December 2021

QIAseq® FastSelect™ Custom Handbook

For rapid RNA removal for RNA-seq library preparation

Sample to Insight

QIAGEN

Contents

Kit Contents

QIAseq FastSelect Custom RNA Removal Kit	
Catalog no.	**333390**
Custom panel no.	**CTFS–#####Z–###**
No. of reactions	**1536**
QIAseq FastSelect Custom RNA Removal reagent	2 x 768 µl

QIAseq FastSelect Beads and Buffer Kit	
Catalog no.	**333299**
No. of reactions	**1536**
FastSelect FH Buffer	16 x 144 µl
Nuclease-free Water	50 ml bottle
QIAseq Beads	55 ml bottle
QIAseq Bead Binding Buffer	54 ml bottle

Shipping and Storage

QIAseq FastSelect Custom RNA Removal Kit is shipped on dry ice. Upon receipt, all components should immediately be stored in a constant-temperature freezer at –30 to –15°C. Under these conditions, the components are stable, without showing any reduction in performance and quality, until the date indicated on the box label.

QIAseq FastSelect Beads and Buffer Kit is shipped on blue ice. Upon receipt, all components should immediately be stored in a constant-temperature refrigerator at 2–8°C. Under these conditions, the components are stable, without showing any reduction in performance and quality, until the date indicated on the box label.

Intended Use

All QIAseq FastSelect products are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety**, where you can find, view, and print the SDS for each QIAGEN kit and kit component.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of QIAseq FastSelect is tested against predetermined specifications to ensure consistent product quality.

Introduction

RNA-focused next-generation sequencing (NGS) enables a thorough investigation of both coding and noncoding RNAs. QIAseq FastSelect Custom is a breakthrough technology that rapidly and efficiently removes over-represented RNAs during NGS library preparation. QIAseq FastSelect Custom can be used alone or in combination with any other QIAseq FastSelect kits. Depending on the combination of QIAseq FastSelect kits used, the QIAseq FastSelect Beads and Buffer Kit (QIAGEN cat. no. 333299) may be required.

In order to determine if the QIAseq FastSelect Beads and Buffer Kit is required, please refer to your custom catalog number and reference Table 1 below. The final three digits of your QIAseq Custom FastSelect (underlined and bolded here: CTFS–#####Z–**###**) are used for determining if you need to follow a protocol that includes a bead cleanup before initiating the reverse transcription step.

Table 1. When combining QIAseq Custom FastSelect with other FastSelect panels, a bead cleanup step may be required. In this case, it may be necessary to purchase the QIAseq FastSelect Beads and Buffer Kit (cat. no. 333299).

FastSelect kit combined with QIAseq FastSelect Custom RNA Removal Kit	**QIAseq FastSelect Custom RNA Removal Kit 333390/CTFS–#####Z–###**	
	### is ≤100	**### is >101**
QIAseq FastSelect –rRNA HMR QIAseq FastSelect –Globin FastSelect –rRNA HMR and Globin FastSelect –rRNA Yeast FastSelect –rRNA Fly FastSelect –rRNA Worm	Bead cleanup is not required	Purchase and use the FastSelect Beads and Buffer Kit (cat. no. 333299)
FastSelect –rRNA Plant FastSelect –rRNA Fish	Purchase and use the FastSelect Beads and Buffer Kit (cat. no. 333299)	Purchase and use the FastSelect Beads and Buffer Kit (cat. no. 333299)
FastSelect –5S/16S/23S rRNA Kit	Use the bead cleanup protocol. Beads and buffers are provided in the FastSelect 5S/16S/23S Kit	Use bead cleanup protocol. Beads and buffers are provided in the FastSelect 5S/16S/23S Kit

QIAseq FastSelect works with existing RNA-seq workflows for the removal of unwanted RNAs during the reverse transcription step of NGS library preparation. QIAseq FastSelect is compatible with a broad range of commercially available stranded library preparation kits (Table 2). Compatible samples include RNA from cells, fresh/frozen tissue, FFPE tissue, whole blood, and serum/plasma samples, including exosomes.

Table 2. QIAseq FastSelect Kit compatibility*

Vendor	Kit	Cat. no.	Total RNA range tested
QIAGEN	QIAseq Stranded Total RNA Lib Kit	180743, 180745	100 ng to 1 µg
	QIAseq Stranded RNA Lib Kit UDI	180450, 180451, 180452, 180453, 180454	
Illumina®	Illumina Stranded Total RNA Prep	20040525, 20040529	1 ng to 1 µg
		20040532, 20040534	25 ng to 1 µg total RNA
	Illumina Stranded mRNA Prep	20020596, 20020597	100 ng to 1 µg
	TruSeq® Stranded Lib Prep	20020594, 20020595	100 ng to 1 µg total RNA
	TruSeq Stranded mRNA Lib Prep		
New England Biolabs	NEBNext® Ultra™ II Directional	E7760S, E7760L	5 ng to 1 µg
Kapa® Biosystems	KAPA RNA HyperPrep	KK8540, KK8541	25 ng to 1 µg

* QIAseq FastSelect is compatible with almost any stranded RNA library prep kit that begins with heat fragmentation of RNA. For questions regarding kits that are not listed, please contact QIAGEN technical support.

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.

- QIAseq FastSelect Beads and Buffer Kit (QIAGEN cat. no. 333299). This is necessary when combining QIAseq FastSelect Custom with certain QIAseq FastSelect catalog kits (refer to Table 1).
- 80% ethanol (made fresh daily)*
- Nuclease-free pipette tips and tubes
- Microfuge tubes (1.5–2 ml)
- PCR tubes (0.2 ml individual tubes or tubes strips) (VWR cat. no. 20170-012 or 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 cat. no. 12331D)

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

Important Notes

- **Important**: Two options are included for each Protocol, no bead cleanup and bead cleanup. Please follow the guidance provided, both in the "Introduction" section and here for determining whether or not a bead cleanup is required.
  - FastSelect Custom alone, –**###** ≤ 450: No bead cleanup is required
  - FastSelect Custom and FastSelect –rRNA HMR, FastSelect –Globin, FastSelect –rRNA HMR and Globin, FastSelect Fly, FastSelect Worm, or FastSelect Yeast
    - –**###** ≤ 100: No bead cleanup is required
    - –**###** > 100: Bead cleanup is required. Purchase of QIAseq FastSelect Beads and Buffer Kit is required.
  - FastSelect Custom and FastSelect –5S/16S/23S
    - Regardless of –**###**, bead cleanup is required. Components of the QIAseq FastSelect Beads and Buffer Kit are included in the QIAseq FastSelect –5S/16S/23S Kit; as a result, purchase of the QIAseq FastSelect Beads and Buffer Kit is not required.
  - FastSelect Custom and FastSelect Plant or FastSelect Fish
    - Regardless of –**###**, bead cleanup is required. Purchase of the QIAseq FastSelect Beads and Buffer Kit is required.
- As part of each "Option 2: bead cleanup" protocol, RNA fragmentation conditions are listed for high-quality RNA (RIN ≥8). If working with RNA with RIN values <8, please refer to Table 3 for fragmentation recommendations. These conditions have been worked out using synthetically fragmented RNA, so they should be used as a general guideline. **Note**: Regardless of the specific fragmentation condition, the temperature ramp-down steps (steps 2–9 in Table 7, Table 11, Table 14, Table 18, Table 22, or Table 27) need to be performed. They need to be performed whether the RNA is high quality, moderate quality, FFPE, or degraded.

Table 3. Fragmentation conditions based on RIN values of input RNA*

RIN value of input RNA	Mean insert size ~175–225 bp	Mean insert size ~275–325 bp
≥8	89°C, 8 min	89°C, 5.5 min
6–7	89°C, 6 min	89°C, 4.5 min
4–5	89°C, 4 min	89°C, 3 min
3	89°C, 2 min	89°C, 2 min
≤2	No fragmentation	No fragmentation

* Regardless of the specific fragmentation condition, the temperature ramp-down steps (steps 2–9 in Table 7, Table 11, Table 14, Table 18, Table 22, or Table 27) need to be performed. They need to be performed whether the RNA is high quality, moderate quality, FFPE, or degraded.

