High density SNP genotyping array for hexaploid wheat and its relatives

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Summary

A lack of genetic diversity between wheat breeding lines has been recognised as a significant block to future yield increases. Wheat breeding and pre-breeding strategies are increasingly using material from wheat ancestors or wild relatives to re-introduce diversity. Where molecular markers are polymorphic between the host and introgressed material, they may be used to track the size and location of the introgressed material through generations of backcrossing. To generate markers for this purpose, sequence capture targeted re-sequencing was carried out for a range of wheat varieties, wheat relatives and wheat progenitors. From these sequences, putative SNPs were identified and used to generate the Axiom® Wheat HD array. A selection of varieties representing a selection of elite wheat breeding material, progenitor species and wild relatives were used to validate the array. The procedures used are described here in detail.

Key Words Wheat, *Triticum aestivum*, SNPs, Genotyping, Array,

1. Introduction

Hexaploid wheat (*Triticum aestivum*) is a globally important crop being the staple food for humans and livestock. Breeding efforts during the Green Revolution resulted in substantial yield increases that saw average yield double from 1.4 to 2.8 t/ha [1]. This yield increase has not been sustained and all countries are experiencing yield stagnation (1). Recent breeding efforts have employed genomic technologies to develop faster and more accurate breeding strategies. These may also be used to identify potentially useful novel alleles outside of the elite wheat gene pool such as landrace varieties, wheat relatives and progenitor species.

Wheat is derived from the hybridization of diploid *Aegilops tauschii* with tetraploid wild emmer, *Triticum turgidum* ssp. *dicoccoides* [2, 3, 4]. Since this hybridization event, early domestication subjected crops to strong selection pressure and often inbreeding resulting in a reduced level of genetic diversity in modern wheat varieties compared to their wild ancestors [5]. This lack of genetic diversity is often cited as a limiting factor in the production of high yielding and stress resistant varieties [6, 7, 8]. It is possible to introduce novel genetic diversity into elite breeding lines through the introgression of material from wheat ancestors or wild relatives [9], a process employed by some pre-breeding programs. Where molecular markers are polymorphic between the host and introgressed material, they may be used to track the size and location of the latter through generations of backcrossing.

Molecular markers are also widely used as a research tool to analyse existing populations. Within wheat, SNP based markers have been used to carry out genome wide associate studies to elucidate the regions of the genome associated with disease resistance [10] and grain yield [11] (Sukumaran *et al*., 1025), for example.

Sequence capture targeted re-sequencing was carried out for a range of wheat varieties and for wheat relatives and progenitors considered to be a potential source of novel variation suitable for introgression into wheat. From these sequences, a large number of putative SNPs between different varieties of hexaploid wheat and between hexaploid wheat and related species were identified [12]. The Axiom® Wheat HD array was designed to validate these SNPs and to map introgressions within the hexaploid wheat genome. A selection of diploid, tetraploid and hexaploids varieties, representing elite wheat breeding material, progenitor species and wild relatives, was used to validate the array (Table 1). The procedures described in depth in this chapter cover the method used to genotype these accessions using the Wheat HD array.

The markers, associated sequence and genotype information relating to the Axiom® Wheat HD array have been made available through an interactive web site. www.cerealsdb.uk.net.

2. Materials

2.1 Plant material

For this study, plants of bread wheat and wheat relatives (Table 1) were grown in a peat based soil in single pots at 15–25 °C with a day-night cycle of 16h light and 8h dark. An approximately 8cm section of the first true leaf was collected from plants six weeks post-germination and stored at -80°C prior to DNA extraction.

2.2 Reagents and Solutions

- RNase A (Thermo Scientific)
- Isopropanol
- DNA Extraction Buffer: 200ml 1MNaCl, 25ml 2M Tris-HCl ph7.5, 50ml 10% SDS, 50ml 0.5M EDTA per litre.
- 25bp ladder (TrackIt, Invitrogen)

The following solutions were made, according to guidelines given in *Axiom® 2.0 Assay Manual Workflow User Guide Rev3*, using reagents supplied by Affymetrix upon the purchase of the Axiom array.

