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A Native PAGE Assay for the Biochemical Characterization of G Protein Coupling to GPCRs

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[Abstract] G protein-coupled receptors (GPCRs) are a large family of membrane-embedded receptors that have diverse roles in physiology and are major drug targets. GPCRs transduce an agonist binding signal across the membrane to activate intracellular heterotrimeric G proteins. The dynamic nature of the receptors and the complexity of their interactions with agonists and G proteins present significant challenges for biochemical studies. Most biochemical/biophysical methods that have been employed to study GPCR-G protein coupling require purified receptors and are technically difficult. Here, we provide a protocol for a relatively simple and time- and cost-effective membrane protein native PAGE assay, to visualize and biochemically characterize agonist-dependent coupling of detergent-solubilized GPCRs to purified G protein surrogate “mini-G” proteins, which stabilize the receptor in an active state. The assay was developed for our studies of the calcitonin receptor-like receptor, a class B GPCR that mediates the actions of calcitonin gene-related peptide and adrenomedullin peptide agonists. It does not require a purified receptor and it can be used in a screening format with transiently-transfected adherent mammalian cell cultures, to quickly identify detergent-stable complexes amenable to study, or in a quantitative format with membrane preparations, to determine apparent affinities of agonists for the mini-G-coupled receptor and apparent affinities of mini-G proteins for the agonist-occupied receptor. The latter provides a partial measure of agonist efficacy. The method should be applicable to other GPCRs, and has the potential to be adapted to the study of other challenging membrane proteins and their complexes with binding partners.

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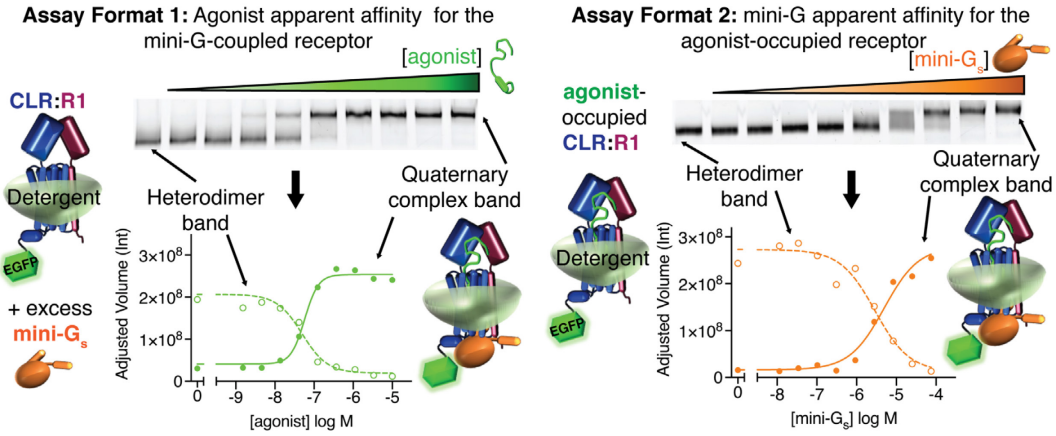
Graphic abstract:



Visualizing agonist-dependent mini-G protein coupling and determining apparent binding affinities using the native PAGE assay quantitative formats.

Keywords: Mini-G, Membrane proteins, G protein-coupled receptors, Native PAGE, Peptide agonist, Thermostability, Detergent solubilization, hrCNE

[Background] G protein-coupled receptors are a family of ~800 membrane-embedded receptors with critical physiologic roles in virtually every system of the human body and they are targeted by approximately one-third of FDA-approved therapeutics (Pierce *et al*., 2002; Sriram and Insel, 2018). Significant advances in biochemical, biophysical, and structural methods for the study of GPCRs have drastically improved our understanding of how an agonist-bound GPCR acts as a guanine nucleotide exchange factor, to activate intracellular heterotrimeric G proteins. Nonetheless, biochemical investigations of receptor-G protein coupling remain challenging due to the dynamic nature of the receptors and the complexity of their interactions with heterotrimeric G proteins, which are regulated by agonist binding to the GPCR, and guanine nucleotide binding to the G protein alpha subunit. Biochemical and biophysical methods that have been used to investigate this interaction include fluorescence- and nuclear magnetic resonance (NMR)-based methods (Yao *et al*., 2009; Gregorio *et al*., 2017; Huang *et al*., 2021). These methods are very powerful, but they are technically challenging and costly and require purified receptors in detergents or nanodiscs.

An alternative to working with G protein heterotrimers for biochemical studies is the use of “mini-G” proteins that were first developed as tools for GPCR structural studies (Carpenter and Tate, 2016; Nehmé *et al*., 2017). Mini-G proteins are minimal G protein alpha subunits that have enhanced stability in detergents and contain mutations that uncouple receptor binding from guanine nucleotide exchange, such that they trap GPCRs in an active state conformation, equivalent to that observed in structures of agonist-bound GPCRs in complex with nucleotide-free heterotrimer. Mini-G proteins are available for each of the four families of G protein alpha subunits. Biochemical assays for assessing mini-G interactions with GPCRs have been reported (Nehmé *et al*., 2017), including a fluorescent saturation

binding assay using immobilized receptor, and an assay based on the fluorescence-detection size-exclusion chromatography (FSEC) technique (Kawate and Gouaux, 2006). These assays work with unpurified receptors in detergent-solubilized lysates and use GFP-tagged mini-G for detection.

