KAPA HiFi™ Real-Time PCR Library Amplification Kit

Kit codes and components	
KK2700 10 x 50 µL reactions (sample kit)	1 x 0.25 ml KAPA HiFi HotStart Real-Time PCR Master Mix (2X) 4 x 1.5 ml Fluorescent Standards
KK2701 50 x 50 µL reactions	1 x 1.25 ml KAPA HiFi HotStart Real-Time PCR Master Mix (2X) 4 x 1.5 ml Fluorescent Standards
KK2702 250 x 50 µL reactions	1 x 6.25 ml KAPA HiFi HotStart Real-Time PCR Master Mix (2X) 4 x 1.5 ml Fluorescent Standards

Storage, handling and specifications

Store kit components protected from light at –20 °C for long-term use. Please refer to Section 4 for full details.

Quick Notes

- 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.
- KAPA HiFi HotStart Real-Time PCR Master Mix (2X) contains the novel KAPA HiFi DNA Polymerase, engineered for increased processivity and high fidelity.
- Optimal amplification for NGS applications corresponds to the region between fluorescent standard 1 and 3. The termination cycle number should be adjusted accordingly without the requirement for performing gel electrophoresis (see Figure 1).
- It is critical that the correct data acquisition temperature is adhered to, to minimize background fluorescence due to inter- and intra-primer interaction (see Table 1).
- When using custom primers that differ in sequence from those listed in Table 1, we recommend performing gradient PCR to optimize the annealing temperature.
- The hot start version of the KAPA HiFi Real-Time PCR Master Mix (2X) is not recommended for use with Nextera™ Sample Prep Kits.

1. Product Description

High fidelity PCR is used to selectively enrich library fragments carrying appropriate adaptor sequences and to amplify the amount of DNA prior to sequencing. During PCR enrichment of libraries, minimizing amplification bias is critical to ensure uniform sequence coverage. 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 over-amplified.

KAPA HiFi Real-Time PCR Library Amplification Kits are designed to address both sources of PCR-induced bias. The novel KAPA HiFi DNA Polymerase, engineered for high fidelity and processivity, is capable of balanced amplification of complex library DNA. Real-time monitoring of library amplification provides additional information required to minimize over-amplification. 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-enrichment gel electrophoresis and identifying inconsistencies in library preparation.
- Seamless integration with KAPA Library Quantification Kits.

KAPA HiFi Real-Time PCR 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 2X 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 1 and 2).

KAPA HiFi HotStart DNA Polymerase is an antibody-based hot start formulation of KAPA HiFi DNA Polymerase, a novel B-family DNA polymerase exhibiting industry-leading performance in comparison with other high-fidelity (B-family) DNA polymerases and polymerase blends. KAPA HiFi DNA Polymerase was engineered for increased affinity to DNA, without the need for accessory protein domains. The intrinsic high processivity of the enzyme results in significant improvements in yield, sensitivity, speed, target length and the ability to amplify difficult amplicons. These enhancements result in lower amplification bias which leads to more uniform sequence coverage. 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 superior accuracy during DNA amplification. The error rate of KAPA HiFi HotStart DNA Polymerase is ~2.8 x 10^{-7}. This fidelity is approximately 100X higher than that of wild-type *Taq* and up to 30X higher than 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 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 are blunt-ended, but may be 3′-dA-tailed for cloning into TA cloning vectors.

2. Overview

Real-time high fidelity amplification of next-generation DNA sequencing libraries.

