invitrogen

Collibri™ 3’ mRNA Library Prep Kit for Illumina®

USER GUIDE

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

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Revision	Date	Description
A.0	16 October 2019	New user guide for Collibri™ 3′ mRNA Library Prep Kit.

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Product information

Product description

Invitrogen™ Collibri™ 3′ mRNA Library Prep Kit is designed for robust construction of cDNA libraries for strand-specific RNA sequencing on the Illumina® NGS (next generation sequencing) platforms.

The kit supports library preparation from polyadenylated RNA within 4.5 hours. The procedure generates one fragment per transcript, resulting in accurate gene expression values, sequences obtained are close to the 3′ end of the transcripts. The majority of inserts are greater than 75 bp in size, corresponding to final library fragment sizes ≥200 bp. Multiplexing of libraries can be carried out using up to 96 single-indexed primers. 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.

As Collibri™ 3′ mRNA Library Prep Kit targets the 3′ end of transcripts it is suitable for various quality RNA samples, including FFPE.

Note: For an overview of the technology used in the Collibri™ 3′ mRNA Library Prep Kit, see "Technology overview" on page 10.

Kit contents and storage

Sufficient reagents for library construction and cleanup are supplied in the Collibri™ 3′ mRNA Library Prep Kit to prepare indexed libraries for 24 or 96 samples.

Component	Cap color		Amount		Storage
			24 preps	96 preps	
Library Prep Kit					
2X Priming Mix	White	○	120 µL	480 µL	–20°C
First Strand Synthesis Buffer	White	○	228 µL	912 µL	
40X First Strand Enzyme Mix	White	○	12 µL	48 µL	
5X RNA Removal Solution	Yellow	●	120 µL	480 µL	
3.5X Hybridization Mix	Red	●	240 µL	960 µL	
10X Second Strand Synthesis Buffer	Red	●	96 µL	384 µL	
40X Second Strand Synthesis Enzyme Mix	Red	●	24 µL	96 µL	
5X Library Amplification Buffer	Blue	●	168 µL	672 µL	
i5 Universal Primer	Blue	●	120 µL	480 µL	
35X Library Amplification Enzyme Mix	Blue	●	24 µL	96 µL	
7X i7 Index Primer Plate[1]	—		5 µL/well (24 wells)	5 µL/well (96 wells)	
Library Cleanup Kit					
Purification Beads	White	○	1.22 mL	4.89 mL	2°C to 8°C **IMPORTANT!** Do not freeze.
Purification Solution	White	○	2.06 mL	8.25 mL	
Elution Buffer	White	○	2.68 mL	10.75 mL	

[1] i7 indices allow up to 24 or 96 samples to be multiplexed, are included in the kit (supplied in 7X Index Primer Plate format). Each well in the 7X Index Primer Plate contains 5 µL of primer, sufficient for amplification/barcoding of one library. See "i7 index sequences and locations in the primer plate" on page 7.

i7 index sequences and locations in the primer plate

i7 index sequences (Table 1) and the location of the indices in the 7X i7 Index Primer 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
7001	CAGCGT	7025	TTTATG	7049	GTGCCA	7073	GACATC
7002	GATCAC	7026	AACGCC	7050	TCGAGG	7074	CGATCT
7003	ACCAGT	7027	CAAGCA	7051	CACTAA	7075	CGTCGC
7004	TGCACG	7028	GCTCGA	7052	GGTATA	7076	ATGGCG
7005	ACATTA	7029	GCGAAT	7053	CGCCTG	7077	ATTGGT
7006	GTGTAG	7030	TGGATT	7054	AATGAA	7078	GCCACA
7007	CTAGTC	7031	ACCTAC	7055	ACAACG	7079	CATCTA
7008	TGTGCA	7032	CGAAGG	7056	ATATCC	7080	AACAAG
7009	TCAGGA	7033	AGATAG	7057	AGTACT	7081	GCAGCC
7010	CGGTTA	7034	TTGGTA	7058	ATAAGA	7082	ACTCTT
7011	TTAACT	7035	GTTACC	7059	GGTGAG	7083	TGCTAT
7012	ATGAAC	7036	CGCAAC	7060	TTCCGC	7084	AAGTGG
7013	CCTAAG	7037	TGGCGA	7061	GAAGTG	7085	CTCATA
7014	AATCCG	7038	ACCGTG	7062	CAATGC	7086	CCGACC
7015	GGCTGC	7039	CAACAG	7063	ACGTCT	7087	GGCCAA
7016	TACCTT	7040	GATTGT	7064	CAGGAC	7088	AGACCA
7017	TCTTAA	7041	CTCTCG	7065	AAGCTC	7089	CGCGGA
7018	GTCAGG	7042	TGACAC	7066	GACGAT	7090	CCTGCT
7019	ATACTG	7043	AAGACA	7067	TCGTTC	7091	GCGCTG
7020	TATGTC	7044	ACAGAT	7068	CCAATT	7092	GAACCT
7021	GAGTCC	7045	TAGGCT	7069	AGTTGA	7093	TTCGAG
7022	GGAGGT	7046	CTCCAT	7070	AACCGA	7094	AGAATC
7023	CACACT	7047	GCATGG	7071	CAGATG	7095	AGGCAT
7024	CCGCAA	7048	AATAGC	7072	GTAGAA	7096	ACACGC

