

Tag-based RNA-Seq sample preparation, for sequencing on the Illumina HiSeq

Galina Aglyamova, Eli Meyer, and Mikhail Matz

aglyamova@austin.utexas.edu, matz@utexas.edu

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

Updated January 1, 2013 and April 2, 2013 to reflect the switch to Illumina HiSeq sequencing platform and to add qPCR-based quantification of the resulting samples.

Updated August 21, 2013 to reflect minor changes in procedure (maximizing cDNA representation).

Updated March 12, 2015 to reflect substitution of qPCR for Picogreen DNA assay for DNA quantification.

At least 100 ng, and ideally 0.5-1 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 synthesize cDNA (steps 1-2). cDNA is amplified.

Day 2: PCR product are cleaned and DNA concentrations are quantified with Picogreen DS DNA assay and sample concentrations are equalized. Short PCR (4 cycles) is performed to incorporate sample-specific barcodes. Samples are pooled, cleaned and size-selection by gel extraction is performed.

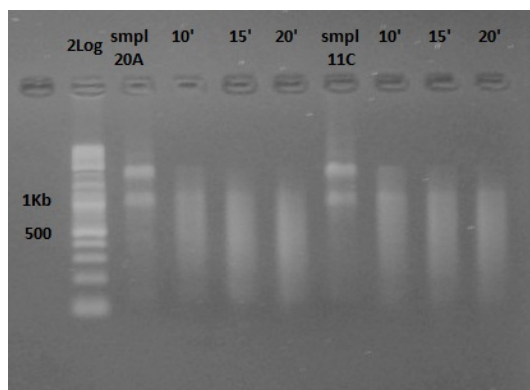
Day 3: After overnight elution from gel, the final DNA concentrations are quantified by Picogreen assay. Optional control PCR is run to confirm size range of the samples.

The sequences of all oligonucleotides used in this protocol are provided at the end of this document.

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). To achieve this concentration, RNA samples can be concentrated by drying in Speedvac (without heating) or by standard ethanol or LiCl precipitation. 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 to fragment the RNA. This can be most easily accomplished in a thermocycler. In our previous work the optimum time has been ~10-15 minutes.
- c. Analyze 100 ng of fragmented RNA alongside the intact RNA from the same sample on a standard (as for DNA) 1% agarose gel to evaluate the extent of RNA fragmentation. The smear must extend all the way up into the region where ribosomal RNA bands were, while the bands themselves should be mostly gone. In the figure on the next page, 15' result is close to the ideal, but in fact all three incubation times are acceptable.



2. First-strand cDNA synthesis

NOTE: Although we have occasional success with amounts as low as 100 ng of fragmented RNA per reaction, we strongly recommend using 0.5-1 µg to ensure adequate representation of all transcripts.

- The following recipe assumes a starting volume of 10 µl (11 µl minus evaporation), so if the volume is lower than this, add water to achieve 10 µl.
- Add 1 µl of the 10 µM oligonucleotide 3ILL-30TV to each well. Incubate at 65°C for 3 minutes in a thermocycler, then transfer immediately onto ice for 2 minutes.
- 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)	
dNTP (10 mM ea)	1
DTT (0.1 M)	2
5X first-strand buffer	4
10 µM S-ILL-swMW	
(RNA oligonucleotide; stored at -80°C)	1
SMARTScribe Reverse Transcriptase	
(Clontech 639537)	1

- Add 9 µl of this master mix to the RNA from (2b), mix thoroughly, and incubate in a thermocycler for one hour at 42°C.
- Incubate at 65°C for 15 minutes to inactivate the RT. Store First Strand cDNA (FS-cDNA) on ice or at -20°C until ready to proceed to the next step.

3. cDNA amplification

- a. Prepare a set of master mixes for small-scale PCR tests: the control amplification (A), lacking the 5'-specific primer, should be empty. 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 2 μ l of template (step 2e), so if you use a different amount of template, adjust the water accordingly.

