March 2024

QIAcuity® OneStep Advanced EvaGreen® Handbook

For quantification of RNA and DNA with intercalating dye-based one-step RT-dPCR

Sample to Insight

Contents

Kit Contents

QIAcuity OneStep Advanced EvaGreen Kit (1 mL)

Catalog no.	250141
4x QIAcuity OneStep Advanced EvaGreen Mastermix (1 mL)	1 tube
100x QIAcuity OneStep Advanced EvaGreen RT Mix (45 µL)	1 tube
Q-Solution	1 tube
QuantiNova Internal Control RNA	1 tube
RNase-Free Water	2 tubes

QIAcuity OneStep Advanced EvaGreen Kit (5 mL)

Catalog no.	250142
4x QIAcuity OneStep Advanced EvaGreen Mastermix (1 mL)	5 tubes
100x QIAcuity OneStep Advanced EvaGreen RT Mix (45 µL)	5 tubes
Q-Solution	5 tubes
QuantiNova Internal Control RNA	1 tube
RNase-Free Water	8 tubes

dPCR Instruments and Nanoplate Formats Compatible with the Kit

Type	Instruments	Instrument cat. nos.	Nanoplate formats	Nanoplate cat. nos.
2 channels	QIAcuity 1-2plex	911001	8.5k 24-Well	250011
			8.5k 96-Well	250021
			26k 8-Well	250031
			26k 24-Well	250001
5 channels	QIAcuity 1-5plex	911021	8.5k 24-Well	250011
	QIAcuity 4-5plex	911042	8.5k 96-Well	250021
	QIAcuity 8-5plex	911052	26k 8-Well	250031
			26k 24-Well	250001

Shipping and Storage

The QIAcuity OneStep Advanced EvaGreen Kit is shipped on dry ice. Upon arrival the kit should be stored protected from light at –30°C to –15°C in a constant-temperature freezer. Under these conditions, the kit is stable without showing any reduction in performance and quality, until the date indicated on the label. Before opening any tube, briefly centrifuge the tube to collect all material at the bottom.

Intended Use

The QIAcuity OneStep Advanced EvaGreen Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety**, where you can find, view, and print the SDS for each QIAGEN kit and kit component.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of QIAcuity OneStep Advanced EvaGreen Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

The QIAcuity OneStep Advanced EvaGreen Kit allows for accurate absolute quantification of RNA and DNA targets with an intercalating dye in one-step RT-dPCR reactions on the QIAcuity digital PCR platform.

Absolute quantification with QIAcuity digital PCR uses the procedure of end-point PCR but splits the PCR reaction into thousands of single partitions. After partitioning, some partitions will contain no copy of the target molecule, some will contain one copy of the target molecule, and some others will contain more than one copy of the target molecule. Following PCR cycling, the amplified target is detected by measuring the fluorescence across reaction partitions. As the template is distributed randomly, Poisson statistics can be used to calculate the average amount of target molecule per valid, analyzable partition. The total amount of target in all partitions of a well is calculated by multiplying the amount of average target DNA per partition with the number of valid partitions. Calculation of target concentration is determined by referring to the volume in all analyzable partitions, that is, partitions which were filled with reaction mix. The total number of filled partitions is identified by a fluorescent dye present in the reaction mix itself. Absolute quantification by dPCR eliminates the need for standard curves to determine the amounts of target RNA or DNA in a given sample.

In the QIAcuity OneStep Advanced EvaGreen® Mastermix, the intercalating dye, EvaGreen, gives users the freedom to detect numerous targets without needing to synthesize unique fluorescently labeled probes for each assay. The kit accommodates a variety of RNA targets, such as total RNA from eukaryotes and prokaryotes, as well as in vitro-transcribed RNA. High specificity and sensitivity in the RT-dPCR reactions are achieved by the use of a novel hot-start RT enzyme and hot-start DNA polymerase, together with a specialized RT-dPCR buffer. This allows room-temperature setup of the RT-dPCR reactions.

The QuantiNova® Internal Control RNA (QN IC RNA) supplied with the kit can be optionally used to monitor for successful reverse transcription.

The QIAcuity OneStep Advanced EvaGreen Kit can be used with any of the QIAcuity dPCR instruments and any of the QIAcuity nanoplates.

Principle and procedure

One-Step RT-dPCR

Use of the 4x QIAcuity OneStep Advanced EvaGreen Master Mix together with the 100x QIAcuity OneStep Advanced EvaGreen RT Mix allows for both reverse transcription and dPCR to take place in a single reaction. All reagents required for both reactions are added at the beginning, so there is no need for a separate reverse transcription reaction before proceeding with dPCR. There is also no need to set up the reaction on ice, owing to the hot-start RT and PCR technologies used in the kit.

100x QIAcuity OneStep Advanced EvaGreen RT Mix

The QIAcuity OneStep Advanced EvaGreen RT Mix contains a hot-start reverse transcriptase for heat-mediated activation of the reverse-transcription step, and an RNase inhibitor to protect RNA samples from degradation. Although the included RNase inhibitor effectively reduces the risk of RNA degradation, template RNA of high quality and purity should be used, and any RNAse contamination should be prevented to ensure reliable RT-dPCR results. The hot-start reverse transcriptase comes loaded with an RT-blocker, rendering the enzyme almost inactive at ambient temperatures. Upon starting the RT-dPCR protocol with the RT step at 50°C, the inhibitor is released from the reverse transcriptase and cDNA synthesis is initiated. This allows room-temperature RT-PCR reaction setup. It also lets users take full advantage of the QIAcuity Four and QIAcuity Eight instruments. Users can run up to four and eight plates in parallel on the QIAcuity Four and QIAcuity Eight instruments, respectively, and obtain the same accurate, sensitive, and specific quantification across all plates.

4x QIAcuity OneStep Advanced EvaGreen Master Mix

The components of the 4x QIAcuity OneStep Advanced EvaGreen Master Mix include QuantiNova DNA Polymerase, a dedicated QIAcuity OneStep Advanced EvaGreen RT-dPCR Buffer and the intercalating dye, EvaGreen. The optimized Master Mix ensures fast RT-dPCR

amplification with high specificity and sensitivity. The QIAcuity OneStep Advanced EvaGreen RT-dPCR Buffer contains an optimized concentration of cations to for optimal RT and DNA polymerase performance. EvaGreen binds all double-stranded DNA molecules, emitting a fluorescent signal upon binding.

Novel, antibody-mediated hot-start mechanism

QuantiNova DNA Polymerase is provided in an inactive state and has no enzymatic activity at ambient or higher temperatures. The enzyme remains completely inactive during the reverse-transcription reaction and does not interfere with it. The antibody-mediated hot-start mechanism prevents the formation and extension of nonspecific RT-dPCR products and primer–dimers during reaction setup, reverse transcription, and the first denaturation step. Therefore, this mechanism ensures higher PCR specificity and accurate quantification. At low temperatures, the QuantiNova DNA Polymerase is kept in an inactive state by the QuantiNova Antibody. After the reverse transcription step and within 2 minutes of raising the temperature to 95°C, the QuantiNova Antibody denatures. The QuantiNova DNA Polymerase is thereby activated, enabling PCR amplification. The hot-start enables rapid and convenient room-temperature setup and allows both RT and dPCR steps to be performed sequentially in a single reaction.

QuantiNova Internal Control RNA

The QN IC RNA is a synthetic RNA supplied with the kit that can optionally be used to monitor for successful RT-dPCR. When used by itself, the QN IC RNA is intended to report instrument failures, chemistry failures, and errors in assay setup. When added to reactions containing sample RNA, quantification of QN IC RNA can be used to assay for the presence of RT-dPCR inhibitors. These include phenol, ethanol, sodium dodecyl sulfate (SDS), or ethylene diaminetetraacetic acid (EDTA) that may be carried over from the lysis and purification steps from the RNA isolation procedure. To detect such inhibition, users must also assemble at least one RT-dPCR reaction that contains only QN IC RNA for comparison purposes.

The QN IC RNA is detected with a 200 bp amplicon. The primer sequences used to amplify QN IC RNA have been bioinformatically validated for non-homology against hundreds of eukaryotic and prokaryotic organisms. Additionally, they have been experimentally tested against a multitude of human, mouse, and rat RNA samples from a variety of tissues and cell lines. To detect QN IC RNA in QIAcuity OneStep Advanced EvaGreen reactions, use the QuantiNova IC SYBR® Green Assay (cat no. SBH1218551, ordered via GeneGlobe) at a 1x final concentration.

The QN IC RNA provided with the kit comes at a concentration of ~$1x10^5$ to $1x10^6$ copies/µL. Users are recommended to add the QN IC RNA to their QIAcuity reactions at a final dilution of 1:1000 (e.g., create a working dilution by diluting the QN IC RNA stock 1:10 in RNase-Free Water, which is then diluted 1:100 in the QIAcuity OneStep Advanced EvaGreen reaction mix.)

Validated, ready-to-use primer sets for gene expression analysis

For optimal results in gene expression analysis, we recommend using QuantiNova LNA PCR Assays for Digital PCR. QuantiNova LNA PCR Assays set new standards in intercalating dye-based gene expression analysis. A proprietary algorithm has been used to design over 1.3 million QuantiNova LNA PCR Assays to provide sensitive, accurate, and effective mRNA and lncRNA analysis. The predesigned assays cover most transcripts in the Ensembl database for human, mouse, and rat genes, enabling in-depth PCR-based gene expression studies. Most of the assays are intron-spanning when possible and detect only RNA. LNA enhancement allows shorter primer designs, which are more flexible to position on the target. As a result, designs can be optimized for specific transcript detection or target differentiation. Additionally, thorough design validation ensures optimal performance and robust detection, and assays for all popular mRNAs and lncRNAs undergo wet-lab validation.

QuantiNova LNA PCR Assays for Digital PCR are easily ordered online at **www.qiagen.com/GeneGlobe**

RNA isolation

An important prerequisite for any gene expression analysis experiment is consistent, high-quality RNA from every experimental sample. Residual traces of proteins, salts, or other contaminants may degrade the RNA or decrease the efficiency of enzyme activities necessary for optimal RT and PCR performance.

Recommended RNA preparation methods

The most important prerequisite for any RNA target analysis experiment is consistent, high-quality RNA from every experimental sample. High-quality total RNA for your experiment can be prepared using one of the methods described below, depending on the biological sample. For optimal results, elute RNA samples in RNase-Free Water.

Important: Do not use DEPC-treated water.

