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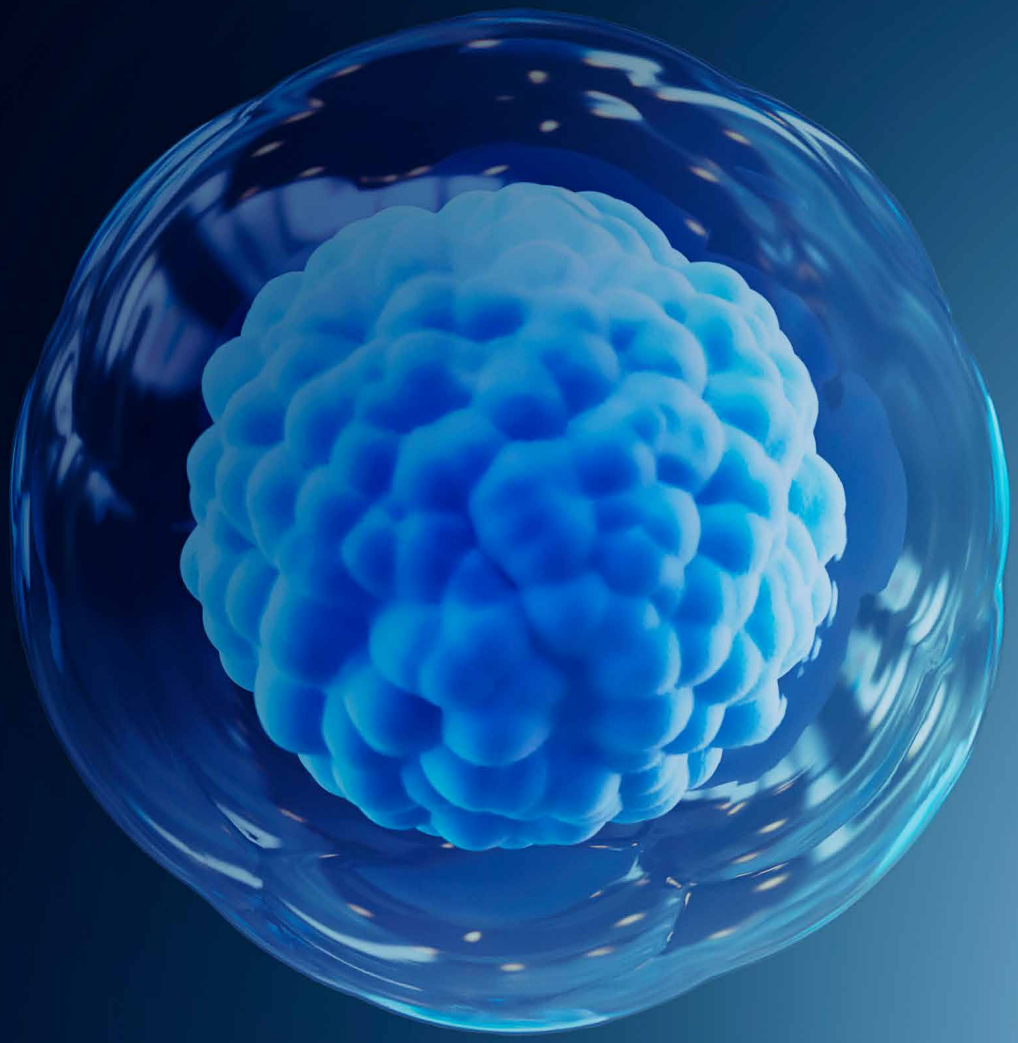


PLURIPOTENT STEM CELL RESOURCE HANDBOOK

Make the connection

gibco

Contents

Introduction

Pluripotent stem cells (PSCs) is a term that encompasses both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs).

Human ESCs (hESCs) are isolated from the inner cell mass of the blastocyst stage of a developing embryo and were first derived in 1998 by Dr. James Thomson [1]. iPSCs are generated via ectopic expression of one or more genes to reprogram an adult somatic cell. They are similar or equivalent to ESCs and were first derived by Dr. Shinya Yamanaka in 2007 [2]. PSCs are characterized by their ability to renew themselves indefinitely and differentiate into almost any cell type when exposed to the right microenvironment.

iPSCs have revolutionized the field of stem cell research by simplifying the generation of patient-specific stem cells that can then be used to model diseases in a dish. These models can be valuable in defining the mechanisms of disease pathology, and thereby play a vital role in drug discovery and identification of therapeutic targets.

Major areas in which PSCs and their derivatives have many potential applications are:

- Regenerative medicine: PSC-derived cells can be used to repair or replace diseased or damaged cells
- Disease research: PSC-derived cells can be useful for modeling various disease conditions
- Drug discovery and development: PSC-derived cells are excellent tools for testing the effects of experimental drugs
- Developmental biology: PSCs and PSC-derived cells provide a system for studying normal development

These applications involve a variety of protocols and require different tools. This handbook serves as a resource for the pluripotent stem cell workflow and provides recommendations for the use of related tools.

Find the solutions to support your stem cell research at **thermofisher.com/stemcells**

Pluripotent stem cell workflow

Whether you're new to stem cell research or expanding into new areas, this educational handbook will help guide your PSC research by providing useful tips, resources, and product recommendations for every step of your PSC workflow.

References

1. Takahashi K, Tanabe K, Ohnuki M et al. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131 (5):861-872.
2. Thomson JA, Itskovitz-Eldor J, Shapiro SS et al. (1998) Embryonic stem cell lines derived from human blastocysts. *Science* 282(5391):1125-1147.

Somatic and progenitor cells

The reprogramming process starts with human cells

Human somatic cells
Blood (CD34+)
Fibroblast
Keratinocyte
Epithelial (corneal, mammary)

Media
StemPro-34 SFM
DMEM/F-12
Advanced DMEM
Keratinocyte SFM

Cell culture plastics
Nunc flasks, dishes, and plates

Reprogram

Reprogram adult cells, such as blood cells, back to a pluripotent state to become iPSCs

Viral
CytoTune-iPS 2.0 Sendai Reprogramming Kit

DNA
Episomal vectors
Epi5 vectors

Transfection
Lipofectamine 3000 Transfection Reagent

Cell culture plastics
Nunclon Delta and ECM-coated plates

thermofisher.com/cellreprogram

Culture

Expand and maintain iPSCs, which require careful attention to culture conditions

Culture media
StemScale PSC Suspension Medium
StemFlex Medium
Essential 8 Medium
Essential 8 Flex Medium
CTS Essential 8 Medium
KnockOut Serum Replacement

Supplements and reagents
RevitaCell Supplement
PSC Cryopreservation Kit

Matrices
Geltrex matrix
Vitronectin (VTN-N) Recombinant Human Protein
rhLaminin-521

Cell culture plastics
Nunclon Delta plates and dishes with matrix coating (2D)
Nunclon Sphera plates and dishes (3D)

thermofisher.com/pscculture

Engineer

Engineer iPSCs to analyze gene and protein expression and to study differentiation and disease pathways

Transfection
Lipofectamine Stem Transfection Reagent
Lipofectamine 3000 Transfection Reagent
Lipofectamine MessengerMAX Transfection Reagent
Lipofectamine CRISPRMAX Transfection Reagent
Neon Transfection System

thermofisher.com/transfection

Gene editing
TrueCut Cas9 Protein v2
TrueGuide Synthetic sgRNA
TrueTag Donor DNA Kits

thermofisher.com/truedesign

Cell culture plastics
Nunclon Delta and ECM-coated plates

Differentiate

Differentiate reprogrammed cells into specific cell types

Ectoderm
PSC Neural Induction Medium
PSC Dopaminergic Neuron Differentiation Kit
CultureOne Supplement
B-27 Plus Neuronal Culture System

Mesoderm
PSC Cardiomyocyte Differentiation Kit

Endoderm
PSC Definitive Endoderm Induction Kit

Media
Essential 6 Medium

Cell culture plastics
Nunclon Delta and ECM-coated plates and dishes (monolayer culture)
Nunclon Sphera plates and dishes (spheroid and organoid cultures)

thermofisher.com/differentiation

Characterize

Multiple methods from basic to more advanced characterization are required throughout, as validation is critical in iPSC research

Tools
TaqMan hPSC Scorecard Panel
PrimeView assays, compatible with the PluriTest bioinformatics assay
KaryoStat and KaryoStat HD Assays
Immunocytochemistry and live staining kits
GeneArt Genomic Cleavage Detection Kit

Instruments
EVOS cell imaging systems
Attune NxT Flow Cytometer
CellInsight CX7 High Content Analysis (HCA) Platform

Cell culture plastics
Nunc Lab-Tek and Lab-Tek II Chamber Slide Systems and Chambered Coverglasses
Nunc Optical Bottom Plates
Nunc Glass Bottom Dishes

thermofisher.com/detectpsc

Section 1

Reprogramming

1.1 Introduction

iPSCs are generated from somatic cells through the forced expression of specific transcription factors that reprogram the cells to a pluripotent state. To date, different sets of reprogramming factors have been tested, along with different types of gene delivery technologies that are associated with varying levels of efficiency and safety (Figure 1.1).

Find the best solution for your reprogramming experiment at **thermofisher.com/reprogramming**

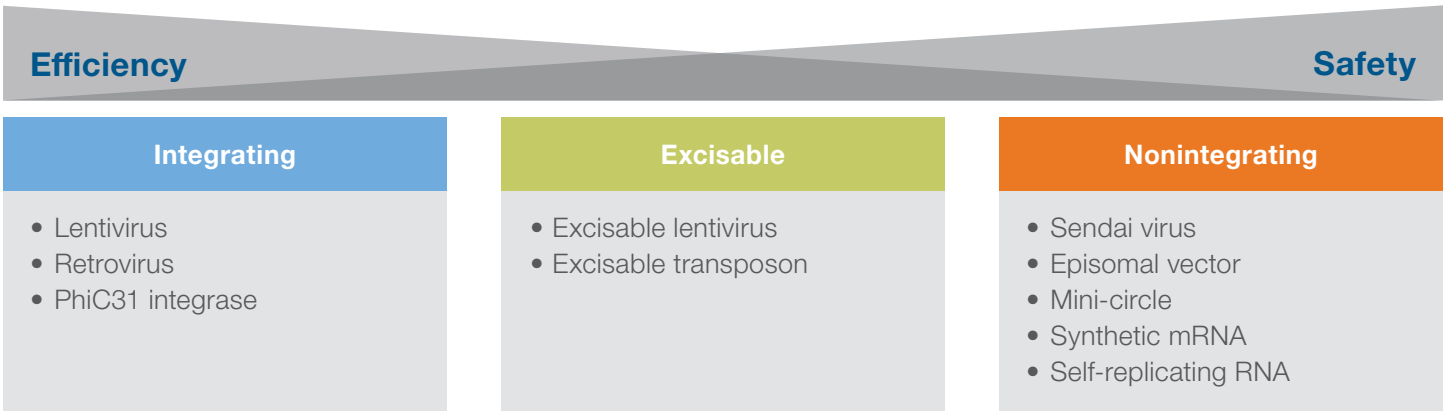


Figure 1.1. Safety and efficiency of various reprogramming technologies. Different reprogramming agents are classified as integrating, excisable, or nonintegrating technologies, which are associated with increasing levels of safety. Under each category, technologies are listed in order of decreasing efficiency.

Traditional reprogramming technologies using lentiviruses or retroviruses involve integration of foreign DNA into the host genome. This can lead to insertional mutagenesis, which can affect the properties of the derived cell lines. The general trend in the field has been towards nonintegrating technologies because they avoid the issue of insertional mutations and generate footprint-free PSCs that do not contain detectable vectors or transgenes.

Two common nonintegrating reprogramming technologies make use of episomal vectors and Sendai virus (SeV). These two technologies are discussed in more detail in this section. Other nonintegrating reprogramming technologies make use of mRNAs, miRNAs, proteins, and other small molecules.

1.2

Choosing a reprogramming method

Different reprogramming technologies have their own advantages and disadvantages that must be weighed when planning an experiment. The main issues to consider include a lab's flexibility in working with viruses, the intended parental somatic cells, the efficiency required in downstream experiments, and the importance of avoiding any chance of genomic integration. Features of the Invitrogen™ Epi5™ Episomal iPSC Reprogramming Kit and the Invitrogen™ CytoTune™-iPS 2.0 Sendai Reprogramming Kit are compared in Table 1.1. Generally, the CytoTune reprogramming kit is great for parental cells that are difficult to reprogram and for experiments that require higher-efficiency reprogramming and footprint-free iPSCs. Epi5 reprogramming vectors work well for parental cells that are easy to reprogram, especially when viral particles cannot be used.

Table 1.1. Episomal and Sendai reprogramming features and selection guide.

	Epi5 iPSC Episomal Reprogramming Kit	CytoTune-iPS 2.0 Sendai Reprogramming Kit
Description	Virus-free, nonintegrating episomal DNA vectors	Nonintegrating RNA virus
Reprogramming efficiency	0.01–0.1%	0.05–1%
Genomic integration–free	Yes, but all DNA vectors have a minor chance of integration	Yes
Virus-free reprogramming	Yes	No
Blood-cell reprogramming	Yes, for limited cell types ($CD34^+$ cells) and with low efficiency	Yes, for many cell types ($CD34^+$ cells, PBMCs, T cells) and with high efficiency
Special equipment required	Neon Transfection System or similar device for blood-cell reprogramming; Lipofectamine 3000 reagent can be used with fibroblasts	None
Reprogramming factors	Oct4, Sox2, Nanog, Lin28, Klf4, L-Myc	Oct4, Sox2, Klf4, c-Myc
Kit format	2 tubes with 20 µL each of: • Tube A: mixture of pCE-hOCT3/4, pCE-hSK (containing Sox2, Klf4), and pCE-hUL (containing L-Myc, Lin28) • Tube B: mixture of pCE-mP53DD and pCXB-EBNA1	3 tubes with 100 µL of each vector: • Polycistronic KOS (Klf4, Oct3/4, Sox2) • c-Myc • Klf4
Transfection or transduction control	None	CytoTune EmGFP Sendai Fluorescence Reporter
Detection of residual reprogramming vector backbones	Endpoint PCR	qPCR, endpoint PCR, or TaqMan hPSC Scorecard Panel

Useful tips

- Parental fibroblasts used for reprogramming should be from an early passage (<P6) with normal growth and karyotype.
- The density of seeded fibroblasts prior to initiation of reprogramming is critical to achieving good reprogramming efficiencies. A confluence of 50–80% is recommended on the day of transfection or transduction.
- Protocols describe reprogramming in 6-well formats. The protocol can be scaled down to a 12-well or 24-well culture dish, albeit with potentially reduced efficiency.
- Besides fibroblasts, a variety of somatic cells can be used for reprogramming. The CytoTune-iPS 2.0 Sendai Reprogramming Kit has been validated for a wide variety of cell types, including:
  - Adult and neonatal dermal fibroblasts
  - Amniotic fluid MSCs
  - Cardiac fibroblasts
  - $CD34^+$ blood cells
  - Mammary epithelial cells
  - Mouse embryonic fibroblasts
  - Nasal epithelial cells
  - Peripheral blood mononuclear cells (PBMCs)
  - Skeletal myoblasts
  - T cells
  - Umbilical vein epithelial cells
  - Urine epithelial cells

Protocols for reprogramming some of these cell types can be found at **thermofisher.com/pscprotocols**

- The Epi5 and CytoTune reprogramming systems are validated for human cells. oriP/EBNA1 vectors are also known to function in canine cells, while their use in murine systems may require additional components. For a current list of publications citing the use of SeV for reprogramming various cell types and species, go to **thermofisher.com/sendaipubs**
- Reprogramming can be carried out on either feeder-dependent or feeder-free culture systems. Typically the efficiency of reprogramming is higher in feeder-dependent systems than under feeder-free conditions because of the more nutrient-rich formulations. View validated protocols for Epi5 and CytoTune reprogramming kits with Gibco™ KnockOut™ Serum Replacement–based media for feeder-dependent systems and Gibco™ Essential 8™ Medium for feeder-free reprogramming at **thermofisher.com/reprogramprotocols**
- Reprogramming with the CytoTune-iPS 2.0 kit can be optimized for maximal reprogramming efficiency by varying the amount of Klf4. Typically, the multiplicity of infection (MOI) for the KOS and c-Myc vectors are maintained at a 1:1 ratio, with the Klf4 vector varied independently. The standard MOI ratio for KOS:c-Myc:Klf4 is 5:5:3, which could be changed to 5:5:6 or 10:10:6 to achieve higher efficiency.
- To optimize transduction of hard-to-transduce cells, we recommend testing different seeding densities using at least two or three different MOI values (e.g., 1, 3, and 9) of the Invitrogen™ CytoTune™ EmGFP Sendai Fluorescence Reporter. The expression of EmGFP in successfully transduced cells is detectable at 24 hours posttransduction by fluorescence microscopy, and reaches maximal levels at 48–72 hours posttransduction. Note that cells infected with SeV will most likely be refractive to further infection. Therefore, trying to use the CytoTune-iPS 2.0 Sendai Reprogramming Kit with cells already transduced with the CytoTune EmGFP Sendai Fluorescence Reporter, or vice versa, is not recommended.

1.3

Reprogramming with episomal vectors

Episomal vectors are circular extrachromosomal DNA molecules that are used to introduce and express exogenous genetic material. They are attractive reprogramming vectors because they carry viral elements that allow the prolonged and controlled expression of reprogramming factors, but they can be transfected into cells without the need for viral packaging.

One popular episomal vector system specifically incorporates the oriP/EBNA1 system derived from the Epstein–Barr virus. The oriP sequence is a cis-acting element that serves as the origin of replication on the pCEP backbone of the reprogramming vectors; EBNA1 codes for a DNA-binding protein that binds to oriP and tethers the plasmids to genomic DNA during replication, allowing one replication per cycle. Together, the oriP and EBNA1 elements ensure the replication and retention of the reprogramming vectors during each cell division, driving high expression of reprogramming genes and allowing iPSC derivation in a single transfection [1]. The loss of the episomal vectors at a rate of ~5% per cell cycle allows the removal of vectors from the iPSCs without any additional manipulation [2]. Therefore, while reprogramming vectors are retained long enough for reprogramming to occur, they are lost over time, so the newly derived iPSCs are footprint-free, lacking transfected DNA and integrated transgenes.

Knockdown of p53 has been shown to improve reprogramming efficiencies [3,4], with the mp53DD dominant negative mutant providing higher-efficiency knockdown compared to traditional short hairpin RNA (shRNA) systems [5]. An improved reprogramming system described by Okita et al. [6] includes episomal vectors carrying reprogramming factors along with mp53DD. In this system, an additional EBNA1 expression vector ensures high expression of reprogramming factors at the early stages of reprogramming.

A complete set of vectors based on the above study is available in the Epi5 Episomal iPSC Reprogramming Kit (Figure 1.2). The kit includes two tubes: a reprogramming vector tube containing a mixture of three plasmids that code for Oct3/4, Sox2, Klf4, L-Myc, and Lin28; and a second tube containing a mixture of two plasmids that code for the p53 dominant-negative mutant and EBNA1.

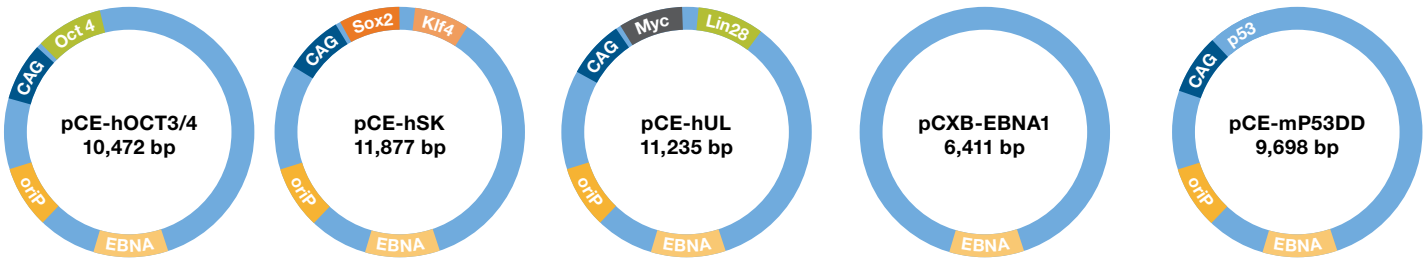


Figure 1.2. Vectors in the Epi5 Reprogramming Kit.

With all of these vectors together, the Epi5 reprogramming system achieves efficiencies of around 0.01 to 0.1% and can be used to reprogram different cell types, including CD34⁺ blood cells. To initiate reprogramming, the kit must be used in combination with a gene delivery system (Figure 1.3).

The Invitrogen™ Neon™ Transfection System allows electroporation of the vectors into most cell types. For fibroblasts, it is possible to achieve efficient reprogramming without electroporation through the use of Invitrogen™ Lipofectamine™ 3000 Transfection Reagent.

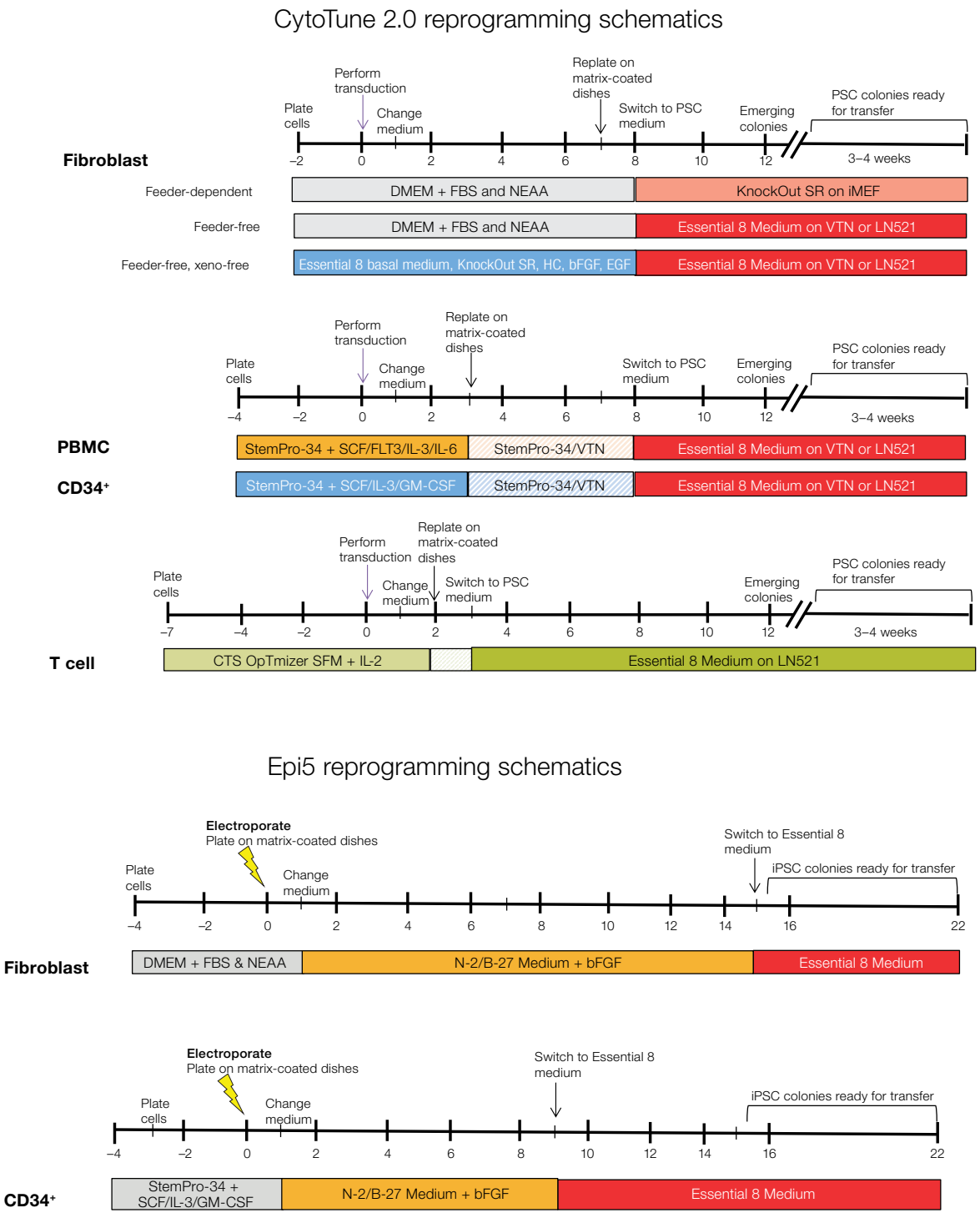


Figure 1.3. A comparison of Sendai and episomal vector reprogramming workflows, starting from various somatic cell types.
HC: hydrocortisone; iMEF: irradiated mouse embryonic fibroblasts; LN521: recombinant human laminin-521; NEAA: nonessential amino acids; VTN: vitronectin; SCF: stem cell factor.

1.4

Reprogramming with Sendai virus (SeV)

SeV is an enveloped virus with a single-chain RNA genome in the minus sense. This genome codes for the structural proteins that form and support the envelope (NP and M); the subunits of RNA polymerase (P and L); hemagglutinin-neuraminidase (HN), which recognizes sialic acid; and fusion protein (F), which, when activated by a protease, fuses the viral envelope with the cell membrane during infection.

There are two main characteristics that make SeV an attractive system for reprogramming. First, it can infect a wide range of cell types from various animal species because SeV infects cells by attaching itself to the sialic acid present on the surface of many different cells. Second, the SeV vectors are made of RNA and remain in the cytoplasm, ensuring that they do not integrate into the host genome or alter the genetic information of the host cell [7-9]. This is in contrast to retroviral vectors that require integration into host chromosomes to express reprogramming genes, or even adenovirus and plasmid vectors that exist episomally and do not require integration but carry the possibility of integrating into host chromosomes by virtue of being DNA-based.

SeV, modified through deletion of the F gene and introduction of temperature sensitivity mutations in SeV proteins (SeV/TSΔF and SeV/TS15ΔF), enables safe and effective delivery and expression of reprogramming genes [7-10]. These modifications prevent transmission and curtail the propagation of the reprogramming vectors. Thus, the viral vectors contained in the cytoplasm are eventually diluted out, leaving footprint-free iPSCs.

Currently, there are two CytoTune reprogramming kits based on the SeV system developed by Fusaki et al. [7]. The CytoTune-iPS 2.0 Sendai Reprogramming Kit contains only three vectors, the first one combining Oct4, Sox2, and Klf4; the second one containing c-Myc; and the third one containing additional Klf4 to achieve higher reprogramming efficiency (Figure 1.4). The CTS CytoTune-iPS 2.1 Sendai Reprogramming Kit also contains three vectors, KOS and Klf4 as in the 2.0 kit, and the third carrying L-Myc, which is reported to be less oncogenic than c-Myc (Table 1.2). The Sendai vectors offered in the CytoTune kits are efficient at reprogramming fibroblasts, PBMCs, CD34⁺ cells, and T cells (workflows shown in Figure 1.3).

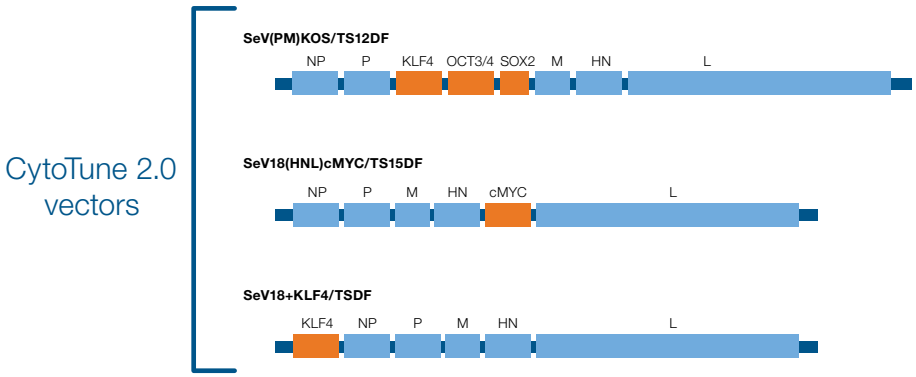


Figure 1.4. Configuration of vectors in the CytoTune-iPS 2.0 Reprogramming Kit.

Table 1.2. Comparison of reprogramming kits.

	CytoTune 2.0	CTS CytoTune 2.1
Efficiency	+++	+
Vectors	KOS, Klf4, c-Myc	KOS, Klf4, L-Myc
Vector formulation	AO protein	AOF stabilizer
Manufacturing standard	Manufactured at a site with a process that was audited for conformity with ISO 9001 standards	Manufactured at a site with a process that was audited for conformity with current Good Manufacturing Practices for medical devices, 21 CFR Part 820 of the regulation
Product use statement	For Research Use Only	For Research Use Only or noncommercial manufacturing of cell-based products for clinical research

While many researchers choose to begin reprogramming with dermal fibroblasts (Figure 1.5), the CytoTune 2.0 kit has proven to be effective at reprogramming multiple somatic cell types, including PBMCs, CD34⁺ cells, and T cells (Figure 1.6).

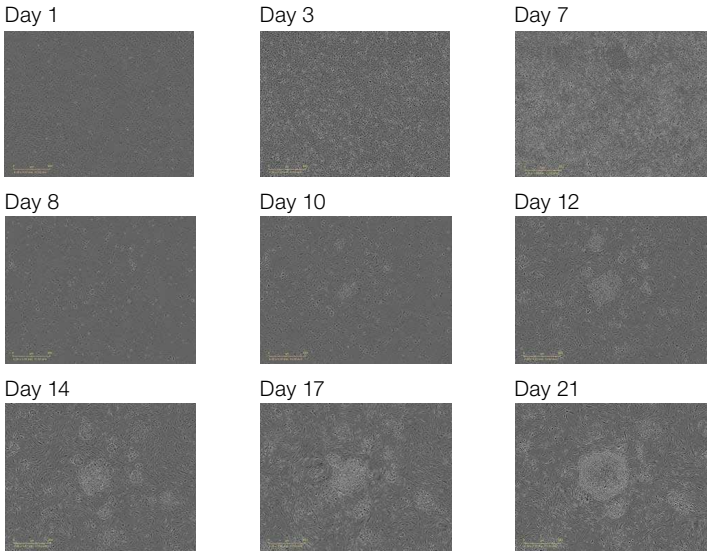


Figure 1.5. A time course of iPSC colony formation. Cells were replated at day 7. Initial colony formation is usually visible by days 10–12. By day 21, large compact colonies will emerge from the background lawn of fibroblasts.

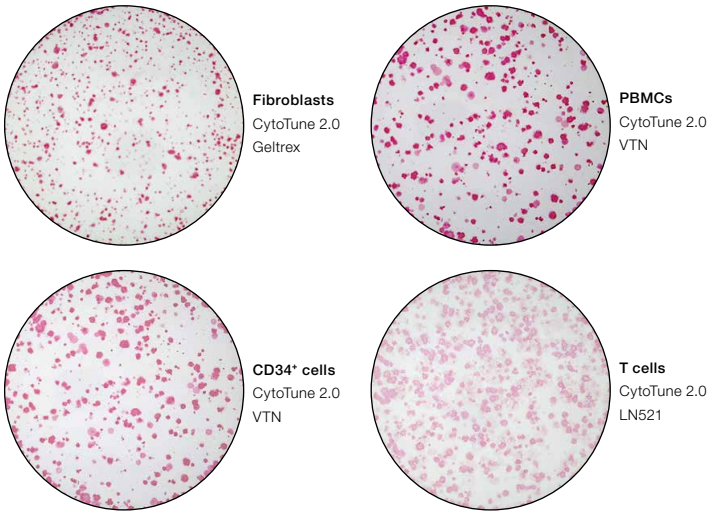


Figure 1.6. Reprogramming of multiple cell types using the CytoTune 2.0 kit. iPSCs were derived from various somatic cell sources using the CytoTune 2.0 kit and were grown under feeder-free conditions using Essential 8 Medium in wells coated with Gibco™ Geltrex™ matrix, vitronectin (VTN), or rhLaminin-521 (LN521). The resulting colonies were stained for alkaline phosphatase expression at day 19–23.

Identification of colony quality

Two primary methods of distinguishing a “good” colony from a “bad” colony include morphology and live staining (Figure 1.7).

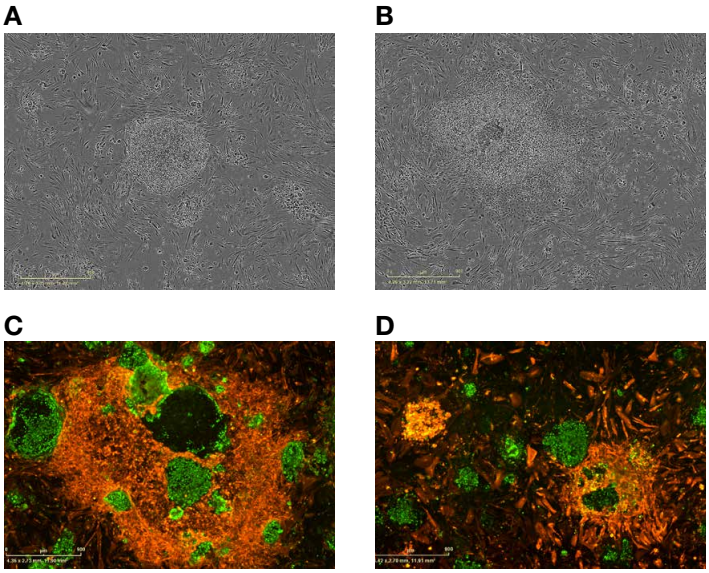


Figure 1.7. Distinguishing quality of colonies. (A) Good-quality colonies are flat and smooth with tight edges. **(B)** Poor-quality colonies are disperse, have heterogeneous morphology, and lack clear edges. **(C)** Good-quality colonies stain positive for TRA-1-60 only (green). **(D)** Poor-quality or partially reprogrammed colonies stain positive for CD44 (red), and may also stain positive for TRA-1-60 (green).

As clones are established, residual SeV is cleared from the population. In general, Sendai vector clearance is complete by passage 10 (Figure 1.8).

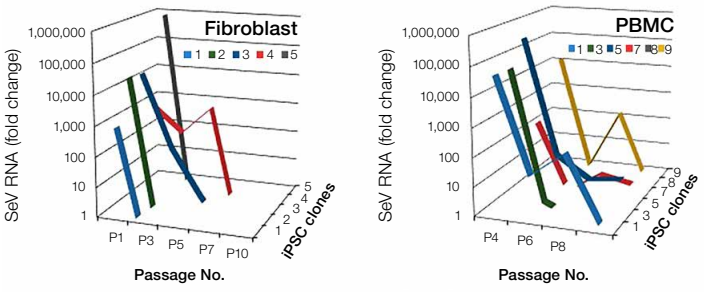


Figure 1.8. Rapid clearance of SeV. iPSC clones derived from fibroblasts or PBMCs using the CytoTune 2.0 kit were expanded, and cells were collected at different passages up to passage 10. RNA was isolated from the clones and analyzed for the presence of SeV using a prevalidated Applied Biosystems™ TaqMan® Gene Expression Assay. Expression values were normalized to untransduced parental somatic cells.

After a stable iPSC line has been generated, it is critical to demonstrate the quality and utility of the line before proceeding to differentiation. Assays such as immunofluorescence, qPCR, and flow cytometry are useful readouts for the ability of the line to produce cells of all three embryonic germ layers (Figures 1.9 and 1.10). Traditional cytogenetic analysis is important to demonstrate normal karyotype (Figure 1.9).

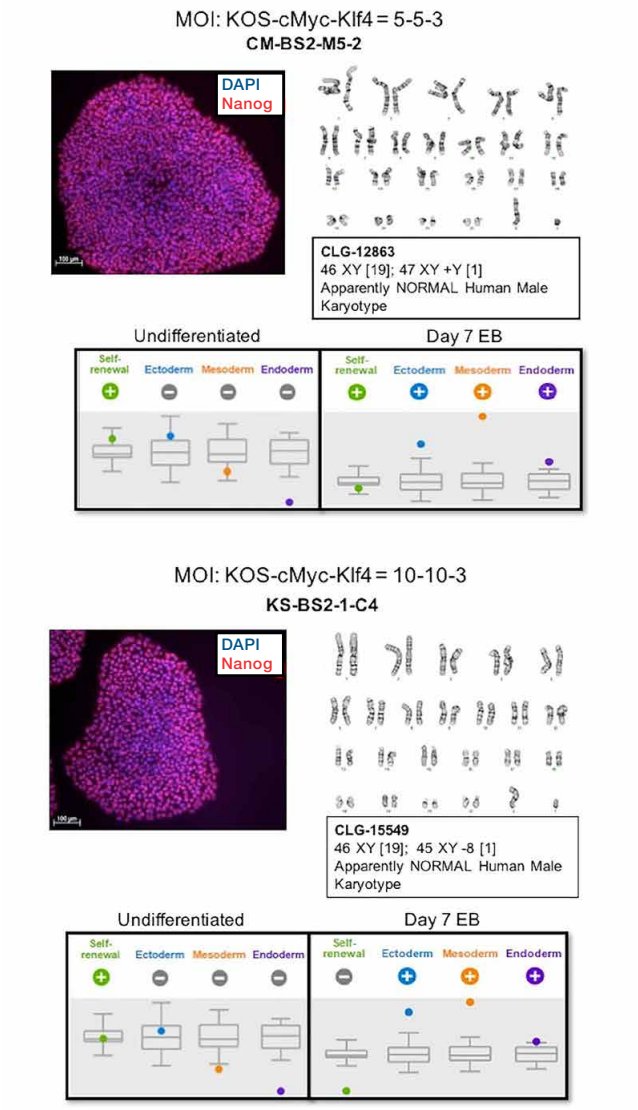


Figure 1.9. Characterization of iPSC clones. Two representative iPSC clones, derived using the CytoTune 2.0 kit at MOI of 5-5-3 (top panel) or 10-10-3 (bottom panel), show pluripotent marker expression (Nanog), normal G-banding karyotype, and the ability to differentiate into representative germ layers as assessed using an Applied Biosystems™ TaqMan® hPSC Scorecard™ Panel.

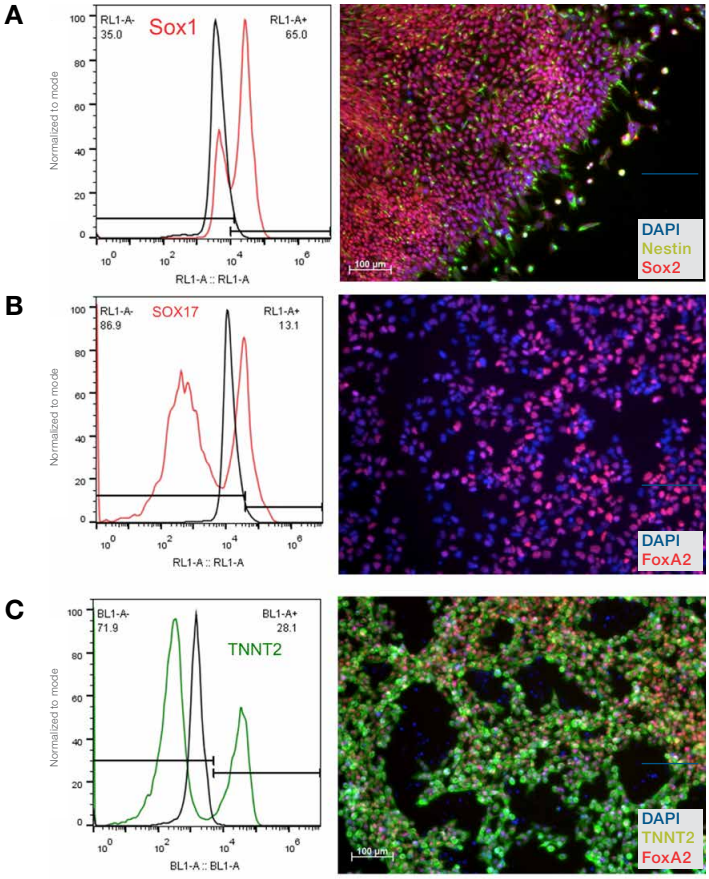


Figure 1.10. Differentiation of iPSC clones. A representative iPSC clone derived using the CytoTune 2.0 kit was subjected to directed differentiation into **(A)** neural stem cells using Gibco™ PSC Neural Induction Medium, **(B)** definitive endoderm using the Gibco™ PSC Definitive Endoderm Differentiation Kit, or **(C)** cardiomyocytes using the Gibco™ PSC Cardiomyocyte Differentiation Kit.

1.5

References

1. Yu J, Hu K, Smuga-Otto K et al. (2009) Human induced pluripotent stem cells free of vector and transgene sequences. *Science* 324(5928):797-801.
2. Nanbo A, Sugden A, Sugden B (2007) The coupling of synthesis and partitioning of EBV's plasmid replicon is revealed in live cells. *EMBO J* 26(19):4252-4262.
3. Hong H, Takahashi K, Ichisaka T et al. (2009) Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. *Nature* 460(7259):1132-1135.
4. Spike BT, Wahl GM (2011) p53, stem cells, and reprogramming: tumor suppression beyond guarding the genome. *Genes Cancer* 2(4):404-419.
5. Kawamura T, Suzuki J, Wang YV et al. (2009) Linking the p53 tumour suppressor pathway to somatic cell reprogramming. *Nature* 460(7259):1140-1144.
6. Okita K, Yamakawa T, Matsumura Y et al. (2013) An efficient nonviral method to generate integration-free human-induced pluripotent stem cells from cord blood and peripheral blood cells. *Stem Cells* 31(3):458-466.
7. Fusaki N, Ban H, Nishiyama A et al. (2009) Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *Proc Jpn Acad Ser B Phys Biol Sci* 85(8):348-362.
8. Li HO, Zhu YF, Asakawa M et al. (2000) A cytoplasmic RNA vector derived from nontransmissible Sendai virus with efficient gene transfer and expression. *J Virol* 74(14):6564-6569.
9. Seki T, Yuasa S, Oda M et al. (2010) Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells. *Cell Stem Cell* 7(1):11-14.
10. Inoue M, Tokusumi Y, Ban H et al. (2003) Nontransmissible virus-like particle formation by F-deficient Sendai virus is temperature sensitive and reduced by mutations in M and HN proteins. *J Virol* 77(5):3238-3246.

