# NEBNext® Library Quant Kit for Illumina®

NEB #E7630S/L

100/500 reactions

Version 3.0_03/20

## Table of Contents



## The Kit Includes

*The volumes provided are sufficient for preparation of up to 100 reactions (NEB #E7630S) 500 reactions (NEB #E7630L). All reagents should be stored at –20°C. Colored bullets represent the color of the cap of the tube containing the reagent.*

NEBNext Library Quant Master Mix

NEBNext Library Quant Quant Primer Mix

NEBNext Library Dilution Buffer (10X)

ROX (Low)

ROX (High)

○ (yellow) NEBNext Library Quant DNA Standard 1

● (green) NEBNext Library Quant DNA Standard 2

● (red) NEBNext Library Quant DNA Standard 3

○ (white) NEBNext Library Quant DNA Standard 4

## Storage of Kit Components

All of the individual components of the NEBNext Library Quant Kit for Illumina are stable at –20°C for two years. When not in use, kit components should be stored at –20°C. All components of the kit, including the combined NEBNext Library Quant Master Mix and Primer Mix, are stable for at least 30 freeze/thaws.

Once the Primer Mix has been added to the NEBNext Library Quant Master Mix (see Protocol page 6), the resulting mix can be stored at –20°C for seven months. For short-term storage, this mix may be stored at 4°C for up to six weeks.

Reactions can be prepared at room temperature, but unused portions of the kit reagents should be kept on ice after thawing them for use.

## Required Materials Not Included

- Nuclease-free water
- qPCR machine
- qPCR plates and seals
- PCR strip tubes or microcentrifuge tubes
- Conical centrifuge tubes

## Applications

The NEBNext Library Quant Kit for Illumina contains enzymes and buffers that are ideally suited for quantitating libraries for next-generation sequencing. Each of these components must pass rigorous quality control standards and are lot controlled, both individually and as a set of reagents.

Lot Control: The lots provided in the NEBNext Library Quant Kit for Illumina are managed separately and are qualified by functional testing. Individual reagents undergo standard enzyme activity and quality control assays, and also meet stringent criteria as described on each individual component page.

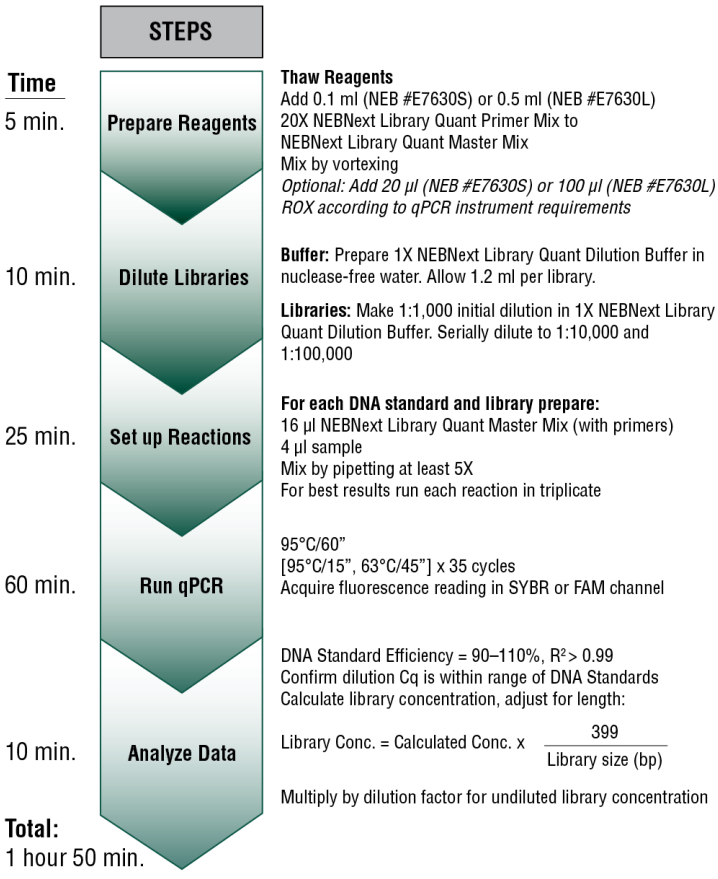
Functionally Validated: Each set of reagents is functionally validated together through qPCR-based library quantitation assays. Reagents pass the functional test by adhering to stringent criteria set for assay efficiency and quantitation cycles (Cq) for each DNA standard and no template control reaction.

For larger volume requirements, customized and bulk packaging is available by purchasing through the OEM/Bulks department at NEB. Please contact OEM@neb.com for further information.

## Introduction

The NEBNext Library Quant Kit for Illumina contains components that are optimized for qPCR-based quantitation of libraries prepared for Illumina next-generation sequencing platforms. Library quantitation is critical for proper cluster generation on the Illumina flow cell. The NEBNext Library Quant Kit contains primers which target the P5 and P7 Illumina adaptor sequences and a set of high-quality, pre-diluted DNA standards to enable reliable quantitation of diluted DNA libraries between 150–1000 bp.

**Figure 1. NEBNext Library Quant Kit for Illumina (NEB #E7630) Workflow.**



## Section 1: Protocol

**Symbols**

*This caution sign signifies a step in the protocol that has multiple paths leading to the same end point but is dependent on a user variable, like the amount of input DNA.*


*This is a point where you can safely stop the protocol and store the samples prior to proceeding to the next step in the protocol.*

**Starting Material:** 5 ng–1 µg fragmented DNA.

### 1.1. Thaw Kit Reagents

Thaw kit components at room temperature. Mix well and centrifuge briefly to collect material from the sides of the tubes. Place reagents on ice.

