A Novel Assay for DNA-Dependent DNA Polymerase Activity

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Abstract

Human, viral, and bacterial DNA polymerases are attractive targets for drug interventions, and as such are candidates for HTS screening efforts. We have developed an assay for DNA-dependent DNA polymerase activity using a new assay platform developed by Meso Scale Discovery™ (MSD™). This platform combines array technologies and electrochemiluminescence detection to achieve ultra-fast, highly sensitive assays in a no-wash format. Polymerase activity results in the extension of a primer containing a ruthenium complex that emits electrochemiluminescence. The assay is exquisitely sensitive to the processivity of the enzyme and only detects full-length products. Several distinct polymerases (Klenow and the HIV RT enzymes) were analyzed in the assay. Several known inhibitors were screened to validate the assay format. We have demonstrated feasibility of assay automation in a 96-well format, and developed protocols to enable minimal manipulations.

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Multi-Array™ Technology

Unified technology platform with instruments, plates and reagents for drug discovery.

Combines the power of microarrays with the sensitivity of electrochemiluminescence.

96-, 384- and 1536 microplate formats.

Multi-Spot™ plates with high density arrays for multiplexing.

Sector HTS™ Instrument: High resolution imaging detection and robotic integration for HTS and large-scale proteomics.

Sector PR™ Instrument: Medium throughput benchtop reader for assay development, cellular and molecular biology, research in therapeutic areas, secondary screening, QC. Assays developed on Sector PR port to Sector HTS.

Sector HTS

Sector PR

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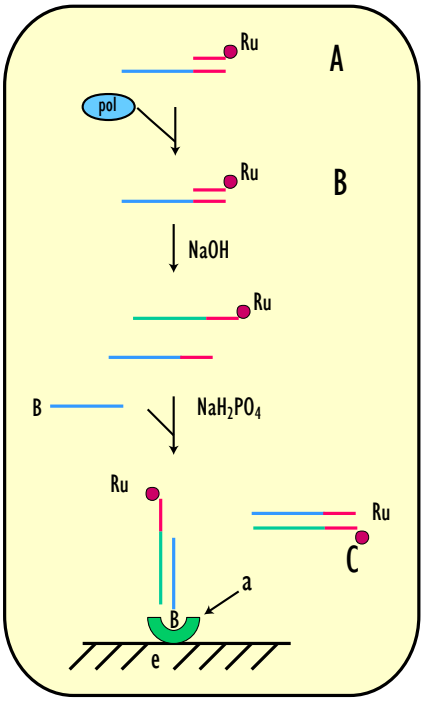
Schematic of the DNA-Dependent DNA Polymerase Assay



The substrate is a heteroduplex containing an annealed region (red) for priming and a template region (blue) for elongation (A). The primer to be elongated has a ruthenium chelate (Ru) attached to the 5' end. After elongation by the DDDP activity of the polymerase (B), the reaction is stopped with NaOH. Subsequently, the reaction is neutralized by the addition of NaH_2PO_4 containing a biotinylated oligonucleotide complementary to the newly synthesized strand. The entire reaction is conducted in a well containing an avidin-coated (a) carbon electrode (e). The biotinylated oligonucleotide serves as a capture probe that tethers the elongated, Ru-containing strand to the electrode. In the standard reaction conditions, a 20ul reaction is conducted using 1nM HIV-RT (Worthington) and 50nM substrate in reaction buffer (10mM Tris pH 7.5, 5mM $MgCl_2$, 3mM DTT, 10uM dNTPs). The reaction is stopped by the addition of 25ul of base to a final concentration of 30mM followed by 25ul of 90mM NaH_2PO_4 containing 4 pMol of biotinylated capture probe. The microtiter plate is agitated throughout the 45 minute capture period. Note that re-annealing of sister strands (C) competes with capture and immobilization of the Ru-labeled species at the surface of the electrode. The data shown here were obtained using HIV-RT. However, with slight changes to the reaction buffer, similar results were obtained with the Klenow polymerase of E. coli and human DNA polymerase beta. The reaction is distinguished from those that measure nucleotide incorporation in that only full-length products capable of hybridizing are detected.

