

## V. Instrument Setup and Thermal Cycling for Genotyping (SNP) Assays

These instructions describe instrument setup and thermal cycling conditions for genotyping assays using the Plexor™ qPCR System. The cycling program will include one cycle with an annealing temperature of 50°C, followed by 40 cycles with an annealing temperature of 60°C. The first round of PCR is performed at the lower annealing temperature; additional rounds of PCR are performed at the higher annealing temperature to increase the specificity of amplification. This cycling program is specific for genotyping primers designed using the Plexor™ Primer Design Software, which can be accessed at: [www.promega.com/plexorresources/](http://www.promega.com/plexorresources/). Instructions for data export from the ABI PRISM® 7700 Sequence Detection Software into the Plexor™ Analysis Software are provided in Section VI.

### V.A. General Setup

! The real-time instrument must be calibrated for the dyes used. A list of compatible dyes is available at: [www.promega.com/plexorresources/](http://www.promega.com/plexorresources/)

1. Open the 7700 Sequence Detection Software.
2. Ensure that “single-reporter, standard plate” is selected.
3. Set instrument exposure time and turn off the ROX Reference standard:
  - a. Select “Show Analysis”.
  - b. Under the Instrument menu, select “Diagnostics” then “Advanced Options” (Figure 9).
  - c. Select the box next to “Set 7700 Exposure Time for Plates” and change the setting to 10.
  - d. Change the reference to “None” and uncheck the box next to “Reference”.

**Note:** This is critical for proper data analysis. Crosstalk or bleed-through of emission from one dye may affect the signal in a second dye.

- e. Select “OK”

! A message will appear telling you to close and restart the program. **Do not close and restart the program** as this will result in loss of these settings. It is important to confirm that the ROX reference standard is turned off each time you start the instrument.

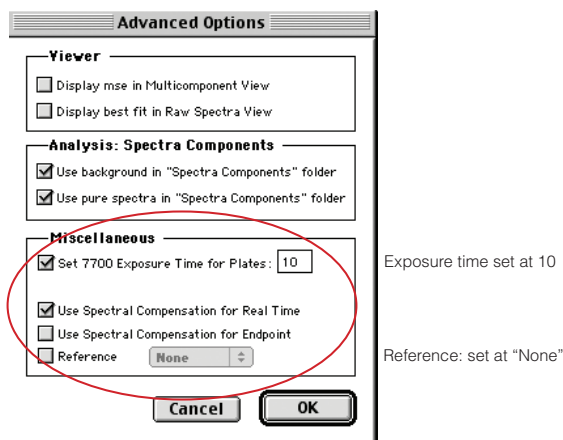


Figure 9. Setting the instrument exposure time and turning off the ROX reference standard.

4. Set up the plate.
  - a. Select "Show Setup".
  - b. Select the dye layer to be used in the Plexor™ assay.
  - c. Select the sample wells to be used then select "Unknown" as the sample type (Figure 10).
 

**Note:** Do not assign sample names or other sample types. The sequence detection software will not export this information. Sample names can be entered in the Plexor™ Analysis Software or copied and pasted into the Plexor™ Analysis Software from a Microsoft® Excel spreadsheet.
  
5. For multiplex assays, set up the additional dye layers. Select the second dye to be used in the Plexor™ assay.
  - a. To add the sample type "unknown" for the second dye, highlight the Sample Type and select "Sample Type Setup" (Figure 11).
  - b. Uncheck the box next to "Quencher".
  - c. Select "Add".
  - d. Type in an acronym, such as UNKN, and use "Unknown" for the name. Select a color (optional).
  - e. Select the appropriated dye in the "Reporter" box.
  - f. Select "OK".



Figure 10. Setup screen.

**Sample Type Setup**

Acronym	Name	Color	Reporter
IPC+	Internal Positive	Yellow	JOE
IPC-	Internal Positive	Blue	JOE
TARG	RelIQ Target	Orange	FAM
ENDO	RelIQ Endogenous	Brown	JOE
STND	Standard	Pink	FAM
UNKN	Unknown	Blue	FAM
NTC	No Template Control	Purple	FAM

**Reference**  
None

**Quencher**  
None

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Figure 11. Example of a new Unknown sample type for the JOE dye layer.

6. Select the sample wells to be used then select "Unknown" as the sample type.

**Note:** Do not assign sample names or other sample types. The sequence detection software will not export this information. Names can be entered in the Plexor™ Analysis Software or copied and pasted into the Plexor™ Analysis Software from a Microsoft® Excel spreadsheet.

