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Invitrogen™ Collibri™ Stranded RNA Library Prep Kit for Illumina™ with Collibri™ H/M/R rRNA Depletion Kit

USER GUIDE

for use with Illumina™ next generation sequencing (NGS) platforms

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ThermoFisher SCIENTIFIC

Manufacturer: Thermo Fisher Scientific Baltics UAB | V. A. Graiciuno 8 | LT-02241 Vilnius, Lithuania

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Revision history: MAN0017584

Revision	Date	Description
C.0	07 January 2020	Update protocol in "Methods" with details for library prep of ~300 bp inserts, add sequences of unique dual indices in "Kit contents and storage"
B.0	17 August 2018	Update the "Required materials" table, add the i5 index sequence needed to pool single index libraries with dual indexed libraries in "Sequence the prepared library".
A.0	07 May 2018	New user guide.

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1. Product information

Product description

Invitrogen™ Collibri™ Stranded RNA Library Prep Kit for Illumina™ with Collibri™ H/M/R rRNA Depletion Kit is designed for robust construction of cDNA libraries for strand-specific RNA sequencing on the Illumina™ NGS (next generation sequencing) platforms.

The Collibri™ Stranded RNA Library Prep Kit with Collibri™ H/M/R rRNA Depletion Kit supports fast library preparation within 6 hours for whole transcriptome libraries starting with 100 ng–1 µg of total human, mouse, or rat RNA. Multiplexing of libraries can be carried out using up to 96 single-indexed or unique dual-indexed primers, enabling single read or paired-end sequencing. For convenience, the kit provides color-coded components for visual tracking of library preparation progress. Inert dyes in the reagents do not interfere with enzymatic reactions and do not compromise library prep and sequencing results. The kit is supplied with rRNA depletion reagents that allow a comprehensive view of the transcriptome through efficient removal of ribosomal RNA.

The Collibri™ Stranded RNA Library Prep Kit with Collibri™ H/M/R rRNA Depletion Kit is suitable for various quality RNA samples, including FFPE.

Note: For an overview of the technology used in the Invitrogen™ Collibri™ Stranded RNA Library Prep Kit, see "Technology overview", page 12.

Kit contents and storage

Sufficient reagents for rRNA depletion, library construction, and library cleanup are supplied in the Collibri™ Stranded RNA Library Prep Kit with Collibri™ H/M/R rRNA Depletion Kit to prepare indexed cDNA libraries for 24 samples (Cat. No. A39003024, A39005024) or 96 samples (Cat. No. A39003096, A39005096).

Component	Cap color	Amount		Storage
		24 preps	96 preps	
Library Prep Kit				
10X Fragmentation Buffer	Blue	24 µL	96 µL	−20°C
RNase III	Yellow	24 µL	96 µL	
RNA End Repair Enzyme	Green	24 µL	96 µL	
Water, nuclease-free	White	1.25 mL	4 × 1.25 mL	
2X Adaptor Mix	Blue	240 µL	4 × 240 µL	
2X Ligation Buffer	Yellow	600 µL	4 × 600 µL	
10X Ligation Enzyme Mix	White	120 µL	4 × 120 µL	
2.5X RT Buffer	Red	960 µL	4 × 960 µL	
10X SuperScript™ IV Enzyme Mix	White	240 µL	4 × 240 µL	
2X Library Amplification Master Mix	Blue	600 µL	2 × 600 µL	
10X Index Primer Mix[1]	—	5 µL/well (24 wells)	5 µL/well (96 wells)	
Library Cleanup Kit				
Dynabeads™ Cleanup Beads	Orange	10 mL	40 mL	2°C to 8°C **IMPORTANT!** Do not freeze.
Wash Buffer (Concentrated)	Blue	4.5 mL	18 mL	
Elution Buffer	White	5 mL	20 mL	
H/M/R rRNA Depletion Kit				
Hybridization Solution	White	0.096 mL	0.384 mL	2°C to 8°C **IMPORTANT!** Do not freeze.
Probe Mix	White	0.120 mL	0.480 mL	
Depletion Beads	Yellow	1.8 mL	7.2 mL	
Conditioning Solution	Green	3.6 mL	14.4 mL	
Depletion Solution	Yellow	6.12 mL	24.48 mL	
Purification Beads	Orange	0.576 mL	2.304 mL	
Purification Solution	Blue	4.176 mL	16.704 mL	
Elution Solution	White	1.008 mL	4.032 mL	

[1] i7 indices pre-mixed with universal or indexed i5 PCR primer, which allow up to 24 or 96 samples to be multiplexed, are included in the kit (supplied in 10X Index Primer Mix Plate format). Each well in the 10X Index Primer Mix contains 5 µL of primer mix, sufficient for amplification/barcoding of one library. See "i7 index sequences and locations in the primer mix plate", page 4, for single indexing and "Unique dual index sequences and locations in the primer mix plate" for dual indexing, page 6.

i7 index sequences and locations in the primer mix plate

i7 index sequences (Table 1) and the location of the indices in the 10X Index Primer Mix Plate for 96 preps (Table 2) and 24 preps (Table 3) are listed in the following tables.

Table 1 Index sequences

Index	Sequence	Index	Sequence	Index	Sequence	Index	Sequence
IX001	ATGCCTAA	IX025	AACTCACC	IX049	ACGTATCA	IX073	AATGTTGC
IX002	GAATCTGA	IX026	GCTAACGA	IX050	GTCTGTCA	IX074	TGAAGAGA
IX003	AACGTGAT	IX027	CAGATCTG	IX051	CTAAGGTC	IX075	AGATCGCA
IX004	CACTTCGA	IX028	ATCCTGTA	IX052	CGACACAC	IX076	AAGAGATC
IX005	GCCAAGAC	IX029	CTGTAGCC	IX053	CCGTGAGA	IX077	CAACCACA
IX006	GACTAGTA	IX030	GCTCGGTA	IX054	GTGTTCTA	IX078	TGGAACAA
IX007	ATTGGCTC	IX031	ACACGACC	IX055	CAATGGAA	IX079	CCTCTATC
IX008	GATGAATC	IX032	AGTCACTA	IX056	AGCACCTC	IX080	ACAGATTC
IX009	AGCAGGAA	IX033	AACGCTTA	IX057	CAGCGTTA	IX081	CCAGTTCA
IX010	GAGCTGAA	IX034	GGAGAACA	IX058	TAGGATGA	IX082	TGGCTTCA
IX011	AAACATCG	IX035	CATCAAGT	IX059	AGTGGTCA	IX083	CGACTGGA
IX012	GAGTTAGC	IX036	AAGGTACA	IX060	ACAGCAGA	IX084	CAAGACTA
IX013	CGAACTTA	IX037	CGCTGATC	IX061	CATACCAA	IX085	CCTCCTGA
IX014	GATAGACA	IX038	GGTGCGAA	IX062	TATCAGCA	IX086	TGGTGGTA
IX015	AAGGACAC	IX039	CCTAATCC	IX063	ATAGCGAC	IX087	AACAACCA
IX016	GACAGTGC	IX040	CTGAGCCA	IX064	ACGCTCGA	IX088	AATCCGTC
IX017	ATCATTCC	IX041	AGCCATGC	IX065	CTCAATGA	IX089	CAAGGAGC
IX018	GCCACATA	IX042	GTACGCAA	IX066	TCCGTCTA	IX090	TTCACGCA
IX019	ACCACTGT	IX043	AGTACAAG	IX067	AGGCTAAC	IX091	CACCTTAC
IX020	CTGGCATA	IX044	ACATTGGC	IX068	CCATCCTC	IX092	AAGACGGA
IX021	ACCTCCAA	IX045	ATTGAGGA	IX069	AGATGTAC	IX093	ACACAGAA
IX022	GCGAGTAA	IX046	GTCGTAGA	IX070	TCTTCACA	IX094	GAACAGGC
IX023	ACTATGCA	IX047	AGAGTCAA	IX071	CCGAAGTA	IX095	AACCGAGA
IX024	CGGATTGC	IX048	CCGACAAC	IX072	CGCATACA	IX096	ACAAGCTA

Table 2 Location of i7 indices in 10X Index Primer Mix Plate for 96 preps

	1	2	3	4	5	6	7	8	9	10	11	12
A	IX001	IX009	IX017	IX025	IX033	IX041	IX049	IX057	IX065	IX073	IX081	IX089
B	IX002	IX010	IX018	IX026	IX034	IX042	IX050	IX058	IX066	IX074	IX082	IX090
C	IX003	IX011	IX019	IX027	IX035	IX043	IX051	IX059	IX067	IX075	IX083	IX091
D	IX004	IX012	IX020	IX028	IX036	IX044	IX052	IX060	IX068	IX076	IX084	IX092
E	IX005	IX013	IX021	IX029	IX037	IX045	IX053	IX061	IX069	IX077	IX085	IX093
F	IX006	IX014	IX022	IX030	IX038	IX046	IX054	IX062	IX070	IX078	IX086	IX094
G	IX007	IX015	IX023	IX031	IX039	IX047	IX055	IX063	IX071	IX079	IX087	IX095
H	IX008	IX016	IX024	IX032	IX040	IX048	IX056	IX064	IX072	IX080	IX088	IX096

Table 3 Location of i7 indices in 10X Index Primer Mix Plate for 24 preps

	1	2	3	4	5	6	7	8	9	10	11	12
A	IX001	IX009	IX017									
B	IX002	IX010	IX018									
C	IX003	IX011	IX019									
D	IX004	IX012	IX020									
E	IX005	IX013	IX021									
F	IX006	IX014	IX022									
G	IX007	IX015	IX023									
H	IX008	IX016	IX024									

Unique dual index sequences and locations in the primer mix plate

UD index sequences (Table 4) and the location of the indices in the 10X Index Primer Mix Plate for 96 preps (Table 5) and 24 preps (Table 6) are listed in the following tables.

