Standard Operating Procedure (SOP)	
SOP Title	qPCR GbS library quantification
SOP number	GbS05
SOP Version	1

1. SCOPE

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.

2. MATERIALS REQUIRED:

2.1 REAGENTS, LIBRARIES AND MATERIALS

Reagents	***Supplier***	***Cat. No.***
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
Buffer Elution Buffer (Buffer EB, 10 mM Tris-Cl, pH 8.5)	Qiagen	19086
Nuclease free water (NFW) - Ambion	Fisher Scientific	AM9930
Prepared Libraries (Generated from Library preparation)		
Pooled amplified GbS samples GRC1.		
Pooled amplified GbS samples GRC2.		
Pooled amplified GbS samples Speciation.		
Materials	***Supplier***	***Cat. No.***
qPCR Instrument (ROCHE LightCycler 480II)	ROCHE	
White 96 well qPCR plate	StarLab	I1402-9909
Optically clear plate seal	StarLab	E2796-9795
Lo-bind Eppendorf tubes (1.5mL)		
Tube/ Microplate vortexer		
Centrifuge (requires a 96 well plate adaptor)		
Tissue wipes (Azowipes)		
Pipette (1000, 200, 20 and 10 µL)		
Filtered tips of various volumes		

3. METHODOLOGY

3.1 REAGENT PREPARATION	

3.1.01	The KAPA SYBR FAST qPCR complete kit this should contain the following:
	• KAPA SYBR FAST qPCR Master Mix (2X), with no passive reference dye
	• Library Quantification DNA Standards 1 - 6 (a 10-fold dilution series of a linear 452 bp template).
	• Library Quantification Primer Premix (10X), containing the following primers:
	  • Primer 1: 5'-AAT GAT ACG GCG ACC ACC GA-3'
	  • Primer 2: 5'-CAA GCA GAA GAC GGC ATA CGA-3'

3.1.02	Defrost the following then vortex and spin down the contents. These should be kept on ice at all times. • Library Quantification DNA Standards 1 – 6 • Library Quantification Primer Premix (10X)
3.1.03	SYBR master mix preparation: NEW KIT To prepare the SYBR master mix add 1mL of the Library Quantification Primer Premix (10X), to the 5mL bottle of KAPA SYBR FAST qPCR Master Mix (2X). To mix the reagents; repeat pipette a few times, then invert the 5mL bottle 10 times, and finally vortex at maximum speed for 5 seconds. Store in a box in the fridge until use, as it is light sensitive. OPEN/USED KIT If the kit has been used previously the SYBR master mix would have already been prepared. So, take the SYBR master mix out of the freezer and allow to thaw. Store in a box in the fridge until use. After use, this Master mix/primer bottle can be frozen and used again.

3.1.04	Prepare the SYBR master reaction mix as follows. The volume of mastermix has been calculated below for 55 wells including excess (to accommodate pipetting errors).

