CellPore™ Unactivated Human Pan T Cell Gene Editing Protocol

Special applications protocol for CellPore™ Transfection Kit 300

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Description

The following protocol describes how to perform CRISPR-Cas9 genome editing in unactivated pan T cells using CellPore™ Transfection Kit 300 (Catalog #100-1020) on CellPore™ Transfection System (Catalog #100-0946). It includes instructions for the isolation and resting of unactivated pan T cells, preparation and delivery of CRISPR-Cas9 ribonucleoprotein (RNP) complexes to unactivated pan T cells using CellPore™ Transfection System, and post-delivery handling and culture recommendations.

Materials Required but Not Provided

PRODUCT	CATALOG #
CellPore™ Transfection Kit 300	100-1020
CellPore™ Transfection System	100-0946
Cas9 nuclease (glycerol-free)	--
Target gRNA (e.g. ArciTect™ sgRNA)	200-0013
EasySep™ Human T Cell Isolation Kit	17951
EasySep™ Buffer	20144
ImmunoCult™-XF T Cell Expansion Medium	10981
Nuclease-free water	e.g. 79001

Preparation of Reagents and Materials

A. SAMPLE PREPARATION

For available fresh and frozen samples, see www.stemcell.com/primarycells.

PERIPHERAL BLOOD

Prepare a peripheral blood mononuclear cell (PBMC) suspension from whole blood by centrifugation over a density gradient medium (e.g. Lymphoprep™, Catalog #07801). For more rapid PBMC preparation, use the SepMate™ RUO (Catalog #86450/86415) or SepMate™ IVD* (Catalog #85450/85415) cell isolation tube.

If using previously frozen PBMCs, incubate the cells with DNase I Solution (Catalog #07900) at a concentration of 100 µg/mL at room temperature (15 - 25°C) for at least 15 minutes prior to labeling and separation. Filter aggregated suspensions through a 37 µm cell strainer (e.g. Catalog #27250) for optimal results.

After preparation, resuspend cells at 5 x 10^7 cells/mL in EasySep™ Buffer. Proceed to section B.

*SepMate™ IVD is only available in select regions where it is registered as an In Vitro Diagnostic (IVD) device for the isolation of mononuclear cells (MNCs) from whole blood or bone marrow by density gradient centrifugation. In all other regions, SepMate™ is available for research use only (RUO).

LEUKAPHERESIS

Wash the peripheral blood leukapheresis sample by adding an equivalent volume of EasySep™ Buffer or phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS). Centrifuge at 300 x g for 10 minutes at room temperature (15 - 25°C). Remove the supernatant and resuspend the cells at 5 x 10^7 cells/mL in EasySep™ Buffer.

NOTE: If cell aggregates, clumps, or strands are visible in the sample tube, filter the cell suspension through a 37 µm cell strainer (e.g. Catalog #27250) before proceeding to section B.

B. EASYSEP™ HUMAN T CELL ISOLATION AND RESTING

1. Isolate human pan T cells from from the sample (prepared in section A) using EasySep™ Human T Cell Isolation Kit (Catalog #17951). For complete instructions, refer to the Product Information Sheet (Document #10000005298).

   NOTE: Ensure isolated T cells are in a single-cell suspension before proceeding. If cell aggregation is observed at this stage, it is not recommended to proceed with the affected donor as this may impact performance and recovery.
2. Centrifuge isolated cells at 500 x g for 5 minutes at room temperature (15 - 25°C).
3. Resuspend viable pan T cells in pre-warmed (37°C) ImmunoCult™-XF T Cell Expansion Medium at 2 - 4 x 10^6 cells/mL in a 50 mL conical tube.
4. Rest pan T cells in the conical tube for 1 hour in a humidified incubator at 37°C and 5% CO_2.

   NOTE: For cryopreserved samples, increasing the resting period to 2 hours is recommended.

Directions for Use

Please read the entire protocol before proceeding.

For complete instructions on using CellPore™ Transfection System, refer to the CellPore™ Transfection System User Reference Manual (Document #10000018433), available at www.stemcell.com, or contact us to request a copy.

A. PREPARATION OF sgRNA WORKING SOLUTION

1. Briefly centrifuge the vial of lyophilized sgRNA before opening.
2. Add nuclease-free water to the vial to a final concentration of 100 µM (see Table 1 for examples). Mix thoroughly.

   NOTE: If not used immediately, aliquot the sgRNA working solution into DNase- and RNase-free microtubes and store at -20°C for up to 6 months. Alternatively, store at -80°C for long-term storage. After thawing the aliquots, use immediately. Do not re-freeze.

