Project:_____ RADseqReady Plate #_____ Library #_____
Name: _____ Date: _____

BestRAD Library Preparation
Based on protocol of Ali *et al.* 2015 (10.1534/genetics.115.183665)
Adapted by Linda Rutledge many times but this version was done on August 16, 2016

Section 1: DNA Standardization

1. Standardize your high quality genomic DNA with 75 ng in 15 uL (ie. 5 ng/uL) in a 96 well plate.

Section 2: Restriction Enzyme Digestion

Materials Required

- ☐ 10x Cutsmart Buffer
- ☐ Molecular Grade Water
- ☐ *Sbf*I-HF Restriction Enzyme (NEB R3642L)
- ☐ 8-well PCR strips
- ☐ 1.5 mL tubes

1. Prepare restriction enzyme (RE) digestion master mix (MM) as follows:

Item	C1	V1 (µL)	C2	V2 (µL)	Total Volume (µL) 96 samples (x110)	Total Volume (µL) 192 samples (x 220)	Total Volume (µL) 384 samples (x 440)
Cutsmart Buffer (or NEBBuffer 4)	10x	1.7	1x	2.0	187	374	748
Molecular Grade Water		0.18		2.0	19.8	39.6	79.2
SbfI-HF enzyme (add last & keep cold)	20,000 U/mL	0.12 (2.4 Units)		2.0	13.2	26.4	52.8
Total		***2.0 µL***			***220 µL***	***440 µL***	***880 µL***

*Note that here we use *SbfI* but other enzymes are possible, especially *PstI*, which gives you more loci but less coverage. But check long protocol for any changes required if you change the enzyme. You may need different BestRAD adapters if you change the enzyme.

2. Add 2 uL of RE digestion MM to each well. Seal well and quick spin plate.
3. Incubate plate at:
    a. 37°C for 60 minutes, then
    b. 80°C for 20 minutes to heat kill RE.
    c. Note: "*sbfI digest*" program on Sirius/Orion in vonHoldt lab.
    d. Note that original UIdaho short protocol killed at 65°C for 20 min.

Project:_____ RADseqReady Plate #_____ Library #_____
Name: _____ Date: _____

Section 3: BestRAD Adapter Ligation

Materials Required

- ☐ Annealed, indexed *Sbf*I bestRAD adapters at 75nM
- ☐ Molecular grade water
- ☐ NEBuffer 4 (NEB B7004S)
- ☐ rATP (100mM) (Fermentas R0441)
- ☐ T4 DNA Ligase (NEB M0202M)
- ☐ T4 DNA Ligase Buffer with 10mM rATP (comes with T4 DNA Ligase)
- ☐ 1.5 mL tubes
- ☐ 8-well PCR strips

1. Add 2 uL of **annealed** indexed *SbfI* BestRAD adapters at **75 nM** (Note that the original short protocol said 1 µM, which is what I assumed we used at UIdaho, but there is a note in the long version and in the published version saying to use 50 nM with *SbfI* because it cuts less frequently). There are 96 different adapters stored in plate format. Set aside while you make the adapter ligation mastermix (AL-MM). Refer to the document "20140917 adapter annealing.docx" on how to anneal adapters.
2. Prepare adapter ligation (AL) mastermix (MM) as follows:

New Protocol utilizing the T4 DNA Ligase Buffer with 10 mM rATP

Item	**C1**	**V1 (µL)**	**C2**	**V2 (µL)**	**Total Volume (µL) 96 samples (x110)**	**Total Volume (µL) 192 samples (x 220)**	**Total Volume (µL) 288 samples (x 330)**
Molecular Grade Water		1.28		2.0	140.8	281.6	422.4
10x T4 DNA Ligase Buffer*	10x	0.56	1x	2.0	61.6	123.2	184.8
T4 DNA Ligase	2,000,000 U/mL	0.16		2.0	17.6	35.2	52.8
Total		***2.0***			***220***	***440***	***660***

* with 10mM rATP

3. Keep adapter ligation (AL-MM) on ice. Divide total volume by 8 and aliquot into PCR strips to facilitate multi-channel addition to plate.
4. Add 2 uL of AL-MM to each well and pipette up and down 2x to mix.
5. Seal and quick spin.
6. Incubate **with heated lid off** at:
    a. 20°C for 60 minutes, then
    b. 65°C for 15 minutes to inactivate ligase
    c. Note: "*rad ligation*" program on Sirius/Orion in vonHoldt lab

Project:_____ RADseqReady Plate #_____ Library #_____
Name: _____ Date: _____

Section 4: Pool, Magnetic Bead Clean Up

Materials Required

- ☐ Freshly make 80% ethanol (from 96% Molecular Grade ethanol e.g. ThermoFisher Scientific BP82021)
- ☐ 8 well PCR strip tubes
- ☐ 1.5 mL microcentrifuge tubes
- ☐ Agencourt AMPure XP Beads (Beckman Coulter, A63881) mixed and at room temperature & Magnetic Rack
- ☐ Low TE ($TE_{0.1}$: 10mM Tris-HCl pH 7.5, 0.1 mM EDTA).