Table 1. Recommended RNA preparation methods

Sample type	Recommendation	Remarks
Cultured cells	Use the QIAwave RNA Mini Kit (cat. no. 74536) or the RNeasy Plus Mini Kit (cat. no. 74134)	–
Tissue samples	Use the QIAwave RNA Mini Kit or the RNeasy® Plus Mini Kit for RNA purification.	Some tissues, such as spleen and thymus, contain very high amounts of DNA, which will overload the gDNA Eliminator spin column. For these tissues, we recommend using the RNeasy Mini Kit (cat. no. 74104) in combination with the RNase-Free DNase Set (cat. no. 79254).
FFPE samples	Use the RNeasy FFPE Kit (cat. no. 73504)	–
Small samples yielding <100 ng total RNA	Use the RNeasy Plus Micro Kit (cat no. 74034)	–
Whole blood samples	Use the PAXgene® Blood RNA Kit (cat. no. 762164) or the QIAamp RNA Blood Mini Kit (cat. no. 52304)	–

Sample type	Recommendation	Remarks
Total RNA isolated using a phenol-based method (e.g., QIAzol® Lysis Reagent)	Purify further using the RNeasy Mini Kit (cat. no. 74104)	Perform the on-column DNase digestion step described in the RNeasy Mini Handbook, www.qiagen.com/HB-0435
Other biological samples	Contact QIAGEN Technical Service	support.qiagen.com

Storage of RNA

Purified RNA may be stored at –30°C to –15°C or –90 to –65°C in RNase-Free Water. Under these conditions, RNA degradation will not be detectable for one year.

Quantification of RNA

For best results, all RNA samples should demonstrate consistent quality according to the following criteria for concentration and purity, as determined by UV spectrophotometry and gel electrophoresis.

The concentration of RNA should be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer (see "Spectrophotometric quantification of RNA", below). For small amounts of RNA, however, it may be difficult to determine amounts photometrically. Small amounts of RNA can be accurately quantified using QIAxcel®, Agilent® 2100 Bioanalyzer, or fluorometric quantification.

Spectrophotometric quantification of RNA

To ensure significance, A_{260} readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 44 µg of RNA per mL (A_{260} =1 → 44 µg/mL). This relation is valid only for measurements at a neutral pH. Therefore, if it is necessary to dilute the RNA sample, this should be done in a buffer with neutral pH. The ratio between the absorbance values at

260 and 280 nm gives an estimate of RNA purity. As discussed below (see "Purity of RNA"), the ratio between absorbance values at 260 nm and 280 nm gives an estimate of RNA purity.

When measuring RNA samples, be certain that cuvettes are RNase free, especially if the RNA is to be recovered after spectrophotometry. This can be accomplished by washing cuvettes with 0.1 M NaOH, 1 mm EDTA, followed by washing with RNase-Free Water. Use the buffer in which the RNA is diluted to zero the spectrophotometer. An example of the calculation involved in RNA quantification is shown below:

Volume of RNA sample = 100 µL

Dilution = 10 µL of RNA sample + 490 µL of 10 mm Tris·Cl, pH 7.0 (1/50 dilution)

Measure absorbance of diluted sample in a 1 mL cuvette (RNase-Free).

A_{260} = 0.2	
Concentration of RNA sample	= 44 µg/mL x A260 x dilution factor
	= 44 µg/mL x 0.2 x 50
	= 440 µg/mL
Total amount	= concentration x volume in milliliters
	= 440 µg/mL x 0.1 mL
	= 44 µg of RNA

Purity of RNA

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV spectrum, such as protein. However, the A_{260}/A_{280} ratio is influenced considerably by pH. Because water is not buffered, the pH and the resulting A_{260}/A_{280} ratio can vary greatly. Lower pH results in a lower A_{260}/A_{280} ratio and reduced sensitivity to protein contamination. For accurate values, we recommend measuring absorbance in 10 mm Tris·Cl, pH 7.5. Pure RNA has an A_{260}/A_{280} ratio of

1.9–2.1* in 10 mm Tris·Cl, pH 7.5. Always be sure to calibrate the spectrophotometer with the same solution used for dilution. For determination of RNA concentration, however, we recommend dilution of the sample in a buffer with neutral pH since the relationship between absorbance and concentration (A_{260} reading of 1 = 44 µg/mL RNA) is based on an extinction coefficient calculated for RNA at neutral pH (see "Spectrophotometric quantification of RNA").

Integrity of RNA

Run an aliquot of each RNA sample on a QIAxcel Connect instrument (cat. no. 9003110) with the QIAxcel RNA QC Kit v2.0 (cat. no. 929104); on an Agilent Bioanalyzer using an RNA 6000 Nano LabChip; or on a denaturing agarose gel. For RNA derived from biological samples (e.g., human cells), verify that there are sharp bands/peaks present for the appropriate ribosomal RNAs (e.g., human 18S and 28S). For synthetic RNA samples (e.g., in vitro transcripts), verify that there are sharp peaks at the size expected for that RNA molecule. Any smearing of the RNA bands or shoulders on the RNA peaks indicate that degradation has occurred in the RNA sample.

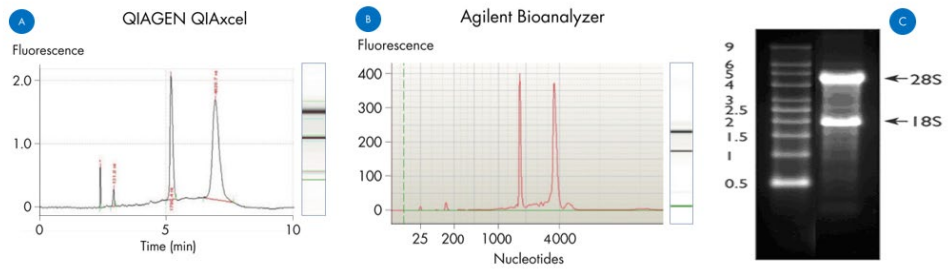


Figure 1. Ribosomal RNA integrity. (**A**): QIAxcel and (**B**): Agilent Bioanalyzer electropherogram of high-quality total RNA from human cells showing sharp peaks for the 18S (left) and 28S (right) ribosomal RNA. Due to the high quality of the RNA, peaks do not have shoulders (especially to the left of each peak). (**C**): Agarose gel electrophoresis shows sharp bands (especially at the bottom of each band) for 28S and 18S ribosomal RNA.

* Values up to 2.3 are routinely obtained for pure RNA (in 10 mM Tris·Cl, pH 7.5) with some spectrophotometers.

Genomic DNA contamination

Unwanted signals generated by contaminating genomic DNA in a sample can be limited by the design of assays. If the gene structure allows, QuantiNova LNA PCR Assays make use of primers that target different exons while spanning a large intron in the mRNA (intron-spanning assay). GeneGlobe® indicates if intron-spanning assay design is not possible by the warning "Important: this assay may detect gDNA".

When using self-designed assays or assays from other commercial providers, it is important to keep these design considerations in mind.

To remove genomic DNA contamination from your RNA samples, we strongly recommend RNA purification using the RNeasy/RNeasy Plus products, including the optional on-column DNase digestion step.

General considerations for using the QIAcuity OneStep Advanced EvaGreen Kit

Poission statistics, dynamic range, and dilutions

The number of transcripts is calculated using Poisson statistics based upon the total number of positive partitions. In extreme cases, that is:

- **Samples with low number of transcripts**: expression values might vary from the true expression as a rare transcript could be lost in the dead volume, or as the rare transcript might not be correctly represented in the fraction of the original RNA that was used for quantification.
- **Samples with high number of transcripts**: with increasing number of transcripts being randomly distributed over the partitions, a partition could contain multiple transcripts. In

extreme cases, when all partitions are positive, it will no longer be possible to conduct any quantification.

- Table 2 provides guidance on the dynamic range of the different QIAcuity Nanoplates for gene expression analyses.

Table 2. Calculated dynamic range of template input amounts for fold change spectra in gene expression assays with the QIAcuity nanoplates

Nanoplate	Reaction volume/ well	Fold change spectrum	Lower limit	Sweet spot (copies/partition)					
			1 Positive partition	0.001	0.01	0.1	0.5	2	
			4.1	35.7	351.7	3517	17,586	70,344	Number of input template molecules per reaction well
8.5k	12 µL	5-fold					X	X	
		20-fold				X	X	X	
		200-fold			X	X	X	X	
		2000-fold		X	X	X	X	X	
		17,000-fold	X	X	X	X	X	X	
			1.7	43.33	433.3	4333	21,667	86,667	Number of input template molecules per reaction well
26k	40 µL	5-fold					X	X	
		20-fold				X	X	X	
		200-fold			X	X	X	X	
		2000-fold		X	X	X	X	X	
		50,980-fold	X	X	X	X	X	X	

Calculations are based on 8500 partitions with 0.34 nL per partition for the 8.5k nanoplate; and 26,000 partitions with 0.91 nL per partition for 26k nanoplates. Depending on the nanoplate used, 8.5k or 26k, fold change spectra cover different amounts of input template molecules per well. With a maximum of 2 copies per partition, the upper limits for input amounts per well are 70,344 template molecules for the 8.5k nanoplate and 86,667 template molecules for the 26k nanoplate. The maximum lower limit of a single positive partition translates into 4.1 and 1.7 template molecules per well and a coverage of fold changes of 17,000 and 50,980 for the 8.5k and 26k nanoplates, respectively.

RNA input amounts: starting recommendation and upper limits

When evaluating gene expression in biological systems like cultured cells or animal models, the abundance of transcript from a particular gene of interest is apt to be unknown. Therefore, using between 0.5 ng and 5 ng total RNA per reaction is recommended as a starting point. In cases where detecting low abundant transcripts requires greater amounts of input RNA, users are advised to add no more than 200 ng of RNA per 8.5k Nanoplate reaction (Figure 2) or 500 ng of RNA per 26k Nanoplate reaction (Figure 3). Adding more than the recommended amount of RNA to a reaction will generate background fluorescence that makes thresholding between positive and negative partitions difficult or impossible.

