Expression Analysis

ExiSEQ LNA™ Quant Kit

Instruction manual v1.0

For product # 800200, 800211, 800212, 800213
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EXIQON
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Product summary

The ExiSEQ LNA™ Quant Kit contains DNA standards, LNA™ probe and LNA™ qPCR primers that are well suited for quantifying Illumina® platform based libraries for Next Generation Sequencing. This kit is based on a dual probe hydrolysis rather than a SYBR® Green based qPCR system, which means that the size distribution of the library is irrelevant in the downstream calculation of the library concentration.

The probe is based on Illumina's® standard Index I primer binding site (i7) and will only work on libraries that use the primer index I design shown on page 7. The ExiSEQ LNA™ Quant Kit can be utilized to measure individual libraries prior to normalization and pooling as well as pool quantifications prior to cluster generation on Illumina® platform flowcells.

Product overview of the ExiSEQ LNA™ Quant Kit

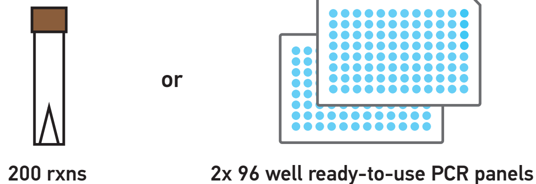


Figure 1.

Available kits

The ExiSEQ LNA™ Quant Kit is available with primers in ready-to-use qPCR plates (panel), covering most qPCR cyclers or in a vial (200rxns) for flexible setups. The ExiSEQ LNA™ Quant Kit consists of the components described in Tables 1 and 2:

Prod. no. 800211-800213 ExiSEQ LNA™ Quant Kit, panel:
The reagents provided are sufficient for preparation of up to 192 reactions or 2x 27 samples, 2x dilution curves and two "No Template Controls" (NTC), all performed in triplicate.

Contents	Amount supplied
ExiSEQ LNA™ Quant 96 well plates with dried down primers and probe	2 plates
ExiSEQ LNA™ Quant Standard Template 1 (20 pM), lyophilized	60 rxns
ExiSEQ LNA™ Quant Standard Template 2 (2 pM), lyophilized	60 rxns
ExiSEQ LNA™ Quant Standard Template 3 (200 fM), lyophilized	60 rxns
ExiSEQ LNA™ Quant Standard Template 4 (20 fM), lyophilized	60 rxns
Dilution buffer	1.2 mL

Table 1.

Prod. no. 800200 ExiSEQ LNA™ Quant Kit, 200rxn:
The reagents provided are sufficient for preparation of up to 200 reactions or 61 samples, 2 dilution curves and two NTC's all performed in triplicate.

Contents	Amount supplied
ExiSEQ LNA™ Quant primers and probe mixture	200 rxns
ExiSEQ LNA™ Quant Standard Template 1 (20 pM), lyophilized	60 rxns
ExiSEQ LNA™ Quant Standard Template 2 (2 pM), lyophilized	60 rxns
ExiSEQ LNA™ Quant Standard Template 3 (200 fM), lyophilized	60 rxns
ExiSEQ LNA™ Quant Standard Template 4 (20 fM), lyophilized	60 rxns
Dilution buffer	1.2 mL

Table 2.

Probe and Primer Pair (Prod no 800200):
The LNA™ probe-primer mix is suspended in water and should be stored at -20 °C. Since the probe is dual labelled it should also be protected from light at all time. If more than 10 freeze-thaw cycles are expected, divide the mixture into smaller aliquots for use.

Standard templates 1, 2, 3 and 4:
Re-suspend all four DNA standards in 250 μL Dilution buffer. Mix well (vortex) and spin briefly to gather the liquid at the bottom of the tube. Let the vials stand on ice for at least 30 minutes before first use.

