Takara Bio USA, Inc.

Library Quantification Kit User Manual

Cat. Nos. 638324, 638325
(050919)

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I. Introduction

A. Summary

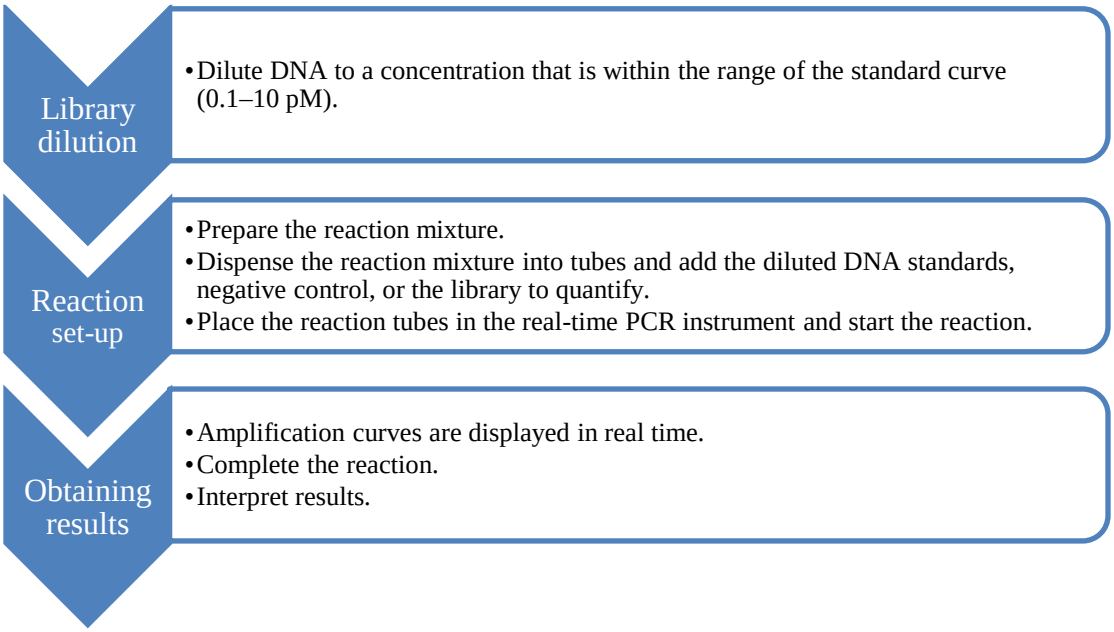
Illumina® next generation sequencing (NGS) platforms (including MiSeq®, Genome Analyzer™ IIx, HiScan®SQ, HiSeq® 2000/1000, and HiSeq 2500/1500) sequence DNA molecules by massively parallel sequencing. Generally, the libraries that are required for NGS analyses are prepared by ligating specific adapters to the ends of prepared DNA fragments. For sequencing, the prepared library is used as a template with primers that recognize the adapters, and then DNA clusters are clonally generated by bridge amplification on a flow cell. The clusters are sequenced using fluorescently labeled nucleotides; the DNA sequence is determined by detection of the fluorescent signal.

Loading the flow cell with an appropriate amount of library DNA is essential to generate clusters at an optimal density. An insufficient amount of library DNA will result in low cluster density and reduced sequencing yield. In contrast, an overabundance of library DNA may increase cluster density and result in poor quality data. Thus, accurate quantification of the library DNA concentration prior to loading on the flow cell is critical to a successful sequencing run.

The Library Quantification Kit uses standards and primers that recognize the adapter sequences used for Illumina's NGS reactions. Sequencing library quantification with this kit is accomplished by qPCR using TB Green® for detection. Amplification products are detected by intercalation of TB Green I in double-stranded DNA. Fluorescence detection in real-time allows quantification of amplification products. This kit includes DNA standards of known concentration to allow absolute quantification of library DNA.

This PCR-based library quantification method enables the specific quantification of DNA molecules that are bound to adapters, the target of sequencing primers. This allows specific measurement of the sequencing library concentration even in samples containing DNA molecules *not* bound to adapters. In addition, this kit can be used to confirm adapter ligation during library preparation.

