# Regulatory RNA qPCR Profiler

## Cat #RA950A-1

## *User Manual*

**Store at -20°C upon arrival**

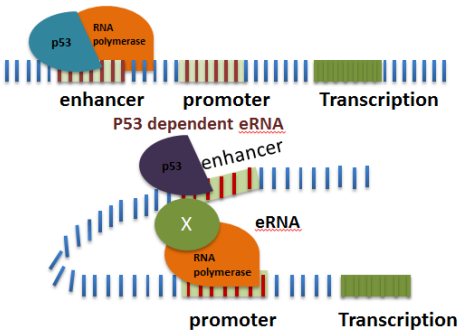
# Contents

# I. Introduction and Background

## A. Overview

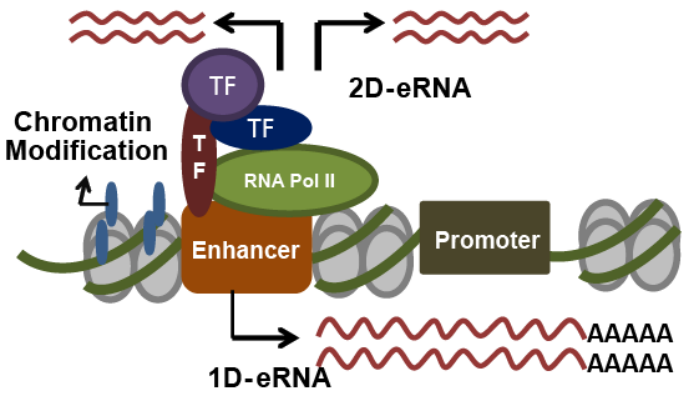
In the era of genome sequencing and transcriptome profiling, scientists have realized that the genome is pervasively transcribed and large fraction of these transcripts do not code for any proteins[1]. These transcripts were initially thought to be non-functional, but scientists have shown that they have regulatory functions and regulate gene expression during development and in disease states [2-4]. These transcripts, commonly known as non-coding RNAs (ncRNAs) comes in several different flavors, such as microRNAs, long non-coding RNAs, and PIWI RNAs. Recently, another class of non-coding RNAs have gained prominence in the RNA community, called enhancer RNAs (eRNAs). Enhancer RNAs are transcribed from enhancers [5] and they function as enhancers to affect gene activation [6]. Similarly, several long non-coding RNAs have shown to have functions similar to enhancer RNAs and have been implicated in gene activation and even inhibition [5]. Aberrant expression of these RNAs has been linked to cancer and other diseases[7]. Their regulatory potential holds promise in the area of identifying novel therapies for diseases and for understanding complex cellular pathways.

## B. Enhancer RNAs (eRNA)




Developmental and disease programs respond to external signals which initiate a regulatory cascade leading to phenotypic consequences for the organism in question. A sequence in the genome that responds to these external cues is called an enhancer [5]. These enhancer sequences bind transcription activators and activate target genes via protein-protein interactions and DNA looping. Michael Greenberg's group in 2010 has shown that in addition to transactivators, RNA polymerase II is recruited to enhancers that have distinct chromatin signatures leading to their transcription. These transcripts are called enhancer RNAs (eRNAs) [8]. Since then, eRNAs have been identified in all cell types that have been studied to date and been shown to have very significant regulatory function in development and disease [6, 7, 9]. For example, scientists have found p53-dependent transcription of some enhancers and the regulation of expression of p53-dependent genes by eRNAs [10]. The cartoon above shows p53 binding to an enhancer sequence and its expression. Expression of the eRNA helps to recruit modulators and helps in the transcription of p53 target genes.

