<u>Protocol: Generating RNA Baits for Capture-based enrichment</u>

Tung lab, Duke University, September 10th, 2015

1. **Extract high quality, genomic DNA (gDNA) from a primary tissue sample.**
   *Note that you do not want to make the baits from the same individual you will ultimately enrich DNA from.

2. **Sonicate** 5 µg of gDNA to ~150 bp (Diagenode Bioruptor)
   Bioruptor conditions: 30 seconds on / 30 seconds off – "H" setting – 45 minutes
   100 ul total volume in 1.5 mL tube
   * be sure to allow bioruptor to cool to 4C prior to shearing, if shearing a lot of samples give the bioruptor a 15 minute break between every hour of shearing to ensure the machine doesn't overheat

3. **Purify with AMPure Beads**
   1. Ensure beads are at room temperature (RT) and resuspended prior to use
   2. Add the following: 180 uL beads + 100 uL sample
   3. Mix thoroughly by pipetting up and down 10 times
   4. Incubate at RT for 5 minutes
   5. Place sample on magnetic stand for 5 minutes at RT or until liquid is completely clear
   6. Remove and discard most of liquid, do not disturb the beads, it is okay if there is a little liquid left over at this time
   7. Keeping the sample on the magnetic stand wash with 200 ul of 80% EtOH.
   8. Remove and discard the EtOH and repeat for a total of 2 washes with 80% EtOH
   9. Keep sample on magnetic stand and allow beads to dry at RT for 15 minutes
   10. Resuspend samples in 50 µL of water and incubate at RT for 5 minutes
   11. Place samples on magnetic stand at RT for 5 minutes or until solution is cleared.
   12. Recover DNA in 50 µL of supernatant and transfer to new tubes.

4. **End-Repair (KAPA Biosystems Library Prep Kit)**
   Mix:

Water (to 100 µL)	35 µL
10X End Repair Buffer	10 µL
End Repair Enzyme Mix	5 µL
Sheared DNA	50 µL

   Incubate at 20°C for 30 minutes on thermomixer

5. **Purify with AMPure Beads** - 180 µL beads, elute into 30 µL H_2O
   1. Ensure beads are at RT and resuspended prior to use
   2. Add the following: 180 uL beads + 100 uL sample
   3. Mix thoroughly by pipetting up and down 10x
   4. Incubate at RT for 5 minutes
   5. Place sample on magnetic stand for 5 minutes at RT or until liquid is completely clear
   6. Remove and discard most of liquid, do not disturb the beads, it is okay if there is a little liquid left over at this time
   7. Keeping the sample on the magnetic stand wash with 200 ul of 80% EtOH.
   8. Remove and discard the EtOH and repeat for a total of 2 washes with 80% EtOH
   9. Keep sample on magnetic stand and allow beads to dry at RT for 15 minutes.
   10. Resuspend samples in 30 ul of water and incubate at RT for 5 minutes
   11. Place samples on magnetic stand at RT for 5 minutes or until solution is cleared.
   12. Recover DNA in 30 ul of supernatant and transfer to new tubes.

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6. A-tail (KAPA Biosystems Library Prep Kit)

Mix:

Water (to 50 μL)	12 μL
10X A-Tailing Buffer	5 μL
A-Tailing Enzyme	3 μL
End-Repaired DNA	30 μL

Incubate at 30°C for 30 minutes on thermomixer

7. Purify with AMPure Beads - 90 μL beads, elute into 30 μL H_2O

1. Ensure beads are at RT and resuspended prior to use
2. Add the following: 90 uL beads + 50 uL sample
3. Mix thoroughly by pipetting up and down 10 x
4. Incubate at RT for 5 minutes
5. Place sample on magnetic stand for 5 minutes at RT or until liquid is completely clear
6. Remove and discard most of liquid, do not disturb the beads, it is okay if there is a little liquid left over at this time
7. Keeping the sample on the magnetic stand wash with 200 ul of 80% EtOH.
8. Remove and discard the EtOH and repeat for a total of 2 washes with 80% EtOH
9. Keep sample on magnetic stand and allow beads to dry at RT for 15 minutes
10. Resuspend samples in 30 ul of water and incubate at RT for 5 minutes
11. Place samples on magnetic stand at RT for 5 minutes or until solution is cleared.
12. Recover DNA in 30 ul of supernatant and transfer to new tubes.

