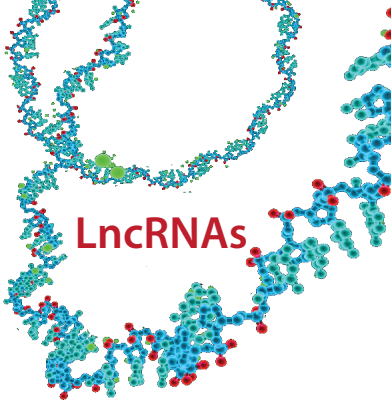


LncProfiler™ qPCR Array Kit

Quantitate long non-coding RNAs (lncRNAs) by real-time qPCR

Cat # RA900A-1, RA910A-1

User Manual

Store kit at -20°C on receipt

Contents

I. Introduction and Background

A. Overview

For the last few decades of the 20th century, the underlying dogma of molecular biology has been that the purpose of RNA is to direct the assembly of proteins from amino acids. A few exceptions to this paradigm were known (for example, ribosomal RNA and transfer RNA, which are functional RNA macromolecules that do not code for protein, or viral genomes that exist as or pass through an RNA phase as part of total genome replication).

Non-coding RNAs (ncRNAs) include the familiar “housekeeping” RNAs (ribosomal, transfer, small nuclear, and small nucleolar RNAs) and the thousands of regulatory RNAs that are the subject of recent intense exploration. Regulatory ncRNAs are arbitrarily classified by size: small ncRNAs (sncRNA) being less than 200 bp, and long ncRNAs (lncRNA) greater than 200 bp. The sncRNAs include other sub-classifications: microRNA (miRNA), endogenous small inhibitor RNA (endo-siRNA), and PIWI-associated RNA (piRNA).

The roles of lncRNAs in the regulation of gene expression and organismal development are diverse and just beginning to be discovered. Biological processes dependent upon lncRNAs include imprinting and gene dosage regulation, stem cell pluripotency, embryonic development and segmentation, hematopoiesis, and neural cell fate determination. LncRNAs may employ a number of mechanisms to impact gene expression via cis and trans processes.

Gene imprinting: While the function of parental gene imprinting is still unclear, lncRNAs have been found to participate in imprinting processes. Imprinting Control Regions (ICRs) are DNA regions that are differentially methylated depending on their parental origins. Unmethylated ICRs cause specific expression of nearby lncRNAs, which then suppress neighboring genes in cis. Airn and Kcnq1ot1 are examples of lncRNAs that cause suppression of paternally inherited genes.
Gene dosage and X chromosome inactivation: The discovery of Xist was one of the defining moments in the realization that ncRNAs can have profound roles in the control of gene expression. Xist is an lncRNA that suppresses the inactive, non-coding X chromosome (Xi) in female cells. In all, 7 ncRNAs are found as part of the X inactivation center on the X chromosome, including Xist. Initially, Xist and its antisense transcript Tsix are expressed on both X chromosomes. However, Tsix expression continues on the X that will remain active (Xa) and this activity recruits DNMT3A to suppress Xist from being transcribed on Xa. On Xi, it is Tsix that is suppressed, potentially via another lncRNA that is part of the X inactivation center, Jpx. With Tsix suppressed, the protein PRC2 is recruited to induce histone modification marks at the 5’ end of Xist. This upregulates Xist expression on Xi and causes further propagation of these silencing marks throughout Xi, which are maintained across the lifetime of the organism.

Embryonic development and segmentation: The expression of HOX genes is also regulated by lncRNAs. Some HOX-related lncRNAs operate in cis, having either enhancing or repressive effects. However, some like the human HOTAIR work in trans, and may function as scaffolds for histone-modifying complexes. It is not clear if trans-acting lncRNAs like HOTAIR are involved in the process of identifying the DNA sites to which the complexes will be recruited, or if that function is retained by the protein elements of the complex.

Stem cell pluripotency: The promoters of more than 100 lncRNAs are bound by stem cell factors. Disruption of these lncRNAs can alter cell differentiation. 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.

Cell fate determination: LncRNAs are implicated in cell fate determination events in multiple cell lineages, including the nervous system. TUG1 is an lncRNA that may enhance rod gene expression and suppress cone gene expression in the developing eye. Evf2 is a mouse lncRNA that appears to have both cis and trans effects to repress Dlx5, Dlx6, and Gad1 during forebrain development.

Dysregulated expression of lncRNAs has been shown to be associated with a broad range of diseases such as Alzheimer's, psoriasis and many cancers. Studying the expression patterns of lncRNAs will be a crucial method to understanding the roles they play in many model systems. SBI has built a sensitive, accurate and robust qPCR array to enable researchers to closely profile the expression changes in the top lncRNAs known to date.

This manual provides details and information necessary to use the LncProfiler™ Kit to tag and convert small non-coding RNAs into detectable and quantifiable cDNAs. The system allows for the ability to quantitate dynamic fold differences of lncRNAs across 20 separate experimental RNA samples. The array plate also includes 5 endogenous RNA assays as normalization signals. To ensure optimal results, please read the entire manual before using the reagents and material supplied with this kit.