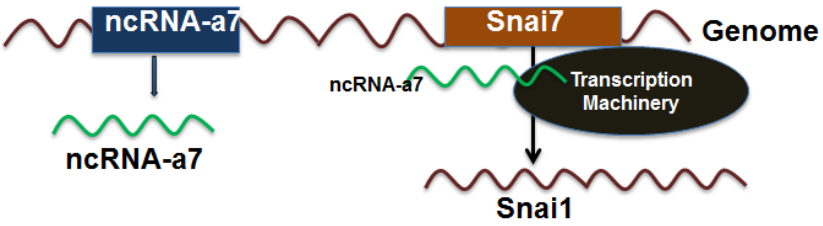
## C. Description of eRNAs




eRNAs are short RNA sequences that are transcribed from active enhancer region of the genome. Active enhancers are marked by high levels of H3K4me1 and H3K4me2 relative to the H3K4me3[11]. There are two types of eRNA, 1D eRNAs and 2D eRNAs. 1D eRNAs are uni-directional RNAs that are polyadenylated but not capped, while 2D eRNAs are bidirectional and capped. eRNAs range in size from 50 to 2000 nt in length.

## D. Regulatory LncRNAs

LncRNAs are long non-coding RNAs that are more than 200 bp long and have a cap and a polyA tail. To date, lncRNAs have been found to exhibit a wide range of functions ranging from signaling, serving as molecular decoys, and guiding ribonucleoprotein complexes to specific chromatin sites and also participate as scaffolds in the formation of complexes. Some lncRNAs are known to affect specific genes by either activating them or inhibiting them. One example is activating non-coding RNAs (ncRNA-a), which are a type of RNA that has enhancer-like functionality. For example, ncRNA-a7 activates expression of its neighboring gene Snai1. Inhibition of ncRNA-a7 abolishes the activation of Snai1.




## E. List of Components

SBI's Regulatory RNA qPCR Profiler contains the following components with enough material to perform 20 cDNA synthesis reactions and sufficient primer stock in the concentrated Primer Array plate to fully profile 20, 96-well qPCR plates as outlined in this manual:

| | | |
|---|---|---|
| 40 µl | 5X PolyA Polymerase Buffer | 10 µl Poly A Reaction (enough for 20 reactions) |
| 10 µl | PolyA Polymerase | |
| 20 µl | 25 mM MnCl2 | |
| 30 µl | 5 mM ATP | |
| 10 µl | Oligo dT Adaptor | 20 µl RT Reaction (enough for 20 reactions) |
| 80 µl | 5X Reverse Transcriptase Buffer | |
| 20 µl | Reverse Transcriptase | |
| 30 µl | 0.1 M Dithiothreitol (DTT) | |
| 50 µl | dNTP Mix | |
| 30 µl | Random Primer Mix | |
| | Array Primers, dried down in Primer plate (400 pmoles); resuspend in 44µl RNase-free Water | |
| 1.2 ml | RNase-free Water | |

The kit is shipped on blue ice and should be stored at -20°C upon arrival. Properly stored kits are stable for 1 year from the date received. The oligonucleotides for the specific target genes are dried-down in the wells of the provided optical qPCR plate. Resuspend the plate in 44µl of RNase-free water, and transfer 2µl of each primer/well to a qPCR plate compatible with the researcher's qPCR instrument.

## F. Additional Required Materials

- Real-time qPCR Instrument
- Instrument-specific optical qPCR plates
- Thermocycler (with heated lid)
- 2X SYBR Green Master Mix
- Thermocycler PCR tubes or plates for end-point reactions
- PCR Master Mix, including *Taq* polymerase for PCR
- 3.0-3.5% Agarose Gel in Tris-Borate EDTA (TBE) or Tris-Acetate EDTA (TAE) Buffer
- DNA Size Ladder with markers from 50 to 2,000 bp (Bio-Rad AmpliSize™ DNA Ladder; Cat. # 170-8200)
- Nuclease-Free $H_20$ (qPCR-certified or equivalent)

# II. Protocol

## A. RT Reaction Setup

**(for 1 RNA sample to be assayed on 1 qPCR plate)**

It is important to start with total RNA that includes the small RNA fraction. For **optimum** signals, perform the following.