- We highly recommend DNase treatment (on-column or in-solution) of total RNA samples before starting your RNA-seq library preparation.
- It is not possible to test the efficiency of the FastSelect reaction by running a portion of the eluate from the bead cleanup on a Bioanalyzer®, TapeStation®, Fragment Analyzer™, etc. FastSelect works by inhibiting reverse transcription of its target RNAs, which does not occur until the first-strand synthesis reaction during library prep.
- QIAseq FastSelect is an inline solution for the removal of unwanted rRNAs during NGS library preparation. The total RNA input is defined by the range of the RNA library kit used. For example, the QIAseq Stranded Total RNA Lib Kit (cat. no. 180743 or 180745) has a total RNA input range of 100 ng – 1 µg. As a result, you would start with 100 ng – 1 µg into the FastSelect reaction.
- The rRNA removal imparted by QIAseq FastSelect is extremely robust, especially when compared to other methods. We recommend to prepare libraries and use the standard protocol for library preparation unless specifically noted in the handbook.
- If the yield of the library is less than other methods, this is often caused by the increased removal of RNA imparted by the QIAseq FastSelect method and is normal. In our experience, adding 2 cycles of library amplification is usually sufficient to increase library yield for all downstream quantification and sequencing applications.
- Depending on the RNA-seq kit and RNA input amounts, adapter–dimers may be observed. If this happens, we recommend that you perform a second bead-based cleanup reaction of the final library.

Protocol: FastSelect with QIAseq Stranded RNA Lib Kits

Important points before starting

- The QIAseq Stranded Total RNA Lib Kit (cat. no. 180743 or 180745) or QIAseq Stranded RNA Lib Kit UDI (cat. no. 180450, 180451, 180452, 180453, or 180454) is required for use with this protocol.
- Refer to Table 1 in the "Introduction" or recommendations in the "Important Notes" sections and choose the appropriate procedural option to perform. Two options, both integrated with QIAseq FastSelect, are provided:
  - **Option 1**: No bead cleanup
  - **Option 2**: Bead cleanup
    - **Important**: Prepare fresh 80% ethanol daily.
    - Prewarm QIAseq Beads and Bead Binding Buffer to room temperature (15–25°C) prior to use. Ensure that the QIAseq Beads are thoroughly mixed at all times: work quickly and resuspend the beads immediately before use. If a delay in the protocol occurs, simply vortex the beads.
- Refer to the *QIAseq Stranded RNA Library Kit Handbook* available at **www.qiagen.com**.

Procedure

Option 1: No bead cleanup

1. Thaw total RNA on ice. Gently mix, briefly centrifuge to collect residual liquid from the sides of the tubes, and return to ice.
2. Prepare the required reagents.
   2a. Thaw QIAseq FastSelect tube(s) as well as 5x RT Buffer and Nuclease-free Water (from the QIAseq Stranded kit) at room temperature. Mix by vortexing and then briefly centrifuge.
3. On ice, prepare the RNA fragmentation/depletion reaction according to Table 4. Briefly centrifuge, mix by pipetting up and down 10 times, and centrifuge briefly again.

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

Table 4. Setup of RNA fragmentation/depletion reaction: No bead cleanup

Component	Volume/reaction
Total RNA (100 ng – 1 µg)	Variable
RT Buffer, 5x*	8 µl
QIAseq FastSelect Custom	1 µl
Cataloged QIAseq FastSelect†	1 µl
ERCC Control‡	Optional
Nuclease-free Water	Bring total reaction volume to 37 µl
Total volume	**37 µl**

* From QIAseq Stranded Total RNA Lib Kit.

† If required, choose the appropriate cataloged QIAseq FastSelect kit(s).

‡ ERCC Control RNA can be added according to the concentrations specified by the manufacturer. If added, the total fragmentation/RNA Removal reaction volume should remain 37 µl.

4. Incubate as described in Table 5, according to input RNA quality and desired insert size.

Table 5. RNA Fragmentation/depletion protocol: No bead cleanup

Input RNA quality	Step	Insert size ~150–250 bp	Insert size ~350 bp
High quality (RIN >9)	1*	15 min at 95°C	3 min at 95°C
Moderate quality (RIN 5–6)	1*	10 min at 95°C	3 min at 95°C
FFPE or degraded sample (RIN <3)	1*	No fragmentation†	No fragmentation†
Steps 2–9 are performed regardless of input RNA quality. They need to be performed whether the RNA is high quality, moderate quality, FFPE, or degraded.	2	2 min at 75°C	2 min at 75°C
	3	2 min at 70°C	2 min at 70°C
	4	2 min at 65°C	2 min at 65°C
	5	2 min at 60°C	2 min at 60°C
	6	2 min at 55°C	2 min at 55°C
	7	2 min at 37°C	2 min at 37°C
	8	2 min at 25°C	2 min at 25°C
	9	Hold at 4°C	Hold at 4°C

* Choose one option for the time on step 1 according to the input RNA quality and desired insert size.

† Also suitable for exosomal RNA or RNA of other origin with a size between 80–500 bp.

Important: Regardless of time and temperature chosen in step 1, steps 2–9 must be performed.

5. Refer to the *QIAseq Stranded RNA Library Kit Handbook* and immediately proceed to "Protocol: First-strand Synthesis".
6. Follow the *QIAseq Stranded RNA Library Kit Handbook* to perform all remaining library construction steps.

Option 2: Bead cleanup

1. Thaw template RNA on ice. Gently mix, briefly centrifuge to collect residual liquid from the sides of the tubes, and then return to ice.
2. Thaw QIAseq FastSelect tube(s), FastSelect FH Buffer, and Nuclease-free Water at room temperature. Mix by vortexing and then briefly centrifuge.
3. On ice, prepare the RNA fragmentation/depletion reaction according to Table 6. Briefly centrifuge, mix by pipetting up and down 10 times, and then centrifuge briefly again.

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

Table 6. Setup of RNA fragmentation/depletion reaction: Bead cleanup

Component	Volume/reaction
Total RNA (100 ng – 1 µg)	Variable
FastSelect FH Buffer	1.5 µl
QIAseq FastSelect Custom	1 µl
Cataloged QIAseq FastSelect*	1 µl
Nuclease-free Water	Bring total reaction volume to 15 µl
Total volume	**15 µl**

* If required, add appropriate cataloged QIAseq FastSelect kit(s).

4. Incubate as described in Table 7.

Important: Regardless of the time and temperature chosen in step 1, steps 2–9 must be performed.

Table 7. RNA fragmentation/depletion protocol: Bead cleanup

Note	Step	Mean insert size ~175–225 bp
RNA fragmentation	1*†	8 min at 89°C*†
Steps 2–9 are performed regardless of input RNA quality. They need to be performed whether the RNA is high quality, moderate quality, FFPE, or degraded.	2	2 min at 75°C
	3	2 min at 70°C
	4	2 min at 65°C
	5	2 min at 60°C
	6	2 min at 55°C
	7	2 min at 37°C
	8	2 min at 25°C
	9	Hold at 4°C

* For high-quality RNA (RIN ≥8), 8 min at 89°C is the basic recommendation. Alternatively, 4 min at 89°C provides an average insert size of ~325–375 bp, and 12 min at 89°C provides an average insert size of ~125–175 bp.

† If working with RNA with RIN values <8, please refer to Table 3 for fragmentation recommendations.

