VAHTS Library Quantification Kit for Illumina

NQ101-NQ106

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Instruction for Use

Version 22.1

Vazyme

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01/Product Description

VAHTS Library Quantification Kit for Illumina is specially designed for accurate quantification of the concentration of a DNA library for Illumina high-performance sequencing platform using SYBR Green qPCR. The principle is to draw a standard curve using DNA Standard 1 - 6, and then calculate the absolute concentration of the library to be tested according to the standard curve. VAHTS SYBR qPCR Master Mix included in this kit is a new-type qPCR mix based on antibody modified hot-start DNA polymerase, which is of high specificity, high amplification efficiency, broad GC content compatibility, and high sensitivity. All the reagents provided in the kit have undergone rigorous quality control and functional testing, to ensure the optimal stability and repeatability of library construction.

02/Storage

Store at -30 ~ -15℃ and transport at ≤0℃. Master Mix and ROX should be protected from light.

▲Reagents can maintain performance within 30 freeze-thaw cycles; if used repeatedly in a short period of time, Library Dilution Buffer and Master Mix can be thawed and temporarily stored at 2 ~ 8℃. Master Mix should be protected from light, and the validity period is three months.

03/Applications

It is applicable for the absolute quantification of the concentration of libraries for Illumina platform. Regardless of the construction method, this product can be used for absolute quantification if the end of the library contains Illumina P5 and P7 flow cell binding sequences. The library should be no more than 1 kb in length and no less than 0.0002 pM in concentration. In addition, this product can also be used to detect the library contamination level in experimental environment. The primer sequences of qPCR Primer Mix are as follows:

Primer 1: 5'-AATGATACGGCGACCACCGA-3'

Primer 2: 5'-CAAGCAGAAGACGGCATACGA-3'

Confirm whether the library can be amplified with the primer pair by sequences above.

05/Notes

For research use only. Not for use in diagnostic procedures.

05-1/Precautions for Pipetting

qPCR is extremely sensitive and the accuracy of pipetting has a significant impact on the reaction results. Please read the following contents carefully before operation:

1. All components should be thawed and mixed thoroughly. Briefly centrifuge to collect liquid to the bottom before use.
2. DNA solution with high concentration is viscous and the DNA molecular dispersion is poor, please avoid directly dilution with a large volume (such as 1:10,000 dilution). Instead, serial dilutions with small volumes are recommended (i.e. dilute 1:100 twice to make a 1:10,000 dilution).
3. Use filtered pipette tips to avoid aerosol contamination.
4. Do not use multichannel pipettors.
5. Change tips between samples to avoid cross contamination.
6. When pipetting, do not immerse the tip too deeply into the solution to avoid liquid sticking to the outside wall of the tip.
7. When expelling the liquids out, keep the tips close to the bottom of the reaction tube.
8. Pipetting for 2 - 3 times after expelling the solution.
9. After completely expelling the liquid out, please check carefully to confirm there is no liquid remains in the tip.

05-2/Library Concentration and Dilution Factor

The library must be diluted to the effective C_T range of standard curve for quantitative reaction. C_T values out of the effective range are not suitable for quantification. If only one dilution of library is assayed, it should be rediluted to an appropriate concentration and repeat the experiment. If more than one dilution of each library is assayed, select C_T values within effective range for concentration calculation. Effective C_T range selection scheme of standard curve can refer to **07/Data Analysis**. Library dilutions can be performed based on previous experiments or using concentrations determined by other assays as a reference.The following table shows the concentration range of the quantitative library:

Molar Concentration:	20 - 0.0002 pM
Mass Concentration:	5.5 - 0.000055 pg/μl
Copies Concentration:	12×10^6 - 12×10^1 copies/μl

05-3/Library Dilution

Dilute the DNA library with an appropriate buffer (Library Dilution Buffer, Vazyme #NQ106; or self-provide buffer whith 10 mM Tris-HCl, pH 8.0 @25℃, 0.05% Tween 20). DO NOT dilute the library with ddH_2O. Use freshly diluted library for quantification. Before qPCR, the diluted library and the thawed DNA Standards should be kept on ice.