**Dilute your RNA to ~100-200 ng/µl**

**Start:** **In a thin-walled PCR tube or PCR-compatible plate well, combine:**

**STEP 1: PolyA Tail**

| | | | |
|---|---|---|---|
| | 5 | µl | Total RNA (~500 ng) |
| | 2 | µl | 5X PolyA Buffer |
| + | 1 | µl | 25mM $MnCl_2$ |
| | 1.5 | µl | 5mM ATP |
| | 0.5 | µl | PolyA Polymerase |
| | 10 | µl | Total in tube |

**Incubate for 30 min. at 37°C**

**STEP 2: Anneal Anchor dT Adaptor**

**Add: + 0.5 µl Oligo dT Adaptor**

**Heat for 5 min. at 60°C**
**Let cool to room temp for 2 min.**

**STEP 3: Synthesize cDNAs**

| | | | |
|---|---|---|---|
| Add: | 4 | µl | 5X RT Buffer |
| | 2 | µl | dNTP mix |
| | 1.5 | µl | 0.1M DTT |
| + | 1.5 | µl | Random Primer Mix |
| | 1 | µl | Reverse Transcriptase |
| | 20.5 | µl | Total in tube |

**Incubate for 60 min. at 42°C**
**Heat for 10 min. at 95°C**

*** The cDNAs can be stored at -20°C. For more sensitive applications, a single phenol: chloroform extraction with ethanol precipitation can be performed on the cDNA to remove proteins, unutilized dNTPs, and primers. Typically, this is not necessary.**

## B. Real-time qPCR Reaction Setup

### 1. Mastermix qPCR Reaction Setup for 1 entire 96-well qPCR plate

To determine the expression profile for the target genes under study, mix the following for 1 entire qPCR plate:

**For 1 entire plate:**

| | | | |
|---|---|---|---|
| | 1,810 | µl | **2X SYBR Green*** qPCR Mastermix buffer |
| | 20 | µl | **User synthesized cDNA** |
| + | 1,670 | µl | **RNase-free water** |
| | 3,500 | µl | Total |

**Aliquot 28µl of Mastermix per well in your qPCR Plate.**

* SBI has tested and recommends SYBR Green Master mix from three vendors:
1. 2x Maxima SYBR GREEN-Fermentas
2. SYBR GreenER™ qPCR SuperMix for ABI PRISM® instrument from Invitrogen (Cat. #s 11760-100, 11760-500, and 11760-02K)
3. RT² Real-Time™ SYBR Green / ROX PCR (Cat. #s PA-012 and PA-112) from SuperArray.

**Resuspend Primers in Primer plate with 44µl RNase-free water per well before use. (The primers are dried-down in the Primer plate)**

**(*Resuspended primers tend to dry during storage. To maximize the use of the primers, we recommend aliquoting 2µl/ well in 20 different qPCR plates and dry them covered with a kimwipe at 37 degrees for 30 mins. Seal the plates and the dried plates can be stored at 4 degrees for over a year. Before use, resuspend in 2 µl of water.)**

**Note: Primers should be mixed well and allowed to be in solution for ~30min before use.**

**Then :**

**Load 2µl per well of each of the Primers from the Primer plate into your qPCR plate (well A1 into qPCR plate A1, etc.)**

The Mastermix contents can be scaled up or down depending upon on your experimental needs. If you want to perform the reactions in triplicate, scale up the reactions by 3-fold and add 3X the RNA input. Or, simply follow the above recipe three times for each of the qPCR plates you want to run as replicates. Once reagents are loaded into the wells, cover the plate with an optical adhesive cover and spin briefly in a centrifuge to bring contents to bottom of wells. Place plate in the correct orientation (well A1, upper left) into the Real-time qPCR instrument and perform analysis run.

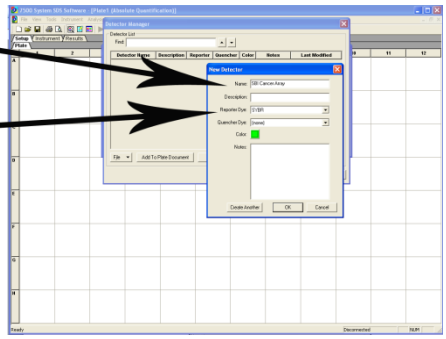
**Use a Multichannel pipette to load the qPCR plate with MasterMix and Primers:** Pour the Mastermix into a reservoir trough and use a 8 or 12 channel pipette to load the entire 96-well qPCR plate with the Mastermix. Then load the primers from the primer plate to the qPCR plate using a separate multichannel pipette.

