BioResearch

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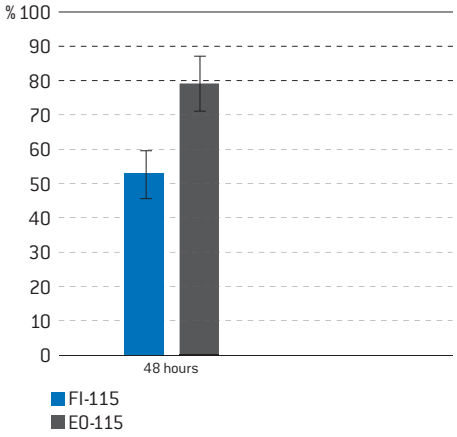
Amaxa™ 4D-Nucleofector™ Protocol for Unstimulated Human T Cells

For 4D-Nucleofector™ X Unit

Unstimulated human T cells (small round lymphoblastoid cells) are a subpopulation of human peripheral blood mononuclear cells (PBMC). PBMC should be purified from fresh human blood samples treated with an anti-coagulant or from leukocyte-enriched buffy coat.

Example for Nucleofection™ of human T cells

Transfection efficiency of fresh unstimulated human T cells 24 hours post Nucleofection™.1 x 10^6 enriched T cells were transfected with program FI-115 (high efficiency) or EO-115 (high functionality) using 0.4 µg pmaxGFP™ Vector in 20 µl Nucleovette™ Strips. Cells were analyzed 48 hours post Nucleofection™ using a FACSCalibur™ [Becton Dickinson]. Cell viability (% PI negative T cells) is approximately 79% (for program EO-115) or 53% (for program FI-115) after 48 hours.Functionality (% of CD25 expression compared to non-transfected control) is usually 59% for EO-115.



Product Description

Recommended Kit(s)–P3 Primary Cell 4D-Nucleofector™ X Kit

Cat. No.	V4XP-3012	V4XP-3024	V4XP-3032
Transfection volume	100 µl	100 µl	20 µl
Size [reaction]	2 x 6	24	2 x 16
Nucleofector™ Solution	2 x 0.675 ml (0.492 ml + 27% overfill)	2.25 ml (1.968 ml + 13% overfill)	0.675 ml (0.525 ml + 22% overfill)
Supplement	2 x 0.15 ml (0.108 ml + 27% overfill)	0.5 ml (0.432 ml + 13% overfill)	0.15 ml (0.115 ml + 22% overfill)
pmaxGFP™ Vector (1 µg/µl in 10 mM Tris pH 8.0)	50 µg	50 µg	50 µg
Single Nucleocuvette™ (100 µl)	12	24	-
16-well Nucleocuvette™ Strips (20 µl)	-	-	2

Storage and stability

Store Nucleofector™ Solution, Supplement and pmaxGFP™ Vector at 4°C. For long-term storage, pmaxGFP™ Vector is ideally stored at -20°C. The expiration date is printed on the solution box. Once the Nucleofector™ Supplement is added to the Nucleofector™ Solution, it is stable for three months at 4°C.

Note

4D-Nucleofector™ Solutions can only be used with conductive polymer Nucleovettes™, i.e. in the 4D-Nucleofector™ and the 96-well Shuttle™ System. They are not compatible with the Nucleofector™ II Device.

Required Material

Note
Please make sure that the entire supplement is added to the Nucleofector™ Solution. The ratio of Nucleofector™ Solution to supplement is 4.5 : 1 (see table 1).

- 4D-Nucleofector™ System (4D-Nucleofector™ Core Unit and 4D-Nucleofector™ X Unit)
- Supplemented 4D-Nucleofector™ Solution at room temperature
- Supplied 100 µl single Nucleocuvette™ or 20 µl 16-well Nucleocuvette™ Strips
- **Compatible tips for 20 µl Nucleocuvette™ Strips:** epT.I.P.S. (US/CDN: Eppendorf North America, Cat. No. 2491.431, Rest of World: Eppendorf AG, Cat. No. 0030073.266), Matrix TallTips® (Matrix Technologies Corp., Cat. No. 7281) or LTS Tips (Rainin Instruments, LLC, Cat. No. SR-L10F, SR/SS-L250S, SR/SS-L300S). Before using other types of pipette tips, please ensure they reach the bottom of the Nucleocuvette™ Wells without getting stuck
- **Supplied pmaxGFP™ Vector, stock solution 1 µg/µl**

Note
For positive control using pmaxGFP™, dilute the stock solution to an appropriate working concentration. Further details are provided in table 3 of this Optimized Protocol. The volume of substrate solution added to each sample should not exceed 10% of the total reaction volume (2 µl for 20 µl reactions; 10 µl for 100 µl reactions).

