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Supplementary Information for

DCyFIR: a high-throughput CRISPR platform for multiplexed G protein-coupled receptor profiling and ligand discovery

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SI References

Other supplementary materials for this manuscript include the following:

Dataset 1. Comprehensive list of yeast strains used and engineered in this study.
Dataset 2. Gene sequences of human GPCRs used in this study.
Dataset 3. Comprehensive list of plasmids used in this study.
Dataset 4. Oligonucleotides used in this study.
Dataset 5. GPCR-Gα coupling comparisons between this study and other sources.

Supplementary Information Text

Discussion

Cost and time savings of the DCyFIR method. Estimating the relative cost of assays is complicated by many factors, such as price differences for consumables and core services across research environments. Here, we estimate the cost savings of the DCyFIR method using microplates as an example.

In this DCyFIR application, 30 receptors (a 300-plex of GPCR-Gα combinations) were screened against a small library of 320 metabolites. These receptors were divided into three screening plex sets as described in the text, a step we learned is not necessary for future experiments. Using DCyFIRscreen, we profiled the three plexes in duplicate against the 320 metabolites, using a total of 6 microplates (384-well format) at a total cost of $36.00. In contrast, screening each GPCR individually in duplicate would have required a minimum of 60 microplates at a total cost of $360.00. Thus, a traditional screen would have cost at least 10 times more than our DCyFIR-based screens. However, we know that the cost savings is actually much greater when we consider the cost of robotic tips, other consumables, and media. Additionally, the cost of DCyFIR-based screens is independent of ligand (very little is needed) and plex size, and only dependent on the size and initial cost of the screening library.

Both the DCyFIRscreen and DCyFIRplex experimental modes offer substantial time savings over traditional GPCR screening assays in done in mammalian models. With yeast, long-term storage (several months) in 96-well deep block plates at 4°C is feasible. Using these source blocks, we can seed fresh 384-well screening plates (n=4 replicates), treat with vehicle and ligand, incubate overnight 30°C. This entire process takes 2-3 days and can be done for thousands of GPCR-Gα reporter strains as needed. After incubation, the plates are read on the plate reader and Z scores and SSMD values are quickly calculated to identify the ligand hits to be interrogated by DCyFIRplex (next). Using the same source blocks mentioned above, we can seed fresh growths that are combined, sorted, and deconvoluted over the course of 3 lab days. Because it takes less than 3 minutes to sort one ligand treatment, we can collect DCyFIRplex profiles for 50 ligands in a typical sorting session, and this could easily be extended if needed.

In summary, the combined DCyFIRscreen and DCyFIRplex runs are typically completed in 5-6 days for thousands of GPCR-Gα reporter strains. We estimate that this 5-day process is equivalent to 6-12 months of lab labor hours using traditional approaches in mammalian cells, assuming many lab members are assigned to the same screening task in parallel. In contrast, the entire DCyFIR process is done by one graduate student using a liquid handling robot as a force multiplier.

Methods

CRISPR transformation reactions. CRISPR edits in yeast are done by co-transforming plasmids containing the CRISPR machinery (Cas9 endonuclease and guide RNA) and DNA payloads containing homology arms typically within 30 bp of the double-stranded DNA break made by Cas9 at the targeted genome locus. For this work we performed these transformations both on an individual basis and in high-throughput format.

Individual CRISPR transformation reactions. Five milliliters of cells were grown to mid-log phase (OD = 0.2-1.0) in YPD medium. Cells were harvested by centrifugation and washed with 5 mL TE buffer (10 mM Tris, 1 mM EDTA), then harvested again and washed with 5 mL lithium acetate mix buffer (LiOAc mix; 10 mM Tris, 1 mM EDTA, 100 mM LiOAc). Cells were harvested by centrifugation and resuspended in 200 µL LiOAc mix buffer. CRISPR vector(s) (300 ng) and DNA payload (4-5 µg) were combined with salmon sperm DNA (100 µg) in a mix with 50 µL cells and 350 µL PEG mix (10 mM Tris, 1 mM EDTA, 100 mM LiOAc, 40% PEG_{3350}). This mixture was incubated at room temperature for 30 minutes before addition of 24 µL DMSO and a 15 minute heat shock at 42 °C. Following heat shock, cells were harvested by centrifugation at 8000 x g for 1 minute, resuspended in 200 µL YPD and spread on selective media plates

poured in petri-dishes with a 100 mm diameter. This protocol is sufficient for 4 individual CRISPR transformations and is scalable.

High-throughput CRISPR transformation reactions. In this work we engineered hundreds of new yeast strains using a miniaturized version of our protocol for "*Individual CRISPR transformation reactions*". Both approaches used the same culture and plate media compositions. For the miniaturized protocol, the necessary CRISPR vector(s) (150 ng) and DNA payload (4-5 µg) for many transformation reactions (typically 80–100 at a time) were combined in a mixture with 50 µL cells and 175 µL PEG mix in individual wells of a 96-well plate (CytoOne CC7672-7596). This mixture was incubated for 30 minutes at room temperature before addition of 12 µL DMSO and a 15 minute heat shock at 42 °C. Cells were harvested by centrifugation and resuspended in 100 µL YPD. 30 µL transformant resuspension was distributed over miniaturized selective media plates poured in 22.1 mm 12-well microplates (CytoOne CC7672-7512). Each step was performed on our Biomek NXp liquid-handling robot driven by our custom Python code. Colonies were picked into SCD-U media.

Counterselection to remove CRISPR vectors. An advantage of CRISPR in yeast is the ability to engineer a genome modification without exhausting available auxotrophic markers. In this work we primarily used CRISPR vectors conferring URA selectivity, which we could remove via counter-selection on 5FOA plates after the desired genome edit was confirmed. In cases where two CRISPR vectors where used, such as gene deletions, the CRISPR vector conferring URA selectivity was removed as usual by counter-selection on 5FOA and the CRISPR vector conferring LEU selectivity was naturally lost after several generations of outgrowth in non-selective media. As a result, the base BY4741 genotype remained unchanged, despite the many changes introduced into the genomes of the GPCR-Gα reporter strains.

CRISPR guide RNA plasmid design. Previously described methods and base CRISPR vectors pML104 (Addgene #67638), pML107 (Addgene #67639), and pT040 (Addgene #67640) (1) were used to construct all genomically-targeted guide RNA plasmids used in this study. Briefly, pML104 and pML107 plasmids both contain the Cas9 endonuclease ORF, a gRNA scaffold flanked by a SNR52 promoter and SUP4 terminator, and an auxotrophic marker (URA and LEU, respectively). Plasmid pT040 contains the same gRNA scaffold as pML104 and pML107, as well as a URA auxotrophic marker.

Design of the X-2 CRISPR-addressable expression cassette. We first generated random 20 base pair (bp) guide sequences using a custom Python program. Using the BLAST algorithm (2), we then tested the uniqueness of each guide sequence against our locally built BLAST database for the updated release of the *S. cerevisiae* BY4741 genome (BY4741_Toronto_2012) available via www.yeastgenome.org (3). Using the established threshold for avoiding off-target Cas9 cutting (4), guide sequences with more than three mismatched bases were identified and used as synthetic unique targeting sites (UnTS). We designed our CRISPR-addressable expression cassette, which we refer to as a landing pad, to include one of these UnTS sequences (5'- TTGCGTAAGTGGCCCCTAGC-3') preceding a protospacer adjacent motif (PAM-site) site (5'- GGG-3') flanked upstream by a constitutive TEF1 promoter (5) and downstream by a CYC1 terminator variant, CYC1b, a corrected version that leads to higher expression output than other CYC1 terminator variants (6). Lastly, we extended the landing pad to include 500 bp homology arms to the known yeast X-2 safe harbor locus on chromosome X (7, 8). We ordered the X-2 landing pad sequence synthetically constructed and cloned into the pMARQ vector by ThermoFisher (Supplementary Dataset 3). The sequence of the X-2 landing pad with homology arms is available in Table S2.

CRISPR DNA payload design. In every CRISPR transformation reaction, the necessary CRISPR vector(s) are co-transformed with a DNA sequence that serves to both 1) patch the locus-specific double-stranded DNA break caused by the Cas9 endonuclease and 2) incorporate the desired gene deletion, edit, replacement, or knock-in. This DNA sequence, which we refer to as a DNA payload, contains both the DNA required for the desired CRISPR change (usually a heterologous gene or gene fragment) and important design features for targeting the intended genome location (homology arms) and preventing continued Cas9 cutting once the genome has been altered PAM-site silencing.

Homology arm design. Our DNA payloads were flanked by homology arms that typically contained 60 bp of genome sequence upstream and downstream of the targeted genome locus. These homology arms were introduced by PCR amplification (using primers with overhangs containing the necessary homology), or by including the sequence homology directly in the designs of synthetic payloads (e.g. gBlocks and synthetic DNA constructs cloned into storage vectors). Based on our empirical observations from thousands of CRISPR edits, we avoid using homology arms less than 60 bp to prevent diminished CRISPR efficiency.

PAM-site silencing design. Once a DNA payload has been integrated into the yeast genome, Cas9 endonuclease will continue to create a double-stranded break at the targeted genome locus if the PAM-site is not removed by the genome edit. Such constant cutting can reduce CRISPR efficiency due to its effect as a cytotoxic stress and as a mechanism for reversing the desired genome edit. In such cases where PAM-site silencing was needed, we prevented continuous genome cutting by including a point mutation in the portion of the homology arm that corresponds to the PAM-site. We call this process PAM-site silencing. In cases where PAM-site silencing occurs within the open reading frame of a protein, a synonymous codon that removes the PAM-site in used in place of the original codon in the homology arm.

The collection of PAM-site silenced homology arms used to deliver the various CRISPR payloads is listed in Supplementary Dataset 4 and Table S2. Deletion, editing, and replacement of all genes was verified by PCR gel electrophoresis using the relevant primers (listed in Supplementary Dataset 4).

Engineering positive controls strains for DCyFIRplex development. To establish our DCyFIRplex FACS gating procedure we used our set of 10 base GPCR-Gα reporter strains (mixed as a 10-plex) as a negative control (inactive bin in Figure 2D). To build our positive control strains for cyan (mTq2) and red (mRuby3 tracer) fluorescence, we first installed a X-2 landing pad into the wild-type BY4741 strain using the same procedure described in "*Installation of the X-2 landing pad*". We next PCR-amplified mTq2 (from a pFA6-link-mTurquoise2-CaURA3MX vector [Addgene #86424]) and mRuby3 (from a pNCS-mRuby3 vector [Addgene #74324]) using primers containing ~45 bp homology arms corresponding to sequences within the TEF promoter and CYC1b terminator of the X-2 landing pad. In a second round of PCR, we used universal primers to extend both homology arms to a final length of 60 bp. The PCR-amplified genes were then installed into their respective X-2 landing pads by co-transformation with the CRISPR vector pML104 X2 UnTS using the approach described in "*CRISPR transformation reactions*". Integration into the X-2 landing pad was confirmed both by PCR and a marked increase in mTq2 or mRuby3 fluorescence using a microplate reader (ClarioStar, BMG LabTech). The resultant genotypes of these strains, which we refer to as DI P1, DI P1 mTq2 and DI P1 mRuby3, are in Supplementary Dataset 1.

Determining G protein transcript levels in the 10 base GPCR-Gα reporter strains. The 10 base GPCR-Gα reporter strains were individually seeded into pH 5.0 Synthetic Complete Low Fluorescence Screening Media (SCD LoFo), grown for several hours, and back-diluted in pH 7.0 of the same medium to achieve an OD_{600} between 1 and 2 after overnight growth. The next morning, these cultures were back-diluted again into SCD LoFo pH 7.0 and grown to an OD_{600} of 1.0. The procedure ensured that the yeast cells were in log phase for multiple divisions before harvesting their mRNA. Cells were then pelleted and frozen at -80°C for later processing using a Zymolyase enzyme (Zymo Research #E1004) to digest the cell wall (37°C for 1 hour), YeaStar high purity RNA extraction column kit (Zymo #R1002), and DNaseI enzyme treatment (Zymo #E1010) to digest unwanted genomic DNA. The resultant RNA samples were serial diluted to a concentration of 5 ng/µL as confirmed by NanoDrop quantitation. mRNA quantification using one step, gene-specific qRT-PCR was performed on a Bio-Rad CFX384 with a SYBR Green 1-step kit (Bio-Rad SYBR Green 2X iTaq #1725151 with Bio-Rad iScript #L002630) and analyzed with Bio-Rad Maestro qPCR software, with two housekeeper genes for normalization, *ALG9* and *TAF10*. One primer set amplified the native and all humanized yeast Gα chimeras. High primer efficiencies above 90% with an R^2 value of 97.8 or higher were confirmed on a linear template standard curve from 7 pg/µL to 70 ng/µL. For each primer set, controls without template confirmed a lack of primer-dimer products and controls without the reverse-transcriptase enzyme confirmed a lack of genomic DNA amplification. Each qRT-PCR reaction was 7.125 µL with 11 ng of template (1.54 ng/µL) with each primer at 300 nM.

Extraction of genomic DNA for qPCR deconvolution. Yeast sample aliquots (100 µL, see "*DCyFIRplex protocol*") were harvested by centrifugation at 8,000 x g for 1 minute, resuspended in 200 µL of genomic DNA lysis buffer (200 mM lithium acetate, 1% SDS), and incubated at 70°C for 10 minutes. Genomic DNA was collected by adding 600 µL of 100% ethanol to each tube of lysed cells and centrifugation at 13,000 x g for 5 minutes. Supernatant was then removed and the pellet of genomic DNA was washed with 600 µL 70% ethanol followed by centrifugation at 13,000 x g for 5 minutes. The resultant pellet of genomic DNA was dried at 70 °C for 10 minutes before a final resuspension in 50 µL nuclease-free H_2O. Genomic DNA was normalized to a final concentration of 10 ng/µL and used as the template for qPCR deconvolution.

qPCR primer design for DCyFIRplex deconvolution. There are several challenges associated with developing qPCR primers with the specificity and performance necessary to deconvolute complex gene mixtures. These primers must bind only one gene sequence in the mixture, avoid non-specific binding to background genomic DNA, produce the desired amplicon size with an optimal melting temperature, and lack the propensity to form secondary structures (e.g. hairpins), primer-dimers, and primer-heterodimers. To address these issues, we created a Python program that utilized the Primer3 module, a Python-specific application programming interface that provides accessibility to the open source primer design software package Primer3 (www.primer3.org). Using this program, we designed forward primers targeting specific C-terminal sequences in each of the 30 GPCRs in our exploratory panel. These forward primers, when combined with a universal reverse primer (targeting the CYC1b terminator in the X-2 landing pad), were designed to produce amplicons between 111 and 200 bp, an ideal size for qPCR analysis. Using this *in silico* design process, we generated and tested hundreds of forward qPCR primers for each GPCR using the following Primer3 settings:
PRIMER_OPT_SIZE: 20,
PRIMER_MIN_SIZE: 18,
PRIMER_MAX_SIZE: 22,
PRIMER_OPT_TM: 58,
PRIMER_MIN_TM: 52,
PRIMER_MAX_TM: 60,
PRIMER_MIN_GC: 20,
PRIMER_MAX_GC: 80,
PRIMER_GC_CLAMP: 1,
PRIMER_MAX_POLY_X: 6,
PRIMER_SALT_MONOVALENT: 50.0,
PRIMER_DNA_CONC: 50.0,
PRIMER_THERMODYNAMIC_ALIGNMENT: 1,
PRIMER_MAX_SELF_ANY_TH: 47.0,
PRIMER_MAX_SELF_END_TH: 47.0,
PRIMER_PAIR_MAX_COMPL_ANY_TH: 47.0,
PRIMER_PAIR_MAX_COMPL_END_TH: 47.0,
and PRIMER_PRODUCT_SIZE_RANGE: [111, 200].
The resultant set of primer candidates for each GPCR were ranked from best to worst by their Primer3 scores and assessed for uniqueness via sequential BLAST queries against locally built BLAST databases for 1) the updated release of *S. cerevisiae* BY4741 genome (BY4741_Toronto_2012) available via www.yeastgenome.org (3) and 2) the set of GPCR sequences comprising the Presto-TANGO library (9). After BLAST filtering, the top 8 primer designs for each GPCR were ordered from ThermoFisher and experimentally validated. In almost all cases, the primer designs met our specificity standards. However, only the top primer design was selected for our deconvolution procedure. The sequences for our final set of designed deconvolution primers are listed in Supplementary Dataset 4.

qPCR DCyFIRplex deconvolution. To achieve an accurate, reproducible, and scalable qPCR deconvolution procedure, we automated all processes using our Biomek NXp liquid-handling robot programmed using in-house Python code. The following procedure describes the process for deconvoluting a 300-plex genomic DNA sample from a single DCyFIRplex experiment. A reaction master mix was created by combining the necessary volumes of qPCR master mix (Bio-Rad Cat. #1725124), universal reverse primer, and template (i.e., the pool of extracted gDNA from a DCyFIRplex profile). In 384-well format, we used the robot to first distribute 3.0 µL of each GPCR forward qPCR primer in duplicate at a concentration of 500 ng/µL. Next, we used the robot to distribute 3.65 µL of reaction master mix to each well, giving a total qPCR reaction volume of 6.65 µL. For 30 receptors in a 300-plex, plus the additional mRuby3 tracer gene, a total of 62 wells were needed. We then removed the microplate from the robot deck, centrifuged it for 1 min at 1000xg to consolidate the samples at the bottom of the microplate wells, sealed the microplate with adhesive film (Applied Biosystems Cat. #4311971), and performed the qPCR experiment using a Bio-Rad CFX384.

