nrStar™ Mouse Functional LncRNA PCR Array

Cat#: AS-NR-004M

Instruction Manual Version 1.0

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I. Introduction

A. Overview

Long non-coding RNAs (LncRNAs) as a non-coding RNA (> 200 nt) biotype can be subgrouped as antisense, enhancer, bidirectional, intronic, sense-overlapping and intergenic (lincRNAs); LncRNAs can act by multiple mechanisms as molecular scaffolds, aid alternative splicing, modify chromatin structure, control mRNA stability, or sponge miRNAs as competing endogenous RNAs (ceRNA). LncRNAs are often specific in expression patterns and regulate a wide range of biological functions, including genomic imprinting, stem cell pluripotency, embryonic development[1], cardic development[2], hematopoiesis, immunity[3], and endocrine systems[4]. Dysregulated expression of lncRNAs has been associated with many diseases such as neurodegenerative, cardiovascular[5], kidney, diabetes and many cancer diseases[6]. Profiling LncRNA expression is important in understanding of their functional roles, unraveling their regulatory molecular mechanisms or identifying LncRNA biomarkers.

Arraystar nrStar™ Mouse Functional LncRNA PCR profiles 185 lncRNA transcripts with known biological functions or disease associations. These well-characterized functional LncRNAs are comprehensively collected based on the results published in scientific publications and from the functional lncRNA databases. It has the most comprehensive and the up-to-date content to represent the functionally known and best studied lncRNAs on the market. It is a sensitive and accurate tool for functional lncRNA profiling, drug targets finding or biomarker validation.

B. LncRNA in biological functions and human diseases

■ Classification of LncRNAs

lncRNAs have been attracting intense research interest. However only a handful of lncRNAs have been characterized thoroughly. According to their genome position in relation with the neighboring protein-coding genes, lncRNAs can be categorized as antisense, enhancer, bidirectional, intronic, and intergenic lncRNAs (**Figure 1**), which are strongly correlated with the molecular mechanism of action. For example, enhancer lncRNAs mediate short-range and long-range interactions between the enhancers from which they are transcribed and other regulatory elements in the genome. The act of lncRNA transcription itself can initiate changes in chromatin accessibility or protein factor binding independent of its gene products. lncRNAs can also be categorized as *nuclear* in the nucleus to regulate gene expression, or *cytosolic* to regulate mRNA stability and translational efficiency through RNA–RNA interactions.

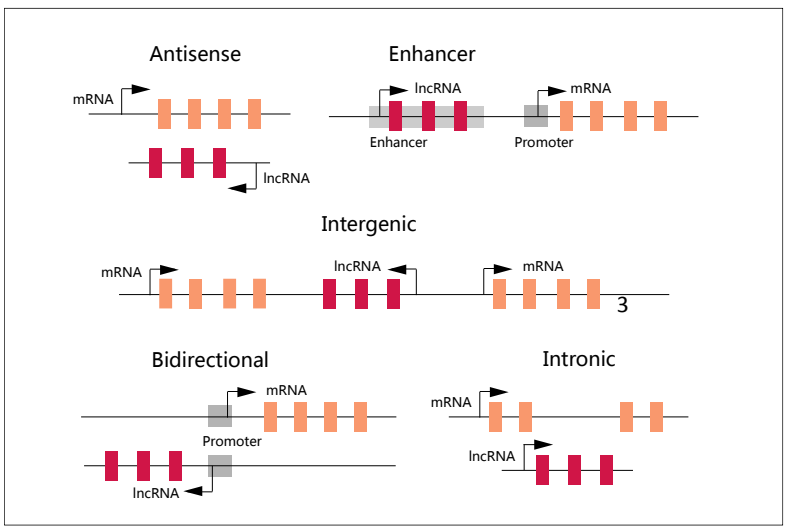


Figure 1 | Classification of lncRNAs according to their genomic location with the neighboring genes. Antisense lncRNAs are transcribed from the antisense strand of protein-coding genes and overlap one or several introns and exons of the sense sequence. Enhancer lncRNAs are located in enhancer regions. Intergenic lncRNAs are located more than 1kb~ 5kb away from the nearest protein-coding genes. Bidirectional lncRNAs are located within 1 kb of promoters in the opposite direction from the protein-coding transcript. Intronic lncRNAs are located in an intron of a coding gene.

■ Mechanisms of action

LncRNAs act through diverse mechanisms that rely on base paring, secondary or tertiary structures. Most lncRNAs are located in the nucleus, where they can act as molecular scaffolds, aid alternative splicing or modify chromatin structures. Some lncRNAs also have functions in the cytoplasm, such as modulating translation, promoting or inhibiting mRNA degradation, or acting as miRNA sponges (**Figure 2**).

