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LightCycler® 480



1. Introduction

This document is intended as a guide to running KASP™ genotyping reactions on the Roche LightCycler 480 (LC480) instrument.

KASP chemistry for allelic discrimination performs well on a Roche LC480-series machine and this step-by-step protocol will enable users to successfully set-up, run and read plates on the LC480. Please be aware that some of the default settings within the LightCycler® software are not suitable for reading KASP and must be changed prior to performing plate runs and analysis of data.

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 once thermal cycling has ended – no useful data will be captured during the thermal cycling protocol.
4. There are two different methods available for SNP analysis on the LC480 system – KASP requires the use of 'Endpoint Genotyping Analysis'.
5. Ensure that you use FAM and HEX fluorophores only for your plate reads*.
*If you prefer to export your data and manipulate in another program, it is advisable to measure the passive reference dye in ROX and use this for normalisation (if the filter set is included in your machine). The LC480 Endpoint Genotyping software cannot use ROX to normalise.
6. The KASP recycling program will often will improve results, especially for assays that are slow to group.
7. The use of white plates for genotyping on the LC480 is recommended.

3. Overview of the procedure

1. Set up the correct detection format for KASP within the LC480 software – see Section 5.1.
2. Prepare and run a thermal cycle 'Experiment' with a plate read step – see Section 5.2.
3. Analyse your endpoint genotyping results to obtain genotyping calls – see Section 5.3.
4. Recycle the plate if necessary – see Section 5.4.



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4. KASP thermal cycling conditions

4.1 KASP thermal cycling conditions

Stage 1

- 94°C - 15 minutes

Stage 2

- 94°C - 20 seconds
- 61°C - 60 seconds¹
- Repeat Stage 2 x9 times (a total of 10 cycles) achieving a final annealing temperature of 55.6°C.

¹Drop -0.6°C per cycle

Stage 3

- 94°C - 20 seconds
- 55°C - 60 seconds
- Repeat Stage 3 x25 times (a total of 26 cycles)

Stage 4

- Add a read step at 37°C for 1 min*

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

- Add read step at 37°C for 1 min*

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

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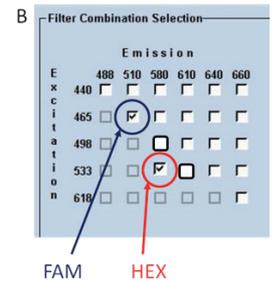
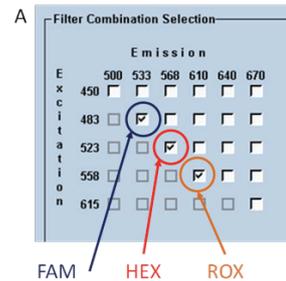
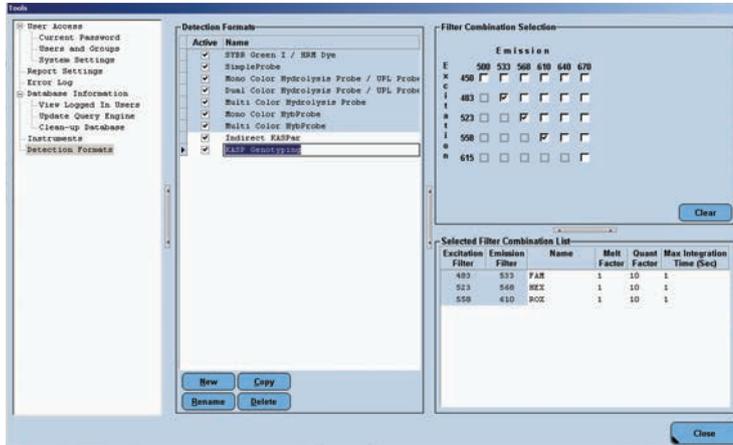
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5. Step-by-Step User Guide

5.1 Create a new protocol in the LightCycler software

- Open the LightCycler software.
- Select 'Overview' from the dropdown menu.
- In the 'Overview' window, click on the 'Open Tools' icon.
- This will open the 'Tools' window. Click on 'Detection formats'.



Please note: this screenshot is taken from the LC480 vI instrument software. The filter combination selection box will be different for the LC480 vII instrument software. See below for more details.

The filter combinations that should be selected for KASP when using the LC480 vI (screenshot A) and the LC480 vII (screenshot B).