Table 2 Location of i7 indices in 7X i7 Index Primer Plate for 96 preps

	1	2	3	4	5	6	7	8	9	10	11	12
A	7001	7009	7017	7025	7033	7041	7049	7057	7065	7073	7081	7089
B	7002	7010	7018	7026	7034	7042	7050	7058	7066	7074	7082	7090
C	7003	7011	7019	7027	7035	7043	7051	7059	7067	7075	7083	7091
D	7004	7012	7020	7028	7036	7044	7052	7060	7068	7076	7084	7092
E	7005	7013	7021	7029	7037	7045	7053	7061	7069	7077	7085	7093
F	7006	7014	7022	7030	7038	7046	7054	7062	7070	7078	7086	7094
G	7007	7015	7023	7031	7039	7047	7055	7063	7071	7079	7087	7095
H	7008	7016	7024	7032	7040	7048	7056	7064	7072	7080	7088	7096

Table 3 Location of i7 indices in 7X i7 Index Primer Plate for 24 preps

	1	2	3	4	5	6	7	8	9	10	11	12
A	7001	7009	7017									
B	7002	7010	7018									
C	7003	7011	7019									
D	7004	7012	7020									
E	7005	7013	7021									
F	7006	7014	7022									
G	7007	7015	7023									
H	7008	7016	7024									

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:	
ProFlex™ 96-well PCR System	4484075
Veriti™ 96-Well Thermal Cycler	4375786

Item	Source
Magnetic rack – one of the following:	
DynaMag™-2 Magnet (for 1.5-mL tubes)	12321D
DynaMag™-96 Side Magnet (for 96-well 0.2-mL plates)	12331D
Other equipment and reagents	
Agilent™ 2100 Bioanalyzer™ instrument[1]	Agilent™, G2938A
Agilent™ High Sensitivity DNA Kit[1]	Agilent™, 5067-4626
Centrifuge with plate rotor	MLS
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
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
Water, nuclease free	R0581 R0582
(Optional) Agilent™ RNA 6000 Pico Kit	Agilent™, 5067-1513
(Optional) Qubit™ 4 Fluorometer	Q33226
(Optional) Qubit™ RNA BR Assay Kit	Q10210
(Optional) RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE	AM1975
(Optional) Collibri™ Library Quantification Kit	A38524100 A38524500
(Optional) ERCC RNA Spike-In Mix	4456740
(Optional) ERCC ExFold RNA Spike-In Mix	4456739
(Optional) Universal Human Reference RNA	QS0639
(Optional) Human Brain Total RNA	AM7962

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

Technology overview

The Collibri™ 3′ mRNA Library Prep Kit contains the Illumina® Read 1 linker sequence in the second strand synthesis primer, hence NGS reads are generated towards the poly(A) tail, directly reflecting the mRNA sequence. This version is recommended for gene expression analysis.

Reverse transcription

The Collibri™ 3′ mRNA Library Prep Kit uses total RNA as an input, therefore there is no need for prior poly(A) enrichment or rRNA depletion. First strand synthesis is initiated by oligodT priming. An oligodT primer containing an Illumina®-compatible sequence at its 5′ end is hybridized to the RNA and reverse transcription is performed.

RNA removal

After the first strand synthesis RNA is removed by heat treatment. During this step the RNA template is degraded. It is essential for efficient second strand synthesis.

Second strand synthesis

Second strand synthesis is initiated by random priming. The random primer contains Illumina®-compatible linker sequence at its 5′ end. During this step the library is converted to dsDNA. Magnetic bead-based purification step is required after second strand synthesis.

Library amplification

The libraries are amplified introducing sequences required for cluster generation. Up to 96 i7 indices can be used in library amplification for multiplexing. Library amplification generates sufficient material for quality control and sequencing.

