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Target-enrichment strategies for next-generation sequencing

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Supplementary Table 1. Error-correcting barcode tags and PCR primers.
Column B contains octamer barcodes, and Column D contains PCR primers that contain these barcodes.
Column C contains the reverse complement of the barcodes given in Column B. This is the sequence that is obtained from the barcodes in the sequencing reaction, and which are robust to errors.

Tag number	Barcode sequence	Single correcting, double & shift detecting octamers	PCR primers
1	ACAAGCTA	TAGCTTGT	CAAGCAGAAGACGGCATACGAGATACAAGCTAGAGATCGGTCTCGGCATTC
2	AAACATCG	CGATGTTT	CAAGCAGAAGACGGCATACGAGATAAACATCGGAGATCGGTCTCGGCATTC
3	ACATTGGC	GCCAATGT	CAAGCAGAAGACGGCATACGAGATACATTGGCGAGATCGGTCTCGGCATTC
4	ACCACTGT	ACAGTGGT	CAAGCAGAAGACGGCATACGAGATACCACTGTGAGATCGGTCTCGGCATTC
5	AACGTGAT	ATCACGTT	CAAGCAGAAGACGGCATACGAGATAACGTGATGAGATCGGTCTCGGCATTC
6	CGCTGATC	GATCAGCG	CAAGCAGAAGACGGCATACGAGATCGCTGATCGAGATCGGTCTCGGCATTC
7	CAGATCTG	CAGATCTG	CAAGCAGAAGACGGCATACGAGATCAGATCTGGAGATCGGTCTCGGCATTC
8	ATGCCTAA	TTAGGCAT	CAAGCAGAAGACGGCATACGAGATATGCCTAAGAGATCGGTCTCGGCATTC
9	CTGTAGCC	GGCTACAG	CAAGCAGAAGACGGCATACGAGATCTGTAGCCGAGATCGGTCTCGGCATTC
10	AGTACAAG	CTTGTACT	CAAGCAGAAGACGGCATACGAGATAGTACAAGGAGATCGGTCTCGGCATTC
11	CATCAAGT	ACTTGATG	CAAGCAGAAGACGGCATACGAGATCATCAAGTGAGATCGGTCTCGGCATTC
12	AGTGGTCA	TGACCACT	CAAGCAGAAGACGGCATACGAGATAGTGGTCAGAGATCGGTCTCGGCATTC
13	AACAACCA	TGGTTGTT	CAAGCAGAAGACGGCATACGAGATAACAACCAGAGATCGGTCTCGGCATTC
14	AACCGAGA	TCTCGGTT	CAAGCAGAAGACGGCATACGAGATAACCGAGAGAGATCGGTCTCGGCATTC
15	AACGCTTA	TAAGCGTT	CAAGCAGAAGACGGCATACGAGATAACGCTTAGAGATCGGTCTCGGCATTC
16	AAGACGGA	TCCGTCTT	CAAGCAGAAGACGGCATACGAGATAAGACGGAGAGATCGGTCTCGGCATTC
17	AAGGTACA	TGTACCTT	CAAGCAGAAGACGGCATACGAGATAAGGTACAGAGATCGGTCTCGGCATTC
18	ACACAGAA	TTCTGTGT	CAAGCAGAAGACGGCATACGAGATACACAGAAGAGATCGGTCTCGGCATTC
19	ACAGCAGA	TCTGCTGT	CAAGCAGAAGACGGCATACGAGATACAGCAGAGAGATCGGTCTCGGCATTC
20	ACCTCCAA	TTGGAGGT	CAAGCAGAAGACGGCATACGAGATACCTCCAAGAGATCGGTCTCGGCATTC
21	ACGCTCGA	TCGAGCGT	CAAGCAGAAGACGGCATACGAGATACGCTCGAGAGATCGGTCTCGGCATTC
22	ACGTATCA	TGATACGT	CAAGCAGAAGACGGCATACGAGATACGTATCAGAGATCGGTCTCGGCATTC
23	ACTATGCA	TGCATAGT	CAAGCAGAAGACGGCATACGAGATACTATGCAGAGATCGGTCTCGGCATTC
24	AGAGTCAA	TTGACTCT	CAAGCAGAAGACGGCATACGAGATAGAGTCAAGAGATCGGTCTCGGCATTC
25	AGATCGCA	TGCGATCT	CAAGCAGAAGACGGCATACGAGATAGATCGCAGAGATCGGTCTCGGCATTC

Tag number	Barcode sequence	Single correcting, double & shift detecting octamers	PCR primers
26	AGCAGGAA	TTCCTGCT	CAAGCAGAAGACGGCATACGAGATAGCAGGAAGAGATCGGTCTCGGCATTC
27	AGTCACTA	TAGTGACT	CAAGCAGAAGACGGCATACGAGATAGTCACTAGAGATCGGTCTCGGCATTC
28	ATCCTGTA	TACAGGAT	CAAGCAGAAGACGGCATACGAGATATCCTGTAGAGATCGGTCTCGGCATTC
29	ATTGAGGA	TCCTCAAT	CAAGCAGAAGACGGCATACGAGATATTGAGGAGAGATCGGTCTCGGCATTC
30	CAACCACA	TGTGGTTG	CAAGCAGAAGACGGCATACGAGATCAACCACAGAGATCGGTCTCGGCATTC
31	CAAGACTA	TAGTCTTG	CAAGCAGAAGACGGCATACGAGATCAAGACTAGAGATCGGTCTCGGCATTC
32	CAATGGAA	TTCCATTG	CAAGCAGAAGACGGCATACGAGATCAATGGAAGAGATCGGTCTCGGCATTC
33	CACTTCGA	TCGAAGTG	CAAGCAGAAGACGGCATACGAGATCACTTCGAGAGATCGGTCTCGGCATTC
34	CAGCGTTA	TAACGCTG	CAAGCAGAAGACGGCATACGAGATCAGCGTTAGAGATCGGTCTCGGCATTC
35	CATACCAA	TTGGTATG	CAAGCAGAAGACGGCATACGAGATCATACCAAGAGATCGGTCTCGGCATTC
36	CCAGTTCA	TGAACTGG	CAAGCAGAAGACGGCATACGAGATCCAGTTCAGAGATCGGTCTCGGCATTC
37	CCGAAGTA	TACTTCGG	CAAGCAGAAGACGGCATACGAGATCCGAAGTAGAGATCGGTCTCGGCATTC
38	CCGTGAGA	TCTCACGG	CAAGCAGAAGACGGCATACGAGATCCGTGAGAGAGATCGGTCTCGGCATTC
39	CCTCCTGA	TCAGGAGG	CAAGCAGAAGACGGCATACGAGATCCTCCTGAGAGATCGGTCTCGGCATTC
40	CGAACTTA	TAAGTTCG	CAAGCAGAAGACGGCATACGAGATCGAACTTAGAGATCGGTCTCGGCATTC
41	CGACTGGA	TCCAGTCG	CAAGCAGAAGACGGCATACGAGATCGACTGGAGAGATCGGTCTCGGCATTC
42	CGCATACA	TGTATGCG	CAAGCAGAAGACGGCATACGAGATCGCATACAGAGATCGGTCTCGGCATTC
43	CTCAATGA	TCATTGAG	CAAGCAGAAGACGGCATACGAGATCTCAATGAGAGATCGGTCTCGGCATTC
44	CTGAGCCA	TGGCTCAG	CAAGCAGAAGACGGCATACGAGATCTGAGCCAGAGATCGGTCTCGGCATTC
45	CTGGCATA	TATGCCAG	CAAGCAGAAGACGGCATACGAGATCTGGCATAGAGATCGGTCTCGGCATTC
46	GAATCTGA	TCAGATTC	CAAGCAGAAGACGGCATACGAGATGAATCTGAGAGATCGGTCTCGGCATTC
47	GACTAGTA	TACTAGTC	CAAGCAGAAGACGGCATACGAGATGACTAGTAGAGATCGGTCTCGGCATTC
48	GAGCTGAA	TTCAGCTC	CAAGCAGAAGACGGCATACGAGATGAGCTGAAGAGATCGGTCTCGGCATTC
49	GATAGACA	TGTCTATC	CAAGCAGAAGACGGCATACGAGATGATAGACAGAGATCGGTCTCGGCATTC
50	GCCACATA	TATGTGGC	CAAGCAGAAGACGGCATACGAGATGCCACATAGAGATCGGTCTCGGCATTC
51	GCGAGTAA	TTACTCGC	CAAGCAGAAGACGGCATACGAGATGCGAGTAAGAGATCGGTCTCGGCATTC
52	GCTAACGA	TCGTTAGC	CAAGCAGAAGACGGCATACGAGATGCTAACGAGAGATCGGTCTCGGCATTC
53	GCTCGGTA	TACCGAGC	CAAGCAGAAGACGGCATACGAGATGCTCGGTAGAGATCGGTCTCGGCATTC
54	GGAGAACA	TGTTCTCC	CAAGCAGAAGACGGCATACGAGATGGAGAACAGAGATCGGTCTCGGCATTC
55	GGTGCGAA	TTCGCACC	CAAGCAGAAGACGGCATACGAGATGGTGCGAAGAGATCGGTCTCGGCATTC