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8. Pre-Anneal Oligos to make T-overhanging adapters

Recipe:
Rehydrate IDT pellets to an initial stock: 150 μM in H_2O.

Mix together:

Adapter Oligo 1:	16.7 μL	(EcoT7dev)
Adapter Oligo 2:	16.7 μL	
10X NEB Buffer 2:	10 μL	(CutSmart)
H_2O:	56.6 μL	

* This dilutes each stock adapter 1:6 and the NEB Buffer 2 1:10, making a 25 μM adapter solution.

Incubate at 95°C for 5 min , then remove and let cool at room temperature (RT) for ~10 min.
Store at room temperature.

9. Oligo/Adapter Design (Only refer to step 9 when new adapters need to be created): This is the Oligo design for step 8.

Promoter for T7 transcription: TAATACGACTCACTATA

The protocol for the IVT kit we use emphasizes importance of having the three additional G's after this sequence; transcription efficiency is supposedly greatly enhanced.

- We have added restriction sites 3' of the promoter
- General oligo schematic:
  - Oligo 1: 5'-[NNN]-[Promoter]-GGG-[RE site]-T-3'

- Oligo 2: Reverse complement of Oligo 1, except no 5' A to match the Oligo 1's 3' T. Also, the 5' end (complementary to Oligo 1's 3' end) has a 5'-phosphate added ("/5Phos/" when ordering from IDT).
- "EcoT7" oligos:
  - EcoOT7dTV Fwd: 5'- GGA AGG AAG GAA GAG A**TA ATA CGA CTC ACT ATA GGG** CCT GGT (T7 promoter in **bold**, restriction site underlined)
  - EcoOT7dTV Rev: 5'-/5Phos/C CAG GCC CTA TAG TGA GTC GTA TTA TCT CTT CCT TCC TTC C
  - T_m=65.2°C, 46 bp

10. Ligate Adapters (KAPA Biosystems)

Mix:

Water	to 50 µL	
5X Ligation Buffer	10 µL	
DNA Ligase	5 µL	
Adapter (25 µM)	1 µL	(this is the adapter made in previous step)
A-tailed DNA	≤ 34 µL	

Incubate at 20°C for 15 minutes

11. **Purify with AMPure Beads** - 90 µL beads, elute into 30 µL H_2O

1. Ensure beads are at RT and resuspended prior to use
2. Add the following: 90 uL beads + 50 uL sample
3. Mix thoroughly by pipetting up and down 10 x
4. Incubate at RT for 5 minutes
5. Place sample on magnetic stand for 5 minutes at RT or until liquid is completely clear
6. Remove and discard most of liquid, do not disturb the beads, it is okay if there is a little liquid left over at this time
7. Keeping the sample on the magnetic stand wash with 200 ul of 80% EtOH.
8. Remove and discard the EtOH and repeat for a total of 2 washes with 80% EtOH
9. Keep sample on magnetic stand and allow beads to dry at RT for 15 minutes
10. Resuspend samples in 30 ul of water and incubate at RT for 5 minutes
11. Place samples on magnetic stand at RT for 5 minutes or until solution is cleared.
12. Recover DNA in 30 ul of supernatant and transfer to new tubes.

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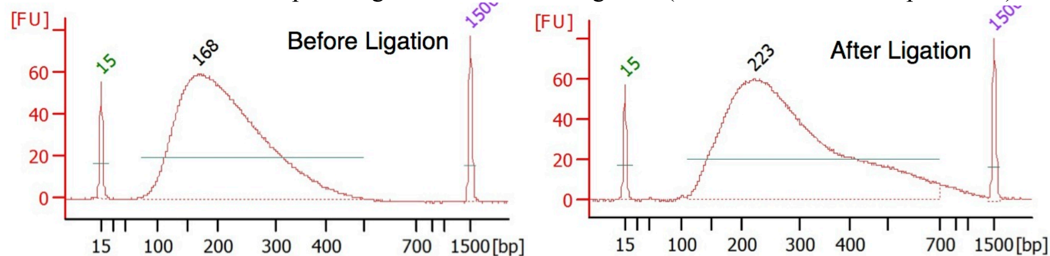
12. **Bioanalyze (DNA 1000)** to verify ligation and to quantify