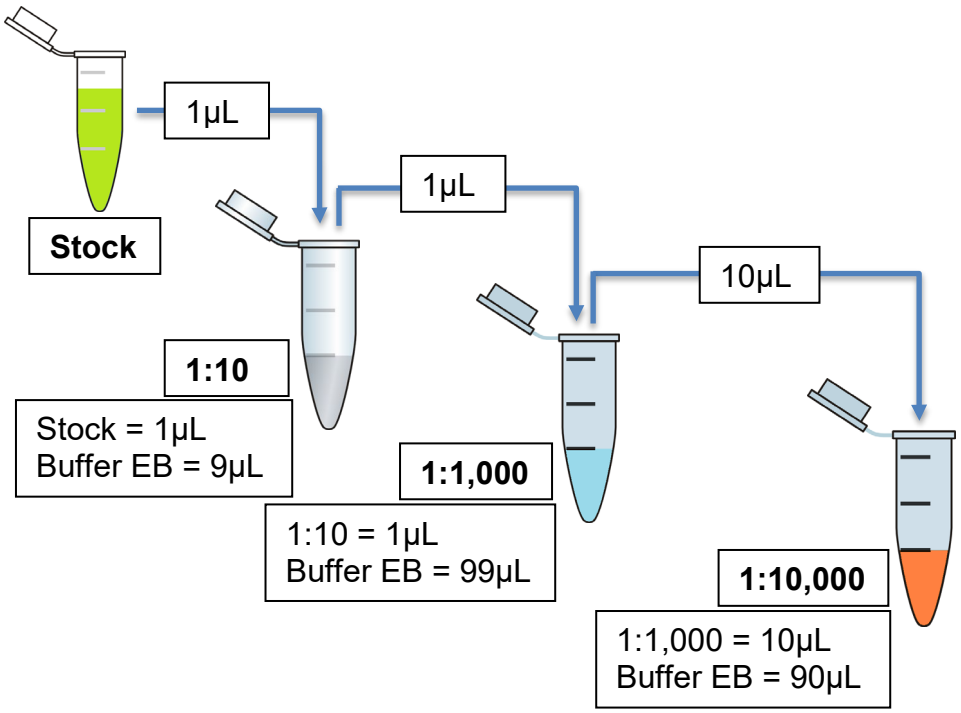
SYBR master reaction mix:		
Consumable	µL/well	55 wells
KAPA SYBR FAST Master Mix Universal (2x)	12	684
NFW	4	228
Total Volume	**16**	**912**

3.2 LIBRARY DILUTION

3.2.01 Create library **serial dilution** (GRC1, GRC2 & Speciation).

Library serial dilution		
Dilution	Library	Diluent (Buffer EB)
1:10	1µL (stock)	9µL
1:1,000	1µL **(1:10)**	99µL
1:10,000	10µL **(1:1,000)**	90µL

Vortex each tube for 10 seconds, and spin down, keeping on ice until use.



3.3 qPCR PLATE LAYOUT AND STANDARDS

The approximate layout of the pPCR quantification plate is shown below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	S6	S6	S6	S5	S5	S5	S4	S4	S4	S3	S3	S3
B	S2	S2	S2	S1	S1	S1				NC	NC	NC
C	GRC1 1:1,000	GRC1 1:1,000	GRC1 1:1,000	GRC1 1:10,000	GRC1 1:10,000	GRC1 1:10,000						
D	GRC2 1:1,000	GRC2 1:1,000	GRC2 1:1,000	GRC2 1:10,000	GRC2 1:10,000	GRC2 1:10,000						
E	SPEC 1:1,000	SPEC 1:1,000	SPEC 1:1,000	SPEC 1:10,000	SPEC 1:10,000	SPEC 1:10,000						
F												
G												
H												

3.3.01	Add 16µL of the "**SYBR master reaction mix**" to each well of the plate, which will be used as part of the assay.
3.3.02	To the corresponding well as indicated above, add the following: • 4µL of each specified standard S1-6 (high to low respectively) in triplicate: **Standard** / Concentration Standard 6 — 0.0002pM Standard 5 — 0.002pM Standard 4 — 0.02pM Standard 3 — 0.2pM Standard 2 — 2pM Standard 1 — 20pM • 4µL of Diluent (Buffer EB) to each of the designated "NC" wells. • 4µL of each library dilution to each of the designated wells.
3.3.03	Cover the plate with an optically clear plate seal; make sure that the wells are sealed by rubbing down the surface with a tissue. Do not use your hands to seal the plate.
3.3.04	Spin the plate down using a benchtop centrifuge (a pulse spin should be adequate).
3.3.05	Return the Kapa kit and library tubes to the freezer.

3.4 EXAMPLE qPCR USING ROCHE LIGHTCYCLER

Introduction – The following example is based upon use of the Roche LightCycler. We appreciate different labs have various types of qPCR machines. Therefore, please follow the guidelines as per specific qPCR machine.

