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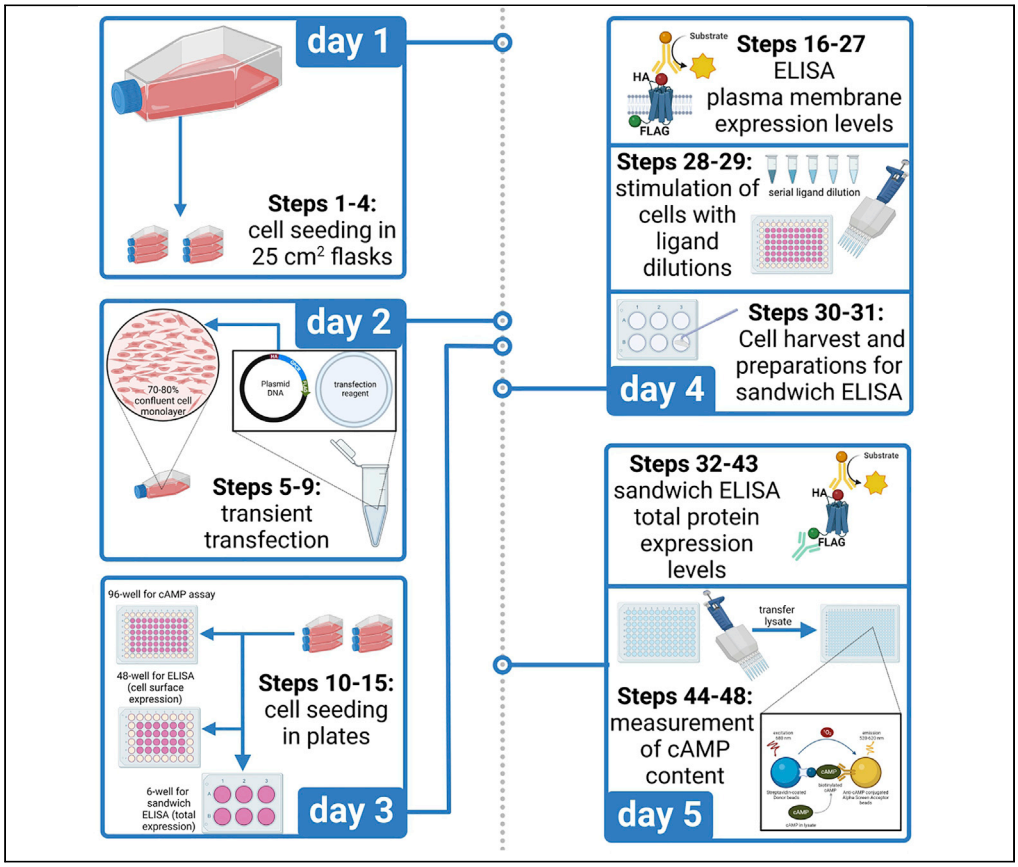
Protocol to characterize $G_{i/o}$ and G_s protein-coupled receptors in transiently transfected cells using ELISA and cAMP measurements



Activation of G_s or $G_{i/o}$ protein-coupled receptors (GPCRs) leads to changes of intracellular cyclic adenosine monophosphate (cAMP) levels. This protocol describes steps for cloning HA- and FLAG-tagged GPCRs, transient transfection of CHO-K1 or HEK293-T cells, and determination of basal and ligand-induced changes in intracellular cAMP levels. We detail enzyme-linked immunosorbent assays to determine relative GPCR plasma membrane and total expression levels.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Cloning of a GPCR with an N-terminal HA-tag and a C-terminal FLAG-tag

Determination of relative expression of a GPCR at the plasma membrane using ELISA

Determination of relative total expression levels of a GPCR using sandwich ELISA

Measurement of intracellular cAMP levels upon ligand stimulation of a GPCR

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Protocol to characterize $G_{i/o}$ and G_s protein-coupled receptors in transiently transfected cells using ELISA and cAMP measurements

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SUMMARY

Activation of G_s or $G_{i/o}$ protein-coupled receptors (GPCRs) leads to changes of intracellular cyclic adenosine monophosphate (cAMP) levels. This protocol describes steps for cloning HA- and FLAG-tagged GPCRs, transient transfection of CHO-K1 or HEK293-T cells, and determination of basal and ligand-induced changes in intracellular cAMP levels. We detail enzyme-linked immunosorbent assays to determine relative GPCR plasma membrane and total expression levels.

