nrStar™ Mouse tRF PCR Array

Cat#: AS-NR-002M-1

Instruction Manual Version 1.0

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I. Introduction

A. Overview

Transfer RNA (tRNA) is an adaptor molecule that decodes mRNA and translates protein. Recent studies have demonstrated that tRNAs also serve as a major source of small non-coding RNAs having distinct and varied functions [1]. These tRNA-derived ncRNAs are not random degradation products, but rather generated through precise biogenesis processes (**Figure. 1**). tRNA-derived ncRNAs can be broadly classified into two main groups: tiRNAs (or tRNA halves) and tRFs (tRNA-derived fragments), with characteristic sizes, nucleotide compositions, functions and biogenesis [1-3].

tRNA halves (tiRNAs) are produced by specific cleavage in the anticodon loop of mature tRNA by angiogenin under various stress conditions to produce 29 - 50 nucleotide 5'-tRNA and 3'-tRNA halves.

tRFs are 16 - 28 nt fragments derived from tRNA or pre-tRNA, which are classified by their sites of origin: (i) tRF-5 derived from the 5' parts of mature tRNAs by cleavage in the D loop. (ii) tRF-3 derived from the 3' part of mature tRNAs by cleavage at the T loop. The 3'end contains a 3'-CCA terminus. (iii) tRF-1 originated from the 3'trailer from the pre-tRNA. The 3'end has poly-U residues at the end. (iv) i-tRF, which does not belong to tRF-5, tRF-3 or tRF-1, is typically derived from the internal region of the mature tRNA.

The nrStar™ Mouse tRF PCR Array profiles 88 tRFs, which are selected from tRFdb database. These tRFs represent the most prevalently detected ones, and thus imply critical bio-functional significances.

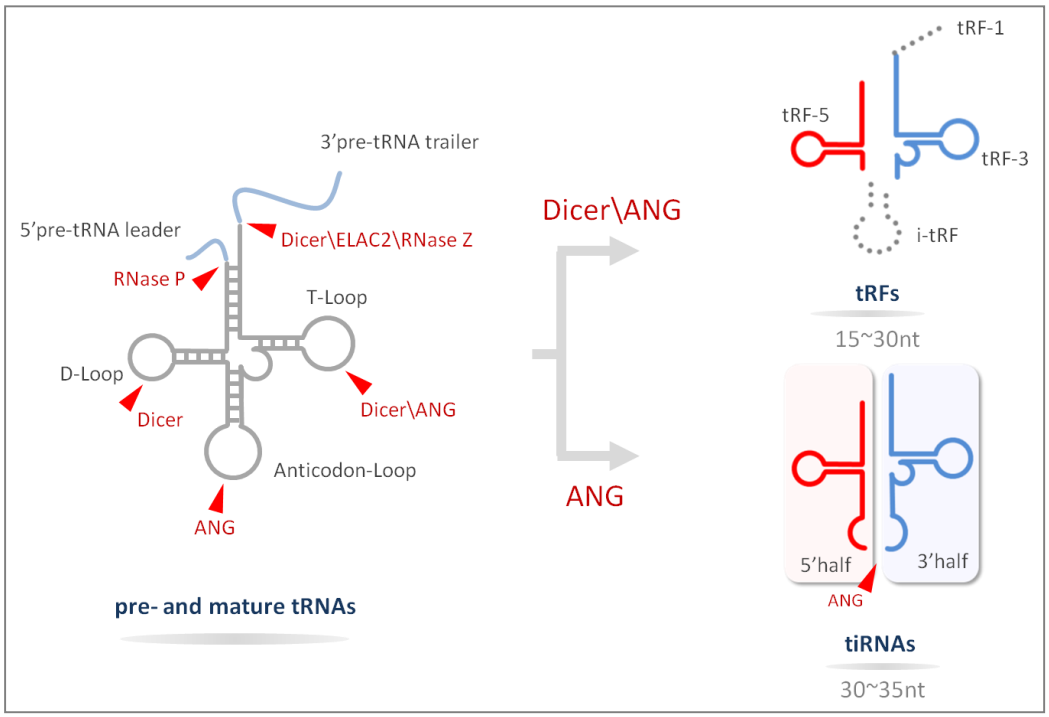


Figure 1. Biogenesis of tRFs&tiRNAs. tRF-1 is generated from the 3'-trailer of primary tRNA. tRF-5, i-tRF and tRF-3 are produced from the 5'-, internal- and 3'-portions of the mature tRNA, respectively. When the cleavage site is within the anticodon loop, two fragments are generated as tiRNAs for the 5'- and 3'- tRNA halves.

B. Biological Functions

tRFs and tiRNAs perform many biological functions as small noncoding RNAs (**Figure.7**). They are known to act as microRNAs in RNA interference (**Figure. 2**); directly inhibit protein synthesis by displacing eIF4G translation initiation factor eIF4G from mRNA on ribosomes [4,5]; bind protein factors such as YBX1 to regulate target mRNA stability (**Figure. 3**); interact with cytochrome c to modulate apoptosis [6]; assemble stress granules in response to stress conditions (**Figure. 4**); sensitize cells to oxidative-stress-induced p53 activation and p53-dependent cell death [7]; alter transcriptional cascades in intergenerational inheritance as paternal epigenetic factors [8,9] (**Figure. 6**).

Figure 2. tRFs have many functional characteristics of a microRNA, such as Dicer-dependent biogenesis, RISC complex formation with Argonaute proteins, and RNA silencing. Some of the catalogued miRNAs directly map to tRFs [10].

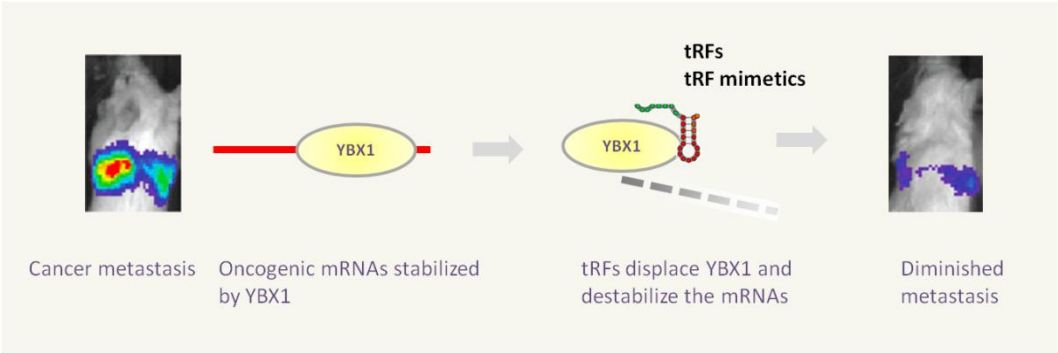


Figure 3. tRFs or the mimetics displace oncogenic RNA binding protein YBX1 and destabilize many cancer driver mRNAs. Consequently, the cancer metastasis is greatly diminished [11].