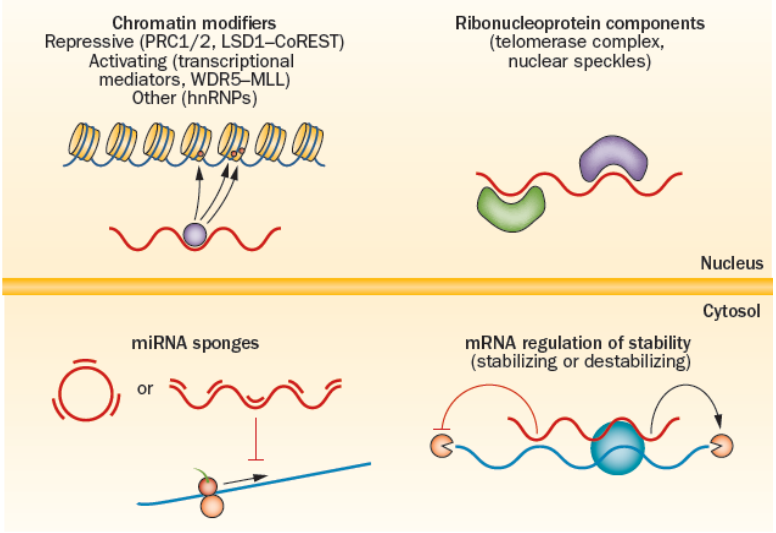


Figure 2 | LncRNA mechanisms action [4]**.** Most lncRNAs are nuclear and at common mechanism of action is via recruitment of chromatin modifiers to DNA. These chromatin modifiers can be either repressive or activating (such as transcriptional mediators) or other modifiers such as hnRNPs as nuclear organization factors. Some lncRNAs bind to specific proteins and act as scaffolds within ribonucleoprotein complexes. In the cytosol, lncRNAs can act at the post-transcriptional level as sponges for miRNAs, therefore inhibiting the actions of miRNAs on mRNAs. A few examples of lncRNAs that affect the half-life of mRNAs by either destabilizing or stabilizing a specific subset of mRNAs have been described. Abbreviations: hnRNP, heterogeneous ribonucleoprotein; miRNA, microRNA; PRC, polycomb repressor complex.

■ Biological functions

The functions of lncRNAs are only now starting to be understood. Central roles for lncRNAs have been uncovered in a diverse set of biological processes, including genomic imprinting, X chromosome inactivation, stem cell differentiation, embryonic development, lipid metabolism and adipogenesis, among many others.

Imprinting and X chromosome inactivation

Genomic imprinting is an important developmental mechanism as most imprinted genes regulate embryonic growth[7]. Several lncRNAs have been described to regulate imprinting. For instance, Airn is essential for the silencing of the *Igf2r/Slc22a2/Slc22a3* gene cluster on the paternal chromosome. The antisense lncRNA *Kcnq1ot1*, which regulates the silencing function of the imprinting control region of *Kcnq1* on the unmethylated paternal chromosome, is another example of lncRNA involved in imprinting. The lncRNA *Xist* also has a role in imprinting. During development in females, *Xist* leads to chromosome-wide repression of gene expression in the inactive X chromosome, whereas genetic information of the active X chromosome is transcribed.

Stem cell differentiation

The promoters of more than 100 lncRNAs are bound by stem cell factors. Disruption of these lncRNAs can alter cell differentiation[8]. One of them, lincRNA-RoR, is involved in the reprogramming of fibroblasts back to a pluripotent state. Thus, lncRNAs are likely to play important roles in both normal development and processes that require maintenance of adult stem cell pools.

Embryonic development

HOX genes encode an evolutionary conserved family of transcription factors that regulate the embryo body plan and that contribute to cell specification in several adult differentiation processes[3]. Several lncRNAs have been shown to be directly involved in the regulation of HOX genes. The lncRNA HOTTIP can bind WDR5, a key component of histone-modifying MLL1 complex, to catalyze activating H3K4me3 marks and maintain gene activation in the HOXA locus. Another lncRNA HOTAIR acts as a repressor of the HOXD cluster by recruiting repressive complex PRC2.

Lipid metabolism and adipogenesis

Recent publications have shown that lncRNAs influence lipid homeostasis by controlling lipid metabolism in the liver and by regulating adipogenesis[9]. APOA1 is a major component of high-density lipoprotein (HDL). APOA1-AS, antisense transcript of APOA1, negatively regulate APOA1 expression in vitro and in vivo. Another lncRNA, NEAT1, regulates PPARγ2 splicing during adipogenesis. It also mediates miR-140 induced adipogenesis.

Hematopoiesis and Immunity

Multiple lncRNAs participate in different stages of immune system development and activation[3]. For example, Lnc-DC controls dendritic cell differentiation by promoting phosphorylation and nuclear translocation of a key DC transcription factor STAT3. The lncRNA PACER is upregulated after LPS stimulation in human macrophages and selectively regulates the expression of its neighboring gene COX-2. Furthermore, lncRNA THRIL, is essential for basal and inducible expression of the inflammatory-cytokine- encoding gene TNF (tumor necrosis factor), through interaction with hnRNP factor.

Cardiac development and heart function

Investigators have profiled cardiac-expressed lncRNAs and identified hundreds of differentially expressed lncRNAs during cardiac development. However, only several of them have been characterized function roles [2]. For example,

BVHT is expressed in cardiac mesoderm and is highly present in embryonic stem cells and cardiomyocytes. Depletion of BVHT severely impairs the capacity of embryonic stem cells to produce differentiated cardiomyocytes. Another lncRNA involved in cardiac lineage commitment is FENDRR. This lncRNA is exclusively expressed in cardiac mesoderm progenitors during development, and controls differentiation of tissues derived from the lateral mesoderm, which gives rise to the ventral body wall and heart.

