PCRs for Sequencing of ModPokI Screen Samples

MATERIALS:

- KAPA Hifi Hotstart ReadyMix (Roche, 07958935001)
- Primers (see below)
- Thermocycler for PCR
- AMPure XP Beads (Beckman Coulter, A63881) or Sera-Mag SpeedBeads Carboxyl Magnetic Beads (VWR, 65152105020250 or 10204-664)
- Qubit (Thermo Fisher Scientific)
- NEBNext Ultra II Q5 Master Mix (NEB, M0544X)
- EBT buffer (10mM Tris-HCl, pH 8.5 with 0.1% Tween 20)

When RNA is used as starting material:
- Direct-zol RNA kit (Zymo Research)
- Maxima H Minus Reverse Transcriptase (Thermo Fisher Scientific, EP0753)
- dNTP Mix 10mM (e.g., Thermo Fisher Scientific, 18427088)
- Oligo(dT) 12-18 Primer (e.g. Thermo Fisher Scientific, 18418012)

When genomic DNA is used as starting material:
- Quick-DNA Kit (Zymo Research)

PROTOCOL:

1. Extract RNA or genomic DNA from T cells using e.g., Zymo Research kits according to the manufacturer’s instructions. When extracting RNA, include the DNase I step into the protocol. When extracting genomic DNA, cDNA synthesis will be skipped and you can directly proceed to step 6 of the protocol.
2. After RNA extraction, quantify concentration.
3. Set up cDNA reaction (scale up the number/volume of reactions as needed to process all the RNA depending on coverage calculations):
   - 500ng RNA
   - 1uL Oligo(dT) primer
   - 1uL dNTP mix
   - 1uL Maxima H Minus Reverse Transcriptase
   - 4uL 5x RT buffer
   - fill up with water to 20uL
4. Thermocycler:
   - 50C 30min
   - 85C 4min
   - 4C forever
5. Purify the product using standard magnetic bead purification, e.g. AMPure XP Beads or Sera-Mag SpeedBeads. Elute in 12.5uL.
6. Verify concentration using Qubit.
7. Set up PCR1 (scale up the number/volume of reactions as needed to process all the cDNA/gDNA depending on coverage calculations):
• 100ng of cDNA or gDNA
• 12.5uL of 2x KAPA Hifi Hotstart ReadyMix
• 30pmol forward primer
• 30pmol reverse primer
• fill up with water to 25uL

8. Thermocycler:
   • 95C 3min
   • 98C 20s
   • 69C 15s
   • 72C 15s
   • Back to step 2, total of 18 cycles
   • 72C 3min
   • 4C forever

9. Purify the PCR product using standard magnetic bead purification, e.g. AMPure XP Beads or Sera-Mag SpeedBeads. Elute in 12.5uL.

10. Verify concentration using Qubit.

11. Set up PCR2:
    • 5ng of PCR1 product
    • 10uL of NEBNext Ultra II Q5 Master Mix
    • 5pmol forward primer
    • 5pmol reverse primer
    • fill up with water to 20uL

12. Thermocycler:
    • 98C 3min
    • 98C 20s
    • 60C 20s
    • 72C 25s
    • Back to step 2, total of 10 cycles
    • 72C 2min
    • 4C forever

13. Purify the PCR product using standard magnetic bead purification, e.g. AMPure XP Beads or Sera-Mag SpeedBeads. Elute in 12.5uL.


15. Dilute to 1nM with EBT, can freeze library at this step.

16. The library is now ready to be sequenced.

PRIMER SEQUENCES:

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<tr>
<th>Primer Name</th>
<th>Sequencing Strategy</th>
<th>Sequence</th>
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<td>Single knockin, 3' barcode</td>
<td>TCGTGGCAGCGTGAGGTGTATAAGAGACGAGGGCTAGAGAGGTTTAGGATCT</td>
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<tr>
<td>Primer Name</td>
<td>Sequencing Strategy</td>
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**PCR2**

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For information on Illumina i7 and i5 sequences, see Illumina website (“Sequences for AmpliSeq for Illumina Panels”).