KAPABIOSYSTEMS

Technical Data Sheet

KAPA Library Preparation Kit

with Real-time PCR Library Amplification for Illumina® Platforms

KR0411 – v5.16

This Technical Data Sheet provides product information and a detailed protocol for the KAPA Library Preparation Kits with Real-time PCR Library Amplification.

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Kapa/*Roche* Kit Codes and Components		
KK8220 *7961847001* 10 reactions	End Repair Enzyme Mix	50 µL
	End Repair Buffer with dNTPs (10X)	100 µL
	A-Tailing Enzyme	30 µL
	A-Tailing Buffer (10X)	50 µL
	DNA Ligase	50 µL
	Ligation Buffer (5X)	100 µL
	KAPA HiFi HotStart Real-time PCR Master Mix (2X)	250 µL
	4X Fluorescent Standards	1500 µL ea
KK8221 *7961855001* 50 reactions	End Repair Enzyme Mix	250 µL
	End Repair Buffer with dNTPs (10X)	500 µL
	A-Tailing Enzyme	150 µL
	A-Tailing Buffer (10X)	250 µL
	DNA Ligase	250 µL
	Ligation Buffer (5X)	500 µL
	KAPA HiFi HotStart Real-time PCR Master Mix (2X)	1250 µL
	4X Fluorescent Standards	1500 µL ea

Quick Notes

- Reaction components should be mixed fresh and used on the same day.
- A bead-based cleanup with KAPA Pure Beads (KK8000, KK8001, KK8002) is recommended for reaction cleanup steps between enzymatic reactions.
- 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.
- To minimize background fluorescence due to inter- and intra-primer interaction, it is critical to adhere to the correct data acquisition temperature.
- For custom primers, a gradient PCR is recommended to optimize annealing temperature. For more information, please contact Technical Support at kapabiosystems.com/support.

Product Description

The KAPA Library Preparation Kit provides all of the enzymes and reaction buffers required for constructing libraries from fragmented dsDNA via the following steps:

1. end repair: produce blunt-ended, 5'-phosphorylated fragments.
2. A-tailing: add dAMP to the 3'-ends of the dsDNA library fragments.
3. adapter ligation: ligate dsDNA adapters with 3'-dTMP overhangs to library fragments.
4. library amplification: real-time amplification of library fragments carrying appropriate adapter sequences on both ends.

Reaction buffers are supplied in convenient, concentrated "master mix" formats comprising all of the required reaction components except oligonucleotide adapters and PCR primers. Similarly, a single enzyme mixture is provided for each step of the library construction process, reducing the number of pipetting steps.

High-fidelity PCR is used to selectively enrich library fragments carrying appropriate adapter sequences and to amplify the amount of DNA prior to sequencing. During PCR enrichment of libraries, minimizing amplification bias is critical to ensure more 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 optimize the number of amplification cycles and minimize over-amplification. The 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 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 engineered for increased processivity and high fidelity. 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 calculated at 1 error in 3.54×10^6 bases covered (2.82×10^{-7}). The SYBR Green I intercalating dye contained within the master mix does not affect the fildelity of the polymerase. DNA fragments generated with KAPA HiFi HotStart ReadyMix may be used for routine downstream analyses or applications, including restriction enzyme digestion and sequencing. PCR products generated with KAPA HiFi HotStart ReadyMix are blunt-ended, but may be 3'-dA-tailed for cloning into TA cloning vectors.

Product Applications

This kit is primarily intended for the construction of genomic, paired-end, and paired-end multiplex (indexed/barcoded) Illumina DNA libraries, but may be used for other applications requiring efficient end-repair, A-tailing, ligation, and/or library amplification steps.

Product Specifications

Shipping and Storage

The enzymes provided in this kit are temperature sensitive, and appropriate care should be taken during shipping and storage. KAPA Library Preparation Kits are shipped on dry ice or ice packs, depending on the destination country. Upon receipt, immediately 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, the kit components will retain full activity until the expiry date indicated on the kit label.