■ Association with human diseases

Recent years, the examination of lncRNA involvement in diseases have got explosive progress. However, this field is still infancy, more efforts are needed to reveal the lncRNA functions in diseases and explore their therapeutic and biomarker potential. In the following part, we summarize the current knowledge of lncRNAs in multiple types of diseases with fatal concern.

Cancer

Cancer is fundamentally a genetic disease that alters cellular information flow to modify cellular homeostasis and promote growth. Genome-wide cancer mutation analyses are revealing an extensive landscape of functional mutations within the noncoding genome, with profound effects on the expression of lncRNAs. While the exquisite regulation of lncRNA transcription can provides signals of malignant transformation, lncRNAs drive many important cancer phenotypes (including growth, proliferation, metastasis and survival) and participate in cancer pathophysiology[6] (**Figure 3**).For instance, in T cell acute lymphoblastic leukemia, the Notch 1 oncogene drives growth in part by inducing lncRNA LUNAR1 to upregulate insulin-like growth factor 1 receptor expression and

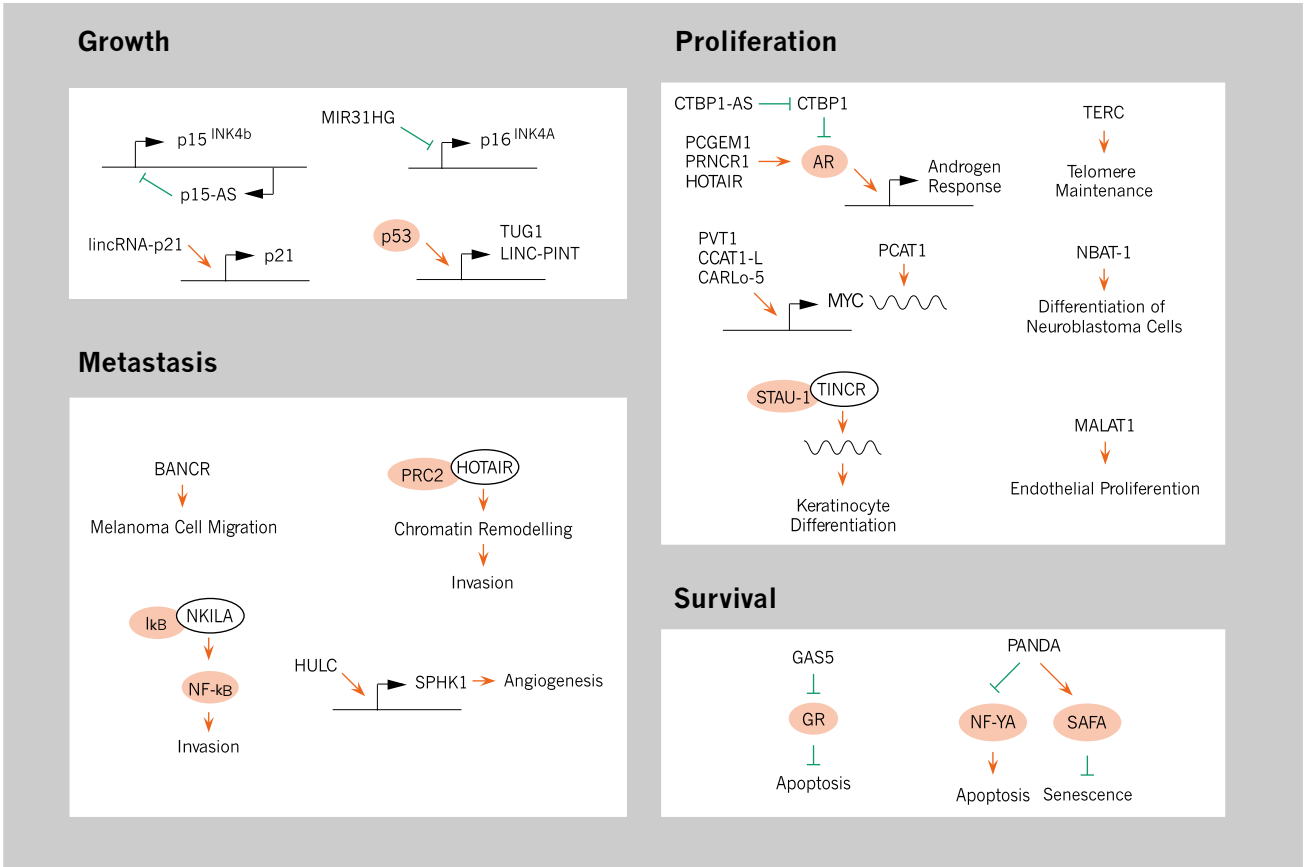


Figure 3 | LncRNAs in Cancer Phenotypes.

signaling. Androgen signaling in prostate cancer also relies on a number of lncRNAs implicated in prostate cancer proliferation that act though direct interaction with the androgen receptor (PCGEM1, PRNCR1, HOTAIR), or by inhibiting repressors of the androgen receptor (CTBP1-AS).

