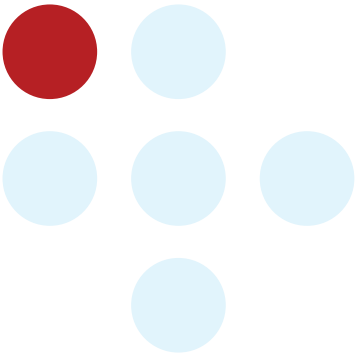
Expression Analysis

ExiLERATE LNA™ qPCR, for mRNA and long non-coding RNA

Instruction manual v1.1

#303301 and #303350

April 2016



EXIQON

Seek Find Verify

Table of contents

Product summary

The ExiLERATE LNA™ qPCR system is designed for sensitive and accurate detection of mRNA and long non-coding RNA (lncRNA) by quantitative real-time PCR using SYBR® Green. The method is based on universal reverse transcription (RT) followed by real-time PCR amplification with LNA™ enhanced primers. The ExiLERATE LNA™ PCR portfolio is comprised of three types of reagent kits; including:

- ExiLERATE LNA™ qPCR, cDNA Synthesis kit
- ExiLERATE LNA™ qPCR, SYBR® Green master mix kit
- ExiLERATE LNA™ qPCR primer sets for mRNA and lncRNA

I. Reagent kits

Starter kit (product # 303350)

This kit contains all reagents required to perform 20 cDNA reactions[1] and 100 10µL PCR reactions. It includes a GAPDH endogenous control primer set.

Contents	
cDNA buffer	200 µL, 5 x concentrated
cDNA enzyme[2]	40 µL, 10 x concentrated
cDNA primers	100 µL, 10 x concentrated
SYBR®Green PCR Master mix	500 µL, 2 x concentrated
ExiLERATE LNA™ qPCR control, GAPDH (hsa, mmu, rno), dried down[3]	200 reactions
Nuclease free water	1.25 mL

cDNA Synthesis Kit, 50 rxns (product # 303301)

This kit contains all reagents required for first-strand cDNA synthesis of mRNA and lncRNA for 50 reactions[1].

Contents	
cDNA buffer	200 µL, 5 x concentrated
cDNA enzyme[2]	100 µL, 10 x concentrated
cDNA primers	100 µL, 10 x concentrated
Nuclease free water	1.25 mL

[1] Number of reactions is based on a standard reaction volume of 20 µL.
[2] The cDNA enzyme contains reverse transcriptase and RNase inhibitor.
[3] Re-suspend in 220 µL Nuclease-free water before first use.

ExiLERATE LNA™ qPCR SYBR® Green Master Mix kit, 2.5 ml (product# 303402)

This kit contains all reagents required for PCR amplification of mRNA and lncRNA. In addition, an endogenous control primer set is provided with this kit for amplification of GAPDH. The reagents are provided in amounts sufficient for 500 reactions of 10 µL.

Contents	
SYBR® Green PCR master mix	2 x 1.25 ml, 2x concentrated
Nuclease free water	2 x 1.0 ml
ExiLERATE LNA™ qPCR control, GAPDH (hsa, mmu, rno), dried down[1)]	200 reactions

ExiLERATE LNA™ qPCR SYBR® Green master mix, 10 ml (product# 303410)

This kit contains all reagents required for PCR amplification of mRNA and non-coding RNA. In addition, a positive control assay is provided with this kit for amplification of endogenously expressed GAPDH mRNA. The reagents are provided in amounts sufficient for 2000 reactions of 10 µL.

Contents	
SYBR® Green PCR master mix	10 ml, 2x concentrated
Nuclease free water	1 x 10 ml
ExiLERATE LNA™ qPCR control, GAPDH (hsa, mmu, rno), dried down[1)]	200 reactions

[1)] Re-suspend in 220 µL Nuclease-free water before first use.

II. Primer Sets

ExiLERATE LNA™ qPCR custom primer set (product # 309998/309999)

ExiLERATE LNA™ qPCR custom primer sets are designed for optimal performance with the ExiLERATE LNA™ qPCR cDNA Synthesis Kit and SYBR® Green master mix. The performance of LNA™ primer sets will be affected if used in combination with less than optimal reagents. The primer sets are supplied in sufficient amounts for 200 or 500 reactions of 10 µL (or 100 and 250 reactions of 20 µL).

Product number	Product name	Amount supplied
309999	ExiLERATE LNA™ qPCR custom primer set	200 reactions
309998	ExiLERATE LNA™ qPCR custom primer set	500 reactions

ExiLERATE LNA™ qPCR Control primer set (product #308000-3080XX)

Endogenous control LNA™ qPCR primer sets are designed for optimal performance with the ExiLERATE LNA™ qPCR cDNA Synthesis Kit and the ExiLERATE LNA™ qPCR SYBR® Green master mix. It is always recommended to use stably expressed endogenous controls for normalization.

