PerfeCTa® NGS Library Quantification Kit for Ion Torrent™ Sequencing Platforms

Cat. Nos.			Size:		**Store at -20°C protected from light**
	95151-500	PerfeCTa NGS Quantification Kit – Ion Torrent		500 x 20-µL reactions	
	95152-500	PerfeCTa NGS Quantification Kit – Ion Torrent / ROX		500 x 20-µL reactions	
	95153-500	PerfeCTa NGS Quantification Kit – Ion Torrent / Low ROX		500 x 20-µL reactions	

Description

Accurate quantification of the number of amplifiable library molecules is one of the most important factors for obtaining high quality read data with next-generation sequencing technologies. The PerfeCTa NGS Library Quantification Kit uses real-time PCR to specifically quantify library molecules that possess the appropriate adapter tag at each end. These are the only suitable template molecules for the emulsion PCR (emPCR) step. The included stabilized, pre-diluted DNA standards and pre-qualified primer set simplifies the library quantification process, minimizes pipetting errors, and ensures reproducible and precise qPCR results, even with dilute samples. The robust qPCR performance of PerfeCTa SYBR® Green SuperMix provides accurate quantification of NGS libraries with varying fragment sizes or GC content.

Instrument Compatibility

Different real-time PCR systems employ different strategies for the normalization of fluorescent signals and correction of well-to-well optical variations. It is critical to match the appropriate qPCR reagent to your specific instrument. Please consult the following table, or visit our web site at www.quantabio.com to find the optimal kit for your instrument platform.

NGS Quant. Cat. No.	qPCR Reagent	Compatible Real-Time PCR Systems
95151-500	PerfeCTa SYBR Green SuperMix	Bio-Rad CFX96™, CFX384™, Opticon™, MiniOpticon™, Chromo4™, iQ™5*, MyiQ™*, iCycler iQ®* (*requires persistent well factors) Cepheid Smart Cycler®; Qiagen/Corbett Rotor-Gene® Ion Torrent® Eco™; Eppendorf Mastercycler® ep realplex Roche Applied Science LightCycler® 480, 96
95152-500	PerfeCTa SYBR Green SuperMix, ROX	Applied Biosystems 7000, 7300, 7700, 7900, 7900HT, 7900HT Fast, StepOne™, StepOnePlus™
95153-500	PerfeCTa SYBR Green SuperMix, Low ROX	Applied Biosystems 7500, 7500 Fast, ViiA™ 7, QuantStudio™ Agilent/Stratagene MX4000™, MX3005P™, MX3000P™

Components

Name	Description	Part Number	Volume
Library Dilution Buffer (10x)	10X concentrated buffer containing 0.1M Tris-HCl (pH 8.0), 1 mM EDTA, and stabilizers	84287	2 x 3 mL
Ion Torrent Primer Mix	Mix of 10 µM (each) of primers that target the Ion Torrent "A" and "trP1" adaptor sequences in 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA. Ion Torrent forward primer: 5'-CCA TCT CAT CCC TGC GTG TC -3' Ion Torrent reverse primer: 5'- CCT CTC TAT GGG CAG TCG GTG AT-3'	84286	0.3 mL
Ion Torrent Standard 1	1 x 10^3 copies/µL linear, dsDNA standard in 1X library dilution buffer	84281	0.1 mL
Ion Torrent Standard 2	1 x 10^4 copies/µL linear, dsDNA standard in 1X library dilution buffer	84282	0.1 mL
Ion Torrent Standard 3	1 x 10^5 copies/µL linear, dsDNA standard in 1X library dilution buffer	84283	0.1 mL
Ion Torrent Standard 4	1 x 10^6 copies/µL linear, dsDNA standard in 1X library dilution buffer	84284	0.1 mL
Ion Torrent Standard 5	1 x 10^7 copies/µL linear, dsDNA standard in 1X library dilution buffer	84285	0.1 mL
qPCR Reagent	2X concentrated master mix for SYBR Green qPCR		
95151-500	PerfeCTa SYBR Green SuperMix	2 x 95054-100	4 x 1.25 mL
95152-500	PerfeCTa SYBR Green SuperMix ROX	2 x 95055-100	4 x 1.25 mL
95153-500	PerfeCTa SYBR Green SuperMix Low ROX	2 x 95056-100	4 x 1.25 mL

Storage and Stability

Kit components are stable for 2 years when stored in a constant temperature freezer at -20°C and protected from light*. For convenience, they may be stored unfrozen at +2 to +8°C for up to 6 months. Repeated freezing and thawing will not affect product performance.

