

CTS[™] CytoTune[™] -iPS Sendai 2.1 Reprogramming Kit USER GUIDE

For efficient, integration-free reprogramming of somatic cells
into induced pluripotent stem cells (iPSC)

Catalog Number A34546
Publication Number MAN0016994
Revision 3.0



Life Technologies Corporation | 7335 Executive Way | Frederick, MD 21704 | USA

For descriptions of symbols on product labels or product documents, go to [thermofisher.com/symbols-definition](https://www.thermofisher.com/symbols-definition).

The information in this guide is subject to change without notice.

DISCLAIMER: TO THE EXTENT ALLOWED BY LAW, THERMO FISHER SCIENTIFIC INC. AND/OR ITS AFFILIATE(S) WILL NOT BE LIABLE FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE, OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING YOUR USE OF IT.

Revision history: Pub. No. MAN0016994

Revision	Date	Description
3.0	17 December 2019	Update user guide with new xeno-free protocols for CD34+ cells and PBMC using CTS™ StemPro™ HSC Expansion Medium
2.0	25 June 2018	Update product SKUs Update protocol Use Corp. entity statement
1.0	13 July 2017	New document

Important Licensing Information: This product may be covered by one or more Limited Use Label Licenses. By use of this product, you accept the terms and conditions of all applicable Limited Use Label Licenses.

TRADEMARKS: All trademarks are the property of Thermo Fisher Scientific and its subsidiaries unless otherwise specified. TaqMan is a trademark of Roche Molecular Systems, Inc., used under permission and license. CytoTune is a trademark of ID Pharma. Essential 8 is a trademark of Cellular Dynamics International. Parafilm is a registered trademark of Bemis Company, Inc. Triton is a trademark of Union Carbide Corporation. TRIzol is a registered trademark of Molecular Research Center, Inc. Polybrene is a registered trademark of Abbott Laboratories Corp.

©2019 Thermo Fisher Scientific Inc. All rights reserved.

Contents

■	CHAPTER 1	Product information	7
	Product description		7
	Contents and storage		7
	Reduced efficiency		7
	Description of the system		8
	Induced pluripotent stem cells (iPSC)		8
	CTS™ CytoTune™ -iPS 2.1 reprogramming system		8
	Sendai virus		8
	CTS™ CytoTune™ 2.1 reprogramming vectors		9
	Advantages of CTS™ CytoTune™ -iPS 2.1 sendai reprogramming kit		9
	Safety features of the system		10
	Sendai virus (SeV) safety information		10
	Non-transmissible CTS™ CytoTune™ 2.1 sendai reprogramming vectors		10
	Biosafety level 2		10
■	CHAPTER 2	Before you begin	11
	Guidelines for generating iPSCs		11
	Experimental guidelines		11
	Positive control		11
	CytoTune™ -EmGFP sendai fluorescence reporter		12
■	CHAPTER 3	Reprogram fibroblasts	13
	Experiment outline (xeno-free)		13
	Workflow		13
	Reprogramming timeline		13
	Reduced efficiency		13
	Reprogram fibroblasts (xeno-free)		14
	Media for reprogramming fibroblasts (xeno-free)		14
	Required materials		14
	Reprogram fibroblasts		15

■	CHAPTER 4	Reprogram PBMCs	20
	Experiment outline (xeno-free)		20
	Workflow		20
	Reprogramming timeline		20
	Reduced efficiency		21
	Reprogram peripheral blood mononuclear cells (PBMCs) (xeno-free)		21
	Media for reprogramming PBMCs (xeno-free)		21
	Required materials		21
	Reprogramming protocol		22
■	CHAPTER 5	Reprogram CD34⁺ cells	27
	Experiment outline (xeno-free)		27
	Workflow		27
	Reprogramming timeline		27
	Reduced efficiency		28
	Reprogram CD34 ⁺ cells (xeno-free)		28
	Media for reprogramming CD34 ⁺ cells (xeno-free)		28
	Required materials		28
	Cells		29
■	CHAPTER 6	Reprogram T-cells	33
	Experimental outline (xeno-free)		33
	Workflow		33
	Reprogramming timeline		33
	Reduced efficiency		33
	Reprogram T-Cells (xeno-free)		34
	Media for reprogramming T-Cells (feeder-free)		34
	Required materials		34
	Reprogramming protocol		35
■	CHAPTER 7	Identify and pick iPSC colonies	39
	Visual identification		39
	Visual identification		39
	Live stain		39
	Live stain with antibodies		39
	Required antibodies		39
	Live stain cells		39
	Pick iPSC colonies		40
	Pick iPSC colonies (feeder-free)		40

■	CHAPTER 8	Generate vector-free iPSCs	42
	Generate vector-free iPSCs		42
	Guidelines for generating vector-free iPSCs		42
	Required materials		42
	Generate vector-free iPSCs		43
	Immunocytochemistry with anti-SeV antibodies		43
	RT-PCR protocol for detecting the SeV genome and transgenes		43
	RT-PCR primer		44
	RT-qPCR		45
■	CHAPTER 9	Troubleshooting	46
■	APPENDIX A	Media and reagents	47
	Prepare media and reagents		47
	SCF (c-kit Ligand), FLT-3 Ligand, IL-3, IL-6, and TPO stock solutions		47
	0.5 mM EDTA in DPBS (50 mL)		47
	Complete fibroblast medium		47
	Complete xeno-free fibroblast medium		47
	Essential 8 [™] Medium (for 500 mL of complete medium)		48
	CTS [™] StemPro [™] HSC Expansion Medium (for 100 mL of complete medium)		48
	OpTmizer [™] CTS [™] T-Cell expansion medium (for 1000 mL of complete medium)		49
	Essential 8 [™] freezing medium		49
■	APPENDIX B	Prepare culture vessels	51
	Coat culture vessels with vitronectin		51
	Vitronectin working concentration		51
	Coat culture vessels with vitronectin		52
	Coat culture vessels with rhLaminin-521		53
	rhLaminin-521 working concentration		53
	Coat culture vessels with rhLaminin-521		54
■	APPENDIX C	Support protocols	55
	CytoTune [™] -EmGFP reporter control transduction		55
	CytoTune [™] -EmGFP sendai fluorescence reporter		55
	Guidelines for using the CytoTune [™] EmGFP sendai fluorescence reporter		55
	Control transduction protocol for adherent cells		55
	Expected results		57
	Passage iPSCs with EDTA		58
	Passaging protocol		58
	Cryopreserve iPSCs		59
	Freeze iPSCs in Essential 8 [™] freezing medium		59

- **APPENDIX D** Ordering information 61
 - Accessory products 61
 - RT-qPCR 63
- **APPENDIX E** Safety 64
 - Chemical safety 65
 - Biological hazard safety 66
- Documentation and support 67**
 - Customer and technical support 67
 - Limited product warranty 67



Product information

Product description

The CTS™ CytoTune™ -iPS 2.1 Sendai Reprogramming Kit contains three CTS™ CytoTune™ 2.1 reprogramming vectors that are used for delivering and expressing key genetic factors necessary for reprogramming somatic cells into iPSCs.

IMPORTANT! This product must be used under Biosafety Level 2 (BL-2) containment with biological safety cabinet and laminar flow hood, and with appropriate personal safety equipment to prevent mucosal exposure/splash. For more information on BL-2 guidelines, see “Biosafety level 2” on page 10.

Contents and storage

Component ^[1]	Cap color	Amount ^[2]	Storage
CTS™ CytoTune™ 2.1 KOS	Orange	0.2 mL	≤ -70°C
CTS™ CytoTune™ 2.1 hL-Myc	Brown	0.2 mL	
CTS™ CytoTune™ 2.1 hKlf4	Red	0.2 mL	

^[1] The titer of each CTS™ CytoTune™ 2.1 reprogramming vector is lot-dependent. For the specific titer of your vectors, refer to the Certificate of Analysis (CoA) available on our website. Go to www.thermofisher.com/cytotune and search for the CoA by product lot number, which is printed on the vial.

^[2] Each vial containing 0.2 mL of one of the CTS™ CytoTune™ 2.1 reprogramming vector at a concentration of $\geq 5 \times 10^7$ cell infectious units/mL (CIU/mL).

Reduced efficiency

For increased safety, the cMyc transgene has been replaced with a less oncogenic variant, L-Myc. This change has been observed to decrease the overall reprogramming efficiency when compared to CytoTune™ 2.0, so steps should be taken to account for this decrease. These steps include plating a higher cell number at Day 3 (PBMCs) and using rhLaminin-521 instead of rhVTN-N as the plating matrix.

Description of the system

Induced pluripotent stem cells (iPSC)

Induced pluripotent stem cells (iPSCs) are genetically reprogrammed adult cells which exhibit a pluripotent stem cell-like state similar to embryonic stem cells while these artificially generated cells are not known to exist in the human body, they show qualities remarkably similar to those of embryonic stem cells (ESC); thus, they are an invaluable new source of pluripotent cells for drug discovery research, cell therapy research, and basic research.

There are multiple methods to generate iPSCs, including retrovirus-mediated gene transduction and chemical induction. While retroviral vectors require integration into host chromosomes to express reprogramming genes, DNA-based vectors such as adenovirus, adeno-associated virus, and plasmid vectors exist episomally and do not require integration; however, they may still be integrated into host chromosomes at certain frequencies. Unlike these vectors, the CTS™ CytoTune™ 2.1 reprogramming vectors do not integrate into the host genome or alter the genetic information of the host cell.

CTS™ CytoTune™ -iPS 2.1 reprogramming system

CTS™ CytoTune™ -iPS 2.1 Reprogramming System uses vectors based on a modified, non-transmissible form of Sendai virus (SeV) to safely and effectively deliver and express key genetic factors necessary for reprogramming somatic cells into iPSCs. In contrast to many available protocols, which rely on viral vectors that integrate into the genome of the host cell, the CTS™ CytoTune™ -iPS 2.1 Reprogramming System uses vectors that are non-integrating and remain in the cytoplasm (i.e., they are zero-footprint). In addition, the host cell can be cleared of the vectors and reprogramming factor genes by exploiting the cytoplasmic nature of SeV and the functional temperature sensitivity mutations introduced into the key viral proteins.

The CTS™ CytoTune™ -iPS 2.1 Sendai Reprogramming Kit contains three SeV-based reprogramming vectors, and are optimized for generating iPSCs from human somatic cells. The reprogramming vectors in this kit have been engineered to increase biological and environmental safety (see “Safety features of the system” on page 10).

Sendai virus

Sendai virus (SeV) is a respiratory virus of mouse and rat, classified as mouse parainfluenza virus type I belonging to the *Paramyxoviridae* family. SeV was first isolated in Japan in the early 1950s and is also called Hemagglutinating Virus of Japan (HVJ). SeV is an enveloped virus of 150–250 nm in diameter whose genome is a single chain RNA (15,384 bases) in the minus sense. Six genes coding for viral proteins are situated sequentially on the genome of the wild-type SeV in the following order (starting from the 3' end):

- Nucleocapsid protein (NP) forms the core nucleocapsid complex with the genome RNA.
- Phosphoprotein (P) is the small subunit of the RNA polymerase.
- Matrix™ protein (M) supports the envelope structure from the inside.
- Fusion™ protein (F) fuses the viral envelope with cell membrane when the virus enters the cell.

Note: The gene encoding the F protein is deleted from the CTS™ CytoTune™ 2.1 reprogramming vectors, rendering them incapable of producing infectious particles from infected cells (see “Non-transmissible CTS™ CytoTune™ 2.1 sendai reprogramming vectors” on page 10).

- Hemagglutinin-Neuraminidase (HN) recognizes the cell surface receptor, sialic acid.
- Large protein (L) is the large subunit of RNA polymerase.

Because SeV infects cells by attaching itself to the sialic acid receptor present on the surface of many different cells, it can infect a wide range of cell types of various animal species. Activation of F protein by a protease is required for the virus-cell fusion process to take place. After infection, the virus goes through genome replication and protein synthesis, and then daughter virus particles are assembled and released.

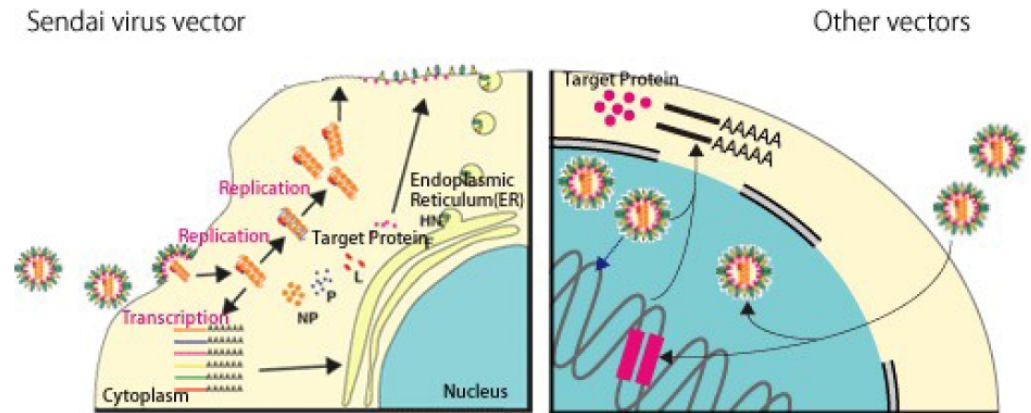


Figure 1 Comparison of the lifecycles of non-integrating SeV vectors and other, integrating vectors

CTS™ CytoTune™ 2.1 reprogramming vectors

The table below lists the CTS™ CytoTune™ 2.1 reprogramming vectors included in the CTS™ CytoTune™-iPS 2.1 Sendai Reprogramming Kit. The reprogramming vectors include the four Yamanaka factors, Oct3/4, Sox2, Klf4, and L-Myc, shown to be sufficient for efficient reprogramming.

CTS™ CytoTune™ Sendai vector	Cap color	Transgene	GenBank™ ID
CTS™ CytoTune™ 2.1 KOS	Orange	Human Klf4 Human Oct3/4 Human Sox2	BC029923.1 NM_002701.4 NM_003106.2
CTS™ CytoTune™ 2.1 hL-Myc	Brown	Human L-Myc	M19720.1
CTS™ CytoTune™ 2.1 hKlf4	Red	Human Klf4	BC029923.1

Advantages of CTS™ CytoTune™ -iPS 2.1 sendai reprogramming kit

- No genotoxicity: CTS™ CytoTune™ 2.1 Sendai reprogramming vectors do not integrate into chromosomes of the target cells and potentially disrupt important genes.
- Wide range of targets: CTS™ CytoTune™ 2.1 Sendai reprogramming vectors are capable of transducing a wide range of cell types in proliferative and quiescent states.
- High transduction efficiency with low multiplicity of infection (MOI).
- Short contact time of virus with target cells is sufficient to establish transduction.
- High level of expression of the transgenes.

- Fast expression of the transgenes: expression is detectable as early as 6–10 hours after transduction, with maximum expression detected more than 24 hours after transduction.
- Zero footprint: the vectors and transgenes can be eliminated from the cells.
- No production of infectious particles by the transduced cells.
- Derived from a virus that is non-pathogenic to humans.

Safety features of the system

Sendai virus (SeV) safety information

Host species: The host species for the Sendai virus (SeV) reported so far are mouse, rat, hamster, and guinea pigs, all of which have been described to be serologically positive.

Transmission: SeV is transmitted by aerosol and contact with respiratory secretions. The virus is highly contagious, but the infection does not persist in immunocompetent animals.

CTS™ CytoTune™ 2.1 Sendai reprogramming vectors: CTS™ CytoTune™ 2.1 Sendai reprogramming vectors in this kit are based on a modified, non-transmissible form of SeV, which has the Fusion™ protein (F) deleted, rendering the virus incapable of producing infectious particles from infected cells.

