3RAD Protocol

1. Restriction Digest and Adapter Ligation

Note: ligation must be performed immediately following restriction digest

Restriction Digest

Reagents

- 20,000 U/mL New England BioLabs Restriction Enzymes
- 10X New England BioLabs CutSmart Buffer
- 2.5 µM Read 1 and Read 2 3RAD Adapters

Final Concentrations

In 15 µL:

- 20-100 ng Sample DNA
- 1X Cutsmart Buffer
- 10 U of read 1 restriction enzyme
- 10 U of read 2 restriction enzyme
- 10 U of adapter dimer restriction enzyme
- 0.33 µM read 1 adapter
- 0.33 µM read 2 adapter

Procedure

1. Pipette 5 µL of DNA at a concentrations of 4-20 ng/µL onto 96 well plate.
2. Prepare enzyme digestion master mix.
3. Add 6 µL master mix to each sample.
4. Add 2 µL of 2.5 µM each adapter.
5. Incubate samples at:
    - No heated lid
    - 37 °C for 1 hour

Enzyme Digestion Master Mix	1X	116X
10X CutSmart Buffer	1.5 µL	174 µL
H_2O	3.0 µL	348 µL

Enzyme 1 20 U/µL	0.5 µL	58 µL
Enzyme 2 20 U/µL	0.5 µL	58 µL
Enzyme 3 20 U/µL	0.5 µL	58 µL
Total volume	6 µL	696 µL

Ligation

Reagents

- 400,000 U/mL New England BioLabs T4 DNA Ligase
- 10X New England Biolabs Ligase Buffer
- Promega 10 mM rATP

Final Concentrations

In 20 µL:

- 100 U of T4 DNA ligase
- 0.25X ligase buffer
- 0.75 mM rATP

Procedure

1. Prepare ligation master mix.
2. Add 5 µL ligation master mix to each sample.
3. Incubate samples at:
    - No heated lid
    - 2 cycles of:
        - 22 °C for 20:00
        - 37 °C for 10:00
    - 80 °C for 20:00

Ligation Master Mix	1X	106X
H_2O	2.75 µL	291.5 µL
10X Ligase Buffer	0.5 µL	53 µL
10 mM rATP	1.5 µL	159 µL
Ligase 400,000 ∪/mL	0.25 µL	26.5 µL

Total volume	5 µL	530 µL

2. Bead Cleanup

1. Pool 5 µL of each sample
2. Mix pooled sample with 1X Speedbeads and briefly vortex.
3. Allow sample to sit for 1 minute.
4. Place sample on magnet stand until solution is completely clear and magnets have been drawn to the side of the tube.
5. Carefully pipette solution from tube and discard.
6. Wash sample twice with 500 µL of 70% ETOH
    i. Add 500 µL of 70% ETOH to tube and let sit for 1 minute.
    ii. Carefully pipette ETOH from tube.
    iii. Repeat once.
    iv. Use toothpick to remove remaining drops of ETOH and let stand until no ETOH remains.
7. Re-suspend in initial volume of TLE Buffer Solution

- Note: Maybe suspend this in smaller volume?

3. One Cycle PCR With iTru5-8N Primer

Reagents

- Kapa HiFI PCR Kit
- 5 µM iTru5 Primer

Final Concentrations

In 50 µL:

- 5 µL of pooled DNA samples
- 1X KAPA HiFi Fidelity Buffer
- 0.3 µM iTru5 Primer
- 0.3 mM each dNTP
- 1 U of KAPA HiFi Hotstart DNA Polymerase

Procedure

1. Prepare PCR master mix.
2. Add 45 µL master mix to six 0.2 mL PCR tubes
3. Add 5 µL pooled DNA to each of the six PCR tubes

4. Incubate samples at:
    - 95 °C for 2:00
    - 98 °C for 0:20
    - 61 °C for 0:15
    - 72 °C for 5:00

PCR Master Mix	1X	6.5X
H_2O	29.5 µL	191.75 µL
5X Kapa HiFi Buffer	10 µL	65 µL
5 µM iTru5-8N Primer	3 µL	19.5 µL
10mM dNTP	1.5 µL	9.75 µL
KAPA Hifi Polymerase	1.0 µL	6.5 µL
Total volume	45 µL	292.5

