QIAGEN Supplementary Protocol

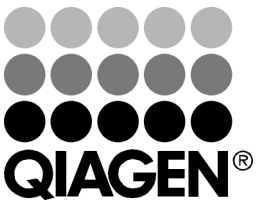
Type-it® HRM® PCR protocol for use with the Applied Biosystems® 7500 Fast System

This protocol shows how to perform detection of gene mutations and SNPs by high-resolution melting (HRM) analysis using the Applied Biosystems 7500 Fast System and the Type-it HRM PCR Kit. The procedure involves 2 steps: initial instrument calibration for EvaGreen® dye and genotyping analysis using HRM technology. This supplementary protocol is divided into 3 protocols which provide information on the following:

- Calibration of the instrument for EvaGreen dye if using the Applied Biosystems 7500 Fast System with the Type-it HRM PCR Kit for the first time
- Genotyping protocol — analysis of gene mutations and microbial genetic differences by HRM: this part of the protocol is recommended for all HRM applications except SNP genotyping
- Genotyping protocol — analysis of SNPs by HRM

IMPORTANT: Please consult the "Safety Information" section in the *Type-it HRM PCR Handbook* before beginning this procedure. For safety information on the additional chemicals mentioned in this protocol, please consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

The Type-it HRM PCR Kit is intended for molecular biology applications. This product is neither intended for the diagnosis, prevention, or treatment of a disease, nor has it been validated for such use either alone or in combination with other products.



Protocol 1: Calibration of the Applied Biosystems 7500 Fast System with software v1.4 for EvaGreen Dye

This Type-it HRM PCR protocol has been optimized for use with the Applied Biosystems 7500 Fast System. If using the Applied Biosystems 7500 Fast System for HRM analysis for the first time, it is necessary to initially calibrate the instrument for each new dye that will be processed. EvaGreen dye (contained in Type-it HRM PCR Master Mix) is recommended as a suitable dye by the instrument manufacturer, making the Type-it HRM PCR Kit an optimal solution for successful HRM analysis on the Applied Biosystems 7500 Fast System.

IMPORTANT: Once instrument calibration has been performed, it is not necessary to repeat it again.

The instrument calibration procedure consists of 3 steps:

- Preparing and running a background calibration plate
- Preparing an EvaGreen dye calibration plate
- Performing 3 runs on the same EvaGreen dye calibration plate: these include an amplification run, EvaGreen dye calibration, and an HRM calibration run

Equipment and Reagents to be supplied by user

- MicroAmp™ Fast Optical 96-Well Reaction Plate with barcode, 0.1 ml (Applied Biosystems, cat. nos. 4346906 and 4366932)
- MicroAmp Optical Adhesive Film (Applied Biosystems, cat. nos. 4360954 and 4311971)
- HRM Calibration Standard, 20x (Applied Biosystems, cat. no. 4396728)
- Distilled water
- Type-it HRM PCR Kit (100) (QIAGEN, cat. no. 206542)

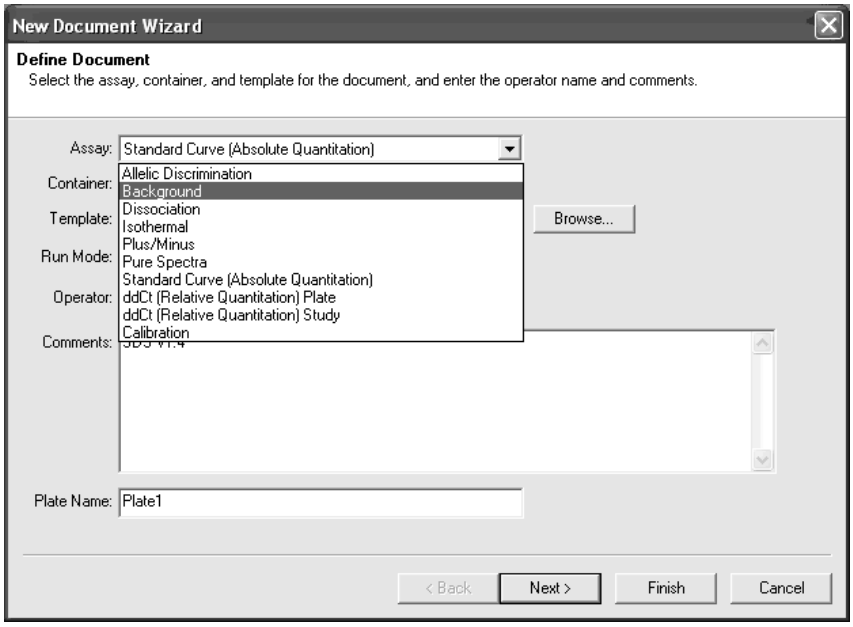
Important points before starting

- The procedures outlined here must be followed to ensure successful results.
- If using the Applied Biosystems 7500 Fast System for HRM analysis, it is necessary to initially calibrate the instrument for each new dye that will be processed. If EvaGreen dye has not yet been calibrated with your instrument, proceed with this calibration protocol. If it has been calibrated with the instrument once, it does not need to be repeated.
- Optimal instrument and HRM analysis settings are a prerequisite for accurate genotyping results. For details, please refer to the manual provided with the Applied Biosystems 7500 Fast System.

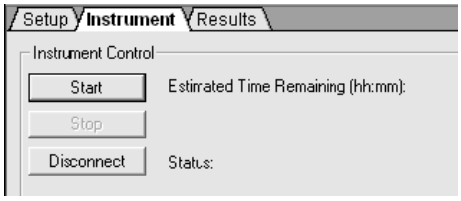
Procedure

Preparing and running a background calibration plate

1. **Add 20 µl RNase-free water to each well of a 96-well reaction plate and load the calibration plate into the cycler.**
2. **From the assay drop-down list, select "Background".**

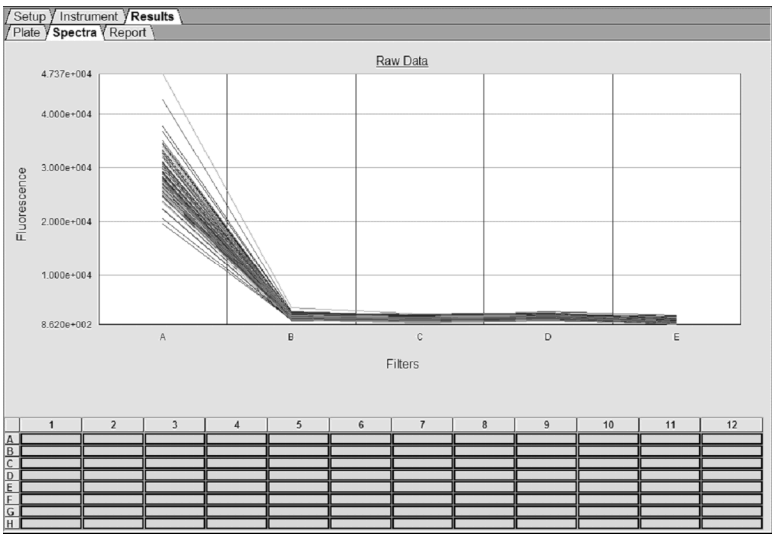


3. **Click "Finish", and select the "Instrument" tab, and click "Start".**
4. **Save the file as "Background Calibration_<today's date>".**



