YourSeq (FT & 3'DGE) Strand-Specific mRNA Library Prep

(version A2)

Catalog No. 23001, 23002, 23003, 23004

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Overview

The YourSeq Library Prep workflow takes advantage of the natural phenomenon of DNA breathing to eliminate several steps which add time and cost to traditional RNA library prep protocols. DNA breathing is the opening and closing of DNA due to thermal fluctuations. During library prep, first-strand cDNA synthesis creates a DNA:RNA hybrid molecule which undergoes DNA breathing at the terminal end. YourSeq takes advantage of openings at the terminal end of this DNA:RNA hybrid to bind the 5-prime adapter to the cDNA in a process called "Breath Capture." This allows the enzymatic synthesis of a second strand of DNA without removal of RNA.

The workflow also implements strictly-enforced strand specificity as a second adapter is added by a DNA dependent DNA polymerase (DNA Pol I). As it primes off of the DNA strand of the DNA-RNA hybrid, it cannot prime off of the RNA strand.

YourSeq (FT & 3'DGE) Strand-Specific mRNA Library Prep Kits can be used to prepare samples for either conventional full-transcript (FT) or 3'-Digital Gene Expression (3'DGE) sequencing. Conventional full-transcript (FT) RNA-seq yields sequencing reads that map to the entire expressed transcriptome. The 3' Digital Gene Expression (3'-DGE) option creates reads mapping just at the 3'-end of the transcript. The reduced complexity associated with 3'-DGE confers the advantage of requiring a quarter to a third less sequencing depth, greatly reducing cost.

YourSeq (FT & 3'DGE) Strand-Specific mRNA Library Prep Kits can be ordered with either combinatorial dual indexes or unique dual indexes. Combinatorial dual-indexing kits allow you to choose from 96 or 384 unique index combinations. Unique dual-indexing kits allow the multiplexing of either 24 or 96 samples at a time without using any index twice. This can help avoid Illumina index hopping, or the incorrect assignment of libraries to the expected index.

Product	Format	Catalog No.
YourSeq (FT & 3'DGE) Strand-Specific mRNA Library Prep 24 CDI	24 rxn Combinatorial Dual Indexes	23001
YourSeq (FT & 3'DGE) Strand-Specific mRNA Library Prep 96 CDI	96 rxn Combinatorial Dual Indexes	23002
YourSeq (FT & 3'DGE) Strand-Specific mRNA Library Prep 24 UDI	24 rxn Unique Dual Indexes	23003
YourSeq (FT & 3'DGE) Strand-Specific mRNA Library Prep 96 UDI	96 rxn Unique Dual Indexes	23004

Kit Components and Storage

YourSeq (FT & 3'DGE) Strand-Specific mRNA Library Prep Kits are for research use only. Not for use in diagnostic procedures. All components are guaranteed stable for 6 months from date of receipt when stored properly. Store the Carboxyl Beads at 4°C after first use. All other components store at -20 °C.

Catalog no.	**23001**	**23002**	**23003**	**23004**	**Storage**
Indexing	CDI	CDI	UDI	UDI	
Reactions	24	96	24	96	
Reagent					
Carboxyl Beads	1.5 ml	6 ml	1.5 ml	6 ml	4°C
FT Priming Mix	75 µl	300 µl	75 µl	300 µl	-20°C
DGE Priming Mix	75 µl	300 µl	75 µl	300 µl	-20°C
Control mRNA	5 µl	5 µl	5 µl	5 µl	-20°C
First Strand Mix	135 µl	540 µl	135 µl	540 µl	-20°C
RT enzyme	15 µl	60 µl	15 µl	60 µl	-20°C
Breath Capture Mix	175 µl	690 µl	175 µl	690 µl	-20°C
5-Prime Adapter	120 µl	480 µl	120 µl	480 µl	-20°C
DNA Pol I	10 µl	30 µl	10 µl	30 µl	-20°C
PCR Mix	264 µl	1056 µl	264 µl	1056 µl	-20°C
Phusion Pol	8 µl	24 µl	8 µl	24 µl	-20°C
Nuclease-Free Water	1.8 ml	1.8 ml	1.8 ml	1.8 ml	-20°C
10 mM Tris HCl	1.8 ml	5 ml	1.8 ml	5 ml	-20°C
Carboxyl Beads Buffer	1.8 ml	6 ml	1.8 ml	6 ml	-20°C
YourSeq Index i5-A	30 µl	120 µl			-20°C
YourSeq Index i5-B	30 µl	120 µl			-20°C
YourSeq Index i5-C	30 µl	120 µl			-20°C
YourSeq Index i5-D	30 µl	120 µl			-20°C
YourSeq i7 1-24 Index Set	1 plate		1 plate		-20°C
YourSeq i5 1-24 Index Set			1 plate		-20°C
YourSeq i7 1-96 Index Set		1 plate		1 plate	-20°C
YourSeq i5 1-96 Index Set				1 plate	-20°C

