General Protocol

for 96-well nucleofection® of suspension cell lines

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Please check the cell line database on our website:
www.amaxa.com/celldatabase

to see if an Optimized Protocol or any customer data exists for your specific cell line or primary tumor cell.
If no such data exists we recommend using our Cell Line Optimization 96-well Nucleofector® Kit.

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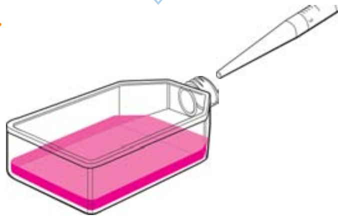
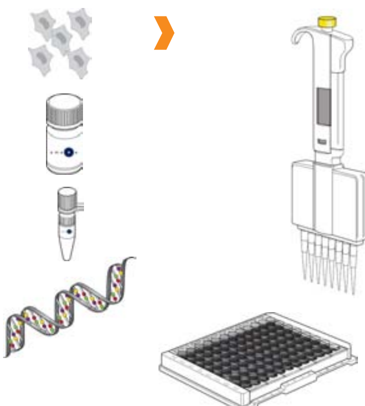
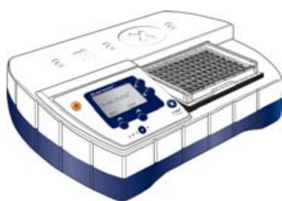
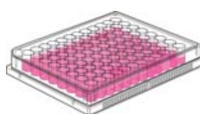
1 Procedure outline & important advice

	Procedure outline	Important advice
1. 	Culturing of cells before nucleofection. (For details see chapter 3.1.)	› For culturing, follow instructions of the cell line supplier. › Passage 1-2 days before nucleofection. › Cells must be in their logarithmic growth phase.
2. 	Combine the cells of interest, DNA or siRNA and the 96-well Nucleofector Solution and transfer to the amaxa 96-well Nucleocuvette™ modules.	**Contents of one nucleofection sample:** › 2×10^5 to 1×10^6 cells › 0.2 - 1 µg plasmid DNA (in 1-2 µl H_2O or TE) or 2 nM - 2 µM siRNA (final concentration, 600 pg - 600 ng/sample) › 20 µl 96-well Nucleofector Solution Avoid leaving cells in 96-well Nucleofector Solution for extended periods of time.
3. 	Insert the Nucleocuvette plate plus lid into the Nucleofector 96-well Shuttle™ and start the nucleofection process. (For details see 3.5.)	› If the optimal Nucleofector program is unknown, we recommend following the instructions of the Cell Line 96-well Optimization Nucleofector Kit. › After completion of the nucleofection process incubate Nucleocuvette plate.
4. 	Rinse the cuvette wells with culture medium and transfer the cells to a culture plate. (For details see 3.5.)	› Add medium and transfer cells to 37°C.

2 Product description

Size	1 x 96	10 x 96	reactions
Kit components	2.025 ml	20.25 ml	**Cell Line 96-well Nucleofector® Solution**
	0.45 ml	4.5 ml	Supplement
	45 µg	45 µg	pmaxGFP™ (0.2 µg/µl in 10 m
	1	10	Nucleocuvette™ plate(s)
Storage and stability	Store Solution and Supplement at 4°C, pmaxGFP™ at 4°C for short term storage or at -20°C for long term storage. The expiry date is printed on the Solution Box.		

Note

96-well Nucleofector Solutions and standard Nucleofector Solutions are not compatible. Please do not use the 96-well Nucleofector Solutions with the standard Nucleofector (100 µl cuvette) and respective programs or vice versa.

3 Protocol

3.1 › Cell culture

For commercially available cell lines we recommend following the instructions of the supplier regarding culture medium and supplements, as well as, passaging and seeding conditions. Best nucleofection results will be obtained with standardized cell culture conditions.

Culture conditions before nucleofection

- Cells should be passaged 1-2 days before nucleofection.
- For nucleofection, cells must be in their logarithmic growth phase.

Note

Contamination of cell culture with mycoplasma is a wide spread phenomenon that might negatively influence experimental results. We recommend the use of Normocin™ [Cat. No. VZA-1001], a new antibiotic formulation specifically developed to protect sensitive cell lines from mycoplasma infection and microbial contaminations. For more information and ordering info see **www.amaxa.com/antibiotics**.

