allow at least 1.5 mL/well) of freshly prepared PBMC medium (see part 1.3.4 for the recipe). Warm it at room temperature.
- Observe the cells under a microscope and optionally take a picture to follow the cell's evolution (See Figure 34).
- Leave the plate under the laminar flow hood for 5-10 minutes to ensure that the cells sediment at the bottom of the well.
- Using a P1000 pipette, gently remove half of the medium (1.5 mL) by remaining on the surface to avoid aspirating cells.
- Gently add the same volume (1.5 mL) of freshly prepared PBMC medium.

NOTE: If cells are present in 0.5 mL removed from the wells, centrifuge the cell suspension at 200 × g for 10 minutes, discard the supernatant, and resuspend the cells in 0.5 mL fresh PBMC medium before adding them back to the plate.

- Incubate the cells at 37°C, 5% CO_2.

Figure 34. Morphology of PBMCs at day -2. There are apparitions of cell clusters. Magnification x40.

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3.4 Day -1: Renew half of the medium

⚠ As PBMCs are non-adherent cells, DO NOT use an aspiration system. ⚠

- Make sure to prepare enough quantity (allow at least 1.5 mL/well) of freshly prepared PBMC medium (see part 1.3.4 for the recipe). Warm it at room temperature.
- Observe the cells under a microscope and optionally take a picture to follow the cell's evolution.
- Leave the plate under the laminar flow hood for 5-10 minutes to ensure that the cells sediment at the bottom of the well.
- Using a P1000 pipette, gently remove half of the medium (1.5 mL) by remaining on the surface to avoid aspirating cells.
- Gently add the same volume (1.5 mL) of freshly prepared PBMC medium.

  NOTE: If cells are present in 0.5 mL removed from the wells, centrifuge the cell suspension at 200×g for 10 minutes, discard the supernatant, and resuspend the cells in 0.5 mL fresh PBMC medium before adding them back to the plate.
- Incubate the cells at 37°C, 5% CO_2.

3.5 Day 0: Transduction

- Make sure to prepare enough quantity (allow at least 1.5 mL/well) of freshly prepared PBMC medium (see part 1.3.4 for the recipe). Warm it at room temperature.
- Make sure to prepare enough quantity (allow at least 0.5 mL per transduction) of freshly prepared PBMC medium (see part 1.3.4 for recipe) **supplemented with 4 µg/mL of hexadimethrine bromide** (see part 1.3.3 to prepare it). Warm it at room temperature.
- For this step, use the Cytotune-iPS 2.0 Sendai Reprogramming kit (Invitrogen, ref: A16517. Please refer to part 1.5 of this SOP to prepare the Sendai virus with the right MOI (5-5-3), aliquoted to transduce 200 000 cells.
- Observe the cells under a microscope and optionally take a picture to follow the cell's evolution (See Fig.6).
- Under the laminar flux hood, flush the well 3-4 times using a P1000 pipette and transfer all the cells from the 12-well plate to a new 15 mL sterile tube.
- Centrifuge the cells at 200 x g for 10 minutes.
- Aspirate the supernatant without disturbing the pellet.
- Resuspend the pellet with 1 mL of freshly prepared PBMC medium.
- Determine the viable cell count using your method of choice (e.g., Countess Automated Cell Counter).
- Calculate the volume of cell suspension needed to have 200 000 PBMCs:

$$\left(\frac{cell\ count\ (cells/mL)}{2\ 000\ 000\ (cells)} = volume\ to\ collect\ to\ have\ 200\ 000\ PBMCs\ (mL)\right)$$

- Plate 200 000 PBMCs in one well of a new 24-well plate. Add PBMC medium to adjust at 0.5 mL of medium in the well.
- Move the plates in several quick, short, back-and-forth and side-to-side motions to ensure uniform distribution of cells.

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⚠ For handle viral compounds, please make sure to apply all the safety measures involved. Wear two pairs of gloves; use an appropriate laminar flux hood, and make sure to bleach all consumables in contact with the virus for at least 24 hours. ⚠

- Take out of the -80°C one aliquot of Sendai Virus (prepared to transduce 200 000 cells, see part 1.5 to prepare it). Thaw it on ice.
- Transfer the virus to a 1.5 mL sterile tube containing enough freshly prepared PBMC medium + hexadimethrine **bromide (4 µg/mL) to have a final volume of 0.5 mL.** Homogenise well using a P1000 pipette.
- Gently add 0.5 mL of this mix to the 200 000 plated cells in the 24-well plate. Avoid bubbles.
- Move the plates in several quick, short, back-and-forth and side-to-side motions to ensure uniform distribution of cells.
- Incubate the cells at 37°C, 5% CO_2 for 24 hours.
- Prepare a dry pellet with the reaming cells: centrifuge at 200 x g for 5 min, remove all the supernatant and freeze the cell pallet at -20°C.

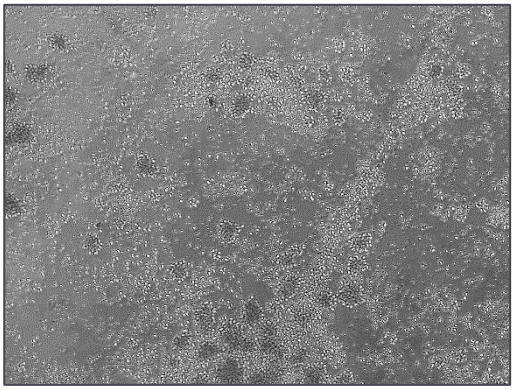

Figure 36: Morphology of PBMCs at day 0 before collecting the cells. Magnification x40.

Figure 35: Morphology of the 200 000 PBMCs seeded in a 24-well plate after Sendai transduction. Magnification x40.

3.6 Day +1: Replace the whole medium

⚠ For handle viral compounds, please make sure to apply all the safety measures involved. Wear two pairs of gloves; use an appropriate laminar flux hood, and make sure to bleach all consumables in contact with the virus for at least 24 hours. ⚠

- Make sure to prepare enough quantity (allow at least 1 mL/well) of freshly prepared PBMC medium (see part 1.3.4 for the recipe). Warm it at room temperature.
- Observe the cells under a microscope and optionally take a picture to follow the cell's evolution.
- Flush and transfer all the transduced cells to a new 15 mL sterile tube using a P1000 pipette.
- Wash the well with 1 mL of Complete StemPro-34 SFM medium (without cytokines). Add it to the same 15 mL tube.
- Centrifuge at 200 x g for 10 minutes.

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- Remove the supernatant using a P1000 and trash it to the bleach.
- Resuspend the pellet with 1 mL of PBMC medium and plate the cells in one well of a new 24-well plate.
- Move the plates in several quick, short, back-and-forth and side-to-side motions to ensure uniform distribution of cells.
- Incubate the cells at 37°C, 5% CO_2.

3.7 Day +2: MEF (feeders) seeding

Prepare 2 B3 dishes per well of reprogrammed cells.

- First, coat the B3 dishes with gelatin 0.1%. To do so, please refer to part 1.4.1.2.
- Prepare an aliquot of the needed volume of MEF medium (See part 1.3.5 for recipe) + P/S-0.5%.
- In a new 15 mL sterile tube, dispense 5 mL of MEF medium + P/S-0.5% and warm it at 37°C.
- Remove the vial of C57BL/6 Mouse Embryonic Fibroblasts, irradiated (Gibco, ref: A34961) from liquid nitrogen storage. Thaw the vial quickly in a 37°C water bath. When only a small ice cube remains in the vial, remove it from the water bath.
- Gently transfer the MEF into the 15 mL sterile tube containing the MEF medium.
- Centrifuge the cells at 200 x g for 5 minutes.
- Aspirate the supernatant without disturbing the pellet.
- Resuspend the pellet with 1 to 4 mL of MEF medium + P/S-0.5%.
- Determine the viable cell count using your method of choice (e.g., Countess Automated Cell Counter).
- Adjust the volume by adding MEF medium + P/S-0.5% to have 1 000 000 cells/mL.
    - If the concentration is inferior to 1 000 000 cells/mL, then centrifuge at 200 x g for 5 minutes, aspirate the supernatant and resuspend the pellet with the appropriate volume of MEF medium to have 1 000 000 cells/mL.
- Depending on the viable cell count, determine the volume of MEF to dispense in each B3 dish to have 25 000 cells/cm².

Example: For an initial concentration of 2 000 000 MEF/mL:

$$\left(\frac{9.6\ (cm^2)\ \times 25\ 000\ (cells)}{2\ 000\ 000\ (cells/mL)} = 120\ \mu L\ (volume\ to\ dispense\ to\ have\ 25\ 000\ MEF/cm^2)\right)$$

- Aspirate gelatin coating from the B3 dishes to use.
- Add 2 mL of MEF medium + P/S-0.5%.
- Add the determined volume of MEF suspension to seed 25 000 MEF/cm² in each B3 dish.
- Move the dishes in several quick, short, back-and-forth and side-to-side motions to ensure uniform distribution of cells.
- Incubate the cells at 37°C, 5% CO_2. (See Figure 37).

3.8 Day +3: PBMCs transfer on MEF feeder cells

- Take out of the incubator the plate from the incubator.
- Flush 2-3 times with a P1000 pipette and transfer all the cells to a new 15 mL sterile tube.

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- Wash the well with 1 mL of Complete StemPro-34 SFM medium + P/S-0.5% (without cytokines). Add it to the same 15 mL tube.
- Complete the 15 mL tube to 1.5 mL/B3 dishes (= 3 mL if 2 B3 per well) with complete StemPro-34 SFM medium + P/S-0.5%.
- Take out of the incubator the B3 dishes with seeded MEF.
- Aspirate the MEF medium from those B3 dishes.
- Dispense 1.5 mL of PBMCs in each B3 dish.
- Move the dishes in several quick, short, back-and-forth and side-to-side motions to ensure uniform distribution of cells.
- Incubate the cells at 37°C, 5% CO_2. (See Figure 38).

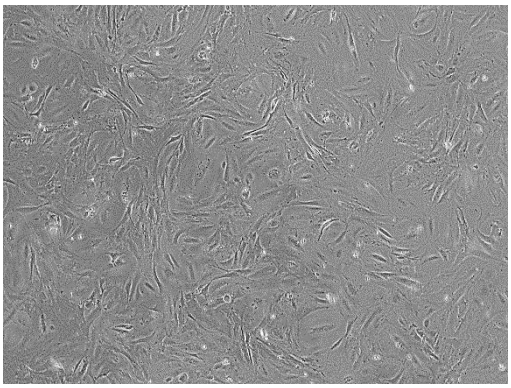

Figure 37. Picture of MEF 24h after seeded on a B3 dishes

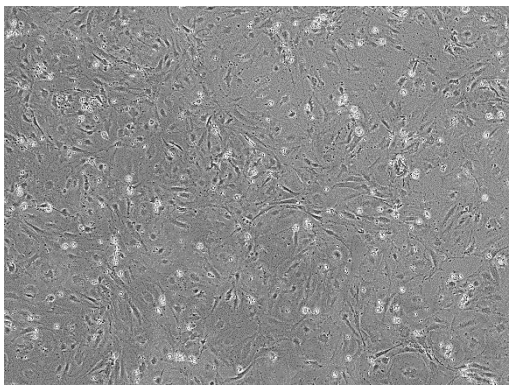

Figure 38. Picture of transfected PBMCs after transfer on MEF

3.9 Day +4: Renew half of the medium

⚠ As PBMCs are non-adherent cells, DO NOT use an aspiration system. ⚠

- Using a P1000 pipette, gently remove 750 µL of medium from the B3 dishes by remaining on the surface to avoid aspirating cells. Do not tilt the dish.
- Prepare an aliquot of the needed volume of Complete StemPro-34 SFM medium + P/S-0.5%.
- Add 750 µL of Complete StemPro-34 SFM medium + P/S-0.5%.
- Incubate the cells at 37°C, 5% CO_2.

3.10 Day +6: Renew half of the medium

⚠ As PBMCs are non-adherent cells, DO NOT use an aspiration system. ⚠

- Prepare an aliquot of the needed volume of Complete StemPro-34 SFM medium + P/S-0.5%.
- Using a P1000 pipette, gently remove 750 µL of medium from the B3 dishes by remaining on the surface to avoid aspirating cells. Do not tilt the dish.
- Add 750 µL of Complete StemPro-34 SFM medium + P/S-0.5%.
- Incubate the cells at 37°C, 5% CO_2.

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3.11 Day +7: Renew half of the medium with Complete TeSR E7 medium

⚠ As PBMCs are non-adherent cells, DO NOT use an aspiration system. ⚠

- At this step, remove half of StemPro-34 SFM medium and replace it by complete TeSR E7 medium. This allow to do a transition between StemPro-34 SFM medium and TeSR E7 medium without disturbing the cells.
- Prepare an aliquot of the needed volume of Complete TeSR E7 medium (See part 1.3.6 for recipe) + P/S-0.5%.
- Using a P1000 pipette, gently remove 750 µL of medium from the B3 dishes by remaining on the surface to avoid aspirating cells. Do not tilt the dish.
- Add 750 µL of Complete TeSR E7 medium + P/S-0.5%.
- Incubate the cells at 37°C, 5% CO_2.

3.12 Day +8: Replace the whole medium with Complete TeSR E7 medium

- Prepare an aliquot of the needed volume of Complete TeSR E7 medium + P/S-0.5%.
- Aspirate and discard the entire medium in the B3 dishes.
- Add 1.5 mL of Complete TeSR E7 medium + P/S-0.5% in each B3 dishes.
- Incubate the cells at 37°C, 5% CO_2.
- Change the medium this way every 2 days.

NOTE: Complete TeSR E7 medium is used for a maximum of 7 days to allow hiPSC colonies to appear. Above those 7 days, use Complete StemMACS iPS-Brew XF medium (See part 1.3.7 for the recipe).

NOTE: Since around day +10, It must appear emerging hiPSC colonies under a microscope and monitor their growth. To follow their evolution, we highly recommend to take a picture of each colony every day. (See Figure 39).

/!\ From day 10, if there are clones emergence, please follow part 3.13. If not, continue with step 3.12 until clones emergence and for 7 days maximum.
For the following step, timeline is indicative. It can vary between reprogramming. Here, it indicates day+12 but clones can emerge at day+11 or day+14 depending of the experiment.

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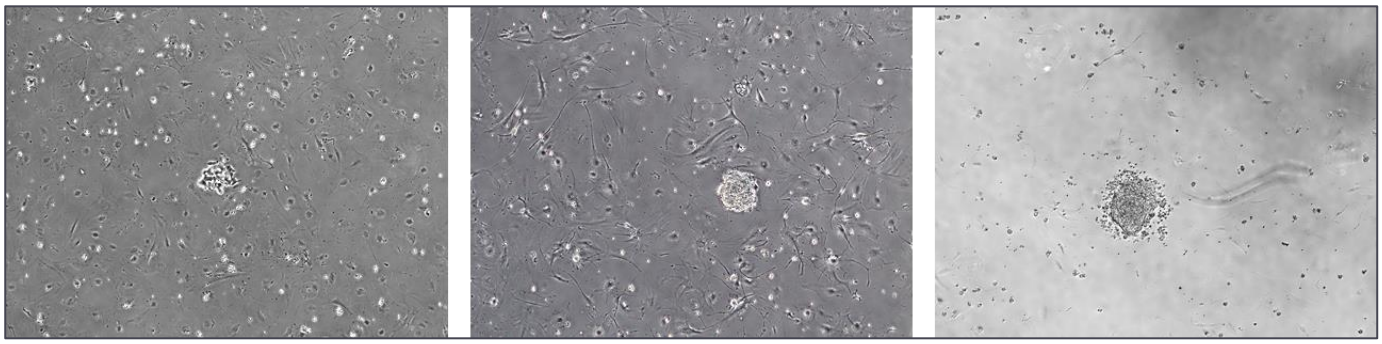

Figure 39. Examples of emerging hiPSC colonies. Magnification x40

3.13 Day +12: Renew half of the medium with Complete StemMACS iPS-Brew XF medium

- Prepare an aliquot of the needed volume of Complete StemMACS iPS-Brew XF medium (See part 1.3.7 for recipe) + P/S-0.5%.
- Using a P1000 pipette, gently remove 750 µL of medium from the B3 dishes by remaining on the surface to avoid aspirating cells.
- Add 750 µL of Complete StemMACS iPS-Brew XF medium + P/S-0.5%.
- Incubate the cells at 37°C, 5% CO_2.

3.14 Day +13: Replace the whole medium with Complete StemMACS iPS-Brew XF medium

- Prepare an aliquot of the needed volume of Complete StemMACS iPS-Brew XF medium + P/S-0.5%.
- Aspirate and discard the entire medium in the B3 dishes.
- Add 1.5 mL of Complete StemMACS iPS-Brew XF medium + P/S-0.5% in each B3 dishes.
- Incubate the cells at 37°C, 5% CO_2.
- Change the medium this way every 2 days.

3.15 Day -1 before P1: MEF (feeders) seeding

- First, coat Center-well organ cell (OC) and B3 dishes with gelatin 0.1%. To do so, please refer to part 1.4.1.2. Choose the number of dishes to coat and seed with MEF depending on the number of hiPSC colonies that appeared.
- In a new 15 mL sterile tube, dispense 5 mL of MEF medium (see part 1.3.5 for recipe) and warm it at 37°C.
- Remove the vial of C57BL/6 Mouse Embryonic Fibroblasts, irradiated (Gibco, ref: A34961) from liquid nitrogen storage. Thaw the vial quickly in a 37°C water bath. When only a small ice cube remains in the vial, remove it from the water bath.
- Gently transfer the MEF into the 15 mL sterile tube containing the MEF medium.
- Centrifuge the cells at 200 x g for 5 minutes.

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- Aspirate the supernatant without disturbing the pellet.
- Resuspend the pellet with 1 mL of MEF medium.
- Determine the viable cell count using your method of choice (e.g., Countess Automated Cell Counter).
- Depending on the viable cell count, determine the volume of MEF to dispense in each OC or B3 dish to have 25 000 cells/cm².

Example: for an initial concentration of 2 000 000 MEF/mL:

Volume for B3 dish: $\left(\frac{\mathbf{9.6}\ (cm^2) \times 25\ 000\ (cells)}{2\ 000\ 000\ (cells/mL)} = 120\ \mu L\ (volume\ to\ dispense\ to\ have\ 25\ 000\ MEF/cm^2)\right)$

Volume for OC dish: $\left(\frac{\mathbf{2.89}\ (cm^2) \times 25\ 000\ (cells)}{2\ 000\ 000\ (cells/mL)} = 36\ \mu L\ (volume\ to\ dispense\ to\ have\ 25\ 000\ MEF/cm^2)\right)$

- Aspirate gelatin coating from OC and B3 dishes to use.
- Add 2 mL of MEF medium in B3 dishes and 0.5 mL in OC dishes.
- Add the determined volume of MEF suspension to seed 25 000 MEF/cm² in each dish.
- Add 2 mL of PBS on the edge of OC dishes.
- Move the dishes in several quick, short, back-and-forth and side-to-side motions to ensure uniform distribution of cells.
- Incubate the cells at 37°C, 5% CO_2.

3.16 P1: Passage of emerging clones

Passage on OC feeders and OC feeders free

Around day +10, there will be emerging hiPSC colonies under a microscope. Here, we consider that one colony is one clone.
Assign a number to emerging clones and monitor their growth. Once a clone is large enough, it will need to be passed in an OC dish containing MEF (OC-MEF) (see Figure 40).
Each emerging clone must be transferred to a different OC-MEF.

Prior to performing the needle cutting, read carefully the following instructions :

- Make sure to be in a comfortable position (to the operator discretion) and test the needle cutting first on a patch of the culture plate edge. This will allow to see a good angle and make small precise cuts.
- Make sure to keep the same needle position and angle when move and rotating the culture ingdish.
- Make the cuts in steady left to right movements if right-handed and the invert if left-handed.
- Always cut the colony from an outer edge across the colony to the other edge.

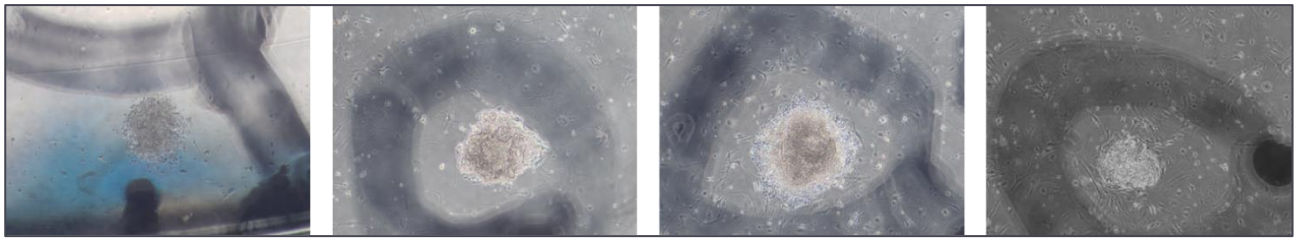

Figure 40 : Examples of hiPSC clone ready to be passed from B3-MEF to OC-MEF. Magnification x40

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- Take out of the incubator an OC-MEF dish early-prepared (See part 3.15).
- Aspirate the medium from the OC-MEF and dispense 0.5 mL of StemMACS iPS-Brew XF. Write the clone number and P1 on the lid.
- Place the B3 dish containing emerging clones to pass under a stereomicroscope under a laminar flux hood.
- Using a sterile needle mounted on a syringe cut the colony in half (See Figure 41).
- Using a p200 pipette, lift-up and aspirate one-half of the clone and dispense it in the OC-MEF.

From this first passage, there is one colony of a clone. The aim is to multiply the number of colonies of the different clones, so, multiply the dishes of the same clone to obtain several colonies of the same clone. To multiply the dishes, it is possible to transfer in a new OC-MEF half of the clone from the B3 dish containing emerging clones if the previously cut part has grown back.