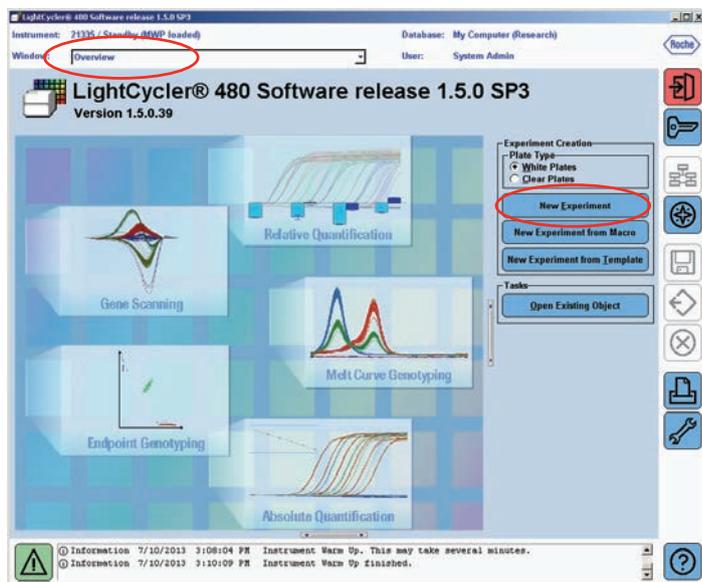
- Click 'New' and give the new format an appropriate new name such as 'KASP genotyping'. Once saved, this detection format can be used for all subsequent experiments.
- There are two versions of the LC480 instrument; version I and version II. These two versions differ in the filter sets that are available / in-built within the instrument.
- For the version I instrument, select boxes 483 – 533 (FAM), 523 – 568 (HEX) and, if the filter is in place, 558 – 610 (ROX) (see screenshot A above).
- For the version II instrument, select boxes 465 – 510 (FAM) and 533 – 580 (HEX) (see screenshot B above). Please note that ROX cannot be read at the same time as FAM and HEX on the version II instrument.
- Selection of the appropriate filters will enable you to select the FAM and HEX channels at a later stage of analysis (see Section 5.3).
- The screenshots above detail the filters that should be selected for both the vI and vII instruments.
- It is important to increase the 'Quant Factor' setting to 10 as this will improve the signal to background fluorescent ratio and hence improve genotyping results. All other settings can remain at 1.
- Click the 'Close' button to return to the 'Overview' screen.

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5.2 Prepare and run a thermal cycle experiment with a plate read step

- Select 'Overview' from the dropdown menu.
- From the LC480 start page in the 'Experiment Creation' box:
 - Select the plate type that you will be using from the 'Plate Type' box
 - Click on the 'New Experiment' experiment button to take you to the program page.



- Select the correct 'Detection Format' from the drop-down menu (the format that you created in Section 5.1).
- Ensure that the correct reaction volume is programmed for your plate type:
 - For 384-well plates, 5 µL reaction volumes should be used.
 - For 96-well plates, 10 µL reaction volumes should be used.
- Please note: once you have set up the KASP program in the LC480 software, save this as a template to re-use in future runs.
- In the program section, add four programs. The analysis mode should be set to 'None' for all programs except Program 4.
- The Programs should be as follows:

Programs		
Program Name	Cycles	Analysis Mode
Program	1	None
Program	10	None
Program	26	None
Program	1	Quantification

- The four 'programs' can be re-named if required e.g. 'Hot-start', 'Touchdown', 'PCR' and 'Plate Read'.
- Please note that the ramp rates shown in the next set of screenshots were the default settings in our software. The default settings for your instrument should be fine for KASP.

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Program 1

- One stage (one cycle): 94°C for 15 minutes

Program Temperature Targets							
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
94	None	00:15:00	4.8		0	0	0

Program 2

- Two stages (10 cycles)
 - 94°C for 20 seconds
 - 61°C for 1 minute. This step is a touch-down. Ensure that the ‘Step Size’ is set to 0.6°C and that the ‘Sec Target’ is set to 55°C.

