NuRNA™ Human Epitranscriptomics PCR Array

Cat#: AS-NM-003

Instruction Manual Version 1.0

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I. Introduction

A. Overview

In recent years, major breakthroughs in RNA-modification-mediated regulation of gene expression have led to the emerging field of epitranscriptomics. RNA modifications, including N6-methyladenosine (m6A), 5-methylcytidine (m5C), inosine (I), pseudouridine (ψ), N1-methyladenosine (m1A) and 5-hydroxylmethylcytidine (hm5C), are intrinsically present in eukaryotic mRNAs and found to influence mRNA metabolism and functions (Fig. 1). Research and knowledge of epitranscriptomic marks and their writers, readers and erasers have recently advanced rapidly. Their mutational changes are coupled with a rapidly growing list of human diseases and disorders. The dynamics of RNA modifications and their writers, readers and erasers have a critical but still poorly understood role in gene regulation. To help quick and convenient analyses of the expression of the writers, readers and erasers of epitranscriptional marks, Arraystar has produced the first commercial PCR panel for profiling the epitranscriptional regulators. The panel contains gene expression qPCR assays for 89 enzymes or proteins in post-transcriptional mRNA modifications, including m1A, m6A, m5C, hm5C, ψ, and I, These experimentally studied or computationally predicted modifiers are comprehensively compiled from research publications and authoritative databases including UniProt and Modomics. Combined with other techniques such as high-throughput LC-MS/MS and next-gen sequencing that profile and map mRNA modifications, this array is an essential and powerful tool to study the functional roles of epitranscriptomic marks in gene regulation and the implications of the dynamic regulation of these marks in biological processes and human disorders.

B. RNA modifications and their functions

More than 100 distinct chemical modifications to RNA have been characterized to date. Many are present in abundant non-coding RNAs, such as ribosomal RNA (rRNA), transfer RNA (tRNA) and small nuclear RNA (snRNA), to maintain the proper RNA structure and functions [1]. Some modifications, for example, N6-methyladenosine (m6A), 5-methylcytidine (m5C), inosine (I), pseudouridine (ψ), N1-methyladenosine (m1A) and 5-hydroxylmethylcytidine (hm5C), are found intrinsically in eukaryotic mRNA and can regulate the metabolism and functions of the mRNAs (Fig. 1). Mutations and dysregulation of the writers, readers and erasers for these modifications are linked to human diseases and disorders [2-4]. The discovery and research in this field have become an emerging science of epitranscriptomics.

■ m6A

m6A is the most abundant internal mRNA modification in eukaryotes, which can modulate mRNA secondary structure, the accessibility of binding-protein complexes, mRNA splicing events, mRNA maturation, nuclear-cytoplasmic transport, translation, and sorting into mRNA metabolism tracks. m6A modification is catalyzed by a methyltransferase complex, or 'writers', which includes at least METTL3, METTL14 and WTAP. m6A can be demethylated and removed by FTO and ALKBH5 as the "erasers". m6A modification is recognized by multiple m6A-specific binding proteins YTHDF1 and YTHDF2 as the 'readers' to affect the metabolism and functions of the m6A-marked mRNAs in various ways. The dynamic regulation of m6A is fundamentally important in the regulation of meiosis and in pluripotency control [4]. The elevated m6A in cancer stem cells

ultimately up-regulates key oncoproteins such as EGFR, which confers a proliferative advantage to promote neoplastic evolution and lead to poorer prognosis. The m6A writers METTL3 [5] and erasers FTO [6] and ALKBH5 [7] are involved in cancer progression. In addition to mammalian RNA, m6A can also be installed on viral RNA to influence viral infection and production.

