Capturing Exons with Molecular Inversion Probes

Generate Probes

Amplify off-array oligonucleotides (MIP precursors) using PCR: (2.5 hrs)

1. Dissolve array-derived MIP precursor oligonucleotides (mixture of 100-mers obtained from Agilent) to a final concentration of 100 nM in Tris-EDTA buffer with a pH of 8 and 0.1 % Tween.

2. Prepare the following 400 µl PCR mix in a 1.5 ml centrifuge tube. Mix and spin down:

Reagent	Volume (µl)	Final Concentration
2x iProof HF PCR master mix (Biorad)	200	1x
Oligo_Fwd_Amp Primer (100 µM)	2	500 nM
Oligo_Rev_Amp Primer (100 µM)	2	500 nM
SYBRGreen I 100 x (Invitrogen)**	1	0.2X
Template (100 nM in 0.1 % Tween)	1	250 pM
Water	194	

Split into 8 x 50 µl reactions in 0.2 ml PCR tubes. One PCR preparation can be expected to yield around 1.5 µg of amplified DNA. **Use SYBR Green when using a real-time thermocycling instrument.

3. Use the following PCR cycling program, ideally on a real-time thermocycling instrument such as the Biorad MJ Mini.

98 °C for 30 seconds

98 °C for 10 seconds
60 °C for 30 seconds x 25 cycles
72 °C for 30 seconds (read plate)

4 °C indefinitely

**We typically stop our reactions after 20 rounds of PCR. When using RT-PCR, stop the reaction slightly before the fluorescence curve plateaus to avoid over-amplification.

4. Combine and clean up PCR reactions on one column using the QIAquick PCR purification kit following the manufacturer's instructions. Elute with 90 µl elution buffer.

5. Use a Qubit High Sensitivity dsDNA Assay Kit to quantify 1 µl of the amplified DNA.

6. Analyze 1 µl amplified DNA on a 6 % TBE PAGE gel (Invitrogen) to verify amplification. Product should appear as a single band at 110 bp, as the primers add an additional 10 bp.

Digest PCR product with nicking restriction endonucleases to generate 70-mer MIPs (7.5 hrs):

7. Add 10 µl of NEB-2 (10x) and 5 µl of Nt.AlwI (10 U / µl; NEB) to 85 µl of PCR product (total volume of 100 µl)

8. Mix and split to two tubes of 50 µl each. Incubate at 37 °C for 3 hours, followed by 80 °C for 20 minutes in a thermocycler

9. Let the temperature drop to 65 °C for at least 1 minute. Add 2.5 µl of Nb.BsrDI (2 U / µl; NEB) to each of the 50 µl reactions

10. Leave at 65 °C for 3 hours, followed by 80°C for 20 minutes

11. Purify two 50 µl digestion reactions on one column using reagents from the QIAquick Nucleotide Removal Kit. Elute each column in 30 µl elution buffer. We have observed yields of 80-90 % for this step.

Quantify usable probe using a denaturing gel (2 hrs):

12. Accurate quantification of usable MIP inside the digested probe mix is important as it determines how much probe mix to add to the capture reaction.

13. Prepare two-fold dilutions of a NEB 100 bp DNA ladder (we used dilutions from 500 ng to 62 ng).

14. Mix 2x TBE-Urea sample buffer (Invitrogen) with 1 µl digested probe and the dilutions made above.

15. Denature DNA by heating to 95 °C for 5 minutes and immediately transferring to ice.

16. Run samples on a precast 6 % TBE-urea denaturing PAGE gel (Invitrogen) for 1 hr at 160 V.

17. Quantify the amount of usable MIP in the digested mixture by comparing the intensity of ladder dilutions with the intensity of the 70 bp band. Use this MIP concentration when determining the volume of probe mix to add to a capture reaction.

Capture Reaction

Note: We have found that it is no longer necessary to gel-purify the single stranded 70 bp MIP from the digested probe mix in a capture reaction. Instead, we use a blocking oligo to limit hybridization of the undigested strand of the MIP precursor (still a 100-mer) to the active 70-mer MIP in the capture reaction, which could potentially interfere with MIP hybridization to genomic DNA targets.

Hybridize probes to genomic DNA (37 hrs):

1. For each sample to capture, add the following reagents in a 0.2 ml PCR tube. The final capture reaction volume is 25 µl. Because there is no size selection of the 70 bp MIP, the volume of probe mix to add is based on the concentration of usable MIP.

Reagent	Volume (µl) per sample	Final Concentration in reaction
750 ng genomic DNA*	3	30 ng / µl*
10 x Ampligase buffer (Epicentre)	2.5	1x
40 ng (2 pmol) of MIP**	3	1.6 ng / µl**
Blocking Oligonucleotide (100 µM)	0.1	0.4 µM
Water	16.4	

   * Additionally, prepare a blank capture reaction containing MIP probe but no gDNA to detect cross contamination.
   ** For a reaction targeting 55,000 regions in the genome. We currently aim for a ratio of MIPs to genomic DNA of 100:1 (i.e. 100 copies of each MIP in the mix for each genomic equivalent). The concentration of MIPs can be adjusted accordingly depending on complexity of the targeting reaction.

