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High Capacity, Detergent Resistant, Covalent Coated Streptavidin FlashPlate® PLUS

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Introduction

FlashPlate white, opaque, 96- and 384-well scintillant coated microplates are suitable as a convenient platform for radioisotope based high throughput screening assays: FlashPlates coated with streptavidin have many applications for homogeneous binding assays and enzyme assays.

We present data comparing the performance of the newly available covalent streptavidin coated FlashPlates with streptavidin FlashPlates coated by adsorption.

1 Materials and Methods

Covalent Coating of FlashPlates with streptavidin was done by a proprietary procedure (patent pending).

Biotin binding assays were performed in Dulbecco's PBS (no Ca^{++} or Mg^{++}) at 5 pmol/ml tracer ([^{3}H]biotin, 41 Ci/mmol, NET721, NEN), and a concentration range of unlabeled biotin, to determine the 50% inhibition of tracer binding (IC_{50}), and at the highest amount of unlabeled biotin, the non-specific binding of the tracer (NSB).

Capacity assays were done using ^{3}H-labeled tracer at 300 pmol/ml for 96-well plates or 800 pmol/ml for 384-well plates. Capacities were calculated by difference between the unbound [^{3}H]biotin, determined by removing the contents of the wells and counting by LSC and the total amount added, also counted by LSC, with corrections for counter efficiency and the specific activity of the tracer. Assay volumes were 200 µl for 96-well plates and 50 µl for 384-well plates. Assay incubations were overnight at room temperature, covered, and shielded from light. The plates were counted on a Packard TopCount®-HTS Microplate Scintillation and Luminescence Counter, model B384V00.

Tyrosine Kinase Assays were performed in 384-well plates. A biotinylated peptide, biotin-EGPWLEEEEEAYGWMDF-amide (Boehringer-Mannheim, PKS-2) was dissolved in PBS to 50 pmol/µl and diluted in assay buffer. Tyrosine kinase (Upstate Biotechnology SRC Kinase $P60^{c\text{-}src}$) was diluted to 0.02 u/µl in the assay buffer containing 8 mM imidazole, pH 7.5, 8 mM glycerophosphate, 200 µM EGTA, 20 mM $MgCl_2$, 1mM $MnCl_2$, 1 mg/ml protease-free BSA. ATP was dissolved in the assay buffer at 300 pmol/µl.

Reaction initiation: 10 µl of each of the above solutions were added to the well in the sequence prepared and incubated for 90 minutes at 30°C.

2 Materials and Methods (cont.)

Reaction detection: 10 µl of 0.8 ng/µl anti-phosphotyrosine antibody (Transduction Laboratories PY20) diluted in assay buffer and 10 µl of [^{125}I]-goat anti-mouse antibody (NEX159, 7.84 µCi/µg, NEN) diluted to 6.5 nCi/µl (1.2 ng/µl) in assay buffer plus 0.03% NP40 were added to each of the wells followed by overnight incubation at 4°C. The plates were counted on a TopCount-HTS.

Reverse Transcriptase assays were done by extending a biotinylated, 20-base DNA oligonucleotide primer (Keystone Labs) annealed to an 89-base RNA template. Recombinant Rnasin® Ribonuclease Inhibitor (Promega Corp.) was added to the DNA primer/RNA template solution at 1500 u/ml. The DNA primer/RNA template complex was combined with a mixture consisting of dATP, dGTP, and dCTP (Promega Corp.) at a final reaction concentration of 25 µM in the reaction buffer (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT), and [^{3}H]dTTP (NET520A, 90-120 Ci/mmol, NEN). The concentration of [^{3}H]dTTP in the reaction was 0.06 µM (0.5 µCi/reaction). This reaction mixture was then added to the streptavidin coated 96-well FlashPlates, 60 µl/well. The reaction was initiated by the addition of recombinant HIV-1 Reverse Transcriptase (NEI490, 10,000-15,000 u/ml, NEN), initial concentration 10 u/µl, 20 µl per well. Reaction conditions were: 37°C, 2.5 hours, with binding measured on a TopCount-HTS.