- Monitor the colony, change medium every 2 days.
- Once colony is ready to be passed, using a sterile needle mounted on a syringe cut the colony in half and cut this half in two to obtain two clumps (See Figure 42).
- Using a p200 pipette, lift-up and aspirate the clumps of the clone and dispense it a new OC-MEF.
- Incubate the cells at 37°C, 5% CO2.
- Once the colonies have adapted well to OC-MEF, i.e. the colonies are growing well after 1-2 passages, switch to OC without MEF (feeder free).
- Whenever obtaining 4-5 matures colonies per clones perform passage on a B3 dish.

NOTE: It is advisable to freeze colonies as early as possible, whenever possible, in order to have a backup.

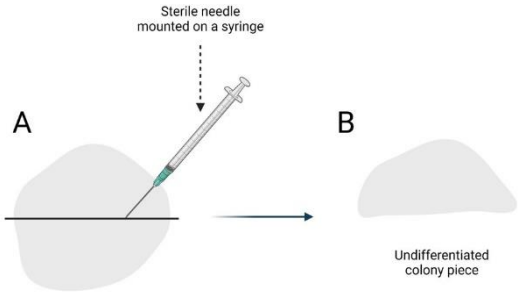

Figure 41 : Cutting of a colony in half by using a sterile needle mounted on a syringe.

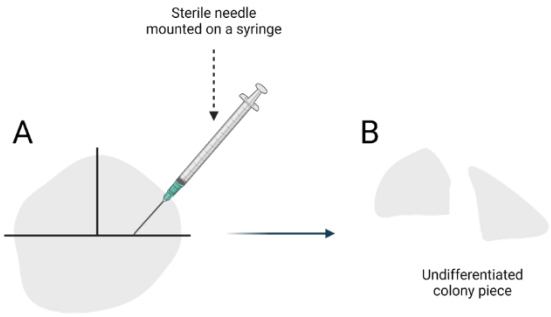

Figure 42 : Cutting of a colony in 2/4 by using a sterile needle mounted on a syringe.

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Passage on B3 feeder free

In order to transfer clones into a B3 dish support a maximum of colonies are needed as the surface of the culture dish is increased. The clones are passed into a B3 dish by mechanical technique (see part *Mechanical passage*, just next) until they are stabilized (usually until passage 10), i.e. when no more differentiated cells are observed. Quality controls are then carried out from this point onwards. To assess these quality controls, please refer to quality controls protocols part. Once all quality controls are validated, please refer to "*Human Induced Pluripotent Stem Cell (hiPSC) Maintenance Protocol*" for hiPS culture.

CLEANING DIFFERENTIATED CELLS

NOTE: The cells must be checked daily and cleaned in case of differentiated cell emergence.

- Place the dish under the hood and the stereomicroscope (See Figure 43A).
- Identify the differentiated colonies or differentiated cells (See Figure 43B, B' Figure 44A, B and C).
- Using a P200 and 200 µL tip, remove all the differentiated colonies or parts of colonies by scraping them (See Figure 43C).
- Aspirate all the medium and cell debris in dishes.
- Wash the cells by adding D-PBS to cover the entire dish. Aspirate and discard D-PBS.
- Add the appropriate volume of room temperature warmed StemMACS iPS-Brew XF.
- The cells are now ready to be passed or to be placed back in the incubator at +37°C, 5% CO_2.

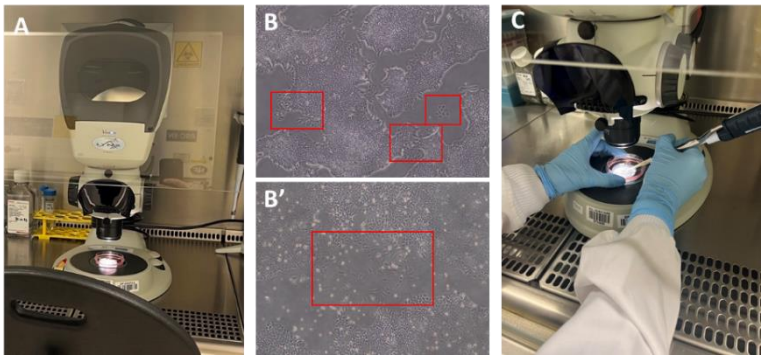

Figure 43: (**A**) Picture of the stereomicroscope under the hood, (**B**) (**B'**) Differentiated cells are surrounded by a red frame, (**C**) P200 pipette cleaning technique.

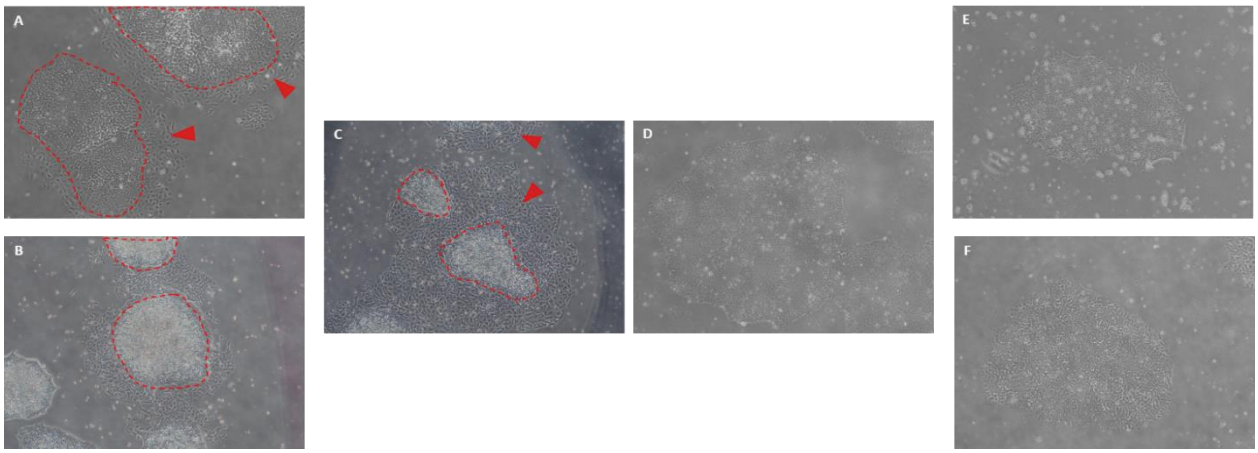

Figure 44: (**A, B, C**) Pictures showing spontaneous differentiation (red arrows) around iPS colonies (surrounded in red) which need to be cleaned. (**D, E, F**) Pictures showing stabilized iPS colonies without differentiation.

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MECHANICAL PASSAGE

- Annotate the cell culture surface coated with matrigel that will receipt the "clumps" from your clone.
- Cut into strips of homogenous size by making parallel straight lines with a sterile needle mounted on a syringe under the stereomicroscope (See Figure 45A).
- Rotate the culture plate at 90° and make again parallel lines as explained in the previous step (See Figure 45B).
- Repeat these last 2 steps as many colonies as required.
- Lift up and scrape the colony pieces of culture dish (See Figure 45C) by using a 200 µL sterile pipette tip.
- Transfer the colony pieces in suspension into the coated culture dishes containing appopriate volume of StemMACS iPS-Brew XF.
- Incubate the cells at 37°C, 5% CO_2 overnight.
- The next day, check that most of the clumps are successfully attached under the stereomicroscope.
- Incubate at 37°C, 5% CO_2 .
- Monitor the cells everyday, remove any differentiated cells if spotted, change the medium every two days and assess the passage whenever colonies are matures (every 5-7 days) (See Figure 46).

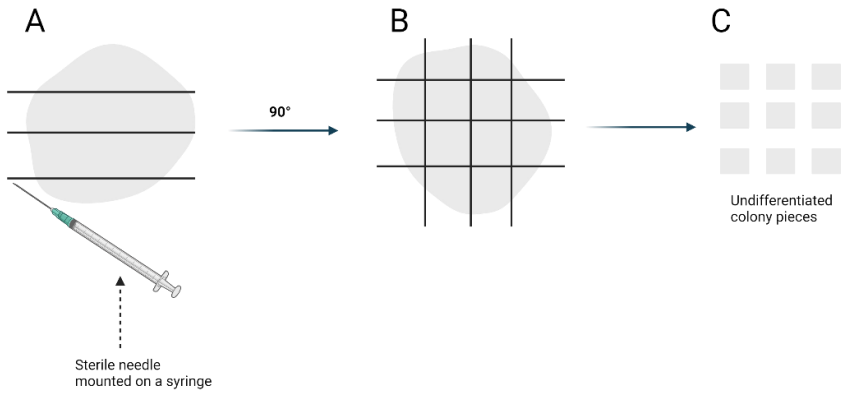

Figure 45: Cutting of a colony by using a sterile needle mounted on a syringe.

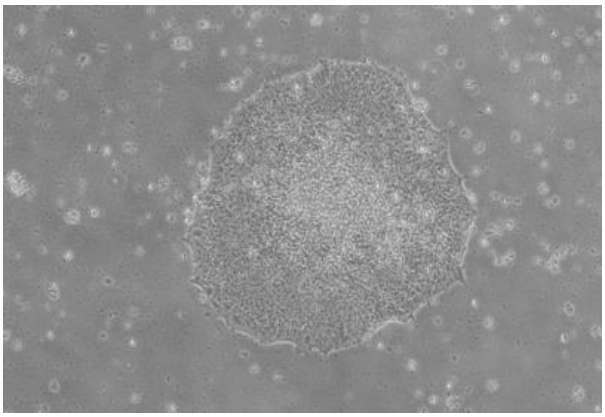

Figure 46 : Colony ready to be passed : center of the colony is more dense (white) evidence that the colony is ready to be passed.

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Annex B - hiPSC Culture

1 References & prerequisites protocols

1.1 Product references

Table 11. References of different products used during the protocol

Products	Suppliers	References
Y-27632 dihydrochloride	Biotechne	1254
Trypan Blue Solution, 0.4%	Gibco	15250061
StemMACS iPS-Brew XF, human kit	Miltenyi	130-104-368
Dulbecco's Phosphate Buffered Saline (D-PBS)	SIGMA	D8537
CryoStor® cell cryopreservation media	SIGMA	C2874
Freezing container, Nalgene® ''Mr. Frosty''	SIGMA	C1562
mTeSR Plus kit	STEMCELL Technologies	100-0276
TrypLE Express	ThermoFisher scientific	12605-010
DMSO	VWR	A3672.0050

1.2 Prerequisites

Figure 47: Chronological representation of main steps for cell culture

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The following standard operating procedure concerns the **hiPSC "clumps-like" maintenance with EDTA (thawing, passage and freezing)** on B6 dishes (see Figure 47). Indeed, as removing differentiated cells from the hiPSC might be needed by scraping them under a stereomicroscope, it is **highly recommended to handle hiPS cells exclusively using B3 or B6 dishes.** However, if alternative cell culture support is used, do not forget to adjust volumes accordingly.

NOTE 1: Always use a sterile technique to maintain hiPS cells.
NOTE 2: For the Matrigel® and vitronectin coating, please refer to "Matrigel_and_Vitronectin_ Matrix_Protocol".

2 Preparation of medium

To maintain hiPS cells in culture, we use two different media: StemMACS iPS-Brew XF (Miltenyi, Ref: 130-104-368) **or** mTeSR Plus (STEMCELL Technologies Ref: 100-0276). The medium is chosen according to the cell line, refer to the "*Specific cell line*_informations" file.

2.1 Reconstitution of StemMACS iPS-Brew XF culture medium

- Thaw StemMACS iPS-Brew XF Supplement at room temperature (15-25°C). Mix thoroughly.
  ⚠ Do not use a water bath to warm supplements.
- Add the entire supplement to the StemMACS iPS-Brew XF basal medium bottle. Mix thoroughly. Bring the medium to room temperature before use.
  ⚠ Do not use a water bath to warm the medium.
- Store reconstituted medium according to 2 options:
  - Option 1: Just after reconstitution of iPS-Brew XF culture medium, dispense 45 mL per new 50 mL sterile tube. Write the medium's name, the batch number and the date on the sterile tube and freeze at -20°C.
  - Option 2: Store medium at 2-8°C for up to 10 days.

  NOTE: The medium contains cytokines that may be altered if warmed at 37°C.

2.2 Reconstitution of mTeSR™ Plus culture medium

- Thaw mTeSR™ Plus 5X Supplement at room temperature (15-25°C) Mix thoroughly.
  ⚠ Do not use a water bath to warm supplements.
- Add the entire supplement to a bottle of mTeSR Plus basal medium. Mix thoroughly. Bring the medium to room temperature before use.
  ⚠ Do not use a water bath to warm the medium.
- Store reconstituted medium according to 2 options:
  - Option 1: Just after reconstitution of mTeSR Plus culture medium, dispense 45mL per new 50mL sterile tube. Write the medium's name, the batch number and the date on the sterile tube and freeze at -20°C.

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- Option 2: Store medium at 2-8°C for up to 10 days.

  NOTE: The medium contains cytokines that may be altered if warmed at 37°C.

2.3 ROCK Inhibitor (Y-27632) aliquot of 10 mM

- Go to bio-techne page: https://www.bio-techne.com/p/small-molecules-peptides/y-27632-dihydrochloride_1254
- In the part "Preparing Stock Solutions for Y-27632 dihydrochloride", enter the batch number, it will give the appropriate volume of DMSO to add to the powder to obtain a 10 mM stock solution.
- Prepare "stocks" aliquots of Y-27632 Rock inhibitor [10 mM] to avoid freezing and thawing cycles (Example: 20 µL/aliquot).

NOTE: ROCK inhibitor enhances the survival of reprogrammed cells. To plate hiPS cells, always add ROCK inhibitor Y-27632 at 1:1000 dilution of the 10 mM stock solution (10 µM final concentration) in mTeSR Plus or StemMACS iPS-Brew XF medium. After 24 hours, change the medium to mTeSR Plus or StemMACS iPS-Brew XF medium ***without ROCK inhibitor.*** *Cells should not be left in Y-27632 for more than 24 hours.*

3 Culture of hiPSC

Table 12. Information concerning the volume of medium or EDTA 0,25 mM to add depending on the used cell culture support. Note that on Fridays, the volume of medium to add is often increased so the cells have enough medium for the whole weekend.

Cell culture support	Working surface (cm^2)	Complete medium volume	Complete medium volume on Fridays	EDTA 0,25 mM volume
35 mm dish (B3 dish)	9,6	2 mL	3 mL	960 µL
60 mm dish (B6 dish)	22,1	4 mL	6 mL	2,2 mL
6-wells	9,6	2 mL	4 mL	960 µL
12-wells	3,8	1 mL	1 mL	380 µL
24-wells	2	500 µL	500 µL	200 µL
T25	25	5 mL	5 mL	2,5 mL
T75	75	13 mL	13 mL	7,5 mL
T225	225	28 mL	30 mL	22,5 mL

NOTE: For hiPSC single-cell maintenance (thawing, passage and freezing) it is ***highly recommended to use B3 or B6 dishes for removing spontaneous differentiation****.*

3.1 Thawing

- Prepare two B6 dishes coated with Corning Matrigel® or vitronectin depending on the cell line. For coating preparation, refer to "Matrigel_and_Vitronectin_Matrix_Protocol".
  For coating and medium information about the cell line, please, refer to ''*Specific cell line*_Informations'' file.

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- Prepare an aliquot with the appropriate volume of mTeSR Plus or StemMACS iPS-Brew XF medium and warm it at 37°C.
- Write the cell name, the number of cell new passage P(n+1), and the date and initials of the manipulator on the dish.
- Switch on the warm bath and set it at +37°C.
- Prepare a 15 mL sterile tube with the name of the cell line written on it and add 5 mL of mTeSR Plus or StemMACS iPS-Brew XF warmed medium.
- Collect the cryovial from nitrogen and place it in dry ice.
- Thaw the cryovial rapidly (< 1 minute) in the 37°C warm bath.
- When only a small ice cube remains, transfer the content of the cryovial to the 15 mL sterile tube containing 5 mL of StemMACS iPS-Brew XF or mTeSR Plus warmed medium.
- Centrifuge 3 minutes at 1 200 rpm.
- Carefully aspirate the supernatant and slowly resuspend the pellet with 1 mL of medium (mTeSR Plus or StemMACS iPS-Brew XF). Don't resuspend the pellet too abruptly to avoid making single-cell resuspension. If needed, add more medium to ensure that the cell count does not exceed 3,000,000 cells/mL.

  NOTE: iPS cells need to be in clusters to proliferate efficiently.
- Perform a cell count using Trypan Blue:
  - Make a mix of 20 µL of cell solution and 20 µL of trypan blue.
  - Load this mix in a counting slide and assess the cell concentration
  - If the concentration exceeds 3,000,000 cells/mL, add more medium to your cells to dilute them. Then, perform the same cell-counting procedure.

    NOTE: In our lab, we use a Countess II automated cell counter.
- Aspirate the coating from Matrigel® or vitronectin-coated B6 dishes previously prepared.
- Add 4 mL per B6 of the appropriate medium (mTeSR Plus or StemMACS iPS-Brew XF) **supplemented with 1/1000 of Y-27632 stock at 10 mM (10 µM final concentration).**
- Plate the two B6 dishes with two different densities of cells (refer to "*Specific cell line*_Informations" file): Calculate the volume of cell suspension to seed regarding the chosen densities, the calculated cell concentration and the B6 dish surface.

  NOTE: For example, for a cell suspension of 1 200 000 cells/mL and to plate a B6 dish of 22,1cm² at 5 000 cells/cm², first calculate de total number of cells needed (22,1 cm² x 5 000 cells = 100 500 cells to plate in the B6). Then divide it by the calculated cell concentration to obtain the volume of cell suspension to dispense in the new B6 (100 500 cells / 1 200 000 cells/mL = 84 µL to dispense).

  NOTE 2: It is important to plate at least two different densities to keep or passage the dish showing the best confluency.
- Move the plates in several, quick and short back-and-forth and side-to-side motions to ensure uniform distribution of cells.
- Incubate cells 24h at 37°C, 5% CO_2.

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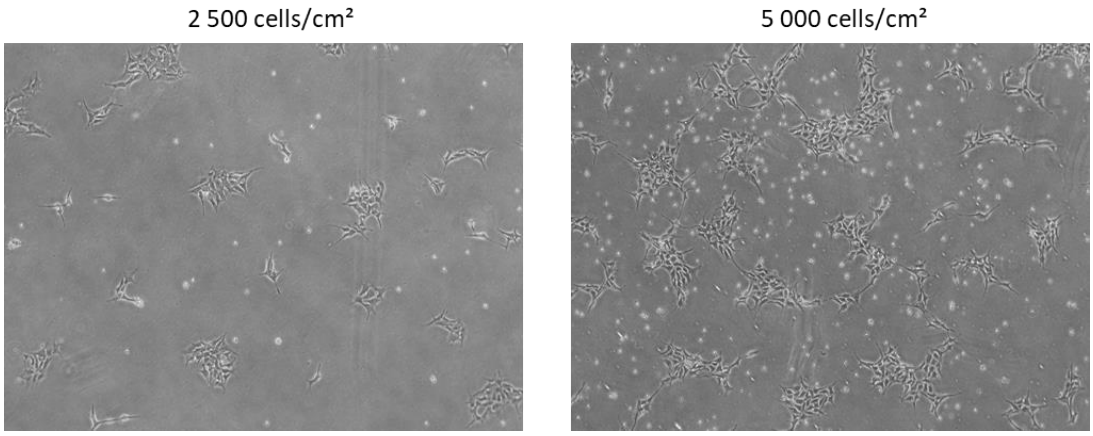

Figure 48: Example of hiPSC density and morphology 24h after plating.

- The next day, check the cell's morphology (see Figure 48) and change the medium to mTeSR Plus or StemMACS iPS-Brew XF **without Rock Inhibitor (Y-27632).**

  NOTE: *Cells should not be left in Y-27632 for more than 24 hours.*
- Monitor the cell growth and change medium every 2 days.

  NOTE: ***Add more medium before the weekend*** *to allow cells to stay during the weekend without medium changing (see Table 12).*
- When cells reach around 80% confluency, split them into new coated B6 dishes.

3.2 Cell Passage

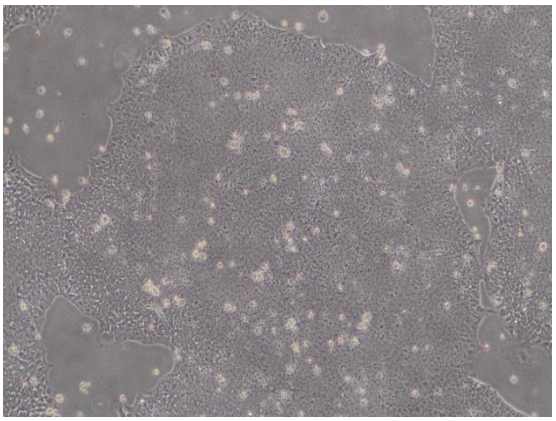

Figure 49: Example of density cells to be passed.

- Check hiPS cells under the microscope, they should reach around 80-90% confluency to be passaged (see Figure 49).
- Prepare two B6 dishes coated with Corning Matrigel® or vitronectin depending on the cell line (Refer to "Matrigel_and_Vitronectin_ Matrix_Protocol" and "*Specific cell line*_Informations" files).
- Write the cell name, date, cell density and passage number on the coated B6 dishes.
- Bring to room temperature the appropriate volume of mTeSR Plus or StemMACS iPS-Brew XF medium before using it.

  ⚠ Do not use a water bath to warm your media.

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- Take out of the incubator the dish containing the cells to pass.
- Aspirate the supernatant and carefully wash the cells by adding D-PBS to cover the entire dish.
- Aspirate the D-PBS.
- Add 0,1 mL of EDTA 0,25 mM per cm² on the cells.
- Incubate the cells with EDTA for 3 to 5 minutes at 37°C until they have the same morphology as in Figure 50B**.**
- Meanwhile, write the cell line name on a new sterile 15 mL tube.
- After those 3 to 5 minutes, check under the microscope if the cells look like in Figure 50B.
- Remove the EDTA by aspirating it.
- Add 4mL of room temperature warmed StemMACS iPS-Brew XF or mTeSR Plus medium to the cells. Mix gently to detach the cells in clumps and transfer the whole suspension into the new 15 mL sterile tube.
- Perform a cell count using Trypan Blue and an automated cell counter :
    - Make a mix of 20 µL of cell suspension and 20 µL of trypan blue.
    - Load this mix in a counting slide and assess the cell concentration using a cell counter.
    - If the concentration exceeds 3 000 000 cells/mL, add more medium to your cells to dilute them. Then, perform the same cell-counting procedure.

