# GenNext™ NGS Library Quantification Kit

NLQ-101 500 reactions

**Store at –20°C, protected from light**

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**CAUTION**

All reagents in this kit are intended for research purposes. Do not use for diagnosis or clinical purposes. Please observe general laboratory precautions and safety procedures while using this kit.



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**FOR RESEARCH USE ONLY. NOT FOR HUMAN OR DIAGNOSTIC USE.**

## [1] Introduction

### Description

GenNext™ NGS Library Quantification Kit is for the SYBR® Green I qPCR-based library quantification of Illumina next-generation sequences. The kit allows the specific and accurate quantification of libraries bearing P5 and P7 adaptors which can be applied to flow cell amplification. It uses the highly efficient qPCR master mix KOD SYBR® qPCR Mix.

### Features

- **Accurate quantification**
  KOD SYBR® qPCR Mix can efficiently amplify GC- and AT-rich fragments of different lengths without bias.

- **Broad dynamic range**
  The kit has a broad dynamic range from 20 pM (Standard DNA 1) to 0.0002 pM (Standard DNA 6).

- **Convenient**
  The kit contains all reagents (KOD SYBR® qPCR Mix, 5× Primer Mix, Standard DNA, and 50× Dilution buffer) needed for the qPCR-based quantification of an NGS library.

### About KOD SYBR® qPCR Mix

KOD SYBR® qPCR Mix is a highly efficient 2× master mix for real-time PCR using SYBR® Green I that is based on the 3′→5′ exonuclease-deficient KOD DNA polymerase. The master mix contains all required components, except for the ROX reference dye and primers (50× ROX reference dye is supplied separately). This product has been optimized for efficiency and robustness in the SYBR® Green assay.

## [2] Components

This kit includes the following components, sufficient for 500 reactions, with a total of 20 µl per reaction. All reagents should be stored at –20°C.

**< KOD SYBR® qPCR mix* >**

| | |
|---|---|
| KOD SYBR® qPCR mix | 1.67 mL × 3 |
| 50× ROX reference dye | 250µL×l |

* KOD SYBR® qPCR mix (Code no. QKD-201) is supplied.

**< Standard & Primer Set >**

| | |
|---|---|
| Standard DNA 1 (20 pM) | 200 µL |
| Standard DNA 2 (2 pM) | 200 µL |
| Standard DNA 3 (0.2 pM) | 200 µL |
| Standard DNA 4 (0.02 pM) | 200 µL |
| Standard DNA 5 (0.002 pM) | 200 µL |
| Standard DNA 6 (0.0002 pM) | 200 µL |
| 5× Primer Mix | 2 mL (2 × 1 mL) |
| 50× Dilution Buffer | 1.7 mL |

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**Notes:**

**KOD SYBR® qPCR mix**

This reagent can be stored, protected from light, at 2–8°C for up to 3 months. For longer storage, it should be kept at –20°C and protected from light. No negative effect was detected following 10 freeze–thaw cycles of KOD SYBR® qPCR Mix. This reagent does not contain the ROX reference dye.

**50× ROX reference dye**

This reagent can be stored, protected from light, at 2–8°C or –20°C. For real-time cyclers that require a passive reference dye, this reagent must be added to the reaction mixture at a concentration of 1× or 0.1×. The master mix solution with the ROX reference dye can be stored, protected from light, at 2–8°C for up to 3 months. For longer storage, it should be kept at –20°C and protected from light. The pre-mixed reagents can be prepared according to the following ratios [4]. Table 2 shows the optimal concentration of the ROX dye.

1× solution
KOD SYBR® qPCR Mix: 50× ROX reference dye = 1.67 mL: 66.8 µL

0.1× solution
KOD SYBR® qPCR Mix: 50× ROX reference dye = 1.67 mL: 6.7 µL

For real-time cyclers that do not require a passive reference dye, KOD SYBR® qPCR Mix can be used without the ROX reference dye.

