Fecal Enrichment Protocol (Library prep and hybridization)

General Notes:

- Do not vortex stock solutions of fDNA (fecal DNA), tap them to mix
- Thaw fDNA aliquots as quickly as possible without going over RT (room temperature)
- Spin fDNA samples down before use and keep on ice
- Places where you can stop overnight will be indicated throughout this protocol
- Transfer to and use low-bind tubes during elution after hybridization

A) Extract fDNA

- Use your preferred fDNA extraction protocol to obtain at least 1µg of fDNA from each sample.

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B) fDNA: Shear to Correct Fragment Size

a) Quantify fDNA using a Nanodrop and divide into 100 µL aliquots for shearing. Split up DNA sample into as many aliquots as needed for a total of 1µg of fDNA. If your sample is at a concentration greater than 10ng/ul, then you will need to dilute it to 100ul with DNase/RNase free H_2O prior to shearing.

b) Turn on the bioruptor and allow it to cool to 4C prior to shearing. If you don't allow it to cool down it can damage the machine.

c) Sonicate fecal DNA to 400 bp using the Bioruptor (30 seconds on / 90 seconds off – H (High) – 20 minutes)

* The bioruptor only runs in 15 minute increments so you will have to do one 15 minute cycle, wait 10 – 15 minutes for the machine to cool down, do one 5 minute cycle, for a total of 20 minutes of shearing

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If your sample is in one tube (i.e., ≤100µL), then skip to section D

i. Vacufuge Sheared fDNA down to 100 µL

- This step depends on how much fDNA (in volume) you have. If you have 100 µL or less then you can skip this step (C) and move on to step (D).

a) Combine all of your 100µl fDNA aliquots from the same sample into one 1.7 mL microcentrifuge tube.

b) Open the lid of the microcentrifuge tube and place a piece of parafilm over the opening. Poke a hole through the parafilm with a 10µL pipette tip.

c) Place sample in vacufuge and vacufuge sample down until you have 100µL. It is OKAY to go below the 100 µL volume just make sure you don't vacufuge your sample dry. You can always add more H_20 to your sample to get it back up to 100µL.

Vacufuge settings:
- Time: going to depend on your sample, if you have a lot of volume it will take an hour or two or maybe more. Check the sample often to ensure the sample does not evaporate completely
- Heat: DO NOT add any heat to the sample
- Setting: V-AQ

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D) Size Selection of Sheared DNA

* Note: For now we are doing an Ampure Bead size selection here, while you lose less DNA with an Ampure size selection than in a gel extraction, the size selection does not result in as tight of a distribution around the desired size.

a) Ampure beads are stored in the 4°C. Take beads out, vortex well, and then place on bench to thaw at room temperature for 30 minutes.

b) Vortex beads well before each use.

c) Add 65 µL of AMPure XP beads to the 100µL sheared fDNA sample and mix well by pipetting up and down at least 10x.

d) Incubate for 5 minutes at room temperature on bench.

e) Quickly spin the tube to collect any remaining beads and sample from sides of tube and place sample on the magnetic stand and sit for 5 minutes OR until supernatant is clear and carefully. TRANSFER the supernatant to a new microcentrifuge tube. Be careful to not disturb the beads that contain the unwanted fragments of fDNA. Discard the beads.

f) Vortex stock of Ampure beads well. Add 25 µL of AMPure beads to the supernatant and mix well and incubate for 5 minutes at room temperature on bench.

g) Quickly spin the tube to collect any remaining beads and sample from sides of tube and place on magnetic stand. Separate beads from supernatant until supernatant is clear (~ 5 minutes). Discard the supernatant. Do not discard any of the beads.

h) Add 200 µL of 80% ethanol (freshly prepared) to the tube while on the magnetic stand and incubate at room temperature for at least 30 seconds and then carefully remove and discard the supernatant.

i) Repeat step h) twice for at total of 3 washes.

j) Air dry the beads for 10 minutes while the tube is on the magnetic stand with the lid open. It may take longer than 10 minutes for the sample to dry. Ensure that the beads do not look wet before eluting fDNA off beads as residual ethanol can inhibit downstream steps.

k) Elute the fDNA target from the beads by adding 57 µL of nuclease free water.

l) Mix well by pipetting or vortexing.

m) Quickly spin tube and place on magnetic stand.

n) After the solution is clear (~ 5 minutes) transfer 56.5 µL of supernatant to a new tube for library prep (be sure to not carry over beads to next steps).

