ROX passive reference dye for troubleshooting real-time PCR

Real-time PCR—also called quantitative polymerase chain reaction (qPCR)—is one of the most powerful and sensitive gene analysis techniques available. Real-time quantitation of DNA and cDNA requires monitoring of a fluorescent signal that reports on the progress of the amplification reaction at each cycle. (For more information, see qPCR education resources at thermofisher.com/understandingct).

A number of conditions may influence real-time PCR results: pipetting errors create variability in volume; evaporation or condensation within each well may change the concentrations of components; and bubbles or droplets may produce spikes in a fluorescent signal at various time points (Figure 1). These events can affect the fluorescent signal from the reporter dye and result in unreliable data. To compensate for common artifacts, researchers might typically include larger numbers of replicates to help ensure statistically robust data and plan for additional time and reagents to repeat questionable results.

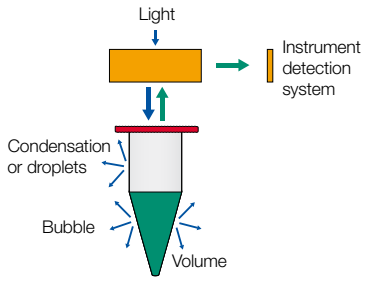

Figure 1. Common sources of process errors that can compromise real-time PCR data.

The role of an optional passive reference dye in real-time PCR

A passive reference dye is an independent, inert dye that exhibits no change in fluorescence within a PCR amplification reaction. This tool offers a number of benefits: correcting for changes in signal within a run due to non–PCR-related artifacts, assisting in troubleshooting questionable data, and normalizing differences within a run (Table 1). In an ideal reaction with perfect samples and reaction mix, reference dye normalization would have no impact on results, since there would be no effects from non–PCR-related events (Figure 2).

Table 1. Appropriate uses for ROX passive reference dye.

What ROX dye can be used for	• Help diagnose evaporation problems • Help diagnose pipetting problems • Help diagnose the presence of droplets • Normalize spurious signals arising from bubbles
What ROX dye can't do	• Fix bad data • Correct for pipetting errors

Applied Biosystems™ ROX™ dye is a proprietary passive reference dye introduced in the 1990s. ROX dye is inert and does not interfere with the fluorescence emission of the reporter dyes commonly used in real-time PCR. Today all Applied Biosystems™ real-time PCR systems are designed to take advantage of the benefits of including a passive reference dye, which are outlined in this paper. In addition, ROX dye is used in all Applied Biosystems™ real-time PCR master mixes to help make troubleshooting real-time PCR experiments easier.

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Despite a decade of use and widespread scientific acceptance, the role of ROX dye as a passive reference in real-time PCR is confused by several commonly held misconceptions, including an erroneous belief that ROX dye is required for PCR analysis with Applied Biosystems instruments. ROX dye–capable real-time PCR instruments are compatible with PCR reagents with and without the passive reference dye (Figures 2 and 3). Moreover, the Applied Biosystems™ real-time PCR analysis software allows users to ignore the ROX dye signal during analysis, if desired. It is also important to understand that ROX dye is not intended to correct for instrument limitations or make an instrument more sensitive, nor does it serve as a fix for "bad" data. Additionally, ROX dye does not compensate for improper pipetting techniques (Table 1).

Multicomponent plot view in Applied Biosystems instruments

The multicomponent plot screen (Figure 4) displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run. To compute the normalized amplification curves (Rn) typically shown in the amplification plots, the reporter dye signal is divided by the ROX dye (passive reference) signal (Figure 5).

When expressed in a multicomponent plot, the ROX dye fluorescence level should remain relatively constant throughout the real-time PCR process (Figure 4, red line), while the reporter dye fluorescence level displays a flat region corresponding to the baseline, followed by a rapid rise in fluorescence as the amplification

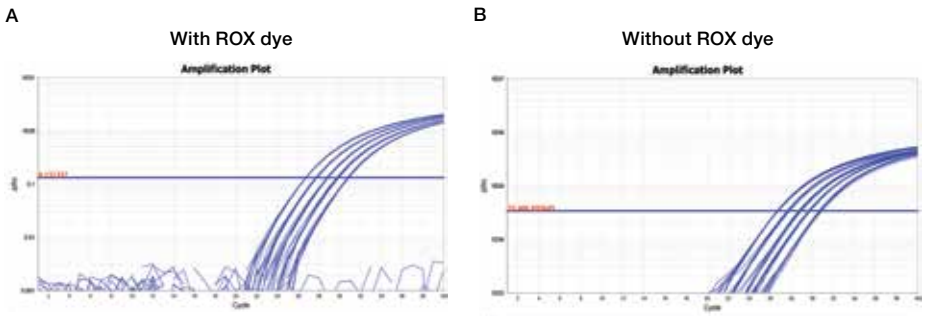

Figure 2. Real-time PCR amplification plots of RNase P serial dilutions, with ROX dye (A) and without ROX dye (B) normalization. Standard deviation of replicates with ROX dye normalization is 0.02, without ROX normalization is 0.03.