## V.B. Thermal Cycling Program

The thermal cycling program is shown in Table 3. This program is specific for genotyping primers designed using the Plexor™ Primer Design software. Each of the genotyping primers includes bases at the 5' end that are not complementary to the target sequence. The annealing temperature for the first round of amplification is 50°C to allow the primer to anneal and be extended. Subsequent rounds are performed using an annealing temperature of 60°C, which is the melting temperature ( $T_m$ ) of the primer pair including the noncomplementary bases. During subsequent rounds of amplification, performed at the higher annealing temperature, the target formed in the first round is amplified. Figure 12 shows the final thermal cycling program.


**Table 3. Thermal Cycling Profile for Genotyping Assays.**

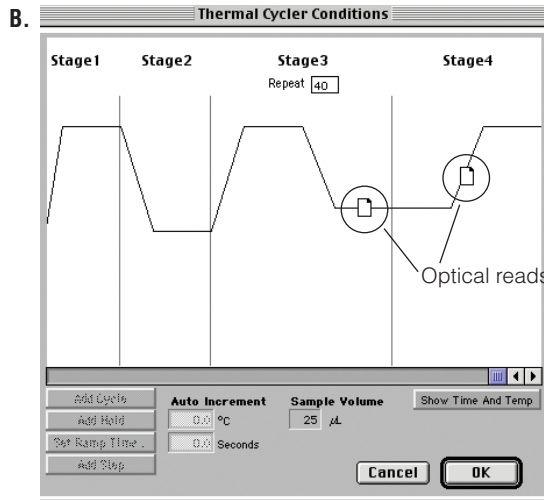
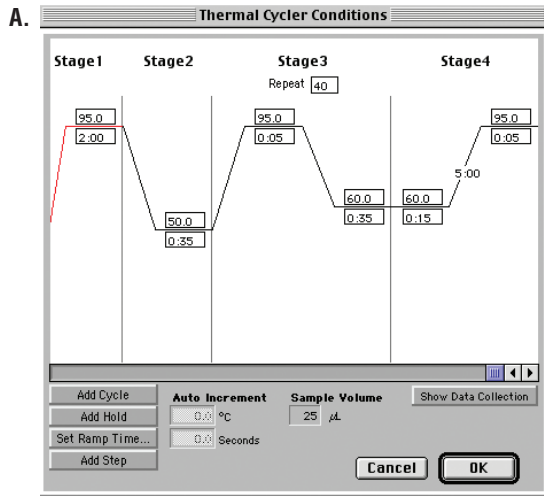
Step	Temperature	Time	Number of Cycles
Initial denaturation	95°C	2 minutes	1 cycle
Annealing and extension:	50°C	35 seconds	1 cycle
Denaturation:	95°C	5 seconds	40 cycles
Annealing and extension:	60°C	35 seconds	
Melt temperature curve:	60°C	15 seconds	1 cycle
	Ramp: 5 minutes		
	95°C	5 seconds	

1. Select “Thermal Cycler Conditions”.
2. Change the sample volume to 25µl.
3. Change Stage 1, to 95.0 for 2:00.
4. Change Stage 2, Step 1 to 50°C for 00:35.
5. Ensure that the repeat number is 1.
6. Change Stage 3, Step 1 to 95.0 for 00:05.
7. Change Stage 3, Step 2 to 60°C for 00:35.
8. Ensure that the repeat number is 40.
9. Select “Add Hold” to add a new stage. Change Stage 4, Step 1 to 60.0 for 00:15.
10. Select “Add Step” to add a new step. Change Stage 4, Step 2 to 95.0 for 00:05.
11. Select “Set Ramp Time” and change the ramp to 5 minutes.
12. Select “Show Data Collection”. Optical reads should be performed during the 60°C extension step in Stage 3 and during the melt curve ramp. Select an optical read icon to delete it from a data collections step, or select the empty step or melt curve ramp to add an optical read.
13. Select “OK”.
14. Choose “File”, then “Save”.

**Note:** The instrument setup and thermal cycling program can be saved as a template for future use. To do so, choose “File”, then “Save”. Select the template icon, and then save the file. This template can be opened by selecting “File”, then “Open plate” and used for future assays.



15. Select “Show Analysis”.
  16. Place the PCR plate into the instrument and immediately begin thermal cycling by selecting “RUN”.
-  Prolonged exposure of the reactions to high temperatures before thermal cycling may adversely affect the final results.



**Figure 12. Thermal cycling program for genotyping assays.**  
**A.** Times and temperatures. **B.** Data collection.

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