

nrStar™ Mouse tRNA PCR Array

Cat#: AS-NR-001M-1

Instruction Manual Version 1.0

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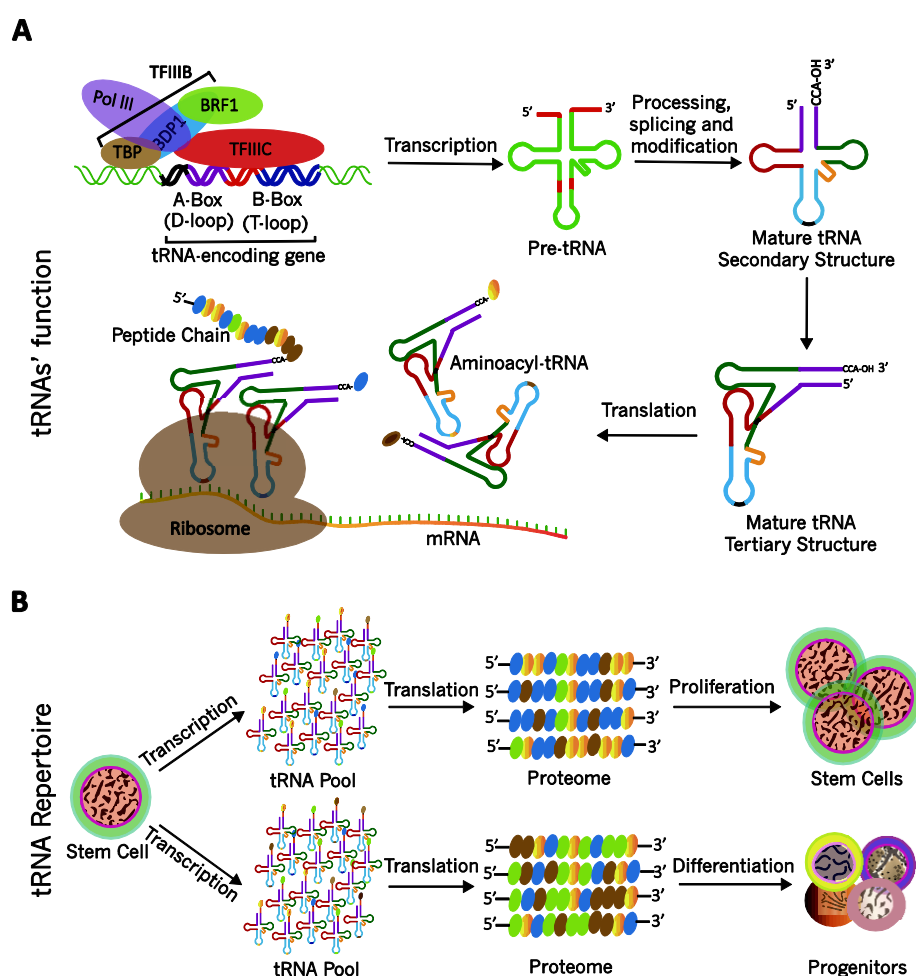
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I. Introduction

A. Overview

Transfer RNAs (tRNAs) are ubiquitous and the most abundant of all small non-coding RNA molecules. As a fundamental component in translation, tRNAs serve as the physical link between the mRNA coding and protein sequences (**Figure 1**). Genomes exhibit substantial variations in their preference for specific codons across their coding sequences. The source of this bias, though still debated, likely reflects selection for translational efficiency and accuracy [1-3]. Alterations of tRNA repertoire affect cell-fate choices during cell development (**Fig. 1B**), and dysregulated tRNAs expression are associated with many disease including cancer. nrStar™ Mouse tRNA PCR Array profiles 166 PCR-distinguishable nuclear tRNA isodecoders and 19 PCR-distinguishable mitochondrial tRNA species in the Mouse tRNA Repertoire by optimized SYBR Green qPCR assays in a PCR panel. The panel covers all the nuclear anti-codons and major mitochondria anticodons compiled in GtRNAdb and tRNAdb database, making it a powerful tool for analyzing the profile of mouse tRNA repertoire. As tRNAs are heavily modified posttranscriptionally, which badly affect tRNA cDNA synthesis efficiency, scientists at Arraystar have developed rtStar™ tRNA Pretreatment & First-Strand cDNA Synthesis Kit to efficiently remove the tRNA modifications and greatly improve the cDNA synthesis quality. With the powerful combination of this kit and the PCR array, researchers can obtain a new level of accuracy on the tRNA pool alterations and gain further insight to interpret the proteome and tRNA-derived fragments.

Figure 1 A. tRNA: Role, Function & Synthesis
B. The effects of tRNA repertoire during cell fate determination



B. tRNA and cell state

A wide variety of biological processes like cell proliferation[4], differentiation[4, 5] and apoptosis[6] are always accompanied with tRNA level variation (**Fig. 1B**). It has been found that codon usage is different between genes serving cell-autonomous functions and genes involved in multicellularity. Proliferation-induced and differentiation-induced tRNAs often carry anti-codons that correspond to the codons enriched among the cell-autonomous and the multicellularity genes, respectively. Because mRNAs of cell-autonomous genes are induced in proliferation and cancer in particular, the concomitant induction of their codon-enriched tRNAs suggests coordination between transcription and translation[4]. According to another study, overexpression of tRNAⁱ (Met) significantly alters the global tRNAs expression profile and results in increased cell metabolic activity and cell proliferation[5]. In addition, tRNA regulates apoptotic sensitivity at the level of cytochrome c mediated apoptosome formation[6]. Microinjection of tRNA can inhibit cytochrome c-induced apoptosis[7]. In sum, alteration of tRNA levels can change the cell state by various mechanisms.

