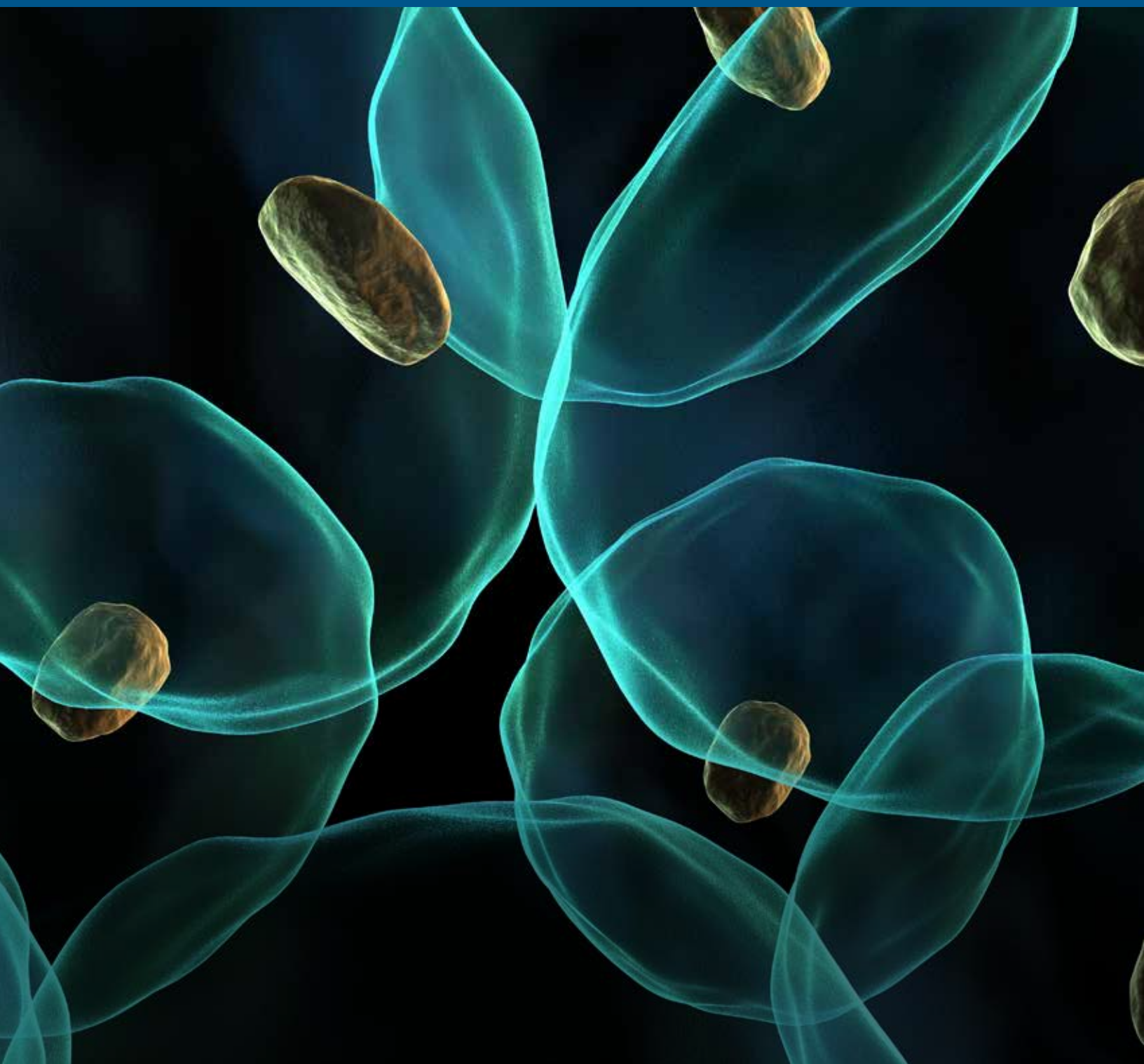


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Pluripotent Stem Cell Handbook

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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 at the University of Wisconsin, Madison¹. 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 in Kyoto, Japan². 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.

Below are the major areas in which PSCs and their derivatives have many potential applications:

- 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

The applications mentioned above 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.

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.



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).

To find the best solution for your reprogramming experiment, go to thermofisher.com/reprogramming

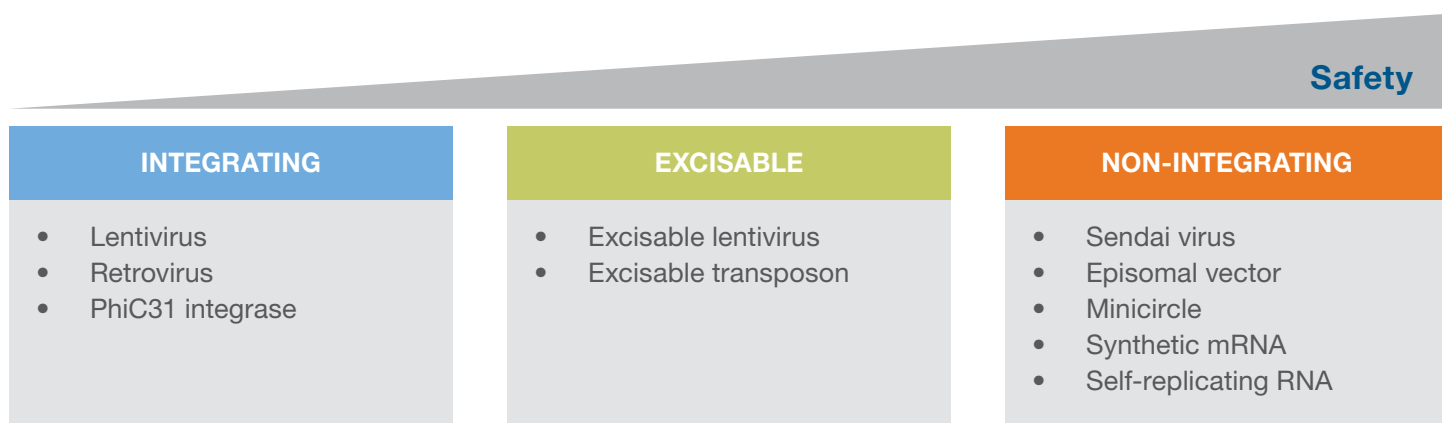


Figure 1.1. Safety and efficiency of various reprogramming technologies. Different reprogramming agents are classified as integrating, excisable or non-integrating technologies, which exhibit increasing levels of safety. Under each category, technologies are listed in order of decreasing efficiency.

Traditional reprogramming technologies using lentivirus or retrovirus involve the 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 non-integrating technologies because they avoid the issue of insertional mutations and generate footprint-free

PSCs that do not contain detectable vectors or transgenes.

Two common non-integrating reprogramming technologies make use of episomal vectors and Sendai virus (SeV). These two technologies are discussed in more detail in this section. Other non-integrating 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 features 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. These features are compared between the Invitrogen™ Epi5™ Reprogramming Kit and Gibco™

CytoTune™-iPS Sendai and CytoTune™-iPS 2.0 Sendai reprogramming kits in Table 1.1. Generally, CytoTune reprogramming kits are 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 Reprogramming Kit	CytoTune and CytoTune 2.0 -iPS Sendai Reprogramming Kits
Description	Virus-free non-integrating episomal DNA vectors	Non-integrating 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 reprogramming; Lipofectamine 3000 can be used with fibroblasts	None
Reprogramming factors	Oct4, Sox2, Nanog, Lin28, Klf4, and L-Myc	Oct4, Sox2, Klf4, c-Myc
Kit format	2 tubes with 20 µl each: 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	CytoTune Kit 4 tubes with 100 µl each: CytoTune Sendai hOct3/4 CytoTune Sendai hSox2 CytoTune Sendai hKlf4 CytoTune Sendai hc-Myc CytoTune 2.0 Kit 3 tubes with 100 µl each: CytoTune 2.0 KOS (containing Klf4, Oct3/4, and Sox2) CytoTune 2.0 hc-Myc CytoTune 2.0 hKlf4
Transfection/ 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 early passage (<P6) with normal growth and karyotype
- Density of seeded fibroblasts prior to initiation of reprogramming is critical to achieve good reprogramming efficiencies. A confluence of 50–80% is recommended on the day of transfection or transduction
- Protocols describe reprogramming in 6-well formats. Protocol can be scaled down to a 12-well or 24-well culture dish, albeit with a potentially reduced efficiency
- Besides fibroblasts, a variety of somatic cells can be used for reprogramming. The Gibco™ CytoTune™-iPS 2.0 Sendai Reprogramming Kit has been validated for a wide variety of cell types, including human fibroblasts, CD34+ cord blood cells, and peripheral blood mononuclear cells. Protocols for reprogramming these cell types can be found at [thermofisher.com/pscprotocols](https://www.thermofisher.com/pscprotocols)
- The Epi5 and CytoTune reprogramming systems are validated for human cells. EBNA/oriP 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, visit [thermofisher.com/sendapubs](https://www.thermofisher.com/sendapubs)
- 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 with Gibco™ KnockOut™ Serum Replacement-based media for feeder-dependent systems and Gibco™ Essential 8™ Medium for feeder-free reprogramming at [thermofisher.com/reprogramprotocols](https://www.thermofisher.com/reprogramprotocols)
- CytoTune 2.0 reprogramming can be optimized for maximal reprogramming efficiency by varying the amount of Klf4. Typically, the multiplicity of infection for the KOS and c-Myc vectors are maintained at a 1:1 ratio, with the Klf4 vector varied independently. The standard 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, it is recommended to test different seeding densities using at least two or three different multiplicity of infection values (e.g., 1, 3, and 9) of 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, it is not recommended to try and use the CytoTune-iPS 2.0 Sendai Reprogramming Kit with cells already transduced with the CytoTune EmGFP Sendai Fluorescence Reporter or vice versa

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 transfection¹. 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 manipulation². 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 shRNA systems⁵. 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.

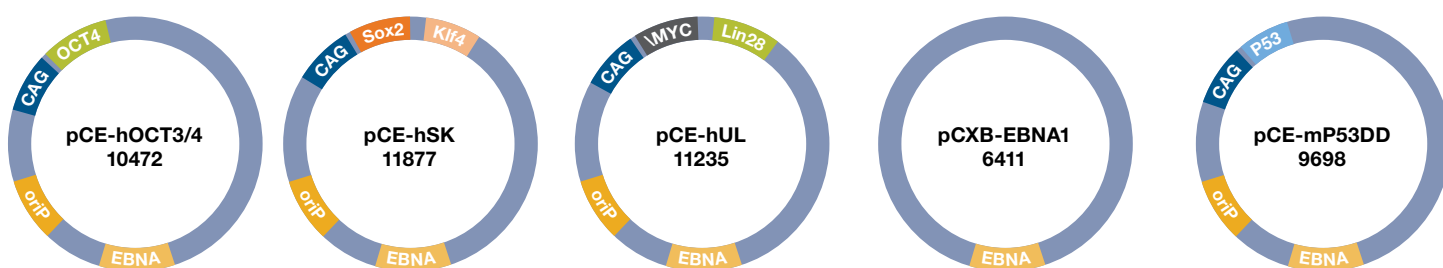


Figure 1.2. Configuration of vectors in the Epi5 Reprogramming Kit.

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.

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.

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 cell⁷⁻⁹. 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

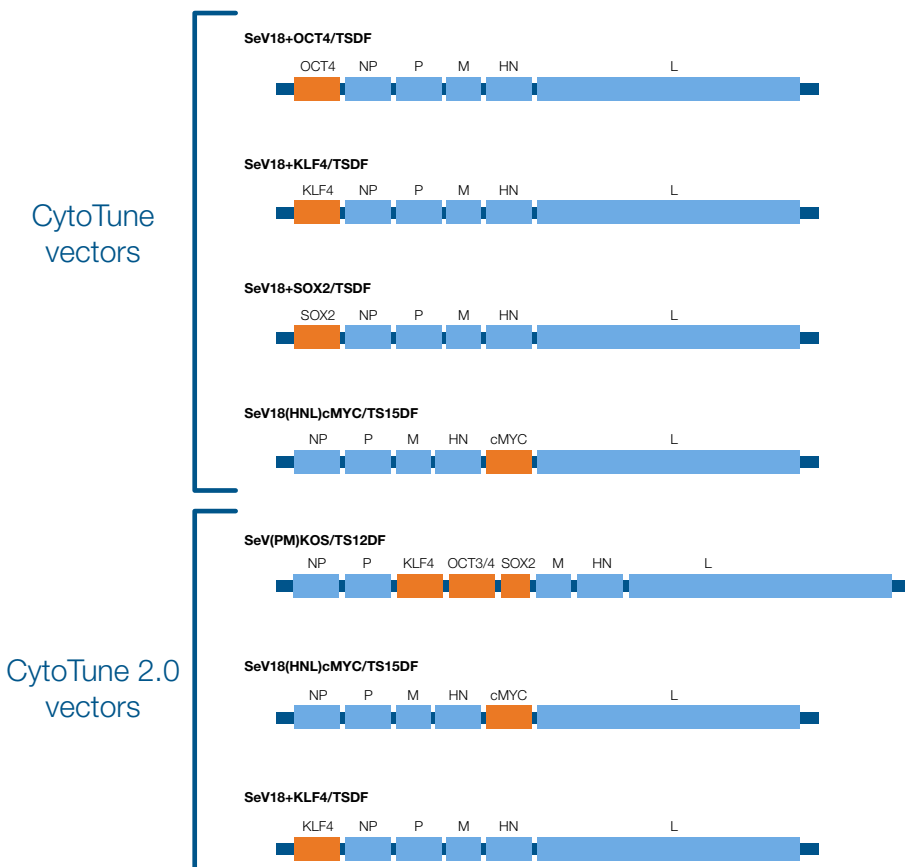


Figure 1.3. Configuration of vectors in the CytoTune Reprogramming Kits.

in SeV proteins (SeV/TS Δ F and SeV/TS15 Δ F), enables safe and effective delivery and expression of reprogramming genes⁷⁻¹⁰. 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 Reprogramming Kit contains four SeV-based reprogramming vectors, each capable of expressing one of the

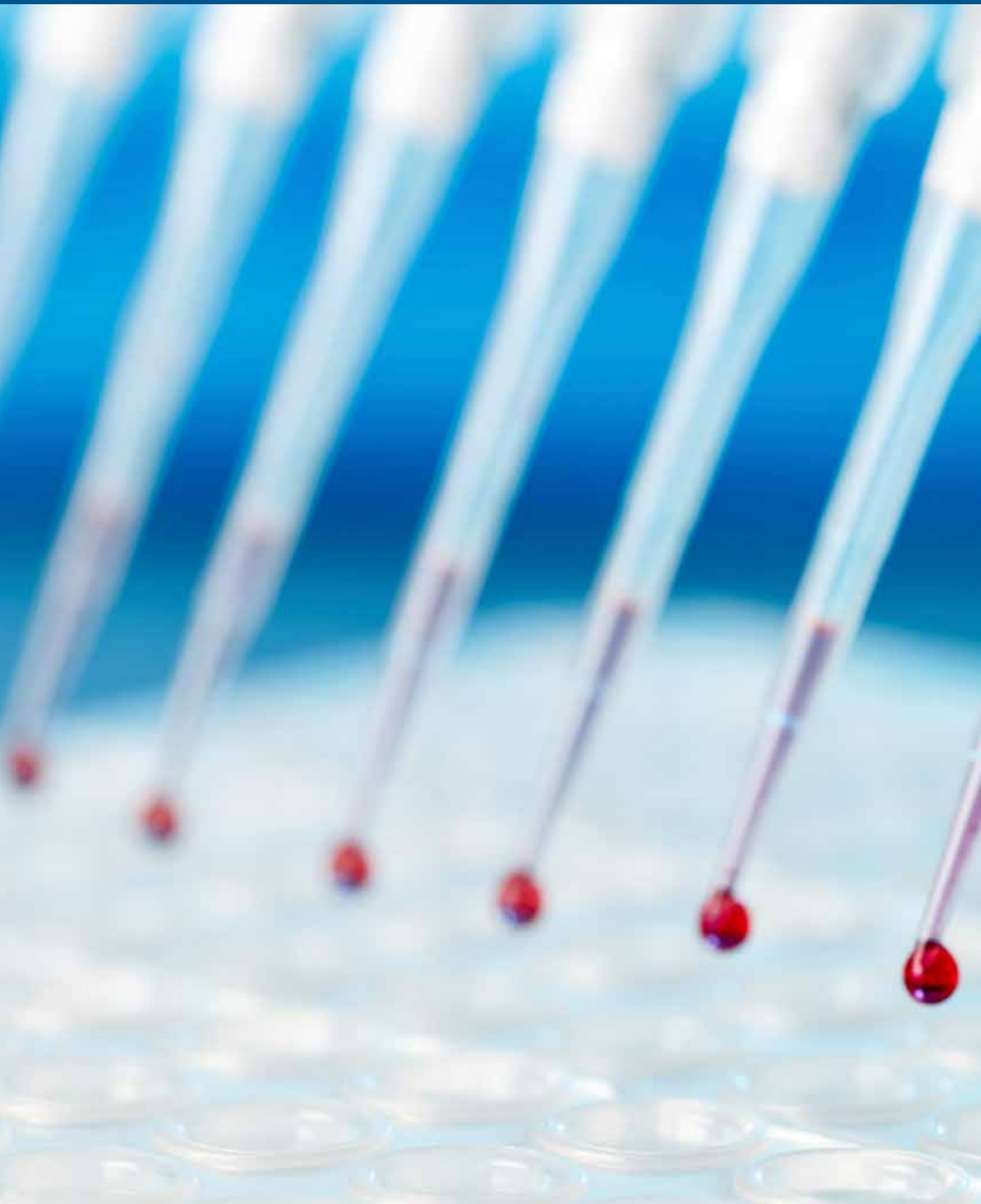
four Yamanaka factors (i.e., Oct4, Sox2, Klf4, and c-Myc) (Figure 1.3). The more recent CytoTune-iPS 2.0 Sendai Reprogramming Kit contains only three vectors, the first one combining Oct4, Sox2, and Klf4; the second one containing c-Myc; and the third one contributing additional Klf4 and RNA polymerase to achieve higher reprogramming efficiency (Figure 1.3). The key differences between the two kits are highlighted in Table 1.2.

Table 1.2. Comparison of reprogramming kits.

	CytoTune kit	CytoTune 2.0 kit
Efficiency	+	+++
Cytotoxicity	++	+
Viral clearance (safety)	~P10	~P3

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

Engineering

Introduction

Broadly, gene engineering or genome editing involves the changing of 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 engineering approaches supports a diverse range of applications (Figures 2.1 and 2.2) 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 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

By systematically targeting different loci in a polygenic disease model, the combination of hPSCs and gene editing can be an exquisitely precise tool to isolate and investigate the contributions of different disease-associated genes to disease pathology.

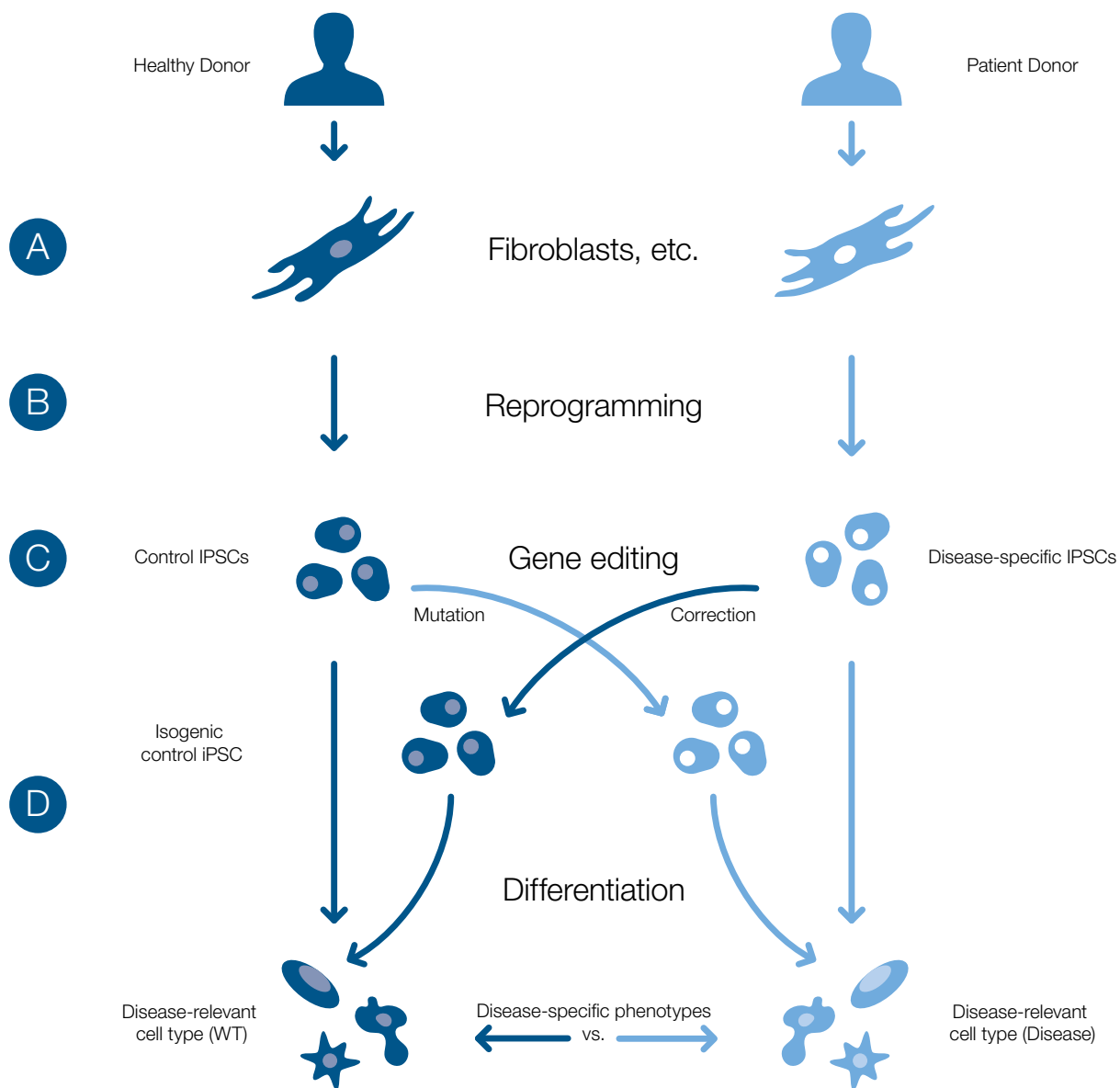


Figure 2.1. Generation of disease-specific and isogenic control (wild type - WT) iPSCs and disease-relevant cell types using gene engineering. Somatic cells such as fibroblasts or blood cells are isolated from healthy or patient donors (**A**) and reprogrammed to generate control and disease-specific iPSCs (**B**). Gene 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 (**C**). 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 (**D**).