04/Components

Components	Size	NQ101	NQ102	NQ103	NQ104	NQ105	NQ106
VAHTS SYBR qPCR Master Mix	4 × 1.25 ml	√	√	—	—	—	—
VAHTS SYBR qPCR Master Mix (Low ROX Premixed)	4 × 1.25 ml	—	—	√	—	—	—
VAHTS SYBR qPCR Master Mix (High ROX Premixed)	4 × 1.25 ml	—	—	√	√	—	—
qPCR Primer Mix	2 × 0.5 ml	√	√	√	√	—	—
50 × ROX Reference Dye 1	200 μl	√	—	—	—	—	—
50 × ROX Reference Dye 2	200 μl	√	—	—	—	—	—
DNA Standard 1 - 6	6 × 96 μl	—	—	—	—	√	—
Library Dilution Buffer	50 ml	—	—	—	—	—	√

Note:

1. NQ101, NQ102, NQ103, and NQ104 are amplification kits containing VAHTS SYBR qPCR Master Mix, qPCR Primer Mix, and individual ROX package (only in NQ101). They are sufficient for 500 reactions (20 μl/rxn). NQ105 is a standard kit containing DNA Standard 1 - 6, which is sufficient for 8 standard curves (3 repeated wells). NQ106 is Library Dilution Buffer (50 ml).
2. Select the appropriate ROX reference dye according to the Realtime PCR instrument used:

No ROX Reference Dye (Type I)	Bio-Rad CFX96, CFX384, iCycler iQ, iQ5, 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 LightCycler 480; Thermo Scientific PikoReal Cycler.
Add Reference Dye 1 (Type II)	Applied Biosystems 5700, 7000, 7300, 7700, 7900, 7900HT, 7900HT Fast; StepOne, StepOnePlus.
Add Reference Dye 2 (Type III)	Applied Biosystems 7500, 7500 Fast, ViiA7; Stratagene MX4000, MX3005P, MX3000P.

Individual packages of both ROX Reference Dye 1 and 2 are provided in NQ101. Therefore, NQ101 is suitable for all quantitative PCR instruments. Use appropriate ROX Reference Dye according to the table above.

NQ102 kit contains no individual or pre-mixed ROX Reference Dye. It is only suitable for Type I qPCR instruments in the table above.

NQ103 kit contains Low ROX (ROX Reference Dye 2) in VAHTS SYBR qPCR Master Mix. It is suitable for Type III qPCR instruments in the table above.

NQ104 kit contains High ROX (ROX Reference Dye 1) in VAHTS SYBR qPCR Master Mix. It is suitable for Type II qPCR instruments in the table above.

05-4/Contaminations and No Template Control (NTC)

1. Inappropriate operation may leads to contaminations in PCR products, which results in inaccurate quantitative results and low credibility. It is recommend to physically isolate the sample preparation area from the template preparation area. Use specialized pipettor and filtered tips. Clean the experimental area regularly (with 0.5% sodium hypochlorite or 10% bleaching agent).
2. Always dispense the DNA Standards from the lowest concentration to the highest (i.e. from DNA Standard 6 to DNA Standard 1). Replace tips after each pipetting.
3. It is highly recommended that NTC is included in each assay, and detect the PCR specificity and possible contamination introduced with the melting curve. The primers used in this kit is based on universal sequences on Illumina platform. It is normal that amplification product and a C_T value appear in NTC, due to inevitable aerosol contamination during repeated library dilution. In this case, determine the effective C_T range of the standard curve firstly according to the NTC negative control (refer to **07/Data Analysis**), and then draw the standard curve and calculate the concentration.

05-5/Baseline Setting of the Amplification Curve

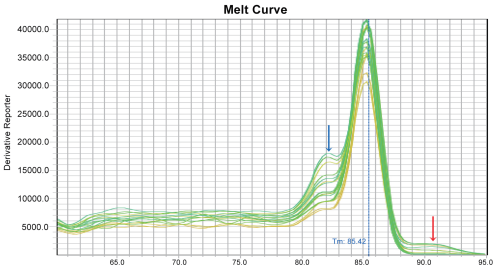
The molar concentration of DNA Standard 1 is significantly higher than that of the conventional qPCR templates, therefore generally, its C_T value is as small as 7 - 9 cycles. While for most qPCR instruments, the default baselines are set at 3 - 15 cycles, which may increase the C_T value of DNA Standard 1 by mistake and may further affects the linear relationship of the standard curve. Manually set the baseline at 1 - 3 cycles to avoid this situation.

