HB220414

Hieff NGS™ DNA Library Quantification Kit for Illumina

Cat# 12302

INSTRUCTION FOR USE

Yeasen Biotechnology (Shanghai) Co., Ltd.

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Product Information

Product Name	Cat#	Specification
Hieff NGS™ Library Quantification Kit for Illumina, qPCR Master Mix	12302ES05	500 T
Hieff NGS™ Library Dilution Buffer	12303ES50	50 mL
Hieff NGS™ Library Quantification Kit for Illumina, DNA Standard1-6	12307ES09	6×96 μL

Product Description

This product is a kit for quantification of high-throughput sequencing library on the lllumina platform. It provides DNA standards, qPCR Master Mix, dilution buffer, quantitative amplification primers and reference dye ROX required for quantification. Among them, the DNA standards contains six 450 bp double-stranded DNA fragments, with a concentration of 20 pM-0.0002 pM. The amplification primers are designed according to the NGS library adapter sequences P5 and P7, which can ensure specific amplification of library with complete adapters. The qPCR Master Mix is a Dye-Based hot-start qPCR master mix, which can efficiently and specifically amplify samples with different lengths, different GC or AT contents, and achieve accurate quantification.

Product Application

This product is designed to accurately quantify libraries prepared for high-throughput sequencing on the lllumina platform. It is suitable for quantifying libraries prepared in any way that match the NGS library adapter sequences P5 and P7, and the concentration is not less than 0.0002 pM. In addition to NGS library quantification, the kit can also be used to detect the degree of library contamination in the experimental environment during the preparation of the lllumina library. The sequences of two primers contained in the qPCR Primer Mix are as follows:

Primer 1：5'-AAT GAT ACG GCG ACC ACC GA-3'

Primer 2：5'-CAA GCA GAA GAC GGC ATA CGA-3'

Whether the library can be amplified by the primer pair can be confirmed in advance by the primer sequence.

Product Component

Components		Volume		
12302-A	Hieff NGS™ SYBR qPCR Master Mix (2×)	5×1 mL	-	-
12302-B	qPCR Primer Mix	2×0.5 mL	-	-
12302-C	50×Low Rox	200 μL		-
12302-D	50×High Rox	200 μL	-	-
12303	Hieff NGS™ Library Dilution Buffer	-	50 mL	-
12307	Hieff NGS™ Library Quantification Kit for Illumina, DNA Standard1-6	-	-	6×96 μL

Category	Instrument Model
No ROX added	Bio-Rad CFX96™, CFX384™, iCycler iQ™, iQ™5, MyiQ™, MiniOpticon™, Opticon®, Opticon 2, Chromo4™; Cepheid SmartCycler®; Eppendorf Mastercycler®ep realplex, realplex 2 s; Illumina Eco qPCR; Qiagen/Corbett Rotor-Gene®Q, Rotor-Gene®3000, Rotor-Gene®6000; Roche Applied Science LightCyclerTM480; Thermo Scientific PikoReal Cycler
Add 50×High Rox	Applied Biosystems 5700, 7000, 7300, 7700, 7900, 7900HT, 7900HT Fast; StepOne™, StepOnePlus™.
Add 50×Low Rox	Applied Biosystems 7500, 7500 Fast, ViiA™7; Stratagene MX4000™, MX3005P™, MX3000P™.

【Note】: Select the appropriate reference dye ROX according to the type of instrument.

Shipping and Storage

All the components are shipped with ice pack and should be stored at -20°C.The qPCR Master Mix and ROX can be stored in dark for 12 months, unless used repeatedly in a short period, in which case the qPCR Master Mix can be thawed and temporarily stored in the dark at 4°C for 3 months.

