Standard Operating Procedure (SOP)	
SOP Title	Laboratory protocol for testing and rebalancing for the new primer panels.
SOP number	GbS02a – 96 well
SOP Version	2

1. SCOPE

This SOP describes the procedure for determining the optimum concentration of each primer pair in a genotyping by sequencing (GbS) reaction for even amplification across the individual targets within a panel.

Before batch processing of samples, a new GbS design requires test amplification of DNA samples using an equimolar pool of primers. This is because the performance of individual primer pairs within a multiplex cannot be predicted *in silico* so must be determined empirically. The test libraries are then Ampure SPRI size selected and purified and following quality control checks pooled before sequencing as a 150PE MiSeq run in an identical manner to that of a production GbS run.

The resultant read data are analysed to provide a result of the level of coverage of each target as a percentage of all on target reads.

Concentrations of each primer pair are subsequently calculated and adjusted to compensate for over- and underperforming primers. A rebalanced pool is prepared and aliquoted for future use as a working panel.

2. MATERIALS REQUIRED:

2.1 REAGENTS AND MATERIALS

Primers		
Stock oligonucleotide GbS panel @ 500µM - GCR1, GCR2, & Speciation (SPEC)		
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
Genomic DNA High Sensitivity D1000 ScreenTape	Agilent	5067-5584
Genomic DNA High-sensitivity reagents	Agilent	5067-5585
KAPA SYBR FAST ABI Prism qPCR Kit, KAPA SYBR FAST qPCR Master Mix (2X) Library Quantification DNA Standards 1 – 6 Library Quantification Primer Premix (10X)	Sigma-Aldrich	KK4605
Samples	***Supplier***	***Cat. No.***
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 (heat sealer)	Bio-Rad	#1814040
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		
Agilent TapeStation System	Agilent	
Agilent – tube vortexer (IKA MS3)	included with TapeStation system	
Agilent consumables (loading tips, optical tube, optics cap, optical 96 well plate and foil seals)	Specific to TapeStation system	
qPCR Instrument (ROCHE LightCycler 480II)	ROCHE	
White 96 well qPCR plate	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, 20 and 1 µL)		
Filtered tips of various volumes (compatible with pipettes and multichannel)		

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 Primer Diluent

The primer diluent (T1E0.1 +0.01% v/v Triton X-100) is used in section 9 for the diluting of the pooled primers to 40nM before aliquoting.

To create the primer diluent do the following: Dilute 10mM Tris/1mM EDTA pH 8 (T10E1) 1in10 with 10mM Tris pH 8 (e.g. Qiagen EB) then spiking in the 0.01%v/v Triton X-100 detergent.

- Add 5mL of 10x TE (pH 8) to a 50mL falcon tube.
- Then add 45mL of EB (10mM Tris).
- Dispense 5µL of neat Triton X-100 onto the inner surface of the tube slowly, using a P10 pipette, i.e. not into the diluent.
- Vortex thoroughly until all the Triton X-100 is in solution.

2.5 Samples and Batch Size

Please note that sample selection is very important. For the *P. falciparum* primer panel only *P. falciparum* positive samples must be used. Furthermore, for the *P. vivax* primer panel only *P. vivax* positive samples must be used.

Input DNA does not require prior quantification or normalisation. Normalisation of library yields are achieved during the PCR itself.

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

Test DNA samples (of a representative type/quality/concentration to that expected to be supplied in production samples):

- All samples must have a volume of 2µL.
- 95 of the 96 wells must have sample (96th well is the negative control).
- Either a minimum of 95 individual samples can be used or 24 samples in multiples across the plate.

A large number of negative/empty wells will generate significant amounts of smaller PCR artefacts (primer dimers), which are problematic to fully remove by size selection and will preferentially sequence.

Therefore, the plate must have all 96 wells used for balancing.

3. LIBRARY PREPARATION

Primers
Plates containing GRC1, GRC2 and Speciation GbS primers. Forward and Reverse primers for each set.

PCR Reagents
QIAGEN Multiplex PCR polymerase (x2)
Stock oligonucleotide GbS panel @ 500μM - GCR1, GCR2, & Speciation (SPEC)
Nuclease free water (NFW)
T0.1E buffer

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		

3.1 Primer Pooling

See Appendix 4.	
3.1.01	Clear out a sufficient work area and wipe down the lab bench surface and pipettes with tissue wipes (Azowipes) or similar.
3.1.02	Defrost the forward and reverse primer plates for each target (GRC1, GRC2 & Speciation @ 500μM).
3.1.03	Once defrosted, gently vortex the plate to ensure the primers are fully re-suspended.
3.1.04	Centrifuge the plate to collect all the liquid at the bottom of the wells.
3.1.05	Add 7.5μL from each forward primer well into the equivalent wells of a new 96-well PCR plate (equivolume primer plate).
3.1.05	Add 7.5μL from each reverse primer well into the equivalent wells of the "equivolume primer plate" The resultant wells of the "equivolume primer plate" should contain 15μL of a 1:1 mixture of forward and reverse primers (at 250μM).
3.1.06	The "equivolume primer plate" can now be used as the stock for both the "equivolume" and "rebalanced" primer pools in order to minimise accumulated pipetting errors.

Equivolume pool	
3.1.07	Centrifuge the plate of paired primers.
3.1.08	Using a 12-channel remove 2µL from each primer pair well and pool into a single column of empty wells on a new 96 well plate.
3.1.09	Then using a single channel combine the subpools together to produce the "equivolume pool" into an appropriately labelled Eppendorf tube.
3.1.10	Calculate the concentration of the individual primers in the pool. Label the Eppendorf with calculated concentration. Concentration of each individual primers = 250µM÷(no. primer pairs) GRC1 = 250/68 = 3.67µM GRC2 = 250/67 = 3.73µM Speciation = 250/2 = 125µM
Working pool	
3.1.11	**Prepare a "working pool" aliquot of the stock at the working concentration (40nM) in a Lo-bind Eppendorf (dilute in T0.1E).** Dilution factor = ([stock]/40nM) GRC1 = (3.67µM x 1000)/40nM = 91.75 Pooled primer pool = 400/91.75 = 4.4µL Diluent (T0.1E) = 400-4.4 = 395.6µL GRC2 = (3.73µM x 1000)/40nM = 93.25 Pooled primer pool = 400/93.25 = 4.3µL Diluent (T0.1E) = 400-4.3 = 395.7µL Speciation (pre-dilution) = 1:25 = 1µL+24µL Diluent (T0.1E) = (125/25) = 5µM Speciation = (5µM x 1000)/40nM = 125 Pooled primer pool = 400/125 = 3.2µL Diluent (T0.1E) = 400-3.2 = 396.8µL
3.1.12	Aliquot the "equivolume pool" and "working pool" into 20µL aliquots and store at -20°C. After this proceed directly to PCR_1 (section 3.2).

Plate Storage: Seal the Forward primer, Reverse primer and mixed "equivolume primer plate" 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.

Plates can be stored at -20°C until required.

3.2 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 5).