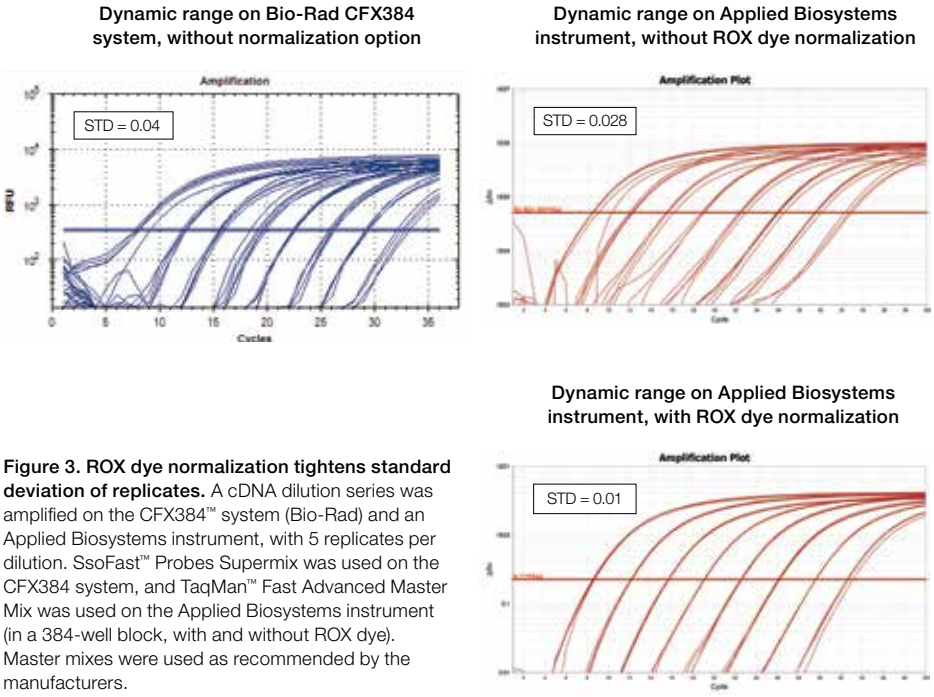

Figure 3. ROX dye normalization tightens standard deviation of replicates. A cDNA dilution series was amplified on the CFX384™ system (Bio-Rad) and an Applied Biosystems instrument, with 5 replicates per dilution. SsoFast™ Probes Supermix was used on the CFX384 system, and TaqMan™ Fast Advanced Master Mix was used on the Applied Biosystems instrument (in a 384-well block, with and without ROX dye). Master mixes were used as recommended by the manufacturers.

proceeds during the exponential phase and a flattening when the reaction plateaus (Figure 4, blue line). If any non-PCR related events arise in a well (e.g., evaporation, droplets, bubbles) the multicomponent plot will present spikes, dips, or other abnormal behavior in both the reporter dye signal as well as in the ROX dye signal.

ROX dye tightens standard deviation of replicates and facilitates quality control

Figure 3 shows the analysis of two plates—set up using TaqMan™ chemistry for a dynamic range—with and without ROX dye as a passive reference, on the Applied Biosystems™ ViiA™ 7 instrument and on the Bio-Rad CFX384™ instrument. The data clearly demonstrate that ROX dye has no impact on average Ct but effectively tightens the standard deviation of replicates (Figure 3). This means that users can run fewer replicates and still have confidence in the resulting data. Irregularities in fluorescence signal can occur in real-time PCR, and for that reason ROX dye has immense value as a troubleshooting tool.

If a reaction fails, analysis of the ROX dye signal in the multicomponent plot may help

discriminate potential causes of abnormal data. A lack of a ROX dye signal would clearly indicate that master mix was not added or the well was empty, whereas large signal discrepancies would indicate a possible double-volume reaction.

The following are examples of simulated situations in which a common experimental error or data artifact was forced in the experiment along with the resulting data plots. The quality control capabilities of ROX dye can help reduce analysis time and increase confidence in results for all users, from novice researchers to the most experienced users.

Common problem 1: bubbles

Bubbles in reaction wells commonly occur when plates are improperly centrifuged. Bubbles can generate an abnormal jump in the fluorescent signal(s) when they expand and burst during cycling. In Figure 6, air bubbles were forced into wells by injecting air with a pipette below the reaction mix surface. If no ROX dye is used to normalize the data, amplification plots display spurious signals in wells containing the forced bubbles (Figure 6A). Without ROX dye, the multicomponent plot (Figure 6A) shows the same spurious signal, but the results from these two curves cannot be properly interpreted. Using the passive reference dye to normalize the data (Figure 6B), the software is able to properly compute the amplification curve using the FAM™ reporter and ROX dye passive reference signals to provide acceptable normalized data. The abnormal jump remains visible in the multicomponent plot (Figure 6B), but the results from all amplification curves can now be interpreted with confidence.

