

SEQuoia Express Stranded RNA Library Prep Kit

Catalog #	Description
12017297	SEQuoia Express Stranded RNA Library Prep Kit, 24 reactions
12017265	SEQuoia Express Stranded RNA Library Prep Kit, 96 reactions

For research purposes only.

Introduction

The SEQuoia Express 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. Driven by a carefully balanced adapters-to-enzyme ratio, the enzyme associates an adapter with a unique molecular identifier and engages with the 3' end of an RNA molecule to initiate cDNA synthesis in a process referred to as template jumping. When the enzyme reaches the 5' end of the RNA molecule, it jumps to the 5' adapter and completes the cDNA synthesis. The end product is a dual-end adapter-tagged cDNA library. This kit captures long RNA transcripts, including mRNA and long noncoding transcripts that are >200 bp.

All kit components are optimized to facilitate efficient library synthesis through the input of a wide range of high-quality total RNA (1 ng–1 µg). The streamlined three-tube workflow is automation friendly and enables high-throughput library construction in less than 3 hours (Figure 1). The kit does not include RNA or the solid phase reversible immobilization (SPRI) beads needed for the purification step.

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

- Differential gene expression profiling of mRNA and long noncoding RNAs
- Gene ontology
- Single nucleotide variation discovery
- Gene fusion identification
- Splice junction identification

Storage and Stability

The SEQuoia Express Stranded RNA Library Prep Kit is guaranteed for 12 months after the shipping date if stored at –20°C (see Table 1). The kit components have been tested to perform per specifications for as many as six freeze-thaw cycles.

Table 1. Kit contents.

Component	Cap Color	24 Reactions, µl	96 Reactions, µl	Storage Temperature
SEQuoia Express Reaction Mix	Violet	600	2 x 1,200	–20°C
SEQuoia Express Enzyme Mix	Orange	120	480	
SEQuoia Express Amplification Mix	Green	600	2 x 1,200	

Library Preparation Workflow

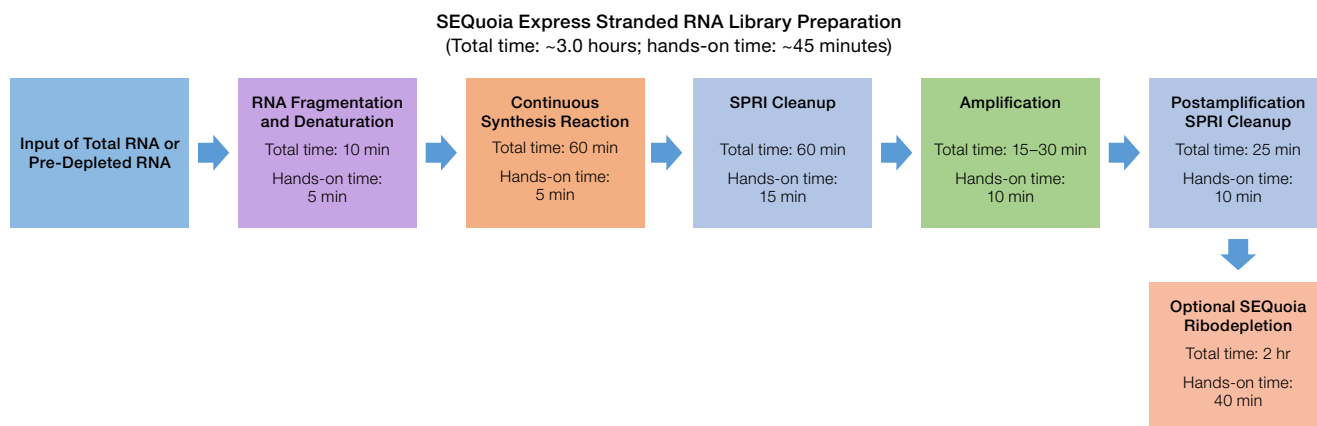


Fig 1. The entire SEQuoia Express library preparation workflow can be completed in less than 3 hours and generates sequence-ready libraries for sequencing on Illumina platforms.