Section 2

Culture

2.1 Introduction

Culturing PSCs requires a compatible combination of media, matrices, and passaging methods that support cell health and pluripotency. When human ESCs were first derived by Thomson et al. in 1998 [1], the ESCs were cultured on mitotically inactivated mouse embryonic fibroblast (MEF) feeder cells with a medium supplemented with fetal bovine serum (FBS). This pioneering work served as the starting point for the development of various PSC culture systems. To reduce the risk of contamination from heterologous proteins and adventitious agents, MEFs in feeder-dependent cultures were replaced with human-derived alternatives, including human fibroblast cells. To further reduce variable components and animal material, FBS was replaced with Gibco™ KnockOut™ Serum Replacement, a supplement for FBS-free, feeder-dependent culture. Eventually, media and matrices were developed to support PSCs completely independent of feeder cells. Coming full circle in 2011, the Thomson lab published a fully defined, feeder-free culture system that supported the undifferentiated growth of PSCs without any animal components; the medium is now available as Gibco™ Essential 8™ Medium. A newer version of this medium, Gibco™ Essential 8™ Flex Medium, is also based on the same formulation.

Clearly, much of the evolution of PSC culture systems was driven by interest in generating PSCs suitable for clinical applications, devoid of nonhuman animal material (xeno-free or XF), or better yet, free from any animal-origin material, human or otherwise (animal origin–free or AOF).

The migration from serum-based media towards more defined media has been valuable for both research and translational applications; more defined media make use of known components at known quantities, and hence perform more consistently. However, not all applications require XF or AOF media and many of the newer, more advanced, and demanding PSC applications actually benefit from a more complex medium. For example, Gibco™ StemFlex™ Medium is richer than Essential 8 Medium and was optimized to support these new and more challenging applications such as single-cell passaging and gene editing.

As the use of PSCs expands in research, drug discovery, and therapeutic applications, the need to produce large numbers of cells in a cost-effective, scalable manner has become pressing. As a result, suspension culture methods that overcome surface area and footprint limitations in adherent culture while maximizing expansion of PSCs have been developed. Hurdles in the development of these methods include the maintenance of pluripotency, successful adaptation from adherent to suspension culture, and limitations in scale. Gibco™ StemScale™ PSC Suspension Medium was developed in response to this need.

Altogether, a variety of “culture systems” now exist to satisfy the progressively stringent requirements of different PSC applications, from basic research to translational applications, from simple cell culture to gene editing.

With a full spectrum of culture systems available, there can be multiple options to satisfy each application’s requirements. Choosing between different options may ultimately depend on other considerations, including cost, workflow, and scalability. This chapter will discuss the features, reagents, and workflows associated with different culture systems. To facilitate the discussion, the culture systems will be divided into feeder-dependent and feeder-free systems and will primarily revolve around the different PSC culture media.

Find the right PSC media for your research at **thermofisher.com/pscculture**

2.2 Feeder-dependent culture systems

Feeder-dependent culture systems generally support pluripotency and cell health using a DMEM-based medium that is supplemented with basic fibroblast growth factor (bFGF) and serum, or more commonly KnockOut Serum Replacement, a more defined and more reliable serum-free alternative that is specifically optimized for PSC culture. As the name implies, feeder-dependent cultures rely on feeder cells to provide many other proteins, most often growth factors and extracellular matrix proteins, that are necessary for PSCs to grow in culture. With the abundance of components to support PSC growth, feeder-dependent culture systems are considered rich and robust and are still widely used years after feeder-free systems have been introduced.

The vast majority of feeder-dependent cultures use MEF feeders that have been irradiated or treated with mitomycin-C to arrest the cell cycle. The most commonly used MEFs are derived from the outbred CF1 mouse strain, but MEFs can be derived from a variety of strains, including inbred C57BL/6 mice or even drug-resistant mice, thereby enabling anything from routine culture to drug selection with feeder-dependent PSCs. The preparation and quality control of the feeder cells is critical, as isolation of the fibroblasts can introduce contaminants like mycoplasmas, and incomplete inactivation can allow MEFs to overgrow and outcompete slower-growing PSCs. A broad selection of pre-isolated, pre-inactivated wild type or drug-resistant Gibco™ MEFs can save the time and trouble of generating feeder cells. The rigorous quality testing helps ensure that they are free of contamination and can truly support PSC culture.

Learn more about Gibco MEFs at **thermofisher.com/gibcomef**

Workflow

Generally, the proper maintenance of PSCs involves daily media changes as well as daily inspections to check the culture's morphology, general health, and confluency. Healthy and undifferentiated PSCs cultured on MEFs have a high nucleus-to-cytoplasm ratio and grow in colonies that are compact and have well-defined edges (Figure 2.1), whereas areas of differentiation contain larger, flatter, and less compact cells (Figure 2.2). Some level of differentiaiton can be expected in cultures when colonies have grown too big or when cultures have become too confluent, particularly when colonies begin to overlap with each other. When this occurs, areas of differentiation can be removed by manual dissection prior to passaging. However, to prevent excessive differentiation, feeder-dependent cultures should be passaged regularly, typically every 3–4 days with a split ratio around 1:4 to 1:6, with actual intervals and ratios adjusted depending on cell line and culture confluency.

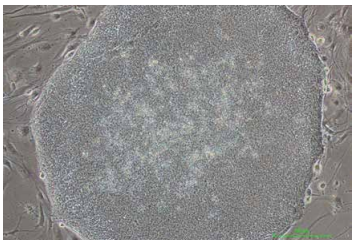


Figure 2.1. Phase-contrast image of H9 hESCs grown on inactivated MEFs. Cells were grown on gelatin-coated plates using medium containing KnockOut Serum Replacement. The ESC colony consists of compact cells, exhibits a well-defined border, and is surrounded by inactivated MEFs with spindle-like morphology (10x magnification).

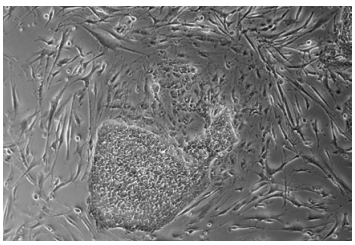


Figure 2.2. Phase-contrast image of differentiating H9 ESC colony on inactivated MEFs. Cells were grown under the same conditions as in Figure 2.1. Part of the colony remains compact and well-defined, but another part shows flatter, loosely arranged cells, indicative of differentiation (5x magnification).

Unlike many other cell types, feeder-dependent PSCs are passaged as cell clumps that are harvested using either enzymatic or mechanical methods. For enzymatic passaging, colonies are incubated with Gibco™ Collagenase Type IV or Gibco™ Dispase II until the edges lift from the plate. They are then completely detached and fragmented into smaller clumps by trituration, with care taken to obtain the optimum fragment size; very small and very large fragments tend to differentiate or fail to attach. Mechanical passaging can be more appropriate for certain cases, such as when picking colonies for expansion. This involves scoring colonies into smaller fragments using a 25-gauge needle and lifting the fragments off the plate with a 200 µL pipette tip so that they can be transferred to a fresh plate. Scoring can also be done for bulk passaging, although scoring a whole plate is tedious and time-consuming.
If mechanical methods are preferred for bulk passaging, the Gibco™ StemPro™ EZPassage™ Tool can provide a quicker, easier alternative. The StemPro EZPassage Tool is a grooved rolling tool that moves across multiple colonies at a time, generating uniform fragments that can then be scraped off the plate with a cell lifter. Regardless of passaging method, plates are typically coated with 0.1% gelatin or the ready-to-use equivalent, Gibco™ Attachment Factor, to facilitate feeder cell attachment and spreading. The feeder cells themselves should be seeded at least a day in advance of culture.

Access detailed protocols for culturing PSCs on feeder cells at **thermofisher.com/cultureprotocols**

Useful tips

- It is possible to skip changing the media for one day if the cells are double-fed the day before. However, this practice should be limited to minimize the stress on cells and to consequently minimize the risk of accumulating karyotypic abnormalities.
- For cultures that are exhibiting high levels of differentiation, it can be possible to save the line by manually picking the undifferentiated colonies and transferring them to a fresh plate of MEFs.

2.3

Feeder-free culture systems

Feeder-free cultures work on the principle of omitting feeder cells and supplementing the remaining culture components to compensate for the nutrients, growth factors, and extracellular matrix proteins that are missing. This is exemplified by the use of MEF-conditioned media to create culture systems that physically do not contain feeders but still contain the soluble factors secreted by feeder cells. However, MEF-conditioned media fail to offer much improvement over using actual feeder cells, and the associated workflow can be even more tedious. As such, studies have parsed the protein contributions of MEFs and investigated the pathways that are critical for pluripotency, with the goal of developing better feeder-free systems. These studies initially led to a first generation of stem cell media that did not require MEFs, neither as feeder cells nor for conditioning media. Next, they led to minimal, defined, xeno-free media like Essential 8 Medium and Essential 8 Flex Medium. Finally, they have resulted in optimized media like StemFlex Medium that support pluripotency and survival despite the stress that PSCs undergo during more demanding applications.

Essential 8 Medium and Essential 8 Flex Medium

In order to develop a more defined medium to support the growth and pluripotency of PSCs more consistently, James Thomson's lab re-examined the composition of an existing feeder-free medium, testing new combinations with fewer components [2]. The result was a defined, xeno-free, feeder-free medium that is now available as Essential 8 Medium. While most feeder-free media formulations consist of more than 20 components, adding complexity, time, and cost, Essential 8 Medium is formulated with only eight components. Furthermore, unlike most feeder-free media, Essential 8 Medium was specifically designed to exclude serum albumin, which is a frequent source of variability. This simple formulation has been extensively tested and has been shown to maintain pluripotency and normal karyotype in multiple PSC lines for over 50 passages. An updated formulation, Essential 8 Flex Medium, has been enhanced to eliminate daily feeding schedules required for most PSC culture maintenance. This medium uses the same wild type bFGF found in the original Essential 8 Medium, but a slightly modified formulation extends the activity of this growth factor, along with other key heat-sensitive components found in PSC media. This allows for a flexible feeding schedule in which feeding can be skipped for up to 3 days in a week, including up to 2 consecutive days, without compromising pluripotency or genetic stability.

CTS Essential 8 Medium

Based on the widely published Essential 8 Medium, we have developed a cell therapy–grade, defined human PSC culture medium. Gibco™ CTS™ Essential 8™ Medium offers all the same benefits of the RUO version but with components not directly derived from animals to support clinical research applications.

CTS Essential 8 Medium offers a best-in-class design for clinical PSC applications. To reduce risks, it is developed under current good manufacturing practice (cGMP) standards in an FDA-registered facility for medical devices. Additionally, CTS Essential 8 Medium offers extensive regulatory documentation and safety testing, including traceability of raw materials and an FDA Drug Master File. This medium is a well-accepted formulation that has been used and referenced extensively in the research market. CTS Essential 8 Medium enables the translational market to seamlessly move from research to clinical applications.

StemFlex Medium

StemFlex Medium is optimized to deliver superior performance in the innovative and challenging applications used in today's stem cell research, such as reprogramming, single-cell passaging, and gene editing. In addition to core performance enhancements, it delivers the convenience of a flexible feeding schedule (including weekend-free options), just like Essential 8 Flex Medium. StemFlex Medium also offers the ability to choose between matrix and passaging reagents best suited for a given application.

Workflow

Passaging PSCs cultured under feeder-free conditions is subject to many of the same considerations and practices as cells that are grown on MEFs. Cells are inspected and fed daily, although there is greater flexibility in this schedule when using Essential 8 Flex Medium or StemFlex Medium. As a guideline, healthy and undifferentiated feeder-free cells grow in colonies (Figure 2.3), just like in feeder-dependent cultures. However, the colonies may appear flatter or less compact, and the colony edges may not be as smooth, especially right after passaging. As with feeder-dependent cultures, overconfluency leads to areas of differentiation (Figure 2.4). These don't typically require manual removal, but it is best to prevent widespread differentiation by passaging regularly, particularly when using passaging methods other than Gibco™ Versene™ Solution or EDTA. Passaging methods using reagents such as Gibco™ TrypLE™ Select or Gibco™ StemPro™ Accutase™ cell dissociation reagents do not differentially dissociate PSCs from the plastic surface and thus also harvest differentiated progeny.

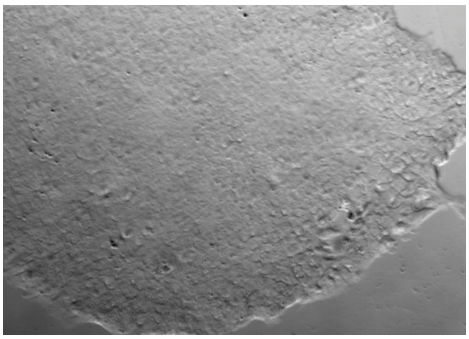


Figure 2.3. Phase contrast image of hiPSCs grown under feeder-free conditions. Gibco™ Human Episomal iPSCs were grown on Geltrex matrix–coated plates using Essential 8 Medium (10x magnification).

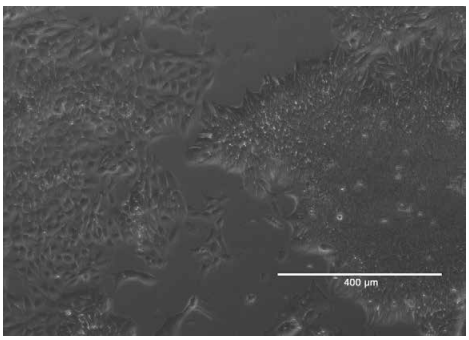


Figure 2.4. Phase contrast image of differentiating hiPSC colony without feeders. Cells were grown under the same conditions as in Figure 2.3. Part of the colony remains compact, but another part shows flatter, loosely arranged cells, indicative of differentiation (5x magnification).

Matrices and passaging methods

Essential 8, Essential 8 Flex, and StemFlex media can be used with a variety of matrices, including Gibco™ Geltrex™ matrix, vitronectin, and rhLaminin-521. Geltrex matrix consists of basement membrane proteins derived from Engelbreth-Holm-Swarm mouse tumors. Unlike Geltrex matrix, vitronectin and rhLaminin-521 are defined, as well as xeno-free recombinant human matrix proteins. The vitronectin substrate specifically uses the VTN-N variant of the protein, which supports hPSC attachment and survival better than the wild type variant when used with Essential 8 Medium [2]. On the other hand, Gibco™ rhLaminin-521 has been proven to promote PSC survival under stressful conditions, even in the absence of small-molecule Rho-associated protein kinase (ROCK) inhibitors [3]. It can be used for routine culture and is particularly useful in stressful applications such as reprogramming, adaptation of PSCs from richer to leaner media, and single-cell clonal outgrowth following fluorescence-activated cell sorting (FACS).

The standard passaging method for cells cultured in Essential 8, Essential 8 Flex, and StemFlex media is nonenzymatic. Colonies are subjected to a short treatment with 0.5 mM EDTA in Gibco™ calcium-free, magnesium-free DPBS, which is also available in a ready-to-use format as Gibco™ Versene™ Solution. Once EDTA has been replaced with the medium, cells are then removed from the plate by gentle pipetting. This results in cell clumps that are transferred to a new plate without further trituration.

Cells are passaged when they reach ~85% confluence. This typically occurs at day 3–7 with split ratios of around 1:6 to 1:20.

For specific applications like gene editing or clonal expansion or to make the workflow more amenable to large-scale culture, it may be necessary to passage cells as single cells using Gibco™ TrypLE™ Select Enzyme or as 2- to 3-cell clusters using StemPro Accutase Cell Dissociation Reagent. The best culture system for supporting such passaging methods would be the combination of the most robust feeder-free medium and matrix—StemFlex Medium and rhLaminin-521. If Geltrex matrix or VTN-N is preferred to be used with StemFlex Medium, single-cell passaging can still be achieved with the use of Gibco™ RevitaCell™ Supplement, a proprietary, chemically defined, animal origin–free formulation that contains antioxidants, free radical scavengers, and a ROCK inhibitor with higher specificity than Y-27632 or thiazovivin. In this protocol, adherent PSCs are dissociated using TrypLE Select Enzyme. Gibco™ RevitaCell™ Supplement is added to the medium during the first 24 hours after passaging, but is no longer required in subsequent media changes.

Note: Do not add additional ROCK inhibitors such as Y-27632 or thiazovivin when using RevitaCell Supplement in the medium.

If a defined, xeno-free medium is required, Essential 8 Medium can also support single-cell passaging when used with rhLaminin-521 or when used with RevitaCell Supplement and VTN-N or Geltrex matrix.

For a guide to the different matrices and passaging methods for StemFlex and Essential 8 media, refer to Table 2.1.

Table 2.1. Guide to choosing matrices and passaging methods for StemFlex and Essential 8 media.

Matrix	Application	Clump passaging (standard recommendation)	2- to 3-cell cluster passaging	Single-cell passaging	
				Passaging reagent	RevitaCell Supplement required?
Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix	Replacement for Matrigel matrix; an ideal economical choice for expansion and maintenance of PSCs	Versene Solution	StemPro Accutase reagent	TrypLE Select Enzyme	Yes
Vitronectin recombinant human protein, truncated (VTN-N)	Used for applications requiring xeno-free and/or defined matrix				Yes
Recombinant human laminin-521 (rhLaminin-521)	Recommended for highest performance in stressful applications, including gene editing and single-cell passaging				No

Useful tips

- It is very important to prewarm complete Essential 8 Medium at room temperature and not in a 37°C water bath. Basic bFGF activity can decline rapidly with repeated temperature changes from 4°C to 37°C.
- ROCK inhibitors can be used with Essential 8 Medium; however, this isn't necessary and they are not routinely used with our clump passaging protocols. If the use of a ROCK inhibitor is desired, it should be added to the medium during the first 24 hours post-passage.
- Cells should not be pretreated with RevitaCell Supplement before passaging. Cells only require RevitaCell Supplement for 18–24 hours after single-cell passaging, with the cells being fed regular, unsupplemented medium for the remainder of the culture.
- RevitaCell Supplement can also be added to growth media during the first 24 hours post-thaw to achieve optimum post-thaw recovery of cryopreserved cells.

2.4

Suspension culture

As the use of PSCs in research, drug discovery, and therapeutic applications continues to expand, the need to create large numbers of high-quality cells in a robust and cost-effective manner becomes ever more pressing. Media that enable scalable culture and support efficient downstream differentiation while minimizing material, labor, and production requirements will greatly facilitate the use of PSCs for these applications. To address these challenges, three-dimensional (3D) suspension culture systems, which offer advantages for scale-up over 2D adherent culture, have been adopted. Notably, suspension cultures have lower overall cost, reduced footprint and hands-on time, and greater compatibility with closed systems compared to adherent culture systems. Furthermore, for the same number of cells grown, suspension culture systems consume less media and plasticware than adherent culture systems (Table 2.2). For these reasons, suspension culture systems are more desirable for generating large quantities of PSCs.

Table 2.2. Scale-up in suspension culture systems (using StemScale PSC Suspension Medium) vs. scale-up in adherent culture systems (using Essential 8 Medium).

	Suspension scale-up			Adherent scale-up		
Total days in culture	**Culture vessel format**	**Average cell yield***	**Medium consumed**	**Culture vessel format**	**Average cell yield***	**Medium consumed**
3 days	6-well plate (single well)	1.5 x 10^6 cells	6 mL	6-well plate (single well)	1.5 x 10^6 cells	6 mL
7 days	6-well plate (full plate)	18 x 10^6 cells	30 mL	100 mm dish	9 x 10^6 cells	48 mL
11 days	100 mL bioreactor	150 x 10^6 cells	200 mL	150 mm dish	22 x 10^6 cells	72 mL
15 days	500 mL bioreactor	750 x 10^6 cells	1,000 mL	Nunc TripleFlask Treated Cell Culture Flask	78 x 10^6 cells	800 mL
19 days	3 L bioreactor	4.5 x 10^9 cells	6,000 mL	2-layer Nunc Cell Factory System	300 x 10^6 cells	1,200 mL
23 days	–	–	–	10-layer Nunc Cell Factory System	1.5 x 10^9 cells	6,000 mL
27 days	–	–	–	30-layer Nunc Cell Factory System	4.5 x 10^9 cells	18,000 mL

* Values are estimated from data based on 4 days of expansion per culture vessel format. Values for the 3 L bioreactor and 30-layer Thermo Scientific™ Nunc™ Cell Factory™ System have been extrapolated from the smaller bioreactor and Nunc Cell Factory System formats listed in the table.

Methods of suspension culture

Suspension culture systems that attempt to overcome the challenges of expandability and scale posed by adherent culture can be categorized into two groups: microcarrier culture and self-aggregating spheroid culture. Microcarrier cultures require cells to be attached to a supportive matrix such as beads, gels, or other rigid porous structures. Although microcarrier cultures provide consistent cell growth, they can be time-consuming to prepare, have limited surface area that prohibits large-scale expansion of PSCs, and can be inconvenient to passage due to the need to dissociate cells from the microcarriers. Self-aggregating spheroids, although relatively easy to maintain, can be difficult to maintain at optimal size, leading to hypoxic necrotic cores and subsequent loss of pluripotency. Nucleation, or formation of spheroids upon initiation of cultures, can be inconsistent across PSC lines; and finally, feeding and passaging can be cumbersome due to the need to use cell strainers during passaging, or the buildup of metabolic waste during nutrient replenishment.

StemScale PSC Suspension Medium

Gibco™ StemScale™ PSC Suspension Medium overcomes challenges of PSC suspension culture by supporting the growth of PSCs in suspension through the self-assembly of spheroids, providing superior expansion while maintaining high viability and consistent spheroid formation across multiple PSC lines (Figure 2.5). Simplified medium exchange and passaging workflows offer scalability in different formats and flexibility in feeding schedule. PSCs expanded in StemScale medium can achieve 5- to 10-fold expansion per passage, maintain pluripotency and normal karyotype across multiple passages, and can be differentiated to the three germ layers.

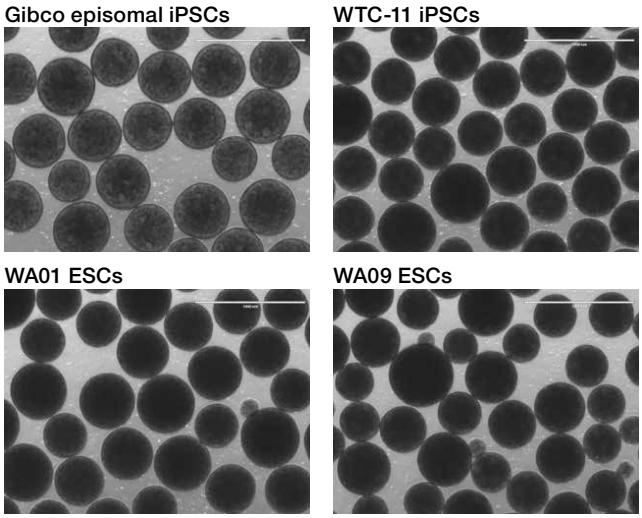


Figure 2.5. Consistent spheroid growth and maintenance of pluripotency. Human ESC and iPSC lines were expanded in complete StemScale medium. Consistent spheroid size and growth were observed across multiple cell lines after 5 days in culture.

Workflow

PSCs maintained in StemScale medium are fed by a 50% medium replacement strategy (Figure 2.6), reducing metabolic waste in the culture while maintaining constant media volumes, which is important to the maintenance of spheroid size. Spheroids are passaged via chemical dissociation, eliminating the use of cell strainers during passaging that can lead to cell loss. Similar to StemFlex Medium and Essential 8 Flex Medium, StemScale medium offers a flexible workflow that allows users to selectively determine when to passage cultures. Feeding days can be skipped, providing the option of a weekend-free passaging schedule.

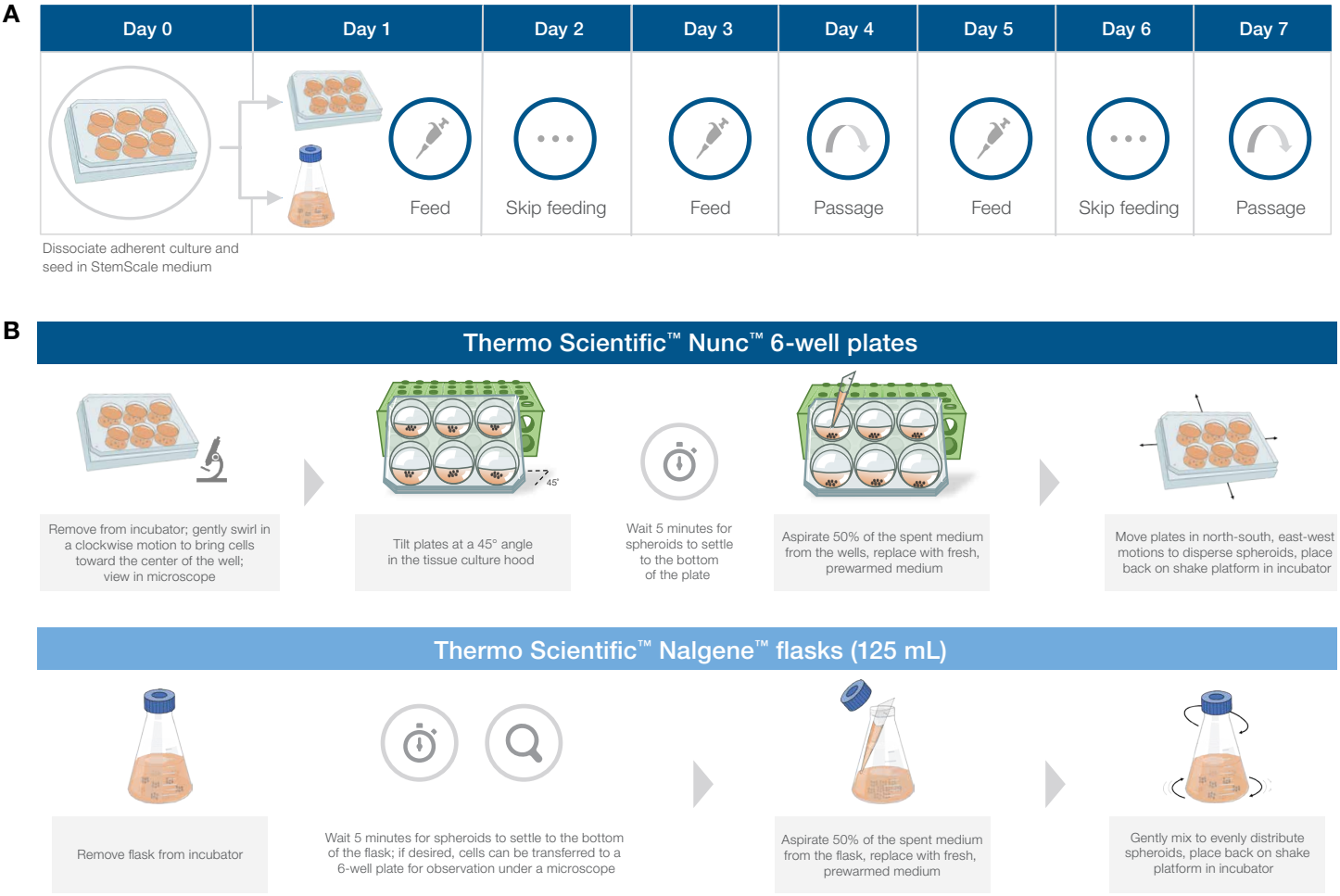


Figure 2.6. Simplified workflow for adapting adherent cultures to suspension cultures using StemScale PSC Suspension Medium. After initiation of cultures in StemScale medium, cells are fed periodically using a 50% medium exchange—every day or **(A)** every other day. **(B)** PSCs cultured in plates or flasks are sedimented via gravity, and 50% of the medium is aspirated and replaced with fresh, prewarmed medium, reducing metabolic waste in the medium. The plate or flask is mixed before placing back on the shake platform in the incubator. **(B,** upper**)** Cells cultured in 6-well plates are first swirled to bring cells toward the center of the well, then the plates are tilted at a 45° angle. Spheroids can be imaged under the microscope directly in a 6-well plate. **(B,** lower**)** A similar protocol is followed with cells grown in shaker flasks. Spheroids can be viewed under a microscope by taking a sample from the flask and transferring it to a 6-well plate. For detailed instructions on transitioning to suspension cultures using StemScale medium, refer to the instructions in the user guide.

Expandability and scalability

The promise of PSC suspension culture is the ability to generate large numbers of PSCs in a short amount of time. In order to achieve this, expansion per passage must be high while allowing users to scale to progressively larger vessels. PSCs cultured in StemScale medium have been shown to achieve 5- to 10-fold expansion per passage and maintain this expansion potential across multiple passages (Figure 2.7). Furthermore, PSCs can be cultured in StemScale medium across multiple vessel types, including 6-well plates, shaker flasks, and small to medium-sized bioreactors (Figure 2.8).

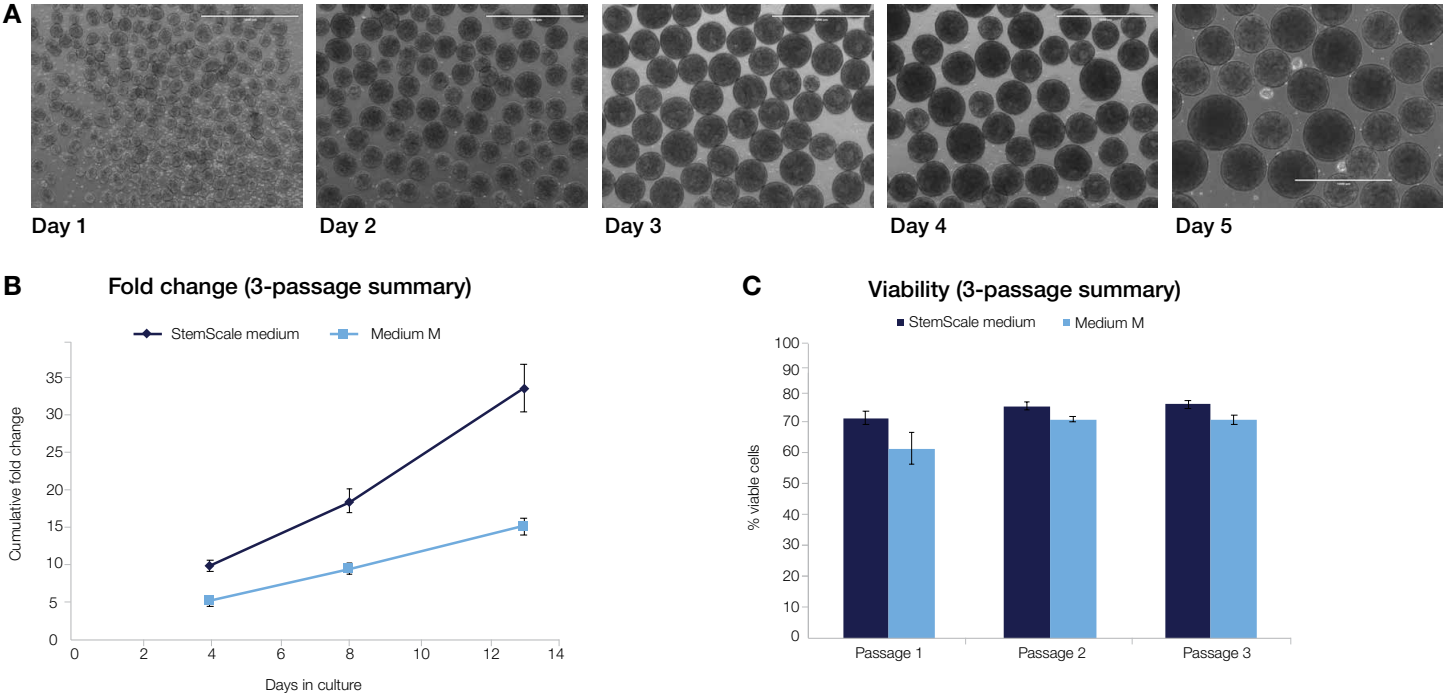


Figure 2.7. StemScale PSC Suspension Medium delivers superior expansion capability. Multiple cell lines were cultured in Thermo Scientific™ Nunc™ non-treated 6-well plates (Cat. No. 150239) in StemScale medium. **(A)** Morphology and growth of PSC spheroids on days 1–5 after initiating cultures in StemScale Medium. **(B)** Fold expansion over 3 passages of PSCs cultured in StemScale medium or in another supplier's Medium M. **(C)** Viability over 3 passages of PSCs cultured in StemScale medium or in Medium M.

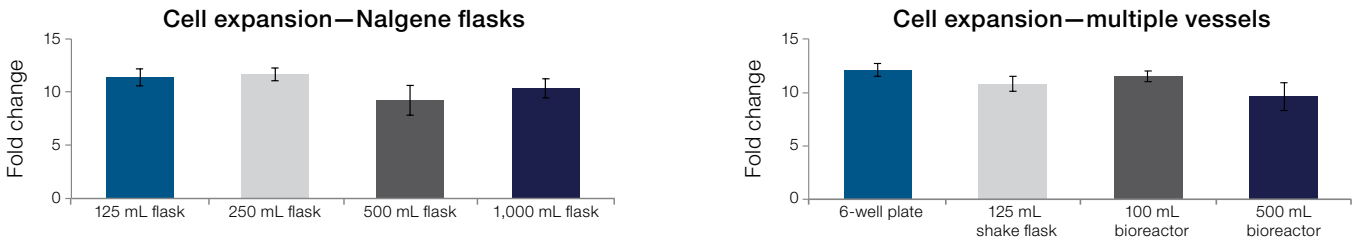


Figure 2.8. StemScale PSC Suspension Medium is compatible with various vessel types and sizes. StemScale medium achieved 9- to 12-fold expansion across multiple vessel types, flasks, and bioreactors. Maintenance of PSC expansion capability was achieved starting in 125 mL Nalgene flasks up to 1,000 mL flasks, and then from Nunc 6-well plates up to a 500 mL bioreactor.

Useful tips

- It is important to use a ROCK inhibitor such as Y-27632 when initiating suspension cultures. This ensures proper nucleation from single cells into spheroids. Do not use RevitaCell Supplement instead of ROCK inhibitor (Y-27632). Y-27632 targets ROCK for inhibition through a mechanism that is more favorable for hPSC spheroid formation with StemScale PSC Suspension Medium.
- Make sure to use a CO_2-resistant shaker. Orbital shakers that are not designed for use in a CO_2 incubator can overheat the culture medium, leading to cell death.
- To avoid over-aggregation of spheroids, cultures should not be removed from the incubator for more than 10 minutes, except during passaging.

Learn more at **thermofisher.com/stemscale**

2.5
Choosing a culture system

As previously mentioned, feeder-dependent cultures have a proven track record of supporting PSC growth and maintaining pluripotency, and are sufficient for many basic research projects. The rich and robust medium makes for forgiving culture conditions that are ideal for novice PSC researchers, and even more experienced researchers may use feeder-dependent cultures as backup or as a point of comparison for feeder-free cultures. However, feeder-dependent cultures do carry certain disadvantages that can discourage prospective users. The undefined components are prone to inconsistent performance. MEFs are of animal origin and pose risks of carrying adventitious agents. Moreover, the workflow is more tedious, involving a longer passaging protocol, requiring significant work to obtain and prepare the feeder cells, often also requiring grooming of cultures to remove areas of spontaneous differentiation. These may be undesirable but tolerable for small-scale work. However, they are extremely difficult to deal with for very large projects.

In contrast, feeder-free systems do not require feeder cell isolation, inactivation, banking, and pre-plating; nor do they require feeder cell removal prior to certain downstream experiments such as molecular analysis or flow cytometry. With these workflow improvements and with the added possibility of performing single-cell passaging, feeder-free systems are generally more amenable to large-scale culture and high-throughput experiments. By eliminating the need for feeders, they also perform more consistently. That said, even among feeder-free cultures systems there can be differences in consistency because some media and matrices do contain less-defined components. In addition, some media contain more components than others, and that equates to not only greater cost and greater complexity, but also greater potential for experiencing inconsistencies in performance.

Due to these considerations, defined and xeno-free minimal culture systems such as Essential 8 Medium and Essential 8 Flex Medium can be more favored for workflows spanning basic research to translational research. In basic research, these systems are more attractive because, in addition to reduced cost, they provide a cleaner background for performing experiments on different biological pathways. These media are also produced according to GMP quality standards for medical devices, which helps increase consistency and reduce burden for future translational research.

While the benefits are abundant, it is important to understand that leaner systems also tend to be more sensitive to stressors, and can be less forgiving of harsh cell manipulations. Furthermore, not all customers require a xeno-free system, and many of today's more complex workflows are not well supported by lean media. The convergence of these factors makes a modern, robust medium like StemFlex Medium extremely attractive. StemFlex Medium addresses the insufficiencies of other media on the market, which were not designed to support the wide variety of modern PSC workflows and applications that are used today. As with all Gibco™ PSC media, StemFlex Medium is manufactured under GMP conditions to offer a consistent and robust product for research applications such as gene editing, single-cell passaging, and clonal expansion, to name just a few.

There are many subtleties to choosing a PSC culture system, and the final choice depends on the research goal. For example, even if the intended application in basic research can ostensibly be satisfied by feeder-dependent systems, feeder-free systems using Essential 8 Medium may still be preferred if the actual experiment requires or can benefit from scalability, consistency, cleaner background, improved workflow, and the potential to skip media changes over entire weekends (when using Essential 8 Flex Medium). However, if the experiment involves heavy cell manipulation or is executed by someone less experienced in PSC culture, a richer medium like StemFlex Medium or a feeder-dependent option like Gibco™ DMEM/F-12 with KnockOut Serum Replacement may offer more benefit. To assist in choosing the appropriate culture system, the advantages and disadvantages of various media are summarized in Table 2.3.

Get additional guidance finding the right PSC culture tools at **thermofisher.com/pscculture**

Table 2.3. Comparison of PSC culture systems.

Media	DMEM/F-12 with KnockOut Serum Replacement	StemScale PSC Suspension Medium	StemFlex Medium	Essential 8 Medium	Essential 8 Flex Medium	CTS Essential 8 Medium
Culture type	Adherent	Suspension	Adherent	Adherent	Adherent	Adherent
Feeder-dependent	Yes	No	No	No	No	No
Defined	No, contains animal-origin components	No, contains animal-origin components	No, contains animal-origin components	Xeno-free	Xeno-free	Components not directly derived from animals
Media complexity	High	Medium	Low	Low	Low	Low
Lot-to-lot variability	Medium	Medium	Medium	Low	Low	Low
Weekend-free feeding	No	Yes	Yes	No	Yes	No
Workflow complexity	High	Low	Low	Low	Low	Low
Scalability	Low	Very high	High	High	High	High
CTS version available*	Yes	Not currently	Not currently	Yes	Not currently	Yes
Manufactured in cGMP-compliant facility	Yes	Yes	Yes	Yes	Yes	Yes

* Gibco™ CTS™ products are designed for clinical research applications. Refer to section 7 for more details.

2.6

Adapting to feeder-free culture systems

Sometimes it is necessary to transition PSCs into a specific feeder-free culture system in order to satisfy changing project requirements or new experimental designs. Several adaptation schemes and protocols enable a smooth transition to StemFlex and Essential 8 media systems (Figure 2.9). PSCs in other feeder-free media like mTeSR™1 Medium (STEMCELL Technologies) can be passaged with Versene Solution directly into StemFlex Medium or Essential 8 Medium with Geltrex matrix. If culturing in Essential 8 Medium with VTN-N is preferred, cells can be passaged into this system after one to two passages in Essential 8 Medium with Geltrex matrix.

Feeder-dependent cultures can be adapted directly into Essential 8 Medium with VTN-N or into StemFlex Medium with Geltrex matrix, but obtaining the right colony fragment size is critical, as large fragments form embryoid bodies while small fragments differentiate upon plating. The recommended approach to obtaining optimum fragment sizes involves harvesting colonies using collagenase IV followed by trituration and, more importantly, two rounds of gravity sedimentation as described in the adaptation protocols. For lines that are difficult to transition or simply to achieve the best results with, cells can be transferred to the medium of choice with rhLaminin-521 for a passage or two before transitioning to Essential 8 Medium with VTN-N or to StemFlex Medium with Geltrex matrix. The combination of Essential 8 Medium and rhLaminin-521 is available as the Gibco™ Essential 8™ Adaptation Kit.