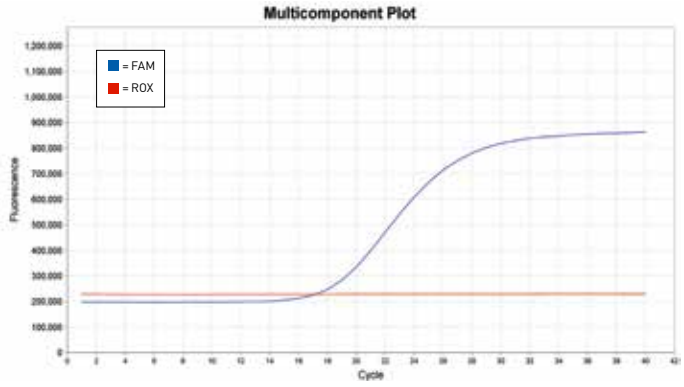

Figure 4. Multicomponent plot using ROX reference dye. Example of expected amplification (blue line) and passive reference (red line) curves.

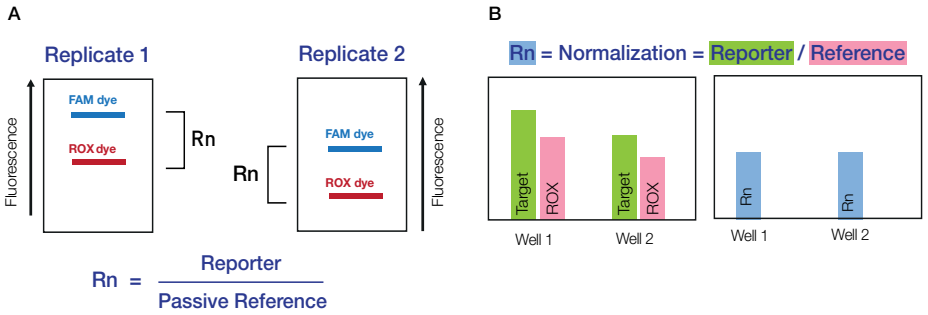

Figure 5. Calculation for ROX dye–enabled normalization. (A) Dividing the reporter signal by the ROX dye signal gives a normalized value (Rn). **(B)** Using Rn values, signals from two different wells can be compared.