*Prolonged exposure of PerfeCTa SYBR Green SuperMix to direct light will photo-bleach the SYBR Green I dye and destroy qPCR product detection.

NGS Library Sample Dilutions

Prepare 1X library dilution buffer with nuclease-free, molecular biology grade water. Do not use DEPC-treated water. Do not use autoclaved water. Properly maintained ultra-filtration systems generally deliver water of suitable quality.

Add 0.1 mL of 10X Library Dilution Buffer to 0.9 mL of nuclease-free water. Mix by vortexing. Larger or smaller quantities of 1X dilution buffer may be prepared depending on your experimental requirements. Each library sample requires approximately 0.3 mL. The 1X dilution buffer is stable at 2-8ºC for 6 months.

The optimal dilutions for qPCR may vary depending on the nature of your NGS sequencing operations. Use the following example as a general guideline. It is important for one of your library dilutions to fall within the linear dynamic range of the supplied DNA standards.

1. Prepare a 1:20 dilution of the library sample:

   Add 2 µL of library sample to a 1.5-mL tube containing 38 µL of 1X dilution buffer

   Mix by vortexing, and then centrifuge to collect contents

2. Prepare 2 successive dilutions for qPCR as follows:

   1:2000 diln. Transfer 2 µL of the 1:20 dilution to a 1.5-mL tube containing 198 µL of 1X dilution buffer.

   Mix by vortexing and then centrifuge to collect contents

   1:20,000 diln. Transfer 5 µL of the 1:2000 dilution to a 1.5-mL tube containing 45 µL of 1X dilution buffer.

   Mix by vortexing and then centrifuge to collect contents

qPCR Reaction Assembly

Preparation of a reaction cocktail is recommended to reduce pipetting errors and maximize assay precision. Use the appropriate PerfeCTa SYBR Green SuperMix for your qPCR instrument (see *Instrument Compatibility* section).

1. Prepare the reaction cocktail:

Component	Volume for complete 20-µL rxn.	Volume for a 30-rxn reaction cocktail	Final Concentration
PerfeCTa SYBR Green SuperMix (2X)	10 µL	300 µL	1x
Ion Torrent Primer Mix (10 µM each)	0.6 µL	18 µL	300 nM (each)
Nuclease-free water	5.4 µL	162 µL	
Template DNA	4 µL	_____	
Final Volume	20 µL	480 µL	

Vortex gently to mix contents. Centrifuge briefly to collect components at the bottom of the reaction tube.

> **Note:** For smaller, or larger, reaction volumes scale all components proportionally. For example: when working with a 384-well plate carry out reactions in 10-µL (final) volumes using 8 µL of reaction cocktail and 2 µL of DNA standard or NGS library dilution.

2. Label 8 x 1.5-mL tubes A to H. Dispense 56 µL of reaction cocktail into each tube.
3. Add 14 µL of the appropriate standard or NGS library dilution (1:2,000 or 1:20,000) to each tube. (See suggested plate loading below)
4. Mix each tube by gently vortexing and centrifuge to collect contents.
5. Dispense 20 µL from each tube into 3 wells for triplicate reactions for each DNA standard concentration or library dilution sample.

   Row A: 3 x 20 µL of 1X dilution buffer (NTC)
   Row B: 3 x 20 µL tube "B": Standard 1 (1E3 copies/µL)
   Row C: 3 x 20 µL tube "C": Standard 2 (1E4 copies/µL)
   Row D: 3 x 20 µL tube "D": Standard 3 (1E5 copies/µL)
   Row E: 3 x 20 µL tube "E": Standard 4 (1E6 copies/µL)
   Row F: 3 x 20 µL tube "F": Standard 5 (1E7 copies/µL)
   Row G: 3 x 20 µL tube "G": 1:2,000 Library Dilution
   Row H: 3 x 20 µL tube "H": 1:20,000 Library Dilution

> **Note:** Alternately, reaction cocktail (without DNA template) can be dispensed into the reaction plate (16 µL / well), and then DNA template added to each well to complete each qPCR reaction. Mix reactions after adding DNA template before PCR amplification.