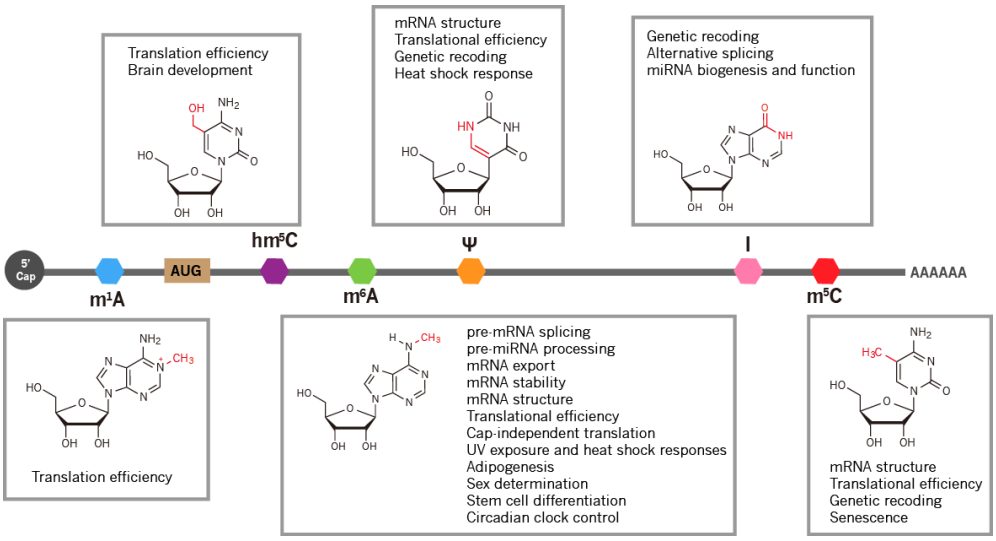


Figure 1. mRNA modifications and their functions.

m5C and hm5C

m5dC is a widespread epigenetic marker in DNA and has been extensively studied in DNA methylation. m5C is also found in abundant noncoding RNAs including tRNA and rRNA. In tRNA, m5C can stabilize the secondary structure and influence the anticodon stem-loop conformation. In rRNA, m5C can affect translational fidelity in ribosome. Two RNA methyltransferases, NSUN2 and DNMT2, catalyze m5C methylation in higher eukaryotes. Recent discoveries have also revealed important and surprising roles for m5C in RNA-mediated epigenetic transgenerational inheritance [8]. In mice, m5C methyltransferase DNMT2 is shown required for RNA-mediated epigenetic alterations in fur color and an overgrowth phenotype. In DNA, hm5dC is an important epigenetic marker generated by oxidation of m5dC catalyzed by the TET protein. The hm5C modification of RNA was first found in the rRNA of wheat seedlings. Recently, both mammalian and Drosophila TET proteins have been shown to oxidize m5C in RNA to hm5C or 5-formylcytidine. Many hm5C peaks are located in the coding sequences [9]. The distribution pattern different from that of m5C suggests hm5C and m5C having different roles .

A-to-I Editing

RNA editing events are widely observed in mRNA, tRNA, rRNA, and miRNA in all kingdoms of life. Adenosine-to-inosine (A-to-I) editing is the most prevalent type of RNA editing in higher eukaryotes and can be catalyzed by the dsRNA-specific adenosine deaminases acting on RNA (ADARs). ADARs have a preference for dsRNA, and A-to-I editing occurs within both the coding sequence and the non-coding regions of an mRNA transcript. A-to-I editing plays numerous roles in modulating gene expression, including recoding codons, altering alternative splicing and regulating miRNA biogenesis and function. Deficiency of A-to-I editing activity

in certain mRNA transcripts has been implicated in amyotrophic lateral sclerosis (ALS) [10] and cancer [11]. Alterations in ADAR editing activity levels have been linked to stem cell maintenance, hereditary autoimmune diseases, and disease progression in cancer, suggesting the potentials of using A-to-I editing as a biomarker of severity for certain diseases or conditions [12].

Pseudouridine

Pseudouridine (ψ), the "fifth nucleotide" of RNA, is overall the most abundant and widespread modification in stable ncRNAs such as rRNA, tRNA and snRNA [13]. ψ is isomerized from uridine, catalyzed by two distinct mechanisms: the RNA-dependent mechanism with the box H/ACA ribonucleoproteins and the RNA-independent mechanism with the 'stand-alone' pseudouridine synthases. In these abundant ncRNAs, ψ plays important roles in regulating their function. For instance, in rRNA, ψ is required for proper rRNA folding and for ribosome translational fidelity [14, 15]; in tRNA, it stabilizes the RNA structure; and in snRNA, it affects snRNP biogenesis and mRNA splicing [16]. Recently, ψ was also found in mRNA, although the biological function of such mRNA pseudouridylation remains enigmatic. Nevertheless, ψ is abundant in mammalian mRNA, with a ψ/U ratio at about 0.2–0.6% in human cells and mouse tissues.

m1A

m1A is prevalent in tRNA and rRNA. Enzymatically, m1A methylation at position 58 of cytoplasmic tRNAs is catalyzed by TRMT6 and the TRMT61A complex; m1A58 in mitochondrial tRNAs is catalyzed by TRMT61B; and m1A at position 9 of metazoan mitochondrial tRNA is catalyzed by Trmt10C. m1A methylation stabilizes the tRNA tertiary structures and affects tRNA folding [17]. In human 28S rRNA, m1A at position 1,322 is modified by RRP8 (also known as NML) and is necessary for proper rRNA biogenesis [17, 18]. Recently, m1A modification was also found in mRNA [19, 20]. The topology of m1A is distinct from that of m6A, and it is significantly enriched at the 5' ends of mRNAs. ALKBH3 is an eraser of mRNA m1A modification.