C. tRNA and diseases

Multiple lines of evidence have associated the disruption of tRNA levels with many diseases. For example, certain dysregulated tRNAs can induce tumorigenesis and cancer progression[5]. Studying tRNA repertoire has become increasingly important in human diseases.

■ Cancer

After cataloging the tRNA repertoire, Gingold et al demonstrated there are different tRNA pools between cancer cells and differentiated cells[4]. tRNAs that are upregulated in differentiated/arrested cells are repressed in proliferating cells. Conversely, tRNAs whose levels are high in proliferating cells become low in differentiated/arrested cells. Cancer cells appear to adjust their tRNA pools to selectively bolster translation of mRNAs that are required for tumor progression. When comparing tRNAs expression in breast tumors versus normal breast tissues, Pavon-Eternod et al found that nuclear- and mitochondrial-encoded tRNAs exhibit distinct expression patterns, indicating the potentials of using tRNAs as biomarkers for breast cancer[8]. Recently, Goodarzi et al also confirmed that specific tRNAs are upregulated in human breast cancer cells as they gain metastatic activity, among which tRNA^{Glu-UUC} and tRNA^{Arg-CCG} are implicated as promoters of breast cancer metastasis. Further studies conclusively showed that tRNA^{Glu-UUC} promotes metastatic progression by directly enhancing EXOSC2 and GRIPAP1 expression [9]. These and other cases have conclusively demonstrated dysregulated tRNA repertoire can promote tumorigenesis and cancer progression[5, 8-15].

■ Huntington's disease

Huntington's disease (HD), a dominantly inherited neurodegenerative disorder caused by the expansion of a CAG-encoded polyglutamine (polyQ) repeat in huntingtin, displays a highly heterogeneous etiopathology and disease onset. Analyses of HD-affected brain tissues revealed traces of polyalanine (polyA) or polyserine (polyS) proteins within the polyQ aggregates. These species probably result from a shift in the Gln-encoding CAG frame to an Ala-encoding -1 GCA frame or a Ser-encoding +1 AGC frame. But what is the role of translational frameshifting in the pathogenesis of polyQ diseases? Girstmair et al found that depletion of tRNA^{Gln-CUG} pairing to the CAG codon was the main cause of -1 frameshifting. In addition, frameshifted proteins form morphologically distinct aggregates in vivo dependent on the Q:A ratio. These results suggested that frameshifting within expanded CAG stretches may contribute to the heterogeneity in the course and onset of HD at both cellular and individual level[16].

■ Virus Infection

Viruses are wholly dependent on the host translation machinery to synthesize their proteins. Consequently, viral

codon usage is thought to be under selective pressure to adapt to the host cell tRNA pool. Since host codon usage generally reflects the host tRNA pool, viral translation should be more efficient when viral codon usage is similar to that of the host genes. In many cases, however, viral codon usage seems poorly adapted to that of its host. After profiling the tRNA repertoire, Pavon-Eternod et al found that influenza A and vaccinia viruses could manipulate tRNA populations to favor translation of their own viral genes[17]. In another research, the codon usage of HIV-1 early genes is similar to that of highly expressed host genes, but codon usage of HIV-1 late genes was better adapted to the altered tRNA pool late in the viral infection[18]. This is a striking example of the virus modulating the tRNA pool to optimize its translation efficiency.

D. Product summary

nrStar™ Mouse tRNA PCR Array profiles 166 PCR-distinguishable nuclear tRNA isodecoders and 19 PCR-distinguishable mitochondrial tRNA species, covering all the nuclear anti-codons and major mitochondria anticodons compiled in GtRNAdb and tRNAdb databases.