### 1.2. Prepare Master Mix + Primer Mix

*NEB #E7630S: Add 100 µl NEBNext Library Quant Primer Mix to the tube of NEBNext Library Quant Master Mix (1.5 ml). Mix by vortexing for 10 seconds. Write the date on the master mix tube to indicate that primer mix has been added.*

*NEB #E7630L: Add 500 µl NEBNext Library Quant Primer Mix to the bottle of NEBNext Library Quant Master Mix (7.5 ml). Mix by vortexing for 10 seconds. Write the date on the master mix bottle to indicate that primer mix has been added.*

*Note:If using ROX for normalization, add to the NEBNext Library Quant Master Mix. Add 20 µl (NEB #E7630S) or 100 µl (NEB #E7630L) ROX and vortex. See Table 1 in Chapter 3 for ROX selection.*

### 1.3. Prepare NEBNext Library Quant Dilution Buffer (1X)

Prepare the NEBNext Library Quant Dilution Buffer (1X) by making a 1:10 dilution of the 10X buffer in nuclease-free water. Prepare sufficient buffer for the desired number of libraries to be quantitated, allowing 1.2 ml for each library.

### 1.4. Prepare Library Dilutions

Prepare an initial 1:1,000 dilution of each library sample in NEBNext Library Quant Dilution Buffer (1X).

1.4.1. Add 1 µl library sample to 999 µl NEBNext Library Quant Dilution Buffer (1X) to create a 1:1,000 dilution.

Prepare two additional dilutions of the diluted Library samples to create 1:10,000 and 1:100,000 dilutions. These two library dilutions will be used for qPCR analysis.

1.4.2. Add 10 µl of the 1:1,000 dilution to 90 µl NEBNext Library Quant Dilution Buffer (1X) (creates 1:10,000 dilution).

1.4.3. Add 10 µl of the 1:10,000 dilution to 90 µl NEBNext Library Quant Dilution Buffer (1X) (creates 1:100,000 dilution).

### 1.5. Prepare qPCR Assays

For best results, we recommend running each DNA standard and library sample in triplicate.

1.5.1. Prepare DNA standards and diluted library samples.

| | |
|---|---|
| NEBNext Library Quant Master Mix (with primers) | 16 µl |
| DNA standard or library dilution | 4 µl |
| Total Volume | 20 µl |

1.5.2. Prepare a no-template control.

| | |
|---|---|
| NEBNext Library Quant Master Mix (with primers) | 16 µl |
| Library Dilution Buffer (1X) | 4 µl |
| Total Volume | 20 µl |

Mix reactions by pipetting sample or buffer at least 5X. Try to minimize bubbles in plate wells, but 1–2 bubbles per well will be removed by heating and not affect results. If replicates are prepared outside of the qPCR plate, load 19 µl per well to minimize bubble formation.

**1.6. Run qPCR Assay in a Real-time Thermal Cycler Using FAM/SYBR Setting**

1.6.1. qPCR cycling conditions

| CYCLE STEP | TEMP | TIME | CYCLES |
|---|---|---|---|
| Initial Denaturation | 95°C | 1 minute | 1 |
| Denaturation<br>Extension | 95°C<br>63°C | 15 seconds<br>45 seconds | 35 |

Note: A denaturation/melt curve can be included if desired, but is optional. See discussion in Section 6.1.

We recommend the "Fast" temperature profile where applicable (e.g. Applied Biosystems® StepOne Plus®, 7500 Fast) if appropriate plates are available. "Standard" mode is compatible with the kit if desired.

1.6.7. Analyze Data and Calculate Library Concentrations

Use the real-time instrument to annotate the standard concentrations as follows:

| SAMPLE NAME | CONCENTRATION (pM) |
|---|---|
| DNA Standard 1 | 10 |
| DNA Standard 2 | 1 |
| DNA Standard 3 | 0.1 |
| DNA Standard 4 | 0.01 |

Confirm PCR efficiency for the DNA standards is 90–110%.

Adjust library concentration for size using:

Adjusted Conc. = Calculated Conc. × 399 / library size (bp)

Calculate concentrations using standard curve generated with qPCR instrument software, or use NEB qPCR webtool to calculate standard curve and library concentrations, available at https://nebiocalculator.neb.com/qPCR

## Section 2
## Experimental Considerations

- qPCR is a sensitive DNA detection method. Proper sterile technique and careful pipetting should be used to avoid DNA contamination and ensure accurate quantitation results.
- This kit is used for quantitation of libraries for Illumina sequencing platforms and includes primers that are complementary to the P5 and P7 sequences. Prior to using this kit, confirm that the library samples being tested include these Illumina adaptor sequences.
- Ensure that all kit components are thawed and mixed prior to use.
- Upon first use, when the NEBNext Library Quant Primer Mix is added to the NEBNext Library Quant Master Mix the date should be recorded on the NEBNext Library Quant Master Mix container. Use this notation to verify that the NEBNext Library Quant Primer Mix was added prior to using the mix in subsequent experiments. Once mixed the combined NEBNext Library Quant Master Mix and NEBNext Library Quant Primer Mix is stable for six weeks at 4°C (mix can be stored without freezing for convenience) or seven months at –20°C.
- For each qPCR reaction add 4 µl of NEBNext Library Quant DNA Standards or diluted library to 16 µl of pre-mixed NEBNext Library Quant Master Mix (with primers).
- We recommend inclusion of a no template control (NTC) reaction in addition the NEBNext Library Quant DNA Standards 1–4. The Cq from the NTC will not be used in quantitation analysis, but serves as a valuable control reaction to ensure performance of the kit and absence of sample contamination.
- Accurate library quantitation relies on careful dilution of library DNA samples. Use a 1X concentration of provided NEBNext Library Quant Dilution Buffer and prepare 1:1,000–1:100,000 dilutions carefully.
- We recommend running triplicate reactions for each standard and library sample. This ensures the most accurate quantitation and enables exclusion of outlier traces due to bubble, plate sealing or other problems.
- Multichannel pipettes can be used with the kit, but care should be taken to ensure consistency of volume when loading both the NEBNext Library Quant Master Mix (with primers) and samples into the qPCR plate. For most accurate and consistent results we recommend single channel pipettes.
- When pipetting into the qPCR plate, it is advisable to avoid the formation of bubbles. Centrifugation of sealed qPCR plates for 5 minutes at 2,500–3,000 rpm is recommended to collect samples at the bottom of wells, but is not required. If 1–2 small bubbles are present at the top of the liquid after loading, the assay can proceed as the bubbles will be removed as the plate is heated for the denaturation step of the PCR cycle.
- If quantifying fewer libraries than require a full plate to be loaded, we recommend avoiding the wells along the edges of the plate, as these can be prone to plate-sealing failures during qPCR runs, resulting in outlier traces or evaporated reagents. When run in triplicate, the failed well does not affect results if excluded from analysis.
- Consult the qPCR instrument manual to verify the appropriate ROX solution to be used. A quick recommendation can be found in Table 1 below, however the appropriate ROX amount should be verified for instruments not listed.
- Use the “SYBR Green” or “SYBR/FAM” channel of the qPCR instrument. Only the single channel plate read is necessary for the NEBNext Library Quant Kit, and selecting a single read often results in faster experiment times.
- Denaturation or melt curves may be included in the qPCR cycling protocol, although this will add time to the workflow and likely not provide helpful information about library quality. Extended discussion of melt curves with examples can be found in Section 6.1.