C. Product summary

NuRNA™ Human Epitranscriptomics PCR Array profiles the gene expression of 89 validated or predicted enzymes or proteins responsible for post-transcriptional mRNA modifications, including m1A, m6A, m5C, hm5C, ψ, and I. These genes are compiled from research publications and authoritative databases including UniProt and Modomics.

■ Array plate layout

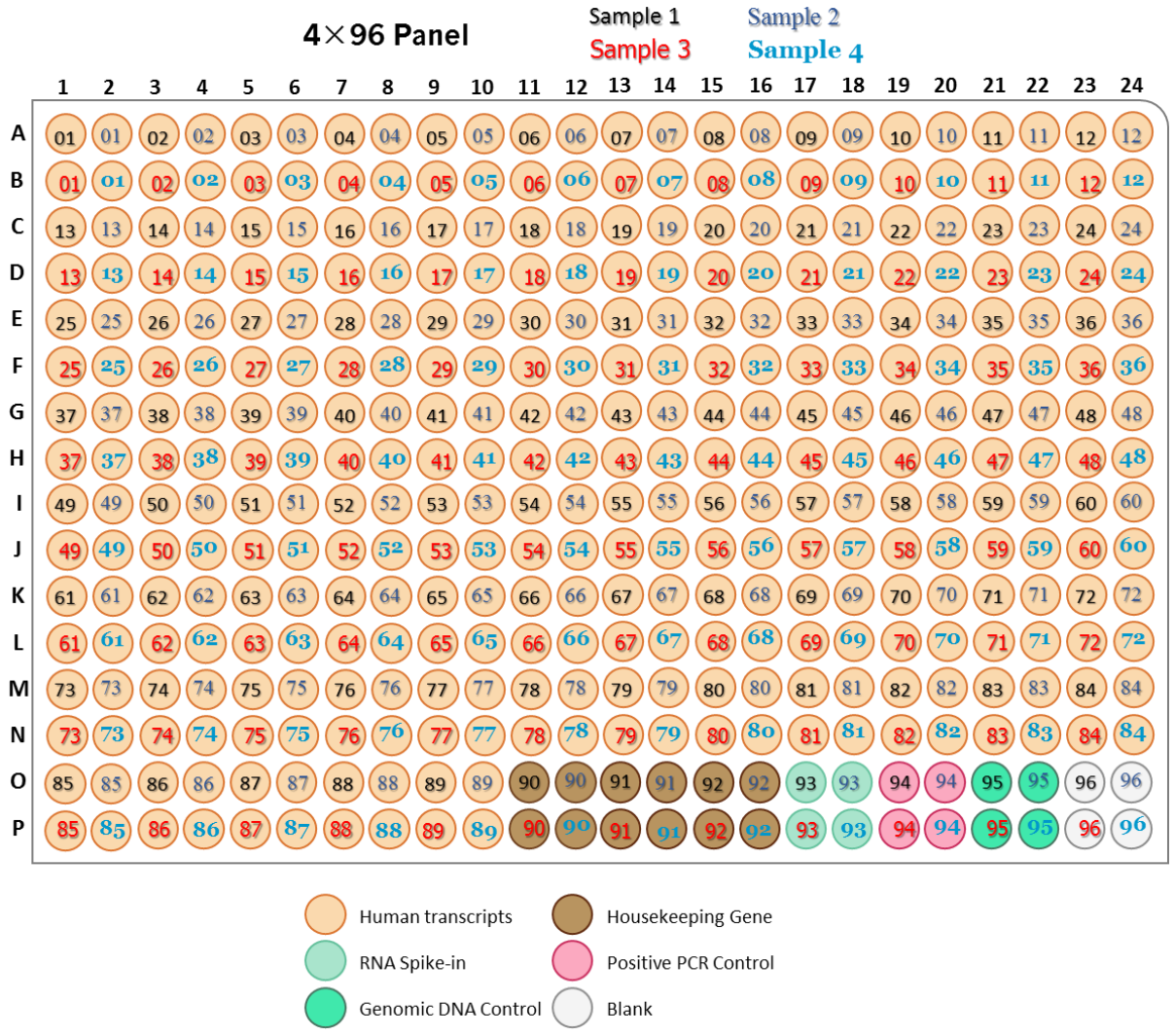


Figure 2. The array plate layout for NuRNA™ Human Epitranscriptomics PCR Array. The well positions are indicated as rows A-P and the columns 1-24. The assays for each sample are numbered from 1-96 as Well numbers, color coded for 4 samples.