5. Add 19.5 µl QIAseq Beads (prewarmed to room temperature and mixed by vortexing) to the 15 µl reaction. Mix thoroughly by vortexing, and then incubate for 5 min at room temperature.
6. Centrifuge in a table-top centrifuge until the beads are completely pelleted (~2 min).
7. Place the tubes/plate on a magnetic rack for 2 min. Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

   Note: You can completely avoid transferring beads by pipetting very slowly.

   Important: Do not discard the beads, because they contain the RNA of interest.
8. Add 15 µl of Nuclease-free Water and 19.5 µl of QIAseq Bead Binding Buffer (prewarmed to room temperature and mixed by vortexing). Mix vigorously by vortexing, and then incubate for 5 min at room temperature.
9. Centrifuge in a table-top centrifuge until the beads are completely pelleted (~2 min).
10. Place the tubes/plate on a magnetic rack for 2 min. Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

    Note: You can completely avoid transferring beads by pipetting very slowly.

    Important: Do not discard the beads, because they contain the RNA of interest.
11. With the beads still on the magnetic stand, add 200 µl of 80% ethanol. Wait for 30 s. Carefully remove and discard the wash.
12. Repeat the ethanol wash.

    Important: Completely remove all traces of ethanol after this second wash. Remove the ethanol with a 200 µl pipette tip first, and then use a 10 µl pipette tip to remove any residual ethanol that will settle.
13. With the beads still on the magnetic stand, air-dry at room temperature for 6–10 min until all liquid has evaporated but without overdrying the beads.

    Note: Visually inspect the pellet to confirm that it is completely dry.
14. Remove the beads from the magnetic stand, and elute the RNA from the beads by adding 31 µl Nuclease-free Water. Mix well by pipetting or vortexing, and then allow the tubes to sit at room temperature for 2 min to appropriately hydrate the beads.
15. Return the tubes/plate to the magnetic rack until the solution has cleared.

16. Transfer 29 µl of the supernatant to clean tubes/plate. Alternatively, the samples can be stored at –90 to –65°C in a constant-temperature freezer.
17. Set up the first-strand synthesis associated with the QIAseq Stranded RNA Lib Kit as described in Table 8. Briefly centrifuge, mix by pipetting up and down 10 times, and then centrifuge briefly again.

Table 8. QIAseq Stranded Total RNA Lib Kit first-stranded synthesis setup

Component	Volume/reaction (µl)
RNA from bead cleanup reaction	29
RT Buffer, 5x*	8
Diluted DTT (0.4 M)*	1
RT Enzyme*	1
RNase Inhibitor*	1
Total volume	**40**

* All designated components are from the QIAseq Stranded Total RNA Lib Kit.

18. Refer to the *QIAseq Stranded RNA Lib Kit Handbook* and immediately proceed to and perform the first-strand protocol incubation in "Protocol: First-strand Synthesis".
19. Follow the *QIAseq Stranded RNA Lib Kit Handbook* to perform all remaining library construction steps.

Protocol: FastSelect with Illumina Stranded Total RNA Prep

Important points before starting

- The Illumina Stranded Total RNA Prep (Illumina cat. no. 20040525, 20040529) is required for use with this protocol.
  Note: Follow the steps outlined below before proceeding to the designated step in the *Illumina Stranded Total RNA Prep Ligation Reference Guide* (1000000124514 v02). By doing this, a stranded total RNA library prep will be performed.
- Refer to Table 1 in the "Introduction" or recommendations in the "Important Notes" sections and choose the appropriate procedural option to perform. Two options, both integrated with QIAseq FastSelect, are provided:
  - **Option 1**: No bead cleanup
  - **Option 2**: Bead cleanup
    - **Important**: Prepare fresh 80% ethanol daily.
    - Prewarm QIAseq Beads and Bead Binding Buffer to room temperature (15–25°C) prior to use. Ensure that the QIAseq Beads are thoroughly mixed at all times: work quickly and resuspend the beads immediately before use. If a delay in the protocol occurs, simply vortex the beads.
- **Important**: It is recommended to test dilution of the Illumina anchors 2-fold compared to what is suggested in the default Illumina protocol.

Procedure

Option 1: No bead cleanup

1. Thaw QIAseq FastSelect tube(s) at room temperature. Mix by vortexing and then briefly centrifuge.

2. To 1 ng – 1 µg of total RNA, which is required to be in a maximum volume of 6.5 µl, add QIAseq FastSelect as follows:
   2a. Add 1 µl of QIAseq FastSelect Custom
   2b. **Optional**: Add 1 µl of cataloged QIAseq FastSelect kit reagent
3. From the Illumina Stranded Total RNA Prep, add ELB (variable µl) to bring the volume of the reaction to 8.5 µl.
4. From the Illumina Stranded Total RNA Prep, add 8.5 µl EPH3 to bring the volume of the reaction to 17 µl.
5. Mix thoroughly by pipetting up and down several times and then briefly centrifuge to collect residual liquid from the sides of the tubes.
6. Incubate in a thermal cycler with a heated lid as described in Table 9.

Table 9. RNA fragmentation/depletion protocol: No bead cleanup

Step	Time and temperature
1	2 min at 94°C
2	2 min at 75°C
3	2 min at 70°C
4	2 min at 65°C
5	2 min at 60°C
6	2 min at 55°C
7	2 min at 37°C
8	2 min at 25°C
9	Hold at 4°C

7. Using the entire 17 µl fragmented/hybridized RNA reaction, refer to the *Illumina Stranded Total RNA Prep Ligation Reference Guide* and immediately proceed to "Synthesize First Strand cDNA."

8. Follow the *Illumina Stranded Total RNA Prep Ligation Reference Guide* to perform all remaining library construction steps.

IMPORTANT 	It is recommended to test dilution of the Illumina anchors 2-fold compared to what is suggested in the default Illumina protocol.

Option 2: Bead cleanup

1. Thaw template RNA on ice. Gently mix, briefly centrifuge to collect residual liquid from the sides of the tubes, and then return to ice.
2. Thaw QIAseq FastSelect tube(s), FastSelect FH Buffer, and Nuclease-free Water at room temperature. Mix by vortexing and then briefly centrifuge.
3. On ice, prepare the RNA fragmentation/depletion reaction according to Table 10. Briefly centrifuge, mix by pipetting up and down 10 times, and then centrifuge briefly again.

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

Table 10. Setup of RNA fragmentation/depletion reaction: Bead cleanup

Component	Volume/reaction
Total RNA (1 ng – 1 µg)	Variable
FastSelect FH Buffer	1.5 µl
QIAseq FastSelect Custom	1 µl
Cataloged QIAseq FastSelect*	1 µl
Nuclease-free Water	Bring total reaction volume to 15 µl
Total volume	**15 µl**

* If required, add appropriate cataloged QIAseq FastSelect kit(s).

4. Incubate as described in Table 11.

   Important: Regardless of time and temperature chosen in step 1, steps 2–9 must be performed.

Table 11. RNA fragmentation/depletion protocol: Bead cleanup

Note	Step	Mean insert size ~175–225 bp
RNA fragmentation	1*†	8 min at 89°C*†
Steps 2–9 are performed, regardless of Input RNA quality. They need to be performed whether the RNA is high quality, moderate quality, FFPE, or degraded.	2	2 min at 75°C
	3	2 min at 70°C
	4	2 min at 65°C
	5	2 min at 60°C
	6	2 min at 55°C
	7	2 min at 37°C
	8	2 min at 25°C
	9	Hold at 4°C

* For high-quality RNA (RIN ≥8), 8 min at 89°C is the basic recommendation. Alternatively, 4 min at 89°C provides an average insert size of ~325–375 bp, and 12 min at 89°C provides an average insert size of ~125–175 bp.

† If working with RNA with RIN values <8, please refer to Table 3 for fragmentation recommendations.