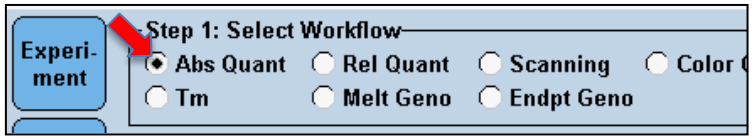
3.4.01	Turn on the PC.
3.4.02	Turn on qPCR machine. The instrument will take a few minutes to warm up.
3.4.03	Open qPCR software.
3.4.04	Create an experiment using the following parameters analysing absolute quantification. Program the thermal profile as shown below:

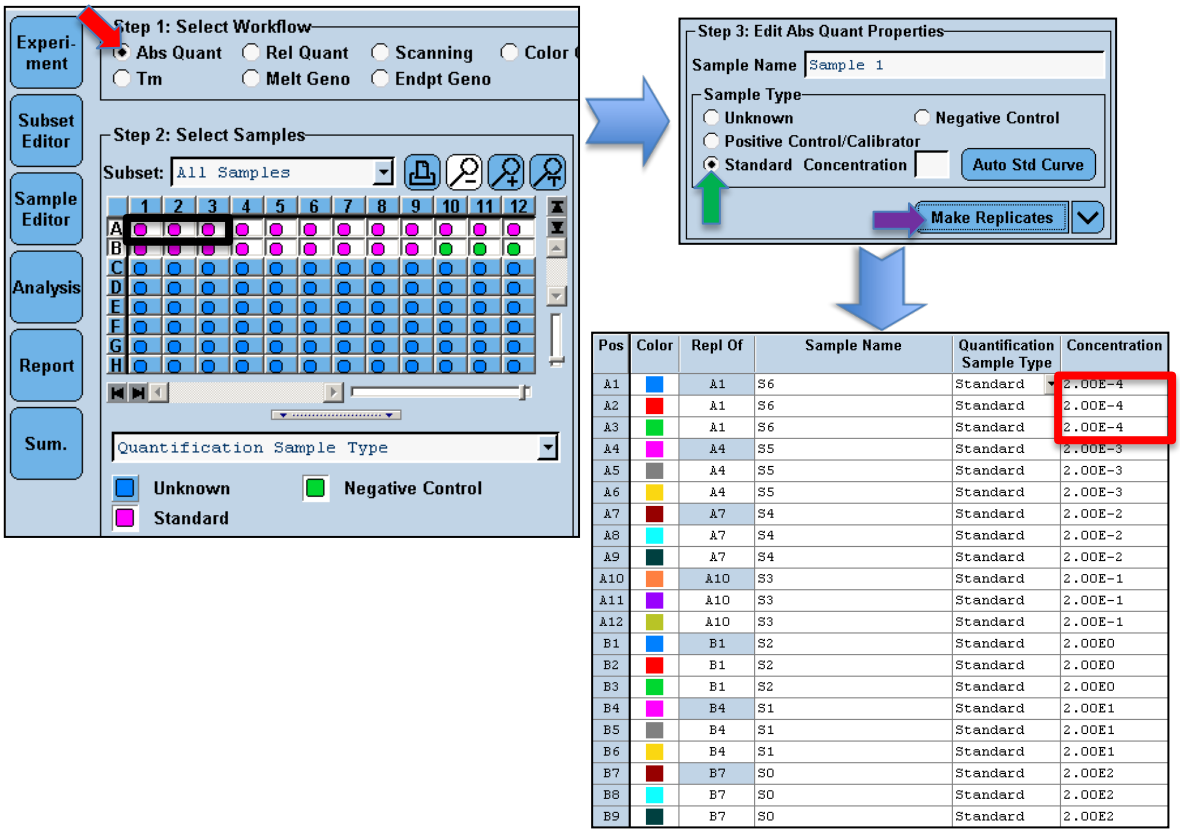
Thermal Profile

Procedure	Temperature	Duration	Comment
Initial denaturation	95°C	15:00 min	
Denaturation	95°C	00:30 sec	} Total of 35 cycles
Annealing / extension / Data acquisition	60°C	00:45 sec[1]	
Storage	4°C	Infinite	

