Receptor Binding Assays with the PerkinElmer Wheat Germ Agglutinin (WGA) coated Image FlashPlate

Authors:

Patrice Robinson
Ryan Johnson
Hao Xie
Steve Hurt

PerkinElmer, Life and Analytical Sciences
Boston, MA 02118 USA

Introduction

An Image FlashPlate is a scintillant coated 384-well low volume microplate suitable for radiometric assays using a variety of isotopes. The low volume well configuration provides a viable platform for ultra high throughput screening assays, with the added advantage of reduced reagent costs and reduced radioactive waste. The plate has a standard microplate footprint that is compatible with current plate and liquid handling equipment. The emission maximum of the scintillant is >600 nm (red-shifted), ideal for imaging on the ViewLux™ ultraHTS Microplate Imager from PerkinElmer Life and Analytical Sciences (PKLAS), enabling data collection of all wells simultaneously increasing throughput to >10,000 compounds per hour.

Wheat germ agglutinin (WGA) is a plant lectin that binds a range of carbohydrates, with highest affinity for N-acetylglucosamine, providing a means to capture receptor membranes. Thus, the WGA coated Image FlashPlate is an excellent platform for capturing membrane preparations for receptor-radioligand binding assays for uHTS applications.

Applications have been developed on the WGA Image FlashPlate measuring the binding interactions of several receptors, including galanin, ORL_1, motilin, and MC4. Optimized reactions buffers, reagent concentrations, incubation times, and washing conditions have all been determined for these receptor/ligand pairs. In addition, these protocols can likely be used, with minor modifications, to rapidly develop a high throughput assay for any receptor-binding assay.

Materials and Methods

Motilin

- Reaction Buffer-50 mM Tris-HCl pH 7.5; 10 mM $MgCl_2$; 1 mM EDTA; 0.1 % BSA; in ddH_20
- WGA Image FlashPlate (PKLAS cat. #RMP111)
- ^{125}I-Motilin (PKLAS cat. #NEX378), 0.75 nM in assay
- Motilin Receptor Membrane (PKLAS cat #RBHMOT), 1.25 µg/well

MC4

- Reaction Buffer-25 mM HEPES pH 7.4, 1.5 mM CaCl2, 1 mM MgSO4, 0.2% BSA in H_20
- WGA Image FlashPlate (PKLAS cat. #RMP111)
- ^{125}I-NDP-α(-MSH (PKLAS cat. # NEX352), 0.5 nM in assay
- MC4 receptor (PKLAS cat. #RBHMC4M), 1.25 µg/well

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Table 1. Receptor specific assay conditions.

Receptor Binding Assay	Membrane Mass (μg/well)	Radioligand Concentration (nM in assay)	Incubation Time (hr)	Aspirate/1X PBS Wash	PEI Blocking
Motilin	1.25	0.75	4	Not necessary	Not necessary
MC4	1.25	0.5	4	Not necessary	Not necessary
ORL_1	1.25	0.5	4	Not necessary	Not necessary
Galanin	0.6	1.0	3	Recommended	Recommended
Alternate Galanin Protocol	1.25	0.5	5	Recommended	Recommended

ORL_1

- Reaction Buffer-50 mM HEPES pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA, 0.1% BSA in H_20
- WGA Image FlashPlate (PKLAS cat. #RMP111)
- ^{125}I-Nociceptin (PKLAS cat. #NEX338), 0.5 nM in assay
- ORL_1 receptor (PKLAS cat. #RBHORLM), 1.25 μg/well

Galanin

- Reaction Buffer- 25 mM Tris-HCl pH 7.5; 10 mM $MgCl_2$; 1 mM EDTA; 0.5% BSA; in ddH_20
- WGA Image FlashPlate (PKLAS cat. #RMP111)
- ^{125}I-Galanin (PKLAS cat. #NEX333), 1.0 nM in assay
- GALR2 Galanin receptor (PKLAS cat. #RBHGALR2)
- Polyethyleneimine (PEI) solution (Sigma cat. #P-182)

The following general guidelines can be used for all receptor/ligand pairs and a modified protocol for the enhanced signal:noise galanin assay is also shown. In all applications tested, washing the Image FlashPlates after incubation increased the signal:noise ratio but is not necessary to obtain suitable values. Details specific to each receptor are given in Table 1.