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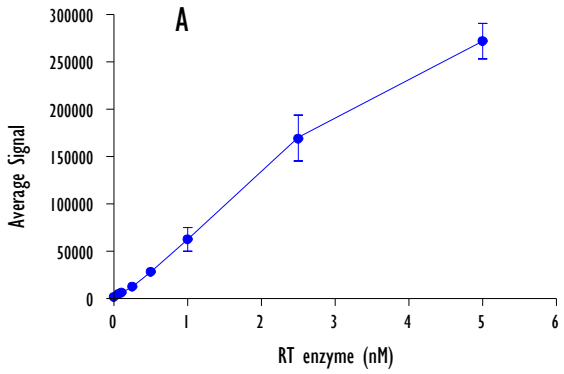
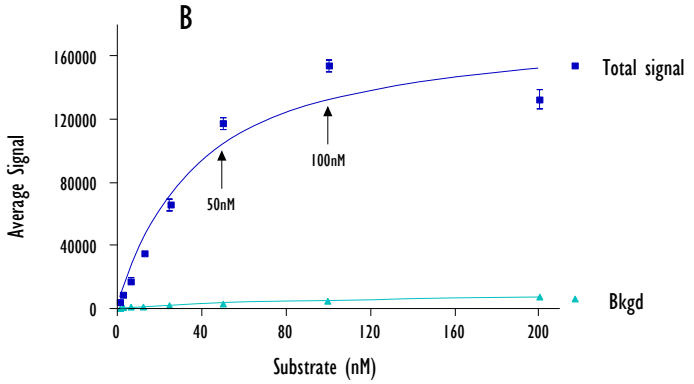
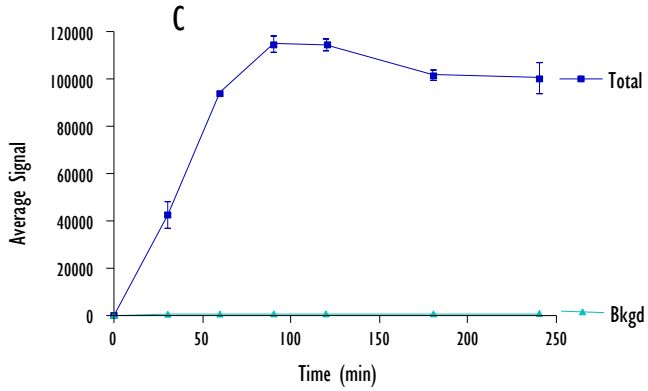
Kinetic Analysis of the Polymerase Reaction







The DNA polymerase reaction was performed under standard conditions except for variation in enzyme (A) and substrate (B) concentrations. The reaction is linear with respect to enzyme concentration over the range studied. The standard reaction conditions were chosen such that the linear dependence on enzyme (A) and time (C) are represented while substrate is present in excess.

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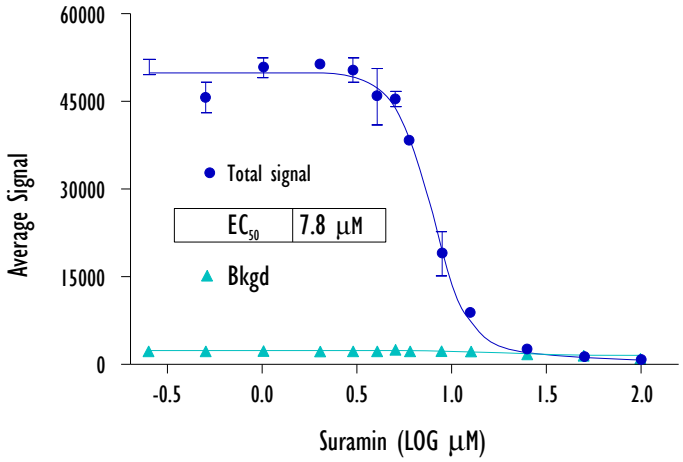
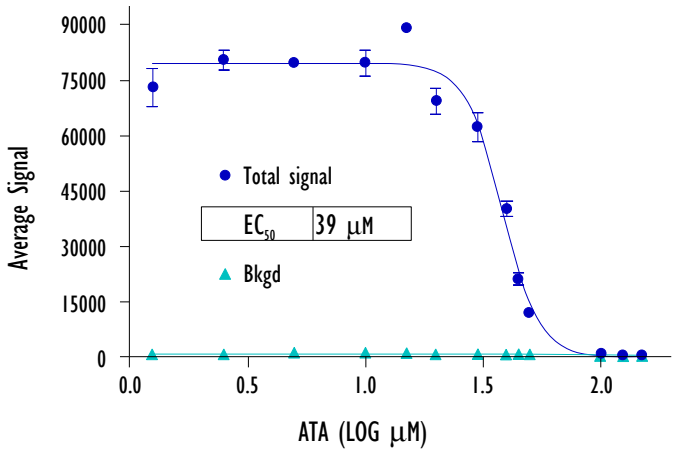
The DNA-Dependent DNA Polymerase Assay Facilitates EC_{50} Determinations