Program Temperature Targets							
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
94	None	00:00:20	4.8		0	0	0
61	None	00:01:00	2.5		55	0.6	0

Program 3

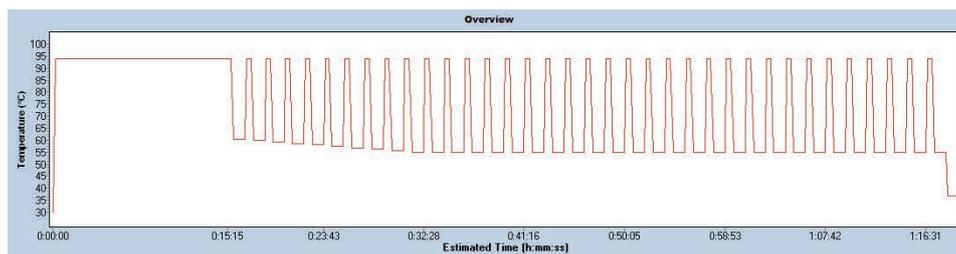
- Two stages (26 cycles)
 - 94°C for 20 seconds
 - 55°C for 1 minute.

Program Temperature Targets							
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
94	None	00:00:20	4.8		0	0	0
55	None	00:01:00	2.5		0	0	0

Program 4

- Two stages
 - 37°C for 1 minute to bring the plate to a suitable temperature for reading KASP (Plates must be read below 40°C).
 - 37°C plate read. For this step, the acquisition mode must be changed from ‘None’ to ‘Single’.

Program Temperature Targets							
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
37	None	00:01:00	2.5		0	0	0
37	Single	00:00:01	4.8		0	0	0



- Click on the ‘Start Run’ button to start the thermal cycle program.

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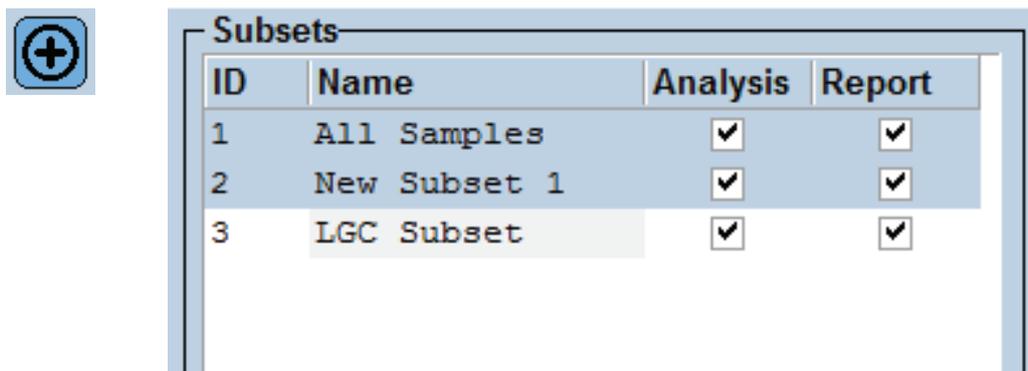
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5.3 Analysis of results

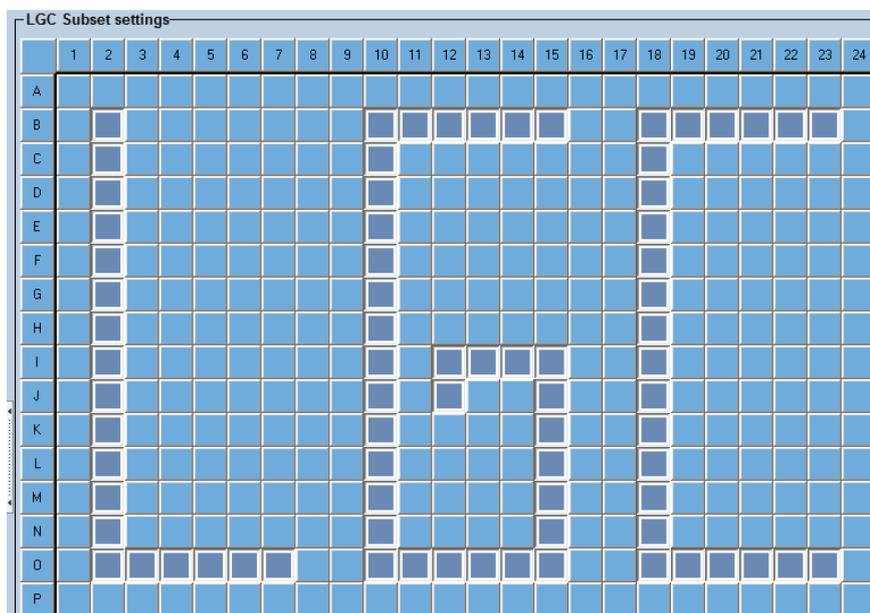
- Once the thermal cycle with plate read is complete, you can proceed to analysis of the data.

