Standard Operating Procedure (SOP)	
SOP Title	Library Preparation (96 well)
SOP number	GbS03
SOP Version	2

1. SCOPE

This SOP describes the laboratory procedure to capture regions surrounding Single Nucleotide Polymorphisms (SNPs) by high-throughput multiplex PCR, for Illumina MiSeq sequencing. This is called Genotyping by Sequencing (GbS). It encompasses the use of in-house validated rebalanced primer panels, which facilitates even amplification across all targeted loci. Substantive normalisation of library yields are achieved within the PCR itself, thus removing any requirement for upfront normalisation of DNA input or post library quantification and custom pooling.

Amplicons included in the two-step PCR protocol described within this SOP are designed to capture a narrow size range (190-250bp inclusive of priming sites) to complement the sequencing length of the MiSeq v2 300 kit (Illumina, San Diego, CA), whilst enabling efficient Ampure XP beads size selection away from contaminating smaller off-target amplification products.

Primers are rebalanced according to GbS testing and rebalancing SOP (see GbS02_Testing_and_balancing). This maximises the evenness of coverage across all amplicons in the multiplex.

For further information on this process, see Appendix 1.

2. MATERIALS REQUIRED:

2.1 REAGENTS AND MATERIALS

Primers		
Rebalanced “working pool” oligonucleotide GbS panel @ 40nM GCR1, GCR2, & Speciation		
i7 & i5 lyophilised GbS primers		
Reagents	***Supplier***	***Cat. No.***
QIAGEN Multiplex PCR polymerase (x2)	Qiagen	206145
Ampure XP beads (SPRI) - Beckman Coulter	Fisher Scientific	NC9959336
Nuclease free water (NFW) - Ambion	Fisher Scientific	AM9930
Ethanol (75%)		
T0.1E buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0)		
Buffer Elution Buffer (Buffer EB, 10 mM Tris-Cl, pH 8.5)	Qiagen	19086
KAPA SYBR FAST ABI Prism qPCR Kit, KAPA SYBR FAST qPCR Master Mix (2X) Library Quantification DNA Standards 1 – 6 Library Quantification Primer Premix (x10)	Sigma-Aldrich	KK4605
Genomic DNA High-sensitivity reagents	Agilent	5067-5585
Samples		
Test DNA sample. 2µL sWGA/PEP amplified DNA		
Materials	***Supplier***	***Cat. No.***
PCR machine		
Microplate heat sealer (PX1)	Bio-Rad	#1814000
Foil pierceable plate seal		
Tissue wipes (azowipes)		
Lo-bind Eppendorf tubes (1.5mL)		
Ice tray/cooling block		
96 well qPCR plate, skirted (AB2800)	Fisher Scientific	10032013
Adhesive PCR Film Polyester plate seals	Thermo Fisher Scientific	AB0558
Tube/ Microplate vortexer		
Magnetic tube rack (Invitrogen DynaMag)	Thermo Fisher Scientific	12321D
Non-Magnetic tube rack		
Centrifuge (requires a 96 well plate adaptor)		
Agilent TapeStation System	Agilent	
Genomic DNA High Sensitivity D1000 ScreenTape	Agilent	5067-5584
qPCR Instrument (ROCHE LightCycler 480II)	ROCHE	
White 96 well qPCR plate - Semi-Skirted	StarLab	I1402-9909
Optically clear plate seal	StarLab	E2796-9795
8-channel Adjustable Multichannel (0.5-10µL)		
12-channel Adjustable Multichannel (10-100µL)		
Pipettes (1000, 200, 100, 20 and 1 µL)		

2.2 PCR_1 PRIMER PANEL

GbS primers (100nmol scale synthesis from IDT, supplied re-suspended in T0.1E at 500µM in a 96 deep well plates); it is assumed that the primers have been ordered with paired forward and reverse primers in separate plates (e.g. forward primers in Plate 1 A1, B1…, reverse primers in Plate 2 A1, B1…)

PCR is sensitive to inhibitors, which may be introduced due to a DNA source, or introduced via DNA extraction. A review article can be found here for further details.

Oyola, S.O., Ariani, C.V., Hamilton, W.L. et al. Whole genome sequencing of Plasmodium falciparum from dried blood spots using selective whole genome amplification. Malar J 15, 597 (2016).

2.3 PCR_2 Primer Panel

Pre-made PCR_2 tag plates as produced as in Tag plate preparation SOP.

2.4 SAMPLES AND BATCH SIZE

Input DNA does not require prior quantification or normalisation. Normalisation of library yields are achieved during the PCR itself. For the human QC plexes, consistent yields are observed from 3-600ng input.

All samples should have a volume of **2µL**.

Each plate must contain at least one negative control (i.e. **2µl of T0.1E**).

Notes - Start PCR_1 setup first thing in the morning to enable processing through to PCR_2 within a working day.

N.B. Highly dilute samples require overnight PCR_1 (see 3.1.10). Overnight PCR_1, plates should be placed on the PCR machine at around 3:30pm. The plates will be ready for PCR_2 setup at 8:30am.

A large number of negative/empty wells generates significant amounts of smaller PCR artefacts (primer dimers), which can be problematic to fully remove by size selection and will preferentially sequence. **Therefore 96-well plates are required to be >80% full.**

3. METHODOLOGY

3.1 METHODOLOGY PCR_1

During PCR_1, genomic priming takes place via a low number of PCR cycles utilising a long annealing stage, which allows the highly dilute primers to specifically anneal to their genomic target (see Appendix 2).