05-6/Size-adjustment calculation

The fluorescence intensity of SYBR Green I, a dye that binds to all double-stranded DNA molecules, is proportional to the molecular weight of DNA molecules. For example, the fluorescence intensity of two 250 bp dsDNA molecules is equivalent to one 500 bp dsDNA molecule. Therefore, it is necessary to adjust the library length according to the length of DNA Standards (452 bp) and the average length of the library (refer to **07/Data Analysis**).

05-7/Melting Curve Analysis

The melting curve is very important for the analysis of the degree of contamination and the confirmation of the maximum effective C_T of the standard curve, and it needs to be analyzed together with the test results of NTC. The melt curves for the DNA Standards displays a characteristic double peak (as shown by the blue arrow). This is due to different positions of DNA Standards (452 bp dsDNA) have different melting temperature and is not indicative of non-specific amplification. In addition, the molar concentrations of DNA Standards 1 - 3 are too high, and the amplification products are too many to melt completely at the Tm. Therefore, it is normal that the melting curves of DNA Standards 1 - 3 sometimes exhibit raising tails (as shown by the red arrow).



05-8/Other Methods for Library Quantification

Methods for library quantification include approaches base on spectrophotometer (e.g. NanoDrop), fluorescent dyes (e.g. Qubit, PicoGreen), electrophoresis (e.g. 2100 Bioanalyzer, TapeStation, LabChip GX), and qPCR. qPCR only measures the libraries with intact dual-index adapters, so it has the most accurate quantification results than other methods. In general, the library concentration determined by qPCR will be slightly lower than that of other methods, and the library concentration roughly measured by other methods can be used as a reference to select an appropriate library dilution for qPCR concentration determination. However, when the library is over-amplified (e.g., too many amplification cycles), a large number of imperfect annealing products (partially double-stranded) in the amplification product will cause the library concentration determined by the qPCR to be higher than other methods. At this time, if the library concentration roughly determined by other methods is used as a dilution reference, the library dilution will be insufficient.

06/Experiment Process

1. Prepare an appropriate volume of Library Dilution Buffer (refer to **05/Notes 05-3/Library Dilution**). Store Library Dilution Buffer at 4℃. Equilibrate it to room temperature for 30 min before use and store at 4℃ after use.
2. Dilute the library. The optimum dilution ratio should be adjusted according to the library concentration. The recommended dilution ratio is 1/1,000 - 1/100,000. At least one additional 2-fold dilution of each library is also recommended (e.g. 1/10,000 and 1/20,000). Library should be kept on ice and diluted freshly before use.
3. Thaw VAHTS SYBR qPCR Master Mix (Without ROX, Low ROX Premixed or High ROX Premixed), qPCR Primer Mix, ROX Reference Dye (Optional) and DNA Standard 1 - 6. Mix thoroughly upside down several times, briefly centrifuged to the bottom of the tube, and placed on ice for use. And store at -20℃ after use.
4. Prepare the reaction solution in a qPCR tube as follows:

VAHTS SYBR qPCR Master Mix (Without ROX, Low or High ROX Premixed)	10.0 μl
qPCR Primer Mix	2.0 μl
ROX Reference Dye 1/2[a]	0.4 μl
DNA Standard 1 - 6 or Diluted Library or ddH_2O[b]	4.0 μl
ddH_2O[c]	To 20.0 μl

a. Only needed when using NQ101. Choose the appropriate ROX according to the qPCR instrument. Do not add it when using other kits and replace with ddH_2O.
b. Add ddH_2O to the NTC reaction tubes, add diluted library to the sample reaction tubes; and add DNA Standards into the standard curve reaction tubes. Always dispense the DNA Standards from the lowest concentration to the highest (i.e. from DNA Standard 6 to DNA Standard 1) to avoid the aerosol contamination .
c. The recommended reaction volume is 20 µl. For a 10 µl reaction system, please reduce the amount of each reagent in proportion.

