July 2020

QIAseq® FastSelect™ –5S/16S/23S Handbook

Bacterial 5S/16S/23S rRNA removal during stranded RNA library preparation

Sample to Insight

QIAGEN

Contents

Kit Contents

QIAseq FastSelect –5S/16S/23S Kit	**(24)**	**(96)**	**(384)**
Catalog no.	**335925**	**335927**	**335929**
Number of reactions	**24**	**96**	**384**
FastSelect 5S/16S/23S	3 x 8 µl	96 µl	4 x 96 µl
FastSelect FH Buffer	3 x 12 µl	144 µl	4 x 144 µl
Nuclease-free Water	1 tube	1 tube	1 tube
QIAseq Beads	10 ml	10 ml	10 ml
QIAseq Bead Binding Buffer	10.2 ml	10.2 ml	10.2 ml

Shipping and Storage

The QIAseq FastSelect –5S/16S/23S Kit is shipped on blue ice. Upon receipt, the FastSelect 5S/16S/23S tube should be immediately stored at –30 to –15°C in a constant-temperature freezer. All remaining components should immediately be stored in a refrigerator at 2–8°C.

Intended Use

The QIAseq FastSelect –5S/16S/23S Kit is intended for molecular biology applications. This product is 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 the QIAseq FastSelect –5S/16S/23S Kit 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. While stranded library preparation is being performed, significantly overrepresented RNAs (such as ribosomal RNA [rRNA]) must be avoided to facilitate optimal read allocation. Prokaryotic RNA profiling presents a unique challenge for rRNA depletion, due to the incredible diversity in rRNA sequences. For complex bacterial samples, hundreds of thousands of 5S, 16S, and 23S sequences need to be removed. While different methods to remove bacterial rRNA are commonly utilized during RNA-seq library preparation, these methods are not designed to be pan-bacteria; they focus exclusively on removing 16S and 23S sequences for a handful of common bacterial species and ignore the 5S rRNA.

QIAseq FastSelect –5S/16S/23S utilizes a novel technology and is a pan-bacterial rRNA-removal kit designed to selectively remove 5S, 16S, and 23S rRNA from complex bacterial community samples. As a result, QIAseq FastSelect –5S/16S/23S rapidly and efficiently removes a broad spectrum of bacterial rRNA during NGS RNA library preparation, thus providing an optimal solution for both single-species isolates and complex community samples for metatranscriptomics analysis. Furthermore, QIAseq FastSelect –5S/16S/23S can be combined with any other QIAseq FastSelect for mixed-species RNA profiling experiments.

Principle and procedure

QIAseq FastSelect –5S/16S/23S is an inline solution for the removal of unwanted bacterial rRNAs during NGS library preparation; the protocol takes approximately one hour. This pan-bacteria–removal technology works by inhibiting reverse transcription of bacterial rRNA, even in difficult samples such as formalin-fixed paraffin-embedded (FFPE) samples. Simply hybridize the FastSelect 5S/16S/23S reagent during the NGS library preparation, heat-fragment the sample (if necessary), cool the reaction from 75°C to 25°C over 14 min, perform

a bead-base cleanup, and continue with the first-strand synthesis of the library prep procedure (Figure 1).

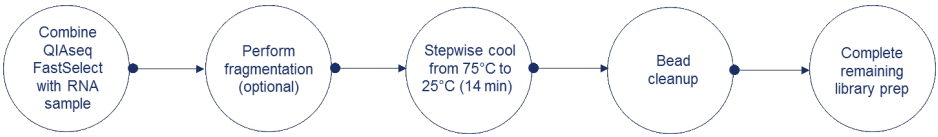

Figure 1. QIAseq FastSelect –5S/16S/23S workflow.

QIAseq FastSelect: Stranded library and sample-type compatibility

QIAseq FastSelect has been tested to be compatible with a variety of commercially available stranded RNA library prep kits provided by QIAGEN, Illumina®, New England Biolabs®, and Roche®, and it works across a broad RNA input range (20 ng – 1 µg of total RNA, depending on the kit). In general, QIAseq FastSelect is compatible with any stranded RNA library prep kit. For questions regarding protocols for kits that are not detailed in the handbook, please contact QIAGEN technical support at **support.qiagen.com**.

