VAHTS Library Quantification Kit for Illumina 2.0

NQ107

Vazyme

Instruction for Use

Version 24.2

Vazyme

Contents

For Research Use Only. Not for use in diagnostic procedures.

01/Product Description

VAHTS Library Quantification Kit for Illumina 2.0 is for absolute, dye-based qPCR quantification of Illumina libraries. The principle is to generate the standard curve using each DNA Standard, then the concentrations of diluted library samples are calculated against the standard curve using absolute quantification. This kit utilizes a novel dye-based qPCR mix VAHTS SYBR qPCR Master Mix 2.0 based on the antibody-mediated hot start DNA polymerase. This component completely premixes amplification reagent, primers and ROX, which has many advantages such as strong specificity, high amplification efficiency, good GC content compatibility, high sensitivity, easy operation and rapid amplification. This kit is therefore suitable for high-throughput sample quantification. All reagents provided in the kit have undergone rigorous quality control and functional testing to ensure the optimal stability and repeatability of different lots.

02/Components

Components	NQ107-01 (500 rxns)
VAHTS SYBR qPCR Master Mix 2.0[a]	4 × 1.25 ml

a. It contains Primer Mix and universal ROX, etc. This component need to be protected from light after unpacking and are stable after 30 freeze-thaw cycles. If used repeatedly for short-term, reagents are stable protected from light at 2 ~ 8ºC for up to 2 months.

03/Storage

Store at -30 ~ -15°C and ship at ≤0°C.

04/Applications

This kit for Illumina platforms is designed for the accurate quantification of libraries prepared for Illumina sequencing. Any library with a concentration >0.0002 pM that contains sequences complementary to the primers in the Primer Premix can be quantified with the kit, irrespective of the library type, how it was constructed, or on which Illumina instrument it will be sequenced. The kit supports quantification of average fragment lengths up to 1 kb. In addition to NGS library quantification, the kit can also be used to detect library contamination in work spaces used during the preparation of Illumina libraries.

The VAHTS SYBR qPCR Master Mix 2.0 provided in the kit has pre-mixed Primer Mix, containing the following primers:

Primer 1: 5'-AATGATACGGCGACCACCGA-3'
Primer 2: 5'-CAAGCAGAAGACGGCATACGA-3'

It is recommended to confirm whether the library can be amplified by these primers through the sequences.

05/Self-prepared Materials

◇ DNA Standard 1 - 6

VAHTS Library Quantification Kit for Illumina DNA Standard 1 - 6 (Vazyme #NQ105).

◇ Library Dilution Buffer

Library Dilution Buffer (Vazyme #NQ106).

06/Notes

06-1/Storage

Since qPCR is a very sensitive technique, the reliability of results is highly dependent on accurate liquid handling. Care must be taken to ensure the highest degree of accuracy when executing this protocol. This can be achieved as follows:

1. Always ensure that reagents are fully thawed before use. Mix thoroughly and briefly centrifuge to collect the solution at the bottom of the tube.
2. Concentrated solutions of DNA may be viscous, making it difficult to accurately dispense small volumes for analysis. Avoid making extremely large dilutions during sample preparation. If samples require very large dilutions to fall within the dynamic range of the assay, it is preferable to perform serial dilutions (e.g., make two consecutive 1:100 dilutions instead of a single 1:10,000 dilution).
3. Take precautions to avoid cross-contamination by using aerosol-resistant barrier tips.
4. If possible, avoid the use of multi-channel pipettes.
5. Always use a new pipette tip to avoid cross-contamination.
6. Avoid placing the pipette tip too far under the reagent surface during aspiration, as this may result in liquid adhering to the outside of the tip.
7. Always try to dispense reaction components as close as possible to the bottom of the tube or well.
8. Flush/rinse pipette tips by pipetting up and down 2 - 3 times after dispensing.
9. Ensure that no residual liquid remains in the tip after dispensing.

06-2/Library Concentration and Dilutions

Libraries and controls must be diluted to fall within the dynamic range of the assay. Any library dilution that amplifies before DNA Standard 1 or after DNA Standard 6 should not be used in library concentration calculations. If only one dilution was included in the assay, it must be repeated with a more appropriate dilution of the library. If multiple dilutions were

included, those that fall within the dynamic range of the assay can be used to quantify the library. For the selection of effective C_T range of standard curves, refer to **08/Data Analysis**. Library dilutions should be based on estimations from previous experience with libraries of the same type, or prepared using similar workflows, and/or on concentration information obtained with other methods during library construction and quality control. The following table shows the range of quantifiable library concentrations:

Molar concentration	20 - 0.0002 pM
Mass concentration	5.5 - 0.000055 pg/μl
Copy number	12×10^6 - 12×10^1 copies/μl

06-3/Library Dilution

It is recommended to dilute DNA using Library Dilution Buffer (Vazyme #NQ106) and never dilute libraries with water, or use the self-prepared diluent (10 mM Tris-HCl, pH 8.0, 25°C, 0.05% Tween 20). Prepare fresh dilutions for each assay and keep dilutions on ice during qPCR setup.

