Protocol for KRAS interlab study

Notes LGC has provided all the samples, Supermix, assays, DG8 cartridges, gaskets, and droplet generation oil required for the study.

The consumables not provided are nuclease-free water, droplet reader oil, foil seal, 2 x Eppendorf twin.tech semi-skirted 96-well microtiter plate (P/N 951020362), removable seal, and Rainin pipettes and tips to transfer droplets (P/N RT-L200F).

Please use all the provided reagents and consumables for this study. If any of the provided items are compromised in any way, please contact LGC prior to proceeding (alexandra.whale@lgcgroup.com).

Three units of each unknown sample (A-D), three units of the negative control (NEG) and one unit of the positive control (POS) have been provided. These are randomly selected from the 300 manufactured units. Each unit contains ~ 100 µL. Upon arrival, please fill in **Table 1** with the unit number found on each tube (e.g. N234 or B45). The sample name corresponds to the 96-well plate layout below and . *qlt* file.

Table 1

Sample	Purpose	Unit number
N1	Negative Control	
N2	Negative Control	
N3	Negative Control	
P	Positive Control	
A1	Unknown Sample	
A2	Unknown Sample	
A3	Unknown Sample	
B1	Unknown Sample	
B2	Unknown Sample	
B3	Unknown Sample	
C1	Unknown Sample	
C2	Unknown Sample	
C3	Unknown Sample	
D1	Unknown Sample	
D2	Unknown Sample	
D3	Unknown Sample	

Protocol

Step 1 Thaw the Supermix and Assays at room temperature, vortex briefly to mix and pulse down in a microfuge.

Assemble the mastermix as detailed in **Table 2**.

This is enough mastermix for all 56 reactions with excess to allow for pipetting loss.

Table 2

Reagent	Volume (µL)
ddPCR Supermix for Probes with no dUTP	750.0
ddPCR Mutation Detection Assay (KRAS G12D)	75.0
ddPCR Mutation Detection Assay (WT for KRAS G12D)	75.0
Total	900.0

Step 2 In an Eppendorf twin.tech semi-skirted 96-well microtiter plate, add 15 µL of the mastermix from **Table 2** to the wells shown in the plate layout below (grey shaded wells).

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Step 3 Thaw samples at room temperature, vortex briefly to mix and pulse down in a microfuge.

Add 10 µL of each sample unit to the wells containing mastermix according to the plate layout below.

This layout enables triplicate reactions to be made for each sample unit. There is excess material in each unit to perform the triplicate reactions.

Add 10 µL of nuclease-free water to the eight NTC reactions instead of sample.

Each well will contain 25 µL total volume to ensure that 20 µL can be loaded into the DG8 cartridge (step 5).

	1	2	3	4	5	6	7	8	9	10	11	12
A	N1	N1	N1	B2	B2	B2	NTC					
B	N2	N2	N2	B3	B3	B3	NTC					
C	N3	N3	N3	C1	C1	C1	NTC					
D	P	P	P	C2	C2	C2	NTC					
E	A1	A1	A1	C3	C3	C3	NTC					
F	A2	A2	A2	D1	D1	D1	NTC					
G	A3	A3	A3	D2	D2	D2	NTC					
H	B1	B1	B1	D3	D3	D3	NTC					

Step 4 Seal the plate with a removable seal and vortex for 5 seconds to mix the reactions.
If no removable seal is avaliable, mix the reactions by pipetting up and down 10 times.
Centrifuge the plate for 1 min at ~ 3,000 x *g* .

Step 5 Please DO NOT use an AutoDG™ for this step.
Create droplets as described in the QX100™/QX200™ User Manual for each of the samples using the following volumes:
Transfer 20 µL of each reaction mix and 70 µL of the provided droplet generation oil into the relevant wells of the DG8 cartridge.
Following droplet generation, transfer 40 µL of droplets into a fresh Eppendorf 96-well twin.tech plate, maintaining the plate layout as in Step 4.
It is strongly recommended that a Rainin multichannel pipette is used to transfer the droplets for this step.

Step 6 Seal the plate with a piercable foil heat seal using a BioRad PX1 PCR plate sealer, for 5 sec @ 180 °C.
Perform the PCR using the thermal cycling conditions described in **Table 3**.

Table 3

Step	Time	Temp (°C)	# of cycles	Ramp rate (°C/sec)
Enzyme activation	10 min	95	1	2
Denaturation	30 sec	94	40	2
Annealing/Extension	1 min	55		2
Signal stabilization	10 min	98	1	2
Hold	forever	4	1	1

Sample volume: 40 µl
Heated lid: 105 °C

Step 7 Open QuantaSoft and import the template file (KRAS.*qlt*) provided from LGC.
Run the droplet reader using the FAM/HEX settings.

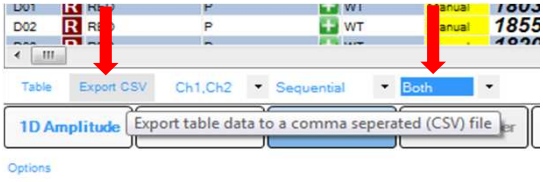
Data Analysis

Step 8 Analyse the data using the positive and negative controls to assist with threshold setting.
The KRAS G12D assay has a FAM probe and so the probe amplitude is in Ch1, the WT for KRAS G12D assay has a HEX probe and so the probe amplitude is in Ch2.
Please DO NOT rename any of the sample wells; the template has been set up specifically to allow comparison between the data generated by the different participants.

Once analysed, save the *.qlp* file under the name of your institution (e.g. LGC_KRAS.*qlp*) and upload the file using the sharefile link provided.

Data Reporting

Step 9 Please complete **Table 4** (reporting form in separate file). Green shaded boxes have drop down menus.
Save the **Table 4** file under the name of your instiution (e.g. LGC_Table 4) and upload with the *.qlp* file.
The comments column is for any extra information you would like to add, e.g. if you want to specify a parameter that does not appear in the drop down menu.
Export the *.csv* table from the QuantaSoft analysis program ensuring that you have selected the 'both' option (highlighted in the screen shot below):



Save the *.csv* file under the name of your institution (e.g. LGC_KRAS.*csv*) and upload with the *.qlp* file.
The *.csv* file can be used for your analysis in another software package (e.g. MS Excel) to allow quantification of the samples
For data reporting, please use the technical and unit replicates to calculate the copies/µL of the WT and G12D in the reaction with the associated 95% confidence intervals. DO NOT rename the sample wells in the *.qlp* file to do this; use the software generated results to do this.

Please also include the calculated fractional abundance and associated 95% confidence intervals of the G12D molecule.
Other analysis files may be uploaded to the sharefile link to assist in explaining the data analysis method. Please include your institution in the file name to aid with identification (e.g. LGC_analysis.*xlsx*).

If you have any questions regarding the data reporting please contact alexandra.whale@lgcgroup.com.