B. Protocol Overview



NOTE: When first using this kit, simultaneously perform quantification of a library that has been previously sequenced. The optimal loading concentration will vary depending on the instrument, reagents, and version of the analytical software. The results from the previously-sequenced library can serve as reference data for determining the optimal loading concentration.

II. List of Components

- **200 rxns Terra™ qPCR Direct TB Green Premix (Cat. No. 638319)**

  NOTE: Protect the contents from light.

  - 5 x 1 ml 2X Terra qPCR Direct TB Green Premix
  - 200 µl ROX Reference Dye LSR (50X)
  - 200 µl ROX Reference Dye LMP (50X)
- **500 rxns Primer Mix Kit (Cat No. 638326; Not sold separately)**
  - 2 x 1 ml 5X Primer Mix
  - 3 x 1 ml EASY Dilution Buffer
  - 3 x 1 ml RNase-Free Water
- **50 rxns DNA Standards for Library Quantification (Cat. No. 638325)**
  - 2 x 50 µl DNA Standard (10 pM)
  - 2 x 50 µl DNA Standard (1 pM)
  - 2 x 50 µl DNA Standard (0.1 pM)
  - 2 x 50 µl DNA Standard (0.01 pM)

NOTE: ROX Reference Dyes are included for analyses with devices that perform between-well correction of fluorescent signal, e.g. Applied Biosystems real-time PCR instruments.

- Use ROX Reference Dye LSR for the ABI PRISM or StepOnePlus systems.
- Use ROX Reference Dye LMP for the 7500 Real-Time or Fast Real-Time PCR Systems.

The Terra qPCR Direct TB Green Premix contains enzymes, TB Green I, buffer, and dNTPs; the Primer Mix Kit contains primers for qPCR and EASY Dilution Buffer for library DNA dilution. The DNA Standard solutions contain specific concentrations of a DNA fragment (538 bp) and are designed specifically for use with this kit. When used as templates for qPCR, the standards will generate 447-bp DNA fragments that can be used to produce a standard curve for determining the concentration of DNA in an Illumina sequencing library.

The components of the Library Quantification Kit (Cat. No. 638324) have been specifically designed to work together and are optimized for this particular protocol. Please do not make any substitutions. The substitution of reagents in the kit and/or modification of the protocol may lead to unexpected results.

Storage Conditions:

- Store the Terra qPCR Direct TB Green Premix and ROX Reference Dyes at –80°C. After thawing, store the Terra qPCR Direct TB Green Premix kit protected from light at 4°C, and use within 6 months.
- Store all other components at –20°C.

III. Additional Materials Required

The following materials are required but not supplied:

- Real-time PCR instrument and tubes for real-time PCR
  - Thermal Cycler Dice™ Real Time System (software Ver. 3.00 or later; Cat. Nos. TP800, TP900)*
  - Applied Biosystems 7000, 7500, or 7500 Fast Real-Time PCR System (Life Technologies)
  - Applied Biosystems StepOnePlus Real-Time PCR System (Life Technologies)
  - LightCycler (Roche)
- 0.2 ml, 8-strip, individual flat cap tubes
- 1000-µl, 200-µl, 20-µl, and 10-µl micropipettes
- Micropipette tips (with hydrophobic filters)
- Tabletop centrifuge
- High-speed, 4ºC microcentrifuge

*Not available in all geographic locations. Check for availability in your region.

IV. General Considerations

1. When operating real-time PCR amplification systems, be sure to follow the instructions for each instrument.
2. If chimeric probes or primers are degraded due to contamination with nuclease, accurate detection will not be possible. Perspiration and saliva are potential sources of nuclease contamination; take appropriate precautions to avoid contamination.
3. The steps in the protocol should be performed in the three physically segregated areas described below.
   - Area 1: Dilute libraries and DNA standards.
   - Area 2: Prepare and dispense reaction mixtures.
   - Area 3: Add library dilutions and DNA standards to the reaction mixture.

   Avoid opening or closing tubes containing amplification products in any of the three areas.
4. With this kit, the amplification reaction and product detection take place simultaneously in real time; thus, there is no need to analyze the amplification products by electrophoresis or another procedure after reaction completion. To avoid contamination, do not remove amplification products from the tubes.

