RNA-Quant™ cDNA Synthesis Kit

Profile any RNA by real-time qPCR

Cat# RA430A-1

User Manual

Store kit at -20°C on receipt

Contents

I. Introduction and Background

A. Overview

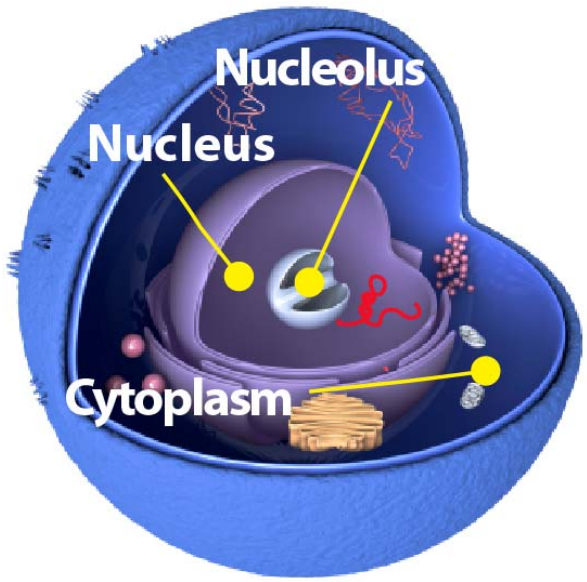
Types of RNAs

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. These are the functions of messenger RNAs (mRNAs). mRNAs code for protein genes, have 5' mG caps and most often have poly-A tails. 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). There are now numerous exceptions that include: microRNAs, small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs) and long non-coding RNAs (lncRNAs). The functions of each class of RNA are briefly summarized in the Table below.

Type of RNA	Function
Messenger RNAs (mRNAs)	Code for Proteins
MicroRNAs (miRNAs)	Regulate mRNA translation
Small Nuclear RNAs (snRNAs)	Bridge splicing events during transcription via the spliceosome
Small Nucleolar RNAs (snoRNAs)	Guide methylation on target RNAs
Long Non-coding RNAs (lncRNAs)	Bind chromatin for epigenome changes and scaffolds for protein complexes
Ribosomal RNAs (rRNAs)	Structural and catalytic subunits of ribosomes

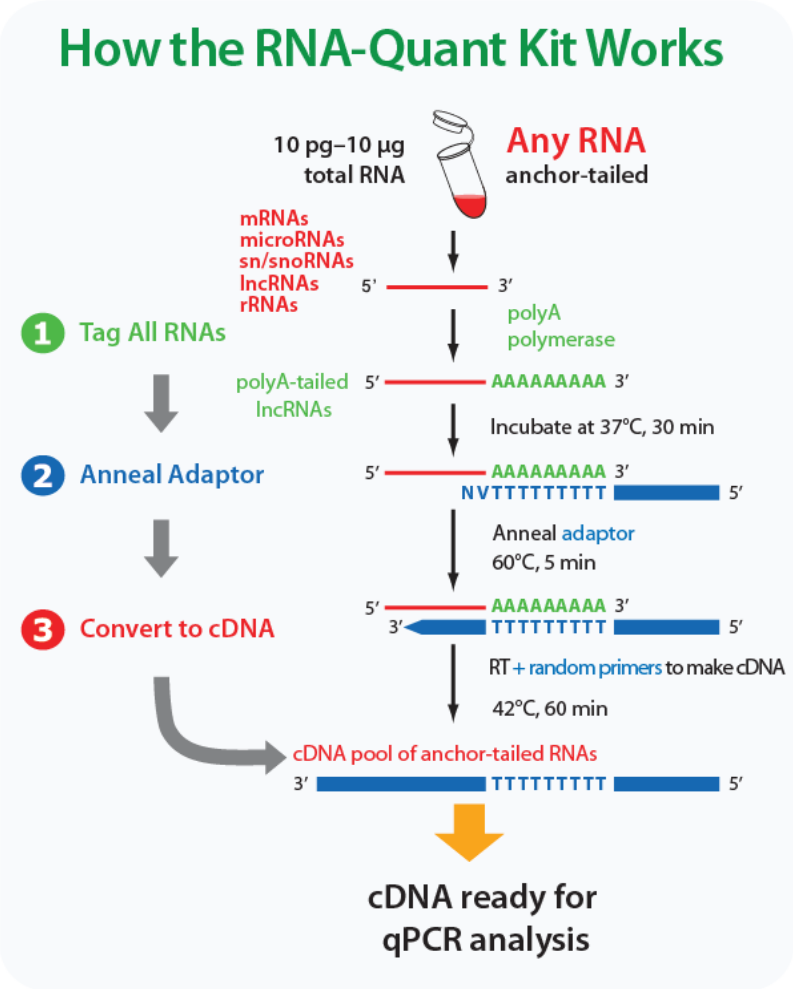
Non-coding RNAs (ncRNAs) include the familiar "housekeeping" RNAs (ribosomal, transfer, small nuclear, and small nucleolar RNAs) and the thousands of regulatory RNAs that are the subject of recent intense exploration. Regulatory ncRNAs are arbitrarily classified by size: small ncRNAs (sncRNA) being less 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).

