Preparing sequencing libraries for gene expression profiling using 3' cDNA tag sequencing (Tag-Seq) – Voss Lab Optimization Protocol

Adapted from Eli Meyer's updated 26 Aug 2016 protocol http://people.oregonstate.edu/~meyere/tools.html

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Overview

RNA is first fragmented to expose 3' ends of transcripts and standardize template lengths across genes.

cDNA is synthesized from the 3' ends of transcripts using oligo-dT primers and template switching activity to attach known sequences at each end of the fragment.

Constructs are amplified and purified, then barcodes and sequencing primer binding sites incorporated through PCR.

Finally, libraries are size-selected using gel purification to select a uniform size distribution appropriate for sequencing on the Illumina platform.

Before you start

- Have 1ug RNA in 8ul Tris buffer
  - (will add 2ul 10 mM MgCl (1:100 dilution of 1M stock) to make a total volume of 10ul)
- Have 100ng in 5ul to compare on frag gel with no MgCl
- RNA should be eluted into **10 mM Tris pH 8.0**.
- Contact O'Corry-Crowe Lab about Qubit availability

Fragmentation (Day 1)

- Add 2ul 10mM MgCl2 just before fragmentation and incubate at 95C for 5-10 minutes based on previous trials (Profile: 'Fragmentation')
- Analyze 100 ng (1ul product in 4ul NFW) from each sample of fragmented RNA, alongside an intact sample of ~100 ng RNA (in 5ul NFW) from the same sample, on a 1% agarose gel to confirm that fragmentation worked as intended or incubate for an additional 5min if needed.

Fragmentation Gel

Product used	Take 1ul of fragmented RNA and pipet to Parafilm with 4ul NFW
Gel	3g Agarose
	285 ml DI
	15 ml 20x SB
	6ul EtBr Single well combs
Load	3ul RNA ladder
	2ul Loading dye on Parafilm
	5ul unfragmented in every other using 10ul multichannel
	5ul product in every other using 10ul multichannel
Run	200 V for 10-15 min (can be up to 300V but make sure to watch dye front)

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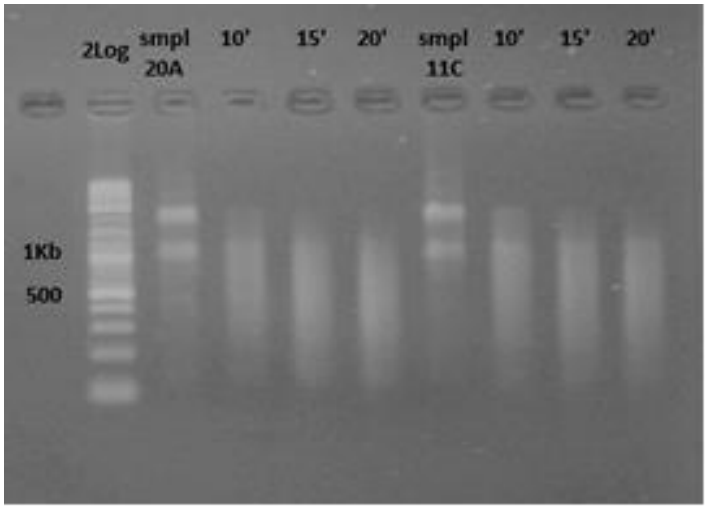
Gel Image: you want to see a distinct smear without bands starting at the subunit region. In the image below, the smear must extend all the way up into the region where ribosomal RNA bands were, while the bands themselves should be mostly gone. In the figure on the next page, 15' result is close to the ideal, but in fact all three incubation times are acceptable. Take out and fragment samples for an addition period of time if not completely fragmented.