- Denaturation Master Mix: 400µl 'Axiom 2.0 Denat Soln' 10X, 3.6ml DNAse-free water*.
- Neutralisation Solution: 'Axiom Neutral Soln' use as supplied.
- Amplification Master Mix: 26ml 'Axiom 2.0 Amp Soln', 578µl 'Axiom 2.0 Amp Enzyme'*.
- Fragmentation Master Mix: 6ml 'Axiom 10X Frag Buffer', 1.35ml 'Axiom Frag Diluent, 131µl 'Axiom Frag Enzyme'*.
- Precipitation Master Mix: 26ml 'Axiom Precipitation Solution 1', 218µl 'Axiom Precipitation Solution 2'*.
- Resuspension Buffer: 'Axiom Resusp Buffer' use as supplied.
- Hybridization Master Mix: 7.8ml 'Axiom Hyb Buffer', 55.6µl 'Axiom Hyb Solution 1', 1ml 'Axiom Hyb Solution 2'*.
- Stain 1 Master Mix: 22.2ml 'Axiom Wash A', 463µl 'Axiom Stain Buffer', 231µl 'Axiom Stain 1A', 231µl 'Axiom Stain 1B'. Prepared soon before use, store away from light.
- Stain 2 Master Mix: 11.1ml 'Axiom Wash A', 231µl 'Axiom Stain Buffer', 115.6µl 'Axiom Stain 2A', 115.6µl 'Axiom Stain 2B'. Prepared soon before use, store away from light.

- Stabilization Master Mix: 10.3ml DNAse-free water, 1.16ml ‘Axiom Stabilize Diluent’, 144.8µl ‘Axiom Stabilize Soln’*.
- Ligation Master Mix: 7.3ml ‘Axiom Ligate Buffer’, 1.45ml ‘Axiom Ligate Soln 1’, 348µl ‘Axiom Ligate Soln 2’, 1.16ml ‘Axiom Probe Mix 1’, 1.16ml ‘Axiom Probe Mix 2’, 174.4µl, ’Axiom Ligate Enzyme’. Prepared immediately prior to use.

NB *These **s**olutions should be prepared prior to use, not made in advance and stored.

2.3 Consumables

Deep well 96-well plates (at least 2.2ml)

PCR machine suitable 96-well plates

Good quality plate seals

Pre-cast 4% agarose eGel (Thermo Fisher Scientific)

Qiagen purification kit (Qiagen)

2.4 Equipment

Multichannel pipettes (8 or 16 channel); P20, p200 and p1200 suggested.

Vortex

Centrifuge capable of up to 2250 RCF

Ovens; at least two, but three would be ideal to prevent cross-contamination.

Freezer set to −20 °C

Mircroplate reader with 260nm (A260) optic module

Microplate shaker

UV Transilluminator

3. Methods

3.1 Array Design

This protocol is for the pre-designed Wheat HD array, however other custom arrays may be designed and genotyping performed in the same manner. As well as custom designs from sequence data, it is possible to generate new arrays from a combination of existing probes. For the Wheat HD array, smaller arrays have already been designed selecting only the probes most useful in wheat breeding [13] and in wide crosses with wild relatives [14]. When designing an array, take care in choosing the probes to include. Re-designs are expensive so it is worthwhile including multiple probes on important targets. Fortunately, there is a good conversion of probes between genotyping platforms enabling the use of pre-validated probes.