Condition		1	2	3	4	5	6	7	8	9	10	11	12
α	A	Ala- AGC-1	Ala- AGC-2	Ala- AGC-3	Ala- AGC-4	Ala- AGC-5	Ala- AGC-6	Ala- AGC-7	Ala- CGC-1	Ala- CGC-1	Ala- GGC-1	Ala- GGC-2	Ala- TGC-1
β	B												
α	C	Arg- TCT-3	Arg- TCT-4	Arg- TCT-5	Arg- TCT-6	Arg- TCT-7	Asn- GTT-1	Asn- GTT-2	Asn- GTT-3	Asp- GTC-1	Asp- GTC-2	Cys- ACA	Cys- GCA-1
β	D												
α	E	Gln- TTG-3	Glu- CTC-1	Glu- CTC-2	Glu- CTC-3	Glu- CTC-4	Glu- CTC-5	Glu- TTC-1	Glu- TTC-2	Gly- ACC-1	Gly- ACC-2	Gly- CCC-1	Gly- CCC-2
β	F												
α	G	His- GTG-3	Ile-AAT- 1	Ile-AAT- 2	Ile-GAT	Ile-TAT- 1	Ile-TAT- 2	Ile-TAT- 3	Ile-TAT- 4	iMet- CAT-1	iMet- CAT-2	iMet- CAT-3	Leu- AAG
β	H												
α	I	Lys- CTT-2	Lys- CTT-3	Lys- CTT-4	Lys- CTT-5	Lys- CTT-6	Lys- CTT-7	Lys- CTT-8	Lys- TTT-1	Lys- TTT-2	Lys- TTT-3	Lys- TTT-4	Met- CAT-1
β	J												
α	K	Pro- TGG-3	Pro- TGG-4	Pro- TGG-5	SeC- TCA	Ser- AGA-1	Ser- AGA-2	Ser- AGA-3	Ser- AGA-4	Ser- CGA-1	Ser- CGA-2	Ser- GCT-1	Ser- GCT-2
β	L												
α	M	Thr- TGT-2	Trp- CCA-1	Trp- CCA-2	Trp- CCA-3	Tyr- GTA-1	Tyr- GTA-2	Tyr- GTA-3	Tyr- GTA-4	Tyr- GTA-5	Tyr- GTA-6	Tyr- GTA-7	Tyr- GTA-8
β	N												
α	O	mtCys- GCA	mtGln- TTG	mtGlu- TTC	mtGly- TTC	mtHis- GTG	mtIle- GAT	mtLeu- 1-TAG	mtLeu- 2-TAA	mtLys- TTT	mtMet- CAT	mtPhe- GAA	mtPro- TGG
β	P												
Condition		13	14	15	16	17	18	19	20	21	22	23	24
α	A	Ala- TGC-2	Ala- TGC-3	Arg- ACG-1	Arg- ACG-2	Arg- ACG-3	Arg- CCG-1	Arg- CCG-2	Arg- CCT-1	Arg- CCT-2	Arg- TCG	Arg- TCT-1	Arg- TCT-2
β	B												
α	C	Cys- GCA-2	Cys- GCA-3	Cys- GCA-4	Cys- GCA-5	Cys- GCA-6	Cys- GCA-7	Gln- CTG-1	Gln- CTG-2	Gln- CTG-3	Gln- CTG-4	Gln- TTG-1	Gln- TTG-2
β	D												
α	E	Gly- CCC-3	Gly- CCC-4	Gly- CCC-5	Gly- GCC-1	Gly- GCC-2	Gly- GCC-3	Gly- TCC-1	Gly- TCC-2	Gly- TCC-3	His- ATG	His- GTG-1	His- GTG-2
β	F												
α	G	Leu- CAA-1	Leu- CAA-2	Leu- CAA-3	Leu- CAA-4	Leu- CAA-5	Leu- CAG	Leu- TAA-1	Leu- TAA-2	Leu- TAG-1	Leu- TAG-2	Leu- TAG-3	Lys- CTT-1
β	H												
α	I	Met- CAT-2	Met- CAT-3	Met- CAT-4	Met- CAT-5	Phe- GAA	Pro- AGG-1	Pro- AGG-2	Pro- AGG-3	Pro- CGG	Pro- GGG	Pro- TGG-1	Pro- TGG-2
β	J												
α	K	Ser- GCT-3	Ser- GGA	Ser- TGA-1	Ser- TGA-2	Ser- TGA-3	Sup- TTA	Thr- AGT-1	Thr- AGT-2	Thr- CGT-1	Thr- CGT-2	Thr- CGT-3	Thr- TGT-1
β	L												
α	M	Tyr- GTA-9	Tyr- GTA-10	Val- AAC-1	Val- AAC-2	Val- CAC-1	Val- CAC-2	Val- CAC-3	Val- CAC-4	Val- GAC	Val- TAC	mtAla- TGC	mtAsn- GTT
β	N												
α	O	mtSer- 2-TGA	mtThr- TGT	mtTrp- TCA	mtTyr- GTA	mtVal- TAC	U6	5S rRNA	18S rRNA	RNA Spike- In	PPC	GDC	Blank
β	P												

Figure 2 The array layout for nrStar™ Mouse tRNA PCR Array**■ Kit Contents****Table 1** List of Mouse tRNAs and the Controls

1	Ala-AGC-1	41	Cys-GCA-6	81	iMet-CAT-1	121	Pro-TGG-3	161	Val-CAC-1
2	Ala-AGC-2	42	Cys-GCA-7	82	iMet-CAT-2	122	Pro-TGG-4	162	Val-CAC-2
3	Ala-AGC-3	43	Gln-CTG-1	83	iMet-CAT-3	123	Pro-TGG-5	163	Val-CAC-3
4	Ala-AGC-4	44	Gln-CTG-2	84	Leu-AAG	124	SeC-TCA	164	Val-CAC-4
5	Ala-AGC-5	45	Gln-CTG-3	85	Leu-CAA-1	125	Ser-AGA-1	165	Val-GAC
6	Ala-AGC-6	46	Gln-CTG-4	86	Leu-CAA-2	126	Ser-AGA-2	166	Val-TAC
7	Ala-AGC-7	47	Gln-TTG-1	87	Leu-CAA-3	127	Ser-AGA-3	167	mtAla-TGC
8	Ala-CGC-1	48	Gln-TTG-2	88	Leu-CAA-4	128	Ser-AGA-4	168	mtAsn-GTT
9	Ala-CGC-2	49	Gln-TTG-3	89	Leu-CAA-5	129	Ser-CGA-1	169	mtCys-GCA
10	Ala-GGC-1	50	Glu-CTC-1	90	Leu-CAG	130	Ser-CGA-2	170	mtGln-TTG
11	Ala-GGC-2	51	Glu-CTC-2	91	Leu-TAA-1	131	Ser-GCT-1	171	mtGlu-TTC
12	Ala-TGC-1	52	Glu-CTC-3	92	Leu-TAA-2	132	Ser-GCT-2	172	mtGly-TTC
13	Ala-TGC-2	53	Glu-CTC-4	93	Leu-TAG-1	133	Ser-GCT-3	173	mtHis-GTG
14	Ala-TGC-3	54	Glu-CTC-5	94	Leu-TAG-2	134	Ser-GGA	174	mtIle-GAT
15	Arg-ACG-1	55	Glu-TTC-1	95	Leu-TAG-3	135	Ser-TGA-1	175	mtLeu1-TAG
16	Arg-ACG-2	56	Glu-TTC-2	96	Lys-CTT-1	136	Ser-TGA-2	176	mtLeu2-TAA
17	Arg-ACG-3	57	Gly-ACC-1	97	Lys-CTT-2	137	Ser-TGA-3	177	mtLys-TTT
18	Arg-CCG-1	58	Gly-ACC-2	98	Lys-CTT-3	138	Sup-TTA	178	mtMet-CAT
19	Arg-CCG-2	59	Gly-CCC-1	99	Lys-CTT-4	139	Thr-AGT-1	179	mtPhe-GAA
20	Arg-CCT-1	60	Gly-CCC-2	100	Lys-CTT-5	140	Thr-AGT-2	180	mtPro-TGG
21	Arg-CCT-2	61	Gly-CCC-3	101	Lys-CTT-6	141	Thr-CGT-1	181	mtSer2-TGA
22	Arg-TCG	62	Gly-CCC-4	102	Lys-CTT-7	142	Thr-CGT-2	182	mtThr-TGT
23	Arg-TCT-1	63	Gly-CCC-5	103	Lys-CTT-8	143	Thr-CGT-3	183	mtTrp-TCA
24	Arg-TCT-2	64	Gly-GCC-1	104	Lys-TTT-1	144	Thr-TGT-1	184	mtTyr-GTA
25	Arg-TCT-3	65	Gly-GCC-2	105	Lys-TTT-2	145	Thr-TGT-2	185	mtVal-TAC
26	Arg-TCT-4	66	Gly-GCC-3	106	Lys-TTT-3	146	Trp-CCA-1	186	U6
27	Arg-TCT-5	67	Gly-TCC-1	107	Lys-TTT-4	147	Trp-CCA-2	187	5S rRNA
28	Arg-TCT-6	68	Gly-TCC-2	108	Met-CAT-1	148	Trp-CCA-3	188	18S rRNA
29	Arg-TCT-7	69	Gly-TCC-3	109	Met-CAT-2	149	Tyr-GTA-1	189	Spike-In
30	Asn-GTT-1	70	His-ATG	110	Met-CAT-3	150	Tyr-GTA-2	190	PPC
31	Asn-GTT-2	71	His-GTG-1	111	Met-CAT-4	151	Tyr-GTA-3	191	GDC
32	Asn-GTT-3	72	His-GTG-2	112	Met-CAT-5	152	Tyr-GTA-4	192	Blank
33	Asp-GTC-1	73	His-GTG-3	113	Phe-GAA	153	Tyr-GTA-5		
34	Asp-GTC-2	74	Ile-AAT-1	114	Pro-AGG-1	154	Tyr-GTA-6		
35	Cys-ACA	75	Ile-AAT-2	115	Pro-AGG-2	155	Tyr-GTA-7		
36	Cys-GCA-1	76	Ile-GAT	116	Pro-AGG-3	156	Tyr-GTA-8		
37	Cys-GCA-2	77	Ile-TAT-1	117	Pro-CGG	157	Tyr-GTA-9		
38	Cys-GCA-3	78	Ile-TAT-2	118	Pro-GGG	158	Tyr-GTA-10		