06-4/Contamination 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 reaction system preparation area. Use specialized pipettes and filtered tips. Clean the experimental area regularly (with RNase, RNA and DNA Remover (Vazyme #R504) or 0.5% sodium hypochlorite or 10% bleaching agent).
2. Always dispense the DNA Standards from the lowest to the highest concentration (i.e., from DNA Standard 6 to DNA Standard 1) and use a fresh tip for each DNA Standard to avoid aerosol contamination.
3. It is highly recommended that NTC is included in each assay, which analysis the PCR specificity and possible contamination with the melting curve. This is due to primer design, which is not optimal for qPCR, but dictated by the Illumina flow cell oligo sequences. And 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 **08/Data Analysis**), and then draw the standard curve and calculate the concentration.

06-5/Baseline Setting

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 baseline is 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.

06-6/Size-adjustment Calculation

The fluorescent signal generated by SYBR Green I is dependent on the total mass of DNA. For example, the fluorescence intensity of two 250 bp dsDNA molecules is equivalent to one 500 bp dsDNA molecule. Therefore, the size-adjustment calculation is a simple multiplication of the concentration derived from the standard curve with the ratio between the size of the DNA Standard (452 bp) and the average fragment size for that particular library (refer to **08/Data Analysis**).

06-7/Melt Curve Analysis

The melting curve is very important for analysing the degree of contamination and the confirmation of the maximum effective C_T of the standard curve, and it It is recommended to plot the melting curve for each experiment. The melt curve 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 amplicons 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).

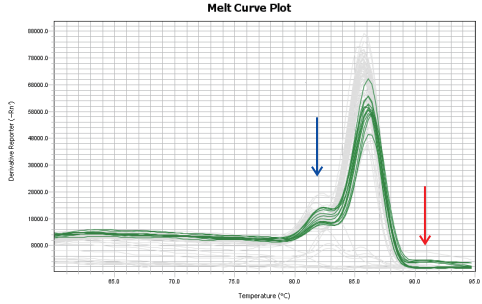

Fig 1. DNA Standards Melt Curve Plot

06-8/Other Quantitative Methods

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 quantified 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 quantification. However, when the library is over-amplified (e.g., too many numbers of amplification cycles), a large number of imperfect annealing products (partially double-stranded) in the amplicons will cause the library concentration quantified by the qPCR to be higher than other methods. At this time, if the library concentration roughly quantified by other methods is used as a dilution reference, the library dilution will be insufficient.

07/Experiment Process

1. Prepare an appropriate volume of DNA dilution buffer (refer to **06-3/Library Dilution**). This buffer can be stored at 4°C. Always equilibrate the buffer to room temperature before use and store at 4°C after use.
2. Prepare the appropriate library dilutions (using DNA dilution buffer). Depending on the expected concentration of the library, 1:1,000 - 1:100,000 dilutions may be appropriate. At least one additional 2-fold dilution of each library is recommended. Prepare fresh library dilutions for each qPCR-based quantification assay. Keep them on ice before use and discard after use.
3. Thaw VAHTS SYBR qPCR Master Mix 2.0 and DNA Standard 1 - 6. Mix thoroughly upside down several times, centrifuge briefly to collect the solution at the bottom of the tube, and place on ice before use. And store at -20°C after use.
4. Prepare the reaction solution in a qPCR tube as follows:

Components	Volume
VAHTS SYBR qPCR Master Mix 2.0[a]	10 μl
DNA Standard 1 - 6 or diluted library or ddH_2O[b]	4 μl
ddH_2O[c]	To 20 μl

a. VAHTS SYBR qPCR Master Mix 2.0 contains pre-mixed primers and universal ROX, which is suitable for all qPCR instruments without adjusting the concentration of ROX on different instruments.

b. Add ddH_2O to the NTC reaction tubes, add diluted library to the sample reaction tubes. Add DNA Standards into the standard curve reaction tubes. Always dispense the DNA Standards from the lowest to the highest concentration (i.e., from DNA Standard 6 to DNA Standard 1) and use a fresh tip for each DNA Standard to avoid aerosol contamination.

c. The reaction volume recommended is 20 μl. If the reaction volume required is 10 μl, the volume of DNA Standards and diluted libraries should be kept at 4 μl to improve accuracy, and VAHTS SYBR qPCR Master Mix 2.0 at 5 μl, ensuring that consumables, pipettes, qPCR plates and reaction volume are compatible with the qPCR instruments.