## 2. Real-time qPCR Instrument Parameters

Follow the guidelines as detailed for your specific Real-time instrumentation. The following parameters tested by SBI were performed on an Applied Biosystems 7300 Real-time PCR System but can also apply to an ABI 7500 or an ABI 7900 96-well system. The details of the thermal cycling conditions used in testing at SBI are below. A screenshot from SBI's ABI7300 Real-time instrument setup is shown below also. Default conditions are used throughout.

**Create a detector**:

1. Create a new Detector
2. Name the Detector (any name will do)
3. Select Reporter Dye as "SYBR"
4. Select Quencher Dye as (none)
5. Highlight all wells and select this new detector to measure the signals

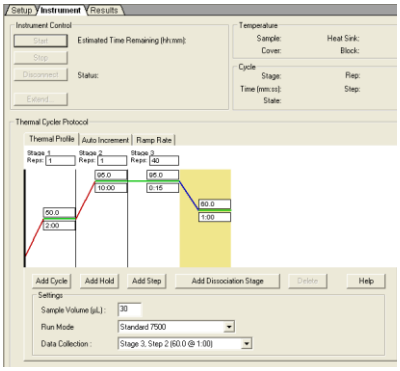


**Instrument Setup**:

**qPCR cycling and data accumulation conditions:**

1. 50°C 2 min.
2. 95°C 10 min.
3. 95°C 15 sec.
4. 60°C 1 min.

(40 cycles of steps 3 and 4), data read at 60°C 15 sec. Step (gold rectangle)




An additional recommendation is to include a melt analysis after the qPCR run to assess the Tm of the PCR amplicon to verify the specificity of the amplification reaction. Refer to the User Manual for your specific instrument to conduct the melt analysis and the data analyses of the amplification plots and Cycle Threshold (Ct) calculations. In general, Cycle thresholds should be set within the exponential phase of the amplification plots with software automatic baseline settings.

# III. Validation Data

## A. Designing of the primers for the Regulatory RNA profiler:

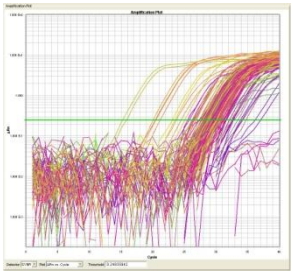
Primers for eRNAs and lncRNAs were designed by taking sequence from Pubmed (http://www.ncbi.nlm.nih.gov/nuccore/) or from published manuscripts. In some cases when Pubmed sequences were not available primers from publications were used. Primers were designed using the Primer III program (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) where forward and reverse primers were designed to get amplicon sizes ranging from 100 to 120 nucleotides. Each primer was checked using the genome browser website (http://genome.ucsc.edu/) to ensure that there are no homology to any other sequence other than the gene of interest.

**Regulatory RNA plate arrangement**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 4q32e | ACSL1 e | AZIN1e | CA12e | Lnc-PERP-1 | Lnc-KLF14-1 | Linc-LAMA4-3 | Lnc-LYZL2-1 | Linc-LAMA4-2 | Lnc-LCP2 1:1 | Lnc-FABP5-2:1 | Lnc-TMTC3-6:2 |
| B | Lnc-IER3-5:2 | Lnc-C21orf33-1 | ERBS3/SBNO2 e | Epha4 e | FKBP5 e | FOXC1 e | GREB1 e | IL1b-e | IL6 e | IRS2 e | KLF6 e | KLK3 e |
| C | MARCKS e | miR-200b e | nanog e | NKX31e | NRIP1e | P2RY2 e | P53BER2 | P53BER4 | PCBP1 e | PGRe | PLZFe | Pou5f1 e |
| D | Ppap2b e | SIAH2e1 | SLC30A4 e | SMAD7 e | SOCS3 e | SOX2 e | TFF1 e | TNFSF8e | Wnt-8b e | XBP1 e | CBR3-AS1 | CCAT1-L |
| E | CD48-AS- | CTBP1-AS | DBE-T | ERIC | FIRRE | GAPlinc | HAS2-AS1 | HOTTIP | iL7-mf-lncRNA | Lnc_DC | lncRNA-508851 | lncRNA-ATB |
| F | LOC100132354 | MRUL | ncRNA-a1-same as FAI1 | ncRNA-a2 | ncRNA-a3 | ncRNA-a4 | ncRNA-a5 | ncRNA-a6 | ncRNA-a7 | ncRuPAR | NONCO2077 | NONCO261 |
| G | NONCO2807 | NONCO2823 | NONCO2913 | NONCO526 | OIP5-mf | Paupar | PCAT-1 | PCGEM1 | PCNA-AS1 | PINT | PRNCR1 | RPLP0P2 |
| H | SENCR | IT1 | LncR1 | Thril | AS1 | BRD4 | FOXO3 | Actin | GAPDH | U6 | 18S rRNA | control |