5. **After the run, the "*Run completed successfully*" message appears.**
6. **Review the background calibration results.**
7. **Click (or select "Analysis Extract Background").**
8. **When the software displays the following message, click "OK".**

9. **Select the "Results" and "Spectra" tabs, and then select all wells in the plate grid as shown below.**



Note: If one or more wells produce raw spectra that exceed 72,000 FSU, the background plate or the sample block could contain a fluorescent contaminant.

Preparing an EvaGreen dye calibration plate

IMPORTANT: The EvaGreen dye calibration plate should be prepared fresh and used immediately. It is important to perform the amplification run, dye calibration, and HRM calibration on the same day that the HRM dye calibration plate is prepared.

1. **Prepare the EvaGreen Dye Calibration Plate according to Table 2.**

Table 2. Composition of EvaGreen Dye Calibration Plate

Component	Volume for 1 reaction	Volume for 110 reactions	Final concentration
HRM PCR Master Mix, 2x	10 µl	1100 µl	1x
HRM Calibration Standard, 20x	1 µl	110 µl	1x
Deionized water	9 µl	990 µl	–
Total volume per reaction	20 µl	2200 µl	–

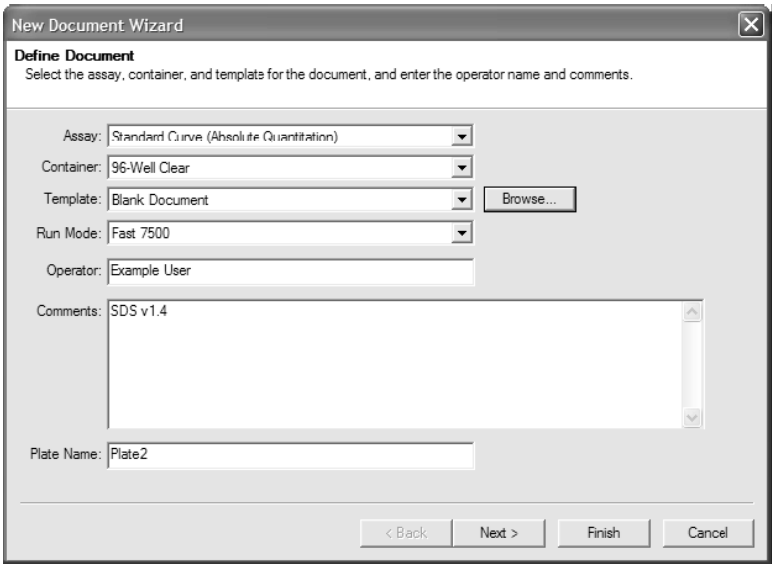
2. **Pipet 20 µl of the mixture into each well of a 96-well reaction plate.**

Amplification run

1. **Run the HRM dye calibration plate to amplify the DNA.**

   IMPORTANT: Perform the next steps (EvaGreen dye calibration and HRM calibration) within 1 h of performing the amplification run.

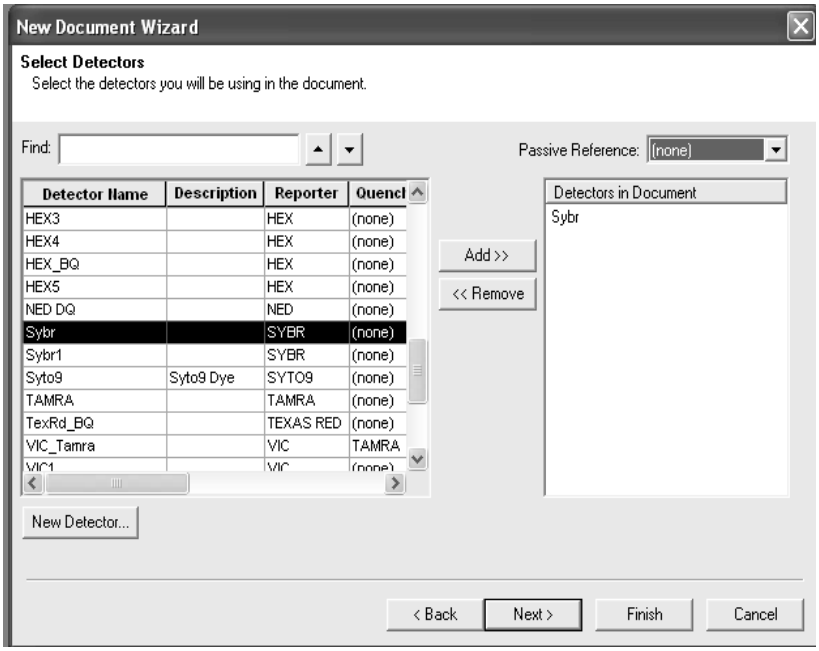
2. **From the "Assay" drop-down list, select "Standard Curve (Absolute Quantitation)".**



