Using Passive Reference Dyes for Normalization and Troubleshooting in qPCR

Introduction

Passive reference dyes are commonly used in qPCR reactions to normalize for non-PCR related fluorescence signal variation. Because the passive reference does not take part in the PCR reaction, the passive reference dye signal is stable throughout the PCR reaction. This provides a reference internal to the reaction to which the reporter dye signal (usually denoted as **R**, for Reporter) can be normalized.

Normalization typically consists of dividing the emission intensity of the reporter dye by the emission intensity of the passive reference (**P**), to obtain a ratio denoted as $\mathbf{R_n}$ (normalized reporter) for a given reaction site: $\mathbf{R_n=(R/P)}$. An $\mathbf{R_n}$ value is calculated for every cycle and every reporter and is typically plotted as an available view of the qPCR amplification data.

Three statements are often made about what the use of a passive reference achieves with respect to normalizing non-PCR related fluorescence variation:

1. **It normalizes for pipetting errors.**
2. **It normalizes for well-to-well optical variations on the instrument.**
3. **It normalizes for changes in concentration or volume of reaction mix during the qPCR run.**

This technical note discusses the accuracy of these statements and explores other benefits of using passive reference dyes in qPCR.

Passive Reference in Practice

Early in the development of qPCR technology, fluorescence excitation sources were often high-powered (laser, halogen lamp) and subject to variability due to power variation and moving optical components. Adding a passive reference provided an additional optical channel, independent of qPCR, that could be used to compensate for this source of variation. This capability was firmly established in the laboratory as standard practice and has remained so long after dramatically more stable, low-power fluorescence excitation sources have come into common use.

The first passive reference introduced was ROX (6-Carboxyl-X-Rhodamine; lmax – 610nm). Adding ROX to the qPCR Master Mix is the most popular incarnation of passive reference normalization; it is so common, in fact, that passive reference normalization is often referred to as “ROX normalization”. (It should be noted that ROX can inhibit PCR if the concentration is too high.)

ROX and Pipetting Errors

It is believed that ROX normalization can help calculate away variations caused by pipetting errors. However, because the passive reference dye is in the Master Mix, the signal will correlate to the amount of Master Mix used, with no relationship to the quantity of the target. This means that template pipetting errors can actually be compounded by this addition of an independent source of noise. Adding a passive reference to the template mix, rather than the Master Mix, would likely have some benefit with respect to normalization. However, because ROX interacts with DNA, this needs some care in practice.

ROX and Bubbles

The most significant contribution of ROX normalization is that it can correct for changes in the sample during PCR due to bubbles. Most detectors are optimized to collect fluorescence signal from the sample when in the expected location at the bottom of the well, so any movement in the sample - such as from an expanding bubble lifting the sample off the well bottom - results in a signal drop, even though the PCR reaction itself is largely unaffected. Correction works because the sample has simply been moved within the optical interogation site by a bubble. Because both the passive reference and sample probe dye are homogeneously distributed through the sample, the fluorescence signals change in proportion with one another. The observed impact is that in the **R** plot there is an abrupt fall in signal at some point in the run, but the $\mathbf{R_n}$ plot is barely affected and the data remains usable. That said, bubble compensation via passive reference only works if it is true that the optical path is the same for both the passive reference and probe and, ideally, that the two signals are collected at the same moment in time.

ROX, Condenstion, and Evaporation

It is sometimes argued that both condensation and evaporative loss can be compensated for with passive reference normalization. However, both condensation and evaporation loss remove water from the reaction mix and disrupt the reaction concentration. (The only difference is that, in the case of condensation, the water is retained on the tube walls and lid, whereas with evaporative loss, the water has escaped the tube entirely.) In both cases, loss of water from the reaction changes the concentration of reaction mix and directly impacts the PCR reaction such that the growth curve will be affected. Passive reference cannot compensate for this effect.

ROX and System Variation over Time

Some excitation sources, such as halogen lamps and lasers, can be expected to require routine replacement or to change output intensity over the instrument lifetime. For example, halogen lamp output will fall over its typical 1000-hour life and the replacement is likely to have a different light energy distribution than its predecessor. Because of these factors, some level of correction for intensity variation is preferred to maintain system performance over time. The preferred method for some manufacturers is passive reference normalization.