PCR Reagents
QIAGEN Multiplex PCR polymerase (x2)
Rebalanced oligo\nucleotide GbS panel 40nM (pool)
Nuclease free water (NFW)

Samples
PEP or sWGA DNA - 2µL

PCR Equipment		
PCR machine	Foil pierceable plate seal	1000 µl pipette
96 well qPCR plate	Centrifuge	200 µl pipette
Ice tray/cooling block	Tissue wipes (Azowipes)	20 µl pipette
Microplate heat sealer	Multichannel (8 or 12)	

<table>
<tr><th colspan="2">Methodology PCR_1 – Pre-PCR room</th></tr>
<tr><td>3.1.01</td><td>Clear out a sufficient work area and wipe down the lab bench surface and pipettes with azowipes or similar.</td></tr>
<tr><td>3.1.02</td><td>Prepare ice tray/cooling block for use after PCR_1.</td></tr>
<tr><td>3.1.03</td><td>Defrost 1 plate per Target containing according to the batch size being processed (see Table 1). Do not forget to label plates.
<table>
<tr><th>Target</th><th>Number of amplicons/panel</th><th>Number of test plates</th></tr>
<tr><td>GRC1</td><td>68</td><td>1</td></tr>
<tr><td>GRC2</td><td>66</td><td>1</td></tr>
<tr><td>Speciation</td><td>2</td><td>1</td></tr>
</table>
Table 1: Number of plates of the same panel that may be sequenced together as a batch (GRC1, GRC2 and Speciation may be pooled for sequencing).</td></tr>
<tr><td>3.1.04</td><td>Pulse centrifuge to ensure all liquid are at bottom of plate(s).</td></tr>
</table>

<table>
<tr><td>3.1.05</td><td>Transfer 2µl of each sample to the required number of plates making sure to copy the source plate identically. Add the negative control (2µl of T0.1E) to the control wells.</td></tr>
<tr><td>3.1.06</td><td>Make the PCR_1 mastermix for the number of plates being processed as shown below (Table 2) in a Lo-bind Eppendorf tubes (1.5mL). The “x110” will be sufficient for a single 96 well plate. This may be prepared at room temperature. N.B. It is important to pipette up and down when adding the primer panel to ensure the entire volume is transferred into the master mix.
<table>
<tr><th></th><th></th><th>1 plate</th><th>2 plates</th></tr>
<tr><th>Components:</th><th>Per well (µL)</th><th>x110 (µL)</th><th>x250 (µL)</th></tr>
<tr><td>NFW</td><td>3.42</td><td>377.6</td><td>855</td></tr>
<tr><td>Qiagen mastermix (x2)</td><td>5.5</td><td>605</td><td>1375</td></tr>
<tr><td>Primer pool (40nM)</td><td>0.068</td><td>7.4</td><td>17</td></tr>
<tr><td>TOTAL</td><td>9.0</td><td>990</td><td>2247</td></tr>
</table>
Table 2: 2µL DNA in sample plate; volumes of mastermix for 1 or 2 plates including excess.</td></tr>
<tr><td>3.1.07</td><td>Gently mix the master mix by vortexing and pulse spin down.</td></tr>
<tr><td>3.1.08</td><td>Add 9µL of PCR_1 master mix to each well of a plate and mix.</td></tr>
<tr><td>3.1.09</td><td>Apply a foil pierceable plate seal on top of the plate, red strip facing upwards.</td></tr>
<tr><td>3.1.10</td><td>Using the plate sealer heat seal the lid to the plate (175 °C for 5 seconds).
NOTE: It is important to use these exact settings, as too high a temperature will melt the top of the wells and too low will lead to incomplete sealing and evaporation during PCR.</td></tr>
<tr><td>3.1.11</td><td>Centrifuge at 1000g for 20 seconds at 4°C</td></tr>
</table>

For standard concentration proceed to 3.1.12a or for highly dilute samples proceed to 3.1.12b

3.1.12a

Transfer to a genotyping PCR machine, tighten the lid to fingertip tight and start the required program using the parameters below (Table 3: PCR_1):

Falciparum (GRC1, Spec and GRC2) PCR_1			
STEP	**Temperature**	**Duration**	**Cycles**
Step 1	**95°C**	15:00 min	
Step 2	**95°C**	00:20 sec	} Total of 5 cycles
Step 3	**51°C**	40:00 min	
Step 4	**60°C**	03:00 min	
Step 5	**4°C**	∞	

Table 3: PCR_1 programs and cycling conditions.

3.1.12b

If the samples are **highly dilute** then these will require an overnight PCR (Table 4: PCR_1):

OVERNIGHT Falciparum (GRC1, Spec and GRC2) PCR_1			
STEP	**Temperature**	**Duration**	**Cycles**
Step 1	**95°C**	15:00 min	
Step 2	**95°C**	00:20 sec	} **Total of 15 cycles**
Step 3	**51°C**	40:00 min	
Step 4	**60°C**	03:00 min	
Step 5	**4°C**	∞	

Table 4: PCR_1 programs and cycling conditions for overnight PCR.
Start PCR_1 at around 3:30pm and will finish at about 8:30 am for PCR_2.

3.1.13	Prepare ice tray/cooling block for use after PCR_1.
3.1.14	Once the PCR machine program reaches the 4°C hold the PCR_1 program is complete, **immediately place plates on ice/cold block.**
3.1.15	Proceed to next stage (see section 3.2); it is recommended that the plate is left at 4°C for no longer than three hours.

3.2 METHODOLOGY PCR_2

The second PCR involves no additional reagents or clean up after the PCR_1. A volume of PCR_1 will be transferred to a dried down dual indexed tag plate. These indexed tag primers anneal to and extend from the 5'-tails incorporated during PCR_1. This enables the capability to multiplex up to 1536 samples per sequencing batch (See Appendix 2).

PCR Reagents
PCR_2 Tag plates (i5 and i7 lyophilised primers)
Nuclease free water (NFW)

PCR Equipment		
Ice tray/cooling block	Foil pierceable plate seal	Centrifuge
PCR machine	Tube/ Microplate vortexer	Multi-channel (10µL)
Microplate heat sealer (PX1)		20µl pipette

Methodology PCR_2 plate preparation	
It is important to continue to PCR_2 as soon as possible after PCR_1 has finished.	
3.2.01	Take the completed PCR_1 plates and PCR_2 tag plates and centrifuge at 2000g for 1 minute (at 4°C) and place back on ice tray/cooling block.
3.2.02	Place the PCR_1 plates containing the test reactions and a pre-prepared PCR_2 96 well tag plates on ice. Make a note of which i5 primers are used, this information is required at the submission stage later.
3.2.03	Remove the seals from the PCR and Tag plates.
3.2.04	Using a 10µL multichannel pipette (8 or 12 channel), transfer 10µL of the PCR_1 reaction volume into the equivalent well of the PCR_2 tag 96 well plates and mix thoroughly (10 times, see Appendix 2). Ensure that the dried tag primers in the PCR_2 plate are fully re-suspended.
3.2.05	Apply a foil pierceable plate seal on top of the plate, red strip facing upwards.
3.2.06	Using the plate sealer heat seal the lid to the plate (175°C for 5 seconds). NOTE: It is important to use these exact settings, as too high a temperature will melt the top of the wells and too low will lead to incomplete sealing and evaporation during PCR.
3.2.07	Keep created PCR_2 Tag plate on ice and transfer to a centrifuge. Centrifuge plates at 2000g for 1 minute (at 4°C). Immediately place back on ice.

