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Running KASP genotyping reactions on the Thermo Scientific PikoReal instrument

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BIOSEARCH™ TECHNOLOGIES
GENOMIC ANALYSIS BY LGC

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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 Thermo Scientific™ PikoReal™ instrument.

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

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 experiment for KASP genotyping – see section 5.1.
2. Select the fluorophores for KASP genotyping – see section 5.2.
3. Programme the thermal cycling conditions – see section 5.3.
4. Programme the plate set-up including sample type – see section 5.4.
5. View the data – see section 5.5.
6. Recycle the plate if necessary – see section 5.6.

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4. KASP thermal cycling conditions

4.1 KASP thermal cycling conditions

Stage 1

1. 94 °C 15 minutes

Stage 2

1. 94 °C 20 seconds
2. 61 °C 60 seconds[1]

    [1]Drop -0.6 °C per cycle

    Repeat stage 2 × 9 times (a total of 10 cycles) achieving a final annealing temperature of 55.6 °C.

Stage 3

1. 94 °C 20 seconds
2. 55 °C 60 seconds

    Repeat stage 3 × 25 times (a total of 26 cycles).

Stage 4

1. Add read step at 37 °C for 1 min*

4.2 KASP recycling conditions

Stage 1

1. 94 °C 20 seconds
2. 57 °C 60 seconds

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

Stage 2

1. Add read step at 37 °C for 1 min*.
2. 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.
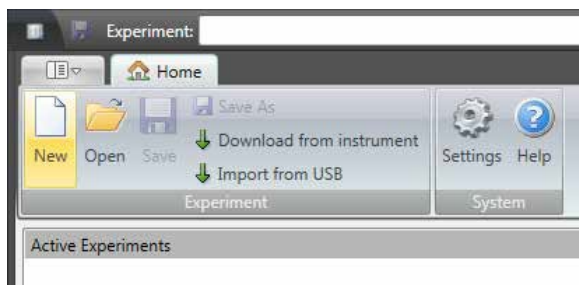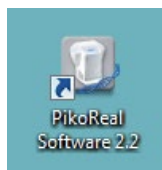
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## 5. Step-by-step user guide

5.1 Create a new experiment for KASP genotyping

- Open the PikoReal software by double-clicking on the desktop icon.
- Click on the 'New' button in the 'Experiment' menu bar.
- A new experiment will be created in the 'Active Experiments' section of the window.

5.2 Select the fluorophores for KASP genotyping

- Click on the 'Protocol' tab to enable selection of the required fluorophores and programming of the KASP thermal cycling conditions.
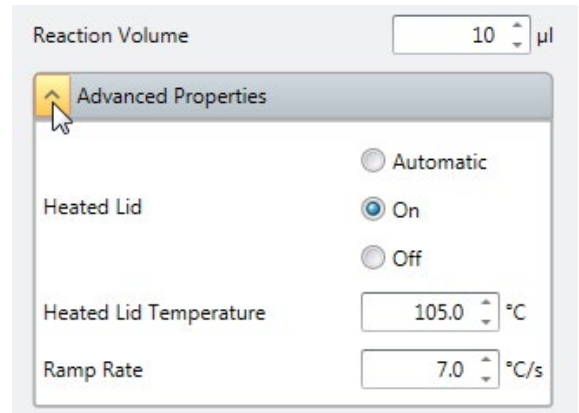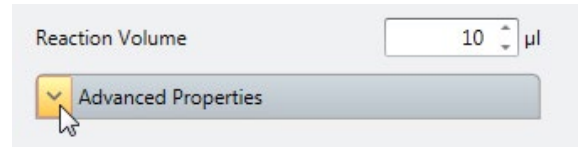- The initial view will contain all available fluorophores and default thermal cycling conditions.
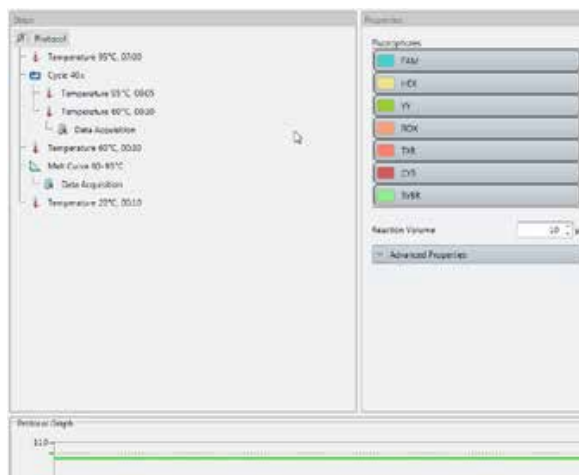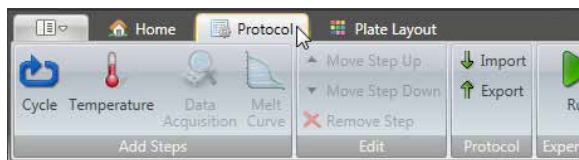
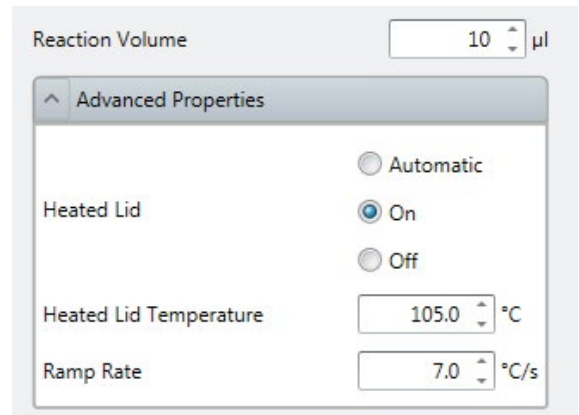Default 'Protocol' tab view within the PikoReal software

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• Expand the 'Advanced properties' box and check that the properties are appropriate for your run.

• For 24-well PikoReal strips, 10 µL KASP reaction volumes are recommended. For 96-well PikoReal strips, 5 µL KASP reactions volumes are recommended. The heated lid should be set to the default setting 'On'. 'Heated Lid Temperature' and 'Ramp Rate' can remain on the default settings.

• By default, all seven available fluorophores are selected within the 'Protocol' tab. For KASP genotyping, the FAM and HEX fluorophores are required. KASP Master mix contains ROX as a passive reference dye. The PikoReal instrument can read ROX but the software does not use this information for normalisation. It is therefore optional to read ROX when running KASP on the PikoReal instrument. Deselect the fluorophores that are not required so that only FAM and HEX (and ROX if desired) are highlighted in grey.
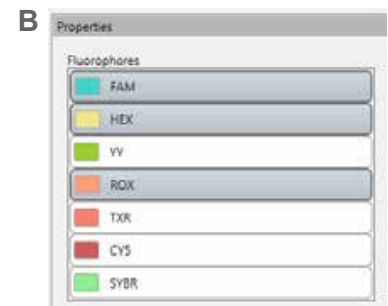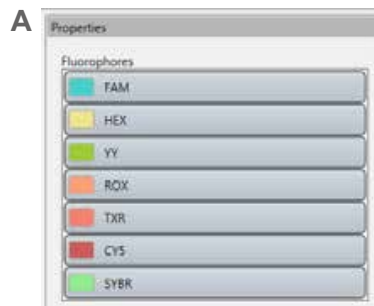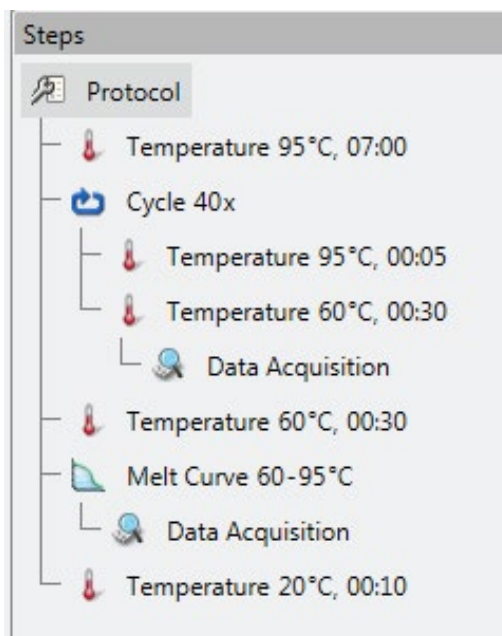

