Shortened KAPA RNA HyperPrep with RiboErase (HMR) workflow†

OVERVIEW

This document contains the shortened KAPA RNA HyperPrep with RiboErase (HMR) workflow. The step numbers are kept the same as the *Instructions for Use* (IfU). All differences are highlighted with a dark red star (★).

Please note that **2nd Post-Ligation Cleanup (Step 11)** was completely eliminated.

†*Roche provides complete support for the standard protocol detailed in the KAPA RNA HyperPrep Kit with RiboErase (HMR) Instructions for Use. For those customers seeking a shorter workflow, we have provided this modified protocol, noting that lower performance may be observed depending upon various factors, such as sample type and/or amount.*

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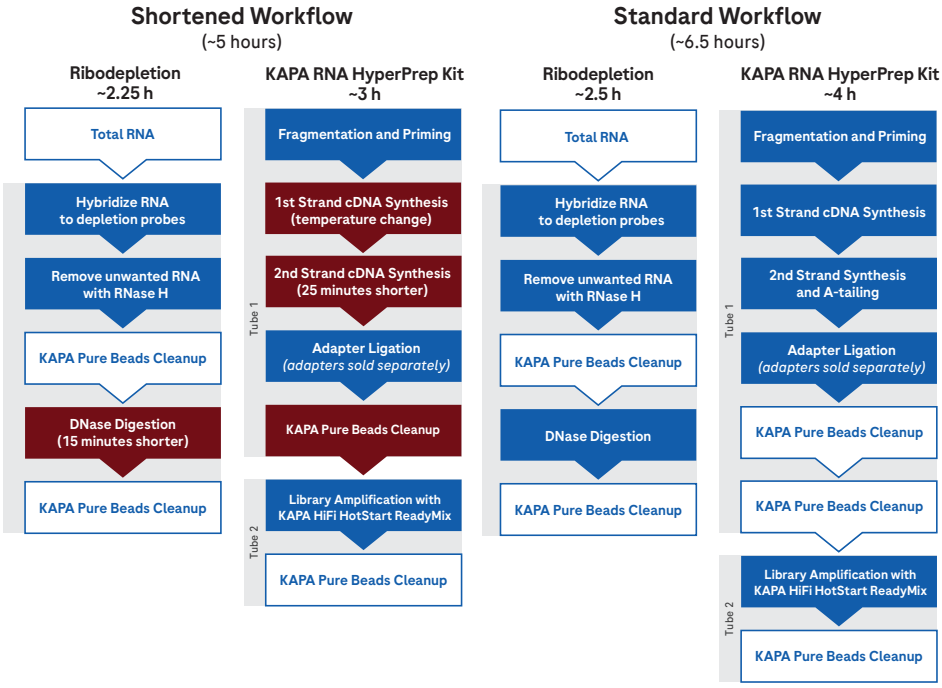
COMPARISON OF THE PROTOCOLS AT A GLANCE



PROTOCOL

1. Reagent Preparation

This protocol takes approximately 5.5 hrs to complete both rRNA depletion or ribodepletion and RNA Library preparation. Ideally, master mixes for the various steps in the process should be prepared as required. For maximum stability and shelf-life, enzymes and reaction buffers are supplied separately in the KAPA RNA HyperPrep Kit with RiboErase (HMR). For a streamlined protocol, a reagent master mix with a minimum of 10% excess is prepared for each of these enzymatic steps, as outlined in Tables 2 – 8 in the IfU. Volumes of additional reagents required for the KAPA RNA HyperPrep Kit with RiboErase (HMR) protocol are listed in Table 9 in the IfU. Always ensure that KAPA Pure Beads and PEG/NaCl Solution are fully equilibrated to room temperature before use.

2. Oligo Hybridization and rRNA Depletion

This protocol requires 25 ng – 1 µg of total RNA, in 10 µL of RNase-free water. Ensure that the oligo hybridization master mix and depletion master mix are prepared as in the two tables on page 23 of the IfU.