5. Failure of any of the auto functions on the real-time PCR amplification system may lead to erroneous results. Set up the real-time PCR amplification system according to the system's operation manual.
6. The primers included in this kit are designed to anneal to the P5 and P7 regions at the ends of Illumina sequencing library fragments, sequences that are required for hybridization with the flow cell (Figure 1). Libraries not recognized by the primers cannot be quantified with this kit. The primers have the following sequences:

   Primer 1: 5' – TGA TAC GGC GAC CAC CGA GA – 3'

   Primer 2: 5' – AAG CAG AAG ACG GCA TAC GA – 3'

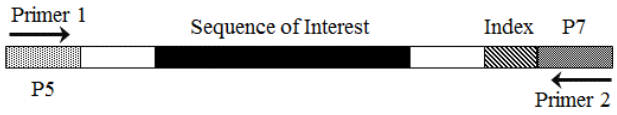


Figure 1. Structure of a typical fragment from an Illumina sequencing library.

V. Library Quantification

A. Protocol: Preparation of DNA Standards

(Perform in Area 1)

Thaw the DNA Standards (0.01–10 pM) at room temperature, mix well by vortexing or other means, then centrifuge briefly and keep on ice.

B. Protocol: Library Dilution

(Perform in Area 1)

1. Dilute an aliquot of the library to be analyzed to a concentration of approximately 1 pM with EASY Dilution. To achieve an accurate final concentration, preparation of a serial dilution series is recommended.

   Optional: If the initial DNA concentration of your library is not available, use serial dilutions of the library (e.g., 10-, 100-, 1000-fold).
2. If the molar concentration of a library is unknown, use the following equations to calculate the concentration (w/v).

   Determine the molecular weight of the library:

   Molecular Weight = Average Library Size (bp)* × 650

   Determine the molar concentration of the library:

   Molar Concentration (nM) = Library Concentration (ng/µl) × 10^6/Molecular Weight

   *Average library size as determined by an Agilent Bioanalyzer or equivalent method.

C. Protocol: Reaction Mixture Preparation

(Perform in Area 2)

1. Mix components of the reaction mixture (except the diluted library sample or DNA Standards) as indicated in Table 1 in a volume sufficient for the number of PCR reactions you plan to perform, plus an additional reaction to compensate for pipetting errors. The minimum number of reactions is the number of samples plus five (DNA Standards and the negative control). Increasing the number of trial runs can increase the accuracy of quantification, therefore, perform all reactions in at least triplicates.

Table 1. Reaction Master Mixes for Different Real-Time PCR Instruments

Reagent[a]	Roche LightCycler	Takara Thermal Cycler Dice Real Time System	ABI PRISM 7000, ABI 7500/7500, and StepOnePlus Real-Time PCR Systems	
	Reagent volume (µl)			
RNase-Free Water	4.0	4.0	3.6	12.0
Terra PCR Direct TB Green Premix (2X)	10.0	10.0	10.0	25.0
5X Primer Mix	4.0	4.0	4.0	10.0
ROX Reference Dye LSR or LMP (50X)[b]	-	-	0.4	1.0
Master mix volume per well	18.0	18.0	18.0[c]	48.0[d]

a) This kit is sufficient for 500 x 20 µl (total volume) reactions.

b) Two different ROX formulations are included for normalization of fluorescent signals on instruments that are equipped with this option. Use ROX Reference Dye LSR with instruments with a 488 nm laser excitation source. Use ROX Reference Dye LMP with instruments whose excitation source is either a lamp or an LED. Be sure to use the formulation that is appropriate for your real-time PCR instrument.

c) Prepare an 18-µl reaction master mix when using 384-well plates or 96-well fast thermal cycling plates.

d) Prepare a 48-µl reaction master mix when using 96-well plates, single tubes, or 8-tube strips.

2. Dispense the appropriate amount of master mix (Table 1) into each well of a PCR plate, each tube, or each PCR capillary tube.
3. **(Perform in Area 3)**
   Add 2 µl of RNase-Free Water (for the negative control; NTC), diluted library samples, or DNA standards into appropriate wells.
4. Seal the plate or tubes according to the procedure recommended for the real-time instrument being used. Centrifuge briefly.

NOTE: Start the reaction within 1 hour of preparation.

D. Protocol: Quantification by Real-Time PCR

(Perform in Area 3)

Program your thermal cycler using the cycling conditions recommended in Table 2. Place the plate/tubes in the real-time PCR instrument and begin cycling.

