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Setting up a Run of Real-**time PCR** cycler

Ro**tor Gene**™ 3000

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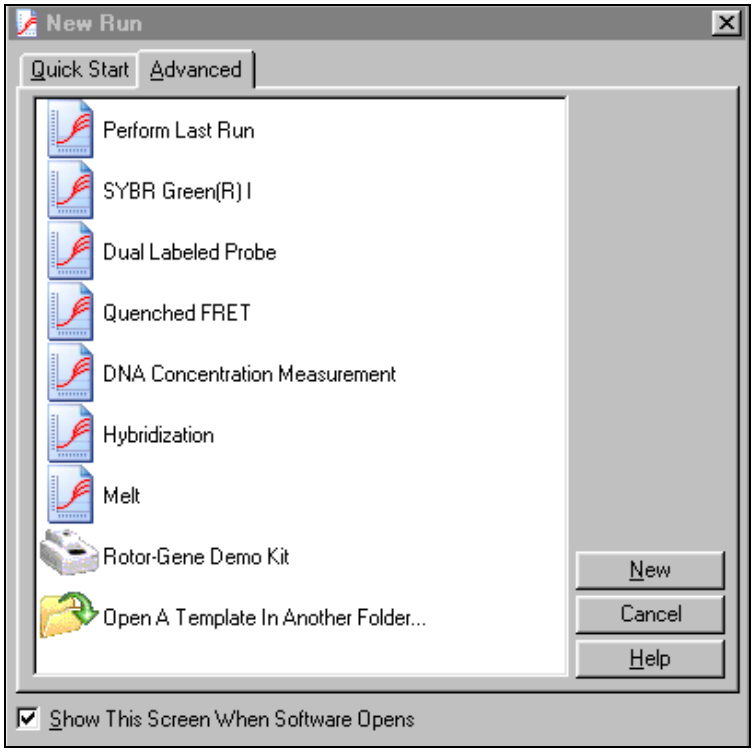
ge**neri bio**tech

OBSAH:

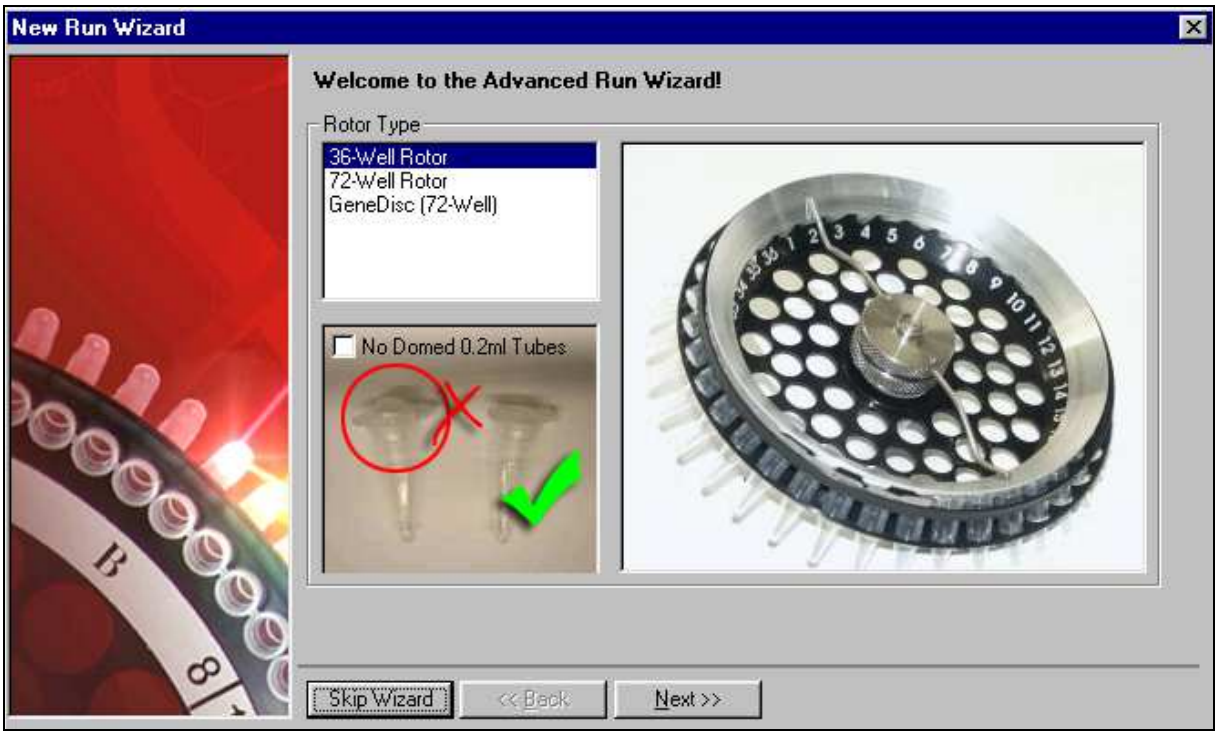
1. Setting of a new temperature profile and running a cycler

1.1. Put samples into the cycler.

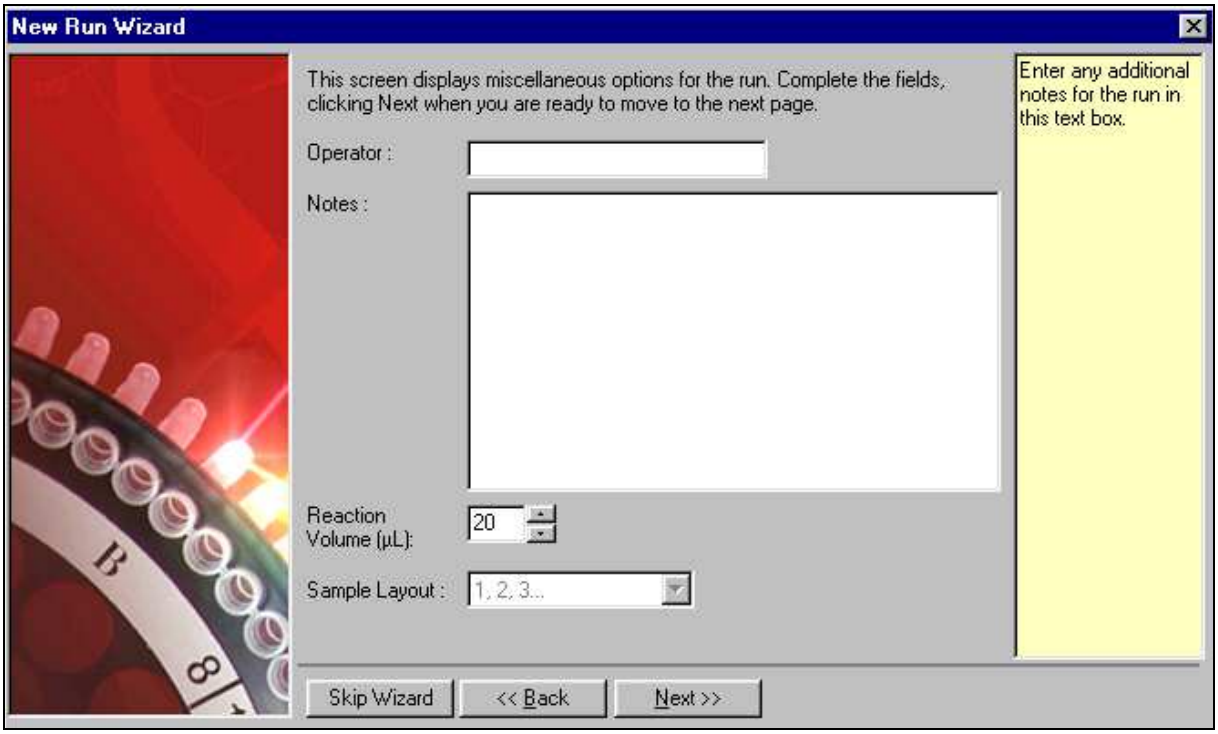
1.2. Run the software of the cycler and select a new run („New“ button) in the „Advanced“ window.



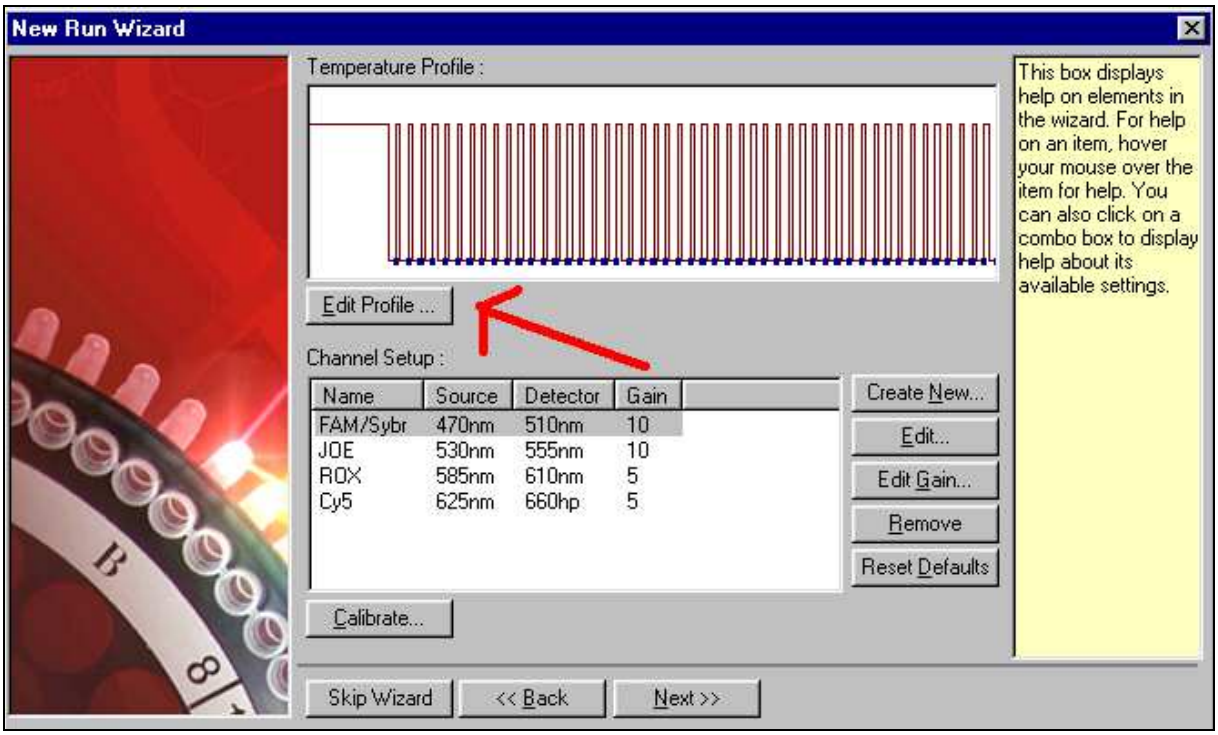
1.3. Select the type of the rotor and tubes. Subsequently click „Next“.