Cautions

I. About operation

1. For your safety and health, please wear lab coats and disposable gloves for operation.
2. It is recommended to physically separate the sample preparation area, reaction system preparation area and sample addition area.
3. It is recommended to perform all operations on ice boxes before performing qPCR testing on instrument.
4. To ensure product quality, avoid repeated freezing and thawing more than 30 times.
5. The sensitivity of qPCR technology is high, and accurate pipetting is crucial to the reliability of the detection results. Accuracy of operation can be guaranteed by paying attention to the following details:
   - 5.a – Make sure that all reagents and samples have been completely thawed and mixed thoroughly before the experiment, and the liquid is collected to the bottom of the tube after a brief centrifugation.
   - 5.b – Use a pipette tip with a filter element as much as possible to avoid aerosol pollution;
   - 5.c – Avoid using Multichannel Pipette;
   - 5.d – The pipette tip should be replaced every time to avoid cross-contamination;
   - 5.e – Avoid the pipette tip going deep into the liquid when sucking the liquid, so as to prevent the liquid from sticking to the outside of the pipette head;
   - 5.f – When discharging liquid, the pipette tip should be as close to the bottom of the reaction tube as possible;
   - 5.g – After discharging the liquid, pipette up and down 2-3 times to rinse the pipette tip;
   - 5.h – After discharging the liquid, check that no liquid remains at the tip of the pipette tip.
6. For research use only!

II. About library dilution

The library needs to be diluted to the effective Ct range of the standard curve for quantification. The dilution ratio can refer to past experience or the concentration determined by other assay methods as a reference (such as NanoDrop®, Qubit® or Bioanalyzer). Note that high-concentration DNA solutions are viscous and have poor dispersion of DNA molecules. Serial dilutions are recommended during the dilution process (for example, two serial 1:100 dilutions are performed instead of only one 1:10000 dilution). The range of library concentrations that can be quantified using this kit is shown in the table below.

Since DNA is easily degraded in a non-buffered environment, it is recommended to use dilution buffer (10 mM Tris-HCl, pH 8.0 [25°C]; 0.05% Tween 20) (cat#12303ES50) to dilute the library to ensure the quality of the assay samples. Never use water as a diluent. When assaying, the library should be diluted and stored on ice. Do not use the diluted library prepared and stored in the past. If the sample needs to be re-assayed, a freshly diluted sample will need to be prepared for testing.

DNA Standard	Molarity	Concentration	Copy Number Concentration
STD 1	20 pM	5.5 pg/μL	12×10^{6} copies/μL
STD 2	2 pM	0.55 pg/μL	12×10^{5} copies/μL
STD 3	0.2 pM	0.055 pg/μL	12×10^{4} copies/μL
STD 4	0.02 pM	0.0055 pg/μL	12×10^{3} copies/μL
STD 5	0.002 pM	0.00055 pg/μL	12×10^{2} copies/μL
STD 6	0.0002 pM	0.000055 pg/μL	12×10^{1} copies/μL

III. Contamination and No-template Controls

1. When performing qPCR experiments, good experimental practices should be ensured to prevent contamination of the work area, reagents, consumables, instruments, DNA standards, etc. It is recommended to physically separate the reaction system preparation area from the template preparation area, and regularly use 0.5% sodium hypochlorite or 10% bleach for each experiment.

2. During the preparation of the reaction system, DNA Standards should be added in the order from low concentration to high concentration (DNA Standard 6 to 1), and new pipette tips should be replaced with each pipetting to avoid aerosol contamination.

3. The NTC negative control reaction should be performed in parallel for each experiment, and the amplification specificity and system contamination degree analysis should be carried out in conjunction with the melting curve. Because the amplification primer sequences are fixed sequences designed by the Illumina® platform rather than qPCR-specific primer sequences, and the number of

amplification cycles is large, it is normal to generate primer-dimer amplification resulting in Ct production for the NTC negative control reaction. Normally, Ct (NTC) > Ct (DNA Standard 6) + 3.

IV. Amplification curve baseline settings

As the molarity of DNA Standard 1 is much higher than that of conventional qPCR templates, its Ct value is often very small. However, most qPCR instruments set 3-15 cycles as Baseline by default, and sometimes the Ct value of DNA Standard 1 is mistaken for the noise background during the measurement, which in turn affects the standard curve creation. Manually setting Baseline to cycle 1-3 can effectively avoid this from happening.