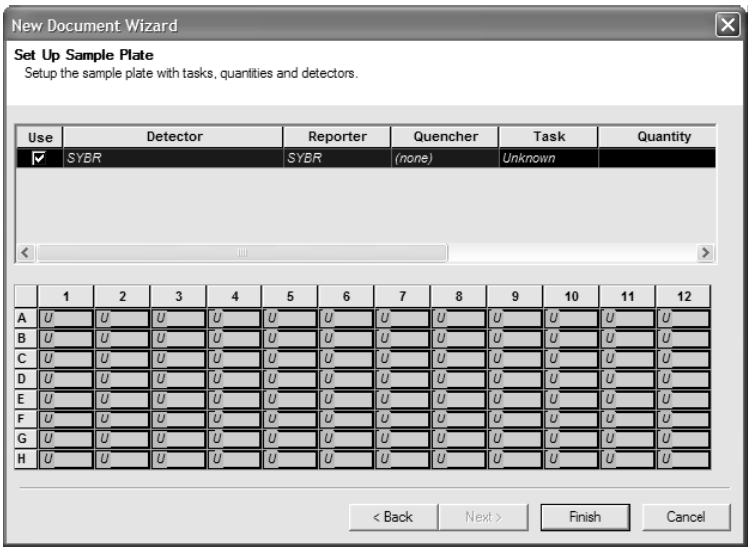
3. **Click next.**

4. **From the table, select SYBR® Green dye as the detector and click "Add".**

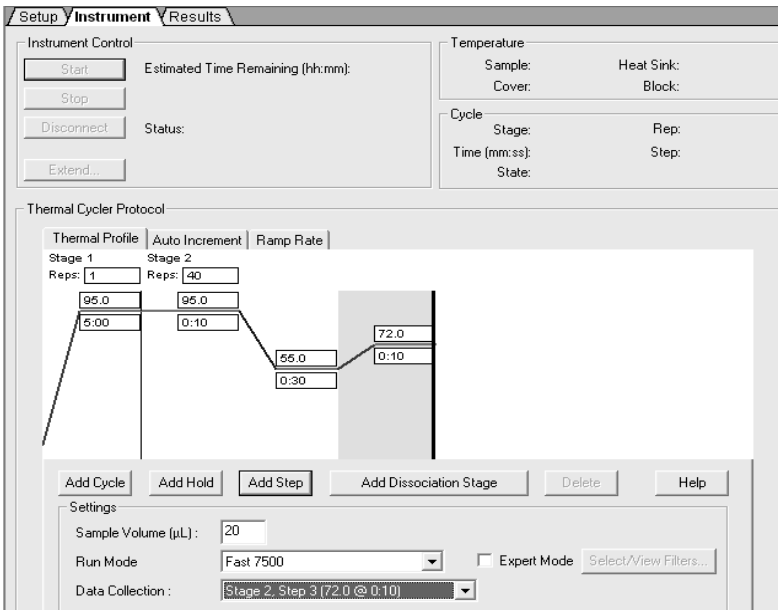
5. **From the "Passive Reference" drop-down list, select "(none)". Then click "Next".**



6. **Set up the sample plate by selecting all wells in the plate grid and selecting "SYBR" as the detector. Then click "Finish".**



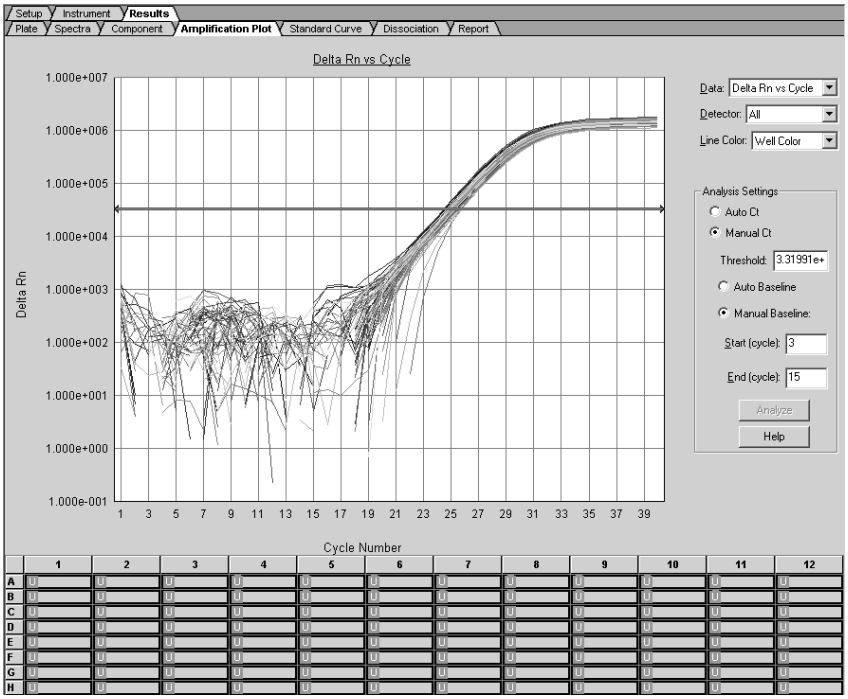
7. **Load the EvaGreen dye calibration plate into the instrument.**
8. **Select the instrument's "Thermal Profile" tab and set the thermal cycler protocol as shown below.**
9. **Set the sample volume to 20 µl.**
10. **Select the "Fast 7500" run mode.**
11. **Click "Start".**



Note: The 3-step cycling protocols for analysis of mutations with the Type-it HRM PCR Kit have been chosen. The calibration has no influence on the Type-it protocol selected later on.

12. **Save the file as "Amplification_<today's date>".**
13. **When the run is complete, the "*Run completed successfully*" message appears.**

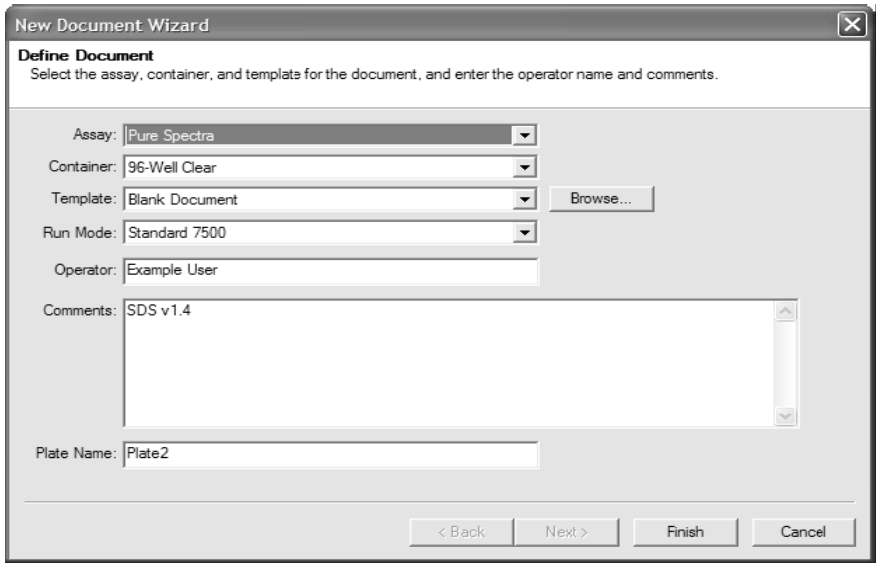
14. **Review the results. Click (Analyze). Click on the "Results" and "Amplification Plot" tabs, and then select all wells in the plate grid. Verify that the samples amplified.**



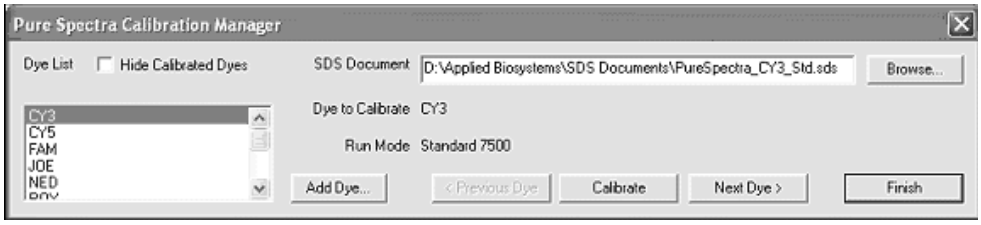
EvaGreen dye calibration

IMPORTANT: Use the same EvaGreen dye calibration plate after amplification a second time to calibrate the Applied Biosystems 7500 Fast System for EvaGreen dye.