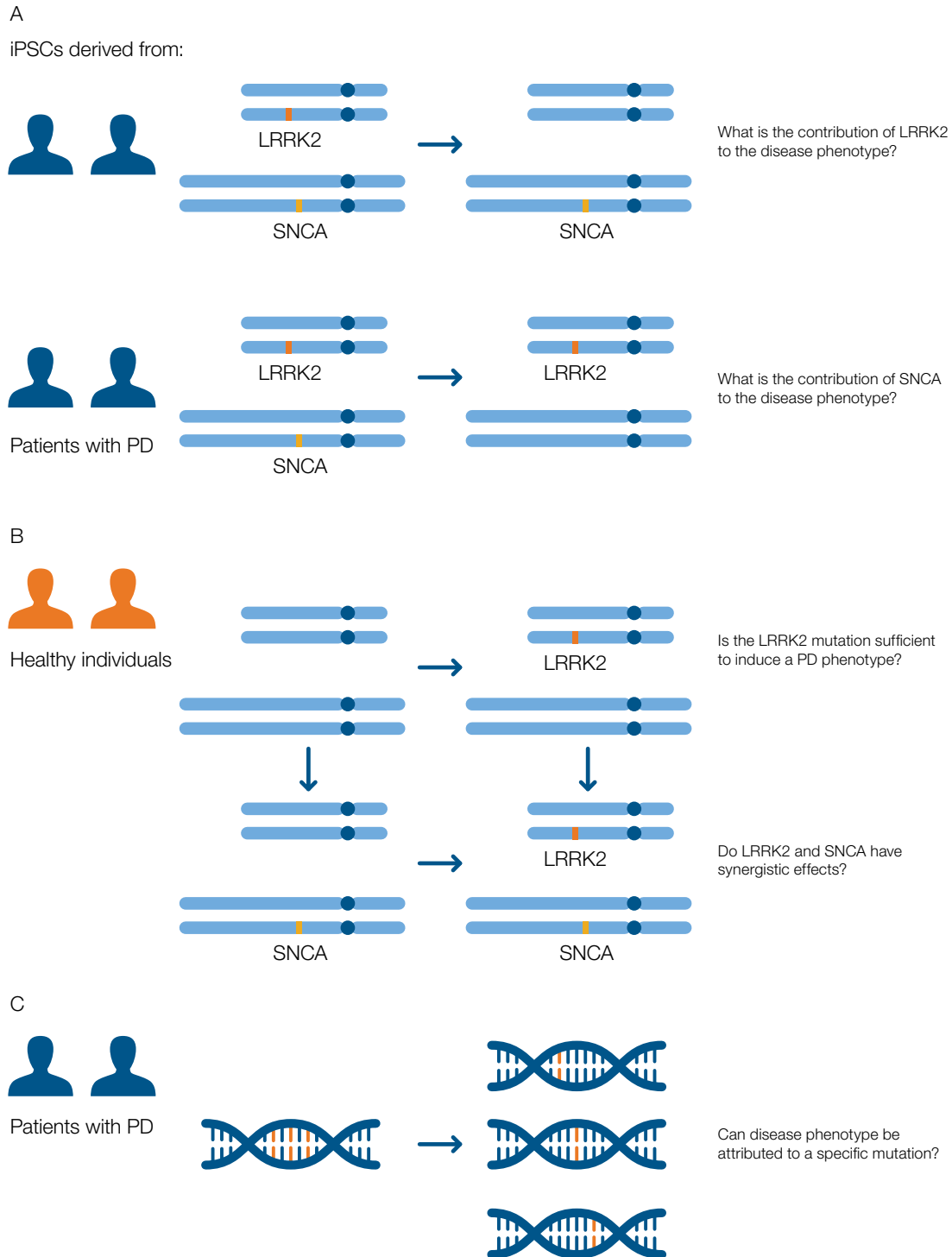


Figure 2.2. Using Parkinson's disease (PD) specific iPSCs to demonstrate how disease models can be used to isolate the effects of individual loci in complex polygenic disorders. PD is associated with mutations in both leucine-rich repeat kinase 2 (LRRK2;red) as well as mutations and polymorphisms of alpha-synuclein (SNCA;orange). **(A)** To determine the contribution of individual disease-specific mutations to disease phenotype, one loci or the other can be selectively corrected in patient-derived iPSCs carrying both mutations. **(B)** To determine whether one mutation is sufficient to induce a disease phenotype, or whether there are synergistic effects, the mutations can be introduced, singly or in combination, in healthy control iPSCs. **(C)** The roles of multiple mutations within a single gene can be deconvoluted by individually introducing distinct mutations using gene engineering technologies.

2.2

Gene engineering tools

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 guide RNA (gRNA) and TAL effectors target nucleases to specific sites in the genome, creating double-strand breaks at desired locations.

The natural repair mechanisms of the cell heal the break by either homologous recombination or non-homologous 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. However, 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 non-functional gene, this approach can be harnessed to rapidly and efficiently generate specific gene knockouts (Figure 2.3).

While until recently, Invitrogen™ GeneArt™ CRISPR gene editing technologies were touted as more efficient but also more prone to off-target effects compared to TAL technologies, recent advances in the tools and reagents available for both gene editing systems have negated some of these differences. A highlight of the benefits and limitations of the two technologies can be found in Table 2.1.

Nuclease-mediated genome editing

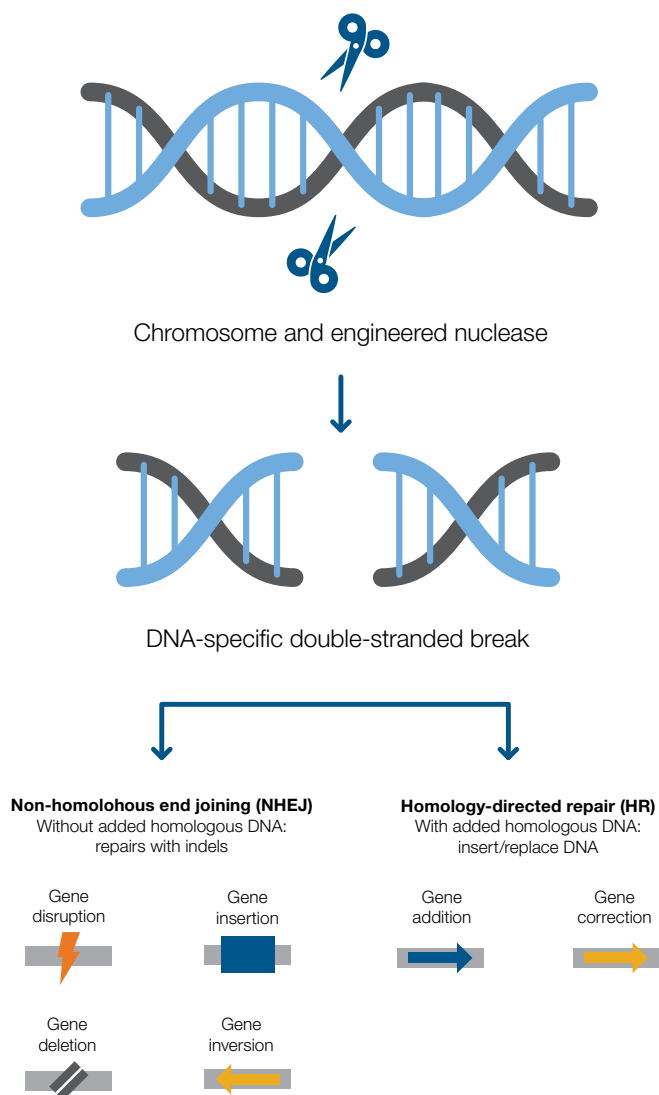


Figure 2.3. Sequence-specific nucleases are used to generate gene-specific double-strand breaks at a locus of interest.

The subsequent repair mechanism via homologous recombination or NHEJ can be harnessed to introduce a variety of stable genetic changes in the host genome.

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GeneArt engineering cell models

Through a partnership with Horizon Discovery, access the largest collection of engineering isogenic cell lines with the latest genome editing tools including CRISPRs, zinc-finger nucleases (ZFNs), and recombinant adeno-associated virus (rAAV) editing for creating both knockout and knock-in models. This expansive collection of isogenic cell lines offers a superior resource for functional characterization of genes and their role in cellular processes, signaling pathways, disease development and progression, and drug response.

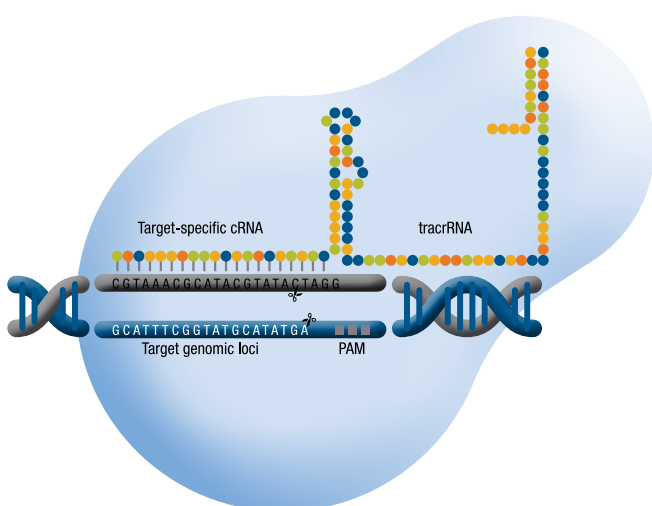
Search for engineered cell lines at

[**thermofisher.com/engineeredcelllines**](https://thermofisher.com/engineeredcelllines)

2.3

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 guide RNA (gRNA) to target the bacterial Cas9 endonuclease to specific genomic loci. Because the gRNA supplies the specificity, changing the target only requires a change in the design of the sequence encoding the gRNA.



The CRISPR-Cas system used in gene editing consists of three components: the Cas nuclease Cas9 (a double-stranded DNA endonuclease), a target complementary CRISPR RNA (crRNA) and an auxiliary trans-activating crRNA (tracrRNA) (Figure 2.4).

Figure 2.4. A CRISPR-Cas9 targeted double-strand 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 gRNA. After the gRNA unit has guided the Cas9 nuclease to a specific genomic locus, the Cas9 protein induces a double-strand 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 guide RNA (gRNA) sequence and delivering it along with the Cas endonuclease to the target cell. Different formats of CRISPR tools are available for specific research needs including: CRISPR-Cas9 all-in-one expression plasmids, CRISPR-Cas9 mRNA and gRNA, Cas9 protein and CRISPR libraries (Figure 2.5).

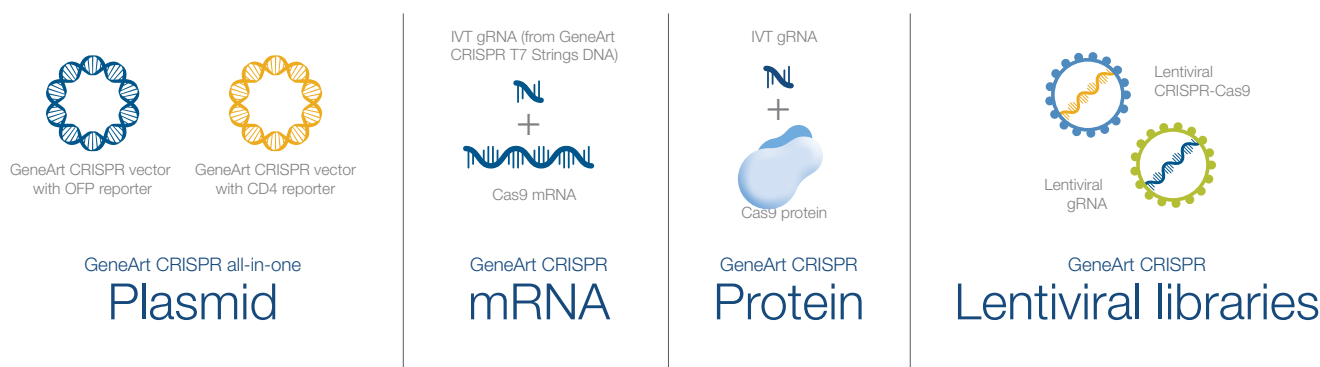


Figure 2.5. Available CRISPR-Cas9 delivery formats.

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 2.6.

PSCs can readily be edited using CRISPR plasmid vectors and mRNA; however, the highest cleavage efficiencies in hPSCs are observed using the Cas9 protein format and *in vitro* transcribed (IVT) gRNA (Figure 2.7). Reference Table 2.1 for a comparison of different CRISPR technologies.

Find out more at thermofisher.com/crispr

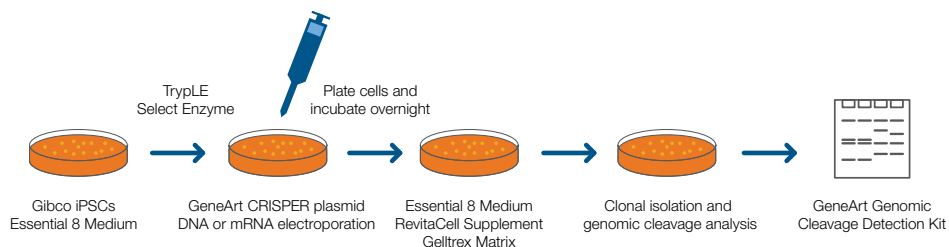


Figure 2.6. CRISPR gene editing workflow.

Electroporation of GeneArt all-in-one plasmid vector and GeneArt CRISPR Nuclease mRNA in Gibco™ hiPSCs, and analysis of genomic cleavage efficiency.

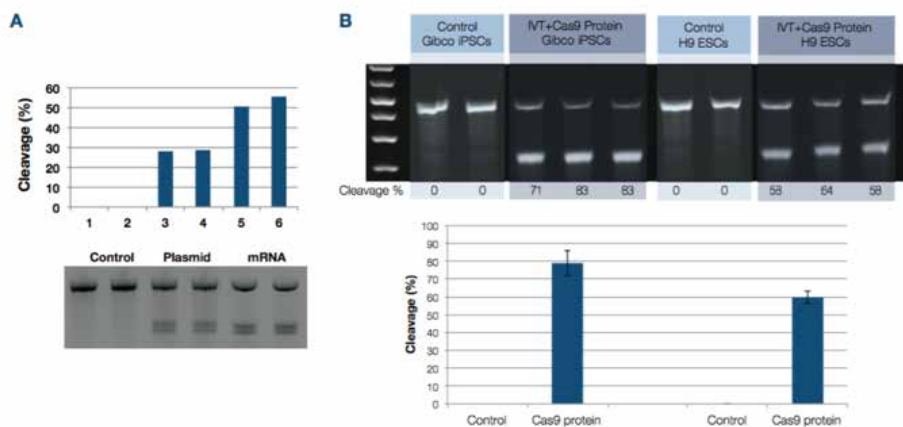


Figure 2.7. Genomic cleavage efficiency in mouse ESCs and human iPSCs/ESCs.

(A) Mouse ESCs were transfected with either GeneArt CRISPR nuclease reporter plasmid (OFF) or GeneArt CRISPR nuclease mRNA along with *in vitro* transcribed gRNA (IVT gRNA) using Lipofectamine 3000 or Lipofectamine MessengerMax respectively; The target loci tested in each case was Rosa26. Cells were assayed for genomic cleavage 48 hours post transfection using GeneArt Genomic Cleavage Detection kit. (B) HPRT loci were targeted in Human iPSCs and H9 ESCs using GeneArt Platinum Cas9 Nuclease (protein) and target specific IVT gRNA. Neon electroporation system was used in each case. Results shown here are for triplicate samples using 10uL Neon electroporation tips. Highest genomic cleavage was achieved using 1,400V, 30 ms pulse width, and 1 pulse. Each well contained 1.4ug Cas9 protein, 300 ng IVT gRNA and 1×10^5 cells.

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Table 2.1. Comparison of CRISPR and TALs technologies.

	CRISPR-Cas9 technology				TAL effector Technology
Product name	GeneArt CRISPR all-in-one plasmid	GeneArt CRISPR mRNA	GeneArt Cas9 protein	GeneArt CRISPR lentiviral libraries	GeneArt TAL effectors
Product benefits	<ul style="list-style-type: none"> All-in-one CRISPR system Reporter-based enrichment 	<ul style="list-style-type: none"> No footprint left behind (no random integration concern) No promoter constraint Ready to use Controlled dosage Fast turnover Microinjection ready Multiplexing and screening capable 	<ul style="list-style-type: none"> No footprint left behind (no random integration concern) No promoter constraint Ready to act Controlled dose Fast turnover Stable RNP complex Microinjection ready Multiplexing and screening capable 	<ul style="list-style-type: none"> Infect dividing and non-dividing cells Stable expression of CRISPR system Loss-of-function screening Large-scale genetic screens 	<ul style="list-style-type: none"> Access to the only TALEN technology that includes the rights under foundational TAL IP Precise Flexible, no design restrictions
Modification options	Gene knockout, gene knock-in	Gene knockout, gene knock-in	Gene knockout, gene knock-in	Loss of function screening	Gene knockout, downregulation (knockdown), integration (knock-in), gene activation
Ease of design	Simple and fast design process	Simple and fast design process with gRNA synthesis kit, no need to clone	Simple and fast design process with gRNA synthesis kit, no need to clone	Ready-to-use lentiviral particles	Flexible, no design restrictions
Multiplexing	N/A	Capable	Capable	High-throughput screening	Rarely used
Design requirement	PAM site (NGG)	PAM site (NGG)	PAM site (NGG)	PAM site (NGG)	Active range of spacing needed for effector activity, no design constraints
Type of recognition	RNA-DNA	RNA-DNA	RNA-DNA	RNA-DNA	Protein-DNA

Useful tips:

- To use the GeneArt CRISPR Nuclease Vector Kit, first design two single-stranded DNA oligonucleotides (24 to 25 bp), one encoding the target-specific crRNA (forward-strand oligonucleotide) and the other its complement (reverse-strand oligonucleotide). Then generate a double-stranded oligonucleotide suitable for cloning into the linearized vector provided in the kit by simply annealing the complementary oligonucleotides
- The design of the single-stranded oligonucleotides is critical to the success of the cloning procedure and to the effectiveness of the construct as a genome editing tool. Guidelines are provided below for choosing a target sequence. Note that these are general recommendations only and exceptions may occur. We recommend that you test more than one target-specific crRNA sequence per locus of interest
 - **Length**—choose a target sequence ranging from 19 to 20 nucleotides in length that is adjacent to an NGG proto-spacer adjacent motif (PAM) sequence on the 3' end of the target sequence. The 5' G required for transcription initiation from the U6 Pol III promoter is already included in the vector overhangs and does not need to be included in the target sequence.
 - **Homology**—make sure that the target sequence does not contain significant homology to other genes, as this can increase off-target effects. Recently published work has shown that gRNA-Cas9 complexes can potentially tolerate one to three or more mismatches, depending on their location in the gRNA.
 - **Orientation**—by choosing a target sequence encoding the sense sequence of the target locus or the antisense sequence, it is possible to generate CRISPR RNA in two possible orientations, provided that it meets the PAM requirements on the 3' end.

1. Choose genomic DNA target sequence:

Choose a 19 to 20 bp target sequence upstream of the NGG PAM site. You can choose a target site either in the sense or antisense strand of the genomic DNA provided it meets the PAM requirements.

2. Top strand oligo design: Add a 5-base GTTTT 3' overhang needed for cloning to the selected 19 to 20 bp target sequence to generate the top strand oligo. Please note that the PAM site is not included in the oligo.

3. Bottom strand oligo design:

Generate reverse complementary sequence specific to the 19 to 20 bp target sequence and add a 5-base CGGTG 3' overhang to generate the bottom strand oligo.

4. Anneal oligos: Anneal top and bottom strand oligos to generate a double-stranded (ds) oligo with compatible ends for cloning into the GeneArt CRISPR Nuclease Vector.

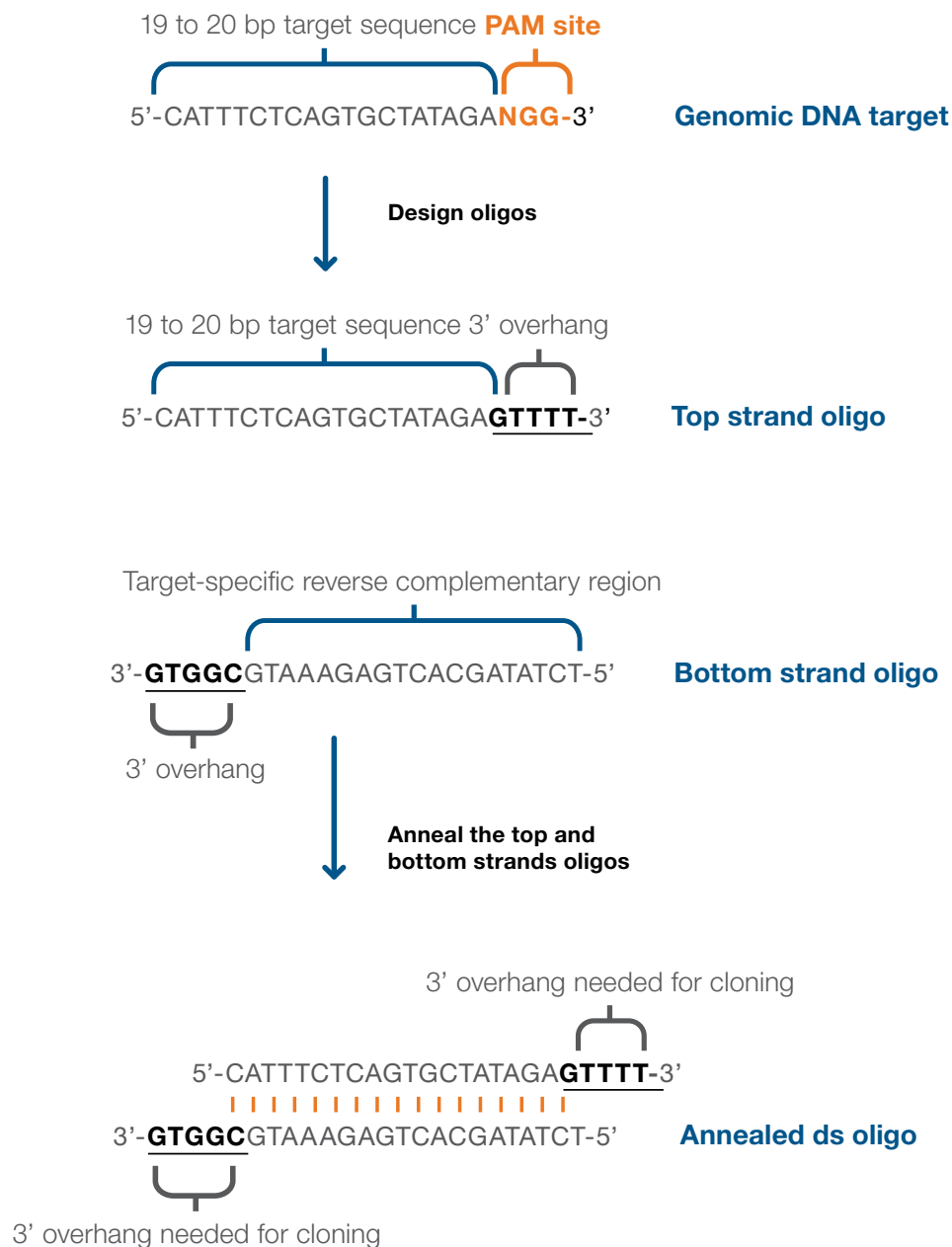


Figure 2.8. An overview of the oligo design workflow.

TAL effectors

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 highly conserved 32 to 34 amino acid repeat sequences except for the amino acids in positions 12 and 13. These two amino acids, called the repeat variable diresidue or RVD, dictate the 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.

The genome editing processes in products such as Invitrogen™ GeneArt™ PerfectMatch TALs use pairs of TALs that are fused to truncated Fok1 nuclease. Fok1 nuclease functions as a homodimer, and creates a double-strand break in the DNA flanked by the TAL 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 2.9).