As described, in each deconvolution run qPCR reactions were done twice for each of the 30 deconvolution primers (and mRuby3 control tracer primer), giving n=2 observations for each GPCR primer. However, in practice, deconvolution was done in triplicate for each agonist treatment using three independent builds of the 300-plex. As a result, we had n=6 observations for each GPCR primer in the deconvolution run. Therefore, all Cq and ΔCq values reported in this work represent an average of n=6 deconvolution runs, with error bars representing SEM of these values. All Cq values were quantified using the Bio-Rad Maestro qPCR software.

NanoString DCyFIRplex deconvolution. In addition to our qPCR-based deconvolution method, we analyzed the same 300-plex samples presented in Figures 2F and 3A using an orthogonal approach known as NanoString (NanoString Technologies, Seattle, WA). Using NanoString, the number of mRNA transcripts can be counted for a given gene using sequence-specific RNA hybridization probes covalently modified with proprietary fluorescent barcodes. Working with NanoString and Integrated DNA technologies as part of NanoString's proof-of-principle program, we designed these probes for our panel of 30 GPCR genes and the mRuby3 tracer gene. We then purified the total RNA from our collection of agonist-treated 300-plex samples using an RNA extraction kit (Zymo #R1002). These RNA samples were normalized to a concentration of 45 ng/µL and 45.0 µL volumes were submitted on ice to NanoString. Samples were run in duplicate on the nCounter Sprint Profiler using 100 ng of RNA, then again using 400 ng of RNA, giving a total of n=4 observations for each GPCR transcript. Qualitatively, both RNA amounts gave the same result; in Figure 3B we present the data only for the 400-ng run. To generate the heat map of normalized transcript counts presented in Figure 3B, we processed the NanoString data in four steps. In step one, data were background subtracted using a minimum value of 20 transcript counts. In step two, data were normalized across the set of GPCRs using the ratio of mRuby3 and GPR4 transcript counts. In step three, duplicate transcript counts for each GPCR were averaged to give a single transcript count value. And in step four, the average transcript count values were normalized across all agonist treatments for a given GPCR. As a result, the agonist responses for each GPCR in the heat map are ranked from zero to 1. Because our NanoString data was collected through the proof-of-principle program, our sample number was limited to the set of receptors and ligands presented in Figure 3B. Consequently, the only receptor/ligand combinations missing from the set are GPR35 with kynurenic acid and CNR2 with 2-AG and HU-210.

Microscopy. We used two microscopy approaches in this study.

Fluorescence microscopy. Consolidated pools of GPCR-Gα strains treated with metabolites were grown in 384-well plates (see *"DCyFIRscreen protocol"* above) for 18 hours to an OD ~ 1.0-2.0. Brightfield and fluorescence images (445/20 nm excitation, 510/40 nm emission, 455 nm longpass dichroic) were captured at 20X magnification using a fluorescence microscope (Echo Revolve, San Diego, USA).

Confocal microscopy. Cells were grown to mid-log phase in SCD LoFo pH 7.0 media. Cells were harvested by centrifugation, washed with sterile H_2O and concentrated to an OD = 20 in SCD LoFo pH 7.0 media. 2 µL cells were added to an agar pad (SCD LoFo pH 7.0 media, 15% agar) on a 75x25x1mm microscope slide (VWR Cat. #16004-422) and covered with a 22x22mm no. 1.5 glass cover slip (VWR Cat. #48366-227). Brightfield and fluorescence images were captured at 63X magnification under oil immersion on a confocal microscope (LSM800, Carl Zeiss, Jena, Germany). Fluorescence images for mTq2 (433 nm excitation, 475 nm emission) and mRuby3 (587 nm excitation, 610 nm emission) (Figure 2B) are depicted as maximum intensity projections (MIPs) of a Z-stack composed of 3-7 slices of 1-3 µm. MIP and image overlay for brightfield and confocal images were processed using the Zeiss Zen software.

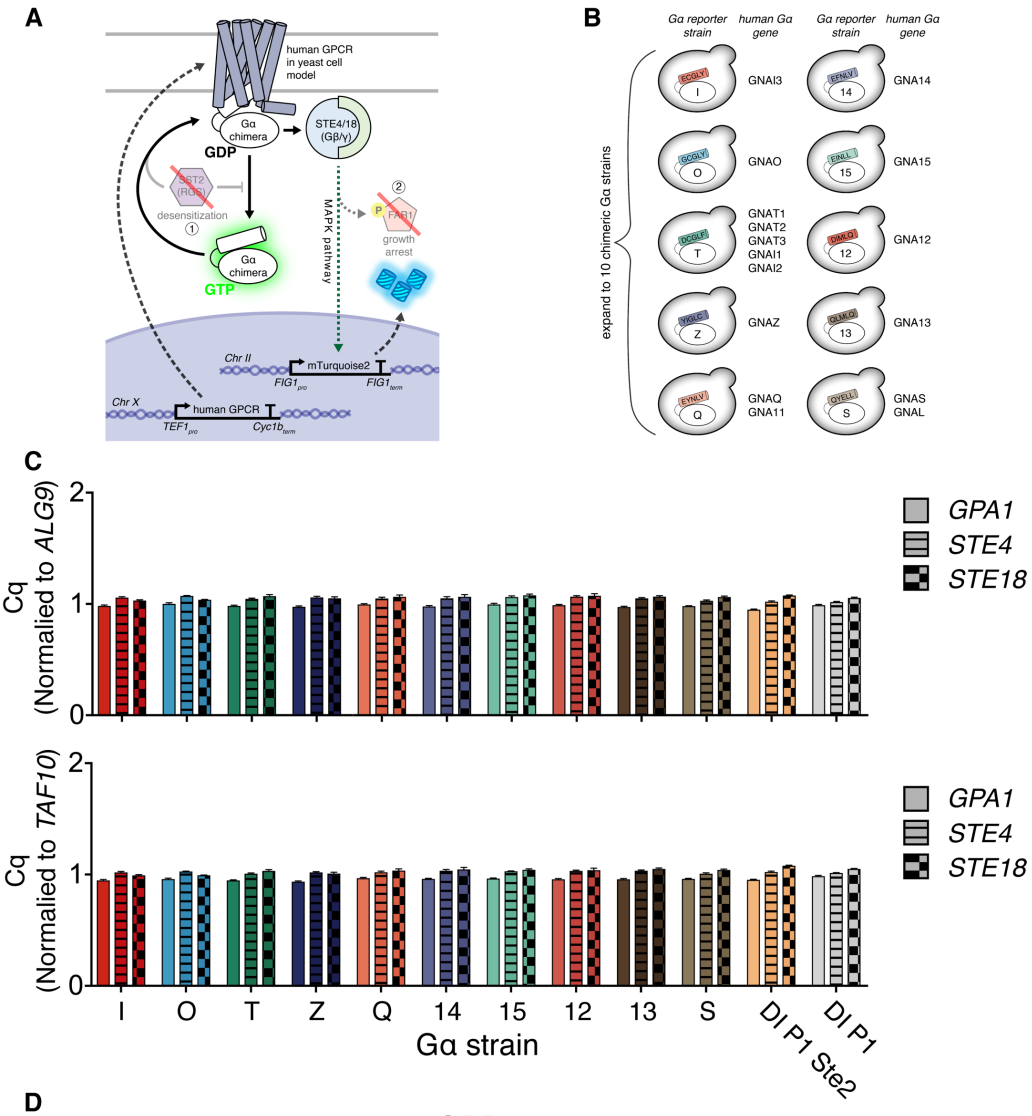
Ligands and metabolite library. All ligands were purchased from Sigma, Cayman, Tocris, and Avanti. The library of 320 endogenous human metabolites was purchased from MedChem Express (HY-L030). A table describing the source and composition of each ligand is available in the Key Resource Table. Lipid stocks in organic solvent were prepared using the general protocol from Avanti.

Titration Analyses. All titration curves were analyzed using Prism software and the pharmacological fitting function log(agonist) vs. response – Variable slope (four parameters) (GraphPad Software, San Diego, USA).

Validating select GPCR-metabolite interactions in mammalian cells using BRET. GPCR-luciferase constructs for expression in mammalian cells were made by appending Rluc8 directly to the receptor C-terminus by subcloning PCR-amplified receptor fragments into pRluc8-N1. GPCR sequences were obtained as a gift from Bryan Roth (Addgene, PRESTO-Tango Kit - #1000000068) (9). Plasmids encoding $G\alpha$ subunits were obtained from cdna.org (Bloomsburg University, Bloomsburg, PA). Plasmids encoding NES-Venus-mGsi, Venus-1-155-$G\gamma_2$ and Venus-155-239-$G\beta_1$ have been described previously (10, 11).

HEK 293 cells (ATCC, Manassas, VA, USA) were propagated in plastic flasks and on 6-well plates according to the supplier's protocol. Cells were transfected in growth medium using linear polyethyleneimine MAX (PEI MAX; MW 40,000) at a nitrogen/phosphate ratio of 20 and were used for experiments 12-48 hours later. Up to 3.0 µg of plasmid DNA was transfected in each well of a 6-well plate. Cells were transfected with a GPCR-Rluc8 and either a mini G protein (NES-Venus-mGsi for HCAR3) or G protein heterotrimers ($G\alpha$ subunit, Venus-1-155-$G\gamma_2$ and Venus-155-239-$G\beta_1$; $G\alpha_{12}$ for GPR35 and $G\alpha_{i1}$ for ADRA2B).

For experiments with mini G proteins cells were washed with DPBS, harvested by trituration, and transferred to opaque black or white 96-well plates containing diluted drug solutions. For assays with nucleotide-free heterotrimers cells were washed with permeabilization buffer containing 140 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 0.1 mM K-EGTA, 20 mM NaHEPES (pH 7.2), harvested by trituration, and permeabilized in the same buffer containing 10 µg ml^{-1} high purity digitonin (EMD Millipore, Burlington, MA). Measurements were made from permeabilized cells supplemented with apyrase (2 U ml^{-1}; Sigma, St. Louis, MO). BRET measurements were made using a Mithras LB940 photon-counting plate reader (Berthold Technologies GmbH, Bad Wildbad, Germany). Coelenterazine h (5 µM; Nanolight, Pinetop, AZ) was added to all wells immediately prior to making measurements. Raw BRET signals were calculated as the emission intensity at 520-545 nm divided by the emission intensity at 475-495 nm. Net BRET was this ratio minus the same ratio measured from cells expressing only the BRET donor.


A
human GPCR
in yeast cell
model
Gα
chimera
STE4/18
(Gβ/γ)
GDP
SST2
(RGS)
desensitization
①
②
FAR1
growth
arrest
MAPK pathway
Gα
chimera
GTP
Chr II
mTurquoise2
FIG1pro
FIG1term
Chr X
human GPCR
TEF1pro
Cyc1bterm
B
Gα reporter strain
human Gα gene
Gα reporter strain
human Gα gene
expand to 10 chimeric Gα strains
I
GNAI3
14
GNA14
O
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STE18
Cq
(Normalied to TAF10)
GPA1
STE4
STE18
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13
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DI P1 Ste2
DI P1
Gα strain
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GPR4
Cq
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MTNR1A
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Figure S1. Engineering and characterizing the base GPCR-Gα reporter strains.
(A) Schematic of the engineered yeast pheromone pathway for studying human GPCRs. A gene encoding a human GPCR is directly integrated into the yeast genome into a synthetic expression cassette at the X-2 locus. Human GPCR signaling through a chimeric yeast Gα protein is prolonged due to the deletion of a negative regulator (RGS protein, Sst2). MAP kinase cascade signaling drives the expression of a bright cyan fluorescent protein, mTq2, that was directly installed into the yeast genome replacing a pheromone-responsive gene, Fig1. Deletion of Far1 prevents cell cycle arrest upon MAPK signaling. (B) The 16 genes encoding human Gα proteins can be represented by 10 degenerate Gα chimeras (10 unique yeast strains), each with the corresponding 5 C-terminal amino acids from humans. (C) qPCR data illustrating similar heterotrimeric G protein expression levels across all 10 GPCR-Gα reporter strains. Data reported as Cq values normalized to two housekeeping genes (*ALG9* and *TAF10*) with error bars representing SEM of n=3-5 technical replicates. (D) qPCR data illustrating similar levels of GPR4 and MTNR1A expressed from the synthetic expression cassette at the X-2 locus across all 10 GPCR-Gα reporter strains. Data reported as Cq values normalized to housekeeping genes (*ALG9* or *TCF9*) with error bars representing SEM of n=3 technical replicates.