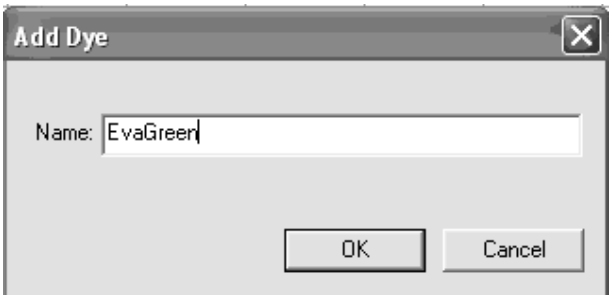
1. **Create a new file. From the "Assay" drop-down list, select "Pure Spectra". Click "Finish". The software opens the "Pure Spectra Calibration Manager" dialog box.**



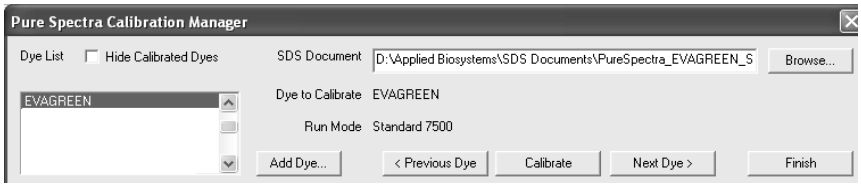
2. **In the "Pure Spectra Calibration Manager" dialog box, click "Add Dye".**



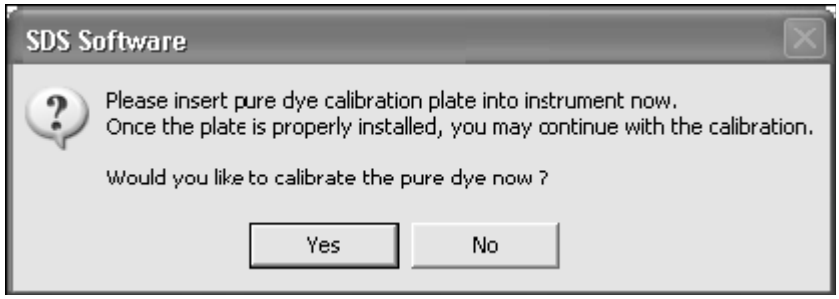
3. **Enter "EvaGreen" and click "OK".**



4. **Select "EvaGreen" from the drop-down list of dyes and click "Calibrate".**

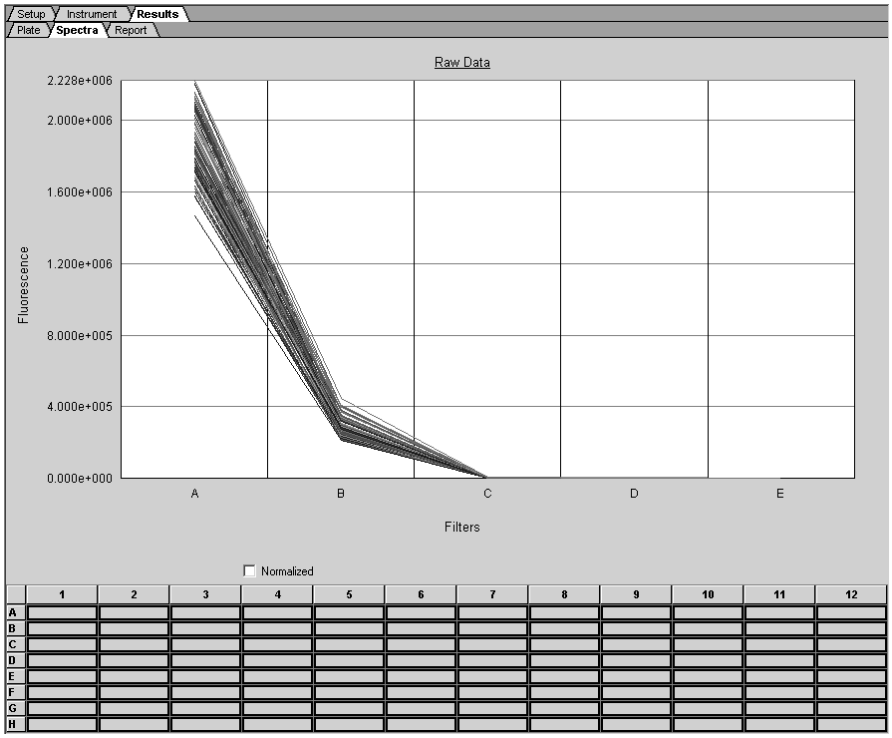


5. **Insert the EvaGreen dye calibration plate a second time into the instrument to perform the dye calibration. At the prompt, click "Yes" to perform the custom dye calibration.**



6. **When the run is complete, click "Finish".**
   The software automatically saves the EvaGreen dye data to a calibration file on the computer hard drive.
7. **Review the results. Click (or select "Analysis" and then "Extract Pure Spectra"). When the software displays the following message, click "OK".**