This manual provides details and information necessary to use the RNA-Quant™ cDNA synthesis kit that is designed to tag and convert any type of RNA into detectable and quantifiable cDNAs. The system allows for the ability to quantitate dynamic fold differences of RNAs across 20 separate experimental RNA samples. The RNA-Quant kit also includes 3 endogenous RNA reference qPCR assays as normalization signals. These assays include miR-16 microRNA, Y RNA lncRNA and GAPDH mRNA. These three qPCR assays will work with both human and mouse RNA samples. To ensure optimal results, please read the entire manual before using the reagents and material supplied with this kit.



Profile any RNA, no matter its identity or localization.

B. RNA-Quant cDNA synthesis overview



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

C. 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	
1.2 ml	RNase-free Water	
600 µl	3' Universal Reverse primer	
50 µl	miR-16 microRNA qPCR assay, 10µM (human and mouse)	
50 µl	Y RNA lncRNA qPCR assay, 10µM (human and mouse)	
50 µl	GAPDH mRNA qPCR assay, 10µM (human and mouse)	

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. SBI recommends using the following SYBR Green reagents:

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

II. Protocol

A. RNA-Quant 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 RNA-Quant cDNAs can be stored at -20ºC. 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 96-well qPCR plate

To determine the expression profile for the RNAs under study, mix the following for 1 well in a 96-well qPCR plate:

For 1 well in 96-well plate (30 µl reaction):

+	15	µl	**2X SYBR Green** qPCR Mastermix buffer
	0.3	µl	**RNA-Quant cDNA (from Step A)***
	0.5	µl	3' Universal Reverse Primer (10 µM)
	14.7	µl	**RNase-free water**
	29	µl	Total

*** Starting RNA amount and amount of cDNA per qPCR reaction**

Starting RNA input	Amount of RQ cDNA to add per rxn
10pg -100 ng	0.5µl to 1µl
100ng – 1 µg	Dilute 1: 100
> 1 µg Dilute	1: 1,000

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

Then :

Load 1µl per well of each of the Control qPCR Primer (10 µM) into your qPCR plate well (miR-16, Y-RNA or GAPDH).

C. Mastermix qPCR Reaction Setup for 384-well qPCR plate

To determine the expression profile for the RNAs under study, mix the following for 1 well in a 384-well qPCR plate:

For 1 well in 384-well plate (6 µl reaction):

	3 µl	**2X SYBR Green** qPCR Mastermix buffer
+	0.06 µl	**RNA-Quant cDNA (from Step A)***
	0.1 µl	3' Universal Reverse Primer (10 µM)
	2.6 µl	**RNase-free water**
	5.7 µl	Total

*** Starting RNA amount and amount of cDNA per qPCR reaction**

Starting RNA input	Amount of RQ cDNA to add per rxn
10pg -100 ng	0.1µl to 0.5µl
100ng – 1 µg	Dilute 1: 500
> 1 µg Dilute	1: 5,000

Aliquot 5µl of Mastermix per well in your qPCR Plate.

Then :

Load 0.2µl per well of each of the Control qPCR Primer (10 µM) into your qPCR plate well (miR-16, Y-RNA or GAPDH).

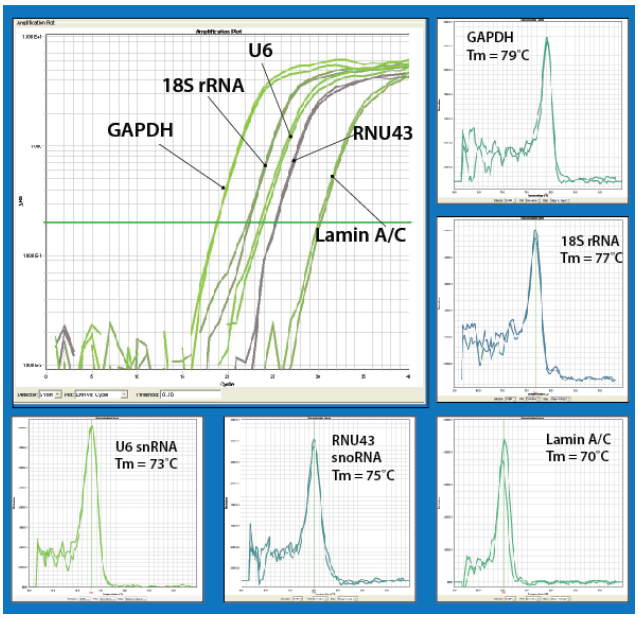
III. Sample Data

A. Profile mRNAs, snRNAs, snoRNAs and rRNAs

Total RNA was harvested from human HT1080 cells using the following protocol. Sample amplification plots and specificity tests using dissociation analyses are shown below.