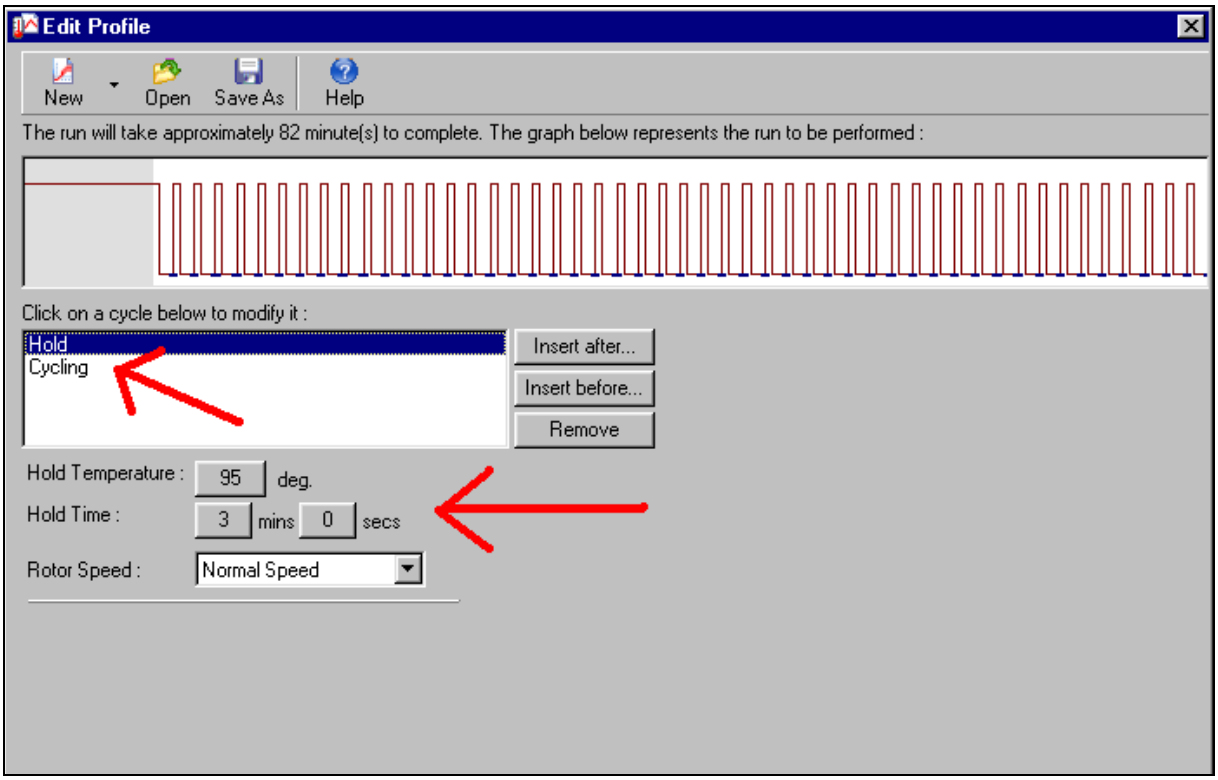
1.4. Set the volume of reaction (usually 20 or 25 µl).



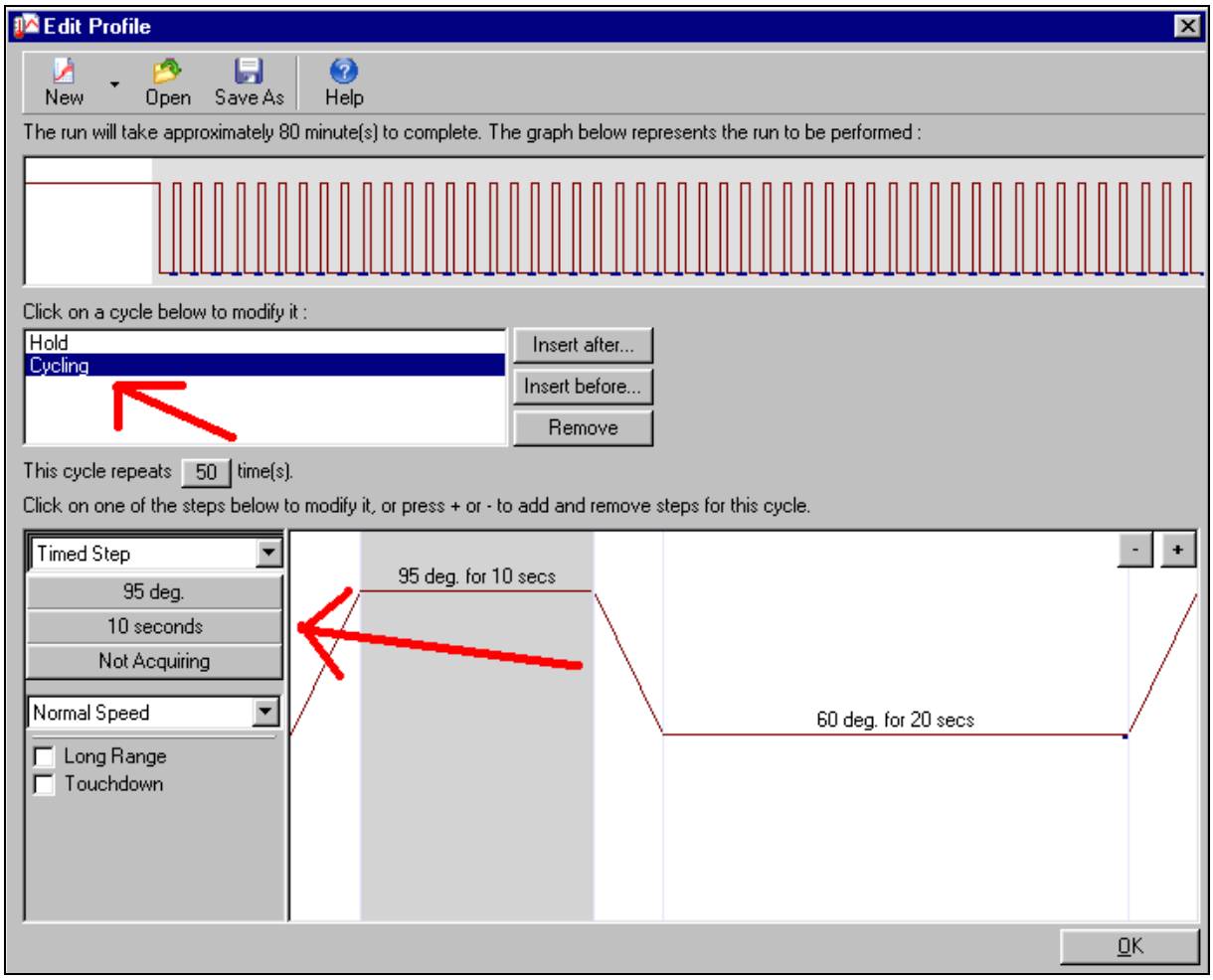
1.5. Set up the temperature profile in the „Edit profile“ window.



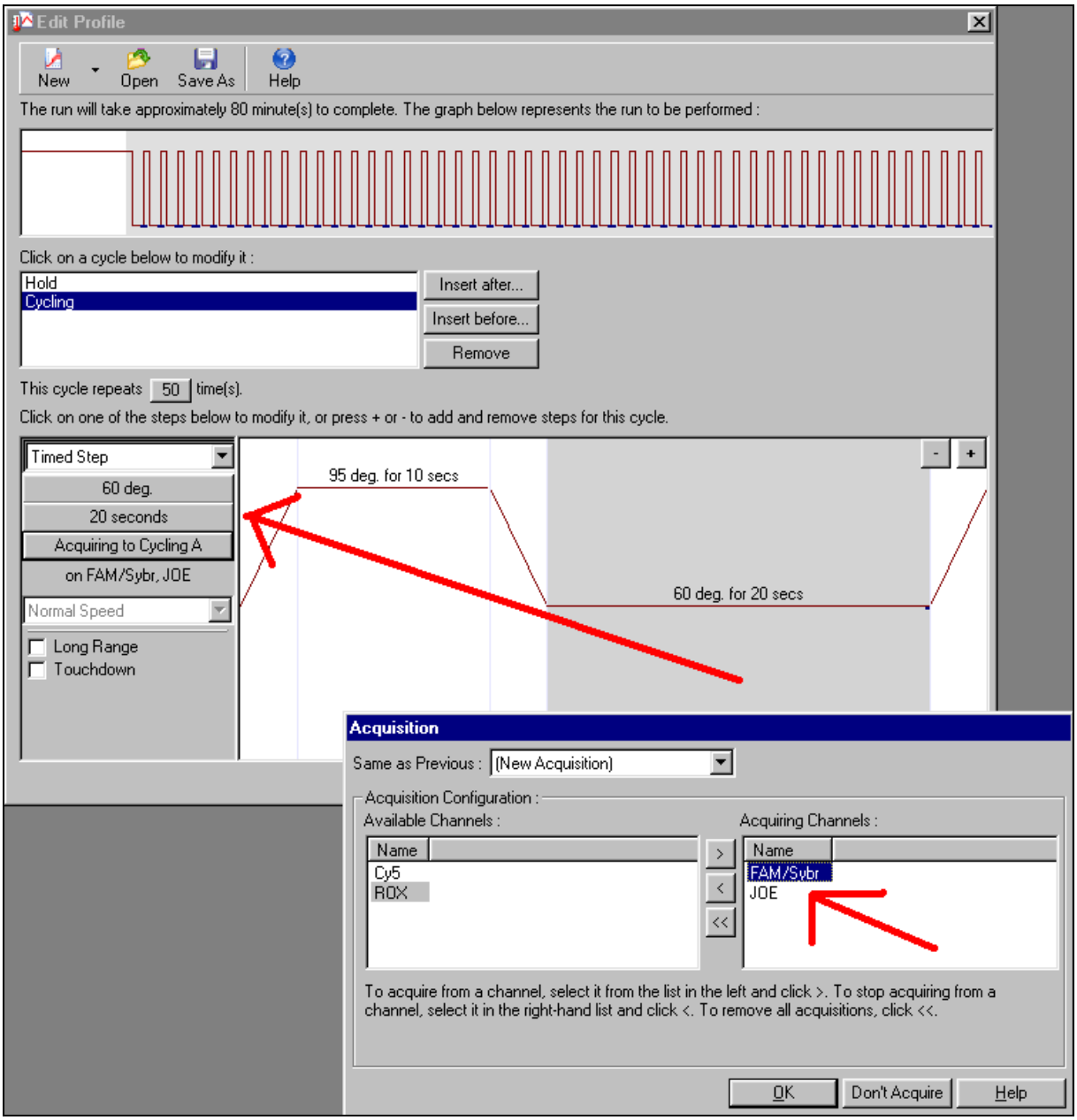
In the „Hold“ tab define the temperature and duration of initial denaturation.