Tag number	Barcode sequence	Single correcting, double & shift detecting octamers	PCR primers
56	GTACGCAA	TTGCGTAC	CAAGCAGAAGACGGCATACGAGATGTACGCAAGAGATCGGTCTCGGCATTC
57	GTCGTAGA	TCTACGAC	CAAGCAGAAGACGGCATACGAGATGTCGTAGAGAGATCGGTCTCGGCATTC
58	GTCTGTCA	TGACAGAC	CAAGCAGAAGACGGCATACGAGATGTCTGTCAGAGATCGGTCTCGGCATTC
59	GTGTTCTA	TAGAACAC	CAAGCAGAAGACGGCATACGAGATGTGTTCTAGAGATCGGTCTCGGCATTC
60	TAGGATGA	TCATCCTA	CAAGCAGAAGACGGCATACGAGATTAGGATGAGAGATCGGTCTCGGCATTC
61	TATCAGCA	TGCTGATA	CAAGCAGAAGACGGCATACGAGATTATCAGCAGAGATCGGTCTCGGCATTC
62	TCCGTCTA	TAGACGGA	CAAGCAGAAGACGGCATACGAGATTCCGTCTAGAGATCGGTCTCGGCATTC
63	TCTTCACA	TGTGAAGA	CAAGCAGAAGACGGCATACGAGATTCTTCACAGAGATCGGTCTCGGCATTC
64	TGAAGAGA	TCTCTTCA	CAAGCAGAAGACGGCATACGAGATTGAAGAGAGAGATCGGTCTCGGCATTC
65	TGGAACAA	TTGTTCCA	CAAGCAGAAGACGGCATACGAGATTGGAACAAGAGATCGGTCTCGGCATTC
66	TGGCTTCA	TGAAGCCA	CAAGCAGAAGACGGCATACGAGATTGGCTTCAGAGATCGGTCTCGGCATTC
67	TGGTGGTA	TACCACCA	CAAGCAGAAGACGGCATACGAGATTGGTGGTAGAGATCGGTCTCGGCATTC
68	TTCACGCA	TGCGTGAA	CAAGCAGAAGACGGCATACGAGATTTCACGCAGAGATCGGTCTCGGCATTC
69	AACTCACC	GGTGAGTT	CAAGCAGAAGACGGCATACGAGATAACTCACCGAGATCGGTCTCGGCATTC
70	AAGAGATC	GATCTCTT	CAAGCAGAAGACGGCATACGAGATAAGAGATCGAGATCGGTCTCGGCATTC
71	AAGGACAC	GTGTCCTT	CAAGCAGAAGACGGCATACGAGATAAGGACACGAGATCGGTCTCGGCATTC
72	AATCCGTC	GACGGATT	CAAGCAGAAGACGGCATACGAGATAATCCGTCGAGATCGGTCTCGGCATTC
73	AATGTTGC	GCAACATT	CAAGCAGAAGACGGCATACGAGATAATGTTGCGAGATCGGTCTCGGCATTC
74	ACACGACC	GGTCGTGT	CAAGCAGAAGACGGCATACGAGATACACGACCGAGATCGGTCTCGGCATTC
75	ACAGATTC	GAATCTGT	CAAGCAGAAGACGGCATACGAGATACAGATTCGAGATCGGTCTCGGCATTC
76	AGATGTAC	GTACATCT	CAAGCAGAAGACGGCATACGAGATAGATGTACGAGATCGGTCTCGGCATTC
77	AGCACCTC	GAGGTGCT	CAAGCAGAAGACGGCATACGAGATAGCACCTCGAGATCGGTCTCGGCATTC
78	AGCCATGC	GCATGGCT	CAAGCAGAAGACGGCATACGAGATAGCCATGCGAGATCGGTCTCGGCATTC
79	AGGCTAAC	GTTAGCCT	CAAGCAGAAGACGGCATACGAGATAGGCTAACGAGATCGGTCTCGGCATTC
80	ATAGCGAC	GTCGCTAT	CAAGCAGAAGACGGCATACGAGATATAGCGACGAGATCGGTCTCGGCATTC
81	ATCATTCC	GGAATGAT	CAAGCAGAAGACGGCATACGAGATATCATTCCGAGATCGGTCTCGGCATTC
82	ATTGGCTC	GAGCCAAT	CAAGCAGAAGACGGCATACGAGATATTGGCTCGAGATCGGTCTCGGCATTC
83	CAAGGAGC	GCTCCTTG	CAAGCAGAAGACGGCATACGAGATCAAGGAGCGAGATCGGTCTCGGCATTC
84	CACCTTAC	GTAAGGTG	CAAGCAGAAGACGGCATACGAGATCACCTTACGAGATCGGTCTCGGCATTC
85	CCATCCTC	GAGGATGG	CAAGCAGAAGACGGCATACGAGATCCATCCTCGAGATCGGTCTCGGCATTC

Tag number	Barcode sequence	Single correcting, double & shift detecting octamers	PCR primers
86	CCGACAAC	GTTGTCGG	CAAGCAGAAGACGGCATACGAGATCCGACAACGAGATCGGTCTCGGCATTC
87	CCTAATCC	GGATTAGG	CAAGCAGAAGACGGCATACGAGATCCTAATCCGAGATCGGTCTCGGCATTC
88	CCTCTATC	GATAGAGG	CAAGCAGAAGACGGCATACGAGATCCTCTATCGAGATCGGTCTCGGCATTC
89	CGACACAC	GTGTGTCG	CAAGCAGAAGACGGCATACGAGATCGACACACGAGATCGGTCTCGGCATTC
90	CGGATTGC	GCAATCCG	CAAGCAGAAGACGGCATACGAGATCGGATTGCGAGATCGGTCTCGGCATTC
91	CTAAGGTC	GACCTTAG	CAAGCAGAAGACGGCATACGAGATCTAAGGTCGAGATCGGTCTCGGCATTC
92	GAACAGGC	GCCTGTTC	CAAGCAGAAGACGGCATACGAGATGAACAGGCGAGATCGGTCTCGGCATTC
93	GACAGTGC	GCACTGTC	CAAGCAGAAGACGGCATACGAGATGACAGTGCGAGATCGGTCTCGGCATTC
94	GAGTTAGC	GCTAACTC	CAAGCAGAAGACGGCATACGAGATGAGTTAGCGAGATCGGTCTCGGCATTC
95	GATGAATC	GATTCATC	CAAGCAGAAGACGGCATACGAGATGATGAATCGAGATCGGTCTCGGCATTC
96	GCCAAGAC	GTCTTGGC	CAAGCAGAAGACGGCATACGAGATGCCAAGACGAGATCGGTCTCGGCATTC

Supplementary Protocol 1

PCR and 96-well library prep standard operating procedure

Adapter preparation

Custom adapters are required. These should be HPLC purified. Adapters are phosphorylated and annealed together, as described [1].

Ind_t 5' ACACTCTTTCCCTACACGACGCTCTTCCGATC*T 3'
Ind_b 5' GATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTC 3'

* indicates phosphorothioate

Sample shearing

1. Take 1 µg of pooled long PCR products and make volume up to 75 µl with water.

2. Shear to approximately 200 bp by acoustic shearing: transfer to a 6 mm × 16 mm AFA fiber vial (Covaris cat. no. 520031).

3. Seal the tube with an 8 mm crimp seal cap (Covaris cat no. 520028) and crimping tool.

4. Shear with a Covaris, using the settings:

Duty cycle	20 %
Intensity	5
Cycle/burst	200
Time	150 sec

5. Transfer samples to 96-well PCR plate, gently spin and keep frozen until used.

End-repair

This protocol converts the overhangs resulting from fragmentation into blunt ends, using T4 DNA polymerase and E. coli DNA polymerase I Klenow fragment. The 3' to 5' exonuclease activity of these enzymes removes 3' overhangs and the polymerase activity fills in the 5' overhangs.

1. Mix the reagents below (all NEB) in a 15ml Falcon Tube, and decant into a reagent reservoir.

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	1X	104X
10x T4 DNA ligase buffer with 10 mM ATP	10 µl	1040 µl
10 µM dNTP mix	4 µl	416 µl
3 U / µl T4 DNA polymerase	5 µl	520 µl
5 U / µl Klenow DNA polymerase	1 µl	104 µl
10 U / µl T4 PNK	5 µl	520 µl

2. Add 25 µl of master mix to each sample using an electronic pipette. Cover the plate with transparent cover, vortex briefly and gently spin down.

3. Incubate plate for 30 min at 20 °C in a thermocycler.

4. While incubating, prepare SPRI beads for the reaction cleanup: allow SPRI beads to come to room temperature for at least 30 minutes. Mix well, and ensure that the beads appear homogeneous and consistent in colour.

5. Add 180 µl of SPRI beads per 100 µl of end-repaired DNA in a 1.5 ml Lo-Bind tube.

6. Vortex and leave at room temperature for 5 minutes.

7. Place tubes in a magnetic rack.

8. Leave for 5 minutes or until sample is clear.

9. Carefully remove the clear solution from the tubes and discard.

10. Dispense 700 µl of 70 % ethanol into each tube while in the magnetic rack taking care not to disturb the magnetic beads. Aspirate and discard ethanol.

11. Repeat the ethanol wash once again (total of two washes).

12. Dry the samples on a heat block (keep the lid of the tube open) at 37 °C for 5 to 10 minutes or until the residual ethanol has evaporated.

13. Add 32 µl of molecular biology grade water, vortex and incubate at room temperature for 2 minutes.

14. Place tubes into the magnetic rack and leave for 2-3 minutes or until sample is clear.

15. Carefully remove the water and retain in a new 1.5 ml Lo-Bind tube.

16. Centrifuge the eluates at 13,000 rpm in a bench top centrifuge for 10 minutes.

17. Transfer eluates to a 96-well plate leaving behind any precipitated beads.

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18. Proceed immediately with A-tailing.

A-tailing

This protocol adds an 'A' base to the 3' end of the blunt phosphorylated DNA fragments, using the polymerase activity of Klenow fragment (3' to 5' exo minus). This prepares the DNA fragments for ligation to the adapters, which have a single 'T' base overhang at their 3' end.

1. Mix the reagents below (all NEB) in a 15ml Falcon Tube, and decant into a reagent reservoir.

	1X	104X
10x NEB buffer 2	5 µl	520 µl
1 mM dATP	10 µl	1040 µl
5 U / µl Klenow fragment (3' to 5' exo^{-})	3 µl	312 µl

2. Add 18µl to each sample using an electronic pipette. Cover the plate with transparent cover, vortex and gently spin.

3. Incubate plate for 30 min at 37 °C in a thermocycler. Clean using SPRI beads, in the 96-well reaction plate (see End-Repair protocol above. Use 90 µl beads for each 50µl reaction, and elute in a mixture of 10 µl EB + 8.5 µl water).

4. Proceed immediately with ligation.

Ligation

This protocol ligates adapters to the ends of the DNA fragments.

1. Mix the reagents below in a 15ml Falcon Tube, and decant into a reagent reservoir.

	1X	104X
2x Quick DNA ligase buffer (NEB)	25 µl	2.6 ml
Adapter oligo mix (see above)	1.5 µl	156 µl

2. Add 26.5 µl to each sample using an electronic pipette. Cover the plate with transparent cover, vortex and gently spin.

3. Pipette 5 µl Quick Ligase (NEB) into each well using a manual pipette. Mix by pipetting, do not cover, and after pipetting in the last column leave for 15 min at room temperature (20 °C).

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4. Clean using AMPure SPRI beads as described above, eluting in 20 µl EB.

5. Run an DNA 1000 chip on an Agilent BioAnalyzer 2100 on a small selection of samples. You should detect smear between 300-1000 bp.

Indexing Enrichment PCR

Indexes can be added to the central region of the reverse PCR primer. The general sequence is:

5’ CAAGCAGAAGACGGCATACGAGAT-INDEX-GAGATCGGTCTCGGCATTC 3’

where INDEX represents an oligonucleotide sequence that is used to identify the sample. Indexes should be selected so that they are maximally different from one another. We typically use 8-base indexes. Indexed primers must be PAGE purified. For an example of a 96-plex set of error-correcting barcodes, and primer sequences, see **Supplementary Table 1**.