6. Seal the plate and briefly centrifuge to collects contents to the bottom of each well.

qPCR Amplification

1. Program your real-time qPCR instrument – reaction plate well assignments.
   a. Define wells containing the DNA standards as "standards" with the appropriate concentration listed above.
   b. Define NGS library dilution samples as "unknowns"
   c. Group replicate reactions to allow the qPCR data analysis program to calculate average Cq and concentration based on the DNA standards.
2. Incubate the reaction plate in a real-time qPCR system as follows:

Initial denaturation:		95ºC, 3 min
PCR cycling (35 cycles)	Denaturation:	95ºC, 10 to 15s
	Annealing:	60ºC, 20s
	Extension:	72ºC, 45s (collect and analyze data)
Dissociation (melt) curve		refer to your qPCR instrument manual (*optional*)

Full activation of the hot-start DNA polymerase occurs within 30 seconds at 95ºC. Initial denaturation times greater than 3 minutes are usually not required. However, amplification of GC-rich libraries may benefit from a longer initial denaturation step (5-10 min) to fully denature the template and minimize the potential for renaturation of highly stable and/or repetitive sequence regions. For AT-rich libraries, use a 2-step cycling protocol with a combined annealing and extension step of 58°C for 1 min.

Data Analysis

The reaction plate should be defined, or annotated, using the stated concentrations for each DNA standard. Since equal volumes of each DNA Standard and library dilution sample were used in each qPCR, a simple trend analysis of the standard curve (log copies/µL concentration vs Cq value) linear regression can be used to calculate the concentration for each library sample. This should be carried out automatically using the absolute (or standard curve) quantification algorithm provided with your qPCR detection system. There is no need to determine the template copy number or template concentration of each qPCR. Please consult your qPCR system documentation.

The Ion Torrent DNA standards and primer mix generate a 183-bp amplicon (51.9% GC). A common problem with some NGS library quantification protocols is the use of DNA standards that are overly concentrated and generate qPCR data that are outside of the linear dynamic range for most qPCR instruments. Improper baseline settings result in compressions between the highest concentrated DNA standards, which in turn give rise to inflated PCR efficiencies and inaccurate library quantification results. The NGS DNA standards supplied with the PerfeCTa NGS Library Quantification Kits have been selected to avoid these artifacts and produce absolute quantification standard curves with exceptionally high linear regression correlation coefficients.

Size-adjusted concentration (copies/µL) for each library dilution sample is obtained as follows:

average concentration (copies/µL) x (183 / average fragment length)

The size-adjusted concentration of the original library sample is obtained by multiplying the value above by the dilution factor used for the qPCR (either 2,000 or 20,000). It is important to only use Cq data from library dilution samples that fall within the linear dynamic range of the DNA Standards.

Use the size-adjusted concentration of the original library stock to determine the appropriate dilution to use for the emPCR. The optimal input amount of library molecules for emPCR is 280 x 10^6 copies/18 µL (or 1.556 x 10^7 copies/µL).

Example: avg. size-adjusted library concentration = 7.44 x 10^9 copies/ µL

Dilution factor = 7.44 x 10^9 ÷ 1.556 x 10^7

= 478

Therefore, mix 1 µL of undiluted library stock with 477 µL of 1X library dilution buffer and use 18 µL for emPCR

Quality Control

Kit components are free of contaminating DNase and RNase. qPCR of the DNA Standards are assayed in triplicate reactions with PerfeCTa SYBR Green SuperMix and must generate a standard curve with $r^2 \geq 0.995$ and a PCR efficiency ≥ 90%. Dissociation curve analysis must present as a single melt peak with the expected Tm.

Limited Label Licenses

The PerfeCTa NGS Library Quantification Kit for Ion Torrent Sequencing Platforms is intended for research use only and is not intended for any animal or human therapeutic or diagnostic use.

Please consult the limited label license statements provided with the included PerfeCTa SYBR Green SuperMix component.

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