Non-transmissible CTS™ CytoTune™ 2.1 sendai reprogramming vectors

SeV vectors used in this kit consist of viral proteins NP, P, M, F (activated), HN, and L, and the SeV genome RNA, from which the F gene is deleted. Because SeV infects cells by attaching itself to cell surface receptor sialic acid, present on the surface of many cell types of different species, the vectors are able to transduce a wide range of cells. However, they are no longer capable of producing infectious particles from infected cells, because the viral genome lacks the F-gene. In addition, the presence of functional mutations such as temperature sensitivity in the amino acid sequence of several SeV proteins (SeV/TSΔF, SeV/TS12ΔF, and SeV/TS15ΔF) renders the vectors easily removable from transduced cells.

Note: SeV vectors used in this kit were developed by ID Pharma and their rights for commercial use are the property of ID Pharma.

Biosafety level 2



WARNING! BIOHAZARD. Although human is not the natural host for the SeV, and the virus has not shown to be pathogenic to humans, appropriate care must be taken to prevent the potential mucosal exposure to the virus. This product must be used under Biosafety Level 2 (BL-2) containment with biological safety cabinet and laminar flow hood, and with appropriate personal safety equipment to prevent mucosal exposure/splash. In the event that the virus comes into contact with skin or eyes, decontaminate by flushing with plenty of water and consult a physician. For more information on BL-2 guidelines, refer to *Biosafety in Microbiological and Biomedical Laboratories*, 5th ed., published by the Centers for Disease Control, which is available for downloading at: www.cdc.gov/od/ohs/biosfty/bmb15/bmb15toc.htm

Guidelines for generating iPSCs

Experimental guidelines

- To maintain sterile culture conditions, carry out all of the procedures in this manual using sterile laboratory practices in a Biosafety Level 2 laminar flow hood.
 - You can use the CTS™ CytoTune™-iPS 2.1 Sendai Reprogramming Kit to reprogram a wide range of cell types in proliferative and quiescent states. However, the reprogramming efficiency may vary between different cell types (~0.01%–1%).
 - For successful reprogramming, transduce your cells using all three reprogramming vectors.
- Note:** For successful reprogramming, all four Yamanaka factors (i.e., Oct4, Sox2, Klf4, and L-Myc) need to be expressed in your host cell.
- Cells that have already been infected with Sendai virus are refractive to further infection by Sendai virus. Therefore, you cannot transduce cells with CTS™ CytoTune™ 2.1 reprogramming vectors that have already been transduced with other Sendai vectors such as the CytoTune™-EmGFP Sendai Fluorescence Reporter or vice versa.
 - One CTS™ CytoTune™-iPS 2.1 Reprogramming Kit of three tubes supplies sufficient reagents to transduce a minimum of 2.0×10^6 cells at MOI=5-5-5 (i.e., KOS MOI=5, hL-Myc MOI=5, hKlf4 MOI=5).
 - The titer of each CTS™ CytoTune™ 2.1 Sendai reprogramming vector is lot-dependent. For the specific titer of your vectors, refer to the Certificate of Analysis (CoA) available on our website. Go to thermofisher.com/cytotune and search for the CoA by product lot number, which is printed on the vial.
 - Viral titers can decrease dramatically with each freeze/thaw cycle. Avoid repeated freezing and thawing of your reprogramming vectors. Viral titer is not guaranteed for kits that have been refrozen or thawed.
 - Prior to starting, ensure that the media are equilibrated to 37°C and appropriately gassed.

IMPORTANT! Peeling of vial labels has been observed during thawing in water bath. If labels come off of tube, cap colors can be used to identify each vector. See “Contents and storage” on page 7.

Positive control

For positive control, we recommend performing a reprogramming experiment with human neonatal foreskin fibroblast cells (strain BJ; ATCC® no. CRL2522). Note that experimental conditions may vary among target cells and need to be optimized for each cell type. The example given in the following protocol does not guarantee the generation of iPSCs for all cell types.

**CytoTune™ -
EmGFP sendai
fluorescence
reporter**

The CytoTune™-EmGFP Sendai Fluorescence Reporter (Cat. No. A16519), available separately, is a fluorescent control vector carrying the Emerald Green Fluorescent Protein (EmGFP) gene. The fluorescent control vector allows you to determine whether your cell line of interest is amenable or refractive to transduction by the Sendai reprogramming vectors, including the vectors from the original CytoTune™ iPS Sendai Reprogramming Kits. We recommend testing your cell lines of interest using the CytoTune™-EmGFP Sendai Fluorescence Reporter before starting your reprogramming experiments.

Note that you cannot transduce cells with CytoTune™ reprogramming vectors that have already been transduced with the CytoTune™-EmGFP Sendai Fluorescence Reporter or vice versa. If you wish to use the CytoTune™-EmGFP Sendai Fluorescence Reporter during reprogramming, you must add it to the cells at the same time as the reprogramming vectors.

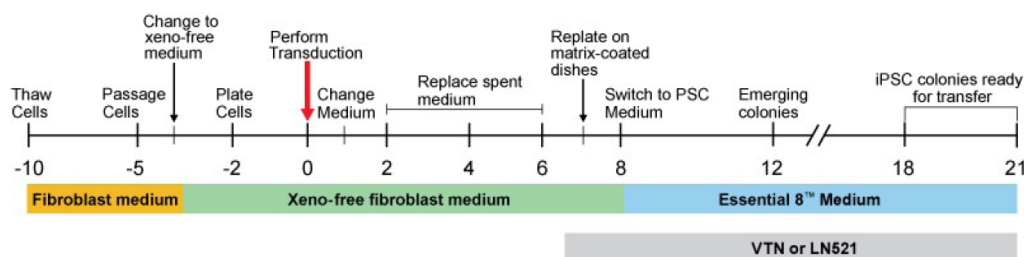
For detailed instructions on using the CytoTune™-EmGFP Sendai Fluorescence Reporter, see “CytoTune™-EmGFP sendai fluorescence reporter” on page 55.

Reprogram fibroblasts

Experiment outline (xeno-free)

Workflow

The major steps required for xeno-free reprogramming of human dermal fibroblast cells using the CTS™ CytoTune™ 2.1 Sendai Reprogramming Kit to generate iPSCs cultured feeder-free on vitronectin-coated (or LN521-coated) culture dishes are shown below. Note that the timeline is provided as a guideline for experimental planning; actual timeline can vary based on the cell type and experimental conditions.



Reprogramming timeline

Day -10: Thaw fibroblasts.

Day -5: Split fibroblasts, change to xeno-free medium.

Day -2: Plate human fibroblasts into at least two wells of a 6-well plate in xeno-free fibroblast medium so that they are 30–60% confluent on the day of transduction (Day 0).

Day 0: Transduce the cells using the CTS™ CytoTune™ 2.1 Sendai reprogramming vectors at the appropriate MOI. Incubate the cells overnight.

Day 1: Replace the medium with fresh complete xeno-free fibroblast medium to remove the CTS™ CytoTune™ 2.1 Sendai reprogramming vectors.

Day 2–6: Replace the spent medium every other day.

Day 7: Plate transduced cells on vitronectin-coated culture dishes in xeno-free fibroblast medium.

Day 8: Change the medium to complete Essential 8™ Medium.

Day 9–28: Replace spent medium every day and monitor the culture vessels for the emergence of iPSC colonies. When iPSC colonies are ready for transfer, perform live staining, and pick and transfer undifferentiated iPSCs onto fresh culture dishes for expansion.

Reduced efficiency

For increased safety, the cMyc transgene has been replaced with a less oncogenic variant, L-Myc. This change has been observed to decrease the overall reprogramming efficiency when compared to CytoTune™ 2.0, so steps should be taken to account for this decrease. These steps include plating a higher cell number Day 7 (HDF), and using rhLaminin-521 instead of rhVTN-N as the plating matrix.

Reprogram fibroblasts (xeno-free)

Media for reprogramming fibroblasts (xeno-free)

For optimal reprogramming of human dermal fibroblast cells using the CTS™ CytoTune™ 2.1 Sendai Reprogramming Kit to generate iPSCs cultured feeder free on vitronectin-coated culture dishes, use the following media at the designated stages of the reprogramming experiment:

- Fibroblast medium (“Complete fibroblast medium” on page 47): Initial thawing of fibroblasts
- Xeno-free fibroblast medium (“Complete xeno-free fibroblast medium” on page 47): Adaptation and expansion of fibroblasts, Plating cells prior to transduction, expansion, posttransduction recovery of cells, plating of transduced cells on vitronectin-coated culture dishes
- Complete Essential 8™ Medium (“Essential 8™ Medium (for 500 mL of complete medium)” on page 48): Expansion of transduced cells on vitronectin-coated culture dishes, live staining and picking of iPSCs

Required materials

Cells and vectors

- CTS™ CytoTune™ 2.1 Sendai reprogramming vectors
Note: For successful reprogramming, you need all three tubes of reprogramming vectors.
- Human fibroblast cells to reprogram
Note: It is critical to use high quality fibroblasts at the lowest possible passage in order to ensure successful reprogramming.
- *Optional:* Human neonatal foreskin fibroblast cells (strain BJ; ATCC® no. CRL2522) as a positive reprogramming control

Media and reagents

- DMEM with GlutaMAX™ -I (high glucose) (Cat. No. 10569-010)
- Fetal Bovine Serum (FBS), ES Cell-Qualified (Cat. No. 16141-079)
- Optional: Penicillin-Streptomycin, liquid (Cat. No. 15140-122)
- DMEM/F-12, HEPES (Cat. No. 11330032)
- CTS™ KnockOut™ SR Xeno-Free (Cat. No. A1099201)
- Sodium Bicarbonate 7.5% solution (Cat. No. 25080094)
- Basic Fibroblast Growth Factor (bFGF) (Cat. No. PHG0264)
- EGF Recombinant Human protein (Cat. No. PHG 0313)
- Hydrocortisone (Sigma Aldrich, Cat. No. H6909)
- TrypLE™ Select Cell Dissociation Reagent (Cat. No. 12563) or 0.05% Trypsin/EDTA (Cat. No. 25300)
- Dulbecco's PBS (DPBS) without Calcium and Magnesium (Cat. No. 14190)
- Essential 8™ Medium (Cat. No. A1517001)
- Vitronectin, truncated recombinant human (VTN-N) (Cat. No. A14700)
- rhLaminin-521 (Cat. No. A29249)
- Coating Matrix Kit (Cat. No. R011K)

Reprogram fibroblasts

The following protocol has been optimized to transduce one well of human dermal fibroblast cells under xeno-free conditions. We recommend that you optimize the protocol for your cell type, and add an appropriate number of conditions/wells to utilize the entire volume of virus. Note that experimental conditions may vary among target cells. The example given in the following protocol does not guarantee the generation of iPSCs for all cell types.

Day –10: Adapt cells to xeno-free fibroblast medium

1. Prior to transducing cells, they should be adapted to xeno-free fibroblast medium for at least two passages. Thaw frozen fibroblast cultures onto a T75 flask using standard thawing procedures, and allow them to recover and reach confluence.
Note: some fibroblasts will experience a decrease in growth rate when cultured in xeno-free medium. This can be partially mitigated when the cells are plated onto a matrix, such as collagen. The Coating Matrix Kit can be used for this purpose. If using, follow manufacturer instructions and coat the necessary culture ware ahead of time. Continue to culture and plate cells on the matrix until Day 7 after transduction, when cells are replated onto VTN or LN521 (step 5 on page 18).
2. When cells are 85–90% confluent, split as follows. Pre-warm 3 mL of TrypLE™ Select reagent, to 37°C.
3. Aspirate medium from cells, and wash 2X with D-PBS. Add 3 mL of warmed TrypLE™ Select, and incubate cells at 37°C for 3–5 minutes.
4. When cells are detached, add 7 mL of pre-warmed fibroblast medium to dilute TrypLE™ Select. Pipette to detach cells from flask, and transfer to a 15 mL conical tube. Wash flask with 4 mL of fibroblast medium, and add wash to cell solution.
5. Centrifuge the cells at $200 \times g$ for 5 minutes.
6. Aspirate medium, and resuspend in 1–2 mL of fibroblast medium.
7. Determine the viable cell count using your method of choice (e.g. Countess™ II Automated Cell Counter).
8. Plate cells into new T75 flask(s) at a ratio of about 1:4–1:6 (plate between 4×10^5 – 3×10^5 cells per T75).
9. After 24 hours, change medium to xeno-free fibroblast medium. Wash flask 2X with 10 mL of D-PBS before adding fresh medium, to remove traces of FBS.
10. Feed cells every other day until cells are 85–90% confluent, usually 3–4 days after splitting.

Day -2: Prepare the cells for transduction

1. Two days before transduction, plate fibroblasts onto at least two wells of a 6-well plate at the appropriate density to achieve between 2×10^5 – 3×10^5 cells per well on the day of transduction (Day 0). One of the wells will be used to count cells for viral volume calculations.

Note: Each CTS™ CytoTune™ 2.1 Sendai Reprogramming Kit supplies sufficient virus to transduce cells in at least 6 wells of a 6-well plate. We recommend using the entire volume of virus.

Note: We recommend about 30–60% confluency on the day of transduction. Because over confluency results in decreased transduction efficiency, we recommend replating your cells to achieve 30–60% confluency if your cells have become over confluent during culturing.

2. Culture the cells for two more days, ensuring the cells have fully adhered and extended.

Day 0: Perform transduction

1. On the day of transduction, warm 1 mL of xeno-free fibroblast medium in a water bath for each well to be transduced.
2. Harvest the cells from one well to perform a cell count. These cells will not be transduced, but will be used to estimate the cell number in the other well(s) plated in Step 1 on page 15.
3. Remove the cells from the 6-well plate using 0.5 mL of TrypLE™ Select reagent, following the procedure recommended by the manufacturer and incubating at room temperature. When the cells have rounded up (1–3 minutes later), add 1 mL of xeno-free fibroblast medium into each well, and collect the cells in a 15-mL conical centrifuge tube.
4. Count the cells using the desired method (e.g., Countess™ II Automated Cell Counter), and calculate the volume of each virus needed to reach the target MOI using the live cell count and the titer information on the CoA.

Note: We recommend initially performing the transductions with MOIs of 5, 5, and 3.

(i.e., KOS MOI=5, hL-Myc MOI=5, hKlf4 MOI=3). These MOIs can be optimized for your application.

Note: The titer of each CTS™ CytoTune™ 2.1 reprogramming vector is lot-dependent. For the specific titer of your vectors, go to thermofisher.com/cytotune and search for the CoA by product lot number, which is printed on the vial. Avoid re-freezing and thawing of the reprogramming vectors since viral titers can decrease dramatically with each freeze/thaw cycle.

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

5. Remove one set of CTS™ CytoTune™ 2.1 Sendai tubes from the $\leq -70^{\circ}\text{C}$ storage. Thaw each tube one at a time by first immersing the bottom of the tube in a 37°C water bath for 10-20 seconds, and then removing the tube from the water bath and allowing it to thaw at room temperature. Once thawed, briefly centrifuge the tube and place it immediately on ice.
6. Add the calculated volumes of each of the three CTS™ CytoTune™ 2.1 Sendai tubes to 1 mL of xeno-free fibroblast medium, pre-warmed to 37°C . Ensure that the solution is thoroughly mixed by pipetting the mixture gently up and down. Complete the next step on page 17 within 5 minutes.
7. Aspirate the medium from the cells, and add the reprogramming virus mixture prepared in Step 18 on page 17 to the well containing the cells. Incubate the cells overnight in a 37°C incubator with a humidified atmosphere of 5% CO_2 .

Day 1: Replace medium and culture cells

1. 24 hours after transduction, replace the medium with fresh xeno-free fibroblast medium.

Note: Depending on your cell type, you should expect to see some cytotoxicity 24–48 hours post-transduction, which can affect >50% of your cells. This is an indication of high uptake of the virus. We recommend that you continue culturing your cells and proceed with the protocol.

2. Culture the cells for 6 more days, changing the spent medium with fresh xeno-free fibroblast medium every other day.

Note: Depending on your cell type, you may observe high cell density before Day 5.

We do not recommend passaging your cells before 7 days post-transduction. You may replace spent medium daily with fresh xeno-free fibroblast medium if cultures become very dense.

Day 7: Plate transduced cells on vitronectin-coated culture dishes

1. Coat a sufficient number of tissue culture dishes (e.g. 6-well, 60-mm, or 100-mm) with vitronectin (see “Vitronectin working concentration” on page 51 for coating protocol).