5. Add 19.5 µl QIAseq Beads (prewarmed to room temperature and mixed by vortexing) to the 15 µl reaction. Mix thoroughly by vortexing, and incubate for 5 min at room temperature.
6. Centrifuge in a table-top centrifuge until the beads are completely pelleted (~2 min).
7. Place the tubes/plate on a magnetic rack for 2 min. Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

   Note: You can completely avoid transferring beads by pipetting very slowly.

   Important: Do not discard the beads, because they contain the RNA of interest.
8. Add 15 µl of Nuclease-free Water and 19.5 µl of QIAseq Bead Binding Buffer. Mix thoroughly by vortexing, and incubate for 5 min at room temperature.

9. Centrifuge in a table-top centrifuge until the beads are completely pelleted (~2 min).
10. Place the tubes/plate on a magnetic rack for 2 min. Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

    Note: You can completely avoid transferring beads by pipetting very slowly.

    Important: Do not discard the beads, because they contain the RNA of interest.
11. With the beads still on the magnetic stand, add 200 µl of 80% ethanol. Wait for 30 s. Carefully remove and discard the wash.
12. Repeat the ethanol wash.

    Important: Completely remove all traces of ethanol after this second wash. Remove the ethanol with a 200 µl pipette tip first, and then use a 10 µl pipette tip to remove any residual ethanol that will settle.
13. With the beads still on the magnetic stand, air-dry at room temperature for 6–10 min until all liquid has evaporated but without overdrying the beads.

    Note: Visually inspect the pellet to confirm that it is completely dry.
14. Remove the beads from the magnetic stand, and elute the RNA from the beads by adding 10.5 µl ELB. Mix well by pipetting or vortexing, and then allow the tubes to sit at room temperature for 2 min to appropriately hydrate the beads.

    Note: FPF Buffer is a component from the Illumina Stranded Total RNA Prep.
15. Return the tubes/plate to the magnetic rack until the solution has cleared.
16. Transfer 8.5 µl of the supernatant to clean tubes/plate. Alternatively, the samples can be stored at –90 to –65°C in a constant-temperature freezer.
17. From the Illumina Stranded Total RNA Prep, add 8.5 µl EPH3 to the 8.5 µl of supernatant, briefly centrifuge, and pipet up and down 10 times to mix.

    Important: Do not perform the DEN_RNA program. The RNA has already been fragmented above.

    Note: EPH3 is a component from the Illumina Stranded Total RNA Prep.
18. Refer to the *Illumina Stranded Total RNA Prep Ligation Reference Guide* and immediately proceed to "Synthesize First Strand cDNA".

19. Follow the *Illumina Stranded Total RNA Prep Ligation Reference Guide* to perform all remaining library construction steps.

IMPORTANT 	It is recommended to test dilution of the Illumina anchors 2-fold compared to what is suggested in the default Illumina protocol.

Protocol: FastSelect with Illumina Stranded mRNA Prep

Important points before starting

- The Illumina Stranded mRNA Prep (Illumina cat. no. 20040532, 20040534) is required for use with this protocol.
  Note: With this protocol, do not perform mRNA purification. Instead, follow the steps outlined below before proceeding to the designated step in the *Illumina Stranded mRNA Prep Ligation Reference Guide* (1000000124518 v02). By doing this, a stranded total RNA library prep will be performed.
- Refer to Table 1 in the "Introduction" or recommendations in the "Important Notes" sections and choose the appropriate procedural option to perform. Two options, both integrated with QIAseq FastSelect, are provided:
  - **Option 1**: No bead cleanup
  - **Option 2**: Bead cleanup
    - **Important**: Prepare fresh 80% ethanol daily.
    - Prewarm QIAseq Beads and Bead Binding Buffer to room temperature (15–25°C) prior to use. Ensure that the QIAseq Beads are thoroughly mixed at all times: work quickly and resuspend the beads immediately before use. If a delay in the protocol occurs, simply vortex the beads.
- **Important**: It is recommended to test dilution of the Illumina anchors 2-fold compared to what is suggested in the default Illumina protocol.

Procedure

Option 1: No bead cleanup

1. Thaw QIAseq FastSelect tube(s) at room temperature. Mix by vortexing and then briefly centrifuge.

2. To 25 ng – 1 µg of total RNA, which is required to be in a maximum volume of 6.5 µl, add QIAseq FastSelect as follows:
   2a. Add 1 µl of QIAseq FastSelect Custom
   2b. **Optional**: Add 1 µl of cataloged QIAseq FastSelect kit
3. From the Illumina Stranded mRNA Prep, add ELB (variable µl) to bring the volume of the reaction to 8.5 µl.
4. From the Illumina Stranded mRNA Prep, add 8.5 µl EPH3 to bring the volume of the reaction to 17 µl.
5. Mix thoroughly by pipetting up and down several times and then briefly centrifuge to collect residual liquid from the sides of the tubes.
6. Incubate in a thermal cycler with a heated lid as described in Table 12.

Table 12. RNA fragmentation/depletion protocol: No bead cleanup

Step	Time and temperature
1	2 min at 94°C
2	2 min at 75°C
3	2 min at 70°C
4	2 min at 65°C
5	2 min at 60°C
6	2 min at 55°C
7	2 min at 37°C
8	2 min at 25°C
9	Hold at 4°C

7. Using the entire 17 µl fragmented/hybridized RNA reaction, refer to the *Illumina Stranded mRNA Prep Ligation Reference Guide* and immediately proceed to "Synthesize First Strand cDNA."

8. Follow the *Illumina Stranded mRNA Prep Ligation Reference Guide* to perform all remaining library construction steps.

IMPORTANT 	It is recommended to test dilution of the Illumina anchors 2-fold compared to what is suggested in the default Illumina protocol.

Option 2: Bead cleanup

1. Thaw template RNA on ice. Gently mix, briefly centrifuge to collect residual liquid from the sides of the tubes, and then return to ice.
2. Thaw QIAseq FastSelect tube(s), FastSelect FH Buffer, and Nuclease-free Water at room temperature. Mix by vortexing and then briefly centrifuge.
3. On ice, prepare the fragmentation/depletion reaction according to Table 13. Briefly centrifuge, mix by pipetting up and down 10 times, and then centrifuge briefly again.

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

Table 13. Setup of RNA fragmentation/depletion reaction: Bead cleanup

Component	Volume/reaction
Total RNA (25 ng – 1 µg)	Variable
FastSelect FH Buffer	1.5 µl
QIAseq FastSelect Custom	1 µl
Cataloged QIAseq FastSelect*	1 µl
Nuclease-free Water	Bring total reaction volume to 15 µl
Total volume	**15 µl**

* If required, add appropriate cataloged QIAseq FastSelect kit(s).

4. Incubate as described in Table 14.

   Important: Regardless of time and temperature chosen in step 1, steps 2–9 must be performed.

Table 14. RNA fragmentation/depletion protocol: Bead cleanup

Note	Step	Mean insert size ~175–225 bp
RNA fragmentation	1*†	8 min at 89°C*†
Steps 2–9 are performed, regardless of Input RNA quality. They need to be performed whether the RNA is high quality, moderate quality, FFPE, or degraded.	2	2 min at 75°C
	3	2 min at 70°C
	4	2 min at 65°C
	5	2 min at 60°C
	6	2 min at 55°C
	7	2 min at 37°C
	8	2 min at 25°C
	9	Hold at 4°C

* For high-quality RNA (RIN ≥8), 8 min at 89°C is the basic recommendation. Alternatively, 4 min at 89°C provides an average insert size of ~325–375 bp, and 12 min at 89°C provides an average insert size of ~125–175 bp.

† If working with RNA with RIN values <8, please refer to Table 3 for fragmentation recommendations.

5. Add 19.5 µl QIAseq Beads (prewarmed to room temperature and mixed by vortexing) to the 15 µl reaction. Mix thoroughly by vortexing, and incubate for 5 min at room temperature.
6. Centrifuge in a table-top centrifuge until the beads are completely pelleted (~2 min).
7. Place the tubes/plate on a magnetic rack for 2 min. Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

   Note: You can completely avoid transferring beads by pipetting very slowly.