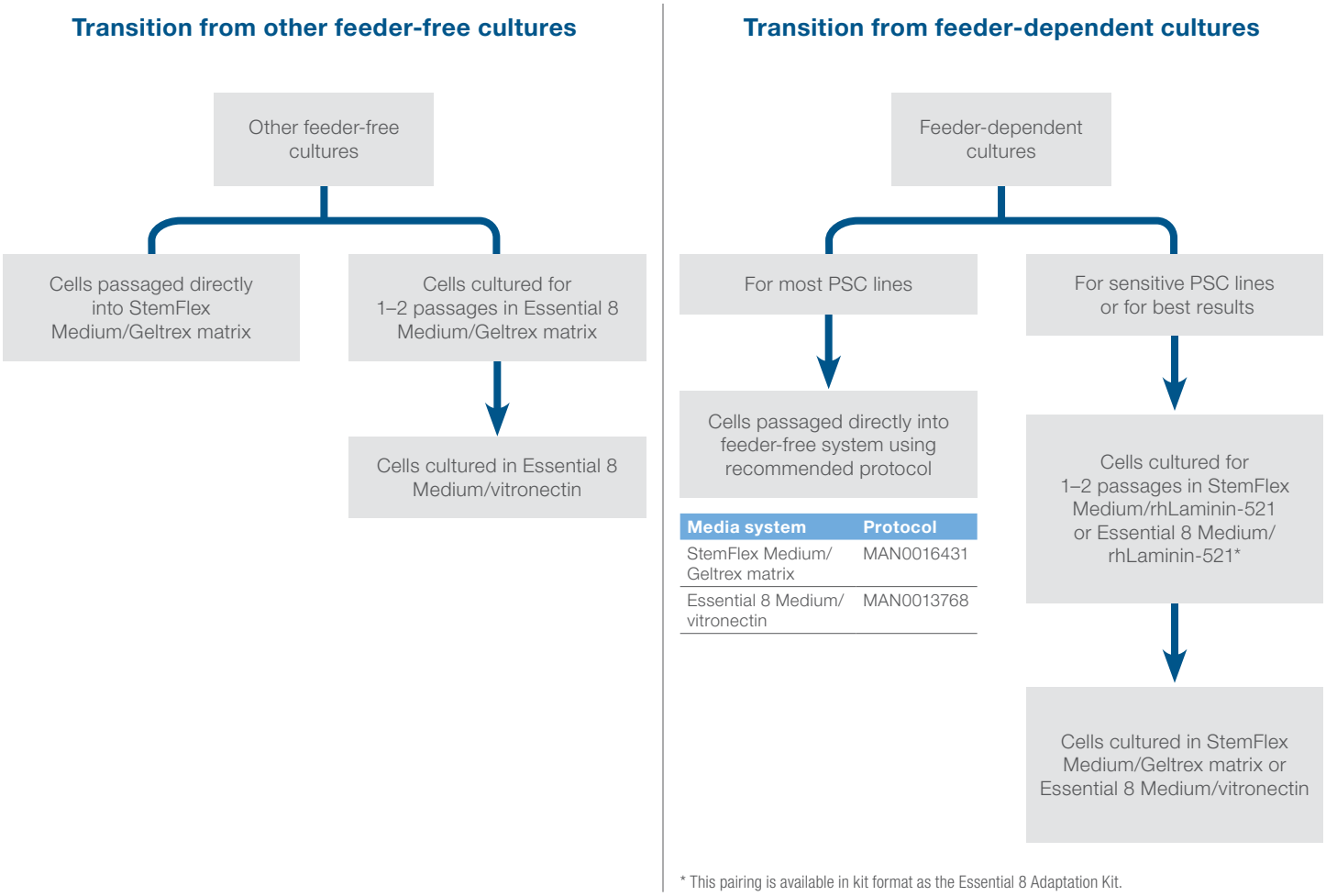


Figure 2.9. Guide for adaptation into StemFlex and Essential 8 media systems. The left side shows the scheme for transitioning PSCs from other feeder-free systems. The right side shows the scheme for adapting from feeder-dependent cultures.

2.7

Cryopreservation

Cryopreservation is an important part of every cell culture workflow. In the PSC workflow, cells are cryopreserved to store backup cultures that can be recovered in case the cells currently in culture are compromised by genetic changes, contamination, excessive cell death, or spontaneous differentiation. They are also frozen to save cell lines for future use, including when projects are temporarily placed on hold or when creating stem cell banks. Finally, PSCs are cryopreserved to enable the transport and sharing of PSC cultures between different facilities. In summary, cryopreservation enhances continuity, longevity, and flexibility of projects, and it improves the availability and dissemination of different PSC lines.

The traditional method for cryopreservation involves freezing PSCs in 10% DMSO, typically by resuspending the cell pellet in PSC medium at half the desired volume, then bringing it up to the full volume with a 2X freezing medium containing 20% DMSO. To minimize the exposure to DMSO, the cryovial is promptly transferred to –80°C in a controlled-rate freezing apparatus that then decreases the temperature gradually by approximately 1°C/min. After 24 hours, the cryovial is transferred for long-term storage to a liquid nitrogen freezer at –200°C to –125°C. When the PSCs need to be thawed, the cryovial is warmed in a water bath until a small sliver of ice remains. Again, to minimize exposure to DMSO and to improve cell survival, the contents are quickly transferred to a conical tube and diluted by adding fresh medium. To avoid osmotic shock, the medium is added dropwise while gently shaking the conical tube. After washing and resuspending in fresh culture medium, the cells are then transferred to a plate and allowed to recover and grow.

Cryopreservation and thawing are stressful for cells, but substituting or supplementing the traditional reagents with the optimized Gibco™ PSC Cryopreservation Kit allows for maximum post-thaw viability and recovery of cryopreserved PSCs. The PSC Cryopreservation Kit comprises a ready-to-use, defined, xeno-free cryopreservation medium and the AOF RevitaCell Supplement, which improves cell survival through antioxidants, free radical scavengers, and a more specific ROCK inhibitor. For a ready-to-use alternative that has been designed for use with a wider variety of cells, one may also use Gibco™ Synth-a-Freeze™ Cryopreservation Medium. This defined medium contains 10% DMSO in a HEPES and sodium bicarbonate buffer, without antibiotics, antimycotics, hormones, growth factors, serum, or protein. Both the PSC Cryopreservation Kit and Synth-a-Freeze Cryopreservation Medium are available as cell therapy–grade products.

To assist you in choosing the best cryopreservation medium, these options are compared in Table 2.4.

Get more information at
thermofisher.com/cryopreservation

Table 2.4. Summary of key characteristics and performance of PSC cryopreservation media.

	PSC Cryopreservation Kit	Synth-a-Freeze Cryopreservation Medium	Homemade cryopreservation medium with DMSO
Ready-to-use	Yes	Yes	No
Recovery component	Yes	No	No
Xeno-free	Yes	Yes	Varies
Manufactured in cGMP-compliant facility	Yes	No	No
CTS version available	Yes	Yes	No
Performance	+++	++	Varies

Useful tips

- For optimum results, collect cells from a healthy, actively growing, high-confluency culture.
- Ensure that differentiated colonies have been removed so that only high-quality PSCs are cryopreserved.
- PSCs may require several passages to recover after cryopreservation. Do not be discouraged if cultures look unhealthy immediately after thawing.

2.8 References

1. Thomson JA, Itskovitz-Eldor J, Shapiro SS et al. (1998) Embryonic stem cell lines derived from human blastocysts. *Science* 282(5391):1145-1147.
2. Chen G, Gulbranson DR, Hou Z et al. (2011) Chemically defined conditions for human iPSC derivation and culture. *Nat Methods* 8:424-429.
3. Rodin S, Antonsson L, Hovatta O et al. (2014) Monolayer culturing and cloning of human pluripotent stem cells on Laminin-521-based matrices under xeno-free and chemically defined conditions. *Nat Protoc* 9(10):2354-2368.

Section 3

Transfection

3.1

Introduction

The ability of stem cells to self-renew and differentiate into various specialized cell types promises to contribute greatly to future applications in regenerative medicine and the development of novel therapeutic treatments. Technologies enabling the genetic manipulation of stem cells support preclinical and clinical research to develop new gene correction and tissue replacement therapies. The ability to subsequently isolate and expand lines of stem cells under feeder-free culture conditions, using systems like StemFlex and Essential 8 media, further enable the movement of stem cell research from the bench to the clinic.

Transfecting stem cells without inhibiting cell viability and cell growth have been shown to be difficult, as the delivery method itself must have little to no effect on the properties of stem cells (e.g., maintenance of pluripotency posttransfection) for downstream assay results to be reliable. Research applications such as gene editing, gene expression, and directed differentiation all require the ability to deliver a variety of constructs into stem cells. Advances in gene editing using the CRISPR-Cas9 system also require the ability to deliver large plasmid constructs or a combination of DNA, RNA, or Cas9 ribonucleoprotein (RNP) complexes.

3.2

Choosing a transfection method and technologies

Different transfection methods and technologies have their own advantages and disadvantages that must be considered. However, nonviral transfection methods are the most widely used methods for delivering foreign molecules into cells (Figure 3.1). The main features to consider when transfecting cells include transfection efficiency, toxicity, versatility, and cost. The products below are recommended, keeping these key considerations in mind (Table 3.1).

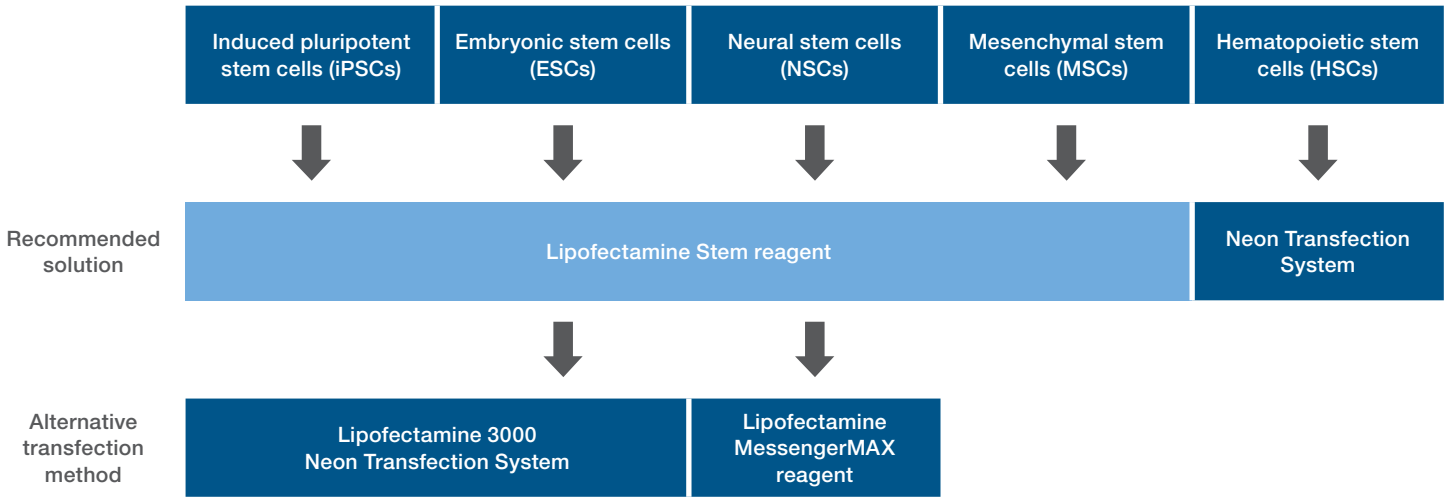


Figure 3.1. Recommended transfection methods by cell type.

Table 3.1. Transfection selection guide for stem cells—recommended payload by product and transfection efficiency by cell type (more blocks represent higher efficiency).

Transfection method	Recommended payloads				Transfection efficiency by cell type			
	DNA	mRNA	RNP (Cas9 protein)	Co-delivery	iPSC	ESC	NSC	MSC
Lipofectamine Stem reagent	✓	✓	✓	✓	■■■■	■■■■	■■■■	■■□□
Lipofectamine 3000 reagent	✓	✓		✓	■■■□	■■■□	■■□□	NA
Lipofectamine MessengerMAX reagent		✓			■■■□	■■■□	■■□□	■■□□
Neon Transfection System	✓	✓	✓	✓	■■■■	■■■■	■■■□	■■■■
Lipofectamine CRISPRMAX reagent			✓		■■■□	Not tested	Not tested	Not tested

3.3

Transfecting DNA

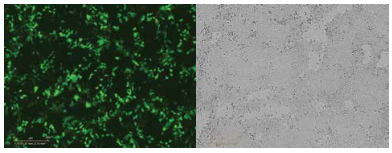
Plasmid DNA remains the most common transgene construct for transfection. Until recently, it was difficult to get high levels of DNA delivery using chemical transfection reagents in stem cells. However, with the introduction of Invitrogen™ Lipofectamine™ Stem Transfection Reagent, efficiency of 60% or higher is easily achieved.

Lipofectamine Stem Transfection Reagent is optimized for efficient DNA delivery into stem cells (human ESCs and iPSCs), with high efficiency (Figure 3.2). This reagent has also been validated to achieve high-efficiency delivery of large plasmid DNA (>10 kb) into PSCs, while supporting their continued proliferation in an undifferentiated state (Figure 3.3).

Applications like gene editing frequently require large plasmids encoding gene-editing constructs or template DNA, and often require electroporation to transfect cells. Lipofectamine Stem reagent provides a complementary alternative to electroporation to introduce a wide range of plasmid DNA into stem cells and is gentler on cells. Lipofectamine Stem reagent is also compatible with a variety of cell culture media, including feeder-free culture systems such as Essential 8 Medium with vitronectin and StemFlex Medium with a Geltrex matrix (Figure 3.4). In addition, human PSCs can be transfected in suspension during re-plating as a convenient alternative to electroporation.

A. Human pluripotent stem cells (Gibco iPSCs)

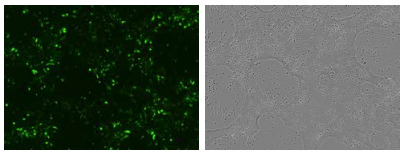
Experimental condition	Recommendation
Delivery platform	Lipofectamine Stem reagent, 1 µL/well
Plate format	24-well plate
DNA	GFP plasmid, 500 ng/well
Medium	Essential 8 Medium
Extracellular matrix	Vitronectin
Cell density	50,000 cells/well



Gibco iPSCs, GFP plasmid transfection efficiency: 75%

B. Human embryonic stem cells (H9 ESCs)

Experimental condition	Recommendation
Delivery platform	Lipofectamine Stem reagent, 1 µL/well
Plate format	24-well plate
DNA	GFP plasmid, 500 ng/well
Medium	Essential 8 Medium
Extracellular matrix	Vitronectin
Cell density	50,000 cells/well



H9 ESCs, GFP plasmid transfection efficiency: 83%

Figure 3.2. High-efficiency DNA transfection with Lipofectamine Stem reagent in human (A) iPSCs and (B) ESCs.

Transfects large DNA constructs

Lipofectamine Stem reagent achieves high-efficiency delivery of large plasmid DNA (>10 kb) into human PSCs, while supporting their continued proliferation in an undifferentiated state in defined and complex feeder-free culture systems.

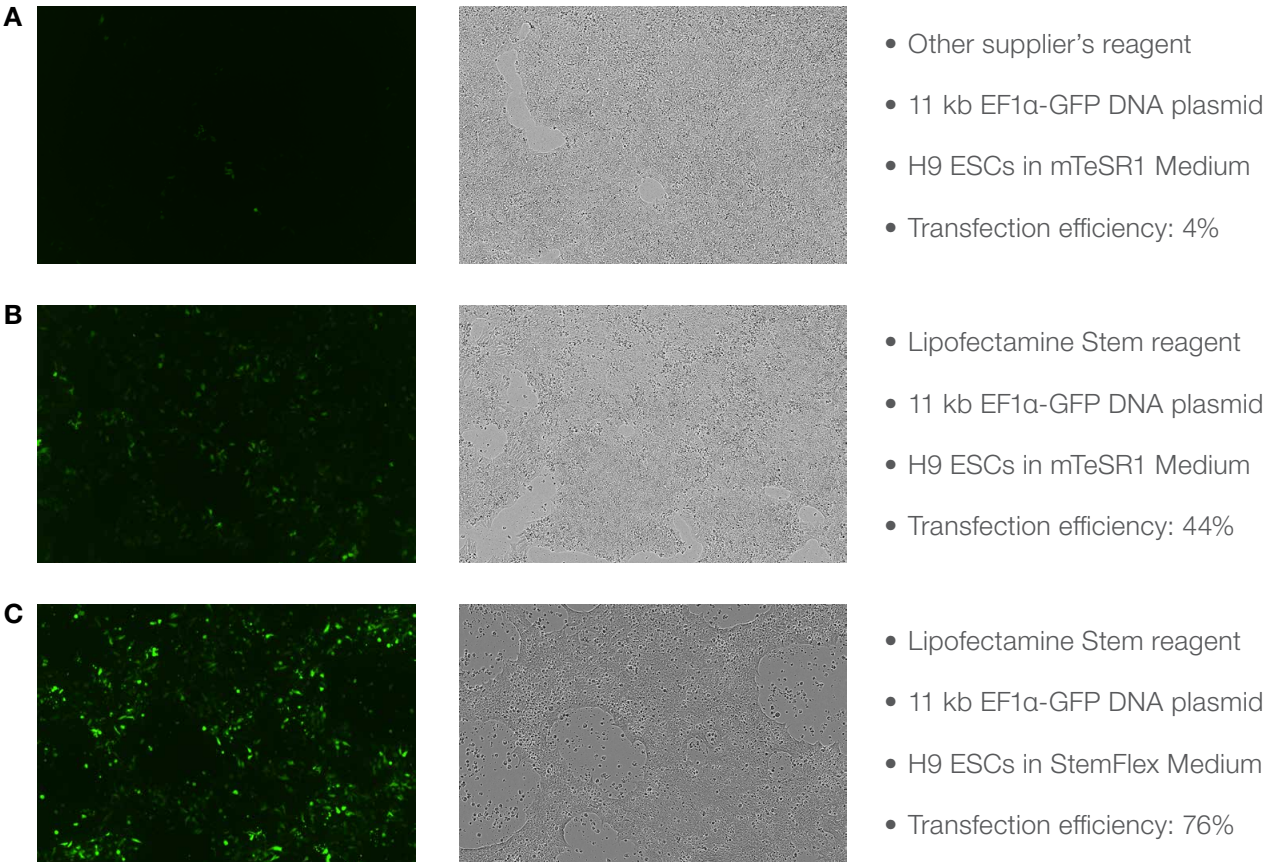


Figure 3.3. Delivery of large DNA constructs with significantly higher efficiency than leading supplier's reagent. (A) H9 ESCs transfected with 11 kb DNA plasmid using a leading DNA delivery reagent in mTeSR™1 Medium. **(B)** H9 ESCs transfected with 11 kb DNA plasmid using Lipofectamine Stem reagent in mTeSR1 Medium. **(C)** H9 ESCs transfected with 11 kb DNA plasmid using Lipofectamine Stem reagent with StemFlex Medium.

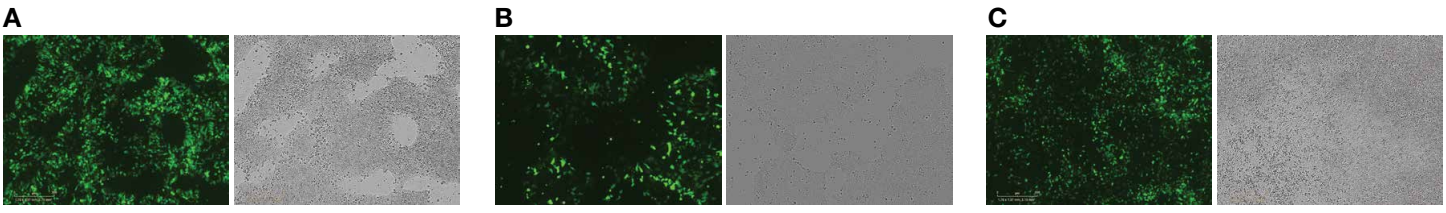


Figure 3.4. Lipofectamine Stem reagent is compatible with a variety of media systems. Results show human PSCs 38–44 hours posttransfection. Human iPSCs plated in 24-well plates were transfected with 1 or 2 µL Lipofectamine Stem reagent in **(A)** StemFlex Medium on Geltrex matrix, **(B)** Essential 8 Medium on vitronectin, and **(C)** mTeSR1 Medium on Geltrex matrix.

Gentle on cells—keeps stem cells viable and healthy

Transfecting stem cells without inhibiting cell viability and cell growth can be challenging due to the sensitivity of these cells. Transfection requires a balancing act between introducing foreign nucleic acids into a cell, and not killing the cell in the process. Lipofectamine Stem reagent delivers low amounts of nucleic acid with high translational efficiency, allowing stem cells to stay healthy and continue proliferating without inducing differentiation. Pluripotent stem cells continue to proliferate with continuous transfection, reaching near-confluency by 48 hours (Figure 3.5).

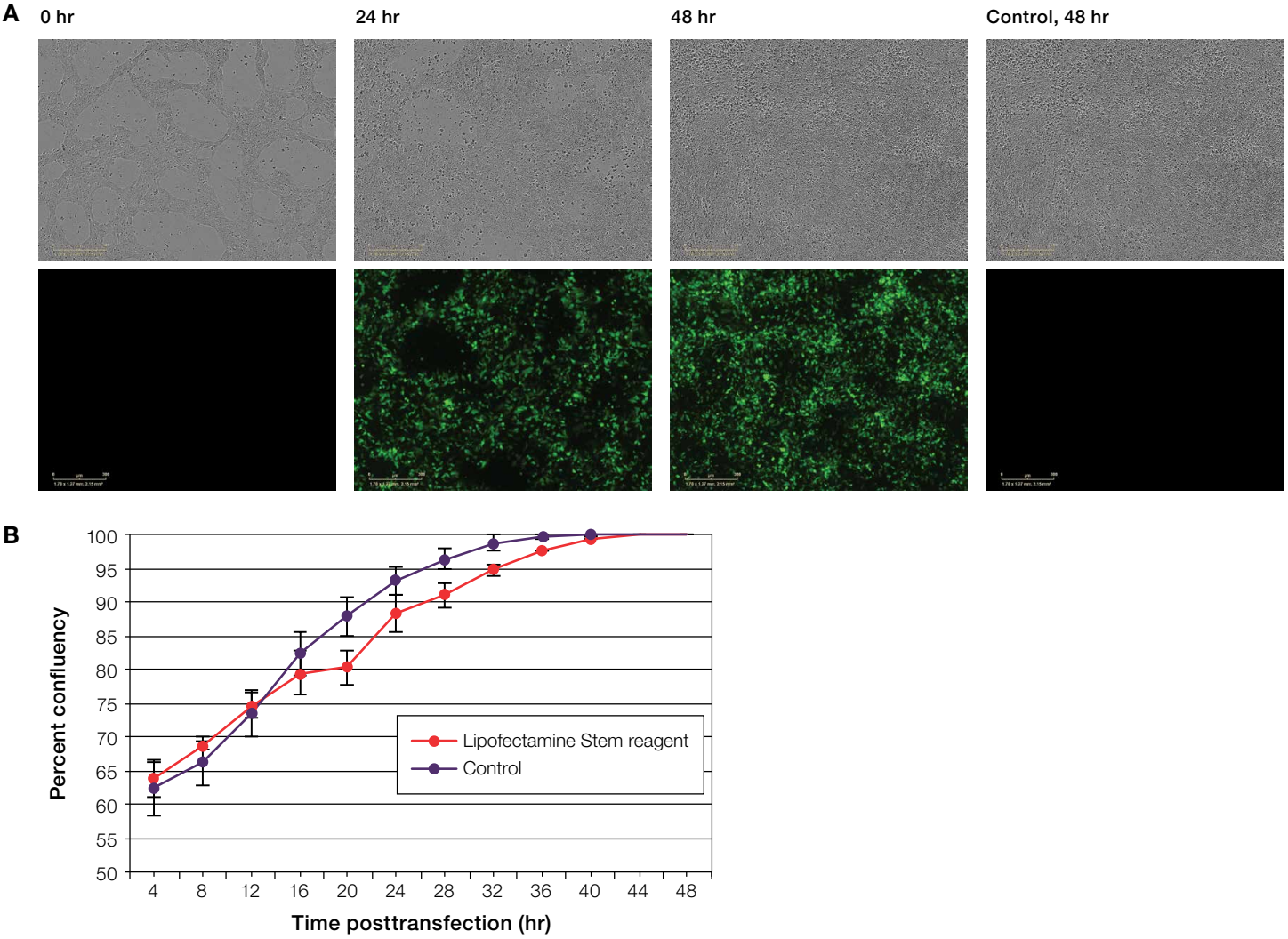


Figure 3.5. Lipofectamine Stem reagent maintains healthy, proliferating stem cells during transfection. (A) Human iPSCs cultured in feeder-free Essential 8 Medium were transfected using Lipofectamine Stem reagent or not transfected (control). Cells remain healthy and viable with normal morphology and continued to proliferate with continuous transfection, reaching confluency by 48 hours. **(B)** Percent area confluency of transfected and untransfected cells.

After transfection with Lipofectamine Stem reagent, PSCs can be serially passaged and expanded, while maintaining pluripotency. Wells of control and transfected Gibco™ Human Episomal iPSCs were passaged in parallel with Gibco™ Versene™ Solution at 48 hours after transfection with Lipofectamine Stem reagent to deliver a GFP DNA plasmid, replated in a 6-well format, and allowed to expand in StemFlex Medium for 4 additional days. They were then passaged again, allowed to expand for 3 more days, and fixed after reaching >75% confluency. Cultures exhibited uniform cell morphology and homogeneous expression of Oct4 after transfection with Lipofectamine Stem reagent and continued culture in StemFlex Medium (Figure 3.6).

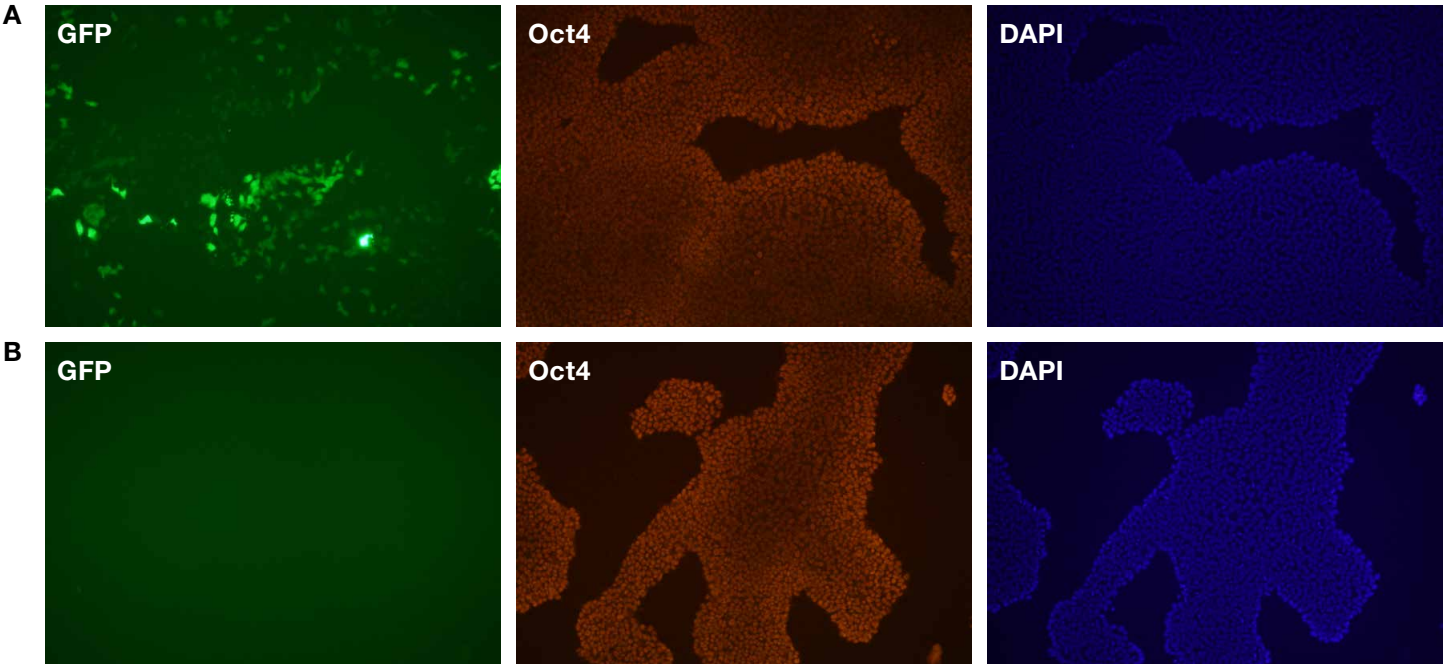


Figure 3.6. PSCs maintain markers of pluripotency after transfection with Lipofectamine Stem reagent and growth in StemFlex Medium. **(A)** Gibco iPSCs transfected with circular plasmid DNA and subsequently expanded for 2 passages. **(B)** Gibco iPSCs not transfected. Gibco iPSCs retain residual GFP expression two passages after transfection of a circular plasmid DNA (**A**, left panel), while continuing to express Oct4 protein (**A**, middle panel) in the nuclei, similar to untransfected Gibco iPSCs grown in parallel in StemFlex Medium (**B**, middle panel).

3.4

Transfecting mRNA

Messenger RNA (mRNA) tends to transfect more efficiently than DNA, making it a great alternative for transfection when working with difficult-to-transfect stem cells. Transfection of mRNA only requires entry into the cell cytoplasm—without the need for translocation to the nucleus for transcription. Since nuclear entry is not necessary with mRNA, it eliminates any risk of integration into the host genome, which can cause insertional mutagenesis and activation of oncogenes; and transfection efficiency is generally higher. Additional benefits include a transient transfection and faster time to protein expression than with DNA. Transfection with mRNA is a useful platform for manipulating cell genotype and phenotype by gene editing and transcription factor–directed differentiation. Having control over the timing, dosage, and stoichiometry of transgene delivery provides a precise way to drive foreign protein production in stem cells.

As shown in Figure 3.7, Lipofectamine Stem Transfection Reagent demonstrates outstanding transfection efficiency in stem cells with low amounts of mRNA.

A. Human H9 ESCs

Experimental condition	Recommendation
Delivery platform	Lipofectamine Stem reagent, 1 µL/well
Plate format	24-well plate
Content delivered	GFP mRNA , 250 ng/well
Medium	Essential 8 Medium
Extracellular matrix	Vitronectin
Cell density	50,000 cells/well

B. H9 ESCs, GFP mRNA transfection efficiency: 79%

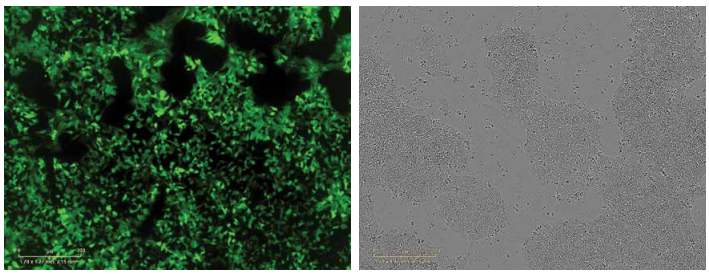


Figure 3.7. High-efficiency transfection of human stem cells with mRNA using Lipofectamine Stem reagent. (A) Experimental conditions for H9 ESCs. **(B)** The cells were examined for GFP expression 24 and 48 hours posttransfection.

3.5

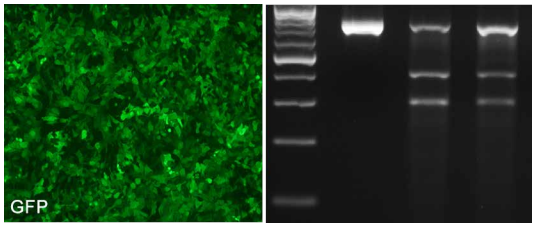
Transfecting RNP complexes

Research applications such as gene editing require the ability to deliver a variety of substrates into cells. Lipofectamine Stem reagent can be used to efficiently co-deliver RNP complexes of Cas9 protein with a guide RNA (gRNA) to support high-efficiency insertion or deletion (indel) formation of your target gene for the purpose of functional knockout (Figure 3.8). Single-stranded DNA constructs can also be mixed with Cas9 mRNA + gRNA or Cas9 protein + gRNA to promote homology-directed repair (HDR) and introduce targeted genomic sequences.

A. Human PSCs (Gibco iPSCs)

Experimental condition	Recommendation
Delivery platform	Lipofectamine Stem reagent, 1 µL/well
Plate format	24-well plate
Cas9 protein	500 ng Cas9 protein + 125 ng gRNA + 50 ng GFP mRNA
Cas9 mRNA	500 ng Cas9 mRNA + 125 ng gRNA + 50 ng GFP mRNA
Medium	Essential 8 Medium
Extracellular matrix	Vitronectin
Cell density	50,000 cells/well

B. iPSCs, GFP mRNA transfection efficiency: 90%



Transfection efficiency: 90%
Cas9 mRNA: 64% indel
Cas9 protein: 44% indel

Figure 3.8. Transfection with Lipofectamine Stem reagent supports high-efficiency gene editing in stem cells. (A) Experimental conditions for human iPSCs. **(B)** (Left) Human iPSCs were cotransfected with Cas9 mRNA, gRNA, and GFP mRNA (not shown) or Cas9 RNP targeting the *EMX1* gene and GFP mRNA. (Right) Genomic cleavage detection of the *EMX1* locus in iPSCs was analyzed 48 hours after transfection.

For certain media systems, such as StemFlex Medium, some of the media components may be inhibitory to lipid-based transfection. For these media systems, the following workflow can be used to accomplish high-efficiency lipid-based delivery using the Lipofectamine Stem reagent (Figure 3.9).

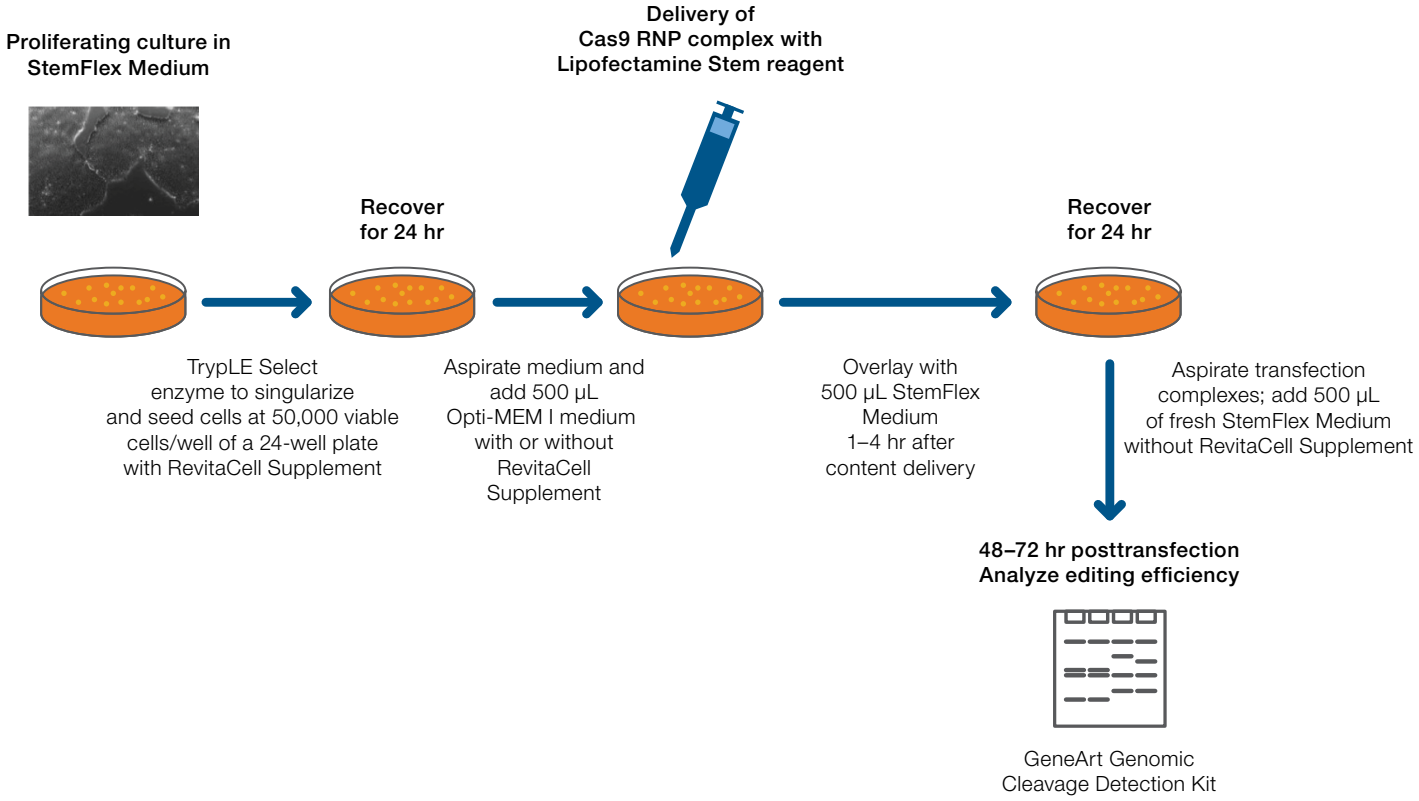


Figure 3.9. Schematic workflow of overlay method for delivery of Cas9 RNP complex using Lipofectamine Stem reagent to PSCs cultured in StemFlex Medium. For detailed instructions, go to **thermofisher.com/lipofectaminestem**.

As shown in Figure 3.10 and Table 3.2, using the previous workflow (Figure 3.9), high-efficiency delivery of Cas9 RNP complex is attained using Lipofectamine Stem reagent with >40% cleavage or indel efficiency being observed using the Invitrogen™ GeneArt™ Genomic Cleavage Detection (GCD) Kit. PSCs are also shown to attain high maintenance of pluripotency as assessed via immunocytochemistry of PSCs stained for Oct4, an intracellular marker of pluripotency.

Table 3.2. Human PSCs (Gibco iPSCs).

Experimental condition	Recommendation
Delivery platform	Lipofectamine Stem reagent, 2 µL/well
Plate format	24-well plate
Content delivered	Cas9 protein/HPRT gRNA (RNP complex) + 150 ng GFP mRNA (as transfection proxy)
Medium	StemFlex Medium
Extracellular matrix	Geltrex
Cell density	50,000 cells/well

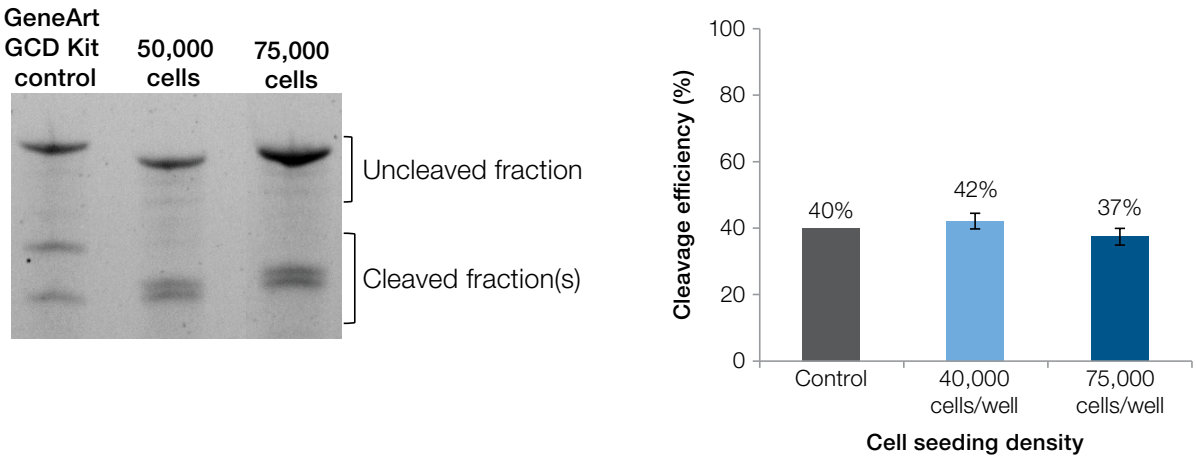


Figure 3.10. GeneArt Genomic Cleavage Detection Kit analysis of cleavage efficiency. The assay was performed 72 hours following transfection of Gibco Human Episomal iPSCs with Cas9 RNP complex via Lipofectamine Stem reagent.

At times, lipid-based reagent solutions are not a viable option. Therefore, we recommend electroporation using the Invitrogen™ Neon™ Transfection System in these instances. StemFlex Medium is a versatile medium, which also supports PSCs in electroporation-based workflows, providing robust recovery of PSCs following electroporation (Figures 3.11 and 3.14), high cleavage efficiency under different electroporation conditions (Figure 3.12), and high maintenance of pluripotency of stem cells following electroporation and recovery (Figure 3.13).

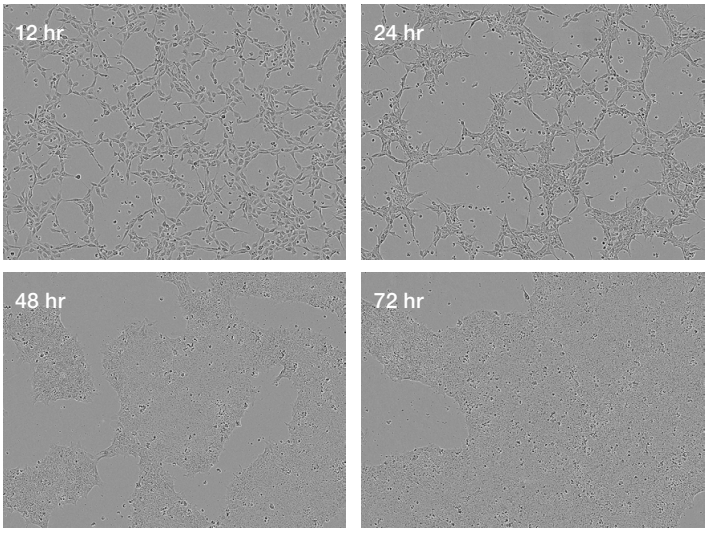


Figure 3.11. Representative images after electroporation showing robust recovery of iPSCs cultured in StemFlex Medium.

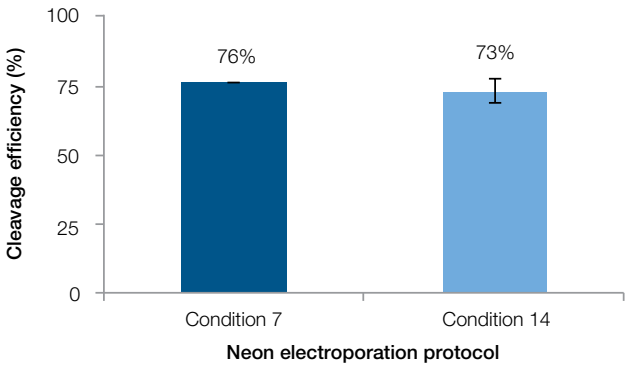
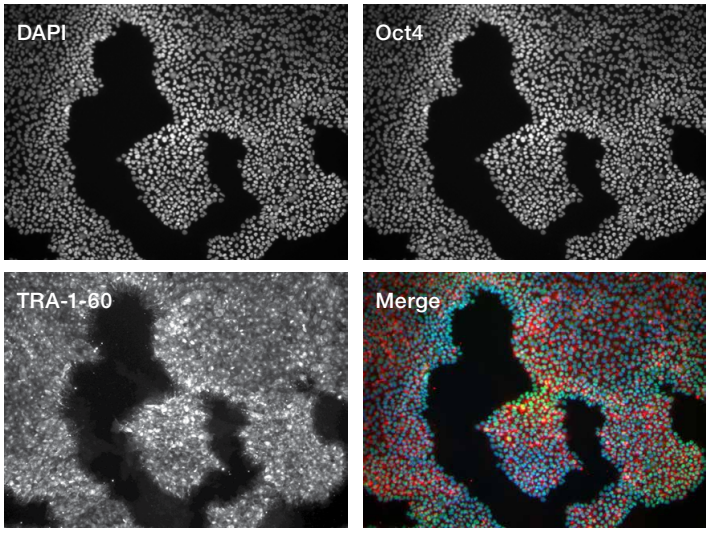


Figure 3.12. Cleavage efficiency of cultures grown in StemFlex Medium ~72 hours after electroporation with Cas9–gRNA complexes targeting the *HPRT* gene.