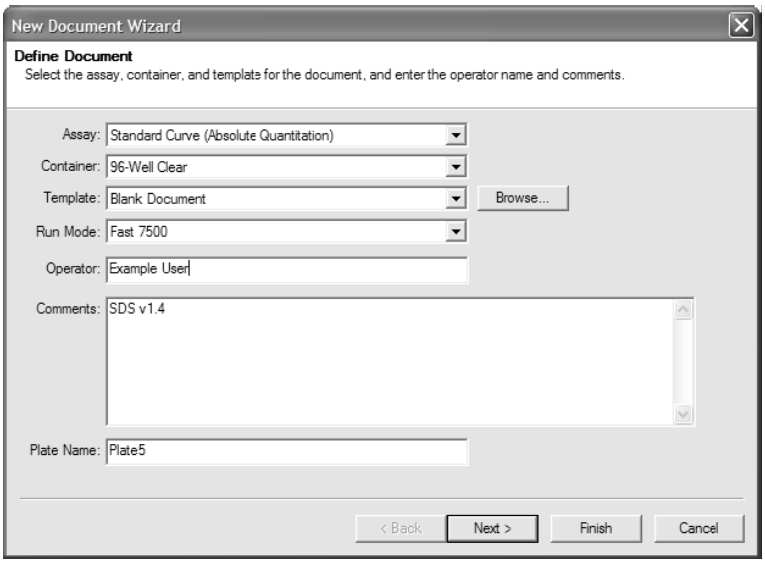
8. **Select the "Results" and "Spectra" tabs, and then select all wells in the plate grid.**
9. **Verify that the peak of your HRM dye appears in Filter A.**



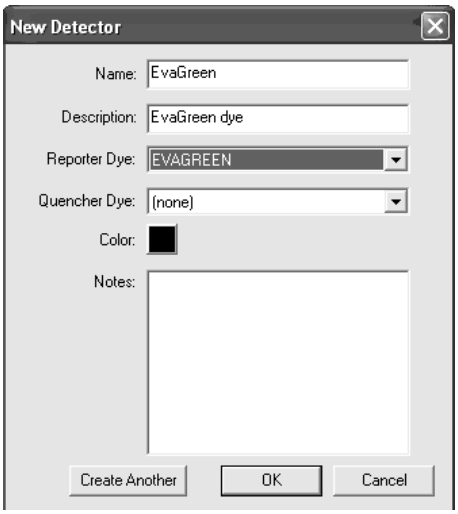
HRM calibration run

IMPORTANT: Use the same EvaGreen dye calibration plate a third time.

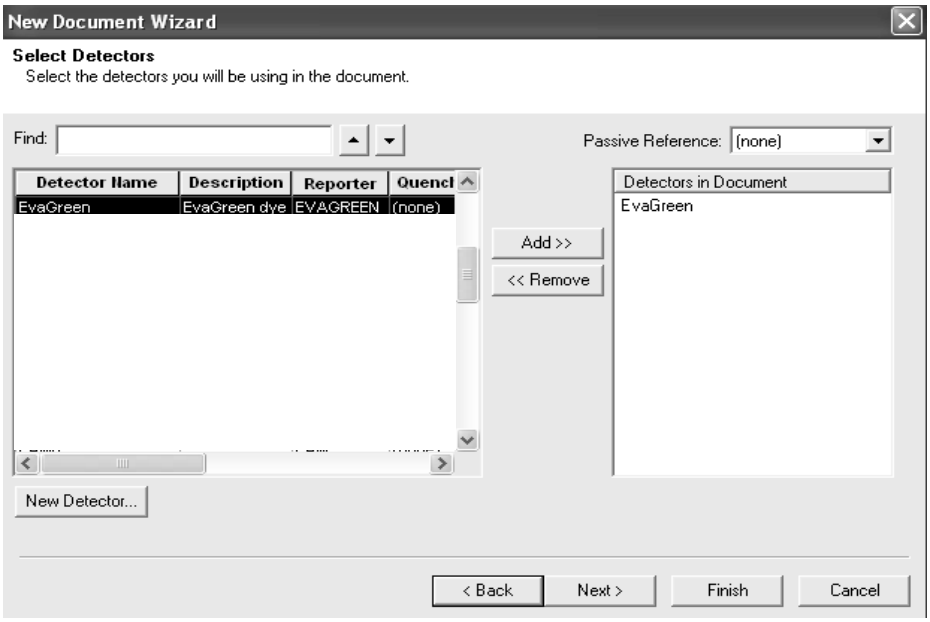
1. **Create a new file. From the "Assay" drop-down list, select "Standard Curve (Absolute Quantitation)". Leave the defaults in the remaining fields.**
2. **Click next.**



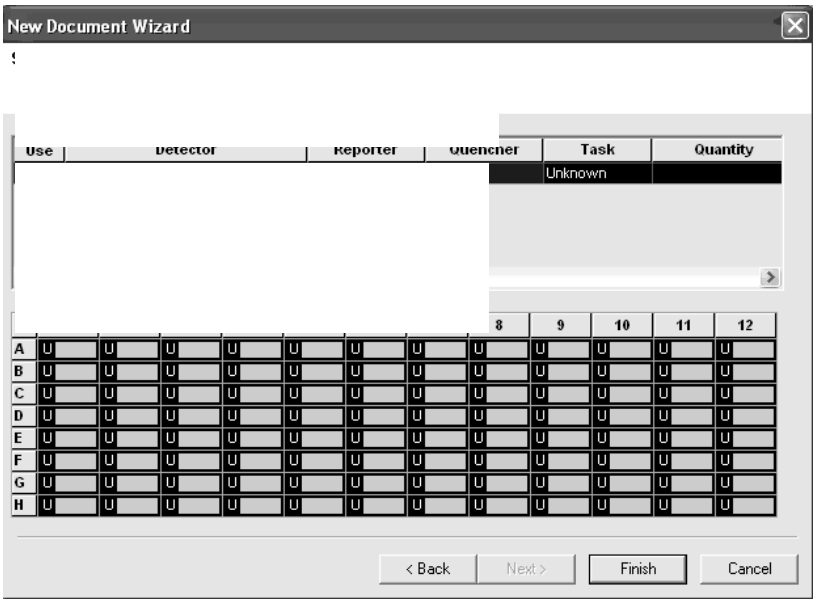
3. **Select "EvaGreen dye" as the detector. Click "New Detector". In the "New Detector" dialog box, enter the appropriate information for the EvaGreen dye being used for HRM analysis, and click "OK".**



