RNA-seq Library Construction with the KAPA mRNA HyperPrep Kit

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1 Works for me

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ABSTRACT

A protocol for making multiplexed RNA-seq libraries using KAPA Biosystems mRNA HyperPrep Kit, optimized for *Saccharomyces cerevisiae*.

GUIDELINES

This protocol is written to use half reactions, which we have found works well for yeast RNA-seq libraries. We suggest piloting half reactions for a small number of samples to determine whether your library yields are sufficient. Reagent amounts can be doubled for full reactions.

MATERIALS

NAME	CATALOG #	VENDOR
Ethanol (100%, Molecular Biology Grade)	BP2818500	Fisher Scientific
KAPA mRNA HyperPrep Kit	KK8514	Kapa Biosystems
KAPA Single Indexed Adapters	KK8700	Kapa Biosystems
AvantGuard 200uL Barrier Tips	AV200-H	MIDSCI
1M Tris-HCl (pH 8.0)	15568025	Thermo Fisher Scientific
RNase-Free Water	10977015	Thermo Fisher Scientific
Ring Magnet Plate - Agencourt SPRIPlate 96R	A29164	Fisher Scientific
Magnetic Rack (0.2mL)	B04000001	Diagenode
Surebeads Magnetic Rack	1614916	Bio-rad Laboratories

MATERIALS TEXT

BEFORE STARTING

Before starting experiment:

(1) Make or have ready RNase-free stock solutions.

(2) 80% Ethanol must be made fresh daily.

(3) All Mastermixes can be prepared 1 day in advance (must be stored at -20ºC). All mastermixes and samples must be stored on ice when not in use.

(4) PEG and KAPA Purebeads must warm up to room temperature for 30 minutes prior to use.

(5) Pre-label 4 sets of RNase-free 0.2 mL microcentrifuge tubes for each sample. Make sure you have RNase-Free 1.7 mL microcentrifuge tubes for mastermixes labeled.

(6) RNase-Free Barrier tips must be used for all tip handling.

(7) When sampes are on the ring magnet all liquid dispensing must be done by carefully pipetting directly above the tube opening (Do not let the pipet tip touch the tube). If liquid is dispensed within the tube, the beads can dislodge from the magnet, which can reduce library yield.

(8) Bead drying can be performed at room temperature either in a Biosafety cabinet, or on the bench next to a flame. Beads will change color when dry (from brown to orange/rust). Bead drying time necessarily depends on the room temperature of the lab.

Prepping mRNA Capture Beads

1

This protocol is written to use half reactions, which we have found works well for yeast RNA-seq libraries. We suggest piloting half reactions for a small number of samples to determine whether your library yields are sufficient. Reagent amounts can be doubled for full reactions.

1. Resuspend the mRNA Capture Beads (stored at 4°C) by gently pipetting (try to prevent foaming).

2. For each library, transfer 26.25 µL of beads (25 µL x 1.05) to an RNase-free 1.7 mL microcentrifuge tube (up to 24 total reactions per one tube).

3. Place the tube with beads on a Surebeads Magnetic Rack (Bio-Rad #1614916) and wait for the sample to clear (~1 min). Carefully pipet and discard the supernatant.

4. Carefully resuspend the beads via pipetting (try to prevent foaming) with equal amount of Bead Binding Buffer (stored at 4°C).

5. Place the tube with beads on the Surebeads Magnetic Rack, wait for sample to clear (~1 min), and carefully pipet and discard the supernatant.

6. Carefully resuspend beads via pipetting (try to prevent foaming) with equal amount of Bead Binding Buffer (stored at 4°C).

mRNA Capture

2

The protocol requires 50 ng to 1 µg of total RNA in 25 µL RNase-Free water.

We recommend 500 ng of total RNA as a starting point.