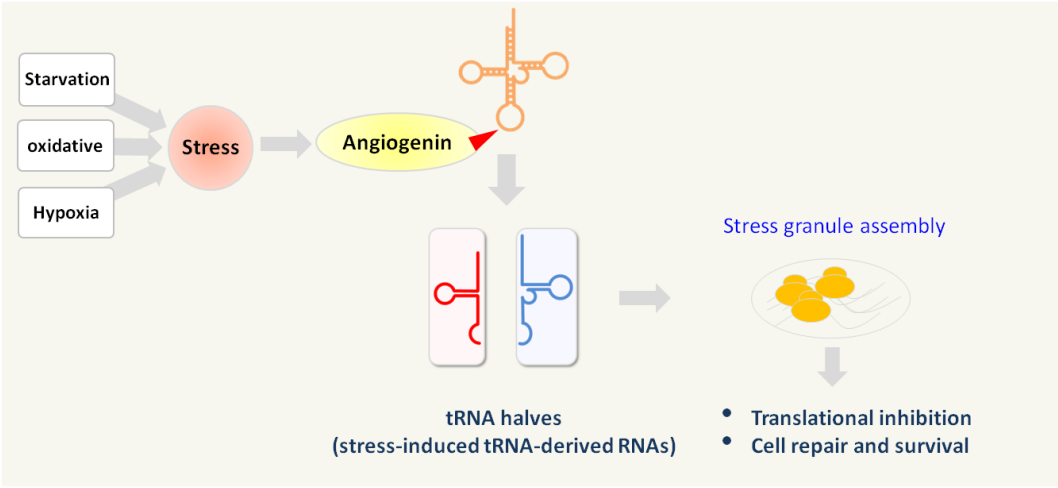


Figure 4. tRNA halves (tiRNAs) are generated by angiogenin cleavage in response to cellular stresses, which promote stress granule assembly and induce translational inhibition,

C. Disease Associations

tRFs&tiRNAs are associated with or are causal factors for disease conditions including cancers, neurodegeneration, and metabolic disorders (**Figure. 5**).

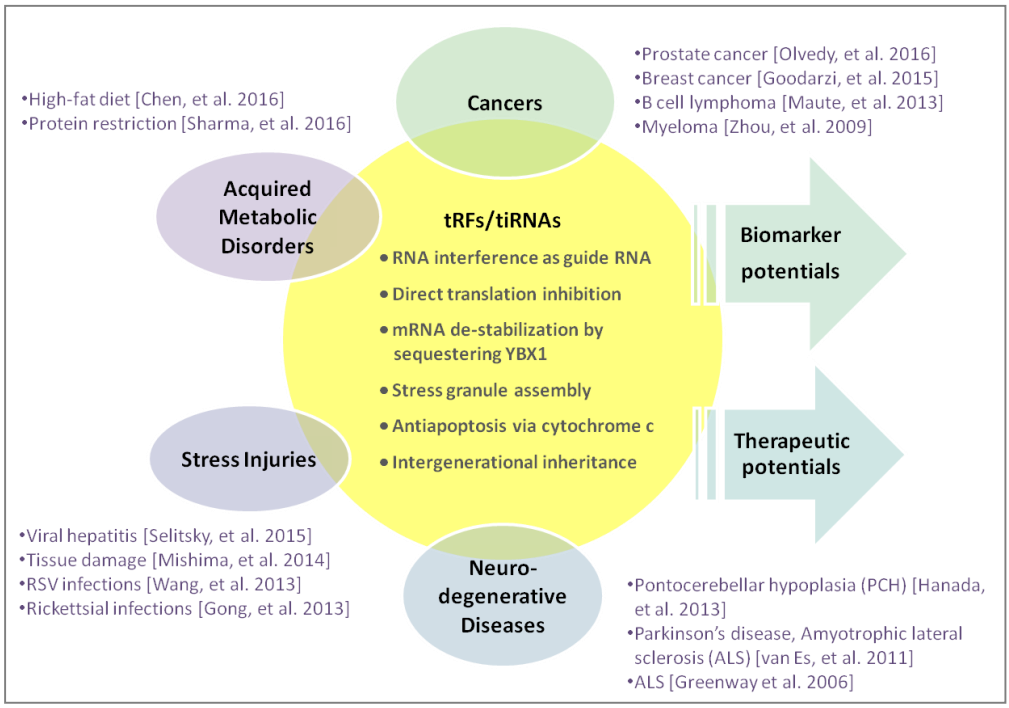


Figure 5. tRF&tiRNA molecular functions and diseases.

Cancers

Differential expression of tRFs&tiRNAs is detected in different cancer cell lines, including the prostate cancer cell lines LNCaP and C4-2. tRFs&tiRNAs levels are elevated by cellular stress conditions and particularly under oxidative stress such as hypoxia [13]. Moreover, a 3'-derived tRF identified in B-cell lymphoma cells possesses the functional characteristics of a guide RNA that suppresses proliferation and modulates response to DNA damage in a miRNA-fashion [14]. By competing YBX1 binding sites and destabilization of oncogenic transcripts, tRFs&tiRNAs act as tumor suppressors [11] (**Figure. 3**). tRF-1001, a 3'tRF derived from pre-tRNA-Ser, is highly expressed in different cancer cell lines, and is required for proliferation of prostate cancer cells [15]. Additionally, ANG produced tiRNAs promote the assembly of stress granules that help cells to survive under adverse conditions. It is possible that ANG-induced tiRNAs directly contribute to ANG-mediated angiogenesis and cancer cell proliferation. Similarly, tiRNAs can help cancer cells to prevent apoptosis by binding to Cyt c [6]. Taken together, these findings strongly suggest a functional role of tRFs&tiRNAs in tumorigenesis.

Acquired metabolic disorder

Increasing evidence indicates that offspring metabolic disorders can result from the father's diet. In a paternal high-fat diet (HFD) mouse model, a subset of sperm tRFs&tiRNAs, mainly from 5'-halves and ranging in size from 30 to 34 nucleotides, exhibit changes in expression profiles and RNA modifications under high fat diet condition. Injection of sperm tRFs&tiRNAs fractions from HFD male into normal zygotes generated metabolic disorders in the F1 offspring and altered gene expression of metabolic pathways in early embryos and islets of F1 offspring, which was unrelated to DNA methylation at CpG-enriched regions. Hence, sperm tRFs&tiRNAs represent a type of paternal epigenetic factor that may mediate intergenerational inheritance of diet-induced metabolic disorder [8] (**Figure. 6**).

Protein restriction in mice also affects small RNA levels in mature sperm, with decreased let-7 levels and increased levels of 5'- tRFs&tiRNAs of glycine tRNAs. tRFs&tiRNAs have been linked to the regulation of expression of transcripts driven by endogenous retroelements active in the preimplantation embryo [9].

