# Reprogramming of Human Fibroblasts on Callisto Automated Cell Culture System Using CytoTune-iPS 2.0 Sendai Reprogramming Kit

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Reprogramming somatic cells to induced pluripotent stem cells (iPSCs) is a critical yet labor- and time- intensive process. Multiple methods have been explored and developed to facilitate the ever-growing interest in iPSC generation. Here we describe a workflow that combines the highly efficient CytoTune™-iPS 2.0 Sendai virus reprogramming kit with Callisto™, an automated microfluidic cell culture platform that enables miniaturization and optimization as well as improved kinetics and efficiency over traditional culture methods. This enables reprogramming using rare cell types or hard-to-obtain specimens.

## Introduction

iPSCs are genetically reprogrammed somatic cells that exhibit a pluripotent stem cell–like state similar to embryonic stem cells. iPSCs have great potential for various disease modeling and cell-based therapies. iPSCs are commonly derived by inducing expression of selected genes via various methods including viruses (retrovirus, lentivirus, Sendai virus), mRNA, and episomal plasmid–mediated gene transduction. Different methods often yield reprogrammed cells with different efficiency and quality. Somatic cells from different donors also vary in their propensity for reprogramming. Manual reprogramming is a time-consuming and labor-intensive process, often resulting in low efficiency. Typical results yield successful reprogramming in only 0.01–1% of all cells. It can take up to three weeks before colonies are clearly visible and ready to be isolated. In addition, the multiplicities of infection (MOIs) may need to be optimized when reprogramming cells from new donors.

The CytoTune-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific, Carlsbad, CA) is a non-integrating system that uses Sendai viral vectors to reprogram somatic cells into iPSCs. The kit contains three reprogramming vectors: KOS (a polycistronic vector containing hKlf4, hOct3/4, hSox2), hc-Myc, and hKlf4. As a result, the relative ratios of the reprogramming factors can be fine-tuned for difficult-to-program donor cells. Only a single application of the vectors is required for successful reprogramming. Sendai virus infects

cells by attaching itself to the sialic acid receptor presented on the surface of many animal cells. Hence, when used for reprogramming it can transduce cells derived from various tissues and donors, including those that are refractory to reprogramming by other methods. The CytoTune 2.0 virus is an RNA virus that replicates itself in the host cell cytoplasm, and the viral clearance occurs spontaneously over several passages. Viral clearance can also be accelerated by culture at higher temperatures (Figure 1).

The Callisto automated cell culture system revolutionizes cell model studies with a fully integrated microfluidic platform for precise combinatorial manipulation of biological pathways. The system's innovative integrated fluidic circuit (IFC) includes 32 independent microfluidic culture chambers that can be controlled for up to three weeks with minimal intervention. Up to 16 reagents can be metered and mixed in any combination on the IFC before they are delivered to specified chambers. Callisto Experiment Planner software enables easy planning, monitoring, and modification of the entire experiment workflow. The instrument controls the temperature, humidity, and gas environment of the cells, automatically changes medium at pre-programmed time points, and logs all experimental steps and conditions. These features make Callisto an attractive platform for optimization of reprogramming using the CytoTune-iPS 2.0 Sendai virus reprogramming kit.

A sample experimental design in which multiple parameters are optimized within a single run is shown in Figure 2. Multiple conditions can be easily set up within one Callisto IFC, including a control transduction with the CytoTune EmGFP Sendai Fluorescence Reporter (often used to assess transduction efficiency by Sendai virus), multiple MOIs of reprogramming factors, and different durations of virus exposure, as well as different analysis workflows. Replicates can be easily programmed, so cells in the same virus treatment group can be harvested from some chambers and expanded off the IFC while other chambers are analyzed by immunostaining or gene expression.

# Materials and Methods

Experiments were designed using Callisto Experiment Planner software. The Callisto IFCs were first treated with recombinant human laminin-521 (50 µg/mL), then loaded with human adult (data not shown) or fetal dermal fibroblasts. Between 100 and 200 human fetal dermal fibroblasts were seeded into each chamber (culture area: 1.25 $mm^2$) and cultured in fibroblast medium overnight to allow good attachment and ~50% confluency before viral transduction. CytoTune-iPS 2.0 Sendai Reprogramming Kit contains three vectors: CytoTune 2.0 KOS, CytoTune 2.0 hc-Myc, and CytoTune 2.0 hKlf4. To optimize the efficiency of reprogramming and maximize the chance of successful reprogramming without significant cell toxicity on one IFC, we set up different chambers to receive different MOIs of the three vectors. MOI describes the ratio of viral particles to cells. A starting MOI ratio of 5:5:3 (KOS:hc-Myc:hKlf4) is typically recommended for new donor cells being reprogrammed with the CytoTune-iPS 2.0 Sendai Reprogramming Kit. However, increasing the MOI two-fold for all vectors or increasing the hKlf4 alone can further enhance reprogramming efficiency. In

