Meyer & Matz RNA-Seq sample preparation for sequencing on the SOLiD System

Updated 26 Jan 2011 to include changes for high-throughput sample preparation. This version of the protocol is optimized for working in 96-well plates.

Updated 19 July 2011 to include magnetic bead based PCR purification.

This protocol describes in detail the procedures used to prepare cDNA fragment libraries for quantitative analysis of gene expression (RNA-Seq) by deep sequencing on the SOLiD System (Applied Biosystems). About 0.6-1 µg of DNAse-treated total RNA is required per sample, and this starting material should be carefully quantified and analyzed by gel electrophoresis prior to beginning these procedures to verify that the RNA is intact, and free of genomic DNA contamination.

The procedure can be reasonably completed within three days. Day 1: RNA is fragmented and used to synthesis cDNA (steps 1-2). Day 2: cDNA is amplified, sample-specific barcodes are incorporated, and size-selection is accomplished by means of gel extraction (steps 3-4). Day 3: the preparations are tested by amplification with the SOLiD primers to verify that the material can be amplified in emPCR for SOLiD sequencing. Finally, prior to sequencing the finished product on the SOLiD System, we recommend confirming the constructs by cloning a small aliquot of the SOLiD-ready product (step 4i) for Sanger sequencing. These additional quality control steps obviously require additional time, but allow the user to detect any major artifacts prior to investing resources in SOLiD sequencing.The sequences of all oligonucleotide primers used in this protocol are provided at the end of this document, to allow synthesis of custom oligos as needed.

1. RNA fragmentation

   NOTE: the buffer in which the original RNA is incubated is critically important for the success of fragmentation, as are the volume and concentration of the RNA. Prior to working with the precious experimental samples, we recommend testing a range of different incubation times to identify the duration that produces the appropriate size range in these samples.

   a. Aliquot 1 µg of total RNA in 10 µl of 10 mM Tris (pH 8.0) in a 96-well PCR plate. If concentrations are not sufficiently high to allow this loading, RNA samples can be concentrated in the Speedvac prior to loading. Set aside an additional sample (~100 ng) of the original intact RNA for comparison with the fragmented samples.

   b. Carefully seal all wells and incubate RNA at 95°C for 5-30 minutes to fragment to the desired size range (100 – 500 bp). This can be most easily accomplished in a thermocycler. In our previous work with grasses the optimum time has been ~10 minutes.

c. Analyze 100 ng from each sample of fragmented RNA, alongside the intact RNA from the same sample, on a standard 1% agarose gel to evaluate the molecular weight of the fragmented RNA.

2. First-strand cDNA synthesis

NOTE: if RNA quantity is not limiting, first-strand cDNA should be synthesized using 1 µg of fragmented RNA. The reaction shown below is intended for ~ 1 µg; this can be doubled or halved as needed if the amount of RNA is very different from 1 µg.

a. Measure the volume remaining after fragmentation using a pipette. The following recipe assumes a starting volume of 9 µl (10 µl minus evaporation), so if the volume is lower than this add additional water to achieve 9 µl.

b. Add 1 µl of the 10 µM oligonucleotide 3SLD-10TV to each well. Incubate at 65°C for 3 minutes in a thermocycler, then transfer immediately onto ice.

c. Prepare a cDNA synthesis master mix. The following volumes are intended for a single reaction, so multiply these values by the number of reactions plus a small amount (~10%) to account for pipetting error.

	(all volumes given in µl)
H_2O	1
dNTP (10 mM ea)	1
DTT (0.1 M)	2
5X first-strand buffer	4
10 µM S-SLD-SW (RNA oligonucleotide; stored at -80°C)	1
SuperScript II Reverse Transcriptase (Invitrogen #18064-022).	1

d. Add 10 µl of this master mix to the RNA from (2b), mix thoroughly, and incubate in a thermocycler for one hour at 42°C.

e. Incubate at 65°C for 5 minutes to inactivate the RT, dilute 1:5 in H_2O, and store on ice or at -20°C until ready to proceed to the next step.