## B. QC and Sample Data

### Regulatory RNA validation test



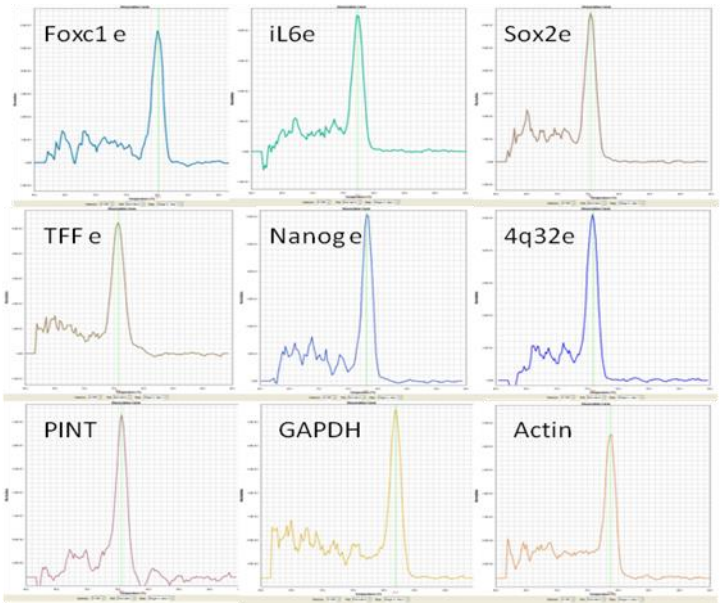
Regulatory RNA qPCR profiler was validated using cDNA from Human Foreskin Fibroblast (HFF) primary cells. Total RNA was converted to cDNA using SBI's RNA-Quant kit (Cat #RA430). The adjacent figure on the left shows real-time amplification plot for the entire plate for HFF cells. The Ct values ranged from 18.47 to 36.30, reflecting approximately a 5-log fold expression detection range.

### Controls

For our reference controls we have provided primers for GAPDH, Actin, U6 and 18S RNAs. Researchers can choose any of the reference controls of their choice. In addition, we have also provided primers for FOXO3 and BRD4. FOXO3 activation potentiates the activation of enhancer RNAs [12] and BRD4 is required for estrogen dependent enhancer activation [13].

### Primer performance

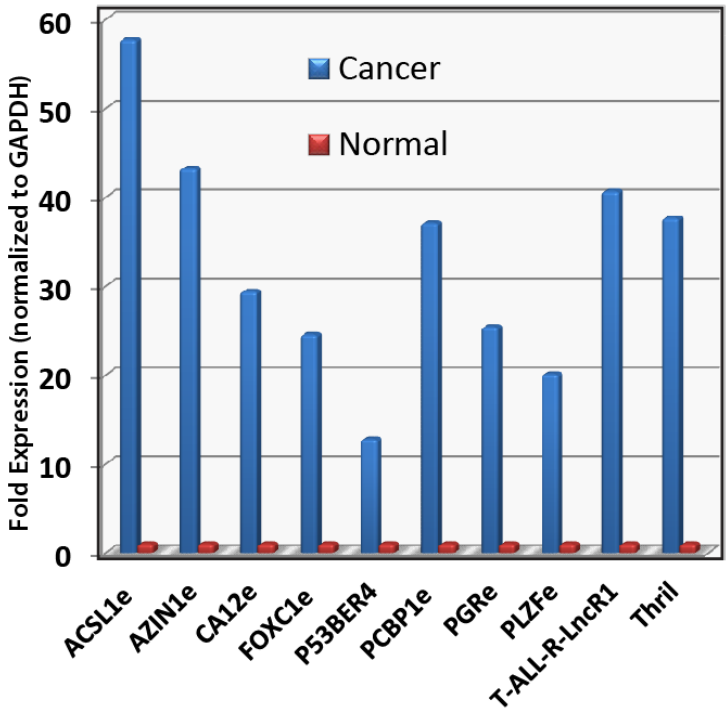
All primers have been validated by using total RNAs from several cell lines and tissues such as HEK293, HT1080, MCF7 ( human breast cancer cell line), human fibroblast cells, human fetal brain, human adult brain, iPSC cells, and neural precursor cells (NPCs). All primers in the assay show a single peak during our dissociation melt analysis. Shown below are representative peaks for several eRNAs and controls.