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

- Design and synthesis of TAL constructs
- 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

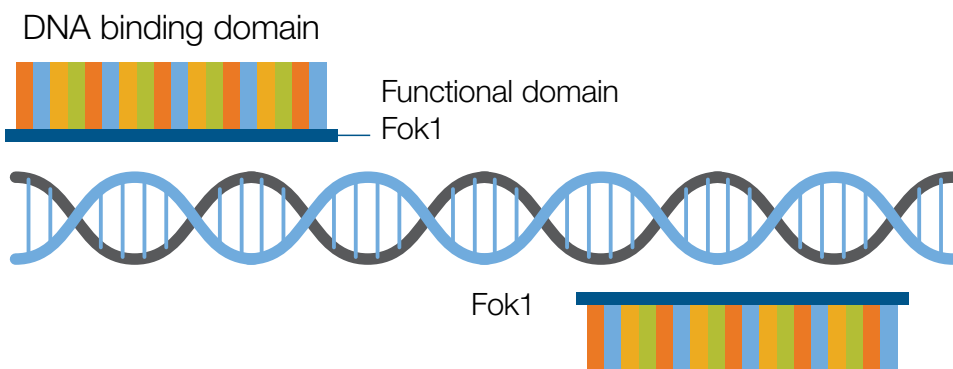


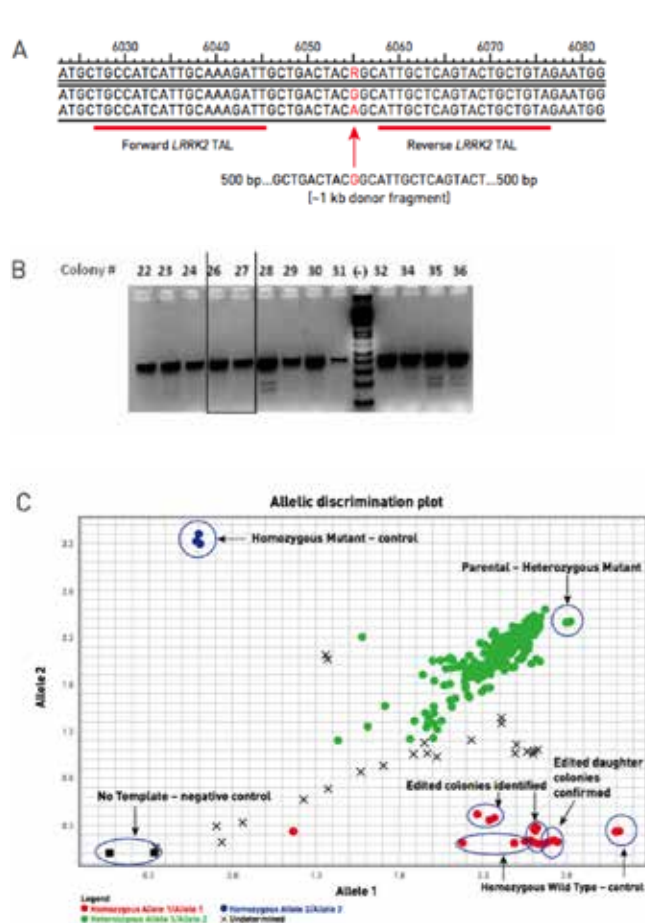
Figure 2.9. GeneArt PerfectMatch TAL technology. A fusion of a precision TAL to a Fok1 nuclease generates a homodimer pair that is designed to bind to genomic sequences flanking the target site and to generate a double-strand break at the desired locus. GeneArt PerfectMatch TALs eliminate the 5' T constraint of natural occurring TALs. GeneArt PerfectMatch TALs allow targeting of any sequences across the genome; 15 to 16 bp spacing between the two TAL effector targets is optimal for GeneArt PerfectMatch TALs.

In a recent proof-of-concept study, Invitrogen™ GeneArt™ TALs were used to correct a LRRK2 G2019S mutation in iPSCs from a Parkinson's disease patient. A review of this study is described below. Leucine-rich repeat kinase 2 (LRRK2) is a large multi-domain protein that contains protein-protein interaction domains flanking a catalytic core that harbors a GTPase and a kinase domain. Although the exact role of the LRRK2 gene in Parkinson's disease is unknown, several mutations in LRRK2 have been linked to the disease, with G2019S being the most common one. Correcting the LRRK2 G2019S mutation back to wild type required editing via homologous recombination, which involved changing one nucleotide from an A back to

the wild type G using a GeneArt TAL pair flanking the region along with a 1 kb donor DNA containing the desired correction (Figure 2.10A). In the initial screen for the LRRK2 correction, 2 out of 140 colonies (1.4%) were positive for editing in the Invitrogen™ GeneArt™ Genomic Cleavage Detection assay (Figure 2.10B, colonies 26 and 27). The colonies were subcloned and rescreened with the Applied Biosystems™ TaqMan™ SNP Genotyping Assay (Figure 2.10C). Ion PGM™ sequencing was performed on positive colonies to confirm clonality of the population (Figure 2.10D).

To read the full study, visit

thermofisher.com/diseasemodels



D

Sample sequenced	Wild type (G)	Mutant (A)
Parental-heterozygous	53	47
LRRK2-edited colony	93	7
Edited daughter colony 1	100	0
Edited daughter colony 2	100	0
Edited daughter colony 3	100	0

Figure 2.10. Generation of Parkinson's disease donor iPSCs with LRRK2 G2019S corrected to wild type. (A) Sequence of LRRK2 G2019S region in the Parkinson's disease line. The binding sites for the TAL pair are underlined in red. The TALs were electroporated into the cells along with a 1 kb purified PCR fragment containing the wild type sequence and 500 bp flanking sequences. (B) Colony screening by GeneArt Genomic Cleavage Detection Assay. Out of the 140 colonies screened, colonies 26 and 27 showed negligible cleavage product due to mismatch, indicating that the heterozygous mutation was mainly corrected to homozygous wild type. (C) A TaqMan SNP Genotyping Assay confirmed that clones 26 and 27 and their daughter colonies contain homozygous wild type allele, as these clones (red) were plotted in the same region as the wild type controls (from wild type donor plasmid or wild type template from HEK 293 cells) on the allelic discrimination plot. (D) Ion PGM sequencing showed progress from the heterozygous state of the parental line (53% G), to a predominantly edited form in colony 26 (93% G), and finally to a homozygous edited state (100% G) in each of three daughter colonies.

Additional TAL functionalities

The predictability with which Invitrogen™ GeneArt™ Precision TAL effectors bind to exact DNA sequences makes it possible to target any sequence in the genome. The choice of the effector domain then determines whether the TAL effector edits, activates, or represses the targeted gene.

Activator function

A GeneArt Precision TAL effector can be designed to function as a transcriptional activator that will increase transcription of a gene near the target TAL effector DNA-binding site (Figure 2.11). To create this site-specific gene activator, a Precision TAL DNA-binding domain is fused to a herpes simplex VP16 activation domain or to VP64, a tetrameric repeat of the VP16 activation domain. When targeted appropriately, these GeneArt Precision TAL activators offer the advantage of expressing all the endogenous splice variants of the target gene in the naturally occurring ratios.

Repressor function

GeneArt Precision TALs can be designed to act as repressors that will down-regulate a targeted gene. To create this site-specific gene repressor, a Precision TAL DNA-binding domain is fused to a Krüppel associated box (KRAB) domain, a potent repressor of transcription.

Both gene activation and repression have been used to reveal the roles played by specific gene products in signaling pathways or in the expression of various other phenotypes.

Custom function

It is possible to deploy an effector domain not currently available from the Thermo Fisher Scientific catalog through a custom services project. This may include constructing a vector that contains both a multiple cloning site (MCS) and a sequence for a custom TAL DNA-binding domain. The service can then insert any protein-coding sequence in frame with the sequence for the TAL DNA-binding domain, and the resulting Precision TAL fusion protein will deliver the chosen

effector to the selected locus in the genome (Figure 2.13). If there is not a clone that codes for the effector available, the gene synthesis services can generate the exact effector domain sequence desired.

Find out more at thermofisher.com/tals

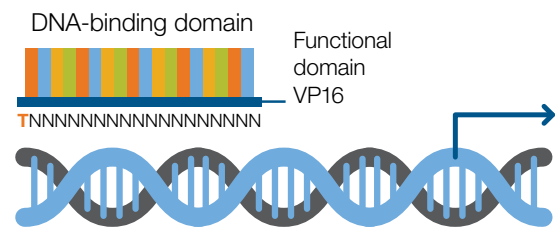


Figure 2.11. Targeted gene activation can be accomplished with a GeneArt Precision TAL protein fused to a VP16 transcription activator domain.

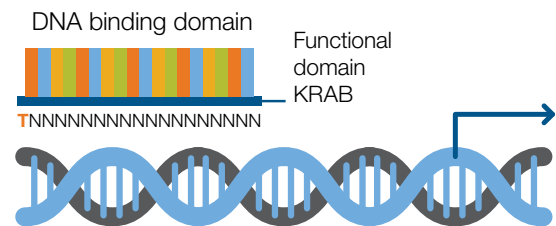


Figure 2.12. Targeted gene repression can be accomplished with GeneArt Precision TAL protein fused to a KRAB transcription repressor domain.

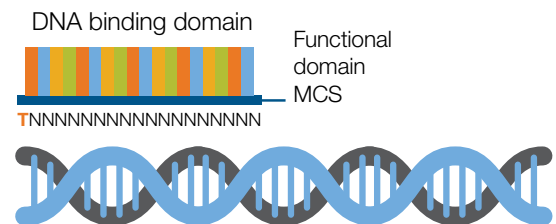


Figure 2.13. Specifically target a custom effector to any locus in the genome with a GeneArt Precision TAL protein fused to the effector domain.

Useful tips

- Design and test at least 2–3 pairs of transcription activator–like (TAL) effectors per gene
- Design the TAL pair to cleave the DNA as close as possible to the desired position
- Design TAL repeats to target 18 or 24 bp of DNA sequence, and design TAL pair targets with 16 bp of spacing in between; then add one N (A, T, G, or C) to the 5′ end of each target sequence
- GC content should be distributed throughout the target site when possible
- Select the TAL vector of interest
- Validate TALs with the Invitrogen™ GeneArt™ Genomic Cleavage Selection Kit and Invitrogen™ GeneArt™ Genomic Cleavage Detection Kit
- Optimize transfection conditions
- Use mRNA instead of DNA
- Test modification efficiency with the GeneArt Genomic Cleavage Detection Kit
- Enrich modified cells with the GeneArt Genomic Cleavage Selection Kit

Table 2.2. Summary of available effector domain.

Effector domain	Functionalities	Applications
Fok1 endonuclease	Gene targeting (truncated TAL)	Silencing Gene editing (i.e., introduction of SNP incorporation of exogenous DNA)
VP16 activator	Activation of transcription (native TAL VP16)	Increasing the expression level of endogenous gene isoforms
VP64 activator	Activation of transcription (native TAL VP64)	Increasing the expression level of endogenous gene isoforms
KRAB repressor	Epigenetic repression of transcription (TAL repressor)	Knockdown of gene expression
MCS	Steric repression and custom design (modified TAL MCS)	Transient knockdown of gene repression Target any locus in the genome with the effector domain of your choice

KRAB = Krüppel-associated box; MCS = multiple cloning site; TAL = transcription activator-like (DNA-binding domain).

2.6

Screening methods for TAL and CRISPR

When using genome editing tools such as CRISPRs or TAL effectors to obtain targeted mutations, it is recommended to determine the efficiency with which these nucleases cleave the target sequence prior to continuing with labor-intensive and expensive experiments.

After gene editing, it is recommended to plate cells at low density to allow for formation of well-separated

clonal colonies. After a one to two week expansion, colonies can be divided in half and harvested. One-half of the colony is maintained for further culture while the other is analyzed to determine whether gene editing events have occurred. For successfully edited colonies, it may be desirable to perform a second round of single-cell dissociation and clonal expansion followed by analysis to ensure that a true clonal population of edited cells is obtained (Figure 2.14).

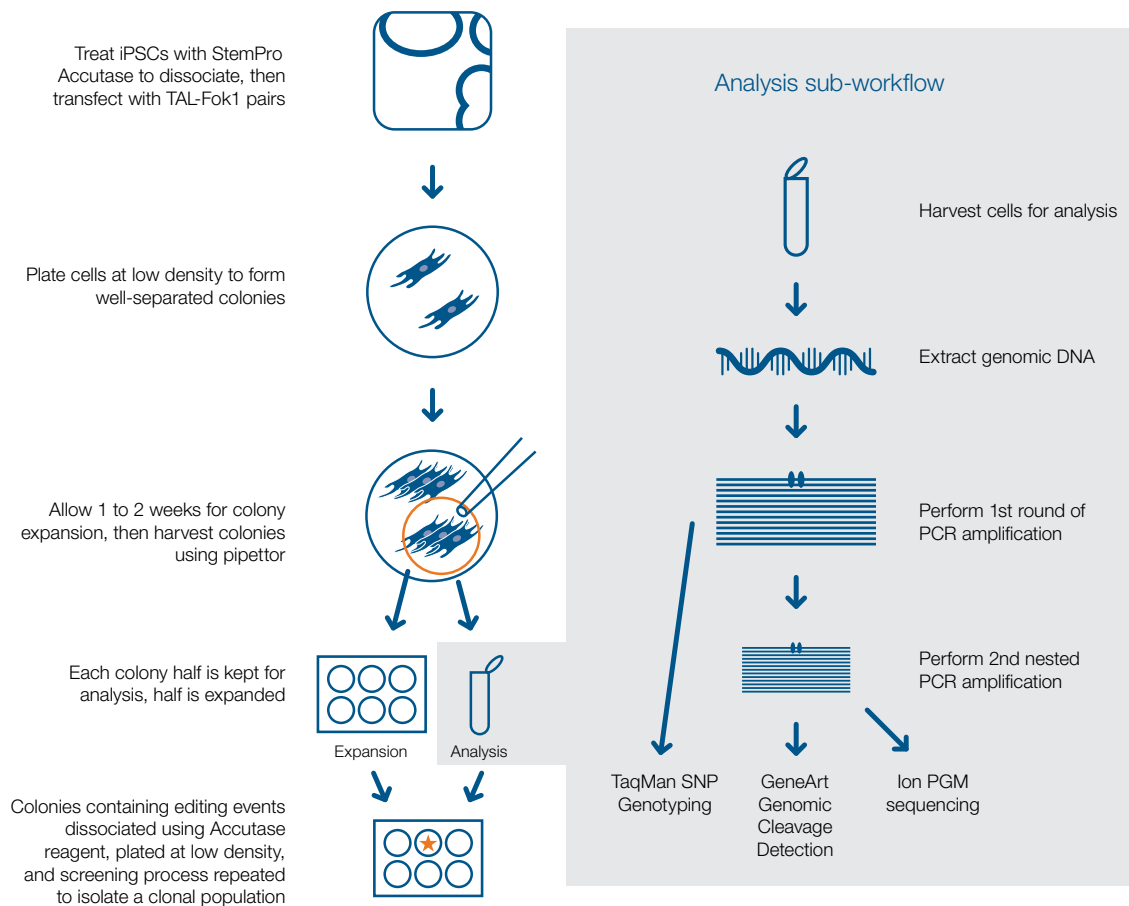


Figure 2.14. Colony screening workflow. Colonies are manually divided in half, with half the cells analyzed and the other half cultured until analysis is complete. PCR of genomic DNA is performed to obtain the sensitivity needed to monitor changes at the target location using the GeneArt Genomic Cleavage Detection Kit and Ion PGM assays. The resulting amplicons are analyzed using the three screening methodologies shown. Note that the sensitivity of the TaqMan SNP Genotyping Assay is such that the PCR steps can be omitted if the other methodologies are not used.

A variety of tools and reagents, including TaqMan SNP Genotyping, GeneArt Genomic Cleavage Selection Kit, GeneArt Genomic Cleavage Detection 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 2.3.

Table 2.3. Comparison of common genomic analysis methodologies.

Methodology	Advantages	Limitations	When to use
GeneArt Genomic Cleavage Selection Kit	<ul style="list-style-type: none"> • Fast, live detection • Visual indication • Proves editing tool works • Allows clone enrichment 	<ul style="list-style-type: none"> • Limited use if editing heterozygous loci to homozygous loci 	<ul style="list-style-type: none"> • Want to visually check functionality of engineered nuclease within 24–48 hours • Enrich for the edited cell population
GeneArt Genomic Cleavage Detection Kit	<ul style="list-style-type: none"> • Inexpensive • Can detect small changes in homozygous state of DNA NHEJ and HDR editing 	<ul style="list-style-type: none"> • Positive result does not indicate whether editing tool works • No enrichment capability • Limited use if editing heterozygous loci to homozygous loci 	<ul style="list-style-type: none"> • Triaging colonies from editing via NHEJ repair
TaqMan SNP Genotyping Assay	<ul style="list-style-type: none"> • Inexpensive • Fast • Clearly distinguishes changes in allele status 	<ul style="list-style-type: none"> • Only detects changes in alleles that the assay is designed for; may not detect indels from NHEJ repair 	<ul style="list-style-type: none"> • Triaging colonies from editing via homologous recombination
Ion PGM sequencing	<ul style="list-style-type: none"> • Can specifically detect all changes in a population • Quantitative results 	<ul style="list-style-type: none"> • Higher cost compared to other assays • Longer workflow 	<ul style="list-style-type: none"> • Best used as a secondary assay, for confirmation and quantitation of editing in populations identified from primary screens

Screening with the GeneArt Genomic Cleavage Selection Kit

The GeneArt Genomic Cleavage Selection Kit is a rapid and reliable tool for detecting functionality of engineered nucleases in transfected cells as well as enriching for modified cells (Figure 2.15). When using engineered nucleases to create double-strand breaks in genomic DNA, it is necessary to know whether or not the designed nucleases are functional. Furthermore, to efficiently screen for modified cells, a way to enrich for the edited cells is also necessary, particularly if the engineered nuclease has low efficiency or the cell line used is difficult to transfect. The GeneArt Genomic Cleavage Selection Kit contains a vector with the orange fluorescent protein (OFP) gene for a quick visual check of the functionality of the engineered nuclease. In addition, the reporter

genes OFP and CD4 can be used to enrich for edited cells. It can be used in conjunction with genome editing tools such as ZFNs, TAL effector nucleases, and CRISPRs.

Useful tips:

- Choose a genome editing tool
- Design 2–4 CRISPR target sequences or TAL pairs and corresponding GCS vectors per gene
- Establish optimal growth conditions for the cell lines being used
- Transfect the GCS vector and editing tool using optimal transfection conditions and reagents
- Use appropriate controls to establish cleavage efficiency
- Screen for cells expressing OFP using fluorescence microscopy or flow cytometry
- Correlate OFP expression with genome editing efficiency on the endogenous loci
- Confirm GCS vector functionality in a workhorse cell line
- Enrich for nuclease-modified cells
- Analyze CD4- or OFP-positive cells

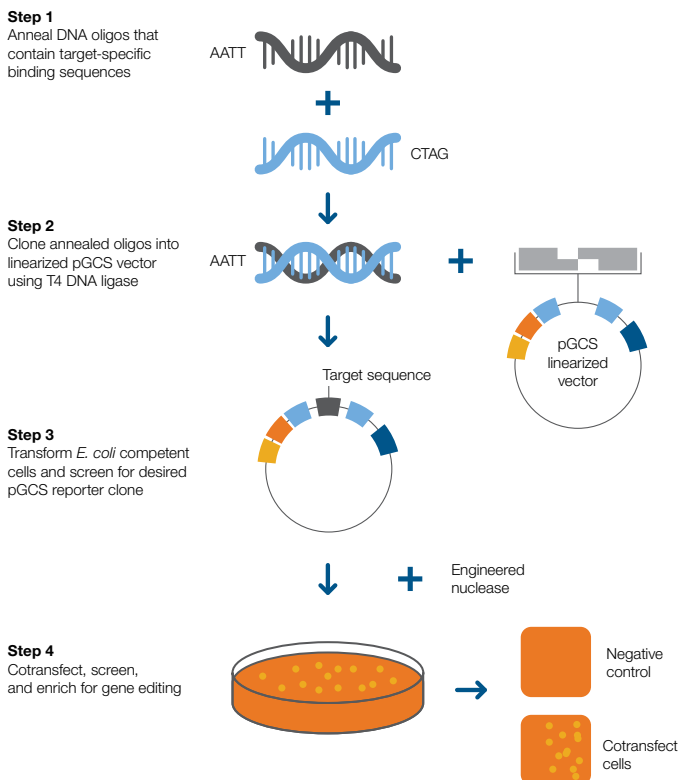


Figure 2.15. OFP reporter and CD4 enrichment. The GeneArt genomic cleavage selection vector has been constructed such that the N-terminal and C-terminal ends of the OFP gene are separated by a cloning site for the target sequence of the programmable nuclease. The upstream sequence coding for the N-terminal portion of the OFP gene contains a region complementary to the 5' end of the C-terminal region of the OFP gene. The CD4 gene is out of frame for expression when the OFP gene is interrupted by the cloning site. When a double-strand break is introduced into the target sequence by the programmable nuclease, the complementary strands from each end sequence of OFP will recombine to restore OFP expression, and the CD4 gene is now in frame for expression. Thus, cleavage by TAL, CRISPR, or zinc finger nucleases can be checked as early as 24 hours posttransfection by simply viewing the transfected cells under a fluorescence microscope.

2.8

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 2.16). 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-strand 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

- Optimize cell culture conditions
- Optimize transfection conditions
- Optimize the amount of nuclease
- Include negative controls
- Prepare cell lysate
- Generate a strong PCR product
- Verify PCR product before proceeding
- Denature and reanneal PCR fragment
- Optimize enzyme digestion
- Optimize gel analysis

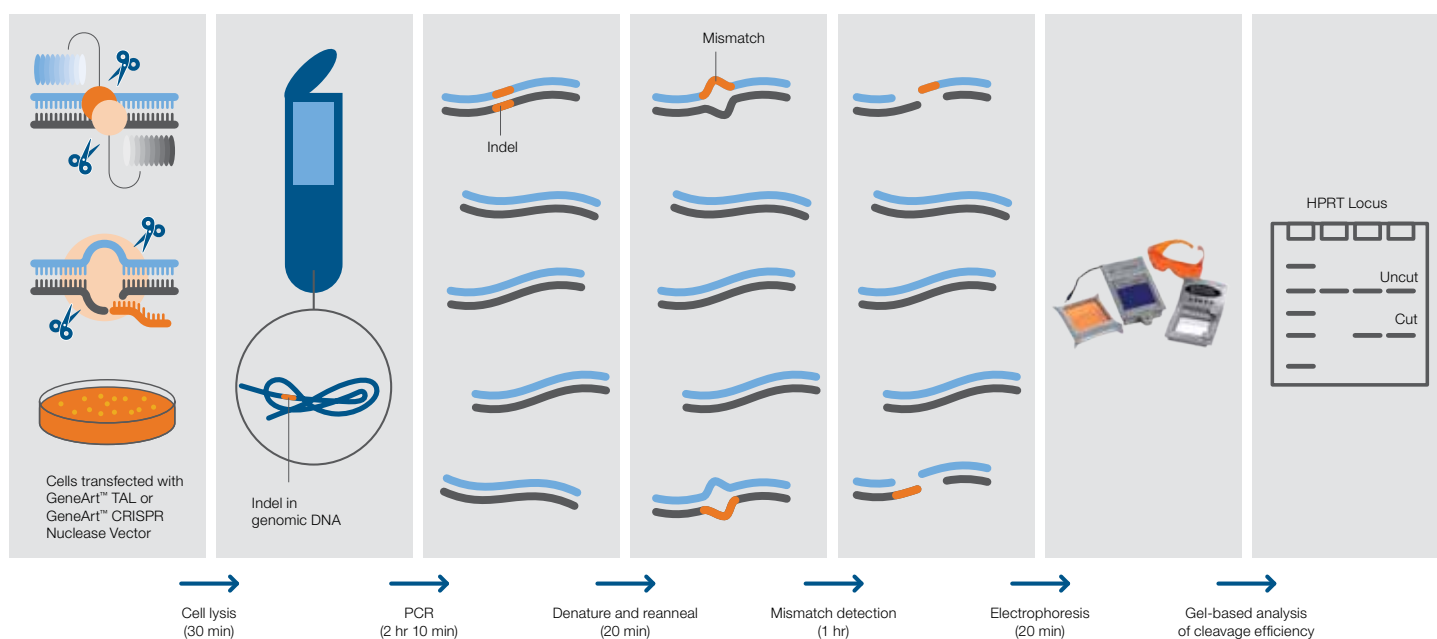


Figure 2.16. GeneArt Genomic Cleavage Detection Assay. To detect either an indel or a mutation within a specific sequence of DNA, the region is first amplified using primers specific for that region. A second nested PCR can be performed to increase sensitivity. After heating the sample and reannealing 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 3

Culture

Introduction

Culturing PSCs requires a compatible combination of media, matrices, and passaging methods that support cell health and pluripotency. A variety of combinations or 'culture systems' developed satisfy the progressively stringent requirements of different PSC applications, from basic research to regenerative medicine. Chemically defined media make use of known components at known quantities, making them valuable for both, research and clinical applications. Xeno-free media contain only human origin components, making them particularly suitable for clinical applications. Feeder-dependent culture systems make use of inactivated fibroblasts and are not chemically defined; nor are they xeno-free unless the feeder is of human origin. In contrast, feeder-free cultures have the potential to be defined and xeno-free, depending on the medium/matrix combination used. Thus, on the least stringent end of the spectrum lie feeder-dependent culture systems containing animal components, which are acceptable for some basic research applications. On the most stringent end lie the completely defined and xeno-free, feeder-free systems that are specifically manufactured and qualified for clinical applications.