Table 1: Recommended ROX amount for indicated instruments used with NEBNext Library Quant Kit.

| qPCR INSTRUMENT | ROX |
|---|---|
| Bio-Rad® iQ™ 5, CFX96, CFX384, Opticon<br>Roche Lightcycler®<br>Qiagen® Rotor-Gene®<br>Eppendorf® Mastercycler®<br>Cepheid® SmartCycler® | Not Recommended |
| Applied Biosystems 7500, QuantStudio®, ViiA7™<br>Agilent Mx™ | Low ROX |
| Applied Biosystems 7000, 7300, 7700, 7900HT, StepOne®, StepOnePlus™ | High ROX |

## Section 3
## Library Quant Kit Protocol

**Starting Material:** 5 ng–1 µg fragmented DNA.

### 3.1. Thaw and Mix Reagents

3.1.1. Thaw the NEBNext Library Quant Master Mix and NEBNext Library Quant Primer Mix. Ensure mixing of NEBNext Library Quant Primer Mix by vortexing for 5 seconds. Centrifuge briefly to collect material from the sides of the tubes. Place reagents on ice.

3.1.2. Thaw the NEBNext Library Quant DNA Standards, tubes 1–4. Mix well by inverting 3–5 times. Centrifuge briefly to collect material from the sides of the tubes. Place on ice.

3.1.3. Thaw the NEBNext Library Quant Dilution Buffer (10X). Mix well by vortexing 10 seconds. Centrifuge briefly to collect material from the sides of the tube. Place on ice.

### 3.2. Preparation of NEBNext Library Quant Master Mix (with primers)

Prepare the NEBNext Library Quant Master Mix (with primers) by adding 100 µl NEBNext Library Quant Primer Mix to a tube of NEBNext Library Quant Master Mix (1.5 ml; NEB #E7630S) or 500 µl NEBNext Library Quant Primer Mix to a bottle of NEBNext Library Quant Master Mix (7.5 ml; NEB #E7630L). Mix by vortexing for 10 seconds.

Record the date on the NEBNext Library Quant Master Mix container to indicate that the NEBNext Library Quant Primer Mix has been added.

This mix can be stored at 4°C for convenience for up to six weeks. For longer term storage, the mix will be stable for seven months at –20°C and for 30 freeze/thaw cycles.

ROX: If using a real-time instrument that uses ROX normalization dye, we recommend adding ROX to the NEBNext Library Quant Master Mix after thawing. Both a low and high concentration of ROX are provided at 100X concentration (refer to Table 1 for ROX recommendations for common qPCR instruments).

For E7630S add 20 µl of ROX to the NEBNext Library Quant Master Mix tube, and for E7630L add 100 µl.

Alternatively, ROX can be added to each reaction if desired (0.2 µl per well in 96 well format and 0.1 µl per well in 384 well format).

### 3.3 Preparation of NEBNext Library Quant Dilution Buffer (1X)

Dilute the NEBNext Library Quant Dilution Buffer (10X) 1:10 with nuclease-free water. Mix by vortexing for 10 seconds. Prepare sufficient buffer for the desired number of libraries to be quantitated, allowing 1.2 ml for each library.

| | |
|---|---|
| NEBNext Library Quant Dilution Buffer | 1 ml |
| Nuclease-free Water | 9 ml |
| Total Volume | 10 ml |

### 3.4 Dilution of Library Samples

Perform an initial 1:1,000 dilution of each library sample in 1X NEBNext Library Quant Dilution Buffer in a nuclease-free tube as described below. Pipet carefully and mix thoroughly by vortexing for 10 seconds. Centrifuge briefly to collect material from the sides of the tube.

3.4.1. Add 1 µl library to 999 µl NEBNext Library Quant Dilution Buffer to create a 1:1,000 dilution.

Prepare two additional dilutions of the library sample to create 1:10,000 and 1:100,000 dilutions as described below. Mix each thoroughly by vortexing for 10 seconds. Centrifuge briefly to collect material from the sides of the tube. These library dilutions will be used for qPCR analysis.

3.4.2. Add 10 µl of the 1:1,000 dilution from Step 1 to 90 µl of 1X NEBNext Library Quant Dilution Buffer to create a 1:10,000 dilution.

3.4.3. Add 10 µl of the 1:10,000 dilution from Step 2 to 90 µl of 1X NEBNext Library Quant Dilution Buffer to create a 1:100,000 dilution.

The range of standard concentrations in the NEBNext Library Quant Kit is 10–0.01 pM. Most library prep protocols will produce libraries with concentrations of 1–200 nM, thus the 1:100,000 dilution will fall in range of the standards for nearly all libraries (e.g. 2 pM, from a 1:100,000 dilution of 200 nM, will amplify near standard 2 in the qPCR run). Libraries prepared

with very low yield will still be quantitated by the kit, with the 1:10,000 and 1:100,000 dilution recommended for libraries expected to be as low as 0.2 nM. The 1:1,000 dilution can be run for lower concentration libraries.

### 3.5 Prepare qPCR Assays

For best results, we recommend running each DNA standard and library sample in triplicate.