We recently reported a novel biochemical assay for GPCR-mini-G coupling based on the high resolution clear native electrophoresis (hrCNE) technique, which is a membrane protein native-PAGE method compatible with fluorescently-labeled proteins (Wittig *et al*., 2007). We developed the assay during the course of our work to understand how calcitonin gene-related peptide and adrenomedullin peptide agonists and RAMP accessory proteins control G protein coupling of the class B GPCR calcitonin receptor-like receptor (CLR) (Roehrkasse *et al*., 2020). The method enables both rapid, cost-effective screening for detergent-stable GPCR-G protein complexes and quantitative studies of agonist-dependent receptor-mini-G coupling. It does not require a purified receptor. Instead, it uses an EGFP-tagged receptor transiently over-expressed in the mammalian HEK293S GnT1$^-$ cell line. In the screening format, adherent cell cultures are directly solubilized with detergent, subjected to a simple centrifugation step, and the supernatants are analyzed by native-PAGE with visualization of the receptor by in-gel fluorescence imaging. Exogenous addition of agonist peptides and purified mini-G yields a mobility shift indicative of complex formation. In the quantitative assay format, crude membrane preparations are used to improve reproducibility and increase throughput. In the first quantitative format, the agonist is varied in the presence of a constant excess of mini-G to generate a binding curve that measures the apparent affinity of the agonist for the mini-G-coupled receptor. In the second quantitative format, mini-G is varied in the presence of a constant receptor-saturating concentration of agonist to generate a binding curve that measures the apparent affinity of mini-G for the agonist-occupied receptor. This second format provides a partial measure of agonist efficacy (Roehrkasse *et al*., 2020).

The native PAGE method proved to be a relatively simple, inexpensive, and highly versatile assay, which allowed us to investigate the biochemistry of agonist and G protein interactions with CLR:RAMP complexes. The screening and quantitative assay formats are described in detail here. We have also found the method to be very useful in a thermostability format, which is not described here, but can be found in our original report (Roehrkasse *et al*., 2020). This assay should be applicable to other GPCRs and may be valuable for the biochemical study of other challenging membrane proteins and their binding partners.

Materials and Reagents

1. Plasticware
   15 ml conical tubes (VWR, catalog number: 76176-950)
   48-well cell culture plate (Corning, Costar, catalog number: 3548)
   Microfuge tubes (VWR, catalog number: 87003-294)
   T-75 culture flask (Corning, catalog number: 43064)
2. Cell lines
   HEK293S GnT1$^-$ (ATCC, catalog number: CRL-3022) (see Note 3)

3. Cell culture reagents
   DMEM with 4.5 g/L Glucose and L-Glutamine (Lonza, catalog number: 12-604Q)
   Fetal Bovine Serum (FBS) (Gibco, catalog number: 16000-044)
   Non-Essential Amino Acids (NEAA) (Gibco, catalog number: 11140-050)
   Penicillin/Streptomcyin (Gibco, catalog number: 15140-122)
   Phosphate Buffered Saline (PBS) (Gibco, catalog number: 10010-023)
   Polyethylenimine, branched (PEI) (Sigma-Aldrich, catalog number: 408727-100 ml)
4. Salts – stored at room temperature
   Ammonium persulfate (APS) (Bio-Rad, catalog number: 1610700)
   Calcium Chloride ($CaCl_2$) (Sigma-Aldrich, catalog number: 223506-500G)
   HEPES Sodium Salt (Sigma-Aldrich, catalog number: H7006-500G)
   Potassium Chloride (KCl) (Sigma-Aldrich, catalog number: P9333-500G)
   Magnesium Chloride ($MgCl_2$) (Sigma-Aldrich, catalog number: 442611-500G)
   Sodium Chloride (NaCl) (EMD Millipore, catalog number: SX0420-5)
   Valproic acid sodium salt (Sigma-Aldrich, catalog number: P4543-25G)
5. Alcohols
   Isoproponol (VWR, BDH, catalog number: BDH1133-4LG)
   Methanol (Supelco, EMD Millipore, catalog number: MX0475-1)
6. Solubilizing chemicals – stored at -20°C
   Lauryl Maltose Neopentyl Glycol (LMNG) (Anatrace, catalog number: NG310-25GM)
   Cholesteryl Hemisuccinate (CHS) (Anatrace, catalog number: CH210-5GM)
7. Other Chemicals
   6-amino hexanoic acid (Sigma-Aldrich, catalog number: 07260-1KG)
   30% Acrylamide/Bis-acrylamide 29:1 (Acryl/Bis) (Bio-Rad, catalog number: 1610156)
   Fatty-Acid-Free BSA (FAF BSA) (PAA Laboratories, catalog number: K35-002)
   Glutathione oxidized (GSSG) (Sigma-Aldrich, catalog number: G4376-10G)
   Glutathione reduced (GSH) (Sigma-Aldrich, catalog number: G4251-25G)
   Imidazole (EMD Millipore, catalog number: 5720-500GM)
   Sodium Hydroxide (NaOH) (VWR, BDH, catalog number: BDH3247-1)
   Tetramethylenediamine (TEMED) (Bio-Rad, catalog number: 161-0800)
   Tricine (Sigma-Aldrich, catalog number: T0377-250G)
   Protease Inhibitor tablets (Thermo Scientific, Pierce, catalog number: A32955)
8. Peptides
   Human α calcitonin gene-related peptide (Bachem, catalog number: 4013281)
   Human adrenomedullin (Bachem, catalog number: 4034489)
   Human adrenomedullin2/intermedin (Bachem, catalog number: 4044529)
9. Enzymes
   Apyrase (New England BioLabs, catalog number: M0398L)
10. Membrane preparations and purified proteins