While the main consideration in selecting a PSC culture system is the intended PSC application and its requirements, a full spectrum of culture systems are available and there can be multiple options satisfying the application's minimum requirements. Choosing between different options may ultimately depend on other considerations, including cost, workflow, scalability, and/or consistency of performance. 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.

3.2

Feeder-dependent culture systems

Feeder-dependent cultures systems generally support pluripotency and cell health using a DMEM-based medium that is supplemented with basic Fibroblast Growth Factor (bFGF) and serum or KnockOut Serum Replacement (KSR), a defined serum-free alternative that is specifically optimized for PSC culture¹. 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 mouse embryonic fibroblast (MEF) feeders that have been irradiated or treated with mitomycin-c to arrest the cell cycle; this “inactivation” prevents the MEFs from overgrowing and outcompeting the slower-growing PSCs. A smaller percentage of feeder-dependent PSC cultures make use of human feeder cells, for example inactivated human foreskin fibroblasts, as a xeno-free alternative that is more suitable for clinical applications. Whether the feeders are of mouse or human origin, they are typically cultured on plates coated with 0.1% gelatin, which is available in a ready-to-use format.

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 clear edges (Figure 3.1), whereas areas of differentiation contain larger, flatter, and less compact cells. Such differentiation is triggered when colonies have grown too big or when cultures have become too confluent, particularly when colonies begin to overlap with each other. 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 to 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.

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 to smaller clumps by trituration, taking care 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 P200 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 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 require coating with gelatin or an Attachment Factor, and then should be seeded with feeder cells at least a day in advance of culture.

To access detailed protocols for culturing PSCs on feeder cells, visit [thermofisher.com/cultureprotocols](https://www.thermofisher.com/cultureprotocols)

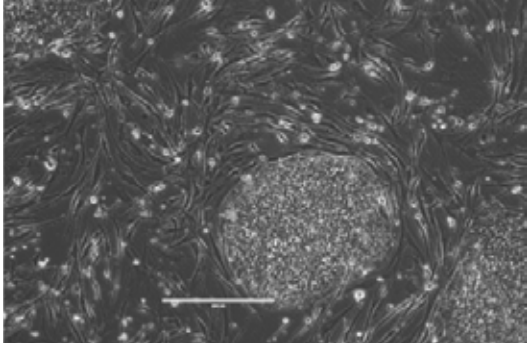


Figure 3.1. Phase contrast image of hiPSCs grown on inactivated MEFs. Cells were grown on gelatin-coated plates using medium containing KnockOut Serum Replacement (4X magnification).

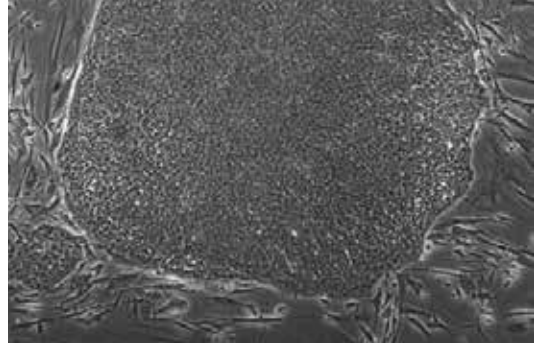


Figure 3.2. Phase contrast image of H9 hESCs grown on inactivated MEFs. Cells were grown on gelatin-coated plates using medium containing KnockOut Serum Replacement. The ESC colony consists of compact cells, exhibits a well-defined border, and is surrounded by inactivated MEFs with spindle cell morphology (10X magnification).

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

3.3

Feeder-free culture systems

Human ESCs may have been first derived under feeder-dependent conditions², but since then tremendous efforts have been made to simplify culture systems and to tailor them for different intended applications, including cell therapy. These efforts are centered on removing the feeder cells and supplementing the remaining culture components to compensate for this change. 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 continued to parse out the protein contributions of MEFs and to investigate the pathways that are critical for pluripotency with the goal of developing better feeder-free systems.

Gibco StemPro hESC Serum and Feeder-free Medium (SFM)

Gibco™ StemPro™ hESC SFM is a feeder-free media that resulted from these studies to optimize feeder-free culture. This fully defined and serum-free medium has been extensively tested and is proven to maintain pluripotency in a growing list of hESC lines, including BG01, BG02, BG03, H1, H9, and BG01V. It has been shown to support hESC and iPSC growth for >50 passages without any signs of karyotypic abnormalities. Furthermore, it maintains the ability of hESCs to differentiate into all three germ line lineages. It is also amenable to large-scale culture and has been used to expand one 60-mm dish of hESCs to over 1×10^{10} cells in 20 days.

StemPro hESC SFM works in combination with Gibco™ CTS™ CELLstart™ Substrate, a defined and xeno-free substrate. However, it is more commonly used with Gibco™ Geltrex™ matrix, which consists of basement membrane proteins derived from Engelbreth-Holm-Swarm mouse tumors. Geltrex matrix is available in both a standard formulation that requires prior dilution as well as a ready-to-use formulation.

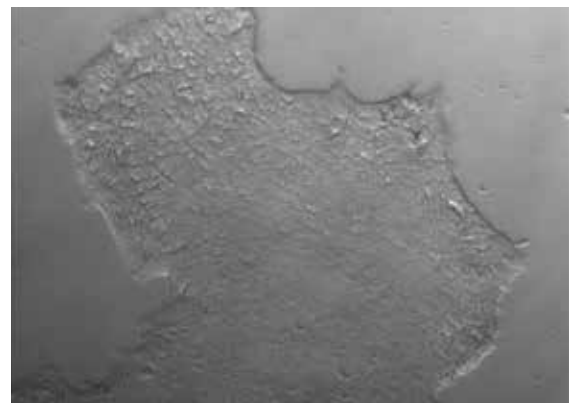
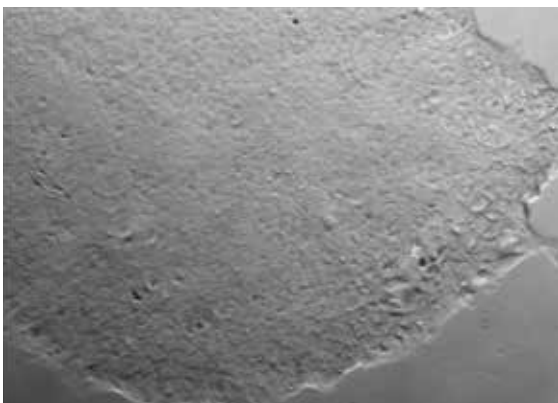


Figure 3.3. Phase contrast images of hiPSCs grown under feeder-free conditions. Gibco Human Episomal iPSCs were grown on Geltrex matrix-coated plates using Essential 8 Medium. (10X magnification).

Gibco Essential 8 Medium and Gibco 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, at the University of Wisconsin, re-examined the composition of an existing feeder-free medium, testing new combinations with fewer components³. The result was a fully 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 comprised of 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 25 passages. Now available, the Gibco™ Essential 8™ Flex Medium Kit has been designed to eliminate daily feeding schedules required for most PSC culture maintenance. This medium extends the activity of key heat-sensitive components found in PSC medium, including bFGF. This formulation allows for a flexible feeding schedule in which feeding can be skipped for up to two consecutive days without compromising pluripotency and genetic stability.

Both, Essential 8 and Essential 8 Flex Media can be used with a variety of matrices, including Geltrex matrix. To complete a defined and xeno-free culture system, these media can be used with Gibco™ Vitronectin substrate. This substrate is comprised of the VTN-N variant of the vitronectin protein, which, as the Thomson lab found, supports hPSC attachment and survival better than wild type vitronectin when used with Essential 8 Medium³.

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. As a guideline, healthy and undifferentiated feeder-free cells grow in colonies, 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 (Figure 3.3). As with feeder-dependent cultures, overconfluency leads to areas of differentiation that can be removed mechanically, but to prevent widespread differentiation, cultures must be passaged regularly.

Cells cultured with StemPro hESC SFM Medium and Geltrex matrix or CTS CELLstart Substrate are clump passaged very similarly to feeder-dependent cultures. They can be mechanically passaged using the EZPassage Tool or enzymatically passaged using Collagenase or Dispase, but these splits are usually done every 4 to 6 days at a split ratio of 1:4 to 1:6. In contrast, cells cultured in Essential 8 or Essential 8 Flex Media with either Geltrex matrix or vitronectin are more compatible with non-enzymatic passaging. 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 with Gibco™ Versene Solution. Once EDTA has been replaced with media, cells are then removed from the plate by gentle pipetting. Unlike the other passaging methods described, this method results in smaller 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 4 to 7 with split ratios of around 1:6 to 1:12.

Single cell passaging

Essential 8 Medium also supports single cell passaging when used in combination with a Rho-associated protein kinase (ROCK) inhibitor. The recommended protocol has been optimized to specifically work with Gibco™ RevitaCell™ Supplement, a proprietary, chemically defined, xeno-free formulation that contains antioxidants, free radical scavengers, and most importantly, a ROCK inhibitor with higher specificity than Y-27632 or Thiazovivin. In this protocol, adherent PSCs are dissociated using Gibco™ TrypLE™ Select Enzyme or Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent. RevitaCell Supplement is added to Essential 8 Medium during the first 18 to 24 hours after passaging, but is no longer required in subsequent media changes.

Single cell passaging is critical for certain applications such as gene editing and clonal expansion. It is also technically simpler than clump passaging, making it more desirable for routine PSC culture and more amenable to large scale culture. Indeed, single cell passaging has been used to enable the large scale culture of up to 1×10^9 PSCs in about 600 mL of Essential 8 Medium in spinner flasks⁴.

For a guide on what matrix and passaging method should be used given a particular choice of medium, reference Figure 3.5.

Useful tips

- It is very important to pre-warm complete Essential 8 Medium at room temperature and not in a 37°C water bath. Basic fibroblast growth factor (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 at the time of post-thaw recovery or during the first 24 hours post-passage.
- The inclusion of either a ROCK inhibitor or blebbistatin improves initial survival and supports a high cloning efficiency, which is also increased by the addition of transferrin and selenium. However, if cells are cultured routinely in medium containing a ROCK inhibitor, it may become necessary to include it afterwards. Such addictive behavior has not been noted for RevitaCell Supplement.
- Cells should not be pre-treated with the RevitaCell Supplement before passaging. Cells only require the RevitaCell Supplement for 18 to 24 hours after single cell passaging, with the cells being fed regular Essential 8 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.

Choosing a culture system

As previously mentioned, feeder-dependent cultures are rich and robust, with a proven track record of supporting PSC growth and maintaining pluripotency, and are sufficient for many basic research projects. However, they carry certain disadvantages that can also 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 and requiring significant work to obtain and prepare the feeder cells, which is undesirable but tolerable for a small scale work and is extremely difficult for very large projects.

Alternatively, 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 for 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 undefined 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, fully-defined and completely xeno-free minimal culture systems such as Essential 8 Medium and Essential 8 Flex Medium

are becoming more favored for workflows spanning basic research to clinical research. In basic research, feeder-free systems are becoming more attractive because, in addition to reduced cost, they provide a cleaner background for performing experiments on different biological pathways. Media produced under GMP and specifically designed for clinical applications can additionally add value through increased consistency and lessening the burden for future clinical research. While the benefits of a leaner system are abundant, it is important to understand they also tend to be more sensitive to stressors, and can be less forgiving of harsh cell manipulations.

There are many subtleties to choosing a PSC culture system and the final choice depends on the research goal. For example, if the intended application in basic research can ostensibly be satisfied by feeder-dependent systems, Essential 8 feeder-free systems may be preferred if the actual experiment requires or can benefit from the 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 StemPro hESC SFM or a feeder-dependent option like Gibco™ DMEM/F12 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 3.1.

For additional assistance on finding the right PSC culture tools for you, visit thermofisher.com/psciculture

Table 3.1. Comparison of PSC culture methods.

Media	DMEM/F-12 with KnockOut Serum Replacement	StemPro hESC SFM	Essential 8 Medium	Essential 8 Flex Medium
Feeder-dependent	Yes	No	No	No
Xeno-free	No	No	Yes	Yes
Defined	No	Yes	Yes	Yes
Media complexity	High	Medium	Low	Low
Lot-to-lot variability	Medium	Medium	Low	Low
Weekend-free feeding	No	No	No	Yes
Workflow complexity	High	Medium	Low	Low
Scalability	Low	High	High	High
CTS version available*	Yes	Not currently	Yes	Not currently

*CTS products are designed for clinical research applications. Refer to section 6 for more details.

Choosing matrices and passaging methods

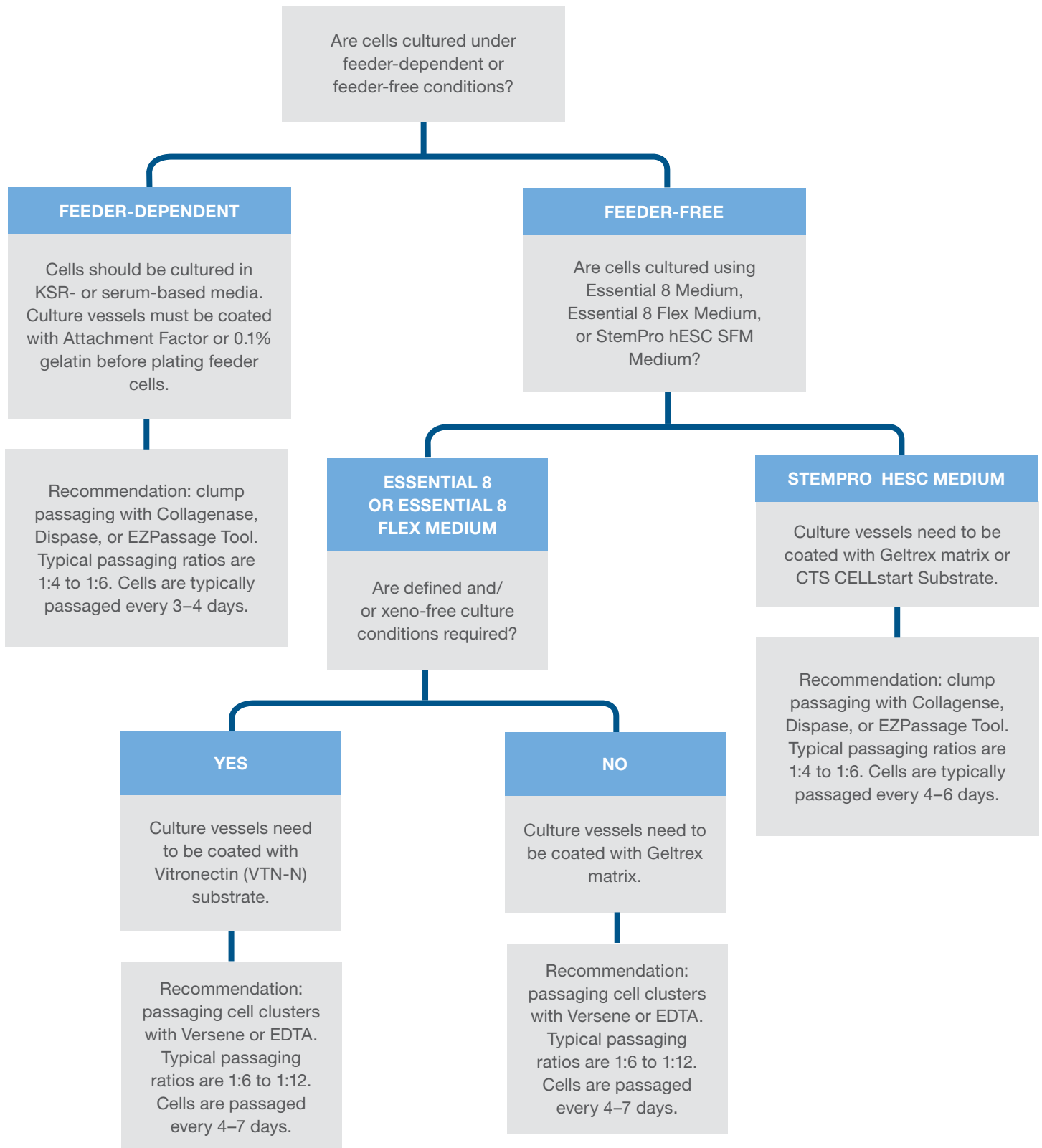


Figure 3.4. Schematic for choosing matrices and passaging methods based on a selected media system.

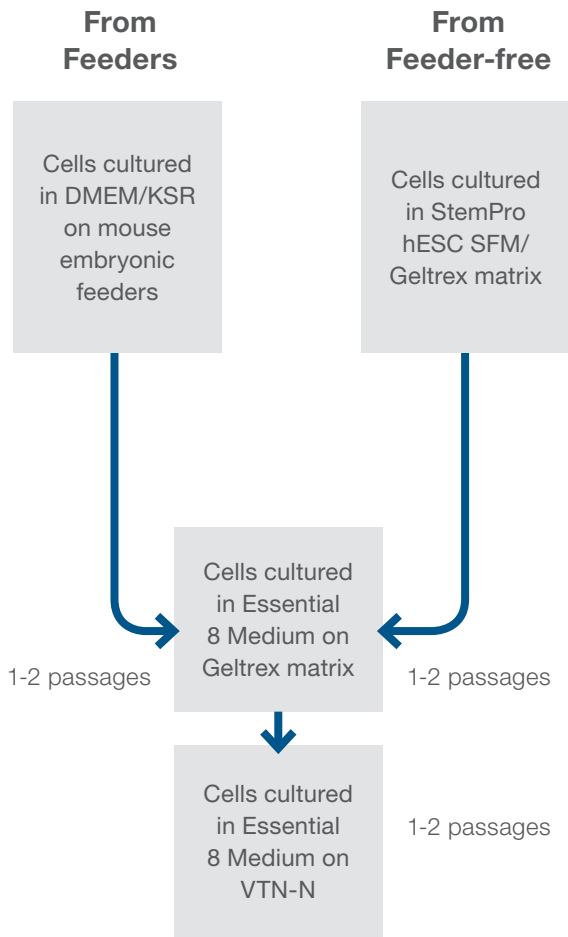
3.4

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. Transitions into much leaner systems can be especially harsh on cells. Typically, cells are placed in intermediate media systems to allow them to slowly acclimate to new environments. Adaptation of feeder-dependent PSCs into Essential 8 Medium with VTN-N can be done after a passage or two in MEF-conditioned medium and Geltrex matrix, plus another passage or two in Essential 8 medium and Geltrex matrix. In contrast, cultures in other medium (like mTeSR™ 1 medium and Matrigel™ Matrix) or in StemPro hESC SFM and Geltrex matrix are already feeder-free. Therefore, a passage or two in Essential 8 medium with Geltrex matrix serves as a sufficient transition (Figure 3.4). Note that in each of these transitions, the passaging method should match the matrix and medium on the dish to be passaged. Furthermore, sensitive cell lines may require more passages at each step of the adaptation (Figure 3.5).

As an alternative to gradual adaptation, one may directly switch media systems, but this often requires special protocols. For example, feeder-dependent cultures can be directly transferred into Essential 8 Medium with VTN-N, 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 fragments sizes involves harvesting colonies using Collagenase IV followed by trituration and, critically, two rounds of gravity sedimentation.

Transitions to Essential 8 culture system



Transitions for cell lines sensitive to changes in culture conditions

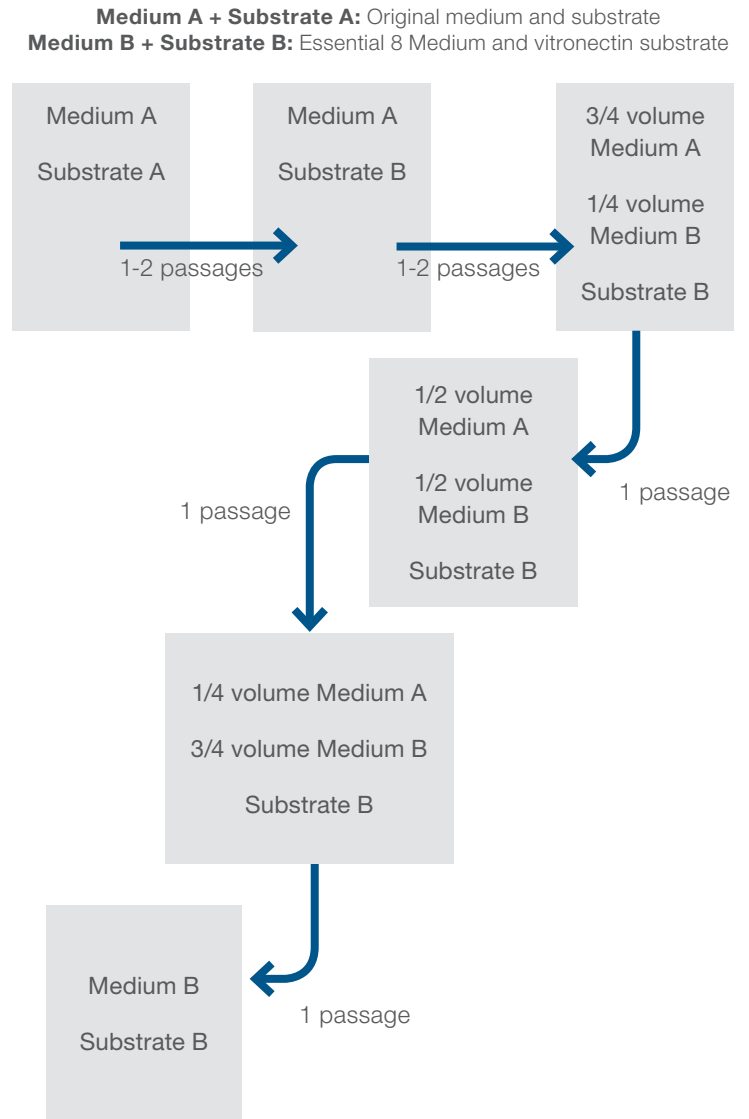


Figure 3.5. Guide for gradual adaptation into feeder-free culture. The left panel shows the scheme for transitioning PSCs into an Essential 8 feeder-free culture system. The right panel provides a more detailed transition scheme for cell lines that are particularly sensitive to changes in culture conditions.

Coming soon!

For an easier transition of feeder-dependent cells to Essential 8 Medium, use Essential 8 Adaptation Kit. Ask a Thermo Fisher Scientific sales representative for details.

3.5

Cryopreservation

Cryopreservation is an important part of any cell culture workflow. In the Gibco™ PSC workflow, cells are cryopreserved to store back-up 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 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 at approximately 1°C per minute. After 24 hours, the cryovial is transferred for long-term storage in a liquid nitrogen freezer at -200°C to -125°C . When the PSCs finally 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 resuspension in fresh culture media, the cells are then transferred to a plate and allowed to recover and grow.