3.2 › Required material

Pipette tips: **Nucleocuvette compatible tips:**
epT.I.P.S.® [Eppendorf, Cat. No. 0030073.266] or
Matrix TallTips® [Matrix Technologies Corp., Cat. No. 7281].

Before using other types of pipette tips, please test ability to reach the bottom of the Nucleocuvette wells without getting stuck.

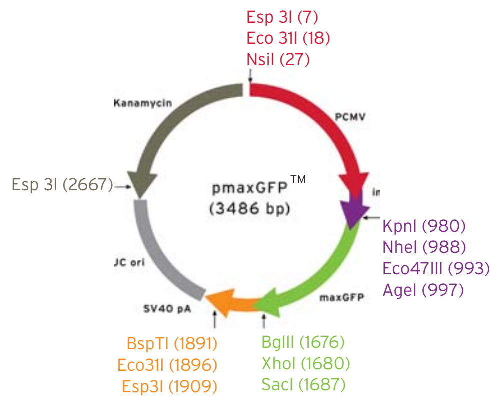
3.3 › DNA preparation and quality

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 QIAGEN EndoFree® Plasmid Kits [Cat. No. 12391 Giga Kit, 12362 Maxi Kit, 12381 Mega Kit). The purified DNA should be resuspended in deionized 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 QIAGEN protocol.

3.4 › Important controls for 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 the 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 control samples to assess the initial quality of cell culture and the potential influences of nucleofection or amount/purity of DNA on cell viability.

control Recommended amount of cells in 96-well Nucleofector Solution with DNA but without application of the program (alternatively: untreated cells) **(Cells + Solution + DNA - program).**

Vector information

If using IRES sequences in your vectors, please remember that the gene encoded 3' of the IRES sequence is usually expressed to a lesser extent than the upstream 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.

3.5 › Nucleofection protocol

Preparation of Nucleofector Solution

Add the Supplement to the 96-well Nucleofector Solution and mix gently.
1 x 96 reactions: 0.45 ml supplement plus 2.025 ml Nucleofector Solution
10 x 96 reactions: 4.5 ml supplement plus 20.25 ml Nucleofector Solution

The 96-well 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

› **2 x 10^5 - 1 x 10^6 cells**
Higher or lower cell numbers may be more suitable for your cells. The optimal cell number can be determined in a titration experiment.

› **0.1 - 0.4 µg plasmid DNA (in 1 - 2 µl H_2O or TE), 0.4 µg pmaxGFP or 2 nM - 2 µM siRNA (600 pg - 600 ng/20 µl)**
Higher or lower DNA amounts may be more suitable for your cells. The optimal DNA amount can be determined in a titration experiment.

› **20 µl Cell Line 96-well Nucleofector Solution**

Note

The volumes and cell numbers indicated below refer to the exact numbers required for the experiment. Please include sufficient extra volume and cells when setting up your pipetting scheme to account for possible losses during pipetting or small discrepencies in pipetted volumes (which may arise from inexactly calibrated pipettes or loose pipette tips).

Preparation of samples

1. Cultivate the required number of cells (**2 x 10^5 - 1 x 10^6** cells per samples).
2. Start Nucleofector 96-well Shuttle Software, **verify device connection** and upload experimental Parameter file (for details see Manual »**Nucleofector 96-well Shuttle System**«).
3. Prepare **0.2 - 1 µg** plasmid DNA, **0.4 µg** pmaxGFP DNA or **2nM - 2 µM** siRNA (600 pg - 600 ng/20 µl) for each sample.
4. Pre-warm the supplemented **Cell Line 96-well Nucleofector Solution** to room temperature.
5. Pre-warm an aliquot of culture medium to 37° C (recommendation: 80 µl per sample[1]).
6. Prepare cell culture plates by filling the intended number of wells with desired amount of culture medium (recommendation 150 µl for 96 well plates[1]). Pre-incubate plates in a humidified 37°C / 5% CO_2 incubator.
7. Take an aliquot of cell culture and count the cells to determine the cell density.
8. Centrifuge for **10 min** at **90xg**. Discard supernatant completely so that no residual medium covers the cell pellet.