Crude membrane preparation from HEK293S GnT1^{-} cells transiently co-expressing tagged MBP-CLR-EGFP and MBP-RAMP, using the pHLsec expression vector (Aricescu *et al*., 2006); the preparation method was published in Roehrkasse *et al*. (2020) – flash-frozen in liquid nitrogen and stored at -80°C. The final preparationss are stored in 25 mM HEPES, pH 7.5, 10% (v/v) glycerol, 25 mM NaCl, 2 mM $MgCl_2$, and 1× protease inhibitor tablet. Protein concentrations are ~4-6 mg/ml with the receptor at ~100 nM.

Purified H_6-SUMO-mini-G proteins (mini-G_s, mini-$G_{s/i}$, and mini-$G_{s/q}$ were used; the present protocol utilizes example data from miniG_s); methods published in Carpenter and Tate (2016 and 2017), Nehmé *et al*. (2017), and Roehrkasse *et al*. (2020) – stored at -80°C. The purified proteins are stored at ~25-30 mg/ml in a buffer of 25 mM HEPES, pH 7.5, 50% (v/v) glycerol, 150 mM NaCl, 0.5 mM DTT, 1 mM $MgCl_2$, and 1 µM GDP (see Notes 1 and 2).

11. High resolution clear native PAGE gel (see Recipes)
12. 4× Binding Buffer (see Recipes)
13. 4× Detergent Buffer (see Recipes)
14. Running Buffers (see Recipes)
15. Culture media (see Recipes)
16. Transfection media (see Recipes)
17. 100 mM GSH and GSSG redox buffer stocks (see Recipes)

Equipment

1. Mini-PROTEAN Electrophoresis apparatus (Bio-Rad, model: 1658001FC)
2. Chemidoc MP imager (Bio-Rad, model: 12003154)
3. CO_2 incubator (NuAire, model: NU-5800)
4. Imager tray (Bio-Rad, catalog number: 12003028)
5. Rocking shaker (Reliable Scientific, model: 55)
6. Tube tumbler (Labnet International, model: H5500)
7. Benchtop Micro centrifuge (Eppendorf, model: 5415R)

Software

1. ImageLab (Bio-Rad, https://tinyurl.com/2v4t44re)
2. Prism (GraphPad, https://www.graphpad.com/)

Procedure

A. Preparation: High resolution clear native PAGE (hrCNE) gels (Wittig *et al*., 2007) and buffer stocks
   1. Prepare hrCNE gels according to the recipe section below (see Recipe 1, Table 2). We used an 8% resolving gel, but other resolving gel percentages could be used (see Note 12).

2. Prepare 4× binding buffer and 4× solubilization buffer stocks according to the recipes below (Recipes 2 and 3).
3. Prepare the hrCNE 20× Cathode and 50× Anode buffers and store them at room temperature in a dark cabinet (Recipe 4).

B. Adherent cell culture – For adherent cell culture screening format (see Note 4)
1. Preparation: Cell media
   a. Culture media (see Recipe 5).
   b. Transfection media (see Recipe 6).
2. Seeding
   a. Seed 120,000 cells/well of HEK293S GnT1$^-$ cells with 250 µl culture media in a cell culture-treated 48-well clear plate.
   b. Incubate the plate at 37°C with 5% CO_2 for 24 h and grow to ~90% confluency.
3. Transient transfection of DNA using PEI
   Prepare plasmid DNA transfection mixtures and add to cells.
   a. Ingredients
      1). DMEM (remaining volume for a total of 30 µl).
      2). Receptor expression plasmid DNA (300 ng total per well) (see Note 5).
      3). PEI for a 1.5:1 ratio to DNA (0.45 µl of 1 mg/ml stock).
      4). 46.67 mM valproic acid (2.8 µl of 500 mM stock).
   b. Assembly and addition to cells
      i. For the transfection mix, combine ingredients numbered 1)-3) to microfuge tubes (order of addition DMEM → DNA → PEI) and mix well by pipetting up and down.
      ii. Incubate for 10 min at room temperature.
      iii. While the transfection mixture is incubating, carefully aspirate media from the 48-well plate and replace with 250 µl of transfection media.
      iv. Add valproic acid (ingredient number 4) to microfuge tubes and mix well.
      v. Transfer 30 µl of transfection mixture to the appropriate well in the 48-well plate, and mix by gently swirling plate.
      vi. Incubate plate at 30°C with 5% CO_2 for 72 h.