Storage of kit components

All of the individual components of the ExiSEQ LNA™ Quant Kit are stable at –20 °C for at least 6 months. Plates are stable at 4 °C for at least 6 months. When not in use, kit components should be stored at –20 °C. All components of the kit will tolerate multiple freeze/thaw cycles. Reactions can be prepared at room temperature, but unused portions of the kit reagents should be kept on ice after thawing them for use and before being returned to storage at –20 °C.

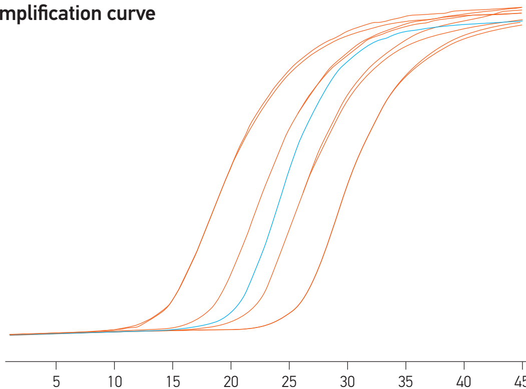
Additional required materials (not included)

- QuantiTect Probe mastermix PCR kit (Cat No./ID 204343, QIAGEN® Inc.)
- Real-time PCR instrument
- qPCR seals matching the instrument
- PCR strip tubes or microcentrifuge tubes
- Centrifuge tubes
- Standard laboratory equipment (pipettes, tips etc.)

Product description

The ExiSEQ LNA™ Quant Kit contains reagents and components that are optimized for real-time PCR based quantification of Next Generation Sequencing libraries for the Illumina® platform. Library quantification is critical for optimal cluster generation on the Illumina® flow cells. The ExiSEQ LNA™ Quant Kit contains LNA™ based primers which target the P5 and P7 Illumina® adaptor sequences, and a dual labeled LNA™ probe that targets the i7 index I sequencing primer binding site. It also contains synthetic DNA template in four concentrations as standards in 10 fold dilutions (20 pM, 2 pM, 0.2 pM and 0.02 pM) to enable reliable quantification of diluted DNA libraries. The use of a probe based qPCR instead of SYBR® based qPCR makes the size distribution of the library irrelevant in the library concentration calculations.

Amplification curves



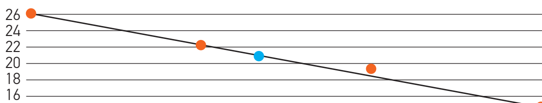


Figure 2. Principle of the ExiSEQ LNA™ Quant Kit. The four dilution standards (representing 10 fold dilutions of a synthetic DNA template, orange) as well as 10000 fold dilutions of real samples (blue) are amplified and Cq values determined. The standard curve is used to convert the Cq values of the samples into concentration values. Since the reactions are based on probe cleavage, no correction for library size is needed.

The sequences of the LNA™ primers are as follows:
ExiSEQ LNA™ Quant forward primer: 5'- AATGATACGGCGACCACCGAGAT -3'
ExiSEQ LNA™ Quant reverse primer: 5'- CAAGCAGAAGACGGCATACGA -3'

The ExiSEQ LNA™ Quant Probe is designed to target the Index I (i7) primer binding site which has the following sequence: AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC

When checking the compatibility of kits with the ExiSEQ LNA™ Quant Kit, note that all libraries that contain the above Index I primer binding site (i7) will be quantified.

The ExiSEQ LNA™ Quant Kit is compatible with the following library preparation kits:

a) QIAGEN® NGS kits for the Illumina® platform (not smallRNA kit)
b) Illumina® TruSeq based libraries (not Nextera™ transposase based kits or smallRNA kits)
c) Agilent® NGS kits for the Illumina® platform (not SureSelectQXT transposase based kits)
d) Bioo Scientific NGS kits for the Illumina® platform
e) Nugen NGS kits for the Illumina® platform

If the adaptor sequence data is missing from vendors of library generation kits check if custom Index I primers are supplied and recommended with the corresponding kit. That is an indication that the ExiSEQ LNA™ Quant Kit will not be able to quantify the libraries.

