Optimization of Probe Performance in Real-Time PCR through an Understanding of Synthesis Impurities

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Introduction

The quality of dual-labeled probes after synthesis and purification dramatically affects the sensitivity and reproducibility of real-time PCR assays. We investigate how several synthesis impurities impact the performance of 5' reporter/3' quencher-labeled oligos. Residual fluorescent contamination is routinely removed using reverse phase HPLC. However, this method is ill-suited to separate the failure sequence impurities that result from poor reporter or base coupling. We show that these quencher-only species don't raise the level of background fluorescence, but instead suppress performance/sensitivity through competition with the dual-labeled probe to bind the target. Use of amidite chemistry combined with methods to screen for failure sequences is a rigorous approach that ensures consistent performance across different syntheses and even different assays.

"Probe quality is a major issue and in our experience is the major reason why an assay fails to perform according to expectation" - Stephen Bustin and Tania Nolan, *A-Z of Quantitative PCR*

Different oligonucleotide vendors apply different approaches to both their probe synthesis and subsequent purification, likely resulting in different degrees of contamination and performance. In our experience, syntheses sporadically suffer from two classes of impurities:

1) Quencher-only failure sequences
- Can be removed by anion exchange HPLC (AX-HPLC)
- Modeled using synthesized BHQ-only failure sequences (BOFS):
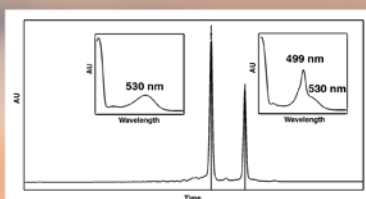
5' - full probe sequence - BHQ1 -3'

2) Reporter-only contamination
- Routinely removed by reverse phase HPLC (RP-HPLC)
- Modeled using synthesized FAM-only failure sequences (FOFS):

5' - FAM - full probe sequence - 3'

Figure 1: AX-HPLC trace of a crude synthesis containing BHQ-only contamination. Inserts: Absorption spectra of the eluted peaks
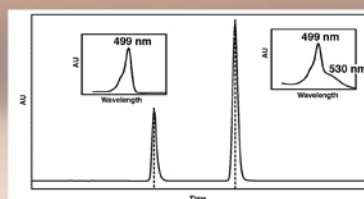
Figure 2: RP-HPLC trace of a crude synthesis containing FAM-only contamination. Inserts: Absorption spectra of the eluted peaks

To investigate the impact on performance from these contaminants, we synthesized artificial impurities (BOFS & FOFS) and spiked real-time PCR reactions at concentrations ranging from 2% - 150% the probe concentration. Using an ABI 7700, six different assays were characterized in this manner, and here we present the results for three of these that typify the group. Because the impurities contain the entire probe sequence, we expect them to bind the target in a manner similar to the probe, reducing the probability that a replication event will release fluorescence.
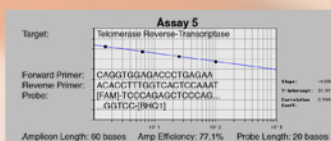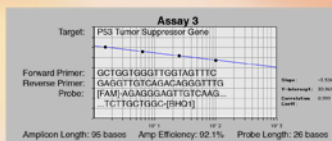

Figure 3: Oligo sequences and reaction characteristics for each assay. Standard curves document a 4-fold dilution series and reveal the amplification efficiency

BHQ-Only Failure Sequence Contamination
- BOFS contamination suppresses the normalized dynamic range of fluorescence (ΔRn) and delays the threshold cycle (CT) of real-time PCR amplifications.
- Probe performance calculated from the final ΔRn values and from the CT values correlates with performance suppression from a probe competition model.
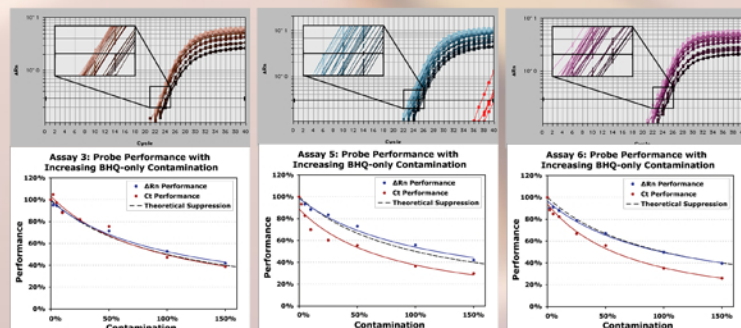- BOFS mildly reduce the detected range of exponential amplification by 1-2 cycles.