Additional materials

- Nanodrop™, Qubit™ or equivalent method to determine DNA concentration
- Agarose gel electrophoresis equipment (If checking library post amplification)
- 2% agarose gel (If checking library post amplification)
- Agilent TapeStation or BioAnalyzer (if checking library post clean-up)
- 80% ethanol
- Vortexer
- Thermal cycler
- Multichannel pipetter (20 - 200 µl)
- Multichannel pipetter (1 - 10 µl)
- Filter tips for pipettes
- 0.2 ml 8-well PCR strip tubes with caps
- Microcentrifuge for 8-well PCR strip tubes with caps
- 500 ul or 1.5 ml microcentrifuge tubes for master mixes
- 96-Well Magnetic Separator for microtiter plates, ex. MagWell Magnetic Separator 96, EdgeBio, cat#57624.

Choose Full-Transcript (FT) or 3' Digital Gene Expression (3'DGE) Sequencing

The YourSeq (FT & 3'DGE) Strand-Specific mRNA Library Prep Kits come with sufficient reagents for two types of RNA-seq libraries. If you are unsure which is best for your needs please read the descriptions below to determine which to use. Note that other than the priming mix used in Section A, Step 1, the two protocols are identical.

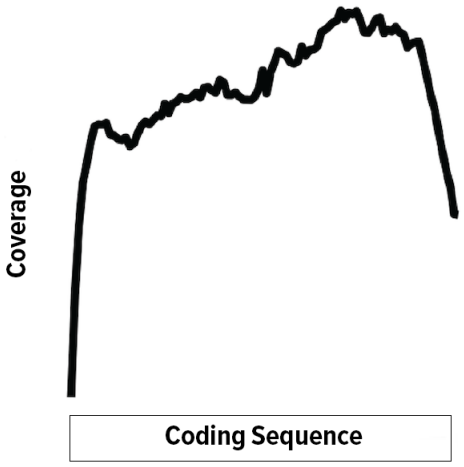
Full Transcript (FT) Sequencing

The Full Transcript (FT) sequencing option is conventional Full Transcript RNA sequencing and is the common type of RNA sequencing that most people are familiar with.

Conventional full-transcript (FT) coverage RNA-seq yields sequencing reads that map to the entire expressed transcriptome. This is achieved through the combination of random RNA fragmentation and subsequent random priming during the cDNA synthesis step. The result is sequencing reads that, when mapped to the reference transcriptome or genome, tile across the exons of expressed transcripts. With conventional RNA-seq, the number of reads mapping to a gene is a function of transcript abundance and transcript length. A long gene, therefore, will have more reads mapping to it than a short gene of similar transcript abundance.

Use Full-transcript RNA sequencing for:

- Differential gene expression for non-annotated genomes
- Genotyping and detection of exonic polymorphisms
- Splice variant analysis
- Detection of gene fusions abundance



3' Digital Gene Expression (3'DGE) Sequencing

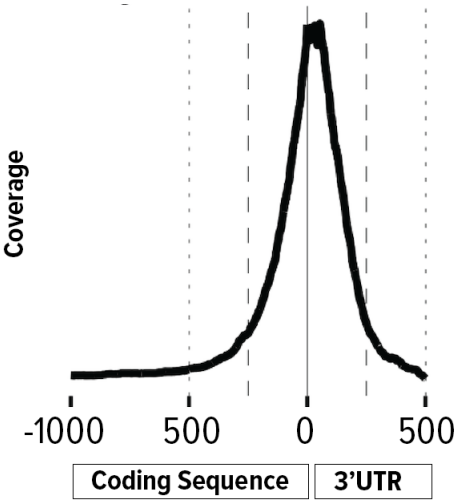
The 3' Digital Gene Expression (3'-DGE) option is optimized for differential gene expression analyses, where the main goal is to identify genes and pathways that are up- or down- regulated in response to some experimental condition.