These LncProfiler qPCR Array comes with all the reagents necessary to tag and long non-coding as well as small RNAs from 20 different total RNA samples into quantifiable cDNA. The kits include assays in pre-formatted plates for well-annotated human, lncRNAs with three endogenous reference RNA controls on each plate. All of the lncRNAs on the qPCR array have validated primer sets for well-annotated lncRNAs that are registered in the lncRNA database created by Dr. John Mattick (www.lncrnadb.org).

Potential functions of lncRNAs

To date, lncRNAs have been found to exhibit a wide range of functions ranging from signaling, serving as molecular decoys, guiding ribonulceoprotein complexes to specific chromatin sites and also participating as scaffolds in the formation of complexes.

I. Signaling

The transcription of certain lncRNAs is very tissue and temporal specific. Their expression can be in response to certain stimuli, such as cellular stress and temperature. Thus, lncRNAs can serve as molecular signals and can act as markers of functionally significant biological events. Examples include imprinting lncRNAs **XIST, AIR and Kcnq1ot1**.

II. Decoys

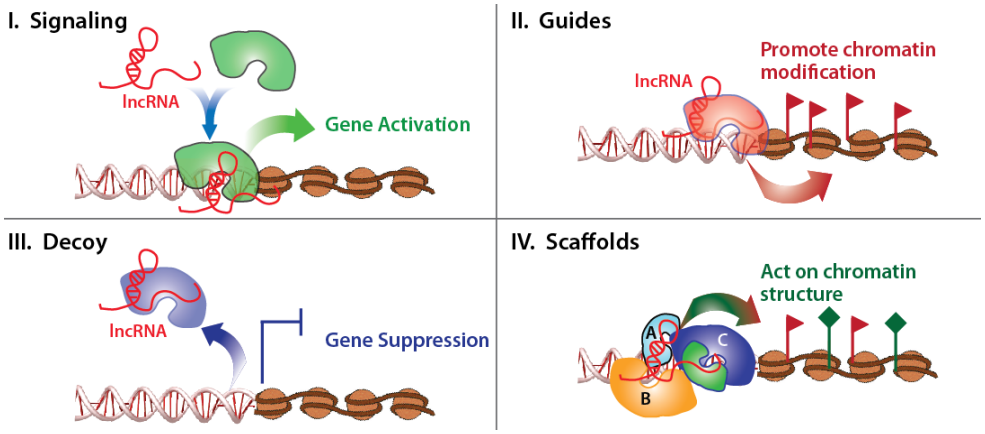
The molecular decoy type of activity takes place when specific lncRNAs are transcribed and then bind to and titrate away protein factors. Decoy lncRNAs can "sponge" protein factors such as transcription factors and chromatin modifiers. This leads to broad changes in the cell's transcriptome. Example is **MALAT1**.

III. Guides

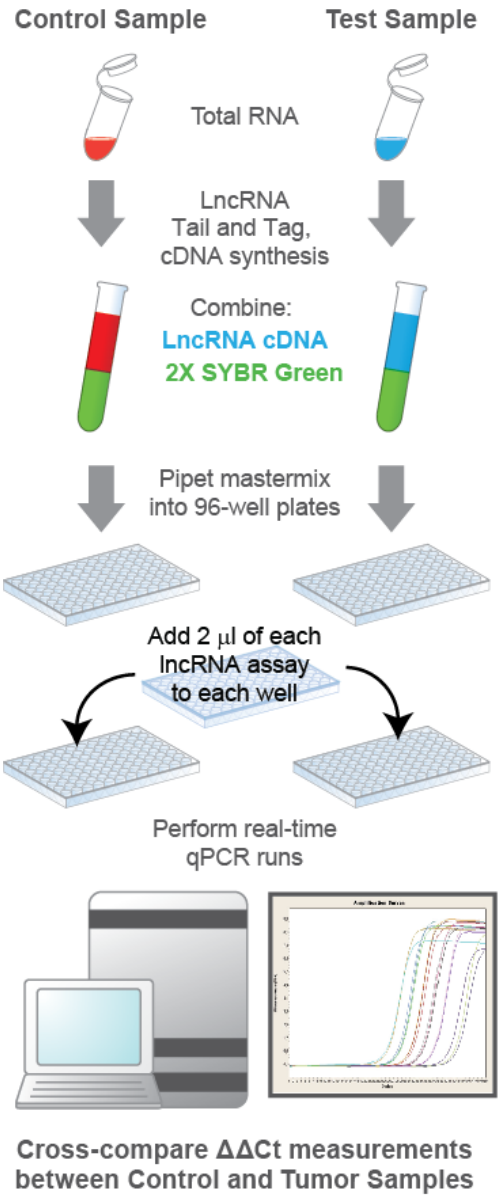
LncRNAs can be molecular guides by localizing particular ribonucleoprotein complexes to specific chromatin targets. This activity can cause changes in gene expression either in *cis* (on neighboring genes) or in *trans* (distantly located genes) that cannot be easily predicted by just the lncRNA sequence itself. Some example lncRNAs that act as guides are **XIST and HOTTIP**.

