Restriction Digest

1. **Place 6 μL of sample DNA in each well of a plate** (DNA should ideally be at a minimum concentration of 20 ng/μL and a maximum concentration of 150 ng/μL (note: this is for small genomes, for large genomes add higher concentrations of DNA). **Keep on ice.**

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

2. **For each sample prepare Master Mix I. Mix by vortexing, and centrifuge.** For these and all other reactions make sure to prepare an excess of mix to accommodate multiple rounds of pipetting, particularly if you are working with whole plates. Because the enzymes are stored in glycerol and other viscous solutions, a substantial volume is lost

Master Mix I (Restriction Digest)

REAGENT	x1	x (150%)
10x CutSmart Buffer	1.00	
1M NaCl	0.52	
Water	0.73	
MseI	0.10	
EcoRI	0.25	

/8 =

through adhesion to the outside of pipette tips. **We suggest making 150% of what you think you will need.**

3. **Add 2.6 *µ*L of the combined Master Mix I to each sample. Always keep cold once the enzymes have been added.** Easiest to divide Master Mix I into **8-strip tubes** and use multichannel pipet.

4. The total reaction volume should be 8.6 *µ*L. **Cover and seal the plate, vortex, centrifuge and incubate at 37 ◦C for 2 hours, followed by 65◦C for 20 minutes** (to inactivate the enzyme) on a thermalcycler with a heated lid. (GBSCUT)

Adaptor Ligation

1. **Thaw MseI and EcoRI adaptors.** Have these adaptors annealed and easily accessible

Master Mix II (Adaptor Ligation)

	x1	x (125%)
MseI adaptor	1.000	
10x T4 buffer	0.322	
1M NaCl	0.050	
T4 DNA ligase	0.1675	
		/8 =

in plate format (for the EcoRI adaptors) (see Sections 2.1.1 and 2.1.2). **We suggest making 125% of what you think you will need.**

2. **Add 1.54 µL of Master Mix II to each restriction-digested reaction.**

3. **Add 1 µL of EcoRI adaptors (with barcodes) to each well on plate.** Note that the MseI adaptor is in master mix II. **Adaptors used =**

4. The total reaction volume should now be 11.14 µL. **Cover and seal the plate, vortex, centrifuge and incubate at 16 °C for 2 hours followed by 65 °C for 10 minutes on a thermalcycler.** (GBSLIG)

5. **Dilute the Restriction-Ligation reaction with 189 µL of water.** Store at 4 °C for a month, or -20 °C for longer.