Handling

Always ensure that components have been fully thawed and thoroughly mixed before use. Keep all reaction components and master mixes on ice whenever possible during handling. Please note that certain components in KAPA Library Preparation Kits (e.g., End Repair Mix, DNA Ligase, buffers containing dNTPs and/or ATP, etc.) are particularly sensitive to temperature and freeze-thaw cycles, and should be handled with special care. 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 -15ºC to -25ºC. 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. Please contact Technical Support at **kapabiosystems.com/support** for more information.

Library Construction Protocol

Materials required but not supplied in this kit:

- Reaction tubes: Reactions may be assembled and processed in PCR plates, PCR tubes, or microcentrifuge tubes.
- Pipette tips: The use of high-quality filter-plugged tips is recommended to prevent contamination of reagents and library samples.
- Reaction cleanup: The use of either KAPA Pure Beads (KK8000, KK8001, KK8002) or Agencourt® AMPure® XP (Beckman Coulter) is recommended.
- Adapters: KAPA Adapters are recommended, however, the kit is also compatible with non-indexed, single-indexed, and dual-indexed adapters for Illumina sequencing.
- Library amplification primers: Library Amplification Primer Mix (10X) (KK2623) is recommended for the amplification of libraries for Illumina sequencing as the primers contain the same sequences that are used for flowcell hybridization.

1. End Repair

1.1 Assemble each end repair reaction in a tube or well of a PCR plate as follows:

Component	Volume
Water to 100 µL	X µL
End Repair Buffer (10X)	10 µL
End Repair Enzyme Mix	5 µL
Sheared dsDNA (1 – 5 µg)	1 – 85 µL
Total volume:	**100 µL**

1.2 Incubate for 30 min at 20ºC.

1.3 Proceed immediately to **End Repair Cleanup** (step 2).

2. End Repair Cleanup

2.1 Ensure that KAPA Pure Beads have been equilibrated to room temperature, and that the beads are fully resuspended before proceeding.

2.2 In the same plate/tube(s), perform a 1.6X bead-based cleanup by combining the following:

Component	Volume
End repair reaction	100 µL
KAPA Pure Beads	160 µL
Total volume:	**260 µL**

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

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

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

2.6 Carefully remove and discard the supernatant.

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

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

2.9 Carefully remove and discard the ethanol.

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

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

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

2.13 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.***

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

2.15 Thoroughly resuspend the beads in 32.5 µL of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5).

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

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

2.18 Transfer the clear supernatant to a new plate/tube(s) and proceed with **A-tailing** (step 3).

SAFE STOPPING POINT

If you are not proceeding to **A-tailing** (step 3) immediately, the protocol can be safely stopped here. Store at -20ºC for ≤7 days.

3. A-tailing

3.1 In the plate/tube(s) containing end-repaired DNA, assemble the A-tailing reaction as follows:

Component	Volume
End-repaired DNA	30 µL
Water	12 µL
A-Tailing Buffer (10X)	5 µL
A-Tailing Enzyme	3 µL
Total volume:	**50 µL**

3.2 Incubate for 30 min at 30ºC.

3.3 Proceed immediately to **A-tailing Cleanup** (step 4).

4. A-tailing Cleanup

4.1 Ensure that KAPA Pure Beads have been equilibrated to room temperature, and that the beads are fully resuspended before proceeding.

4.2 In the same plate/tube(s), perform a 1.8X bead-based cleanup by combining the following:

Component	Volume
A-tailing reaction	50 µL
KAPA Pure Beads	90 µL
Total volume:	**140 µL**

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

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

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

4.6 Carefully remove and discard the supernatant.

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

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

4.9 Carefully remove and discard the ethanol.

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

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

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

4.13 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.***

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

4.15 Thoroughly resuspend the beads in 32.5 µL of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5).

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

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

4.18 Transfer the clear supernatant to a new plate/tube(s) and proceed with **Adapter Ligation** (step 5)

SAFE STOPPING POINT

If you are not proceeding to **Adapter Ligation** (step 5) immediately, the protocol can be safely stopped here. Store at -20ºC for ≤7 days.