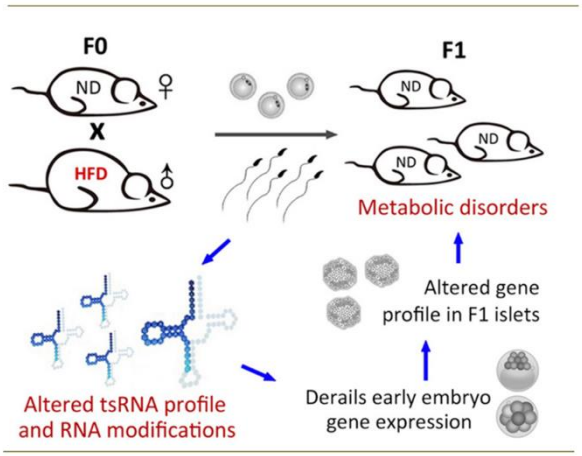


Figure 6. tRNA-derived small RNAs (tsRNAs, mostly tRNA halves) have altered expression profiles and RNA modifications in the sperms of high fat diet fed mice. The sperm tsRNAs confer the metabolic disorder phenotypes in the offspring by oocyte injection. This tsRNA paternal epigenetic factor mediates intergenerational inheritance by modulating embryonic gene transcription that cascades into the adulthood [8].

Neurological disorders

A number of neurological disorders are caused by defects in tRNA metabolism and tRNA processing enzymes (e.g. ANG). Angiogenin mutants with reduced RNase activity are implicated in the pathogenesis of Amyotrophic Lateral Sclerosis (ALS), a fatal neurodegenerative disease [16]. A subset of ALS-associated ANG mutants was also found in Parkinson's Disease (PD) patients [17]. Further links are strengthened between ANG-induced tRNA halves, cellular stress and neurodevelopmental disorders [18]. Loss of RNA kinase CLP1 activity results in accumulation of a

novel set of small RNA fragments, derived from aberrant processing of tyrosine pre-tRNA. These tRFs&tiRNAs sensitize cells to oxidative stress induced p53 activation and p53-dependent neuronal cell death, leading to motor neuron loss, muscle denervation and respiratory failure [7]. Another example is the mutations in the cytosine-5 RNA methyltransferase NSun2 that cause microcephaly and other neurological abnormalities. Loss of cytosine-5 RNA methylation of tRNA in the absence of NSun2 increases the ANG binding and cleavage of tRNA leading to an accumulation of 5'-halves, which reduces protein translation rates and activates stress pathways leading to reduced cell size and increased apoptosis of cortical, hippocampal and striatal neurons [19].

Pathological stress injuries

Stress imparted by hypoxia, nutrient deprivation, oxidative conditions and metabolic imbalance can damage cells and promote disease. These stresses stimulate production of tRNA halves. In animal models of tissue damage (e.g., toxic injury, irradiation and ischemic reperfusion), the production of tRNA halves is correlated with the degree of tissue damage, for example, a change in tRNA conformation that promotes ANG-mediated production of tRNA halves [1]. Increased expression of 5'-halves has been associated with viral and rickettsial infections, and may serve to prevent apoptosis and promote cell survival. tRFs&tiRNAs, specifically 30-35 nucleotide long 5'-halves, are abundant in non-malignant liver and significantly increased with chronic viral hepatitis [18].

D. Biomarker Potentials

The composition and abundance of tRFs&tiRNAs are highly dependent on the cell type and disease condition [20]. In particular, tRNA and tRF&tiRNA populations are highly enriched in biofluids, sometimes more so than microRNAs [13,21,22] (**Figure. 7A**). Although miRNAs have been current main focus as candidate biofluid-based biomarkers, the high stability and abundance of tRFs&tiRNAs in body fluids, the involvement in pathological processes, the demonstrated differential expression in solid tumors and hematological malignancies, and their power to discriminate cancer patients from healthy controls open the prospect for development of minimally invasive, tRFs&tiRNAs-based biomarker tests. For example, the tRF profiles have been shown to discriminate triple-negative, triple positive breast cancer cells from the normal controls in PLS-DA analyses [20] (**Figure. 7B,C**). The ratio of tRFs&tiRNAs has also been demonstrated as a good indicator of cancer progression-free survival and a candidate prognostic marker [13].

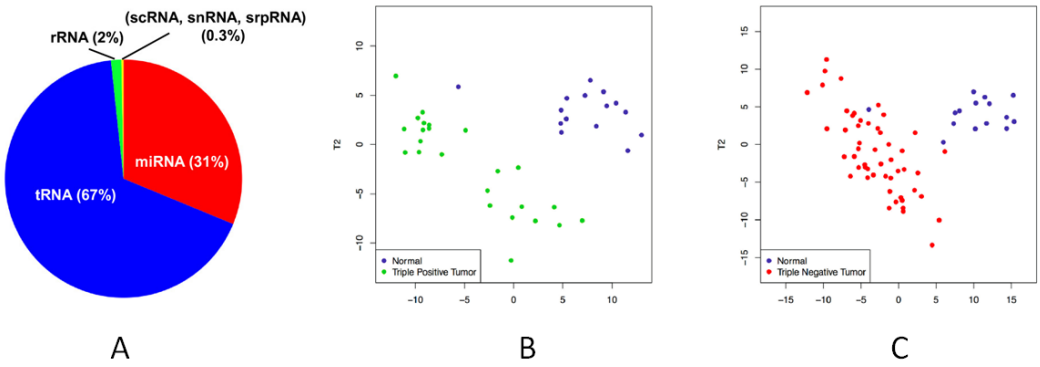


Figure 7. The abundant presence of tRNA derived fragments in serum [21] (A). tRF profiles discriminate triple-negative (B), triple positive breast cancer cells (C) from the normal controls in PLS-DA analyses.

II. Product Summary

A. Kit Contents

The nrStar™ Mouse tRF PCR Array profiles 88 tRFs selected from tRFdb database. To ensure high data quality, the panel includes 7 control sets for tRFs to better quantify and normalize the qPCR data. cDNA synthesis and PCR efficiency are evaluated by the RNA Spike-in control (SP) and Positive PCR control (PPC). Potential genomic DNA contamination is monitored by using the genomic DNA control (GDC). Three stably expressed small RNA genes are included in the array as the quantification references for tRFs.

B. Description of the Control Assays

There are four types of control assays built in the nrStar™ Mouse tRF PCR Array. Their uses and meanings are explained below.

- **SP** (Spike-in control): An RNA spike-in control is added in the RNA sample during the first-strand cDNA synthesis. The SP control assay indicates the overall success and the efficiency of the reactions beginning from the adaptor ligation, cDNA synthesis to the final qPCR. Any problem(s) in these steps will result in a failed or compromised SP outcome.

- **CT** (small RNA Reference Control): Three stably expressed small RNA genes U6 (**CT1**), miR-93-5p (**CT2**), and 5S rRNA (**CT3**) are included in the array as the quantification references for tRFs.

- **PPC** (Positive PCR control): Two replicates of an artificial DNA and the PCR primer pairs to indicate the qPCR amplification efficiency. A Ct value greater than 25 is an indication of low qPCR amplification efficiency. More importantly, the PPC are used as inter-plate calibrator (IPC) between PCR plate runs to give the same Ct value for the calibrator, thereby reducing run-to-run variance. Inter-plate calibration can easily be performed with the data analysis software available on our website *www.arraystar.com*.