C. Preparation: Day of the experiment (screening format or quantitative format)
1. Prepare the 1× Cathode and 1× Anode buffers by diluting the 20× and 50× stocks in ddH_2O and chill at 4°C for several hours prior to running the native gel, add detergent system to the Cathode buffer.
   a. 1× Cathode buffer (see Recipe 4).
   b. 1× Anode buffer (see Recipe 4).
2. Thaw 4× binding buffer and 4× detergent buffer stocks, apyrase, and peptide agonists on ice.
3. Make 1× binding buffer with 0.1 mg/ml FAF-BSA: For 500 µl total volume, combine 372.5 µl of

ddH$_2$O with 125 µl 4× binding buffer and 2.5 µl 20 mg/ml FAF-BSA (dissolved in ddH$_2$O).

4. Dilute 500 U/ml apyrase stock
   a. Screening Format: 1:10 in binding buffer with FAF-BSA to 50 U/ml and keep on ice.
   b. Quantitative Formats: 1:100 in binding buffer with FAF-BSA to 5 U/ml and keep on ice.
5. Make 4× detergent buffer with 0.2 U/ml apyrase: For 200 µl total volume, combine 199.2 µl 4× detergent buffer with 0.8 µl 50 U/ml apyrase dilution (see Note 6).
6. Thaw membrane prep and mini-G protein on ice right before setting up the assay.
7. Make 100 mM GSH and GSSG redox buffer stocks (see Recipe 7) (see Note 7):
8. Use the 100 mM GSH and 100 mM GSSG stocks to make working stocks for a specific assay format.
   a. Screening Format
      2.4 mM GSH/0.48 mM GSSG stock (for 100 µl: 97.12 µl ddH$_2$O, 2.4 µl of 100 mM GSH, 0.48 µl of 100 mM GSSG).
   b. Quantitative Formats
      50 mM GSH/10 mM GSSG stock (for 100 µl: 40 µl ddH$_2$O, 10 µl of 100 mM GSSG, 50 µl of 100 mM GSH).

D. Adherent cell culture screening format

1. Binding Reaction
   The final reaction is composed of 25 µl 4× detergent buffer and 75 µl binding reaction by volume; the binding reaction is assembled in microfuge tubes prior to introduction to the transfected cells in the 48-well plate.
   a. Ingredients (in order of addition)
      1). 1× Binding buffer (remaining volume for a total of 75 µl).
      2). 200 µM GSH/40 µM GSSG (6.25 µl of 2.4 mM GSH/0.48 mM GSSG stock).
      3). 4% v/v Glycerol (6 µl from 50% v/v stock).
      4). 66.66 µM mini-G protein (the volume depends on mini-G stock concentration).
      5). 13.33 µM peptide agonist (the volume depends on peptide stock concentration).
   b. Assembly and addition to cells
      i. Combine ingredients numbered 1)-4) and mix well.
      ii. Incubate for 30 min on ice, to allow for reduction of the mini-G protein.
      iii. Add the peptide agonist (ingredient number 5) to the desired reactions and mix well.
      iv. Aspirate media from cells in the 48-well plate.
      v. Wash wells with 250 µl 1× PBS.
      vi. Aspirate PBS and place plate on ice.
      vii. Add 75 µl of binding reaction to each well of cells, swirl plate to mix.
      viii. Incubate the 48-well plate on ice for 30 min.
2. Solubilization
   a. Add 25 µl of 4× Detergent buffer ± 0.2 U/ml apyrase to each well and swirl plate to mix (see

Note 6).

b. Place the plate on a rocker at 4°C and allow cells to solubilize for 2 h.

c. Pipette lysates to pre-chilled microfuge tubes on ice.

d. Analyze by hrCNE gel electrophoresis and image gel as in Procedure F-G.

E. Quantitative Assay Formats

1. Quantitative assay binding format 1 (Figure 1) – agonist apparent affinity for the mini-G-coupled receptor

a. The final reaction is composed of ¼ 4× detergent buffer and ¾ binding reaction by volume; the binding reaction is assembled from a mastermix and agonist serial dilutions, to produce the final desired concentrations (see Table 1 for concentrations at each step).

b. To maximize reproducibility, combine reaction components that will be equal in all reactions in a mastermix (MM) (receptor membrane prep, an excess of the mini-G protein, and apyrase); GSH and GSSG are added with the mini-G to keep the mini-G protein reduced (see Note 7).

c. Mastermix:

i. Ingredients (in order of addition with volumes for 400 µl MM shown)

1). ddH_2O (remaining Volume for a total of 400 µl).

2). 1× Binding buffer (100 µl of 4× stock).

3). 0.1 mg/ml FAF-BSA (2 µl of 20 mg/ml).

4). 400 µM GSH/80 µM GSSG (3.2 µl of 50 mM GSH/10 mM GSSG stock).

5). 0.133 U/ml apyrase (10.64 µl of 5 U/ml stock) (see Note 8).

6). 133.5 µM mini-G protein (the volume depends on mini-G stock concentration) (see Note 9).

7). 26.67 nM receptor membrane prep (106.68 µl of 100 nM stock).

ii. Assembly

Combine ingredients numbered 1)-6) and mix well.