Sample images of a successful ligation (notice the shift in the peak size):



13. Digest ligated DNA library with duplex-specific nuclease (DSN)

(Note: You need to rehydrate DSN before)
Duplex Specific Nuclease (DSN) from Axxora.com (EVN-EA001, 002, or 003)

Because this procedure uses very small volumes, there are a few things to consider:

- Be sure to add reagents directly to reaction volume; make sure nothing is stuck to the side of the tube!
- Consider doing several replicates of each sample that you digest. This way, if you make any mistakes or have any reaction failures later on, you'll have more digested DNA to spare.

DNA Denaturation Mix:

2 µL DNA, representing 50-500 ng (from previous step 30 ul – can do 14-15 rxns)

1 µL 4X hybridization buffer

- 200 mM HEPES pH 7.5
- 2 M NaCl
- 0.8 mM EDTA

1 µL human Cot-1 DNA (1 µg/µL)

Incubate denaturation mix

- 98°C for 3 minutes
- hold at 65°C

After 4 hours of incubation/annealing at 65°C, add in this order:

- 4 µL H_2O, pre-warmed to 70°C
- 1 µL 10X DSN Buffer, pre-warmed to 70°C (can combine H20 and DSN buffer prior to the 70C incubation)
- 1 µL DSN (1 U/µL)

After 20 more minutes of incubation at 65°C, add:

- 5 µL 2X DSN Stop Solution (10 mM EDTA), pre-warmed to 70°C

14. Purify DNA with AMPure Beads: 36 µL beads, elute into 10 µL H_2O

1. Ensure beads are at RT and resuspended prior to use
2. Add the following: 36 uL beads + 15 uL sample
3. Mix thoroughly by pipetting up and down 10x
4. Incubate at **RT for 15 minutes** (notice that this is longer than the typical cleanup)
5. Place sample on magnetic stand for 15 minutes at RT or until liquid is completely clear
6. Remove and discard most of liquid, do not disturb the beads, it is okay if there is a little liquid left over at this time
7. Keeping the sample on the magnetic stand wash with 200 ul of 80% EtOH.
8. Remove and discard the EtOH and repeat for a total of 2 washes with 80% EtOH
9. Remove sample from magnetic stand and allow beads to dry at RT for 15 minutes.
10. Resuspend samples in 30 ul of water and incubate at RT for 2 minutes
11. Place samples on magnetic stand at RT for 5 minutes or until solution is cleared.
12. Recover DNA in 10 ul of supernatant and transfer to new tubes.

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15. Klenow Fill-in of DSN-digested DNA

Mix:

15 µM EcoOT7dTV Fwd Primer	0.5 µL
10X NEB Buffer 2	2.5 µL
10 mM dTNPs	0.5 µL
Water	10 µL

DNA	10 µL

Incubate:
94°C, 2 minutes to melt DNA
Ramp -1°C/sec to 35°C, hold for 2 minutes to anneal
Ramp -0.5°C/sec to 25°C
Hold at 25°C for at least 45 seconds, but no more than 6 minutes. During this time:
Add 1 µL (5U) Klenow DNA Polymerase (NEB M0210S)
Spin down condensation on tube, if necessary
37°C, 90 minutes
75°C, 20 min (heat inactivation)
(* In addition or alternatively, reaction can also be stopped with 2.5 µL 0.5M EDTA pH 8.0)

