

Guidelines for the “Advance to Next Stage” feature on the SmartCycler[®] II System

Cepheid Technical Support

Overview

The SmartCycler Advance to Next Stage (ANS) feature allows you to set up a protocol that will automatically advance to the next protocol stage after a threshold crossing occurs in a designated channel. This feature can be used to increase instrument throughput or stop reactions after a threshold crossing by advancing to an unused stage. The ANS feature can decrease time to result and potentially reduce the formation of non-specific products by advancing to a melt curve stage. This feature allows you to increase your multiplexing capabilities when used in combination with temperature-controlled primer-limiting applications by advancing to another thermal cycling stage.

This SmartNote covers the following topics:

- Using the SmartCycler Advance to Next Stage feature
- General guidelines for using the Advance to Next Stage feature
- Advancing to an Unused Stage
- Advancing to a Melt Curve Stage
- Advancing to another Thermal Cycling Stage

General guidelines for using the Advance to Next Stage feature

Always completely optimize the analysis settings, especially the threshold setting and curve analysis, before using the Advance to Next Stage feature. If the threshold is set too low, the reaction might cross the threshold before the log linear phase of amplification. Likewise, if the threshold is set too high, the reaction might cross the threshold too late.

- Specify an optimum number of cycles to wait after the threshold crossing. The valid range is between 2 and 9 cycles.
- When advancing to an Unused, Melt Curve or Temperature Cycle stage, evaluate several protocols that use a different number of cycles to wait after the threshold crossing. The longer you wait after the threshold crossing, the more you

will be able to evaluate amplification efficiency and curve shape.

- When advancing to a Melt Curve stage, try several protocols that use a different number of cycles to wait after the threshold crossing to determine if enough amplified product has been made to produce a large enough melt peak. In general, it is best if the melt peak is at least 10 fluorescence units in height. If you advance to a Melt Curve stage too soon, you may not have enough amplified product to produce an adequate size melt peak.
- If you are using the 2nd Derivative Curve Analysis, always wait at least two cycles after the threshold crossing before advancing to the next stage to allow for the calculation of the C_t .
- If the threshold crossing occurs at the end of the thermal cycling stage, the software will add the

number of cycles you specified as a wait period to the total number of cycles. For example, if the Temperature Cycle stage is set to repeat for 45 cycles and the threshold crossing occurs at cycle 44, the software will continue to cycle past 45 cycles. If you specified five cycles for the wait period, the software would not advance to the next stage until cycle 50.

Using the SmartCycler Advance to Next Stage feature

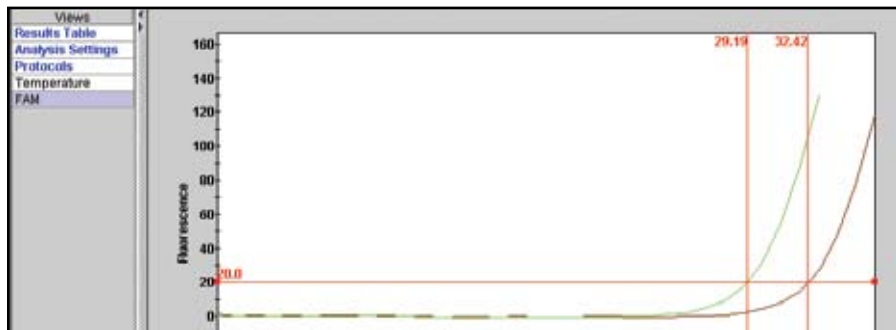
General steps for using Advance to Next Stage are as follows (see figure 1):
See the SmartCycler System Operator Manual for specific instructions on how to define a protocol. Define a new thermal cycling protocol or edit an existing protocol.