Table 4 Unique dual (i7 and i5) index sequences

UDI Index primer mix name	i7 index	i5 index for entry on sample sheet (NovaSeq™, MiSeq™, HiSeq™ 2000/2500)	i5 index for entry on sample sheet (MiniSeq™, NextSeq™, HiSeq™ 3000/4000, HiSeq™ X) [1]
UDI001	CCTTCTAC	GGCGAATA	TATTCGCC
UDI002	GGTCGTAT	GTTGCATG	CATGCAAC
UDI003	CGTAGACA	TCGTAGAC	GTCTACGA
UDI004	ATGTCACG	AGACAGCT	AGCTGTCT
UDI005	CAAGAAGC	AGGTCTCA	TGAGACCT
UDI006	CACGGATA	CTGAACAG	CTGTTCAG
UDI007	ACAGGATG	ACGTTGTC	GACAACGT
UDI008	ACACAACC	GGCTCAAT	ATTGAGCC
UDI009	TGCTGACT	GACACAGA	TCTGTGTC
UDI010	ATCGGAGA	AGACACAG	CTGTGTCT
UDI011	ATTAGCGG	TGGTTCAC	GTGAACCA
UDI012	TAGCCACT	CGATGGAT	ATCCATCG
UDI013	AGCACACA	CCACAGAA	TTCTGTGG
UDI014	GTTAAGCG	TGTGTCAG	CTGACACA
UDI015	GGTTGGTT	ATGGCGAT	ATCGCCAT
UDI016	AACGCATG	TCGACGAA	TTCGTCGA
UDI017	TAGTCAGC	TAGGCTAC	GTAGCCTA
UDI018	ACTGATGC	ACACCTCT	AGAGGTGT
UDI019	CTATGTGG	GAATAGGC	GCCTATTC
UDI020	ATAGTCGG	GAATCAGG	CCTGATTC
UDI021	AAGTACGC	CGCATTAC	GTAATGCG
UDI022	CAACAGGT	CGCAACAT	ATGTTGCG
UDI023	GGATCACA	GATGGCAA	TTGCCATC
UDI024	ACTAAGCC	TACCGTGA	TCACGGTA
UDI025	AAGTGGCT	TCGTGCAT	ATGCACGA
UDI026	CCATCGTA	CGTGCTAA	TTAGCACG
UDI027	CGACAATC	AATGACGG	CCGTCATT
UDI028	GTTGGCTT	ACAAGAGC	GCTCTTGT
UDI029	AGTGAGGA	ACCGCTAT	ATAGCGGT
UDI030	TTCATGCG	AGTGCTGT	ACAGCACT

UDI Index primer mix name	i7 index	i5 index for entry on sample sheet (NovaSeq™, MiSeq™, HiSeq™ 2000/2500)	i5 index for entry on sample sheet (MiniSeq™, NextSeq™, HiSeq™ 3000/4000, HiSeq™ X) [1]
UDI031	TTATGGCC	GTACTAGC	GCTAGTAC
UDI032	TCGATGCT	CGGTCTAT	ATAGACCG
UDI033	CGTAACGA	ATCCACGA	TCGTGGAT
UDI034	CTGTATGC	GTCATCTG	CAGATGAC
UDI035	GTTACGGT	TGTCTAGC	GCTAGACA
UDI036	ACATGCCA	TCAACGGT	ACCGTTGA
UDI037	CTATACCG	CATCTCGA	TCGAGATG
UDI038	TATGGTCC	ATCGATGG	CCATCGAT
UDI039	TCAGGCTA	TGTAAGGC	GCCTTACA
UDI040	TAGTGGTG	CACATGGT	ACCATGTG
UDI041	CAGTGATC	CATGTTGG	CCAACATG
UDI042	ATCCGCTT	GCATCTGA	TCAGATGC
UDI043	GTTGTCGA	ATCCAACG	CGTTGGAT
UDI044	GTTCTTGG	ATTCTCGC	GCGAGAAT
UDI045	ATGCTGGT	GACGATGT	ACATCGTC
UDI046	GTGATCCA	TTGAGACG	CGTCTCAA
UDI047	GACAATCG	GTAGATGC	GCATCTAC
UDI048	GGTATTCC	GTGTGTGT	ACACACAC
UDI049	AGGACCTA	GCTACACA	TGTGTAGC
UDI050	CATTCGTG	AATCGACG	CGTCGATT
UDI051	GCTTCATC	TCACATGC	GCATGTGA
UDI052	ACACGTGA	CAGTCACA	TGTGACTG
UDI053	AGTCTTCG	TTAGAGCG	CGCTCTAA
UDI054	CGATACGT	ATGAGTGC	GCACTCAT
UDI055	CCTCATCA	GATTGGCA	TGCCAATC
UDI056	GGTTCTTG	CATCAACC	GGTTGATG
UDI057	AGAGCTTC	GAGAGACT	AGTCTCTC
UDI058	CTAACCGT	ATTGGCCA	TGGCCAAT
UDI059	TCCACTCA	GTATTGCG	CGCAATAC
UDI060	GCATGTTG	CTAAGACC	GGTCTTAG
UDI061	GCAACTTC	CCAACACT	AGTGTTGG
UDI062	TACGTCGT	TTGTTCCG	CGGAACAA
UDI063	GATGTCTG	TTCTCACC	GGTGAGAA
UDI064	TTGCGTTC	GAAGAGCT	AGCTCTTC
UDI065	TACTAGCG	TCCTTGGT	ACCAAGGA

UDI Index primer mix name	i7 index	i5 index for entry on sample sheet (NovaSeq™, MiSeq™, HiSeq™ 2000/2500)	i5 index for entry on sample sheet (MiniSeq™, NextSeq™, HiSeq™ 3000/4000, HiSeq™ X) [1]
UDI066	GCAGTTCA	TTACGTCG	CGACGTAA
UDI067	CACTTCTC	TTCCTTCC	GGAAGGAA
UDI068	AAGCCTGT	CTTCTGCT	AGCAGAAG
UDI069	TTGACCTG	CTCTCTCA	TGAGAGAG
UDI070	GATCCTCA	TGAAGGTG	CACCTTCA
UDI071	TCCAACTG	CACACATC	GATGTGTG
UDI072	TAGCTGTC	GAGCATCT	AGATGCTC
UDI073	CTAGACTC	TGGCTTCA	TGAAGCCA
UDI074	GCCAAGAA	ACTAGGTG	CACCTAGT
UDI075	TCACTCAC	ACCGTATC	GATACGGT
UDI076	TCTCAAGG	AACCGTCT	AGACGGTT
UDI077	ATGGTCAC	GACTTGTG	CACAAGTC
UDI078	GATCAGTG	AAGCTAGG	CCTAGCTT
UDI079	CGTTGAAG	TGGCGATA	TATCGCCA
UDI080	CTTCCAAC	GTGTACTG	CAGTACAC
UDI081	TCCGAGAT	CGTACGTT	AACGTACG
UDI082	GGTACGAA	CCGCTATA	TATAGCGG
UDI083	GAGGTAAC	CAGGTGTT	AACACCTG
UDI084	TACCGGAT	CGGTACTA	TAGTACCG
UDI085	CTCCTGAA	ACACTCTG	CAGAGTGT
UDI086	TAGGAGAG	TGCTTGTC	GACAAGCA
UDI087	TGGCTGAT	GCAAGCTT	AAGCTTGC
UDI088	GTGAGTAG	GGATGCTA	TAGCATCC
UDI089	AACGTGAC	AGTGGTTG	CAACCACT
UDI090	CCGTTGAT	CGATGTTC	GAACATCG
UDI091	AGCCGTAA	TGTCGCTT	AAGCGACA
UDI092	TCAGTAGG	GAAGCCTA	TAGGCTTC
UDI093	GGATACAC	ACCTGTTC	GAACAGGT
UDI094	GTCACCAT	GTCTCCTA	TAGGAGAC
UDI095	TGAACCAC	CGACTCTT	AAGAGTCG
UDI096	CCGCATAT	TGCACTTC	GAAGTGCA

[1] Sequencing on the MiniSeq™, NextSeq™, HiSeq™ 3000/4000, and HiSeq™ X systems follow a different dual-indexing workflow than other Illumina™ systems, which require the reverse complement of the i5 index adaptor sequence.

