Microarray-Based Quantification of Cellular tRNAs

Sebastian Kirchner, Robert Rauscher, Andreas Czech, Zoya Ignatova

Abstract

This protocol describes the microarray-based quantification of human tRNAs. The tRNA microarray consists of 40 full-length tDNA probes recognising all 54 nuclearly encoded human isoacceptor tRNAs[1]. The array has been shown to reliably distinguish tRNA isoacceptors with a sequence difference of more than 8 nucleotides. Deacetylated tRNAs are selectively labeled with a fluorescent oligonucleotide hairpin which is covalently attached to the single-stranded 3' NCCA overhang, a commen feature of all tRNAs[2]. For comparison, differently labeled tRNAs from two different conditions or cell/tissue types are labeled with the oligonucleotide hairpins with two different colors, mixed and hybridized onto the tRNA microarray. Ratios between the two different fluorescent signals are then used for relative comparison of tRNAs between the two samples. The assay has also be applied to other eukaryotic or prokaryotic organisms. For more information please contact Prof. Zoya Ignatova (University of Hamburg, Hamburg, Germany). However, this protocol only describes the quantification of human tRNAs. Example of the successful usage of the tRNA array can be found in Ref. 3 and 4.

References

1. Dittmar KA, Goodenbour JM, Pan T. Tissue-specific differences in human transfer RNA expression. *PLoS Genetics*. 2006, 2(12):e221. DOI: 10.1371/journal.pgen.0020221

2. Dittmar KA, Mobley EM, Radek AJ, Pan T. Exploring the Regulation of tRNA Distribution on the Genomic Scale. *Journal of Molecular Biology*. 2004, 337:31–47. DOI: 10.1016/j.jmb.2004.01.024

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

4. Czech A, Wende S, Mörl M, Pan T, Ignatova Z. Reversible and Rapid Transfer-RNA Deactivation as a Mechanism of Translational Repression in Stress. *PLoS Genetics*. 2013, 9(8):e1003767. DOI: http://dx.doi.org/10.1371/journal.pgen.1003767

Citation: Sebastian Kirchner, Robert Rauscher, Andreas Czech, Zoya Ignatova Microarray-Based Quantification of Cellular tRNAs. **protocols.io**

dx.doi.org/10.17504/protocols.io.hfcb3iw

Published: 29 Mar 2017

Before start

This assay is based on a custom-made microarray not yet comercially available. Arrays are printed onto (3-glycidyloxypropyl)trimethoxysilane (Sigma) activated glas slides using a contact-spotter

(QArray 2, Moleculare Devices). tRNA probes are arrayed in 384 well plates with a final concentration of 10 µM in 3× SSC/0.01% SDS in a total volume of 25 µl. After spotting, microarrays are incubated overnight at 4°C in a humidified atmosphere and subsequently dried for 30 min at 30°C. Arrays should be stored in a dry atmosphere at room temperature until use. tDNA probes are spotted in blocks of 100, whereby each tDNA probe is present 2x in each block. Probes targeting three tRNA standards are represented 3x in each block. Each microarray itself consists of 12 blocks. Thus, each tDNA probe is in total spotted 24x and each tRNA standard probe is spotted 36x on each array, respectively. Sequences of tDNA probes targeting the human tRNAome can be found in the attached Excel file. For more information on the microarray design or information on microarrays available for other organisms (mouse, *E. coli*, *B. subtilis*, etc.) please contact Prof. Zoya Ignatova (University of Hamburg, Hamburg, Germany) directly.

Protocol

Extraction of tRNAs

Step 1.

tRNAs-containing total RNA can be extracted using various procedures, each depending on the type of cells which will be analyzed and the experimental setup. We routinely extract total RNA from 10^7 mammalian cells or 100 mg tissue samples using TRIzol (ThermoFisher, #15596018). For more information on the TRIzol Reagent please consult the ThermoFisher homepage. Please see the attached file for the standard TRIzol extraction protocol. RNA extraction protocols selecting against small RNAs should be avoided (e.g. LiCl based extraction).