        NOTE: In our lab, we use a Countess II automated cell counter.
- Calculate the volume of cell suspension needed to plate the chosen densities (refer to "*Specific cell line*_Informations" file).

    NOTE: For example, for a cell suspension of 1 200 000 cells/mL and want to plate a B6 dish of 22,1 cm² at 5 000 cells/cm², first calculate de total number of cells needed (22,1 cm² x 5 000 cells = 100 500 cells to plate in the B6). Then divide it by the calculated cell concentration to obtain the volume of the cell suspension to dispense in the new B6 (100 500 cells/1 200 000 cells/mL = 84 µL to dispense).
- Aspirate the corning Matrigel® or vitronectin coating in each B6 dish (or other chosen cell culture support).
- Add 4 mL of room temperature warmed StemMACS iPS-Brew XF or mTeSR Plus medium per B6 dish.
- Resuspend the cell suspension by gently inverting the tube several times to avoid breaking the clumps obtained by the EDTA action (see Figure 50C).
- Add the calculated volume of cell suspension on each B6 dish containing culture medium.
- Move the plates in several quick, short, back-and-forth and side-to-side motions to ensure uniform distribution of cells.
- Check the dishes under the microscope to ensure that the clumps obtained are not too small (see Figure 50C). If it is the case add ROCK inhibitor at 10 µM final concentration.
- Incubate cells 24h at 37°C, 5%CO_2.
- The next day, check the cell morphology (see Figure 51) and change the medium to mTeSR Plus or StemMACS iPS-Brew XF **without ROCK inhibitor** if added during the seeding.
- Monitor the cell growth and change medium every 2 days.

    *NOTE: **Add more medium before the weekend** to allow cells to stay during the weekend without medium changing (see Table 12).*

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- When cells reach around 80% of confluence split them into a new coated B6 dish.

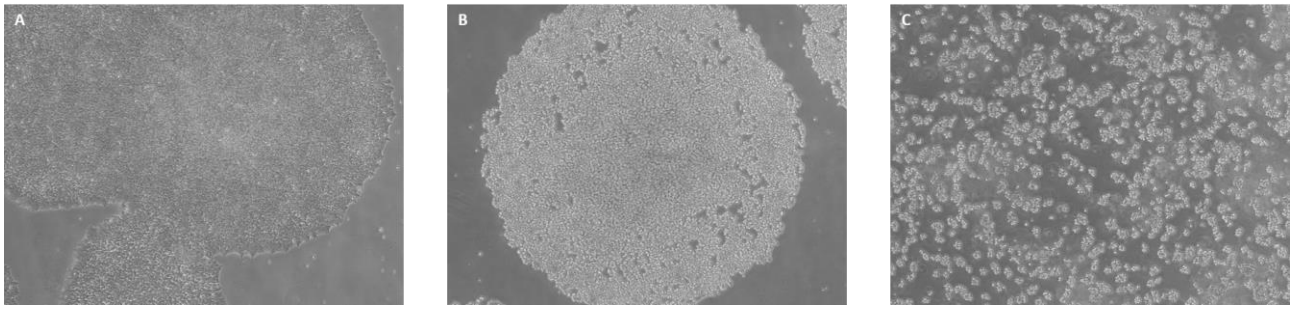

Figure 50: (**A**) iPS colony before addition of EDTA 0,25 mM ; (**B**): iPS colony after incubation at 37°C with EDTA ; (**C**): Clumps of iPS after addition of medium on EDTA incubated colonies.

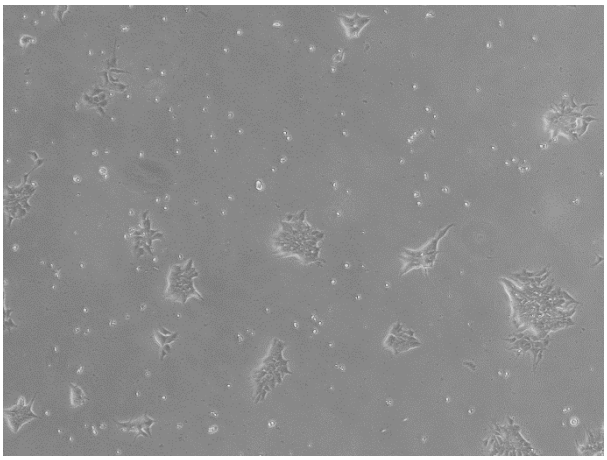

Figure 51: iPS colonies 24h after EDTA passage.

3.3 Cleaning hiPSC

NOTE: The cells must be checked daily and cleaned in case of differentiated cell emergence.

- Place the dish under the hood and the stereomicroscope (See Figure 52A).
- Identify the differentiated colonies or differentiated cells (See Figure 52B, B' and Figure 53A, B and C).
- Using a P200 and 200 µL tip, remove all the differentiated colonies or parts of colonies by scraping them (See Figure 52C).
- Aspirate all the medium and cell debris in dishes.
- Wash the cells by adding D-PBS to cover the entire dish. Aspirate and discard D-PBS.
- Add the appropriate volume (see Table II) of room temperature warmed StemMACS iPS-Brew XF or mTeSR Plus medium.
- The cells are now ready to be passed or to be placed in the incubator at +37°C, 5% CO_2.

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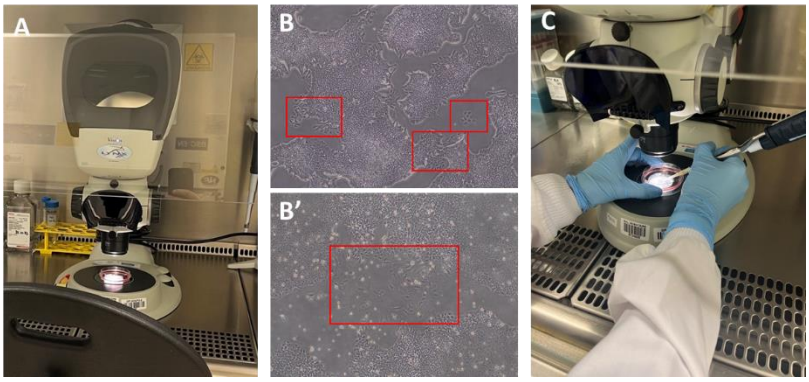

Figure 52: (**A**) Picture of the stereomicroscope under the hood, (**B**) (**B'**) Differentiated cells are surrounded by a red frame, (**C**) P200 pipette cleaning technique.

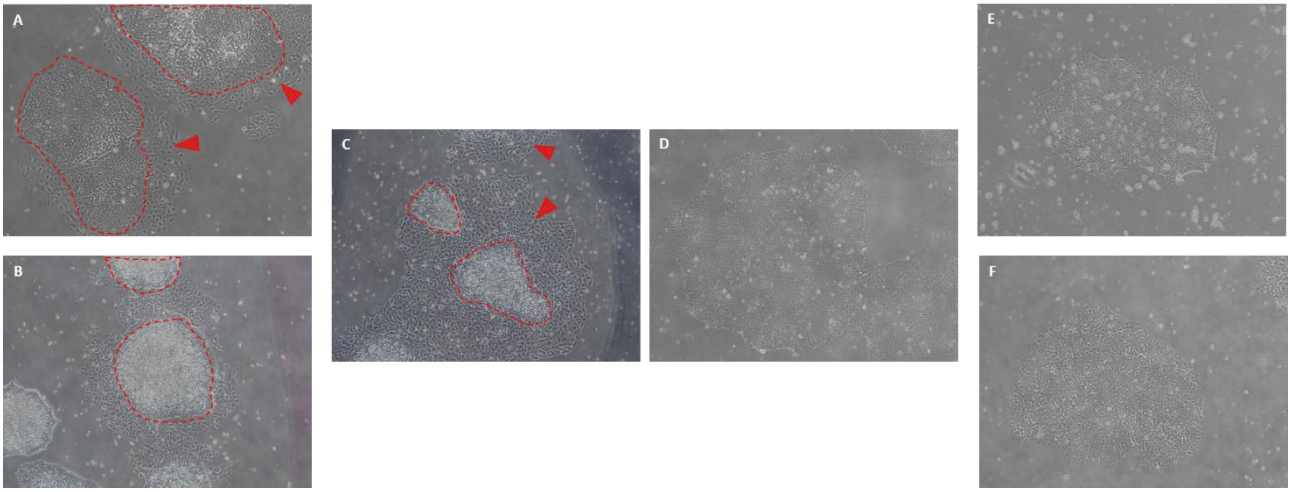

Figure 53: (**A, B, C**) Pictures showing spontaneous differentiation (red arrows) around iPS colonies (surrounded in red) which need to be cleaned. (**D, E, F**) Pictures showing stabilized iPS colonies without differentiation.

3.4 Cell Freezing

- Check under the microscope that the cells reached around 80-90% confluency (See Figure 49).
- Prepare the cryovials by writing the cell line name, the passage, the date, the coating, the medium and the initials of the manipulator.
- Check that the "Mr Frosty" (SIGMA, Ref: C1562) contains enough isopropanol (to be changed after 5 times usage).
- Put the "Mr frosty" at 4°C at least 30 minutes before freezing hiPS cells.
- Thaw at room temperature appropriate volume of mTeSR Plus or StemMACS iPS-Brew XF medium at room temperature before using.

  ⚠ Do not use a water bath to warm your media.
- Take out of the incubator the B6 dish containing the cells to freeze.

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- Aspirate the supernatant and carefully wash the cells by adding D-PBS to cover the entire cell culture support.
- Aspirate and discard the D-PBS.
- Add 0,1 mL of EDTA 0,25 mM per cm^2 on the cells.
- Incubate the cells with EDTA for 3 to 5 minutes at 37°C until they have the same morphology as in Figure 50B**.**
- Meanwhile, write the cell line name on a new sterile 15 mL tube.
- After those 3 to 5 minutes, check under the microscope if the cells look like in Figure 50B.
- Remove the EDTA by aspirating it.
- Add 4mL of room temperature warmed StemMACS iPS-Brew XF or mTeSR Plus medium to the cells. Mix gently to detach the cells in clumps and transfer the whole suspension into the new 15 mL sterile tube.
- Perform a cell count using Trypan Blue and a cell counter :
    - Make a mix of 20 µL of cell solution and 20 µL of trypan blue.
    - Load this mix in a counting slide and assess the cell concentration using a cell counter.
    - If the concentration exceeds 3,000,000 cells/mL, add more medium to your cells to dilute them. Then, perform the same cell-counting procedure.

        NOTE: In our lab, we use a Countess II automated cell counter.
- Depending on the number of cells obtained, calculate the number of cryovials freeze counting 1,000,000 cells per cryovial.

    NOTE: For example, for 3 000 000 cells/mL in 4 mL of medium, that makes : 3 000 000 cells x 4 mL = 12 000 000 cells in the tube, and fill: 12 000 000 cells / 1,000,000 cells = 12 cryovials.
- Centrifuge the cells for 3 minutes at 1 100 rpm.
- Carefully aspirate the supernatant.
- Resuspend the pellet in appropriate volume of CryoStore® (SIGMA, Ref: C2874). Usually 1 mL per cryovial.

    NOTE: For example, to freeze 12 000 000 cells, resuspend the pellet in 12 mL of CryoStore® and dispense 1 mL of it in 12 different cryovials.
- Transfer immediately the cryovials to the "Mr Frosty".
- Transfer immediately the "Mr Frosty" to -80°C during 24h minimum (7 days maximum).
- Transfer the cryovials into the nitrogen storage system.

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Annex C - hiPSC Karyotyping & SNPs Genotyping

1 hiPSC quality controls

For hiPS characterization, we perform the following quality controls: karyotyping, Single Nucleotide Polymorphism analysis, embryonic bodies assay, flow cytometry, immunofluorescence staining and RT-qPCR.

Table 13. References of the different equipment, reagents and supplies used during the protocol.

Article	Supplier	Reference	Link
Equipment			
Dry oven			
Cytogenetic Drying Chamber	Thermotron	CDS-5	Product
Water bath			
Inverted phase-contrast microscope			
Coplin jars			
Reagents			
Colchicine 20 mg/L	Eurobio Scientific	CCHCLC00-JA	Product
DMEM, high glucose, GlutaMAX™ Supplement	Gibco	61965-026	Product
Fetal Bovine Serum	Sigma-Aldrich	F7524	Product
D-PBS	Sigma-Aldrich	D8537-500ML	Product
TryplExpress	Gibco	12605-010	Product
Acetic acid 100%	VWR	20104.298	Product
Methanol	VWR	20847.295	Product
UltraPure 20X SSC Buffer	Invitrogen	15557-036	Product
NaOH			
Ethanol 100%			
24XCyte Human Multicolor FISH probe	MetaSystems Probes	D-0125	Product
SlowFadeTM Gold antifade reagent with DAPI	Invitrogen	S36938	Product
Tween			
QIAamp DNA Blood Mini kit	Qiagen	51104	Product
Supplies			
15 mL sterile tube	Falcon	352096	
SuperFrost Microscope slides Ground 90° Pink Tab	Epredia	AG00008232E01MNZ20	Product
Coverslips 24x24 mm	Menzel-Gläser	630-2104	Product
Coverslips 24x60 mm	Epredia	24X60-2-601640G	Product
1.5 mL Eppendorf			
Flexible mounting adhesive (glue)			

1.1 Karyotyping quality control

This quality control allows us to ensure that the hiPS generated by Sendaï reprogramming don't show chromosomal abnormalities.
To perform this control cells should have reached at least passage 10 after reprogamming and 90% confluency on a 22,1 cm² Petri dish.

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Step 1: Cell fixation

- Add 50 µL of Colchicine 20 mg/L (Eurobio Scientific, #CCHCLC00-JA) per mL of cell culture medium directly in the Petri dish to obtain a 1 µg/mL Colchicine concentration.

  This step will block the dividing cells in metaphase
- Incubate at 37°C, 5% CO_2 for 90 minutes.
- During the incubation time, prepare 8 mL of hypotonic solution for one 22,1 cm² Petri dish fixation: 2 mL of DMEM, high glucose, GlutaMAX™ Supplement (Gibco, #61965-026) + 10% FBS (Sigma-Aldrich, #F7524) + 6 mL of ultrapure water (1/4 dilution).
- Warm this hypotonic solution at 37°C in an incubator.
- After 90 minutes of incubation, remove the medium with colchicine, wash the cells twice with D-PBS (Sigma-Aldrich, #D8537-500ML) and add 2 mL of TryplExpress (Gibco, #12605-010).
- Incubate at 37°C, 5% CO_2 for 3-4 minutes until cell detachment.
- Add 8 mL of DMEM, high glucose, GlutaMAX™ Supplement + 10% FBS to recover the cells.
- Homogenize the cell suspension and transfer it into a 15 mL sterile tube annotated with all the information about the cell line fixed (name, clone number, passage, date).
- Centrifuge at 1 200 rpm for 3 minutes.
- Aspirate supernatant and resuspend gently the cell pellet in 8 mL of warmed hypotonic solution previously prepared.
- Incubate the tube horizontally at 37°C, 5% CO_2 for 27 minutes precisely.
  /!\ After this step, manipulate under a chemical hood /!\
- During the incubation time, prepare the Carnoy fixative solution : 3 volumes of methanol + 1 volume of acetic acid.

  ex: for 40 mL, 30 mL of methanol + 10 mL of acetic acid
- After the 27 minutes of incubation, add 2 mL of Carnoy fixative solution directly in the 15 mL tube containing the cell suspension in hypotonic solution for 8 mL of it.
- Centrifuge at 1 200 rpm for 5 minutes at room temperature.
- Remove the supernatant and resuspend briefly the pellet in 5 mL of Carnoy fixative solution.
- Centrifuge at 1 200 rpm for 5 minutes at room temperature.
- Repeat the two last steps.
- Remove the supernatant, and add 5 mL of Carnoy fixative solution.
- Store the cells vertically at +4°C.

  Fixed cells can be stored for up to 2-3 weeks at +4°C and several years at -20°C.

Step 2: Cell spreading

The following steps are to be performed at least one day after cell fixation.
Before performing the following steps, start the cytogenetic drying chamber system and ensure that it will reach the correct temperature and hygrometry (25°C, 40% humidity).

- Resuspend the cells by inverting the tube.
- Centrifuge at 1 200 rpm for 5 minutes at room temperature.
- Remove supernatant using a Pasteur pipette, and let a small volume of Carnoy fixative solution (more or less five times the pellet size) and perform a resuspension of the pellet.

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- Annotate two glass slides (SuperFrost Microscope slides Ground 90° Pink Tab, Epredia, #AG00008232E01MNZ20) with the name of the cell line, number of the clone, passage, date and name of the manipulator (ex: DMD FEAD Clone 1 P11 03/05/24 BM).
- Under the cytogenetic drying chamber, homogenize the cell suspension and spread 30 µL of it on each glass slide.
- Let the glass slides dry under the cytogenetic drying chamber for 10 minutes.
- Check the quantity and quality of the metaphases under an inverted phase-contrast microscope.

Step 3: mFISH staining

Before performing the following steps, be sure that all the following solutions are ready to use and in sufficient volume :

- SSC 0.1X (250 µL UltraPure 20X SSC Buffer (Invitrogen, #15557-036) + 49.750 mL D-PBS)
- SSC 2X (5 mL UltraPure 20X SSC Buffer (Invitrogen, #15557-036) + 45 mL D-PBS)
- NaOH 0.07M
- Ethanol 70% (35 mL ethanol 100% + 15 mL Milli-Q water)
- Ethanol 95% (47.5 mL ethanol 100% + 2.5 mL Milli-Q water)
- Ethanol 100%

Before the experiment, warm a water bath at 70°C and warm a plastic Coplin jar, filled with SSC 2X solution, in it.

- When the SSC 2X solution reaches 70°C, add the glass slides to the plastic Coplin jar for 30 minutes.
- During this time, prepare a 1.5 mL Eppendorf containing 6 µL of 24XCyte Human Multicolor FISH probe (MetaSystems Probes, ref. D-0125-060-DI) per glass slide to treat and warm it in the water bath at 75°C for 5 minutes.
- Cool down the Eppendorf on ice and then, keep it in the dry oven at 37°C.
- Prepare different Coplin jars containing: SSC 0.1X at room temperature, NaOH 0.07 M at room temperature, SSC 0.1X at +4°C, SSC 2X at +4°C, ethanol 70% at room temperature, ethanol 95% at room temperature and ethanol 100% at room temperature.
- After the 30 minutes of incubation in SSC 2X solution at 70°C, let the solution with the slide cool down at room temperature to reach 37°C and then, immerge successively for 1 minute precisely in the different Coplin jars in the following order :
    - SSC 0.1X at room temperature
    - NaOH 0.07M at room temperature
    - SSC 0.1X at 4°C
    - SSC 2X at 4°C
    - Ethanol 70% at room temperature
    - Ethanol 95% at room temperature
    - Ethanol 100% at room temperature
- Let the glass slides dry.
- Add 6 µL of pre-warmed 24XCyte Human Multicolor FISH probe in the middle of the cell droplet.
- Drop a coverslip 24x24 mm (Menzel-Gläser, #630-2104) on the top of the cell droplet and wait for the probe to diffuse.

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- If any air bubbles are remaining, remove them by applying a little pressure on the cover slip.
- Seal the coverslip with glue to prevent the probe from drying out.
- Place the glass slide in a humid and light-proof chamber and incubate it at 37°C overnight in a dry oven.

The next day, before the experiment, warm a water bath at 72°C and warm a Coplin jar, filled with SSC 0.4X solution, in it.

- When the SSC 0.4X solution reaches 72°C, remove the glue cover the glass gently from the glass slide and immerse it in the SSC 0.4X bath for 2 minutes protected from light.
- Fill a Coplin jar with 2SSC + Tween 0.05% at room temperature.
- Immerge the glass slide in the 2SSC + Tween 0.05% bath for 2 minutes protected from light.
- Wash the glass slide in a Coplin jar filled with distilled water and dry it, always protected from light.
- Be sure the glass slide is well dry, if this is not the case, dry it with a tissue.
- Add 25 µL of SlowFadeTM Gold antifade reagent with DAPI (Invitrogen, #S36938) in the middle of the glass slide.
- Drop a 24x60 mm coverslip (Epredia, #24X60-2-601640G) on the top of the droplet and wait for the liquid to spread.
- Remove bubbles by applying a little pressure on the coverslip.
- Store glass slide horizontally at +4°C.

Step 4: Metaphasis capture and analysis

- Capture 50 metaphases using MetaSystems device coupled to an AxioImager Zeiss Z2 microscope with Matefer software equipped with a camera cool cube and 10X and 63X objectives.
- Analyse the images using MetaSystems ISIS software for mFISH.
- Perform the full karyotype of at least 30 metaphases using the MetaSystems software.

1.2 Single-Nucleotide Polymorphism Analysis

This quality control allows us to ensure that the hiPSC generated using Sendaï reprogramming does not show chromosomal anomalies that are not detected by mFISH. If possible, genotype of hiPS cells should be compared to the genotype of the cells that have been reprogrammed (PBMCs or other cell types).
To perform this control have a dry cell pellet of a minimum 1 000 000 cells which should have reached at least passage 10.

Step 1: Genomic DNA extraction

For this step, we use the QIAamp DNA Blood Mini kit from Qiagen (ref. 51104).
Before the manipulation, be sure that the following reagents are all ready to use:

- QIAGEN Protease stock solution: When using the QIAamp DNA Blood Mini Kit (50), pipet 1.2 mL protease solvent* into the vial containing lyophilized QIAGEN Protease, as indicated on the label.
- Buffer AL: Mix Buffer AL thoroughly by shaking before use.
- Buffer AW1: Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle.

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- Buffer AW2: Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle.

10 minutes before starting the extraction, heat a heating block to 56°C.