Cryopreservation and thawing is 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 is comprised of a ready-to-use, defined, xeno-free cryopreservation medium and the RevitaCell Supplement, which improves cell survival through antioxidants and a ROCK inhibitor. For clinical applications or simply 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.

To assist in choosing the best cryopreservation medium, these options are compared in Table 3.2.

For more information, visit

thermofisher.com/cryopreservation

Useful tips

- For optimum results, collect cells from a high-confluence well of an actively growing culture to ensure that cells will be in mid-log phase of growth.
- 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.

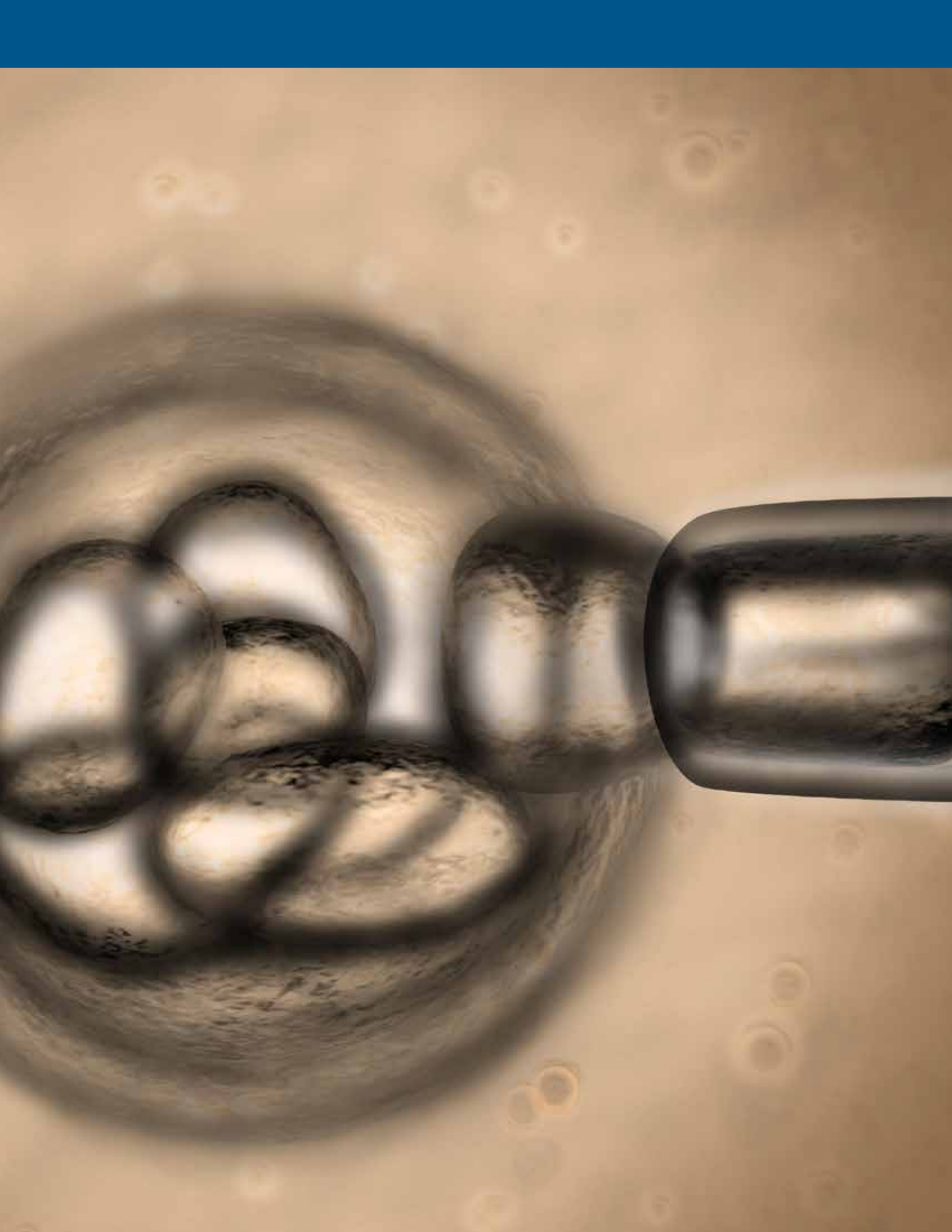
Table 3.2. Summary of key characteristics and performance of PSC cryopreservation media.

	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
cGMP-compliant facility	Yes	No	No
CTS version available	Not currently available		No
Performance	+++	++	Varies

3.7

References

1. Amit M, Carpenter MK, Inokuma MS et al. (2000). Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev Biol* 227(2): 271 - 278.
2. Thomson JA, Itskovitz-Eldor J, Shapiro SS et al. (1998). Embryonic stem cell lines derived from human blastocysts. *Science* 282(5391): 1145 - 1147.
3. Chen G, Gulbranson DR, Hou Z et al. (2011). Chemically defined conditions for human iPSC derivation and culture. *Nat Methods* 8(5):424 - 429.
4. Wang Y, Chou BK, Downey S et al. (2013). Scalable expansion of human induced pluripotent stem cells in the defined xeno-free E8 medium under adherent and suspension culture conditions. *Stem Cell Res* 11(3):1103 - 1116.



Section 4

Differentiation

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 cells or conceivably any other cell type in the body.

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 4.1).

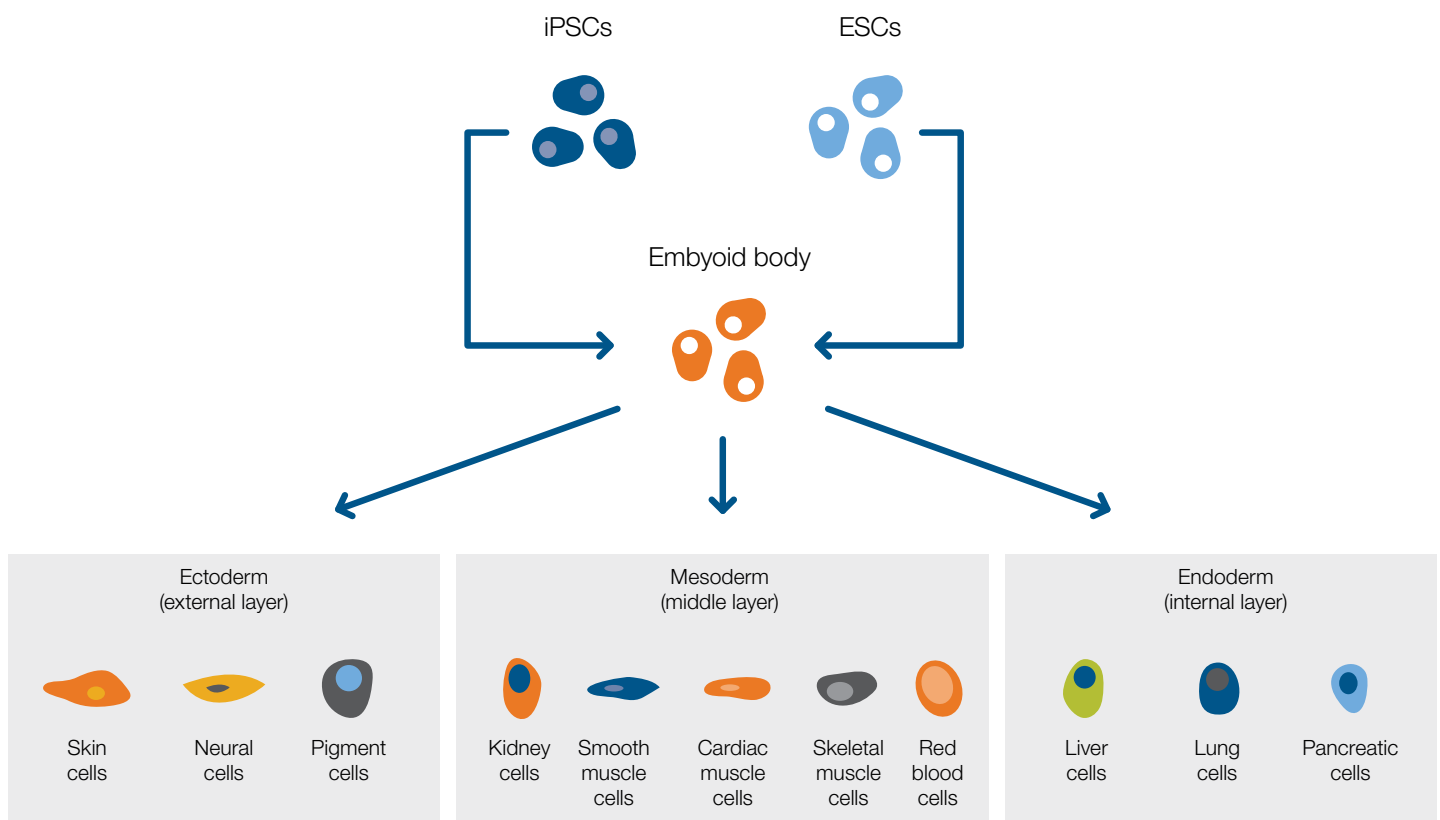


Figure 4.1. Differentiation of PSCs to different lineages via an EB intermediate.

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 fetal 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 to 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:

- **High 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*?
- **A defined protocol**—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 for 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**—if there is interest in potential clinical applications, researchers may want to ask whether a protocol uses GMP-grade reagents or, if not, can readily be converted to GMP-grade conditions down the line. Starting with either a GMP-compatible protocol or one in which RUO reagents can readily be replaced with GMP versions can save significant time, effort, and cost that would be required to adapt and optimize protocols at a later timepoint when the project is ready to proceed to the clinic.

View complete differentiation portfolio at [thermofisher.com/differentiation](https://www.thermofisher.com/differentiation)

4.2

Neural differentiation

iPSC-derived neural stem cells (NSCs) are particularly attractive due to their utility for a wide range of applications in disease modeling, drug discovery, and cell therapy.

At the NSC stage, cells are still mitotic and can be expanded and banked for later use. The multipotency of NSCs means that they possess the capacity to differentiate into multiple glial and neuronal subtypes depending on the maturation conditions to which they are exposed.

Early protocols for NSC induction relied on EB intermediates, stromal coculture, 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.

PSC Neural Induction Medium

Gibco™ PSC Neural Induction Medium is a serum-free medium that provides high-efficiency neural induction of hPSCs (Figure 4.2) in only 7 days. 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 (Figure 4.3). 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 4.4).

For more information, go to thermofisher.com/nscdiff

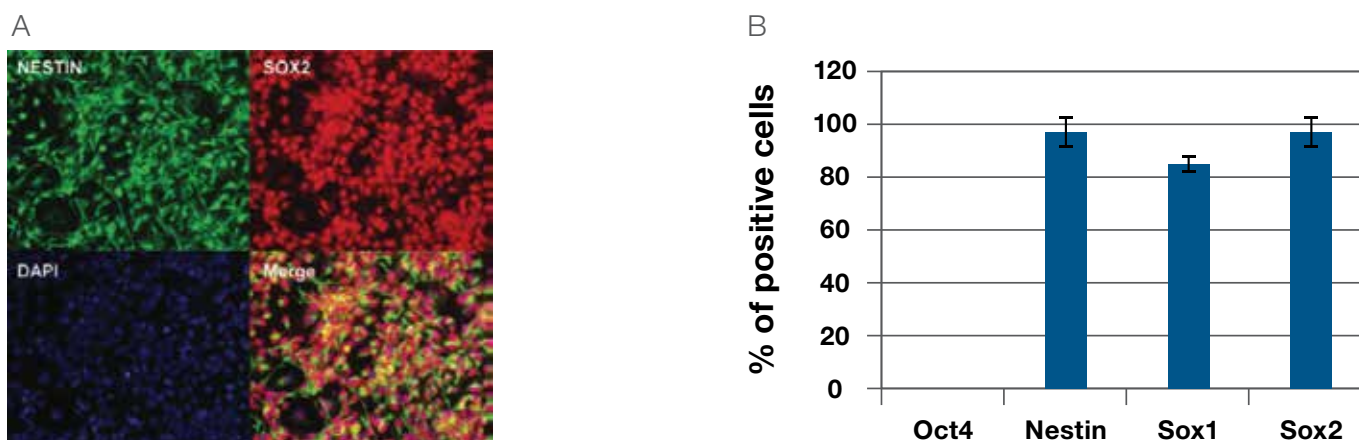
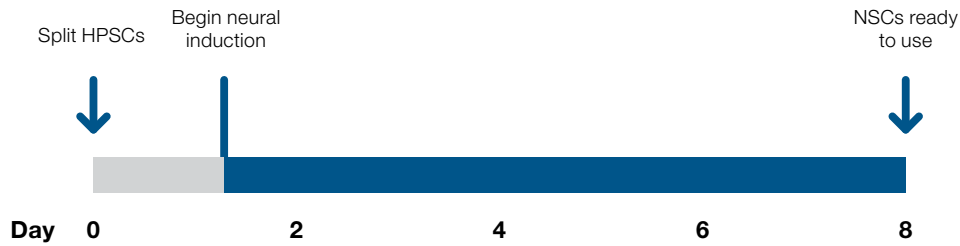


Figure 4.2. At day 7 of neural induction using Gibco PSC Neural Induction Medium, H9 embryonic stem cell induced P0 NSCs were dissociated and re-plated on Geltrex coated plates overnight. Cells were then fixed and stained with pluripotent marker Oct4 and neural markers including Nestin, Sox2, and Sox1. **(A)** The re-plated P0 NSCs were positive for neural marker Nestin (green) and Sox2 (red). Cell nuclei were stained with DAPI (blue). **(B)** The quantification of stained markers showed that less than 1% of P0 NSCs were positive for pluripotent marker Oct4 and more than 80% of P0 NSCs were positive for neural markers Nestin, Sox2, and Sox1.

PSC Neural Induction Medium



Other methods of generating NSC

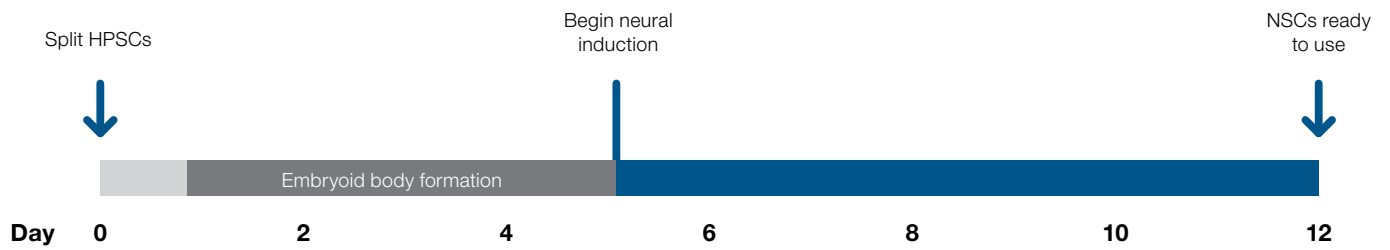


Figure 4.3. Unlike other methodologies, PSC Neural Induction Medium does not require the intermediary step of embryoid body (EB) formation which adds time, labor, and variability.

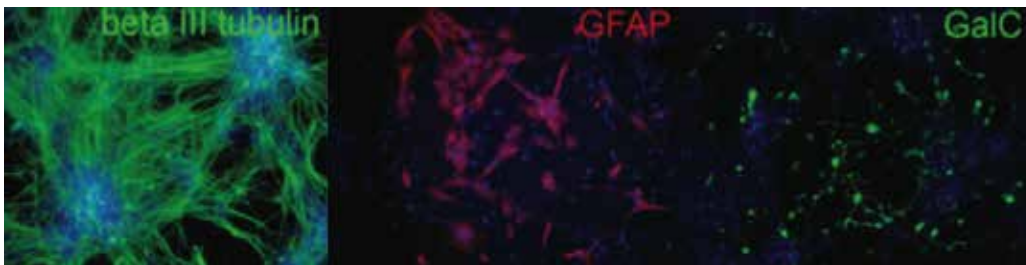


Figure 4.4. Neural Stem Cells (NSCs) generated using PSC Neural Induction Medium have high expression of NSC markers and can be further differentiated into other neural cell types.

4.3

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, for cardiotoxicity screening during drug development.

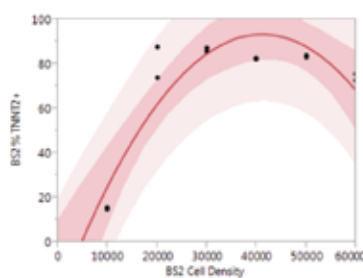
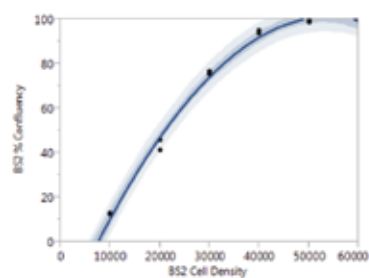
PSC Cardiomyocyte Differentiation Kit

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 (Figure 4.5). 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.

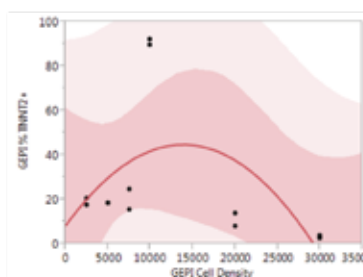
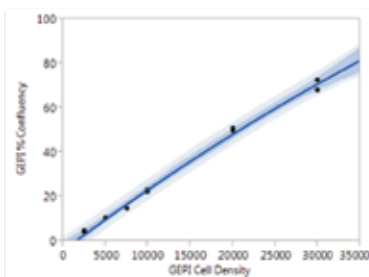
Comprised of three 1X media that require no thawing or mixing, each medium is used consecutively over a total of 14 days (Figure 4.6), resulting in functional cardiomyocytes that express relevant physiological markers, contract in culture, and can be subsequently maintained in culture for more than 15 days.

Find more information at
thermofisher.com/cardiadiff

iPSC line derived from BJ fibroblasts using CytoTune



Gibco Human Episomal iPSC line derived from CD34+ cord blood cells using EBNA-based episomal system



H9 hESC

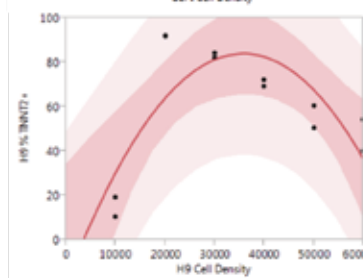
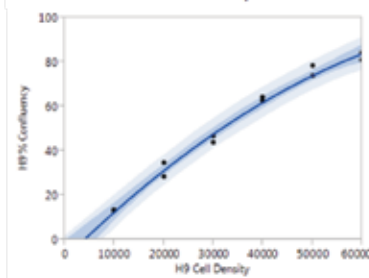


Figure 4.5. Efficiency across multiple PSC lines. Seeding density is crucial for optimal iPSC cardiomyocyte differentiation. TrypLE-dissociated PSC lines were used for setup of these studies. For two CytoTune-derived lines, BS2 iPSC was observed to be promiscuous at higher density. For the Human Episomal iPSC line, it was also found to be optimal at a specific density. For hESC, H9 was observed to be promiscuous at various densities. JMP Profiler tool identified optimal seeding density for efficient differentiation of different PSC lines.

Cardiomyocyte Maintenance Medium (prototype)

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™ Molecular Probes™ Human Cardiomyocyte Immunocytochemistry Kit enables optimal image-based analysis of two key cardiomyocyte markers: NKX2.5 and TNNT2/cTnT. It is the only kit that offers superior imaging 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.

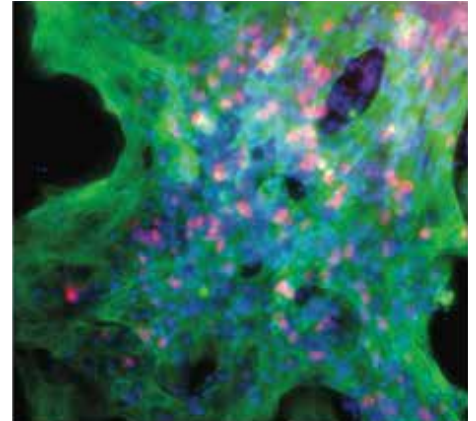


Figure 4.6. iPSCs were differentiated for 14 days using the PSC Cardiomyocyte Differentiation Kit. The cells were stained using the Human Cardiomyocyte Immunocytochemistry Kit for the following markers NKX2.5 (red) for early cardio mesoderm and TNNT2/cTnT (green) for cardiomyocytes along with DAPI nuclear DNA.

4.4

Definitive endoderm

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 and Wnt.

PSC Definitive Endoderm Induction Kit

The Gibco™ PSC Definitive Endoderm Induction Kit consists of two xeno-free media that enable efficient induction of hPSC to definitive endoderm (Figure 4.7). Unlike other methods that require multiple components and take 5 or more days, the PSC Definitive Endoderm Induction Kit enables generation of $\geq 90\%$ CXCR4+/PDGFR α -definitive endoderm cells with only two components in just 2 days (Figure 4.8).

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 key markers SOX17 and FOXA2 across multiple PSC lines (Figure 4.9) and is capable of differentiating to downstream lineages (Figure 4.10).

Find out more at thermofisher.com/defendo

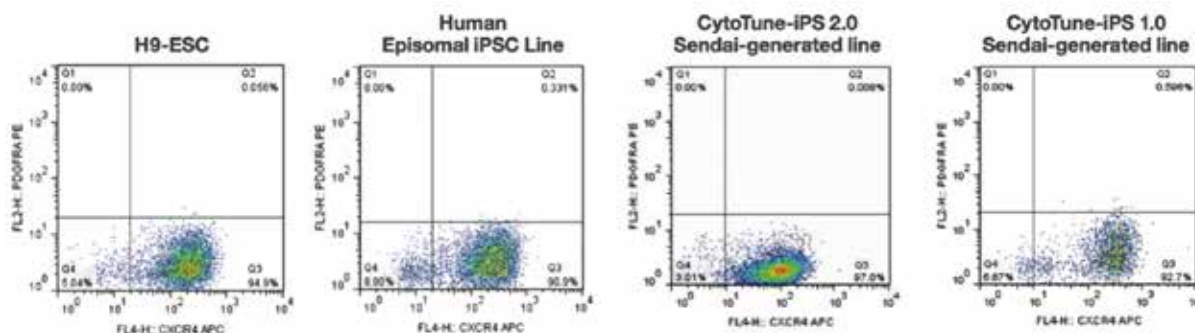
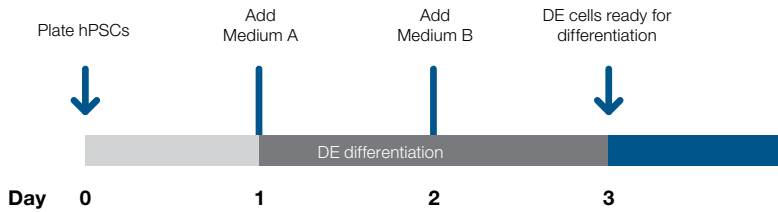


Figure 4.7. The PSC Definitive Endoderm Induction Kit produces definitive endoderm populations with high efficiency ($\geq 90\%$) across hESC and iPSC lines, including cell lines reprogrammed using episomal vectors or CytoTune kits. Representative dot plots show CXCR4+/PDGFR α -cell populations derived from various cell lines. For each experiment, unstained cells were used to set quadrant gates.