3.2 DNA extraction

- Prior to use, store extraction buffer for 1 hour in a pre-heated (65°C) oven.
- Take an approximately 8cm section of the first true leaf (*see* **Note 1**) and immediately freeze using liquid nitrogen. Store at −20 °C prior to DNA extraction.
- Using a mortar and pestle, grind frozen leaf tissue into a fine powder. Add small quantities of liquid nitrogen throughout to keep the leaf tissue frozen and brittle.
- Transfer tissue to a solvent-safe, 15ml centrifuge tube and add 5ml of DNA extraction buffer.
- Invert several times and then incubate at 65°C for 20 minutes; invert once more after the first 10 minutes.
- Allow the centrifuge tube to cool to room temperature and add 5ml phenol. Invert several times and centrifuge at 3900 RCF for 20 minutes.
- Using a large volume pipette, carefully transfer the top aqueous phase to a fresh solvent-safe, 15ml centrifuge tube. Add 3ml chloroform, invert several times and centrifuge at 3900 RCF for 10 minutes.
- As before, carefully transfer the top aqueous phase to a fresh solvent-safe 15ml tube. Add 3ml isopropanol, invert several times and centrifuge at 3900 RCF for 20 minutes.
- Carefully invert over the sink to remove the waste isopropanol solution; pellet should appear pale white and clean. Allow the centrifuge tube to remain inverted on lab roll for approximately 10 minutes.
- When there is no longer an odour from the residual isopropanol, add 200µl water and vortex to suspend the pellet.

3.3 RNase treatment

- Pre-heat an oven or water bath to 37°C.
- Quantify samples using a fluorometric method such as Qubit and, where there is great variation in concentration, dilute to a uniform 50ngµl^{-1}.
- Add 1µl of RNase to 150µl of 50ngµl^{-1} DNA. Briefly vortex and centrifuge each sample and incubate at 37°C for 50 minutes.
- Cool to room temperature and continue to DNA purification.

3.4 DNA Purification

Samples are purified using the protocol suggested by the QIAquick PCR purification kit (QIAquick Spin Handbook, 2008) (*see* **Note 2**).

- Add 750µl of Buffer PB to 150µl sample DNA at 50ngµl^{-1} and transfer to the QIAquick column. Centrifuge for 60 seconds at 14,100 RCF and discard the waste solution.
- Add 750µl of Buffer PE to the QIAquick column and centrifuge for 60 seconds at 14,100 RCF. Discard the waste solution and centrifuge again for 60 seconds at 14,100 RCF without any additional buffer.
- Replace the bottom component of the QIAquick column with a microcentrifuge tube. Cut the hinge lid from the microcentrifuge tube before use to prevent it causing friction in the centrifuge.
- Add 30µl of Buffer EB directly to the bottom of the QIAquick column and allow to stand for 60 seconds before a final centrifugation for 60 seconds at 14,100 RCF.

3.5 Sample Preparation

The Wheat HD Axiom array exists as a two array set (550491 and 550492). The following stages must be carried out simultaneously for two 96-well sample plates with identical sample layouts (*see* **Notes 3-4**).

- Quantify samples and dilute to a uniform 10ngµl^{-1} (*see* **Note 5**).
- Carefully transfer 20-22µl of 10ngµl^{-1} DNA to each well. Ensure that the same sample has the same location in each plate (A and B) (*see* **Note 6**).
- Briefly centrifuge to spin down samples.

3.6 Amplification

- Pre-heat the oven to 37°C. (*see* **Note 7**).
- Using a multichannel pipette, carefully add 20µl of the Denaturation Master Mix to each well (*see* **Note 8**). Set a timer for 10 minutes. Seal the plates well, vortex to mix and centrifuge briefly to bring down any solution from the sides of the well. Allow the plates to sit at room temperature for the remaining time.
- At the end of 10 minutes, use a multichannel pipette to add 130µl of the supplied neutralisation solution ('Axiom Neutral Soln') to each well. Work in the same row and plate order as previously. Seal the plates well, vortex to mix and centrifuge to spin down solute.
- Carefully add 230µl Amplification Master Mix to each well. Seal the plates well, vortex to mix and centrifuge to spin down solute. Place in the pre-heated 37°C oven and allow to incubate for 23 hours. This incubation time can be increased if difficulty in extracting sample resulted in less than the recommended starting amount of DNA being used.

3.7 Fragmentation

- Pre-heat one oven to 65°C and another to 37°C. (*see* **Note 7**).
- At the end of the 23 hour amplification incubation, transfer both sample plates to the 65°C for 20 minutes to stop the amplification reaction.
- After 20 minutes transfer both sample plates to the 37°C oven for 45 minutes.
- Using a multichannel pipette, carefully add 57µl of the Fragmentation Master Mix to each well. Set a timer for 30 minutes. Seal the plates well, vortex to mix and centrifuge to spin down solution before returning to the 37°C oven.
- Be prepared to add 19µl of the supplied 'Stop Solution' to each well after exactly 30 minutes. Using a multichannel pipette, work in the same row and plate order as previously. Seal the plates well, vortex to mix and centrifuge to spin down prior to precipitation.

