



FLASH NEWS

PerkinElmer™ Products

- Radioreceptor Assay Applications

USE OF FLASHPLATE™ FOR RADIORECEPTOR ASSAYS

FlashPlate is a versatile tool which can be used for non-separation assays of receptors, both membrane bound and soluble. The following procedures have been reported in the literature, developed at DuPont NEN or by customers. This bulletin contains general information, followed by specific information for endothelin receptor A, endothelin receptor B, and IL-1 receptor.

I. GENERAL PROCEDURE

Materials:

- Basic FlashPlate (SMP-200) for most applications
- Streptavidin FlashPlate (SMP-103) for use with biotinylated receptors or membrane preparations.
- Phosphate-buffered saline (PBS). This buffer is used for diluting the receptor preparation prior to coating and also can be used as the assay buffer.
- 1-2% Bovine Serum Albumin in phosphate-buffered saline (Blocking Buffer). This is used to block all of the areas of the FlashPlate that are not already coated with protein, and reduces the non-specific binding. Because some assays do not need this step, the use of this buffer should be validated for each assay.
- TopSeal-A™ Plate Covers (DuPont # SMP-201) for sealing the FlashPlates prior to counting.

Methods:

I. General Considerations for Coating Membrane Bound Receptors

- A. It is possible to coat membrane bound receptors onto polystyrene and have them retain their full activity (2). But no one method works for all receptors. Start with the simplest method of coating membranes directly onto SMP-200, Basic FlashPlate (Example II). Alternatively, the double capture methods such as a pre-coat of poly-lysine (Example III), antisera to membrane proteins, or streptavidin (with SMP-103 as described in Example IV) for use with biotinylated receptors or membranes can be tried.

- B. Membrane preparations have only a small percent of the total protein as the receptor. Though the interior of membranes is hydrophobic, the exposed portions of membrane preparations are hydrophilic. This can reduce the binding to polystyrene inner surface of the FlashPlate.
- C. Consider the impact of any additives used in dilution or storage buffers for membrane preparations (i.e. BSA, glycerol). Additional protein such as BSA frequently sticks more avidly than do membranes and is usually in very high concentration relative to the membrane protein. Do not use detergents in the buffers or use detergent-treated membrane preparations for direct coating onto SMP-200. The detergent very effectively blocks all hydrophobic regions of the membranes and prevents binding of the membranes to polystyrene surfaces (2).
- D. Coating of microplates is a very inefficient extraction method. Only a small percentage of the total protein presented in the well for binding attaches, even with ideal proteins. It may be necessary to use as much or more membrane preparation than is used in an optimized filtration assay.
- E. Whenever possible, use ^{125}I ligands to optimize the binding of receptors and then switch to ^3H if desired. The higher energy of the ^{125}I emission as well as the higher specific activity of the tracer make it easier to use with membrane preparations.
- F. For some receptors it may be possible to use the FlashPlate as a reaction vessel (i.e. add receptor, ligand and tracer all at the same time) and centrifuge the FlashPlate at low speed to collect the receptors against the well wall or to allow to settle briefly before counting. Aspiration and washing are not needed prior to counting (Example I.)

II. General Considerations for Coating Pure Receptors or Soluble Receptors

- A. Receptors not in membranes, such as purified receptors or soluble receptors, are generally easier to coat onto polystyrene surfaces as long as detergent was not used in the purification or used in the assay buffers. Pure receptors do not have other proteins present that will compete for the binding. Many receptors have hydrophobic regions which should bind to the polystyrene of the microplates.
- B. Direct coating of the pure receptor can be optimized by starting with a receptor concentration of 5 ug/ml in PBS and adding more or less depending on the binding results. Direct coating can inactivate some or all of the pure receptor and may provide steric hindrance in the binding assay.
- C. Non-neutralizing antisera to the receptor may first be coated (5 ug/ml) on SMP-200 to improve the binding of the pure receptor and prevent inactivation of the receptor. The receptor may be more accessible to the solution as well and this may improve the binding kinetics.
- D. The pure receptor may be biotinylated (reagents and methods from Pierce, Inc.) and bound to the streptavidin coated FlashPlate Plus (SMP-103) (see Example IV).