16. Run DNA on a gel

a) Prepare a 2% agarose cell (e.g., 100mL TAE + 2g agarose)
b) Add all of DSN-digested DNA (~25 µL) per well
c) Excise and purify the 200-300 bp region using Zymoclean Gel DNA Recovery Kit (or other Gel DNA purification kits)
*Note: If you chose to do replicate digestions, you may want to pool replicate samples together after the gel-purification
d) Weigh eppendorf tube with gel fragment and record the weight. Make sure to weigh an empty eppendorf tube and subtract the weight to get the weight of the gel fragment.
e) Add 3 volumes of ADB to each volume of gel. (exp: 100 mg gel slice, add 300 ul of ADB).
f) Incubate samples at 55C in thermomixer for 10 minutes (do not incubate over 60C). Vortex occasionally to break up agarose pieces. Can incubate a little longer if agarose pieces are not dissolving.
g) Load entire sample into a Zymo-Sping Column in a Collection Tube.
h) Centrifuge at full speed (> 10,000g) for 1 minute. Discard flow through.
i) Add 200 ul of DNA Wash Buffer to the column and centrifuge for 1 minute. Repeat Wash step 1X.
j) Spin sample for 1 minute with nothing in it to ensure all DNA Wash Buffer is out of column.
k) Place Zymo Spin Column in a new 1.5 ml tube. Add 11 ul of water (pre heat water to 55C) directly to the membrane and allow it to incubate for 5 minutes prior to spinning.
l) Spin down for 1 minute. Keep flow through (~10µL).

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17. PCR-Amplify the DSN-digested DNA

*To assure sufficient DNA for later do replicate PCRs of 3-5 µL DNA per well

Reagents:
Notes: (Use a high-fidelity polymerase. The recipe shown is for the HiFi polymerase in KAPA's Library Prep Kit.)

1. For primers, use two oligos that each make up approximately half of the "Fwd" oligo used to make the T7 adapter (the oligo with the overhanging T)
2. To amplify from the EcoT7 adapter, use these (note that the last ten nt from #1 and the first ten nt from #2 are identical)
    a. **EcoT7 PCR 1**: 5'-GGA AGG AAG GAA GAG ATA ATA CGA CTC ACT
    b. **EcoT7 PCR 2**: 5'-TAC GAC TCA CTA TAG GGC CTG GT
    c. T_m=58.7-58.8°C

Mix:

2X HiFi RM	25 µL	
Primer 1 (25 µM)	1 µL	(EcoOT7 PCR 1)

Primer 2 (25 μM)	1 μL	(EcoOT7 PCR 2)
DSN-Digested DNA	3-5 μL	
Water	to 50 μL total reaction volume	

PCR Conditions:

1) 98°C – 45 seconds
2) 98°C – 15 seconds
3) 65°C – 30 seconds
4) 72°C – 30 seconds
5) Repeat steps 2-4 15x for a total of 16 cycles
6) 72°C – 1 minute
7) 10°C – ∞

18. Purify DNA with AMPure Beads - 90 μL beads, elute into 30 μL H_2O

(If you chose to do replicate PCRs, pool replicate eluates)

1. Ensure beads are at RT and resuspended prior to use
2. Add the following: 90 uL beads + 50 uL sample
3. Mix thoroughly by pipetting up and down 10 x
4. Incubate at RT for 5 minutes
5. Place sample on magnetic stand for 5 minutes at RT or until liquid is completely clear
6. Remove and discard most of liquid, do not disturb the beads, it is okay if there is a little liquid left over at this time
7. Keeping the sample on the magnetic stand wash with 200 ul of 80% EtOH.
8. Remove and discard the EtOH and repeat for a total of 2 washes with 80% EtOH
9. Keep on magnetic stand and allow beads to dry at RT for 15 minutes.
10. Resuspend samples in 30 ul of water and incubate at RT for 5 minutes
11. Place samples on magnetic stand at RT for 5 minutes or until solution is cleared.
12. Recover DNA in 30 ul of supernatant and transfer to new tubes.

19. Bioanalyze (DNA 1000) - to verify that products are of desired size, and to record molarity

*This should be about same size as adapter ligated sample

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20. *In vitro* transcription and subsequent purification

- Using Speedvac, adjust volume of DNA libraries to at least 100 nM (even better to go up to ~125-150 nM). Ambion MEGAShortscript kit protocol suggests to aim for ~75-100 nM, but due to the PCR primers used to amplify this DNA template we expect only ~1/2 of the strands to contain the full T7 promoter. So the higher the concentration, the better.

