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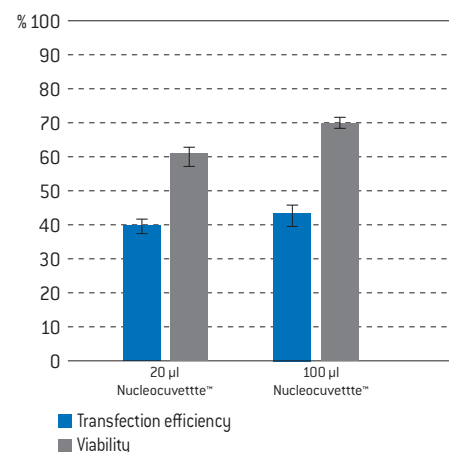
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# Amaxa™ 4D-Nucleofector™ Protocol for Rat Hippocampal or Cortical Neurons For 4D-Nucleofector™ X Unit—Transfection in suspension

Isolated from embryonic (E17-18) or neonatal rats (P0-2) and cultured as mixed glial cells

## Example for Nucleofection™ of rat cortical neurons

Freshly isolated rat cortical neurons (E18) were transfected using P3 Primary Cell Nucleofector™ Solution, high viability program CU-133 and 0.4 µg pmaxGFP™ Vector (in 20 µl Nucleocuvette™ Strips) or 2 µg pmaxGFP™ Vector ((in 100 µl Nucleocuvettes™). Post Nucleofection™, cells were cultured in poly-L-ornithine coated 96-well plates and after 1 DIV analyzed using a FACSCalibur™ [Becton Dickinson]. Depending on preparation transfection efficiency ranges between 30–60 %. Viability was normalized to untransfected control.



## 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 Nucleovettes™ (conductive polymer cuvettes), i.e. in the 4D-Nucleofector™ System and the 96-well Shuttle™ Device. 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. 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 1µg/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
- **Solution for coating:** 1 mg/ml poly-L-lysine [PLL; Sigma] dissolved in borate buffer, sterilized by filtration (as an alternative to PLL, poly-D-lysine can be used as well for cultivation over more than 3 days); 10 µg/ml laminin solution [Invitrogen, Cat. No. 23017-015]; PBS Poly-L-lysine (PLL) and laminin coated glass coverslips [Marienfeld; 15 mm] or PLL coated 96-well culture plates
- **Dissection solution:** 500 ml HBSS [Lonza; Cat. No. 10-508F], 5 ml penicillin/streptomycin [Lonza; Cat. No. 17-602E], 5 ml 1 M MgCl<sub>2</sub>, 3.5 ml 1 M Hepes (pH 7.3) and 5 ml 200 mM L-glutamine; sterilized by filtration and pre-cooled on ice before use
- **Trypsin solution:** 1 mg/ml trypsin [Sigma; Cat. No. T4799] in calcium and magnesium-free HBSS [Lonza; Cat. No. 10-543F] or Trypsin/EDTA mixture [Lonza; Cat. No. 17-161E]; HBSS [Lonza; Cat. No. 10-508F] for washing
- **Culture medium (for embryonic neurons):** PNGM™ BulletKit™ [Lonza; Cat. No. CC-4461] including PNB™ Basal Medium and PNGM™ SingleQuot™ Supplements (GA-1000, final concentration 0.1%; NSF-1, final concentration 2%; L-glutamine, final concentration 2 mM). The medium is supposed to be serum free. On occasion 5% horse serum or FCS serum can be used transiently during plating of the cells

