Protocols and Tips for Success with the EvaEZ™ Fluorometric Polymerase Activity Assay Kit

EvaEZ™ is a fluorescence-based assay that allows you to quantitate DNA polymerase activity without using radioisotopes. While the assay is simple to use, there are many factors that can affect the quality of your data. In this article, we've outlined our advice for getting the best results using our EvaEZ™ Assay with your enzyme.

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Introduction

The EvaEZ™ Assay is a ready-to-use assay mix that uses Biotium's EvaGreen® Dye to detect the formation of dsDNA when a DNA polymerase catalyzes the extension of the primed substrate in the reaction mix (see Figure 1). The rate of increase of fluorescence is positively correlated to the activity of polymerase. In this tech tip, we show results obtained using Taq polymerase, but the assay can be used with other DNA polymerases such as *Pfu*, Vent, Phusion®, *Bst*, phi29, MMLV, AMV, SuperScript®, T4 DNA polymerase, T7 DNA polymerase, Klenow, and *E. coli* DNA polymerase I.

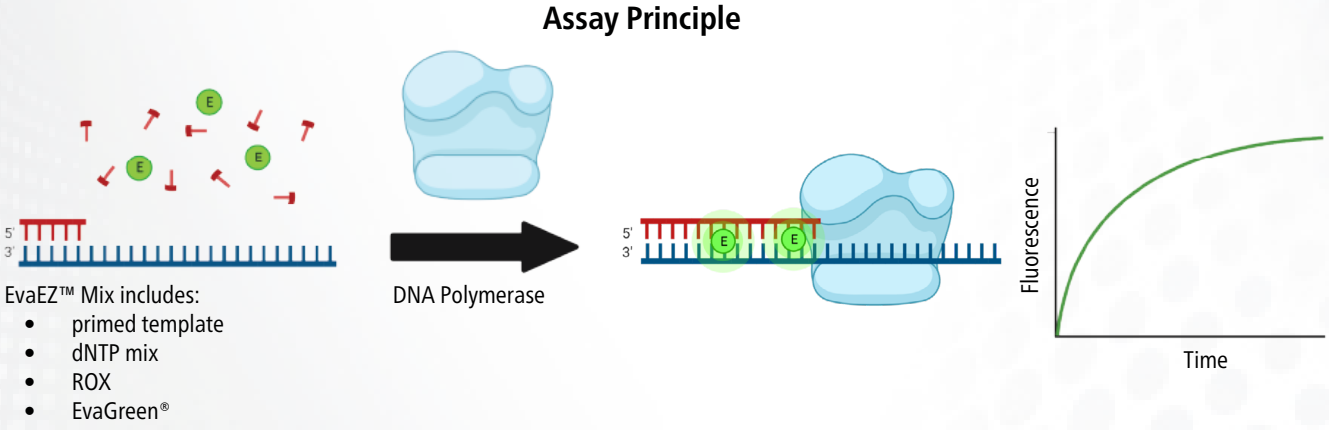

Figure 1. Schematic showing the principle behind the EvaEZ™ Fluorometric Polymerase Activity Assay. The assay mix contains all reaction components, you only need to add your enzyme.

Technical Tips

1. Tips for setting up your instrument

a. We recommend using a real-time qPCR instrument for performing the EvaEZ™ Assay.

- If you have a choice between using plates and tubes, using a plate can help reduce variability because it allows you to use multichannel pipettors for assay setup, and to quickly load your samples into the instrument to better control the assay temperature.
- If it is an option for your instrument, using ROX normalization can reduce variability in the assay. See Tip 1e.

b. Alternatively, you may be able to use a fluorescence microplate reader for the assay. Some customers have reported better reproducibility between replicates using a microplate format compared to single-tube qPCR. A few variables and properties of the plate reader need to be considered:

- Make sure the plate reader can read green fluorescence (e.g., FITC channel, Ex/Em: 485/530 nm) and can heat to the required temperature for your enzyme.
- Use plate seals to avoid evaporation.
- Be aware that plate readers may provide less uniform temperature between the outer and inner wells of the plate, and on the top or lid compared to a PCR instrument. Differences in reaction temperature will become more pronounced with increased reaction volumes.

c. If your instrument takes time to heat up to temperature, we recommend pre-heating to minimize delay or lag at the start of the reaction. PCR instruments that are capable of rapid temperature changes, like the Rotor-Gene® Q, may not require pre-heating; see example run profiles (Tip 1f on page 3).

d. We usually run the assay at a constant temperature, acquiring data in the SYBR® Green channel every 5 seconds for 60 minutes total run time.

- See Section 6 (Optimizing assay linearity and temperature) on page 14 for information on optimal assay temperature.
- If you are using the standard curve method (Protocol A in the Product Information Sheet) you may not need to run the assay for a full 60 minutes.
- If you are determining absolute nucleotide incorporation (Protocol C in the Product Information Sheet) then you must run the assay until maximum fluorescence is reached and the amplification curve plateaus for your highest enzyme concentration. This may take longer than 60 minutes depending on the enzyme and reaction conditions. See Figure 7 on page 12.

e. EvaEZ™ Assay Mix contains high ROX. We recommend using ROX normalization to reduce data variation:

- For qPCR instruments that require a reference dye for normalization, ensure that ROX is selected.
- For low ROX instruments, we recommend testing whether ROX normalization can be used with EvaEZ™; ROX normalization works well with EvaEZ™ Assay Mix on QuantStudio™ 5, even though low ROX is recommended for that instrument (refer to Tip 1.f.v. on page 3). If your instrument cannot perform ROX normalization with EvaEZ™ Assay Mix, turn ROX normalization off or use non-normalized SYBR® channel data for analysis.
- For instruments that do not require a passive reference dye, ROX normalization is optional; you can perform the assay with ROX normalization turned off or using non-normalized SYBR® channel data.

Technical Tips

1. Setting up your instrument (cont.)

f. Example instrument run profiles for EvaEZ™ Assay with Taq polymerase:

Qiagen Rotor-Gene® Q instrument settings:

i. Set up a single stage profile: 54°C, 5 seconds x 300 cycles, acquire to Cycling A. Green.

ii. Open the software to your profile so the instrument is ready to start.

Note: Because the Rotor-Gene® Q rapidly adjusts temperature, we do not find it necessary to pre-heat the instrument before adding the tubes.

iii. Place your tubes in the instrument and start the run immediately.

iv. View the raw fluorescence channel (Cycling A. Green) or use the exported "Data From Channel Cycling A. Green" to view the EvaEZ™ curves.

Thermo Fisher QuantStudio™ 5 qPCR instrument settings:

i. Set up a run profile with the following settings:

- Chemistry: SYBR® Green Reagents
- Passive Reference: ROX
- Volume: 20 uL, Lid: 105°C
- 2-Stage Method:
  - PCR stage 1, step 1: 54°C 5 seconds x 1, data collection: OFF, Pause at 54°C after 1 cycle
  - PCR stage 2, step 1: 54°C 5 seconds x 305 cycles, data collection: ON

ii. Click "Start Method" and allow the instrument to come to temperature and pause.

iii. On the instrument, press the "Eject" icon to open sample drawer.

iv. On the instrument, scroll to the page with the "Resume" button. Place tubes/plate in instrument, then immediately click "Resume" to begin acquiring data.

v. View the Multicomponent plot to view the EvaEZ™ curves (SYBR® channel) and ROX reference curves to check that ROX signal is similar for all samples and constant across the run, and that there are no abnormal spikes or dips in the EvaGreen® curves.

vi. View the "Rn" plot or use the exported "Amplification Data" to view the EvaEZ™ curves.