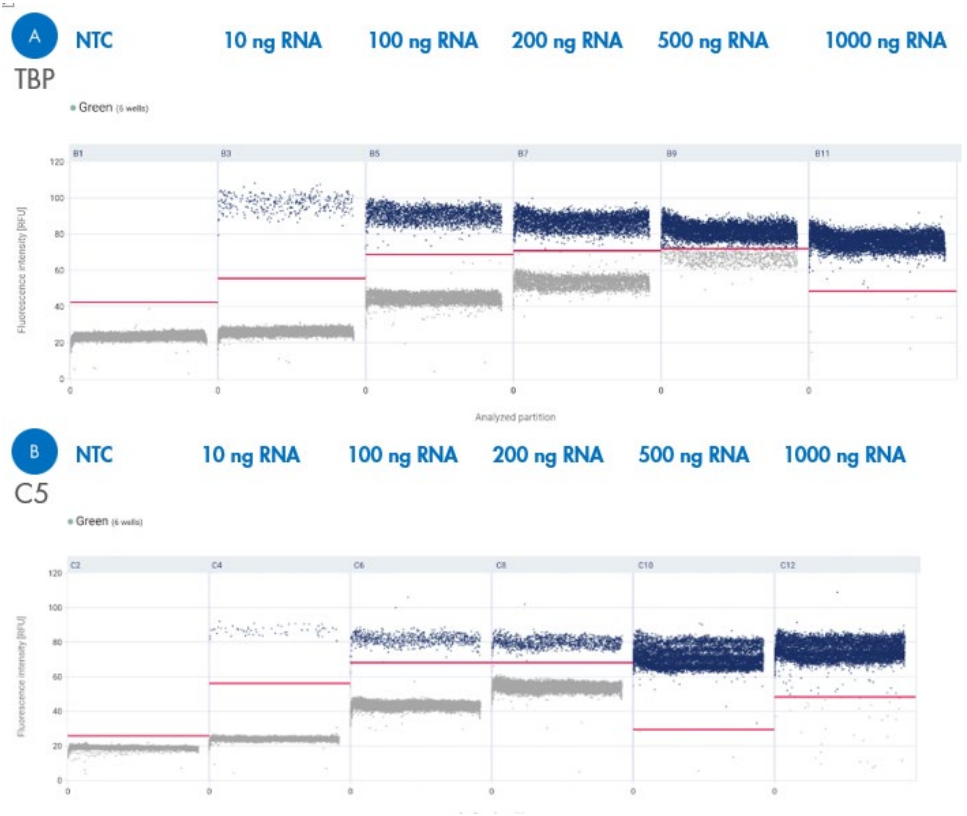


Figure 2. RNA amounts should not exceed 200 ng per reaction when using 8.5k Nanoplates. QuantiNova LNA Assays targeting TBP (**Figure 2A**) and C5 (**Figure 2B**) mRNA were used in QIAcuity OneStep Advanced EvaGreen reactions in 8.5k Nanoplates following recommended cycling protocols. Between 10 to 1000 ng of total RNA isolated from cultured human leukocytes were used as template per reaction. In reactions containing more than 200 ng total RNA, the separation between negative partitions (gray) and positive partitions (dark blue) decreased such that thresholding between positive and negative partitions became (**A**) difficult or (**B**) impossible.

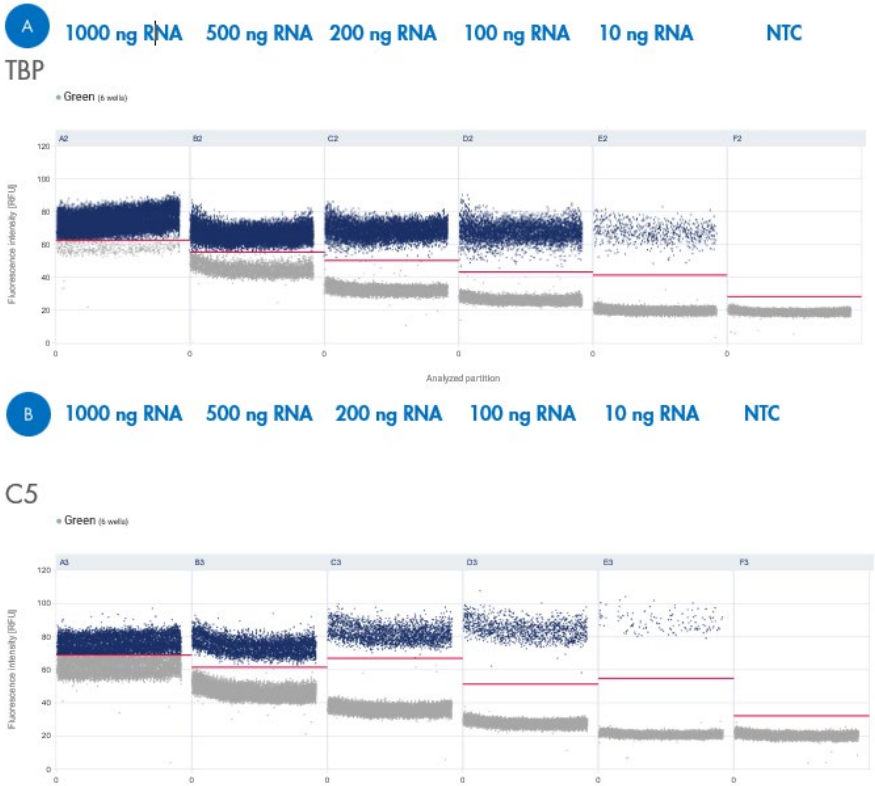


Figure 3. RNA amounts should not exceed 500 ng per reaction when using 26k Nanoplates. QuantiNova LNA Assays targeting TBP (**Figure 3A**) and C5 (**Figure 3B**) mRNA were used in QIAcuity OneStep Advanced EvaGreen reactions in 26k Nanoplates following recommended cycling protocols. Between 10 to 1000 ng of total RNA isolated from cultured human leukocytes were used as template per reaction. In reactions containing more than 500 ng total RNA, the separation between negative partitions (gray) and positive partitions (**dark blue**) decreased such that thresholding between positive and negative partitions became (**A**) difficult or (**B**) impossible.

Recommendations for usage of QIAcuity Nanoplates in gene expression analyses

The different QIAcuity Nanoplate configurations accommodate a wide range of applications, from the Nanoplate 26k 8-well format with higher volumes for high sensitivity and broad dynamic range to the Nanoplate 8.5k 96-well format with lower volumes that offers high throughput.

For selecting the ideal QIAcuity Nanoplate for your application, please refer to Table 2, page 18.

Normalization and recommended controls

The efficiency of RNA extraction can vary from sample to sample and from experiment to experiment. It is therefore crucial to normalize expression values obtained for a sample against a reference gene.

> **Note**: It is assumed that reference genes do not change from sample to sample. It is nevertheless recommended to confirm that the reference gene that you are using does not change between the different conditions that you are analysing.

The reference gene for normalization should exhibit a similar expression level as the gene that you are planning to analyze. In this way, you can assess the expression values of both gene of interest and reference gene using the same dilution of your sample of interest. This minimizes concentration differences caused by pipetting errors or the variability conferred by the pipette that you are using. Refer to "Appendix C" (page 47) for more information on the expected concentration of housekeeping genes detected by QuantiNova LNA PCR Assays.

Equipment and Reagents to be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

- Genomic RNA isolation kit (check Table 1 on page 13 for RNA purification kit recommendations)
- Digital PCR Instrument; the table on page 4 indicates the appropriate dPCR cyclers.
- QIAcuity dPCR Nanoplates; the table on page 4 indicates the compatible nanoplates.
- Multichannel pipettor
- Nuclease-free pipette tips and tubes
- Standard 96-well PCR pre-plate

Important Notes

For accurate and reproducible dPCR results, it is essential to avoid contamination of the assay with foreign RNA and DNA, especially PCR products from previously run nanoplates. The most common sources of RNA and DNA contamination are the products of previous experiments and highly concentrated template dilutions.

To maintain a working environment free of RNA and DNA contamination, we recommend the following precautions:

- Wear gloves throughout the procedure. Use only fresh PCR-grade labware (tips and tubes).
- Use sterile pipette tips with filters.
- Store and extract positive materials (specimens, positive controls, and amplicons) separately from all other reagents.
- Physically separate the workspaces used for dPCR setup and post-dPCR processing operations. Decontaminate your dPCR workspace and labware (pipets, tube racks, etc.) with UV light before each new use to render any contaminated DNA ineffective in dPCR through the formation of thymidine dimers or with 10% bleach to chemically inactivate and degrade any DNA.
- Do not open any previously run and stored dPCR nanoplate. Removing the sealer foil from dPCR nanoplate releases dPCR product DNA into the air where it can contaminate the results of future experiments. In the event of contamination ensure that any affected labware and bench surfaces are decontaminated.
- Do not remove the dPCR nanoplate from its protective sealed bag until immediately before use.

Protocol: Universal Protocol for OneStep RT-digital PCR with QIAcuity OneStep Advanced EvaGreen Kit

This universal protocol is optimized for the quantification of RNA and DNA targets using the QIAcuity OneStep Advanced EvaGreen Kit in a singleplex reaction using QIAGEN's QIAcuity instruments for digital PCR (dPCR) in 26k nanoplates and 8.5k nanoplates (cat. nos. 250001, 250011, 250021, 250031). Follow this protocol when using self-designed assays or assays from third parties. To use QuantiNova LNA PCR Assays from QIAGEN with the QIAcuity OneStep Advanced EvaGreen Kit, refer to the dedicated protocols provided in this handbook.

Important points before starting

- Refer to Appendix D (page 51) for guidance on assay design and experimental setup for the QIAcuity platform.
- The QIAcuity OneStep Advanced EvaGreen Kit has been specially formulated with a hot-start RT enzyme and hot-start DNA polymerase, allowing users to assemble reactions at room temperature (15–25°C), and to run up to four or eight plates in parallel on the QIAcuity Four or QIAcuity Eight instruments, respectively.
- Pipetting accuracy and precision affect the consistency of results. Be sure that all pipettes and instruments have been checked and calibrated according to the manufacturer's recommendations. Ensure that bubbles will not be introduced into the wells of the QIAcuity Nanoplate during pipetting.
- To control for any potential environmental contamination, it is required to run at least one No Template Control (NTC) reaction for each assay used.
- The QuantiNova Internal Control RNA (QN IC RNA) supplied with the kit can be used optionally as a reverse transcription and amplification control. To do so, users must separately purchase the QuantiNova IC SYBR Green Assay (cat no. SBH1218551, ordered via GeneGlobe) and use it a final concentration of 1x.

- The QN IC RNA comes at a concentration of ~1x105 to 1x106 copies/µL. Users are recommended to add the QN IC RNA to their QIAcuity reactions at a final dilution of 1:1000 (e.g., create a working dilution by diluting the QN IC RNA stock 1:10 in
- RNase-Free Water, which is then diluted 1:100 in the QIAcuity OneStep Advanced EvaGreen reaction mix.)
- Refer to the dedicated protocol for using QuantiNova LNA PCR Assays with the QIAcuity OneStep Advanced EvaGreen Kit.

Things to do before starting

- Determine RNA concentration, integrity, and purity (refer to "RNA isolation" section in the Introduction, page 13)
- Thaw template, primers, and QIAcuity OneStep Advanced EvaGreen Kit reagents on ice (4°C). After thawing, mix gently by repeated pipetting or quick vortex, followed by a brief centrifugation to settle the liquids.

Procedure

1. Place the 100x OneStep Advanced Reverse Transcription Mix on ice. Thaw the 4x QIAcuity OneStep Advanced EvaGreen Master Mix, template RNA, primers, Q-Solution, and RNase-Free Water. Vigorously mix the QIAcuity OneStep Advanced EvaGreen Master Mix and the individual solutions. Centrifuge the tubes briefly to settle the liquids.
2. Prepare a Master Mix according to Table 3 and the desired Nanoplate format.

