nrStar™ Human tRNA PCR Array

Cat#: AS-NR-001H-1

Instruction Manual Version 2.0

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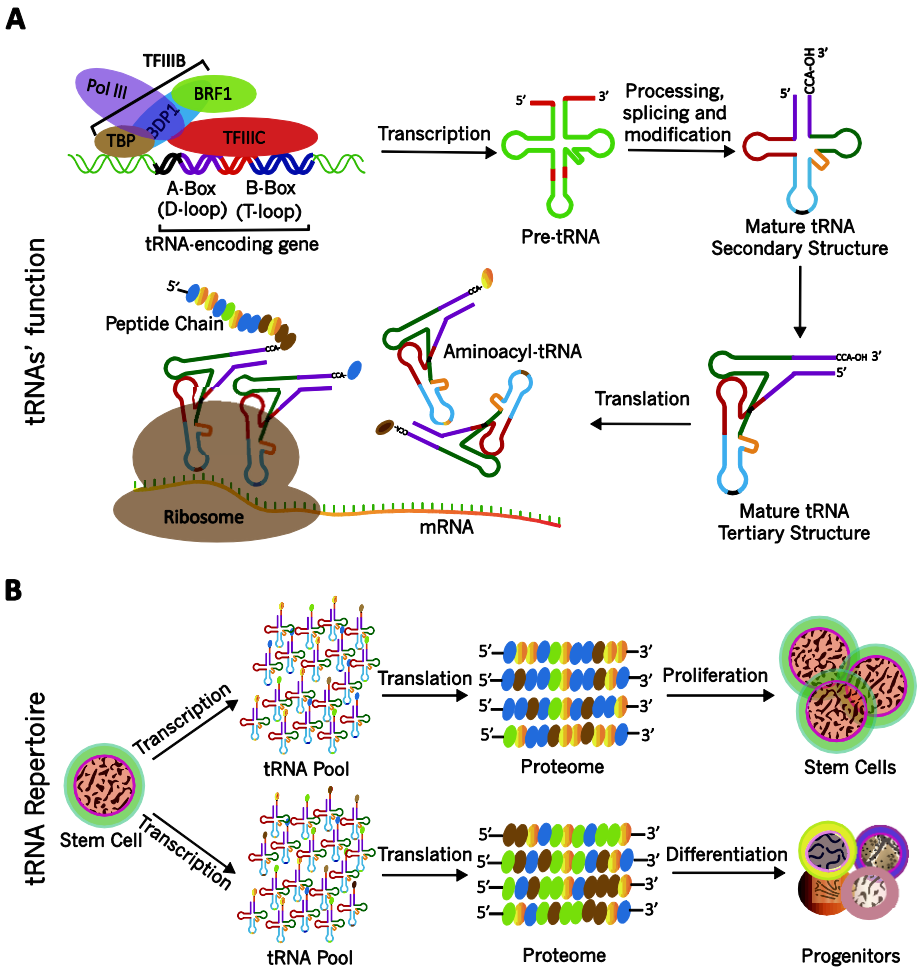
Table of Contents

