



# KAPA HiFi HotStart Uracil+ ReadyMix PCR Kit

KR0413 – v5.17

## Product Description

KAPA HiFi HotStart is a novel B-family DNA polymerase, engineered to have increased affinity for DNA, without the need for accessory proteins or DNA-binding domains. The intrinsic high processivity of the enzyme results in significant improvement in yield, speed and sensitivity when compared with wild-type B-family DNA polymerases and polymerase blends. When used for the amplification of next-generation sequencing (NGS) libraries, KAPA HiFi HotStart DNA Polymerase exhibits high yields with minimal amplification bias and provides extremely uniform sequence coverage.

The read-ahead function of proofreading DNA polymerases detects pro-mutagenic uracil residues in the template strand and prevents further strand extension, thereby reducing or completely inhibiting PCR amplification. In KAPA HiFi HotStart Uracil+ DNA Polymerase, this uracil-binding pocket is inactivated to enable the amplification of uracil-containing DNA. The enzyme shows the same high yield, low GC-bias and coverage uniformity as the unmodified KAPA HiFi HotStart DNA Polymerase, making it particularly advantageous for applications employing bisulfite DNA conversion, which typically produces low concentrations of AT-rich DNA.

KAPA HiFi HotStart Uracil+ DNA Polymerase has 5'→3' polymerase and 3'→5' exonuclease (proofreading) activity, but no 5'→3' exonuclease activity. The strong 3'→5' exonuclease activity results in superior accuracy during DNA amplification. A proprietary antibody inactivates the polymerase until the first cycle of denaturation, minimizing spurious amplification products that may result from nonspecific priming events during reaction setup and initiation, and increasing overall reaction efficiency.

KAPA HiFi HotStart Uracil+ ReadyMix (2X) is a ready-to-use cocktail containing all components required for PCR, except primers and template. The ReadyMix contains 0.2 mM of each dNTP (dATP, dCTP, dGTP, dTTP), and does not contain dUTP.

## Product Applications

### Amplification of bisulfite-converted DNA

Sodium bisulfite treatment converts unmethylated cytosines to uracil, while methylated cytosines remain unconverted. Uracil is replaced by thymine during subsequent PCR amplification, allowing base-pair resolution of DNA methylation via sequencing. KAPA HiFi HotStart Uracil+ ReadyMix provides high yields and robust amplification of bisulfite-converted DNA.

### Kapa/Roche Kit Codes and Components

<b>KK2800</b> 07959044001	KAPA HiFi HotStart Uracil+ ReadyMix (2X)*	0.25 mL
<b>KK2801</b> 07959052001	KAPA HiFi HotStart Uracil+ ReadyMix (2X)*	1.25 mL
<b>KK2802</b> 07959079001	KAPA HiFi HotStart Uracil+ ReadyMix (2X)*	6.25 mL

\* Contains 2.5 mM MgCl<sub>2</sub> at 1X

### Quick Notes

- KAPA HiFi HotStart provides high fidelity NGS library amplification with the highest yield, lowest bias, and most uniform sequence coverage available. These characteristics are retained in KAPA HiFi HotStart Uracil+, a modified version of KAPA HiFi HotStart, engineered to tolerate uracil residues.
- KAPA HiFi HotStart Uracil+ ReadyMix contains all components for PCR, except primers and template.
- Suitable for amplification of bisulfite-converted NGS libraries (Section 1). Optimal cycle numbers should be determined empirically.
- Suitable for methylation-specific PCR, or standard amplification of bisulfite-converted DNA (Section 2).
- Can be used with UDG to prevent false positives due to carryover amplicon contamination.
- Due to the AT-rich nature of bisulfite-converted DNA, ensure that annealing temperatures and MgCl<sub>2</sub> concentrations are optimal.

The KAPA HiFi HotStart Uracil+ ReadyMix is especially well-suited for NGS applications, providing reduced amplification bias and increased amplification efficiency of bisulfite-converted libraries. Library amplification with KAPA HiFi HotStart Uracil+ ReadyMix provides dramatic improvements in coverage depth uniformity, and more complete representation across reference sequences.

### Amplification of damaged DNA samples

Cytosine deamination occurs spontaneously over long periods of time, and more rapidly at elevated temperatures, and results in the accumulation of uracil in DNA and among free nucleotides. When other proofreading enzymes fail, KAPA HiFi Uracil+ DNA Polymerase may allow high-fidelity amplification from damaged DNA templates containing uracil.