Methodology PCR_2 – Post-PCR room

PLEASE NOTE AT THIS STAGE IF POSSIBLE IT IS ADVISABLE TO PROCEED TO THE POST-PCR ROOM

3.2.08 Leave the plate on ice.

If the PCR machine is capable to running a nesting program proceed to 3.2.09a. However, if a nesting function is not available proceed to 3.2.09b.

3.2.09a **Pre-heat** the PCR machine by closing the lid and starting the PCR machine with the required program using the parameters below (Table 5: PCR_2).

GRC1 / GRC2 / Speciation (Subcycling PCR_2)				
Step 1	95°C	∞		
Step 2	95°C	00:20 sec		Total of 31 cycles
Step 3	68°C	00:15 sec	Total of 4 cycles	
Step 4	60°C	00:15 sec		
Step 5	68°C	03:00 min		
Step 6	4°C	∞		

Table 5: PCR_2 programs and cycling conditions

3.2.09b If the PCR machine does not have a nested function then use the following parameters (Table 6).

GRC1 / GRC2 / Speciation (Subcycling PCR_2)			
Step 1	95°C	∞	
Step 2	95°C	00:20 sec	Total of 31 cycles
Step 3	68°C	00:15 sec	
Step 4	60°C	00:15 sec	
Step 5	68°C	00:15 sec	
Step 6	60°C	00:15 sec	
Step 7	68°C	00:15 sec	
Step 8	60°C	00:15 sec	
Step 9	68°C	00:15 sec	
Step 10	60°C	00:15 sec	
Step 11	68°C	03:00 min	
Step 12	4°C	∞	

Table 6: PCR_2 programs and cycling conditions

3.2.10 Once the temperature has been reached, open the lid and place the plate on the PCR machine and then close.

3.2.11 Select “continue/skip/next” to move to Step 2 to begin PCR_2.

3.2.12 The PCR can remain on the PCR machine overnight at 4°C without detriment.

3.2.13 Once the PCR is complete, you can either move directly to pooling or plates may be stored at -20°C for up to one week.

As the Ampure XP beads size selection is extremely sensitive to errors in pipetting volumes, it is recommended to ensure that the pool volume carried forward into size selection is a constant 100µL.

3.3 POOLING, PURIFICATION & SIZE SELECTION.

Size selection and clean-up is performed with a 2 x Ampure XP beads. Under these conditions the majority of small primer dimers and unincorporated primers <170bp will remain in the supernatant whilst the desired amplicons bind to the beads (see Appendix 3).

Reagents
Ampure XP beads (SPRI)
75 % ethanol
Buffer EB

Equipment		
Centrifuge	Tube/ Microplate vortexer	1000µL pipette
Magnetic tube rack		200µL pipette
Non-Magnetic tube rack		100µL pipette
1.5ml Lo-bind Eppendorf tubes		20µl pipette

Pooling of PCR products	
3.3.01	Remove Ampure XP beads from fridge and allow to warm to room temperature (approx. 30min).
3.3.02	Take plates off the PCR machine and place on ice.
3.3.03	Label 3 new 1.5ml Lo-bind Eppendorf tubes and label them • TARGET pool stock (e.g. GRC1 pool stock).
3.3.04	Transfer the contents of the 96 well plate into a fresh 96 well plate using an multi-channel pipette (either 8 or 12). Mix and transfer to the labelled 1.5ml Lo-bind Eppendorf tube (GbS pool stock) using a pipette and discard the PCR_2 plate. **NOTE: When pooling keep each Target plate separate.**
3.3.05	At this stage, it is possible to view the size profile of the pool by analysing on an Agilent TapeStation (Agilent High Sensitivity D1000 Kit). An example size profile is shown in Appendix 5.

Size selection and Clean-up	
3.3.06	Vortex Ampure XP beads to re-suspend
3.3.07	Accurately transfer 100µL of pooled PCR product into a new 1.5ml Lo-bind Eppendorf. Make sure the tube is clearly labelled with target name (e.g. GRC1).
3.3.08	Then add 75µL of Ampure XP beads and close the lids.
3.3.09	Vortex to mix and pulse spin down to collect all of the liquid at the bottom of the tube.
3.3.10	Place on a non-magnetic rack at room temperature for 5 minutes.
3.3.11	Transfer tube to a magnetic tube rack until clear (approx. 3-4min).
3.3.12	Once the supernatant is clear and all beads are captured on the side of the magnet, remove and discard the supernatant. Be careful not to disturb or aspirate the beads.
3.3.13	Using a 1000µL pipette and keeping the tube on the rack, wash the beads by adding 700µL of 75% ethanol, being careful not to disturb the beads captured on the magnet.
3.3.14	After 30 seconds, carefully remove and discard the ethanol
3.3.15	Repeat the ethanol wash once more (3.3.13 and 3.3.14)
3.3.16	Close the tube lid and pulse centrifuge to draw final residual ethanol to the bottom and remove.
3.3.17	With the lid open, air dry on a magnetic rack at room temperature for approximately 2 minutes.
3.3.18	Off magnet, re-suspend the beads in 105µl of Buffer EB and vortex to mix.
3.3.19	Incubate **off-magnet** for 5 minutes to release the target product into solution. Spin down if any splashes have occurred on the side of the tube.
3.3.20	Transfer to the magnetic rack.
3.3.21	Label 3 new 1.5ml Lo-bind Eppendorf's (1 per Target): First TARGET size selections and the relevant I5 primer (e.g. 1st GRC1-T5-1).
3.3.22	Once the supernatant is clear (approx. 3-4min) and beads are captured on the magnet, transfer all of the supernatant containing the purified size selected PCR products to the lo bind 1.5ml Eppendorf tube labelled “1st size selections"