Image A shows the default fluorophore list with all seven available fluorophores selected. Image B shows the fluorophore list amended for KASP genotyping, with only FAM, HEX and ROX selected.

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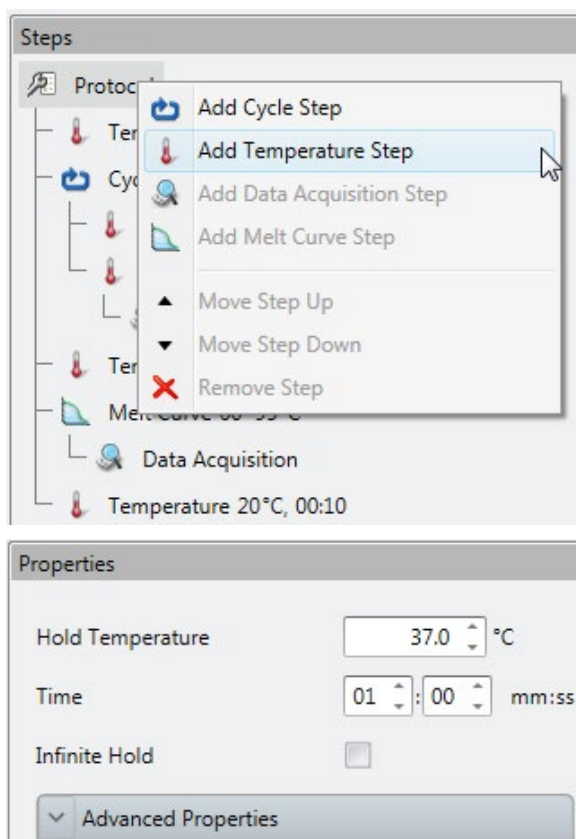
5.3 Programme the KASP thermal cycle on the PikoReal

- Within the 'Protocol' tab, edit the thermal cycle to the conditions required for KASP genotyping. The stages of the KASP thermal cycling programme are detailed in section 4.1.
- The default cycling conditions in the PikoReal software will be as shown in the protocol tree opposite.

Steps

Protocol
- Temperature 95°C, 07:00
- Cycle 40x
  - Temperature 95°C, 00:05
  - Temperature 60°C, 00:30
    - Data Acquisition
- Temperature 60°C, 00:30
- Melt Curve 60-95°C
  - Data Acquisition
- Temperature 20°C, 00:10

5.3.1 Programme a baseline read at 37 °C for 1 minute.

- Right click on 'Protocol' at the top of the protocol tree, and 'Add temperature step'. Edit the parameters on the right-hand side of the window.

Steps

Protocol
- Ter   Add Cycle Step
- Cyc   Add Temperature Step
  - Add Data Acquisition Step
  - Add Melt Curve Step

  - Move Step Up
  - Move Step Down
- Ter   X   Remove Step
- Mer
  - Data Acquisition
- Temperature 20°C, 00:10

Properties

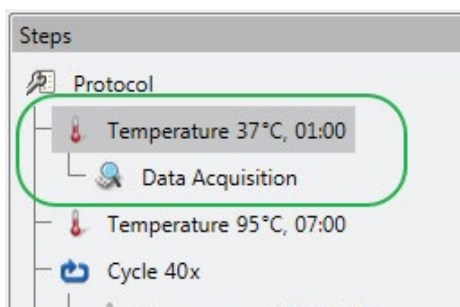| Hold Temperature | 37.0 | °C |
| Time | 01 : 00 | mm:ss |
| Infinite Hold | ☐ | |

∨ Advanced Properties

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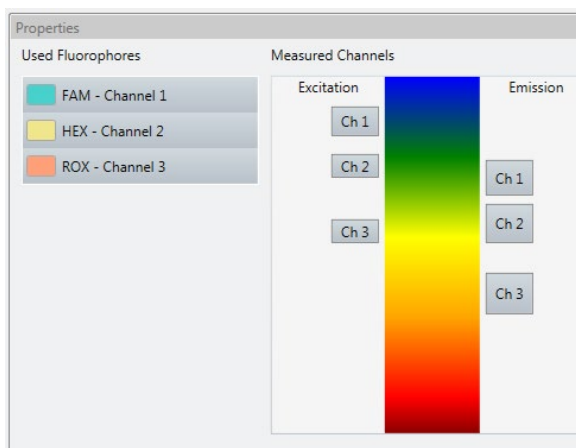Running KASP genotyping reactions on the Thermo Scientific PikoReal instrument

- Add a data acquisition step to this temperature step by right clicking on the temperature branch in the protocol tree and selecting 'Add data acquisition step'.
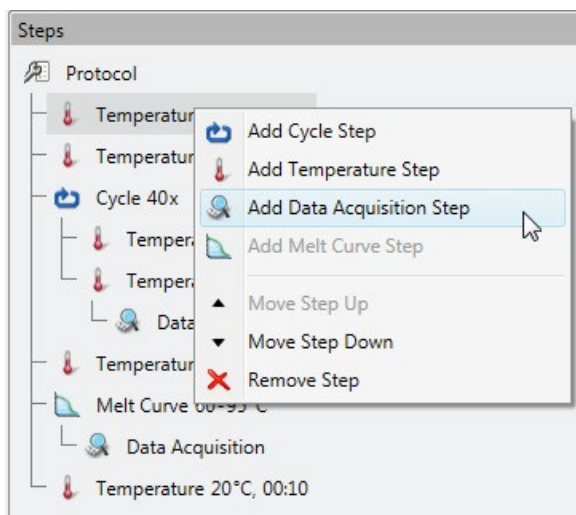
- The protocol tree will then appear as shown (the added step is outlined in green).