3.8 Precipitation

- Add 240µl of the Precipitation Master Mix to each well. Seal the plates well, vortex to mix and spin down. The samples should now be pale blue in colour.
- Using a large volume multichannel pipette, add 600µl of isopropanol to each well. To thoroughly mix, carefully aspirate and dispense several times until the solution in each well is uniformly cloudy.

- Use lab roll to remove any excess liquid from the top of the plate to prevent contamination between wells. Seal the plates well and incubate at -20°C for 16-24 hours.

3.9 Re-suspension.

- Pre-chill a centrifuge to 4°C and one post-amplification oven to 37°C.
- Centrifuge both plates simultaneously at 2250 RCF for 40 minutes. Pale blue-white pellets should be visible in each well.
- Remove the seal from the sample plate and carefully invert over the sink to remove the waste isopropanol solution. The pellets should not dislodge if the plate is inverted with a smooth action; take care not to knock or shake the plate as this could result in pellet loss.
- Allow the plates to remain inverted on lab roll for 5 minutes. The tissue will rapidly become saturated, so it is recommended to replace the tissue several times.
- After 5 minutes, move sample plates to the 37°C oven to dry for a further 20 minutes. Where possible, do not exceed 20 minutes drying time as this will affect the resuspension of the pellet.
- Remove any excess liquid from the top of the plate with lab roll and add 35µl of the supplied resuspension buffer ('Axiom Resusp Buffer') to each well.
- Seal well and use a plate shaker to shake the plates for 10 minutes. This can be extended as required to fully re-suspend the pellets. Occasionally, the addition of an extra 5µl-10µl of resuspension buffer may be required if the pellet remains present after 20 minutes.
- When fully suspended, spin down both plates and transfer the contents to a 96-well, PCR plate. Add 80µl of the Hybridization Master Mix to each well. Seal well, vortex to mix and spin down.
- As this stage, it is possible to remove a small amount from each well to confirm the success of the amplification and fragmentation reactions. This is recommended as repeated sample preparation is preferable to poor array data.
- The hybridization plates can be safely stored at -20°C until the sample quality has been established.

3.10 Quality Control.

To confirm the successful amplification and fragmentation of sample DNA, whole plate quantification is suggested using an optical plate reader. All, or a substantial subset, of the samples may be run on a gel to confirm fragment size.

- Transfer 3µl from each well of the hybridization plates to new 96-well 'Dilution plate' and dilute with 33µl of DNase-free water. Seal well, vortex to mix and spin down.
- Further dilute the Dilution Plate samples 1:10 with DNase-free water and use an OD260 optical plate reader to quantify all samples simultaneously. Methods may vary depending on the plate reader used. After multiplication by the dilution factor of 120, the plate median DNA should be over 1000ug. If the plate median is considerably below this, poor genotyping will result.
- Further dilute the dilution plate samples 1:40 with gel loading dye and load 20µl sample per well onto a pre-cast eGel (4% agarose, double-comb cassette). This allows for 48 samples per gel minimising gel to gel variation.
- Add 15µl of a 25bp ladder (1:10 dilution TrackIt, Invitrogen) to the flanking wells of each row.
- Run gel using the pre-set EG protocol for 22 minutes and visualise immediately using a UV transilluminator. The post-fragmentation size should be between 125bp and 25bp (Figure 1). Larger fragments will negatively affect the hybridization to the array.

3.11 Denaturation

The following steps relate to the Gene Titan equipment (*see* **Note 9**). Sample denaturation is not to be carried out for both plates simultaneously. The Gene Titan software will indicate when plate B should be loaded (approximately 8 hours after plate A). It is recommended that plate B is stored at -20°C until required.

