Additional Protocol

PerfeCTa® NGS Library Quantification Kit for Illumina® Sequencing Platforms

This additional protocol provides an alternative preparation of the reaction cocktail other than recommended in the Instruction For Use provided for PerfeCTa NGS Quantification Kit for Illumina Sequencing Platforms.

Additional protocol

To each 1.25 mL tube of PerfeCTa SYBR Green SuperMix add:

1. 75 µL of 10 µM Illumina Primer Mix
2. 175 µL of water
3. Total volume = 1.5 mL
4. Store at 4°C or at -20°C
5. Add 12 µL of master mix per well and complete the reactions to 20 µL with 4 µL of water and 4 µL of Illumina Standard or diluted library sample as template.

The reactions can also be run at 10µl- for this use half volume in step 5.

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.
   b. Define NGS library dilution samples as "unknowns"
   c. Group replicate reactions to allow the qPCR data analysis program to calculate average Cq and concentrations 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 15 s
	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. Increased extension time (1 min) may improve performance for libraries with longer (up to 1 kb) average insert sizes. For AT-rich libraries, use a 2-step cycling protocol with a combined annealing and extension step of 60°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 pM concentration vs Cq value) linear regression can be used to calculate the pM 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.

Data Analysis (continued)

The Illumina DNA standards and primer mix generate a 426-bp amplicon (48.8% 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 (pM) for each library dilution sample is obtained as follows:

average concentration (pM) x (426 / 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 to determine the appropriate dilution to use for loading the flow cell for the bridge amplification. The optimal loading concentration may vary for different sample types or library construction methods, but in general, 5 to 10 pM generates an appropriate cluster density.

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