STANDARD OPERATING PROTOCOL · EnGeL-MOLB-KAPA

Illumina library quantification by qPCR (KAPA Library Quantification Kit)

Protocol ID	EnGeL-MOLB-KAPA
Version	1.2.0
Date	2026-11-20
Status	draft
Area	Molecular biology (MOLB)
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Licence	Text: CC BY 4.0 · Code: MIT
Main hazards	 KAPA Library Quantification Kit: may cause damage to organs (H371; kit safety data sheet)

1 Purpose and scope

This protocol determines the molar concentration of Illumina sequencing libraries (pooled amplicon libraries) by qPCR, before dilution for sequencing. It is used at the end of library preparation (EnGeL-MOLB-MB). It measures only molecules carrying the Illumina P5/P7 adapter sequences.

2 Principle

The library is diluted into the range of six DNA standards of known concentration. Library and standards are amplified with primers that bind the P5 and P7 adapter sequences, using a SYBR Green qPCR master mix. The Ct values of the standards give a standard curve, from which the concentration of each library dilution is read and corrected for the difference in size between the standard (452 bp) and the library (KAPA Library Quantification Kit, Roche).

3 Before you start

Item	Details
Hands-on time	1 h
Total time	2 h 30 min
Sample	1 µL of the pooled, bead-cleaned library (for the 1:1 000 intermediate dilution); average fragment size measured on the TapeStation; PicoGreen or Qubit concentration known
Prepare in advance	Combined Master Mix + Primer Mix (first use of the kit); plate layout; standards thawed on ice
Key safety points	See Safety and waste

4 Materials

4.1 Equipment

- qPCR instrument: QuantStudio 3 (Applied Biosystems)
- Pipettes and filter tips
- Vortex mixer and mini-centrifuge

4.2 Consumables

- qPCR plate and optical seal
- Low-binding tubes

4.3 Reagents and kits

- KAPA Library Quantification Kit for Illumina (Roche) [TO CONFIRM: catalogue number], containing:
  - KAPA SYBR FAST qPCR Master Mix (2X)
  - Primer Mix (10X)
  - DNA Standards 1–6
- Library dilution buffer: 10 mM Tris-HCl pH 8.0–8.5 + 0.05% Tween 20 (called “TE pH = 8” in the source) [TO CONFIRM: name and recipe]
- PCR-grade water
- Positive control, 200 nM [TO CONFIRM: product and catalogue number]

5 Reagent preparation

- **Combined Master Mix + Primer Mix:** see the first steps of the Procedure (first use of the kit only).
- **Library dilution buffer:** 10 mM Tris-HCl pH 8.0–8.5 + 0.05% Tween 20 [TO CONFIRM: preparation, storage and shelf life].

6 Procedure

6.1 Before starting

- Clean the working space and material with bleach and water and leave the UV light on for at least 15 min.
- Use filter tips at all steps.
- Clean forceps and spatulas with 3% sodium hypochlorite and 96% ethanol between samples.

6.2 Quantification

Kit preparation (first use)

1. Add the 1 mL Primer Mix (10X) to the 5 mL bottle of SYBR FAST Master Mix (2X); vortex briefly.
2. Store the combined mix at −20 °C (or 4 °C for short-term use, per kit insert); keep away from light.
3. Thaw the standards on ice, vortex and spin down. Do not dilute the standards further.

Library dilutions (serial, in library dilution buffer)

Dilution	Make from	Volume taken (µL)	Dilution buffer (µL)	Use
1:1 000	Pool	1	999	Intermediate only
1:10 000	1:1 000	100	900	Run in qPCR
1:20 000	1:1 000	50	950	Run in qPCR
1:100 000 (optional)	1:10 000	10	90	If PicoGreen > c. 50 nM

4. Prepare the dilutions above. Vortex and spin each dilution before taking the next step. Check against the PicoGreen value: the diluted library should be about 0.2–20 pM, inside the standard range.

Positive control: treat the positive control like a library. It starts at 200 nM and is diluted in the same series: 1:1 000 (intermediate), then 1:10 000 and 1:20 000, which should give about 20 pM and 10 pM in the reaction.

Reaction set-up (20 µL per well)

Component	Per well (µL)
Master Mix + Primer Mix (combined)	12
PCR-grade water	4
Diluted library, standard or NTC buffer	4

5. Prepare a master mix of 12 µL combined mix + 4 µL water per well, plus 10% excess.
6. Dispense 16 µL per well, then add 4 µL of diluted library, standard or dilution buffer (for the NTC).
7. Seal, spin briefly, and keep the plate in the dark until loading.