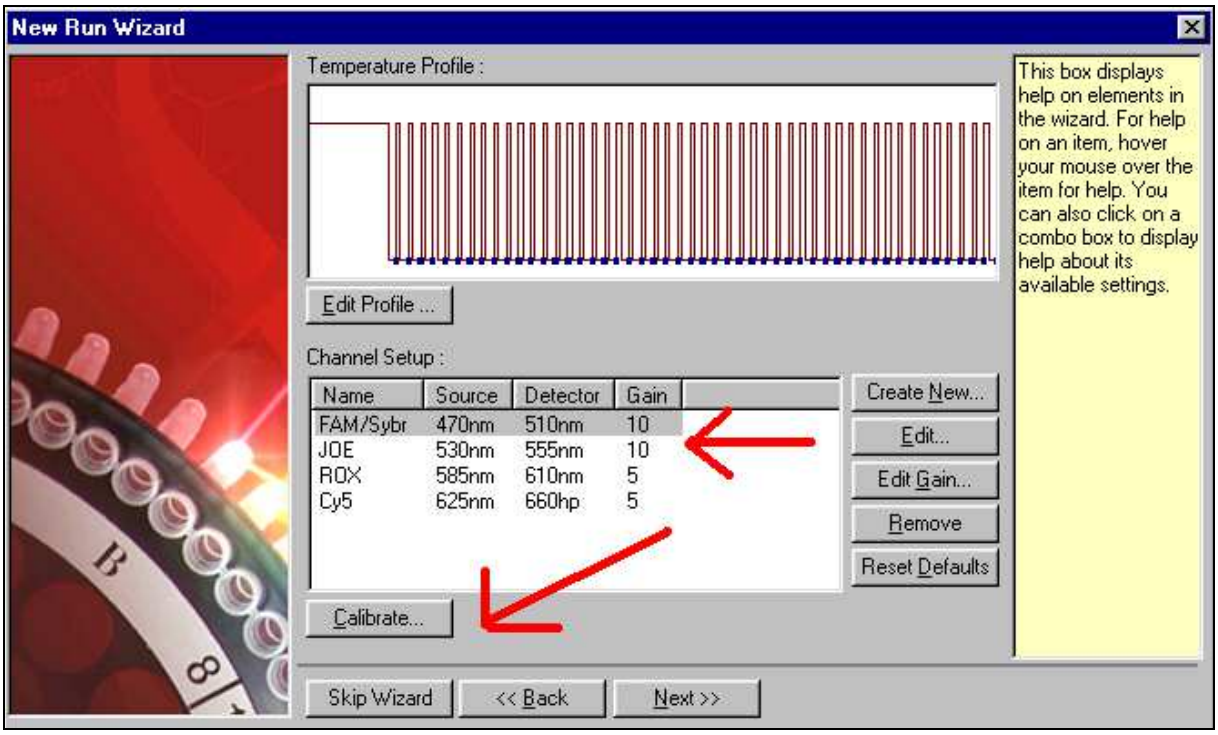
In the „Cycling“ tab set up the temperature and duration of denaturation and subsequent annealing step. It is possible to define the number of repeats as well.



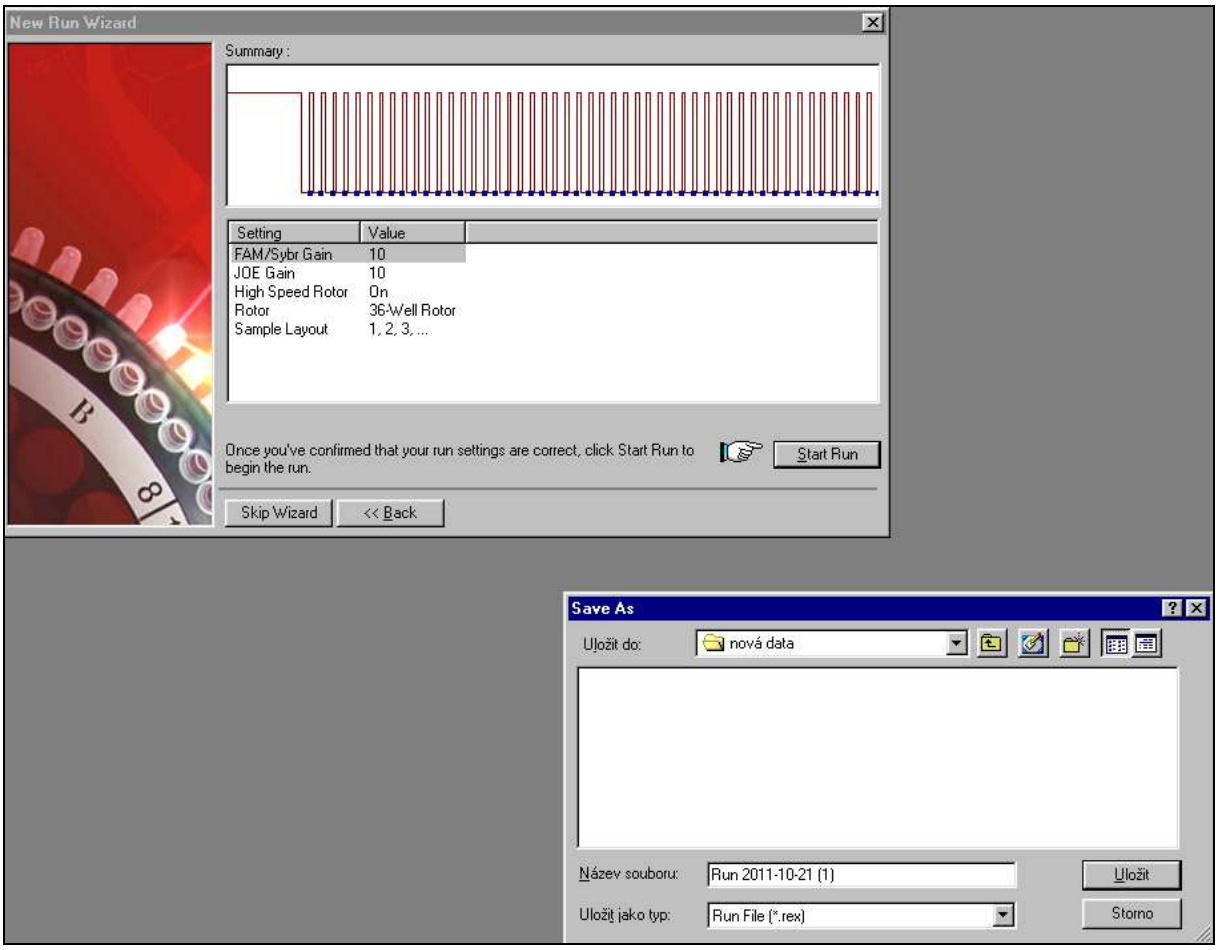
1.6. Set up fluorescence acquisition in the appropriate channels. Mark the area of annealing (grey color) and click the „Acquiring to Cycling A“ button. In the „Acquisition“ window move required channel from „Available Channels“ to „Acquiring Channels“.



1.7. If you need to perform a calibration, follow the manufacturer's instructions (the gain should not differ extremely between channels).

