applied biosystems

RNA Quantification Kit

USER GUIDE

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ThermoFisher SCIENTIFIC

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Revision	Date	Description
A.0	13 March 2018	Initial release in Thermo Fisher Scientific document control system. Supersedes legacy Affymetrix™ publication number 703388. Updated manufacturer.

Contents

1 Introduction

Product information

Target preparation assay kits, such as the GeneChip™ Pico and WT Pico Kits, allow expression profiling using as little as 100 pg of total RNA (equivalent to approximately 10 cells). These low amounts of RNA require you to accurately measure the quantity of input sample RNA that is used in the target preparation assay for optimal performance. However, at these low RNA concentrations it becomes difficult to accurately quantify the RNA with standard UV absorbance or fluorescent dye-based techniques.

The RNA Quantification Kit uses *Power* SYBR™ Green RNA-to-C_T™ *1-Step* Kit to measure levels of the consistently expressed 18S ribosomal RNA in human, mouse, and rat samples. The amount of 18S ribosomal RNA target in the unknown samples is then scaled against a Control HeLa Total RNA standard curve for accurate quantification that fails using conventional techniques. The concentration of any unknown sample can be interpolated from the standard curve that is based on the quantification cycle (Cq) of the sample. This protocol recommends running at least two replicate reactions per sample. Test for DNA contamination by including a no RT added control reaction.

Power SYBR™ Green RNA-to-C_T™ *1-Step* Kit is a ready-to-use master mix for real-time, quantitative analysis of RNA templates in a single reaction format. The kit contains two tubes: *Power* SYBR™ Green RT-PCR Mix (2X) and RT Enzyme Mix (125X). The 125X RT Enzyme Mix is a blend of reverse transcriptase and RNase Inhibitor. The *Power* SYBR™ Green RT-PCR Mix (2X) contains chemically-modified AmpliTaq Gold™ DNA Polymerase, ultrapure nucleotides, SYBR™ Green, and ROX™ passive reference dye in an optimized buffer formulation for quantitative, real-time reverse transcription PCR detection (RT-qPCR). One kit is sufficient for running 1 plate of 6 point standards and 21 unknown samples or 2 plates of 6 point standards and 9 unknown samples per plate in duplicate reactions.

In addition to the*Power* SYBR™ Green RNA-to-C_T™ *1-Step* Kit, the RNA Quantification Kit also includes Control HeLa Total RNA for standard curve preparation and an 18S rRNA Primer Mix, providing an assay range of 0.002–200 pg/µL total RNA.

Kit contents and storage

Table 1 RNA Quantification Kit (Cat. No. 902905)

Contents	Amount (100-reaction kit)	Storage [1]
Power SYBR™ Green RT-PCR Mix (2X)	1 mL	–20°C
RT Enzyme Mix (125X)	20 µL	
10X 18S rRNA Primer Mix (78 nt)	220 µL	
Control HeLa Total RNA (100 ng/µL)	5 µL	

[1] Refer to label for expiration date.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Item	Source
Microcentrifuge	MLS
Pipette	MLS
Real-Time PCR instrument	MLS
Spectrophotometer and fluorometer • NanoDrop™ UV-Vis Spectrophotometer • Optional: NanoDrop™ Fluorospectrometer • Optional: Qubit™ 2.0 Fluorometer	Thermo Fisher Scientific
Vortex Mixer	MLS
Nuclease-free aerosol-barrier tips	MLS
Nuclease-free 1.5 and 0.2 mL tubes or plates	MLS
Real-Time PCR consumables (plates, stripes and respective sealing options)	MLS
Nuclease-free water	MLS

2 RNA Quantification Kit

Procedural notes

Sample preparation

Purify total RNA

RNA samples must be free of genomic DNA and we recommend including a DNase treatment with the RNA purification method. The contaminating genomic DNA could cause over-estimation of the RNA amount. We strongly recommend against the use of nucleic acid-based carriers during RNA purification because many have been shown to produce cDNA product in first-strand synthesis reaction. Select a purification method or commercially available kit that it is appropriate for your sample type and amount.

