

Chapter 2

Competitive Binding Assay for the G-Protein-Coupled Receptor 30 (GPR30) or G-Protein-Coupled Estrogen Receptor (GPER)

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Abstract

The role of 2-methoxyestradiol is becoming a major area of investigation because of its therapeutic utility, though its mechanism is not fully explored. Recent studies have identified the G-protein-coupled receptor 30 (GPR30, GPER) as a high-affinity membrane receptor for 2-methoxyestradiol. However, studies aimed at establishing the binding affinities of steroid compounds for specific targets are difficult, as the tracers are highly lipophilic and often result in nonspecific binding in lipid-rich membrane preparations with low-level target receptor expression. 2-Methoxyestradiol binding studies are essential to elucidate the underlying effects of this novel estrogen metabolite and to validate its targets; therefore, this competitive receptor-binding assay protocol was developed in order to assess the membrane receptor binding and affinity of 2-methoxyestradiol.

Key words 2-Methoxyestradiol, GPR30, G-protein-coupled receptor 30, GPER, G-protein-coupled estrogen receptor, Endoplasmic reticulum membrane, Radioligand binding, Competitive binding

1 Introduction

2-Methoxyestradiol (2ME2) is a final end product of estradiol (E2) metabolism. While the affinity of E2, notably 17- β estradiol, for estrogen receptor α and β (ER α /ER β) has been demonstrated conclusively, 2ME2 has 2000-fold less activation potential for classical genomic estrogen receptors [1]. Estrogen may also induce cellular responses through non-genomic pathways through receptors such as G-protein coupled estrogen receptor (GPER), also called G-protein-coupled receptor 30 (GPR30), a G α_i -associated G-protein-coupled receptor [2]. In our own studies, we have found that 2ME2 retains significant binding affinity for this receptor, even though it lacks the same affinity for inducible nuclear estrogen receptors [3]. Additionally, 2ME2 is capable of inducing a number of cellular responses primarily through this receptor. In the literature, it is suggested that this receptor may be localized in

one of three places, either the endoplasmic reticulum (ER), plasmalemma, or golgi apparatus [4–6]. In any case, for one to assess the potential binding affinity of a ligand to this novel receptor, membrane preparation is required. Studies aimed at establishing the binding affinities of steroid compounds for specific targets are difficult, as the tracers are highly lipophilic and often result in non-specific binding in lipid-rich membrane preparations with low-level target receptor expression [7]. This chapter describes a competitive receptor binding assay protocol to assess the membrane receptor binding and affinity of 2-methoxyestradiol for the membrane estrogen receptor, GPR30. The following protocol was originally developed for use on rat liver epithelial cells, although the method should be compatible with any cell type for which GPR30 binding affinity must be determined.

2 Materials

2.1 Reagents

1. [^3H]-2-Methoxyestradiol (2ME2) (American Radiolabeled Chemicals Inc., 50 Ci/mmol).
2. Unlabeled 2ME2 (16.5 mM in DMSO).
3. GPR30-specific antagonist, G15 (1.3 mM in DMSO).
4. Trypsin solution, 10 \times (25 g porcine trypsin peptide/L HBSS).

2.2 Total Cell Membrane Isolation

1. 50 mM Tris-HCl, pH 7.4.
2. Wheaton glass dounce tissue grinder with “B” pestle.
3. Hanks’ balanced salt solution (HBSS).
4. Rubber policeman.
5. Binding buffer without bovine serum albumin: 50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 4 mM KCl, 1 mM CaCl_2 , 10 $\mu\text{g}/\text{mL}$ bacitracin, and 2 mg/mL dextrose.
6. Low-speed and high-speed centrifuges and centrifuge tubes.

2.3 Endoplasmic Reticulum Membrane Isolation

1. Phosphate-buffered saline (PBS), pH 7.4.
2. 0.05 % porcine trypsin and 0.02 % EDTA in PBS.
3. Trypsin-neutralizing solution: soybean trypsin inhibitor.
4. MTE buffer: 270 mM mannitol, 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, Complete Mini Protease inhibitor cocktail tablet (Roche), 1 mM phenylmethanesulfonylfluoride (PMSF) (100 mM stock in ethanol) (*see Note 1*).
5. Wheaton glass Dounce tissue grinder with “B” pestle.
6. Discontinuous sucrose gradient: 1.3, 1.5, and 2.0 M sucrose in 10 mM Tris-HCl, pH 7.0.

