ddRAD protocol

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08/06/2021

Annealing of adapters

Adapter P1 will bind *HindIII* ends, and adapter P2 will bind *SphI* ends. Both adapters need to be annealed separately. Oligos are asumed to be suspended to 100 μM concentrations. The annealing buffer stock at 10× concentration should be 100 mM Tris HCl (pH 8), 500 mM NaCl, 10 mM EDTA (Peterson 2012).

Table 1: 10× Annealing Buffer Stock, 200 μl

Reagent	Amount to add (μl)	Final concentration (mM)
Tris-HCl 1M pH 8.0	20	100
EDTA 0.5M	4	10
NaCl 1M	100	500
Water	76	NA

Both adapters can be at a final concentration of 2.5 μM. We will use 1 μl per sample. We can prepare 500 μl of annealed stock of each adapter, to have them in a comfortable excess.

Table 2: **Annealing reaction**. 500 μl of 2.5 μM annealed adapter. Two separate reactions must be prepared, for *HindIII* P1 and for *SphI* P2 adapters.

Reagent	Amount to add (μl)
Top oligo 100 μM	12.5
Bottom oligo 100 μM	12.5
10× Annealing buffer stock	50.0
Water	425.0
Total	500.0

- **Adapter P1**: Mix 12.5 μl *HindIII*-P1.1 100 μM and 12.5 μl of *HindIII*-P1.2 100 μM oligos, 50 μl of 10× annealing buffer stock (see above), and 425 μl of water.
- **Adapter P2**: Mix 12.5 μl *SphI*-P2.1 100 μM and 12.5 μl of *SphI*-P2.2 100 μM oligos, 50 μl of 10× annealing buffer stock (see above), and 425 μl of water.

Anneal oligos in a thermal cycler; 95°C 5 min, followed by a 0.1°C/s ramp down to 20°C. Keep small aliquots of annealed adapters in the freezer.

Double digestion

Salas-Lizana and Oono (2018) set up digestion reactions in small volumes (9 μl), where DNA constitutes 67% of the volume (in there in TE, which contain 1 mM Na^+ and 10 mM Cl^-, I think). On top of that, they add 0.45 μl of 1 M NaCl, reaching a salt concentration above 150 mM. That is higher than the salt concentration of a NEBuffer 3.1 (100 mM NaCl), where *SphI*-HF and *HindIII*-HF have 10% activity. That is, our enzymes are much more sensitive to salt than those used by Salas-Lizana and Oono (2018). Thus, we need to increase the volume of the digestion reaction to prevent salt-inhibition of the enzymes. The recommendation is to keep DNA volume below 25% of the total volume.

I assume enzymes are at 20000 U/ml. In general, 10 U of enzyme (1 μl) are supposed to be enough for 1 μg of DNA. We are using up to 300 ng of DNA in the reaction. If the number of units required are proportional to the amount of DNA, 0.3 μl of each enzyme (3 U) should be enough for 300 ng. We will use 0.5 μl. Being in 50% glicerol, that brings the final glicerol concentration to 2%, which is lower than the maximum recommended of 5%.

Table 3: Double digestion master mix. Multiply volumes by 1.2 times the number of samples. The total volume of master mix per sample is 18 μl

Component	Volum per reaction (μl)
10× rCutSmart Buffer	2.4
SphI-HF	0.5
HindIII-HF	0.5
Nuclease-free water	14.6

1. Prepare the double digestion master mix (see table above) for 1.2× the number of samples.
2. Mix 18 μl of double digestion master mix with 6 μl of sample DNA in each PCR tube or well.
3. Centrifuge briefly (spin down?) and incubate at 37°C for 4 hours in a thermal cycler with a heated lid. Note: both enzymes are "Time-Saver qualified" (according to NEB), so they are supposed to do the job in 15 minutes.
4. Do **not** heat inactivate the enzymes, because adapters are designed not to reconstitute the restriction target sites upon ligation.

Ligation of adapters

We will add the ligation reaction components in two times: first the adapters and then the T4 DNA ligase. Note that Salas-Lizana and Oono (2018) put everything together in a single Ligation Master Mix. However, I want to mix the annealed adapters with the digested genomic DNA before adding the T4 DNA ligase. Otherwise, the ligase would start working on the adapters.

Let ATP thaw at room temperature and keep in ice to minimize degradation. Resuspend the CutSmart buffer at room temperature as well. Keep adapters and the whole mixture cool to keep adapters annealed. Prepare the two mixtures separately. Volumes are set to ease pippetting.

Table 4: **Adapter Mix**. Multiply volumes by 1.2 times the number of samples. The total volume of adapter mix per sample is 5.00 μl.

Component	Volume per reaction (μl)
HindIII-P1 adapter 2.5 μM	1.0
SphI-P2 adapter 2.5 μM	1.0
CutSmart buffer 10×	0.5
Water	2.5

Table 5: **T4 DNA Ligase Mix**. Multiply volumes by 1.2 times the number of samples. The total volume of T4 DNA ligase mix per sample is 5.00 μl.

Component	Volume per reaction (μl)
ATP 10 mM	3.40
CutSmart buffer 10×	0.50
T4 DNA ligase 400000 U/ml	0.64
Water	0.46

1. Add 5 μl of the adapter mix to every digested sample. Mix well and incubate at room temperature for 5-10 minutes. Return to ice.
2. Add 5 μl of the T4 DNA ligase mixture to every sample. Mix well and incubate at 16°C overnight (or for at least 2 hours). The total reaction volume will be 34 μl.

Clean up with magnetic beads

PCR amplification with incorporation of Nextera indices

Pooling in equimolar proportion

Size selection

Peterson, Jesse N. AND Kay, Brant K. AND Weber. 2012. "Double Digest Radseq: An Inexpensive Method for de Novo Snp Discovery and Genotyping in Model and Non-Model Species." *PLOS ONE* 7 (5). Public Library of Science: 1–11. https://doi.org/10.1371/journal.pone.0037135.

Salas-Lizana, Rodolfo, and Ryoko Oono. 2018. "Double-Digest Radseq Loci Using Standard Illumina Indexes Improve Deep and Shallow Phylogenetic Resolution of Lophodermium, a Widespread Fungal Endophyte of Pine Needles." *Ecology and Evolution* 8 (13): 6638–51. https://doi.org/10.1002/ece3.4147.