Workflow overview

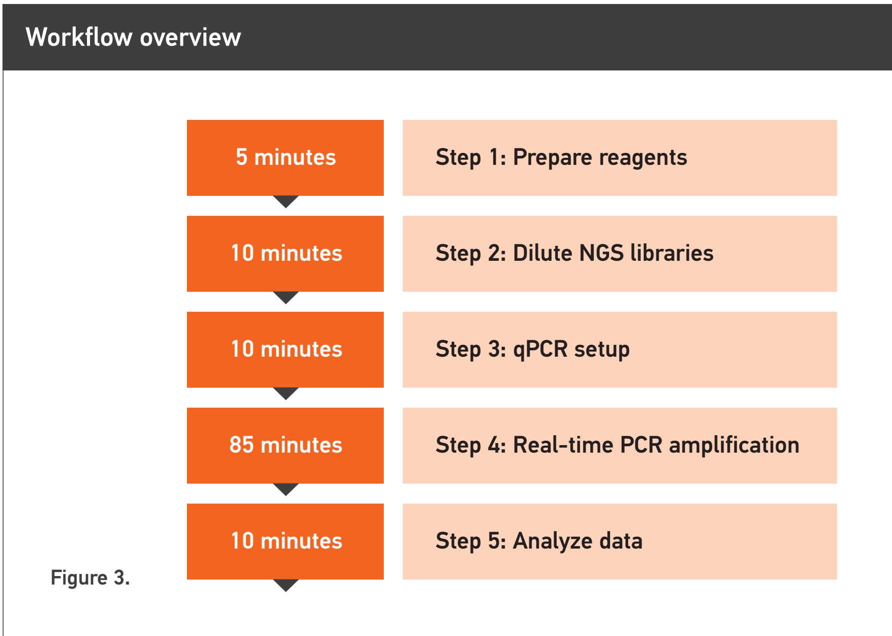


Figure 3.

Plate layout

It is recommended to run triplicates of both samples and standards. Standards (Template 1, 2, 3 and 4) and No Template Control =NTC (water) are best placed in the upper left corner of the plate, as demonstrated in Figure 4 if the Exiqon Library Concentration Calculator is to be used for the calculation of library concentrations.

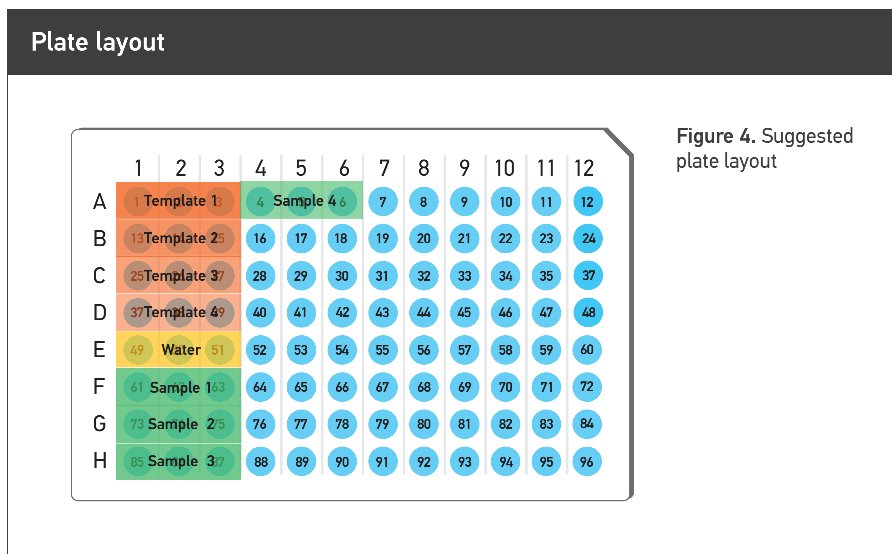


Figure 4. Suggested plate layout

Protocols

1: Procedure for setting up individual reactions (#800200)

Step 1
Prepare reagents

Starting Material: 1 ng – 1 µg DNA libraries for the Illumina® Next Generation Sequencing Platform.
Thaw the QuantiTect Probe Mastermix (see page 5) and ExiSEQ LNA™ Quant standards as well as the libraries to be quantified. Ensure mixing of standards by inverting 3-5 times and spin down briefly. Do not vortex the Mastermix. Place reagents on ice.