General Protocol

- *Dispense 10 μl membrane solution*
- *Dispense 10 μl competitor/ compound/tracer solutions*
- *RT incubation*
- *Image, or aspiration and wash followed by imaging on the ViewLux™.*

Modified Galanin Protocol

- *Dispense 20 μl membrane solution*
- *2 hr RT incubation*
- *Aspirate wells*
- *Dispense 20 μl competitor/ compound/tracer solutions*
- *3 hr RT incubation*
- *Aspirate, 1X PBS wash, aspirate. Image on the ViewLux™.*

PEI Blocking Protocol

- *Dispense 20 μl 0.1% PEI solution*
- *Incubate overnight covered, RT*
- *Aspirate, wash 3 times with 1X PBS*

Results

Motilin

A functional assay for the binding of ^{125}I-Motilin (PKLAS cat. #NEX378) to the Motilin receptor was developed for use on the WGA Image FlashPlate. ^{125}I-Motilin ligand and motilin membrane were first titered to determine the optimal concentration for such assays. Figure 1 indicates that a membrane concentration of 1.25 μg/well will result in the greatest ligand binding. Optimum ^{125}I-Motilin concentration was then determined to be 0.75 nM as shown in Figure 2.

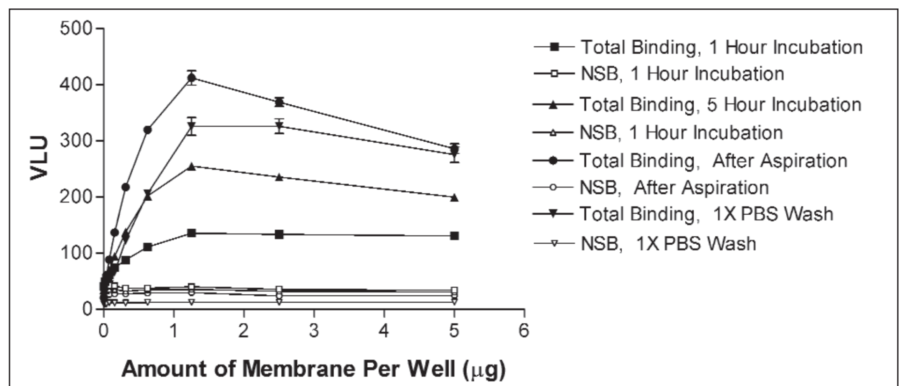

Figure 1. Binding of 125I-Motilin to Motilin Receptor on WGA Image FlashPlate.

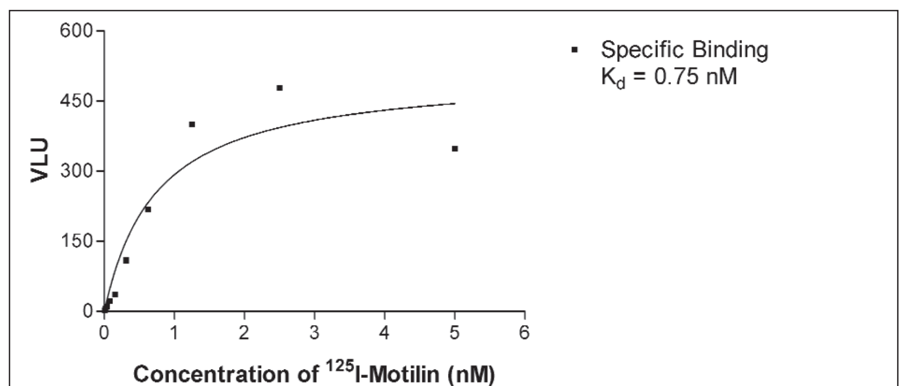

Figure 2. Titration of ^{125}I-Motilin on WGA Image FlashPlate.

