

IV. Instrument Setup and Thermal Cycling for One-Step qRT-PCR

These instructions describe instrument setup and thermal cycling conditions for cDNA quantitation using the Plexor™ One-Step qRT-PCR System. The thermal cycling program includes the initial incubation for the reverse transcription. Thermal cycling programs described in this manual are optimized to work with primers designed using the Plexor™ Primer Design Software, which can be accessed at: 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.

IV.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/

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 5).
 - 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.** Closing the program 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.

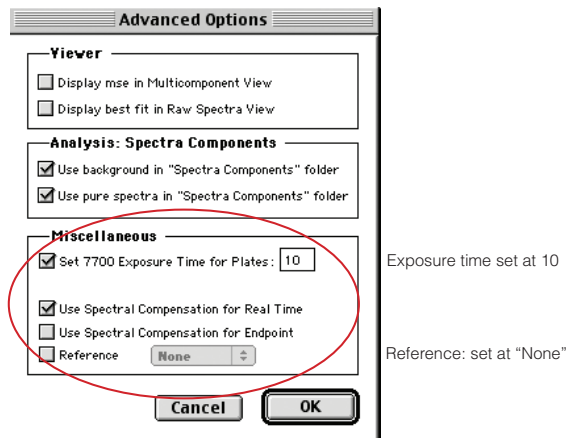


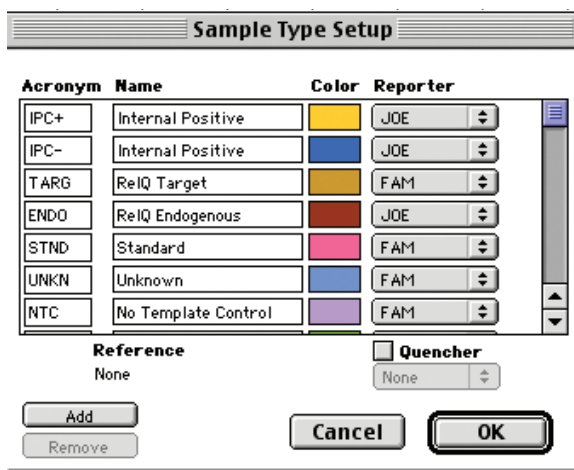
Figure 5. Setting the instrument exposure time and turning off the ROX reference standard.

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 6).
- 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 7).
 - 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”.
 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.



Figure 6. Setup screen.



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

Buttons: Add, Remove, Cancel, OK

Figure 7. Example of a new “Unknown” sample type for the JOE dye layer.

IV.B. Thermal Cycling Program

The one-step qRT-PCR thermal cycling program is shown in Table 2. Primers designed using the Plexor™ Primer Design Software have an annealing temperature of approximately 60°C. Figure 8 shows the final thermal cycling program.

Table 2. One-Step qRT-PCR Thermal Cycling Program.


Step	Temperature	Time	Number of Cycles
Reverse transcription reaction ¹	45°C	5 minutes	1 cycle
Initial denaturation and inactivation of the ImProm-II™ Reverse Transcriptase	95°C	2 minutes	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	

¹The length of incubation for the reverse transcription reaction can be increased to up to 30 minutes. Longer incubation times can lead to increased sensitivity but also higher background.

1. Select "Thermal Cycler Conditions".
2. Change the sample volume to 25 μ l.
3. Change Stage 1 to 45.0 for 5:00.
4. Change Stage 2 to 95.0 for 2:00.
5. Change Stage 3, Step 1 to 95.0 for 00:05.
6. Change Step 2 to 60°C for 00:35.
7. Ensure that the repeat number is 40.
8. Select "Add Hold" to add a new stage. Change Stage 4, Step 1 to 60.0 for 00:15.
9. Select "Add Step" to add a new step. Change the Stage 4, Step 2 to 95.0 for 00:05.
10. Select "Set Ramp Time" and change the ramp to 5 minutes.
11. 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.
12. Select "OK".
13. 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.



14. Select "Show Analysis".
 15. 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.

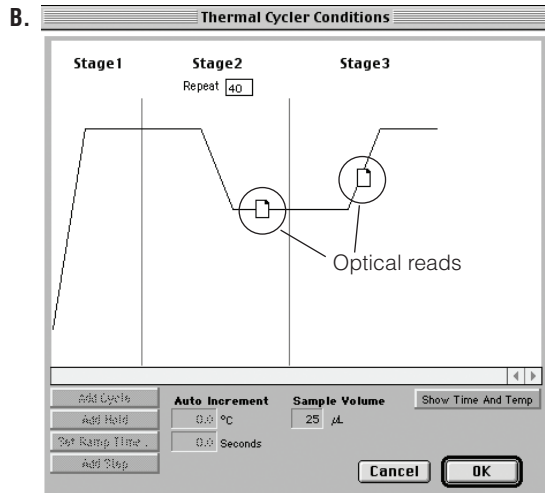
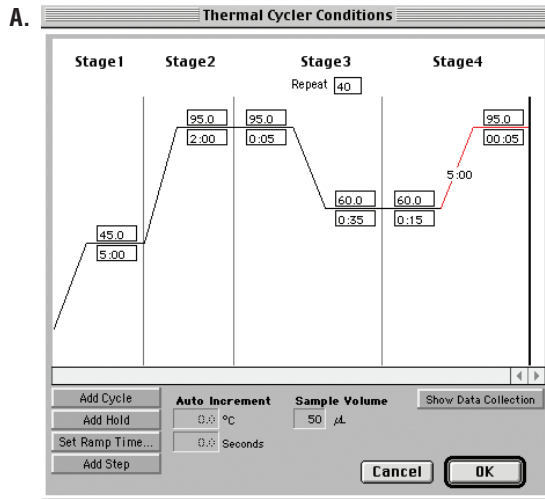


Figure 8. One-step qRT-PCR thermal cycling program.
 A. Times and temperatures. B. Data collection.