Manual

Running KASP genotyping reactions on the QIAGEN Rotor-Gene Q instrument

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BIOSEARCH™ TECHNOLOGIES

GENOMIC ANALYSIS BY LGC

Manual

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Contents

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1. Introduction

This document is intended as a guide to running KASP™ genotyping reactions on the QIAGEN Rotor-Gene® Q instrument.

KASP chemistry for allelic discrimination performs well on a QIAGEN Rotor-Gene Q instrument and this step-by- step protocol will enable users to successfully set-up, run and read plates on the QIAGEN Rotor-Gene Q.

2. Tips and suggestions before you start

1. Optimal cycling conditions for KASP require a touchdown 2-step PCR protocol. The cycling conditions for most assays will be as described in this manual (section 4), although you must check the cycling conditions included in your assay information pack to ensure that your assay does not have any specific cycling conditions.
2. KASP is an endpoint chemistry and will require a final read once the PCR amplification steps have been completed. Completed KASP reactions must be read below 40 °C.
3. Data capture should only be performed at the end of the thermal cycle programme – no useful data will be captured during the thermal cycling protocol.
4. The KASP recycling programme will often improve results, especially for assays that are slow to form clusters.

3. Overview of the procedure

1. Create a new run for KASP genotyping – see section 5.1.
2. Programme the thermal cycling conditions and read step – see section 5.2.
3. Edit the samples included in the run – see section 5.3.
4. View the data – see section 5.4.
5. Recycle the reactions if required – see section 5.5.

4. KASP thermal cycling conditions

4.1 Standard KASP thermal cycling conditions

Stage 1

- 94 °C - 15 minutes

Stage 2

- 94 °C - 20 seconds
- 61 °C - 60 seconds[1]
- Repeat Stage 2 × 9 times (a total of 10 cycles) achieving a final annealing temperature of 55.6 °C.

[1]Drop -0.6 °C per cycle

Stage 3

- 94 °C - 20 seconds
- 55 °C - 60 seconds
- Repeat Stage 3 × 25 times (a total of 26 cycles)

Stage 4

- Cool the reactions to 35 °C (suggested 2 minutes)*

Stage 5

- Add a read step at 35 °C for 30 sec*

4.2 KASP recycling conditions

Stage 1

- 94 °C - 20 seconds
- 57 °C - 60 seconds
- Repeat steps 1-2 twice (a total of 3 cycles)

Stage 2

- Cool the reactions to 35 °C (suggested 2 minutes)*

Stage 3

- Add read step at 35 °C for 1 min*

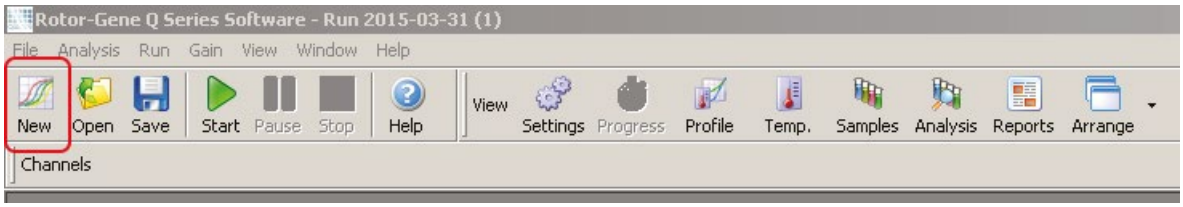
*KASP cannot be read at temperatures above 40 °C.

Please note: We can provide a setup file for this machine for standard KASP thermal cycling. Please contact the technical support team (details at the end of this document) for further information.

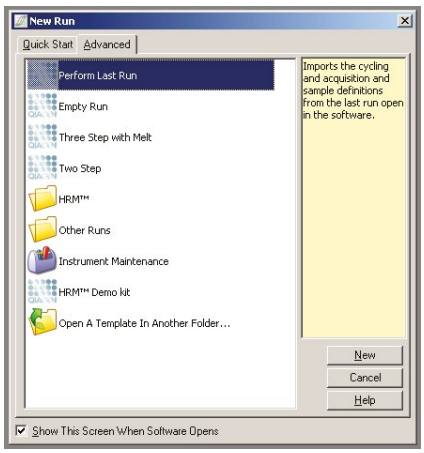
5. Step-by-step user guide

5.1 Create a new run for KASP genotyping

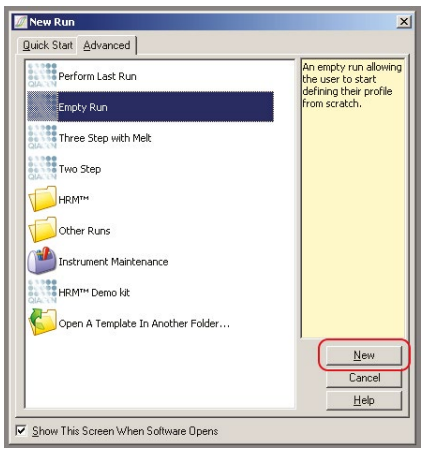
- Open the Rotor-Gene Q software by double-clicking on the desktop icon.
- Click on the ‘New’ button in the top menu bar.



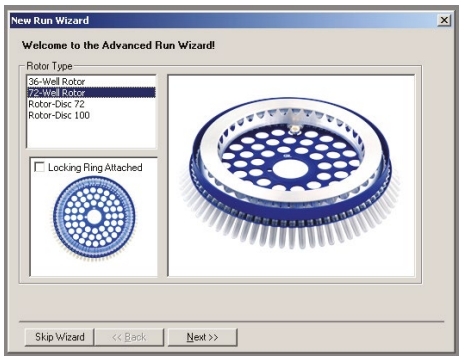
- This will open the ‘New Run’ window. (Please note that this window may open automatically when the software first launches depending upon your default settings).



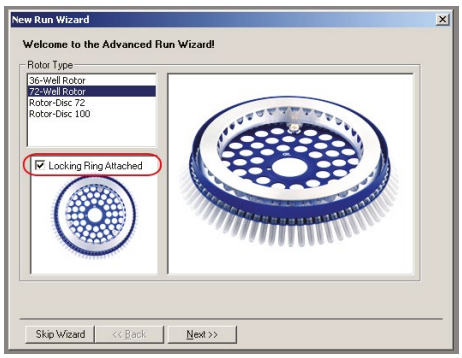
- On the ‘Advanced’ tab, select ‘Empty Run’ and press ‘New’.



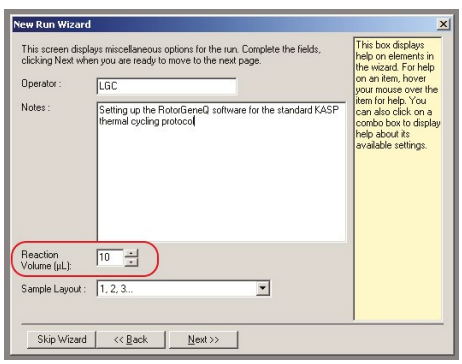
- You will then need to select the appropriate rotor type for the run that you plan to perform on your instrument.