Table 1. Reconstitution of sgRNA to 100 µM*

sgRNA (nmol)	VOLUME OF NUCLEASE-FREE WATER (µL)
1.5	15
10	100
50	500

* 100 µM is equal to 100 pmol/µL

B. PREPARATION OF CRISPR-CAS9 RNP COMPLEX MIX

1. To prepare the RNP Complex Mix, combine the components in the order listed in Table 2 in a sterile microcentrifuge tube. Adjust volumes according to the number of reactions, including controls required.

   NOTE: For best results, the total volume of cargo added should not exceed 10% of the reaction volume.

Table 2. Preparation of RNP Complex Mix for a 50 µL or 100 µL Reaction

REAGENT	FOR 0.5 - 10 x 10^6 CELLS (50 µL REACTION)		FOR > 10 - 25 x 10^6 CELLS (100 µL REACTION)	
	VOLUME (µL)	AMOUNT (pmol)	VOLUME (µL)	AMOUNT (pmol)
10 mg/mL Cas9 nuclease	0.97	60	1.94	120
100 µM sgRNA	1.52	150	3.04	300
CellPore™ Delivery Medium	7.51	---	5.02	---
Total Volume	10	---	10	---

NOTE: The above examples provide the required volumes for a 1:2.5 Cas9:sgRNA ratio. It is highly recommended to optimize the Cas9:sgRNA ratio and Cas9 amount for each gene target (see Notes and Tips).

2. Mix thoroughly by gently pipetting up and down. Avoid introducing bubbles.
3. Incubate RNP Complex Mix at room temperature (15 - 25°C) for 15 minutes.

   NOTE: If not used immediately, keep on ice until use. Allow the RNP Complex Mix to warm to room temperature for 5 minutes prior to transfection.

C. PREPARATION OF REACTION MIXTURE

Each CellPore™ Delivery Cartridge 300 can process 0.5 - 25 x 10^6 unactivated pan T cells per reaction. The following example is for preparing one Reaction Mixture (unactivated pan T cells + RNP Complex Mix + [optional] CellPore™ FITC-Dextran). Adjust volumes accordingly based on the number of reactions required. Include a small excess to account for pipetting error.

1. Prepare a sufficient volume of T cell culture medium (e.g. ImmunoCult™-XF T Cell Expansion Medium) for the number of reactions and cell culture wells required. Warm to 37°C before use.

   NOTE: To improve cell viability, supplement complete medium with 5% human AB serum and/or 10 ng/mL human recombinant IL-2 (e.g. Catalog #78036). If desired, add antibiotics immediately before use (e.g. 50 µg/mL gentamicin).
2. After resting the isolated pan T cells (Preparation of Reagents and Materials, section B), transfer 0.5 - 25 x 10^6 cells to a sterile RNAse-free microcentrifuge tube.
3. Centrifuge the cells at 500 x g for 5 minutes at room temperature (15 - 25°C).
4. Aspirate supernatant and resuspend the cell pellet in CellPore™ Delivery Medium as follows:
   - For 0.5 - 10 x 10^6 cells, resuspend in 40 µL.
   - For > 10 - 25 x 10^6 cells, resuspend in 90 µL.

   OPTIONAL: CellPore™ FITC-Dextran may be co-delivered as a positive control to indicate successful cargo delivery. Adjust the resuspension volume above to accommodate for the additional volume of CellPore™ FITC-Dextran if applicable (Table 3).
5. Add 10 µL of RNP Complex Mix (prepared in section B) to the resuspended pan T cells (± CellPore™ FITC-Dextran) according to Table 3.

Table 3. Volumes for Preparing 50 µL or 100 µL of Reaction Mixture

COMPONENT	VOLUMES (µL) FOR 0.5 - 10 x 10^6 CELLS		VOLUMES (µL) FOR > 10 - 25 x 10^6 CELLS	
Resuspended Pan T Cells	37.5	40	85	90
2 mg/mL CellPore™ FITC-Dextran (optional)	2.5	---	5	---
RNP Complex Mix	10		10	
Total Volume	50		100	

6. Gently mix the Reaction Mixture by pipetting up and down. Avoid introducing bubbles. Immediately proceed to section D.

D. DELIVERY OF RNP COMPLEXES TO UNACTIVATED PAN T CELLS

1. Remove the Cartridge Insert of a new CellPore™ Delivery Cartridge 300 (Figure 1) and add warm T cell culture medium (prepared in section C step 1) to the Collection Tube such that total volume of T cell culture medium + the Reaction Mixture is 200 µL.