PCR Reagents
Primer Panel "equivolume working pool" (40nM)
QIAGEN Multiplex PCR polymerase (x2)
Buffer EB
Nuclease free water (NFW)

Samples
Test DNA samples (sWGA or PEP 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		1µL pipette

Methodology PCR_1 – Pre-PCR room	
3.2.01	Clear out a sufficient work area and wipe down the lab bench surface and pipettes with tissue wipes (Azowipes) or similar.
3.2.02	Prepare ice tray/cooling block for use during PCR_1.
3.2.03	Aliquot **2µL** of **each** of the **test DNA samples** – a minimum 24 individual samples in multiples spaced over the 96 well plate (please ensure you meet minimum plate coverage - see section 2.5)
3.2.04	Add 2µL of buffer EB to the one or two empty wells (negative control wells).
3.2.05	Spin the plate down in the centrifuge to ensure that all liquid is collected at the bottom of the wells.
Note - Start PCR_1 setup first thing in the morning to enable processing through to PCR_2 within a working day.	

<table>
<tr><td>3.2.06</td><td>Make the PCR_1 mastermix for the number of plates being processed as shown below (Table 1) in a Lo-bind Eppendorf tubes (1.5mL). The “x110” will be sufficient for a single 96 well plate.

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>3.433</td><td>377.6</td><td>855</td></tr>
<tr><td>Qiagen MM (x2)</td><td>5.500</td><td>605</td><td>1375</td></tr>
<tr><td>“working pool” (40nM) 1/133</td><td>0.068</td><td>7.48</td><td>17</td></tr>
<tr><td>TOTAL</td><td>9</td><td>990</td><td>2247</td></tr>
</table>
Table 1: 2μL DNA in sample plate; volumes of mastermix for 1 or 2 plates including excess.</td></tr>
<tr><td>3.2.07</td><td>Gently mix the master mix by vortexing and pulse spin down.</td></tr>
<tr><td>3.2.08</td><td>Add 9μL of PCR_1 master mix to each well of a plate and mix.</td></tr>
<tr><td>3.2.09</td><td>Apply a foil pierceable plate seal on top of the plate, red strip facing upwards.</td></tr>
<tr><td>3.2.10</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>3.2.11</td><td>Centrifuge at 1000g for 20 seconds at 4°C</td></tr>
<tr><td>3.2.12</td><td>Transfer to a PCR machine, tighten the lid and start the required program using the parameters below (Table 2: PCR_1):

<table>
<tr><th colspan="4">Standard PCR_1 – GRC1, GRC2, SPEC</th></tr>
<tr><td>Step 1</td><td>95°C</td><td>15:00 min</td><td></td></tr>
<tr><td>Step 2</td><td>95°C</td><td>00:20 sec</td><td rowspan="2">} Total of 5 cycles</td></tr>
<tr><td>Step 3</td><td>51°C</td><td>40:00 min</td></tr>
<tr><td>Step 4</td><td>60°C</td><td>03:00 min</td><td></td></tr>
<tr><td>Step 5</td><td>4°C</td><td>∞</td><td></td></tr>
</table>
Table 2: PCR_1 programs and cycling conditions.</td></tr>
<tr><td>3.2.13</td><td>Prepare ice tray/cooling block for use after PCR_1.</td></tr>
<tr><td>3.2.14</td><td>Once the PCR Machine reaches “4°C” the PCR_1 program is complete, <u>immediately place on ice/cold block.</u></td></tr>
<tr><td>3.2.15</td><td>Proceed to next stage (see section 3); it is recommended that the plate is left at 4°C for no longer than three hours.</td></tr>
</table>

3.3 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 5).

PCR Reagents
PCR_2 Tag plates (i5 and i7 lyophilised primers) **– 400nM**
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	Tissue wipes (Azowipes)	20μl pipette

Methodology PCR_2 plate preparation	
It is important to continue to PCR_2 as soon as possible after PCR_1 has finished.	
3.3.01	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.
3.3.02	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.
3.3.03	Remove the seals from the PCR and Tag plates.
3.3.04	Using a 10μL multichannel pipette (either 8 or 12 channel), transfer 10μL of the PCR_1 reaction volumes into the equivalent well of the PCR_2 tag 96 well plates and mix thoroughly (mix by pipetting 10 times). Ensure that the dried tag primers in the PCR_2 plate are fully re-suspended.
3.3.05	Apply a foil pierceable plate seal on top of the plate, red strip facing upwards.
3.3.06	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.
3.3.07	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

3.3.08	Leave the plate on ice.
3.3.09	Pre-heat the PCR machine by tightening the lid and starting the PCR machine with the required program using the parameters below (Table 3: PCR_2).

GRC1 / GRC2 / SPEC (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 3: PCR_2 programs and cycling conditions

3.3.10	Once the temperature has been reached, loosen the lid and place the plate on the PCR machine and then tighten the lid.
3.3.11	Select continue/skip to move to Step 2 to begin the PCR_2 program.
3.3.12	The PCR can remain on the PCR machine overnight at 4°C without any impact on the downstream steps.
3.3.13	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.

3.4 POOLING, PURIFICATION & SIZE SELECTION.

Size selection takes place via a 0.75x SPRI. Under these conditions the majority of small primer dimers and unincorporated primers <170bp will remain in the supernatant whilst the desired amplicons bind onto the beads (see Appendix 6).

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

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

Pooling of PCR products	
3.4.01	Remove Ampure XP beads from fridge and allow to warm to room temperature (approx. 30min).
3.4.02	Take plates off the PCR machine and place on ice.
3.4.03	Label 3 new 1.5ml Lo-bind Eppendorf tubes and label them • TARGET pool stock (e.g. GRC1 pool stock).
3.4.04	Transfer the contents of the 96 well plate from PCR2 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.**
3.4.05	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 Section 4 & Appendix 7.

Size selection and Clean-up	
3.4.06	Vortex Ampure XP beads to re-suspend
3.4.07	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).
3.4.08	Then add 75µL of Ampure XP beads and close the lids.
3.4.09	Vortex to mix and pulse spin down to collect all of the liquid at the bottom of the tube.
3.4.10	Place on a non-magnetic rack at room temperature for 5 minutes.
3.4.11	Transfer tube to a magnetic tube rack until clear (approx. 3-4min).
3.4.12	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.
3.4.13	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.
3.4.14	After 30 seconds, carefully remove and discard the ethanol
3.4.15	Repeat the ethanol wash once more (3.4.13 and 3.4.14)
3.4.16	Close the tube lid and pulse centrifuge to draw final residual ethanol to the bottom and remove.
3.4.17	With the lid open, air dry on a magnetic rack at room temperature for approximately 2 minutes.
3.4.18	Off magnet, re-suspend the beads in 105µl of Buffer EB and vortex to mix.
3.4.19	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.
3.4.20	Transfer to the magnetic rack.
3.4.21	Label 3 new 1.5ml Lo-bind Eppendorf tubes (1 per Target): First TARGET size selections and the relevant I5 primer (e.g. 1st GRC1-T5-1).
3.4.22	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 TARGET selection".

Final size selection on ELUTION (see Appendix 4)	
3.4.23	Vortex Ampure XP beads to re-suspend.
3.4.24	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).
3.4.25	Add 75µL of Ampure XP beads to the “2nd TARGET selection” Eppendorf and close the lid.
3.4.26	Vortex to mix and pulse spin down to collect all of the liquid at the bottom of the tube.
3.4.27	Place on a non-magnetic rack at room temperature for 5 minutes.
3.4.28	Transfer tube to a magnetic tube rack until clear (approx. 3-4min).
3.4.29	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.
3.4.30	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.
3.4.31	After 30 seconds, carefully remove and discard the ethanol
3.4.32	Repeat the ethanol wash once more (3.4.30 and 3.4.31).
3.4.33	Close the tube lid and pulse centrifuge to draw final residual ethanol to the bottom and remove.
3.4.34	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.
3.4.35	Re-suspend beads with 22µL of Buffer EB by pipetting up and down.
3.4.36	Transfer to non-magnetic rack and incubate “off magnet” for 2 minutes.
3.4.37	Transfer the tube containing eluted size selected libraries to the magnetic rack.
3.4.38	Allow supernatant to clear as beads are captured on the magnet (approx. 3-4min).
3.4.39	Transfer supernatant containing the purified size selected PCR products to 1.5ml Lo-bind Eppendorf tube labelled “TARGET Pool” (e.g. GRC1 pool).
3.4.40	The **remainder of the pooled PCR stocks** should be stored at -20°C.