Table 3. Preparing the QIAcuity OneStep Advanced EvaGreen RT-dPCR reaction mix

Component	Volume/reaction		
	Template RNA (added at step 4)†	Template RNA (added at step 4)†	Template RNA (added at step 4)†
4x OneStep Advanced EvaGreen	3 µL	10 µL	1x
Master Mix	0.12 µL	0.4 µL	1x
100x OneStep Advanced RT Mix (Reverse Transcription)	0.6 µL	2 µL	0.75 µM forward primer
20x primer mix	0.75 µM reverse primer		
Q-Solution*	1 µL	3.3 µL	–
RNase-Free Water	Variable	Variable	–
Template RNA (added at step 4)†	Variable	Variable	–
Total reaction volume	**12 µL**	**40 µL**	

* The use of Q-Solution is strongly recommended, but the PCR reaction will work if users choose to omit it. Q-solution is especially beneficial for use with amplicons >150 bp in length, GC-rich amplicons, and RNA targets containing challenging secondary structures. The reaction mix may become faintly cloudy when Q-solution is added. However, this has no impact on PCR performance and should largely resolve when the reaction mix reaches its final 1x concentration.

† Appropriate template amount depends on various parameters.

3. Vortex the reaction mix well. Dispense appropriate volumes of the reaction mix into the wells of a standard 96-well PCR pre-plate.

   Note: The pre-plate may be assembled at room temperature.
4. Add template RNA to wells containing the reaction mix. Thoroughly mix the template RNA with the reaction mix by pipetting up and down.

One-step RT-dPCR protocol for all QIAcuity instruments

1. Transfer the contents of each well in the pre-plate to the wells of a Nanoplate.
2. Seal the Nanoplate properly using the QIAcuity Nanoplate Seal provided in the QIAcuity Nanoplate Kits.

3. Place the Nanoplate into the QIAcuity instrument and start the RT-dPCR program. Recommended cycling conditions for two-step and three-step RT-dPCR are described in Table 4 and Table 5. Imaging Settings should be programmed according to Table 6.

Table 4. Two-step QIAcuity RT-dPCR cycling program

Step	Time	Temperature (°C)
Reverse Transcription	40 min	50
RT Enzyme Inactivation	2 min	95
2-step cycling (40 cycles)	–	–
Denaturation	10 s	95
Combined annealing/extension	30 s	55–60*
Cooling Down	5 min	40

* Temperature during annealing/extension and number of cycles might vary depending on assay type. As a starting point, we recommend that users first begin with 40 cycles and an annealing/extension temperature of 58°C.

Table 5. Three-step QIAcuity RT-dPCR cycling program

Step	Time	Temperature (°C)
Reverse Transcription	40 min	50
RT Enzyme Inactivation	2 min	95
3-step cycling (40 cycles)	–	–
Denaturation	15 s	95
Annealing	15 s	55–60*
Extension	15 s	72
Cooling Down	5 min	40

* Temperature during annealing and number of cycles might vary depending on assay type. As a starting point, we recommend that users first begin with 40 cycles and an annealing temperature of 58°C.

Table 6. Imaging Settings

Channel	Exposure Duration	Gain
Green	200 ms	3

Protocol: QuantiNova LNA PCR Assays with the QIAcuity OneStep Advanced EvaGreen Kit

This protocol is optimized for the quantification of mRNA/lncRNA targets (cDNA) using the QuantiNova LNA PCR Assays (cat. nos. 249990 and 249992) with the QIAcuity OneStep Advanced EvaGreen Kit (cat. nos. 250141 and 250142) in a singleplex reaction using QIAcuity instruments for digital PCR (dPCR).

Important points before starting

- The QIAcuity OneStep Advanced EvaGreen Kit has been specially formulated with a hot-start RT enzyme and hot-start DNA polymerase, allowing users to assemble reactions at room temperature, and to run up to four or eight plates in parallel on the QIAcuity Four or QIAcuity Eight instruments, respectively.
- Pipetting accuracy and precision affect the consistency of results. Be sure that all pipettes and instruments have been checked and calibrated according to the manufacturer's recommendations. Ensure that bubbles will not be introduced into the wells of the QIAcuity Nanoplate during pipetting.
- To control for any potential environmental contamination, it is required to run at least one No Template Control (NTC) reaction for each assay used.
- The QuantiNova Internal Control RNA (QN IC RNA) supplied with the kit can be used optionally as a reverse transcription and amplification control. To do so, users must separately purchase the QuantiNova IC SYBR Green Assay (cat no. SBH1218551, ordered via GeneGlobe) and use it a final concentration of 1x.
- The QN IC RNA comes at a concentration of ~$1x10^5$ to $1x10^6$ copies/µL. Users are recommended to add the QN IC RNA to their QIAcuity reactions at a final dilution of 1:1000 (e.g., create a working dilution by diluting the QN IC RNA stock 1:10 in RNase-Free Water, which is then diluted 1:100 in the QIAcuity OneStep Advanced EvaGreen reaction mix.)

Things to do before starting

- Determine RNA concentration, integrity, and purity (refer to “RNA Isolation”, page 13) section in the Introduction)
- Thaw template, primers, and QIAcuity OneStep Advanced EvaGreen Kit reagents on ice (4°C). After thawing, mix gently by repeated pipetting or quick vortex, followed by a brief centrifugation to settle the liquids.

Procedure

Reaction mix setup

1. Place the 100x OneStep Advanced Reverse Transcription Mix on ice. Thaw the 4x QIAcuity OneStep Advanced EvaGreen Master Mix, template RNA, primers, Q-Solution, and RNase-Free Water. Vigorously mix the QIAcuity OneStep Advanced EvaGreen Master Mix and the individual solutions. Centrifuge the tubes briefly to settle the liquids.
2. Prepare a Master Mix according to Table 7 and the desired Nanoplate format.

Table 7. Preparing the QIAcuity OneStep Advanced EvaGreen RT-dPCR reaction mix

Component	Volume/reaction		
	Nanoplate 8.5k (24-well and 96-well)	Nanoplate 26k (8-well and 24-well)	Final concentration
4x OneStep Advanced EvaGreen Master Mix	3 µL	10 µL	1x
100x OneStep Advanced RT Mix (Reverse Transcription)	0.12 µL	0.4 µL	1x
QuantiNova LNA PCR Assay (10x)	1.2 µL	4 µL	1x
Q-Solution*	1 µL	3.3 µL	–
RNase-Free Water	Variable	Variable	–
Template RNA (added at step 4)†	Variable	Variable	–
Total reaction volume	**12 µL**	**40 µL**	

* Adding Q-Solution to PCR reactions is strongly recommended for all users, but the PCR reaction will work if it is omitted. The reaction mix may become faintly cloudy when Q-solution is added. However, this has no impact on PCR performance and should largely resolve when the reaction mix reaches its final 1x concentration.

† Appropriate template amount depends on various parameters.

3. Vortex the reaction mix well. Dispense appropriate volumes of the reaction mix into the wells of a standard 96-well PCR pre-plate.

   Note: The pre-plate may be assembled at room temperature.

4. Add template RNA to wells containing the reaction mix. Thoroughly mix the template RNA with the reaction mix by pipetting up and down.

One-step RT-dPCR protocol for all QIAcuity instruments

1. Transfer the contents of each well in the pre-plate to the wells of a Nanoplate.
2. Seal the Nanoplate properly using the QIAcuity Nanoplate Seal provided in the QIAcuity Nanoplate Kits.
3. Place the Nanoplate into the QIAcuity instrument and start the RT-dPCR program described in Table 8. Imaging Settings should be programmed according to Table 9.

Table 8. QIAcuity RT-dPCR cycling program for QuantiNova LNA PCR Assays

Step	Time	Temperature (°C)
Reverse Transcription	40 min	50
RT Enzyme Inactivation	2 min	95
2-step cycling (40 cycles)	–	–
Denaturation	10 s	95
Combined annealing/extension	30 s	58
Cooling Down	5 min	40

Table 9. Imaging settings

Channel	Exposure Duration	Gain
Green	200 ms	3

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: **www.qiagen.com/FAQ/FAQList.aspx**. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, visit **www.qiagen.com**).

		Comments and suggestions
Weak or no signal detected		
a)	Incorrect programming of the QIAcuity dPCR instrument	Compare the temperature profile with the protocol described in the handbook.
b)	PCR extension time too short	Use the extension time specified in the protocol. If desired, increase PCR extension time to 60 or 90 seconds
c)	RT-dPCR was inhibited	Use the recommended RNA isolation method and closely follow the manufacturer's instructions. QIAGEN offers dedicated sample preparation kits for RNA extraction. If inhibition persists, perform a serial dilution of RNA sample to dilute out inhibitor. Optionally, use the QN IC RNA provided with the kit to monitor for RT-dPCR inhibition
d)	Incorrect setup of the PCR reaction mix	Ensure that reactions were set up according to the reaction mix preparation tables in the handbook (Table 3 and Table 7). Repeat the dPCR run, if necessary.
e)	The storage conditions for one or more kit components did not comply with the instructions given in "Shipping and Storage".	Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary.

		Comments and suggestions
f)	Insufficient starting template	Increase the amount of template RNA. This can be achieved by adding more template to the reaction mix. If volume constraints prevent users from adding more RNA to 8.5k Nanoplate reactions, try using 26k Nanoplates, which allow users to add up to more than 3 times the volume of sample per reaction. If RNA concentrations are limiting, perform new RNA extractions using greater amounts of starting material or elute RNA in smaller volumes of elution buffer.
g)	Too much starting template	Use less template. Too much starting template may result in complete saturation of the nanoplate well, such that all partitions are positive for PCR amplification. This will result in 1D scatterplots that contain just one cluster of partitions. In certain circumstances, this may give the impression that all the partitions are negative. If too much starting template is suspected, perform a serial dilution until the quantification of the target lands within the dynamic range of the nanoplate used. When possible, use a positive control at a concentration in the middle of the dynamic range of the nanoplate. This will generate 1D scatterplots with clearly distinguishable clusters of positive and negative partitions. The RFU values of the positive and negative partitions can be used to determine if a well is saturated with template or contains no target.

Observed quantification of RNA is lower than expected

a)	RNAse contamination occurred during PCR setup	Repeat the PCR with new reagents. If possible, seal the PCR array/close the PCR tubes directly after addition of the sample to be tested. Make sure that workspace and instruments are decontaminated for nucleases at regular intervals.
b)	RNase contamination occurred during extraction	Repeat the extraction and PCR of the sample to be tested using new reagents. Make sure that workspace and instruments are decontaminated for RNAses at regular intervals.

