KAPABIOSYSTEMS

Technical Data Sheet

KAPA HiFi HotStart Real-Time Library Amplification Kit

KR0409 – v10.19

This Technical Data Sheet provides product information and a detailed protocol for the KAPA HiFi HotStart Real-Time Library Amplification Kit.

The document applies to KAPA HiFi HotStart Real-Time Library Amplification Kit (07959028001), and KAPA HiFi HotStart Real-Time Library Amplification Standards Kit (07959036001).

Contents

Product Description 2

Product Specifications 2

Overview 3

Library Amplification Protocol 4

Restrictions and Liabilities. 6

Note to Purchaser: Limited Product Warranty 6

Note to Purchaser: Limited License 6

Kapa/*Roche* Kit Codes and Components		
KK2702 *07959028001* (250 x 50 µL reactions)	KAPA HiFi HotStart Real-Time PCR Master Mix (2X) Fluorescent Standards 1 – 4	1 x 6.25 mL 4 x 1.5 mL
KK2709 *07959036001*	Fluorescent Standards 1 – 4	4 x 1.5 mL

Quick Notes

- Real-time monitoring of library amplification provides a means for carefully controlling the number of cycles used when amplifying libraries prepared from precious samples.
- KAPA HiFi HotStart Real-Time PCR Master Mix (2X) contains KAPA HiFi DNA Polymerase, engineered for increased processivity and high fidelity, in a formulation optimized for real-time PCR.
- The error rate of KAPA HiFi DNA Polymerase (as determined by 454 sequencing) is 1 error per 3.6 x 10^6 nucleotides incorporated.
- The KAPA HiFi HotStart Real-Time Library Amplification Kit is compatible with KAPA Library Amplification Primer Mix Kit (07958994001; sold separately) and recommended for the real-time amplification of Illumina® libraries flanked by P5 and P7 flow cell sequences.
- Optimal amplification for NGS applications corresponds to the region between Fluorescent Standards 1 and 3. The termination cycle number should be adjusted accordingly without the requirement for performing gel electrophoresis (see Figure 1B, p. 3).
- KAPA HiFi HotStart Real-Time PCR Master Mix (2X) and Fluorescent Standards 1 – 4 are light-sensitive and should be protected from light during storage, thawing, and reaction setup.
- To minimize background fluorescence due to inter- and intra-primer interaction, it is critical to use the correct data acquisition temperature.
- Performing a gradient PCR to optimize the annealing temperature is recommended when using custom primers.
- The KAPA HiFi HotStart Real-Time Library Amplification Kit is compatible with the Nextera® Sample Preparation protocol. Use a denaturation temperature of 98°C.

KAPA HiFi HotStart Real-Time Library Amplification Kit

Technical Data Sheet

Product Description

KAPA HiFi HotStart Real-Time Library Amplification Kits are designed for the amplification of next-generation sequencing NGS libraries prepared for Illumina sequencing. In order to maximize sequence coverage uniformity, it is critical to minimize library amplification bias. Amplification bias occurs when a DNA polymerase is unable to amplify all targets within a complex population of library DNA with equal efficiency. Bias is further exacerbated when libraries are excessively amplified.

KAPA HiFi DNA Polymerase is designed for low-bias, high-fidelity PCR, and is the reagent of choice for NGS library amplification.[1,2,3,4] Real-time monitoring of library amplification provides a means for carefully controlling the number of cycles used when amplifying libraries prepared from precious samples. Benefits of performing high-fidelity, real-time PCR for next-generation sequencing library amplification include:

- Real-time monitoring of amplification allows precise control over the optimal number of PCR cycles.
- Real-time amplification workflows are amenable to automation.
- Real-time amplification plots provide quality metrics for individual enriched libraries, eliminating expensive and time-consuming post-amplification gel electrophoresis and identifying inconsistencies in library preparation.
- Seamless integration with KAPA Library Quantification Kits.

KAPA HiFi HotStart Real-Time Library Amplification Kits contain KAPA HiFi HotStart Real-Time PCR Master Mix (2X), a ready-to-use cocktail containing all components for PCR, except primers and template. The master mix contains KAPA HiFi HotStart DNA Polymerase in a proprietary reaction buffer, dNTPs, $MgCl_2$ (2.5 mM at 1X), SYBR® Green I dye and stabilizers. Four fluorescent standards are supplied, and are used to define a window for optimal amplification (Figures 1B and 2).