Table 5 Location of unique dual indices in 10X Index Primer Mix Plate, UD indexes for 96 preps

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDI001	UDI009	UDI017	UDI025	UDI033	UDI041	UDI049	UDI057	UDI065	UDI073	UDI081	UDI089
B	UDI002	UDI010	UDI018	UDI026	UDI034	UDI042	UDI050	UDI058	UDI066	UDI074	UDI082	UDI090
C	UDI003	UDI011	UDI019	UDI027	UDI035	UDI043	UDI051	UDI059	UDI067	UDI075	UDI083	UDI091
D	UDI004	UDI012	UDI020	UDI028	UDI036	UDI044	UDI052	UDI060	UDI068	UDI076	UDI084	UDI092
E	UDI005	UDI013	UDI021	UDI029	UDI037	UDI045	UDI053	UDI061	UDI069	UDI077	UDI085	UDI093
F	UDI006	UDI014	UDI022	UDI030	UDI038	UDI046	UDI054	UDI062	UDI070	UDI078	UDI086	UDI094
G	UDI007	UDI015	UDI023	UDI031	UDI039	UDI047	UDI055	UDI063	UDI071	UDI079	UDI087	UDI095
H	UDI008	UDI016	UDI024	UDI032	UDI040	UDI048	UDI056	UDI064	UDI072	UDI080	UDI088	UDI096

Table 6 Location of unique dual indices in 10X Index Primer Mix Plate, UD indexes for 24 preps

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDI001	UDI009	UDI017									
B	UDI002	UDI010	UDI018									
C	UDI003	UDI011	UDI019									
D	UDI004	UDI012	UDI020									
E	UDI005	UDI013	UDI021									
F	UDI006	UDI014	UDI022									
G	UDI007	UDI015	UDI023									
H	UDI008	UDI016	UDI024									

Required materials not supplied

For the Safety Data Sheet (SDS) of any chemical not distributed by Thermo Fisher Scientific, contact the chemical manufacturer. Before handling any chemicals, refer to the SDS provided by the manufacturer, and observe all relevant precautions.

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Item	Source
Thermal cycler with heated lid, such as: • Veriti™ 96-Well Thermal Cycler • ProFlex™ 96-well PCR System	 • 4375786 • 4484075
Agilent™ 2100 Bioanalyzer™ instrument[1]	Agilent, G2938A
Agilent™ High Sensitivity DNA Kit[1]	Agilent, 5067-4626
Agilent™ RNA 6000 Pico Kit	Agilent, 5067-1513
Magnetic rack – one of the following: • Invitrogen™ DynaMag™-2 Magnet (for 1.5-mL tubes) • Invitrogen™ DynaMag™-96 Side Magnet (for 96-well 0.2-mL plates)	 • 12321D • 12331D
Benchtop microcentrifuge	MLS
Vortex mixer	MLS
Heating block and/or thermomixer	MLS
0.2-mL nuclease-free PCR tubes or 96-well 0.2-mL PCR plates	MLS
Nuclease-free 1.5-mL tubes, such as Eppendorf™ DNA LoBind™ Tubes, 1.5-mL	Eppendorf, 022431021
Cooling rack for 0.2-mL PCR tubes/plates	MLS
Calibrated single-channel or multi-channel pipettes (1 µL– 1,000 µL)	MLS
Nuclease-free pipette tips	MLS
Ethanol 96–100%, molecular biology grade	MLS
(Optional) Qubit™ 4 Fluorometer	Q33226
(Optional) Qubit™ RNA BR Assay Kit	Q10210
(Optional) RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE	AM1975
(Optional) Globin mRNA depletion reagents – one of the following: • GLOBINclear™ Kit, human, for globin mRNA depletion • GLOBINclear™ Kit, mouse/rat, for globin mRNA depletion	 • AM1980 • AM1981

[1] You can also use comparable method to assess the quality of prepared library.

(Optional) Invitrogen™ Collibri™ Library Quantification Kit	• A38524100 • A38524500
(Optional) ERCC RNA Spike-In Mix	4456740
(Optional) ERCC ExFold RNA Spike-In Mix	4456739
(Optional) Human Brain Total RNA	AM7962
(Optional) Invitrogen™ Collibri™ Library Amplification Master Mix with Primer Mix	• A38540050 • A38540250

Technology overview

The Collibri™ Stranded RNA Library Prep Kit with Collibri™ H/M/R rRNA Depletion Kit combines the SuperScript™ IV Reverse Transcriptase, Dynabeads™ magnetic beads, and Platinum™ SuperFi™ DNA Polymerase to enable the generation of high quality sequencing-ready libraries.

Hybridization and ligation to the Adaptor Mix

Following rRNA depletion and fragmentation, RNA sample is hybridized with helper Adaptor Mix, which is a set of RNA/DNA oligonucleotides with single-stranded degenerate sequence at one end and a defined sequence at the other end. Ligation enzyme mix is then added to the mixture to ligate the hybridized adaptors.

Reverse transcription

Next, RNA population with ligated adaptors is reverse transcribed using the SuperScript™ IV Enzyme Mix to generate cDNA. cDNA is then PCR amplified, which introduces full-length Illumina™-compatible sequencing adaptors and generates ready-to-sequence libraries compatible with single-read or paired-end sequencing.

Indexing PCR

The Index Primer Mix plate contains 24 or 96 barcoded PCR primers. Indices are introduced during the library amplification step using the Library Amplification Master Mix (see pages 4-9 for index sequences).

Cleanup and quantification

Optimized cleanup steps efficiently remove residual primers and adapter/primer dimers while preserving high library yields. For best results, we recommend qPCR-based quantifications of libraries using the Invitrogen™ Collibri™ Library Quantification Kit before proceeding to sequencing.

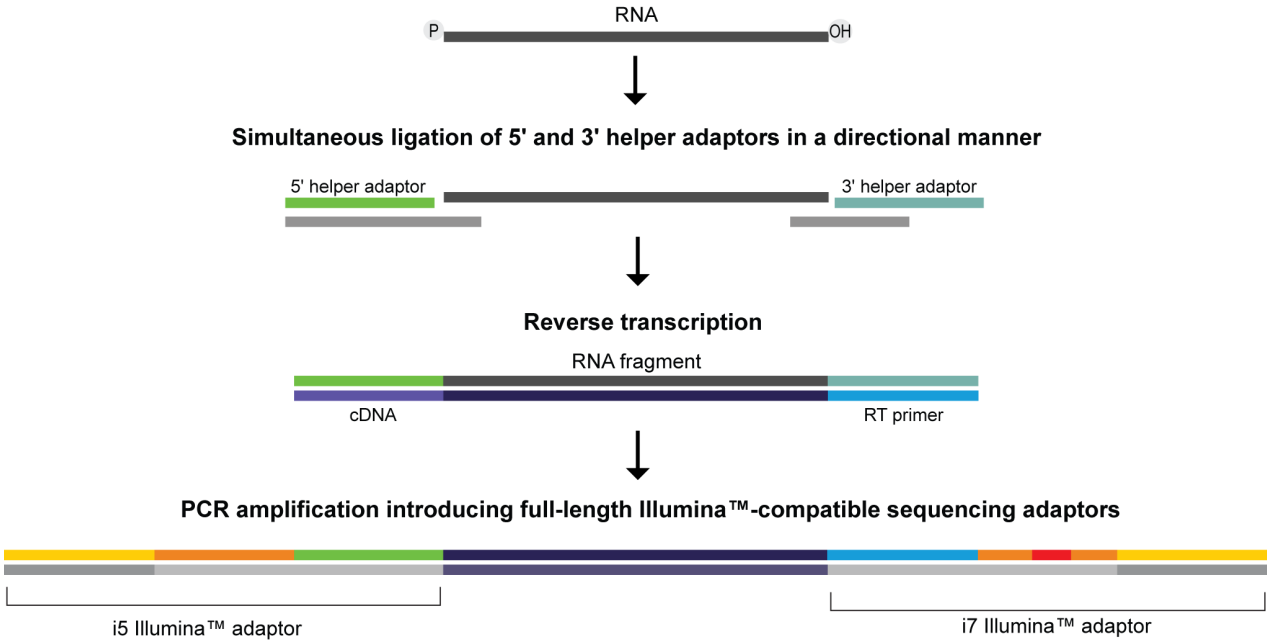

Figure 1 Simplified schematic representation of technology used in the Collibri™ Stranded RNA Library Prep Kit

2. Methods

Workflow

Figure 2 illustrates the Collibri™ Stranded RNA Library Prep Kit workflow to construct sequencing-ready cDNA libraries for strand-specific RNA sequencing.

