

# ddPCR™ Library Quantification Kit for Ion Torrent

Catalog #	Description
186-3041	<b>ddPCR Library Quantification Kit for Ion Torrent</b> , 200 x 20 µl reactions, includes ddPCR Supermix for Probes (no dUTP) (2 x 1 ml vials), ddPCR library quantification assay (1 x 200 µl vial), for quantification of Ion Torrent libraries using the QX100™ or QX200™ Droplet Digital™ PCR System

**For research purposes only.**

## Description

The ddPCR Library Quantification Kit for Ion Torrent provides highly precise measurements to quantify Ion Torrent libraries prior to sequencing, without the need for standards. An added benefit of Droplet Digital PCR (ddPCR) is the ability to assess library quality by viewing fluorescence amplitude plots of droplet populations that discern features such as well-constructed libraries and adapter-adapter species.

## Contents

ddPCR Supermix for Probes (no dUTP) (2 x 1 ml vials) and ddPCR library quantification assay (1 x 200 µl vial) sufficient for 200 x 20 µl reactions.

## Storage and Stability

The ddPCR Library Quantification Kit is stable for 18 months when stored at -20°C. Once thawed, it can be stored at 4°C for up to 2 weeks.

**Important:** Repeated freezing and thawing of the kit are not recommended.

## Directions for Use

### Reaction Setup

Thaw all kit components at room temperature. Mix thoroughly by vortexing and centrifuging twice to ensure homogeneity because a concentration gradient may form during -20°C storage.

- For accurate library quantification, the sample should target a ddPCR concentration of 100–5,000 copies/µl for best performance
- Assaying 3 different dilution points (for example, 10<sup>-4</sup>, 10<sup>-5</sup>, and 10<sup>-6</sup> from an enriched library) will typically ensure that a reliable concentration measurement is obtained; modified TE buffer (20 mM Tris-HCl, 0.1 mM EDTA, pH 8) is recommended for serial dilutions
- If large variations in concentrations occur with highly diluted libraries, we recommend adding polyA (part number 10108626001, Roche Applied Science) or tRNA (part number R5636, Sigma-Aldrich Co.) to a final concentration of 100 ng/µl in the TE dilution buffer and PCR mixture to mitigate DNA loss due to nonspecific binding to plastic consumables

**Note:** The dilution guidelines in Tables 1 and 2 are based on libraries built according to Ion Torrent standard library preparation protocols. Additional dilution points may be required for accurate quantification if alternate protocols are employed.

**Table 1. Ion AmpliSeq and genomic DNA (gDNA) libraries for assaying 10<sup>-4</sup>, 10<sup>-5</sup>, and 10<sup>-6</sup> dilutions.**

Dilution Step	Tube 1	Tube 2	Tube 3	Tube 4
Sample into TE buffer	5 µl of stock into 495 µl TE	5 µl of Tube 1 into 495 µl TE	10 µl of Tube 2 into 90 µl TE	10 µl of Tube 3 into 90 µl TE
Dilution	100x	100x	10x	10x
Final dilution	10 <sup>-2</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>

For RNA-Seq libraries, the quality of input RNA, purification methods, and kits for library construction can contribute to differences in the final library concentration, and thus RNA-Seq libraries may require additional dilution steps to achieve the desirable range for quantification (100–5,000 copies/µl). The dilution guidelines in Table 2 are designed to capture at least one dilution point in the quantification range.

**Table 2. RNA-Seq libraries for assaying 10<sup>-4</sup>, 10<sup>-6</sup>, 10<sup>-8</sup>, and 10<sup>-10</sup> dilutions.**

Dilution Step	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
Sample into TE buffer	5 µl of stock into 495 µl TE	5 µl of Tube 1 into 495 µl TE	5 µl of Tube 2 into 495 µl TE	5 µl of Tube 3 into 495 µl TE	5 µl of Tube 4 into 495 µl TE
Dilution	100x	100x	100x	100x	100x
Final dilution	10 <sup>-2</sup>	10 <sup>-4</sup>	10 <sup>-6</sup>	10 <sup>-8</sup>	10 <sup>-10</sup>

Prepare a ddPCR master mix for the appropriate number of reactions needed according to the guidelines in Table 3.

**Note:** Duplicate reaction mixtures are recommended for each dilution point to be assayed, for a total of six reactions per library.