5.3.1 Define the plate layout (when full plates have not been run)

- If you have not run a full plate, you will need to define the subset of wells that contain samples.
- Click on the **'Subset Editor'** button on the left hand side of the screen.
- Click on the plus (+) symbol at the bottom of the window to add a new subset.
- This will add a subset to the list of subsets. You can change the name from the default **'New Subset 1'** if desired.



- In the plate layout view, select all of the wells that contain samples. N.B. If the wells are not adjacent to each other, you will need to press **'Ctrl'** on the keyboard whilst selecting all of the wells. Once all the required wells are highlighted (white), click **'Apply'**. The subset of wells will turn blue and your subset will be saved.



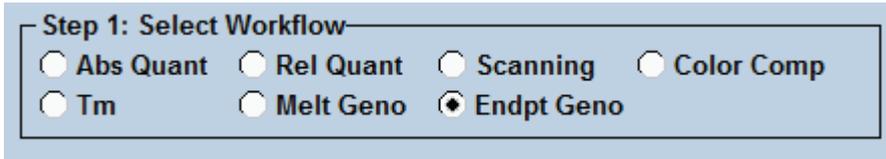
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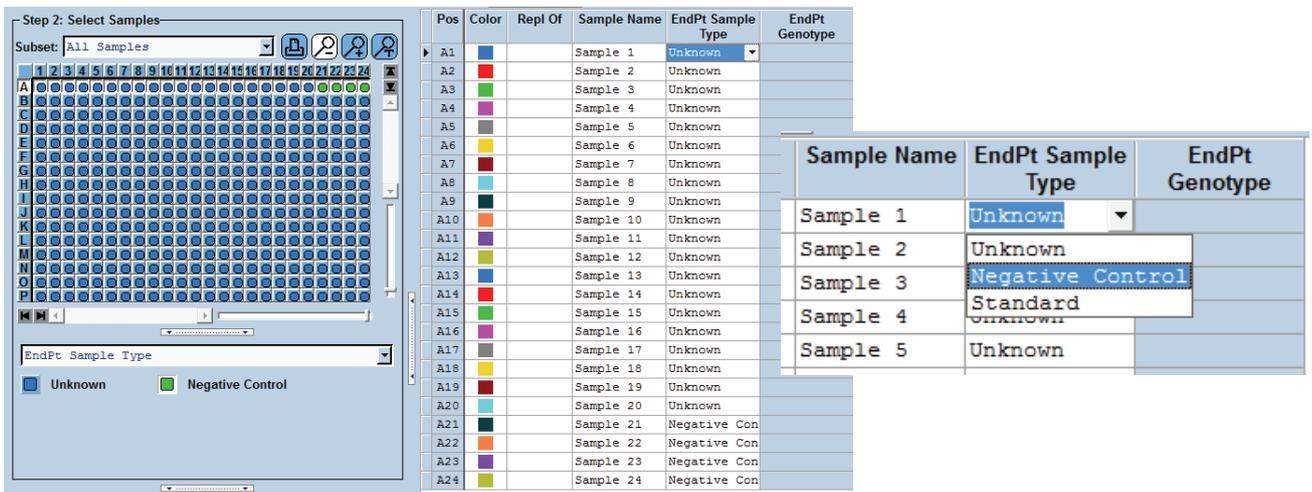
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5.3.2 Define the samples

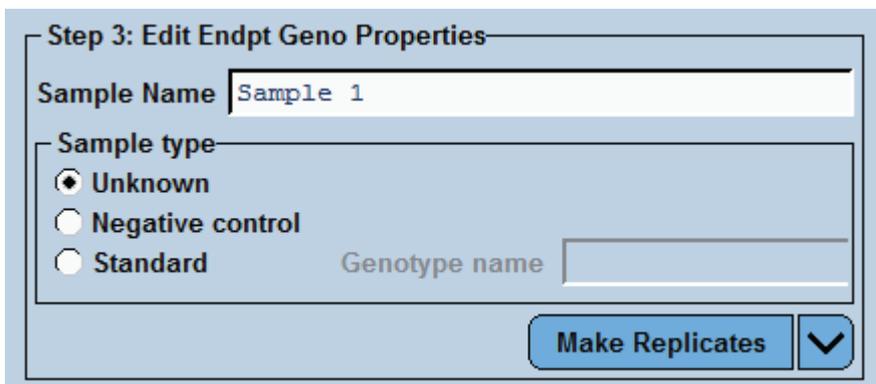
- Click on the ‘**Sample Editor**’ button on the left hand side of the screen.
- In the ‘**Step 1: Select Workflow**’ box, select ‘**Endpt Geno**’ to enable the endpoint genotyping options.