Suramin



AurintricarboxylicAcid



The effects of suramin and aurintricarboxylic acid (ATA), two inhibitors known to affect the reverse transcriptase (RNA-dependent DNA polymerase) activity of the HIV RT enzyme, were tested for effects on the DNA-dependent DNA polymerase activity of this multifunctional enzyme. Our results generated EC_{50} values for both inhibitors as indicated.

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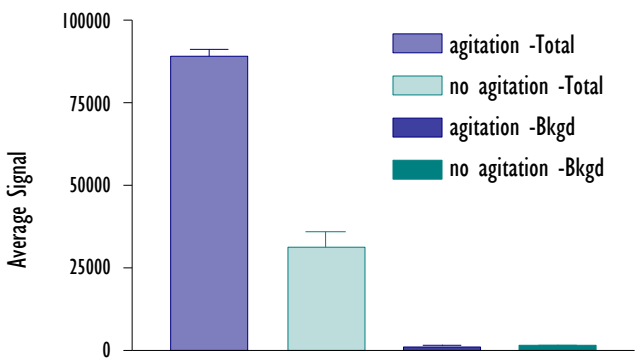
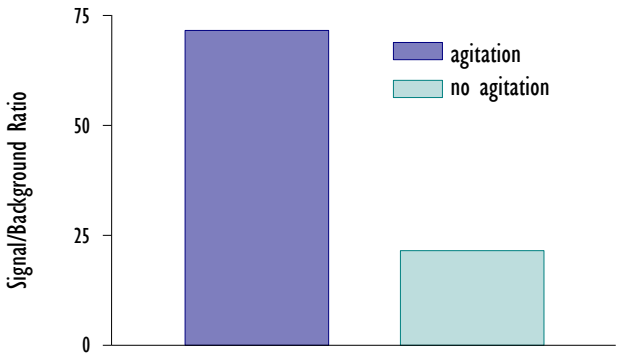
A Simplified Protocol that Omits Agitation Retains a High Signal/Background Ratio

Total Signal



Signal/Background



The standard DNA-dependent DNA polymerase assay was conducted except the protocol eliminated agitation during the capture step. To compensate for the lack of agitation, the capture time was extended to 3 hours. In the absence of agitation, there is a significant decrease in total signal obtained, despite the longer capture time allowed. This decreased signal is likely the result of increased sister strand re-annealing (see schematic). The signal/background ratio also decreases. However, the signal/background ratio remains high (22) despite the elimination of the agitation step. The results support the notion that an automated assay without agitation can be executed.