The common forward primer (HPLC purified) has the sequence:

5’ AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT 3’

1. Dilute indexed primers to 10 µM with water.

2. Pipette 2 µl of each library into a well of a 96-well plate.

3. Add 6 µl of 10 µM indexed primer to each well.

4. Prepare a master mix of all other reagents:

	1X	52X
10X PFx buffer (Invitrogen)	10µl	520µl
2.5 mM dNTPs	20µl	1040µl
50 mM $MgSO_4$	4µl	208µl
10 µM Forward primer	6µl	312µl
Platinum PFx Polymerase (Invitrogen)	1µl	52µl
Water	51µl	2652µl

5. Mix, and add 92 µl master mix to each well. The PCR is performed using the following program :

   94 °C for 2 min

   94 °C for 15 sec

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68 °C for 45 sec x 12 cycles

4°C indefinitely

6. Clean PCR reactions SPRI beads, as described above, eluting in 18 µl EB.

7. Run an DNA 1000 chip on an Agilent BioAnalyzer 2100 on a small selection of samples. You should detect smear between 300-1000 bp.

Normalization

Normalize PCR products by qPCR before pooling. The primers used for the qPCR are locus-specific, designed to amplify one of the long PCR products in the original pool. In this way, adapter dimers are not problematic. As a concentration standard, the most concentrated library from the 6 random libraries quantified by Agilent Bioanalyzer 2100 in the preceding step, is used.

1. Dilute 2µl of the chosen concentration standard 10x, 100x and 1,000x with EB buffer.

2. Dilute all other libraries 80x in EB.

3. Perform qPCRs in duplicate, using a 2x SybrGreen master mix.

4. Pool products in equimolar ratios.

Size selection

1. Load sample pools on a 2% agarose gel in 1X TBE, and are electrophoresed against a Low Molecular Weight ladder (NEB), at 5V cm^{-1} for approximately 1 hour.

2. From each lane, cut a 250-450 bp gel slice is cut and extract the DNA.

3. Quantify pooled libraries by SYBRGreen qPCR [2].

4. Sequence using standard primers for reads 1 and 2. Indexes are sequenced after read 1, using a custom primer.

Ind_seq 5’ AAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTC 3’

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References

1. Kozarewa I. *et al.* Amplification-free Illumina sequencing-library preparation facilitates improved mapping and assembly of (G+C)-biased genomes. *Nat Methods* **6**, 291-295 (2009).

2. Quail M.A., Swerdlow H. & Turner D.J. Improved protocols for the illumina genome analyzer sequencing system. *Curr Protoc Hum Genet* **Chapter 18**, Unit 18 12 (2009).

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Supplementary Protocol 2

Molecular Inversion Probe standard operating procedure

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.

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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.

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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)	Final

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	per sample	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

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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

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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	AGGACCGGATCAACTxxxxxxxxxxxxxxxxxxxxCTTCAGCTTCCCGATA TCCGACGGTAGTGTyyyyyyyyyyyyyyyyyyyyCATTGCGTGAACCGA
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

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Supplementary Protocol 3

Hybrid capture library prep standard operating procedure (array OR solution capture)

Shearing samples to 100-300bp

For array capture, dilute 20 µg genomic DNA to a total volume of 100 µl with water.
For solution capture, dilute 3 µg genomic DNA to a total volume of 100 µl with water.

1. Mix and transfer to a 6mmx16mm AFA fibre vial (Covaris cat. no. 520031).

2. Seal the tube using an 8mm metal crimp seal cap (Covaris, cat no. 520028) and crimping tool.

3. Shear with a Covaris, using the settings:

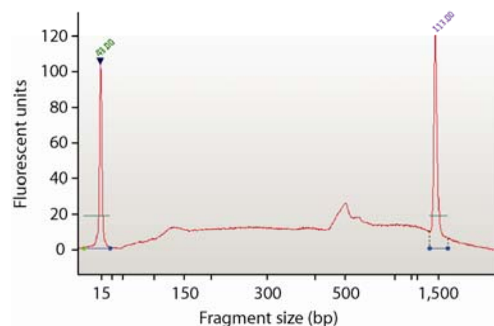
Duty cycle	20 %
Intensity	5
Cycle/burst	200
Time	120 sec

4. Remove the sample from the machine. Open the vial and transfer the sample into a fresh 1.5 ml Eppendorf lo-bind tube. Keep samples on ice.

5. Run 1 µl on an Agilent Bioanalyzer 2100 chip to check the quantity of fragmented DNA and to confirm the success of the fragmentation.

Impure DNA may shear badly. If there is any doubt about the purity of the sample, perform an ethanol precipitation before shearing (Figure 1).

a) without ethanol precipitation



b) with ethanol precipitation

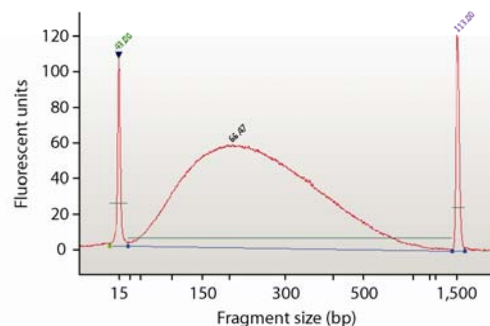


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Figure 1: Impure DNA sample sheared using identical Covaris settings a) before and b) after ethanol precipitation.

Purification after Fragmentation

Use one column for each 10 µg of DNA. For 10-20 µg DNA, divide equally between two columns. Elute with 45 µl EB per 5 µg bound DNA.

1. Add 5 x volume of Buffer PB to the fragmented DNA (for 100 µl of fragmented DNA add 500 µl buffer). Vortex.

2. Pipette 750 µl of mix per column. Pipette slowly and make sure that all the liquid comes out of the pipette tip. Centrifuge the samples for 1 minute at 13,000 rpm in a benchtop centrifuge.

3. Discard the flow-through from the collection tube. Repeat step 2 if you have any buffer mix left.

4. To wash, add 750 µl of Buffer PE to each of the columns. Centrifuge for 1 minute as above. Discard the flow-through and centrifuge again for a further minute.

5. Leave the tubes in a rack with the lids open to dry for 2 minutes.

6. Transfer the column to a clean labelled 2 ml lo-bind Eppendorf tube. For each 5 µg of bound DNA, add 47 µl of EB buffer to the centre of the column and leave for 1 minute. Centrifuge for 1 minute as above.

7. Combine eluates, if applicable. You should have a volume of 45 µl for every 5 µg in the original sample (approximately 2 µl of buffer is retained by the column).

End Repair

This protocol converts the overhangs resulting from fragmentation into blunt ends, using T4 DNA polymerase and E. coli DNA polymerase I Klenow fragment. The 3' to 5' exonuclease activity of these enzymes removes 3' overhangs and the polymerase activity fills in the 5' overhangs.

1. Prepare a master mix containing the following reaction mix per 5 µg sample, plus a 10 % excess (round the mass of sample UP to the nearest 5 µg):

   Water 30 µl

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10x T4 DNA ligase buffer with 10mM ATP	10 µl
10mM dNTP mix	4 µl
3U/ µl T4 DNA polymerase	5 µl
5U/ µl Klenow DNA polymerase	1 µl
10U/ µl T4 PNK	5 µl

2. Mix, and aliquot 55 µl of master mix into each sample tube containing the 45 µl of eluate from the previous step. Mix well and spin down.

3. Incubate for 30 minutes at room temperature (20-25 °C).

4. Clean up using one QIAquick PCR column for up to 10 µg of DNA, as described above. Elute in 34 µl EB per 5 µg DNA. This gives ~32 µl of eluate, because approximately 2 µl of buffer is retained by the column.

Addition of 'A' Bases to the 3' End of the DNA Fragments

This protocol adds an 'A' base to the 3' end of the blunt phosphorylated DNA fragments, using the polymerase activity of Klenow fragment (3' to 5' exo minus). This prepares the DNA fragments for ligation to the adapters, which have a single 'T' base overhang at their 3' end.

1. Prepare a master mix containing the following reaction mix per 5 µg sample, plus a 10 % excess (round the mass of sample UP to the nearest 5 µg):

10x Klenow buffer	5 µl
1 mM dATP	10 µl

2. Mix, and aliquot 15 µl of master mix into each sample tube containing the 32 µl of end-repaired sample. Mix well and spin down.

3. Add 3 µl 5 U/ µl Klenow exo (3' to 5' exo minus). Mix and spin down.

4. Incubate for 30 minutes at 37 °C in a hot block.

5. Clean up using one QIAquick MinElute columns per 5 µg of DNA, eluting in 12 µl of EB buffer per column, in a 1.5 ml lo-bind Eppendorf tube. This gives ~10 µl of eluate, because approximately 2 µl of buffer is retained by the column.

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Ligation of Adapters to DNA Fragments

This protocol ligates adapters to the ends of the DNA fragments. The procedure uses a 10:1 molar ratio of adapter to DNA insert, based on a starting quantity of 5 µg of DNA before fragmentation. The quantities below are given per 5 µg of DNA. Adjust as appropriate.

1. Prepare a master mix containing the following reaction mix per 5 µg sample, plus a 10 % excess (round the mass of sample UP to the nearest 5 µg):

2 x DNA ligase buffer	25 µl
Illumina PE Adapter oligo mix	10 µl

2. Mix and aliquot 30 µl master mix into each sample tube containing the 10 µl of A-tailed sample. Mix and spin down.

3. Add 10 µl 2,000 U/ µl T4 DNA ligase. Mix and spin down.

4. Incubate for 15 minutes at room temperature (20-25 °C).

5. Clean ligated samples and C_0t1 DNA with SPRI beads, eluting in 50 µl water. Use a 5 x excess of C0t1 for array capture and a 20 x excess for solution capture.

Alternatively, if doing a pre-hyb PCR (see below), clean up using a QIAquick PCR column as described above, eluting in 50 µl EB.

SPRI bead cleanup

Allow SPRI beads to come to room temperature for at least 30 minutes. Reagents need to be mixed well prior to use and should appear homogeneous and consistent in colour.

1. Add 90 µl of SPRI beads per 50 µl of adapter ligated sample in a 1.5 ml Lo-bind Eppendorf tube.

2. Vortex and leave at room temperature for 5 minutes.

3. Place tubes in a magnetic rack.

4. Leave for 5 minutes or until sample is clear.

5. Carefully remove the clear solution from the tubes and discard.

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6. Dispense 700 µl of 70 % ethanol into each tube while in the magnetic rack taking care not to disturb the magnetic beads. Aspirate and discard ethanol.

7. Repeat the ethanol wash once again (total of two washes).

8. Dry the samples on a heat block (keep the lid of the tube open) at 37 °C for 5 to 10 minutes or until the residual ethanol has evaporated.

9. Add 50 µl of molecular biology grade water, vortex and incubate at room temperature for 2 minutes.

10. Place tubes into the magnetic rack and leave for 2-3 minutes or until sample is clear.

11. Carefully remove the water and retain in a new 1.5 ml lo-bind Eppendorf tube.

12. Repeat step 9-12 once more, retaining the water in the same 1.5 ml lo-bind tube. Total volume of elute should be 100 µl.

