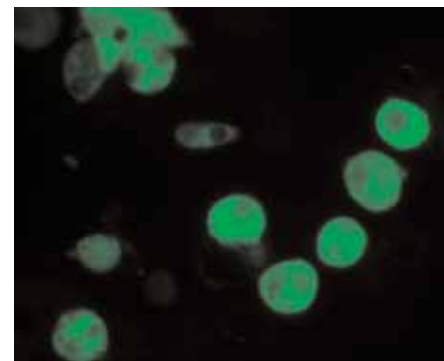
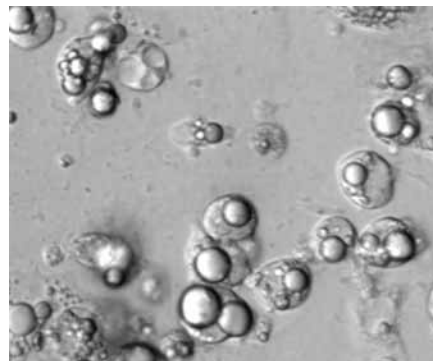
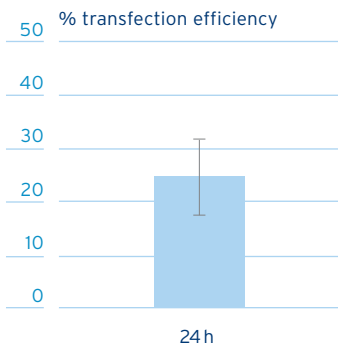


Cell Line Nucleofector® Kit L

for 3T3-L1 (adipocytes) [ATCC]

Cell type	Origin	Mouse embryonal fibroblast [ATCC® CL-173™; frozen vial], differentiated into adipocytes.
	Morphology	Fibroblast-like before differentiation. Adipocyte-like after differentiation.

Example for nucleofection® of 3T3-L1 (adipocytes) cells.



Average transfection efficiencies of 3T3-L1 (adipocytes) cells. Cells were nucleofected with program **A-33/A-033** and 2 µg of pmaxGFP™. **24 hours** post nucleofection, the cells were analyzed by flow cytometry. Cell viability (% compared to un-nucleofected control) is around 90 % 24h post nucleofection.

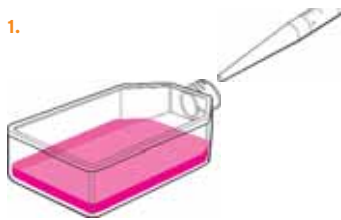
3T3-L1 (adipocytes) cells were nucleofected using the Cell Line Nucleofector Kit L, program **A-33/A-033** and 2 µg of pmaxGFP. **24 hours** post nucleofection the cells were analyzed by fluorescence microscopy.

Chapter	Contents
1	Procedure outline & important advice
2	Product description
3	Protocol
	3.1 › Required reagents
	3.2 › DNA preparation and quality
	3.3 › Preparation of 6-well plates, culture and differentiation
	3.4 › Important controls and vector information
	3.5 › Nucleofection protocol
4	Recommended literature

1

Procedure outline & important advice

1.



Procedure outline

Culturing of cells before nucleofection.
(For details see 3.3.)

Important advice

- › Cells should be seeded with a density of 5×10^5 cells per 162 cm^2 flask.
- › Let cells grow to absolute confluency within 5 days and start differentiation at this point.
- › Differentiate cells for **10 days** before nucleofection.

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:

- › 2×10^6 cells (optimal cell number)
- › $2 \mu\text{g}$ highly purified plasmid DNA (in max. $5 \mu\text{l}$) 2 nM - $2 \mu\text{M}$ siRNA (final concentration, 3 ng - $3 \mu\text{g}/\text{sample}$)
- › $100 \mu\text{l}$ Nucleofector Solution L

Process each sample separately to avoid storing the cells longer than 15 min in Nucleofector Solution L.

3.

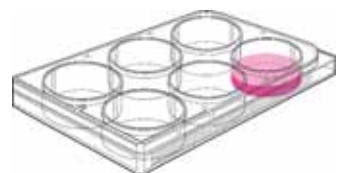


Choose the cell-type specific program. Insert the cuvette into the Nucleofector and press the "X" button to start the program.
(For details see 3.5.)

- › Optimal Nucleofector program: **A-33*/A-033****

- › * 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.
- › Transfer directly to 37°C .

