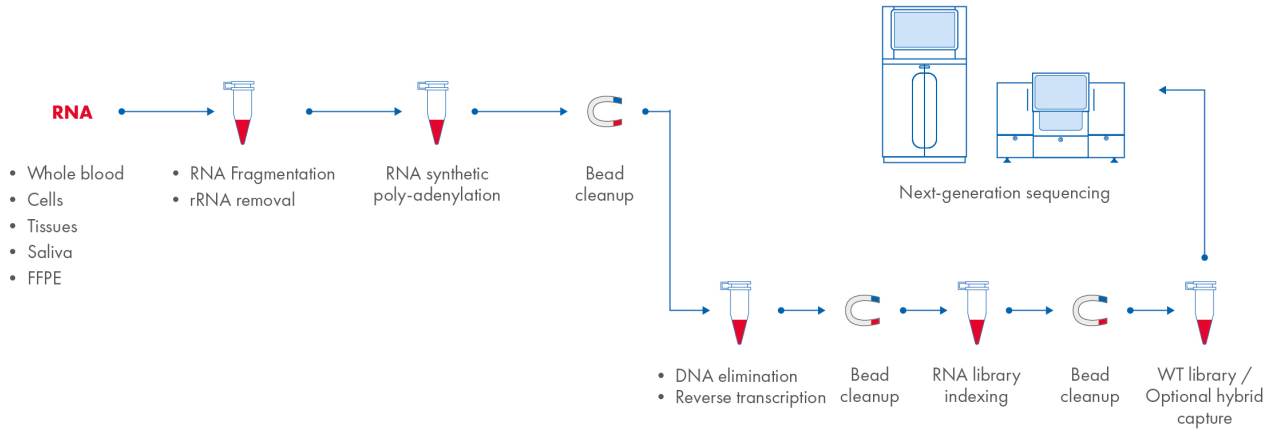
June 2025

Supplementary Protocol

QIAseq® Multimodal RNA Kit

For the construction of WTS libraries using the QIAseq Multimodal RNA Kit (cat. nos. 335012 and 335015) and QIAseq Multimodal RNA UDI (cat. nos. 334822 and 334825).

QIAseq Multimodal DNA/RNA Library Kit workflow



Equipment and Reagents to be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs) available from the product supplier.

The following are required:

- 80% ethanol (made fresh daily)*
- Nuclease-free pipette tips and tubes
- 1.5 mL LoBind® tubes (Eppendorf®, cat. no. 022431021)
- PCR tubes (0.2 mL individual tubes [VWR®, cat. no. 20170-012] or tube strips [VWR, cat. no. 93001 118]) or plates
- Ice
- Microcentrifuge
- Thermal cycler
- Magnet for bead cleanups:
  - **Tubes**: MagneSphere® Technology Magnetic Separation Stand (Promega, cat. no. Z5342)
  - **Plates**: DynaMag™-96 Side Magnet (Thermo Fisher Scientific Inc., cat. no. 12331D)
- QIAxcel® Connect System: QIAxcel DNA High Resolution Kit

*Do not use denatured alcohol, which contains other substances, such as methanol or methylethylketone.

Protocol: RNA Fragmentation

Important points before starting

- Set up reactions on ice.
- Do not vortex any reagents or reactions.
- Use MRNA index plates, either MRNA-24X or MRNA-96AX.
- Prepare fresh 80% ethanol daily.
- Ensure that the QIAseq Beads are thoroughly mixed at all times. This necessitates working quickly and resuspending the beads immediately before use. If a delay in the protocol occurs, simply vortex the beads.

Procedure

1. Thaw nucleic acid sample(s) on ice. Gently mix, briefly centrifuge to collect residual liquid from the sides of the tubes, and then return to ice.
2. Prepare the reagents required for the fragmentation.
   a. Thaw 10x FX Buffer, QIAseq FastSelect –rRNA HMR (8) at room temperature (15–25°C).
   b. Mix by flicking the tube, and then centrifuge briefly.
3. Dilute an aliquot of QIAseq Fastselect –rRNA HMR 1:10 with Nuclease-free Water. Mix by flicking the tube, and then centrifuge briefly.
4. On ice, prepare the fragmentation mix according to Table 1. Briefly centrifuge, mix by pipetting up and down 20 times, and then briefly centrifuge again.

   Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than what is required for the total number of reactions.

Table 1. Reaction mix for nucleic acid fragmentation of RNA samples

Component	Volume/reaction (µL)
RNA sample	Variable
10x FX Buffer	2
QIAseq FastSelect –rRNA HMR (diluted 1:10)	1
Nuclease-free Water	17 – Variable
Total	**20**

5. Program the thermal cycler according to Table 2. Use the instrument's heated lid.

   Important: The thermal cycler must be prechilled and paused at 4°C.