FAM-Only Failure Sequence Contamination
- FOFS contamination produces jagged amplification traces that vary widely in their ΔRn and CT values.
- In contrast to BOFS contamination, the detection of the exponential phase is dramatically obscured due to elevated baseline fluorescence.
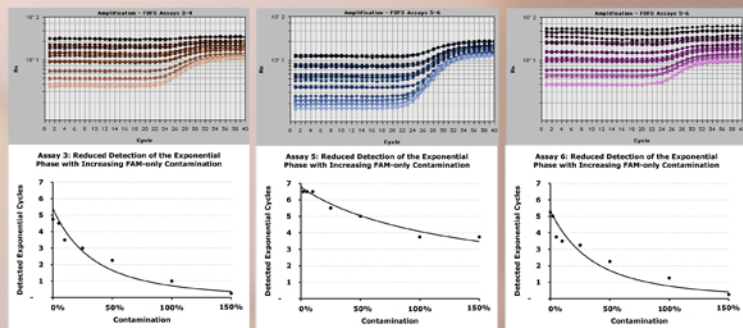

Figure 4: Top: Semi-Log plots of ΔRn vs. Cycle # document the amplifications of each assay. Increasing levels of BOFS contamination are represented by more darkly-colored traces. Inserts magnify the range of CT values where amplifications crosses the threshold. Bottom: Performance suppression is calculated from ΔRn values as follows: Performance = $\Delta Rn_{CONTAMINATED} / \Delta Rn_{PURE}$. Performance is calculated from CT values as follows: Performance = $1/(E^{\wedge}(\Delta CT_{contaminated-pure}))$ where E is the amplification efficiency

Figure 5: Top: Semi-Log plots of Rn vs. Cycle # document the amplifications of each assay. Increasing levels of FOFS contamination are represented by more darkly-colored traces. Bottom: The detected number of exponential cycles are recorded for each amplification trace. A cycle was deemed to be exponential if its fluorescence intensity was above the baseline noise and was ≥ 1.4 when divided into the intensity of the subsequent cycle.

Methods for Screening Against Synthesis Impurities

- BOFS contamination can be separated and removed by AX-HPLC, but co-elutes during RP-HPLC analysis.
- BOFS contamination can still be detected during reverse-phase HPLC by comparing the absorption spectrum of an eluted peak to that of a library of pure probe spectra. The resulting match coefficient of this comparison is a center-weighted average distance of the spectra.
- Concentration differences between library standards and experimental samples will impact the value of the match coefficient.
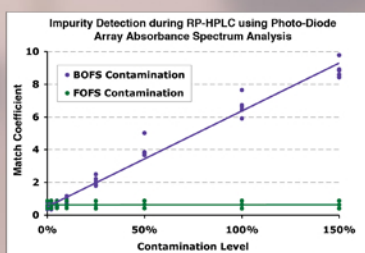
- FOFS contamination is currently removed by Reverse-Phase HPLC.
- For applications where this purification stringency will not be applied, FOFS contamination can still be screened against by comparing the absorbances of nucleic acid and BHQ1, looking for the absence of this label.
- We measured nucleic acid at 260nm and BHQ1 at 567, 583, and 596nm, where FAM has no overlapping absorbance.
- To boost the accuracy of this analysis, the probe's extinction coefficient at 260 nm must be accounted for.

Figure 6: The resulting match coefficient of five different probe sequences at varying levels of BOFS and FOFS contamination

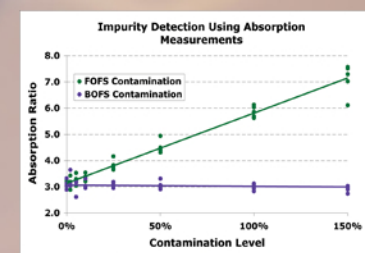**Figure 7:** FOFS contamination is revealed by taking (250,000 / Ext. Coeff.) x (A_{260} / (A_{567} + A_{583} + A_{596}))

Conclusion

While both FAM-only and BHQ-only contaminants impact the reproducibility and sensitivity of real-time PCR assays, FAM-only failure sequences represent the more severe because of their effect on the depth and uniformity of amplification traces, and should be avoided whenever possible. Different assays demonstrate different sensitivities to each impurity, perhaps due to the contribution of the labels upon the stability of probe annealing. To manufacture high-performing probes, synthesis impurities can be diminished from the outset by utilizing amidite chemistry with its superior labeling efficiency over ester coupling. For applications such as molecular diagnostics, which demand highly reproducible probe performance, a regime of AX-HPLC followed by RP-HPLC will ensure purity, but is expensive and greatly reduces the yield of dual-labeled probe. For those applications that don't demand rigorous purification, such as validating microarray results, we have presented inexpensive methods for detecting contamination that provide a forecast of probe performance.