Technical Tips

2. Tips for setting up your samples

a. Selection of dilution buffer: Enzymes are commonly stored in buffers containing 50% glycerol and other stabilizing ingredients. When performing a dilution series, we prefer to make the dilutions in the same enzyme storage buffer as the enzyme stock solution, to ensure that all the samples have the same final concentration of glycerol and other buffer components. For example, the concentration of glycerol in the assay may affect the lag phase of the amplification curves, and therefore it is important to maintain the same concentration in all samples.

b. Keep all reagents and sample tubes or plates chilled during assay setup to prevent enzyme activity before the samples are brought to the assay temperature.

c. Try to avoid introducing bubbles during reaction mixing (see Tip 8f on page 16). We recommend performing a centrifugation step after reaction setup to reduce bubbles, but this should be done as quickly as possible to avoid warming the samples.

d. For PCR instruments, the standard recommended reaction volume is 20 uL, but other reaction volumes may be used. Data quality may improve with larger volume if reactions are well mixed, and temperature is evenly controlled. However, in microplate readers, reaction temperature may be more uneven with higher volumes so a smaller volume may improve data quality.

   Note: If you are calculating final nucleotides synthesized, the equation will need to be adjusted accordingly to account for volume. See calculations in Section 5 (Calculating nucleotide incorporation) on page 11.

e. We recommend testing 2-3 dilutions of your unknown sample to ensure that the concentration falls within the linear range of the assay. If you get different activity calculations for different dilutions of the same sample, that is an indication that some of your dilutions may not be within the assay linear range. An overloaded assay may report as zero activity if the assay reaches plateau before the first data point is read (see Figure 3 on page 7 for example of curve profiles).

f. We recommend performing all dilutions independently, rather than making serial dilutions, which can propagate pipetting error from the initial dilution. We find we achieve the greatest accuracy when diluting enzyme from a concentrated stock and diluting into varying volumes of diluent to avoid using different pipettes to measure the enzyme volumes. We see noisier data when we create an intermediate stock solution to use for dilutions, possibly due to variable mixing or adsorption of protein to plastics.

3. Performing an assay with a titration of standard enzyme

Also see Protocol A in the Product Information Sheet.

Important: You must include the standard curve in the same assay as your unknown samples. It is not possible to directly compare standard curve data between assays.

3.1. Make a dilution series of your standard enzyme in your buffer of choice (see Tip 2a on page 4 and Tip 8f on page 16). We usually perform the dilutions shown in Table 1 using a Taq polymerase stock at 5 U/uL (5000 U/mL).

Note: We do not recommend performing a serial dilution to make the standard curve (see Tip 2f on page 4).

Tube	Volume of Taq (5000 U/mL)	Volume of buffer	Resulting concentration
1	4.8 uL	10.2 uL	1.6 U/uL
2	2.4 uL	12.6 uL	0.8 U/uL
3	1 uL	11.5 uL	0.4 U/uL
4	1 uL	24 uL	0.2 U/uL
5	1 uL	49 uL	0.1 U/uL
6	1 uL	99 uL	0.05 U/uL
7	1 uL	199 uL	0.025 U/uL

Table 1. Example dilution series for Taq polymerase.

3.2. Prepare the dilutions of your unknown samples (see Tip 2e on page 4 and Table 1 for example).

3.3. If necessary, pre-heat the instrument to get it ready for the run (see Tip 1c on page 2).

3.4. Prepare working mix by combining 2X EvaEZ™ Assay Mix (Cat. No. 29051) with dH_2O at a ratio of 10 uL EvaEZ™ + 9 uL dH_2O for each sample to be tested. Vortex to mix completely. Keep the working mix chilled on ice or in a cooling block.

For example, to have enough working mix for 7 standards and 3 unknown sample dilutions in triplicate, mix 320 uL of 2X EvaEZ™ Assay Mix with 288 uL of dH_2O.

Note: This is enough working mix for two extra tubes, to account for volume loss during pipetting. Always make excess working mix so you don't run short (see Tip 8d on page 16).

3.5. Prepare your reaction tubes or plate on a cooling block. Pipette 19 uL of chilled working mix to each tube or well.

3.6. Add 1 uL of each standard or sample per tube or well (see Tip 8f on page 16).

3.7. After dispensing standards and samples, pipette up and down to mix, taking care to not introduce bubbles (see Tip 8e on page 16).

3.8. Place your tubes or plate in the instrument and start the run.

4. Calculating initial slopes and graphing standard curves

4.1. Graph the EvaEZ™ fluorescence amplification curves (fluorescence vs. cycle number) (See Figure 2).

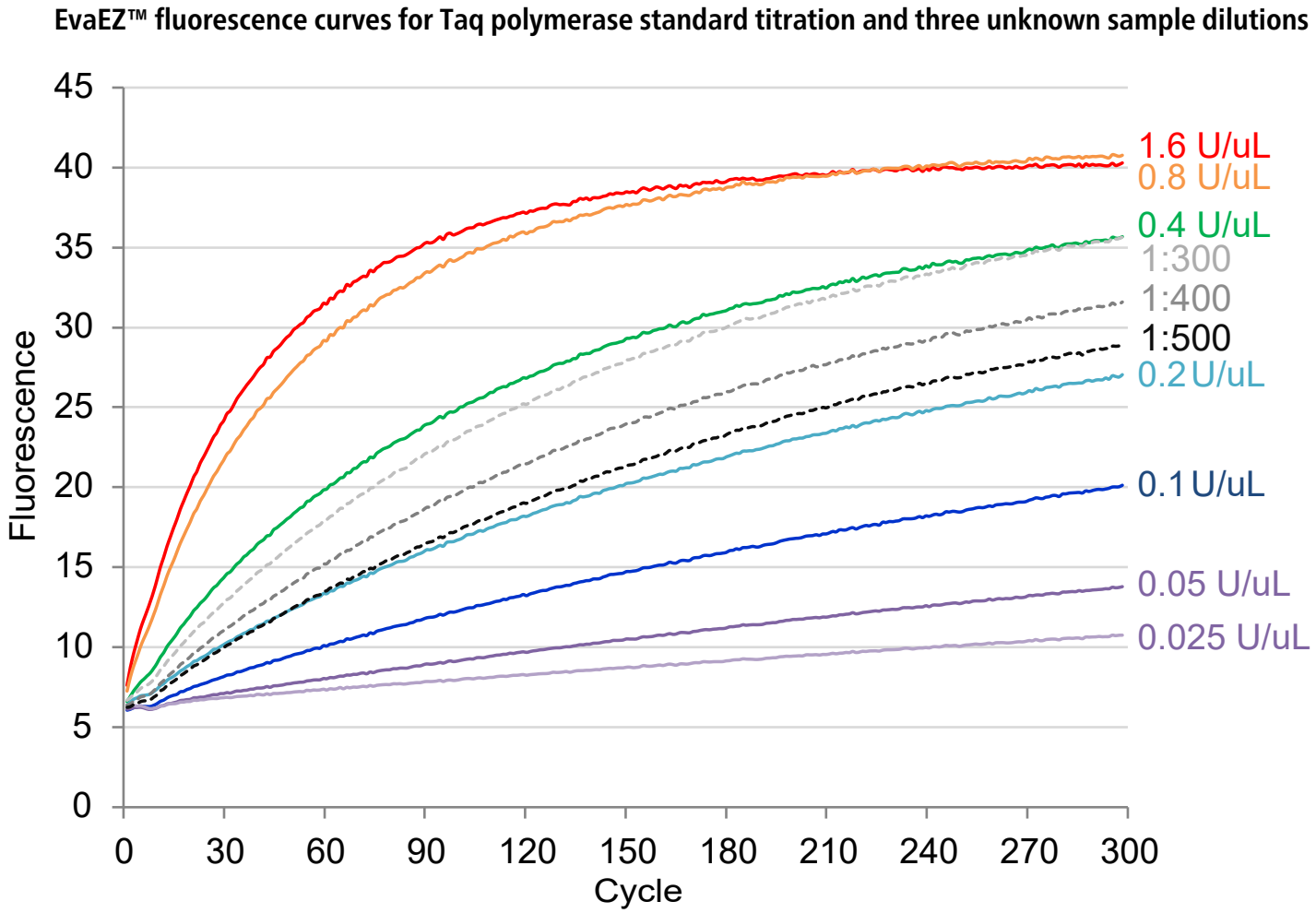

Figure 2. EvaEZ™ amplification curves for Taq polymerase. The assay was measured in the SYBR® Green channel on a Rotor-Gene® Q qPCR instrument. Averaged values for triplicate samples of a Taq dilution series ranging from 1.6 U/uL (red) to 0.025 U/uL (violet) are shown. The amplification curves for three dilutions of an experimental sample with unknown concentration are shown in gray (dashed lines).