1. Remove AMPure beads from fridge, protect from light, place on mixer and mix at least until they are at room temperature.
2. **Prepare a small volume (5-10 mL) of 80% ethanol** (e.g. mix 4.167 mL of 96% ethanol with 0.833 mL molecular grade water to get 5mL 80% ethanol)
3. **Pool** 4 uL from each uniquely indexed sample (in our case half the plate – columns 1 – 6 and then columns 7 – 12 – because we don't want to have too much liquid in the tube for mag-bead clean up) into 8 well PCR strips. (Note that the amount depends on the desired ng per sample). This will give you 192 uL of sample in each 1.5 mL tube.
4. **Pool** all the samples from each 8 well PCR strip into a separate 1.5 mL tube. (Note that if you are doing 1 plate you will have 2 tubes, each filled with 48 uniquely barcoded samples).
5. **Clean the DNA with 1:1.5 X Agencourt AMPure XP beads** (Beckman Coulter, A63881). (288 uL of beads because 192 x 1.5 = 288). (Note that published protocol recommends 1X).
    a. Add 288 uL Agencourt AMPure Beads to each tube. Gently mix by pipetting and quick spin.
    b. Incubate the sample at room temperature for 10 minutes to bind DNA to the beads.
    c. Place the tube on a magnetic rack (**LIDS CLOSED**) and wait until the liquid is clear to capture the beads (10 minutes).
    d. With tube on rack, carefully open lids, remove and discard the supernatant (in hazardous waste because beads are stored in sodium azide).
    e. Keep the tube on the rack and add 400-500 uL (cover beads) freshly made 80% ethanol to wash the beads.
    f. Incubate at room temperature for 30 – 60 seconds.
    g. Leave tube on rack; carefully remove ethanol & discard.
    h. Repeat steps e – g again. Remove the residual ethanol with a small pipette tip. Do not disturb beads.
    i. Dry the beads at room temperature for about 5 minutes. Do not overdry the beads.
    j. Close lids and remove the tubes from the magnetic rack.
    k. Add 130 uL Low TE. Mix by pipetting and quick spin.
    l. Place on magnet for 10 minutes.
    m. Remove supernatant and place in 1.5 mL tube. **KEEP SUPERNATANT!**

Project:_____ RADseqReady Plate #_____ Library #_____
Name: _____ Date: _____

Section 5: Covaris Sonication to Selected Size Fragment

Materials Required

- ☐ Glass Covaris tubes (available by Covaris LE220 in Princeton Core for use with PN500282 rack)
  1. Sonicate samples for 400bp on Covaris LE 220 in Princeton Core Facility. **NOTE: Testing showed that the settings for 400 bp actually sheared for 600 bp and that settings for 300 bp sheared for 400 bp (see figure at right). So we want to target 400 bp but that required the Covaris LE220 settings to be at the 300 bp suggestion (See settings below)**
  2. Transfer 130uL back to 1.5 mL tube.

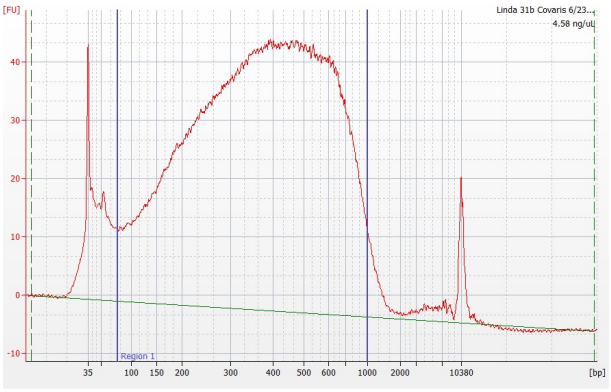


Figure 1. Bioanalyzer results from shearing of pooled DNA after settings changed to "300 bp" settings on LE220.