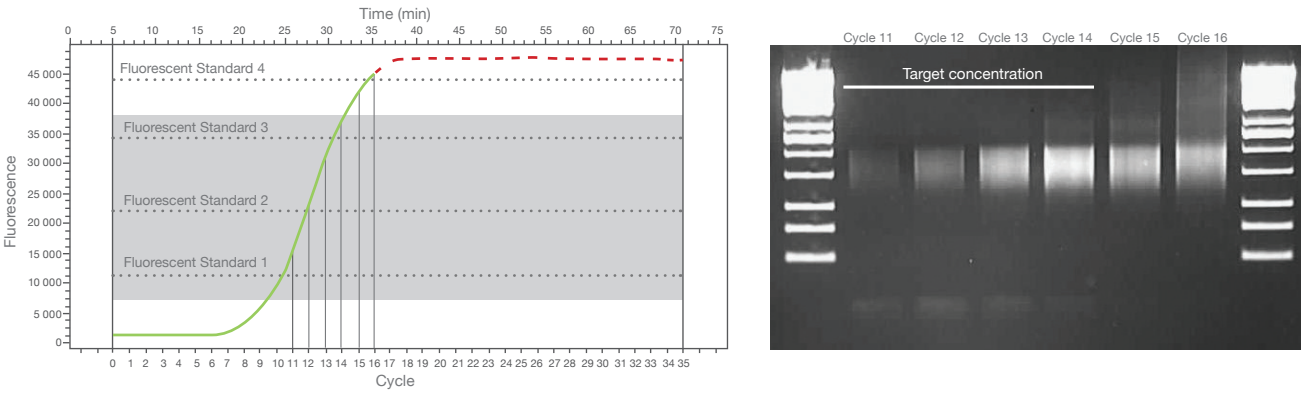

Figure 1. Libraries are amplified using a SYBR® Green-based real-time, high fidelity PCR master mix (left panel). Four triplicate wells of the PCR plate contain fluorescent reference standards representing a range of distinct DNA concentrations. Reactions terminated between standards 1 and 3 represent the optimal library amplification range (grey box), depicted here from cycle 10-14. Gel image of a typical library stopped at different amplification cycles (right panel). Low and high molecular weight artifacts increase progressively with additional cycles.

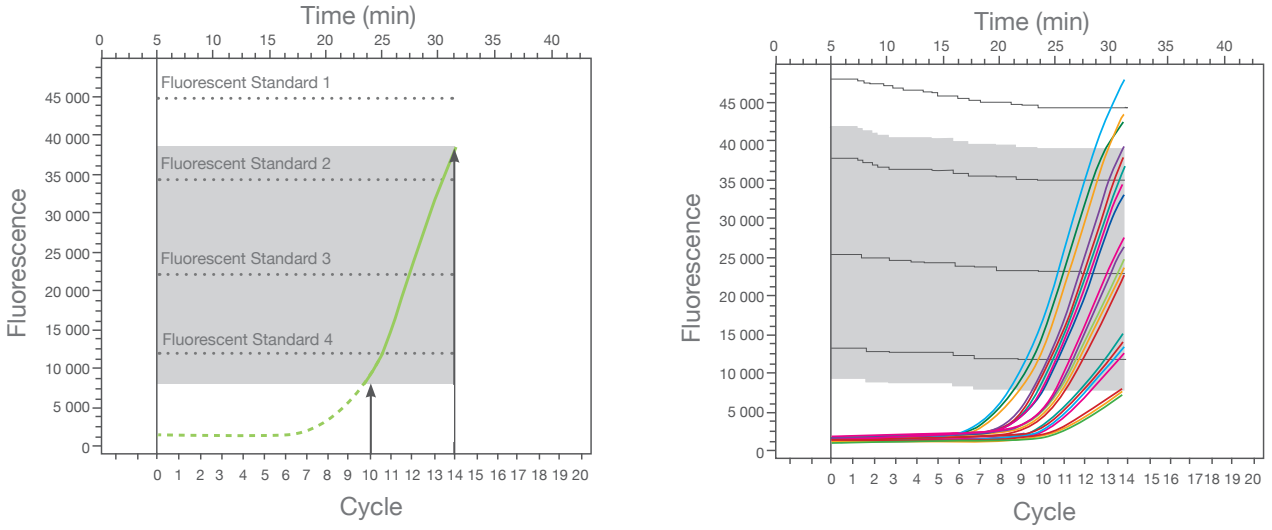

Figure 2. (Left panel) Superimposed amplification plots for reactions terminated at the lower bound (hashed line, cycle 10) or upper bound (solid line, cycle 14) of the targeted concentration range (grey box). Library amplification reactions should ideally be terminated anywhere within the indicated target concentration range. (Right panel) Example of real-time high fidelity amplification of multiple libraries. 20 libraries, spanning a ~64-fold concentration range (6 cycles), were simultaneously amplified and terminated after 14 cycles. 14 of the 20 libraries fall within the targeted amplification range. The remaining 6 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.