First-strand cDNA synthesis (Day 1)

1. Confirm the volumes of fragmented RNA remain at least 9 µl after losses to evaporation during fragmentation, and replace with additional NFW if necessary to bring to a final volume of 9 µl.
2. Add 1 µl of the primer 3ILL-20TV (at 10 µM) to each well. Incubate at 65°C for 3 minutes in a thermocycler (Profile: 'cDNA synthesis step 1'), then transfer immediately onto ice.
3. Prepare a cDNA synthesis master mix

cDNA Synthesis

Component	**Reaction Vol (ul)**	**Total Vol 90 rxns**
Nuclease Free Water	3	270
dNTP (10mM ea)	1	90
5X first-strand buffer	4	360
10uM ILL-4N-TS	1	90
Tetro Reverse Transcriptase	1	90
Total	**10**	**900**
Fragmented RNA	9	
10uM 3ILL-20TV oligo; incubate with RNA at 65C for 3min before adding MM	1	90

4. Add 10 µl of this master mix to each sample of fragmented RNA on ice, mix thoroughly, and incubate in a thermocycler for one hour at 45°C (Profile: 'cDNA synthesis step 2')
5. Incubate at 85°C for 5 minutes to inactivate the RT (Included in 'cDNA synthesis step 2'), and **store on ice or at -20°C** until ready to proceed to the next step.

----------End of Day 1----------

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cDNA amplification (Day 2)

- Prepare a set of master mixes for test-scale PCR tests using 4-5 samples. This recipe assumes 2 µl of template, so if you use a different amount of template, adjust the water accordingly.

cDNA Amplification Test PCR						
Component	**B**	**Total Vol 6 rxns**	**C**	**Total Vol 6 rxns**	**D**	**Total Vol 6 rxns**
Nuclease Free Water	12.7	**76.2**	12.7	**76.2**	12.1	**72.6**
dNTP (10mM ea)	0.5	**3**	0.5	**3**	0.5	**3**
5X Q5 reaction buffer (2 tubes)	4	**24**	4	**24**	4	**24**
10uM 5ILL oligo	0.6	**3.6**	0	**0**	0.6	**3.6**
10uM 3ILL-20TV oligo	0	**0**	0.6	**3.6**	0.6	**3.6**
Q5 DNA Polymerase	0.2	**1.2**	0.2	**1.2**	0.2	**1.2**
Total	**18.0**	**108**	**18.0**	**108**	**18**	**108**
First-strand cDNA	2ul		2ul		2ul	

2. For each sample, prepare three PCR tubes labeled B-D. Add 18 µl of the appropriate master mix to each tube.
3. Add 2 µl of the FS-cDNA prepared above.
4. Amplify in a thermocycler using the following profile: 'cDNA amplification test PCR'

   98°C 30 sec, (98°C 10 sec, 60°C 30 sec, 72°C 30 sec) X 10 cycles

5. Sample 5 µl from each reaction after 13, 16, 19, and 22 cycles
6. After sampling each reaction at each of 3 cycle numbers (e.g. 13, 16, 19, and 22 cycles), load each sample on a 2% agarose gel.

cDNA Amplification Test Gel

Product used	Take a subset of samples (4-6) that encompass all the levels of quality from your fragmentation gel
Gel	2% Agarose Gel 300mL (large)
	6g Agarose
	285 ml DI
	15 ml 20x SB
	6ul EtBr
	Single well combs
Load	3ul DNA ladder
	2ul loading dye on Parafilm
	5ul product
Run	150 V for 15-20 min

Ideally, reaction D should show a smear ranging from ~100-500 bp, and nothing should be visible in reactions B and C.

If nothing is detected in reaction D, you can repeat the reactions at higher cycle numbers (up to a maximum of 20), or with additional template.

If product ever appears in reactions C, this indicates too much template, too many cycles, or contamination in one or more reagents. Troubleshoot the FS- cDNA.

A small amount of product in reaction B is OK, since the 3ILL-20TV oligo omitted in that reaction is still present at low concentrations in the FS-cDNA. The goal at this stage is to

identify the minimum cycle number that produces a visible smear in reaction D while remaining reasonably clean in reactions B-C.

7. After determining the optimum amount of template and number of cycles, prepare a larger prep-scale reaction for each cDNA sample as follows. This recipe assumes 10 µl of template (i.e. scaled up five-fold from the default amount in test-scale PCR), so if you use more than 2 µl template in test-scale PCR adjust the template and water accordingly.

cDNA Amplification Final PCR

Component	Reaction Vol (ul)	Total Vol 90 rxns
Nuclease Free Water	60.5	5445
dNTP (10mM ea)	2.5	225
5X Q5 reaction buffer	20	1800
10uM 5ILL oligo	3	270
10uM 3ILL-20TV oligo	3	270
Q5 DNA Polymerase	1	90
Total	**90**	**8100**
First-strand cDNA	10ul	

8. After full scale PCR run 5ul on a 2% Agarose gel

cDNA Amplification Final Gel

Gel	2% Agarose Gel 300mL (large)
	6g Agarose
	285 ml DI
	15 ml 20X SB
	6ul EtBr
	Single well combs
Load	3ul DNA ladder
	2ul loading dye on parafilm
	5ul product
Run	150 V for 15-20 min

9. Purify PCR products with the GeneJet PCR Purification kit. Be sure to include any optional steps intended to include low-molecular-weight fragments (<200 bp).