Final size selection on ELUTION (see Appendix 4)	
3.3.23	Vortex Ampure XP beads to re-suspend.
3.3.24	Transfer 100µl of the “First TARGET selections" to a new Eppendorf. Label the new Eppendorf with “Second TARGET selection” and the relevant I5 primer (e.g. 2nd GRC1-T5-1).
3.3.25	Add 75µL of Ampure XP beads to the “2nd TARGET selection” Eppendorf. Vortex, pulse centrifuge and leave at room temperature for 5 minutes.
3.3.26	Transfer tube to a magnetic tube rack until clear (approx. 3-4min).
3.3.27	Once the supernatant is clear and all beads are captured on the side of the magnet, remove and discard the supernatant. Be careful not to disturb or aspirate the beads.
3.3.28	Using a 1000µL pipette and keeping the tube on the rack, wash the beads by adding 700µL of 75% ethanol, being careful not to disturb the beads captured on the magnet.
3.3.29	After 30 seconds, carefully remove and discard the ethanol.
3.3.30	Repeat the ethanol wash once more (3.3.29 and 3.3.30)
3.3.31	Close the tube lid and pulse centrifuge to draw final residual ethanol to the bottom and remove.
3.3.32	With the lid open, air dry on a magnetic rack at room temperature for approximately 2 minutes. NOTE: At this point, the size selected DNA will be dried onto the beads.
3.3.33	Re-suspend beads with 22µL of Buffer EB by pipetting up and down.
3.3.34	Transfer to non-magnetic rack and incubate “off magnet” for 2 minutes.
3.3.35	Transfer the tube containing eluted size selected libraries to the magnetic rack.
3.3.36	Allow supernatant to clear as beads are captured on the magnet (approx. 3-4min).
3.3.37	Transfer supernatant containing the purified size selected PCR products to 1.5ml Lo-bind Eppendorf tube labelled “TARGET Pool” (e.g. GRC1 pool).
3.3.38	The **remainder of the pooled PCR stock** should be stored at -20°C.

4. QUALITY CONTROL

4.1 AGILENT TAPESTATION

The efficiency of the clean-up and size selection can be assessed by running 1μL of the “TARGET Pool” on an Agilent TapeStation using an Agilent High Sensitivity Kit.

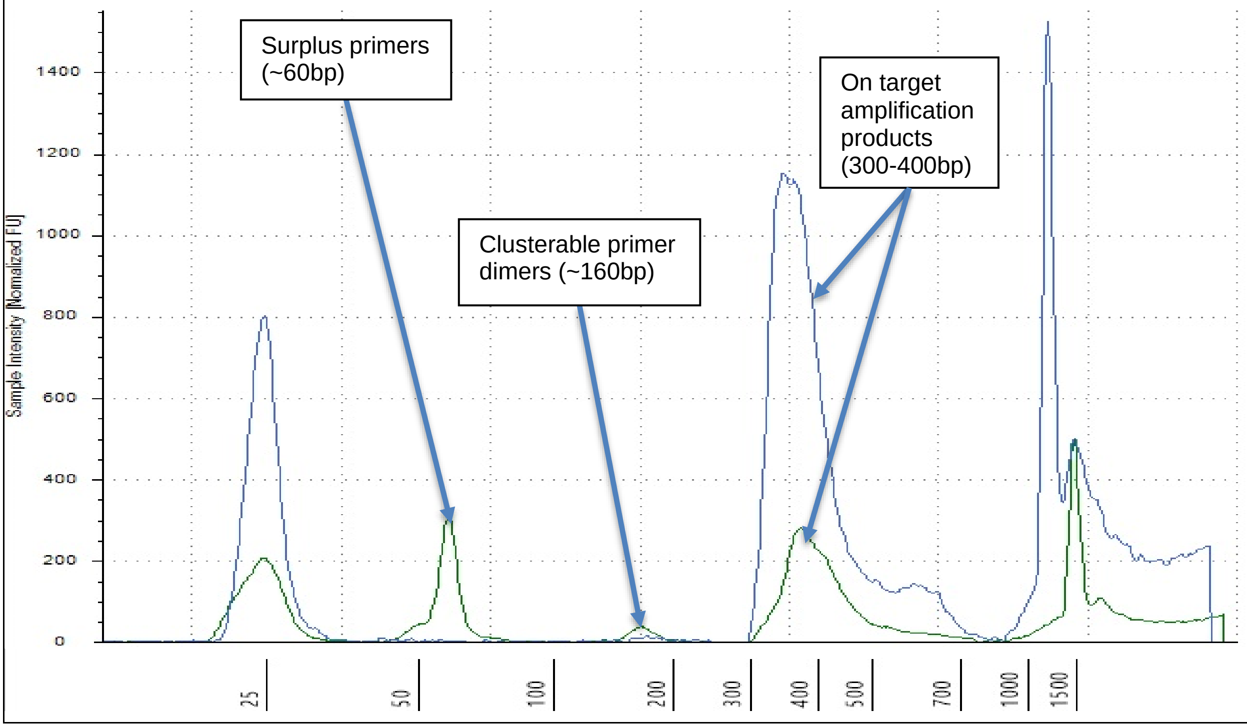
Note: small peak at ~60bp, are the surplus primers and the small peak at ~160bp, are the result of primer dimers between first round genomic PCR primers. Due to their size, these will preferentially cluster during sequencing and result in loss of on-target data.

If the peaks are still present post clean-up and size selection then repeat Ampure XP beads size selection until the ~160bp peak is barely visible by TapeStation analysis.

See Agilent TapeStation SOP.

4.1.01	Assess the efficiency of the size selection by running on an Agilent TapeStation using an Agilent DNA High Sensitivity Kit, while keeping tubes on ice.
4.1.02	A typical profile obtained of an Ampure XP size selected MxPCR, run on an Agilent TapeStation High Sensitivity DNA chip is shown in Figure 1.

Figure 1. A typical profile obtained of an Ampure XP size selected GbS pool, run on an Agilent TapeStation High Sensitivity DNA chip. The green profile shows the amplification product pre-clean up and size selection. The surplus primers and primer dimers are present. The Blue profile shows the amplification products post clean-up and size selection. Note surplus primers and primer dimers have been removed.