- In the ‘**Step 2: Select Samples**’ box, choose your sample subset from the drop down menu, if applicable.
- You can now identify those wells that contain ‘**Unknown**’ samples and those that contain ‘**Negative Controls**’. To edit the sample type of a well, first click on the well. Details of the sample will be displayed on the right hand side of the screen. In the ‘**EndPt Sample Type**’ column, use the drop down menu to change the sample type. The colour of the well in the plate layout will change accordingly.

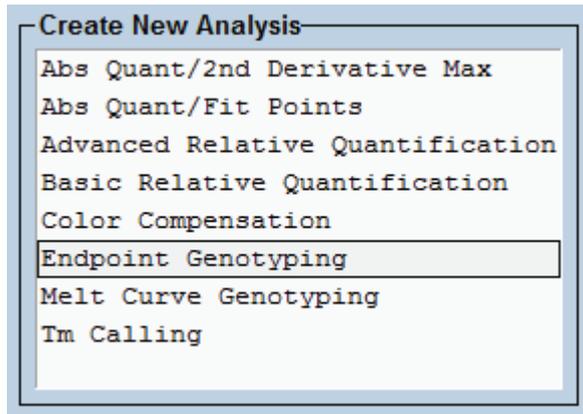


- The ‘**Step 3: Edit Endpt Geno Properties**’ box can be used to change the sample type and sample name for selections of wells. Highlight the relevant section of wells e.g. the whole of row A, and then choose the sample type. Edit the sample name in the box, if required.

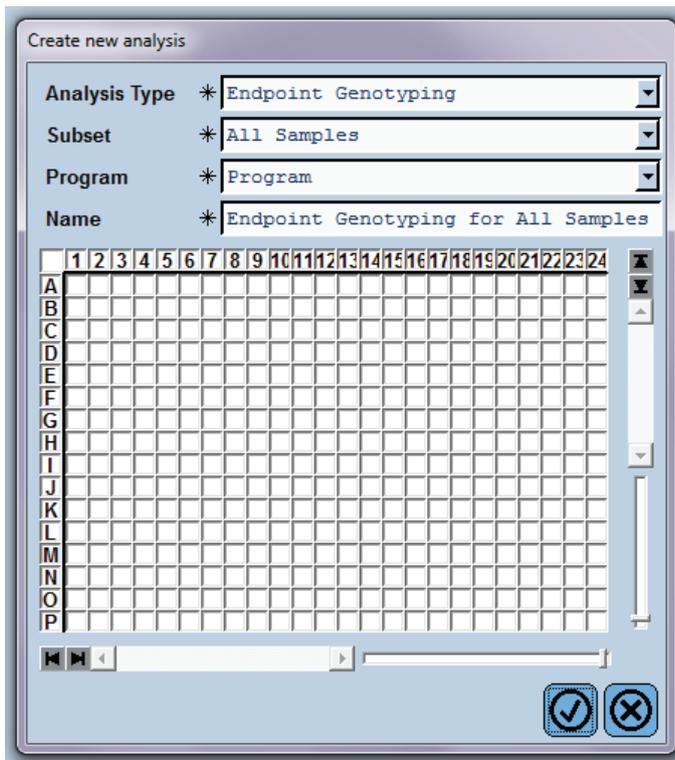


5.3.3 View the genotyping plot

- Click on the 'Analysis' button on the left hand side of the screen.
- In the 'Create New Analysis' box, select 'Endpoint Genotyping'.



