

# SEQuoia™ Complete Stranded RNA Library Prep Kit

Catalog #	Description
17005726	<b>SEQuoia Complete Stranded RNA Library Prep Kit</b> , 24 reactions
17005710	<b>SEQuoia Complete Stranded RNA Library Prep Kit</b> , 96 reactions

For research purposes only.

## Introduction

The SEQuoia Complete Stranded RNA Library Prep Kit provides all of the components required to make an RNA sequencing (RNA-Seq) library for Illumina® platforms. The kit includes SEQzyme, a proprietary engineered enzyme that couples the addition of adapters with cDNA synthesis, enabling a one-tube continuous synthesis reaction. The kit captures all types of RNA (both long and short fragments) from limited and low-quality samples including liquid biopsy and formalin-fixed paraffin-embedded (FFPE) tissue samples. The streamlined workflow minimizes the number of pipetting steps and greatly reduces the overall protocol time to less than 4 hours.

All kit components are optimized to facilitate efficient library synthesis using a wide range of input RNA (1 µg–100 pg) and are compatible with automated next-generation sequencing (NGS) library construction. The kit does not include RNA or the solid phase reversible immobilization (SPRI) beads needed for the purification step.

The SEQuoia Complete Stranded RNA Library Prep Kit is ideal for a wide range of RNA-Seq applications, including:

- Differential gene expression (DGE) analysis
- microRNA and other noncoding RNA analysis
- Whole transcriptome analysis, which includes both small and large RNA
- Liquid biopsy analysis
- Splice junction identification
- Single nucleotide variation discovery
- Biomarker discovery

## Storage and Stability

The SEQuoia Complete Stranded RNA Library Prep Kit is guaranteed for 12 months after the shipping date if stored properly (see Table 1).

**Table 1. Kit contents.**

Component	Cap Color	24 Reactions, µl	96 Reactions	Storage Temperature
Reagent A Fragmentation Mix	Red	48	192 µl	-20°C
Reagent B End Repair Enzyme	Yellow	48	192 µl	
Reagent C Poly(A) Buffer	Violet	570	2 x 1.1 ml	
Reagent D Poly(A) Polymerase	Blue	30	120 µl	
Reagent E SEQzyme Mix	Orange	120	480 µl	
Reagent F Amplification Mix	Green	600	2 x 1.2 ml	
Purification Beads*	Gray	480	2 x 960 µl	+4°C

\* Purification Beads ship and are stored separately from the rest of the kit.

## Required Materials Not Provided

The following reagents are required but not supplied. These materials have been validated with this protocol. Substitutions may not produce ideal results.

- SEQuoia RiboDepletion Kit (catalog #17006487)
- SEQuoia Dual Indexed Primers Set (#12011928) or SEQuoia Dual Indexed Primers Plate (#12011930)
- 0.2 ml RNase- and DNase-free, low-binding PCR tube strips and cap strips (#TBC0802 or #TBC1202)
- RNase-free filtered pipet tips: 10, 20, and 200 µl
- SPRIselect Reagent from Beckman Coulter, Inc.
- 10 mM Tris-HCl, pH 8, 1x TE buffer, or water (RNase- and DNase-free)
- 80% ethanol (prepare fresh)

## Optional Materials Not Provided

- ddPCR™ Library Quantification Kit for Illumina® TruSeq® (#1863040)
- Agilent 2100 Bioanalyzer System and RNA 6000 Nano or Pico Kit
- Agilent 2100 Bioanalyzer System High Sensitivity DNA Kit

### Equipment List

- Thermal cycler for accurate incubation temperatures
- Calibrated single-channel and multichannel pipets: 10, 20, and 200  $\mu$ l
- Magnetic rack for small-scale separation of magnetic beads in 0.2 ml tubes
- Vortexer
- Microcentrifuge for 0.2 ml tubes
- 96-well PCR cooling rack
- Ice bucket