Note: rhLaminin-521 can be substituted for vitronectin; see “Coat culture vessels with rhLaminin-521” on page 53 for coating protocol.

2. Seven days after transduction, fibroblast cells are ready to be harvested and plated on vitronectin-coated culture dishes. Remove the medium from the fibroblasts, and wash cells once with D-PBS.
3. To remove the cells from the 6-well plate, use 0.5 mL of TrypLE™ Select reagent, following the procedure recommended by the manufacturer and incubate at room temperature. When the cells have rounded up (1–3 minutes later), add 5 mL of xeno-free fibroblast medium into each well, and collect the cells in a 15-mL conical centrifuge tube.

Note: Because the cells can be very sensitive to dissociation enzymes at this point, minimize exposure time and incubate the cells at room temperature.

4. Centrifuge the cells at $200 \times g$ for 4 minutes, aspirate the medium, and resuspend the cells in an appropriate amount of xeno-free fibroblast medium.
5. Count the cells using the desired method (e.g., Countess™ II Automated Cell Counter), and seed the vitronectin-coated (or LN521-coated) culture dishes with 5×10^4 – 2×10^5 cells per well of a 6-well and incubate overnight in a 37°C incubator with a humidified atmosphere of 5% CO₂.

Note: Reprogramming efficiencies will typically be lower when using xeno-free conditions, so the number of cells plated should be increased accordingly.

We recommend plating at least two to four different densities per well. Depending on your cell type, you may need to plate most of your cells on the same plate to ensure sufficient numbers of colonies.

Note: It is strongly recommended to set aside cells at this point for RNA extraction to be used as a positive control in the RT-PCR or qPCR detection of the CytoTune™ vectors (see “RT-PCR protocol for detecting the SeV genome and transgenes” on page 43). It is very important to include this positive control when performing detection of the CytoTune™ vectors.

Day 8 to 28: Feed and monitor the cells

1. 24 hours later, change the medium to complete Essential 8™ Medium (see “Essential 8™ Medium (for 500 mL of complete medium)” on page 48), and replace the spent medium every day thereafter.
2. Starting on Day 8, observe the plates every other day under a microscope for the emergence of cell clumps indicative of reprogrammed cells.

3. Three to four weeks after transduction, colonies should have grown to an appropriate size for transfer. When the colonies are ready for transfer, perform live staining using Tra1-60 or Tra1-81 for selecting reprogrammed colonies (see “Live stain” on page 39).

Note: We typically harvest colonies closer to three weeks to avoid differentiation.

4. Manually pick undifferentiated iPSC colonies (see “Pick iPSC colonies” on page 40) and transfer them onto prepared vitronectin-coated 6- or 12-well culture plates for further expansion or analysis.

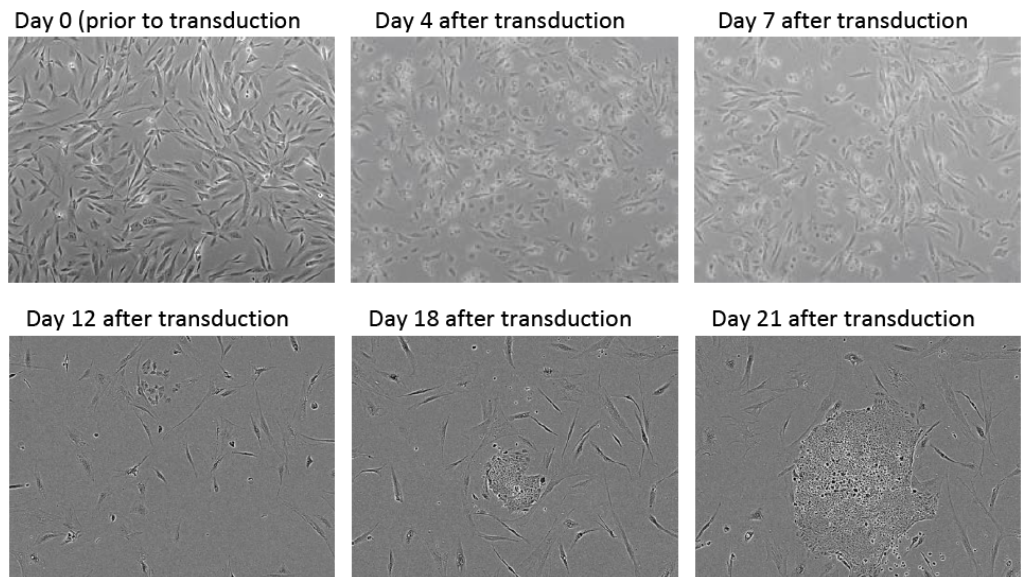
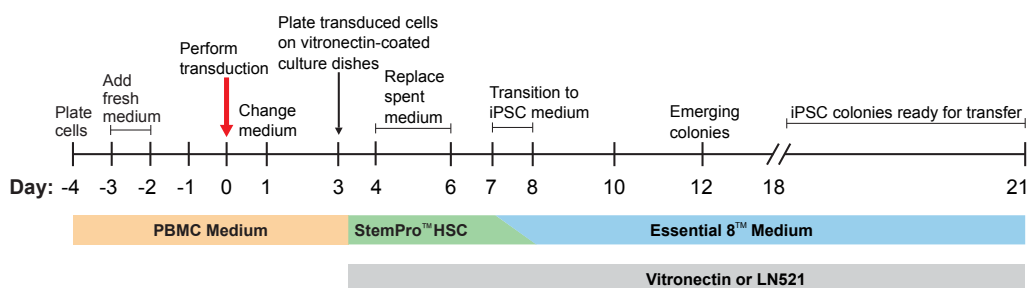


Figure 2 Human dermal fibroblasts reprogrammed using the CTS™ CytoTune™ iPS 2.1 Sendai Reprogramming Kit. Human dermal fibroblasts reprogrammed using the CTS™ CytoTune™ iPS 2.1 Sendai Reprogramming Kit according to the protocol, and plated on to recombinant human laminin-521 seven days after transduction. 50X magnification phase contrast images were taken at the indicated number of days post-transduction.

Experiment outline (xeno-free)

Workflow

The major steps required for reprogramming peripheral blood mononuclear cells (PBMCs) using the CTS™ CytoTune™ -iPS 2.1 Sendai Reprogramming Kit to generate iPSCs cultured feeder-free on vitronectin-coated (or LN521-coated) culture dishes are shown below. Note that the timeline is provided as a guideline for experimental planning; actual timeline can vary based on the cell type and experimental conditions.



Reprogramming timeline

Day -4: Plate peripheral blood mononuclear cells (PBMCs) at 5×10^5 cells/mL to the middle section of a 24-well plate in complete PBMC medium.

Day -3 to -1: Replace half of the medium with 0.5 mL of fresh complete PBMC medium.

Day 0: Transduce the cells using the CTS™ CytoTune™ 2.1 Sendai reprogramming vectors at the appropriate MOI. Incubate the cells overnight.

Day 1: Replace the medium with fresh complete PBMC medium to remove the CTS™ CytoTune™ 2.1 Sendai reprogramming vectors.

Day 3: Plate the transduced cells on vitronectin-coated culture dishes in complete CTS™ StemPro™ HSC Expansion Medium without cytokines.

Day 4–6: Replace spent complete CTS™ StemPro™ HSC Expansion Medium without cytokines every other day.

Day 7: Start transitioning into Essential 8™ Medium by replacing half of the CTS™ StemPro™ HSC Expansion Medium without cytokines with Essential 8™ Medium.

Day 8: Replace the entire medium with Essential 8™ Medium to conclude the transitioning, and continue culturing cells on vitronectin-coated culture dishes.

Day 9–28: Replace spent medium with fresh Essential 8™ Medium every day and monitor the culture vessels for the emergence of iPSC colonies. When iPSC colonies are ready for transfer, perform live staining, and pick and transfer undifferentiated iPSCs onto fresh vitronectin-coated culture dishes for expansion.

Reduced efficiency For increased safety, the cMyc transgene has been replaced with a less oncogenic variant, L-Myc. This change has been observed to decrease the overall reprogramming efficiency when compared to CytoTune™ 2.0, so steps should be taken to account for this decrease. These steps include plating a higher cell number at Day 3 (PBMCS) and using rhLaminin-521 instead of rhVTN-N as the plating matrix.

Reprogram peripheral blood mononuclear cells (PBMCS) (xeno-free)

Media for reprogramming PBMCS (xeno-free)

For optimal reprogramming of PBMCS using the CTS™ CytoTune™ -iPS 2.1 Sendai Reprogramming Kit to generate iPSCs cultured on VTN or LN521, use the following media at the designated stages of the reprogramming experiment:

- **PBMC medium** (CTS™ StemPro™ HSC Expansion Medium containing cytokines (i.e., SCF, IL-3 IL-6, FLT3)“CTS™ StemPro™ HSC Expansion Medium (for 100 mL of complete medium)” on page 48): Plating cells prior to transduction, expansion, post-transduction recovery of cells
- **CTS™ StemPro™ HSC Expansion Medium without cytokines** (“CTS™ StemPro™ HSC Expansion Medium (for 100 mL of complete medium)” on page 48): Plating of transduced cells on vitronectin-coated culture dishes
- **Complete Essential 8™ Medium** (“Essential 8™ Medium (for 500 mL of complete medium)” on page 48): Expansion of transduced cells on vitronectin-coated culture dishes, live staining and picking of iPSCs

Required materials

Cells and vectors

- CTS™ CytoTune™ 2.1 Sendai reprogramming vectors
Note: For successful reprogramming, you need all three tubes of reprogramming vectors.
- Peripheral blood mononuclear cells (PBMCS) to reprogram
Note: You can use PBMCS extracted from fresh blood by a conventional method (i.e., Ficoll™-Paque purification) or frozen PBMCS.
- *Optional:* Human neonatal foreskin fibroblast cells (strain BJ; ATCC® no. CRL2522) as a positive reprogramming control
Note: If you are using this as a control, follow the protocol for reprogramming fibroblasts within this manual (Chapter 3, “Reprogram fibroblasts”).

Media and reagents

- CTS™ StemPro™ HSC Expansion Medium (Cat. No. A4222301)
- SCF (C-Kit Ligand), Recombinant Human (Cat. No. PHC2111)
- FLT-3 Ligand, Recombinant Human (Cat. No. PHC9414)
- IL-3, Recombinant Human (Cat. No. PHC 0034)
- IL-6, Recombinant Human (Cat. No. PHC0065)
- *Optional:* Penicillin-Streptomycin, Liquid (Cat. No. 15140-122)
- *Optional:* Polybrene™ Hexadimethrine Bromide (Sigma Aldrich, Cat. No. H9268)
- Dulbecco's PBS (DPBS) without Calcium and Magnesium (Cat. No. 14190)
- Essential 8™ Medium (Cat. No. A1517001)

Reprogramming protocol

- Vitronectin, truncated recombinant human (VTN-N) (Cat. No. A14700)
- Nunc™ 14 mL Round-Bottom Tubes (Cat. No. 150268)

The following protocol has been optimized for peripheral blood mononuclear cells (PBMCs) isolated through density gradient centrifugation via Ficoll™-Paque. We recommend that you optimize the protocol for your cell type, and add an appropriate number of conditions/wells to utilize the entire volume of virus. Note that experimental conditions may vary among target cells. The example given in the following protocol does not guarantee the generation of iPSCs for all cell types.

Day -4: Seed PBMCs

1. Four days before transduction, remove vial(s) of PBMCs from liquid nitrogen storage. Thaw the vial quickly in 37°C water bath. When only a small ice crystal remains in the vial, remove it from the water bath. Spray the outside of the vial with 70% ethanol before placing it in the cell culture hood.
2. Gently transfer the PBMCs into a 15-mL conical tube. Slowly (drop-wise) add 5–10 mL pre-warmed complete PBMC medium (see “CTS™ StemPro™ HSC Expansion Medium (for 100 mL of complete medium)” on page 48) to the cell suspension. Remove an aliquot of cells to count and determine cell viability.

Note: PBMC medium consists of complete CTS™ StemPro™ HSC Expansion Medium containing the appropriate cytokines; aliquot the cytokines and add fresh daily.

3. Centrifuge the cell suspension at $200 \times g$ for 10 minutes, discard the supernatant, and resuspend the cells in complete PBMC medium to 5×10^5 cells/mL.
4. Add 1 mL per well to the middle section of a 24-well plate to prevent excessive evaporation of the medium during incubation.

Note: Use at least 4 wells (i.e. 2×10^6 cells) to ensure a sufficient number of cells on Day 0.

5. Incubate the cells in a 37°C incubator with a humidified atmosphere of 5% CO₂.

Day -3 to -1: Observe cells and add fresh medium

Feed the cells daily, gently remove 0.5 mL of the medium from each well, and replace it with 0.5 mL of fresh complete PBMC medium, trying not to disturb the cells. If cells are present in 0.5 mL removed from the wells, centrifuge the cell suspension at $200 \times g$ for 10 minutes, discard the supernatant, and resuspend the cells in 0.5 mL fresh PBMC medium before adding them back to the plate.

Note: Some cell death is generally observed the first day after the thaw. Some cells may adhere to the surface of the tissue culture plate. Proceed with the cells in suspension, and leave behind any attached cells. Cells will not proliferate, but should maintain stable cell number for the first few days (PBMCs contain a variety of cells, and the current media system is only targeting a small population).

Day 0: Count cells and perform transduction

1. Count the cells using the desired method (e.g., Countess™ II Automated Cell Counter), and calculate the volume of each virus needed to reach the target MOI using the **live cell count** and the titer information on the CoA.

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

Note: We recommend initially performing the transductions with MOIs of 5, 5, and 3 (i.e., KOS MOI=5, hL-Myc MOI=5, hKlf4 MOI=3). These MOIs can be optimized for your application.

Note: The titer of each CTS™ CytoTune™ 2.1 reprogramming vector is lot-dependent. For the specific titer of your vectors, go to thermofisher.com/cytotune and search for the CoA by product lot number, which is printed on the vial. Avoid re-freezing and thawing of the reprogramming vectors since viral titers can decrease dramatically with each freeze/thaw cycle.

2. For each transduction, pipette 2.5×10^5 – 5×10^5 cells into a round bottom tube.
3. Remove CTS™ CytoTune™ 2.1 Sendai tubes from the $\leq -70^\circ\text{C}$ storage. Thaw each tube one at a time by first immersing the bottom of the tube in a 37°C water bath for 10–20 seconds, and then removing the tube from the water bath and allowing it to thaw at room temperature. Once thawed, briefly centrifuge the tube and place it immediately on ice.
4. Add the calculated volumes of each of the three CTS™ CytoTune™ 2.1 Sendai tubes to 1 mL of PBMC medium, pre-warmed to 37°C . Ensure that the solution is thoroughly mixed by pipetting the mixture gently up and down. Complete the next within 5 minutes.
5. Add the reprogramming virus mixture prepared in step 4 to the round bottom tube containing PBMCs prepared in step 2. Total volume should now be between 1–1.5 mL. Place cap tightly onto the tube, and wrap with Parafilm™ film. Centrifuge the cells and virus at $1000 \times g$ for 30 minutes at room temperature. Once the centrifugation is complete, add an additional 1 mL of PBMC medium to the tube, re-suspend the cells, and transfer them to 1 well of a 12-well plate (total volume should now be between 2–2.5 mL). Incubate the plate overnight in a 37°C incubator with a humidified atmosphere of 5% CO_2 .

Note: Although this centrifugation step is not required, it significantly increases the transduction and reprogramming efficiencies. If the centrifugation step is omitted, transductions can be performed in a 24-well plate using 0.3 mL of total volume of cells, virus, and medium. Adding $4 \mu\text{g/mL}$ of Polybrene™ to the medium at the time of transduction may increase transduction efficiencies, but should only be used if the centrifugation step is not performed.