Pre-incubate for 30 min on ice, to allow for reduction of the mini-G protein.

Add the receptor membrane prep and mix well by pipetting up and down.

d. Agonist dilutions:

i. Dilute the agonist peptide to 2.67× of the final desired highest concentration in 1× binding buffer with 0.1 mg/ml FAF-BSA (see Table 1 for concentrations) in microfuge tubes on ice.

ii. Serially dilute the agonist peptide 3-fold in 1× binding buffer with 0.1 mg/ml FAF-BSA (*e.g.*, dilute 10 µl of the agonist dilution in 20 µl of 1× binding buffer with 0.1 mg/ml FAF-BSA).

e. Binding reaction: Assemble the binding reaction by combining equal volumes (15 µl + 15 µl) of the agonist dilution and mastermix.

f. Incubate binding reaction on ice for 30 min and proceed to solubilization (Step E3).

2. Quantitative assay binding format 2 – mini-G apparent affinity for the agonist-occupied receptor
   a. The final reaction is composed of ¼ 4× detergent buffer and ¾ binding reaction, as in assay format 1; the binding reaction is assembled from a mastermix and miniG serial dilutions to produce the final desired concentrations (see Table 1).
   b. To maximize reproducibility, combine reaction components that will be equal in all reactions in a mastermix (MM) [10 nM of the receptor complex, an excess (10 μM) of the agonist (see Note 14)]; GSH and GSSG are added with the mini-G in the serial dilution, to keep the mini-G protein reduced.
   c. Mastermix:
      i. Ingredients (in order of addition with volumes for a 400 μl MM shown)
         1). ddH_2O (remaining Volume for a total of 400 μl).
         2). 1× Binding buffer (100 μl of 4× stock).
         3). 0.1 mg/ml FAF-BSA (2 μl of 20 mg/ml).
         4). 0.133 U/ml apyrase (10.64 μl of 5 U/ml stock) (see Note 8).
         5). 26.67 μM agonist (the volume depends on agonist stock concentration).
         6). 26.67 nM receptor complex (106.68 μl of 100 nM stock).
      ii. Assemble all ingredients in the order indicated and mix well.
   d. Mini-G dilutions:
      i. Dilute the mini-G protein to 2.67× of the final desired highest concentration (200 μM) in 1× binding buffer with 0.1 mg/ml FAF-BSA.
      ii. Add a 3-fold molar excess of GSH/GSSG (600 μM), mix well, and pre-incubate for 30 min on ice, to reduce mini-G.
      iii. Serially dilute the mini-G 3-fold in 1× binding buffer with 0.1 mg/ml FAF-BSA (*e.g.*, dilute 10 μl of the agonist dilution in 20 μl of 1× binding buffer with 0.1 mg/ml FAF-BSA); note that the GSH/GSSG is diluted proportionally with the mini-G protein.
   e. Binding reaction: Assemble the binding reaction by combining equal volumes (15 μl + 15 μl) of the mini-G dilution and mastermix.
   f. Incubate binding reaction on ice for 30 min, and proceed to solubilization (Step E3).

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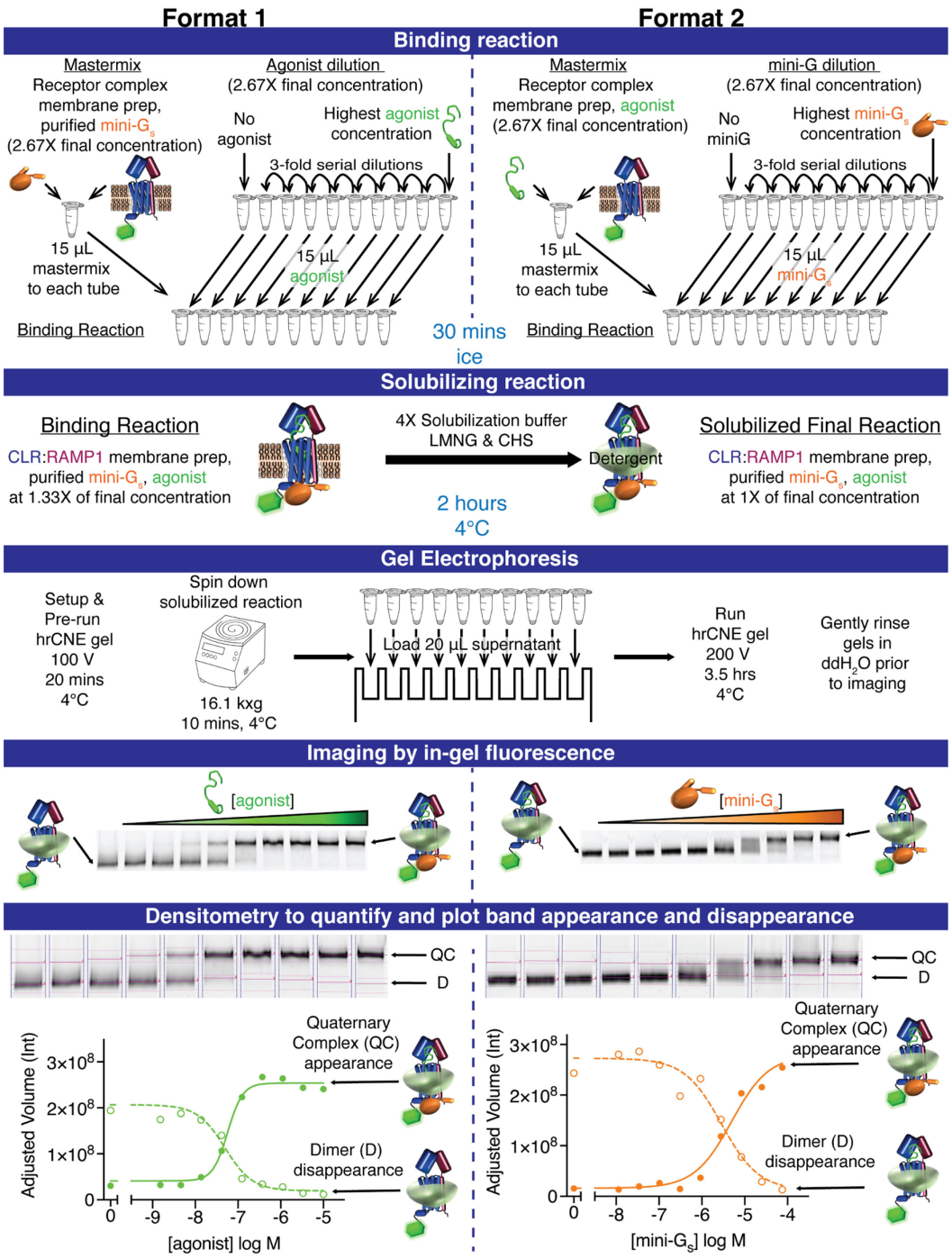