Cleanup and quantification

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

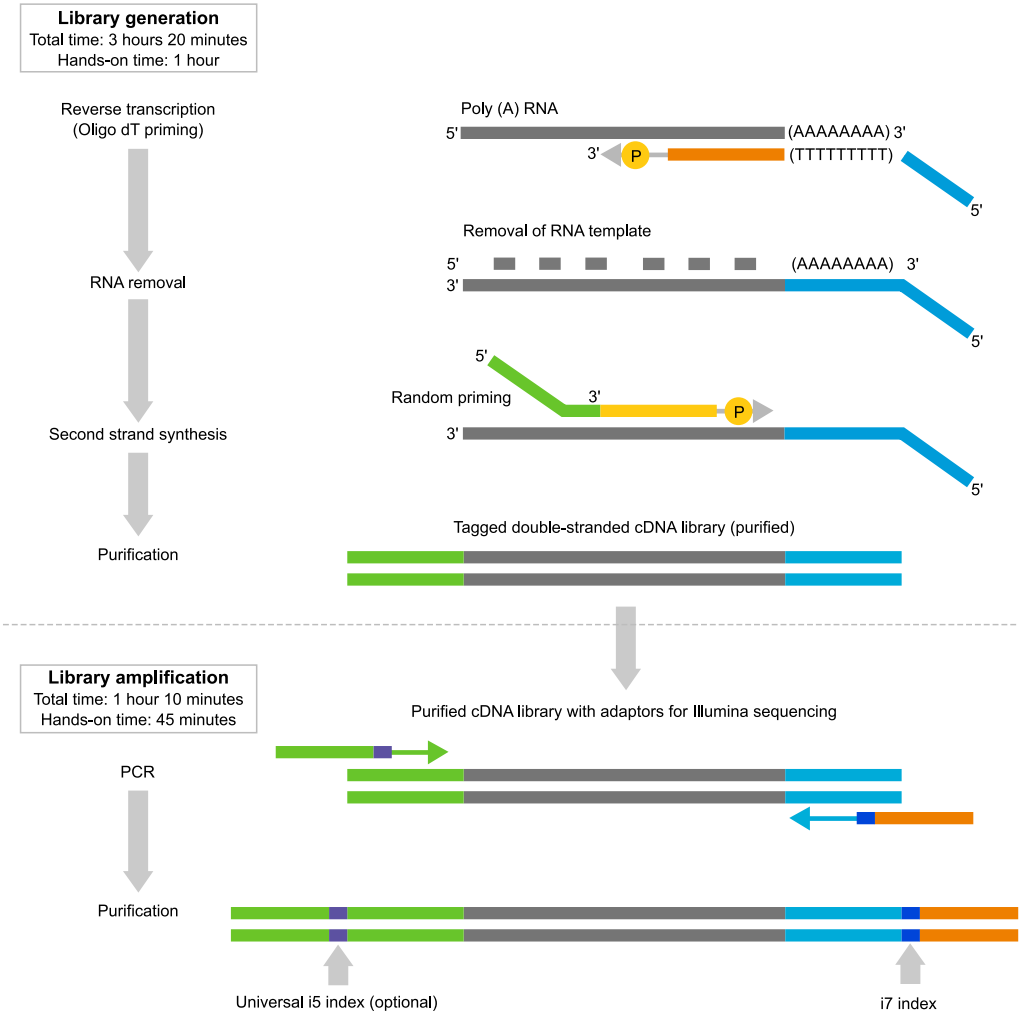

Figure 1 Simplified schematic representation of technology used in the Collibri™ 3' mRNA Library Prep Kit

Workflow

Figure 2 illustrates the Collibri™ 3′ mRNA Library Prep Kit workflow to construct sequencing-ready cDNA libraries.