The simultaneous phosphorylation and capture of a biotinylated peptide on streptavidin coated FlashPlates was done in the following manner: Cyclic AMP dependent protein kinase (PKA), catalytic subunit, 60-100 u/µl, (Promega Corp), was combined with [γ-^{33}P] ATP (NEG302H, 1,000-3000 Ci/mmol, NEN) in assay buffer (40 mM Tris-HCl, pH 7.4, 20 mM Mg acetate, 10 µM ATP) and added to the plates, 50 µl/well and 20 µl/well, 96-well and 384-well respectively. Note: reactions were done with 10 units of PKA and 1 µCi of tracer/well for both 96- and 384-well streptavidin FlashPlates. Reactions were initiated by addition of the biotinylated substrate, kemptide, (LRRASLG), (Alpha Diagnostic International) at a concentration of 100 pmol in 50 µl/well for 96- and 384-well plates. The plates were incubated at room temperature for 2.5 hours. The reaction was stopped by aspiration and 2 washes with PBS and the plates counted on a TopCount-HTS.

3 Results/Discussion

A much higher concentration of unlabeled biotin was required to inhibit 50% of the [^{3}H]biotin tracer binding to covalent coated streptavidin 96-well FlashPlates than adsorption coated streptavidin 96-well FlashPlates. This reflects the higher biotin binding capacity of the covalent streptavidin coated FlashPlates (see Figure 1).

Covalent coupling of streptavidin to 96- and 384-well FlashPlates increases the biotin binding capacity of the wells by more than 3-fold (see Figure 2).

Covalent coupling of streptavidin to FlashPlates also increases the detergent resistance of the surface compared with adsorption coated streptavidin FlashPlates. In one case, the effect was quite dramatic (see Figure 3a and Table 1). DMSO enhances biotin binding when included in the assay (see Table 1).

Covalent streptavidin coated FlashPlates are, like the adsorbed version of the product, sensitive to 100% DMSO.

Two of the three enzymatic assays presented are homogeneous. In every case the signal was higher on the covalently coupled streptavidin FlashPlate which reflects the higher biotinylated substrate binding capacity of these plates.

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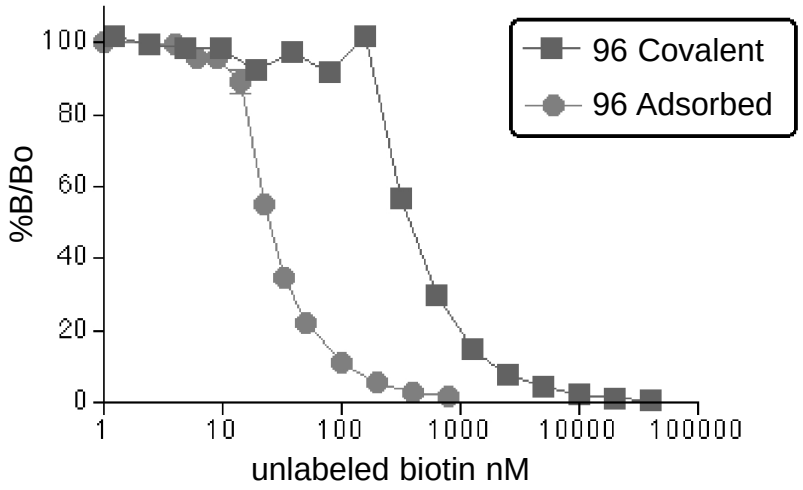

Figure 1: Competition curve of 5 pmol/ml [^{3}H]biotin tracer with added unlabeled biotin. Binding comparison of adsorption and covalent coated streptavidin FlashPlates. The 50% inhibition of tracer binding (IC_{50}) by unlabeled biotin was 300 pmol/ml for a 96-well plate and 500 pmol/ml for a 384-well plate (data not shown).