Neurodegenerative diseases

Multiple lncRNAs play important roles in brain development, neuron function and maintenance and central nervous system (CNS) development. Moreover, lncRNA involvement in neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) are becoming increasingly evident[10]. BACE1-AS, which transcribed from the antisense protein-coding BACE 1 gene, is highly expressed in AD patients. Unlike the other natural antisense transcripts forming the duplex complex with the sense of coding mRNA to inhibit mRNA translation, BACE1-AS play the function by increasing BACE1 mRNA stability then generating additional Aβ42 through a post-transcriptional feed-forward mechanism, implying that BACE1-AS may drive AD-associated pathology, directly implicate in the increased abundance of Aβ42 in AD.

Cardiovascular diseases

A variety of lncRNAs have been demonstrated to significantly influence cardiac diseases such as heart failure and myocardial infarction[5]. In a study of lncRNA expression in a mouse model of myocardial infarction, the researchers found two lncRNAs were upregulated — myocardial infarction-associated transcript 1 (*Mirt1*) and *Mirt2*. The expression of these lncRNAs peaked 24 h after myocardial infarction and returned to baseline after 2 days. In mouse models of cardiac pressure overload and myocardial infarction, reactivation of the fetal cardiac genetic programme was accompanied by increased expression of fetal cardiac enhancer-associated lncRNA transcripts, suggesting that this specific type of lncRNA might have a central role in driving the progression of heart failure following myocardial infarction.

Kidney diseases and diabetes mellitus

Kidney disease frequently occurs in the setting of diabetes mellitus[5]. miRNA-coding lncRNAs, such as the intergenic lncRNA plasmacytoma variant translocation gene (*PVT1*), might have a role in kidney disease. *PVT1* was identified by genome-wide association studies conducted to identify genetic variants that contribute to end-stage renal disease in patients with type 2 diabetes mellitus. High-glucose treatment induced the expression of *PVT1* as well as fibronectin 1 (FN1), collagen type IV α1, transforming growth factor β1 (TGF-β1) and plasminogen activator inhibitor-1 (PAI-1; otherwise known as SERPINE1) in human kidney mesangial cells. In turn, *PVT1* silencing resulted in a significant downregulation of these factors.

LncRNAs in clinic

It is now widely understood that lncRNAs, serving as signals of specific cellular states or readouts of active cellular programs, could identify cellular pathologies such as cancer, provide prognostic value, or even inform therapeutic options for patients. Spatial, temporal, and disease-associated regulation of expression suggest that lncRNA can be powerful and effective biomarkers. There has been numerous studies demonstrate the potential biomarker utility of lncRNA (**Table 1**). LncRNA *MT-LIPCAR,* was differentially expressed in patients with left ventricular remodelling compared to those without remodelling, and this differential expression was subsequently validated in 788 patients with cardiac remodelling and heart failure, comprising three independent cohorts. High circulating levels of *MT-LIPCAR* independently predicted adverse cardiac remodelling and this association was not confounded by other

predictive markers of cardiovascular death. This study demonstrates the feasibility of detecting and amplifying circulating lncRNAs in large, independent cohorts of patients with heart failure.

LncRNA	Disease Association	Biomarker application
CDKN2B-AS1	Coronary artery disease[11, 12]	Risk factor
CDKN2B-AS1	Myocardial infarction[12]	Risk factor
KCNQ1OT1	Myocardial infarction[13]	Predicts left ventricular dyfunction
MIAT	Myocardial infarction[14]	Risk factor
MT-LIPCAR	Myocardial infarction[15]	Predicts postinfarct remodelling
SOX2OT	Alzheimer's disease[16]	Risk factor
H19	Parkinson's Disease[17]	Risk factor
HULC	Hepatocellular carcinoma[18]	Diagnosis and monitoring
FALEC	Ovarian cancer[19]	Predicts poor prognosis
CCAT1	Colorectal cancer[20]	Predicts therapeutic responsiveness

Table 1 | LncRNAs with potential biomarker applications.