RNA quantification

UV absorbance measurement is the most common and convenient method for determining ng/µL RNA quantity. Absorbance at 260 nm is used to measure the amount of nucleic acid present in the sample and concentration can be calculated using the 260-nm reading and a conversion factor based on the extinction coefficient for each nucleic acid (A_{260} of 1.0 = 40 µg/mL for RNA). In addition, the absorbance at 230 nm and 280 nm can be used to determine the number of other contaminants that can interfere with an accurate A_{260} nm reading and subsequent enzymatic reactions. However, UV absorbance readings are not accurate when used to quantify low concentration samples. For example, the lower detection limit for the NanoDrop™ spectrophotometer is 2 ng/µL.

Fluorescent dye-based quantification offers an alternative to UV absorbance for determining pg/µL RNA quantity. In this method, a nucleic acid sample along with a series of standards of known concentrations are incubated with a fluorescent dye that bind to dsDNA, ssDNA, or RNA, resulting in increased fluorescence at a wavelength specific to the dye being used. However, one disadvantage to using fluorescent dye-based methods is the absence of specificity with the caveat that some fluorescence dyes offer higher template specificity than others. Therefore, the presence of DNA in an RNA sample can result in an over-estimation of nucleic acid concentration. Specific detection of RNA can be achieved using an RNA-specific dye (for example, Qubit™ RNA HS Assay Kit and Qubit™ 2.0 Fluorometer), but the quantification lower detection limit is 250 pg/µL.

Lastly, measurement of RNA concentrations by a Bioanalyzer can be less accurate, especially at RNA concentrations less than 25 ng/µL. The RNA 6000 Nano Kit can be used for RNA quantification of high concentration samples (25 to 500 ng/µL) but the RNA 6000 Pico Kit must not be used for quantification of low concentration samples.

Equipment preparation

Program the Real-Time PCR thermal cycler.

- 1 cycle of:
  - 50°C for 10 minutes
  - 95°C for 10 minutes
- 35 cycles of:
  - 95°C for 15 seconds
  - 60°C for 60 seconds. Acquire real-time fluorescence data during this step.

Reagent preparation and kit component handling guidelines

- Properly chill essential equipment such as cooling blocks and reagent coolers before use.
- Enzymes and Reagents: Mix by gently vortexing the tube followed by a brief centrifuge to collect contents of the tube and then keep on ice.
- Prepare master mixes for each step of the procedure to save time, improve reproducibility, and minimize pipetting errors.
- Avoid pipetting solutions less than 2 µL in volume to maintain accuracy and precision.
- Return the components to the recommended storage temperature immediately after use.
- Ensure that all temperature transitions to incubation temperatures are rapid and/or well-controlled to help maintain consistency across samples.

RNase contamination prevention

RNase contamination in reagents and the work environment results in failure to generate amplified targets. Follow these guidelines to minimize contamination:

- Wear disposable gloves and change them frequently.
- Clean and decontaminate work areas and instruments, including pipettes, with commercially available decontamination reagents.
- Avoid touching surfaces or materials that could introduce RNase.
- Use RNase-free filter tips and microcentrifuge tubes.
- Use a work area that is designated for RNA work.

Prepare RNA sample

The Control RNA (100 ng/µL HeLa total RNA) is used as the standard in the RNA Quantification Assay by RT-qPCR for determining the concentration of total RNA. Ensure thorough coverage of your quantification range by preparing dilutions to cover the expected range of RNA concentrations in your samples. We recommend preparing 6-point 10-fold serial standard curve between 0.01 pg–1 ng per reaction, which corresponds to 0.002–200 pg/µL.

1. On ice, serially dilute stock Control HeLa Total RNA with nuclease-free water.

   Avoid pipetting solutions less than 2 µL in volume to maintain accuracy and precision. Use the following tables as a guide for the dilutions. Five microliters of the diluted RNA are needed for each replicate reaction.