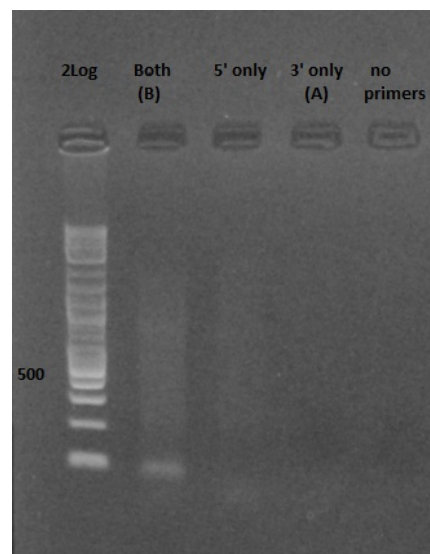
(Volumes given in μ l)

	A	B
H ₂ O	12.2	11.8
dNTP (2.5 mM ea)	2	2
10X PCR buffer	2	2
10 μ M 5ILL oligo	0	0.4
10 μ M 3ILL-30TV oligo	0.4	0.4
Titanium Taq polymerase (Clontech #639208)	0.4	0.4

- b. For each of the original RNA samples, prepare two PCR tubes labeled A and B. Add 18 μ l of the appropriate master mix to each tube.
- c. Add 2 μ l FS-cDNA (step 2e).
- d. Amplify using the following profile:
95°C 5 min, (95°C 40 sec, 63°C 2 min, 72°C 1 min) X 13 cycles
after which save 3 μ l aliquots from all tubes.
- e. Put the tubes back into the thermocycler; perform additional 2 cycles:
95°C 40 sec, 63°C 2 min, 72°C 1 min,
and once again save 3 μ l aliquots from all tubes.
- f. Repeat step (3e) two more times (until the total number of PCR cycles is 19).
- g. Run the saved aliquots on a standard 1% agarose gel. The desired pattern (see figure) is a smear of cDNA (~100-1200 bp) which should be just becoming visible in reaction B, while nothing should be detectable in the reaction A.

NOTES:

- If you started with large amount (1 μ g) of total RNA you might see a carry-over degraded RNA smear on the gel, in both A and B reactions. Do not confuse it with the PCR product! Make sure the product actually accumulates as you are adding more cycles.



- Different samples might require slightly different number of cycles, this is OK since all the potential biases due to PCR amplification will be removed at the data analysis stage by discarding PCR duplicates.

- Very important: if a smear is not produced after 19 cycles, the representation of the cDNA is not adequate for RNA-seq; you must optimize previous stages. Ideal RNA-seq results can be obtained for samples that are amplified in 15 cycles or less, 17 cycles is OK.

- h. Once the optimum number of cycles has been determined, prepare a single large-scale reaction for each cDNA sample as follows.

(all volumes given in μl)	
H ₂ O	32
dNTP (2.5 mM ea)	5
10X PCR buffer	5
10 μM 5ILL oligo	1
10 μM 3ILL-30TV oligo	1
Titanium Taq polymerase (Clontech #639208)	1
First-strand cDNA	5

95°C 5 min, (95°C 40 sec, 63°C 2 min, 72°C 1 min) X N cycles

- i. Perform as in step (3d) but for the determined optimal number of cycles for each sample. Run 5 μl of the product on a gel to verify that the reaction worked.
- j. Purify PCR products using PCR-clean up kit (Fermentas K0702), according to the manufacturer's instructions.
- k. Quantify the purified products by Quant_IT Picogreen DS DNA Kit (Life Technologies P7589) according to the manufacturer's protocol. We used SpectraMax M2 plate reader and Costar assay plates 96 well, no lid, flat bottom, non-treated black with black bottom (Corning 3650) or clear bottom (Corning 3631). See the end of this protocol for details.
- l. Prepare 30 μl of the purified PCR products diluted to 5 $\text{ng } \mu\text{l}^{-1}$ (in 10 mM tris HCl pH 8, or the elution buffer from the PCR-cleanup kit). It's extremely important to put the same amount of template into the barcoding PCR.

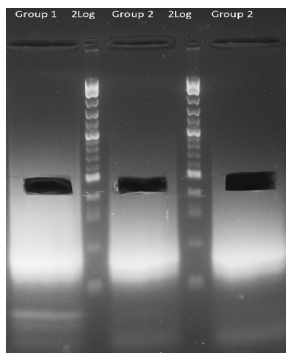
4. Barcoding and size selection

- a. Prepare a large-scale reaction with 50 ng template (10 μl of the pooled purified cDNA, step 3l) in 30 μl total volume. The recipe below 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	15
dNTP (2.5 mM ea)	3
10X PCR buffer	3
* TruSeq_Un1 (10 μM)	0.6
Titanium Taq polymerase	0.6

(*) We use four different variations of Illumina Universal Oligo: TruSeq_Un1, TruSeq_Un2, TruSeq_Un3, TruSeq_Un4, so each sample is barcoded from both ends. It's convenient to prepare four master mixes, one for each TruSeq_Uni oligo.