1. Transfer 500 ng of RNA to an RNase-free PCR tube, and bring the volume up to 25 µL with RNase-free water.

2. Add 25 µL of prepped mRNA Capture Beads to PCR tubes containing total RNA. Thoroughly mix via gently pipetting.

3. Place tubes in thermocycler and run:

Step	Temperature	Duration
1st mRNA Capture	65°C	2 min
Cool	20°C	5 min

4. Remove tubes and place on magnetic rack (Diagenode #B04000001) and incubate at room temperature (RT) until sample is clear (~1 min). Use a pipettor to carefully remove supernatant.

4. Remove tubes from rack and gently resuspend in 100 µL mRNA Bead Wash Buffer.

5. Place tubes on magnetic rack (0.2 mL) and incubate at RT until sample is clear (~1 min). Use a pipettor to carefully remove supernatant.

7. Remove tubes from magnetic rack and gently resuspend in 25 µL RNase-free water.

8. Place tubes in thermocycler and run:

Step	Temperature	Duration
2nd mRNA Capture	70°C	2 min
Cool	20°C	5 min

9. Add 25 µL Bead Binding Buffer to the tubes. Mix thoroughly via gentle pipetting.

10. Incubate tubes at 20°C for 5 min.

11. Place tubes on magnetic rack and incubate at RT until sample is clear (~1 min). Use a pipettor carefully remove supernatant.

12. Remove tubes from rack and gently resuspend in 100 µL mRNA Bead Wash Buffer.

13. Again, place tubes on magnetic rack and incubate at RT until sample is clear (~1 min). Use a pipettor carefully remove supernatant.

mRNA Elution, Fragmentation, and Priming

3

1. Prepare Fragment, Prime, and Elute (FPE) Mastermix.

FPE Mastermix (per sample, if doing more than one sample make 1.05 times the number of samples)
5.5 µL RNase-Free water
5.5 µL Fragment, Prime, and Elute Buffer (2X)

Incubation times and temperatures will vary depending on your desired fragment size.
For 100-200 bp, incubate at 94ºC for 8 minutes
For 201-300 bp, incubate at 94ºC for 6 minutes
For 301-400 bp, incubate at 85ºC for 6 minutes

2. Add 11 µL FPE Mastermix to each sample and thoroughly resuspend via pipetting.

This is a safe stopping point.
FPE resuspended samples may be stored at 4ºC ≤ 24 hrs. Freezing samples will damage beads. DO NOT FREEZE.

3. Have a thermocycler pre-incubated to desired temperature (placing samples in a cold thermocycler and ramping up to the temperature can cause over fragmentation, as samples are exposed to additional heating as the thermocycler gets up to temperature).

4. Place ring magnet (Fisher Scientific #A29164) next to thermocycler.

5. Place FPE-resuspended sample tubes in a pre-warmed thermocycler and incubate for desired fragment size using a timer (time must be exact. Any longer or shorter will result in differences in fragment sizes).

6. Once the incubation is complete, immediately place tubes on ring magnet.

7. Using a pipettor, carefully transfer 10 µL supernatant to a fresh RNase-free PCR tube.

8. Place tube on ice and proceed immediately to 1st Strand Synthesis.

1st Strand Synthesis

4 1. Prepare 1st Strand Synthesis Mastermix.

1st Strand Synthesis Mastermix (per sample, if doing more than one sample make 1.05 times the number of samples)
5.5 µL 1st Strand Synthesis Buffer
0.5 µL KAPA Script

2. Add 5 µL 1st Strand Synthesis Mastermix to the tube containing 10 µL fragmented RNA.

3. Keeping tubes on ice, gently mix thoroughly via pipetting.

4. Place tubes in a thermocycler and run:

Step	Temperature	Duration
Primer Extension	25ºC	10 min
1st Strand Synthesis	42ºC	15 min
Enzyme Inactivation	70ºC	15 min
HOLD	4ºC	∞

5. Place tubes on ice.

6. Proceed immediately to 2nd Strand Synthesis and A-tailing.

2nd Strand Synthesis and A-tailing

5 1. Prepare 2nd Strand Synthesis and A-tailing Mastermix.