3.5.1. Prepare DNA standards and diluted library samples.

| | |
|---|---|
| NEBNext Library Quant Master Mix (with primers) | 16 µl |
| DNA standard or library dilution | 4 µl |
| Total Volume | 20 µl |

3.5.2. We recommend inclusion of a no template control (NTC) reaction in addition to the 4 DNA Standards. The Cq from the NTC will not be used in quantitation analysis, but serves as a valuable control reaction to ensure performance of the kit and absence of sample contamination. Prepare a no-template control as follows:

| | |
|---|---|
| NEBNext Library Quant Master Mix (with primers) | 16 µl |
| Library Dilution Buffer (1X) | 4 µl |
| Total Volume | 20 µl |

Mix reactions by pipetting sample or buffer at least 5X. Try to minimize bubbles in plate wells, but 1–2 bubbles per well on the top of the liquid will be removed by heating and not affect results.

If using a 384-well or other instrument with small reaction volumes (e.g. 10 µl) then simply reduce the above volumes accordingly. For 10 µl reactions use 8 µl of NEBNext Library Quant Master Mix (with primers) and 2 µl of NEBNext Library Quant DNA Standards, library dilution or NEBNext Library Quant Dilution Buffer:

| | |
|---|---|
| NEBNext Library Quant Master Mix (with primers) | 8 µl |
| DNA standard, library dilution or dilution buffer | 2 µl |
| Total Volume | 10 µl |

### 3.6 Load the qPCR Plate

Prepare the qPCR plate as desired, with a recommended layout shown below.

- If reactions were not prepared in the final qPCR plate, transfer them into the appropriate plate for the real-time instrument being used.
- Although most bubbles will pop during thermal cycling, it is best to avoid bubble formation.
- If desired, include a no template control (NTC) assay in the plate in triplicate.
- Include qPCR assays for the 4 prediluted NEBNext Library Quant DNA Standards 1–4 in triplicate.
- Include qPCR assays for at least 2 dilutions of each library sample (1:10,000 and 1:100,000), with 3 dilutions each if desired.

  Note: Standards and NTC require 15 wells of the plate, leaving 81 for libraries. This enables quantitation of 9 libraries with 3 dilutions each loaded on the plate, or 13 libraries with 2 dilutions.
- Seal the qPCR plate, and if desired, centrifuge at 2500–3000 rpm for 2 minutes to collect material at the bottom of wells.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | STANDARD 1 10 pM | STANDARD 1 10 pM | STANDARD 1 10 pM | LIBRARY 2 1:10,000 | LIBRARY 2 1:10,000 | LIBRARY 2 1:10,000 | LIBRARY 6 1:10,000 | LIBRARY 6 1:10,000 | LIBRARY 6 1:10,000 | LIBRARY 10 1:10,000 | LIBRARY 10 1:10,000 | LIBRARY 10 1:10,000 |
| B | STANDARD 2 1 pM | STANDARD 2 1 pM | STANDARD 2 1 pM | LIBRARY 2 1:100,000 | LIBRARY 2 1:100,000 | LIBRARY 2 1:100,000 | LIBRARY 6 1:100,000 | LIBRARY 6 1:100,000 | LIBRARY 6 1:100,000 | LIBRARY 10 1:100,000 | LIBRARY 10 1:100,000 | LIBRARY 10 1:100,000 |
| C | STANDARD 3 0.1 pM | STANDARD 3 0.1 pM | STANDARD 3 0.1 pM | LIBRARY 3 1:10,000 | LIBRARY 3 1:10,000 | LIBRARY 3 1:10,000 | LIBRARY 7 1:10,000 | LIBRARY 7 1:10,000 | LIBRARY 7 1:10,000 | LIBRARY 11 1:10,000 | LIBRARY 11 1:10,000 | LIBRARY 11 1:10,000 |
| D | STANDARD 4 0.01 pM | STANDARD 4 0.01 pM | STANDARD 4 0.01 pM | LIBRARY 3 1:100,000 | LIBRARY 3 1:100,000 | LIBRARY 3 1:100,000 | LIBRARY 7 1:100,000 | LIBRARY 7 1:100,000 | LIBRARY 7 1:100,000 | LIBRARY 11 1:100,000 | LIBRARY 11 1:100,000 | LIBRARY 11 1:100,000 |
| E | NTC | NTC | NTC | LIBRARY 4 1:10,000 | LIBRARY 4 1:10,000 | LIBRARY 4 1:10,000 | LIBRARY 8 1:10,000 | LIBRARY 8 1:10,000 | LIBRARY 8 1:10,000 | LIBRARY 12 1:10,000 | LIBRARY 12 1:10,000 | LIBRARY 12 1:10,000 |
| F | LIBRARY 1 1:10,000 | LIBRARY 1 1:10,000 | LIBRARY 1 1:10,000 | LIBRARY 4 1:100,000 | LIBRARY 4 1:100,000 | LIBRARY 4 1:100,000 | LIBRARY 8 1:100,000 | LIBRARY 8 1:100,000 | LIBRARY 8 1:100,000 | LIBRARY 12 1:100,000 | LIBRARY 12 1:100,000 | LIBRARY 12 1:100,000 |
| G | LIBRARY 1 1:100,000 | LIBRARY 1 1:100,000 | LIBRARY 1 1:100,000 | LIBRARY 5 1:10,000 | LIBRARY 5 1:10,000 | LIBRARY 5 1:10,000 | LIBRARY 9 1:10,000 | LIBRARY 9 1:10,000 | LIBRARY 9 1:10,000 | LIBRARY 13 1:10,000 | LIBRARY 13 1:10,000 | LIBRARY 13 1:10,000 |
| H | | | | LIBRARY 5 1:100,000 | LIBRARY 5 1:100,000 | LIBRARY 5 1:100,000 | LIBRARY 9 1:100,000 | LIBRARY 9 1:100,000 | LIBRARY 9 1:100,000 | LIBRARY 13 1:100,000 | LIBRARY 13 1:100,000 | LIBRARY 13 1:100,000 |

### 3.7 Run the qPCR Plate in a Real-time Thermal Cycler

3.7.1. Run the qPCR assay following the cycling conditions listed below:

| CYCLE STEP | TEMP | TIME | CYCLES |
|---|---|---|---|
| Initial Denaturation | 95°C | 1 Minute | 1 |
| Denaturation<br>Extension | 95°C<br>63°C | 15 seconds<br>45 seconds | 35 |

Ensure a plate read is included at the end of the extension step. Use the SYBR or SYBR/FAM setting on the real-time instrument. When ROX is used as normalization dye, verify that ROX is selected in the appropriate setting.