- b. Aliquot 30 μl of master mix to each well, then add 10 μl of the appropriate barcode oligo (1 μM), and 10 μl of 5 $\text{ng } \mu\text{l}^{-1}$ cleaned PCR product (step 3l).
- c. Amplify using the following profile:
95°C 5 min, (95°C 40 sec, 63°C 2 min, 72°C 1 min) X 4 cycles
- d. Run 5 μl of each product on 2% agarose gel to confirm that amplification across all samples was successful and uniform (as it should be if quantification and dilutions at the previous stage were precise). If just a few of the samples are lagging behind, it is OK to add 1-2 more cycles just to those, but then make sure to run them on gel again alongside a couple of evenly-amplified samples.
- e. Pool 20 μl from each sample in groups of 5-8 (depending on the total number of samples in the experiment). Makes sure the pools all comprise the same (or nearly same) number of samples. Concentrate the pools into 50 μl using PCR-clean up kit (Fermentas K0702), according to the manufacturer's instructions.
- f. Prepare a gel for size selection. This preparative gel should be 2% agarose in 1X TBE buffer, with SYBR Green I nucleic acid gel staining dye (Invitrogen # S7563) added according to the manufacturers' instructions (1:10,000 dilution). Be sure to use very wide and large volume combs to allow loading of the 50 μl mix +10 μl loading dye into a single well.



- g. Load samples and run the gel slowly, at 5 volts cm^{-1} , for 70 -90 minutes (or until marker bands in the 100 - 500bp size range are well separated on your gel system). Use blue-light gel illuminator to safely cut out the required size range (400-500bp). Cut only the middle of

the lane, leave the edges (see picture above). Slice each cut-out piece into 4-5 fragments and put them into a new 0.5 ml tube.

- h. Add 20 μ l of nuclease-free water to the tubes containing gel slices, make sure the water and gel pieces are in contact, and incubate overnight at 4°C to let the DNA diffuse out of the gel. No further purification procedures are necessary; simply use the water eluate in the subsequent steps. Alternatively use QIAquick Gel Extraction Kit (QIAGEN 28704).

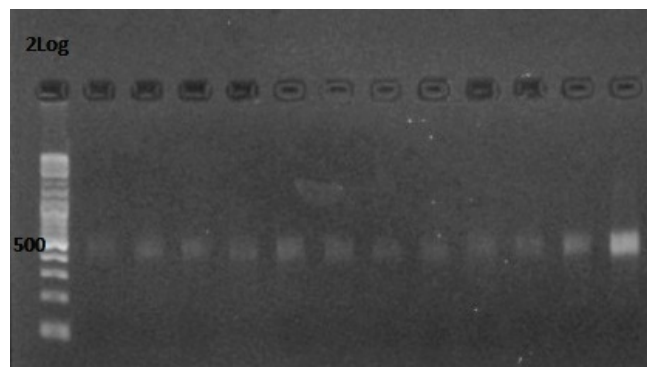
5. Quantification for mixing on the same HiSeq lane

NOTE: For checking quality of eluted DNA we do PCRs to verify the product size on gel; it should be the same as the band we cut out and no additional products. Second, for mixing the barcoded samples together in equal proportions we perform Picogreen DS DNA assay which gives absolute value of DNA concentrations.

- a. For quality check prepare a PCR master mix according to the following recipe. The volumes are given for a single reaction, so multiply these values by the total number of reactions plus a small additional amount to account for pipetting error.

(volumes given in μ l)	
H ₂ O	6.4
dNTP (2.5 mM ea)	1
10X PCR buffer	1
IC2-P7 primer (10 μ M)	0.2
IC1-P5 primer (10 μ M)	0.2
Titanium Taq polymerase	0.2

- b. Add 1 μ l of gel-extracted final product DNA template (step 4h) to each reaction, for a total reaction volume of 10 μ l.
- c. Amplify using the following profile:
95°C 5 min, (95°C 40 sec, 63°C 1 min, 72°C 1 min) X 10-12 cycles
Run 3 μ l on gel. The size of the product should match the size you aiming when cut a band for gel-extraction.



- d. Run Quant-IT picogreen DS DNA assay (Life Technologies P7589) to determine the final concentrations of the eluted product in order to mix libraries in equal proportions.