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™ Human tRNA PCR Array profiles 163 PCR-distinguishable nuclear tRNA isodecoders and 22 PCR-distinguishable mitochondrial tRNA species by optimized SYBR Green qPCR assays in a PCR panel. The panel covers all the anti-codons compiled in GtRNAdb and tRNAdb database, making it a powerful tool for analyzing the profile of human tRNA repertoire. As tRNAs are heavily modified posttranscriptionally, which badly affect tRNA cDNA synthesis efficiency, scientists at Arraystar have developed rtStar™ tRNA-optimized 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 multi- cellularity 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 $tRNA^{Glu\text{-}UUC}$ and $tRNA^{Arg\text{-}CCG}$ are implicated as promoters of breast cancer metastasis. Further studies conclusively showed that $tRNA^{Glu\text{-}UUC}$ 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 frame- shifting in the pathogenesis of polyQ diseases? Girstmair et al found that depletion of $tRNA^{Gln\text{-}CUG}$ 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™ Human tRNA PCR Array profiles 163 PCR-distinguishable nuclear tRNA isodecoders and 22 PCR-distinguishable mitochondrial tRNA species, covering all the anti-codons 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-CGC-1	Ala-CGC-2	Ala-CGC-3	Ala-TGC-1	Ala-TGC-2	Ala-TGC-3	Ala-TGC-4	Ala-TGC-5
β	B												
α	C	Arg-TCT-2	Arg-TCT-3	Asn-GTT-1	Asn-GTT-2	Asn-GTT-3	Asn-GTT-4	Asn-GTT-5	Asn-GTT-6	Asn-GTT-7	Asp-GTC-1	Asp-GTC-2	Asp-GTC-3
β	D												
α	E	Gln-CTG-4	Gln-CTG-5	Gln-CTG-6	Gln-CTG-7	Gln-TTG-1	Gln-TTG-2	Gln-TTG-3	Gln-TTG-4	Glu-CTC	Glu-TTC-1	Glu-TTC-2	Glu-TTC-3
β	F												
α	G	His-GTG-1	His-GTG-2	His-GTG-3	Ile-AAT-1	Ile-AAT-2	Ile-AAT-3	Ile-AAT-4	Ile-AAT-5	Ile-AAT-6	Ile-GAT	Ile-TAT	Leu-AAG-1
β	H												
α	I	Leu-TAA-5	Leu-TAG-1	Leu-TAG-2	Leu-TAG-3	Lys-CTT-1	Lys-CTT-2	Lys-CTT-3	Lys-CTT-4	Lys-CTT-5	Lys-CTT-6	Lys-CTT-7	Lys-CTT-8
β	J												
α	K	Pro-AGG-2	Pro-AGG-3	Pro-CGG-1	Pro-CGG-2	Pro-CGG-3	Pro-TGG-1	Pro-TGG-2	Pro-TGG-3	Sec-TCA-1	Sec-TCA-2	Sec-TCA-3	Ser-AGA-1
β	L												
α	M	Thr-CGT-3	Thr-CGT-4	Thr-CGT-5	Thr-TGT	Trp-CCA-1	Trp-CCA-2	Trp-CCA-3	Tyr-ATA	Tyr-GTA	Val-AAC-1	Val-AAC-2	Val-AAC-3
β	N												
α	O	mt-Gln-TTG	mt-Glu-TTC	mt-Gly-TCC	mt-His-GTG	mt-Ile-GAT	mt-Leu-TAA	mt-Leu-TAG	mt-Lys-TTT	mt-Met-CAT	mt-Phe-GAA	mt-Pro-TGG	mt-Ser-GCT
β	P												