IV. Scaffolds

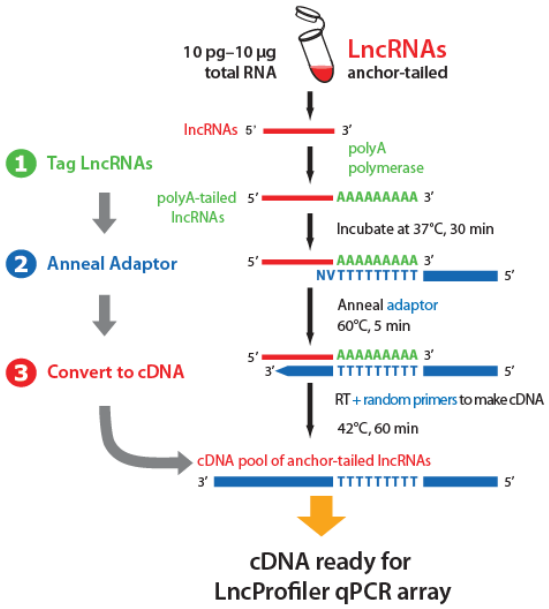
Assembly of complex protein complexes can be supported by lncRNAs, linking factors to together to form new functions. Some lncRNAs possesses different domains that bind distinct protein factors that altogether, may impact transcriptional activation or repression. Some examples of scaffold lncRNAs are **HOTAIR and ANRIL**.



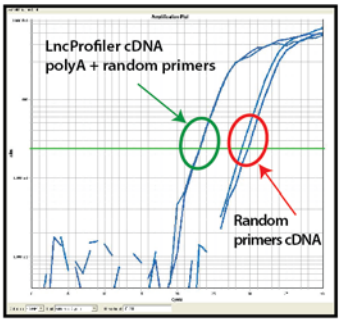
B. LncProfiler qPCR profiler workflow

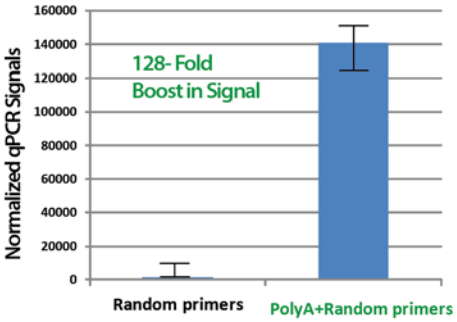


C. How the LncProfiler cDNA synthesis works



The initial polyadenylation step greatly enhances cDNA synthesis yields of lncRNAs (over 100-fold) and enables the usage of small RNAs (like U6 and RNU43) to be included as reference controls on the qPCR array.





D. List of components

40 µl	5X PolyA Polymerase Buffer	10 µl Poly A Reaction (enough for 20 reactions)
10 µl	PolyA Polymerase	
20 µl	25 mM $MnCl_2$	
30 µl	5 mM ATP	
10 µl	Oligo dT Adaptor	20 µl RT Reaction (enough for 20 reactions)
80 µl	5X Reverse Transcriptase Buffer + Random Primer Mix	
20 µl	Reverse Transcriptase	
30 µl	0.1 M Dithiothreitol (DTT)	
50 µl	dNTP Mix	
3500 µl	2X SYBR Green master Mix *	(enough for 20 profiles)
	Array Primers, dried down in Primer plate (200 µmoles); resuspend in 42µl RNase-free Water	
1.2 ml	RNase-free Water	

***Catalog# RA910A-1 contains all of the components listed above.**

***Catalog# RA900A-1 has all of the above components except for the 2X SYBR Green reagent.**

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 lncRNAs are dried-down in the wells of the optical qPCR plates. Resuspend in 10µl RNase-free water. SBI recommends using the LncProfiler qPCR array with the following SYBR Green reagents:

- 2X Maxima® SYBR Green with Rox (Cat# K0223) from Fermentas.
- Power SYBR Master Mix® (Cat. #s 4368577, 4367650, 4367659, 4368706, 4368702, 4368708, 4367660) from Applied Biosystems.

II. Protocol

A. LncProfiler cDNA reaction setup

(for 1 RNA sample to be assayed on qPCR 96-well plates)

It is important to start with total RNA that includes the lncRNA fraction. RNA input can be as low as 1-2 μg total. For optimum signals, perform the following.

Dilute your RNA to ~200-400 ng/μl

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

STEP 1:
PolyA Tail

5 μl Total RNA (~1-2 μg)
2 μl 5X PolyA Buffer
\+ 1 μl 25mM $MnCl_2$
1.5 μl 5mM ATP
0.5 μl PolyA Polymerase
10 μl

↓

Incubate for 30 min. at 37°C

↓

STEP 2:
Anneal Anchor dT Adapter

Add: + 0.5 μl Oligo dT Adapter

↓

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

Done!