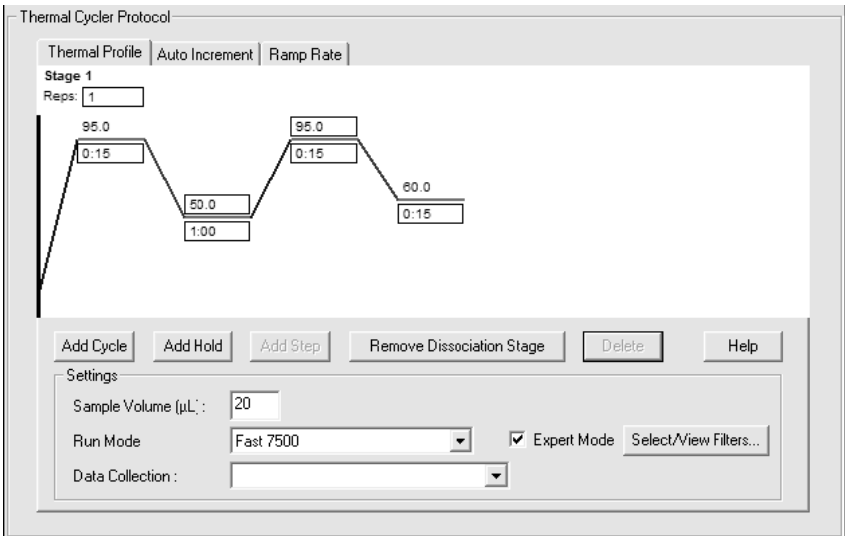
4. **Select "EvaGreen" dye from the table, and click "Add".**
5. **From the "Passive Reference" drop-down list, select "(none)".**
6. **Click "Next".**



7. **Set up the sample plate by selecting all wells in the plate grid and selecting "EvaGreen" dye as the detector.**
8. **Click "Finish".**

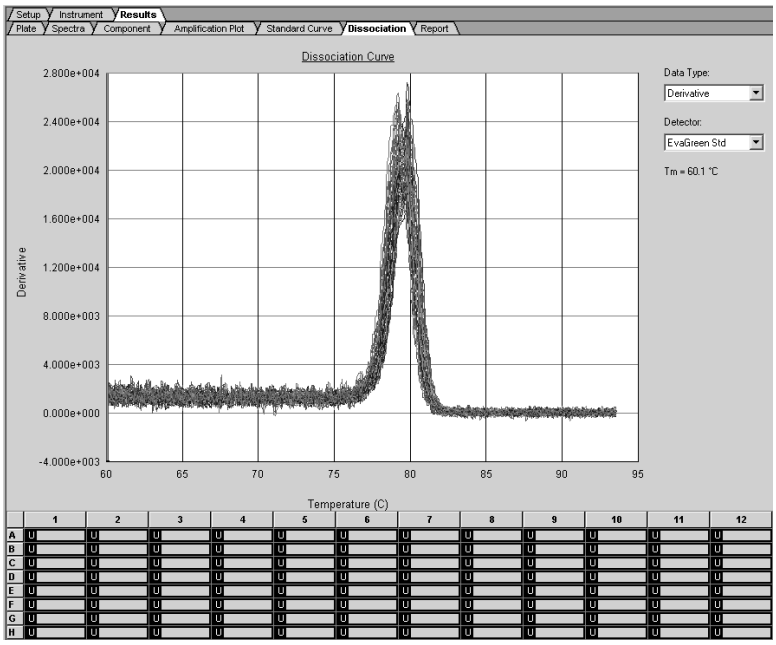


9. **Use the same EvaGreen calibration plate to perform the HRM calibration.**
10. **Centrifuge the HRM dye calibration plate, and then load the plate into the instrument.**
11. **Set the thermal cycler protocol as follows. Delete the default "Thermal Profile", and then add a dissociation stage (new Stage 1).**



12. **Check the "Expert Mode" box.**
13. **Click "Select/View Filters". In the "Filter Selection" dialog box, check only the "Filter A" box, and click "OK".**

14. **Click "Start". Save the file as "HRMCalibration_<dye name>_<today's date>".**
15. **When the run is complete, the "*Run completed successfully*" message appears.**
16. **Review the results.**
17. **Click  (Analyze).**
18. **Select the "Results" and "Dissociation" tabs and select all wells in the plate grid.**
19. **Verify that a single peak appears in the graph.**



When starting the HRM software for the first time, you will be prompted to open this file: (HRMCalibration_<dye name>_<today's date>).

Protocol 2: Genotyping protocol — analysis of gene mutations and microbial genetic differences by HRM

This section of the protocol must be followed when analyzing gene mutations or microbial genetic differences using the Type-it HRM PCR Kit and the Applied Biosystems 7500 Fast System. For more information, including protocols for use with the Rotor-Gene Q®, Rotor-Gene 6000, and LightCycler® 480 instruments, please refer to the *Type-it HRM PCR Handbook* at www.qiagen.com/Products/Type-itHRMPCRKit.aspx.

IMPORTANT: The initial calibration procedure (pages 2–13 of this supplementary protocol) must be performed to achieve successful results.

Important points before starting

- This optimized protocol must be followed to ensure successful results.
- Always use a primer concentration of 0.7 µM.
- No optimization of the Mg^{2+} concentration or the annealing temperature is required.
- Always start with the cycling conditions specified in this protocol.
- Optimal instrument and HRM analysis settings are a prerequisite for accurate genotyping results. For details, please refer to the manual provided with the Applied Biosystems 7500 Fast System.

Procedure

1. **Thaw 2x HRM PCR Master Mix, primer solutions, RNase-free water, template DNAs, and control DNAs (optional).**

   Note: It is important to mix the solutions completely before use to avoid localized concentrations of salt.

2. **Prepare a reaction mix according to Table 3.**

   Note: It is recommended to prepare a volume of reaction mix 10% greater than that required for the total number of reactions to be performed. Reaction setup can be done at room temperature (15–25°C). However, it is recommended to keep the individual reagents, samples, and controls on ice.

3. **Mix the master mix thoroughly and dispense appropriate volumes into the wells of a PCR plate.**

4. **Add equal amounts and volumes of template DNA (1–50 ng genomic DNA or 1–50 pg microbial DNA; same amount for each sample) to the individual wells and mix thoroughly.**

   Note: Add sufficient DNA so that all samples show C_T values below 30. Samples should not differ by more than three C_T values.

Table 3. Reaction components using 2x HRM PCR Master Mix for HRM analysis of mutations and microbial genetic variations

Component	Volume per 20 µl reaction*	Final concentration
Reaction mix		
2x HRM PCR Master Mix	10 µl	1x
10 µM primer mix†	1.4 µl	0.7 µM forward primer 0.7 µM reverse primer
RNase-free water	Variable	–
Template DNA (added at step 4)	Variable (equal volume for all reactions)	Eukaryotic: 1–50 ng DNA/reaction Microbial: 1–50 pg DNA/reaction (use equal amounts for each reaction)
Total volume per reaction	**20 µl***	**–**

* If your real-time cycler requires a final reaction volume other than 20 µl, adjust the amount of master mix and all other reaction components accordingly.

† A 10 µM primer mix consists of 10 µM forward primer and 10 µM reverse primer.