Rather than use random priming, the cDNA synthesis step for 3'-DGE RNA-seq libraries uses an adapter with an oligo(dT) to prime off the transcript's poly(A) tail. This means that 3'-DGE reads tend to map only to the 3'-end of the transcript. Since there is a theoretical 1-to-1 correlation between transcript molecule and cDNA molecule, transcript length is irrelevant and the number of reads mapping to a gene is solely a function of transcript abundance. A long gene, therefore, will have the same number of reads mapping to it as will a short gene of similar transcript abundance.

The reduced complexity associated with 3'-DGE confers one of the biggest advantages. Transcript counting means that less sequencing depth is needed for a robust analysis compared to conventional RNA-seq. Also, paired-end sequencing does not add value for 3'-DGE. Therefore, a researcher who might sequence conventional RNA-seq libraries at 20-30 million pairs of paired-end reads per sample can reduce cost by sequencing those same samples as 3'-DGE libraries at 3-10 million single reads per sample.

Use 3'DGE RNA sequencing for:

- Differential gene expression for well-annotated genomes
- To improve annotation by identifying expressed genes and their 3'-UTRs
- Genotyping and detection of polymorphisms in the 3'-UTR
- Detection and characterization of alternative polyadenylation



Assay Protocol

Read the entire protocol before use.

Section A: RNA Fragmentation and cDNA Priming

1. For each mRNA Sample, dilute 5-100 ng mRNA with Nuclease-Free Water for a total volume of 7.5 µl in a 0.2 ml PCR strip reaction tube. Note that total RNA contains approximately 3-5% mRNA. For 5-100 ng mRNA we recommend starting with at least 100 ng - 5 µg of total RNA and doing either poly(A) enrichment or ribosomal depletion of the total RNA. For further guidance contact technical support.

   Optional Controls: It's recommended to include one positive control reaction consisting of 1 µl of the included Control mRNA diluted with 6.5 µl Nuclease-Free Water and one negative control reaction consisting of 7.5 µl Nuclease-Free Water.

Reagent	mRNA Sample	Positive Control	Negative Control
mRNA	5-100 ng	1 µl	-
Nuclease-free water	up to 7.5 µl	6.5 µl	7.5 µl
FT Priming Mix OR* DGE Priming Mix	2.5 µl	2.5 µl	2.5 µl
Total Volume	10 µl	10 µl	10 µl

2. Add 2.5 µl Priming Mix (FT or DGE, see Page 4) to the 7.5 µl diluted mRNA & mix well by pipetting.
3. Briefly centrifuge reaction tubes to ensure that all of each sample is at the bottom of its tube.
4. Incubate in thermal cycler for fragmentation and first-strand priming using the fragmentation program:

Step	Temperature	Incubation time
1	25°C	1 sec
2	94°C	1.5 min
3	30°C	1 min
4	20°C	4 min
5	20°C	Hold

Do not put on ice. Proceed directly to Section B.

Important: Place Carboxyl Beads at room temperature for at least 30 min before use. Carboxyl Beads are light sensitive and should be protected from light when not used.

Section B: cDNA Synthesis

1. Make enough cDNA Synthesis Cocktail for all samples. For each sample, add 0.5 µl RT Enzyme + 4.5 µl First Strand Mix into a single microcentrifuge tube (Calculate using 10% additional volume).
2. Add 5 µl cDNA Synthesis Cocktail to each Fragmented/Primed mRNA Sample (from Step A.4) for a total reaction volume of 15 µl. Mix well by pipetting. Briefly centrifuge reaction tubes to ensure that all of each sample is at the bottom of its tube.

Reagent	Volume (per reaction)
cDNA Synthesis Cocktail	5 µl
Fragmented/Primed mRNA (from Step A.4)	10 µl
Total Volume	15 µl

3. Incubate in thermal cycler for the reverse transcription program as shown below.

Step	Temperature	Incubation time
1	25°C	10 min
2	42°C	50 min
3	50°C	10 min
4	70°C	10 min
5	4°C	Hold

4. Resuspend Carboxyl Beads (at room temperature) by vortexing.
5. Add 22.5 µl Carboxyl Beads to each sample. Mix well by pipetting.
6. Let beads in samples stand 5 minutes at room temperature.
7. Place tubes on magnetic rack and remove supernatant.
8. Without resuspending pellet, wash beads twice on magnetic stand.

   a. Add 200 µl of 80% ethanol.

   b. Pipette up and down 5 times.

   c. Remove and discard ethanol.

   d. Repeat steps a-c.