Surplus primers (~60bp)

Clusterable primer dimers (~160bp)

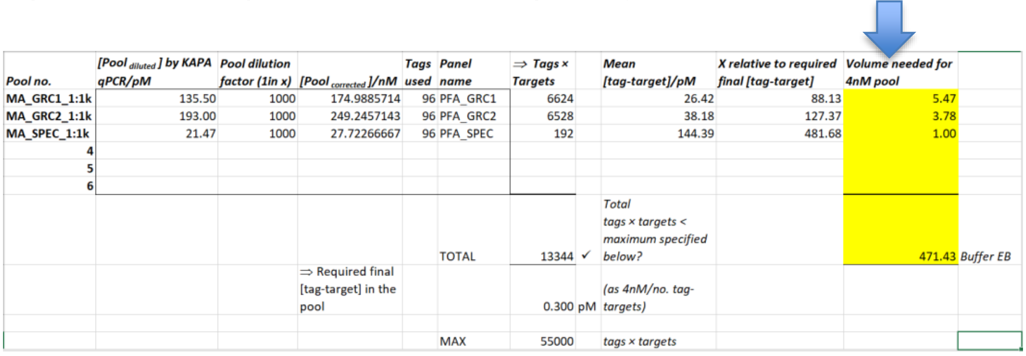
On target amplification products (300-400bp)

4.2 PCR QUANTIFICATION

Library quantification is performed by amplifying the set of five pre-diluted DNA Standards and diluted library samples by qPCR, using the KAPA SYBR FAST qPCR mastermix and primers targeting the Illumina P5 and P7 flow cell oligo sequences. The average Cq score for each DNA Standard is plotted against log10 (concentration in pM) to generate a standard curve. The concentrations of diluted library samples are then calculated against the standard curve, using absolute quantification.

See qPCR Quantification SOP.

5. MISEQ POOL CALCULATOR

<table>
<tr><td>5.0.01</td><td>Open the excel spreadsheet “flexible pooling calculator”

<table>
<tr><th>Pool no.</th><th>[Pool $_{\text{diluted}}$] by KAPA qPCR/pM</th><th>Pool dilution factor (1in x)</th><th>[Pool $_{\text{corrected}}$]/nM</th><th>Tags used</th><th>Panel name</th><th>⇒ Tags × Targets</th><th></th><th>Mean [tag-target]/pM</th><th>X relative to required final [tag-target]</th><th>Volume needed for 4nM pool</th><th></th></tr>
<tr><td>MA_GRC1_1:1k</td><td>135.50</td><td>1000</td><td>174.9885714</td><td>96</td><td>PFA_GRC1</td><td>6624</td><td></td><td>26.42</td><td>88.13</td><td>5.47</td><td></td></tr>
<tr><td>MA_GRC2_1:1k</td><td>193.00</td><td>1000</td><td>249.2457143</td><td>96</td><td>PFA_GRC2</td><td>6528</td><td></td><td>38.18</td><td>127.37</td><td>3.78</td><td></td></tr>
<tr><td>MA_SPEC_1:1k</td><td>21.47</td><td>1000</td><td>27.72266667</td><td>96</td><td>PFA_SPEC</td><td>192</td><td></td><td>144.39</td><td>481.68</td><td>1.00</td><td></td></tr>
<tr><td>4</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>5</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>6</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td>TOTAL</td><td>13344</td><td>✓</td><td>Total tags × targets < maximum specified below?</td><td></td><td>471.43</td><td>Buffer EB</td></tr>
<tr><td></td><td></td><td></td><td>⇒ Required final [tag-target] in the pool</td><td></td><td></td><td>0.300</td><td>pM</td><td>(as 4nM/no. tag-targets)</td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td>MAX</td><td>55000</td><td></td><td>tags × targets</td><td></td><td></td><td></td></tr>
</table></td></tr>
<tr><td>5.0.02</td><td>For each sample, enter their name under the “pool No.”</td></tr>
<tr><td>5.0.03</td><td>For each sample, enter the quantity mean, as determined by the qPCR under the “[pool $_{\text{diluted}}$] by KAPA qPCR/pM”.</td></tr>
<tr><td>5.0.04</td><td>For each sample enter the dilution factor under the “Pool dilution factor (1in x)”</td></tr>
<tr><td>5.0.05</td><td>For each sample, enter 96 under “Tags used”.</td></tr>
<tr><td>5.0.06</td><td>For each sample, select panel from the drop down menu in “panel name”.
• PFA_GRC1
• PFA_GRC2
• PFA_SPEC</td></tr>
<tr><td>5.0.07</td><td>Number of Tags and Targets is calculated:
Check that the “TOTAL” (e.g. 13,344) is less than the “MAX” “tags x targets” (55,000).
If the number exceeds the “MAX” (55,000), then adjust the constituents of the pool to reduce the number.
If the number is lower than the “MAX” (55,000) then a “✓” will appear and proceed to preparation of the 4nM pool.</td></tr>
<tr><td>5.0.08</td><td>Completing these fields will give you the total volume needed for your pool (blue arrow).”</td></tr>
<tr><td>5.0.09</td><td>Label a new Eppendorf with “GbS MiSeq Pool” and add the quantity of each pool and Buffer EB as calculated by the Excel spreadsheet.</td></tr>
</table>

6. MISEQ SUBMISSION

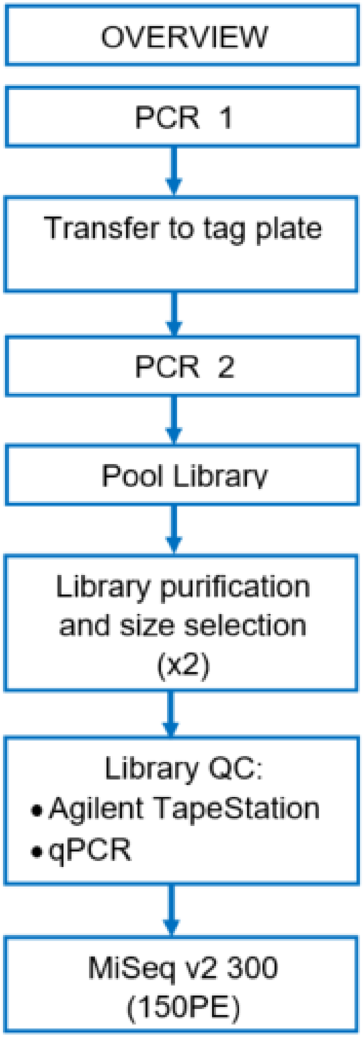
6.0.01	Material ready for sequencing.

