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A Homogeneous Receptor Binding Assay for HTS on FlashPlate® PLUS

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Introduction

NEN® FlashPlates are scintillant coated micro-well plates allowing homogeneous binding assays to be developed in familiar 96-well and 384-well formats. FlashPlates coated with Wheat Germ Agglutinin (WGA) have been developed for use in homogeneous receptor assays. WGA is a plant lectin having specificity for the simple carbohydrate N-acetylglucosamine. WGA FlashPlate PLUS captures glycoproteins and membrane preparations having similar carbohydrate residues, which are recognized by the WGA. Both 96- and 384-well WGA FlashPlate PLUS products have been developed. This report describes a simple "mix and measure" type of homogeneous binding assay which is robust, easy to perform and readily automated.

The homogeneous and miniaturized format of this platform makes it well suited for high throughput screening, and several GPCR assays have been validated in both 96- and 384-well formats. Data is presented showing saturation and competitive binding results for a number of receptors with [^{125}I]-labeled ligands. Precision characteristics, solvent and detergent resistance, and stability of the plates is discussed.

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Results

The standard method for performing receptor binding assays is by filtration. A saturation curve was determined for Human Angiotensin II receptor and [^{125}I]-[Sar1, Ile^{8}]-Angiotensin II using the 96-well WGA FlashPlate PLUS and a Millipore MultiScreen® Filter Plate. The assay conditions were identical. Figures 1a and 1c show the binding results for the WGA FlashPlate PLUS. The K_d determined for the assay was 0.082 nM (Figure 1c). This value is within two-fold of the reported K_d for this receptor and ligand (0.16 nM). Generally, experimental results within an order of magnitude are considered acceptable.

The Filter Plate assay performed at the same time also showed acceptable agreement to the reported K_d value for Angiotensin II (Figures 1b and 1d).

Figure 2 demonstrates that a competition assay can also be successfully performed in the WGA FlashPlate PLUS. The same receptor and ligand system as described above were run, using the homologous unlabeled ligand to the tracer as an inhibitor and comparing to Human Angiotensin II as the unlabeled inhibitor. The potencies of these molecules are comparable when compared with K_d values obtained from filtration assay.

Figures 3a and 3b show the same Angiotensin II receptor system (see Figures 1a and 1c) run in 384-well WGA FlashPlate PLUS products. The K_d (Figure 3b, 0.193 nM) shows good agreement to the reported value of 0.16 nM.

WGA FlashPlate PLUS products were also tested by a [^{125}I]-glycophorin binding assay. Glycophorin contains carbohydrates recognized by WGA. The purpose of this assay is to assess the coating precision of the plates during production. Table 1 shows the intra-plate and inter-plate precision results from an experiment that compared Angiotensin II receptor binding to the glycophorin assay on the same plates. Using a concentration of unlabeled Angiotensin II or unlabeled glycophorin that was close to the IC_{50} concentration, the mean intra-plate CV of the Angiotensin assay was 7.2% and the glycophorin assay was 8.3%. The results indicate comparable precision between these two methods and suggest typical performance characteristics.

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Results (continued)

One of the major advantages of FlashPlate assays is the ability to format homogeneous assays, thus significantly reducing handling and processing steps compared to alternate methods such as filter plates. However, aspiration is possible with FlashPlate assays, if desired; in some cases, aspiration of the assay buffer from the FlashPlate wells at the end of the assay can often improve the signal-to-noise ratio. In some situations this may allow an assay to be formatted which would otherwise have a signal-to-noise ratio which is too low. Table 2 illustrates the improvement of the signal-to-noise ratio in an Angiotensin II assay. The same plate was read before and after aspiration. The signal-to-noise ratio improved by over four-fold for some conditions.

A number of receptors have been tested on WGA FlashPlate PLUS products. Figure 4 shows the specific binding for a Galanin receptor assay on the 384-well WGA FlashPlate. The agreement between the reported K_d (0.1 nM) and the determined K_d (0.17 nM) is excellent for this assay.

DMSO and detergent resistance has been determined for the WGA FlashPlate PLUS products. DMSO in the assay buffer at 10% does not inhibit the binding of glycoprotein to WGA. The plates are not stable to 100% DMSO added directly to the plates.