2.1 Program a thermocycler as follows:

Step	Temp	Duration
Hybridization	95°C	2 min
Ramp down to 45°C at -0.1°C/sec		
Pause	45°C	HOLD
Depletion	45°C	30 min
HOLD	4°C	HOLD

2.2 Assemble rRNA Hybridization reactions as follows:

Component	Volume
Total RNA in water	10 µL
Hybridization master mix at room temperature (page 23, IfU)	10 µL
Total Volume	**20 µL**

2.3 Place samples in the pre-programmed thermocycler and execute the program.

2.4 Ensure the depletion mastermix containing RNase H is added while the samples are kept at 45°C in the thermocycler. When program reaches the pause step at 45°C, add the following to each 20 µL hybridization reaction and mix thoroughly by pipetting up and down multiple times.

Component	Volume
Depletion master mix at room temperature (page 23, IfU)	5 µL
Total Volume	**25 µL**

2.5 Resume the cycling program to continue with the depletion step (45°C for 30 min).

2.6 Proceed immediately to **rRNA Depletion Cleanup (Step 3)**.

3. rRNA Depletion Cleanup

3.1 Perform a 2.2X bead-based cleanup by combining the following:

Component	Volume
rRNA depleted RNA	25 µL
KAPA Pure Beads	55 µL
Total Volume	**80 µL**

3.2 Thoroughly resuspend the beads by pipetting up and down multiple times.

3.3 Incubate the tubes at room temperature for 5 min to bind RNA to the beads.

3.4 Place the tubes on a magnet to capture the beads. Incubate until the liquid is clear.

3.5 Carefully remove and discard 75 µL of supernatant.

3.6 Keeping the tubes on the magnet, add 200 µL of 80% ethanol.

3.7 Incubate the tubes on the magnet at room temperature for more than 30 sec.

3.8 Carefully remove and discard the ethanol.

3.9 Keeping the tubes on the magnet, add 200 µL of ethanol.

3.10 Incubate the tubes on the magnet at room temperature for more than 30 sec.

3.11 Remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

3.12 Dry the beads at room temperature for 3 – 5 min or until all the ethanol has evaporated.

3.13 Proceed immediately to **DNase Digestion (Step 4)**.

4. DNase Digestion

Note: Places in this protocol that are different than IfU are pointed out by this dark red star.

4.1 Assemble the DNase Digestion reaction as follows using the master mix made using the table on page 25 in the IfU:

Component	Volume
Beads with rRNA depleted RNA	–
DNase Digestion master mix at room temperature (page 25, IfU)	22 µL
Total Volume	**22 µL**

4.2 Thoroughly resuspend the beads by pipetting up and down multiple times.

4.3 Incubate the tubes at room temperature for 3 min to elute RNA off the beads.

4.4 Place the tubes on the magnet to capture the beads—incubate until the liquid is clear.

4.5 Carefully transfer 20 µL of supernatant into new tubes. Discard the tubes with beads.

4.6 Incubate the tubes with supernatant using the following protocol:

Step	Temp	Duration
DNase Digestion	37°C	15 min
HOLD	4°C	HOLD

4.7 Proceed immediately to **DNase Digestion Cleanup (Step 5)**.

5. DNase Digestion Cleanup

5.1 Perform a 2.2X bead-based cleanup by combining the following:

Component	Volume
DNase Treated RNA	20 µL
KAPA Pure Beads	44 µL
Total Volume	**64 µL**

5.2 Thoroughly resuspend the beads by pipetting up and down multiple times.

5.3 Incubate the tubes at room temperature for 5 min to bind RNA to the beads.

5.4 Place the tubes on a magnet to capture the beads. Incubate until the liquid is clear.

5.5 Carefully remove and discard 60 µL of supernatant.

5.6 Keeping the tubes on the magnet, add 200 µL of 80% ethanol.

5.7 Incubate the tubes on the magnet at room temperature for more than 30 sec.

5.8 Carefully remove and discard the ethanol.

5.9 Keeping the tubes on the magnet, add 200 µL of ethanol.

5.10 Incubate the tubes on the magnet at room temperature for more than 30 sec.

5.11 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 the ethanol has evaporated.