- **GDC** (Genomic DNA Control): The control assay consists of PCR primers for an untranscribed genomic region. Non-RT sample or RNA sample are added during the qPCR Process. The Ct values should be greater than 35. A positive GDC signal indicates the array result may have been compromised with genomic DNA contamination.

C. Array Layout

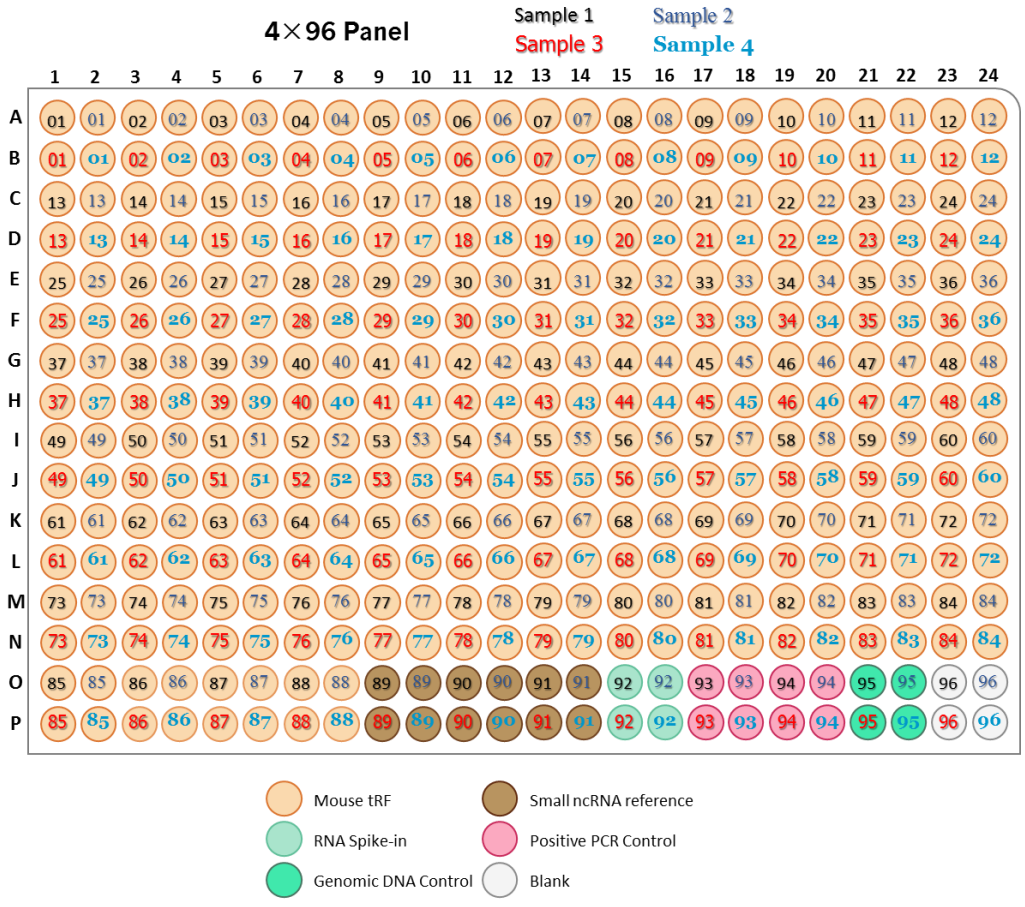


Figure 8. nrStar™ Mouse tRF PCR Array layout.

D. List of tRFs and Controls

1	1003	25	3017a	49	3038a	73	5011a
2	1006	26	3017b	50	3038b	74	5011b/5012b
3	1008	27	3019a	51	3039a	75	5012b
4	1009	28	3019b/3020b	52	3041b/3042b	76	5013a
5	1010	29	3021a	53	3043a	77	5013b
6	1015	30	3023b	54	3043b	78	5013b/5017b/5018b
7	1016	31	3024b/3046b	55	3044a	79	5014a
8	1019	32	3025a	56	3044b	80	5014a/5015a
9	1020	33	3025b	57	3045a	81	5014b
10	1026	34	3026a	58	3046a	82	5016a
11	1035	35	3027a	59	3047b	83	5019a
12	3001b	36	3028/3029b	60	3048a	84	5019b
13	3002a/3035a	37	3028b/3029b/3040b/3045b	61	3050a	85	5020b/5021a
14	3003a	38	3029a	62	3051a	86	5022a
15	3004b	39	3030a	63	3052a	87	5022b
16	3005a	40	3031a	64	5001a/5001b/5010a	88	5023b/5024b
17	3006a	41	3031b	65	5002b/5004b	89	U6
18	3009a	42	3032a	66	5005a/5006b	90	miR-93-5p
19	3009b	43	3032b	67	5005b	91	5S rRNA
20	3010a	44	3033a	68	5006a	92	RNA Spike-in
21	3010b	45	3033b	69	5006c	93	PPC
22	3011a	46	3034a	70	5007a	94	PPC
23	3011b	47	3036a	71	5009a	95	GDC
24	3012b	48	3036b/3037b	72	5009b	96	Blank

Figure 9. List of tRFs and controls

E. Shipping and Storage

nrStar™ PCR Panels are shipped at ambient temperature, on ice, or on dry ice depending on the destination and accompanying products. Store at –20℃ upon receipt. The contents are stable for at least 6 months.

F. Additional Required Equipment & Reagents

- Thermal cycler
- Real time qPCR instrument, compatible with 384-well format
- rtStar™ First-Strand Synthesis Kit (3' and 5' adaptor) (Cat# AS-FS-003)
- Arraystar™ SYBR Green qPCR Master Mix (Cat# AS-MR-006-5)
- Nuclease free PCR-grade water

III. Protocol

IMPORTANT: Please read through this entire protocol before your first experiment. Prepare a workspace and use labwares free of DNA, RNA, and nuclease contamination. Wear gloves while performing this protocol.