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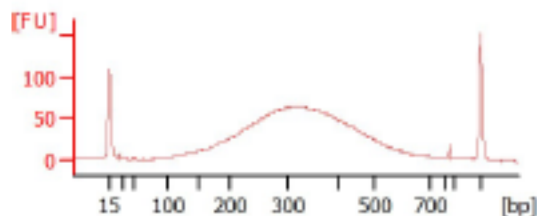
THIS IS AN OPTIONAL STEP

E) Bioanalyze (DNA 1000) the purified fragments to assess quality/concentration

- Here you are looking to ensure that (i) you still have your fDNA, (ii) that you completed a successful size selection, and (iii) that you still have enough fDNA to continue through the library prep process.
- Alternatively, you can quantify 1µL of your size-selected sample using a Qubit fluorometer or nanodrop.

Sample Bioanalyzer image:



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F) NEBNext Ultra DNA Library Prep Kit for Illumina (#E7370 and #E7335)

NEBNext End Prep

a) Mix together the following in a PCR tube:

End Prep Enzyme Mix (green) – 3 µL
End Repair Reaction Buffer (10X) (green) – 6.5 µL
Fragmented DNA – 55.5 µL

Total Volume = 65 µL

b) Mix by pipetting and spin down to collect liquid from sides of tube.

c) Place in thermocycler with a heated lid on and run the following program:
30 minutes @ 20C
30 minutes @ 65C
Hold at 4C

Adaptor ligation

a) Add the following components directly to the End Prep reaction mixture and mix well:

Blunt / TA Ligase Master Mix (red cap) – 15 µL
NEBNext Adaptor for Illumina (red cap) – 2.5 µL
Product #: (singleplex NEB #E7350, Multiplex #E7335, #E7500)
Ligation Enhancer (red cap) – 1 µL
Total Volume = 83.5 µL

b) Mix by pipetting and spin down to collect liquid from sides of tube.

c) Incubate at 20C for 15 minutes in thermocycler.

d) Add 3 µL of USER enzyme to the ligation mixture from step c) while the sample remains in the thermocycler.

e) Mix well by pipetting and incubate for 37C for 15 minutes.

Ampure bead cleanup

a) Ampure beads are stored in the 4°C. Take beads out, vortex well, and then place on bench to thaw at room temperature for 30 minutes.

b) Vortex beads before each use.

c) Add **86.5 µL** of AMPure XP beads to the ligation reaction and mix well by pipetting up and down at least 10x.

d) Incubate for 5 minutes at room temperature.

e) Quickly spin the tube and place on the magnetic stand and sit for 5 minutes OR until supernatant is clear and carefully remove and discard the supernatant. Be careful to not disturb the beads that contain DNA.

f) Add 200 µL of 80% ethanol (freshly prepared) to the tube while on the magnetic stand and incubate at room temperature for at least 30 seconds and then carefully remove and discard the supernatant.

g) Repeat step f) twice for at total of 3 washes.

h) Air dry the beads for 10 minutes while the tube is on the magnetic stand with the lid open.

i) Elute the DNA target from the beads by adding 24µL of nuclease free water

j) Mix well by pipetting or vortexing.

k) Quickly spin tube and place on magnetic stand.

l) After the solution is clear (~ 5 minutes) transfer 23 μL to a new PCR tube for amplification (be sure to not carry over any beads to next steps).