**Table 3. Preparation of ddPCR master mix stock solution.**

Component	Volume/Reaction	Final Concentration
2x ddPCR Supermix for Probes (no dUTP)	10 µl	1x
20x ddPCR library quantification assay	1 µl	1x
RNase-/DNase-free water	5 µl	—
<b>Total volume</b>	<b>16 µl</b>	<b>1x</b>

**Table 4. Ion AmpliSeq and gDNA libraries: ddPCR mixture layout in a 96-well plate for assaying 10<sup>-6</sup>, 10<sup>-5</sup>, and 10<sup>-4</sup> dilutions.**

	1	2	3	4	5	6	7	8	9	10	11	12
<b>A</b>	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC
<b>B</b>	Lib 1 (10 <sup>-6</sup> )	Lib 1 (10 <sup>-6</sup> )	Lib 1 (10 <sup>-5</sup> )	Lib 1 (10 <sup>-5</sup> )	Lib 1 (10 <sup>-4</sup> )	Lib 1 (10 <sup>-4</sup> )	Lib 8 (10 <sup>-6</sup> )	Lib 8 (10 <sup>-6</sup> )	Lib 8 (10 <sup>-5</sup> )	Lib 8 (10 <sup>-5</sup> )	Lib 8 (10 <sup>-4</sup> )	Lib 8 (10 <sup>-4</sup> )
<b>C</b>	Lib 2 (10 <sup>-6</sup> )	Lib 2 (10 <sup>-6</sup> )	Lib 2 (10 <sup>-5</sup> )	Lib 2 (10 <sup>-5</sup> )	Lib 2 (10 <sup>-4</sup> )	Lib 2 (10 <sup>-4</sup> )	Lib 9 (10 <sup>-6</sup> )	Lib 9 (10 <sup>-6</sup> )	Lib 9 (10 <sup>-5</sup> )	Lib 9 (10 <sup>-5</sup> )	Lib 9 (10 <sup>-4</sup> )	Lib 9 (10 <sup>-4</sup> )
<b>D</b>	Lib 3 (10 <sup>-6</sup> )	Lib 3 (10 <sup>-6</sup> )	Lib 3 (10 <sup>-5</sup> )	Lib 3 (10 <sup>-5</sup> )	Lib 3 (10 <sup>-4</sup> )	Lib 3 (10 <sup>-4</sup> )	Lib 10 (10 <sup>-6</sup> )	Lib 10 (10 <sup>-6</sup> )	Lib 10 (10 <sup>-5</sup> )	Lib 10 (10 <sup>-5</sup> )	Lib 10 (10 <sup>-4</sup> )	Lib 10 (10 <sup>-4</sup> )
<b>E</b>	Lib 4 (10 <sup>-6</sup> )	Lib 4 (10 <sup>-6</sup> )	Lib 4 (10 <sup>-5</sup> )	Lib 4 (10 <sup>-5</sup> )	Lib 4 (10 <sup>-4</sup> )	Lib 4 (10 <sup>-4</sup> )	Lib 11 (10 <sup>-6</sup> )	Lib 11 (10 <sup>-6</sup> )	Lib 11 (10 <sup>-5</sup> )	Lib 11 (10 <sup>-5</sup> )	Lib 11 (10 <sup>-4</sup> )	Lib 11 (10 <sup>-4</sup> )
<b>F</b>	Lib 5 (10 <sup>-6</sup> )	Lib 5 (10 <sup>-6</sup> )	Lib 5 (10 <sup>-5</sup> )	Lib 5 (10 <sup>-5</sup> )	Lib 5 (10 <sup>-4</sup> )	Lib 5 (10 <sup>-4</sup> )	Lib 12 (10 <sup>-6</sup> )	Lib 12 (10 <sup>-6</sup> )	Lib 12 (10 <sup>-5</sup> )	Lib 12 (10 <sup>-5</sup> )	Lib 12 (10 <sup>-4</sup> )	Lib 12 (10 <sup>-4</sup> )
<b>G</b>	Lib 6 (10 <sup>-6</sup> )	Lib 6 (10 <sup>-6</sup> )	Lib 6 (10 <sup>-5</sup> )	Lib 6 (10 <sup>-5</sup> )	Lib 6 (10 <sup>-4</sup> )	Lib 6 (10 <sup>-4</sup> )	Lib 13 (10 <sup>-6</sup> )	Lib 13 (10 <sup>-6</sup> )	Lib 13 (10 <sup>-5</sup> )	Lib 13 (10 <sup>-5</sup> )	Lib 13 (10 <sup>-4</sup> )	Lib 13 (10 <sup>-4</sup> )
<b>H</b>	Lib 7 (10 <sup>-6</sup> )	Lib 7 (10 <sup>-6</sup> )	Lib 7 (10 <sup>-5</sup> )	Lib 7 (10 <sup>-5</sup> )	Lib 7 (10 <sup>-4</sup> )	Lib 7 (10 <sup>-4</sup> )	Lib 14 (10 <sup>-6</sup> )	Lib 14 (10 <sup>-6</sup> )	Lib 14 (10 <sup>-5</sup> )	Lib 14 (10 <sup>-5</sup> )	Lib 14 (10 <sup>-4</sup> )	Lib 14 (10 <sup>-4</sup> )