2nd Strand Synthesis Mastermix (per sample, if doing more than one sample make 1.05 times the number of samples)
15.5 µL 1st Strand Synthesis Buffer
1 µL KAPA Script

2. Add 15 µL 2nd Strand Synthesis Mastermix to the tube containing 15 µL 1st Strand Synthesized samples.

3. Place tubes in a thermocycler and run:

Step	Temperature	Duration
2nd Strand Synthesis	16ºC	30 min
A-tailing	62ºC	10 min
HOLD	4ºC	∞

5. Place tubes on ice.

6. Proceed immediately to Adapter Ligation.

Adapter Ligation

6 1. Dilute Adapters.

Dilute adapters according to quantity of starting RNA concentration.

Quantity of RNA	Adapter stock concentration
50 ηg - 499 ng	1.5 µM
500 ηg - 1000ng	7 µM

I stared with 500 ng of total RNA and used 1.5 µM adapter concentration.

2. Prepare Adapter Ligation Mastermix.

Adapter Ligation Mastermix (per sample, if doing more than one sample make 1.05 times the number of samples)
20 µL Ligation Buffer (viscous, pipet slowly)
5 µL DNA Ligase

3. Add 2.5 µL diluted adapters to 30 µL 2nd Strand Synthesized samples.

4. Keeping tubes on ice, gently mix thoroughly via pipetting.

5. Add 22.5 µL Adapter Ligation Mastermix to the 32.5 µL adapter containing 2nd Strand Synthesis samples.

6. Keeping tubes on ice, gently mix thoroughly via pipetting.

7. Incubate at 20°C for 15 min.

8. Proceed immediately to 1st Post-Ligation Cleanup.

1st Post-Ligation Cleanup

7

KAPA Pure Beads must be warmed up to RT (~30 min)

All ethanol washes will be done on the ring magnet (Fisher #A29164)

1. Add 35 µL KAPA Pure Beads to 55 µL Adapter ligated samples and mix thoroughly via pipetting.

2. Incubate at RT for 5 min.

3. Place tubes on ring magnet (Fisher #A29164). Incubate until liquid is clear (~1 min).

4. Carefully, without touching the beads, use a pipettor to remove the supernatant (using Avant #AH200-H - skinnier tips than normal barrier tips).

5. Carefully, dispense 100 µL 80% ethanol into the tube, making sure to not touch the tube with the pipet tip (see "Before Start" in Guidelines & Warnings).

6. Incubate at RT for 30 sec.

7. Carefully, without touching the beads, use a pipettor to remove the supernatant (using Avant #AH200-H - skinnier tips than normal barrier tips).

8. Carefully, dispense 100 µL 80% ethanol into the tube, making sure to not touch the tube with the pipet tip.

9. Incubate at RT for 30 sec.

10. Carefully, without touching the beads, use a pipettor to remove the supernatant (using Avant #AH200-H - skinnier tips than normal barrier tips).

11. Using a fresh pipet tip, remove residual ethanol.

12. Dry beads at RT for 5 min (over-drying beads may reduce library yield).

13. Remove tubes from ring magnet.

14. Using a pipettor thoroughly resuspend beads in 25 µL RNase-Free 10 mM Tris-HCl (pH 8.0-8.5).

15. Incubate tubes at RT for 2 min.

This is a safe stopping point.

Resuspended beads can be stored at 4°C for ≤24hrs. DO NOT FREEZE beads, as this will cause dramatic loss of DNA.

16. Proceed to 2nd Post-ligation Cleanup.

2nd Post-ligation Cleanup

8

PEG/NaCl must be warmed up to RT (~30 min)

All ethanol washes will be done on the ring magnet (Fisher #A29164).

1. Add 17.5 µL PEG/NaCl to 25 µL 1st Post-ligation cleaned samples and mix thoroughly via pipetting.

2. Incubate at RT for 5 min.

3. Place tubes on ring magnet (Fisher #A29164). Incubate until liquid is clear (~1 min).

4. Carefully, without touching the beads, use a pipettor to remove the supernatant (using Avant #AH200-H - skinnier tips than normal barrier tips).

