# nrStar™ Human tRF&tiRNA PCR Array

Cat#: AS-NR-002

## 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™ Human tRF&tiRNA PCR Array profiles 185 tRFs&tiRNAs, of which 118 are derived from tRF and tiRNA database and the other 67 from recently published papers. These tRFs&tiRNAs represent the most prevalently detected ones, and thus imply critical bio-functional significances.

To ensure high data quality, the panel includes 7 control sets for tRFs&tiRNAs 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 nuclear or small nucleolar RNA genes are included in the array as the quantification references for tRFs&tiRNAs.

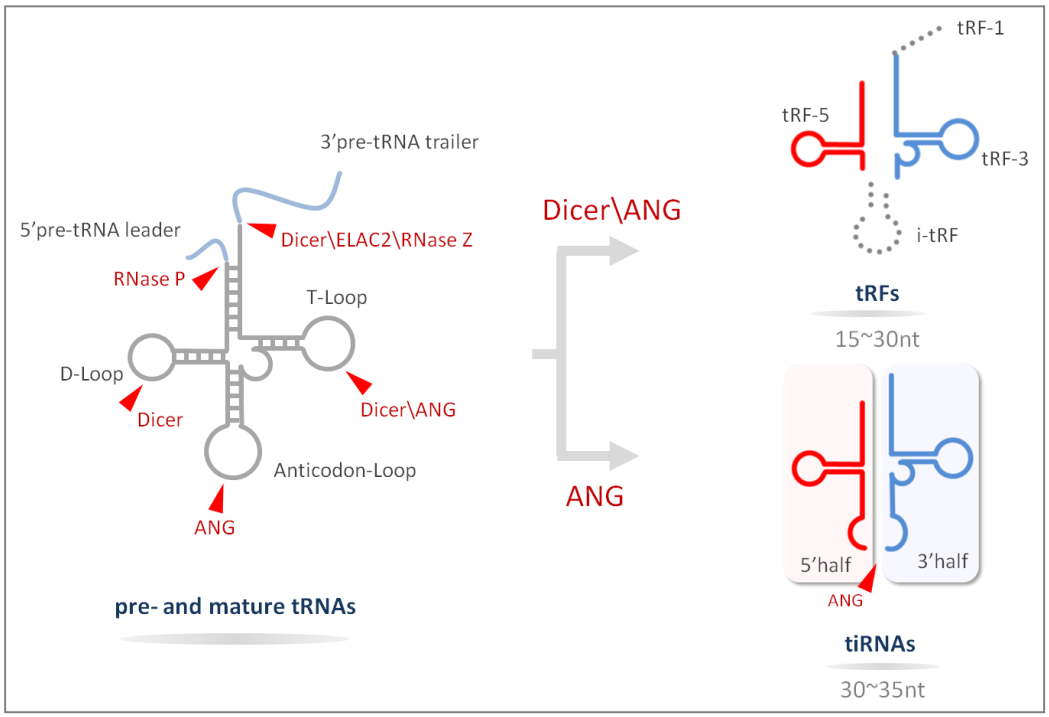


**Figure 1.** Biogenesis of tRFs&tiRNAs. tRF-1 is generated from the 3'-trailer of primary tRNA. tRF-5, i-tRF and tRF-3 are produced from the 5'-, internal- and 3'-portions of the mature tRNA, respectively. When the cleavage site is within the anticodon loop, two fragments are generated as tiRNAs for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

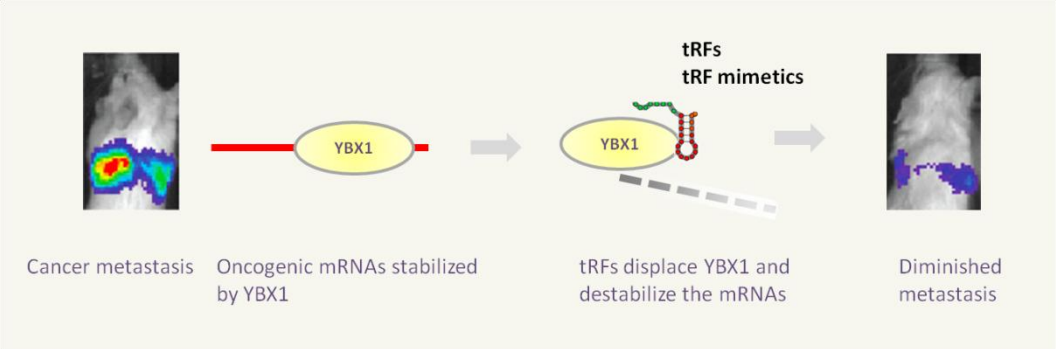


**Figure 3.** tRFs or the mimetics displace oncogenic RNA binding protein YBX1 and destabilize many cancer driver mRNAs. Consequently, the cancer metastasis is greatly diminished [11].