C. Product summary

■ Kit Contents

Figure 4 The array layout for nrStar™ Mouse Functional LncRNA PCR Array

Condition		1	2	3	4	5	6	7	8	9	10	11	12
A	A	1700007 L15Rik	4930503 E24Rik	4930570 G19Rik-1	4930570 G19Rik-2	5430416 N02Rik	5S-OT	6430411 K18Rik	9530018 H14Rik	Abhd11os	Airn-1	Airn-2	Airn-3
B	B												
A	C	B130024 G19Rik	Borg	Bvht	C130071 C03Rik-1	C130071 C03Rik-2	C2dat1	C730029 A08Rik	C730036 E19Rik	CAIF	Chaer	Chast	Crxos
B	D												
A	E	Emx2os	Esrp2-as-1	Esrp2-as-2	Esrp2-as-3	Evx1as	Fendrr-1	Fendrr-2	Firre	Ftx-1	Ftx-2	G730013 B05Rik-1	G730013 B05Rik-2
B	F												
A	G	Gm2694-2	Gm30731	Gm32592	Gm6644	Gm6768	H19	Halr1	Hm629797	Hotair	Hottip-1	Hottip-2	Hoxa11os
B	H												
A	I	Lnc13	lnc-31	lncKdm2b	lnc-LFAR1	lncLGR	lncOL1	Lncpint-1	Lncpint-2	Lncpint-3	lncPrep	lncRNA033862	lncRNA-ACOD1
B	J												
A	K	Miat-1	Miat-2	Mir124a-1hg	Mir142hg	Mira	Mirt1	Mirt2	Morrbid-1	Morrbid-2	Morrbid-3	Msx1os	Munc
B	L												
A	M	Platr14-1	Platr14-2	Pluto	Ptgs2os2	Rian	Rmrp	Rmst	Rr18	Rroid	Rubie	Sghrt-1	Sghrt-2
B	N												
A	O	TK99129	Tog	Trp53cor1	Tsix	Ttc39aos1	Tug1-1	Tug1-2	Tug1-3	Uchl1os	Uph	Vax2os-1	Vax2os-2
B	P												

Condition		13	14	15	16	17	18	19	20	21	22	23	24
A	A	AK028007	AK038798	AK044955	AK081227	AK133808	AK139328	AK143294	AK143693	AK153778	alncRNA-EC7	APOA4-AS	Atp2a2
B	B												
A	C	Cyp4b1-ps2	Dalir	Digit	Dio3os	Dleu2	Dlx1as	Dlx4os	Dlx6os1	Dreh	Dworf	E130102 H24Rik	Egr2-AS
B	D												
A	E	Gas5	Gfra1	Gm12919	Gm13865	Gm14155	Gm15050	Gm15054	Gm15834	Gm16551	Gm16845-1	Gm16845-2	Gm2694-1
B	F												
A	G	Hoxaas3	Huc1	Jpx	Kcnq1ot1	LeXis-1	LeXis-2	linc1242	linc1257-1	linc1257-2	linc1368	LincRNA-Gm4419-1	LincRNA-Gm4419-2
B	H												
A	I	lncRNA-Nfkb2-1	lncRNA-Nfkb2-2	lncRNA-Smad7-1	lncRNA-Smad7-2	lncRNA-Smad7-3	lnc-Smad3	LOC105246506	Malat1	Mdrl	Meg3-1	Meg3-2	Mhrt
B	J												
A	K	Myhas	Nctc1	Neat1-1	Neat1-2	Nespas	Nkx2-2os	np_17856	np_5318	Panct1	PAPAS	Paupar	Pinc
B	L												
A	M	Six3os1-1	Six3os1-2	Six3os1-3	Six3os1-4	Six3os1-5	Six3os1-6	Smn-AS1	Snhg3	Snhg5	Sox2ot	Srsf9	Tdpx-ps1
B	N												
A	O	Wt1os	Xist-1	Xist-2	Yam-1	Zfhx2os	**ACTB**	**GAPDH**	**18S RNA**	**RNA Spike-In**	**PPC**	**GDC**	**Blank**
B	P												

All 185 functional lncRNAs chosen for the array are based on results from published in multiple scientific publications, or from the most updated databases. The controls on the array plate include six housekeeping genes for qPCR normalization, one RNA Spike-In for overall reactions efficiency, three positive PCR control (PPC) for PCR efficiency and inter-plate calibration, and Genomic DNA Control (GDC) for genomic DNA contamination detection.

Description of the control assays

nrStar™ Mouse Functional 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.

- **HK** (Housekeeping genes; Internal Controls): 3 mouse housekeeping genes ACTB, GAPDH and 18S rRNA are included as the internal qPCR normalization references. 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 DNAs 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 tool 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.

