APPLICATION NOTE

OnPoint Reagent Services

Scintillation Proximity Assay (SPA): Custom Assay Development and Membrane Validation

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Abstract

G protein-coupled receptors (GPCRs) represent attractive drug targets because they participate in a wide range of physiological processes and are believed to play a role in a variety of disease states. At least a third of all prescription drugs on the market today are molecules acting as agonists or antagonists of the GPCR. Receptor-ligand interaction studies are pivotal in the successful identification of drug candidates. Despite numerous advances in nonradioactive technologies, radioligand binding assays remain one of the most commonly used technologies for analysis of receptor-ligand interaction in High Throughput Screening (HTS) and Structure Activity Relationship (SAR) studies. Among the different radioligand binding assays developed, Scintillation Proximity Assay (SPA) is a well established technology, offering the advantage of being homogeneous (no separation of the bound and unbound radiotracer), and therefore easily amenable for miniaturization and automation. In addition to traditional filtration and FlashPlate® assays, PerkinElmer has also developed SPA binding conditions for a large number of receptors. Custom development of SPA assays can be performed utilizing PerkinElmer's OnPoint Reagent Services team.

Introduction

Scintillation Proximity Assay (SPA) is a technology whereby binding reactions can be assayed without the washing or filtration procedures normally used to separate bound from free fractions. Assays are performed using radioactive labels that emit electrons with only a short range (about 10 µm) in water. When bound close to a solid scintillator surface by the binding reaction, they are able to transfer electron energy to the scintillator to produce photons detectable with a scintillation counter. Electrons emitted from labeled molecules not bound close to the surface dissipate their energy in the medium and are not detected. Thus, the bound fraction is detected specifically without separation of the solution from the solid support. The radioisotopes ^{3}H and ^{125}I, which are commonly used in ligand binding assays and radioimmunoassays (RIA), emit electrons with the low energies required for SPA. In summary, many of these binding assays can be adapted to this new method, avoiding the usual filtration or washing procedures. SPA is particularly attractive because it is often used as a high volume screening procedure, typically in drug discovery, and high throughput and ease of automation are required for cost-effectiveness. Additional attractive features of SPA are that the progress of binding reactions can be monitored in time, and that the bound fraction can be measured while in equilibrium with the free fraction.

PerkinElmer®
For the Better

Scintillation Binding Assay Development

Typical requirements for a robust assay are: ligand depletion < 10%, S/N > 4, Z' factor > 0.5 and signal stability > 4 hours.

In order to achieve those goals, the following parameters are routinely examined:

I. Reagents	a. Receptor (membranes) b. Radioligand
II. Assay format	a. Bead type b. Ratio Beads:Membranes c. Assay plate d. Assay assembly
III. Assay conditions	a. Assay buffer and temperature b. Incubation time-Signal stability c. Solvent Interference
V. Assay Miniaturization	
VI. Pharmacology	

I. Reagents

Quality reagents are crucial in the successful development of SPA.

Ia. Receptors

PerkinElmer offers instant access to the most extensive portfolio of pharmacologically characterized GPCR membrane preparations. Membrane Target Systems® are quality-assured frozen membranes from cells that express recombinant or endogenous receptors.

Ib. Radioligands

A pioneer in the development of essential tools for receptor ligand binding, PerkinElmer now offers over 400 NEN® ^{3}H and ^{125}I ligands with new products continuously introduced. Each of our ultra-pure radioligands is fully characterized for pharmacological action and validated in receptor binding assay.