this experiment, MOI ratios of 10:10:12, 10:10:6, 5:5:3, and 2.5:2.5:1.5 were tested. Virus stocks were diluted in the culture medium and loded onto the IFC according to the reagent loading map generated from Callisto Experiment Planner. Vectors were automatically dosed for 6 or 12 hours according to the experiment design. Instead of depositing the reagents in single dose as for traditional cell culture, reprogramming viruses were delivered into the cell culture chambers at intervals defined by the user using the Callisto Experiment Planner software. By default, cells were dosed every 2 hours, meaning chambers dosed for 6 hours received three deliveries each with 1/3 of the total number of virus particles. IFCs were unloaded periodically for imaging and reagent refreshing. Cells were switched to Gibco™ Essential 8™ (E8) Medium or Essential 8 Flex Medium (data not shown) on day 4 of transduction. SSEA-4 and Tra-1-81 live staining was performed on IFC at various time points starting at 2 days after infection. From day 3 to day 7 after virus infection, cells were harvested live from some chambers using TrypLE™ and collagenase mix (or Accumax™ and collagenase mix) and then expanded in Essential 8 Medium in 48-well cell culture dishes. Cells from other chambers were lysed at different time points and lysates collected for qRT-PCR. The remaining chambers were fixed with 2% paraformaldehyde and stained with anti-human Oct4, Sox2, and Nanog antibodies.

# Results

Fibroblasts were transduced with Sendai virus at high efficiency on Callisto IFC, as assessed by EmGFP reporter expression (data not shown) and by antibody staining against the transgenes. As shown in Figure 2, 24 hours after infection, expression of the reprogramming factors Oct4 and Sox2 could be detected in as many as 80% of the cells.

Reprogramming of fibroblasts with the CytoTune-iPS 2.0 Sendai Reprogramming Kit on the Callisto IFC occurs with enhanced efficiency and kinetics compared to traditional adherent cultures. At only 2 days after transduction, live staining detected expression of SSEA4 on a small number of cells in culture chambers treated with the reprogramming factors. By day 5, SSEA4-positive cells increased in number and started to aggregate and cells started staining positive for Tra-1-81 (or Tra-1-60, data not shown). From day 6 many stem cell–like colonies were visible. As shown in Figure 3, live staining on day 9 revealed multiple colonies strongly expressing TRA-1-81 and SSEA4. qRT-PCR results from lysates harvested from selected chambers indicated up-regulation of pluripotent cell markers including Oct4, Sox2, Nanog, and Klf4 by day 8 (Figure 4). Finally, when reprogrammed cells were fixed on day 9 and stained with antibodies against Oct4, Sox2, and Nanog, double- or triple-positive cells were readily observed, indicating cell fate conversion from fibroblast toward pluripotent cells (Figure 5).

At different time points during reprogramming, cells from some chambers were harvested and replated onto either recombinant laminin-521 or mouse embryonic fibroblast–coated culture dishes. Tight colonies emerged on both substrates within one week of replating, showing morphology of characteristic pluripotent cell colonies (Figure 6). Reprogramming

efficiency from human neonatal fibroblasts could be as high as 8%, as indicated by the number of SSEA4/Tra-1-81-positive colonies.

Different input cells were found to require different optimal viruses to achieve high-efficiency and low-toxicity reprogramming. For example, we found that a MOI ratio of 5:5:3 (KOS:hc-Myc:hKlf4) worked best for BJ fibroblasts, while 10:10:6 worked best for human fetal fibroblasts. Overall, combinatorial experiments including multiple conditions on one IFC maximized the success rate of obtaining fully reprogrammed and expandable iPS cells.

# Discussion

Pairing the highly robust and efficient CytoTune-iPS 2.0 Sendai Reprogramming Kit with the Callisto automated, microfluidic-based cell culture platform allows for exquisite optimization of the reprogramming process. In addition to requiring minimal starting material, which can often be of concern for projects relying on precious samples, Callisto presents some additional features that make it a great platform for reprogramming.