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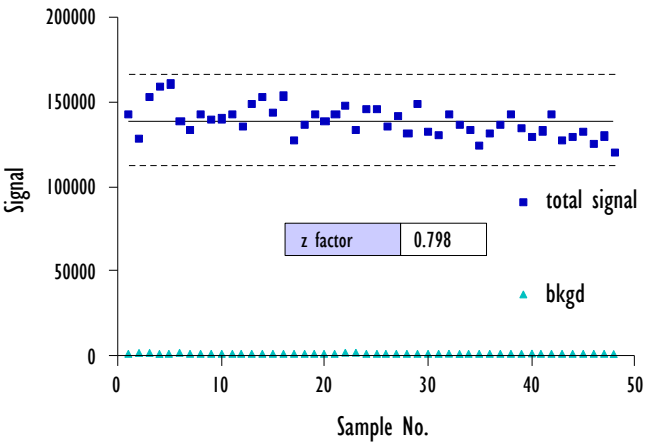
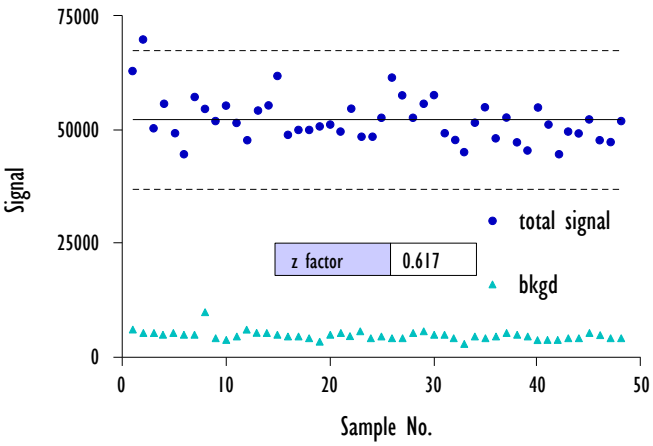
Validation of Alternative Assay Formats for HTS

+ wash, + agitation



-wash, -agitation



Two workflow protocols were compared for performance based on Z-factor scores. The standard assay was compared to an assay in which there were no washes. In the no-wash assay, the agitation during capture was eliminated and the capture time was extended to 3 hours. In both cases, the assays were executed in an automated fashion in which a Rapid-Plate™ (Zymark) and a Multidrop™ (Labsystems) were used for reagent delivery, aspiration and washing, where appropriate. The HIV RT enzyme was used, though similar results have been obtained with the Klenow polymerase for non-washed and washed formats. Acceptable Z-factor values (>0.5) support the notion that both protocols can be used in HTS.

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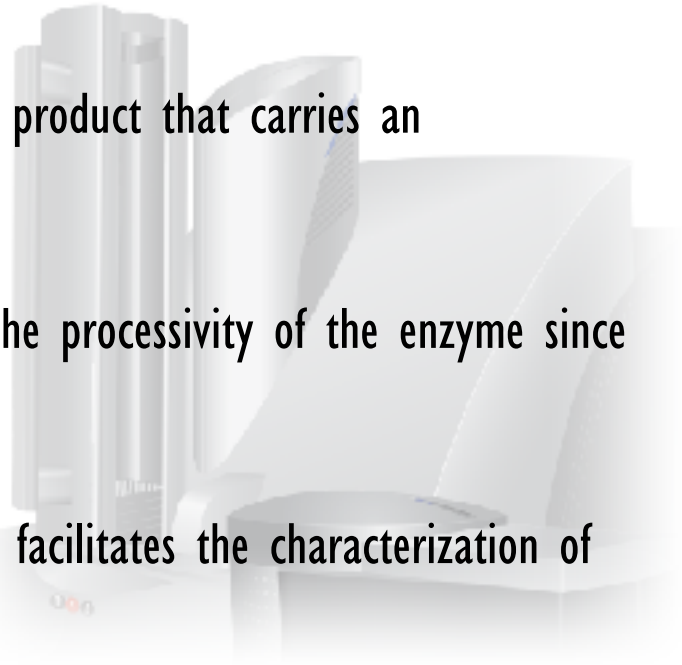
Conclusion

A DNA-dependent DNA polymerase assay has been developed on a novel platform that utilizes disposable carbon electrodes arrayed in microtiter plates.

A DNA capture probe immobilizes the product that carries an electrochemiluminescent reporter.

The assay is particularly sensitive to the processivity of the enzyme since only full-length products are detected.

The assay is compatible with HTS and facilitates the characterization of EC_{50} values of inhibitors.



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