III. Assay Conditions

- A. Start any experiment to optimize conditions with what is working in other assays with the same membrane or receptor preparation (i.e. filtration). To obtain a good signal, at least 10 fmol of receptor should be in each well. It will probably not be necessary to increase the amount of radioactivity to detect binding.
- B. Use the previously established incubation times as a starting point as well. If the incubation is at room temperature, kinetic studies may be set up directly on the counter.
- C. Typical assay buffers are those used in radioreceptor assays with filtration separation, e.g.,
 - 50 mM Tris, pH 7.4, with 1 mM CaCl₂, 0.1% BSA, 0.1% Bacitracin
 - or - 50 mM mM Tris, pH 7.4 with 5 mM MgCl₂, 5 mM KCl, 5 mM EDTA, 1.5 mM CaCl₂
- D. Non-specific binding may limit the signal to noise ratio achievable. Aspiration is not usually required but could be useful for decreasing non-specific binding.

Example:

I. **Receptor Assays Without Precoating the FlashPlate with Receptor**

A. Method

- 1) 50 ul of the cell fragment preparation [human Endothelin B receptor expressed in CHO cells (DuPont NEN Cat. Item CRM012) diluted 1:10 in PBS is mixed with 50 ul of the tracer [40,000 cpm of ¹²⁵I-Endothelin-1 (DuPont NEN Cat. Item NEX259) and 50 ul of the cold ligand [1 uM for NSB] or drug in assay buffer to be tested directly in the FlashPlate. (Optimum results are achieved with FlashPlate precoated with polylysine as in III.)
- 2) The FlashPlate is allowed to incubate 4 hours to overnight at room temperature.
- 3) The FlashPlate may be centrifuged at 800xg for 10 minutes at 4°C, though this is not usually necessary.
- 4) The FlashPlate is counted with or without aspirating the supernatant, depending on which method gives the best signal to noise ratio and highest signal.

Example:

II. **Receptor Assays Using FlashPlate Precoated With Receptor(1,2)**

A. Coating Method (Centrifuge Method (1,2))

- 1) Endothelin A receptor membrane preparation (see Appendix) was diluted to 0.05 mg/ml protein in PBS pH 7.5 and 100 ul was added to the FlashPlate.
- 2) The FlashPlate is centrifuged at 800xg for 10 minutes at 4°C .
- 3) The FlashPlate is blocked by the addition of 5 mg/ml BSA in PBS to each well and incubated for 30 minutes at room temperature.
- 4) The coated FlashPlates are stored frozen.

B. Coating Method (incubation method)

- 1) Add Endothelin B receptor membrane preparation to the FlashPlate (100 ul).
- 2) Let incubate at 4°C overnight.
- 3) Decant and use immediately or store sealed at 4°C. For best results, do not block or rinse the plates.

C. Assay

- 1) Add 50 µl of ¹²⁵I-Endothelin-1 (25,000-30,000 cpm/50 µl)
- 2) Add 50 µl of buffer or competitor (NSB = 500 nM ET-1)
- 3) Incubate 2 hours at 37°C.
- 4) Count before and after aspirating the contents of the wells.

D. Results

| | Assay (centrifuge method) | Assay (incubation method) | |
|--------------|---------------------------------------|-------------------------------------|--------------------------|
| | Without Aspiration (cpm) [ref. #1] | Without Aspiration (cpm) | With Aspiration (cpm) |
| Bo | 1591 | 1218 | 1546 |
| NSB | 151 | 180 | 90 |
| Signal/Noise | 10.5 | 6.8 | 17.18 |

Example:

III. Receptor Assays With Precoated FlashPlates

A. Precoating Method With Poly D-Lysine and Polyethylenimine (PEI)

- 1) Prepare 100 ug/ml of Poly D-Lysine (m.w. 50,000) or 0.1% PEI. (PEI is supplied as a 50% aqueous solution.)
- 2) Add 200 ul of the solution to each well of a FlashPlate.
- 3) Incubate overnight at room temperature.
- 4) Decant and wash twice with PBS.
- 5) Store dry at 4°C.

B. Receptor Coating Method

- 1) Add Endothelin B receptor membrane preparation to the FlashPlate (100 ul).
- 2) Let incubate at 4°C overnight.
- 3) Decant and use immediately or store sealed at 4°C. It is not necessary to block or rinse the plates.

C. Assay

- 1) Add 50 µl of ¹²⁵I-Endothelin-1 (25,000-30,000 cpm/50 µl)
- 2) Add 50 µl of buffer or competitor (NSB = 500 nM ET-1)
- 3) Incubate 2 hours at 37°C.
- 4) Count before and after aspirating of the contents of the wells.