Condition		13	14	15	16	17	18	19	20	21	22	23	24
α	A	Arg-ACG-1	Arg-ACG-2	Arg-ACG-3	Arg-CCG-1	Arg-CCG-2	Arg-CCT-1	Arg-CCT-2	Arg-CCT-3	Arg-CCT-4	Arg-TCG-1	Arg-TCG-2	Arg-TCT-1
β	B												
α	C	Cys-GCA-1	Cys-GCA-2	Cys-GCA-3	Cys-GCA-4	Cys-GCA-5	Cys-GCA-6	Cys-GCA-7	Cys-GCA-8	Cys-GCA-9	Gln-CTG-1	Gln-CTG-2	Gln-CTG-3
β	D												
α	E	Gly-CCC-1	Gly-CCC-2	Gly-CCC-3	Gly-GCC-1	Gly-GCC-2	Gly-GCC-3	Gly-GCC-4	Gly-TCC-1	Gly-TCC-2	Gly-TCC-3	Gly-TCC-4	Gly-TCC-5
β	F												
α	G	Leu-AAG-2	Leu-CAA-1	Leu-CAA-2	Leu-CAA-3	Leu-CAA-4	Leu-CAA-5	Leu-CAA-6	Leu-CAG	Leu-TAA-1	Leu-TAA-2	Leu-TAA-3	Leu-TAA-4
β	H												
α	I	Lys-TTT-1	Lys-TTT-2	Lys-TTT-3	Met-CAT-1	Met-CAT-2	Met-CAT-3	Met-CAT-4	iMet-CAT	Phe-GAA-1	Phe-GAA-2	Phe-GAA-3	Pro-AGG-1
β	J												
α	K	Ser-AGA-2	Ser-CGA-1	Ser-CGA-2	Ser-GCT-1	Ser-GCT-2	Ser-GCT-3	Ser-TGA-1	Ser-TGA-2	Thr-AGT-1	Thr-AGT-2	Thr-CGT-1	Thr-CGT-2
β	L												
α	M	Val-AAC-4	Val-AAC-5	Val-CAC-1	Val-CAC-2	Val-TAC-1	Val-TAC-2	Val-TAC-3	mt-Ala-TGC	mt-Arg-TCG	mt-Asn-GTT	mt-Asp-GTC	mt-Cys-GCA
β	N												
α	O	mt-Ser-TGA	mt-Thr-TGT	mt-Trp-TCA	mt-Tyr-GTA	mt-Val-TAC	**U6**	**5S rRNA**	**18S rRNA**	**RNA Spike-In**	**PPC**	**GDC**	**Blank**
β	P												

Figure 2 The array layout for nrStar™ Human tRNA PCR Array

■ Kit Contents

Table 1 List of Human tRNAs and the Controls

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

38	Cys-GCA-2	78	Ile-AAT-3	118	Phe-GAA-2	158	Val-AAC-5
39	Cys-GCA-3	79	Ile-AAT-4	119	Phe-GAA-3	159	Val-CAC-1
40	Cys-GCA-4	80	Ile-AAT-5	120	Pro-AGG-1	160	Val-CAC-2

Description of the control assays

nrStar™ Human 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 avaliable 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. The contents are stable for at least 6 months.