Gene Jet Protocol: (Day 2)

1. Prep 0.6ml tubes for all the samples and transfer remaining PCR product (95ul).
2. Add 95ul (1:1 volume) Binding Buffer to completed PCR mixture
    a. Mix thoroughly
    b. Check the color of the solution
        i. If yellow: the pH is optimal for DNA binding
        ii. If orange or violet: add 10ul of 3M sodium acetate, pH 5.2 and mix
        iii. The color will then become yellow
3. Add 95ul (1:2 volume) 100% isopropanol, mix thoroughly
4. Transfer up to 800ul (~300ul) of the solution to the GeneJet purification column

5. Centrifuge for 30-60 sec at >12,000 X g
6. Add 700ul of Wash Buffer (diluted with ethanol as described in protocol)
7. Centrifuge for 30-60 sec at 12,000 X g
    a. Discard flow-through and place the purification column back into the collection tube

8. Centrifuge the empty GeneJet purification column for an additional 1min to completely remove any residual wash buffer
9. Transfer the GeneJet purification column to a clean 1.5ml microcentrifuge tube
10. Add 25ul Elution Buffer (pre-heated 65C in incubator) to the center of the column, let incubate ~5 minutes
11. Centrifuge for 1 min at 12,000 X g
12. Discard the GeneJet purification column and store the purified DNA at -20C or on ice.

cDNA Quantification (Day 2)

10. Quantify by QuBit in the O'Corry-Crowe Lab – contact Heidi and/or Jeremie for availability.
    *You need at least 50-70 ng cDNA (total) for each sample at this stage.
    Store DNA at -20 until next steps

Qubit Protocol for 40 Samples

1) Set out stds & soln to be measured >30min beforehand, must be room temp				
2) Prepare working stock (Reagent:Buffer dilution of 1:200)				
#Samples	80			
#Standards	2			
	82	x 200 =	16,400	Total ul working stock
			82	ul Reagent
			16,318	ul Buffer* (Light sensitive)
4) Add 2 ul sample to 198 ul working stock for each sample				
5) Vortex all tubes for 3 seconds, be careful not to create bubbles				
6) Incubate all tubes at RT for 2 minutes				
7) Measure on Qubit				
Notes: Only label top of tubes, not sides Do not mix working solution in glass container Can use anywhere from 1-20 ul sample Assay temperature is extremely sensitive, all reagents and samples must be room temp Make sure assay volume is correct after each run				

----------End of Day 2----------

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Adaptor extension and barcoding (Day 3)

1. Test-scale PCRs are conducted to verify yield and specificity of the reaction, using a variety of primer combinations to control for artifacts that may arise from contamination.
    a. 6-8 samples
    b. For each sample to be tested, dilute an aliquot to 10 ng μl^{-1} so that all templates are at the same concentration.
2. Prepare four separate master mixes for test-scale PCR.
    a. Aliquot 8ul of each MM into each well
    b. Add 2 µl of the cDNA previously quantified and diluted to 10 ng µl-1 (above) to each of these four reactions (B-D) for a total volume in each reaction of 10 µl.

