

Multiplexed Library Preparation

adapted from [Parchman et al. \(2012\)](#)

all microcentrifuge vials can hold 1500µL volume

Digestion:

- Start by preparing the master mix (MM1). If you are preparing 2 plates multiply the reagents by two.
- Take out the reagents from -20 freezer and let them thaw. Once thawed place on ice. Don't take out the enzymes (EcorI and Msel).
- Vortex and centrifuge all reagents, only centrifuge the enzymes.

Making Master Mix I (MMI) for Digestion (150% of what you will need)

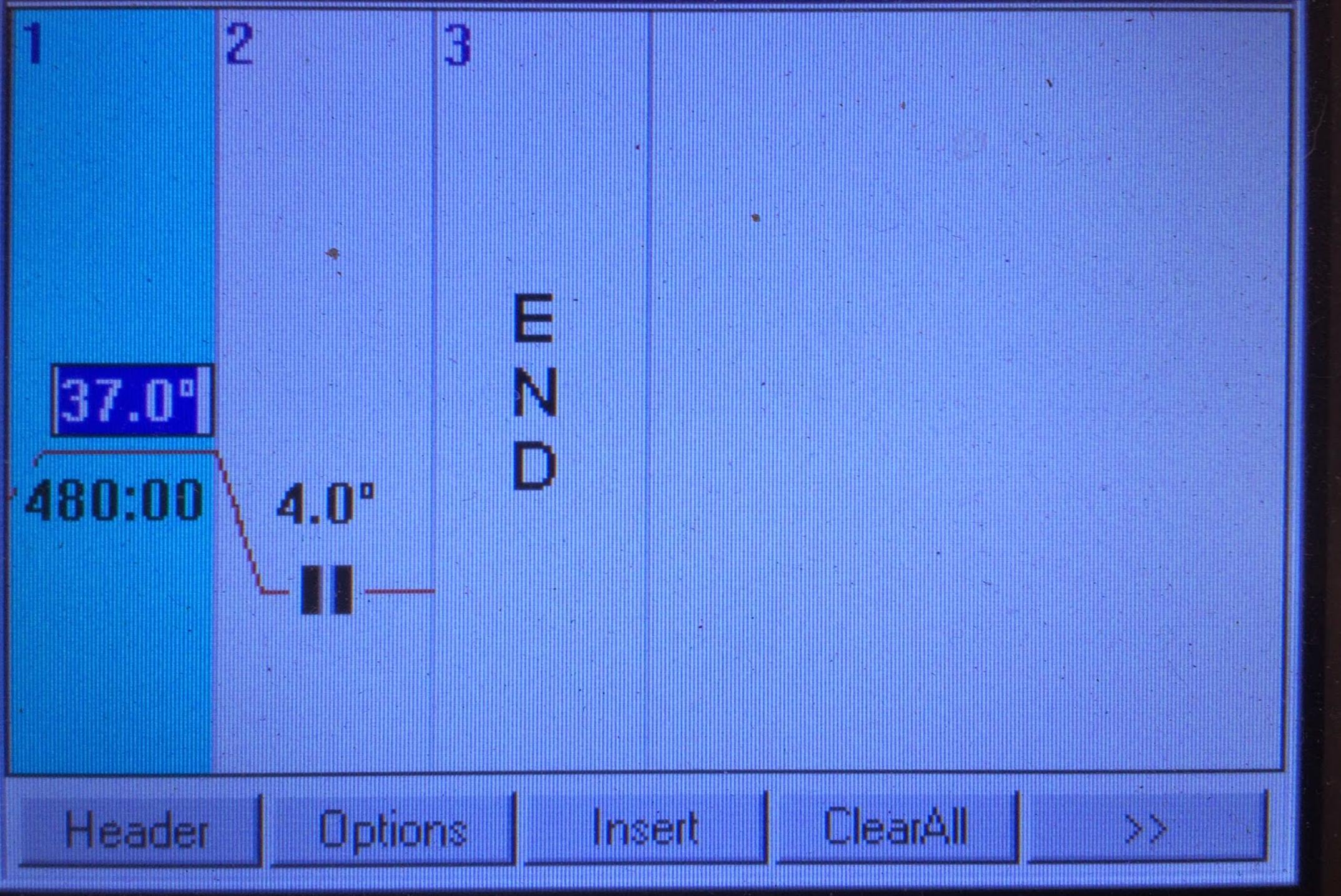
This is good for one 96-well plate. Once made, store in -20°C freezer. Always label with made-on date, MMI will expire in time (use within 3 months).

