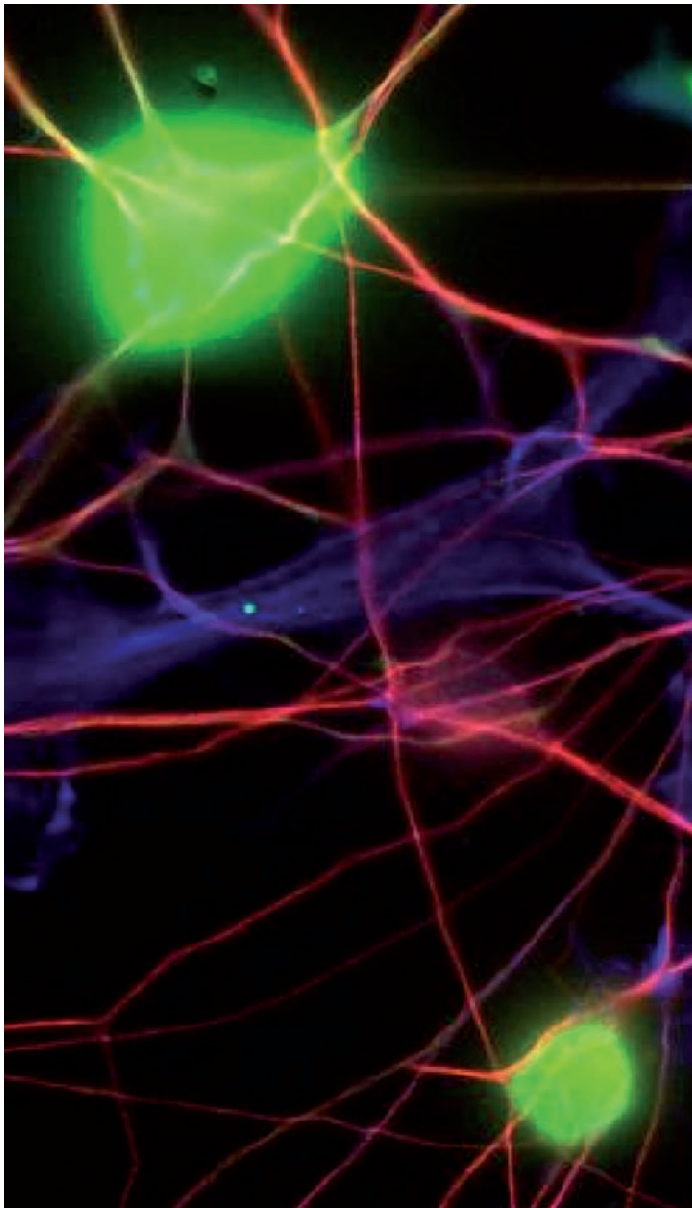


White Paper:

Efficient Transfection of shRNA-encoding Plasmids into Mammalian Neurons



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Introduction

Transfection methods are widely used to study miscellaneous aspects of cell biology¹. The transfection of plasmids encoding tagged proteins, for instance, allows the visualization of the location and behavior of proteins in living cells. With the discovery of RNA interference (RNAi), it is now also possible to specifically prevent the production of certain proteins within a cell via the transfection of plasmids encoding short-hairpin RNAs (shRNAs)^{2,3}. Transfection of shRNA-encoding expression plasmids has several advantages over a transfection with siRNAs. For one, it ensures the knock-down of the targeted protein over longer periods of time via the prolonged expression of the shRNA. Moreover, by including inducible elements in the promoter, the expression of an shRNA can be switched on and off as desired. Finally, constructs can be designed for the stable integration of the shRNA-encoding sequence into the genome. As a consequence, such approaches will greatly accelerate our understanding of gene function in a cellular context.

While transfections are generally easy and fast to perform in dividing cells, transfecting post-mitotic cells can be very challenging. This is a particular concern with neurons, which tend to be more sensitive than other cell types to any form of stress, be it physical or chemical. As a consequence, a number of transfection protocols that can achieve comparatively high transfection rates in dividing cells, such as certain forms of lipid-based transfection methods (lipofection), are not well suited when working with neurons. Other procedures, for instance the Ca²⁺-phosphate co-precipitation⁴, which are generally less stressful to neurons, tend to achieve only low to moderate transfection efficiencies¹. This poses a problem for approaches where high transfection efficiencies are required.