5. Adapter Ligation

KAPA Adapters (supplied separately) are recommended for use with the KAPA Library Preparation Kit. However, the kit is also compatible with non-indexed, single-indexed, and dual-indexed adapters that are routinely used in Illumina TruSeq®, Roche® NimbleGen™ SeqCap™ EZ, Agilent® SureSelect, and other similar library construction and target capture workflows. Custom adapters that are of a similar design and are compatible with TA-ligation of dsDNA may also be used, remembering that custom adapter designs may impact library construction efficiency.

5.1 In the plate/tube(s) containing the A-tailed DNA, assemble the adapter ligation reaction as follows:

Component	Volume
A-tailed DNA	30 µL
Adapter (30 µM)	5 µL
Ligation Buffer (5X)*	10 µL
DNA Ligase*	5 µL
Total volume:	**50 µL**

*The buffer and ligase enzyme should preferably be premixed and added in a single pipetting step. Premixes are stable for ≤24 hrs at room temperature, for ≤3 days at 4°C, and for ≤4 weeks at -20°C.

5.2 Incubate for 15 min at 20ºC.

5.3 Proceed immediately to **1st Post-ligation Cleanup** (step 6).

6. 1st Post-ligation Cleanup

Depending on requirements and chosen workflow, either one post-ligation cleanup followed by size-selection or two post-ligation cleanups may be performed.

6.1 Ensure that KAPA Pure Beads have been equilibrated to room temperature, and that the beads are fully resuspended before proceeding.

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

Component	Volume
Adapter ligation reaction	50 µL
KAPA Pure Beads	50 µL
Total volume:	**100 µL**

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

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

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

6.6 Carefully remove and discard the supernatant.

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

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

6.9 Carefully remove and discard the ethanol.

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

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

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

6.13 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.***

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

6.15 Thoroughly resuspend the beads in an appropriate volume of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5).

Recommended volumes are as follows:

If proceeding to **2nd Post-ligation Cleanup** (step 7), resuspend the beads in 50 µL.

If proceeding to **Size Selection** (step 8), resuspend the beads in 100 µL, and omit **2nd Post-ligation Cleanup** (step 7).

For a **safe stopping point**, resuspend the beads in either 50 µL or 100 µL (as required), and store at 4°C for ≤24 hrs.

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

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

6.18 Transfer the clear supernatant to a new plate/tube(s) and proceed with either **2nd Post-ligation Cleanup** (step 7) or **Size Selection** (step 8).

7. 2nd Post-ligation Cleanup

7.1 Ensure that KAPA Pure Beads have been equilibrated to room temperature, and that the beads are fully resuspended before proceeding.

7.2 In the plate/tube(s) containing adapter ligated library from the first cleanup reaction, perform a 1X bead-based cleanup by combining the following:

Component	Volume
Purified, adapter-ligated DNA	50 µL
KAPA Pure Beads	50 µL
Total volume:	**100 µL**

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

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

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

7.6 Carefully remove and discard the supernatant.

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

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

7.9 Carefully remove and discard the ethanol.

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

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

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

7.13 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.***

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

7.15 Thoroughly resuspend the beads in 32.5 µL of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5).

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

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

7.18 Transfer the clear supernatant to a new plate/tube(s) and proceed with the **Library Amplification Protocol** (p. 8).

8. Size Selection

Size selection requirements vary widely for different sequencing applications. If required, any commonly used bead- or gel-based size selection technique may be integrated in the KAPA Library Preparation Kit workflow.

The double-sided size selection (0.6X - 0.8X) procedure described here is designed for selection of adapter-ligated fragments approximately 250 – 450 bp in length. If you wish to select a different range of fragment lengths, please consult the **KAPA NGS Library Preparation Technical Guide** or contact Technical Support at **kapabiosystems.com/support**.

8.1 Perform the first size cut (0.6X) by combining the following:

Component	Volume
Purified, adapter-ligated DNA	100 µL
KAPA Pure Beads	60 µL
Total volume:	**160 µL**

8.2 Thoroughly resuspend the beads by vortexing and/or pipetting up and down multiple times.

8.3 Incubate the plate/tube(s) at room temperature for 5 – 15 min to bind library fragments larger than ~450 bp to the beads.

