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ZYMO RESEARCH

RNA - Seq



Made Simple™

Zymo-Seq SwitchFree™ 3' mRNA Library Kit

For simple and efficient mRNA expression analysis.

Highlights

- **Simplest protocol:** RNA to library in less time with easy handling driven by the SwitchFree™ technology.
- **High performance:** Built-in Unique Molecular Identifiers (UMIs) allow for accurate deduplication maximizing unique reads.
- **Low input compatible:** Utilize as little as 10 ng total RNA without prior mRNA enrichment.

Catalog Numbers:
R3008 & R3009



Scan with your smart-phone camera to
view the online protocol/video.



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Table of Contents

Product Contents	01
Specifications	02
Product Description	03
Protocol	04
Buffer Preparation.....	04
Before Starting	04
Section 1: cDNA Synthesis	05
Section 2: Adapter Ligation.....	07
Section 3: Library Amplification.....	08
Appendices	10
A: Select-a-Size MagBead Clean-up Protocol	10
B: Unique Dual Index (UDI) Primer Sets	11
C: Library Characterization	12
D: Considerations for Sequencing	13
E: Bioinformatic Analysis	14
Ordering Information	16
Complete Your Workflow	17
Notes	18
Guarantee	21

Product Contents

Zymo-Seq SwitchFree™ 3' mRNA Library Kit	R3008 (12 preps)	R3009 (96 preps)	Storage Temperature
PolyA R1 Reagent	60 µL	60 µL × 8	-80 °C
R2 Reagent	120 µL	120 µL × 8	-80 °C
Reaction Clean-up Solution	50 µL	50 µL × 8	-80 °C
3'mRNA L Reagent	180 µL	180 µL × 8	-80 °C
ZymoTaq™ PreMix	300 µL	300 µL × 8	-80 °C
Zymo-Seq™ UDI Primer Sets (Indexes 1-12) ¹	20 µL / Index	-	-80 °C
Zymo-Seq™ UDI Primer Plate (Indexes 1-96) ²	-	10 µL / Index	-80 °C
Select-a-Size MagBead Concentrate	300 µL	1.5 mL	4 °C
Select-a-Size MagBead Buffer	10 mL	50 mL	4 °C
Library Binding Solution	1 mL	10 mL	4 °C
DNA Wash Buffer (concentrate)	6 mL	48 mL	Room Temp.
DNA Elution Buffer	1 mL x 2	16 mL	Room Temp.
DNase/RNase-Free Water	1 mL × 2	10 mL	Room Temp.
Instruction Manual	1	1	-

1 The provided **Zymo-Seq™ UDI Primer Set** (Indexes 1-12) (Cat. No. D3008) contains 12 pre-mixed unique dual index barcode primers in 1.5 mL tubes. Please refer to **Appendix B** for primer specifications and index sequences.

2 The provided **Zymo-Seq™ UDI Primer Plate** (Indexes 1-96) (Cat. No. D3096) contains 96 pre-mixed unique dual index barcode primers in a 96-well plate format. Please refer to **Appendix B** for primer specifications and index sequences.

Specifications

- **RNA Input** – Compatible with 10 – 500 ng of total RNA.
- **Input Quality** – RNA should be free of DNA contamination and enzymatic inhibitors, with A260/A280 and A260/A230 ≥ 1.8 . RNA with lower purity ratios (A260/A280 and A260/A230) should be treated with DNase I and purified with the **RNA Clean & Concentrator™ (Cat. No. R1013)** prior to processing. RNA should be suspended in water, TE, or a low-salt buffer.
 - Intact RNA (RNA Integrity Number or RIN ≥ 8.0) performs optimally with this kit.
 - For degraded RNA input, e.g., those with a size distribution peaking at ≤ 500 nt, or RIN < 4 , see [Section 1, Step 4](#) for the suggested modification. Additionally, use a higher amount of input whenever possible.
- **Processing Time** – ~4 hours¹
- **Sequencing Platform Compatibility** – Libraries are compatible with all Illumina® sequencing platforms except HiSeq® X.²
- **Equipment Needed (user provided)** – Thermal cycler with heated lid, magnetic stand for 0.2 mL PCR tubes, and microcentrifuge for 0.2 mL PCR tubes and 1.5 mL microcentrifuge tubes.
- **Library Storage** – Libraries eluted in DNA Elution Buffer (provided) may be stored at $\leq 4^{\circ}\text{C}$ overnight or $\leq -20^{\circ}\text{C}$ for long-term storage.