Note:

- Up to 5 µL of RNA sample can be added to a reaction. If RNA sample is less than 5 µL, add nuclease-free water to make up the difference.
- Avoid pipetting solutions less than 2 µL in volume to maintain accuracy and precision.

Table 2 Example of control HeLa total RNA dilution preparation.

Stock concentration	Volume of RNA sample	Volume of nuclease-free water	Final concentration	HeLa total RNA per PCR reaction (5 µL, pg/rxn)
On the day of the experiment, thaw 100 ng/µL stock tube and perform dilutions.				
Pre-dilution: 100 ng/µL	2 µL	98 µL	2,000 pg/µL	
Standard 1: 2 ng/µL	4 µL	36 µL	200 pg/µL	1,000 pg
Standard 2: 200 pg/µL	4 µL	36 µL	20 pg/µL	100 pg
Standard 3: 20 pg/µL	4 µL	36 µL	2 pg/µL	10 pg
Standard 4: 2 pg/µL	4 µL	36 µL	0.2 pg/µL	1 pg
Standard 5: 0.2 pg/µL	4 µL	36 µL	0.02 pg/µL	0.1 pg
Standard 6: 0.02 pg/µL	4 µL	36 µL	0.002 pg/µL	0.01 pg

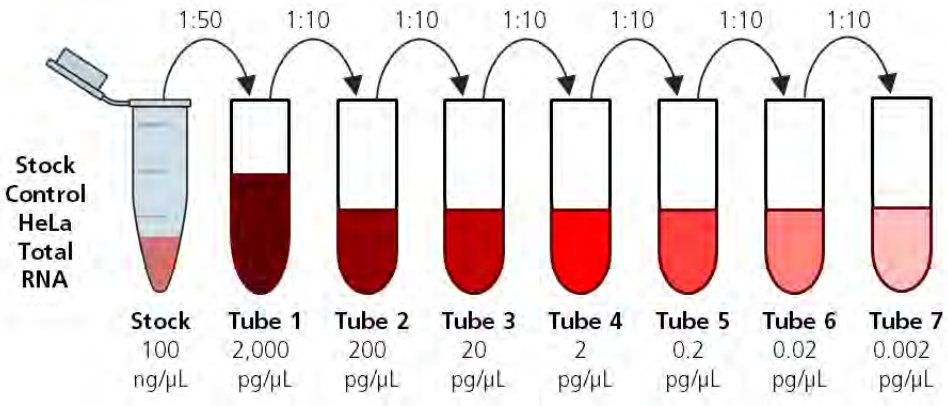

Figure 1 Serial dilution.

2. On ice, transfer 5 µL of each diluted Control HeLa Total RNA sample, at least in duplicate, to each real-time PCR tube or well.

3. On ice, transfer up to 5 µL of each unknown sample, at least in duplicate, to each real-time PCR tube or well. Bring volume up to 5 µL with nuclease-free water if less than 5 µL of sample is used in a reaction.

   Note:

   - RNA samples must be kept frozen and thawed immediately before use.
   - Always prepare fresh-dilution of Control HeLa Total RNA.
   - Use nuclease-free tubes and nuclease-free water to prepare all the dilutions including unknown samples (not included).
   - After each step, mix the Control HeLa Total RNA dilutions thoroughly by gently vortexing followed by a fast centrifuge to collect contents of the tube.

Set up real-time PCR

IMPORTANT!

- Run all test samples and Control HeLa total RNA on the same plate. This practice allows the calculation of quantification cycle differences for each sample relative to Control HeLa total RNA
- For genomic DNA detection, run all test samples without adding RT Enzyme Mix on the same plate. This is a no RT negative control that allows for the detection of genomic DNA contamination for each sample.
- For best results, we recommend at least two technical replicates per sample. It is highly recommended to prepare a master mix of Power SYBR™ components, primer mix, and nuclease-free water.
- It is highly recommended to make a master mix for at least ten reactions to reduce pipetting errors.
- We recommend using 20 µL reaction volumes because accurate and reproducible setup of 10 µL reaction in 96-well plates using multi-channel pipettes can be challenging.