- Pipet 20 µL of QIAGEN Protease into the bottom of a 1.5 mL Eppendorf.
- Add 200 µL sample to the Eppendorf. Use up to 200 µL of cells in PBS.
  If the sample volume is less than 200 µL, add the appropriate volume of PBS.
- Add 200 µL Buffer AL to the sample. Mix by pulse-vortexing for 15 s.
  To ensure efficient lysis, the sample and Buffer AL must be mixed thoroughly to yield a homogeneous solution.
  If the sample volume is larger than 200 µL, increase the amount of QIAGEN Protease and Buffer AL proportionally (for example, a 400 µL sample will require 40 µL QIAGEN Protease and 400 µL Buffer AL.
  NOTE: Do not add QIAGEN Protease directly to Buffer AL.
- Incubate at 56°C for 10 minutes.
- Add 200 µL ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s.
  If the sample volume is greater than 200 µL, increase the amount of ethanol proportionally; for example, a 400 µL sample will require 400 µL of ethanol.
- Transfer all the volume in a QIAamp Mini column placed into a 2 mL collection tube.
- Centrifuge at 6 000 x *g* (8 000 rpm) for 1 minute at room temperature.
- Place the QIAamp Mini column into a new 2 mL collection tube and discard the tube containing the filtrate.
- Apply 750 µL Buffer AW1 to the QIAamp Mini column.
- Centrifuge at ≥ 6 000 x *g* (8 000 rpm) for 1 minute at room temperature.
- Place the QIAamp Mini column into a new 2 mL collection tube and discard the tube containing the filtrate.
- Apply 750 µL Buffer AW2 to the QIAamp Mini column.
- Centrifuge at ≥ 6 000 x *g* (8 000 rpm) for 1 minute at room temperature.
- Place the QIAamp Mini column into a new 2 mL collection tube and discard the tube containing the filtrate.
- Centrifuge at ≥ 11 000 rpm for 2 minutes to dry the membrane completely.
- Place the QIAamp Mini column in a clean 1.5 mL Eppendorf annotated with "*gDNA*", cell line name, passage and date of the extraction.
- Add 100 µL Buffer AE or distilled water to the QIAamp Mini column.
- Incubate at room temperature for 1 minute.
- Centrifuge at 6 000 x *g* (8 000 rpm) for 1 minute at room temperature.
- Store gDNA at -20°C.

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Step 2: Genomic DNA quantification using NanoDrop 8 spectrophotometer

- Select "*DNA*" on the software.
- Load 2 µL of RNAse-free water on the read cell of the NanoDrop 8 spectrophotometer using a 10 µL sterile pipette to make the blank.
- Click on the "*Blank*" button on the software.
- Homogenize the DNA sample by tapping the tube and pipetting up and down several times.
- Load 2 µL of the DNA sample on the read cell of NanoDrop using a 10 µL sterile pipette.
- Click on the "*Measure*" button on the software.

The software will give the DNA concentration and 2 ratios (A_{260}/A_{280} and A_{260}/A_{230}).
Write the DNA concentration on the tube.

Step 3: Sending of a genomic DNA sample to Integragen for chip reading

Send 30 µL of 100 ng/µL gDNA sample to Integragen to perform hybridization using Illumina Infinium Core24.
The raw data will be sent back for analysis with Genome Studio software V2.0.5.

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Annex D - hiPSC/Myoblast/Myotube qPCR

1 References

Table 14. References of different media and supplements used during the protocol.

Article	Supplier	Reference	Link
Qiagen RNeasy Mini Kit (50)	Qiagen	74104	Product
100 mM dATP Solution	Invitrogen	55082	
100 mM dCTP Solution	Invitrogen	55083	Product
100 mM dGTP Solution	Invitrogen	55084	
100 mM dTTP Solution	Invitrogen	55085	
3 µg/µL Random Primer	Invitrogen	48190011	Product
500 ng/µL Oligo(dT)$_{18}$ Primers	Invitrogen	SO131	Product
SuperScript III RT (200 U/µL)	Invitrogen	18080093	
0.1 M DTT	Invitrogen	Y00147	Product
5X First Strand Buffer	Invitrogen	Y02321	
RNase-free water			
Luminaris HiGreen Low ROX qPCR Master Mix (2X)	ThermoScientific	K0971	Product
Dulbecco's Phosphate Buffered Saline (D-PBS)	Sigma	D8537	Product
Eppendorf PCR Tubes 0.2 mL	Eppendorf		
Eppendorf 0.5 mL	Eppendorf		

2 Experiment objectives

To characterize hiPS, myoblasts and myotubes cells, we should control if pluripotency and myogenic specific genes are expressed or not according to the cell stage. To do so, we perform reverse transcription on RNA extracted from those different cell types to obtain cDNA to perform qPCR on targeted genes.
In this document, these different steps will be detailed.

3 RNA Extraction

RNA extraction can be performed on fresh cells or frozen dry pellets.
For this part of the protocol we follow the instructions of "*Protocol: Purification of Total RNA from Animal Cells Using Spin Technology*" from the user manual provided with the RNeasy Mini Kit (ref. 74104) from Qiagen.
The following protocol will provide information for extraction from one well of a 6-well plate.

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- Be sure that your cells reach at least 80% confluency before performing RNA extraction.
- Before starting, please be sure that every buffer in the kit has sufficient volume to perform the extraction.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of absolute ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- Prepare the different columns and Eppendorf needed for the extraction and annotate them ("*RNA*", cell line name and date of the extraction).
- Prepare a sufficient volume of 70% ethanol in a sterile tube.
- Remove the media from your cells.
- Wash the cells 2 times with 500 µL of D-PBS (Sigma, #D8537).
- Add 350 µL of RLT Buffer, provided with the RNeasy Mini Kit, per well of a 6-well plate.
- Briefly scratch the well with a P1000 tip.
- Flush 2-3 times and transfer the 350 µL of RLT Buffer into a QIAshredder spin column placed in a 2 mL collection tube.
- Centrifuge the tube for 1 minute at 11,000 rpm.
- Discard the QIAshredder spin column and keep the collection tube.
- Add 1 volume (= 350 µL) of 70% ethanol to the homogenized lysate.
- Mix well by pipetting.
- Transfer up to 700 µL of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 mL collection tube (supplied).
- Close the lid gently, and centrifuge for 30 s at ≥8,000 x g (≥10,000 rpm). Discard the flow-through.
- Add 700 µL Buffer RW1 to the RNeasy spin column.
- Close the lid gently, and centrifuge for 30 s at ≥8,000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.
- After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.
- Add 500 µL Buffer RPE to the RNeasy spin column.
- Close the lid gently, and centrifuge for 30 s at ≥8,000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.

  NOTE: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use.
- Add 500 µL Buffer RPE to the RNeasy spin column.
- Close the lid gently, and centrifuge for 2 min at ≥8,000 x g (≥10,000 rpm) to wash the spin column membrane.

  The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.
- Place the RNeasy spin column in a new 2 mL collection tube (supplied), and discard the old collection tube with the flow-through.

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- Close the lid gently, and centrifuge at full speed for 1 min.
  Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column.
- Place the RNeasy spin column in a new 1.5 mL collection tube (supplied). Add 30 µL RNAse-free water directly to the spin column membrane.
- Close the lid gently, and centrifuge for 1 min at ≥8,000 x g (≥10,000 rpm) to elute the RNA.
- Place the 1.5 mL collection tube directly on ice after centrifugation.
- Perform an RNA quantification.

4 RNA quantification using NanoDrop 8 spectrophotometer

- Load 2 µL of RNAse-free water on the read cell of the NanoDrop 8 spectrophotometer using a 10 µL sterile pipette to make the blank.
- Click on the "*Blank*" button on the software.
- Homogenize the RNA sample by tapping the tube and pipetting up and down several times.
- Load 2 µL of the RNA sample on the read cell of NanoDrop using a 10 µL sterile pipette.
- Click on the "*Measure*" button on the software.
- The software will give the RNA concentration and 2 ratios (A_{260}/A_{280} and A_{260}/A_{230})

5 cDNA obtention by Reverse Transcriptase PCR

Before this step, calculate the volume of RNA to use for each sample to obtain 10 µL of RNA suspension at 50 ng/µL (500 ng in 10 µL).

Formula for the volume of RNA to take: $\frac{\boldsymbol{Final\ volume\ (\mu L) \times Final\ concentration\ (ng/\mu L)}}{\boldsymbol{RNA\ concentration\ (ng/\mu L)}}$

Example: for a RNA sample with a concentration of 325 ng/µL

$\frac{10\ \mu L \times 50\ ng/\mu L}{325\ ng/\mu L}$ = 1.54 µL of RNA sample to add in a sterile eppendorf

10 µL - 1.54 µL of RNA = 8.46 µL of RNase-free water to complete

For the following steps, RNA samples, 10 mM dNTP mix, 3 µg/µL random hexamers, 500 ng/µL oligo(dT)$_{18}$, 5X FS buffer, 0.1 M DTT, SuperScript III RT enzyme and RNase-free water are required.
Before starting, please, ensure to have enough 10 mM dNTP mix to experiment. If not, prepare a new solution: for a 100 µL solution, dilute 10 µL of 100 mM dATP solution (Invitrogen, #55082) + 10 µL of 100 mM dCTP solution (Invitrogen, #55083) + 10 µL of 100 mM dGTP solution (Invitrogen, #55084) + 10 µL of 100 mM dTTP (Invitrogen, #55085) + 60 µL of RNase-free water.

- Annotate sterile Eppendorf PCR Tubes 0.2 mL with the name of the sample and the date of the experiment.
- Add the appropriate volumes of RNase-free water and RNA sample previously calculated in the appropriate Eppendorf PCR Tubes 0.2 mL.

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- Prepare the first mix of reverse transcription (Mix RT1) following the volume presented in Table 15.

Table 15. Volumes for preparation of mix RT1

	Volume for 1 tube (µL)	Volume for Y tube (µL)
10 mM dNTP mix	1	Y x 1 µL
3 µg/µL Random Hexamers	0.2	Y x 0.2 µL
500 ng/µL Oligo(dT)$_{20}$	1	Y x 1 µL
Total volume (µL)	2.2	Y x 2.2 µL

- Add 2.2 µL of mix RT1 in each RNA sample mix.
- Place the Eppendorf PCR Tubes 0.2 mL tube containing 10 µL of RNA sample mix + 2.2 µL of mix RT1 in the thermocycler and launch a cycle with the following steps:
  - 65°C for 5 minutes
  - 4°C ; ∞
- During the thermocycling time, prepare the second mix of reverse transcription (Mix RT2) following volumes presented in Table 16.

Table 16. Volumes for preparation of mix RT2

	Volume for 1 tube (µL)	Volume for Y tube (µL)
5X First Strand Buffer	4	Y x 4 µL
0,1M DTT	2	Y x 2 µL
SuperScript III RT (200 U/µL)	1	Y x 1 µL
RNase-free water	1	Y x 1 µL
Total volume (µL)	8 µL	Y x 8 µL

- When thermocycling is done, add 8 µL of mix RT2 per 0.5 mL tube and launch a new thermocycler cycle following these steps:
  - 25°C for 10 minutes
  - 55°C for 60 minutes
  - 75°C for 15 minutes
  - 4°C ; ∞
- Dilute the sample at 1:10 with RNase-free water.
  For the 20 µL cDNA obtained with this protocol, add 180 µL of RNase-free water.
- cDNA can be stored at -20°C for years.

6 qPCR using QuantStudio 12K Flex system from Applied Biosystems

The following steps are described for qPCR performed in a 384-wells plate and a final reading using the QuantStudio 12K Flex system from Applied Biosystems. If any other plate or device is used, please, adjust the volume and consumables accordingly.

Before performing the qPCR, prepare a plate plan with a sample name and probe mix to add to the different wells to use (see Figure 54 for an example).

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		1	2	3	4	5	6	7	8	9
A	hiPS	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1
B	Myoblast	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2
C	Myotube	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3
		NANOG			***OCT4***			***DESMIN***		
D	hiPS	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1
E	Myoblast	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2
F	Myotube	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3
		MYOD			***MYOG***			***DP427M***		
G	hiPS	Sample 1	Sample 1	Sample 1						
H	Myoblast	Sample 2	Sample 2	Sample 2						
I	Myotube	Sample 3	Sample 3	Sample 3						
		18S								

Figure 54 : Plate plan example for qPCR on cDNA from hiPS, myoblast and myotubes cells

First qPCR to perform is Sendaï non-integration check (See Table 17 and 18 for expected results and primers sequences).
In a second time, after obtaining myoblast and myotube cells, it is recommended to perform qPCR on hiPS, myoblast and myotube RNA on the same plate in order to compare expressions normalized on hiPS cells expression. It is expected to observe loss of expression of pluripotency markers (*NANOG* and *OCT4*) and increase expression of myogenic markers (*DESMIN*, *MYOD*, *MYOG* and *DP427M*) in the myoblast and myotube cells compare to hiPS cells.

The first step of this protocol is the preparation of the different Luminaris HiGreen probe mixes targeting different genes according to the quality control to assess (see Table 17 and 18). *Human 18S* is used as housekeeping gene to normalize gene expression.

Table 17. Genes to assess according to quality control

Quality control assessed	Genes to target
hiPS pluripotency	*NANOG, OCT4*
Sendaï non-integration	*C-MYC, KOS, SEV, KLF4*
Myoblasts cells characterization	*NANOG, OCT4, DESMIN, MYOD, MYOG, DP427M*
Myotubes cells characterization	*NANOG, OCT4, DESMIN, MYOD, MYOG, DP427M*

Table 18. Primers sequences for the different genes assessed by qPCR

Gene	Primers sequence
NANOG	Forward : 5' - CAAAGGCAAACAACCCACTT - 3' Reverse : 5' - TCTGCTGGAGGCTGAGGTAT - 3'
OCT4	Forward : 5' - CCTCACTTCACTGCACTGTA - 3' Reverse : 5' - CAGGTTTTCTTTCCCTAGCT - 3'
SEV-C-MYC	Forward : 5' - TAACTGACTAGCAGGCTTGTCG - 3' Reverse : 5' - TCCACATACAGTCCTGGATGATGATG - 3'
SEV-KOS	Forward : 5' - ATGCACCGCTACGACGTGAGCGC - 3' Reverse : 5' - ACCTTGACAATCCTGATGTGG - 3'
SEV	Forward : 5' - GGATCACTAGGTGATATCGAGC - 3' Reverse : 5' - ACCAGACAAGAGTTTAAGAGATATGTATC - 3'
SEV-KLF4	Forward : 5' - TTCCTGCATGCCAGAGGAGCCC - 3' Reverse : 5' - AATGTATCGAAGGTGCTCAA - 3'
DESMIN	Forward : 5' - ATTGGAGGACCGATTTGCC - 3' Reverse : 5' - TCACCGTCTTCTTGGTATGGA - 3'

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MYOD	Forward : 5' - TATGGAGCTACTGTCGCCAC - 3' Reverse : 5' - GAGTGCTCTTCGGGTTTCAG - 3'
MYOG	Forward : 5' - TAAGGTGTGTAAGAGGAAGTCG - 3' Reverse : 5' - CCACAGACACATCTTCCACTGT - 3'
DP427M (Dystrophin)	Forward : 5' - GTGGGAAGAAGTAGAGGACTGT - 3' Reverse : 5' - TCCTGTAGGTCACTGAAGAGGT - 3'
Human 18S (housekeeping gene)	Forward : 5' - TCTTCAGTCCGCTCCAGGTCT - 3' Reverse : 5' - GAGGATGAGGTGGAACGTGT - 3'

Before starting the experiment, be sure that enough mix of 10 µM primer forward + primer reverse is available. If not, do a new mix.

- Dilute 10 µL of 10 mM primer forward and 10 µL of 10 mM primer reverse in 80 µL of RNase-free water to obtain a 10 µM mix of primers forward + reverse.

For the preparation of the probe mix, refer to Table 19. Keep the mix on ice!

Table 19. Volumes for probe mix preparation

Reagent	Volume for 1 well (µL)	Volume for Y wells (µL)
Mix primer forward + primer reverse (10 µM)	0.15	0.15 x (Y + 3 security wells)
Luminaris HiGreen Low ROX qPCR Master Mix (2X)	5	5 x (Y + 3 security wells)
RNase-free water	2.35	2.35 x (Y + 3 security wells)
Total	**7.5**	**7.5** x **(Y + 3 security wells)**

- Prepare the different probe mixes (see Table 19) and keep them on ice.
- Dispense 2.5 µL of cDNA previously obtained (see part 5) per well according to the plate plan.
    - Dispense each cDNA in triplicate.
- Dispense 7.5 µL of probe mix per well according to plate plan.
- Seal the plate with sealing foil and centrifuge it to ensure every drop goes to the bottom of the wells.
- Transfer the plate to the QuantStudio 12K Flex system.

QuantStudio 12K Flex software settings

- Switch on the QuantStudio 12K Flex system.
- On the screen, touch the ejecting tray button.
- Put the plate in the tray and click on the same button.
- Open QuantStudio 12K Flex software on the computer.
- In the section "*Set Up*", click on "*Experiment Setup*".
- In the section "*Setup - Experiment Properties*":
    - Enter the experiment name.
    - Select the instrument used, here, QuanStudio 12 Flex System.
    - Block: 384-well.
    - Experiment type: Standard curve
    - Reagent: SYBR® Green Reagents.
    - Properties: Standard.
- In the section "*Define*", enter the different targets and samples present on the plate.

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- In the section "*Assign*", assign the different targets and samples according to the plate plan.
- In the section "*Run Method*", make sure the reaction volume per well is set to 10 µL and check that the amplification program is set as follows:
    - Hold Stage: 95°C for 20 seconds.
    - PCR Stage: 95°C for 1 second and 60°C for 20 seconds.
      Number of cycles = 40
    - Melt Curve Stage: 95°C for 15 seconds, 60°C for 1 minute and 95°C for 15 seconds.
- In the section "*Run*", click on "*Start Run*".
- Wait for the run to complete, 1 hour and 42 minutes for a 384-wells plate on the QuantStudio 12 Flex system.
- On the screen, touch the ejecting tray button.
- Throw the plate and close the tray with the same button.
- On the computer, in the section "*Analysis*", check the amplification plots and melt curve plots are good.
- Save the file and in the section "*Export*", click on the "*Export*" button.

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Annex E - hiPSC Immunofluorescence

1 References & experiment objectives

1.1 Product references

Table 20. References of different products used during the protocol.

Products	Suppliers	References
Dulbecco's Phosphate Buffered Saline without calcium chloride and magnesium chloride (D-PBS)	Sigma-Aldrich	D8537
Paraformaldehyde 16% Aqueous Solution EM Grade	Electron Microscopy Sciences	15710
Triton™ X-100	Sigma-Aldrich	X100
Bovine Serum Albumin	Sigma-Aldrich	A9647

Table 21. References of antibodies used for the protocol

Antibody	Suppliers	References	Specificity	Isotype	Species	Dilution	Diluant
Nanog (D73G4)	Cell Signaling	4903	Human	IgG	Rabbit	1/250	Saturation buffer
Oct-4A (C30A3)	Cell Signaling	2840	Human	IgG	Rabbit	1/250	Saturation buffer
Anti-Rabbit IgG Alexa Fluor™ 555	ThermoFisher Scientific	A-21429	Rabbit	IgG	Goat	1/500	Saturation buffer
Hoechst 33342, trihydrochlorure, trihydrate	ThermoFisher Scientific	H3570	-	-	-	1/2 000	Saturation buffer

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1.2 Experiment objectives

It is essential to check the high expression of pluripotency markers such as OCT4 and NANOG on hiPS cells before performing any experiment and every 10 passages.
For this procedure, hiPS cells must be seeded beforehand in 24 wells coated with matrigel or vitronectin depending on the cell line to at least three different densities to obtain isolated clusters of cells on the day of the fixation. Only 3 conditions (NANOG staining, OCT4 staining and unstained control) are tested in 3 separate wells in this procedure but it is highly recommended to seed extra wells/densities with cells as hIPS are very fragile and can detach from the support.

NOTE 1: Always use a sterile technique to maintain hiPS cells.

NOTE 2: For the Matrigel® and vitronectin coating please refer to "Matrigel_and_Vitronectin_ Matrix_Protocol".

NOTE 3: For hiPS seeding please refer to" Human Induced Pluripotent Stem Cell (hiPSC) Maintenance Protocol"

2 Preparation of solution

2.1 4% paraformaldehyde (4% PFA)

/!\ Manipulate paraformaldehyde under a chemical hood. /!

- Dilute Paraformaldehyde 16% Aqueous Solution EM Grade (Electron Microscopy Sciences, #15710) in D-PBS (Sigma-Aldrich, #D8537) to obtain a solution at 4%.
  For example, to prepare 40 mL of PFA 4%, add 10 mL of PFA 16% into 30 mL of D-PBS.
- Solution can be stored at +4°C up to one month. Cool PFA at RT before use.

2.2 Permeabilization buffer (Triton 0.5%)

- Dilute Triton™ X-100 (Sigma-Aldrich, #X100) in D-PBS to obtain a solution at 0.5%.
  For example, to prepare 40 mL of Triton 0.5%, add 200 µL of Triton™ X-100 into 39.8 mL of D-PBS.
- Solution can be stored at RT.

2.3 Saturation buffer (D-PBS + 1% BSA)

- Weight Bovine Serum Albumin (BSA) powder (Sigma-Aldrich, #A9647) to prepare a 1% solution and add the appropriate volume of D-PBS.
  For example, to prepare 10 mL of saturation solution, weigh out 0.1g of BSA and add 10 mL of D-PBS.
  NOTE: to allow quicker dissolution of BSA in D-PBS, incubate the solution at +4°C for 5-10 minutes.
- Solution can be stored at +4°C 1-2 weeks.

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3 Staining protocol

The following protocol has been developed for the use of a Z3 epifluorescence microscope. We therefore seed hiPS cells in a 24-well plate as a support. If another device is used and need to use other support such as a microscope slide, please adjust the volume and consumables accordingly.
As the hiPS cells are delicate, please be gentle during all stages of the protocol to prevent cell detachment.