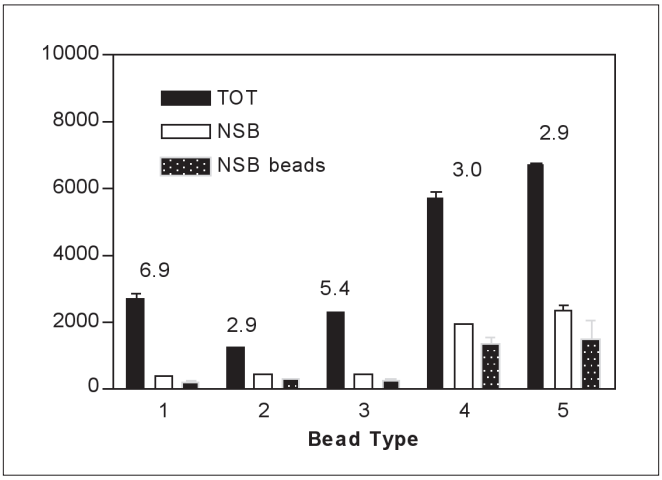
II. Assay Format

The receptor studied, the radioligand used and the final throughput required will deeply impact the selection of the assay format.

IIa. Selection of Bead Type

Different SPA-bead types are commercially available: PVT (polyvinyltoluene) and YtSi beads with different surface coupling molecules. Typically five bead types are compared in order to maximize the receptor capture on the beads with minimal interaction of the radioligand.

In the example shown below, PVT_WGA beads were found to give the best assay window.



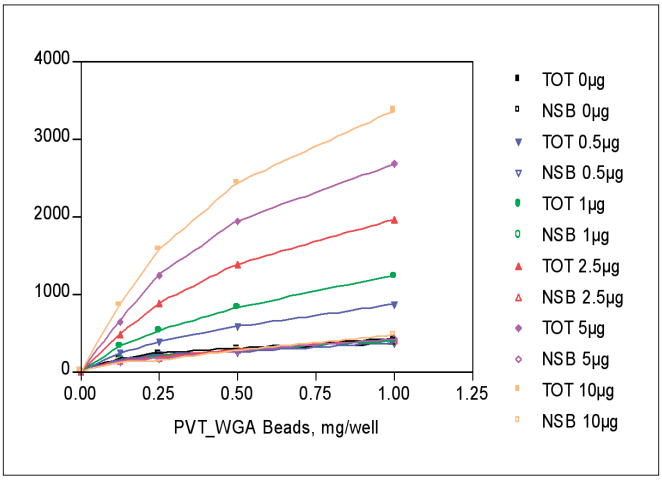
Selection of SPA Bead Type

Membranes expressing the GPCR of interest are incubated with a fixed amount of radioligand and with five bead types (1= PVT_WGA, 2 = PVT_PEI _WGA Type A, 3 = PVT_PEI_WGA Type B, 4 = YtSi_WGA, 5 = YtSi-Polylysine).

Non specific binding is determined by co-incubation with a large excess of cold ligand. Bead non-specific binding is determined by co-incubation of the radioligand only with the beads.

IIb. Selection of the Optimal Beads:Membrane Ratio.

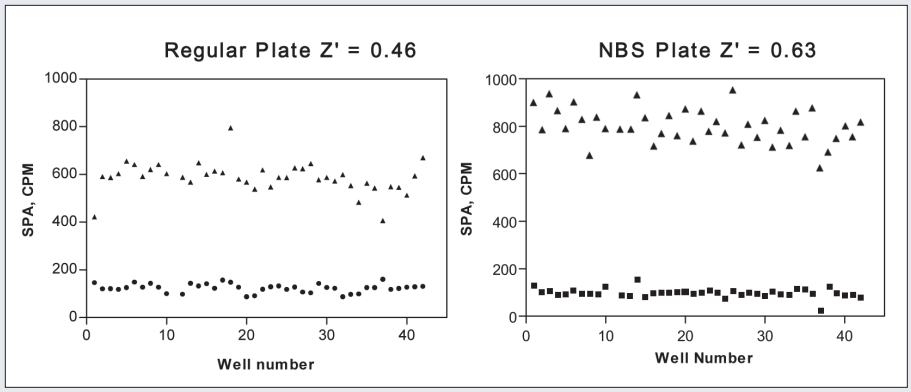
In order to optimize the assay – especially when developing an HTS assay – amounts of membranes and beads are kept at a minimum level, with radioligand bound kept below 10% to avoid ligand depletion.