Day 1: Replace medium and culture cells

1. The next day, remove the cells and medium from the culture plate and transfer to a 15-mL centrifuge tube. Rinse the well gently with 1 mL of medium to ensure most of the cells are harvested.
2. Remove the CTS™ CytoTune™ 2.1 Sendai viruses by centrifuging the cell suspension at $200 \times g$ for 10 minutes, aspirating the supernatant, and resuspending the cells in 0.5 mL of complete PBMC medium per well of a 24-well plate.
Note: The cells may have drastic cell death (>60%); continue with the protocol using the **live** cell count. For the first 48 hours, observe the cells under the microscope for changes in cell morphology as a validation of transduction. Expect large, aggregated cells.
Note: To prevent attachment of any cells prior to plating onto vitronectin, it may be beneficial to use a low attachment 24-well plate.
3. Culture the cells at 37°C in a humidified atmosphere of 5% CO₂ for 2 days. No media change is required during this time.

Day 3: Plate cells on vitronectin-coated culture dishes

1. Coat a sufficient number of tissue culture dishes (e.g. 6-well, 60-mm, or 100-mm) with vitronectin (see “Coat culture vessels with vitronectin” on page 51 for coating protocol).
Note: rhLaminin-521 can be substituted for vitronectin; see “Coat culture vessels with rhLaminin-521” on page 53 for coating protocol.
2. Count the cells using the desired method (e.g., Countess™ II Automated Cell Counter) and seed the 6-well vitronectin-coated culture plates with 1×10^4 – 1×10^5 live cells per well in 2 mL of complete CTS™ StemPro™ HSC Expansion Medium without the cytokines.
Note: It may be necessary to plate more than two different densities, as the reprogramming efficiencies of PBMCs can vary widely between donors. If sufficient cells are available, it is recommended to plate 4–6 different densities, ranging from 2×10^4 – 2×10^5 cells per well of a 6-well plate.
Note: It is strongly recommended to set aside cells at this point for RNA extraction to be used as a positive control in the RT-PCR or qPCR detection of the CytoTune™ vectors. It is very important to include this positive control when performing detection of the CytoTune™ vectors.
3. Incubate the cells at 37°C in a humidified atmosphere of 5% CO₂.

Day 4–6: Replace spent medium

Every other day, gently remove 1 mL (half) of the spent medium from the cells and replace it with 1 mL of fresh complete CTS™ StemPro™ HSC Expansion Medium without cytokines and without disturbing cells.

Note: The transduced PBMCs may only be loosely attached for the first few days after plating. Be sure to perform media changes gently during this time. If cells are present in 1 mL removed from the wells, centrifuge the cell suspension at $200 \times g$ for 10 minutes, discard the supernatant, and resuspend the cells in 1 mL fresh PBMC medium before adding them back to the plate.

Day 7: Start transitioning cells to Essential 8™ Medium

1. Prepare Essential 8™ Medium. See “Essential 8™ Medium (for 500 mL of complete medium)” on page 48.
2. Remove 1 mL (half) of CTS™ StemPro™ HSC Expansion Medium from the cells and replace it with 1 mL of Essential 8™ Medium to start the adaptation of the cells to the new culture medium.

Day 8 to 28: Feed and monitor the cells

1. 24 hours later (day 8), change the full volume of the medium to Essential 8™ Medium, and replace the spent medium every day thereafter.

Note: Plated PBMCs should have fully attached by this point. Any cells that have not attached will likely never do so.

2. Starting on day 8, observe the plates every other day under a microscope for the emergence of cell clumps indicative of reprogrammed cells (see Figure 3).

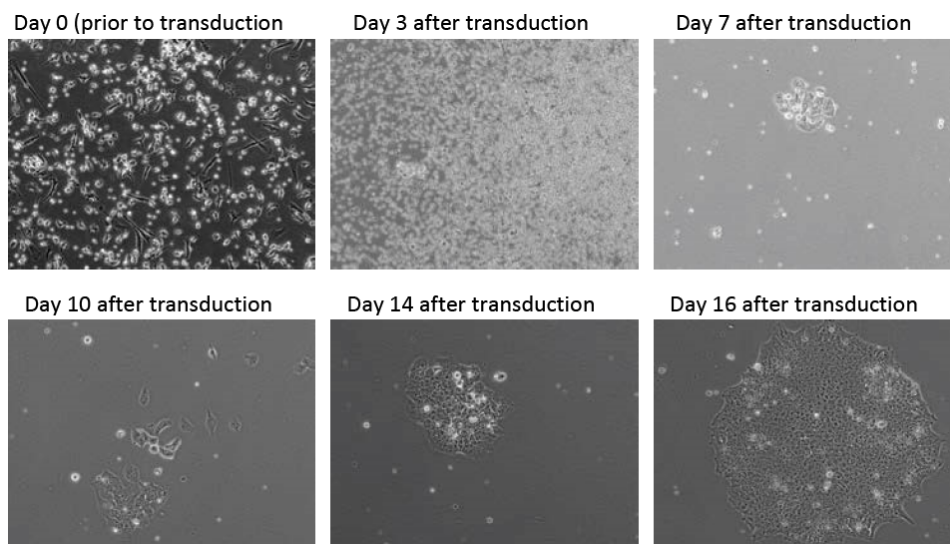


Figure 3 PBMcs reprogrammed using the CTS™ CytoTune™-iPS 2.1 Sendai Reprogramming Kit.

PBMcs reprogrammed using the CTS™ CytoTune™ 2.1 Sendai Reprogramming Kit according to the protocol, and plated on to recombinant human laminin-521 3 days after transduction. 100X magnification phase contrast images were taken at the indicated number of days post-transduction.

3. By day 15 to 21 after transduction, colonies should have grown to an appropriate size for transfer.

Note: We typically harvest colonies closer to 3 weeks to avoid differentiation.

4. When colonies are ready for transfer, perform live staining using Tra1-60 or Tra1-81 for selecting reprogrammed colonies if desired (see “Live stain” on page 39).

Manually pick colonies and transfer them onto prepared vitronectin-coated 6- or 12-well culture plates (see “Pick iPSC colonies” on page 40).

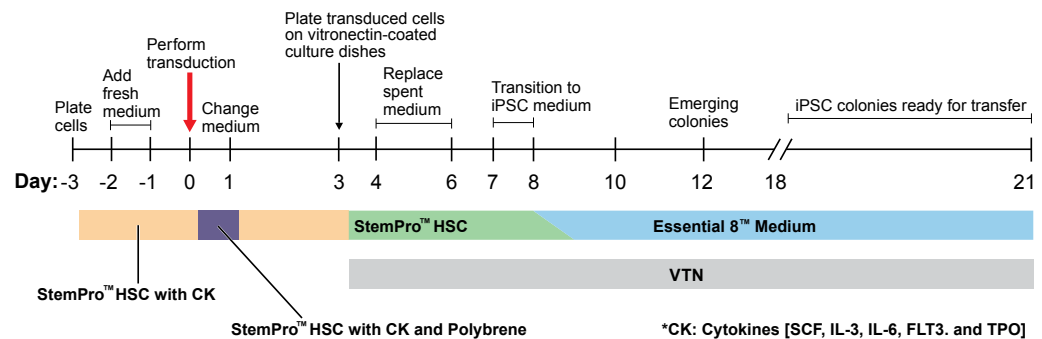
Reprogram CD34⁺ cells

Experiment outline (xeno-free)

Workflow

The major steps required for reprogramming CD34⁺ cells using the CTS[™] CytoTune[™] - iPS 2.1 Sendai Reprogramming Kit to generate iPSCs cultured on vitronectin-coated (or LN521-coated) culture dishes are shown below.

Note: The timeline is provided as a guideline for experimental planning; actual timeline can vary based on the cell type and experimental conditions.



Reprogramming timeline

Day -3: Plate CD34⁺ cells into at least two wells of a 24-well plate in complete CTS[™] StemPro[™] HSC Expansion Medium containing cytokines (i.e., SCF, IL-3, IL-6, FLT3, and TPO).

Day -1: Replace 0.5 mL of spent medium with 0.5 mL of fresh complete CTS[™] StemPro[™] HSC Expansion Medium containing cytokines.

Day -2: Replace 0.5 mL of spent medium with 1 mL of fresh complete CTS[™] StemPro[™] HSC Expansion Medium containing cytokines.

Day 0: Transduce the cells using the CTS[™] CytoTune[™] 2.1 Sendai reprogramming vectors at the appropriate MOI in CTS[™] StemPro[™] HSC Expansion Medium containing cytokines and 4 µg/mL of Polybrene[™]. Incubate the cells overnight.

Day 1: Replace the medium with fresh CTS[™] StemPro[™] HSC Expansion Medium containing cytokines (no Polybrene[™]) to remove the CTS[™] CytoTune[™] 2.1 Sendai reprogramming vectors.

Day 3: Plate the transduced cells on vitronectin-coated culture dishes in complete CTS[™] StemPro[™] HSC Expansion Medium without cytokines.

Day 4-6: Replace half of the spent medium with fresh complete CTS[™] StemPro[™] HSC Expansion Medium without cytokines every other day.

Day 7: Start transitioning into Essential 8[™] Medium by replacing half of the CTS[™] StemPro[™] HSC Expansion Medium without cytokines with complete Essential 8[™] Medium.

Day 8: Replace the entire medium with Essential 8™ Medium to conclude the transitioning and continue culturing cells on vitronectin-coated culture dishes.

Day 9–28: Replace spent medium with fresh Essential 8™ Medium every day and monitor the culture vessels for the emergence of iPSC colonies. When iPSC colonies are ready for transfer, perform live staining, and pick and transfer undifferentiated iPSCs onto fresh vitronectin-coated culture dishes for expansion.

Reduced efficiency For increased safety, the cMyc transgene has been replaced with a less oncogenic variant, L-Myc. This change has been observed to decrease the overall reprogramming efficiency when compared to CytoTune™ 2.0, so steps should be taken to account for this decrease. These steps include plating a higher cell number at Day 3 (CD34⁺ cells), and using rhLaminin-521 instead of rhVTN-N as the plating matrix.

Reprogram CD34⁺ cells (xeno-free)

Media for reprogramming CD34⁺ cells (xeno-free)

For optimal reprogramming of CD34⁺ cells using the CTS™ CytoTune™ -iPS 2.1 Sendai Reprogramming Kit to generate iPSCs cultured on vitronectin-coated plates, use the following media at the designated stages of the reprogramming experiment:

- **CTS™ StemPro™ HSC Expansion Medium containing cytokines (i.e., SCF, IL-3 IL-6, FLT3, and TPO)** (“CTS™ StemPro™ HSC Expansion Medium (for 100 mL of complete medium)” on page 48): Plating cells prior to transduction, post-transduction recovery of cells
- **CTS™ StemPro™ HSC Expansion Medium containing cytokines + 4 µg/mL of Polybrene™**: Transduction
- **CTS™ StemPro™ HSC Expansion Medium without cytokines** (“CTS™ StemPro™ HSC Expansion Medium (for 100 mL of complete medium)” on page 48): Plating of transduced cells on vitronectin-coated culture dishes
- **Complete Essential 8™ Medium** (“Essential 8™ Medium (for 500 mL of complete medium)” on page 48): Expansion of transduced cells on vitronectin-coated culture dishes, live staining and picking of iPSCs

Required materials

Cells and vectors

- CTS™ CytoTune™ 2.1 Sendai reprogramming vectors
Note: For successful reprogramming, you need all three tubes of reprogramming vectors.
- CD34⁺ cells to reprogram
- *Optional:* Human neonatal foreskin fibroblast cells (strain BJ; ATCC® no. CRL2522) as a positive reprogramming control

Note: If you are using this as a control, follow the protocol for reprogramming fibroblasts within this manual (Chapter 3, “Reprogram fibroblasts”).

Media and reagents

- CTS™ StemPro™ HSC Expansion Medium (Cat. No. A4222301)
- Recombinant Human SCF Lyophilized (Cat. No. PHC2111)
- Recombinant Human IL-3 Lyophilized (Cat. No. PHC0031)
- Recombinant Human IL-6 Lyophilized (Cat. No. PHC0065)
- Recombinant Human FLT3 Lyophilized (Cat. No. PHC9414)
- Recombinant Human TPO Lyophilized (Cat. No. PHC9511)
- *Optional:* Penicillin-Streptomycin, Liquid (Cat. No. 15140-122)
- Polybrene™ Hexadimethrine Bromide (Sigma Aldrich, Cat. No. H9268)
- Dulbecco's PBS (DPBS) without Calcium and Magnesium (Cat. No. 14190)

Cells

The following protocol has been optimized for CD34⁺ cells derived from the human umbilical cord blood of mixed donors. We recommend that you optimize the protocol for your cell type, and add an appropriate number of conditions/wells to utilize the entire volume of virus. Note that experimental conditions may vary among target cells. The example given in the following protocol does not guarantee the generation of iPSCs for all cell types.

Day -3: Seed cells

1. Three days before transduction, remove one vial of CD34⁺ cells from the liquid nitrogen storage tank.
2. Briefly roll the cryovial between hands to remove frost, and swirl it gently in a 37°C water bath to thaw the CD34⁺ cells.
3. When only a small ice crystal remains in the vial, remove it from water bath. Spray the outside of the vial with 70% ethanol before placing it in the cell culture hood.
4. Pipet the thawed cells gently into a 15-mL conical tube.
5. Add 10 mL of pre-warmed complete CTS™ StemPro™ HSC Expansion Medium (see “CTS™ StemPro™ HSC Expansion Medium (for 100 mL of complete medium)” on page 48) drop-wise to the cells. Gently mix by pipetting up and down.
Note: Adding the medium slowly helps the cells to avoid osmotic shock.
6. Centrifuge the cell suspension at 200 × g for 10 minutes.
7. Discard the supernatant and resuspend the cells in 1 mL of complete CTS™ StemPro™ HSC Expansion Medium containing cytokines (i.e., SCF, IL-3, IL-6, FLT3, and TPO) (see “CTS™ StemPro™ HSC Expansion Medium (for 100 mL of complete medium)” on page 48).
8. Place 0.5 mL each of cell suspension into two wells of a 24-well plate and incubate at 37°C in a humidified atmosphere of 5% CO₂.

Note: We recommend using the wells in the middle section of the 24-well plate to prevent excessive evaporation of the medium during incubation.

Day -2: Observe cells and add fresh medium

Two days before transduction, add 0.5 mL of fresh complete CTS™ StemPro™ HSC Expansion Medium containing cytokines without disturbing the cells. If cells are present in 0.5 mL removed from the wells, centrifuge the cell suspension at 200 × g for 10 minutes, discard the supernatant, and resuspend the cells in 0.5 mL fresh medium before adding them back to the plate.

Day -1: Observe cells and add fresh medium

One day before transduction, gently remove 0.5 mL of medium and add 1 mL of fresh complete CTS™ StemPro™ HSC Expansion Medium containing cytokines without disturbing the cells.

Day 0: Count cells and perform transduction

1. Count the cells using the desired method (e.g., Countess™ II Automated Cell Counter), and calculate the volume of each virus needed to reach the target MOI using the **live cell count** and the titer information on the CoA.

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

Note: We recommend initially performing the transductions with MOIs of 5, 5, and 3 (i.e., KOS MOI=5, hL-Myc MOI=5, hKlf4 MOI=3). These MOIs can be optimized for your application.

Note: The titer of each CTS™ CytoTune™ 2.1 reprogramming vector is lot-dependent. For the specific titer of your vectors, go to thermofisher.com/cytotune and search for the CoA by product lot number, which is printed on the vial. Avoid re-freezing and thawing of the reprogramming vectors since viral titers can decrease dramatically with each freeze/thaw cycle.

2. Harvest the cells and seed the necessary number of wells of a 24-well plate in a minimal volume (~100 μL) with 1.0×10^5 cells/well for transduction.
3. Remove one set of CTS™ CytoTune™ 2.1 Sendai tubes from the -80°C storage. Thaw each tube one at a time by first immersing the bottom of the tube in a 37°C water bath for 10–20 seconds, and then removing the tube from the water bath and allowing it to thaw at room temperature. Once thawed, briefly centrifuge the tube and place it immediately on ice.
4. Add the calculated volumes of each of the three CTS™ CytoTune™ 2.1 Sendai viruses to 0.4 mL of pre-warmed CTS™ StemPro™ HSC Expansion Medium containing cytokines and 4 $\mu\text{g/mL}$ of Polybrene™. Ensure that the solution is thoroughly mixed by pipetting the mixture gently up and down. Complete the next step within 5 minutes.
5. Add the reprogramming virus mixture (from step 4) to the well(s) containing cells (from step 2). Incubate the cells at 37°C in a humidified atmosphere of 5% CO_2 overnight.