Regarding samples, QIAseq FastSelect has been verified to be compatible with a variety of total RNA samples isolated from single-species bacterial clones and/or complex meta-bacterial samples, cells, fresh/frozen tissue, FFPE tissue, whole blood, and serum/plasma samples, including exosomes. From control bacterial communities, such as the Gut Microbiome Whole cell Mix (ATCC® MSA-2006™), 70–90% depletion of rRNA is observed. From single species, such as *E. coli* Total RNA (Thermo Fisher Scientific cat. no. AM7940), greater than 95% depletion of rRNA is routinely observed.

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.

- 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)
- If also removing mammalian rRNA: QIAseq FastSelect –rRNA HMR Kit (cat. no. 334386, 334387, or 334388)
- If also removing mammalian globin mRNA: QIAseq FastSelect –Globin Kit (cat. no. 334376, 334377, or 334378)
- If also removing mammalian rRNA and globin mRNA: QIAseq FastSelect –rRNA/Globin Kit (cat. no. 335376, 335377, 335378)
- If also removing Plant rRNA: QIAseq FastSelect -rRNA Plant Kit (cat no. 334315, 334317, 334319)
- If also removing Yeast rRNA: QIAseq FastSelect -rRNA Yeast Kit (cat no. 334215, 334217, 334219)

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

Important Notes

- FastSelect 5S/16S/23S will appear cloudy. This is completely normal and not a cause for concern. Incubate at 37°C for 5 min and then vortex to dissolve the precipitate.
- We highly recommend DNase treatment (on-column and in-solution) of total RNA samples.
- QIAseq FastSelect is an inline solution for the removal of unwanted bacterial rRNAs during NGS library preparation. The total RNA input is defined by the range of total RNA input dictated by the stranded RNA library kit. 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.
- FastSelect FH Buffer has been optimized for both the fragmentation of total RNA as well as the hybridization of the FastSelect reagent for its intended targets. It should not be replaced by a different buffer.
- As part of each protocol, RNA fragmentation conditions are listed for high-quality RNA (RIN ≥8). If working with RNA with RIN values <8, please refer to Table 1 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 3, Table 6, Table 8, or Table 11) need to be performed. They need to be performed whether the RNA is high quality, moderate quality, FFPE, or degraded.

Table 1. 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 3, Table 6, or Table 8) need to be performed. They need to be performed whether the RNA is high quality, moderate quality, FFPE, or degraded.

- QIAseq FastSelect –5S/16S/23S can be combined with any or all available FastSelect products. As examples:
  - For mixed bacterial/mammalian samples, QIAseq FastSelect –5S/16S/23S is fully compatible with QIAseq FastSelect –rRNA HMR (to remove mammalian rRNA), QIAseq FastSelect –Globin (to remove mammalian globin mRNA).
  - For mixed bacterial/plant samples, QIAseq FastSelect –5S/16S/23S is fully compatible with QIAseq FastSelect –rRNA Plant (to remove plant rRNA).
  - For mixed bacterial/yeast samples, QIAseq FastSelect –5S/16S/23S is fully compatible with QIAseq FastSelect –rRNA Yeast (to remove yeast rRNA).
- A convenient stopping point in the FastSelect workflow is at the end of the bead cleanup. The samples can be stored at –90 to –65°C in a constant-temperature freezer.
- 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 bacterial rRNA, which does not occur until the first-strand synthesis reaction during library prep.
- 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: QIAseq FastSelect –5S/16S/23S with the QIAseq Stranded RNA Lib Kit

Important points before starting

- The QIAseq Stranded Total RNA Lib Kit (cat. no. 180743 or 180745) is required for use with this protocol. For more information, please refer to the *QIAseq Stranded Total RNA Lib Kit Handbook* available at **www.qiagen.com/HB-2465**.
- This protocol has been tested with 100 ng – 1 µg of total RNA.
- **Note**: FastSelect 5S/16S/23S will appear cloudy. This is completely normal and not a cause for concern. The procedure begins with a step to dissolve the precipitate.
- With this protocol, you can also add QIAseq FastSelect –rRNA HMR, QIAseq FastSelect –Globin, QIAseq FastSelect –rRNA Plant, and/or QIAseq FastSelect –rRNA Yeast. Any or all FastSelect products can be combined.
- **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**: For library amplification with the QIAseq Stranded RNA Lib Kit, 2 additional cycles of library amplification must be performed.