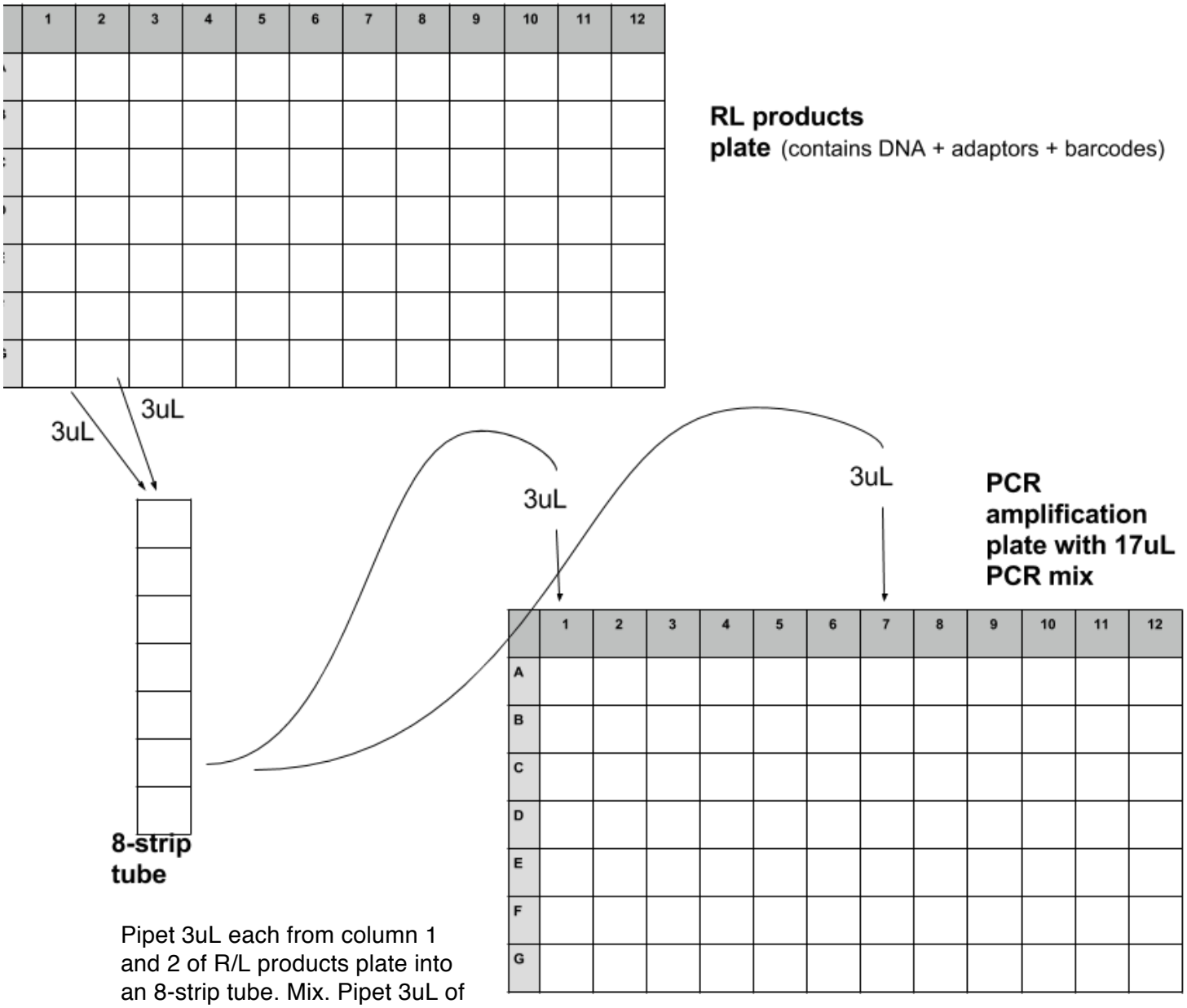
PCR amplification

PCR Mix (17uL mix per rxn, then will add 3uL R/L product to each)

	x1	x (110%)
Water	10.4	
5x Iproof buffer (HF)	4.0	
dNTP (10mM)	0.4	
MgCl2 (50mM)	0.4	
Pre-mixed PCR Primers (5uM each)	1.3	
Iproof taq	0.2	
DMSO	0.3	
		/8 =

1. This PCR step uses the Illumina PCR primers to amplify fragments that have our adaptors+barcodes ligated onto the ends. To ameliorate stochastic differences in PCR production of fragments in reactions, we run two separate 20 µL reactions per restriction-ligation product, and later combine them. Pairs of individuals can be pooled prior to PCR to reduce time and costs. (More likely to get stochastic variation from 1 PCR rxn with 1 DNA template vs. a combined 2 PCR rxns with the 2 DNA templates.) See chart on following page. (**Make ~110% needed.**) **See pipetting diagram on following page!**

2. **Thermalcycler profile for this PCR: 98◦C for 30s; 30 cycles of: 98◦C for 20s, 60◦C for 30s, 72◦C for 30s; final extension at 72◦C for 10 min.; 4◦C infinity.** **(GBSPCR1) (~ 1hr. 20mins.)**



Pipet 3uL each from column 1 and 2 of R/L products plate into an 8-strip tube. Mix. Pipet 3uL of this mix into column 1 and 3uL into column 7 of PCR plate. Using same 8-strip tube (all samples are barcoded already), continue with cols. 3 & 4 of R/L plate into strip tube and mix and then place into cols. 2 and 8 of PCR plate, etc.

Extra PCR step

1. This PCR step adds Illumina PCR primers, dNTPs, and iproof buffer and executes a final ong PCR cycle. The purpose of this step is to attempt to convert single stranded template remaining from the first PCR to double stranded. This typically results in libraries that have a better distribution of fragments in the correct size range, presumably

because it removes single stranded molecules that interfere with the precision of the electrophoresis step below.

2. **Add 2.125 *µ*L of the mix directly to each reaction immediately after the PCR step above is completed.** (All are barcoded, so you can use a single tip.) (Make 130%)

3. Thermalcycler profile for this PCR cycle: 98◦C for 3 min; 60◦C for 2 mins, and 72◦C for 10 min.

Extra PCR Step

	x1	x (130%)
5x Iproof buffer	0.425	
dNTP (10mM)	0.40	
Pre-mixed PCR Primers, 5uM each	1.30	
		/8 =

Sample Pooling and Visualization

1. Use multichannel pipet to transfer 15uL from each sample into an 8-strip tube. Then transfer the 8 wells of the strip tube into a single 2mL tube.

2. Run 15uL of the pooled samples on an ~1% agarose gel for visualization. Should have a nice “smear” around the 200-1,000bp range. If gel looks good, then proceed to BluePipin.

3. Run on BluePipin: contact Aaron Thomas: aaron.thomas@usu.edu. You will need to send about 65uL total. (30uL per lane gets ran). **Size range = 300-400bp**. Provide an empty, labeled tube for the product. He will also run a few uL of the final product on the Bioanalyzer. Arrange for sample drop-off, payment, etc.