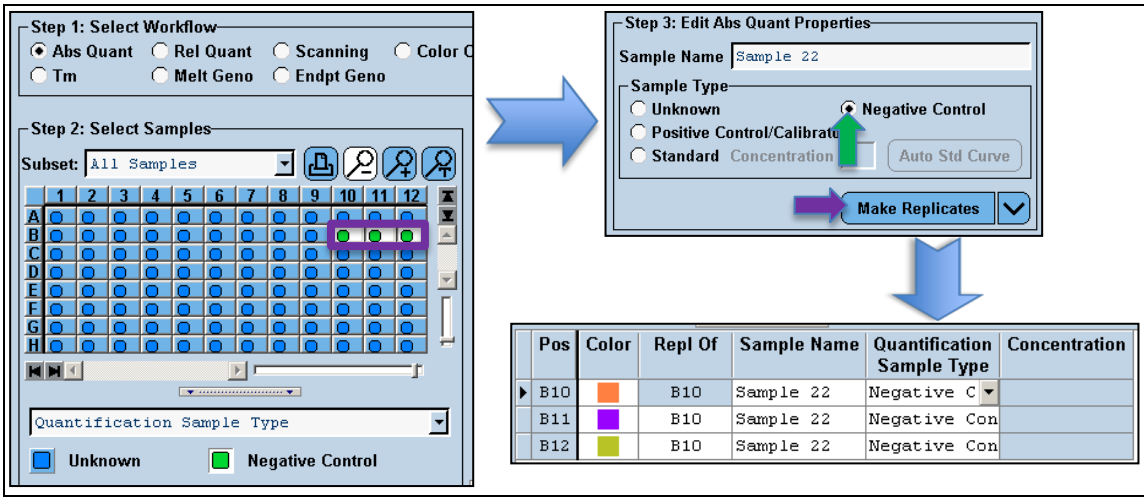
[1] This can be increase to 90 seconds for long insert libraries (>700bp)

3.4.05	Once complete move to plate template input.
3.4.06	In data workflow select the “Abs Quant” option (red arrow below).