1. Check the box next to **Advance to Next Stage** at the bottom of a protocol Stage area.
2. Enter the number of cycles to wait before automatically advancing to the next stage. The valid range is 0 to 9 cycles.
3. Enter the emission channel to monitor.
4. In the next Temperature Cycle stage, select one of the following:
 - Unused** — Stops a reaction after the threshold crossing.
 - Melt Curve** — Discriminates between specific and non-specific products if you are using an intercalating dye, and melts molecular beacons probes and Scorpion™ probes.
 - 2, 3, 4, or 5- Temperature Cycle** — Uses the temperature-controlled primer-limiting application or different temperature cycles if you are multiplexing more than four targets.
5. Save the new protocol.

Figure 1: An example protocol for advance to an Unused stage.



Figure 2: An example protocol for advance to an Unused stage.



Advancing to an Unused Stage

Advancing to an Unused stage allows you to increase instrument throughput and decrease the time to result by automatically stopping a reaction after a threshold crossing occurs in a designated emission channel. The software will monitor the designated channel for a threshold crossing, wait for a specified number of cycles and then advance to the Unused stage. After the protocol advances to the Unused stage, the site will stop and will be available to start a new reaction without having to wait for the entire run to finish. This can increase your lab efficiency and instrument throughput by allowing you to run more samples in a single day and making it easier for multiple researchers to use the instrument at the same time.

To define a protocol to advance to an Unused stage:

1. Open the SmartCycler® Software.
2. In the SmartCycler window, click **Define Protocols**. The **Define Protocols** window appears.
3. Click **New Protocol**.

4. Enter the temperature and time for Stage 1 (typically a Hold stage).
5. Enter the temperature and time for Stage 2 (typically a Temperature Cycle stage)
6. Check the box next to **Advance to Next Stage** at the bottom of the Stage 2 area.
7. Enter the number of cycles (0–9) to wait before automatically advancing to the next stage. If you are using the 2nd derivative curve analysis, it is important to wait at least 2 cycles after the threshold crossing before advancing to the next stage.
8. Enter the emission channel to monitor for a threshold crossing. Refer to the SmartCycler Operator Manual for a table of supported dyes and their corresponding emission channels.
9. Select **Unused** (default) in the next protocol stage.
10. Click **Save Protocol**.

The example in Figure 3 shows two reactions that stopped three cycles after the threshold crossing. After a reaction crosses the threshold and advances to an Unused stage, it will stop and the reaction site can be used to start another reaction immediately.

Figure 3: Typical results for reactions that used the advance to an unused stage feature.

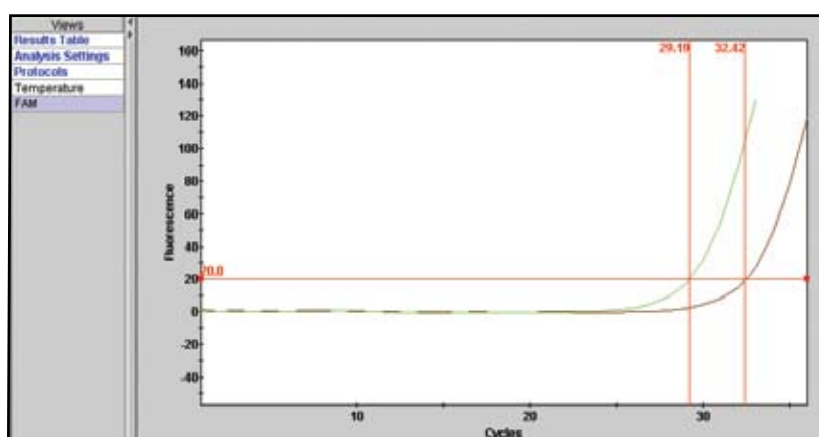


Figure 4: An example protocol for advance to a Melt Curve stage.