Amaxa® Nucleofector® Technology (Nucleofection®), a specialized form of electroporation, has been shown to result in very high transfection

efficiencies for primary mammalian neurons, while ensuring good cell viability⁵. However, the efficiency of the transfection can vary depending on the plasmid used. Especially certain pSUPER and pSUPERIOR Vectors encoding shRNAs proved to be notoriously difficult to transfect with high efficiency.

High transfection rates of shRNA plasmids are, however, essential for quantitative approaches, e.g. when determining the level of down-regulation of the target protein via quantitative western blot analyses to assess the efficiency of an siRNA-mediated knock-down. In such cases, the proteins produced by untransfected cells in the culture can obstruct the assessment of the level of residual protein in transfected, shRNA-expressing cells. As a consequence, the actual degree of down-regulation of the target protein as well as any other changes in the proteome of transfected cells could be underestimated or even be undetectable.

This problem prompted us to develop an optimized Amaxa[®] Nucleofection[®] Protocol to reliably transfect different shRNA plasmids into primary mammalian neurons with high efficiency.

Material and Methods

Isolation of Hippocampal Neurons

Primary neurons from 17 day-old embryonic (E17) rat brains were prepared as described^{5,6}.

Vectors

Freshly isolated neurons were transfected with the following plasmids: (i) pmaxGFP[®] Vector (Lonza) as a control or (ii) pSUPERIOR shRNA Encoding Vectors (OligoEngine; Seattle, WA) targeting rat Barentsz (Btz) mRNA, rat Pumilio 2 (Pum2) mRNA, rat Staufen1 (Stau1) mRNA and rat Staufen2 (Stau2) mRNA and (iii) pDsRed monomer-C1 RFP (CMV promoter, BD PharMingen); see ref. 7 for details. All shRNA constructs were cloned into the pSUPERIOR Vector, which, in addition to the shRNA, co-expresses Enhanced Green Fluorescent protein (EGFP). Expression of EGFP was used to assess the transfection of individual cells under an Axiovert[®] 200M Fluorescence Microscope (Zeiss, Germany).

Nucleofection[®]

Nucleofection[®] was performed as described⁵, with the following modifications: 0.5–2 × 10⁶ neurons, and subsequently 3–30 µg of plasmid DNA, were added to a total of 100 µl of the Nucleofector[®] Solution for each transfection reaction. The Nucleofection[®] was carried out with either of the following pre-defined programs: O-003, C-009, X-001, X-003, AK-009, and AL-007 (included in Amaxa[®] Nucleofector[®] II Device, serial version "S"; software version S4-4 or higher Lonza).

Western Blots and Immunofluorescence

For western blots, 2 × 10⁶ transfected E17 rat hippocampal neurons were seeded into 6 cm Petri dishes (Nunc), cultured for 4 days as

described (ref. 6) and analyzed for cell survival, morphological signs of differentiation (including the expression pattern of F-actin and tubulin) as well as the expression of the proteins targeted by the respective siRNAs via immunofluorescence and quantitative western blotting (for details on the antibodies and other labels used to detect protein expression, please see ref. 7). Western blots were quantified using the Odyssey[®] Infrared Imaging System (LI-COR, Bad Homburg, Germany).

Results

In order to optimize the transfection efficiencies of plasmids encoding shRNAs in mammalian neurons, we chose four different shRNA plasmids, which had previously shown varying efficiencies with Nucleofection[®]. shBarentsz (shBtz) and shPumilio2 (shPum2) both tended to transfect with efficiencies of no more than 2% prior to the optimization. shStaufen1 (shStau1), and shStaufen2 (shStau2) showed transfection efficiencies of 8% and 12%, respectively. We identified three parameters that are crucial for obtaining high Nucleofection[®] Efficiencies when using plasmids coding for shRNAs: the amount of plasmid DNA used, the purity of the DNA and the Nucleofector[®] Program employed⁷.

First, we tested whether increasing the amount of DNA used for the Nucleofection[®] Reaction can significantly improve the transfection efficiency without compromising cell viability. Past protocols generally employed 3 µg to maximally 5 µg of plasmid DNA. In our experiments, we raised this amount to up to 40 µg without observing any adverse effects on the survival and/or differentiation of the hippocampal neurons. Increasing the plasmid DNA concentration did, however, substantially improve the efficiency of Nucleofection[®].