- Defrost the sample plate and allow the appropriate array to acclimatise to room temperature.
- Centrifuge sample plate and using a PCR machine with a hot-lid function, denature at 95°C.
- The suggested protocol is as follows:
    - 95 °C 10 min
    - 48 °C 3 min
    - 48 °C hold
- The sample plate is ready to be transferred to the Gene Titan after the three minute 48°C step. A hold step is used to maintain the sample plate at 48°C should any delays or technical difficulties be encountered.
- Using a multichannel pipette, carefully transfer the contents of each well to a Gene Titan readable 'Hybridization Tray' and load into the Gene Titan and the appropriate array following the on-screen instructions.

3.12 Gene Titan Reagent Preparations

- Make up the Stabilisation Master Mix, Ligation Master Mix and both Stain 1 and 1 Master Mix up as described in Reagents and Solutions immediately prior to use. As the Ligation Master Mix involves the least stable enzyme it is suggested that this is left until last.
- Use a static gun to remove potential charge on all Gene Titan readable stain trays and lids. Failure to remove static will lead to reagent adhering to the lid and being lost from the tray.
- Using two stain trays for Stain 1 and one stain tray for the other reagents, add 105µl of the Master Mix to each well. Ensure that no bubbles are present as this will interfere with the application of the reagents onto the array.

3.13 Data Analysis

Data analysis is carried out using the proprietary but freely available 'Axiom Analysis Suite' software.

- Select the correct array type from the drop down menu. The Wheat HD array has an A and B option which are genotyped separately. Take care to upload .CEL files from the corresponding A or B plate (*see* **Note 10**).
- Select the correct analysis and prior files. These will vary with array but may also vary within projects. Genotype calls are generated by the Axiom GT1 clustering algorithm which uses the observed data and specified priors file to do so. The default Wheat HD priors (Axiom_BristolW_A.r2.AxiomGT1 and Axiom_BristolW_B.r2.AxiomGT1) were designed based on the clustering behaviour of 475 wheat and wheat relative lines.
- Use the 'Import CEL files' option to select the appropriate files. The Gene Titan will generate a number of files, some are intermediary and some will only become relevant during troubleshooting. The files required for generating genotype data are the .CEL files, each of which represents one sample. It is possible to combine .CEL files from multiple projects as long as the array type remains the same; for example, .CEL files generated using the Wheat HD array cannot be genotyped simultaneously with the Wheat Breeders Array. For accurate clustering, at least 96 samples are required in one project.
- Adjust the Threshold Configurations for the project. Defaults exist for some levels of ploidy, but changes should be made to suit the needs of the project. For example, the default call rate threshold is 97%, but for a set containing both wheat and wheat relatives, a call rate threshold of 80% may be used. The Wheat HD array contains a range of probes which are

polymorphic between different material of different ploidy (elite hexaploid, wheat relatives, landraces). As such, there is no wheat accession which could result in a 100% call rate and so the cut-off for a 'good' call rate is lowered.

- Select the output folder and batch name then select 'Run Analysis'. The analysis may take some time to run, depending on the number and size of the files.
- The genotypes are exported in a file called 'AxiomGT1.calls.txt', each row of which represents a SNP on the array and each column a sample. The genotypes are encoded numerically (Table 2).

The software uses a number of clustering properties to classify the SNPs into performance categories (Figure 2) (*see* **Note 11**).

1. Poly High Res: Polymorphic and co-dominant. Three clusters consisting of one heterozygous group and two homologous groups. Each cluster will contain at least two samples.
2. No Minor Hom: Polymorphic and dominant. Two clusters, one heterozygous group and one homologous group.
3. Off-Target Variant (OTV): Four clusters consisting of one heterozygous group and two homologous groups.
4. Mono High Res: Monomorphic single cluster.
5. Call Rate Below Threshold: The SNP call rate is below the defined threshold while other cluster properties are above threshold.
6. Other: One or more of the cluster properties are below threshold.