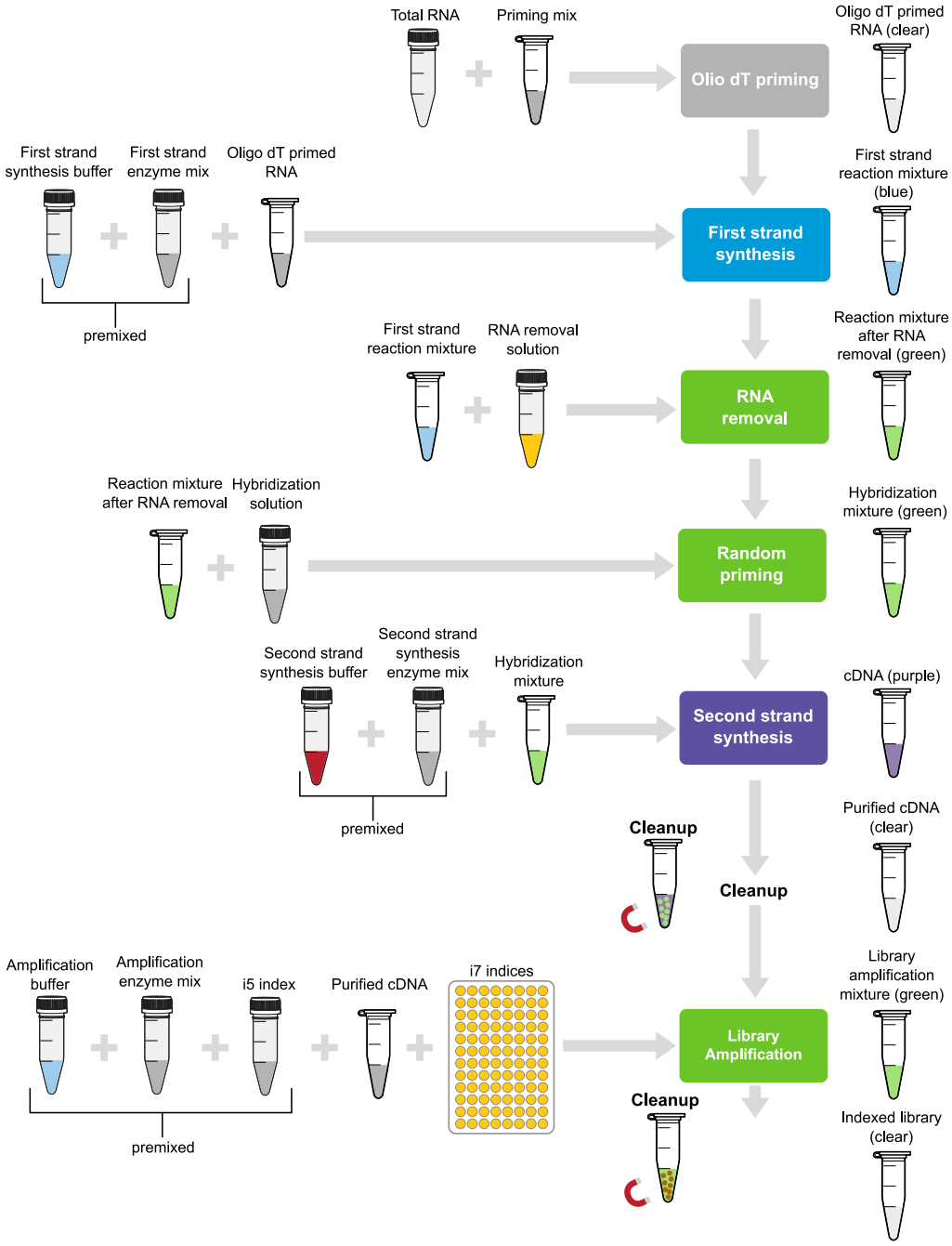

Figure 2 Collibri™ 3′mRNA Library Prep Kit workflow

Note: Kit components are colored with inert dyes to provide a visual control of the proper workflow progress – reaction mix changes color in different stages ensuring that right component is added.

Methods

Important procedural guidelines

Guidelines for RNA sample type and amount

- The Collibri™ 3′ mRNA Library Prep Kit is suitable for library preparation using 100 pg– 500 ng of total RNA.
- Inputs of >200 ng are recommended for efficient detection of low abundant transcripts. As a starting point, the protocol with 500 ng total RNA is recommended.
- The maximum recommended input is 500 ng.
- The minimum recommended input amounts of high-quality total RNA are 100 pg for Collibri™ 3′ mRNA Library Prep. When using input amounts ≤1 ng of total RNA, PCR cycle optimization is required and including a no-input control is strongly recommended.
- Lower RNA inputs (≤10 ng), and low quality RNA samples require protocol modifications “Modified protocol for low input / low quality / FFPE RNA samples“ on page 17, including adjusting the number of PCR cycles
- 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.).
- Concentrate RNA in volumes <5 µL by ethanol precipitation, bead purification, or column-based methods before library preparation.
- We recommend that you assess the quality and size distribution of the input RNA using the Agilent™ RNA 6000 Pico assay (Agilent™, Cat. No. 5067-1513). Most microfluidics platforms will carry out an automated peak analysis and generate a quality score (RIN). As Collibri™ 3′ mRNA Library Prep Kit targets the 3′ end of transcripts even RNAs with a lower RIN are suitable as input material.
- Mitochondrial ribosomal RNAs (mt-rRNAs) are polyadenylated and hence will also be reverse transcribed and converted into a cDNA library. mt-rRNAs can make up 1 - 2 % of the reads when using a 3′ mRNA-Seq protocol. Optional an rRNA depletion method, which also removes mt-rRNAs can be used before starting the Collibri™ 3′ mRNA library preparation if it is essential to remove mt-rRNA transcripts.
- 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. For RNA extraction from FFPE samples, we recommend using the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Cat. No. AM1975). The DV200 value which measures the percentage of RNAs larger than 200 nt in the sample, is a better measure of quality for highly degraded RNA and FFPE RNA samples with very low RIN scores.

Guidelines for RNA controls

- We recommend that you add ERCC RNA Spike-In Control Mixes to the input RNA before library preparation. 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 thus causing ERCC to be overrepresented in the final library.
- For positive RNA control, we recommend using Universal Human Reference RNA (Cat. No. QS0639) or Human Brain Total RNA (Cat. No. AM7962).

Guidelines for bead handling

- Store the Purification Beads included in the Collibri™ 3′ mRNA Library Prep Kit at 2℃ to 8℃.
- Thoroughly resuspend the Purification Beads before usage. 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.
- When fully separated, the supernatant should be completely clear and the beads collected at one point or as a ring along the wall of the tube / well.

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® Hi-Seq™ 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 4 illustrates possible pooling strategies for 4-channel sequencing systems.

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

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

	Good		Bad
7008	TGTGCA	7023	CACACT
7016	TACCTT	7031	ACCTAC
7024	CCGCAA	7039	CAACAG
7032	CGAAGG	7055	ACAACG
	++++++		---+-+

First strand cDNA synthesis – reverse transcription

Required materials

Components from the Collibri™ 3′ mRNA Library Prep Kit:

- 2X Priming Mix
- First Strand Synthesis Buffer
- 40X First Strand Synthesis Enzyme Mix

Other materials:

- Total RNA (100 pg to 500 ng)
- Water, nuclease-free

Standard input protocol

Use this protocol when using >10 ng of total RNA. Protocol modifications are recommended for low input (≤10 ng), low quality, and FFPE RNA samples, see "Modified protocol for low input / low quality / FFPE RNA samples" on page 17.

1. Mix 10–500 ng of total RNA in a volume of 5 µL, with 5 µL 2X Priming Mix in a PCR tube or plate. Mix well by pipetting, spin down to collect the liquid at the bottom of the tube.

2. Denature prepared mixture for 3 min at 85°C in a thermocycler and then cool down to 42°C. Leave the reaction mixture at 42°C until step 4.

3. Prepare a First Strand Synthesis Master Mix containing 9.5 µL First Strand Synthesis Buffer and 0.5 µL First Strand Synthesis Enzyme Mix. Mix well, spin down, and pre-warm for 2–3 minutes at 42°C.

   ATTENTION: Do not cool Master Mixes on ice.