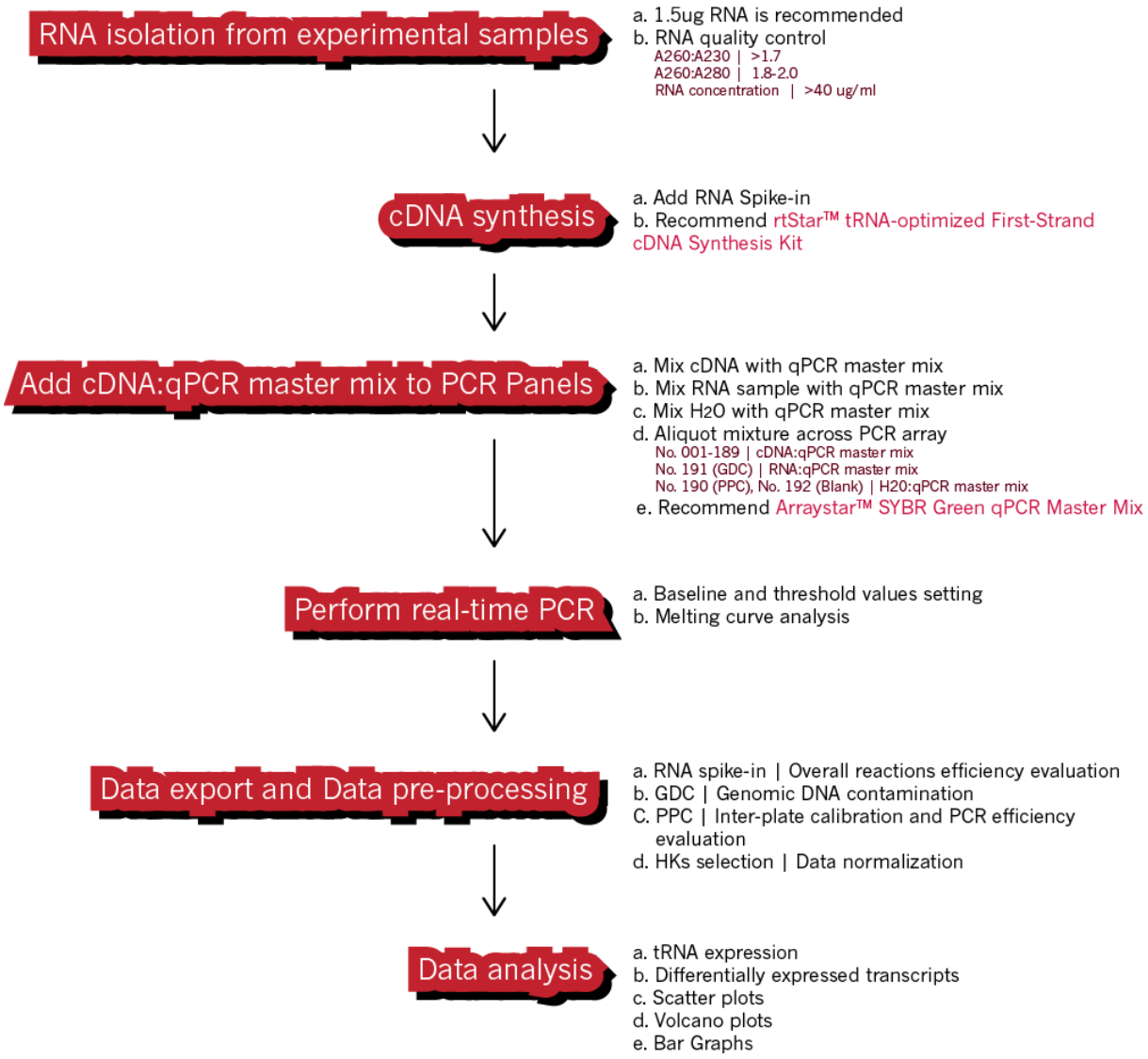
Additional Required Equipment

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

Additional Required Reagents

- rtStar™ tRNA-optimized 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.

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-optimized 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.

■ RNA demethylation

1. Prepare reagents

   Gently thaw the Demethylation Reaction buffer (5×) and Nuclease-free Water, and immediately place on ice. Mix by vortexing. Immediately before use, remove the Demethylase from the freezer, mix by flicking the tubes and place on ice. Spin down all reagents.

2. Combine reagents

   Combine reagents orderly according to the following table. If performing RNA demethylation on multiple RNA samples, it is recommended to prepare a demethylation master mix of the Demethylation Reaction Buffer (5×), Demethylase and Nuclease-free Water (in the proportion indicated in Table). Considering the pipetting losses, 10% excess of all reagents is recommended.

Nuclease-free Water	Variable
Demethylation Reaction Buffer (5×)	10 μl
Demethylase	5 μl
RNase Inhibitor	1 μl
Input RNA	≤5 μg
Total volume	50 μl

3. Perform & stop RNA demethylation reaction

   Incubate the above mix at 37°C for 100 min. Then orderly add 40 μl Nuclease-free Water and 10 μl Demethylation Stop Buffer (5×) to terminate the reaction.

4. RNA precipitation

   a. Add 400 μl phenol: chloroform to the sample. Mix well by inverting. Incubate at room temperature for 10 min. Centrifuge at 12,000 rpm for 10min.

   b. Carefully pipette off top layer to RNase-free tube and discard bottom to phenol waste.

   c. Add 400 μl chloroform to sample, mix well then microfuge at 12,000 rpm for 10 min.

   d. Carefully pipette off top layer to RNase-free tube and discard bottom to phenol waste.

   e. Add 1ml isopropanol to the aqueous phase. Mix well by inverting. Incubate at room temperature for 10 min. Centrifuge at 12,000 rpm for 10 min.

   f. Remove the supernatant from the tube, leaving only the RNA pellet.

   g. Add 1ml 75% ethanol (in DEPC-treated water). Mix well by inverting.

   h. Centrifuge the tube at 7,500 rpm for 5min at 4°C. Discard the wash.

   i. Vacuum or air dry the RNA pellet for 5–10min.

   j. Resuspend the RNA pellet in 12 μl Nuclease-free Water.

   k. Incubate in a water bath or heat block set at 55–60°C for 10–15 min.