- The properties of the 'Data Acquisition' step should correspond to the fluorophores that you selected in section 5.2 and will ensure that the correct fluorophores are read to generate KASP genotyping results.
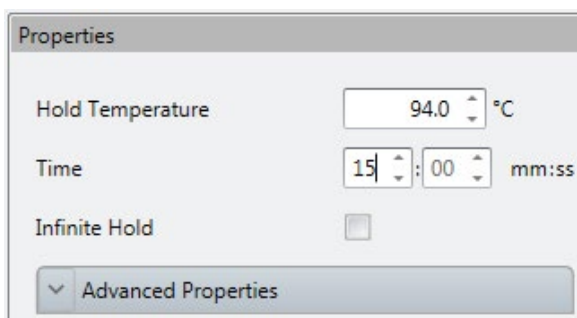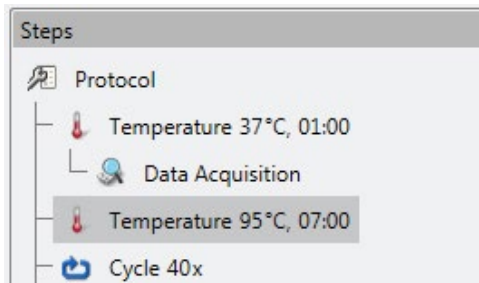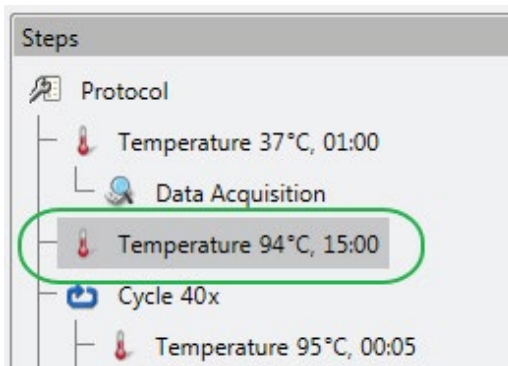
5.3.2 Programme the activation stage (stage 1):

- 94 °C 15 minutes.

Click on the second temperature branch in the protocol tree and edit the parameters on the right hand side of the window.

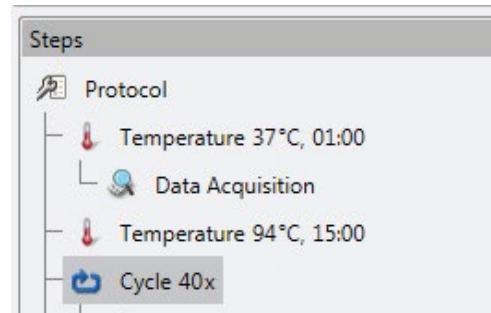- The protocol tree will then appear as shown (the edited branch is outlined in green).

### 5.3.3 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 'Cycle' branch in the protocol tree, and edit the parameters on the right-hand side of the window.

**Please note:** to programme the temperature touchdown stage, you will need to do the following:

- Set the total number of cycles for this step to 10. N.B. A drop of -0.6 °C per cycle will achieve a final annealing temperature of 55.6 °C.

- Ensure that the temperatures and times are programmed correctly for each of the two steps of the amplification stage – these can be edited in the 'Properties' box.

- **To set the temperature decrement:** Click on the 'Temperature 61 °C, 01:00' step and expand the 'Advanced Properties' box. Set the 'Temperature Change Per Cycle' to 0.6 °C and tick the 'Temperature Decrement' box to ensure that the temperature decreases each cycle rather than increases.
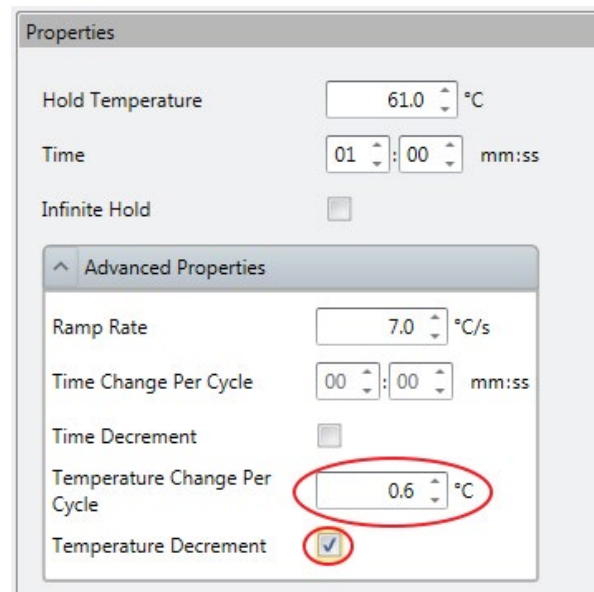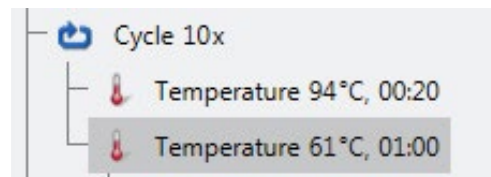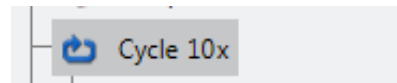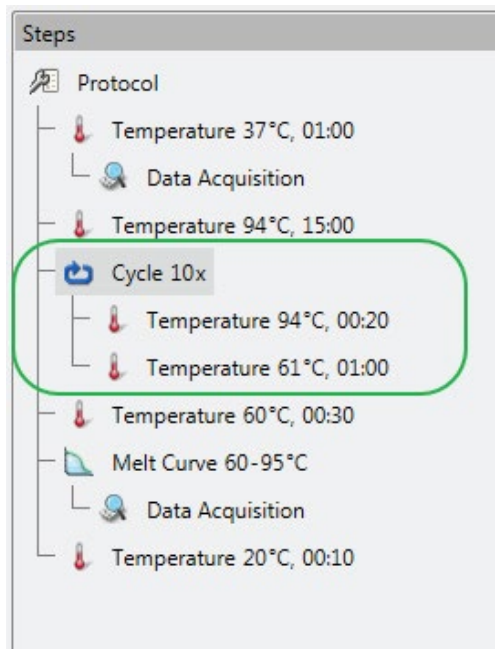
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- Remove the 'Data Acquisition' step for the touchdown stage. KASP genotyping reactions only produce meaningful data at the end of the thermal cycle and when read below 40 °C. To remove this step, right click on the step and select 'Remove Step'.
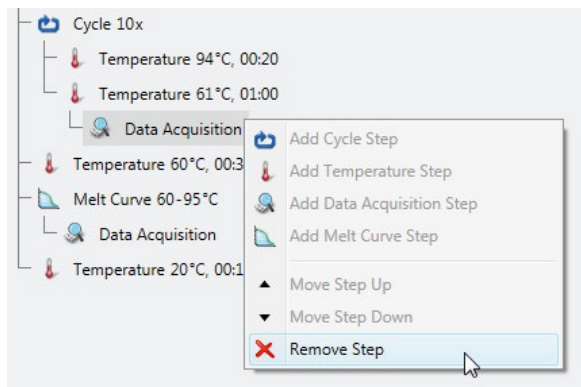
- The protocol tree will then appear as shown (the edited step is outlined in green).
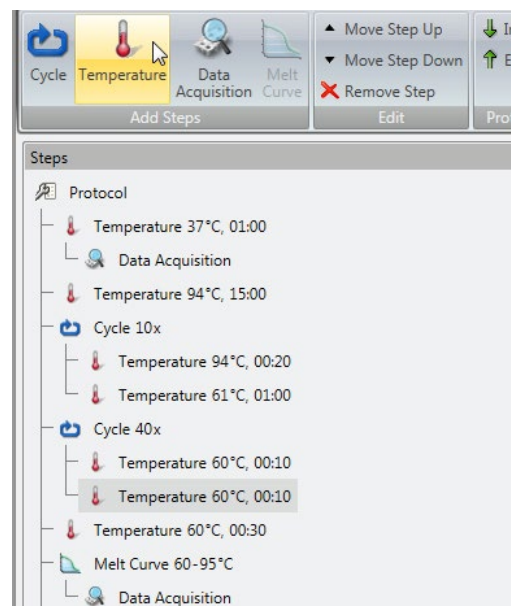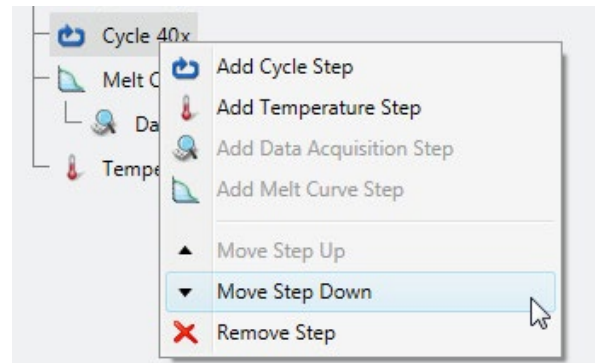
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5.3.4 Programme the amplification stage (Stage 3) (26 cycles):