Product number	Product name	Amount supplied
308000	HOTAIR (hsa) PCR primer set, ExiLERATE	200 reactions
308001	ACTB (hsa, mmu, rno) PCR primer set, ExiLERATE	200 reactions
308002	PPIA (hsa, mmu, rno) PCR primer set, ExiLERATE	200 reactions
308003	MALAT1 (hsa) PCR primer set, ExiLERATE	200 reactions
308004	PGK1 (hsa, mmu, rno) PCR primer set, ExiLERATE	200 reactions
308005	GAPDH (hsa, mmu, rno) PCR primer set, ExiLERATE	200 reactions
308006	U6 snRNA (hsa) PCR primer set, ExiLERATE	200 reactions
308007	SNORD38B (hsa) PCR primer set, ExiLERATE	200 reactions
308008	SNORD44 (hsa) PCR primer set, ExiLERATE	200 reactions
308009	SNORD48 (hsa) PCR primer set, ExiLERATE	200 reactions
308010	SNORD49A (hsa) PCR primer set, ExiLERATE	200 reactions
308011	SNORA66 (hsa) PCR primer set, ExiLERATE	200 reactions
308012	5S rRNA (hsa,mmu) PCR primer set, ExiLERATE	200 reactions
308013	U6 snRNA (mmu, rno) PCR primer set, ExiLERATE	200 reactions
308014	RNU5G (hsa, mmu, rno) PCR primer set, ExiLERATE	200 reactions
308015	RNU1A1 (hsa, mmu, rno) PCR primer set, ExiLERATE	200 reactions
308016	SNORD65 (mmu) PCR primer set, ExiLERATE	200 reactions
308017	SNORD68 (mmu) PCR primer set, ExiLERATE	200 reactions
308018	SNORD110 (mmu) PCR primer set, ExiLERATE	200 reactions

Storage

Primers
The LNA™ primers are shipped dried down at room temperature and can be stored between +4°C and -20°C. Under these conditions, they are stable for at least 6 months. After re-suspension, it is recommended to store LNA™ primer sets in aliquots at -20°C to avoid repeated freeze-thaw cycles.

ExiLERATE LNA™ qPCR cDNA synthesis and ExiLERATE SYBR® Green master mix kits
These kits are shipped on dry ice in polystyrene containers and should be stored at -15°C to -25°C. Do not store in a frost-free freezer. Under these conditions, all components are stable until the expiry date on the package or vial.

Additional required materials

Reagents not supplied
- ROX or other passive reference dye (required on some PCR cyclers)

Materials and Equipment not supplied
- Nuclease-free PCR tubes or plates for use with individual assays
- Nuclease-free, aerosol barrier pipette tips
- Nuclease-free, low nucleic acid binding (siliconized) microcentrifuge tubes
- Sealing foils for PCR plates
- Micro-centrifuge and plate centrifuge
- Heating block, thermal cycler or other incubators
- Real-time PCR instrument

Recommended accompanying products

Exiqon recommends the Exiqon GenEx qPCR software for comprehensive and convenient data analysis. GenEx includes a wizard for import of data and offers advanced methods to analyze real-time qPCR data in a few simple steps. The software includes tools for selection and validation of reference genes, data pre-processing and comprehensive statistical analyses. For more information and to download a free trial, please go to www.exiqon.com/qpcr-software. The following Exiqon GenEx products are available as either perpetual or time limited licenses:

Exiqon GenEx Pro - GenEx Professional with Exiqon qPCR wizard, qPCR analysis software (Industrial or Academic license)

Exiqon GenEx Enterprise GenEx Enterprise with Exiqon qPCR wizard, qPCR analysis software (Industrial or Academic license)

Exiqon recommends the miRCURY™ RNA Isolation kits for purification of total RNA that includes the small RNA (microRNA) fraction. The following kits are available:

miRCURY™ RNA Isolation Kit – Cell & Plant
Provides a rapid method for purification of total RNA from cultured animal cells, small tissue samples, blood, bacteria, yeast, fungi and plants.

miRCURY™ RNA Isolation Kit – Tissue
Specifically designed for purification of total RNA from tissue samples.

miRCURY™ RNA Isolation Kit – FFPE
Non-toxic paraffin removal and efficient isolation of total RNA from archived samples.

miRCURY™ RNA Isolation Kit – Biofluids
Kit for purification of low abundant RNAs from samples such as serum, plasma, urine and CSF. NB! Only RNA molecules <1000nt's efficiently isolated.

See Tip 1 & 2

Before starting the Experiment

Important note

RNA work requires specific handling and precautions to prevent RNase contamination of the reagents and degradation of the RNA sample. Find information on how to handle RNA in the tips section. The tips section also provides simple guidelines for good laboratory practice to ensure optimal performance of PCR experiments.

Before setting up a real-time PCR experiment, there are a number of practical experimental design parameters that should be considered:

RNA input - The ExiLERATE LNA™ qPCR protocol is optimized for use with 20-200 ng total RNA per cDNA synthesis reaction. The exact amount of total RNA needed may vary depending on the mRNA /non-coding RNA expression levels in the cells or tissue to be analyzed. For highly expressed mRNAs it is possible to use down to 1 pg total RNA as starting material. For weakly expressed mRNAs it may be possible to use up to 5 µg of total RNA; however, in samples with high amounts of PCR inhibitors (e.g. FFPE tissue samples), this may not be feasible. Finally, inhibitors may be present in RNA preparations from certain samples e.g. serum and plasma. Prior to conducting a larger RNA profiling study, it is recommended to optimize the amount of input RNA to the RT reaction in order to avoid that the target RNAs are not expressed above detection limit, or inhibition occurs sporadically throughout the data set.

Information on how to extract and handle RNA can be found in the tips section. In short, total RNA should be prepared using a method that preserves all RNA species. DNase treatment may be necessary.