For instance, the percentage of transfected cells could be increased from 2% to 29% for shBtz and from 2% to 33% for shPum2 when 30 µg of plasmid DNA was used instead of 3 µg (Figure 1A). Similar results were obtained when Stau1 and Stau2 were targeted (not shown). These results were also confirmed by the corresponding knock-down efficiencies when pooled protein from an entire culture was analyzed. Pum2 protein levels were down-regulated by 85% when the Nucleofection[®] Reaction was performed with 30 µg of shRNA plasmid DNA. By contrast, only 3.5% of the protein was down-regulated when 3 µg of the same plasmid were used (Figure 1B). For Btz, the knock-down efficiency improved from 30% (3 µg) to 60% (30 µg). For a discussion on why the apparent transfection efficiency does not match to the observed down-regulation in a fully predictable manner, see ref. 7.

An important consideration when using high concentrations of DNA is that it might damage the highly sensitive neurons. Our analyses showed, however, that the cells remained healthy after Nucleofection[®] even with the highest concentrations of plasmid DNA used in these experiments and showed all the morphological hallmarks of differentiation of hippocampal neurons in culture. After 3 days in vitro (DIV), for example, they had sprouted neurites, one of which was already longer than the others and went on to develop into the future axon. Moreover, the

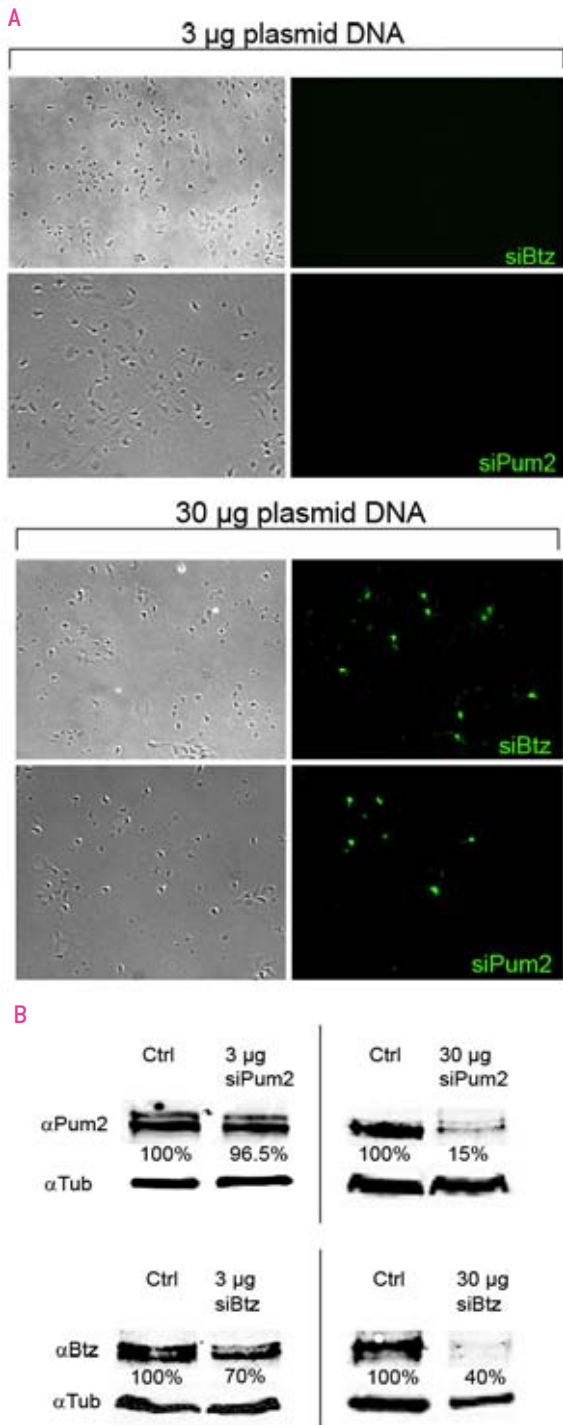


Figure 1. Increasing the concentration of plasmid DNA in Nucleofection[®] Reactions results in higher efficiencies of transfection and RNAi knock-down. **(A)** Phase contrast and fluorescence images of cultured hippocampal neurons transfected with 3 μg resp. 30 μg of plasmid DNA coding for shRNAs targeting the mRNAs for Barentsz (siBtz) and Pumilio 2 [Pum2]. The 0-003 program was used for Nucleofection[®]. Scale bar: 20 μm . **(B)** Quantification of the efficiencies of the knock-down via quantitative Western blot analysis. Control: untransfected neurons (Ctrl). (Figure adapted from ref. 7. Copyright 2009, John Wiley & Sons. Reprinted with permission of John Wiley & Sons.).