3.1 Fixation (4% PFA)

- Prepare 4% Paraformaldehyde according to part 2.1 of this protocol.
- **Carefully** aspirate the supernatant and wash the cells with 300 µL of D-PBS/well.
  To avoid cell detachment do not place the D-PBS directly on the cells but on the side of the wells.
- **Carefully** aspirate the D-PBS.
- Under a chemical hood, **carefully** add 300 µL of 4% paraformaldehyde previously prepared.
  To avoid cell detachment do not place the PFA directly on the cells but on the side of the wells.
- Incubate for 10 minutes at room temperature under chemical hood.
- **Carefully** aspirate and discard the paraformaldehyde in a specific CMR waste container and wash it 3 times with 300 µL of D-PBS.
  To avoid cell detachment do not place the D-PBS directly on the cells but on the side of the wells.
- After the last washing step, **carefully** add 800 µL of D-PBS.
  To avoid cell detachment do not place the D-PBS directly on the cells but on the side of the wells.
- Check under a microscope the presence of cells in the wells.
- If staining is not carried out the same day, seal the plate with Parafilm and store at 4°C for a maximum of 2 weeks.

3.2 Permeabilization (Triton 0.5%)

- **Carefully** aspirate the D-PBS and add 300 µL of permeabilisation buffer (Triton 0.5%, see part. 2.2).
  To avoid cell detachment do not place the permeabilisation buffer directly on the cells but on the side of the wells.
- Incubate for 10 minutes at room temperature.
- After incubation, do not wash the cells; go straight to the saturation step.

3.3 Saturation (D-PBS + 1% BSA)

- **Carefully** aspirate the permeabilization buffer and add 300 µL of saturation buffer (D-PBS + 1% BSA) previously prepared.
  Do not put directly the saturation buffer on the cells as it can cause detachment.
- Incubate 1h at room temperature.
- During saturation, prepare the different primary antibody solutions:

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- NANOG: 1:250 of NANOG (D73G4) antibody (Cell Signaling, #4903) in saturation buffer.
  For example, for 1 well: Add 1.8 µL of NANOG antibody in 450 µL of saturation buffer.
- OCT4: 1:250 of OCT4 (C30A3) antibody (Cell Signaling, #2840) in saturation buffer.
  For example, for 1 well: Add 1.8 µL of OCT4 antibody in 450 µL of saturation buffer.

NOTE: Because the antibodies are of the same species, do not co-label with NANOG and OCT4.

3.4 Staining with NANOG and OCT4 antibodies

Day 1: Primary antibody

- Before performing the staining design plate plan.
- **Carefully** aspirate the saturation buffer.
- **Carefully** add 300 µL of previously prepared antibody solution, one antibody per well according to the plate plan.
  Do not add the antibody solution directly to the cells as it can cause detachment, but carefully on the edge of the well.
- **Carefully** add 300 µL of saturation buffer in one well to perform a negative control (unstained well).
  Do not add the saturation buffer directly on the cells as it can cause detachment, but carefully on the edge of the well.
- Seal the plate with Parafilm® and incubate overnight at 4°C.

Day 2: Secondary antibody

- **Carefully** aspirate the primary antibody solution from the wells.
- **Carefully** wash 3 times with 300 µL of D-PBS.
  To avoid cell detachment do not place D-PBS directly on the cells but on the side of the wells.
- **Carefully** add 300 µL of secondary antibody mix according to the plate plan:
  - NANOG well: 1:500 of anti-rabbit IgG AlexaFluor™ 555 antibody (ThermoFischer Scientific, #A-21429) + 1:2000 Hoechst 33342, trihydrochlorure, trihydrate (ThermoFischer Scientific, #H3570) in saturation buffer.
  - OCT4 well: 1:500 of anti-rabbit IgG AlexaFluor™ 555 antibody (ThermoFischer Scientific, #A-21429) + 1:2000 Hoescht 33342, trihydrochlorure, in saturation buffer.
  - Unstained well: 1:500 of AlexaFluor 555 antibody + 1:2000 Hoescht 33342, trihydrochlorure, in saturation buffer.
- Incubate 1h at room temperature **protected from light**.
- **Carefully** aspirate and wash 3 times with 300 µL of D-PBS.
  To avoid cell detachment do not add D-PBS directly on the cells but on the side of the wells.
- Keep the plate protected from light.
- Acquisition is done on a Z3 microscope using DAPI (Hoechst) and DsRed (AlexaFluor 555) channels at 20X magnification with ZEN software.
  - Unstained cells (unstained well) should be negative on the DsRed channel and positive on the DAPI channel.
  - NANOG stained cells should be positive on DAPI and DsRed channels (see Figure 55).
  - OCT4 stained cells should be positive on DAPI and DsRed channels (see Figure 55).

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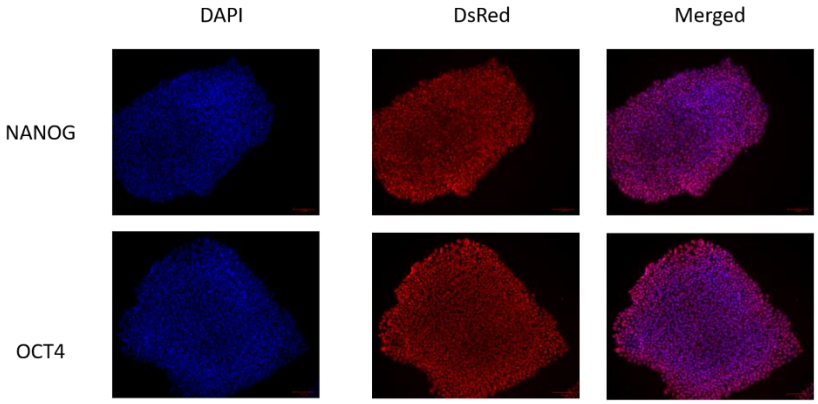

Figure 55: NANOG and OCT4 staining on hiPS cell line 20X magnification.

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Annex F - hiPSC Cytometry

1 References & experiment objectives

1.1 Product references

Table 22. References of different products used for the protocol.

Products	Suppliers	References
Dulbecco's Phosphate Buffered Saline without calcium chloride and magnesium chloride (D-PBS)	Sigma Aldrich	D8537
TrypLE Express	ThermoFisher scientific	12605-010
DMEM/F-12, GlutaMAX™Supplement	Gibco	31331028
Fetal Bovine Serum	Sigma Aldrich	F4135
Trypan Blue Solution, 0.4%	Gibco	15250061

Table 23. References of antibodies used for the protocol.

Antibody	Suppliers	References	Specificity	Isotype	Species	Clone	Final Dilution
TRA-1-81-AF647	Biolegend	330706	Human	IgM	Mouse	TRA-1-81	1/25
Isotype ctrl IgM-AF647	Biolegend	401618	Human	IgM	Mouse	MM-30	1/250
SSEA4-PE	Miltenyi	130-122-914	Human	IgG1	Recombinant	REA101	1/50
Isotype ctrl IgG1-PE	Miltenyi	130-113-438	Human	IgG1	Recombinant	REA293	1/50

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1.2 Experiment objectives

The following procedure describes the flow cytometry technique to verify the co-expression of two pluripotency markers at the hiPSC surface. The aim is to validate the pluripotency character of these cells and it should be performed every 10 passages.
For this procedure, is needed 200 000 cells per tested condition and 4 conditions separated in 4 wells. To obtain enough cells (around 1 000 000 cells), we usually seed a B6 dish coated with vitronectin or Matrigel, 3-4 days before the experiment, and assess flow cytometry when cells have reached 80% confluency. For these steps, please refer to *"Matrigel_and_Vitronectin_Matrix_Protocol" and "Human Induced Pluripotent Stem Cell (hiPSC) Maintenance Protocol".*

2 Preparation of solution

The solutions and antibody mixes must be prepared extemporaneously.

2.1 Flow cytometry buffer (D-PBS + 2% FBS)

FBS (SIGMA, Ref: F4135) and D-PBS (SIGMA, Ref: D8537) are required for the following steps.
For the preparation of the flow cytometry buffer, please, follow these instructions:

- Thaw FBS at room temperature. Mix thoroughly.
- Before the experiment, calculate the volume of flow cytometry buffer needed.
  For example, to assess the 4 conditions on one hiPS cell line, prepare 9ml of flow cytometer buffer.
- In a new 50 mL tube, dilute the FBS in D-PBS 1X to obtain a 2% FBS solution (e.g.: 1 mL of FBS in 49 mL of D-PBS.).
- Store at +4°C and put it on ice when using it to keep it cool.

2.2 Antibodies mixes

For each hiPS cell line, to analyse, 55 µL of three different antibody mixes must be prepared. First mix only contains flow cytometer buffer to identify cells of interest, two mixes are FMO (Fluorescence Minus One) controls needed for cytometer calibration and last mix contains the two specific antibodies to assess the co-expression.

- Mix Unstained: Flow cytometry buffer
- Mix FMO (PE): isotype control IgG1-PE + TRA-1-81-AF647
- Mix FMO (AF647): SSEA-4-PE + isotype control IgM-AF647
- Mix Double Staining: SSEA4-PE + TRA-1-81-AF647

To prepare those mixes, follow the procedure below. The volumes given are for assessing one cell line. If performing more than one cell line at a time, increase the volume accordingly. See Table 23 to use the appropriate final dilutions.

- Put 5 new 0.5 mL tubes on ice.
- Put the 4 antibody stock tubes on ice (See Table 23 for references).
- Put cold flow cytometry buffer on ice (See part 2.1 for the recipe).

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Using one of the 5 tubes, perform a 1:10 dilution in cold flow cytometry buffer **only on Isotype IgM-AF647 Antibody** (Biolegend, ref: 401618): 1 µL of antibody in 9µL of cold flow cytometry buffer. Mix well.

- Annotate the 4 remaining empty tubes writing: Unstained, FMO_PE; FMO_AF647 or DS.
- Referring to Table 24, add appropriate volume of cold flow cytometry buffer in each tube (See Table 24).
- Referring to Table 23, add the appropriate volume of each antibody to the corresponding tube. ***For Isotype IgM-AF647 pay attention to use the 1:10 dilution prepared just before.***
- Vortex the tubes and keep them on ice, protected from light.

Table 24. Volumes for antibody mix preparation.

	Iso IgG1-PE **dilution 1:50** (Miltenyi, 130-113-438)	SSEA4-PE **dilution 1:50** (Miltenyi, 130-122-914)	Iso IgM-AF647 (diluted 1:10) **dilution 1:25** (Biolegend, 401618)	TRA-1-81-AF647 **dilution 1:25** (Biolegend, 330706)	Flow Cytometry buffer
Tube Mix unstained	/	/	/	/	**55 µL**
Tube Mix FMO_PE	1.1 µL	/	/	2.2 µL	**51.7 µL**
Tube Mix FMO_AF647	/	1.1 µL	2.2 µL	/	**51.7 µL**
Tube Mix DS	/	1.1 µL	/	2.2 µL	**51.7 µL**

3 Staining protocol

- Check under a microscope that the hiPSC have reached 80% confluency.
- Aspirate the supernatant and carefully wash the cells by adding D-PBS to cover the entire dish.
- Aspirate the D-PBS.
- Add 2mL of TrypLE Express (Gibco, ref: 12605-010) to the cells.
- Incubate the cells with TrypLE Express for 5 minutes at 37°C.
- Meanwhile, write the cell line name on a new sterile 15mL tube.
- Check the dish under a microscope to be sure that all the cells are detached.
- Carefully dissociate the cells by performing up and down aspirations with a 1000µL pipette.
- Add 4mL of DMEM/F-12, GlutaMAX™Supplement (Gibco, ref: 31331028) + 10%-FBS medium.
- Centrifuge 5 minutes at 900 rpm.
- Carefully aspirate the supernatant without disturbing the pellet.
- Resuspend the pellet in 1 to 3mL of DMEM/F-12, GlutaMAX™Supplement+10%-FBS medium with a 1 000 µL pipette and add more medium if needed to ensure that the cell count does not exceed 3 000 000 cells/mL.
- Determine the viable cell count using your method of choice (e.g., Countess Automated Cell Counter).
- Calculate the volume of cell suspension needed to collect 200 000 cells:

$$\left(\frac{cell\ count\ (cells/mL)}{200\ 000\ (cells)} = volume\ to\ collect\ to\ have\ 200\ 000\ cells\ (mL)\right)$$

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- Dispense this volume in 4 wells of a 96-well plate. **From this step, cells should remain on ice as much as possible.**
- Add 1 mL of cold flow cytometry buffer in each of these 4 wells.
- Centrifuge the plate for 5 minutes at 900 rpm.
- Discard the supernatant by inverting the plate.
- Resuspend the pellet with antibodies mixes (See table 24):
    - 1 well with 50 µL of Mix unstained
    - 1 well with 50 µL of Mix FMO_PE
    - 1 well with 50 µL of Mix FMO_AF647
    - 1 well with 50 µL of Mix DS
- Incubate plate for 30 minutes **on ice protected from light.**
- Add 1mL of cold flow cytometry buffer/well.
- Centrifuge plate 5 minutes at 900 rpm.
- Discard the supernatant by inverting the plate.
- Resuspend the pellet with 200 µL of cold flow cytometry buffer, to avoid bubbles.
- Keep the plate **on ice and protected from light.**
- Samples are ready for flow cytometry acquisition.

4 Flow cytometry acquisition

- Make sure to calibrate the flow cytometer.
- The acquisition required FSC; SSC; PE and AF647 (=APC) channels.
- Read the unstained cells to adjust FSC and SSC channel voltages. The population of interest should be in the middle of the graph (See P1 gate in Figure 56). Gate also the single cell population (See P2 gate in Figure 56)
- Read the FMO_PE-stained cells to adjust the PE channel voltage. Those cells determine your negative cells for PE-stained cells. Place the positivity threshold to have between 0.5% and 1% of positive cells.
- Read the FMO_AF647 stained cells to adjust the APC channel voltage. Those cells determine your negative cells for AF647 stained cells. Place the positivity threshold to have between 0.5% and 1% of positive cells.
- Using the determined settings, start the acquisition for the 4 wells, and set the stopping gate on P2 with at least 30,000 events. See Figure 56 for the gating strategy.
- A percentage greater than or equal to 80% of double-positive cells for SSEA4/TRA-1-81 is required to admit the pluripotency of the cells.

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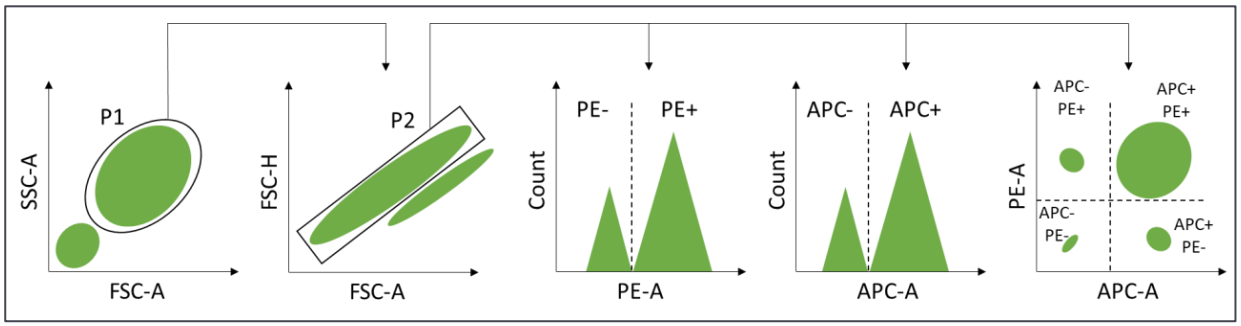

Figure 56: Presentation of the gating strategy to assess the co-expression of SSEA4 and TRA-1-81 on hiPSC. Green shape = cells; black shape = gate; dotted line = positivity threshold.

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Annex G - Myoblast Differentiation

1 References & prerequisites protocols

1.1 Product references

Table 25. References of different media and supplements used during the protocol.

Products	Suppliers	References
Y-27632 dihydrochloride	Biotechne	1254
ClearLine cell strainers 70µM white	Dutscher	141379C
DMEM/F-12 with HEPES	Gibco	11330-032
Trypan Blue Solution, 0.4%	Gibco	15250061
Optional: DMEM, low glucose, pyruvate	*Gibco*	*11885084*
Penicillin/Streptomycin	Life Technologies	15140122
STEMdiff™ Myogenic Progenitor Supplement A	STEMCELL Technologies	100-0152
STEMdiff™ Myogenic Progenitor Supplement B	STEMCELL Technologies	100-0153
STEMdiff™ Myogenic Progenitor Supplement C	STEMCELL Technologies	100-0154
STEMdiff™ Myogenic Progenitor Supplement D	STEMCELL Technologies	100-0155
Collagenase Type IV (1 mg/mL)	STEMCELL Technologies	7909
DMEM with 1000 mg/L D-Glucose	STEMCELL Technologies	36253
MyoCult™ -SF Expansion Supplement Kit (Human)	STEMCELL Technologies	05982
TryplExpress	ThermoFisher Scientific	12605-010
DMSO	VWR	A3672.0050

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1.2 Prerequisites

Figure 57: Chronological representation of main steps for cell culture.

The following standard operating procedure concerns the differentiation of human induced pluripotent stem cells (hiPSC) into myogenic progenitor cells using the STEMCELL Technologies protocol (See Figure 57).

It is essential to check that hiPS cells have high expression of pluripotency markers such as OCT4, Nanog, TRA-1-81, and SSEA-4 before performing any experiment. It is also not advisable to start any experiment with cells that have just been thawed as they take a week to recover from it.

NOTE 1: Always use a sterile technique to perform myogenic differentiation.

NOTE 2: For the Matrigel® coating, please refer to "Matrigel and Vitronectin Matrix Protocol".

NOTE 3: For the thawing and seeding of hiPSC, refer to "Human Induced Pluripotent Stem Cell (hiPSC) Single Cell Maintenance Protocol".

1.3 hiPS cell density test

To start the differentiation protocol, the colonies of cells must contain approximately 10 - 30 cells. To have a better idea of the range of density to use, we recommend to perform a density test. Plate cells into 6-well plates, coated with Matrigel®, between 5 000 to 20 000 cells/cm². The density can be more or less depending on the hiPS cell line growth.

NOTE 1: The hiPS cells must be passed at least once after thawing before the cell density test.

NOTE 2: Only use coated Matrigel® *surface to perform cell density test and differentiation protocol.*

The following protocol concerns the preparation of a 6-well plate from a B6 dish. If using alternative culture support, adjust volumes accordingly.

- Prepare 6-well coated plate with Matrigel® (See "*Matrigel and Vitronectin Matrix Protocol*").
- Carry out cells from B6 dish to 6-well coated plate passaging (See "*Human Induced Pluripotent Stem Cell (hiPSC) Single Cell Maintenance Protocol*").
  - *NOTE: During the suspension of the pellet following the centrifugation step, doing only one or two up and down will allow to have multiple colonies of cells and no single cells the following day.*
- Following the cell count step (See "*Human Induced Pluripotent Stem Cell (hiPSC) Single Cell Maintenance Protocol*" Part 3.2), discard the coating, add 2 mL/well of hiPSC medium on a 6-wells plate, and add the appropriate volume of cell suspension on wells.
- Move the plate in several quick, short, back-and-forth and side-to-side motions to ensure uniform distribution of cells.

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- The remaining hiPS cells should be used to prepare B6 dishes to keep cell line in culture (See "SOP Human Induced Pluripotent Stem Cell (hiPSC) Single Cell Maintenance Protocol" Part 3.2).
- Monitor the cells the following day and choose the density that contains 10-30 cells/colony. If 10-30 cells/colony is not reached, repeat the test by increasing or decreasing the densities (see Figure 58).

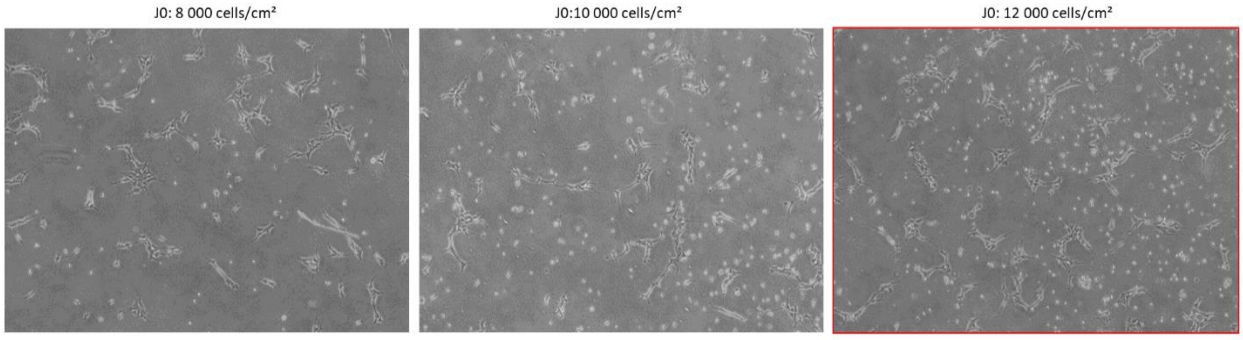

Figure 58 : Cell density test: On J0, densities 8 000 cells/cm² and 10 000 cells/cm² were not selected because they contain very few colonies of 10-30 cells. Density 12 000 cells/cm² was selected because it contains enough colonies of 10-30 cells.

2 Myogenic differentiation

2.1 Preparation of differentiation media

Preparation of medium A, B and C

For the following steps, DMEM/F-12 with HEPES (Gibco, ref: 11330-032), penicillin/streptomycin (Life Technologies, ref: 15140122) and STEMdiff™ Myogenic Progenitor Supplement A (STEMCELL Technologies, ref: 100-0152), STEMdiff™, Myogenic Progenitor Supplement B (STEMCELL Technologies, ref: 100-0153) or STEMdiff™ Myogenic Progenitor Supplement C (STEMCELL Technologies, ref: 100-0154) are required.

- Thaw STEMdiff™ Myogenic Progenitor Supplement A, B or C at room temperature (15 - 25°C). Mix thoroughly.