A. Protocol Overview

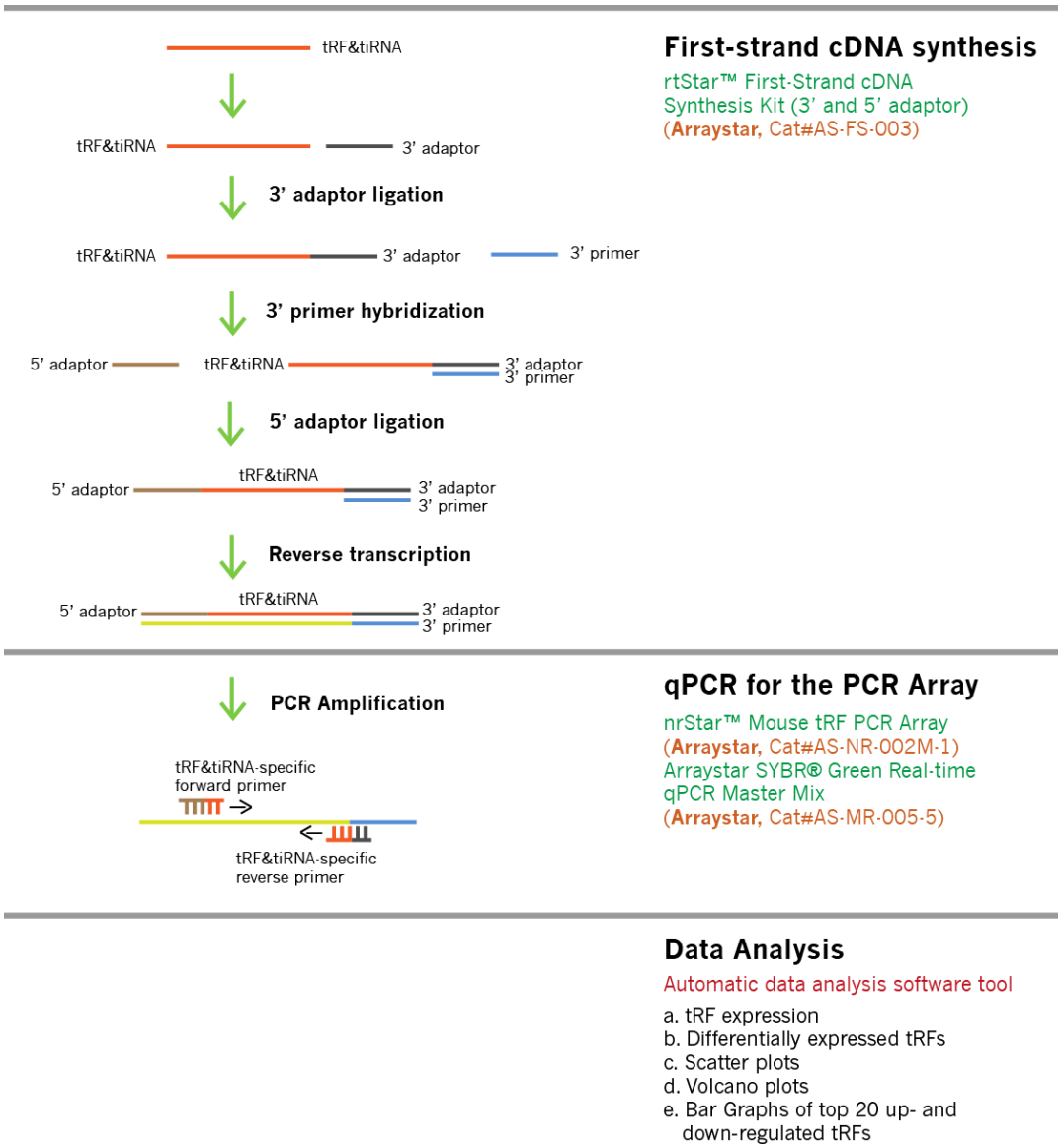


Figure 10. qPCR array workflow of tRFs

B. RNA Sample Preparation and Quality Control

FOR BEST RESULTS FROM THE PCR ARRAY, RNA samples must meet the QC standards of integrity and purity from protein, organics, genomic DNA contamination and excessive RNA degradation. You can measure the RNA concentration and purity by UV spectrophotometry. You can also check 18S and 28S ribosomal RNA as an indicator of RNA integrity by denaturing gel electrophoresis or by using an Agilent BioAnalyzer.

- A260:A230 ratio greater than 1.7

- A260:A280 ratio between 1.8 and 2.0
- Total RNA concentration greater than 40 ng/μL

Eliminating genomic DNA contamination is essential for accurate tRFs expression profiling by qPCR, which is particularly important for tRFs at low expression levels. Due to the presence of pseudogenes, even cross-intron primers are not a guarantee of avoiding amplification from contaminating genomic DNA. The Genomic DNA Control (GDC) in the PCR Array specifically detects potential genomic DNA contamination. A Ct for GDC less than 35 indicates the presence of genomic DNA contamination that may compromise the qPCR results.

C. First-strand cDNA Synthesis

Total RNA samples should be extracted by a method that can recover tRF fraction (13-30 nt), for example, TRIzol Reagent method.

High quality cDNA synthesis is vital for the following qPCR performance. rtStar™ First-Strand Synthesis Kit (3' and 5' adaptor) (Cat# AS-FS-003), is specifically optimized and essential for tRF & tiRNA PCR Array. Please refer to the Instruction Manual of the Kit for its use.

■ Ligate the 3´ Adaptor

Note: For smaller amounts of total RNA at 100 ng, reduce the use of 3´Adaptor by diluting 1:2 in Nuclease-free Water.

Note: Add 40 μL and 30 μL Nuclease-free Water to RNA Spike-in tube and Reverse Transcription Primer tube respectively. Mix by vortexing and spin down. Leave on ice for 20 ~ 30 min to fully dissolve them. Vortex again, then spin down.

1. Combine the reagents in a RNase-free 200 μL PCR tube according to the order in the table:

Nuclease-free Water	variable
Input RNA	1.0-7.3 μL
3' Adaptor	1.0 μL
RNA Spike-in	0.5 μL
Total volume	8.8 μL

2. Incubate the mix at 70°C for 2 min. Transfer the tube to ice.
3. Ligate 3' Adaptor to the RNA by adding the Components:

3' Ligation Reaction Buffer (2X)	7.2 μL
3' Ligation Enzyme Mix	1.0 μL

RNase Inhibitor	1.0 µL
Total volume	9.2 µL

4. Incubate at 25°C for 1 hour.

   Note: To improve the ligation efficiency for end-methylated RNA such as piRNA, longer incubation at lower temperature (e.g.16°C for 18 hrs) may be used. However, some concatemerization products may occur.

Hybridize the Reverse Transcription Primer

The excess of the unligated 3' Adaptor is hybridized with Reverse Transcription Primer to prevent adaptor-dimer formation. The double stranded DNA duplexes are not substrates for T4 RNA Ligase 1 and will not ligate to 5' Adaptor in the 5' Adaptor Ligation step.

Note: For smaller amounts of total RNA at 100 ng, reduce the use of Reverse Transcription Primer by diluting 1:2 in Nuclease-free Water.

5. Add the following reagents to the tube from step 4 and mix well:

Nuclease-Free Water	1.0 µL
Reverse Transcription Primer	1.0 µL
Total volume now should be	20 µL

6. Hybridize the primers at 75°C for 5 min, 37°C for 15 min and 25°C for 15 min.

Ligate the 5´ Adaptor

Note: For smaller amounts of total RNA at 100 ng, reduce the use of 5' Adaptor by diluting 1:2 in Nuclease-free water. Store unused 5' adaptor at -80°C.