- 94 °C 20 seconds
- 55 °C 60 seconds

Please note: to programme the amplification stage, you will need to do the following:

- Add an additional 'Cycle' branch to the default thermal cycle that is pre-programmed within the software. Click on the 'Cycle' symbol at the top of the window and an additional 'Cycle' stage will be added to the thermal cycle (if the 'Cycle' button is greyed out, click on the protocol tree to enable this button to be used). Ensure that this additional 'Cycle' stage is added at the same 'level' as the touchdown cycle stage.

- NB. If the new cycle stage is added too far up or down in the tree, right click on the stage and select 'Move Step Up' or 'Move Step Down' as appropriate.

- Add temperature steps to this 'Cycle' stage by clicking on the 'Temperature' symbol at the top of the window. Do this twice to add two steps as required for KASP thermal cycling.
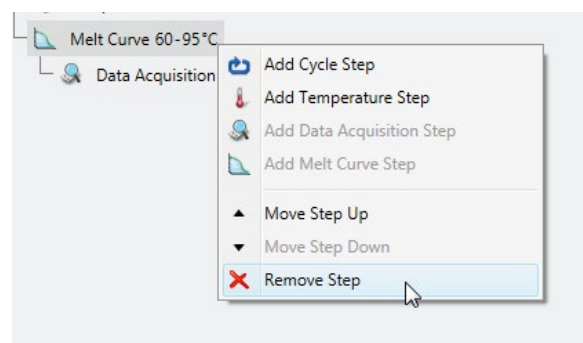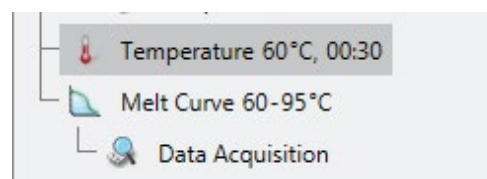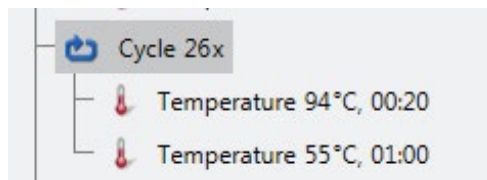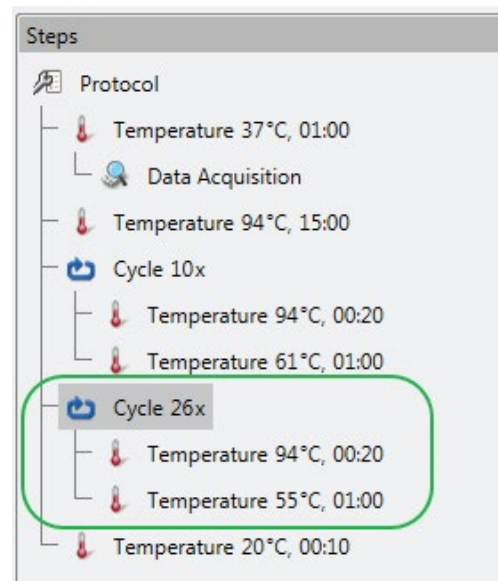
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- Set the number of cycles for the amplification stage to 26 (the default is 40).
- Ensure that the temperatures and times are programmed correctly for each of the two steps of the amplification stage – these can be edited in the 'Properties' box.
- Remove the default 'Temperature 60 °C, 00:30' and 'Melt Curve 60-95 °C' steps from the end of the thermal cycle as these are not required.

  To remove these steps, right click on each step and select 'Remove Step'.

- After programming the amplification stage (Stage 3) the protocol tree will then appear as shown (the edited step is outlined in green).

5.3.5   Programme the plate read stage (stage 4) (1 cycle):

Click on the final 'Temperature' branch in the protocol tree and edit the parameters on the right-hand side of the window.

- Edit the final default temperature branch ('Temperature 20 °C, 00:10') to 'Temperature 37 °C, 01:00'. This will ensure that the KASP genotyping reactions are at the correct temperature for fluorescent signal to be read.
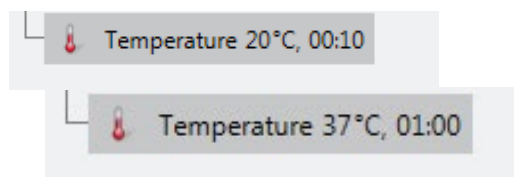
- Add a data collection step to this plate read branch by clicking on the 'Data Aquisition' symbol at the top of the window.
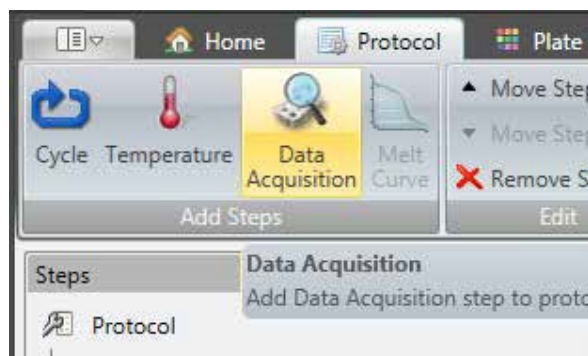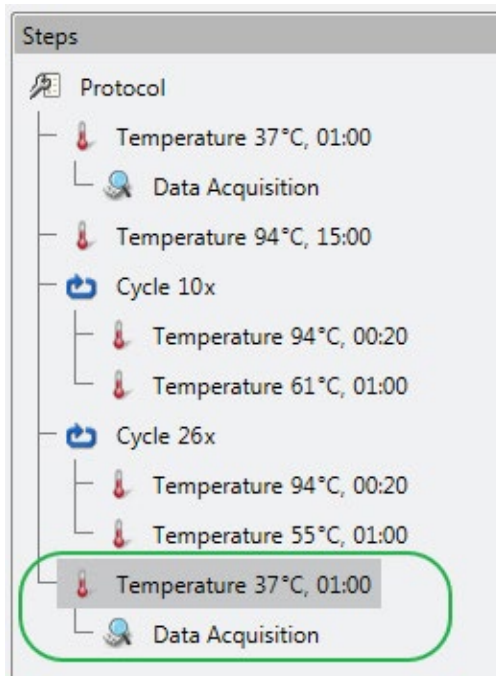