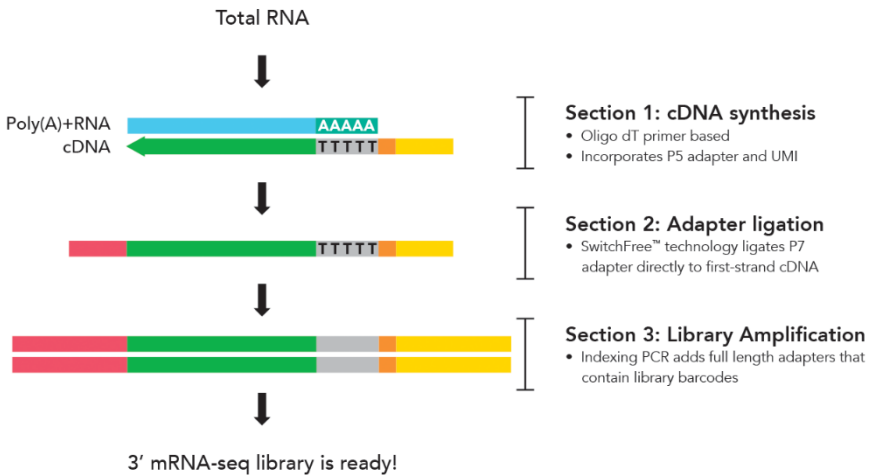
¹ Estimated based on processing ≤ 8 samples at a time. Handling more samples simultaneously may require longer processing time.

² Illumina® originally limits the applications on HiSeq® X exclusively for whole-genome libraries. Please confirm with the sequencing service provider for acceptability and additional details if expecting to sequence Zymo-Seq SwitchFree™ 3' mRNA libraries on HiSeq® X Series sequencers.

Product Description

The **Zymo-Seq SwitchFree™ 3' mRNA Library Kit** provides a simple workflow to prepare mRNA libraries in about 4 hours using as little as 10 ng of total RNA. This kit features built-in unique molecular identifiers (UMIs) within the workflow, allowing accurate evaluation of duplication rate and gene expression quantification. With enriched coverage at the 3' end of the poly(A)⁺ transcripts, this kit provides a great tool for protein-coding gene counting and differential gene expression analysis. This kit contains all the necessary reagents required for 3' mRNA library preparation.

Zymo-Seq SwitchFree™ 3' mRNA Library Kit Workflow



Quickly Prepare Libraries with Minimal Hands-on Time

Section	Thermal cycler/ Incubation Time	Hands-on Time ¹
1	43 min	25 min
2	50 min	25 min
3	~ 60 min	30 min
Total Processing Time	~ 4 hours	

¹ The hands-on time estimates are based on processing ≤8 samples at a time. Handling more samples simultaneously may require more hands-on time.