PSC Definitive Endoderm Induction Kit



STEMdiff™ Definitive Endoderm Kit

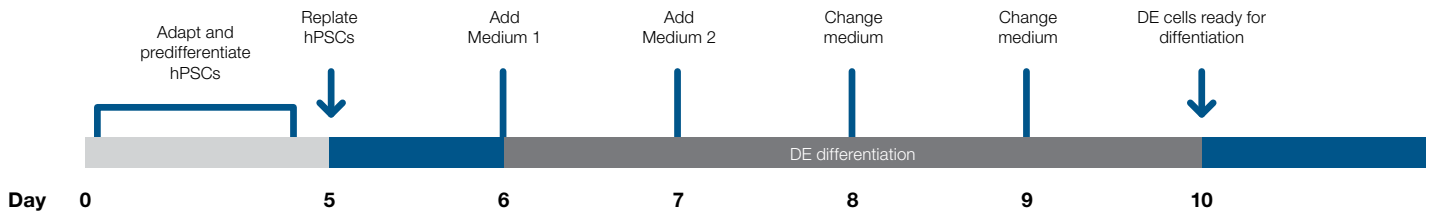


Figure 4.8. Compared to other differentiation protocols, the PSC Definitive Endoderm Induction Kit produces cells in up to 50% less time and requires no predifferentiation or mixing of media.

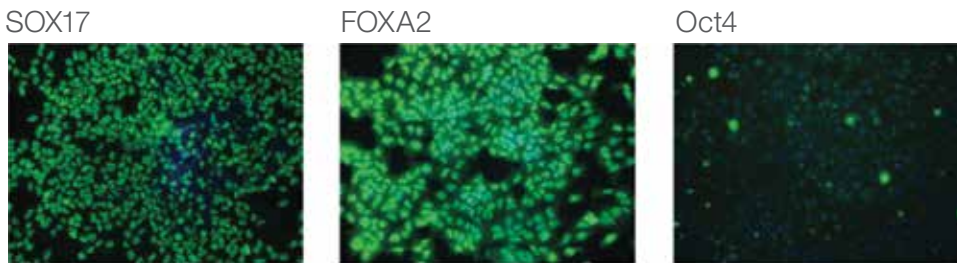


Figure 4.9. Immunocytochemistry of hESCs treated with the PSC Definitive Endoderm Induction Kit. At day 3, induced cells were immunostained for the endodermal transcription factors SOX17 and FOXA2 and the pluripotent marker Oct4. Nuclei were counterstained with DAPI (blue) to assess total cell numbers.

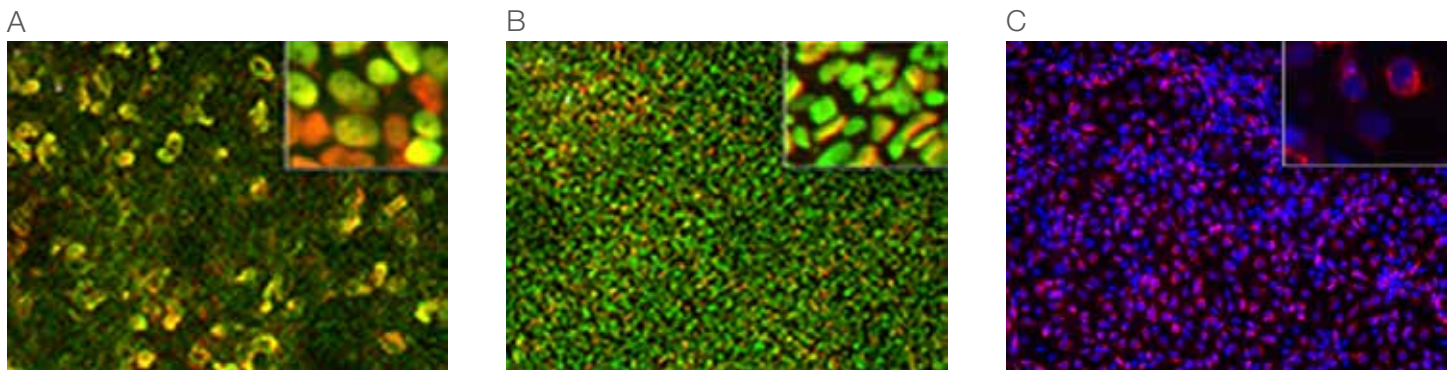


Figure 4.10. Definitive endoderm can be differentiated to downstream lineages. H1 ESCs were treated with PSC Definitive Endoderm Induction Kit media and differentiated into functional cells **(A)** midgut/hindgut (nuclei, blue; FoxA2, green; Cdx2, red), **(B)** pancreatic endoderm (nuclei, blue; FoxA2, green; Pdx1, red), and **(C)** liver bud progenitors (nuclei, blue; AFP, red) that express relevant physiological markers. Data credited to LT Ang, KM Loh, and B Lim of the Genome Institute of Singapore.

Useful tips

Definitive endoderm differentiation:

- It is critical to use high-quality hPSCs (with minimal or no differentiated colonies) that are karyotypically normal, confirmed to exhibit pluripotency markers, and are routinely passaged every three days for at least three passages before starting differentiation. Additionally, we recommend that the PSC line not be used past 100 passages.
- Depending on the cell reprogramming technology, incomplete conversion of adult cell type into induced pluripotent state may lead to the generation of refractory cell lines that are unable to differentiate into some lineages. We recommend inclusion of a positive control cell line like the H7 or H9 hESC line to assess the ability of your iPSC line to differentiate into your cell type of interest.

Neural differentiation:

- 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 4.11.
- Culture conditions for NSC differentiation frequently also generate a contaminating population of neural crest, a highly proliferative and migratory population of cells that *in vivo* gives rise to a range of cell types including peripheral neurons and glia, bone, cartilage, and melanocytes. Neural crest contaminants can be identified by their expression of CD271 (NGFR/p75) and HNK-1. The maintenance medium for the PSC Neural Induction Medium does not promote the expansion of neural crest contaminants; however, NSC cultures generated via other protocols may exhibit significant levels of neural crest contamination. If these flat and highly migratory cells are observed in the dish, they should be removed by positive or negative selection to avoid overpopulation of the culture by these highly proliferative cells.

Cardiomyocyte differentiation:

- Singularizing PSCs for differentiation to cardiomyocytes allows better seeding and confluence estimates, resulting in more consistent results well-to-well and overall better differentiation of difficult-to-differentiate lines. A recommended protocol for plating singularized cells for cardiomyocyte differentiation can be found at [thermofisher.com/cardioidiffprotocol](https://www.thermofisher.com/cardioidiffprotocol)

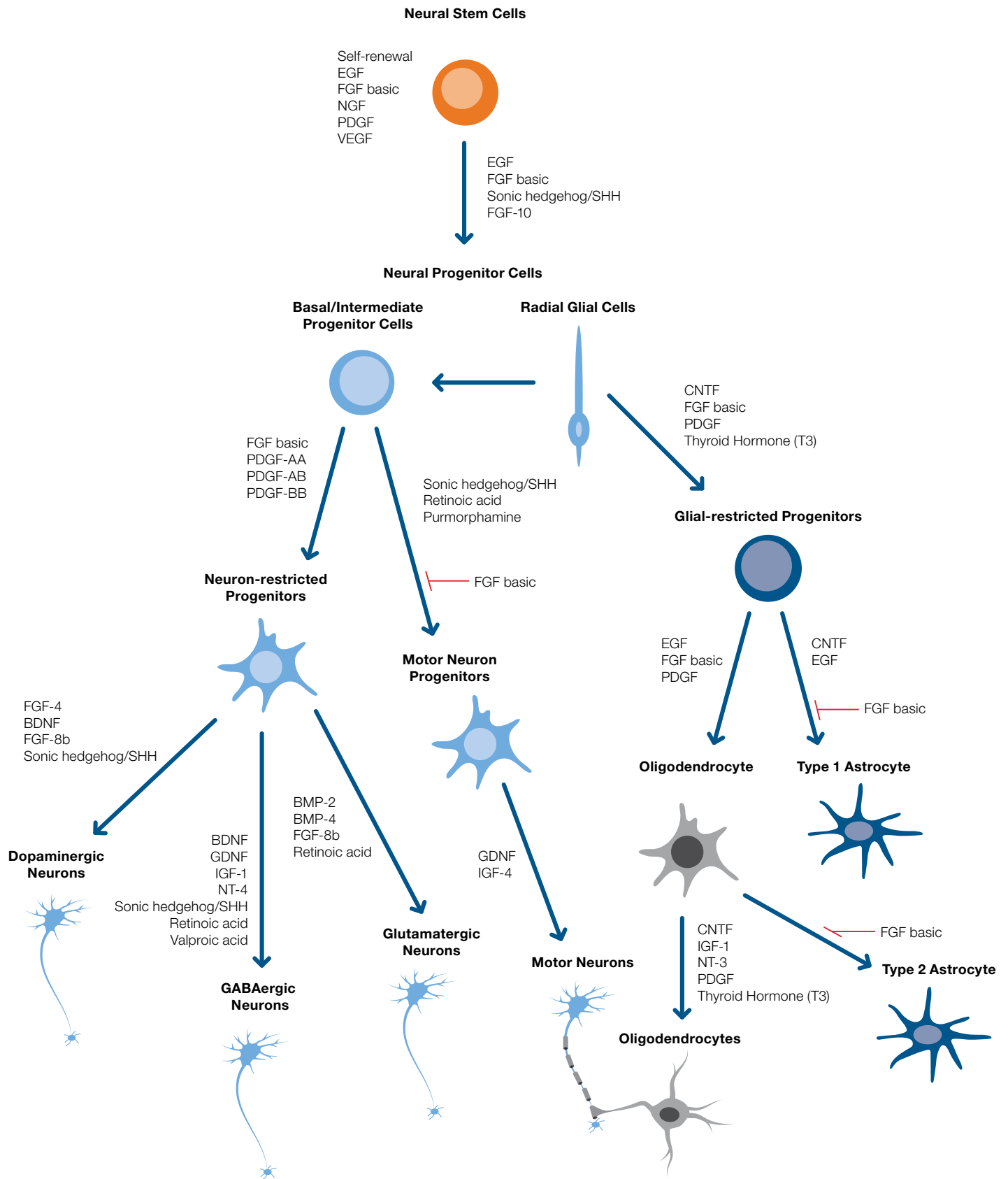


Figure 4.11. Summary of key signaling pathways regulating the differentiation of NSCs to specific neural and neuronal subtypes.

Differentiation functional 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.

Neuronal functional and cell health assays

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 multielectrode arrays to determine neuronal subtype-specific 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 4.1) in the presence of various cell stressors to assess neural cell health (Figure 4.12). Additional levels of complexity can be obtained with all of these assays by coculturing neurons and glial cells to isolate cell-autonomous from nonautonomous disease phenotypes or to determine neuroprotective effects of glia.

Table 4.1. 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	Fluorescent 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
MitoSOX Red Mitochondrial Superoxide Indicator	Mitochondrial superoxide levels	Increased red fluorescence with increased mitochondrial superoxide levels

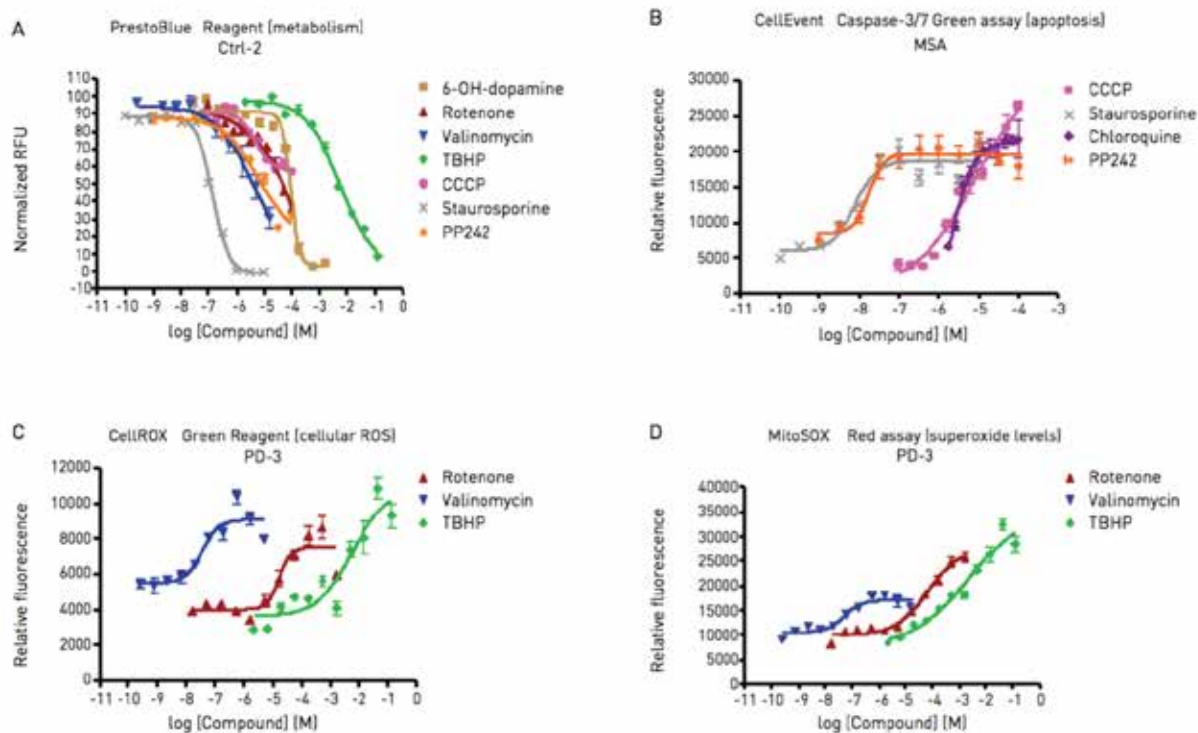


Figure 4.12. A panel of functional assays was used to assess the health of NSCs in response to various cell stressors. iPSC lines were derived from a Parkinson's disease (PD)-affected donor (PD-3), one multiple systems atrophy (MSA)-affected donor, and two age-matched, healthy control individuals (Ctrl-1 and Ctrl-2) and differentiated into a neural stem cell (NSC) population using PSC Neural Induction Medium. The derived NSCs were expanded on 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 CELLstart Substrate-coated 384-well assay plates for evaluation by four high-throughput assays. A Tecan Safire™ reader was used to measure fluorescence. Representative results are shown for **(A)** the PrestoBlue assay on Ctrl-2, demonstrating the expected loss in metabolic activity with an increase in the concentration of stressors added, **(B)** the CellEvent Caspase-3/7 Green assay on MSA, demonstrating the expected increase in apoptosis with an increase in the concentration of stressors added, **(C, D)** the multiplexed CellROX Green assay and MitoSOX Red assay on PD-3, demonstrating the expected increase in oxidative stress with an increase in the concentration of stressors added.

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 (Figure 4.13).

Cardiomyocyte beating patterns can also be measured using multielectrode arrays and parameters such as peak count, peak frequency, peak amplitude, peak width, and decay time can be determined in disease versus control cells or in the presence or absence of cardiotoxic compounds (Figure 4.14).

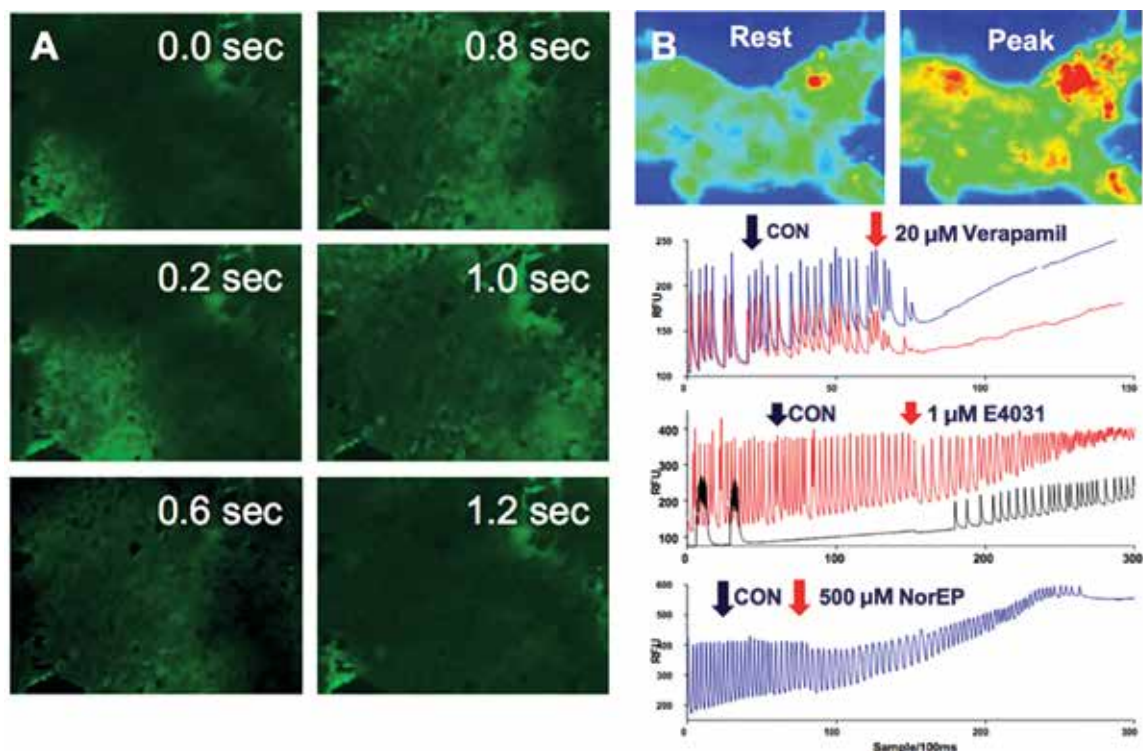


Figure 4.13. Fluo-4 NW Calcium Assay imaging. (A) H9-derived cardiomyocytes were labeled with the Fluo-4 Calcium Imaging Kit, exchanged with Cardiomyocyte Maintenance Medium and imaged on an EVOS FL Auto Imaging System at 4X magnification using liteCam™ HD software capturing 30 frames per second. (B) Calcium imaging of cells and no wash. Spontaneous calcium transients were imaged at 100 ms intervals with a TILL Polychrome (FEI Systems) and regions of interest captured for plotting vs. time. Carrier control or drug containing solution was added as indicated from a 10X stock in Live Cell Imaging Solution.

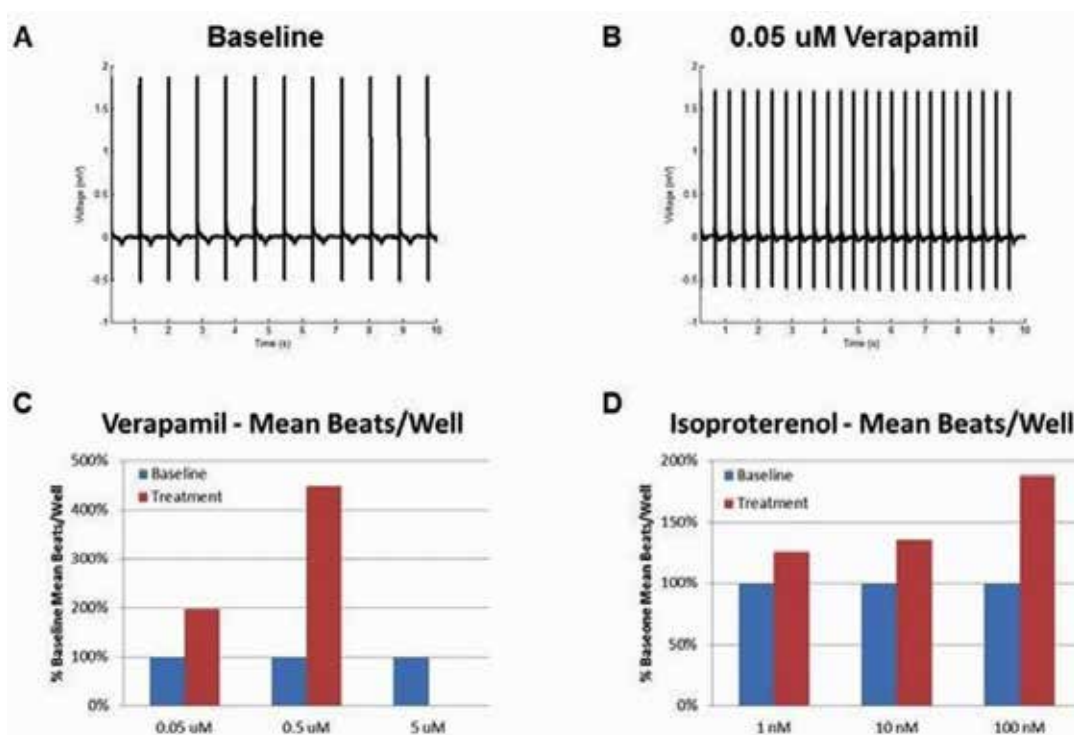
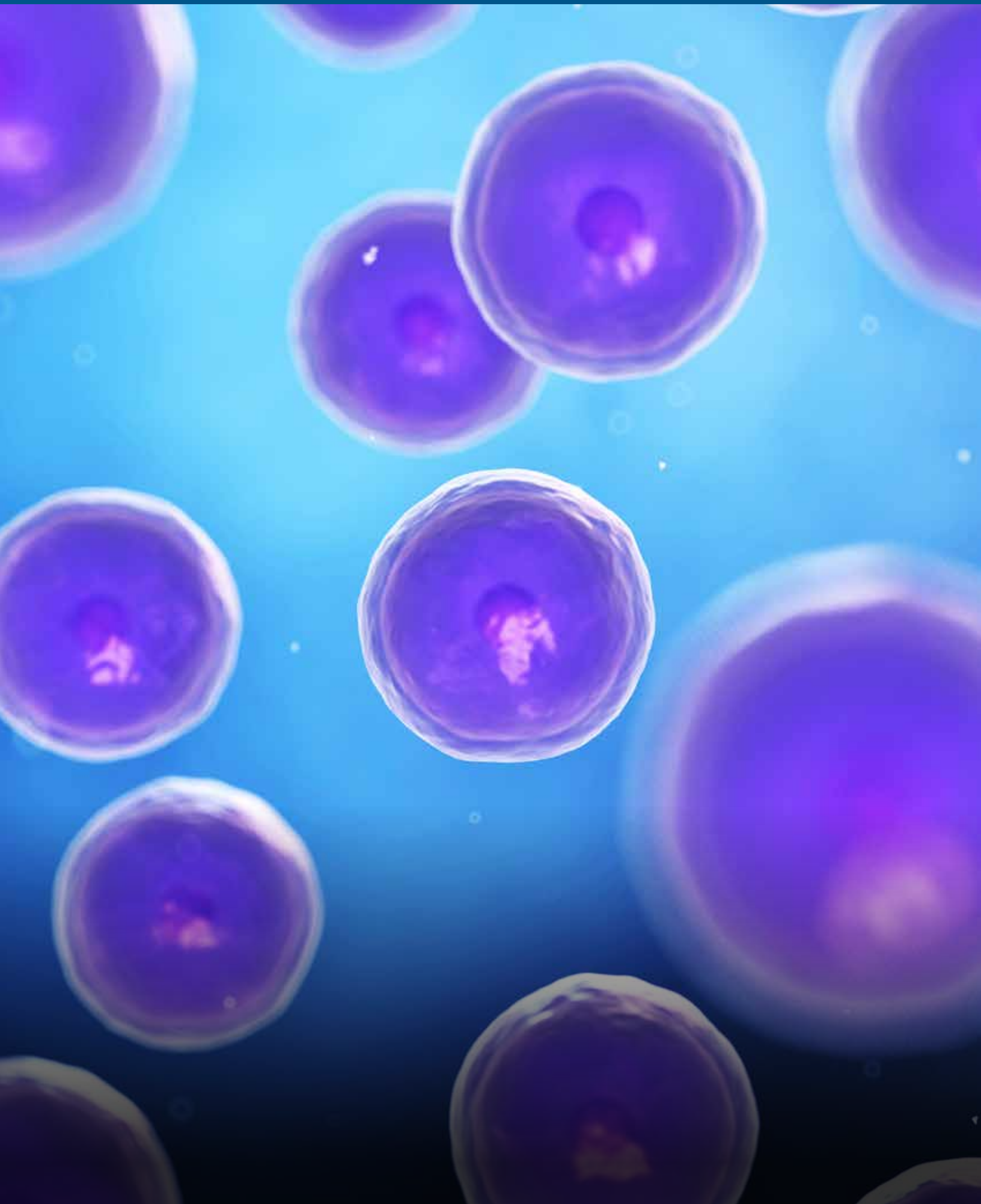


Figure 4.14. Cardiomyocyte response to known cardiotoxicants, showing that cardioactive compounds can modulate PSC-derived cardiomyocyte contraction. After stabilization of electrode activity, spontaneously contracting cardiomyocytes plated at 4×10^4 cells/well on fibronectin-coated multielectrode arrays wells, signals at baseline and in response to drug treatment were averaged over a three-minute period. **(A, B)** Cardiomyocyte waveform under baseline or verapamil treatment conditions. **(C)** Effect of verapamil, an L-type Ca^{2+} channel blocker, on the spontaneous beat rate of H9-derived cardiomyocytes; at the highest dose level, cardiomyocyte contraction ceased. **(D)** Effect of isoproterenol, a beta-adrenergic receptor agonist, on the spontaneous beat rate of H9-derived cardiomyocytes.