Component	Volume (+10%)				
	1 library	6 libraries	24 libraries	96 libraries	N libraries
First Strand Synthesis Buffer (blue)	10.45 µL	62.7 µL	250.8 µL	1003.2 µL	N × 10.45 µL
40X First Strand Synthesis Enzyme Mix	0.55 µL	3.3 µL	13.2 µL	52.8 µL	N × 0.55 µL
Total volume (blue mixture):	**11 µL**	**66 µL**	**264 µL**	**1056 µL**	**N × 11 µL**

4. Spin down denaturated RNA samples from step 2, place them back to 42°C. Add 10 µL prewarmed First Strand Synthesis Master Mix prepared at step 3. Mix by pipetting, briefly spin down the samples and incubate the reactions for 15 minutes at 42°C.

Modified protocol for low input / low quality / FFPE RNA samples

1. Prepare a First Strand Synthesis Master Mix containing 5 µL 2X Priming Mix, 9.5 µL First Strand Synthesis Buffer, 0.5 µL First Strand Synthesis Enzyme Mix. Mix well, spin down, and pre-warm for 2–3 minutes at 42°C.

Component	Volume (+10%)				
	1 library	6 libraries	24 libraries	96 libraries	N libraries
2X Priming Mix	5.5 µL	33 µL	132 µL	528 µL	N × 5.5 µL
First Strand Synthesis Buffer (blue)	10.45 µL	62.7 µL	250.8 µL	1003.2 µL	N × 10.45 µL
40X First Strand Synthesis Enzyme Mix	0.55 µL	3.3 µL	13.2 µL	52.8 µL	N × 0.55 µL
Total volume (blue mixture):	**16.5 µL**	**99 µL**	**396 µL**	**1584 µL**	**N × 16.5 µL**

2. Add 15 µL of the pre-warmed First Strand Synthesis Master Mix to each 5 µL RNA sample, mix well, spin down briefly and incubate the reaction for 60 minutes at 42°C.

IMPORTANT! Do not cool the samples below room temperature after reverse transcription. Proceed immediately to RNA Removal step.

RNA removal

Required materials

5X RNA Removal Solution from the Collibri™ 3′ mRNA Library Prep Kit.

Standard protocol

Note: At this point 3.5X Hybridization Mix should be placed at 37 ℃ to thaw. If precipitate is visible, incubate and mix until buffer components dissolve completely.

1. Add 5 µL of RNA Removal Solution to 20 µL reaction mixture after first strand synthesis. Mix well, spin down briefly.

Component	Volume
First Strand Synthesis Reaction Mixture (blue)	20 µL
RNA Removal Solution (yellow)	5 µL
Total volume (green mixture):	**25 µL**

2. Incubate for 10 minutes at 95°C, then cool down to 25°C. Proceed immediately to second strand synthesis step.

Modified protocol for ≤1 ng total RNA input

IMPORTANT! Incubation time should be reduced to 5 min at 95°C, then using input lower than 1 ng of total RNA.

Second strand synthesis

Required materials

Components from the Collibri™ 3′ mRNA Library Prep Kit:

- 3.5X Hybridization Mix
- 10X Second Strand Synthesis buffer
- 40X Second Strand Synthesis Enzyme Mix

Perform second strand synthesis

Note: At this point Purification Beads, Purification Solution and Elution Buffer should be placed at room temperature to equilibrate.

1. Add 10 µL of 3.5X Hybridization Mix to each reaction. Mix well by pipetting and spin down.
2. Incubate for 1 minute at 98°C in a thermocycler and slowly cool down to 25°C by setting the ramp rate to 0.5°C/second (10–15 % of the maximum rate for most thermocyclers). Incubate the reaction for 30 min at 25°C.
3. Prepare the Second Strand Synthesis Master Mix containing 4 µL 10X Second Strand Synthesis Buffer and 1 µL 40X Second Strand Synthesis Enzyme Mix.

Component	Volume (+10%)				
	1 library	6 libraries	24 libraries	96 libraries	N libraries
10X Second Strand Synthesis Buffer (red)	4.4 µL	26.4 µL	105.6 µL	422.4 µL	N × 4.4 µL
40X Second Strand Synthesis Enzyme Mix	1.1 µL	6.6 µL	26.4 µL	105.6 µL	N × 1.1 µL
Total volume (orange mixture):	**5.5 µL**	**33 µL**	**132 µL**	**528 µL**	**N × 5.5 µL**

4. Add 5 µL Second Strand Synthesis Master Mix to Hybridization reaction mixture from step 2.

Component	Volume
Hybridization Reaction Mixture (green)	35 µL
Second Strand Synthesis Master Mix (orange)	5 µL
Total volume (purple mixture):	**40 µL**

5. Incubate for 15 minutes at 25°C, briefly spin down.

Safe stopping point

Proceed with cleanup or store samples at –20°C.

Purify the double stranded cDNA

Required materials

Components from the Collibri™ 3′ mRNA Library Prep Kit (equilibrated to room temperature):

- Purification Beads
- Purification Solution
- Elution Buffer

Other materials and equipment:

- 96% ethanol.
- Magnetic rack (see "Required materials not supplied" on page 8)

Before you begin

- Prepare fresh 80% Ethanol solution.
- Ensure that all components are at room temperature.
- Gently vortex the Purification Beads to completely resuspend the magnetic beads in the solution.
- If the libraries were stored at −20°C, ensure that they are thawed and equilibrated to room temperature, and spun down before starting the protocol.