*** The lncRNA 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, unused dNTPs and primers, typically this is not necessary.**

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

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

For 1 entire plate:

+	1,750	µl	**2X SYBR Green** qPCR Mastermix buffer
	20	µl	**LncRNA cDNA (from Step A)**
	1,730	µl	**RNase-free water**
	3,500	µl	Total

Aliquot 29µl of Mastermix per well in your qPCR Plate.

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

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.)

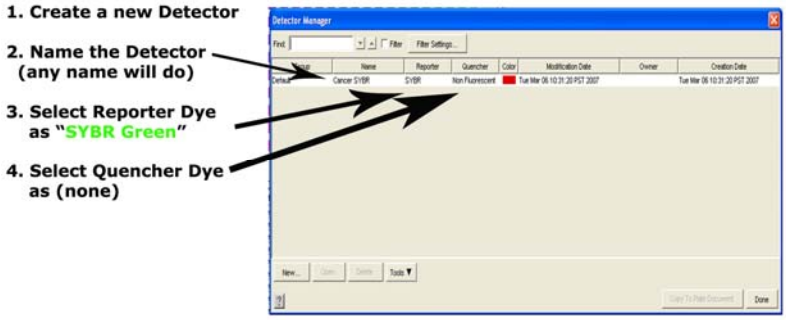
The Mastermix contents can be scaled up or down depending upon on your experimental needs. If you want to perform the reactions in triplicate, scale up the cDNA synthesis reactions by 3-fold and add 3X the RNA input. Or, simply follow the above recipe three times for each of the qPCR plates you want 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 7300/7500 Real-time PCR System but can also apply to any other 96-well systems. The details of the thermal cycling conditions used in testing at SBI are below. A screenshot from the Real-time instrument setup is shown below also. Default conditions are used throughout.

Create a detector:



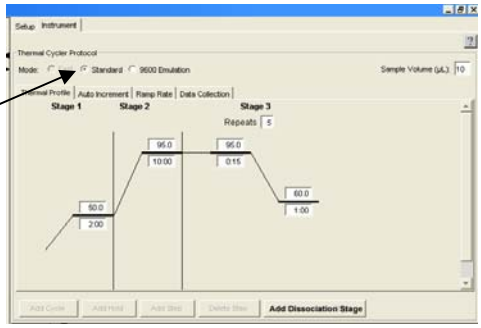
Instrument setup:

qPCR cycling and data accumulation conditions:

Standard Protocol

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

(40 cycles of Stage 3), data read at 60°C 1 min. Step.



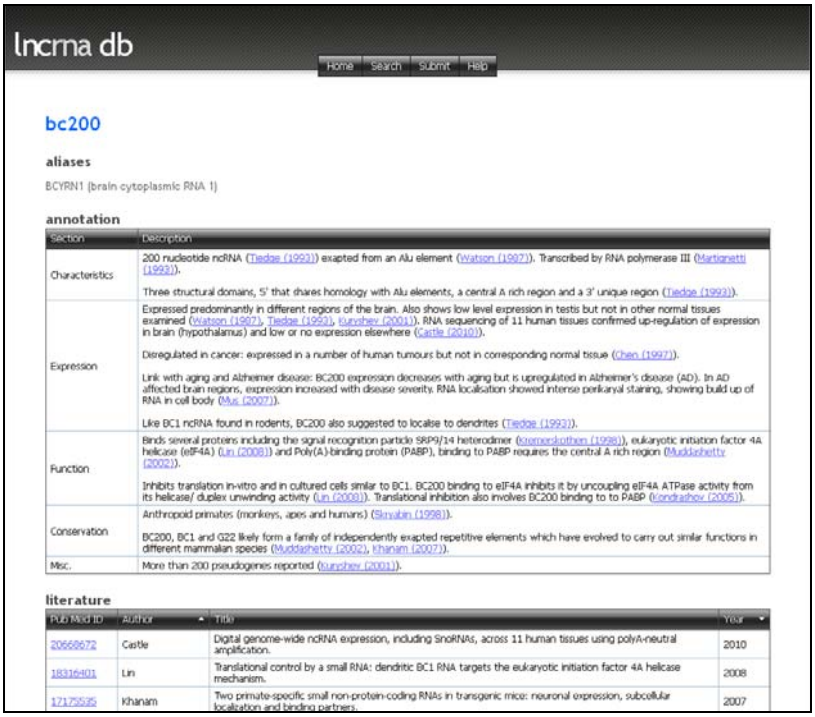
An additional recommendation is to include a **Dissociation Stage** after the qPCR run to assess the Tm of the PCR amplicon to verify the specificity of the amplification reaction. Refer to the User Manual for your specific instrument to conduct the melt analysis and the data analyses of the amplification 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.

C. How the lncRNA-specific primers are designed for detection and quantitation in the qPCR array

SBI's LncProfiler is complete cDNA synthesis kit combined with a 96-well based qPCR assay set. The qPCR assays have been validated across numerous cell types for robust and specific performance. Some lncRNAs have endogenouse polyA tails, while other lncRNAs do not. To enhance qPCR assay performance, the cDNA synthesis kit includes reagents to polyadenylate all lncRNAs before cDNA conversion with the oligo dT adaptor and random primers. SBI's lncRNA qPCR assays are derived from published primer sequences and others were designed in-house for robust performance and all amplicons are below 200 bp in size.