7. Aliquot 1.1 × number of samples (µL) of 5' Adaptor in a nuclease-free 200 µL PCR tube.
8. Denature the adaptor at 70°C for 2 min and then immediately chill on ice. Use the denatured 5' Adaptor within 30 minutes.
9. Add the following components to the Reverse Transcription Primer hybridized 3' Adaptor ligation product from Step 6 and mix well:

5´ Adaptor (denatured)	1.0 µL
10 mM ATP	2.5 µL
5´ Ligation Reaction Buffer	0.5 µL
5´ Ligation Enzyme Mix	1.0 µL

Total volume	25 µL

10. Incubate at 25°C for 1 hr.

■ Perform Reverse Transcription

11. Mix the components in a RNase-free 200 µL PCR tube:

Adaptor Ligated RNA	25 µL
First-Strand Synthesis Reaction Buffer	8.0 µL
0.1 M DTT	3.0 µL
2.5 mM dNTP Mix	2.0 µL
RNase Inhibitor	1.0 µL
Reverse Transcriptase	1.0 µL
Total volume	40 µL

12. Incubate at 45°C for 60 min and chill on ice. The cDNA may be used immediately for qPCR. If not used immediately, heat inactivate the enzyme at 70°C for 15 min and store at -20°C.
13. OPTIONAL. To check the synthesized cDNA quality, reconstitute the RNA Spike-in qPCR Primer Mix in 200 µl nuclease-free water. Use 2 µl RNA Spike-in qPCR Primer Mix with 2 µl cDNA, 5 µl SYBR Green Master Mix, and 1 µl Nuclease-free Water. Run the PCR program described in "Running Real-Time PCR Detection" below. A *Ct* value < 30 for the RNA spike-in indicates a successful tRF cDNA synthesis.

D. Perform qPCR for the PCR array

NOTE: The fellow operations are designed for one sample. If repetitive experiments are planned, the volume of the reagent should be accordingly increased. To make it easier to understand, we take Sample1 in Figure 8 for example.

1. Dilute the cDNA in Nuclease-free Water. If 1.5 µg input RNA is used with rtStar™ First-Strand cDNA Synthesis Kit (3' and 5' adaptor) (Cat#AS-FS-003), the dilution factor is 1:10. Mix well and spin down. The diluted cDNA is used as the qPCR template in the wells for tRF Transcript assays, Internal Control References, and Spike-in External Controls.
2. For GDC Controls, combine 1.0 µl NRT (no RT) sample or 1.0 µl RNA sample, 5.0 µl SYBR Green Master Mix, and 4 µl Nuclease-free Water. Mix well and spin down.
3. For Blank Controls, combine 20 µL SYBR Green Master Mix and 20 µL Nuclease-free Water. Mix well and spin down.

4. Use Arraystar SYBR Green Real-Time Quantitative PCR Master Mix to prepare the qPCR Master Mix. There are total of 96 wells of PCR reaction. Some extra amount is included for consumption by the liquid dispensing operation. Prepare the cocktail according to the following table.

SYBR Green Master Mix	500 μl
diluted cDNA template	400 μl
ddH_2O	100 μl
total volume	1000 μl

5. Loading the PCR Array plate.

   NOTE: In order to better understand the fellow operations, we take Sample1 (in **Figure 8**) for example. If repetitive experiments are planned, it is important to note that the reagents should load to the related well number corroding to **Figure 8** and **9**.

   a. CAREFULLY remove the plate seal from the PCR Array;

   b. Add 10 μl of the cocktail from STEP 4 to each PCR Array plate well (except No.93-No.96; i.e. well O17, well O19, well O21, well O23);

   c. Add10 μl GDC Mixture aliquot from STEP 2 into the No.95 (well O21) to detect genomic DNA contamination.

   d. Add 10 μl Blank Mixture aliquot from STEP 3 into the No.93 (well O17), No.94 (well O19) and No.96 (well O23).

   e. CAREFULLY but tightly seal the PCR Array plate with the optical adhesive cover. Be sure that no bubbles appear in any of the wells. To remove bubbles, tap the plate gently on the bench top or centrifuge the plate briefly.

   f. Keep the plate on ice while setting up the PCR program described in "Running Real-Time PCR Detection" below.

6. Running Real-Time PCR Detection

Cycles	Temperature	Time
1	95 ℃	10 minutes
40	95 ℃	10 seconds
	60 ℃	1 minutes
Melting curve analysis		

E. Data Pre-processing and Data Analysis

Pre-process the qPCR run data according to the qPCR instrument manufacturer's instructions. If using baseline and threshold method for Ct calculations, such as with ABI 7900HT, the optimal baseline and threshold manual settings applied consistently across all assays on the plate are preferred over the software automatic settings for better reliability and accuracy.

Inspect the melting curve analysis of the post-PCR products to verify the amplification specificity. If the melting curve has multiple peaks or poor peak morphology deviated from that of the known single amplicon product, it may indicate non-specific off-target amplification or primer dimer formation, which will compromise the quantification. In such cases, gel electrophoresis or DNA fragment analysis can be performed to verify whether the product is from a single correct amplicon.

Export the raw Ct values to Excel. Free, easy-to-use data analysis software is available online, please refer to *http://www.arraystar.com* for detailed instruction. The data analysis procedures include:

■ Data Pre-processing

1. Set all Ct values ≥ 35 or N/A (not detected) to 35. From this point on, any Ct value equal to 35 is considered a negative result.

2. Examine the Ct values of the Genomic DNA Control (GDC) wells. If the value is 35, no genomic DNA contamination is detected and no action is needed. If the value is less than 35, genomic DNA contamination is evident and the result may be compromised.

3. Before initiating the data analysis, the RNA spike-in wells are compared. Outlier samples (Ct >20) may be identified and considered for exclusion in the further data analysis.

4. Inter-plate calibration (IPC) can be performed using the PPC assay replicates either with the data analysis software or manually. For each plate, verify that the replicates have Ct standard deviation ≤ 0.5. If this is not the case, exclude the outlier if it can be identified. Calculate the average of the replicates for each plate, the overall average (average of IPC values from all plates). The calibration factor for each plate is calculated as the difference between the plate average and the overall average:

$$calibration\ factor = IPC(plate\ n) - IPC(overall)$$

5. The Ct value is corrected with the calibration factor as:

$$Ct_{RNA} = Ct_{RNA}(Raw\ value, plate\ n) - IPC(plate\ n) + IPC(overall)$$

or

$$Ct_{RNA} = Ct_{RNA}(Raw\ value, plate\ n) - calibration\ factor$$

	Plate 1	Plate 2	Plate 3
Trf-1001	20.26	20.83	20.44
IPC Plate average	16.70	16.70	16.40
IPC overall average	16.60	16.60	16.60
Calibration factor	0.10	0.10	-0.20
Trf-1001 (Calibrated)	20.16	20.73	20.64

6. Calculate the ΔCt for each tRFs in the plate.

$$\Delta Ct_{RNA} = Ct_{RNA} - \ average(Ct_{HKs})$$

- Where average (Ct_{HKs}) is the average of the Ct values derived from the 3 control reference. These most stably expressed tRF reference were selected from a broad range of samples by our stringent algorithm that evaluates the optimal properties and the number for endogenous controls.