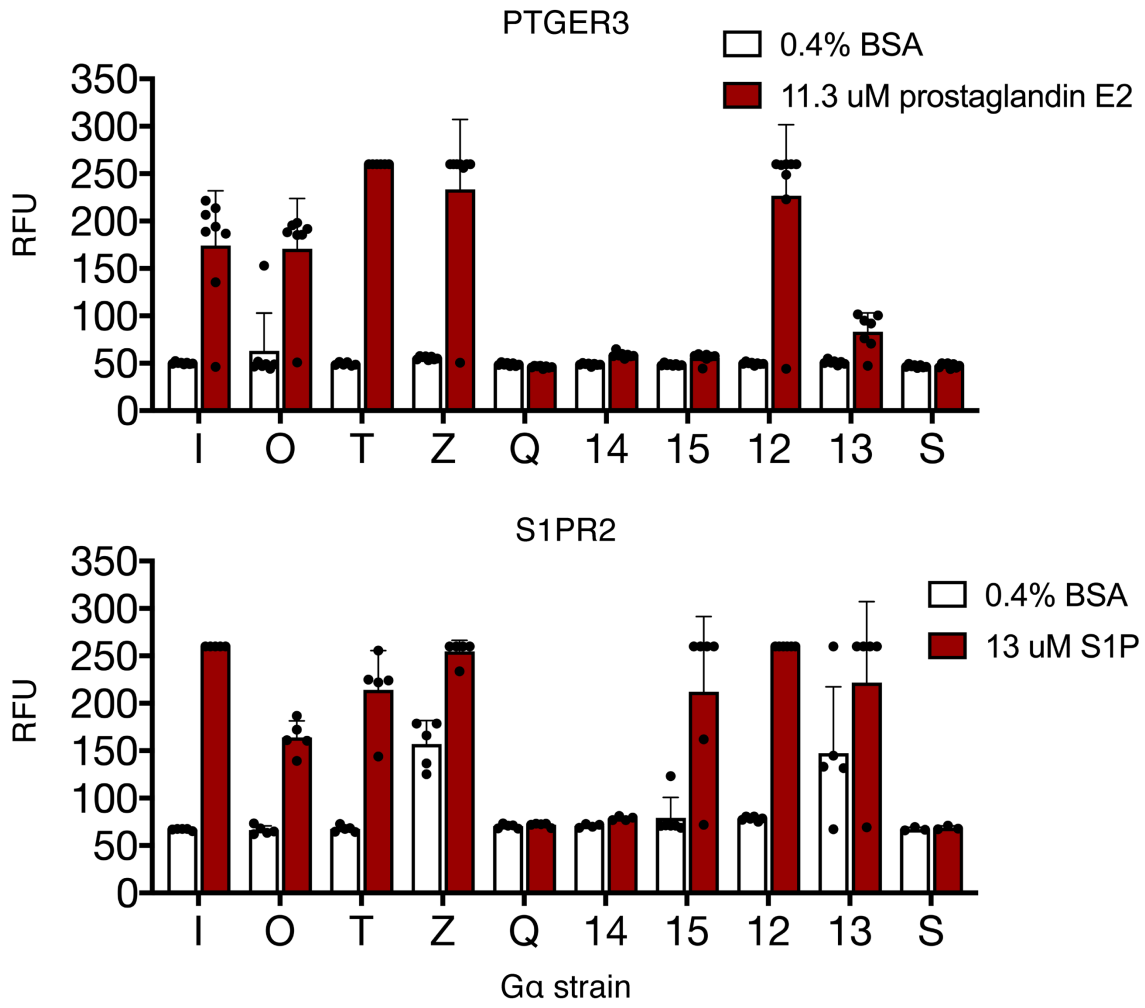


Figure S2. Raw DCyFIR screening data from multiple colonies of select receptors. Raw DCyFIR screening data of PTGER3 and S1PR2 with their endogenous ligands. Error bars represent SD of at least n=3 individual PCR-verified clones.

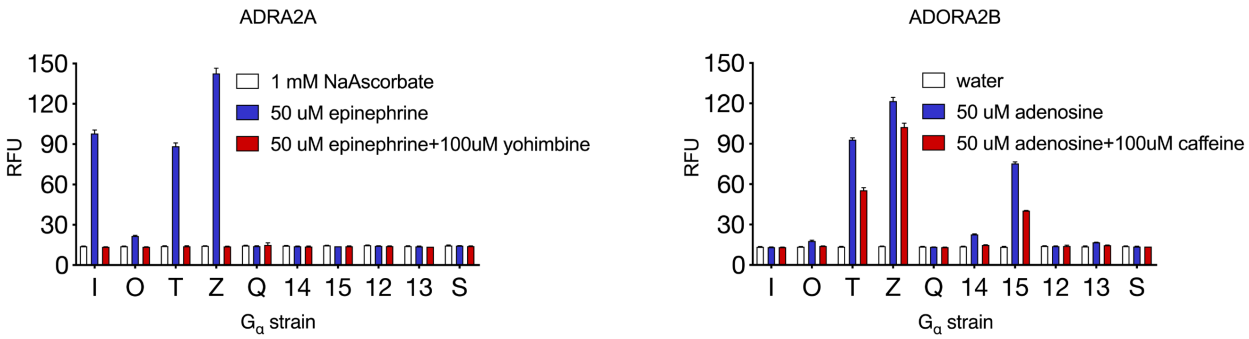


Figure S3. DCyFIRscreen data illustrating antagonism of select receptors. DCyFIRscreen analysis of ADRA2A (left) and ADORA2B (right) treated with their endogenous agonist (blue bars) in the presence of an antagonist (red bars). Error bars represent SD of n=4 experimental replicates.

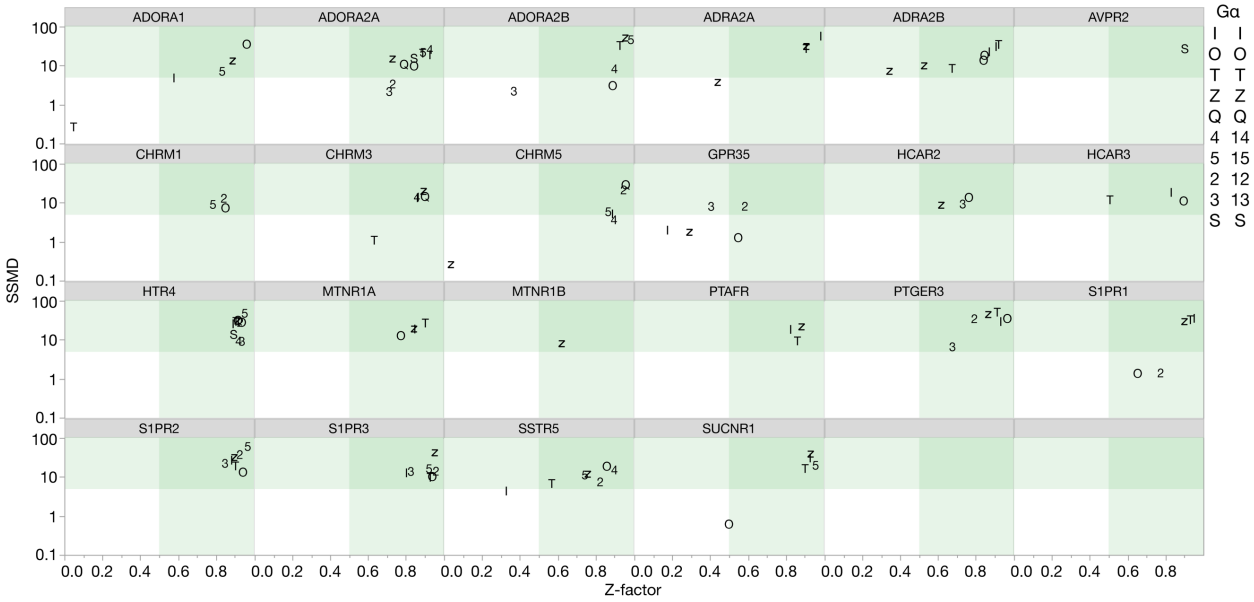


Figure S4. Demonstrating the low signal-to-noise level of the DCyFIR approach. Agonist responses for 22 GPCRs (220 GPCR-Gα reporter strains) analyzed by SSMD and Z-factor (see equations) for n=4 experimental replicates. SSMD is plotted on a log scale y-axis, while Z-factor is plotted on the x-axis. For each GPCR, one or more Gα coupling strains provides "excellent" screening suitability (green regions), both by SSMD (SSMD > 5) and Z-factor (Z-factor > 0.5).

Equations:

Equation 1. SSMD with unequal variance (12):

$$SSMD = \frac{\mu_t - \mu_u}{\sqrt{\sigma_t^2 + \sigma_u^2}}$$

Equation 2. Z-factor (Z') (13):

$$Z' = 1 - \frac{3(\sigma_t + \sigma_u)}{|\mu_t - \mu_u|}$$

Where μ_t = average RFU of treated, μ_u = average RFU of untreated, σ_t = standard deviation RFU of treated, σ_u = standard deviation RFU of untreated.

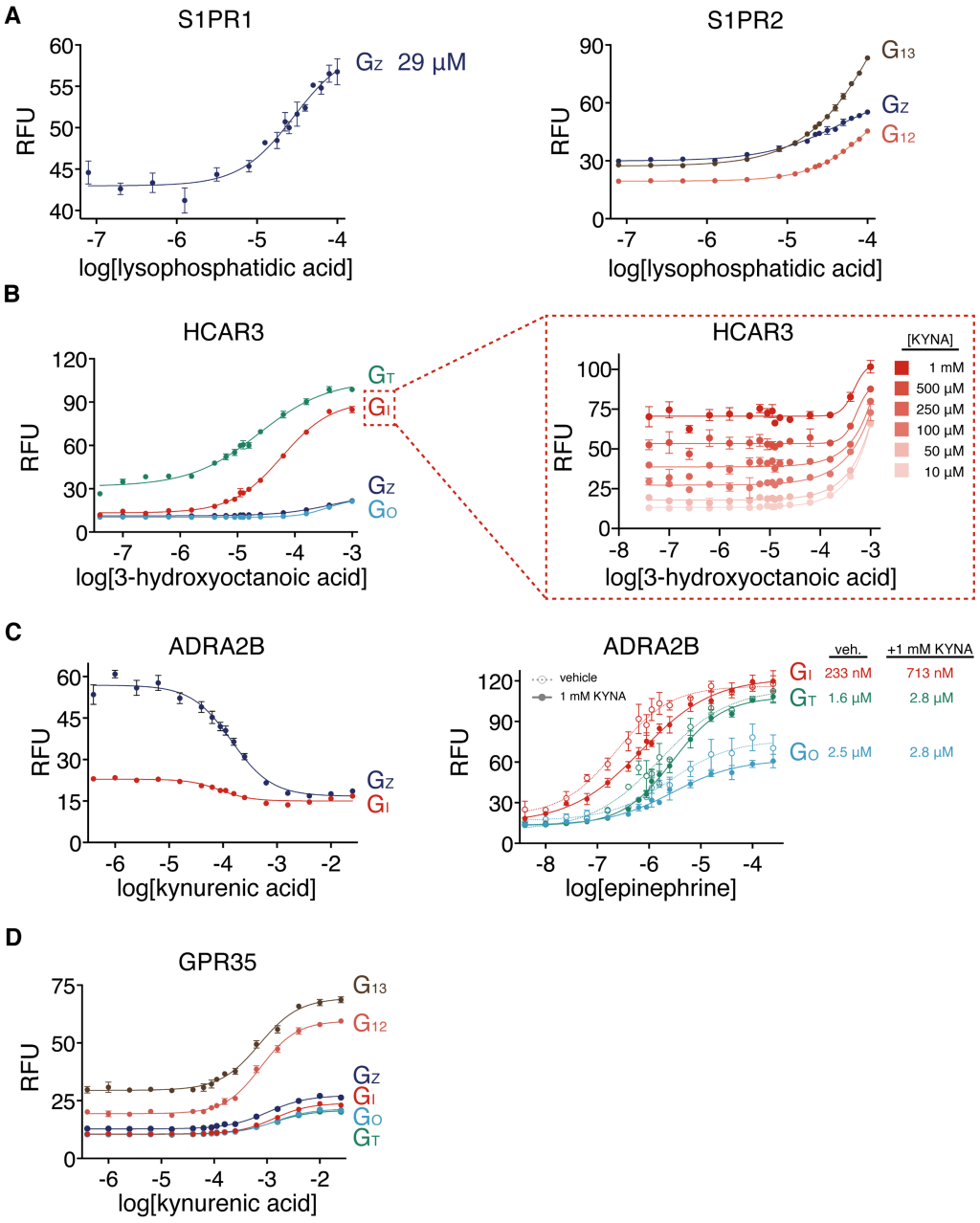
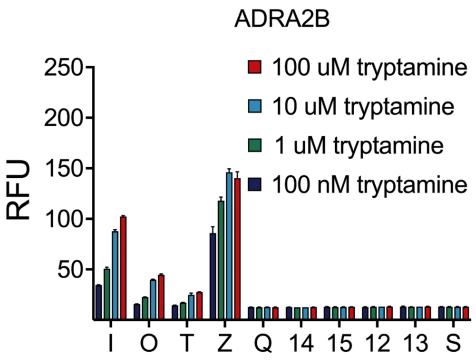
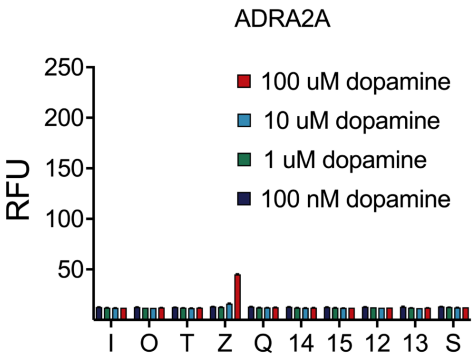
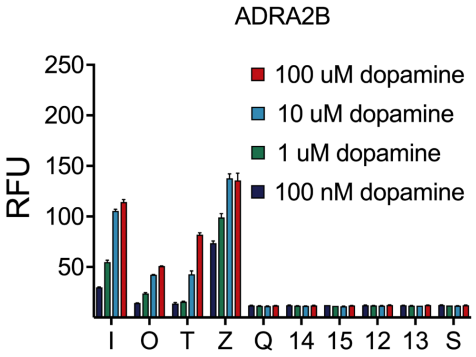


Figure S5. Follow-up titrations and control experiments for new ligand discoveries.
(A) Dose-response curves for S1PR1 and S1PR2 with lysophosphatidic acid (LPA). Error bars represent SD of n=4 experimental replicates. (B) Dose-response curves for HCAR3 with 3-hydroxyoctanoic acid (left) and 3-hydroxyoctanoic acid in the presence of kynurenic acid (right). Error bars represent SD of n=4 experimental replicates. (C) Dose-response curves for ADRA2B with kynurenic acid (left) and epinephrine (dashed line, open circles) in the presence of kynurenic acid (solid line, closed circles) (right). Error bars represent SD of n=4 experimental replicates. (D) Dose-response curves for GPR35 with kynurenic acid. Error bars represent SD of n=4 experimental replicates.