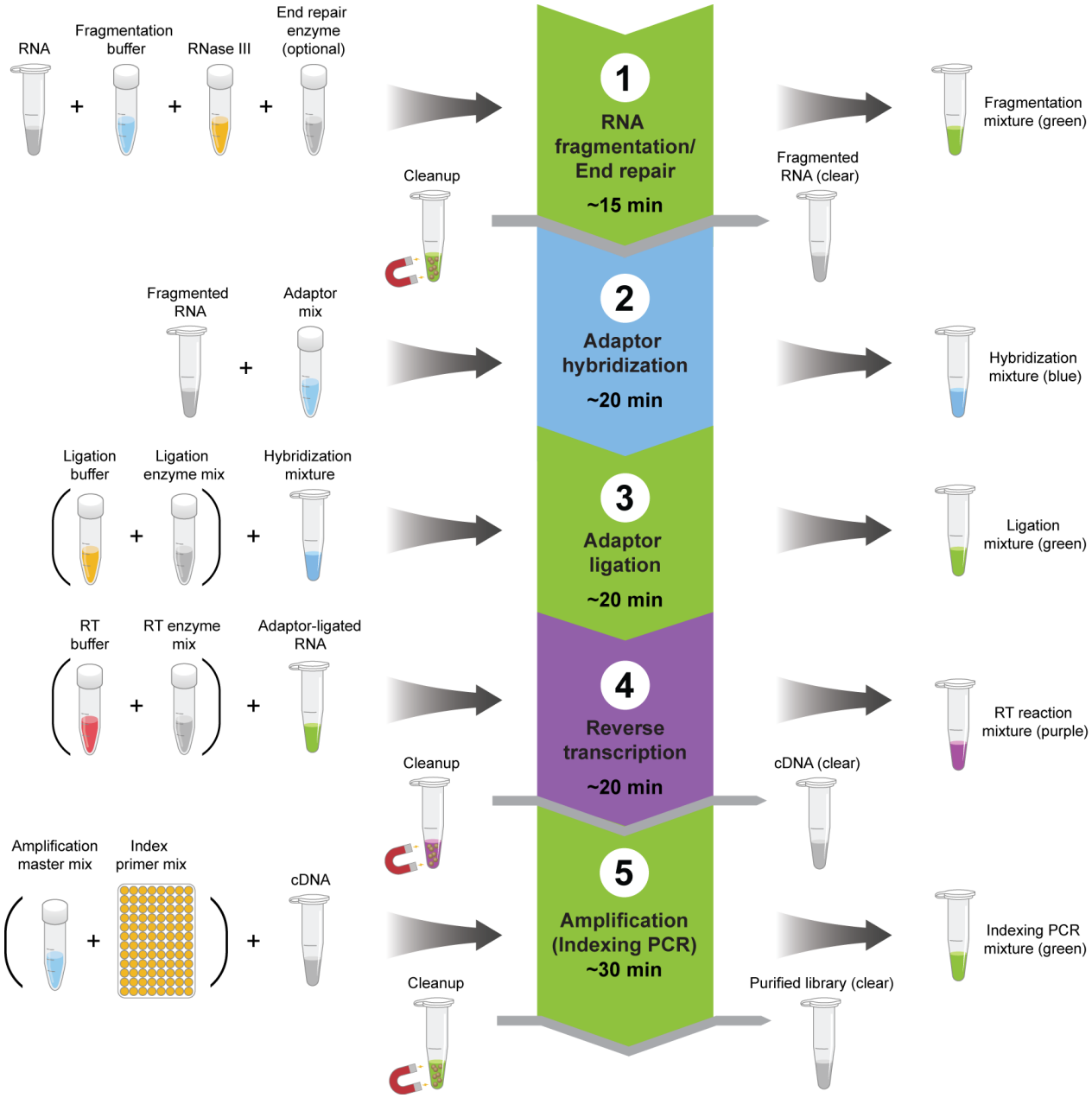

Figure 2 Collibri™ Stranded RNA Library Prep Kit workflow

Note: Colored components of the kit provide visual control of the proper workflow progress – reaction mix changes color in every step ensuring that right component is added.

Important procedural guidelines

Guidelines for RNA sample type and amount

- The Collibri™ Stranded RNA Library Prep Kit with Collibri™ H/M/R rRNA Depletion Kit is suitable for library preparation starting with 100 ng–1 µg of total RNA. For best results, we strongly recommend that you use up to 500 ng of total RNA. Note that higher amounts of input RNA can result in slightly higher ratios of chimeric reads.
- For accurate quantitation of input RNA, we recommend using the Qubit™ RNA BR Assay Kit (Cat. No. Q10210).
- Ensure that the RNA sample is free of salts or organic contaminants (phenol, ethanol, etc.).
- Before rRNA depletion, concentrate RNA in volumes >26 µL to 26 µL by ethanol precipitation, bead purification, or column-based methods. If you are using previously mRNA-enriched or rRNA-depleted sample directly for library preparation, use 8 µL of sample volume.
- We recommend that you assess the quality and size distribution of the input RNA before rRNA depletion step using the Agilent™ RNA 6000 Pico assay (Agilent, Cat. No. 5067-1513).
- The quality of RNA extracted from formalin-fixed paraffin embedded (FFPE) tissues is highly variable due to crosslinking, chemical modification, and fragmentation that can occur during the fixation process. Library prep results can vary depending on the input amount and quality of FFPE RNA. Increasing the input amount (up to 1 µg) and/or optimizing the number of PCR cycles can improve library construction with difficult FFPE samples. For RNA extraction from FFPE samples, we recommend using the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Cat. No. AM1975).
- To prepare rRNA-depleted RNA from the total RNA sample, use the Collibri™ rRNA Depletion Kit (included in the Collibri™ Stranded RNA Library Prep Kit with Collibri™ H/M/R rRNA Depletion Kit). The Collibri™ rRNA Depletion Kit enables efficient removal of 28S, 18S, 5.8S, 45S, 5S, mt16S, and mt12S ribosomal RNA from 100 ng–1 µg of human, mouse, or rat total RNA.

Note: rRNA depletion reagents included in the Collibri™ H/M/R rRNA Depletion Kit are not suitable for bacterial or plant samples.

Guidelines for RNA controls

- We recommend that you add ERCC RNA Spike-In Control Mixes to the input RNA before rRNA depletion for whole transcriptome library generation. The ERCC RNA Spike-In Control Mixes (not included in the kit) provide a set of external RNA controls that enable performance assessment (dynamic range, lower limit of detection, fold-change response) of a variety of technology platforms used for gene expression experiments. For detailed information, refer to the *ERCC RNA Spike-In Control Mixes User Guide* (Pub. No. 4455352), available at **thermofisher.com**.
- Do **not** use the ERCC RNA Spike-In Control Mixes with highly degraded samples such as FFPE RNA. High quality ERCC RNA can outcompete lower quality RNA population for the ligation of adaptors, thus causing ERCC to be overrepresented in the final library.
- For positive RNA control, we recommend using Human Brain Total RNA (Cat. No. AM7962).

Guidelines for bead handling

- Store the magnetic beads included the Library Cleanup Kit and the rRNA Depletion Kit (included in the Collibri™ Stranded RNA Library Prep Kit with Collibri™ H/M/R rRNA Depletion Kit) at 2°C to 8°C.
- Thoroughly resuspend the magnetic beads before use. You can resuspend the beads by pipetting up and down several times or by vortexing. When properly resuspended, the bead solution should have a uniform brown color with no visible clumping on the walls or at the bottom of the tube.
- The beads are superparamagnetic and are collected by placing the reaction plate or tube in a magnetic stand. The time required for complete separation varies depending on the strength of your magnet, tube thickness, viscosity of the solution, and the proximity of the tube to the magnet.

Guidelines for PCR optimization

For the optimization of PCR cycles or library re-amplification use Invitrogen™ Collibri™ Library Amplification Master Mix with Primer Mix (Cat. Nos. A38540050, A38540250).

Guidelines for index balancing

In 2-channel sequencing systems (Illumina™ NextSeq™, MiniSeq™, and NovaSeq™ instruments), a green laser is used to sequence A/T and a red laser to sequence A/C (signal in both red and green indicate A base), and absence of any signal represents G (see Figure 3). For 2-channel sequencing, index reads must begin with at least one base other than G in either of the first two cycles.

4-channel sequencing systems (Illumina™ HiSeq™ and MiSeq™ instruments) use green lasers to sequence G/T and red lasers to sequence A/C (see Figure 3). At each cycle at least one of two nucleotides for each color channel need to be read to ensure proper registration. Always use at least two unique and compatible barcodes for each index sequenced. Table 7 illustrates possible pooling strategies for 4-channel sequencing systems.

Figure 3 Registration scheme for 2-channel and 4-channel sequencing systems

Table 7 Examples of proper and improper index combinations in the same 4-channel sequencing run

	Good		Bad
IX001	ATGCCTAA	IX025	AACTCACC
IX012	GAGTTAGC	IX044	ACATTGGC
IX035	CATCAAGT	IX068	CCATCCTC
IX066	TCCGTCTA	IX091	CACCTTAC
	+++++++		---++++-

Before you begin

- Place the Purification Beads and the Purification Solution from the rRNA Depletion Kit, and the Dynabeads™ Cleanup Beads, Wash Buffer, and Elution Buffer from the Library Cleanup Kit at room temperature before starting the protocol to give them enough time to equilibrate to room temperature.
- Prepare fresh 80% ethanol solution to wash the Purification Beads (see step 6, "Purify the rRNA-depleted sample", page 18).
- Pre-heat the thermomixer to 75°C for the rRNA depletion protocol.
- Add the appropriate amount of 96–100% ethanol (35 mL for 24 prep kit, 140 mL for 96 prep kit) to the Wash Buffer before using it for the first time.

Deplete the rRNA from the total RNA sample

The Collibri™ rRNA Depletion Kit (included in the Collibri™ Stranded RNA Library Prep Kit with Collibri™ H/M/R rRNA Depletion Kit) allows the removal of ribosomal RNA (rRNA) from human, mouse, and rat total RNA samples and is fully compatible with the Collibri™ Stranded RNA Library Prep Kit. Total RNA samples are treated using a set of affinity probes for specific depletion of rRNA sequences. Number and positioning of probes are designed for applicability towards intact as well as fragmented input RNA. Reagents in the rRNA Depletion Kit remove 28S, 18S, 5.8S, 45S, 5S, mt16S, and mt12S ribosomal sequences.