2.4 Radioligand-Binding Assay

1. Binding buffer with BSA: 50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 4 mM KCl, 1 mM CaCl₂, 10 µg/mL bacitracin, 0.25 % BSA, and 2 mg/mL dextrose (*see Note 2*).
2. Wash buffer: 50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 4 mM KCl, 1 mM CaCl₂, 10 µg/mL bacitracin, and 2 mg/mL dextrose (*see Note 3*).
3. Borosilicate disposable culture tubes.
4. Millipore circular nitrocellulose membrane (0.45 µm).
5. Millipore filtration unit (e.g., Model 1225 sampling manifold vacuum).
6. Scintillation fluid (Scintiverse®).
7. Scintillation counting vials.

3 Methods

Following are two specific methods, one for radioligand binding on total membrane extracts [8], and the other for assessing the binding of a given ligand (for our purposes we have used [³H] 2ME2) for ER membrane localized GPR30 receptors [9]. All steps of membrane isolation must be performed at 4 °C or on ice, while radioligand binding may proceed at room temperature (22 °C).

3.1 Total Cell Membrane Preparation

1. Grow cells (rat liver epithelial cells [3]) to 90 % confluence in 100 mm cell culture plates under conditions required by the experiment.
2. Wash the cells 2× with HBSS.
3. Harvest cells in 1 mL HBSS using a rubber policeman.
4. Homogenize the cells using a Wheaton glass Dounce tissue grinder using a “B” pestle for ten strokes.
5. Collect the homogenate and centrifuge at 1000×g for 7 min.
6. Transfer the supernatant to a fresh tube and centrifuge at 45,000×g for 20 min.
7. Resuspend the membrane pellet in 50 mM Tris-HCl, pH 7.4 and centrifuge at 45,000×g for 20 min.
8. Suspend the resulting pellet in binding buffer without BSA and use immediately for radioligand binding study or store in aliquots at -80 °C (*see Note 4*).
9. Determine the protein concentration before the binding assay (We use the Bio-Rad protein assay based on the Bradford method).

3.2 Preparation of Endoplasmic Reticulum Membranes

1. Grow cells (rat liver epithelial cells [3]) to 90 % confluence in a 75 cm² cell culture flask under conditions required by the experiment.
2. Wash the cells 1× with PBS.
3. Trypsinize the cells for 3 min in 1 mL of trypsin at 37 °C.
4. Add 1 mL of trypsin inhibitor and mix at room temperature.
5. Collect the cells in 8 mL PBS (yielding 10 mL total volume) and transfer to a 15 mL centrifuge tube.
6. Centrifuge cells at 2500×*g* for 5 min.
7. Resuspend the cell pellet in 4 mL MTE buffer.
8. Homogenize the cell suspension using a Wheaton glass Dounce tissue grinder using a “B” pestle for ten strokes.
9. Centrifuge the resultant homogenate at 700×*g* for 10 min at 4 °C.
10. Collect the supernatant and centrifuge at 15,000×*g* for 10 min at 4 °C.
11. Prepare a discontinuous sucrose gradient: first layer the densest sucrose solution (2.0 M) at the bottom of the high-speed centrifuge tube, then layer the intermediate solution (1.5 M), then the least dense (1.3 M), creating three distinct layers of proportionate volumes. It is important to allow the sucrose solution to run slowly down the inside of the high-speed centrifuge tube.
12. Collect the supernatant and layer on top of the discontinuous sucrose gradient. The centrifugation requires a balanced rotor; therefore, split the supernatant sample into two parts and prepare two gradient centrifuge tubes with the same mass [9]. Centrifuge at 100,000×*g* for 45 min at 4 °C.
13. Collect the purified ER membrane (white circular band embedded in the sucrose gradient), resuspend in binding buffer, and immediately proceed with radioligand-binding assay.
14. Alternatively, you may store aliquots of the purified ER membrane at –80 °C until needed (*see* **Note 4**).
15. Determine the protein concentration before the binding assay (we use the Bio-Rad protein assay based on Bradford method).

3.3 [³H] 2-Methoxyestradiol-Binding Assay

1. Membrane samples isolated in Subheading 3.1 or 3.2 are placed in disposable borosilicate tubes in a volume of 50 µL binding buffer (*see* **Note 5**).
2. Add 400 µL of binding buffer with BSA.
3. Add 3.3 pmol [³H] 2ME2 (200 nCi) in a volume of 50 µL of binding buffer (*see* **Note 6**).
4. Incubate the reaction mixture for 1 h at 22 °C. Total reaction volume should not exceed 500 µL.

5. Pass the reaction volume through a presoaked membrane filter using a Millipore filtration unit (*see* **Note 7**).
6. Wash the membrane 3× each with 2 mL wash buffer without BSA.
7. Transfer the filter to a scintillation counting vial.
8. Add 10 mL Scintiverse® scintillation fluid and mix thoroughly.
9. After 1 h, proceed to counting on a Beckman scintillation spectrometer or other suitable platform.
10. Counts (disintegrations per minute, dpm) represent total radioligand binding to the membranes.