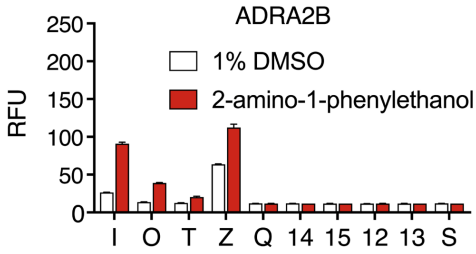
A

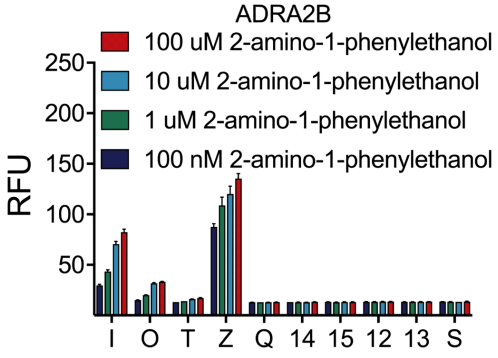

ADRA2B
100 uM tryptamine
10 uM tryptamine
1 uM tryptamine
100 nM tryptamine
RFU
250
200
150
100
50
I O T Z Q 14 15 12 13 S

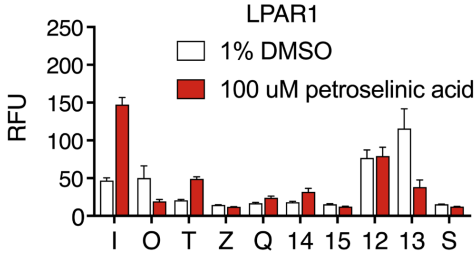

ADRA2A
100 uM dopamine
10 uM dopamine
1 uM dopamine
100 nM dopamine
RFU
250
200
150
100
50
I O T Z Q 14 15 12 13 S

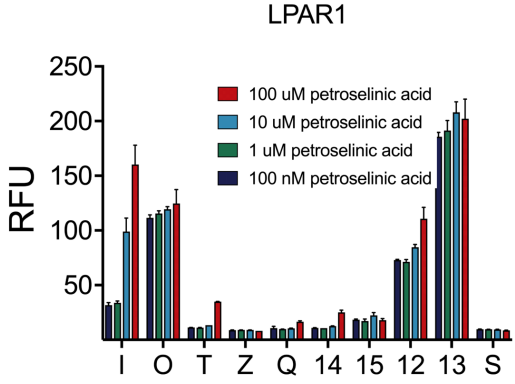

ADRA2B
100 uM dopamine
10 uM dopamine
1 uM dopamine
100 nM dopamine
RFU
250
200
150
100
50
I O T Z Q 14 15 12 13 S

B


ADRA2B
1% DMSO
2-amino-1-phenylethanol
RFU
250
200
150
100
50
0
I O T Z Q 14 15 12 13 S


ADRA2B
100 uM 2-amino-1-phenylethanol
10 uM 2-amino-1-phenylethanol
1 uM 2-amino-1-phenylethanol
100 nM 2-amino-1-phenylethanol
RFU
250
200
150
100
50
I O T Z Q 14 15 12 13 S


LPAR1
1% DMSO
100 uM petroselinic acid
RFU
250
200
150
100
50
0
I O T Z Q 14 15 12 13 S


LPAR1
100 uM petroselinic acid
10 uM petroselinic acid
1 uM petroselinic acid
100 nM petroselinic acid
RFU
250
200
150
100
50
I O T Z Q 14 15 12 13 S

B(cont.)

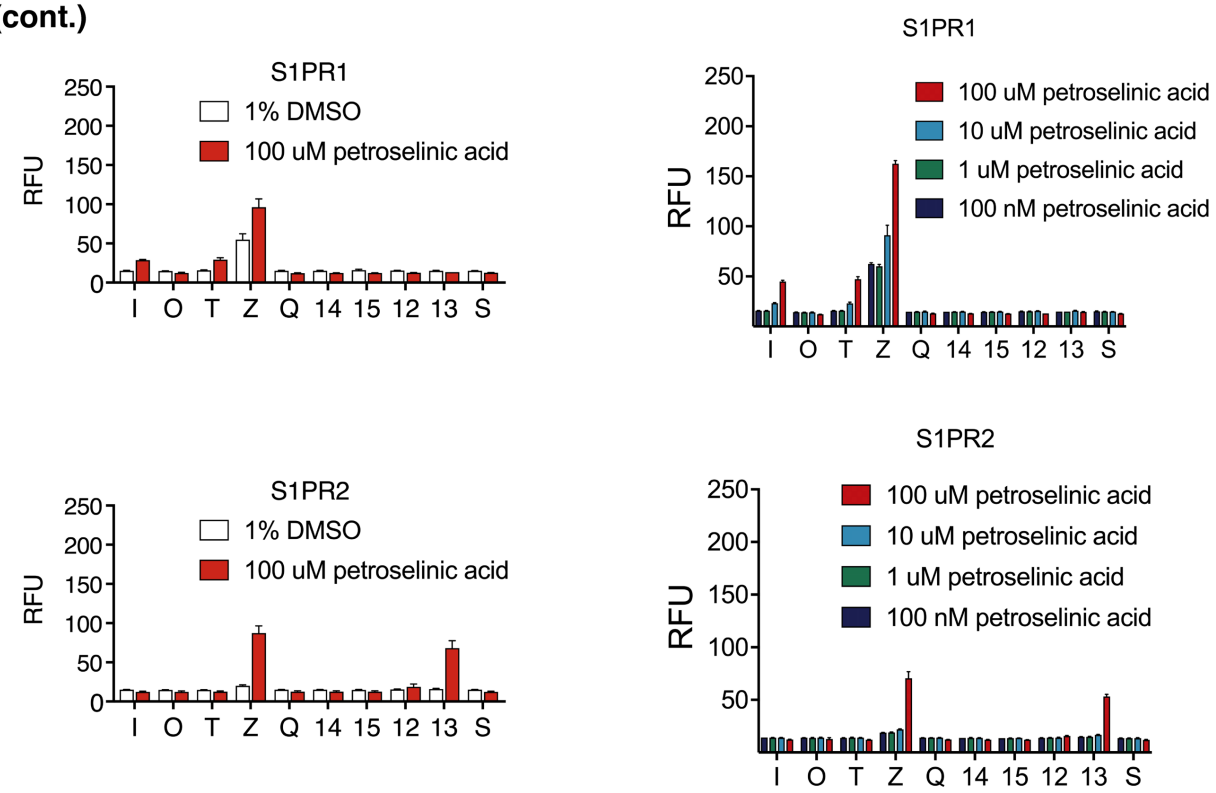


Figure S6. DCyFIRscreen profiles and coarse titrations to confirm new metabolite agonists and allosteric modulators. (A) Coarse 4-point titrations for HTR4 and ADRA2B with tryptamine, and ADRA2A and ADRA2B with dopamine. Error bars represent SD of n=4 experimental replicates. (B) DCyFIRscreen profiles and coarse 4-point titrations for new metabolite interactions (2-amino-1-phenylethanol and petroselinic acid). Error bars represent SD of n=4 experimental replicates.

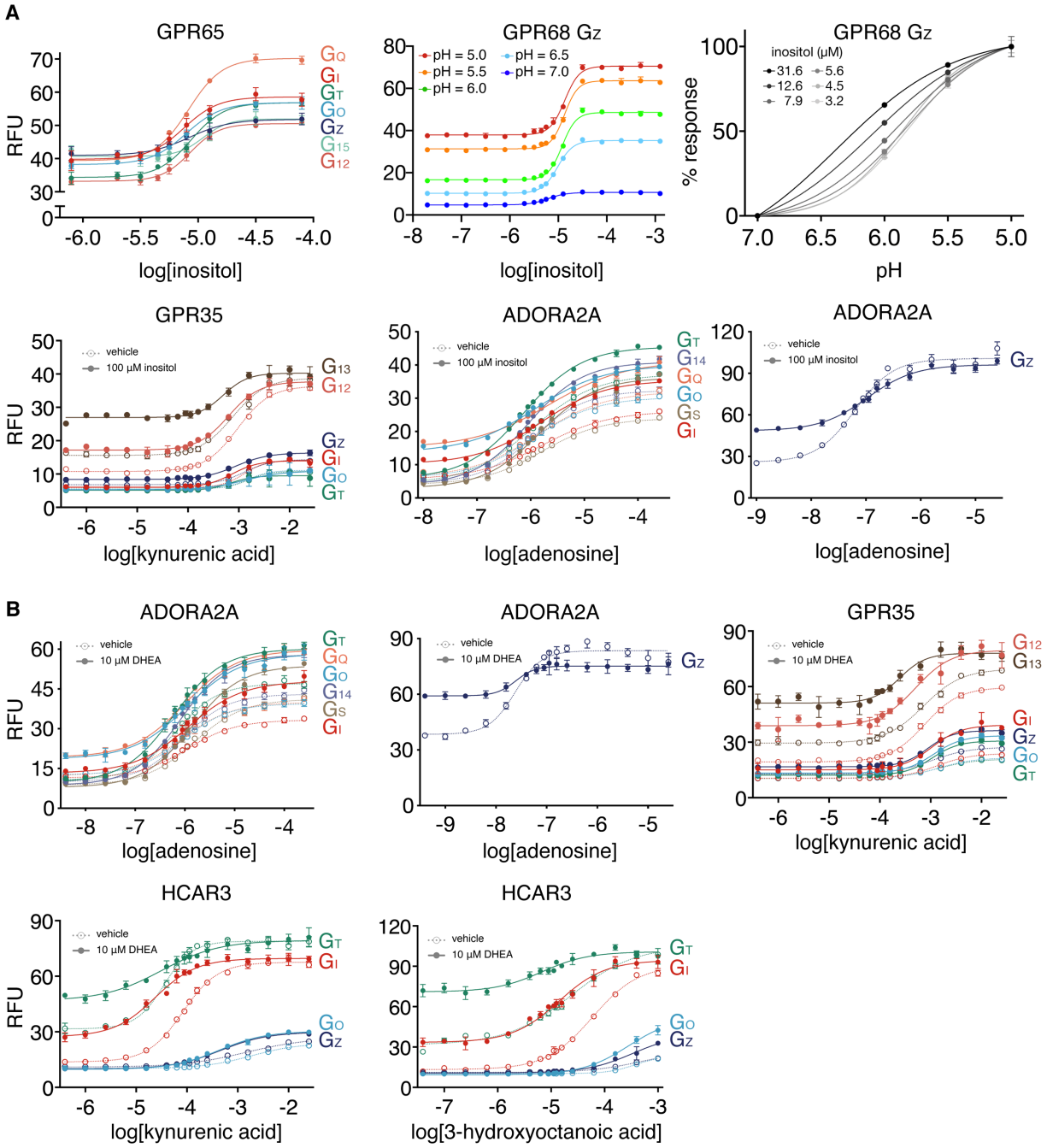
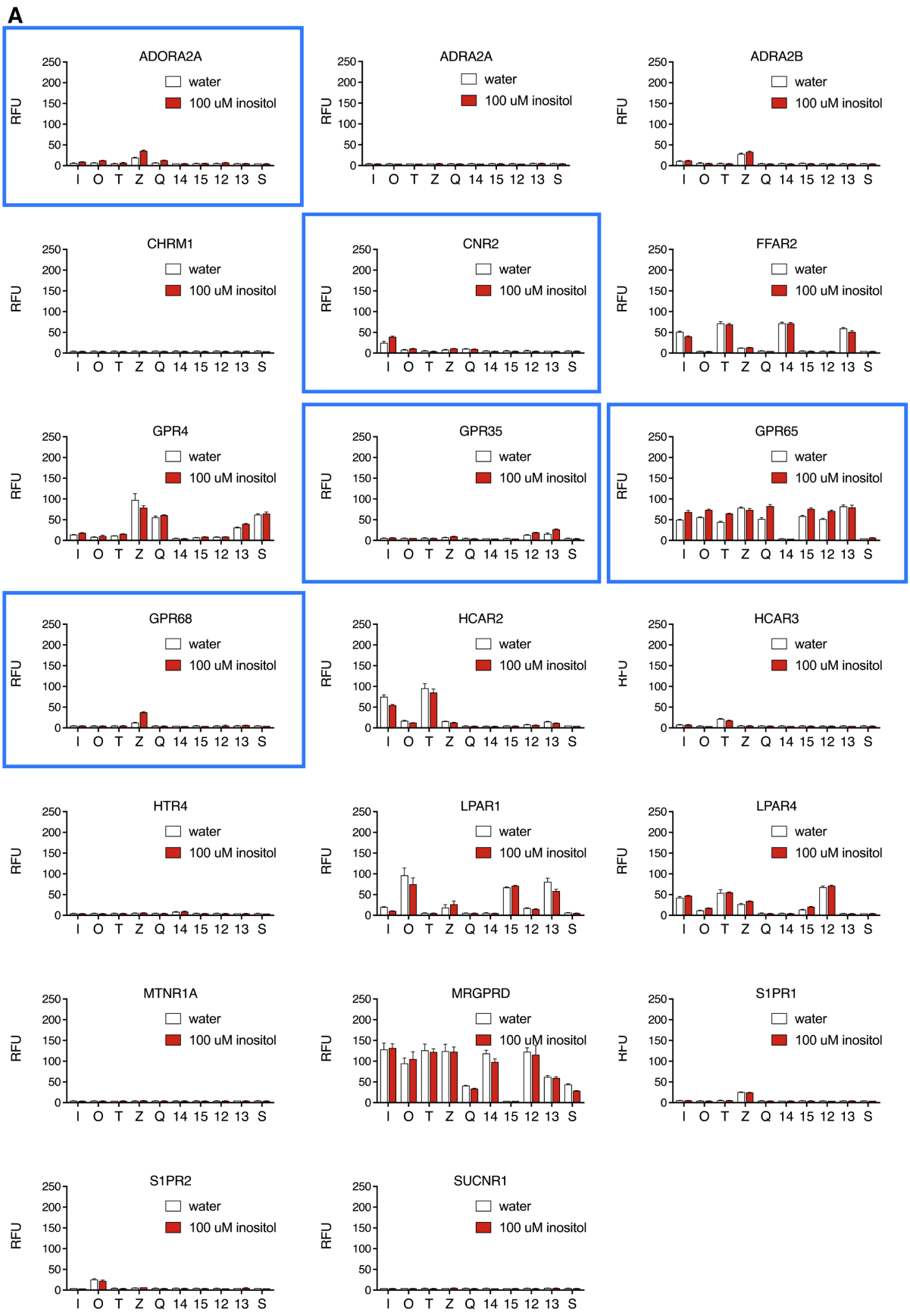
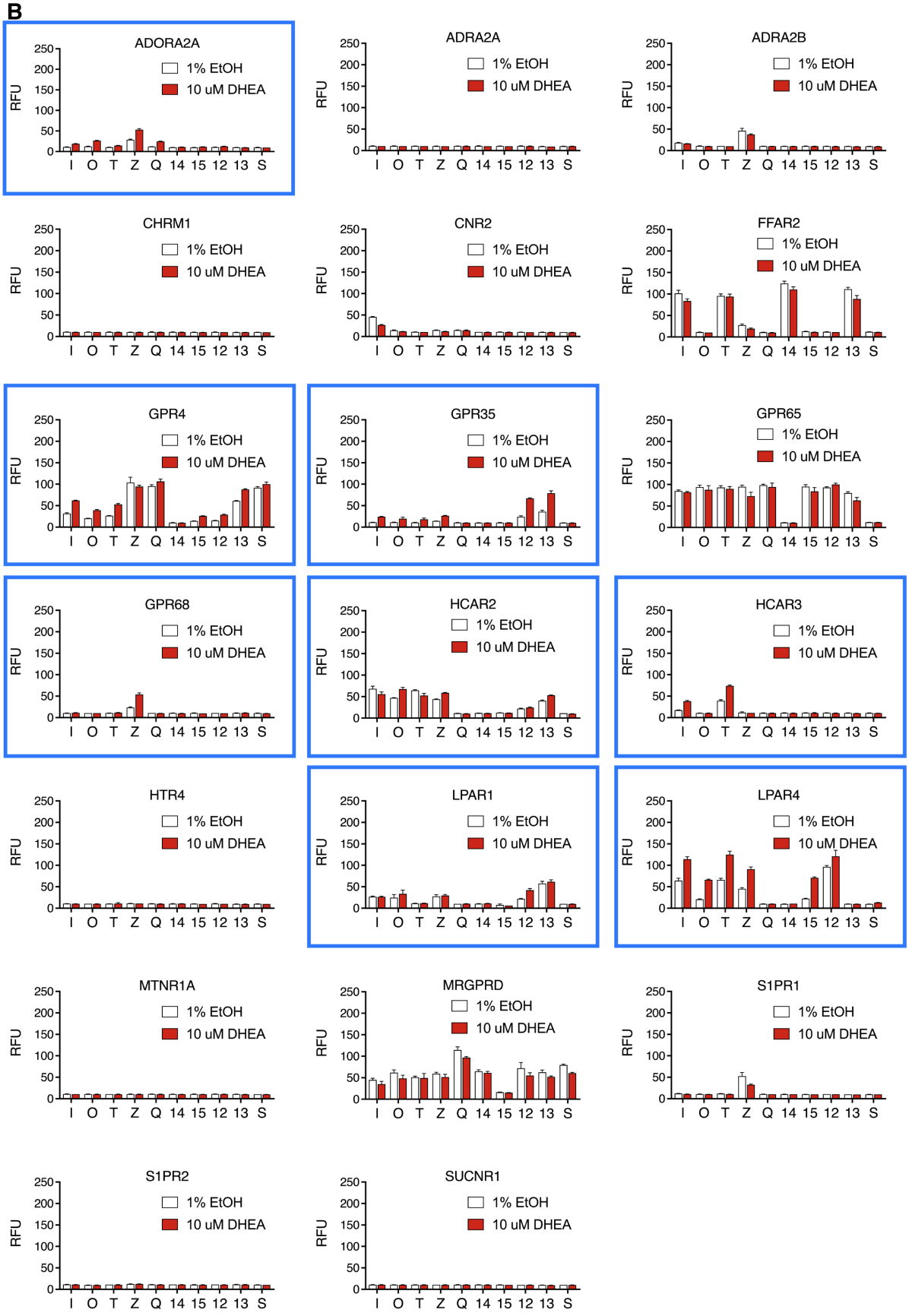