13. Centrifuge the eluate at 13,000 rpm in a bench top centrifuge for 10 minutes

14. Transfer the sample to a new 1.5 ml lo-bind Eppendorf tube leaving behind any precipitated beads.

15. Quantify 1 µl of the library using an Agilent DNA 1000 chip on a Bioanalyzer 2100 and proceed to hyb, following the manufacturer's recommended protocols.

Optional step: pre-hyb PCR

Performing a small number of PCR cycles before hybridisation can improve robustness, particularly for clinical samples, and will simplify sample indexing. Amplify each 50 µl adapter-ligated library by dividing between 4 PCR reactions.

1. Prepare a master mix containing the following reaction mix per sample, plus a 10 % excess:

10 µM PE2.1	10 µl
10 µM PEV2.2	10 µl
2 x Phusion HF master mix	100 µl
water	30 µl

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2. Mix and aliquot 150 µl of master mix into each 50 µl adapter-ligated library. Mix and spin down.

3. Aliquot into four 200 µl PCR tubes (50 µl each), and perform the following temperature cycling:

98 °C	2 minutes	
98 °C	20 seconds	
65 °C	30 seconds	x 6 cycles
72 °C	30 seconds	
72 °C	5 minutes	
4 °C	indefinitely	

4. Combine all 4 reactions and clean up with SPRI beads (see above), adding 360 µl beads to the 200 µl PCR reactions, and eluting in 50 µl water.

Quantify 1 µl of library using an Agilent DNA 1000 chip on a Bioanalyzer 2100 and proceed to hybridization (Supplementary Protocol 4 or 5).

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Supplementary Protocol 4

Array capture standard operating procedure

This protocol is for hybridization of adapter-ligated or PCR-amplified library DNA, so must be performed after Hybrid Capture Protocol 1.

Sample preparation

Following SPRI bead cleanup or pre-hyb PCR (see Hybrid Capture Protocol 1), lyophilize libraries using a SpeedVac.

1. Add 5.4 µl of molecular biology grade water to each sample to rehydrate.
2. Vortex and centrifuge.
3. Place sample in 70 °C heat block for 10 minutes.
4. Vortex and spin down.
5. To each sample, add:

2 x Hybridization Buffer (Nimblegen)	9.0 µl
Hybridization Component A	3.6 µl

6. Vortex and spin down.
7. Place each sample in a 95 °C heat block for 10 minutes.
8. Spin down.
9. Store at 42 °C (in hyb station tube slots) until ready for hybridization.

Prepare slides and mixers

1. Remove the mixer from the packaging (must be used within 30 minutes of opening). Blow compressed gas across the mixer and slide.

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2. Position the precision mixer alignment tool (PMAT) so that the hinge is on the left and then open it.

3. Snap the mixer onto the two alignment pins on the lid of the PMAT, with the tab end of the mixer towards the inside hinge and the mixer's adhesive gasket exposed.

4. While pushing back the plastic spring with a thumb, place the slide in the base of the PMAT so that the barcode is on the right and the corner of the slide sits against the plastic spring. Remove your thumb and make sure the spring is engaging the corner of the slide and the entire slide is registered to the edge of the PMAT closest to you. Gently blow compressed gas across the mixer and slide.

5. Using forceps remove the backing from the adhesive gasket and close the lid of the PMAT so that the gasket makes contact with the slide. Lift the lid while applying pressure through the hole in the lid of the PMAT to free the mixer-slide assembly from the pins.

6. Remove the mixer-slide assembly from the PMAT. Place the mixer-slide assembly on a smooth, dark flat surface.

NOTE: *You can place the mixer-slide assembly on the back of a 42 °C heating block to facilitate adhesion of the mixer to the slid.*

7. Rub the Mixer Brayer over the mixer just hard enough to adhere the adhesive gasket and remove any bubbles. Start in the centre of the array and rub outwards. The adhesive gasket will become clear when fully adhered to both surfaces.

NOTE: *Mark the sample area on the other side of the working surface on the glass slide!*

8. Place the mixer-slide assembly in the slide bay of the Hybridization System. Make sure the assembly is seated completely within the bay.

Load and hybridize samples

NOTE: *Before loading samples onto the hyb station ensure that the array area is marked on the back of the slide.*

1. Using a Gilson Microman M100 pipette, slowly dispense the sample into the fill port. Dispense slowly to avoid introducing bubbles.

2. Tap the slide to remove any bubbles, dry any sample leaking from the ports with a clean tissue.

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3. Adhere a seal tab over each fill and vent port on the mixer and rub the seal gently with the blunt end of the forceps to ensure a tight seal.

4. Close the bay clamp.

5. Turn the MAUI hyb station on/off with the mixing on/off switch. The 2 green mix mode lights on the front panel will flash for 5 seconds. Press the B mix mode switch to select the B mix mode while the light is flashing. The hyb system will recognise the slide in each occupied bay (the red indicator light will change to green if occupied).

6. Hybridize the sample to the array for 72 hours at 42 °C.

Washing arrays after hybridisation

Preparing the Wash Solutions

1. Dilute the concentrated wash solutions from the wash buffer kit. **NB:** Shown below are the quantities needed for 1 array. Volumes should be adjusted for the number of arrays to be processed. Wash buffers I, II & III come in 10X concentrated form and the stringent buffer in 2X concentrated form.

Wash Solution	Volume required (of diluted)
Wash buffer I	32 ml
Wash buffer II	164 ml
Wash buffer III	32 ml
Stringent buffer	64 ml

Preparation of the Elution and Clean-up Solutions

Method note: The 125 mM sodium hydroxide (NaOH) and 20 % acetic acid should both be prepared freshly prior to using them in the elution and clean up. Molecular biology grade water (H_2O) should be used for preparing the solutions.

2. Prepare 125 mM sodium hydroxide as detailed below. **NB:** The amounts below are for processing 1 array. The volumes must be adjusted to reflect the number of arrays being processed.

Solution	Volume H_2O	Volume 10 M NaOH

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125 mM NaOH	987.5 µl	12.5 µl

3. Prepare 20 % acetic acid as detailed below. **NB:** The amounts below are for processing 1 array. The volumes must be adjusted to reflect the number of arrays being processed.

Solution	Volume H_2O	Volume glacial acetic acid
20 % acetic acid	400.0 µl	100.0 µl

4. Open a QIAquickPCR purification kit and take out the buffers to prepare them for use, see below:

Buffer	Prepare by
PE	Adding 24 ml of 100 % molecular biology grade ethanol
PB	Adding 120 µl pH indicator I to the 30 ml bottle of PB buffer
EB	Requires no further preparation

Washing the Arrays

1. Ensure that there are the wash solutions as detailed below are prepared, aliquoted into falcon tubes where necessary and preheated if needed before starting the washing procedure.

NB: These quantities are for one array and must be adjusted to reflect the number of arrays to be processed.

Number of aliquots	Aliquot volume (ml)	Wash solution	Container	Temperature (°C)
1	100	Wash buffer II	Ready for reservoir	42
2	32	Wash buffer II	Labeled 50ml falcon tube	20
2	32	Stringent buffer	Labeled 50ml falcon tube	47.5
1	32	Wash buffer I	Labeled 50ml falcon tube	20
1	32	Wash buffer III	Labeled 50ml falcon tube	20

2. Label a new disposable reservoir with the sample number for each of the arrays to be washed. Remove the array from the hybridisation station and place into the reservoir. Cover the array with the 100 ml of preheated wash buffer II (see below).

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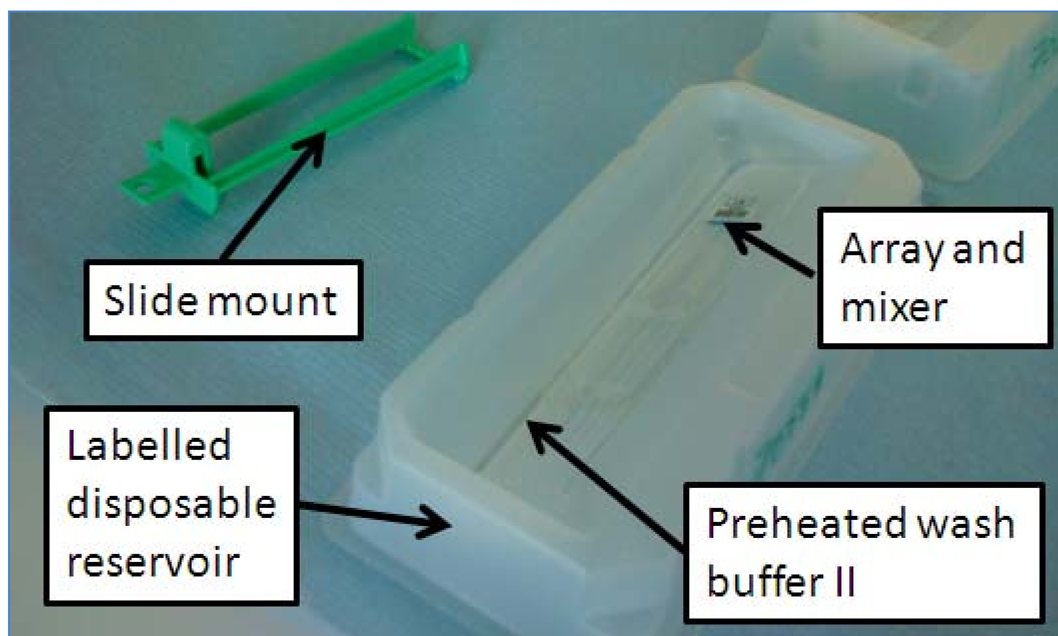
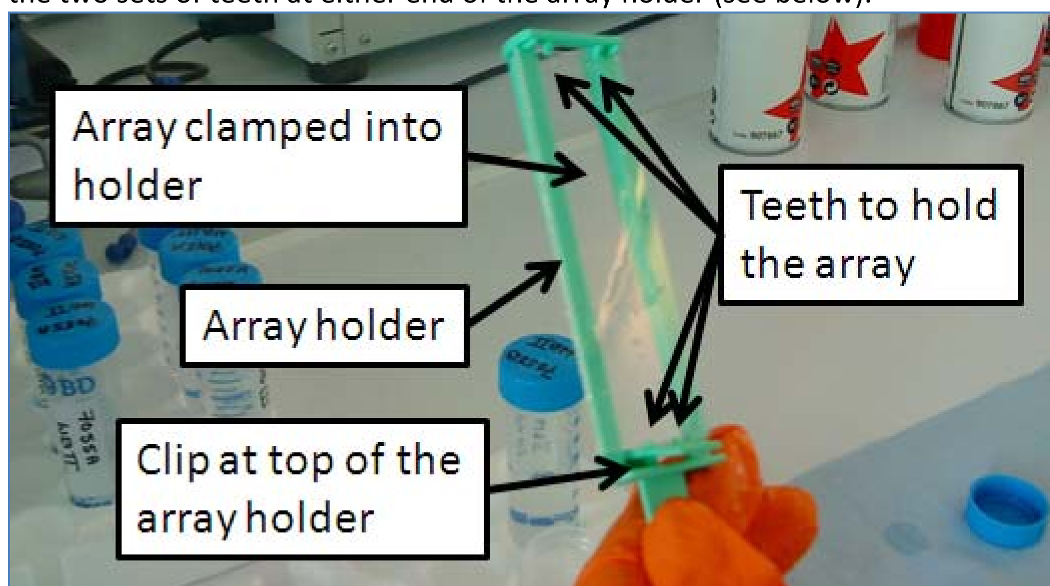


3. Carefully peel off the mixer from the array taking care not to touch the area of the array under the mixer. The mixer may now be discarded.