Note: Aug5, 2016: Covaris did testing and realized the published protocol below for this model was inaccurate. They recommend settings closer to 300bp except with shorter duration of 50s. I found that the 60s shearing was better than that so the setting should be:

PIP(W)	DF(%)	CPB	Time(s)
450	30	200	60

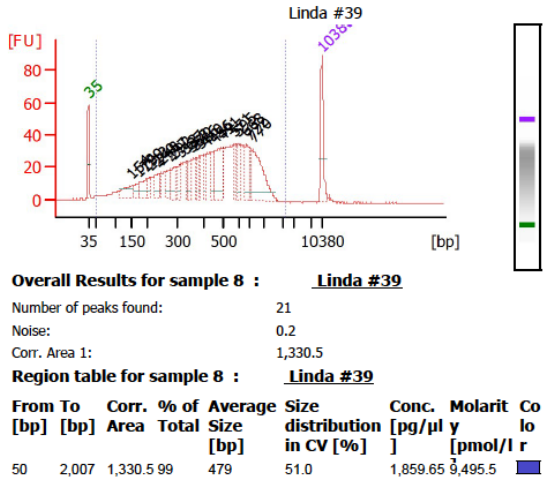


Figure 2. Bioanalyzer results from shearing of pooled DNA after settings changed to "300 bp" settings on LE220 with only 50s

Quick Guide:

DNA Shearing with LE220 Focused-ultrasonicator

This Quick Guide provides DNA Shearing protocols when using microTUBE, microTUBE-50, microTUBE-15 or miniTUBE and a Covaris LE220 Focused-ultrasonicator.

130 µl sample volume - from 150 to 1,500 bp
microTUBE AFA Fiber Crimp-Cap
8 microTUBE Strip
96 microTUBE Plate

Target BP (Peak)	150	200	300	400	500	900	1500
Duty Factor	30%	30%	30%	15%	15%	5%	5%
Peak Incident Power PIP (W)	450	450	450	450	450	450	450
Cycles per Burst	200	200	200	200	200	200	200
Treatment Time (s)							
Racks (PN 500282 and 500191)	420	175	60	63	46	77	17
96 microTUBE plate (PN 520078)	490	190	80	100	75	118	20
Temperature (°C)	7	7	7	7	7	7	7
Water Level	6	6	6	6	6	6	6
Sample volume (µl)	130	130	130	130	130	130	130

Additional Optional Step: You can check shearing efficiency on one of your samples at this point to ensure the correct sample size. Shearing at 400 bp is important for downstream workflow.

Project:_____ RADseqReady Plate #_____ Library #_____
Name: _____ Date: _____

Section 6: Streptavidin Bead Binding Assay
Note from Michael Miller: We resuspend the beads each wash by pipetting. The biotin-streptavidin interaction is the strongest known non-covalent interaction. You have to use hot phenol to dissociate it. There's no way resuspending the beads would cause the fragments to fall off.

Materials Required
- ☐ Dynabeads M-280 Streptavidin (Invitrogen 11205D); room temperature and resuspended by mixing well.
- ☐ 37°C water bath or incubator
- ☐ 56°C heat block/ incubator (to warm 1X BW buffer for second round of washes)
- ☐ 2X Binding and Wash Buffer (2X BW Buffer: 10 mM Tris-HCl (pH 7.5), 1 mM EDTA pH 8.0, 2M NaCl)
- ☐ 1X binding and wash buffer (1X BW Buffer: 5 mM Tris-HCl (pH 7.5), 0.5 mM EDTA pH 8.0, 1 M NaCl) – you can dilute 2x BW Buffer 1:1 with Molecular Grade Water
- ☐ 1X NEB Buffer 4 (It comes as 10X – dilute 1:9 with Molecular Grade Water)
- ☐ Agencourt AMPure XP Beads
- ☐ Low TE (10 mM tris-HCl pH 7.5, 0.1 mM EDTA)
- ☐ *SbfI*-HF restriction enzyme

