tRNA Knock-Down in Mammalian Cells using Short Hairpin RNAs

Sebastian Kirchner, Robert Rauscher, Andreas Czech, Zoya Ignatova

Abstract

This protocol describes the knock-down of specific tRNA(s) in mammalian cells using short hairpin RNA (shRNA)-based RNA interference (RNAi). We have used this method successfully to silence specific tRNAs in in HeLa[1] and N2a cells[2].

References

1. Kirchner *et al*. Alteration of Protein Function by a Silent Polymorphism Linked to tRNA Abundance. *PLoS Biology.* 2017, *in press.*

2. Girstmair *et al*. Depletion of Cognate Charged Transfer RNA Causes Translational Frameshifting within the Expanded CAG Stretch in Huntingtin. *Cell Reports*. 2013, 3(1):148-59. DOI: http://dx.doi.org/10.1016/j.celrep.2012.12.019

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Before start

shRNA expression vectors can be generated using the pSUPER siRNA expression vector system from OligoEngine. The pSUPER plasmid contains unique BglII and HindIII restriction site which can be used for integration of anealed forward and reverse shRNA oligonucleotides. This positions the shRNA oligonucleotide immediately downstream of the polymerase-III H1-RNA gene promoter, enabling expression of the desired shRNA. A T5 (5 consecutive thymidines) termination signal follows immediately downstream of the HindIII cloning site. For more details please see the attached pSUPER manual from OligoEngine.

The following example outlines the generation of a pSUPER-based shRNA expression vector targeting the human $tRNA^{Thr}$(CGU):

1. *shRNA design*

Please see the attached document for exemplary targeting of the human $tRNA^{Thr}$(CGU).

2. *Annealing of shRNA DNA oligonucleotides*

shRNA-forming oligonucleotides (see step 1.) can be ordered as single stranded DNA oligonucleotides which are then annealed to form a double-stranded DNA molecule. The shRNA encoding dsDNA oligonucleotide contains 5' overhangs corresponding to BglII and HindIII restriction sites.

3. *Linearizing the pSUPER vector*

Linearize the pSUPER vector using BglII and HindIII restriction enzymes and gel purify the cut vector.

4. *Ligation and transformation*

The shRNA-encoding dsDNA oligonucleotide is then cloned into the linearized pSUPER vector. Prior to transformation, the ligation mixture is treated with BglII to remove all incorrectly ligated products. Ligation mixtures are then transformed into competent *E. coli*.

Note: The BglII 5' overhang of the shRNA encoding dsDNA oligonucleotide actually corresponds to a BamHI restriction site within the pSUPER vector, and thus destroys the BglII site upon ligation. Hence, correctly annealed constructs do not contain a BglII restriction site.

5. *Selection of positive clones*

Positive clones are indetified using EcoRI and HindIII restriction analysis or integration PCRs.

6. *Purification of shRNA expression plasmids*

Purify shRNA expression plasmids using the EndoFree Plasmid Maxi Kit (Qiagen, 12362) and resuspend the plasmid DNA in endotoxin-free H_2O. Store purified plasmids at a concentration of 1 µg/µl at -20°C until use.

Protocol

Construction and purification of shRNA bearing plasmids

Step 1.

For the generation and purification of pSUPER-based shRNA expression plasmids please refere to the 'Before Start Instructions' section of this protocol.

Transfection of shRNA-pSUPER

Step 2.

Seed cells (e.g. 200,000 HeLa cells) into a 3.5 cm cell culture dish in DMEM (Dulbecco's Modified Eagle Medium, PAN-Biotech, #P04-03500; supplemented with 10% FCS and 2 mM L-glutamine) 24h prior to transfection and incubate at 37°C in a humidified atmosphere with 5% CO_2.

Note: Seeding conditions need to be adjusted depending on the used cell type. This protocol describes the shRNA mediated knock-down of Thr-tRNAs in HeLa cells. Other cells successfully transfected with shRNAs are N2a mouse neuroblastoma cells. However, for tRNA delivery into N2a cells we used the jetPRIME transfection reagent (PolyPlus, #114). Please refere to Girstmair et al., Cell Reports, 2013 for more details.

Transfection of shRNA-pSUPER

Step 3.

At the day of transfection (cells should have reached 70-80% confluency) prepare transfection mix as follows: For each 3.5 cm cell culture dish, add 2 µg of shRNA pSUPER plasmid and 7 µl PEI (1 mg/ml) to 100 µl opti-MEM (ThermoFisher, #31985062).

PEI (1 mg/ml):

Dissolve polyethylenimine (PEI, linear [25,000], Polysciences, #23966) in endotoxin-free H_2O preheated to 80°C to a concentration of 1 mg/ml.

Cool the mixture to room temperature, adjust pH to 7.0, filtersterilize and store in 1 ml aliquots at -20°C. For short-term store at 4°C.

Note: The amount of shRNA bearing plasmids to be transfected as well as the amount of PEI depends on the specific experimental setup, the used cell line and the efficiency of the tRNA silencing. Optimal amounts need to be empirically determined (DNA:PEI ratios are normally kept at 3:1). However, a complete tRNA knock-down can be detrimental to mammalian cells and the level of optimal tRNA silencing needs to carefully titrated. Usually, tRNA silencing of up to 40% still allows basic translation functions. Further silencing is not recommended, although it can dependend on the used system. We have also successfully co-transfected shRNA bearing plasmids together with protein-coding plasmids. Protein expression plasmids can be added together with the shRNA pSUPER plasmids prior to incubation. For co-transfection we use 1 µg of shRNA-pSUPER and 1 µg of the protein expression plasmid and 7 µl PEI (1 mg/ml).

Transfection of shRNA-pSUPER

Step 4.

Vortex the transfection mix for 10 s, spin briefly and incubate 30 min at 22°C.

DURATION
00:30:00

Transfection of shRNA-pSUPER

Step 5.

While incubating, exchange the culture medium with 1.9 ml fresh DMEM (supplemented with 10% FCS and 2 mM L-glutamine).

Transfection of shRNA-pSUPER

Step 6.

Once incubation is completed, add transfection mixture dropwise to cells and incubate cells for 48 h at 37°C in a humidified atmosphere with 5% CO_2. Cells can then be further manipulated as necessary.

Note: The incubation time which is required to successfully silence the tRNA species of interest depends on the experimental setup and the used cell line. The efficiency of the tRNA knock-down can be analysed by qRT-PCR or Northern blotting. Exemplary results can be seen in Kirchner et al., PLoS Biology, 2017 and Girstmair et al., Cell Reports, 2013.