Purify the cDNA

1. Add 16 µL of Purification Beads to ds cDNA sample. Mix well, briefly spin down to collect all the droplets and incubate for 5 minutes at room temperature.
2. Place the tube/plate on a magnetic stand and let the beads collect for 2–5 min or until the supernatant is completely clear.
3. Remove and discard supernatant without removing tube / plate from the magnet. Make sure not to disturb the beads.

   Note: If the pellet of magnetic particles was disturbed, mix the sample and let the beads settle to the side of the tube or plate on the magnet again.
4. Add 40 µL of Elution Buffer to the sample, resuspend magnetic beads fully and incubate for 2 minutes at room temperature.
5. Add 56 µL of Purification Solution to the beads / Elution Buffer mixture. Mix thoroughly and incubate for 5 minutes at room temperature.

   Note: For low input/low quality/FFPE RNA add only 48 µL of Purification Solution.
6. Place the tube/plate onto magnetic rack and let the beads collect for 2–5 min or until the supernatant is completely clear.
7. Remove and discard supernatant without removing tube/plate from the magnet. Make sure not to disturb the beads.
8. Keeping the tube / plate on the magnetic rack add 120 µL of 80% Ethanol and incubate for 30 seconds. Remove and discard the supernatant.

   IMPORTANT! Leave the plate in contact with the magnet as beads should not be resuspended during this washing step.

9. Repeat washing step for a total of two washes. Remove the supernatant completely, as traces of ethanol can inhibit subsequent reactions.

   Note: To remove the residual ethanol, briefly centrifuge the tube or plate, place it back on the magnetic rack, then carefully remove any remaining supernatant with a pipette without disturbing the pellet.

10. Keeping the reaction tube or plate on the magnet, air dry the magnetic particles for 2–5 minutes at room temperature or until there are no droplets of ethanol left on the walls of the tube or plate.

    IMPORTANT! Dry the beads at room temperature only and do not let the beads dry too long (visible cracks appear), this will negatively influence the elution and the resulting library yield.

11. Add 20 µL of Elution Buffer, resuspend magnetic beads fully and incubate for 2 minutes at room temperature.

12. Place the tube/plate on a magnetic rack and let the beads collect for 2–5 minutes or until the supernatant is completely clear.

13. Transfer 17 µL of the supernatant into a fresh PCR tube or plate. Do not transfer any beads.

Safe stopping point

Proceed with library amplification or store samples at −20°C.

Library amplification (Single indexing PCR)

Required materials

Components from the Collibri™ 3′ mRNA Library Prep Kit:

- 5X Library Amplification Buffer
- i5 Universal Primer
- 35X Library Amplification Enzyme Mix
- 7X i7 Index Primer Plate

Amplify the cDNA

1. Prepare PCR Master Mix containing 7 µL 5X Library Amplification Buffer, 5 µL i5 Universal Primer and 1 µL 35X Library Amplification Enzyme Mix.

Component	Volume (+10%)				
	1 library	6 libraries	24 libraries	96 libraries	N libraries
5X Library Amplification Buffer	7.7 µL	46.2 µL	184.8 µL	739.2 µL	N × 7.7 µL
i5 Universal Primer	5.5 µL	33 µL	132 µL	528 µL	N × 5.5 µL
35X Library Amplification Enzyme Mix	1.1 µL	6.6 µL	26.4 µL	105.6 µL	N × 1.1 µL
Total volume (blue mixture):	**14.3 µL**	**85.8 µL**	**343.2 µL**	**1372.8 µL**	**N × 14.3 µL**

2. Add 13 µL of PCR Master Mix to each tube containing 17 µL of purified cDNA sample.

3. Add 5 µL of selected indices from 7X i7 Index Primer Plate to each PCR reaction.

Component	Volume
Purified cDNA	17 µL
PCR Master Mix (blue)	13 µL
7X i7 Index Primer (yellow)	5 µL
Total volume (green mixture):	**35 µL**

IMPORTANT! Before use, centrifuge the 7X i7 Index Primer Plate to collect all the droplets to the bottom of the well and to avoid cross-contamination of indices.

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

Stage	Number of cycles[1]	Temperature	Time
Initial denaturation	1	98°C	30 seconds
Denature	21–25 (500 pg of input RNA) 17–20 (10 ng of input RNA) 14–17 (100 ng of input RNA) 11–14 (500 ng of input RNA)	98°C	10 seconds
Anneal		65°C	20 seconds
Extend		72°C	30 seconds
Final extension	1	72°C	1 minute
Hold	1	10°C	hold

[1] The number of PCR cycles depends on the starting amount of RNA (i.e., input RNA). These values are provided as a reference. Sample type influences the optimal cycle number hence the optimization for number of PCR cycles is recommended.

Library purification

Required materials

Components from the Collibri™ 3′ mRNA Library Prep Kit (equilibrated to room temperature):

- Purification Beads
- Purification Solution
- Elution Buffer

Other materials and equipment:

- 96% ethanol.
- Magnetic rack (see “Required materials not supplied“ on page 8)

Before you begin

- Prepare fresh 80% Ethanol solution.
- Ensure that all components are at room temperature.
- Gently vortex the Purification Beads to completely resuspend the magnetic beads in the solution.
- If the libraries were stored at –20°C, ensure that they are thawed and equilibrated to room temperature, and spun down before restarting the protocol.