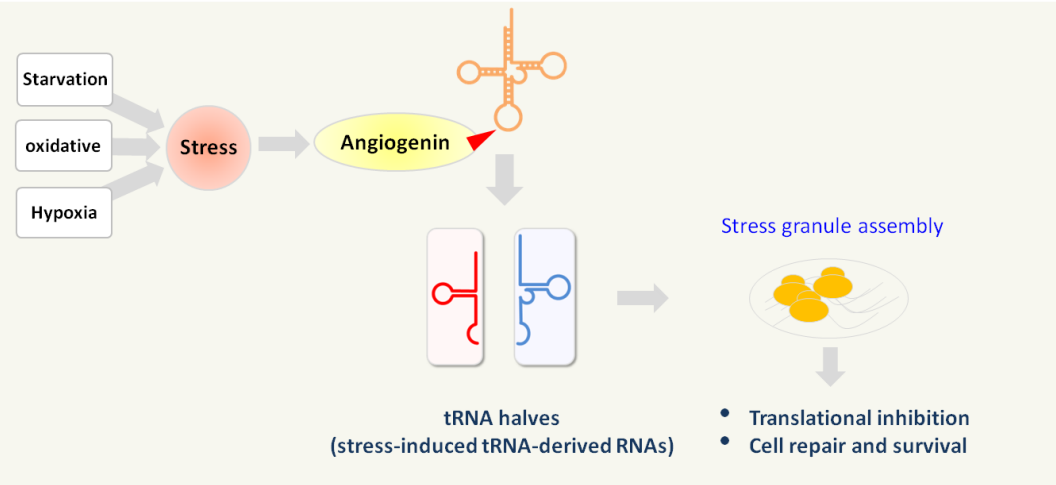


**Figure 4.** tRNA halves (tiRNAs) are generated by angiogenin cleavage in response to cellular stresses, which promote stress granule assembly and induce translational inhibition,