## C. Differential expression of regulatory RNAs

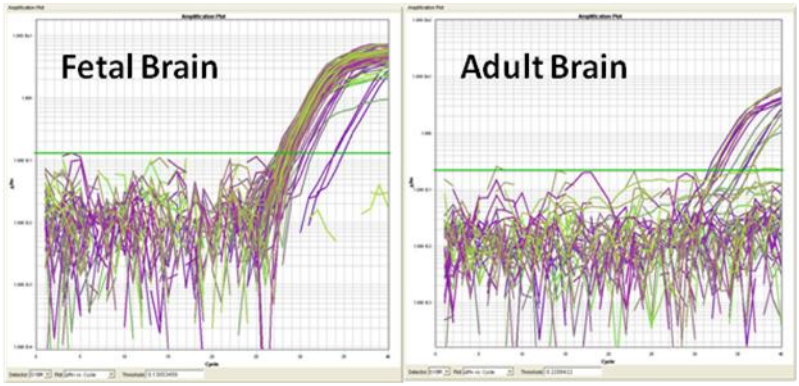
### I. Overexpression of regulatory RNA in cancer

Regulatory RNAs or eRNAs are aberrantly expressed in cancer cells, disease or during development. We found that there is a significant over expression of regulatory RNAs in cancer cells compared to normal cells. The figure below shows representative eRNAs and lncRNAs that are significantly up regulated in cancer cells. RNA from HFF (normal) and HT1080 fibrosarcoma (cancer) cells were taken, converted to cDNA and the cDNA was profiled for expression across the regulatory RNA profiler.




## II. Overexpression of Regulatory RNA during development

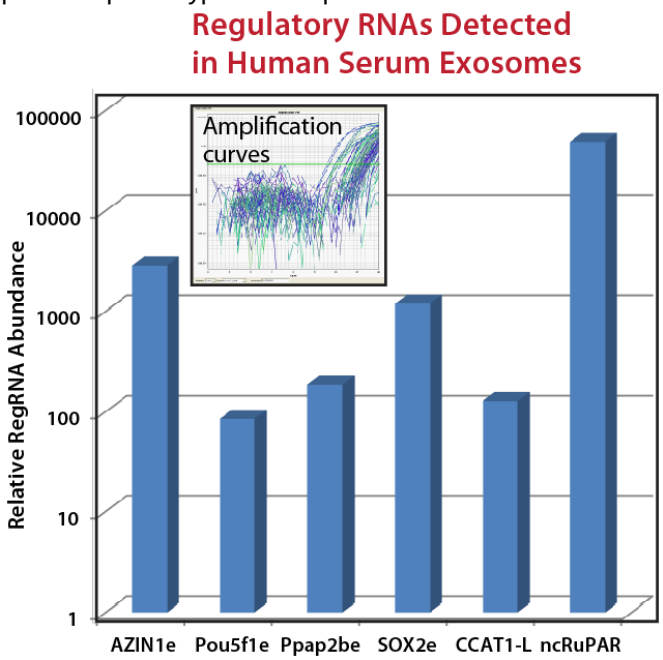
We have also explored if regulatory RNA expression was changed during the development process. We took total RNA from adult and fetal brain, converted them to cDNA and compared its expression. Intriguingly, most of the characterized regulatory RNAs, especially enhancer RNAs, were found to be poorly expressed in adult brain compared to fetal brain (see figure below), which may be indicative of potential suppression of regulatory RNAs during human brain development.