1. Thaw the*Power* SYBR™ Green RT-PCR Mix (2X) at room temperature. Keep RT Enzyme Mix and RNA samples on ice. Mix thoroughly, briefly centrifuge to collect tube contents and then place on ice.

2. Assemble all reaction tubes or plates on ice.

3. The following table is the suggested protocol when nine RNA samples plus the six diluted Control HeLa Total RNA are run side-by-side.

Table 3 PCR reaction master mix preparation.

Component	Volume for 1-well replicate	Volume for 2 replicates of 6 standards and 9 samples[1]	Volume for 2 replicates of 9 samples no RT control[1]
Power SYBR™ Green RT-PCR Mix (2X))	10 µL	330 µL	198 µL
Enzyme Mix (125X)[2]	0.2 µL	6.6 µL	0 µL
10X 18S rRNA Primer Mix (78 nt)	2 µL	66 µL	39.6 µL
Nuclease-free water	2.8 µL	92.4 µL	59.4 µL
Total volume	**15 µL**	**495 µL**	**297 µL**

[1] With 10% overage to account for pipetting inaccuracies.
[2] Replace with water for no RT negative control.

4. Gently vortex the master mix for a few seconds, quick spin, then return the tube to ice.

5. On ice, add 15 µL of the PCR Reaction Master Mix to each (5 µL) real-time PCR tube or well containing the diluted RNA sample for a final reaction volume of 20 µL.

   Note: The PCR Reaction Master Mix maybe added before adding the diluted RNA samples.

6. Cap tubes or seal plates with optically transparent caps or film.

7. Mix tubes or plates by gentle vortexing, then spin to collect contents without bubbles (for example, 2–5 minutes at 1,000–2,000 × *g*).

The following table shows recommended cycling conditions:

Table 4 Real-time thermal cycler protocols.

Standard cycling protocols
1 cycle of: 50°C for 10 minutes: Reverse transcription of RNA by reverse transcriptase to generate cDNA
1 cycle of: 95°C for 10 minutes: AmpliTaq Gold™ DNA Polymerase activation and reverse transcriptase inactivation
35 cycles of: 95°C for 15 seconds 60°C for 60 seconds: Acquire real-time fluorescence data during this step
Melt-Curve Analysis: Recommended to distinguish specific products from nonspecific ones. Consult the thermal cycler manual for details.

3 Data analysis

To calculate quantification data from RT-qPCR amplification curves, the results must be plotted as a linear regression of the quantification cycle (Cq) values versus the log of the RNA quantities to generate a standard curve (Table 5, Figure 2). The slope of the line is a measure of the efficiency of the assay (Figure 2). Slopes between –3.1 and –3.9 are considered acceptable (110% and 80% efficient, respectively), although a slope of –3.32 is indicative of 100% efficiency. The R2 is a measure of the performance of the assay, and is the coefficient of correlation between the data that are generated and the results that are expected under ideal conditions (Figure 2). The R2 should be higher than 0.95, which means that >95% of the total variation in the input samples that make up the standard curve can be explained by the relationship between the Cqs that is obtained and their respective concentrations.

The following equation is used to determine quantification data from Cq value of unknown sample:

$$\text{Quantity} = 10^{(\text{Cq} - \text{y-intercept}) / \text{Slope}}$$

Calculate the concentration of unknown samples based on the standard curve and resulting linear equation. The final concentrations (for example, picograms/microliter) of the unknown samples are based on the calculated quantity that is derived from the linear equation, the sample volume that is used per reaction, and any dilution that has been made.