# C. Human Diseases

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

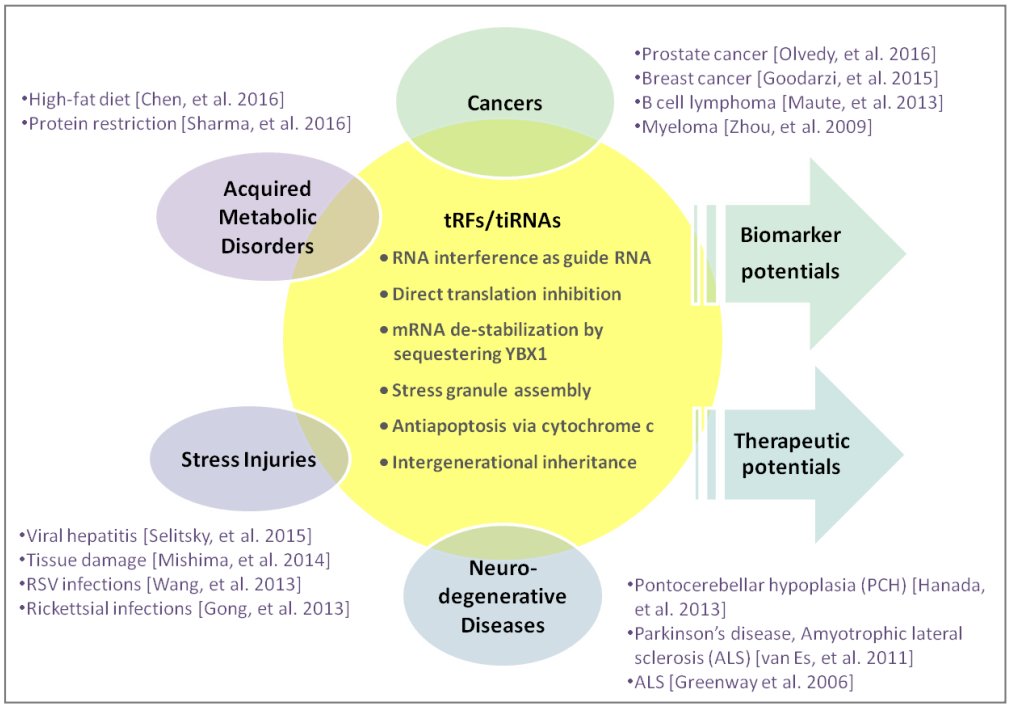


**Figure 5.** tRF&tiRNA molecular functions and diseases.

## 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].

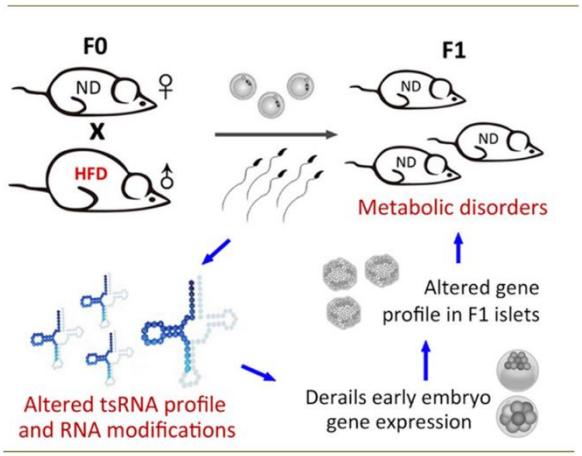


**Figure 6.** tRNA-derived small RNAs (tsRNAs, mostly tRNA halves) have altered expression profiles and RNA modifications in the sperms of high fat diet fed mice. The sperm tsRNAs confer the metabolic disorder phenotypes in the offspring by oocyte injection. This tsRNA paternal epigenetic factor mediates 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].

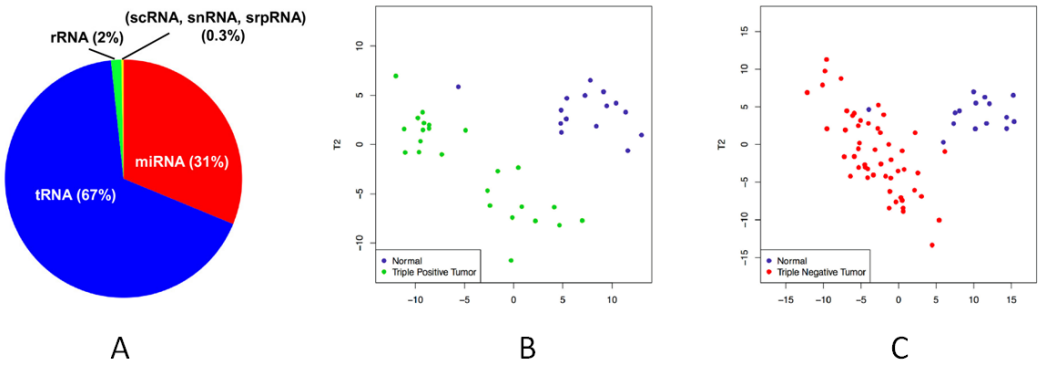


**Figure 7.** The abundant presence of tRNA derived fragments in serum [21] (A). tRF profiles discriminate triple-negative (B), triple positive breast cancer cells (C) from the normal controls in PLS-DA analyses.

# II. Product Summary

## A. Kit Contents

The nrStar™ Human tRF&tiRNA PCR Array profiles 185 tRFs&tiRNAs, of which 101 are derived from tRF and tiRNA database and the other 84 from recently published papers. To ensure high data quality, the panel includes 7 control sets for tRFs&tiRNAs 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 nuclear or small nucleolar RNA genes are included in the array as the quantification references for tRFs&tiRNAs.

## B. Description of the Control Assays

There are four types of control assays built in the nrStar™ Human tRF&tiRNA 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 (Protocol Step B2). 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 nuclear or small nucleolar RNA genes RNU6 (**CT1**), SNORD43 (**CT2**), and SNORD95 (**CT3**) are included in the array as the quantification references for tRFs&tiRNAs.

- **PPC** (Positive PCR control): 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 (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.