5. Perform qPCR with the following cycling protocol

Stage 1	Pre-denaturation	Rep: 1	95°C	30 sec
Stage 2	Cycles	Reps: 35	95°C	10 sec
			60°C	30 sec
Stage 3	Melt curve[a]	Rep: 1	95°C	15 sec
			60°C	60 sec
			95°C	15 sec

a. Various instruments show different melt curve collection procedures, just using default settings.

08/Data Analysis

08-1/Standard Curve

1. Replicate data points should differ by ≤0.2 cycles. Filter the original C_T of DNA Standards 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 value 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 value 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 value 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 is indicated the serious contamination of the assay. It is necessary to replace all the components of the assay, and repeat the tests.

3. Generate standard curve.

The C_T within the effective range (as Y axis) for each DNA Standard is plotted against Log[pM] (as X axis) to generate a standard curve. The coefficient of determination R^2 should not be less than 0.99, and the slope of the standard curve should be -3.1 and -3.6 (indicating the amplification efficiency is between 90% and 110%). If the standard curve does not meet these criteria, calculated library concentration is not reliable and the experiment needs to be repeated.

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.

08-2/Library Concentration Calculation

We recommend exporting qPCR data to the Vazyme Library Quantification Data Analysis Template (vazymeglobal.com/Resoure/Tools/Library Quantification Data Analysis) to perform the calculations to determine the undiluted library concentration.

1. Replicate data points should differ by ≤0.2 cycles. Filter the original C_T of DNA Standards 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. Calculate 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 calculate the concentration. If the diluted library falls outside the standard curve, can not use the C_T value.

3. Calculate the size-adjusted concentration (in pM) for each dilution of every library according to the following formula.

The average size-adjusted concentration of each library dilution (pM) = [452 bp/Average fragment length of the library (bp)] × Concentration of the diluted library (pM).

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

Original library concentration (nM) = The average size-adjusted concentration of each library dilutionn (pM) × Dilution factor/1,000.

09/Examples

1. Sample preparation

Using VAHTS Universal DNA Library Prep Kit for Illumina V3 (Vazyme #ND607) to prepare two

DNA libraries with the insert size about 300 bp (total library size is about 420 bp). The size and concentration of the libraries are analysed by Agilent Bioanalyzer 2100 High Sensitivity DNA Assay, refer to Table 2.

2. Library dilution

An initial 1:10,000 dilution and one additional 2-fold (i.e., 1:20,000) dilution of each library was prepared.

a. Replicate data points should differ by ≤0.2 cycles. Filter the original C_T of DNA Standards. Remove the third data of DNA Standard 1 and the first 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. The average C_T values for each DNA Standard is plotted against log[pM] to generate a standard curve.

Table 1. C_T values of DNA Standards

DNA Standard	Log[pM]	C_T	Average C_T	Log[pM]
1	Log[20]	8.18 8.23 ~~7.87~~	8.20	-
2	Log[2]	11.74 11.69 11.71	11.71	3.50
3	Log[0.2]	15.21 15.22 15.23	15.22	3.51
4	Log[0.02]	18.76 18.70 18.66	18.71	3.49
5	Log[0.002]	22.12 22.07 22.10	22.10	3.39
6	Log[0.0002]	~~25.74~~ 25.56 25.55	25.55	3.46
NTC	-	Undetermined 34.84 33.64	34.24	-

* ΔC_T should be between 3.1 and 3.6.

3. Library concentration calculation

a. Outliers (>0.2 C_T difference) for the 1/10,000 dilution of Library 1 and of Library 2 were excluded, and calculate the average C_T (Table 2, Row 5).

b. Calculate the concentration of the diluted library based on the standard curve (Table 2, Row 7).

c. Refer to the average size of the library, calculate the size-adjusted concentration of each library dilution (Table 2, Row 8).

d. Calculate the initial library concentration of each dilution (Table 2, Row 9) and the average initial library concentration (Table 2, Row 11) based on the dilution factor.

