# Supplementary Methods

## BEA: bead-emulsion amplification

### Primer and probe list

Note that the last 20 base pairs of these primers (lower case) are complementary to the 3’ of the dual-biotinylated primer on the magnetic beads.

### Pre-BEA amplification

|  |  |
| --- | --- |
| Name | Sequence |
| F-ACH-88bp | 5´-GAG CTG GTG GAG GCT GAC GA-3` |
| R-ACH\_BA | 5´-aga gca gga ccc caa agg acc agc AGG CAG CTC AGA ACC TGG TA-3` |
| F-TDII\_BA3 | 5´-GTG CTG GTG ACC GAG GAC AAC G-3` |
| R-TDII\_BA | 5´-aga gca gga ccc caa agg acc agc CAG GCG TCC TAC TGG CAT GA-3` |

### Bead preparation

|  |  |
| --- | --- |
| Name | Sequence |
| Bead-ACH-TDII-SNP\_R2 | 5´-/52-Bio//iSp9/tat gtc ttt ctc tca cat aaA GAG CAG GAC CCC AAA GGA CCA GC-3` |

### Aquaeous phase

|  |  |
| --- | --- |
| Name | Sequence |
| F-ACH-88bp | 5´-GAG CTG GTG GAG GCT GAC GA-3` |
| R-ACH-R93-SNP\_R2 | 5´-aga gca gga ccc caa agg acc agc CCA CCA CCA GGA TGA ACA GGA AG-3` |
| F-TDII-3 | 5´-CGG GAC GTG CAC AAC CTC GAC TAC-3` |
| R-TDII-BA | 5´-aga gca gga ccc caa agg acc agc CAG GCG TCC TAC TGG CAT GA-3` |

### Labeling probes

|  |  |
| --- | --- |
| Name | Sequence |
| ACH wt 488 | 5´-/Alex488N/AGG CAT CCT CAG C\*T\*A\*C\*G-3` |
| ACH mut TX592 | 5´-/TexRd-XN/CAG GCA TCC TCA GC\*T\*A\*C\*A-3` |
| TDII wt 647 | 5´-/Alex647N/CAC AAC CTC GAC TAC TAC\*A\*A\*G\*A-3` |
| TDII mut 532 | 5´-/Alex532N/ACA ACC TCG ACT ACT AC\*A\*A\*G\*G-3` |

### Dye switch labeling probes

|  |  |
| --- | --- |
| Name | Sequence |
| ACH wt TX592 | 5´-/TexRd-XN/ AGG CAT CCT CAG C\*T\*A\*C\*G-3` |
| ACH mut 488 | 5´-/Alex488N/CAG GCA TCC TCA GC\*C\*A\*C\*A-3` |
| TDII wt 532 | 5´-/Alex532N/CAC AAC CTC GAC TAC TAC\*A\*A\*G\*A-3` |
| TDII mut 647 | 5´-/Alex647N7ACA ACC TCG ACT ACT AC\*A\*A\*G\*G-3` |

## Preparation of emulsion

### Oil phase

* Weigh Formulation Aid, Fluid, and Silicone oil in a ratio of 4:3:3 based on the composition below (Dow Corning/Sigma Aldrich)
* Mix the oil phase by vortexing for 10 seconds.
* Centrifuge the oil phase at 3000 rpm. Alternatively, allow the oil phase to settle for 5 minutes or until bubbles dissipate, omitting the centrifugation step.

|  |  |
| --- | --- |
| Preparation of the oil phase | |
| Components | x 1 |
| DC 5225 Formulation Aid (40% w/w) (Dow Corning) | 400 µg |
| DC749 Fluid \*Dow Corning (30% w/w) | 300 µg |
| Ar20 Silicon Oil (30% w/w), Sigma Aldrich | 300 µg |
| Sum | 1000 µg |

|  |  |  |  |
| --- | --- | --- | --- |
| Preparation of the MOCK mix | | | |
| Stock solutions | Components | End concentrations | x 1 | |
|  | dH2O |  | 151.2 µL | |
| 10x | Titanium Taq buffer | 1x | 24 µL | |
| 10 mM | MgSO4 | 2.5 mM | 60 µL | |
| 10% | BSA | 0.1% | 2.4 µL | |
| 1% | Tween 80 | 0.01% | 2.4 µL | |
| Sum |  |  | 240 µL | |