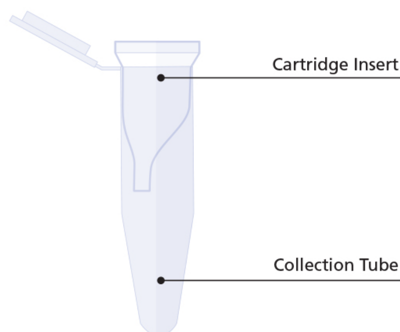

Figure 1. Each CellPore™ Delivery Cartridge 300 Comprises a Cartridge Insert and a Collection Tube

2. Re-insert the Cartridge Insert into the Collection Tube and transfer the entire volume of the Reaction Mixture into the Cartridge Insert. Always insert the pipette tip to the bottom of the Cartridge Insert when dispensing the sample (Figure 2).

   NOTE: Do not centrifuge the Delivery Cartridge at this stage as this will lead to loss in delivery performance. Gently tap the Delivery Cartridge several times to collect volume at the bottom if necessary.

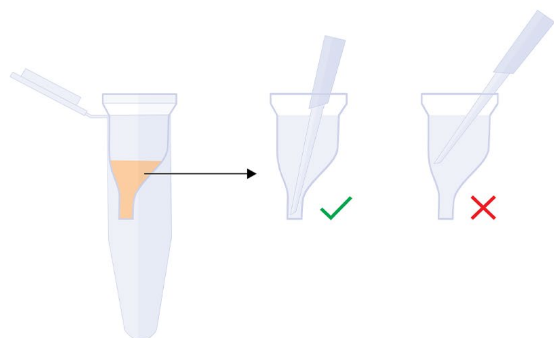

Figure 2. Proper Pipetting Technique for CellPore™ Delivery Cartridge

3. Close the cap and ensure the Cartridge Insert is securely placed in the Collection Tube.
4. Place the Delivery Cartridge into the Cartridge Holder of CellPore™ Transfection System.
5. Set instrument pressure and run time. Select Run.

   NOTE: The recommended pressure range for unactivated pan T cells is 50 - 90 psi. When delivering to freshly isolated pan T cells, a starting delivery pressure of 70 psi is recommended. A run time of 3 seconds is sufficient for all reactions. For complete instructions on performing sample runs, refer to the CellPore™ Transfection System User Reference Manual (Document #10000018433), available at www.stemcell.com, or contact us to request a copy.
6. Once the run is complete, retrieve the Delivery Cartridge from the instrument. The cell sample should be at the bottom or side of the collection tube.

   NOTE: It is recommended to spin down the Cartridge in a mini centrifuge for a few seconds for full volume recovery.
7. Remove and discard the Cartridge Insert. Cap the Collection Tube and place in a humidified incubator at 37°C and 5% CO_2 for 2 hours.

   NOTE: A rest period of < 2 hours may result in decreased viability.

E. CELL CULTURE

1. Following the 2-hour rest period, perform a viable cell count; culture pan T cells at a density of 2 - 4 x 10^6 cells/mL in T cell culture medium (prepared in section C step 1).

   NOTE: If proceeding with T cell activation using ImmunoCult™ Human T Cell Activators (e.g. Catalog #10970 or #10971), refer to the applicable PIS (Document #10000000464 or #10000000465) for details regarding cell culture requirements.
2. Transfer cells to an appropriate culture plate and incubate in a humidified incubator at 37°C and 5% CO_2 until ready for analysis.

Notes and Tips

- Cells isolated from older PBMCs or leukapheresis samples (> 48 hours post-draw) may result in lower viability for some donors. Editing results may be donor-dependent.
- When using cryopreserved cells, care must be taken to quickly and fully wash away cryoprotectants (e.g. DMSO) from the thawed cell suspension before proceeding.
- Appropriate controls include: Cas9 only, sgRNA only, scrambled sgRNA, mock (no cargo), and untreated.
- Titration of Cas9 RNP may be required in order to obtain optimal editing efficiencies. Similarly, titration of the Cas9:sgRNA ratio (from 1:1 - 1:8) may also be required.
- Reaction volumes lower than 50 µL may result in reduced performance.
- A glycerol-free formulation of Cas9 nuclease may result in improved editing efficiency in certain applications.
- Best results are obtained when limiting prolonged cell exposure to ambient temperature conditions. Consider keeping unused cells in a humidified incubator at 37°C and 5% CO_2 when performing larger experiments.
- Undelivered cargo may lead to lower cell viability in certain cell culture systems. Therefore, cells may require washing with an appropriate culture medium prior to plating for downstream culture.
- This protocol is not optimized for use with stimulated human T cells.