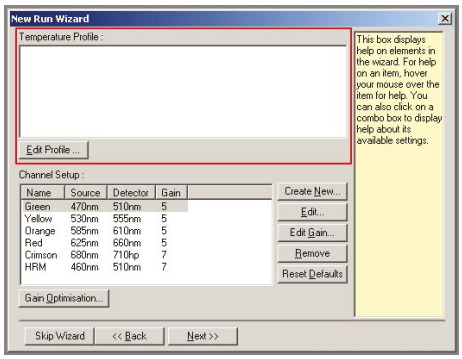
- Before pressing 'Next', it is essential to tick the 'Locking Ring Attached' box. Once this is ticked, press 'Next' to progress through the wizard.



- The next window requires input of miscellaneous information regarding the run. It is essential to specify a reaction volume to proceed through the wizard. Press 'Next'.



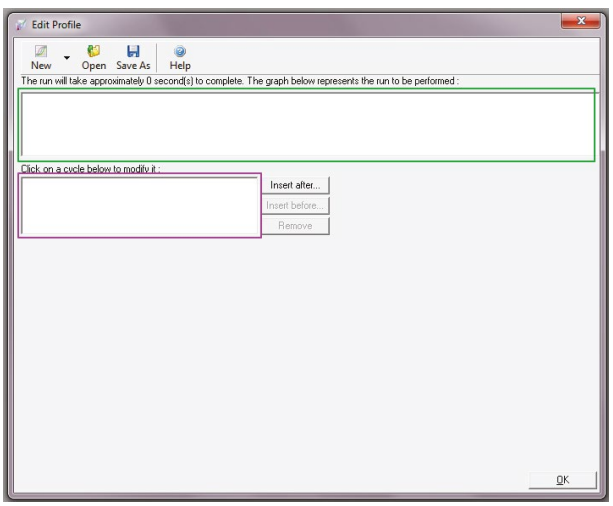
- The next window contains an empty temperature profile box. Section 5.2 of this guide details how to programme the KASP thermal cycle within the Rotor-Gene Q software.



5.2 Programme the thermal cycling conditions and read step

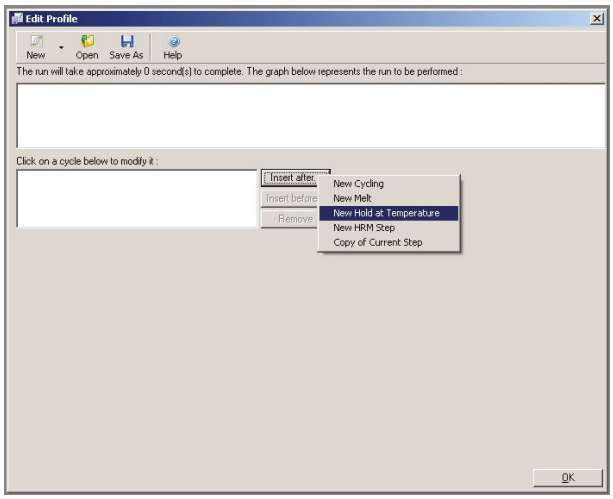
- After completing the steps outlined in section 5.1 of this guide, you should have an open window of the 'New Run Wizard' containing a blank 'Temperature Profile' box. Click on the 'Edit Profile' button to open a new window within which the temperature profile can be programmed.

Once programming is complete, the top box (outlined in green) will contain the thermal profile graph and the lower box (outlined in purple) will contain a list of the steps that have been programmed in the thermal cycle.

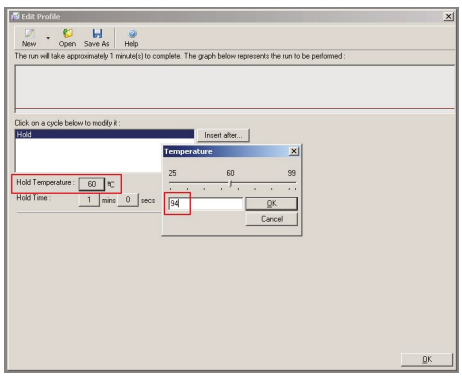


5.2.1 Programme the activation stage (Stage 1): 94 °C; 15 minutes

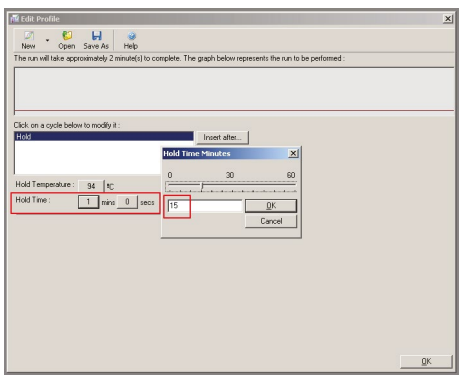
- Click on the ‘Insert after…’ button and select ‘New Hold at Temperature’. A stage called ‘Hold’ will be added to your thermal cycle protocol.



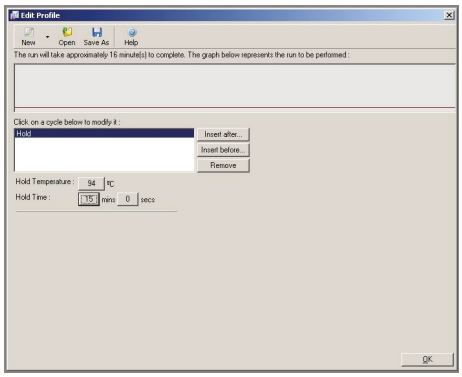
- Click on the ‘Hold Temperature’ button, edit this to 94 °C and press ‘OK‘.



- Click on the ‘Hold Time’ button, edit this to 15 min and press ‘OK‘.



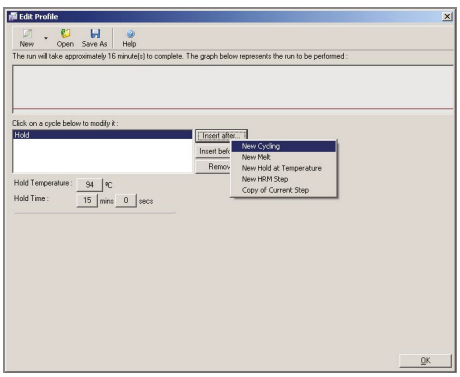
- The correctly programmed stage (called ‘Hold’) should appear as shown below:

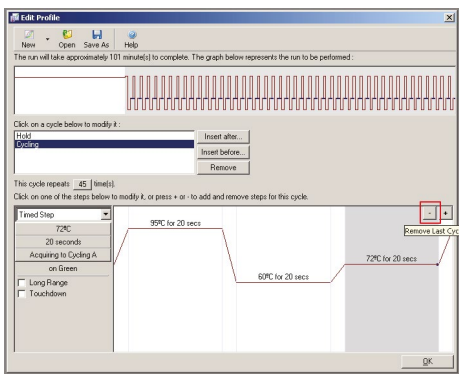


5.2.2 Programme the touchdown stage (Stage 2) (10 cycles):

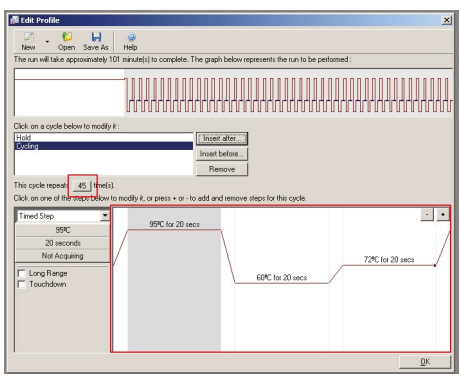
- 94 °C - 20 seconds
- 61 °C - 60 seconds (drop -0.6 °C per cycle)

- Click on the ‘Insert after…’ button and select ‘New Cycling’. A default cycling stage in the Rotor-Gene Q software contains 45 cycles of 3-step PCR – this requires editing for the KASP thermal cycling protocol.

