April 25, 04

HEK293E transfected with FLAG-SmGPCR-EYFP-Rat β-arrestin2-Rluc

Zeocin (Invitrogen) stock is 100mg/ml

Puromycin (Ivivogen) stock is 10mg/ml

Culture medium:
DMEM (high glucose), 5% FBS, 2mM L-Gln, 100U/ml Penicillin, 100µg/ml Streptomycin (i.e. 1:100 of the stock), 10µg/ml Zeocin and 0.25µg/mlPuromycin.

Thus, to prepare 50ml enrich medium:
- 47ml DMEM (Glucose rich)
- 2.5ml FBS (i.e. 5%)
- 0.5ml of Gln/Pen/Strept cocktail (i.e. 1:100 of the stock of 10,000U, µg/ml of pen/strept)
- 5µl Zeocin stock
- 1.25µl Puromycin

Note: Both Zeocin and Puromycin are needed in the culture medium since Rat β-arrestin2-Rluc is under Zeocin selection while FLAG-SmGPCR-EYFP is controlled by Puromycin pressure.

These antibiotics are not required in the freezing medium and should be omitted.

Freezing medium:
DMEM (high glucose), 20% FBS, 2mM L-Gln, 100U/ml Penicillin, 100µg/ml Streptomycin (i.e. 1:100 of the stock), and 10% DMSO.

Thus, to prepare 10ml enrich medium:
- 2ml FBS
- 1ml DMSO
- 7ml DMEM (Glucose rich)
- 100µl of Gln/Pen/Strept cocktail

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Solubilization buffer (to extract SmGPCR) for Western blot analysis:

100mM Ammonium sulfate; 20mM Tris-HCl (pH7.5); 100mM NaCl; 10% Glycerol; 1% (w/v) detergent and protease inhibitor cocktail. Thus, prepare all the components except the detergent and protease inhibitors. Take portions of the solubilization buffer and add 1% detergents (i.e. CHAPS, C8∅, Digitonin ..etc) plus protease inhibitors. Detergent MUST be used fresh.

To prepare 50ml of the solubilization buffer (without a detergent or PrtInh):
- 0.2922g NaCl (58.44g/mol)
- 0.6607g $(NH_4)_2SO_4$ (132.14g/mol)
- 0.1211g of Tris (121.14g/mol), add HCl to adjust pH to 7.5
- 5ml Glycerol
- H_2O to 50ml

To specify the detergents, add 1% detergent in labeled tubes (i.e. 0.02g of a detergent in 2ml of above prepared buffer).

Procedure of SmGPCR extraction:

(1) Wash cells that have reached 90-95% confluent with 8ml of cold PBS (on the edges, to avoid cell detachment).

(2) Aspirate the medium.

(3) Add 5ml of cold PRS containing 4mM EDTA [i.e. 0.7444g of sodium EDTA (372.2g/mol) in 500ml of PBS]. Incubate for 5min at RT and detach the cells by gentle pipetting. Pool cell plates together.

(4) Spin at 200xg for 5min at RT to pellet cells.

(5) solubilize in 1ml of detergent containing buffer in a microfuge. Rotate gently for 1hr at 4C, using end-over-end rotor. Avoid fast rotating since this causes cell pelleting and hindering the solubilization process.

(6) Spin at 14,000 rpm for 30min (use bench centrifuge) at 4C to pellet debris and nuclei.

(7) Transfer the supernatant into a new, pre-chilled tube. Measure protein content by Bradford method (use 2 and 5µl). Use in SDS-PAGE or store at -80C. Expected yield is 1.5-2.0mg per 100mm dish.

See Fadi's attachment in the next pages

HEK293E cells co-expressing FLAG-SmGPCR-EYFP and Rat β-arrestin2-Rluc

Enclosed a 1)T-150 flask containing HEK293E cells that stably (chromosomally integrated) co-express FLAG-SmGPCR-EYFP and Rat β-arrestin2-Rluc. 2) FLAG-SmGPCR-EYFP-pIRESpuro3 (~0.4 ug/ul), 3) 0.1 g (or less) of CHAPS (Sigma# C3023-5G), 4) 0.1g Digitonin (Calbiochem# 300410), 5) a vial of Zeocin (100 mg/ml; Invitrogen), 6) 1 vial of Puromycin (10 mg/ml) INVIVOGen # ant-pr-1; distributed by NEDICORP: tel (514) 733-1900), 7) pIRESpuro3 (Clontech, for Joe). Below is a summary/ background of this cell line. Also included are 2 vials one containing Zeocin and the other contains Puromycin (store at 4C for few months, long term at –20C) that should provide you with starting reagents until you order new vials.