4. LIBRARY QUALITY CONTROL

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, is the surplus primers and the small peak at ~160bp, is 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 ~60bp and ~160bp peaks are barely visible by TapeStation analysis.

See Agilent TapeStation SOP.

5. LIBRARY QUANTIFICATION

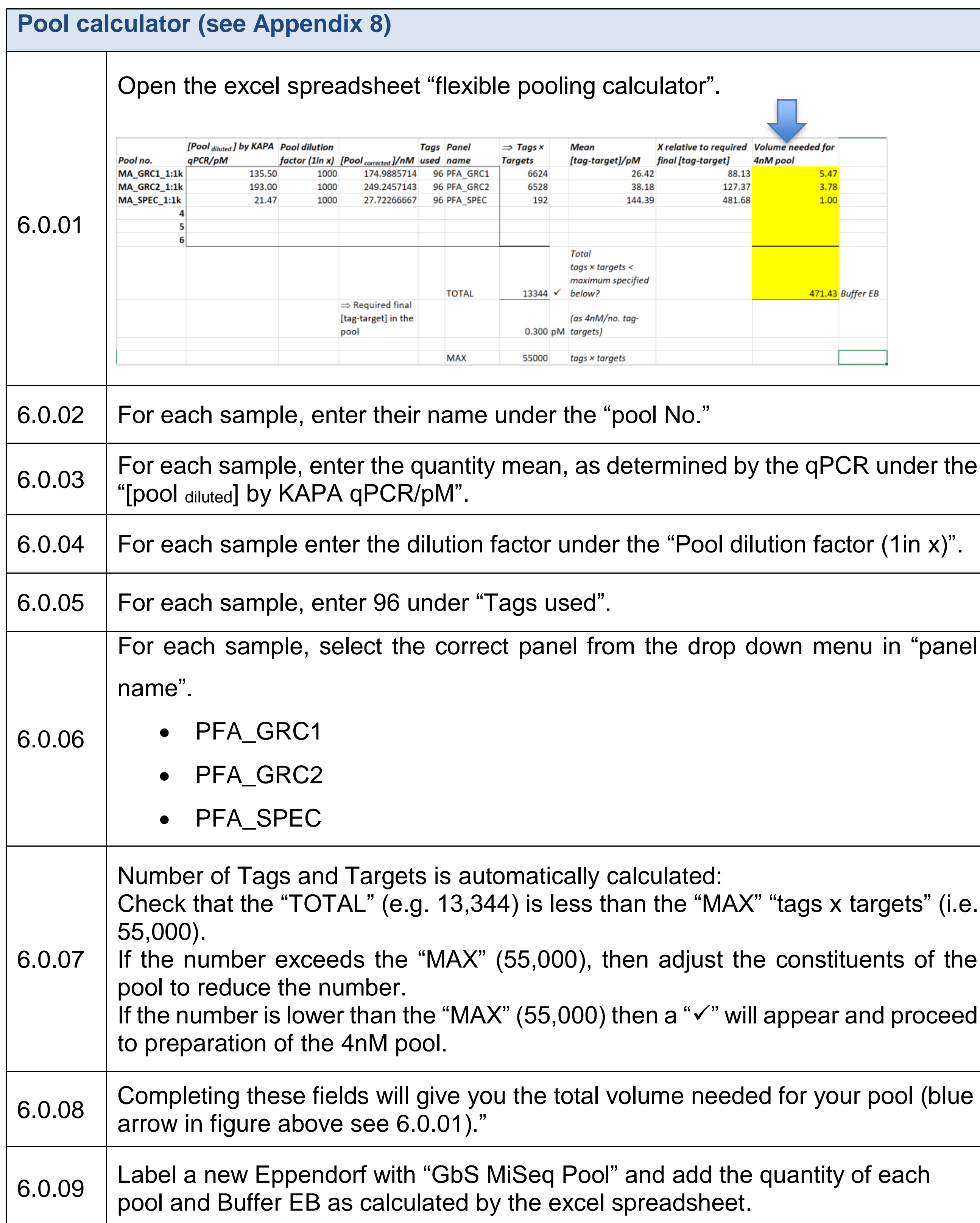
Library quantification is performed by amplifying the set of five pre-diluted DNA Standards and diluted library samples by qPCR, using the KAPA SYBR FAST qPCR mastermix and primers targeting the Illumina P5 and P7 flow cell oligo sequences. The average Cq score for each DNA Standard is plotted against 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.

6. MISEQ POOL CALCULATOR

Pool calculator (see Appendix 8)

6.0.01 Open the excel spreadsheet “flexible pooling calculator”.



Pool no.	[Pool $_{diluted}$] by KAPA qPCR/pM	Pool dilution factor (1in x)	[Pool $_{corrected}$]/nM	Tags used	Panel name	⇒ Tags × Targets		Mean [tag-target]/pM	X relative to required final [tag-target]	Volume needed for 4nM pool	
MA_GRC1_1:1k	135.50	1000	174.9885714	96	PFA_GRC1	6624		26.42	88.13	5.47	
MA_GRC2_1:1k	193.00	1000	249.2457143	96	PFA_GRC2	6528		38.18	127.37	3.78	
MA_SPEC_1:1k	21.47	1000	27.72266667	96	PFA_SPEC	192		144.39	481.68	1.00	
4											
5											
6											
					TOTAL	13344	✓	Total tags × targets < maximum specified below?		471.43	Buffer EB
			⇒ Required final [tag-target] in the pool			0.300	pM	(as 4nM/no. tag-targets)			
					MAX	55000		tags × targets			

Step	Instruction
6.0.02	For each sample, enter their name under the “pool No.”
6.0.03	For each sample, enter the quantity mean, as determined by the qPCR under the “[pool $_{diluted}$] by KAPA qPCR/pM”.
6.0.04	For each sample enter the dilution factor under the “Pool dilution factor (1in x)”.
6.0.05	For each sample, enter 96 under “Tags used”.
6.0.06	For each sample, select the correct panel from the drop down menu in “panel name”. • PFA_GRC1 • PFA_GRC2 • PFA_SPEC
6.0.07	Number of Tags and Targets is automatically calculated: Check that the “TOTAL” (e.g. 13,344) is less than the “MAX” “tags x targets” (i.e. 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.
6.0.08	Completing these fields will give you the total volume needed for your pool (blue arrow in figure above see 6.0.01).”
6.0.09	Label a new Eppendorf with “GbS MiSeq Pool” and add the quantity of each pool and Buffer EB as calculated by the excel spreadsheet.

7. MISEQ SUBMISSION

MiSeq Submission	
7.0.01	Material ready for sequencing. See Illumina MiSeq Sequencing SOP.

8. DATA ANALYSIS

Data analysis	
8.0.01	Following the MiSeq run proceed to testing and balancing data analysis SOP.