Procedure

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. Prepare the reagents required for the RNA fragmentation and QIAseq FastSelect rRNA removal:
   2a. Bring FastSelect 5S/16S/23S, FastSelect FH Buffer, and Nuclease-free Water to room temperature.
   2b. **Important**: Only for FastSelect 5S/16S/23S, incubate the tube at 37°C for 5 min, and then vortex to dissolve the precipitate.
   2c. Mix all other reagents vigorously by vortexing, and then briefly centrifuge.
3. On ice, prepare the fragmentation/depletion reaction according to Table 2. 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 2. Setup of fragmentation/depletion reactions

Component	Volume/reaction
Total RNA (100 ng – 1 µg)	Variable
FastSelect FH Buffer	1.5 µl
FastSelect 5S/16S/23S*	1 µl
Nuclease-free Water	Bring total reaction volume to 15 µl
Total volume	**15 µl**

* If needed, add 1 µl QIAseq FastSelect –rRNA HMR, 1 µl QIAseq FastSelect –Globin, 1 µl QIAseq FastSelect –rRNA Plant, and/or 1 µl QIAseq FastSelect –Yeast. To keep the total volume at 15 µl, correspondingly reduce the Nuclease-free Water by the volume of additional FastSelect products that have been added.

4. Incubate as described in Table 3.

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

Table 3. Combined QIAseq fragmentation and FastSelect hybridization protocol

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 1 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 4. Briefly centrifuge, mix by pipetting up and down 10 times, and then centrifuge briefly again.

Table 4. QIAseq Stranded Total RNA Lib Kit first-stranded synthesis setup

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

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

18. Refer to the *QIAseq Stranded Total 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 Total RNA Lib Kit Handbook* to perform all remaining library construction steps.

IMPORTANT 	For library amplification, 2 additional cycles of library amplification must be performed.

Protocol: QIAseq FastSelect –5S/16S/23S with TruSeq Stranded Library Preparation

Important points before starting

- The TruSeq® Stranded mRNA Library Prep (Illumina cat. no. 20020594, 20020595) 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 *TruSeq Stranded mRNA Reference Guide* (1000000040498). By doing this, a stranded total RNA library prep will be performed.
- This protocol has been tested with 100 ng – 1 µg of total RNA.
- **Note**: FastSelect 5S/16S/23S will appear cloudy. This is completely normal and not a cause for concern. The procedure begins with a step to dissolve the precipitate.
- With this protocol, you can also add QIAseq FastSelect –rRNA HMR, QIAseq FastSelect –Globin, QIAseq FastSelect –rRNA Plant, and/or QIAseq FastSelect –rRNA Yeast. Any or all FastSelect products can be combined.
- **Important**: Prepare fresh 80% ethanol daily.
- Prewarm QIAseq Beads and Bead Binding Buffer to room temperature 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**: When performing TruSeq library prep, it is highly recommended to dilute the Illumina adapters 2-fold compared to what is suggested in the default Illumina protocol.

Procedure

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. Prepare the reagents required for the RNA fragmentation and QIAseq FastSelect rRNA removal:
   2a. Bring FastSelect 5S/16S/23S, FastSelect FH Buffer, and Nuclease-free Water to room temperature.
   2b. **Important**: Only for FastSelect 5S/16S/23S, incubate the tube at 37°C for 5 min, and then vortex to dissolve the precipitate.
   2c. Mix all other reagents vigorously by vortexing, and then briefly centrifuge.
3. On ice, prepare the fragmentation/depletion reaction according to Table 5. 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 5. Setup of fragmentation/depletion reactions

Component	Volume/reaction
Total RNA (100 ng – 1 µg)	Variable
FastSelect FH Buffer	1.5 µl
FastSelect 5S/16S/23S*	1 µl
Nuclease-free Water	Bring total reaction volume to 15 µl
Total volume	**15 µl**

* If needed, add 1 µl QIAseq FastSelect –rRNA HMR, 1 µl QIAseq FastSelect –Globin, 1 µl QIAseq FastSelect –rRNA Plant, and/or 1 µl QIAseq FastSelect –Yeast. To keep the total volume at 15 µl, correspondingly reduce the Nuclease-free Water by the volume of additional FastSelect products that have been added.