Plate layout (96-well): Standards 1–6 in triplicate (18 wells); NTC (dilution buffer as template) in duplicate (2 wells); positive control and each library at 1:10 000 and 1:20 000 in triplicate (6 wells each). With the positive control, up to 11 libraries fit on one plate (18 + 2 + 6 + 6 × 11 = 92 wells). In the software, enter the standards as *Standard* with quantities 20, 2, 0.2, 0.02, 0.002 and 0.0002 pM (S1–S6).

Cycling

Step	Temperature	Time	Cycles
Initial denaturation	95 °C	5 min	1
Denaturation	95 °C	30 s	35
Annealing/extension + read	60 °C	45 s	
Melt curve	65 °C, then to 95 °C at 0.15 °C/s	15 s at each temperature	1

Ramp rate 1.6 °C/s for all other steps (instrument settings in use).

8. Run the plate on the qPCR instrument (QuantStudio 3) with the cycling above.
9. Export the results as .csv (tab *Export*) and check the run against the run QC criteria before calculating.

6.3 After finishing

- Clean the bench with 70% ethanol.
- Empty the small bench bin into the right waste bin.
- Wash your hands.
- Turn off equipment and lights.
- Lock doors, fridge and freezer.

7 Calculations

On the *Results* sheet of the exported file, use the **Quantity Mean** (pM) reported for each dilution:

$$c_{\mathrm{lib}} = \frac{Q \times \frac{452}{L} \times D}{1000}$$

- c_{lib}: library concentration (nM)
- Q: Quantity Mean reported by the software for that dilution (pM)
- 452: size of the kit DNA standard (bp)
- L: average fragment size of the library (bp), from the TapeStation
- D: dilution factor (e.g. 20 000; dimensionless)
- 1000: conversion from pM to nM

Calculate c_{lib} for each valid dilution, then average them.

Worked example (illustrative values): Quantity Mean 1.5 pM at 1:20 000, library size 595 bp: 1.5 × (452 / 595) × 20 000 / 1000 = 22.8 nM.

8 Quality control

Accept the run before calculating:

Check	Accept
Standard curve efficiency	90–110% (slope −3.6 to −3.1)
Standard curve R^2	≥ 0.99
ΔCt between consecutive standards	c. 3.3 (3.1–3.6)
Replicate Ct SD	≤ 0.2–0.3; omit a clear outlier
NTC	Undetermined
ΔCt between 1:10 000 and 1:20 000	c. 1.0 (0.8–1.2)
Sample Ct	Within the Ct range of Standards 1–6
Melt curve	Single peak, no primer-dimer peak
Positive control (200 nM)	Measured within ±20% of 200 nM (160–240 nM)