NOTE: The operating procedures vary with each real-time PCR amplification system. For detailed operating procedures, refer to the user manual for your system.

Table 2. Recommended Cycling Conditions

Reaction Cycles	Roche LightCycler[b]	Takara Thermal Cycler Dice Real Time System	ABI PRISM 7000, 7500/7500 Fast, & StepOnePlus Real-Time PCR Systems
	Thermal cycling conditions for each instrument		
Initial Denaturation[a] (1 Cycle)	98°C 2 min	98°C 2 min	98°C 2 min
qPCR (35 Cycles)	98°C 10 sec 60°C 15 sec 68°C 45 sec	98°C 10 sec 60°C 15 sec 68°C 45 sec	98°C 10 sec 60°C 15 sec 68°C 45 sec
Melting/Dissociation Curve (1 Cycle)	Melting/Dissociation Curve[c]	Melting/Dissociation Curve[c]	Melting/Dissociation Curve[c]

a) Initial denaturation at 98°C for 2 min is necessary to denature the hot start antibody.

b) Ramp speed 20°C/sec.

c) Follow the manufacturer's instructions.

E. Protocol: Data Analysis

1. After the reaction is complete, verify the amplification and melting curves, and confirm that all dissociation curves obtained from the DNA Standards have a single peak at approximately the same temperature.
2. Plot the average C_t (SDM) values for the four DNA Standards versus concentration (10, 1, 0.1, and 0.01 pM, in log scale) to generate a standard curve. Typically, the coefficient of determination (R^2) should be ≥0.98, and the reaction efficiency should be ≥90% (Figure 2).

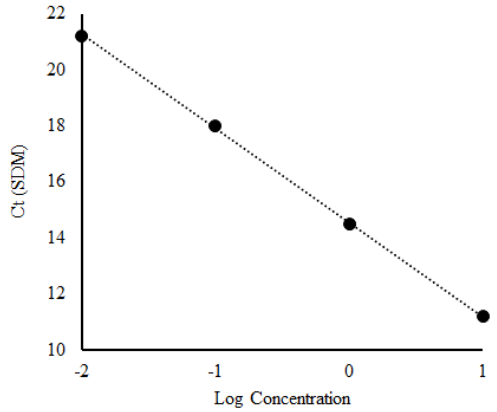


Figure 2. Typical standard curve.

3. Using the average C_t (SDM) values obtained from the diluted library samples, determine the corresponding concentration (pM) from the standard curve generated in Step 2. Only use C_t values that are below the C_t of the NTC.

4. Calculate the library concentration according to the formula below:

$$\text{Library Concentration (nM)} = \frac{[\text{Library concentration (pM) from Step 3}] \times [\text{Dilution factor}] \times [447\ \text{bp}^*]}{[1{,}000] \times [\text{Average Library Size (bp)}]}$$

*447 bp is the size of the DNA fragment amplified from the DNA Standards

Appendix A: Sequencing Recommendations

A. Determining the Loading Concentration for Sequencing

- The loading concentration of the library varies depending on the sequencing platform, reagents, and the version of analytical software used. Quantify a library that has been sequenced previously, and use the result as a reference to determine the optimal loading concentration.
- Library Quantification Kit is compatible with DNA libraries of varying GC-content and fragment length (the largest DNA fragment tested with the Library Quantification Kit was 773 bp). If a similar library that has been sequenced is available, include it as a control in the quantification to further improve the accuracy in determining the loading concentration.
- For additional recommendations, contact Illumina, Inc.

B. Multiplexed Sequencing

- When pooling libraries, any slight differences in pipetting can result in variations in the number of sequencing reads. To pool libraries that differ greatly in concentration, dilute the higher concentration library to a concentration comparable to the more dilute library before pooling.

Appendix B: Experimental Examples

A. Sequencing of Libraries with Different GC Contents

Genomic DNA libraries from *Escherichia coli* (GC content, 50.8%), *Thermus thermophilus* (GC content, 69.5%), and *Saccharomyces cerevisiae* (GC content, 38.1%) were prepared with the TruSeq® DNA Sample Prep Kit v2 (Illumina, Cat. No. FC-121-2001). The libraries were quantified using the Library Quantification Kit, pooled to yield a loading concentration of approximately 3.5 pM per library, and sequenced in a multiplexed sequencing run (Figure 3).

