BioResearch

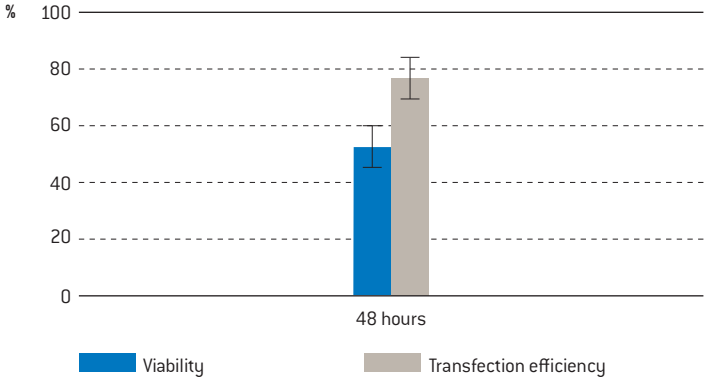
Lonza

Amaxa™ HT Nucleofector™ protocol for unstimulated human T cells

Cell description

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 of Nucleofection™ of human T cells



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

Product description

Recommended kits

P3 primary cell HT Nucleofector™ kits

Cat. No.	V5SP-3002
Size (reactions)	2×384
P3 primary cell HT Nucleofector™ solution	22.5 ml
Supplement	5.0 ml
pmaxGFP™ vector (1.0 µg/µl in 10 mM Tris pH 8.0)	50 µg
384-well Nucleocuvette™ plate(s)	2

Cat. No.	V5SP-3010
Size (reactions)	10×384
P3 primary cell HT Nucleofector™ solution	90 ml
Supplement	20 ml
pmaxGFP™ vector (1.0 µg/µl in 10 mM Tris pH 8.0)	150 µg
384-well Nucleocuvette™ plate(s)	10

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

HT Nucleofector™ solutions can only be used with conductive polymer cuvettes, i.e. in the HT Nucleofector™, the 96-well Shuttle™ device and in the 4D-Nucleofector™ system. They are not compatible with the Nucleofector™ II/2b device.

Required material

Note
Please make sure that the entire supplement is added to the Nucleofector™ solution.

- HT Nucleofector™ system
- Supplemented HT Nucleofector™ solution at room temperature
- Supplied 384-well Nucleocuvette™ plate(s)
- Supplied pmaxGFP™ vector, stock solution 1 µg/µl

Note
Volume of substrate solution added to each sample should not exceed 10% of the total reaction volume (2 µl for 20 µl reactions). For positive control using pmaxGFP™ vector, please dilute the stock solution to reach the appropriate working concentration.

- Substrate of interest, highly purified, preferably by using endotoxin free kits; A260 : A280 ratio should be at least 1 : 8
- 384-well culture plates or culture plates of your choice
- 384-well Nucleocuvette™ plates are best handled with an automated liquid handling system. If manual pipetting is required please use compatible tips: 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 Instrument, 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
- 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)
- 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-CD28bHU); 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)
- Pre-warm appropriate volume of culture media at 37°C (94 µl per sample)
- Appropriate number of cells (1×10^6 cells per sample; 5×10^5 cells can be used with slightly reduced transfection efficiency and viability; at even lower cell numbers transfection efficiency and viability are significantly decreased)

1. Pre Nucleofection™

Notes
- 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).
- Transfection results may be donor-dependent.
- For preparation, do not perform protocols using hypo-osmolar buffers. This may lead to high cell mortality after Nucleofection™.
- 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™.
- 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 µg/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 750×g 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 350×g 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 160×g for 15 minutes at 4°C. Remove the supernatant carefully

1.9 Resuspend the cell pellet in 25 ml PBS/BSA and centrifuge at 300×g 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™

One Nucleofection™ sample contains

- 1×10^6 cells
- 0.2–1 µg plasmid DNA (in 1–2 µl H_2O or TE) or 0.4 µg pmaxGFP™ vector or 30–300 nM siRNA (0.6–6 pmol/sample); if cells are going to be stimulated post Nucleofection™ use 0.2–0.4 µg plasmid DNA for Nucleofection™
- 20 µl P3 primary cell HT Nucleofector™ solution