■ Kit Contents

Table 1 List of qPCR assays for gene expression of the human epitranscriptomic factors and the control assays.

1	ADAR	25	TRMT10C	49	HNRNPC2	73	PUS1
2	ADARB1	26	TRMT6	50	YTHDC1	74	PUS3
3	ADARB2	27	TRMT61A	51	YTHDC2	75	PUS7
4	ADAD1	28	TRMT61B	52	YTHDF1	76	TRUB1
5	ADAD2	29	ALKBH3	53	YTHDF2	77	PUS10
6	ADAT1	30	ALKBH1	54	YTHDF3	78	PUS7L
7	ADAT2	31	NSUN2	55	ELAVL1	79	PUSL1
8	ADAT3	32	TRDMT1	56	KIAA1429	80	RPUSD1
9	TDG	33	MECP2	57	METTL14	81	RPUSD2
10	SMUG1	34	UHRF1	58	METTL3	82	RPUSD3
11	CHTOP	35	DNMT1	59	METTL4	83	RPUSD4
12	ERH	36	DNMT3A	60	WTAP	84	TRUB2
13	HMCES	37	DNMT3B	61	DGCR8	85	RBM15
14	MEP50	38	DNMT3L	62	ALKBH5	86	RBM15B
15	MGME1	39	NOP2	63	FTO	87	SRSF2
16	NEIL1	40	NSUN3	64	RNGTT	88	EIF3A
17	PRMT1	41	NSUN4	65	RNMT	89	EIF3B
18	PRMT5	42	NSUN5	66	CMTR1	90	GAPDH
19	THYN1	43	TET1	67	CMTR2	91	ACTB
20	WDR76	44	TET2	68	DKC1	92	18S RNA
21	WT1	45	TET3	69	GAR1	93	RNA Spike-in
22	EGR1	46	HNRNPA2	70	NAF1	94	PPC
23	HSD17B10	47	HNRNPB1	71	NHP2	95	GDC
24	KIAA0391	48	HNRNPC1	72	NOP10	96	Blank

■ Description of the control assays

NuRNA™ Human Epitranscriptomics PCR Array includes a series of external and internal controls as described below.

- **HK** (Housekeeping Genes; Internal Controls): 3 human housekeeping genes GAPDH, ACTB, and 18S RNA are included as the internal qPCR normalization references. Arraystar PCR system provides multiple reference genes selected from 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 starting 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 corresponding 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.

- **Blank** (Blank Control): The background reading from the SYBR Green Master Mix.

Shipping and Storage

Arraystar 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.

