MSD® GPCR-Ligand Binding Assay Demonstration Kit: Melanocortin 5

MULTI-ARRAY™ 96 Small Spot Plate

I. Materials Included

	Storage

MSD Catalog Items

❑ MSD Read Buffer T, surfactant free (4x)	RT
❑ MSD Blocker A	4ºC

R&D Custom Items

❑ MULTI-ARRAY PEI 96 Small Spot Plate (High Bind)	RT
❑ MC5 receptor-containing cell membranes	- 80ºC
❑ Control membranes	- 80ºC
❑ MC5 Binding Buffer (10X)	4ºC
❑ MSD TAG™ labeled NDP-α-MSH	4ºC
❑ NDP-α-MSH (unlabeled)	4ºC

II. Other Materials & Equipment (not supplied)

- ❑ Deionized water for diluting Binding Buffer and Read Buffer
- ❑ 37°C water bath or incubator for quick thawing membranes
- ❑ Automated or manual multi-channel liquid handling equipment that must accurately deliver 2 µL to a precise location in X, Y, Z coordinates within each well of a 96-well small spot plate
- ❑ Hand pipettes, tubes, source plates, tips, etc. for making serial dilutions of ligand, and the required sizes will depend upon the scale of experiment
- ❑ Syringe (1 or 3 mL, depending upon size of experiment) with a 26 gauge, 5/8 inch needle
- ❑ Adhesive plate seals and/or cover plates

III. Preparation & General Notes:

Prepare working stock of MC5 Binding Buffer:

1. Approximately 10 mL is required per 96-well plate.
2. Dilute 10X MC5 Binding Buffer to 1X with deionized water.

Prepare membranes:

1. Determine the number of wells to be used in the experiment. Each will receive 2 µL (0.5 µg) of diluted MC5 receptor-containing or control membranes. Remember to include extra for "dead" volume in the pipetting technique when performing calculations for membrane preparations. A 10-12 µL cushion per well is recommended when using automation with a source plate, or a 10-15% excess volume per well when hand-spotting membranes.
2. Quick-thaw membranes in a 37°C water bath for one minute.
3. Collect aliquots to one tube (if using multiple stock tubes) and process 6 times using a syringe with a 26G 5/8 needle, keeping the flat end of the needle against the sidewall of the tube.
4. Dilute membranes from the stock concentration of 1.5 µg/µL to 0.25 µg/µL (1:6) in cold 1X MC5 Binding Buffer.
5. Keep on ice until ready to dispense.

Prepare unlabeled ligand for competition curve (if desired):

1. Determine the number of points required for a competition curve and the desired number of replicates. To demonstrate a good competition curve, sixteen 3-fold dilutions, starting at 10 µM, are recommended. Each well will receive 3 µL of the unlabeled ligand titrations, and this amount does not account for "dead" volume in the pipetting technique (see above). Figure 1 illustrates a suggested plate format.
2. Prepare the working dilution of the unlabeled NDP-α-MSH ligand to 8.3X the final assay concentration using 1X MC5 Binding Buffer ***with 3% (w/v) MSD Blocker A added (30 mg/mL)***. Note: From this 8.3X working dilution, 3 µL/well will be added with a total assay volume of 25 µL/well yielding the final 1X ligand.
3. Prepare 1:3 serial dilutions of unlabeled ligand fifteen times using the **1X MC5 Binding Buffer-3% Blocker A**, including a zero-ligand final point.

Dilute labeled ligand:

1. Each well will receive 20 µL of labeled ligand for competition curves or 23 µL for saturation-binding curves. Remember to include calculations for extra ligand solution to account for the "dead" volume in pipetting technique.
    a. For cold competition curves, dilute MSD TAG labeled NDP-α-MSH to a 1.25 nM working concentration in 1X MC5 Binding Buffer with ***3.0% (w/v) MSD Blocker A added (30 mg/mL)***. Note: The final assay concentration will be 1 nM labeled ligand.

Notes:

MSD MULTI-ARRAY plates are compatible with most binding buffers. A wide variety of binding buffers have been tested. The buffer supplied has been optimized for the biological reagents provided.