4. Calculating initial slopes and graphing standard curves (cont.)

4.2. Find the linear region of fluorescence increase. In the first few cycles (the lag phase), fluorescence may be flat or erratic for some reactions. Fluorescence then typically increases linearly for several cycles before the higher concentration curves begin to plateau (see Figure 3). Choose the range of cycles where the fluorescence increase appears to be most linear. For Taq polymerase, this is typically a span of 10-15 cycles anywhere between cycles 5-30.

Note: Use the same range of cycles for all curves in the same experiment, do not determine a separate linear region for different enzyme concentrations.

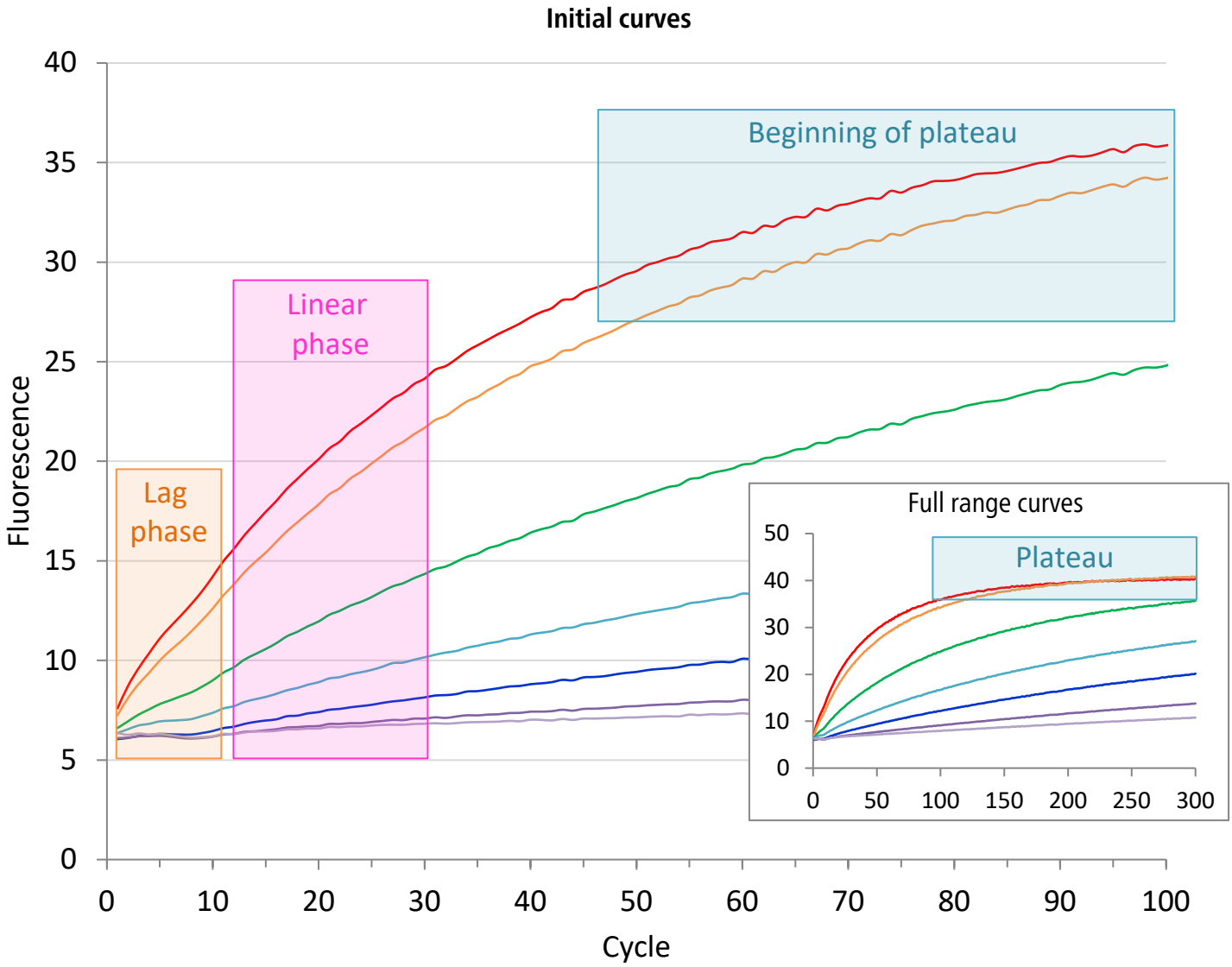

Figure 3. Detail of EvaEZ™ amplification curves from Figure 2 showing cycles 0 to 100, highlighting the lag phase, the linear phase, and the beginning of the plateau phase. The assay was measured in the SYBR® Green channel on a Rotor-Gene® Q qPCR instrument. Averaged values for triplicate samples of a Taq dilution series ranging from 1.6 U/uL (red) to 0.025 U/uL (violet) are shown. The inset shows the full range of the data, where the complete plateau of the fluorescence signal for the highest enzyme concentrations can be seen.

4. Calculating initial slopes and graphing standard curves (cont.)

4.3. Verify that the y-intercept of the standards and samples are similar across all concentrations; in other words, that all samples start from a similar baseline, as in Figure 3 on page 7 and Figure 8 on page 13.

If the y-intercept is much higher for a few of the samples, that is an indication that these samples warmed up before the assay was started, or that the enzyme concentration is too high. Subsequently, the cycling of these samples is asynchronous to the others, and you will not be able to accurately determine a comprehensive linear phase that is inclusive of all samples. See Figure 11 on page 17 for an example of curves with inconsistent y-intercepts.

4.4. You may verify that the curves are linear for the cycle range you selected by graphing that range separately and fitting linear trendlines to the data (see Figure 4).