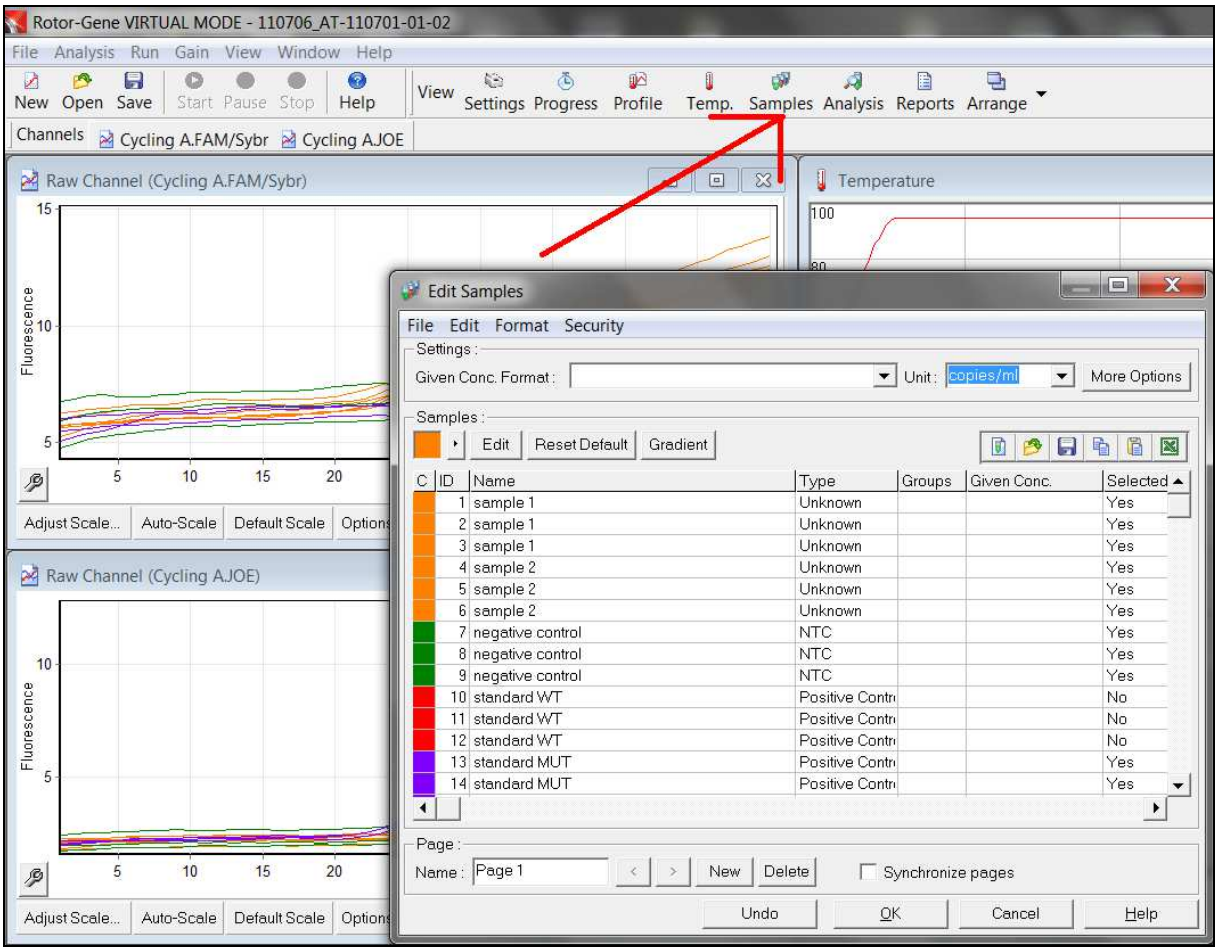


1.8. Save the run to a folder and start the analysis.



2. Definition of samples

2.1. Once the run has started, it is automatically loaded into the sample editor that allows you to define your samples (it is possible to edit the samples afterwards through the "Samples" button in the main menu).



Methods of allelic discrimination analysis

Quantification of Gene Expression

This analysis is based on assessment of Ct values obtained through manual threshold setting by the user. Each fluorescent channel needs to be evaluated individually.

Scatter Graph Analysis

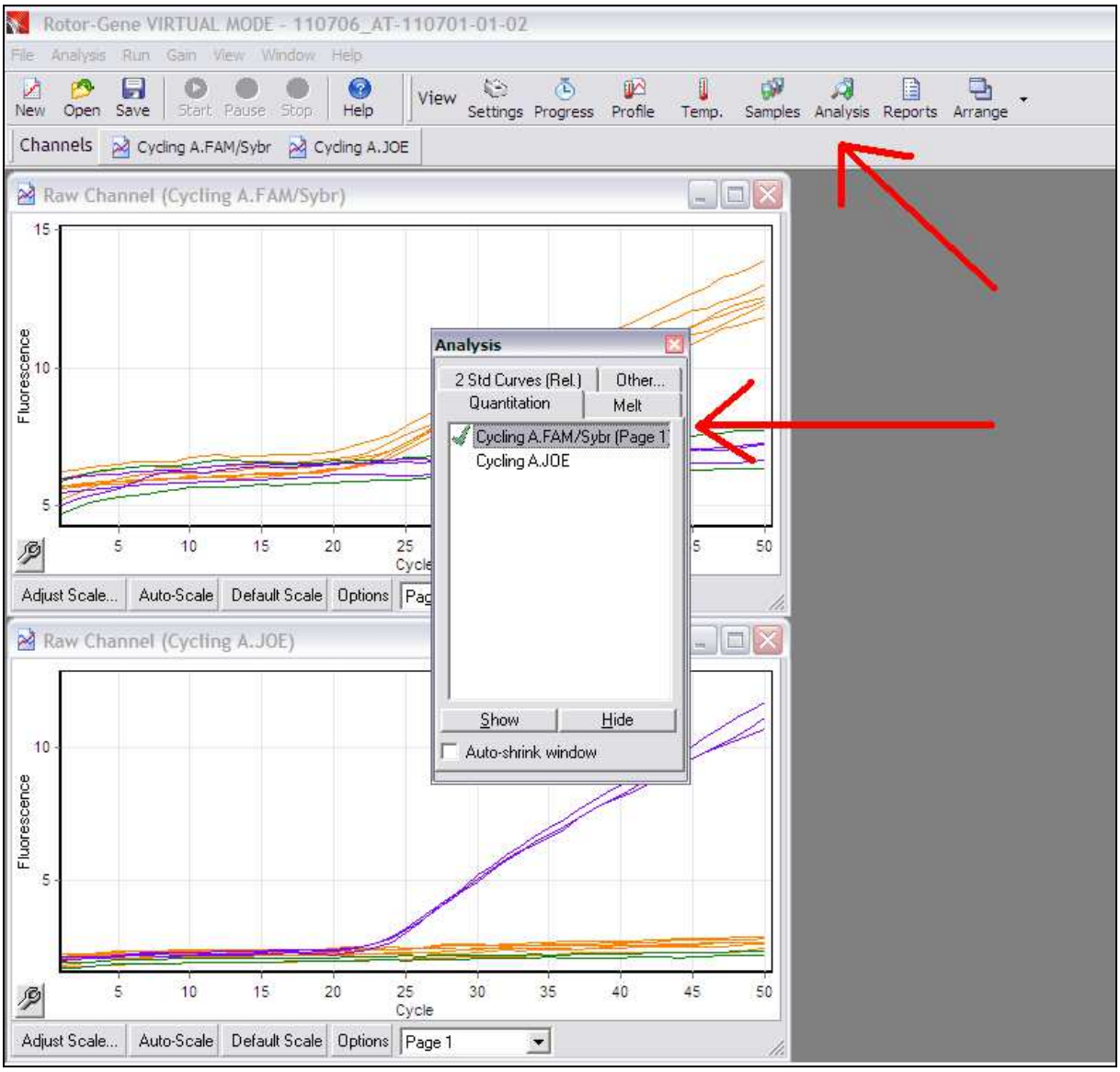
This type of analysis enables quick and simple evaluation of obtained values through a chart. Software can classify samples according to their genotype. In this case it is not possible to set the threshold value manually.

Allelic Discrimination

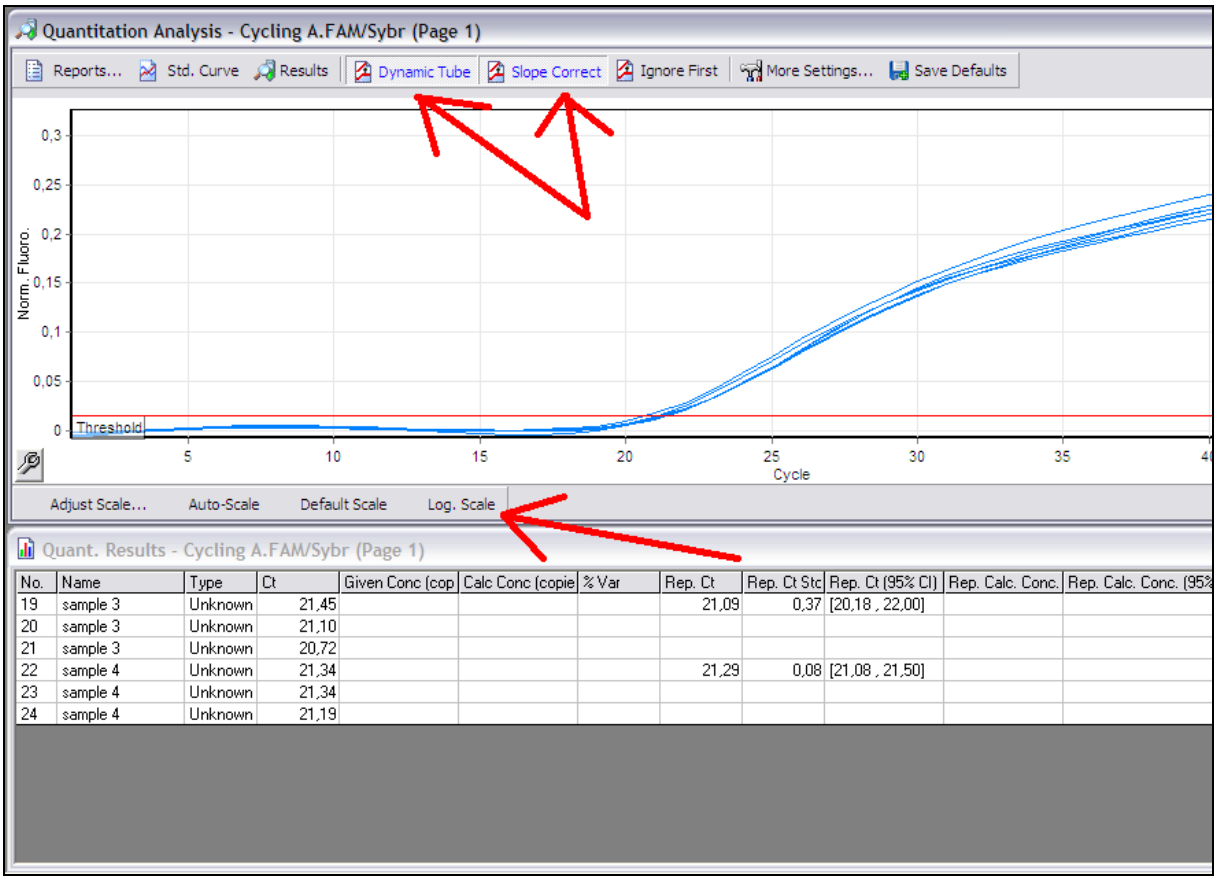
This evaluation is based on terminal fluorescence (the fluorescence acquired in the last cycle of qPCR). The threshold value can be adjusted manually.