- **Culture medium (for adult neurons):** PNB™-A BulletKit™ [Lonza; Cat. No. CC-4512; for adult neurons] including PNB™ Basal Medium and PNGM™-A SingleQuot™ Supplements (GA-1000, final concentration 0.1%; NSF-1, final concentration 2%; L-glutamine, final concentration 2 mM; additional components are included in the PNGM™-A SingleQuots™ Kit to adjust for unique growth conditions required for optimal growth and survival of adult neurons). The medium is supposed to be serum free. On occasion 5% horse serum or FCS serum can be used transiently during plating of the cells. Optionally 5 µM Ara C [EMD Calbiochem; Cat. No. 251010] may be used 24 hours after plating to inhibit the proliferation of glial cells, which are more abundant in preparations from postnatal brains
- **Recovery medium (optional):** Low calcium medium, e.g. RPMI [Lonza; Cat. No. 12-167F]
- Prewarm appropriate volume of culture medium to 37 °C (see table 2)
- Appropriate number of cells/sample (see table 2)

## 1. Pre Nucleofection™

### Note

This protocol only gives an outline for the isolation and culture of primary rat hippocampal and cortical neurons. Please refer to more detailed protocols in the literature before starting the experiments. A selection of references is given at the end of this document.

### Coating of 96-well culture plates

- 1.1 Add sufficient volume of poly-L-lysine solution to each well to cover the bottom surface
- 1.2 Incubate in a humidified 37 °C/5% CO<sub>2</sub> incubator overnight
- 1.3 Wash 2x with sterile water and dry
- 1.4 Fill each dish with culture medium and return to the incubator for at least 12 hours and up to 2 weeks

### Preparation of coverslips (optional)

- 1.5 Place tissue-culture treated coverslips into an appropriate culture dish (e.g. one slide per well of 12-well plate)
- 1.6 Add 400 µl poly-L-lysine solution and incubate in a humidified 37 °C/5% CO<sub>2</sub> incubator overnight
- 1.7 Wash 2x with sterile water and dry
- 1.8 Incubate coverslips in 400 µl laminin solution in a humidified 37 °C/5% CO<sub>2</sub> incubator over night
- 1.9 Wash 2x with sterile PBS. For more details please refer to Zeitelhofer M et al. 2007 (see reference list at the end of this document)

### Preparation of dissociated hippocampal or cortical neurons for Nucleofection™

- 1.10 Separate heads from rat embryos (E17-18) or early postnatal rats (P0-2)

- 1.11 Dissect brains from the skull and transfer them into a Petri dish with pre-cooled dissection solution
- 1.12 Cut brains along midline and extract hippocampi or cortices
- 1.13 Store hippocampi or cortices in at least 10 ml dissection solution in Falcon tubes on ice
- 1.14 Centrifuge at 80xg for 5 minutes and carefully remove supernatant (alternatively, if experienced, remove dissection solution by very careful decanting)
- 1.15 Add 1.5 ml trypsin solution and incubate for 10–20 minutes at 37 °C
- 1.16 After trypsinization, centrifuge at 80xg for 5 minutes and carefully remove supernatant (alternatively, if experienced, remove dissection solution by very careful decanting)
- 1.17 Wash two times with HBSS
- 1.18 After the second wash, add 1.5 mL of culture medium, prewarmed to 37 °C
- 1.19 Triturate about 20–30x with a fire-polished Pasteur pipette until all pieces of tissue are homogeneously dispersed
- 1.20 Triturate a second time for exactly 1 minute with a fire-polished Pasteur pipette
- 1.21 Add 5 ml of culture medium and count cells
- 1.22 Continue at step 2.1 of the Nucleofection™ Protocol

## 2. Nucleofection™

For Nucleofection™ Sample contents and recommended Nucleofector™ Program, please refer to Table 3.

- 2.1 Please make sure that the entire supplement is added to the Nucleofector™ Solution
- 2.2 Start 4D-Nucleofector™ System and create or upload experimental parameter file (for details see device manual)
- 2.3 Select/Check for the appropriate Nucleofector™ Program (see table 3)
- 2.4 Prepare cell culture plates by filling appropriate number of wells with desired volume of recommended culture media (see table 5) and pre-incubate/equilibrate plates in a humidified 37 °C/5% CO<sub>2</sub> incubator
- 2.5 Pre-warm an aliquot of culture medium to 37 °C (see table 4)
- 2.6 Prepare plasmid DNA or pmaxGFP™ Vector or siRNA (see table 3)
- 2.7 Count an aliquot of the cells and determine cell density
- 2.8 Centrifuge the required number of cells (see table 3) at 80xg for 10 minutes at room temperature. Remove supernatant completely
- 2.9 Resuspend the cell pellet carefully in room temperature 4D-Nucleofector™ Solution (see table 3)
- 2.10 Prepare mastermixes by dividing cell suspension according to number of substrates
- 2.11 Add required amount of substrates to each aliquot (max. 10% of final sample volume)
- 2.12 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.