Unlabeled NDP-α-MSH sticks to labware. If preparing a competition curve, it is important to dilute into 3% MSD Blocker A to obtain accurate IC_{50} values.

Labeled NDP-α-MSH and unlabeled NDP-α-MSH can be stored at 4 °C for up to 2 weeks. Ligands can be aliquoted and placed at -20 °C for long-term storage.

For other GPCR systems, it is recommended to optimize the concentration of Blocker A (added to the Binding Buffer) through a titration experiment.

b. For a saturation-binding curve, eight, 2-fold serial dilutions starting at 16 nM are recommended to produce a complete curve. Prepare the working dilution of MSD TAG labeled NDP-α-MSH to 1.09X the final assay concentration in 1X MC5 Binding Buffer containing **3.0% (w/v) MSD Blocker A (30 mg/mL)**. Prepare 1:2 serial dilutions of labeled ligand seven times in **1X MC5 Binding Buffer-3% MSD Blocker A**, including a zero-ligand final point.
Note: From the 1.09X working dilutions, 23 µL/well will be added with a total assay volume of 25 µL/well yielding the final 1X ligand.

Dilute Read Buffer:

1. Determine the total number of wells to be used in the experiment. Each well will receive 125 µL of 1.2X Read Buffer.
2. Dilute 4X MSD Read Buffer T (surfactant-free) to 1.2X with deionized water (12 mL 4X Read Buffer T without surfactant per 40 mL total volume).

IV. Detailed Instructions:

Saturation-Binding Assay Protocol:

Begin with an MSD MULTI-ARRAY PEI 96 Small Spot Plate (High Bind). No pre-treatment is necessary.

1. Carefully deliver 2 µL/well of *quick-thawed, syringe-processed, and diluted* membranes directly to the center of the working electrode, taking care to ensure the droplet is contained to the electrode surface.

2. Seal or cover, and incubate at room temperature for 1 hour.

3. Dispense 23 µL/well of the MSD TAG labeled NDP-α-MSH titrations.

4. Seal or cover, and incubate at room temperature for 1 hour.

5. Dispense 125 µL/well of the diluted 1.2X MSD Read Buffer T (surfactant free) and analyze *immediately* with the SECTOR™ Imager.

Competition-Binding Assay Protocol:

Begin with an MSD MULTI-ARRAY PEI 96 Small Spot Plate (High Bind). No pre-treatment is necessary.

1. Carefully deliver 2 µL/well of *quick-thawed, syringe-processed, and diluted* membranes directly to the center of the working electrode, taking care to ensure the droplet is contained to the electrode surface.

2. Seal or cover, and incubate at room temperature for 1 hour.

3. Dispense 3 µL/well of the unlabeled NDP-α-MSH titrations. The plate may be incubated while stacked with a cover plate at room temperature for 1 hour to simulate HTS workflow - inconsequential for demonstration purposes.

4. Dispense 20 µL/well of diluted MSD TAG labeled NDP-α-MSH solution.

5. Seal or cover, and incubate at room temperature for 1 hour.

6. Dispense 125 µL/well of diluted 1.2X MSD Read Buffer T (surfactant free) and analyze *immediately* with the SECTOR Imager.

Figure 1. Suggested plate format for saturation and competition binding curves using a MULTI-ARRAY 96 Small Spot Plate.

Saturation-Binding — ***MSD TAG labeled NDP-α-MSH***

Cold Competition — ***unlabeled NDP-α-MSH***

	1	2	3	4	5	6	7	8	9	10	11	12
A	0nM		0nM		0nM		0nM		4.57nM		4.57nM	
B	0.25nM		0.25nM		0.00209nM		0.00209nM		13.7nM		13.7nM	
C	0.5nM		0.5nM		0.00627nM		0.00627nM		41nM		41nM	
D	1nM		1nM		0.0188nM		0.0188nM		123nM		123nM	
E	2nM		2nM		0.0565nM		0.0565nM		370nM		370nM	
F	4nM		4nM		0.169nM		0.169nM		1,111nM		1,111nM	
G	8nM		8nM		0.508nM		0.508nM		3,333nM		3,333nM	
H	16nM		16nM		1.52nM		1.52nM		10,000nM		10,000nM	
	MC5		HEK		MC5		HEK		MC5		HEK	