a m a × a now a Lonza company www.lonza.com

Mouse ES Cell Nucleofector® Kit

for Mouse Embryonic Stem Cells

Cell type		
	Origin	Cells derived from mouse blastocysts.
	Morphology	Round cells growing in clumps.

Important remarks!

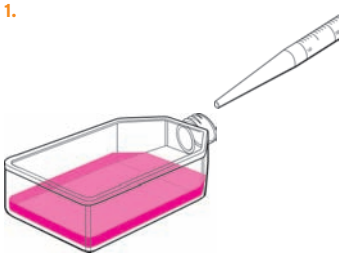
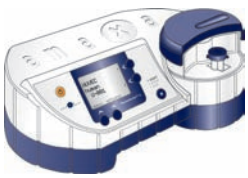
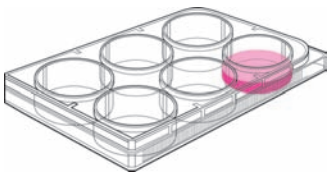
1. This protocol only gives an outline for mouse ES cell culture. Please refer to more detailed protocols in the literature in chapter 4 before starting the experiments.

2. Culture and nucleofection® conditions vary depending on which ES cell line you use.

3. Lines tested so far:

   - CCE
   - D3
   - E14
   - EB5
   - LF2
   - R1
   - RW4

1 Procedure Outline and Important Advice

	Procedure outline	Important advice
1. 	Preparation of cells. (For details see 3.4.)	› Use DMEM containing 15% FCS and leukaemia inhibitory factor (LIF). › ES cells should be in an early growth phase in well-formed colonies. › Feed cultures 4 - 12 hours prior to nucleofection®. › Cell should preferably be cultured on gelatin-coated plates and not on feeder cells.
2. 	Combine the cells of interest, DNA or siRNA and the appropriate cell-type specific Nucleofector® Solution and transfer to an amaxa certified cuvette. (For details see 3.5.)	**Contents of one nucleofection® sample:** › Optimal cell number: **2 - 5 x 10^6** › Plasmid DNA: **2 - 20 µg** plasmid DNA or **2 µg** pmaxGFP® › siRNA: start with **30 - 300 nM** › Nucleofector® Solution: **100 µl** Mouse ES Cell Nucleofector® Solution › Avoid leaving the cells in Nucleofector® Solution for extended periods of time (longer than 15 min).
3. 	Choose the cell-type specific program, insert the cuvette into the Nucleofector® and press the start button »**X**«. (For details see 3.5.)	› Optimal Nucleofector® program: **A-13*/A-013**, A-23*/A-023****, **A-24*/A-024**** or **A-30*/A-030**** **As nucleofection® conditions vary depending on ES cell line and culture conditions, we recommend to test all four programs.** › * for Nucleofector® I Device › ** for Nucleofector® II Device
4. 	Rinse the cuvette with culture medium using an amaxa certified pipette. Transfer the cells into the culture dish. (For details see 3.5.)	› Using an amaxa certified pipette, immediately remove sample from the cuvette with 500 µl prewarmed medium. › Transfer directly to 37°C.

2 Product Description

Cat. No.	**VPH-1001**
Kit components	2.25 ml **Mouse ES Cell Nucleofector® Solution**
	0.5 ml Supplement
	10 µg pmaxGFP® (0.5 µg/µl in 10 mM Tris pH 8.0)
	25 certified cuvettes
	25 plastic pipettes
Size	25 reactions
Storage and stability	Store Nucleofector® Solution, Supplement and pmaxGFP® at 4°C.
	For long term storage pmaxGFP® is ideally stored at -20°C.
	The expiry date is printed on the Solution Box

3 Protocol

3.1 › Required reagents

Medium DMEM containing 15% FCS and leukemia inhibitory factor (LIF).