Protocol

Buffer Preparation

- ✓ Add 24 mL of 100% ethanol (26 mL of 95% ethanol) to the 6 mL **DNA Wash Buffer** (concentrate) (provided with Cat. No. R3008) or 192 mL of 100% ethanol (208 mL of 95% ethanol) to the 48 mL **DNA Wash Buffer** (concentrate) (provided with Cat. No. R3009).
- ✓ Prepare the **Select-a-Size MagBeads** at least 5 days in advance of library preparation. For optimal performance, use the assembled **Select-a-Size MagBeads** within 60 days.
 1. Add 300 μ L of the **Select-a-Size MagBead Concentrate** to every 10 mL of the **Select-a-Size MagBead Buffer**.
 - a. For Cat. No. R3008, 10 mL of **Select-a-Size MagBeads** can be prepared in its entirety.
 - b. For Cat. No. R3009, 50 mL of **Select-a-Size MagBeads** can be prepared in its entirety, or multiple 10-mL **Select-a-Size MagBead** aliquots can be prepared as needed for processing smaller numbers of samples at a time.
 2. Resuspend the mixture by pipetting up and down and vortexing. Store at 4°C – 8 °C.

Before Starting:

- ✓ Set the thermal cycler lid temperature to 100-105°C for each program unless instructed otherwise.
- ✓ Thaw and store all -80°C reagents on ice prior to use. Mix reagents by flicking and centrifuge briefly.
- ✓ Allow the **Select-a-Size MagBeads** to equilibrate to room temperature for \geq 30 minutes prior to use.
- ✓ Resuspend the **Select-a-Size MagBeads** immediately before each use by vigorously inverting and vortexing until homogenous.
- ✓ Avoid multiple freeze-thaws of the -80°C components. Make aliquots as necessary.
- ✓ Using a multichannel pipette to transfer reagents will minimize hands-on time when processing multiple samples.
- ✓ **For new users, please read Appendix A: Select-a-Size MagBead Clean-up Protocol (page 10) carefully before proceeding.**

Section 1: cDNA Synthesis

1. Set up the following thermal cycler program (reaction volume: 24 μ L).

Step	Temperature	Time
1	95°C	3 min
2	4°C	hold
3	45°C	20 min
4	42°C	hold
5	37°C	15 min
6	98°C	5 min
7	4°C	hold

2. Transfer the input RNA (10 – 500 ng and \leq 5 μ L) into a 0.2 mL PCR tube. Use **DNase/RNase-Free Water** to raise the volume to 5 μ L. Place the sample on ice.
3. Add 5 μ L of **PolyA R1 Reagent** to the sample tube and mix thoroughly by pipetting. Centrifuge briefly.
4. *For degraded RNA input¹, skip Section 1, Step 4 and proceed immediately to Section 1, Step 5.* For intact RNA input, place the sample tube in the thermal cycler and run Steps 1-2 of the program. DO NOT remove the tube from the thermal cycler at the Step 2 hold.
5. Without removing the tube, add 10 μ L of **R2 Reagent** to the sample and mix thoroughly by pipetting.²
6. Close the thermal cycler lid and run Steps 3-4 of the program. DO NOT remove the tube from the thermal cycler at the Step 4 hold. *During this incubation, allow the **Reaction Clean-up Solution** to equilibrate to room temperature.*
7. Without removing the tube from the thermal cycler, add 4 μ L of **Reaction Clean-up Solution** to the sample and mix thoroughly by pipetting. Quickly remove the tube from the thermal cycler and centrifuge briefly to remove air bubbles. Immediately return the tube to the thermal cycler.
8. Close the thermal cycler lid and run Steps 5-7 of the program.

Continue Section 1 protocol on the next page.

¹ Please refer to **Specifications** on page 2 for guidance on evaluating input RNA quality.

² If needed, quickly remove the tube from the thermal cycler and centrifuge briefly to collect the reaction and remove air bubbles. Immediately return the tube to the thermal cycler to continue.

9. Remove the tube from the thermal cycler. Add 26 μL of 95% ethanol to the tube to raise the volume to 50 μL .
10. Follow the clean-up protocol (**Appendix A**, page 10) using 110 μL of **Select-a-Size MagBeads**. Elute with 10 μL of **DNA Elution Buffer**.

This is a safe stopping point. Purified cDNA can be safely stored at -20°C overnight. Thaw samples on ice before proceeding to the next section.