3. Allelic Discrimination evaluation - Quantification mode

3.1. In the menu of the „Analysis“ mode select „Quantitation“, subsequently mark appropriate fluorescent channel and click „Show“.

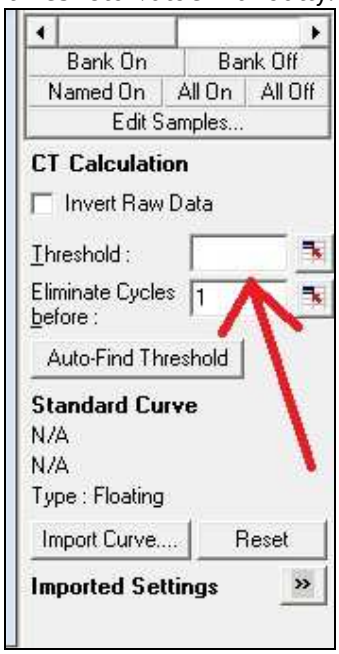


3.2. In the „Quantitation Analysis“ window click „Dynamic Tube“ and „Slope Correct“, then select „Log. Scale“ function.



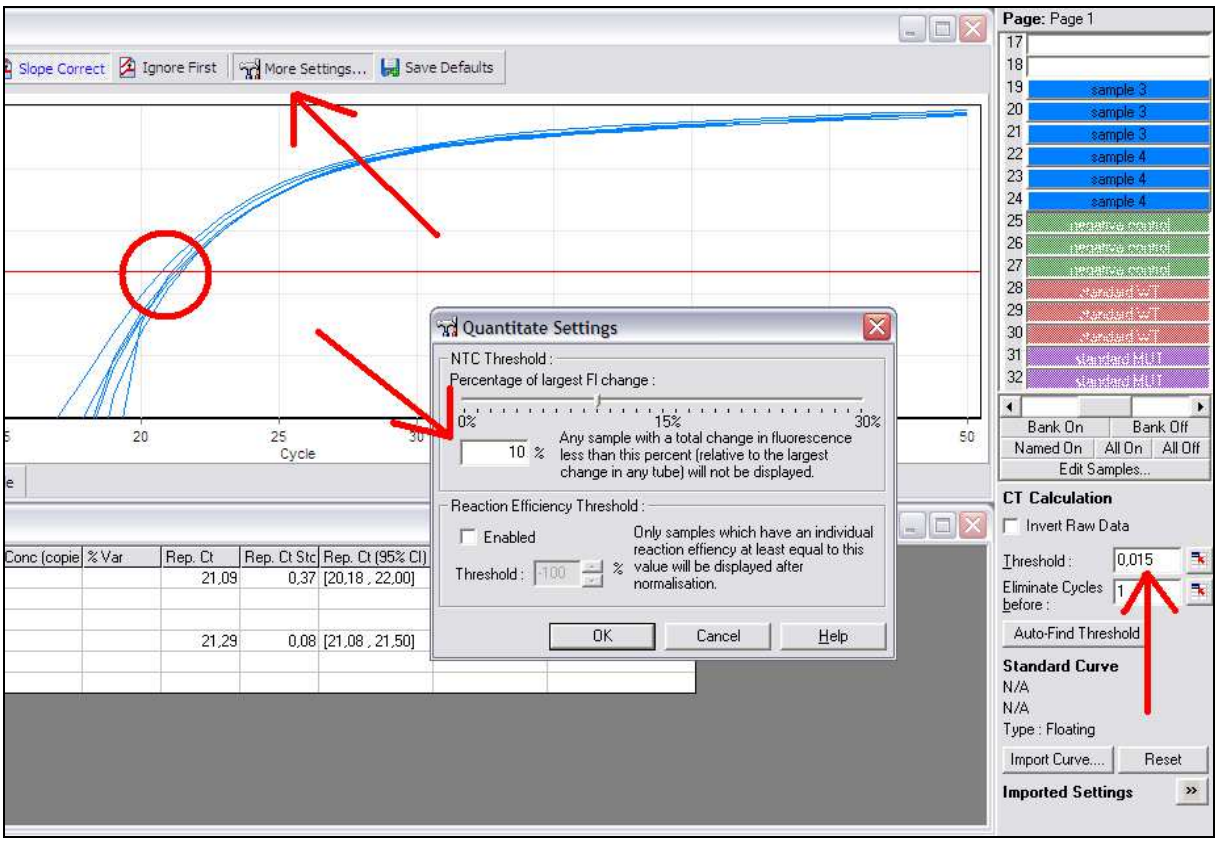
No.	Name	Type	Ct	Given Conc (cop	Calc Conc (copie	% Var	Rep. Ct	Rep. Ct Stc	Rep. Ct (95% CI)	Rep. Calc. Conc.	Rep. Calc. Conc. (95%
19	sample 3	Unknown	21,45				21,09	0,37	[20,18 , 22,00]		
20	sample 3	Unknown	21,10								
21	sample 3	Unknown	20,72								
22	sample 4	Unknown	21,34				21,29	0,08	[21,08 , 21,50]		
23	sample 4	Unknown	21,34								
24	sample 4	Unknown	21,19								

3.3. In the lower right part of the window it is possible to define the threshold value manually. In case the table does not appear automatically, click on the chart.

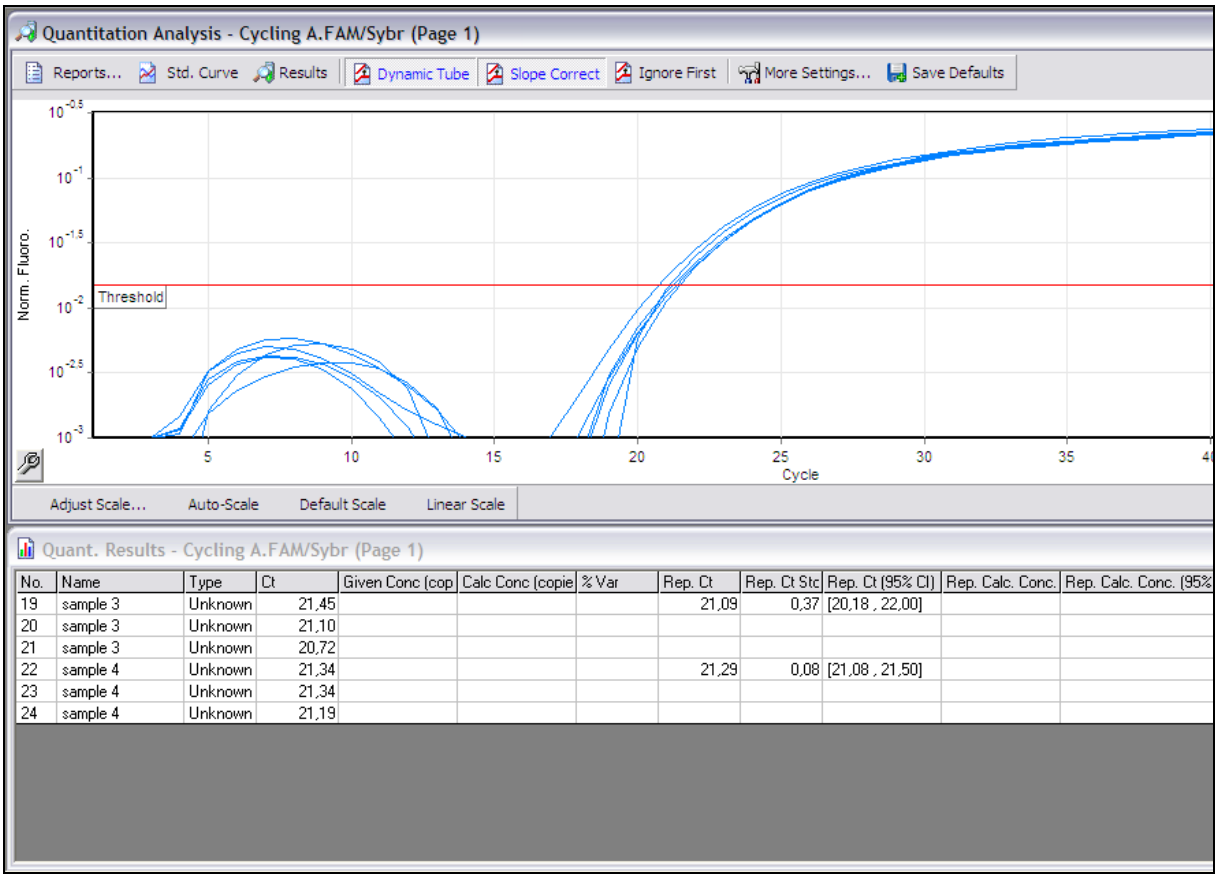


3.4. The threshold line for Ct calculation needs to be set in the middle of the linear part of the logarithmic curves, subsequently click „More Settings...“ and define NTC threshold in the range of 1 – 20 % (gradually increase the value for noise elimination). Perform this setup for each fluorescent channel individually (display only standard curves) and use this setup later during the samples evaluation.