   After removing ethanol from second wash: spin tubes for 15 seconds, place on magnetic rack, and remove residual supernatant with a 20 µl pipette. Allow pellets to dry for 2 min.

   Warning: Avoid significantly over-drying beads; otherwise, beads may clump and be difficult to homogenize.

   Note: The protocol can be paused at any point where the beads are in 80% Ethanol at the second wash. Simply store the strip tubes/plates at -20ºC, even for an extended period of many weeks, if necessary.

Section C: 5'-Adapter Sequence Addition

1. Thaw 5-Prime Adapter and bring to room temperature. Shake and briefly centrifuge tube.
2. With tubes on magnetic rack, add 4 µl room-temperature 5-Prime Adapter to each dry bead pellet. It is not necessary to resuspend the beads at this point.
3. Make enough Breath Capture Cocktail for all samples. For each sample, add 0.25 µl DNA Pol I + 5.75 µl Breath Capture Mix into a single microcentrifuge tube (Calculate using 10% additional volume). Pipette up and down to mix.
4. Add 6 µl Breath Capture Cocktail to each bead pellet for a total reaction volume of 10 µl. Mix well and resuspend the beads by pipetting.
5. Incubate in thermal cycler at 25ºC for 15 minutes.

Step	Temperature	Incubation time
1	25°C	15 min

6. Add 15 µl of Carboxyl Beads Buffer to each sample. Mix well and resuspend the beads by pipetting.
7. Let resuspended beads stand 5 minutes at room temperature.
8. Place tubes on magnetic rack and remove supernatant.
9. Without resuspending pellet, wash beads twice on magnetic stand.

   a. Add 200 µl of 80% ethanol.

   b. Pipette up and down 5 times.

   c. Remove and discard ethanol.

   d. Repeat steps a-c.

   After removing ethanol from second wash: spin tubes for 15 seconds, place on magnetic rack, and remove residual supernatant with a 20 µl pipette. Allow pellets to dry for 2 min.

   Warning: Avoid significantly over-drying beads; otherwise, beads may clump and be difficult to homogenize.

   Note: The protocol can be paused at any point where the beads are in 80% Ethanol at the second wash. Simply store the strip tubes/plates at -20ºC, even for an extended period of many weeks, if necessary.
10. Resuspend pellet in 10 µl 10 mM Tris HCl.
11. Repeat steps 6-9.
12. Resuspend pellet in 12 µl 10 mM Tris HCl.
13. Place tubes on magnetic rack and transfer 10 µl of the cDNA-containing supernatant to fresh tubes. Be careful to avoid bead carryover.

For Combinatorial Dual-Indexing (CDI):

Proceed to Section D-CDI, page 9.

For Unique Dual-Indexing (UDI):

Proceed to Section D-UDI, page 10.

Section D-CDI: Enrichment and Indexing, Combinatorial Dual-Indexing (CDI)

YourSeq (FT & 3'DGE) Strand-Specific Library Prep 24 CDI: The 24 rxn kit contains 24 YourSeq Index i7 (1-24) oligos and 4 YourSeq Index i5 (A,B,C, or D) oligos. For each sample you'll be pairing one Index i7 (1-24) oligo with one Index i5 (A,B,C, or D) oligo. There are 96 possible combinations of i7 and i5.

YourSeq (FT & 3'DGE) Strand-Specific Library Prep 96 CDI: The 96 rxn kit contains 96 YourSeq Index i7 (1-24) oligos and 4 YourSeq Index i5 (A,B,C, or D) oligos. For each sample you'll be pairing one Index i7 (1-96) oligo with one Index i5 (A,B,C, or D) oligo. There are 384 possible combinations of i7 and i5.

Note: Set up enrichment at room temperature. Hot start is not necessary.