Figure 1. Quantitative Assay Formats Overview.

Depiction of key steps of the quantitative hrCNE gel assay format corresponding to Procedure C-G and the Data analysis in the protocol.

Table 1. Buffer and binding reaction components concentrations at various stages of the protocol.

Assay Format 1: Agonist affinity	**Concentrations at various stages of the protocol**			
	Mastermix (2.67×)	Agonist dilution (2.67×)	Binding Reaction (1.33×)	Final (after solubilization) (1×)
Binding buffer[1]	1×	1×	1×	1×
FAF-BSA	0.1 mg/ml	0.1 mg/ml	0.1 mg/ml	0.075 mg/ml
GSH/ GSSG[2]	400 µM/80 µM	N/A	200 µM/40 µM	150 µM/30 µM
Receptor membrane prep	26.7 nM	N/A	13.3 nM	10 nM
Agonist	N/A	26.7 µM - 0 µM	13.3 µM - 0 µM	10 µM - 0 µM
Mini-G protein	133.5 µM	N/A	66.7 µM	50 µM
Apyrase	0.133 U/ml	N/A	0.067 U/ml	0.05 U/ml
Assay Format 2: mini-G affinity	**Concentrations at various stages of the protocol**			
	Mastermix (2.67×)	mini-G dilution (2.67×)	Binding Reaction (1.33×)	Final (after solubilization) (1×)
Binding buffer[1]	1×	1×	1×	1×
FAFBSA	0.1 mg/ml	0.1 mg/ml	0.1 mg/ml	0.075 mg/ml
GSH/GSSG[2]	N/A	600 µM/120 µM - 0 µM	300 µM/60 µM - 0 µM	225 µM/45 µM - 0 µM
Receptor membrane prep	26.7 nM	N/A	13.3 nM	10 nM
Agonist	26.7 µM	N/A	13.3 µM	10 µM
Mini-G protein	N/A	200 µM - 0 µM	100 µM - 0 µM	75 µM - 0 µM
Apyrase	0.133 U/ml	N/A	0.067 U/ml	0.05 U/ml

[1]Binding buffer: 25 mM HEPES, pH 7.5, 140 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$ + Pierce Protease Inhibitor (PI).

[2]GSH:GSSG is at 5:1 molar ratio throughout, and GSH:miniG ratio is at a 3:1 ratio throughout for mini-G_s and mini-$G_{s/q}$; for additional information about mini-$G_{s/i}$ see Roehrkasse *et al*. (2020).

3. Solubilization
   a. Mix the thawed 4x detergent buffer by pipetting up and down.
   b. Add 10 µl of 4× detergent buffer (Recipe 3) to each of the binding reactions; this will bring all concentrations to 1× of the final concentration
   c. Place the microfuge tubes on a tube tumbler in a cold room (4°C), and allow reactions to solubilize for 2 h (see Note 10).
   d. Final buffer conditions:
      25 mM HEPES, pH 7.5
      140 mM NaCl
      10 mM KCl
      1 mM $MgCl_2$
      2 mM $CaCl_2$

0.5% w/v LMNG/0.05% w/v CHS

Pierce Protease Inhibitor at 0.538×

Note: This is from buffer stocks only, the final solution also includes components from the mini-G storage buffer, the amount added varies with assay format, as well as the membrane preparation storage buffer.The final concentrations of glycerol in our samples vary from 1% v/v in samples without added mini-G protein to 3% in samples with the highest amount of mini-G protein.