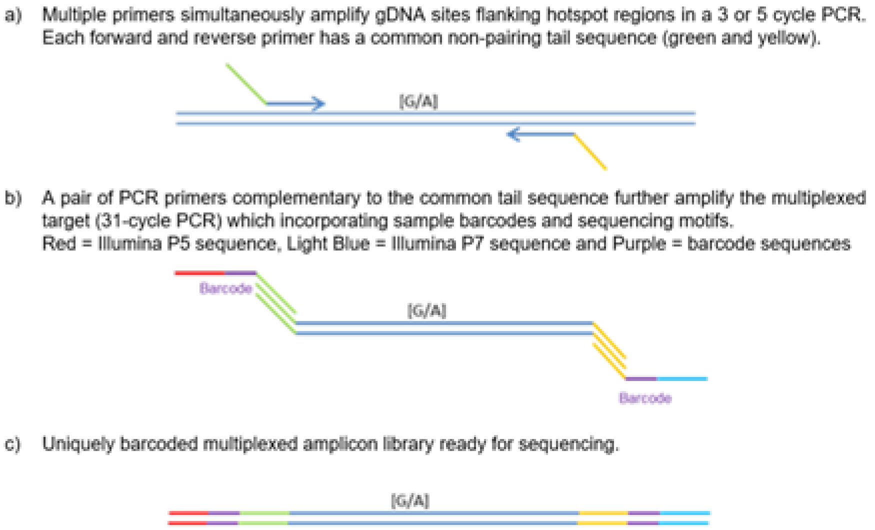
APPENDIX 1 – OVERVIEW

Amplicons included in the two-step PCR protocol described within this SOP are designed to capture a narrow size range (190-250bp inclusive of priming sites) to complement the sequencing length of the MiSeq v2 300 kit (Illumina, San Diego, CA), whilst enabling efficient Ampure XP size selection away from contaminating smaller off-target amplification products.

The first PCR reaction involves a limited cycle PCR during which a low concentration of seeding primers specifically anneal to the genomic template. Low primer concentrations combined with long annealing times act to reduce target to target variation whilst maximising specificity. All gene specific forward and reverse primers, each have a common 5'-tail which are priming sites in the second PCR (see overview and schematic).

The second PCR involves no additional reagents or clean-up after the first PCR, aside from transfer of a volume of the reaction to a dried down dual indexed tag plate. These indexed tag primers anneal to and extend from the 5'-tails incorporated during PCR_1. This enables the capability to multiplex up to 1536 samples per sequencing batch. In practice, the number of samples that may be combined in any one sequencing pool is dictated by the number of amplicons in the GbS panel.



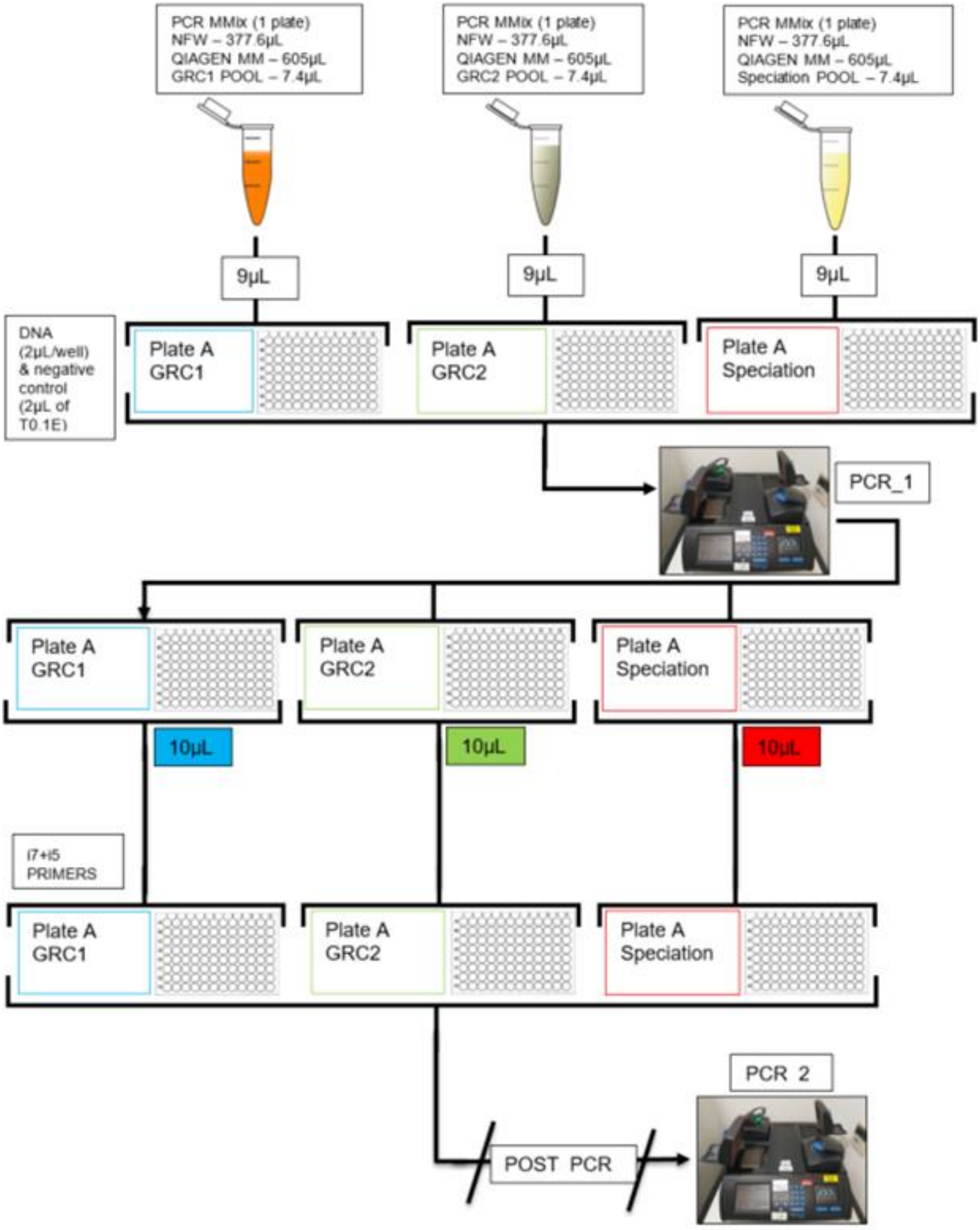

a) Multiple primers simultaneously amplify gDNA sites flanking hotspot regions in a 3 or 5 cycle PCR. Each forward and reverse primer has a common non-pairing tail sequence (green and yellow).