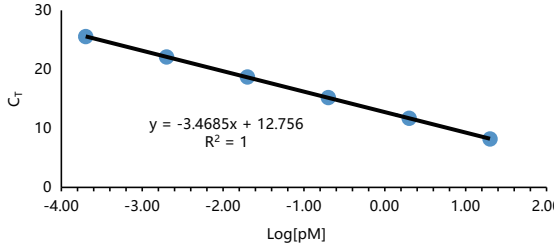

Fig 2. Standard Curve

Table 2. Library Quantitative Data

	Parameter	Library 1		Library 2	
1	Average fragment length (Bioanalyzer)	420 bp		433 bp	
2	Estimated concentration (Bioanalyzer)	21.30 ng/μl = 82.08 nM		6.78 ng/μl = 25.33 nM	
3	Dilutions for qPCR	1/10,000	1/20,000	1/10,000	1/20,000
4	Triplicate C_T values	9.58 ~~9.24~~ 9.49	10.57 10.55 10.58	11.46 ~~11.63~~ 11.44	12.52 12.43 12.48
5	Average C_T	9.54	10.57	11.45	12.48
6	ΔC_T	1.03		1.03	
7	Average concentration for sample dilution calculated using standard curve (pM)	8.47	4.28	2.38	1.20
8	Average size-adjusted concentration for library dilution (pM)	9.11	4.60	2.48	1.25
9	Average final calculated concentration of undiluted library dilution (nM)	91.13	92.10	24.82	25.10
10	Deviation between final concentrations calculated from different dilutions	1.06%		1.11%	
11	Working concentration	91.62 nM = 23.78 ng/μl		24.96 nM = 6.68 ng/μl	

10/FAQ & Troubleshooting

◇ Efficiency not in specified range (90 - 110%)

① If C_T (NTC) - C_T (DNA Standard 6) <3 or C_T (DNA Standard 6) - C_T (DNA Standard 5) <3.1, and the efficiency above 100% may be indicative of contamination. Examine melt curves to determine the source of contamination (DNA standard or library DNA). When generating 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 by contamination and use the remaining C_T values to plot the standard curve.

② Baseline setting may delay C_T value for DNA Standard 1, affecting efficiency. Adjust baseline manually to 1 - 3 cycles.

③ Poor pipetting accuracy.

◇ R^2 <0.99

① Poor pipetting accuracy.

② Ensure that all reagents are thoroughly mixed before use.

◇ Standard curve is not spaced correctly

① If C_T (DNA Standard 6) - C_T (DNA Standard 5) <3.1 is indicative of contamination. Examine melt curves to determine whether contaminant is DNA standard or library DNA.

② If C_T (DNA Standard 2) - C_T (DNA Standard 1) <3.1 may be indicative of problems with background subtraction. Adjust the baseline manually to 1 - 3 cycles.

③ If ΔC_T of >3.6 points to poor reaction efficiency. Ensure that all reagents are thoroughly mixed before use. Confirm that all reaction components were added at the correct concentration, and that the correct cycling protocol was used.

④ Severe light exposure of VAHTS SYBR qPCR Master Mix 2.0 will reduce total fluorescence and may result in a delay of C_T values, resulting in ΔC_T values of >3.6.

◇ Poor reproducibility of between replicates

① Poor pipetting accuracy.

② Ensure that all reagents are thoroughly mixed before use.

◇ ΔC_T of library dilutions is not within expected range (0.9 - 1.1 for 2-fold dilutions)

① Poor pipetting accuracy.

② Ensure that all reagents are thoroughly mixed before use.

③ Library is challenging to amplify, i.e., extremely GC- or AT-rich, or has an average fragment length >1 kb.

④ Library has degraded. Prepare fresh library dilutions for each qPCR-based quantification assay. Keep them on ice before use and discard after use.

◇ Concentrations calculated from different library dilutions differ by more than 10%

① Poor pipetting accuracy.

② Ensure that all reagents are thoroughly mixed before use.

③ Library is challenging to amplify, i.e., extremely GC- or AT-rich, or has an average fragment length >1 kb.

④ Library has degraded. Prepare fresh library dilutions for each qPCR-based quantification assay. Keep them on ice before use and discard after use.

◇ Library dilutions do not fall within dynamic 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.

◇ DNA standard 1 amplification plot appears abnormal

① Baseline setting may delay C_T value for DNA Standard 1, affecting efficiency. Adjust baseline manually to 1 - 3 cycles.

◇ DNA standards amplify, but libraries do not, or very late amplification of libraries

① Library does not contain the appropriate adapter sequences for quantification primers to bind to.

② Gross error with initial dilution: It is recommended to decrease the dilution factor and repeat the experiment.

③ Library has degraded: Prepare fresh library dilutions for each qPCR-based quantification assay. Keep them on ice before use and discard after use.

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