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 Dual Indexed Primers Set (12 vials of unique dual indexed primers, catalog #12011928) or SEQuoia Dual Indexed Primers Plate (96-well plate of unique dual indexed primers, #12011930)
- SEQuoia RiboDepletion Kit, 24 reactions (#17006487)
- 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 μ l
- SPRIselect Reagent (Beckman Coulter, Inc., #B23318)
- Low TE Buffer, pH 8.0 (for example, IDTE Buffer, Integrated DNA Technologies, Inc., #11050109)
- Nondenatured 80% ethanol (prepare fresh)
- ddPCR Library Quantification Kit for Illumina® TruSeq® (#1863040)
- Agilent RNA 6000 Nano or Pico Kit (Agilent Technologies, Inc., #5067-1513 or 5067-1511)
- Agilent High Sensitivity DNA Kit (Agilent Technologies, #5067-4626)
- Qubit Assay Tubes (Thermo Fisher Scientific Inc., #Q32856)
- Qubit 1X dsDNA Assay Kit, high sensitivity (Thermo Fisher Scientific, #Q33231)
- DEPC-treated water

Equipment List*

- C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module (#1851197), or equivalent, for accurate incubation temperatures
- CFX96 Touch Deep Well Real-Time PCR System (#1854095)
- Calibrated single-channel and multichannel pipets: 10, 20, and 200 μ l
- NEBNext Magnetic Separation Rack (New England Biolabs, Inc., #S1515S)
- Vortexer
- Microcentrifuge for 0.2 ml tubes
- 96-well PCR cooling rack
- Agilent 2100 Bioanalyzer System (Agilent Technologies)
- Qubit 4 Fluorometer (Thermo Fisher Scientific, #Q33238)
- Ice bucket

* All products can be obtained from Bio-Rad Laboratories, Inc. unless otherwise noted.

Important Considerations — Please Read Before Starting

- Confirm all kit components are present and the required consumables and equipment, including the SPRI beads and unique dual indexed primers, are available
- Thaw all reagents on ice and set up reactions on ice unless otherwise noted
- For total RNA extraction, PureZOL RNA Isolation Reagent (Bio-Rad, #7326880) is recommended
- 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
- Avoid multiple freeze-thaw cycles of input RNA to preserve the integrity of RNA
- DNase treatment to remove genomic DNA from the starting material is recommended
- Use a thermal cycler for each incubation step to ensure thermal accuracy. Program the thermal cycler with each of the protocols prior to starting library preparation
- Prepare fresh 80% ethanol for SPRI cleanup
- Assess the quality of the input RNA by running the RNA sample on an Agilent RNA 6000 Nano or Pico Chip to determine the RNA integrity number (RIN ≥ 7 indicates high-quality intact RNA and is recommended for the best results) or an Agilent Bioanalyzer or TapeStation System to determine DV₂₀₀

Note: DV₂₀₀ represents the percentage of the RNA fragments that are larger than 200 nucleotides.

- Perform post-library ribodepletion using the SEQuoia RiboDepletion Kit. Alternatively, ribosomal RNA (rRNA) can be depleted from total RNA prior to library construction
- 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
- When multiplexing samples together for sequencing, note the indices used for each sample and make sure the indices used in a pool are unique for demultiplexing
- There are several safe stopping points, which are identified in the protocol
- Always use the most current version of this product insert, which can be downloaded at [bio-rad.com/SEQExpress](https://www.bio-rad.com/SEQExpress)

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
Fragmentation	1	98	Hold
	2	98	8 min
	Immediately remove and place on ice for 2 min		
Continuous synthesis	1	30	Hold
	2	30	60 min
	3	4	Hold
Amplification	1	98	Hold
	2	98	1 min
	3	98	20 sec
	4	61	15 sec
	5	72	20 sec
	6	Repeat steps 3–5 n times (Refer to Tables 7 and 8 for number of PCR cycles [n])	
	7	72	1 min
	8	4	Hold

* Use a heated lid set to 105°C and set the sample volume to 50 µl.