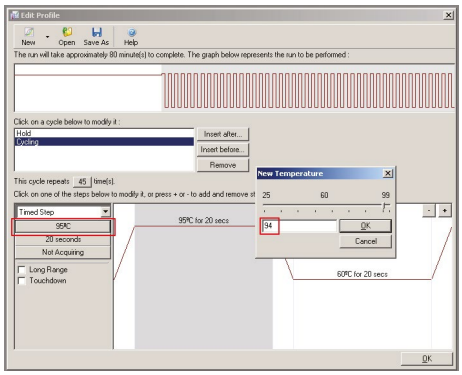




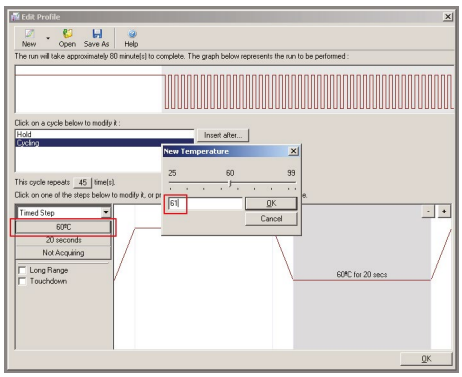
- Remove the third step of the default cycling stage by clicking on the step and subsequently clicking the minus (-) icon.



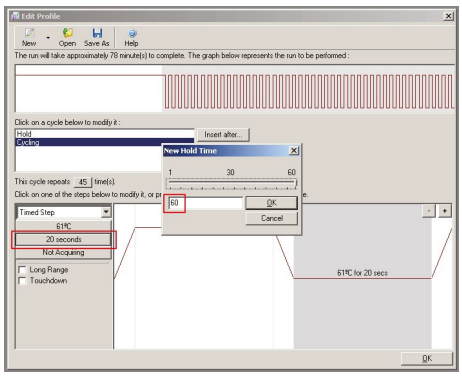
- Edit the temperature for the first step of the touchdown cycling stage to 94 °C. To do this, highlight the step in the image (it will appear grey) and press the temperature button (default is ‘95 °C’). In the window that opens, change the temperature to 94 °C and press ‘OK’.



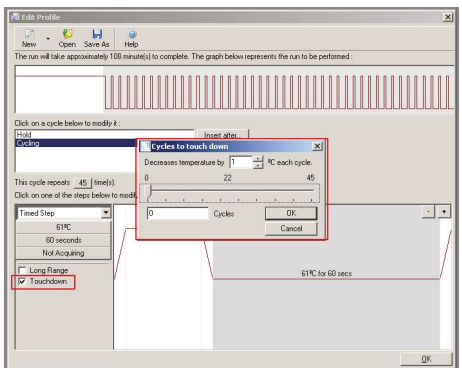
- Ensure that the time for the first step of the touchdown cycling stage is set to 20 seconds (this is the default).
- Edit the temperature for the second step of the touchdown cycling stage to 61 °C. To do this, highlight the step in the image (click on the step and it will appear grey) and press the temperature button (default is ‘60 °C’). In the window that opens, change the temperature to 61 °C and press ‘OK’.



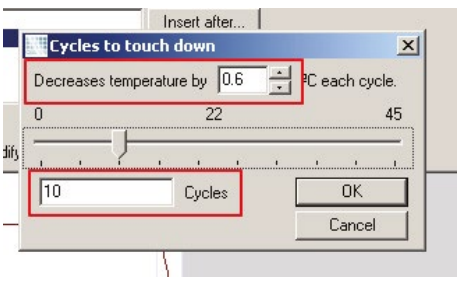
- Edit the time for the second step of the touchdown cycling stage to 1 minute. Press the time button (default is 20 seconds) and, in the window that opens, change the time to 60 seconds and press ‘OK’.



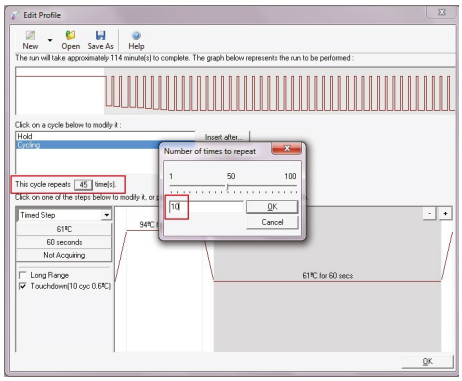
- With the second step of the touchdown cycling stage highlighted (grey, 61 °C for 20 seconds), click to put a tick in the ‘Touchdown’ box. This will open the ‘Cycles to touch down’ window.



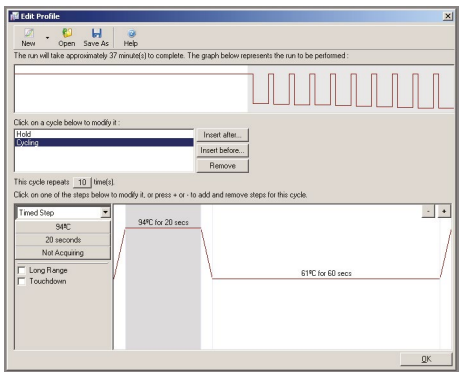
- Edit the temperature decrease to 0.6 °C each cycle and ensure that the number of cycles is set to 10. Press ‘OK’.



- Reduce the total number of cycles for this cycling stage to 10 cycles.



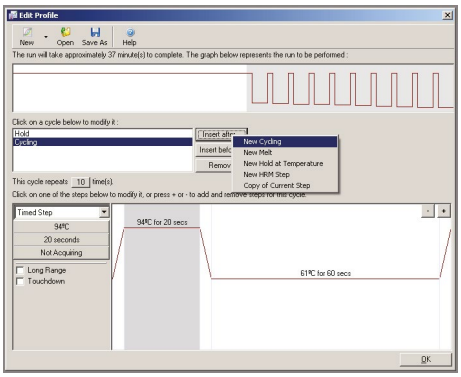
- The correctly programmed stage (called ‘Cycling’) should appear as shown below:



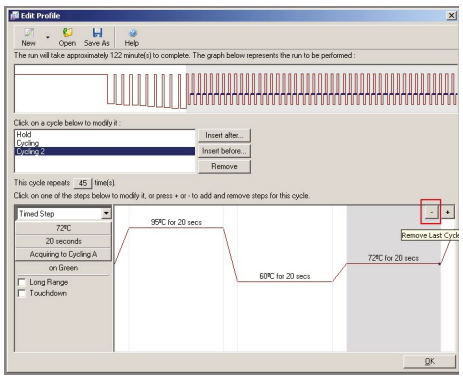
5.2.3 Programme the amplification stage (Stage 3) (26 cycles):

- 94 °C - 20 seconds
- 61 °C - 60 seconds (drop -0.6 °C per cycle)

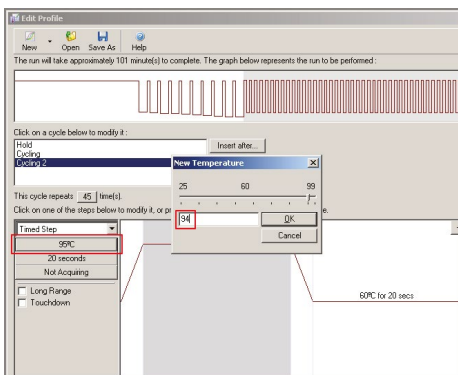
- Click on the ‘Insert after…’ button and select ‘New Cycling’. A default cycling stage in the Rotor-Gene Q software contains 45 cycles of 3-step PCR – this requires editing for the KASP thermal cycling protocol.