Stage 1			Stage 2				Stage 3			
Hold			Repeat 45 times.				Melt Curve			
Temp	Secs	Optics	2-Temperature Cycle				Start	End	Optics	Deg/Sec
95.0	150	Off	Deg/Sec	Temp	Secs	Optics	60.0	95.0	Ch1	0.2
			NA	95.0	15	Off				
			NA	60.0	30	On				
			<input checked="" type="checkbox"/> Advance to Next Stage							
			3 Cycles after Ch 1 Ct.							

Advancing to a Melt Curve Stage

Advancing to a Melt Curve allows you to automatically advance to a Melt Curve stage before the reaction has finished which can decrease your time to result and potentially limit or reduce the amplification of non-specific products and primer-dimers that can occur at the end of a PCR reaction. It is imperative that the assay design and thermal cycling conditions are optimized first and that you use the shortest annealing/extension times possible. After the assay is completely optimized, you can try to further reduce the occurrence of these non-specific products by advancing to a Melt Curve stage between 0 and 9 cycles after the threshold crossing. At this point in the reaction, it is less likely that many non-specific products have been amplified. In addition, by advancing to a Melt Curve stage after the threshold crossing, you can take advantage of reduced cycling time by using available sites to start new reactions.

To define a protocol to advance to a Melt Curve stage:

1. Open the SmartCycler Software.
2. In the SmartCycler window, click **Define Protocols**. The **Define Protocols** window appears.
3. Click **New Protocol**.
4. Enter the temperature and time for Stage 1 (typically a Hold stage).
5. Enter the temperature and time for Stage 2 (typically a Temperature Cycle stage).

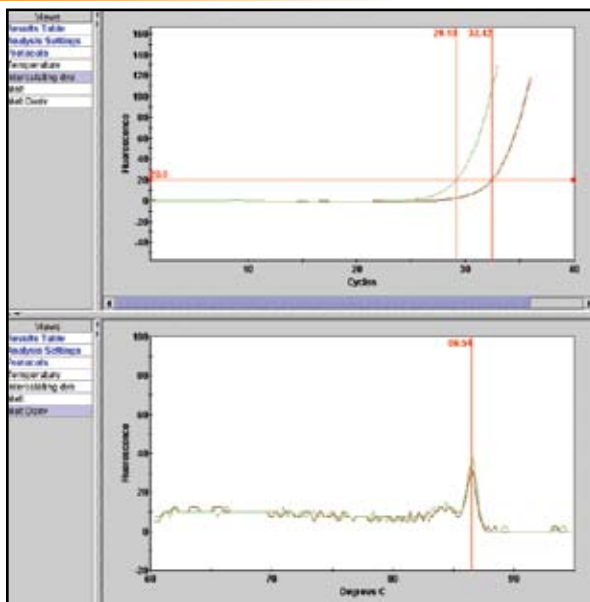
6. Check the box next to **Advance to Next Stage** at the bottom of the Stage 2 area.
7. Enter the number of cycles to wait before automatically advancing to the next stage.

Note: When advancing to a Melt Curve stage, try several protocols that use a different number of cycles to wait after the threshold crossing to determine if enough amplified product has been made to produce a large enough melt peak. In general, it is best if the melt peak is at least 10 fluorescence units in height. If you advance to a Melt Curve stage too soon, you may not have enough amplified product to produce an adequate size melt peak.

8. Enter the emission channel to monitor for a threshold crossing. Most intercalating dyes will be detected in channel 1. Refer to the SmartCycler Operator Manual for a table of supported dyes and their corresponding emission channels.
9. Select **Melt Curve** for Stage 3. Enter the start and end temperature, emission channel and the melt speed.
10. Click **Save Protocol**

The reactions displayed in Figure 5 stopped three cycles after the threshold crossing and advanced to a melt curve. This example clearly shows that there is a single melt peak at 86.54 degrees for both reactions.

Figure 5: Example results for two reactions that advanced to a melt curve three cycles after the threshold crossing.