5. Program the real-time cycler according to Table 4.

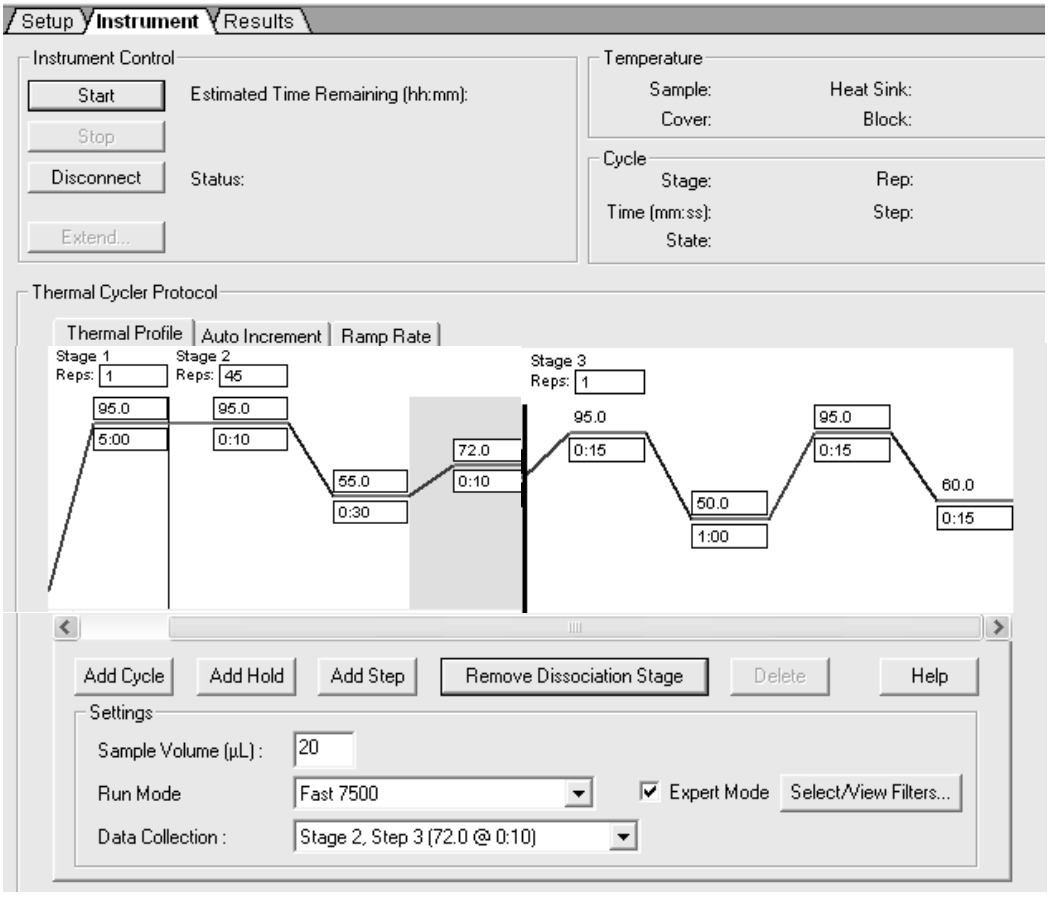
Note: Check the real-time cycler's user manual for correct instrument setup.

Table 4. Optimized cycling protocol for analyzing mutations and microbial genetic variations by HRM analysis on the Applied Biosystems 7500 Fast System

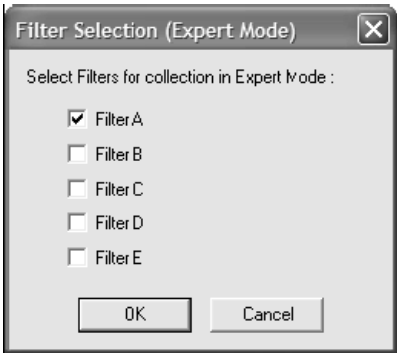
Step	Time	Temperature	Additional comments
			Important: Choose detection format: EvaGreen/HRM Dye
Initial PCR activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step
3-step cycling:			**Important: Optimal performance is only assured using these cycling conditions**
Denaturation	**10 s**	**95°C**	
Annealing	**30 s**	**55°C**	
Extension	**10 s**	**72°C**	Activate "single" fluorescence data acquisition Suitable for PCR products up to 350 bp. For PCR products >350 bp, use 1 s extension time per 25 bp of PCR product length.
Number of cycles	45		10–50 ng template DNA or 10–50 pg microbial DNA
	50		1–9 ng template DNA or 1–9 pg microbial DNA
HRM			**Analysis mode:** Melting curve
	15 s	95°C	
	1 min	50°C	
	15 s	95°C	
	15 s	60°C	

6. **Click the "Instrument" tab and change the preinstalled cycling protocol as described in the following steps.**

   Note: Add a dissociation stage to the end of the thermal profile (Stage 3), and then set the parameters as shown on page 17.



7. Click the "Select/View Filters" dialog field. In the "Filter Selection" box, check only "Filter A" and click "OK".



8. Place the PCR plate in the real-time cycler and start the PCR cycling program.

9. After the run, perform HRM analysis using the HRM software.

Note: See the HRM software manual for details.

Protocol 3: Genotyping protocol — analysis of SNPs by HRM

This section of the protocol must be followed when analyzing SNPs using the Type-it HRM PCR Kit and the Applied Biosystems 7500 Fast System. For more information, including protocols for use with Rotor-Gene Q, Rotor-Gene 6000, and LightCycler 480 instruments, please refer to the *Type-it HRM PCR Handbook* at www.qiagen.com/Products/Type-itHRMPCRKit.aspx.

IMPORTANT: The initial calibration procedure (page 2–13 of this supplementary protocol) must be performed to achieve successful results.

Important points before starting

- This optimized protocol must be followed to ensure successful results.
- Always use a primer concentration of 0.7 µM.
- No optimization of the Mg^{2+} concentration or the annealing temperature is required.
- Always start with the cycling conditions specified in this protocol.
- Optimal instrument and HRM analysis settings are a prerequisite for accurate genotyping results. For details, please refer to the manual provided with the Applied Biosystems 7500 Fast System.

Procedure

1. **Thaw 2x Type-it HRM PCR Master Mix, primer solutions, RNase-free water, template DNAs, and control DNAs (optional).**

   Note: It is important to mix the solutions completely before use to avoid localized concentrations of salt.

2. **Prepare a reaction mix according to Table 5.**

   Note: It is recommended to prepare a volume of reaction mix 10% greater than that required for the total number of reactions to be performed.

   Note: Reaction setup can be performed at room temperature (15–25°C). However, it is recommended to keep the individual reagents, samples, and controls on ice.