**Standard DNA**

Six pre-diluted standard DNAs (20, 2, 0.2, 0.02, 0.002, and 0.0002 pM) are supplied. The standard DNA contains the P5 and P7 flow cell oligo sequences used in the library for Illumina next-generation sequencing. The amplicon size is 452 bp.

**5× Primer Mix**

The vial contains the following primers which can amplify targets containing P5 and P7 flow cell oligo sequences.

Forward primer: 5′-ATACGGCGACCACCGAGATC-3′
Reverse primer: 5′-CAAGCAGAAGACGGCATACGAG-3′

The mixture of 5× Primer Pix, 50× ROX reference dye, and KOD SYBR® qPCR Mix should be stored according to the conditions described above for KOD SYBR® qPCR Mix.

**50× Dilution Buffer**

1× Dilution Buffer can be used in the library dilution step. The buffer can be stored at 4°C.

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## [ 3 ] Dilution of library

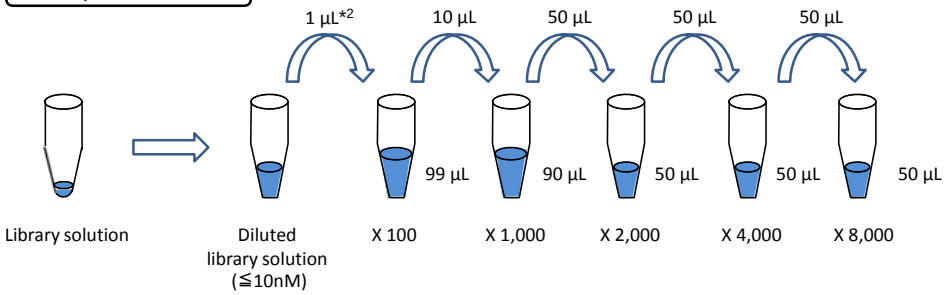
(1) Preparation of 1× Dilution Buffer

1× Dilution Buffer can be prepared by diluting 50× Dilution Buffer 1:50. The composition of 1× Dilution Buffer is as follows: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1% Tween 20. 1× Dilution Buffer can be stored at 4°C. The buffer should only be used in the library dilution step.

(2) Dilution of libraries

The libraries should be diluted so that the Ct values will be in the range of standard DNA 1–6 (20–0.0002 pM = 5.5–0.000055 pg/µl).
First, the library should be diluted ≤10 nM[*1] (≤2.7 ng/µl) using 1× dilution buffer, then diluted 100, 1,000, 2,000, 4,000, and 8,000 times. The diluted libraries (×1,000–×8,000) should be analyzed.

Example of dilution




*1 Libraries should be diluted to ≤10 nM based on the absorbance measured by a spectrophotometer such as NanoDrop™, Qubit®, or Bioanalyzer. If the average size of the library is 450 bp, 2.7 ng/µl library corresponds to 10 nM. If the average size of the library is 225 bp, 2.7 ng/µL library corresponds to 20 nM (10 [nM] × 450 [bp] / 225 [bp]).

*2 Small volumes of solution should be dispensed carefully and accurately.

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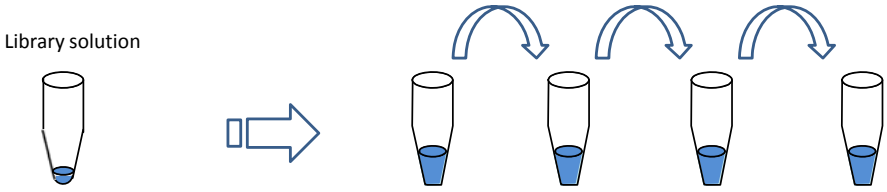
Table I can be referred to when the library concentration cannot be estimated.