We recommend the “Fast” temperature cycling profile where applicable (e.g. Applied Biosystems StepOnePlus, 7500 Fast). The kit can also be run in “Standard” temperature mode if desired, but this will result in a longer time required for the PCR protocol to be completed.

## Section 4
## Data Analysis

The NEB qPCR webtool (https://nebiocalculator.neb.com/qPCR) can be used to assist with data analysis

### 4.1 Use the Real-time Thermal Cycler Software to Annotate the Concentration of the DNA Standards as Follows:

Table 4.1: Concentration of standards

| SAMPLE NAME | CONCENTRATION (pM) |
|---|---|
| DNA Standard 1 | 10 |
| DNA Standard 2 | 1 |
| DNA Standard 3 | 0.1 |
| DNA Standard 4 | 0.01 |

Once the appropriate wells are set as "Standard" confirm that the efficiency for the NEBNext Library Quant DNA Standards (derived from the slope of the linear fit of the DNA standard curve plotted on a log-scale x-axis) is 90–110%. The coefficient of determination ($R^2$) for the linear fit of the standards should be ≥ 0.99.

### 4.2 Determine the Concentration of the Library Samples

- Obtain the concentration (in triplicate) of each diluted library sample from the qPCR thermal cycler using the standard curve generated by DNA standards 1–4. As a guide in Table 4.2, obtain values for 1a-1c and 2a-2c.
- Calculate the average concentration of the 1:10,000 and 1:100,000 library dilution from the triplicates as indicated (1 and 2 in the table below).

  Note: If the 1:10,000 dilution falls outside of the range of the standards, i.e. has a Cq value lower than that of DNA Standard 1, do not use that dilution to calculate concentration. Use only the 1:100,000 dilution. Similarly, if the Cq of the 1:100,000 dilution is greater than that of DNA Standard 4, use only the 1:10,000 dilution.
- Adjust each concentration for size, using the average size of the library normalized by the size of the standard fragment (399 bp).
- Calculate the concentration of the undiluted library stock by multiplying by the appropriate dilution factor (10,000 or 100,000).

Table 4.2: Concentration determination of library samples

| LIBRARY SAMPLE | CONC. (pM) FROM THERMAL CYCLER FOR EACH REPLICATE | AVE. CONC. (pM) | SIZE ADJUSTED CONC. (pM) | CONC. OF UNDILUTED LIBRARY (pM) |
|---|---|---|---|---|
| Library Dilution 1 1:10,000 | 1a | 1 | 1 x 399/(Ave. library size) | $1_{adj}$ x 10,000 |
| Library Dilution 2 1:1,000,000 | 2a | 2 | 2 x 399/(Ave. library size) | $2_{adj}$ x 100,000 |

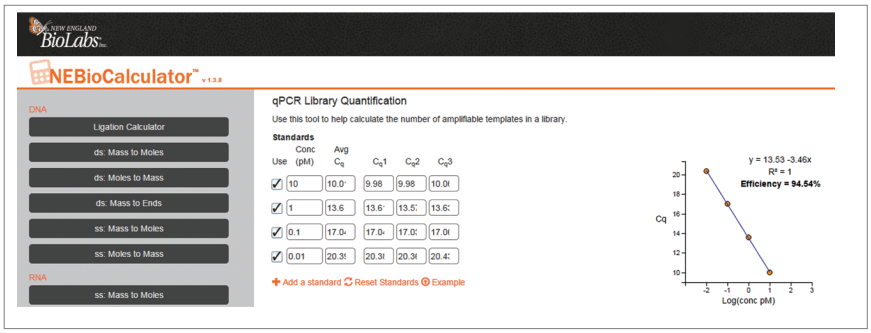
### 4.3 Recommendations

- If one of the triplicates of a diluted library sample (e.g. 1a, 1b, or 1c) is an outlier (> 0.5 Cq from the other replicates), then the data from this outlier should be excluded from the data analysis.
- For accurate quantitation, it is essential to have at least one library dilution that falls within the range of the DNA Standards 1–4. If all qPCR traces for the diluted library sample fall outside the range of the standards, then the qPCR assay should be repeated with greater (if faster than standard 1) or lower (if slower than standard 4) dilution.
- The traces for the NTC may show unexpected amplification due to contamination of library targets, primer-dimers, or amplified products if the qPCR assays have been performed repeatedly. As long as the Cq value for the NTC is far enough away from the dynamic range of the DNA Standards (e.g. Cq of NTC should be > 30), the NTC amplification can be ignored as it will not have any effect on library quantitation. If the NTC shows faster amplification, new NEBNext Library Quant Dilution Buffer should be prepared for the next runs.

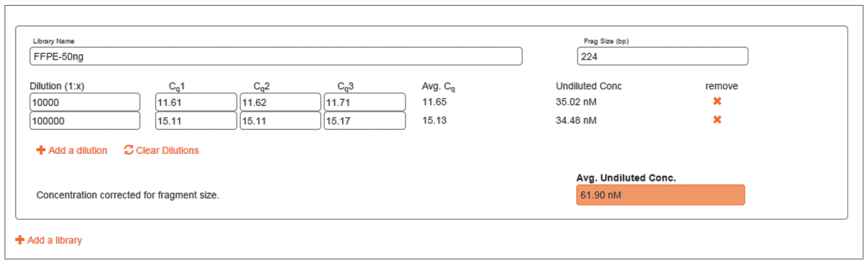
### 4.4 NEBioCalculator

For efficient and convenient analysis of qPCR data from the NEBNext Library Quant Kit, we recommend using the NEBNext Library Quant qPCR tool available from the NEBioCalculator webtools menu (at https://nebiocalculator.neb.com/qPCR). To use the tool, simply open a web browser to the webtool page and have available the Cq's from the qPCR experiment.