  ⚠ Do not use a water bath to warm the supplements.

NOTE: If not used immediately, store at 2 - 8°C for up to 4 weeks. Alternatively, aliquot and store at -20°C. Do not exceed the shelf life of the components. After thawing aliquots, use immediately. Do not re-freeze.

- Dilute supplement A, B or C 1:100 in DMEM/F-12 with HEPES + 0.1% penicillin/streptomycin. Mix thoroughly. Warm to room temperature before use.

  ⚠ Do not use a water bath to warm your prepared media.

NOTE: If not used immediately, store at 2 - 8°C for up to 4 weeks.

Preparation of medium D

For the following steps, DMEM/F-12 (with HEPES) (Gibco, ref: 11330-032), penicillin/streptomycin (Life Technologies, ref: 15140122) and STEMdiff™ Myogenic Progenitor Supplement D (STEMCELL Technologies, ref: 100-0155) are required.

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- Thaw STEMdiff™ Myogenic Progenitor Supplement D at room temperature (15 - 25°C) or overnight at 4°C. Mix thoroughly.

  ⚠ Do not use a water bath to warm the supplement.

NOTE: *If not used immediately, aliquot and store at -20°C. Do not exceed the shelf life of the components. After thawing aliquots, use immediately. Do not re-freeze*.

- Dilute supplement D 1:5 in DMEM/F-12 (with HEPES) + 0.1% penicillin/streptomycin. Mix thoroughly. Warm to room temperature before use.

  ⚠ Do not use a water bath to warm your prepared medium.

NOTE: *If not used immediately, store at 2 - 8°C for up to 4 weeks*.

2.2 Differentiation protocol

The following protocol allows the transition from human induced pluripotent cells to myogenic progenitors in 30 days. Four media are required for the differentiation protocol: *medium A* on days 0 and 1, *medium B* on days 2 and 3, *medium C* on days 4 and 5 and medium D from day 6 to the end of the differentiation (See Figure 59). At day 30, an *Expansion medium* is required for amplification of the differentiated cells (See Part 3).

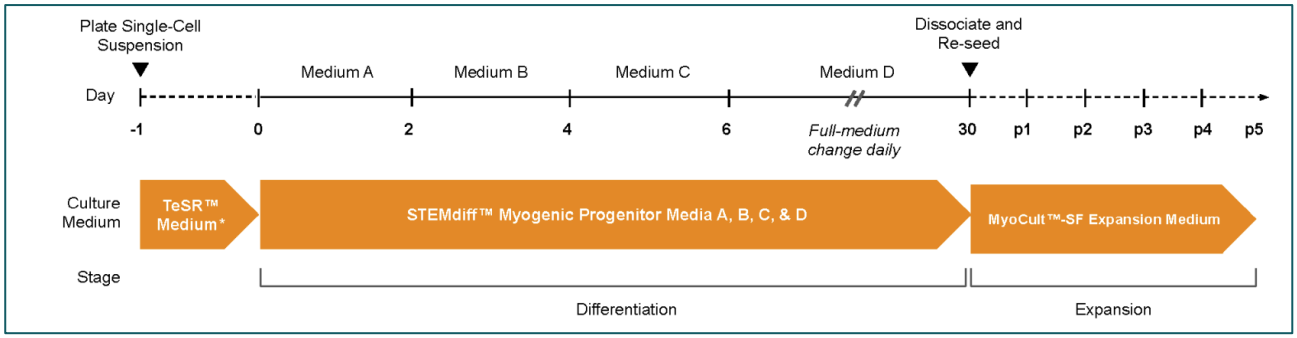

Figure 59. Protocol diagram.

Medium change is mandatory when switching from one medium to another on days 2, 4, and 6 which is why coming on the weekend on differentiation launch week is inevitable. For example, to plate cells on Monday and start differentiation protocol on Tuesday, change to medium C on Saturday (See Figure 60).

Figure 60. Example of chronological protocol diagram of differentiation with media volume.

The following protocol concerns differentiation protocol into a 6-wells plate. If using alternative culture support, adjust volumes accordingly.

Day-1: Cell plating

- Carry out seeding hiPS cells in a 6-wells plate according to the "1.3 hiPS cell density test".

NOTE: *Even if the density test has been carried out, it is recommended to test close densities to make sure to have the right cell distribution at D0.*

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Day 0-1: Shift and refresh of Medium A

On day 0, monitor cells under the microscope (see Figure 61), if cell density is sufficient (10-30 cells/colony):

- Prepare medium A for 2 days: 2 mL/well/day (See Part 2.1.1 of this protocol).
- Bring to room temperature the prepared medium A.

  ⚠ Do not use a water bath to warm your prepared medium.
- Discard medium from the wells and display 2 mL/well of medium A.
- Incubate cells 24h at 37°C, 5% CO_2.
- Store the remaining medium A at +4°C.

On day 1, monitor cells under the microscope (see Figure 62), and observe cell morphology. Cultures should contain small colonies. Then:

- Bring to room temperature medium A prepared the day before.

  ⚠ Do not use a water bath to warm your prepared medium.
- Discard medium from the wells and display 2 mL/well of medium A.
- Incubate cells 24h at 37°C, 5% CO_2.

NOTE: If the medium cannot be refreshed on day 1, add twice the volume of medium A on day 0.

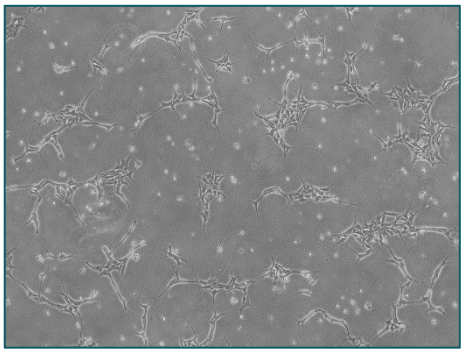

Figure 61. Day 0 of hiPSC culture from STEMCELL Technologies protocol.

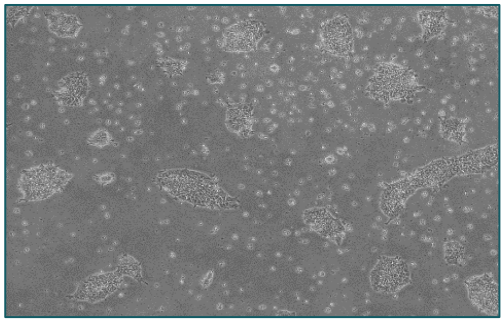

Figure 62. Day 1 of hiPSC myogenic differentiation

Day 2-3: Shift and refresh of Medium B

On day 2, monitor cells under the microscope (see Figure 63) and assess cells for confluency. Cultures **should be approximately 80% confluency**.

⚠ If cells are <80% confluent, do not continue protocol; adjust hiPSC seeding density and plate cells to obtain optimal confluency.

Then:

- Prepare medium B for 2 days: 2 mL/well/day (See Part 2.1.1 of this protocol).
- Bring to room temperature the prepared medium B.

  ⚠ Do not use a water bath to warm your prepared medium.
- Discard medium from the wells and display 2 mL/well of medium B.
- Incubate cells 24h at 37°C, 5% CO_2.
- Store the remaining medium B at +4°C.

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On day 3, monitor cells under the microscope (see Figure 64) and:

- Bring to room temperature medium B prepared the day before.

  ⚠ Do not use a water bath to warm your prepared medium.
- Discard medium from the wells and display 2mL/well of medium B.
- Incubate cells 24h at 37°C, 5% CO_2.

NOTE: If the medium cannot be refreshed on day 3, add twice the volume of medium B on day 2.

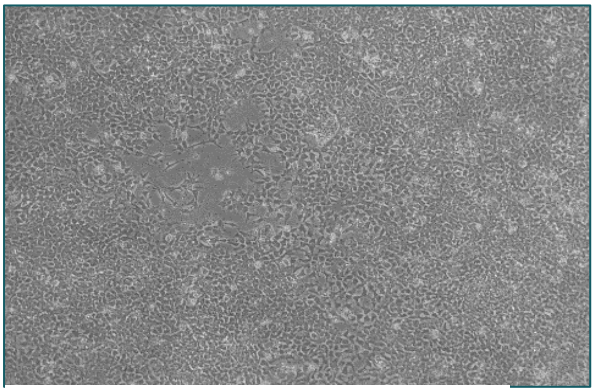

Figure 63. Day 2 of hiPSC myogenic differentiation with confluence >80%.

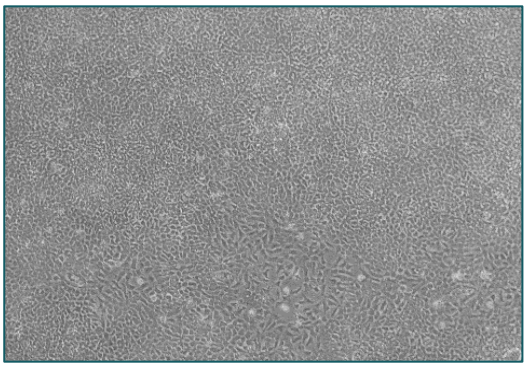

Figure 64. Day 3 of hiPSC myogenic differentiation.

Day 4-5: Shift and refresh of Medium C

On day 4, monitor cells under a microscope and:

- Prepare medium C for 2 days: 2 mL/well/day (See Part 2.1.1 of this protocol).
- Bring to room temperature the prepared medium C.

  ⚠ Do not use a water bath to warm your prepared medium.
- Discard medium from the wells and display 2 mL/well of medium C.
- Incubate cells 24h at 37°C, 5% CO_2.
- Store the remaining medium C at +4°C.

On day 5, monitor cells under the microscope (see Figure 65) and:

- Bring to room temperature medium C prepared the day before.

  ⚠ Do not use a water bath to warm your prepared medium.
- Discard medium from the wells and display 2mL/well of medium C.
- Incubate cells 24h at 37°C, 5% CO_2.

NOTE: If the medium cannot be refreshed on day 5, add twice the volume of medium C on day 4.

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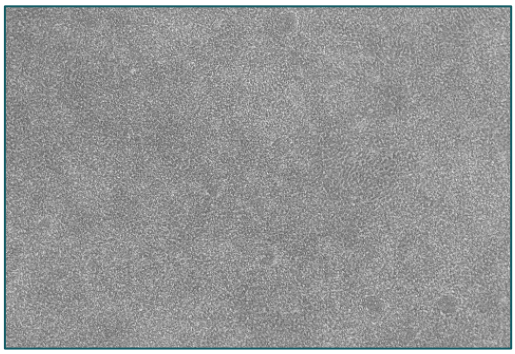

Figure 65. Day 5 of hiPSC myogenic differentiation.

Day 6-29: Shift in Medium D

On day 6, monitor cells under a microscope and:

- Prepare medium D for 24 days: 2 mL/well/day (See Part 2.1.2 of this protocol).
- Bring to room temperature the prepared medium D. **⚠ Do not use a water bath to warm your prepared medium.**
- Discard medium from the wells and display 2 mL/well of medium D.
- Incubate cells 24h at 37°C, 5% CO_2.

NOTE: Formation of 3D structures should be present between day 7 and day 10. During this time, cells can detach from the support at the edges of the wells but will re-adhere quickly. To avoid total detachment, discard and add the medium D very carefully by using a 1000 µL pipette starting day 7.

From day 7 to day 29, monitor cells under the microscope (see Figure 66-68) and refresh to medium D every day until day 29:

- Bring to room temperature the medium D already prepared.

  ⚠ Do not use a water bath to warm your prepared medium.
- Discard medium from the wells and display 2 mL/well of medium D.
- Incubate cells at 37°C, 5% CO_2.

NOTE 1: Cells should develop into a dense, multi-layered, and tightly packed culture (Figures 64 and 65). Medium will turn yellow during the later stages of the culture period (Day 10 onward).

NOTE 2: To avoid changing medium on weekends add 6 mL/well on Friday afternoons and change to 2 mL/well on Monday mornings.

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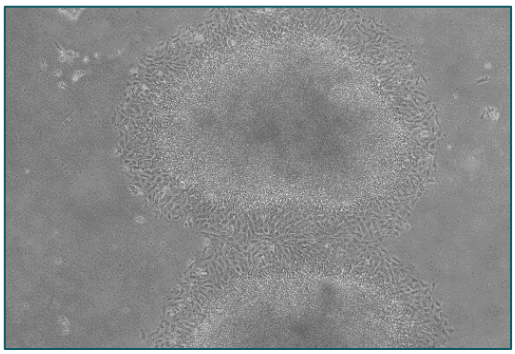

Figure 67. Day 7 of hiPSC myogenic differentiation.

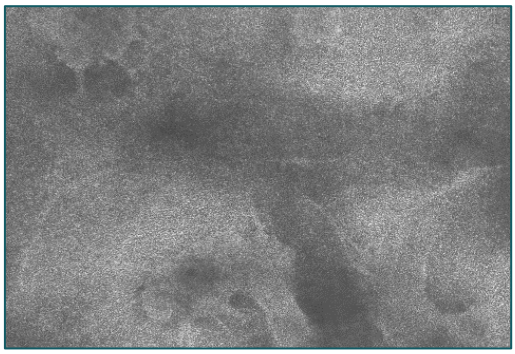

Figure 66. Day 24 of hiPSC myogenic

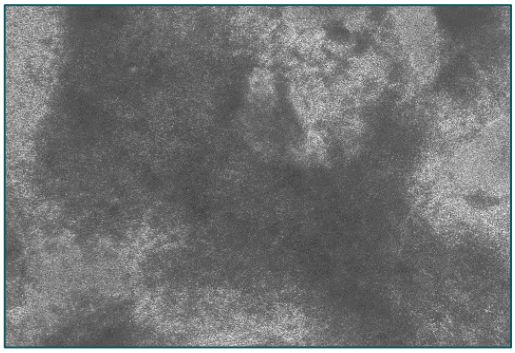

Figure 68. Day 30 of hiPSC myogenic differentiation.

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3 D-30: Dissociation and first passage

At day 30, the myogenic differentiation protocol is ending. The following procedure describes the first passage of the differentiated cells from the 6-well plate to the T225 flask, which corresponds to **passage P1**. The passages after P1 allow cell expansion and refer to another protocol untitled ''Expansion of hiPSC Derived Myoblasts StemCell Protocol''.

3.1 Preparation of medium

Expansion medium

For the following steps, DMEM with 1000 mg/L D-Glucose medium (STEMCELL Technologies, ref: 36253) and MyoCult™-SF Expansion 10X Supplement (Human) (STEMCELL Technologies, ref: 05982) are required. The medium needs to be prepared extemporaneously for each manipulation.

- Thaw MyoCult™-SF Expansion 10X Supplement (Human) at room temperature. Mix thoroughly. **⚠ Do not use a water bath to warm the supplement.**
- Before the experiment, calculate the volumes of DMEM medium and MyoCult™-SF Expansion 10X Supplement (Human) needed to obtain a 1X Supplement solution.

  NOTE: For example, for a complete T225 flask, prepare 28.5 mL of expansion medium: 28.5 mL / 10 = 2.85 mL of MyoCult™-SF Expansion 10X Supplement (Human). 28.5 mL - 2.85 mL = 25.65 mL of DMEM medium.
- Prepare your medium mix in a sterile tube or sterile bottle. Mix thoroughly.

  NOTE: For the DMEM medium, use either reference from STEMCELL Technologies (Ref: 36253) or Gibco (Ref: 11885084), compositions are the same and there is no notifiable difference in the growth of the cells.

Preparation of Collagenase IV

- Thaw Collagenase IV (1000 mg/mL) overnight at 2-8°C (STEMCELL Technologies, Ref: 7909). Mix thoroughly.
- Dispense into aliquots (1 mL) and store at -20°C.

Preparation of ROCK inhibitor (Y-27632) aliquot of 10mM

- Go to bio-techne page: https://www.bio-techne.com/p/small-molecules-peptides/y-27632-dihydrochloride 1254.
- In the part "Preparing Stock Solutions for Y-27632 dihydrochloride", enter the number of batches and add the appropriate volume of DMSO to have a 10 mM of stock solution.
- Prepare in advance "stocks" aliquots of Y-27632 Rock inhibitor [10 mM] to avoid freezing and thawing cycles (Example: 20 µL/aliquot).

  NOTE: ROCK inhibitor is used at 10 µM, corresponding to a 1:1000 dilution, directly in the culture medium.

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3.2 First passage (P1) of myogenic progenitors (day 30)

- Coat the appropriate number of T225 with corning Matrigel® (See "Matrigel and Vitronectin Matrix Protocol").

  NOTE: If working with two wells for the differentiation, prepare four T225 flasks (= two T225 per well).

- Write the cell name, date, and passage number **(P1)** on the coated flasks with Matrigel®.
- Prepare an adequate volume of MyoCult™-SF Expansion Medium (See Part 3.1 of this protocol).
- Remove the medium from the 6-wells plate. Gently wash the cells with 2 mL of D-PBS (Without Ca^{2+} and Mg^{2+}) per well. Remove and discard D-PBS.
- Add 0.5 mL/well of TrypLE™ Express. Incubate at 37°C and 5% CO_2 for 6 minutes.
- Add 200 µL/well of Collagenase Type IV (STEMCELL Technologies, Ref: 7909). Using a sterile 1000µL pipette, gently triturate culture by pipetting up and down for 2 minutes. Incubate at 37°C and 5% CO_2 for 4 minutes.
- Add 3 mL/well of DMEM/F-12 (with HEPES) medium. Continue to triturate culture by pipetting for 1-2 minutes with the 1000 µL pipette tip.
- Using a 5 mL serological pipette, transfer the cell suspension to a 70 µm strainer placed on top of a 50 mL sterile tube. Wash the well with an additional 1 mL of DMEM/F-12 medium and transfer it to the strainer.
- Centrifuge at 300g for 5 minutes.
- Remove supernatant carefully with a pipette-Aid or cell culture pump without disturbing the pellet.
- Add to the pellet 1 mL of warmed at room temperature MyoCult™-SF Expansion Medium **supplemented with rock inhibitor 10 µM Y-27632.** Mix gently to resuspend the pellet. ⚠ **Do not use a water bath to warm your prepared medium.**
- Perform a cell count using Trypan Blue and an automated cell counter:
  - Make a mix of 20 µL of cell solution and 20 µL of trypan blue.
  - Load this mix in a counting slide and assess the cell concentration using a cell counter.
  - If the concentration exceeds 3 000 000 cells/mL, add more medium to your cells to dilute them.

    NOTE 1: In our lab, we use a Countess II automated cell counter.
    NOTE 2: Expected cell yield is approximately 1 - 4 x 10^6 cells/well. If there is a very high percentage of dead cells, do not worry.
    NOTE 3: Cell counting is only informative and does not influence seeding volumes.
- Put 28 mL of MyoCult™-SF Expansion Medium **with 10 µM ROCK inhibitor Y-27632** (dilution 1:1000 of the solution stock 10 mM) in each flask and add an appropriate volume of cell suspension.

  NOTE: Split the cell suspension by the number of wells performed for the differentiation. For example, for 4 mL of cell suspension and have 2 wells out of a 6-wells plate, add 1 mL of cell suspension in four T225 flasks.
- Move the flask in several quick, short, back-and-forth and side-to-side motions to ensure uniform distribution of cells.
- Incubate the flasks at 37°C and 5% CO_2 for 24h.

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- The next day change the medium to warm at room temperature MyoCult™-SF Expansion **without ROCK inhibitor.**

  ⚠ Do not use a water bath to warm your prepared medium.
- Monitor every day the morphology of the cells (see Figure 69) and change the medium every 2 days.
- Once the cells reach 80% confluency, follow "Expansion of hiPSC Derived Myoblasts StemCell Protocol" for myoblasts expansion, cell cryoconservation and dry pellet assessing for RNA or DNA extraction.

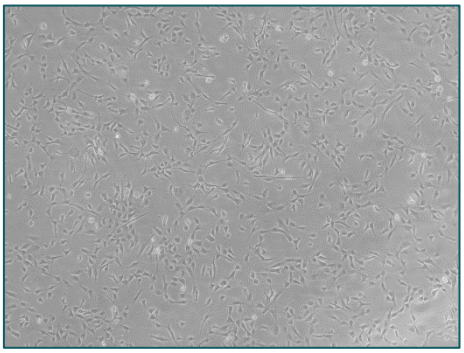

Figure 69. Myoblasts cell morphology at P1 D+5.

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Annex H - Myoblast Culture

Figure 70. Chronological representation of main steps for cell culture

1 References

Table 26. References of different media and supplements used during the protocol

Article	Supplier	Reference	Link
MyoCult™-SF Expansion 10X Supplement (Human)	STEMCELL Technologies	05982	Product
DMEM with 1000 mg/L D-Glucose	STEMCELL Technologies	36253	Product
DMEM/F-12, GlutaMAX™ Supplement	Gibco	31331028	Product
Optionnal : DMEM, low glucose, pyruvate	*Gibco*	*11885084*	*Product*
Corning® Matrigel® hESC-Qualified Matrix, LDEV-free, 5 mL	Corning	354277	Product

2 Matrigel coating

All myoblast cells need Matrigel coating as a support for culture.

2.1 Preparation of Matrigel aliquots

Warning: Matrigel sets very quickly if it is not cold. **Always work on ice!**

- Thaw the bottle of Corning® Matrigel® hESC-Qualified Matrix, LDEV-free, 5 mL (Corning, ref. 354277) in a tray filled with ice at 4°C overnight.
- Place sterile tips and sterile Eppendorf to use for aliquoting at -20°C overnight.
- On Corning's website, click on ''quality certificate". Enter the product reference and batch number of your Matrigel's bottle and click on "Download certificate". In this certificate is indicated the "Dilution factor", divide it by two, to obtain the volume in µL to aliquot per Eppendorf.

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- For the aliquoting, use cold tips and cold Eppendorf. Keep Matrigel on ice and place your Eppendorf on ice even when they are empty.
- Immediately stock Matrigel's aliquots at -20°C.

2.2 Matrigel's coating of cell culture support

For this part, please refer to "Matrigel and Vitronectin Matrix Protocol".