Section 2: Adapter Ligation

11. Create a thermal cycler incubation program for 3 min at 95°C for a reaction volume of 10 µL.
12. Place the tube containing the 10 µL eluate from Section 1, Step 10 into the thermal cycler and complete the 3-min incubation. Immediately transfer the tube to ice and incubate for 2 minutes.
13. Create a thermal cycler incubation program for 45 min at 25°C for a reaction volume of 25 µL. Set the lid temperature to 40°C.
14. Add 15 µL of **3'mRNA L Reagent** to the tube containing the 10 µL eluate. Mix thoroughly by pipetting and centrifuge briefly.
15. Place the tube in the thermal cycler and complete the 45-min incubation.¹
16. Remove the tube from the thermal cycler. Add 25 µL of **DNase/RNase-Free Water** to the tube to raise the volume to 50 µL.
17. Follow the clean-up protocol (**Appendix A**, page 10) using 100 µL of **Select-a-Size MagBeads**. Elute with 15 µL of **DNA Elution Buffer**.

This is a safe stopping point. Purified DNA can be safely stored at -20°C overnight. Thaw samples on ice before proceeding to the next section.

¹ Ensure that the thermal cycler temperature has reached 25°C (lid temperature at 40°C) before starting this incubation. If it is still cooling down, leave the sample tube on ice until the thermal cycler is ready.

Section 3: Library Amplification

18. Set up the following thermal cycler program (reaction volume: 50 μL). Adjust the total cycles based on the RNA input amount as listed on the right side of the table below.¹

Step	Temperature	Time	
1	95°C	10 min	
2	95°C	30 sec	} 500 ng = 13-14 cycles 100 ng = 15-17 cycles 10 ng = 19-21 cycles
3	58°C	30 sec	
4	72°C	60 sec	
5	--- Go to Step 2 ---		
6	72°C	7 min	
7	4°C	hold	

19. Add 10 μL of the appropriate **Index Primer Set²** to the tube containing the 15 μL eluate from Section 2, Step 17. Mix thoroughly by pipetting.
20. Add 25 μL of **ZymoTaq Premix** to the tube. Mix thoroughly by pipetting and centrifuge briefly.
21. Place the tube in the thermal cycler and run the program above. *During this incubation, allow the **Library Binding Solution** to equilibrate to room temperature.*
22. Remove the tube from the thermal cycler. Add 50 μL of **DNase/RNase-Free Water** to the tube to raise the volume to 100 μL .
23. Add 80 μL of **Select-a-Size MagBeads** to the tube. Mix thoroughly by pipetting until homogenous. Incubate for 5 minutes at room temperature.
24. Place the sample on a magnetic stand for 5 minutes, or until the beads have fully separated from the solution. Without dislodging the bead pellet, aspirate slowly and discard the supernatant.
25. Remove the sample from the magnetic stand. Add 100 μL of **DNA Elution Buffer** to the beads and mix thoroughly by pipetting up and down until homogenous.

Continue Section 3 protocol on the next page.

¹ Please note that the optimal PCR cycle number can vary depending on the quality of the input total RNA and should be optimized prior to working with precious samples.

² Please refer to **Appendix B** for guidance on selecting compatible index primer sets for multiplexing.

26. Add 80 μL of **Library Binding Solution** to the tube. Mix thoroughly by pipetting until homogenous. Incubate for 5 minutes at room temperature.
27. Follow the clean-up protocol (**Appendix A**, page 10) starting from Step 2. Elute with 15 – 20 μL of **DNA Elution Buffer**.

The eluate is your final 3' mRNA-seq library.¹ Libraries may be stored at 4°C overnight or $\leq -20^\circ\text{C}$ for long-term storage.

¹ Please refer to **Appendix C** for characterizing libraries generated with the kit.