1. Wash Dynabeads
    a. Transfer 30 uL Dynabeads to new 1.5 mL tube. Repeat for # of pools.
    b. Add 70 uL 2x BW buffer and vortex for 5 seconds.
    c. Quick spin and place on magnet and let beads aggregate to side (1 minute with lids closed).
    d. Remove supernatant and discard.
    e. Repeat wash once more with 100 uL 2X BW buffer.
2. Resuspend, Bind, & Wash Dynabeads (resuspend each time by pipetting)
    a. Remove tube from magnet and resuspend Dynabeads in 130 uL **2X BW buffer** (equivalent to volume of sheared DNA from Step 5). Mix well by pipetting.
    b. Add full volume of sheared DNA to beads. Pipette up and 5 - 10 times to mix.
    c. Incubate at room temperature for 20 minutes, with gentle vortexing for 3 seconds every 5 minutes.
    d. Quick spin, place tube on magnetic rack for 2 minutes, remove SN, resuspend beads in 150 uL **1X BW buffer**. Mix by gentle pipetting 10x.
    e. Quick spin and place on magnet and let beads aggregate to side (2 minutes)
    f. Remove SN and discard.
    g. Repeat wash with **1x BW buffer.**
    h. Repeat 2 additional washes (resuspending each time either by gentle pipetting or gentle vortex) with 150 uL of 56°C 1X BW buffer (heated in heat block ahead of time).

Project:_____ RADseqReady Plate #_____ Library #_____
Name: _____ Date: _____

3. Liberate DNA from Dynabeads
    a. Remove from rack and resuspend beads+DNA in 100 uL of **1X NEB4 Buffer**
    b. Place tube on magnetic rack and remove SN once beads have aggregated to side of the tube and liquid becomes clear.
    c. Repeat a-b once.
    d. Resuspend beads and bound DNA in 40 uL **1X NEBuffer 4**
    e. Add 2 uL of *Sbf* I enzyme to resuspended beads + DNA. Note that the bestRAD adapters are designed to have this cut site so it liberates DNA of interest from the beads during the next incubation step. Mix with gentle vortex.
    f. Incubate at 37°C for 60 minutes in **water bath** with gentle vortex after 30 minutes.
    g. Quick spin, place tube on magnetic rack 5 minutes.
    h. **KEEP supernatant** – put in new 1.5 mL tube.
    i. Do a 1:1.5X Ampure bead clean up;
        i. Add 60 uL AMPure beads (well mixed and at room temperature) to each sample
        ii. Gentle mix/spin and incubate at room temperature for 10 minutes.
        iii. Put on magnet for 5 minutes.
        iv. Remove and discard supernatant.
        v. Leave tube on magnet and add 200 uL freshly made 80% ethanol.
        vi. Incubate for 30 seconds.
        vii. Remove and discard supernatant.
        viii. Repeat steps v – vii.
        ix. Let air dry for 5 minutes.
        x. Remove tubes from rack and add 57 uL Low TE.
        xi. Mix/spin. Let sit for a couple minutes.
        xii. Put on rack for 5 minutes.
        xiii. **KEEP SUPERNATANT** and put in new 1.5 mL tubes.
    j. Quantify 2 uL of the elute with HS Qubit. Quantification is usually low (between 0.1 – 0.2 ng/uL).

Sample ID	Volume Quantified (uL)	Concentration (ng/uL)

Project:_____ RADseqReady Plate #_____ Library #_____
Name: _____ Date: _____

Section 7: NEBNext Ultra II Library Prep
Follow the instructions for NEBnext Ultra II DNA Library Kit for Illumina with the following notes & details

PDF found at: https://www.neb.com/~/media/Catalog/All-Products/9CBC60D62F5A4D3AB3970664E099CC73/Datacards%20or%20Manuals/manualE7645.pdf

Materials Required

- ☐ NEBNext Ultra II DNA Library Prep Kit (E7645S/L)
- ☐ PCR tubes
- ☐ AMPure XP beads at room temperature and well mixed
- ☐ Molecular Grade Water
- ☐ Freshly made 80% ethanol
- ☐ 1.5 mL tubes

Notes:

STEP 1: NEBNext End Prep

Step 1.1
1. We have 55 uL but use only 50 uL of this in the PCR.

Step 1.2
1. It is important to mix well according to directions and give a quick spin prior to PCR.

Step 1.3
1. PCR program: NEB_NEXT_END_PREP – be sure to check settings and the heated lid is set to 75^{o}C
2. Do not store overnight in freezer or fridge – proceed to ligation to avoid DNA loss.

STEP 2: Adapter Ligation
Step 2.1
1. Based on your last Qubit you should be able to estimate adapter concentration, however, sometimes the Qubit is not super accurate and so for these libraries a standard 1uM of adapter is good because we expect to typically have just under 5ng of input DNA. Adapter can be diluted in 10 mM Tris HCl by combining 10 uL of 1M TrisHCl (ph 7.5) with 990 uL molecular grade water. Adapter is at 15 uM concentration so you may need to dilute to 1 uM by mixing 2 uL of 15 uM adapter with 28 uL of 10mM TrisHCl.