4. Incubate as described in Table 6.
   Important: Regardless of time and temperature chosen in step 1, steps 2–9 must be performed.

Table 6. Combined QIAseq fragmentation and FastSelect hybridization protocol

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 1 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: QIAseq FastSelect –5S/16S/23S with the NEBNext Ultra II Directional Library Prep Kit

Important points before starting

- The NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina (New England Biolabs cat. no. E7760S, E7760L) is required for use with this protocol. For more information, refer to the *NEBNext Ultra II Directional RNA Library Prep Kit for Illumina* instruction manual (Version 2.2_05/19).
- This protocol has been tested with 20 ng – 1 µg of total RNA.
- **Note**: FastSelect 5S/16S/23S will appear cloudy. This is completely normal and not a cause for concern. The procedure begins with a step to dissolve the precipitate.
- With this protocol, you can also add QIAseq FastSelect –rRNA HMR, QIAseq FastSelect –Globin, QIAseq FastSelect –rRNA Plant, and/or QIAseq FastSelect –rRNA Yeast. Any or all FastSelect products can be combined.
- **Important**: Prepare fresh 80% ethanol daily.
- Prewarm QIAseq Beads and Bead Binding Buffer to room temperature 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.

Procedure

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. Prepare the reagents required for RNA fragmentation and QIAseq FastSelect rRNA removal:
   - 2a. Bring FastSelect 5S/16S/23S, FastSelect FH Buffer, and Nuclease-free Water to room temperature.
   - 2b. **Important**: Only for FastSelect 5S/16S/23S, incubate the tube at 37°C for 5 min, and then vortex to dissolve the precipitate.
   - 2c. Mix all other reagents vigorously by vortexing, and then briefly centrifuge.

3. On ice, prepare the fragmentation/depletion reaction according to Table 7. 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 7. Setup of fragmentation/depletion reactions

Component	Volume/reaction
Total RNA (20 ng – 1 µg)	Variable
FastSelect FH Buffer	1.5 µl
FastSelect 5S/16S/23S*	1 µl
Nuclease-free Water	Bring total reaction volume to 15 µl
Total volume	**15 µl**

* If needed, add 1 µl QIAseq FastSelect –rRNA HMR, 1 µl QIAseq FastSelect –Globin, 1 µl QIAseq FastSelect –rRNA Plant, and/or 1 µl QIAseq FastSelect –Yeast. To keep the total volume at 15 µl, correspondingly reduce the Nuclease-free Water by the volume of additional FastSelect products that have been added.

4. Incubate as described in Table 8.
   Important: Regardless of time and temperature chosen in step 1, steps 2–9 must be performed.

Table 8. Combined QIAseq fragmentation and FastSelect hybridization protocol

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 1 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 9. Briefly centrifuge, mix by pipetting up and down 10 times, and then centrifuge briefly again.

Table 9. 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: QIAseq FastSelect –5S/16S/23S with the KAPA RNA HyperPrep Kit

Important points before starting

- The KAPA RNA HyperPrep Kit (Roche cat. no. KK8540, KK8541) is required for use with this protocol. For more information, refer to the *KAPA RNA HyperPrep Kit Technical Data Sheet* (KR1350 – v2.17).
- This protocol has been tested with 25 ng – 1 µg of total RNA.
- **Note**: FastSelect 5S/16S/23S will appear cloudy. This is completely normal and not a cause for concern. The procedure begins with a step to dissolve the precipitate.
- With this protocol, you can also add QIAseq FastSelect –rRNA HMR, QIAseq FastSelect –Globin, QIAseq FastSelect –rRNA Plant, and/or QIAseq FastSelect –rRNA Yeast. Any or all FastSelect products can be combined.
- **Important**: Prepare fresh 80% ethanol daily.
- Prewarm QIAseq Beads and Bead Binding Buffer to room temperature 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**: When performing KAPA library prep, it is highly recommended to dilute the KAPA adapters 1.5-fold compared to what is suggested in the default KAPA protocol.

Procedure

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. Prepare reagents required for RNA fragmentation and QIAseq FastSelect rRNA removal:
   - 2a. Bring FastSelect 5S/16S/23S, FastSelect FH Buffer, and Nuclease-free Water to room temperature.
   - 2b. **Important**: Only for FastSelect 5S/16S/23S, incubate the tube at 37°C for 5 min, and then vortex to dissolve the precipitate.
   - 2c. Mix all other reagents vigorously by vortexing, and then briefly centrifuge.