Ratio beads:membrane

Increasing amounts of membranes (0, 0.5, 1.0, 2.5, 5.0 and 10 µg) are incubated with increasing amounts of SPA beads (0, 0.25, 0.5 and 1 mg/well) and a fixed amount of radioligand. Non-specific binding is determined by co-incubation with a large excess of unlabeled competitor.

The best ratio was found to be 0.5 mg of beads to 5mg of membrane.



Comparison of regular and NBS plates: Z' values were compared on regular and NBS plates.

Total binding (receptor + radioligand + SPA beads) and non-specific binding (receptor + radioligand + SPA beads + excess cold ligand) are measured (n=42) and Z' are calculated using Zhang's formula ($Z' = 1- (3\sigma_{TOT} + 3\sigma_{NSB})/(\mu_{TOT} - \mu_{NSB})$).

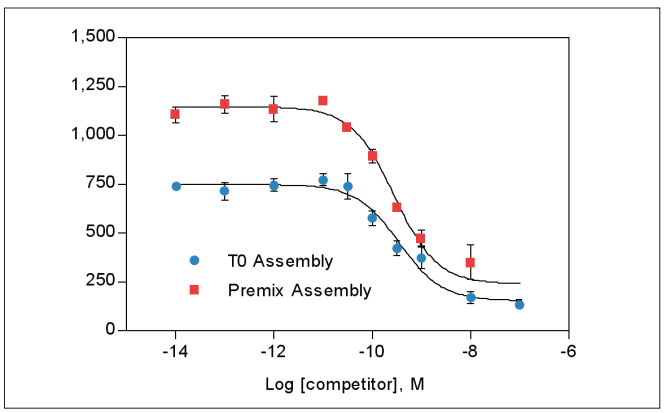
IIc. Assay Plate

Several assay plate formats are commercially available. Final throughput will govern the selection of 96- or 384-well plates. Furthemore, non-specific binding of the radioligand to the plate plastic may require the selection of special plates such as NBS plates.

IId. Assay Assembly

The order of addition of the different reagents in the assay may affect assay performance as well as ease of automation.

The most commonly used order of addition, called T0, requires four successive additions (unlabeled ligand or buffer, membranes, radioligand and finally beads) which make the assay less amenable to automation. In the following figure, the T0 assembly was compared to an assay assembly starting with a 30 minute pre-incubation of the beads with the membranes and premixing of the unlabeled and labeled ligand before dispensing into a 96 well plate. This type of assembly offers the advantage of having only two addition steps into the assay plate containing the compounds. In the example given below, the assay window is higher when using the premixing assembly.



Comparison of T0 Assembly and Premix

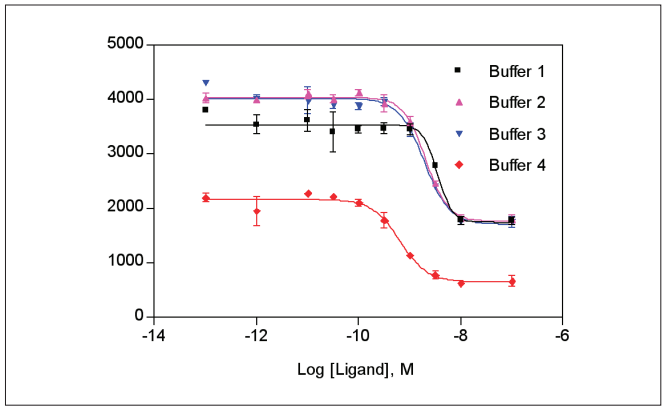
Competition binding isotherms were performed with either sequential addition of unlabeled ligand or buffer, membranes, radioligand and finally beads (T0 assembly); or premix of the beads with the membranes and of the unlabeled and labeled ligand before addition into the 384-well plate.

III. Assay Conditions

IIIa. Temperature and Assay Buffer

Typically, receptor binding assays are performed at room temperature.