For complete details on the use and execution of this protocol, please refer to Schulze et al. (2022).[1]

BEFORE YOU BEGIN

This protocol uses transiently transfected CHO-K1 and HEK293-T cells and is applicable for both $G\alpha_s$- and $G\alpha_{i/o}$-coupled receptors. For determination of relative plasma membrane and total expression levels of GPCRs using ELISA, N-terminal and C-terminal tags have been introduced. This experimental setup enables the comparison of receptor mutants, orthologs or paralogs, and screening for potential agonists. Information about endogenous GPCR expression levels or GPCR trafficking cannot be inferred with this protocol. Further, N-terminal tags may affect plasma membrane expression and C-terminal tags may interfere with GPCR signaling.

The protocol may be adapted for the determination of intracellular cAMP levels upon ligand stimulation of endogenously GPCR-expressing cell lines, but cell numbers and incubation times need to be optimized. For ELISA, antibodies directly targeting the GPCR of interest may be used, although very rigorous controls are necessary to exclude the detection of unspecific antibody binding.

Cloning your GPCR of interest with N-terminal HA- and C-terminal FLAG tag

A prerequisite for the analyses of your GPCR of interest using the described ELISA approach are an N-terminal hemagglutinin (HA) epitope (YPYDVPDYA) and a C-terminal FLAG-tag (DYKDDDDK) added to the open reading frame (ORF). As a template for the described PCR-based overlapping fragment approach, the GPCR-ORF as PCR product or already cloned in a plasmid are suitable. All PCR reactions are performed using Q5 High-Fidelity DNA Polymerase. All PCR products are evaluated using agarose gel electrophoresis. Detailed protocols for basic molecular biology methods are available here: https://www.addgene.org/protocols/#basicmolecularbiology.

Design primers as depicted in Figure 1 and as follows.

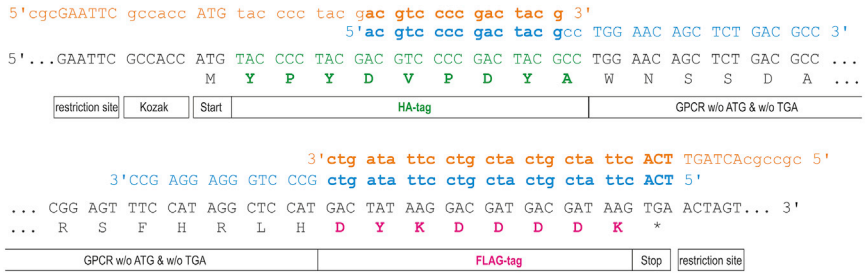

Figure 1. Primer design strategy to generate constructs with an N-terminal hemagglutinin (HA) epitope and a C-terminal FLAG-tag added to the open reading frame of your GPCR of interest

1. First PCR reaction:
   a. sense primer = HA-adapter primer (blue).

   Note: This primer has an overlap with the GPCR of interest from its second codon (i.e., excluding ATG, highlighted in blue with CAPITAL letters) and 5′ to that an overlap to the HA-tag (shown in blue with lower case letters).

   b. antisense primer = FLAG-adapter primer (blue).

   Note: This primer has an overlap to the GPCR of interest starting from the second last codon (i.e., excluding the stop, depicted in blue and CAPITAL letters) and 5′ to that an overlap to the complete FLAG-tag introducing a stop codon (highlighted in blue and bold).

   Note: Use https://tmcalculator.neb.com/ to calculate the T_m values. Each of the overlaps to the GPCR of interest has a calculated T_m of 68°C. 5 µL of the cleaned-up PCR product serves as the template for the second PCR reaction.