5.13 Proceed immediately to **RNA Elution Fragmentation and Priming (Step 6)**.

6. RNA Elution Fragmentation and Priming

RNA depleted of rRNA is eluted from the beads in Fragment, Prime, and Elute Buffer (1X) and fragmented to the desired size by incubation at a high temperature.

6.1 Prepare the required volume of Fragment, Prime, and Elute buffer (1X) by combining the following at room temperature:

Component	1 Library
FPE Buffer (2X)	11
RNase-Free Water	11
Total Volume	**22**

6.2 Thoroughly resuspend the beads with purified, DNase-treated RNA in 22 µL of Fragment, Prime, and Elute Buffer (1X) by pipetting up and down multiple times.

6.3 Incubate the tubes at room temperature for 3 minutes to elute RNA off the beads.

6.4 Place the tubes on the magnet to capture the beads. Incubate until liquid is clear.

6.5 Carefully transfer 20 µL of supernatant into new tubes. Discard the tubes with beads.

6.6 Place the tubes in a thermocycler and carry out the fragmentation and priming program as follows:

Input RNA Type	Desired mean library insert size (bp)	Fragmentation
Intact	100 – 200	8 min at 94°C
	200 – 300	6 min at 94°C
	300 – 400	6 min at 85°C
Partially Degraded	100 – 300	1 – 6 min at 85°C
Degraded (e.g., FFPE)	100 – 200	1 min at 65°C

6.7 Place the tubes on ice and proceed immediately to **1st Strand Synthesis (Step 7)**.

★ 7. 1st Strand Synthesis

7.1 On ice, assemble the 1st strand synthesis reaction (page 28, IfU) as follows:

Component	Volume
Fragmented, Primed RNA	20 µL
1st Strand Synthesis master mix (page 28, IfU)	10 µL
Total Volume	**30 µL**

7.2 Keeping the tubes on ice, mix thoroughly by pipetting up and down several times.

7.3 Incubate the tubes using the following protocol:

Step	Temp	Duration
Primer Extension	★ 20°C	10 min
1st Strand Synthesis	42°C	15 min
Enzyme Inactivation	70°C	15 min
HOLD	4°C	HOLD

7.4 Place the tubes on ice and proceed immediately to **2nd Strand Synthesis and A-tailing (Step 8)**.

★ 8. 2nd Strand Synthesis and A-tailing

8.1 On ice, assemble the 2nd strand synthesis and A-tailing reaction (page 29, IfU) as follows:

Component	Volume
1st Strand Synthesis Product	30 µL
2nd Strand Synthesis & A-tailing master mix (page 29, IfU)	30 µL
Total Volume	**60 µL**

8.2 Keeping the tubes on ice, mix thoroughly by pipetting the reaction up and down several times.

8.3 Incubate the tubes using the following protocol:

Step	Temp	Duration
2nd Strand Synthesis	★ 42°C	★ 5 min
A-tailing	62°C	10 min
HOLD	4°C	HOLD

8.4 Place the tubes on ice and proceed immediately to **Adapter Ligation (Step 9)**.

★ 9. Adapter Ligation

9.1 Dilute the adapters in preparation for ligation, targeting the following concentrations:

Quantity of Starting Material	Starting Material Quality	Adapter Stock Concentration
25 – 49 ng	Partially Degraded or FFPE derived	★ 0.15 µM
	High Quality	★ 0.15 µM
50 – 499 ng	Partially Degraded or FFPE derived	1.5 µM
	High Quality	1.5 µM
500 – 1000 ng	Partially Degraded or FFPE derived	1.5 µM
	High Quality	7 µM

9.2 On ice, set up the adapter ligation reaction (page 30, IfU) as follows:

Component	Volume
2nd Strand Synthesis Product	60 µL
Adapter Ligation master mix (page 30, IfU)	45 µL
Diluted Adapter Stock	5 µL
Total Volume	**110 µL**