Purify the amplified libraries

1. Add 35 µL of Purification Beads to each reaction. Mix well, briefly spin down to collect all the droplets and incubate for 5 minutes at room temperature.

   Note: for low input/low quality/FFPE RNA add 31.5 µL of Purification Beads.

2. Place the tube/plate on a magnetic stand and let the beads collect for 2–5 min or until the supernatant is completely clear.

3. Remove and discard supernatant without removing tube/plate from the magnet. Make sure not to disturb the beads.

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

4. Add 30 µL of Elution Buffer to the sample, resuspend magnetic beads fully and incubate for 2 minutes at room temperature.

5. Add 30 µL of Purification Solution to the beads/Elution Buffer mixture. Mix thoroughly and incubate for 5 minutes at room temperature.

6. Place the tube/plate onto magnetic rack and let the beads collect for 2–5 min or until the supernatant is completely clear.

7. Remove and discard supernatant without removing tube/plate from the magnet. Make sure not to disturb the beads.

8. Keeping the tube/plate on the magnetic rack add 120 µL of 80% Ethanol and incubate for 30 seconds. Remove and discard the supernatant.

   IMPORTANT! Leave the plate in contact with the magnet as beads should not be resuspended during this washing step.

9. Repeat washing step for a total of two washes. Remove the supernatant completely, as traces of ethanol can inhibit subsequent reactions.

   Note: To remove the residual ethanol, briefly centrifuge the tube or plate, place it back on the magnetic rack, then carefully remove any remaining supernatant with a pipette without disturbing the pellet.

10. Keeping the reaction tube or plate on the magnet, air dry the magnetic particles for 2–10 minutes at room temperature or until there are no droplets of ethanol left on the walls of the tube or plate.

    IMPORTANT! Dry the beads at room temperature only and do not let the beads dry too long (visible cracks appear), this will negatively influence the elution and the resulting library yield.

11. Add 20 µL of Elution Buffer, resuspend magnetic beads fully and incubate for 2 minutes at room temperature.

12. Place the tube/plate on a magnetic rack and let the beads collect for 2–5 minutes or until the supernatant is completely clear.

13. Transfer 18 µL of the supernatant into a fresh tube or plate. Do not transfer any beads.

Safe stopping point

Proceed with quality control, quantification and sequencing or store samples at −20°C.

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)

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. Analyze 1 µL of the prepared library using the appropriate chip on the Agilent™ 2100 Bioanalyzer™ instrument with the Agilent™ High Sensitivity DNA Kit.
2. Using the 2100 Expert software, perform a smear analysis to determine the average library length. Set the range to 160 - 1,000 bp to include the whole library size distribution, and to exclude any linker-linker artifacts (150 bp), or overcycling bumps (>1,000 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

Collibri™ 3′ mRNA libraries are intended for a high degree of multiplexing, and hence libraries do not need to be extensively amplified. Library yield, shape, and average insert size may vary depending on the type of input sample (e.g., FFPE samples typically produce shorter libraries than high quality Universal Human Reference RNA (UHRR), see Figure 4). The majority of inserts are greater than 75 bp in size, corresponding to final library fragment sizes ≥200 bp.

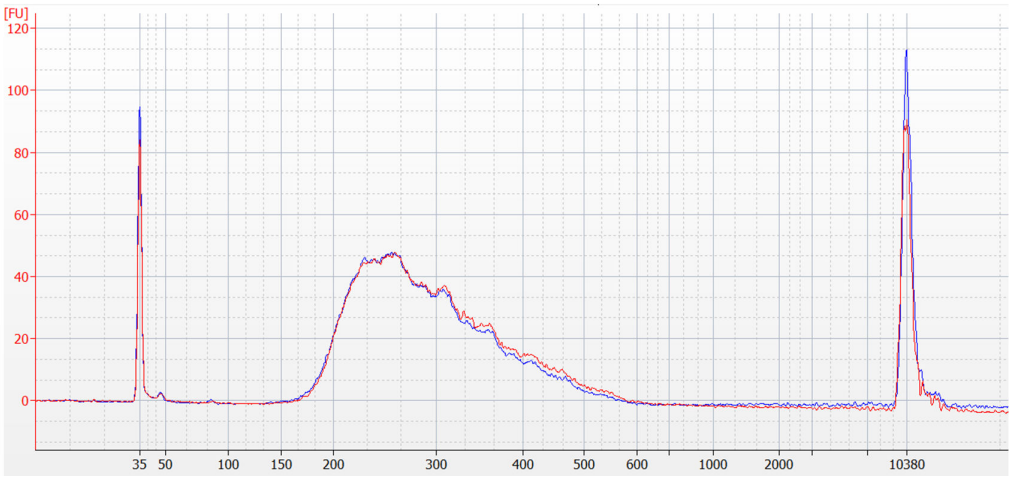

Figure 4 Typical Agilent™ 2100 Bioanalyzer™ instrument trace of libraries prepared from Universal Human Reference RNA using the Collibri™ 3′ Library Prep Kit. Two replicates of libraries prepared from 500 ng total RNA (reverse transcription for 15 minutes at 42°C for both, RNA removal using 10 minute incubation at 95°C, 13 PCR cycles).