Step 2
Dilute NGS libraries

Libraries should be diluted in nuclease free water or low TE buffer in 1:10000, fold dilutions. Libraries of high concentrations can be diluted further and low concentration libraries can be diluted less.

Step 3
qPCR setup

When setting up multiple reactions it is recommended to prepare a master mix working-solution of the PCR primers, qPCR Mastermix and water in the proportions indicated in Table 3.
The following procedure is recommended:

1. Prepare the required amount of working-solution (see Table 3) and place it on ice. It is recommended to include excess of all reagents to compensate for pipetting excess material.
2. Place the relevant volume of primer:mastermix working-solution 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 diluted sample/standard to each tube/well.
4. Seal the tubes/plate with optical sealing as recommended by the instrument manufacturer.

Reagent	Volume (µL)
QuantiTect Probe qPCR Mastermix (2x)	10
Diluted sample or standard	4
ExiSEQ LNA™ Quant Probe/PCR primer mix	2
Nuclease free water	4
Total volume	20

Table 3. Real-time PCR setup, per 20 µL reaction

Step 4
Real-time PCR amplification

Perform real-time PCR amplification according to Table 4 using FAM / SYBR settings without melting curve analysis.

Process step	Instrument settings
Polymerase Activation/Denaturation	95 °C, 15 min
Amplification	40 amplification cycles at 94 °C, 15 sec 60 °C, 1 min

Table 4. Real-time PCR cycle conditions

Step 5
Data analysis

Transfer the data over to the Library Concentration Calculator (download from exiqon.com/NGS-library-quant) and calculate the samples' concentrations based on the standard curve.

Or use the real-time PCR instrument's internal software to annotate the standard concentrations as follows:

Standard template 1 20 pM
Standard template 2 2 pM
Standard template 3 0.2 pM
Standard template 4 0.02 pM

Confirm that the efficiency is approximately 90-110 % and collect the calculated concentrations for the given samples and recalculate the dilution factors of the samples. Refer to the instrument's software instructions regarding how to use the software.

2: Procedure for a 96 well plate setup (ready-to-use panel)

Step 1
Prepare reagents

Starting Material: 1 ng – 1 µg DNA libraries for the Illumina® Next Generation Sequencing Platform.
Thaw the QuantiTect Mastermix (see page 5) and ExiSEQ LNA™ Quant standards as well as the libraries to be quantified. Ensure mixing of standards by inverting 3-5 times and spin down briefly. Do not vortex the Mastermix. Place reagents on ice.

Note: If using a real-time instrument that uses ROX normalization dye, it is recommend to add ROX to the Quantitect Master Mix after thawing.

Step 2
Dilute NGS libraries

Libraries should be diluted in nuclease free water or low TE buffer in 1:10000 fold dilutions. Libraries of high concentrations can be diluted further and low concentration libraries can be diluted less.

Step 3
qPCR setup

When setting up multiple reactions it is recommended to prepare a master mix working-solution of the qPCR Mastermix and water in the proportion indicated in Table 5.
The following procedure is recommended:

1. Prepare the required amount of working-solution (see Table 5) and place it on ice. It is recommended to include excess of all reagents to compensate for pipetting excess material.
2. Place the relevant volume of working-solution in PCR the wells (see Table 5) and spin plate briefly in a centrifuge (1500g for 1 minute), to remove air bubbles.
3. Add diluted sample/standard to each tube/well.
4. Seal the plate with optical sealing as recommended by the instrument manufacturer.