9. Resuspend cell pellet with room temperature Cell Line 96-well Nucleofector Solution (volumes as specified below).
   Recommendations for nucleofection of:
   One or few substrates (DNAs or RNAs) in multiplicates:
   - resuspend cell pellet in 96-well Nucleofector Solution (20 µl per sample).
   - 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 96-well Nucleocuvette modules.

   Multiple substrates (e.g. library transfection):
   - Alternative A:
     - resuspend cell pellet in 96-well Nucleofector Solution (20 µl per sample).
     - pipette substrates into a separate sterile U- or V-bottom 96-well microtiter plate to a maximum of 2 µl per well.
     - add 20 µl of cell suspension to substrates.
     - transfer 20 µl of cells with substrates into the wells of the 96-well Nucleocuvette modules.
   - Alternative B:
     - resuspend cell pellet in 96-well Nucleofector Solution (15 µl per sample).
     - pipette substrates into a separate sterile U- or V-bottom 96-well microtiter plate to a maximum of 2 µl per well.
     - adjust to 5 µl with 96-well Nucleofector Solution.
     - transfer substrates into the wells of the 96-well Nucleocuvette modules.
     - ransfer 15 µl of cells into the wells of the 96-well Nucleocuvette modules.

   It is advisable to pre-dispense each cell suspension into a sterile round-bottom 96-well plate or to pipet from a pipetting reservoir for multi-channel pipettes.
   Use a multi-channel or single-channel pipette with suitable pipette tips (see chapter 3.1). Inappropriate pipette tips can get stuck in the wells of the Nucleocuvette modules.
   Work as quickly as possible, leaving cells in 96-well Nucleofector Solution for extended periods of time may lead to reduced transfection efficiency and viability.

   Make sure the sample covers the bottom of the well, if necessary gently tap the Nucleocuvette plate. Avoid air bubbles while pipetting.

Nucleofection

10. Place lid onto the frame.
11. Place 96-well Nucleocuvette plate with lid into the retainer of the 96-well Shuttle. Well »**A1**« must be in upper left position.
12. Start 96-well nucleofection process by either:
    - Pressing »**Upload and start**« in the 96-well Shuttle Software (please refer to Manual)
    - or pressing »**Upload**« in the 96-well Shuttle Software and then the »**Start**« button at the 96-well Shuttle (please refer to Manual).

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13. After complete program execution and retainer opening, carefully remove the 96-well Nucleocuvette plate from the retainer.
14. Incubate Nucleocuvette plate 10 min at room temperature[2].
15. Resuspend cells with desired volume of pre-warmed medium (maximum cuvette volume 200 µl). Mix cells by gently pipetting up and down two to three times.
    Recommendation for 96-well plates: Resuspend cells in 80 µl of pre-warmed medium[1].
16. Plate desired amount of cells in culture system of your choice (96-well to 12-well plate).
    Recommendation for 96-well plates: Transfer 50 µl of resuspended cells to 150 µl pre-warmed medium prepared in 96-well culture plates[1].

Cultivation post nucleofection post nucleofection

17. Incubate cells in a humidified 37°C/5% CO_2 incubator.
    Following nucleofection, gene expression should be analyzed at different times. Depending on the gene, expression is often detectable after 4-8 hours. If this is not the case, the incubation period may be prolonged to 24 or 48 hours.

Notes

1. The indicated cell numbers and volumes have been found to produce optimal 96-well 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. When nucleofecting lower cell numbers you will likely need to transfer a larger volume of resuspended cells.
2. In single cases it may be advantageous to omit the incubation of the Nucleocuvette plate before resuspending cells with medium.

4 Recommended literature

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

* Please note that amaxa's Nucleofector® Technology is not intended to be used for diagnostic purposes or for testing or treatment in humans.

* The Nucleofector® Technology, comprising Nucleofection® Process, Nucleofector® Device, Nucleofector® Solutions, Nucleofector® 96-well Shuttle™ System and 96-well Nucleocuvette™ plates and modules are covered by patent and/or patent-pending rights owned by amaxa.

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