A



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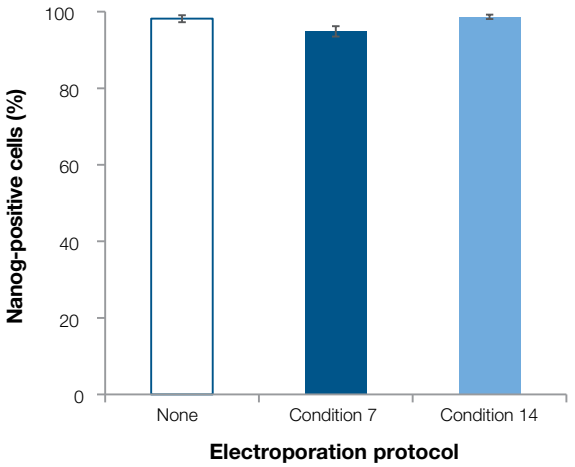

Figure 3.13. Maintenance of pluripotency of iPSCs cultured in StemFlex Medium after electroporation and recovery. Cultures transfected with Cas9–gRNA complexes targeting the *HPRT* gene were assessed by **(A)** qualitative immunocytochemistry of Oct4 and TRA-1-60 expression and **(B)** quantitative assessment of Nanog expression via flow cytometric analysis.

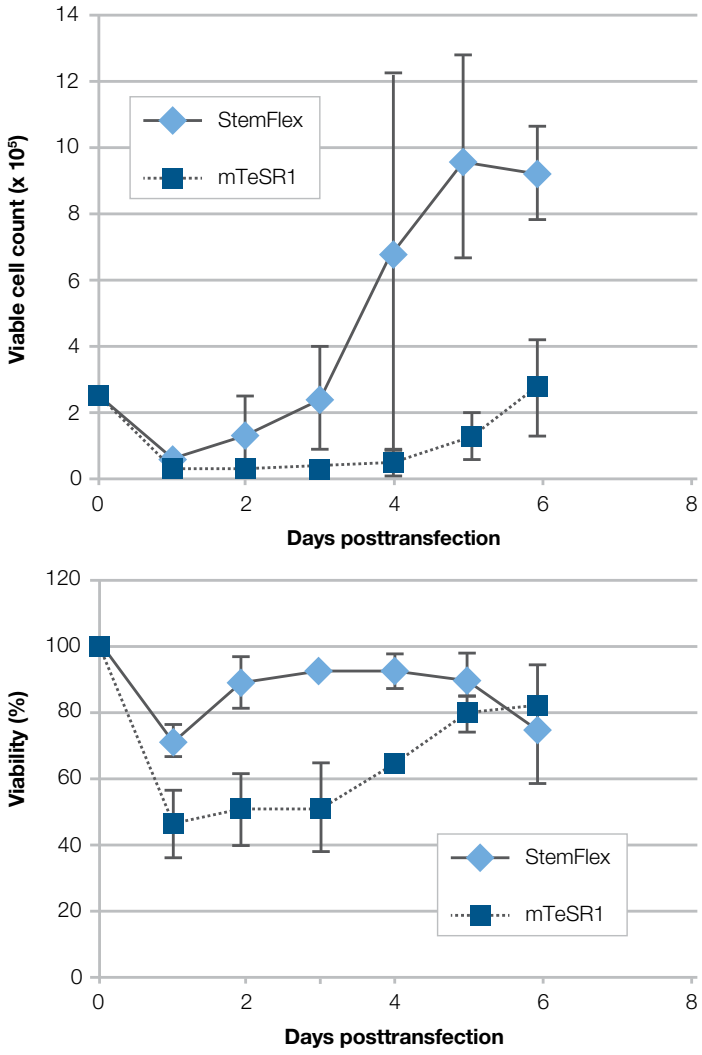

Figure 3.14. Cell growth and viability following electroporation of Cas9 plasmid and donor plasmid into ESI-017 cells.

Useful tips

- In addition to vitronectin, PSCs can be successfully grown and transfected in Essential 8 and StemFlex media on rhLaminin-521, as well as Geltrex matrix.
- PSCs passaged with Versene Solution should be triturated into small clumps of 3–5 cells. Colonies broken into too large clusters (>10 cells) will only transfect well around the outer edges. Alternatively, use Gibco™ TrypLE™ Select Enzyme as recommended for use with the StemFlex Medium system.
- Lipofectamine Stem reagent complexes should be made in Gibco™ Opti-MEM™ I Reduced Serum Medium and can be added directly to stem cells in culture medium growing with or without serum. **Note:** an overlay method is required for StemFlex Medium–cultured PSCs.
- While the optimal timing of transfection (24–72 hours) of Cas9 RNP for maximal indel formation may need to be determined for each gene of interest, it is not necessary to remove transfection complexes. An equal volume of additional Essential 8 Medium should be added to the wells the following day without interfering with the transfection, if stem cell cultures need to be transfected for 48 hours. For cultures in StemFlex medium, removal of complexes 24 hours post-delivery is recommended.
- The amount of Lipofectamine Stem reagent required for optimal transfection depends upon the amount of stem cells plated and the amount of DNA used (see recommended amounts below).
  - Proliferating stem cell cultures need room to expand during transfection, so plate the recommended starting cell numbers to achieve 30–60% confluency on the day of transfection.
  - Using lower amounts of DNA, mRNA, or RNP complexes can yield improved expression while minimizing risks of cytotoxicity from excess foreign constructs and erroneous cleavage events.
- In instances where poor lipid-based transfection is observed, the Neon Transfection System may be used as an alternative method.

Section 4

Genome editing

4.1 Introduction

Broadly, gene engineering or genome editing involves changing an organism's DNA through sequence disruption, replacement, or addition. While approaches for genetic manipulation of mouse ESCs have been widely used for decades in the generation of transgenic mouse models, recent advances in genome editing technologies now make this a tool that can readily be applied to hPSCs.

The capacity of hPSCs to self-renew and differentiate makes them ideally suited for generating both disease models and cells at the scale needed for drug development and cell therapy applications. The ability to genetically modify hPSCs further increases their usefulness for both research and clinical applications, enabling the generation of models for genetically complex disorders.

The dovetailing of iPSC and genome editing approaches supports a diverse range of applications (Figure 4.1), including:

- Generation of disease models by introducing known mutations in control iPSCs
- Generation of isogenic controls by correcting mutations in patient-specific iPSCs
- Testing the disease relevance of implicated genes by selectively knocking down or knocking out loci in wild type iPSCs
- Deconvoluting contributions of multiple loci in polygenic diseases by selectively correcting individual loci
- Generation of lineage-specific reporter lines
- Generation of gene-corrected disease-relevant cell types for cell replacement therapies

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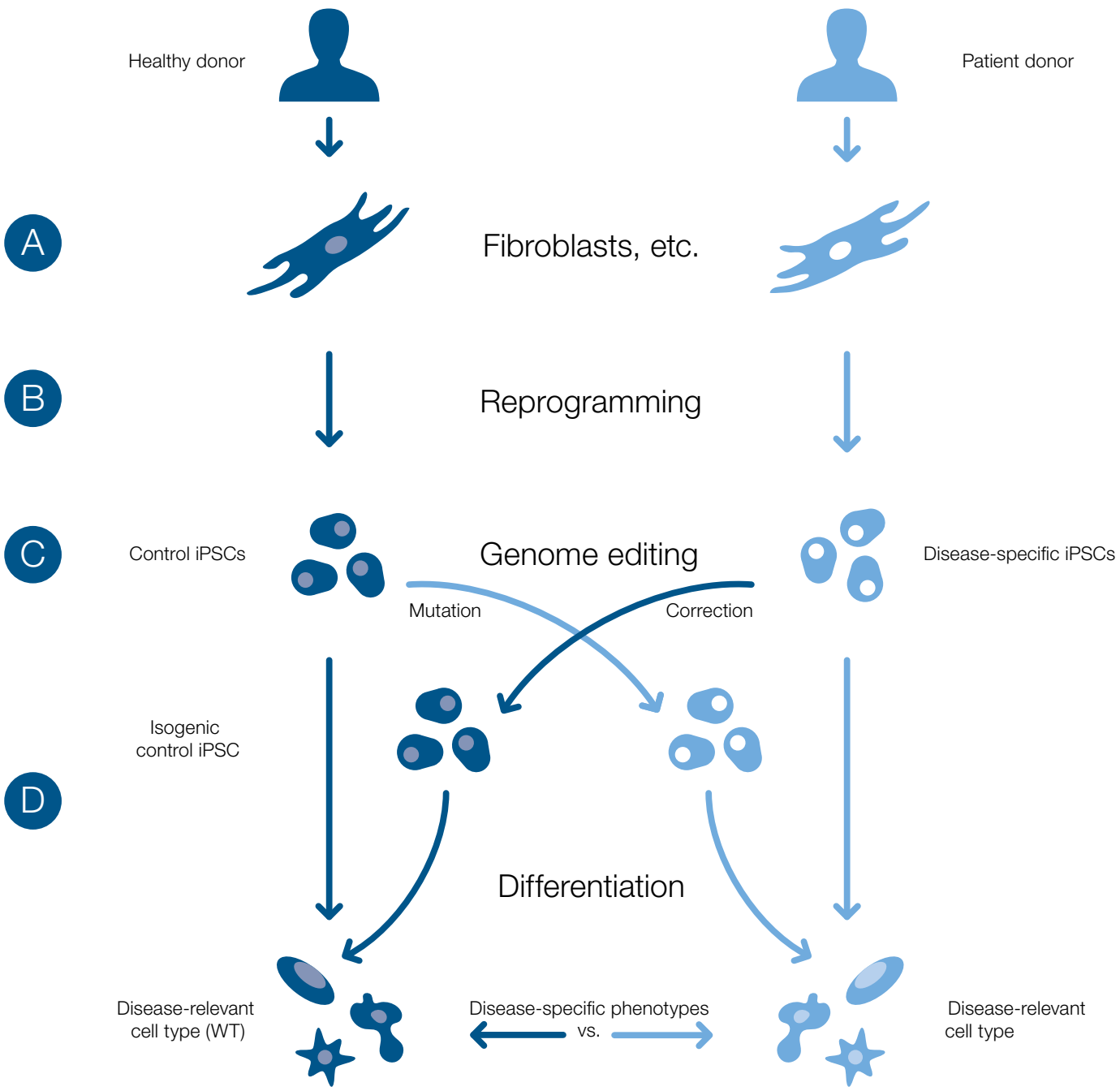


Figure 4.1. Generation of disease-specific and isogenic control (wild type, WT) iPSCs and disease-relevant cell types using genome editing. (A) Somatic cells such as fibroblasts or blood cells are isolated from healthy or patient donors and **(B)** reprogrammed to generate control and disease-specific iPSCs. **(C)** Genome editing can be used to introduce disease-relevant mutations into control iPSCs to generate disease-specific iPSCs. Alternatively, gene correction can be used to generate isogenic controls from disease-specific iPSCs. **(D)** Disease phenotypes can potentially be quantified by comparing the behavior of disease-relevant cell types, such as neurons or cardiomyocytes derived from control and patient-specific iPSCs, in functional assays.

The design of a successful genome editing project will be impacted by several choices, starting with the choice of a genome editing tool. Further factors that need to be considered include the delivery method, cell culture system, and editing validation technologies (Figure 4.2).

Thermo Fisher Scientific provides a suite of tools optimized to help ensure a high level of success. These will be addressed in greater detail in the following sections.

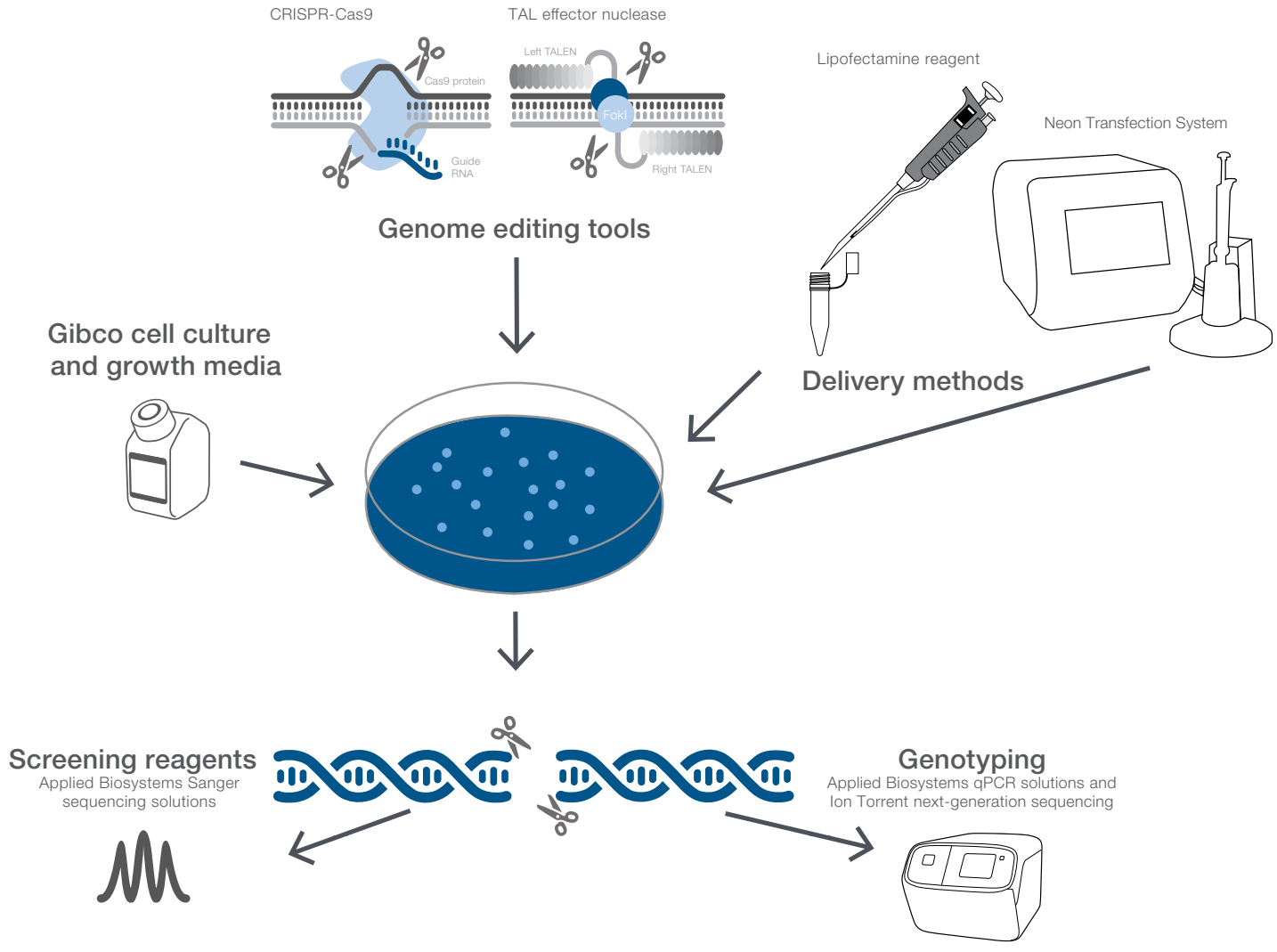


Figure 4.2. Factors affecting genome editing outcomes in iPSCs.

4.2

Genome editing tools

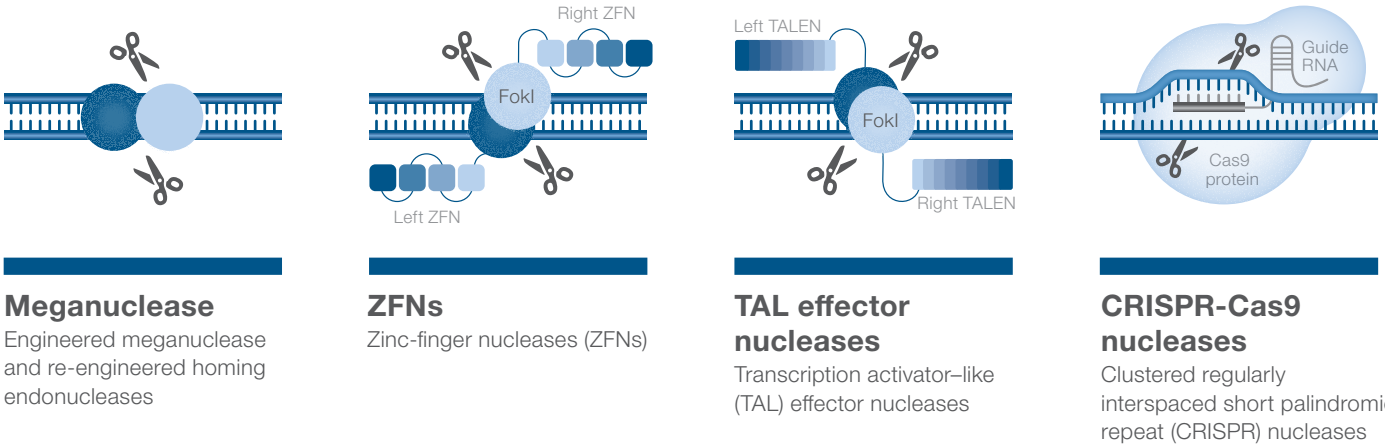
Genome editing used to be a laborious and inefficient process, using either random mutagenesis or older technologies such as zinc finger nucleases that were difficult to design and target to specific sites in the genome. Recent advances in gene engineering tools now allow exquisite precision and control in a user-friendly workflow.

Genome editing is now routinely being achieved through the use of technology derived from clustered regularly interspaced short palindromic repeats (CRISPRs) and transcription activator–like (TAL) effectors. CRISPR-Cas9 guide RNA (gRNA) and TAL effectors target nucleases to specific sites in the genome, creating double-stranded breaks at desired locations (Figure 4.3).

The natural repair mechanisms of the cell heal the break by either homologous recombination or nonhomologous end-joining (NHEJ). Homologous recombination is more precise because it requires a template for repair.

By providing the cell with a synthetic template containing a sequence of interest, for example a disease-specific mutation, the researcher can introduce this sequence into the genome. In contrast, double-strand break repair by NHEJ is more error-prone, frequently introducing errors such as small insertions or deletions (indels). Since the resulting frameshift often leads to a nonfunctional gene, this approach can be harnessed to rapidly and efficiently generate specific gene knockouts.

Originally, CRISPR-Cas9 genome editing technologies were considered to be more efficient but also more prone to off-target effects when compared to TAL technologies. Recent advances in the tools and reagents available for both gene editing systems have negated some of these differences, and CRISPR-Cas9 and TAL technologies are now both widely used in a broad range of applications. A highlight of the benefits and limitations of the two technologies can be found in Table 4.1.



Meganuclease
Engineered meganuclease and re-engineered homing endonucleases

ZFNs
Zinc-finger nucleases (ZFNs)

TAL effector nucleases
Transcription activator–like (TAL) effector nucleases

CRISPR-Cas9 nucleases
Clustered regularly interspaced short palindromic repeat (CRISPR) nucleases

Figure 4.3. Available genome editing technologies.

Genome editing learning center
Explore the genome editing support center to find answers, information, and resources to support iPSC research. Read through frequently asked questions, view on-demand webinars, download the latest application notes, or check out tips and tricks.

Access all resources at **thermofisher.com/genomeedit101**

Table 4.1. Comparison of CRISPR and TAL technologies.

	CRISPR-Cas9 technology	TAL effector technology
	A fast, simple, and precise method to perform gene edits	Flexible editing, targeting any gene in the cell with all-around freedom
Modification options	• Gene deletion (knockout) • Integration (knock-in)	• Gene deletion (knockout) • Integration (knock-in)
Type of recognition	RNA–DNA	Protein–DNA
Requirement for recognition	PAM site (NGG at the end of the 20 bp target sequence)	Active range of spacing needed for activity
Areas of application	• Gene function • Gene modulation • Gene tagging • Pathway engineering • Metabolic engineering • Protein production	• Gene function • Gene modulation • Gene tagging • Pathway engineering • Metabolic engineering • Protein production
Limitations	• Off-target effects • Sensitive to methylation • PAM dependence may limit optimal targeting for HDR	• Difficult to design • Blocked by CpG methylation • Inhibited by chromatin structure • Large size for delivery
Off-target effects	Moderate	Low
Multiplexing	Capable	Rarely used

Ease of design and construction—CRISPR-Cas9 gene editing relies on the formation of an RNA–DNA complex. Targeting of a new site simply requires the design and generation of a 20 nt gRNA that provides specificity to the system. This can be achieved both readily and cost effectively, which is often particularly appealing to academic scientists. On the other hand, TAL effector target recognition occurs as a result of protein–DNA interactions between the TAL effector proteins and the DNA target sequence. Editing of a new site, therefore, requires the design and construction of a new protein specific to that sequence. Although the process of TAL effector protein cloning has been greatly streamlined through the availability of repeat combinations of modules, this can still present a significant pain point for users.

Target site selection—While the nature of TAL effector targeting means that TAL pairs can be engineered to target virtually any position in the genome, CRISPR functionality requires that the gRNA target sequence immediately precedes a three-base PAM sequence, typically NGG. This is not typically an issue when designing knockouts, where there is greater flexibility in the exact gene region being targeted. However, this can severely limit the availability of sites that are amenable to homologous recombination using donor DNA, since the editing efficiency will depend on the distance from the PAM site. Therefore, when designing an elegant knock-in experiment, TAL effectors could be a better tool to use.

Specificity—CRISPR-Cas9 tools have a comparatively high tolerance for mismatches between the gRNA and intended DNA target, which can negatively impact specificity. However, tools such as Invitrogen™ TrueCut™ Cas9 Protein v2, rather than DNA- or RNA-based systems, have a reduced half life and a more acute effect once introduced into cells, and this can greatly reduce off-target effects. TALs have traditionally been thought of as having fewer off-target effects due to the system's longer DNA-binding sites, which reduce the likelihood of homology in other areas of the genome. The high degree of specificity inherent to TAL effector–based gene editing is particularly important for potential cell therapy applications, for example in the use of CAR T cells.

Efficiency—The high cleavage efficiencies achieved with CRISPR-Cas9 may be a deciding factor for many R&D applications. This feature also makes CRISPR-Cas9 suitable for high-throughput applications using lentiviral-based CRISPR libraries, as an alternative to RNAi-based screening, and further makes it amenable to simultaneous targeting of multiple sites. The efficiency of editing with TAL pairs can be impacted by the system's sensitivity to CpG methylation. This can render some TAL pairs ineffective if they target areas with high levels of methylation.

Delivery—Both CRISPR-Cas9 and TAL technologies can be enabled via lipid transfection and electroporation.

Licensing—For researchers pursuing commercial applications of their gene edited products, it is worth noting that TAL technologies provide a clear licensing path for this purpose. The intellectual property landscape relating to CRISPR-Cas9 editing has been less clear. It may still be some time before clear guidelines for the appropriate commercial use of CRISPR-Cas9 tools become available. For more information about licensing, contact us at **outlicensing@thermofisher.com**

4.3

CRISPR-Cas9 technology

Genome editing uses engineered nucleases in conjunction with endogenous repair mechanisms to alter the DNA in a cell. The CRISPR-Cas9 system takes advantage of a short gRNA to direct the bacterial Cas9 endonuclease to a specific genomic locus. Because the gRNA supplies the specificity, changing the target only requires a change in the design of the sequence encoding the gRNA.

The CRISPR-Cas9 system is composed of a DNA endonuclease called Cas9 and a short, noncoding gRNA that has two molecular components: a target-specific CRISPR RNA (crRNA) and an auxiliary trans-activating crRNA (tracrRNA). These components are commonly concatenated to form a long single guide RNA (sgRNA). The gRNA guides the Cas9 protein to a specific genomic locus via base pairing between the crRNA sequence and the target sequence within the genomic locus (Figure 4.4).

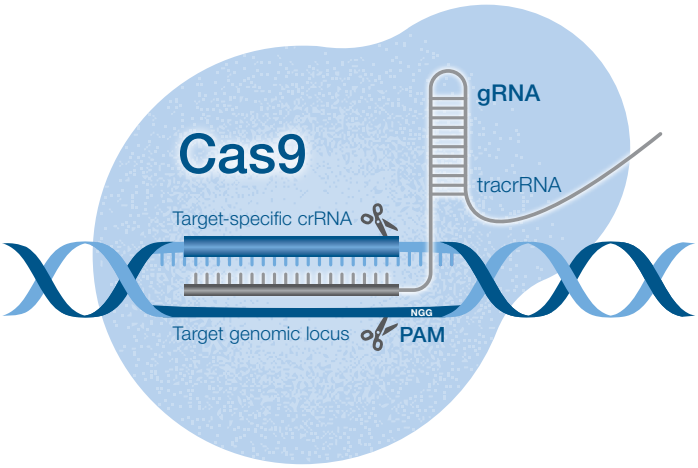


Figure 4.4. A CRISPR-Cas9 targeted double-stranded break. Cleavage occurs on both strands, 3 bp upstream of the NGG PAM sequence at the 3′ end of the target sequence. The specificity is supplied by the gRNA, and changing the target only requires a change in the design of the sequence encoding the crRNA portion of the gRNA. After the gRNA unit has guided the Cas9 nuclease to a specific genomic locus, the Cas9 protein induces a double-stranded break at the specific genomic target sequence.

With their highly flexible yet specific targeting, CRISPR-Cas9 systems can be manipulated and redirected to become powerful tools for genome editing. CRISPR-Cas9 technology permits targeted gene cleavage and gene editing in a variety of cells, and because the endonuclease cleavage specificity in CRISPR-Cas9 systems is guided by RNA sequences, editing can be directed to virtually any genomic locus by engineering the gRNA sequence and delivering it along with the Cas9 endonuclease to the target cell.

Looking to get started with CRISPR-Cas9–based genome editing?
Check out the Invitrogen™ TrueDesign™ Genome Editor, our free online software for designing genome editing experiments.

Learn more at **thermofisher.com/truedesign**

CRISPR-Cas9 reagent formats

Both components of the CRISPR-Cas9 system—the Cas9 nuclease and the gRNA—need to be delivered to cells for gene editing. Different experimental requirements are supported by a variety of formats of CRISPR-Cas9 tools, including: Cas9 protein, Cas9 mRNA, lentiviral Cas9, synthetic gRNA, and lentiviral gRNA.

See Table 4.2 for a comparison of different Cas9 nuclease formats.

Table 4.2. Comparison of Invitrogen™ Cas9 nuclease reagents.

CRISPR-Cas9 technology				
Product name	**Cas9 iPSC line**	**Cas9 mRNA**	**TrueCut Cas9 Protein v2**	**LentiArray lentiviral Cas9 nuclease**
Product benefits	• Stably expressed Cas9 • Robust editing efficiency • Simple gRNA delivery via lipofection • Editing in differentiated progeny • Large-scale genetic screens	• No footprint left behind (no random integration concern) • No promoter constraint • Ready to use • Controlled dosage • Fast turnover • Microinjection ready • Multiplexing and screening capable	• No footprint left behind (no random integration concern) • No promoter constraint • Ready to use • Controlled dosage • Fast turnover • Stable RNP complex • Microinjection ready • Multiplexing and screening capable	• Infect dividing and nondividing cells • Stable expression of Cas9 nuclease • Ready-to-use, high-titer lentiviral particles • Large-scale genetic screens

TrueCut Cas9 Protein v2

The highest cleavage efficiencies in hPSCs are observed using TrueCut Cas9 Protein v2 with TrueGuide synthetic sgRNA. TrueCut Cas9 Protein v2 is a wild type Cas9 protein that has been designed to deliver consistently higher editing efficiency across a range of gene targets and cell types.

- High editing efficiency in all tested cell lines, including standard, immune, primary, and stem
- Up to 2x higher editing efficiency in difficult targets, compared to that obtained with kits from other suppliers
- High quality—manufactured under strict ISO 13485 quality standards
- Validated protocols—achieve success faster using protocols optimized for an extensive list of cell types

CRISPR gRNA

The design, production, and delivery of high-quality gRNAs are critical to achieving a successful result when using a CRISPR-Cas9 system for gene editing. CRISPR gRNAs are available in multiple formats, including transfection-ready synthetic gRNAs and lentiviral expression systems (Table 4.3).

Table 4.3. Available formats of Invitrogen™ CRISPR gRNAs.

	TrueGuide Synthetic gRNA	LentiArray Lentiviral gRNA
Product benefits	• Predesigned sgRNAs for human and mouse • Upload your own sequence for custom sgRNA synthesis • TrueDesign online tool assists with custom designs for precision gene editing (e.g., knock-in, SNP edits, tagging)	• Predesigned gRNAs for human • Ready-to-use lentiviral particles, no transfection reagent needed • Antibiotic resistance marker enables selection of gRNA-expressing cells
Format	Synthetic sgRNA in individual tubes or arrayed in 96-well plates	High-titer lentiviral particles in individual tubes, custom collections in 96-well plates, or predefined collections for screening
Application	Knockout or knock-in	Knockout or library screening
Species	Predesigned human and mouse, custom synthesis supports any species	Human
Delivery method	Lipid-mediated transfection or electroporation	Lentiviral transduction
Recommended Cas9 format	TrueCut Cas9 Protein v2 or delivery into Cas9-expressing cells	LentiArray Cas9 Lentivirus co-transduction or delivery into Cas9-expressing cells
Controls	Positive and negative controls available	Positive and negative controls available

TrueGuide Synthetic gRNA

Invitrogen™ TrueGuide™ Synthetic gRNAs are ready-to-transfect synthetic gRNAs designed and validated to work with the Invitrogen™ suite of genome editing tools to provide consistent, high-efficiency editing. TrueGuide Synthetic gRNAs offer the reagents required to introduce your specific edit into your cell line and have demonstrated high-efficiency editing in primary and stem cells.

TrueDesign Genome Editor

Easily create accurate and more successful genome editing experiments with the TrueDesign Genome Editor. Our free design tool has an intuitive point-and-type interface that provides superior flexibility and support for creating genome edits. The simple step-by-step program allows you to:

- Generate a complete CRISPR-Cas9 design in minutes
- Edit up to 30 bases in any human gene using CRISPR-Cas9 or TALEN technology to create knockouts, SNPs, or amino acids changes
- Add a GFP or RFP tag and create adapter primers for Invitrogen™ TrueTag™ Donor DNA Kits to produce a donor template without cloning

CRISPR-Cas9 gene editing workflow

Once a specific CRISPR format has been selected, it is introduced into the target cells via lipid-mediated transfection or electroporation. Cells are plated at low density to allow for expansion of clonal colonies. These are then selected and screened for gene editing events. A sample workflow is shown in Figure 4.5.

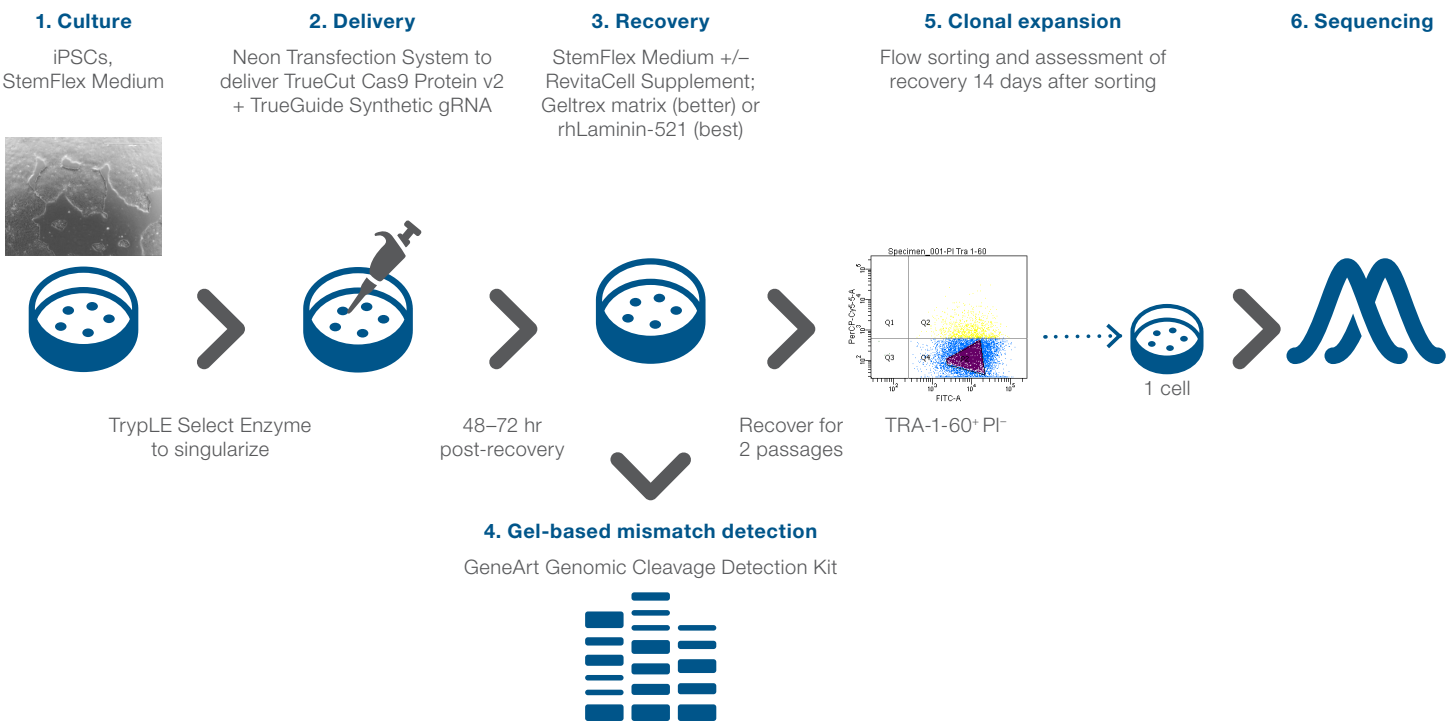


Figure 4.5. Standard gene editing workflow using CRISPR-Cas9 technology. Following the expansion of hPSCs with Gibco™ StemFlex™ Medium, cells are singularized and electroporated using the Invitrogen™ Neon™ Transfection System to introduce precomplexed TrueCut Cas9 Protein v2 and TrueGuide Synthetic gRNA. Cells recover in StemFlex Medium in the presence or absence of Gibco™ RevitaCell™ Supplement on either Gibco™ Geltrex™ matrix or rhLaminin-521. Following 48–72 hours of recovery, cleavage efficiency is assessed using the Invitrogen™ GeneArt™ Genomic Cleavage Detection Kit. Pending successful cleavage, cells are recovered and expanded for 2 passages prior to clonal expansion. At this time, viable PSCs are flow-sorted based on expression of TRA-1-60 and the absence of PI expression. Subsequently, cells are plated at 1 cell/well in StemFlex Medium on rhLaminin-521–coated 96-well plates. Following 14 days of recovery, successful clonal expansion is determined, followed by confirmation of successful gene editing of clonally established cell lines through sequencing.

New to CRISPR?

Check out our CRISPR hands-on workshop. Our experienced team has designed a comprehensive four-day CRISPR workshop comprising both lectures and hands-on laboratory work at our state-of-the-art training facility.

In addition, you will receive a detailed course guide containing all lecture materials, laboratory protocols, troubleshooting tips, and more to help you get started right away.

Find out more at **thermofisher.com/crisprworkshop**

While gene editing in workhorse cell lines like 293T cells has become quite routine, successful editing of PSCs can still require significant optimization. In addition to optimizing the delivery of Cas9 and gRNA, the recovery following electroporation and later clonal expansion can be particularly challenging in PSCs. Below we outline our best practices that will maximize your chances of success.

1. Culture—While the maintenance of PSCs leading up to gene editing may seem like a routine step, having your cells adapted to a medium that will support the survival of PSCs during the bottlenecks in the gene editing workflow is particularly critical. StemFlex Medium has been specifically developed for optimal performance in these challenging applications. It is a feeder-free medium that eliminates the need for enzymatically or mechanically removing mouse embryonic fibroblasts (MEFs) that can otherwise lower transection efficiencies. Unlike other feeder-free culture systems, StemFlex Medium supports robust cell survival following singularization and transfection with a Cas9–gRNA complex, even in the absence of ROCK inhibitors. It also supports clonal expansion from a single cell and is compatible with every-other-day feed schedules, which not only reduces workload prior to gene editing but is also a critical step during the clonal expansion stage.

2. Delivery—Delivery of Cas9 and the target gRNA can be achieved via either lipid transfection using reagents such as Lipofectamine Stem Transfection Reagent. However, the highest cleavage efficiencies are obtained using TrueCut Cas9 Protein v2 and TrueGuide Synthetic gRNA RNP transfection via electroporation with the Neon Transfection System (Figures 4.6 and 4.7).

Optimal Neon system settings can vary depending on the culturing conditions, cell lines being used, and content being delivered. These may even differ between PSC lines. We therefore recommend that users test all 24 electroporation conditions using the optimization protocol and empirically determine the best program for their particular cell line, considering both editing efficiency and cell viability (Figure 4.8).

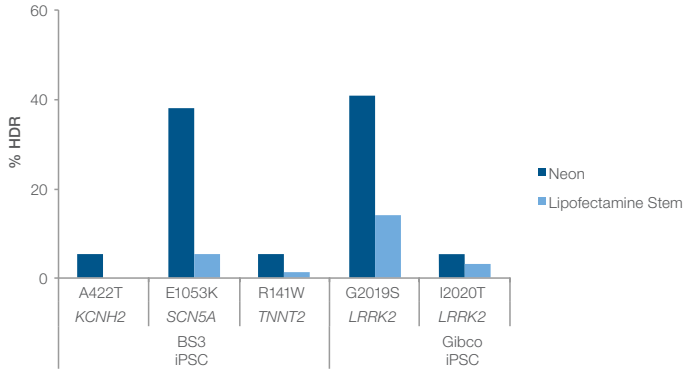


Figure 4.6. A comparison between Neon Transfection System and Lipofectamine Stem reagent–based delivery of editing tools. Gibco™ episomal and BS3 iPSC lines were edited in the indicated genomic loci using TrueCut Cas9, IVT gRNAs, and single-stranded oligo donors.

	Cas9 version and delivery method		
Target	**Cas9 protein Lipofectamine Stem**	**Cas9 protein Neon**	**Stable Cas9 Neon**
KCNH2 A422T	0	5	7
SCN5A E1053K	5	38	41
TNNT2 R141W	1	5	11
LRRK2 G2019S	14	41	26
LRRK2 I2020T	3	5	4

Figure 4.7. IVT gRNA delivered with single-stranded oligo donor, percent SNP (measured by NGS).

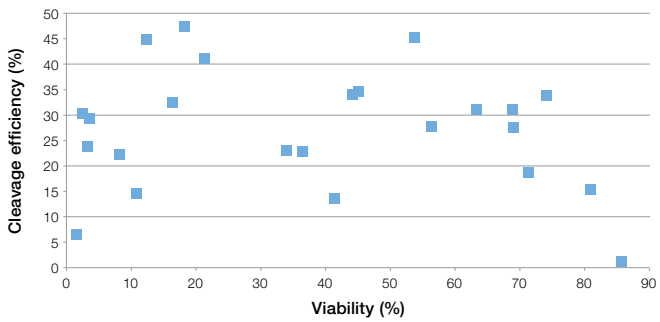


Figure 4.8. Optimization of the electroporation conditions using the Neon system.

3. Recovery—For many scientists, post-electroporation recovery with Cas9 RNP presents the first significant bottleneck during gene editing of PSCs. Not only do cells undergo the stressful electroporation process, but the protocol necessitates the singularization of cells prior to electroporation. StemFlex Medium addresses this pain point by allowing for optimal recovery following electroporation with Cas9 RNP complex (Figure 4.9).

4. Analysis—Prior to continuing with clonal expansion, it is recommended to first confirm successful cleavage at the target site. This allows for the identification of the condition with the best editing efficiency, which can then be used for further cell line generation. Please see Section 4.5, "Screening methods for TAL and CRISPR" for additional details.

5. Clonal expansion—The establishment of a successfully edited clonal PSC line is undoubtedly the most challenging step of the gene editing workflow. Cells can be plated at limiting dilution, however to reduce the number of wells screened, we recommend flow sorting and plating of viable, pluripotent cells (TRA-1-60^{+}, PI^{-}) at 1 cell/well of a 96-well plate (Figure 4.10). StemFlex Medium is the only medium tested that allows for establishment of a clonal line from a single cell under these stressful conditions in the absence of ROCK inhibitor. Typically, 20–50% clonal recovery is achieved using this medium.

6. Sequencing—The correct editing of the desired target sequence in clonal populations can be confirmed via Sanger sequencing at the site of interest or via Ion PGM™ sequencing to simultaneously confirm the absence of off-target effects. Please see section 4.5, "Screening methods for TAL and CRISPR", for additional details.