Fragmentation Step

Note: Thaw the SEQuoia Express Reaction Mix (violet 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.

1. Preheat the thermal cycler to 98°C and hold at that temperature.
2. Prepare 1 ng–1 µg of high-quality total RNA or pre-ribodepleted RNA.
3. Bring the sample volume to 20 µl by adding RNase-free water and transfer to a 0.2 ml PCR tube or strip tube (Table 3).
4. Add 25 µl of the SEQuoia Express Reaction Mix (violet cap) to the input sample (Table 3).
5. Pulse vortex the reactions. Centrifuge briefly to collect the reactions at the bottom of the tubes. Place the tubes on ice.
6. Incubate the reactions at 98°C for 8 min (200–300 bp fragment size).

Note: This recommendation is for highly intact RNA (RIN ≥7). The fragmentation conditions should be optimized for degraded samples.
7. Immediately place the reactions on ice for 2 min.
8. Proceed to Continuous Synthesis Step.

Table 3. Fragmentation reaction setup.

Component	Volume, µl
Input RNA sample	20
SEQuoia Express Reaction Mix (violet cap)	25
Total volume	45

Continuous Synthesis Step

Note: Remove the SEQuoia Express Enzyme Mix (orange cap) from –20°C storage 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.

1. Preheat the thermal cycler to 30°C and hold at that temperature.
2. Add 5 µl of SEQuoia Express Enzyme Mix (orange cap) to each reaction tube (Table 4).
3. Pulse vortex the reactions. Centrifuge briefly to collect the reactions at the bottom the tubes.
4. Incubate the reactions in a thermal cycler at 30°C for 60 min.

Note: This is a safe stopping point. Optional: Store the reactions at –20°C overnight.

Table 4. Continuous synthesis reaction setup.

Component	Volume, µl
Fragmentation reaction	45
SEQuoia Express Enzyme Mix (orange cap)	5
Total volume	50

SPRI Cleanup (I)

Note: Thoroughly mix and resuspend SPRIselect Reagent before use.

1. Add 60 µl (1.2x) of SPRIselect Reagent to 50 µl of each continuous synthesis reaction.
2. Mix well by pipetting up and down five to ten times or seal the tubes and vortex.
3. Incubate at room temperature for 5 min.
4. Briefly centrifuge to collect the contents to bottom of the tubes and place on a magnetic rack for 5 min or until the solution is clear.

Note: Keep the tubes on the magnetic rack until step 10.
5. Carefully aspirate and discard the clear solution without disturbing the beads.
6. Wash the beads by dispensing 200 µl of freshly prepared 80% ethanol to each tube and incubating for 30 sec.
7. Carefully aspirate off the ethanol and discard it.
8. Repeat steps 6 and 7 for a total of two ethanol washes.
9. Allow the beads to air dry at room temperature by leaving them on the benchtop with the lids open for 1–3 minutes, until the ethanol has completely evaporated. Use a 20 µl pipet to remove any residual ethanol.

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

10. Remove the tubes from the magnetic rack.
11. Resuspend the pellet in 30 µl of Low TE Buffer.
12. Mix well by pipetting up and down five to ten times.
13. Incubate the tubes at room temperature for 2 min.
14. Quickly centrifuge to collect any droplets from the sides or tops of the tubes.
15. Place the tubes on a magnetic rack for 5 min or until the solution is clear.
16. Transfer 28 µl of the supernatant to a fresh PCR tube or strip tube.

SPRI Cleanup (II)

1. Add 33.6 µl (1.2x) of SPRIselect Reagent to 28 µl of the supernatant.
2. Mix well by pipetting up and down five to ten times or seal the tubes and vortex.
3. Incubate at room temperature for 5 min.
4. Briefly centrifuge to collect the contents to bottom of the tubes and place on a magnetic rack for 5 min or until the solution is clear.

Note: Keep the tubes on the magnetic rack until step 10.