1. Make enough Enrichment PCR Cocktail for all samples, adding 0.2 µl Phusion Polymerase + 7.8 µl PCR Mix into a single tube for each sample (Calculate using 10% additional volume). Pipette up and down to mix.
2. For each sample, add 8 µl of the Enrichment PCR Cocktail to an empty tube of a PCR strip.
3. Add 1 µl of YourSeq i7 Index oligo and 1 µl of a YourSeq i5 (A,B,C,D) Oligo to each PCR tube so that no combination is used twice.
4. Add the 10 µl of cDNA Supernatant (from Step C.13) to each PCR tube for a total reaction volume of 20 µl. Mix well by pipetting.

Reagent	Volume (per reaction)
Enrichment PCR Cocktail	8 µl
YourSeq Index i7 oligo	1 µl
YourSeq Index i5 A,B,C, or D oligo	1 µl
cDNA Supernatant	10 µl
Total Volume	20 µl

Proceed to Section D: Enrichment and Indexing cont., page 11.

Section D-UDI: Enrichment and Indexing, Unique Dual-Indexing (UDI)

YourSeq (FT & 3'DGE) Strand-Specific Library Prep 24 UDI: The 24 rxn kit contains 24 YourSeq Index i7 (1-24) oligos and 24 YourSeq Index i5 (1-24) oligos. For each reaction you'll be pairing one Index i7 (1-24) oligo with one Index i5 (1-24) oligo, never using any index twice. There are 24 unique dual-index pairs.

YourSeq (FT & 3'DGE) Strand-Specific Library Prep 96 UDI: The 96 rxn kit contains 96 YourSeq Index i7 (1-96) oligos and 96 YourSeq Index i5 (1-96) oligos. For each sample you'll be pairing one Index i7 (1-96) oligo with one Index i5 (1-96) oligo. There are 96 unique dual-index pairs.

Note: Set up enrichment at room temperature. Hot start is not necessary.

1. Make enough Enrichment PCR Cocktail for all samples, adding 0.2 µl Phusion Polymerase + 7.8 µl PCR Mix into a single tube for each sample (Calculate using 10% additional volume). Pipette up and down to mix.
2. For each sample, add 8 µl of the Enrichment PCR Cocktail to an empty tube of a PCR strip.
3. Add 1 µl of a YourSeq i7 Index oligo and 1 µl of a YourSeq i5 Oligo to each PCR tube so that no index oligo is used more than once.
4. Add the 10 µl of cDNA Supernatant (from Step C.13) to each PCR tube for a total reaction volume of 20 µl. Mix well by pipetting.

Reagent	Volume (per reaction)
Enrichment PCR Cocktail	8 µl
YourSeq Index i7 oligo	1 µl
YourSeq Index i5 oligo	1 µl
cDNA Supernatant	10 µl
Total Volume	20 µl

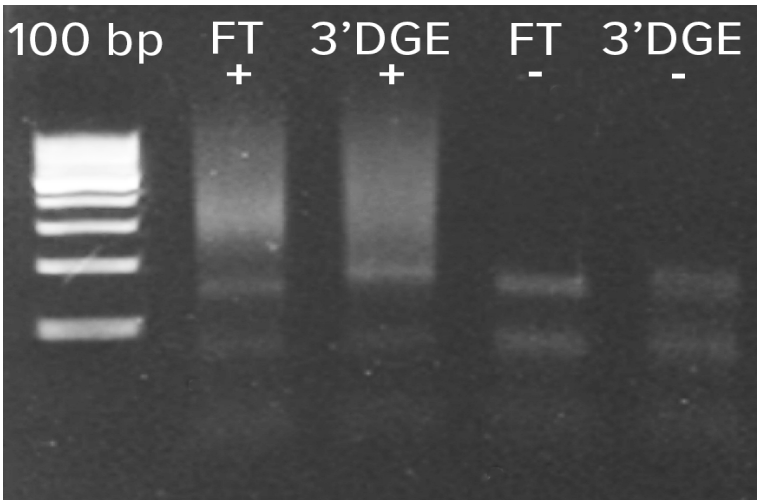
Proceed to Section D: Enrichment and Indexing cont., page 11.

Section D: Enrichment and Indexing cont.

5. **Optional:** Transfer 10 µl of the 20 µl enrichment PCR mix from Step 4 to a second strip of PCR tubes. This can be done without changing tips after mixing. The second set of PCR reactions can be stored at -20ºC for later use if additional PCR cycles are required.
6. Incubate in thermal cycler for enrichment and adapter extension as shown below. Depending on your particular samples the number of cycles may be more or less. Start with 14 cycles.