4. Holding the array sides only, remove the array from the reservoir and clip it into an array holder by pressing down on the top clip of the holder. The array must go under the two sets of teeth at either end of the array holder (see below).



5. Place the array-holder assembly into a labelled 50 ml Falcon tube containing room temperature wash buffer II (32.0 ml). Ensure that the array holder is orientated with the clip at the top of the tube (see below). Replace the lid of the Falcon tube.

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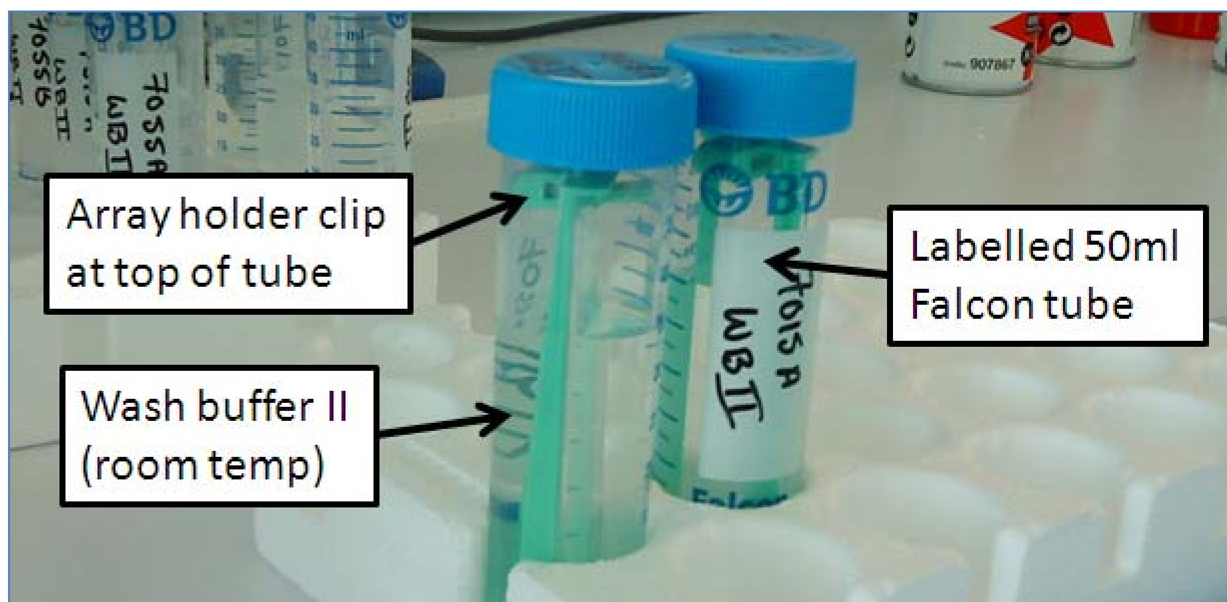

6. Invert the Falcon tube containing the array-holder assembly ten times pausing for 1 second after each inversion before inverting again.

7. Remove the array-holder assembly from the Falcon tube and place into a new labelled 50 ml Falcon tube containing preheated stringent buffer (32.0 ml at 47.5 ^{o}C). Replace the lid of the Falcon tube.

8. Invert the Falcon tube containing the array-holder assembly 10 times pausing for 1 second after each inversion before inverting again. When 10 inversions have been completed place the falcon tube with the array-holder assembly into a water bath at 42.0 ^{o}C for 5 minutes.

9. Following the incubation in the water bath invert the Falcon tube containing the array-holder assembly a further ten times pausing for 1 second after each inversion before inverting again.

10. Remove the array-holder assembly from the Falcon tube and place into a new labelled 50 ml Falcon tube containing a second aliquot of preheated stringent buffer (32.0 ml at 47.5 ^{o}C). Replace the lid of the Falcon tube.

11. Invert the Falcon tube containing the array-holder assembly 10 times pausing for 1 second after each inversion before inverting again. When 10 inversions have been completed place the falcon tube with the array-holder assembly into a water bath at 42.0 ^{o}C for 5 minutes.

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12. Following the incubation in the water bath invert the Falcon tube containing the array-holder assembly a further ten times pausing for 1 second after each inversion before inverting again.

13. Remove the array-holder assembly from the Falcon tube and place into a new labelled 50 ml Falcon tube containing preheated wash buffer I (32.0 ml). Replace the lid of the Falcon tube.

14. Invert the Falcon tube containing the array-holder assembly for 2 minutes pausing for 1 second after each inversion before inverting again.

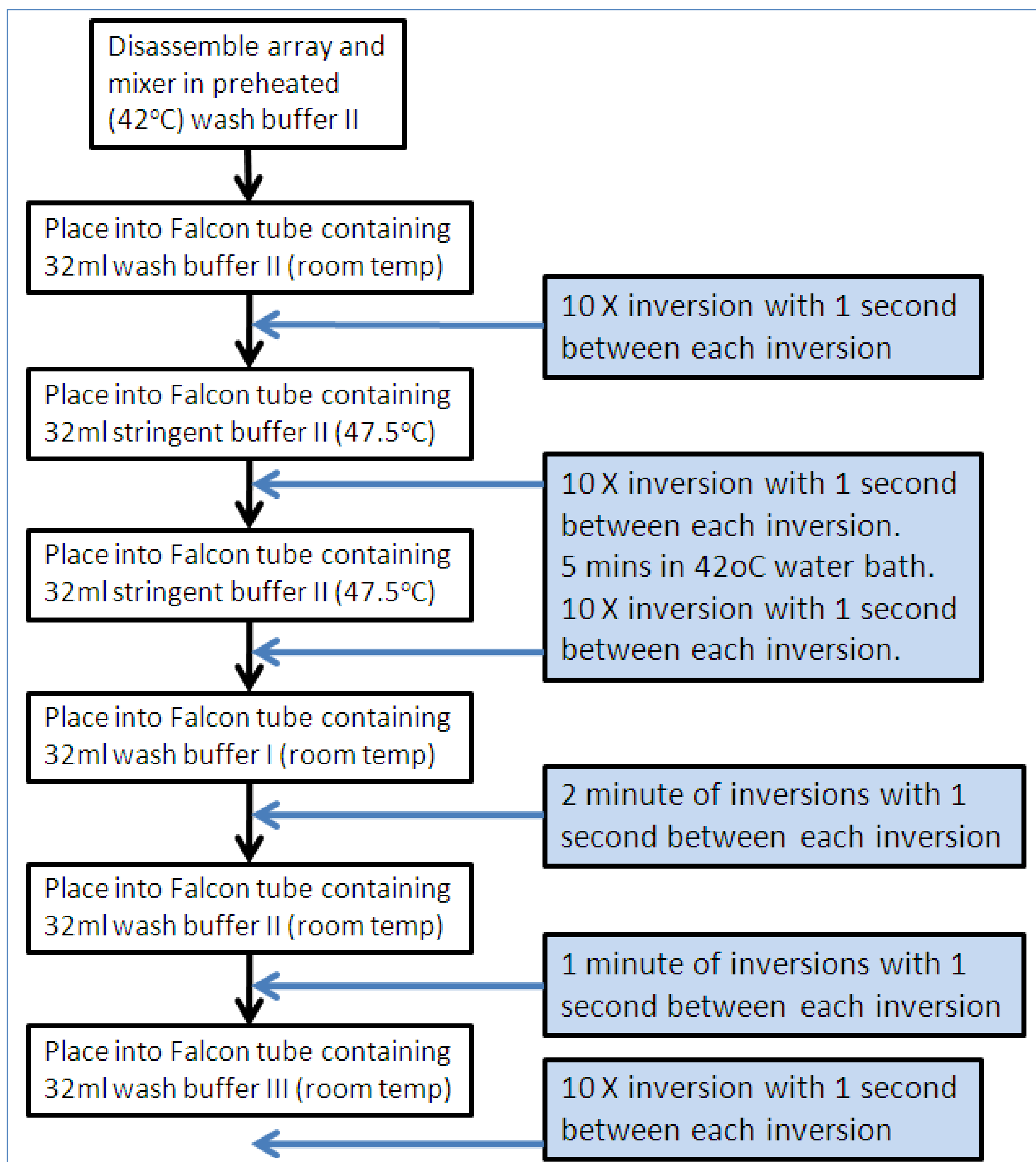
15. Remove the array-holder assembly from the Falcon tube and place into a new labelled 50 ml Falcon tube containing preheated wash buffer II (32.0 ml). Replace the lid of the Falcon tube.

16. Invert the Falcon tube containing the array-holder assembly for 1 minute pausing for 1 second after each inversion before inverting again.

17. Remove the array-holder assembly from the Falcon tube and place into a new labelled 50 ml Falcon tube containing preheated wash buffer III (32.0 ml). Replace the lid of the Falcon tube.

18. Invert the Falcon tube containing the array-holder assembly ten times pausing for 1 second after each inversion before inverting again (see below for flow diagram of washes).