Compared to a traditional cell culture plate, microfluidic culture chambers have a substantially higher surface area-to-volume ratio. On one hand, this keeps the adherent cells growing on the surface of the culture chamber closer to nutrient and stimulations in the medium. On the other hand, since the amount of medium on top of each section of surface is small, nutrients get depleted quickly, so medium replacement needs to happen more frequently compared to traditional well plates or flasks. On the Callisto IFC, the default for feeding/dosing cells is 2 hours. Because of this higher frequency of medium change, fresh medium and fresh virus particles can be deposited in each dosing cycle. Cycled dosing combined with on-IFC mixing of reagents prior to cell incubation affords a more even distribution of reagents than manual pipetting into well plates. Finally, since the virus particles are delivered more evenly each time with smaller doses, cell toxicity is lower. In combination, these features—increased surface area-to-volume ratio, even and repetitive exchange of virus particles, and lower toxicity—result in more favorable kinetics for uptake of the Sendai virus. Faster uptake of the virus leads directly to a more efficient activation of pluripotency genes such as SSEA4 compared to the standard well plate format.

The data presented here demonstrates improved reprogramming efficiency, enhanced reprogramming kinetics, and ready optimization of viral MOIs that can be easily adapted to different cell types.

# Figures

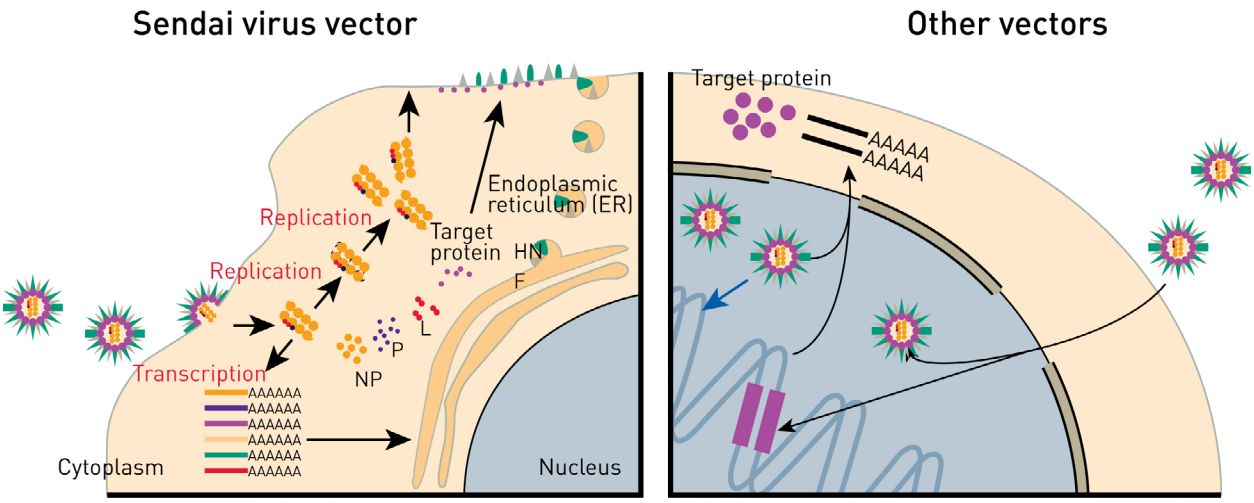


Figure 1. Comparison of the life cycles of non-integrating Sendai virus vectors and other, integrating vectors. Sendai virus is an RNA-based respiratory mouse parainfluenza type I virus of mouse and rat, belonging to the Paramyxoviridae family. It replicates exclusively in the cytoplasm, eliminating any risk of genomic integration. The fusion (F) gene was deleted in the CytoTune-iPS Sendai Reprogramming system to allow for retention of infectivity while preventing the production of infectious particles.

| Conditions | MOI | EmGFP (CIU/uL) | hKOS/ hc-Myc (CIU/uL) | hKlf4 (CIU/uL) | Time (hrs) | Chamber # | % Oct4+/Sox2+ |
|---|---|---|---|---|---|---|---|
| 1 | | --- | --- | --- | --- | 1, 9, 17, 25 | --- |
| 2 | 1 | 875 | --- | --- | 6 | 2, 10, 18, 26 | --- |
| 3 | 1.25:1.25:0.75 | --- | 2000 | 600 | 6 | 3, 11, 19, 27 | ~20% |
| 4 | 2.5:2.5:1.5 | --- | 4200 | 1200 | 6 | 4, 12, 20, 28 | ~30% |
| 5 | 5:5:3 | --- | 8400 | 2500 | 6 | 5, 13, 21, 29 | ~50% |
| 6 | 10:10:6 | --- | 8400 | 2500 | 12 | 6, 14, 22, 30 | ~80% |
| 7 | 5:5:6 | --- | 8400 | 5000 | 6 | 7, 15, 23, 31 | ~80% |
| 8 | 10:10:12 | --- | 8400 | 5000 | 12 | 8, 16, 24, 32 | ~80% |