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

8.5 Carefully transfer 155 µL of supernatant containing library fragments smaller than ~450 bp to a new plate/tube(s). ***It is critical that no beads are transferred with the supernatant.***

8.6 Discard the plate/tube(s) with beads, to which library fragments larger than ~450 bp are bound.

8.7 Perform the second size cut (0.8X) by combining the following:

Component	Volume
Supernatant from first cut	155 µL
KAPA Pure Beads	20 µL
Total volume:	**175 µL**

8.8 Thoroughly resuspend the beads by vortexing and/or pipetting up and down multiple times.

8.9 Incubate the plate/tube(s) at room temperature for 5 – 15 min to bind library fragments larger than ~250 bp to the beads.

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

8.11 Carefully remove and discard the supernatant, which contains library fragments smaller than ~250 bp.

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

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

8.14 Carefully remove and discard the ethanol.

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

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

8.17 Carefully remove and discard.

8.18 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.***

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

8.20 Thoroughly resuspend the beads in 25 µL of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5).

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

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

8.23 Transfer the clear supernatant containing size-selected DNA to a new plate/tube(s) and proceed with the **Library Amplification Protocol** (p. 8), QC, and/or sequencing, as appropriate.

Library Amplification Protocol

1. Reagent Preparation

1.1 Thaw the primers (sold separately) required for PCR enrichment, 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 the 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.

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 adapter-ligated library DNA to each enrichment PCR reaction. The optimal cycle number is dependent on the volume and concentration of library material added to each 50 µL PCR reaction. 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 the 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
Library Amplification Primer Mix (10X)*	5 µL
Adapter-ligated library DNA	20 µL
Total volume:	**50 µL**

*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.

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.

3. Cycling Protocol

- 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 72ºC.

Amplify using the following cycling protocol:

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

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

*The optimal cycling number will depend upon the volume and concentration of adapter-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. 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 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:
  - 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 of the library is achieved before terminating the real-time reaction.

Note: 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.

5. Bead-based 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 7). Store purified, amplified libraries at 4ºC for 1 – 2 weeks, or at -20ºC.

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.

Integrate KAPA Real-time 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.

Appendix:

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

1A

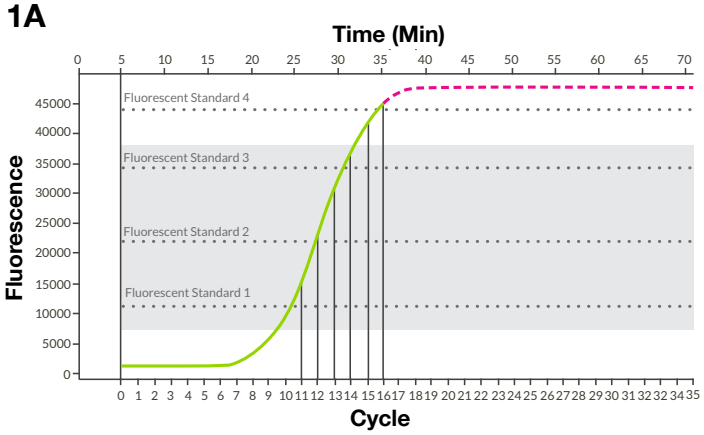

Figure 1A. Libraries are amplified using a SYBR® Green-based real-time, high-fidelity PCR master mix. Four triplicate wells of the PCR plate contain fluorescent reference standards representing a range of distinct DNA concentrations. Reactions terminated between Standards 1 and 3 represent the optimal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

1B

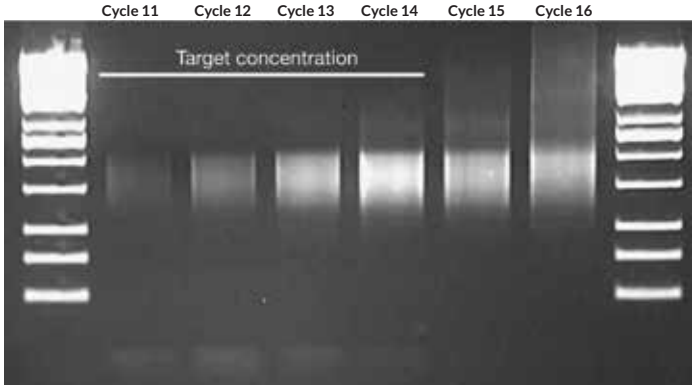

Figure 1B. Gel image of a typical library stopped at different amplification cycles. Low and high molecular weight artifacts increase progressively with additional cycles.