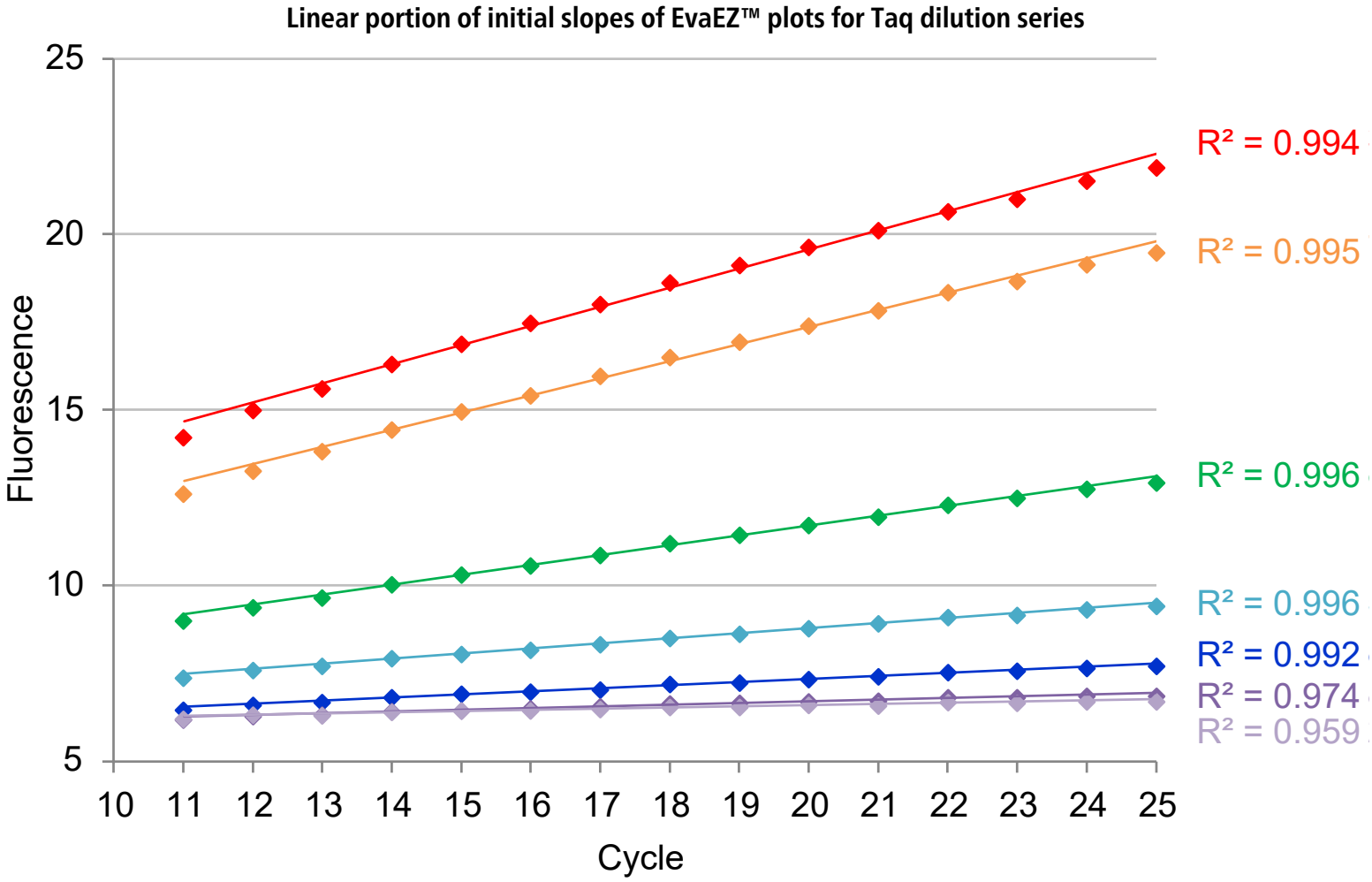

Figure 4. Initial slopes for EvaEZ™ curves shown in Figure 2 and Figure 3, with R^2 values for the linear trendlines.

4. Calculating initial slopes and graphing standard curves (cont.)

4.5. Use the linear region of the curve (see Figure 3), to calculate the initial slope for each enzyme concentration.

Slope = (ending fluorescence - starting fluorescence)/number of cycles

4.6. Graph the initial slope vs. enzyme concentration (see Figure 5).

Note: You may use any units that you wish for concentration (for example, U/mL or U/uL) as long you use them consistently throughout your calculations. However, concentration units that result in higher numbers (for example, 1600 U/mL as opposed to 1.6 U/uL) may return a very small number for the slope of the standard curve equation, which may be less convenient for formatting the trendline (see Notes to step 4.8 on next page).

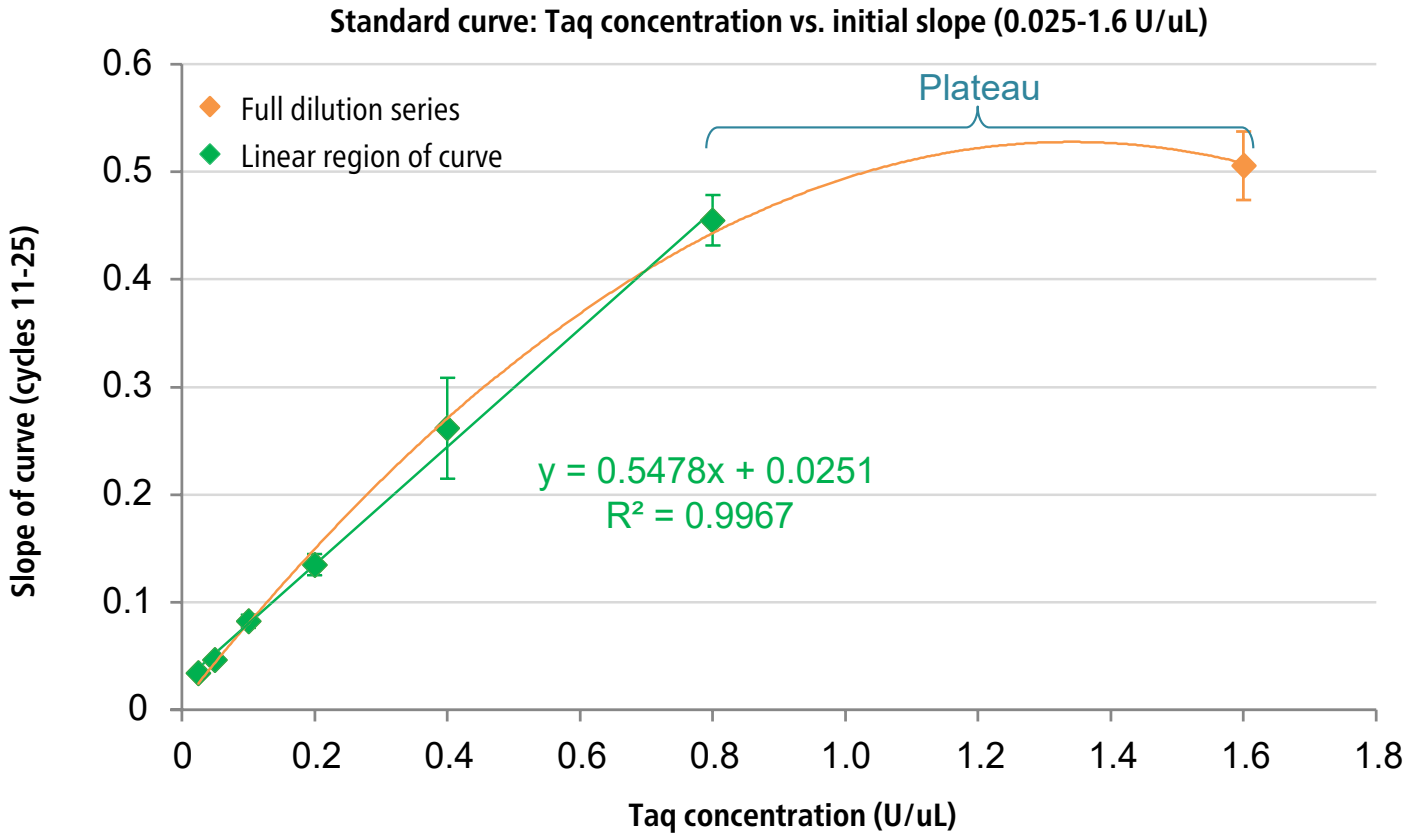

Figure 5. Plot of Taq concentration vs. slope for the EvaEZ™ initial slopes shown in Figure 4, showing linear increase in initial slope up to 0.8 U/uL, with activity reaching plateau before 1.6 U/uL. Each point is the average of triplicate samples; error bars show standard deviation.

4. Calculating initial slopes and graphing standard curves (cont.)

4.7. Determine which of the dilutions are in the linear range of the calibration curve. For example, dilutions in the linear range should show an approximately 2-fold increase in slope for every 2-fold increase in enzyme concentration. Any dilutions that show a plateau in signal must be excluded from analysis to fit a linear trendline to the data. For example, in Figure 5 on page 9, the dilution series curve shows a plateau after 0.8 U/uL, so the 1.6 U/uL point must be excluded to fit a linear trendline.

4.8. Fit a linear trendline to the selected dilution series (see Figure 6). This is the working concentration range of the EvaEZ™ Assay for this enzyme at this temperature.

Note: In the standard EvaEZ™ Assay, background is not subtracted from fluorescence values, therefore, **do not** set the intercept of the trendline to zero.

Note: If you are using Excel and the values you use result in a very small number for the slope of the trendline, the trendline may show a slope of zero. If this occurs, right-click the trendline label, choose "format trendline label," then format the number with more decimal places until it shows a positive number. You can also calculate slope using Excel functions such as "Linest", "Slope", and "Intercept".