- The properties of the 'Data Acquisition' step should correspond to the fluorophores that you selected in section 5.2 and will ensure that the correct fluorophores are read to generate KASP genotyping results.
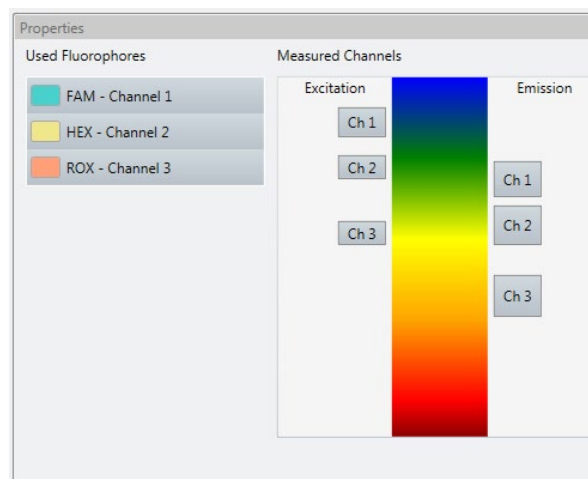
- After programming the plate read stage (Stage 4) the protocol tree will then appear as below (the edited step is outlined in green).

5.3.6 The thermal cycling protocol

Once the thermal cycling protocol for KASP has been correctly programmed, it can be viewed in the left-hand tree and in graphical format at the bottom of the window. The graphical format displays the approximate run time for the thermal cycling protocol - this should be around 01:22:00 (run time outlined in green in graphical format below).
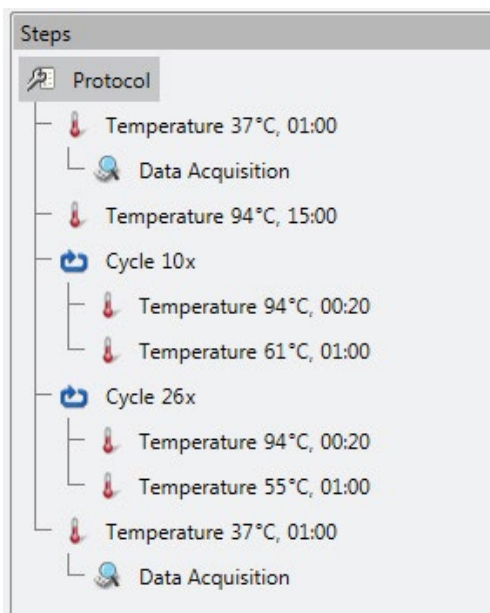
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5.4 Programme the plate layout for the experiment and start the run

Once the KASP thermal cycling protocol has been programmed correctly, click on the 'Plate Layout' tab to define the plate parameters. Please note that the plate layout can also be edited during or after the run.

- Click on the 'Pipette' symbol at the top of the window to enter the pipetting mode.

- Define the appropriate pipette content for the KASP genotyping reaction plate. Click on the FAM fluorophore to move it from the 'Fluorophore' list into the 'Pipette Content' list. The fluorophore will move to the 'Pipette Content' list and the box will be filled in with the appropriate colour.
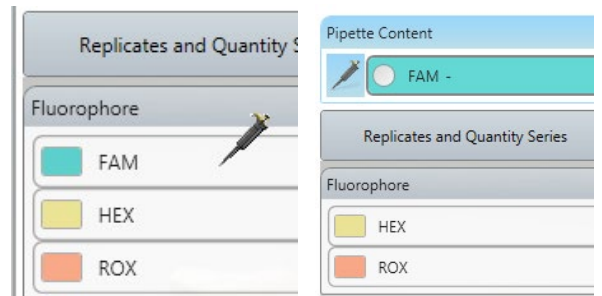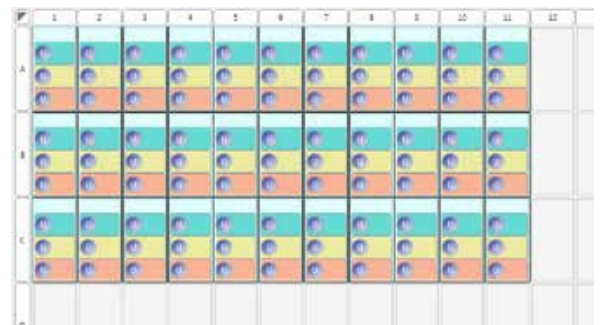

- Once a fluorophore is in the 'Pipette Content' list, the 'Sample Type' options will become available. Click on 'Unknown' from this list, and it will be added to the FAM fluorophore in the 'Pipette Content' list (shown by the purple circle containing the letter U).
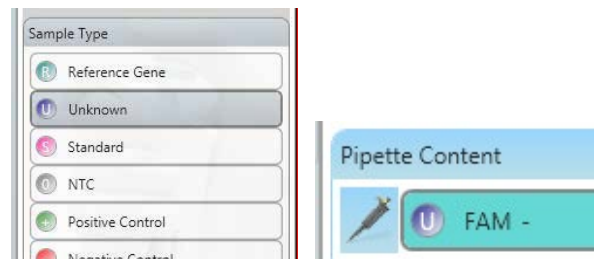
- Repeat this process for HEX and ROX (if you have chosen to read this) fluorophores. The 'Pipette Content' list should then contain all three fluorophores, each with the 'Unknown' sample type assigned to them.
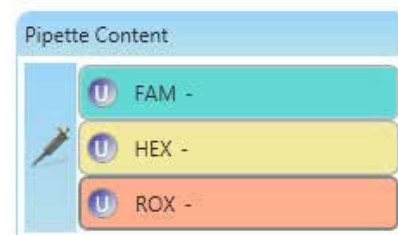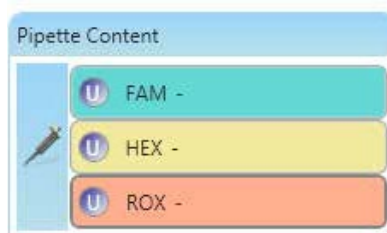

- 'Pipette' the content into the wells on the plate layout by clicking on the relevant wells (i.e. those that will contain DNA samples).


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- In the 'Pipette Content' list, highlight the FAM fluorophore by clicking on it. Click on 'NTC' within the 'Sample Type' list and the fluorophore will now display a grey circle with the letter O. Repeat this for the HEX and ROX fluorophores.

- 'Pipette' the content into the wells on the plate layout by clicking on the relevant wells (i.e. those that contain the no template controls).
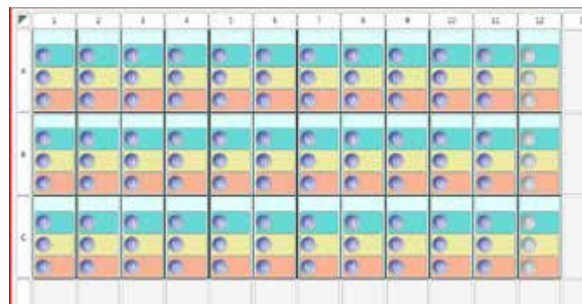

- In the PikoReal plate layout, it is important to add an AD standard for each of the expected genotypes. An AD standard is a well containing DNA of a known genotype, and the software uses this information to automatically call the genotypes of the unknown samples. Without AD standards, genotyping clusters will still be apparent on the cluster plot, but the software will not automatically assign genotypes and colour-code the datapoints. It is possible to manually assign genotypes based on clusters, in the absence of controls, using the 'Virtual Standards' function within the analysis tab (See section 5.5.2 for more details).