## C. Array Layout

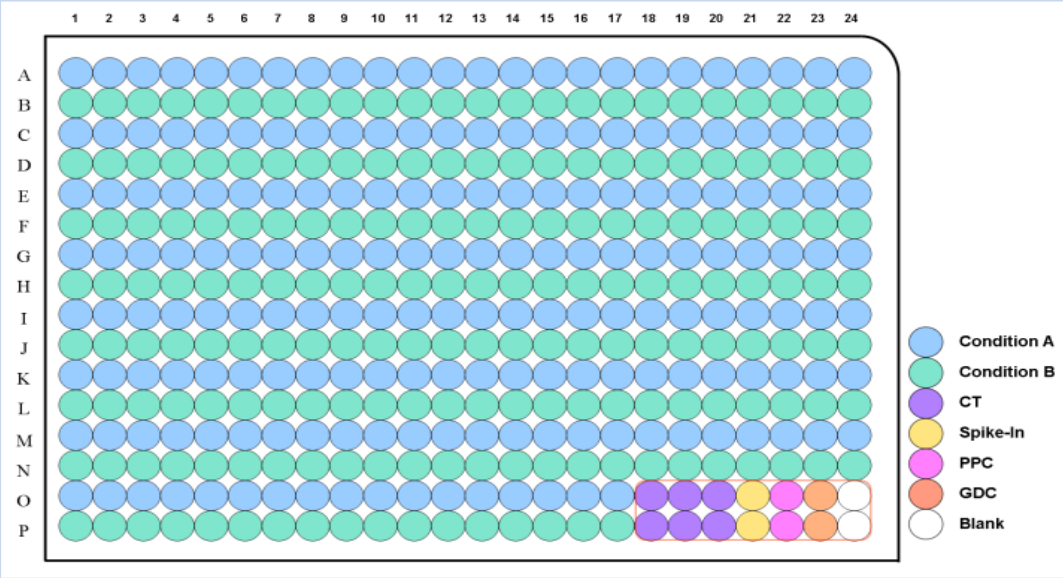


**Figure 8.** nrStar™ Human tRF&tiRNA PCR Array layout

# D. List of tRFs&tiRNAs and Controls

| Condition | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | A | 3'tiR_007_GluTTC (n) | 3'tiR_012_ArgCCT (n) | 3'tiR_026_GlnCTG (n) | 3'tiR_028_HisGTG (mt) | 3'tiR_037_ArgCCG (n) | 3'tiR_056_ValTAC (mt) | 3'tiR_060_MetCAT (n) | 3'tiR_063_ArgCCT (n) | 3'tiR_075_GluTTC (mt | 3'tiR_078_ArgTCT (n) | 3'tiR_080_ProTGG (mt) | 3'tiR_082_ThrTGT (mt) |
| B | B | | | | | | | | | | | | |
| A | C | tiRNA-5033-GluTTC-1 | tiRNA-5029-GlyGCC-2 | tiRNA-5034-ValCAC-2 | tiRNA-5034-ValCAC-3 | tiRNA-5030-HisGTG-1 | tiRNA-5030-GluTTC-1 | tiRNA-5033-GluTTC-2 | tiRNA-5030-LysCTT-2 | tiRNA-5029-ProAGG | tiRNA-5031-GluTTC-1 | tiRNA-5031-HisGTG-1 | tiRNA-5031-GluCTC-1 |
| B | D | | | | | | | | | | | | |
| A | E | tiRNA-5032-GluTTC-1 | tiRNA-5035-GluCTC | tiRNA-5030-SerGCT-3 | tiRNA-5030-SerGCT-1 | tiRNA-5031-HisGTG-2 | tiRNA-5029-AlaAGC-1 | tiRNA-5032-LysCTT-1 | 1001 | 1003 | 1004 | 1005 | 1006 |
| B | F | | | | | | | | | | | | |
| A | G | 1031 | 1032 | 1033 | 1035 | 1036 | 1037 | 1038 | 1039 | 1040 | 1041 | 1042 | 3002B |
| B | H | | | | | | | | | | | | |
| A | I | 3009B | 3011/12A | 3016/18/22B | 3017A | 3017B | 3019/20/21B | 3026B | 3027/28B | 3019A | 3020/21A | 3022A | 3026/27/28A |
| B | J | | | | | | | | | | | | |
| A | K | 5013B | 5015/17A | 5016A | 5019A | 5019B | 5020/21A | 5020B | 5021B | 5022A | 5022B | 5023B | 5024A |
| B | L | | | | | | | | | | | | |
| A | M | TRF250 | TRF272/274 | TRF273 | TRF293/294 | TRF305/306/307 | TRF308 | TRF312 | TRF316 | TRF318 | TRF320 | TRF321 | TRF322 |
| B | N | | | | | | | | | | | | |
| A | O | TRF393 | TRF396 | TRF417 | TRF457 | TRF460 | TRF462 | TRF463 | TRF466/468/469/471/472/473 | TRF490 | TRF492 | TRF493 | TRF511 |
| B | P | | | | | | | | | | | | |