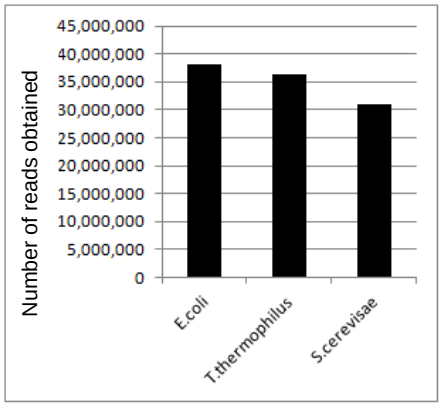


Figure 3. Number of reads obtained from multiplex sequencing of libraries from samples with varying GC contents.

B. PCR-Free Library Sequencing

Libraries prepared with the TruSeq DNA Sample Prep Kit v2 (Illumina, Cat. No. FC-121-2001) were used as standard libraries (Figure 4, Standards A through F). A PCR-free library was prepared according to Kozarewa *et al*. PCR-free library preparations create libraries with less bias; however, without library enrichment by PCR, assessment of the concentration of such a library requires qPCR. The PCR-free library was quantified using the Library Quantification Kit, pooled with the standard libraries to yield a loading concentration of 0.9 pM per library, and sequenced in a multiplexed sequencing run (Figure 4).

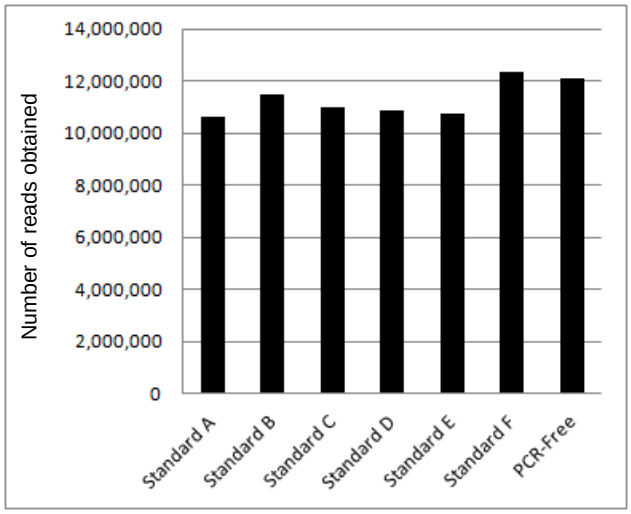


Figure 4. Number of reads obtained from multiplex sequencing of a PCR-free library.

Reference

Kozarewa, I., Ning, Z., Quail, M.A., Sanders, M.J., Berriman, M., & Turner, D.J. (2009) Amplification-free Illumina sequencing-library preparation facilitates improved mapping and assembly of GC-biased genomes. *Nature Methods* **6**(4):291–295.

Appendix C: Troubleshooting Guide

Table 3. Troubleshooting Guide

Problem	Possible Explanation	Solution
Amplification in the no template control (NTC) (FAM/SYBR filter).	Contamination may have occurred.	Decontaminate the instruments and laboratory area used to prepare reaction mixtures, then repeat the reaction.
No amplification in the DNA standard reactions.	PCR failed to work properly.	It is possible that the primers or DNA standards are degraded. Repeat the reaction.
No amplification in the diluted library sample reactions.	PCR failed to work properly.	1. The sample may have contained a reaction inhibitor. Dilute the sample and perform the reaction again. Alternatively, prepare the sample again, and perform the reaction on the newly prepared sample. 2. It is possible that the primers supplied with this kit do not recognize the adapters of the prepared library. For details, please see IV. General Considerations.
The R^2 value of the standard curve is <0.98.	The DNA standards may have been added to the reaction mixture improperly.	Repeat the reaction.
The amplification efficiency (Eff) of the standard curve was <90%.	The reaction solution (2X Terra qPCR Direct TB Green Premix) may be degraded, or the reaction may have contained a reaction inhibitor.	Repeat the reaction with a new tube of reaction solution.
The concentration of the diluted library is outside of the range of the standard curve (0.01–10 pM).		Increase or decrease library dilution as needed and repeat the quantification.

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