

Reverse Transcriptase Assay on FlashPlate[®] PLUS

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Introduction

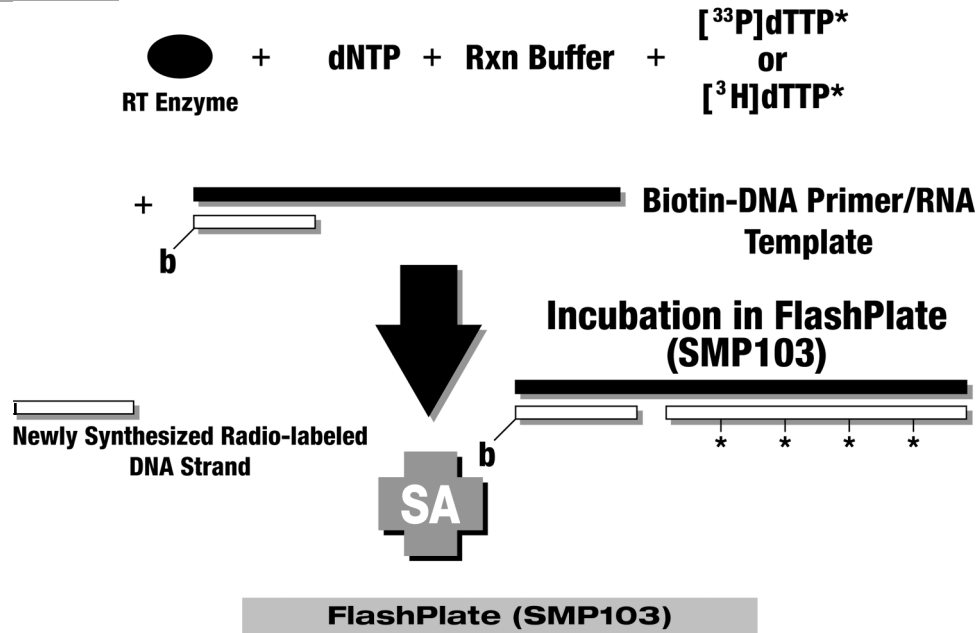
FlashPlates are scintillant coated microplates designed for radiometric high throughput screening assays. FlashPlates can be coated with antibodies, chelated metals, enzyme substrates and other proteins for capture and functional assays. Here we demonstrate a polymerase assay formatted on a Streptavidin FlashPlate. In this assay a biotinylated DNA oligonucleotide acts as a primer for reverse transcriptase (RT) on an RNA template. The signal is generated by incorporation of labeled dTTP into the newly synthesized strand of DNA. This label can be either ^3H or ^{32}P . We examined assay performance relative to enzyme and labeled dTTP concentration as well as incubation time and temperature. Inhibition of the reaction is demonstrated with dideoxynucleotides.

Materials and Methods

Reverse transcriptase was used to extend a biotinylated, 20-base DNA oligonucleotide primer (Keystone Labs) annealed to an 89-base RNA template. A reaction mixture was prepared by adding Recombinant RNasin[®] ribonuclease inhibitor (Promega Corp.) to the DNA primer/RNA template solution at 1500 units per ml, with DTT added for a final reaction concentration of 1 mM. The DNA primer/RNA template complex was combined with a dNTP mixture consisting of dATP, dGTP, and dCTP (Promega Corp.), each at a final reaction concentration of 25 μM , buffer (50 mM Tris, pH 8.0, 10 mM MgCl_2), and either [Methyl,1',2', ^3H] dTTP (NEN, NET520A, 90-120 Ci/mmol), or [α - ^{32}P]dTTP (NEN305H, 2000-4000 Ci/mmol). The concentration of [^3H]dTTP in the reaction was 60 nM (0.5 μCi per reaction). The concentration of [^{32}P]dTTP in the reaction was 3.125 nM (0.5 μCi per reaction). This reaction mixture was then added to 96-well streptavidin FlashPlates in a volume of 60 μl per well. The reaction was initiated by the addition of recombinant HIV-1 Reverse Transcriptase (NEN, NEI490) in a volume of 20 μl per well at 0.2 units per reaction, unless otherwise indicated. Controls consisted of the reaction mixture without the addition of RT. The final 96-well assay volume was 80 μl per well. Unless otherwise indicated, the reaction was performed at 37°C. For the purpose of comparing binding capacities, the 384-well FlashPlate format was compared to the 96-well format by adding identical reagents and volumes to both plates. For inhibition studies with the nucleoside analog 2',3'-dideoxycytidine 5'-triphosphate (Pharmacia), assays were set up as follows: 10 μl of inhibitor solution, then 10 μl RT at 0.2 units per reaction, then 60 μl of reaction mixture, followed by incubation for 1 hour at 37°C; controls consisted of reaction mixture without RT, as well as reaction mixture with inhibitor, without RT. Assays utilizing [^{32}P]dTTP were terminated through an aspiration and two wash steps. Assays performed with [^3H]dTTP are homogeneous and did not require aspiration or wash steps.