Step 2.2
1. Make sure you mix well and quick spin as per instructions

Step 2.3
1. Make sure turn the heated lid off for ligation.

Project:_____ RADseqReady Plate #_____ Library #_____
Name: _____ Date: _____

STEP 3: Size Selection & Bead Cleanup
A general rule to follow is that after a bead clean up you can store in fridge overnight. But you should not store end prep and ligations in fridge overnight. If you have to store before a bead cleanup then store at -20^{o}C. However, if the bead cleaned product is going to be stored more than 12 hours then put it in the freezer.

Step 3.1

1. Even though size selection for low volumes of DNA are not recommended by NEB, we still do it. Select for 300 – 400 bp insert. Note that the Ultra II ligation Mastermix has more PEG in it so the bead ratios are different. AmpureXP beads can be used and they are the same as the SPRIselect beads, it is just that SPRIselect beads are more expensive because they are guanteed to be verified for size selection.
2. Note that when I did this I only had 85 uL of product so I added 11.5 uL of molecular grade water to get the 96.5 uL for size selection.
3. Use 25 uL for 1st bead selection and 10 uL for 2nd bead selection

Step 3.1

1. Ignore this section

STEP 4: Follow section 4.1 (NEBNext multiplex oligos for Illumina Set 1, E7335 OR NEBNext singleplex Oligos for Illumina (NEB 7350).
If you need to combin 2 indices be sure to check for compatibility – for example, use index 6 with index 12. Consult Illumina sequencing for combinatorial indexing strategy.

Step 4.1
Follow section 4.1 (NEBNext multiplex oligos for Illumina Set 1, E7335 OR NEBNext singleplex Oligos for Illumina (NEB 7350). I used index primer 7 and the universal primer for both 39 and 40 because there are 96 barcodes already attached. If you need to combine 2 indices be sure to check for compatibility – for example, use index 6 with index 12. Consult Illumina sequencing for combinatorial indexing strategy.

Step 4.1.2

1. Mix well.

Step 4.1.3

1. I used 12 cycles based on Table 4.2. Although this kit is supposed to be super efficient and require fewer cycles, I thought the input DNA was closer to 0.5 ng (based on qubit) and wanted to ensure enough product. 12 cycles seemed to work well.

Step 4.2

1. Ignore this section (except for Table 4.2 which may be useful to consult for estimating cycles).

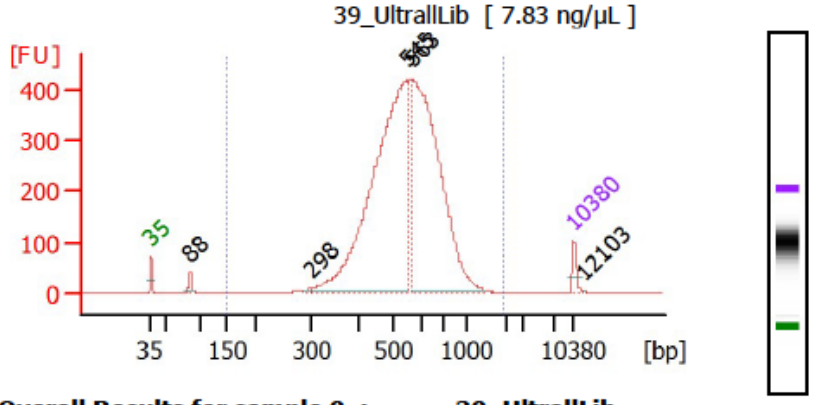
Project:_____ RADseqReady Plate #_____ Library #_____
Name: _____ Date: _____

STEP 5: Cleanup of PCR Amplification

Follow this section exactly, **except in section 5.8 elute in 35 uL 0.1x TE so you can Qubit 2 uL.**

1. Quantify 2 uL of product with Qubit HS Assay. Expect 5 – 10 ng/uL, but anything over 3 ng/uL is OK. Keep remaining for sequencing. This is your final, complete sequencing library to use for preparation of sample submission to sequencing facility.

Sample ID	Volume Quantified (uL)	Concentration (ng/uL)



Overall Results for sample 9 : 39_UltrallLib

Number of peaks found:	5
Noise:	0.2
Corr. Area 1:	6,470.7

Region table for sample 9 : 39_UltrallLib

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
149	1,983	6,470.7	99	559	24.8	7,751.42	22,513.5	

This shows a Bioanalyzer result prior to standardizing and combining with LIB40.