ASSESSING VIABILITY AND DELIVERY EFFICIENCY

The following fluorochrome-conjugated antibodies and dyes are recommended in order to facilitate analysis of gene-edited T cells:

- Anti-Human CD45 Antibody, Clone HI30 (Catalog #60018)
- Anti-Human CD3 Antibody, Clone UCHT1 (Catalog #60011)
- Anti-Human CD4 Antibody, Clone SK3 (Catalog #60122)
- Anti-Human CD8a Antibody, Clone SK1 (Catalog #60125)
- Viability Dye, including 7-AAD (Catalog #75001) or Propidium Iodide (Catalog #75002)

Data

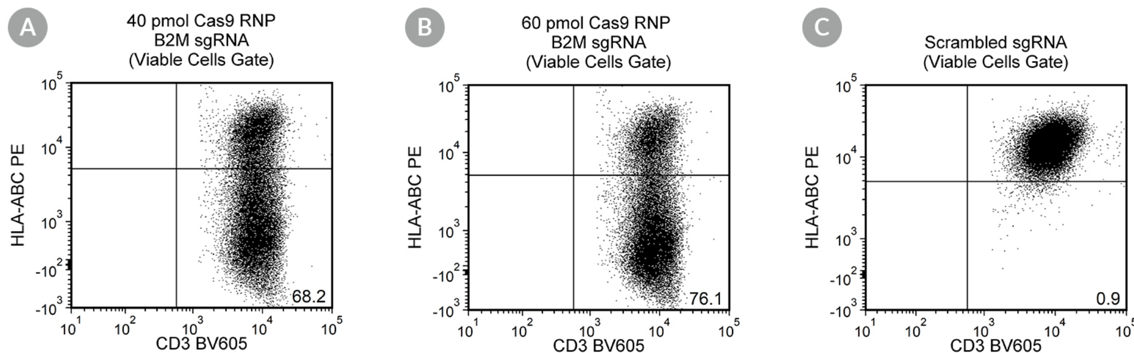

Figure 3. CRISPR/Cas9-Mediated Knockout of Surface Receptor MHC-I via *B2M* Gene Editing in Primary Human T Cells Using CellPore™ Transfection System.
Cas9 RNP complex was delivered to 2.5 x 10^6 human pan T cells (isolated from a fresh leukapheresis sample) using CellPore™ Transfection System. After 2 days of culture, T cells were assayed for MHC-I marker expression by flow cytometry (using HLA-ABC antibody). The frequency of surface marker knock-out was (A) 69.7 ± 9.3% with 40 pmol or (B) 81.3 ± 1.7% with 60 pmol Cas9 complexed with sgRNA targeting the β-2 microglobulin (*B2M*) gene. A non-targeting scrambled sgRNA measured (C) 1.0 ± 0.5% knock-out. Data are shown as mean ± SD; n = 5 - 7 donors. Representative flow cytometry data for one donor is shown.

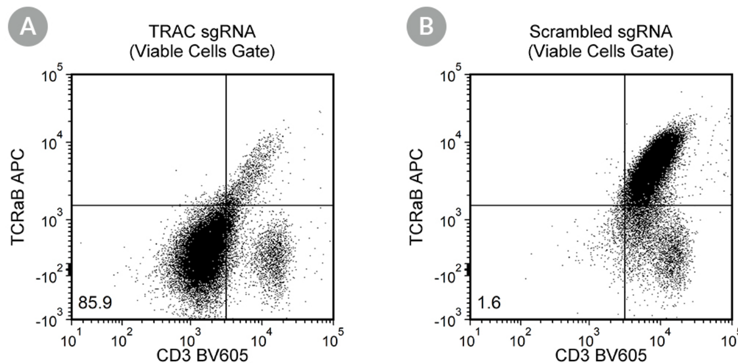

Figure 4. CRISPR/Cas9-Mediated Knockout of Surface T Cell Receptor Alpha/Beta Via *TRAC* Gene Editing in Human Primary T Cells Using CellPore™ Transfection System
Cas9 RNP complex (40 pmol) was delivered to 2.5 x 10^6 human pan T cells (isolated from a fresh leukapheresis sample) using CellPore™ Transfection System. A partial-medium change was performed 72 hours post-delivery. After 6 days of culture, T cells were assayed for T cell receptor alpha/beta (TCRαβ) marker expression by flow cytometry. The frequency of TCRαβ/CD3 surface marker knock-out was (A) 85.7 ± 3.2% with sgRNA targeting the T cell receptor alpha constant (TRAC) gene, or (B) 3.6 ± 2.5% using a non-targeting scrambled sgRNA (5 donors; mean ± SD). Representative flow cytometry data for one donor is shown.

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