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Schematic of the Reverse Transcriptase Assay on FlashPlate



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Time Course of Reverse Transcriptase Assay on 96-well Streptavidin FlashPlate

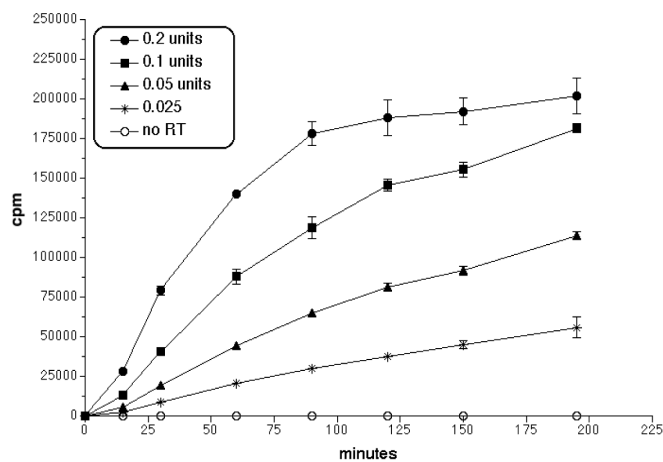


Figure 1: Reverse Transcriptase assay performance at several enzyme concentrations: 0.2 units, 0.1 units, 0.05 units, and 0.025 units per reaction. This assay was done with $[^{33}\text{P}]\text{dTTP}$. The reaction was terminated through aspiration and wash steps.

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Reverse Transcriptase Assay on 96-well Streptavidin FlashPlate Titration with Enzyme

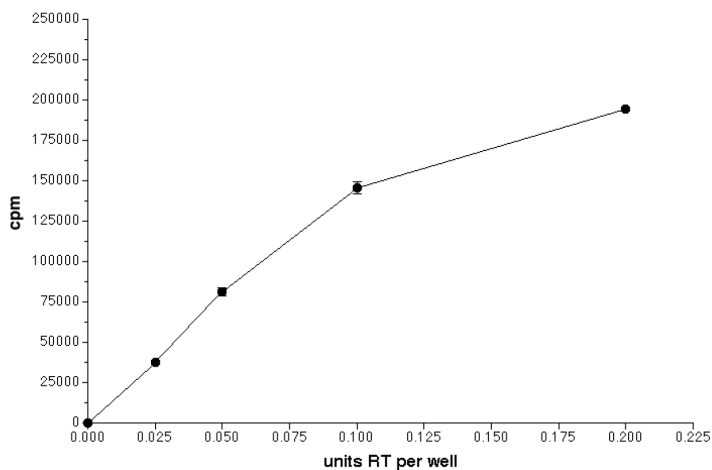


Figure 2: Reverse Transcriptase assay performance over a range of enzyme concentrations from 0.025 units to 0.2 units per reaction. The assay was done with [³³P]dTTP and terminated after 2 hours through aspiration and wash steps.

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Reverse Transcriptase Assay on 96-well Streptavidin FlashPlate Titration with [³³P]dTTP

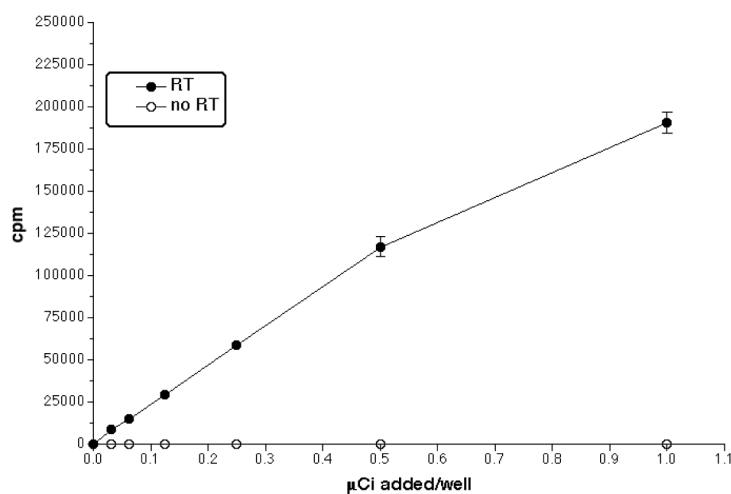


Figure 3: The Reverse Transcriptase Assay was run with a series of concentrations of [³³P]dTTP. The enzyme was used at 0.2 units per reaction. The assay was incubated for 2 hours and terminated with an aspiration and wash step.