- Substrate of interest, highly purified, preferably by using endotoxin-free kits; A260:A280 ratio should be at least 1.8
- Cell culture plates of your choice
- **For isolation:** Ficoll-Paque™ Plus (GE Healthcare; Cat. No. 17-1440-03); PBS containing 0.5% (w/v) BSA (PBS/BSA)
- **For enrichment (optional):** Pan T Cell Isolation Kit II (Miltenyi Biotec; Cat. No. 130-091-156) or RosetteSep™ Isolation Kit for human T cells (StemCell Technologies, Cat. No 15021)
- **For coating of plates (optional for post Nucleofection™ stimulation):** Anti-Human CD3 MAB (OKt 3; eBioscience, Cat. No. 14-0037-82) and Anti-Human CD28 MAB (5E8; Research Diagnostics Inc., Cat. No. 10R-CD28bHUµg/µl); control antibody (purified mIgG(K); BD-Pharmingen, Cat. No. 554 721); antibodies should be diluted in carbonate buffer (32 mM Na_2CO_3/16 mM $NaHCO_3$) from 100 ng/µl stock solutions directly before use; Immuno™ Plate C96 Maxi Sorp™ (Nunc, Cat. No.: 430 341)
- **Culture medium:** Clonetics™ Lymphocyte Growth Media-3 LGM-3® for serum-free culture (Lonza, Cat.No. CC-3211) or BioWhittaker® IMDM media for addition of 10% serum (Lonza, Cat.No. BE12-722F)
- Prewarm appropriate volume of culture medium to 37°C (see table 2)
- Appropriate number of cells/sample (see table 2)

1. Pre Nucleofection™

Notes
1. This protocol is designed for fresh unstimulated primary human T cells from whole PBMCs. Depending on application T cells can be further enriched (see below)
2. Transfection results may be donor-dependent
3. For preparation, do not perform protocols using hypo-osmolar buffers. This may lead to high cell mortality after Nucleofection™
4. For freshly isolated cells no cultivation is required prior to Nucleofection™. For cryopreserved cells we recommend incubating the thawed cells for 1–2 hours at 37°C in culture medium before Nucleofection™
5. For Nucleofection™ of stimulated T cells, please refer to the Optimized Protocol for Stimulated Human T Cells

Coating of culture plates (optional for stimulation post Nucleofection™)

1.1 Incubate each well with 1 ml (for 6-well) or 50 µl (for 96-well; Nunc Immuno™ Plate C96 Maxi Sorp™) of a solution of Anti-Human CD3 MAB at a final concentration of 1 µg/ml and Anti-Human CD28 MAB at a final concentration of 2 µg/ml (or with a solution of a control antibody (purified mIgG(K)) at a final concentration 3 ug/ml)at 37°C/5% CO_2 for 5 hours

1.2 Wash the wells carefully three times with PBS/BSA

Blood samples

1.3 Fresh human blood treated with an anticoagulant (e.g. heparin, citrate, ACD-A) or alternatively, leukocyte-enriched buffy coat not older than 8 hours. The samples should be diluted with 2–4 volumes of PBS containing 0.5% BSA (PBS/BSA)

Preparation of PBMC

1.4 Pipet 15 ml Ficoll-Paque™ Plus in a 50 ml conical tube

1.5 Overlay Ficoll- Paque™ Plus with 35 ml blood sample and centrifuge at 750xg for 20 minutes at 20°C in a swinging-bucket rotor without brake

1.6 Remove the upper layer leaving the mononuclear cell layer undisturbed at the interphase. Carefully transfer the interphase cells (lymphocytes and monocytes) to a new 50 ml conical tube

1.7 Add PBS/BSA to 50 ml mark, mix and centrifuge at 350xg for 10 minutes at 4°C. Remove the supernatant carefully

1.8 Resuspend the cell pellet in 25 ml of PBS/BSA and centrifuge at 160xg for 15 minutes at 4°C. Remove the supernatant carefully

1.9 Resuspend the cell pellet in 25 ml PBS/BSA and centrifuge at 300xg for 10 minutes at 4°C. Remove the supernatant carefully

1.10 Resuspend cell pellet in 5 ml PBS/BSA and count the cells

Note
Purified PBMC may be stored at 4°C overnight in PBS/BSA, but this can cause a significant loss of transfection efficiency.

Enrichment of T cells (optional)

1.11 Primary human T cells can be further enriched by using Pan T Cell Isolation Kit II (Miltenyi) or RosetteSep™ Isolation Kit for human T cells (StemCell Technologies) according to the manufacturer's protocol

2. Nucleofection™

2.1 For Nucleofection™ Sample contents and recommended Nucleofector™ Program, please refer to Table 3
2.2 Please make sure that the entire supplement is added to the Nucleofector™ Solution
2.3 Start 4D-Nucleofector™ System and create or upload experimental parameter file (for details see device manual)
2.4 Select/Check for the appropriate Nucleofector™ Program (see table 3)
2.5 Prepare cell culture plates by filling appropriate number of wells with desired volume of recommended culture media (see table 4) and pre-incubate/equilibrate plates in a humidified 37°C/5% CO_2 incubator
2.6 Pre-warm an aliquot of culture medium to 37°C (see table 4)
2.7 Prepare plasmid DNA or pmaxGFP™ Vector or siRNA (see table 3)
2.8 Count an aliquot of the cells and determine cell density
2.9 Centrifuge the required number of cells (see table 3) at 200xg for 10 minutes at room temperature. Remove supernatant completely
2.10 Resuspend the cell pellet carefully in room temperature 4D-Nucleofector™ Solution (see table 3)
2.11 Prepare mastermixes by dividing cell suspension according to number of substrates
2.12 Add required amount of substrates to each aliquot (max. 10% of final sample volume)
2.13 Transfer mastermixes into the Nucleocuvette™ Vessels

Note
As leaving cells in Nucleofector™ Solution for extended periods of time may lead to reduced transfection efficiency and viability it is important to work as quickly as possible. Avoid air bubbles while pipetting.