KAPA HiFi HotStart DNA Polymerase is an antibody-based hot start formulation of KAPA HiFi DNA Polymerase, a B-family DNA polymerase exhibiting extremely high fidelity in comparison with other high-fidelity DNA polymerases and polymerase blends. In the HotStart formulation, a proprietary antibody inactivates the polymerase until the first denaturation step. This eliminates spurious amplification products resulting from non-specific priming events during reaction setup and initiation and increases overall reaction efficiency.

KAPA HiFi HotStart DNA Polymerase has 5'→3' polymerase and 3'→5' exonuclease (proofreading) activities, but no 5'→3' exonuclease activity. The strong 3'→5' exonuclease activity results in extremely high accuracy during DNA amplification. The error rate of KAPA HiFi HotStart DNA Polymerase is ~2.8 x 10^{-7}.

This fidelity is approximately 100 times higher than that of wild-type *Taq* DNA polymerase, and up to 10 times higher than that of other B-family DNA polymerases and polymerase blends. The presence of SYBR Green I dye in the reaction does not compromise fidelity. DNA fragments generated with KAPA HiFi HotStart Real-Time PCR Master Mix (2X) may be used for routine downstream applications, including restriction enzyme digestion and sequencing. PCR products generated with KAPA HiFi HotStart Real-Time PCR Master Mix (2X) are blunt-ended, but may be 3'-dA-tailed for cloning into TA-cloning vectors.

1. Oyola, S.O., et al., *BMC Genomics* **13**, 1 (2012).
2. Quail, M.A., et al., *Nature Methods* **9**, 10 (2012).
3. Quail, M.A., et al., *BMC Genomics* **13**, 341 (2012).
4. Ross, M.G., et al., *Genome Biology* **14**, R51 (2013).

Product Specifications

Shipping and Storage

The components provided in this kit are temperature sensitive, and appropriate care should be taken during shipping and storage. KAPA HiFi HotStart Real-Time Library Amplification Kits are shipped on dry ice or ice packs, depending on the destination country. Upon receipt, store the entire kit at -15ºC to -25ºC in a constant-temperature freezer. KAPA HiFi HotStart Real-Time PCR Master Mix (2X) and Fluorescent Standards 1 – 4 are light sensitive and should be protected from light during storage, thawing, and reaction setup. When stored under these conditions and handled correctly, all kit components will retain full activity until the expiry date indicated on the kit label.

Handling

Keep all reaction components and master mixes on ice whenever possible during handling. Minimize exposure of KAPA HiFi HotStart Real-Time PCR Master Mix (2X) and Fluorescent Standards 1 – 4 to direct light. Exposure to direct light for an extended period of time may result in loss of fluorescent signal intensity. KAPA HiFi HotStart Real-Time PCR Master Mix (2X) contains isostabilizers and may not freeze solidly, even when stored at -15˚C to -25˚C. Nevertheless, always ensure that KAPA HiFi HotStart Real-Time PCR Master Mix (2X) is fully thawed and has been vortexed before use.

Quality Control

All kit components are subjected to stringent functional quality control, are free of detectable contaminating exo-and endonuclease activities, and meet strict requirements with respect to DNA contamination. Each batch of KAPA HiFi HotStart DNA Polymerase is confirmed to contain <2% contaminating protein (Agilent Protein 230 Assay). Please contact Technical Support at www.sequencing.roche.com/support for more information.

Overview

Real-time High-fidelity Amplification of Next-generation DNA Sequencing Libraries

To minimize over-amplification and associated unwanted artifacts (Figure 1A), the number of amplification cycles should be optimized to produce a sufficient amount of amplified library for the next step in the workflow (hybridization capture or sequencing), plus the amount needed for library QC and/or archiving. Real-time monitoring of library amplification provides a means for carefully controlling the number of cycles used when amplifying libraries prepared from precious samples. Optimal amplification for NGS applications corresponds to the region between Fluorescent Standards 1 and 3 (Figure 1B, Figure 2A, B).