### Prevention of amplicon contamination with UDG

KAPA HiFi Uracil+ DNA Polymerase readily incorporates dUTP during amplification, and can therefore be used in conjunction with uracil-DNA-glycosylase (UDG) to prevent carryover contamination. dUTP is added to PCRs so that amplicons that may contaminate subsequent reactions are removed by digestion with UDG prior to amplification.

## Section 1: Protocol for bisulfite-converted NGS library amplification

### 1. Library Amplification

Library Amplification Primer Mix (10X) (KK2623), sold separately, is designed to eliminate or delay primer depletion during library amplification reactions performed with KAPA HiFi HotStart Uracil+ ReadyMix (2X). The primer mix is suitable for the amplification of all Illumina libraries flanked by the P5 and P7 flow cell sequences. Primers are supplied at a 10X concentration of 20  $\mu\text{M}$  each, and have been formulated as previously described. User-supplied primer mixes may be used in combination with incomplete or custom adapters. For guidelines on the formulation of user-supplied library amplification primers, please contact Technical Support at [sequencing.roche.com/support](http://sequencing.roche.com/support).

- 1.1 Assemble each library amplification reaction as follows:

Component	Volume
KAPA HiFi HotStart Uracil+ReadyMix (2X)	25 $\mu\text{L}$
Library Amplification Primer Mix (10X)*	5 $\mu\text{L}$
Adapter-ligated library DNA	20 $\mu\text{L}$
<b>Total volume:</b>	<b>50 <math>\mu\text{L}</math></b>

\*Or another suitable 10X library amplification primer mix. The recommended final concentration of each primer in the library amplification reaction is 0.5 – 2  $\mu\text{M}$ .

- 1.2 Mix thoroughly and centrifuge briefly.

- 1.3 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 <sup>2</sup>
Annealing <sup>1</sup>	60°C	30 sec	
Extension	72°C	30 sec	
Final extension	72°C	1 min	1
HOLD	4°C	$\infty$	1

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

<sup>2</sup>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.

- 1.4 Proceed directly to **Post-amplification Cleanup** (step 2).

### 2. Post-amplification Cleanup

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

Component	Volume
Library amplification reaction product	50 $\mu\text{L}$
KAPA Pure Beads	50 $\mu\text{L}$
<b>Total volume:</b>	<b>100 <math>\mu\text{L}</math></b>

- 2.2 Mix thoroughly by vortexing and/or pipetting up and down multiple times.
- 2.3 Incubate the plate/tube(s) at room temperature for 5 – 15 min to bind DNA to the beads.
- 2.4 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
- 2.5 Carefully remove and discard the supernatant.
- 2.6 Keeping the plate/tube(s) on the magnet, add 200  $\mu\text{L}$  of 80% ethanol.
- 2.7 Incubate the plate/tube(s) on the magnet at room temperature for  $\geq 30$  sec.
- 2.8 Carefully remove and discard the ethanol.
- 2.9 Keeping the plate/tube(s) on the magnet, add 200  $\mu\text{L}$  of 80% ethanol.
- 2.10 Incubate the plate/tube(s) on the magnet at room temperature for  $\geq 30$  sec.
- 2.11 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.
- 2.12 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.**
- 2.13 Remove the plate/tube(s) from the magnet.
- 2.14 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.
- 2.15 Incubate the plate/tube(s) at room temperature for 2 min to elute DNA off the beads.
- 2.16 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
- 2.17 Transfer the clear supernatant to a new plate/tube(s). Store purified, amplified libraries at 2°C to 8°C for 1 – 2 weeks, or at -15°C to -25°C.

## Section 2: Protocol for Standard PCR

**IMPORTANT!** The KAPA HiFi HotStart Uracil+ ReadyMix contains an engineered B-family (proofreading) DNA polymerase and uniquely-formulated buffers, and requires specialized reaction conditions. If these conditions are not adhered to, reaction failure is likely. Refer to **Important Parameters** for more information.