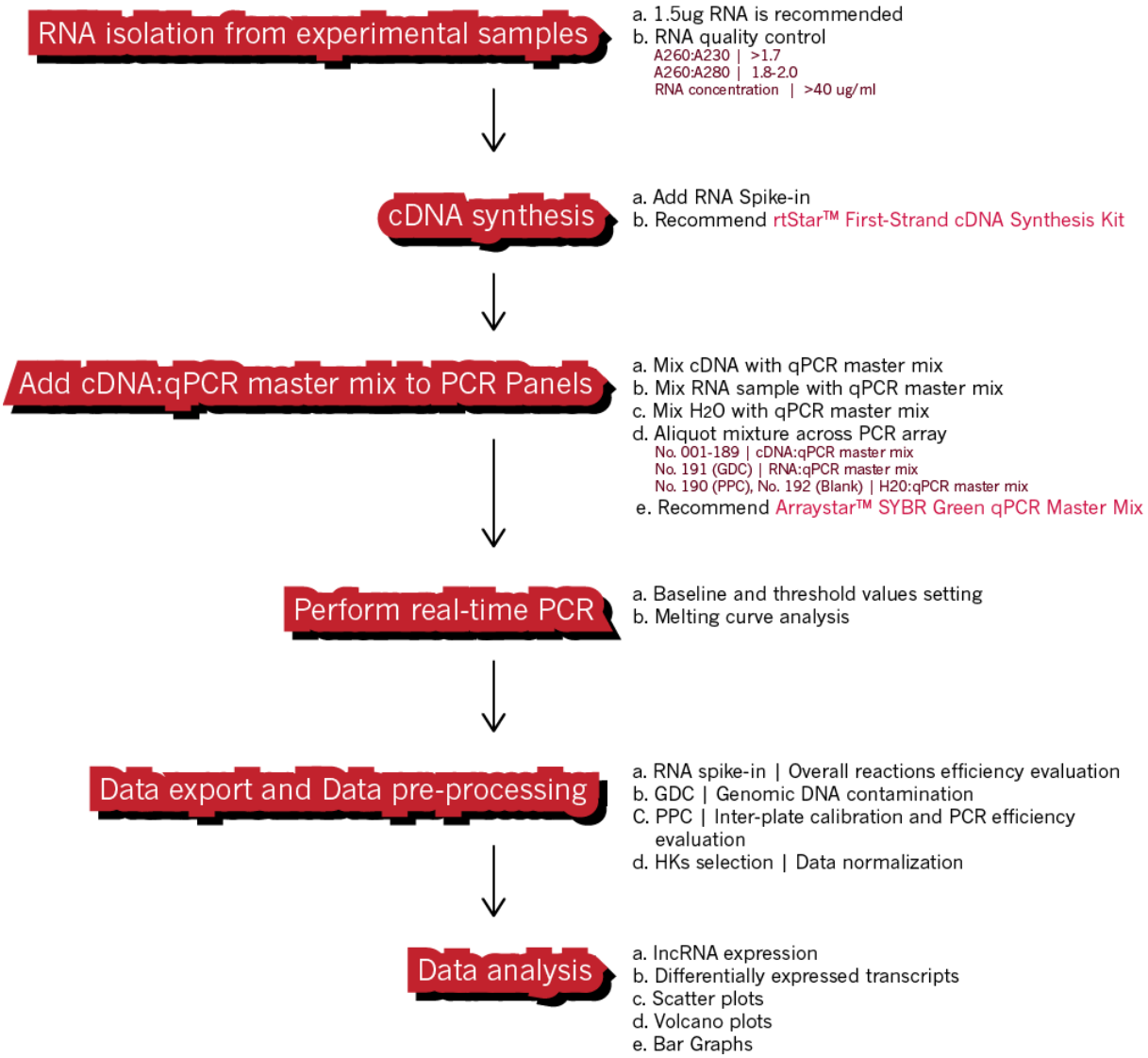
Additional Required Equipment

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

Additional Required Reagents

- rtStar™ First-Strand cDNA Synthesis Kit (Cat# AS-FS-001)
- Arraystar™ SYBR Green qPCR Master Mix (Cat# 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 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

The recommended input total RNA amount is 1.5 μg. Lower amounts may reduce the assay sensitivity particularly for genes at lower expression levels.

B. First-strand cDNA synthesis

Use the same amount of total RNA in this reaction for every sample. High quality cDNA synthesis is vital for the following qPCR performance. We highly recommend using rtStar™ First-Strand cDNA Synthesis Kit (Cat# AS-FS-001), which is specifically optimized for and fully compatible with the nrStar™ PCR Arrays.

> **NOTE:** The first time to use this kit, please reconstitute the RNA spike-in by adding 50 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.

1. Mix the following components in a 200 μL PCR tube for each sample.

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

Random Primers	1.0 µl
2.5 mM dNTP Mix	1.0 µl
RNA Spike-in	2.0 µl
Template Total RNA	10.0 µl
Total volume	14.0 µl

2. Incubate in a thermal cycler at 65°C for 5 min, and then immediately chill on ice for at least 1 min. Collect the contents of the tube by brief centrifugation.

3. Add the following components directly to the product from STEP 2. The final volume will be 20 µL.

5 × RT Reaction buffer	4.0 µl
RNase Inhibitor	1.0 µl
Reverse Transcriptase	1.0 µl
Total volume	20 µl

4. Incubate at 25°C for 10 min, followed by 30 min at 45°C

5. Terminate the reactions at 85°C for 5 min. Hold the finished First Strand cDNA Synthesis Reaction on ice until the next step. OK to store overnight 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™ First-Strand cDNA Synthesis Kit (Cat#AS-FS-001), 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 µl SYBR Green Master Mix, and 4 µl Nuclease-free Water. Mix well and spin down.
3. For Blank Controls, combine 15 µL SYBR Green Master Mix and 15 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 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 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
KCNQ1OT1	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
KCNQ1OT1 (Calibrated)	20.16	20.73	20.64

5. Calculate the ΔCt for each RNA 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 HK genes. These most stably expressed housekeeping reference genes were selected from a broad range of samples by our stringent algorithm that evaluates the optimal properties and the number for endogenous controls.