Appendix 1 – GRC1 Primers

Well	Name	Forward sequence	Reverse Sequence
A1	CRT_220	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATCTTTTGAAACACAAGAAGmAA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTATTTCCCTTGTCATGTTTGAmAA
B1	CRT_326	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAGCATGGGTAAGAAGCTTAmUA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTCCTCTTGTATGTATCAACGTmUT
C1	CRT_371	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGGTACAACGTATCATATTTmUA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTACGAACAAGCCATTTGATATmUA
D1	CRT_72_74_75_76	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTAACAGATGGCTCACGTTmUA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTGAGTTTCGGATGTTACAAAAmCT
E1	DHFR_16_51_59	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTTTTCGATATTTATGCCATAmUG	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTACATTATCCACAGTTTCTTTmGT
F1	DHFR_306	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGATGATGATGAAGAAGAAGAmUG	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTCGATCACTTTGTTTATTTCmCA
G1	DHPS_436_437	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTTTGTTGAACCTAAACGTmGC	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTATAATTGGTTTCGCATCAmCA
H1	K13_resistance_1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATGAATTTAGAACTTCGCCAmUT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTCCATATGCCTTATTAGAAGmCT
A2	K13_resistance_3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCATAGCTGATGATCTAGGmGG	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTCTGAGGTGTATGATCGTTTAmAG
B2	K13_resistance_5	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTAGACATAGGTGTACACATAmCG	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTCTTAGATAGGGATAGTGAGmUT
C2	MDR1_86	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGTATGTGCTGTATTATCAGmGA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTCATGAAATTGTCCATCTTGAmUA
D2	Pf_PF3D7_1460900-1_Pf3D7_14	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCCCAAAAGACAATAAGAAAmGA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTGCAAGAGTACTGTTTTATTTmCG
E2	Pf3D7_01_v3_145515_294I_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCCTGAGTTTTAAGTGAATGmAA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTAGTTTGTTGATGAAAGAAAGmGA
F2	Pf3D7_01_v3_180554_D714N	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACACCCAGAAATATAAATGGAmAC	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTCAAAACGACGTTATTACAmAT
G2	Pf3D7_01_v3_535211_2521F_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAAATAATAAGAACAACGATGmCT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTCACTCATCAATTAATTGTTCmAT
H2	Pf3D7_02_v3_470013_G75E_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAATATGTTGTTTGTGTGGTCmAT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTGCCATGATAAACCTTTTCmAT
A3	Pf3D7_02_v3_714480_D258G	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGAGGTGATCACTATGTTTAmUT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTCATCCAAGGTTTTGTCTTmAC
B3	Pf3D7_03_v3_155697_150P_B	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACGTCTCATTACCAAATTCAmAA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTTATCAACTGAAGGTACAAmCG
C3	Pf3D7_03_v3_656861_129V_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATTATTATCATCACAATGGTmUG	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTAGTATGAAGGGTGATGATACmUT
D3	Pf3D7_04_v3_1037656_2776I	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTAAATGCTGAAGATGAACCAmAG	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTATGGTAAATAAAGTGAGTGTmGC
E3	Pf3D7_04_v3_139051_K438N_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAAATTCTAGTAGTAAATACAmCA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTGCATATTCTTTTAAATCGTTTmCT
F3	Pf3D7_04_v3_426436_D560A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGGAGTTGGCTGTAATAATTmUT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTCTTCCATGACAAGGAAGAATmUA
G3	Pf3D7_04_v3_531138_A992E_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCTCATCCATATGTATCCACmUC	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTACAAAAGCAGCAATACACATmAT
H3	Pf3D7_04_v3_881571_1081R_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACAAGGTACATTATTATGGAAmAC	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTAATATTATATGCAACACCCAmCC
A4	Pf3D7_05_v3_1204155_1338I_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGATGAGTGGAATAAAATTTCAmGA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTGTCATATTCGAAGGATCAAAmCT
B4	Pf3D7_05_v3_172801_E218K_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATTATTGGACGTTCAGAAACmUG	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTGGTTCCAATACTAATATGGATmGG
C4	Pf3D7_05_v3_369740_907L_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCCTCCTATAATCATAGTGAAmUG	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTGTCATTATTATTTGATCTTmUT
D4	Pf3D7_06_v3_1289212_125T_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGGGAACTATTTAGATTACAmAA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTTCCAAATTTCCCTTTACTmGT
E4	Pf3D7_06_v3_900278_P696S_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGTTCATCCTTTATTTCAACAmGA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTGTCCATTGGATTTAATTCCmUC
F4	Pf3D7_07_v3_1066698_G483S_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCATTATGTTTAAAGGAAAGmGA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTGTGTTCACAAATATCTTCATmGT

Well	Name	Forward sequence	Reverse Sequence
G4	Pf3D7_07_v3_1256331_L321F_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTAATATAAATATGTATGATGGAAmGG	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTTCACTTTCTTGAACTACAmCC
H4	Pf3D7_07_v3_1358910	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGGAGGTTATTCAACTAAGmGC	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTAGCAAAAATTCTCCATGCmCA
A5	Pf3D7_07_v3_619957_675R_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCTATGCTAAAACTGAACGAmAA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTGTGTTATATGGAAGAAAAGmAT
B5	Pf3D7_07_v3_704373_389E	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAAGGATTAAGGAGAAACACmAG	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTATCCCTATCTCTTTCTCTTTmCC
C5	Pf3D7_08_v3_1056829_L474I_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGACAATATGGCTAGTAACAmGT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTATCATCATTGTCTAAAGCTTmCG
D5	Pf3D7_08_v3_1314831_1342K_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGAAGATTTTAAAGAAGAAGAATmGT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTCCCTTATATTCCATTCTTmUT
E5	Pf3D7_08_v3_150033_1315I_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATCAACAAGACGTTTCTGATmUT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTGGTATAACACGTTCCAATACmUT
F5	Pf3D7_08_v3_399774_421K_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTTCATCATTGTGTTGAATGmUC	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTGCTCTTGTGAATGAAAGTmUA
G5	Pf3D7_08_v3_417335_R244K_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGGATCATATCTTCGGACTTmUT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTACAGTAGAGCAAACAAAAGAmAA
H5	Pf3D7_08_v3_549993	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGTGCTTGTACAAATAATCmCA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTATCCTATAACGAGGTATTCmCA
A6	Pf3D7_09_v3_452690_1018I	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCAGGAACCATACTTTTGTTmUA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTATATTTCGACCTGCTTCAAmUG
B6	Pf3D7_09_v3_900277_1534E_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGATTTGGAAATAACTGATGmAA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTGTCCATGTTTAAATGCTTTmUG
C6	Pf3D7_10_v3_1383789_N114H	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTTAGGTTGGTTAGAATGGAmGT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTATGAACCCAACTTTCTTTACmAT
D6	Pf3D7_10_v3_1386850_927K_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTTAAATATATTGAAGACGTmGG	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTGAGGACAAGGAAAATAATAmCA
E6	Pf3D7_10_v3_361684	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACTTCATCAGCATTTTCAAmCC	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTCTCATTCATTAGGTGGTGmGT
F6	Pf3D7_11_v3_1006911_D124E_B	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTAATTTTGCAAAATAGCGTmGG	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTCGTTTTGGTCATTCATGTCTmUA
G6	Pf3D7_11_v3_1020397_G700E_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTATAATGCATGTGTCACCTmUC	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTACAGAAACATTAAACACAACmCA
H6	Pf3D7_11_v3_1295068_E405K	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTAATTGGGGAAATTATAAmCA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTCCCTTCATCATCATTATCATmUT
A7	Pf3D7_11_v3_1815412_E765Q	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATGAGTTGTTATATTCATGTGmCT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTAACATATGCTGCAGATTTTGTmUA
B7	Pf3D7_11_v3_1935031_I139L	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGATGTTCTTTTATGAAATCAmCA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTACGTCAATACAAGAATTAACmCA
C7	Pf3D7_11_v3_477922_H147Y_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAACAACCTTAGCATTGATTGmAT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTATATTGACTTTCATCATTTmGA
D7	Pf3D7_12_v3_1667593_2381N_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGCTGCTGAATATACATATCmUG	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTCTTTTCTAATTCCTTTTCmCA
E7	Pf3D7_12_v3_2171901_V140D_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGAAGATTCTAAGGAACAAAmAT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTCTGATTCCACAAAAGAAGAAmGA
F7	Pf3D7_12_v3_858501_Q469K	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAAATAGAAAATTTGCCACATmGC	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTAGACCCCTGTTTTAGTAAGAmAT
G7	Pf3D7_12_v3_974663	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAGAAGGAAGACCTTGTTmGT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTAGAATCCTTAAGAGACTGTmUT
H7	Pf3D7_13_v3_1056452_1234D	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTTTCATCGCAGGAAAATACmUT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTCGTCATTAATGTGTTTATTmCA
A8	Pf3D7_13_v3_1419519	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCTTCTTGACCAAACATAmAT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTAAGTACAAAAACCAGmCA
B8	Pf3D7_13_v3_1867630_M4911I	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTCCGATTGGTAGTTATACAmGA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTAAAGGAGATGTATCATTTGCmAC
C8	Pf3D7_13_v3_2377887_2002S_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATTAAAGAAGAATGTGGAGCmUG	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTGGAACGGCAAGGATATTTTAmAT
D8	Pf3D7_13_v3_2573828_I1153M	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCTTCATAACATATAGCAGCmAT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTATGACACCAATCGTTAATCmAC
E8	Pf3D7_13_v3_388365_S1236R	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGTCTTTGTCTCTTTTCCTTmUC	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTATCCAACTCAAAACAGCTTAmUT