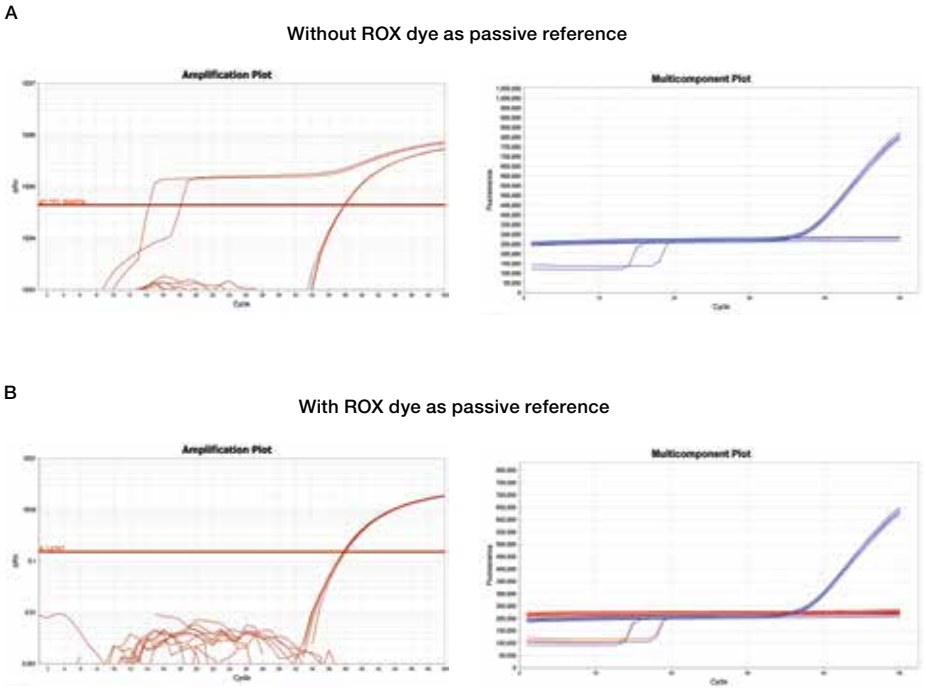

Figure 6. Including ROX dye allows the user to distinguish PCR-related aberrations from non–PCR-related aberrations. (A) When air bubbles were intentionally forced into the wells with a pipette, the amplification plots without ROX dye normalization showed grossly variable C_t values for replicates in the experiment. On the multicomponent plot, the intentionally created irregularities of some of the replicate wells can be seen, but in the absence of ROX dye it is not possible to determine if the source of the irregularity is PCR-related or not. This makes it difficult to determine if the results are valid. **(B)** The same amplification plot—analyzed with ROX as passive reference—shows good amplification. The multicomponent plot shows both FAM and ROX dye signals increasing suddenly, indicating a non–PCR-related event. Because ROX dye was used as passive reference, the data can be normalized to account for the jump in fluorescence, and accurate amplification values can still be obtained.

Common problem 2: evaporation and droplets

Evaporation problems may arise when plates are improperly sealed with an optical cover (e.g., when the sealing cover is misaligned on the plate, the adhesive cover is wrinkled, or not enough pressure is applied). To illustrate the effect of evaporation on qPCR results and how helpful ROX dye is for troubleshooting, a hole was purposely punched in the adhesive cover on one of the 16 replicate wells (Figure 7) on two identical plates. These plates were then run on an Applied Biosystems instrument (Figure 7A) as well as another instrument (Figure7B) that does not use ROX dye for normalization. On the amplification plot of the plate run on the Applied Biosystems instrument, we can see differences in the curves. The multicomponent plot view of the same plate shows an increase in the ROX dye signal, characteristic of an evaporation problem. The amplification plot of the plate run on an another vendor's instrument shows an aberration (Figure 7B), but there is no way for the user to determine if the abnormal signal is related to the PCR reaction or not. ROX dye normalization enables scientists to more easily track down the source of the problem by visualizing and deducing the source of the trouble in the multicomponent plot. With this knowledge, the user can choose whether or not to disregard the results from one well rather than rerun the reaction.

Conclusion

The simulated conditions described in this paper represent examples of common situations that may arise in any laboratory's real-time PCR experiments. Given the prevalence of slight variations and artifacts associated with real-time PCR systems, there is value in using ROX dye as a normalizing and troubleshooting agent when analyzing real-time PCR data. All Applied Biosystems real-time PCR instruments have the capability to run and analyze experiments with or without ROX dye, and even allow the ROX dye signal to be removed from the analysis post-run, if desired. Without ROX dye or the multicomponenting capability of Applied Biosystems real-time PCR instruments, many sources of error may go undetected and, as a result, data may be misinterpreted or discarded altogether. ROX dye enables users of Applied Biosystems instruments to run fewer replicates and have higher confidence in the resulting real-time PCR data.

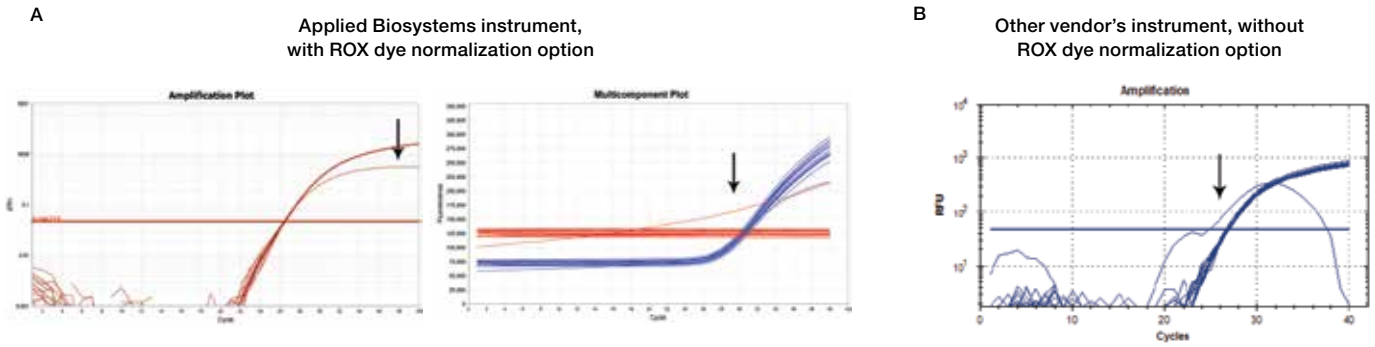

Figure 7. ROX dye makes it easier to distinguish between PCR-related and non-PCR-related events, such as evaporation in a well. (A) On the Applied Biosystems instrument, evaporation in one well (in this case, caused by a punctured plate cover) can be visualized. With ROX dye normalization the system calculates an approximate amplification curve, but the multicomponent plot shows that there is a problem with the well and can be used for troubleshooting. **(B)** The same experimental setup run on another vendor's instrument that lacks the capability of normalizing data with ROX dye results in indecipherable data.

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