Day 1: Replace medium and culture cells

1. Remove the CTS[™] CytoTune[™] 2.1 Sendai viruses by centrifuging the cells at $200 \times g$ for 10 minutes. Aspirate and discard the supernatant.
2. Resuspend the cells in 0.5 mL of complete CTS[™] StemPro[™] HSC Expansion Medium containing cytokines (see “CTS[™] StemPro[™] HSC Expansion Medium (for 100 mL of complete medium)” on page 48) in the 24-well plate.
3. Incubate the cells in at 37°C in a humidified atmosphere of 5% CO₂ for two days.

Day 3: Plate cells on vitronectin-coated dishes

1. Coat a sufficient number of tissue culture dishes (e.g. 6-well, 60-mm, or 100-mm) with vitronectin (see “Coat culture vessels with vitronectin” on page 51 for coating protocol).

Note: rhLaminin-521 can be substituted for vitronectin (see “Coat culture vessels with rhLaminin-521” on page 53 for coating protocol).

2. Count the cells using the desired method (e.g., Countess[™] II Automated Cell Counter) and seed the vitronectin-coated dishes with at least two different densities between 2×10^4 – 2×10^5 CD34⁺ cells per well of a 6-well in 2 mL of complete CTS[™] StemPro[™] HSC Expansion Medium without cytokines.

Note: It is strongly recommended to set aside cells at this point for RNA extraction to be used as a positive control in the RT-PCR or qPCR detection of the CytoTune[™] vectors). It is very important to include this positive control when performing detection of the CytoTune[™] vectors.

3. Incubate the cells at 37°C in a humidified atmosphere of 5% CO₂ for three days.
4. Replace half of the spent medium every other day. Gently remove 1 mL of medium from the cells and replace with 1 mL of complete CTS[™] StemPro[™] HSC Expansion Medium without cytokines.

Note: The transduced cells may only be loosely attached for the first few days after plating. Be sure to perform media changes gently during this time. If cells are present in 1 mL removed from the wells, centrifuge the cell suspension at $200 \times g$ for 10 minutes, discard the supernatant, and resuspend the cells in 1 mL fresh medium before adding them back to the plate.

Day 7: Transition to Essential 8[™] Medium

1. Remove 1 mL of medium from the cells and add 1 mL of Essential 8[™] Medium (see “Essential 8[™] Medium (for 500 mL of complete medium)” on page 48) to transition the cells to the new culture medium.
2. Incubate the cells in a 37°C, 5% CO₂ incubator overnight.

Day 8 to 28: Feed and monitor the cells

1. The next day, remove the spent medium completely and replace with 2 mL of Essential 8[™] Medium. Replace spent medium daily.
Note: Plated cells should have fully attached by this point. Any cells that have not attached will likely never do so.
2. Starting on Day 8, observe the plates every other day under a microscope for the emergence of cell clumps indicative of reprogrammed cells.
Note: For BJ fibroblasts (positive control), colony formation is normally observed on Day 12 post-transduction. However, depending on cell type, it may take up to 4 weeks before colonies are seen.
3. Day 15 to 20 after transduction, colonies should have grown to an appropriate size for transfer.
Note: We recommend harvesting colonies closer to 3 weeks to avoid differentiation.
4. When colonies are ready for transfer, perform live staining using Tra1-60 or Tra1-81 for selecting reprogrammed colonies if desired (see “Live stain” on page 39).
5. Manually pick colonies and transfer them onto prepared vitronectin-coated 12- or 6-well culture dishes (see “Pick iPSC colonies” on page 40).

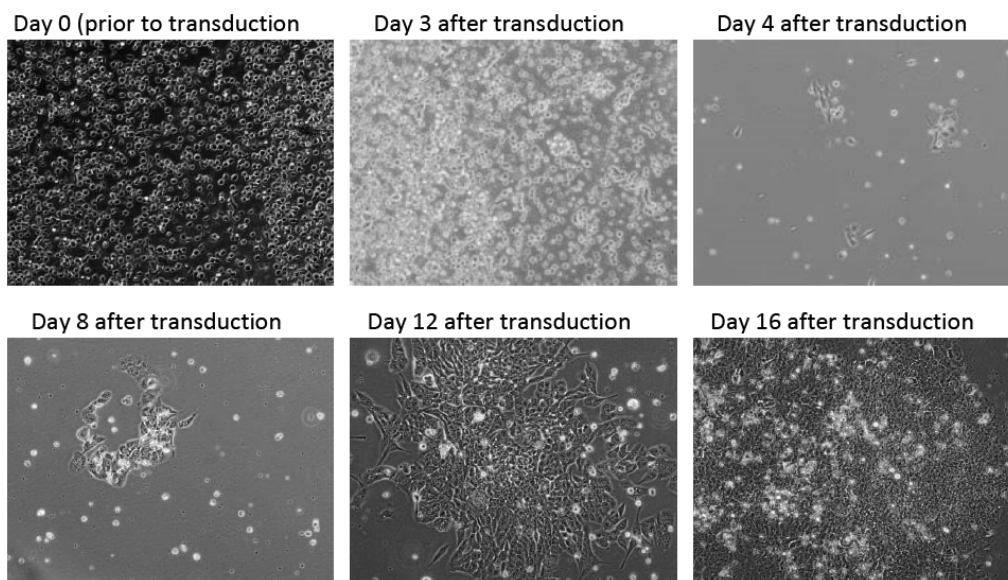
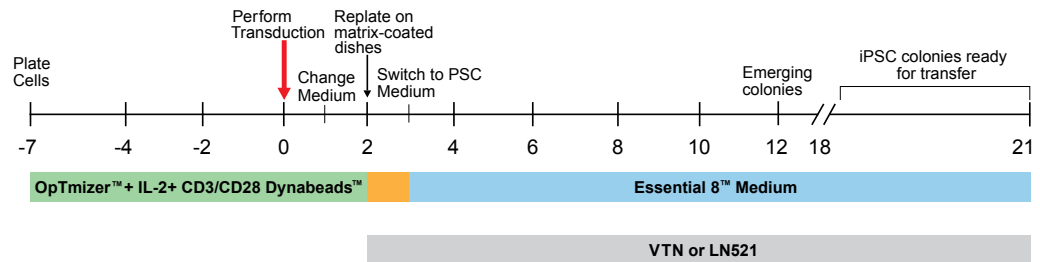


Figure 4 CD34⁺ cells reprogrammed using the CTS[™] CytoTune[™] -iPS 2.1 Sendai Reprogramming Kit. CD34⁺ cells reprogrammed using the CTS[™] CytoTune[™] -iPS 2.1 Sendai Reprogramming Kit according to the protocol, and plated on to recombinant human laminin-521 three days after transduction. 100X magnification phase contrast images were taken at the indicated number of days post-transduction.

Experimental outline (xeno-free)

Workflow

The major steps required for reprogramming T-Cells using the CTS™ CytoTune™ iPS 2.1 Sendai Reprogramming Kit to generate iPSCs cultured feeder-free on vitronectin-coated (or LN521-coated) culture dishes are shown below. Note that the timeline is provided as a guideline for experimental planning; actual timeline can vary based on the cell type and experimental conditions.



Reprogramming timeline

Day -7: Isolate T-cells, or thaw previously isolated T-cells. Activate with CD3/CD28 Dynabeads™.

Day -4: Replace half of the medium with fresh OpTmizer™ + IL-2.

Day -2: Count cells, add fresh OpTmizer™ + IL-2 to bring concentration to 1×10^6 cells/mL.

Day 0: Transduce the cells using the CTS™ CytoTune™ 2.1 Sendai reprogramming vectors at the appropriate MOI. Incubate the cells overnight.

Day 1: Replace the medium with fresh OpTmizer™ + IL-2 to remove the CTS™ CytoTune™ 2.1 Sendai reprogramming vectors.

Day 2: Remove CD3/CD28 Dynabeads™. Plate the transduced cells on vitronectin-coated (or LN521-coated) culture dishes in OpTmizer™ + IL-2.

Day 3: Change medium to Essential 8™ Medium.

Day 4–21: Replace spent medium with fresh Essential 8™ Medium every day and monitor the culture vessels for the emergence of iPSC colonies. When iPSC colonies are ready for transfer, perform live staining, and pick and transfer undifferentiated iPSCs onto fresh vitronectin-coated culture dishes for expansion.

Reduced efficiency

For increased safety, the cMyc transgene has been replaced with a less oncogenic variant, L-Myc. This change has been observed to decrease the overall reprogramming efficiency when compared to CytoTune™ 2.0, so steps should be taken to account for this decrease. These steps include plating a higher cell number at Day 2 (T cells), and using rhLaminin-521 instead of rhVTN-N as the plating matrix.

Reprogram T-Cells (xeno-free)

Media for reprogramming T-Cells (feeder-free)

For optimal reprogramming of T-Cells using the CTS™ CytoTune™ -iPS 2.1 Sendai Reprogramming Kit to generate iPSCs cultured on VTN or LN521, use the following media at the designated stages of the reprogramming experiment:

- **T-Cell Medium (OpTmizer™ + 100 U/mL IL-2)** (“OpTmizer™ CTS™ T-Cell expansion medium (for 1000 mL of complete medium)” on page 49): Plating cells prior to transduction, expansion, post-transduction recovery of cells, plating onto VTN or LN521.
- **Complete Essential 8™ Medium** (“Essential 8™ Medium (for 500 mL of complete medium)” on page 48): Expansion of transduced cells on vitronectin-coated (or LN521-coated) culture dishes, live staining and picking of iPSCs.

Required materials

Cells and vectors

- CTS™ CytoTune™ 2.1 Sendai reprogramming vectors
Note: For successful reprogramming, you need all three tubes of reprogramming vectors.
- T-Cells to reprogram
Note: You can use T-Cells extracted from fresh blood by a conventional method (i.e. magnetic bead isolation/enrichment) or frozen T-Cells.
- *Optional:* Human neonatal foreskin fibroblast cells (strain BJ; ATCC® no. CRL2522) as a positive reprogramming control
Note: If you are using this as a control, follow the protocol for reprogramming fibroblasts within this manual (Chapter 3, “Reprogram fibroblasts”).

Media and reagents

- CTS™ OpTmizer™ T Cell Expansion SFM™, bottle format (Cat. No. A1048501)
- IL2 Recombinant Human Protein (Cat. No. PHC0021)
- Dynabeads™ Human T-Expander CD3/CD28 (Cat. No. 11141D)
- Dulbecco's PBS (DPBS) without Calcium and Magnesium (Cat. No. 14190)
- Dulbecco's PBS (DPBS), Calcium, Magnesium (Cat. No. 14040133)
- Essential 8™ Medium (Cat. No. A1517001)
- Vitronectin, truncated recombinant human (VTN-N) (Cat. No. A14700)
- rhLaminin-521 (Cat. No. A29249)

Reprogramming protocol

The following protocol has been optimized for T-Cells isolated from whole blood, (or leukapheresis product), through magnetic bead isolation or enrichment. Cells can be freshly isolated, or thawed (after being frozen following isolation). We recommend that you optimize the protocol for your cell type, and add an appropriate number of conditions/wells to utilize the entire volume of virus. Note that experimental conditions may vary among target cells. The example given in the following protocol does not guarantee the generation of iPSCs for all cell types.

Day -7 to -5: Seed T-Cells

1. Five to seven days before transduction, isolate T-Cells from whole blood (or leukapheresis product) using standard isolation procedures, such as the Dynabeads™ Human T-Expander CD3/CD28, following manufacture's instructions. If cells are frozen, thaw cells using standard thawing procedures.
2. Suspend the cells in T-Cell medium to a concentration of 0.5×10^6 cells/mL. If T-cells were isolated using using Dynabeads™ Human T-Expander CD3/CD28, skip step 3 and go directly to step 4.
3. Add Dynabeads™ Human T-Expander CD3/CD28 at a ratio of 3:1 (3 beads to 1 cell).
4. Plate the cells into an appropriate culture dish. For a 6-well plate, add 4 mL of cells (approximately 2×10^6 cells) per well.
5. Incubate the cells in a 37°C incubator with a humidified atmosphere of 5% CO₂.

Day -4: Add fresh medium

Gently remove 0.5 mL of the medium from each well, and replace it with 1.5 mL of fresh T-Cell medium.

Day -2: Count cells, add fresh medium

1. Count the cells. If using an automated counting method (e.g. Countess™ II Automated Cell Counter), Dynabeads™ Human T-Expander CD3/CD28 should first be removed from a small sample of cells to generate an accurate count.
2. Add medium to bring the cells to a concentration of 0.5×10^6 live cells/mL. Plate cells onto additional wells or plates, if necessary (maximum volume for each well of a 6-well plate should be 5 mL).

Day 0: Count cells and perform transduction

1. Count the cells using the desired method and calculate the volume of each virus needed to reach the target MOI using the live cell count and the titer information on the CoA. If using an automated counting method (e.g. Countess™ II Automated Cell Counter), Dynabeads™ Human T-Expander CD3/CD28 should first be removed from a small sample of cells to generate an accurate count.

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

Note: We recommend initially performing the transductions with MOIs of 5, 5, and 3 (i.e., KOS MOI=5, hL-Myc MOI=5, hKlf4 MOI=3). These MOIs can be optimized for your application.

Note: The titer of each CTS™ CytoTune™ 2.1 reprogramming vector is lot-dependent. For the specific titer of your vectors, go to [thermofisher.com/cytotune](https://www.thermofisher.com/cytotune) and search for the CoA by product lot number, which is printed on the vial. Avoid re-freezing and thawing of the reprogramming vectors since viral titers can decrease dramatically with each freeze/thaw cycle.

2. For each transduction, add 3×10^5 T-cells, in a minimal volume, to one well of a tissue culture-treated 12-well plate.
3. Remove CTS™ CytoTune™ 2.1 Sendai tubes from the -80°C storage. Thaw each tube one at a time by first immersing the bottom of the tube in a 37°C water bath for 10–20 seconds, and then removing the tube from the water bath and allowing it to thaw at room temperature. Once thawed, briefly centrifuge the tube and place it immediately on ice.
4. For each transduction, add the calculated volumes of each of the three CTS™ CytoTune™ 2.1 Sendai tubes to 1 mL of T-Cell medium, pre-warmed to 37°C . Ensure that the solution is thoroughly mixed by pipetting the mixture gently up and down. Complete the next step on page 36 within 5 minutes.
5. Add the virus mixture prepared in step 4 to the well(s) of cells prepared in step 2. Incubate the plate overnight in a 37°C incubator with a humidified atmosphere of 5% CO_2 .

Day 1: Replace medium and culture cells

1. The next day, remove the cells and medium from the culture plate and transfer to a 15-mL centrifuge tube. Rinse the well gently with 1 mL of T-Cells medium to ensure most of the cells are harvested.
2. Remove the CTS™ CytoTune™ 2.1 Sendai viruses by centrifuging the cell suspension at $200 \times g$ for 10 minutes, aspirating the supernatant, and resuspending the cells in 1.0 mL of T-Cell medium per well of a 12-well plate. Return cells to the same well they came from in the 12-well plate.
3. Return the plate to the incubator overnight.

Day 2: Plate cells on vitronectin-coated (or LN521-coated) culture dishes

1. Coat a sufficient number of tissue culture dishes (e.g. 6-well, 60-mm, or 100-mm) with vitronectin (see “Vitronectin working concentration” on page 51 for coating protocol).

Note: rhLaminin-521 (LN521) can be substituted for vitronectin; see “rhLaminin-521 working concentration” on page 53 for coating protocol. The plating efficiency of transduced T-Cells is typically much higher on LN521 than VTN, and this yields more iPSC colonies at the end of the experiment.