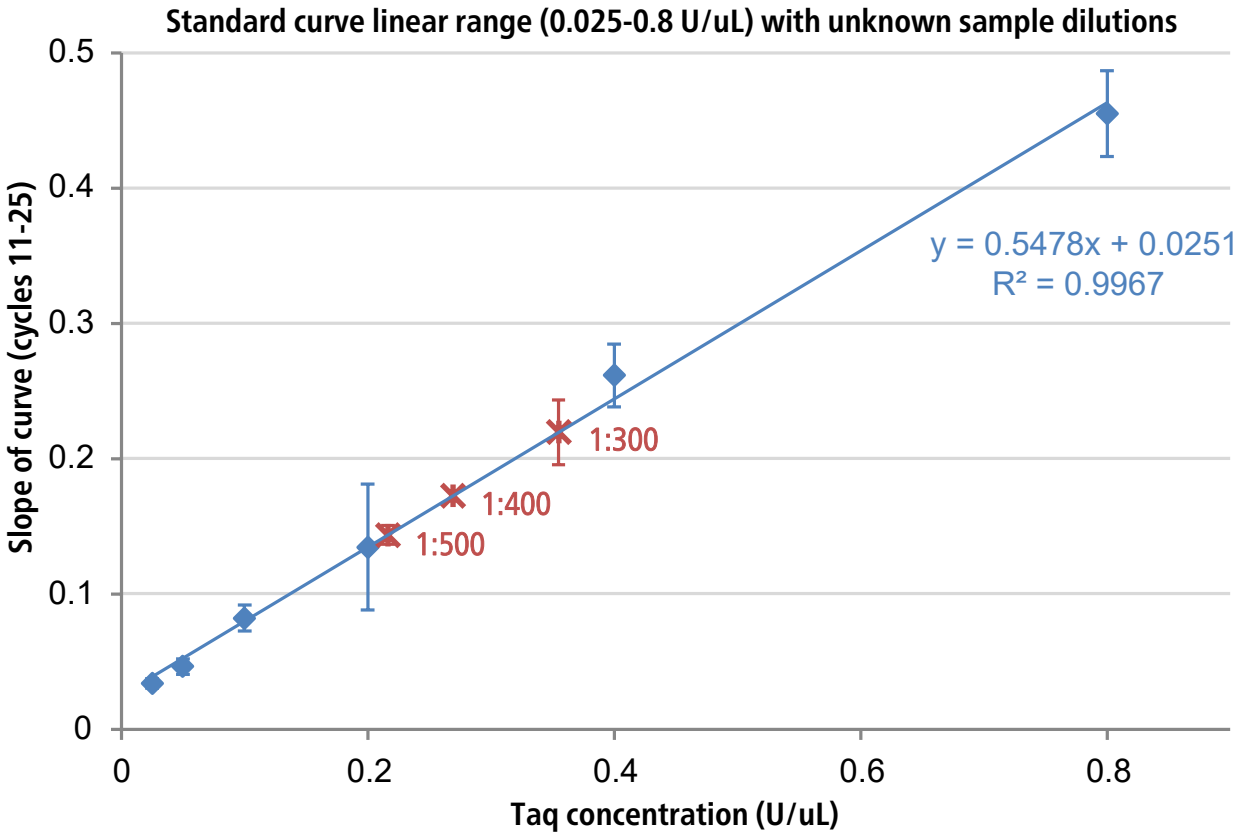

Figure 6. Taq standard curve in the linear range of 0.025-0.8 U/uL, with a linear trendline showing R^2 value and line equation. Data points for each unknown sample dilution are also shown (red asterisks). Each point is the average of triplicate samples; error bars show standard deviation.

4.9. Use the trendline equation to calculate the concentration of your unknown sample:

$y = mx + b$

where: y: is the initial slope of the EvaEZ™ amplification curve for your unknown

x: is the Taq concentration in the sample

m: is the slope of your standard curve

b: is the y-intercept of your standard curve

4. Calculating initial slopes and graphing standard curves (cont.)

In our example data, the initial slope of the EvaEZ™ curve for the 1:300 dilution was found to be 0.219. We can calculate the concentration of the unknown sample dilution using the linear trendline from the Taq dilution series (shown in Figure 6 on page 10):

$y = 0.5478x + 0.0251$

Initial slope = 0.5478 (Concentration) + 0.0251

0.219 = 0.5478 (Concentration) + 0.0251

Concentration = (0.219 - 0.0251)/ 0.5478 = 0.355 U/uL

By multiplying the calculated concentration by the dilution factor (300), the concentration of Taq in the unknown sample stock solution is calculated to be 106.5 U/uL.

4.10. We recommend performing a few different dilutions of your sample to make sure that the concentration falls within the linear range of the assay. In our example data, three different dilutions were made for the unknown sample, and they all fell within the linear range. The calculated concentration of the stock solution from each of these dilutions shows good agreement (see Table 2), further validating the linearity of the assay in this test.

Unknown sample dilution	Initial slope	Calculated concentration (U/uL)	Multiply by dilution factor	Concentration of unknown sample (U/uL)
1:300	0.219	0.355	300	106.5
1:400	0.173	0.269	400	107.8
1:500	0.144	0.216	500	108.2

Table 2. Calculated concentrations for three dilutions of Taq polymerase from EvaEZ™ activity curves shown in Figure 5.

5. Calculating nucleotide incorporation

Also see Protocol C in the Product Information Sheet.

The activity of an enzyme can be quantitated directly (without a reference standard) by calculating the number of nucleotides incorporated in the reaction. The maximum number of nucleotides that can be incorporated in a 20 uL EvaEZ™ reaction with a saturating concentration of enzyme is 270 pmol. By comparing EvaEZ™ fluorescence signal of a reaction in the linear range of the assay to a reaction with saturating enzyme, one can calculate units of activity based on nucleotide incorporation.

Note: 60 minutes is the recommended assay time for Taq polymerase, your enzyme may require a longer or shorter assay time. The equation will need to be adjusted accordingly to account for the change. See calculations in Step 5.4 on page 12.

5.1. Perform the EvaEZ™ Assay with a series of dilutions of your test DNA polymerase as in Section 3, but also include a no enzyme control. Be sure to extend your titration to include enzyme concentrations that result in a saturating signal with a clear plateau phase (see Figure 3 on page 7).

5.2. Determine the initial slopes of the amplification curves according to Section 4. Plot initial slope vs. dilution to see the linear range of the dilution series as in Figure 5 on page 9.

5.3. Select three curves for analysis (see Figure 7 on page 12).

a. Test sample with enzyme concentration within the linear range of the curve and no plateau before 60 minutes

b. Saturated sample showing signal plateau at or before 60 minutes, and maximal fluorescence

c. Zero enzyme control

5. Calculating nucleotide incorporation (cont.)

5.4. Determine the initial slope within the linear portion of the curve for the test sample (sample a). Multiply the initial slope by 60 to calculate the change in fluorescence over 60 minutes. This is ΔF.

$$\Delta F = \text{initial slope} * 60$$

5.5. Calculate the maximum fluorescence change over 60 minutes by subtracting the endpoint fluorescence at 60 minutes of the no enzyme control (sample c) from the endpoint fluorescence at 60 minutes of the saturating enzyme sample (sample b). This is ΔFmax. See Figure 7 on this page.

$$\Delta Fmax = \text{saturating enzyme fluorescence at 60 min} - \text{no enzyme fluorescence at 60 min}$$

5.6. The number of nucleotides synthesized by the polymerase at the concentration in the linear range in 60 minutes under kit conditions is calculated as follows:

$$(\Delta F/\Delta Fmax) * 270 \text{ pmol}$$

Figure 8 and Table 3 on page 13 show examples of nucleotide incorporation calculation for three different lots of EvaEZ™ Polymerase Activity Assay Mix.