   Important: Do not discard the beads, because they contain the RNA of interest.
8. Add 15 µl of Nuclease-free Water and 19.5 µl of QIAseq Bead Binding Buffer. Mix thoroughly by vortexing, and incubate for 5 min at room temperature.
9. Centrifuge in a table-top centrifuge until the beads are completely pelleted (~2 min).

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

    Note: You can completely avoid transferring beads by pipetting very slowly.

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

11. With the beads still on the magnetic stand, add 200 µl of 80% ethanol. Wait for 30 s. Carefully remove and discard the wash.

12. Repeat the ethanol wash.

    Important: Completely remove all traces of ethanol after this second wash. Remove the ethanol with a 200 µl pipette tip first, and then use a 10 µl pipette tip to remove any residual ethanol that will settle.

13. With the beads still on the magnetic stand, air-dry at room temperature for 6–10 min until all liquid has evaporated but without overdrying the beads.

    Note: Visually inspect the pellet to confirm that it is completely dry.

14. Remove the beads from the magnetic stand, and elute the RNA from the beads by adding 10.5 µl ELB. Mix well by pipetting or vortexing, and then allow the tubes to sit at room temperature for 2 min to appropriately hydrate the beads.

    Note: ELB Buffer is a component from the Illumina Stranded mRNA Library Prep

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

16. Transfer 8.5 µl of the supernatant to clean tubes/plate. Alternatively, the samples can be stored at –90 to –65°C in a constant-temperature freezer.

17. From the Illumina Stranded mRNA Prep, add 8.5 µl EPH3 to the 8.5 µl of supernatant, briefly centrifuge, and pipet up and down 10 times to mix.

    Important: Do not perform the DEN_RNA program. The RNA has already been fragmented above.

    Note: EPH3 is a component from the Illumina Stranded mRNA Prep

18. Refer to the *Illumina Stranded mRNA Prep Ligation Reference Guide* and immediately proceed to "Synthesize First Strand cDNA".

19. Follow the *Illumina Stranded mRNA Prep Ligation Reference Guide* to perform all remaining library construction steps.

IMPORTANT 	It is recommended to test dilution of the Illumina anchors 2-fold compared to what is suggested in the default Illumina protocol.

Protocol: FastSelect with TruSeq Stranded Library Preparation

Important points before starting

- The TruSeq Stranded mRNA Library Prep (Illumina cat. no. 20020596 or 20020597) is required for use with this protocol.
  Note: With this protocol, do not perform mRNA purification. Instead, follow the steps outlined below before proceeding to "Synthesize First Strand cDNA" in the *TruSeq Stranded mRNA Reference Guide*. By doing this, a stranded, total RNA library preparation will be performed.
- Refer to Table 1 in the "Introduction" or recommendations in the "Important Notes" sections and choose the appropriate procedural option to perform. Two options, both integrated with QIAseq FastSelect, are provided:
  - **Option 1**: No bead cleanup
  - **Option 2**: Bead cleanup
    - **Important**: Prepare fresh 80% ethanol daily.
    - Prewarm QIAseq Beads and Bead Binding Buffer to room temperature (15–25°C) prior to use. Ensure that the QIAseq Beads are thoroughly mixed at all times: work quickly and resuspend the beads immediately before use. If a delay in the protocol occurs, simply vortex the beads
- **Important**: It is highly recommended to dilute the Illumina adapters 2-fold compared to what is suggested in the default Illumina protocol.
- Refer to the TruSeq Stranded mRNA Reference Guide (1000000040498).

Procedure

Option 1: Bead cleanup

1. Thaw QIAseq FastSelect tube(s) at room temperature. Mix by vortexing and then briefly centrifuge.

2. To 100 ng – 1 µg of total RNA, which is required to be in a maximum volume of 5 µl, add QIAseq FastSelect as follows:

   2a. Add 1 µl of QIAseq FastSelect Custom

   2b. **Optional**: Add 1 µl of cataloged QIAseq FastSelect kit

3. From the TruSeq Stranded mRNA Library Prep, add FPF Buffer to bring the volume of the reaction to 20.5 µl.

4. Mix thoroughly by pipetting up and down several times and then briefly centrifuge to collect residual liquid from the sides of the tubes.

5. Incubate in a thermal cycler with a heated lid as described in Table 15.

   Important: Table 16 can be consulted to adjust RNA insert size. Irrespective of time at 94°C, steps 2–9 listed in Table 15 must be performed.

Table 15. RNA fragmentation/depletion protocol: No bead cleanup

Step	Time and temperature
1*	8 min at 94°C*
2	2 min at 75°C
3	2 min at 70°C
4	2 min at 65°C
5	2 min at 60°C
6	2 min at 55°C
7	2 min at 37°C
8	2 min at 25°C
9	Hold at 4°C

* The initial step at 94°C can be modified to permit longer RNA insert sizes. Refer to Table 11 for recommendations.
Note: The remaining steps 2–9 are performed regardless of the time at 94°C.

Table 16. Fragmentation time at 94°C for alternative RNA insert sizes

Time at 94°C*	Range of insert length (bp)	Median insert length (bp)	Average final library size (Bioanalyzer bp)
0 min	130–350	200	467
1 min	130–310	190	439
2 min	130–290	185	410
3 min	125–250	165	366
4 min	120–225	160	326
8 min	120–210	155	309
12 min	115–180	140	272

* The remaining steps 2–9 from Table 15 must be performed regardless of the time at 94°C.

6. Use 17 µl of the fragmented/hybridized RNA, refer to the *TruSeq Stranded mRNA Reference Guide* and immediately proceed to "Synthesize First Strand cDNA."

   Note: From the *TruSeq Stranded mRNA Reference Guide*, the procedural step "Place the RBP plate on the magnetic stand and wait until the liquid is clear (~5 min)" is not applicable.

7. Follow the *TruSeq Stranded mRNA Reference Guide* to perform all remaining library construction steps.

IMPORTANT 	It is highly recommended to dilute the Illumina adapters 2-fold compared to what is suggested in the reference guide.

Option 2: Bead cleanup

1. Thaw template RNA on ice. Gently mix, briefly centrifuge to collect residual liquid from the sides of the tubes, and then return to ice.
2. Thaw QIAseq FastSelect tube(s), FastSelect FH Buffer, and Nuclease-free Water at room temperature. Mix by vortexing and then briefly centrifuge.

3. On ice, prepare the fragmentation/depletion reaction according to Table 17. Briefly centrifuge, mix by pipetting up and down 10 times, and then centrifuge briefly again.

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

Table 17. Setup of RNA fragmentation/depletion reaction: Bead cleanup

Component	Volume/reaction
Total RNA (100 ng – 1 µg)	Variable
FastSelect FH Buffer	1.5 µl
QIAseq FastSelect Custom	1 µl
Cataloged QIAseq FastSelect*	1 µl
Nuclease-free Water	Bring total reaction volume to 15 µl
Total volume	**15 µl**

* If required, add appropriate cataloged QIAseq FastSelect kit(s).

4. Incubate as described in Table 18.

   Important: Regardless of time and temperature chosen in step 1, steps 2–9 must be performed.

Table 18. RNA fragmentation/depletion protocol: Bead cleanup

Note	Step	Mean insert size ~175–225 bp
RNA fragmentation	1*†	8 min at 89°C*†
Steps 2–9 are performed, regardless of Input RNA quality. They need to be performed whether the RNA is high quality, moderate quality, FFPE, or degraded.	2	2 min at 75°C
	3	2 min at 70°C
	4	2 min at 65°C
	5	2 min at 60°C
	6	2 min at 55°C
	7	2 min at 37°C
	8	2 min at 25°C
	9	Hold at 4°C

* For high-quality RNA (RIN ≥8), 8 min at 89°C is the basic recommendation. Alternatively, 4 min at 89°C provides an average insert size of ~325–375 bp, and 12 min at 89°C provides an average insert size of ~125–175 bp.