Required materials

Use components from the rRNA Depletion Kit:

- Hybridization Solution
- Probe Mix
- Depletion Beads
- Conditioning Solution
- Depletion Solution
- Purification Beads
- Purification Solution
- Elution Solution

Other materials and equipment:

- 80% ethanol, molecular biology grade

  Note: Prepare fresh ethanol solution before each use.
- Magnetic rack (see "Required materials not supplied", page 10)
- Thermomixer set to 75°C

Hybridize total RNA with the Probe Mix

1. Set-up the hybridization reaction for each total RNA sample:

Component	Volume
Total RNA sample (100 ng–1 µg)	26 µL
Hybridization Solution	4 µL
Probe Mix	5 µL
Total volume:	**35 µL**

2. Vortex the hybridization mixture gently, then denature for 5 minutes in a thermomixer set to 75°C under gentle agitation at 400 rpm.
3. Decrease the temperature to 60°C and incubate for 30 minutes under gentle agitation at 400 rpm.
4. During the incubation step, condition the Depletion Beads (page 18). For multiple samples, we recommend batched processing.

Condition the Depletion Beads

1. Thoroughly resuspend the Depletion Beads, then transfer 75 µL of the bead suspension per RNA sample to a fresh tube.
2. Place the tubes with the Depletion Bead suspension in the magnetic rack for 2–5 minutes. Keeping the tubes on the magnet, remove the supernatant and discard.
3. Resuspend the Depletion Beads in 75 µL of Conditioning Solution per sample, then incubate for 2 minutes in the magnetic rack. Keeping the tubes on the magnet, remove the supernatant and discard.
4. Repeat step 3.
5. Resuspend the Depletion Beads in 75 µL of Depletion Solution per sample, then incubate for 2–5 minutes in the magnetic rack. Keeping the tubes on the magnet, remove the supernatant and discard.
6. Repeat step 5 twice.
7. Resuspend the Depletion Beads in 30 µL of Depletion Solution per sample.

Deplete the rRNA from the sample

1. Briefly centrifuge the hybridized sample (from step 4, page 17), then add 30 µL of freshly conditioned Depletion Beads. Mix by pipetting up and down 8 times.

   IMPORTANT! Avoid creating bubbles during mixing.

2. Place the tubes back in the thermomixer, then incubate for 15 minutes at 60°C under gentle agitation at 400 rpm.
3. Briefly centrifuge the tubes, then place them in the magnetic rack for 5 minutes.
4. Transfer 60 µL of supernatant from each rRNA-depleted sample to a separate, fresh tube.

Purify the rRNA-depleted sample

1. To each rRNA-depleted sample, add 24 µL of Purification Beads and 108 µL of Purification Solution, mix well, then incubate for 20 minutes at room temperature.
2. Place the tubes in the magnetic rack for 5–10 minutes. Keeping the tubes on the magnet, remove the supernatant and discard.
3. Remove the tubes from the magnet, add 30 µL of Elution Solution, mix well, then incubate for 2 minutes at room temperature.
4. Add 66 µL of Purification Solution, mix well, then incubate for 5 minutes at room temperature.
5. Place the tubes in the magnetic rack for 2–5 minutes. Keeping the tube on the magnet, remove the supernatant and discard.
6. Keeping the tubes on the magnet, add 120 µL of 80% ethanol solution, then incubate at room temperature for 30 seconds. Carefully remove the supernatant using a pipette and discard.

   IMPORTANT! Do **not** resuspend the magetic beads in the ethanol solution.

7. Repeat step 6.

8. Keeping the tubes on the magnet, air dry the magnetic beads for 5–10 minutes at room temperature or until there are no droplets of ethanol left on the walls of the tubes.

   IMPORTANT! Do **not** over-dry the magnetic beads by prolonged incubation for more than 10 minutes. Over-drying significantly decreases elution efficiency.

9. Remove the tubes from the magnet, add 12 µL of Elution Solution, mix well, then incubate for 2 minutes at room temperature.
10. Place the tubes in the magnetic rack for 2–5 minutes. Keeping the tubes on the magnet, carefully remove 10 µL of the supernatant from each tube and transfer into a fresh tube.

Note: We recommend that you assess the quality of the depleted RNA using the Agilent™ RNA 6000 Pico Kit (see page 10) before proceeding to library preparation and sequencing. You can use up to 2 µL of the depleted sample for quality assessment.

Expected results

Figure 4 shows a typical Agilent™ 2100 Bioanalyzer trace of an rRNA-depleted sample analyzed using the Agilent™ RNA 6000 Pico Kit.

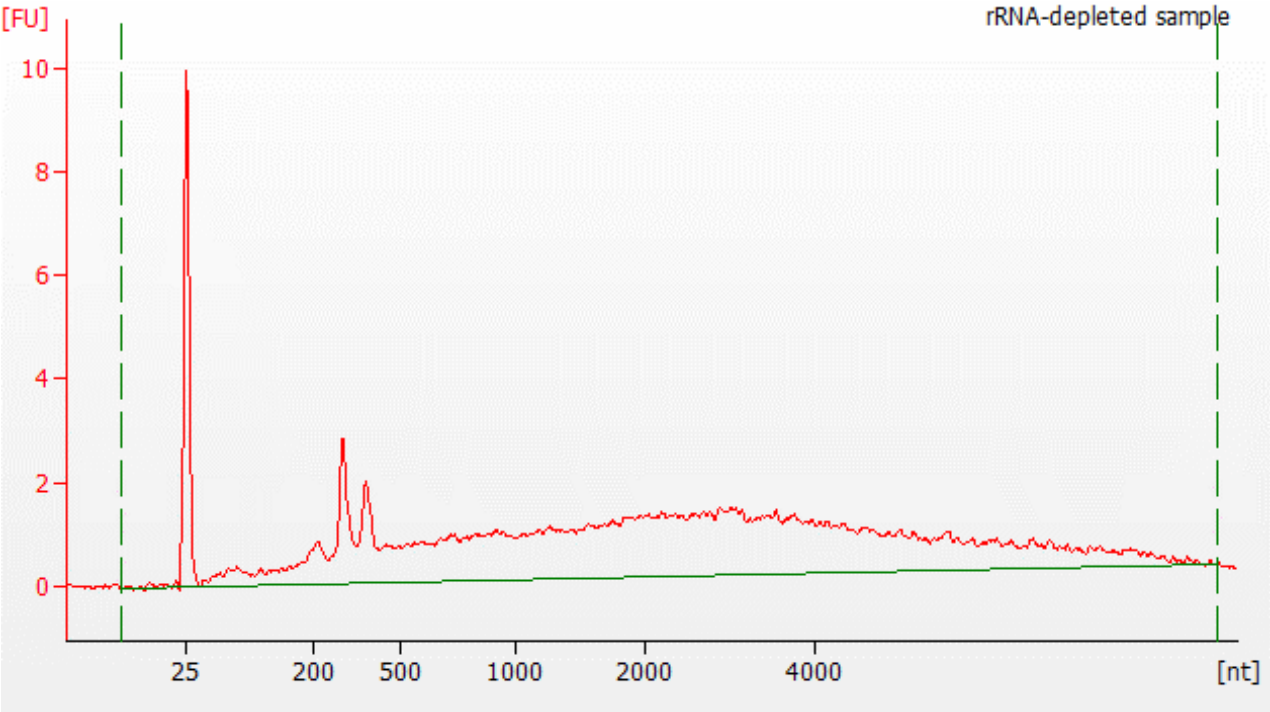

Figure 4 Typical Agilent™ 2100 Bioanalyzer trace of rRNA-depleted sample.

STOPPING POINT. Store the rRNA-depleted RNA samples at –70°C, or immediately proceed to the fragmentation step (page 20).

Fragment the RNA using RNase III

Required materials

Use components from the Library Prep Kit:

- Nuclease-free Water
- 10X Fragmentation Buffer
- RNase III
- RNA End Repair Enzyme (for degraded samples such as FFPE RNA only)

Fragment the RNA using RNase III

1. On ice or a cooling rack, assemble a reaction for each RNA sample in a 0.2-mL PCR tube or PCR plate:

Component	Volume
RNA sample (rRNA-depleted)	8 µL
10X Fragmentation Buffer (blue)	1 µL
RNase III (yellow)	1 µL
Total volume (green mixture):	**10 µL**

IMPORTANT! To reduce fragmentation variability, accurately pipet 1 µL of 10X Fragmentation Buffer and 1 µL of RNase III to each sample. **Do not prepare** a master mix that contains only 10X Fragmentation Buffer and RNase III.

For degraded samples (e.g. FFPE), add 1 µL of RNA End Repair Enzyme directly into the fragmentation reaction mixture. To ensure that the final reaction volume is still 10 µL, use 7 µL of FFPE RNA (instead of 8 µL).

2. Flick the tube or pipet up and down several times to mix, then centrifuge briefly to collect the liquid in the bottom of the tube.
3. Incubate the reaction mixture in a thermal cycler.

Desired insert length	Fragmentation reaction conditions
~150 bp	30°C, 10 minutes
~300 bp	30°C, 3 minutes

Note: After indexing, PCR adaptors will add ~130 bp to the insert size.

4. **Immediately** after the incubation, add 20 µL of nuclease-free water to stop the reaction, then place the fragmented RNA on ice.