V. Melting curve analysis

The melting curve is very important when performing contamination analysis with the NTC negative control and confirming the maximum effective Ct of the standard curve. It is recommended to perform the melting curve acquisition step for each experiment. All melting curves generated by the DNA Standards for this product exhibit a characteristic single peak.

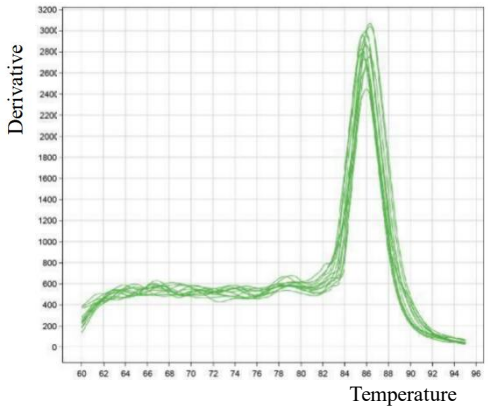

Figure 1. Library standard melting curve

VI. Other quantitative methods

There are various methods for NGS library concentration determination, such as a spectrophotometer (Nanodrop™, etc.), fluorescent dye method (Qubit PicoGreen, etc.), electrophoresis detection method (2100 Bioanalyzer, TapeStation, LabChip GX, etc.) and qPCR detection method. Since qPCR assays can only measure libraries with completes adapters in double-ends, the library concentrations measured are similar to or lower than those quantified by other methods. However, when the library is over-amplified, there will be a large number of non-target annealing products, resulting in a higher concentration of the library detected by qPCR than that quantified by other methods. At this time, if the library concentration roughly determined by other methods is used as a dilution reference, there will be insufficient library dilution.

Experimental method

Other reagent materials need to be prepared:
Hieff NGS™ Library Dilution Buffer（Cat#12303ES50）;
Hieff NGS™ Library Quantification Kit for Illumina, DNA Standard1-6（Cat#12307ES09）

I. Thaw reagents

Remove Hieff NGS™ qPCR Mix, Primer Mix, DNA Standards 1-6, ROX Dye (if needed) from refrigerator, thaw on ice, library diluent (10 mM Tris-HCl, pH 8.0; 0.05% Tween 20) was removed and equilibrated to room temperature. After each reagent is thawed, mix thoroughly, centrifuge briefly, and place on ice for later use.

II. Dilute library

The library to be tested was diluted appropriately using library diluent (10 mM Tris-HCl, pH 8.0 [25°C]; 0.05% Tween 20). The

recommended dilution is 1:1000-1:100000. For high-concentration libraries, three additional 2-fold dilution gradients can be set. For example, if the library is diluted by 1:10000, the additional dilution ratios of 1:20000, 1:40000, and 1:80000 can be set to ensure that the measured value is within the range of standard curve provided by the kit.

III. Set up reaction system

Prepare the following system in a reaction tube, invert or vortex to mix, and centrifuge briefly to collect the reaction solution to the bottom of the tube.

Table 2. qPCR reaction system

Components name	Volume (μL)	Recommended order of addition
Hieff NGS™ SYBR qPCR Master Mix (2×)	10	②
qPCR Primer Mix	2	③
Diluted DNA Library/DNA Standards 1-6/ddH_2O*	4	④
ddH_2O	4	①
Total	20	

【Notes】: 1) At least 3 technical replicates should be set for each reaction;

2) A 20 μL reaction system is recommended. If a 10 μL reaction is required, reduce the components of the system in equal proportions;

3) *Add ddH_2O to the negative control reaction tube; add the diluted library to the sample reaction tube; add DNA Standards to the standard curve reaction tube;

4) Select the appropriate ROX according to the instrument, the recommended amount is 0.4 μL, and the amount of ddH_2O should be reduced in the corresponding system.