4. Notes

1. Different accessions and tissues types may be used depending on the research interest of the investigator. As the Wheat HD array was designed to accommodate diverse breeding material (Table 1), it is possible to use a range of wheat related species on the same array.
2. RNAase treatment and purification of samples are carried out to minimise any potential interference from impurities in the sample. In practice, we have found the protocol to be very robust and generate unambiguous genotype scores for untreated DNA extracts. Note, however, that if RNAase treatments are omitted, then quantification by spectrophotometer such as Nanodrop may over-estimate the amount of DNA present.
3. Use two 2.2ml deep-well 96-well plates labelled 'A' and 'B' to ensure sample preparation is carried out in the same order each time.
4. The Wheat HD sample preparation involves two sample plates and the ability to balance a centrifuge at every step. If you are using a single-plate array, it is recommended that you prepare the appropriate balances in advance to prevent delays in the protocol (Table 3).
5. The protocol appears quite robust and may tolerate samples outside of the 20-22µl of 10ngµl^{-1} parameters if necessary.
6. During the preparation of sample plates, it is possible to make a mistake resulting in non-identical sample plate layouts. As long as each sample is present on each plate (A and B) the correct sample locations may be recorded on the Gene Titan sample submission sheet and the resulting data will still be correct. Unlike many other genotyping methods, a negative control is not required to generate an accurate genotype call. In fact, where negative controls are included, the genotyping software will identify this as a poor quality sample and remove it from the analysis.
7. Where possible, carry out pre- and post-amplification steps in separate locations and using separate pipettes to prevent contamination of future pre-amplified samples.
8. For all stages involving both samples plates, work quickly and methodically. Pipette in the same plate and column order each time to reduce differences in incubation times between samples.
9. This Gene Titan is unique to the Axiom array, so is not as ubiquitous as the other items of equipment used throughout the method. It may be necessary to transport the prepared sample plate to another facility which has access to a Gene Titan. Ensure the sample plates are well sealed and frozen for transit.

10. The clustering algorithm determines genotype more accurately where all three clusters are observed. If data from multiple Wheat HD arrays are to be compared, better results will be obtained by analysing all the 'A' .CEL files and all the 'B' .CEL files simultaneously rather than attempting to compare outputs for multiple projects.
11. The SNP performance classification is based on traits such as SNP call rate and cluster spread which can vary depending on the samples used. A SNP may be assigned a different performance category depending on the set of samples used. See Figure 2 for an example of each performance category.

5. References

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Figure Captions

Figure 1 Agarose gel showing amplified samples. The red line indicates a fragment size of 125bp. After a successful fragmentation, the DNA should be below this point at a range of 125bp to 25bp.

Figure 2:

Table Captions

Table 1 Wheat species and wheat relatives used on the 820K HD Array.

Table 2 Numerical called given in the AxiomGT1.calls.txt file and their genotype equivalent.

Table 3 Plate weight required to balance sample plate at each stage of sample preparation. Weights are additional to the base weight of the plate which will vary with manufacturer.

Tables

Table 1

Accession	Genome
Hexaploid	
108 *Triticum aestivum* elite varieties	ABD
27 *Triticum aestivum* landrace accessions	ABD
1 *Thinopyrum intermedium*	SJJ
133 Avalon x Cadenza mapping population	ABD
64 Savannah x Rialto mapping population	ABD
60 Synthetic x Opata mapping population	ABD
32 Deletion Lines	
20 Nullisomic Lines	
Tetraploid	
8 *Triticum turgidum* ssp. *Durum*	AB
1 *Triticum timopheevii*	AG
1 *Aegilops peregrina* (*Ae. variabilis*)	SU
Diploid	
1 *T. monococcum* ssp. *aegilopoides* (*T. urartu*)	A
11 *Ae. tauschii* accessions	D
1 *Thinopyrum elongatum*	E
1 *Thinopyrum bessarabicum*	J
1 *Secale cereale*	R
1 *Aegilops speltoides*	S
1 *Aegilops markgrafii* (*Ae. caudata*)	T
1 *Amblyopyrum muticum* (*Ae. mutica*)	T
Decaploid	
1 *Thinopyrum ponticum*	JJJJsJs

Table 2

Axiom Genotype	
-1	Null
0	AA
1	AB
2	BB

Table 3

Sample Preparation Stage	Additional Weight
Empty Plate	-
After Denaturation Solution	-
After Neutralisation Solution	15g
After Amplification Master Mix	37g
After Fragmentation Master Mix	42g
After Fragmentation Stop Solution	42g
End of Precipitation	124g
Resuspension	~5g but varies with pellet size