### Important Considerations — Read Before Starting

- Thaw all reagents on ice and set up reactions on ice unless otherwise noted
- Proper care should be taken while handling RNA. Where needed, use RNase-free water, keep the workspace clean using RNase removal products, and use RNase-free plastic consumables. The reactions, unless otherwise specified in the protocol, should be kept in closed tubes and on ice
- Avoid multiple freeze-thaw cycles of input RNA to preserve the integrity of RNA
- To maximize library diversity, use an RNA sample preparation that is compatible with large, small, and degraded RNA fragments. For cultured cells, we recommend using either the SingleShot Lysis Kit (#1725080) or the PureZOL RNA Isolation Reagent (#7326880). For other sample types, such as fresh frozen tissue, we recommend PureZOL Reagent. Column-based RNA purification is not recommended unless the kit can capture the small RNA efficiently
- Use a thermal cycler for each incubation step to ensure thermal accuracy. Program the thermal cycler with each of the protocols prior to starting
- Assess the quality of the input RNA by running the RNA sample with an Agilent RNA 6000 Nano or Pico Kit to determine the RNA integrity number (RIN)
- For degraded samples (such as FFPE tissue) with a RIN <2, reduce the fragmentation incubation temperature to 70°C and incubate for no longer than 1 min
- Deplete ribosomal RNA (rRNA) after library construction, using the SEQuoia RiboDepletion Kit to construct a more complex library that produces a richer dataset more representative of the whole transcriptome. At fragmentation step 6, follow the instructions for using total RNA as the input sample.
 

**Note:** This kit is also compatible with RNA samples that have been enriched for mRNA or depleted of rRNA prior to library construction. At fragmentation step 6, follow the instructions for using pre-depleted RNA as the input sample.

- The suggested number of amplification cycles in the Library Amplification section should be used as a guideline and should be optimized by the user to avoid under- or overamplification
- There are several safe stopping points, which are identified in the protocol
- Always use the most current revision of this manual, which can be downloaded at [bio-rad.com/SEQuoiaComplete](http://bio-rad.com/SEQuoiaComplete)

### Programming the Thermal Cycler

Before starting the reaction, program the thermal cycler protocols for each step separately, or combine them into one continuous protocol with infinite holds that delineate the steps (Table 2).

**Table 2. Thermal cycler protocols.\***

Protocol Step	Step	Temperature, °C	Time, min
Fragmentation	1	94 or 70	Hold
	2	94 or 70	1–10
Immediately place on ice			
End repair	1	37	Hold
	2	37	20
	3	70	4
	4	4	Hold
Poly(A) tailing	1	16	Hold
	2	16	5
	3	70	2
	4	4	Hold
Continuous synthesis	1	34	Hold
	2	34	60
	3	4	Hold

\* Use a heated lid set to 105°C and set the sample volume to 50  $\mu$ l.

### Fragmentation Step

**Note:** Thaw Reagent A (red cap) on ice. Pulse vortex and quickly centrifuge to collect any droplets from the sides or tops of the tubes. Store on ice until step 4.

- Preheat thermal cycler to 94 or 70°C and hold at that temperature.
- Prepare 1  $\mu$ g–1 ng of total RNA if depleting rRNA using the SEQuoia RiboDepletion Kit after library construction. Alternatively, prepare 1  $\mu$ g–100 pg of depleted RNA if using a pre-library depletion method.
- Bring sample volume to 16  $\mu$ l by adding RNase-free water and transfer to a 0.2 ml PCR tube or strip tube.
- Add 2  $\mu$ l of Reagent A Fragmentation Mix (red cap) to each sample.
- Mix the reactions thoroughly by vortexing 5–10 sec. Centrifuge briefly to collect the reactions at the bottom of the tubes.

- Incubate the reactions at 94°C for an empirically determined period of time to achieve the desired fragment size. Use the recommendations in Table 3 as an initial guide if using total RNA as input sample and the SEQuoia RiboDepletion Kit for post-library depletion. Use the recommendation in Table 4 if using depleted RNA as the input library with optional pre-library depletion method.

**Important:** Fragmentation condition should be used as a guideline and should be optimized by the user to avoid under- or overfragmentation. Depletion of rRNA-derived fragments from a library is most efficient when insert size is >300 bp. Therefore, shorter incubation times will be required if depleting rRNA after library construction.

- Immediately place the reactions on ice for 2–5 min.

**Table 3. Incubation recommendation for fragmentation step when using total RNA.**

RNA Integrity Number	Desired Mean Insert Size, bp	Fragmentation Protocol	
		Temperature, °C	Time, min
>7	>300	94	2–3
2–7	100–300*	94	Up to 1
<2	100–200*	70	Up to 1

\* For optimal post-library ribodepletion efficiency, the mean insert size should be >300 bp.