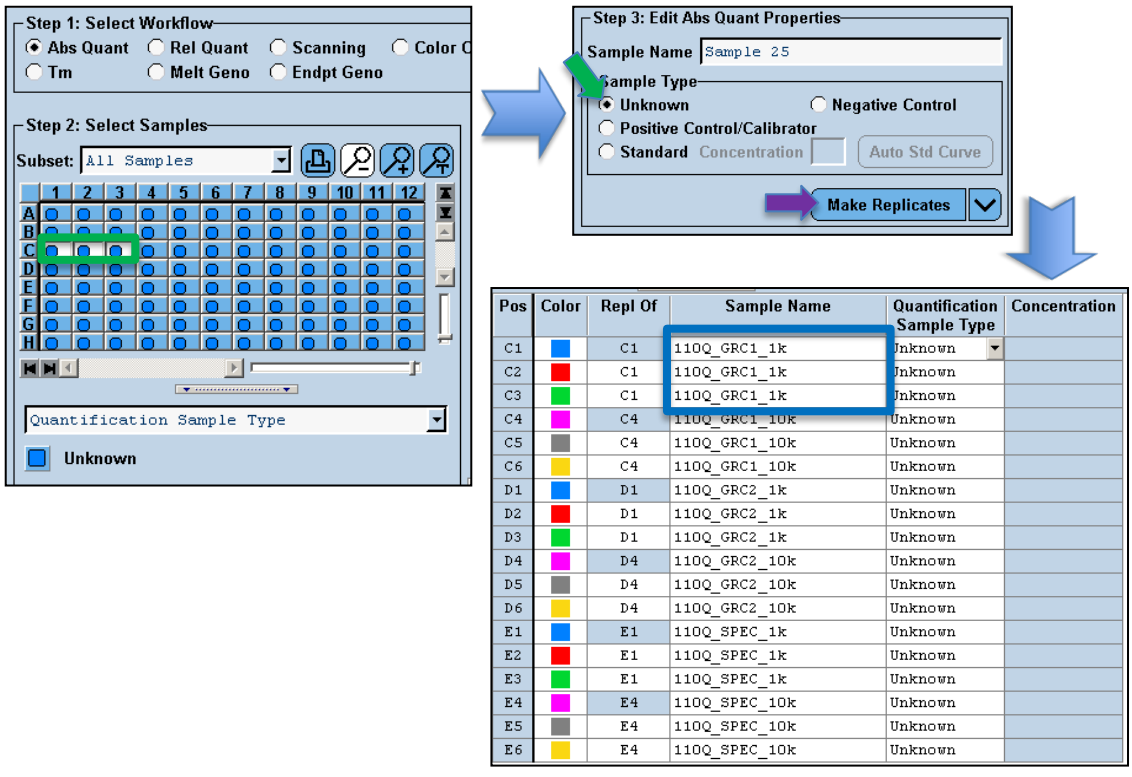




3.4.07	Select sample boxes and highlight wells in triplicate (A1, A2, & A3, black box). Confirm that the sample will be a standard by select “Standard concentration” as the sample type (green arrow). Then select “make duplicate” so that the wells are linked during analysis (purple arrow). In the “concentration” cells (red box) add the corresponding concentrations.
3.4.08	Repeat 3.4.07 for remainder of the standard concentrations.



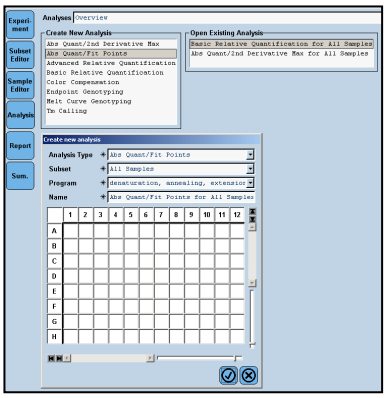
3.4.09	Select sample boxes and highlight wells in triplicate (B10, B11 & B12, purple box). Confirm that the sample will be negative controls by selecting “Negative Control” as the sample type (green arrow). Then select “make duplicate” (purple arrow).

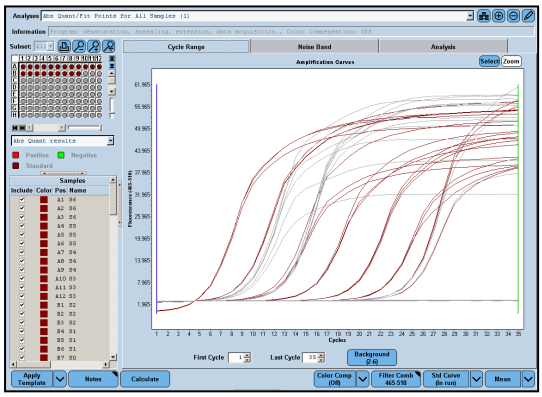


3.4.10	Select sample boxes and highlight wells in triplicate (C1, C2 & C3, green box). Confirm that the sample will be an unknown by select “unknown” as the sample type (green arrow). Then select “make duplicate” (purple arrow).

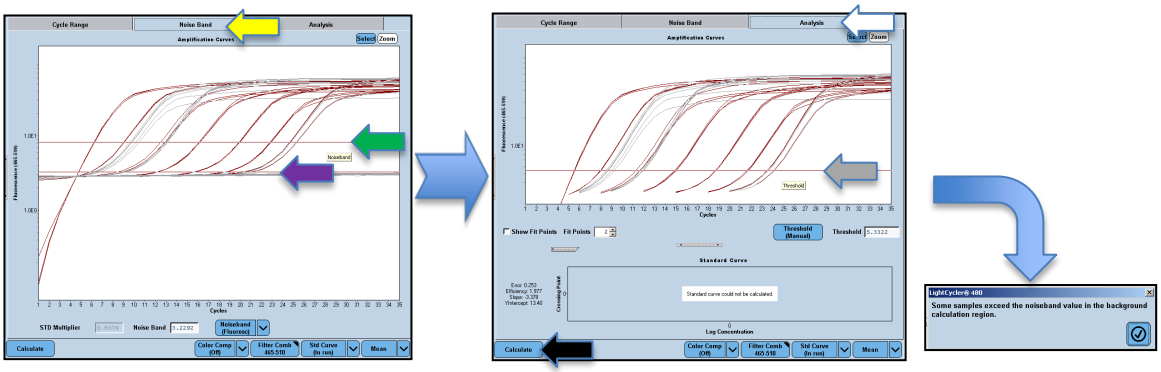
3.4.11	In the comments box add the unique sample ID that's relates to those specific wells (blue box).
3.4.12	Repeat for the remainder of the triplicate wells making sure that the correct unique sample ID is associated to each set of wells.
3.4.13	Open qPCR machine and place the PCR plate on the tray. Making sure that the plate is orientated correctly. Then close the qPCR machine.
3.4.14	Save the run file using a suitable file name (include plate ID/number).
3.4.15	Select save and start the qPCR programme.