Advancing to another Thermal Cycling Stage

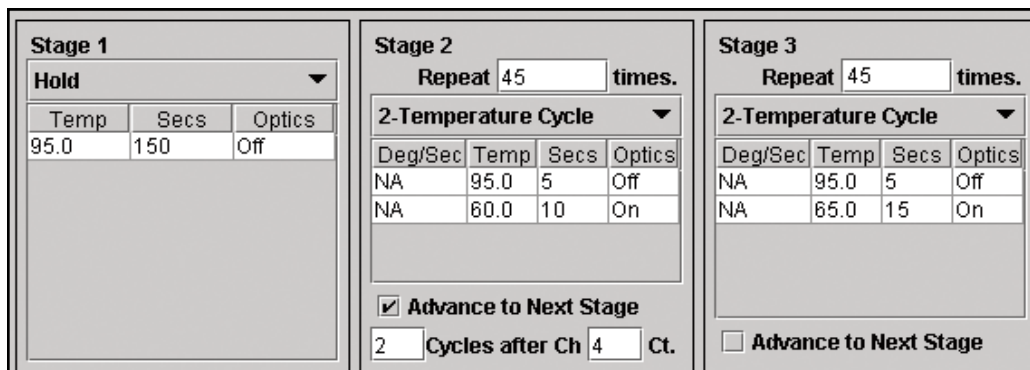
It can be difficult to multiplex targets that vary greatly in copy number because the more abundant target will generally dominate the reaction and impede the amplification of the lower copy target. However, it is possible to use temperature to control primer-binding activity in order to detect a highly abundant target (such as an endogenous control)

and a lower copy target in a single reaction without

compromising dynamic range and sensitivity. Automatically advancing from one cycling stage to another after a threshold crossing allows you to take advantage of the temperature-controlled primer-limiting application to multiplex a wider range of targets and to detect more than four targets in a single reaction. This is achieved by designing the primers and probe for the highly abundant target to anneal at a lower temperature than the primers and probes for the lower copy targets. The assay is initially run using the lower annealing temperature so that the abundant target is amplified and crosses the threshold. After the abundant target crosses the threshold, the protocol will advance to another thermal cycling stage that has a higher and more stringent annealing temperature. The change in temperature will stop the amplification of the abundant target and allow the amplification of the lower copy targets without competition from the abundant target. It is important that the abundant target uses a lower annealing temperature because lower annealing temperatures are not as stringent. Otherwise, if the low copy targets use the lower annealing temperature it may be possible for the abundant target to amplify at this temperature also and suppress the amplification of the low copy targets.

The same principle applies if you want to multiplex more than four targets in a single reaction. The SmartCycler System uses four independent LED and

Figure 6: An example protocol for advance to another thermal cycling stage.



filter combinations for excitation and detection so in order to multiplex five targets you need to use one reporter dye for two different targets. In addition, you also need to design the multiplex reaction to use the temperature-controlled primer-limiting application. Table 1 shows an example of a five target reaction that uses the FAM reporter dye for two different targets. Note, four of the target reactions are designed to anneal at 65 degrees and the internal control was designed to anneal at 60 degrees.

Target	Reporter Dye	Annealing Temperature
Target 1	FAM	65°C
Target 2	Alexa 546	65°C
Target 3	Texas Red	65°C
Target 4	Alexa 647	65°C
Internal Control	FAM	60°C

Please refer to the Guidelines for Temperature-controlled primer-limiting Applications, SmartNote 6.6 for a more detailed description and instructions on how to design an assay with the temperature-controlled primer-limiting application and multiplexing with more than four targets.

To define a protocol to advance to another thermal cycling stage:

1. Open the SmartCycler Software.
2. In the SmartCycler window, click **Define Protocols**. The **Define Protocols** window appears.
3. Click **New Protocol**.
4. Enter the temperature and time for Stage 1 (typically a Hold stage).
5. Enter the temperature and time for Stage 2 (typically a Temperature Cycle stage).
6. Check the box next to **Advance to Next Stage** at the bottom of the Stage 2 area.
7. Enter the number of cycles to wait before automatically advancing to the next stage.
8. Enter the emission channel to monitor for a threshold crossing. Refer to the SmartCycler Operator Manual for a table of supported dyes and their corresponding emission channels. Select **2-Temperature Cycle** or **3-Temperature Cycle** for Stage 3.
9. Enter the thermal cycling protocol with the higher annealing temperature.
10. Click **Save Protocol**.