| Condition | | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | A | 3'tiR_088_LysCTT (n) | 5003/4C | 5008C | 5009C | 5016C | 5026/27C | TRF62 | TRF315 | TRF327 | TRF353 | TRF419 | tiRNA-5033-ProTGG-1 |
| B | B | | | | | | | | | | | | |
| A | C | tiRNA-5034-GlyCCC-1 | tiRNA-5034-GluTTC-1 | tiRNA-5033-LysTTT-1 | tiRNA-5032-LysTTT-1 | tiRNA-5031-PheGAA | tiRNA-5034-ValTAC-3 | tiRNA-5030-GlnTTG-3 | tiRNA-5029-GlyGCC-3 | tiRNA-5035-GluTTC-1 | tiRNA-5035-GluTTC-2 | tiRNA-5034-GluTTC-2 | tiRNA-5035-GluTTC-3 |
| B | D | | | | | | | | | | | | |
| A | E | 1007 | 1010 | 1012 | 1013 | 1015 | 1020 | 1025 | 1026 | 1027 | 1028 | 1029 | 1030 |
| B | F | | | | | | | | | | | | |
| A | G | 3004B | 3006B | 3031B | 3033A | 3030A | 3030B | 3002A | 3003A | 3003B | 3006A | 3008A | 3008B |
| B | H | | | | | | | | | | | | |
| A | I | 3029A | 3031A | 5001A | 5001B | 5002A | 5002B | 5008B | 5009A | 5009B | 5010A | 5011A | 5012B |
| B | J | | | | | | | | | | | | |
| A | K | 5026A | 5026/27B | 5028/29A | 5028/29B | 5032A | 5032B | TRF21-26 | TRF63 | TRF23 | TRF205 | TRF208 | TRF223 |
| B | L | | | | | | | | | | | | |
| A | M | TRF323/324/326 | TRF337-339 | TRF347 | TRF351 | TRF354 | TRF356/359 | TRF368 | TRF366 | TRF365 | TRF373 | TRF374 | TRF375 |
| B | N | | | | | | | | | | | | |
| A | O | TRF524 | TRF533/534 | TRF537 | TRF546/547 | TRF550/551 | RNU6 | SNORD 43 | SNORD 95 | Spike-In | PPC | GDC | Blank |
| B | P | | | | | | | | | | | | |

**Figure 9.** List of tRFs&tiRNAs and controls

# E. Shipping and Storage

nrStar™ Human tRF&tiRNA PCR Array 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