Supplementary Protocol 4:
Array capture standard operating procedure



19. The array is now ready for the elution process.

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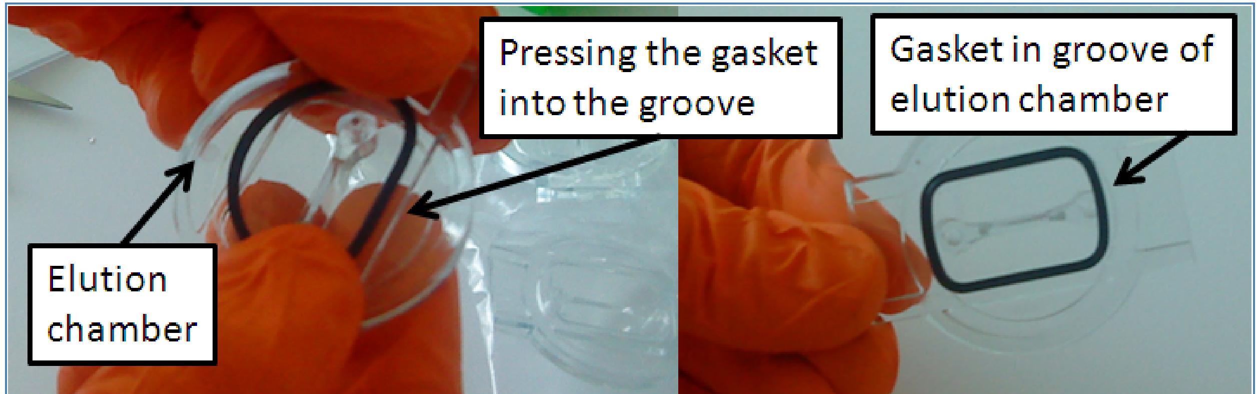
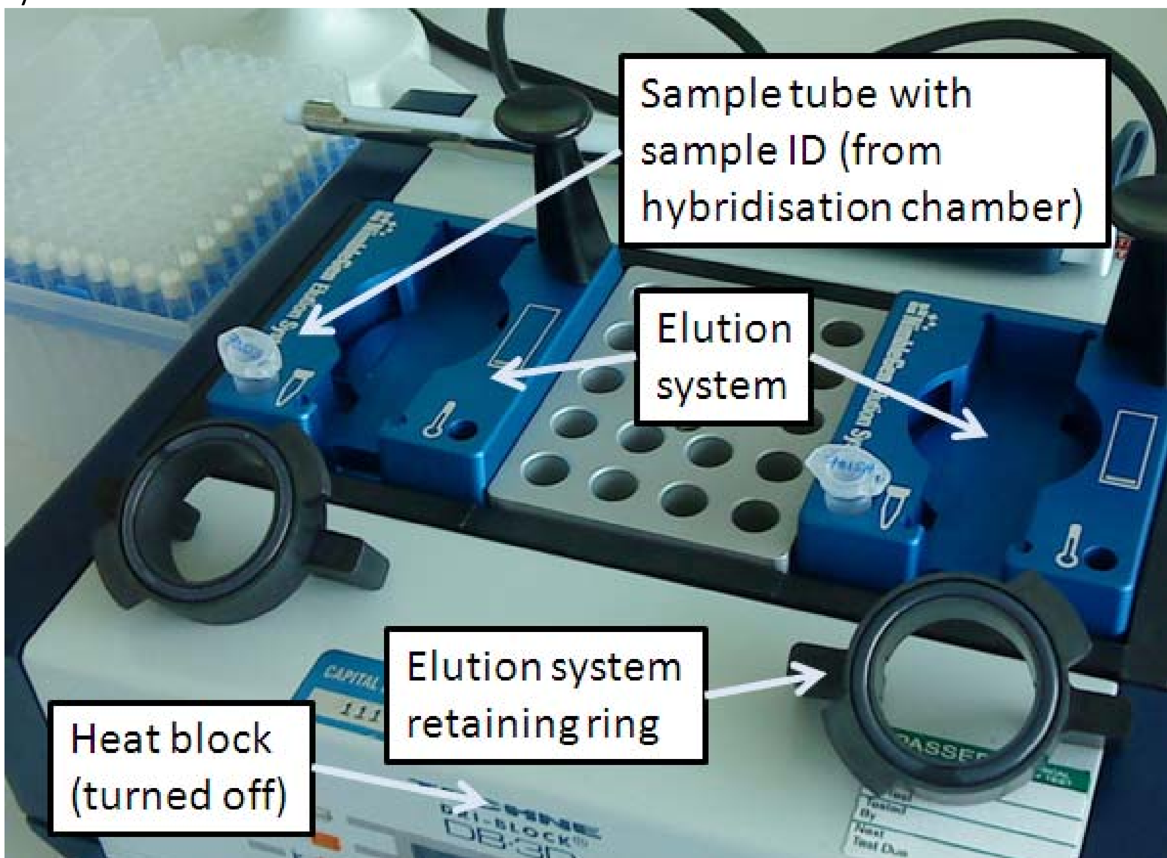
Eluting the DNA Using Sodium Hydroxide (NaOH)

1. Unwrap a new, clean EL1 elution chamber and rubber gasket. Prepare the elution chamber by pressing the gasket into the grove around the edge (see below). Once prepared replace the elution chamber into the plastic packaging until needed.



2. Place the elution system blocks into a heat block that is turned off and at room temperature for stability (see below) or place them onto the bench top. Place an elution system retaining ring ready to lock down the elution chamber near to the elution system.



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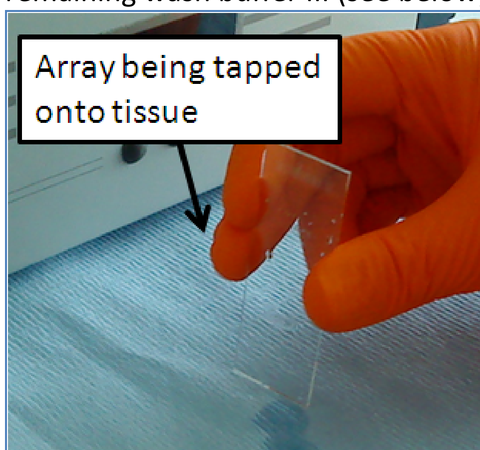
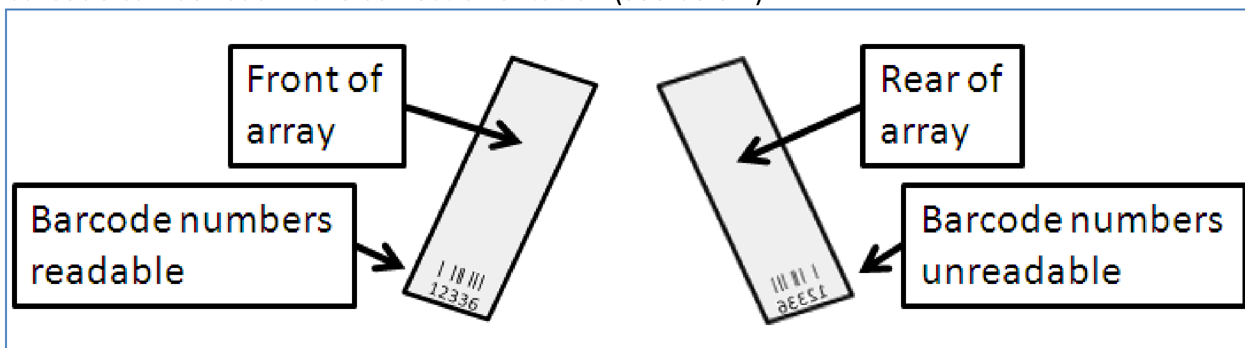
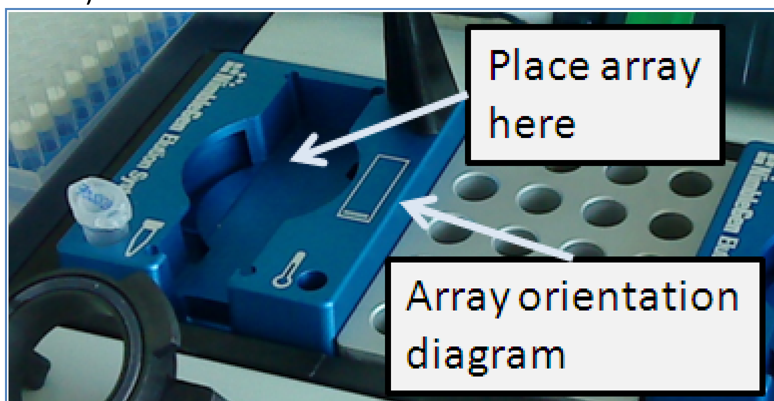
3. Remove the slide from the wash buffer III (final wash above). Unclip the array from the array holder by bending back the clip at the top of the holder. Whilst only holding the sides of the array, gently tap the bottom of the array onto some tissue to remove any remaining wash buffer III (see below)



4. Look at the array barcode (which is etched into the glass) to determine the front and back faces of the array. The front face is the side through which the numbers of the barcode can be read in the correct orientation (see below).



5. Wipe the rear face of the array with a low-lint tissue. Insert the array into the elution system with the barcode orientated as shown on the side of the elution system (see below).



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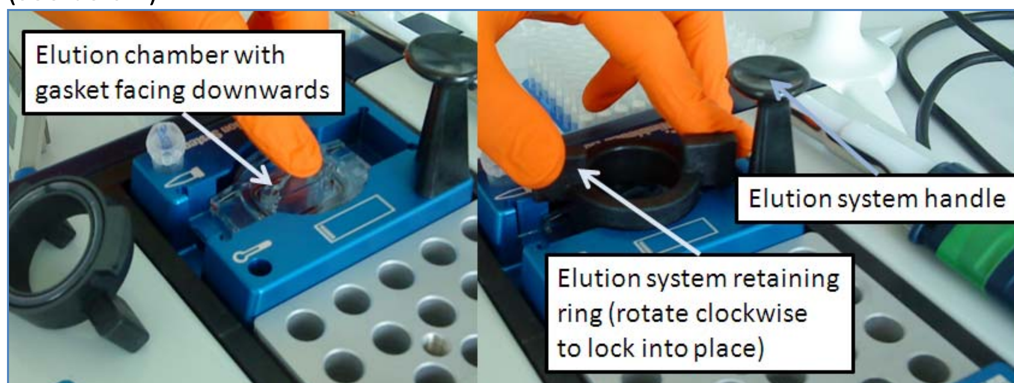
6. Place the elution chamber (prepared as above – Step 1) into the round section of the elution system orientated so that the gasket forms a seal on the array. Place the elution system retaining ring over the elution chamber and twist clockwise to lock into position (see below).



7. Tilt the elution system upwards by pulling on the elution system handle until it is locked in place.

8. Label a clean Eppendorf LoBind 1.5 ml tube with the sample name. Pipette 900 µl of the freshly prepared 125 mM NaOH into the tube.

9. Using a pipette introduce the freshly prepared sodium hydroxide (NaOH) solution (section 'Preparation of the Elution and Clean-up Solutions' above) into the elution chamber by placing the pipette tip into the bottom port of the elution chamber (see below). Introduce the liquid slowly and keep adding liquid until liquid can be seen rising up the top (vent) port of the elution chamber. **NB:** For 2.1 M arrays about 900 µl of NaOH is needed.

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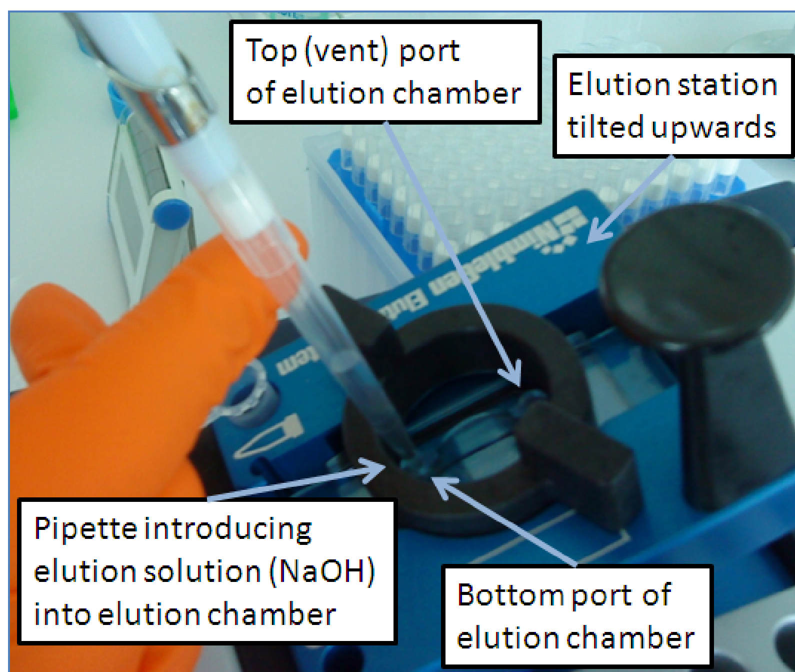

10. Leave the pipette tip in the bottom vent of the elution chamber to prevent any elution liquid from leaking out of the elution chamber. Return the elution system to the horizontal position and remove the pipette tip. Return any unused NaOH to the labelled 1.5 ml Eppendorf LoBind tube that it came from.