Figure 7: Example results for a reaction that used advanced to next stage in combination with temperature-controlled primer-limiting.

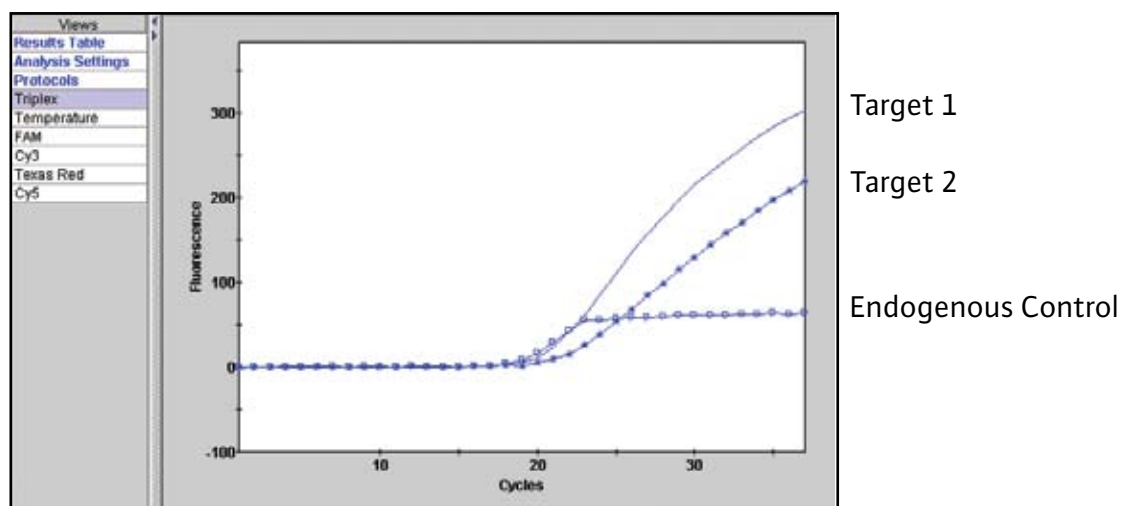


Figure 7 is an example of a reaction that used the Advance to Next Stage feature in combination with temperature-controlled primer-limiting. In figure 6, the Advance to Next Stage feature was used to quench a highly abundant endogenous control (EC) to allow the amplification of two lower copy targets. Target 1 and Target 2 ($T_m = 70^\circ\text{C}$) were designed to amplify at a higher temperature than

the endogenous control ($T_m = 60^\circ\text{C}$) so that the EC reaction would be quenched after the protocol advanced to the next Temperature Cycle stage. After the EC crossed the threshold and the protocol advanced to the next stage, the EC reaction was quenched and the target reactions were able to amplify.

References

- Raja, S., El-Hefnawy, T., Kelly, L., Chestney, M., Luketich, J., and Godfrey, T. 2002. *Clinical Chemistry* 48:8 1329–1337. Temperature-controlled Primer Limit for Multiplexing of Rapid, Quantitative Reverse Transcription-PCR Assays: Application to Intraoperative Cancer Diagnostics
- Kozma, L. 2004. Sentinel Lymph Node Detection of Breast Cancer Presentation
- Kozma, L., Xi, L., Swenson, D., Peralta, J., Ly, C., Rodgers, R., McMillan, B., Godfrey, T., 2005. A Dual Termination Strategy Increases the Linear Dynamic Range of a Multiplex RT-PCR Assay. AACC.

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