Normalization

When running individual assays it is important to consider how the data will be normalized. We always recommend to include several internal reference genes in your study and validate which ones are stably expressed in the sample type being investigated. Potential reference genes include but are not limited to Beta-actin (ACTB) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

See Tip 10

Excess volumes required for pipetting

Liquid handling with pipettes or pipetting robots require excess volumes of reagents due to loss during pipetting. The loss depends on the available pipetting system but losses in the range from 10% -25% are not uncommon. The protocol in the current instruction manual reflects the required reaction volumes and pipetting volumes should be adjusted according to accommodate the pipetting loss of the available pipetting system.

ROX

ROX is a passive reference dye used by some real-time PCR cyclers to obtain a robust read over the entire array of wells in a 96- or 384-well PCR plate. The requirement for ROX is instrument dependent and it is recommended to follow the instrument manufacturers' guidelines on this.

See Tip 7

ABI instruments

The default settings on ABI real-time PCR cyclers are not suitable for running ExiLERATE LNA™ qPCR. Settings need to be changed from automatic to manual background and threshold settings to obtain valid PCR data, see Tip 10. Furthermore, if the dataset is to be analyzed using the GenEx software, it is important that the experiment is set up as an AQ experiment, not RQ. To ensure correct settings, download the instrument settings file at www.exiqon.com/sds.

See Tip 10

Protocol

This protocol is used for conducting first-strand cDNA synthesis and real-time PCR, using the validated endogenous control and custom designed individual assays.

Before using the ExiLERATE LNA™ qPCR primer set for the first time, the primers must be re-suspended. Please refer to the table below for appropriate volumes.

Table 1. Dilution of PCR primer sets

Product number	Amount supplied	Volume for re-suspension of primers
309999	200 reactions	220 µL nuclease free water
309998	500 reactions	550 µL nuclease free water
308000-3080XX	200 reactions	220 µL nuclease free water

Re-suspend the primers by adding the appropriate volume of nuclease free water (see table above). Mix by vortexing and spin down. Leave on ice for 20-30 minutes.

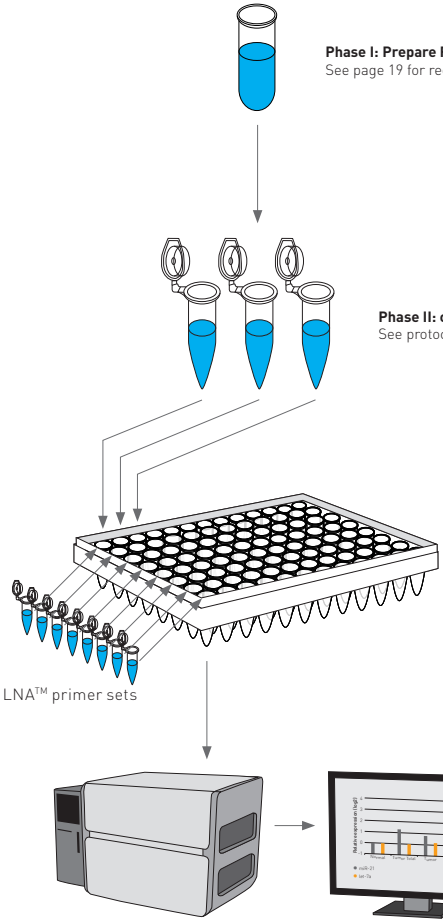
Additional required materials:

- 96- or 384-well plate real time PCR cycler
- Thermocycler for first-strand cDNA synthesis
- 96/384 well plates or tube strips compatible with available real time PCR cycler
- Micro centrifuge
- Swing bucket centrifuge for 96/384 well plates

Checklist:

- Have you considered excess volumes required for using liquid handling robotics - see page 10.
- ROX: The ExiLERATE LNA™ qPCR SYBR® Green master mix does not include the ROX passive reference dye. Please follow instrument manufacturers' recommendations.
- ABI instruments: The use of manual background and threshold settings is necessary for obtaining correct qPCR data. Make sure to have the optimal settings by downloading the instrument settings file at www.exiqon.com/sds. Furthermore, if the data is to be analyzed using GenEx, the experiment must be set up as an AQ experiment, not RQ.
- Have you optimized the input amount to the RT reaction in order to allow detection and avoid inhibition?

Workflow for individual primer sets (per sample)



Phase I: Prepare RNA sample
See page 19 for recommendations

Phase II: cDNA synthesis
See protocol page 14.

Phase III: real-time PCR amplification
See protocol page 16.
- Prepare adequate amount of pre-mixed primer + PCR Master mix and distribute into wells
- Add cDNA to all primer sets to be analyzed

Phase IV: Data analysis
See data analysis guide online
- Export data for further analysis
- Data pre-processing, normalization and statistical analysis

Protocol

The ExiLERATE LNA™ qPCR protocol is a two-part protocol consisting of:

1. First-strand cDNA synthesis (Step 1-5).
2. Real-time PCR amplification (Step 6-11).

Important: Keep reagents and reactions on ice (or at 4°C) at all times.

First strand synthesis

Step 1 Dilute template RNA	It is possible to use from 1 pg to 5 µg of input RNA, depending on how highly expressed your RNA of interest is. For most purposes, an input of 20-200 ng to an RT reaction will work well. For 20 ng input, adjust the concentration to 5 ng/µl using nuclease free water; for 200 ng input, adjust the concentration to 50 ng/µl.
Step 2 Prepare reagents	Gently thaw the cDNA buffer, cDNA primers, and nuclease- free water and immediately place on ice. Mix by vortexing. Immediately before use, remove the cDNA enzyme from the freezer, mix by flicking the tubes and place on ice. Spin down all reagents.