2. Remove the Dynabeads™ Human T-Expander CD3/CD28 from the cells. Count the cells using the desired method (e.g., Countess™ II Automated Cell Counter) and seed the 6-well vitronectin-coated culture plates with 2×10^4 – 2×10^5 live cells per well in 2 mL of T-Cell medium.

Note: It may be necessary to plate more than two different densities, as the reprogramming efficiencies of T-Cells can vary widely between donors. If sufficient cells are available, it is recommended to plate 4–6 different densities, ranging from 2×10^4 – 2×10^5 cells per well of a 6-well plate.

Note: It is strongly recommended to set aside cells at this point for RNA extraction to be used as a positive control in the RT-PCR or qPCR detection of the CytoTune™ vectors. It is very important to include this positive control when performing detection of the CytoTune™ vectors.

3. Incubate the cells at 37°C in a humidified atmosphere of 5% CO₂.

Day 3: Transition cells to Essential 8™ Medium

1. Prepare Essential 8™ Medium (see “Essential 8™ Medium (for 500 mL of complete medium)” on page 48).
2. Remove T-Cell Medium, and replace with Essential 8™ Medium.

Note: The transduced T-Cells may only be loosely attached for the first 7 days after plating. Be sure to perform media changes gently during this time. If cells are present in medium removed from the wells, centrifuge the cell suspension at $200 \times g$ for 10 minutes, discard the supernatant, and resuspend the cells in 1 mL fresh Essential 8™ Medium before adding them back to the plate. Any cells that have not attached by 7 days after plating are unlikely to ever attach.

Days 4 to 21: Feed and monitor the cells

1. Replace the spent medium with fresh Essential 8™ Medium daily.
2. Starting on day 6, observe the plates every other day under a microscope for the emergence of cell clumps indicative of reprogrammed cells.

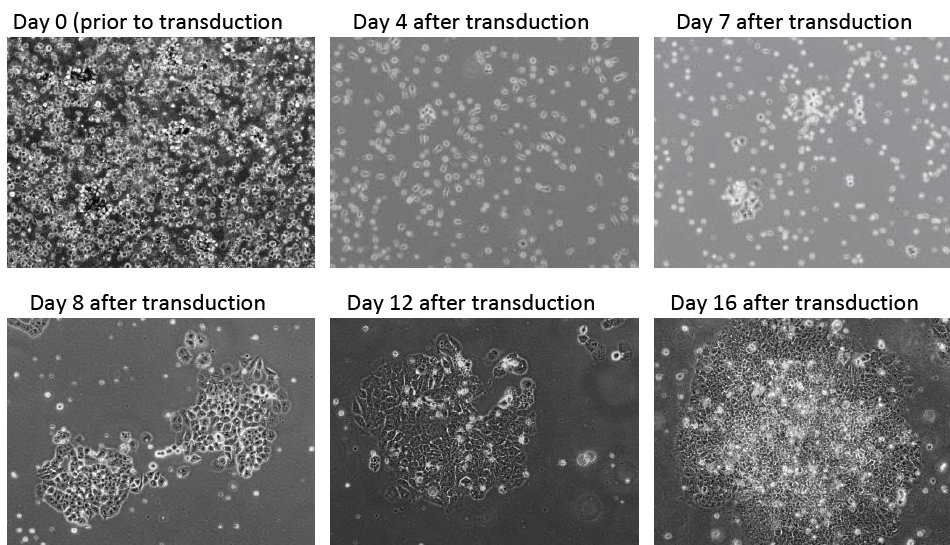


Figure 5 T-cells reprogrammed using the CTS™ CytoTune™ -iPS 2.1 Sendai Reprogramming Kit. T-cells reprogrammed using the CTS™ CytoTune™ -iPS 2.1 Sendai Reprogramming Kit according to the protocol, and plated on to recombinant human laminin-521 two days after transduction. 100X magnification phase contrast images were taken at the indicated number of days post-transduction.

3. By day 15 to 21 after transduction, colonies should have grown to an appropriate size for transfer.
Note: We typically harvest colonies around Day 16–18 to avoid differentiation and overgrowth of colonies.
4. When colonies are ready for transfer, perform live staining using Tra1-60 or Tra1-81 for selecting reprogrammed colonies if desired (see page 39).
5. Manually pick colonies and transfer them onto prepared vitronectin-coated 6- or 12-well culture plates (see Chapter 7, “Identify and pick iPSC colonies”).



Identify and pick iPSC colonies

Visual identification

Visual identification

By Day 21 post-transduction, the cell colonies on the culture dishes will have become large and compact, covering the majority of the surface area of the culture dish. However, only a fraction of these colonies will consist of iPSCs, which exhibit a hESC-like morphology characterized by a flatter cobblestone-like appearance with individual cells clearly demarcated from each other in the colonies (see Figure 5 on page 38). Therefore, we recommend that you perform live staining with Tra1-60 or Tra1-81 antibodies that recognize undifferentiated iPSCs.

Live stain

Live stain with antibodies

One of the fastest and most reliable methods for identifying a reprogrammed colony is live staining with Tra1-60 or Tra1-81 antibodies that recognize undifferentiated iPSCs and enable the identification of reprogrammed cells from a variety of human cell types.

Note: Other methods of identifying iPSCs (such as alkaline phosphatase staining) are also acceptable.

Required antibodies

TRA-1-60 Mouse anti-human mAb, Alexa Fluor™ 488 Conjugate Kit for Live Cell Imaging (Cat. No. A25618) or TRA-1-60 Mouse anti-human mAb, Alexa Fluor™ 594 Conjugate Kit for Live Cell Imaging (Cat. No. A24882)

Live stain cells

If live-stained cells are to be used for further culture, be sure to use antibodies that are sterile (filter sterilize as necessary) and work aseptically.

1. Centrifuge the dye-conjugated antibody solution (e.g., 2 minutes at $10,000 \times g$) and only use the supernatant.

Note: This step minimizes transferring protein aggregates that may have formed during storage, thereby reducing non-specific background staining.

2. Add a 1:50 volume of the dye-conjugated antibody directly to the cell culture medium of the cells to be stained (see Table 1 on page 40), then mix by gentle swirling.
3. Incubate for 30 minutes at 37°C.
4. Remove the staining solution and gently wash the cells 2–3 times with FluoroBrite™ DMEM (Cat. No. A1896701).

- For optimal results, image the cells immediately (i.e., within 30 minutes).

Note: To continue culturing the cells, replace the FluoroBrite™ DMEM with fresh cell culture medium and return the cells to the 37°C incubator.

Table 1 Recommended volumes for staining protocol

Culture format	No. of tests	Staining volume	50X antibody
96-well plate	200	50 µL/well	1 µL
48- well plate	100	100 µL/well	2 µL
24- well plate	50	200 µL/well	4 µL
12- well plate	25	400 µL/well	8 µL
6- well plate	10	1 mL/well	20 µL
35-mm dish	10	1 mL/dish	20 µL
60-mm dish	5	2 mL/dish	40 µL
100-mm dish	2	5 mL/dish	100 µL
4-well chamber slide	25	400 µL/well	8 µL
8-well chamber slide	50	200 µL/well	4 µL

Pick iPSC colonies

Pick iPSC colonies (feeder-free)

- Place the culture dish containing the reprogrammed cells under an inverted microscope and examine the colonies under 10X magnification.
- Mark the colony to be picked on the bottom of the culture dish.
Note: We recommend picking at least 10 distinct colonies by the end of each reprogramming experiment and expanding them in separate 12- or 6-well vitronectin-coated culture plates (see below).
- Transfer the culture dish to a sterile cell culture hood (i.e., biosafety cabinet) equipped with a stereomicroscope.
- Using a 25 gauge 1½ inch needle, cut the colony to be picked into 4–16 pieces in a grid-like pattern.
- Using a 200 µL pipette, transfer the cut pieces onto a vitronectin-coated 12- or 6-well culture plate (see “Vitronectin working concentration” on page 51) containing complete Essential 8™ Medium (“Essential 8™ Medium (for 500 mL of complete medium)” on page 48).
- Incubate the vitronectin-culture plate containing the picked colonies in a 37°C incubator with a humidified atmosphere of 5% CO₂.

7. Allow the colonies to attach to the culture plate for 48 hours before replacing the spent medium with fresh complete Essential 8™ Medium. After that, change the medium every day.
8. When the colonies cover ~85% of the surface area of the culture vessel, they are ready for passaging. Passage the colonies using 0.5 mM EDTA prepared in Dulbecco's Phosphate-Buffered Saline (DPBS) without calcium or magnesium (see "Passage iPSCs with EDTA" on page 58).

Note: Enzymes such as collagenase and dispase do not work well with cells cultured in Essential 8™ Medium on vitronectin-coated plates. Use of these enzymes for passaging cells results in compromised viability and attachment.

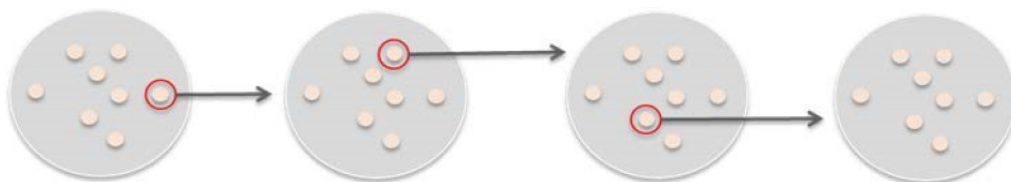
9. Continue to culture, expand, and maintain the reprogrammed colonies in complete Essential 8™ Medium until you have frozen cells from two 60-mm plates (see "Freeze iPSCs in Essential 8™ freezing medium" on page 59).

Generate vector-free iPSCs

Generate vector-free iPSCs

Guidelines for generating vector-free iPSCs

- The time needed to derive vector-free iPSCs may vary depending on culture and passage conditions. In the case of human neonatal foreskin fibroblast cells (strain BJ), it takes about 1–2 months after gene transduction to obtain iPSCs free of CTS™ CytoTune™ 2.1 Sendai reprogramming vectors.
- To obtain virus-free clones faster, we recommend that you perform single colony subcloning for the first few passages (minimum 5) instead of bulk or pooled-clone passaging.
- To perform single colony subcloning, pick from a single colony to transfer to another 6-well plate (Passage 1). From Passage 1, pick a single colony and transfer to another 6-well plate (passage 2) and so forth. We recommend subcloning for 5 passages and then testing for virus free iPSCs.



Required materials

- Dulbecco's PBS (DPBS) without Calcium and Magnesium (Cat. No. 14190)
- Sendai virus HN Monoclonal Antibody (1A6); (eBioscience™; Cat. No. 14-6494-82)
- Alexa Fluor™ 488 goat anti-mouse IgG (H+L) antibody (Cat No. A11001) or Alexa Fluor™ 594 goat anti-mouse IgG (H+L) antibody (Cat No. A11005)
- TRIzol™ LS reagent (Cat. No. 10296-010)
- SuperScript™ VILO™ cDNA Synthesis Kit (Cat. No. 11754-050)
- AccuPrime™ SuperMix I (Cat. No. 12342-010)

Generate vector-free iPSCs

1. When passaging iPSC colonies, prepare duplicate plates; one for immunostaining and one for further passaging.
2. Perform immunostaining on one plate using anti-SeV antibodies.
3. If any colonies stain positive, perform cell cloning on the other duplicate plate.
4. Repeat immunostaining with anti-SeV antibodies on the cloned colonies until all colonies in a plate are negative.
5. If all colonies are negative for anti-SeV antibodies, passage the cells and confirm the absence of the CTS™ CytoTune™ 2.1 Sendai reprogramming vectors by RT-PCR (see “RT-PCR protocol for detecting the SeV genome and transgenes”), or real-time RT-PCR.

Immunocytochemistry with anti-SeV antibodies

1. Wash cells once with D-PBS
2. Fix the cells in 4% paraformaldehyde for 5 minutes at room temperature.
3. Wash cells twice with D-PBS.
4. Add the anti-SeV antibody (Sendai virus HN Monoclonal Antibody (1A6), eBioscience™; Cat. No. 14-6494-82) diluted in 0.1% Triton™ X-100 in D-PBS to the cells and incubate for 1 hour at 37°C.
5. Remove the antibody solution. Wash the cells 3 times with D-PBS.
6. Add the secondary antibody diluted in 0.1% Triton™ X-100 in D-PBS to the cells and incubate for 1 hour at 37°C.
7. Remove the secondary antibody solution from the dish. Wash the cells 3 times with D-PBS.
8. Visualize the cells under a fluorescence microscope.

RT-PCR protocol for detecting the SeV genome and transgenes

1. Extract the total RNA from 5×10^6 iPSCs using the TRIzol™ Reagent (Cat. No. 15596-026) following the instructions provided with the reagent. As a positive control, use cells set aside during the reprogramming procedure.
Note: It is important to use cells set aside during the reprogramming experiment (e.g. 7 days after transduction for fibroblast, or 4 days after transduction for PBMCs) as a valid positive control. Early-passage iPSC may already have cleared one or more of the vectors and are therefore not a suitable positive control.
2. Synthesize cDNA using 1 µg of RNA (from previous step) and the High-Capacity cDNA Reverse Transcription Kit (Cat. No. 4368813) following the instructions provided with the kit.
Note: Because the CTS™ CytoTune™ 2.1 Sendai reprogramming vectors are based on SeV, which is an RNA virus, reverse transcription is required for detecting the presence of the SeV genome in your reprogrammed cells.

- After completion of the cDNA synthesis reaction, adjust the volume to 250 µL with nuclease-free water. The cDNA sample can be used immediately or stored at –20°C for later use. Carry out the PCR using 5 µL of diluted cDNA from the and AccuPrime™ SuperMix I (Cat. No. 12342-010) with the parameters below. For the RT-PCR primer sequences and the expected product size, see “RT-PCR primer”.

Step	Temperature	Time	Cycles
Denaturation	95°C	30 seconds	30–35
Annealing	55°C	30 seconds	
Elongation	72°C	30 seconds	

- Analyze the PCR products using 2% agarose gel electrophoresis.

If you still detect CTS™ CytoTune™ 2.1 Sendai virus in your iPSC lines after more than 10 passages, and have performed RT-PCR or qPCR to show that hKlf4 is absent from your cells (this vector does not have the temperature sensitive mutation), then you can perform a temperature shift to remove KOS and/or the L-Myc vector(s). CTS™ CytoTune™ 2.1 Sendai L-Myc may persist in the cells longer than the other CTS™ CytoTune™ 2.1 Sendai reprogramming vectors. However, because this vector contains a temperature sensitivity mutation, you can enhance its removal and obtain complete absence of Sendai virus by incubating your cells at 38–39°C for 5 days.

RT-PCR primer

Use the following RT-PCR primer sets to detect the SeV genome and transgenes **sets** in cells reprogrammed using the CTS™ CytoTune™ 2.1 Sendai reprogramming vectors.

Target	Primer sets	Product size
SeV	Forward: GGA TCA CTA GGT GAT ATC GAG C ^[1] Reverse: ACC AGA CAA GAG TTT AAG AGA TAT GTA TC ^[1]	181 bp
KOS	Forward: ATG CAC CGC TAC GAC GTG AGC GC Reverse: ACC TTG ACA ATC CTG ATG TGG ^[1]	528 bp
Klf4	Forward: TTC CTG CAT GCC AGA GGA GCC C Reverse: AAT GTA TCG AAG GTG CTC AA ^[1]	410 bp
L-Myc	Forward: GAG AAG AGG ATG GCT ACA GAG A Reverse: GAC GTG CAA CTG TGC TAT CT ^[1]	237 bp

^[1] Primer contains SeV genome sequences. Pairing of these primers with transgene-specific primers allows specific detection of transgenes carried by the CTS™ CytoTune™ 2.1 Sendai reprogramming vectors.