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PCR Amplificaiton

a) Mix the following components in sterile strip tubes (PCR tubes):

Adaptor ligated DNA fragments (from previous steps) – 23 μL
NEBNext High Fidelity 2X PCR Master Mix (blue) – 25 μL
Index Primer (blue) – 1 μL
Universal PCR Primer (blue) – 1 μL

Total Volume = 50 μL

b) Spin down briefly before placing in thermocycler.

c) PCR conditions:

1) 98°C – 30 seconds
2) 98°C – 10 seconds
3) 65°C – 30 seconds
4) 72°C – 30 seconds
5) repeat steps 2-4 for a total of 6 cycles (NOTE: if you can get >100ng using fewer cycles, then we advise doing fewer cycles and multiplexing your samples)
6) 72°C – 5 minutes
7) 10°C – ∞

Ampure Bead Clean Up

a) Ampure beads are stored in the 4°C. Take beads out, vortex well, and then place on bench to thaw at room temperature for 30 minutes.

b) Vortex beads well before each use.

c) Add 50 μL of AMPure XP beads to the ligation reaction and mix well by pipetting up and down at least 10X.

d) Incubate for 5 minutes at room temperature.

e) Quickly spin the tube and place on the magnetic stand and sit for 5 minutes OR until supernatant is clear and carefully remove and discard the supernatant. Be careful to not disturb the beads that contain DNA.

f) Add 200 μL of 80% ethanol (freshly prepared) to the tube while on the magnetic stand and incubate at room temperature for at least 30 seconds and then carefully remove and discard the supernatant.

g) Repeat step f) once for a total of 2 washes.

h) Air dry the beads for 10 minutes while the tube is on the magnetic stand with the lid open.

i) Elute the DNA target from the beads by adding 25 μL of nuclease free water

j) Mix well by pipetting or vortexing.

k) Quickly spin tube and place on magnetic stand.

l) After the solution is clear (~ 5 minutes) transfer 24 μL of the supernatant to a new tube (do not transfer any beads to the next steps).

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G) Bioanalyze (High Sensitivity DNA chip) to verify correct size and to measure concentration of each library

*Note 1: Alternatively, if you know the average fragment size (which should be ~300-400bp), then you can use a Qubit dsDNA HS Assay kit to calculate the concentration and molarity of each sample.

**Note 2: You need to have at least 100ng of fDNA at this step. We highly advise that you have 500ng total, but we have done the hybridization with as low as 100ng. These low-input hybridizations are not as effective, which is why we advise that you use 500ng.

**Note 3: If you are multiplexing multiple samples together, then you should have a total of 500ng across all of your samples. For example, if you are multiplexing 5 samples together, then you want to ensure that you have at least 100ng of each sample, so that they can be pooled in equimolar amounts, with the least concentrated sample being your limiting sample. It is possible to multiplex with <500ng in the pool of samples, but we do not advise it.

If multiplexing samples, then you must do the following before proceeding to step A:

- Pool your samples in equimolar amounts using the molarity concentrations calculated at the beginning of step G. Do not pool more than 500ng total DNA. For example, if you are pooling 5 samples, do not add more than 100ng of each sample to the pool. This pool is now your "sample" in the following steps.

a) Vacufuge the sample to adjust volume to ~3.5μl. The concentration of the sample should be no more than 150 ng/μl.

Before placing each tube in vacufuge (V-AQ), open the tube and cover it tightly with parafilm, then punch a small hole in the film with a 10-μL pipet tip).

It's okay to go a little below your target volume and add more water to adjust, but try to avoid allowing the library to completely evaporate. If your sample goes completely dry it may not work.