Step 3
Combine reagents according to Table 2

Note: remember to calculate necessary excess volume for pipetting and robotic dead volume.

If performing first-strand cDNA synthesis on multiple RNA samples, it is recommended to prepare an RT working solution of the cDNA buffer, cDNA primers, water, cDNA enzyme (in the proportion indicated in the first four lines of Table 2).
The following procedure is recommended:
1. Prepare the required amount of RT working solution and place it on ice.
2. Dispense RT working solution into nuclease free tubes.
3. Dispense template RNA in each tube.

Table 2 – Reverse transcription reaction setup

Reagent	Volume (µL), RT reaction
cDNA buffer	4
Nuclease-free water	8
cDNA primers	2
cDNA enzyme	2
Template total RNA (5-50 ng/µL)	4
Total volume	20

Step 4
Mix and spin reagents

Mix the reaction by very gentle vortexing or pipetting to ensure that all reagents are thoroughly mixed. After mixing, spin down.

Step 5
Incubate and heat inactivate[1]

- Incubate for 10 min at 25°C followed by 15 min at 50°C
- Heat-inactivate the reverse transcriptase for 5 min at 85°C
- Immediately cool to 4°C.
- Store at 4°C or freeze.

Note: For RNA templates that are GC-rich or have a large amount of secondary structure, the reaction temperature can be increased from 50°C to 65°C.

Real-time PCR amplification

Step 6
Prepare reagents for real-time PCR

Before using the primer sets for the first time, re-suspend as described on page 11. Place cDNA (from Step 5), nuclease-free water, re-suspended primers and ExiLERATE LNA™ qPCR SYBR® Green master mix on ice and thaw for 15-20 min. Protect the SYBR® Green master mix vials from light. Immediately before use, mix the SYBR® Green master by pipetting up and down. The rest of the reagents are mixed by vortexing and spun down.

Step 7
Dilute cDNA template 80x in nuclease free water[2]

Immediately before use, dilute only the amount of cDNA template needed for the planned real-time PCR reactions 80x in nuclease free water (e.g. add 395 µL nuclease free water to each 5 µL of reaction). It is important that "low-nucleic acid binding" tubes or plates are used. It is not recommended to store the 1:80 dilution of cDNA.

Recommendation: Include a passive reference dye in the cDNA dilution if advised by instrument manufacturer. Please note that the ExiLERATE LNA™ qPCR SYBR® Green master mix does not include ROX. The amount of ROX required is instrument dependent and it is important to refer to the manufacturer's recommendations when deciding how much ROX to use, see Tip 7.

Step 8
Combine ExiLERATE LNA™ qPCR SYBR® Green master mix, PCR primer mix and cDNA according to Table 3

Mix thoroughly

Note: remember to calculate necessary excess volume for pipetting and robotic dead volume.

When multiple real-time PCR reactions are performed with the same mRNA primer set, it is recommended to prepare a PCR master mix of the PCR primers and the ExiLENT LNA™ qPCR SYBR® Green master mix (in the proportion indicated in Table 3).
The following procedure is recommended:

1. Prepare the required amount of PCR master mix (see Table 3) and place it on ice. It is recommended to include excess of all reagents in the master mix to compensate for pipetting excess material.
2. Place the relevant volume of PCR master mix in PCR tubes/wells (see Table 3) and spin tubes/plate briefly in a centrifuge (1500g for 1 minute), to remove air bubbles.
3. Add cDNA template to each tube/well.

Table 3 – Real-time PCR reaction, pr. 10 µL reaction[3]

Reagent	Volume (µL)
SYBR® Green PCR master mix	5
ExiLERATE LNA™ qPCR primer mix[4]	1
Diluted cDNA template	4

Note: Following these instructions, 1/400th of the RNA added to the cDNA reaction is used in one PCR reaction, i.e. starting with 20 ng RNA in a 20 µl RT reaction would result in 0.05 ng RNA per 10 µl qPCR reaction.

ABI instrument user?

Apply manual baseline and threshold settings! Go to www.exiqon.com/sds to download settings

Step 9
Mix and spin reagents

Mix the reaction by gentle pipetting to ensure that all reagents are mixed thoroughly. After mixing cap tubes or strips or seal the plate with optical sealing as recommended by the manufacturer. Spin down in a centrifuge (1500g for 2 minutes). The experiment can be paused at this point. Store the reactions at 4°C protected from light for up to 16 hours.

Step 10
Real-time PCR amplification

Perform real-time PCR amplification followed by melting curve analysis according to Table 4.

Table 4 – Real-time PCR cycle conditions

Process step	Settings, LC480 instrument[5]	Settings, other instruments[3]
Polymerase Activation/Denaturation	95°C, 10 min	95°C, 10 min
Amplification	45 amplification cycles at 95°C, 10 s 60°C, 1 min, Optical read	40 amplification cycles at 95°C, 10 s 60°C, 1 min, Optical read
Melting curve analysis[6]	Yes	Yes

Step 11
Analyze data

Perform initial data analysis using the software supplied with the real-time PCR instrument to obtain raw Cq values (Cp or Ct, depending on PCR instrument). If you are using an ABI instrument, please note that it is not recommended to use auto Ct settings. Furthermore, if the data is to be analyzed using GenEx, the experiment must be set up as an AQ experiment, not RQ. For a guide on how to set manual baseline and threshold, refer to Tip 9 in the tips section. If you are using a Roche LC480 instrument, we recommend analysis using the 2nd derivative method. For tips on normalization, see Tip 10.