Table 2. Incubation conditions for nucleic acid fragmentation

Step	Incubation temperature (°C)	Incubation time for RNA (min)
1	4	1
2	65	30
3	60	2
4	55	2
5	37	5
6	25	5
7	4	Hold

6. Transfer the tubes/plate wells prepared in step 3 to the prechilled thermal cycler and resume the program.
7. Upon completion, allow the thermal cycler to return to 4°C.
8. Place the samples on ice and immediately proceed to "Protocol: RNA Polyadenylation".

Protocol: RNA Polyadenylation

Important points before starting

- The product from "Protocol: RNA Fragmentation" is the starting material for this protocol.
- Set up reactions on ice.
- Do not vortex any reagents or reactions.

Procedure

1. Prepare the reagents required for the polyadenylation.
   a. Thaw PAP Dilution Buffer, 10x, and ATP Solution on ice.
   b. Mix by flicking the tube, and then centrifuge briefly.

   Note: T4 Polynucleotide Kinase and PAP Enzyme should be removed from the freezer and placed on ice just before use. After use, immediately return the enzymes to the freezer.
2. Prepare the RNA polyadenylation mix according to Table 3. Briefly centrifuge, mix by pipetting up and down 20 times, and then briefly centrifuge again.

   Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than what is required for the total number of reactions.

Table 3. Reaction mix for RNA polyadenylation

Component	Volume/reaction (µL)
Fragmentation reaction (already in tube)	20
ATP Solution	1.25
T4 Polynucleotide Kinase	1
Diluted PAP Enzyme (1 U/µL)*	1
Nuclease-free Water	1.75
Total	**25**

* Ensure PAP Enzyme has been diluted from its stock 5 U/µL concentration to 1 U/µL using 1x PAP Dilution Buffer.

3. Incubate the reactions in a thermal cycler according to Table 4. Use the instrument's heated lid.

Table 4. Incubation conditions for RNA polyadenylation

Step	Temperature (°C)	Time (min)
1	4	1
2	30	10
3	4	Hold

4. After the reaction, add 75 µL of Nuclease-free Water to make a total volume of 100 µL.
5. Add 90 µL QIAseq Beads and mix by vortexing.
6. Incubate for 5 min at room temperature.

7. Place the tubes/plate wells on a magnetic rack for 2 min (for tubes) or 10 min (for plates). Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

   Important: Do not discard the beads because they contain the RNA of interest.

   Note: For plates, the following may improve performance. After 8 min, remove 90 µL supernatant. Leave it on the magnetic stand for 2 min and remove 90 µL supernatant. Then, briefly centrifuge the plate, return it to the magnetic stand for 1 min, and use a 10 µL pipette to remove the remaining supernatant.

8. With the beads still on the magnetic stand, add 200 µL of 80% ethanol. Rotate the tube 2–3 times or move the plate side-to-side between the 2 column positions of the magnet to wash the beads. Carefully remove and discard the wash.

9. Repeat the ethanol wash.

   Important: Completely remove all traces of the ethanol after the second wash. Remove the ethanol with a 200 µL pipette tip first, spin down briefly, and then use a 10 µL pipette tip to remove any residual ethanol.

10. With the beads still on the magnetic stand, air-dry at room temperature for 5–10 min.

    Note: Visually inspect the pellet to confirm that it is completely dry. If the pellet is not completely dry, additional drying time may be required.

11. Remove the beads from the magnetic stand, and then elute the RNA from the beads by adding 10 µL of Nuclease-free Water. Mix well by pipetting.

12. Return the tubes/plate wells to the magnetic rack until the solution has cleared.

13. Transfer 7.5 µL of the eluate to clean tubes/plate wells.

14. Proceed to "Protocol: RNA Reverse Transcription".

    Alternatively, the samples can be stored at –30°C to –15°C in a constant-temperature freezer.

Protocol: RNA Reverse Transcription

Important points before starting

- The product from “Protocol: RNA Polyadenylation” is the starting material for this protocol.
- Set up reactions on ice.
- Do not vortex any reagents or reactions.
- Prepare fresh 80% ethanol daily.
- Ensure that the QIAseq Beads are thoroughly mixed at all times. This necessitates working quickly and resuspending the beads immediately before use. If a delay in the protocol occurs, simply vortex the beads.