39	Cys-GCA-4	79	Ile-TAT-3	119	Pro-TGG-1	159	Val-AAC-1
40	Cys-GCA-5	80	Ile-TAT-4	120	Pro-TGG-2	160	Val-AAC-2

■ Description of the control assays

nrStar™ Mouse tRNA PCR Array includes a series of external and internal controls for effective correction and normalization of sample and qPCR variabilities. In addition, Positive PCR Control and Genomic DNA Control are included to monitor the experiment process and the quality of RNA sample. These controls are described below.

- **Ref** (small ncRNA Control Reference; Internal Controls): Three stably expressed small ncRNA genes U6 (Ref 1), 5S rRNA (Ref 2), and 18S rRNA (Ref 3) are included in the array as the quantification references for tRNA. nrStar™ PCR system provides multiple reference genes selected among commonly used reference genes by using a stringent bioinformatic algorithm, which offers the flexibility of choosing the most valid reference gene(s) for qPCR normalization for your sample types.
- **RNA Spike-In** (External Control): One External RNA Spike-In Mix is added in the RNA sample **prior to the first strand cDNA synthesis**. The RNA Spike-In control assay indicates the overall success and the efficiency of the reaction beginning from the cDNA synthesis to the final qPCR. Any problem(s) in these steps will result in a failed or compromised RNA Spike-In outcome. RNA spike-in assay results for samples are compared and outliers or failed reactions may be identified and excluded from further data analysis.
- **PPC** (Positive PCR control): One 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 (IPC) 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.

■ Shipping and Storage

nrStar™ PCR Arrays are shipped at ambient temperature, on ice, or on dry ice depending on the destination and accompanying products. Store at –20°C upon receipt. When stored properly, the contents are stable for up to 1 year after delivery.