RT-qPCR

As an alternative to endpoint RT-PCR, use the following pre-validated TaqMan™ assays to detect the presence of CTS™ CytoTune™ 2.1 Sendai reprogramming vectors by RT-qPCR:

Target	Taqman assay
SeV	Mr04269880_mr
KOS	Mr04421257_mr
Klf4	Mr04421256_mr
L-Myc	Mr04944276_mr

Troubleshooting

Observation	Possible cause	Recommended action
Cytotoxic effect observed after transduction	Viral load is too high.	Decrease the volume of CTS™ CytoTune™ 2.1 vector or increase the starting cell number.
Too many colonies on the plate	Too many cells plated	Decrease the number of cells plated after transduction.
No PSC colony formation	Insufficient amount of virus used	Check the volume of the CTS™ CytoTune™ 2.1 vector and the starting cell number. Changing the MOI may improve the results.
		We suggest initially increasing the MOI of Klf4 to improve efficiency (e.g., KOS MOI = 5, L-Myc MOI =5, Klf4 MOI =6). If efficiencies are still too low, increase the MOI of KOS and L-Myc, while maintaining a 1:1 ratio between the two (e.g. KOS MOI = 10, L-Myc MOI = 10, Klf4 =6).
		Not all cell types will be reprogrammed with the same efficiency. Check the levels of protein expression in your cell type using TaqMan™ Protein Assays (see page 44 for ordering information).
		Do not re-freeze thaw or aliquot virus. Viral titer is not guaranteed for kits re-frozen or thawed.
Too few iPSC colony compare BJ fibroblasts	Cell type is not efficiently reprogrammable.	Not all cell types will have the same reprogramming efficiency. Increase the number of the cells plated.
		If reprogramming a new cell type, use the CytoTune™ -EmGFP Sendai Fluorescence Reporter to assess uptake of Sendai virus (see “CytoTune™ -EmGFP reporter control transduction” on page 55).
iPSC colonies look differentiated	iPSC colonies transferred to vitronectin-coated dishes too late	Perform staining earlier and transfer iPSC colony to fresh vitronectin or rhLaminin-521 coated plates.



Media and reagents

Prepare media and reagents

SCF (c-kit Ligand), FLT-3 Ligand, IL-3, IL-6, and TPO stock solutions

SCF (c-kit Ligand), FLT-3 Ligand, IL-3, IL-6, and TPO are supplied lyophilized. Prepare stock solutions as described in their specific product inserts and store small aliquots frozen. Thaw at time of use.

0.5 mM EDTA in DPBS (50 mL)

1. To prepare 50 mL of 0.5 mM EDTA in DPBS, aseptically mix the following components in a 50-mL conical tube in a biological safety cabinet:

DPBS without Calcium and Magnesium	50 mL
0.5 M EDTA	50 μ L

2. Filter sterilize the solution.

The solution can be stored at room temperature for up to six months.

Complete fibroblast medium

To prepare 100 mL of complete fibroblast medium, aseptically mix the following components:

DMEM	89 mL
FBS, ESC-Qualified	10 mL
MEM™ Non-Essential Amino Acids Solution, 10 mM	1 mL

Complete fibroblast medium can be stored at 2–8°C for up to 4 weeks.

Complete xeno-free fibroblast medium

1. To prepare 500 mL of complete xeno-free fibroblast medium, aseptically mix the following components:

DMEM/F-12, HEPES	436 mL
Sodium Bicarbonate, 7.5% solution	3.2 mL
Knockout SR, xeno-free	50 mL
bFGF (10 μ g/mL)	0.6 mL
EGF (1mg/mL)	0.5 mL
Hydrocortisone (50 μ M solution)	10 mL

2. Filter Sterilize the solution

Complete xeno-free fibroblast medium can be stored at 2–8°C for up to 2 weeks.

Essential 8™ Medium (for 500 mL of complete medium)

1. Thaw frozen Essential 8™ Supplement at 2–8°C overnight before using it to prepare complete medium. **Do not thaw the frozen supplement at 37°C.**
2. Mix the thawed supplement by gently inverting the vial a couple of times, remove 10 mL from the bottle of Essential 8™ Basal Medium, and then aseptically transfer the entire contents of the Essential 8™ Supplement to the bottle of Essential 8™ Basal Medium. Swirl the bottle to mix and to obtain 500 mL of homogenous complete medium.
3. Complete Essential 8™ Medium can be stored at 2–8°C for up to 2 weeks. Before use, warm complete medium required for that day at room temperature until it is no longer cool to the touch. **Do not warm the medium at 37°C.**

CTS™ StemPro™ HSC Expansion Medium (for 100 mL of complete medium)

CTS™ StemPro™ HSC Expansion Medium is used for xeno-free reprogramming of CD34+ cells, as well as PBMCs. CTS™ StemPro™ HSC Basal Medium requires supplementation with CTS™ StemPro™ HSC Supplement. Also, depending on the cell type and protocol step, the medium may need to be supplemented with the appropriate indicated cytokines. Follow the procedure below to prepare 100 mL of complete CTS™ StemPro™ HSC Expansion Medium.

1. Thaw CTS™ StemPro™ HSC Supplement (50X) at room temperature for at least 1 hour or overnight at 4°C
2. Equilibrate the CTS™ StemPro™ HSC Supplement (50X) to room temperature for at least 30 minutes, and no more than 3 hours. Gently invert vial until supplement is completely homogenous before preparing aliquots or adding to CTS™ StemPro™ HSC Basal Medium. (Optional) - Once CTS™ StemPro™ HSC Supplement (50X) is thawed and mixed until it is completely homogenous, prepare single use aliquots and refreeze at –20°C. Avoid multiple freeze-thaw cycles.
3. For 100 mL of complete medium: Aseptically add 2 mL of thawed CTS™ StemPro™ HSC Supplement (50X) to 98 mL of room temperature CTS™ StemPro™ HSC Basal Medium and thoroughly mix by gently inverting several times.
4. Prepare complete medium immediately prior to use in culture.
5. Depending on the cell type, add the following cytokines to the indicated final concentration to an aliquot of medium on the day of use:

PBMC cytokine	Final concentration
SCF	100 ng/mL
FLT-3	100 ng/mL
IL-3	20 ng/mL
IL-6	20 ng/mL

CD34+ cytokine	Final concentration
SCF	100 ng/mL
FLT-3	100 ng/mL
IL-3	50 ng/mL
IL-6	20 ng/mL
TPO	100 ng/mL

OpTmizer™ CTS™ T-Cell expansion medium (for 1000 mL of complete medium)

OpTmizer™ CTS™ T-Cell Expansion Medium is used for xeno-free reprogramming of T-cells. OpTmizer™ CTS™ T-Cell Expansion Basal Medium requires supplementation with OpTmizer™ CTS™ T-Cell Expansion Supplement, and L-glutamine.

- For complete 1X medium, aseptically add to OpTmizer™ CTS™ T-Cell Expansion Basal Medium before use:
 - 26 mL/L of OpTmizer™ CTS™ T-Cell Expansion Supplement.
 - 10 mL/L of 200 mM L-glutamine solution for a final concentration of 2 mM.
- Place the OpTmizer™ CTS™ T-Cell Expansion Basal Medium, OpTmizer™ CTS™ T-Cell Expansion Supplement and thawed L-Glutamine solution under a sterile laminar flow hood.
- Remove the caps and using a sterile pipette, remove 26 mL of OpTmizer™ CTS™ T-Cell Expansion Supplement and add to 1 L of OpTmizer™ CTS™ T-Cell Expansion Basal Medium. Discard pipette.
- Using a new sterile pipette, remove 10 mL of L-Glutamine solution and add to 1 L of OpTmizer™ CTS™ T-Cell Expansion Basal Medium. Discard pipette.
- Replace the caps tightly and swirl gently to mix the complete OpTmizer™ CTS™ T-Cell Expansion SFM™.
- Add the following cytokines to the indicated final concentration to an aliquot of medium on the day of use:

T-Cells Cytokine	Final concentration
IL-2	100 U/mL

Essential 8™ freezing medium

- Pre-warm the required volume of Essential 8™ Medium at room temperature until it is no longer cool to the touch. **Do not warm the medium in a 37°C water bath.**
- Prepare Essential 8™ Freezing Medium. For every 1 mL of freezing medium needed, aseptically combine the components listed below in a sterile 15-mL tube:

Complete Essential 8™ Medium	0.9 mL
DMSO	0.1 mL



Appendix A Media and reagents

Prepare media and reagents

3. Place the tube with Essential 8™ Freezing Medium on ice until use. Discard any remaining freezing medium after use.

Note: Alternatively, PSC Cryopreservation medium can be used for freezing iPSCs.



Prepare culture vessels

Coat culture vessels with vitronectin

Vitronectin working concentration

The optimal working concentration of vitronectin is cell line dependent and must be determined empirically. We recommend using a final coating concentration of $0.5 \mu\text{g}/\text{cm}^2$ on the culture surface. Prior to coating culture vessels, calculate the working concentration of vitronectin using the formula below and dilute the stock appropriately. See Table 2 for culture surface area and volume required.

$$\text{Working conc.} = \text{Coating conc.} \times \frac{\text{Culture surface area}}{\text{Volume required for surface area}}$$

$$\text{Dilution factor} = \frac{\text{Stock concentration (0.5 mg/mL)}}{\text{Working concentration}}$$

For example, to coat a 6-well plate at a coating concentration of $0.5 \mu\text{g}/\text{cm}^2$, you will need to prepare 6 mL of diluted vitronectin solution ($10 \text{ cm}^2/\text{well}$ surface area and 1 mL of diluted vitronectin/well; see Table 2) at the following working concentration:

$$\text{Working conc.} = 0.5 \mu\text{g}/\text{cm}^2 \times \frac{10 \text{ cm}^2}{1 \text{ mL}} = 0.5 \mu\text{g}/\text{mL}$$

$$\text{Dilution factor} = \frac{0.5 \text{ mg/mL}}{5 \mu\text{g}/\text{mL}} = 100\text{X (i.e., 1:100 dilution)}$$

Table 2 Vitronectin Coating Reagent volumes (per well or per dish)

Culture vessel	Surface area	Volume of diluted vitronectin solution
6-well plate	10 cm^2 per well	1.0 mL/well
12-well plate	4 cm^2 per well	0.4 mL/well
24-well plate	2 cm^2 per well	0.2 mL/well
35-mm dish	10 cm^2	1.0 mL
60-mm dish	20 cm^2	2.0 mL
100-mm dish	60 cm^2	6.0 mL

Culture vessel	Surface area	Volume of diluted vitronectin solution
T-25 flask	25 cm ²	2.5 mL
T-75 flask	75 cm ²	7.5 mL

Coat culture vessels with vitronectin

Instructions for coating a 6-well culture plate with vitronectin at a coating concentration of 0.5 µg/cm² are provided below. For volumes used in other culture vessels, see Table 2. To calculate the working concentration of vitronectin used with other coating concentrations and to determine the appropriate dilution factor, use the equations on “Vitronectin working concentration” on page 51.

1. Upon receipt, thaw the vial of vitronectin at room temperature and prepare 60-µL aliquots of vitronectin in polypropylene tubes.
Freeze the aliquots at -80°C or use immediately.
2. To coat the wells of a 6-well plate, remove a 60-µL aliquot of vitronectin from -80°C storage and thaw at room temperature. You will need one 60-µL aliquot per 6-well plate.
3. Add 60 µL of thawed vitronectin into a 15-mL conical tube containing 6 mL of sterile DPBS, no calcium, no magnesium (Cat. No. 14190) at room temperature. Gently resuspend by pipetting the vitronectin dilution up and down.

Note: This results in a working concentration of 5 µg/mL (i.e., a 1:100 dilution).

4. Add 1 mL of the diluted vitronectin solution to each well of a 6-well plate (see Table 2 for the recommended volumes for other culture vessels). When used to coat a 6-well plate (10 cm²/well) at 1 mL/well, the final concentration will be 0.5 µg/cm².

5. Incubate the coated plates at room temperature for 1 hour.

Note: The culture vessel can now be used or stored at 2–8°C wrapped in laboratory film for up to a week. Do not allow the vessel to dry. Prior to use, pre-warm the culture vessel to room temperature for at least 1 hour.

6. Aspirate the vitronectin solution and discard. It is not necessary to rinse off the culture vessel after the removal of vitronectin. Cells can be passaged directly onto the vitronectin-coated culture vessels.

Coat culture vessels with rhLaminin-521

rhLaminin-521 working concentration

The optimal working concentration of rhLaminin-521 is cell line dependent and must be determined empirically. We recommend using an initial coating on the culture surface. Prior to coating culture concentration of 0.5 µg/cm vessels, calculate the working concentration according to the formula below and dilute the stock appropriately. See Table 3 for culture surface area and volume required.

$$\text{Working conc.} = \text{Coating conc.} \times \frac{\text{Culture surface area}}{\text{Volume required for surface area}}$$

$$\text{Dilution factor} = \frac{\text{Stock concentration (100 µg/mL)}}{\text{Working concentration}}$$

Table 3 rhLaminin-521 Coating Reagent volumes (per well or per dish)

Culture vessel (surface area)	Volume of diluted rhLaminin-521 solution
6-well (10 cm ²)	2 mL
12-well (4 cm ²)	0.8 mL
24-well (2 cm ²)	0.4 mL
35-mm (10 cm ²)	2 mL
60-mm (20 cm ²)	4 mL
100-mm (60 cm ²)	12 mL

For example, to coat a 6-well plate at a coating concentration of 0.5 µg/cm², you will need to prepare 12 mL of diluted rhLaminin-521 solution (10 cm²/well surface area and 2 mL of diluted rhLaminin-521/well; see Table 3) at the following working concentration:

$$\text{Working conc.} = 0.5 \mu\text{g/cm}^2 \times \frac{10 \text{ cm}^2}{2 \text{ mL}} = 2.5 \mu\text{g/mL}$$

$$\text{Dilution factor} = \frac{100 \mu\text{g/mL}}{2.5 \mu\text{g/mL}} = 40\text{X (i.e., 1:40 dilution)}$$

Coat culture vessels with rhLaminin-521

Instructions for coating a 6-well culture plate with rhLaminin-521 at a coating concentration of $0.5 \mu\text{g}/\text{cm}^2$ are provided below. For volumes used in other culture vessels, see Table 3. To calculate the working concentration of rhLaminin-521 used with other coating concentrations and to determine the appropriate dilution factor, use the equations above.

1. Upon receipt, thaw the vial of rhLaminin-521 slowly at 2°C to 8°C , mix by gentle trituration, and prepare usage size aliquots in polypropylene tubes. Freeze aliquots at -30°C to -10°C or store aliquots at 2°C to 8°C for up to 3 months.
2. To coat the wells of a 6-well plate, add 300 μL aliquot of rhLaminin-521 into a 15-mL conical tube containing 12 mL of sterile DPBS containing calcium and magnesium (Cat. No. 14040). Gently resuspend by pipetting the rhLaminin-521 dilution up and down.

Note: This results in a working concentration of $2.5 \mu\text{g}/\text{mL}$ (i.e., a 1:40 dilution).

3. Add 2 mL of the diluted rhLaminin-521 solution to each well of a 6-well plate (refer to Table 3 for the recommended volumes for other culture vessels). When used to coat a 6-well plate ($10 \text{ cm}^2/\text{well}$) at 2 mL/well, the final coating concentration will be $0.5 \mu\text{g}/\text{cm}^2$.
4. Incubate the plates in a 37°C , 5% CO_2 for 2 hours for efficient coating.
Note: Alternatively, the plate can be coated at 2°C to 8°C overnight. Do not allow the culture vessel to dry. Prior to use, pre-warm the culture vessel to room temperature.
5. Aspirate the rhLaminin-521 solution and discard. It is not necessary to rinse off the culture vessel after the removal of rhLaminin-521. Cells can be passaged directly onto the rhLaminin-521-coated culture vessels.



Support protocols

CytoTune™ -EmGFP reporter control transduction

CytoTune™ - EmGFP sendai fluorescence reporter

The CytoTune™ -EmGFP Sendai Fluorescence Reporter (Cat. No. A16519), available separately, is a control vector carrying the Emerald Green Fluorescent Protein (EmGFP) gene. The fluorescent control vector allows you to determine whether your cells of interest are amenable or refractive to transduction by Sendai reprogramming vectors. We recommend testing your cell line interest using the CytoTune™ -EmGFP Sendai Fluorescence Reporter before starting your reprogramming experiments.