Data analysis

1. Calculate the ΔΔCt for each RNA

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

or

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

2. Calculate the fold change 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 1.5 fold change.

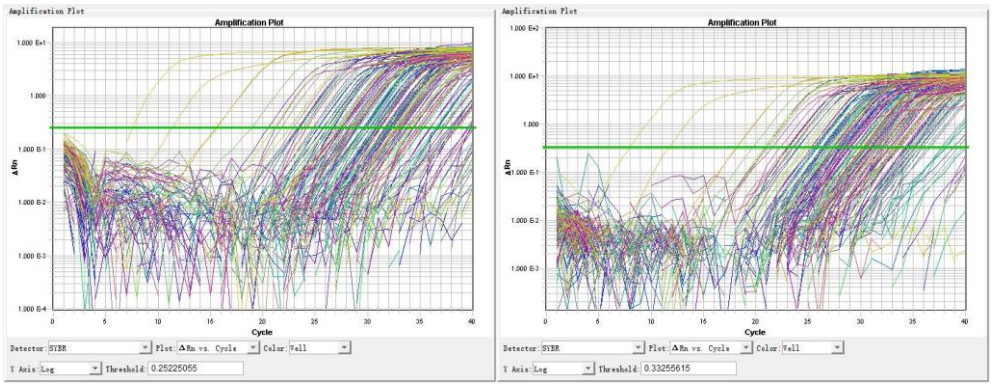
4. Other analyses such as scatter plots, volcano plots, and bar graph of expression differences for the lncRNAs are performed and included in the standard analysis package.

III. Quality Control and Sample Data

A. Mouse Functional LncRNA PCR Array validation

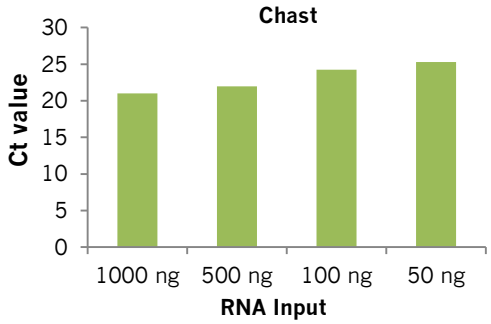
■ Real-time qPCR Validation

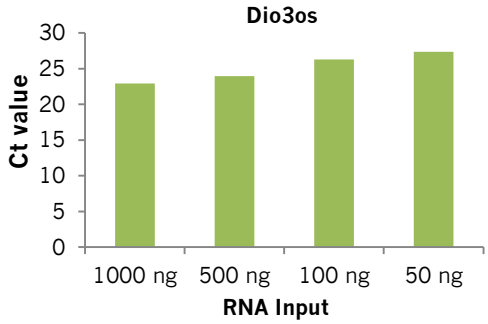
The performance of Mouse Functional LncRNA PCR Panel was tested using a cohort of mouse tissues. The extracted RNA samples were converted to cDNA using rtStar™ First-Strand cDNA Synthesis Kit. The cDNAs were profiled using the PCR array according to aforementioned protocol without modification. The results are charted on the real-time amplification plots for the entire plate for mouse tissues.



■ Sensitivity Test

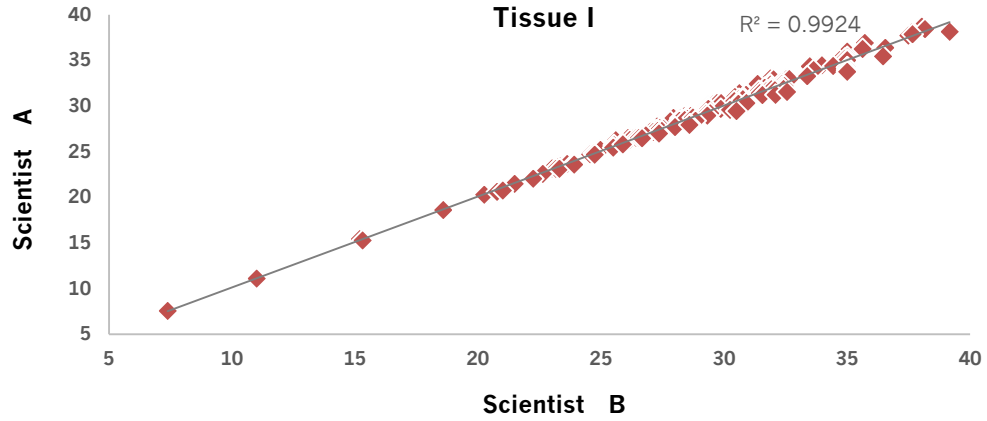
The decreasing input amounts of total RNAs from mouse tissues were converted to cDNAs and profiled by the PCR arrays. The Ct values were determined using the software default automatic baseline and Ct settings. lncRNA Dleu2 and Chast transcripts were detected at Ct values of 25.2 and 27.0 respectively, with the input RNA amount as low as 50 ng.