- First enter the Cq values for the 4 standards into the standards boxes at the top of the page. The Cq average for each standard is then plotted against the known concentrations to calculate the PCR Efficiency and standard curve for library concentration determination. An example dataset is shown below:




- For each quantitated library, enter the size, dilution factor, and Cq for each well containing the library. Multiple dilutions for the same library are averaged together for highest quantitation accuracy. Size correction is calculated automatically.




- For each additional library, click the “Add a library” tab and enter the appropriate information and Cq data.

# Section 5
# Expected Results

## 5.1 Typical results from a NEBNext Quant Kit Assay

Figure 1 shows typical qPCR traces generated with DNA Standards 1–4, a no template control, and a diluted Illumina library using either a Bio-Rad CFX96 Touch™ (top) or Applied Biosystems 7500 Fast with ROX normalization (bottom). The DNA library was diluted 1:100,000 as described in the protocol above. In these examples, the qPCR trace generated by the diluted library fell within the range of the qPCR traces from the DNA standards. The NTC was well separated from the last DNA standard (Standard 4) and the efficiency was calculated to be 94.4% (top) or 95.9% (bottom). The concentration of the example library was determined using the measured standard curves (right), adjusted by a size normalization factor (standard size divided by library size as measured by BioAnalyzer; 399/310 base pairs), and multiplied by the dilution factor (100,000) to give an undiluted library stock concentration of 69.8 nM (top) or 69.6 nM (bottom). For both instruments and software, default thresholds and calculation methods were used.

Figure 1a: Bio-Rad CFX96 Touch; Linear amplification plot, No ROX normalization

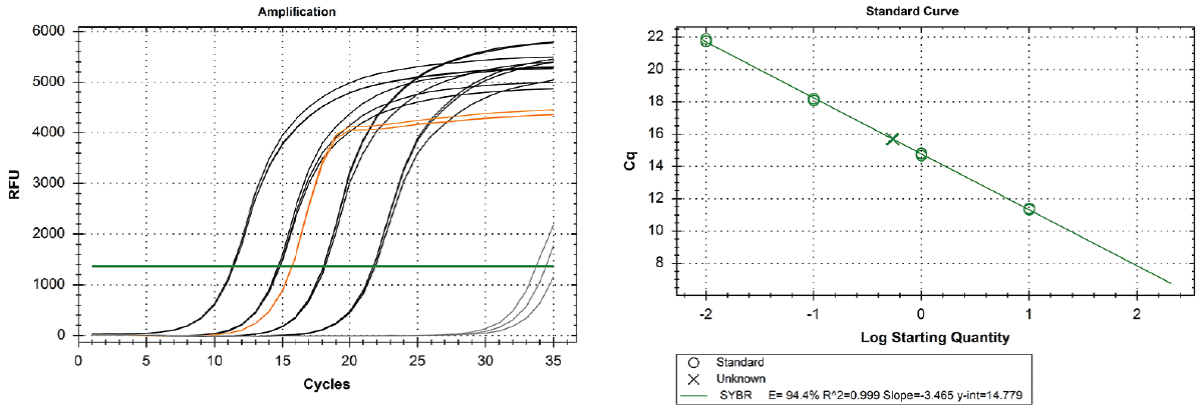
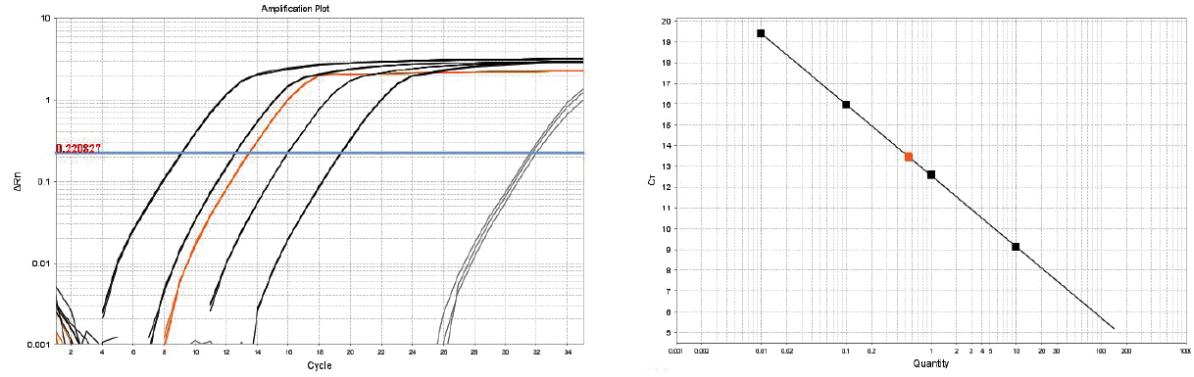



Figure 1b: Applied Biosystems 7500 Fast; Log amplification plot, ROX



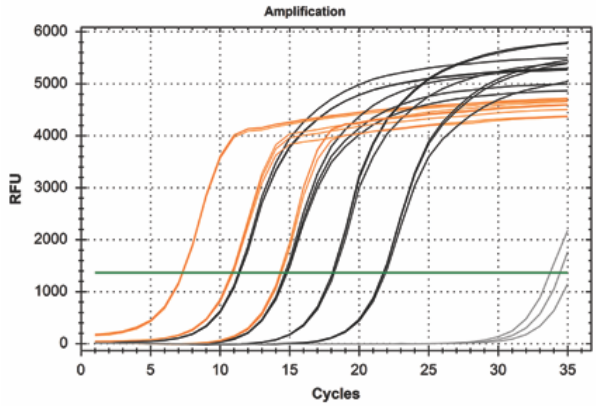

*Typical results from the NEBNext Library Quant Kit on a Bio-Rad CFX96 Touch (Figure 1a) and an Applied Biosystems 7500 Fast real-time qPCR instrument (Figure 1b). Amplification curves are shown on the left and resulting standard curves on the right. All default settings were used on both platforms. ABI 7500 Fast assay included 1X ROX (Low Concentration) and the ROX normalization.*