Mix *at room temperature* (unless noted otherwise, reagents are from Ambion MEGAShortscript Kit, AM1354):

H_2O	to 20 μL	
T7 10X Reaction Buffer	2 μL	
T7 ATP Solution (75 mM)	2 μL	
T7 CTP Solution (75 mM)	2 μL	
T7 GTP Solution (75 mM)	2 μL	
T7 UTP Solution (75 mM)	1.33 μL	
Biotin-UTP (50 mM)	1.00 μL	*from the kit: **Illumina, BU6105H**
Template DNA (at least 100 nM)	≤7.67 μL	
T7 Enzyme Mix	2 μL	

Incubate at 37°C, 4 hours

21. Digest DNA

Add 1 µL TURBO DNase

Incubate at 37°C, 15 minutes

22. Purify with Ambion MEGAClear Kit, AM1908

1. Bring the RNA sample to 100 µL with Elution Solution. Mix gently but thoroughly.
2. Add 350 µL of Binding Solution Concentrate to the sample. Mix gently by pipetting.
3. Add 250 µL of 100% ethanol to the sample. Mix gently by pipetting.
4. Apply the sample to a centrifuge filter:
    a. Insert a Filter Cartridge into 1 of the Collection and Elution Tubes supplied.
    b. Pipet the RNA mixture onto the Filter Cartridge.
    c. Centrifuge for ~15 sec to 1 min, or until the mixture has passed through the filter. Centrifuge at RCF 10,000 x g. Spinning faster than this may damage the filters.
    d. Discard the flow-through and reuse the Collection and Elution Tube for the washing steps.
5. Wash with 2 x 500 µL Wash Solution. Make sure that the ethanol has been added to the Wash Solution Concentrate before using it.
    a. Apply 500 µL Wash Solution. Draw the Wash Solution through the filter as in the previous step.
    b. Repeat with a second 500 µL aliquot of Wash Solution.
    c. After discarding the Wash Solution, continue centrifugation for at least 10–30 sec to remove the last traces of Wash Solution.
6. Elute RNA using either one of the methods described below; they are equivalent in terms of RNA recovery.
7. RNA elution
    a. Pre-heat 110 µL of Elution Solution per sample to 95°C.
    b. Apply 35 µL of the pre-heated Elution Solution to the center of the Filter Cartridge, close the cap of the tube and centrifuge for 1 min at room temperature (10,000 x g) to elute the RNA.
    c. To maximize RNA recovery, repeat this elution procedure with a second pre-heated 35 µL aliquot of Elution Solution. Collect the eluate into the same Collection/Elution Tube.
    d. Should yield for a total product that is 70 ul.

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23. Quantify with Nanodrop

Using Nanodrop estimated concentration (ng/µL), estimate molarity, assuming that your RNA are ~250 bases

1 ng/µL of 1 kb RNA is ~1.5 nM, so your nM = (1.5 nM × *n* ng/µL)/0.250 kb

More simply, **nM= 6 × *n* ng/µL,** where *n* is the concentration estimated from the nanodrop

24. Restriction Digestion of the RNA Baits

*RNA digestion adapted from Murray et al, Nuc. Acids Res. 2010; doi: 10.1093/nar/gkq702

*Complete steps 24 and 25 for 3 sets of RNA baits – so when it comes to do the MEGAclear clean up you will pool a total of 60 ul of RNA bait product and then will bring it to 100 ul by adding 40 ul of elution solution

Recipe:

Mix:

RNA (at least 100 pmol)	14.4 µL
10X NEB Buffer 4	1.6 µL

Incubate at 90°C for 5 minutes, then cool to RT on the bench for ~10 minutes

25. RE digestion

To use all of the annealed RNA, add the below mix directly to the tube that you used for annealing:

Mix

Water	0.6 µL (or to 20 µL)
BSA (1 mg/mL)	2.0 µL
Annealed RNA	16 µL
10X NEB Buffer 4	0.4 µL (note that there is already NEB buffer in the annealed RNA)
*Eco*O109I Enzyme	1.0 µL