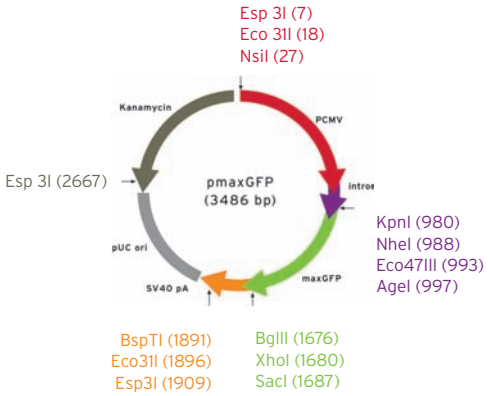
3.2 › DNA preparation and quality

The quality and the concentration of DNA used for nucleofection® plays a central role for the efficiency of gene transfer. We strongly recommend the use of high quality products for plasmid purification like EndoFree® Plasmid Kits [Qiagen® Cat. No. 12391 Giga Kit, 12362 Maxi Kit, 12381 Mega Kit). The purified DNA should be resuspended in deionised water or TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) with a concentration between 1 - 5 µg/µl. Please check the purity of each plasmid preparation by measurement of the A260:A280 ratio, according to the Qiagen protocol.

3.3 › Important controls and vector information

Positive control

We strongly recommend establishing the Nucleofector® Technology with the positive control vector **pmaxGFP®** as provided in this kit. pmaxGFP® encodes the green fluorescent protein (GFP) from copepod *Pontellina p.* Just like eGFP expressing cells, maxGFP® expressing cells can easily be analyzed by fluorescence microscopy or flow cytometry to monitor transfection efficiency.



Negative control

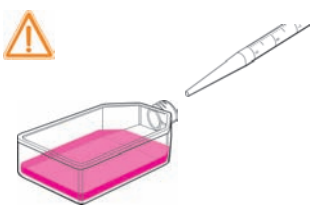
We recommend you always perform two control samples to assess the initial quality of cell culture and the potential influences of nucleofection® or amount/purity of DNA on cell viabilty.

Control 1 Recommended amount of cells in Nucleofector® Solution with DNA but without application of the program (alternatively: untreated cells) (**Cells + Solution + DNA - program)**

control 2 Recommended amount of cells in Nucleofector® Solution without DNA with application of the program **(Cells + Solution - DNA + program)**

Vector information

If using IRES sequences in your vectors, please remember that the gene encoded downstream (3') of the IRES sequence is usually expressed to a lesser extent than the upstream (5´) gene, and in some cell types may not be expressed at all. As alternatives we suggest either: co-transfecting two (or more) plasmids, using one plasmid with each gene under the control of its own promoter, or making a GFP fusion.



3.4 › Cell culture

Please select the culture medium as recommended for your ES cell line. Also, the **amount of LIF has to be adapted** e.g. depending on the cell line or cell culture conditions (see Hogan *et al*., chapter 4).

Note

Contamination of cell culture with mycoplasma is a widely spread phenomenon that might negatively influence experimental results. We recommend the use of Primocin™ [Cat. No. VZA-1021], a new antibiotic formula specifically developed to protect sensitive primary cells from mycoplasma infection and microbial contaminations. Add it directly to the cell culture medium without further need of Pen/Strep or other antibiotics. For more information and ordering info see **www.amaxa.com/antibiotics**.

3.5 › Nucleofection® protocol

Preparation of Nucleofector® Solution

Add 0.5 ml Supplement to 2.25 ml Nucleofector® Solution and mix gently.
The Nucleofector® Solution is now ready to use and is stable for 3 months at 4°C.
Note the date of addition on the vial.

One nucleofection® sample contains

- Optimal cell number: **2 - 5 x 10^6**
  (Minimal cell number: 1 x 10^6 cells (a lower cell number may lead to a major increase in cell mortality). Maximum cell number: 6 x 10^6
- Plasmid DNA: 2 - 20 µg plasmid DNA (in 1 - 5 µl H_2O or TE) for stable linearized DNA or 2 µg pmaxGFP®
- siRNA: 30 - 300 nM of siRNA (start range)
- Nucleofector® Solution: 100 µl Mouse ES Cell Nucleofector® Solution

For an initial experiment we recommend using 30 and 300 nM siRNA as a minimum. Depending on target and cell type, the minimum effective siRNA concentration may range between 1 nM and 1 µM. For optimal knockdown we propose to perform a time course experiment (mRNA: 12 - 72 hours, protein/phenotype: 24 - 96 hours) in addition.

For more details about the nucleofection® of siRNA:
www.amaxa.com/RNAi

Note

The DNA to cell ratio has to be adapted depending on the size and characteristic of your construct.