IV. Set up qPCR running program

Place the reaction tube in the qPCR instrument, set the qPCR reaction program according to the following conditions, and run it (the melting curve can be based on the default of the instrument).

Table 3. qPCR reaction program

Stage	Temperature(℃)	Time	Cycles
Pre-denaturation	95	5 min	1 cycle
Cyclic reaction	95	30 sec	35 cycles
	60	45 sec*	
Melting curve	95	15 sec	1 cycle
	60	60 sec	
	95	15 sec	

Note: *When the average length of the library is >700 bp, it is recommended to extend the extension time to 90 sec.

V. Data Analysis

1. Standard curve making

1.a – According to the principle of Ct value difference between duplicate wells ≤0.2, filter the original Ct value of DNA Standards, and calculate the average Ct value;

1.b – Refer to the Ct value of the NTC negative control to confirm the effective range of the Ct value of the standard curve. Judging according to the following principles, use the Ct value within the effective range (as the ordinate) and Log[pM] (as the abscissa) to draw the standard curve. The quality control indicators for the standard curve are as follows:

The △Ct of two adjacent DNA standards should be between 3.1 and 3.6; the amplification efficiency E should be between 90 and 110%; the correlation coefficient $R^2 \geq 0.99$ which meet the technical parameters requirements.

Ct value of NTC	Maximum valid Ct value	Valid Standard Gradient
Ct (NTC) > Ct (DNA STD 6) + 3	Ct (STD 6)	STD 1~6
Ct (DNA STD 6) + 3 > Ct (NTC)> Ct (DNA STD 5) + 3	Ct (STD 5)	STD 1~5
Ct (DNA STD 5) + 3 > Ct (NTC)> Ct (DNA STD 4) + 3	Ct (STD 4)	STD 1~4
Ct (DNA STD 4) + 3 > Ct (NTC)	System indicated is seriously polluted, and all components need to be replaced.	

2. Library concentration calculation

The Ct value of the diluted library can only be used for concentration calculation if it is within the dynamic range of the valid Ct value of the standard curve:

2.a – Filter the original data according to the principle that the Ct value difference of the duplicate wells is ≤0.2, and calculate the average Ct value of each diluted library. If the Ct value difference between any three duplicate wells exceeds 0.2, the experiment needs to be repeated;

2.b – Bring the average Ct value of each diluted library into the standard curve, and calculate the concentration (in pM);

2.c – In order to avoid the influence of fragment length on the absolute quantification of the library, according to the principle that the fluorescence intensity generated after the library fluorescent dye SYBR® Green I binds to DNA is proportional to the DNA molecular weight, the length of the diluted library is adjusted by the following formula (in pM);

$$\frac{\text{DNA standard size (450bp)}}{\text{Average library length (bp)}} \times \text{Concentration of diluted library (pM)}$$

2.d – Calculate the original concentration (nM) of each library:

$$\text{Original library concentration (nM)} = \text{Adjusted library concentration (pM)} \times \frac{\text{Dilution multiples}}{1000}$$

2.e – Determine the working concentration of the library (ng/μL):

$$\text{Original library concentration (nM)} = \frac{?\ \text{ng}/\mu\text{L} \times 10^6}{660 \times \text{Average fragment size (bp)}}$$

Experimental example

I. Library preparation and dilution

Use the Hieff NGS™ OnePot DNA Library Prep Kit for Illumina (Cat#12204) to prepare 2 DNA libraries with a total length of about 340 bp, and analyze the average fragment size and approximate concentration of the amplified libraries with reference to other library quantification methods (see Table 2, p. 1 and 2).

To indicate the reliability of the calculated concentrations, for serial 2-fold dilutions of one sample, Ct values between 0.9 and 1.1 are expected to be controllable, indicating that the difference in library concentration calculated from two or more dilutions methods< 10%. The two library prepared above was diluted 1/10000 (twice 1/100) and another 2-fold 1/20000 dilution (1/10000 dilution and then 1 fold). Detection was performed using the Yeasen library quantification kit, 3 replicates for each gradient of standards and library dilutions (including NTC) should be performed.