Expected results

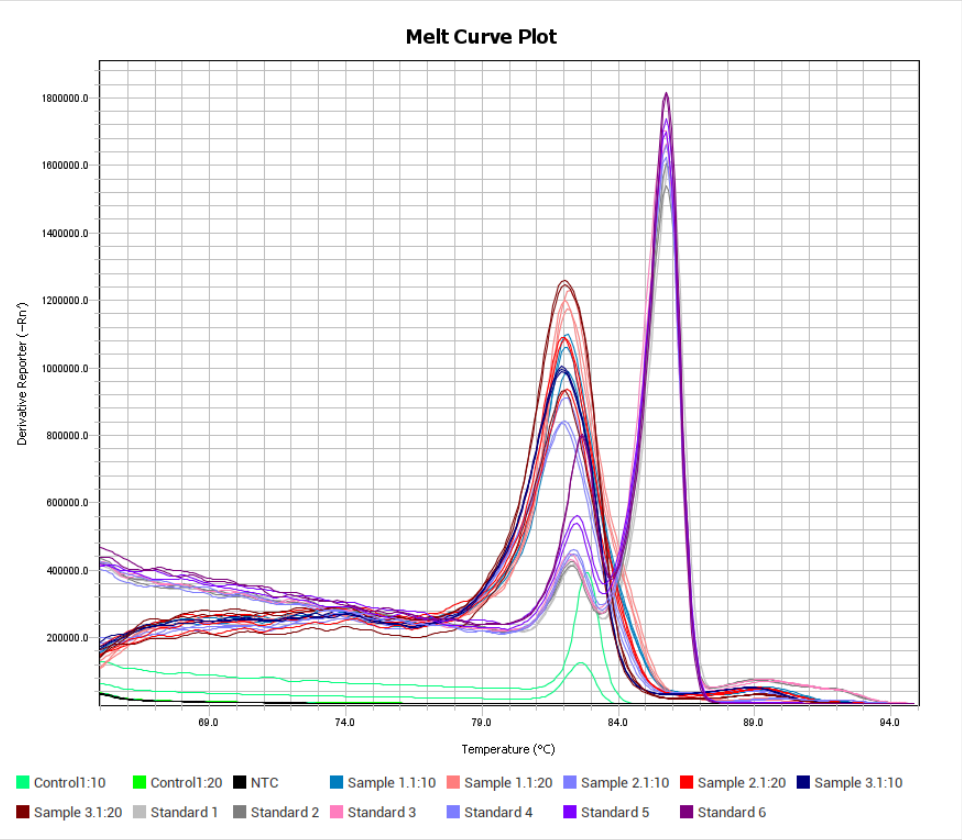

Figure 1: Melt curve of a good run: the libraries (here three pools at 1:10 000 and 1:20 000, labelled “1:10” and “1:20” in the software) and the positive control give one peak each, the DNA standards a single peak at a slightly higher temperature, and the NTC stays flat. Data: EnGeL-UC (Andreia Quaresma).

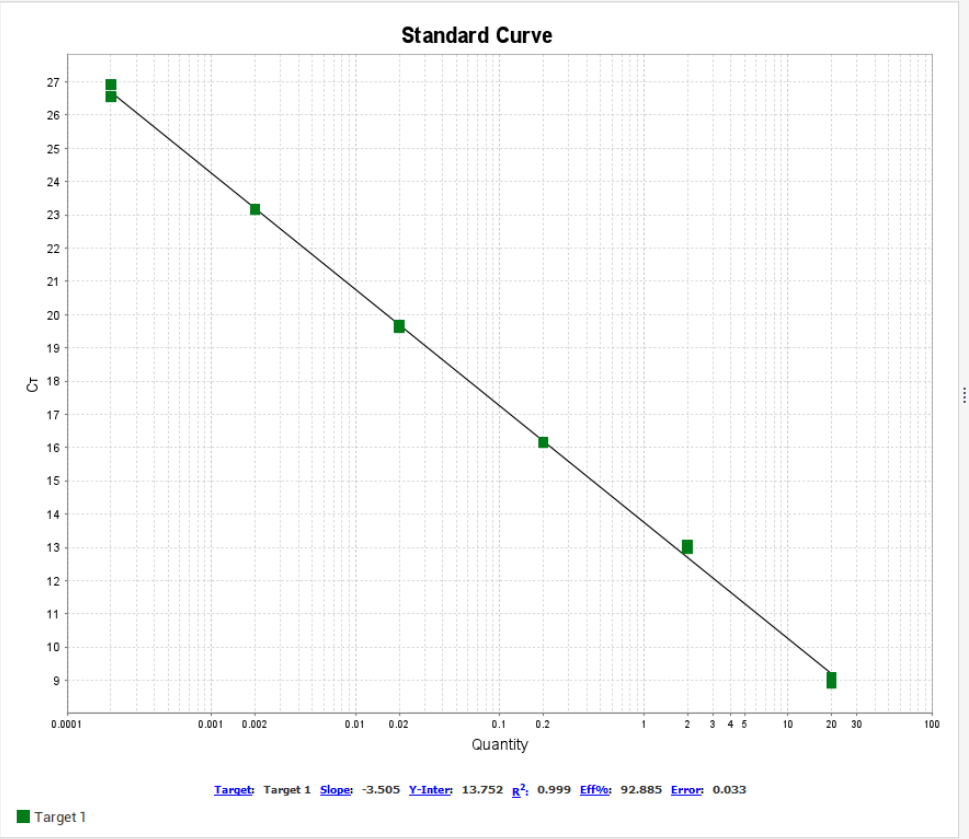

Figure 2: Standard curve of a good run (Standards 1–6 in triplicate): slope −3.505, R^2 0.999, efficiency 92.9%, within the acceptance criteria above. Data: EnGeL-UC (Andreia Quaresma).

9 Data and metadata

- **Outputs:** exported qPCR results (.csv); library concentration (nM) per library, averaged over valid dilutions.
- **Where:** the *Library_QC* sheet of the Metabarcoding Lab Pipeline workbook [TO CONFIRM] and the lab book; instrument use in the QuantStudio 3 user log.