3. On ice, prepare the fragmentation/depletion reaction according to Table 10. 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 10. Setup of fragmentation/depletion reactions

Component	Volume/reaction
Total RNA (25 ng – 1 µg)	Variable
FastSelect FH Buffer	1.5 µl
FastSelect 5S/16S/23S*	1 µl
Nuclease-free Water	Bring total reaction volume to 15 µl
Total volume	**15 µl**

* If needed, add 1 µl QIAseq FastSelect –rRNA HMR, 1 µl QIAseq FastSelect –Globin, 1 µl QIAseq FastSelect –rRNA Plant, and/or 1 µl QIAseq FastSelect –Yeast. To keep the total volume at 15 µl, correspondingly reduce the Nuclease-free Water by the volume of additional FastSelect products that have been added.

4. Incubate as described in Table 11.

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

Table 11. Combined QIAseq fragmentation and FastSelect hybridization protocol

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 1 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 at 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 and/or protocols in this handbook or sample and assay technologies (for contact information, visit **www.qiagen.com**).

	Comments and suggestions
I don't know how long to fragment my RNA	
The fragmentation time is dependent on the desired insert size.	For high-quality RNA (RIN ≥8), 8 min at 89°C is recommended. Using different types of RNA with RIN values ≥8, we expect 4 min at 89°C to provide an average insert size of ~325–375 bp, and 12 min at 89°C provides an average insert size of ~125–175 bp. For additional recommendations, please refer to Table 1.
Adapter–dimer observed in final library QC	
Depending on kit and RNA input amount, adapter–dimers may be observed.	Perform a second cleanup reaction of the final library.
Library yield is lower than expected	
The rRNA removal imparted by QIAseq FastSelect is extremely robust, especially when compared to other methods.	Unless otherwise noted, we recommend to amplify the libraries using the number of cycles suggested by the chosen library prep kit, which is based on the starting amount of total RNA. If the yield is too low, adding 2 cycles is usually sufficient to increase library yield for all downstream quantification and sequencing applications.
I don't know how to quantitate my final NGS library	
Various methods exist to quantitate the final NGS library.	In general, we highly recommend using a qPCR method with a standard curve. While Qubit®, TapeStation, and Bioanalyzer are convenient, they are highly variable and subject to limitations such as peak width, salts and oligos, and other leftover reagents during library construction. While convenient and often used during a standard workflow to save time, these methods fail to quantitate the part of the library that will cluster effectively on your flow cell. As an alternative to qPCR, a low pass sequencing reaction can also be used.

Ordering Information

Product	Contents	Cat. no.
QIAseq FastSelect –5S/16S/23S Kit	Bacteria rRNA removal reagent; available in 24, 96, or 384 reactions	335925 335927 335929
QIAseq FastSelect –rRNA HMR Kit	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	Globin mRNA removal reagent: supports human, mouse, and rat; available in 24, 96, or 384 reactions	334376 334377 334378
QIAseq FastSelect –rRNA/Globin Kit	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 –rRNA Plant Kit	Cytoplasmic, mitochondrial and chloroplast rRNA removal reagent: supports plant; available in 24, 96, or 384 reactions	334315 334317 334319
QIAseq FastSelect –rRNA Yeast Kit	Cytoplasmic and mitochondrial rRNA removal reagent: supports yeast; available in 24, 96, or 384 reactions	334215 334217 334219

Product	Contents	Cat. no.
QIAseq Stranded Total RNA Lib Kit	For stranded RNA-seq sequencing library prep reactions: fragmentation, reverse transcription, second-strand synthesis + end-repair + A-addition, adapter ligation, CleanStart PCR enrichment, and QIAseq Beads for library cleanups; available in 24 and 96 reactions	180743 180745

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
10/2019	Initial release
12/2019	Clarified storage condition. Added protocol for using QIAseq FastSelect –5S/16S/23S with the KAPA RNA HyperPrep Kit.
07/2020	Clarified storage condition. Included 37°C incubation for FastSelect 5S/16S/23S as a default step in each procedure. Included options to include FastSelect –rRNA Plant and/or FastSelect –rRNA Yeast in each procedure. Replaced all precise storage temperatures with temperature ranges.

Notes

Notes

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