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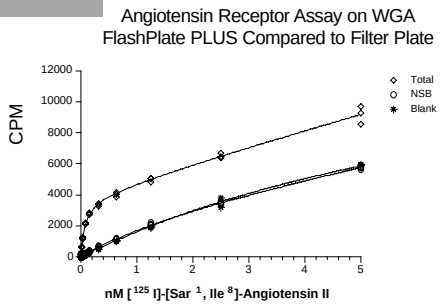

Figure 1a: WGA FlashPlate, 96-Well (SMP105) – Total Binding to the Angiotensin Receptor.

Using a Millipore Filter Plate, the same assay conditions as described in Figure 1a were used to set up a saturation assay.

Increasing concentrations of [^{125}I]-[Sar1, Ile^{8}]-Angiotensin II tracer (NEN, NEX248, 2200 Ci/mmol) were added together with a constant amount of 2 µg/well cloned Human Angiotensin II receptor subtype 2 from HeLa Cells (NEN, CRM069) to a 96-well WGA FlashPlate. The total assay volume was 170 µl per well. The assay buffer was 50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA and 0.1% BSA. The plate was read on a Packard TopCount® counter after 15 hours (overnight) incubation. Triplicate determinations were made for each condition. The mean CPM +/- the standard deviation are plotted. 'Total' represents CPM from receptor + tracer; 'NSB' represents CPM from receptor + tracer + 10 µM unlabeled ligand; 'Blank' represents CPM from wells with tracer only.

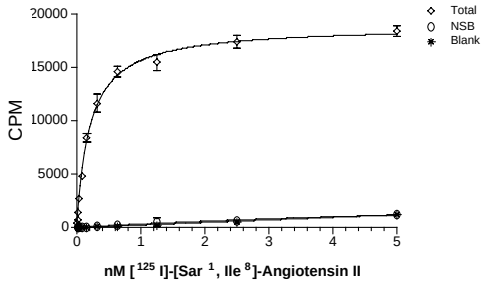

Figure 1b: 96-Well Filter Plate – Total Binding to the Angiotensin Receptor.

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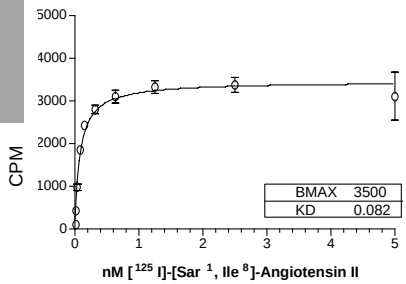
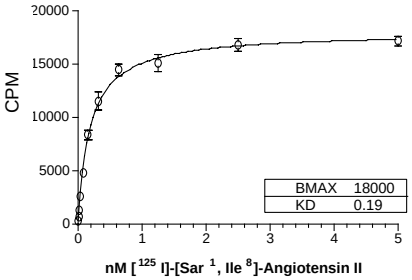


Figure 1c: WGA FlashPlate, 96-Well (SMP105) – Specific Binding to the Angiotensin Receptor.
The NSB CPM was subtracted from the Total CPM to give specific binding in the plate described in Figure 1a. The B_{max} (in CPM) and K_d (in nM) were determined from the non-linear regression fit of the following equation using Prism software from GraphPad:

$$CPM = [ligand] \left(\frac{B_{max}}{K_d + [ligand]} \right)$$

The K_d reported for this receptor and ligand is 0.16 nM. A K_d determined experimentally is generally accepted as agreeing with previously determined values if it is within an order of magnitude.

Figure 1d: 96-Well Filter Plate – Specific Binding to the Angiotensin Receptor.
The specific binding parameters for the Filter Plate results were calculated as described in Figure 1c.



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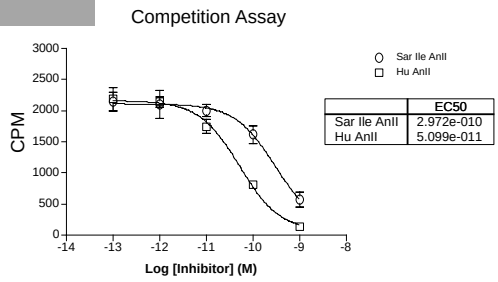
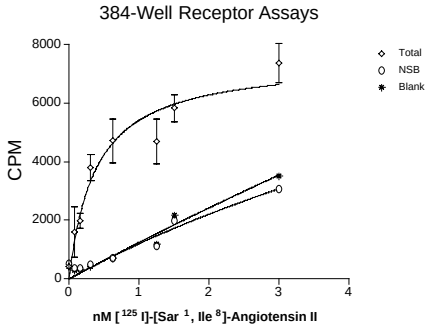