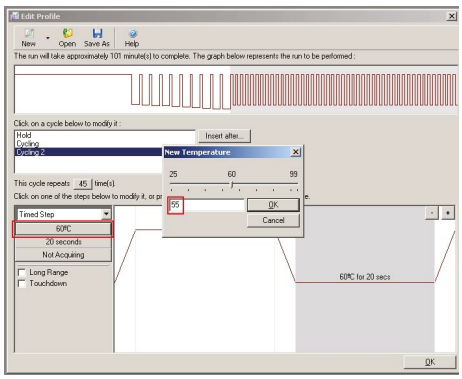
- A third stage called ‘Cycling2’ will be added to your protocol.
- Remove the third step of the default cycling stage by clicking on the step and subsequently clicking the minus (-) icon.



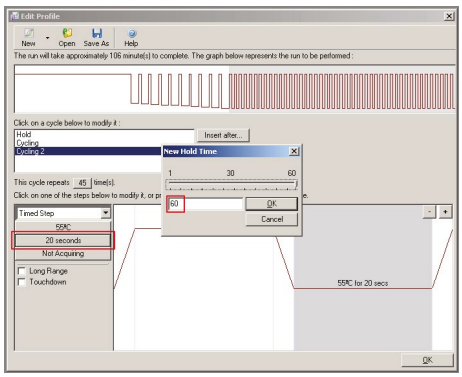
- Edit the temperature for the first step of the second cycling stage to 94 °C. To do this, highlight the step in the image (click on the step and it will appear grey) and press the temperature button (default is ‘95 °C’). In the window that opens, change the temperature to 94 °C and press ‘OK’.



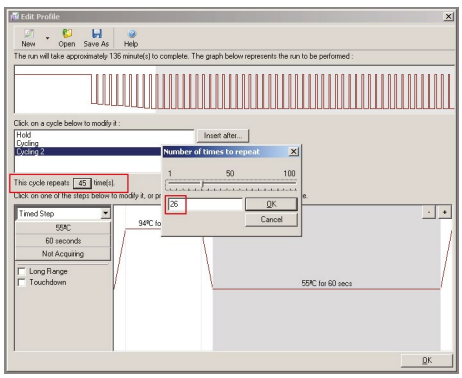
- Ensure that the time for the first step of the Cycling2 stage is set to 20 seconds (this is the default).
- Edit the temperature for the second step of the Cycling2 stage to 55 °C. To do this, highlight the step in the image (it will appear grey) and press the temperature button (default is ‘60 °C’). In the window that opens, change the temperature to 55 °C and press ‘OK’.



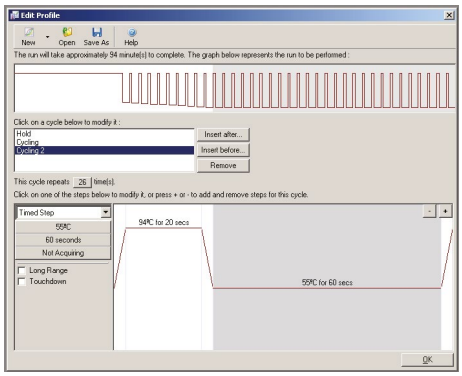
- Edit the time for the second step of the Cycling2 stage to 1 minute. Press the time button (default is 20 seconds) and, in the window that opens, change the time to 60 seconds.



- Reduce the total number of cycles for this cycling stage to 26 cycles and press ‘OK’.

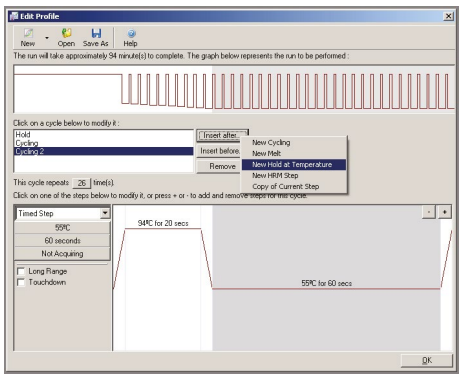


- The correctly programmed stage (called ‘Cycling2’) should appear as shown below:

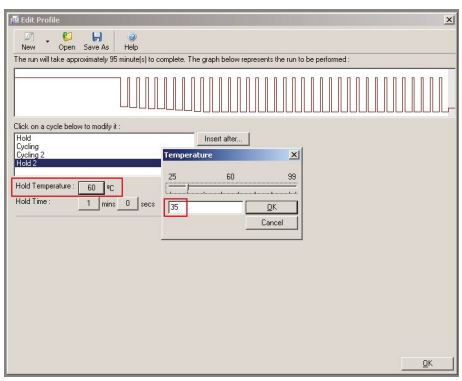


5.2.4 Programme the plate cooling stage (Stage 4) (1 cycle):

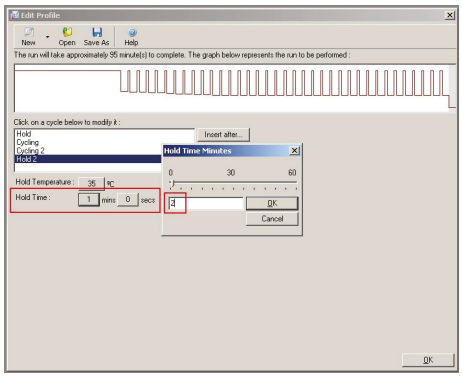
- Click on the ‘Insert after…’ button and select ‘New Hold at Temperature’. A fourth stage called ‘Hold2’ will be added to your thermal cycle protocol.



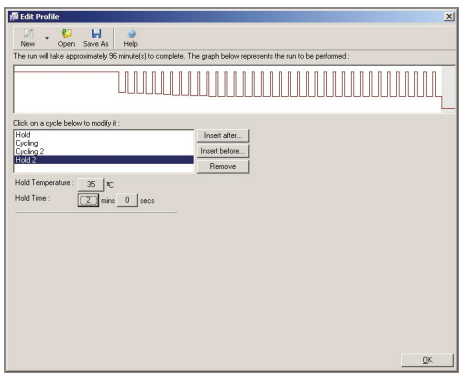
- Click on the ‘Hold Temperature’ button, edit this 35 °C and press ‘OK’. Please note: Completed KASP reactions must be cooled to below 40 °C as KASP chemistry cannot be read above 40 °C.



- Click on the ‘Hold Time’ button, edit this to 2 minutes and press OK.