Section 5

Characterization

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.

Visit [thermofisher.com/characterization](https://www.thermofisher.com/characterization) to find the right monitoring and detection tools for your research.

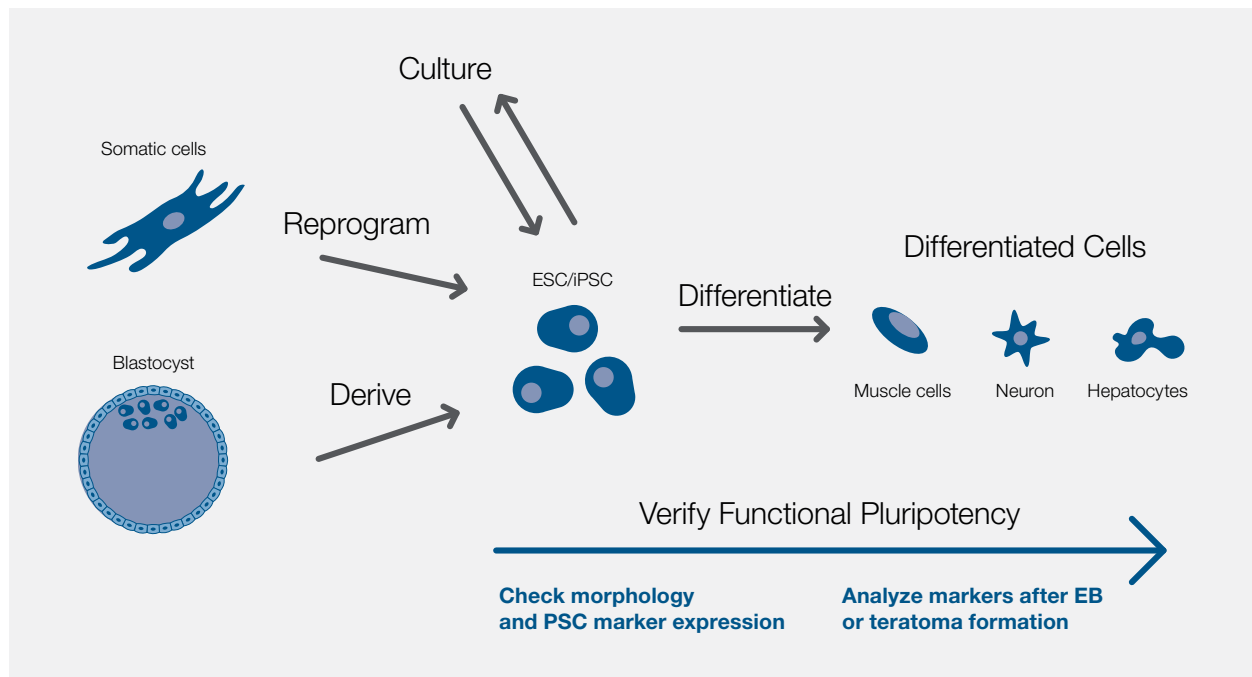


Figure 5.1. PSC characterization practices. Characterization is performed to check the functional pluripotency of newly derived PSC lines.

During the derivation of iPSCs and ESCs, characterization is performed to confirm that a pluripotent line has truly been obtained (Figure 5.1). 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 scale-up scheme and the allotment of cells is shown in Figure 5.2.

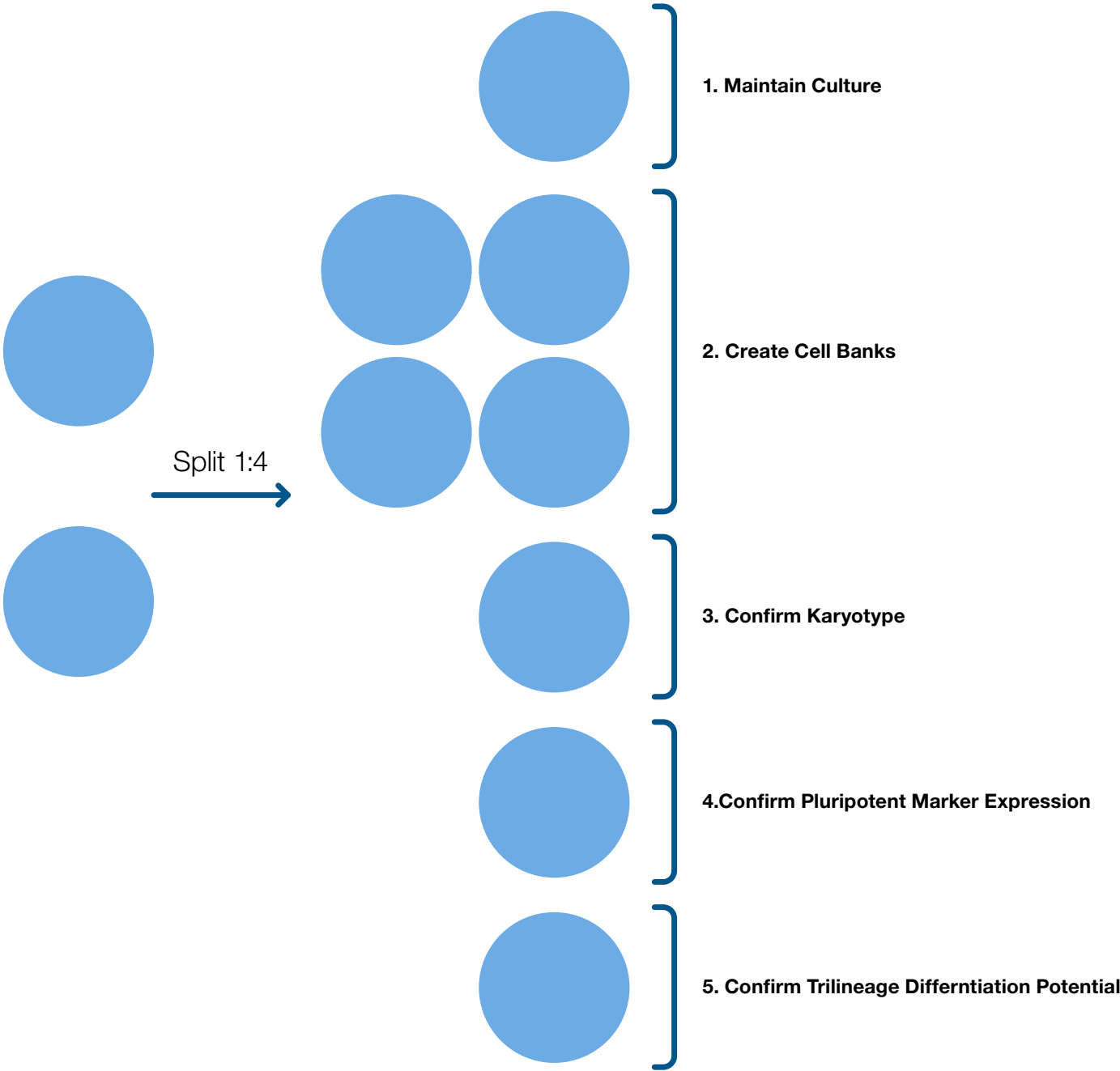


Figure 5.2. Generation of undifferentiated and differentiated cells for karyotyping, cell banking, and characterization. It is recommended that newly derived iPSC lines be scaled up to about seven 6 cm dishes and a T25 flask in order to maintain the culture while karyotyping, performing marker analyses, and creating cell banks. The same approach can be used for PSCs grown on feeders or under feeder-free conditions.

Detecting self-renewal marker expression

Live staining methods

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 or 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

AP is an enzyme that is upregulated in PSCs. AP expression can be detected using the Invitrogen™ Molecular Probes™ Alkaline Phosphatase Live Stain, which consists of a substrate that selectively fluoresces as a result of AP activity¹. 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 5.3).

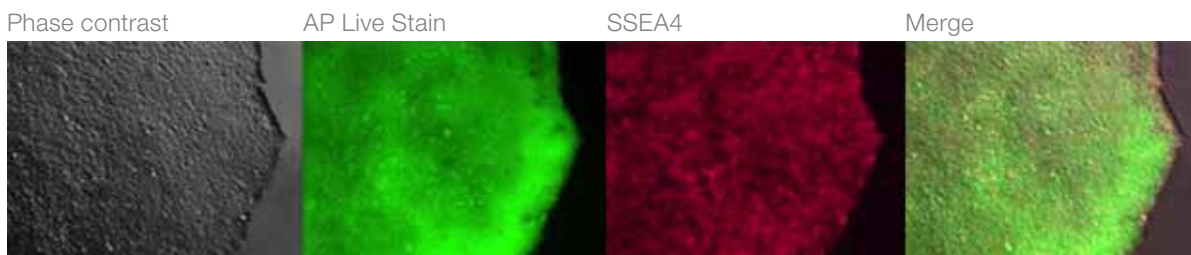


Figure 5.3. Detection of AP in live PSCs. Feeder-free PSCs were analyzed using the AP Live Stain (green), with counterstaining done using an antibody for the PSC marker SSEA4 (red).

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, 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 reprogramming⁴. 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 marker³ (Figure 5.4).

Both TRA-1-60 and CD44 can be detected using live imaging kits that are designed to maximize the signal-to-noise ratio and allow continued culture of cells through the use of live-qualified Molecular Probes™ Alexa Fluor™ dye-conjugated antibodies and optically clear Gibco™ FluoroBrite™ DMEM (Figures 5.5 and 5.6). Both are available with three different fluorophores to accommodate commonly used fluorescence filters and can be used for two applications:

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

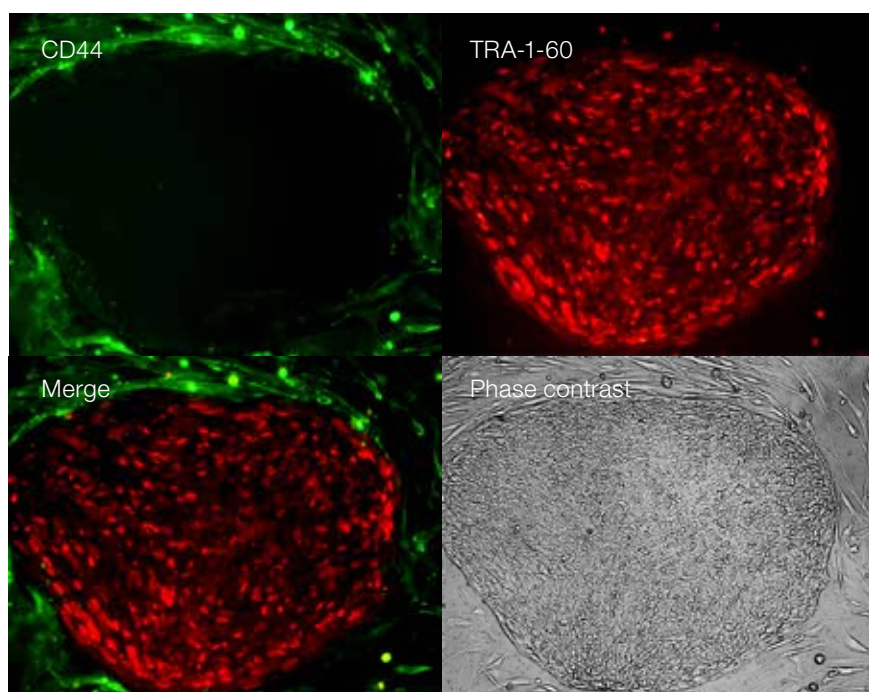


Figure 5.4. Live cell imaging of iPSCs. iPSC colony cultured on mouse embryonic feeder layer and stained using Alexa Fluor conjugated antibodies for fibroblast marker CD44 and PSC marker TRA-1-60. Imaging was performed after replacing the staining medium with FluoroBrite DMEM imaging medium.

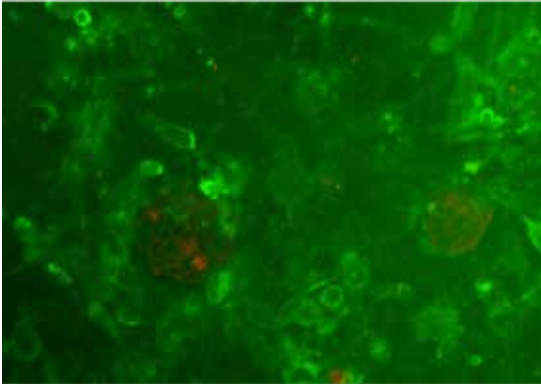


Figure 5.5. Standard media contain phenol red and other sources of background fluorescence.

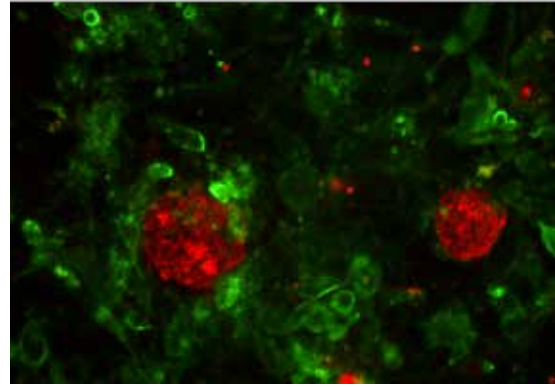


Figure 5.6. FluoroBrite DMEM included in the live imaging kits exhibits optical clarity similar to PBS and supports cell survival post-staining.

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 as SSEA4 and TRA-1-60 (Figure 5.7). Antibodies that can be used

for this purpose include a monoclonal SSEA4 antibody conjugated to Alexa Fluor 647 and an unconjugated monoclonal TRA-1-60 antibody used with a secondary antibody like Alexa Fluor 594 Goat Anti-Mouse IgG (H+L) Antibody.

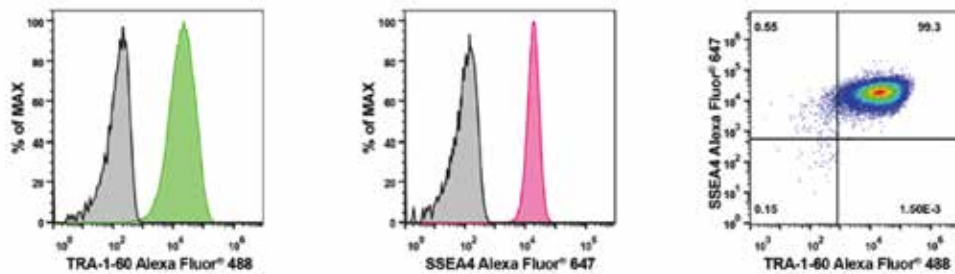


Figure 5.7. Flow cytometry analysis of a feeder-free PSC culture. Isotype controls (gray) are used to determine the percentage of cells expressing TRA-1-60 (green) and SSEA4 (magenta). Dual staining permits the quantification of the number of cells in the culture that are expressing both markers. Typically, >95% SSEA4+/TRA-1-60+ cells are expected. Flow cytometry was performed using the Attune NxT 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 the transcription factors OCT4 and SOX2, which are known to play key roles in maintaining pluripotency² (Figure 5.8). 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™ Molecular Probes™ 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 the specific and simultaneous assessment of two markers at a time.

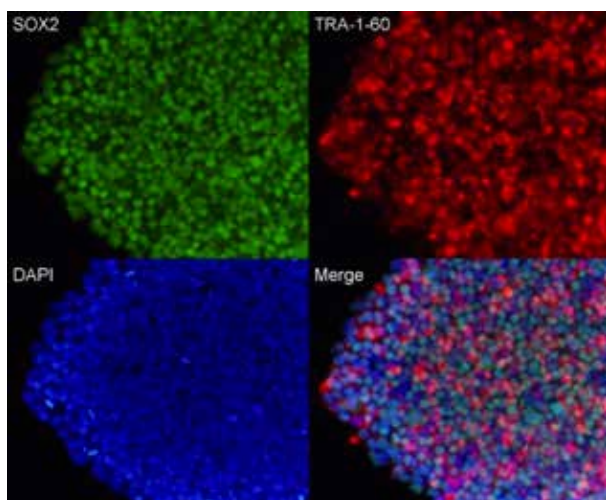


Figure 5.8. Analysis of intracellular markers in fixed PSCs. iPSCs derived from CD34+ cord blood were grown under feeder-free conditions using Essential 8 Medium in wells coated with vitronectin. The cells were stained for pluripotency markers SOX2 (green) and TRA-1-60 (red) using the PSC 4-Marker Immunocytochemistry Kit.

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 to 21 days and

are typically transferred into adherent cultures after the first few days. Although the differentiation of EBs occurs under non-physiological conditions, EB formation has advantages over the teratoma formation not only because it takes much less time, but also because it 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, alpha-fetoprotein (AFP) for endoderm, and beta-III tubulin (TUBB3/TUJ1) for ectoderm (Figure 5.9)³. These three markers can be detected using the Invitrogen™ Molecular Probes™ 3-Germ Layer Immunocytochemistry Kit, which includes a complete set of high performance primary and secondary antibodies, a nuclear DNA stain, and premade buffers for an optimized staining experiment.

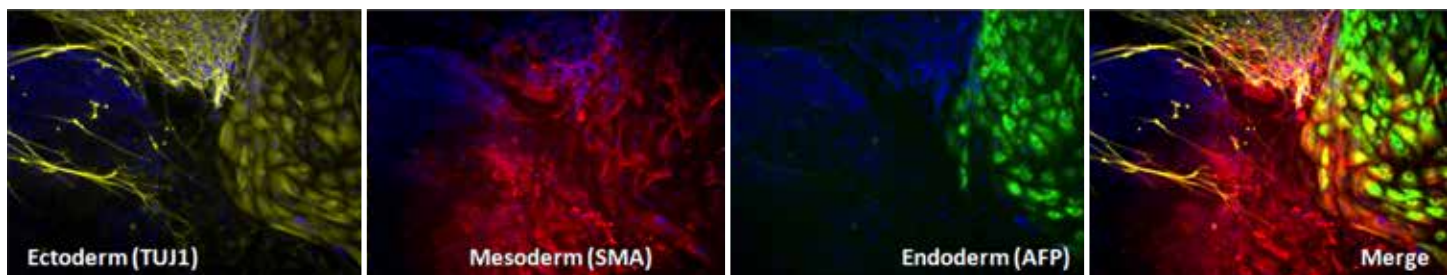


Figure 5.9. Cellular analysis of EBs. EBs from H9 ESCs were allowed to spontaneously differentiate for 23 days prior to staining beta-III tubulin (TUJ1, yellow), smooth muscle actin (SMA, red), and alpha-fetoprotein (AFP, green) against a DAPI nuclear counterstain (blue) using the 3-Germ Layer Immunocytochemistry Kit (A25538).

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 5.10). 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.

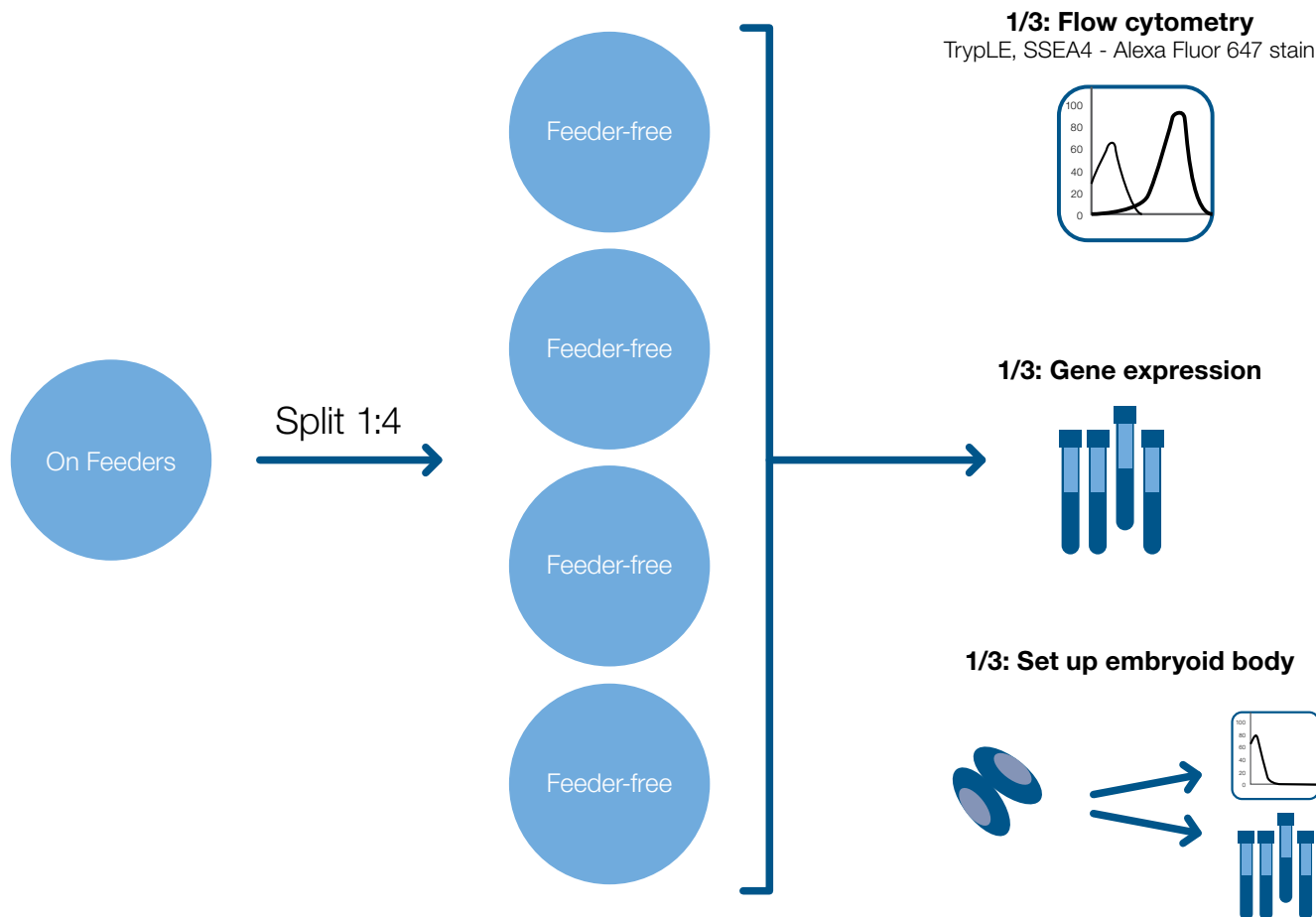


Figure 5.10. Generation of undifferentiated and differentiated PSCs for molecular analysis. When performing molecular analyses, it is best to simultaneously check the purity and quality of the PSCs, as well as confirm the differentiation of the EBs through flow cytometry. Scaling up to about four 6 cm dishes generates sufficient cells for both molecular and flow analyses.