Procedure

1. Prepare the reagents required for sample pre-treatment.

   Note: Buffer GE2 gDNA elimination buffer should be removed from the freezer and placed on ice just before use. After use, immediately return the tube to the freezer.

2. Prepare the sample pretreatment mix according to Table 5. Briefly centrifuge, mix by pipetting up and down 20 times, and then briefly centrifuge again.

   Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than what is required for the total number of reactions.

Table 5. Reaction mix for sample pretreatment

Component	Volume/reaction (µL)
Sample aliquot (from "Protocol: RNA Polyadenylation")	7.5
Buffer GE2 gDNA elimination buffer	2
Total	**9.5**

3. Incubate the reactions in a thermal cycler according to Table 6. Use the instrument’s heated lid.

Table 6. Incubation conditions for sample pretreatment

Step	Incubation temperature (°C)	Incubation time (min)
1	42	5
2	75	10
3	4	Hold

4. Prepare the reagents required for reverse transcription.

   a. Thaw US RT Buffer, 5x; DTT (100 mM); dNTP (10 mM); MM RNA RT Primer; and MM RNA TSO at room temperature.

   b. Mix by flicking the tubes and then centrifuge briefly.

      Note: The RNase Inhibitor and EZ Reverse Transcriptase should be removed from the freezer and placed on ice just before use. After use, immediately return the enzymes to the freezer.

5. To the treated sample, prepare the reverse transcription mix according to Table 7. Briefly centrifuge, mix by pipetting up and down 20 times, and then briefly centrifuge again.

   Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than what is required for the total number of reactions.

Table 7. Reaction mix for reverse transcription

Component	Volume/reaction (µL)
Treated Sample	9.5
US RT Buffer, 5x	4
DTT (100 mM)	0.5
dNTP (10 mM)	2
MM RNA RT Primer	1
MM RNA TSO	1
RNase Inhibitor	0.5
EZ Reverse Transcriptase	1.5
Total	**20**

6. Incubate the reactions in a thermal cycler according to Table 8. Use the instrument's heated lid.

Table 8. Incubation conditions for reverse transcription

Step	Incubation temperature (°C)	Incubation time (min)
1	4	1
2	42	90
3	70	10
4	4	1
5	4	Hold

7. Add 20 µL Nuclease-Free Water and 44 µL QIAseq Beads and mix by vortexing or by pipetting up and down several times.
8. Incubate for 5 min at room temperature.
9. Place the tubes/plate wells on a magnetic rack for 2 min (for tubes) or 10 min (for plates).

   After the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

   Important: Do not discard the beads because they contain the DNA of interest.
10. With the beads still on the magnetic stand, add 200 µL of 80% ethanol. Rotate the tube 2–3 times or move the plate side-to-side between the 2 column positions of the magnet to wash the beads. Carefully remove and discard the wash.
11. Repeat the ethanol wash.

    Important: Completely remove all traces of the ethanol after this second wash. Remove the ethanol with a 200 µL pipette tip first, spin down briefly, and then use a 10 µL pipette tip to remove any residual ethanol.
12. With the beads still on the magnetic stand, air-dry at room temperature for 5–10 min.

Note: Visually inspect the pellet to confirm that it is completely dry. If the pellet is not completely dry, additional drying time may be required. Ethanol carryover to the next universal PCR step will affect PCR efficiency.

13. Remove the beads from the magnetic stand, and then elute the DNA from the beads by adding 42 µL Nuclease-free Water.
14. Return the tubes/plate wells to the magnetic rack until the solution has cleared.
15. Transfer 40 µL of the eluate to clean tubes/plate wells.
16. Add 44 µL QIAseq Beads and mix by vortexing or by pipetting up and down several times.
17. Incubate for 5 min at room temperature.
18. Place the tubes/plate wells on a magnetic rack for 2 min (for tubes) or 10 min (for plates). After the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

    Important: Do not discard the beads because they contain the DNA of interest.
19. With the beads still on the magnetic stand, add 200 µL of 80% ethanol. Rotate the tube 2–3 times or move the plate side-to-side between the 2 column positions of the magnet to wash the beads. Carefully remove and discard the wash.
20. Repeat the ethanol wash.

    Important: Completely remove all traces of the ethanol after this second wash. Remove the ethanol with a 200 µL pipette tip first, spin down briefly, and then use a 10 µL pipette tip to remove any residual ethanol.
21. With the beads still on the magnetic stand, air-dry at room temperature for 5–10 min.