H) Hybridization/"Pull-Down" (adopted from SureSelect protocol and Gnirke et al 2009 Nature Biotech, doi: 10.1038/nbt.1523)

For clarity in the protocol below, the 50 μM "Index Block Reagent" (IBR) mentioned below is explained here.
1. The IBR is an equimolar mixture of 6 oligonucleotides:
   1. AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
   2. AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT
   3. CAAGCAGAAGACGGCATACGAG
   4. CTCGTATGCCGTCTTCTGCTTG
   5. GTGACTGGAGTTCAGACGTGTGCTCTTCCGAT/3ddC/
   6. AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC
2. Resuspend each oligo in water to 300 μM, and mix equal volumes of each together to yield a 50μM solution

3. When ordering more, the oligos need to be HPLC-purified, and the #5 needs to have the indicated 3'-dideoxy-cytosine.

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a) Prepare three mixes, in this order: 1) hybridization ("**A**"), 2) capture ("**C**"), and 3) block ("**B**")
NOTE: this needs to be made fresh each time.

"A" - Hybridization Buffer (2X) (use 20μL/library prep)
Mix at room temperature (per DNA library):
10X SSPE – add 500 μL of 20X SSPE
10X Denhardt's Solution – add 200 μL of 50X Denhardt's Solution
10 mM EDTA – add 100 μL 10 mM EDTA
0.2% SDS – add 200 μL of 1 % SDS

Will make 1000 µL total of Hybridization Buffer

(* The concentration listed first is the concentration of reagent when in working solution. Be sure to add the amount of each reagent that says add ‘x µL’ amount. For example: You will add 500 µL of 20X SSPE concentration to make your working solution A. Once you add all reagents together SSPE will be at a 10X SPPE concentration.)

b) Incubate **“A”** at room temperature while preparing other buffers. If a precipitate forms, incubate at 65°C for ~5 minutes on thermomixer.

“C” - Capture Mix
Mix on ice (per DNA library):

SUPERase-In	1 µL (20 U)
RNA Baits (750 ng)	≤ 6 µL
Water	up to 7 µL

c) Incubate **“C”** on ice while preparing last buffer

“B” - Block Mix
Mix at room temperature (per DNA library):

Human C_0t-1 DNA (1 mg/mL)	2.5 µL
Salmon Sperm DNA (1 mg/mL)	2.5 µL
Index Block Reagent (50 µM)	0.6 µL

For brevity, the three mixes above will be referred to with single-letter abbreviations:
Hybridization Buffer: **"A"**
Capture Mix: **"C"**
Block Mix: **"B"**

d) In PCR tubes add 5.6 µL of **“B”** to one tube for each library being pulled-down.

e) To the same wells that **“B”** was added to, add 3.4 µL of fDNA library.

f) Incubate in **“B” + Sample** in thermocycler:
95°C for 5 minutes
hold at 65°C

g) After 5 minutes at 65°C, add 20 µL **"A"** to separate PCR tubes and place in thermocycler.

h) After 5 minutes at 65°C, add 7 µL **"C"** to separate PCR tubes and place in thermocycler.

i) After 2 more minutes at 65°C, move 13 µL of the preheated **"A"** (toss out to the remaining 7 µL of “A”), **"C"** wells, then all of the **"B"** to the **"C"** wells. Make sure to mix well by pipetting. **Do not take out of thermocycler to spin down or pipette. Start the incubation time here.**

j) Incubate for 48 hours at 65C.

k) Near end of incubation (~ 15 minutes before incubation is to end), wash Dynal MyOne Streptavidin T1 beads (Invitrogen), 50 µL per library:

You will be washing the Dynal MyOne Streptavidin T1 beads with binding buffer. The recipe for Binding Buffer is listed below.