First strand cDNA synthesis

NOTE: The recommended amount of starting material can vary from 10 pg to 5 μg of total RNA according to the expression of interested RNA.

5. Prepare reagents

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

   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.

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.

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-optimized 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_2O	200 μl
Total volume	2000 μl

5. Loading the PCR Array plate.

   NOTE: The reagents should load to the related well number corroding 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. Add10 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 ℃	10 minutes
40	95 ℃	15 seconds
	60 ℃	60 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:

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

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
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.

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

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 abroad range of samples by our stringent algorithm that evaluates the optimal properties and the number for endogenous controls.

Data analysis

1. Calculate the ΔΔCt for each tRNA

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

or

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

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

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

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.

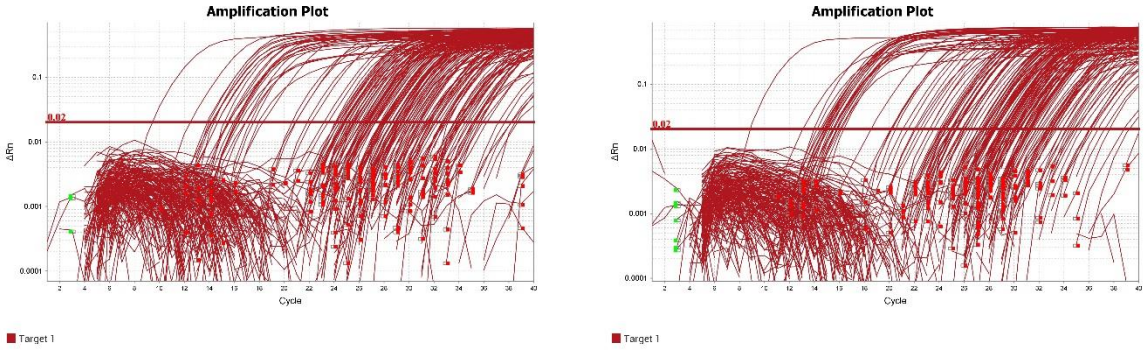
4. Other analyses such as scatter plots, volcano plots, list of differentially expressed genes and bar graph of expression differences for the tRNAs are performed and included in the standard analysis package.

III. Quality Control and Sample Data

A. nrStar™ Human tRNA PCR Array validation

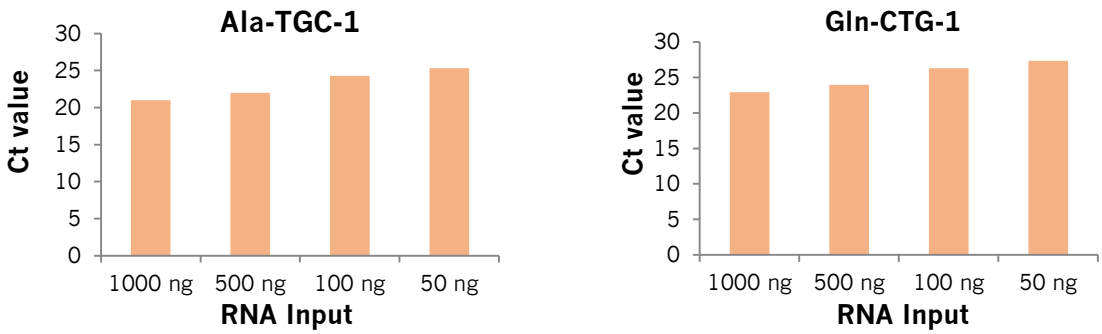
Real-time qPCR Validation

The performance of Human tRNA Panel was tested using a cohort of pancreatic carcinoma and para-carcinoma tissues. The extracted RNA samples were converted to cDNA using rtStar™ tRNA-optimized 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 pancreatic carcinoma and para-carcinoma tissues.