II. Generation of standard curve

The 3 replicates and mean Ct values for 6 DNA standards and NTC are shown in the table below.

Table 4. Ct values for DNA standards

STD #	Conc (pM)	Ct	Av Ct	Δ Ct*
1	20	7.45	7.56	3.42
		7.59		
		7.65		
2	2	10.88	10.98	3.33
		10.99		
		11.07		
3	0.2	14.27	14.31	3.41
		14.27		
		14.40		
4	0.02	17.66	17.72	3.54
		17.71		
		17.79		
5	0.002	21.21	21.26	3.41
		21.27		
		21.30		
6	0.0002	24.63	24.67	-
		24.70		
		~~24.85~~		
NTC	-	30.70	30.	
	-	31.03		
	-	ND		

*For 10-fold dilution series, ΔCt should be between 3.1~3.6.

According to the principle of Ct difference between replicate wells ≤ 0.2, obvious outliers (STD#6, the third replicate Ct value) were excluded, and a standard curve was generated (see Figure 2 for information). The quality control indicators for the standard curve are as follows:

① The ΔCt of two adjacent DNA standards should be between 3.1 and 3.6;

② The amplification efficiency E should be between 90 and 110%;

③ Correlation coefficient $R^2 \geq 0.99$ which meet the technical parameters requirements.

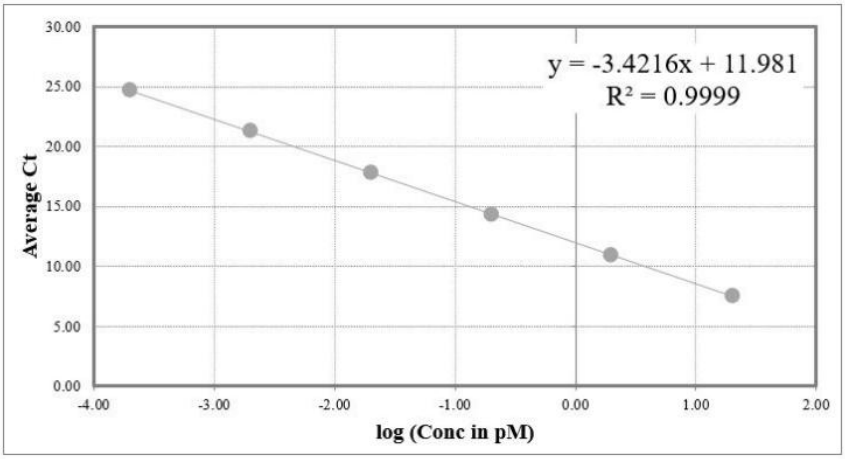

Figure 2. Generated by Yeasen Library Quantification Kit

III. Calculation of library concentration

Table 2. Quantitative data table based on qPCR

	Parameters	Library 1		Library 2	
1	average fragment length (Bioanalyzer)	340 bp		354 bp	
2	estimated concentration (Qubit)	36.20ng/μL=161.32nM		20.20 ng/μL=86.46nM	
3	Dilution for qPCR	1:10K	1:20K	1:10K	1:20K
4	Ct values of three times	8.91	9.83	9.72	10.71
		8.79	9.9	9.76	10.77
		8.80	9.92	~~9.52~~	10.80
5	Mean Ct value	8.83	9.88	9.74	10.76
6	ACt	1.05		1.02	
7	Calculate the mean concentration of sample dilutions using the standard curve (pM)	8.32	4.10	4.52	2.27
8	Length-corrected library concentration (pM)	11.06	5.45	5.77	2.90
9	Initial library concentration (nM)	110.56	109.08	57.69	58.08
10	Quantitative difference in final library concentration calculated at different dilutions	1.4%		0.7%	
11	Working concentration	109.82 nM=24.64ng/μL		57.89 nM=13.53 ng/μL	