### 1. Prepare the PCR master mix

- 1.1 KAPA HiFi HotStart Uracil+ reactions **MUST** be set up on ice since the high proofreading activity of the enzyme will result in rapid primer degradation at room temperature.
- 1.2 Ensure that all reagents are properly thawed and mixed.
- 1.3 Prepare a PCR master mix containing the appropriate volume of all reaction components common to all or a subset of reactions to be performed.
- 1.4 Calculate the required volumes of each component based on the following table:

Component	25 $\mu$ L reaction <sup>1</sup>	Final conc.
PCR-grade water	Up to 25 $\mu$ L	N/A
2X KAPA HiFi HotStart Uracil+ ReadyMix <sup>2,3</sup>	12.5 $\mu$ L	1X
10 $\mu$ M Forward Primer	0.75 $\mu$ L	0.3 $\mu$ M
10 $\mu$ M Reverse Primer	0.75 $\mu$ L	0.3 $\mu$ M
Template DNA <sup>4</sup>	As required	As required

<sup>1</sup> Reaction volumes may be adjusted between 10–50  $\mu$ L. For volumes other than 25  $\mu$ L, scale reagents proportionally. Reaction volumes >50  $\mu$ L are not recommended.

<sup>2</sup> KAPA HiFi HotStart Uracil+ ReadyMix contains 2.5 mM MgCl<sub>2</sub> (1X). Additional MgCl<sub>2</sub> may be added separately, but is unlikely to be required.

<sup>3</sup> KAPA HiFi HotStart Uracil+ ReadyMix contains 0.3 mM of each dNTP (1X), and 0.5 U of KAPA HiFi HotStart Uracil+ DNA Polymerase (per 25  $\mu$ L reaction) in a proprietary reaction buffer.

<sup>4</sup> Use <100 ng genomic DNA (10–100 ng) and <1 ng less complex DNA (0.1–1 ng) per 25  $\mu$ L reaction as first approach.

### 2. Set up individual reactions

- 2.1 Transfer the appropriate volumes of PCR master mix, template and primer to individual PCR tubes or wells of a PCR plate.
- 2.2 Cap or seal individual reactions, mix and centrifuge briefly.

## 3. Run the PCR

3.1 Perform PCR with the following cycling protocol:

Step	Temperature	Duration	Cycles
Initial denaturation <sup>1</sup>	95°C	3 min	1
Denaturation <sup>2</sup>	98°C	20 sec	15–35 <sup>6</sup>
Annealing <sup>3,4</sup>	60–75°C	15 sec	
Extension <sup>5</sup>	72°C	15–60 sec/kb	
Final extension	72°C	1 min/kb	1

<sup>1</sup> Initial denaturation for 3 min at 95°C is sufficient for most applications. Use 5 min at 95°C for GC-rich targets (>70% GC content).

<sup>2</sup> KAPA HiFi HotStart Uracil+ ReadyMix has a higher salt concentration than conventional PCR ready-mixes, which affects DNA melting. To ensure that complex and GC-rich targets are completely denatured, use a temperature of 98°C for denaturation during cycling.

<sup>3</sup> In addition to DNA melting, high salt also affects primer annealing. The optimal annealing temperature for a specific primer set is likely to be different (higher) than when used in a conventional PCR ready-mix. An annealing temperature gradient PCR is recommended to determine the optimal annealing temperature with the KAPA HiFi HotStart Uracil+ ReadyMix. If gradient PCR is not feasible, anneal at 65°C as a first approach.

<sup>4</sup> Two-step cycling protocols with a combined annealing/extension temperature in the range of 68–75°C and a combined annealing/extension time of 30 sec/kb may be used.

<sup>5</sup> Use 15 sec extension per cycle for targets  $\leq$ 1 kb, and 30–60 sec/kb for longer fragments, or to improve yields.

<sup>6</sup> For highest fidelity, use  $\leq$ 25 cycles. In cases where very low template concentrations or low reaction efficiency results in low yields, 30–35 cycles may be performed to produce sufficient product for downstream applications.

## Important Parameters: Standard PCR

### Amplification of bisulfite-converted DNA

Amplification of bisulfite-treated DNA can be problematic due to DNA damage arising from the harsh conditions required for near-complete conversion of unmethylated cytosines. Conversion conditions should be optimized to minimize the extent of DNA damage while ensuring sufficient conversion efficiency.

Because bisulfite treatment converts cytosines to uracils, which are subsequently substituted with thymines during PCR, bisulfite-converted DNA is typically much more AT-rich than the original source DNA. This should be considered carefully when designing PCR primers, and cycling parameters such as annealing temperature may require special attention.