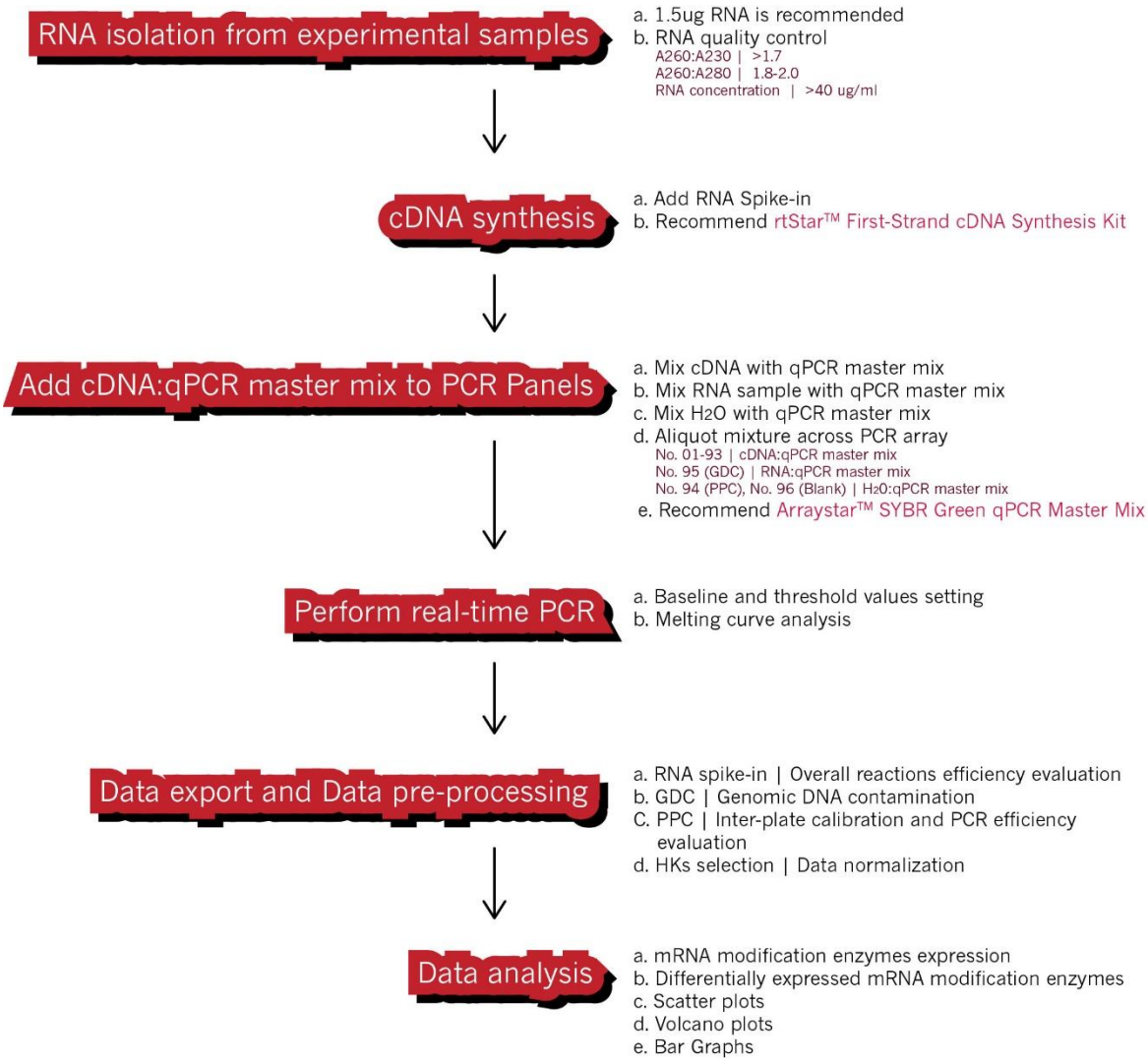
Additional Required Equipment

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

Additional Required Reagents

- rtStar™ First-Strand cDNA Synthesis Kit (Cat# AS-FS-001)
- Arraystar SYBR® Green qPCR Master Mix (ROX+) (AS-MR-006-5)
- Nuclease-free PCR-grade water

D. 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. The RNA must be free of excessive RNA degradation and contamination of protein, organics, or genomic DNA. You can measure the RNA concentration and purity by UV spectrophotometry. You can also check the 18S and 28S ribosomal RNAs 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. We highly recommend using rtStar™ First-Strand cDNA Synthesis Kit (Cat# AS-FS-001), which is specifically optimized for and fully compatible with the Arraystar PCR Arrays.

1. Get the reagents ready

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

   NOTE: *The RNA Spike-in in the kit is supplied as dried down. When using the kit first time, 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. Briefly vortex and spin down again.*

2. Annealing Mix for RT primer

Set up the Annealing Mix according to the following Table. For multiple RNA samples, prepare an Annealing Mix by multiplying each component volume with the number of samples. It is recommended to include 10% extra volume to account for pipetting loss.

Per RNA sample:

Oligo(dT)$_{18}$, or Random Primers	1.0 µL
dNTP Mix	1.5 µL
RNA Spike-in	1.0 µL
Template Total RNA	10.5 µL
Total volume	**14.0 µL**

3. Incubate in a thermal cycler at 65°C for 5 min, and then immediately chill on ice for at least 1 min. Briefly spin down the contents in the tube.

4. cDNA Synthesis Mix

   Prepare cDNA Synthesis Mix according to the table below. For multiple RNA samples, multiply each component volume with the number of the samples, plus 10% extra volume to account for pipetting loss.

   Per RNA sample:

5 × RT Reaction Buffer	4.0 µL
0.1 M DTT	1.0 µL
RNase Inhibitor	0.6 µL
Reverse Transcriptase	0.4 µL
Total volume	**6.0 µL**

5. Add all the material from STEP B3 to the above cDNA Synthesis Mix for a total volume of 20 µL. Vortex and spin down briefly. Incubate at 25°C for 5–10 min, followed by 50 min at 50°C.

6. Terminate the reactions at 85°C for 5 min. Chill on ice.

7. **OPTIONAL**: The cDNA synthesis can be checked by Spike-in assay before the PCR array. Reconstitute the RNA Spike-in qPCR Primer Mix in 100 µL nuclease-free water. Mix 1 µL RNA Spike-in qPCR Primer Mix, 2 µL cDNA, 5 µL SYBR Green Master Mix, and 2 µL Nuclease-free Water. Run the PCR program described in "Running Real-Time PCR Detection" in STEP C.6. A *Ct* value < 30 for the RNA Spike-in indicates a successful cDNA synthesis.