3.5. For genotype analysis - threshold values of both fluorescent channels must be equal.



3.6. In the „Quant. Results“ tab read Ct values.

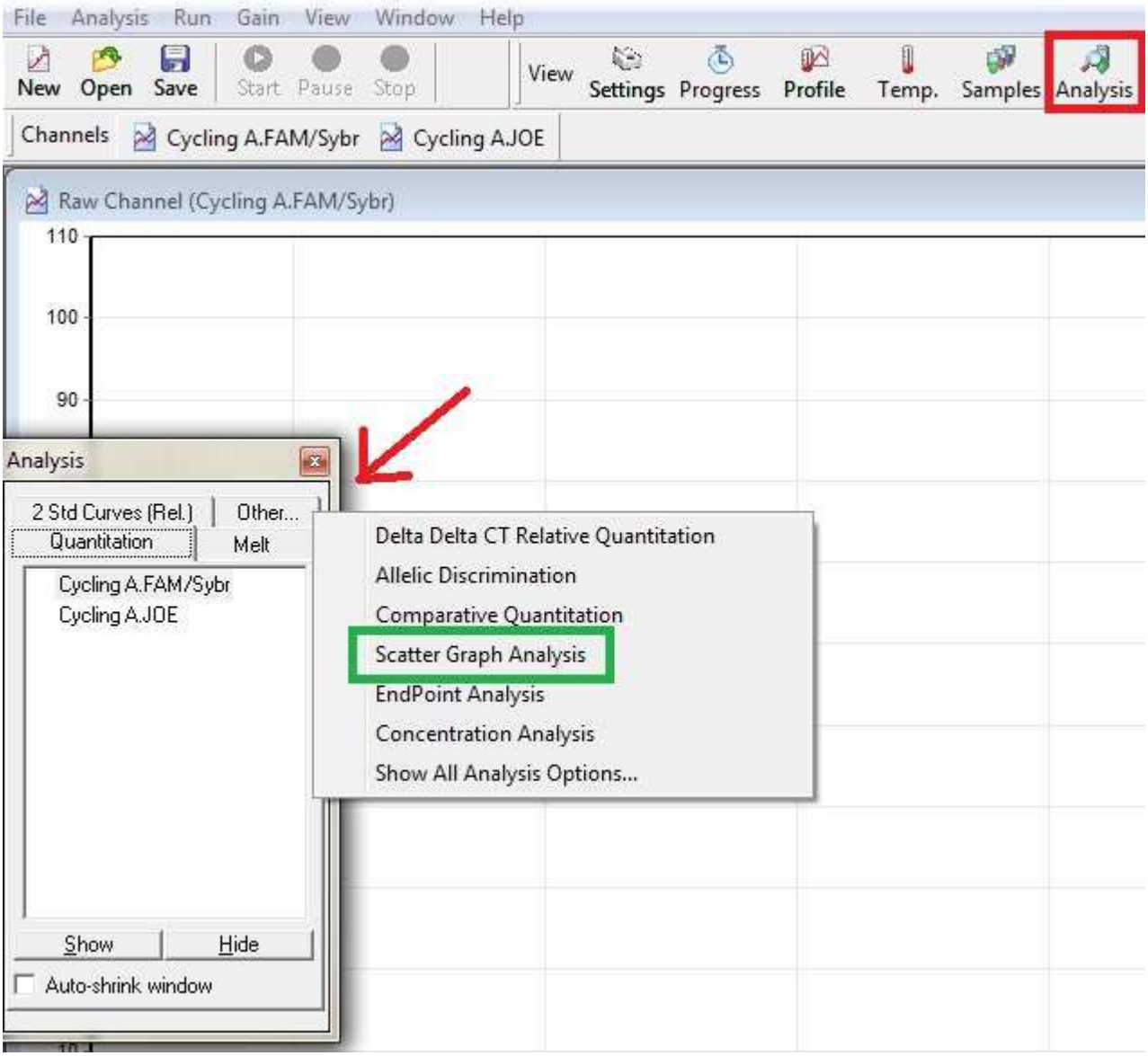


No.	Name	Type	Ct	Given Conc (cop	Calc Conc (copie	% Var	Rep. Ct	Rep. Ct Stc	Rep. Ct (95% CI)	Rep. Calc. Conc.	Rep. Calc. Conc. (95%
19	sample 3	Unknown	21,45				21,09	0,37	[20,18 , 22,00]		
20	sample 3	Unknown	21,10								
21	sample 3	Unknown	20,72								
22	sample 4	Unknown	21,34				21,29	0,08	[21,08 , 21,50]		
23	sample 4	Unknown	21,34								
24	sample 4	Unknown	21,19								

For Ct reading in another fluorescent channel (e.g. in cases of multiplex PCR, genotype analysis and/or internal positive control) mark required channel in „Analysis / Quantitation“ mode, then follow the process as described above (chapter 3.1).

4. Allelic Discrimination evaluation - Scatter Graph Analysis mode

4.1. Run the program and click the „Analysis“ button. In the „Analysis“ menu select the tab „Other“ then the „Scatter Graph Analysis“.

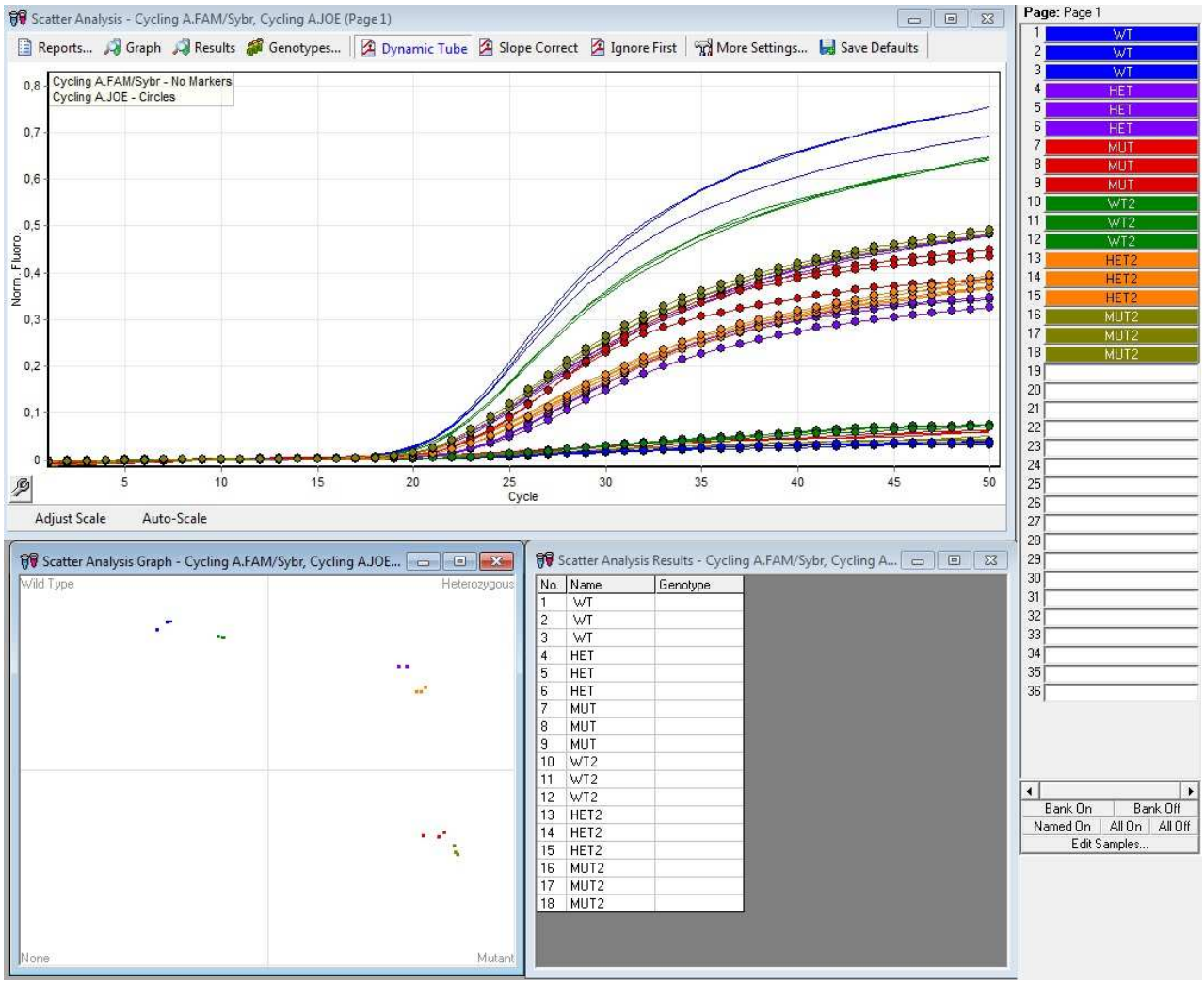


4.2. Mark both the fluorescent channels and click „Show“ button.

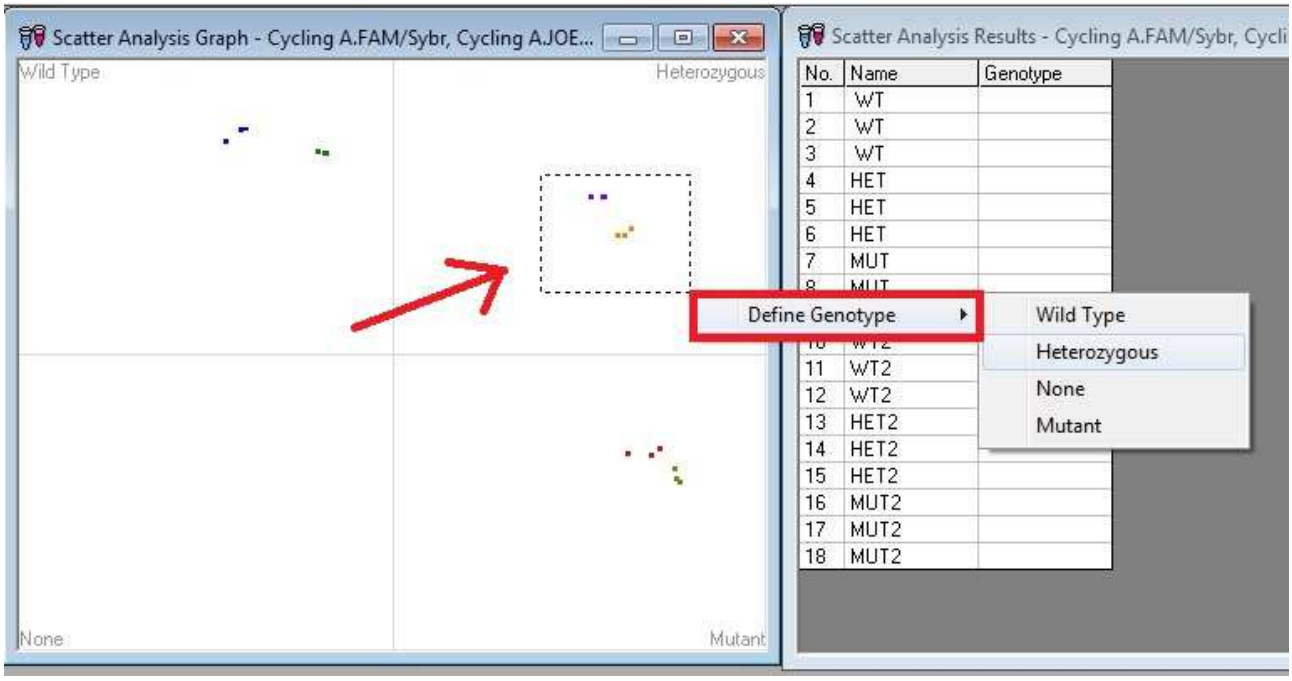
4.3. The allelic discrimination analysis is displayed.