Project:_____ RADseqReady Plate #_____ Library #_____
Name: _____ Date: _____

Section 8: Standardizing Libraries and Sending for Sequencing at Princeton
See https://htseq.princeton.edu for details about sequencing.
Materials Required
☐ Libraries from Section 7
☐ 0.1% Tween EB buffer (EB Buffer is Qiagen EB Buffer)
☐ Screw cap low volume tubes.

1. We send our RADseq libraries to Lewis-Sigler Institute (LSI) at Princeton University. Request 5% PhiX spike in the RAD libraries to ensure maximal reads. On the Illumina Hi-Seq, request 2 lanes of sequencing for each library to get the expected 200 Million reads. As of April 12, 2016 the cost is $2690.
2. We typically submitted 10nM in 20 uL of 0.1% Tween EB (Use Qiagen EB buffer) but it now state in iLab that they want 5ng/uL in 20 uL. For the Canis project we are continuing with the standard 2.6 ng/ul but for new projects people should consider revamping to 5 ng/uL and also doing the nM conversion based on a bioanalyzer output of mean fragment size.
3. Convert PCR product concentrations to nM (see nM Conversion Calculator.xlsx).

Sample ID	(C1) (ng/uL)	V1 (uL)	C2 (ng/uL)	V2 (uL)	Fragment Size (bp)	DNA (nM)	Volume of Standardized Sample (uL)	Volume of 0.1% Tween EB (uL)
39	7.83	3.32	2.6	10	400			6.68
40	8.31	3.13	2.6	10	400			6.87
								Total 13.55 uL

4. For 400 bp we will prepare samples at 10 nM total in 20 uL 0.1% Tween EB.
    a. There are several ways to accomplish this, but here is one way
    b. To a new sequencing submission screw cap tube, add enough volume of each sample to make 2.6 ng/uL in 10 uL. Then add volume of 0.1% Tween EB to top up to 20 uL (an example is shown in the above table).
5. Label tube appropriately, and follow sample submission guidelines at on iLab Sequencing Core Service Request. Be sure to charge the correct chartstring.
6. Print the sample submission form and place in Ziploc bag with library and deliver to the sequencing core freezer in the "Libraries for Sequencing" box in the mini freezer.
7. Data can be downloaded by logging in and following instructions at: https://htseq.princeton.edu.

Project:_____ RADseqReady Plate #_____ Library #_____
Name: _____ Date: _____

Reagents List

Low TE ($TE_{0.1}$: 10mM Tris-HCl pH 7.5, 0.1 mM EDTA)
Materials:

- Molecular Grade Water
- 1M Tris-HCl (pH 7.5)
- 0.5M EDTA (pH 8.0)

To make 100 mL of Low TE, remove 1.02 mL (1 mL plus 20 uL) from a 100 mL bottle of Molecular Grade Water. Then add 1 mL of 1M Tris-HCl (pH 7.5) and 20 uL 0.5M EDTA. Mix by inversion. It is also *probably* fine to just dilute regular 1X TE in a 1:9 ratio with molecular grade water. But then you end up with 1mM Tris-HCl and 0.1 mM EDTA. It is the EDTA concentration that is the most important here.

80% Ethanol
Materials:

- 96% Molecular Grade Ethanol (Fisher BP82021)
- Molecular Grade Water (Fisher BP2484100)
- 5mL tubes or 15 mL conical tubes

Make this fresh each day you are doing a bead clean up or size selection.
Mix 4.167 mL of 96% ethanol with 0.833 mL of molecular grade water.

2X Binding and Wash Buffer

Reagent	C1	V1 (uL)	C2 (mM)	V2 (uL
Tris HCl pH 7.5	1000	1000	10	100000
EDTA pH 7.5	500	200	1	100000
NaCl	5000	40	2	100000
Molecular Grad Water		98760		

Instructions: Remove 1240 uL of water from a 100 mL bottle of molecular grade water, then add the other reagents to that bottle. Label and aliquot appropriately.

0.1% Tween EB
Materials:

- Tween 20 (Fisher BP337-100)
- EB Buffer (Qiagen 19086)

Tween is really viscous so it is difficult to pipette 1 uL accurately. So, first make a 10% solution with the 100-1000 uL pipette: Eg. mix 1 mL of Tween 20 with 9 mL Buffer EB. Then make a 0.1% Tween EB solution by combining 100 uL of 10% Tween EB with 9900 uL of EB Buffer. This gives you 0.1% Tween EB.