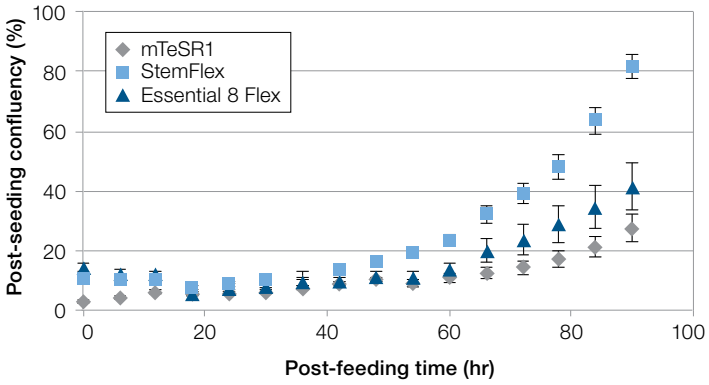


Figure 4.9. StemFlex Medium supports up to 2-fold faster recovery following gene editing. PSCs expanded in various media formulations were singularized using Gibco™ TrypLE™ Select Enzyme and subjected to delivery of a Cas9 protein/HPRT gRNA complex via electroporation. Upon seeding at 100,000 viable cells per well in the absence of Rho-associated protein kinase (ROCK) inhibitor, it was shown that StemFlex Medium supported optimal recovery of cells from this stressful event.

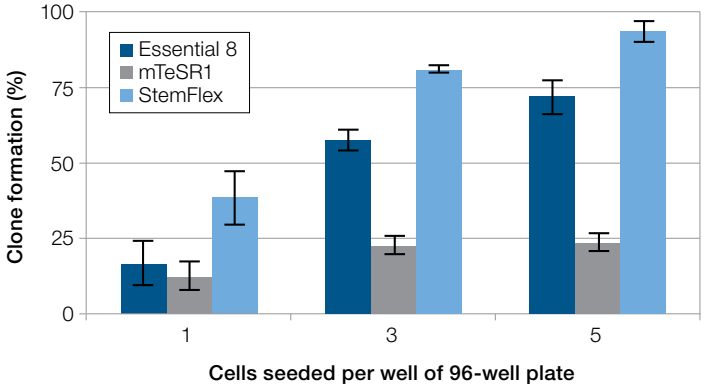
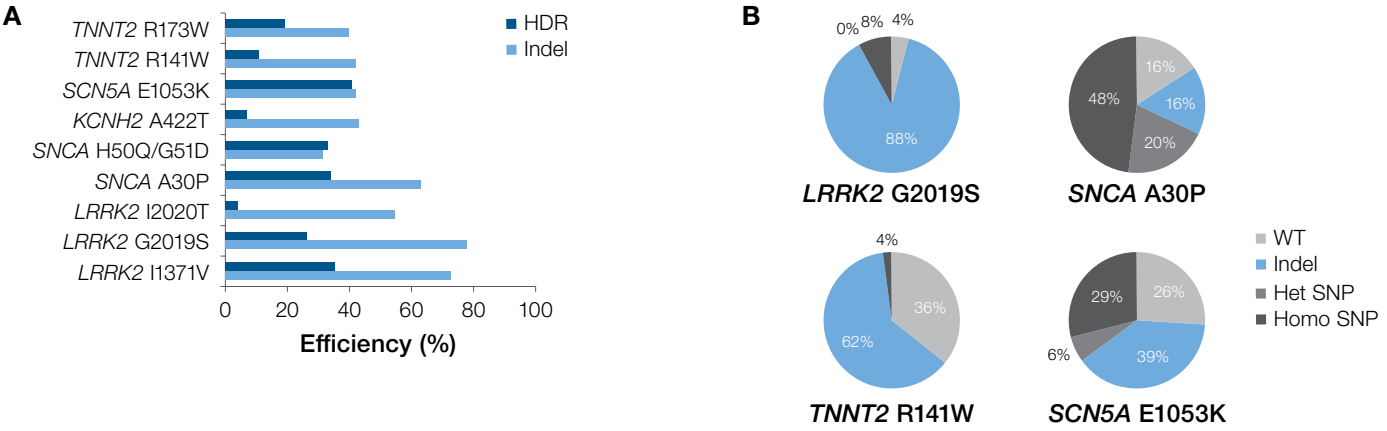


Figure 4.10. StemFlex Medium supports recovery of PSCs from flow sorting, demonstrating as much as 5-fold improvement in clonal expansion following single-cell passaging in the presence of ROCK inhibitor. PSCs expanded in StemFlex Medium on the rhLaminin-521 substrate for >3 passages, were singularized using TrypLE Select enzyme, flow-sorted for live pluripotent stem cells (TRA-1-60^{+}, PI^{-}), and seeded at 1, 3, or 5 cells per well of a 96-well plate. Following plating, cells were fed with fresh medium every 3 days, and the percentage of wells attaining >5% confluency by day 14 was assessed via whole-well imaging on the IncuCyte™ ZOOM system (Essen BioScience).

CRISPR-Cas9 gene editing of a disease-relevant target

In a proof-of-principle study, disease-causing mutations were introduced into a hiPSC line stable expressing Cas9. A number of cardiac and Parkinson's disease–specific mutations were introduced using the Neon Transfection System by delivery of gRNA and a single-stranded oligo donor carrying the SNP to be modified (Figure 4.11). Varying editing efficiencies were observed both for indel formation and HDR-mediated SNP introduction in pools generated for the mutations in the different genomic loci (Figure 4.11A). Isolation of single-cell clones via FACS yielded different distributions for the presence of wild type, indel, heterozygous, and homozygous edits for each of the targets, and in every case, a homozygous clone was identified regardless of the efficiency in the edited pools (Figure 4.11B). Through sequencing on the Ion PGM™ System, clonality can then be verified quantitatively by calculating allele ratios from over 10,000 reads. Wild type or homozygote clones would have ~100% of one allele, and a heterozygote would have ~50% of one allele and ~50% of the other. These clonal lines can then be differentiated into desired cell types to model the disease of interest.

For a detailed walkthrough of how to design similar SNP experiments and how to transfect these materials, download our application note at **thermofisher.com/truedesign**



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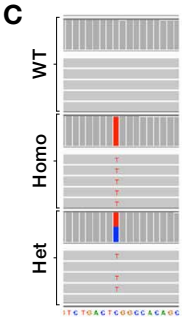


Cell line	*SCN5A* E1053K WT	*SCN5A* E1053K Homo	*SCN5A* E1053K Het	*TNNT2* R141W WT	*TNNT2* R141W Homo	*TNNT2* R141W Het	*LRRK2* G2019S WT	*LRRK2* G2019S Homo	*LRRK2* G2019S Het	*SNCA* A30P WT	*SNCA* A30P Homo	*SNCA* A30P Het
WT alleles (%)	98	2	52	98	1	NA	100	5	NA	100	1	52
Edited alleles (%)	2	98	45	2	99	NA	0	95	NA	0	99	48

Figure 4.11. Generation of disease models through the introduction of SNPs into a wild type iPSC overexpressing Cas9. (A) Indel and homology-driven repair efficiency for the indicated disease targets. **(B)** WT/indel/homozygous SNP/heterozygous SNP distribution of clonal lines isolated from genome edited pools via single-cell FACS. **(C)** Next-generation sequencing analysis to confirm clonality of isolated hiPSC lines.

4.4

TAL effector technology

Transcription activator–like (TAL) effector proteins are plant pathogenic bacterial proteins that bind to specific DNA sequences and act as transcription factors during plant pathogenesis. The TAL DNA-binding domain contains a highly conserved 32–34 amino acid repeat sequence except the amino acids in positions 12 and 13. These two amino acids, called the repeat variable diresidue (RVD), dictates specificity of each repeat to a single specific nucleotide within the target sequence. Because of the modular domain structure and well-defined amino acid–to-nucleotide code, fusion proteins containing TALs conjugated with various functional domains can be targeted to very specific loci within the genome.

Invitrogen™ FlexCut TALEN™ mRNA Pairs encode two fusion proteins each consisting of a target-specific TAL effector and truncated FokI nuclease. After translation of the FlexCut TALEN mRNA Pair in the cell, FokI nuclease functions as a homodimer and creates a double-stranded break in the DNA flanked by the TAL effector binding sites. In the absence of DNA that shares homology across the region containing the break, the cell's natural machinery will attempt to repair the break by NHEJ, which can lead to indels. In protein-coding regions, these indels can cause frameshift mutations that can result in a gene disruption (knockout).

When this break is created in the presence of DNA that shares homology across the region, homology-directed repair can occur, which allows the added DNA to be incorporated at the site of the break. In this manner, specific bases or sequences can be introduced within user-defined locations within the genome (Figure 4.12).

A sample workflow for gene editing of iPSCs after culturing using TALs involves the following steps (Figure 4.13):

- Design and synthesis of TAL constructs and *in vitro* transcription of FlexCut TALEN mRNA Pairs
- Transfection and electroporation of iPSCs with TAL constructs in the presence or absence of donor DNA
- Clonal recovery of cells
- Picking and screening of colonies to determine successful cleavage and editing
- Expansion, characterization, and banking of successfully edited clones

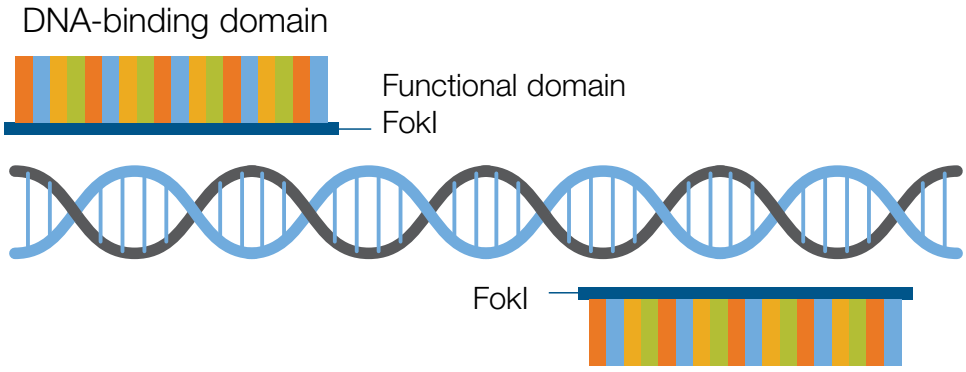


Figure 4.12. FlexCut TALEN mRNA Pairs technology. A fusion of a TAL effector to a FokI nuclease generates a homodimer pair that is designed to bind to genomic sequences flanking the target site and to generate a double-stranded break at the desired locus. FlexCut TALEN mRNA Pairs eliminate the 5′ T constraint of naturally occurring TALs. FlexCut TALEN mRNA Pairs allow targeting of any sequences across the genome.

Useful tips

- Design and test at least 2–3 pairs of FlexCut TALEN mRNA per gene
- Design the FlexCut TALEN pair to cleave the DNA as close as possible to the desired position
- Design each FlexCut TALEN mRNA to target 18 bp of DNA sequence, and design TAL pair targets with 16 bp of spacing in between
- GC content should be distributed throughout the target site when possible
- Order ready-to-use FlexCut TALEN mRNA from our Custom Services team at **GEMservices@thermofisher.com**

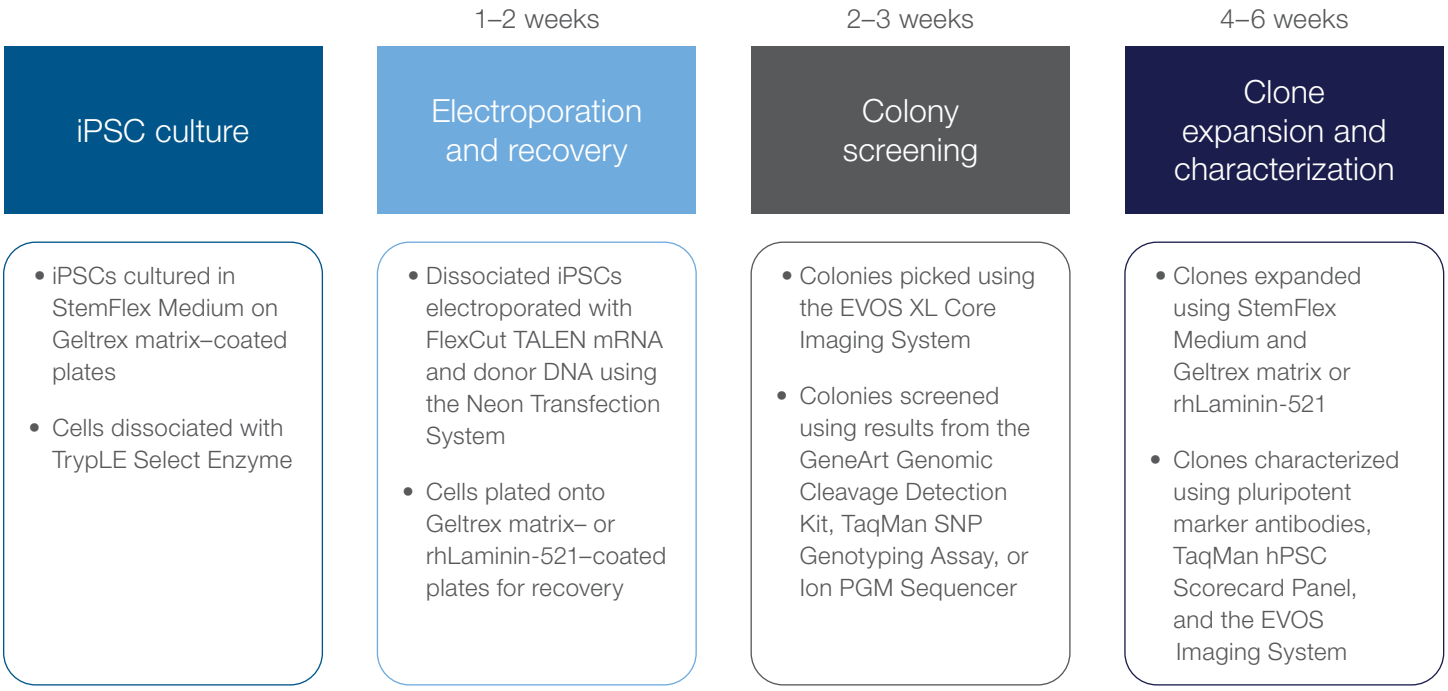


Figure 4.13. Workflow for genome editing of iPSCs using TAL technology. Feeder-free iPSCs cultured in StemFlex Medium and on Geltrex matrix–coated plates are treated with RevitaCell Supplement before dissociation with TrypLE Select Enzyme. Dissociated cells are electroporated with TAL constructs with or without donor DNA using the Neon Transfection System, and then plated at a low density onto Geltrex matrix– or rhLaminin-521–coated plates for recovery. After 1–2 weeks, colonies are picked, screened, and selected based on results obtained with the GeneArt Genomic Cleavage Detection Kit, Applied Biosystems™ TaqMan® SNP Genotyping Assay, or Ion PGM Sequencer. The final clones are expanded and characterized to confirm pluripotency and genomic integrity prior to banking.

4.5

Screening methods for TAL and CRISPR gene editing

When using genome editing tools such as TAL pairs or CRISPR-Cas9 to obtain targeted mutations, it is recommended that you determine the efficiency with which these nucleases cleave the target sequence prior to continuing with labor-intensive and expensive clonal expansion steps.

After gene editing, single-cell clones can be easily derived using single-cell sorting. Relying on StemFlex Medium, rhLaminin-521 substrate, and RevitaCell Supplement, single cells can be deposited and expanded in 96-well plates and expanded for ~2 weeks. Formed clones can then be consolidated and expanded for banking and screening for the occurrence of the genome editing event (Figure 4.14). This approach does not require a second round of clonal isolation and is therefore superior to low-density plating and manual picking.

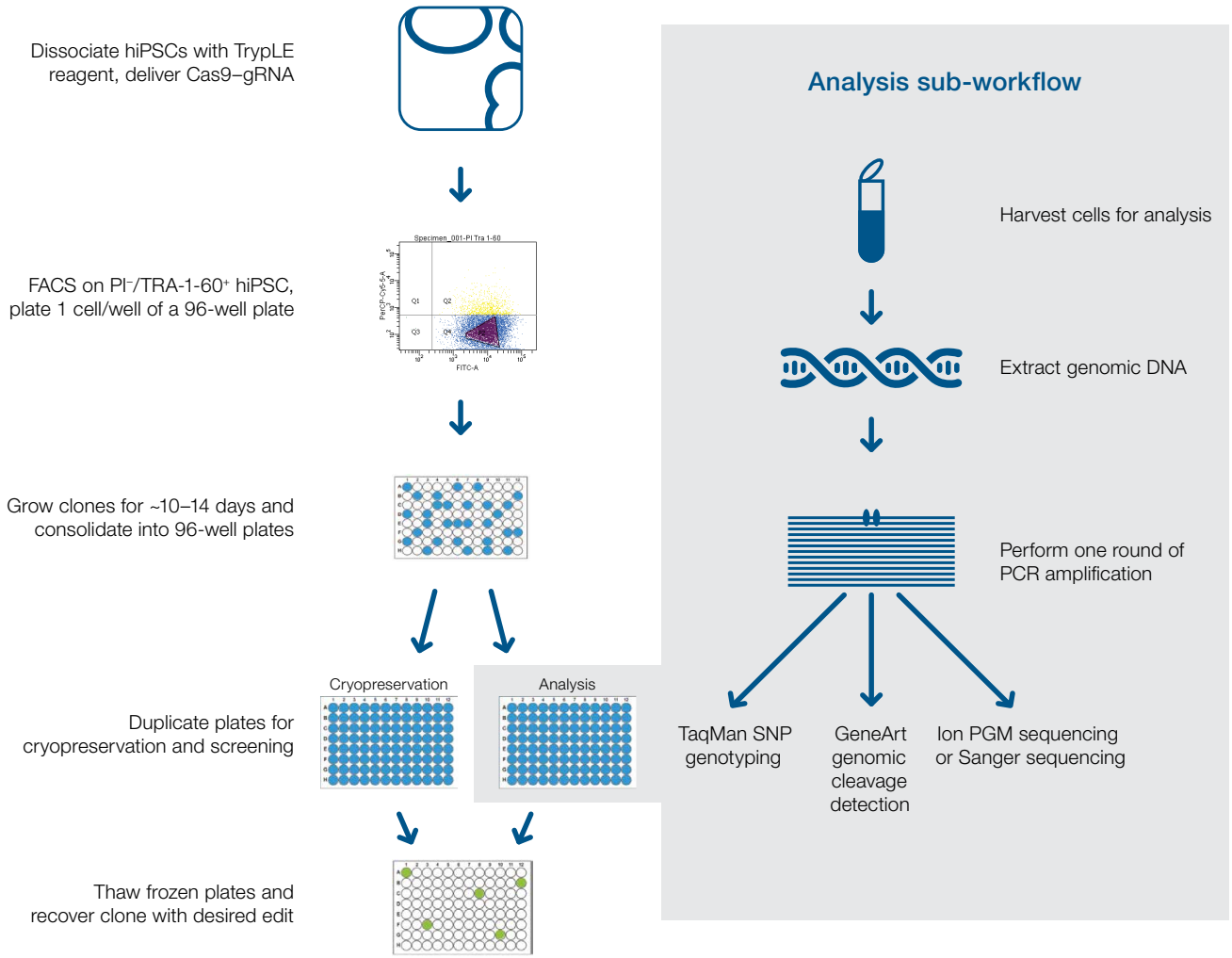


Figure 4.14. Colony screening workflow. After genome editing of singularized hiPSCs, clonal lines can be generated through a FACS-based approach. Single viable pluripotent stem cells are deposited into 96-well plates and allowed to grow for 10–14 days in StemFlex Medium on rhLaminin-521 substrate. At that point, consolidated 96-well plates can be cryopreserved and screened for the genome edit with the indicated assays.

A variety of tools and reagents, including the TaqMan SNP Genotyping Assay, Applied Biosystems™ SeqScreener Gene Edit Confirmation Application for Sanger Sequencing, GeneArt Genomic Cleavage Detection Kit, and Ion PGM sequencing, can be used to quickly determine which cells have been successfully edited. A comparison of these technologies is presented in Table 4.4.

Table 4.4. Comparison of common genomic analysis methodologies.

Methodology	Advantages	Limitations	When to use
Sanger sequencing with SeqScreener Gene Edit Confirmation App	• Detects sequence changes in pools and clonal populations • Fast • Inexpensive	• Limited quantification	• Screening for transfection and gRNA efficiency • Triaging colonies via homologous recombination • Best used to screen isolated single-cell clones
GeneArt Genomic Cleavage Detection Kit	• Inexpensive • Can detect small changes in homozygous state of DNA in NHEJ and HDR editing	• Positive result does not indicate whether editing tool works • Limited use if editing heterozygous loci to homozygous loci	• Triaging colonies from editing via NHEJ repair
TaqMan SNP Genotyping Assay	• Inexpensive • Fast • Clearly distinguishes changes in allele status	• Only detects changes in alleles that the assay is designed for; may not detect indels from NHEJ repair	• Triaging colonies from editing via homologous recombination
Ion PGM sequencing	• Can specifically detect all changes in a population • Quantitative results	• Higher cost compared to other assays • Longer workflow	• Best used as a secondary assay, for confirmation and quantitation of editing in populations identified from primary screens

Screen gene editing outcomes with the SeqScreener Gene Edit Confirmation Application

The SeqScreener Gene Edit Confirmation Application is an app available through the Thermo Fisher™ Connect Platform used to determine the spectrum and frequency of targeted mutations generated in a pool of cells by genome editing tools such as CRISPR-Cas9. This app will screen and verify gene editing results obtained using Sanger sequencing technology.

SeqScreener software enables you to:

- Apply an algorithm that accurately reconstructs the spectrum of insertions and deletions (indels) from the sequence traces
- Screen populations of edited cells to understand and quickly identify ideal edited clones (Figure 4.15)
- Identify the detected indels and their frequencies (Figure 4.16)
- Detect designed mutations generated by homologous recombination using a donor template
- Characterize results from both knock-in and knockout experiments
- Analyze results from a pool or a single clone

Learn more at **thermofisher.com/genomeeditconfirmce**

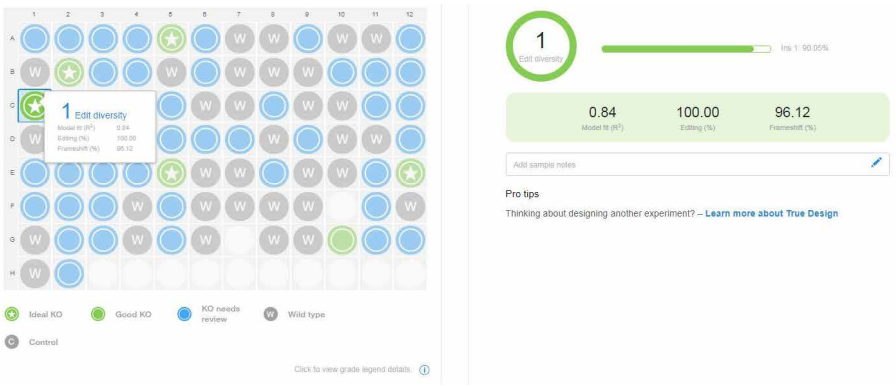


Figure 4.15. SeqScreener software allows you to quickly review plates of Sanger sequencing data from gene editing experiments to determine which wells have successful knockout, knock-in, or wild type results at a glance. The tool will categorize results based on software presets to highlight Ideal wells, as well as provide suggestions on wells that may require additional analysis.

Figure 4.16. When reviewing a single well, SeqScreener software allows for a detailed view of individual indel repair events through the data deconvolution software. The user can view a distribution of indel events, relative to the CRISPR-Cas9 cut site, as well as the relative frequency of specific insertion or deletion events. The percent frameshift will be calculated depending on the number of indel events that could cause the codon reading frame to be disrupted.

Screening with the GeneArt Genomic Cleavage Detection Kit

The GeneArt Genomic Cleavage Detection Kit provides a relatively quick, simple, and reliable assay that allows the assessment of the cleavage efficiency of genome editing tools at a given locus (Figure 4.17). A sample of the edited cell population is used as a direct PCR template with primers specific to the targeted region. The PCR product is then denatured and reannealed to produce heteroduplex mismatches where double-stranded breaks have occurred, resulting in indel introduction. The mismatches are recognized and cleaved by the detection enzyme. Using gel analysis, this cleavage is both easily detectable and quantifiable.

Find out more at **thermofisher.com/genedetect**

Useful tips

- Prepare plenty of cells for your lysate
- Generate and verify a strong PCR product
- Denature and reanneal PCR fragments using a programmable ramping thermal cycler
- Optimize gel electrophoresis conditions

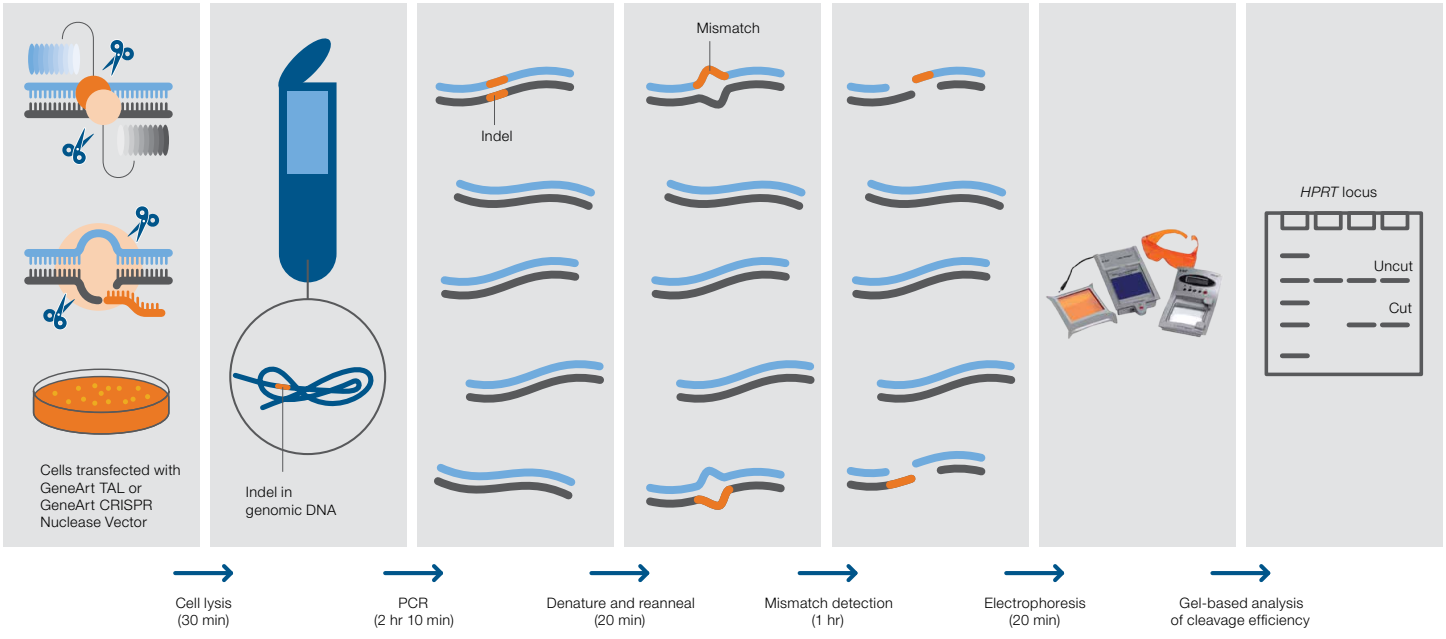


Figure 4.17. GeneArt Genomic Cleavage Detection Assay. To detect either an indel or a mutation within a specific sequence of DNA, the region is first amplified using primers specific for that region. A second nested PCR can be performed to increase sensitivity. After heating the sample and reannealing the PCR products, amplicons containing indels or other changes in sequence will result in the formation of heteroduplexes with amplicons containing unmodified sequences. When these heteroduplexes are treated with an endonuclease that only cleaves in the presence of a mismatch, two pieces of DNA of known size are generated, which can be detected by agarose gel electrophoresis.

Section 5

Differentiation

5.1 Introduction

The generation of iPSCs is often an intermediate step to reach the real experimental goals. The purpose of PSCs in this case is to take advantage of the proliferative capacity and pluripotency of iPSCs to generate virtually unlimited numbers of mature, differentiated cell types, including neurons, cardiomyocytes, beta (β) cells, or conceivably any other cell type in the body (Figure 5.1).

These PSC-derived cells can be used in a range of applications such as:

- Modeling human embryonic development
- As a source of difficult-to-isolate cells for basic research and disease modeling
- Drug screening applications
- Cell replacement therapy

The differentiation of PSCs to a specific lineage is obtained by timed exposure to specific conditions via growth factors, small molecules, and substrates that mimic the sequential events that occur during embryonic development.

Early differentiation protocols relied on the formation of embryoid bodies (EBs), which are 3D aggregates of cells that allowed for spontaneous differentiation. The differentiation from EBs in these earlier protocols could be biased by exposure to growth factors that promoted differentiation of one lineage over another.

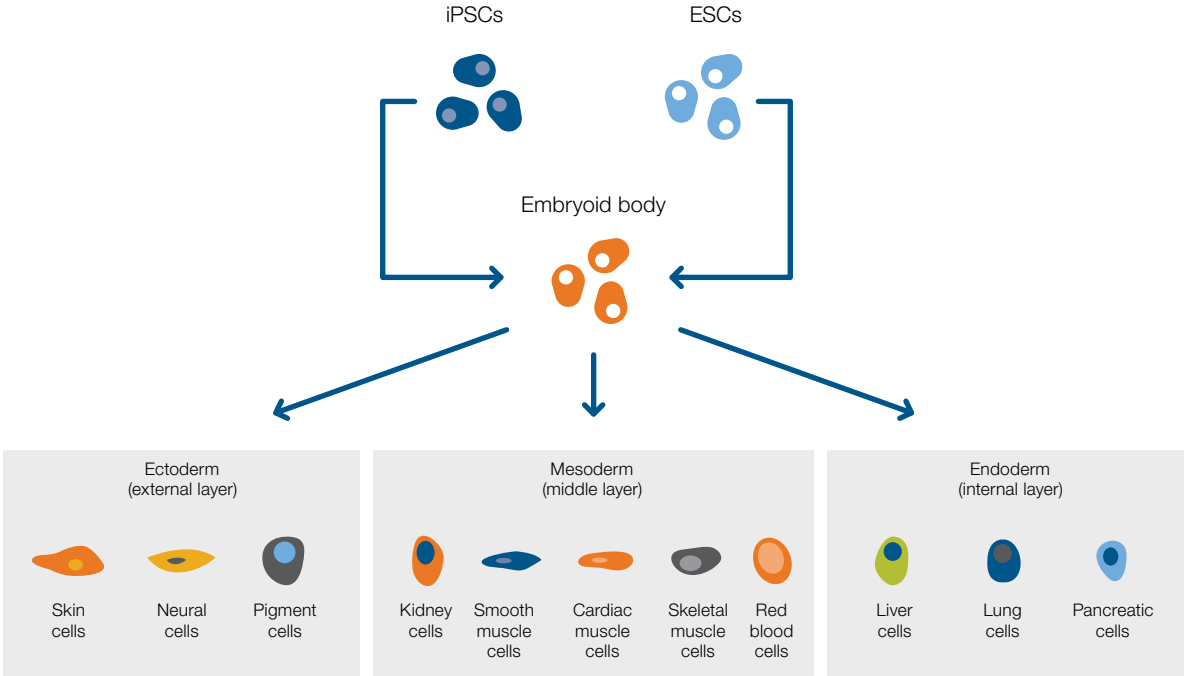


Figure 5.1. Differentiation of PSCs to different lineages via an EB intermediate.

More recently, differentiation protocols have become increasingly defined. Most bypass the EB formation step, which effectively created a black box within which signaling events controlling differentiation were poorly understood.

Instead, recent protocols tend towards adherent culture, in which cells are exposed to a temporarily defined combination of small molecules. The replacement of growth factors with potent small molecules allows for differentiation that is not only more cost effective but also more efficient.

As protocols have become more defined, the understanding of signaling events required to specify a given cell type has become increasingly complex. The focus in evaluating differentiation protocols now more typically revolves around the validity and functionality of the generated cells. How accurately does the iPSC-derived cell type recapitulate the behavior of the primary cell *in vitro* and *in vivo*? Does it express markers associated with the cell lineage? Does it perform as expected in functional assays? And in many cases, most importantly, does it integrate and function *in vivo* when transplanted into an animal model?

A final consideration pertains to the maturity of iPSC-derived cells. Cells derived via differentiation of PSCs will by default exhibit a fetal or neonatal phenotype. This may manifest itself via expression of fetus-associated markers, such as fetal globins in iPSC-derived erythrocytes or alpha-fetoprotein in iPSC-derived hepatocytes. Conversely, the expression of adult markers may be low or absent in iPSC-derived cells, such as cytochrome P450 levels in iPSC-derived hepatocytes.

This may be a concern for drug screening and cell therapy applications or for researchers studying late-onset disorders such as neurodegenerative diseases like Alzheimer's disease or Parkinson's disease, since disease-specific phenotypes may not manifest themselves in fetal cells.

Ongoing research in this field is exploring ways of aging and maturing iPSC-derived cell types *in vitro*, and it can be expected that more approaches to this problem will be uncovered in the near future. Until this time, it is recommended that you keep this caveat in mind and plan around its impact on downstream research.

Some of the key considerations to take into account when developing a differentiation protocol, adapting a published protocol from the literature, or choosing a commercially available differentiation kit include:

- **Quality of cells**—Does the final cell population express the markers associated with the cell type *in vivo*? Does it perform as expected in functional assays *in vitro* and *in vivo*?
- **Protocol and components**—Does the protocol use defined media and substrates, or does it include components such as serum or BSA? Does it involve an EB formation step or coculture with a stromal cell line? These factors can introduce variability into a differentiation protocol and make standardization and optimization difficult.
- **Speed**—How quickly is the desired cell population obtained?
- **Efficiency**—How high is the yield of the desired cell population? Are a considerable number of undesired "contaminating" cell types also obtained?
- **Reproducibility**—Are cells and efficiencies obtained consistently across multiple experiments and among different users?

- **Robustness**—Does the protocol work efficiently and consistently across multiple ESC and iPSC lines? Some protocols were developed with a small set of lines and adaptation to different lines may require significant optimization.
- **Cost**—Does the protocol require significant amounts of expensive recombinant growth factors or specialized tissue culture plates?
- **User friendliness**—How many different media are required, and how often must cells be passaged or otherwise manipulated? Does the protocol involve labor-intensive picking steps, such as with neural rosettes?
- **Scalability**—Can the protocol readily be scaled up for the production of high volumes of cells? Is the cost of media prohibitive? Is the culture system with respect to plate format or manual manipulation requirements not amenable to larger scales?
- **Bankability**—Can cells be frozen as mature cells or at an intermediate stage to establish a bankable population, or must they be derived fresh every time?
- **GMP compatibility**—Gibco™ media and reagents are manufactured in ISO 13485–certified facilities and in conformity with good manufacturing practices (GMP) for medical devices to ensure product performance, consistency, and reliability. For potential clinical applications, researchers should consider these criteria when choosing reagents, which can help save time, effort, and costs when a project is ready to proceed to clinical trials.

View the complete differentiation portfolio at
thermofisher.com/differentiation

5.2

Neural differentiation

The derivation of neural cells, including not just neurons but also glial cells such as astrocytes and oligodendrocytes, can be achieved from PSCs via a neural stem cell (NSC) intermediate (Figure 5.2). The NSC is a proliferative population that can readily be expanded and banked for further use. NSCs are multipotent and possess the capacity to give rise to different neuronal subtypes and glial cells, depending on the lineage-specific maturation conditions to which they are exposed. The bankability and multipotency of iPSC-derived NSCs make this an attractive approach for a number of applications including disease modeling, drug discovery, and cell therapy.

Early protocols for NSC specification or induction relied on EB intermediates, stromal co-culture, and/or formation of rosette structures that required manual isolation prior to expansion. These protocols were poorly defined, inefficient, and labor intensive. More recently, protocols relying on adherent cultures differentiated under defined conditions have been developed that allow for rapid and highly efficient induction of NSC populations.

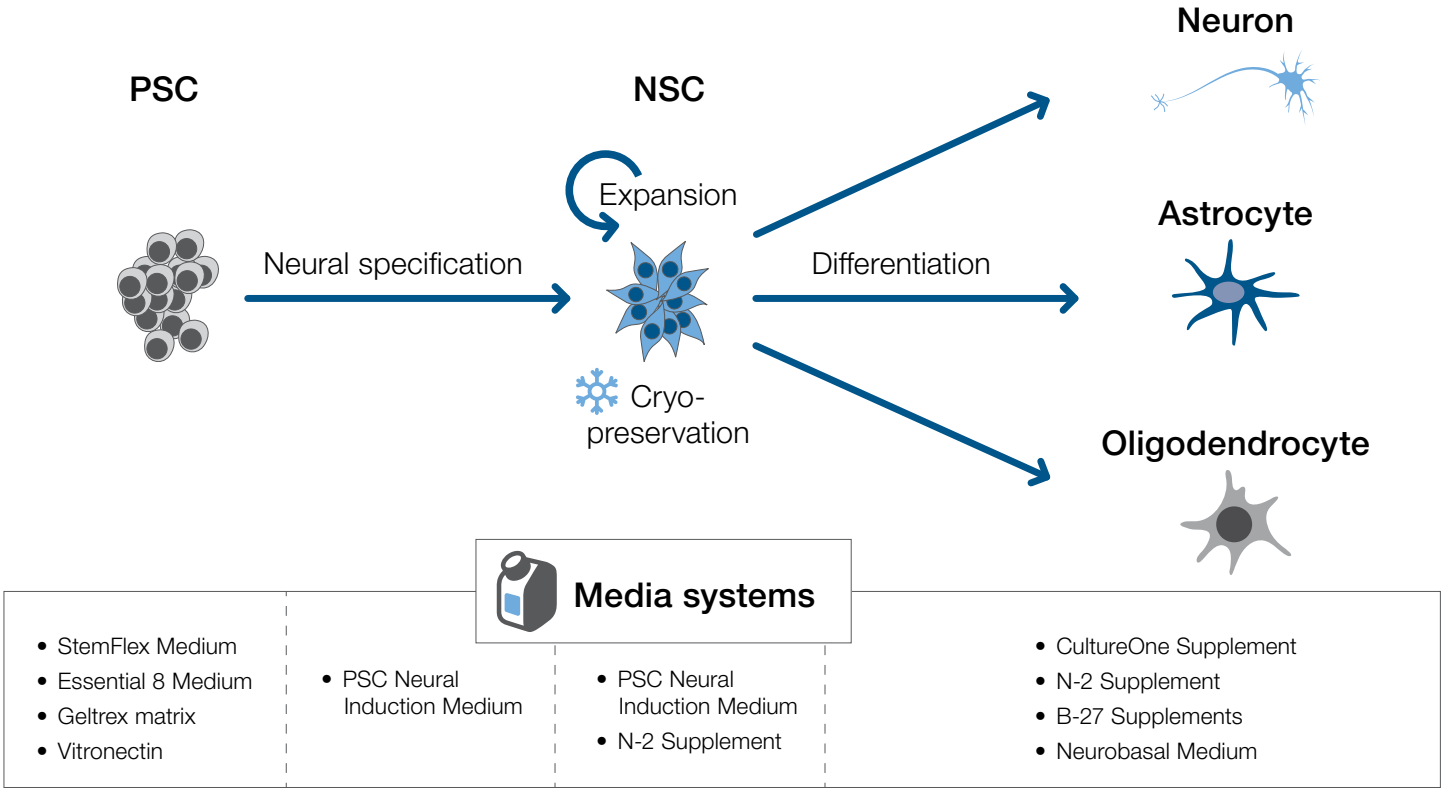


Figure 5.2. The generation of neural cell types from PSCs via an NSC intermediate. Neural specification of PSCs allows for the generation of an expandable NSC population that can further be differentiated to give rise to neurons, astrocytes, and oligodendrocytes via exposure to different lineage-specific signaling factors. Cells at the NSC stage can also be readily cryopreserved, making this a well-suited population for banking large numbers of cells. The media systems to support these different cell populations and transitions are also shown.

Neural induction

Gibco™ PSC Neural Induction Medium is a serum-free medium that provides high-efficiency neural induction of hPSCs in only 7 days (Figure 5.3). Unlike other methodologies, use of PSC Neural Induction Medium does not require the intermediary step of EB formation, thus avoiding added time, labor, and variability.

High-quality NSCs generated using PSC Neural Induction Medium have high expression of NSC markers and can be cryopreserved, expanded, and further differentiated into other neural cell types (Figure 5.4).

Find more information at **thermofisher.com/nscdiff**

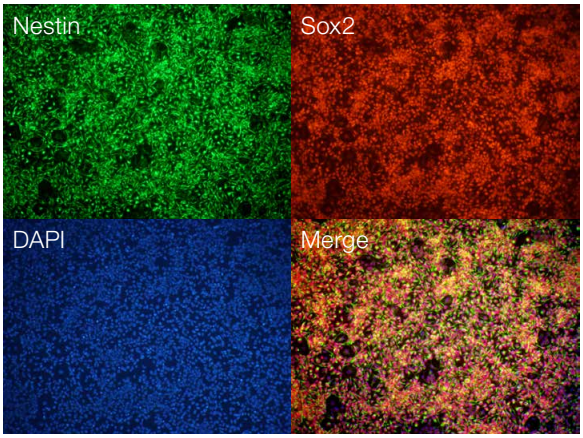


Figure 5.3. Staining of markers after neural induction of iPSCs. At day 7 of neural induction using PSC Neural Induction Medium, H9 embryonic stem cell–induced P0 NSCs were dissociated and replated on Gibco™ Geltrex™ matrix–coated plates overnight. Cells were then fixed and stained for neural markers, including nestin and Sox2. The replated P0 NSCs were positive for nestin (green) and Sox2 (red). Cell nuclei were stained with DAPI (blue).