LINK: https://www.thermofisher.com/uk/en/home.html

Measuring RNA concentration and integrity

Step 2.

We routinely determine the total RNA concentration using a NanoDrop 2000 spectrophotometer. In case of a detectable contamination with phenol (resulting in a low 260/230 absorption ratio), total RNA can be further cleaned with RNA Clean & Concentrator-5 columns (Zymo Research, #R1015).

Note: This measurement does not distinguish between DNA and RNA molecules. Contamination of total RNA with genomic DNA (gDNA) following TRIzol extraction can be problematic for down-stream reactions. However, we have not noticed any changes in the performance of our microarrays in the presence of low level gDNA contamination. Genomic DNA can be removed by DNase I treatment.

The usage of high quality RNA as input material is a key prerequisite for a reliable and meaningful tRNA microarray analysis. RNA integrity can be assessed by running the extracted total RNA on a RNA 6000 Nano chip (Agilent) using a 2100 Bioanalyzer (Agilent). For a reliable microarray analysis only RNA with a RNA Integrity Number (RIN) >8 should be used. Alternatively, RNA integrity can be analysed using a denaturing formamide/agarose gel.

In vitro transcription of tRNA standards

Step 3.

In order to reliably quantify tRNAs, microarray samples are spiked-in with *in vitro* transcribed tRNA serving as internal standards. Each microarray is spotted with probes complementary to the tRNA standards to allow for their quantification. Sequences of the tRNA standards used in our study are (5'→3'):

S. cerevisiae $tRNA^{Phe3}$:
GCGGATTTAGCTCAGTTGGGAGAGCGCCAGACTGAAGATCTGGAGGTCCTGTGTTCGATCCACAGAATTCGCA

E. coli $tRNA^{Lys2}$:
GGGTCGTTAGCTCAGTTGGTAGAGCAGTTGACTTTTAATCAATTGGTCGCAGGTTCGAATCCTGCACGACCCA

E. coli $tRNA^{Tyr2}$:
GGTGGGGTTCCCGAGCGGCCAAAGGGAGCAGACTGTAAATCTGCCGTCATCGACTTCGAAGGTTCGAATCCTT
CCCCCA

For *in vitro* transcription of tRNAs please refere to our protocol 'Upregulating tRNA in Mammalian Cells through Transfection of In Vitro Transcribd tRNAs'. tRNA standards should be refolded and stored at -80°C until further use.

PROTOCOL

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

CONTACT: Sebastian Kirchner

Generation of tDNA Template

Step 3.1.

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 3.2.

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

Generation of tDNA Template

Step 3.3.

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 3.4.

Generation of tDNA Template

Step 3.5.

Incubate mixture for 40 min at 37°C.

DURATION
00:40:00

Generation of tDNA Template

Step 3.6.

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 3.7.

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

Generation of tDNA Template

Step 3.8.

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

DURATION
00:04:00

Generation of tDNA Template

Step 3.9.

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

In vitro tRNA transcription

Step 3.10.

In vitro tRNA transcription

Step 3.11.

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 3.12.

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

DURATION
07:00:00

In vitro tRNA transcription

Step 3.13.

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 3.14.

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 3.15.

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 3.16.

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 3.17.

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 3.18.

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 3.19.

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 3.20.

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 3.21.

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 3.22.

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 3.23.

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.

Step 4.

In order to achieve the accurate quantification of tRNAs, standards should be added already at this step prior to deacetylation of the isolated tRNAs. For each microgram of total RNA, 0.8 pmol of each tRNA standard are added. Routinely, we start the deacetylation reaction with 1-2 µg total RNA.