- Label a 1.5mL microcentrifuge tube with **MMI** and into it pipette:
 - 36 µL ultrapure H₂O
 - 86.4µL 1mg/mL BSA ... in freezer, must first dilute the stock of 20mg/mL to 1mg/mL using ultrapure H₂O
 - 86.4µL 1M NaCl ...on shelf above wet-lab prep area
 - 165.6µL 10X T4 Buffer ... in the freezer
 - 17.28µL Msel (10000 u/ml) ... keep in the freezer until ready to add
 - 40.32µL EcoRI (20000 u/ml) ... keep in the freezer until ready to add

Digestion Protocol:

1. Once the MM1 has been prepared, vortex and centrifuge Master Mix I vials in microcentrifuge for 5s.
2. For each sample, place 6µL of genomic DNA into the assigned well of a 96-well plate
3. Add 3µL of Master Mix I to each well
4. Cover plate with sticker (i.e. microseal), seal by running finger along each row and along each column to seal wells on every side
 - i. Label sticker with fat-tipped marker “Digested [Sample X] [date] [initials]”
 - ii. Label the plate itself in a similar manner
 - iii. Do not label with ‘Digestion’, label with ‘digested’ to emphasize past tense.
5. Vortex plate for 5s.
6. Centrifuge at 1000xG for 20s to pull all liquid to the bottom of the wells
7. Place in Thermocycler and start the Digest Cycle (8 hours run time)

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8. Once cycle has completed, place in 4°C refrigerator in the section labeled "Ready for Ligation".

Ligation:

Making Master Mix II (MMII) for Ligation (125% of what you will need)

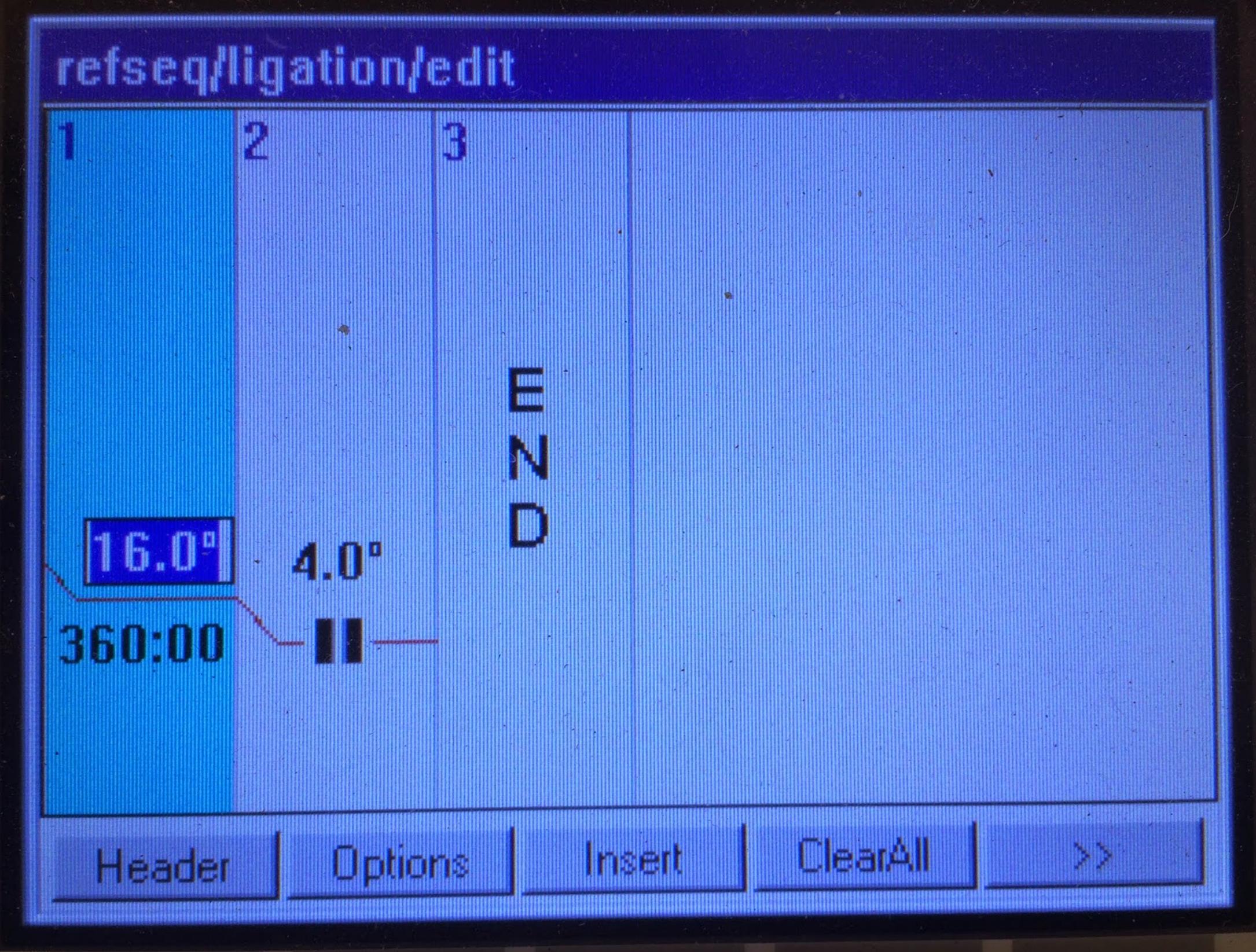
This is good for one 96-well plate. Once made, store in -20°C refrigerator. Always label with made-on date, MMII will expire in time. *Note that the official protocol calls for addition of Msel adaptor to Master Mix II, we DO NOT add it to this Master Mix since it is included in the ligation protocol below*