Oct4/Sox2 staining at 24hr

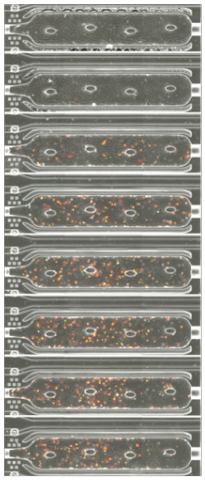

Figure 2. An experiment set up for optimizing transduction and reprogramming efficiency on Callisto. EmGFP viral vectors were used as control to test the transfection efficiency after 24 hours (data not shown). CytoTune 2.0 KOS, CytoTune 2.0 hc-Myc, and CytoTune 2.0 hKlf4 viral vectors were mixed on a Callisto IFC at different MOI combinations. Cells were fixed after 24 hours and stained with Oct4 and Sox2 antibodies to determine transduction efficiency. Up to 80% of the cells showed strong expression for both pluripotent markers.

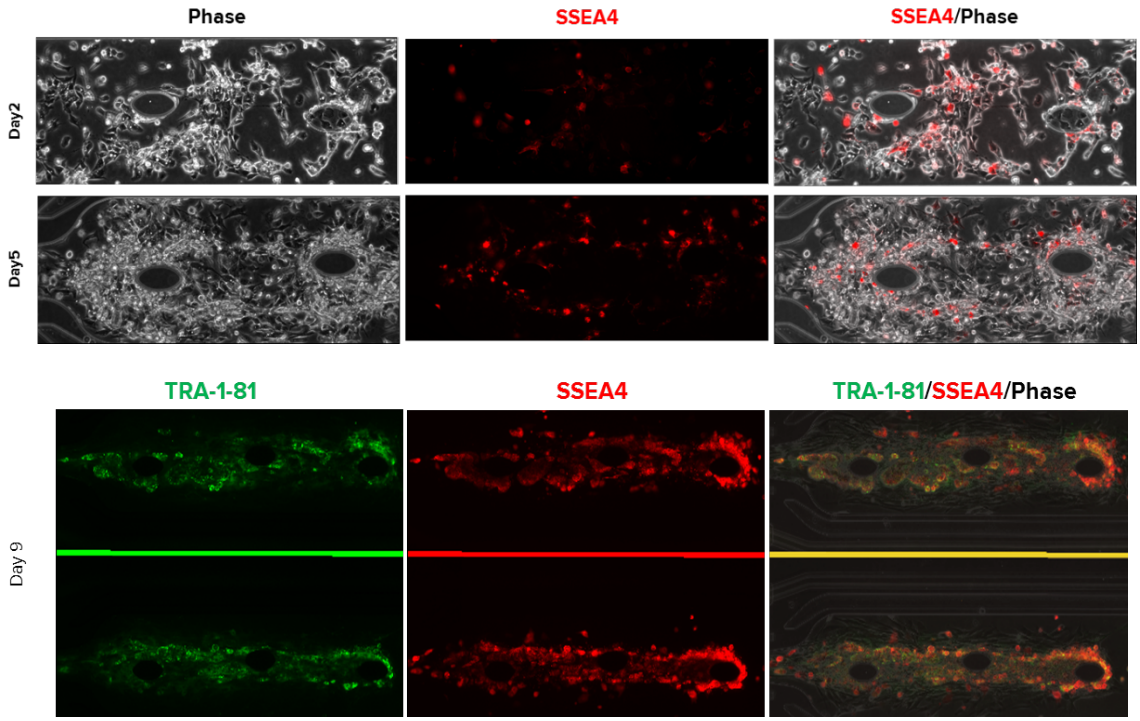


Figure 3. Live staining with SSEA4 and TRA-1-81 showing emerging pluripotent cells and colonies. The first pluripotent surface marker detected was SSEA4 at day 2. By day 5, stronger expression of SSEA4 was detected in more cells. One week after the initial transduction, small colonies were seen throughout the culture chambers. SSEA4 and TRA-1-81 staining was detected in the colony-like cell aggregates.