Barcoding test PCR

Component	B	Total Vol 10 rxns	C	Total Vol 10 rxns	D	Total Vol 10 rxns
Nuclease Free Water	3.7	**37**	3.7	**37**	1.7	**17**
dNTP (10mM ea)	0.2	**2**	0.2	**2**	0.2	**2**
5X Q5 reaction buffer (2 tubes)	2	**20**	2	**20**	2	**20**
Q5 DNA Polymerase	0.1	**1**	0.1	**1**	0.1	**1**
5' TruSeq barcode	2	**20**	0	**0**	2	**20**
3' BCXX barcode	0	**0**	2	**20**	2	**20**
Total	**8**	**80**	**8**	**80**	**8**	**80**
Diluted PCR template	2		2		2	

3. Amplify in a thermocycler using the following profile: 'Barcoding test PCR'

    98°C 30 sec, (98°C 10 sec, 60°C 30 sec, 72°C 30 sec) X 4 cycles

4. After 4 cycles check 5 µl of the PCR products for all reactions on a gel.

    Ideally, reaction D should show a smear ranging from ~200-600 bp, and nothing should be visible in reactions B and C.

    If nothing is detected in reaction D, you can continue the reaction for additional cycles (up to a total of 6), or repeat the reaction with additional template.

    Reactions B and C should remain relatively clean regardless of cycle numbers. If you observe products in these reactions it probably indicates contamination of the reagents or libraries.

Barcoding Test Gels

Product used	Take a subset of samples (~6-10) that encompass all the levels of quality from your fragmentation gel
Gel	2% Agarose Gel 300mL (large)
	6g Agarose
	285 ml DI
	15 ml 20x SB
	6ul EtBr
	Single well combs
Load	3ul DNA ladder
	2ul loading dye on Parafilm
	5ul product
Run	150 V for 15-20 min

7. When the optimum number of cycles and volume of template have been determined, conduct a full-scale reaction based on those parameters, using with 50 ng template in 50 µl total volume. The following master mix assumes the use of 10 µl of template per 50 ul reaction. *Thaw one full set of 14 barcodes and 4 Universal and Nanodrop before use. Select 10 individual barcodes that are the most accurate (near 1uM) for use.

Final Barcoding PCR

Component	Reaction Vol (ul)	Total Vol 90 rxns
Nuclease Free Water	26.5	2385
dNTP (10mM ea)	1	90
5X Q5 reaction buffer	10	900
Q5 DNA Polymerase	0.5	45
Total	**38**	3420
5' TruSeq barcode (10 uM)	1	
3' BCXX barcode (10 uM)	1	
purified cDNA diluted to 5ng/ul	10	

8. Aliquot 38 µl of master mix to each well, then add 1 µl of each sample-specific barcode oligo (at 10 µM), and 10 µl of purified cDNA (diluted to 5 ng μl^{-1}).
*Be sure to record your barcode assignments at this step!

10. Amplify these reactions using the same profile and cycle number as determined above
- Check 5ul product on gel

Barcoding Final Gel

Gel	2% Agarose Gel 300mL (large)
	6g Agarose
	285 ml DI
	15 ml 20X SB
	6ul EtBr
	Single well combs
Load	3ul DNA ladder
	2ul loading dye on Parafilm
	5ul product
Run	150 V for 15-20 min

Gel purification (Day 3)

In this step sequencing constructs are gel-purified to eliminate residual genomic DNA and primer dimers. You will need to prep a 1.5 ml tube for each sample's gel slice.

1. Use the prepared aliquots of loading dye diluted 1:10 with 50% glycerol to minimize dye carryover.
2. Prepare a 2% agarose gel. Use a thick comb that can accommodate 100 µl volumes, this can be the double wide combs on the large 300ml gel box.
3. Combine each 50 µl PCR product with 10 µl of diluted loading buffer, load entire mixture into the gel, and run the gel long enough for good resolution in the 50-500 bp range.
4. View the gel on a blue LED transilluminator to verify the presence of target bands and adequate separation of molecular weight standards to resolve bands in the 50 to 500 bp range. Typically ~5 cm run distance (well to dye front) is sufficient.

5. Cut out the target molecular weight range (200-300 bp), being careful to cut the same range for each sample.

Gel Purification and Size Selection

Gel	2% Agarose Gel 300mL (large)
	6g Agarose
	285 ml DI
	15 ml 20X SB
	30ul SYBR
	Double well combs
Load	6ul DNA ladder
	10ul DNA loading buffer diluted to 1:10 using a 50% glycerol solution with NFW
	50ul product
Run	150 V for 15-20 min
	Typically ~5cm run distance from well to dye front is sufficient
Cut	Cut out 200-300 bp range
	Use razor blade to cut an entire row out
	View and image using Blue Light box and glasses
	Use razor blade to cut line just above desired size range straight across and same for just below
	Make single cut between each sample lane
	Turn off light and place each slice in 2ml tube

6. Transfer each gel slice into a 1.5ml microcentrifuge tube and add 40 µl NFW.
7. Centrifuge tubes 1 min at high speed to bring gel into contact with the water. Incubate at 4°C overnight to maximize yield.