5. Carefully aspirate and discard the clear solution without disturbing the beads.
6. Wash the beads by dispensing 200 µl of freshly prepared 80% ethanol to each tube and incubating for 30 sec.
7. Carefully aspirate off the ethanol and discard it.
8. Repeat steps 6 and 7 for a total of two ethanol washes.
9. Allow the beads to air dry at room temperature by leaving them on the benchtop with the lids open for 1–3 minutes. Use a 20 µl pipet to remove any residual ethanol.

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

10. Remove the tubes from the magnetic rack.
11. Resuspend the pellet in 25 µl of Low TE Buffer.
12. Mix well by pipetting up and down five to ten times.
13. Incubate the tubes at room temperature for 2 min.
14. Quickly centrifuge to collect any droplets from the sides or tops of the tubes.
15. Place the tubes on a magnetic rack for 5 min or until the solution is clear.
16. Transfer 22.5 µl of the supernatant 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)

Note: Thaw the SEQuoia Express 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 5).

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 Product Insert for index sequence information (1000011932; bio-rad.com/SEQDIP).

If using the individual dual indexed primers provided in vials, assemble the library amplification reactions on ice as described in Table 5.

Table 5. Amplification reaction setup.

Component	Volume, µl
SEQuoia Dual Indexed Primers	2.5
SEQuoia Express Amplification Mix (green cap)	25
cDNA library sample	22.5
Total volume	50

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. Seal and pulse vortex the reactions.
2. Spin the tubes or plate to remove any air bubbles and collect the reaction mixture at the bottom of the wells.
3. Set the heated lid to 105°C. Amplify the library using the thermal cycling protocol in Table 6.

Table 6. Amplification protocol for thermal cycler.

Step	Temperature, °C	Time, sec	PCR Cycles
Denaturation	98	60	1
Amplification	98	20	See Tables 7 and 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/µl. The number of PCR cycles is dependent upon the amount and quality of input RNA. Tables 7 and 8 provide general guidelines and can be used as starting points for optimization.

Table 7. PCR cycle recommendations if input for library construction is total RNA.

Input Total RNA	Number of PCR Cycles
1 µg	7
100 ng	10
10 ng	13
1 ng	16

Table 8. PCR cycle recommendations if input is enriched mRNA or pre-ribodepleted RNA.

Input Enriched mRNA or Pre-Ribodepleted RNA*	Number of PCR Cycles
1 µg	10
100 ng	13
10 ng	16
1 ng	19

* Initial input total RNA that is used for the pre-ribodepletion workflow.

Postamplification Cleanup

Note: Thoroughly mix and resuspend SPRIselect Reagent before use.

1. Add 45 µl (0.9x) of SPRIselect Reagent to 50 µl of the library amplification reaction.
2. Mix well by pipetting up and down five to ten times or seal the tubes and vortex.
3. Incubate at room temperature for 5 min.
4. Briefly centrifuge to collect the contents to bottom of the tubes and place on a magnetic rack for 5 min or until the solution is clear.

Note: Keep the tubes on the magnetic rack until step 10.

5. Carefully aspirate and discard the clear solution without disturbing the beads.
6. Wash the beads by dispensing 200 µl of freshly prepared 80% ethanol to each tube and incubating for 30 sec.
7. Carefully aspirate off the ethanol and discard it.
8. Repeat steps 6 and 7 for a total of two ethanol washes.
9. Allow the beads to air dry at room temperature by leaving them on the benchtop with the lids open for 1–3 minutes. Use a 20 µl pipet to remove any residual ethanol.

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

10. Remove the tubes from the magnetic rack.
11. Resuspend the pellet in 20 µl of Low TE Buffer.
12. Mix well by pipetting up and down five to ten times.
13. Incubate the tubes at room temperature for 2 min.
14. Quickly centrifuge to collect any droplets from the sides or tops of the tubes.

15. Place the tubes on a magnetic rack for 5 min or until the solution is clear.
16. Transfer 18 µl of the supernatant to a fresh PCR tube or strip tube.
17. Store the libraries at 4°C overnight or at –20°C if storing for longer than 24 hr.