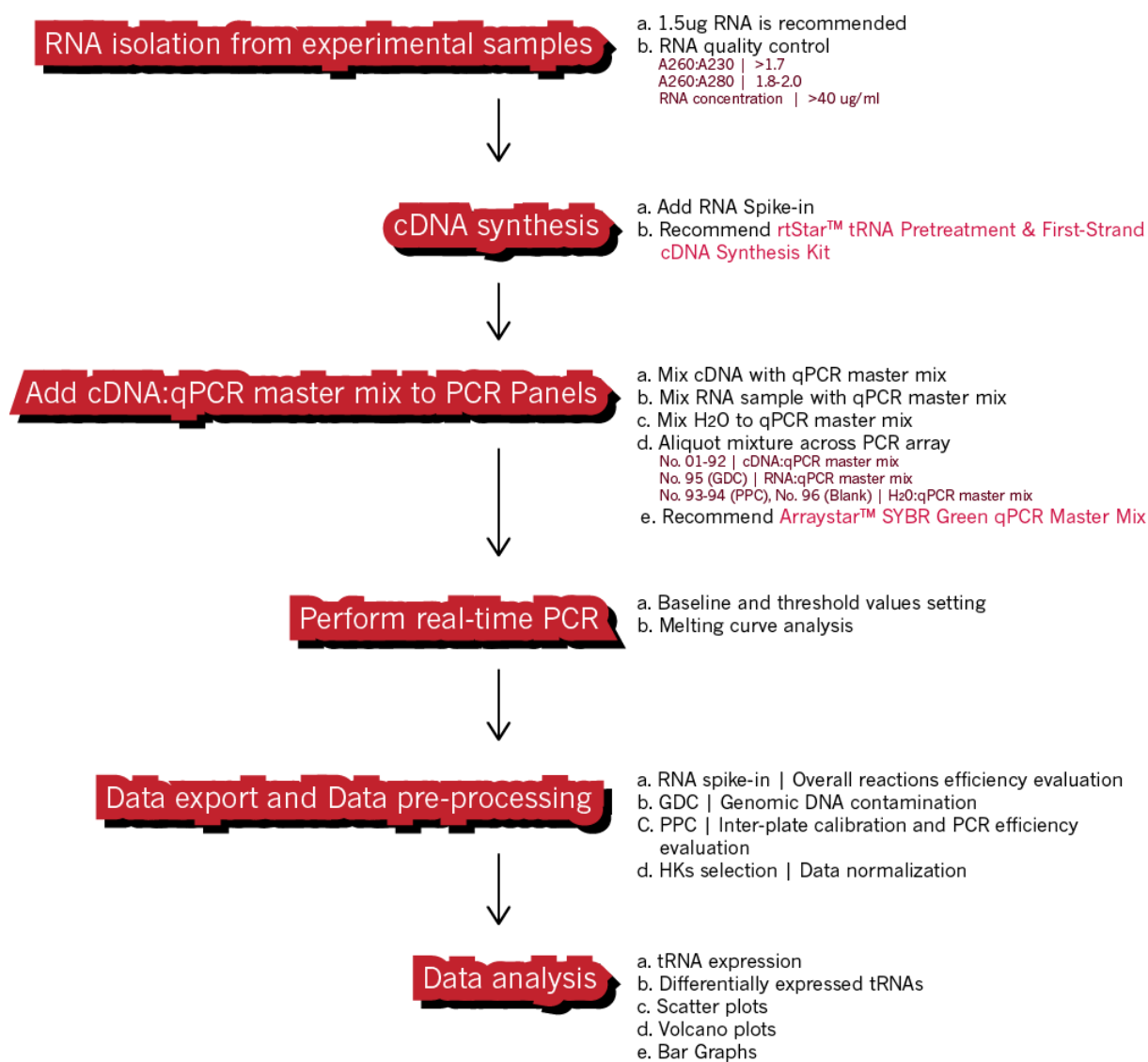
■ Additional Required Equipment

- Thermal cycler
- Real time qPCR instrument, compatible with 384-well format

■ Additional Required Reagents

- rtStar™ tRNA Pretreatment & First-Strand cDNA Synthesis Kit (Cat# AS-FS-004)
- Arraystar SYBR® Green qPCR Master Mix(ROX+) (AS-MR-006-5)
- Nuclease-free PCR-grade water

E. Protocol overview



II. 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. 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 gene expression profiling by qPCR, which is particularly important for genes 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.

nrStar™ Mouse tRNA PCR Array operates on total RNA input amount in the range (50 ng - 5 ug) per array. A good amount of input RNA is better for the assay sensitivity and quantification accuracy/dynamic/linear range. A low amount of input RNA reduces the sensitivity and increases false negatives. The input RNA amount also depends on the abundance of the target transcripts of interest: more input RNA is required for low abundance transcripts, and vice versa. An optimal input amount in the operating range is decided between data quality and sample availability. For first-time use, an input total RNA amount at 1.5 ug is recommended to start with. Input RNA less than 100 ng can result in high false negatives, particularly for genes expressed at low levels.

B. First-strand cDNA synthesis

Use the same amount of total RNA in this reaction for every sample. High quality cDNA synthesis is vital for the following qPCR performance. Because of the disincentive effects of tRNA modification on cDNA synthesis, we highly recommend using **rtStar™ tRNA Pretreatment & First-Strand cDNA Synthesis Kit** (Cat# AS-FS-004) for tRNA first strand cDNA synthesis, which is specifically optimized for and fully compatible with the nrStar™ tRNA PCR Array. It can efficiently remove the modifications and greatly improve cDNA synthesis quality so that obtain more accurate tRNA expression data. The condition in the RNA demethylation step may cause some RNA degradation and loss.

■ RNA demethylation

1. Prepare reagents

Gently thaw all of the kit components except for the Reverse Transcriptase and Demethylase. Mix by brief vortexing, spin down all reagents, and immediately place on ice.

NOTE: The first time to use this kit, please reconstitute the RNA spike-in by adding 200 μl Nuclease-free Water to the tube. Mix by vortexing and spin down. Leave on ice for 20 ~ 30 min to fully dissolve the RNA spike-in. Vortex again, then spin down.

2. Prepare Demethylation Reaction Buffer (4×)

Prepare fresh Demethylation Reaction Buffer (4×) by combine the DRB-C1, C2, C3, C4, C5 with 1:1:1:1:1 volume and keep on ice. The total required volume of Demethylation Reaction Buffer (4×) is $1.1 \times 25 \times$ number of samples (μl).

NOTE: Prepare fresh Demethylation Reaction Buffer (4×) each time before use.

3. Set up Demethylation Master Mix

Combine reagents orderly according to the following table. Before its immediate use, remove the Demethylase from the freezer, mix by flicking the tube, do not vortex. Briefly spin down the content and place on ice. If performing RNA demethylation on multiple RNA samples, it is recommended to prepare a demethylation master mix of the Demethylation Reaction Buffer (4×), Demethylase and RNase Inhibitor (in the proportion indicated in Table). Considering the pipetting losses, 10% excess of all reagents is recommended.

Demethylation Reaction Buffer (4×)	25 μl
Demethylase	5 μl
RNase Inhibitor	2 μl
Total volume	32 μl

4. Perform & stop RNA demethylation reaction

Combine the reagents per demethylation reaction according to the following table in the order shown. Vortex briefly to mix, and spin down.

