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Monitoring cell-surface expression of GPCR by ELISA

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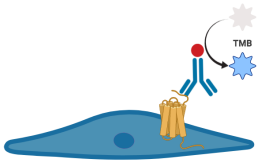

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Protocol status: Working
We use this protocol and it's working

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Abstract

Quantifying cell surface expression of G Protein-Coupled Receptors (GPCRs) can be exteremly important for the expression of mutant receptors. Herein we report a useful Enzyme-Linked Immunosorbent Assay (ELISA) for the cell-surface detection of a HA-tagged version of a GPCR.

Materials

MATERIALS

- NESTLE CARNATION Instant Non-fat Dry Milk
- Poly-l-lysine, 0.1% (wt/vol) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P8920
- Falcon® 24-Well Flat-Bottom Plate, Tissue Culture-Treated 50 Plates **STEMCELL Technologies Inc. Catalog #**38021
- HEK293 **ATCC Catalog #**CRL-1573
- Lipofectamine 3000 **Thermo Fisher Scientific Catalog #**L3000015
- Opti-MEM™ I Reduced Serum Medium **Thermo Fisher Catalog #**31985062
- Sterile Water **Wisent Bioproducts Catalog #**809-115-CL
- PBS 1X **Wisent Bioproducts Catalog #**311-011-CL
- Trypsin 0.25% / EDTA 2.21 mM in HBSS **Wisent Bioproducts Catalog #**325-043-EL
- SafeSeal tube 5mL **Sarstedt Catalog #**72.701
- Formaldehyde Reagent Grade **Bioshop Catalog #**FOR201
- 3 3′5 5′-Tetramethylbenzidine Liquid Substrate Supersensitive for ELISA **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T4444-100ML
- Anti-HA-Peroxidase High Affinity **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**12013819001
- FBS (Fetal Bovine Serum) Premium Quality Endotoxin **Wisent Bioproducts Catalog #**080-150
- DMEM 4.5g/L glucose with L-glutamine sodium pyruvate and phenol red **Wisent Bioproducts Catalog #**319-005-CL
- HEPES 1M Free acid **Wisent Bioproducts Catalog #**330-050-EL
- Penicillin (5000IU) / Streptomycin (5000µg/mL) sterile filtered for cell culture **Wisent Bioproducts Catalog #**450-200-EL

Endotoxin-free purified plasmidic DNA encoding for HA-tagged GPCRs

Tris-Buffered Saline (TBS, containing 20mM Tris–HCl pH 7.5 and 150mM NaCl)

Day 1 - Cell Culture & Transfections

1 **Coat 24-well plates with Poly-L-Lysine** (this need to be done in a biological safety cabinet to ensure sterility).

1.1 Add 300 µL of [M] 0.1 mg/mL Poly-L-Lysine solution in each well of the 24-well plate and incubate 00:10:00 at Room temperature .
This can be done using a 300µL multichannel pipet fitted with 4 tips

1.2 Remove the Poly-L-Lysine solution (this solution can be re-used up to 4 times to coat cell culture plasticware).
This can be done using a 300µL multichannel pipet fitted with 4 tips

1.3 Rinse the wells twice with 300 µL of sterile water.

1.4 Let dry the 24-well plate without lid under the biological safety cabinet for 00:20:00 before seeding cells.

> Note
>
> Poly-L-Lysine-coated plates can be stored for several weeks at Room temperature befor use.

2 **Prepare transfections of plasmids encoding HA tagged-GPCRs** (this need to be done in a biological safety cabinet to ensure sterility).

> Note
>
> In the case you want to compare expression of a mutant receptor, positive and negative controls are needed to normalize the results. The positive control should be the wild-type receptor and the negative control should be cells transfected with the empy vector (MOCK cells).

For 3 well of the 24-well plate:

2.1 Add 300 µL of Opti-MEM into a sterile 5mL tube.

2.2 Add 1.5 µg of plasmidic DNA encoding for the desired HA tagged-GPCR to the tube containing Opti-MEM and mix.

2.3 Add 3 µL of P3000 Reagent to the tube and mix.

2.4 Add 2.25 µL of Lipofectamine 3000 to the tube, mix, and incubate at Room temperature for 00:15:00 .

3 **Prepare HEK293 cells for transfection** (this need to be done in a biological safety cabinet to ensure sterility).