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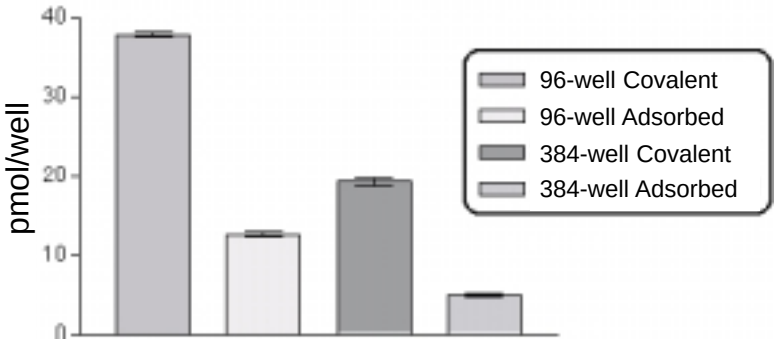

Figure 2: Biotin binding capacity comparison for adsorbed and covalent coated streptavidin FlashPlates. [^{3}H]Biotin tracer at 300 pmol/ml, 200 µl/well for 96-well plates and at 800 pmol/ml, 50 µl/well for 384-plates. [^{3}H]Biotin binding was determined by LSC quantitation of unbound tracer.

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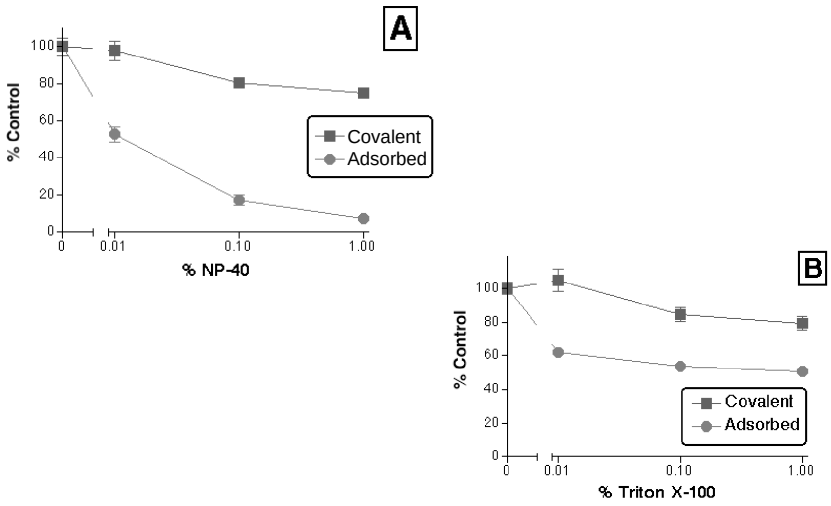

Figure 3: IC_{50} binding of 5 pmol/ml [^{3}H]biotin plus 300 pmol/ml unlabeled biotin in the presence of the indicated amount of NP40 (A) or Triton X-100 (B), assay volume 200 µl/well, 96-well plates.

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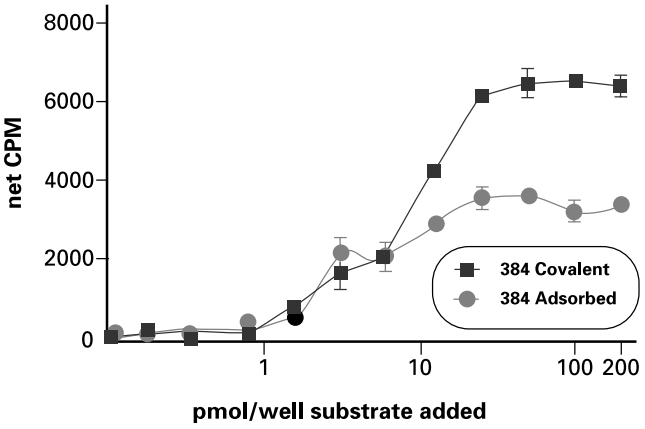

Figure 4: Tyrosine kinase assays on adsorption and covalent coated 384-well streptavidin coated FlashPlates. The reaction mixture consisted of a biotinylated 17 amino acid peptide substrate, tyrosine kinase, and ATP. Reaction detection was performed by the further addition of anti-phosphotyrosine antibody and [^{125}I]-goat anti-mouse antibody. Control wells contained all the assay reagents except the substrate. Control CPM values were deducted from the CPM values of experimental wells.