1A

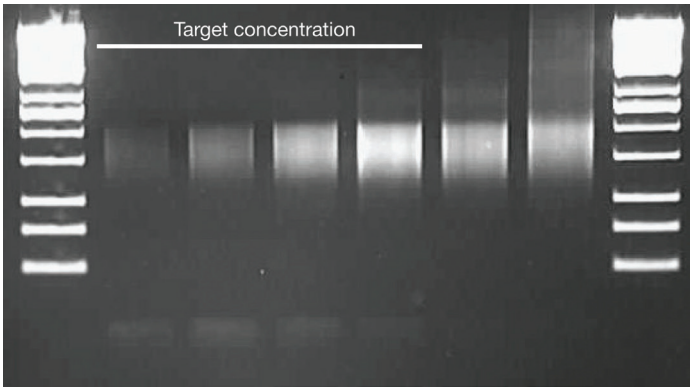

Figure 1A. Gel image of a typical library stopped at different amplification cycles. High molecular weight artifacts increase progressively with additional cycles.

1B

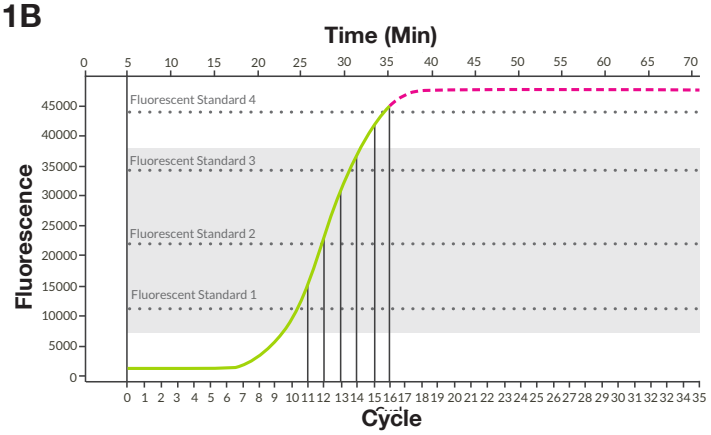

Figure 1B. Libraries are amplified using a SYBR® Green-based real-time, high-fidelity PCR master mix. Four triplicate wells of the PCR plate contain fluorescent reference standards representing a range of distinct DNA concentrations. Reactions terminated between Standards 1 and 3 represent the optimal library amplification range (grey box), depicted here from cycle 10 – 14.

2A

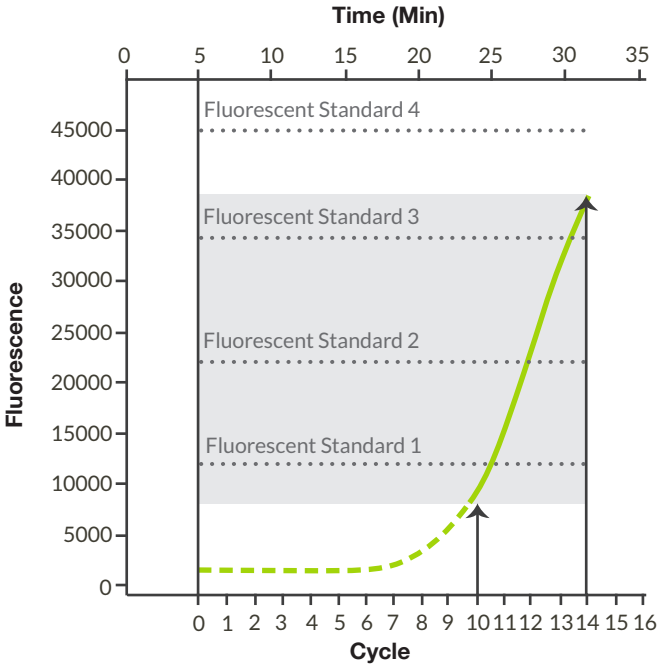

Figure 2A. Amplification plots for reactions terminated at the lower bound (dashed green line, cycle 10) or upper bound (solid green line, cycle 14) of the targeted concentration range (grey box). Library amplification reactions should ideally be terminated anywhere within the indicated target concentration range.