IMPORTANT! Proceed immediately to the next step, "Purify the fragmented RNA" (page 21). Do **not** leave the fragmented RNA on ice for more than 1 hour.

Purify the fragmented RNA

Required materials

Use components from the Library Cleanup Kit, equilibrated to room temperature:

- Dynabeads™ Cleanup Beads
- Wash Buffer (diluted with 96–100% ethanol)

Other materials and equipment:

- 96–100% ethanol, molecular biology grade
- Magnetic rack (see "Required materials not supplied", page 10)
- Water, nuclease-free
- Heating block at 65°C

Before you begin

- Ensure that 96–100% Ethanol was added to Wash Buffer before first use.
- Ensure that the Dynabeads™ Cleanup Beads and Wash Buffer are at room temperature.
- Pre-heat the heating block to 65°C.
- Gently vortex the Dynabeads™ Cleanup Beads to completely resuspend the magnetic beads in the solution.

Purify the fragmented RNA

1. Mix the RNA fragmentation reaction mixture (30 µL) with 60 µL of Dynabeads™ Cleanup Beads and 60 µL of ethanol (96–100%) by pipetting or vortexing until you have obtained a homogenous suspension.
2. Briefly centrifuge the tube or plate to collect all the droplets at the bottom, then incubate for 10 minutes at room temperature.
3. After incubation, briefly centrifuge the tube or plate to collect the droplets at the bottom, then place the tube or plate in the magnetic rack for 2 minutes or until the beads have formed a tight pellet.
4. Keeping the reaction tube or plate in the magnetic rack, carefully remove and discard the supernatant using a pipette. Ensure that all of the supernatant is removed.

   Note: If the pellet of magnetic beads was disturbed, mix the sample and let the beads settle to the side of the tube or plate on the magnet again.

5. Keeping the reaction tube or plate on the magnet, add 200 µL of Wash Buffer (pre-mixed with ethanol), then incubate for 30 seconds at room temperature.

   IMPORTANT! Do not resuspend the magnetic beads in Wash Buffer.

6. Carefully remove and discard the supernatant using a pipette.
7. To remove the residual ethanol, briefly centrifuge the reaction tube or plate, place it back in the magnetic rack, then carefully remove any remaining supernatant with a pipette without disturbing the pellet.

8. Keeping the reaction tube or plate on the magnet, air dry the magnetic beads for 1 minute at room temperature or until there are no droplets of ethanol left on the walls of the tube or plate.

   IMPORTANT! Do not over-dry by prolonged incubation for more than 5 minutes. Over-drying significantly decreases the elution efficiency.

9. Add 12 µL of nuclease-free water to each sample, remove the tube or plate from the magnetic rack, then mix well by pipetting or vortexing until all of the beads are fully resuspended.
10. Place the tube or plate in the heating block at 65°C and incubate for 5 minutes.
11. Place the tube or plate in the magnetic rack for 2 minutes or until the beads have formed a tight pellet. Wait for the solution to clear before proceeding to the next step.
12. Keeping the tube on the magnet, collect 10 µL of the supernatant for each sample into a new 0.2-mL PCR tube or plate.

STOPPING POINT. Store the fragmented RNA samples at –70°C, or immediately proceed to the hybridization and ligation step (page 23).

Hybridize and ligate the adaptors

Required materials

Use components from the Library Prep Kit:

- 2X Adaptor Mix
- 2X Ligation Buffer
- 10X Ligation Enzyme Mix

Other materials and equipment:

- Fragmented and purified RNA (from step 12, page 22)
- Thermal cycler with heated lid (see "Required materials not supplied", page 10)

Hybridize and ligate the adaptors

1. Prepare the RNA-adaptor hybridization reaction mixture in the same 0.2-mL PCR tube or plate containing the purified, fragmented RNA sample (from step 12, page 22):

Component	Volume
Fragmented, purified RNA sample (clear)	10 µL
2X Adaptor Mix (blue)	10 µL
Total volume (blue mixture):	**20 µL**

2. Pipet or vortex the hybridization reaction mixture to mix it thoroughly.
3. Run the hybridization reaction in a thermal cycler using a cooling ramp rate of –0.5°C/second:

Temperature	Time
65°C	10 minutes
20°C	5 minutes

4. Prepare a ligation master mix with 5% of excess volume to compensate for pipetting error:

Component	Volume (+5%)				
	1 library	6 libraries	24 libraries	96 libraries	N libraries
2X Ligation Buffer (yellow)	26.25 µL	157.5 µL	630 µL	2520 µL	N × 26.25 µL
Ligation Enzyme Mix (clear)	5.25 µL	31.5 µL	126 µL	504 µL	N × 5.25 µL
Total volume (yellow mixture):	**31.5 µL**	**189 µL**	**756 µL**	**3024 µL**	**N × 31.5 µL**

IMPORTANT! If the 2X Ligation Buffer contains a precipitate, warm the tube at 37°C for 2–5 minutes or until the precipitate is dissolved. 2X Ligation Buffer is very viscous; pipet slowly to dispense it accurately.

5. Add the RNA ligation reagents (ligation master mix from step 4, page 23) to the same reaction tube or plate containing the hybridization reaction mixture (from step 3, page 23):

Component	Volume
RNA-adaptor hybridization mixture (blue)	20 µL
Ligation master mix (yellow)	30 µL
Total volume (green mixture):	**50 µL**

6. Vortex the ligation reaction mixture to mix it thoroughly, then centrifuge it briefly to collect all the droplets at the bottom.
7. Incubate the ligation reaction in a thermal cycler for 15 minutes at 20°C.

IMPORTANT! Set the temperature of the thermal cycler lid to match the block temperature, turn OFF the heated lid, or leave the thermal cycler open during the incubation.

Perform reverse transcription (RT)

Required materials

Use components from the Library Prep Kit:

- 2.5X RT Buffer
- 10X SuperScript™ IV Enzyme Mix

Other materials and equipment:

- Adaptor-ligated RNA mixture (from step 7, page 24)
- Thermal cycler with heated lid (see "Required materials not supplied", page 10)

Perform reverse transcription

1. Prepare a reverse transcription master mix with 5% of excess volume to compensate for pipetting error:

Component	Volume (+5%)				
	1 library	6 libraries	24 libraries	96 libraries	N libraries
2.5X RT Buffer (red)	42 µL	252 µL	1008 µL	4032 µL	N × 42 µL
10X SuperScript™ IV Enzyme Mix (clear)	10.5 µL	63 µL	252 µL	1008 µL	N × 10.5 µL
Total volume (red mixture):	**52.5 µL**	**315 µL**	**1260 µL**	**5040 µL**	**N × 52.5 µL**

2. Add the reverse transcription reagents to the same reaction tube or plate containing the ligation reaction mixture (from step 7, page 24):

Component	Volume
Adaptor-ligated RNA mixture (green)	50 µL
Reverse transcription master mix (red)	50 µL
Total volume (purple mixture):	**100 µL**

3. Vortex the reverse transcription reaction mixture to mix it thoroughly, then centrifuge it briefly to collect all the droplets at the bottom.
4. Incubate the reverse transcription reaction in a thermal cycler with the lid temperature set to 85–95°C.

Temperature	Time
50°C	10 minutes
85°C	5 minutes

Purify the cDNA

Required materials

Use components from the Library Cleanup Kit, equilibrated to room temperature:

- Dynabeads™ Cleanup Beads
- Wash Buffer (diluted with 96–100% ethanol)
- Elution Buffer

Other materials and equipment:

- 96–100% ethanol, molecular biology grade
- Magnetic rack (see "Required materials not supplied", page 10)

Before you begin

- Ensure that 96–100% Ethanol was added to Wash Buffer before first use.
- Ensure that the Dynabeads™ Cleanup Beads, Wash Buffer, and Elution Buffer are at room temperature.
- Gently vortex the Dynabeads™ Cleanup Beads to completely resuspend the magnetic beads in the solution.

Purify the cDNA

1. Mix the cDNA sample (100 µL) (from step 4, page 25) with 60 µL of Dynabeads™ Cleanup Beads and 40 µL of ethanol (96–100%) by pipetting or vortexing until you have obtained a homogenous suspension.
2. Briefly centrifuge the tube or plate containing the cDNA and bead mixture to collect all the droplets at the bottom, then incubate for 10 minutes at room temperature.
3. After incubation, briefly centrifuge the tube or plate to collect the droplets at the bottom, then place the tube or plate in the magnetic rack for 2 minutes or until the beads have formed a tight pellet.
4. Keeping the tube or plate in the magnetic rack, carefully remove and discard the supernatant using a pipette. Ensure that all of the supernatant is removed.

   Note: If the pellet of magnetic beads was disturbed, mix the sample and let the beads settle to the side of the tube or plate on the magnet again.

5. Remove the tube or plate from the magnetic rack, then add 40 µL of Elution Buffer.
6. Pipet or vortex to mix, briefly centrifuge to collect all the droplets at the bottom, then incubate for 1 minute at room temperature.
7. Add 100 µL of fresh Dynabeads™ Cleanup Beads and 60 µL of ethanol (96–100%) directly to the bead suspension in Elution Buffer, then pipet or vortex to mix until a homogenous suspension is obtained.
8. Briefly centrifuge the cDNA and bead suspension to collect all the droplets at the bottom, then incubate for 10 minutes at room temperature.
9. After incubation, briefly centrifuge the tube or plate to collect the droplets at the bottom, then place the tube or plate in the magnetic rack for 2 minutes or until the beads have formed a tight pellet.