4. Bead Cleanup

1. Pool iTru5-8N PCR product
2. Mix pooled sample with 2X Speedbeads and briefly vortex.
3. Allow sample to sit for 1 minute.
4. Place sample on magnet stand until solution is completely clear and magnets have been drawn to the side of the tube.
5. Carefully pipette solution from tube and discard.
6. Wash sample twice with 500 µL of 70% ETOH
    i. Add 500 µL of 70% ETOH to tube and let sit for 1 minute.
    ii. Carefully pipette ETOH from tube.
    iii. Repeat once.
    iv. Use toothpick to remove remaining drops of ETOH and let stand until no ETOH remains.
7. Re-suspend in 35 µL TLE Buffer Solution

5. Two Primer Amplification With P5 & iTru7 Primers

Reagents

- Kapa HiFI PCR Kit
- 5 µM iTru7 Primer
- 5 µM P5 Primer

Final Concentrations

In 50 µL:

- 5 µL of pooled DNA samples
- 1X KAPA HiFi Fidelity Buffer
- 0.3 µM iTru7 Primer
- 0.3 µM P5 Primer
- 0.3 mM each dNTP
- 1 U of KAPA HiFi Hotstart DNA Polymerase

Procedure

1. Prepare PCR master mix.
2. Add 45 µL master mix to six 0.2 mL PCR tubes
3. Add 5 µL pooled DNA to each of the six PCR tubes
4. Incubate samples at:
    - 95 °C for 2:00
    - 6 cycles of:
        - 98 °C for 0:20
        - 61 °C for 0:15
        - 72 °C for 0:30
    - 72 °C 5:00

PCR Master Mix	1X	6.5X
H_2O	26.5 µL	172.25 µL
5X Kapa HiFi Buffer	10 µL	65 µL
5 µM iTru7 Primer	3 µL	19.5 µL
5 µM P5 Primer	3 µL	19.5 µL
10mM dNTP	1.5 µL	9.75 µL
KAPA Hifi Polymerase	1.0 µL	6.5 µL
Total volume	45 µL	292.5

6. Bead Cleanup

1. Pool P5-iTru7 PCR products into single tube

2. Mix pooled sample with 2X Speedbeads and briefly vortex.
3. Allow sample to sit for 1 minute.
4. Place sample on magnet stand until solution is completely clear and magnets have been drawn to the side of the tube.
5. Carefully pipette solution from tube and discard.
6. Wash sample twice with 500 µL of 70% ETOH
    i. Add 500 µL of 70% ETOH to tube and let sit for 1 minute.
    ii. Carefully pipette ETOH from tube.
    iii. Repeat once.
    iv. Use toothpick to remove remaining drops of ETOH and let stand until no ETOH remains.
7. Re-suspend in 35 µL TLE Buffer Solution

7. Size Selection

Procedure

Size select DNA with BluePippin

8. Final Amplification with P5 and P7 Primers

Reagents

- Kapa HiFI PCR Kit
- 5 µM P5 Primer
- 5 µM P7 Primer

Final Concentrations

In 50 µL:

- 5 µL of pooled DNA samples
- 1X KAPA HiFi Fidelity Buffer
- 0.3 µM P5 Primer
- 0.3 µM P7 Primer
- 0.3 mM each dNTP
- 1 U of KAPA HiFi Hotstart DNA Polymerase

Procedure

1. Prepare PCR master mix.
2. Add 45 µL master mix to six 0.2 mL PCR tubes
3. Add 5 µL pooled DNA to each of the six PCR tubes
4. Incubate samples at:

- 95 °C for 2:00
- 12 cycles of:
  - 98 °C for 0:20
  - 61 °C for 0:15
  - 72 °C for 0:45
- 72 °C 5:00

PCR Master Mix	1X	6.5X
H_2O	26.5 µL	172.25 µL
5X Kapa HiFi Buffer	10 µL	65 µL
5 µM P5 Primer	3 µL	19.5 µL
5 µM P7 Primer	3 µL	19.5 µL
10mM dNTP	1.5 µL	9.75 µL
KAPA Hifi Polymerase	1.0 µL	6.5 µL
Total volume	45 µL	292.5

9. Bead Cleanup

1. Pool P5-P7 PCR products into single tube
2. Mix pooled sample with 1X Speedbeads and briefly vortex.
3. Allow sample to sit for 1 minute.
4. Place sample on magnet stand until solution is completely clear and magnets have been drawn to the side of the tube.
5. Carefully remove pipette solution from tube.
6. Wash sample twice with 500 µL of 70% ETOH
   i. Add 500 µL of 70% ETOH to tube and let sit for 1 minute.
   ii. Carefully pipette ETOH from tube.
   iii. Repeat once.
   iv. Use toothpick to remove remaining drops of ETOH and let stand until no ETOH remains.
7. Re-suspend in 35 µL 10mM Tris-HCl Buffer Solution