**Table 1** Typical library type and recommended dilution range

| Library type | Recommended dilution range |
|---|---|
| Library prepared by PCR for whole-genome sequencing, ChIP-seq, RNA-seq, or Amplicon-seq | ×10,000, ×20,000, ×40,000, ×80,000 |
| Library prepared by target capture method | ×100,000, ×200,000, ×400,000, ×800,000 |
| Library prepared without PCR for whole-genome sequencing etc.<br><br>(1) Library from 100 ng – 1 μg DNA input<br>(2) Library from 10 ng – 100 ng DNA input<br>(3) Library from ≤10 ng DNA input | <br><br>(1) ×5,000, ×10,000, ×20,000, ×40,000<br>(2) ×100, ×200, ×400, ×800<br>(3) ×2, ×4, ×8 |




| | | | | |
|---|---|---|---|---|
| Library prepared with PCR | X 10,000 | X 20,000 | X 40,000 | X 80,000 |
| Library prepared with Target capture method | X 100,000 | X 200,000 | X 400,000 | X 800,000 |
| Library prepared without PCR | | | | |
| (1) Library from 100 ng - 1 μg DNA input | X 5,000 | X 10,000 | X 20,000 | X 40,000 |
| (2) Library from 10 ng - 100 ng DNA input | X 100 | X 200 | X 400 | X 800 |
| (3) Library from =10 ng DNA input | X 2 | X 4 | X 8 | |

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## [4] Protocol

### 1. Reaction mixture set-up

| Reagent | Final concentration |
|---|---|
| DW | 1.6/ 1.96 µL |
| KOD SYBR® qPCR Mix | 1 0 µL |
| 50× ROX reference dye | 0.4 µL / 0.04 µL* |
| 5× primer mix | 4 µL |
| DNA solution | 4 µL |
| Total | 2 0 µL |

* 50× ROX reference dye must be added when using real-time cyclers that require a passive reference dye (i.e., Applied Biosystems, Agilent), according to Table 2. Table 2 shows the optimum concentration of the ROX reference dye. This dye is not necessary for real-time cyclers that do not require a passive reference dye.

**Table 2** Recommended ROX dye concentration

| Real-time cycler | Optimal dye concentration (dilution ratio) |
|---|---|
| Applied Biosystems 7000, 7300, 7700, 7900HT StepOne™, StepOnePlus™ etc. | 1× (50:1) |
| Applied Biosystems 7500, 7500Fast, Agilent Technologies cyclers (Option) etc. | 0.1× (500:1) |
| Roche cyclers, Bio-Rad cyclers, BioFlux cyclers Rotor-Gene etc. | Not required |

**Notes**

The pre-mixed reagents can be prepared according to the following ratios:

1× solution
KOD SYBR® qPCR Mix: 50× ROX reference dye = 1.67 mL: 66.8 µL

0.1× solution
KOD SYBR® qPCR Mix: 50× ROX reference dye = 1.67 mL: 6.7 µL

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## 2. PCR cycling conditions

The following table shows the recommended thermal conditions. The melting/ dissociation curve analytical conditions should be determined according to the instruction manual of the real-time cycler.

| <3-step cycle> | Temperature | Time | Ramp |
|---|---|---|---|
| Pre-denaturation | 98°C | 2 min*1 | Maximum |
| Denaturation: | 98°C | 10 s | Maximum |
| Annealing | 65°C*2 | 10 s | Maximum |
| Extension: | 68°C | 30 s*2 | Maximum |
| (35 cycles) | | | |
| | <data collection should be set at the extension step> | | |
| Melting / dissociation curve analysis | | | |

*1 Pre-denaturation can be completed within 2 min because of the anti-KOD antibody hot-start PCR system.

*2 The extension time should be set to 45 s when the average library size is ≥600 bp.

## 3. Reduction of non-specific amplification

Specificity may be improved using the following cycling conditions when non-specific amplification is detected by melting curve analysis.

| <2-step cycle> | Temperature | Time | Ramp |
|---|---|---|---|
| Pre-denaturation | 98°C | 2 min | Maximum |
| Denaturation: | 98°C | 10 s | Maximum |
| Extension: | 68°C | 30 s | Maximum |
| (35 cycles) | | | |
| | <data collection should be set at the extension step> | | |
| Melting / dissociation curve analysis | | | |

The peak around 82°C in the melting curve assay may be caused by the presence of adaptor dimers. These can be distinguished by measuring their size using capillary or gel electrophoresis. They are generated as a by-product during the library construction step. Because they prevent the accurate quantification of libraries, they should be removed by library re-purification.