Appendices

Appendix A: Select-a-Size MagBead Clean-up Protocol

Before starting:

- ✓ Allow the **Select-a-Size MagBeads** to equilibrate to room temperature for ≥ 30 minutes prior to use.
 - ✓ Resuspend the magnetic particles immediately before use by vigorously shaking or vortexing the **Select-a-Size MagBeads** until homogenous.
1. Add the indicated volume of **Select-a-Size MagBeads** to each sample. Mix thoroughly by pipetting until homogenous. Incubate for 5 minutes at room temperature.
 2. Place the sample on a magnetic stand for 5 minutes, or until the beads have fully separated from the solution. Without dislodging the bead pellet, aspirate slowly and discard the supernatant.
 3. While the sample is still on the magnetic stand, add 200 μL of **DNA Wash Buffer** without disturbing the bead pellet. Aspirate slowly and discard the supernatant without dislodging the bead pellet. Repeat this step for a total of 2 washes.
 4. While the sample is still on the magnetic stand, keep the tube cap open to air-dry the beads. After 1 minute, aspirate any residual **DNA Wash Buffer** that has collected at the bottom of the tube. Continue to air-dry until the bead pellet appears matte without cracking. See [footnote 1](#).
 5. Remove the sample from the magnetic stand. Add the indicated volume of **DNA Elution Buffer** to the beads and mix thoroughly by pipetting up and down until homogenous.
 6. Place the sample on the magnetic stand for 1-2 minutes or until the eluate is clear. Transfer the eluate to a new 0.2 mL PCR tube for each sample.

¹ The optimal air-dry time can vary depending on the humidity and temperature. **Optimally dried beads should appear matte without cracking.** Start with 5 minutes of air-dry time and adjust the time as needed to achieve optimally dried beads. Wash buffer carryover from insufficiently dried beads, and/or overdried, cracked beads can reduce nucleic acid recovery.

Appendix B: Unique Dual Index (UDI) Primer Sets

Indexes in the **Zymo-Seq™ UDI Primer Set (indexes 1-12)** are dispensed in 1.5 mL tubes (D3008), and indexes in the **Zymo-Seq™ UDI Primer Plate (Indexes 1-96)** are dispensed in single-use foil-sealed 96-well plates (D3096). Indexes come as pre-mixes, and the forward and reverse primers are provided at 5 µM total concentration (2.5 µM each).

The complete [index sample sheet](#) is available for download [here](#) (USA Only), or by visiting the Documents section of the **Zymo-Seq SwitchFree™ 3' mRNA Library Kit** product page at www.zymoresearch.com.

Primer Sequences:

Forward Primer Sequence (i5):

5'-AATGATACGGCGACCACCGAGATCTACACNNNNNNNNACACTCTTCCCTACACGACGCTCTTCCGATCT-3'

Reverse Primer Sequence (i7):

5'-CAAGCAGAAGACGGCATAACGAGATNNNNNNNNGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'

Note: NNNNNNNN correspond to the “Bases in Adapter” columns in the [index sample sheet](#) mentioned above.

UDI Primer Plate (D3096) Setup:

To use UDI primers, choose ≥ 2 sets down a column not across a row.

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDI_01	UDI_09	UDI_17	UDI_25	UDI_33	UDI_41	UDI_49	UDI_57	UDI_65	UDI_73	UDI_81	UDI_89
B	UDI_02	UDI_10	UDI_18	UDI_26	UDI_34	UDI_42	UDI_50	UDI_58	UDI_66	UDI_74	UDI_82	UDI_90
C	UDI_03	UDI_11	UDI_19	UDI_27	UDI_35	UDI_43	UDI_51	UDI_59	UDI_67	UDI_75	UDI_83	UDI_91
D	UDI_04	UDI_12	UDI_20	UDI_28	UDI_36	UDI_44	UDI_52	UDI_60	UDI_68	UDI_76	UDI_84	UDI_92
E	UDI_05	UDI_13	UDI_21	UDI_29	UDI_37	UDI_45	UDI_53	UDI_61	UDI_69	UDI_77	UDI_85	UDI_93
F	UDI_06	UDI_14	UDI_22	UDI_30	UDI_38	UDI_46	UDI_54	UDI_62	UDI_70	UDI_78	UDI_86	UDI_94
G	UDI_07	UDI_15	UDI_23	UDI_31	UDI_39	UDI_47	UDI_55	UDI_63	UDI_71	UDI_79	UDI_87	UDI_95
H	UDI_08	UDI_16	UDI_24	UDI_32	UDI_40	UDI_48	UDI_56	UDI_64	UDI_72	UDI_80	UDI_88	UDI_96