D. Results

A. Endothelin A- CHO Cell Membranes (100 µg/ml or 11 fmol/well)

| | PEI Precoat | Poly-D-Lysine |
|-----------------|-------------|---------------|
| Bo (cpm) | 1000 | 1000 |
| NSB | 95 | 98 |
| Signal to Noise | 10.5 | 10.2 |

B. Endothelin B- CHO Cell Membranes (27.5 µg/ml or 12.6 fmol/well)

| | PEI Precoat | Poly-D-Lysine | Basic FlashPlate |
|-----------------|-------------|---------------|------------------|
| Bo (cpm) | 1054 | 1490 | 836 |
| NSB | 228 | 215 | 190 |
| Signal to Noise | 4.6 | 6.9 | 4.4 |

IV. Receptor Assays Using Pure Receptors With Streptavidin FlashPlate Plus, SMP103 (3)

A. Biotinylation of Soluble Recombinant IL-1 Receptor

- 1) React pure soluble receptor with succinimidyl-biotin (available from Pierce) using methods recommended by Pierce.
- 2) Remove the unreacted biotin by size-exclusion chromatography or dialysis.

B. Coating Procedure (using Streptavidin Coated FlashPlates: SMP-103)

- 1) Block the Streptavidin coated FlashPlate with 1% BSA in PBS for 30 min. at room temperature.
- 2) Add 200 ng of biotinylated receptor per well in 100 ul volume.
- 3) Incubate one hour at room temperature.
- 4) Aspirate and rinse twice with 1% BSA in PBS.
- 5) Store at 4^oC

C. Assay Procedure

- 1) Add 50 µl of ¹²⁵I-IL-1β (1 nM) (DuPont NEN Cat. Item NEX232).
- 2) Add 50 µl of Buffer (PBS) or cold IL-1β.
- 3) Incubate 1 hour at 25^oC.
- 4) Count.

D. Results

Coating of the biotinylated receptor onto the Streptavidin coated FlashPlate counts observed (with no washing)

| | |
|-----------------|------|
| Bo (cpm) | 2118 |
| NSB (cpm) | 480 |
| Signal to Noise | 4.4 |

Notes:

- a) Coating of antibody to receptor on the Basic FlashPlate and then capturing the receptor did work.
- b) Direct coating of this receptor on the Basic FlashPlate did not work.

APPENDIX

I. Preparation of Membrane Fractions from Cells Producing Cloned Receptors (1)

- a) Cells are homogenized in a Polytron homogenizer in 50 mM Tris-Cl, pH 7.4 in the presence of soybean trypsin inhibitor (5 ug/ml), 1,10-phenanthroline (1mM), benzamidine (1mM), bacitracin (100 ug/ml) and sucrose (3 mM) [Buffer A].
- b) The cell fragments are centrifuged at 1500xg for 10 minutes at 4°C.
- c) The supernatant is collected and centrifuged again at 40,000xg for 30 minutes.
- d) The pellet is washed once in Buffer A.
- e) The pellet is resuspended in Buffer A using a hand held homogenizer to get uniform resuspension.

NOTE: With small modification, this method has been used for preparation of membrane receptors from tissue.(2).

II. Preparation of Cell Fragments (DuPont CRM-011 and CRM-012)

Disrupt cells by drawing up and down in a pipet, then vortexing. Store frozen in assay buffer (listed above in Appendix I a).

III. Preparation of Crude Membrane Fractions

- a) Disrupt the cells or tissue as described in Appendix I with short bursts for 30 seconds with a Polytron in buffer plus protease inhibitors.
- b) Centrifuge at 100 x g for 15 minutes at 4°C. Resuspend the pellet in 5 ml of buffer and homogenize in the Polytron (step a).
- c) Centrifuge at 300 x g for 20 minutes at 4°C. Decant the supernatant into centrifuge tubes for the high speed rotor and discard the pellet.
- d) Centrifuge at 10,000 x g for 10 minutes at 4°C. Wash this pellet twice by resuspending and centrifuging at 10,000 x g.
- e) Resuspend the pellet in storage buffer and store at -70°C.

REFERENCES:

- 1) Holland, J.D., Singh, P., Brennand, J.C. & Garman, A.J., "A nonseparation microplate receptor binding assay." *Analytical Biochem.* 222 (1994) 516-518.
- 2) Nichols, J.S., LeVine, H., Smith, G.F.H., Wypij, D.M. and Wiseman, J.S., "Determination of endothelin by an immobilized receptor assay utilizing a 96 well format" *Biochemical and Biophysical Methods* 25 (1992) 173-184.
- 3) Methodology and results are a personal communication from Helen Yeoman and Rick Chris at Selectide, Tucson, AZ.

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