Step	Temperature	Incubation time	Cycles
1	98°C	30 sec	1
2	98°C	10 sec	14
	65°C	30 sec	
	72°C	30 sec	
3	72°C	5 min	
4	4°C	Hold	1

7. **Optional:** Run 2 µl of PCR reaction on a 2% Agarose gel to check amplification. See examples of FT & 3'DGE libraries for positive (Control mRNA) and negative (Nuclease-Free Water) controls below:



8. If desired amplification is obtained, proceed to Section E: Final Library Cleanup. Otherwise, use the extra PCR reactions (from Step 5) with an increased or decreased number of cycles.

Section E: Final Library Cleanup

1. Resuspend fresh Carboxyl Beads by vortexing.
2. Add 1.2 volumes of resuspended Carboxyl Beads to each sample (i.e. 9.6 µl Carboxyl Beads for 8 µl of enrichment product). Mix well by pipetting.
3. Let beads in samples stand 5 minutes at room temperature.
4. Place tubes on magnetic rack and remove supernatant.
5. Without resuspending pellet, wash beads twice on magnetic stand.

   a. Add 200 µl of 80% ethanol.

   b. Pipette up and down 5 times.

   c. Remove and discard ethanol.

   d. Repeat steps a-b.

   After removing ethanol from second wash: spin tubes for 15 seconds, place on magnetic rack, and remove residual supernatant with a 20 µl pipette. Allow pellets to dry for 2 min.

   Warning: Avoid significantly over-drying beads; otherwise, beads may clump and be difficult to homogenize.
6. Resuspend pellet in 10 µl 10mM Tris HCl.
7. Add 12 µl of Carboxyl Beads Buffer to each sample. Mix well and resuspend the beads by pipetting.
8. Let beads in samples stand 5 min at room temperature.
9. Place tubes on magnetic rack and remove supernatant.
10. Without resuspending pellet, wash beads twice on magnetic stand.

    a. Add 200 µl of 80% ethanol.

    b. Pipette up and down 5 times.

    c. Remove and discard ethanol.

    d. Repeat steps a-b.

    After removing ethanol from second wash: spin tubes for 15 seconds, place on magnetic rack, and remove residual supernatant with a 20 µl pipette. Allow pellets to dry for 2 min.

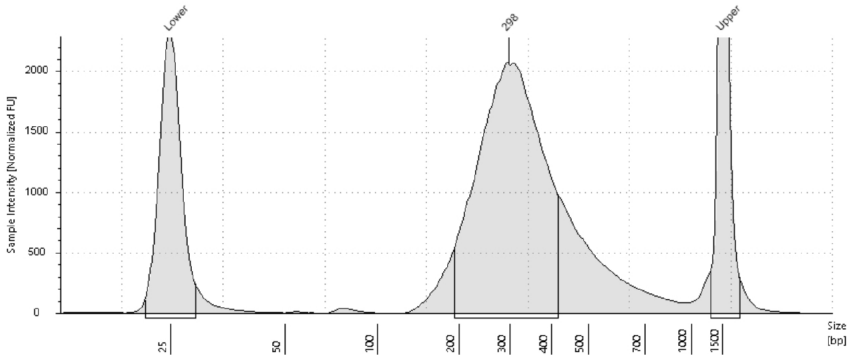
    Warning: Avoid significantly over-drying beads; otherwise, beads may clump and be difficult to homogenize.
11. Resuspend pellet in 12 µl 10mM Tris HCl.
12. Place tubes on magnetic rack and transfer 10 µl of the library-containing supernatant to fresh tubes. Be careful to avoid bead carryover.