3. **Mix the reaction mix thoroughly and dispense appropriate volumes into the wells of a PCR plate.**

4. **Add equal amounts and volumes of template DNA (1–50 ng genomic DNA; same amount for each sample) to the individual wells and mix thoroughly.**

   Note: Add sufficient DNA so that all samples show C_T values below 30. Samples should not differ by more than three C_T values.

Table 5. Reaction components using 2x HRM PCR Master Mix for HRM analysis of SNPs

Component	Volume per 20 µl reaction*	Final concentration
Reaction mix		
2x HRM PCR Master Mix	10 µl	1x
10 µM primer mix†	1.4 µl	0.7 µM forward primer 0.7 µM reverse primer
RNase-free water	Variable	–
Template DNA (added at step 4)	Variable (equal volume for all reactions)	Eukaryotic: 1–50 ng DNA/reaction Microbial: 1–50 pg DNA/reaction (use equal amounts for each reaction)
Total volume per reaction	**20 µl***	**–**

* If your real-time cycler requires a final reaction volume other than 20 µl, adjust the amount of master mix and all other reaction components accordingly.

† A 10 µM primer mix consists of 10 µM forward primer and 10 µM reverse primer.

5. Program the real-time cycler according to Table 6.

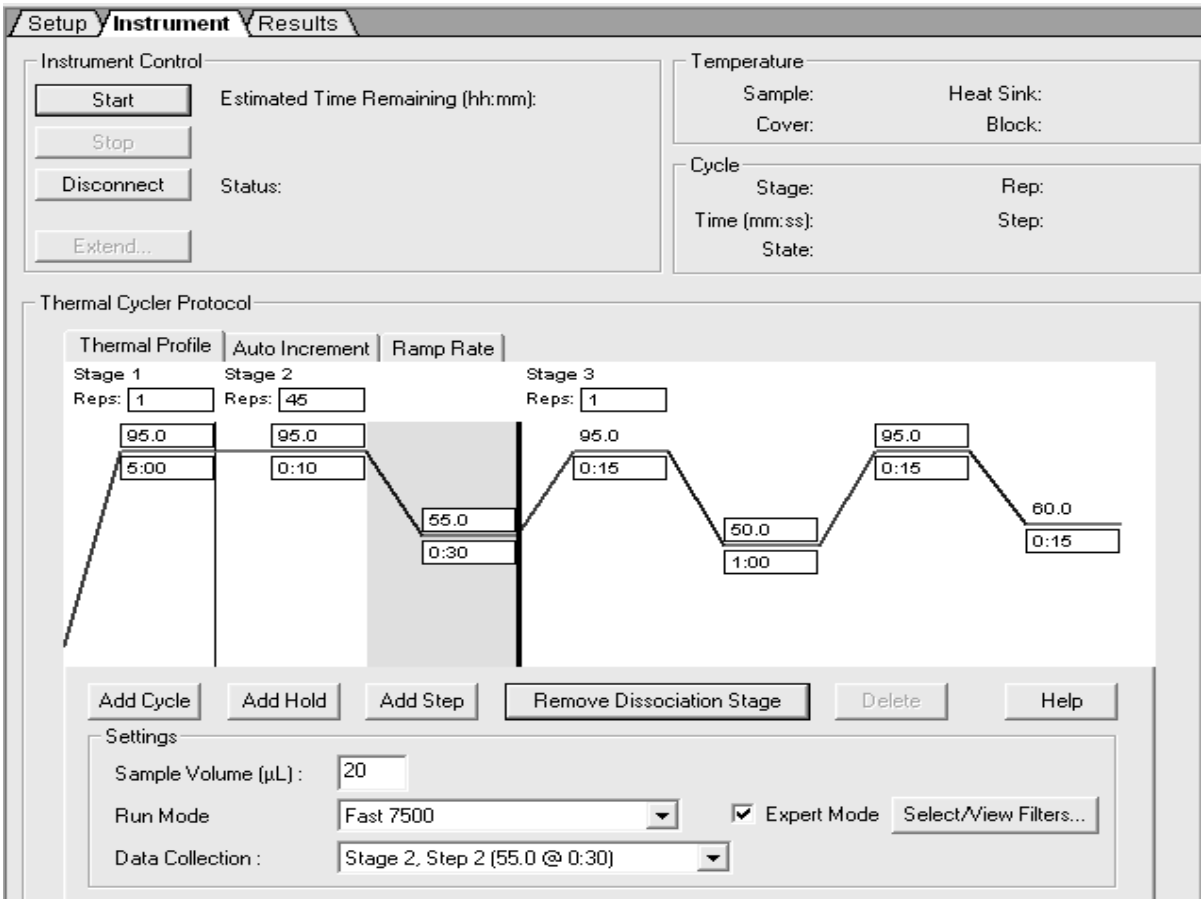
Note: Check the real-time cycler's user manual for correct instrument setup.

Table 6. Optimized cycling protocol for analyzing SNPs by HRM analysis on the Applied Biosystems 7500 Fast System

Step	Time	Temperature	Additional comments
			Important: Choose detection format: EvaGreen/HRM Dye
Initial PCR activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step
2-step cycling:			**Important: Optimal performance is only assured using these cycling conditions**
Denaturation	**10 s**	**95°C**	
Annealing/Extension	**30 s**	**55°C**	Activate "single" fluorescence data acquisition. Suitable for PCR products up to 200 bp. For PCR products >200 bp, use additional 1 s extension time per 25 bp of PCR product length.
Number of cycles	45		10–50 ng template DNA or 10–50 pg microbial DNA
	50		1–9 ng template DNA or 1–9 pg microbial DNA
HRM			Analysis mode: Melting curve
	15 s	95°C	
	1 min	50°C	
	15 s	95°C	
	15 s	60°C	

6. **Click the "Instrument" tab and change the preinstalled cycling protocol as described in the following steps.**

   Note: Add a dissociation stage to the end of the thermal profile (Stage 3), and then set the parameters as shown on page 21.



7. Click the "Select/View Filters" dialog field. In the "Filter Selection" dialog box, check only "Filter A" and click "OK".

8. Place the PCR plate in the real-time cycler and start the PCR cycling program.

9. After the run, perform HRM analysis using the HRM software.

Note: See the HRM software manual for details.

QIAGEN handbooks can be requested from QIAGEN Technical Service or your local QIAGEN distributor. Selected handbooks can be downloaded from www.qiagen.com/literature.

Material safety data sheets (MSDS) for any QIAGEN product can be downloaded from www.qiagen.com/Support/MSDS.aspx.

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