Well	Name	Forward sequence	Reverse Sequence
F8	Pf3D7_14_v3_137622_1179V_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACCATTCATATTTTCTAGGTTTmGT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTACGAAATTTATGAAGATGAGmCA
G8	Pf3D7_14_v3_1757603_D1365G	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTAAATGACGAAGAACTAGCmUA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTCTTCAATATGTAAATCATTCAmCA
H8	Pf3D7_14_v3_2164225_2830S_B	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCTACAAGGAGAAATGACAAmAT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTCATCTGTAGAATCGGTAAGmAA
A9	Pf3D7_14_v3_2733656_557C_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCAAAACCGTTTCATCTACAmAT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTAAAAGATTCAGCATCGCTAmUC
B9	Pf3D7_14_v3_3126219	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAACAAGAGAATCAGGGCAmAT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTCATCTCTAGGTAATAACGTACmCA
C9	PlasII_ref	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGAAAATGCTCTTTTCACCmUT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTCATCTAAATTCTGCAACATTTTmAT
D9	PlasIV_ref	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGACAATGCTTTATTTACAmUT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTCATTCATATCTCTAAAGAATTTmAT

GRC1 = 68 Primers in total.

APPENDIX 2 – GRC2 PRIMERS

Well	Name	Forward sequence	Reverse Sequence
A1	CRT_271	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTTCCAATTGTTCACTTCTTmGT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTATTTTACCTCTACGACTGTmGT
B1	CRT_356	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGTTAGTTGTATACAAGGTCmCA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTACGTTGTACCATCATAAACAmUT
C1	CRT_97	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTTTGCTAAAAGAACTTTAAAmCA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTGGTAGGTGGAATAGATTCmUC
D1	DHFR_108_164	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTTGTAGTTATGGGAAGAACmAA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTATTCTTGATAAACAACGGAAmCC
E1	DHPS_613	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAATGGAATACCTCGTTATAGmGA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTCATTTTGTTGTTCATCATmGT
F1	EXO_415	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAATTTACCTGAAGACGTTAAmAA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTATCCCATTCGATATCTATACmCT
G1	K13_resistance_2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCATTATCAATACCTCCAACAmAC	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTATCGTATGAAAGCATGGGTmAG
H1	K13_resistance_4	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAATTACTTGAAACATACCATmAC	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTATAGGTGGATTTGATGGTGmUA
A2	K13_resistance_6	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGGGTATAGTTAACGGATTTmCT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTAAAATTGTTGATGCAAATATmUG
B2	MDR1_1034_1042	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGCATTTAGTTCAGATGATmGA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTCCAAACCAATAGGCAAAACTmAT
C2	MDR1_1226_1246	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCTGCAGAAGATTATACTGTmAT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTACTAACACGTTTAACATCTTmCC
D2	MDR1_184	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTTACATATGCCAGTTCCTTmUT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTGCAACAGTTCTTATTCCCATmUA
E2	Pf_PF3D7_1318100_Pf3D7_13	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGATTGAGGACAAATTACATmGT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTCATATAGTTGTAGGGGAGGTmAG
F2	Pf_PF3D7_1447900_Pf3D7_14_v	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTATCCATCTCATTTGCTTTTmGT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTGAATAGTTGATAGAGGTACCmGA
G2	Pf3D7_01_v3_179347_311G_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCACATATCCAGCCCTCAmAA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTGCTTGTGTTATTAATCCTGTmAA
H2	Pf3D7_01_v3_283144_H664D_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATGGATTAAACGCTGATATGmUC	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTATCATCATTTTCCTCATCATmCG
A3	Pf3D7_02_v3_376222_K1929E_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGATGTGATTTCTCTACGAACmAA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTAGGTATAACTTCACGAAATTmUT
B3	Pf3D7_02_v3_529709_F487L	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAAAAGACAAGAGTACAAAAGmGA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTACCAGCTATTTCTTGATATGmUG
C3	Pf3D7_02_v3_839620_260L	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGAATGATTTGAAAATTGCAmCT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTAAATCGTGTGCATCCATTATmUT
D3	Pf3D7_03_v3_548178_R2L_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGGGTAAGTTACATTCTTCTmCA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTGTTCATTATGATTAATTATATGmCT
E3	Pf3D7_04_v3_1102392_E808D_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTAGAGGTGTTGATGTTAATATmGG	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTATCATCAAAATATACATCCCCmAC
F3	Pf3D7_04_v3_110442_G285E_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATTGGAAAATTCATGTAGTGmCA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTAGGAAAGACAGCAACAATAAmAA
G3	Pf3D7_04_v3_286542_H586N	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTATATAATGCGTCTCTGTCCmAT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTGAAAAGGAAACAAATAAGGmAT
H3	Pf3D7_04_v3_529500_1477Y_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAATAGGAGACAGAATTTGATmUC	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTAAGAATTCCTTAGGACACGmUA
A4	Pf3D7_04_v3_648101_51V	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAATTTCACATTATACCTTCCmAA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTGATACAAATTCATATAAATAmCA
B4	Pf3D7_04_v3_891732_R4468S	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCATTCCTCCTAGTTTAAACCmAA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTGAGCAAATTCTTGAGCATTAmAC
C4	Pf3D7_05_v3_350933	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGCATCATTTTGCmUC	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTATATATAATTCAACAACAmGC
D4	Pf3D7_05_v3_796714_396K_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGTTAATGAAAAGAACCCAAmAA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTATGGTGTTTTACGTTTTAGmGG
E4	Pf3D7_06_v3_1282691_803K_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAAATGTACAAGTGAGGAGTmUA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTGCTTCATTTGTAAATAAAATGmCT
F4	Pf3D7_06_v3_574938_I2934L_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGACGATGATGAAAACATGAmAT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTACATTTATACATGCCCAACAmAT

Well	Name	Forward sequence	Reverse Sequence
G4	Pf3D7_07_v3_1044052_686K_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGAACACGCTTATCATTTATmCT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTATGACAACCAAAATGAAGGAmAT
H4	Pf3D7_07_v3_1213486_S543N_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATTTCTCCTAACTTACGTCAmGT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTAACAAACACATACATAAAACmCC
A5	Pf3D7_07_v3_1308383_G1945R	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCATTACCTTTACCTTTTCCmUC	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTGATTATGATGATGATGACmGA
B5	Pf3D7_07_v3_1359218_K388N_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGTAAATTTGAATGGCATACmGT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTCACGCTTTTCCTTAATTGmUA
C5	Pf3D7_07_v3_461139	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTATATATCACAACAATATTTAmGA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTCTGAGATTATTTAATTCTTCTTTmAC
D5	Pf3D7_07_v3_635985_T598A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTACTGCGCCTATCATTATTmAT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTAAAAGGGATGGAGATTACAAmUG
E5	Pf3D7_08_v3_1313202_799F_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGAATAAATGGATTGAGAGAmCA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTATCTTGCAATCCATCTTTTmCC
F5	Pf3D7_08_v3_339406_1283C_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTATTTCAAATATCCAACCGCmUT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTATAGATGGAAAGCAACCTAmCA
G5	Pf3D7_08_v3_413067_1044V_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAATGTGTACGAGAAACATCmAC	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTCATAAGTTTCTTCTAAATAGATAmGT
H5	Pf3D7_09_v3_1379145_R398Q_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCCATTTCAATCTTTTCGTTTmAT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTCAGGATGTGTTATGGTATCmAA
A6	Pf3D7_09_v3_163977_403D_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTCATTCATCAAGTGATCCAmAA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTGTAACTAATGGTTTTGCAACmAC
B6	Pf3D7_09_v3_599655_E654D_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATCATGACAAAGAAACATGTmGG	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTGTAGATTGTTCATATCTTCmAT
C6	Pf3D7_10_v3_1385894_815P_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCATTCCACATGTACGTAAAGmAA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTGAGTGATAAACCTGCTTATmCT
D6	Pf3D7_10_v3_317581_311I_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTATCTTGTCTCATGTCATGmUG	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTCAGCTTCTATACCTAATGCTmUG
E6	Pf3D7_10_v3_336274_I1677V_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGCTATAGTCATGATCATCmGT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTACCTGGAGATTCATGTAATTmCA
F6	Pf3D7_11_v3_1018899_1199L_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAAAAGGTTCACAGAATATTmUT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTGTGGTATAAGTAACATAAGTAmGT
G6	Pf3D7_11_v3_1294107_84A_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTATCCAATAGACCACGAmAG	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTAAGTCTATATTGAGTTCCAGmCT
H6	Pf3D7_11_v3_1802201_450S	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGTATTTTCCTTTTCCCTGmAT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTAAGGAGATAGTTTACCTGGTmUT
A7	Pf3D7_11_v3_1935227_R73S_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGGATCTAAGGATGTAATTTmGT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTAGGAATTCAAGAACTTTAGCmAT
B7	Pf3D7_11_v3_408668	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCACAGGATGATGAATATAATAAmCT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTGACGGACATTCTTTCAATmGC
C7	Pf3D7_11_v3_828596_K240E_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGTCCTTTTATATCTGTTTGmCT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTGCATACATTAATAAAAGTGAmCA
D7	Pf3D7_12_v3_1663492_1014E_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGTATGCACCGATTTTCTATmAT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTATATTTTCGAATCAGCGCTAmUT
E7	Pf3D7_12_v3_1934745_241L_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACCTGGAATATTATCACCTTmCT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTGACAAATCATGAAAATCAAmGA
F7	Pf3D7_12_v3_857245_E50G_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTAAGATGTCCTATCAAGACGmUT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTACGACATACTGCTATTTATGmUT
G7	Pf3D7_13_v3_1233218_N277S_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGGTTCTACATTTTCCAGATmUT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTACCAACTTTGTAAAGCTGTAmAA
H7	Pf3D7_13_v3_1466422	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTTACTTGTAACAGTGCATmCA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTGTATACGTCGTCATAATTmGG
A8	Pf3D7_13_v3_159086_21R	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCTAACAACACACTTTTATTCmCT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTCTACTTGGTAGGTATACGTCmAT
B8	Pf3D7_13_v3_2161975_D252V	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCCAATTCCATGTATATTCATmCA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTATATGAACGAACTGGACAAmGA
C8	Pf3D7_14_v3_107014_215K	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCTATTCCATACAACATCAAmCA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTAAAGGTATACATGGTATATCmAT
D8	Pf3D7_14_v3_2355751_H1589Q	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTATCCAAACCCATTTAACGmAG	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTAAATGTTTCGTTATATATGAmCA
E8	Pf3D7_14_v3_2625887_M238I_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCTTATTCGATCTGTACCTTmCA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTAAAGCTTGGTTAGAAGATTTmCT

Well	Name	Forward sequence	Reverse Sequence
F8	Pf3D7_14_v3_3046108_417V_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAGGAAATGAGATAATTGACmGT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTTTCTCATTGGGACGGAmUT
G8	Pf3D7_14_v3_438592_N348T_A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAGAAAATGAATCTATGCCTmCA	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTATGTGGTCAAGTGATGTATCmAT
H8	PlasI_ref	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGAACAAGCCGTTTTTACCmUT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTCATCTAAACCTTCAAAGAATTTmGT
A9	PlasIII_ref	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGAACAAGCCGTTTATTCCmAT	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTCACTTGCAGATTCTACGAATTGmAT
B9	W1421_Pf3D7_14_v3_289610_T4	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTAGGTGACCCATTTATGmAG	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTTAGCTTTAGCATCATTCAmCG

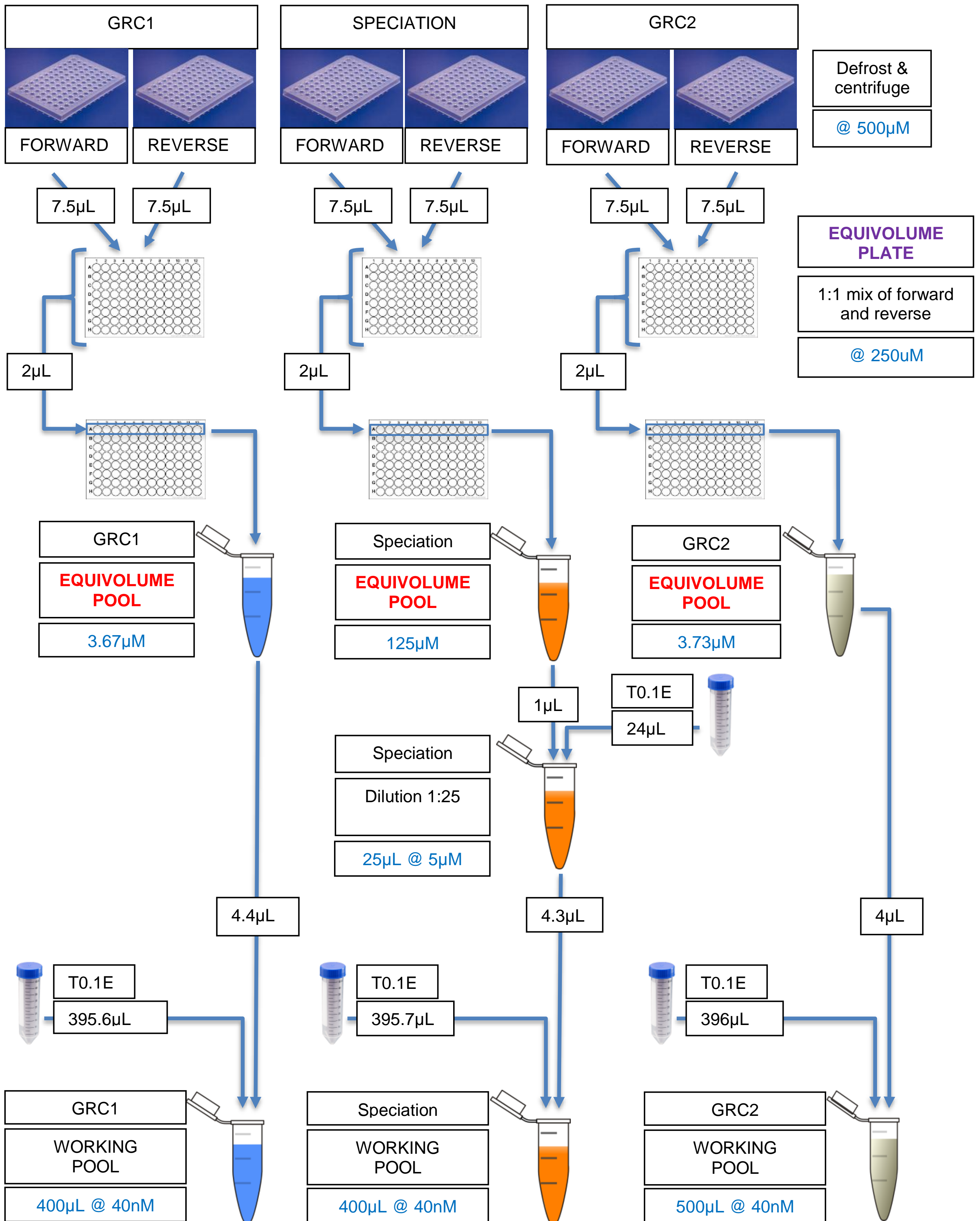
GRC2 = 66 Primers in total.

APPENDIX 3 – SPECIATION PRIMERS

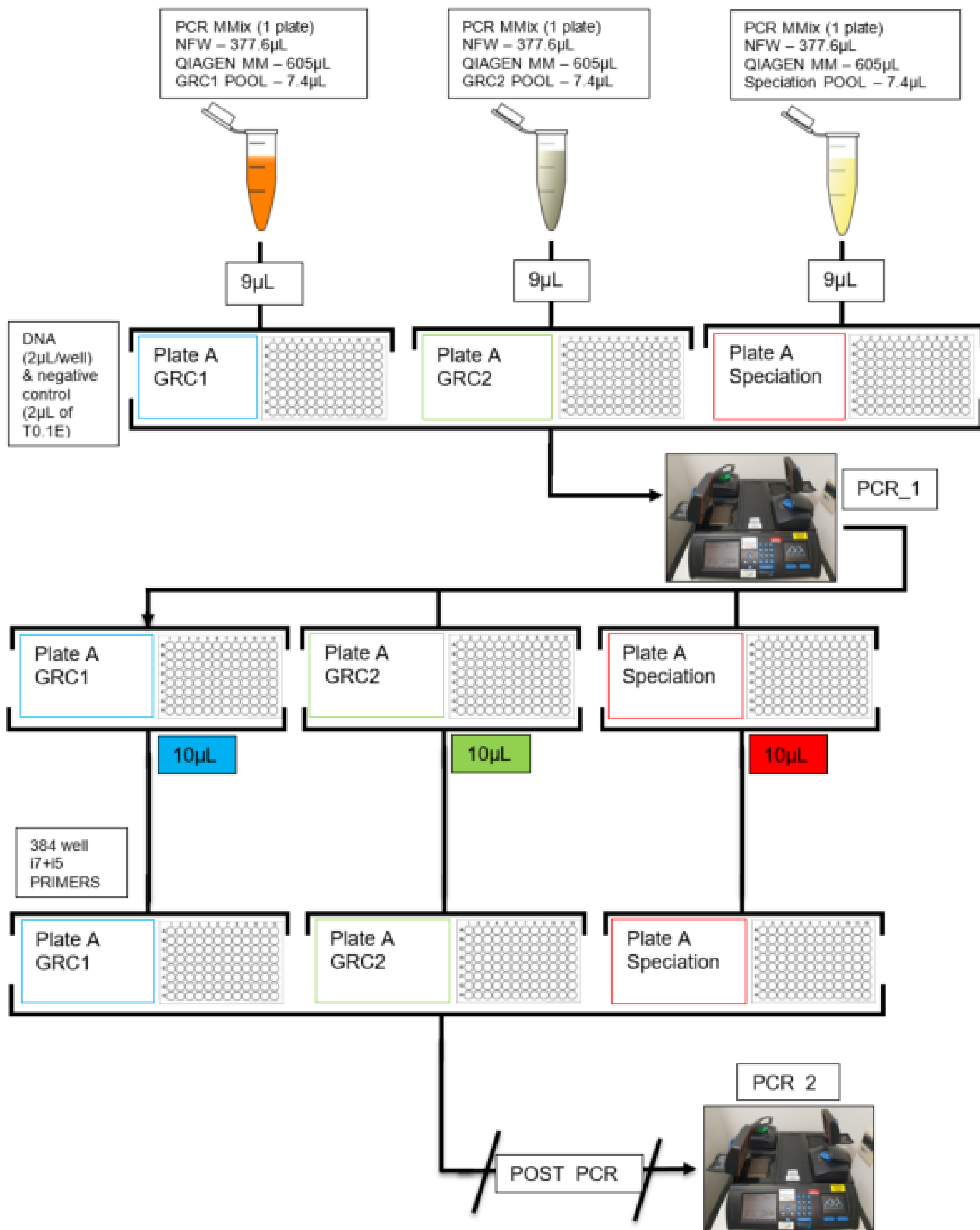
Well	Name	Forward sequence	Reverse Sequence
A1	Spec1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACCATCCAATTTGATTmGG	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTCTTGAGGCAGTTTGTTmCC
A2	Spec2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTAGCGTGTATTGTTGCCTmUG	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTCCTTTAATGTAGTTTCCTCACAmGC

SPEC = 2 Primers in total.