----------End of Day 3----------

8. Freeze at -80°C for at least 30 minutes.
9. Centrifuge at maximum speed (~20,000 X g) for 10 minutes.
10. Press gel slice against side of tube using pipette tip, and withdraw supernatant (at least 60 µl should be recovered). If less than 60 µl is accessible, repeat centrifugation. Transfer the supernatant to a new PCR tube plate.

Combining libraries for sequencing (Day 4)

Finally, the libraries are pooled in equal ratios in an effort to sequence all samples at equal coverage.

1. Prepare a 1:100 dilution of each library by combining 2 µl of the eluted library (above) with 198 µl NFW using the multichannel pipette
2. To quantify each library using qPCR, prepare a master mix. Complete a replicate of 2 rounds per sample.

qPCR for Combining libraries

Component	Reaction Vol (ul)	Total Vol 90 rxns
Nuclease Free Water	4.3	387
SYBR qPCR master mix	7.5	675
ILL-Lib1 10uM	0.6	54
ILL-Lib2 10uM	0.6	54
Total	**13**	
library diluted 1:100	2	

3. Pipette 13 µl qPCR master mix into each well of a PCR plate (Use Real-Time qPCR Plates and Optical Tape), then add 2 µl of each diluted library to the appropriate well.
4. Conduct qPCR using program 'qPCR Quantification' and calculate C_T for each sample.
5. Repeat steps 2-4 with a second plate containing the second half of the samples.
6. To determine volumes of each library for the combined pool:
    a. Rank samples from lowest to highest C_T and identify reference sample (sample with highest C_T)
    b. Calculate proportion of each library to sequence as:

$$P_L = 2 \wedge [C_T \text{ (sample)} - C_T \text{ (reference)}]$$

    c. Calculate the volume of each library to use as:

$$V = P_L * 60 \text{ µl}$$

Note: If you've chosen a reference sample with a very high CT (suggesting a failed library prep) relative to the others, very low volumes (<2 µl) may be calculated at this step. If so, choose the next sample (i.e. next lowest CT) as reference instead, and continue adjusting choice of reference until reasonably high volumes are calculated for the majority of samples.

Note: As a rough rule of thumb, the pool of combined samples used for a single lane of sequencing should be <u>at least</u> 200-500 µl at this stage (it may be substantially higher).

7. Combine libraries using the volumes calculated from qPCR to produce a pool for sequencing.

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8. Illumina sequencing typically requires templates in ≤20 µl volume at ≥2 nM concentration. The pooled libraries produced above are typically too dilute for sequencing. To purify and concentrate these libraries, they may be precipitated with isopropanol (below) or using a commercial PCR cleanup kit.

9. To clean and precipitate:

   a. add 0.1 volumes of 3M sodium acetate and 3 volumes 100% isopropanol. Incubate overnight at -20°C. (If pool volume > 450 µl, reaction will not fit in 2 mL tube and must be split into replicates)

   b. Centrifuge 30 minutes at maximum speed, 4°C.

   c. Remove isopropanol by **pouring carefully**, pellets are very likely to slide off. Pipette remaining isopropanol that doesn't pour out. Add 150 µl cold 70% ethanol and flick gently to wash pellet. Centrifuge for 10 minutes at maximum speed, 4°C.

   d. Remove ethanol with pipette. Dry pellet 10-15' sitting up in the hood, covered with a kim-wipe. Resuspend in 20 µl NFW (final pool should be 20 µl, so adjust accordingly if performing replicates or need to nanodrop).

   e. Incubate 15-30 minutes at 55°C to resuspend pellet. Transfer into 1.5 mL safelock tube and combine replicates if needed.

10. (Optional) Quantify your pooled library using a method sufficiently precise and sensitive to quantify concentrations in the 1-10 ng μl^{-1} range. You need a concentration of **at least 1.0 ng μl^{-1}** at this stage to have enough for sequencing.