We recommend performing normalization and further data analysis with the Exiqon GenEx qPCR analysis software (www.exiqon.com/mirna-pcr-analysis). Please refer to our data analysis guide for recommendations.

See Tip 9 & 10

[1] The protocol can be interrupted at this stage. The undiluted cDNA may be kept at -20°C for up to 5 weeks (optional store at 4°C for up to 4 days). It is recommended that synthesized cDNA is stored in 'low-nucleic acid binding' tubes or plates.
[2] Adjust volumes to accommodate your in-house liquid handling system volume loss when pipetting.
[3] If using a 96-well cycler with a minimum recommended volume of 20 µL (as some ABI instruments), then use 10 µL reaction volume and set the instrument settings at 20 µL.
[4] The PCR primer mix must be prepared from the LNA™ qPCR primer set prior to real-time PCR set-up, see page 10.
[5] Five additional amplification cycles are required when using the LC480 instrument to allow collection of assay data with Cp-values up to 40.
[6] Melting curve analysis of the PCR product(s) is recommended to verify specificity and identity of the amplification reaction. Melting curve analysis is an analysis step built into the software of instruments. Please follow the instructions provided by the supplier. Note: The *Tm* of a PCR product depends on buffer composition, salt concentration and the PCR instrument.

Tips to protocol

Tip 1. RNA extraction and template preparation

Purification and preparation of high quality total RNA from a biological sample is the first critical step for a successful expression profiling analysis of mRNA and lncRNAs. Therefore, the method used for RNA sample preparation is critical to the success of the experiment. The following points should be considered before starting the experiment:

- We recommend using the miRCURY ™ RNA Isolation kit for isolation of total RNA that contains the small RNA fraction, allowing analysis of short and long RNA species from the same sample preparation.
- The comparison of samples prepared using different RNA isolation methods is not recommended.
- The isolation of RNA and the reaction steps preceding real-time PCR should be performed in rooms separate from where real-time PCR experiments will take place in order to avoid contamination with amplicon.
- It is recommended that the integrity of isolated RNA be assessed before proceeding with quantitative real-time PCR experiments. This may be performed either on the Agilent 2100 Bioanalyzer or by denaturing gel electrophoresis.
- If necessary, treat RNA preparations with DNases to remove contaminating DNA that may interfere with the real-time PCR result.
- Purified total RNA should be dissolved in nuclease-free water at a stock concentration of at least 1 µg/µl. See recommendations for storage conditions in Tip 2.

Tip 2. General guidelines for handling and storage of RNA

The following precautions should be taken to prevent RNase contamination and degradation of the RNA sample and reagents:

- Always wear disposable gloves and change them frequently.
- Use nuclease-free, low nucleic acid binding plasticware and filter barrier pipette tips.
- Keep tubes capped when possible. Always spin tubes before opening.
- For long-time storage, RNA may be stored at -80˚C. Avoid repeated freezing and thawing cycles.

Tip 3. Good PCR laboratory practice

To reduce the risk of contaminating PCRs with "old" PCR amplicons, and consequently obtain false results:

- Always wear a clean lab coat. Use separate lab coats for RNA sample preparation, cDNA synthesis and when setting up PCR reactions or handling PCR products.
- Change gloves often, especially whenever you suspect they may have been contaminated.
- Establish and maintain designated areas for PCR set-up, PCR amplification, and gel electrophoresis of PCR products.

PCR products into the PCR set-up area:

- Spin down all reaction and sample tubes before opening. Open and close all reagent and sample tubes carefully, trying not to splash or spray PCR samples.
- Keep reactions and components capped whenever possible.
- Use filter barrier pipette tips to avoid aerosol-mediated contamination of your pipetting device.
- Clean laboratory benches and equipment regularly.

Tip 4. Keep reagents and reactions cool at all times

In order to ensure optimal performance of the ExiLERATE LNA™ qPCR system it is important that the reagents and reactions are kept on ice (or at 4°C) as much as possible during the protocol (apart from reaction steps specifically involving raised temperatures).

Tip 5. First strand cDNA synthesis

Store cDNA samples in nuclease-free low nucleic acid-binding (siliconized) micro centrifuge tubes.

Tip 6. Recommended controls

The following controls are recommended and should be included in the experimental set-up:

- Reverse transcription/no enzyme controls, i.e. first-strand cDNA synthesis reactions performed without the Enzyme mix. If a PCR product is amplified from this control reaction it indicates genomic DNA or PCR product contamination of the template RNA.
- Non-template controls in the real-time PCR amplification, i.e. real-time PCR reactions performed without any cDNA template. This control will reveal PCR product contamination of the reaction.
- Blank purification or carrier only, i.e. when purifying RNA in the presence of carrier RNA (such as for serum and plasma samples). This control will reveal any non-specific signals originating from the carrier RNA alone.

Tip 7. Passive reference dye (ROX)

Many real-time PCR instruments will only produce reliable results when a passive reference dye such as ROX is added to the PCR reaction. The reference dye is used to normalize signals from individual PCR wells in order to enable comparison of real-time PCR amplification signals across entire PCR plate.