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Passive Reference Normalization in Eco

Eco excites at two optical wavelengths, one blue and one green; data is collected at four emission wavelengths, two measured with blue excitation and two with green. This improves detection sensitivity compared to a system with blue excitation only, by improving performance with dyes towards the red end of the spectrum, such as CY5.

Eco collects data simultaneously for all wells for any given emission channel. Eco can read a mix of up to four dyes in the optical range by treating the four emission channel measurements as a four point spectrum and mathematically deconvolving the spectra into component parts.

Eco uses very-long-life LEDs as the excitation source; the LEDs are not expected to change, fail, nor decay during the lifetime of the instrument. Therefore, compensation for run-to-run energy decay is unnecessary and neither is there a need to correct for significant shifts in excitation variability due to lamp replacement and degradation.

Eco uses off-axis excitation optics meaning that the excitation and emission use different optical paths. The benefits are that it minimizes moving parts for user installation, reliability, and robustness and avoids the optical energy loss associated with a beam splitter / dichroic. However, this design means that ROX normalization must be implemented on the Eco system in a different manner. Because the excitation path for the blue LEDs is biased to one side of the tube and the green excitation is biased to the other, it is possible that a bubble could form in the sample that is much more strongly visible in one channel than the other. For example, assume the reporter is FAM (excited by blue) and the passive reference is ROX (excited by green). If a bubble forms mostly in the blue excitation path, then the fall in blue signal would be a larger proportion of the initial signal than the fall in ROX, so the ratio would also fall and compensation would be inadequate.

Passive Reference Implementation in the Eco Software v3.1

Eco excites the ROX passive reference dye with a green light source generating a passive reference signal ($\mathbf{P_{gr}}$) that is optically independent of the blue excitation light source used to generate signal from the most common reporter dyes ($\mathbf{R_{bl}}$). Therefore, simply generating $\mathbf{R_n=R_{bl}/P_{gr}}$ increases spread in the data because the excitation energies are not paired. To overcome this spread there is a need to calculate a value for **P** that eliminates the introduced spread, while retaining the diagnostic power of the passive reference.

This is accomplished in Eco Software version 3.1 by applying a calculation to the passive reference, similar to the base-line algorithm used on the reporter data, to generate a baseline-corrected **P** value, $\mathbf{P_{corr}}$. The value of $\mathbf{P_{corr}}$ removes variability due to the system, but if a non-PCR related data shift occurs (caused by bubbles, evaporation, or poor mixing), it will remain visible in the passive reference data.

Conclusion

Use of a passive reference introduces an additional source of low-level system noise into qPCR data independent of the instrument system being used. However, many labs have determined that the improved ability to identify, diagnose, and partially compensate for non-PCR related variability is worth the price.

Diagnostic Power of a Passive Reference Channel

A passive reference signal can correct for:

- **Bubbles forming in the well during PCR (depending on optical design)**

Can be used to diagnose, but not corrrect for:

- **Bubbles forming in the well during PCR**
- **Some pipetting errors**
- **Poor mixing**
- **Evaporative loss**

Cannot:

- **Normalize for pipetting errors**
- **Normalize for changes in concentration during PCR due to evaporative loss**
- **Normalize for well-to-well optical variations of the instrument**

Does Eco Need Passive Reference Normalization?

Eco does not experience optical detection variations during a run so passive reference normalization is not required for this role.

Compensation or normalization for bubbles forming in the well can work well in some systems, but it is questionable whether this is a good idea in a microplate-based system because bubbles can make a real impact on the thermal profile of the sample, consequently impacting PCR efficiency. Changes in the data may be caused by both optical and actual PCR efficiency changes.

In summary, Eco does not need passive reference correction because:

- **Eco optical detection does not change over time**
- **Eco optics are calibrated in the factory to specific dye concentrations**

For Eco, the benefits of the current implementation are primarily diagnostic for bubble formation, evaporation, and poor mixing. Typically a quick review of the passive reference channel data to look for drift, step changes, and other anomalies can be helpful in diagnosing evaporation (caused, for example, by an improperly placed adhesive seal), bubbles (caused, for example, by a failure to centrifuge the plate prior to PCR), and poor pre-PCR mixing.

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