Figures

Figure 1

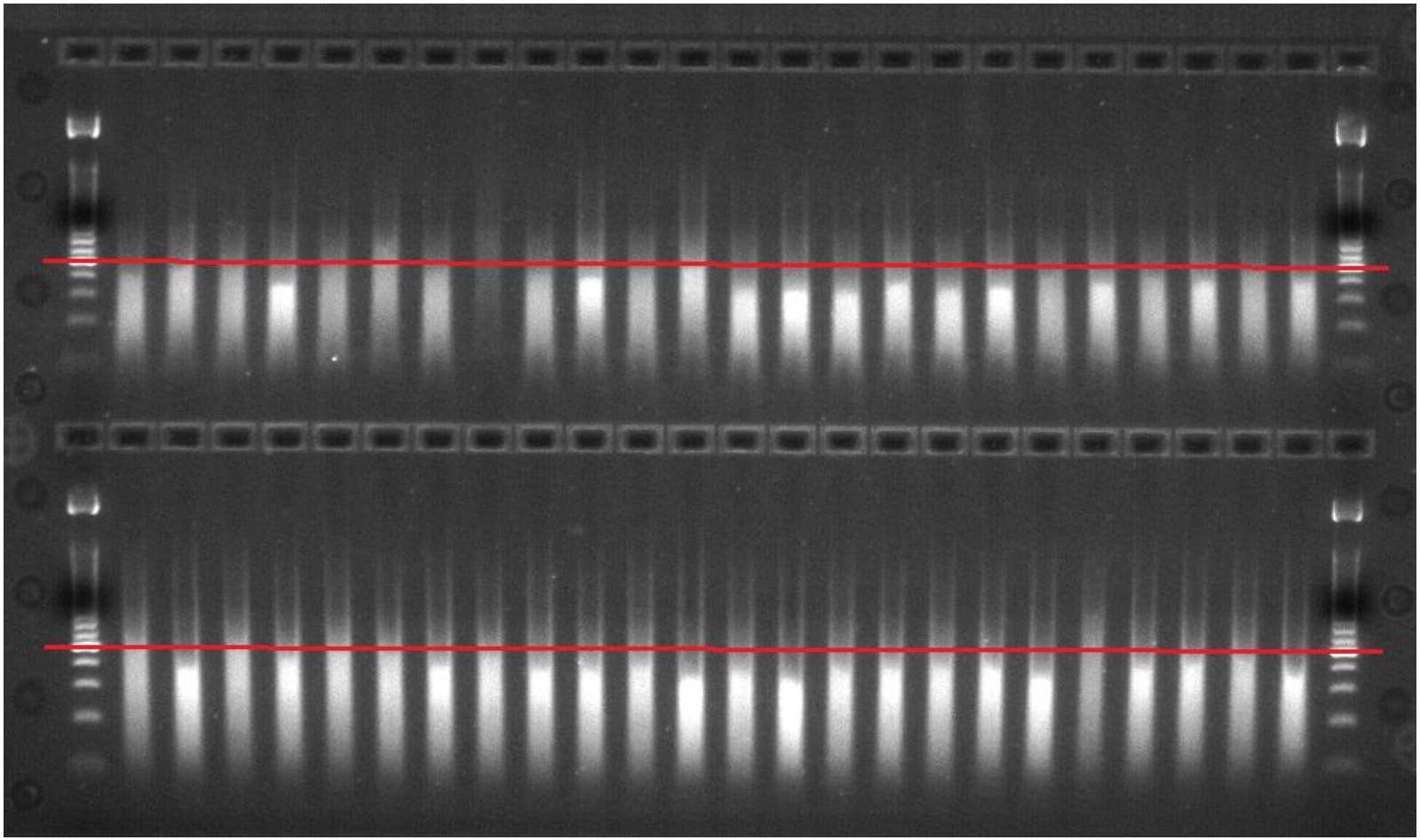
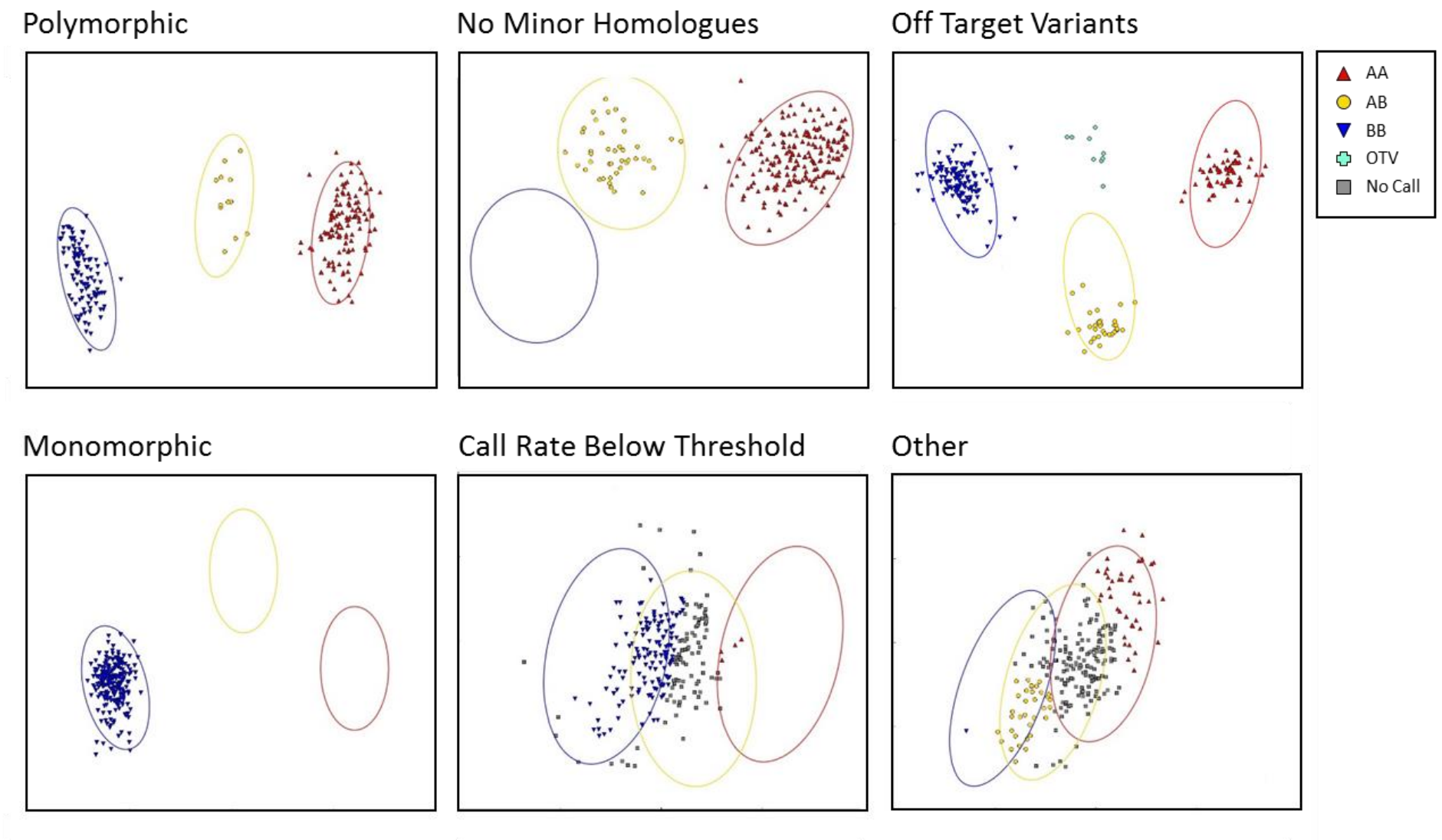


Figure 2


Polymorphic
No Minor Homologues
Off Target Variants
AA
AB
BB
OTV
No Call
Monomorphic
Call Rate Below Threshold
Other