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Temperature Dependence of Reverse Transcriptase Assay on 96-well Streptavidin FlashPlate

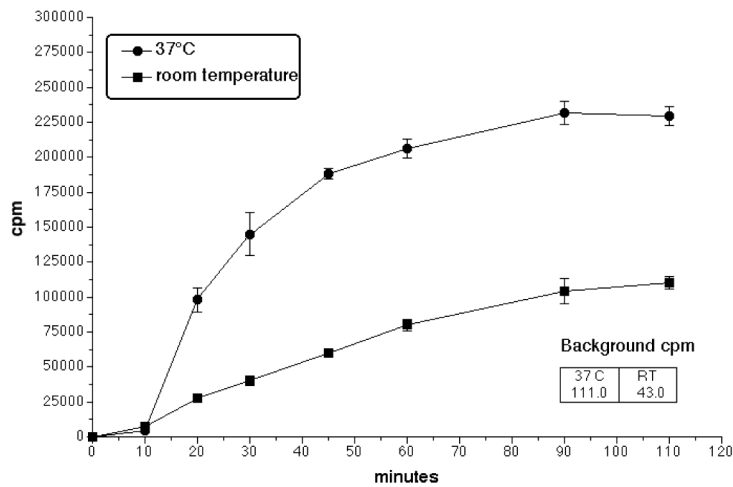


Figure 4: Reverse Transcriptase Assay incubated at 37°C and room temperature. The enzyme was used at 0.2 units/reaction and the [³³P]dTTP was 0.5 μCi/reaction. The assays were incubated at the two temperatures and the reaction terminated at the times indicated through aspiration and wash steps.

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[³³P]dTTP Format

Condition	Assay point	Average CPM	Replicates	SD	% CV	Signal:Noise
no aspiration	RT	105405	48	14713	14.0	3.6
	no RT	29585	42	2661	9.0	
aspirated	RT	116796	48	4768	4.1	8.1
	no RT	14379	42	1253	8.7	
1 wash	RT	103879	48	4028	3.9	532.7
	no RT	195	42	52	26.7*	
2 washes	RT	98092	48	4850	4.9	3772.8
	noRT	26	42	30	115.4*	

* very low CPMs cause this value to be high.

[³H]dTTP Format

Assay point	Average CPM	Replicates	SD	% CV	Signal:Noise
RT	36848	4	637	1.7	100
no RT	367	4	57	15	

Table 1: Performance characteristics of reverse transcriptase assay on 96-well FlashPlate.

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Inhibition of Reverse Transcriptase Activity with Nucleoside Analog

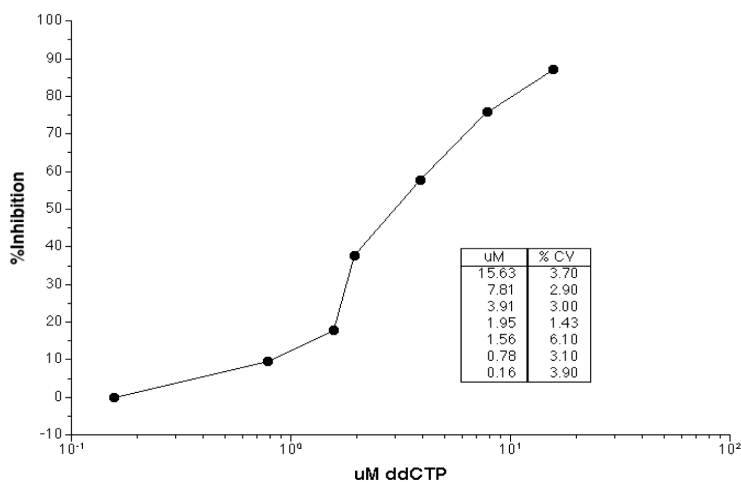


Figure 5: Reverse Transcriptase activity was inhibited with the nucleoside analog dideoxynucleotide (ddCTP). Reverse Transcriptase was added to each well followed by the addition of the inhibitor. The reaction was initiated by addition of reaction mixture (primer/template complex, nucleotides, and [³³P]dTTP). The assay was incubated at 37°C for one hour and terminated through aspiration and wash steps. Background values for absence of RT and absence of RT in the presence of inhibitor were 2 and 4 CPM, respectively.