3.5 qPCR – Roche Analysis

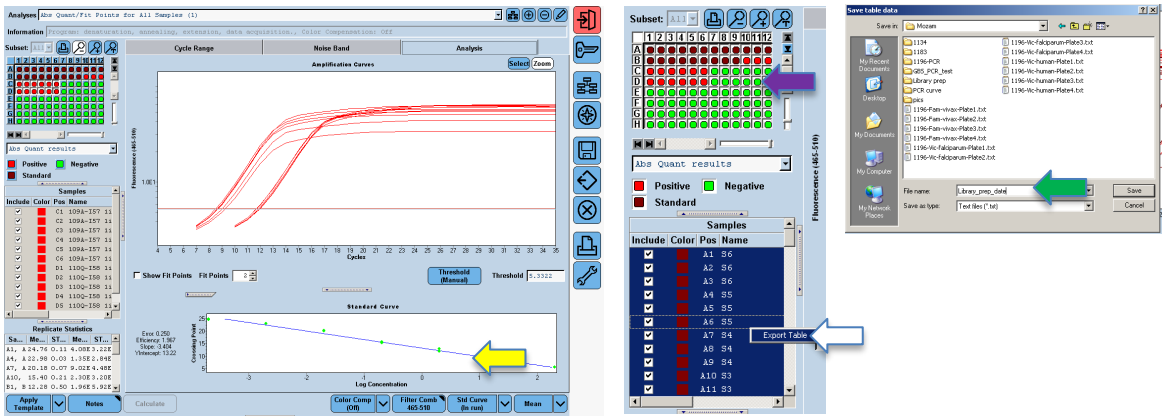




3.5.01	Analyse the new data using “Abs Quant/Fit points” and “Basic relative Quantification for all samples”.
3.5.02	Run the analysis and proceed to the analysis screen.