For each 20 µl deacetylation reaction combine the following: 1-5 µg total RNA, 0.8 pmol of each tRNA standard/µg total RNA, and 2 µl Tris/HCl (1 M, pH 9.0). Adjust reaction volume with RNase-free H_2O to obtain a total volume of 20 µl.

Deacetylation of tRNA ends

Step 5.

DURATION

00:45:00

Deacetylation of tRNA ends

Step 6.

For precipitation, add one volume neutralisation buffer (100 mM NaOAc, 100 mM NaCl, pH 4.8) and add 2.7 volumes 100 % ethanol. Incubate for 30 min at -80°C.

DURATION

00:30:00

Deacetylation of tRNA ends

Step 7.

Pellet RNA by centrifugation for 30 min at 21,000 x g (4°C) and resuspend RNA in 10 µl RNase-free H_2O. Store deacetylated RNA at -80°C or proceed directly to tRNA labeling.

Fluorescent labeling of tRNA ends

Step 8.

For comparative quantification, tRNAs from two different cell lines/conditions are selectively labeled either with Cy3 or Atto647 labeled oligonucleotide hairpins. The sequence of the fluorescently labeled 25-mer oligonucleotides is (5'→3'):

pCGCACUGCdTdTXdTdTdGdCdAdGdTdGdCdGdTdGdGdN

X denotes a dT nucleotide labeled either with Cy3 or Atto647. Oligonucleotide hairpins are ordered lyophilised from Mycrosynth and are reconstituted in TE buffer (pH 8.0) at a concentration of 90 µM. Oligonucleotides are stored at -80°C protected from light.

Step 9.

For labeling, prepare the following 2x labeling mix: 2 µl 10x T4 ligation buffer (NEB, #M0202), 3 µl DMSO (Sigma, #D8418), 1 µl Cy3/Atto647 labeled 25-mer oligonucleotide (90 µM, Microsynth), 1 µl T4 DNA ligase (NEB, #M0202) and 3 µl H_2O.

Note: In previous attempts we used Cy5 instead of Atto647. However, in our hands Cy5 appears to be highly ozone sensitive and hence not suitable to be used for tRNA microarrays.

Fluorescent labeling of tRNA ends

Step 10.

Combine 10 µl of deacetylated tRNAs with 10 µl 2x labeling mix and incubate for at least 16h at 16°C in the dark.

Fluorescent labeling of tRNA ends

Step 11.

The following steps should be performed in the dark to avoid any photobleaching of the fluorescent dyes.

For recovery of labeled tRNAs, perform phenol-chloroform purification. Therefore, add 80 µl Crush & Soak buffer to each ligation mixture, vortex and add 100 µl phenol/chloroform/IAA (Carl Roth, #156.3).

Crush & Soak buffer:

50 mM KOAc

200 mM KCl

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

Fluorescent labeling of tRNA ends

Step 12.

Centrifuge for 15 min at 21,000 x g (4°C). Recover upper aquaeous phase and add 2.7 volumes 100% ethanol. Incubate for 30 min at -80°C.

Fluorescent labeling of tRNA ends

Step 13.

Pellet RNA by centrifugation for 30 min at 21,000 x g (4°C) and resuspend RNA in 20 µl RNase-free H_2O. Store labeled RNA at -80°C or proceed directly to tRNA labeling.

Note: tRNA labeling efficieny can be analysed at this point. Therefore, analyse 2 µl of fluorescently labeled tRNAs on a denaturing 10% TBE-PAGE. Labeled tRNAs should display a size of approx 100 nts, which should be clearly distinguishable from unlabeled tRNA migrating with a size of 70-80 nts. Labeling efficiency can be quantified by comparing the ratio between the band intensity of labeled and unlabeled tRNAs.

tRNA hybridization

Step 14.

Hybridization of fluorescently labeled tRNA samples onto tRNA microarrays is performed using a Hyb4 Microarray Hybridization System (Digilab). Please refer to the manufacture's homepage for more information (see link).