Figure 2: Angiotensin II Competition Curves on 96-Well WGA FlashPlate.
Receptor, buffer and tracer were the same as described for Figure 1a. The receptor was added at 2 µg/well. Two nonradioactive ligands were competed against the tracer. Human Angiotensin II and [Sar1, Ile^8]-Angiotensin II.
Tracer, cold ligands and receptor were added simultaneously. The assay was incubated overnight before reading on the TopCount. Error bars represent the mean CPM + 1 X SD. N = 5 for each point. Curve fitting and EC_{50} determination were performed using Prism software from GraphPad.

Figure 3a: WGA FlashPlate, 384-Well (SMP411) – Total Binding to the Angiotensin Receptor.
Receptor assay in this and the following figure were done as described in Figure 1a with the exception that the assay volume was reduced to 50 µl per well and 384-well WGA FlashPlates were used. The assay volume and amount of receptor added was thus approximately 30% of that added to the 96-well WGA FlashPlates.



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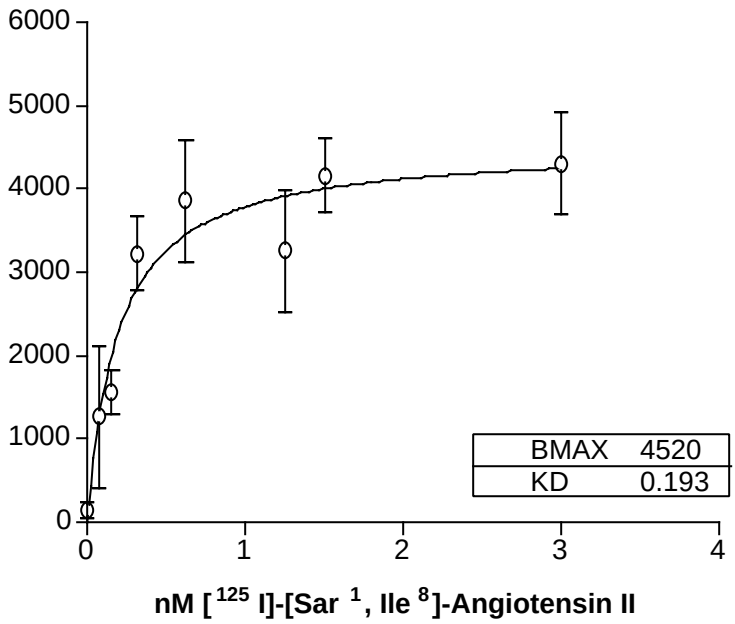

Figure 3b: Angiotensin Specific Binding on 384-Well WGA FlashPlate (HX). This is the same data as in Figure 3a, corrected to show specific binding (Total CPM - NSB). Data was analyzed and plotted as described for Figure 1c.

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Precision

The following table demonstrates that the CVs obtained from the QC assay are comparable to those of a receptor assay.

Intra- and Inter-Plate CVs for Angiotensin and Glycophorin

		Angiotensin Receptor Assay				Glycophorin QC Assay			
Plate	**Condition**	**mean**	**SD**	**CV**	**n**	**mean**	**SD**	**CV**	**n**
1	B_0	2706	90	3.3%	24	6074	244	4.0%	24
2	B_0	2733	78	2.8%	24	6425	225	3.5%	24
3	B_0	2714	104	3.8%	24	5901	220	3.7%	24
1	IC_{50}	1203	98	8.1%	24	1929	220	11.4%	24
2	IC_{50}	1186	88	7.4%	24	2171	139	6.4%	24
3	IC_{50}	1222	75	6.1%	24	1959	139	7.1%	23

				mean	**inter-**			**mean**	**inter-**
Grand	**Condition**	**mean**	**SD**	**CV**	**CV**	**mean**	**SD**	**CV**	**CV**
Means	B_0	2718	14	3.3%	0.5%	6133	267	3.7%	4,4%
	IC_{50}	1204	18	7.2%	1.5%	2019	132	8.3%	6.5%