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## [ 5 ] Data Analysis

(1) Calculation of the concentration of the diluted library

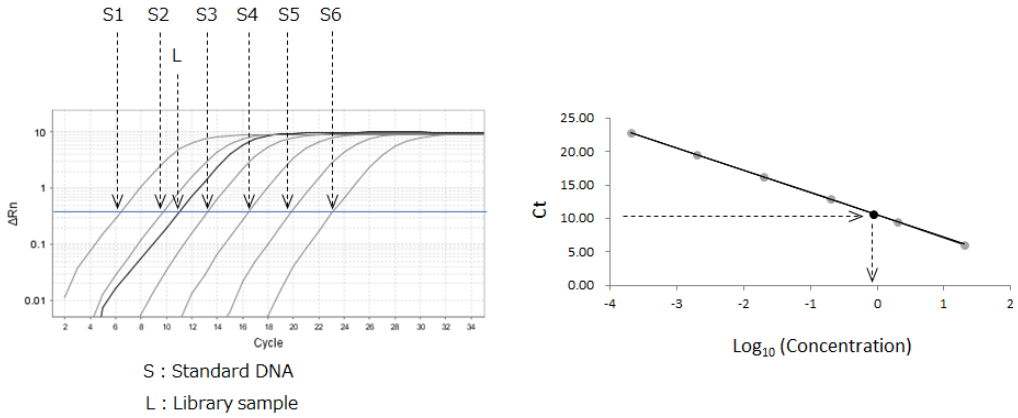
The standard curve should be prepared by plotting the Ct values against the logarithm of the DNA concentrations (20–0.0002 pM). Abnormal Ct values should be excluded. The library concentration should be determined using the Ct values obtained from a diluted library solution that has the maximum concentration in the dynamic range of the assay.

(2) Determination of the library concentration

The concentration of the library should be determined using the following formula when the concentration is estimated using the standard DNA (452 bp).

$$\text{Library concentration (nM)} = \frac{[\text{Library concentration (pM) from (1)}] \times [452]^{*} \times [\text{dilution factor}]}{[1{,}000] \times [\text{Average fragment length of library in bp}]}$$

* Size of DNA standard in bp




S : Standard DNA

L : Library sample

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## [ 6 ] Troubleshooting

| Symptom | Cause | Solution |
|---|---|---|
| Low cluster density | Inappropriate preparation of standard curve | Clearly abnormal Ct values should be excluded from preparation of the standard curve. |
| | Insufficient pipetting | All pipetting steps should be performed carefully and thoroughly. |
| | Inappropriate dilution of libraries | The library solution should be diluted so that its Ct values will be in the range of standard DNA 1–6. |
| | Generation of non-specific amplification | See [4] 3 |
| | Adaptor dimers in the library | See [4] 3 |
| No amplification from libraries | Non-Illumina NGS library | The product is only for use with Illumina NGS libraries bearing P5 and P7 adaptor sequences. |
| Low fluorescent intensity or distorted amplification curve | Excessive ROX reference dye | Excessive ROX reference dye results in a low signal intensity in the SYBR® Green assay. Rox reference dye should be used according to [4] 1. |
| | Insufficient data collection time | Too short a data collection time results in an insufficient fluorescent intensity. An appropriate extension time (45–60 s) can improve this. |
| | Insufficient reaction volume | Too small a reaction volume tends to enhance detection errors. The appropriate reaction volume should be used according to the instruction manual of the real-time cycler. |

## [ 7 ] Related products

| Product name | Package | Code No. |
|---|---|---|
| High efficient real-time PCR master mix for SYBR® Green I assay **KOD SYBR® qPCR Mix** | 1.67 mL × 3 | QKD-201 |

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