10. Keeping the reaction tube or plate in the magnetic rack, carefully remove and discard the supernatant using a pipette. Ensure that all of the supernatant is removed.

    Note: If the pellet of magnetic beads was disturbed, mix the sample and let the beads settle to the side of the tube or plate on the magnet again.

11. Keeping the tube or plate on the magnet, add 200 µL of Wash Buffer (pre-mixed with ethanol), then incubate for 30 seconds at room temperature.

    IMPORTANT! Do not resuspend the magnetic beads in Wash Buffer.

12. Carefully remove and discard the supernatant using a pipette.
13. Repeat steps 11–12.
14. To remove the residual ethanol, briefly centrifuge the tube or plate, place it back in the magnetic rack, then carefully remove any remaining supernatant with a pipette without disturbing the pellet.
15. Keeping the reaction tube or plate on the magnet, air dry the magnetic beads for 1 minute at room temperature or until there are no droplets of ethanol left on the walls of the tube or plate.

    IMPORTANT! Do not over-dry by prolonged incubation for more than 5 minutes. Over-drying significantly decreases the elution efficiency.

16. Remove the tube or plate from the magnetic rack, add 22 µL of Elution Buffer, then vortex to mix well.
17. Briefly centrifuge the tube or plate to collect all the droplets at the bottom, then incubate for 1 minute at room temperature.
18. Place the tube or plate in the magnetic rack for 2–3 minutes or until the beads have formed a tight pellet. Wait for the solution to clear before proceeding to the next step.
19. Without removing the tube or plate from the magnetic rack, collect 20 µL of the supernatant for each sample into a new 0.2-mL PCR tube or plate.

    Note: If the pellet of magnetic beads was disturbed, mix the sample and let the beads settle to the bottom of the tube or plate on the magnet again.

STOPPING POINT. Store the cDNA samples at –20°C, or immediately proceed to the library amplification step (page 28).

Amplify the cDNA (Indexing PCR)

Required materials

Use components from the Library Prep Kit:

- 2X Library Amplification Master Mix
- 10X Index Primer Mix

Other materials and equipment:

- Purified cDNA (from step 19, page 27)
- Thermal cycler with heated lid (see "Required materials not supplied", page 10)

Amplify the cDNA

1. For each cDNA sample, set up the PCR reaction mixture in the same PCR tube or plate containing the purified cDNA sample (from step 19, page 27):

Component	Volume
cDNA	20 µL
2X Library Amplification Master Mix (blue)	25 µL
10X Index Primer Mix (yellow)	5 µL
Total volume (green mixture):	**50 µL**

IMPORTANT! Before use, briefly centrifuge the 10X Index Primer Mix plate to avoid cross-contamination of indices.

2. Run the reactions in a thermal cycler with the lid temperature set to 105°C:

Stage	Number of cycles[1]	Temperature	Time
Activate the enzyme	1 cycle	98°C	30 seconds
Denature	9–11 cycles for 500–1000 ng of input total RNA 10–12 cycles for 250–500 ng of input total RNA 12–14 cycles for 100–250 ng of input total RNA	98°C	10 seconds
Anneal		60°C	30 seconds
Extend		72°C	30 seconds
Final extension	1 cycle	72°C	1 minute
Hold	1 cycle	4°C	hold

[1] The number of PCR cycles depends on the starting amount of RNA (i.e., input RNA).

Purify the amplified cDNA

Required materials

Use components from the Library Cleanup Kit, equilibrated to room temperature:

- Dynabeads™ Cleanup Beads
- Wash Buffer (diluted with 96–100% ethanol)
- Elution Buffer

Other materials and equipment:

- 96–100% ethanol, molecular biology grade
- Magnetic rack (see "Required materials not supplied", page 10)

Before you begin

- Ensure that 96–100% Ethanol was added to Wash Buffer before first use.
- Ensure that the Dynabeads™ Cleanup Beads, Wash Buffer, and Elution Buffer are at room temperature.
- Gently vortex the Dynabeads™ Cleanup Beads to completely resuspend the magnetic beads in the solution.

Purify the amplified cDNA

1. Mix the PCR reaction mixture (50 µL) (from step 2, page 28) with 65 µL of Dynabeads™ Cleanup Beads by pipetting or vortexing until you have obtained a homogenous suspension.

   Note: To obtain libraries with an average insert size of ~300 bp, use 40 µL of Dynabeads™ Cleanup Beads at this step.

2. Briefly centrifuge the tube or plate containing the amplified cDNA and bead mixture to collect all the droplets at the bottom, then incubate for 5 minutes at room temperature.
3. After incubation, briefly centrifuge the tube or plate to collect the droplets at the bottom, then place the tube or plate in the magnetic rack for 2 minutes or until the beads have formed a tight pellet.
4. Keeping the tube or plate on the magnetic rack, carefully remove and discard the supernatant using a pipette. Ensure that all of the supernatant is removed.

   Note: If the pellet of magnetic beads was disturbed, mix the sample and let the beads settle to the side of the tube or plate on the magnet again.

5. Remove the tube or plate from the magnetic rack, then add 50 µL of Elution Buffer.
6. Pipet or vortex to mix, briefly centrifuge to collect all the droplets at the bottom, then incubate for 1 minute at room temperature.
7. Add 75 µL of fresh Dynabeads™ Cleanup Beads directly to the bead suspension in Elution Buffer, then pipet or vortex to mix until a homogenous suspension is obtained.
8. Briefly centrifuge the amplified cDNA and bead suspension to collect all the droplets at the bottom, then incubate for 5 minutes at room temperature.
9. After incubation, briefly centrifuge the tube or plate to collect the droplets at the bottom, then place the tube or plate in the magnetic rack for 2 minutes or until the beads have formed a tight pellet.

10. Keeping the reaction tube or plate in the magnetic rack, carefully remove and discard the supernatant using a pipette. Ensure that all of the supernatant is removed.

    Note: If the pellet of magnetic beads was disturbed, mix the sample and let the beads settle to the side of the tube or plate on the magnet again.

11. Keeping the tube or plate on the magnet, add 200 µL of Wash Buffer (pre-mixed with ethanol), then incubate for 30 seconds at room temperature.

    IMPORTANT! Do not resuspend the magnetic beads in Wash Buffer.

12. Carefully remove and discard the supernatant using a pipette.
13. Repeat steps 11–12.
14. To remove the residual ethanol, briefly centrifuge the tube or plate, place it back in the magnetic rack, then carefully remove any remaining supernatant with a pipette without disturbing the pellet.
15. Keeping the reaction tube or plate on the magnet, air dry the magnetic beads for 1 minute at room temperature or until there are no droplets of ethanol left on the walls of the tube or plate.

    IMPORTANT! Do not over-dry by prolonged incubation for more than 5 minutes. Over-drying significantly decreases the elution efficiency.

16. Remove the tube or plate from the magnetic rack, add 22 µL of Elution Buffer, then mix well by vortexing.
17. Briefly centrifuge the tube or plate to collect all the droplets at the bottom, then incubate for 1 minute at room temperature.
18. Place the tube or plate in the magnetic rack for 2–3 minutes or until the beads have formed a tight pellet. Wait for the solution to clear before proceeding to the next step.
19. Without removing the tube or plate from the magnetic rack, transfer 20 µL of the supernatant to a new tube or plate for storage.

    Note: If the pellet of magnetic beads was disturbed, mix the sample and let the beads settle to the side of the tube or plate on the magnet again.

STOPPING POINT. Store the amplifed cDNA at –20°C, or immediately proceed to the next step (page 31).

Assess the size distribution of the amplified cDNA

Required materials

- Agilent™ 2100 Bioanalyzer™ instrument (Agilent, Cat. No. G2938A)
- Agilent™ High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626)
- Nuclease-free water

Note: You can also use comparable method to assess the yield and size distribution of the prepared libraries.

Analyze the size distribution of the amplified cDNA library

1. Remove 1 µL from each prepared cDNA library (i.e., purified amplified cDNA from step 19, page 30), and dilute it 2–5-fold in nuclease-free water.
2. Analyze 1 µL of the diluted cDNA library using the appropriate chip on the Agilent™ 2100 Bioanalyzer™ instrument with the Agilent™ High Sensitivity DNA Kit.
3. Using the 2100 Expert software, perform a smear analysis to determine the average library length using a size range of 150–1000 bp.

Note: For instructions on how to perform the smear analysis, refer to the *Agilent™ 2100 Bioanalyzer™ Expert User's Guide* (Agilent, Pub. No. G2946-90004).

Expected results

Typical average library length obtained using the Collibri™ Stranded RNA Library Prep Kit protocol for ~150 bp inserts is in the range of 300–450 bp.