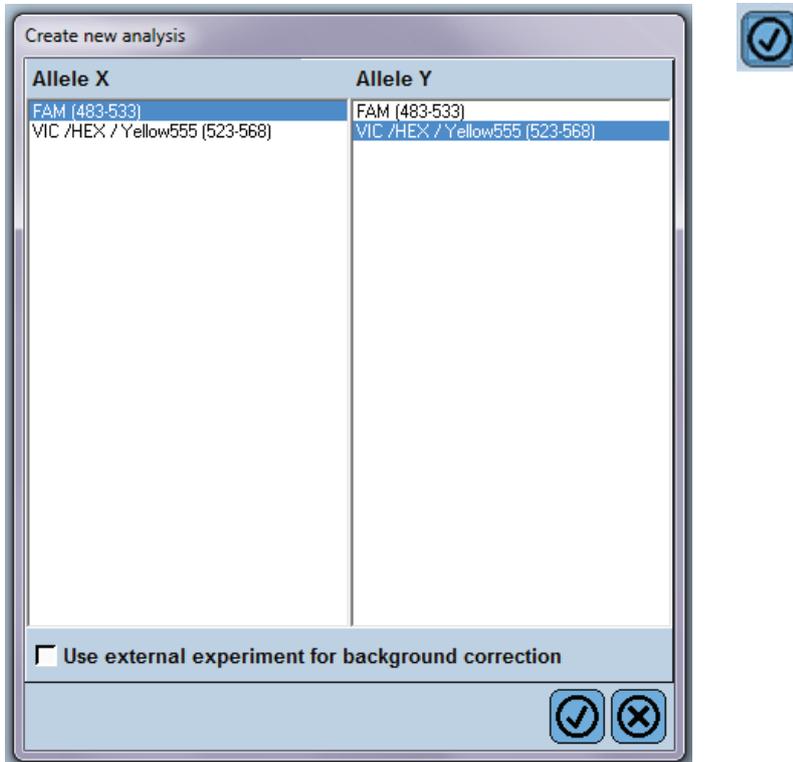
- This will open a 'Create new analysis' window.



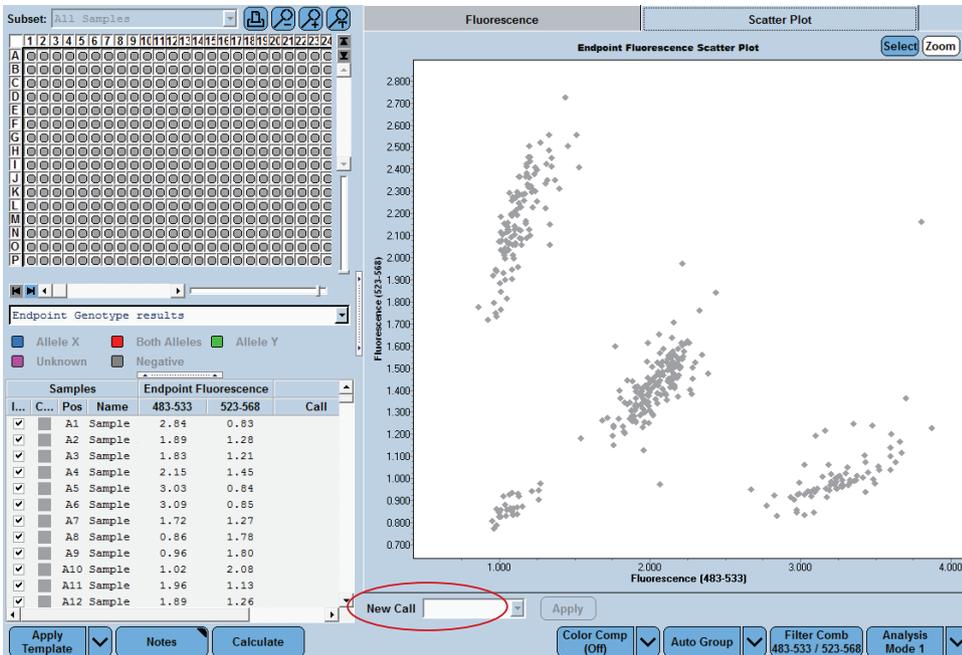
- In this new window, you can select a subset of samples to analyse (if applicable).
- Click on the tick button.



- You will then be asked to select the fluorophores for Allele X and Allele Y. Select 'FAM' for Allele X and 'VIC / HEX' for Allele Y, and click on the tick button.



- The software will then display your endpoint genotyping results in the main window.