### MgCl<sub>2</sub> concentration

KAPA HiFi HotStart Uracil+ ReadyMix contains a final (1X) MgCl<sub>2</sub> concentration of 2.5 mM, which is sufficient for most applications. Applications which are likely to require higher MgCl<sub>2</sub> concentrations include long PCR (>10 kb) and AT-rich PCR, as well as amplification using primers with a low GC content (<40%). Note that bisulfite-converted DNA is typically very AT-rich, as are the primers used for amplification of these templates; this application may therefore require additional MgCl<sub>2</sub>.

### Prevention of amplicon contamination with UDG

For effective removal of carryover contamination, dUTP must be added routinely to PCR reactions with KAPA HiFi HotStart Uracil+ ReadyMix. Typically, dUTP is added to a concentration of 0.2 mM, but short AT-poor amplicons may require up to 0.3 mM dUTP for effective removal using UDG. For amplicons >600 bp, a lower dUTP concentration ( $\leq 0.1$  mM) may be required for efficient amplification. Low amplification efficiencies may result from incomplete UDG inactivation, in which case a longer initial denaturation may be required to inactivate the UDG prior to cycling. Refer to the manufacturer's recommendations for optimal UDG concentration, and incubation/inactivation conditions.

### Denaturation

Due to the high salt concentration of the KAPA HiFi HotStart Uracil+ ReadyMix, it is important to use appropriate denaturation parameters. An initial denaturation time of 2–5 min at 95°C is recommended to ensure that complex template DNA is fully denatured before the first primer annealing step. Use 5 min for complex, genomic DNA and/or GC-rich targets, and at least 45 sec for less complex templates such as purified viral or plasmid DNA.

### Annealing temperature

Due to the high salt concentration of the KAPA HiFi HotStart Uracil+ ReadyMix, the optimal annealing temperature for a given primer set is usually different when compared with other PCR buffer systems. When using the kit with a specific primer pair for the first time, determine the optimal annealing temperature with annealing temperature gradient PCR. We recommend a gradient from 60–72°C, although some assays may require even higher annealing temperatures. For assays with optimal annealing temperatures of 68°C or higher, 2-step cycling may be performed at the optimal annealing temperature. Optimal annealing temperatures below 60°C are typically rare, but may be necessary when using primers with a high AT content, as is often the case with amplification of bisulfite-converted DNA.

If a gradient PCR is not feasible, use an annealing temperature of 60°C as a first approach, and adjust the annealing temperature based on the results obtained:

- If a low yield of only the specific product is obtained, lower the annealing temperature in 1–2°C increments.
- If nonspecific products are formed in addition to the specific product, increase the annealing temperature in 1–2°C increments.
- If no product is formed (specific or nonspecific), reduce the annealing temperature by 5°C.  $MgCl_2$  concentration may have to be increased.

If only nonspecific products are formed (in a ladder-like pattern), increase the annealing temperature by 5°C.

### Amplicon length

KAPA HiFi HotStart Uracil+ ReadyMix can amplify targets up to 18 kb in length from simple purified template such as plasmid DNA, and up to 15 kb in length from high quality (i.e. not bisulfite-treated), complex genomic DNA. For efficient amplification of fragments  $\geq 10$  kb, higher template concentrations, and optimization of the  $Mg^{2+}$  concentration, may be required.

### Primer and template DNA quality

Primers should be designed to minimize the possibility of primer-dimer formation, self-priming, or nonspecific priming. Primer pairs should have similar theoretical melting temperatures ( $T_m$ ), and should have a GC content of 40–60%, although this may not be feasible for bisulfite-converted template DNA. Primers with a GC content >60% may require higher denaturation temperatures or longer denaturation times, while primers with a GC content <40% may require lower annealing temperatures and increased  $MgCl_2$  concentrations.

Template DNA quality has a significant impact on the success of PCR amplification. Degraded, damaged, or sheared template DNA is usually problematic. While KAPA HiFi HotStart Uracil+ ReadyMix tolerates uracil, deamination of dCMP to dUMP in the DNA template will generate G/C to A/T mutations during amplification.

**NOTE:** Always dilute and store primers and DNA in a buffered solution (e.g. 10 mM Tris-HCl, pH 8.0 – 8.5) instead of PCR-grade water, and minimize freeze-thaw cycles to limit degradation and maintain primer quality.

## Important Parameters: Library Amplification

### Cycle number

Excessive library amplification should be avoided to minimize the following adverse effects:

- increased duplicate reads
- uneven coverage depth and sequence dropout
- chimeric library inserts
- nucleotide substitutions
- heteroduplex formation.