3. Library amplification protocol

1: Preparation

- Thaw the primers required for PCR enrichment (see Table 1 for details) and a tube of KAPA HiFi HotStart Real-Time PCR Master Mix (2X), and fluorescent standards 1 - 4 at room temperature.
- Mix and briefly centrifuge the thawed KAPA HiFi HotStart Real-Time PCR Master Mix (2X), primer and fluorescent standards 1 - 4 for 5 seconds at 600 xg.
- Thaw and briefly centrifuge the adaptor-ligated, size-separated purified library DNA for 5 seconds at 600 xg.
- Pre-program the real-time thermal cycler using the recommended cycling protocol supplied in Table 1 for the specific set of library amplification PCR primers.

2: Reaction setup

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 add sufficient adaptor-ligated library DNA to each enrichment PCR reaction. The optimal cycle number is dependant on the volume and concentration of library material added to each 50 uL PCR reaction. High background fluorescence may result if >100 ng dsDNA template is added per 50 µL real-time PCR reaction.

To each reaction add the following components changing tips after each pipetting step. Consult Table 1 for the suggested reaction setup for specific library preparation protocols.

2.1: Sample setup

- 25 µL KAPA HiFi HotStart Real-Time PCR Master Mix (2X).
- Primer mix or each individual primer.
- Purified adaptor-ligated library DNA.
- Make up to 50 µL with PCR-grade water.

2.2: Fluorescent standard setup

- Add 50 µL of each fluorescent standard in triplicate to wells of the real-time PCR plate.
- Seal each reaction, mix gently and centrifuge for 5 seconds at 600 xg.

3: Cycling protocol

Refer to Table 1 for the thermal cycling protocol for specific library types.

- If conventional end-point PCR has previously been used successfully and the same amount and type of library is added to the KAPA HiFi HotStart Real-Time PCR reactions, then 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 72 ºC.

4: Clean up PCR

- After enrichment PCR, clean up each reaction using either Agencourt AMPure XP beads (Beckman Coulter Genomics part # A63881) or Qiagen MinElute PCR Purification Kit (Qiagen, part # 28004).

For advanced troubleshooting or assistance with reaction setup or optimization e-mail **support@kapabiosystems.com**.

3. Library amplification protocol

5: Validate library

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 to sequence after PCR purification.
- If the linear amplification profile of the library is significantly above fluorescent standard 3 at the end of qPCR cycling, then the library has been over-amplified. This may lead to 1) amplification bias, 2) higher error rates, and/or 3) 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:

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

NOTE: It is critical to terminate the reaction directly after data acquisition at 72°C **and before the tube ramps to 95°C** for the start of the next cycle. This will ensure that the enriched library DNA remains double-stranded for efficient downstream purification.

To verify the size of the PCR enriched fragments, check the size distribution by performing gel electrophoresis.

5.1: 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.

Integrate KAPA HiFi Real-Time PCR Library Amplification Kit with the appropriate KAPA Library Quantification Kit (KK4824, KK4835, KK4844, KK4854) to accurately quantify the number of PCR-competent molecules. If libraries have been terminated between fluorescent standards 1 – 3, a single 1:1,000 dilution of each library will be required for library quantification using the KAPA Library Quantification Kits.