Binding buffer:

1M NaCl	(MW – 58.44g)
10 mM Tris-HCl, pH 7.5	(MW - 157.60 g/mol)
1 mM EDTA (10mM EDTA – 2.9224 g EDTA > 1 L ddH2O)	(MW- 292.25g)

To make 1L:

58.44 g NaCl
1.576 g Trizma HCl
0.29225 g EDTA
Fill to 1 L of water

l) Make sure Dynal MyOne Streptavidin T1 beads beads are resuspended well before use, vortex well (these beads are different than the Ampure beads. They DO NOT need to be at room temperature 30 minutes prior to use, you can start your protocol steps immediately).

m) Wash beads by adding 200 µL of binding buffer, mix by pipetting, place on magnetic rack for 1 minute or until clear , discard supernatant, remove from magnetic stand and resuspend in 200 µL binding buffer

n) Repeat step (m) 2x times for a total of 3 washes. And then resuspend in 200 µL of binding buffer.

o) At end of the 48hr incubation, take each library out and quickly centrifuge to collect all liquid from the side and top of the tube. Then add each library to an aliquot of beads resuspended in 200 µL binding buffer (step n), keeping the library at 65°C until added to beads. (i.e. if you started a 48 hr incubation at 11AM on Monday then you get the beads out and wash them around 11AM on Wednesday).

- Bring your tube of beads in binding buffer over to the thermocycler and pipette hybridized libraries right from the thermocycler to tube of beads in binding buffer and pipette up and down several times.

*AGAIN: Pay attention to sample volume at this point, DO NOT let sample drop below 20 µL from incubation. If sample has dropped too much below 20 µL it is unlikely that the hybridization will work.

p) Incubate tubes on thermomixer at room temperature for 30 minutes (with mixer set to 700 RPM).

q) Place tubes in magnet rack and remove supernatant. Be careful not to disturb or remove the beads.

r) Add 500 µL low stringency wash buffer #1 (1X SSC/0.1% SDS) and resuspend with a vortex. Spin down briefly to get any liquid off the sides of the tube.

Wash buffer #1: 0.1g SDS + 5 mL 20X SSC + 95 mL ddH20 (total 100 mL)
OR (0.5 mL 10% SDS + 2.5 mL 20X SSC + 47 mL ddH20)

s) Incubate 15 minutes at room temp with constant shaking on the thermomixer (700 rpm)

t) Place tubes in Dynal magnet rack until cleared and remove supernatant.

u) Either here or earlier on you should take out a 1.7 mL centrifuge tube and fill it with 1500 µL of wash buffer #2 (per sample) and place on thermomixer. Turn thermomixer up to 65C. You need to have wash buffer #2 at 65C to be able to use in step u) so do this as soon as possible.

v) Wash 3X with 500 µL pre-warmed (65.C) high stringency wash buffer #2 (0.1X SSC/0.1% SDS), including 10 min's incubation per wash at 65°C with constant shaking (use thermomixer 700 rpm)
(i.e. Add buffer > incubate > stand> take supernatant > repeat 2x)

Wash buffer #2: 0.1g SDS + 0.5 mL 20X SSC + 99.5 mL ddH20 (total 100 mL)
OR (0.5 mL 10% SDS + 0.25 mL 20X SSC + 49.25 mL ddH20)

w) Before the end of the third wash, transfer the resuspended beads to a new, low-bind tube. Then place it on the magnet. After third wash, take care to remove all wash buffer in the tube (this includes all suds etc.)

x) Resuspend beads with 50 µL 0.1 M NaOH

Elution buffer: 4g NaOH + 1 L of ddH20

y) Incubate for 10 minutes at room temperature on lab bench, then separate beads/supernatant with magnet

z) Transfer supernatant to new tube, and add (or include in new tubes before transferring supernatant) 70 µL 1 M Tris-HCl, pH 7.5. " Neutralization Buffer"

1 M Tris-HCl – 39.997 g Trizma HCL > 1 L ddH20

Purify with AMPure Beads: 216 µL beads, elute into 24 µL H_2O

a) Ampure beads are stored in the 4°C. Take beads out, vortex well, and then place on bench to thaw at room temperature for 30 minutes.

b) Vortex beads well before each use.

c) Add **216 µL** of AMPure XP beads to the ligation reaction and mix well by pipetting up and down at least 10 x. Vortex AMPure breads to resuspend.