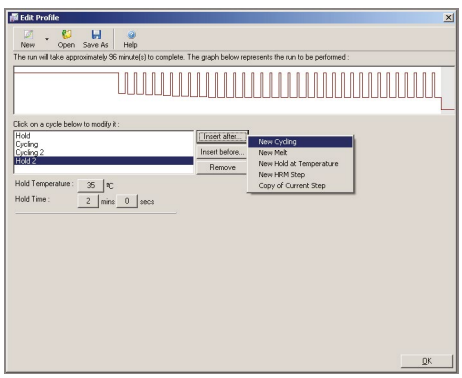


- The correctly programmed stage (called ‘Hold2’) should appear as shown below:

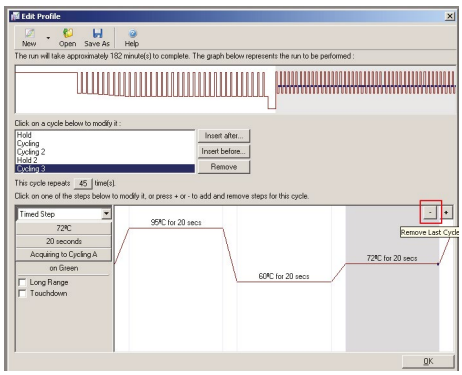


5.2.5 Programme the plate read stage (Stage 5) (1 cycle):

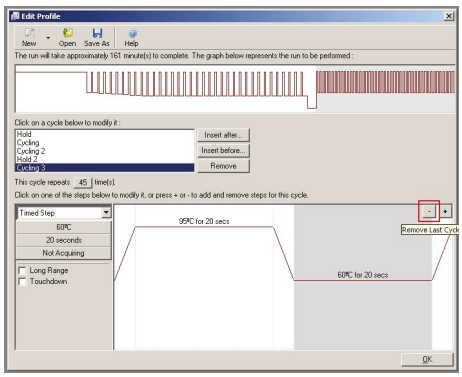
- Click on the ‘Insert after…’ button and select ‘New Cycling’. A fifth stage called ‘Cycling3’ will be added to your thermal cycle protocol.



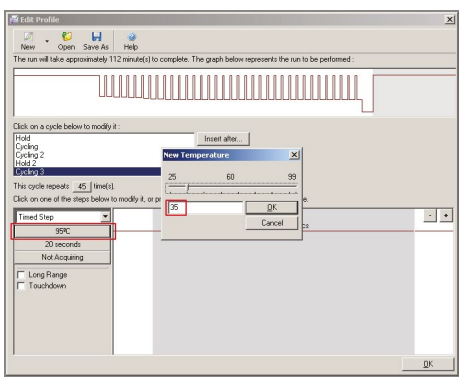
- Remove the third step of the default cycling stage by clicking on the step and subsequently clicking the minus (-) icon.



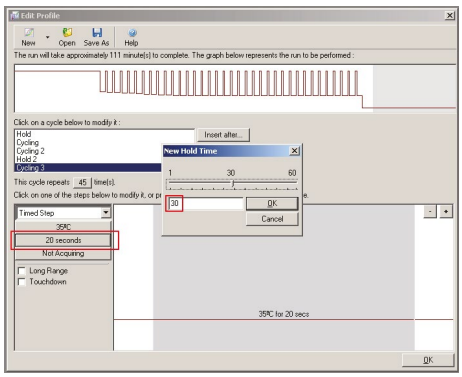
- Remove the second step of the default cycling stage by clicking on the step and subsequently clicking the minus (-) icon.



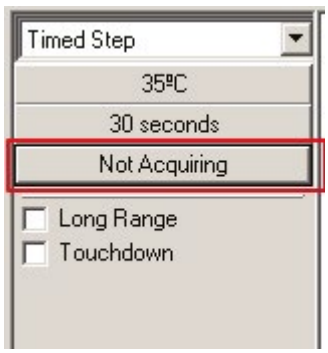
- Click on the temperature button, edit this 35 °C and press OK.

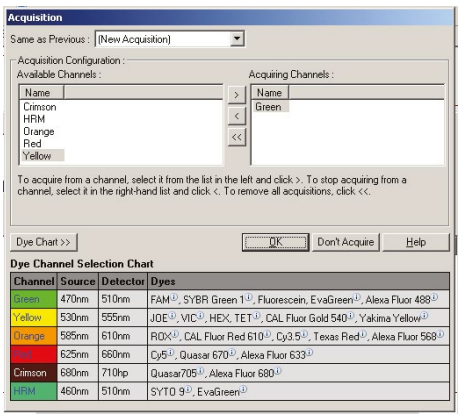


- Click on the time button, edit this to 30 seconds and press OK.

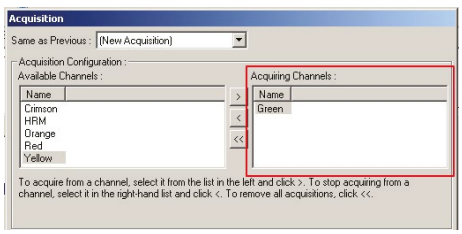


- To define the parameters for reading the completed KASP reactions, click on the ‘Not Acquiring’ button. This will open the ‘Acquisition’ window.

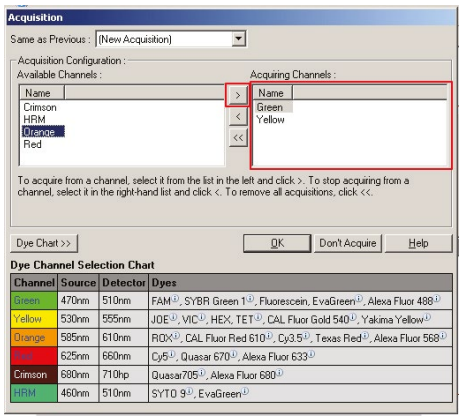




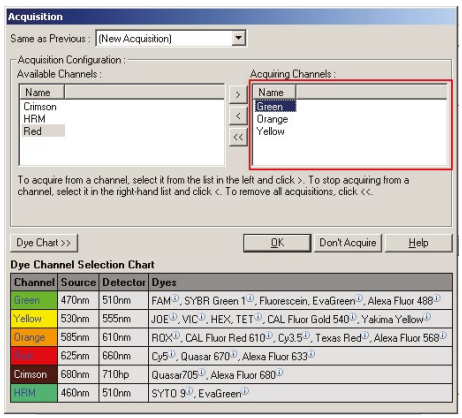
- KASP uses the fluorophores FAM and HEX for distinguishing genotypes. The passive reference dye ROX is also used to allow normalisation of variations in signal caused by differences in well-to-well liquid volume.
- The ‘Green’ channel is included in the ‘Acquiring Channels’ box by default. This channel detects FAM.



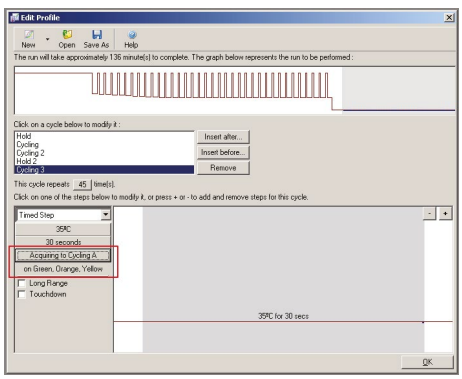
- To programme the instrument to read HEX, click on ‘Yellow’ in the available channels list, and then the right arrow button (>) to move it to the ‘Acquiring Channels’ box.



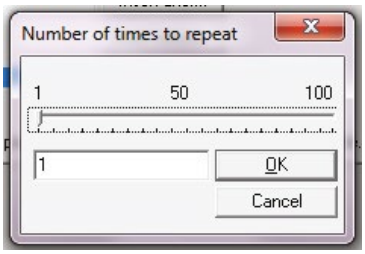
- To programme the instrument to read ROX, click on ‘Orange’ in the available channels list, and then the right arrow button (>) to move it to the ‘Acquiring Channels’ box. Press ‘OK’.