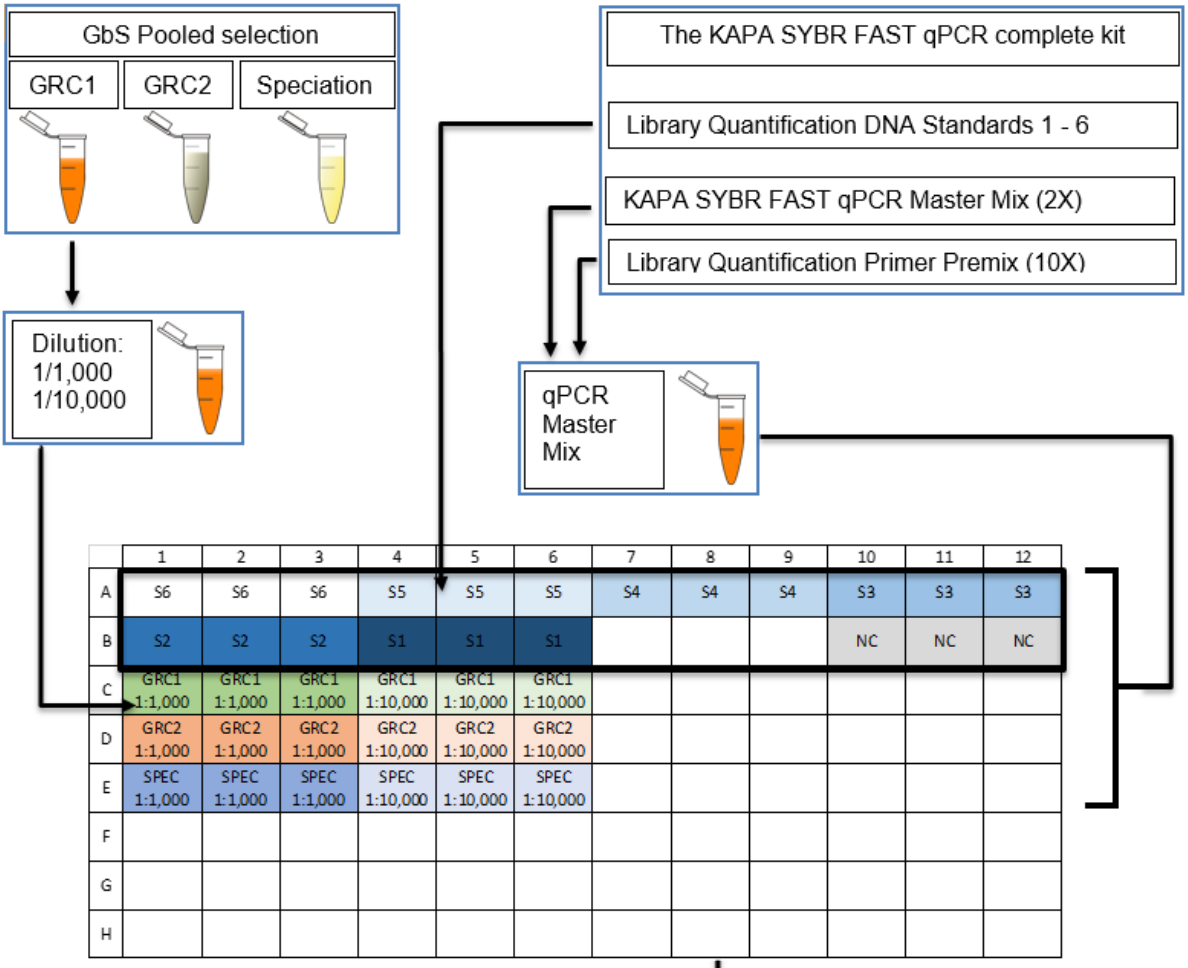


3.5.03	Select the “Noise Band” tab (yellow arrow).
3.5.04	Adjust the “Noise Band” (green arrow) down to a suitable level (purple arrow).
3.5.05	Once a suitable level has been chosen, select the “Analysis” tab (white arrow).
3.5.06	Adjust the “Threshold” to the linear segment of the curve (grey arrow).
3.5.07	Select “Calculate” (black arrow). A warning box will appear which can be dismissed by selecting “OK”. 



3.5.09	The unknowns will be calculated using the standards (yellow arrow).
3.5.10	Select all of the wells (purple arrow).
3.5.11	Then right click on the samples and select “Export Table” (white arrow). The file name window will then open and add a suitable file name (include plate number). Select save.
3.5.12	Analyse the extracted data to produce the library concentration.

APPENDIX 1 – LIBRARY QUANTIFICATION



	1	2	3	4	5	6	7	8	9	10	11	12
A	S6	S6	S6	S5	S5	S5	S4	S4	S4	S3	S3	S3
B	S2	S2	S2	S1	S1	S1				NC	NC	NC
C	GRC1 1:1,000	GRC1 1:1,000	GRC1 1:1,000	GRC1 1:10,000	GRC1 1:10,000	GRC1 1:10,000						
D	GRC2 1:1,000	GRC2 1:1,000	GRC2 1:1,000	GRC2 1:10,000	GRC2 1:10,000	GRC2 1:10,000						
E	SPEC 1:1,000	SPEC 1:1,000	SPEC 1:1,000	SPEC 1:10,000	SPEC 1:10,000	SPEC 1:10,000						
F												
G												
H