A competition curve was generated for motilin receptor binding over time, comparing the effects of aspirating and washing the wells after sufficient incubation. These results are shown in Figure 3 and indicate that the optimal incubation time for motilin receptor binding is between 2 and 5 hours. Signal to noise ratios after this incubation period ranged between 7-8.5 and the z' values are consistently above 0.8. However, Figure 3 illustrates that the added steps of washing and aspirating the plates post incubation will increase the signal:noise ratio by approximately 2 fold.

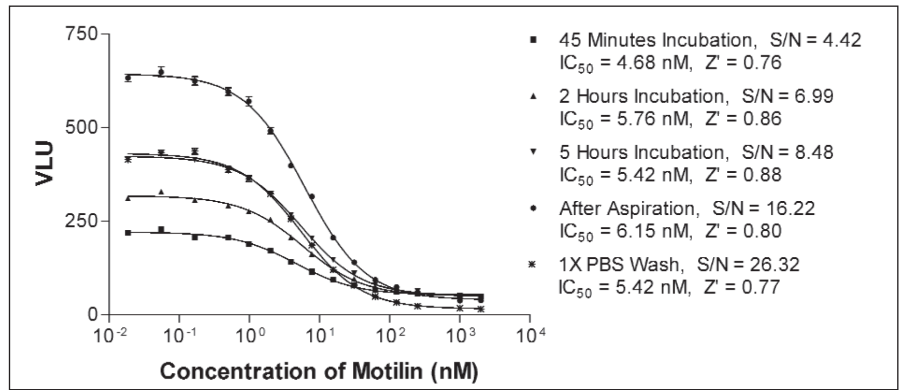

Figure 3. Motilin Competition on WGA Image FlashPlate.

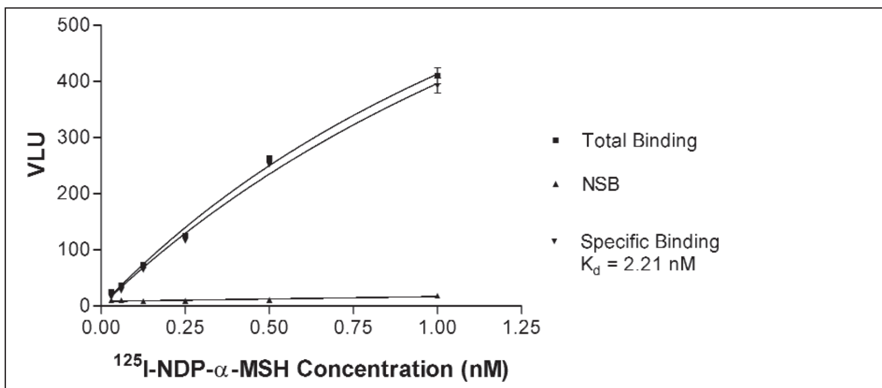

Figure 4. MC4 Receptor Saturation Curve on WGA Image FlashPlates.

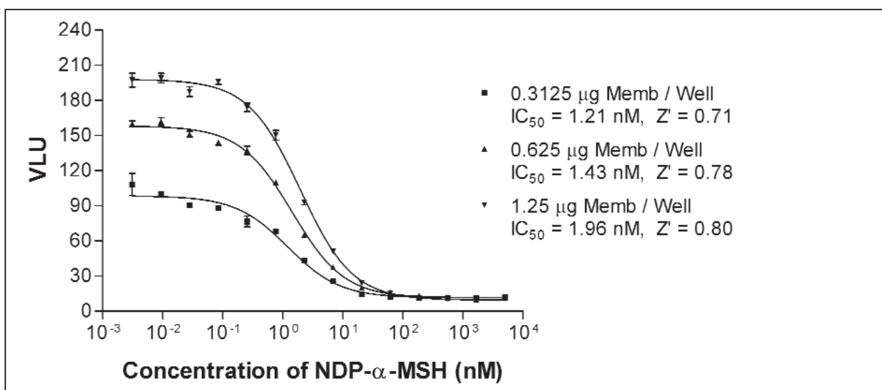

Figure 5. MC4 Receptor Competition Assay Confirming the Optimal Concentrations of Receptor and ^{125}I-NDP-α-MSH.