- Click on a well containing a sample of known genotype e.g. homozygous FAM (X:X), and switch to the 'Select' mode (rather than 'Pipette' mode).
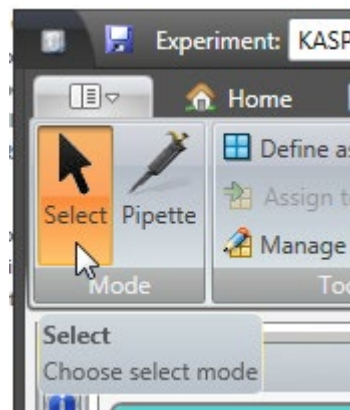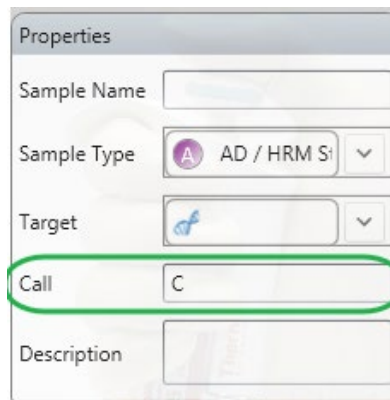
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- For the highlighted well, change the sample type for the relevant fluorophore(s) to 'AD/HRM standard'. Genotype information must also be added to the 'Call' box for the relevant fluorophore(s), otherwise the software will not automatically call the genotypes
  - For a homozygous FAM (X:X) positive control, first click on the FAM fluorophore in the 'Fluorophores' box.
  - Change the sample type to 'AD/HRM standard' using the drop down menu in the 'Properties' box.
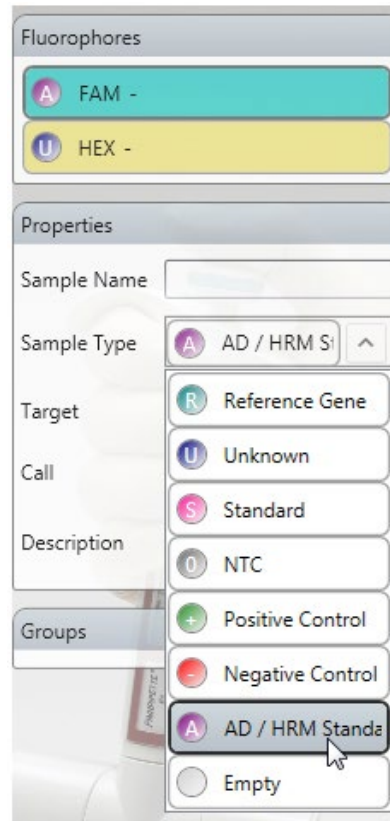
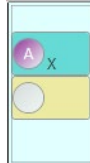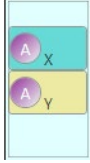 - In the 'Call' box, define the genotype for the FAM allele e.g. 'C'.

  - Click on the HEX fluorophore in the 'Fluorophores' box
  - As this is the homozygous FAM (X:X) positive control, change the sample type for the HEX fluorophore to 'Empty'. No call information is therefore required for this fluorophore.
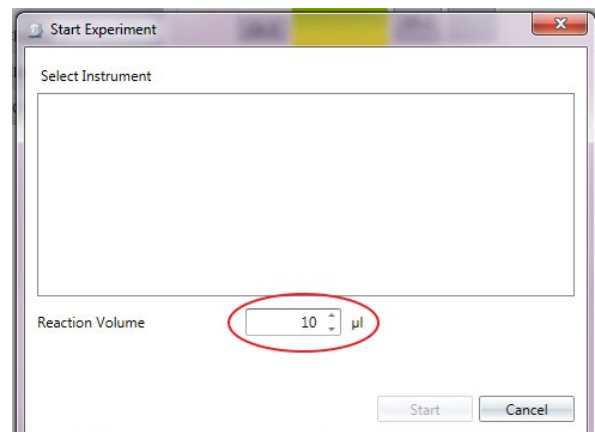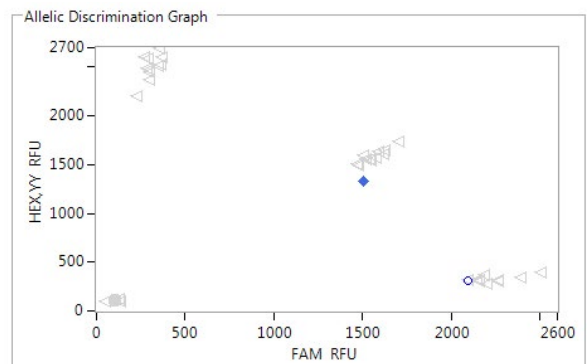
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- The table below details how to programme AD standard wells for each of the possible genotypes

Genotype (call)	FAM sample type and call	HEX sample type	Well in plate layout
Homozygous FAM (X:X)	AD/HRM Standard Call: X (or equivalent base e.g. A, T, C, G)	Empty Call: n/a	
Heterozygous (X:Y)	AD/HRM Standard Call: X (or equivalent base e.g. A, T, C, G)	AD/HRM Standard Call: Y (or equivalent base e.g. A, T, C, G)	
Homozygous HEX (Y:Y)	Empty Call: n/a	AD/HRM Standard Call: Y (or equivalent base e.g. A, T, C, G)	

- If 'AD Standards' are not defined for each potential genotype prior to data analysis, the 'Allelic Discrimination Graph' will display the datapoints in clusters, but these will not be colour-coded or automatically assigned genotypes.
- If it is not possible to define AD standards when setting up the plate layout, 'Virtual Standards' can be defined during the data analysis (see section 5.5.2).
- To start the run, return to the 'Protocol' tab and click on the 'Run' symbol (green triangle) at the top of the window.
- A 'Start Experiment' window will open (as long as you have already saved your experimental set-up file). Select your instrument from the list and ensure that the correct reaction volume is programmed for the plate.

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5.5   Analysis of results

### 5.5.1   Performing data analysis (with defined positive controls)

- **Please note:** if you have not been able to define positive controls (as detailed in section 5.4), please see section 5.5.2 for information about defining virtual standards during the analysis process.

- Once the PCR programme and plate read have completed, an 'Analysis' tab will appear at the top of the window, adjacent to the 'Plate Layout' tab.

- As KASP is an endpoint chemistry, an 'RFU Based' allelic discrimination analysis method should be used.

- Within the 'Analysis' tab, either:
  - Click on the 'RFU Based' button and select the sample group (e.g. 'All Wells').


    OR

  - Right click on the 'Analysis' item in the left-hand tree and select 'Add', then 'Allelic Discrimination (RFU) and then the sample group (e.g. 'All wells').
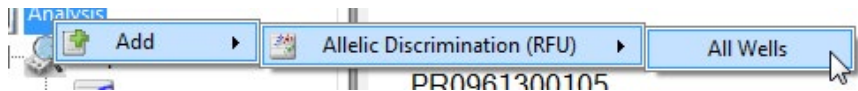
- Click on the newly created 'Allelic Discrimination' item in the left-hand tree to view the data and settings.