Note

Ideally, cells were seeded at a density of 3 million cells per 10cm-pretri dish 48h before transfection to ensure a high transfection rate.

3.1 Remove culture media and rinse cells with PBS.

3.2 Add 1 mL of 0.25% Trypsin to a 10-cm petri dish and incubate for 00:02:00 at 37 °C .

3.3 Add 5 mL of complete DMEM (10% FBS, 20mM HEPES, Penicilin/Streptomycin) to the petri dish and dissociate cells by pipeting up and down.

3.4 Count cells using an automated cell counter or a hemacytometer.

Equipment

MOXI Z Mini	NAME
Automated Cell Counter	TYPE
Orflo	BRAND
MXZ001	SKU
https://www.orflo.com/product_p/mxz001.htm	LINK

3.5 Adjust cell concentration to 150,000 cells/mL.

3.6 Add 2 mL of the cell suspension at 150,000 cells/mL to the 5mL tube containing the plasmidic DNA and mix gently.

3.7 Dispense 450 µL of the mix of cell and plasmidic DNA to the desired wells of the Poly-L-Lysine-coated 24-well plate.

3.8 Incubate at 37 °C in humidified chamber at 5% CO2 for 48:00:00 .

Day 3 - ELISA

4 **Detection of cell surface expression by ELISA** (this part of the protocol can be done on the wet bench).

Note

Buffer and reagents in this part of the protocol can be dispensed to the 24-well plate using combitips and a single channel repeater.

Equipment

M4 Repeater	NAME
Multidispense pipet	TYPE
Eppendorf	BRAND
4982000322	SKU
https://online-shop.eppendorf.ca/CA-en/Manual-Liquid-Handling-44563/Manual-Pipetting--Dispensing-44564/Repeater-M4-PF-44619.html	LINK

4.1 Remove cell culture media and wash each well with 400 µL of PBS.

4.2 Fix cells using 400 µL of [M] 3.7 Mass Percent formaldehyde in TBS for 00:05:00 at Room temperature .

4.3 Rinse cells three times with 500 µL of TBS.

4.4 Block non-specific sites using 400 µL of [M] 3 Mass Percent non-fat dry milk disolved in TBS for 00:30:00 under gentle orbital agitation.

Note

In case non-fat dry milk is not suitable for blocking non-specific sites, a solution of [M] 1 Mass Percent of Bovine Serum Albumine in TBS can be used. The same solution must also be used for the incubation of the antibody.

4.5 Remove blocking solution and add 250 µL of 1/1000 dilution of HRP-linked anti-HA antiboby diluted in [M] 3 Mass Percent non-fat dry milk in TBS for 03:00:00 under gentle orbital agitation.

> Note
>
> This incubation step can also be done Overnight at 4 °C under gentle orbital agitation.

4.6 Remove antibody solution and wash each well three times with 500 µL of TBS.

4.7 Add 250 µL of Room temperature 3, 3′,5 ,5′-Tetramethylbenzidine (TMB) Liquid Substrate, Supersensitive, for ELISA and incubate under gentle orbital agitation for 2 to 15 min (until the color of your positive control turn intense blue).

4.8 Stop the TMB reaction by adding 250 µL of [M] 2 Molarity (M) Hydroclhoric Acid (HCl).

4.9 Transfer 100 µL of the colorimetric reaction to a flat-bottom transparent 96-well plate.

4.10 Read the absorbance at 450nm using a multimode plate reader.

Equipment	
Mithras2 LB943	NAME
Multimode plate reader	TYPE
Berthold	BRAND
LB943	SKU

Results analysis

5 As this quantification of cell surface expression is a semi-quantitative method it should not be presented as raw OD_{450nm} values but rather as a percentage of expression compared to the positive control (or wild-type receptor).

To normalize the results, average the OD_{450nm} of the positive control and the OD_{450nm} of the negative control and apply the following formula:

$$y = \frac{x - \overline{x_{min}}}{\overline{x_{max}} - \overline{x_{min}}} \times 100$$

Normalization formula

y = normalized value
x = OD_{450nm} value of the sample
x_{min} = mean OD_{450nm} value of the negative control
x_{max} = mean OD_{450nm} value of the positive control