		Comments and suggestions
c)	Assay design is suboptimal	Using the QIAcuity OneStep Advanced EvaGreen reagents and the relevant assay and template, run a set of RT-qPCR reactions on any standard qPCR instrument with a variant of the QIAcuity cycling program. In the modified protocol, the standard QIAcuity RT-dPCR cycling program should be followed by a melt curve analysis step. If an RT-qPCR cycler with a temperature gradient block is available, users can further modify the RT-qPCR program to investigate the performance of their assay. The annealing/extension step from QIAcuity cycling protocol should be programmed to generate a temperature gradient ranging from 50–62°C. This will allow users to test identical reactions across a range of annealing/extension temperatures. If the melt curve analysis indicates the assay generates specific amplification products, users may increase primer concentrations from 0.75 µM to 1 µM. This may improve RT efficiency. If the melt curve analysis indicates that the assay generates unspecific PCR products under standard cycling conditions, users should redesign the assay. Unspecific, secondary RT-PCR products can lead to underquantification of the RNA targets. QIAGEN offers QuantiNova LNA PCR Custom Assays for Digital PCR (cat. no. 249910) at GeneGlobe. If assay redesign is not possible, users should reduce primer concentrations and/or adjust the temperature of the annealing/extension step in RT-dPCR program if possible. Refer to Appendix E (page 53) for further information.
d)	Q-solution omitted from reaction setup	Add Q-solution to reaction setup.
e)	Incomplete reverse transcription	Increase length of RT step in RT-dPCR program from 40 minutes to 60 minutes. For particular assays, reducing the temperature of the RT step from 50°C to temperatures between 46°C and 48°C can improve the efficiency of the RT step. Refer to Appendix E (page 53) for further information.
f)	Template has adhered to surfaces of storage container	RNA samples are prone to adhere to the surfaces of the containers in which they are stored. This is particularly true for dilute RNA samples. If possible, store RNA undiluted in tubes specifically designed to minimize the binding of RNA to their surface (e.g., LoBind tubes.)
g)	RNA sample stored at improper temperature	At minimum, RNA should be stored at –20°C. For long term storage, –80°C is recommended.
h)	RNA sample has been frozen and thawed too much	RNA samples should avoid repeated freeze thaw cycles. If possible, distribute RNA samples into smaller aliquots.

		Comments and suggestions
Observed quantification of RNA is higher than expected		
a)	Assay design is suboptimal	Using the QIAcuity OneStep Advanced EvaGreen reagents and the relevant assay and template, run a set of RT-qPCR reactions on any standard qPCR instrument with a variant of the QIAcuity cycling program. In the modified protocol, the standard QIAcuity RT-dPCR cycling program should be followed by a melt curve analysis step. If an RT-qPCR cycler with a temperature gradient block is available, users can further modify the RT-qPCR program to investigate the performance of their assay. The annealing/extension step from QIAcuity cycling protocol should be programmed to generate a temperature gradient ranging from 50–62°C. This will allow users to test identical reactions across a range of annealing/extension temperatures. If the melt curve analysis indicates that the assay generates unspecific PCR products under standard cycling conditions, users should redesign the assay. Unspecific secondary PCR products can lead to an overquantification of RNA targets. QIAGEN offers QuantiNova LNA PCR Custom Assays for Digital PCR at GeneGlobe. If assay redesign is not possible, users should reduce primer concentrations and/or adjust temperature of the annealing/extension step in RT-dPCR program if possible. Refer to Appendix E (page 53) for further information.
Signal present for the negative control template or in NTC (copies/µL >0)		
a)	Contamination occurred during PCR setup	Repeat the PCR with new reagents. Make sure to pipet the positive controls last. Make sure that workspace and instruments are decontaminated at regular intervals.
b)	Contamination occurred during extraction	Repeat the extraction and PCR of the sample to be tested using new reagents. Make sure that workspace and instruments are decontaminated at regular intervals.

		Comments and suggestions
c)	Assay design suboptimal	Use the QIAcuity OneStep Advanced EvaGreen reagents with the relevant assay, positive control template, negative control template (if available), and H_2O as input for a no template control (NTC). Run a set of RT-qPCR reactions on any standard qPCR instrument with a variant of the QIAcuity cycling program. In the modified protocol, the standard QIAcuity RT-dPCR cycling program should be followed by a melt curve analysis step. If an RT-qPCR cycler with a temperature gradient block is available, users can further modify the RT-qPCR program to investigate the performance of their assay. The annealing/extension step from QIAcuity cycling protocol should be programmed to generate a temperature gradient ranging from 50–62°C. This will allow users to test identical reactions across a range of annealing/extension temperatures. If the melt curve analysis indicates the assay generates specific amplification products with template, but unspecific products in the NTC reaction, users should try decreasing primer concentrations from 0.75 µM to 0.5 µM or lower. This may eliminate the presence of signal in the NTC reaction. If the melt curve analysis indicates that the assay generates unspecific PCR products with template, users should redesign the assay. QIAGEN offers QuantiNova LNA PCR Custom Assays for Digital PCR at GeneGlobe. If assay redesign is not possible, users should reduce primer concentrations and/or adjust temperature of the annealing/extension step in RT-dPCR program if possible. Refer to Appendix E (page 53) for further information.
d)	Q-solution omitted from reaction setup	Add Q-solution to reaction setup.
Low quality 1D Scatterplots (i.e. rainy, poor separation between negative and positive partitions)		
a)	Incorrect programming of the QIAcuity dPCR instrument	Compare the temperature profile and imaging settings with those described in the handbook.
b)	PCR was inhibited	Use the recommended RNA isolation method and closely follow the manufacturer's instructions. QIAGEN offers dedicated sample preparation kits for RNA extraction. If inhibition persists, perform a serial dilution of RNA sample dilute out inhibitor.
c)	Incorrect setup of the PCR reaction mix	Ensure that reactions were set up according to the reaction mix preparation tables in the handbook (Table 1 and Table 7). Repeat the dPCR run, if necessary.

		Comments and suggestions
d)	Suboptimal RT-dPCR program	Try the following modifications for your PCR program: • If using an RT-PCR cycling program with 2-step cycling, try switching to a 3-step cycling program • Lower the annealing/extension temperature if confident that PCR products generated at this lower temperature will be specific (see above and refer to Appendix E, page 53) • Increase the length of the annealing/extension step to 60 or 90 seconds • Include 5 additional cycles in the PCR program
e)	QIAcuity OneStep EvaGreen mix has been stored in the light	The intercalating EvaGreen dye is photosensitive and will bleach over time if exposed to too much light. This will lead to lower signal intensity in the 1D scatterplots.

Contact Information

For technical assistance and more information, please see our Technical Support Center at **www.qiagen.com/Support**, call 00800-22-44-6000, or contact one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit **www.qiagen.com**).

Appendix A: Gene Expression Data Analysis in the QIAcuity Software Suite

You can access the "Gene Expression" analysis feature of the QIAcuity Software Suite by selecting the **Analyse** option. A new window will open. In the top right you will find the button for the **Gene Expression** (Figure 4).

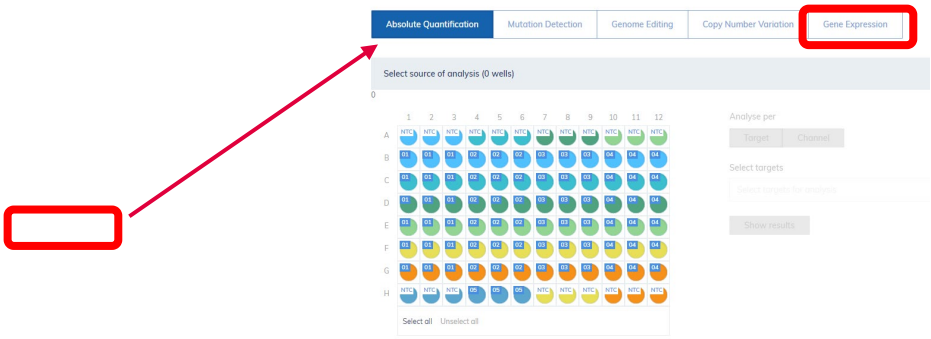


Figure 4. Navigation to second-level analysis for gene expression in the QIAcuity Software Suite.

First select the wells that you would like to analyze. Wells can be selected or unselected by a mouse click or you can also select multiple wells by moving over the wells while clicking the left mouse button (Figure 5).

Next, specify the reference sample in the first pull-down menu. All other gene expression data generated with the remaining samples will be compared against this sample. Fold changes/gene regulation will be reported in the final output.

After that, a target of interest (TOI) needs to be selected. TOIs are assays that are not used as a reference gene. Once a TOI has been selected, users must select one or more "Reference targets".

If the selected “Reference sample” is comprised of wells and hyperwells with the same sample ID, the “Reference selection” drop down menu will become active. Users will then need to choose between the hyperwell or “Non-hyperwell, all replicates” for their reference sample.

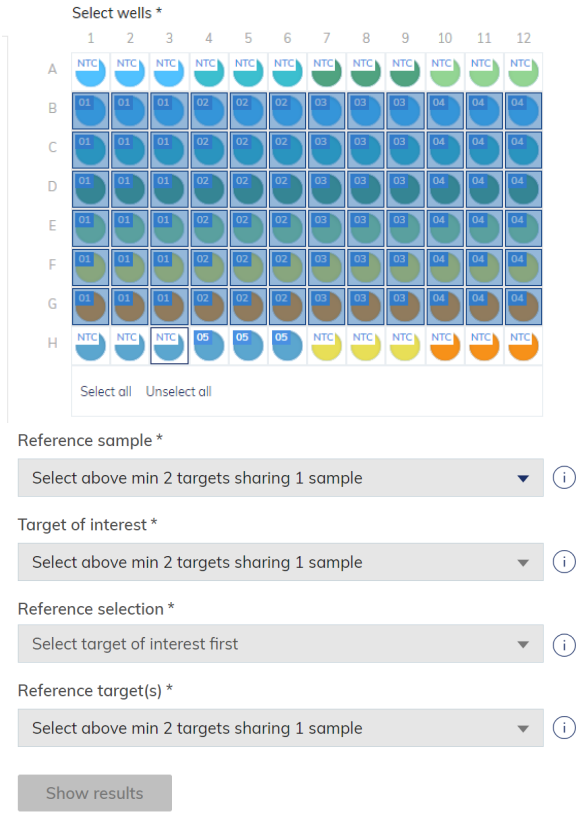


Figure 5. Gene expression can be quantified by selecting multiple wells, TOIs, and reference targets.