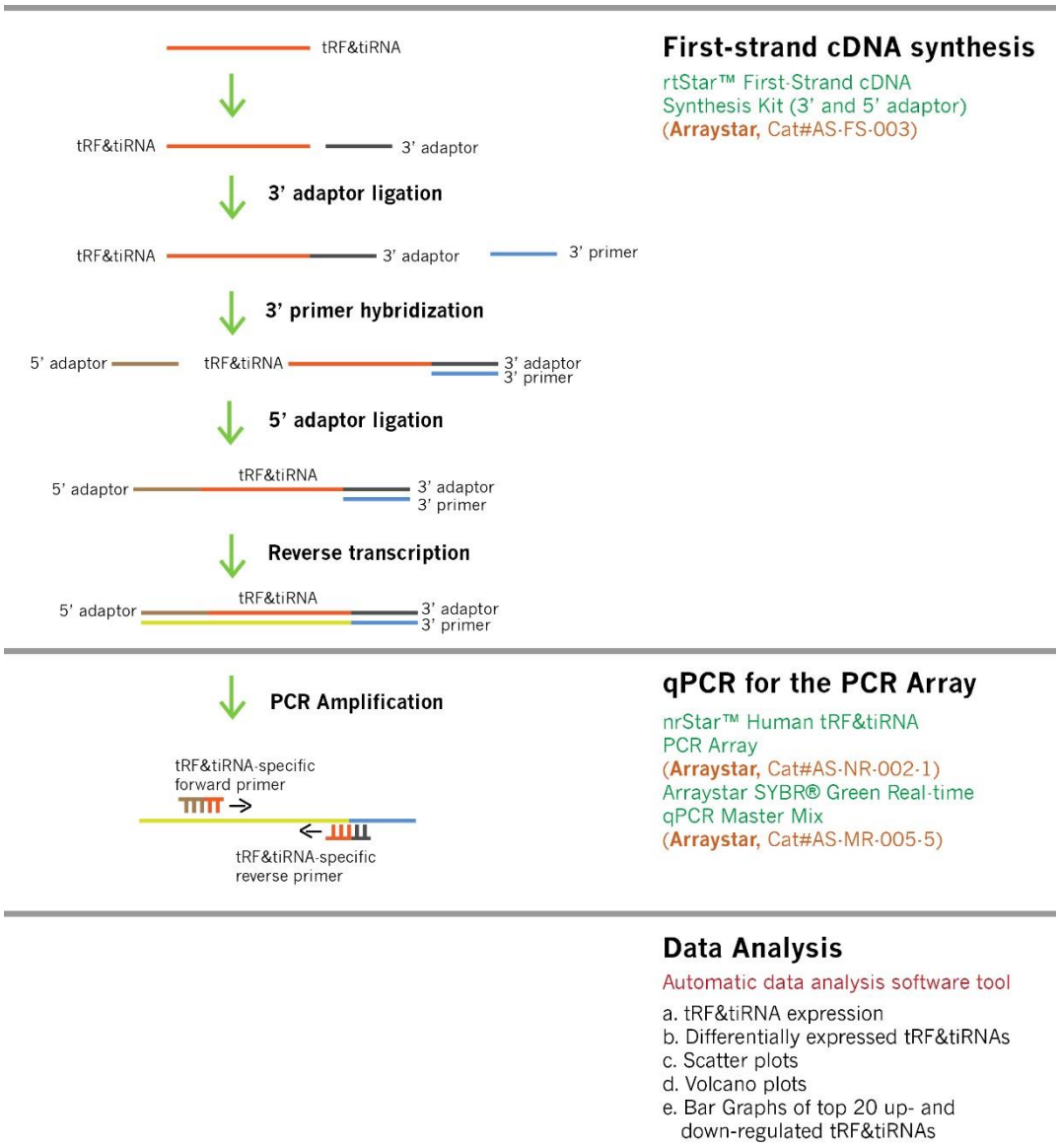


**Figure 10.** qPCR array workflow of tRFs&tiRNAs

## 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&tiRNAs expression profiling by qPCR, which is particularly important for tRFs&tiRNAs 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&tiRNA fraction (13-50 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

1. Dilute the cDNA in nuclease free water. If rtStar™ First-Strand Synthesis Kit (3' and 5' adaptor) (Cat# AS-FS-003) is used for the cDNA synthesis with 10 ng - 2.0 µg total RNA

sample as the starting material, dilute the cDNA product 1/20 in water. The diluted material is used as the qPCR template.

2. For GDC Controls, combine 1 µL NRT (no RT) sample or 1 µL RNA sample, 5 µL SYBR Green Master Mix, and 4 µL Nuclease-free water. Mix well and spin down, and dispense 10 µL to the GDC well.

3. For Blank Controls, combine 5 µL SYBR Green Master Mix and 5 µL Nuclease-free water. Mix well and spin down, and dispense 10 µL to the Blank well.

4. Use Arraystar™ SYBR Green qPCR Master Mix (Cat# AS-MR-006-5) to prepare qPCR Master Mix for each sample per qPCR well. There are two replicates with total 384 reactions in a 384-well qPCR array plate. Add some extra (x1.1) reactions as needed by the liquid handling operation. Multiply 190 wells (384 ÷ 2 · 2) with the individual amounts of the components in the table below and prepare a qPCR Mix.

| | |
|---|---|
| SYBR Green Master Mix | 1045 µL |
| Diluted cDNA template | 800 µL |
| $ddH_2O$ | 245 µL |
| Total volume | 2090 µL |

**Note:** Total volume: $(384 \div 2^{replicates} \cdot 1^{GDC} \cdot 1^{blank}) \times 10\ \mu L \times 1.1 = 2090\ \mu L$

5. Dispense 10 µL of the Mix uniformly to each of the rest 190 wells on the qPCR array plate.

6. Run the qPCR using the following program. Consult the instructions for the instrument for details.

| Cycles | Temperature | Time |
|---|---|---|
| 1 | 95℃ | 10 minutes |
| 40 | 95℃ | 10 seconds |
| | 60℃ | 1 minute |
| 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&tiRNAs 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 tRF&tiRNA. These most stably expressed tRF&tiRNA 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&tiRNAs 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 Human tRF&tiRNA PCR Panel was tested using a cohort of 10 cell line 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 the cell lines.

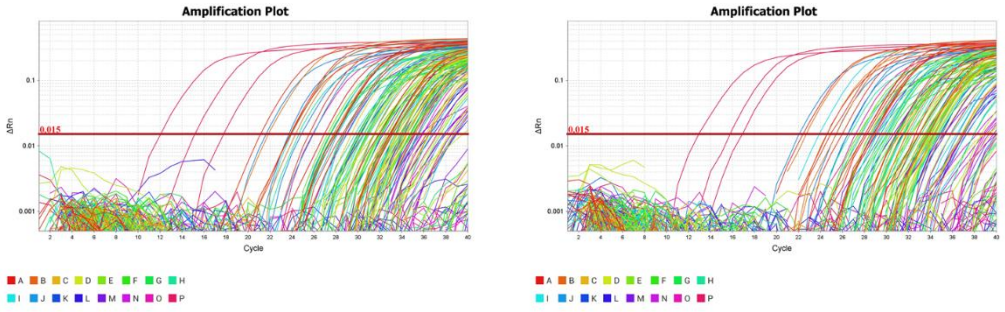


**Figure 11.** Real-time qPCR Validation of LN-299 (left) and LN-18 (right)

## ■ Sensitivity Test

The decreasing input amounts of total RNAs from human cancer 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. tRF-1001 and tRF-3002B were detected at Ct values of 25.2 and 27.0 respectively, with the input RNA amount as low as 50 ng.