d) Incubate for 5 minutes at room temperature.

e) Quickly spin the tube and place on the magnetic stand and sit for 5 minutes OR until supernatant is clear and carefully remove and discard the supernatant. Be careful to not disturb the beads that contain DNA.

f) Add 200 µL of 80% ethanol (freshly prepared) to the tube while on the magnetic stand and incubate at room temperature for at least 30 seconds and then carefully remove and discard the supernatant.

g) Repeat step f) once for a total of 2 washes.

h) Air dry the beads for 10 minutes while the tube is on the magnetic stand with the lid open.

i) Elute the DNA target from the beads by adding 24 µL of nuclease free water

j) Mix well by pipetting or vortexing.

k) Quickly spin tube and place on magnetic stand.

l) After the solution is clear (~ 5 minutes) transfer 23 µL to a new tube and store in -20 C until used (be sure to not carry over any beads to next steps).

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I) PCR Amplification

a)
IF **YOU ARE *NOT* MULTIPLEXING** YOUR SAMPLES:
Mix the following components in sterile strip tubes (PCR tubes):

Hybridized Library fragments (from previous steps) – 23 µL
NEBNext High Fidelity 2X PCR Master Mix (blue) – 25 µL
Universal PCR Primer (blue) – 1 µL
Index Primer (blue) – 1µL

Total Volume = 49 µL

IF **YOU ARE MULTIPLEXING** YOUR SAMPLES:
Mix the following components in sterile strip tubes (PCR tubes):

Hybridized Library fragments (from previous steps) – 23 µL
NEBNext High Fidelity 2X PCR Master Mix (blue) – 25 µL
Illumina TruSeq PCR Primer Cocktail – 2 µL

Total Volume = 49 µL

*NOTE: You must use a complete primer cocktail with index-free P5 and P7 primers (e.g. Illumina TruSeq PCR primer cocktail). The design of the NEB index primers is such that the P7 primer is attached to a specific index and thus cannot be used to amplify multiplexed libraries. You may use any PCR master mix appropriate for amplifying adaptor-ligated libraries.

b) Spin down briefly before placing in thermocycler.

c) PCR conditions:

1) 98°C – 30 seconds
2) 98°C – 10 seconds
3) 65°C – 30 seconds
4) 72°C – 30 seconds
5) Repeat steps 2-4 for a total of 12 cycles
6) 72°C – 5 minutes
7) 10°C – ∞

J) Ampure Cleanup

a) Ampure beads are stored in the 4C. Take beads out, vortex well, and then place on bench to thaw at room temperature for 30 minutes.

b) Vortex beads well before each use.

c) Add **<u>50 µL</u>** of AMPure XP beads to the ligation reaction and mix well by pipetting up and down at least 10 x. Vortex AMPure breads to resuspend.

d) Incubate for 5 minutes at room temperature.

e) Quickly spin the tube and place on the magnetic stand and sit for 5 minutes OR until supernatant is clear and carefully remove and discard the supernatant. Be careful to not disturb the beads that contain DNA.

f) Add 200 µL of 80% ethanol (freshly prepared) to the tube while on the magnetic stand and incubate at room temperature for at least 30 seconds and then carefully remove and discard the supernatant.

g) Repeat step f) once for a total of 2 washes.

h) Air dry the beads for 10 minutes while the tube is on the magnetic stand with the lid open.

i) Elute the DNA target from the beads by adding 20 µL (can do 10-15µl if you want a higher concentration) of nuclease free water (10mM Tris-HCl, pH 8.0 or 0.1X TE)

j) Mix well by pipetting or vortexing.

k) Quickly spin tube and place on magnetic stand.

l) After the solution is clear (~ 5 minutes) transfer 20 µL to a new tube and store in -20 C until used (be sure to not carry over any beads to next steps).

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K) Bioanalyze (High Sensitivity chip) to verify correct size and to measure concentration for sequencing.