Once you click **Show results**, four different analysis results will be provided:

- **List**: A list showing the difference in gene expression of the "Target of interest" for all sample combinations (fold change and fold regulation). This list can be exported as a CSV file.
- **Concentration diagram**: A bar chart showing the expression level for the "Target of interest" and "Reference target" in all samples.
- **Point diagram**: A diagram showing the fold change of the "Target of interest" in all samples.
- **Heatmap**: A heatmap showing the differences in gene expression in each well of the nanoplate.

For detailed information on the different analysis options and applied calculations, refer to the *QIAcuity User Manual* on **www.qiagen.com**

Appendix B: Data Analysis Using Limit of Blank (LOB) Calculations

Principle for Data Analysis

In digital PCR, detection and quantification of target RNA and DNA molecules is based on copies/µL values obtained from individual PCR reactions in nanoscale volumes called partitions. Individual partitions are generated by splitting a PCR reaction over which the target molecules are randomly distributed. After partitioning, some partitions will contain no copy of the target molecule, some will contain one copy of the target molecule, and some others will contain more than one copy of the target molecule. Since the target molecules are randomly distributed, the Poisson distribution can be used to calculate the average number of copies of the target molecule per partition. The total number of copies of the target molecule in all valid partitions of a well is calculated by multiplying the average number of copies of the target molecule per partition by the number of valid partitions. The QIAcuity Software Suite calculates the target DNA molecule concentration in the reaction in copies/µL by referring to the known volume of a valid partition.

The appropriate data analysis method in the QIAcuity Suite depends on the application. For relative profiling or comparing two different populations (i.e. healthy versus disease, time 0 versus time 30 days), the normalization method is recommended. For identification of RNA species, the NTC method is used. The NTC method allows for higher sensitivity of detection by measuring the background of the assay.

Normalization method

The normalization method is used for the relative profiling or comparison between two populations. To account for different starting amounts of RNA in a sample, separate reference

assays are used to normalize the sample input. Established assays, such as reference assays from QIAGEN, targeting stably expressed RNAs can be used.

To calculate the normalized copies/µL values of each sample, the copies/µL values of the replicates for each reference and target assay are averaged. The normalized copies/µL value of the target molecule is obtained by dividing the average copies/µL value of the target molecule by the average copies/µL value of the reference assay. With the normalized copies/µL values the fold increase or decrease in abundance is calculated based on the formula:

$$\text{Fold change} = \text{Log}_2(\text{normalized copies/µL}^{\text{Sample1}} / \text{ normalized copies/µL}^{\text{Sample2}})$$

As an example for the calculation of abundance fold changes between samples using reference genes for normalization Table 16 depicts dPCR analysis results for two samples analysed with a target of interest-specific assay and a reference assay. Each Sample was analysed in triplicates.

Sample 1:

Average copies/µL target Sample 1 = (68+73+69)/3=70 copies/µL

Average copies/µL reference Sample 1 = (172+165+164)/3=167 copies/µL

Normalized abundance target Sample 1 = 70/167 = 0.419

Sample 2:

Average copies/µL target Sample 2 = (111+105+114)/3=110 copies/µL

Average copies/µL reference Sample 2 = (79+84+86)/3=83 copies/µL

Normalized abundance target Sample 2 = 110/83 = 1.325

Fold change in Sample 2 compared to Sample 1:

$$FC^{\text{Sample2}} = \text{Log}_2(1.325/0.419) = 1.661$$

Table 10. Example dPCR data for 2 samples analysed with a target and a reference assay.

Nanoplate Well	Sample/NTC/Control	Reaction Mix	Channel	Concentration (copies/µL)
A1	S1-replicate1	target assay	GREEN	68
A2	S1-replicate1	ref assay	GREEN	172
A3	S1-replicate2	target assay	GREEN	73
A4	S1-replicate2	ref assay	GREEN	165
A5	S1-replicate3	target assay	GREEN	69
A6	S1-replicate3	ref assay	GREEN	164
A7	S2-replicate1	target assay	GREEN	111
A8	S2-replicate1	ref assay	GREEN	79
A9	S2-replicate2	target assay	GREEN	105
A10	S2-replicate2	ref assay	GREEN	84
A11	S2-replicate3	target assay	GREEN	114
A12	S2-replicate3	ref assay	GREEN	86

Second level analyses of fold changes normalized against reference assays can be done in the QIAcuity Software Suite using the Gene Expression Plugin. With this feature, one can select single and multiple reference genes for normalization. For further details refer to the gene expression chapter in the *QIAcuity User Manual Extension: QIAcuity Application Guide* (**www.qiagen.com/HB-2839**).

NTC method

The NTC method allows for higher sensitivity of detection by measuring the background of the assay expressed as the limit of blank (LOB). The assay background and/or low levels of contamination due to environmental factors can be corrected for by performing a dPCR reaction using No Template Control (NTC) as the sample. NTC is used to establish a threshold for the number of positive partitions above which a target in the sample can be considered as positively detected. This threshold is called the limit of detection (LOD) and can vary between assays and sample types. As an example, Table 11 shows the number of detected copies/µL in 4 NTC and 4 Sample replicates. The LOD is calculated as follows:

Average number of measured copies/µL in NTC ($mean^{NTC}$) = (0+0.055+0)/3=0.018

Standard deviation of measured copies/µL in NTC (SD^{NTC}) = 0.026

Limit of Blank (LOB) = $mean^{NTC}$ +1.645*(SD^{NTC}) = 0.018+1.645*0.026 = 0.061

Average number of measured copies/µL in Sample ($mean^{Sample}$) = (0.268+0.386+0.377)/3 = 0.344

Standard deviation of measured copies/µL in Sample (SD^{Sample}) = 0.054

Limit of Detection (LOD) = LOB +1.645*(SD^{Sample}) = 0.061+1.645*0.054 = 0.149 copies/µL

LOD in total copies per reaction (8.5k nanoplate) = LOD * 12 = 0.149*12 = 1.79 copies

LOD in total copies per reaction (26k nanoplate) = LOD * 40 = 0.149*40 = 5.96 copies

For this particular example the target can be considered to be present in the sample, as the average measured copies/µL value ($mean^{Sample}$) is >0.149.

For cases of ($mean^{Sample}$) < LOD, the sample should be considered negative for the target.

Table 11. Example of detected positive partitions for 3 NTC and 3 Sample replicates.

Replicate	Measured Copies/µL
NTC Replicate 1	0
NTC Replicate 2	0.055
NTC Replicate 3	0
Sample Replicate 1	0.268
Sample Replicate 2	0.386
Sample Replicate 3	0.377

Appendix C: Choosing Suitable Reference Genes from QuantiNova LNA PCR Portfolio

To provide users with guidance on picking suitable reference genes for their experiments, the reference gene assays from the QuantiNova LNA PCR Assay portfolio available from GeneGlobe were used in QIAcuity OneStep Advanced EvaGreen reactions in 8.5k Nanoplates using 10 ng, 1 ng, and 0.1 ng of the appropriate human, mouse, or rat RNA as template. The results are summarized in the tables below.

It is important to note that every biological sample is different. The values presented in the following tables will most likely not match values users obtain with their own samples. The data are strictly intended to help users select references genes with suitable expression levels that fall within the dynamic range of their experimental setup.

Table 12. QuantiNova LNA PCR Assays (Human)

Human (*Homo sapiens*)			Copies/µL in 8.5k Nanoplate Reaction			
mRNA	Assay Name	GeneGlobe ID	10 ng RNA	1 ng RNA	0.1 ng RNA	copies/ng RNA
ACTB	HS_ACTB_2475367	SBH1220543	Sat.	3981	417	48,908
B2M	HS_B2M_2476237	SBH1220550	Sat.	5517	574	67,569
GAPDH	HS_GAPDH_2475369	SBH1220545	12,988	1322	140	16,076
GUSB	HS_GUSB_2476238	SBH1220551	496	47	4	550
HGDC	HS_HGDC_2467744	SBH1218553	Und.	Und.	Und.	Not determined
HPRT1	HS_HPRT1_2475370	SBH1220546	531	56	7	703
HSP90AB1	HS_HSP90AB1_2476242	SBH1220555	8045	821	84	9844
LDHA	HS_LDHA_2521431	SBH1225370	3795	347	39	4470
MALAT1	HS_MALAT1_2476243	SBH1220556	Und.	Und.	Und.	Not determined
MTOR	HS_MTOR_1736510	SBH0492696	200	19	2	226
NONO	HS_NONO_2521432	SBH1225371	Und.	Und.	Und.	Not determined
PGK1	HS_PGK1_2475128	SBH1220304	200	23	2	256
PPIA	HS_PPIA_2476239	SBH1220552	9643	955	98	11,606
PPIH	HS_PPIH_2521433	SBH1225372	281	28	2	320
PTEN	HS_PTEN_2521489	SBH1225378	1402	140	14	1673
RN7SK	HS_RN7SK_2476246	SBH1220559	Sat.	Sat.	Sat.	Not determined
RPLP0	HS_RPLP0_2476240	SBH1220553	7288	758	78	9072
RPLP1	HS_RPLP1_2476241	SBH1220554	16,521	1719	177	20,952
SNORA73A	HS_SNORA73A_2521434	SBH1225373	Sat.	15,627	1601	192,087

Human XpressRef Universal Total RNA (cat. no. 338112) was used as RNA template

Sat. = Samples were at or near saturation

Und. = No RNA was detected for this target

Table 13. QuantiNova LNA PCR Assays (Mouse)

Mouse (*Mus musculus*)			Copies/µL in 8.5k Nanoplate Reaction			
mRNA	Assay Name	GeneGlobe ID	10 ng RNA	1 ng RNA	0.1 ng RNA	copies/ng RNA
ACTB	MM_ACTB_2476263	SBM1220560	Sat.	4088	424	49,987
B2M	MM_B2M_1919401	SBM0675336	Sat.	2615	278	32,370
GAPDH	MM_GAPDH_2476265	SBM1220562	Sat.	5679	609	70,615
GUSB	MM_GUSB_2476266	SBM1220563	501	51	5	624
HPRT	MM_HPRT_2521490	SBM1225379	3069	306	32	3739
HSP90AB1	MM_HSP90AB1_2476267	SBM1220564	Sat.	1710	174	20,714
LDHA	MM_LDHA_2287078	SBM1042883	10,720	1105	118	13,442
MGDC	MM_MGDC_2467745	SBM1218554	3	Und.	Und.	4
MTOR	MM_MTOR_2476268	SBM1220565	1291	139	14	1633
PGK1	MM_PGK1_2521435	SBM1225374	1108	130	14	1510
PPIH	MM_PPIH_2521436	SBM1225375	364	38	3	423
PTEN	MM_PTEN_2476269	SBM1220566	3509	382	37	4394
RPLP0	MM_RPLP0_2476264	SBM1220561	8463	953	95	11,005
RPLP1	MM_RPLP1_2521437	SBM1225376	Sat.	2118	213	25,505

A pool combining equal amounts of total RNA from mouse liver, kidney, and brain were used as template