**Table 4. Incubation recommendation for fragmentation step when using pre-depleted RNA.**

RNA Integrity Number	Desired Mean Insert Size, bp	Fragmentation Protocol	
		Temperature, °C	Time, min
>7	100–200	94	10
	200–300	94	8
	300–400	94	5
	400–600	94	3
2–7	100–300	94	1–3
<2	100–200	70	Up to 1

### End Repair Step

- Preheat thermal cycler to 37°C and hold at that temperature.
- Add 2 µl Reagent B End Repair Enzyme (yellow cap) to each reaction tube.
- Mix the reactions thoroughly by vortexing 5–10 sec. Centrifuge briefly to collect the reactions at the bottom of the tubes.
- Incubate the reactions in a thermal cycler using the end repair protocol (Table 2).
- Immediately place the reactions on ice for 2–5 min.

### Poly(A) Tailing Step

Due to reagent viscosity and the low volume of Reagent D Poly(A) Polymerase required, it is recommended that a master mix be made and immediately dispensed into the reaction tube. Do not store the master mix at 4°C; the enzyme will lose activity over time.

- Preheat thermal cycler to 16°C and hold at that temperature.
- Prepare enough poly(A) master mix for all reactions + 10% to account for pipetting loss (Table 5).

**Table 5. Poly(A) master mix.**

Reagent	Volume per Reaction, µl
Reagent C	23.75
Poly(A) Buffer (violet cap)	
Reagent D	1.25
Poly(A) Polymerase (blue cap)	

- Add 25 µl poly(A) master mix to each reaction.
- Mix the reactions thoroughly by gently vortexing for 5–10 sec. Centrifuge briefly to collect the reactions at the bottom of the tubes.
- Immediately after mixing, incubate the reactions in a thermal cycler using the poly(A) tailing protocol (Table 2).
- Immediately place the reactions on ice for 2–5 min.

### Continuous Synthesis Step

**Note:** Remove Reagent E (orange cap) from –20°C just before use in step 2. Pulse vortex and quickly centrifuge to collect any droplets from the sides or tops of the tubes. Store on ice during use.

- Preheat thermal cycler to 34°C and hold at that temperature.
- Add 5 µl of Reagent E SEQzyme Mix (orange cap) to each reaction tube.
- Mix the reactions thoroughly by vortexing 5–10 sec. Centrifuge briefly to collect the reactions at the bottom of the tubes.
- Incubate the reactions in a thermal cycler using the continuous synthesis protocol (Table 2).

**Note:** This is a safe stopping point. Store reactions at 4°C overnight or at –20°C if storing for longer than 24 hr.

**cDNA Purification**

**Note:** Allow the Purification Beads (gray cap) to come to room temperature before use.

1. Vortex the Purification Beads to resuspend them in solution.
2. Add 20 µl of beads to each reaction tube.
3. Mix well by pipetting up and down several times.
4. Incubate at room temperature for 20 min.
5. Place the tubes on a magnetic strip for 5–10 min until the solution is clear.
6. Transfer the supernatant containing the library to a fresh PCR tube or strip tube.

**Note:** This is a safe stopping point. Store the reactions at 4°C overnight or at –20°C if storing for longer than 24 hr.

**cDNA Concentration**

The SPRIselect Reagent cleanup protocol is a guideline and can be customized to purify RNAs of specific sizes. For example, the ratio of beads to reaction mix can be changed to capture only small RNA or exclusively large RNA.

**Note:** Allow the SPRIselect Reagent to come to room temperature before use.

1. Add 112 µl (1.6x) of SPRIselect Reagent to the supernatant collected at the end of the cDNA purification step.
  2. Mix well by pipetting up and down several times.
  3. Incubate at room temperature for 5–8 min.
  4. Centrifuge briefly to collect the reactions at the bottom of the tubes.
  5. Place the tubes on a magnetic strip for 5–10 min until the solution is clear.
- Note:** Keep the tubes on the magnetic strip through step 10.
6. Aspirate the cleared solution and discard it.
  7. Wash the beads by dispensing 200 µl of freshly prepared 80% ethanol to each tube and incubating for 30 sec.
  8. Carefully aspirate off the ethanol and discard it.
  9. Repeat steps 7 and 8.
  10. Allow the beads to air dry at room temperature by leaving them on the benchtop with the lids open for 1–4 min.

**Caution:** Do not let the beads overdry and do not dry at elevated temperatures. Doing so will result in poor sample recovery.

11. Remove tubes from the magnetic strip.
12. Add 24 µl of 10 mM Tris, pH 8.0, to each tube.
13. Mix well by pipetting up and down several times.