11. Incubate the array in the elution system at room temperature for 10 minutes.

12. Following the incubation pipette out eluted DNA and NaOH from the elution chamber and return to the unused, excess NaOH in the labelled Eppendorf LoBind 1.5 ml tube.

13. Twist the elution system retaining ring anti-clockwise to release the elution chamber which can now be discarded. If necessary the elution system can be tilted again and any remaining NaOH be pipetted out and added to the rest of the eluted DNA and NaOH. The array may now be disposed of also. Clean the elution systems and retaining ring with Azo-wipes before storage.

14. Label two 1.5 ml Eppendorf LoBind tubes with the sample details and add 16 µl of 20 % acetic acid and 500 µl of PB buffer to each. Pipette up and down 10 times to mix thoroughly.

15. Split the eluate (~900 µl) and pipette about 450 µl into each of the labelled 1.5 ml Eppendorf LoBind tubes containing the acetic acid-PB buffer mixes. When first added the eluates sit on the top of the acid/buffer mixes and purple layers form at the top of the tubes (see below). Mix the solutions by pipetting up and down 10 times. The solutions should now be yellow, if it is purple the add 20 % acetic acid, 1 µl at a time mixing after each addition until it turns yellow.

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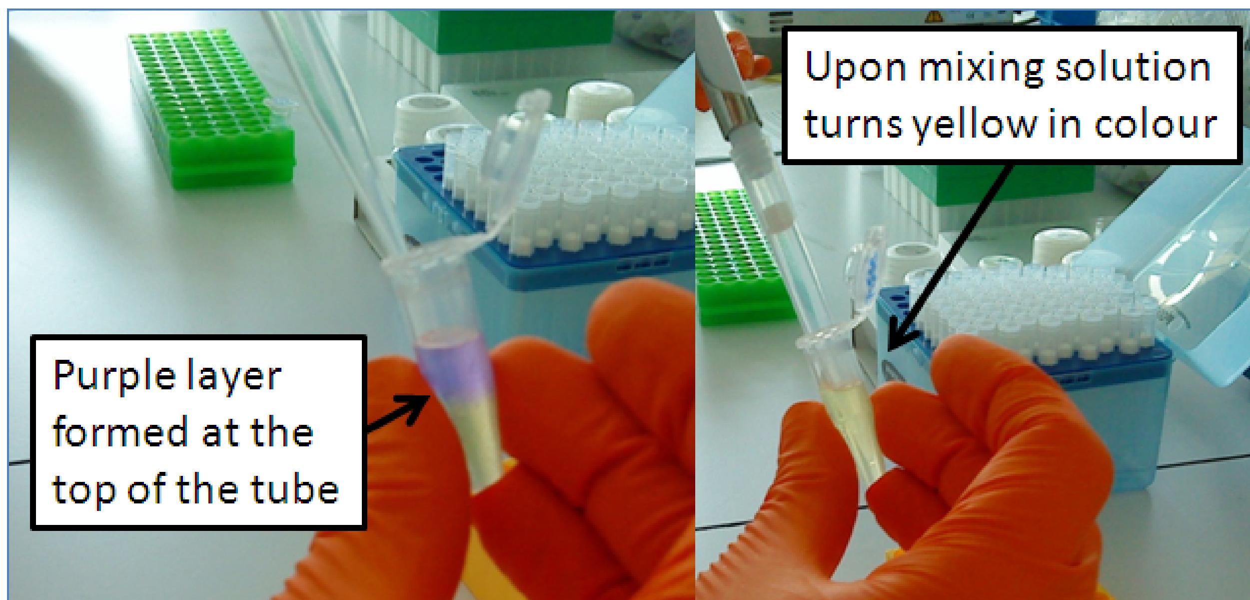

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16. Remove a QIAquickMinElute column (already seated in a collection tube) from the kit stored in the fridge and place into a tube rack on the bench. Label the lid of the column with the sample identifying information that appears on the eluted sample tube lids.

17. Carefully pipette 750 µl of the first DNA-PB buffer-acetic acid mixes into the appropriately labelled column ensuring that all of the liquid is dispensed from the pipette tip and that the tip does not contact the membrane of the column. **NB:** At this point there will be a column with 750 µl of DNA-PB buffer-acetic acid mix loaded onto it and a 1.5 ml Eppendorf containing the remaining 216 µl of DNA-PB buffer-acetic acid mix and a second 1.5 ml Eppendorf containing 966 µl of DNA-PB buffer-acetic acid mix. Close the lid of the first 1.5 ml Eppendorf LoBind tube and keep on the bench until step 23.

18. Close the lid of the column and centrifuge it at 13,000 rpm for 1 minute.

19. Remove the column from the centrifuge and discard the liquid (eluate) that has been spun into the collection tube in which the column was seated. The column may be re-seated into the same collection tube that it was spun in after discarding the eluate.

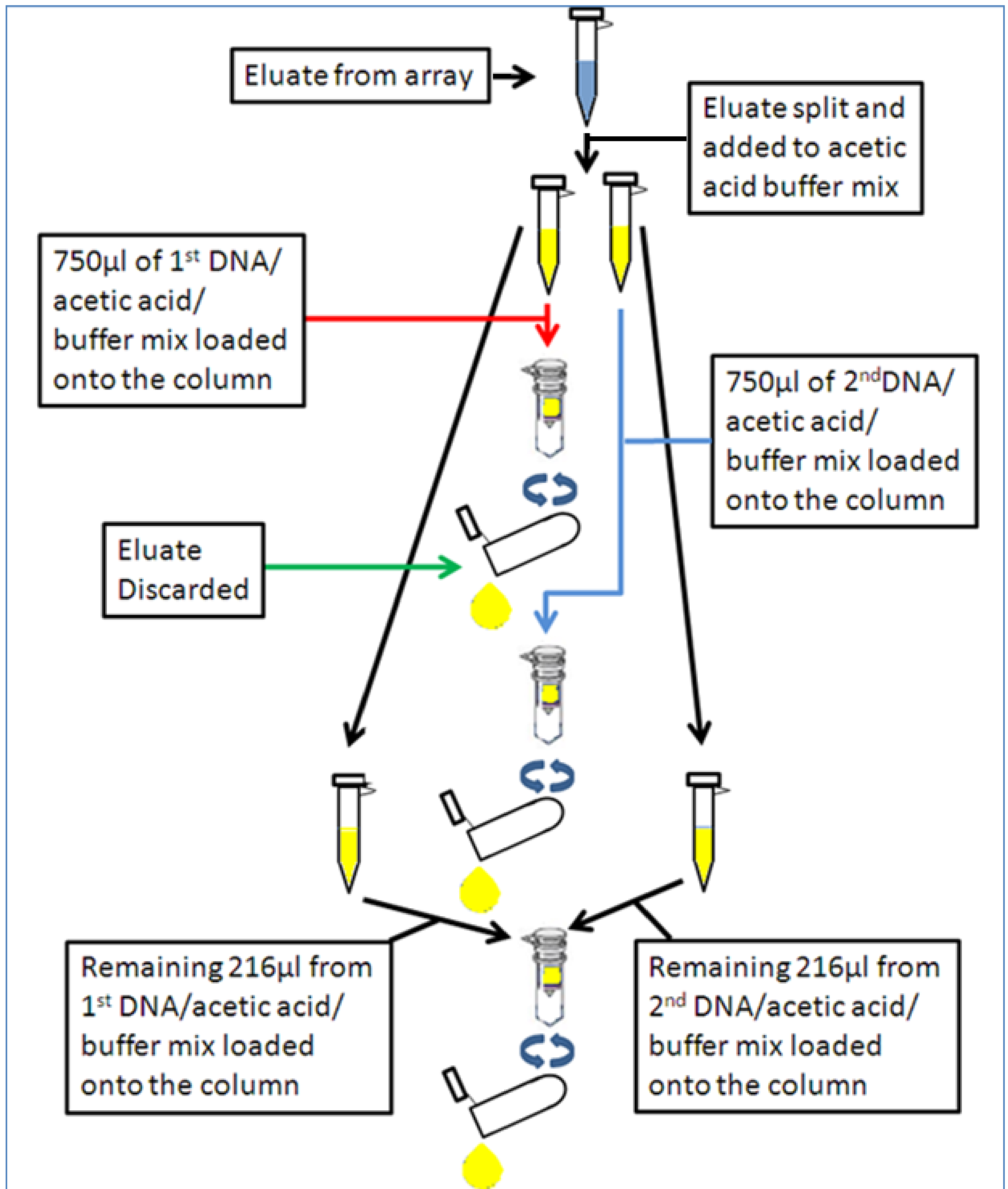
20. Carefully pipette 750 µl of the second DNA-PB buffer-acetic acid mixes from the second 1.5 ml Eppendorf LoBind tube into the appropriately labelled column. **NB:** At this point there will be a column with a second aliquot (750 µl) of DNA-PB buffer-acetic acid mix loaded onto it and two 1.5 ml Eppendorfs containing 216 µl of DNA-PB buffer-acetic acid mix each. Close the lid of the second 1.5 ml Eppendorf LoBind tube and keep on the bench until step 23.

21. Close the lid of the column and centrifuge it at 13,000 rpm for 1 minute.

22. Remove the column from the centrifuge and discard the liquid (eluate) that has been spun into the collection tube in which the column was seated. The column may be re-seated into the same collection tube that it was spun in after discarding the eluate.

23. Carefully pipette the two remaining 216 µl aliquots of the DNA-PB buffer-acetic acid mixes from both of the 1.5 ml Eppendorf tubes into the appropriately labelled column.

Supplementary Protocol 4:
Array capture standard operating procedure



24. Close the lids of the column and centrifuge it at 13,000 rpm for 1 minute.

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25. Remove the column from the centrifuge and discard the eluate that has been spun into the collection tube in which the column was seated. The column may again be re-seated into the same collection tube that it was spun in after discarding the eluate.