3. cDNA amplification

NOTE: it is important not to over-amplify the cDNA at this stage, to avoid artifacts and distortion of expression ratios. The fewer cycles used, the better. If a visible smear is not produced within 17-20 cycles, try repeating the PCR with additional template (diluted FS-cDNA from step 2e), up to a maximum of 3 µl, and correspondingly less water in the master mix. If no smear is detected with 3 µl template and 20 cycles, this indicates a problem with the first-strand cDNA synthesis.

a. Prepare a set of master mixes for small-scale PCR tests. The following volumes are intended for a single reaction each, so multiply these values by the total number of reactions plus a small additional amount to account for pipetting error. This recipe assumes 3 µl of template, so if you use a different amount of template, adjust the water accordingly.

(Volumes given in µl)

	A	B	C	D
H_2O	12.6	12.2	12.2	11.8
dNTP (2.5 mM ea)	2	2	2	2
10X PCR buffer	2	2	2	2
10 µM 5SLD oligo	0	0.4	0	0.4
10 µM 3SLD-10TV oligo	0	0	0.4	0.4
Titanium Taq polymerase (Clontech #639208)	0.4	0.4	0.4	0.4

b. For each of the original RNA samples, prepare four PCR tubes labeled A-D. Add 18 µl of the appropriate master mix to each tube.

c. Add 2 µl diluted FS-cDNA from (2e).

d. Amplify in a thermocycler using the following profile:
95°C 5 min, (95°C 40 sec, 63°C 1 min, 72°C 1 min) X 17 cycles

e. After 17 cycles check 5 µl of the PCR products for all reactions on a standard 1% agarose gel. A “smear” of cDNA (~100-500 bp) should be faintly visible in reaction D, and nothing should be detected in the reactions A-C. If nothing is detected in reaction D, you can continue the reaction for additional cycles (up to a maximum of 20), or repeat the reaction with additional template (up to a maximum of 3 µl). If product ever appears in reactions A-C, this indicates too

much template, too many cycles, or contamination in one or more reagents. The goal at this stage is to identify the minimum cycle number that produces a visible smear in reaction D while remaining clean in reactions A-C.

f. Once the optimum amount of template and number of cycles have been determined (3e), prepare a single large-scale reaction for each cDNA sample as follows. This recipe assumes 15 µl of template, so if you use more template adjust the water accordingly.

	(all volumes given in µl)
H_2O	59
dNTP (2.5 mM ea)	10
10X PCR buffer	10
10 µM 5SLD oligo	2
10 µM 3SLD-10TV oligo	2
Titanium Taq polymerase (Clontech #639208)	2

g. After PCR, check 5 µl of the product on a gel to verify that the reaction worked as expected before freezing or purifying the product.
h. Purify PCR products using the NucleoMag 96 PCR Cleanup Kit, according to the manufacturer's instructions.
i. Working in a 96-well plate or PCR strip, split each PCR into two 50-µl portions.
j. Add 6 µl P-beads and 138 µbuffer MP1 to each well and mix thoroughly by back pipetting.
k. Seal plate and place on shaker for 5 minutes at room temperature (~200 rpm).
l. Remove seal from plate, being careful to avoid contamination. The plate can be spun down briefly if required to concentrate all the liquid in the bottom of the well.
m. Place plate on magnetic separator plate and hold undisturbed for 1 minute to collect beads. Remove supernatant without disturbing beads.
n. Wash beads with 250 µl MP2; repeat step m.
o. Wash beads with 250 µl MP3; repeat step m.
p. Repeat step o. On this final rinse combine the two aliquots of each sample by suspending each in 125 µl of MP3, then combining into a single pool of 250 µl. Be careful to remove all visible supernatant after magnetic separation.
q. Dry the beads 10 minutes at room temperature.

r. Add 25 µl elution buffer (MP4) to the dried beads and mix well by back pipetting. Seal plate and hold on shaker for 5 minutes at room temperature (~200 rpm).
s. Place plate on magnetic separator plate and hold undisturbed for 1 minute to collect beads. Collect eluted DNA without disturbing beads, and transfer into a new 96-well plate or PCR strip.
t. Quantify the purified products by OD260 (Nanodrop).