5. Run the following program for qPCR:

Stage 1	Pre-denaturation	Rep：1	95℃	5 min
Stage 2	Cycles[a]	Reps：35	95℃	30 sec
			60℃	45 sec
Stage 3	Melting Curve[b]	Rep：1	95℃	15 sec
			60℃	60 sec
			95℃	15 sec

a. If the average length of the library is above 600 bp, the annealing time should be extended from 45 sec to 90 sec.
b. Program for melting curve may vary across qPCR instruments. Please select the default program.

07/Data Analysis

07-1/Standard Curve

1. The difference of C_T value between replicates should be less than 0.2. Filter the original C_T and calculate the average C_T value.

The difference of C_T value between 3 replicates should be less than 0.2. If the C_T value of one replicate is significantly different from that of the two others, this C_T should be discard when calculate the average C_T value. If the difference between replicates is >0.2, repeat the assay with particular focus on improving pipetting accuracy.

2. Confirm the effective C_T range refer to the C_T of NTC.

If C_T (NTC) >C_T (DNA Standard 6) + 3, the C_T of DNA Standard 6 is the maximum effective C_T. Generate the standard curve using the C_T values of DNA Standard 1 - 6.

If C_T (DNA Standard 6) + 3>C_T (NTC) >C_T (DNA Standard 5) + 3, the C_T of DNA Standard 5 is the maximum effective C_T. Generate the standard curve using the C_T values of DNA Standard 1 - 5.

If C_T (DNA Standard 5) + 3>C_T (NTC) >C_T (DNA Standard 4) + 3, the C_T of DNA Standard 4 is the maximum effective C_T. Generate the standard curve using the C_T values of DNA Standard 1 - 4.

To guarantee the accuracy of quantification, please use at least four C_T values of DNA Standards to generate the standard curve. If C_T (DNA Standard 4) + 3>C_T (NTC), it's indicated the serious contamination of the assay. It's necessary to replace all the components of the assay, and repeat the tests.

3. Generate standard curve

Choose the C_T within the effective range (as Y axis) and the corresponding Log[pM] in the following table (as X axis) to generate the standard curve. The correlation coefficient R^2 should not be less than 0.99, and the slope should be -3.1 ~ -3.6 (indicating the amplification efficiency lies between 90% and 110%). If the standard curve parameters are poor, repeat the assay.

Name	Molar Concentration	Mass Concentration	Log[pM]
DNA Standard 1	20 pM	5.5 pg/µl	Log[20]
DNA Standard 2	2 pM	0.55 pg/µl	Log[2]
DNA Standard 3	0.2 pM	0.055 pg/µl	Log[0.2]
DNA Standard 4	0.02 pM	0.0055 pg/µl	Log[0.02]
DNA Standard 5	0.002 pM	0.00055 pg/µl	Log[0.002]
DNA Standard 6	0.0002 pM	0.000055 pg/µl	Log[0.0002]

▲ The concentration listed in the table is not the final reaction concentration. Keep the volume of DNA Standards and diluted library the same, and there is no need to convert the final concentration of the reaction.

07-2/Determination of Library Concentration

1. The difference of C_T value between replicates should be less than 0.2. Filter the original C_T and calculate the average C_T value.

The difference of C_T value between 3 replicates should be less than 0.2. If the C_T value of one replicate is significantly different from that of the other two, this C_T should be discard when calculate the average C_T value. If the difference between replicates is >0.2, repeat the assay with particular focus on improving pipetting accuracy.

2. Determination the concentration (pM) of the diluted library according to the standard curve.

Only when the C_T value of diluted library is within the effective C_T range of standard curve, it can be used to determine the concentration. Do not use the C_T value which is out of range.