1- Parent cell line: HEK293E (INVITROGEN): These cells stably express the EBNA1 gene which allows plasmids with an EBV ori (e.g. pCEP4; INVITROGEN) to replicate up to 90 copies. Plasmids such as pCDNA3, which carry only the SV40 ori, do not replicate in these cells (in contrast to HEK293T or COS7 cells). The HEK293E cells are resistant to geneticin (G418). *These cells were NOT included in this package. Stable cell lines listed below were generated using the HEK293E parental line.*
2- β-arrestin2-Rluc-293E: Single cell cloning was performed to select for HEK293E cells that stably integrated the Rat β-arrestin2-Rluc. Selection was done with Zeocin (25 ug/ml, INVITROGEN).
3- FLAG-SmGPCR-EYFP/β-arrestin2-Rluc-293E: Clone #I from 2) which expresses optimal levels of /β-arrestin2-Rluc was transfected with FLAG-SmGPCR-EYFP-pIRESpuro3 construct. Polyclonal β-arrestin2-Rluc-293E cells stably expressing FLAG- SmGPCR-EYFP were selected by Puromycin (1ug/ml; Clontech or ResGENE). This polyclonal line was subjected to FACS where by high expressers of EYFP were sorted out (150,000 in a 12 well were sorted; the selected expression level represented 20% of the total fluorescing population; see attached FACS document). The FACS polyclonal line – which is sent to you- is named as Sm-EYFP-Is.

Culture:
1– Sm-EYFP-Is: Grow in DMEM (high glucose) supplemented with 5% FBS, 2 mM L-glutamine, 100U/ml pen/strep and 10 ug/ml ZEOCIN, 0.25 ug/ml PUROMYCIN. NOTE ALWAYS KEEP THE PEN AND STREP ANTI BACTERIAL AGENTS (we had some problems n the lab with bacterial contamination). Usually I prepare 50 ml Falcon and add to it 2.5 ul Puromycin and 5 ul of Zeocin + complete medium (you may scale that to your 500 ml bottle)

NOTE:
1) The stable cell line has just been established; therefore, it's common to still see cell debris and some dead floating cells.
2) It's crucial to always split and culture the stock cells under Puromycin- and Zeocin selection in order to ensure optimal expression (especially for puromycin resistant

cells, since the SmGPCR-EYFP gene and the Puromycin gene are transcribed from the same mRNA (pIRESpuro3 is a bicistronic vector; Clontech). In case the cells are to be used for transfection, the cells should be split the day before without the selection drugs.
The above cells double ~ every 24 hrs and should be split every 3-4 days. It's best to split the cells at a dilution of **no more than 1:8** whenever they are ~ 95% confluent (i.e, ~ every 3 days). BEST RESPONSES ARE USUALLY OBTAINED FROM CELLS THAT ARE < 95% CONFLUENT SINCE HEK293 CELLS ARE CONTACT INHIBITED (happens at confluency). Do not culture the cells for more than 20 additional passages. ***Therefore, stocks should be generated and frozen ASAP. I suggest that you should should use 1/8th of the T-150 flask that I am sending to seed in a 100 mm dish and the t freez the rest as indicated below (enough to make 4 ampules).***