Incubate at 37°C for 4 hours
Heat-inactivate: 65°C for 20 minutes

26. Purify RNA with MEGAClear Kit (same protocol as above)

1. Bring the RNA sample to 100 µL with Elution Solution. Mix gently but thoroughly.
2. Add 350 µL of Binding Solution Concentrate to the sample. Mix gently by pipetting.
3. Add 250 µL of 100% ethanol to the sample. Mix gently by pipetting.
4. Apply the sample to a centrifuge filter:
    a. Insert a Filter Cartridge into 1 of the Collection and Elution Tubes supplied.
    b. Pipet the RNA mixture onto the Filter Cartridge.
    c. Centrifuge for ~15 sec to 1 min, or until the mixture has passed through the filter. Centrifuge at RCF 10,000 x g. Spinning faster than this may damage the filters.
    d. Discard the flow-through and reuse the Collection and Elution Tube for the washing steps.
5. Wash with 2 x 500 µL Wash Solution. Make sure that the ethanol has been added to the Wash Solution Concentrate before using it.
    a. Apply 500 µL Wash Solution. Draw the Wash Solution through the filter as in the previous step.
    b. Repeat with a second 500 µL aliquot of Wash Solution.
    c. After discarding the Wash Solution, continue centrifugation for at least 10–30 sec to remove the last traces of Wash Solution.
6. Elute RNA using either one of the methods described below; they are equivalent in terms of RNA recovery.
7. RNA elution
    a. Pre-heat 110 µL of Elution Solution per sample to 95°C.
    b. Apply 35 µL of the pre-heated Elution Solution to the center of the Filter Cartridge, close the cap of the tube and centrifuge for 1 min at room temperature (10,000 x g) to elute the RNA.
    c. To maximize RNA recovery, repeat this elution procedure with a second pre-heated 35 µL aliquot of Elution Solution. Collect the eluate into the same Collection/Elution Tube.
    d. Should yield for a total product that is 70 ul.

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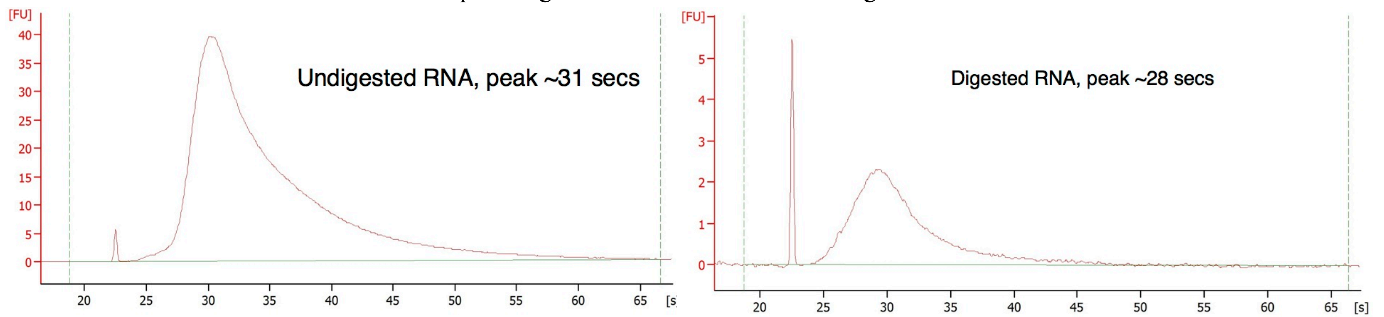
27. Bioanalyze (RNA 6000, "Eukaryote Total RNA")

Check for digestion by Bioanalyzing both undigested and digested RNAs

1. The "Eukaryote Total RNA" assay runs each well a few seconds slower, allowing better visualization of a size shift

2. Sample images of successful EcoO109I digestion:



Given this, the sample images above suggest a shift from ~300 nt to ~200 nt. The EcoT7 adapter, which is on both sides of each fragment, is 46 nt (so ~100nt combined)

Store RNA at -80°C