2A

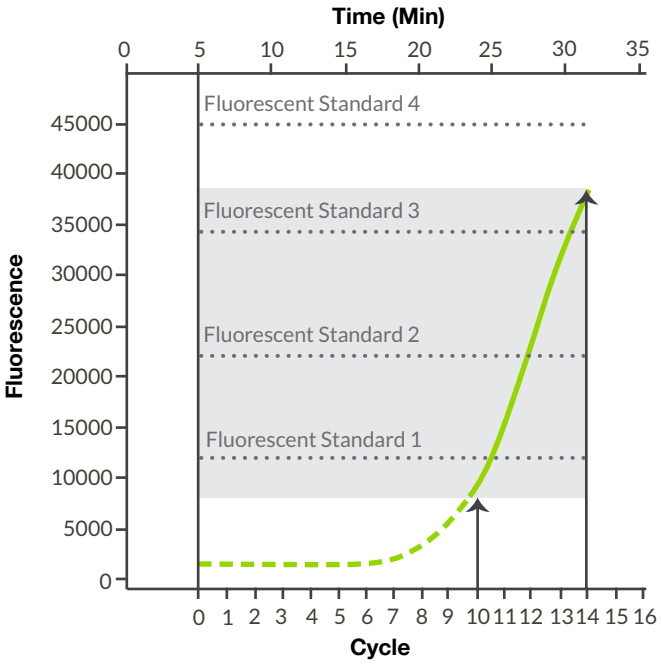

Figure 2A. Superimposed amplification plots for reactions terminated at the lower bound (hashed line, cycle 10) or upper bound (solid line, cycle 14) of the targeted concentration range (grey box). Library amplification reactions should ideally be terminated anywhere within the indicated target concentration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

2B

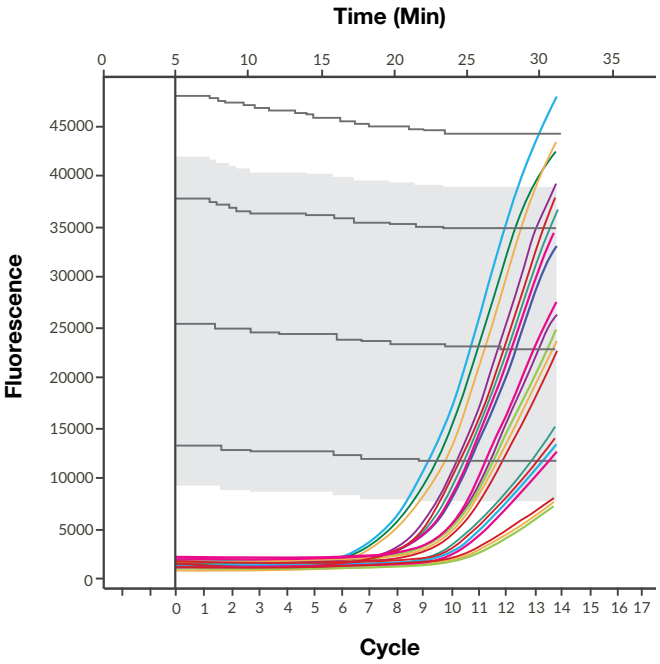

Figure 2B. Example of real-time high fidelity amplification of multiple libraries. 20 libraries, spanning a ~64-fold concentration range (6 cycles), were simultaneously amplified and terminated after 14 cycles. 14 of the 20 libraries fall within the targeted amplification range. The remaining 6 libraries 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.

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