- 2.13 Gently tap the Nucleocuvette™ Vessels to make sure the sample covers the bottom of the cuvette
- 2.14 Place Nucleocuvette™ Vessel with closed lid into the retainer of the 4D-Nucleofector™ X Unit. Check for proper orientation of the Nucleocuvette™ Vessel
- 2.15 Start Nucleofection™ Process by pressing the “Start” on the display of the 4D-Nucleofector™ Core Unit (for details, please refer to the device manual)
- 2.16 After run completion, carefully remove the Nucleocuvette™ Vessel from the retainer
- 2.17 Incubate Nucleocuvette™ 10 minutes at room temperature
- 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)

### Optional (in case of very high mortality)

- 2.20 If very high mortality is observed, a recovery step can be useful: immediately after Nucleofection™, add 100–300 µl pre-equilibrated recovery medium to the cuvette (instead of the standard culture media) and gently transfer it to a reaction tube
- 2.21 Place the cell suspension in incubator for 5–10 minutes (=“Recovery Step”)
- 2.22 Transfer the sample into the prepared culture dish with the coated coverslip and continue at 3.1 of protocol

## 3. Post Nucleofection™

- 3.1 Incubate the cells in humidified 37 °C/5% CO<sub>2</sub> incubator until analysis
- 3.2 Optionally (in case of much debris): Replace half of the medium with fresh culture medium after 2–4 hours
- 3.3 Optionally (in case of much debris): Replace the medium completely with fresh culture medium after 24 hours
- 3.4 After 24–48 hours of incubation viability of cells can be evaluated by proportion of cells attached to the cover slips. Depending on the gene, expression is often detectable after 6–8 hours and can be observed up to 12–14 days after Nucleofection™
- 3.5 After 3 days transfer coverslips to glial support culture
- 3.6 Replace half of the culture medium with fresh medium once a week

## Additional Information

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

[www.lonza.com/nucleofection-citations](http://www.lonza.com/nucleofection-citations)

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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)	800 µl	255 µl
Cell number per Nucleofection™ Sample	4–5 x 10 <sup>6</sup> cells (Minimal cell number is 1 x 10 <sup>6</sup> cells, a lower cell number may lead to a major increase in cell mortality; maximum cell number is 6 x 10 <sup>6</sup> cells)	2.5 x 10 <sup>5</sup> cells (Cell numbers down to 5 x 10 <sup>4</sup> or up to 5 x 10 <sup>5</sup> can be used with slightly reduced transfection efficiency and viability, at even lower cell numbers viability is strongly decreased)

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

	100 µl Single Nucleocuvette™	20 µl Nucleocuvette™ Strip
Cells	4–5 x 10 <sup>6</sup>	2.5 x 10 <sup>5</sup>
Substrate*	pmaxGFP™ Vector	2 µg
	or plasmid DNA (in H <sub>2</sub> O or TE)	1–3 µg
	or siRNA	30–300nM siRNA (3–30 pmol/sample)
P3 Primary Cell 4D-Nucleofector™ X Solution	100 µl	20 µl
Program	CU-133 (for high viability)	CU-133 (for high viability)
	EM-110 (for high expression levels)	EM-110 (for high expression levels)

\* Volume of substrate should comprise maximum 10 % of total reaction volume

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

	100 µl Single Nucleocuvette™	20 µl Nucleocuvette™ Strip*
12-well culture plate	300 µl	-
96-well culture plate	-	175 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