NOTE: If no particular reference gene(s) are designated as the quantification reference, all the CTs can be averaged and used in the above formula, but only if the difference between the averaged values is less than 1 cycle when comparing the two groups.

■ Data Analysis

7. Calculate the $\Delta\Delta Ct$ between two samples or groups for a gene:

$$\Delta\Delta Ct = \Delta Ct(sample\ 1) - \ \Delta Ct(sample\ 2), \text{ between samples}$$

or

$$\Delta\Delta Ct = \Delta Ct(group\ 1) - \ \Delta Ct(group\ 2), \text{ between groups}$$

Where sample1 or group1 is the control and sample2 or group2 is the experimental.

8. Calculate the fold change from group1 to group2 for a gene as:

$$Fold\ Change = 2^{-\Delta\Delta Ct}$$

NOTE: If the fold-change is greater than 1, the result may be reported as a fold up-regulation. If the fold-change is less than 1, the negative reciprocal may be reported as a fold down-regulation.

9. When comparing profiling differences between two groups (such as disease versus control) with biological replicates, the statistical significance of the difference can be estimated as p-value by *t*-test. RNAs having fold changes ≥ 1.5 and *p*-values ≤ 0.05 are selected as the significantly differentially expressed RNAs.

   NOTE: Fold change is related to biological effect size. Ranking by fold change is preferred over *p*-value. qPCR as commonly used in confirmation has a limit of quantification of 0.5 ΔCt, which is equivalent to approximately 1.5 folds change.

10. Other analyses such as scatter plots, volcano plots, list of differentially expressed genes and bar graph of expression differences for the tRFs are performed and included in the standard analysis package.

IV. Quality Control and Sample Data

A. Quality Control

■ Real-time qPCR Validation

The performance of Mouse tRF PCR Panel was tested using a cohort of 10 mouse tissue samples. The extracted RNA samples were converted to cDNA using rtStar™ First-Strand Synthesis Kit (3' and 5' adaptor) (Cat# AS-FS-003). The cDNAs were profiled using the PCR array according to aforementioned protocol without modification. The results are charted on the real-time amplification plots for the entire plate for mouse tissues.

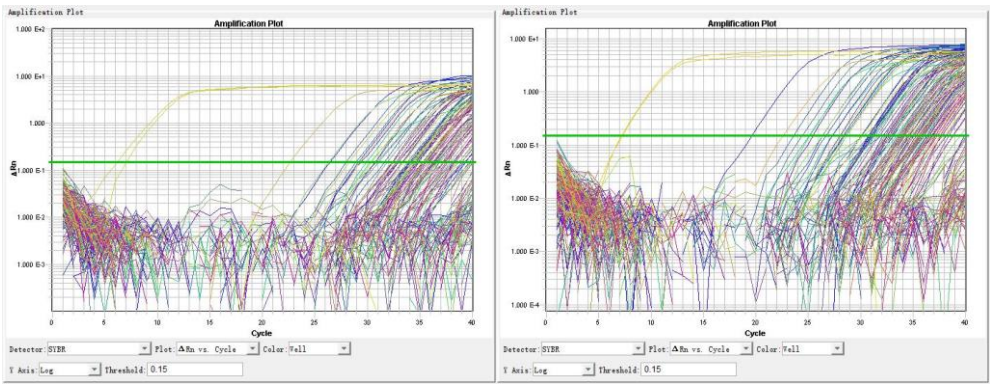


Figure 11. Real-time qPCR Validation of mouse tissue A (left) and B (right)

■ **Sensitivity Test**

The decreasing input amounts of total RNAs from mouse tissues were converted to cDNAs and profiled by the PCR arrays. The Ct values were determined using the software default automatic baseline and Ct settings. tRF-1016 and tRF-3009a were detected at Ct values of 25.2 and 27.0 respectively, with the input RNA amount as low as 50 ng.

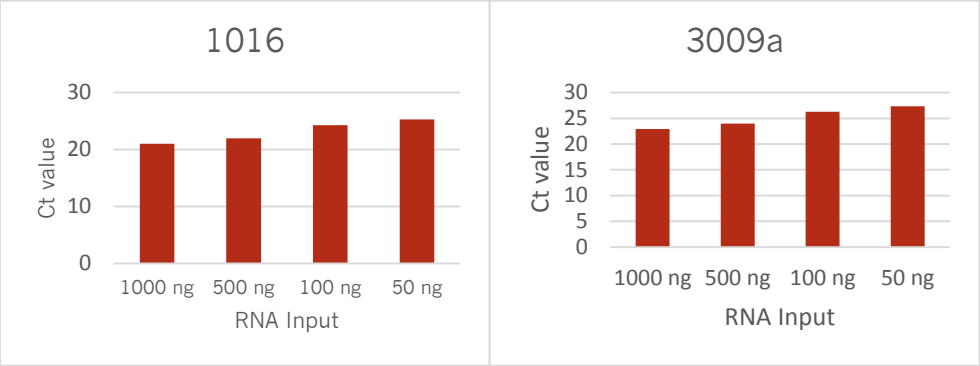
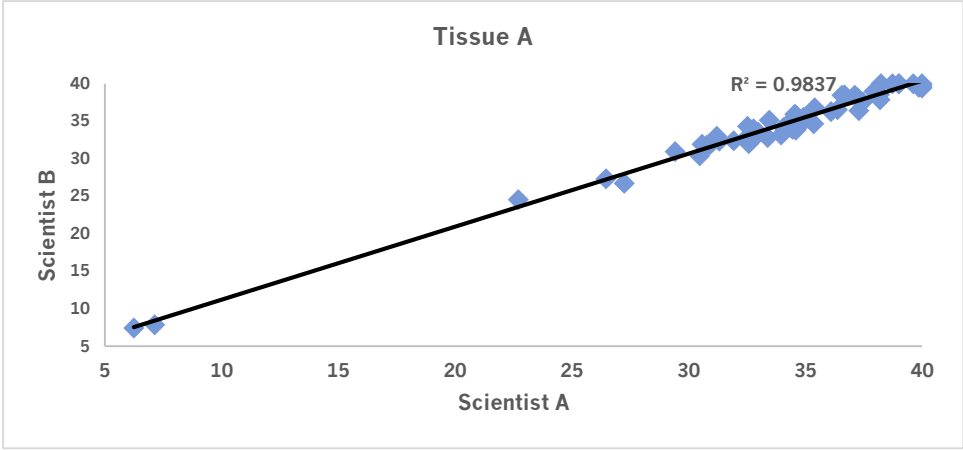


Figure 12. Sensitivity test of two tRFs.

■ **Reproducibility Test**

Two independent runs of Mouse tRF PCR Array were conducted by two different scientists A and B at two different times using two different mouse tissues. The results demonstrate a high degree of reproducibility with correlation R^2>0.98.