- Label a 1.5mL microcentrifuge with "MMII"
- Take out the reagents from -20 freezer and let them thaw. Once thawed place on ice using the black bucket and ice in autoclave/equipment room. Don't take out the enzymes (T4 DNA ligase).
- Vortex and centrifuge all reagents for a few seconds. Only centrifuge the enzymes.
- Into MM2 pipette:
 - 8.64µL ultrapure H₂O
 - 12µL 10X T4 DNA Ligase Buffer
 - 6µL 1M NaCl
 - 6µL 1mg/mL BSA (not the concentrated BSA)
 - 20.1µL T4 DNA Ligase

Ligation Protocol

1. Before you start make sure there is enough annealed Ecor1 and Msel annealed adaptors. If not, follow the method available on GitHub-EckertLab protocols.
2. Let the annealed adaptors thaw. Once thawed either place them in the fridge or on ice.
3. Once thawed, spin digested product in centrifuge at 1000xG for 20s.

4. Spin Master Mix II vials (labeled as MMII or MM2) in the microcentrifuge for 5s.
5. Add 0.6 μ L of Master Mix II to each well
6. Spin the thawed annealed EcoRI and Msel plates at 1000xG for 20s.
7. Orient EcoRI and Msel plates in steps 6-7 to match ligation plate orientation
8. Did you orient the plates from Step 5??
9. Are you sure you oriented the plates correctly??
10. Using the multichannel pipette, add 1 μ L of annealed EcoRI adaptor to each well (this should be a plate)
 - i. Always check to make sure each tip has liquid, some wells may be empty
 - ii. If empty you CANNOT PULL FROM OTHER WELLS!
 - iii. Once complete, replace tin sticker and label with fat-tipped marker. Note on label if there are any empty wells.
 - iv. Check to make sure writing on plate (not sticker) is not fading. If so, rewrite using fine-tipped marker on another part of plate. Don't write over!
11. Using the multichannel pipette, add 1 μ L of annealed Msel adaptor to each well (this should be a plate)
 - i. Always check to make sure each tip has liquid, some wells may be empty.
 - ii. If empty, you can pull from other wells YOU CANNOT DO THIS WITH ECORI
 - iii. Once complete, replace tin sticker and label with fat-tipped marker. Note on label if there are any empty wells.
 - iv. Check to make sure writing on plate (not sticker) is not fading. If so, rewrite using fine-tipped marker on another part of plate. Don't write over!
12. Cover ligation plate with sticker, seal by running finger along each row and along each column to seal wells on every side
 - i. Label with fat-tipped marker "*Ligated [Sample X] [date] [initials]*"
 - ii. Label the plate itself in a similar manner
 - iii. Do not label with 'ligation', label with 'ligated' to emphasize past tense.
13. Vortex plate for 5s.
14. Centrifuge ligation plate at 1000xG for 20s to pull all liquid to the bottom of the wells
15. Place in Thermocycler and start the Ligation cycle (6 hours run time)



