Upregulating tRNAs in Mammalian Cells through Transfection of In Vitro Transcribed tRNAs

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Abstract

This protocol describes the transfection of mammalian cells with *in vitro* transcribed, unmodified, non-charged tRNAs. We have used this method successfully to raise intrinsic tRNA concentrations in HeLa[1] and N2a cells[2]. Furthermore, tRNAs synthesised as described in this protocol have been shown to be translation competent[1].

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

Considerations for tDNA oligo design:

1. Single stranded tDNA oligonucleotides encoding the desired tRNA sequence and the T7 promoter sequence (5'-TAATACGACTCACTATAG-3') are annealed and the resulting 5' overhangs are subsequently filled-up in order to create a linear dsDNA molecule. This dsDNA molecule then serves as template for the subsequent tRNA transcription, whereby the T7 polymerase then starts transcription at the last base of the T7 promoter (i.e. G) and then transcribes 5' → 3' using the opposite strand as a template. Hence, the last nucleotide of the T7 promoter sequence (i.e. G) will be the first base of the synthesised tRNA. It should be noted that therefore only tRNAs starting with a G as first base can be transcribed using the T7 promoter.
2. The length of the overlapping parts between 5' and 3' tDNA oligonucleotides should be approx. 20 nucleotides.
3. Example of human $tRNA^{Thr}$(UGC) tDNA oligonucleotide:
   Full-length $tRNA^{Thr}$(UGC)
   GGCGCGGTGGCCAAGTGGTAAGGCGTCGGTCTCGTAAACCGAAGATCACGGGTTCGAACCCCGTCCGT GCCTcca

+ T7 promoter
TAATACGACTCACTATAGGCGCGGTGGCCAAGTGGTAAGGCGTCGGTCTCGTAAACCGAAGATCACG
GGTTCGAACCCCGTCCGTGCCTcca
Fwd tDNA oligonucleotide
TAATACGACTCACTATAGGCGCGGTGGCCAAGTGGTAAGGCGTCGGTCTCGTAAACC
Rev tDNA oligonucleotide (reverse complement)
tggAGGCACGGACGGGGTTCGAACCCGTGATCTTCGGTTTACGAGACCGACGCCT

Protocol

Generation of tDNA Template

Step 1.

For annealing of overlapping single stranded tDNA oligonucleotides combine: 9.6 µl forward ssDNA oligonucleotide (100 µM), 9.6 µl reverse ssDNA oligonucleotide (100 µM), 4 µl Tris-HCl (200 mM, pH 7.5) and 16.8 µl H_2O.

Step 2.

Incubate 2 min at 95°C, followed by 3 min at 22°C. Store on ice until further use.

Generation of tDNA Template

Step 3.

To fill-in 5' single-stranded tDNA overhangs to form blunt ends prepare Reverse Transcription master mix on ice as follows: 40 µl 5x Reverse transcription buffer (ThermoFisher, #EP0441), 8 µl dNTPs (10 mM), 4 µl Revert Aid H Minus reverse transcriptase (200 U/µl; ThermoFisher, #EP0441) and 108 µl H_2O.

Step 4.

Generation of tDNA Template

Step 5.

Incubate mixture for 40 min at 37°C.

DURATION
00:40:00

Generation of tDNA Template

Step 6.

For tDNA purification, add one volume Crush & Soak buffer and two volumes phenol/chloroform/IAA, vortex 30 s and centrifuge for 5 min at 21,000 x g (4°C).

Crush & Soak buffer:

50 mM KOAc

200 mM KCl

Adjust pH to 7.0, filter sterilize, aliquot and store at -20°C.

Generation of tDNA Template

Step 7.

Recover upper aqueous phase and precipitate tDNA with 2.7 Vol 100% EtOH for 30 min at -80°C.

Generation of tDNA Template

Step 8.

Pellet tDNA for 40 min at 21,000 x g (4°C) and remove supernatant.

DURATION

00:04:00

Generation of tDNA Template

Step 9.

Resuspend tDNA pellet in 50 µl nuclease-free H_2O and determine concentration. Store tDNA at -20°C.

In vitro tRNA transcription

Step 10.

In vitro tRNA transcription

Step 11.

Combine 2 µg tDNA and 25 µl transcription mix and add nuclease-free H_2O to a final volume of 50 µl. For preparative tRNA synthesis (e.g. for tRNA transfection) transcription should be scaled up to 500 µl reactions.