2

Product description

Cat. No.	
Kit components	2.25 ml Cell Line Nucleofector Solution L 0.5 ml Supplement 1 30 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**

Culture Medium Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose (DMEM) [ATCC; Cat. No. 30-2002], 90%; bovine calf serum (FBS), 10% [ATCC; Cat. No. 30-2030].

Differentiation Media

- (I) DMEM [ATCC; Cat. No. 30-2002], 10% fetal calf serum (FSC) [ATCC; Cat. No. 30-2020], 0.86 µM (5 µg/ml) human insulin [Sigma-Aldrich; Cat.No. I 9278]; 0.25 µM dexamethasone [Sigma-Aldrich; Cat.No. D-1756] and 0.5 mM Iso-butyl-methyl-xanthine (IBMX) [Sigma-Aldrich; Cat.No. I 5879].
- (II) DMEM [ATCC; Cat. No. 30-2002], 10% FSC [ATCC; Cat. No. 30-2020], 0.86 µM (5 µg/ml) human insulin.
- (III) DMEM [ATCC; Cat. No. 30-2002], 10% FSC [ATCC; Cat. No. 30-2020].

Trypsin Treatment 2.5 mg/ml Trypsin; 1 mg/ml EDTA in PBS (**5x**).



Preparation of 6 well plates for cultivation after nucleofection

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 QIAGEN® EndoFree® Plasmid Kits [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 QIAGEN protocol.

3.3 › Preparation of 6-well plates, culture and differentiation

1. To prepare a 2.5 mg/ml collagen stock solution, dissolve the collagen (Type I; Sigma Cat. No. C-7661) in 0.2% sterile acetic acid by stirring at room temperature for 4 hours.
2. Add 20 µl collagen stock and 1 ml 30% ethanol per well and coat 6-well plates for 24 hours at room temperature under a laminar flow.
3. Wash 2 x with PBS.
4. Dry plates for about 15 min under a laminar flow.

Note: Gelatin coated plates may also be used. In this case prewarm a 2% gelatin solution (20 mg/ml, gelatin solution type B from bovine skin [Sigma, Cat. No. G1393]) at 37°C for 30 min. Then dissolve gelatin in PBS at 1:8 (final conc.: 2.5 mg/ml). Add 2 ml of diluted gelatin to each well and coat 6-well plates for 30 min at room temperature under laminar flow. Exhaust non-coated gelatin and dry plates for about 15 min. under a laminar flow.

Culture of undifferentiated 3T3-L1 cells

Culture conditions Renew medium every 2-3 days.

Passage interval Cells should be passaged at 80% confluency.
Split cells 3 times a week. **Avoid confluency!**

Seeding conditions 4 x 10⁵ cells per 75 cm² flask (see ATCC protocol).

Differentiation

Culture conditions before differentiation

- › Cells should be seeded with a density of 5 x 10⁵ cells per 162 cm² flask.
- › Let cells grow to 80 - 100% confluency within 5 days and start differentiation at this point.
- › To start differentiation change medium to Differentiation Medium I (day 0 of differentiation).
- › On day 2 of differentiation renew Differentiation Medium I.
- › On day 4 of differentiation switch to Differentiation Medium II for another 2 days.

- › On day 6 of differentiation switch to Differentiation Medium III for another 4 days.
- › Cells should be differentiated for 10 days.
- › **Use only 10 day differentiated 3T3-L1 adipocytes for nucleofection.**

Note: Cells differentiated for less than 10 days can also be transfected using the same nucleofection parameters as described in this protocol. However, differentiation of less than 10 days will result in incompletely differentiated adipocytes.

Note

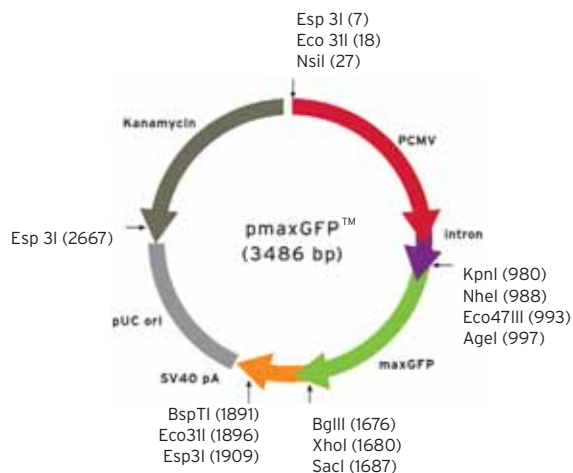
Contamination of cell culture with mycoplasma is a wide spread phenomenon that might negatively influence experimental results. We recommend the use of Normocin™ [Cat. No. VZA-1001], a new antibiotic formulation specifically developed to protect sensitive cell lines from mycoplasma infection and microbial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

3.4

› **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 viability.