## Section 6
## Troubleshooting Guide

| OBSERVATION | POSSIBLE CAUSES | SUGGESTED SOLUTIONS |
|---|---|---|
| qPCR traces full outside the range of the NEBNext Library Quant DNA Standards 1–4 (Figure 2) | Library dilutions are higher concentration than the range of DNA standards (10–0.01 pM) | Omit data for qPCR traces that fall outside the dynamic range of the DNA standards<br>If all qPCR traces fall outside the range of DNA standards, use a different dilution scheme |
| All qPCR traces show low or no amplification | Reagent omitted from qPCR assay<br>Reagent added improperly to qPCR | Verify all steps of the protocol were followed correctly |
| | Incorrect cycling protocol | Refer to the proper qPCR cycling protocol in this user manual |
| | Incorrect reporter dye selected for the qPCR thermal cycler | Select FAM/SYBR on the qPCR instrument |
| | Reagents are contaminated or degraded | Confirm the expiration dates of the kit reagents<br>Verify proper storage conditions provided in this user manual<br>Rerun the qPCR assay with fresh reagents |
| qPCR trace for triplicate data has different shape than the others (Problematic trace should be excluded from analysis) | Improper pipetting during qPCR assay set-up | Ensure proper pipetting techniques |
| | qPCR plate film has lost its seal, causing evaporation in the well. The resulting qPCR trace may show significantly different fluorescence values relative to its replicates | Ensure the qPCR plate is properly sealed before inserting into the qPCR thermal cycler. Exclude problematic trace(s) from data analysis |
| | Poor mixing of reagents during qPCR set-up | Make sure all reagents are properly mixed after thawing them |
| | Bubbles cause an abnormal qPCR trace | Avoid bubbles in the qPCR plate<br>Centrifuge the qPCR plate prior to running it in the thermal cycler<br>Exclude problematic trace(s) from data analysis |
| qPCR traces for the DNA standards are as expected but those for the library samples show little or no amplification | Library samples are incompatible with this kit | Verify that library samples contain Illumina adaptors having P5 and P7 sequences |
| | Library samples diluted incorrectly | Redilute the library samples |

| OBSERVATION | POSSIBLE CAUSES | SUGGESTED SOLUTIONS |
|---|---|---|
| DNA standard curve has a poor correlation coefficient/efficiency of the DNA standard curve falls outside the 90–110% range | Presence of outlying qPCR traces | Omit data produced by qPCR traces that are clearly outlets<br>Omit data from traces that fall outside the dynamic range of the DNA standards (Figure 2) |
| | Improper pipetting during qPCR assay set-up | Ensure that proper pipetting techniques are used |
| | Reaction conditions are incorrect | Verify that all steps of the protocol were followed |
| | Bubbles cause an abnormal qPCR trace | Avoid bubbles in the qPCR plate<br>Centrifuge the qPCR plate prior to running it in the thermal cycler |
| | Poor mixing of reagents | After thawing, make sure all reagents are properly mixed |
| | Threshold is improperly set for the qPCR traces | Ensure the threshold is set in the exponential region of qPCR traces<br>Refer to the user manual of the qPCR thermal cycler to manually set the threshold |
| Melt curve shows single, sharp peak at 81.5°C | Library contains significant amount of adaptor dimers | Cleanup or size select the library to remove adaptor dimers<br>If desired, library quantitation can be repeated with an extension temperature of 68°C and melt curve evaluated. If library contains >5% adaptor dimers, the melt curve peak at 81.5°C will decrease. |
| No template control qPCR trace is within 4 cycles of the last DNA standard | NEBNext Library Quant Master Mix/NEBNext Library Quant Primer Mix has been stored improperly | Verify proper storage conditions provided in this manual |
| | NEBNext Library Quant Dilution Buffer or other component is contaminated with amplified library | Discard previously used 1X NEBNext Library Quant Dilution Buffer, re-make with new aliquot of nuclease free-water, and repeat. If NTC is still with 4 Cq of standard 4, a new NEBNext Library Quant Kit should be used. |