APPENDIX 4 – PRIMER POOL:

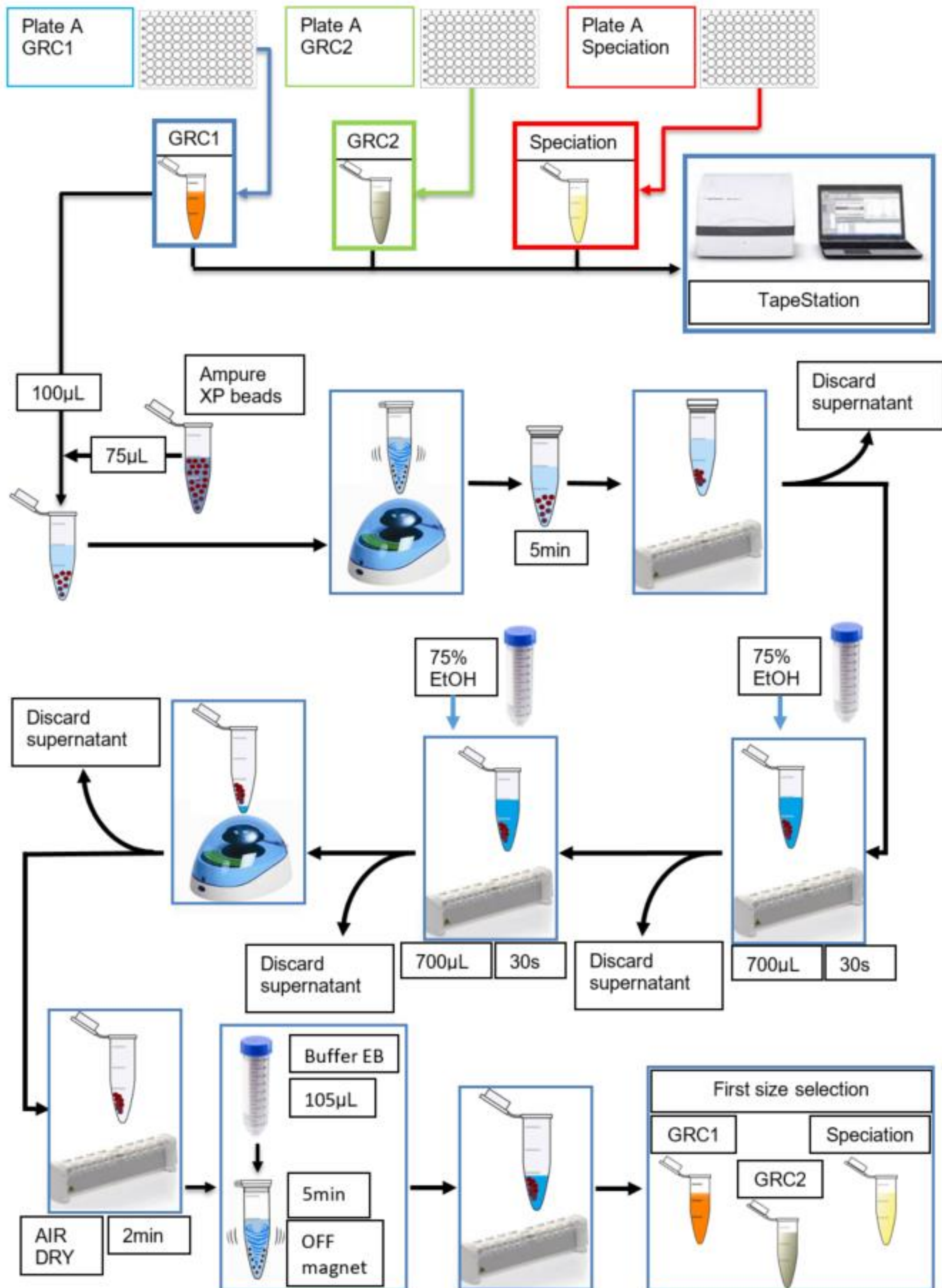


APPENDIX 5 – PCR_1 & PCR_2

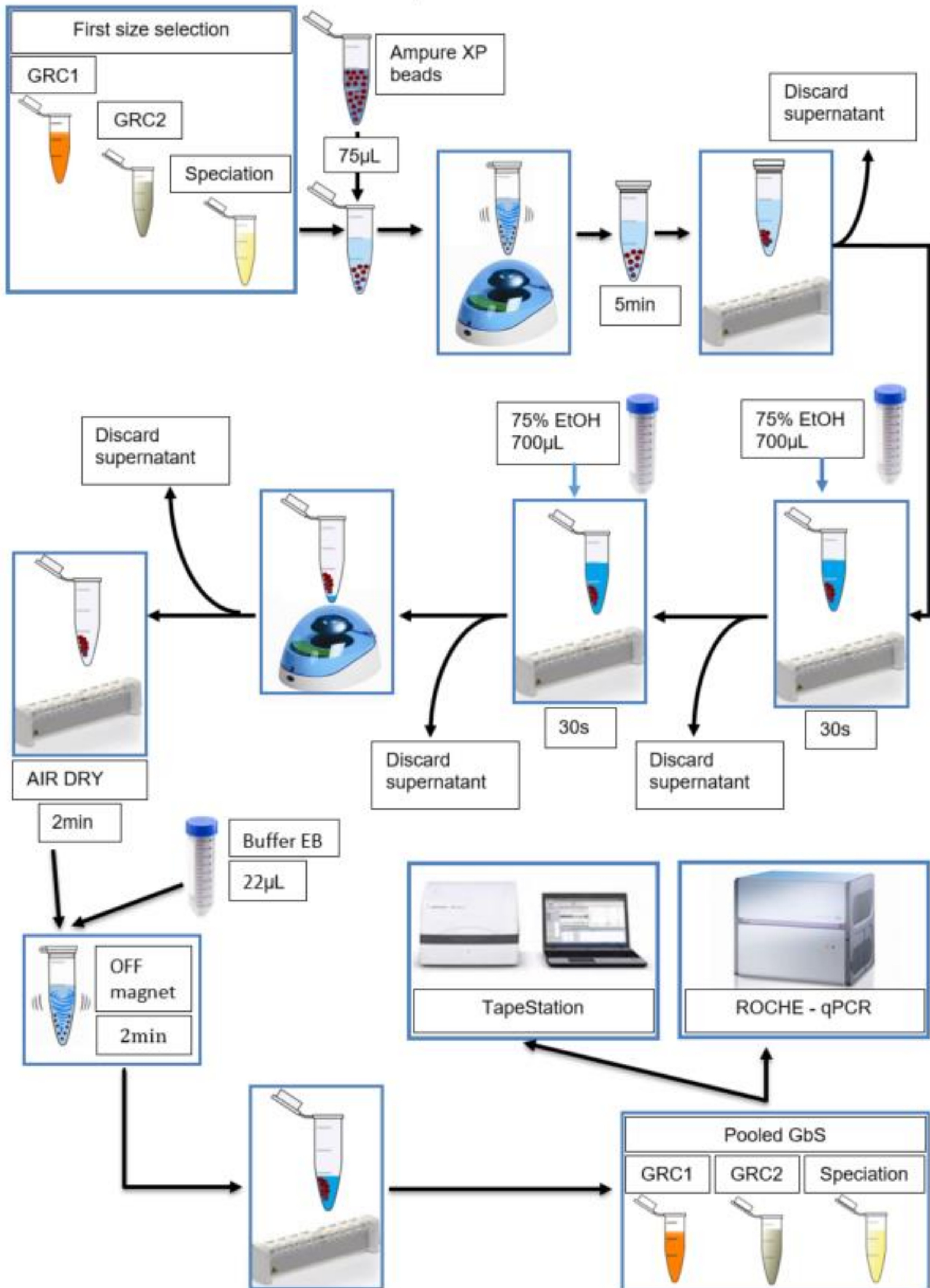


STORAGE - Once the PCR is complete overnight at 4°C without detriment, move directly to pooling, or plates may be stored at -20°C for up to one week

APPENDIX 6 – CLEAN-UP & SIZE SELECTION



Appendix 7 – Final size selection & Elution



Appendix 8 – Primer pooling