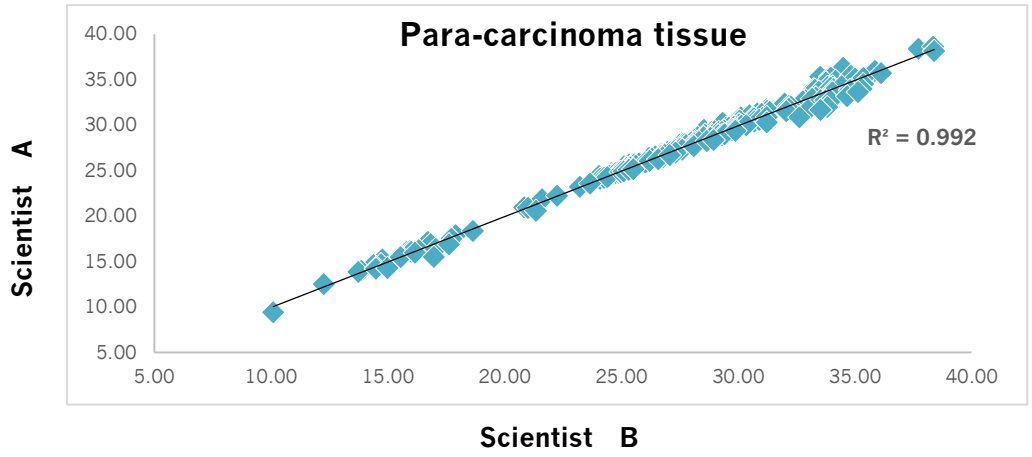
Sensitivity Test

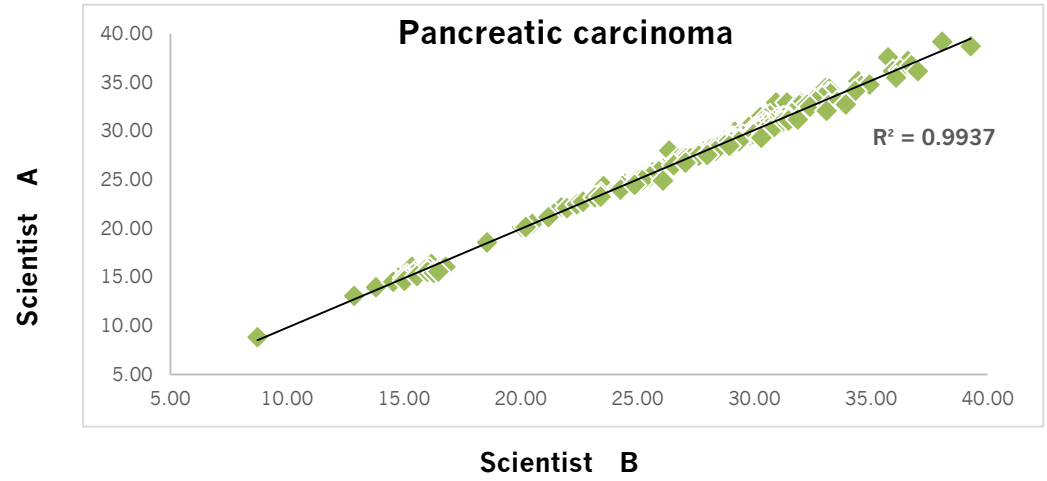
The decreasing input amounts of total RNAs from human glioblastoma cell lines were converted to cDNAs and profiled by the PCR arrays. The Ct values were determined using the software default automatic baseline and Ct settings. Ala-TGC-1 and Gln-CTG-1 transcripts were detected at Ct values of 25.2 and 27.0 respectively, with the input RNA amount as low as 50 ng.