4. Adaptor extension and size selection

NOTE: Because the size distribution of templates is a critical factor for successful emulsion PCR, any templates intended for sequencing on the SOLiD System should be carefully size-selected prior to emPCR. The directions below outline a simple procedure for selecting fragments ranging from 150-200 bp in size that does not require any special equipment. Other methods of size selection could be substituted provided they achieve this same size range.

a. First, four test-scale PCRs are prepared for each sample to verify yield and specificity of the reaction, each using 10 ng PCR product (step 3, above) as template. It may be useful to dilute an aliquot from each sample to be tested so that all templates are at the same concentration. (e.g. you could prepare a 5 ng μl^{-1} solution for each, and use 2 µl of that dilution as a template).

b. Prepare a small amount of diluted barcode oligo (1 µM), with a different barcode used for each sample. Be sure to write down which barcode is assigned to each sample at this stage, since this cannot be easily determined later in the process.

c. Prepare four separate master mixes for small-scale test PCR. The following volumes are for a single reaction, so multiply these values by the total number of samples plus a small additional amount to account for pipetting error. The values shown here assume the use of 2 µ diluted PCR product as template, so if you change this be sure to change the volume of water accordingly.

	(volumes given in µl)			
	A	B	C	D
H2O	5.8	5.6	3.8	3.6
dNTP (2.5 mM ea)	1	1	1	1
10X PCR buffer	1	1	1	1
Multiplex-P1 oligo (10 µM)	0	0.2	0	0.2
Titanium Taq polymerase	0.2	0.2	0.2	0.2
Master mix per reaction	8	8	6	6
Barcode oligo (1 µM)	0	0	2	2

d. Aliquot the specified amount of master mix into each well, then add diluted (1 µM) barcode oligos to reactions C and D as specified in the above table.

e. Add 2 µl of the diluted cDNA (steps 3l, 4b) to each of these four primer combination reactions (A-D). The total reaction volume is 10 µl.

f. Amplify in a thermocycler using the following profile:
   95°C 5 min, (95°C 40 sec, 63°C 1 min, 72°C 1 min) X 4 cycles

g. After 4 cycles check 5 µl of the PCR products for all reactions on a gel. The ideal result is a faint smear in reaction D, with no visible product in reactions A-C. If products appear in these control reactions, consider using less template. If nothing is detected in reaction D, add 1-2 more cycles and check the results on a gel. If no product is visible before 6 cycles, repeat the reaction with a larger volume of template (in our experience this has never been required). A small amount of product in the controls can be tolerated, but if reactions A-C are comparable in intensity to reaction D something is wrong.

h. When the optimum number of cycles and volume of template have been determined, prepare a large-scale reaction based on those parameters with 50 ng template in 50 µl total volume. The following master mix assumes the use of 10 µl of template per 50 µl reaction; if you adjust this template volume be sure to adjust the volume of water accordingly. This recipe is for a single

reaction, so multiple these values by the number of samples to be prepared plus a small additional amount for pipetting error.

	(volumes given in µl)
H2O	27
dNTP (2.5 mM ea)	5
10X PCR buffer	5
Multiplex-P1 oligo (10 µM)	1
Titanium Taq polymerase	1

i. Aliquot 39 µl of master mix to each well, then add 1 µl of the appropriate barcode oligo (10 µM), and 10 µl of PCR product (step 3l).

j. Amplify these reactions using the same profile and cycle number as determined above.

k. Prepare a gel for size selection. This preparative gel should consist of 2% agarose in 1X TBE buffer, with SYBR Safe DNA staining dye (Invitrogen # S33102) added according to the manufacturers' instructions (1:10,000 dilution). A low-molecular weight ladder is required for accurate selection of the appropriate sizes; we recommend pBR322 DNA-MspI Digest (New England Biolabs # N3032S) or Low MW Ladder (NEB, #N3233S). Be sure to use large volume combs to allow loading of the entire 50 µl reaction into a single well.