3 Preparation of myoblasts culture medium

For the following steps, DMEM with 1000 mg/L D-Glucose medium (STEMCELL Technologies, ref. 36253) and MyoCult™-SF Expansion 10X Supplement (Human) (STEMCELLTechnologies, ref. 05982) are needed.

The medium needs to be prepared extemporaneously for each manipulation.

- Thaw MyoCult™-SF Expansion 10X Supplement (Human) at room temperature. Mix thoroughly.
- Before the experiment, calculate the volumes of DMEM medium and MyoCult™-SF Expansion 10X Supplement (Human) needed to obtain a 1X Supplement solution.
    - Example: for a complete 96-well plate with 100 µL of medium per well, prepare:
      (96 wells + 10 security wells) x 100 µL = 10.6 mL of medium mix
      10.6 mL / 10 = 1.06 mL of *MyoCult™-SF Expansion 10X Supplement* (Human)
      10.6 mL - 1.06 mL = 9.54 mL of *DMEM medium*
- Prepare your medium mix in a sterile Falcon or bottle. Mix thoroughly.

NOTE: for the DMEM medium, use either reference from STEMCELL (ref. 36253) or Gibco (ref. 11885084), compositions are the same and there are no notifiable differences in the growth of the cells.

4 Thawing of myoblasts cells

Before thawing:

- Prepare the appropriate volume of the expansion medium (see part 3.1 Preparation of Expansion medium) and incubate it at room temperature (**never warm it in a warm bath !**).
- Prepare coated cell culture support (see part 2. Matrigel coating) and write the name of the cell, number of passages (Pn+1), date and name of the preparer.
- Warm water bath at 37°C.
- Prepare a Falcon with the name of the cell line written on it and add 4,5 mL of room-temperature Expansion medium to it.

- Collect cryovial from nitrogen and place it in dry ice.
- Thaw the cryovial by placing it in the warm bath pre-heated at 37°C.
- When the cryovial is completely thawed, transfer the entire volume (500 µL) of the vial in the Falcon pre-filled with 4,5 mL of Expansion medium.
- Centrifuge 5 minutes at 1200 rpm.
- Aspirate supernatant and resuspend the pellet in 1 mL of Expansion medium.

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- Perform a numeration of the cells.
- Prepare a mix of cells + Expansion medium at the desired concentration(s) for seeding.

  NOTE: See the "*Specific cell line*_informations" file for seeding information.
- Discard the coating remaining volume of the cell culture support to use for seeding.
- Seed cells in prepared cell culture support.
- Incubate at 37°C, 5% CO_2.

  NOTE: Change medium every 2 days to keep the cells in culture for more than 3 days.

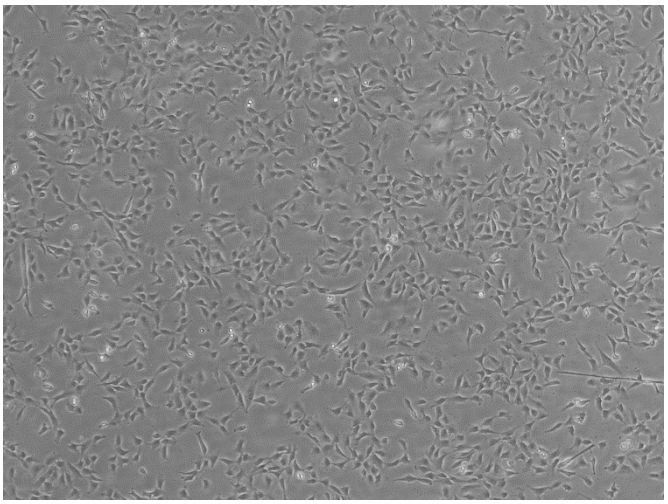

Figure 71 : Example of myoblasts morphology

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Annex I - Myoblast/Myotube Immunofluorescence

1 References & experiment objectives

1.1 Product references

Table 27. References of different products used during the protocol.

Products	Suppliers	References
Dulbecco's Phosphate Buffered Saline without calcium chloride and magnesium chloride (D-PBS)	Sigma-Aldrich	D8537
Paraformaldehyde 16% Aqueous Solution EM Grade	Electron Microscopy Sciences	15710
Triton™ X-100	Sigma-Aldrich	X100
Bovine Serum Albumin	Sigma-Aldrich	A9647
Corning® 96-well Flat Clear Bottom Black Polystyrene TC-treated Microplates	Corning	3904

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Table 28. References of primary antibodies used for the protocol

Antibody	Suppliers	References	Specificity	Isotype	Species	Dilution	Diluent
DESMIN, clone 1H16	R&D System	AF3844	Human	IgG	Goat	1/200	Saturation buffer
MYH1E Antibody (MF 20)	DSHB	MF20-c	Human	MIgG2b	Mouse	1/50	
MYOD antibody [HL1372]	Abcam	ab307805	Human	IgG	Rabbit	1/1000	
MYOG Antibody (F5D)	DSHB	F5D	Human	MIgG1	Mouse	1/50	
PAX7 Antibody	DSHB	PAX7	Human	MIgG1	Mouse	1/50	
CD56 Antibody, anti-human, PE, REAfinity™	Miltenyi	130-113-312	Human	IgG1	Recombinant	1/100	

Table 29. References of secondary antibodies used for the protocol

Antibody	Suppliers	References	Specificity	Isotype	Species	Dilution	Diluent
Donkey anti-Goat Secondary Antibody, Alexa Fluor™ 488	ThermoFisher Scientific	A-11055	Goat	IgG	Donkey	1/1000	Saturation buffer
Donkey anti-Mouse Secondary Antibody, Alexa Fluor™ 488	ThermoFisher Scientific	A-21202	Mouse	IgG	Donkey	1/1000	
Donkey anti-Rabbit Secondary Antibody, Alexa Fluor™ 488	ThermoFisher Scientific	A-21206	Rabbit	IgG	Donkey	1/1000	
Hoechst 33342, trihydrochlorure, trihydrate	ThermoFisher Scientific	H3570	-	-	-	1/2000	
REA Control Antibody (S), human IgG1, PE, REAfinity (= IgG-PE)	Miltenyi	130-113-434	-	IgG	-	1/100	

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1.2 Experiment objectives

Once the hiPS cells have been differentiated into myogenic progenitor cells using the STEMCELL protocol, it is mandatory to check the expression of myogenic cell markers. The following procedure concerns the staining of the myogenic markers DESMIN, MF-20, MYOG, MYOD, PAX7 and CD56 using immunofluorescence.

For this procedure, myoblast cells must be seeded beforehand in two 96-well black plates (Corning, #3904) coated with matrigel at densities ranging from 10,000 to 15,000 cells/cm². When both plates reached 80% of confluency one plate is fixed with ParaFormAldehyde (PFA, see parts 2.1 and 2.2 for recipes and 3.1 for technical explanations) to stain myoblasts (myoblasts plate) and the other is used for the final differentiation of 7 days into myotubes (myotubes plate). This second plate will be fixed following the same protocol as the myoblasts plate. For these steps, please refer to «*Matrigel and Vitronectin Matrix Protocol*», «*Cell Culture Protocol and References for MyoBlasts cells*» and «*Cell Culture Protocol for Myotubes Cells Derived from hiPSC*» protocols.

In the following procedure, 10 conditions (DESMIN, MF20, MYOD, MYOG, PAX7, CD56, unstained goat antibody, unstained mouse antibody, unstained rabbit antibody, CD56 control IgG-PE) are tested in 10 different wells in both myoblasts and myotubes plate. It is highly recommended to seed extra wells with cells as myoblasts and myotubes are very fragile and can detach from the support.

2 Preparation of solution

2.1 8% paraformaldehyde (8% PFA)

/!\ Manipulate paraformaldehyde under a chemical hood. /!

- Dilute Paraformaldehyde 16% Aqueous Solution EM Grade (Electron Microscopy Sciences, #15710) in D-PBS (Sigma-Aldrich, #D8537) to obtain a solution at 4%.

  For example, to prepare 40 mL of PFA 8%, add 20 mL of PFA 16% into 20 mL of D-PBS.
- Solution can be stored at +4°C up to one month. **Cool PFA at RT before use**.

2.2 4% paraformaldehyde (4% PFA)

/!\ Manipulate paraformaldehyde under a chemical hood. /!

- Dilute Paraformaldehyde 8% prepared in 2.1 in D-PBS (Sigma-Aldrich, #D8537) to obtain a solution at 2%.

  For example, to prepare 40 mL of PFA 4%, add 20 mL of PFA 4% into 20 mL of D-PBS.
- Solution can be stored at +4°C up to one month. **Cool PFA at RT before use**.

2.3 Permeabilization buffer (Triton 0.1%)

- Dilute Triton™ X-100 (Sigma-Aldrich, #X100) in D-PBS to obtain a solution at 0.1%.

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For example, to prepare 40 mL of Triton 0.1%, add 40 µL of Triton™ X-100 into 39.6 mL of D-PBS.

- Solution can be stored at RT.

2.4 Saturation buffer (D-PBS + 1% BSA)

- Weight Bovine Serum Albumin (BSA) powder (Sigma-Aldrich, #A9647) to prepare a 1% solution and add the appropriate volume of D-PBS.

  For example, to prepare 10 mL of saturation solution, weigh out 0.1g of BSA and add 10 mL of D-PBS.

  Note: to allow quicker dissolution of BSA in D-PBS, incubate the solution at +4°C for 5-10 minutes.
- Solution can be stored at +4°C for 1-2 weeks.

3 Staining protocol

The following protocol has been developed for the use of high-throughput imager ImageXpress type. We therefore seed myoblast cells in two 96-well plates as support. If using another device and need to use other support, please adjust the volumes and consumables accordingly.
As the myoblasts and myotubes are very fragile, please be gentle during all stages of the protocol to prevent cell detachment.

3.1 Pre-fixation and fixation

- Prepare 8% paraformaldehyde according to part 2.1 of this protocol.
- Prepare 4% paraformaldehyde according to part 2.2 of this protocol.
- **Carefully** aspirate the supernatant and add 50 µL of D-PBS/well.

  To avoid cell detachment do not place the D-PBS directly on the cells but on the side of the wells.
- Under a chemical hood, to prefix the cells, **carefully** add 50 µL of 4% paraformaldehyde previously prepared.

  To avoid cell detachment do not place the PFA directly on the cells but on the side of the wells.
- Incubate for 10 minutes at room temperature under chemical hood.
- Under a chemical hood**, carefully** add 100 µL of 8% paraformaldehyde previously prepared.

  To avoid cell detachment do not place the PFA directly on the cells but on the side of the wells.
- Incubate for 10 minutes at room temperature under chemical hood.
- **Carefully** aspirate and discard the paraformaldehyde in a specific CMR waste container and wash the cells 3 times with 100 µL of D-PBS.

  To avoid cell detachment do not place the D-PBS directly on the cells but on the side of the wells.

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- After the last washing step, **carefully** add 100 µL of D-PBS.
  To avoid cell detachment do not place the D-PBS directly on the cells but on the side of the wells.
- Check under a microscope the presence of cells in the wells.
- If staining is not carried out the same day, seal the plates with Parafilm® and store them at 4°C for 2 weeks.
- Table 30 presents an overview of the different treatment applications according to each target.

Table 30. Overview of the following steps

Target	Permeabilization	Saturation	Primary antibody host	Secondary antibody	Control
CD56	No	Yes	Already coupled	None	IgG-PE
DESMIN	Yes	Yes	Goat	Anti-goat	No primary + anti-goat
MF20	Yes	Yes	Mouse	Anti-mouse	No primary + anti-mouse
MYOD	Yes	Yes	Rabbit	Anti-rabbit	No primary + anti-rabbit
MYOG	Yes	Yes	Mouse	Anti-mouse	No primary + anti-mouse
PAX7	Yes	Yes	Mouse	Anti-mouse	No primary + anti-mouse

3.2 Permeabilization (Triton 0.1%)

This step is only carried out for DESMIN, MF20, MYOG, MYOD and PAX7 markers and their unstained controls.

- Before this step, design a plate plan.
- Note that for CD56, permeabilization is not needed as it is an outer surface marker. For this condition, follow the protocol but add D-PBS instead of a permeabilization buffer.
- **Carefully** aspirate the D-PBS and add 100 µL of permeabilization buffer (Triton 0.1%, see part. 2.3 for recipe) on wells according to the plates plan.
  To avoid cell detachment do not place the permeabilization buffer directly on the cells but on the side of the wells.
- Incubate for 10 minutes at room temperature.
- After incubation, do not wash the cells; go straight to the saturation step.

3.3 Saturation (D-PBS + 1% BSA)

- **Carefully** aspirate the permeabilization buffer or D-PBS and add 100 µL of saturation buffer (D-PBS + 1% BSA) previously prepared according to the plates plan.
  Do not put directly the saturation buffer on the cells as it can cause detachment.
- Incubate 1h at room temperature.
- During saturation, prepare the different primary antibody solutions, counting 60 µL of solution per well:
  - DESMIN: 1:200 of DESMIN (R&D Systems, #AF3844) antibody in saturation buffer.

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For example, if assessing 2 wells: Add 0.6 µL of DESMIN antibody in 119.4 µL of saturation buffer.

- MF20: 1:50 of MF20 (DSHB, #MF20-c) antibody in saturation buffer.

  For example, if assessing 2 wells: Add 2.4 µL of MF20 antibody in 117.6 µL of saturation buffer.
- MYOD: 1:1000 of MYOD (Abcam, #ab307805) antibody in saturation buffer.

  For example, if assessing 2 wells: Add 0.12 µL of MYOD antibody in 119.9 µL of saturation buffer.
- MYOG: 1:50 of MYOG (DSHB, #F5D) antibody in saturation buffer.

  For example, if assessing 2 wells: Add 2.4 µL of MYOG antibody in 117.6 µL of saturation buffer.
- PAX7: 1:50 of PAX7 (DSHB, #PAX7) antibody in saturation buffer.

  For example, if assessing 2 wells: Add 2.4 µL of PAX7 antibody in 117.6 µL of saturation buffer.

Prepare CD56-PE antibody away from light.

- CD56 anti-PE: 1:100 of CD56 (Miltenyi, #130-113-312) antibody in saturation buffer.

  For example, if assessing 2 wells: Add 1.2 µL of CD56 antibody in 118.8 µL of saturation buffer.

3.4 Staining with DESMIN, MF20, MYOD, MYOG, PAX7 and CD56 antibodies.

Day 1: Primary antibody

- **Carefully** aspirate the saturation buffer.
- **Carefully** add 60 µL of previously prepared antibody solution, one antibody per well according to the plate plan.

  Do not add the antibody solution directly to the cells as it can cause detachment, but carefully on the edge of the well. Because some antibodies are of the same species, do not label the markers.
- Because we use antibodies from 3 different species, **carefully** add 60 µL of saturation buffer in three wells to perform negative controls (1 unstained well for each species).

  Do not add the saturation buffer directly on the cells as it can cause detachment, but carefully on the edge of the well.
- Because the CD56 antibody is coupled to a fluorochrome, seal and protect plates from light, and incubate overnight at 4°C.

Day 2: Secondary antibody

- **Protected from light, carefully** aspirate the primary antibody solution from the wells.
- **Protected from light, carefully** wash 3 times with 100 µL of D-PBS.

  To avoid cell detachment do not place D-PBS directly on the cells but on the side of the wells.
- **Protected from light, carefully** add 100 µL of secondary antibody mix :

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- DESMIN wells: 1:1000 of Donkey anti-Goat-488 (ThermoFisher Scientific, #A-11055) + 1:2000 Hoechst 33342, trihydrochlorure, trihydrate (ThermoFischer Scientific, #H3570) in saturation buffer.
- MF20, MYOG and PAX7 wells: 1:1000 of Donkey anti-Mouse-488 (ThermoFisher Scientific, #A-21202) + 1:2000 Hoechst 33342, trihydrochlorure, trihydrate in saturation buffer.
- MYOD wells: 1:1000 of Donkey anti-Rabbit-488 (ThermoFisher Scientific, #A-21206) + 1:2000 Hoechst 33342, trihydrochlorure, trihydrate in saturation buffer.
- CD56 wells: 1:2000 Hoechst 33342, trihydrochlorure, trihydrate in saturation buffer.
- Unstained goat antibody well: 1:1000 of Donkey anti-Goat-488 + 1:2000 Hoechst 33342, trihydrochlorure, trihydrate in saturation buffer.
- Unstained mouse antibody well: 1:1000 of Donkey anti-Mouse-488 + 1:2000 Hoechst 33342, trihydrochlorure, trihydrate in saturation buffer.
- Unstained rabbit antibody well: 1:1000 Donkey of anti-Rabbit-488 + 1:2000 Hoechst 33342, trihydrochlorure, trihydrate in saturation buffer.
- CD56 control IgG-PE well: 1:500 of IgG-PE antibody (REA Control Antibody (S), human IgG1, APC, REAfinity; Miltenyi, #130-113-434) + 1:2000 Hoechst 33342, trihydrochlorure, trihydrate in saturation buffer.

- Incubate 1h at room temperature **protected from light**.
- **Protected from light, carefully** aspirate and wash 3 times with 100 µL of D-PBS.
  To avoid cell detachment do not add D-PBS directly on the cells but on the side of the wells.
- Keep the plates protected from light.
- Acquisition is done on ImageXpress imager at 20X magnification with MetaXpress software using DAPI (Hoechst), FITC (Alexa Fluor™ 488) and Cy3 (PE). For interpretation of the results, see Table 31.

Table 31. Expected results of staining

Biomarkers assessed	Expected expression
CD56	Expressed in both cell types
DESMIN	Higher in myotubes than in myoblasts
MF20	Very high in myotubes
MYOD	Higher in myotubes than in myoblasts
MYOG	Higher in myotubes than in myoblasts
PAX7	Same in myoblasts and myotubes
Unstained goat antibody	No staining on the FITC channel
Unstained mouse antibody	No staining on the FITC channel
Unstained rabbit antibody	No staining on the FITC channel
Unstained CD56	No staining on Cy3 channel

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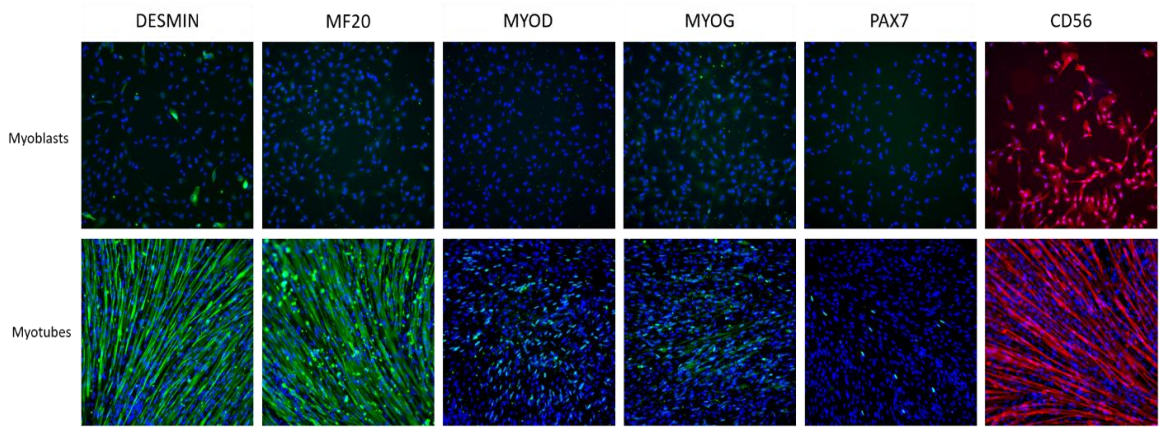

Figure 72. Images from Quality Control Immunofluorescence experiment on myoblasts and myotubes cells

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Annex J - Myoblast Cytometry

1 References & experiment objectives

1.1 Product references

Table 32. References of different products used for the protocol.

Products	Suppliers	References
Dulbecco's Phosphate Buffered Saline without calcium chloride and magnesium chloride (D-PBS)	Sigma Aldrich	D8537
TrypLE Express	ThermoFisher scientific	12605-010
DMEM/F-12, GlutaMAX™Supplement	Gibco	31331028
Fetal Bovine Serum	Sigma Aldrich	F4135
Trypan Blue Solution, 0.4%	Gibco	15250061

Table 33. References of antibodies used for the protocol.

Antibody	Suppliers	References	Specificity	Isotype	Species	Clone	Final Dilution
CD56 Antibody, anti-human, PE, REAfinity	Miltenyi	130-113-874	Human	IgG1	Recombinant	REA196	1/50
CD82 Antibody, anti-human, APC, REAfinity	Miltenyi	130-101-311	Human	IgG1	Recombinant	REA221	1/50
REA Control Antibody (S), human IgG1, PE, REAfinity	Miltenyi	130-113-438	Human	IgG1	Recombinant	REA293	1/11
REA Control Antibody (S), human IgG1, APC, REAfinity	Miltenyi	130-113-434	Human	IgG1	Recombinant	REA293	1/50

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1.2 Experiment objective

The following procedure describes the flow cytometry technique to verify the co-expression of two myoblast surface markers. The aim is to validate myoblasts cells derived from hiPS cells generated using STEMCELL myogenic differentiation protocol.
For this procedure, 200,000 cells are tested per tested condition and have 4 conditions separated in 4 wells. To obtain enough cells (around 1 000 000 cells), we usually seed a B6 dish coated with Matrigel at 15 000 cells/cm², 2-3 days before the experiment, and assess flow cytometry when cells have reached 80% confluency. For these steps, please refer to *"Matrigel and Vitronectin Matrix Protocol" and "Cell Culture protocol and References for MyoBlasts".*

> *NOTE:* Flow cytometry phenotyping is only performed on myoblast cells because myotubes cells are too fragile and burst when detached from the support.

2 Preparation of solution

The solutions and antibody mixes must be prepared extemporaneously.