14. Incubate the tubes at room temperature for 2 min.
15. Centrifuge briefly to collect the reactions at the bottom of the tubes.
16. Place the tubes on a magnetic strip for 5–10 min until the solution is clear.
17. Transfer 22.5 µl of the supernatant containing the cDNA library to a fresh PCR tube or strip tube.

**Note:** This is a safe stopping point. Store the reactions at 4°C overnight or at –20°C if storing for longer than 24 hr.

**Library Amplification (Indexing PCR)**

Sample indices are used to distinguish pooled libraries from each other after sequencing. SEQuoia Dual Indexed Primers are available separately as a set of 12 ready-to-use unique dual indexed oligos in vials (#12011928) or as 96 unique dual indexed oligos dispensed to a PCR plate (#12011930). Refer to the SEQuoia Dual Indexed Primers Instruction Manual for index sequence information (10000119322).

If using the individual dual indexed primers provided in vials, assemble on ice the library amplification reactions as described in Table 6.

**Note:** Thaw Reagent F Amplification Mix (green cap) on ice. Pulse vortex and quickly centrifuge to collect any droplets from the sides or tops of the tubes. Store on ice until amplification reaction setup (Table 6).

**Table 6. Amplification reaction setup.**

Component	Volume, µl
SEQuoia Dual Indexed Primers	2.5
Reagent F Amplification Mix (green cap)	25
cDNA library	22.5

If using the SEQuoia Dual Indexed Primers Plate, add the cDNA library and amplification mix directly to the wells of a 96-well PCR plate. Using a multichannel pipet, pierce the foil seal of the SEQuoia Dual Indexed Primers Plate and transfer 2.5 µl from each well to the corresponding wells on the sample 96-well PCR plate.

1. Mix well by pipetting up and down several times.
2. Spin the tubes or plate to remove any air bubbles and collect the reaction mixture at the bottom of the wells.
3. Amplify the library using the thermal cycling protocol in Table 7.

**Table 7. Amplification protocol for thermal cycler.**

Step	Temperature, °C	Time, sec	PCR Cycles
Denaturation	95	180	1
Amplification	98	20	See Table 8
	61	15	
	72	20	
Final extension	72	60	1
Hold	4	Hold	1

**Note:** Overamplification can result in artifacts such as amplification bias, increased duplicate rates, and chimeras. To minimize artifacts, optimize the number of PCR amplification cycles so that the final library concentration is between 1 and 20 ng/ $\mu$ l. The number of PCR cycles is dependent upon the amount and quality of input RNA. Table 8 provides general guidelines and can be used as a starting point for optimization. Typically, the SEQuoia Complete Stranded RNA Library Prep Kit requires three to five fewer cycles compared to other commercially available kits, due to a high conversion rate.

**Table 8. PCR cycle recommendations.**

Input RNA	Number of PCR Cycles
1 $\mu$ g	6
100 ng	9
10 ng	12
1 ng	15
100 pg	18

### Postamplification Cleanup

**Note:** Allow the SPRIselect Reagent to come to room temperature before use.

1. Add 60  $\mu$ l (1.2x) of SPRIselect Reagent to 50  $\mu$ l of the library amplification reaction.
2. Mix well by pipetting up and down several times.
3. Incubate at room temperature for 5–8 min.
4. Centrifuge briefly to collect the reactions at the bottom of the tubes.
5. Place the tubes on a magnetic strip for 5–10 min until the solution is clear.
6. Aspirate the cleared solution and discard it.
7. Wash the beads by dispensing 200  $\mu$ l of freshly prepared 80% ethanol to each tube and incubating for 30 sec.
8. Carefully aspirate off the ethanol and discard it.

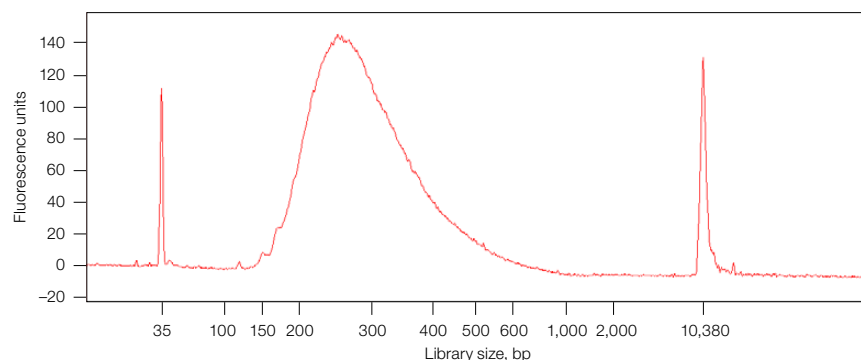
9. Repeat steps 7 and 8.
10. Allow the beads to air dry at room temperature by leaving them on the benchtop with the lids open for 1–4 min.
 