2.14 Gently tap the Nucleocuvette™ Vessels to make sure the sample covers the bottom of the cuvette
2.15 Place Nucleocuvette™ Vessel with closed lid into the retainer of the 4D-Nucleofector™ X Unit. Check for proper orientation of the Nucleocuvette™ Vessel
2.16 Start Nucleofection™ Process by pressing the "Start" on the display of the 4D-Nucleofector™ Core Unit (for details, please refer to the device manual)
2.17 After run completion, carefully remove the Nucleocuvette™ Vessel from the retainer
2.18 Resuspend cells with pre-warmed medium (for recommended volumes see table 5). Mix cells by gently pipetting up and down two to three times. When working with the 100 µl Nucleocuvette™ use the supplied pipettes and avoid repeated aspiration of the sample
2.19 Plate desired amount of cells in culture system of your choice (for recommended volumes see table 5)

3. Post Nucleofection™

3.1 Incubate the cells in a humidified 37°C/5% CO_2 incubator until analysis. Gene expression or down regulation, respectively, is often detectable after only 4–8 hours. If this is not the case, the incubation period may be prolonged to 24 or 48 hours
3.2 Medium change 6 hours post Nucleofection™ leads to an increased viability after transfection. Spin the culture dishes for 8 minutes at 140xg in a tissue culture centrifuge. Carefully remove the medium and add fresh pre-equilibrated culture medium
3.3 Stimulation (optional): Please do not add stimuli immediately after Nucleofection™ as this may lead to increased cell mortality. For stimulation by antiCD3/antiCD28, transfer cells to an antibody coated 6-well plate or Nunc MaxiSorp™ 96-well plate (see chapter 1) containing fresh medium 5 hours post Nucleofection™, and incubate cells for another 43 hours. Alternatively you may add fresh medium containing IL-2 or other suitable stimuli 4 –12 hours post Nucleofection™

Additional Information

For an up-to-date list of all Nucleofector™ References, please refer to:
www.lonza.com/nucleofection-citations

For more technical assistance, contact our Scientific Support Team:

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Table 1: Volumes required for a single reaction

	100 µl Single Nucleocuvette™	20 µl Nucleocuvette™ Strip
Volume of Nucleofector™ Solution	82 µl	16.4 µl
Volume of Supplement	18 µl	3.6 µl

Table 2: Required amounts of cells and media for Nucleofection™

	100 µl Single Nucleocuvette™	20 µl Nucleocuvette™ Strip
Culture medium per sample post Nucleofection™ (for transfer and culture)	2 ml	230 µl
Cell number per Nucleofection™ Sample	5–10 x 10^6 cells (Minimal cell number: 7 x 10^5 cells, a lower cell number may lead to a major decrease of cell viability; maximal cell number: 2 x 10^7 cells)	1 x 10^6 cells (5 x 10^5 cells can be used with slightly reduced transfection efficiency and viability; at even lower cell numbers transfection efficiency and viability are significantly decreased)

Table 3: Contents of one Nucleofection™ Sample and recommended program

	100 µl Single Nucleocuvette™	20 µl Nucleocuvette™ Strip
Cells	5–10 x 10^6 cells	1 x 10^6 cells
Substrate* pmaxGFP™ Vector	2 µg	0.4 µg
or plasmid DNA (in H_2O or TE)	1–5 µg	0.2–1 µg
or siRNA	30–300nM siRNA (3–30 pmol/sample)	30–300nM siRNA (0.6–6 pmol/sample)
P3 Primary Cell 4D-Nucleofector™ Solution	100 µl	20 µl
Program	FI-115 (for high efficiency) or EO-115 (for high functionality)	FI-115 (for high efficiency) or EO-115 (for high functionality)

Table 4: Culture media volumes required for post Nucleofection™ Steps

	100 µl Single Nucleocuvette™	20 µl Nucleocuvette™ Strip*
12-well culture plate	1.5 ml	-
96-well culture plate	-	160 µl
Culture medium to be added to the sample post Nucleofection™	500 µl	80 µl

* Maximum cuvette volume 200 µl

Table 5: Recommended volumes for sample transfer into culture plate

	100 µl Single Nucleocuvette™	20 µl Nucleocuvette™ Strip*
Culture medium to be added to the sample post Nucleofection™	500 µl	80 µl
Volume of sample transferred to culture plate	complete sample (use supplied pipettes)	40 µl

* Maximum cuvette volume 200 µl