MOCK amplification MIX stored at room temperature until needed.

|  |  |  |  |
| --- | --- | --- | --- |
| Preparation of the aqueous phase | | | |
| Stock Solutions | Components | End concentrations | x 1 |
|  | dH2O |  | 85.6 µL |
| 10x | Titanium Taq Buffer | 1x | 15 µL |
| 50 mM | MgCl2 | 8 mM | 24 µL |
| 10 mM | dNTP`s | 1 mM | 15 µL |
| 500 µM | Forward-ACH-88bp | 9 µM | 2.7 µL |
| 5 µM | Reverse-ACH-R-93-SNP\_R2 | 50 nM | 1.5 µL |
| 500 µM | Forward-TDII-3 | 9 µM | 2.7 µL |
| 5 µM | Reverse-TDII-OUT | 50 nM | 1.5 µL |
| 50x | Titanium Taq Polymerase |  | 2 µL |
| Sum |  |  | 150 µL | 630 µL |

### Emulsification in TissueLyser II (BioRad)

* Place one 5mm steel bead in a 2mL round-bottom tube.
* Add 650 µL of the oil phase and 240 µL of MOCK mix to the tube. Seal the tube with Parafilm.
* Position the tubes in the TissueLyser II adaptors and select the appropriate TissueLyser program mixing the oil and MOCK mix at 25Hz for 5min with a 5mm steel bead. The aqueous phase is added next and a second TissueLyser step is performed at 15Hz for 5mm without steel bead. The second step helps to create larger aqueous emulsion droplets which work as microreactors in the PCR reaction. The surfactant Tween80 and the BSA in the MOCK mix enable the stabilization of the emulsion.
* Aliquot 80-100 µL of the emulsion into smaller 200 µL PCR tubes.
* Place the tubes in the thermocycler and execute the PCR program outlined below:

|  |  |  |
| --- | --- | --- |
| Program: BEA72 | | |
| Temperature | Time | |
| 94°C | 2 min. | |
| 94°C | 15 sec. | x 55 |
| 65°C | 15 sec. |
| 72°C | 35 sec. |
| 72°C | 2 min. | |
| 8°C | forever | |

### Breakage of emulsion

* Upon completion of the emulsion PCR, combine the contents of the 200 µL tubes evenly into 2 siliconized 2 mL round-bottom tubes. Add approximately 1.5 mL of ethanol to each tube.
* Seal the tubes with parafilm. Break the emulsion by applying 30Hz for 5 minutes in the TissueLyser II, followed by centrifugation at 17000xg for 2 minutes.
* Remove the supernatant (SN) and rinse again with ~500 µL ethanol.
* Fill up the tube with ethanol, homogenize again at 30Hz for 2 minutes, and centrifuge for 2 minutes at 17000g.
* Place the beads back in the magnetic particle concentrator (MPC), remove the SN, fill up with NXS buffer, and repeat the wash at 30Hz for 1 minute in the TissueLyser II.
* After centrifuging at 17000xg for 1 minute, remove the SN, and rinse the beads with TE buffer.
* Add 500 µL of freshly prepared 0.1M NaOH solution from a 2M stock to the bead pellet on the MPC, and incubate for 1 minute at room temperature (see Note 37).
* Remove the NaOH solution and rinse the beads again with ~500 µL TE buffer.
* Beads can then be labeled immediately or stored in TE buffer at 4°C.

### Labelling of the beads

* Prepare the labelling solution by combining 41.5 µL Millipore water, 5 µL Titanium Taq buffer, 1 µL dNTPs, 0.5 µL Titanium Taq polymerase, and 0.5 µL of each of the 4 labelling probes.
* Place the tubes on the magnetic particle concentrator (MPC), remove the TE buffer, resuspend the beads in 50 µL labelling solution, transfer the entire content into a 200 µL PCR tube, and run the thermocycler with the specified conditons/program.
* Meanwhile, prepare 500 µL of 1E buffer (10mM Tris-HCl (pH 7.4), 50mM KCl, 2mM EDTA, and 0.01% Triton-X100) in an empty 1.5 mL reaction tube. After the program has finished, pipette directly quickly into the prepared reaction tubes containing 1E buffer
* Centrifuge at 9000xg for 1 minute and place the tube in the MPC.
* Remove the supernatant (SN) and wash with ~500 µL TE buffer.