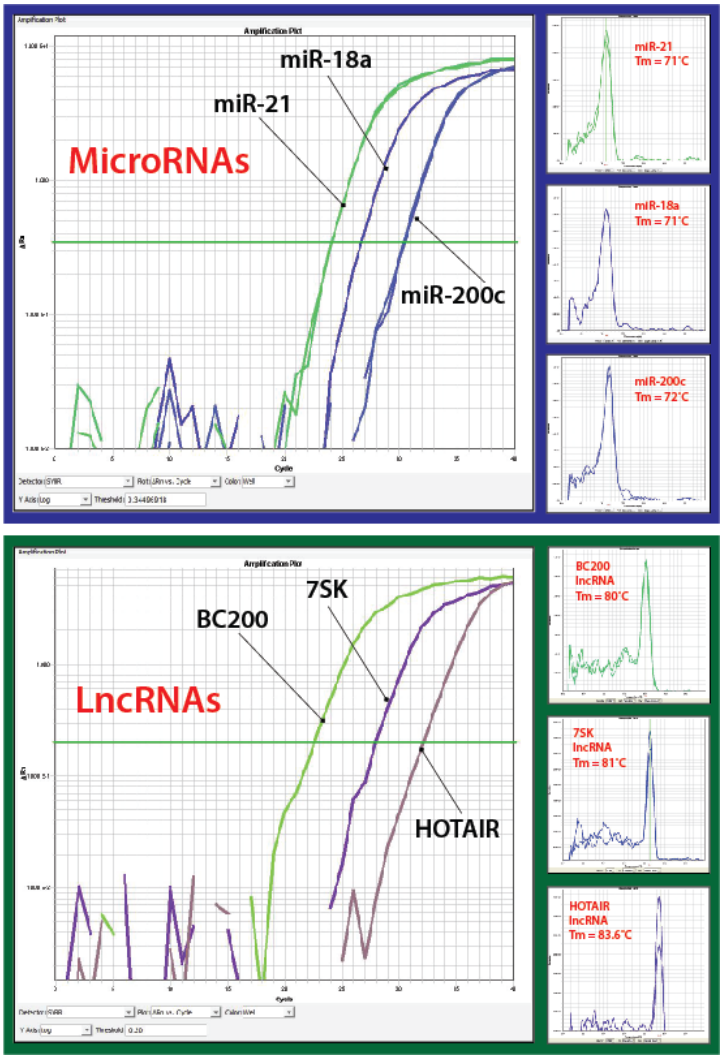
Protocol per one well of 6-well plate

1. Confluent cells in a 6-well, remove media
2. Add 1ml Trizol directly to cells on plate
3. Incubate at Room temperature 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 4ºC
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 RNA-Quant cDNA synthesis (~2µg)



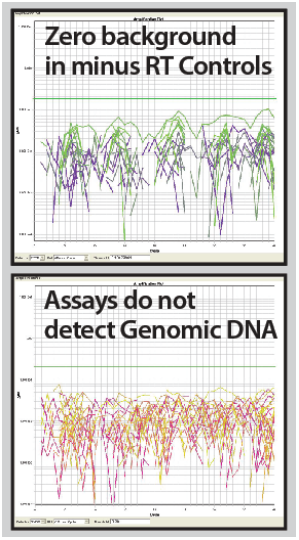
B. Profile microRNAs and lncRNAs

The same cDNA from IIIA. above was used to profile microRNAs and lncRNAs by qPCR. Sample amplification plots and specificity tests using dissociation analyses are shown below.



C. Clean assay design with no background

The poly-A tailing and tagging method in the RNA-Quant system generates cDNA that is highly unique and when used with the 3' Universal reverse primer does not produce any signals in minus RT controls and does not detect any residual genomic DNA. 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 and microRNA 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 qPCR assays do not show any amplification signals even with spiked-in genomic DNA. Profile with confidence and only detect your RNAs of interest.



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. RNA Technical References (selected)

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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 RNA-Quant™ 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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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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