Reproducibility Test

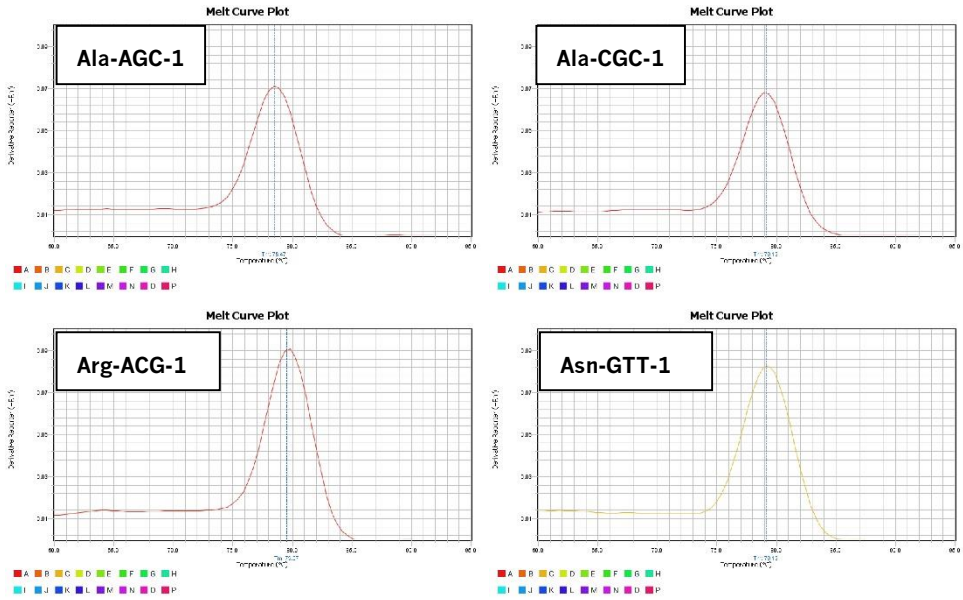
Two independent runs of nrStar™ Human tRNA PCR Array were conducted by two different scientists A and B at two different times using two different tissues. The results demonstrate a high degree of reproducibility with correlation R^2>0.98.





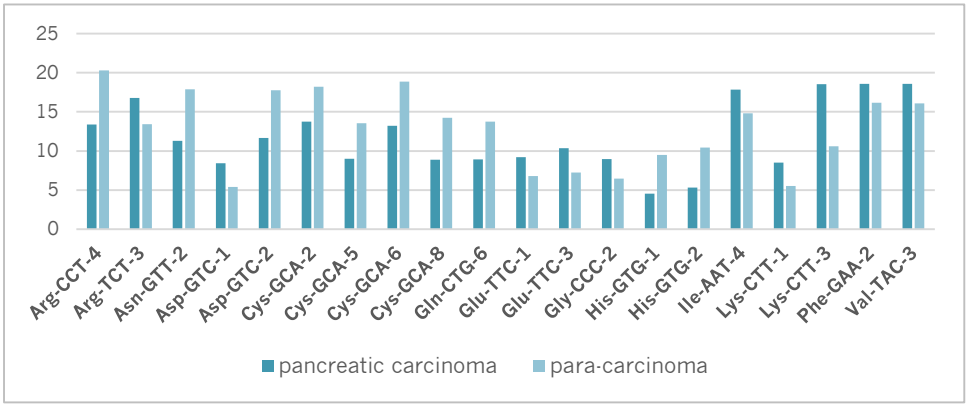
Specificity Test

The amplification products of transcripts of Ala-AGC-1, Ala-CGC-1, Arg-ACG-1, and Asn-GTT-1 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 Human tRNA transcript levels in tissues

The sample data were generated from RNAs extracted from human cancer tissues. The normalization was carried out using the average of the Internal Control Reference genes. Ct for the gene transcripts in pancreatic carcinoma and para-carcinoma tissues are graphed in the bar chart below.



IV. 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 STEP C.6 from 60°C to 50°C.
Baseline and threshold settings	• Follow the instructions of the qPCR system manufacturer. • Contact their technical support as necessary.

V. References

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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™ Human 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.