All qPCR assays are designed to detect Human lncRNAs that are annotated in Dr. John Mattick's LncRNA database.
http://www.lncrnadb.org/

Below is a screenshot of the lncRNA database interface, the example is for the bc200 lncRNA database entry where you can find useful information about its discovery, expression, function, conservation as well as some citations for the lncRNA.



lncrna db

Home | Search | submit | Help

bc200

aliases

BCYRN1 (brain cytoplasmic RNA 1)

annotation

Section	Description
Characteristics	200 nucleotide ncRNA (Tiedge (1993)) exapted from an Alu element (Watson (1997)). Transcribed by RNA polymerase III (Martignetti (1993)). Three structural domains, 5' that shares homology with Alu elements, a central A rich region and a 3' unique region (Tiedge (1993)).
Expression	Expressed predominantly in different regions of the brain. Also shows low level expression in testis but not in other normal tissues examined (Watson (1997), Tiedge (1993), Kuryshev (2001)). RNA sequencing of 11 human tissues confirmed up-regulation of expression in brain (hypothalamus) and low or no expression elsewhere (Castle (2010)). Dieregulated in cancer: expressed in a number of human tumours but not in corresponding normal tissue (Chen (1997)). Link with aging and Alzheimer disease: BC200 expression decreases with aging but is upregulated in Alzheimer's disease (AD). In AD affected brain regions, expression increased with disease severity. RNA localisation showed intense perikaryal staining, showing build up of RNA in cell body (Mus (2007)). Like BC1 ncRNA found in rodents, BC200 also suggested to localise to dendrites (Tiedge (1992)).
Function	Binds several proteins including the signal recognition particle SRP9/14 heterodimer (Kremerskothen (1998)), eukaryotic initiation factor 4A helicase (eIF4A) (Lin (2008)) and Poly(A)-binding protein (PABP), binding to PABP requires the central A rich region (Muddashetty (2002)). Inhibits translation in-vitro and in cultured cells similar to BC1. BC200 binding to eIF4A inhibits it by uncoupling eIF4A ATPase activity from its helicase/ duplex unwinding activity (Lin (2008)). Translational inhibition also involves BC200 binding to to PABP (Kondrashov (2005)).
Conservation	Anthropoid primates (monkeys, apes and humans) (Skryabin (1998)). BC200, BC1 and G22 likely form a family of independently exapted repetitive elements which have evolved to carry out similar functions in different mammalian species (Muddashetty (2002), Khanam (2007)).
Misc.	More than 200 pseudogenes reported (Kuryshev (2001)).

literature

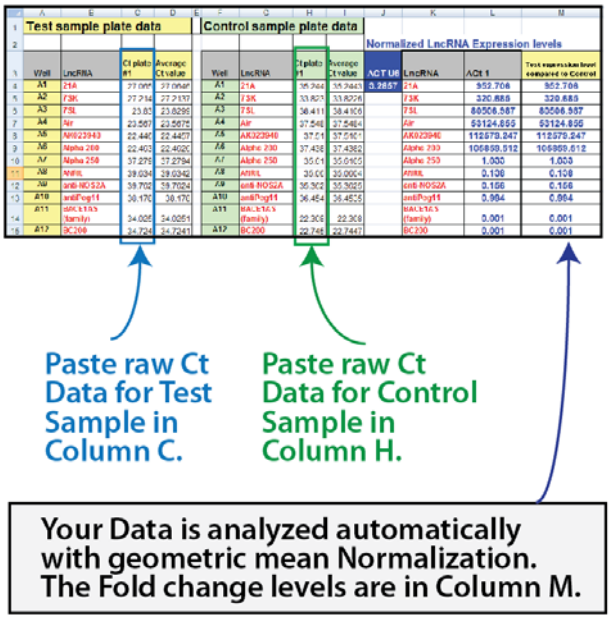
Pub Med ID	Author	Title	Year
20668672	Castle	Digital genome-wide ncRNA expression, including SnoRNAs, across 11 human tissues using polyA-neutral amplification.	2010
18316401	Lin	Translational control by a small RNA: dendritic BC1 RNA targets the eukaryotic initiation factor 4A helicase mechanism.	2008
17175535	Khanam	Two primate-specific small non-protein-coding RNAs in transgenic mice: neuronal expression, subcellular localization and binding partners.	2007

D. LncProfiler qPCR array contents

The qPCR array plate contains assays for 90 lncRNAs and also includes 5 endogenous reference RNAs as normalization signals. Please see the SBI website to download the qPCR array arrangement and ΔΔCT analysis software. **www.systembio.com/LncRNA**