To minimize over-amplification and associated unwanted artifacts, the number of amplification cycles should be optimized to ensure a sufficient amount of amplified library for the next step in the workflow (capture or sequencing), plus the amount needed for library QC and/or archiving. Depending on the sequencing application and degree of multiplexing, 100 ng – 1.5  $\mu$ g of amplified library is typically required.

The number of cycles typically required will vary, depending on input amount and quality. Size selection of libraries at any part in the library construction process results in significant loss of material and as a result, 2–4 additional cycles are required for workflows which include a size-selection step prior to library amplification.

### Primer depletion and library over-amplification

In library amplification reactions (set up according to the recommended protocol), primers are typically depleted before dNTPs. When DNA synthesis can no longer take place due to primer depletion, subsequent rounds of DNA denaturation and annealing result in the separation of complementary DNA strands, followed by imperfect annealing to non-complementary partners. This presumably results in the formation of so-called “daisy-chains” or tangled knots, comprising large assemblies of improperly annealed, partially double-stranded, heteroduplex DNA. These species migrate slower and are observed as secondary, higher molecular weight peaks during the electrophoretic analysis of amplified libraries. However, they are typically comprised of library molecules of the desired length, which are separated during denaturation prior to target enrichment (capture) or cluster amplification. Since these heteroduplexes contain significant portions of single-stranded DNA, over-amplification leads to the under-quantification of library molecules with assays employing dsDNA-binding dyes. qPCR-based library quantification methods, such as that employed by the **KAPA Library Quantification Kit**, quantify DNA by denaturation and amplification, thereby providing a more accurate measurement of the amount of adapter-ligated molecules—even if the library was over-amplified.

### Reaction Cleanups

This protocol has been validated for use with either KAPA Pure Beads or Agencourt® AMPure® XP (Beckman Coulter®). Solutions and conditions for DNA binding and size selection may differ if other beads are used.

Observe all the storage and handling recommendations for KAPA Pure Beads or AMPure XP. Equilibration to room temperature is essential to achieve specified size distribution and yield of libraries. Beads will settle gradually; always ensure that they are fully resuspended before use.

***To ensure optimal DNA recovery, it is critical that the DNA and the KAPA Pure Beads are thoroughly mixed before the DNA binding incubation.***

The volumes of 80% ethanol used for bead washes may be adjusted to accommodate smaller reaction vessels and/or limited pipetting capacity, but it is important that the beads are entirely submerged during the wash steps. ***Always use freshly prepared 80% ethanol.***

It is important to remove all ethanol before proceeding with subsequent reactions. However, over-drying of beads may make them difficult to resuspend, resulting in a dramatic loss of DNA. ***Drying of beads at 37°C is not recommended.***

Where appropriate, DNA should be eluted from beads in elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5). Elution of DNA in PCR-grade water is not recommended, however, libraries constructed for target capture must be eluted and stored in PCR-grade water to facilitate drying of DNA prior to probe hybridization.

Please refer to the **KAPA Pure Beads Technical Data Sheet** for additional detailed information and protocols.

## Product Specifications

### Shipping, storage and handling

KAPA HiFi HotStart Uracil+ ReadyMix is shipped on dry ice or ice packs, depending on the country of destination. Upon arrival, store kit components at -15°C to -25°C in a constant-temperature freezer. When stored under these conditions and handled correctly, full activity of the kit is retained until the expiry date indicated on the kit label. KAPA HiFi HotStart Uracil+ ReadyMix contains isostabilizers and may not freeze solidly, even when stored at -15°C to -25°C. This will not affect the shelf-life of the product.

Always ensure that the product has been fully thawed and mixed before use. Reagents may be stored at 2°C to 8°C for short-term use (up to 1 month). Return to -15°C to -25°C for long-term storage. Provided that all components have been handled carefully and not contaminated, the kit is not expected to be compromised if left (unintentionally) at room temperature for a short period of time (up to 3 days). Long-term storage at room temperature and 2°C to 8°C is not recommended. Please note that reagents stored at temperatures above -15°C to -25°C are more prone to degradation when contaminated during use, and therefore storage at such temperatures is at the user's own risk.

### Quality Control

Each batch of KAPA HiFi HotStart Uracil+ DNA Polymerase is confirmed to contain <2% contaminating protein (Agilent Protein 230 Assay). KAPA HiFi HotStart Uracil+ ReadyMix is subjected to stringent quality control tests, is free of contaminating exo- and endonuclease activity, and meets strict requirements with respect to DNA contamination levels.



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