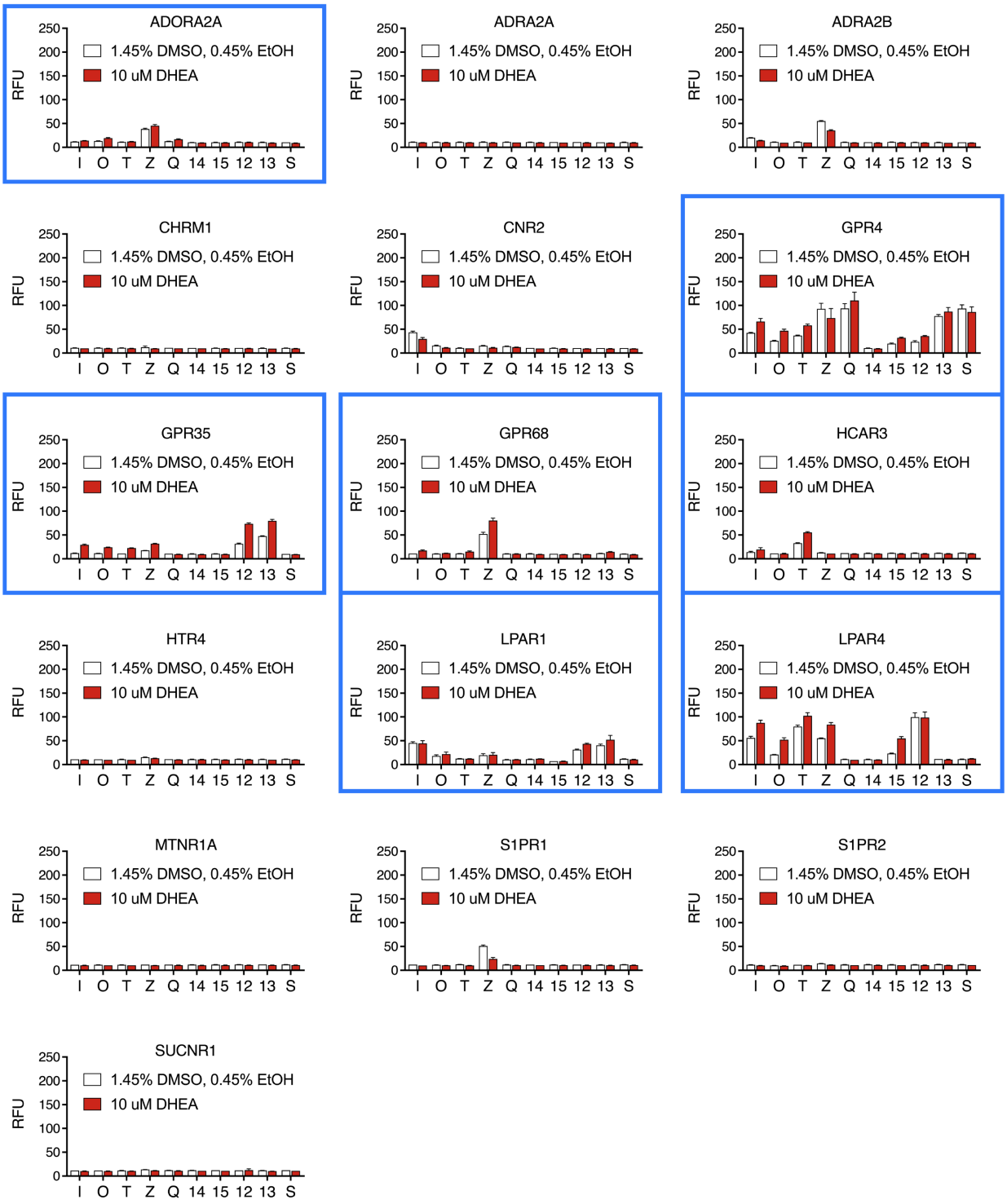
Figure S7. Detailed titrations for new metabolite allosteric modulators. (A) Dose-response curves for GPR65, GPR68, GPR35, and ADORA2A in the presence of inositol. Error bars represent SD of n=4 experimental replicates. (B) Dose-response curves for ADORA2A, GPR35, and HCAR3 (dashed lines, open circles) in the presence of DHEA (solid lines, closed circles). Error bars represent SD of n=4 experimental replicates.


A
ADORA2A
ADRA2A
ADRA2B
CHRM1
CNR2
FFAR2
GPR4
GPR35
GPR65
GPR68
HCAR2
HCAR3
HTR4
LPAR1
LPAR4
MTNR1A
MRGPRD
S1PR1
S1PR2
SUCNR1
water
100 uM inositol
RFU
0
50
100
150
200
250
I O T Z Q 14 15 12 13 S

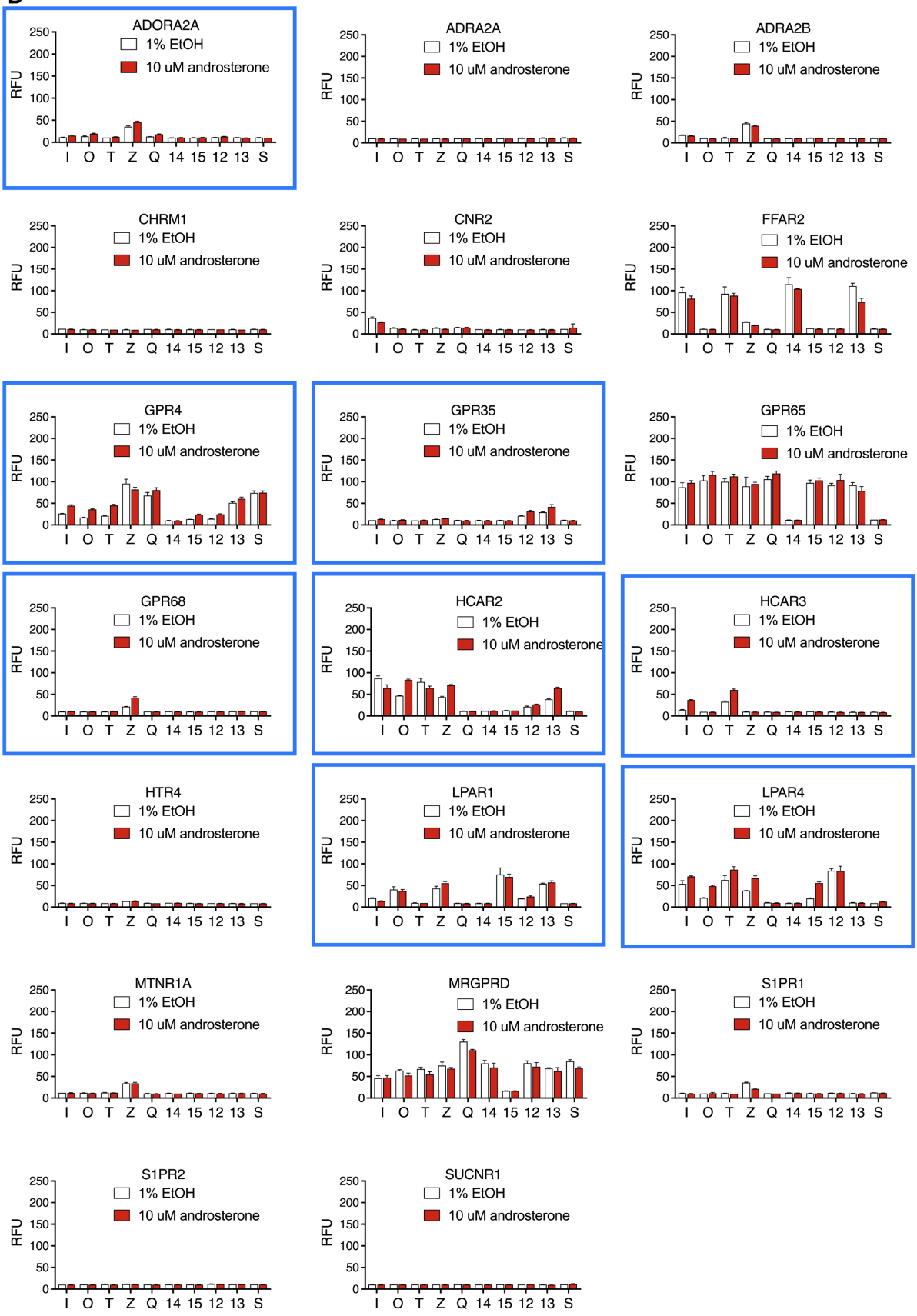
B


ADORA2A
ADRA2A
ADRA2B
CHRM1
CNR2
FFAR2
GPR4
GPR35
GPR65
GPR68
HCAR2
HCAR3
HTR4
LPAR1
LPAR4
MTNR1A
MRGPRD
S1PR1
S1PR2
SUCNR1
1% EtOH
10 uM DHEA
RFU
0
50
100
150
200
250
I O T Z Q 14 15 12 13 S

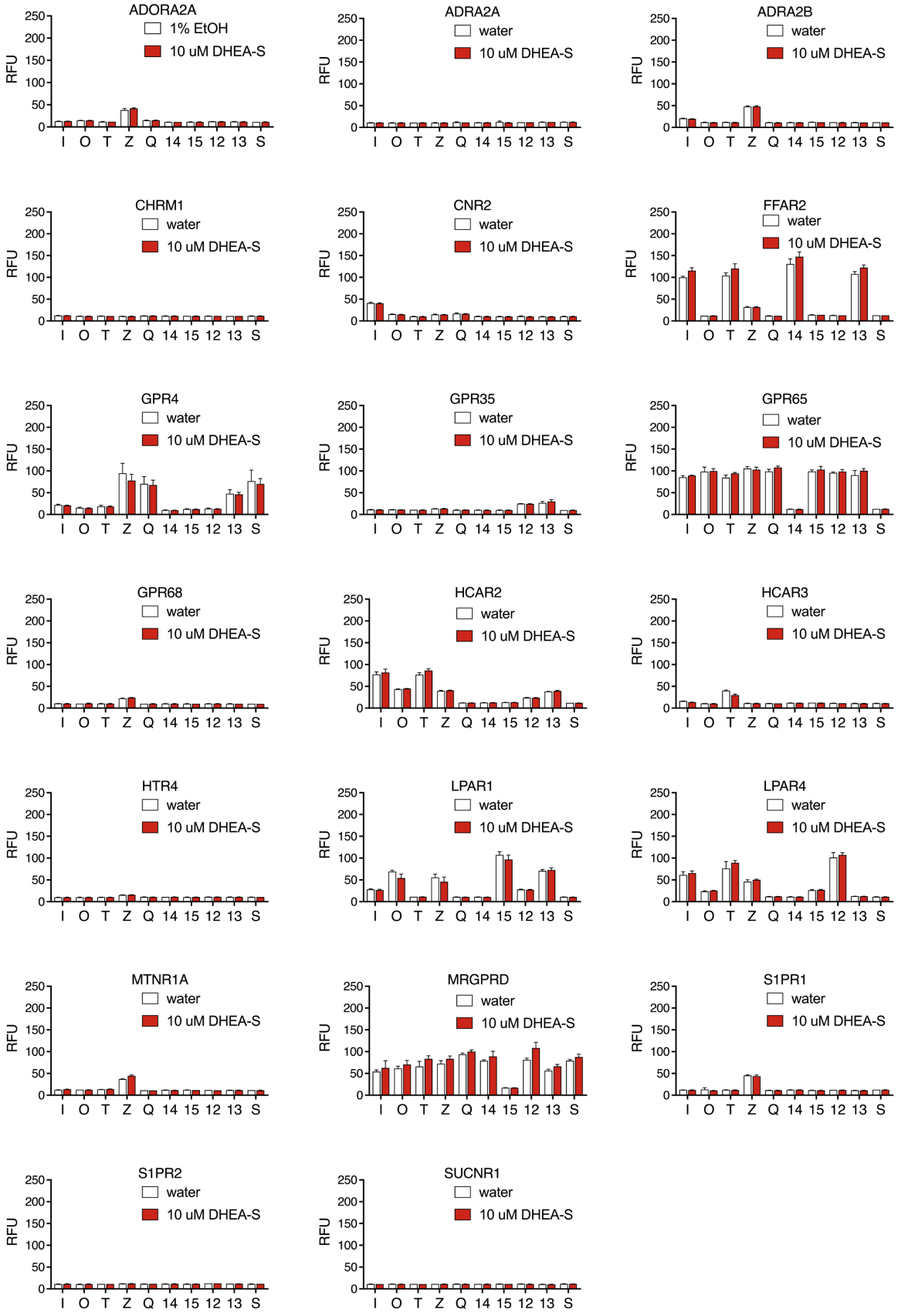
C


ADORA2A
ADRA2A
ADRA2B
CHRM1
CNR2
GPR4
GPR35
GPR68
HCAR3
HTR4
LPAR1
LPAR4
MTNR1A
S1PR1
S1PR2
SUCNR1
1.45% DMSO, 0.45% EtOH
10 uM DHEA
RFU
0
50
100
150
200
250
I O T Z Q 14 15 12 13 S

D


ADORA2A
ADRA2A
ADRA2B
CHRM1
CNR2
FFAR2
GPR4
GPR35
GPR65
GPR68
HCAR2
HCAR3
HTR4
LPAR1
LPAR4
MTNR1A
MRGPRD
S1PR1
S1PR2
SUCNR1
1% EtOH
10 uM androsterone
RFU
0
50
100
150
200
250
I O T Z Q 14 15 12 13 S

