Simple Qualitative and Quantitative Methods for Measuring Residual Activity of "Hot-Start" Enzyme Preparations

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ABSTRACT

PCR precision, specificity, sensitivity and yield of "Hot-Start" DNA polymerases are often directly related to the effectiveness of the method used to impart the "Hot Start" feature as well as the stability of the resulting product. For example, "Hot-Start" enzymes containing elevated levels of residual (not rendered dormant by chemical modification) polymerase activity (RPA) have been implicated in failures in some low copy and multiplex assays. Standard methods for quantifying RPA present in "Hot-Start" DNA polymerases or "Hot-Start" DNA polymerase Master Mixes involve tedious and time-consuming protocols based on direct measurement of alpha-P^{32} dNTP incorporation. Measuring fractions of a percent of residual activity in a Master Mix formulation is rather challenging, given the dilution of enzyme, even with a radiolabel. We have developed a set of non-radioactive, PCR-based assays useful for the detection and quantification of a wide range of RPA levels present in "hot start" DNA polymerase preparations and Master Mixes.

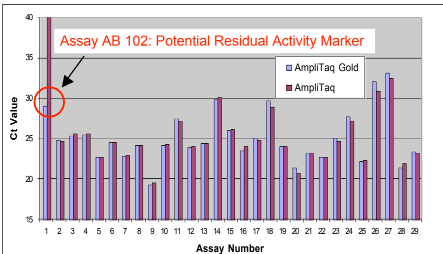
One-hundred fifty real-time assays were designed to have variation in performance. These assays were screened for sensitivity to AmpliTaq Gold® DNA Polymerase RPA using master mixes containing either AmpliTaq® DNA polymerase or AmpliTaq Gold® DNA polymerase and comparing agarose gel electrophoresis band patterns. Assays demonstrating higher amplicon yields with diminished side products due to the "Hot-Start" were further characterized by titrating AmpliTaq Gold Universal PCR Master Mix with increasing amounts of AmpliTaq DNA Polymerase. Three assays showed clear-cut gel patterns that are useful for estimating incremental RPA in the 0-10% ranges. Another assay demonstrated high incremental sensitivity to RPA in the 0-1% range. SYBR® Green PCR end-point melting curve analyses of 0% RPA (no added AmpliTaq® DNA polymerase) samples showed a major amplicon peak (Tm=81°C) and minor dimer peak (Tm=74°C). At 1% AmpliTaq® DNA polymerase addition the dimer peak increased while the amplicon peak completely disappeared. This phenomenon was exploited to develop a highly reproducible, quantitative SYBR Green assay method for RPA detection in the 0-1% range that can be performed on Applied Biosystems Sequence Detection platforms.

MATERIALS AND METHODS

1. Real-time assays were designed to have variation in performance. Real-time PCR and SYBR Green PCR were performed in 25 µL reactions on the ABI PRISM® 7900 HT Sequence Detection System using the TaqMan Universal PCR Master Mix and SYBR Green PCR reagents.
2. Template cDNA was derived from Raji male RNA using the Applied Biosystems High Capacity cDNA Archive Kit.
3. PCR Products were analyzed on 4% agarose gels

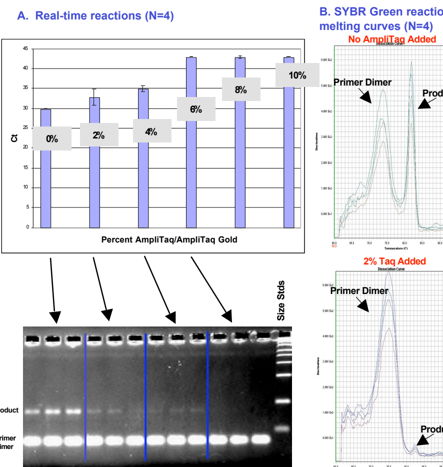
RESULTS

Figure 1. Real-time Assays that were Designed to have Variation in Performance were Screened for Residual Activity Markers using AmpliTaq® DNA Polymerase (purple bars) or AmpliTaq Gold® DNA Polymerase (blue bars).



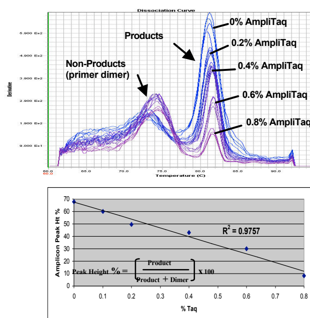
150 duplicate reactions were performed with either AmpliTaq DNA Polymerase or AmpliTaq Gold DNA Polymerase. One assay ("102") from the group shown above demonstrating a significant delta Ct was re-tested adding incremental amounts of AmpliTaq DNA polymerase and analyzed by gel electrophoresis or SYBR Green PCR dissociation curve analysis (Figure 2).

Figure 2. AmpliTaq® DNA Polymerase Titrations of Real-Time PCR and SYBR Green PCR Reactions Containing AmpliTaq Gold® DNA Polymerase.



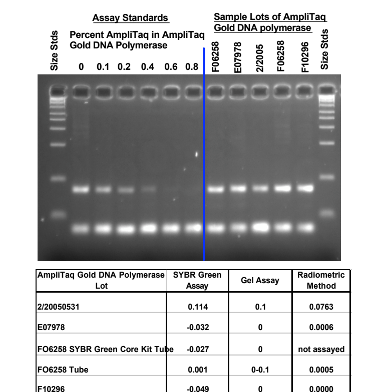
Adding incremental amounts of AmpliTaq® DNA polymerase to TaqMan® Universal Master Mix PCR reactions containing AmpliTaq Gold DNA polymerase yields a "dose response" increase in Ct and corresponding reduction in product also observed on gels and by end-point melting curve analysis of similar SYBR Green PCR reactions.