Reagent	Volume (µL)
QuantiTect probe qPCR Mastermix (2x)	10
Nuclease free water	6
Diluted sample or standard	4
Total volume	20

Table 5. Real-time PCR setup, per 20 µL reaction

Step 4
Real-time PCR amplification

Perform real-time PCR amplification according to Table 6 using FAM / SYBR settings without melting curve analysis.

Process step	Instrument settings
Polymerase Activation/Denaturation	95 °C, 15 min
Amplification	40 amplification cycles at 94 °C, 15 sec 60 °C, 1 min

Table 6. Real-time PCR cycle conditions

Step 5
Data analysis

Transfer the data over to the Library Concentration Calculator (download from exiqon.com/NGS-library-quant) and calculate the samples' concentrations based on the standard curve.

Or use the real-time PCR instruments internal software to annotate the standard concentrations as follows:

Standard template 1 20 pM
Standard template 2 2 pM
Standard template 3 0.2 pM
Standard template 4 0.02 pM

Confirm that the efficiency is approximately 90-110 % and collect the calculated concentrations for the given samples and recalculate the dilution factors of the samples. Refer to the instrument's software instructions regarding how to use the software.

Tips to protocol

Tip 1. Library preparation – Dilutions

Libraries are diluted in nuclease free water. Up to 1 ml for each library is needed. Most types of libraries are to be diluted between 10000 and 100000 fold. If you are unsure about your library concentration, we then recommend that three 1:1 dilutions will be interrogated on the qPCR platform, for example 10000, 20000 and 40000 fold dilutions.

Perform an initial 10000 fold dilution by making two 1:100 dilutions of each library sample in nuclease free water.

Add 2 µL sample to 198 µL nuclease free water. Mix well by vortexing and spin down.

Add 2 µL of the diluted sample (1.) to 198 µL nuclease free water. Mix well by vortexing and spin down. Mark this dilution 1:10000 (2.).

Add 100 µL diluted sample (2.) to 100 µL nuclease free water. Mix well by vortexing and spin down. Mark this dilution 1:20000.

Add 100 µL diluted sample (3.) to 100 µL nuclease free water. Mix well by vortexing and spin down. Mark this dilution 1:40000.

Most regular library preparations will have enough material to be diluted down to 160000 and still be within the titration curve. Note that sample dilutions outside the dilution curves should not be used to quantify the samples, since they may be outside the assay's linear range.

Tip 2. Data analysis

Many real-time instruments have software to determine standard curves within plates by annotating the standards and their concentrations within the plate as well as sample dilution factors. They can be used directly for quantifying libraries since the quantification is size independent.

Cq values can also be extracted as text format and imported into the Library Concentration Calculator (available at exiqon.com/NGS-library-quant). Simply copy-paste names and well positions as seen in the calculator into the import sheet. Use the flowchart in the template to calculate the samples' concentrations.

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 amplifi cation, and gel electrophoresis of PCR products
- Never bring amplified 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

Related products

Exiqon offers a broad variety of tools enabling new discoveries concerning the expression, function and spatial distribution of RNA:

ExiSEQ NGS sample QC kit – small RNA/microRNA
The essential QC kit for evaluating RNA samples prior to microRNA/small RNA NGS library preparation, as well as assessing NGS performance post-sequencing. Essential for challenging samples with low RNA content such as biofluids. Ensure optimal RNA sequencing results.

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Exiqon's microRNA qPCR system offers the best available combination of performance and ease-of-use on the microRNA real-time PCR market. The combination of a Universal RT reaction and LNA™-enhanced PCR primers results in unmatched sensitivity and specificity. The Ready-to-use microRNA PCR panels enable fast and easy microRNA expression profiling. Take advantage of the tailored Universal RT microRNA PCR spike in kit to monitor the performance of your PCR.

miRCURY LNA™ microRNA Inhibitors, Power Inhibitors and Family 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 for unrivalled potency. Available for *in vitro* and *in vivo* studies.

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