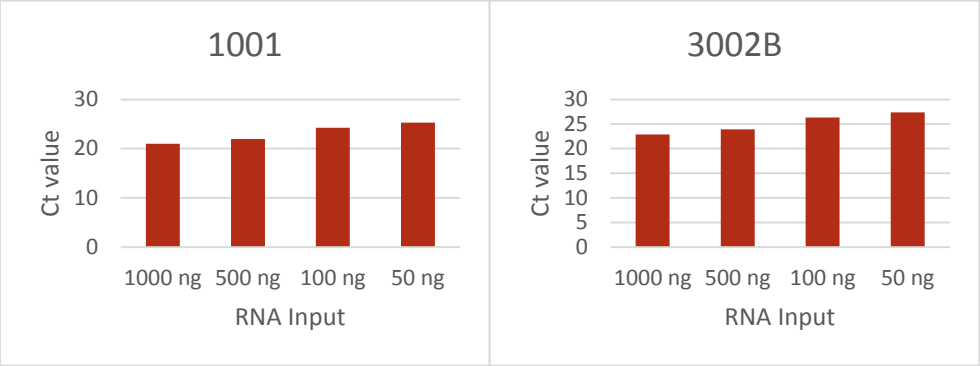


**Figure 12.** Sensitivity test of two tRFs&tiRNAs.

## ■ Reproducibility Test

Two independent runs of Human tRF&tiRNA 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.

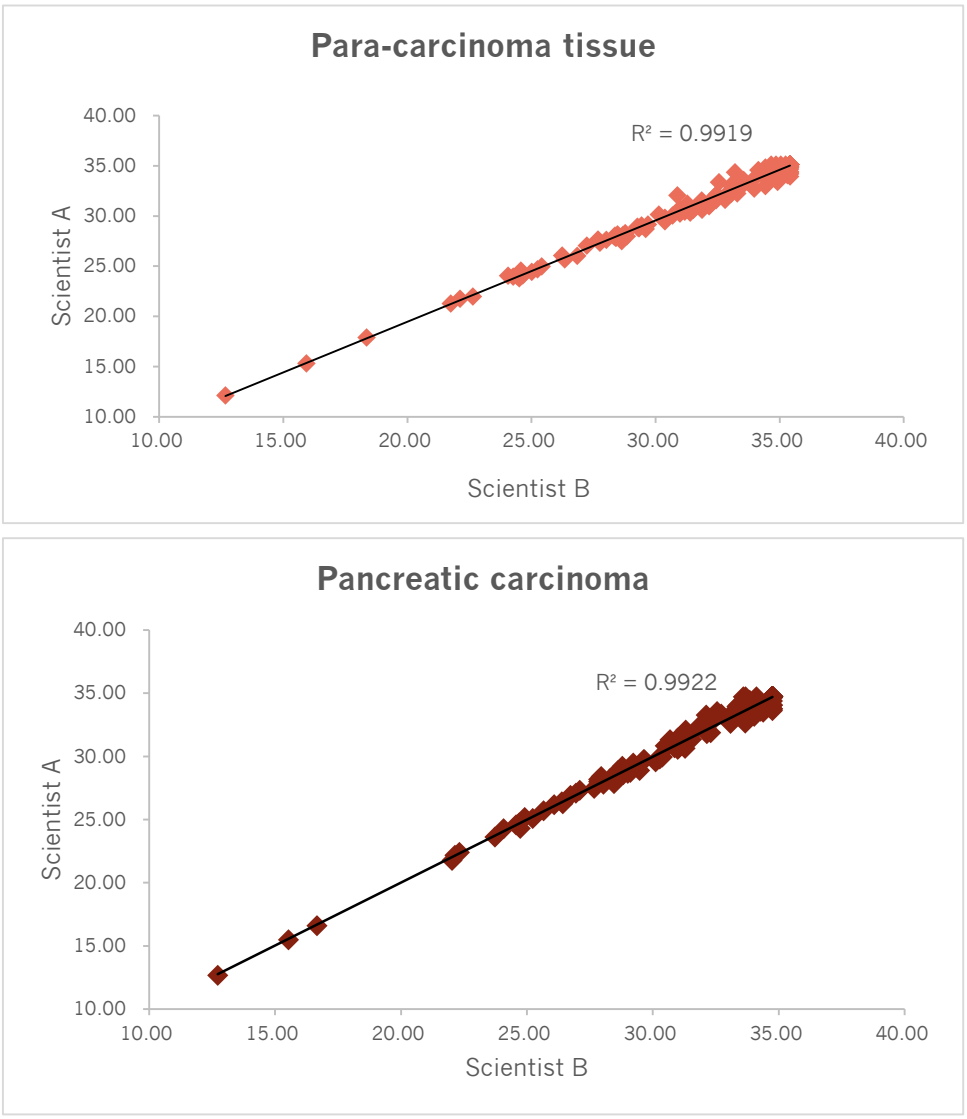


**Figure 13.** Reproducibility test in pancreatic carcinoma and para-carcinoma tissue

■ **Specificity Test**

The amplification products of 4 tRF&tiRNA 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.