In the upper window you can assess the FAM/Sybr and JOE/HEX (with circles) curves. The x axis presents the number of cycles and the y axis shows values of fluorescence.

The Scatter Analysis Graph is displayed in the lower left window. Each sample is assigned to a group of WT, MUT or HET.

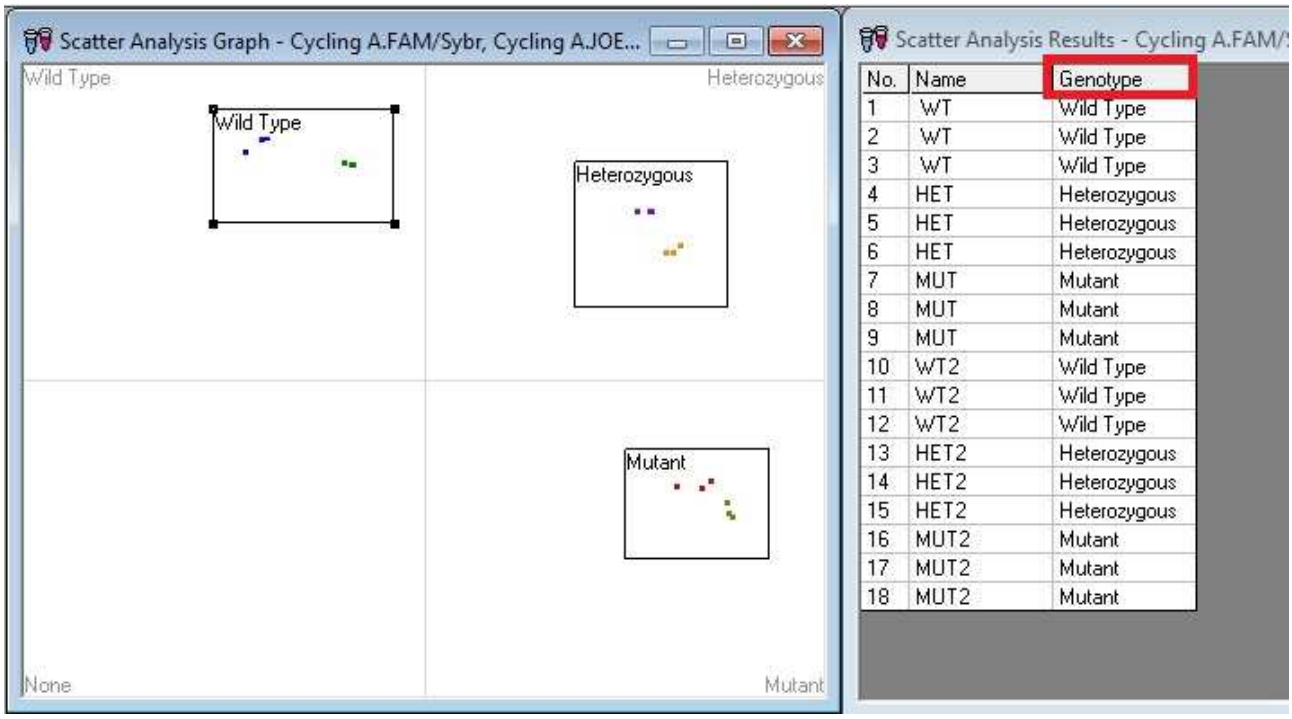


4.4. For allelic discrimination evaluation we recommend first only positive controls signals (WT/MUT/HET) be displayed in the „Scatter Analysis Graph“. Subsequently select a group of each of them to define their genotype through the right mouse button (e.g. WT is detected in FAM/Sybr channel and is displayed in the upper left part of the chart („Scatter Analysis Graph“).



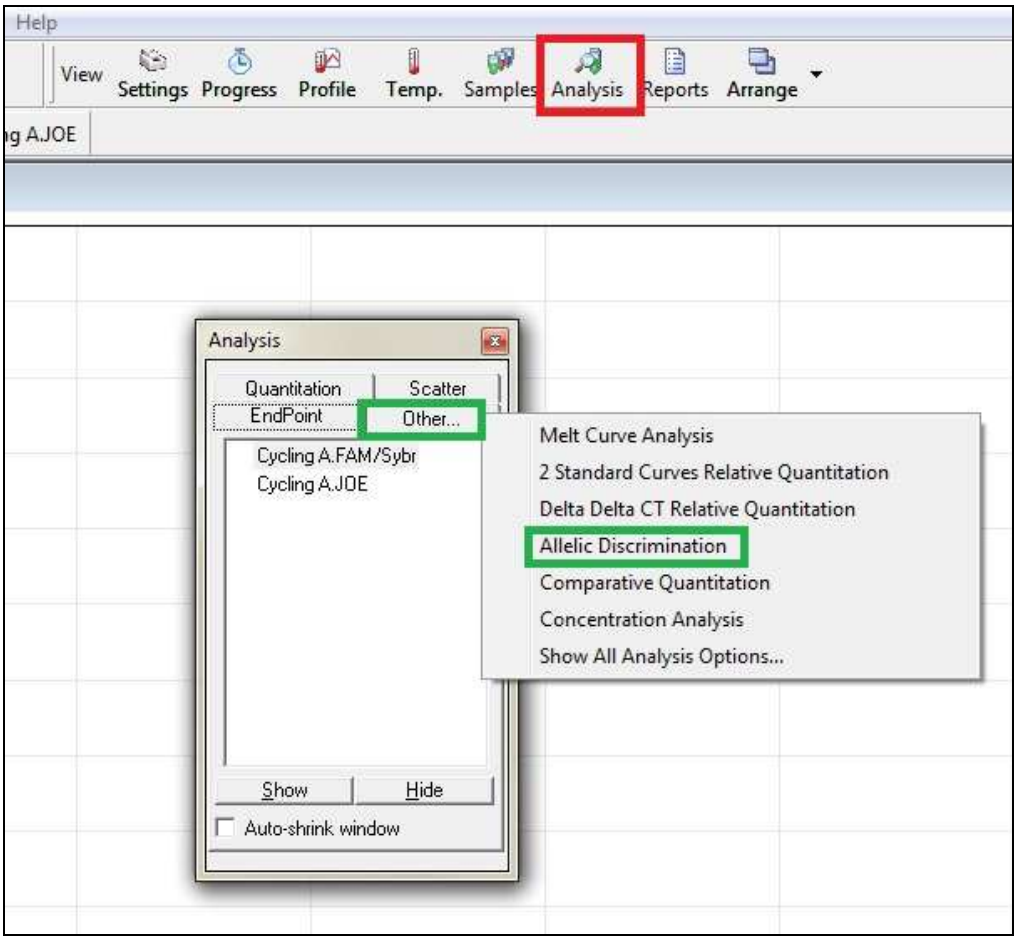
4.5. After genotype definition, the results of analysis are displayed in the tab „Scatter Analysis Results“.

4.6. Use the genotype setting for all the samples evaluation. Areas for genotype definition adapt according to the scatter of the samples in the chart.



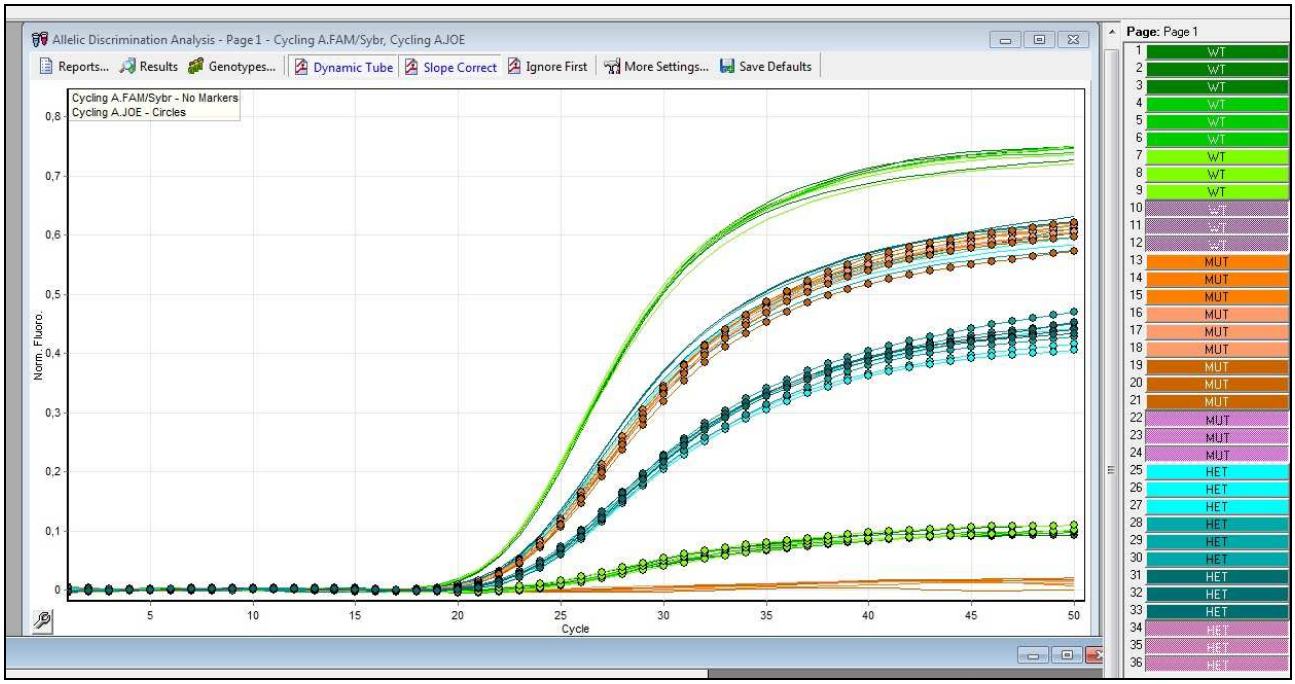
5. Allelic discrimination evaluation – Allelic Discrimination mode

5.1. Run the program and click the „Analysis“ button. In the „Analysis“ window open „Other...“ tab and select „Allelic Discrimination“.