2B

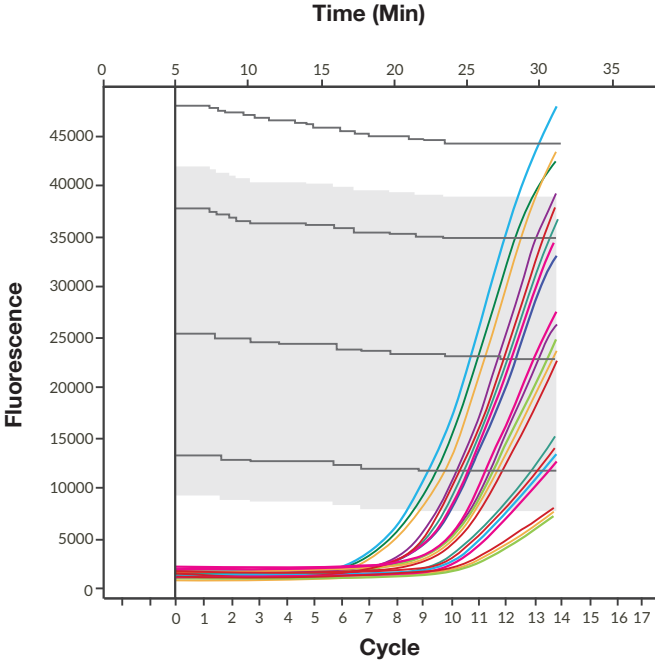

Figure 2B. Example of real-time high fidelity amplification of multiple libraries. Twenty libraries, spanning a ~64-fold concentration range (6 cycles), were simultaneously amplified and terminated after 14 cycles. Fourteen of the 20 libraries fall within the targeted amplification range. The remaining six libraries could either be used as-is, noting that they may be outside the optimal concentration range, or they could be re-amplified individually, or in high- or low-concentration groups.

Library Amplification Protocol

Step 1: Reagent Preparation

1.1 Thaw the library amplification primers, a tube of KAPA HiFi HotStart Real-Time PCR Master Mix (2X) and Fluorescent Standards 1 – 4 at room temperature.

NOTE: KAPA HiFi HotStart Real-Time PCR Master Mix (2X) thaws easily, however, due to its high viscosity it is important to vortex well before use. The Fluorescent Standards should be thawed for at least 15 min before use.

1.2 Mix and briefly centrifuge the thawed KAPA HiFi HotStart Real-Time PCR Master Mix (2X), primer, and Fluorescent Standards 1 – 4.

1.3 Thaw and briefly centrifuge the adapter-ligated, size-separated, purified library DNA.

1.4 Pre-program the real-time thermocycler.

Step 2: Reaction Setup

NOTE: Each plate must contain a set of Fluorescent Standards 1 – 4 (each loaded in triplicate) in addition to a single, 50 µL real-time PCR reaction for each library requiring amplification.

In order to maintain optimal library diversity it is necessary to include sufficient adapter-ligated library DNA to each real-time library amplification reaction. The optimal cycle number is dependent on the volume and concentration of library material used. High background fluorescence may result if >100 ng dsDNA template is added per 50 µL real-time PCR reaction. To ensure accurate results avoid overfilling of wells, bubbles in reactions, or anything else that could distort the fluorescent signal.

2.1 Assemble each library amplification reaction as follows:

Component	Volume
KAPA HiFi HotStart Real-Time PCR Master Mix (2X)	25 µL
KAPA Library Amplification Primer Mix (10X)[1]	5 µL
Adapter-ligated library DNA[2]	20 µL
Total volume:	**50 µL**

[1] Or another suitable 10X library amplification primer mix. The recommended final concentration of each primer in the library amplification reaction is 0.5 – 2 µM. KAPA Library Amplification Primer Mix is sold separately (07958994001)

[2] Purified library DNA. Subject DNA library to a post-ligation cleanup and/or size selection prior to performing real-time amplification.

2.2 Add 50 µL of each fluorescent standard in triplicate to wells of the real-time PCR plate.

2.3 Seal the plate, mix gently, and centrifuge briefly.

Step 3: Cycling Protocol

- If conventional end-point PCR was previously performed and yielded satisfactory results, and the same amount and type of library is being used in the KAPA HiFi HotStart Real-Time PCR reaction, program the real-time thermocycler with the same number of cycles as previously used.
- It is important to ensure that data acquisition is performed at 72ºC.