Genomic DNA contamination of each sample is evaluated by calculating quantification cycle differences in the presence and absence of reverse transcriptase (ΔC_q DNA = Cq (without RT) - Cq (with RT)). If a No RT Control sample has a Cq value 10 cycles higher than an RT Test sample, then the No RT Control sample contained approximately 1,000-fold less target sequence. Because the target template in the No RT Control would be genomic DNA, the conclusion would be that 0.1% of the amplification in the RT Test sample comes from the genomic DNA in the sample, which is negligible compared to the amplification of the RNA-derived cDNA target sequence. If the amount of genomic DNA in RNA samples is unacceptably high,

repeat the RNA purification using a new purification method or perform a DNase treatment.

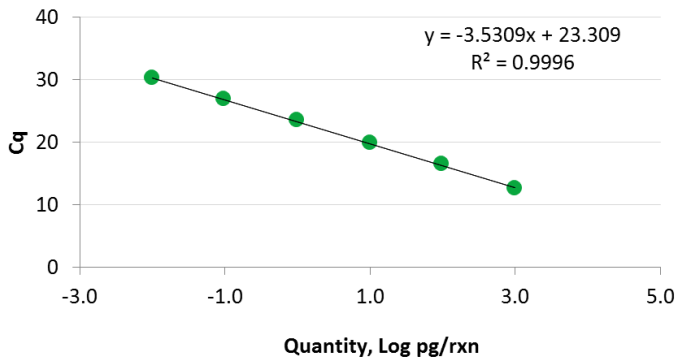

Figure 2 Example of control HeLa total RNA standard curve.

Table 5 Log values of the quantities of diluted HeLa total RNA samples.

Tube	HeLa Total RNA dilution final concentration	HeLa Total RNA per PCR reaction (pg/rxn)	Log10 HeLa Total RNA per PCR reaction (pg/rxn)
1	200 pg/µL	1,000	3.0
2	20 pg/µL	100	2.0
3	2 pg/µL	10	1.0
4	0.2 pg/µL	1	0.0
5	0.02 pg/µL	0.1	–1.0
6	0.002 pg/µL	0.01	–2.0

A Microsoft™ Excel™ template for concentration calculation can be downloaded from our website.

A

Troubleshooting

Observation	Possible cause	Recommended action
Efficiency not in specified range (80–110%)	For high efficiency (or slope is higher than –3.1), the problem is likely related to template.	High efficiency could be due to too much cDNA in the reaction, which can cause inhibition at the higher end of the dilution series. Use less starting template starting at the highest dilution point.
	For low efficiency (or slope is lower than –3.9), the problem is likely related to reaction mix.	Your samples can contain PCR inhibitors—Repurify RNA with a new RNA purification kit, check A_{260}/A_{280} reading (should be near to 2.0), and/or dilute your template and retest again.
		Your PCR primer and/or probe design might not be optimal—optimize your primer/probe design.
		Inaccurate sample and reagent pipetting—ensure that pipettes are calibrated and working properly.
		The standard curve cannot have been properly analyzed.
R^2 value is <0.95	Poor handling.	Ensure that all reagents are thoroughly mixed before use.
	Instrument-related issue.	Ensure that the correct reference dye was used.
Poor replicates reproducibility	Poor handling.	Ensure that all reagents are thoroughly mixed before use.
	Instrument-related issue.	Ensure that the correct reference dye was used.
Dilutions do not fall in range of standard curve	Wrong dilution factor used.	Dilutions that amplify outside the range of the standard curve must not be used.
		Repeat the assay with a more appropriate dilution factor.
No amplification of control	Poor handling.	Ensure that all reagents are thoroughly mixed before use.
		Avoid RNase contamination.
	Reagents were compromised during shipping or storage.	Ask for replacement of reagents from vendor.
	No RT Control sample has a Cq value less than 10 cycles higher than an RT Test sample.	Repeat the RNA purification using a new purification method or perform DNase treatment.
	RNA sample has high gDNA contamination.	Repeat the RNA purification using a new purification method or perform DNase treatment.

B

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

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  - Safety Data Sheets (SDSs; also known as MSDSs)

    Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

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