Cell FREEZING: Cells from a 90% confluent T-150 flask should be rinsed in 5-10 ml of sterile PBS, and then trypsinized by incubating for ~ 3-5 min in 2.5 ml of 0.025% Trypsin (EDTA) (should be at RT or 37C). Cells are then detached with 10 ml of culture medium (NO selection drugs) and then centrifuged at RT for 5 min at 200 xg (1,400 rpm). The pellet is then resuspend in 4-5 ml (best to have ~ 4-5 million/ml) of ice cold freezing solution (DMEM complete medium WITHOUT Zeocin or Puromycin, which contains 20% FBS and 10% DMSO; note once you prepare the freezing medium it's best to pass it through a 0.2um filter to ensure sterility), aliquoted into cryogenic vials (1ml/tube), and transferred (in a freezing box or a Styrofoam box) to –70C for 24 hrs, then to liquid nitrogen tank for long term storage. To thaw cells, quickly transfer an ampule to 37C water bath for few minutes, sterilize the exterior by spraying with 70% ethanol, then add to a 100 mm dish containing 10 ml complete medium WITH NO PUROMYCIN OR ZEOCIN. Allow cells to attach for 4-5 hrs then gently aspirate the medium (to remove the DMSO) and add fresh complete DMEM (also NO PUROMYCIN OR ZEOCIN). Next day add medium with PUROMYCIN AND/OR ZEOCIN.

WESTERN BLOT

Grow Sm-EYFP-Is and control HEK293E cells in 100 mm dish so that they're ~ 90% - 95% confluent on the day of the experiment.

- Gently wash the cells once with 8 ml of cold PBS (4C or ice-cold). NOTE: HEK293 cells are loosely attached and could lift up easily if the buffer was forced on the cells (add the buffer gently on the edge of the plate).
- After discarding the wash buffer (may use vacuum pump to aspirate the medium), add 5 ml of ice cold PBS containing 4 mM EDTA. Incubate at RT for 5 min. and detach the cell by gentle pippeting. Transfer the cells to 15 ml Falcon tube. Pool cells from 2 plates together (total volume is 10 ml), then split into 2 tubes. Spin at 200 xg for 5 min at RT to pellet the cells.
- Solubilize the pellet in 1 ml of ice-cold solubilization solution (add and transfer to 1.5 ml micro-centrifuge tube); rotate gently for 1 hr at 4C (end-over-end; note, if you rotate fast could end up pelleting the cells and hindering the solubilization process).
  Solubilization buffer: 100 mM $(NH4)_2SO4$, 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10% glycerol, 1% (w/v) detergent, and Protease Inhibitor Mixture

(may use an aliquote prepared from Tablets from Roche Molecular Biochemicals; PMSF alone is not enough).
I am including 0.1 g of 2 detergents to be tested: 1) CHAPS (Sigma #C3023-5G) and 2) DIGTONIN (Calbiochem)); It's best to prepare the solubilization solution fresh every time you'll use it. Therefore, you could weigh 0.02 g of each detergent and dissolve that in 2 ml of buffer (enough for 2 plates). Store the left over detergent powder at RT for later use.

Pellet debris and cell nuclei by centrifuging at top speed at 4C (use a microcentrifuge, 14,000 rpm) for 30 min.

- Gently transfer the supernatant to pre-chilled tubes (make sure to leave at least 50 ul on top of the pellet). Measure protein content by BRADFORD (test 2 and 5 ul to secure being in the linear range). Use directly or freez in aliquotes at –80C. Expected yield ~ 1.5 – 2mg / 100 mm dish.

WESTERN BLOT:

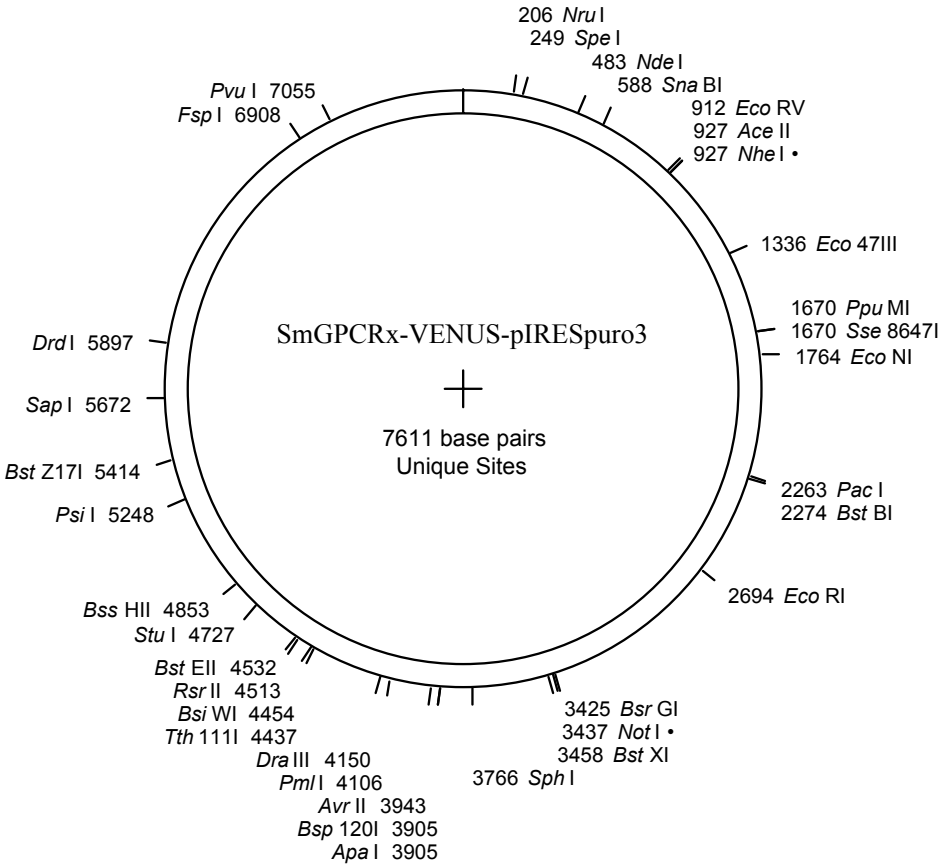
1- SDS-PAGE
For each solubilization condition use 10 and 30 ug of proteins/ lane. Add SDS loading buffer containing 100 mM DTT (or b-mercapto ethanol) to the solubilized proteins and incubate at 37C for 30 min (**DO NOT BOIL**). Load the samples on 10% SDS-PAGE cells such that each group will be duplicated (so that you'd be able to cut the blot and use one half with antiFLAGM2 and the other half with your antiSmGPCR).
2- Transfer to PVDF membranes (nitrocellulose also works)
GPCRs are best transferred at high voltage. At NIH we routinely used 100V for 1 hr. At UM, people fix voltage and run at 250 mA (gives also ~ 80-100V) for 90 min. This requires an open transfer system which could be cooled by an ice pack (e.g., BIORAD's) and also a special power supply. The NOVEX closed system that you've could not run at high voltage (I guess you use 30V). I remember when I was at NIH I compared the transfer of M3 receptor for 1 hr with the open Biorad at 100V vs. the Novex at 30V (1 hr) and 50V(2 hrs). The western blot revealed ~ 3 fold more transfer with the BIORAD than NOVEX at 30V; however, when the transfer was done with the NOVEX for 2 hrs at 50V then the transfer was almost as good as the BIORAD. **NOTE: TO TRANSFER AT 50V WITH THE CLOSED NOVEX SYSTEM YOU SHOULD USE COLD TRANSFER BUFFER (4C) AND SET THE TRANSFER IN THE COLD ROOM TO AVOID OVER-HEATING.**
3- WESTERN BLOT: Suggested protocol; if you've one that works better you may use that.

- Wash the filter with dH2O (Mq filter) for 2 min
- Block the blot for 1 hr at RT with TBS-T (Tween 0.05%) containing 3% skim milk
- Incubate with primary antibody for 1 hr (e.g., antiFLAG M2 at 1ug/ml; SIGMA# F3165,) in TBS-T containing 3% skim milk.
- Wash the blot 2 x for 10 min. with TBS-T (3% skim milk)
- Add secondary antibody for 1 hr at RT (use the right species; mouse vs rabbit): 1:10,000 if you use ECL; 1:80,000 if you use ECL+ or any of the super signal reagents (check manufacturer's recommendation).
- Wash 4 x 5 min. with TBS-T
- Develop

SEQUENCING OF FLAG-Sm-EYFP-pIRESpuro3:
Sequence 5' end using a T7 promoter primer; sequence the rest using internal oligos to SmGPCR spaced at 450 bp intervals. NO NEED TO SEQUENCE THE EYFP REGION SINCE WE KNOW IT'S EXPRESSED.

Good luck,
Fadi, April 04

FLAG-SmGPCR-VENUS-pIRESpuro3 sequence information:
Note: the VENUS is actually EYFP with 5 aa changes (that's why I referred to the construct as EYFP based).