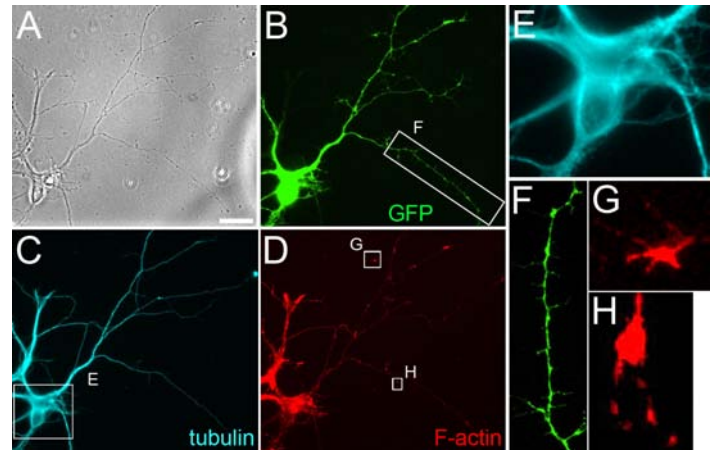


Figure 2. Increasing the concentration of plasmid DNA does not affect neuronal viability and differentiation. **(A-H)** Hippocampal neurons transfected with 30 μg of the shBtz-encoding plasmid. 8 DIV following transfection, the actin cytoskeleton was visualized via staining with Alexa-546-coupled phalloidin **(D, G, H)** and the tubulin cytoskeleton with a specific antibody staining **(C, E)**. Both cytoskeletons are indistinguishable from those of untransfected neurons (not shown). **(G, H)** Higher magnification images of growth cones show the typical concentration of F-actin at the leading edges. **(B)** GFP signal showing the overall morphology of the transfected neuron. **(F)** The boxed region shows a dendrite with protrusions typically seen in hippocampal neurons at this stage. Figure adapted from ref. 7; reprinted with permission from the Journal of Neuroscience Research.

morphology of the cytoskeleton in transfected cells, as visualized via staining for F-actin and tubulin, was indistinguishable from that in untransfected neurons **(Figure 2A-H)**.

Importantly, while increasing the DNA concentration resulted in higher transfection efficiencies, this effect only occurred up to 30 μg of plasmid DNA used. At this concentration, the efficiency reached a plateau and could not be improved by further increasing the amount of plasmid DNA employed.

In a second set of experiments, we examined the importance of DNA purity for the success of a Nucleofection[®] Reaction. These experiments confirmed that the removal of bacterial endotoxins is crucial to the survival of hippocampal neurons in Nucleofection[®] Experiments. Next, determined if neurons transfected with DNA isolated with different commercially available kits for the purification of expression plasmids from *E. coli* showed any differences in their survival rates and/or transfection efficiencies. Briefly, while slight differences were observed, the plasmid DNA purified with any of the four kits tested, resulted in comparable cell survival and Nucleofection[®] Rates (for details on the kits used, see ref. 7). This suggests that, aside from choosing a kit that reliably removes endotoxins, the choice of kit used to purify the DNA is not critical.

Finally, we tested different Nucleofector[®] Programs developed by Lonza to be especially suitable for the transfection of mammalian neurons. The use of alternative programs resulted in even higher transfection