- Use the plate layout at the bottom left of the window to highlight the wells that you are interested in i.e. highlight the wells that contain your reactions. Selected wells will be outlined with dark grey. If you do not select the required wells, every well will be plotted on the allelic discrimination plot, even if it does not contain any fluorescent data.
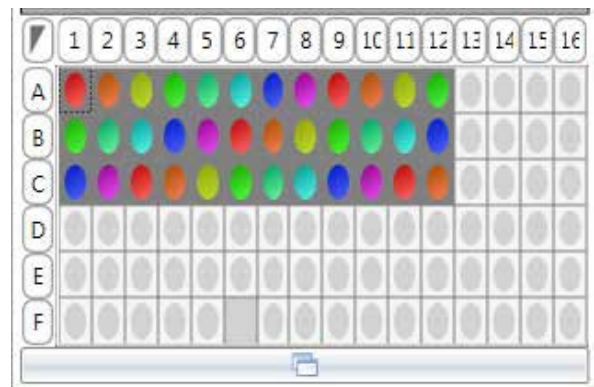
Manual

Running KASP genotyping reactions on the Thermo Scientific PikoReal instrument

- The default analysis settings for the allelic discrimination plot will be shown on the right-hand side of the window and should be similar to those in the screenshot below. NB. Click on the 'Advanced < <' button to show the 'Line Reference Point' and 'Distance Weight Ratio' options.
- Ensure that 'DA1' is selected for Baseline RFU, and 'DA2' is selected for Endpoint RFU. This will mean that the pre-PCR read data is used for the baseline values, and that the post-PCR read data is used for the endpoint analysis.

Default 'Settings' and 'Advanced' settings box

- To view the data correctly on the genotyping results plate layout at the top right of the window, you may need to adjust the 'Distance Weight Ratio' within the 'Advanced < <' settings options. We would suggest reducing this value to 1.00 in the first instance as we found that this meant that only the wells of interest were included on the genotyping results plate layout.

Amended 'Settings' and 'Advanced' settings box, with 'Distance Weight Ratio' adjusted to 1.00

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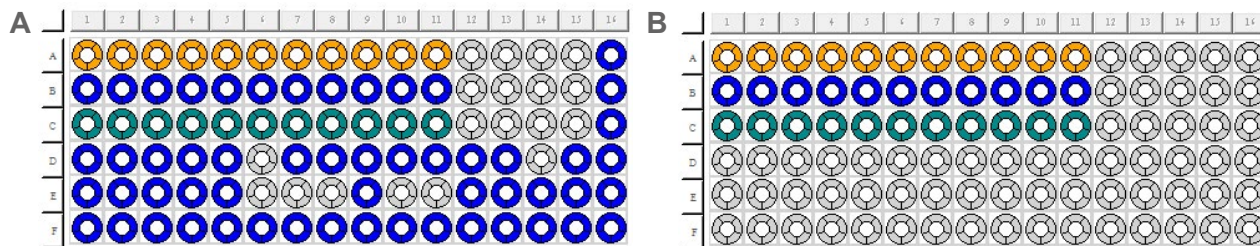Running KASP genotyping reactions on the Thermo Scientific PikoReal instrument


Image A shows the plate layout view with 'Distance Weight Ratio' set to the default of 5.00. Image B shows the plate layout with the 'Distance Weight Ratio' adjusted to 1.00. The adjustment means that only the wells containing completed KASP reactions are assigned a genotype and plotted on the cluster plot.

- An alternative option to adjusting the 'Distance Weight Ratio' is to use the 'Sample Groups' option within the PikoReal software. This allows several wells on a plate to be combined into groups for analysis and will be particularly beneficial if running more than one assay per plate. It is beyond the scope of this manual to explain this in detail, but a full explanation and instructions can be found within the 'Help' section of the PikoReal software and in the Thermo Scientific PikoReal manual.
- The data will be plotted on a cluster plot in the top centre of the window. Each data point on the plot represents one of the wells on the reaction plate. If the software is able to automatically call the genotypes of the samples, the data points will be colour-coded. In the example below, the two homozygote groups are green and orange, and the heterozygote samples are blue.
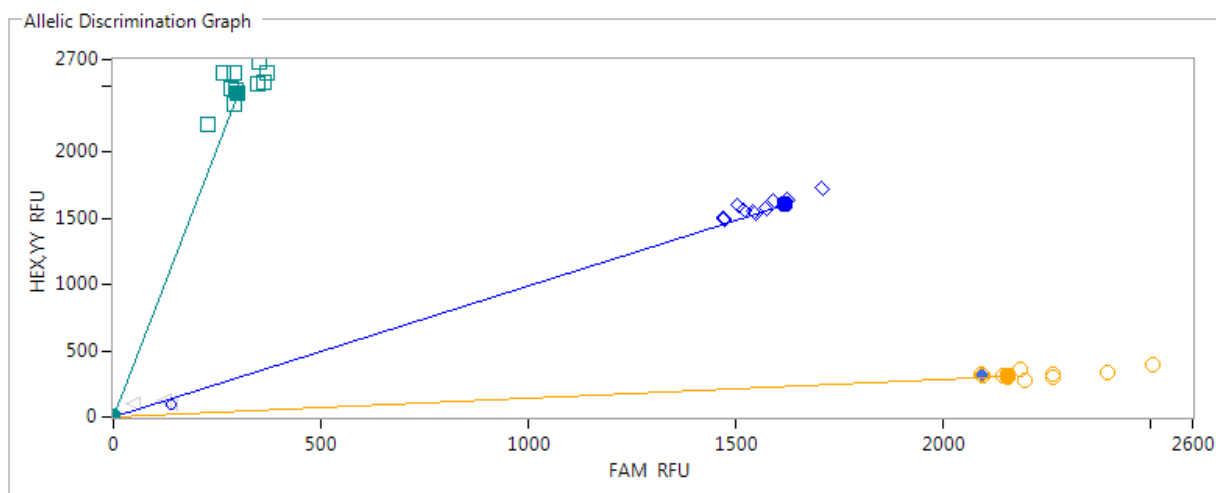

- The data will also be detailed in a table at the bottom centre of the window. This will include the fluorescent values for FAM and HEX, plus the genotyping call that has been assigned to each sample.
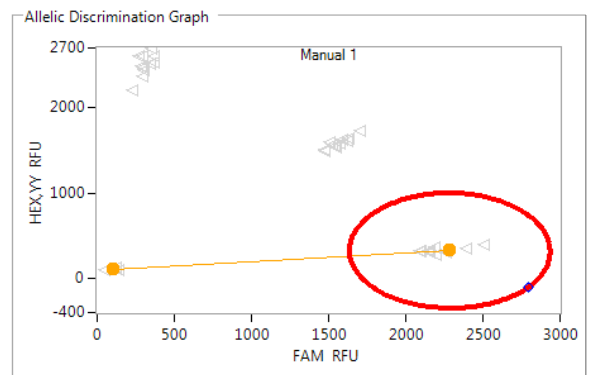
5.5.2 Defining virtual standards