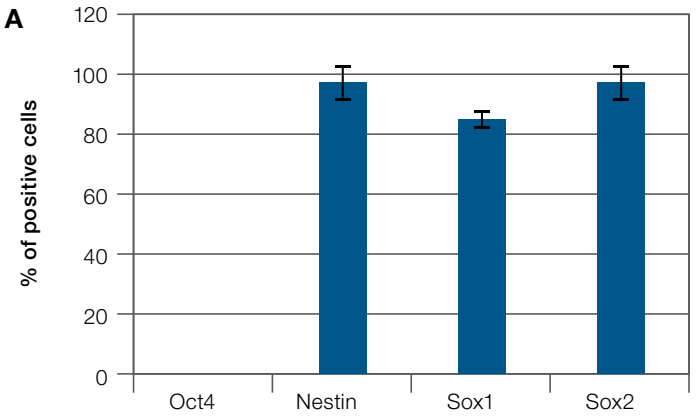
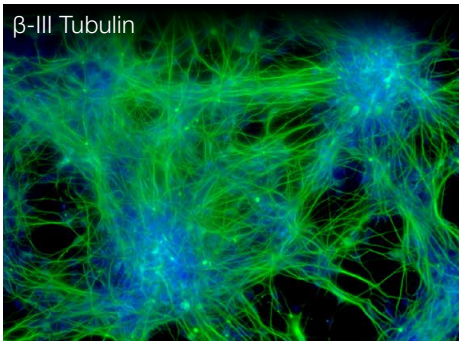
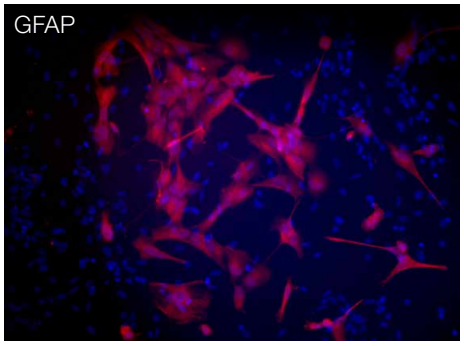


Figure 5.4. Confirmation of NSC markers and differentiation into neural cell types. NSCs generated using PSC Neural Induction Medium have high expression of NSC markers and can be cryopreserved, expanded, and further differentiated into other neural cell types. **(A, above)** Quantification of stained markers showed that less than 1% of P0 NSCs were positive for the pluripotent marker Oct4 and more than 80% of P0 NSCs were positive for the neural markers nestin, Sox1, and Sox2. **(B, below)** NSCs generated using PSC Neural Induction Medium can be further differentiated into three neural cell types of the central nervous system.

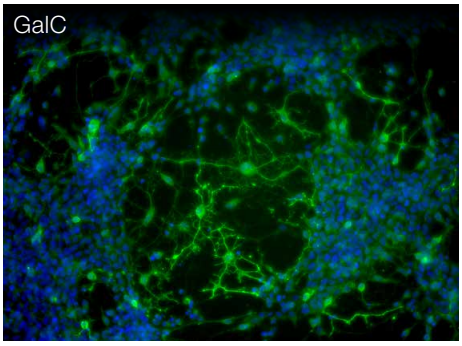
B



Neurons



Astrocytes



Oligodendrocytes

Maturation

To further mature NSCs to specific downstream lineages such as oligodendrocytes, astrocytes, or neuronal subtypes, NSCs must be exposed to additional lineage-specific maturation factors. These conditions must be determined and optimized for each cell type. Key signaling pathways involved in lineage specification are summarized in Figure 5.5.

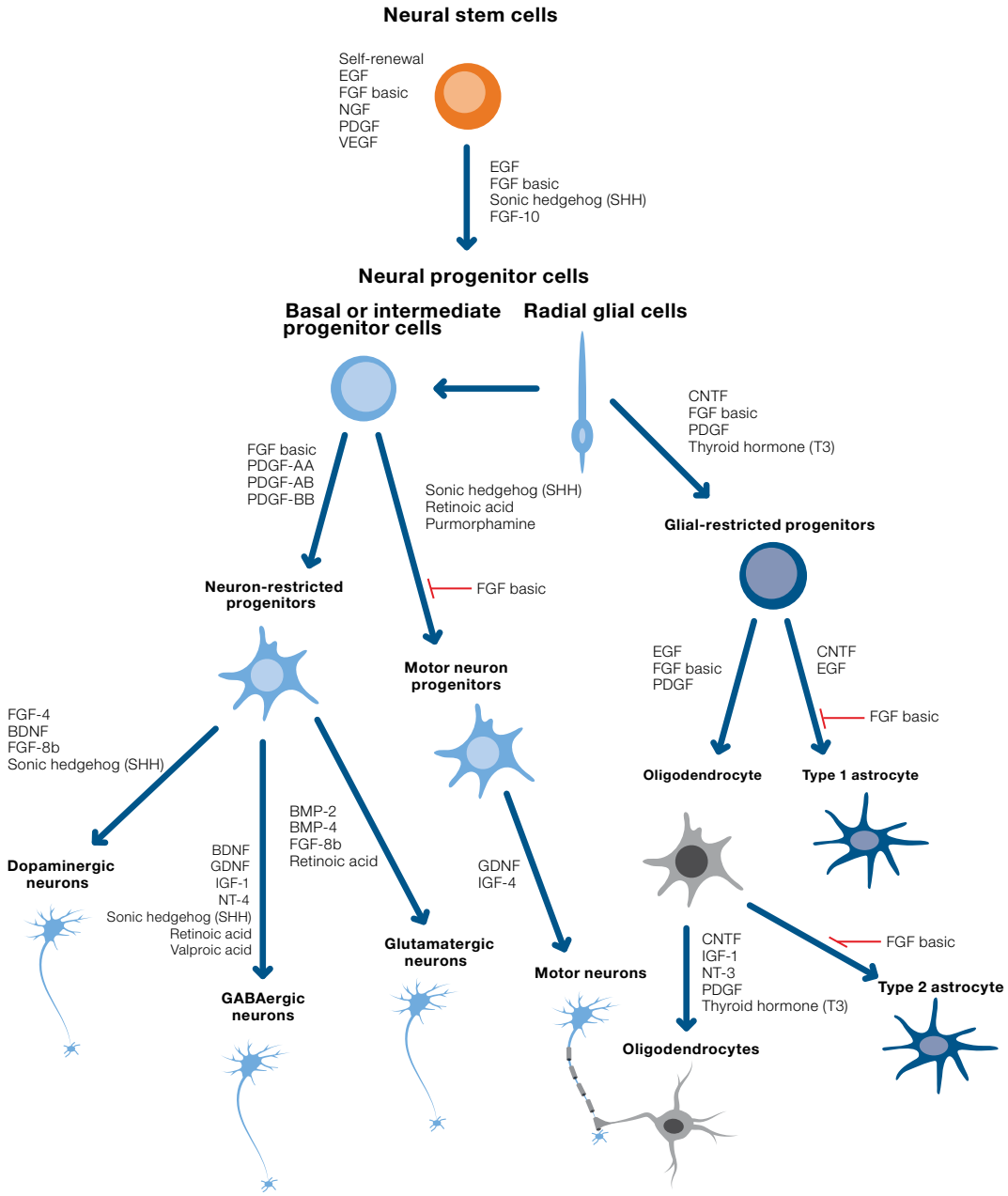


Figure 5.5. Summary of key signaling pathways regulating the differentiation of NSCs to specific neural and neuronal subtypes.

Differentiated neurons from human pluripotent stem cell (hPSC)–derived NSCs enable scientists to study human neural diseases from a large and diverse patient population like never before. However, during and after differentiation, the neuronal cell cultures are typically contaminated with proliferating neural progenitor cells that form clumps and make it nearly impossible to perform critical downstream assays on mature neurons.

Gibco™ CultureOne™ Supplement can be added to any conventional neuronal differentiation medium to eliminate more than 75% of contaminating neural progenitor cells with minimal cell death and no effect on kinase-mediated pathways. The resulting superior cultures of evenly distributed, differentiated neurons enable improved downstream assays, accelerated neuronal maturation, and maintenance in culture for 5 weeks or more (Figures 5.6 and 5.7).

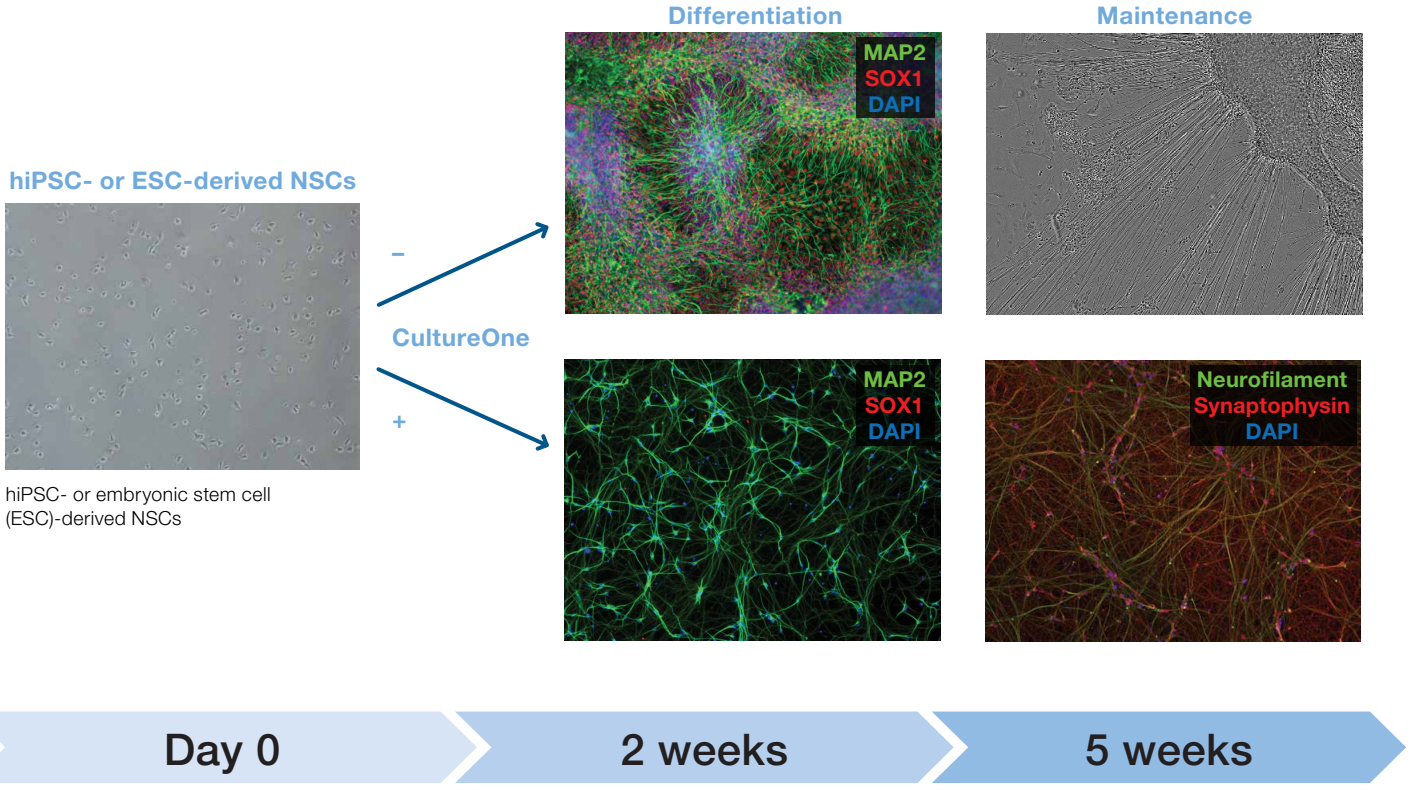


Figure 5.6. The addition of CultureOne Supplement allows for superior neural cell cultures. In conventional NSC differentiations, cultures will overgrow and consist of a mixed population of progenitor cells (SOX1) and differentiated neurons (MAP2). Overgrowth of the progenitor cells will lead to clump formation that, with extended maintenance, results in cells peeling off the culture plate. The addition of CultureOne Supplement eliminates progenitors in NSC differentiations, resulting in superior cultures consisting of differentiated neurons. Without overgrowth or clump formation, these cultures can readily be maintained for 5 weeks or longer.

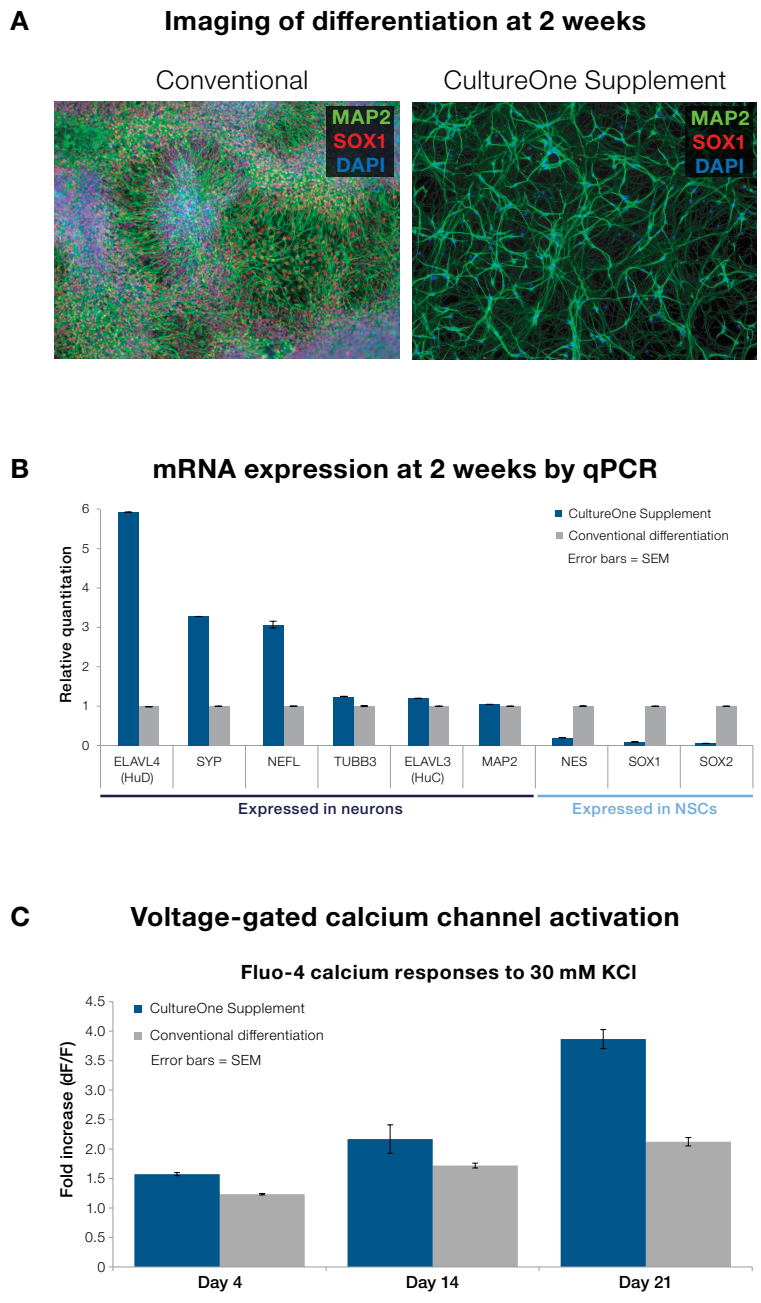


Figure 5.7. CultureOne Supplement enables improved imaging, RNA expression, and electrophysiology assays. (A) After 2 weeks of differentiation with CultureOne Supplement, images show evenly distributed, differentiated neurons (MAP2$^+$) with >75% reduction in NSCs (SOX1$^+$) and cell clumps compared to the conventional differentiation methods. **(B)** With CultureOne Supplement, cells at 2 weeks of differentiation had increased neuronal mRNA expression, reduced NSC mRNA expression, and exhibited higher spike rates as measured by multi-electrode array (MEA, not shown). **(C)** Neurons differentiated from NSCs with CultureOne Supplement showed an increase in cytosolic calcium when depolarized with KCl, meaning they express significantly higher numbers of voltage-gated calcium ion channels, which are an important marker for neuronal maturity and excitability. This, along with the longer neurites after 2 weeks of differentiation, demonstrates that CultureOne Supplement accelerates neuronal maturation.

B-27 Plus Neuronal Culture System

The most cited neural cell culture system consists of Gibco™ B-27™ Supplement and Gibco™ Neurobasal™ Medium. Originally optimized for long-term culture of rat hippocampal and cortical neurons, this combination has been shown, over two decades of research, to be suitable for a wide range of other neural applications including PSC-derived NSCs and neurons.

However, as the desire for more reliable and biologically relevant models has increased, so too has the necessity for a next-generation media system that can maintain and mature optimal densities of functional neurons over longer periods of time *in vitro*. The Gibco™ B-27™ Plus Neuronal Culture System, which includes the B-27 Plus Supplement and Neurobasal Plus Medium, features an optimized formulation, upgraded manufacturing process, and more stringent quality control for raw materials and final product. These improvements enable increased neuronal survival by more than 50%, accelerated neurite outgrowth, improved electrophysiological activity, and maturation of neurons (Figures 5.8 and 5.9).

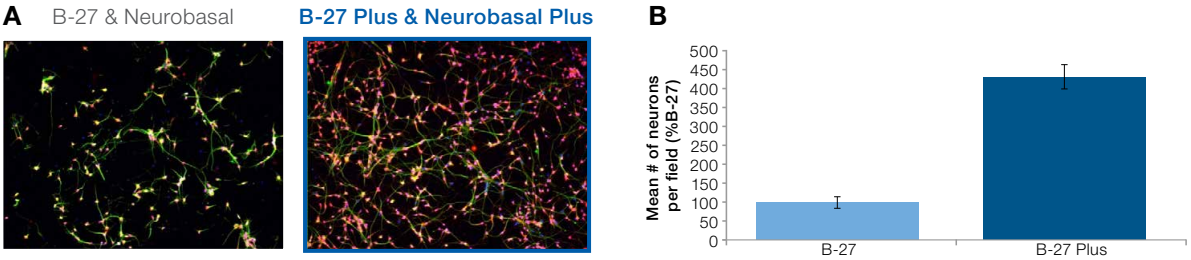


Figure 5.8. B-27 Plus Neuronal Culture System enables superior survival of human stem cell–derived neurons. Cryopreserved HIP Neurons (MTI-GlobalStem) were thawed in classic Neurobasal Medium with B-27 Supplement and plated onto polyethyleneimine-coated 96-well plates into two volumes of the listed media. Neurons were maintained for 4 weeks with half fluid changes two times per week. **(A)** Neurons were immunostained with neuronal dendritic marker, MAP2 (green), neuronal cell body marker, HuC/D (red), and nuclei were counterstained with DAPI (blue). **(B)** Comparability studies indicate that the B-27 Plus Neuronal Culture System is a significantly superior medium compared to the classic B-27–supplemented Neurobasal Medium, with improved neuronal survival and health in long-term cultures.

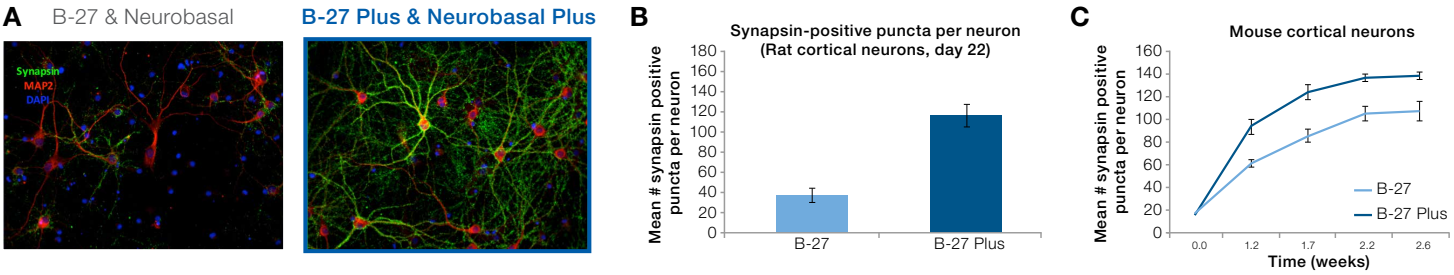


Figure 5.9. Enhanced and accelerated neuronal maturation in the B-27 Plus Neuronal Culture System. (A) Rat cortical neurons at day 22 were stained with dendritic marker MAP2 (red), synapsin 1/2 to label presynaptic terminals (green), and DAPI as a counterstain (blue). **(B)** Neurons maintained in the B-27 Plus Neuronal Culture System had significantly higher numbers of synapsin-positive puncta. **(C)** Cryopreserved mouse cortical neurons were thawed in classic B-27–supplemented Neurobasal Medium and plated onto poly-D-lysine–coated 96-well plates. Neurons were maintained for ~3 weeks in B-27/Neurobasal or B-27 Plus/Neurobasal Plus media systems following the suppliers' recommended protocols. Neurite outgrowth was quantitated on an IncuCyte™ analysis system (Essen BioScience) from differential interference contrast images taken at the time points specified. The B-27 Plus Neuronal Culture System significantly accelerates neurite outgrowth over the first few weeks compared to the classic Neurobasal Medium with B-27 Supplement.

Dopaminergic neuron differentiation

Midbrain dopaminergic (DA) neurons derived from hPSCs provide a viable alternative to primary human neurons for disease modeling and drug screening.

While a neuronal population expressing DA markers can be derived from NSCs, it is necessary to proceed via a floor plate intermediate to generate functional, midbrain-specified DA neurons.

The Gibco™ PSC Dopaminergic Neuron Differentiation Kit enables the differentiation of pluripotent stem cells (PSCs) to midbrain DA neurons that secrete dopamine and exhibit spontaneous action potentials. Unlike other protocols or commercially available solutions to differentiate PSCs to DA neurons (which can be biologically restrictive, lengthy, or ill-defined), our kit enables increased flexibility, speed, and scalability, all while retaining proper biological relevance (Figures 5.10 and 5.11).

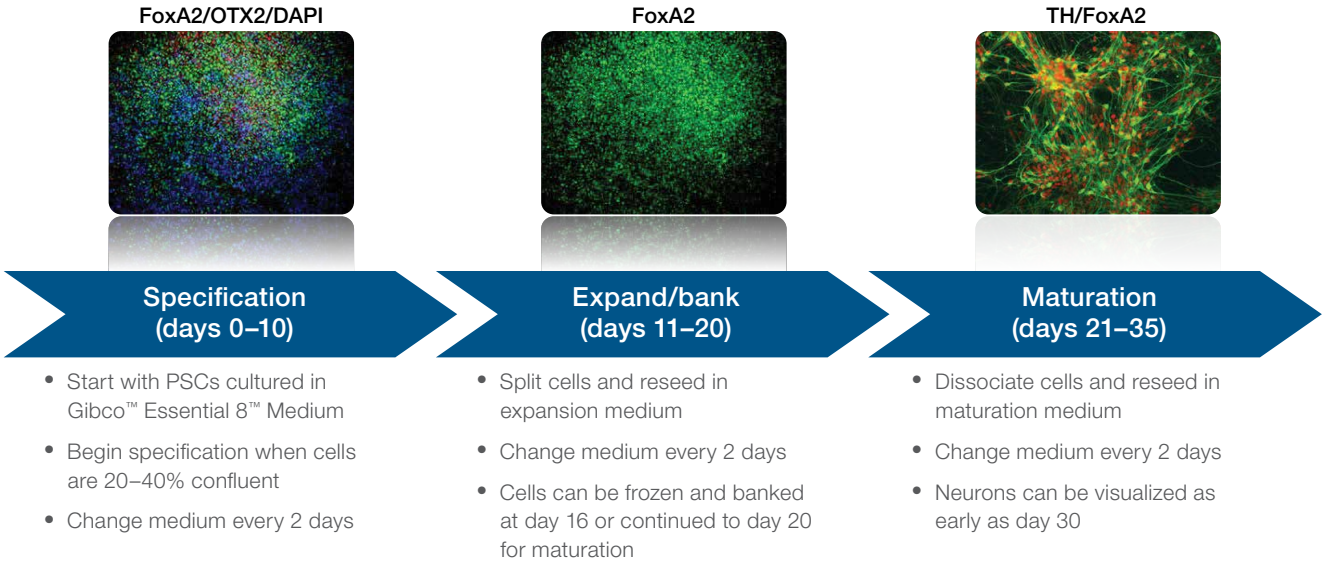


Figure 5.10. Simplified workflow diagram. Pluripotent stem cells cultured in Essential 8 Medium can be specified to the midbrain floor plate, expanded and banked, then matured to midbrain DA neurons in 35 days. Floor plate–derived midbrain progenitors can be expanded up to 10 passages.

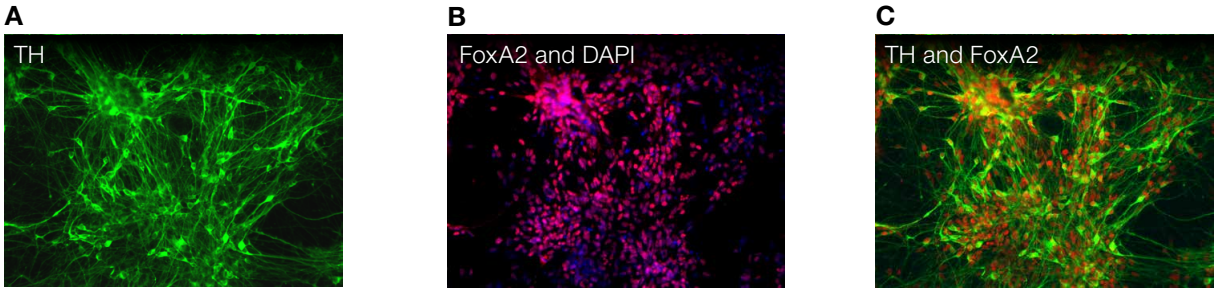


Figure 5.11. Representative images of mature DA neurons. The images were obtained from cells stained with reagents provided in the Invitrogen™ Human Dopaminergic Neuron Immunocytochemistry Kit after 14 days of maturation of floor plate progenitor cells in Dopaminergic Neuron Maturation Medium. The majority of the TH-expressing neurons also coexpressed FoxA2. Cell stains used in these images include **(A)** anti-TH (green), **(B)** anti-FoxA2 (red), and Invitrogen™ NucBlue™ stain (a DAPI nuclear DNA stain) (blue). **(C)** Merged image.

Neuronal functional and cell health assays

iPSCs are powerful tools for disease modeling. They allow researchers to study disease-specific phenotypes in the disease-relevant cell type established from patient-specific iPSCs. The ease with which isogenic controls can be generated via gene editing further allows researchers to eliminate the effects of donor variability and, with high confidence, identify subtle disease-specific phenotypes. However, this requires the availability of assays to interrogate relevant phenotypes.

Neurons are a complex cell type amenable to a variety of cell type–specific assays. Most characteristically, the electrophysiological activity of iPSC-derived neurons can be measured via patch-clamp assays or using multi-electrode arrays (MEAs) to determine neuronal subtype–specific action potential (AP) activity or to assess the effects of neurotoxic compounds.

NSCs can be subjected to a panel of assays compatible with high-throughput methods (Table 5.1) in the presence of various cell stressors to assess neural cell health (Figure 5.12). Additional levels of complexity can be obtained with all of these assays by co-culturing neurons and glial cells to isolate cell-autonomous disease phenotypes from nonautonomous ones, or to determine neuroprotective effects of glia.

Table 5.1. Selected assays that can be used to measure different aspects of neural cell health.

Assay	Parameter measured	Increased readout
PrestoBlue Cell Viability Reagent	Metabolic activity	Increased red fluorescence with higher metabolism
CellEvent Caspase-3/7 Green Detection Reagent	Caspase-3/7–dependent apoptosis	Increased green fluorescence in apoptotic cells
CellROX Green Reagent	Cellular oxidative stress	Increased green fluorescence with increased cellular reactive oxygen species (ROS)
MitoSOX Red Mitochondrial Superoxide Indicator	Mitochondrial superoxide levels	Increased red fluorescence with increased mitochondrial superoxide levels

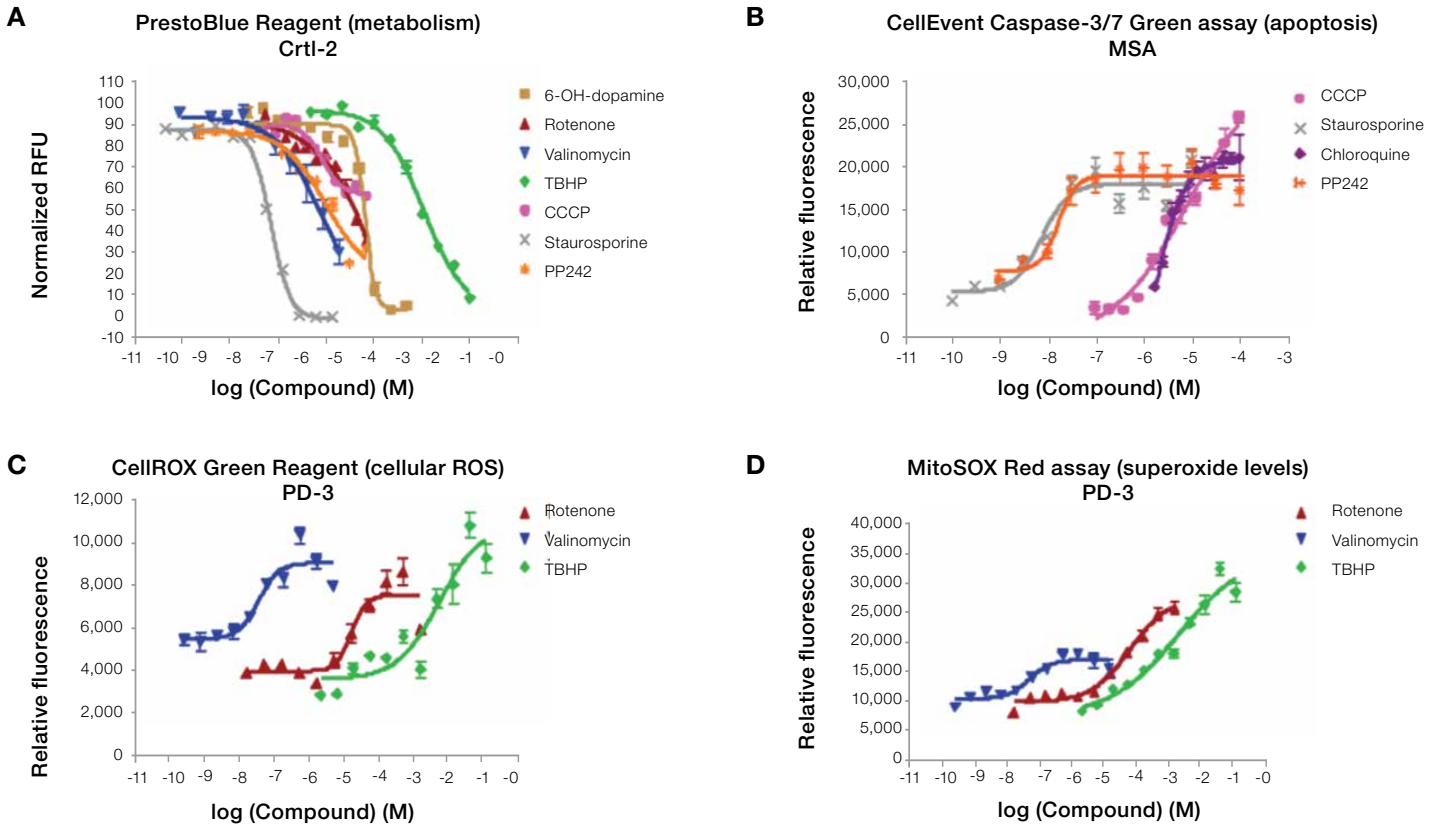


Figure 5.12. A panel of functional assays was used to assess the health of NSCs in response to various cell stressors. iPSC lines were derived from a Parkinson's disease (PD)-affected donor (PD-3), one multiple systems atrophy (MSA)-affected donor, and an age-matched, healthy control individual (Ctrl-2), and differentiated into a NSC population using PSC Neural Induction Medium. The derived NSCs were expanded on Gibco™ CTS™ CELLstart™ Substrate in Neural Expansion Medium for seven passages followed by Gibco™ StemPro™ NSC SFM for another four passages. The NSCs were harvested and plated in CTS CELLstart Substrate–coated 384-well assay plates for evaluation by four high-throughput assays. A Tecan Safire™ reader (Tecan Group Ltd.) was used to measure fluorescence. Representative results are shown for **(A)** the Invitrogen™ PrestoBlue™ assay on Ctrl-2, demonstrating the expected loss in metabolic activity with an increase in the concentration of stressors added, **(B)** the Invitrogen™ CellEvent™ Caspase-3/7 Green assay on NSCs derived from the MSA-affected donor, demonstrating the expected increase in apoptosis with an increase in the concentration of stressors added, and **(C, D)** the multiplexed Invitrogen™ CellROX™ Green assay and MitoSOX™ Red asay on PD-3, demonstrating the expected increase in oxidative stress with an increase in the concentration of stressors added.

5.3

Cardiomyocyte differentiation

Few functional behaviors are as impressive as the spontaneous rhythmic contractions of iPSC-derived cardiomyocytes.

Human iPSC-derived cardiomyocytes serve as a particularly important system for studying inherited cardiomyopathies, as studies in animal models have largely been limited by significant differences in human and rodent cardiac electrophysiological properties. It should, however, be noted that iPSC-derived cardiomyocytes exhibit a fetal phenotype.

Applications of iPSC-derived cardiomyocytes include disease modeling, cell replacement therapy (for example following myocardial infarction), and, increasingly, cardiotoxicity screening during drug development.

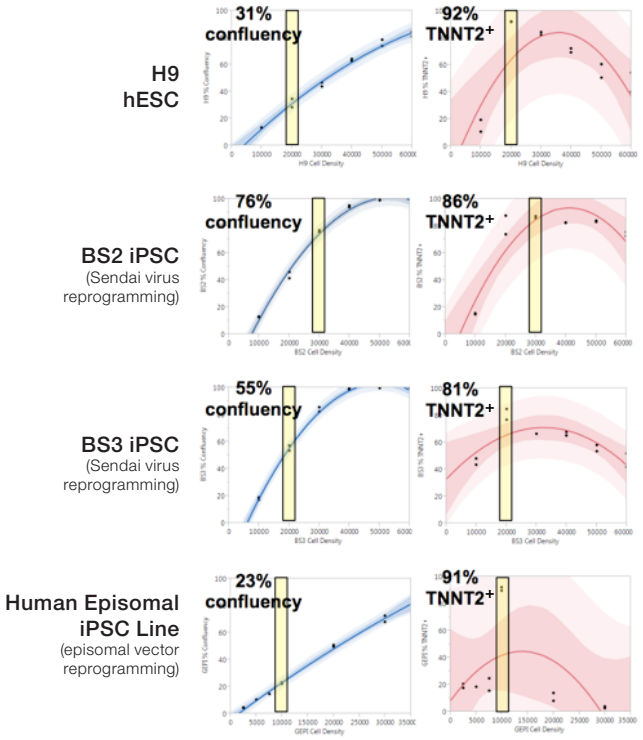


Figure 5.13. Efficient differentiation across multiple PSC lines. Seeding density is crucial for optimal PSC cardiomyocyte differentiation. PSCs dissociated with Gibco™ TrypLE™ reagent were used for setup of these studies. Of two lines derived by reprogramming with the Invitrogen™ CytoTune™-iPS 2.1 Sendai Reprogramming Kit (BS2 and BS3), BS2 iPSCs were observed to be promiscuous at higher density. The Gibco™ Human Episomal iPSC Line was also found to be optimal at a specific density. H9 hESCs were observed to be promiscuous at various densities. The JMP™ Profiler tool identified optimal seeding densities for efficient differentiation of different PSC lines.

PSC Cardiomyocyte Differentiation Kit

The Gibco™ PSC Cardiomyocyte Differentiation Kit consists of a set of serum-free and xeno-free media that enable efficient differentiation of hPSCs to contracting cardiomyocytes in as few as 8 days. Unlike other methods that require multiple components and longer assay duration, the PSC Cardiomyocyte Differentiation Kit can be used to generate cardiomyocytes from PSCs in a ready-to-use media format and in less time.

The kit contains three 1X media that require no thawing or mixing, and each medium is used consecutively over a total of 14 days, resulting in functional cardiomyocytes that express relevant physiological markers, contract in culture, and can be subsequently maintained in culture for more than 15 days.

With optimized conditions, high yields of TNNT2-expressing cardiomyocytes can be generated across a range of ESC and iPSC lines (Figure 5.13). For iPSC lines that may be more difficult to differentiate, our supplemental enrichment protocol based on metabolic selection can improve yield by 10–30%. You can find both our standard kit protocol and the enrichment protocol at **thermofisher.com/cardiacdiff**

Cardiomyocyte Maintenance Medium

Gibco™ Cardiomyocyte Maintenance Medium is a serum-free and xeno-free medium that is capable of maintaining cardiomyocytes that have been differentiated using the PSC Cardiomyocyte Differentiation Kit. This medium is included in the kit, but is also sold separately for researchers wanting to maintain differentiated cardiomyocytes in culture for extended periods of time.

Human Cardiomyocyte Immunocytochemistry Kit

The Invitrogen™ Human Cardiomyocyte Immunocytochemistry Kit enables optimal image-based analysis of two key cardiomyocyte markers: NKX2.5 and TNNT2/cTnT (Figure 5.14). It is the only kit that offers superior imaging reagents for cardiomyocytes in one box, with a complete set of primary and secondary antibodies, a nuclear DNA stain, and all of the premade buffers to enable an optimized staining experiment.

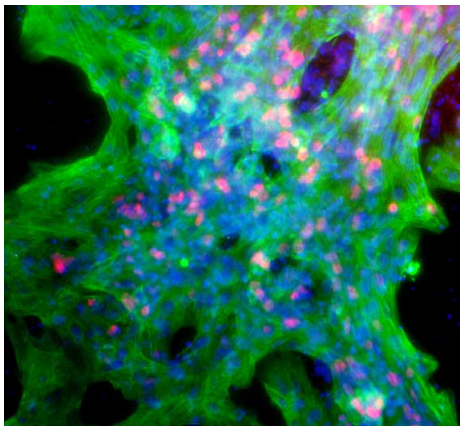


Figure 5.14. iPSCs differentiated for 14 days using the PSC Cardiomyocyte Differentiation Kit. The cells were stained using the Human Cardiomyocyte Immunocytochemistry Kit for the following markers: NKX2.5 (red) for early cardiomesoderm, TNNT2/cTnT (green) for cardiomyocytes, and DAPI for nuclear DNA.

Cardiomyocyte functional assays

The phenotypic and electrophysiological characteristics of iPSC-derived cardiomyocytes are comparable to their primary cell counterparts. The beating syncytium that spontaneously forms is particularly amenable to characterization and analysis. Contractions are accompanied by oscillating intracellular calcium levels that can be measured using calcium-sensitive dyes, and the response to cardiotoxic compounds can be quantified (Figures 5.15 and 5.16). The introduction of disease-associated SNPs via genome editing (see section 4) also enables the use of this system for disease modeling (Figure 5.17).

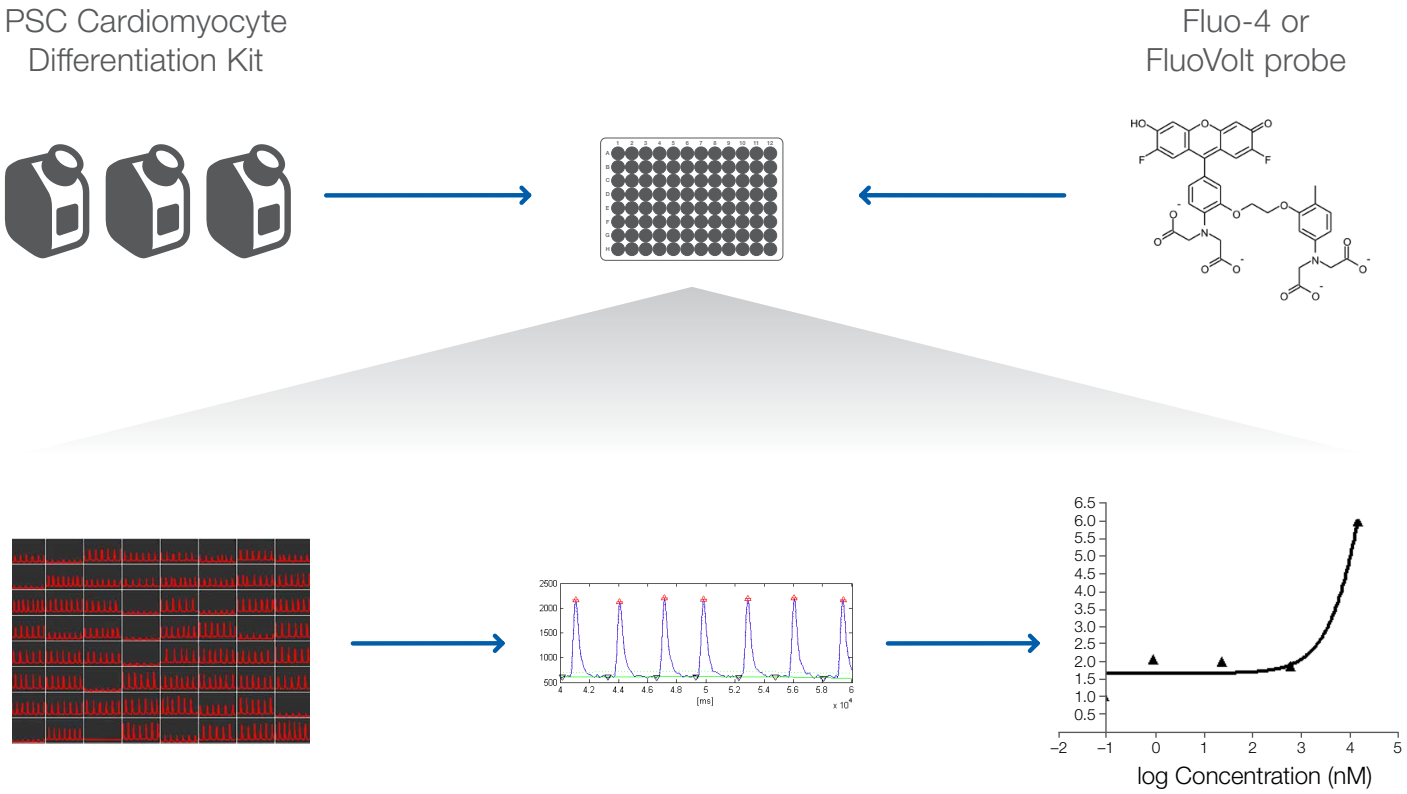


Figure 5.15. A high-throughput assay for functional measurements on hiPSC-derived cardiomyocytes was developed using cardiomyocytes generated with the PSC Cardiomyocyte Differentiation Kit. After differentiation, cardiomyocytes are replated into 96- or 384-well plates and loaded with Invitrogen™ fluo-4 dye or FluoVolt™ probe to measure calcium flux or electrical activity, respectively. Signal transients can then be recorded, analyzed, and turned into meaningful data. This assay can be used for cardiac safety screens or disease model screens.