2.1 Please make sure that the entire supplement is added to the Nucleofector™ solution
2.2 Start HT Nucleofector™ software, verify device connection and upload experimental parameter file (for details refer to the HT Nucleofector™ manuals)
2.3 Select the appropriate HT Nucleofector™ program: **FI-115-BB** (for high efficiency) or **EO-115-DB** (for high functionality)
2.4 Prepare cell culture plates by filling appropriate number of wells with desired volume of culture medium, e.g. 54 µl for one well of a 384-well plate and pre-incubate/equilibrate plates in a humidified 37°C/5 % CO_2 incubator
2.5 Pre-warm an aliquot of culture medium to 37°C (40 µl per sample see note at the end of this chapter)
2.6 Prepare 0.2–1 µg plasmid DNA or 0.4 µg pmaxGFP™ DNA. For siRNA experiments we recommend to start using 30–300 nM (0.6–6 pmol/sample) siRNA
2.7 Count the cells and determine cell density
2.8 Centrifuge the required number of cells (1×10^6 cells per sample) at 200×g for 10 minutes at room temperature
2.9 Resuspend the cell pellet carefully in 20 µl room temperature HT Nucleofector™ solution per sample

A: One or several substrates (DNAs or RNAs) in multiples
- Prepare mastermixes by dividing cell suspension according to number of substrates
- Add required amount of substrates to each aliquot (max. 2 µl per sample)
- Transfer 20 µl of mastermixes into the wells of the 384-well Nucleocuvette™ plates

B: Multiple substrates (e.g. library transfection)
- Pipette 20 µl of cell suspension into each well of a sterile U-bottom 384-well microtiter plate
- Add 2 µl substrates (maximum) to each well
- Transfer 20 µl of cells with substrates into the wells of the 384-well Nucleocuvette™ plates

Note
It is advisable to pre-dispense each cell suspension into a sterile round-bottom 384-well plate or to pipet from a pipetting reservoir for multi-channel pipettes. Use a liquid handling system or at least a multi-channel pipette with suitable pipette tips. 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.

2.10 Briefly shake the 384-well Nucleocuvette™ plate with an appropriate microtiter plate shaker to make sure the sample covers the bottom and sides of the wells without air bubbles. Alternatively thoroughly tap the 384-well Nucleocuvette™ plate
2.11 Place 384-well Nucleocuvette™ plate with closed lid onto the carousel of the plate handler of the HT Nucleofector™. Well "A1" must be in upper left position
2.12 Start Nucleofection™ process clicking "Start" in the HT Nucleofector™ Software (for details refer to the HT Nucleofector™ manuals)
2.13 After run completion, carefully remove the 384-well Nucleocuvette™ plate from the carousel
2.14 Resuspend cells with desired volume of pre-warmed culture medium (maximum cuvette volume 60 µl). Mix cells by gently pipetting up and down two to three times. Recommendation for 384-well plates: Resuspend cells in 40 µl of pre-warmed media*
2.15 Plate desired amount of cells in culture system of your choice. Recommendation for 384-well plates: Transfer 6 µl of resuspended cells to 54 µl pre-warmed media

*** Note**
The indicated cell numbers and volumes have been found to produce optimal Nucleofection™ results in most cases, however, depending on your specific needs you may wish to test an extended range of cell numbers. cell numbers and volumes can be adapted such that fewer cells are transferred or duplicate plates can be seeded.

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 140×g 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

Up-to-date List of all Nucleofector™ references

www.lonza.com/nucleofection-citations

Technical assistance and scientific support

USA/Canada
Tel 800 521 0390 (toll-free)
Fax 301 845 8338
scientific.support@lonza.com

Europe and Rest of World
Tel +49 221 99199 400
Fax +49 221 99199 499
scientific.support.eu@lonza.com

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Lonza Cologne GmbH–50829 Cologne, Germany