b) A pair of PCR primers complementary to the common tail sequence further amplify the multiplexed target (31-cycle PCR) which incorporating sample barcodes and sequencing motifs.
Red = Illumina P5 sequence, Light Blue = Illumina P7 sequence and Purple = barcode sequences

c) Uniquely barcoded multiplexed amplicon library ready for sequencing.

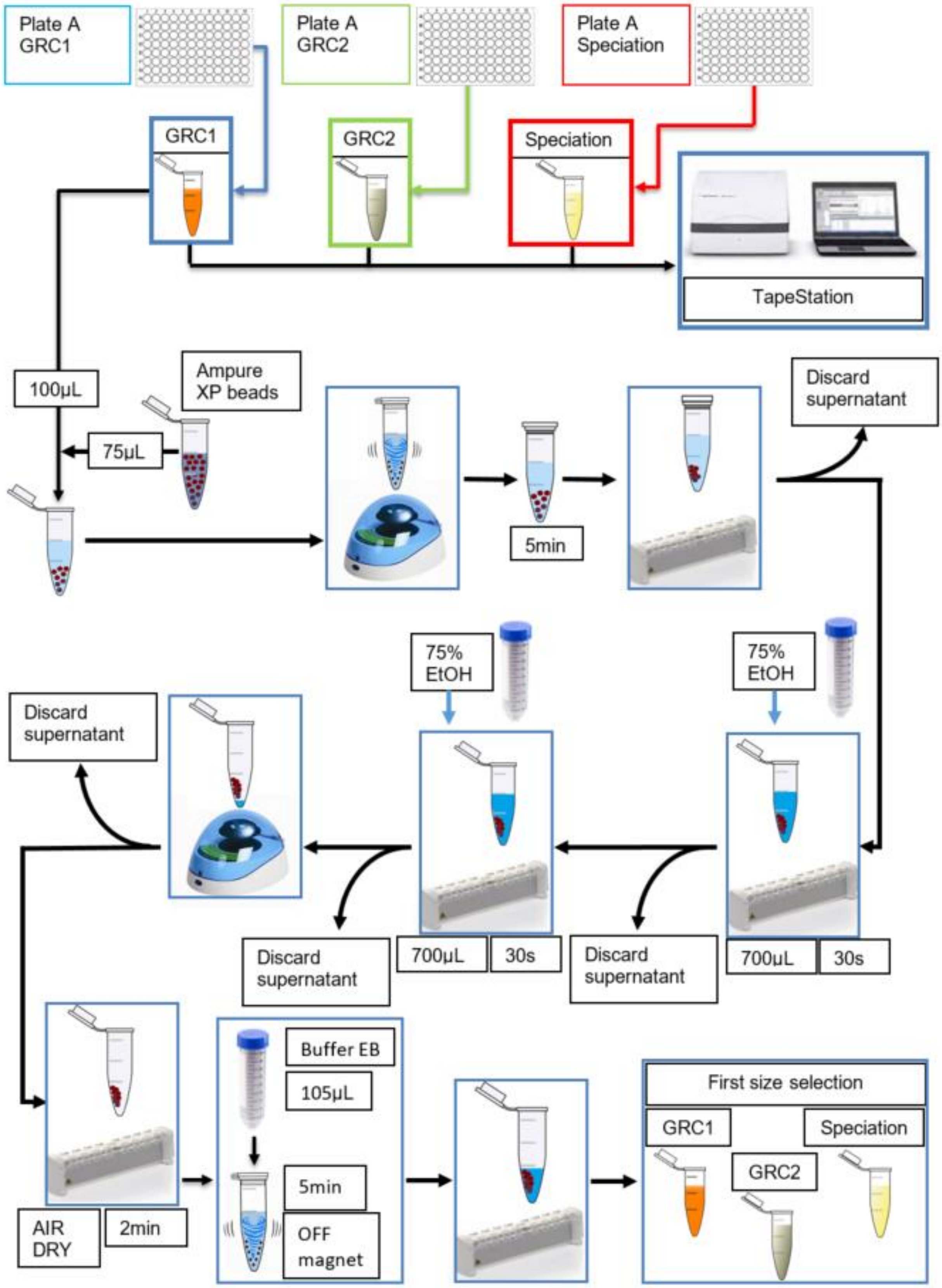
Schematic of GbS

APPENDIX 2 – PCR_1 & PCR_2

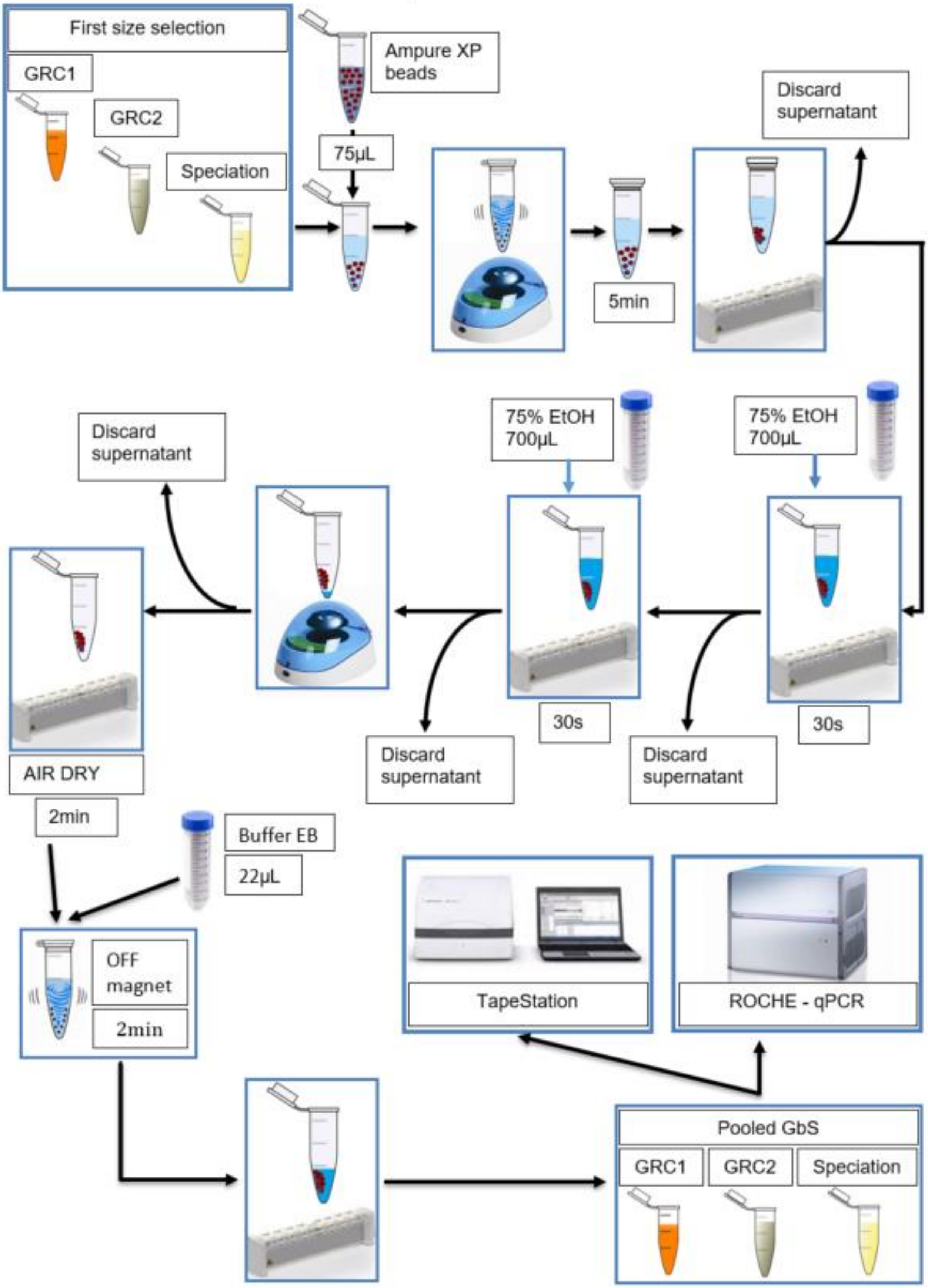


STORAGE - Once the PCR is complete overnight at 4°C without detriment, move directly to pooling, or plates may be stored at -20°C for up to one week