Figure 3. SYBR Green Dissociation Curves (N=4) for Reactions Containing AmpliTaq Gold® DNA Polymerase Titrated with 0-0.8% AmpliTaq DNA Polymerase.



Plotting product peak height as a percent of both peak heights yields a linear curve from which the % residual activity of samples can be measured. Alternatively, semi-quantitative gel assay results can be obtained by comparing the ratio of product to non-product (primer-dimer) band intensity relative to a set of standards (see below).

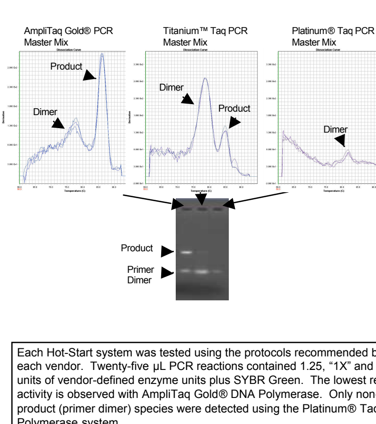
Figure 4. Accuracy of the Quantitative SYBR Green and Semi-Quantitative Gel Methods Compared to the Standard Radiometric Method. Samples are Commercial Lots of AmpliTaq Gold® DNA Polymerase



AmpliTaq Gold DNA Polymerase Lot	SYBR Green Assay	Gel Assay	Radiometric Method
2/20050531	0.114	0.1	0.0763
E07978	-0.032	0	0.0006
FO6258 SYBR Green Core Kit Tube	-0.027	0	not assayed
FO6258 Tube	0.001	0-0.1	0.0005
F10296	-0.049	0	0.0000

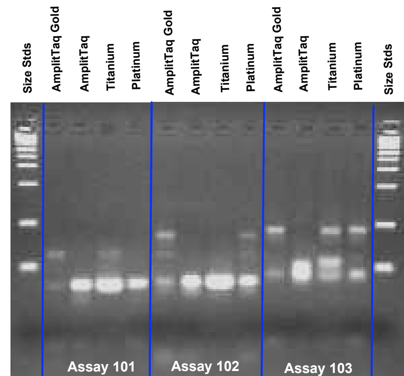
SYBR Green results <0 reflect lower sample residual activity compared to the standard. Good correlation is observed between the agarose gel electrophoresis and SYBR Green methods and between these and the radiometric method considering the lower limit of detection of the radiometric method.

Figure 5. Residual Activity of Hot-Start Master Mixes Compared: AmpliTaq Gold® DNA Polymerase, Titanium™ Taq DNA Polymerase and Platinum® Taq DNA Polymerase. Assay "102" using Vendor Recommended Enzyme Concentration & Conditions.



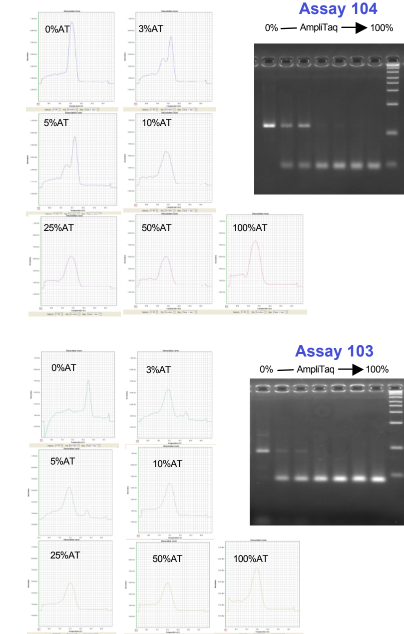
Each Hot-Start system was tested using the protocols recommended by each vendor. Twenty-five µL PCR reactions contained 1.25, "1X" and 0.5 units of vendor-defined enzyme units plus SYBR Green. The lowest residual activity is observed with AmpliTaq Gold® DNA Polymerase. Only non-product (primer dimer) species were detected using the Platinum® Taq DNA Polymerase system.

Figure 6. Residual Activity of Hot-Start Master Mixes Compared: AmpliTaq Gold® DNA Polymerase, Titanium™ Taq DNA Polymerase and Platinum® Taq DNA Polymerase. One Unit/25 µL Reaction Using Vendor Recommended Conditions).



Each Hot-Start system was tested using the protocols recommended by each vendor. Polymerase activity was measured by the radiometric method and adjusted to 1 unit per reaction. The lowest residual activity is observed with AmpliTaq Gold® DNA Polymerase.

Figure 7. AmpliTaq® DNA Polymerase Titration of Assay 103 and One Other Assay (104) Sensitive to Hot-Start Residual PCR Activity.



CONCLUSIONS

A collection of PCR gel assays and a sensitive quantitative SYBR Green PCR assay were developed to measure Taq residual activity (i.e., not rendered dormant by chemical or other modification) present in Hot-Start Taq DNA polymerases, including AmpliTaq Gold® DNA polymerase. The most sensitive method measures residual activity in the 0-0.1% range using either the agarose gel electrophoresis method or SYBR Green approach. These new methods are approximately two orders of magnitude lower in sensitivity compared to the standard radiometric method but are much faster and less tedious to perform. Four lots of AmpliTaq Gold® DNA polymerase analyzed by the new methods gave results similar to the radiometric method within the limits of detection. Using these methods the **AmpliTaq Gold® DNA Polymerase was shown to out-perform the Titanium™ Taq DNA Polymerase and Platinum® Taq DNA Polymerase systems** based on amplification of specific PCR product and suppression of formation of non-specific PCR products.

ACKNOWLEDGEMENTS

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