It is recommended to determine whether your real-time PCR instrument has this type of requirement. The amount of ROX to include in the PCR reaction depends on the requirements of the real-time PCR instrument and must be adjusted accordingly. Please follow the supplier's instructions for preparation and concentrations of ROX solutions. Typically, real-time PCR instruments that allow excitation at individual wavelengths for individual dyes (most filter wheel based instruments) require less ROX than instruments that use a single excitation wavelength for all fluorophores (most laser based instruments use excitation at 488 nm). See table 5, for recommended ROX concentrations.

It is important to note that excessive amounts of ROX may inhibit the PCR reaction. It may be recommended to perform a titration of ROX amounts in order to determine the optimal concentration for a particular instrument- system combination. It is possible to use the GAPDH Control primers (provided in the ExiLERATE LNA™ qPCR SYBR® Green master mix kit) to perform such titrations.

An alternative option for normalization of data from panels (profiling a high number of targets) is to normalize against the global mean; that is, the average of all expressed targets. This can be a good option in samples with a high call-rate (expressed RNAs), but should be used with caution in samples with low call-rates. It is also not a good option in samples for which the general RNA expression level is changed.

For further information on normalization and references, we recommend our data-analysis guide available online as well as the guide to RNA normalization from www.gene-quantification.de.

Table 5. Recommended ROX concentrations

Instrument	ROX concentration
ABI 7900HT	300-500 nM
ABI 7900HT FAST	300-500 nM
ABI Viia7	30-50 nM
ABI Viia7 FAST	30-50 nM
ABI 7500 FAST	30-50 nM
ABI StepOnePlus	300-500 nM
ABI 7300	300-500 nM
ABI 7500	30-50 nM
ABI 7700	300-500 nM
ABI 7000	300-500 nM

Tip 8. Inter-plate calibration

When setting up large scale experiments with several PCR plates involved, individual run differences may be observed. One way of avoiding these having an effect on the results is setting up the experiment in such a way that all samples for one gene are run on the same plate. However, this is not always feasible. When Cq values for one gene have to be compared across plates, it is recommended to employ an inter-plate calibrator.

An inter-plate calibrator is a template-assay combination which is the same in all plates, and always located in the same well across different plates. This can then be used to calibrate all plates to give the same Cq value for the calibrator, thereby reducing run-to-run variance.

Tip 9. Guidelines for real-time PCR data collection using ABI instruments

On cyclers using baseline and threshold values for Cq (Ct) calculations, Such as ABI 7900HT, it is important that the proper settings are used. Use of the automatic function of the software for these settings does not seem to produce optimal results for SYBR® Green based assays. Often the baseline is set erroneously on non-detected assays, and this in turn gives false positives, therefore do not use automatic settings. Another issue to consider when using automatic settings is that the settings may differ between plates resulting in data that cannot be compared directly. Inter-plate calibration may not fully resolve this issue, since each assay has a separately calculated baseline and threshold. Instead, both threshold and baseline should be set manually, applying the same settings for all assays on the plate.

The following principles should be applied to manual baseline and threshold settings:

Baseline:
The baseline should be calculated in the cycle interval before the amplification takes off (see Figure 1).

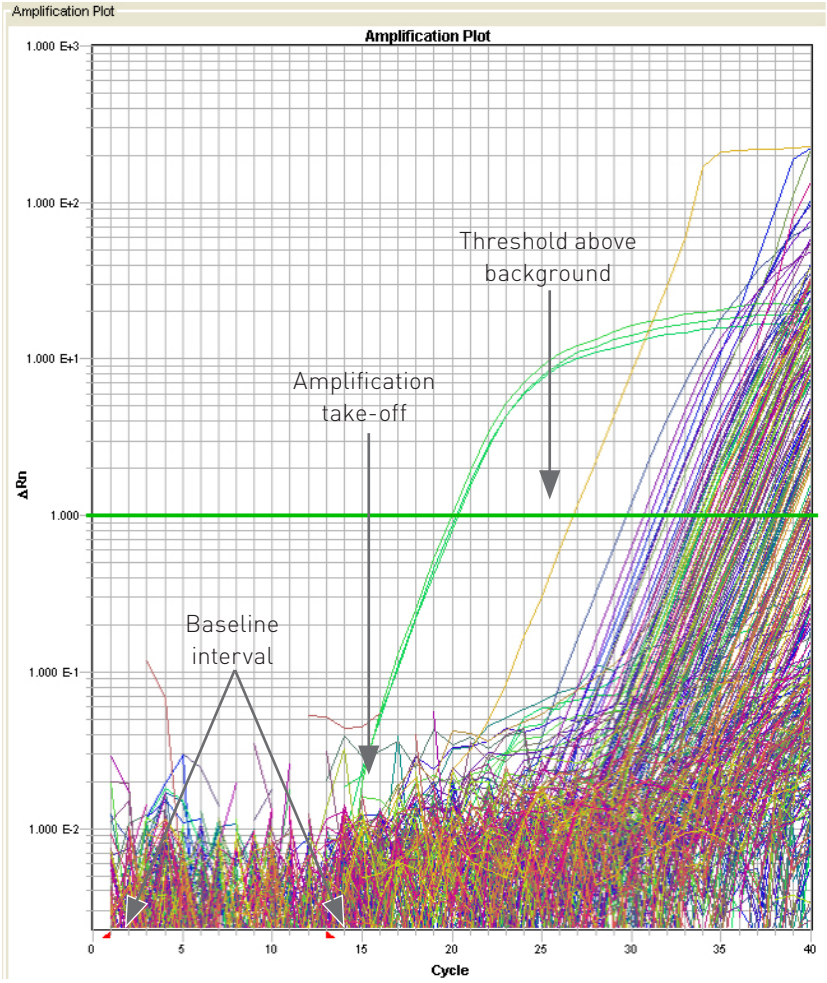
Threshold:
The threshold should then be set with the Y-axis in log scale where all assays are in the log linear phase, and the threshold above background for all assays (see Figure 1).