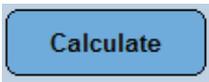


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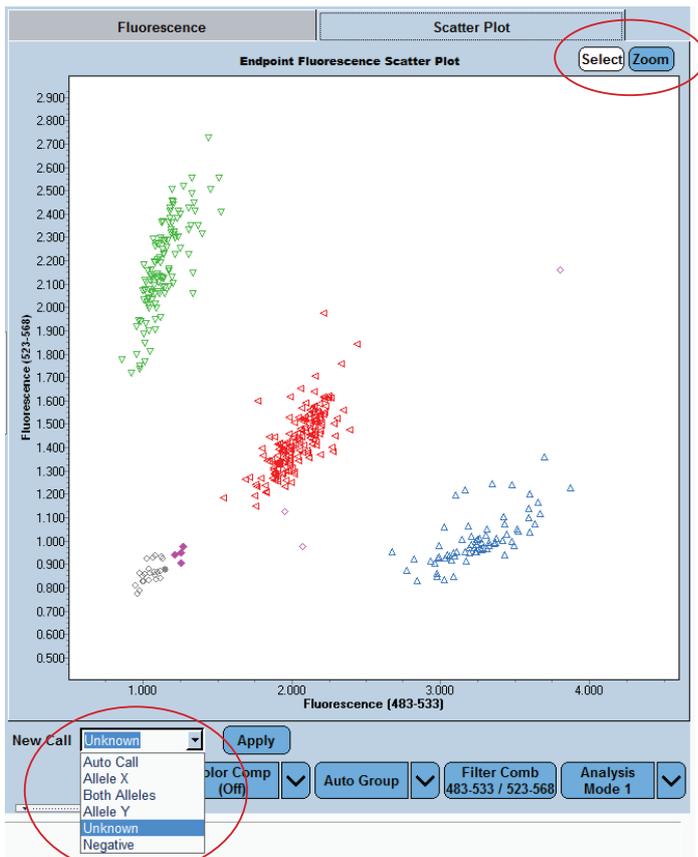
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- The window consists of a plate layout, a table view, and the genotyping scatter plot. The relative sizes of all of these components can be adjusted if required.
- To assign genotypes to your samples, click on the **‘Calculate’** button.



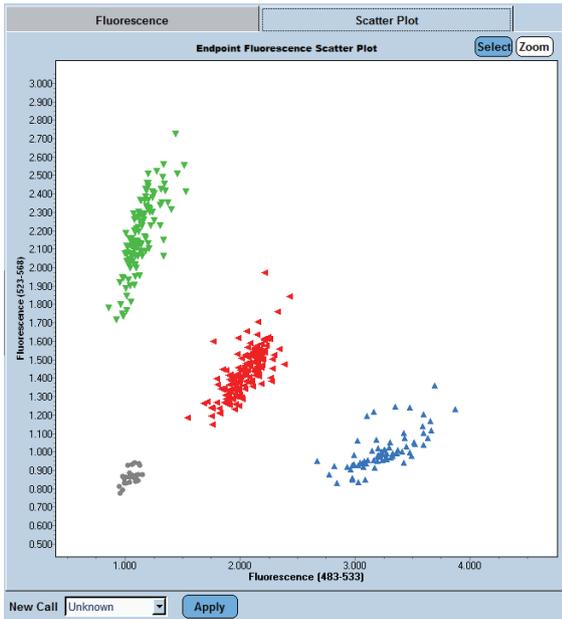
- The software will then automatically assign genotyping calls to your samples based upon their position in the scatter plot. The data points in the scatter plot and the plate layout will become coloured based upon their call.
- You can change the automatic calls for individual samples by selecting the sample in the plate layout view, changing the call in the **‘New Call’** box and clicking **‘Apply’**.



- Alternatively, you can draw around the desired samples using the cursor, and then change the call for all samples at once. Ensure that the **‘Select’** button is white before you select samples (if it is blue, click on it to turn it to white).
- The **‘Zoom’** button can be used to zoom in on a specific region of the plot. The **‘Zoom’** button must be white to enable you to do this (if it is blue, click on it to turn it to white).

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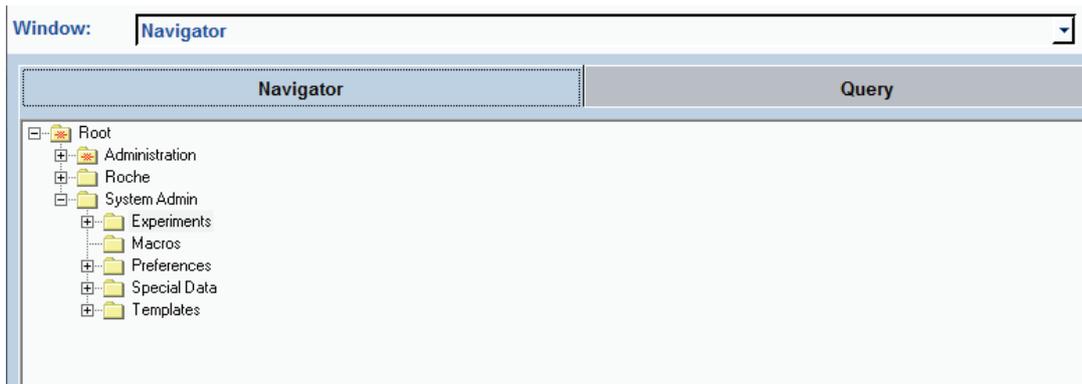
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- If you wish to omit any wells from the analysis, re-assign these wells as **'Unknown'** and then choose to view only the Allele X, Both Alleles, Allele Y and Negative samples on the plot. This can be done by clicking on the relevant buttons below the plate view.