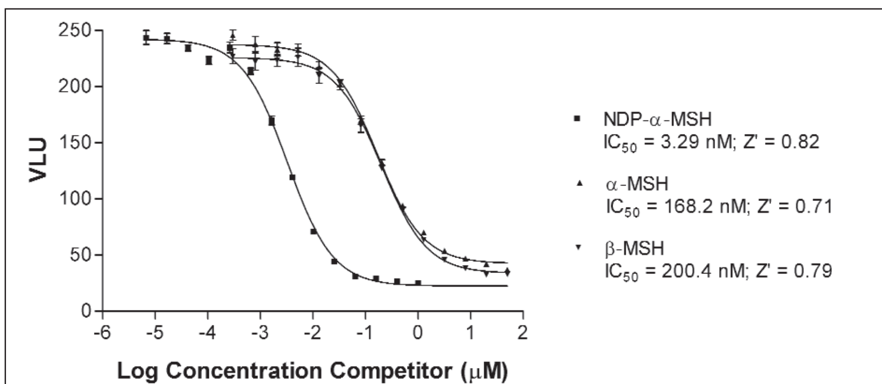

Figure 6. MC4 Receptor Competition Curve with NDP-α-MSH α-MSH, and β-MSH as Competitors.

MC4

The binding of ^{125}I-NDP-α-MSH to 1.25 µg/well MC4 receptor (PKLAS cat. #RBHMC4M) demonstrates excellent signal:noise ratios across a wide range of ligand concentrations, as shown in Figure 4. All tracer concentrations from 1.0 nM to 0.125 nM gave signal:noise ratios higher than 8 and z' values greater than 0.65. It was also determined that the receptor concentrations greater than 1.25 µg/well, did not result in higher total counts.

Competition curves with unlabeled I-NDP-α-MSH were generated at multiple receptor concentrations as shown in Figure 5. It was determined that concentrations as low as 0.3 µg/well of MC4 membrane could be used in this application to achieve a desirable signal:noise ratio and a z' value above 0.70. Furthermore, it was demonstrated that aspirating and washing with 1X PBS would increase the signal:noise, but not necessarily result in an increased z' value. Comparable IC50 values for I-NDP-α-MSH were calculated at all membrane concentrations tested.

Competition of α-MSH and β-MSH with the ^{125}I-NDP-α-MSH was also measured in this assay as shown in Figure 6. While z' values for these assays are all greater than 0.7, the Kd values for the α-MSH and β-MSH are significantly shifted to the right as compared to I-NDP-α-MSH. This observation is consistent with results determined by filtration assays.

ORL_1

A receptor binding assay was developed to study the binding of ^{125}I-Nociceptin (PKLAS cat. #NEX338) to the ORL_1 receptor (PKLAS cat.# RBHORLM). Receptor and ligand (^{125}I-Nociceptin) were titrated to deduce the optimal assay concentrations. As shown in Figure 7, 1.25 μg/well receptor and 0.5 nM ^{125}I-Nociceptin were found to be the most viable concentrations. All results were generated after a 4-hour incubation and an aspirate/1X PBS wash/aspirate step, as this was shown to yield the optimum signal:noise values.

Typical z' and Kd values for Nociceptin binding were determined to be 0.75 and 0.95nM respectively, via competition assays as shown in Figure 8.