E


ADORA2A
1% EtOH
10 uM DHEA-S
ADRA2A
water
10 uM DHEA-S
ADRA2B
water
10 uM DHEA-S
CHRM1
water
10 uM DHEA-S
CNR2
water
10 uM DHEA-S
FFAR2
water
10 uM DHEA-S
GPR4
water
10 uM DHEA-S
GPR35
water
10 uM DHEA-S
GPR65
water
10 uM DHEA-S
GPR68
water
10 uM DHEA-S
HCAR2
water
10 uM DHEA-S
HCAR3
water
10 uM DHEA-S
HTR4
water
10 uM DHEA-S
LPAR1
water
10 uM DHEA-S
LPAR4
water
10 uM DHEA-S
MTNR1A
water
10 uM DHEA-S
MRGPRD
water
10 uM DHEA-S
S1PR1
water
10 uM DHEA-S
S1PR2
water
10 uM DHEA-S
SUCNR1
water
10 uM DHEA-S
RFU
0
50
100
150
200
250
I O T Z Q 14 15 12 13 S

F

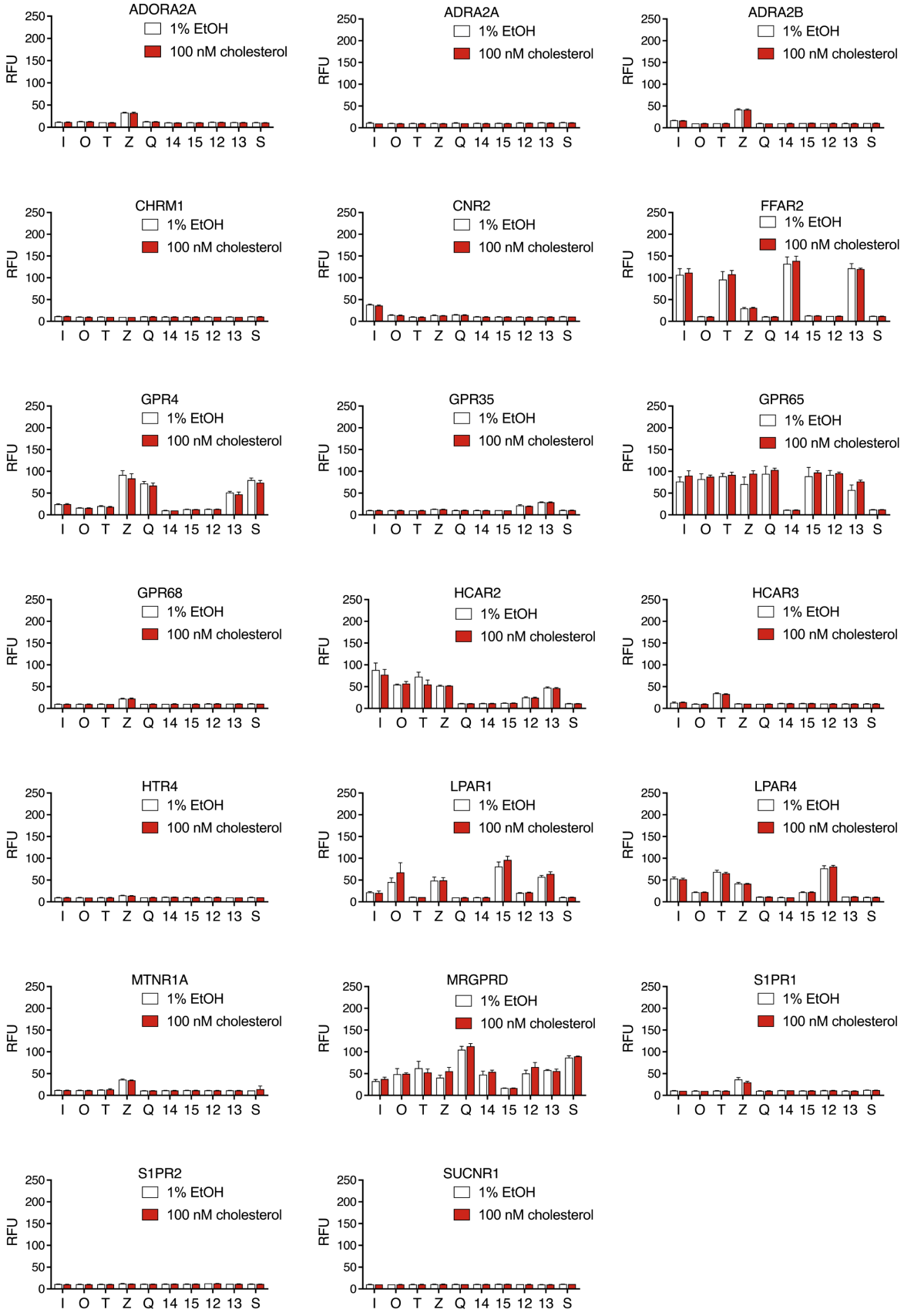
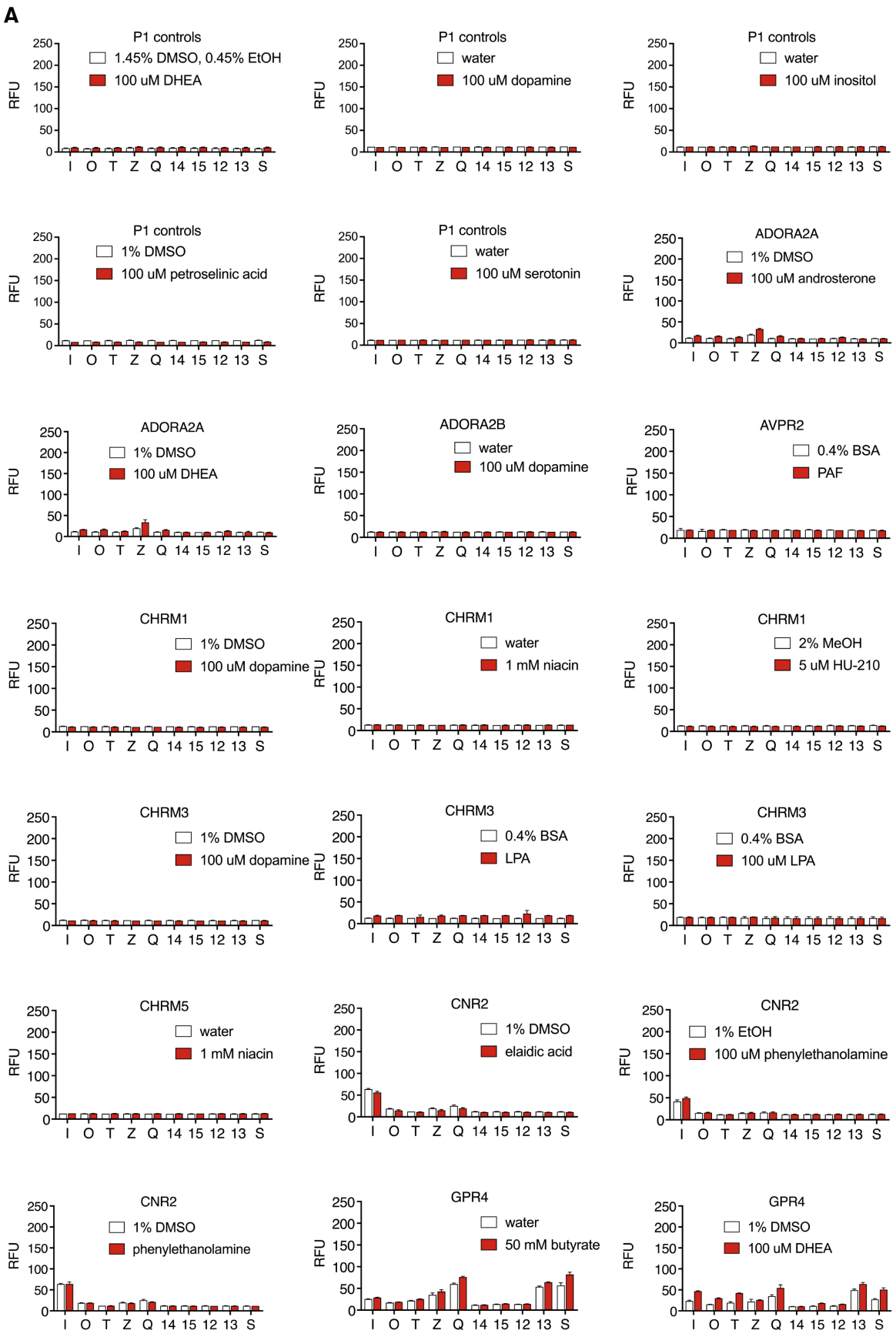
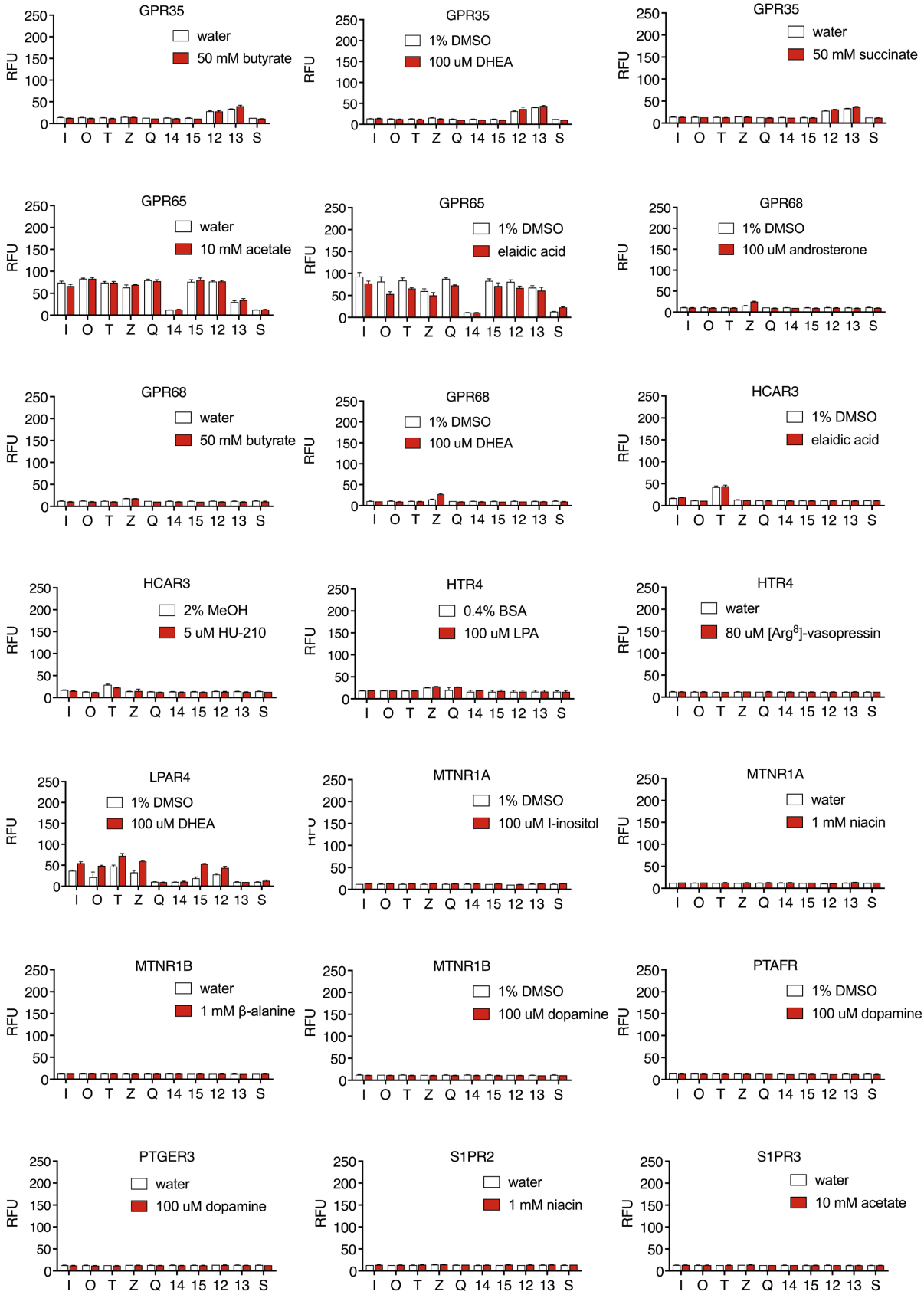

ADORA2A
ADRA2A
ADRA2B
CHRM1
CNR2
FFAR2
GPR4
GPR35
GPR65
GPR68
HCAR2
HCAR3
HTR4
LPAR1
LPAR4
MTNR1A
MRGPRD
S1PR1
S1PR2
SUCNR1
1% EtOH
100 nM cholesterol
RFU
0
50
100
150
200
250
I O T Z Q 14 15 12 13 S

Figure S8. Control DCyFIRscreen profiles for new metabolite allosteric modulators. (A) DCyFIRscreen profiles for GPCR-Gα reporter strains treated with inositol. Error bars represent SD of n=4 experimental replicates. Blue boxes indicate PAM interactions. (B) DCyFIRscreen profiles for GPCR-Gα reporter strains treated with DHEA in EtOH. Error bars represent SD of n=4 experimental replicates. Blue boxes indicate PAM interactions. (C) DCyFIRscreen profiles for GPCR-Gα reporter strains treated with DHEA in DMSO/EtOH. Error bars represent SD of n=4 experimental replicates. Blue boxes indicate PAM interactions. (D) DCyFIRscreen profiles for GPCR-Gα reporter strains treated with androsterone. Error bars represent SD of n=4 experimental replicates. Blue boxes indicate PAM interactions. (E) DCyFIRscreen profiles for GPCR-Gα reporter strains treated with DHEA-S. Error bars represent SD of n=4 experimental replicates. (F) DCyFIRscreen profiles for GPCR-Gα reporter strains treated with cholesterol. Error bars represent SD of n=4 experimental replicates.


A
P1 controls
1.45% DMSO, 0.45% EtOH
100 uM DHEA
P1 controls
water
100 uM dopamine
P1 controls
water
100 uM inositol
P1 controls
1% DMSO
100 uM petroselinic acid
P1 controls
water
100 uM serotonin
ADORA2A
1% DMSO
100 uM androsterone
ADORA2A
1% DMSO
100 uM DHEA
ADORA2B
water
100 uM dopamine
AVPR2
0.4% BSA
PAF
CHRM1
1% DMSO
100 uM dopamine
CHRM1
water
1 mM niacin
CHRM1
2% MeOH
5 uM HU-210
CHRM3
1% DMSO
100 uM dopamine
CHRM3
0.4% BSA
LPA
CHRM3
0.4% BSA
100 uM LPA
CHRM5
water
1 mM niacin
CNR2
1% DMSO
elaidic acid
CNR2
1% EtOH
100 uM phenylethanolamine
CNR2
1% DMSO
phenylethanolamine
GPR4
water
50 mM butyrate
GPR4
1% DMSO
100 uM DHEA
RFU
0
50
100
150
200
250
I O T Z Q 14 15 12 13 S

A (cont.)


GPR35
water
50 mM butyrate
GPR35
1% DMSO
100 uM DHEA
GPR35
water
50 mM succinate
GPR65
water
10 mM acetate
GPR65
1% DMSO
elaidic acid
GPR68
1% DMSO
100 uM androsterone
GPR68
water
50 mM butyrate
GPR68
1% DMSO
100 uM DHEA
HCAR3
1% DMSO
elaidic acid
HCAR3
2% MeOH
5 uM HU-210
HTR4
0.4% BSA
100 uM LPA
HTR4
water
80 uM [Arg8]-vasopressin
LPAR4
1% DMSO
100 uM DHEA
MTNR1A
1% DMSO
100 uM l-inositol
MTNR1A
water
1 mM niacin
MTNR1B
water
1 mM β-alanine
MTNR1B
1% DMSO
100 uM dopamine
PTAFR
1% DMSO
100 uM dopamine
PTGER3
water
100 uM dopamine
S1PR2
water
1 mM niacin
S1PR3
water
10 mM acetate
RFU
I O T Z Q 14 15 12 13 S

A (cont.)