Note: Nucleotide incorporation will depend on the reaction conditions used, which are always provided for specific activity values, for example: pmol dNTP incorporated in 60 minutes at 54°C in EvaEZ™ Polymerase Activity Assay. Enzyme activity can be compared for different EvaEZ™ Assays done under the same conditions but cannot be directly compared to a radiolabeled nucleotide incorporation assay performed with different buffer, time, temperature, or assay detection methods.

Illustration of ΔF and ΔFmax determination

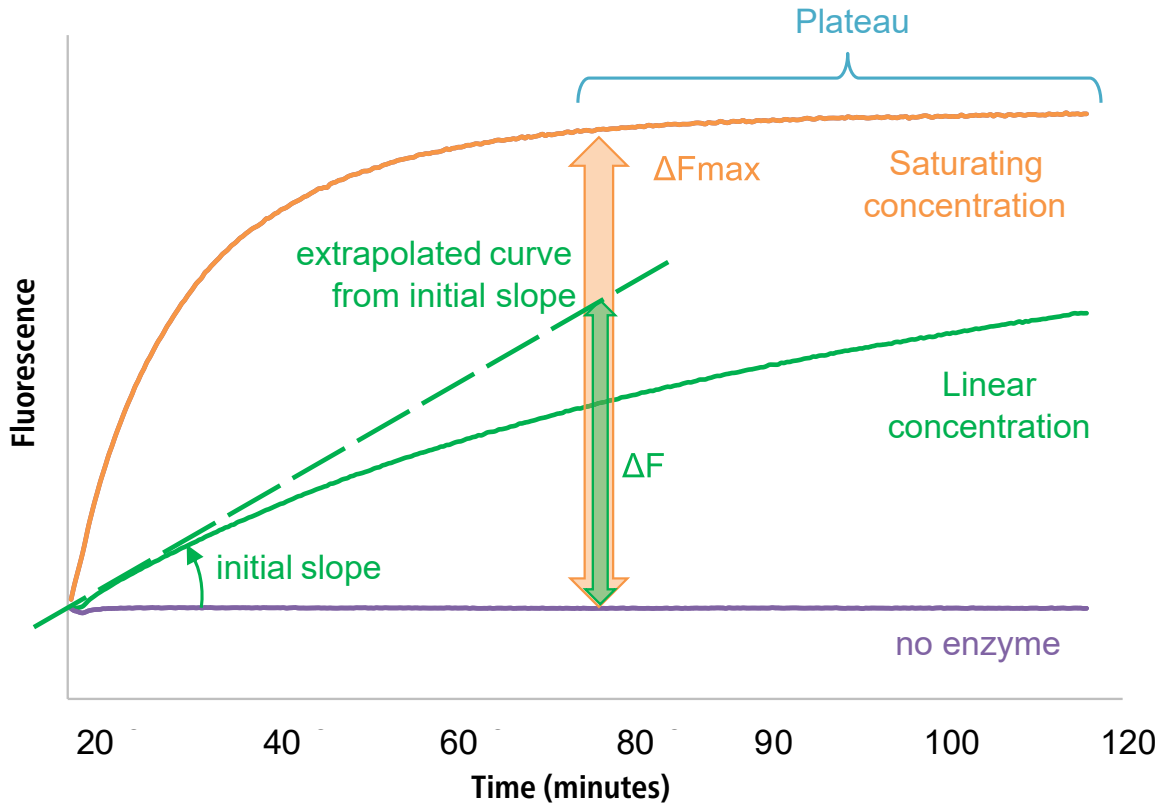

Figure 7. Illustration of ΔF and ΔFmax determination for the nucleotide incorporation calculation. The saturating sample should reach plateau before 60 minutes, while the no enzyme control should be flat, with no evidence of activity. A test sample should be selected that falls within the linear range of the assay with no plateau before 60 minutes.

Figures

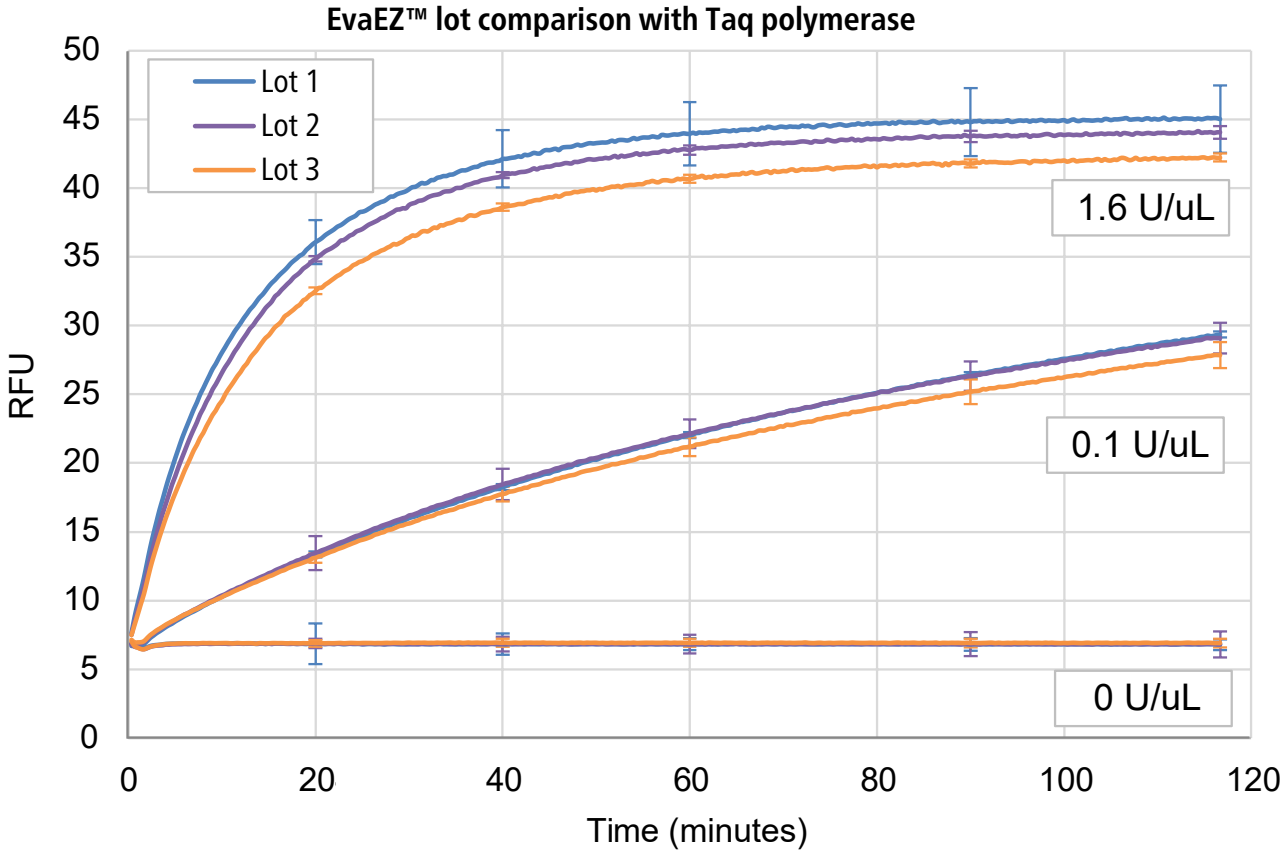

Figure 8. Comparison of three lots of EvaEZ™ Assay Mix for nucleotide incorporation determination using Taq polymerase. The assay was performed at 54°C in Rotor-Gene® Q. Error bars represent standard deviation for triplicate reactions.

EvaEZ™ Assay Mix Lot	ΔF 60 min (100 U/mL)	ΔFmax 60 min (100 U/mL)	ΔF/ΔFmax	ΔF/Fmax * 270 pmol
Lot 1	23.54	37.12	0.63	171.22
Lot 2	23.08	35.94	0.64	173.37
Lot 3	21.57	33.57	0.64	172.60

Table 3. Calculated nucleotide incorporation for the same vial of Taq polymerase from activity curves for three different lots of EvaEZ™ shown in Figure 8 (EvaEZ™ Assay at 54°C for 60 minutes).