Demethylation Master Mix	32 μl
Input RNA	$\leq 5 \mu\text{g}$
Nuclease-free water	x μl
Total volume	100 μl

Incubate the above mix at 25°C for 2h. Then orderly add 100 μl Nuclease-free water and 200 μl phenol:chloroform to terminate the reaction and perform the RNA purification procedure immediately.

5. RNA purification

- a. Mix well the tube from step 4 by inverting. Incubate at room temperature for 10 min. Centrifuge at 12,000 rpm for 10 min.
- b. Carefully transfer the top layer by a micropipette to a RNase-free tube. Discard the bottom liquid phase to waste.
- c. Precipitate the RNA from the aqueous phase by mixing with equal volume of Isopropanol. Vortex the mixture and place the tube for 30 min at -80°C. Centrifuge at 12,000 rpm for 10 min.
- d. Remove the supernatant from the tube, leaving only the RNA pellet.
- e. Add 1 ml 75% ethanol (in DEPC-treated water). Mix well by inverting.
- f. Centrifuge the tube at 7,500 rpm for 5 min at 4°C. Discard the wash.
- g. Vacuum dry under centrifugation or air dry the RNA pellet for 5–10 min.
- h. Resuspend the RNA pellet in 11 µl Nuclease-free Water.
- i. Incubate at 55–60°C for 10–15 min to fully dissolve the RNA.

■ First strand cDNA synthesis

6. Combine Annealing Mix according to Table

If performing first-strand cDNA synthesis on multiple RNA samples, it is recommended to prepare an Annealing Mix of the Random Primer, dNTP Mix and RNA Spike-in (in the proportion indicated in Table). 10% excess volume for pipetting losses is recommended.

Random Primers	1 µl
dNTP Mix	1 µl
RNA Spike-in	1 µl
Template Total RNA	10 µl
Total volume	13 µl

7. Incubate in a thermal cycler at 65°C for 5 min, and then immediately place on ice for at least 1 min. Collect the contents of the tube by brief centrifugation.
8. Combine cDNA Synthesis Mix

cDNA Synthesis Mix is recommended to prepare for multiple RNA samples. It includes the components in the following table. 10% excess volume for pipetting losses is recommended. Before its immediate use, remove the Reverse Transcriptase from the freezer, mix by flicking the tube, do not vortex. Briefly spin down the content and place on ice.

RT Reaction Buffer (5×)	4 µl
RNase Inhibitor	1 µl
Reverse Transcriptase	1 µl
Nuclease-free Water	1 µl
Total volume	7 µl

9. Add cDNA Synthesis Mix to the tube from STEP 7. Vortex the sample briefly to mix, and collect by brief centrifugation. Incubate at 25°C for 10 min, followed by 50 min at 45°C
10. Terminate the reactions at 85°C for 5 min. Chill on ice.
11. 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 tRNA cDNA synthesis.

NOTE: The cDNA synthesis product can proceed directly to PCR or can be stored at -20°C.

C. 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.

1. Dilute the cDNA in Nuclease-free Water. If 1 µg input RNA is used with rtStar™ tRNA Pretreatment & First-Strand cDNA Synthesis Kit (Cat#AS-FS-004), the dilution factor is 1:40. Mix well and spin down. The diluted cDNA is used as the qPCR template in the wells for tRNA Assays, Housekeeping gene Internal Controls, and Spike-in External Control.
2. For GDC Controls, combine 1.0 µl NRT (mock cDNA synthesis reaction without the reverse transcriptase) sample or 1.0 µl RNA sample (without cDNA synthesis), 5.0 µl SYBR Green Master Mix, and 4.0 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. Prepare the qPCR Mix according to the Table below. There are total of 192 wells of PCR reaction. Some extra amount is included for consumption by the liquid dispensing operation.

SYBR Green Master Mix	1000 µl
diluted cDNA template	800 µl
ddH ₂ O	200 µl
Total volume	2000 µl

5. Loading the PCR Array plate.

NOTE: The reagents should load to the related **well number** corrodng to **Figure 2** and **Table 1**.

- 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.190-No.192**);
- c. Add 10 µl GDC Mixture aliquot from STEP 2 into the **No.191** to detect genomic DNA contamination.
- d. Add 10 µl Blank Mixture aliquot from STEP 3 into the **No.190 and No.192**.

- 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 °C	5 minutes
40	95 °C	10 seconds
	60 °C	40 seconds
Melting curve analysis		

D. 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 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 greater than 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 >25) 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:

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

The Ct value is corrected with the calibration factor as

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

or

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

	Plate 1	Plate 2	Plate 3
Ala-TGC-1	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
Ala-TGC-1 (Calibrated)	20.16	20.73	20.64

5. Calculate the ΔCt for each tRNA in the plate.

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

Where average (Ct_{Refs}) is the average of the Ct values derived from the multiple reference genes. Three most stably expressed small ncRNA Control References were selected from a broad range of samples by our stringent algorithm that evaluates the optimal properties and the number for endogenous controls.

■ Data analysis

1. Calculate the $\Delta\Delta Ct$ for each tRNA

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

or

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

2. Calculate the fold changes for each gene from sample 1 to sample 2 or group 1 to group 2 as following:

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

NOTE: By convention, if the fold change is greater than 1, the result is reported as a fold up-regulation. If the fold change is less than 1, its negative inverse is reported as a fold down-regulation.

3. 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 ≥ 2 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 2 fold change.