1. Before starting, clean the plastic parts forming the hybridization chamber with RNase *AWAY* Decontamination Reagent (ThermoFisher, #10328011) and subsequently rinse extensively with RNase-free H_2O. Dry the cleaned chamber parts, assemble the hybridization chambers using dummy slides and place them in the Hyb4 Microarray Hybridization Machine.
2. Wash the machine using the pre-programmed washing protocol.
3. Once the cleaning procedure is completed, place the required number of microarray slides upright into a beaker filled with boiling RNase-free H_2O and boil for 2-5 min.
4. Dry the microarray slides, remove dummy slides from the hybridization chambers and insert the microarray slides. *Note: the Hyb4 Microarray Hybridization System allows the simultaneous hybridization of 4 independent microarrays.*
5. The system is now ready to perform the hybridization reaction and subsequent wash steps.

LINK:
http://https://www.digilabglobal.com/

tRNA hybridization

Step 15.

Prior to the hybridization reaction, mix Cy3 and Atto647 labeled tRNAs as follows: Combine 1-2 µg RNA containing Cy3 labeled tRNAs, 1-2 µg RNA containing Atto647 labeled tRNAs, 1.5 µl poly (A) (20 mg/ml, GE Healtcare, #27-4110-01) and 3 µl UltraPure Salmon Sperm DNA Solution (10 mg/ml; ThermoFisher, #15632011). Add PerfectHyb Plus solution (Sigma, #H7033) to a final volume of 180 µl. Keep away from light.

Note: For exact quantification equal amounts of labeled tRNAs must be combined.

tRNA hybridization

Step 16.

Perform the hybridization reaction as follows (cover Hyb4 machine with foil in order to prevent photobleaching):

1.	O-Ring conditioning	75 °C	2 min
2.	Introduce sample	60 °C	hold
3.	Denature sample	90 °C	5 min
4.	Hybridization (agitation on)	60 °C	16 h
5.	Wash I	50 °C	flow 10 s, hold 20 s
6.	Wash II	42 °C	flow 10 s, hold 20 s
7.	Wash III	42 °C	flow 10 s, hold 20 s

Wash buffer 1: 2x SSC, 0.1 % SDS
Wash buffer 2: 0.1x SSC, 0.1 % SDS
Wash buffer 3: 0.1x SSC

20x SSC buffer:

3 M NaCl

300 mM Tri-sodium citrate

Adjust pH to 7.0, filter sterilize and store at 4°C. (Prepare under RNase-free conditions with RNase-free reagents.)

tRNA hybridization

Step 17.

Note: Perform the following steps protected from light to avoid photobleaching.

After hybridization reaction is finished, remove the microarrays carefully from the hybridization chambers. Place array slides in a 50 ml Falcone tube and wash 2x with wash buffer 3 under gentle agitation for 5 min (room temperature).

tRNA hybridization

Step 18.

Place microarrays in a fresh 50 ml Falcon tube and dry by centrifugation at 200 x g for 15 min (room temperature).

tRNA hybridization

Step 19.

Place the dried microarrays in a slide box (wrapped in foil to avoid photobleaching) and store at 4°C until readout.

Microarray readout and analysis

Step 20.

Cy3 and Atto647 fluorescence intensities of individual spots are quantified using a GenPix 4200A microarray scanner (Molecular Devices). Cy3 fluorescence is recorded at 532 nm excitation and Atto647 at 635 nm excitation using the GenePix Pro 7 software (Molecular Devices). The 'Wavelength Channel Color' option should be set to 'Rainbow 2' to allow optimal spot detection. For analysis, background subtracted mean Cy3 and Atto647 fluorescence values are used. Mean spot intensities of all 12 blocks are recorded and average values for each tRNA probe across all blocks and spots are determined. tRNA level are then calculated as Atto647/Cy3 ratios. Exemplary results can be seen in Kirchner *et al*., *PLoS Biology*, 2017.