3.4 Competitive Binding for Determining [³H] 2ME2 Specificity

1. In order to determine nonspecific binding of the membranes, preincubate with 1 μM unlabeled 2ME2 in binding buffer for 10 min before the addition of the [³H] 2ME2 radioligand.
2. Proceed through **steps 3–9** in Subheading **3.3**.
3. Specific [³H] 2ME2 binding is defined as that portion of the total binding displaced by unlabeled 2ME2.

3.5 Competitive Binding for Determining GPR30 Specificity

1. Preincubate membranes with 10 μM G15 (GPR30-specific antagonist) in binding buffer 10 min before the addition of the [³H] 2ME2.
2. Proceed through **steps 3–9** in Subheading **3.3** to determine receptor specificity.
3. Specific [³H] 2ME2 binding to GPR30 is defined as that portion of the total binding displaced by G15.

3.6 Binding Affinity (K_d) Calculation

1. In order to determine the affinity (K_d) of 2ME2 for GPR30, incubate a series of membrane preparations with increasing concentration of [³H] 2ME2 in binding buffer (for example: 0.25, 0.5, 1.0, 2, 4, 8, 12, 25, and 50 nM).
2. Each reaction should be paired with a matching condition that was preincubated with 100–1000-fold unlabeled 2ME2. This will allow determination of nonspecific binding.
3. Obtain counts for each condition and calculate specific binding as described in Subheading **3.4**.
4. Convert obtained specific DPM values to appropriate molar concentration. For example, a value of 4.4×10^5 counts should be the equivalent of 3.3 pmol of [³H] 2ME2. (This assumes specific activity of the isotope at 50 μCi/nmol, and 2.2×10^6 DPM/μCi.)
5. Plot concentration of bound [³H] 2ME2 (y -axis) by the concentration of free [³H] 2ME2 (x -axis)—(*see* Table **1** and Fig. **1**).
6. Using GraphPad Prism software, or another appropriate statistical analysis tool, analyze the data in nonlinear regression equation of one-site saturation binding: $Y = (B_{\max} \times X) / (K_d + X)$.

Table 1
The numbers calculated and expressed in this table are entirely theoretical

Free [³ H] 2ME2 (nM)	Bound [³ H] 2ME2 (fmol/mg protein)
0.25	95.45
0.5	381.18
1.0	656.00
2.0	1361.54
4.0	2560.81
6.0	3363.63
12.0	5636.36
25.0	8763.00
50.0	12,210.00

Conversion of DPM to fmol was performed assuming specific activity at 2.2×10^6 DPM/ μ Ci and 50 μ Ci/nmol. Protein was held constant at 0.1 mg for all reactions

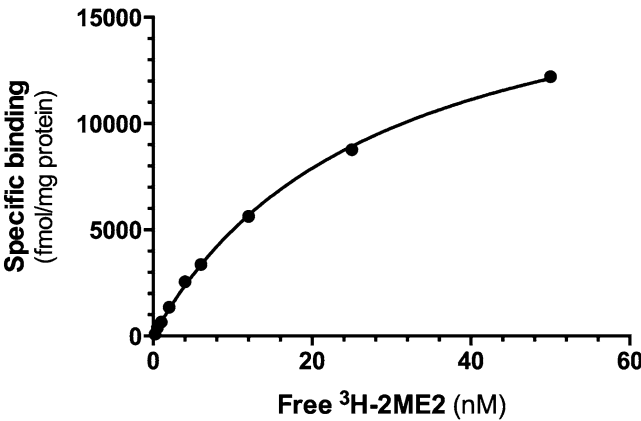


Fig. 1 Saturation binding isotherm plot of values provided in Table 1. As this is only an example using arbitrary numbers for demonstrative purposes, deviation from these calculations of B_{max} and K_d in an actual experiment can be expected

4 Notes

The methods described in this chapter were optimized in our laboratory for our particular cell type; therefore, these notes may be of interest to those who wish to adapt this protocol in other cell lines.

1. MTE buffer must be prepared fresh each day. 100 mM PMSF stock solution may be stored at room temperature.
2. Binding buffer must be prepared fresh each day before the binding assay.

3. Wash buffer and binding buffer without BSA must be prepared separately.
4. Binding affinity of membranes kept at -80°C will probably be reduced over time.
5. Minimum protein content should be above $50\text{ }\mu\text{g}$ and up to $200\text{ }\mu\text{g}$ for a single reaction.
6. In the final reaction volume of 500 mL , 3.3 pmol is the equivalent of a 6.6 nanomolar solution of $[^3\text{H}] 2\text{ME2}$.
7. Membranes must be soaked for 1 h in binding buffer with BSA prior to use.

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