   NOTE: *The cDNA synthesis product can proceed immediately to PCR or can be stored at -20°C for later use.*

C. Perform qPCR on the PCR array

Each sample uses 96-wells on the array. One 384-well PCR panel has 4 identical arrays for up to 4 samples, as color coded in the plate layout (Fig. 2). The following protocol is written for one sample.

1. Dilute the cDNA in Nuclease-free Water. If 1.5 μg input RNA is used with rtStar™ First-Strand cDNA Synthesis Kit (Cat# AS-FS-001), the dilution factor is 1:20 for a total volume of 400 μL. Mix well and spin down.

2. For the assays for Epitranscriptomic Transcripts, Internal Reference Controls and Spike-in External Controls (Wells 1-93), prepare the qPCR Mix according to the table below. Each qPCR reaction volume is 10 μL. Some extra amount has been included for consumption by liquid dispensing operation.

   Per sample:

Arraystar SYBR Green qPCR Master Mix	500 μL
diluted cDNA template	400 μL
ddH_2O	100 μL
Total volume	**1000 μL**

3. For GDC Control (Well 95), combine 1.5 μL NRT (no reverse transcription) sample or 1.5 μL RNA sample, 7.5 μL SYBR Green Master Mix, and 6 μL Nuclease-free Water. Mix well and spin down.

4. For Blank Controls (Wells 94, 96), combine 10 μL SYBR Green Master Mix and 10 μL Nuclease-free Water. Mix well and spin down.

   Load the above mixes to the PCR Array plate:
   NOTE: *The following procedure is written for "**Sample1**" in Figure 2 as an example. For other samples on the panel, it is very important to load the mixes to the corresponding well numbers by the color coding depicted in Table 1 and Figure 2.*

   a. Carefully peel the original seal off the PCR plate;

   b. Add 10 μL aliquot of the Mix from STEP C.2 to Wells 1-93 (Wells 94 to 96, *i.e.* well positions O19, O21, and O23, are reserved for the mixes of other controls);

   c. Add10 μL GDC Mix from STEP C.3 to Well 95 (well positions O21) for genomic DNA contamination detection.

   d. Add 10 μL Blank Mix from STEP 4 into Well 94 (well position O19) and Well 96 (well position O23).

e. Carefully but tightly seal the PCR plate with an optical adhesive cover. Be sure that no air 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 before running the PCR program.

6. Running Real-Time PCR program

Cycles	Temperature	Time
1	95°C	10 minutes
40	95°C	10 seconds
	60°C	1 minute
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 obtained by manual setting should be applied consistently across all assays on the plate. This is preferred over individual assay software automatic setting for better reliability and accuracy.

Inspect the post-PCR melting curves to verify the PCR amplification specificity. A short qPCR amplicon should typically have one single sharp melt peak. If the melting curve has additional multiple peaks or poor peak morphology, it may indicate the presence of non-specific, off-target amplification or primer dimers having different melting temperatures. Non-specific amplification will compromise the quantification. Sometimes, a single longer amplicons may have multiple melting subdomains, in such cases, gel electrophoresis or DNA fragment analysis can be performed to verify whether the product is in fact a single correct amplicon.

Export the raw Ct values to Excel. Free, easy-to-use data analysis software tool is available from www.arraystar.com for download. The data analysis procedures include the following:

■ 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 Controls (GDC). If the value is ≥ 35, no genomic DNA contamination is detected and no action is needed. If the value < 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. Samples with Spike-in Ct > 25 may be identified as outliers and excluded for further 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 PPC replicates have Ct standard deviation ≤0.5. If this is not the case, exclude the outlier sample if it can be identified. Calculate the average of the IPC replicates on each plate and the average of IPC values of all plates (IPC overall). The calibration factor for each plate is the difference between the per 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

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

For example:

	Plate 1	Plate 2	Plate 3
ELP3	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
ELP3 (Calibrated)	20.16	20.73	20.64

5. Calculate the ΔCt for each mRNA 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 multiple housekeeping genes. Three most stably expressed housekeeping genes 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 mRNA

$\Delta\Delta Ct = \Delta Ct(sample\ 1) - \Delta Ct(sample\ 2)$, when comparing *sample 1* with *sample 2*

or

$\Delta\Delta Ct = \Delta Ct(group\ 1) - \Delta Ct(group\ 2)$, when comparing averaged *Group 1* with averaged *Group 2*

2. Convert ΔΔCt to fold change:

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

NOTE: *If FC ≥ 1, the gene expression is up-regulated. If FC < 1, the expression is down-regulated and, by convention, its negative inverse is written as FC. For example, if FC = 0.2, then FC = -1/FC = -5.*

3. The statistical significance of the differential expression between two groups with biological replicates can be estimated by *t*-test. $|FC| \geq 2$ and $p \leq 0.05$ is often used as the cutoffs for differential expression.