Common problems and solutions

Common problems	Possible cause and solutions
Amplification efficiency is not in the range of 90%-110%	If Ct (NTC) - Ct (DNA STD 6) < 3 or Ct (DNA STD6) - Ct (DNA STD 5) < 3.1, and the calculated amplification efficiency exceeds 100%, it indicates that the reaction system is contaminated. The source of contamination (library contamination or DNA STD contamination) should be confirmed based on the melting curve of the NTC negative control.
	Inappropriate Baseline settings, manually adjust the baseline as 1-3 cycles.
	Low pipetting accuracy, repeat the experiment to ensure that all reagents are completely dissolved and mixed before use.
Correlation coefficient R^2 < 0.99	Low pipetting accuracy, repeat the experiment to ensure that all reagents are completely dissolved and mixed before use.
	Instrument related issues, make sure the instrument is used with the appropriate ROX.
The standard curve is not uniformly distributed	Ct (DNA STD 6) - Ct (DNA STD 5) < 3.1, indicating contamination of the reaction system. Confirm the source of contamination (library contamination or DNA STD contamination) based on the melting curve of the NTC negative control.
	Ct (DNA STD 2) - Ct (DNA STD 1) < 3.1, indicating an improper baseline setting. Manually adjust the baseline as 1-3 cycles. ΔCt > 3.6 between DNA STDs, suggesting low amplification efficiency. Verify that all reagents are fully thawed and mixed thoroughly before use; Verify that all components are at the correct concentrations and that the reaction procedures are correct. Prolonged exposure to strong light will lead to a decrease in the fluorescence value of the qPCR Master Mix, resulting in a ΔCt > 3.6. Reagents should be stored as recommended in the dark.
Low repeatability	Low pipetting accuracy, repeat the experiment to ensure that all reagents are completely dissolved and mixed before use.

	Instrument related issues, make sure the instrument is used with the appropriate ROX.
Library diluted ΔCt is not within a reasonable range (eg 2x Dilute library, ΔCt is 0.9-1.1)	Low pipetting accuracy, repeat the experiment to ensure that all reagents are completely dissolved and mixed before use. Libraries are difficult to amplify. Libraries with high GC/AT content or longer than 1 kb have low amplification efficiency. Library degradation. The library should be freshly diluted for immediate usage, and the diluted library should be kept on ice for later use.
The calculated initial library concentration differs by more than 10% for each dilution of the library.	Low pipetting accuracy, repeat the experiment to ensure that all reagents are completely dissolved and mixed before use. Libraries are difficult to amplify. Libraries with high GC/AT content or lengths greater than 1 kb were poorly amplified. Library degradation. The library should be freshly diluted for immediate usage, and the diluted library should be kept on ice for later use.
The Ct value of the diluted library is not within the the standard Ct of standard curve	Library concentrations were calculated using Ct values within the valid range. When Ct (diluted library) < Ct (DNA STD 1), it indicates that the library dilution is not enough, and the library dilution should be increased to repeat the experiment. Ct (diluted library) > Ct (DNA STD 6), indicating that the library dilution is too high, the dilution should be reduced and the experiment should be repeated. Recommended dilution is 1:1000-1:100000. For high-concentration libraries, three additional 2-fold dilution gradients can be set. For example, if the library is diluted by 1:1000, the additional dilution ratios of 1:2000, 1:4000, and 1:8000 can be set to ensure that the measured value is within the range provided by the kit. within the standard curve range.
Abnormal amplification curve of DNA Standard 1	Inappropriate Baseline settings, manually adjust the baseline as 1-3 cycles.
DNA Standards have amplification, but the library does not or the Ct is too large	Library adapter sequence error. Check whether the sequence of the end of the library matches the primer sequence provided by the kit. Over diluted, need to reduce the dilution factor and repeat the experiment. Library degradation. The library should be freshly diluted for immediate usage.

BRING VALUES TO CUSTOMERS
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