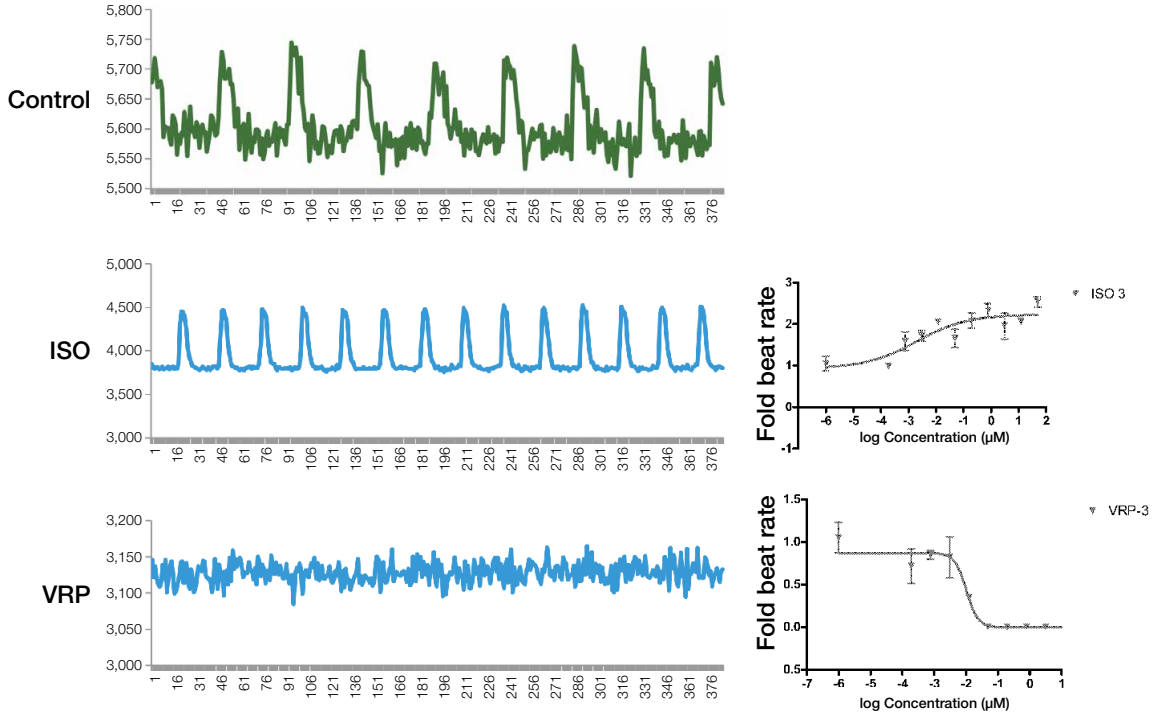


Figure 5.16. Use of the high-throughput functional assay for cardiomyocytes to study the effect of known compounds. Representative fluo-4 traces after treating cardiomyocytes with the β-adrenergic stimulator isoproterenol (ISO) or the calcium channel blocker verapamil (VRP) are shown. Data from these traces can then be used to generate dose-response curves for the different features of a contracting cardiomyocyte such as beat rate as shown.

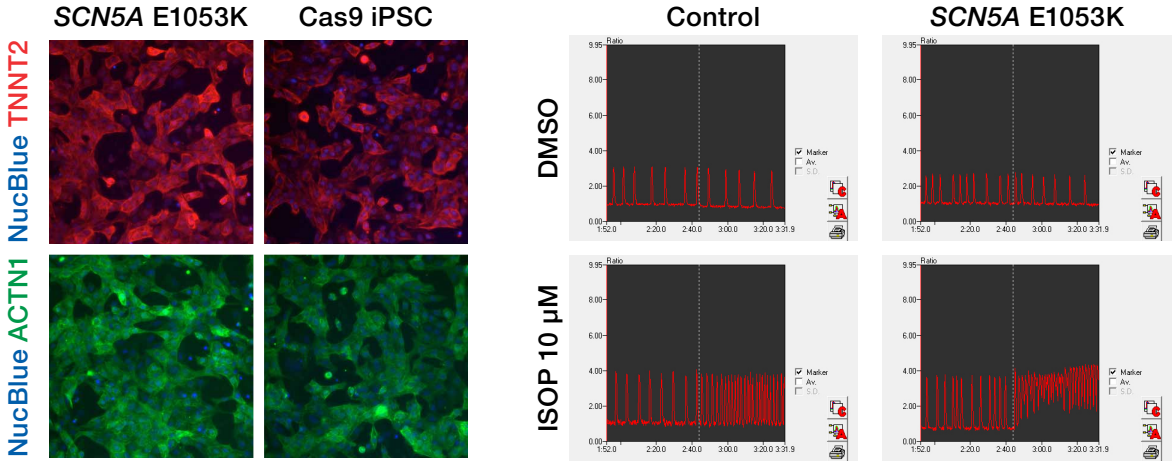


Figure 5.17. Functional effect on cardiomyocytes after introducing a homozygous SNP change in *SCN5A* (E1053K) to mimic Brugada syndrome *in vitro*. Cardiomyocytes with the homozygous SNP change are morphologically normal (left panel) and behave similarly to control cardiomyocytes under standard culture conditions (DMSO, right panel). As shown through use of the high-throughput functional assay, beta-adrenergic stimulation of cardiomyocytes using isoproterenol (ISOP, right panel) causes arrhythmias in ~50% of wells scored, which is similarly observed in patients with Brugada syndrome.

5.4

Definitive endoderm differentiation

Definitive endoderm encompasses an intermediate population of cells that gives rise to downstream lineages including pancreas, liver, and gut. As with many other lineages, it has been found that the generation of functionally relevant mature cell types is best achieved through a differentiation protocol that recapitulates the stepwise differentiation during embryonic development, including the passage through a definitive endoderm intermediate.

Downstream lineages have applications in modeling and cell therapy for a wide range of diseases, including diabetes for pancreatic beta cells and metabolic disorders for hepatocytes. iPSC-derived hepatocytes additionally have potential utility for hepatotoxicity studies during the drug discovery process.

Traditional protocols for definitive endoderm induction can be costly due to the requirement for activin protein and Wnt signaling.

PSC Definitive Endoderm Induction Kit

The Gibco™ PSC Definitive Endoderm Induction Kit consists of two xeno-free media that enable efficient induction of hPSCs to definitive endoderm (Figure 5.18). Unlike other methods that require multiple components and take 5 or more days, the PSC Definitive Endoderm Induction Kit enables generation of ≥90% CXCR4⁺/PDGFRα⁻ definitive endoderm cells with only two components in just 2 days (Figure 5.19).

Each medium is supplied in a 1X complete formulation, requiring no mixing of additional components, and the resultant definitive endoderm shows >90% high expression of the key markers Sox17 and FoxA2 across multiple PSC lines (Figure 5.20) and is capable of differentiating to downstream lineages (Figure 5.21).

Find out more at **thermofisher.com/defendo**

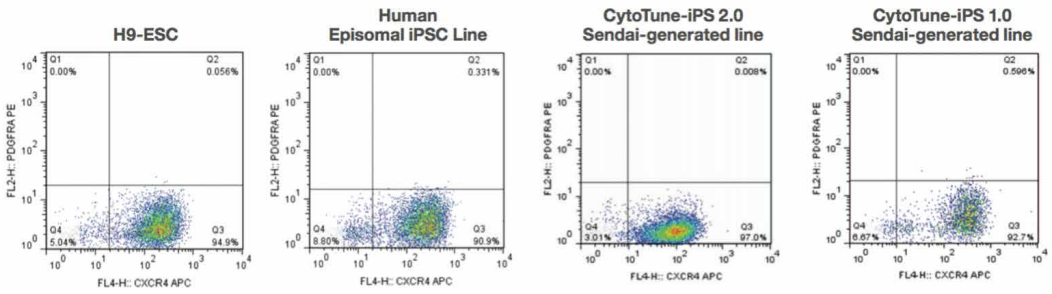


Figure 5.18. The PSC Definitive Endoderm Induction Kit produces definitive endoderm populations with high efficiency (≥90%) across hESC and iPSC lines, including cell lines reprogrammed using episomal vectors or CytoTune-iPS kits. Representative dot plots show CXCR4⁺/PDGFRα⁻ cell populations derived from various cell lines. For each experiment, unstained cells were used to set quadrant gates.

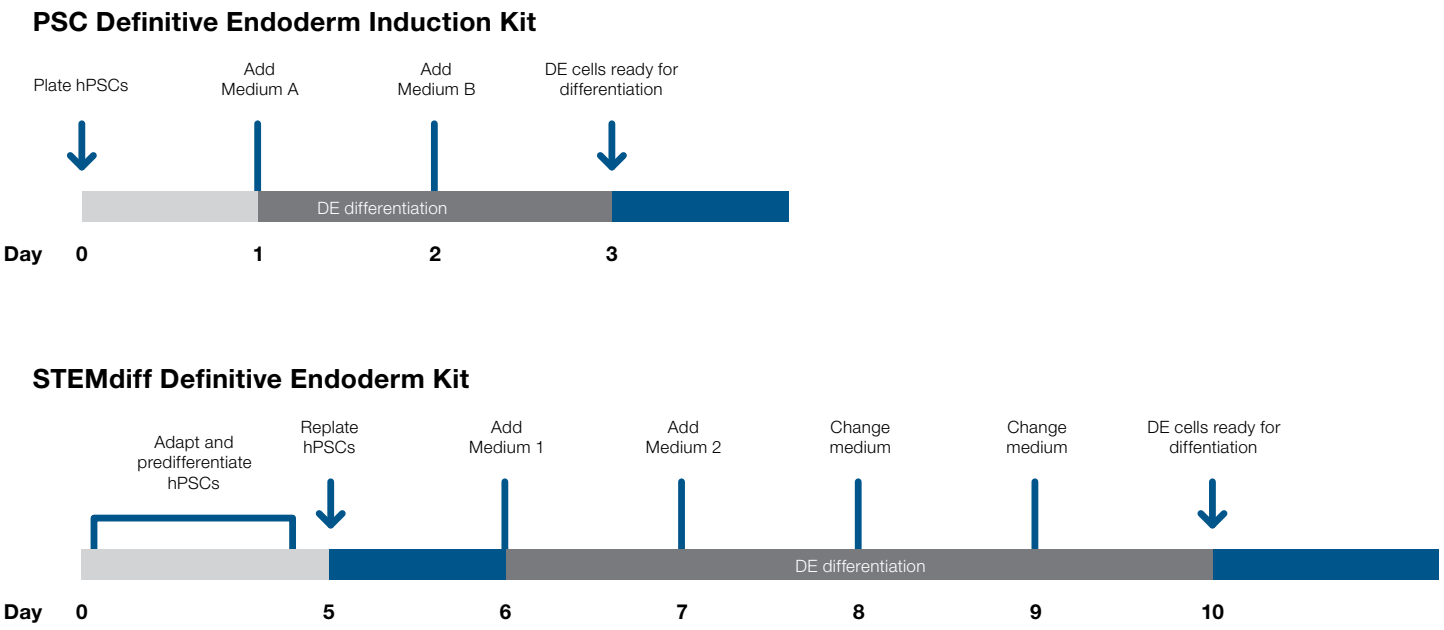


Figure 5.19. Compared to other differentiation protocols, the PSC Definitive Endoderm Induction Kit produces cells in up to 50% less time and requires no predifferentiation or mixing of media.

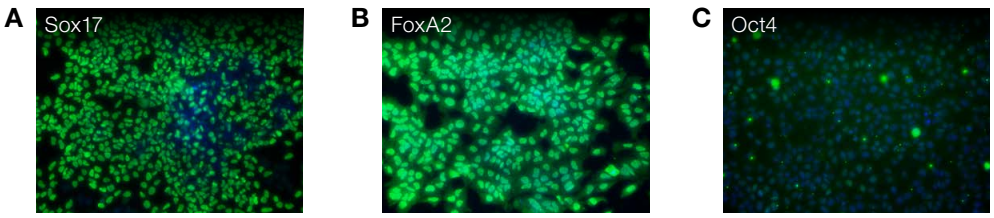


Figure 5.20. Immunocytochemistry of hESCs treated with the PSC Definitive Endoderm Induction Kit. At day 3, induced cells were immunostained for the endodermal transcription factors Sox17 and FoxA2 and the pluripotent marker Oct4. Nuclei were counterstained with DAPI (blue) to assess total cell numbers.

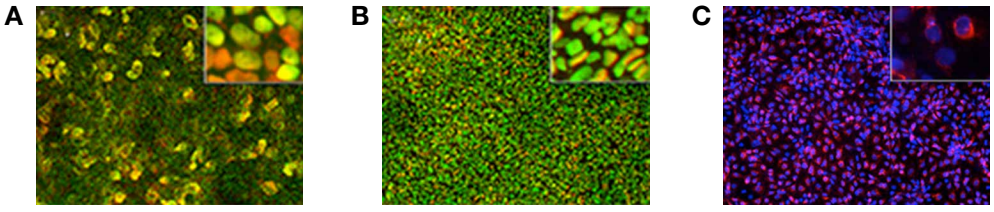


Figure 5.21. Definitive endoderm can be differentiated to downstream lineages. H1 ESCs were treated with the PSC Definitive Endoderm Induction Kit media and differentiated into functional cells that express relevant physiological markers: **(A)** midgut/hindgut (nuclei, blue; FoxA2, green; Cdx2, red); **(B)** pancreatic endoderm (nuclei, blue; FoxA2, green; Pdx1, red); **(C)** liver bud progenitors (nuclei, blue; AFP, red). Data credited to LT Ang, KM Loh, and B Lim of the Genome Institute of Singapore.

Section 6

Characterization

6.1 Introduction

With recent technological advances, iPSCs can now be derived from various somatic cells using different reprogramming methods and can be cultured with different media and matrices. As diverse PSC lines are derived and cultured under different conditions, there is a need for reliable characterization methods to confirm the quality of the PSCs.

Current PSC characterization practices consist of a panel of assays primarily testing functional pluripotency and detecting abnormalities that can affect cell behavior and safety.

Find the right monitoring and detection tools for your research at **thermofisher.com/characterization**

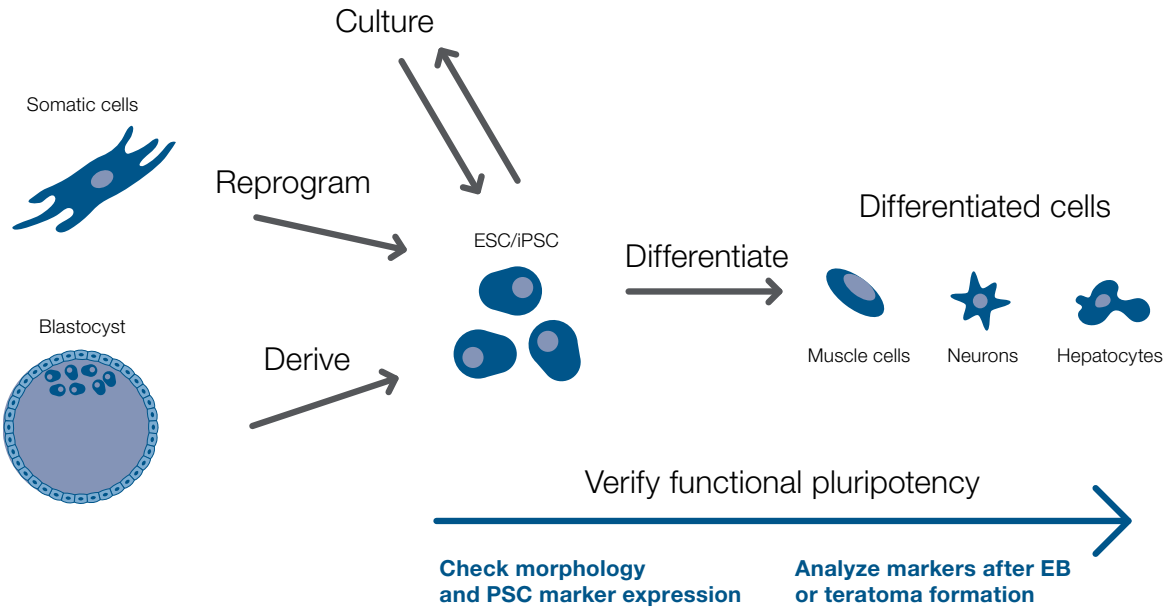


Figure 6.1. PSC characterization practices. Characterization is performed to check the functional pluripotency of newly derived PSC lines.

During the derivation of iPSCs and ESCs, characterization is performed to confirm that a pluripotent line has truly been obtained (Figure 6.1). During routine maintenance and after significant manipulations like gene editing, the goal of characterization is to ensure that the fundamental properties of the PSCs have not changed. In this section, basic and commonly used PSC characterization practices are described in the context of reprogramming and the derivation of a new iPSC line. Note that new iPSC lines require karyotyping and often undergo cell banking in addition to the characterization described. Scale-up of the culture is necessary in order to generate enough cells for all of these processes. An example of a scale-up scheme and allotment of cells is shown in Figure 6.2.

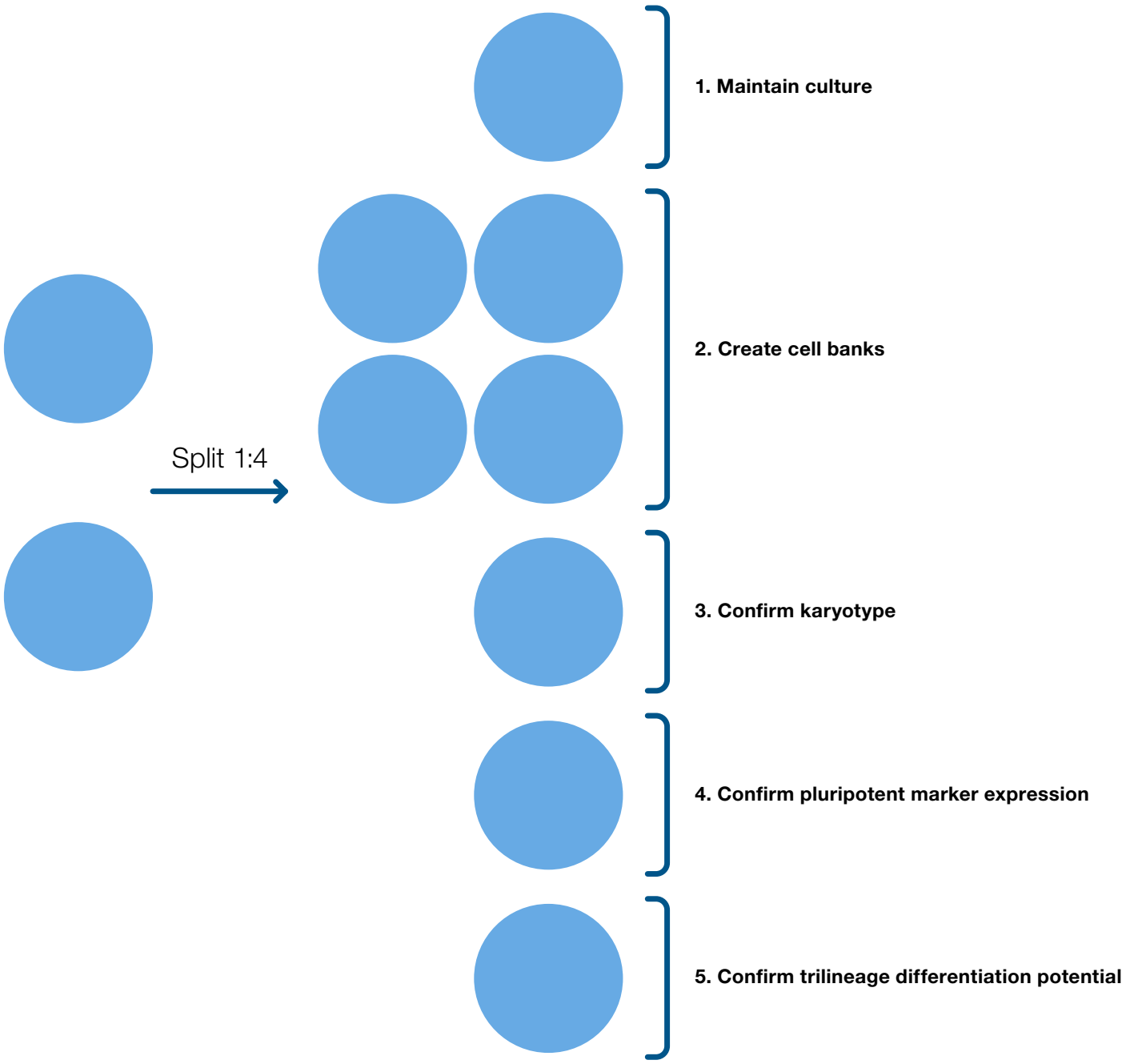


Figure 6.2. Generation of undifferentiated and differentiated cells for karyotyping, cell banking, and characterization. It is recommended that newly derived iPSC lines be scaled up to about seven 6 cm dishes and a T-25 flask in order to maintain the culture while karyotyping, performing marker analyses, and creating cell banks. The same approach can be used for PSCs grown on feeders or under feeder-free conditions.

6.2

Assessing genetic stability

G-band karyotyping

Genetic instability is a known issue during long-term cell culture. So, verifying the absence of major chromosomal aberrations is a critical quality control step when reprogramming or maintaining PSCs. The most common practice is to use G-banding for karyotyping (Figure 6.3), which reveals aneuploidy and large chromosomal abnormalities. Normally, 20 cells are analyzed. The appearance of less than 10% nonclonal aberrations or artifacts is acceptable.

Array CGH and genome sequencing

Behavior-changing genetic alterations are not limited to the large chromosomal abnormalities detected by G-banding. To detect smaller genetic abnormalities, it is necessary to use higher-resolution methods like array comparative genomic hybridization (array CGH) and genome sequencing.

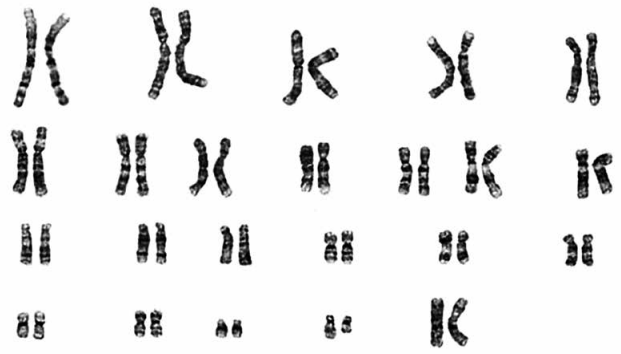


Figure 6.3. G-banding results for a PSC line possessing a karyotype of 46 normal chromosomes in 20 cells analyzed. Analysis was performed by Cell Line Genetics.

KaryoStat and KaryoStat HD Assays

The Applied Biosystems™ KaryoStat™ and KaryoStat™ HD Assays are array-based alternatives to G-banding that offer whole-genome coverage for accurate detection of chromosomal abnormalities (Figure 6.4). Free software enables simple analysis that doesn't require specialized cytogenetics expertise. In addition, the same assay gives genotyping (sample ID) results as well.

Find out more at **thermofisher.com/karyostat**

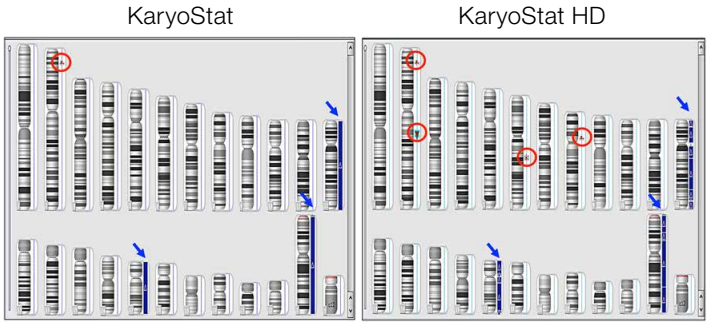


Figure 6.4. Analysis with KaryoStat and KaryoStat HD Assays. The assays detect trisomy of chromosomes 12, 17, and X in BG01V, a human embryonic stem cell line with an abnormal karyotype. In addition, both assays detect a loss on chromosome 2 that was not detected by G-banding. The KaryoStat HD Assay also revealed three additional losses at chromosomes 2, 6, and 8.

6.3

Detecting self-renewal marker expression

Undifferentiated PSCs and their differentiated derivatives can be identified through careful observation of cellular morphology. For example, elongated fibroblasts subjected to reprogramming protocols transform into more compact PSCs that have high nucleus-to-cytoplasm ratios and form three-dimensional colonies with well-defined edges when grown on feeders.

Ideally, each of the colonies picked for further culture and analysis contains only fully reprogrammed, bona fide pluripotent cells. However, in reality, the colonies that emerge include both partially and fully reprogrammed cells that can appear indistinguishable even to the well-trained eye. The visualization of PSC markers increases the likelihood of obtaining a fully reprogrammed iPSC line. These PSC markers can be identified through the detection of PSC-specific enzymatic activity, through live cell immunofluorescence against PSC surface markers, or through fixed-cell immunocytochemistry using intracellular PSC markers.

Live alkaline phosphatase (AP) staining

Alkaline phosphate (AP) is an enzyme that is upregulated in PSCs. AP expression can be detected using the Invitrogen™ Alkaline Phosphotase Live Stain, which consists of a substrate that selectively fluoresces as a result of AP activity [1]. This method for differential AP staining is both quick and reversible and helps preserve the viability of the cells. AP Live Stain can thus be used to discriminate stem cells from feeder cells or parental cells during reprogramming (Figure 6.5).

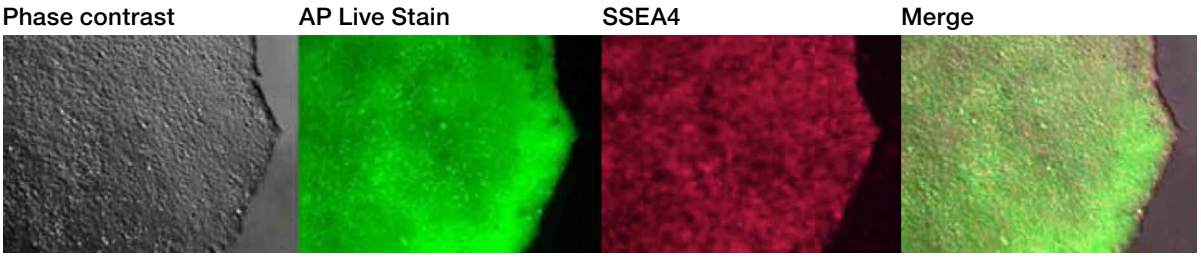


Figure 6.5. Detection of AP in live PSCs. Feeder-free PSCs were analyzed using the AP Live Stain (green), with counterstaining done using an antibody for the PSC marker SSEA4 (red).

Live-cell immunostaining

More specific cell staining can be achieved using antibodies against established markers. Surface proteins like the positive PSC markers SSEA4, TRA-1-60, and TRA-1-81, and the negative PSC markers CD44 and SSEA1, are particularly useful because they can be stained quickly while keeping cells in culture [2,3]. Of the positive PSC markers, TRA-1-60 is thought to be the most stringent, because it is upregulated later on during reprogramming [4]. On the other hand, CD44 is found on many differentiated cell types, but is absent from PSCs. The presence of CD44 on fibroblasts and partially reprogrammed cells, as opposed to PSCs, increases confidence in picking colonies for expansion during reprogramming, especially when it is combined with a positive PSC marker [3] (Figure 6.6).

Both TRA-1-60 and CD44 can be detected using live-cell imaging kits that are designed to maximize the signal-to-noise ratio and allow continued culture of cells through the use of live cell–qualified Invitrogen™ Alexa Fluor™ dye–conjugated antibodies and optically clear Gibco™ FluoroBrite™ DMEM (Figures 6.7 and 6.8). These kits are available with three differnt fluorophores to accommodate commonly used fluorescence filters and can be used for two applications:

- Monitoring the reprogramming process and distinguishing between partially reprogrammed and fully reprogrammed colonies when picking colonies for expansion
- Detection of self-renewal or pluripotency markers for routinely cultured hESCs and hiPSCs

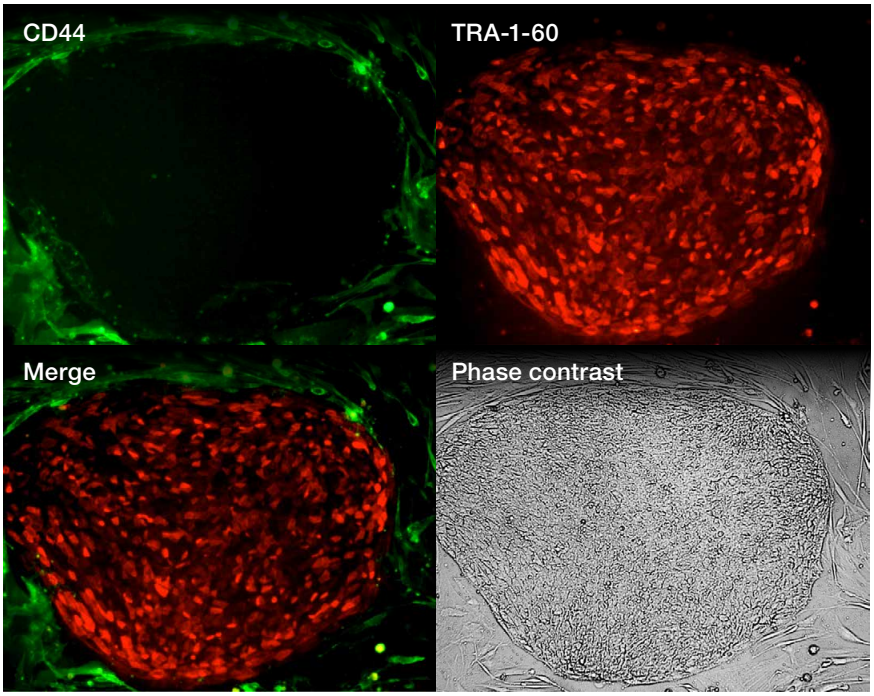


Figure 6.6. Live-cell imaging of iPSCs. iPSC colony cultured on mouse embryonic feeder layer and stained using Alexa Fluor dye–conjugated antibodies for fibroblast marker CD44 and PSC marker TRA-1-60. Imaging was performed after replacing the staining medium with FluoroBrite DMEM imaging medium.

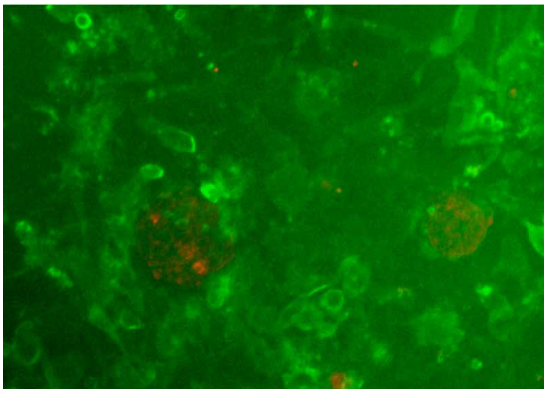


Figure 6.7. Standard media contain phenol red and other sources of background fluorescence.

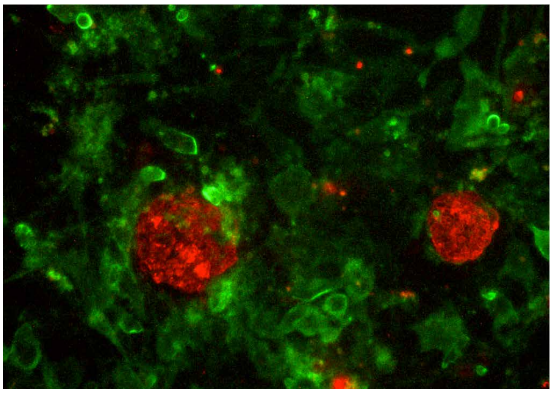


Figure 6.8. FluoroBrite DMEM included in the live-cell imaging kits exhibits optical clarity similar to PBS and supports cell survival post-staining.

Flow cytometry

While the staining and imaging approaches described are qualitative, flow cytometry provides a quantitative measure of how many cells are expressing the markers and at what level, revealing any downregulation of the markers or heterogeneity in the population. It is most common to perform flow cytometry using surface markers such SSEA4 and TRA-1-60 (Figure 6.9). Antibodies that can be used for this purpose include a monoclonal SSEA4 antibody conjugated to Invitrogen™ Alexa Fluor™ 647 dye and an unconjugated monoclonal TRA-1-60 antibody used with a secondary antibody like Invitrogen™ Alexa Fluor™ 594 Goat Anti–Mouse IgG (H+L) Antibody.

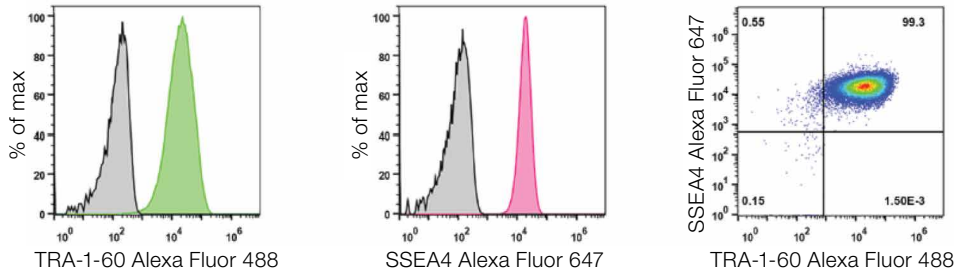


Figure 6.9. Flow cytometry analysis of a feeder-free PSC culture. Isotype controls (gray) are used to determine the percentage of cells expressing TRA-1-60 (green) and SSEA4 (magenta). Dual staining permits the quantification of the number of cells in the culture that are expressing both markers. Typically, >95% SSEA4⁺/TRA-1-60⁺ cells are expected. Flow cytometry was performed using the Invitrogen™ Attune™ NxT Flow Cytometer with blue/red lasers.

Fixed-cell immunostaining

To increase confidence in the quality of an iPSC clone, it is recommended to confirm the expression of not just one or two, but multiple PSC markers. Well-established markers include human PSC–specific surface markers such as SSEA4 and TRA-1-60 and transcription factors Oct4 and Sox2, which are known to play key roles in maintaining pluripotency [2] (Figure 6.10). Since these are intracellular proteins, staining for these markers requires fixation and permeabilization, which necessitates termination of the culture while a duplicate culture of the clone is maintained.

The Invitrogen™ PSC Immunocytochemistry Kits enable optimal image-based analysis of up to four key markers of hPSCs: Oct4, Sox2, SSEA4, and TRA-1-60. These immunocytochemistry kits include a complete set of primary and secondary antibodies, a nuclear DNA stain, and premade buffers for optimized staining of fixed PSCs. The antibodies included in the kit have been validated for high performance and multiplexing ability, allowing for specific and simultaneous assessment of two markers at a time.

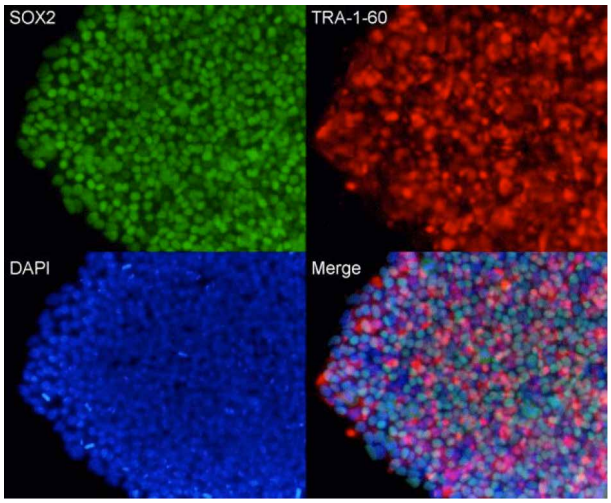


Figure 6.10. Analysis of intracellular markers in fixed PSCs. iPSCs derived from CD34+ cord blood were grown under feeder-free conditions using Gibco™ Essential™ 8 Medium in wells coated with vitronectin. The cells were stained for the pluripotency markers Sox2 (green) and TRA-1-60 (red) using the Invitrogen™ PSC 4-Marker Immunocytochemistry Kit.

PluriTest-compatible PrimeView assays

Flow cytometry and immunostaining, while widely used methods, can only assess a limited number of pluripotency-associated markers. In contrast, Applied Biosystems™ PluriTest™-compatible PrimeView™ Global Gene Expression Profile Assays assess pluripotency through whole-transcriptome analysis. The technology leverages PrimeView gene expression data in combination with the PluriTest analysis tool (Figure 6.11), which is a well-established method for verifying pluripotency with more than 16,000 samples analyzed. The assay utilizes bioinformatics analysis based on the work of Müller et al. [5].

Find more information on PluriTest analysis at **thermofisher.com/primeview**

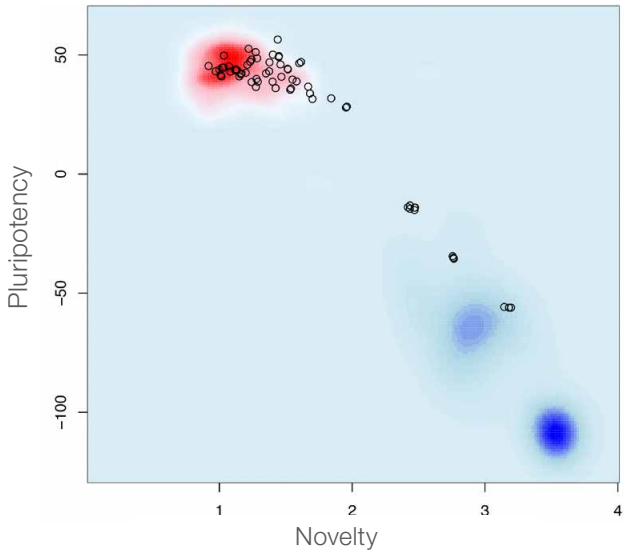


Figure 6.11. Output of PluriTest Online Analysis Tool showing clustering of pluripotent (red) and nonpluripotent (blue) samples.

6.4

Evaluating differentiation potential

Analyzing iPSCs and confirming the presence of self-renewal gene products or the absence of parental somatic gene products is important, but not sufficient, for verifying the functional pluripotency of a newly derived iPSC line. The other critical test is to confirm trilineage potential or the ability of the iPSCs to differentiate into cells of the three embryonic germ layers: ectoderm, mesoderm, and endoderm. This can be done *in vivo* through teratoma formation or, more commonly, through embryoid body (EB) formation in culture.

Teratoma formation involves injecting PSCs into mice and allowing them to proliferate and differentiate into the three lineages over 6–30 weeks, depending on the protocol. On the other hand, EB formation involves culturing PSC aggregates in suspension, in the absence of bFGF. These aggregates are allowed to spontaneously differentiate over 7–21 days and are typically transferred into adherent cultures after the first few days. Although the differentiation of EBs occurs under nonphysiological conditions, EB formation has advantages over teratoma formation because it takes much less time and is less laborious, and EBs are easier to analyze.

Cellular analysis

Common markers for analyzing differentiation in EBs include smooth muscle action (SMA) for mesoderm, α-fetoprotein (AFP) for endoderm, and β-III tubulin (TUBB3/TUJ1) for ectoderm (Figure 6.12) [6].

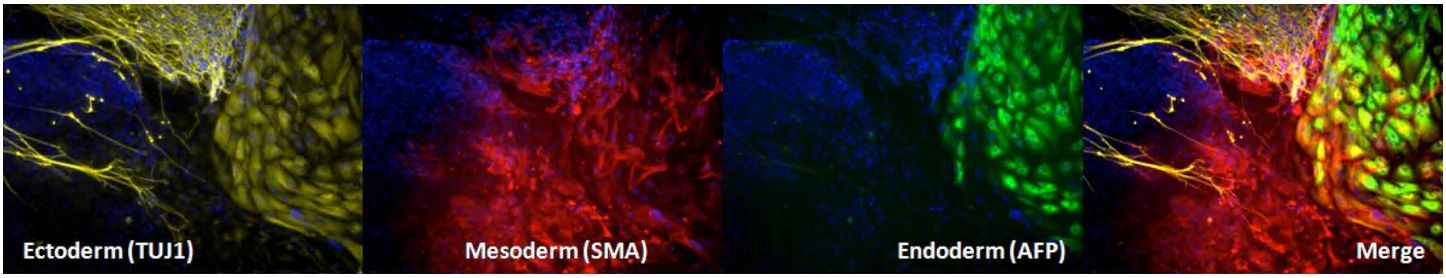


Figure 6.12. Cellular analysis of EBs. EBs from H9 ESCs were allowed to spontaneously differentiate for 23 days prior to staining β-III tubulin (TUJ1, yellow), smooth muscle actin (SMA, red), and α-fetoprotein (AFP, green) against a DAPI nuclear counterstain (blue).