Figure 2



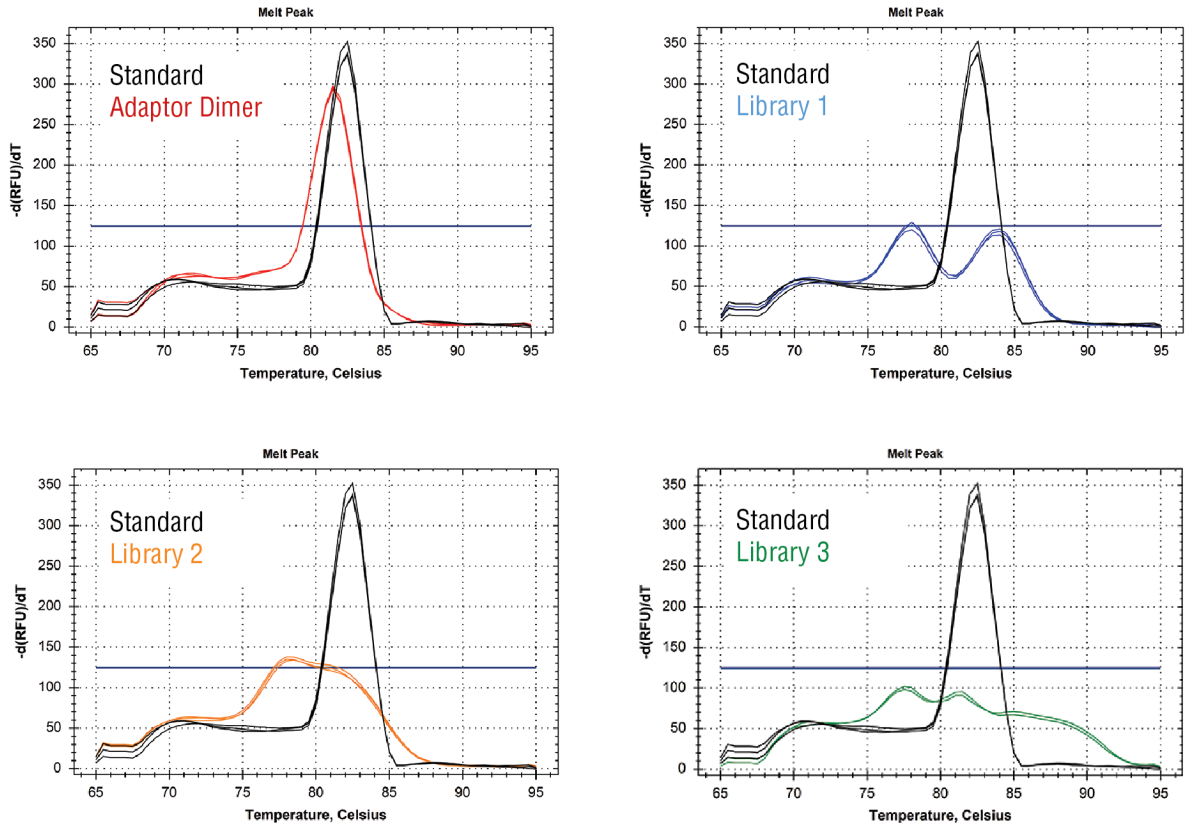

*Results from a high-concentration DNA library where the 1:1,000 and 1:10,000 dilutions fell outside the range of DNA standards (black). Though the calculated concentrations for the 1:10,000 and 1:100,000 dilutions (resulting undiluted stock concentrations of 157.2 nM and 157.0 nM, respectively) were very similar, we recommending using dilutions that fall within the range of the DNA standards. Here, and for other libraries at high concentration, the 1:100,000 dilution should be used.*

### 6.1 Melt Curve Analysis

For typical qPCR experiments with intercalating dyes, melt curves are a useful metric to analyze product specificity and quality. However, we do not require including a melt curve in the NEBNext Library Quant Kit Protocol. Amplification in the kit will represent the diversity of the library, resulting in melt curves with broad or multiple peaks (see examples below). The DNA Standard produces a single, sharp peak due to amplification of a single 399 bp species, but amplified libraries will show a wide range of melt behaviors. These products are the results of 35 cycles of PCR amplification and may not accurately reflect the nature of the library.

One benefit of the melt curve is identification of a significant amount of adaptor dimers in the prepared library. If adaptor dimers are suspected, a melt curve can be included after the PCR cycles. A characteristic melt peak will be seen if adaptor dimers are present at > 5% of the total library concentration, with a single, sharp peak centered at 81.5°C in the negative derivative melt view. An example of adaptor dimer melt curve is shown below. Multiple or broad melt peaks are very common with libraries, and should not be a cause for concerns about library quality (examples shown in Figure 3; despite multiple and broad peaks, all libraries passed all quality metrics for cluster density and sequence quality). Only if a single, sharp 81.5°C peak is observed should you consider cleanup of adaptor dimers. If desired, libraries displaying an adaptor dimer melt peak can be further characterized with the NEBNext Library Quant Kit. qPCR can be repeated using only the libraries of interest, but with the extension temperature increased to 68°C.

Figure 2




*Melt curves showing the signature sharp 81.5°C peak indicative of adaptor dimers (red, top) and examples of diverse melt curves obtained from high quality Illumina libraries (bottom). Broad and multiple peaks are commonly observed with the NEBNext Library Quant Kit, as the kit maintains library diversity during amplification. All libraries shown were sequenced and passed stringent quality score metrics for both cluster density and sequence quality.*

## Kit Components

NEB #E7630S Table of Components

| NEB # | PRODUCT | VOLUME |
|---|---|---|
| E7640A | NEBNext Library Quant Master Mix | 1.5 ml |
| E7639A | NEBNext Library Quant Primer Mix | 0.1 ml |
| E7633A | NEBNext Library Dilution Buffer (10X) | 2 x 1.0 ml |
| E7634A | NEBNext Library Quant DNA Standard 1 (10 pM) | 0.024 ml |
| E7635A | NEBNext Library Quant DNA Standard 2 (1 pM) | 0.024 ml |
| E7636A | NEBNext Library Quant DNA Standard 3 (0.1 pM) | 0.024 ml |
| E7637A | NEBNext Library Quant DNA Standard 4 (0.01 pM) | 0.024 ml |
| E7638A | ROX (Low) | 0.02 ml |
| E7641A | ROX (High) | 0.02 ml |

NEB #E7630L Table of Components

| NEB # | PRODUCT | VOLUME |
|---|---|---|
| E7640AA | NEBNext Library Quant Master Mix | 7.5 ml |
| E7639AA | NEBNext Library Quant Primer Mix | 0.5 ml |
| E7633AA | NEBNext Library Dilution Buffer (10X) | 2 x 7.5 ml |
| E7634AA | NEBNext Library Quant DNA Standard 1 (10 pM) | 0.12 ml |
| E7635AA | NEBNext Library Quant DNA Standard 2 (1 pM) | 0.12 ml |
| E7636AA | NEBNext Library Quant DNA Standard 3 (0.1 pM) | 0.12 ml |
| E7637AA | NEBNext Library Quant DNA Standard 4 (0.01 pM) | 0.12 ml |
| E7638AA | ROX (Low) | 0.1 ml |
| E7641AA | ROX (High) | 0.1 ml |

## Revision History

| REVISION # | DESCRIPTION | DATE |
|---|---|---|
| 1.0 | N/A | |
| 1.1 | In the section for experimental considerations the statement "If 1-2 small bubbles are present in plate wells" is changed to "If 1-2 small bubbles are present at the top of the liquid".<br>In the section for NEBNext Library Quant Kit Protocol; Preparation of NEBNext Library Quant Master Mix (with primers) the statement "500 µl NEBNext Library Quant Master Mix to a bottle of" is changed to "500 µl NEBNext Library Primer Master Mix to a bottle of". Also in this same section, moved the text about "Adding ROX" from page 16 to page 6, Section 3.2, “Preparation of NEBNext Library Quant Master Mix (with primers)” and added volumes of ROX (if added) per well.<br>In the section NEBNext Library Quant Kit Protocol; Prepare qPCR Assays the statement "1-2 bubbles per well" is changed to "1-2 bubbles at the top of the liquid". | |
| 1.2 | Update workflow figure. Total time is 1 hour 50 minutes. | 2/18 |
| 2.0 | Convert to new template format. | 7/19 |
| 3.0 | Update legal text | 3/20 |