Note: The optimal threshold value may vary between individual machines and experiments.

Important note

If ROX passive reference dye has not been used in the PCR reactions, make sure the SDS software is set-up without reference dye correction.

Figure 1. Manual Baseline and Threshhold settings



Tip 10. Quick guide to normalization of RNA qPCR experiments

The purpose of normalization is to remove technical and biological variation between samples that is not related to the biological changes under investigation. Proper normalization is critical for the correct analysis and interpretation of results from real-time PCR experiments. The most commonly used option for normalization is to use stably expressed reference genes.

A number of validated reference gene assays are offered from Exiqon. In general it is recommended to test several endogenous control candidates (reference genes) before setting up the actual RNA expression analysis. These candidates should be chosen among genes that can be expected to be stably expressed over the whole range of samples being investigated. They can be chosen based on literature or pre-existing data (e.g. next generation sequencing analysis).

All reference gene candidates should be empirically validated for each study. A number of different software packages exist for evaluating the optimal nature and number of endogenous controls, and for applying multiple endogenous controls for normalizing target expression. One such option is the GenEx software from MulitD, sold through Exiqon with a special application for Exiqon PCR panels. GenEx incorporates both GeNorm and Normfinder for finding the optimal reference genes, and is easy and intuitive to use for the actual normalization.

For further information on normalization and references, we recommend our data analysis guide available online as well as the guide to RNA normalization from www.gene-quantification.de

Troubleshooting guide

Problem	Suggestion
PCR signal in samples amplified from first-strand synthesis reactions performed without reverse transcriptase	This typically indicates contamination of the template RNA with genomic DNA. Perform DNase treatment of the RNA sample. If this does not solve the problem, RNA samples or other reagents may be contaminated with PCR products.
PCR signal in no-template PCR reaction	This typically indicates contamination of the cDNA template or PCR reagents with amplified PCR product. Exposing the reactions to elevated temperatures (i.e. room temperature) during any part of the protocol increases the risk of background signals. It is important that the reagents and assembled reactions are kept cool (on ice or 4°C) at all times (see Tip 4 for details).
Generated signals are weak	• On some real-time PCR cyclers, gain-settings are adjustable. Make sure the gain settings of your real-time PCR cycler have been set to accommodate the signals generated from the specific assay. • RNA samples may contain PCR inhibitors. Further purification or an alternative RNA extraction method may be necessary. Check positive controls.
No fluorescent signal is detected during the PCR	Confirm that you have a PCR product by running an aliquot of your PCR reaction on an agarose gel.
No fluorescent signal detected during the PCR, but a PCR amplicon can be detected by agarose gel electrophoresis	• Check that the filter in the real-time PCR cycler was set to either SYBR® Green or FAM/FITC. • Check that the optical read is at the correct step of the real-time PCR cycles. • Adjust the baseline in the real-time PCR cycler software.

FAQs

What is the recommended experimental set up for ExiLERATE LNA™ qPCR system?
It is generally accepted that the reverse transcription (RT) reaction gives rise to more variation than the PCR reaction. It is therefore advisable to perform replicate RT reactions, ideally 3 separate reactions with 1-2 PCR reactions for each RT. It is further recommended to always include at least three biological replicates (separate RNA extractions) of each sample type in order to allow statistical analysis of the results. If small changes in mRNA expression are expected, it may be necessary to include more replicates to ensure a significant result. In general it is recommended that replicates should be included at any stage during sample procurement, processing, RNA isolation, etc. that could give rise to variation between samples.

A tech note on guidelines for setting up microRNA qPCR experiments can be downloaded at www.exiqon.com/miRNA-qPCR-guidelines. Many of the recommendations in this guide also apply for mRNA analysis.

What kind of real-time PCR instruments is ExiLERATE LNA™ qPCR compatible with?
ExiLERATE LNA™ qPCR is compatible with all instruments capable of reading green fluorophores such as fluorescein/FITC/FAM and SYBR® Green. The system has been tested and found to work on real-time PCR instruments from several leading suppliers of this type of instrument.

What kind of settings should I use on my real-time PCR instrument?
If your real-time PCR instrument supports fluorophores such as fluorescein/FITC/FAM or SYBR® Green your instrument must be set to detect these fluorophores.

Is ExiLERATE LNA™ qPCR compatible with other SYBR® Green master mixes?
We do not recommend using other SYBR® Green master mixes for real-time PCR analysis with the LNA™ PCR primer sets. The primer sets have been optimized and validated using the ExiLERATE LNA™ qPCR SYBR® Green master mix and the performance of the primer sets will be compromised by using a different master mix (which may contain different salt and/or enzyme concentrations).

My RNA is already enriched for mRNA, how much should I use in the real-time PCR experiments?
The ExiLERATE LNA™ qPCR system is developed for use with total RNA and it is not required to do any mRNA enrichment prior to qPCR.