Note: The efficiency of the in vitro tRNA transcription can vary greatly, dependant on the tRNA sequence. tRNAs starting with another nucleotide than G cannot be transcribed using the T7 promoter.

In vitro tRNA transcription

Step 12.

Incubate for 7h (or overnight) at 37°C

DURATION

07:00:00

In vitro tRNA transcription

Step 13.

Purify tRNAs on a denaturing 10% TBE-PAGE (in case larger transcription reactions have been prepared, transcription mixtures need to be distributed into several slots of the polyacrylamid gel), visualize by UV-shadowing, cut tRNA bands and elute with Crush & Soak buffer overnight.

Note: In vitro transcribed tRNAs should appear as a distinct band, with a molecular weight lower than the input tDNA template (containing the T7 promoter sequence). Purification of tRNAs should be be done under RNase-free conditions.

In vitro tRNA transcription

Step 14.

Remove gel particles by centrifugation (5 min, 4°C, top speed), precipitate with one volume 100% isopropanol at -20°C for 30 min and pellet tRNA by centrifugation (21,000 x g, 4°C, 40 min).

In vitro tRNA transcription

Step 15.

Wash tRNAs 1x with 80% ethanol and resuspend tRNAs in 30 µl nuclease free H_2O. Store tRNAs at -80°C until use.

Refolding and storage of in vitro transcribed tRNAs

Step 16.

For refolding, denature tRNAs at 95°C for 2 min, place at 22°C for 3 min and incubate for further 5 min at 37°C. tRNAs should be stored at -80°C.

Note: The refolding has to be done only once with the complete tRNA stock. The structure is preserved during subsequent storage at -80°C and additional refolding is not necessary. Dilute tRNA stock to a useful working-stock concentration in RNase-free H_2O and aliquot in order to avoid repeated freeze-thaw cycles of the tRNA stocks.

Transfection of in vitro transcribed tRNAs

Step 17.

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 transfection of tRNAs into human HeLa cells. Other cells successfully transfected with in vitro transcriped tRNAs 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 in vitro transcribed tRNAs

Step 18.

At the day of transfection (cells should have reached 70-80% confluency) thaw refolded tRNAs on ice.

For each 3.5 cm cell culture dish, add tRNAs to 50 µl opti-MEM (ThermoFisher, #31985062) **(Tube1)**.

*Note: The amount of tRNAs to be transfected depends on the specific experimental setup, the cell line and the intrinsic tRNA concentration. The optimal tRNA amount needs to be empirically determined. With this protocol, 38 to 600 ng tRNAs have been successfully transfected into HeLa cells. We have also successfully co-transfected in vitro transcribed tRNAs together with protein-coding plasmids. Protein expression plasmids can be added together with the tRNA to **Tube1** prior to incubation. However, high intracellular level of uncharged tRNAs can be harmfull for mammalian cells. The amount of transfected tRNAs hence has to be carefully titrated.*

Add 5 µl Lipofectamin 2000 Transfection Reagent (ThermoFisher, #11668027) to 50 µl opti-MEM **(Tube2)**.

Note: Please consult the ThermoFisher homepage for detailed information on the handling of Lipofectamin 2000 Transfection Reagent. Lipofectamin should be handled under sterile conditions and stored at 4°C. Vortexing of Lipofectamin should be avoided.

Step 19.

Vortex **Tube1** and **Tube2** for 10 s, spin briefly and incubate 5 min at 22°C separately (without shaking).

Transfection of in vitro transcribed tRNAs

Step 20.

Add the content of **Tube2** to **Tube1**, vortex for 10 s, spin briefly and incubate 30 min at 22°C (without shaking).

*Note: The content of **Tube2** has to be added to **Tube1**, not vice versa.*

Transfection of in vitro transcribed tRNAs

Step 21.

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

Transfection of in vitro transcribed tRNAs

Step 22.

Once incubation is completed, add tRNA-Lipofectamin mixture dropwise to cells and incubate for 4 h

at 37°C in a humidified atmosphere with 5% CO_2.

Note: The optimal incubation time might vary between different cell types. Cells with a longer doubling time might require a longer incubation time to allow tRNA uptake.

Transfection of in vitro transcribed tRNAs

Step 23.

Replace medium with fresh medium and incubate for further 20 h at 37°C in a humidified atmosphere with 5% CO_2. Cells can then be further manipulated as necessary.

Note: tRNA transfection efficiency should 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.