l. Load samples and run the gel until marker bands in the 50-200 bp size range are well separated. Illuminate the gel very briefly (< 30 seconds total exposure time) on a UV-transilluminator set at low intensity, for just long enough to mark the appropriate region (180-250 bp) with a clean razor blade. Turn off the UV light and carefully cut out the marked region, transferring it into a microcentrifuge tube.

m. Extract the cDNA from this gel slice by adding 40 µl of nuclease-free water to the tube, spinning down briefly to bring water and gel slice into contact, and incubating overnight at 4°C. This product is now ready for sequencing on the SOLiD system. However, before proceeding with sequencing, we strongly recommend the following quality control steps (step 5, below).

5. Amplification of size-selected fragments

NOTE: this step is a useful test of the final constructs, but is not required for SOLiD sequencing, which only requires about 0.5-1 pg of input cDNA. The goal of this step is to verify that the constructs produced in this procedure include the intended primer binding sites, are free of artifacts e.g. poly-A inserts, and fall within the appropriate range of molecular weights.

a. Quantify the cDNA constructs from step 4n, using 0.5X TBE as the blank, and calculate the volume required to use 5 ng as template for the following reaction. Prepare a small volume of diluted template for each sample such that the same volume can be used in each reaction.

b. Prepare a PCR master mix. The following volumes are for a single reaction, so multiply these values by the total number of reactions plus a small additional amount to account for pipetting error. The values shown here assume a cDNA concentration of 5 ng μl^{-1}; the volumes of cDNA and water should be adjusted according to the concentration of cDNA in your samples:

	(volumes given in µl)
H_2O	5.6
dNTP (2.5 mM ea)	1
10X PCR buffer	1
Lib-PCR1 oligo (10 µM)	0.2
Lib-PCR2 oligo (10 µM)	0.2
Titanium Taq polymerase	1

c. Add 5 ng of cDNA template to each reaction (from 4n), for a total reaction volume of 10 µl.

d. Amplify in a thermocycler using the following profile:
95°C 5 min, (95°C 40 sec, 63°C 1 min, 72°C 1 min) X 15 cycles

e. After 15 cycles check 3 µl of the PCR products for all reactions on a gel. If a clean, single band at 180-250 bp is detected the constructs are correct. If no product is visible, amplify for an additional 1-2 cycles and check the product on a gel. If no product is visible before 18 cycles, this suggests a problem; consider repeating the PCR with additional template.

f. Once the constructs pass the PCR tests in (5e) they are ready for SOLiD sequencing. The purified template from step (4n) is the material to be sequenced. We strongly recommend cloning and sequencing a small number of clones by Sanger sequencing (n=6-24), to verify that no major artifacts are present (e.g., adaptor concatenation or excessive poly-A tracks), prior to sequencing this material on the SOLiD System.

Sequences of oligonucleotide primers used in this protocol

Our custom primers

3SLD-10TV	CTGCTGTACGGCCAAGGCGAATTTTTTTTTTV
S-SLD-SW	ACCCCAUGGGGCUCUCUAUGGGCAGUCGGUGAUGGG (*Note: This custom RNA oligo should be stored in multiple aliquots at -80°C to prevent degradation of this labile and expensive reagent*)

Standard SOLiD System primers

Adapted from the manufacturers bulletin PN 4448274 Rev B.

5SLD	CTCTCTATGGGCAGTCGGTGAT (*Note: a truncated version of AB's Multiplex PCR primer 1)*
Bar-coded adaptor P2	CTGCCCCGGGTTCCTCATTCTCTAAGCCCCTGCTGTACGGCCAAGGCG (*Note: barcode is underlined. Other six-base barcodes can be generated as needed*)
Multiplex PCR primer 1	CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGAT
Lib PCR 1	CCACTACGCCTCCGCTTTCCTCTCTATG
Lib PCR 2	CTGCCCCGGGTTCCTCATTCT