   NOTE: Fold change is related to biological effect size. Ranking by fold change is preferred over *p*-value. qPCR has a sensitivity of detecting $\Delta Ct \geq 0.5$, which is about ≥ 1.5 fold change.

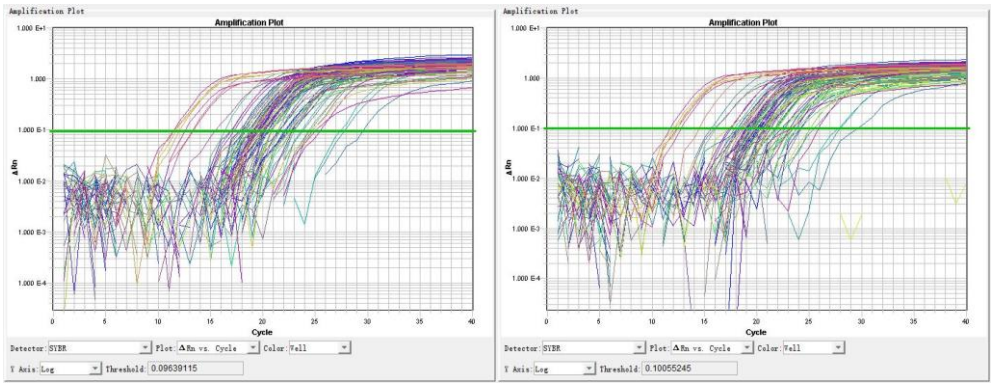
4. Other analyses such as scatter plots, volcano plots, list of differentially expressed genes and bar graph of expression differences for the Epitranscriptomic transcript are performed and included in the standard analysis package.

III. Quality Control and Sample Data

A. NuRNA™ Human Epitranscriptomics PCR Array validation

■ Validation qPCR

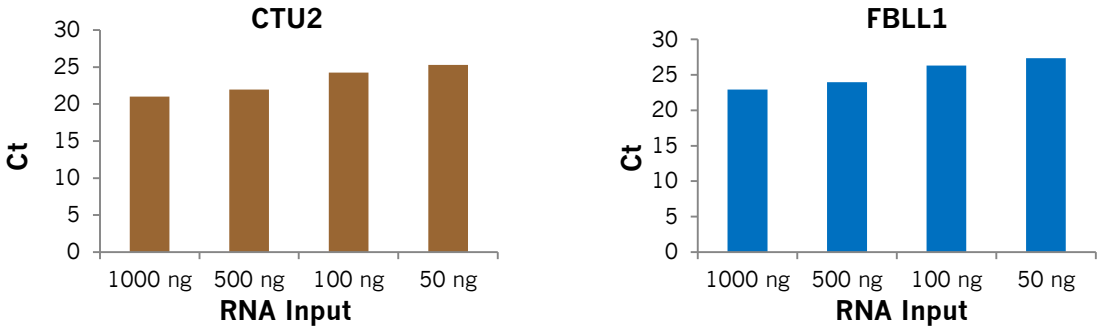
The performance of NuRNA™ Human Epitranscriptomics Panel was tested using a cohort of pancreatic carcinoma and para-carcinoma tissues. The extracted RNA samples were converted to cDNA using rtStar™ First-Strand cDNA Synthesis Kit (Cat#AS-FS-001). The cDNAs were profiled using the PCR array according to aforementioned protocol without modification. The results are charted on the real-time amplification plots for the entire plate for the cell lines.