9.3 Keeping the tubes on ice, thoroughly mix by pipetting up and down several times.

9.4 Incubate the tubes at 20°C for 15 min. Proceed immediately to **Post-Ligation Cleanup (Step 10)**.

★ 10. Post-Ligation Cleanup

10.1 Perform a 0.7X bead-based cleanup by combining the following:

Component	Volume
Adapter-Ligated DNA	110 µL
KAPA Pure Beads	★ 77 µL
Total Volume	**187 µL**

10.2 Thoroughly resuspend the beads by vortexing.

10.3 Incubate the tubes at room temperature for 5 – 15 min to bind DNA to the beads.

10.4 Place the tubes on a magnet to capture the beads. Incubate until the liquid is clear.

10.5 Carefully remove and discard 182 µL of supernatant.

10.6 Keeping the tubes on the magnet, add 200 µL of 80% ethanol.

10.7 Incubate the tubes on the magnet at room temperature for more than 30 sec.

10.8 Carefully remove and discard the ethanol.

10.9 Keeping the tubes on the magnet, add 200 µL of ethanol.

10.10 Incubate the tubes on the magnet at room temperature for more than 30 sec.

10.11 Remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

10.12 Dry the beads at room temperature for 3 – 5 min or until all the ethanol has evaporated.

10.13 Remove the tubes from the magnet.

★ 10.14 Thoroughly resuspend the beads in 22 µL of 10 mM Tris-HCl (pH 8 – 8.5).

★ 10.15 Incubate the tubes at room temperature for 2 min to elute DNA off the beads.

★ 10.16 Transfer 20 µL of the clear supernatant to new tubes.

10.17 Proceed immediately to **Library Amplification (Step 12)**.

★ **Note: 2nd Post-ligation Cleanup (Step 11)** has been removed and is not required.

12. Library Amplification

12.1 Assemble each library amplification reaction (page 36, IfU) as follows:

Component	Volume
Purified, Adapter-Ligated DNA	20 µL
Library Amplification master mix (page 36, IfU)	30 µL
Total Volume	**50 µL**

12.2 Mix well by pipetting up and down several times.

12.3 Amplify the library using the following thermocycling profile:

Step	Temp	Duration	Cycles
Initial Denaturing	98°C	45 sec	1
Denaturation	98°C	15 sec	Refer to Table 2 on page 37, IfU)
Annealing	60°C	30 sec	
Extension	72°C	30 sec	
Final Extension	72°C	1 min	1
HOLD	HOLD	HOLD	1

12.4 Proceed to **Library Amplification Cleanup (Step 13)**.

13. Library Amplification Cleanup

13.1 Mix thoroughly by vortexing.

13.2 Incubate the tubes at room temperature for 5 – 15 min to bind DNA to the beads.

13.3 Place the tubes on a magnet to capture the beads. Incubate until the liquid is clear.

13.4 Carefully remove and discard 95 µL of supernatant.

13.5 Keeping the tubes on the magnet, add 200 µL of 80% ethanol.

13.6 Incubate the tubes on the magnet at room temperature for more than 30 sec.

13.7 Carefully remove and discard the ethanol.

13.8 Keeping the tubes on the magnet, add 200 µL of ethanol.

13.9 Incubate the tubes on the magnet at room temperature for more than 30 sec.

13.10 Remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

13.11 Dry the beads at room temperature for 3 – 5 min or until all the ethanol has evaporated.

13.12 Thoroughly resuspend the beads in 22 µL of 10 mM Tris-HCl (pH 8 – 8.5).

13.13 Incubate the tubes at room temperature for 2 min to elute DNA off the beads.

13.14 Place the tubes on the magnet to capture the beads. Incubate until the liquid is clear.

13.15 Transfer 20 µL of the clear supernatant to a new plate/tube(s), and store the purified, amplified libraries at 2°C to 8°C (≤1 week), or at -15°C to -25°C.

Reference: This document is based on *Instructions for Use for KAPA RNA HyperPrep Kit with RiboErase (HMR), Illumina® Platforms, KR1351 – v6)* with changes made where the methods are done differently.

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