Sat. = Samples were at or near saturation

Und. = No RNA was detected for this target

Table 14. QuantiNova LNA PCR Assays (Rat)

Rat (*Rattus norvegicus*)			Copies/µL in 8.5k Nanoplate Reaction			
mRNA	Assay Name	GeneGlobe ID	10 ng RNA	1 ng RNA	0.1 ng RNA	copies/ng RNA
ACTB	RN_ACTB_2476285	SBM1220560	Sat.	2870	186	28,395
B2M	RN_B2M_2476286	SBM0675336	Sat.	3337	346	40,808
GUSB	RN_GUSB_2476293	SBM1220562	358	37	4	467
HPRT1	RN_HPRT1_2521438	SBM1220563	Und.	Und.	Und.	Not determined
HSP90AB1	RN_HSP90AB1_2521491	SBM1225379	12,232	1293	144	15,835
LOC108351137	RN_LOC108351137_2476292	SBM1220564	Sat.	3564	393	44,947
MTOR	RN_MTOR_2476294	SBM1042883	902	103	11	1206
PPIA	RN_PPIA_2476287	SBM1218554	Sat.	3753	400	46,523
PPIH	RN_PPIH_2476288	SBM1220565	476	50	6	620
RGDC	RN_RGDC_2467746	SBM1225374	Und.	Und.	Und.	Not determined
RPLP0	RN_RPLP0_2476289	SBM1225375	10,676	1149	118	13,607
RPLP1	RN_RPLP1_2476290	SBM1220566	Sat.	2727	282	33,307
TBP	RN_TBP_2476291	SBM1220561	223	25	3	310

A pool combining equal amounts of total RNA from rat liver, kidney, and brain were used as template.

Sat. = Samples were at or near saturation

Und. = No RNA was detected for this target

Appendix D: Transfer of qPCR Assays to dPCR and Design Guidance for Custom Designed Assays

The QIAcuity OneStep Advanced EvaGreen Kit can be used with custom designed assays or assays that have already been successfully used in qPCR. Important factors for success in dPCR include the design of optimal primer pairs, the use of appropriate primer concentrations, and the correct storage of primers. It is particularly important to minimize nonspecific annealing of primers. When designing assays, the following aspects should be followed:

T_m of primers

- The T_m of all primers should be 58–62°C and within 2°C of each other.
- Avoid repetitions of 4 or more of the same nucleotide, especially of guanidine.

Primer sequence

- Primers should have a length of 18–30 nucleotides with a GC content of 30–70%.
- Primer specificity should always be checked by performing a BLAST search. Ensure that primer sequences are unique within the sample.
- Primers should not be complementary to each other.
- Avoid complementarity of 2 or 3 bases at the 3′ ends of primer pairs to minimize primer-dimer formation.

Storage

- Lyophilized primers should be dissolved in a small volume of low-salt buffer to give a concentrated stock solution (e.g., 100 µM). It is recommended to use Buffer TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) for standard primers.
- Primers should be stored in small aliquots at –20°C. Avoid repeated freeze-and-thaw cycles.

Transfer of qPCR assays to dPCR

- All rules for proper real-time PCR assay design apply to dPCR.
- Care should be taken that the recommended cycling conditions and primer concentrations for dPCR are selected.
- For optimization of suboptimal performing assays, a temperature gradient during the annealing steps can be run on any real-time PCR instrument using the QIAcuity OneStep EvaGreen PCR chemistry.

Additional information can be found in the *QIAcuity Application Guide*:
www.qiagen.com/HB-2839

Appendix E: Optimizing Assay Performance with Primer Concentration and Cycling Parameters

Quantification with intercalating dye-based PCR systems can pose particular challenges for specific and consistent quantification, especially if assay design is suboptimal. While the formulation of the QIAcuity OneStep Advanced EvaGreen Kit has gone to great lengths to ensure specific and consistent amplification for the majority of assays, users may still need to optimize their experimental setups.

If users observe inconsistent target quantification, evidence for unspecific PCR products, or signal in no template control (NTC) reactions with a particular assay, the first line of optimization is to redesign the assay. QIAGEN offers QuantiNova LNA PCR Custom Assays for Digital PCR at GeneGlobe.

If assay redesign is not an option, modulating assay concentration and RT-dPCR cycling parameters can help users achieve the desired PCR performance.

Modulating Assay Concentration

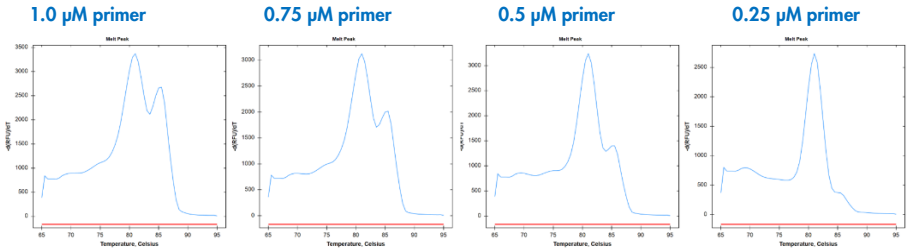
Unspecific amplification products

A final concentration of 0.75 µM in QIAcuity OneStep Advanced EvaGreen reactions is the default recommendation for assays. However, if users suspect that unspecific amplification products are interfering with their results, we recommend reducing the assay concentration to 0.5 µM or below. To test which assay concentration works best, users can assemble QIAcuity OneStep Advanced EvaGreen reactions with a variety of assay concentrations and run the RT-PCR reactions in a qPCR cycler with a program containing a melt curve analysis at the end. Below is an example of such an experiment, where assay X showed unspecific, secondary amplification products when 0.75 µM assay was used (Figure 6). Reducing the concentration

of the assay to 0.25 µM largely eliminated the secondary products in the qPCR melt curve analysis, which was reflected by lower quantification of target X in a multiplate RT-dPCR workflow.

A

Assay X



Observed copies/µL	Plate 1	Plate 2	Plate 3	Plate 4
1.00 µM Assay X	1422	1408	1426	1450
0.75 µM Assay X	1324	1320	1376	1351
0.50 µM Assay X	1195	1194	1273	1216
0.25 µM Assay X	910	969	1007	1004

Figure 6. Reducing assay concentrations can resolve issues with unspecific amplification products. (**A**) QIAcuity OneStep Advanced EvaGreen reaction mixes containing Assay X and 50 ng human RNA were cycled in a qPCR instrument using a program with a melt curve analysis step at the end. The melt curve analysis was used to inspect the specificity of PCR products depending on primer concentration. At higher assay concentrations (0.5–1 µM) secondary PCR products could be observed in the melting curves. However, reducing assay concentrations to 0.25 µM largely eliminated the presence of unspecific products. The impact of these unspecific PCR products on target quantification could be observed in multiplate RT-dPCR reactions. Compared to RT-dPCR reactions using 0.25 µM Assay X, the unspecific PCR products generated in RT-dPCR reactions with higher concentrations of Assay X artificially boosted quantification by up to 40%.

Signal in NTC reactions

If users observe signal in their NTC reactions and can definitively rule out template contamination, modulating the assay concentration can often times greatly reduce or completely eliminate signal in NTC reactions. For these experiments, it is best to evaluate assay titrations directly in a QIAcuity RT-dPCR run. As shown below (Figure 7), varying degrees of signal in NTC reactions was observed when four assays were used at a final concentration of 1 µM. When the assay concentrations were reduced to a final concentration of 0.5 µM, NTC signal was dramatically lowered or undetectable. Reducing the assay concentrations came at minimal cost to the quantification of the target RNA.

	Copies/µL observed in NTC		Target quantification relative to 1 µM Assay (%)	
	1 µM Assay	0.5 µM Assay	1 µM Assay	0.5 µM Assay
Assay 1	0.25	0.00	100	97
Assay 2	1.52	0.25	100	93
Assay 3	6.90	0.53	100	87
Assay 4	3.38	0.78	100	86

Figure 7. Reducing assay concentration can address issues with unwanted signal in NTC reactions. QIAcuity OneStep Advanced EvaGreen reaction mixes were assembled with four assays (Assays 1–4) at a final concentration of 1 µM or 0.5 µM. NTC reactions were run in parallel to reactions containing 50 ng human RNA. At higher assay concentrations (1 µM) varying degrees of signal in the NTC reactions could be observed. However, reducing assay concentrations to 0.5 µM was sufficient to diminish or eliminate this signal. Importantly, lowering assay concentration from 1 µM to 0.5 µM only minimally impacted target quantification.

Inconsistent quantification in multiplate workflows

For some assays, we have observed that minimal RT activity while plates wait to be cycled in multiplate workflows can lead to differences in quantification between the first and last plate. In such cases, use the RT-qPCR approach described above, this time increasing assay concentrations to 1 µM. If the assay is producing specific PCR products in the melt curve analysis, users can use the higher assay concentration for their reactions. Users can also increase the duration of the RT incubation step (e.g., 60 min at 50°).

RT-dPCR Cycling Conditions: Annealing Temperatures

Unspecific amplification

The recommended cycling conditions provided in this handbook should work with most assays. However, suboptimal cycling conditions for a given assay may result in the generation of unspecific PCR products. Therefore, users may need to test which cycling conditions work best with their assays. To do so, users should assemble QIAcuity OneStep Advanced EvaGreen reaction mixes and run them in a qPCR cycler with temperature gradient function. Program the qPCR cycler with the appropriate QIAcuity RT-dPCR cycling settings followed by a melt curve analysis. For the annealing/extension step, the gradient should extend from 50°C to 62°C. By looking at the Cq values and melt curve analysis, users can find an annealing/extension temperature that works best with their assay. Below are data from such an experiment (Figure 8). For Assay M and N, secondary amplification products were observed when a 58°C annealing/extension step was used. However, increasing the annealing/extension temperature to 61°C resolved the issue.