■ Sensitivity

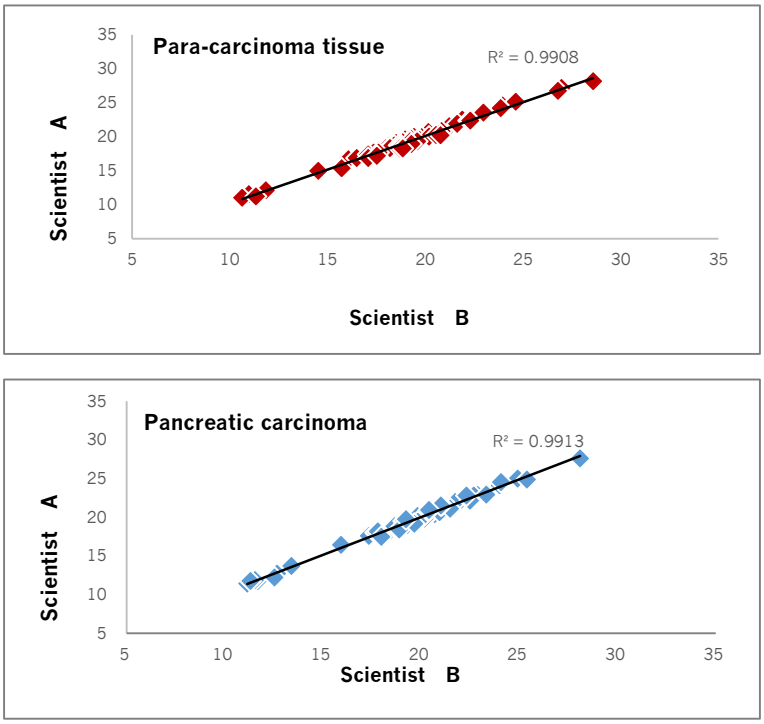
The decreasing input amounts of total RNAs from human glioblastoma cell lines were converted to cDNAs and profiled by the PCR arrays. The Ct values were determined using the software default automatic baseline and

Ct settings. CTU2 and FBLL1 transcripts were detected at Ct values of 25.4 and 27.5 respectively, with the input RNA amount as low as 50 ng.



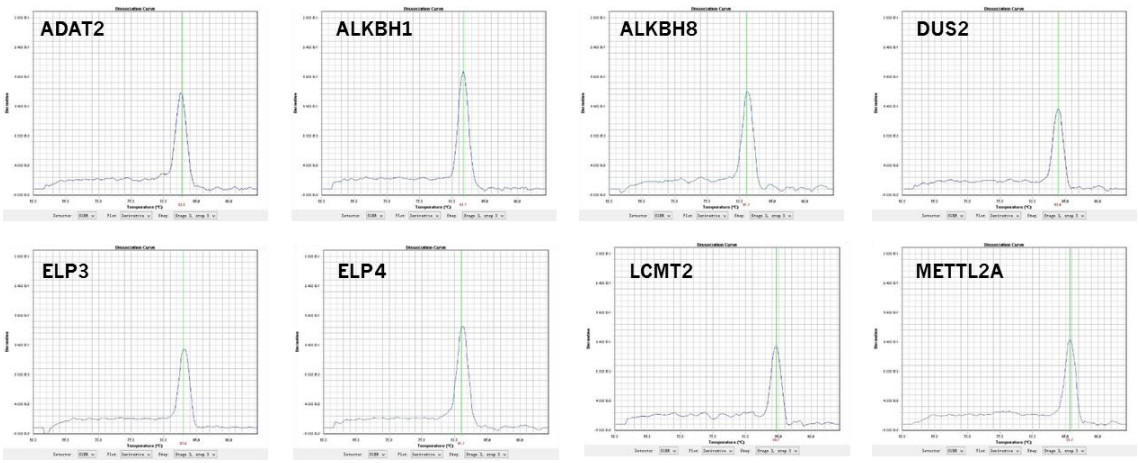
Reproducibility

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



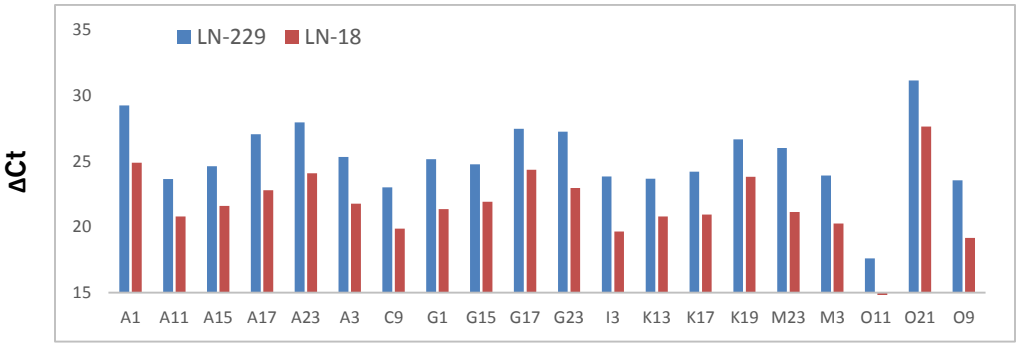
Specificity

The amplification products of transcripts of ADAT2, ALKBH1, ALKBH8, DUS2, ELP3, ELP4, LCMT2, and METTL2A 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 epitranscriptomic transcripts levels in cell lines

The sample data were generated from RNAs extracted from two glioblastoma cell lines LN-229 and LN-18. The normalization was carried out using the average of the housekeeping genes. ΔCt for the gene transcripts (well positions) in LN-229 vs. LN-18 cells 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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