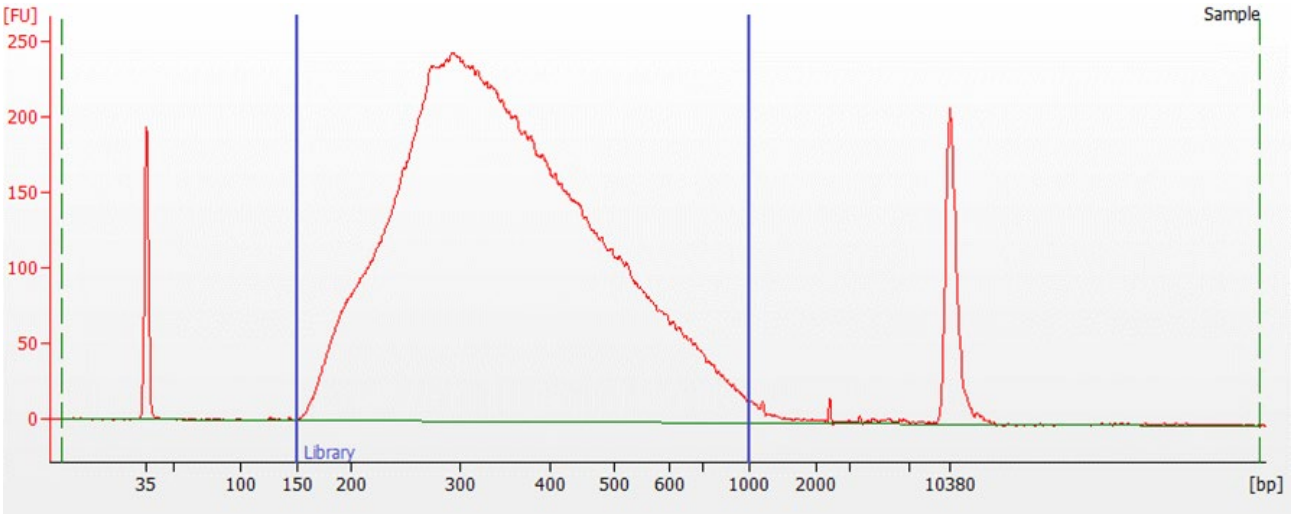

Figure 5 Typical Agilent™ 2100 Bioanalyzer trace of a 5-fold diluted library prepared from Universal Human Reference RNA using the Collibri™ Stranded RNA Library Prep Kit protocol for ~150 bp inserts.

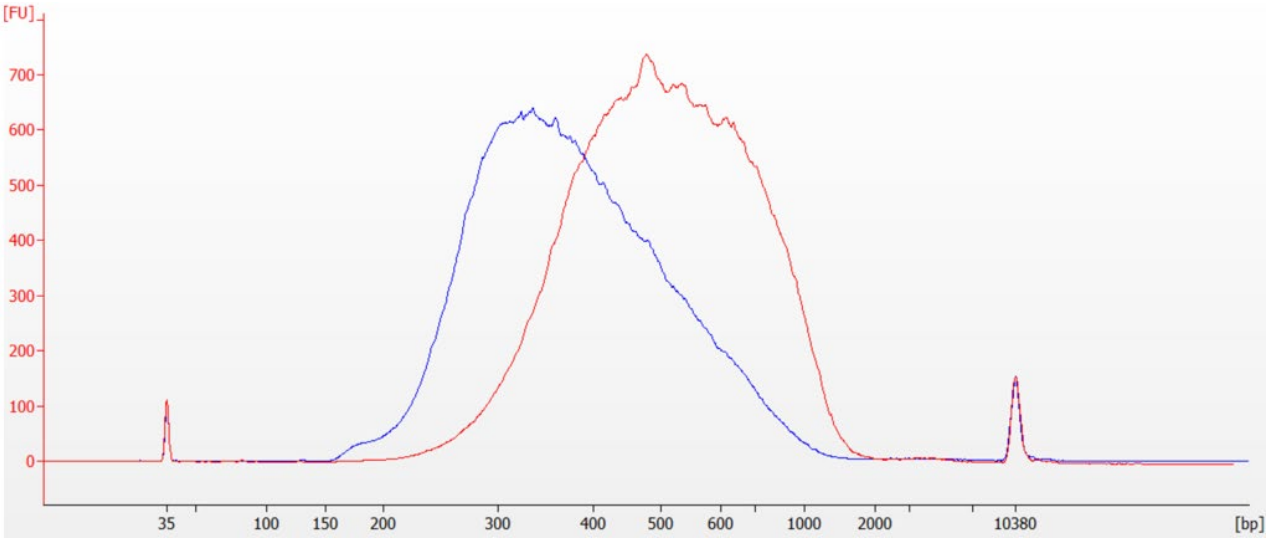

Figure 5 Typical Agilent™ 2100 Bioanalyzer trace of libraries prepared from Universal Human Reference RNA using Collibri™ Stranded RNA Library Prep Kit protocol for ~150 bp inserts (blue profile) and protocol for ~300 bp inserts (red profile).

Next steps

Quantify the prepared library by qPCR

We strongly recommend that you perform qPCR quantification of prepared libraries using the Invitrogen™ Collibri™ Library Quantification Kit (available separately from Thermo Fisher Scientific, Cat. Nos. A38524100, A38524500) before proceeding to sequencing.

Typical sequencing-ready library concentration obtained using the Collibri™ Stranded RNA Library Prep Kit is in the range of 30–70 nM. If more sequencable material is needed, optimize the number of PCR cycles to obtain the desired yield.

Sequence the prepared library

Denature, dilute, and load the libraries according to the standard guidelines appropriate for the Illumina™ NGS platform you are using.

To pool single indexed libraries prepared with the Collibri™ Stranded RNA Library Prep Kit with dual indexed libraries, you will need to indicate i5 index sequence in the sample sheet. For single indexed samples prepared with Collibri™ Stranded RNA Library Prep Kit, use the following i5 index sequence:

- `TCTTTCCC` for MiSeq™, HiSeq™ 2000, HiSeq™ 2500, and NovaSeq™ 6000 instruments.
- `AGATCTCG` for MiniSeq™, NextSeq™, iSeq™ 100, HiSeq™ X, HiSeq™ 3000, and HiSeq™ 4000 instruments.

Do **not** perform Index 2 (i5) Read when sequencing single indexed libraries prepared with the Collibri™ Stranded RNA Library Prep Kit only.

Data analysis

Libraries prepared from polyadenilated mRNAs can produce 2–8% of poly(A) sequence-containing reads. If poly(A) tails are not of research interest, we recommend that you trim the poly(A) sequences using BBDuk from the BBTools suite. Use the following BBDuk settings:

```
ktrim=r k=23 mink=11 hdist=1 minlength=50 maxns=1 tpe tbo
qtrim=r trimq=15
```

As a reference for adaptor and poly(A) trimming use following settings:

```
>for_adapter
AGATCGGAAGAGCACACGTCTGAACTCCAGTCA
>rev_adapter
AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
>polyT
TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
>polyA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

Note: BBTools is a suite of fast, multithreaded bioinformatics tools designed by the Joint Genome Institute of DOE Office of Science for the analysis of DNA and RNA sequence data. BBTools is open source and free for unlimited use.

BBDuk is a specific tool within the BBTools suite that was developed to combine most common data-quality-related trimming, filtering, and masking operations into a single high-performance tool. It filters or trims reads for adapters and contaminants using *k*-mers.

For more information on BBTools and to download BBTools software and documentation, go to **https://jgi.doe.gov/data-and-tools/bbtools/**.

Appendix A: Troubleshooting

Observation	Possible cause	Recommended action
Residual rRNA peaks are visible on the Agilent™ Bioanalyzer™ trace after the rRNA depletion procedure	Impurities in starting material.	Residual chaotropic salts, metal ions, and organic solvents can negatively impact the efficiency of rRNA depletion protocol. Assess the quality of the starting material using UV-Vis spectrophotometer.
Low yield and/or poor size distribution obtained in the amplified library	Low recovery rates after cleanup.	• Make sure that the Dynabeads™ Cleanup Beads suspension is brought to room temperature and the magnetic beads are thoroughly resuspended before use. • Make sure that the appropriate volume of ethanol is added to the bottle of concentrated Wash Buffer before first use. • Do **not** resuspend magnetic beads in Wash Buffer during the wash steps.
	Suboptimal number of PCR cycles.	Increase the number of PCR cycles.
Extremely low yield and/or no PCR products	Enzymatic reaction or purification step after RNA fragmentation failed.	• Check the size distribution and yield of the fragmented RNA after the RNase III treatment and cleanup steps using the Agilent™ 2100 Bioanalyzer RNA Pico Assay. • Minimize the time spent above –20°C for the 10X Ligation Enzyme Mix and 10X SuperScript™ IV Enzyme Mix.
Residual adapters (~60 bp) or adapter dimers (~150 bp) are visible on the Agilent™ Bioanalyzer™ trace.	Inefficient cleanup after the reverse transcription or PCR steps.	Bring the sample volume to 50 µL with the Library Cleanup Kit Elution Buffer and repeat the post-PCR cleanup procedure.
The color of reaction mixture after the setup of fragmentation reaction is not green.	Some of the colored reaction components were not added or were added in wrong quantities.	• Make sure to pipet accurate volumes of reagents. • Make sure to pipet the viscous RNase III carefully to aspirate the correct volume. • The color of reaction mixture after the setup of ligation reaction should be green. • Make sure to pipet the viscous Ligation Buffer carefully to aspirate the correct volume.
The color of reaction mixture after the setup of reverse transcription reaction is not purple.		
The color of reaction mixture after the setup of PCR reaction is not green.		

Appendix B: Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at: **www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf**
- World Health Organisation (WHO), *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at: **www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf**

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  - Safety Data Sheets (SDSs; also known as MSDSs)

  Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

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