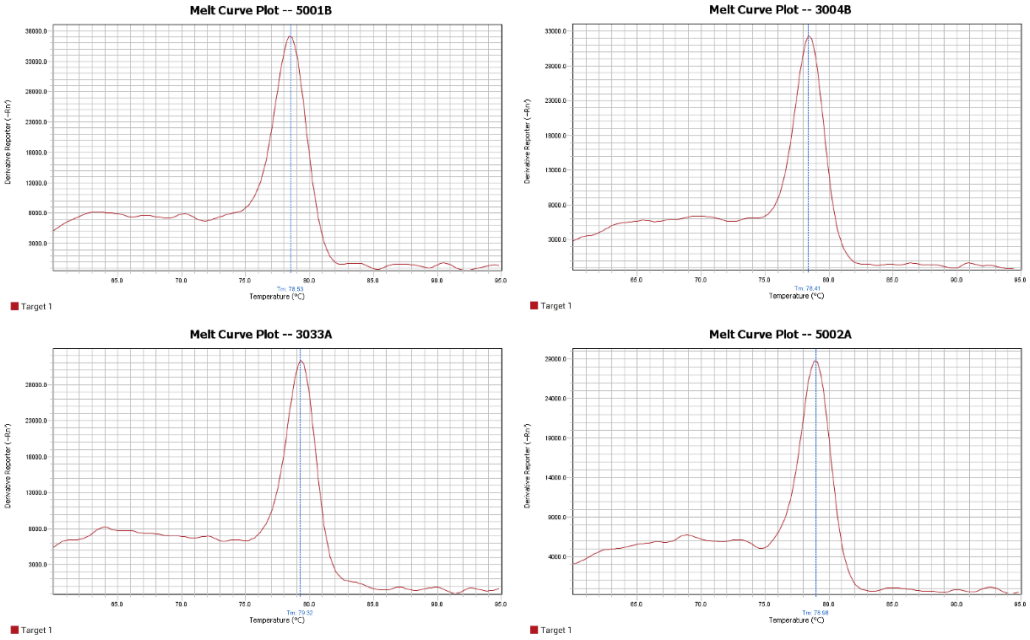


**Figure 14.** Melt curve plot of 4 tested tRFs&tiRNAs

## B. Sample Data

The sample data were generated from RNAs extracted from pancreatic carcinoma and para-carcinoma tissue. The normalization was carried out using the average of the reference genes. ΔCt for the tRF&tiRNA in pancreatic carcinoma (Test) vs. para-carcinoma tissue (Control) are graphed in the bar chart below.

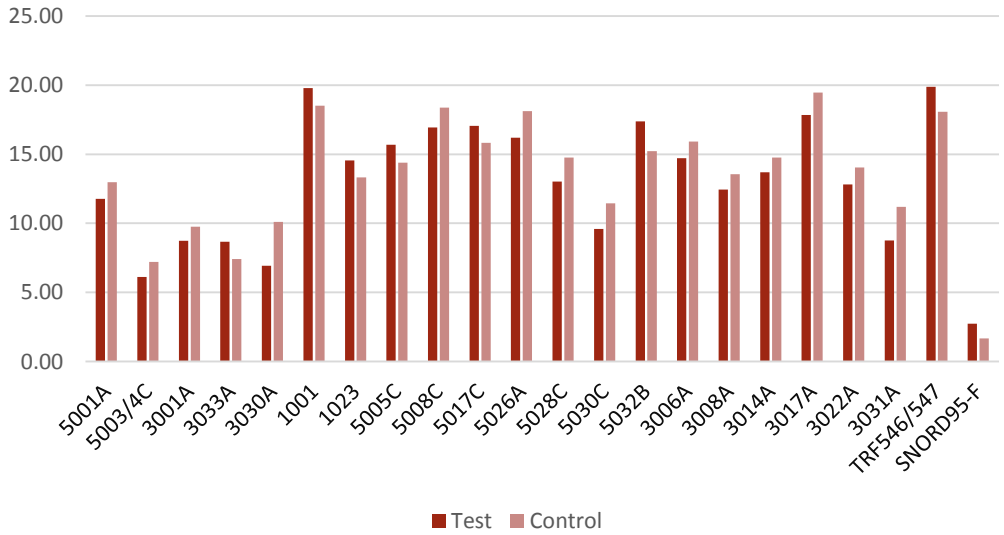


**Figure 15.** Differently expressed tRFs&tiRNAs between carcinoma (Test) and para-carcinoma tissue (Control).

# 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™ Human tRF&tiRNA 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.