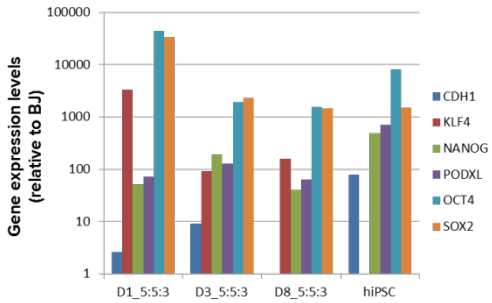


Figure 4. qRT-PCR showing upregulation of pluripotent genes in reprogrammed cells. Gene expression was dynamic during the first week of reprogramming. After transduction, transgenes Oct4, Sox2, and Klf4 were expressed at extremely high level, likely reflecting direct expression of the viral vector. By day3, however, expression of Oct4 and Sox2 genes decreased and maintained similar expression through day 8.

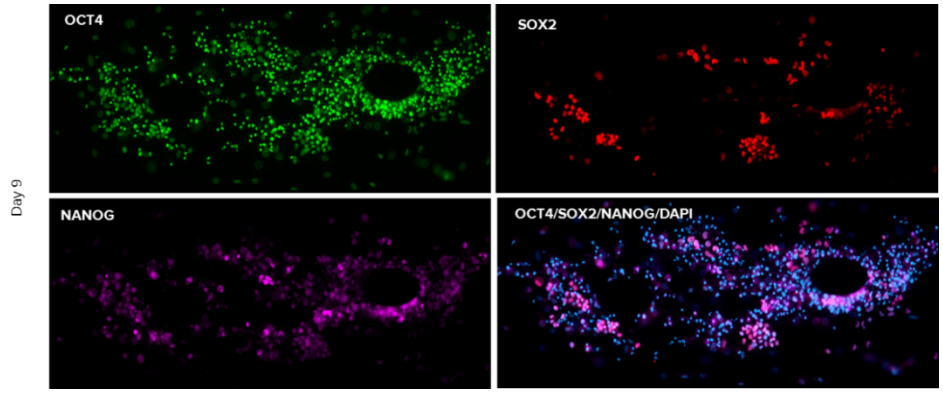


Figure 5. Immunostaining with antibodies against pluripotent markers Oct4, Sox2, and Nanog showing pluripotent stem cell colonies forming 9 days after virus transduction. Strong co-localization of the three genes was detected in tight embryonic stem cell–like colonies.

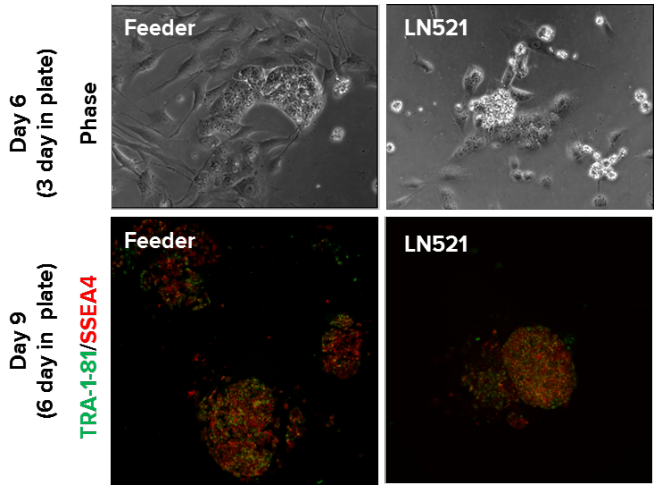


Figure 6. Colonies forming within one week after replating of reprogrammed cells. Reprogrammed cells from select chambers were harvested live and replated onto feeder layer or recombinant laminin 521–coated well plates. Small colonies were visible 1 to 2 days after replating, and SSEA4 and TRA-1-81 staining showed strong expression of these two pluripotent genes.

Table 1. Product information

| Product | Vendor | Part Number |
|---|---|---|
| CytoTune-iPS 2.0 Sendai Reprogramming Kit | Thermo Fisher Scientific | A16517 (1-pack)<br>A16518 (3-pack) |
| CytoTune EmGFP Sendai Fluorescence Reporter | Thermo Fisher Scientific | A16519 |
| rhLaminin-521 | Thermo Fisher Scientific | A29248 (0.1 mg)<br>A29249 (10 x 0.1 mg) |
| Essential 8 Medium | Thermo Fisher Scientific | A1517001 |
| Essential 8 Flex Medium Kit | Thermo Fisher Scientific | A2858501 |
| Callisto IFC—5 IFCs | Fluidigm | 100-9997 |
| Callisto Reagent Kit | Fluidigm | 100-8682 |
| Callisto Accessories Kit | Fluidigm | 101-0003 |
| Callisto Lysis Reagent Kit | Fluidigm | 100-8688 |

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