5.2. Mark both the fluorescent channels (through Shift key) and click the „Show“ button.

5.3. The Allelic Discrimination analysis is displayed. In the upper window you can asses FAM/Sybr and JOE/HEX (with circles) curves. The x axis presents the number of cycles, the y axis shows values of fluorescence.

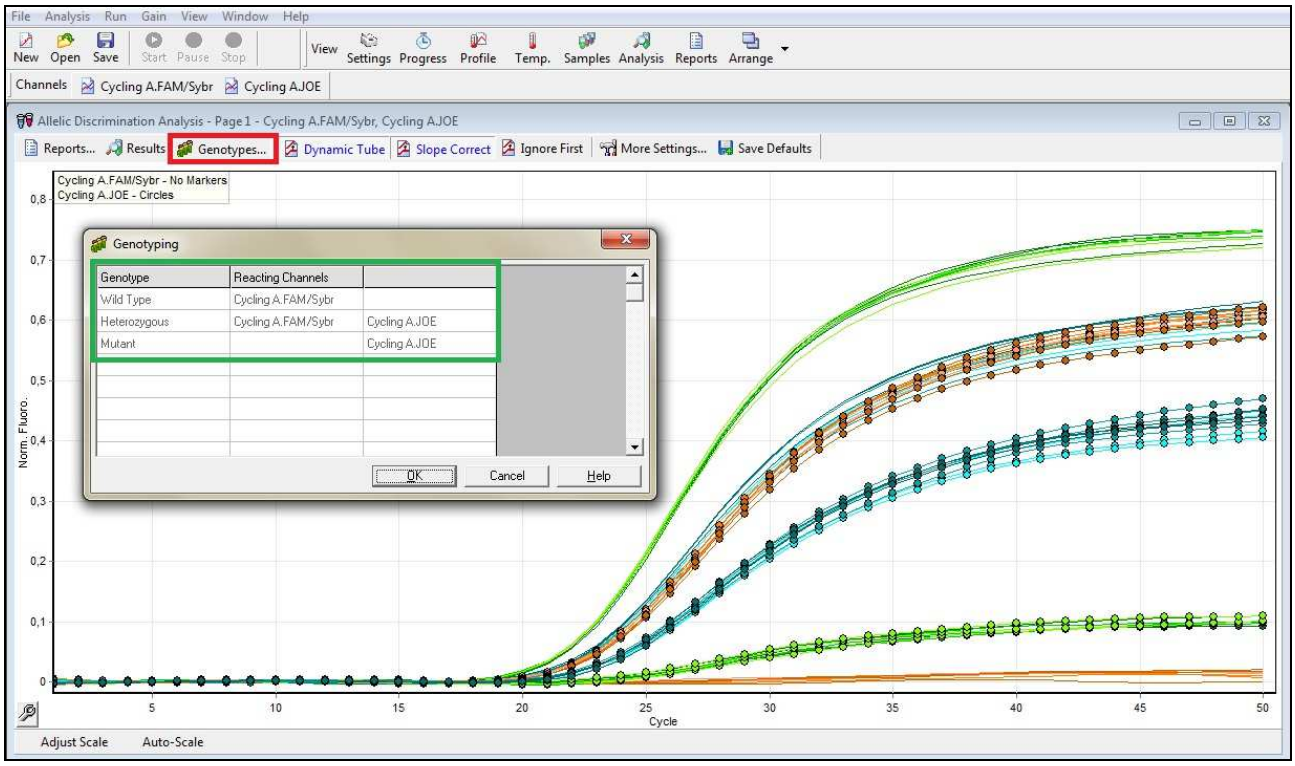


5.4. The genotype of the sample can be defined automatically according to its final fluorescence. First it is necessary to assign fluorescent channels with particular genotypes.

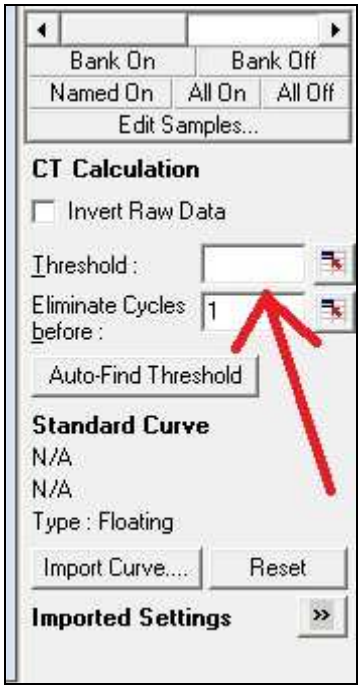
In the window „Allelic Discrimination Analysis“ select „Genotypes“, then in „Genotyping“ window select fluorescent channels for all the genotypes.

Note: Wild-type is detected in the FAM/Sybr channel
Mutant is detected in JOE/HEX channel
Heterozygote is detected simultaneously in FAM/Sybr and JOE/HEX channels

Results of the analysis are displayed in the tab (see 5.8.)

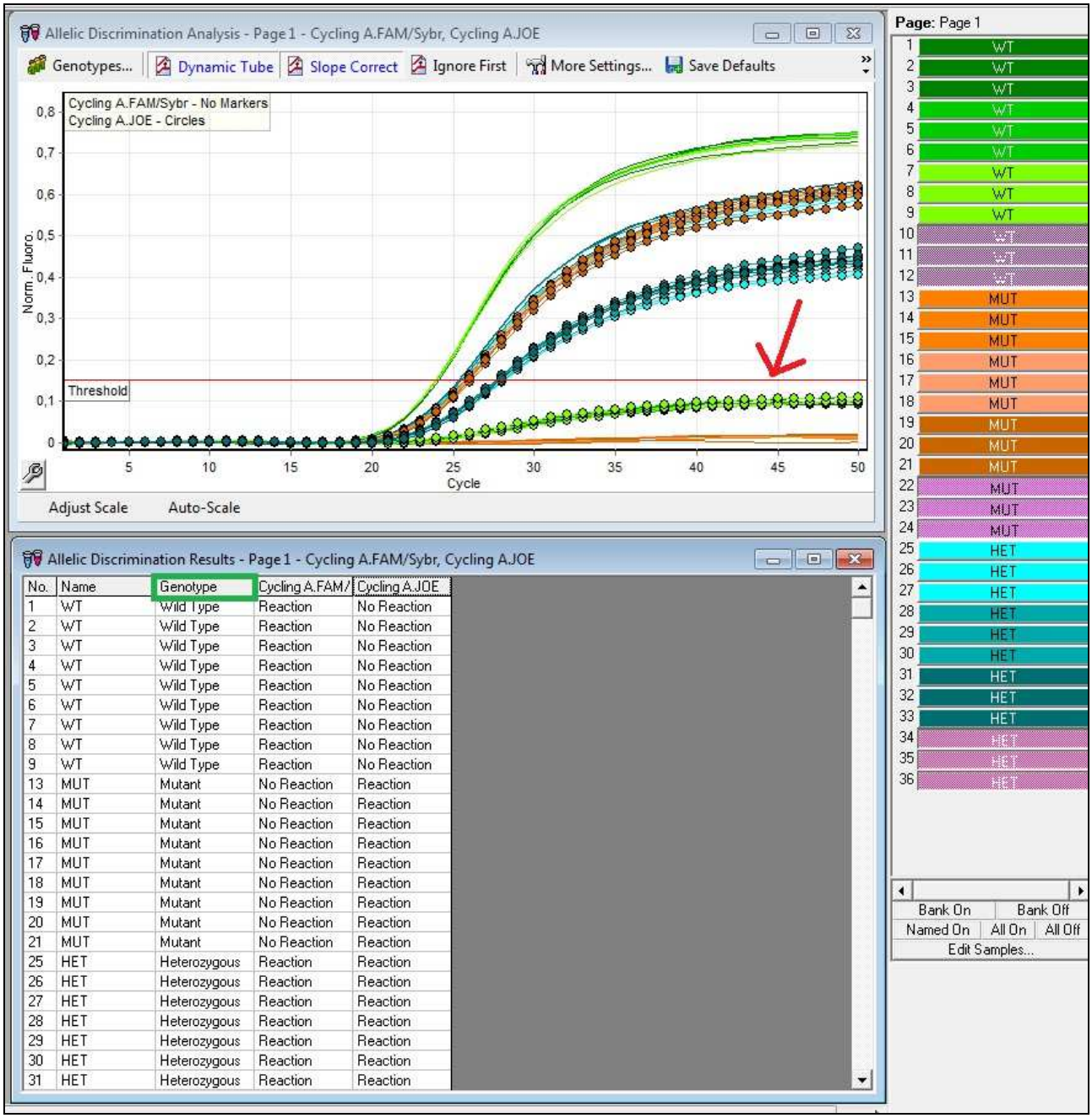


5.5. In the lower right part of the window, it is possible to set the threshold value by the user. When the tab is not displayed automatically, click on the graph.



5.6. For allelic discrimination evaluation we recommend first only positive controls signals (WT in JOE/HEX channel MUT in FAM/Sybr channel) be displayed. Subsequently the threshold line needs to be set just above the non-specific curves.

5.7. In case of evaluation of more than one mutation monitored in one run it is necessary to set the threshold value individually according to appropriate positive controls. Use this particular setup only for assessment of samples tested on the respective mutation.



5.8. In the lower left part of the window the tab with genotypes of individual positive controls is displayed. The DNA samples are assigned to genotypes in the same way.