**Caution:** Do not let the beads overdry and do not dry at elevated temperatures. Doing so will result in poor sample recovery.
11. Add 20  $\mu$ l of 10 mM Tris, pH 8.0, to each tube.
12. Mix well by pipetting up and down several times.
13. Incubate the tubes at room temperature for 2 min.
14. Centrifuge briefly to collect the reactions at the bottom of the tubes.
15. Place the tubes on a magnetic strip for 5–10 min until the solution is clear.
16. Transfer 18  $\mu$ l of supernatant to a fresh PCR tube.
17. Store the reactions at 4°C overnight or at –20°C if storing for longer than 24 hr.

### Assess Quality and Quantity of Library Preparation

To achieve the highest-quality sequencing data, it is important to accurately quantify libraries to create optimum cluster densities. It is also imperative to assess the quality of the library to confirm insert size and minimal adapter-adapter products. For highly precise quantification and qualification of a library preparation, use the ddPCR Library Quantification Kit for Illumina TruSeq (#1863040). This kit enables the assessment of library quality by viewing fluorescence amplitude plots of droplet populations that discern features such as well-constructed libraries and adapter-adapter species.

Alternatively, use Qubit or a qPCR library quantification kit to assess library concentration and a bioanalyzer to assess size distribution (Figure 1). These methods, however, are subject to bias due to nonspecificity.



**Fig. 1. Example of bioanalyzer trace following use of SEQuoia Complete Stranded RNA Library Prep Kit.** Human placental RNA (100 ng) was run on an Agilent 2100 Bioanalyzer System DNA chip. Average library size: 300–400 bp.

## Deplete rRNA from an RNA-Seq Library Using SEQuoia RiboDepletion Kit

To achieve optimal depletion efficiency, quantify and assess the average fragment size of the library preparation reaction prior to starting the depletion protocol. The average fragment size should be greater than 380 bp and the input amount should be 0.1–20 ng of library cDNA. If depleting multiple libraries in a single ribodepletion reaction, combine equimolar ratios of the indexed libraries so that the final quantity of total cDNA library fragments does not exceed 20 ng per ribodepletion reaction. Refer to the SEQuoia RiboDepletion Kit product insert for complete details (10000132295).

### Sequencing Recommendations

- A variation of paired-end sequencing is recommended: sequence 50–150 bp from the 5' end (the R1 read) and 8 bp from the 3' end (the R2 read) of each fragment. The R2 read will contain an 8-base random sequence that can be used as a unique molecular identifier (UMI), which enables the identification of PCR duplicates. Sequencing beyond the 8-base UMI will yield little information due to the presence of the poly(A) tail that was artificially added as part of the library preparation. In addition, sequencing beyond the UMI will degrade the R2 sequencing quality score. Even though the sequence is generated from both ends of each library fragment, only the R1 read will align to the reference genome, which should be sufficient for full transcript coverage
- Alternatively, single-end sequencing can be performed if the use of the 8 bp UMI (the R2 read) is not desired. In this case, sequence 50–150 bp from the R1 read
- Recommended read length: R1 reads 75 bp in length are typical but can be adjusted for differential gene expression analysis. For example, R1 reads 50 bp in length for small RNA or R1 reads 150 bp in length when studying larger RNA
- Recommended read depth: ideal read depth should be determined empirically. Because libraries constructed using the SEQuoia Complete Stranded RNA Library Prep Kit are more complex due to the presence of small RNA fragments, the ideal read depth will likely be up to 40% greater than what is required for libraries that are composed solely of long RNA biotypes. Typically, 20–30 million reads per sample are ideal for the whole transcriptome coverage
- Go to [bio-rad.com/SEQDIP](https://bio-rad.com/SEQDIP) for information about dual indexed primer sequences

## Alignment and Analysis Guidelines

The SEQuoia Complete Stranded RNA Library Prep Kit captures both long and short RNA. That being so, secondary analysis requires a novel solution to simultaneously process both short and long RNA reads.