    Note: Visually inspect the pellet to confirm that it is completely dry. If the pellet is not completely dry, additional drying time may be required. Ethanol carryover to the next universal PCR step will affect PCR efficiency.
22. Remove the beads from the magnetic stand, and then elute the DNA from the beads by adding 22 µL Nuclease-free Water.
23. Return the tubes/plate wells to the magnetic rack until the solution has cleared.
24. Transfer 20 µL of the eluate to clean tubes/plate wells.
25. Proceed to "Protocol: RNA Library Indexing".

    Alternatively, the samples can be stored at –30°C to –15°C in a constant-temperature freezer.

Protocol: RNA Library Indexing

Important points before starting

- The product from "Protocol: RNA Reverse Transcription" is that starting material for this protocol.
- Set up reactions on ice.
- Do not vortex any reagents or reactions.
- Use MRNA index plates, either MRNA-24X or MRNA-96AX.
- Prepare fresh 80% ethanol daily.
- Ensure that the QIAseq Beads are thoroughly mixed at all times. This necessitates working quickly and resuspending the beads immediately before use. If a delay in the protocol occurs, simply vortex the beads.

Procedure

1. Prepare the reagents required for RNA Library indexing.
   a. Thaw HiFi Ultra Buffer, 5x; MM F-R Primer Mix, and MRNA-24X or mRNA-96AX index plate at room temperature.
   b. Mix by either flicking the tube or vortexing the index plate, and then centrifuge briefly. Plate should be centrifuged at 1000 x *g* for 1 min.

   Note: HiFi Ultra Polymerase should be removed from the freezer and placed on ice just before use. After use, immediately return the enzyme to the freezer.
2. Prepare the reactions according to Table 9. Briefly centrifuge, mix by pipetting up and down 20 times, and then briefly centrifuge again.

   Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than what is required for the total number of reactions.

Table 9. Reaction mix for RNA indexing

Component	Volume/reaction (µL)
Sample aliquot (from "Protocol: RNA Reverse Transcription")	20
HiFi Ultra Buffer, 5x	8
MM F-R Primer Mix	1.6
Nuclease-free Water	3.9
Well from MRNA-24X or MRNA-96AX index plate*	2.5
HiFi Ultra Polymerase	4
Total	**40**

*Ensure proper technique to prevent cross-contamination. Additionally ensure that every sample has a unique index and that no well is used twice.

3. Program a thermal cycler as described in Table 10, using cycle numbers described in Table 11.

   Important: Set up the ramp rate of the thermal cycler at ≤2°C/s.

Table 10. Cycling conditions for RNA indexing

Step	Time	Temperature (°C)
Hold	2 min	98
2-step cycling		
Denaturation	20 s	98
Annealing/Extension	1 min	60
Cycle number	See Table 11	
Hold	3 min	72
Hold	∞	4

Table 11. Cycle number recommendations for RNA indexing, based on original sample input

RNA Input (ng)	Standard Sample (cycle)	FFPE sample (cycle)
2	25	–
10	23	–
20	22	–
100	20	23
200	19	22
500	–	22

4. After the reaction is complete, add 36 µL QIAseq Beads, and then mix by vortexing or pipetting up and down several times.
5. Incubate for 5 min at room temperature.
6. Place the tubes/plate well on a magnetic rack for 2 min (for tubes) or 10 min (for plates) to separate the beads from the supernatant.

   After the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

   Important: Do not discard the beads because they contain the DNA of interest.
7. With the beads still on the magnetic stand, add 200 µL of 80% ethanol. Rotate the tube 2–3 times or move the plate side-to-side between the 2 column positions of the magnet to wash the beads. Carefully remove and discard the wash.
8. Repeat the ethanol wash twice, for a total of 3 washes.

   Important: Completely remove all traces of the ethanol after this second wash. Remove the ethanol with a 200 µL pipette tip first, spin down briefly, and then use a 10 µL pipette tip to remove any residual ethanol.
9. With the beads still on the magnetic stand, air-dry at room temperature for 5–10 min.

   Note: Visually inspect the pellet to confirm that it is completely dry. If the pellet is not completely dry, additional drying time may be required.
10. Remove the beads from the magnetic stand, and then elute the DNA from the beads by adding 24 µL Nuclease-free Water. Mix well by pipetting.
11. Return the tube/plate wells to the magnetic rack until the solution has cleared.

12. Transfer 22 µL of the supernatant to clean tubes/plate wells.
13. The library is now ready for sequencing or hybrid capture. Proceed to "Recommendations: Library QC & Quantification" *in QIAseq Multimodal DNA/RNA Library Kit Handbook*. Alternatively, the samples can be stored at –30°C to –15°C in a constant-temperature freezer.

Document Revision History

Date	Description
06/2025	Initial release

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