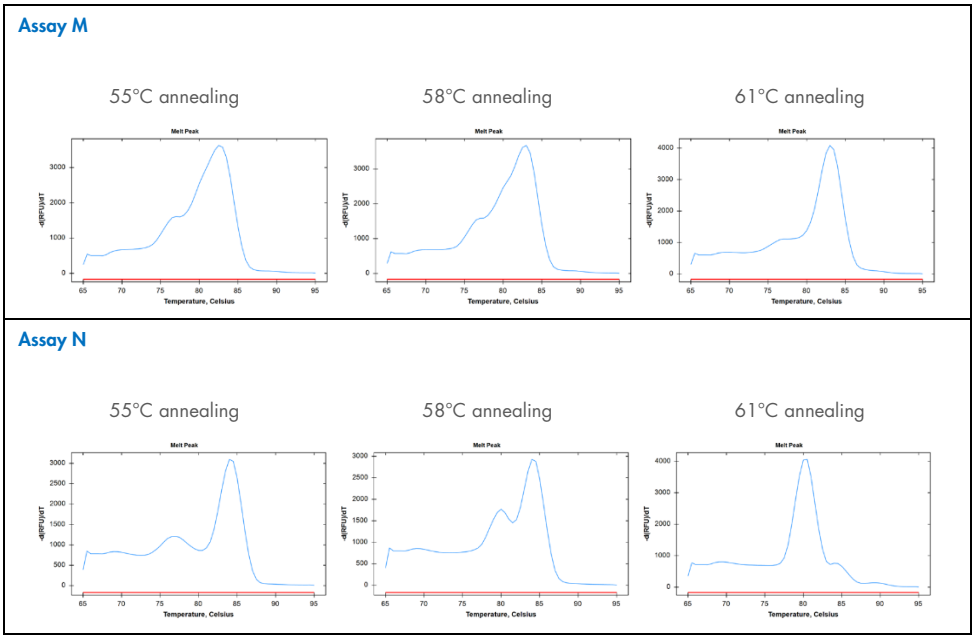


Figure 8. Adjusting the annealing temperature in PCR cycling programs can eliminate unspecific PCR products. QIAcuity OneStep Advanced EvaGreen reaction mixes containing either Assay M or Assay N and 50 ng human RNA were cycled in a qPCR instrument using a program with a temperature gradient during the annealing/extension step and a melt curve analysis step at the end. Melt curve analysis was used to inspect the specificity of PCR products generated with Assays M and N. At lower temperatures (55°C and 58°C) secondary PCR products could be observed in the melting curves for both assays. However, increasing the annealing extension temperature to 61°C largely eliminated the presence of unspecific products.

RT-dPCR Cycling Conditions: Reverse transcription temperature

Suboptimal reverse transcription (RT) efficiency

The recommended cycling conditions provided in this handbook should work with most assays. However, for particular assays, the default setting of a 50°C RT step described throughout this handbook could result in suboptimal reverse transcription efficiency. Therefore, users may need to determine if other RT conditions work better with their assays. To do so, users should assemble QIAcuity OneStep Advanced EvaGreen reaction mixes and run them in a qPCR cycler with temperature gradient function. Program the qPCR cycler with the appropriate

QIAcuity RT-dPCR cycling settings followed by a melt curve analysis. The RT step of the RT-qPCR program should be set to run over a gradient ranging from 40°C to 50°C. If the melt curve analysis indicates that specific PCR products are generated with RT temperatures below 50°C, users can evaluate these adjusted settings in QIAcuity RT-dPCR reactions. Below are data from such an experiment, where multiple assays showed improved RT efficiency when RT incubation temperatures were lowered (Figure 9). However, not all assays will benefit from lowering RT temperatures.

Target quantification relative to program with 40 min RT step at 50°C

RT Condition	Assay A (%)	Assay B (%)	Assay C (%)	Assay D (%)	Assay E (%)	Assay F (%)	Assay G (%)	Assay H (%)
50°C for 40 min	100	100	100	100	100	100	100	100
48°C for 40 min	124	113	142	125	99	92	100	93
46°C for 40 min	108	103	133	98	95	79	85	80
44°C for 40 min	93	72	100	60	84	54	69	62

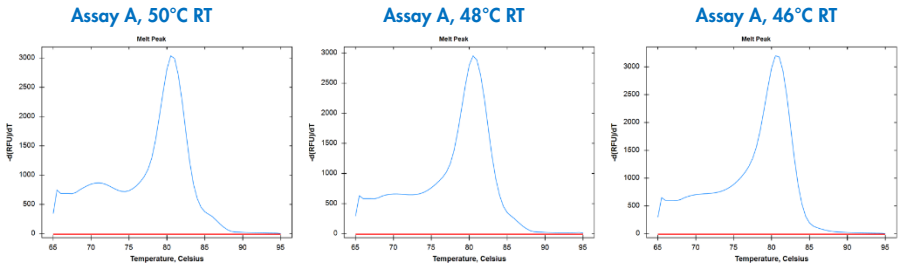


Figure 9. Modulating the temperature of the RT step can improve RT efficiency for some but not all assays. (**A**) Eight human RNA targets (**Assay A to Assay H**) were quantified using RT-dPCR programs with four different RT temperatures (50°C, 48°C, 46°C, and 44°C). Relative to RT-dPCR programs with a 50°C RT step, quantification for some assays increased when lower RT temperatures were used (**Assays A to Assay D**). For other assays, quantification was either unchanged or dramatically reduced (**Assays E to Assay H**). (**B**) QIAcuity OneStep Advanced EvaGreen reaction mixes containing Assay A and 50 ng human RNA were cycled in a qPCR instrument using a program with a temperature gradient during the RT step, and a melt curve analysis step at the end. Melt curve analysis was used to inspect the specificity of PCR products generated with Assay A. The higher quantification of Assay A using a QIAcuity RT-dPCR program with the RT step set to 48°C instead of 50°C is not the result of unspecific PCR products.

Ordering Information

Product	Contents	Cat. no.
QIAcuity OneStep Advanced EvaGreen Kit (1mL)	OneStep Advanced EG Master Mix (4x), OneStep RT Mix (100x), 20 µl QN Internal Control RNA Q-Solution, RNase-free water; for 100 reactions in Nanoplate 26k and 333 reactions in Nanoplate 8.5k	250141
QIAcuity OneStep Advanced EvaGreen Kit (5mL)	OneStep Advanced EG Master Mix (4x), OneStep RT Mix (100x), 20 µl QN Internal Control RNA Q-Solution, RNase-free water; for 500 reactions in Nanoplate 26k and 1666 reactions in Nanoplate 8.5k	250142
Accessories		
QIAcuity Nanoplate 26k 8-well (10)	10 QIAcuity Nanoplate 26k 8-well, 11 Nanoplate Seals	250031
QIAcuity Nanoplate 26k 24-well (10)	10 QIAcuity Nanoplate 26k 24-well, 11 Nanoplate Seals	250001
QIAcuity Nanoplate 8.5k 24-well (10)	10 QIAcuity Nanoplate 8.5k 24-well, 11 Nanoplate Seals	250011
QIAcuity Nanoplate 8.5k 96-well (10)	10 QIAcuity Nanoplate 8.5k 96-well, 11 Nanoplate Seals	250021
Nanoplate Seals (11)	11x Nanoplate Seals	250099
QIAcuity One, 2plex Device	One-plate digital PCR instrument (cat.no 911000 QIAcuity One, 2plex instrument) for detecting up to 2 fluorescent dyes, roller, USB flash	911001

Product	Contents	Cat. no.
	memory and QIAcuity Software Suite: includes 1 preventive maintenance visit. 1 year warranty on labor, travel, and parts also included.	
QIAcuity One, 5plex Device	One-plate digital PCR instrument (cat.no 911020 QIAcuity One, 5plex instrument) for detecting up to 5 fluorescent dyes, roller, USB flash memory and QIAcuity Software Suite: includes 1 preventive maintenance visit. 1 year warranty on labor, travel, and parts also included.	911021
QIAcuity Four Platform System	our-plate digital PCR instrument (cat.no. 911040 QIAcuity Four instrument) for detecting up to 5 fluorescent dyes, notebook computer, barcode scanner, roller, USB flash memory and QIAcuity Software Suite: includes installation, training, and 1 preventive maintenance visit.1 year warranty on labor, travel, and parts.	911042
QIAcuity Eight Platform System	Eight-plate digital PCR instrument (cat.no. 911050 QIAcuity Eight instrument) for detecting up to 5 fluorescent dyes, notebook computer, barcode scanner, nanoplate roller, USB flash memory and QIAcuity Software Suite: includes installation, training, and 1 preventive maintenance visit. 1 year warranty on labor, travel,	911052

Product	Contents	Cat. no.
Related products		
QIAcuity One-Step Advanced Probe PCR Kit (1 mL)	1 mL OneStep Advanced Probe Master Mix (4x), 45 µL OneStep RT Mix (100x), 1 mL Enhancer GC, 20 µL QN Internal Control RNA, 2 x 1.9 mL RNase-free water; for 100 reactions in Nanoplate 26k and 333 reactions in Nanoplate 8.5k	250131
QIAcuity One-Step Advanced Probe PCR Kit (5 mL)	5 x 1 mL OneStep Advanced Probe Master Mix (4x), 5 x 45 µL OneStep RT Mix (100x), 5 x 1 mL Enhancer GC, 1 x 20 µL QN Internal Control RNA, 8 x 1.9 mL RNase-free water; for 500 reactions in Nanoplate 26k and 1666 reactions in Nanoplate 8.5k	250132
QuantiNova LNA PCR Assay (200)†	Predesigned mRNA/lncRNA-specific primer mixture in a single tube; for 200 qPCR reactions or 400 dPCR reactions	249990
QuantiNova LNA PCR Assay (750)†	Predesigned mRNA/lncRNA-specific primer mixture in a single tube; for 750 qPCR reactions or 1500 dPCR reactions	249992
For recommended RNA preparation methods		
QIAwave RNA Mini Kit (250)*	250 RNeasy Mini Spin Columns, Waste Tubes (2 mL), RNase-free Reagents, and Buffers	74536

Product	Contents	Cat. no.
RNeasy Plus Mini Kit (50)	For 50 minipreps: RNeasy Mini Spin Columns, gDNA Eliminator Spin Columns, Collection Tubes, RNase-Free Water, and Buffers	74134
RNeasy Plus Micro Kit (50)	For 50 micropreps: RNeasy MinElute Spin Columns, gDNA Eliminator Spin Columns, Collection Tubes, Carrier RNA, RNase-Free Water, and Buffers	74034
RNeasy Mini Kit (50)	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 mL and 2 mL), RNase-free Reagents, and Buffers	74104
RNeasy FFPE Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes, Proteinase K, RNase-Free DNase I, DNase Booster Buffer, RNase-Free Buffers, RNase-Free Water	73504
QIAamp RNA Blood Mini Kit (50)	For 50 RNA preps: 50 QIAamp Mini Spin Columns, 50 QIAshredder Spin Columns, Collection Tubes (1.5 mL and 2 mL), RNase-free reagents, and buffers	52304

* More sizes available in **www.qiagen.com**
† More information at **www.geneglobe.qiagen.com**

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Document Revision History

Revision	Description
10/2023	Initial release.
03/2024	• Removed the following protocols and product references: o QuantiTect Primer Assays with the QIAcuity OneStep Advanced EvaGreen Kit o RT² qPCR Primer Assays with the QIAcuity OneStep Advanced EvaGreen Kit • Updated the "Ordering Information" section and aligned products information with **www.qiagen.com** • Updated the name of the Ctrl_QNIC_1_SG QuantiTect Primer Assay

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03/2024 HB-3478-002