Table 1. Comparison of CV of Receptor Assay to CV of Glycophorin QC Method. A receptor assay and the [125I]-glycophorin QC assay were compared on the same plates. Three WGA FlashPlates (lot 051299) were assayed in an overnight incubation. Angiotensin II receptor (CRM069) was added at 1 µg/well. Both Angiotensin and glycophorin were tested with tracer only ("B_0" condition; [125I]-[Sar1, Ile8] Angiotensin, NEX248; [125I]-glycophorin, custom iodination) and with tracer plus cold ligand at approximately the concentration that would give 50% inhibition (IC_{50} condition; [Sar1, Ile8] Angiotensin at 0.16 nM; glycophorin at 10 µg/ml). This resulted in 24 wells per condition on each of 3 plates. The average intra-plate CVs are given at the bottom of the table. The inter-plate CVs are calculated from the mean intra-plate CV for each condition (n=3).

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Effect of Aspiration of S/N in a 96-Well WGA FlashPlate Receptor Assay

Tracer Conc. nM	WGA S/N	WGA Aspirated S/N
5.000	1.6	4.0
2.500	1.9	6.0
1.250	2.4	8.4
0.625	3.6	13.6
0.313	5.2	23.0
0.156	7.8	36.3
0.078	11.4	37.0
0.039	11.0	36.5
0.020	8.0	22.9
0.010	7.2	19.3
0.005	4.5	6.8

Table 2: Effect of aspiration of S/N on Angiotensin II Receptor Assay.
The S/N was calculated for an Angiotensin II receptor assay (S/N = Total CPM divided by the CPM in the presence of 10 µM nonradioactive ligand). The assay was set up as described for Figure 1. The WGA column and the WGA Aspirated column represent data from the same plate before and after aspirating the liquid in the wells after the assay incubation.

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Examples of Other Receptors on WGA FlashPlates

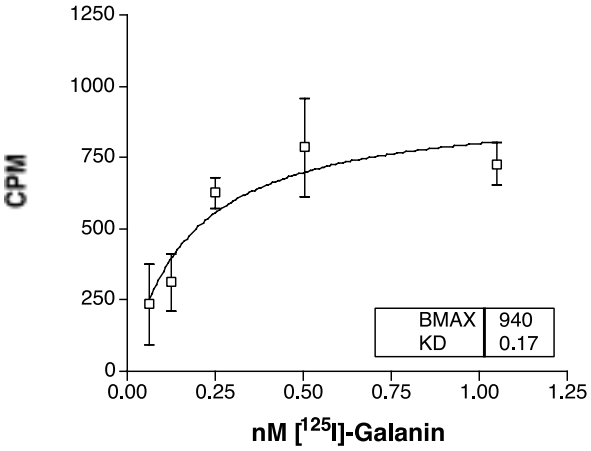

Figure 4: Galanin receptor on 384-Well WGA FlashPlate.
Cloned Human Galanin receptor (CRM067) was added at 0.25 µg/well in 50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$ and 0.5% BSA. Increasing concentrations of [^{125}I]-(porcine) Galanin (NEN, NEX243, 2200 Ci/mmol) were added to a series of wells for an overnight room temperature incubation. The total assay volume was 50 µl, with all reagents added together. The results were read on the TopCount. The reported K_d is 0.1 nM. The results were analyzed as described in Figure 1c.

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DMSO
Concentrations between 10 and 50% DMSO in the assay buffer had minimal (less than 20% reduction of CPM compared to the control) effect on the binding of a test glycoprotein to WGA FlashPlates. Increasing concentrations and exposure times were associated with a greater effect on the CPM bound.

Detergents
None of the following detergents prevented the binding of [^{125}I]-glycophorin to WGA FlashPlates when present in the assay buffer at concentrations from 0.05% to 1.0%:

Tween 20
Triton X-100
Zwittergent

Detergents may not be compatible with membrane receptor preparations or other assay components. All assay conditions should be made with the same buffer formulation.

Stability
Plates have a year stability when stored at 4 degrees.

Conclusions

Homogeneous receptor binding assays have been demonstrated in both 96-well and 384-well WGA FlashPlate PLUS products. The K_d determined from WGA FlashPlate results are comparable to previously reported literature values as well as to filter plate results. Competition assays can be run in these plates. The order of potency for the inhibitors is in agreement with the values obtained from filtration assays. Precision and stability characteristics should allow the use of these plates in HTS applications.

Acknowledgements

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