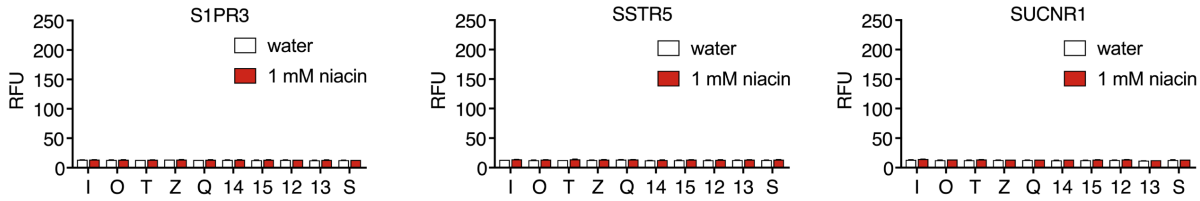


Figure S9. Control DCyFIRscreen profiles to assess DCyFIRplex sensitivity. (A) Control DCyFIRscreen profiles for receptors with low dCq values from DCyFIRplex profiling. Error bars represent SD of n=4 experimental replicates.

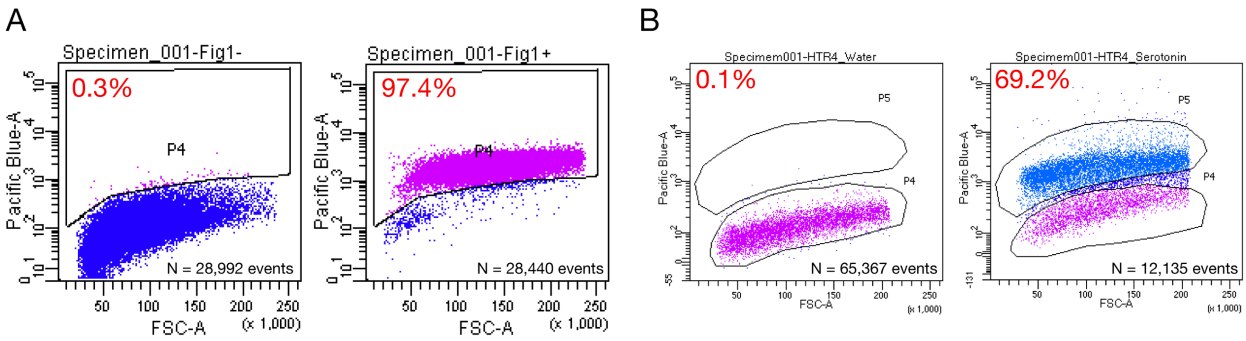


Figure S10. Quantification of FACS gating performance. (A) FACS dot plots illustrating the false positive (A, left) and false negative (A, right) rates of cells expressing the endogenous yeast GPCR, Ste2, treated with α-factor. (B) FACS dot plots illustrating the false positive (B, left) and false negative (B, right) rate of a multiplex consisting of all 10 Gα reporter strains expressing the human serotonin receptor, HTR4, treated with serotonin.

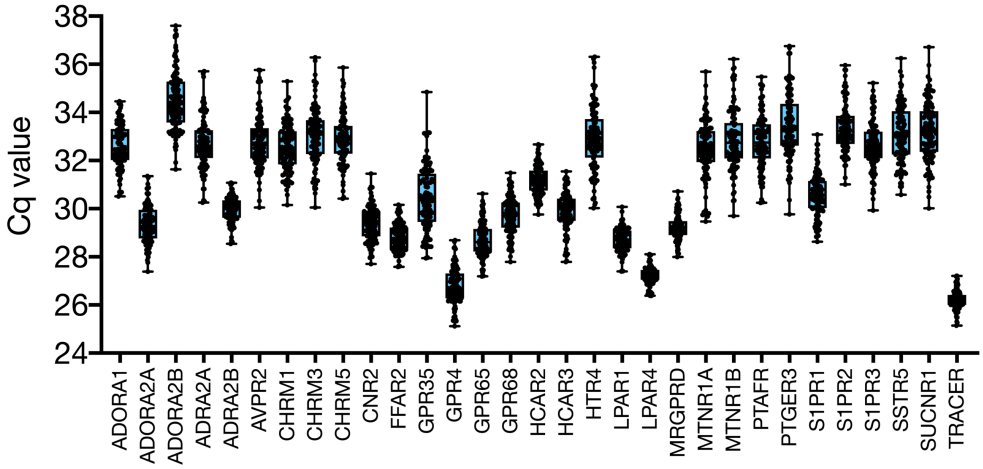


Figure S11. Robustness of the 301-plex composition and performance over 100 independent DCyFIRplex deconvolution experiments. Background Cq values for all 301 strains from 100 individual DCyFIRplex qPCR deconvolution experiments.

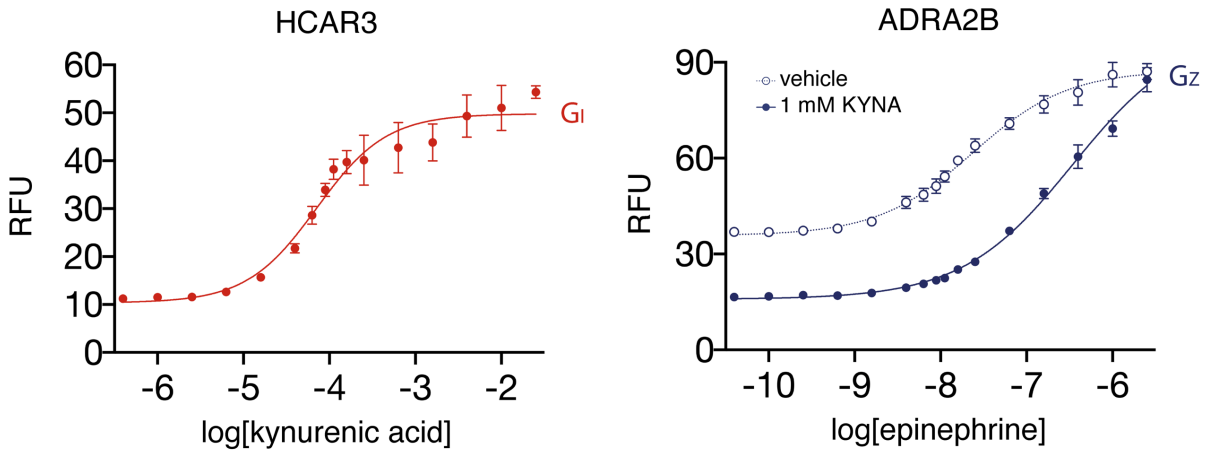


Figure S12. Titrations for multiple colonies of select receptors. Titration of HCAR3 with kynurenic acid (left) and ADRA2B with epinephrine (dashed line, open circles) and at a fixed kynurenic acid concentration of 1 mM (solid line, closed circles) (right). Error bars represent SD of n=4 individual colonies for each receptor.

Table S1. Synthetic DNA constructs used in this study.

Construct name	Type	Sequence (5'-3')
FAR1 DP	gBlock	TGTCTTGAGAGTGTATATTATCTTATCTATTCAAAAAATTTCTATTTACTTTTATATTTCTT GACCATCCTTTACACAAAGTCTATAGATCCACTGGAAAGCTTCGTGGGCGTAAGAAGG CAATCTATTATAGTTCGGGAATCGAGGCCCGTATTTCGAGGCTTTTGCTTTTCCTTTTTT TTTTTTCGTTTCTCCACGTCTATACTACGCAATGACTGAATATATATAATGCGTCGTAAA TAGCAGTTAATGTATAAATA
Ste2 DP	gBlock	CTTCAAAGCAATACGATACCTTTTCTTTTCACCTGCTCTGGCTATAATTATAATTGGTTA CTTAAAAATGCACCGTTAAGAACCATATCCAAGAATCAAAATCAAAATTTACGGCTTTG AAAAAGTAATTTCGTGACCTTCGGTATAAGGTTACTACTAGATTCAGGTGCTCATCAGA TGCACCACATTCTCTATAAAAAAA
GPA1_chimera_GNAI3	gBlock	TTTTTGAGCTTGAATAAGACAAACAAACCAATCTACGTGAAACGAACCTGCGCTACCGA TACCCAAACTATGAAGTTCGTATTGAGTGCAGTCACTGATCTAATCATCCAGCAAAACC TTAAAGAATGTGGTTTGTATTGAAGGAACTGTATAATTAAAGTAGTGTTTAGATACGTAA ATTCTGTTTCCGAAGATGCAAGAAGGAGCAGCAGCACCAGAAAAAATTACTATTTTTCT TCTCCATTAGAGTCTATGATGGAA
GPA1_chimera_GNAO	gBlock	TTTTTGAGCTTGAATAAGACAAACAAACCAATCTACGTGAAACGAACCTGCGCTACCGA TACCCAAACTATGAAGTTCGTATTGAGTGCAGTCACTGATCTAATCATCCAGCAAAACC TTAAAGGTTGTGGTTTGTATTGAAGGAACTGTATAATTAAAGTAGTGTTTAGATACGTAA ATTCTGTTTCCGAAGATGCAAGAAGGAGCAGCAGCACCAGAAAAAATTACTATTTTTCT TCTCCATTAGAGTCTATGATGGAA
GPA1_chimera_GNAT123_I12	gBlock	TTTTTGAGCTTGAATAAGACAAACAAACCAATCTACGTGAAACGAACCTGCGCTACCGA TACCCAAACTATGAAGTTCGTATTGAGTGCAGTCACTGATCTAATCATCCAGCAAAACC TTAAAGATTGCGGTTTGTTTTGAAGGAACTGTATAATTAAAGTAGTGTTTAGATACGTAA ATTCTGTTTCCGAAGATGCAAGAAGGAGCAGCAGCACCAGAAAAAATTACTATTTTTCT TCTCCATTAGAGTCTATGATGGAA
GPA1_chimera_GNAZ	gBlock	TTTTTGAGCTTGAATAAGACAAACAAACCAATCTACGTGAAACGAACCTGCGCTACCGA TACCCAAACTATGAAGTTCGTATTGAGTGCAGTCACTGATCTAATCATCCAGCAAAACC TTAAATATATCGGTTTGTGCTGAAGGAACTGTATAATTAAAGTAGTGTTTAGATACGTAA ATTCTGTTTCCGAAGATGCAAGAAGGAGCAGCAGCACCAGAAAAAATTACTATTTTTCT TCTCCATTAGAGTCTATGATGGAA
GPA1_chimera_GNAQ_11	gBlock	TTTTTGAGCTTGAATAAGACAAACAAACCAATCTACGTGAAACGAACCTGCGCTACCGA TACCCAAACTATGAAGTTCGTATTGAGTGCAGTCACTGATCTAATCATCCAGCAAAACC TTAAAGAATATAATTTGGTTTGAAGGAACTGTATAATTAAAGTAGTGTTTAGATACGTAAA TTCTGTTTCCGAAGATGCAAGAAGGAGCAGCAGCACCAGAAAAAATTACTATTTTTCTT CTCCATTAGAGTCTATGATGGAA
GPA1_chimera_GNA14	gBlock	TTTTTGAGCTTGAATAAGACAAACAAACCAATCTACGTGAAACGAACCTGCGCTACCGA TACCCAAACTATGAAGTTCGTATTGAGTGCAGTCACTGATCTAATCATCCAGCAAAACC TTAAAGAATTTAATTTGGTTTGAAGGAACTGTATAATTAAAGTAGTGTTTAGATACGTAAA TTCTGTTTCCGAAGATGCAAGAAGGAGCAGCAGCACCAGAAAAAATTACTATTTTTCTT CTCCATTAGAGTCTATGATGGAA
GPA1_chimera_GNA15	gBlock	TTTTTGAGCTTGAATAAGACAAACAAACCAATCTACGTGAAACGAACCTGCGCTACCGA TACCCAAACTATGAAGTTCGTATTGAGTGCAGTCACTGATCTAATCATCCAGCAAAACC TTAAAGAAATTAATTTGTTGTGAAGGAACTGTATAATTAAAGTAGTGTTTAGATACGTAAA TTCTGTTTCCGAAGATGCAAGAAGGAGCAGCAGCACCAGAAAAAATTACTATTTTTCTT CTCCATTAGAGTCTATGATGGAA
GPA1_chimera_GNA12	gBlock	TTTTTGAGCTTGAATAAGACAAACAAACCAATCTACGTGAAACGAACCTGCGCTACCGA TACCCAAACTATGAAGTTCGTATTGAGTGCAGTCACTGATCTAATCATCCAGCAAAACC TTAAAGATATTATGCTACAATGAAGGAACTGTATAATTAAAGTAGTGTTTAGATACGTAA