2. Denature at 95 °C for 10 minutes.

3. Incubate at 60 °C for at least 36 hours to hybridize MIPs to gDNA.

Circularize captured exons: (1 day)

4. Prepare a mix of ligase and polymerase enzymes to add to each capture reaction:

Reagent	Volume (µl) per sample	Final Concentration in capture reaction
10 x Ampligase buffer (Epicentre)	0.45	1x
10 U / µl Stoffel** (Applied Biosystems)	2	0.8 U / µl
100 U / µl Ampligase** (Epicentre)	1	4 U / µl

0.25 mM dNTP**	1.25	12 µM

Prepare this mix on ice, and keep cold before adding 4.7 µl into the capture reaction.

5. Incubate at 60 °C for an additional 24 hours to allow for gap-fill and ligation to circularize captured regions.

Exonuclease select for circularized product: (1hr)

6. Prepare a mix of exonucleases to add to each capture reaction in order to remove uncaptured gDNA, excess probe and blocking oligonucleotide:

Reagent	Volume (µl) per sample	Final Concentration in reaction
Exo I 20 U / µl	2	1.7 U / µl
Exo III 100 U / µl	2	8.3 U / µl

7. Reduce the temperature of the capture reaction to 37 °C and allow it to incubate for at least one minute before adding 4 µl of exonuclease mix.

8. Incubate for 15 minutes at 37 °C.

9. Inactivate exonuclease enzymes by heating reaction at 95 °C for 2 minutes.

10. Use 5 µl of the reaction product as the template for PCR. There is no need to purify the reaction product before PCR.

Amplify and Verify Captured Product:

1. Prepare the following PCR mix in a 1.5 ml centrifuge tube. Mix and spin down.

Reagent	Volume (µl) per sample	Final Concentration in capture reaction
iProof PCR master mix (2x)	25	1x
SLXA_Paired_End_CP2_Fwd (100 µM)	0.25	500 nM
SLXA_Paired_End_CP2_Rev (100 µM)	0.25	500 nM
SYBR Green I 100X (Invitrogen)	0.25	0.5X
Water	19.25	

2. Add 45 µl of master mix to 5 µl of each sample to obtain a total reaction volume of 50 µl. Mix gently and spin down.

3. Amplify on a RT-PCR machine using the following conditions:

   98 °C for 30 seconds

   98 °C for 10 seconds
   60 °C for 30 seconds x 25 cycles
   72 °C for 60 seconds (read plate)

   **When using RT-PCR, stop the reaction slightly before the fluorescence curve plateaus to avoid overamplification.

4. Purify each sample reaction on one column QIAquick PCR Purification column following the manufacturer's instructions. Elute each column in 30 µl EB buffer.

5. Use a Qubit HS dsDNA Assay Kit to quantify 1 µl of the amplified DNA.

6. Analyze 2 µl of the amplified DNA on a PAGE gel as described in step 6 to validate that the amplified product is of the expected size range. Currently, we aim for a uniform gap-fill size of 112 bp for all targets. Assuming 20 bp targeting arms, paired-end 76 bp reads enable full coverage of this gap-fill size. At this step, we consequently expect a tight band centered at 245 bp (49 bp primer (SLXA_Paired_End_CP2_Fwd) + 20 bp targeting arm + 112 bp gap-fill + 20 bp targeting arm + 44 bp primer (SLXA_Paired_End_CP2_Rev) = 245 bp).

7. Samples are now ready for analysis using the Illumina Genome Analyzer. Use PE_Capture_Sequencing and PE_Rev_Capture_Sequencing primers for sequencing

Timeline: (4 days)

Generate Probes (Steps 1-17): 12 hrs
Amplify off-array oligonucleotides using PCR: (2.5 hrs)
Digest oligonucleotides: (7.5 hrs)
Quantify usable probe using a denaturing gel: (2 hrs)

Capture Exons (Steps 18-28): 3 days
Hybridize probes to genomic DNA: (1.5 days)
Circularize captured exons: (1 day)
Exonuclease select for circularized product: (1 hr)

Amplify and Verify Captured Product (Steps 29-35): 4 hrs

Oligonucleotide Sequences:

Oligonucleotide Name	Sequence (5'→3')
General Format of MIP precursors (100-mers); x's and y's indicate variable targeting arm sequence	AGGACCGGATCAACTxxxxxxxxxxxxxxxxxxxCTTCAGCTTCCCGATA TCCGACGGTAGTGTyyyyyyyyyyyyyyyyyyyCATTGCGTGAACCGA
Oligo_Fwd_Amp	TGCCTAGGACCGGATCAACT
Oligo_Rev_Amp	GAGCTTCGGTTCACGCAATG
SLXA_Paired_End_CP2_Fwd	AATGATACGGCGACCACCGAGATCTACACGCACGATCCGACGGTA GTGT
SLXA_Paired_End_CP2_Rev	CAAGCAGAAGACGGCATACGAGATCCGTAATCGGGAAGCTGAAG
PE_Capture_Sequencing Primer	ACACGCACGATCCGACGGTAGTGT
PE_Rev_Capture_Sequencing Primer	CATACGAGATCCGTAATCGGGAAGCTGAAG
Blocking Oligonucleotide	CTTCAGCTTCCCGATATCCGACGGTAGTGT