Assay buffers are kept as simple as possible and the buffer optimization is an iterative process. Depending on the individual receptor-ligand system, different reagents may be required to achieve reasonable assay performance.



Comparison of Assay Buffers

Competition binding experiments were performed with 4 different assay buffers.

Buffer 1: 25 mM HEPES, 5 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4

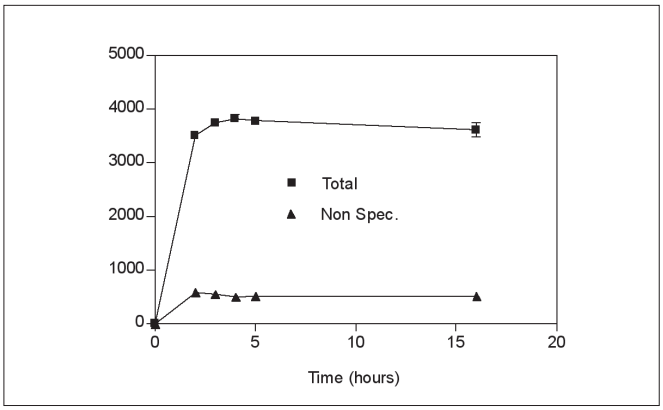
Buffer 2: Buffer 1 + 0.2% protease free BSA

Buffer 3: Buffer 2 + 10 µg/ml saponin

Buffer 4: Buffer 2 + 100 mM NaCl

IIIb. Incubation Time and Assay Stability

Experiments need to be performed to determine when a stable signal is obtained and how long the signal can be maintained. The stable signal is a combination of the reaction between receptor and ligand reaching steady state and bead settling down. Ideally a stable signal should be reached within one hour and maintained overnight.

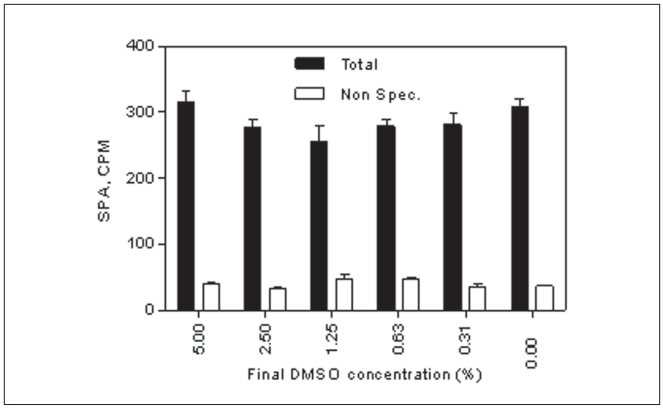


Incubation Time and Assay Stability

Total binding (receptor + radioligand + SPA beads) and non-specific binding (receptor + radioligand + SPA beads + excess cold ligand) measured at different times by repetitive counting on the TopCount® Microplate Scintillation and Luminescence Counter.

IIIc. Solvent Tolerance

Most compounds are stored in organic solvent, DMSO being the most commonly used. It is therefore necessary to investigate the potential interference of the solvent in the receptor-ligand reaction. In the figure shown below, the reaction was performed in up to 5% DMSO without noticeable impact on the assay performance.



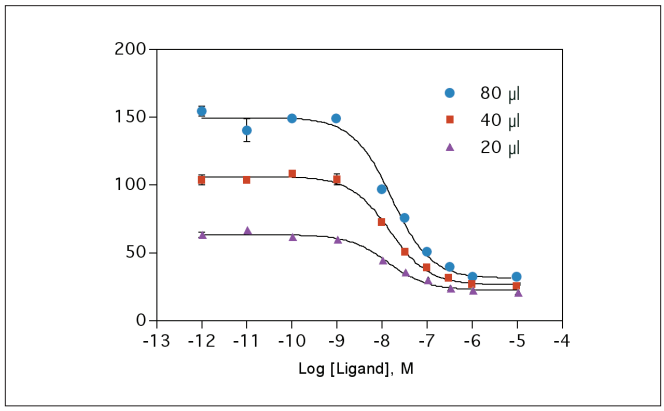
DMSO Tolerance

Total binding (receptor + radioligand + SPA beads) and non-specific binding (receptor + radioligand + SPA beads + excess cold ligand) measured in the presence of increasing concentration of DMSO.