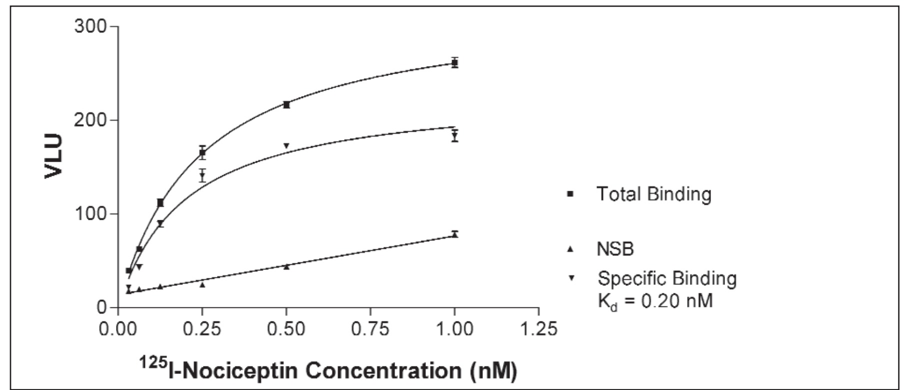

Figure 7. ORL_1 Receptor Saturation Curve on WGA Image FlashPlate.

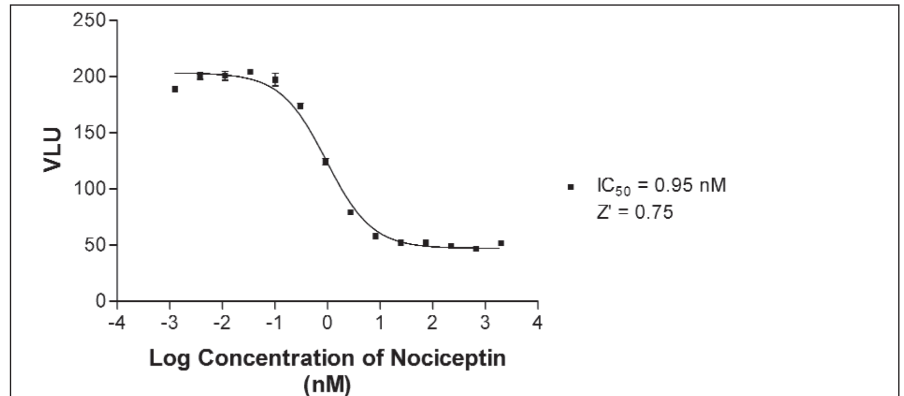

Figure 8. ORL_1 Receptor Competition Assay on WGA Image FlashPlate. Incubation time = 4 hr. S:NSB = 4.4.

Galanin

A functional assay for the binding of ^{125}I-Galanin (cat. #NEX333) to the GALR2 Galanin receptor (RBHGALR2M) was developed.

Experiments indicated that the ^{125}I-Galanin tracer was sticky, i.e. interacting with the FlashPlate, resulting in high NSB and low signal:background. However, it was determined these interactions can be negated by blocking the WGA Image FlashPlate with 0.1% PEI solution prior to assaying. Using this protocol, the galanin receptor was titrated to determine the optimal receptor concentration.

Various buffer components were tested to assess their effectiveness at increasing the signal:noise ratio. Figure 12 compares competition profiles for this assay with buffer supplemented with PEG and two differing BSA concentrations. The results from the competition analysis clearly indicate the advantage of supplementing the reaction buffer with 0.5% BSA. The 0.5% BSA supplement increased the total Bo, decreased non-specific binding and yielded a signal:noise ratio of approximately 4. Furthermore, it was found that aspirating and washing with 1X PBS increased the signal:noise ratio and resulted in an increased z' value.