6. Optimizing assay linearity and temperature

The optimal temperature for maximizing the linear range of the EvaEZ™ Assay may be different than the reported optimal temperature for the enzyme you are testing. Using a lower temperature may delay the plateau in signal and allow a wider linear assay range. Figure 9 shows a comparison of different assay temperatures for the same sample of Taq polymerase. Even though Taq is reported to be most catalytically active between 70-75°C, we found that performing the EvaEZ™ Assay at 54°C gave the widest linear range.

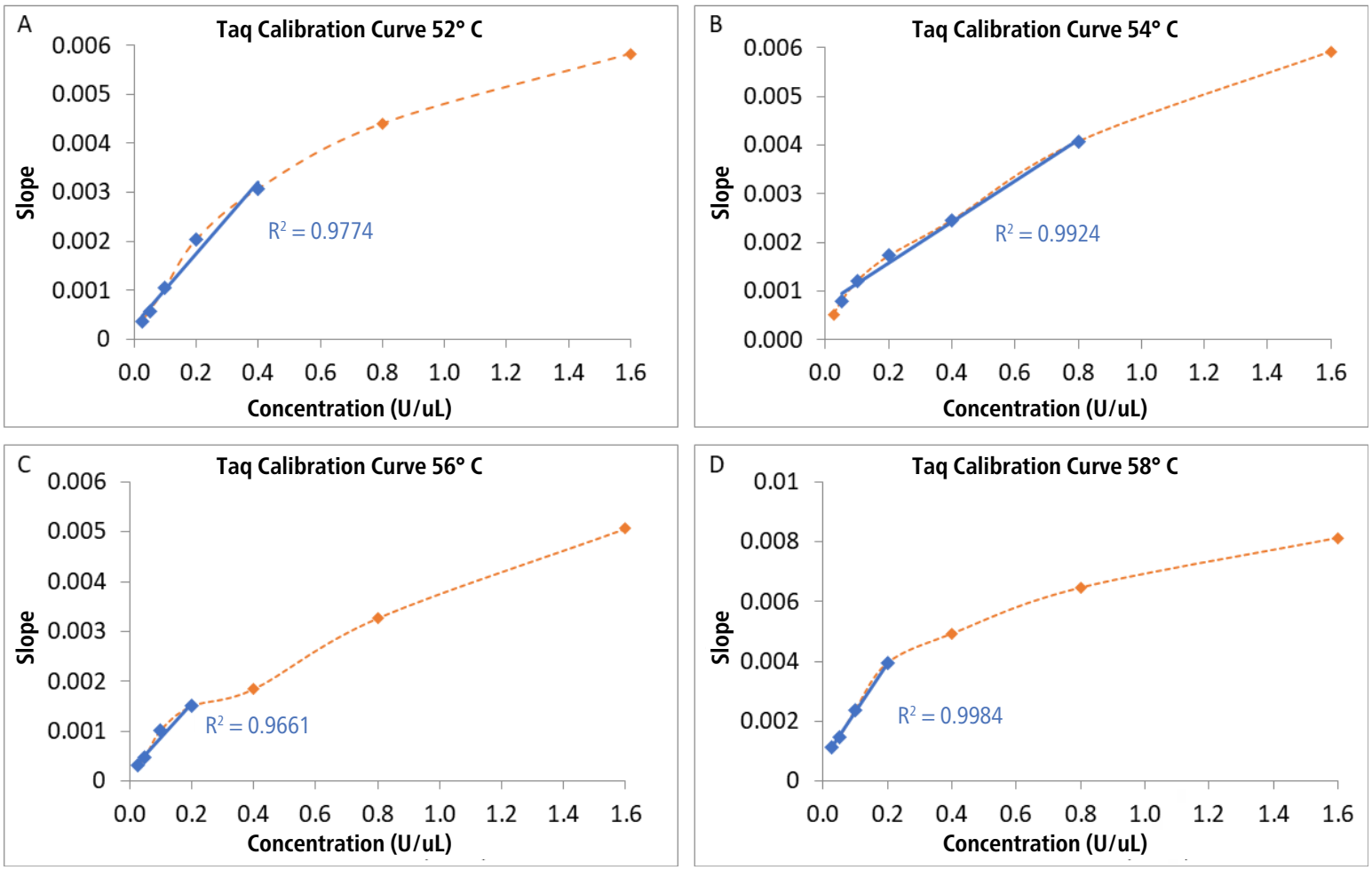

Figure 9. Comparison of EvaEZ™ Assay linear range using different assay temperatures for Taq polymerase. Using a lower assay temperature can delay signal plateau and increase assay linear range. Assays were performed using Taq polymerase on the QuantStudio™ 5.

7. Using hotstart polymerase in the EvaEZ™ Assay

When assaying hotstart polymerase, heat the enzyme to activate it before adding the enzyme to the EvaEZ™ Assay Mix. For our chemically modified Cheetah™ HotStart Taq DNA polymerase, we usually dilute the Taq to less than 1.6 U/uL in Taq storage buffer and then heat at 95°C for 5 minutes before adding to the reaction tubes.

Note: The concentration and buffer composition of the enzyme solution can affect how quickly and how completely it undergoes heat activation. If the enzyme is at a high concentration in a buffer containing 50% glycerol, it may activate more slowly than when the enzyme is in reaction buffer.

To evaluate the effectiveness of hotstart inhibition of enzyme activity, perform an EvaEZ™ Assay at room temperature with a sample of heat-activated enzyme and a sample of unheated, hotstart-inactivated enzyme. The hotstart-inactivated polymerase should show minimal increase in fluorescence at room temperature over the course of ~60 minutes while the heat-activated enzyme should show an increase in fluorescence (see Figure 10).

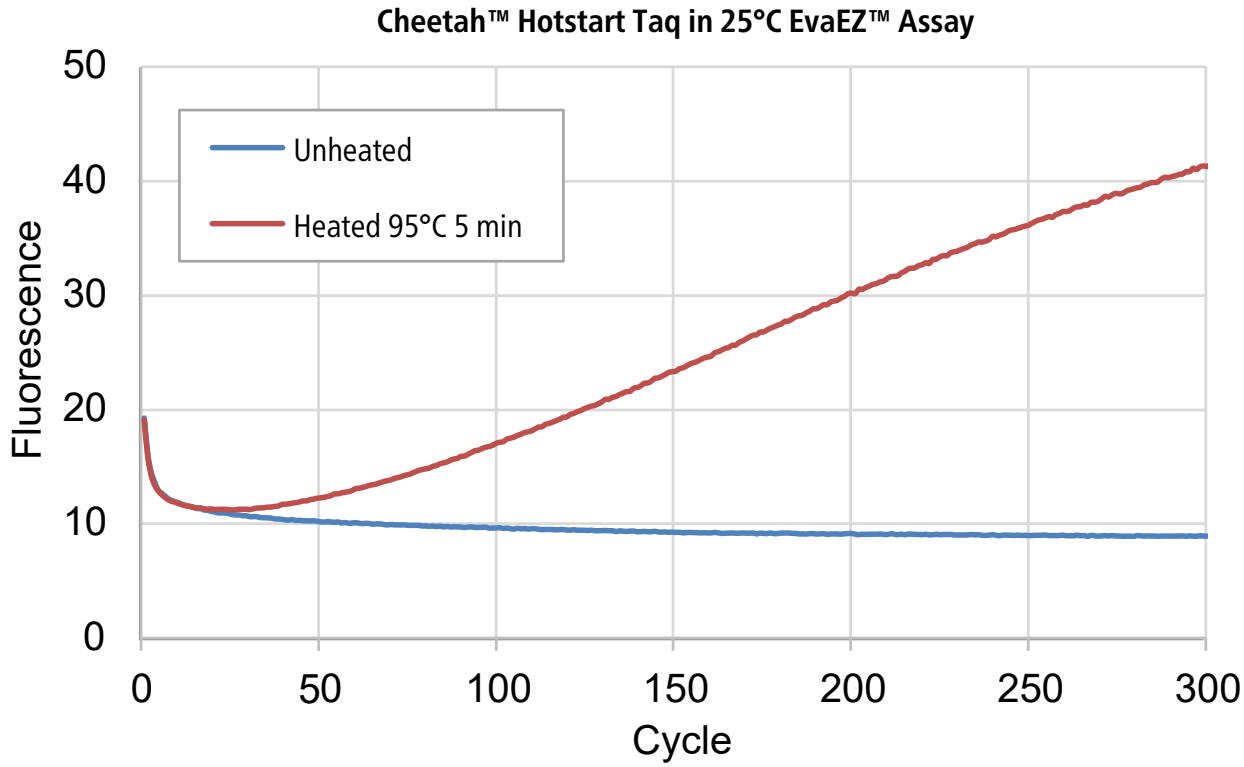

Figure 10. EvaEZ™ Assay used to evaluate hotstart inactivation of Taq polymerase. Cheetah™ HotStart Taq DNA polymerase shows no activity in the EvaEZ™ Assay at 25°C over the course of ~60 minutes (305 cycles on Rotor-Gene® Q) unless heat activated before being added to assay.