IV. Assay Miniaturization and Robotic Assembly

When setting up an HTS assay, reduction of volume (and therefore of the material used in the reaction) and automation are the major concerns, while maintaining the robustness of the assay as much as possible.

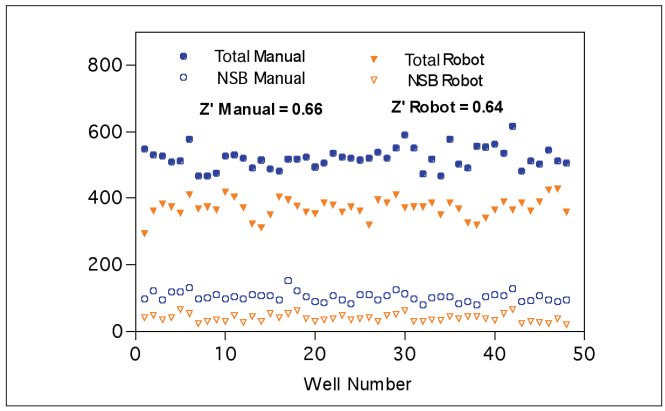
In the following example, we started with stock solutions and made an assembly into a final volume of 80 µl, 40 µl (volume reduced by 2 and material reduced by 2) and 20 µl (volume reduced by 4 and material reduced by 4).



Assay Miniaturization

Starting from single stock solutions of receptor, radioligand and beads, competition binding assays were assembled in final volume of 80, 40 and 20 µl.

Z' value of the same assay assembled manually and automatically with a liquid handling device (MiniTrak™, PerkinElmer) were compared in order to validate the performance of the assay.

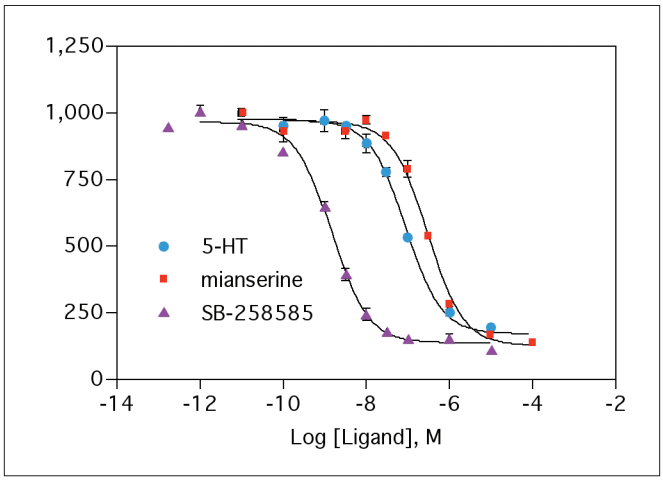


Comparison of Manual and Automated Assembly

Total binding (receptor + radioligand + SPA beads) and non-specific binding (receptor + radioligand + SPA beads + excess cold ligand) are measured (n=48) and Z' are calculated using Zhang's formula (see above)

V. Pharmacology

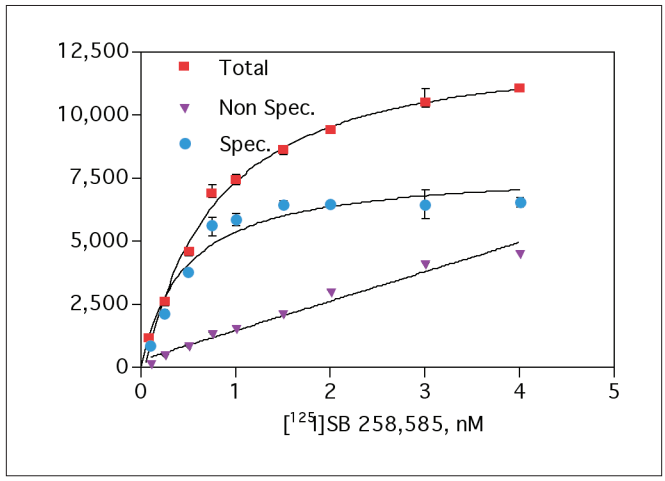
Finally, a saturation binding experiment is performed to estimate the affinity of the radioligand for the receptor (Kd) and the maximal number of receptor binding sites (Bmax). Also, a heterologous competition binding experiment to determine the affinity of reference ligands of the receptor (Ki) is performed.