	1	2	3	4	5	6	7	8	9	10	11	12
A	21A	7SK	7SL	Air	AK023948	Alpha 280	Alpha 250	ANRIL	anti-NOS2A	antiPeg11	BACE1AS	BC200
B	CAR Intergenic	DHFR upstream	Dio3os	DISC2	DLG2AS	E2F4 antisense	EgoA	EGO B	Emx2os	Evf1 and EVF2	GAS5-family	Gomafu
C	H19	H19 antisense	H19 upstream	HAR1A	HAR1B	HOTAIR	HOTAIRM1	HOTTIP	Hoxa11as	HOXA3as	HOXA6as	HULC
D	IGF2AS	IPW	Jpx	Kcnq1ot1	KRASP1	L1PA16	p21	RoR	SFMBT2	VLDLR	LOC285194	LUST
E	Malat1	mascRNA	MEG3	MEG9	MER11C	ncR-uPAR	NDM29	NEAT1	Nespas	NRON	NTT	p53 mRNA
F	PCGEM1	PR antisense	PRINS	PSF inhibiting	PTENP1	RNCR3	SAF	SCA8	snaR	SNHG1	SNHG3	SNHG4
G	SNHG5	SNHG6	Sox2ot	SRA	ST7OT	TEA ncRNAs	Tmevpg1	TncRNA	Tsix	TUG1	UCA1	UM9-5
H	WT1-AS	Xist	Y RNA-1	Zeb2NAT	Zfas1	Zfhx2as	18S rRNA	RNU43	GAPDH	LAMIN A/C	Human U6	No assay control

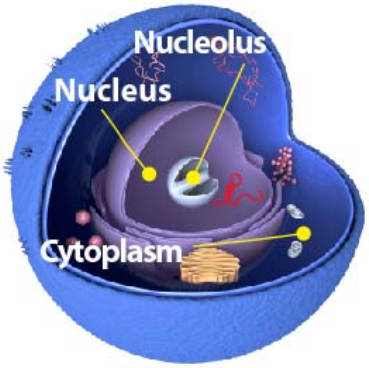
Performing ΔΔCt data analysis



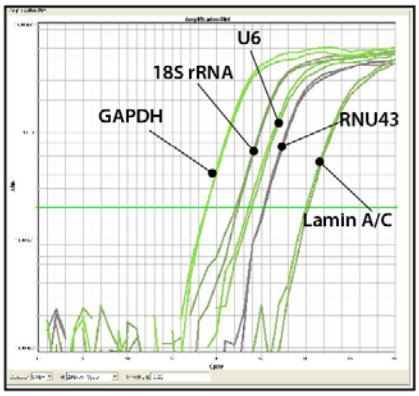
Five Subcellular reference controls

LncRNAs can localize and function in the nucleolus, nucleus and in the cytoplasm. The LncProfiler qPCR array includes RNA reference controls to allow for subcellular fractionation studies to identify and profile three separate subcellular compartments.

· Nucleolus: SnoRNA RNU43 (and some 18S rRNA)
· Nucleus: Small Nuclear splicing snRNA U6B
· Cytoplasm: GAPDH, Lamin A/C and 18S rRNA



Profile your lncRNAs wherever they are located within the cell.

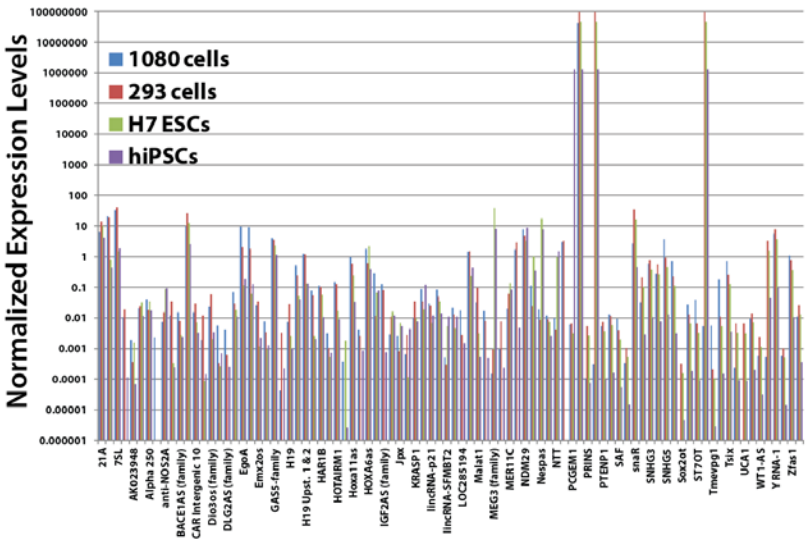


Example of reference control amplification plots. Results may vary depending upon the cell types analyzed.