NheI-EcoRI = signal peptide-FLAG-SmGPCR(no stop)
EcoRI – NotI = VENUS (enhanced EYFP; Nagai et al., Nature Biotech 2002)
NheI/NotI digestion = 2510 and 5101 bp (to verify the construct)
gacggatcgggagatctcccgatcccctatggtcgactctcagtacaatc
tgctctgatgccgcatagttaagccagtatctgctccctgcttgtgtgtt
ggaggtcgctgagtagtgcgcgagcaaaatttaagctacaacaaggcaag
gcttgaccgacaattgcatgaagaatctgcttagggttaggcgttttgcg
ctgcttcgcgatgtacgggccagatatacgcgttgacattgattattgac
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gcttatcgaaattaatacgactcactatagggagacccaagcttggtacc
gagctcggatcgatatctgcggcctagctagccacc(**This is the start of the cds: small caps after ATG represent signal peptide followed by FLAG**)ATGaagacgatcat
cgccctgtcctacatcttctgcctggtgttcgccgactacaaggacgatg
atgacgcc[**cds of SmGPCR starts; and Ala was added after ATG to introduce NcoI site**] ATGgctCAGTACATCAACAAGACCAGCCTGAACAGCAGCGTC
ATCCCCGATAGCCTGATCAAGAGCTGGATCCTGAGCAACCCTATCATCAA
GTGGACCATCTCCCTGTTCCTGATCATCGCCACTGGCACCACATTCTTCG
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CGTGCTGGTGCTGCCTCTGGCTATCGTGAGACAGAACCTGGGATATTGGC
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CATCGCCATCTCTAGACCCATGAAATATTTTACAAAGAGGACCAGGTTCA
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CTGCTCCCCTTCGTGGGCGGCAATCAACACACACTGGGCCTGGGATCTTG
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TATATATGCATTTTTTAAGAAGGAATATGCCAAGTCATTTAAGTATATCA
TTCAAGTTAACAAGTGGAATATTAAGAGCTACAACCATAAGgggaattct
ggtggaggcggatct[**Start of VENUS cds**]
ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGT
GGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCA
GCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTG
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GGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGC
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ACTACAACAGCCACAACGTCTATATCaccGCCGACAAGCAGAAGAACGGC
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t
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cgacacggaaatgttgaatactcatactcttccttttcaatattattga
agcatttatcagggttattgtctcatgagcggatacatatttgaatgtat
ttagaaaaataaacaaatagggttccgcgcacatttccccgaaaagtgc
cacctgacgtc

Translation: Signal peptide is in bold, FLAG is underlined; an Ala insertion at the begining of SmGPCR is bolded and underlined. An inrame flexible linker GNSGGGS that separates SmGPCR from VENUS is shown. **Calculated M.W = 95 kDa**

MKTIIALSYIFCLVFADYKDDDDAM**A**QYINKTSLNSSVIPDSLIKSWILSNPII
KWTISLFLIIATGTTFFGNLLIILAFITNSRLRRITDQYIVSLAVADLLVSVLVLP
LAIVRQNLGYWPFESDRLCQFWLSANIVLCMASILNLCCISLDRYIAISRPMKY
FTKRTRFTASTMIAVAWILPLITMLLPFVGGNQHTLGLGSCHITYNKAYRIYSS

IVGFFGPFLLIAYIYLRVFWIIKHRLKVLQITNIKLSSLKKPKSHIKATRKPAPIII
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NHKHPFDSFRSDDILNEEIDRNYRQKILDIKMNKTDREFSLTSQDHVELDFPEP
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NKLGDKQLINSSKLPSLTSFSIDHIKKLRRLKSLPNFKTEYNKTEQSQKNLIYY
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GYFNSICNPFIYAFFKKEYAKSFKYIIQVNKWNIKSYNHK**[GNSGGGGS]**MVS
KGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKLICTTGKLPV
PWPTLVTTLGYGLQCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNY
KTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYITADKQK
NGIKANFKIRHNIEDGGVQLADHYQQNTPIGDGPVLLPDNHYLSYQSALSKDP
NEKRDHMVLLEFVTAAGITLGMDELYK*