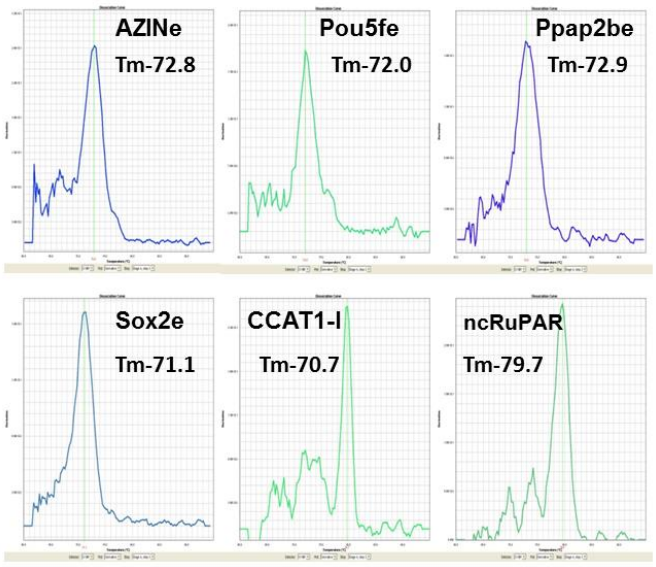

## III. Detection of Regulatory RNA in Serum Exosomes

Regulatory RNAs are only expressed in cellular total RNA, but have been found to be present in extracellular microvesicles known as exosomes. Exosomes are nano-sized vesicles secreted by all cell types that transport RNAs and proteins between cells. These vesicles are present in biofluids such as serum. In this experiment, exosomes were isolated from human serum using SBI's ExoQuick (cat # EXO5A-1) and exoRNA was isolated using SeraMir Kit (cat # RA800A-1). Approximately 50ng of total exo-RNA (as measured by NanoDrop UV-Vis) was utilized as input for the cDNA synthesis reaction using the RNA-Quant kit (cat # RA430A-1). Expression of Regulatory RNA in exosomes was profiled using the regulatory RNA qPCR array and data normalized to the geometric mean of the plate signals. Sample qPCR data for selected regulatory RNAs are shown below.

It is unclear of the significance of these regulatory RNAs in extracellular shuttles such as exosomes, however, due to the nature of exosomes as mediators for cell-cell signaling, it is possible they can mediate intercellular signaling with possible phenotypic consequences.




Shown below: Dissociation analysis of selected qPCR assays shown in bar graph above.



# IV. Troubleshooting

| Problem | Possible Solution |
|---|---|
| Too much background in qPCR signals | Use much less cDNA in the SYBR Green Mastermix. |
| No qPCR signals | 1. Did you select SYBR Green as the Detector's Reporter Dye?<br>2. Did the internal controls work?<br>3. Use more cDNA in Mastermix.<br>4. Check Mastermix contents and try a subset with internal control assays.<br>5. Also try lowering the Annealing Temperature to 50ºC. |
| How do I select the Threshold level for Ct analysis? | Typically, place the threshold setting in the upper third of the exponential phase of the amplification curve. Also, see the User Manual for your specific instrument or contact their technical support team for guidance. |

# V. References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# VI. Technical Support

For more information about SBI products and to download manuals in PDF format, please visit our web site:

**http://www.systembio.com**

For additional information or technical assistance, please call or email us at:

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Technical Support: tech@systembio.com
Ordering Information: orders@systembio.com

# VII. Licensing and Warranty Statement

## Limited Use License

Use of the Regulatory RNA Profiler Kit (*i.e.*, the "Product") is subject to the following terms and conditions. If the terms and conditions are not acceptable, return all components of the Product to System Biosciences (SBI) within 7 calendar days. Purchase and use of any part of the Product constitutes acceptance of the above terms.

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SBI has pending patent applications related to the Product. For information concerning licenses for commercial use, contact SBI.

## Limited Warranty

SBI warrants that the Product meets the specifications described in the accompanying Product Analysis Certificate. If it is proven to the satisfaction of SBI that the Product fails to meet these specifications, SBI will replace the Product or provide the purchaser with a refund. This limited warranty shall not extend to anyone other than the original purchaser of the Product. Notice of nonconforming products must be made to SBI within 30 days of receipt of the Product.

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