3. Library amplification protocol (cont.)

Table 1. Recommended reaction setup and cycling parameters for KAPA HiFi HotStart Real-Time PCR Master Mix (2X) reactions:

Type of Illumina Library	Reaction Setup			Cycling Protocol	
	Component	Final Conc.	Volume/50 µL rxn	Step	
Genomic DNA ChIP	PCR grade water		As needed	Denaturation	45 sec at 98 °C
	2X KAPA HiFi HS RT-PCR MM	1X	25 µL	Cycling*	15 sec at 98 °C 30 sec at 65 °C 30 sec at 72 °C Acquire Data
	PCR Primer 1.1	500 nM	1 µL		
	PCR Primer 2.1	500 nM	1 µL		
	Library DNA		As needed		
PE	PCR grade water		As needed	Denaturation	45 sec at 98 °C
	2X KAPA HiFi HS RT-PCR MM	1X	25 µL	Cycling*	15 sec at 98 °C 30 sec at 65 °C 30 sec at 72 °C Acquire Data
	PE PCR Primer 1.0	500 nM	1 µL		
	PE PCR Primer 2.0	500 nM	1 µL		
	Library DNA		As needed		
PE Multiplex	PCR grade water		As needed	Denaturation	45 sec at 98 °C
	2X KAPA HiFi HS RT-PCR MM	1X	25 µL	Cycling*	15 sec at 98 °C 30 sec at 65 °C 30 sec at 72 °C Acquire Data
	PE PCR Primer InPE 1.0	500 nM	1 µL		
	PE PCR Primer InPE 2.0	10 nM	1 µL		
	PCR Primer Index 1 - 12	500 nM	1 µL		
	Library DNA		As needed		
GEX Small RNA	PCR grade water		As needed	Denaturation	45 sec at 98 °C
	2X KAPA HiFi HS RT-PCR MM	1X	25 µL	Cycling*	15 sec at 98 °C 30 sec at 60 °C 30 sec at 72 °C Acquire Data
	Primer GX1	500 nM	0.5 µL		
	Primer GX2	500 nM	0.5 µL		
	Library DNA		As needed		
TruSeq DNA TruSeq RNA	2X KAPA HiFi HS RT-PCR MM	1X	25 µL	Denaturation	45 sec at 98 °C
	PCR Primer Cocktail (PPC)	500 nM each	5 µL	Cycling*	15 sec at 98 °C 30 sec at 60 °C 30 sec at 72 °C Acquire Data
	Library DNA		20 µL		

* The optimal cycling number is determined by the volume and concentration of adaptor-ligated, size-separated purified library DNA added to each enrichment PCR reaction. Typically this is in the 10-18 cycle range but may require optimization.

4. Storage, handling and specifications

4.1 Shipping, storage and handling

KAPA HiFi Real-Time PCR Library Amplification Kits are shipped on dry ice or ice packs, depending on the country of destination. Upon receipt, store the entire kit at -20 °C in a constant-temperature freezer. When stored under these conditions and handled correctly, all kit components will retain full activity until the expiry date indicated on the kit.

The KAPA HiFi HotStart Real-Time PCR Master Mix contains isostabilizers and may not freeze solidly, even when stored at -20 °C. Nevertheless, always ensure that the KAPA HiFi HotStart Real-Time PCR Master Mix is fully thawed and has been vortexed before use.

Miminmize exposure of the 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. Always ensure that the product has been completely thawed and mixed before use.

KAPA HiFi HotStart Real-Time PCR Master Mix (2X) may be stored at 4 °C for regular, short-term use (up to 1 month). Provided that it has been handled carefully and not contaminated, the Master Mix is not expected to be compromised if left (unintentionally) at room temperature for short periods of time (up to 3 days). Long-term storage at room temperature or 4 °C is not recommended. Please note that reagents stored above -20 °C are more prone to degradation when contaminated by the user; storage at such temperatures is therefore at the user's own risk.

4.2 Quality control

KAPA HiFi DNA Polymerase and its proprietary HotStart antibody are extensively purified through the use of multiple chromatography steps. The final formulation contains <2% contaminating protein, as determined in an Agilent Protein 230 Assay. Each batch of Master Mix is subjected to stringent quality control tests, is free of contaminating exo- and endonuclease activities and meets strict requirements with respect to DNA contamination.

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