Preparation of samples

1. Use ES cells that are in an **early growth phase** in **well-formed** colonies. Feed cultures **4 - 12 hours** prior to transfection.
2. Cells should preferably be **cultured on gelatin-coated plates** and not on feeder cells. If cells have been cultured with feeder cells remove feeder cells as completely as possible. To do this, remove media and rinse cells once with PBS. Detach cells from the plate by trypsinization (0.05% trypsin in PBS), quench trypsin with 5-fold media addition and briefly spin cells down, plate onto a single 10 cm feeder-free dish and allow ES/feeder cell population to sit for 30 min, collect the non-adherent cells.
3. Spin down the number of cells required for nucleofection® for 5 min at 80 xg and 4°C to completely remove the culture medium.
4. Resuspend and rinse pellet once in PBS, spin down as done before.
5. Pre-warm the supplemented Mouse ES Cell Nucleofector® Solution to room temperature.
6. Parallel to the centrifugation step, prepare tubes containing 2 - 20 µg of the linearized DNA of interest in 10 µl supplemented Mouse ES Cell Nucleofector® Solution. We recommend to purify the DNA by agarose gel electrophoresis with ethidium bromide or crystal violet staining after linearization. For siRNA we recommend to start using 30 nM and 300 nM for each sample. Depending on cell type, the minimum effective siRNA concentration can range between 1 nM and 1 µM. To validate optimal conditions for knock-down we recommend performing a time course (for mRNA analysis: 12 - 72 hours, for protein/phenotype analysis: 24 - 96 hours)
7. Prepare Eppendorf tubes containing 0.5 ml culture medium and pre-warm to 37°C.
8. Prepare the required number of 10 cm plates covered with mouse neomycin resistant feeder layers or coated with gelatin and add sufficient amount of culture medium, pre-warm to 37°C.
9. Resuspend cells in 90 µl Mouse ES Cell Nucleofector® Solution for per sample.

Nucleofection®

10. Mix 90 µl cell suspension with 2 - 20 µg iDNA or appropriate amount of siRNA in 10 µl Mouse ES Cell nucleofection® Solution by pipetting three times up and down and transfer it into an amaxa certified cuvette. Make sure that the sample covers the bottom of the cuvette, avoid air bubbles between the electrodes. Close cuvette with the blue cap.
11. Insert the cuvette into the cuvette holder and rotate the carousel clockwise to the final position. Select program **A-13/A-013, A-23/A-023, A-24/A-024** or **A-30/A-030** (see Nucleofector® Manual for details).). Insert the cuvette into the cuvette holder (Nucleofector® I: rotate carousel to final position) and press the »X« button to start the program.
12. When the display shows »OK« (nucleofection® process is completed) take the cuvette out of the holder. Add 500 µl of the pre-warmed culture medium to the cuvette immediately and transfer the sample to the tubes. To transfer the cells from the cuvettes, we strongly recommend using the plastic pipettes provided in the kit

to prevent damage and loss of cells. Avoid transfer of pieces of precipitate attached to the wall or bottom of the cuvette.
Note: Avoid leaving the cells in Nucleofector® solution for extended periods of time (longer than 15 minutes), as this may reduce cell viability.

13. Press the »X« button to reset the Nucleofector®.
14. Repeat steps 10 - 13 for the remaining samples.

Cultivation post nucleofection®

15. After nucleofection®, plate cells on gelatin-coated dishes or over feeder layers in well equilibrated medium such that 40 - 100 clones are obtained in each plate. As an example, nucleofection® of 1 million R1 cells with program A-23/A023 yields about 80 - 100 clones. If aggregates have formed during the treatment disperse them carefully.
16. Culture cells for 24 - 48 hours in a humidified incubator at 37°C.
17. Remove medium and begin selection using 300 - 500 µg/ml G418 in culture medium.
18. Continue selection for five days changing medium on the third, fourth and fifth day. When the plate has cleared due to cell death the resistant clones become visible and may be picked for clonal propagation or analyzed.

4 Recommended Literature

Additional references

1. Hogan, Constantini and Lacy, »Manipulating the mouse embryo«, Cold Spring Harbor Laboratory Press.

For an up-to-date list of all Nucleofector® references, please refer to:
www.amaxa.com/citations

amaxa AG
Europe/World
Scientific Support
+49 (0)221-99199-400
scientific-support@amaxa.com

› www.amaxa.com

amaxa Inc.
USA
Scientific Support
888-632-9110 (toll free)
scientific-support.US@amaxa.com