5. Carefully, dispense 100 µL 80% ethanol into the tube, making sure to not touch the tube with the pipet tip.

6. Incubate at RT for 30 sec.

7. Carefully, without touching the beads, use a pipettor to remove the supernatant (using Avant #AH200-H - skinnier tips than normal barrier tips).

8. Carefully, dispense 100 µL 80% ethanol into the tube, making sure to not touch the tube with the pipet tip.

9. Incubate at RT for 30 sec.

10. Carefully, without touching the beads, use a pipettor to remove the supernatant (using Avant #AH200-H - skinnier tips than normal barrier tips).

11. Using a fresh pipet tip, remove residual ethanol.

12. Dry beads at RT for 5 min (over-drying beads may reduce library yield).

13. Remove tubes from magnet.

14. Using a pipettor, thoroughly resuspend beads in 11 µL RNase-Free 10 mM Tris-HCl (pH 8.0-8.5).

15. Incubate tubes at RT for 2 min.

16. Place tubes on magnetic rack (0.2mL). Incubate until liquid is clear (~1 min).

17. Transfer the liquid (10 µL) to a new RNase-Free PCR tube, being careful to not transfer any beads.

This is a safe stopping point.

Eluted libraries can be stored at 4ºC for ≤1 week or at -20ºC for ≤1 month.

18. Proceed to Library Amplification.

Library Amplification

9 1. Prepare Library Amplification Mastermix.

Library Amplification MAstermix
12.5 µL KAPA HiFi HotStart ReadyMix
2.5 µL Library Amplifcation Primer Mix

For 500 ng starting RNA, 9 cycles worked well. This should be determined empirically.

2. Add 15 µL Library Amplification Mastermix to 10 µL 2nd Post-ligation cleaned samples. Mix thoroughly via pipetting.

3. Place samples in thermocycler and run:

Step	Temperature	Duration	Cycles
Intial Denaturation	98ºC	45 sec	1
Denaturation	98ºC	15 sec	\
Annealing	60ºC	30 sec	9
Extension	72ºC	30 sec	/
Final Extension	72ºC	1 min	1
HOLD	4ºC	∞	

4. Proceed immediately to Library Amplification Cleanup.

Libary Amplification Cleanup

10

KAPA Pure Beads must be warmed up to RT (~30 min)

All ethanol washes will be done on the ring magnet (Fisher #A29164).

1. Add 25 µL KAPA Pure Beads to 25 µL Library Amplified samples and mix thoroughly via pipetting.

2. Incubate at RT for 5 min.

3. Place tubes on ring magnet (Fisher #A29164). Incubate until liquid is clear (~1 min).

4. Carefully, without touching the beads, use a pipettor to remove the supernatant (using Avant #AH200-H - skinnier tips than normal barrier tips).

5. Carefully, dispense 100 µL 80% ethanol into the tube, making sure to not touch the tube with the pipet tip

6. Incubate at RT for 30 sec.

7. Carefully, without touching the beads, use a pipettor to remove the supernatant (using Avant #AH200-H - skinnier tips than normal barrier tips).

8. Carefully, dispense 100 µL 80% ethanol into the tube, making sure to not touch the tube with the pipet tip

9. Incubate at RT for 30 sec.

10. Carefully, without touching the beads, use a pipettor to remove the supernatant (using Avant #AH200-H - skinnier tips than normal barrier tips).

11. Using a fresh pipet tip, remove residual ethanol.

12. Dry beads at RT for 5 min (over-drying beads may reduce library yield).

13. Remove tubes from ring magnet.

14. Using a pipettor thoroughly resuspend beads in 11 µL RNase-Free 10 mM Tris-HCl (pH 8.0-8.5).

15. Incubate tubes at RT for 2 min.

16. Place tubes on magnetic rack (0.2 mL). Incubate until liquid is clear (~1 min).

17. Transfer clear (no beads) 10 µL to a new RNase-Free PCR tube.

RNA-Seq library is completed. Quantify and check quality using an Agilent TapeStation or BioAnalyzer.