† If working with RNA with RIN values <8, please refer to Table 3 for fragmentation recommendations.

5. Add 19.5 µl QIAseq Beads (prewarmed to room temperature and mixed by vortexing) to the 15 µl reaction. Mix thoroughly by vortexing, and incubate for 5 min at room temperature.
6. Centrifuge in a table-top centrifuge until the beads are completely pelleted (~2 min).
7. Place the tubes/plate on a magnetic rack for 2 min. Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

   Note: You can completely avoid transferring beads by pipetting very slowly.

   Important: Do not discard the beads, because they contain the RNA of interest.
8. Add 15 µl of Nuclease-free Water and 19.5 µl of QIAseq Bead Binding Buffer. Mix thoroughly by vortexing, and incubate for 5 min at room temperature.
9. Centrifuge in a table-top centrifuge until the beads are completely pelleted (~2 min).
10. Place the tubes/plate on a magnetic rack for 2 min. Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

    Note: You can completely avoid transferring beads by pipetting very slowly.

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

11. With the beads still on the magnetic stand, add 200 µl of 80% ethanol. Wait for 30 s. Carefully remove and discard the wash.
12. Repeat the ethanol wash.

    Important: Completely remove all traces of ethanol after this second wash. Remove the ethanol with a 200 µl pipette tip first, and then use a 10 µl pipette tip to remove any residual ethanol that will settle.
13. With the beads still on the magnetic stand, air-dry at room temperature for 6–10 min until all liquid has evaporated but without overdrying the beads.

    Note: Visually inspect the pellet to confirm that it is completely dry.
14. Remove the beads from the magnetic stand, and elute the RNA from the beads by adding 19 µl FPF Buffer. Mix well by pipetting or vortexing, and then allow the tubes to sit at room temperature for 2 min to appropriately hydrate the beads.

    Note: FPF Buffer is a component from the TruSeq Stranded mRNA Library Prep.
15. Return the tubes/plate to the magnetic rack until the solution has cleared.
16. Transfer 17 µl of the supernatant to clean tubes/plate. Alternatively, the samples can be stored at –90 to –65°C in a constant-temperature freezer.
17. Refer to the *TruSeq Stranded mRNA Reference Guide* and immediately proceed to and perform "Synthesize First Strand cDNA".

    Note: From the *TruSeq Stranded mRNA Reference Guide*, the procedural step "Place the RBP plate on the magnetic stand and wait until the liquid is clear (~5 minutes)" is not applicable.
18. Follow the *TruSeq Stranded mRNA Reference Guide* to perform all remaining library construction steps.

IMPORTANT 	It is highly recommended to dilute the Illumina adapters 2-fold compared to what is suggested in the default Illumina protocol.

Protocol: FastSelect with NEBNext Ultra II Directional Library Prep Kit

Important points before starting

- The NEBNext® Ultra II Directional RNA Library Prep Kit for Illumina (NEB cat. no. E7760S or E7760L) is required for use with this protocol.
- Refer to Table 1 in the "Introduction" or recommendations in the "Important Notes" sections and choose the appropriate procedural option to perform. Two options, both integrated with QIAseq FastSelect, are provided:
  - **Option 1**: No bead cleanup
  - **Option 2**: Bead cleanup
    - **Important**: Prepare fresh 80% ethanol daily.
    - Prewarm QIAseq Beads and Bead Binding Buffer to room temperature (15–25°C) prior to use. Ensure that the QIAseq Beads are thoroughly mixed at all times: work quickly and resuspend the beads immediately before use. If a delay in the protocol occurs, simply vortex the beads.
- Refer to the *NEBNext Ultra II Directional RNA Library Prep Kit for Illumina Instruction Manual* (Version 3.1).

Procedure

Option 1: No bead cleanup

1. Thaw QIAseq FastSelect tube(s) at room temperature. Mix by vortexing and then briefly centrifuge.
2. Referring to Section 4 from the *NEBNext Ultra II Directional RNA Library Prep Kit for Illumina Instruction Manual*, perform the following in place of steps 4.1.1 through 4.1.4:

   2a. Assemble the fragmentation and priming reaction described in Table 19 on ice in a nuclease-free tube.

Table 19. NEBNext Ultra II Stranded fragmentation and priming mix

Component	Volume/reaction
Total RNA (5 ng–1 µg)	4 µl
(lilac) NEBNext First Strand Synthesis Reaction Buffer*	4 µl
(lilac) Random Primers*	1 µl
Total volume	**9 µl**

* From NEBNext Ultra II Directional Library Prep Kit.

2b. To the assembled fragmentation and priming mix, add QIAseq FastSelect as follows:
- 1 µl of QIAseq FastSelect Custom
- **Optional**: Add 1 µl of cataloged QIAseq FastSelect kit

2c. Mix thoroughly by pipetting up and down several times and then briefly centrifuge to collect residual liquid from the sides of the tubes

2d. Incubate in a thermal cycler with a heated lid as described in Table 20, according to your input RNA quality.

Important: Regardless of time and temperature chosen in step 1, steps 2–9 must be performed.

Table 20. RNA fragmentation/depletion protocol: No bead cleanup

Step	Intact RNA (RIN >7)	Partially degraded RNA (RIN 2–6)
1	15 min at 94°C	7–8 min at 94°C
2	2 min at 75°C	2 min at 75°C
3	2 min at 70°C	2 min at 70°C
4	2 min at 65°C	2 min at 65°C
5	2 min at 60°C	2 min at 60°C
6	2 min at 55°C	2 min at 55°C
7	2 min at 37°C	2 min at 37°C
8	2 min at 25°C	2 min at 25°C
9	Hold at 4°C	Hold at 4°C

3. Refer to the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina Instruction Manual and immediately proceed to "First Strand cDNA Synthesis Reaction".

   Note: "First Strand cDNA Synthesis Reaction" is chapter 4.2 in Version 3.1 of the instruction manual.

4. Follow the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina Instruction Manual to perform all remaining library construction steps.

Option 2: Bead cleanup

1. Thaw template RNA on ice. Mix gently, centrifuge briefly to collect residual liquid from the sides of the tubes, and return to ice.
2. Thaw QIAseq FastSelect tube(s), FastSelect FH Buffer, and Nuclease-free Water at room temperature. Mix by vortexing and then briefly centrifuge. Prepare the reagents required for RNA fragmentation and QIAseq FastSelect rRNA removal.
3. On ice, prepare the RNA fragmentation/depletion reaction according to Table 21. Briefly centrifuge, mix by pipetting up and down 10 times, and then centrifuge briefly again.

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

Table 21. Setup of RNA fragmentation/depletion reaction: Bead cleanup

Component	Volume/reaction
Total RNA (5 ng – 1 µg)	Variable
FastSelect FH Buffer	1.5 µl
QIAseq FastSelect Custom	1 µl
Cataloged QIAseq FastSelect*	1 µl
Nuclease-free Water	Bring total reaction volume to 15 µl
Total volume	**15 µl**

* If required, add appropriate cataloged QIAseq FastSelect kit(s).

4. Incubate as described in Table 22.

   Important: Regardless of time and temperature chosen in step 1, steps 2–9 must be performed.

Table 22. RNA fragmentation/depletion protocol: Bead cleanup

Note	Step	Mean insert size ~175–225 bp
RNA fragmentation	1*†	8 min at 89°C*†
Steps 2–9 are performed, regardless of Input RNA quality. They need to be performed whether the RNA is high quality, moderate quality, FFPE, or degraded.	2	2 min at 75°C
	3	2 min at 70°C
	4	2 min at 65°C
	5	2 min at 60°C
	6	2 min at 55°C
	7	2 min at 37°C
	8	2 min at 25°C
	9	Hold at 4°C

* For high-quality RNA (RIN ≥8), 8 min at 89°C is the basic recommendation. Alternatively, 4 min at 89°C provides an average insert size of ~325–375 bp, and 12 min at 89°C provides an average insert size of ~125–175 bp.