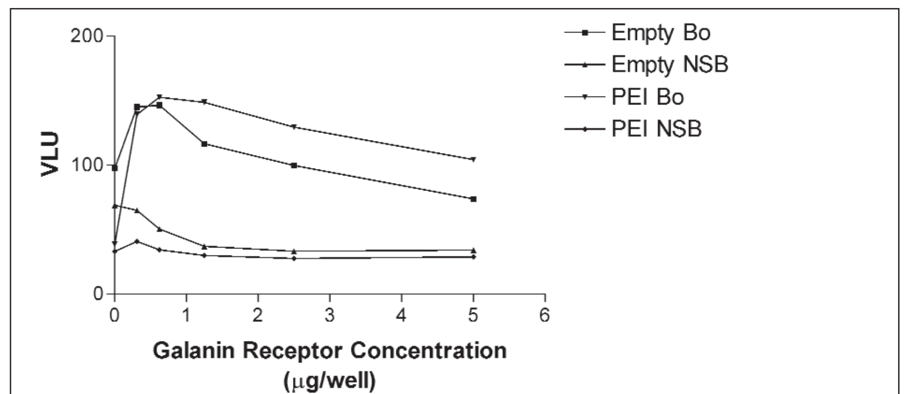

Figure 9. Titration of Galanin Receptor on 0.1% PEI Blocked and Unblocked WGA Image FlashPlates.

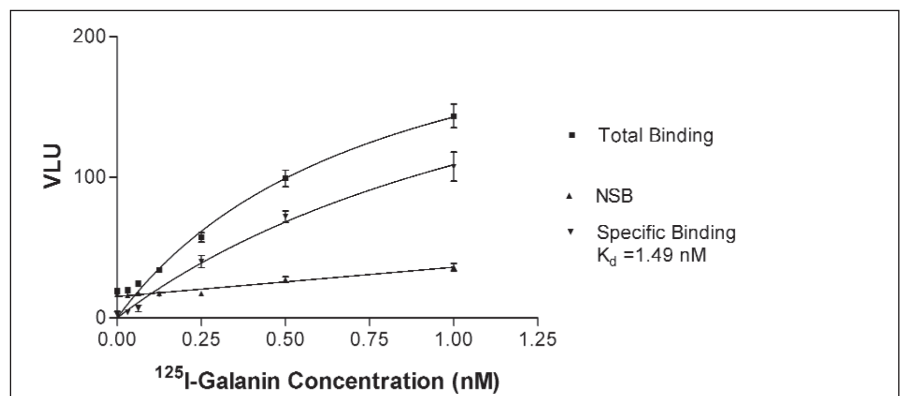

Figure 10. Galanin Receptor Binding Assay on a 0.1% PEI Blocked WGA Image FlashPlate.

An alternative protocol, which requires more manipulation, was developed for the galanin receptor assay on the WGA Image FlashPlate. The modifications in the galanin protocol lead to increased signal to noise ratios if necessary. An initial incubation of 2 hours with 20 µl of receptor at room temperature is followed by aspiration, then the ^{125}I-Galanin and competitor are added, followed by another incubation for 3 hours. The plates should then be imaged. The optimum reagent concentrations for this method have been determined to be 1.25 µg/well of membrane and 0.5 nM ^{125}I-Galanin. This method can increase the signal:noise ratio to a value greater than 9 as shown in Figure 13. The plate was imaged on the ViewLux over 3 hours, aspirated and then washed with 1X PBS.

Discussion

The WGA Image FlashPlate is an excellent high throughput platform for receptor binding assays. The optimum assay conditions for 4 receptors outlined herein demonstrate excellent precision and pharmacology as illustrated in Table 2. For troublesome receptor/ligand pairs, such as galanin, certain assay modifications were found to be very effective. These include addition of BSA to reaction buffer and pre-blocking of the WGA Image FlashPlate with a PEI solution. These modifications will likely translate into other receptor/ ligand pairs to successfully improve assay performance. Receptor binding assays in this format have the added advantage of compatibility with liquid handling, as well as a throughput allowed by imaging with the ViewLux™ of approximately 10,000 wells per hour.