Appendix C: Library Characterization

Libraries should be visualized by running an agarose gel or using an automated electrophoresis instrument (e.g., Agilent TapeStation®, Bioanalyzer®, etc.) to determine that the correct library size is present. Below is an example of a library prepared with this kit and characterized using the High Sensitivity D1000 ScreenTape® on Agilent TapeStation® 4150 (Figure 1).

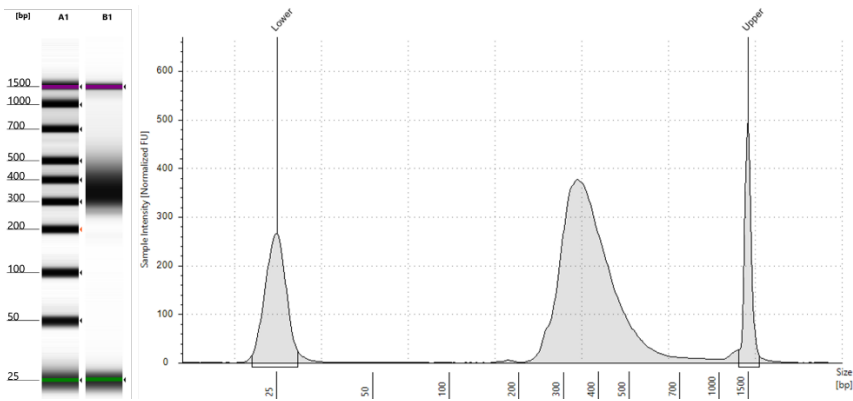


Figure 1. Agilent 4150 TapeStation® HSD1000 electropherogram of a typical Zymo-Seq SwitchFree™ 3' mRNA Library using 10 ng of HeLa total RNA (RIN > 9) and indexed using 19 PCR cycles. Fragment sizes mainly range from 250 to 700 bp. The peaks at 25 and 1500 bp are the interior standards of the High Sensitivity D1000 tape.

The kit is designed to minimize the formation of adapter dimers. If significant dimers show up at ~ 180 bp on the size distribution profile, a bead clean-up is recommended to remove the dimers for better sequencing quality. To remove the dimers, use **DNase/RNase-Free Water** to raise the library volume to 100 µL. Then follow the clean-up protocol in **Appendix A**, page 10 using 85 µL of the **Select-a-Size MagBeads** and elute with 15 – 20 µL of **DNA Elution Buffer**.

Appendix D: Considerations for Sequencing

Instrument Compatibility

The libraries prepared with this kit are compatible with all Illumina® sequencing platforms except the HiSeq® X Series.¹ Follow the instruction manual of the specific sequencer to determine important parameters such as library loading concentrations and PhiX spike-in percentages. The Zymo-Seq SwitchFree™ 3' mRNA libraries are considered low-complexity due to the oligo dT region.

Library Structure

The libraries prepared with this kit have the structure shown below.



Sequencing Specifications

We recommend the following sequencing specifications. Briefly, Read 1 provides UMI information and Read 2 provides the transcript sequence.

Read 1	Index 1	Index 2	Read 2
25 cycles	8 cycles	8 cycles	≥ 50 cycles

Read 1 will sequence the 8-nucleotide UMI, the 6 non-T random nucleotides, and the oligo dT region sequentially before sequencing the insert region. Therefore, it is recommended to assign no more than 25 cycles to Read 1. This is sufficient to allow for cluster identification, quality metrics calculation, and coverage of UMI information while avoiding more low-quality bases associated with sequencing through the oligo dT region.