† If working with RNA with RIN values <8, please refer to Table 3 for fragmentation recommendations.

5. Add 19.5 µl QIAseq Beads (prewarmed to room temperature and mixed by vortexing) to the 15 µl reaction. Mix thoroughly by vortexing, and incubate for 5 min at room temperature.
6. Centrifuge in a table-top centrifuge until the beads are completely pelleted (~2 min).
7. Place the tubes/plate on a magnetic rack for 2 min. Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

   Note: You can completely avoid transferring beads by pipetting very slowly.

   Important: Do not discard the beads, because they contain the RNA of interest.
8. Add 15 µl of Nuclease-free Water and 19.5 µl of QIAseq Bead Binding Buffer (prewarmed to room temperature and mixed by vortexing). Mix vigorously by vortexing, and incubate for 5 min at room temperature.
9. Centrifuge in a table-top centrifuge until the beads are completely pelleted (~2 min).

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

    Note: You can completely avoid transferring beads by pipetting very slowly.

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

11. With the beads still on the magnetic stand, add 200 µl of 80% ethanol. Wait 30 s. Carefully remove and discard the wash.

12. Repeat the ethanol wash.

    Important: Completely remove all traces of ethanol after this second wash. Remove the ethanol with a 200 µl pipette tip first, and then use a 10 µl pipette tip to remove any residual ethanol that will settle.

13. With the beads still on the magnetic stand, air-dry at room temperature for 6–10 min until all liquid has evaporated but without overdrying the beads.

    Note: Visually inspect the pellet to confirm that it is completely dry.

14. Remove the beads from the magnetic stand, and elute the RNA from the beads by adding 7 µl Nuclease-free Water. Mix well by pipetting or vortexing, and then allow the tubes to sit at room temperature for 2 min to appropriately hydrate the beads.

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

16. Transfer 5 µl of the supernatant to clean tubes/plate. Alternatively, the samples can be stored at –90 to –65°C in a constant-temperature freezer.

17. Set up the first-strand synthesis associated with the NEBNext Ultra II Directional RNA Library Prep Kit as described in Table 23. Briefly centrifuge, mix by pipetting up and down 10 times, and then centrifuge briefly again.

Table 23. NEBNext Ultra II Directional RNA Library Prep Kit first-stranded synthesis setup

Component	Volume/reaction
RNA from bead cleanup reaction	5 µl
(lilac) NEBNext First Strand Synthesis Reaction Buffer*	4 µl
(lilac) Random Primers*	1 µl
(brown) NEBNext Strand Specificity Reagent*	8 µl
(lilac) NEBNext First Strand Synthesis Enzyme Mix*	2 µl
Total volume	**20 µl**

* All designated components are from the NEBNext Ultra II Directional RNA Library Prep Kit.

18. Refer to the *NEBNext Ultra II Directional RNA Library Prep Kit for Illumina Instruction Manual* and immediately proceed to and perform step 4.2.3 under "First Strand cDNA Synthesis Reaction".
19. Follow the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina Instruction Manual to perform all remaining library construction steps.

    Important: If starting with 20 ng or less of total RNA, 2 additional cycles of library amplification must be performed.

Protocol: FastSelect with KAPA RNA HyperPrep Kit

Important points before starting

- The KAPA RNA HyperPrep Kit (Roche cat. no. KK8540 and KK8541) is required for use with this protocol.
- Refer to Table 1 in the "Introduction" or recommendations in the "Important Notes" sections and choose the appropriate procedural option to perform. Two options, both integrated with QIAseq FastSelect, are provided:
  - **Option 1**: No bead cleanup
  - **Option 2**: Bead cleanup
    - **Important**: Prepare fresh 80% ethanol daily.
    - Prewarm QIAseq Beads and Bead Binding Buffer to room temperature (15–25°C) prior to use. Ensure that the QIAseq Beads are thoroughly mixed at all times: work quickly and resuspend the beads immediately before use. If a delay in the protocol occurs, simply vortex the beads
- **Important**: It is highly recommended to dilute the KAPA adapters 1.5-fold compared to what is suggested in the default KAPA protocol.
- Refer to the KAPA RNA HyperPrep Kit Technical Data Sheet (KR1350 – v2.17).

Procedure

Option 1: No bead cleanup

1. Thaw QIAseq FastSelect tube(s) at room temperature. Mix by vortexing and then briefly centrifuge.
2. From the KAPA RNA HyperPrep Kit, prepare the fragmentation and priming mix described in Table 24 at room temperature in a nuclease-free tube.

Table 24. KAPA RNA HyperPrep fragmentation and priming mix

Component	Volume/reaction (µl)
Total RNA (25 ng–1 µg)	9*
Fragment, prime, and elute buffer (2X)†	10
Total volume	**19**

* Reduce volume to 8 µl if removing rRNA and globin.

† From KAPA RNA HyperPrep Kit.

3. To the assembled fragmentation and priming mix, add QIAseq FastSelect as follows:
   3a. 1 µl of QIAseq FastSelect Custom
   3b. **Optional**: Add 1 µl of cataloged QIAseq FastSelect
4. Mix thoroughly by gently pipetting the reaction up and down several times and then briefly centrifuge to collect residual liquid from the sides of the tubes.
5. Incubate in a thermal cycler with a heated lid as described in Table 25 according to your input RNA quality.

   Important: Regardless of time and temperature chosen in step 1, steps 2–9 must be performed.

Table 25. RNA fragmentation/depletion protocol: No bead cleanup

Input RNA type	Step	Time and temperature
Intact	1*	**Choose**: 8 min at 94°C *or* 6 min 94°C *or* 6 min at 85°C
Partially degraded	1†	1–6 min at 85°C
Degraded (e.g., FFPE)	1‡	No fragmentation
Steps 2–9 are performed regardless of input RNA quality. They need to be performed whether the RNA is high quality, moderate quality, FFPE, or degraded.	2	2 min at 75°C
	3	2 min at 70°C
	4	2 min at 65°C
	5	2 min at 60°C
	6	2 min at 55°C
	7	2 min at 37°C
	8	2 min at 25°C
	9	Hold at 4°C

* Choose one option, depending if you want a desired mean library insert size of 100–200 bp (8 min at 94°C), 200–300 bp (6 min 94°C) or 300–400 bp (6 min at 85°C).
† For a desired mean library insert size of 100–300 bp.
‡ For a desired mean library insert size of 100–200 bp.

6. Refer to the *KAPA RNA HyperPrep Kit Technical Data Sheet* and immediately proceed to "1st Strand Synthesis", section 3 in v2.17.
7. Follow the *KAPA RNA HyperPrep Kit Technical Data Sheet* to perform all remaining library construction steps.

IMPORTANT 	It is highly recommended to dilute the KAPA adapters 1.5-fold compared to what is suggested in the default KAPA protocol.

Option 2: Bead cleanup

1. Thaw template RNA on ice. Gently mix, briefly centrifuge to collect residual liquid from the sides of the tubes, and return to ice.
2. Thaw QIAseq FastSelect tube(s), FastSelect FH Buffer, and Nuclease-free Water at room temperature. Mix by vortexing and then briefly centrifuge Prepare the reagents required for RNA fragmentation and QIAseq FastSelect rRNA removal.
3. On ice, prepare the RNA fragmentation/depletion reaction according to Table 26. Briefly centrifuge, mix by pipetting up and down 10 times, and centrifuge briefly again.