Next steps

Quantify the prepared library by qPCR

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

Sequence the prepared library

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

It is not recommended to multiplex Collibri™ 3′ mRNA libraries with libraries prepared with other kits in the same sequencing lane.

IMPORTANT! Paired-end sequencing is not recommended for Collibri™ 3′ mRNA libraries as the quality of Read 2 would be very low due to the poly(T) stretch at the beginning of Read 2. In case Collibri™ 3′ mRNA libraries are sequenced in paired-end mode, read 2 should be discarded and downstream data analysis should be performed using only read 1. Read 1 read quality is not adversely affected in paired-end runs.

Data analysis

- Demultiplexing can be carried out by the standard Illumina® pipeline. i7 index sequences indicated in a section: “Kit contents and storage”.
- We recommend the use of a general fastq quality control tool such as FastQC or NGS QC Toolkit to examine the quality of the sequencing run. These tools can also identify over-represented sequences, which may optionally be removed from the dataset.
- Use trimmer software (e.g., the Cutadapt command-line program) to trim the reads to remove adapter sequences, poly(A) / poly(T) sequences, and low quality nucleotides. Reads that are too short (i.e., <20 nt) or have generally low quality scores should be removed from the set.
- In addition, for Collibri™ 3′ mRNA libraries, as second strand synthesis is based on random priming, there is a higher proportion of mismatches over the first 12 nt of the reads. For Collibri™ 3′ mRNA data we therefore recommend using an aligner that can perform soft-clipping of the read ends (e.g., STAR aligner) during alignment, or increasing the number of allowed mismatches to 14. Alternatively, trimming the first 12 nt of Read 1 can be performed prior to alignment when using a more stringent aligner (e.g., HISAT2). While trimming the read can decrease the number of reads of suitable length for alignment, the absolute number of mapping reads may increase due to the improved read quality.
- After filtering and trimming, reads can be aligned with a short read aligner to the reference genome. We recommend the use of STAR aligner for mapping Collibri™ 3′ mRNA data. The reads may not land in the last exon and span a junction hence splice-aware aligners should be used. Bowtie2 or BWA can also be used for mapping against a reference transcriptome.

- Mapping only the 3′ end of transcripts requires an annotation that covers the 3′ untranslated region (UTR) accurately. Conservative annotations might decrease the power of correct gene quantification after mapping. For some gene annotations it might be an advantage to extend the 3′ UTR annotation further downstream in order to assign the mapped read correctly.

  Command line examples for data analysis workflow steps:

  Trimming:

  cutadapt -m 20 -O 20 -n 2 -a "polyA=A{20}" -a "QUALITY=G{20}" input.fastq.gz | cutadapt -m 20 --nextseq-trim=10 -a "truseq=A{18}AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC" - | cutadapt -m 20 -O 20 -g "truseq=A{18}AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC" --discard-trimmed -o output_trimmed.fastq.gz -

  Alignment (using prebuild STAR genome):

  STAR --readFilesCommand zcat --genomeDir "PREBUILD_GENOME_DIR" --genomeLoad LoadAndRemove --readFilesIn "output_trimmed.fastq.gz" --outFilterType BySJout --outFilterMultimapNmax 20 --alignSJoverhangMin 8 --alignSJDBoverhangMin 1 --outFilterMismatchNmax 999 --outFilterMismatchNoverLmax 0.6 --alignIntronMin 20 --alignIntronMax 1000000 --alignMatesGapMax 1000000 --outSAMtype BAM –outFileNamePrefix "ALIGNMENT/"

- Optionally, data analysis can be done using the **Genialis Data Analysis Software**.

A Troubleshooting

Observation	Possible cause	Recommended action
Low yield and/or poor size distribution obtained in the amplified library	Low recovery rates after cleanup.	• Make sure that the Purification Beads are brought to room temperature and the magnetic particles are thoroughly resuspended before use. • Do **not** resuspend magnetic particles in Ethanol during the wash steps.
	Suboptimal number of PCR cycles.	Optimize the number of PCR cycles.
Extremely low yield and/or no PCR products	Used input RNA of lower quality.	• Check the quality of used total RNA using microfluidics assay such as Agilent™ RNA 6000 Pico assay. • Prolong First Strand Synthesis reaction to 1 hour.
Residual adaptors or adaptor dimers are visible on Agilent™ Bioanalyzer™ trace.	Inefficient cleanup after the second strand synthesis or PCR steps.	Repurification is recommended. • Adjust the total sample volume to 20 µL using Elution Buffer or nuclease-free water. • Add 0.9 volume of Purification Beads (20 µL of sample mixed with 18 µL of Purification Beads). • Continue with amplified libraries purification protocol from step 2.
The color of First Strand Synthesis reaction mixture is not blue.	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 solutions carefully to aspirate the correct volume.
The color of RNA removal reaction mixture is not green.		
The color of Second Strand Synthesis reaction mixture is not purple.		
The color of reaction mixture after the setup of PCR reaction is not green.		

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, and so on). 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 sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer 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 needed) 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.

WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

WARNING! 4L Reagent and Waste Bottle Safety. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, 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:
  https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2009-P.pdf
- World Health Organization, *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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  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

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

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.