Technical Tips

8. Tips for improving assay accuracy

a. Ensure the enzyme solution is well mixed by pipetting up and down; do not vortex. Centrifuge briefly to collect the solution at the bottom of the tube.

b. Try to pipette from the surface of the enzyme solution to prevent excess solution from collecting on the outside of the tip. Carefully examine the tip after pipetting and remove any beaded solution from the exterior.

c. Keep the working mix and sample tubes chilled before beginning the reaction to minimize enzyme activity before data is collected. Pre-heat the instrument if necessary (see Tip 1d on page 2). Place the samples in the instrument and start the read as quickly as possible to collect the initial amplification data.

d. Be sure to make enough reaction solution for all your samples before you begin dispensing it into sample tubes. If you run out of reaction solution, do not make more in the middle of setup. Slight differences in concentration between the two separate dilutions of EvaEZ™ will introduce variability between samples. We recommend always preparing excess reaction solution to allow for volume loss during pipetting, so you don't run short while setting up your samples.

e. Avoid introducing bubbles into sample wells/tubes. When pipetting and up and down to mix, only pipette a fraction of the volume to avoid adding bubbles. To get rid of bubbles, try centrifuging the tubes or plate. Another method for removing bubbles is to use a solvent wash bottle filled with ethanol, **with the inner straw removed** so it does not dispense liquid. Use the bottle to gently blow alcohol vapor over the top of the plate to alter surface tension and pop bubbles.

f. In general, pipetting introduces variability, especially at the extremes of the pipette volume range. Use calibrated pipettes and low retention tips. Choose a pipette so that your required volume is in the middle of the pipette range. Use reverse pipetting for viscous samples like enzymes in 50% glycerol. Do not use reverse pipetting for non-viscous solutions, it will be less accurate.

g. Work with independent replicate dilutions and avoid serial dilutions when possible.

h. If possible, use the ROX in the EvaEZ™ mix to normalize fluorescent signal (see Tip 1e on page 2). Always confirm ROX signal is steady throughout the assay.

i. Only select the required channels for analysis on your PCR machine (i.e., green fluorescence channel for EvaGreen® signal, and ROX channel for normalization (if applicable). Acquiring data in unneeded detection channels may increase cycling time and reduce data collection capabilities.

j. Include more replicates (for example, use triplicate instead of duplicate samples).

k. Check that all your samples intercept the y-axis at a similar point; in other words, they should start from the same low baseline. If some samples show a large y-intercept value (or high baseline), that indicates that the reaction started before you began acquiring data, and you will not be able to accurately determine the initial slope for that sample. This may be caused when samples warm up before you begin the assay read, or if enzyme concentration is too high. We recommend including a control reaction without enzyme to empirically determine the baseline value. See Figure 11 on page 17 for an example of inconsistent baseline.

l. For nucleotide incorporation calculations, make sure the sample you use for ΔFmax determination is saturating. If your test samples have higher endpoint fluorescence than the plateau value for the saturating sample, that is an indication that there may have been interference in the saturating sample reaction, possibly from high enzyme concentration outside the linear range of the assay, incomplete mixing, or bubbles. See Figure 11 on page 17 for an example of inconsistent signal saturation.

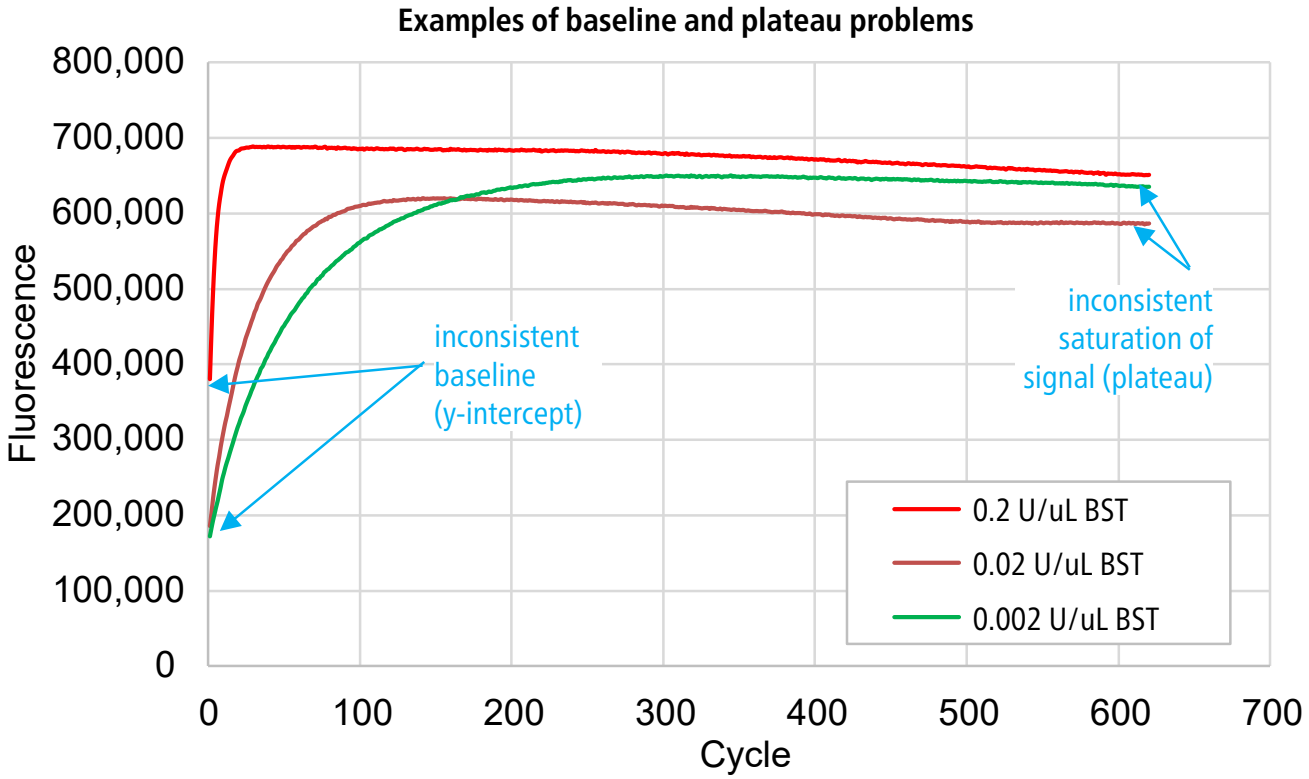

Figure 11. Examples of inconsistent baseline or plateau curve profiles, which are indicative of non-linear assay conditions. See Tips 8k and 8l for more information. Data shown from an EvaEZ™ Assay with *Bst* polymerase at 65°C. The inconsistencies in these profiles are likely due to high enzyme concentration exceeding the linear range of the assay.

Additional Resources
Please visit the EvaEZ™ product page to see the Product Information Sheet, FAQs, publications, and related products.