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

Table 26. Setup of RNA fragmentation/depletion reaction: Bead cleanup

Component	Volume/reaction
Total RNA (25 ng – 1 µg)	Variable
FastSelect FH Buffer	1.5 µl
QIAseq FastSelect Custom	1 µl
Cataloged QIAseq FastSelect*	1 µl
Nuclease-free Water	Bring total reaction volume to 15 µl
Total volume	**15 µl**

* If required, add appropriate cataloged QIAseq FastSelect kit(s).

4. Incubate as described in Table 27.

   Important: Regardless of the time and temperature chosen in step 1, steps 2–9 must be performed.

Table 27. RNA fragmentation/depletion protocol: Bead cleanup

Input RNA quality	Step	Mean insert size ~175–225 bp
RNA fragmentation	1*†	8 min at 89°C*†
Steps 2–9 are performed regardless of input RNA quality. They need to be performed whether the RNA is high quality, moderate quality, FFPE, or degraded.	2	2 min at 75°C
	3	2 min at 70°C
	4	2 min at 65°C
	5	2 min at 60°C
	6	2 min at 55°C
	7	2 min at 37°C
	8	2 min at 25°C
	9	Hold at 4°C

* For high-quality RNA (RIN ≥8), 8 min at 89°C is the basic recommendation. Alternatively, 4 min at 89°C provides an average insert size of ~325–375 bp, and 12 min at 89°C provides an average insert size of ~125–175 bp.

† If working with RNA with RIN values <8, please refer to Table 3 for fragmentation recommendations.

5. Add 19.5 µl QIAseq Beads (prewarmed to room temperature and mixed by vortexing) to the 15 µl reaction. Mix thoroughly by vortexing, and then incubate for 5 min at room temperature.
6. Centrifuge in a table-top centrifuge until the beads are completely pelleted (~2 min).
7. Place the tubes/plate on a magnetic rack for 2 min. Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

   Note: You can completely avoid transferring beads by pipetting very slowly.

   Important: Do not discard the beads, because they contain the RNA of interest.
8. Add 15 µl of Nuclease-free Water and 19.5 µl of QIAseq NGS Bead Binding Buffer (prewarmed to room temperature and mixed by vortexing). Mix vigorously by vortexing, and then incubate for 5 min at room temperature.
9. Centrifuge in a table-top centrifuge until the beads are completely pelleted (~2 min).
10. Place the tubes/plate on a magnetic rack for 2 min. Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

    Note: You can completely avoid transferring beads by pipetting very slowly.

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

11. With the beads still on the magnetic stand, add 200 µl of 80% ethanol. Wait for 30 s. Carefully remove and discard the wash.
12. Repeat the ethanol wash.

    Important: Completely remove all traces of ethanol after this second wash. Remove the ethanol with a 200 µl pipette tip first, and then use a 10 µl pipettor to remove any residual ethanol that will settle.
13. With the beads still on the magnetic stand, air-dry at room temperature for 6–10 min, until all liquid has evaporated but without overdrying the beads.

    Note: Visually inspect the pellet to confirm that it is completely dry.
14. Remove the beads from the magnetic stand, and elute the DNA from the beads by adding 12 µl Nuclease-free Water + 10 µl Fragment, Prime, and Elute Buffer (2x). Mix well by pipetting or vortexing, and then allow the tubes to sit at room temperature for 2 min to appropriately hydrate the beads.

    Note: The Fragment, Prime, and Elute Buffer (2x) is from the KAPA RNA HyperPrep Kit.
15. Return the tube/plate to the magnetic rack until the solution has cleared.
16. Transfer 20 µl of the supernatant, which is the "Fragmented, primed RNA" to clean tubes/plate. Alternatively, the samples can be stored at –90 to –65°C in a constant-temperature freezer.
17. Refer to the *KAPA RNA HyperPrep Kit Technical Data Sheet* and proceed directly to "1st Strand Synthesis", section 3 in v2.17, and perform step 3.1.

    Note: There is no need to perform steps 2.2, 2.3, and 2.4, because the RNA has already been fragmented during the FastSelect procedure.
18. Follow the *KAPA RNA HyperPrep Kit Technical Data Sheet* to perform all remaining library construction steps.

IMPORTANT 	It is highly recommended to dilute the KAPA adapters 1.5-fold compared to what is suggested in the default KAPA protocol.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page in our Technical Support Center: **www.qiagen.com/FAQ/FAQList.aspx**. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information or protocols in this handbook (for contact information, visit **support.qiagen.com**).

	Comments and suggestions
Adapter–dimer observed in final library QC	
Depending on the library kit and RNA input amount, adapter–dimers may be observed.	Perform a second cleanup of the final library using the same beads-to-sample ratio as in the first cleanup.

Ordering Information

Product	Contents	Cat. no.
QIAseq FastSelect –rRNA HMR Kit (24), (96), (384)	Cytoplasmic and mitochondrial rRNA removal reagent: supports human, mouse, and rat; available in 24, 96, or 384 reactions	334386 334387 334388
QIAseq FastSelect –Globin Kit (24), (96), (384)	Globin mRNA removal reagent: supports human, mouse, and rat; available in 24, 96, or 384 reactions	334376 334377 334378
QIAseq FastSelect –rRNA/Globin Kit (24), (96), (384)	Cytoplasmic and mitochondrial rRNA removal reagent and globin mRNA removal reagent: supports human, mouse, and rat; available in 24, 96, or 384 reactions	335376 335377 335378
QIAseq FastSelect –5S/16S/23S Kit	Bacteria rRNA removal reagent; available in 24, 96, or 384 reactions	335925 335927 335929
QIAseq FastSelect Epidemiology Kit	Cytoplasmic and mitochondrial rRNA removal reagent (supports human, mouse, and rat) and bacteria rRNA removal reagent; available in 24, 96, or 384 reactions	333272 333275 333277
QIAseq FastSelect –rRNA Plant Kit (24), (96), (384)	Cytoplasmic, mitochondrial and chloroplast rRNA removal reagent: supports plant; available in 24, 96, or 384 reactions	334315 334317 334319
QIAseq FastSelect –rRNA Yeast Kit (24), (96), (384)	Cytoplasmic and mitochondrial rRNA removal reagent: supports yeast; available in 24, 96, or 384 reactions	334215 334217 334219
QIAseq FastSelect –rRNA Worm Kit (24), (96)	Cytoplasmic and mitochondrial rRNA removal reagent: supports worm; available in 24 or 96 reactions	333242 333245

Product	Contents	Cat. no.
QIAseq FastSelect –rRNA Fish Kit (24), (96)	Cytoplasmic and mitochondrial rRNA removal reagent: supports fish; available in 24 or 96 reactions	333252 333255
QIAseq FastSelect –rRNA Fly Kit (24), (96)	Cytoplasmic and mitochondrial rRNA removal reagent: supports fly; available in 24 or 96 reactions	333262 333265
QIAseq Stranded RNA Lib Kit UDI (24), (96)	Stranded RNA-seq sequencing library preparation: fragmentation, reverse transcription, second-strand synthesis + end-repair + A-addition, adapter ligation, CleanStart® PCR enrichment, and QIAseq Beads for library cleanups; available in 24 or 96 reactions with unique dual sample index adapters	180450 180451 180452 180453 180454
QIAseq Stranded mRNA Select Kit (24), (96)	Stranded RNA-seq sequencing library preparation reactions: mRNA enrichment of total RNA, fragmentation, reverse transcription, second-strand synthesis + end-repair + A-addition, adapter ligation, CleanStart PCR enrichment, and QIAseq Beads for library cleanups; available in 24 or 96 reactions	180773 180775

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

Document Revision History

Date	Changes
06/2021	Initial revision
12/2021	Changed the symbols (less than/greater than sign) modifying "###" of bead cleanup.

Limited License Agreement for QIAseq FastSelect Custom RNA Removal Kit and QIAseq FastSelect Beads and Buffer Kit

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