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16. Once cycle has completed, place in 4°C refrigerator in section labeled "Ready for PCR"
17. As soon as ligation is done add 189 μ L of 0.1X TE buffer (NOT TAE) to each well to dilute the products and also avoid samples being evaporated.
18. If you think you will not be using these products for a while place them in -20°C.

PCR

Making 380 μ L of 5 μ M PCR primers for Master Mix III

This is enough for all three vials of MM3 needed. Once made, store in -20°C freezer. Always label with made-on date, MMIII will expire in time.

- Label a 1.5mL microcentrifuge tube with "PCR Primers"
- Vortex and centrifuge the thawed Primers (F and R) before preparing the PCR Primers.
- Into it pipette:
 - 10 μ L 100 μ M IIIpcr F Primer
 - 10 μ L 100 μ M IIIpcr R Primer
 - 360 μ L ultrapure H₂O

Making 100 μ L of 10mM dNTP mix for Master Mix III

This is enough for all three vials of MM3 needed. Once made, store in -20°C freezer. Always label with made-on date, dNTPs will expire in time.

- Label a 1.5mL microcentrifuge tube with "dNTP Mix"
- Vortex and centrifuge the thawed dNTPs before preparing the dNTP mix.
- Into it pipette:
 - 2.5 μ L of each 100mM dNTP (A, C, T, G)

- 90µL ultrapure H₂O

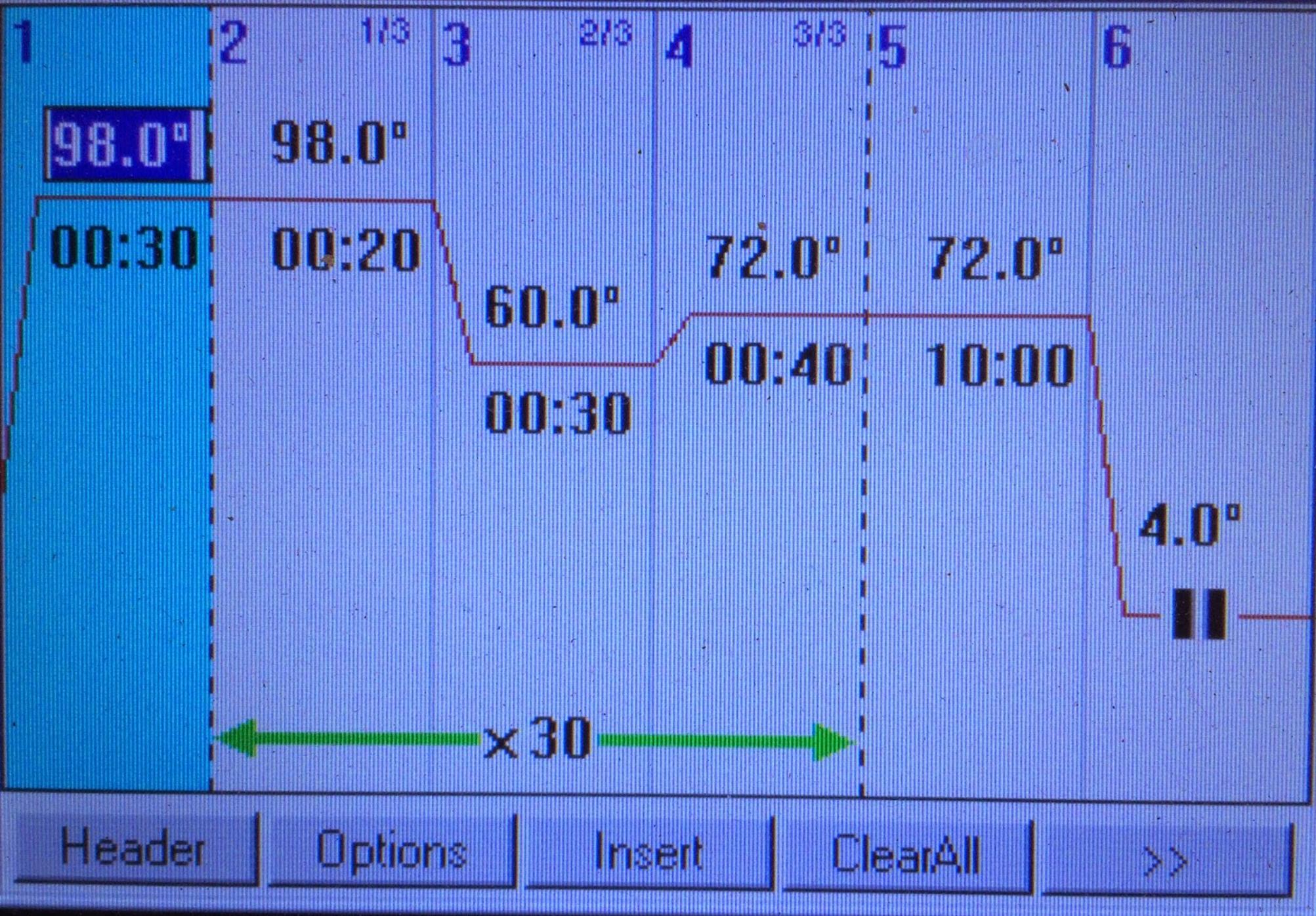
Making Master Mix III for PCR (125% if what you will need)