Section F: Library QC

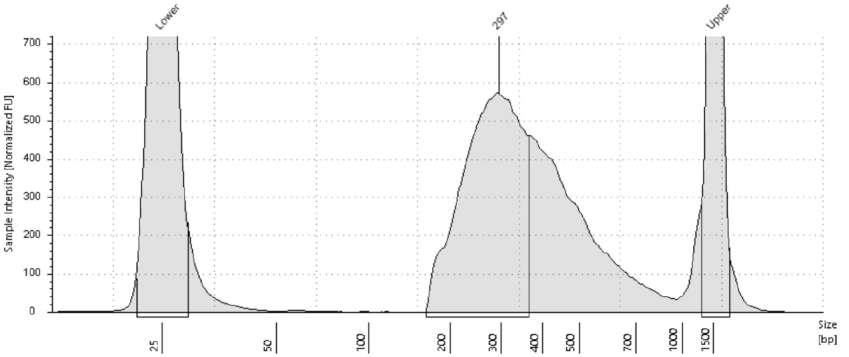
You may evaluate libraries to confirm sufficient recovery from beads and the absence of adapter contamination. If adapter contaminants are observed, an additional wash of the pooled libraries can be done to ensure removal of any remaining adapter contaminants.

Below are example libraries created using 5 µl of the included Control mRNA and following either the Full Transcript or 3' Digital Gene Expression protocol. Libraries were run on an Agilent TapeStation with the D1000 Screen Tape Assay.

Example Full-Transcript (FT) Library



Example Digital Gene Expression (3'DGE) Library



Appendix

Section G: Sequencing Guidelines

Combinatorial Dual Indexed (CDI) Libraries

Libraries from the combinatorial dual-indexing (CDI) version of the kit can be sequenced as either single-read (Read 1) or paired-end reads (Read 1 + Read 2), with a unique combination of Index 1 (i7) and Index 2 (i5) per sample.

The 24 reaction, CDI version of the kit comes with 24 Index 1 (i7) adapters and 4 Index 2 (i5) adapters. The 96 reaction, CDI version of the kit comes with 96 Index 1 (i7) adapters and 4 Index 2 (i5) adapters.

Adapter sequences for your kit can be downloaded from the product page at:

https://www.activemotif.com/catalog/1358/yourseq-mrna-library-prep#documents

Illumina's dual-indexing sequencing workflow requires that the reverse complement of the Index 2 (i5) adapter sequence be entered into the Sample Sheet for some systems. Use the correct adapter sequence for your instrument as shown in the spreadsheet.

Unique Dual-Indexed (UDI) Libraries

Libraries from the Unique dual-indexing version (UDI) of the kit can be sequenced as either single-read (Read 1) or paired-end reads (Read 1 + Read 2), with a unique Index 1 (i7) and unique Index 2 (i5) per sample.

The 24 reaction, UDI version of the kit comes with 24 Index 1 (i7) adapters and 24 YourSeq Index 2 (i5) adapters, for 24 unique dual-index pairs. The 96 reaction, UDI version of the kit comes with 96 Index 1 (i7) adapters and 96 Index 2 (i5) adapters, for 96 unique dual-index pairs. No index should be used more than one time. This indexing strategy is used to combat index hopping. Index hopping is a phenomenon associated with Illumina sequencers that utilize patterned flow cells (including HiSeq X/3000/4000 and NovaSeq) whereby a small percentage of reads get assigned to the wrong bar-code. Unique dual indexing allows the removal during demultiplexing of unexpected combinations caused by index hopping. As a result, you only keep the reads actually associated with your samples.

Adapter sequences for your kit can be downloaded from the product page at:

https://www.activemotif.com/catalog/1358/yourseq-mrna-library-prep#documents

Illumina's dual-indexing sequencing workflow requires that the reverse complement of the Index 2 (i5) adapter sequence be entered into the Sample Sheet for some systems. Use the correct adapter sequence for your instrument as shown in the spreadsheet.

Troubleshooting Guide

Problem/question	Possible cause	Recommendation
Low library yields	Not enough PCR cycles	Increase number of library amplfication PCR cycles.
	mRNA input too low	Increase amount of input.
Bioanalyzer peak around 70 bp	Indexing primers carried over from library amplification	Indexing primers can cluster but cannot be sequenced. Generally not an issue if peak is a small fraction compared to main library peak.
Bioanalyzer peak around 140 bp	Adapter dimers	Adapter dimers contain complete adapter sequences and so can cluster on flow cell and sequence. If ratio of adapter dimer peak is low compared to main library peak, do nothing. If the peak is high percentage of main library peak, cleanup the library again.
Second Bioanalzyer peak approximately 2X the main library peak	Overcycling	Reduce number of library amplification PCR cycles.

Technical Services

If you need assistance at any time, please call Active Motif Technical Service at one of the numbers listed below.

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