### Array and Scan of the Beads

* To analyze the beads, arrange them within a polyacrylamide matrix (PAA gel) immobilizing them in a monolayer onto a slide to maintain positional information over consecutive washing and scanning steps.
* Start by treating object slides with a layer of Gamma-Methalcryloxypropyl-trimethoxysilan by preparing the polyacrylamide gel. In advance, pipette 4 µL of 20% Rhinohide in Acrylamide (37/1) and 4 µL TE buffer together in 200 µL PCR tubes, while adding TEMED and APS shortly before bead arraying.

|  |  |  |
| --- | --- | --- |
| Resuspend in TE buffer and array immediately or store the beads until the arraying step is carried out. PAA gel preparation | | |
| Component | Percentage | Amount |
| TE-Buffer |  | 4 µL |
| Rhinohide in AA (37/1) | 20 % | 4 µL |
| TEMED | 5 % | 1.25 µL |
| APS | 0.5 % | 1.25 µL |

* On the magnetic particle concentrator (MPC), remove TE buffer from the beads, take them out of the MPC, and let the beads air dry for a maximum of 5 minutes.
* Add 1.25 µL 5% TEMED and 1.25 µL 0.5% APS to the already prepared Rhinohide-in-Acrylamide/TE buffer-mix. Flick the tube, take 1.9 µL of the mixture, add the PAA gel to the beads, mix by pipetting up and down, and transfer beads to the slide.
* Cover the bead drop with a cover slip (12x12mm), apply soft pressure to spread the gel over the entire area of the cover slip, creating the desired monolayer. Be cautious to avoid air bubble formation.
* Wait approximately 5 minutes until the gel is polymerized. Carefully remove the 12x12mm cover slip. Place a few drops of TE buffer on the sample, cover the array with another cover slip (24x40x1mm), and remove excess liquid with a tissue. Seal the slide with rubber cement glue.
* Scan the array with an epifluorescent microscope using ~300 raster positions. Capture three (or five) images at each raster position in different fluorescent channels and the bright field mode with the 12-bit 4K CCD camera. The bright field image is essential to infer the bead area during image analysis.
* If scanning more than two polymorphisms, the array can be washed, the fluorophores stripped off, and the array labeled with a new set of probes, as explained in the next step (additional probing and imaging of the array).

### Additional Probing and Imaging of the Array

* The probes on the arrayed beads can be stripped off, and the beads can be labeled again with another set of probes. This is important to verify the mutants using a dye-switch approach.
* Prepare the dye switch solution by mixing Titanium Taq buffer 1x, 0.2mM dNTPs, and 1µM each of A592 ACHwt, A488 ACHmut, A532 TDIIwt, and A647 TDIImut, along with Titanium Taq polymerase at 0.02U/µL.
* Remove the glue from the cover slip sealed on the slide and then the cover slip (24x40x1mm) by adding a few drops of TE buffer around the edges. The cover slip quickly floats on the excess TE buffer and can be lifted without bead loss.
* Cover the array with TE buffer and use an in situ PCR block or adaptor to strip off the probes at 94°C for ~1 minute. Ensure the gel is covered with TE buffer at all times.
* Remove the slide with the array from the in situ block and rinse with TE buffer.
* Let the excess water air-dry on the array. Place the hybridization chamber as centrally as possible on the array and pipette 100 µL dye switch solution into the hybridization chamber through the holes at two of the corners. Before sealing the holes with adhesive films, remove all air bubbles by gently tapping on the surface of the hybridization chamber.
* Place the slide on the in situ block and incubate for an initial denaturation step at 95°C for 2 minutes, followed by a probe annealing at 63°C for 5 minutes, and an extension at 72°C for 5 minutes, and a final extension at 75°C indefinitely.
* When removing the slide from the hot in situ block (at ~75°C), immediately strip off the hybridization chamber, and place the slide in a Coplin jar filled with 1E buffer to wash off all non-extended allele-specific probes.
* Rinse the array with TE buffer, cover it again with a cover slip (24x40x1mm), and seal it with rubber cement glue.
* When scanning the array for a second or other consecutive time, place the slide at the same position on the slide holder of the microscope and check with Metamorph how well the first image of the first scan aligns with the bead positions of the second scan. If the offset is too large, you can adjust the x- and y-positions of the scan area defined in Metamorph for a perfect image overlay.