APPENDIX 3 – CLEAN-UP & SIZE SELECTION

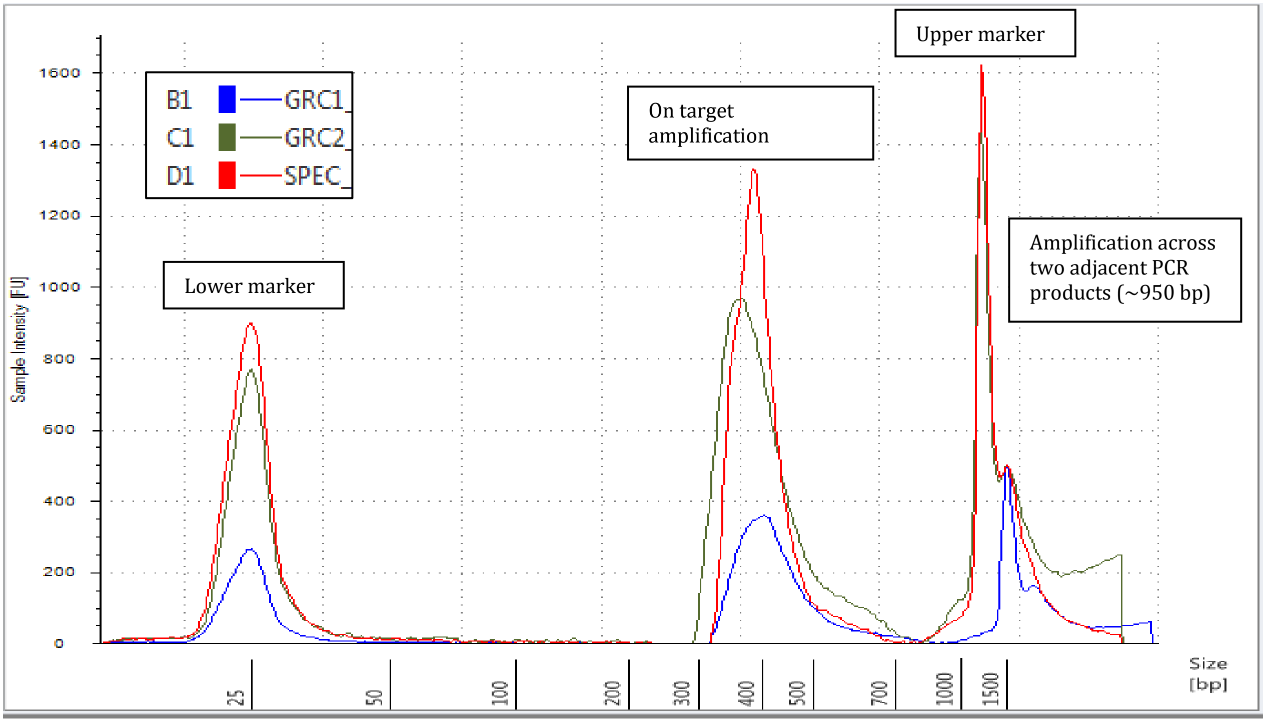


APPENDIX 4 – FINAL SIZE SELECTION & ELUTION



APPENDIX 5 – AGILENT TAPESTATION

An example size profile is shown below. A typical GbS product profile analysed following purification on an Agilent TapeStation. The blue trace is the GRC1, the green trace is the GRC2 and the red trace the Speciation.



Upper marker

On target amplification

Amplification across two adjacent PCR products (~950 bp)

Lower marker