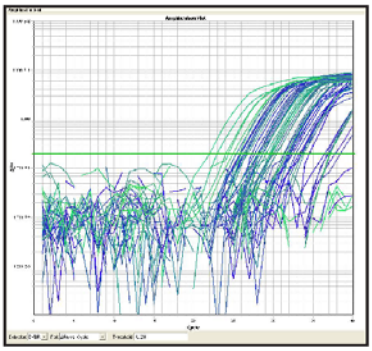
III. Sample Data

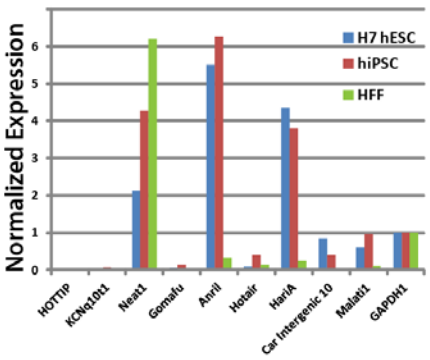
A. LncProfiler qPCR Array sample data

The LncProfiler qPCR arrays was tested across Human HT1080 lung cancer cells, HEK293 embryonic kidney cells, H7 human embryonic stem cells and Human induced Pluripotent stem cells (hiPSCs). The hiPSCs were made using standard Yamanaka retroviruses for Oct4, Sox2, Klf4 and c-Myc. Intriguingly, there are similar but not identical lncRNA expression patterns between H7 stem cells and hiPSC cells. The lncRNA expression patterns differ significantly between HEK293 cells and HT1080 lung cancer cells. The LncProfiler qPCR array offers over 10 log-fold of expression range detection.



To explore the lncRNA expression pattern differences between H7 stem cells and iPSCs, we next profiled the source cells that were used to make the iPSCs (HFF cells). The LncProfiler qPCR Array was tested using 2 μg total RNA extracted from H7 human embryonic stem cells (H7 hESC), human induced pluripotent stem cells (hiPSC) and human foreskin fibroblast cells (HFF). The sample RNAs were converted to cDNA LncProfiler Kit. The resulting cDNAs were tested using about 10 ng cDNA per well. Shown below are the resulting Real-time amplification plots for selected data. The Ct data were normalized and shown as Normalized Expression levels in the Bar Graph below for some selected data. The H7 and iPS cells profile was similar (as expected). The iPS source cell's (HFF) lncRNA expression pattern was very different from the stem cells, especially for Anril, HariA, Car Intergenic 10 and Malat1.





Protocol per one well of 6-well plate

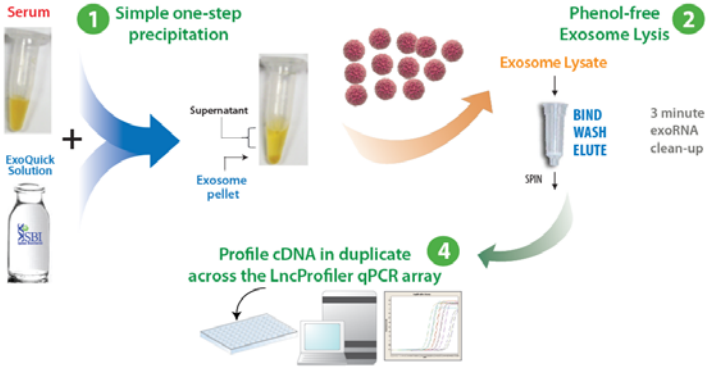
1. Confluent cells in a 6-well, remove media
2. Add 1ml Trizol directly to cells on plate
3. Incubate at Room for 5 minutes for complete lysis
4. Collect Trizol/cell mixture and transfer to 1.5ml tube
5. Add 200 μl Chloroform, vortex 15 seconds
6. Centrifuge mixture for 15 minutes at 4°C
7. Collect aqueous layer and transfer to fresh 1.5 ml tube
8. Add equal volume (~250 μl) Isopropanol, mix by inversion

9. Precipitate RNA overnight at -20ºC
10. Centrifuge at 13,000 rpm for 20 minutes
11. Remove supernatant
12. Wash 1X with 500 µl 80% Ethanol
13. Centrifuge again for 5 minutes at 13,000 rpm
14. Remove supernatant and let air dry 5 minutes
15. Resuspend RNA pellet in 50 µl water (RNase-free)
16. Use 5 µl of RNA per cDNA synthesis

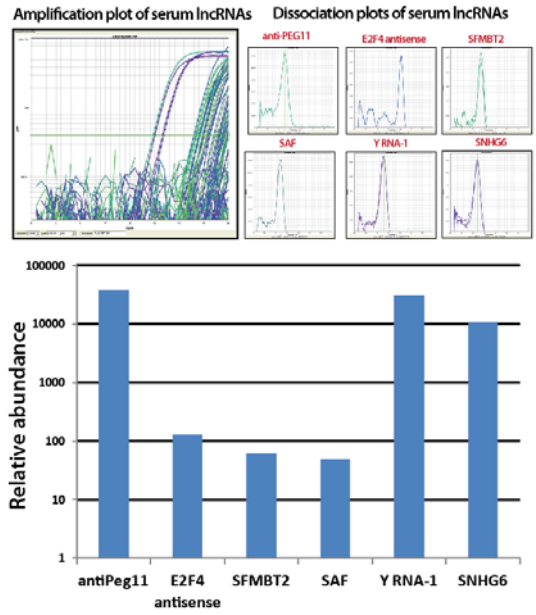
LncRNAs are present in serum exosomes

There is high interest in discovering and developing useful RNA-based biofluid markers. The RNAs in patient fluids are present in circulating exosomes. Exosomes are 40 - 100 nm membrane vesicles secreted by most cell types in vivo and in vitro. Exosomes are found in blood, urine, amniotic fluid, malignant ascite fluids and contain distinct subsets of microRNAs depending upon the tumor from which they are secreted. We wanted to test whether lncRNAs may be present in circulation exosomes as well by using the LncProfiler qPCR array. We precipitated exosomes from a human pooled serum sample (1ml) using SBI's ExoQuick™ exosome precipitation reagent (cat#EXOQ5A-1). The exosome vesicles were then lysed and the exosomal RNA purified using SBI's SeraMir™ kit (cat#RA806A-1). The resulting exoRNA was converted to cDNA using the LncProfiler cDNA synthesis kit. The cDNA was tested in duplicate across all of the LncProfiler qPCR array assay set.