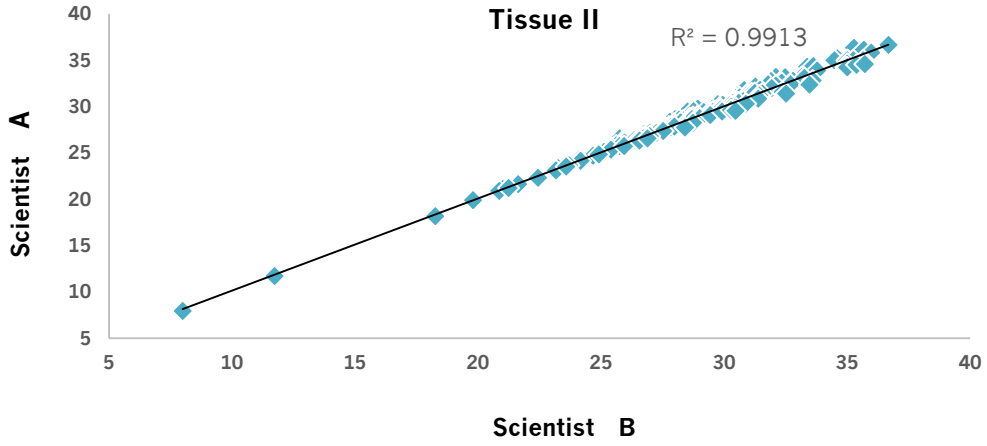




■ Reproducibility Test

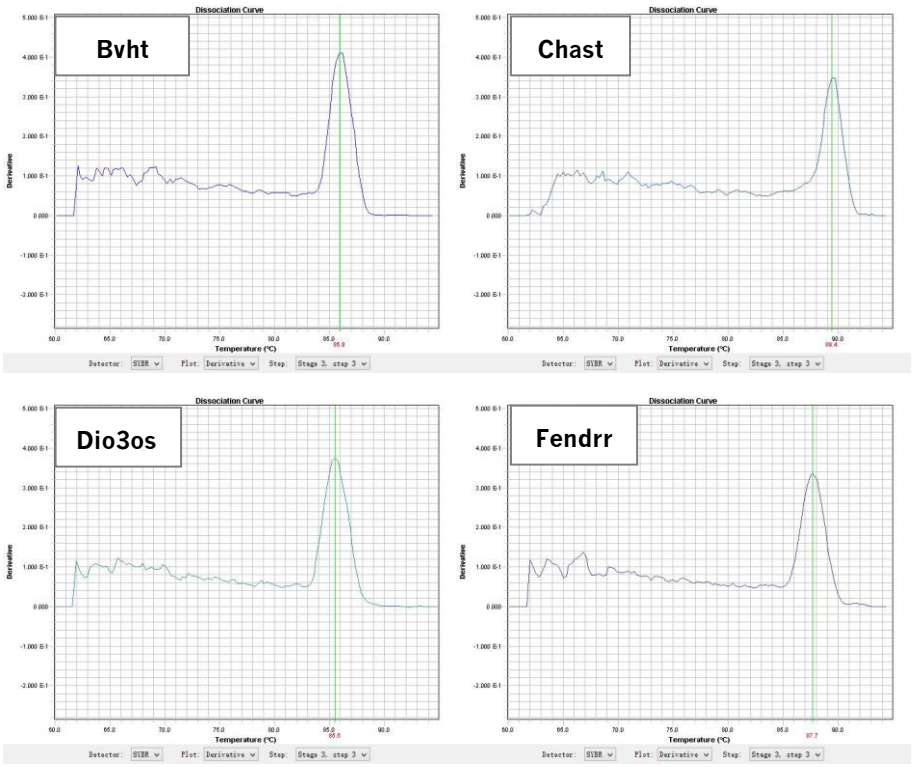
Two independent runs of Mouse Functional PCR Array were conducted by two different scientists A and B at two different times using two different mouse tissues. The results demonstrate a high degree of reproducibility with correlation R^2>0.98.





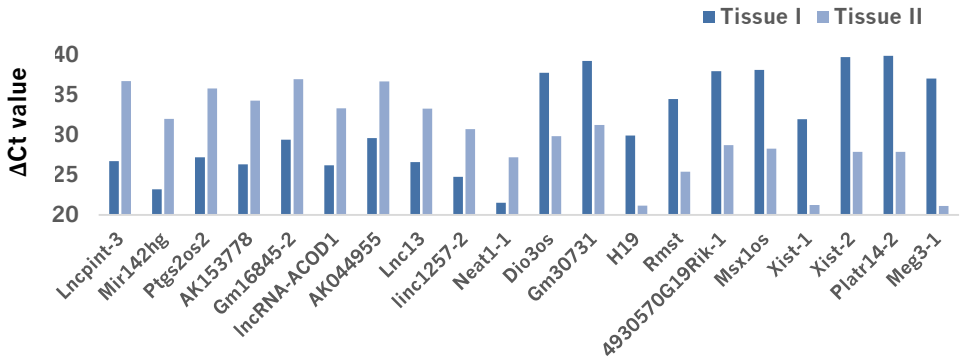
Specificity Test

The amplification products of transcripts and the isoforms of Bvht, Chast, Dio3os, Fendrr genes 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.



B. Sample data: Analysis of Mouse Functional lncRNA levels in tissues

The sample data were generated from RNAs extracted from two different tissues. The normalization was carried out using the average of the housekeeping genes. ΔCt for the gene transcripts (well positions) in tissue I vs. tissue II 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™ Mouse Functional LncRNA 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.