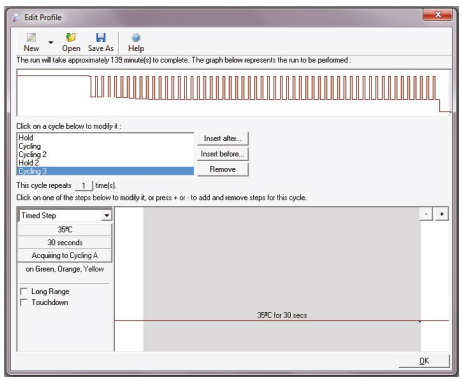
- In the ‘Edit Profile’ window, the acquisition button now states ‘Acquiring to Cycling A’. It also lists the dyes that have been selected below the button (i.e. Green, Orange and Yellow).



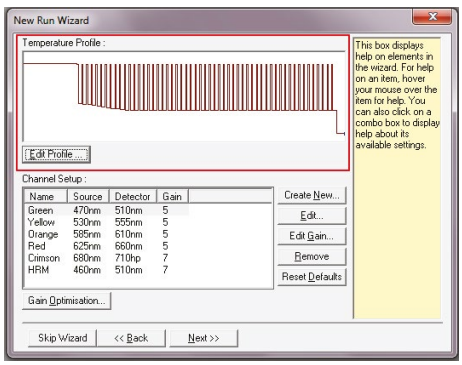
- The ‘Cycling3’ stage is set to repeat 45 times by default. Click on the number of cycles button, reduce this to 1 cycle, and press ‘OK’.



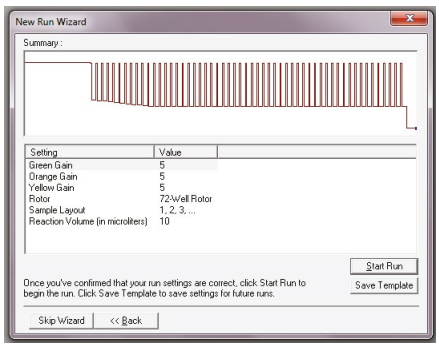
- The correctly programmed stage (called ‘Cycling3’) should appear as shown below:



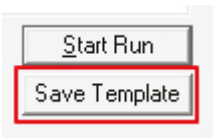
- After ‘Cycling3’ is programmed, press ‘OK’ at the bottom right of the ‘Edit Profile’ window.
- This will then enable you to see a summary of the temperature profile (graphical format). The gains settings for each channel are also shown – these can be left as the default settings. Press ‘Next’.

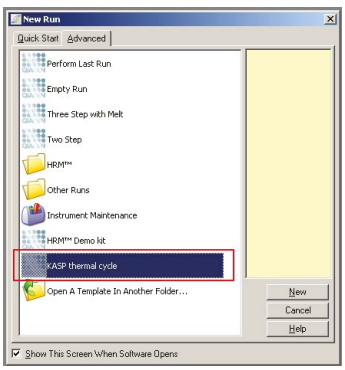


- The final window of the ‘New Run Wizard’ provides a summary of the thermal profile, and the rotor and read settings.

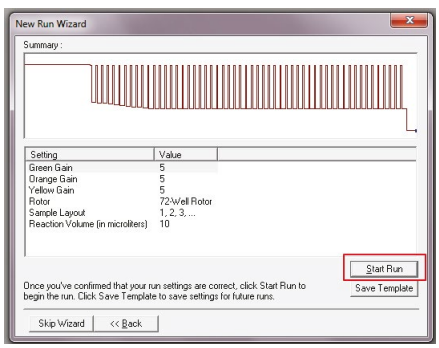


- At this stage, it may be helpful to save the KASP thermal cycle as a template that can be used for future KASP genotyping experiments. To do this, click on the ‘Save Template’ button. You will then be prompted to give the template a suitable name. By saving it as a template, it will then be available in the ‘New Run’ window in the future.





- To start the run, press the ‘Start Run’ button.



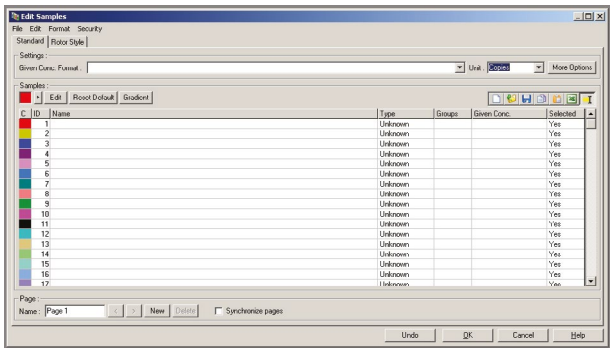
- You will be prompted to choose a suitable location to save the completed run file in.
- It is then possible to edit the sample information for the run, before the run commences. Alternatively, sample information can be edited whilst the run is in progress, as detailed in section 5.3.

5.3 Edit the samples included within the run

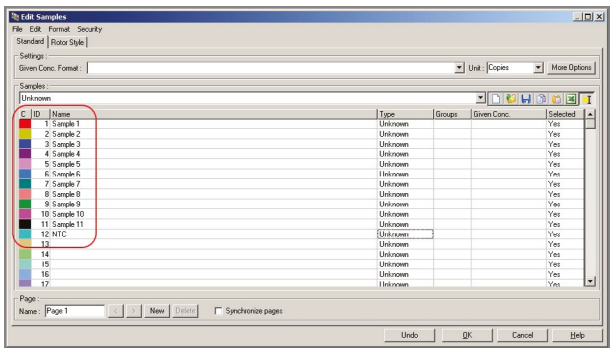
Whilst the KASP genotyping reactions are running on the Rotor-Gene Q, the samples can be defined within the software.

- In the main Rotor-Gene Q window, click on the ‘Samples’ button in the top menu bar.

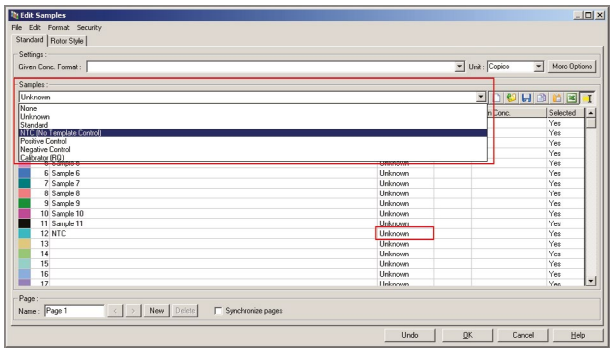
- Alternatively, you can click on the ‘Edit Samples’ button at the bottom of the sample list (right hand side of Rotor-Gene Q window).
- Both of these options will open the ‘Edit Samples’ window.

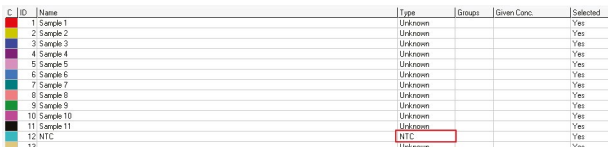


- Complete the ‘Name’ and ‘Type’ fields for each individual sample, according to the experiment that you are running.
- Sample names can be typed directly into the box next to the sample ID number.