There are three main steps to processing and analyzing SEQuoia Complete Stranded RNA data:

### FASTQ Preprocessing

- Trimming of SEQuoia Complete specific poly(A) tails
- Optional quality trimming

### Alignment

- Optional UMI-based PCR deduplication
- Single-pass alignment of all reads, both short and long RNA, to a conjoined annotation set of the transcriptome and known microRNAs using the STAR aligner

### Feature Counting

- Assignment of aligned reads to exons
- Single output for all mapped reads with raw and normalized counts (transcripts per million [TPM], reads per kilobase of transcript per million mapped reads [RPKM], fragments per kilobase of transcript per million mapped fragments [FPKM])

Bio-Rad offers two streamlined bioinformatic workflow options that ensure consistent, high-quality data analysis. The parameters and settings of both options are identical.

#### Option 1: Web-Based Platform

[SeqSense.bio-rad.com](https://SeqSense.bio-rad.com)

This secure, web-based environment provides an integrated preconfigured data analysis workflow that allows users to perform complex analyses with only a few mouse clicks and to visualize and further interrogate their results.

#### Option 2: Docker Container

[github.com/BioRadOpenSource/SEQuoia-Complete](https://github.com/BioRadOpenSource/SEQuoia-Complete)

A command line application packaged with required accessories, such as libraries and other dependencies, is available to run on your infrastructure (cloud or on-premises).

### Trimming Guidelines

A read trimming step is recommended before proceeding with any quality metric calculations or downstream analyses. The SEQuoia Complete Kit adapters contain a single cytosine nucleotide base that appears immediately upstream of the insert and a poly(A) sequence immediately following the insert. To trim the cytosine base and the poly(A) tail, use Cutadapt, which runs on Linux, macOS, and Windows.

[cutadapt.readthedocs.io/en/stable/guide.html](https://cutadapt.readthedocs.io/en/stable/guide.html)

Work with the reads in a FASTQ file (compressed or uncompressed). The reads can be trimmed by running Cutadapt from the command line: `cutadapt -u 1 -a A{10} -m 15 -o output_file.fastq.gz input_file.fastq.gz` (see Table 9 for description of the command line).

**Table 9. Command line.**

Component	Description
-u 1	Directs Cutadapt to trim the first base (5') of the read
-a A{10}	Directs Cutadapt to trim any poly(A) track and all following bases in the read. The poly(A) track must be at least 10 bases long (unless it appears truncated at the end of the read) and contain no more than 1 error (that is, a nonadenine base). Poly(A) removal is important
-m 15	Removes reads from the FASTQ file that are shorter than 15 bases after trimming

### Library Molecular Structure

The SEQuoia Complete Stranded RNA Kit libraries will retain strand information (see example in Figure 2). The second strand should be used during alignment and analysis.



**Fig. 2. SEQuoia Complete Stranded RNA Kit library.**

### Assessment of Residual rRNA

Use SeqSense NGS Data Analysis Software to process the FASTQ file and generate a SEQuoia Analysis Report. The percentage of rRNA reads present in the dataset can be calculated by dividing the **Ribosomal Bases by PF Bases** and multiplying by 100. Ribosomal Bases and PF Bases are both reported in the Alignment section of the SeqSense Analysis Report.

### Quality Control

The SEQuoia Complete Stranded RNA Library Prep Kit undergoes extensive quality control testing to ensure functionality and lot-to-lot consistency. This product is free of detectable contaminants, including DNase and RNase activities.

### Bio-Rad Technical Support

The Bio-Rad Technical Support department in the U.S. is open Monday through Friday, 5:00 AM to 5:00 PM, Pacific time.

Phone: 1-800-424-6723, option 2

Email: [support@bio-rad.com](mailto:support@bio-rad.com) (U.S./Canada only)

For technical assistance outside the U.S. and Canada, contact your local technical support office or click

**Contact us at [bio-rad.com](http://bio-rad.com).**

### Related Products

Catalog #	Description
17006487	<b>SEQuoia RiboDepletion Kit</b> , 24 reactions
12011928	<b>SEQuoia Dual Indexed Primers Set</b> , 12 vials of unique dual indexed primers, 96 reactions
12011930	<b>SEQuoia Dual Indexed Primers Plate</b> , 96-well plate of unique dual indexed primers, 96 reactions
1863040	<b>ddPCR Library Quantification Kit for Illumina TruSeq</b>

Visit [bio-rad.com/SEQuoiaComplete](http://bio-rad.com/SEQuoiaComplete) for more information.

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