Amplify using the following cycling protocol:

Step	Temp.	Duration	Cycles
Initial denaturation	98°C	45 sec	1
Denaturation	98°C	15 sec	Minimum required for optimal amplification (10 – 18 cycles)[2]
Annealing[1]	60°C	30 sec	
Extension	72°C	30 sec	
Final extension	72°C	1 min	1
HOLD	4°C	∞	1

[1] Optimization of the annealing temperature may be required for non-standard (i.e., other than Illumina TruSeq®) adapter/primer combinations.

[2] The optimal cycling number will depend upon the volume and concentration of adapter-ligated library DNA added to each real-time PCR reaction. Typically, this is in the 10 – 18 cycle range but may require optimization.

Step 4: Data Analysis and Interpretation

Initially, the raw data (i.e., **not background subtracted**) linear real-time amplification plots can be used as a built-in quality metric to validate the level of amplification of each amplified library.

- If the linear amplification profile of the library is significantly below Fluorescent Standard 1 at the end of qPCR cycling, then it is unlikely that there will be sufficient library material for sequencing after PCR purification.
- If the linear amplification profile of the library is significantly above Fluorescent Standard 3 at the end of qPCR cycling, the library has been excessively amplified. This may lead to:
  - amplification bias,
  - higher error rates, and/or
  - the presence of chimeric PCR products.

This data is also useful as a quality control metric for identifying inconsistencies during library preparation between multiple libraries.

NOTE: the amplification plots can also be used in real-time to select the optimal cycle without a pre-programmed termination cycle. To do this:

- Program 30 cycles into the real-time thermocycler.
- After starting the real-time thermocycler, wait until the desired fluorescence level is achieved before terminating the real-time reaction.

If the above approach is followed, it is critical to terminate the reaction directly after data acquisition at 72ºC and before the tube ramps to 95ºC for the start of the next cycle. This will ensure that the enriched library DNA remains double-stranded for efficient downstream purification.

Step 5: Post-amplification Cleanup

5.1 In the library amplification plate/tube(s), perform a 1X bead-based cleanup by combining the following:

Component	Volume
Library amplification reaction product	50 µL
KAPA Pure Beads	50 µL
Total volume:	**100 µL**

5.2 Mix thoroughly by vortexing and/or pipetting up and down multiple times.

5.3 Incubate the plate/tube(s) at room temperature for 5 – 15 min to bind DNA to the beads.

5.4 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

5.5 Carefully remove and discard the supernatant.

5.6 Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

5.7 Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.

5.8 Carefully remove and discard the ethanol.

5.9 Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

5.10 Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.

5.11 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

5.12 Dry the beads at room temperature for 3 – 5 min, or until all of the ethanol has evaporated. ***Caution: over-drying the beads may result in reduced yield.***

5.13 Remove the plate/tube(s) from the magnet.

5.14 Thoroughly resuspend the beads in an appropriate volume of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5) or PCR-grade water. ***Always use PCR-grade water if proceeding to target capture.***

5.15 Incubate the plate/tube(s) at room temperature for 2 min to elute DNA off the beads.

5.16 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

5.17 Transfer the clear supernatant to a new plate/tube(s) and proceed with **Library Quantification** (step 6). Store purified, amplified libraries at 2°C to 8°C for 1 – 2 weeks, or at -15°C to -25°C.

Step 6: Library Quantification

Accurate quantification of amplifiable library molecules is critical for the efficient use of next-generation sequencing platforms. Overestimation of library concentration results in lower cluster density after bridge PCR. Underestimation of library concentration results in too many clusters on the flow cell, which can lead to poor cluster resolution. Both scenarios result in suboptimal sequencing capacity. Accurate library quantification is equally important when pooling indexed libraries for multiplexed sequencing to ensure equal representation of each library.

Libraries amplified with the KAPA HiFi HotStart Real-Time Library Amplification Kit may be quantified using the KAPA Library Quantification Kits to accurately determine the concentration of sequencing competent molecules. If real-time library amplification was terminated in the cycle range defined by Fluorescent Standards 1 – 3, a single 1:1000 dilution of each library will be sufficient for library quantification using the KAPA Library Quantification Kits. Refer to the KAPA Library Quantification Technical Data Sheet (KR0405) for more information and a detailed protocol.

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