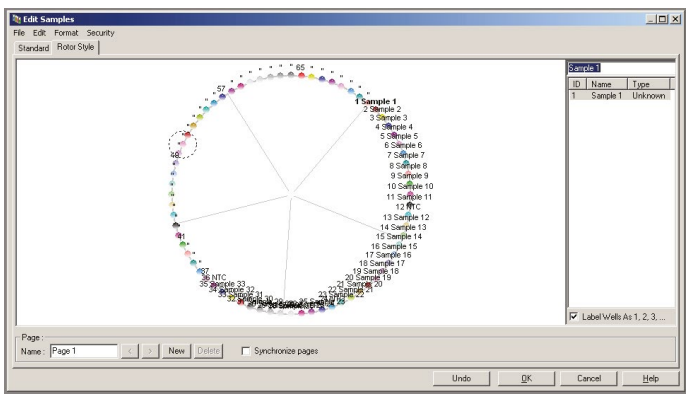


- DNA samples that are to be genotyped should contain ‘Unknown’ in the ‘Type’ field. No template controls (NTC) should be defined as such within the ‘Type’ field. This can be done by clicking on the corresponding ‘Type’ box and selecting ‘NTC’ from the drop down ‘Samples’ menu above the table.



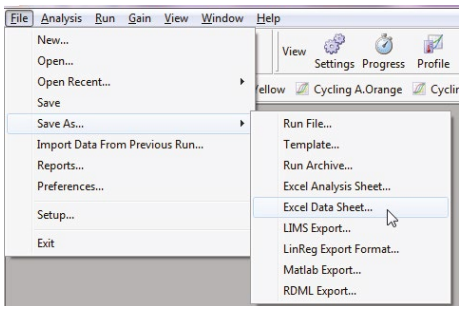


- It is also possible to view the sample information in a rotor format, with sample names corresponding to their position within the rotor.

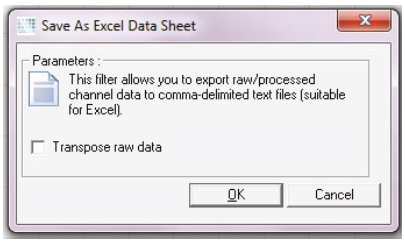


5.4 View and analyse the data

- It does not appear to be possible to directly analyse endpoint genotyping data within the QIAGEN Rotor-Gene Q software.
- At the end of the run, click on the 'File' menu at the top of the window. Select 'Save As…' and choose 'Excel Data Sheet'.



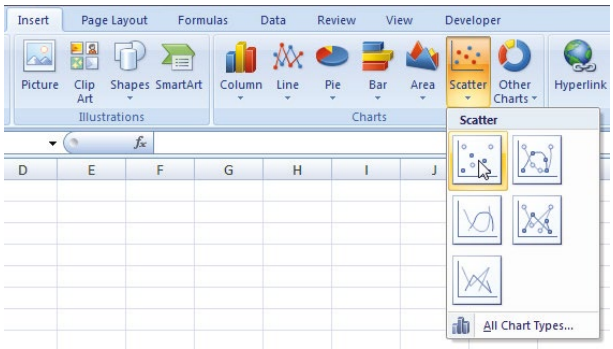
- You will then be prompted to choose an appropriate location to save the MS Excel file to.
- A message window will then open giving you the option to choose to transpose the raw data before exporting. LGC Biosearch Technologies suggest exporting the raw data rather than transposing it first (i.e. do not tick the 'Transpose raw data' box).



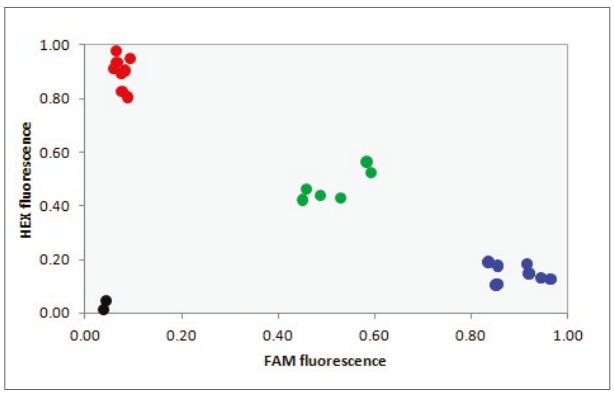
- Press 'OK'.
- The resulting MS Excel file will contain run information, and the raw fluorescent read values for each sample.
- The 'Green' channel data gives the FAM fluorescence values for each sample.
- The 'Yellow' channel data gives the HEX fluorescence values for each sample.
- The 'Orange' channel data gives the ROX fluorescence values for each sample.
- To normalise the data, the raw values for FAM and the raw values for HEX should be divided by the corresponding ROX values.

FAM	HEX	ROX	Normalised FAM	Normalised HEX
GREEN	YELLOW	ORANGE	FAM ÷ ROX	HEX ÷ ROX
3.886	1.505	1.514	2.566	0.994
3.616	1.436	1.276	2.833	1.125
4.173	1.628	1.785	2.337	0.912
3.922	1.483	1.533	2.557	0.967
3.951	1.598	1.471	2.685	1.086
4.280	1.695	1.857	2.305	0.913
4.457	1.729	1.944	2.292	0.889
4.275	1.648	1.759	2.431	0.937
3.029	5.004	2.233	1.356	2.241
2.962	4.901	2.065	1.434	2.374
2.884	4.739	2.116	1.363	2.239
3.144	5.375	2.221	1.415	2.420
2.568	4.032	1.404	1.829	2.872
2.728	4.342	1.709	1.596	2.541
2.627	4.275	1.623	1.619	2.634

- To view a genotyping cluster plot, highlight the ‘Normalised FAM’ and the ‘Normalised HEX’ data and select ‘Scatter’ from the ‘Charts’ section of the ‘Insert’ menu.



- A cluster plot of the data will then be inserted into your MS Excel workbook and can be used to determine the genotypes of your samples. Ensure that the X and Y axes are scaled comparably to prevent misinterpretation of the data.
- A typical cluster plot, with three clear genotyping clusters and no template controls at the origin (no amplification), will look similar to the figure below:



5.5 Recycle the reactions if required

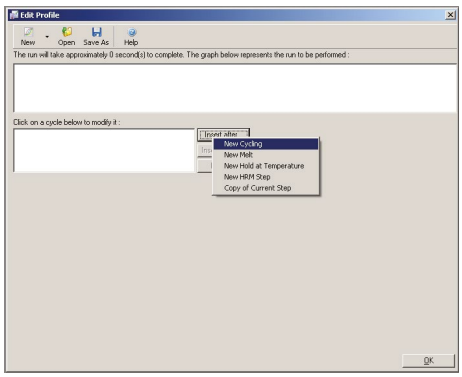
- If your data has not formed tight clusters after the initial thermal cycling protocol, you can recycle the reactions in the QIAGEN Rotor-Gene Q and perform a second post-read.
- Follow the instructions in section 5.1 to create a new empty run for KASP recycling.
- The KASP recycling conditions are:

Stage 1

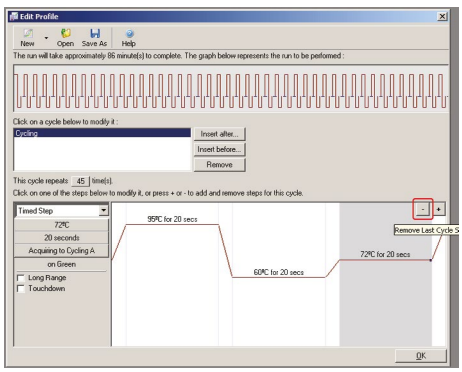
- 94 °C - 20 seconds
- 94 °C - 20 seconds

Repeat steps 1-2 twice (a total of 3 cycles)

- To programme this, first add a ‘New Cycling’ stage to the thermal profile within the empty run. A stage called ‘Cycling’ will be added to the thermal profile.