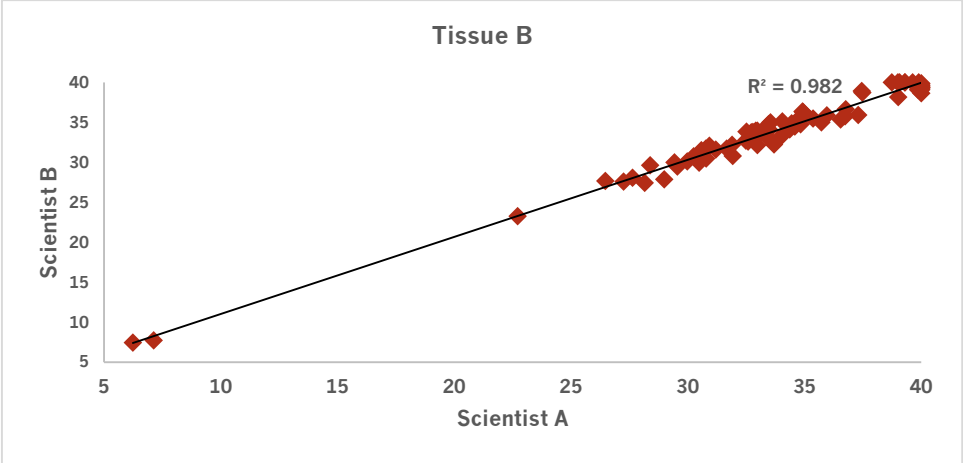


Figure 13. Reproducibility test in tissue A and tissue B

■ Specificity Test

The amplification products of 4 tRF were analyzed by melting curves, all of which had single sharp melting peaks. The results demonstrate the high amplification specificities for the transcript isoforms with the assays on the array.

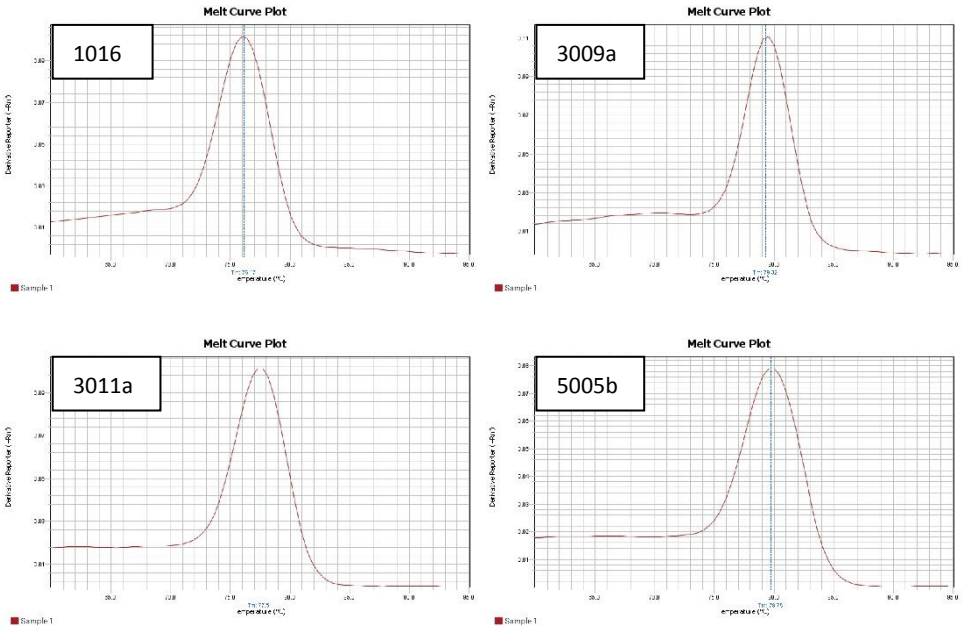


Figure 14. Melt curve plot of 4 tested tRF

B. Sample Data

The sample data were generated from RNAs extracted from two different mouse tissues. The normalization was carried out using the average of the reference genes. ΔCt for the tRF in mouse tissue A vs. mouse tissue B are graphed in the bar chart below.

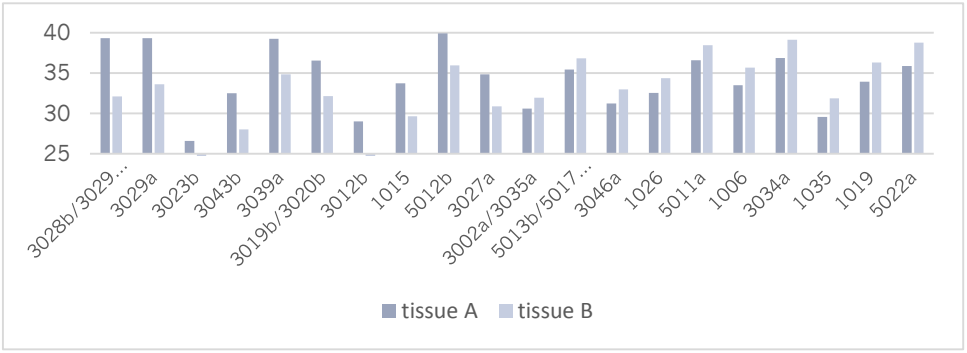


Figure 15. Differently expressed tRFs between mouse tissue A and Mouse tissue B

V. Troubleshooting

Problem	Possible solution
qPCR background too high	Reduce the amount of cDNA used in the SYBR Green Master Mix
No qPCR signals	Inspect if the Internal Controls have valid qPCR signal
	Set SYBR Green as the Detector's Reporter Dye
	Use more cDNA in the Master Mix
	Lower the annealing temperature in Protocol D - 4 from 60°C to 50°C
Baseline and threshold settings	Follow the instructions of the qPCR system manufacturer
	Contact their technical support as necessary

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VII. Technical Support

For additional information, manual download or technical assistance, please visit our website at www.arraystar.com, or contact us at:

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9430 Key West Ave #128

Rockville, MD 20850, USA

Tel: 888-416-6343

Fax: 240-238-9860

Email: support@arraystar.com

VIII. Terms and Conditions

By purchasing and using any part of the nrStar™ Mouse tRF PCR Array, you agree to accept the following terms and conditions.

■ Product Use Limitation

Except as otherwise agreed in writing, all products are sold to end-users for research purposes only, and not for human or animal therapeutic or diagnostic use. We do not submit our products for regulatory review by any government body or other organization for clinical, therapeutic or diagnostic use. You are solely responsible for the way you use the products in compliance with applicable laws, regulations, and governmental policies.

The purchase of Product does not grant any right to use such Product in the practice of any methods covered by Arraystar intellectual property rights. You may not perform compositional, structural, functional or other analysis of our products, or undertake deconvolution or reverse engineering with respect to our products.

■ Product Warranty

Arraystar warrants that the Product will meet the specifications stated on the technical data sheet for that product, and agrees to replace the product free of charge if the product does not conform to the specifications. Notice for non-conformity and request for replacement must be given within 30 days of receipt of Products. In consideration of the above warranty by Arraystar, the buyer agrees to and accepts the following conditions: That the buyer's sole remedy shall be to obtain replacement of the product from Arraystar; and

Arraystar Inc. shall not be responsible for replacing Product that has been improperly stored, handled, or used by buyer or End-User.

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