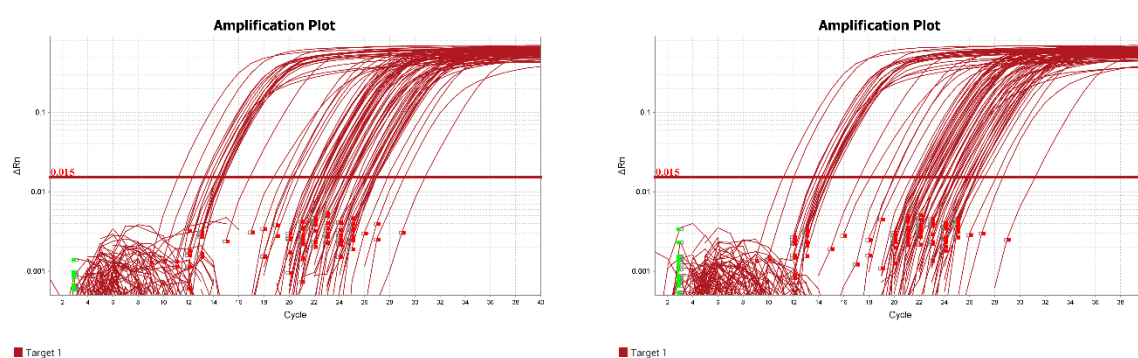
4. Other analyses such as scatter plots, volcano plots, list of differentially expressed genes and bar graph of expression differences for the tRNAs are performed and included in the standard analysis package.

III. Quality Control and Sample Data

A. nrStar™ Mouse tRNA PCR Array validation

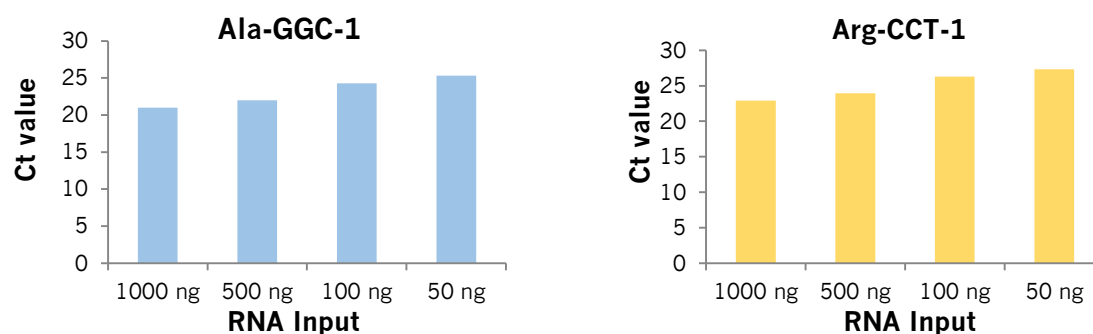
■ Real-time qPCR Validation

The performance of Mouse tRNA Panel was tested using a cohort of mouse tissues. The extracted RNA samples were converted to cDNA using rtStar™ tRNA Pretreatment & First-Strand cDNA Synthesis Kit (Cat#AS-FS-004). The cDNA 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 the mouse tissues.



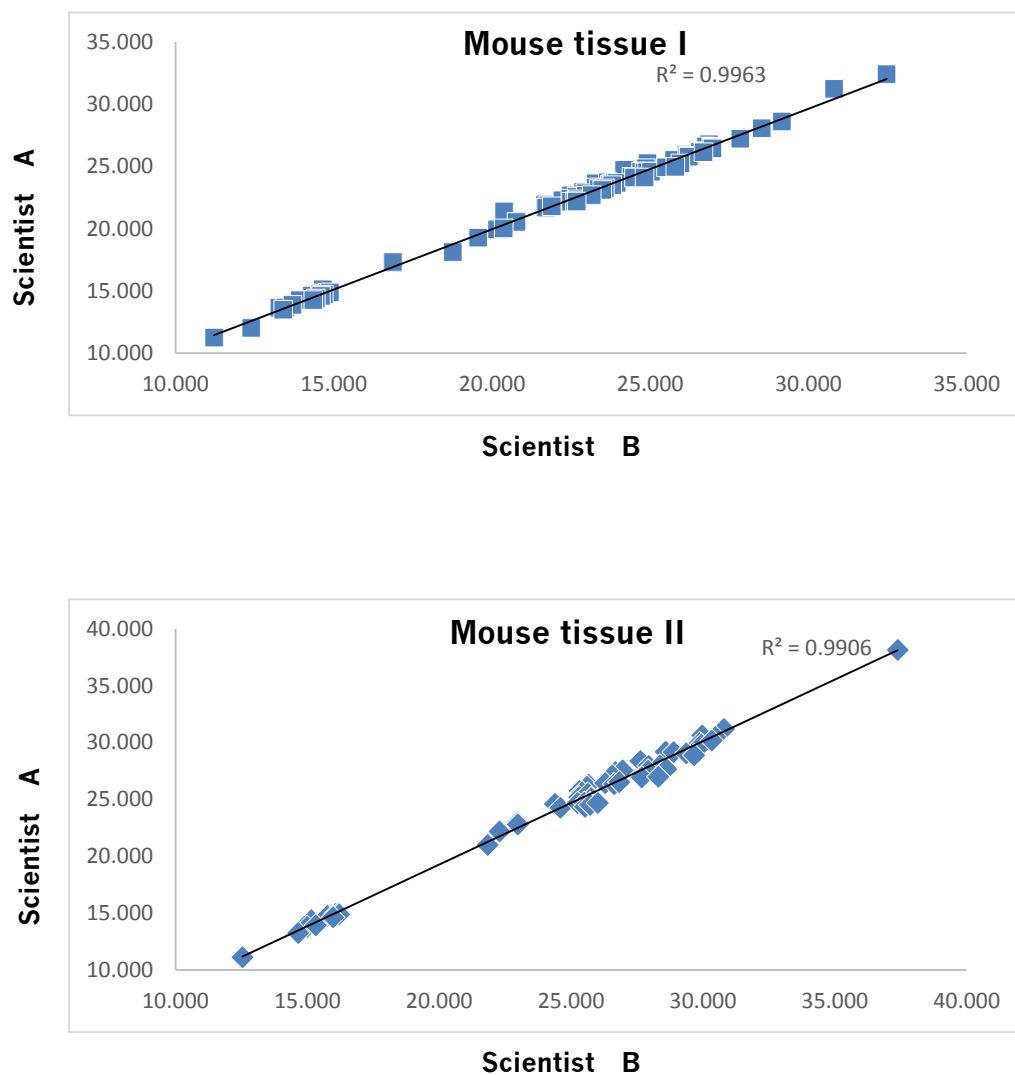
■ Sensitivity Test

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. Ala-GGC-1 and Arg-CCT-1 transcripts were detected at Ct values of 25.2 and 27.0 respectively, with the input RNA amount as low as 50 ng.