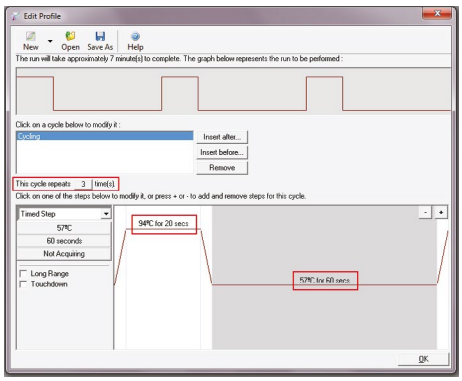


- Remove the third step of the default cycling stage by clicking on the step and subsequently clicking the minus (-) icon.

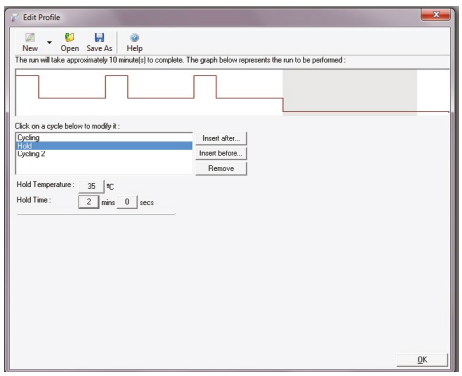


- Edit the temperature for the first step of the second cycling stage to 94 °C. To do this, highlight the step in the image (click on it and it will appear grey) and press the temperature button (default is ‘95 °C’). In the window that opens, change the temperature to 94 °C and press ‘OK’.
- Ensure that the time for the first step of the cycling stage is set to 20 seconds (this is the default).
- Edit the temperature for the second step of the touchdown cycling stage to 57 °C. To do this, highlight the step in the image (click on it and it will appear grey) and press the temperature button (default is ‘60 °C’). In the window that opens, change the temperature to 57 °C and press ‘OK’.
- Edit the time for the second step of the touchdown cycling stage to 1 minute. Press the time button (default is 20 seconds) and, in the window that opens, change the time to 60 seconds and press ‘OK’.
- Reduce the total number of cycles for this stage to 3.

- The edited ‘Cycling’ stage should then appear as shown below:

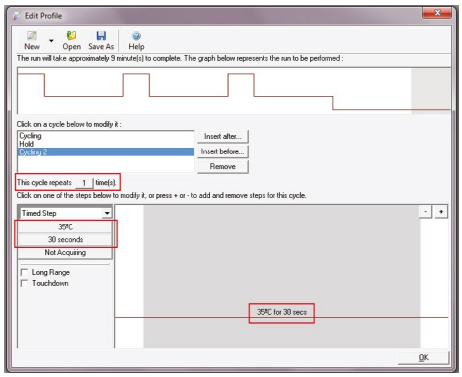


- Click on the ‘Insert after…’ button and select ‘New Hold’. A second stage called ‘Hold’ will be added to your thermal cycle protocol.
- Edit the ‘Hold Temperature’ of this stage to 35 °C and ensure that the ‘Hold Time’ is 2 minutes. This stage will ensure that the reactions are cooled to below 40 °C prior to performing the fluorescent read.

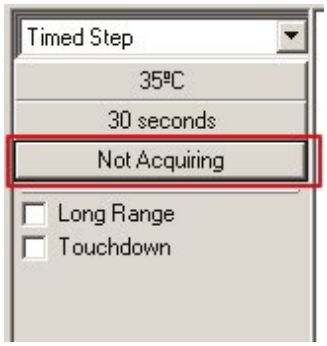


- Click on the ‘Insert after…’ button and select ‘New Cycling’. A third stage called ‘Cycling2’ will be added to your thermal cycle protocol.
- Remove the second and third steps of the default cycling stage by clicking on the each of the steps in turn and subsequently clicking the minus (-) icon.
- Click on the temperature button, edit this 35 °C and press OK
- Click on the time button, edit this to 30 seconds and press OK.

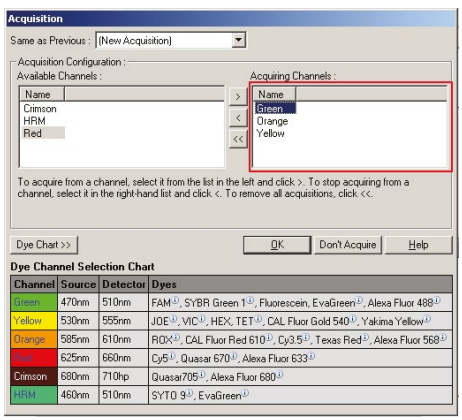
- Reduce the number of repeats for this cycle to 1.



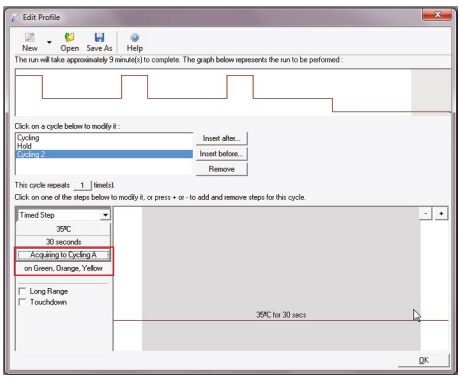
- To define the parameters for reading the completed KASP reactions, click on the ‘Not Acquiring’ button. This will open the ‘Acquisition’ window.



- To programme the instrument to read FAM, HEX and ROX, ensure that ‘Green’, ‘Yellow’ and ‘Orange’ are all moved to the ‘Acquiring Channels’ box.



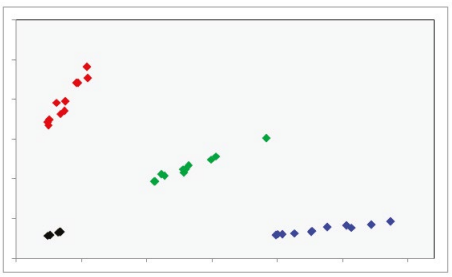
- The correctly programmed stage (called ‘Cycling2’) should appear as shown below:



- The recycling programme can be repeated until tight clusters have formed (re-read the reactions after each recycling programme).
- Once results have been read, completed KASP reactions are stable at room temperature for up to one week as long as the reaction tubes remain well sealed.
- If it is found that recycling is always necessary for a particular assay, extra cycles can be added to the PCR amplification stage (i.e. 29 cycles instead of 26).

5.6 Running the KASP trial kit on the QIAGEN Rotor-Gene Q

- If you have requested a KASP trial kit to run on your QIAGEN Rotor-Gene Q instrument, please follow the protocol included with the kit to set up your reaction plate. This Rotor-Gene Q manual can be used to help you to programme the instrument to run the trial kit reactions.
- After running the KASP thermal cycle, the trial kit reactions should produce data similar to the figure below.



Results from the KASP trial kit reaction plate when run on the QIAGEN Rotor-Gene Q instrument using the standard KASP thermal cycle. Data has been exported and plotted using the scatter plot function in MS Excel.

Further support

For any queries about this manual, please contact techsupport@lgcgroup.com.

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