		ATTCTGTTTCCGAAGATGCAAGAAGGAGCAGCAGCACCAGAAAAAATTACTATTTTTCT TCTCCATTAGAGTCTATGATGGAA
GPA1_chimera_GNA13	gBlock	TTTTTGAGCTTGAATAAGACAAACAAACCAATCTACGTGAAACGAACCTGCGCTACCGA TACCCAAACTATGAAGTTCGTATTGAGTGCAGTCACTGATCTAATCATCCAGCAAAACC TTAAACAATTGATGTTGCAATGAAGGAACTGTATAATTAAAGTAGTGTTTAGATACGTAA ATTCTGTTTCCGAAGATGCAAGAAGGAGCAGCAGCACCAGAAAAAATTACTATTTTTCT TCTCCATTAGAGTCTATGATGGAA
GPA1_chimera_GNAS_L	gBlock	TTTTTGAGCTTGAATAAGACAAACAAACCAATCTACGTGAAACGAACCTGCGCTACCGA TACCCAAACTATGAAGTTCGTATTGAGTGCAGTCACTGATCTAATCATCCAGCAAAACC TTAAACAATATGAATTGTTGTGAAGGAACTGTATAATTAAAGTAGTGTTTAGATACGTAA ATTCTGTTTCCGAAGATGCAAGAAGGAGCAGCAGCACCAGAAAAAATTACTATTTTTCT TCTCCATTAGAGTCTATGATGGAA
CHRM1 Δil3	gBlock	ATGAATACAAGCGCTCCTCCAGCGGTTTCCCCAAATATTACGGTGCTGGCACCTGGG AAAGGACCATGGCAAGTTGCTTTTATCGGTATCACCACCGGCTTGCTCTCCTTGGCAA CAGTCACGGGCAATTTGCTCGTACTGATCTCATTTAAGGTGAACACAGAGCTGAAAAC AGTGAACAACTACTTCTTGTTGAGTTTGGCCTGTGCGGACCTTATTATCGGAACGTTTT CAATGAATCTGTATACAACCTATCTCCTGATGGGCCACTGGGCGCTGGGAACCCTTGC TTGCGACCTTTGGTTGGCCCTTGATTACGTGGCGAGCAACGCAAGCGTCATGAATCTC CTGTTGATATCATTTGACAGGTATTTTTCTGTTACCCGGCCCCTGAGCTACAGAGCTAA ACGAACTCCACGACGCGCTGCACTGATGATTGGTCTGGCCTGGCTGGTCAGTTTTGT CCTGTGGGCCCCTGCCATACTGTTTTGGCAGTACCTGGTGGGAGAGCGCACCGTGCT TGCAGGTCAGTGCTATATACAGTTTCTGTCTCAGCCCATAATCACATTCGGCACCGCG ATGGCGGCATTTTACTTGCCTGTGACTGTTATGTGTACTCTGTATTGGCGAATTTACCG CGAAACCGAGAATAGAGCCAGGGAACTTGCTGCACTGCAGGGTTCAGAGACAAAGGA ACAGCTGGCGAAGCGGAAAACCTTCAGCCTCGTGAAGGAAAAGAAAGCCGCACGAA CCCTGTCCGCTATCTTGCTGGCTTTCATACTGACCTGGACGCCTTATAACATTATGGTG CTGGTCTCCACCTTTTGTAAAGATTGCGTCCCAGAGACACTTTGGGAGCTGGGGTACT GGCTGTGCTATGTGAACTCCACAATTAACCCAATGTGTTATGCCTTGTGTAATAAAGCG TTCAGAGATACATTCCGGCTTCTCCTTCTGTGCCGGTGGGATAAGAGGCGCTGGCGC AAAATCCCCAAACGGCCCGGGTCAGTTCACAGAACACCTAGCAGACAATGTTAA
CHRM3 Δil3	gBlock	ATGACGCTGCATAATAATTCAACCACAAGCCCTCTGTTCCCTAACATCAGTAGTAGCTG GATACACTCTCCTAGCGACGCCGGACTTCCTCCCGGAACCGTGACCCACTTTGGGTC ATATAACGTCTCTCGGGCCGCAGGCAATTTTTCTTCCCCAGATGGGACAACCGACGAT CCGCTCGGCGGCCACACTGTATGGCAGGTGGTTTTCATAGCCTTTCTGACCGGCATT CTGGCTCTGGTCACAATCATCGGCAATATTCTGGTAATTGTGTCCTTCAAGGTAAACAA ACAACTGAAGACTGTCAATAACTACTTTCTGCTTAGTTTGGCATGCGCCGATCTGATTA TCGGAGTGATAAGTATGAATCTCTTCACTACTTACATCATTATGAACAGATGGGCTCTG GGTAACTTGGCTTGCGACCTGTGGCTCGCAATTGACTATGTCGCAAGCAACGCCAGT GTGATGAACCTTCTGGTTATTAGCTTCGACCGCTATTTTAGCATTACACGCCCTCTGAC CTACAGAGCGAAACGCACTACAAAGCGGGCAGGAGTTATGATCGGCCTGGCCTGGGT CATTTCCTTCGTGCTCTGGGCACCAGCCATCTTGTTCTGGCAGTATTTCGTCGGTAAG AGGACAGTCCCACCAGGCGAGTGCTTCATCCAATTTTTGTCCGAGCCAACCATAACAT TCGGCACAGCCATCGCGGCATTTTACATGCCAGTGACTATCATGACCATTCTGTACTG GAGAATCTACAAAGAAACCGAAAAGCGGACAAAGGAGTTGGCAGGGCTTCAGGCATC AGGCACAGAGACCCGGTCACAGATAACCAAGCGCAAGCGAATGTCACTCGTGAAAGA AAAAAAAGCAGCACAGACATTGAGCGCCATCTTGCTCGCTTTTATCATAACATGGACA CCCTACAATATCATGGTCCTCGTGAACACTTTCTGCGACTCCTGTATTCCTAAGACTTT CTGGAACCTCGGCTACTGGCTGTGCTATATCAACAGTACAGTTAACCCTGTGTGCTAC GCTCTCTGTAACAAGACCTTTAGGACCACGTTCAAGATGCTCCTCCTGTGCCAGTGTG ACAAAAAGAAACGACGAAAGCAGCAATATCAGCAGCGACAGAGTGTGATCTTTCATAA ACGGGCGCCAGAACAAGCGCTCTAA

CHRM5 Δil3	gBlock	ATGGAGGGCGACTCATATCACAATGCCACGACTGTGAACGGCACACCCGTCAACCAC CAGCCACTTGAAAGGCACAGGCTTTGGGAGGTAATAACCATAGCCGCAGTGACTGCA GTAGTTAGTCTGATTACCATTGTTGGCAATGTATTGGTCATGATTTCTTTTAAGGTTAAC AGCCAGCTGAAGACCGTGAATAATTATTATCTCCTTTCCCTGGCATGCGCTGACTTGAT CATCGGGATCTTTAGCATGAACCTGTATACTACGTACATCCTGATGGGGCGCTGGGCA CTGGGTTCATTGGCATGTGACTTGTGGCTTGCGCTGGATTATGTTGCATCTAACGCCA GCGTAATGAATCTTCTCGTGATATCCTTTGACAGATACTTCTCTATTACACGGCCCCTG ACGTACAGAGCCAAAAGGACCCCCAAACGAGCAGGCATCATGATTGGACTGGCTTGG CTCATATCCTTCATACTCTGGGCCCCTGCGATCCTCTGTTGGCAGTATCTCGTGGGGA AAAGGACTGTGCCCCTGGACGAATGCCAGATCCAGTTCCTGAGTGAGCCCACAATAA CTTTTGGGACCGCCATAGCTGCTTTCTATATTCCGGTATCCGTTATGACCATCCTTTAC TGCAGGATCTACAGAGAGACAGAGAAAAGGACCAAAGATTTGGCTGATCTTCAGGGG TCTGATTCTGTGTCTACTAAAGGACTCAACCCGAATCCCAGCCATCAGATGACCAAAC GCAAGCGGGTAGTGCTGGTTAAAGAAAGGAAAGCTGCACAGACTCTGAGCGCGATTC TGCTGGCCTTTATCATTACTTGGACCCCCTATAACATCATGGTGCTCGTATCTACTTTC TGTGACAAGTGCGTCCCCGTGACCCTGTGGCACCTGGGCTACTGGCTGTGCTACGTT AATTCAACAGTGAATCCCATCTGTTATGCCCTTTGTAACCGGACCTTTCGCAAAACCTT TAAGATGCTGCTTTTGTGTAGGTGGAAGAAGAAAAAAGTCGAAGAAAAGCTTTACTGG CAGGGCAATAGCAAATTGCCATAA
X2-LP-CYC1b	PCR product	TCCATTTCTTTTTCCTCGGGCAGAGAAACTCGCAGGCAACTTGCTCTCGAAGTGGTCA CGCACACACCATAGCTTCAAAATGTTTCTACTCCTTTTTTACTCTTCCAGATTTTCTCGG ACTCCGCGCATCGCCGTACCACTTCAAAACACCCAAGCACAGCATACTAAATTTCCCC TCTTTCTTCCTCTAGGGTGTCGTTAATTACCCGTACTAAAGGTTTGGAAAAGAAAAAAG AGACCGCCTCGTTTCTTTTTCTTCGTCGAAAAAGGCAATAAAAATTTTTATCACGTTTCT TTTTCTTGAAAATTTTTTTTTTGATTTTTTTCTCTTTCGATGACCTCCCATTGATATTTAA GTTAATAAACGGTCTTCAATTTCTCAAGTTTCAGTTTCATTTTTCTTGTTCTATTACAACT TTTTTTACTTCTTGCTCATTAGAAAGAAAGCATAGCAATCTAATCTAAGTTTTAATTACAA TTGCGTAAGTGGCCCCTAGCGGGGCAGTGGTAATTAGTTATGTCACGCTTACATTCAC GCCCTCCTCCCACATCCGCTCTAACCGAAAAGGAAGGAGTTAGACAACCTGAAGTCT AGGTCCCTATTTATTTTTTTTAATAGTTATGTTAGTATTAAGAACGTTATTTATATTTCAAA TTTTTCTTTTTTTTCTGTACAAACGCGTGTACGCATGTAACATTATACTGAAAACCTTGCT TGAGAAGGTTTTGGGACGCTCGAAGGCTTTAATTTGGCATAATCGGCCCTCACAGAGG GATCCCGTTACCCATCTATGCTGAAGATTTATCATACT
fig1-mTq2	PCR product	GCTTGTCTTTGGTAGAAGAAATTATAGTAAACAAACAAACAAACAAACAAAAAAAAAAAA AAAAAAatgtctaaaggtgaagaattgtttactggtgttgttccaattttggttgaattggatggtgatgttaatggtcataa attttctgtttctggtgaaggtgaaggtgatgctacttatggtaaattgactttgaaatttatttgtactactggtaaattgccag ttccatggccaactttggttactactttgtcttggggtgttcaatgttttgctagatatccagatcatatgaaacaacatgatttt tttaaatctgctatgccagaaggttatgttcaagaaagaactatttttttttaaagatgatggtaattataaaactagagctgaa gttaaatttgaaggtgatactttggttaatagaattgaattgaaaggtattgattttaaagaagatggtaatattttgggtcata aattggaatataattatttttctgataatgtttatattactgctgataaacaaaaaaatggtattaaagctaattttaaaattaga cataatattgaagatggtggtgttcaattggctgatcattatcaacaaaatactccaattggtgatggtccagttttgttgcca gataatcattatttgtctactcaatctgctttgtctaaagatccaaatgaaaaaagagatcatatggttttgttggaatttgttac tgctgctggtattactttgggtatggatgaattgtataaataaTTTTATCCTCAAATAAACATATAAGTTTTGA GCGGATATTTCAGAATGTCAATTTTTAAAAGTAA
fus1-mTq2	PCR product	TACGACATCCTTTATCTTTTTTCCTTTAAGAGCAGGATATAAGCCATCAAGTTTCTGAAA ATCAAAatgtctaaaggtgaagaattgtttactggtgttgttccaattttggttgaattggatggtgatgttaatggtcataa attttctgtttctggtgaaggtgaaggtgatgctacttatggtaaattgactttgaaatttatttgtactactggtaaattgccag ttccatggccaactttggttactactttgtcttggggtgttcaatgttttgctagatatccagatcatatgaaacaacatgatttt tttaaatctgctatgccagaaggttatgttcaagaaagaactatttttttttaaagatgatggtaattataaaactagagctgaa gttaaatttgaaggtgatactttggttaatagaattgaattgaaaggtattgattttaaagaagatggtaatattttgggtcata aattggaatataattatttttctgataatgtttatattactgctgataaacaaaaaaatggtattaaagctaattttaaaattaga

		cataatattgaagatggtggtgttcaattggctgatcattatcaacaaaatactccaattggtgatggtccagttttgttgcca gataatcattatttgtctactcaatctgctttgtctaaagatccaaatgaaaaaagagatcatatggttttgttggaatttgttac tgctgctggtattactttgggtatggatgaattgtataaataaTGAAAATAATATTGACGTTCGCATTTAATC TATACCTATAATTCTGTACTTATATACTGTTCCTT

Table S2. Fit parameters and uncertainties for select titrations.

Figure	Receptor	Strain	Ligand 1	Ligand 2	pEC50 Ligand 1	pEC50 error	Emax (RFU)	Emax error (RFU)
4C	MTNR1A	Z	serotonin		4.2	0.038	82218	2089.0
4C	ADRA2B	I	serotonin		4.8	0.021	84795	821.3
4C	ADRA2B	O	serotonin		4.9	0.020	38858	336.9
4C	ADRA2B	Z	serotonin		5.0	0.022	181109	1500.0
4D	HCAR3	I	kynurenic acid		4.1	0.011	109926	549.0
4D	HCAR3	T	kynurenic acid		4.4	0.014	129928	500.2
4D	GPR35	12	kynurenic acid		3.0	0.017	70252	572.4
4D	GPR35	13	kynurenic acid		3.1	0.026	72374	734.7
4D	ADRA2B	Z	kynurenic acid		3.8	0.029	56911	629.6
4D	ADRA2B	Z	epinephrine		7.8	0.029	153775	1591.0
4D	ADRA2B	Z	epinephrine	kynurenic acid [1 mM]	7.0	0.034	79017	2520.0
4D	ADRA2B	Z	epinephrine	kynurenic acid [500 uM]	7.4	0.026	78185	1525.3
4D	ADRA2B	Z	epinephrine	kynurenic acid [100 uM]	8.0	0.048	88291	1542.3
4D	ADRA2B	Z	epinephrine	kynurenic acid [50 uM]	8.3	0.086	87085	1859.2
5D	HTR4	I	tryptamine		4.4	0.045	28300	684.9
5D	HTR4	T	tryptamine		4.6	0.022	45296	549.8
5D	HTR4	Z	tryptamine		5.3	0.021	73156	858.4
5D	HTR4	15	tryptamine		4.4	0.038	25452	480.5
5D	ADRA2B	I	tryptamine		5.4	0.018	95788	781.6
5D	ADRA2B	O	tryptamine		5.3	0.023	45772	505.2
5D	ADRA2B	T	tryptamine		5.2	0.033	29602	389.2
5D	ADRA2B	Z	tryptamine		5.8	0.035	181020	1489.0
5D	ADRA2A	Z	dopamine		4.3	0.020	50015	787.6
5D	ADRA2B	I	dopamine		5.6	0.014	124721	734.9
5D	ADRA2B	O	dopamine		5.3	0.019	73531	726.2
5D	ADRA2B	T	dopamine		4.9	0.017	105894	1125.0
5D	ADRA2B	Z	dopamine		6.0	0.035	169797	1469.0
6A	ADRA2B	I	phenylethanolamine		5.9	0.050	57975	719.2
6A	ADRA2B	O	phenylethanolamine		5.9	0.050	21150	230.4
6A	ADRA2B	Z	phenylethanolamine		6.2	0.086	86593	1045.0
6A	S1PR2	Z	petroselinic acid		4.9	0.004	65549	863.4
6A	S1PR2	13	petroselinic acid		4.9	0.002	52542	335.8
6A	S1PR1	I	petroselinic acid		5.0	0.007	49717	666.6

6A	S1PR1	T	petroselinic acid		5.0	0.008	48822	822.3
6A	S1PR1	Z	petroselinic acid		5.1	0.011	146456	2913.0
6A	LPAR1	I	petroselinic acid		5.1	0.028	189886	3080.0
6A	LPAR1	T	petroselinic acid		4.6	0.015	36716	729.2
6A	LPAR1	12	petroselinic acid		4.8	0.019	130642	1713.0
6B	GPR65	Q	H+	inositol [31.6 uM]	6.6	0.141	103	4.3
6B	GPR65	Q	H+	inositol [12.6 uM]	6.1	0.058	103	4.8
6B	GPR65	Q	H+	inositol [7.9 uM]	5.8	0.036	108	4.2
6B	GPR65	Q	H+	inositol [5.6 uM]	5.8	0.042	108	5.0
6B	GPR65	Q	H+	inositol [4.5 uM]	5.9	0.031	106	3.6
6B	GPR65	Q	H+	inositol [3.2 uM]	6.0	0.037	106	3.9
6B	ADORA2A	15	adenosine		5.9	0.020	40980	387.7
6B	ADORA2A	15	adenosine	inositol	6.0	0.019	47239	397.3
6B	GPR35	13	kynurenic acid		3.1	0.030	38789	474.0
6B	GPR35	13	kynurenic acid	inositol	3.3	0.048	40271	434.8
6B	ADORA2A	15	adenosine		6.1	0.018	60602	495.8
6B	ADORA2A	15	adenosine	DHEA	6.2	0.013	70241	402.7
6B	GPR35	13	kynurenic acid		3.1	0.027	69478	701.6
6B	GPR35	13	kynurenic acid	DHEA	3.6	0.052	78028	979.4
6B	HCAR3	I	kynurenic acid		4.1	0.011	67562	368.9
6B	HCAR3	I	kynurenic acid	DHEA	4.6	0.036	69634	531.4
6B	HCAR3	I	3-hydroxyoctanoic acid		4.2	0.021	90877	1159.0
6B	HCAR3	I	3-hydroxyoctanoic acid	DHEA	4.9	0.034	94439	1129.0

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