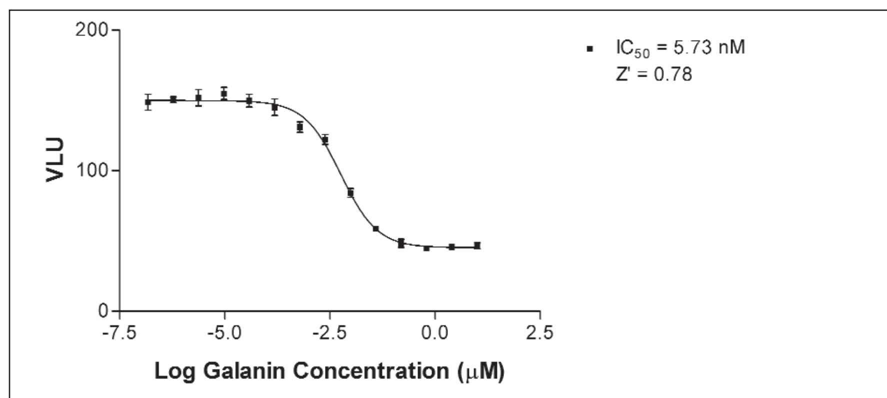

Figure 11. Galanin Receptor Competition Assay on 0.1% PEI Blocked WGA Image FlashPlate.

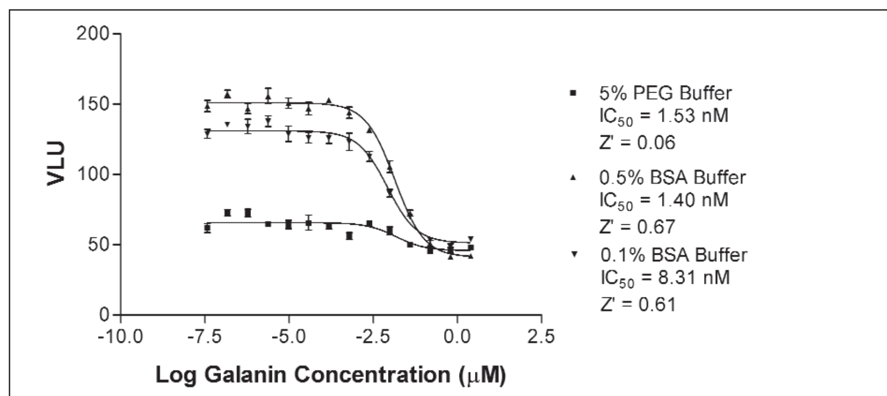

Figure 12. Galanin Receptor Competition Assay and Buffer Comparison on a 0.1% PEI Blocked WGA Image FlashPlate.

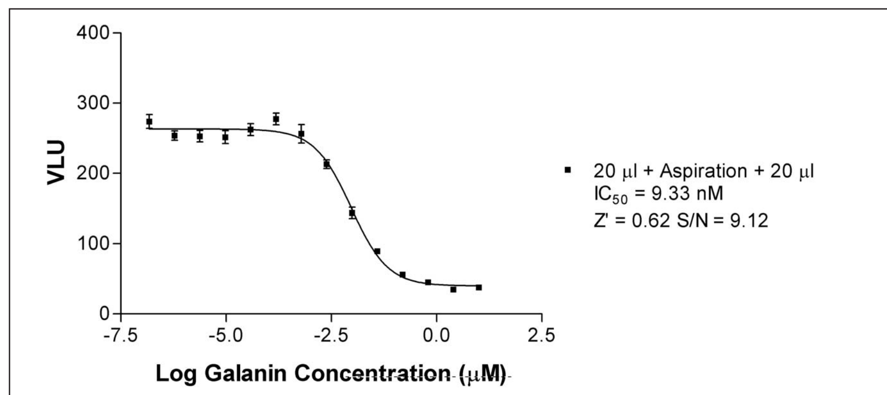

Figure 13. Galanin R2 Receptor Competition Assay on a 0.1% PEI Blocked WGA Image FlashPlate by the Alternate Galanin receptor assay protocol.

Table 2. Typical Receptor Binding assay results.

Receptor Binding Assay	S/B	Radioligand Kd (nM)	Cold competitor IC50 (nM)	Z'
Motilin	8.5	0.75	8.0	0.8
MC4	10	2.2	2.0	0.8
ORL_1	9	0.2	1.0	0.75
Galanin	3	0.4	5.7	0.78
Alternate Galanin Protocol	9			0.62

PerkinElmer Life and
Analytical Sciences
710 Bridgeport Avenue
Shelton, CT 06484-4794 USA
Phone: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

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