Guidelines for using the CytoTune™ EmGFP sendai fluorescence reporter

- Transducing your cell line of interest using the CytoTune™ -EmGFP Sendai Fluorescence Reporter allows you to determine whether or not the cells can be transduced by the Sendai virus vectors; it **does not** indicate the cell line's capability to be reprogrammed.
- Different cell types require different MOIs to express detectable levels of EmGFP. As such, cells should be transduced using a range of different MOIs. We suggest initially transducing your cells with at least 2–3 different MOIs (e.g. 1, 3, and 9).
- Expression of EmGFP should be detectable at 24 hours post-transduction by fluorescence microscopy, and reach maximal levels at 48–72 hours.
- The titer of the CytoTune™ EmGFP vector is lot-dependent. For the specific titer of the vector, refer to the Certificate of Analysis (CoA) available on our website. See thermofisher.com/cytotune and search for the CoA by product lot number, which is printed on the vial.
- Avoid re-freezing and thawing of the CytoTune™ -EmGFP Sendai Fluorescence Reporter since viral titers can decrease dramatically with each freeze/thaw cycle.

Control transduction protocol for adherent cells

Day –1 to –2: Prepare the cells for transduction

1. 1–2 days before transduction, plate the cells of interest onto the necessary number of wells of a multi-well plate at the appropriate density to achieve 30–60% confluency on the day of transduction (Day 0). One extra well can be used to count cells for viral volume calculations.
2. Culture the cells for one to two more days, ensuring the cells have fully adhered and extended.



Day 0: Perform transduction

1. On the day of transduction, warm an appropriate volume of cell culture medium for each well to be transduced (e.g., 0.5 mL for each well of a 12-well plate) in a 37°C water bath.
2. Harvest cells from one well of the multi-well plate and perform a cell count. These cells will not be transduced, but will be used to estimate the cell number in the other well(s) plated in Step 1 on page 55.

Note: This step is optional and is performed to obtain more accurate MOI calculations. If exact MOIs are not needed, a rough estimate of the number of cells in the well (based on plating density and growth rates) will also suffice.

3. Count (or estimate) the cell number using the desired method (e.g., Countess™ II Automated Cell Counter), and calculate the volume of the virus needed to reach the target MOI(s). Titer information can be found on the CoA.

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

4. Remove one tube of CytoTune™ -EmGFP Sendai Fluorescence Reporter from the –80°C storage. Thaw the vector by first immersing the bottom of the tube in a 37°C water bath for 5–10 seconds, and then removing the tube from the water bath and allowing its contents to thaw at room temperature. Once thawed, briefly centrifuge the tube and place it immediately on ice.
5. Add the calculated volume of CytoTune™ -EmGFP Sendai Fluorescence Reporter to the pre-warmed cell culture medium prepared in Step 3 on page 56. Ensure that the solution is thoroughly mixed by pipetting the mixture gently up and down. Complete the next step on page 56 within 5 minutes.
6. Aspirate the cell culture medium from the cells, and add the solution prepared in Step 7 on page 56 to the well. Incubate the cells in a 37°C, 5% CO₂ incubator overnight.

Day 1: Replace medium and culture cells

1. 24 hours after transduction, replace the medium with fresh cell culture medium.
Note: Depending on your cell type, you should expect to see some cytotoxicity 24–48 hours post-transduction, which can affect >50% of your cells. This is an indication of high uptake of the virus. We recommend that you continue culturing your cells and proceed with the protocol.
2. Visualize the cells on a fluorescence microscope using a standard FITC filter set. EmGFP expression should be visible in some cells (expression will reach maximum levels between 48–72 hours).

Day 2+: Replace medium and culture cells

1. 48 hours after transduction, replace the medium with fresh cell culture medium.
2. Visualize the cells on a fluorescence microscope using a standard FITC filter set. EmGFP expression should be much brighter than Day 1, and should be visible in many cells (see Figure 6).

Expected results

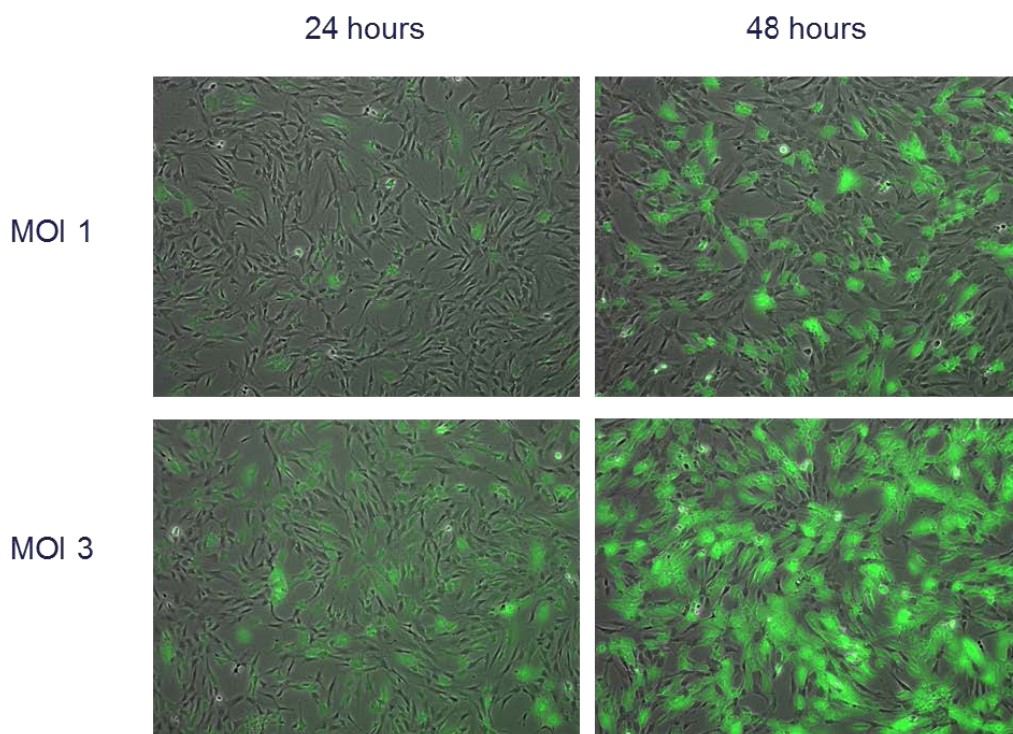


Figure 6 BJ HDFn cells transduced with the CytoTune™-EmGFP Sendai Fluorescence Reporter at the indicated MOI (1 or 3) and at the indicated time post-transduction (24 or 48 hours).



Passage iPSCs with EDTA

Passaging protocol

1. Pre-warm complete Essential 8™ Medium (see “Essential 8™ Medium (for 500 mL of complete medium)” on page 48) and vitronectin-coated culture vessels (see “Vitronectin working concentration” on page 51) to room temperature.
2. Aspirate the spent medium from the vessel containing PSCs and rinse the vessel twice with DPBS, no calcium, no magnesium (see Table 4 for the recommended volume).
3. Add 0.5 mM EDTA in DPBS to the vessel containing PSCs (see Table 4). Swirl the vessel to coat the entire cell surface.
4. Incubate the vessel at room temperature for 5 to 8 minutes or at 37°C for 4 to 5 minutes. When the cells start to separate and round up, and the colonies appear to have holes in them when viewed under a microscope, they are ready to be removed from the vessel.
5. Aspirate the EDTA solution, and add pre-warmed complete Essential 8™ Medium to the vessel (see Table 4).
6. Remove the cells from the well(s) by gently squirting medium and pipetting the colonies up. Avoid creating bubbles. Collect cells in a 15-mL conical tube. There may be obvious patches of cells that were not dislodged and left behind. **Do not scrape the cells from the dish in an attempt to recover them.**

Note: Work with no more than 1 to 3 wells at a time, and work quickly to remove cells after adding Essential 8™ Medium to the well(s), which quickly neutralizes the initial effect of the EDTA. Some cell lines re-adhere very rapidly after medium addition, and must be removed 1 well at a time. Others are slower to re-attach, and may be removed 3 wells at a time.
7. Add an appropriate volume of pre-warmed complete Essential 8™ Medium to each well of a vitronectin-coated 6-well plate so that each well contains 2 mL of medium after the cell suspension has been added. See Table 4 for the recommended volumes for other culture vessels.
8. Move the vessel in several quick figure eight motions to disperse the cells across the surface of the vessels. Place the vessel gently into the 37°C, 5% CO₂ incubator and incubate the cells overnight.



- Feed the PSC cells beginning the second day after splitting. Replace the spent medium daily.

Note: It is normal to see cell debris and small colonies after passage.

Table 4 Plating volumes (per well or per dish)

Culture vessel	Surface area	DPBS	0.5 mM EDTA in DPBS	Complete Essential 8™ Medium
6-well plate	10 cm ² /well	2 mL/well	1 mL/well	2 mL/well
12-well plate	4 cm ² /well	1 mL/well	0.4 mL/well	1 mL/well
24-well plate	2 cm ² /well	0.5 mL/well	0.2 mL/well	0.5 mL/well
35-mm dish	10 cm ²	2 mL	1 mL	2 mL
60-mm dish	20 cm ²	4 mL	2 mL	4 mL
100-mm dish	60 cm ²	12 mL	6 mL	12 mL

Cryopreserve iPSCs

Freeze iPSCs in Essential 8™ freezing medium

Follow the following protocol to cryopreserve iPSCs maintained in Essential 8™ Medium on vitronectin-coated culture dishes by freezing them in Essential 8™ freezing medium (see “Essential 8™ freezing medium” on page 49 for recipe).

- Prepare the required volume of fresh Essential 8™ freezing medium and place it on ice until use.

Note: Discard any remaining Essential 8™ freezing medium after use.

Note: PSC Cryopreservation Medium can be used as an alternative to the Essential 8™ freezing medium, using the same protocol described here.

- Aspirate the spent medium from the dish using a Pasteur pipette, and rinse the cells twice with DPBS, no calcium, no magnesium (see Table 5).
- Add 0.5 mM EDTA solution to the dish. To adjust the volume of EDTA for various dish sizes; see Table 5. Swirl the dish to coat the entire cell surface.
- Incubate the dish at room temperature for 5–8 minutes or at 37°C for 4–5 minutes. When the cells start to separate and round up, and the colonies appear to have holes in them when viewed under a microscope, they are ready to be removed from the vessel.
- Aspirate the EDTA solution with a Pasteur pipette.
- Add 1 mL of ice-cold Essential 8™ freezing medium to each well of a 6-well plate. To adjust the volume of Essential 8™ freezing medium for various dish sizes, see Table 5.



7. Remove the cells by gently squirting the colonies from the well using a 5-mL glass pipette. Avoid creating bubbles. Collect the cells in a 15-mL conical tube and place on ice.
8. Resuspend the cells gently. Aliquot 1 mL of the cell suspension into each cryovial.
9. Quickly place the cryovials containing the cells in a cryo-freezing container to freeze the cells at 1°C per minute and transfer them to –80°C overnight.
10. After overnight storage at –80°C, transfer the cells to a liquid nitrogen tank vapor phase for long term storage.

Table 5 Plating volumes (per well or per dish)

Culture vessel	Surface area	DPBS	0.5 mM EDTA in DPBS	Essential 8™ freezing medium
6-well plate	10 cm ² /well	2 mL/well	1 mL/well	2 mL/well
12-well plate	4 cm ² /well	1 mL/well	0.4 mL/well	1 mL/well
24-well plate	2 cm ² /well	0.5 mL/well	0.2 mL/well	0.5 mL/well
35-mm dish	10 cm ²	2 mL	1 mL	2 mL
60-mm dish	20 cm ²	4 mL	2 mL	4 mL
100-mm dish	60 cm ²	12 mL	6 mL	12 mL



Ordering information

Accessory products

Unless otherwise indicated, all materials are available through [thermofisher.com](https://www.thermofisher.com).

Product	Amount	Cat. No.
CytoTune™ -iPS products		
CytoTune™ -iPS 2.0 Sendai Reprogramming Kit	1 × 3 vials	A16517
CytoTune™ -EmGFP Sendai Fluorescence Reporter	1 vial	A16519
Media, sera, and reagents		
DMEM with GlutaMAX™ -I (high glucose)	500 mL	10569-010
Essential 8™ Medium	1 kit	A1517001
CTS™ OpTmizer™ T Cell Expansion SFM™, bottle format	1000 mL	A1048501
CTS™ StemPro™ HSC Expansion Medium	500 mL	A4222301
Fetal Bovine Serum (FBS), ES-Cell Qualified	500 mL	16141-079
Dulbecco's PBS (DPBS) without Calcium and Magnesium	500 mL	14190-144
MEM™ Non-Essential Amino Acids Solution (10 mM)	100 mL	11140-050
Dulbecco's PBS (DPBS), Calcium, Magnesium	500 mL	14040133
CTS™ KnockOut™ SR Xeno-Free Kit	100 mL	A1099201
Sodium Bicarbonate 7.5% solution	100 mL	25080094
Basic Fibroblast Growth Factor (bFGF)	10 µg	PHG0264
EGF Recombinant Human protein	1 mg	PHG0313
Hydrocortisone (50 µM)	10 mL	Sigma-Aldrich™, H6909
SCF (C-Kit Ligand) Recombinant Human Protein	100 µg	PHC2111
FLT3 Ligand Recombinant Human Protein	10 µg	PHC9414
IL3 Recombinant Human Protein	10 µg	PHC0034
IL6 Recombinant Human Protein	10 µg	PHC0065
Recombinant Human TPO Lyophilized	100 µg	PHC9511
Dynabeads™ Human T-Expander CD3/CD28	10 mL	11141D



Product	Amount	Cat. No.
IL2 Recombinant Human Protein	100 µg	PHC0021
GlutaMAX™ -I Supplement	100 mL	35050-061
Penicillin-Streptomycin, liquid	100 mL	15140-122
Cells		
Human Dermal Fibroblasts, neonatal (HDFn)	1 vial	C-004-5C
Matrices and dissociation reagents		
Vitronectin, truncated human recombinant (VTN-N)	1 mL	A14700
rhLaminin-521	100 µg	A29249
0.05% Trypsin/EDTA Solution (1X)	100 mL	25300-054
TrypLE™ Select Cell Dissociation Reagent	100 mL	12563-011
UltraPure™ 0.5 M EDTA, pH 8.0	4 × 100 mL	15575-020
Versene Solution	100 mL	15040-066
Antibodies		
TRA-1-60 Mouse anti-human mAb, Alexa Fluor™ 488 Conjugate Kit for Live Cell Imaging	1 kit	A25618
TRA-1-60 Mouse anti-human mAb, Alexa Fluor™ 594 Conjugate Kit for Live Cell Imaging	1 kit	A24882
CD44 Rat anti-human/mouse mAb, Alexa Fluor™ 488 Conjugate Kit for Live Cell Imaging	1 kit	A25528
Equipment		
Countess™ II Automated Cell Counter	1 unit	AMQAX1000
StemPro™ EZPassage™ Disposable Stem Cell Passaging Tool	10 units	23181-010
Reagents for RT-PCR		
TRIzol™ LS reagent	100 mL	10296-010
High-Capacity cDNA Reverse Transcription Kit	1000 reactions	4368813
AccuPrime™ SuperMix I	200 reactions	12342-010



RT-qPCR

Use the following pre-validated TaqMan™ assays to detect the SeV genome and transgenes in cells reprogrammed using the CTS™ CytoTune™ 2.1 Sendai reprogramming vectors by RT-qPCR:

Target	TaqMan™ assay
SeV	Mr04269880_mr
KOS	Mr04421257_mr
Klf4	Mr04421256_mr
L-Myc	Mr04944276_mr



Safety



WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
 - Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, see the “Documentation and Support” section in this document.
-

Chemical safety



WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety



WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.



WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
<https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2009-P.pdf>
 - World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf
-

Documentation and support

Customer and technical support

Visit **thermofisher.com/support** for the latest service and support information.

- Worldwide contact telephone numbers
- Product support information
 - Product FAQs
 - Software, patches, and updates
 - Training for many applications and instruments
- Order and web support
- Product documentation
 - User guides, manuals, and protocols
 - Certificates of Analysis
 - Safety Data Sheets (SDSs; also known as MSDSs)

Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