Minimum to record

- Library / pool ID and EnGeL label
- Run date, operator, instrument (QuantStudio 3)
- KAPA kit lot number
- Dilutions run; Quantity Mean per dilution; library size used (bp); final concentration (nM)
- Run QC values (efficiency, R^2, NTC)

10 Safety and waste

Follow the manufacturer's instructions and safety data sheets for each reagent. Biological samples are potentially hazardous and are handled following the facility's procedures. EnGeL lab rules:

- Before you enter: wear your lab coat, use appropriate gloves, closed shoes only, tie back long hair.
- Not allowed: eating or drinking; touching phones or laptops with contaminated gloves; bags or coats on benches.
- Label all reagents (name, concentration, date) and treat all chemicals as hazardous.

Waste

EnGeL waste streams (choose the right bin):

- **Biological solid waste** (black bin, white bag): contaminated gloves, contaminated disposable materials, solid biological laboratory waste, contaminated PPE. No liquids or sharps.
- **Specific hazardous waste** (red bin, red bag): chemicals and pharmaceuticals designated for this waste stream, specific hazardous laboratory waste, anatomical material; sharps only in a rigid sharps container. No loose sharps.
- **Biological liquid waste** (jerrican, UN 3291): liquid biological waste and contaminated biological liquids only. Use a funnel; rinse the original container and the funnel with dH_2O and discard the rinse water in the same jerrican.

For this protocol: used qPCR plates, tips and tubes go to biological solid waste (black bin) [TO CONFIRM].

11 Troubleshooting

Problem	Likely cause	Solution
Diluted library outside the standard range	Wrong dilution for the library concentration	Use the PicoGreen value to choose dilutions; add the 1:100 000 dilution if PicoGreen > c. 50 nM.
ΔCt between dilutions not c. 1.0	Pipetting or dilution error	Repeat the dilutions, vortexing and spinning each one.
Extra melt-curve peak	Primer dimers or non-specific product	[TO CONFIRM] see also the kit technical data sheet (Roche, KR0405).
Signal in the NTC	Contamination	[TO CONFIRM]

12 Remarks

The standard is 452 bp; the size correction uses the average fragment size measured on the TapeStation.

13 References

Roche (n.d.). KAPA Library Quantification Kit – Illumina Platforms. Technical data sheet KR0405, v8.17. Troubleshooting: https://rochesequencingstore.com/wp-content/uploads/2017/10/KAPA-Library-Quantification-Kit-for-Illumina.pdf

14 Version history and citation

Version	Date	Change	By
1.0.0	2026-11-16	First EnGeL-UC version, from the lab protocol “KAPA qPCR library quantification” (2026).	Luís Cunha
1.0.1	2026-11-17	Contributors: Luísa Fraga and Andreia Quaresma.	Luís Cunha
1.1.0	2026-11-17	Updated from Andreia Quaresma’s v1.0 (2026-11-05): times, sample volume, positive control treated like a library (200 nM, 1:1 000 then 1:10 000 and 1:20 000), diluted library range 0.2–20 pM, melt-curve and ramp settings, plate capacity, hazard	Luís Cunha

Version	Date	Change	By
		H371 (GHS08), technical data sheet KR0405 v8.17, expected-results figures (melt curve, standard curve).	
1.1.1	2026-11-17	Authorship: all who contributed are authors, with their roles; lead author first where identified, reviewer (Luís Cunha) last.	Luís Cunha
1.2.0	2026-11-20	Positive-control acceptance criterion confirmed by Luís Cunha: within ±20% of 200 nM (160–240 nM).	Luís Cunha

How to cite

Quaresma A, Fraga L, Cunha L (2026). Illumina library quantification by qPCR (KAPA Library Quantification Kit). EnGeL-UC protocol EnGeL-MOLB-KAPA, version 1.2.0. Zenodo. DOI [TO CONFIRM].

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15 Open questions

1. Authorship (author or contributor model for the handbook, pending); DOI.
2. Kit catalogue number.
3. Positive control: product and catalogue number.
4. Library dilution buffer: the source calls it “TE pH = 8” but gives 10 mM Tris-HCl + 0.05% Tween 20 (no EDTA). Name, preparation and storage?
5. Where results are recorded (Lab Pipeline *Library_QC* sheet?).
6. Troubleshooting: action for extra melt-curve peaks and for signal in the NTC.
7. Waste route for used plates.