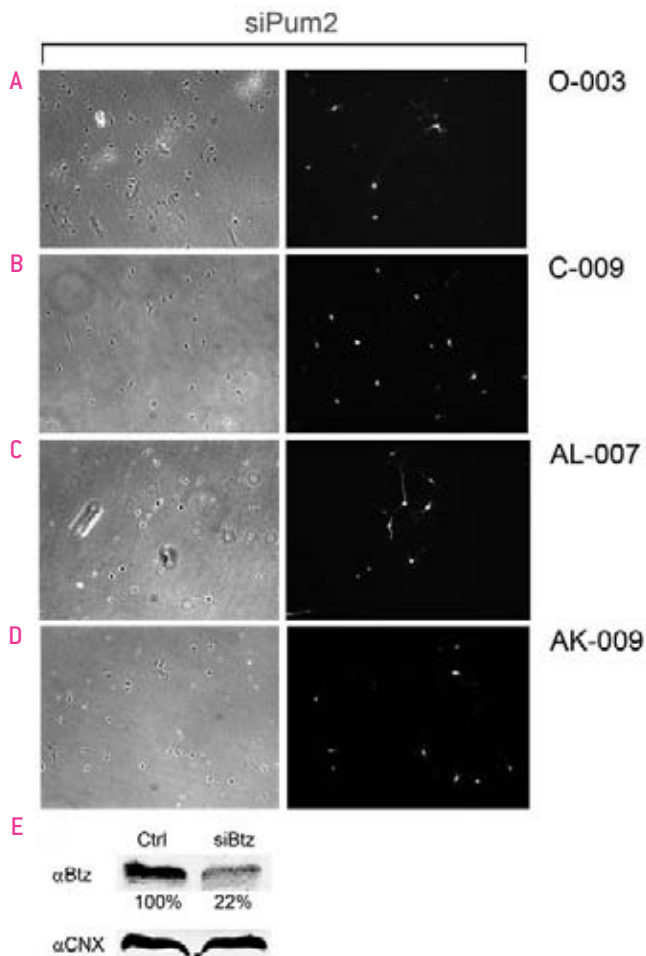


Figure 3. New Nucleofector® Programs achieve higher transfection efficiencies. Hippocampal neurons transfected with 30 µg of the siPum2 plasmid using the established (O-003, **A**) or new Nucleofector® Programs (C-009: **B**, AL-007: **C** and AK-009: **D**; all included in Nucleofector® II Device, serial version "S", software version S4-4 or higher). Phase contrast and fluorescence images of cultures are shown. Neurons transfected with programs O-003 (**A**) and C-009 (**B**) resulted in survival rates between 77%-100% and medium to good transfection efficiencies between 24%-53%. Program AK-009 (**D**) yielded good transfection rates (60%-81%) while ensuring good cell viability (45%-61%). (**E**) Western blot analyses of neuronal cultures transfected with 30 µg of shBtz using the AK-009 program. The knock-down efficiency for shBtz increased from 60% to 78% when the AK-009 instead of O-003 was used. (Figure adapted from ref. 7. Copyright 2009, John Wiley & Sons. Reprinted with permission of John Wiley & Sons.)

efficiencies than could be obtained with the increase of the DNA amount alone. In this study, we evaluated several programs and compared the transfection efficiencies and cell survival rates to those observed when the current recommended program (O-003) is used for transfecting hippocampal neurons.

Nucleofection® with programs AK-009, AL-007, X-001 and X-003 resulted in significantly higher transfection efficiencies than program O-003. While program O-003 typically resulted in transfection efficiencies of

between 25% (30 µg of shBtz plasmid DNA) to 33% (30 µg of shStau2 plasmid DNA), using either of these new programs yielded efficiencies of 46-82% [Figure 3]. Especially when neurons were transfected with the programs AL-007 and AK-009, very high transfection efficiencies of 70-80% were achieved. In general, however, the survival rate of the neurons transfected with these new programs was reduced by about 50%. While this may pose a problem when large numbers of cells are required, it should not hamper experiments for which only a high proportion of transfected cells is needed, such as the quantification of knock-down efficiencies.

Program C-009, by contrast, yielded slightly lower transfection rates than programs AK-009, AL-007, X-001 and X-003 (around 50%). It did, however, result in a significantly higher cell viability of approximately 80% of the levels observed with program O-003. This suggests that C-009 is the program of choice when large absolute numbers of transfected cells are required, albeit at the expense of transfection efficiency.

Conclusions

Our experiments show that increasing the amount of plasmid DNA used in a Nucleofection® Reaction, using an endotoxin-free DNA preparation and choosing the appropriate program, can significantly increase the Nucleofection® Efficiency of shRNA-encoding plasmids in mammalian neurons. These steps also allow for a highly efficient transfection of notoriously difficult to transfect plasmids and reliably achieve these high transfection efficiencies while simultaneously maintaining good survival rates. This allows knock-down experiments to be carried out in primary mammalian neurons on a large scale and with a quantifiable read-out, opening up the possibility to perform, for example, RNAi-based screening approaches.

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