Molecular analysis

Cellular analyses like immunostaining are low-throughput methods that are limited to the detection of markers for which antibodies are available. In contrast, molecular analyses may allow the quantitative analysis of many markers at one time, thereby complementing the cellular data. Such molecular analyses are best done using both undifferentiated and differentiated cells (Figure 6.13).

qPCR is currently the most popular method for molecular analysis, and predesigned Applied Biosystems™ TaqMan® assays offer ideal tools for rapid screening and analysis of gene expression.

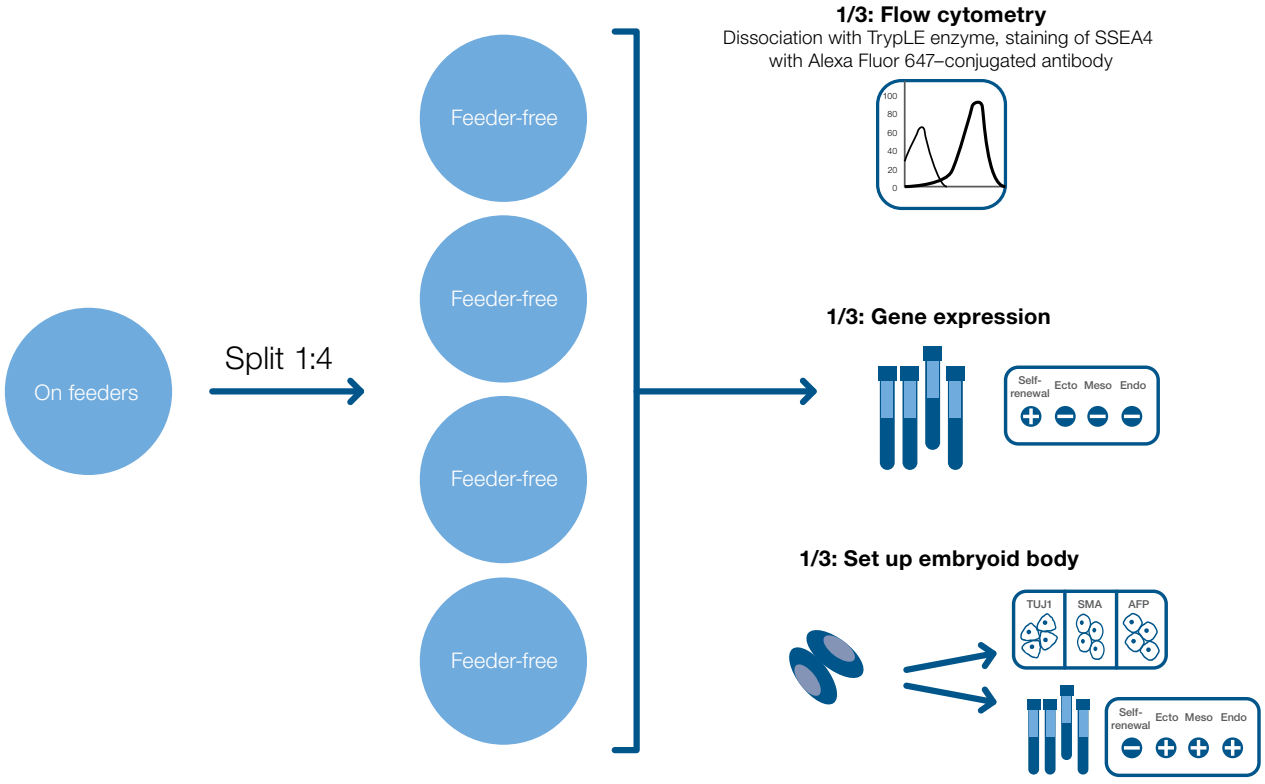
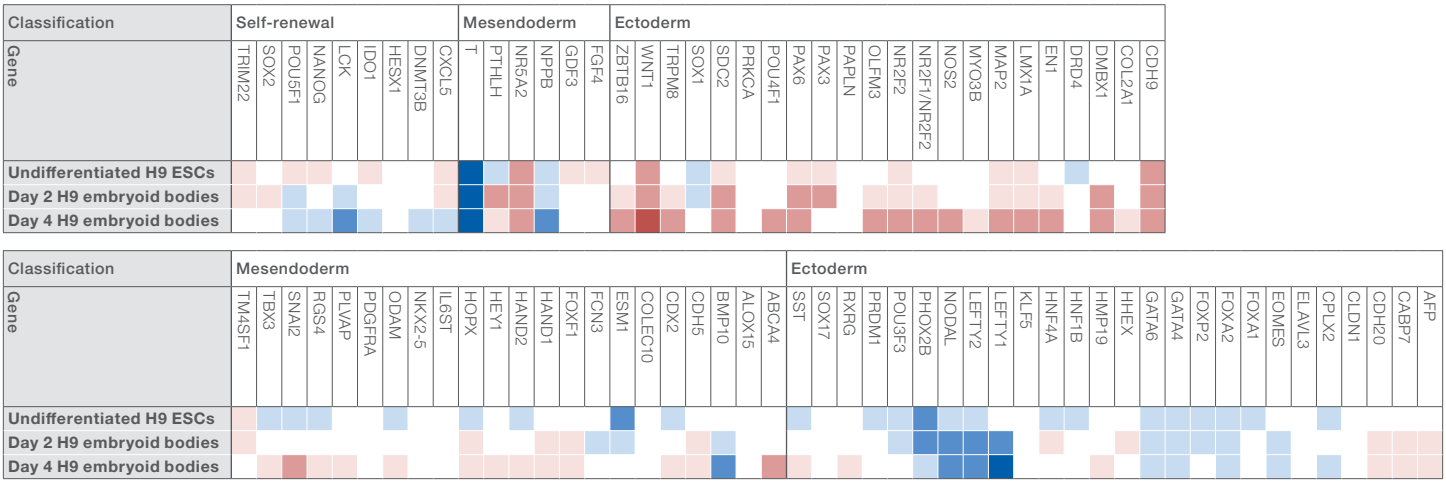


Figure 6.13. Generation of undifferentiated and differentiated PSCs for molecular analysis. When performing molecular analyses, it is best to simultaneously check the purity and quality of the PSCs, as well as confirm the differentiation of the EBs through flow cytometry. Scaling up to about four 6 cm dishes usually generates sufficient cells for both molecular and flow analyses.

The Applied Biosystems™ TaqMan® hPSC Scorecard™ Panel utilizes RT-qPCR, but offers a higher-throughput analysis by employing a panel of 93 gene expression assays, including 9 self-renewal genes, 74 germ layer–specific genes, 10 housekeeping genes, and even an assay to confirm clearance of the SeV backbone from iPSCs after reprogramming with the Invitrogen™ CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Figure 6.14). The assay utilizes bioinformatics analysis based on the work of Bock et al. [7].



Fold change (fc) legend

fc > 100	Upregulated
10 < fc ≤ 100	
2 < fc ≤ 10	
0.5 ≤ fc ≤ 2	Comparable
0.1 ≤ fc < 0.5	
0.01 ≤ fc <0.1	
fc < 0.01	Downregulated
Omitted	

Figure 6.14. TaqMan hPSC Scorecard analysis of an EB formation time course using H9 ESCs. 93 genes are analyzed as part of the TaqMan hPSC Scorecard Panel. Colors correlate to the fold change in expression relative to the reference set. Markers of the undifferentiated state are downregulated over the course of EB formation, shown by the blue shading. Markers of the three germ layers are upregulated over the course of EB formation, shown by the red shading.

The analysis software facilitates interpretation of the data by statistically comparing the gene expression profile to a reference set of well-characterized ESC and iPSC lines. The software then scores the expression of self-renewal genes and trilineage markers (Figure 6.15). As such, this permits not only the analysis of undifferentiated PSCs, but also the derivative EBs to determine functional pluripotency [8]. By providing a more comprehensive and sophisticated analysis of EBs rather than just the confirmation of a few differentiation markers via immunostaining, the TaqMan hPSC Scorecard assay enables a more reliable and consistent method for quantifying the differentiation potential of PSCs, and makes EB formation an increasingly attractive alternative to time-consuming and laborious teratoma formation assays.

To learn more about the TaqMan hPSC Scorecard assay and software, go to **thermofisher.com/scorecard**

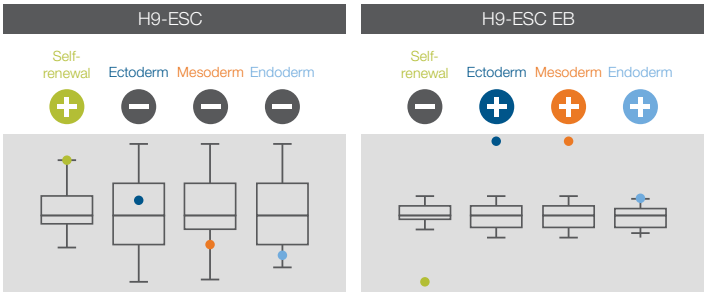


Figure 6.15. TaqMan hPSC Scorecard assay results. H9 ESCs and H9 ESC-derived EBs were analyzed using the TaqMan hPSC Scorecard assay. The comparison of self-renewal and germ-layer marker expression against the reference standards is summarized in box plots and in simple pass/fail scores.

Useful tips

Cellular analysis

- **Live AP staining**—AP is a differential marker that is expressed more robustly in undifferentiated cells relative to unreprogrammed parental fibroblast cells or fibroblasts in the feeder layer. The washing steps in the staining protocol are critical for observing the differential staining pattern. Note that AP is not an ideal method to distinguish between undifferentiated and early-differentiating cells.
- **Live-cell and fixed-cell immunostaining**—Feeder-free cultures need to be handled with caution while staining and washing. Excessive or harsh washes can dislodge the cells from the plate, leading to peeling away of the cells.
- **Flow cytometry**—It is recommended that cultures be harvested as single cells using TrypLE Express Enzyme prior to antibody staining. Alternatively, live monolayer feeder-free cultures can be stained with antibodies; after images are captured, cells can be harvested using TrypLE Express Enzyme for flow cytometry.
- **Evaluating differentiation potential**—Obtaining the right size of EBs is essential for achieving successful trilineage differentiation. Since feeder-dependent PSCs grow in thick three-dimensional colonies, while feeder-free PSCs grow in flatter colonies, it is important to use the appropriate harvesting procedures that will result in the optimal EB size.

Molecular analysis

- The TaqMan hPSC Scorecard Panel measures the potential for self-renewal and trilineage differentiation: **thermofisher.com/scorecard**
- Since PCR is a global analysis, it is critical to ensure the high quality of the undifferentiated cells and EBs at day 7 of differentiation. If the medium or differentiation method deviates from the proposed method, evaluation of a time course with 2–3 time points of differentiation (e.g., days 7, 10, and 14) is recommended.

- MEFs constitute a small percentage (~20%) of feeder-dependent PSC cultures. Although selective harvesting using collagenase reduces MEF carryover, and the gene panel is specific to human genes with minimal cross-reactivity with mouse cells, it is recommended that feeder-dependent cells be cultured for one passage on Geltrex matrix and MEF-conditioned medium to eliminate MEFs prior to analysis. If cells are cultured on human feeders, at least two passages are necessary to ensure complete removal of human feeders, which can alter the gene expression signature.
- One well of a 6-well dish, or about 5 x 10^5 cells, are recommended for the standard RNA extraction method using Invitrogen™ TRIzol™ Reagent. However, as few as 100,000 cells can be used with TRIzol Reagent, and as few as 15,000 cells can be used with the Invitrogen™ Cells-to-C_T™ Kit or the CellsDirect™ One-Step RT-qPCR Kit.
- When reprogramming, it is recommended that stable and homogeneous iPSC clones at passage 8 or higher be used for molecular analysis.
- Early-passage iPSC clones reprogrammed with SeV can be assessed for dilution of the virus by detecting the presence of SeV (the SeV backbone). Inclusion of parental somatic cells as a control to eliminate SeV cross-reactivity with the parental line is recommended. TaqMan hPSC Scorecard Panel results for the somatic primary cells would show a low self-renewal score, with one or more lineages showing high expression. Results that indicate the presence or absence of SeV in established iPSC clones do not have an impact on pluripotency.

6.5

References

1. Singh U, Quintanilla RH, Grecian S et al. (2012) Novel live alkaline phosphatase substrate for identification of pluripotent stem cells. *Stem Cell Rev* 8(3):1021–1029.
2. International Stem Cell I, Adewumi O, Aflatoonian B et al. (2007) Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nat Biotechnol* 25(7):803–816.
3. Quintanilla RH, Asprer JS, Vaz C et al. (2014) CD44 is a negative cell surface marker for pluripotent stem cell identification during human fibroblast reprogramming. *PLos One* 9(1):e85419.
4. Chan EM, Ratanasirintrawoot S, Park IH et al. (2009) Live cell imaging distinguishes bona fide human iPS cells from partially reprogrammed cells. *Nat Biotechnol* 27:1033–1037.
5. Müller FJ, Schuldt BM, Williams R et al. (2011) A bioinformatic assay for pluripotency in human cells. *Nat Methods* 8:315–317.
6. Quintanilla RH (2013) Cellular characterization of human pluripotent stem cells. *Methods Mol Biol* 997:179–190.
7. Bock C, Kiskinis E, Verstappen G et al. (2011) Reference Maps of human ES and iPS cell variation enable high-throughput characterization of pluripotent cell lines. *Cell* 144(3):439–452.
8. Fergus J, Quintanilla R, Lakshmipathy U (2014) Characterizing pluripotent stem cells using the TaqMan® hPSC Scorecard™ Panel. *Methods Mol Biol* 1307:25–37.

Section 7

Applications for cell therapy

7.1 Introduction

While it may seem that the promise of stem cells in cell replacement therapies has long been on the horizon, the first hESCs were only isolated in 1998 [1], and the first human iPSC was generated in 2007 [2]. In less than two decades, researchers have:

- Identified and streamlined conditions for maintenance and expansion of human PSCs
- Discovered an approach for generating PSCs from human somatic cells
- Optimized protocols for differentiation to clinically relevant cell types
- Developed tools for characterization of those cells
- Introduced human mesenchymal stem cell and hematopoietic stem cell therapies into clinical trials for different disease indications—both allogeneic and autologous
- Introduced and commercialized T cells as a new paradigm to treat cancer via CAR T cell therapy

The next step in moving cell therapies from the bench to the bedside is to translate this process to a clinically compatible system. This means that ancillary materials for clinical applications will need to be generated under GMP so that quality control measures are in place to ensure patient safety.

cGMP refers to the regulations established by the European Medicines Agency and the US Food and Drug Administration as a means of ensuring that products meet preset standards for quality and safety. These standards encompass both the manufacturing and testing of the final product. Key requirements include the traceability of raw materials and the adherence to validated standard operating procedures.

The use of animal origin–free components (e.g., media, substrates, and reagents used in the expansion, passaging, and cryopreservation of cells) significantly reduces the risk of inducing immune reactions against animal proteins and exposure to adventitious agents.

The most desirable materials are those that already have regulatory approvals. However, these are not always available and/or suitable. In those cases, additional testing may be needed to ensure the safety and quality of reagents.

Qualifying and validating reagents for use in cell therapy manufacturing can be a complicated and time-consuming process, requiring multiple levels of testing and documentation to support appropriate risk assessment of incoming materials. Qualifying reagents and raw materials begins with the following:

- Obtaining documentation to demonstrate traceability of components and potential contact with animal-sourced materials
- Establishing assays for the detection of adventitious agents, including bacterial and fungal contamination, mycoplasmas, endotoxins, and viral agents
- Incorporating procedures for inactivation or removal of infectious agents or toxic impurities when necessary
- Developing quality control and quality assurance systems to appropriately assess the risk of using incoming raw materials in the clinical manufacturing process

As the field matures, an increasing number of off-the-shelf prequalified reagents are becoming available that will make the task of qualifying materials easier for the teams responsible for ensuring quality and safety of cellular therapies. In fact, the field may soon develop a complete clinical-grade set of solutions that encompasses the full workflow from iPSC generation to final cell product.

Find solutions and support for your PSC therapy needs at **thermofisher.com/ctsstemcells**

7.2

Translating research into clinical evaluation

There has been immensely exciting potential demonstrated in the cell therapy field in a number of disease states, most notably the recent clinical successes in oncology and gene-modified CAR T therapy commercialization. The promise of cell therapy is bright, and developing technologies for raw materials, isolation, expansion, differentiation, cryopreservation, serum-free media, and large-scale manufacturing protocols will be the drivers to bring this promise to reality.

The following sections are intended to provide a broad overview of several considerations needed to translate a basic research program forward to clinical evaluation. Consulting with an expert in regulatory affairs, preferably with experience in cell therapy, is highly advisable as research is transitioned to the clinic.

Materials, manufacturing, and process considerations

The first step is to ensure current methods of isolating and expanding cells of interest are consistent and can support reproducible results in preclinical models. The selection of the model and protocol are critical because the data generated at this phase will support the investigational new drug (IND) application. Therefore, the raw materials used to isolate, expand, reprogram, engineer, or differentiate cells of interest are also critical because they will form the basis of the protocols that will get translated into cGMP processes.

Changing critical components, including cytokines, small molecules, serum, media, and culture systems that may affect the biology and phenotypes of therapeutic cells, is considered high-risk after the completion of preclinical evaluations. Discovering that a common material used on the bench presents a risk profile would require finding an alternative while filing your IND.

This discovery could significantly delay clincial programs and could even change the profile of the therapeutic cells and the clinical application. For these reasons, understanding the sourcing, quality, and risk profiles, and having as much information as possible about the components being used, are of paramount importance to generating preclinical data sets.

Once raw materials, which have been obtained with proper regulatory documentation, consistently generate cells that are characterized to match a profile correlated to a clinical benefit, you can begin to think about the manufacturing process and whether it has the potential to scale. Scalability will be critical for realizing the number of cells needed to treat a patient population. Selecting and developing a scalable manufacturing process is also critical for raw material selection, since cell phenotypes can also be affected by the culture systems used to generate them. For example, changing from planar culture to suspension culture can significantly impact cells. Finally, having characterization tools with the necessary sensitivity and resolution to detect process or raw material changes is a critical adjunct to the raw materials and culture system selection.

Preparing your regulatory submission

It is appropriate to begin assembling the components of an IND once raw materials with acceptable risk profiles have been selected, a process with line of sight to future scale-up has been developed, and characterization methods that ensure production of intended cells have been confirmed. The chemistry, manufacturing, and controls (CMC) section of the IND will describe everything needed to manufacture cells, including:

- The procedure used to obtain tissue or other cell sources and how it is transported to the lab or clinic
- The raw materials used during the process and how suitability is verified
- The plasticware and pipettes used
- Preparation of final cells for storage and delivery to the patient
- The sterility, mycoplasma, endotoxin, and final release testing used to characterize incoming materials and the final product

Other parts of the IND include the clinical protocol that will be followed to administer the cell therapy to the patient as well as the pharmacology and toxicology data developed to show cells are safe for administration.

Prior to gathering all of the information to prepare an IND, scheduling of at least one pre-IND meeting with the FDA is recommended, to advise them of clinical intent and to present current thoughts and intentions about moving forward. These pre-IND meetings are critical to prepare a program towards clinical evaluation.

Moving through the clinical phases

As preparation for clinical trials begins, quality control, quality assurance, regulatory affairs, process development, and manufacturing teams should be established to interact with the FDA or other regulatory agencies. These teams will oversee the implementation of quality systems, batch records, personnel training, change control systems, and all other aspects of cGMP needed to ensure that the final cell therapy products are safe and in compliance with regulations. Clinical trials progress from small patient number safety studies in phase 1, to slightly larger efficacy studies during phase 2, and on to pivotal phase 3 studies that will test the manufacturing systems intended to support commercial production and hopefully demonstrate clinical efficacy in large patient populations. During this progression, quality will be tightened and systems validated to demonstrate reproducible production at scale, and with an acceptable cost of goods, to provide for a reasonable profit margin and reimbursement profile to ensure commercial success.

7.3
Cell Therapy Systems products

The Gibco™ Cell Therapy Systems™ (CTS™) portfolio offers a broad array of high-quality products designed for use in clinical research applications, including media, reagents, growth factors, enzymes, selection beads, and devices, which are manufactured in compliance with 21 CFR Part 820 Quality System Regulation and/or are certified to ISO 13485 and/or ISO 9001. Regardless of the type and source of cells, the CTS portfolio offers tools to help with every step of the cell therapy workflow and enables progress through each stage of clinical development and scale-up (page 97).

CTS products are designed to help you translate your cell therapy to clinical applications with extensive safety testing and traceability documentation required for regulatory review, so you can transition your cell therapy to the clinic with confidence. CTS products are supplied with unified documentation, including:

- Access to authorization letters for the FDA Drug Master File
- Certificates of Analysis
- Certificates of Origin
- 9 CFR–compliant adventitious viral testing

See more information on cell therapy products at **thermofisher.com/celltherapy**

Pluripotent stem cell therapy workflow solutions

Isolate

Isolation
(T cells) CTS Dynabeads CD3/CD28

$CD34^+$ isolation beads

Media
(T cells) CTS OpTmizer medium

Dissociation/ passaging reagents
CTS TrypLE Select Enzyme

Reprogram

Viral
CTS CytoTune-iPS 2.1 Sendai Reprogramming Kit

Transfection
Lipofectamine Stem Transfection Reagent

Lipofectamine 3000 Transfection Reagent

Electroporation
Neon Transfection System

Culture

Media
CTS Essential 8 Medium

CTS KnockOut SR XenoFree Medium

CTS KnockOut DMEM

CTS KnockOut DMEM/F-12

CTS Synth-a-Freeze Medium

Custom media and services

Reagents and supplements
CTS RevitaCell Supplement

CTS PSC Cryopreservation Kit

CTS PSC Cryomedium

CTS Versene Solution

Matrices
CTS Vitronectin

rhLaminin-521

Engineer

Genome editing
TrueCut Cas9 Protein v2

TrueGuide Synthetic gRNAs

TrueDesign Genome Editor

TrueTag Donor DNA Kits

Electroporation
Neon Transfection System

Single-use technologies
BioProcess Containers (BPCs)

Transfer assemblies

Static bags

Rocker bags

Differentiate

Kits and supplements
PSC Cardiomyocyte Differentiation Kit

PSC Dopaminergic Neuron Differentiation Kit

PSC Definitive Endoderm Induction Kit

CTS N-2 Supplement

CultureOne Supplement

Media
CTS Essential 6 Medium

CTS Neurobasal Medium

CTS Neurobasal-A Medium

Wash, fill, finish, and cryopreserve

Wash
CTS DPBS

Cryopreservation medium
CTS Synth-a-Freeze Medium

Cryogenic storage and logistics
Banking services

Cold-chain logistics solutions

Transition your cell therapy to the clinic with confidence

As you move from basic cell therapy research to the clinic, high-quality GMP-manufactured cell therapy ancillary materials and proper documentation are essential to getting it right the first time. CTS products, services, and support provide a proven choice for clinical stem cell therapy and manufacturing.

CTS products have cell therapy–specific and gene therapy–specific intended use statements, extensive safety testing, and proactive regulatory documentation. We take these measures to provide quality ancillary materials to help minimize risk, ease the burden on your quality systems, and support your regulatory submission—making CTS products the superior choice as you transition from the bench to the clinic.

Discover
- Gibco™ media and reagents
- Cell engineering solutions
- Broad characterization portfolio

Translate
- CTS media and reagents
- Custom media and process development services
- Clinical trial support

Commercialize
- Scale-up and scale-out solutions
- cGMP-manufactured ancillary materials
- Global cryogenic logistics services

GMP manufacturing

- Ancillary materials are manufactured in conformity with cGMP for medical devices, 21 CFR Part 820, USP<1043>, and Ph Eur 5.2.12
- Our ancillary material manufacturing sites are FDA-registered and ISO 13485– and ISO 9001–certified

Testing and regulatory

- Traceability documentation—including Drug Master Files and/or Regulatory Support Files, and Certificates of Origin
- Extensive safety testing—including sterility, endotoxin, and mycoplasma

Expert support

- Knowledgeable regulatory support team will provide comprehensive regulatory product support from research through commercialization
- Experienced cell therapy professionals available to help answer your questions

7.4

Custom services and scalability

As process development defines manufacturing systems and operating parameters needed to produce a consistent product at a phase-appropriate scale, customized containers or media may allow for optimization. Filling media into bags with appropriate connectors that facilitate manufacturing and help close the system to potential contaminants can lead to more robust processes. In some instances, slight adjustments to media and feed systems can also help the process. Gibco media can be produced in formats that meet process and scale-up requirements through preparation and progress of clinical evaluations.

If you are interested in custom CTS services, please send an inquiry to **custommedia@thermofisher.com**

Large-scale cGMP custom media

For large-scale clinical or commercial biomanufacturing applications, rely on our validated cGMP custom services.

- Liquid in batches from 10 to 10,000 L
- Dry powder media (DPM) in batches from 1 to 8,000 kg
- Advanced Granulation Technology™ (AGT™) media in batches from 50 to 6,000 kg

Custom packaging options

Receive your Gibco™ custom media in the packaging that best suits your needs. We have many different options for liquid and powder media in a variety of package sizes—available in both bottles and bags—to manage small-, intermediate-, and large-scale needs.

cGMP manufacturing sites

We maintain two primary Gibco™ cell culture manufacturing locations—in the US and Scotland—and three primary Gibco™ serum and/or protein product manufacturing locations—in the US, New Zealand, and Australia. For reliable global service and contingency planning, we welcome visits and audits of our facilities to help oversee regulatory approvals of your products and services.

Process development custom services

Choose the Gibco™ Custom Services team to help reduce process development inefficiencies, and improve time and cost performance using our latest technologies.

MediaExpress and Rapid Research services

Gibco™ MediaExpress™ and Rapid Research services are specifically designed for small-scale, non-cGMP custom orders when speed matters most. We offer Gibco product quality in small batches for quick turnaround and smooth transitions to cGMP scale-up.

7.5

References

1. Thomson JA, Marshall VS (1998) Primate embryonic stem cells. *Curr Top Dev Biol* 38:133–165.
2. Takahashi K, Tanabe K, Ohnuki M, et al. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131(5):861–872.

Section 8

CellModel Services

8.1 Introduction

Built on the stem cell innovations introduced throughout the past decade, Invitrogen™ CellModel™ Services enable stem cell scientists to reach their desired outcomes faster. These services offer stem cell researchers choices at every stage of their research, including innovative tools that make stem cell projects easier to bring in-house and a custom services offering that utilizes an experienced team of stem cell professionals.

Advantages of CellModel Services include:

- **Expertise**—team of stem cell specialists to deliver desired results
- **Full transparency**—detailed protocols provided after project completion to demonstrate how each milestone was achieved and to document which tools were utilized
- **High-quality products**—all high-quality reagents and media used within the CellModel Services can be purchased and used to facilitate post-service projects
- **Exceptional support**—experienced custom service project manager provides exceptional support and frequent communication

Available stem cell services

Choose the combination of tools and services to meet research needs:

- **Reprogramming**—human fibroblasts or blood cells reprogrammed in 4–6 months with top clones expanded, cryopreserved, and characterized
- **Engineering**—genetic modification of PSCs to create models with disease-relevant mutations
- **Differentiation**—PSCs differentiated into NSCs and many other terminal lineages in as little as 3 to 6 weeks
- **Characterization**—confirm pluripotency, karotype, gene expression, cell identity, and more
- **Assay development**—develop customized assays to interrogate your disease-relevant model
- **Screening**—compound screening for your stem cell–based discovery projects, utilizing our complete high-throughput screening capabilities

Experienced service providers

CellModel Services are delivered by scientists from the Custom Biology team, which has helped researchers with cellular engineering and custom assay development projects for over a decade. Additionally, the Discovery Services project management team—responsible for the management of hundreds of cell line generation and assay development projects—is uniquely suited to manage project milestones and proactively communicate updates from day one to project completion.

Find out more at **thermofisher.com/cellmodel**

We offer choices at every stage of the stem cell workflow.
Choose the services that best fit your research needs.

Note: We are continually expanding our service capabilities and offerings. Please reach out to your local sales specialist to see how we can help achieve your project goals.

8.2

Case study: Engineered iPSCs as hypoimmunogenic cell grafts for universal transplant

Research goal: To generate hypoimmunogenic iPSCs through the inactivation of MHC Class I and II genes and overexpression of CD47 for use in a downstream transplant study.

The researcher first conducted this study with mouse iPSCs and later with human iPSCs.

- Mouse iPSCs (customer supplied)
- Human iPSCs (Gibco™ Cas9 iPSCs)

Alternatively, the Custom Biology team can also reprogram supplied somatic cells as part of the Reprogramming Service.

The Custom Biology team then generated hypoimmunogenic iPSC models by performing the following:

- Knockout of MHC Class I and II genes (*B2M* and *CIITA*) using CRISPR-Cas9
- Overexpression of CD47 gene using viral transgenic approach

The engineered cell lines were characterized before delivery: normal karyotype (KaryoStat service) and pluripotency (TaqMan hPSC Scorecard service).

Using the engineered cell lines, the researcher differentiated them into endothelial-like cells and cardiomyocytes for downstream *in vivo* study.

Alternatively, the Custom Biology team can also perform the differentiation using our proprietary Gibco™ differentiation kits and reagents as part of the Differentiation Service.

The differentiated cells were later used as cell grafts in transplant studies and evaluated for survival and structural change in allogeneic recipients.

Results: From this study, the researcher was able to show that engineered cell grafts successfully evaded immune rejection and survived long-term without the use of immunosuppression in mismatched allogeneic recipients (Figure 8.1).

This result further demonstrated the Custom Biology team's ability to generate engineered cell lines to specifications and enable the researcher's project goal.

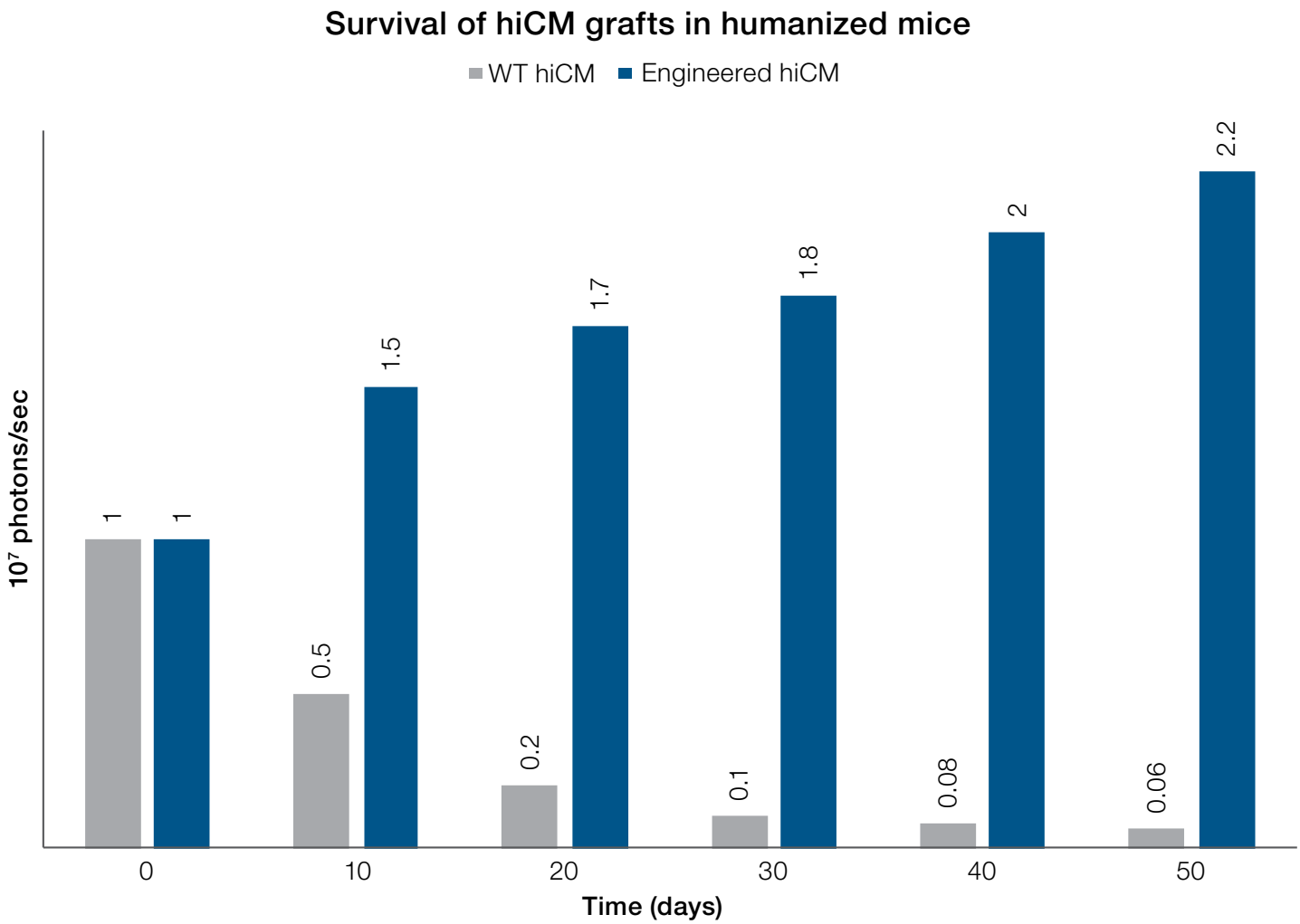


Figure 8.1. Long-term survival of hypoimmunogenic cell grafts observed in allogeneic model.

Section 9

Gibco PSC training courses

9.1

Introduction

Whether you are new to pluripotent stem cell research or need a refresher course, our digital courses provide detailed, step-by-step training so you feel confident using stem cells in your research.

Developed by our stem cell experts, the self-guided digital courses are structured to virtually guide you through a variety of stem cell techniques. Each course includes written instruction, how-to videos, and interactive quizzes to test your knowledge.

All courses can be accessed for free at **thermofisher.com/psctraining**

We have also developed virtual training labs for your convenience.

The PSC Culture Virtual Lab is an introduction to stem cell culture covering many topics, including:

- Coating wells to culture PSCs
- Preparing new cultures of PSCs
- Follow-up culture and characterizing pluripotency

Access the PSC culture lab and others at **thermofisher.com/pscvirtuallab**

Section 10

Ordering information

Product	Cat. No.
Reprogramming	
CTS CytoTune-iPS 2.1 Sendai Reprogramming Kit	A34546
CytoTune EmGFP Sendai Fluorescence Reporter	A16519
CytoTune-iPS 2.0 Sendai Reprogramming Kit	A16517
Epi5 Episomal iPSC Reprogramming Kit	A15960
Episomal iPSC Reprogramming Vectors	A14703
Culture	
Attachment Factor Protein	S006100
bFGF Recombinant Human Protein	13256-029
CF1 Mouse Embryonic Fibroblasts, irradiated (2 million cells)	A34180
CF1 Mouse Embryonic Fibroblasts, irradiated (4 million cells)	A34181
Collagenase, Type IV	17104-019
CTS Essential 8 Medium	A2656101
Dispase II	17105-041
DMEM/F-12, GlutaMAX supplement	10565-018
DPBS, calcium, magnesium	14040
DPBS, no calcium, no magnesium	14190
Essential 6 Medium	A1516401
Essential 8 Adaptation Kit	A25935
Essential 8 Flex Medium Kit	A2858501
Essential 8 Medium	A1517001
Geltrex hESC-Qualified, Ready-to-Use, Reduced Growth Factor Basement Membrane Matrix	A15696-01
Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix	A1413301
Human Episomal iPSC Line	A18945
KnockOut Serum Replacement	10828-028
Mouse (ICR) Inactivated Embryonic Fibroblasts	A24903
PSC Cryopreservation Kit	A26446-01
Recovery Cell Culture Freezing Medium	12648-010
RevitaCell Supplement	A2644501
rhLaminin-521	A29248
StemFlex Medium	A3349401
StemPro Accutase Cell Dissociation Reagent	A1110501
StemPro EZPassage Disposable Stem Cell Passaging Tool	23181-010
StemPro hESC SFM	A1000701
StemScale PSC Suspension Medium	A4965001
Synth-a-Freeze Cryopreservation Medium	A12542-01
TrypLE Select Enzyme, no phenol red	A1217701
UltraPure 0.5 M EDTA, pH 8	15575-020
Versene Solution	15040-066
Vitronectin (VTN-N) Recombinant Human Protein, Truncated	A14700

Product	Cat. No.
Transfection	
Lipofectamine 3000 Transfection Reagent	L3000015
Lipofectamine CRISPRMAX Cas9 Transfection Reagent	CMAX00015
Lipofectamine MessengerMAX Transfection Reagent	LMRNA015
Lipofectamine Stem Transfection Reagent	STEM00001
Neon Transfection System	MPK5000
Genome editing	
GeneArt CRISPR Nuclease mRNA	A29378
GeneArt CRISPR Nuclease Vector with CD4 Enrichment Kit	A21175
Genomic Cleavage Detection Kit	A24372
Ion PGM Sequencer	Contact geneartsupport@thermofisher.com
LentiArray Cas9 Lentivirus, 1 x 10^7 TU/mL	A32064
LentiArray CRISPR Positive Control Lentivirus, human HPRT, 1 x 10^7 TU/mL	A32056
LentiArray CRISPR Positive Control Lentivirus, human HPRT, with GFP, 1 x 10^7 TU/mL	A32060
LentiArray CRISPR Negative Control Lentivirus, human non-targeting, 1 x 10^7 TU/mL	A32062
LentiArray CRISPR Negative Control Lentivirus, human non-targeting, with GFP, 1 x 10^7 TU/mL	A32063
LentiArray CRISPR Negative Control Lentivirus, human non-targeting, 1 x 10^7 TU/mL	A32327
LentiArray Lentiviral sgRNA, 1 x 10^6 TU/mL	A32042
TaqMan SNP Genotyping Assay	Contact geneartsupport@thermofisher.com
TrueCut Cas9 Protein v2 (1 mg/mL)	A36497
TrueGuide sgRNA Negative Control, non-targeting 1	A35526
TrueGuide sgRNA Positive Control, AAVS1 (human)	A35522
TrueGuide sgRNA Positive Control, CDK4 (human)	A35523
TrueGuide sgRNA Positive Control, HPRT1 (Human)	A35524
TrueGuide sgRNA Positive Control, Rosa26 (mouse)	A35525

Product	Cat. No.
Differentiation	
B-27 Plus Neuronal Culture System	A3653401
B-27 Plus Supplement (50X)	A3582801
B-27 Supplement (50X), serum free	17504044
CultureOne Supplement	A3320201
Neurobasal Medium	21103049
Neurobasal Plus Medium	A3582901
PSC Cardiomyocyte Differentiation Kit	A2921201
PSC Cardiomyocyte Maintenance Medium	A2920801
PSC Definitive Endoderm Induction Kit	A3062601
PSC Dopaminergic Neuron Differentiation Kit	A3147701
PSC Neural Induction Medium	A1647801
StemPro NSC SFM	A1050901
Characterization	
Alkaline Phosphatase Live Stain	A14353
Alpha-Fetoprotein Monoclonal Antibody (mouse IgG1), eBioscience	14-6583-80
Alpha-Smooth Muscle Actin Monoclonal Antibody (mouse IgG2a)	MA5-11547
Beta-III Tubulin Monoclonal Antibody (mouse IgG2a)	MA1-118
CD44 Alexa Fluor 488 Conjugate Kit for Live Cell Imaging	A25528
CellsDirect One-Step RT-qPCR Kit	11753-100
EVOS M7000 Imaging System	AMF7000
Fluo-4 Calcium Imaging Kit	F10489
FluoroBrite DMEM	A1896701
GeneChip PrimeView Global Gene Expression Profile Assay	905400
Goat Anti–Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 594 Conjugate	A-11005
Human Cardiomyocyte Immunocytochemistry Kit	A25973
Human Neural Stem Cell Immunocytochemistry Kit	A24354
KaryoStat Assay	905403
KaryoStat HD Assay	905404
Pluripotent Stem Cell 4-Marker Immunocytochemistry Kit	A24881
Pluripotent Stem Cell Immunocytochemistry Kit (Oct4, SSEA4)	A25526
PrimeView 16 Global Gene Expression Profile Assay	905402
SSEA-4 Monoclonal Antibody, Alexa Fluor 647 Conjugate	SSEA421

Product	Cat. No.
Characterization (continued)	
TaqMan Gene Expression Cells-to-C_T Kit	4399002
TaqMan hPSC Scorecard Kit, Fast 96-well	A15871
TaqMan hPSC Scorecard Panel, 384-well	A15870
TaqMan hPSC Scorecard Panel, Fast 96-well	A15876
TaqMan Array Human Stem Cell Pluripotency Panel	4385344
TRA-1-60 Alexa Fluor 488 Conjugate Kit for Live Cell Imaging	A25618
TRA-1-60 Alexa Fluor 555 Conjugate Kit for Live Cell Imaging	A24879
TRA-1-60 Alexa Fluor 594 Conjugate Kit for Live Cell Imaging	A24882
TRA-1-60 Monoclonal Antibody	41-1000
TRIzol Reagent	15596018
Cell therapy	
CTS CELLstart Substrate	A1014201
CTS CytoTune-iPS 2.1 Sendai Reprogramming Kit	A34546
CTS DPBS, without calcium chloride, without magnesium chloride	A12856
CTS Essential 6 Medium	A4238501
CTS Essential 8 Medium	A2656101
CTS GlutaMAX-I Supplement	A1286001
CTS Immune Cell Serum Replacement	A2596-01
CTS KnockOut DMEM	A1286101
CTS KnockOut DMEM/F-12	A13708-01
CTS KnockOut SR XenoFree Medium	12618-012
CTS PSC Cryomedium	A4238801
CTS PSC Cryopreservation Kit	A4239301
CTS RevitaCell Supplement	A4238401
CTS StemPro MSC SFM	A1033201
CTS Synth-a-Freeze Medium	A1371301
CTS TrypLE Select Enzyme	A1285901
CTS Versene Solution	A4239101
CTS Vitronectin (VTN-N) Recombinant Human Protein	A27940

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Find out more at **thermofisher.com/stemcells**

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