The Applied Biosystems™ TaqMan™ hPSC Scorecard™ Panel utilizes qRT-PCR, 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 CytoTune-iPS Sendai Reprogramming Kit (Figure 5.11). The assay utilizes bioinformatics analysis based on the work of Bock et al.⁵ 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 5.12).

As such, this permits the analysis of not only undifferentiated PSCs, but also the derivative EBs to determine functional pluripotency⁶. 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, visit [thermofisher.com/scorecard](https://www.thermofisher.com/scorecard)

Colors correlate to the fold change in expression of the indicated gene relative to the undifferentiated reference set.

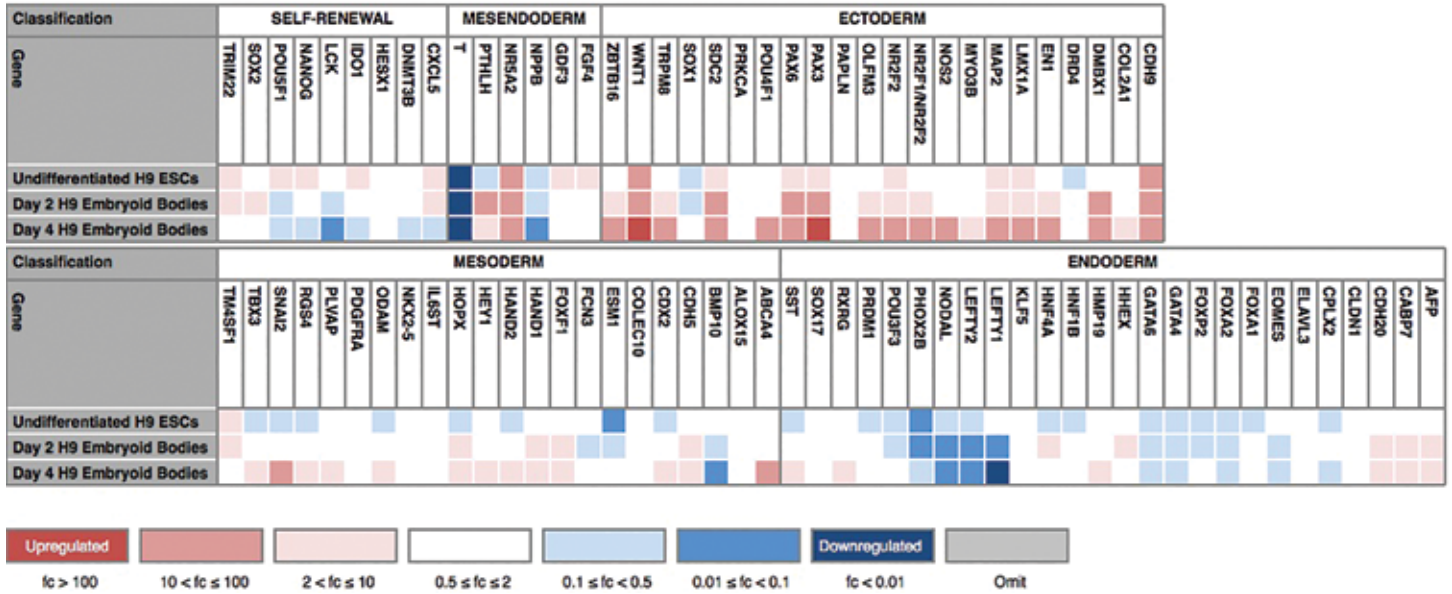


Figure 5.11. TaqMan hPSC Scorecard analysis of an EB formation time course using H9 ESCs. 93 genes are analyzed as part of the TaqMan hPSC Scorecard Panel. Colors correlate to the fold change in expression relative to the reference set. Markers of the undifferentiated state are downregulated over the 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.

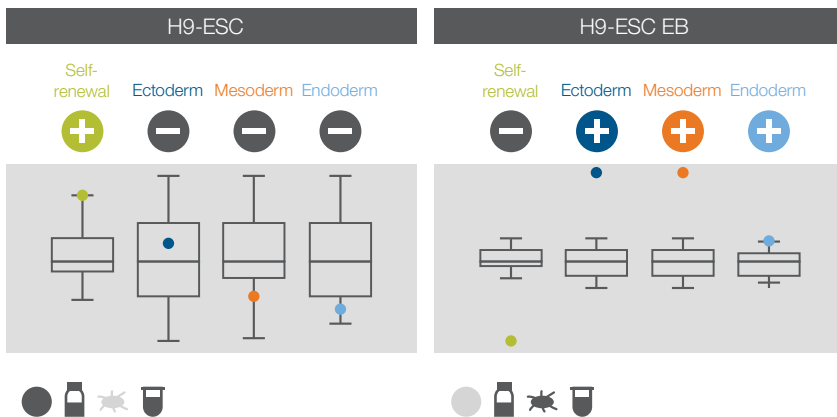


Figure 5.12. TaqMan hPSC Scorecard assay results. H9 ESCs and H9 ESC-derived EBs were analyzed using the TaqMan hPSC Scorecard assay. The comparison of self-renewal and germ layer marker expression against the reference standards is summarized in box plots and in simple pass/fail scores.

Useful tips

Cellular analysis

- Live AP staining: AP is a differential marker that is expressed more robustly in undifferentiated cells relative to unprogrammed 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 analysis.
- 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.
- 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 0.5 million cells are recommended for the standard RNA extraction method using Invitrogen™ Ambion™ TRIzol™ Reagent. However, as few as 100,000 cells can be used with TRIzol Reagent or as few as 15,000 cells can be used with the Ambion™ Cells-to-C_T™ Kit or the Ambion™ 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). It is recommended to include parental somatic cells as a control to eliminate SeV cross-reactivity with the parental line. TaqMan hPSC Scorecard 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.

Molecular analysis

- The TaqMan hPSC Scorecard Panel measures the potential for self-renewal and trilineage differentiation: [thermofisher.com/scorecard](https://www.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, it is recommended to evaluate a time course with 2 to 3 time points of differentiation (e.g., days 7, 10, and 14).

5.4

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.
3. 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.
4. 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.
5. 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.
6. 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.
7. Fergus J, Quintanilla R, Lakshmipathy U (2014). Characterizing pluripotent stem cells using the TaqMan™ hPSC Scorecard™ Panel. *Methods Mol Biol* 1307:25 - 37.



Section 6

Applications for Cell Therapy

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 1998¹, and the first human iPSC was generated in 2007². 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 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 good manufacturing practice (GMP) so that quality control measures are in place to ensure patient safety.

cGMP refers to a quality assurance system that is defined by both the European Medicines Agency and the US Food and Drug Administration as a means of ensuring that clinical-grade cells and products meet preset standards for quality and safety in cell transplantation. 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 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, mycoplasma, endotoxin, 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 pre-qualified 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.

6.2

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 coming from gene modified CAR T Cell immunotherapeutic programs. 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 prepared for the clinic.

Materials, manufacturing, and process considerations

The first thing needed 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

clinical programs and could even change the profile of the therapeutic cells and the clinical application. For these reasons, understanding the sourcing, quality, risk profiles, and having as much information as possible about the components being used is paramount to generating pre-clinical 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, begin to think about the manufacturing process and whether it has the potential to scale. Scale 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

Once raw materials with acceptable risk profiles have been selected, a process providing line of sight to future scale-up is developed, and characterization methods that ensure production of intended cells is confirmed, it is appropriate to start pulling the components of an IND together. 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/clinic
- The raw materials used during the process and how suitability is verified

- The plastic ware 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, it is recommended to schedule at least one pre-IND meeting with the FDA, advising 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.

6.3

Cell Therapy Systems (CTS) products

The Gibco™ Cell Therapy Systems (CTS™) brand 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 ISO 9001. Regardless of the type and source of cells, CTS offers tools to help with every step of the cell therapy workflow and enables progress through each stage of clinical development and scale-up.

CTS products are designed to minimize the risk of contamination and variability in clinical research and provide the required documentation for regulatory review.

All CTS products are supplied with unified documentation including:

- Certificates of Analysis
- Certificates of Origin
- Access to authorization letters for the FDA Drug Master File

For more information on cell therapy products, visit [thermofisher.com/celltherapy](https://www.thermofisher.com/celltherapy)

CTS products for clinical research offer:**Harmonized documentation**

- Traceability documentation including Certificates of Analysis, Certificates of Origin and Drug Master File (DMF). DMFs include full product formulation information.
- Reduced time in preparing investigational new drug (IND) submission
- CTS product labeling and intended use statements

**Seamless transition from research to clinic**

- Defined formulations to minimize lot-to-lot variability
- Scalable cGMP conditions for all products
- Complementary RUO products
- Extensive QA testing for sterility, endotoxin, adventitious agent and mycoplasma on most products

**Expert consultation**

- Regional, accessible technical support experts
- Experienced global professionals to help navigate regulatory processes from research to commercial phase
- Cell therapy expertise to answer questions related to using CTS products for clinical applications
- More than 50 years of Gibco media experience

6.4

Custom Services and scalability

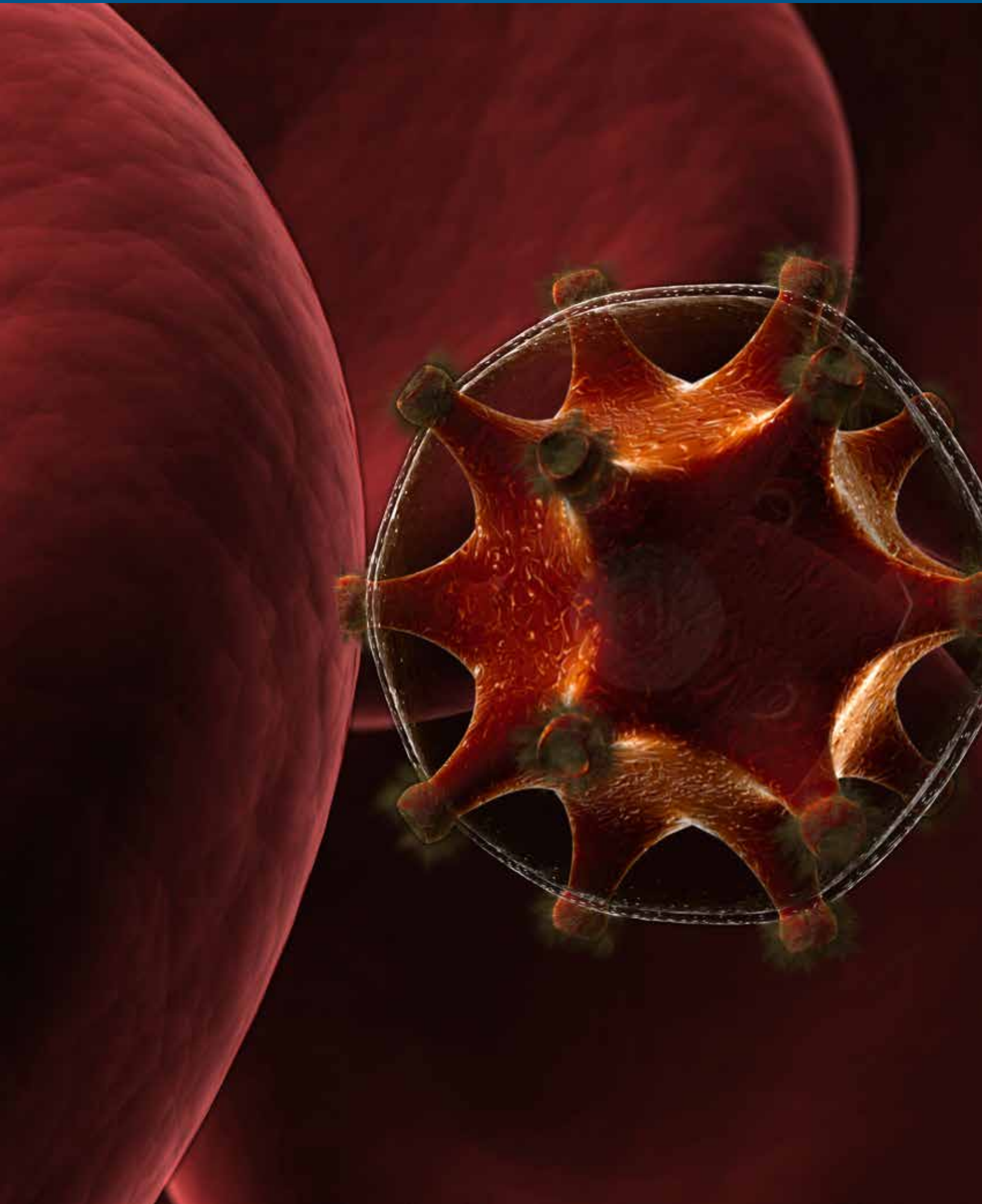
As process development defines manufacturing systems and operating parameters needed to produce a consistent product at a phase appropriate scale, customized containers or media may allow for optimization. Filling media into bags with appropriate connectors that facilitate manufacturing and help close 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 interested in custom CTS services, please send an inquiry to custommedia@thermofisher.com

6.5

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.
3. Chen G, Gulbranson DR, Hou Z, et al. (2011). Chemically defined conditions for human iPS cell derivation and culture. *Nat Methods* 8(5): 424 - 429.



Section 7

CellModel Services

Built on the stem cell innovations introduced throughout the past decade, 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 it easier to “bring in-house” and a custom services offering that utilizes an experienced team of stem cell professionals

Advantages of CellModel services include:

- Detailed protocols provided after project completion to demonstrate each milestone was achieved and to document which tools were utilized
- Availability of all the reagents and media used within the CellModel Service can be purchased and used to facilitate post-service projects
- Exceptional support and frequent project communication provided by a team with extensive experience delivering custom services

Available stem cell services

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

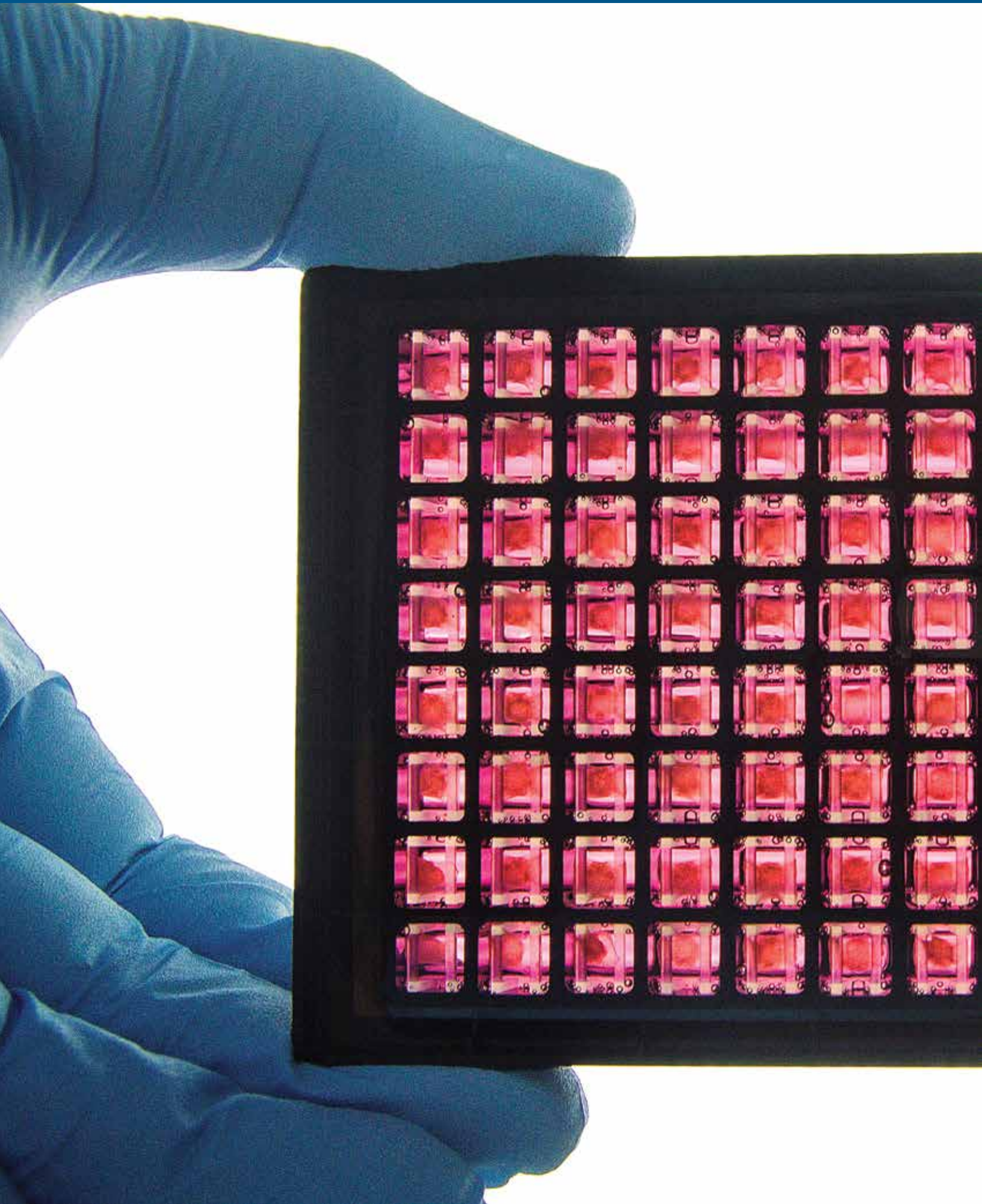
- **Reprogramming**—human fibroblasts or blood cells reprogrammed in 4 to 6 months with top clones expanded, cryopreserved, and characterized

- **Differentiation**—PSCs differentiated into NSCs and terminal lineages
- **Characterization**—confirm pluripotency, gene expression, cell identity, and more
- **Assay development**—customized for validation of stem cell-based assays for interrogation of disease-relevant biology
- **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, who have helped researchers with cellular engineering and custom assay development projects for years. 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.

For more information, go to thermofisher.com/cellmodel



Section 8

Gibco Stem Cell Training Courses

Gibco Stem Cell Training Courses are held at one of three stem cell research centers located in either Carlsbad, CA, USA, Frederick, MD, USA, or Glasgow, UK. These centers provide course attendees with hands-on stem cell training in techniques for culturing and characterizing hESCs and iPSCs, as well as reprogramming techniques for the creation of iPSCs. Whether new to PSC research or in need of a refresher course, experienced R&D scientists provide detailed stem cell training so researchers can feel confident using stem cells in their research.

Training course agenda topics:

- Basic maintenance and care of hESCs and iPSCs
- Freezing, thawing, plating, and passaging techniques
- Culturing PSCs under feeder-dependent and feeder-free conditions
- Reprogramming and identification of iPSCs
- Differentiation and characterization methods for PSCs

Specialized training support:

Each training workshop is structured as a three-day course with both lecture and hands-on laboratory work. Specialized, experienced trainers will guide attendees through a variety of stem cell techniques and work with them one-on-one to help ensure success.

Get more information on the training courses at thermofisher.com/lifelab



Section 9

Ordering information

Ordering Information

Product	Cat. No.
Reprogramming	
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
Lipofectamine 3000 Transfection Reagent	L3000-015
Neon Transfection System	MPK5000
Engineering	
GeneArt CRISPR Nuclease mRNA	A25640
GeneArt CRISPR Nuclease Vector with CD4 Enrichment Kit	A21175
GeneArt CRISPR Nuclease Vector with OFP Reporter Kit	A21174
GeneArt CRISPR T7 Strings DNA	Contact geneartsupport@thermofisher.com
GeneArt CRISPR U6 Strings DNA	Contact geneartsupport@thermofisher.com
GeneArt Genomic Cleavage Detection Kit	A24372
GeneArt Genomic Cleavage Selection Kit	A27663
GeneArt PerfectMatch TALs	Contact geneartsupport@thermofisher.com
GeneArt Precision TALs	Contact geneartsupport@thermofisher.com
GeneArt TALs	Contact geneartsupport@thermofisher.com
Ion PGM Sequencer	Contact geneartsupport@thermofisher.com
Lipofectamine MessengerMAX Transfection Reagent	LMRNA001
TaqMan SNP Genotyping Assay	Contact geneartsupport@thermofisher.com

Product	Cat. No.
Culture	
Basic Fibroblast Growth Factor.	13256-029
Collagenase IV	17104-019
Dispase II	17105-041
DMEM/F-12 with GlutaMAX	10565-018
Essential 6 Medium	A1516401
Essential 8 Flex Medium Kit	A2858501
Essential 8 Medium	A1517001
Geltrex LDEV-Free hESC-qualified Reduced Growth Factor Basement Membrane Matrix	A1413301
Geltrex hESC-qualified, Ready-to-Use, Reduced Growth Factor Basement Membrane Matrix	A15696-01
Gibco Attachment Factor	S006100
Human Episomal iPSC Line	A18945
Knockout Serum Replacement	10828-028
Mouse Inactivated Embryonic Fibroblasts (MEFs)	A24903
RevitaCell Supplement	A2644501
StemPro Accutase Cell Dissociation Reagent	A1110501
StemPro EZPassage Disposable Stem Cell Passaging Tool	23181-010
StemPro hESC SFM	A1000701
TrypLE Select Enzyme	A1217701
UltraPure 0.5M EDTA	15575-020
Versene Solution	15040-066
Vitronectin (VTN-N) Recombinant Human Protein, Truncated	A14700

Product	Cat. No.
Cryopreservation	
PSC Cryopreservation Kit	A26446-01
Recovery Cell Culture Freezing Medium	12648-010
Synth-a-Freeze Cryopreservation Medium	A12542-01
Differentiation	
CellEvent Caspase-3/7 Green Detection Reagent	C10423
CellROX Green Reagent	C10444
EVOS FL Auto Imaging System	AMAFD1000
Fluo-4 Calcium Imaging Kit	F10489
MitoSOX Red Mitochondrial Superoxide Indicator	M36008
PrestoBlue Cell Viability Reagent	A13261
PSC Cardiomyocyte Differentiation Kit	A2921201
PSC Definitive Endoderm Induction Kit	A27654-SA
PSC Neural Induction Medium	A1647801
StemPro NSC SFM	A1050901
Characterization	
3-Germ Layer Immunocytochemistry Kit	A25538
Alkaline Phosphatase Live Stain	A14353
CD44 Alexa Fluor 488 Conjugate Kit for Live Cell Imaging	A25528
TaqMan™ Gene Expression Cells-to-C _T ™ Kit	4399002
CellsDirect One-Step RT-qPCR Kit	11753-100
FluoroBrite DMEM	A1896701
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
Pluripotent Stem Cell 4-Marker Immunocytochemistry Kit	A24881
Pluripotent Stem Cell Immunocytochemistry Kit (OCT4, SSEA4)	A25526

Product	Cat. No.
Characterization (continued)	
Pluripotent Stem Cell Immunocytochemistry Kit (SOX2, TRA-1-60)	A25525
SSEA-4 Antibody, Alexa Fluor 647 Conjugate	SSEA421
TaqMan hPSC Scorecard Kit, 384-well	A15872
TaqMan hPSC Scorecard Kit, Fast 96-well	A15871
TaqMan hPSC Scorecard Panel, 384-well	A15870
TaqMan hPSC Scorecard Panel, Fast 96-well	A15876
TaqMan Human Stem Cell Pluripotency Array	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 Antibody	41-1000
TRIzol Reagent	15596018
Cell therapy	
CTS B-27 Supplement, XenoFree	A1486701
CTS CELLstart Substrate	A1014201
CTS Essential 8 Medium	Contact cts@thermofisher.com
CTS GlutaMAX-I Supplement	A1286001
CTS Immune Cell Serum Replacement	A2596-01
CTS KnockOut DMEM/F-12	A13708-01
CTS KnockOut SR XenoFree Medium	12618-012
CTS StemPro MSC SFM	A1033201
CTS Synth-a-Freeze Cryopreservation Medium	A1371301
CTS TrypLE Select Enzyme	A1285901

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