2.1 Flow cytometry buffer (D-PBS + 2% FBS)

FBS (SIGMA, Ref: F4135) and D-PBS (SIGMA, Ref: D8537) are required for the following steps.
For the preparation of the flow cytometry buffer, please, follow these instructions:

- Thaw FBS at room temperature. Mix thoroughly.
- Before the experiment, calculate the volume of flow cytometry buffer needed.
  For example, if assessing the 4 conditions on one myoblast cell line, prepare 9 mL of flow cytometer buffer.
- In a new 50 mL tube, dilute the FBS in D-PBS 1X to obtain a 2% FBS solution (e.g.: 1 mL of FBS in 49 mL of D-PBS.).
- Store at +4°C and put it on ice when using it to keep it cool.

2.2 Antibodies mixes

For each myoblast cell line, to analyse, 55 µL of three different antibody mixes must be prepared. The first mix only contains a flow cytometer buffer to identify cells of interest, two mixes are FMO (Fluorescence Minus One) controls needed for cytometer calibration and the last mix contains the two specific antibodies to assess the co-expression.

- Mix Unstained: Flow cytometry buffer
- Mix FMO (PE): isotype control IgG1-PE + CD82-APC
- Mix FMO (APC): CD56-PE + isotype control IgG1-APC
- Mix double staining: CD56-PE + CD82-APC

To prepare those mixes, follow the procedure below. The volumes given are for assessing one cell line. If performing more than one cell line at a time, increase the volume accordingly. See Table 33 to use the appropriate final dilutions.

- Put 4 new 0.5 mL tubes on ice.

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- Put the 4 antibody stock tubes on ice (See Table 33 for references).
- Put cold flow cytometry buffer on ice (See part 2.1 for the recipe).
- Annotate the 4 empty tubes writing: Unstained, FMO_PE; FMO_APC or DS.
- Referring to Table 34, add the appropriate volume of cold flow cytometry buffer in each tube (See Table 34).
- Referring to Table 34, add the appropriate volume of each antibody to the corresponding tube.
- Vortex the tubes and keep them on ice, protected from light.

Table 34. Volumes for antibody mix preparation.

	Iso IgG1-PE dilution 1:11 (Miltenyi 130-113-438)	CD56-PE dilution 1:50 (Miltenyi 130-113-874)	Iso IgG1-APC dilution 1:50 (Miltenyi 130-113-434)	CD82-APC dilution 1:50 (Miltenyi 130-101-311)	Flow Cytometry buffer
Tube Mix unstained	/	/	/	/	**55 µL**
Tube Mix FMO_PE	5 µL	/	/	1.1 µL	**48.9 µL**
Tube Mix FMO_APC	/	1.1 µL	1.1 µL	/	**52.8 µL**
Tube Mix DS	/	1,1 µL	/	1.1 µL	**52.8 µL**

3 Staining protocol

- Check under a microscope that myoblast cells have reached 80% confluency.
- Aspirate the supernatant and carefully wash the cells by adding D-PBS to cover the entire dish.
- Aspirate the D-PBS.
- Add 2 mL of TrypLE Express (Gibco, ref: 12605-010) to the cells.
- Incubate the cells with TrypLE Express for 5 minutes at 37°C.
- Meanwhile, write the cell line name on a new sterile 15 mL tube.
- Check the dish under a microscope to be sure that all the cells are detached.
- Carefully dissociate the cells by performing up and down aspirations with a 1000 µL pipette.
- Add 4 mL of DMEM/F-12, GlutaMAX™Supplement (Gibco, ref: 31331028) + 10%-FBS medium.
- Centrifuge 5 minutes at 900 rpm.
- Carefully aspirate the supernatant without disturbing the pellet.
- Resuspend the pellet in 1 to 3mL of DMEM/F-12, GlutaMAX™Supplement+10%-FBS medium with a 1 000 µL pipette and add more medium if needed to ensure that the cell count does not exceed 3 000 000 cells/mL.
- Determine the viable cell count using your method of choice (e.g., Countess Automated Cell Counter).
- Calculate the volume of cell suspension needed to collect 200 000 cells:

$$\left(\frac{cell\ count\ (cells/mL)}{200\ 000\ (cells)} = volume\ to\ collect\ to\ have\ 200\ 000\ cells\ (mL)\right)$$

- Dispense this volume in 4 wells of a 96-well plate. **From this step, cells should remain on ice as much as possible.**

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- Add 1mL of cold flow cytometry buffer in each of these 4 wells.
- Centrifuge the plate for 5 minutes at 900 rpm.
- Discard the supernatant by inverting the plate.
- Resuspend the pellet:
    - 1 well with 50 µL of Mix unstained
    - 1 well with 50 µL of Mix FMO_PE
    - 1 well with 50 µL of Mix FMO_APC
    - 1 well with 50 µL of Mix DS
- Incubate plate for 30 minutes **on ice protected from light.**
- Add 1 mL of cold flow cytometry buffer/well.
- Centrifuge plate 5 minutes at 900 rpm.
- Discard the supernatant by inverting the plate.
- Resuspend the pellet with 200 µL of cold flow cytometry buffer, to avoid bubbles.
- Keep the plate **on ice and protected from light.**
- Samples are ready for flow cytometry acquisition.

4 Flow cytometry acquisition

- Make sure to calibrate the flow cytometer.
- The acquisition required FSC; SSC; PE and APC channels.
- Read the unstained cells to adjust FSC and SSC channel voltages. The population of interest should be in the middle of the graph (See P1 gate in Figure 73). Gate also the single cell population (See P2 gate in Figure 73).
- Read the FMO_PE-stained cells to adjust the PE channel voltage. Those cells determine your negative cells for PE-stained cells. Place the positivity threshold to have between 0.5% and 1% of positive cells.
- Read the FMO_APC stained cells to adjust the APC channel voltage. Those cells determine your negative cells for APC stained cells. Place the positivity threshold to have between 0.5% and 1% of positive cells.
- Using the determined settings, start the acquisition for the 4 wells, and set your stopping gate on P2 with at least 30 000 events. See Figure 73 for the gating strategy.
- A percentage greater than or equal to 90% of double-positive cells for CD56/CD82 is required to admit the pluripotency of the cells.

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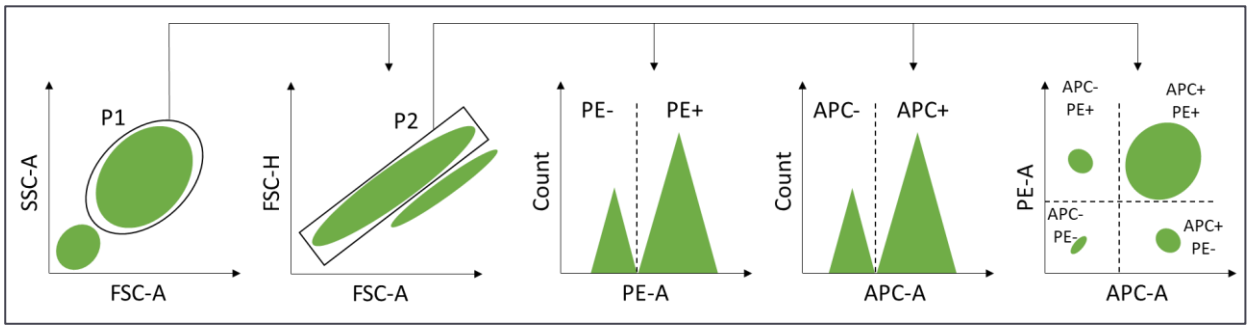

Figure 73. Presentation of the gating strategy to assess the co-expression of CD56 and CD82 on myoblast cells. Green shape = cells; black shape = gate; dotted line = positivity threshold.

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Annex K - Myotube Differentiation

1 References and prerequisites protocols

1.1 Product references

Table 35. References of different media and supplements used during the protocol

Article	Supplier	Reference	Link
MyoCult™ Differentiation Kit (Human)	STEMCELL Technologies	05965	Product
DMEM/F-12, GlutaMAX™ Supplement	Gibco	31331028	Product
Corning® Matrigel® hESC-Qualified Matrix, LDEV-free, 5 mL	Corning	354277	Product

1.2 Prerequisites protocols

Myotubes are derived from myoblasts, so, for this protocol, seed myoblasts in the cell culture support of your choice a minimum **3 days before** starting this experiment (see chronological representation in *Figure 74*). For the thawing and seeding of myoblasts, please refer to the "*Cell Culture Protocol and References for myoblasts cells*" file.

NOTE: Change medium every 2 days if keeping the cells in culture for more than 3 days.

Figure 74: Chronological representation of main steps for cell culture

1.3 Important information

Myotubes **can't** be frozen or passaged, they are **useful only for experiment ends** (Immunofluorescence, RNA extraction, compounds treatment …). Take into consideration the time it takes to obtain these cells in your experiment schedule: a minimum of 10 days to start the experiment from frozen myoblasts.

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2 Preparation of myotubes culture medium

For the following steps, MyoCult™ Differentiation Basal Medium (Human) (STEMCELL Technologies, ref. 05966) and MyoCult™-SF Differentiation 20X Supplement (Human) (STEMCELL Technologies, ref. 05967) from the MyoCult™ Differentiation Kit (Human) (STEMCELL Technologies, ref. 05965) are required.

The medium needs to be prepared extemporaneously for each manipulation.

- Thaw MyoCult™ Differentiation 20X Supplement (Human) at room temperature. Mix thoroughly.
- Before the experiment, calculate the volumes of MyoCult™ Differentiation Basal Medium and MyoCult™ Differentiation Kit (Human) needed to obtain a 1X Supplement solution :
    - Example: for complete 96-well plates with 100 µL of medium per well, prepare :
      (96 wells + 10 security wells) x 100 µL = 10.6 mL of Differentiation medium solution
      10.6 mL / 20 = 0.53 mL of *MyoCult™ Differentiation 20X Supplement* (Human)
      10.6 mL - 0.53 mL = 10.07 mL of *MyoCult™ Differentiation Basal Medium*
- Prepare your medium mix in a sterile Falcon or bottle. Mix thoroughly.

3 Switch of medium from Expansion medium to Differentiation medium

NOTE 1: The myoblasts should be seeded a minimum of **3 days before** this experiment (refer to "Cell Culture Protocol and References for myoblasts cells" and "*Specific cell line*_informations" files for the seeding).
NOTE 2: When the myoblasts reach 80% confluency, proceed to the switch of the medium.

For that, simply remove the Expansion medium from the cells and replace it with an equivalent volume of Differentiation medium.
Incubate the cells for 1 week at 37°C, 5% CO_2 (See morphology *Figures 75-80*).
Check your cells every day to be sure that the process of differentiation is going well.
After 7 days, the myotubes cells are ready to use.

NOTE 1: Change medium every 5 days
NOTE 2: Myotubes **can't** be frozen!

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Images below show the evolution of the morphology from myoblasts to myotubes at different time points:

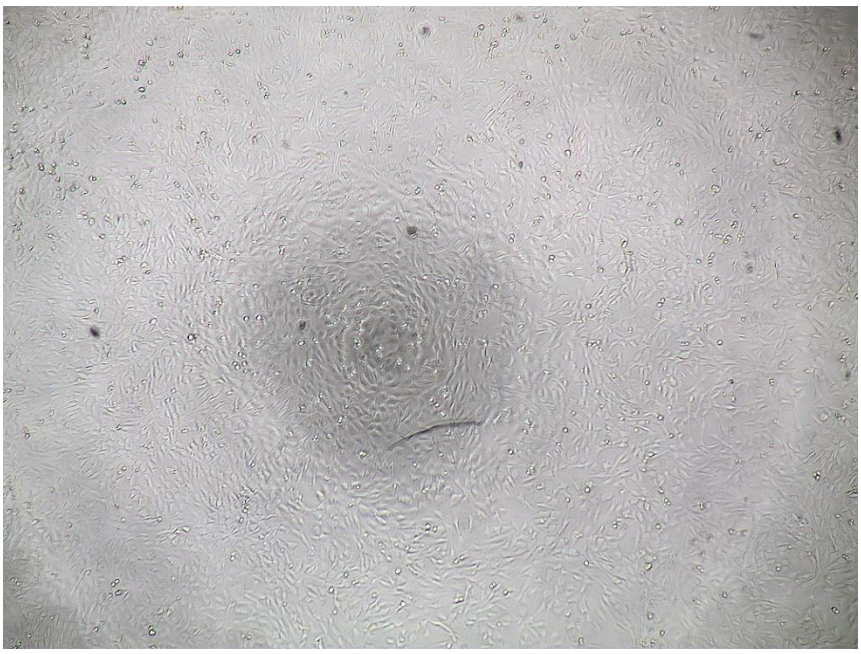

Figure 75 : Myoblasts at 80% confluency ready to be differentiated in myotubes

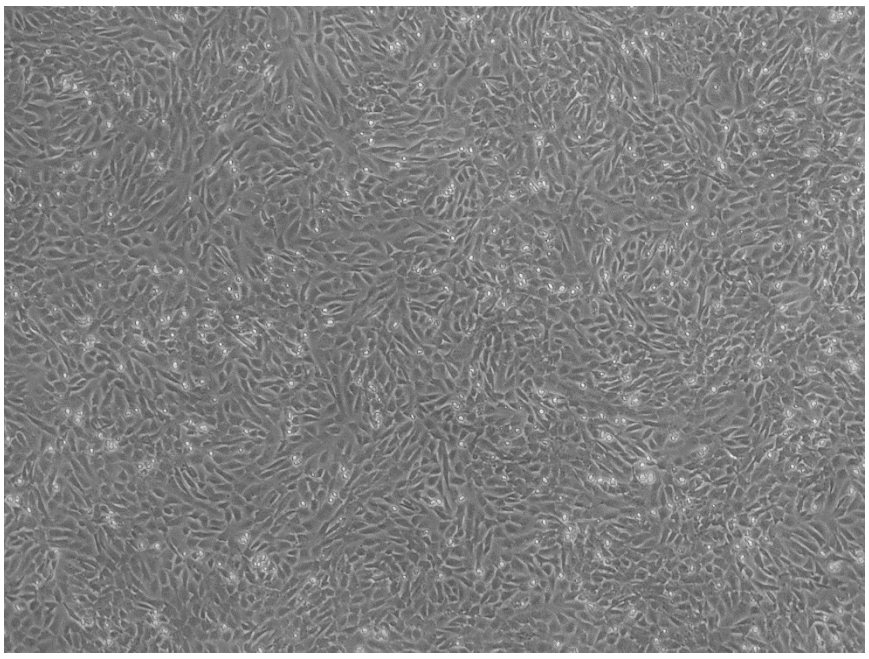

Figure 76 : Cells 1 day after switch of medium - They still have a morphology of myoblasts

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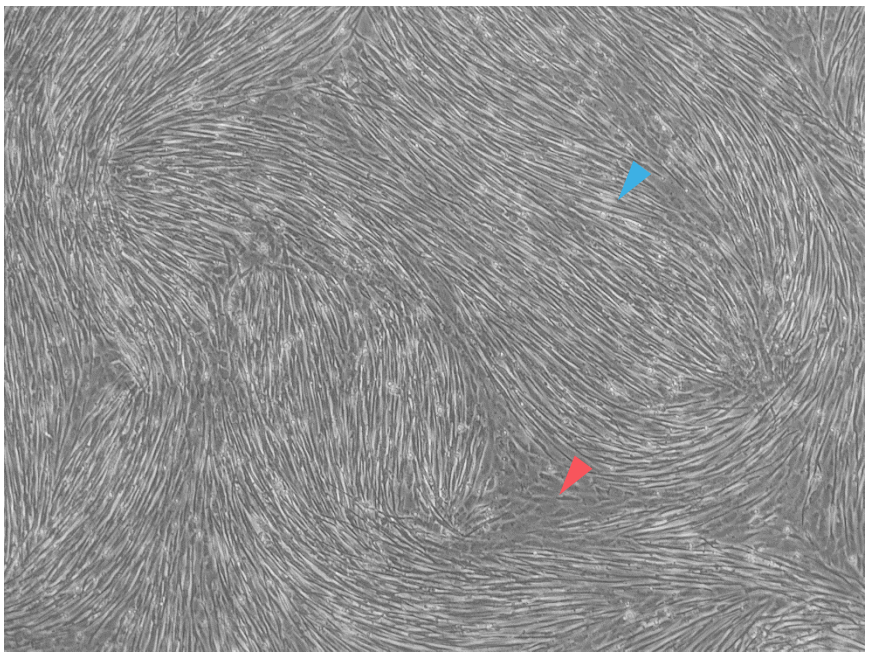

Figure 77 : Cells 4 days after switch of medium - We can see apparition of myotubes.
Legend : : Myotubes : Myoblasts

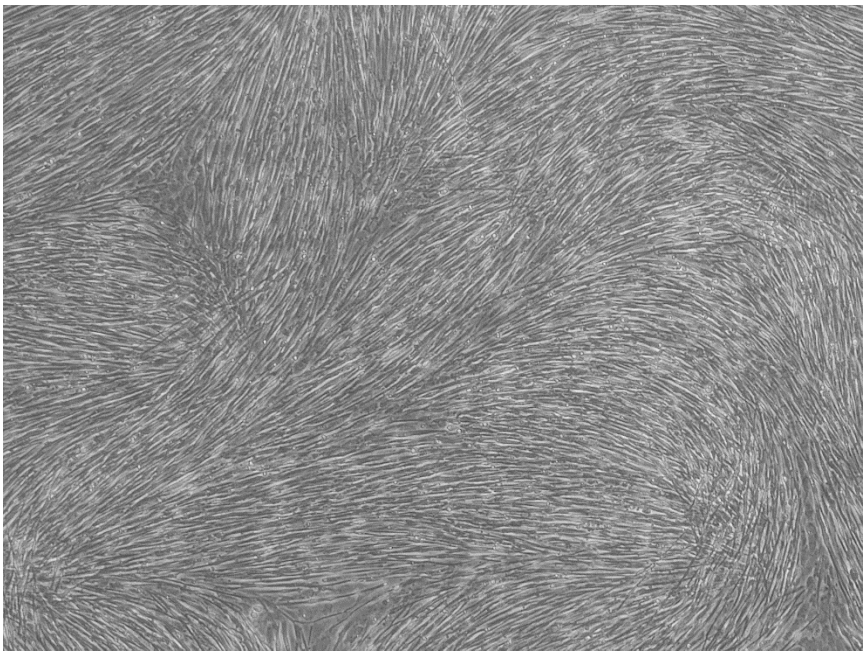

Figure 78 : Cells 5 days after switch of medium - Almost all the cells are differentiated in myotubes

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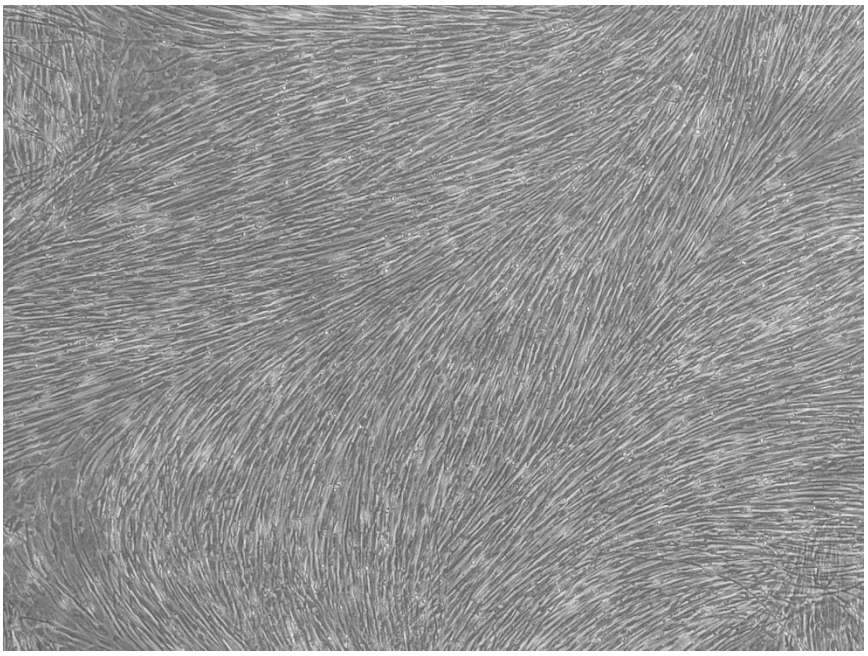

Figure 80 : Cells 6 days after switch of medium - All the cells are differentiated in myotubes and myotubes are well fused

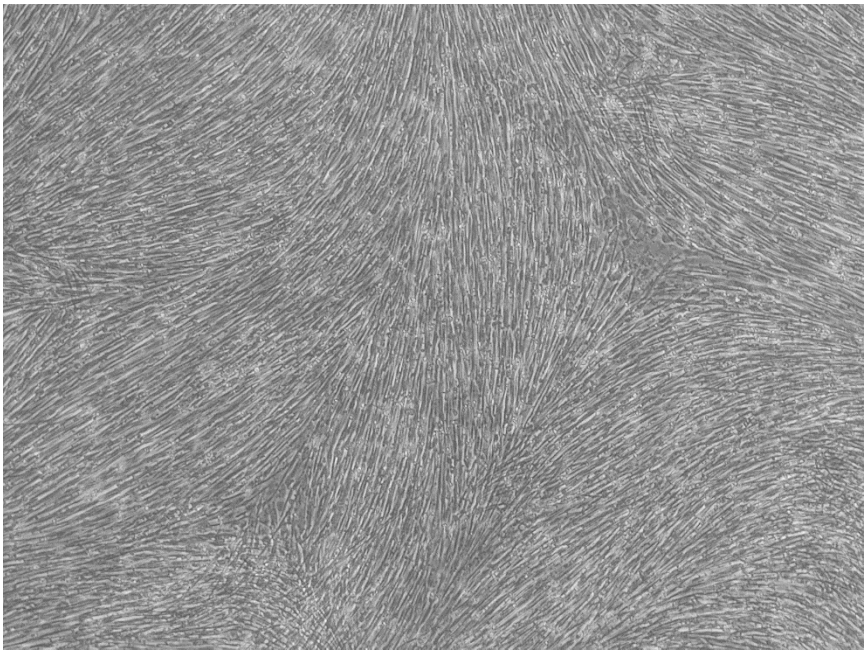

Figure 79 : Cells 7 days after switch of medium - All the myotubes are well fused. The experiment ends at this point.

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