Saturation Binding Experiment

Measurement of the total binding and non-specific binding with increasing amounts of radioligand in the optimum conditions defined with all the above assays.

K_d = 0.46 ± 0.11 nM B_{max}= 2.2 pmol/mg protein



Competition Binding Experiment

Fixed amounts of receptor, radioligand and beads are incubated in the presence of increasing amounts of cold ligands in the optimum assay conditions.

pIC_{50} of -7.05, -6.52 and -8.83 were obtained for 5-HT, mianserin and SB258585 respectively.

Receptor	Subtype	Cat Number	Reference Ligand	pIC_{50} ± SD
5-Hydroxyptamine	5-HT_{2C} edited	ES-313-M	Mianserin	7.87 ± 0.18
	5-HT_6	ES-316-M	SB 258,585	8.83 ± 0.04
Acetylcholine	M_1	ES-210-M	4-DAMP	7.59 ± 0.10
Adrenoceptors	α_{2A}	ES-030-M	Rauwolscine	8.22 ± 0.05
	α_{2C}	ES-032-M	Rauwolscine	8.48 ± 0.11
Bradykinin	B_2	ES-090-M	Bradykinin	8.62 ± 0.04
Calcitonin	$CGRP_1$	ES-420-M	CGRP	9.60 ± 0.07
Chemokine	CCR6	ES-139-M	MIP-3α	9.72 ± 0.10
	CXCR2	ES-145-M	IL-8	9.30 ± 0.08
	CXCR3	ES-142-M	I-TAC	9.31 ± 0.07
Ghrelin	ghrelin	ES-410-M	Ghrelin	9.39 ± 0.06
Histamine	H_1	ES-390-M	Pyrilamine	8.26 ± 0.14
	H_3	ES-392-M	R-α-Me-Histamine	8.27 ± 0.09
Melanocortin	MC_4	ES-191-M	NDP-α-MSH	8.50 ± 0.09
	MC_5	ES-194-M	NDP-α-MSH	9.29 ± 0.03
Melanin Conc. Hormone	MCH_1	ES-370-M	[Phe^{13},Tyr^{19}]MCH	9.88 ± 0.12
Melatonin	MT_2	ES-621-M	2-Iodo-Melatonin	9.99 + 0.09
Neuromedin U	NMU1	ES-450-M	NMU 25	9.48 ± 0.04
Opioid	NOP	ES-230-M	Nociceptin	9.51 ± 0.05
Prokineticin	PKR_1	ES-750-M	MIT-1	9.51 ± 0.02
	PKR_2	ES-751-M	MIT-1	9.85 ± 0.04
Prostanoid	DP_1	ES-560-M	PGD_2	8.04 ± 0.08
	EP_2	ES-562-M	PGE_2	7.45 ± 0.11
	EP_4	ES-563-M	PGE_2	7.84 ± 0.10
Tachykinin	NK2	ES-251-M	[Nle]NKA	7.14 ± 0.07
Somatostatin	sst4	ES-524-M	SRIF28	8.84 ± 0.07
Vasopressin	V_{1A}	ES-361-M	[Arg8]Vasopresssin	8.67 ± 0.06

References

Receptor Binding Assays , Assay Guidance Manual Version 5.0, 2008, Eli Lilly and NIH Chemical Genomics Center
J-H Zhang et al. (1999) J. BioMolecular Screening, 4, 67-73

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