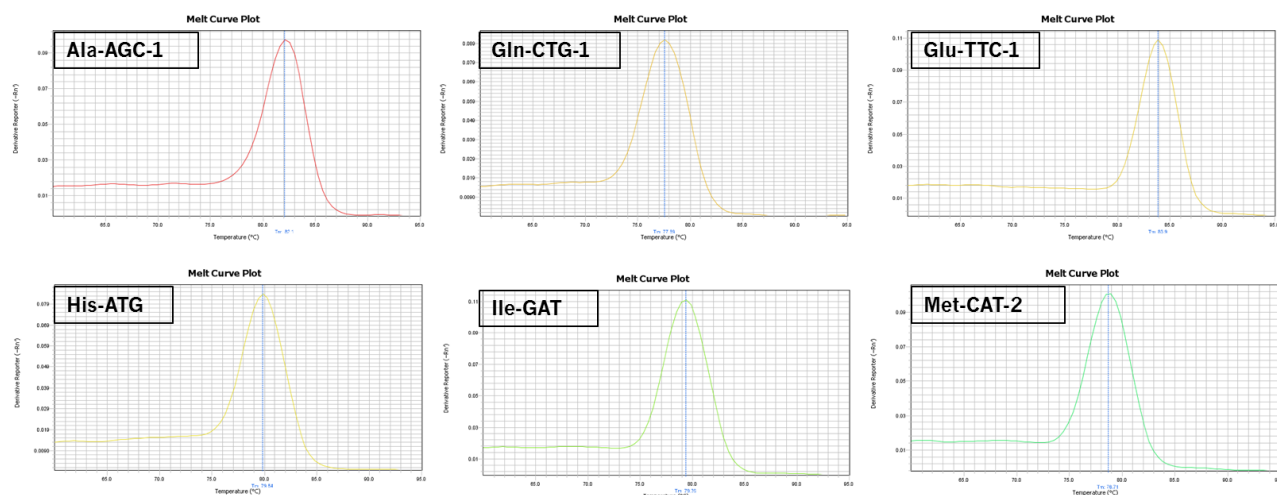
■ Reproducibility Test

Two independent runs of nrStar™ Mouse tRNA 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 $R^2 > 0.98$.



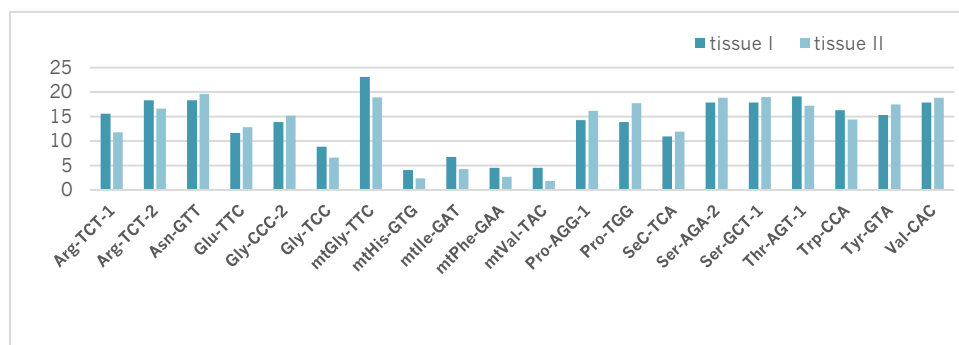
■ Specificity Test

The amplification products of transcripts of Ala-AGC-1, Gln-CTG-1, Glu-TTC-1, His-ATG, Ile-GAT, Met-CAT-2 were analyzed by melting curves, all of which had single sharp melting peaks. The results demonstrate the high amplification specificities for the transcripts with the assays on the array.



B. Sample data: Analysis of Mouse tRNA transcript levels in mouse tissues

The sample data were generated from RNAs extracted from mouse tissues. The normalization was carried out using the average of the Internal Control Reference genes. Ct for the gene transcripts in mouse tissue I vs. mouse tissue II are graphed in the bar chart below.



IV. Troubleshooting

Problem	Possible solution
qPCR background too high	<ul style="list-style-type: none"> Reduce the amount of cDNA used in the SYBR Green Master Mix.
No qPCR signals	<ul style="list-style-type: none"> 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 STEP C.6 from 60°C to 50°C.
Baseline and threshold settings	<ul style="list-style-type: none"> Follow the instructions of the qPCR system manufacturer. Contact their technical support as necessary.

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VI. 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

VII. Terms and Conditions

By purchasing and using any part of the nrStar™ Mouse tRNA 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.

Arraystar, its Agencies and Representatives disclaim liability of any kind whatsoever, including, without limitation, liability for quality, performance, merchantability and fitness for a particular purpose arising out of the use, or inability to use the product. In no event shall Arraystar be liable for claims for any other damages, whether direct, incidental, foreseeable, consequential, or special (including but not limited to loss of use, revenue or profit), whether based upon warranty, contract, tort (including negligence) or strict liability arising in connection with the sale or the failure of products to perform in accordance with the stated specifications.

Arraystar disclaims any and all responsibility and liability for any injury or damage which may be caused by the failure of purchaser or end-user to follow said guidelines and specific product literature. It is the user's responsibility to determine and to adopt safety precautions as may be necessary.