- You will need to make 3 vials of this Master Mix for the two 96 well plates. Once made, store in -20°C refrigerator. Always label with made-on date, dNTPs and polymerase will expire in time.
- Vortex and centrifuge the thawed reagents before adding them to the MM3 vials. Don't vortex or thaw the polymerase.
- Once thawed place all reagents on ice except DMSO.
- When vial is empty, spin down before throwing away, use any remaining. Polymerase is expensive!
- Label 3 microcentrifuge vials with MM3 on lid AND side of vial. Into each place:
 - 773.6µL ultrapure H₂O (770µL + 3.6µL)
 - 320µL 5X Iproof HF Buffer (green vial)
 - 32µL 10 mM dNTP Mix
 - 32µL 50mM MgCl₂
 - 106.4µL 5µM PCR Primers
 - 12µL DMSO (6µL + 6µL)
 - 16µL Iproof Taq Polymerase (DO NOT ADD UNTIL READY TO PLATE!)
- Vortex then spin down the vials of MM3 using the microcentrifuge for 5-10s.

PCR Protocol

1. If opening a new box of ingredients, label with date and your initials with fine-tipped permanent marker
2. Centrifuge ligated-diluted samples at 1000xG for 20s to pull down liquid.
3. If your ligated products are not diluted with 1xTE you can add it now.
4. Get two new 96-well plates and label ON THE PLATE with fine-tipped permanent marker "PCR Product [sample] [date] [initials]"
5. Orient these plates in the same direction as ligated plate!
 - i. Using the multichannel pipette, add 4µl of the diluted, ligated, samples to the 2 NEW plates – DO NOT REUSE PIPETTE TIPS except for the same row across the two plates
6. Add 16µl of Master Mix III to each well
7. Place tin sticker on new plates and label with fat-tipped marker "PCR Product [sample] [date] [initials]"
 - i. Do not label with just 'PCR', label with 'PCR Product' to emphasize past tense.
8. Centrifuge at 1000xG for 20s to pull all liquid to the bottom of the wells
9. Place plates in Thermocyclers and start the PCR cycles (55.5 minutes run time)

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10. Once cycles complete, pool samples into a sterile 15mL blue-capped centrifuge tube.
 - i. Place in 4°C refrigerator in section labeled "Ready for gel extraction"
11. Perform [Gel Extraction Protocol](#) following completion of PCR