F. Gel Electrophoresis (see Notes 11 and 12)
   1. Set up the hrCNE gel in a cold room (4°C) with the pre-chilled cathode and anode buffers prepared in Procedure C.
   2. Pre-run gel at 100 V for 20 min to allow the detergent to enter the gel.
   3. Place the 1.5 ml microfuge tubes from Steps D2 or E3 in a microfuge pre-chilled to 4°C.
   4. Spin at 16,100 × *g* for 10 min at 4°C in the microcentrifuge (Eppendorf 5415R).
   5. Load 20 µl of each reaction supernatant into the wells. This can be a challenging step and may take some practice because no loading dye is used.
   6. Run the gel at 200 V for 3.5 h.

G. Image the gel by in-gel fluorescence
   1. Gently separate the gel from glass plates and rinse three times in ddH_2O.
   2. Image with the BioRad ChemiDoc MP imager, using the blot/UV/stain-free imaging plate.
   3. Preset program: ProQ Emerald 488 for EGFP fluorescence.
   4. Exposure times vary depending on band intensity. For quantitative gels, the optimal exposure time was determined and all gels were imaged for the same time; 80-120 s is a reasonable starting point.

H. Quantify band intensity using the Bio-Rad Image laboratory software
   1. Tutorial videos are available from Bio-Rad on YouTube: https://www.youtube.com/watch?v=IV_P47ScoYo&list=PLrAEgIY86I6zytNeMPGBhvXnKIm5MS-6J&index=3&t=8s.
   2. Use the Image tools to straighten/rotate the image as needed.
   3. Use the lane and band tool to define lanes and select bands
      a. Manually assign 10 lanes and adjust the lanes to align with the gel.
      b. Use the “add bands” function to select bands for all dimer and quaternary complex bands.
      c. In the area where the bands are not easily visible, you can look at the lane profile tool to see faint bands.
   4. Use the lane profile view to select/adjust the width of the band peaks.
      a. Background can be adjusted and excluded as needed, by using the background selection.

b. Review the width for each band and adjust as needed. Pay attention to band widths, to ensure that they are uniform.

5. Export data using the analyze data function.

Data analysis

Plot the adjusted volume determined by densitometry against the agonist (format 1) or mini-G (format 2) concentration on a log scale. Fit the agonist and mini-G binding data using a 3 or 4 parameter logistic equation by nonlinear regression, and determine a pEC_{50} (quaternary complex appearance), or pIC_{50} value (dimer disappearance), to determine the apparent binding affinities (see Note 13). Both the appearance of the quaternary complex band, and the disappearance of the dimer band can be plotted. We have seen good agreement between the pEC_{50} and pIC_{50} values, if the mini-G/agonist affinity is sufficiently high to fully visualize the transition from the dimer to the quaternary complex.

Notes

1. We produced the mini-G proteins as SUMO-fusion proteins originally for purification purposes, but we found that the fusions worked better than free mini-G proteins for some receptor complexes, presumably because their larger size yielded a better mobility shift. Different forms of mini-G may need to be tested to find the best mobility shift.
2. Purification of mini-G_{12} has been reported (Nehmé *et al*., 2017). We did not use this protein in our assays, but we assume it would work as well.
3. The HEK293S GnT1$^-$ cell line lacks N-acetyl-glucosaminyltransferase I activity, and therefore produces homogenous N-glycans, which enables sharp defined bands in the gels.
4. Use the appropriate sterile technique in a cell culture hood.
5. We used CLR that was tagged at the N-terminus with maltose-binding protein (MBP) and at the C-terminus with EGFP. The RAMP subunit was also N-terminally tagged with MBP. MBP seemed to allow more defined bands on the gels, but this may not be necessary in all cases. In addition, the flexible receptor C-tail was truncated. The vector used for expression was the pHLsec vector previously described (Aricescu *et al*., 2006).
6. 4x Detergent buffer + 0.2 U/ml apyrase was used in wells that contained mini-G. 4× Detergent buffer w/o apyrase was used in all other wells.
7. The mini-G proteins have several solvent-exposed cysteine residues that have a tendency to form intermolecular disulfide bonds when stored at high concentrations. We used the GSH/GSSG redox buffer to reduce the mini-G, without damaging our receptors and peptide agonists, which contain several disulfide bonds. In some cases, simpler use of a low concentration of DTT or TCEP to reduce mini-G may be possible.

8. In the original paper, we added apyrase as described for the quantitative formats, but we have since moved to adding the apyrase during the solubilization step, as described for the adherent screening format.
9. We have used both 25 and 50 μM final mini-G concentrations.
10. The 30 min binding reaction and 2 h solubilization were sufficient to reach equilibrium for most of the interactions we studied, but longer times may be needed in some cases. This should be tested on a case-by-case basis.
11. It is beneficial to have designated equipment for native gels, to avoid possible carry-over of residual SDS from previous SDS-PAGE gel runs.
12. The acrylamide percentage used for the hrCNE resolving gels should be optimized. For our purposes, the 8% resolving gel worked well. Gradient gels can also be considered.
13. Interactions between agonists, GPCRs, and G proteins are complex and have allosteric effects in both directions. In addition, there is the possibility of perturbation of the equilibrium during electrophoresis. For these reasons, we describe the affinities derived from the quantitative formats as “apparent binding affinities”.
14. Agonist concentration should be chosen to saturate the receptor (100× K_D if possible).