Purifying exosome RNAs and profiling lncRNAs

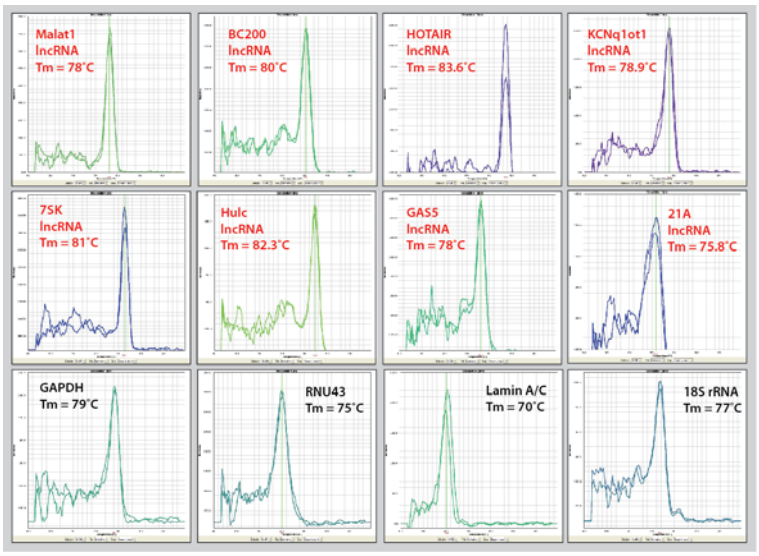


Discover new biofluid lncRNA biomarkers using the LncProfiler qPCR array kit

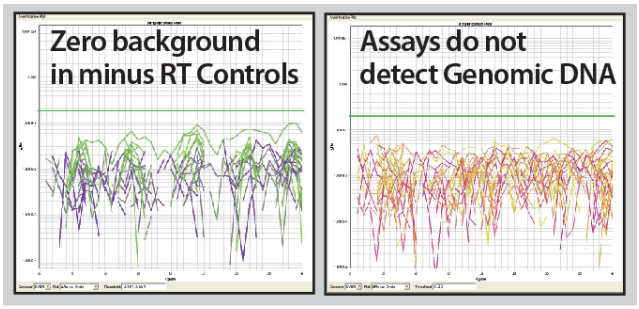


B. Specificity Tests

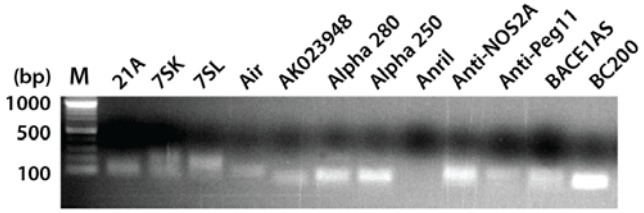
Dissociation analysis
Sample dissociation analyses for reference controls and some lncRNA qPCR assays performed in duplicate.



Clean assay design with no background
Total RNA was prepared from human HT1080 cells in culture. As a control, 2µg of this RNA was checked in a mock cDNA synthesis reaction where the reverse transcriptase (RT) was left out. The sample was then tested across the LncRNA Profiler qPCR assays. Separately, we spiked in 10ng of human genomic DNA and tested this sample with the qPCR assays as well. There is ZERO background in the minus RT controls and the LncRNA Profiler assays do not show any amplification signals even with spiked-in genomic DNA. Profile with confidence and only detect lncRNAs.



Gel analysis
Sample gel analyses for selected lncRNA qPCR assay amplicon products from HFF cDNA and separated on a 1.5% agarose gel and stained with ethidium bromide.



IV. Troubleshooting

Problem	Possible Solution
Too much background in qPCR signals	Use much less cDNA in the SYBR Green Mastermix.
No qPCR signals	Did you select SYBR Green as the Detector's Reporter Dye? Did the controls work? Use more cDNA in Mastermix. Check Mastermix contents and try a subset with the controls as a positive control. Also try lowering the Annealing Temperature to 55ºC.
How do I select the Threshold level for Ct analysis ?	Typically place the threshold setting in the center of the exponential phase of the amplification curve. Also see the User Manual for your specific instrument or phone their technical support team for guidance.

V. LncRNA Technical References (selected)

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VIII. Technical Support

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

http://www.systembio.com

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IX. Licensing and Warranty Statement

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