Lib, library; NTC, no template control.

- Pipet 16 µl of the ddPCR master mix into each well of a 96-well plate using a multichannel pipettor.
- Pipet 4 µl of the appropriate library dilution and 4 µl of modified TE buffer for no template controls (NTCs) into the wells of a 96-well plate (see example plate layout in Table 4).
- Seal the plate with an adhesive seal.
- Vortex the plate well, then follow with brief centrifugation. Repeat.
- Generate droplets with the QX100 or QX200 Droplet Generator by pipetting 20 µl of ddPCR mixture first into the sample wells followed by 70 µl of droplet generation oil into the oil wells, according to the droplet generator protocol. After droplet generation, carefully transfer droplets into a clean 96-well plate for sealing with the PX1™ PCR Plate Sealer, thermal cycling (see protocol in Table 5), and subsequent reading of droplets in the QX100 or QX200 Droplet Reader.

**Table 5. Cycling protocol for Bio-Rad's C1000 Touch™ Thermal Cycler.\***

Cycling Step	Temperature, °C	Time	Ramp Rate	Number of Cycles
Enzyme activation	95	10 min		1
Denaturation	95	30 sec		40
Annealing/ extension	56	1 min	Approximately 2.0°C/sec	
Enzyme deactivation	98	10 min		1
Hold (optional)	12 or 4	∞		1

\* Use a heated lid set to 105°C and set the sample volume to 40 µl.

**Note:** For longer insert libraries, GC-rich libraries, or libraries showing poor separation of positive from negative droplets, it may be useful to increase the annealing/extension time to 1.5 min/cycle.

### Data Acquisition and Analysis

- After thermal cycling, place the sealed ddPCR plate into the QX100 or QX200 Droplet Reader.
- Open QuantaSoft™ Software and set up the plate template according to the recommended plate layout under Setup. The example in Table 4 shows the layout for Ion AmpliSeq and gDNA libraries with the three recommended dilution points in duplicate.
- For QuantaSoft Software Version 1.3.2 or earlier, choose **ABS** as the experiment type in the well editor. Select **Ch1 Unknown** in Assay 1 and leave Assay 2 as **Unused**. Click **Run** and select the **FAM/HEX** dye set.
- For QuantaSoft Software Version 1.4.0 or later, choose **ABS** as the experiment type and **ddPCR Supermix for Probes (no dUTP)** as the supermix type in the well editor. Select **Ch1 Unknown** in Target 1 and leave Target 2 as **Unused**. Click **Run** and select the **FAM/HEX** dye set.
- After data acquisition, select the dilutions corresponding to a library in the well selector under Analyze. Examine the automatic thresholding applied to the amplitude data, paying particular attention to the separation between the positive and negative droplets in the 1-D amplitude plot. If necessary, thresholds can also be set manually by applying either the single well or multiwell threshold between the positive and negative droplets using both the amplitude and histogram plots. Record the concentration as the total positive library concentration (includes both properly adapted library inserts and adapter-adapter species).
- Repeat the analysis for each library.

### Stock Library Concentration Calculations

The stock concentration of each library can be backcalculated using the ddPCR values that fall between 100 and 5,000 copies/ $\mu$ l. If quantification values lie outside this range, it will be necessary to assay additional dilution points or add polyA or tRNA to mitigate potential DNA loss due to nonspecific binding to plastics. Remember to account for the dilution factors, including the fivefold dilution of the ddPCR reaction (4  $\mu$ l of the serial dilution was added to 16  $\mu$ l of supermix stock).