Recipes

1. High resolution clear native PAGE gel (see Table 2)
   a. Stock solution of 0.1 M Imidazole (pH 7.0)/2 M 6-amino hexanoic acid can be prepared in a larger volume (250 ml) and stored in the dark at room temperature.
   b. Selection of the resolving gel percentage will depend on the size of the protein complex to be visualized.
   c. At room temperature in a 15 ml conical tube, combine ddH_2O, 0.1 M Imidazole (pH 7.0)/2 M 6-amino hexanoic acid, and 30% Acryl:Bis and mix well to combine.
   d. Add the APS and TEMED, mix well and immediately cast the resolving gel.
   e. Top the gel off with isopropanol and allow the gel to solidify.
   f. Pour off the isopropanol and gently wipe clean with a tissue.
   g. Assemble stacking gel as done with the resolving gel, cast with a 10 well comb, and allow the gel to solidify.
   h. Gels can be stored at 4°C for several days wrapped in moist paper towels surrounded by plastic wrap.

Table 2. hrCNE gel recipe.

	Resolving			Stacking
% Acryl:Bis	**8%**	**10%**	**12%**	**6%**
ddH_2O	4.74 ml	4.06 ml	3.4 ml	2.29 ml
0.1 M Imidazole (pH 7.0)/2 M 6-amino hexanoic acid	2.5 ml	2.5 ml	2.5 ml	1 ml
30% Acryl:Bis	2.66 ml	3.34 ml	4 ml	0.67 ml
10% APS	100 µl	100 µl	100 µl	40 µl
TEMED	4 µl	4 µl	4 µl	4 µl
Total Volume	10 ml	10 ml	10 ml	4 ml

2. 4× Binding Buffer

   100 mM HEPES, pH 7.5, 560 mM NaCl, 40 mM KCl, 4 mM $MgCl_2$, 8 mM $CaCl_2$ + 1× Pierce Protease Inhibitor (PI) – 10 ml stock

   Stock solutions

   1 M HEPES (pH 7.5) stock

   5 M NaCl

   1 M KCl

   1 M $MgCl_2$

   1 M $CaCl_2$

   Store HEPES at 4°C

   All others can be stored at room temperature.

   For a 10 ml stock solution

   1 ml of 1 M HEPES, pH 7.5

   1.12 ml of 5 M NaCl

   0.4 ml of 1 M KCl

   0.04 ml of 1 M $MgCl_2$

   0.08 ml of 1 M $CaCl_2$

   7.36 ml of ddH_2O

   1 Pierce™ Protease Inhibitor Mini Tablets (1× in 10 ml)

   Add all ingredients to a 15 ml conical tube with the Pierce protease inhibitor tablet and vortex to combine (the protease inhibitor tablets are difficult to dissolve).

   Store at -20°C in 1 ml aliquots.

3. 4× Detergent Buffer

   25 mM HEPES, pH 7.5, 140 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 2% w/v LMNG/0.2% w/v CHS

Stock solutions

Same stock solutions as in Recipe 2; detergent stock: 10% w/v LMNG/1% w/v CHS in ddH_2O, stored in 1 ml aliquots at -20°C.

For a 10 ml stock solution

0.25 ml of 1 M HEPES, pH 7.5
0.28 ml of 5 M NaCl
0.1 ml of 1 M KCl
0.01 ml of 1 M $MgCl_2$
0.02 ml of 1 M $CaCl_2$
2 ml of 10% w/v LMNG/1% w/v CHS
7.34 ml of ddH_2O
1 Pierce™ Protease Inhibitor Mini Tablets (1× in 10 ml)

Add all ingredients ***except detergent*** to a 15 ml conical tube with the Pierce protease inhibitor tablet and vortex to combine (the protease inhibitor tablets are difficult to dissolve).
Add the detergent and gently, but thoroughly, mix to combine.
Store at -20°C in 1 ml aliquots.

4. Running Buffers
   1× Cathode buffer: 50 mM Tricine, 7.5 mM Imidazole, pH 7.0, 0.01% w/v LMNG/0.001% w/v CHS
   1× Anode buffer: 25 mM Imidazole, pH 7.0
   20× Cathode buffer stock: 1 M Tricine, 150 mM Imidazole, pH 7.0
   50× Anode buffer stock: 1.25 M Imidazole, pH 7.0
5. Culture media
   DMEM
   10% FBS
   1× NEAA
6. Transfection media
   DMEM
   2% FBS
   1× NEAA
   50 U/ml Penicillin
   50 µg/ml Streptomycin
7. 100 mM GSH and GSSG redox buffer stocks (see Note 7)
   a. Weigh GSH and GSSG powder and add to separate 15 ml conical tubes.
   b. Add ddH_2O to the final desired concentration and vortex to dissolve.
   c. Adjust the pH to neutral by the addition of NaOH.

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Competing interests

None of the authors have any competing interests to disclose.

Ethics

This protocol utilizes a standard immortalized human cell line and did not involve human or animal subjects.

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