Assess Quality and Quantity of Final Library

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 (Agilent High Sensitivity DNA Chip running in the Agilent 2100 Bioanalyzer System [Figure 2]). These methods, however, are subject to bias due to nonspecificity.

For SEQuoia Express Libraries constructed using total RNA, proceed to the SEQuoia RiboDepletion Kit Product Insert (10000132295; bio-rad.com/SEQRiboDepletion).

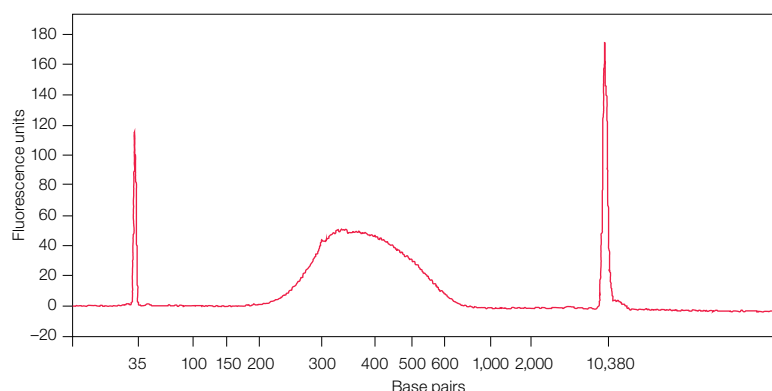


Fig. 2. Example of SEQuoia Express library bioanalyzer trace. Human placental RNA (100 ng) with post-library ribodepletion, carried out with the SEQuoia RiboDepletion Kit, was run on an Agilent 2100 Bioanalyzer System. Average library size: 370 bp.

Sequencing and Multiplexing Recommendations

- Sequencing depth and read length should be determined based on application and prior experience. Standard whole transcriptome (>200 bp) experiments range from 15 to 30 million paired-end reads/sample
- SEQuoia dual indexed primer sequences for the sample sheet can be obtained from the product insert (1000011932; bio-rad.com/SEQDIP). Read 1 (R1) corresponds to the sense strand (Figure 3)
- The first 8 bases on read 2 (R2) consist of a random tag sequence that can be used as a unique molecular identifier (UMI) for identification of PCR duplicates (Figure 3)

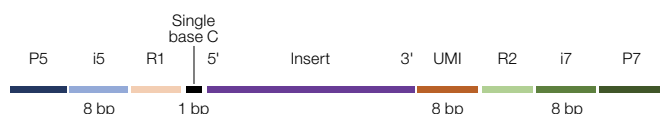


Fig. 3. Final library structure.

Secondary Data Analysis

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

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: Analysis Toolkit

github.com/digitalbiology/Sequoia_express_toolkit

To use the toolkit, a virtual environment is required to run the software. Access the GitHub link for detailed instructions on setup for your infrastructure (cloud or on-premises).

Quality Control

The SEQuoia Express 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. Go to bio-rad.com/en-us/life-science-research/support/certificate-of-analysis for a certificate of analysis.

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 (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.

Related Products

Catalog #	Description
Consumables	
12011928	SEQuoia Dual Indexed Primers Set , 12 vials of unique dual indexed primers, 96 reactions
12011930	SEQuoia Dual Indexed Primers Plate , 96-well plate of unique dual indexed primers, 96 reactions
17006487	SEQuoia RiboDepletion Kit , 24 reactions
1863040	ddPCR Library Quantification Kit for Illumina TruSeq
TBC0802	0.2 ml 8-Tube PCR Strips and Domed Cap Strips , high profile, clear
TBC1202	0.2 ml 12-Tube PCR Strips and Domed Cap Strips , high profile, clear
Instruments	
1851197	C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module
1854095	CFX96 Touch Deep Well Real-Time PCR System

Visit bio-rad.com/SEQuoiaExpress for more information.

BIO-RAD and DDPCR are trademarks of Bio-Rad Laboratories, Inc. in certain jurisdictions. All trademarks used herein are the property of their respective owner.