Picogreen assay Protocol:

- 1) Place 100ul 1X TE into all first column wells except B1
- 2) Add 150ul of stock curve (@ 2ug/ml) into B1
- 3) Serially dilute standards by taking 50ul of B1, mixing into C1, taking 50ul of C1, mixing into D1, and so on until taking 50ul from H1 and throwing it out.
- 4) To all sample wells, add 98ul of 1X TE.
- 5) Add 2ul sample DNA to sample wells.
- 6) Mix Pico Green Master mix: 99.5ul 1XTE + 0.5ul PicoGreen for one sample. Multiply accordingly (plus 8 wells for DNA standard).
- 7) Add 100ul of master mix to all standard and sample wells, bringing up final volumes in each well to 200.
- 8) Turn on the plate reader (SpectraMaxM2 in our case).
- 9) Open software (SoftMaxPro V5 in our case).
- 10) Select the premade program and run it. Read the fluorescence (excitation 480nm, emission 520nm).
- 11) Save the data into txt file, assemble the results in Excel in two-column form – well, reading - save it as comma-delimited (.csv) file. The file must contain all A1-H1 wells (blank and calibrators) plus an arbitrary number of sample wells, in any order.
- 12) Use picogreen.R script to calculate sample concentrations (ng/ul in the original sample).

oligo	use	Sequence, 5'-3'	notes
3ILL-30TV	cDNA synthesis and amplification	ACGTGTGCTCTCCGATCTAATTTTTTTTTTTTTTTTTTTTTT	V=[ACG]
S-III-swMW	cDNA synthesis	ACCCCAUGGGGCUACACGACGCUUCUCCGAUCUNNMWGGG	RNA oligo; M=[AC], W=[AU]
5ILL	cDNA amplification	CTACACGACGCTCTCCGATCT	
ILL-BC23	Barcoding	CAAGCAGAAGACGGCATAACGAGATCCACTCGTGACTGGAGTTCAGACGTGTGCTCTCCGAT	the barcode is underlined
ILL-BC24	Barcoding	GCTACC	only the barcode
ILL-BC25	Barcoding	ATCAGT	only the barcode
ILL-BC26	Barcoding	GTCAT	only the barcode
ILL-BC27	Barcoding	AGGAAT	only the barcode
ILL-BC28	Barcoding	CTTTG	only the barcode
ILL-BC29	Barcoding	TAGTTG	only the barcode
ILL-BC30	Barcoding	CCGGTG	only the barcode
ILL-BC31	Barcoding	ATCGTG	only the barcode
ILL-BC32	Barcoding	TGAGTG	only the barcode
ILL-BC33	Barcoding	CGCCTG	only the barcode
ILL-BC34	Barcoding	GCCATG	only the barcode
ILL-BC35	Barcoding	AAAATG	only the barcode
ILL-BC36	Barcoding	TGTTGG	only the barcode
ILL-BC37	Barcoding	ATTCCG	only the barcode
ILL-BC79	Barcoding	ACGCGG	only the barcode
ILL-BC80	Barcoding	AGGGCG	only the barcode
ILL-BC81	Barcoding	CTGCAG	only the barcode
ILL-BC82	Barcoding	AACTTC	only the barcode
ILL-BC83	Barcoding	GGGTGC	only the barcode
ILL-BC84	Barcoding	TCCTGC	only the barcode
ILL-BC85	Barcoding	CGCGGC	only the barcode
ILL-BC86	Barcoding	ACCGCC	only the barcode
ILL-BC87	Barcoding	TAATAC	only the barcode
ILL-BC88	Barcoding	CACGTA	only the barcode
ILL-BC89	Barcoding	ATGTGA	only the barcode
ILL-BC90	Barcoding	TATAGA	only the barcode
ILL-BC91	Barcoding	TTTGCA	only the barcode
ILL-BC92	Barcoding	GTGCCA	only the barcode
ILL-BC93	Barcoding	CTAACA	only the barcode
ILL-BC94	Barcoding	ATAGAA	only the barcode
TruSeq-Mpx-2n	Barcoding	AATGATACGGCGACCAACGAAAAATACACTCTTCCCTACACGACGCTCTCCGAT	extends the linker at the 5' of the cDNA
TruSeq_Un1	Barcoding	AATGATACGGCGACCAACGAGATCTACAC ATCACG ACACTCTTCCCTACACGACGCTCTCCGATCT	
TruSeq_Un2	Barcoding	AATGATACGGCGACCAACGAGATCTACAC ACTTGA ACACTCTTCCCTACACGACGCTCTCCGATCT	
TruSeq_Un3	Barcoding	AATGATACGGCGACCAACGAGATCTACAC TAGCTT ACACTCTTCCCTACACGACGCTCTCCGATCT	
TruSeq_Un4	Barcoding	AATGATACGGCGACCAACGAGATCTACAC GGCTAC ACACTCTTCCCTACACGACGCTCTCCGATCT	
IC-P7	qPCR, final check	CAAGCAGAAGACGGCATAACGA	
IC-P5	Final check	AATGATACGGCGACCAACGA	