Samples of enriched mRNAs are difficult to quantitate accurately making it very tricky to ensure the same amount of sample is added to each reaction. If necessary, a total RNA equivalent should be used for the enriched sample, e.g. use a proportional amount of enriched sample resulting from 20 ng of total RNA. It may be necessary to try a couple of different amounts of enriched sample to ensure that the results fall within the linear range of the assay.

Related products

Exiqon offers a broad variety of tools enabling new discoveries concerning the expression, function and spatial distribution of microRNAs as well as lncRNA and mRNA analysis:

Antisense LNA™ GapmeRs
Antisense LNA™ GapmeRs are potent antisense oligonucleotides used for highly efficient inhibition of mRNA and lncRNA function. Designed using advanced algorithms, the RNase H-activating gapmers offer excellent performance and high success rate.

mRNA and lncRNA *in situ* hybridization
Use LNA™-enhanced detection probes for your RNA *in situ* hybridization and Northern blot to achieve superior sensitivity and improved specificity compared to longer DNA oligonucleotides and riboprobes.

miRCURY LNA™ microRNA Inhibitors and Power Inhibitors
Unravel the function of microRNAs by microRNA inhibition. Sophisticated LNA™ design ensures potent inhibition of all microRNAs regardless of their GC content. Chemically modified, highly stable Power Inhibitors afford unrivalled potency.

miRCURY LNA™ microRNA Inhibitor Library
For genome-wide high throughput screening of microRNA function.

miRCURY LNA™ microRNA Mimics
Highly potent mature LNA™-enhanced microRNA mimics with unique triple RNA strand design

Please refer to LNA™ Custom qPCR when describing a procedure for publication using this product.

Notice to purchaser
Exiqon, LNA™ and miRCURY are registered trademarks of Exiqon A /S, Vedbaek, Denmark. SYBR® Green is a licensed trademark of Invitrogen. All other trademarks are the proper ty of their respective owners.

Locked-nucleic Acids (LNA™s) are protected by the following US patents and corresponding patent claims outside the US: US Pat No. 6,268,490, US Pat No. 6,770,74 8, US Pat No. 7.572.582, US Pat No. 8.153.365 and other applications and patents owned or licensed by Exiqon A /S. Products are provided to buyers for research use only. The products in their original or any modified form may be used only for the buyer's internal research purposes and not for commercial, diagnostic, therapeutic, or other use, including contract research. The buyer may not provide products to third par ties in their original or any modified form. The purchase of products does not include or carry an implied right or license for the buyer to use such products in their original or any modified form in the provision of services to third par ties, and a license must be obtained directly from Exiqon A /S for such uses.

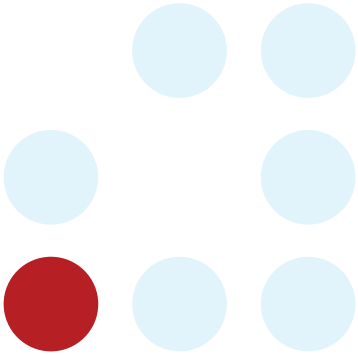
Limited license
Use of this product is covered by one or more of the following US patents and corresponding patent claims outside the US: 5,994,056 and 6,171,785. The purchase of this product includes a limited, nontransferable immunity from suit under the foregoing patent claims for using only this amount of product solely in Contract Research, including reporting results of purchaser's activities for a fee or other commercial consideration, and also for the purchaser's own internal research. No right under any other patent claim is conveyed expressly, by implication, or by estoppel. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Life Technologies, 850 Lincoln Centre Drive, Foster City, California 94 404, USA.

Furthermore, this product is provided under an agreement between Molecular Probes, Inc., a wholly owned subsidiary of Invitrogen Corporation, now Thermo Fischer and EXIQON. The manufacture, use, sale or import of this product is subject to one or more U.S. Patents and corresponding international equivalents. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer, where such research does not include testing, analysis or screening services for any third par ty in return for compensation on a per test basis. The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third par ty or other wise use this product or its components or materials made using this product or its components for Commercial Purposes. Commercial Purposes means any activity by a par ty for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. For information on purchasing a license to this product for purposes other than research, contact Life Technologies Corporation, Inc., Business Development, 29851 Willow Creek Road, Eugene, OR 97402. Tel: (541) 465-8 300, Fax: (541) 335-0354.

Further, the purchase of this product includes a limited, non-transferable license under specific claims of U.S. Patent Nos. 6,174,670 and 6,569,627, owned by the University of Utah Research Foundation and licensed to Roche Diagnostics GmbH and Idaho Technology, Inc., to use only the enclosed amount of product according to the specified protocols. No right is conveyed, expressly, by implication, or by estoppel, to use any instrument or system under any claim of U.S. Patent Nos. 6,174,670 and 6,569,627, other than for the amount of product contained herein.

For life science research use only. Not for use in diagnostic procedures.

Notes



Outside North America
Exiqon A/S · Skelstedet 16
DK-2950 Vedbaek · Denmark
Phone +45 45 660 888
Fax +45 45 661 888

North America
Exiqon Inc. · 12 Gill Street, Suite 1650
Woburn, MA 01801 · United States
Phone (781) 376 4150
Fax (781) 376 4152

exiqon.com

EXIQON
Seek Find Verify

13-0120 - 924079 - v1.1 - 04/2016