- Please note that the ROX values cannot be used for normalisation in the LC480 software; the data must be exported to an alternative analysis program if normalisation is required.
- To export data, you must return to the **'Navigator'** window within the LC480 software.



- Locate the experiment that you wish to export and click on the **'Export'** button at the bottom of the screen. You can then choose the file format that you prefer (*ixo, *txt, *xml).

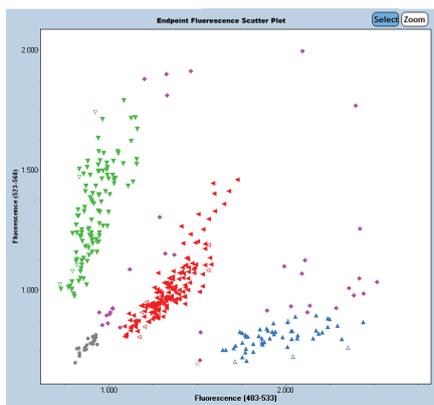


5.4 Recycling your assay plate

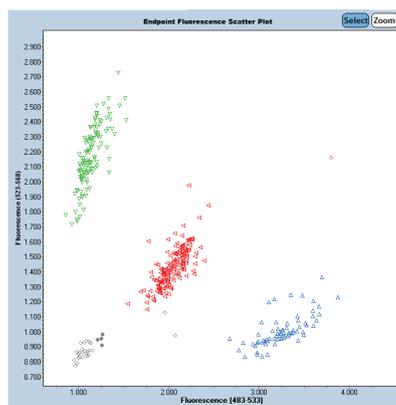
- If your data has not formed tight clusters after the initial thermal cycling protocol, you can recycle the plate on the LC480 and re-read it.

As before:

- Create a new experiment (see Section 5.2). Use the following KASP recycle thermal cycling conditions:
 - 94°C for 20 seconds
 - 57°C for 1 minute
 - Repeat steps 1-2 twice (a total of three cycles).
 - 37°C for 1 minute followed by data acquisition.
 - Once the recycle program has finished, proceed with data analysis as detailed in Section 5.3.



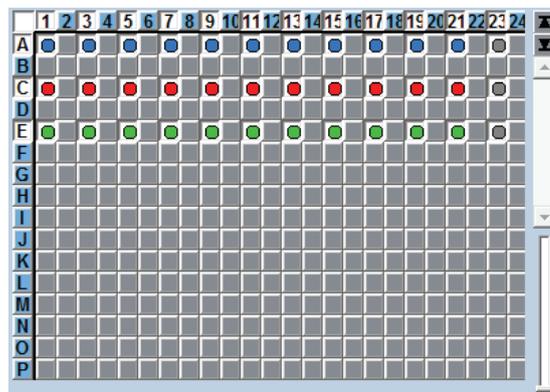
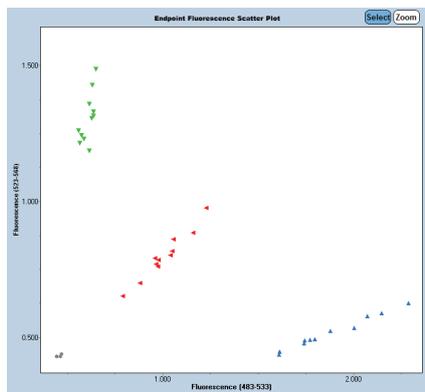
Genotyping results after the standard KASP thermal cycle. The pink samples cannot be assigned to a cluster, and clusters are quite spread out.



Genotyping results after completion of an additional three cycles (One recycle program). The clusters are much tighter and many more samples can be confidently assigned a genotype.

5.5 Running the KASP Trial Kit on the Roche LC480

- If you have requested a KASP Trial Kit to run on your Roche LC480 machine, please follow the protocol included with the kit to set up your reaction plate. This LC480 manual can be used to help you to programme the instrument to run the Trial Kit reaction plate.
- After the initial KASP thermal cycle, the Trial Kit reaction plate should produce data similar to that in the figures below.



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