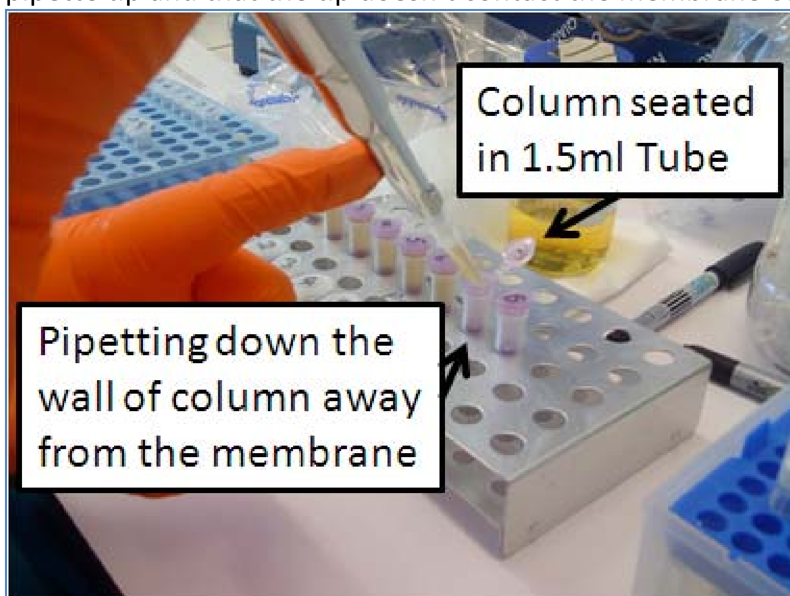
26. Using a clean pipette tip, pipette 750 µl of PE buffer (from the QIAquickPCR purification kit) into the column ensuring that all of the liquid is dispensed from the pipette tip and that the tip doesn't contact the membrane of the column.

27. Close the lid of the column and centrifuge it at 13,000 rpm for 1 minute.

28. Remove the column from the centrifuge and discard the eluate that has been spun into the collection tube in which the column was seated. This collection tube may now be discarded. Place the column into a clean 1.5 ml Eppendorf LoBind tube and place into a rack on the bench.

29. Leaving the column lid open, incubate the column and tube in the rack on the bench for 2 minutes to dry. While the columns are drying a clean 1.5 ml Eppendorf LoBind tube with the sample identifier information written on the column lid and leave in a rack on the bench.

30. Transfer the column into the appropriately labelled clean 1.5 ml Eppendorf LoBind tube from step 29. Using a clean pipette tip add 34 µl of EB buffer (from the QIAquickPCR purification kit) into the column ensuring that all of the liquid is dispensed from the pipette tip and that the tip doesn't contact the membrane of the column (see below).



31. Close the lids of the columns and leave the columns on the bench for 1 minute.

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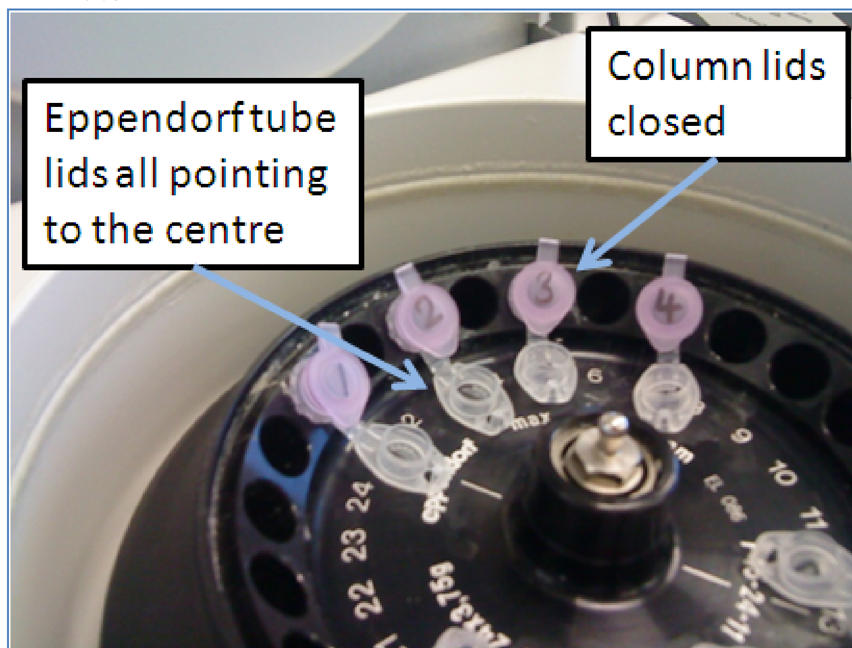
32. Arrange the tube in the centrifuge rotor so that all of the Eppendorf LoBind tube lids all point towards the centre (see below), this will help prevent the Eppendorf LoBind tube lid from breaking off during the centrifugation. Centrifuge the column at 13,000 rpm for 1 minute.



33. Remove the columns and 1.5 ml Eppendorf LoBind tubes from the centrifuge and **DO NOT** discard the eluate that has been spun into the collection tube. The QIAquickMinElute column can now be discarded.

Proceed to the Post-hyb PCR (Supplementary Protocol 6).

Supplementary Protocol 4:
Array capture standard operating procedure

Supplementary Protocol 5

Solution capture standard operating procedure (Agilent SureSelect protocol)

This protocol is for hybridization of adapter-ligated or PCR-amplified library DNA, so must be performed after Hybrid Capture Protocol 1.

Sample preparation

Following SPRI bead cleanup (see Hybrid Capture Protocol 1), lyophilize 500 ng library + 7.5 µg C_0t1 DNA using a SpeedVac..

1. Add 3.4 µl of molecular biology grade water to each sample to rehydrate.

2. Vortex and spin down.

3. To each sample, add:

   SureSelect Block #1 2.5 µl
   SureSelect Block #2 2.5 µl
   SureSelect Block #3 0.6 µl

4. Prepare Hybridization Buffer as follows. Volume for 1 capture:

   SureSelect Hyb #1 25 µl
   SureSelect Hyb #2 1 µl
   SureSelect hyb #3 10 µl
   SureSelect Hyb #4 13 µl

   Note: Do NOT keep on ice.

5. Incubate 40 µl of Hybridization Buffer and library (from step 3.) at 95 °C for 5 minutes and 65 °C for at least 5 minutes. Keep at 65 °C until RNA baits are prepared (see below).

6. Dilute RNAse Block in 1:1 ratio with nuclease-free water and add 1 µl to 5 µl (500 ng) of RNA baits. Incubate for 2 min at 65 °C.

Hybridization

1. Mix 13 µl of hybridization buffer with RNA baits.

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2. Add the 9 µl DNA library to the hyb buffer - RNA bait mixture.

3. Seal plate with the Greiner film and incubate for 24 hours at 65 °C with a heated lid at 105 °C.

Washing and elution

Selection with magnetic beads

1. Prepare magnetic beads by washing with SureSelect Binding buffer 3 times and resuspend in 200 µl of SureSelect Binding buffer. Incubate the hybrid-capture-bead solution on a Nutator for 30 minutes at room temperature (20 °C).

2. Mix hyb. mixture with beads and separate the beads by removing the supernatant.

3. Resuspend beads in SureSelect Wash Buffer #1.

4. Incubate the samples for 15 minutes at room temperature. Separate the beads and buffer on a Dynal magnetic separator and remove the supernatant.

5. Mix the beads in pre-warmed (65 °C) 500 µL SureSelect Wash Buffer #2. Incubate the samples for 10 minutes at 65 °C. Remove wash buffer and repeat these steps for 3 times.

6. Mix the beads in 50 µL SureSelect Elution Buffer. Incubate the samples for 10 minutes at room temperature. Separate the beads and buffer on a Dynal magnetic separator. Add 50 µL of SureSelect Neutralization Buffer.

7. Desalt the capture solution with a Qiagen MinElute PCR purification column, eluting in 34 µL buffer EB.

Proceed to the Post-hyb PCR (Supplementary Protocol 6).

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Supplementary Protocol 6

Hybrid capture eluate PCR standard operating procedure (for array and solution capture eluates)

This protocol is for post-elution amplification of captured DNA from an array or solution capture (i.e. following on from Supplementary Protocol 4 or 5).

PCR primers:
PE.1 = 5′ AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC*T 3′
PE.2 = 5′ CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATC*T 3′

* indicates phosphorothioate. Both primers are HPLC purified.

1. Prepare the PCR master mix for 1 sample:

10 x PCR Buffer	5 µl
25 mM $MgCl_2$	4 µl
2.5 mM dNTPs	4 µl
10 µM PE.1	2.5 µl
10 µM PE.2	2.5 µl
Platinum® *Pfx* DNA Polymerase (Invitrogen)	0.4 µl
Sample (from capture)	31.6 µl

The total volume of the final reaction should be 50 µl.

2. Vortex briefly and spin down.

3. Run on thermocycler with the following program:

94 °C for 5 min

94 °C for 15 sec
58 °C for 30 sec x 18 cycles
72 °C for 30 sec

72 °C for 5 min
4 °C indefinitely

4. Transfer PCR product into a 1.5ml Lo-Bind tube. Run the sample on an Agilent DNA 1000 chip on a Bioanalyzer 2100. If PCR was successful, proceed to the SPRI clean up:

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SPRI bead cleanup

Allow SPRI beads to come to room temperature for at least 30 minutes. Reagents need to be mixed well prior to use and should appear homogeneous and consistent in colour.

1. Take 90 µl of SPRI beads and add them to the 50 µl of PCR sample in a 1.5 ml Lo-Bind tube.
2. Vortex and hold at room temperature for 5 minutes.
3. Place tube in the magnetic rack and leave for 5 minutes or until sample is clear.
4. Carefully remove the clear solution from the tubes and discard.
5. Dispense 500 µl of 70 % ethanol into each tube while in the magnetic rack taking care not to disturb the magnetic beads. Aspirate and discard ethanol.
6. Repeat the ethanol wash once again. Total of two washes.
7. Dry the samples on a heat block (keep the lid of the tube open) at 37 °C for 5 – 10 minutes or until the residual ethanol has evaporated.
8. Add 50 µl of molecular biology grade water, vortex and incubate at room temperature (20 °C) for 2 minutes.
9. Place tubes into the magnetic rack and leave for 2-3 minutes or until sample is clear.
10. Carefully remove the water and retain in a new 1.5 ml Lo-Bind tube.
11. Repeat step 9 -12 once more, retaining the water in the same 1.5 ml Lo-Bind tube. Total volume of elute should be 100 µl.
12. Put the tube into the magnetic tool for 10 min.
13. Transfer the sample to a new 1.5 ml Lo-Bind tube leaving behind any precipitated beads.

After SPRI clean up, quantify by qPCR [1] and sequence.

References

1. Quail M.A., Swerdlow H. & Turner D.J. Improved protocols for the Illumina genome analyzer sequencing system. *Curr Protoc Hum Genet* **Chapter 18**, Unit 18 12 (2009).

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