3. Perform length correction of diluted library concentration (pM) according to the following formula.

Corrected concentration of diluted library (pM) = [452 bp/average library length (bp)] × concentration of diluted library (pM)

4. Caculate the original library concentration (nM) according to the following formula.

Concentration of original library (nM) = Corrected concentration of diluted library (pM) × dilution factor/1,000

08/Examples

1. Starting Materials

Two DNA libraries with insertion length of about 350 bp (the total length of the library is about 470 bp) are prepared using VAHTS Nano DNA Library Prep Kit for Illumina (Vazyme #ND601). The length and concentration of the library are detected by Agilent Bioanalyzer 2100 High Sensitivity DNA Assay (refer to Table 2).

2. Library Dilution

Both the two libraries are diluted in 1/10,000 (serial dilution in 1/100 for twice) and 1/20,000 (dilute the 1/10,000 library in 1/2).

a. The difference of C_T value between replicates should be less than 0.2. Filter the original C_T of DNA Standards. Remove the third data of DNA Standard 6 (Table 1, "C_T" column), and calculate the average C_T (Table 1, "Average C_T" column).

b. Refer to the C_T of NTC, it can be inferred that the C_T of DNA Standard 6 is the maximum effective C_T. Generate a standard curve (Figure 1) by plotting the average C_T values of DNA Standard 1 - 6 against molar concentrations (Table 1,"Log [pM]").

Table 1. C_T Values of DNA Standards

DNA Standard	Log[pM]	C_T	Average C_T	ΔC_T*
1	Log[20]	8.37 8.36 8.27	8.33	-
2	Log[2]	11.83 11.74 11.78	11.78	3.45
3	Log[0.2]	15.26 15.28 15.12	15.22	3.44
4	Log[0.02]	18.78 18.73 18.59	18.70	3.48
5	Log[0.002]	22.16 22.05 22.10	22.10	3.40
6	Log[0.0002]	25.51 25.47 ~~25.17~~	25.49	3.39
NTC	-	30.87 31.26 30.73	30.95	-

* ΔC_T should be between 3.1 and 3.6.

3. Calculation of Library Concentration

a. The difference of C_T value between replicates should be less than 0.2. Filter the original C_T values of diluted library. Remove the second data of Library 2, 1:20,000 dilution (Table 2, "Library 2, 1/20,000" column). Calculate the average C_T (Table 2, Row 5).

b. Calculate the concentration of diluted libraries (Table 2, Row 7) according to the standard curve.

c. Calculate corrected concentration of the diluted library according to the average length of the library (Table 2, Row 8).

d. Calculate original concentration of diltued library (Table 2, Row 9) and the average original concentration of library (Table 2, Row 11) according to dilution factors.

Fig 1. Standard Curve

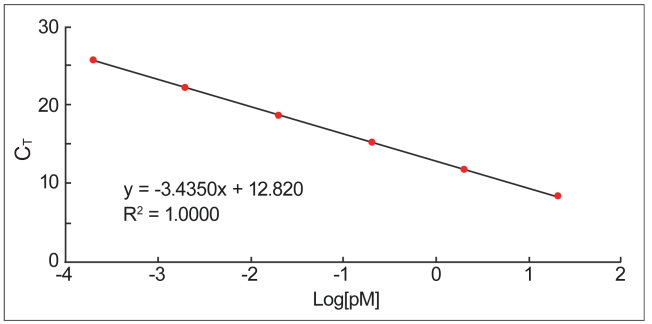


Table 2. Library Quantitative Data

Row	Parameters	Library 1		Library 2	
1	Average Length of Library (Bioanalyzer)	475 bp		470 bp	
2	Estimated Concentration (Bioanalyzer)	25.74 ng/μl = 82.08 nM		36.22 ng/μl = 116.77 nM	
3	Dilution Factor	1/10,000	1/20,000	1/10,000	1/20,000
4	C_T of Replicates	9.91 9.95 9.88	10.82 10.93 10.89	9.15 9.24 9.25	10.31 ~~10.03~~ 10.28
5	Average C_T	9.91	10.88	9.21	10.30
6	ΔC_T	0.97		1.09	
7	Concentration of Diluted Library (pM)	7.03	3.67	11.24	5.42
8	Concentration of Diluted Library (pM) after Correction	6.69	3.49	10.81	5.21
9	Original Library Concentration (nM)	66.93	69.86	108.14	104.16
10	Deviation Between Libraries at Different Dilutions	4.4%		3.8%	
11	Average Concentration of Original Library	68.40 nM = 21.45 ng/μl		106.15 nM = 32.93 ng/μl	

09/FAQ & Troubleshooting

◇ The amplification efficiency is beyond the range of 90% - 110%.