**Example:** A library at the  $10^{-6}$  dilution yielded 2,000 copies/ $\mu$ l in the ddPCR mixture. Multiply 2,000 by  $10^6$  and by 5 to account for the dilution ( $2,000 \times 10^6 \times 5 = 10^{10}$  copies/ $\mu$ l of original stock library). To obtain nM:  $10^{10}$  copies/ $\mu$ l  $\times 10^6 \mu$ l/L/ $6.023 \times 10^{23}$  copies/mole =  $1.66 \times 10^{-8}$  M or 16.6 nM.

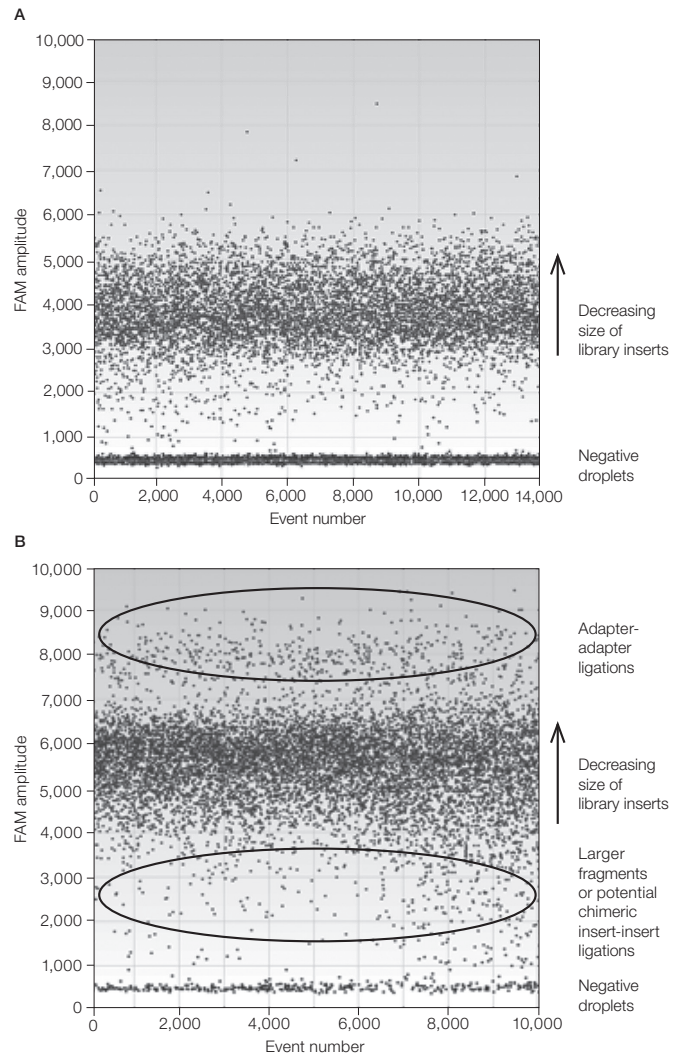
### Library Input on Ion OneTouch System for Clonal Amplification

Optimal loading of the input library was determined to be 4–8 pM for Ion AmpliSeq, gDNA, and RNA-Seq applications. The recommended loading concentration of the total positive library on the Ion OneTouch System, as determined by ddPCR quantification, is 6 pM for each library.

### Library Quality Assessment

When looking at the 1-D amplitude plot of the library (Figure 1), inserts of varying sizes within the library can be observed along the y-axis of the FAM fluorescence amplitude, with larger fragments near the negative droplets. This characteristic pattern allows the quality of the library to be measured. At the highest point in the plot there may be a population that appears separate from the bulk of the library (Figure 1B). This population consists of adapter-adapter ligations with no inserts. Below the insert population are more rare additional adapter or multiple insert ligations.

To learn more about Bio-Rad's solutions for next-generation sequencing library quantification, visit [www.bio-rad.com/web/ddPCRLibraryQuantKitIon](http://www.bio-rad.com/web/ddPCRLibraryQuantKitIon).



**Fig. 1.** Ion RNA-Seq (A) and Ion AmpliSeq (B) libraries quantified using the ddPCR Library Quantification Kit for Ion Torrent.

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Bio-Rad's real-time thermal cyclers are covered by one or more of the following U.S. patents or their foreign counterparts owned by Eppendorf AG: U.S. Patent Numbers 6,767,512 and 7,074,367.