- 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 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 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	› 2 x 10⁶ cells › 2 µg plasmid DNA (in 1-5 µl H₂O or TE) or 2 µg pmax GFP or 2 nM - 2 µM siRNA › 100 µl Nucleofector Solution L
	For more details about the nucleofection of siRNA: www.amaxa.com/RNAi

Preparation of samples

1. **Use 10 day differentiated 3T3-L1 adipocytes for nucleofection.**
2. Prepare **2 µg** DNA or **2 nM - 2 µM** siRNA (final concentration, 3 ng- 3 µg/sample) for each sample.
3. Pre-warm the supplemented **Nucleofector Solution L** to room temperature.
4. Prepare collagen-coated 6-well plates (please refer to chapter 3.3) by filling appropriate number of wells with 2 ml of Differentiation Medium III [DMEM, FCS]. Pre-incubate plates in a humidified 37°C/5% CO₂ incubator for at least 30 min.
5. Remove the medium from the cell culture flask and store it in 50 ml tubes.
6. Add 4% glycerol to the stored medium and mix vigorously.
7. Wash cells once with PBS.
8. Harvest the cells with 5 x Trypsin/EDTA for 10 min at 37°C.
9. Stop trypsinization with stored culture medium supplemented with 4% glycerol.
10. Centrifuge the cells at **90xg** at **room temperature** for **10 min**. Discard supernatant.
11. Wash cells with 10 ml fresh Differentiation Medium III supplemented with 4% glycerol.



Nucleofection

12. Take an aliquot of cell suspension and count the cells to determine the cell density.
13. Centrifuge the required number of cells (**2 x 10⁶** cells per nucleofection sample) at **90xg** at **room temperature** for **10 min**. Discard supernatant completely so that no residual medium covers the cell pellet.
14. Resuspend the pellet in room temperature Nucleofector Solution **L** to a final concentration of **2 x 10⁶ cells/100 µl**. Avoid storing the cell suspension longer than **15 min** in Nucleofector Solution as this reduces cell viability and gene transfer efficiency. **Important: Steps 15-19 should be performed for each sample separately.**
15. Mix 100 µl of cell suspension with **2 µg** DNA or **2 nM - 2 µM** siRNA (final concentration, 3 ng- 3 µg/sample).
16. Transfer the nucleofection sample into an amaxa certified cuvette. Make sure that the sample covers the bottom of the cuvette, avoid air bubbles while pipetting. Close the cuvette with the blue cap.
17. Select the appropriate Nucleofector program, **A-33/A-033** (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.
18. **To avoid damage to the cells remove the sample from the cuvette immediately after the program has finished** (display showing "OK"). Take the cuvette out of the holder. 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. Add 500 µl of the pre-warmed culture medium and transfer the sample into the prepared 6-well plates.
19. Press the "X" button to reset the Nucleofector.
20. Repeat steps 15-19 for the remaining samples.
21. Incubate cells in a humidified 37°C/5% CO₂ 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 up to 24 hours.



Cultivation after nucleofection

4

Recommended literature

For an up-to-date list of all Nucleofector references, please refer to:

www.amaxa.com/citations

- * amaxa's Nucleofector® process, Nucleofector® device and Nucleofector® Solutions are covered by PCT applications PCT/EP01/07348, PCT/DE02/01489, PCT/DE02/01483 and other pending patents and domestic or foreign applications corresponding thereto.
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- * This kit contains a proprietary nucleic acid coding for a proprietary copepod fluorescent protein intended to be used as a positive control with this amaxa product only. Any use of the proprietary nucleic acid or protein other than as a positive control with this amaxa product is strictly prohibited. USE IN ANY OTHER APPLICATION REQUIRES A LICENSE FROM EVROGEN. To obtain such a license, please contact Evrogen at license@evrogen.com.
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