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Reverse Transcriptase Assay on Streptavidin FlashPlates using Tritiated Substrate

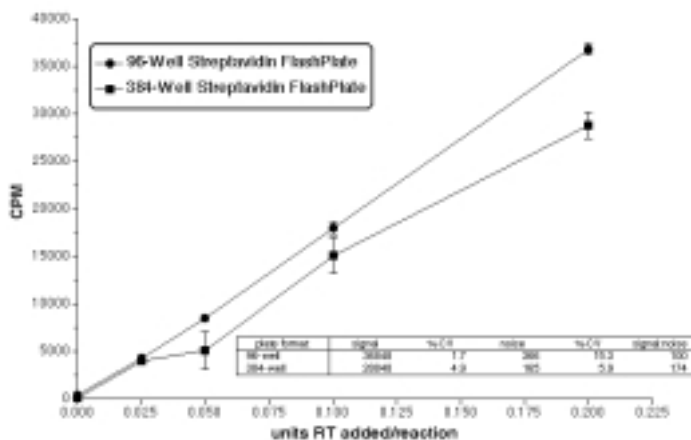


Figure 6: Reverse Transcriptase assay was performed using [³H]dTTP at 0.06 μM, 0.5 μCi per reaction. The enzyme concentration was 0.2 units per reaction. The assay was incubated at 37°C for 2.5 hours. The 96-well and 384-well formats were compared. The same reaction reagents were added to both plates; total assay volume for both plates was 80 μl per well. No wash or aspiration steps are required for the [³H]dTTP format.

Signal and Inhibited Samples

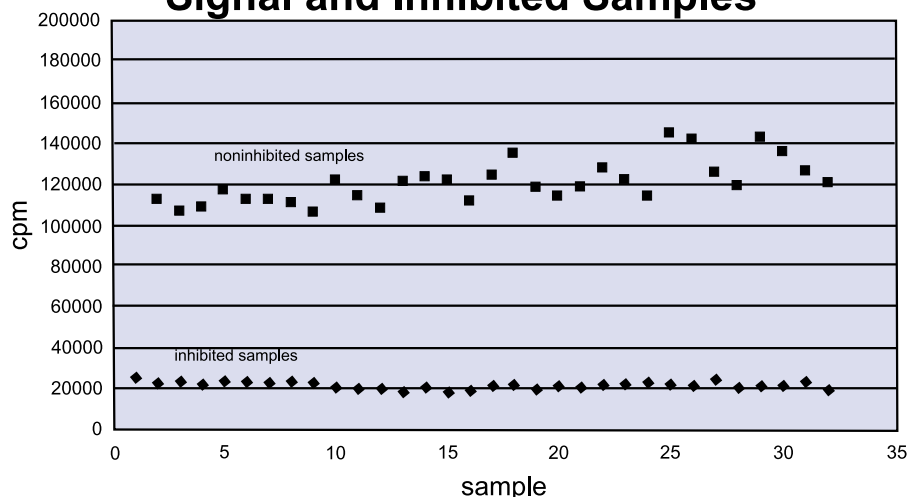


Figure 7: Values for inhibited and non-inhibited samples. The enzyme concentration was 0.2 units per reaction. The assay was done with [³³P]dTTP and terminated after one hour with aspiration and wash steps. The inhibitor concentration was 15.6 μM in the reaction.

Results and Discussion

The reverse transcriptase assay performance has been examined on both 96- and 384-well streptavidin FlashPlates in formats using either ³H or ³³P labeled substrate. The signal-to-noise ratio in the [³³P] format is > 500:1 with an aspiration and one wash step; > 3000:1 with 2 wash steps. The [³H] format is homogeneous, and does not require washing. The signal-to-noise ratio in the [³H] format is 28:1. The %CV values in both ³³P and ³H formats, 96-well plate, is < 10% (table 1).

The assay can be performed at 37°C or room temperature. The reaction rate and final signal is lower at room temperature, but a signal-to-noise ratio > 2000 can be achieved (figure 4). The assay can easily detect an enzyme concentration of 0.025 units per reaction (figure 2). The signal can be increased by adding more [³³P]dTTP per reaction, while the background remains < 0.5% of signal at 1.0 μCi added per reaction (figure 3). The reaction rate and amount of signal are affected by the enzyme concentration (figure 1). *In vitro* inhibition of RT activity has been demonstrated with the triphosphate form of nucleoside analogs; here the addition of dideoxycytidine triphosphate demonstrated 50% inhibition at 3 μM final reaction concentration, at one hour incubation at 37°C (figure 5).

The high signal-to-noise ratio, reproducibility, and flexibility of reverse transcriptase assay on FlashPlate makes it a useful tool in high throughput screening for drugs that affect polymerases.

There is a patent pending on Streptavidin FlashPlate PLUS products. FlashPlate is a registered trademark of Packard Instrument Company exclusively licensed to PerkinElmer Life Sciences, Inc. FlashPlate is protected under U.S. Patent 5,496,502 and foreign equivalents, to all of which PerkinElmer Life Sciences, Inc. holds an exclusive, worldwide license.



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