It is possible to sequence longer for Read 1 (e.g., 100 bp or 150 bp paired-end sequencing) when other libraries of high complexity are sequenced on the same lane with the Zymo-Seq SwitchFree™ 3' mRNA libraries.

Read 2 will provide the transcript sequences.

¹ Illumina® originally limits the applications on HiSeq® X exclusively for whole-genome libraries. Please confirm with the sequencing service provider for acceptability and additional details if expecting to sequence Zymo-Seq SwitchFree™ 3' mRNA libraries on HiSeq® X Series sequencers.

Appendix E: Bioinformatic Analysis

Extracting UMIs

The UMIs can be extracted from Read 1 and assigned to the corresponding Read 2 with publicly available tools such as UMI-tools.¹

Trimming Reads

Generally, it is sufficient to trim Read 2 with the Illumina® TruSeq® adapter sequence: “AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT”.

When the read length is longer than the average library insert size, Read 2 will possibly sequence into the oligo dT sequence, the random hexamer, and even the UMI sequence. Additionally, poly A tail may also present at the end of Read 2. These additional bases can be trimmed to potentially improve alignment. An example using Cutadapt² for such 2-step trimming is shown as below.

```
cutadapt -a A{8}B{6}N{8}AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT -o  
adapterTrimmed.fastq.gz sample.fastq.gz
```

```
cutadapt -a "A{100}" -o completeTrimmed.fastq.gz adapterTrimmed.fastq.gz
```

We highly recommend discarding Read 1 after UMI extraction in any bioinformatic analysis because of the poor sequence quality associated with the oligo dT region.

In the unlikely event that Read 1 is required for downstream analysis, please trim the random hexamer and the oligo dT sequences from the beginning of Read 1 in addition to trimming the Illumina® TruSeq® adapter sequence, “AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC”, from the end of Read 1.

Aligning Reads

The transcript sequence is provided by Read 2. Thus, single-end alignment using UMI-assigned Read 2 is preferred.

¹ UMI-tools is a publicly available software and accessible at <https://github.com/CGATOxford/UMI-tools>.

² Cutadapt is a publicly available trimmer and accessible at <https://github.com/marcelm/cutadapt>.

Library Strand Information

The Read 2 sequence will be sense to the mRNA from which it originates. Therefore, the strandness of the library should be set as forward stranded when using only Read 2 for alignment and subsequent analysis.

Using UMIs

The UMI information can be used to deduplicate the aligned reads for more accurate evaluation of duplicate rate and gene expression quantification. Publicly available tools such as UMI-tools¹ can be used for this purpose with its “dedup” command.

Once trimming, alignment, and optional deduplication with UMI information are completed, no other special consideration is needed for subsequent analysis.

¹ UMI-tools is a publicly available software and accessible at <https://github.com/CGATOxford/UMI-tools>.

Ordering Information

Product Description	Catalog No.	Size
Zymo-Seq SwitchFree™ 3' mRNA Library Kit	R3008	12 preps
	R3009	96 preps
Zymo-Seq UDI Primer Set (Index 1-12)	D3008	12 sets
Zymo-Seq UDI Primer Plate (Index 1-96)	D3096	96 sets

Individual Kit Components	Catalog No.	Amount
ZymoTaq PreMix	E2003	50 rxns
	E2004	200 rxns
DNA Wash Buffer (concentrate)	D4003-2-6	6 ml
	D4003-2-24	24 ml
	D4003-2-48	48 ml
DNA Elution Buffer	D3004-4-1	1 ml
	D3004-4-4	4 ml
	D3004-4-10	10 ml
	D3004-4-16	16 ml
	D3004-4-50	50 ml
DNase/RNase-Free Water	W1001-1	1 ml
	W1001-4	4 ml
	W1001-6	6 ml
	W1001-10	10 ml
	W1001-30	30 ml

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