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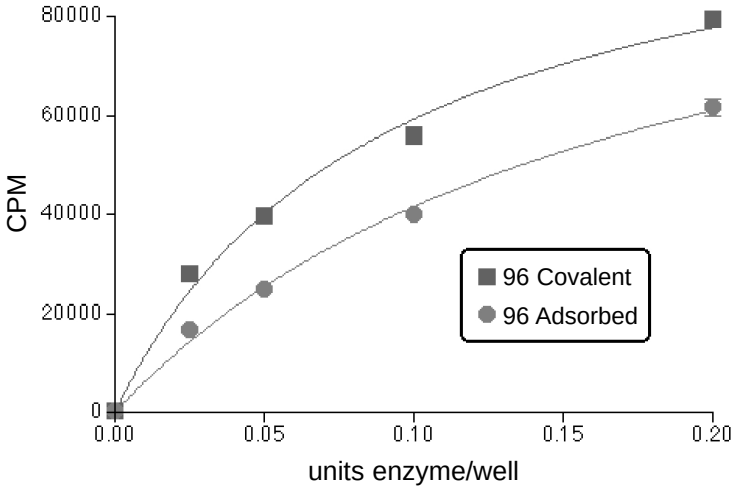

Figure 5: Reverse transcriptase assays on adsorption and covalent coated 96-well streptavidin coated FlashPlates. The assay extended a 20-base biotinylated DNA primer annealed to an 89-base RNA template in an equimolar mixture of dATP, dCTP, dGTP, plus [^{3}H]dTTP, RNAse inhibitor, and buffer (0.05 M Tris-HCl, pH 8.0, 10 mM $MgCl_2$ and 1 mM DTT).

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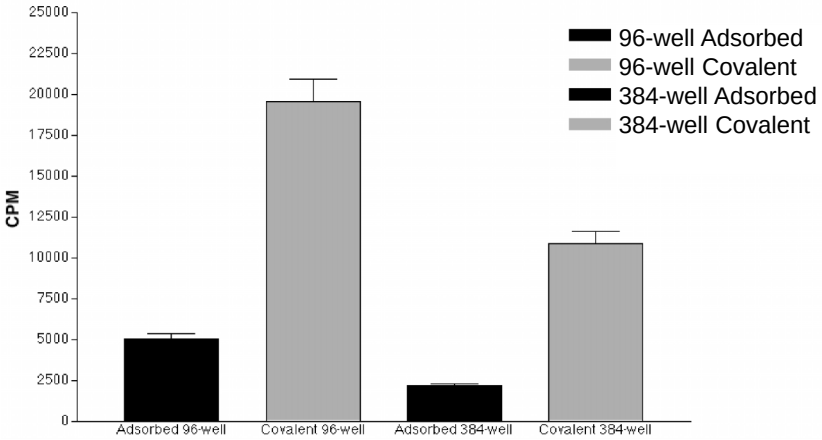

Figure 6: Simultaneous cAMP dependent protein kinase (PKA) phosphorylation (tracer, [γ-^{33}P]ATP) and capture of biotinylated kemptide on adsorption and covalent coated streptavidin FlashPlates. Four matching controls had all the reaction components except PKA and were less than 200 cpm. Reaction/capture time was 2.5 hours followed by aspiration, 2 washes and counting.

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Assay Contains	Covalent	Adsorbed
10% DMSO	118.8%	119.6%
1% Tween 20	100.8%	73.2%
1% Zwittergent	94.6%	66.4%
1% SDS	88.4%	61.3%
1% Triton X-100	79.1%	50.8%
1% NP40	74.9%	7.4%

Table 1: Maximum tested detergent/DMSO concentrations in IC_{50} biotin binding assays, adsorbed vs. covalent 96-well streptavidin FlashPlates. The assay conditions were 5 pmol/ml [^{3}H]biotin plus 300 pmol/ml unlabeled biotin at 200 µl/well in PBS/detergent or DMSO solution. Reported values are relative to a control without detergent. The data was calculated as cpm test condition/cpm zero detergent or DMSO cpm X 100.

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