- To define virtual standards during data analysis, right click on a datapoint that is clearly located within a cluster, and choose 'Virtual Standard', then 'Add Virtual Standard' from the options.
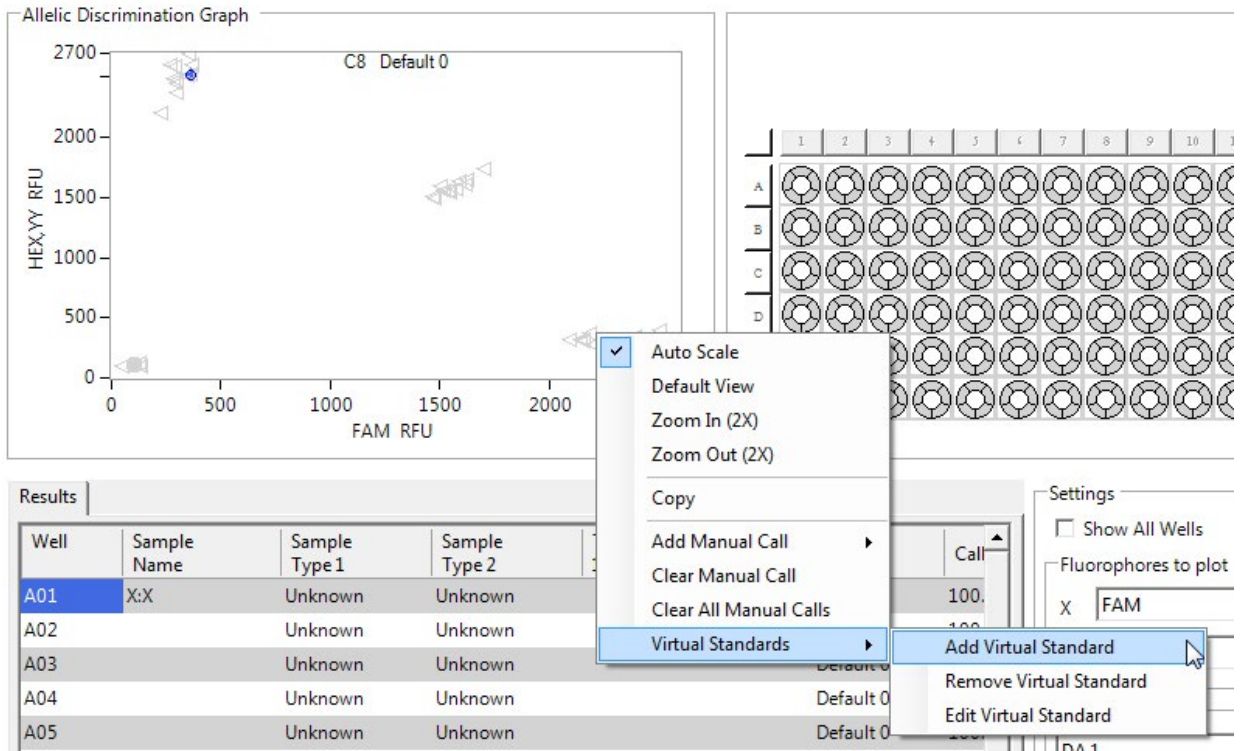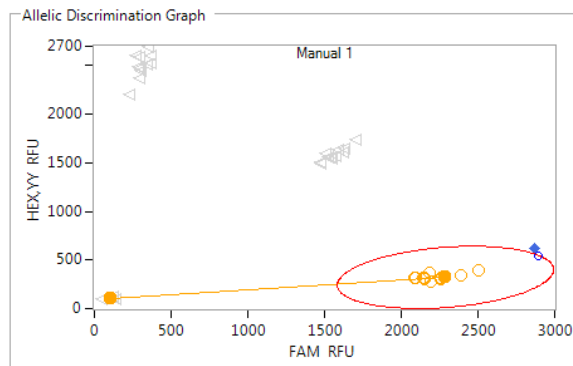

- A virtual standard datapoint will be added to the cluster plot.

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- Click on the red outline to adjust the size, appropriate to the cluster of your datapoints. The software will then assign the appropriate genotype to all datapoints within the cluster.

- Repeat this for the remaining two clusters on the plot.
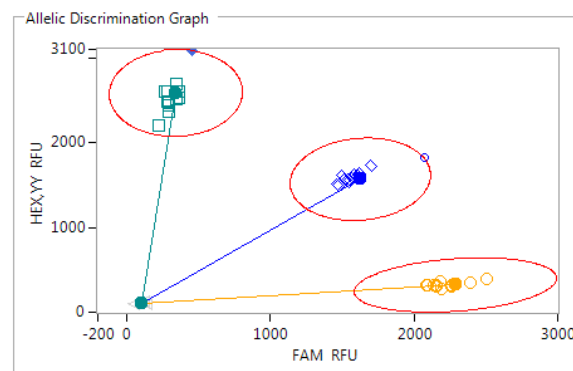
## 5.6   Recycle your reaction plate if required

- If your data has not formed tight clusters after the initial thermal cycling protocol, you can recycle the plate in the Thermo Scientific PikoReal or on a Peltier block and perform a second post-read.
- Please contact technical support (techsupport@lgcgroup.com) for advice on how to programme the PikoReal for recycling.

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5.7   Running the KASP trial kit on the Thermo Scientific PikoReal

- If you have requested a KASP trial kit to run on your Thermo Scientific PikoReal instrument, please follow the protocol included with the kit to set up your reaction plate. This PikoReal manual can be used to help you to programme the instrument to run the trial kit reaction plate.
- After running the KASP thermal cycle on the PikoReal instrument, the trial kit reaction plate should produce data similar to the figure below.
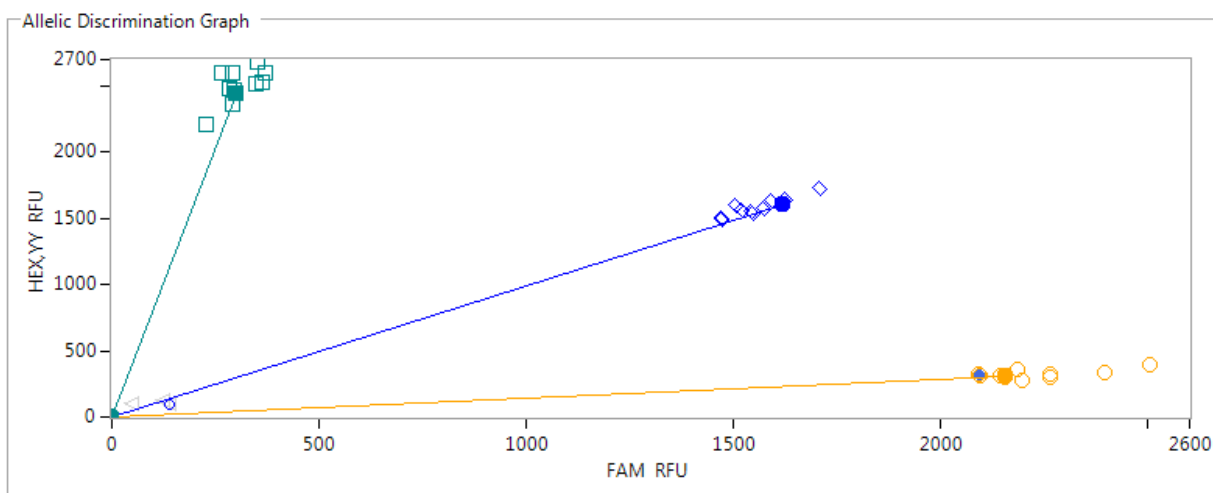
Results from the KASP trial kit reaction plate when run on the Thermo Scientific PikoReal instrument using the standard KASP thermal cycle

6. Further support

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

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