① The qPCR reaction system is contaminated if C_T (NTC) - C_T (DNA Standard 6) <3 or C_T (DNA Standard 6) - C_T (DNA Standard 5) <3.1, while the calculated amplification efficiency is above 100%. Determine the source of contamination (Standard DNA or library DNA) by examining the melting curve of NTC. When drawing the standard curve, the effective C_T value range of the standard curve should be determined according to the C_T value of NTC. Discard the C_T values affected by contamination and use the remaining C_T values to plot the standard curve.

② Inappropriate baseline setting may delay C_T value of DNA Standard 1, further affecting the calculation of amplification efficiency. Manually adjust the baseline to 1 - 3 cycles.

③ Poor pipetting accuracy.

◇ R^2<0.99.

① Poor pipetting accuracy.

② All reagents should be thawed and mix thoroughly before use.

③ Ensure that the appropriate ROX reference dye was used.

◇ The amplification curve of standards are not evenly distributed.

① If C_T (DNA Standard 6) - C_T (DNA Standard 5) <3.1, the reaction system is contaminated. Determine the source of contamination (Standard DNA or library DNA) by examining the melting curve of NTC.

② If C_T (DNA Standard 2) - C_T (DNA Standard 1) <3.1, the baseline setting is inappropriate. Manually adjust the baseline to 1 - 3 cycles.

③ If ΔC_T among DNA Standards is >3.6, the amplification efficiency is poor. Ensure that all reagents are thoroughly thawed and mixed before use. Confirm that all reaction components were added at the correct concentration, and that the correct cycling protocol was used.

④ Prolonged exposure to strong light will reduce the fluorescence signal of VAHTS SYBR qPCR Master Mix, resulting in ΔC_T >3.6. Protect the reagent from light.

◇ Poor reproducibility between replicates.

① Poor pipetting accuracy.

② All reagents should be thawed and mix thoroughly before use.

③ Ensure that the appropriate ROX reference dye was used.

◇ ΔC_T of library dilutions is not within expected range.

① Poor pipetting accuracy.

② All reagents should be thawed and mix thoroughly before use.

③ The library is difficult to amplify, e.g. the library is extremely GC/AT-rich or has a long average fragment length (>1 kb).

④ The library has degraded. Prepare fresh dilutions of library and keep on ice during reaction setup.

◇ The difference of calculated original library concentration at different dilutions is more than 10%.

① Poor pipetting accuracy.

② All reagents should be thawed and mix thoroughly before use.

③ The library is difficult to amplify, e.g. the library is extremely GC/AT-rich or has a long average fragment length (>1 kb).

④ The library has degraded. Prepare fresh dilutions of library and keep on ice during reaction setup.

◇ Library dilutions fall outside of effective range of standard curve.

① If C_T (diluted library) <C_T (DNA Standard 1), the library dilution is not sufficient, usually happening in over-amplified libraries. Increase library dilution factor and repeat quantification reaction.

② If C_T (diluted library) >C_T (DNA Standard 6), the library is over-diluted or the library preparation fails. The C_T value of a conventional library with a dilution factor around 1/10,000 should not exceed the C_T value of DNA Standard 6. Decrease the library dilution factor and repeat quantification reaction.

◇ The C_T of DNA Standard 1 appears abnormal.

① Inappropriate baseline setting may delay C_T value of DNA Standard 1, further affecting the calculation of amplification efficiency. Manually adjust the baseline to 1 - 3 cycles.

② Ensure that the appropriate ROX reference dye was used.

◇ Samples of DNA Standards is amplified, but sample of libraries are not amplified or the C_T value is large.

① The library molecules do not contain the appropriate adapter sequences for the quantification primers to bind to.

② The library is over-diluted. Decrease the library dilution factor and repeat quantification reaction.

③ The library has degraded. Prepare fresh dilutions of library and keep on ice during reaction setup.