PCR reaction master mix

Reagent	Amount
DNA template	100 ng
Q5 High-Fidelity DNA Polymerase	0.5 µL
sense primer = HA-adapter primer (10 µM)	2.5 µL
antisense primer = FLAG-adapter primer (10 µM)	2.5 µL
5× Q5 reaction buffer	10 µL
dNTPs (2.5 mM)	4 µL
ddH_2O	to 50 µL

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	20 s	35
Annealing	68°C	30 s	
Extension	72°C	90 s	
Final extension	72°C	5 min	1
Hold	10°C	forever	

2. Second PCR reaction:
   a. sense primer = HA-universal primer (orange).

Note: This primer has an overlap with the PCR product generated from the first PCR (highlighted in orange and bold). 5′ to this area the primer introduces the rest of the HA-tag, the start-codon, the Kozak-sequence, and the site for a restriction enzyme (in Figure 1 EcoRI).

b. antisense primer = FLAG-universal primer (orange).

Note: This primer has an overlap with the PCR product generated from the first PCR (highlighted in orange and bold). 5′ to the described overlap the primer introduces the site for a restriction enzyme, in Figure 1 SpeI).

Note: Use https://tmcalculator.neb.com/ to calculate the T_m values. Each of the overlaps to the GPCR of interest has a calculated T_m of 65°C.

PCR reaction master mix

Reagent	Amount
DNA template = PCR product from first PCR	5 µL
Q5 High-Fidelity DNA Polymerase	0.5 µL
sense primer = HA-universal primer (10 µM)	2.5 µL
antisense primer = FLAG-universal primer (10 µM)	2.5 µL
5× Q5 reaction buffer	10 µL
dNTPs (2.5 mM)	4 µL
ddH_2O	to 50 µL

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	20 s	35
Annealing	65°C	30 s	
Extension	72°C	90 s	
Final extension	72°C	5 min	1
Hold	10°C	forever	

Institutional permissions

Not applicable.

Cell culture of CHO-K1 and HEK293-T cells

3. Cultivate CHO-K1 and HEK293-T cells at 37°C in a humidified 5% CO_2 incubator.
   a. Cultivate CHO-K1 cells in DMEM/ F12 and HEK293-T in DMEM.
   b. Supplememt all media with 10% fetal bovine serum and 1% Penicillin/ Streptomycin.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-HA-Peroxidase, High Affinity from rat IgG1 monoclonal (1:1000)	Sigma-Aldrich	12013819001RRID: AB_390917
Anti-FLAG M2 antibody produced in mouse (1:100)	Sigma-Aldrich	F3165RRID: AB_259529

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
GPCR open reading frames generated from genomic DNA or purchased as synthetic DNA	See references[1–8]	N/A
HA-adapter primer-S (human GPR84)	Schulze et al.[1]	acgtccccgactacgccTGGAACAGCTCTGACGCC
FLAG-adapter primer-AS (human GPR84)	Schulze et al.[1]	TCActtatcgtcatcgtccttatagtcATGGAGCCTATGGAA
HA-universal primer *EcoRI*-S	Schulze et al.[1]	cgcGAATTCgccaccATGtacccctacgacgtccccgactacg
FLAG-universal primer *SpeI*-AS	Schulze et al.[1]	cgccgcACTAGTTCActtatcgtcatcgtccttatagtc
Chemicals, peptides, and recombinant proteins		
10× HBSS	Thermo Fisher Scientific	14065056
1× D-PBS	Thermo Fisher Scientific	14190144
3-Isobutyl-1-methylxanthine (IBMX)	Sigma-Aldrich	I5879
Bovine serum albumin (BSA)	Sigma-Aldrich	A3294
Citric acid	Sigma-Aldrich	251275
Deoxynucleotide (dNTP) Solution Set	NEB	N0446
Dulbecco's Modified Eagle Medium (DMEM)	Thermo Fisher Scientific	41966029
Dulbecco's Modified Eagle Medium (DMEM)/ F-12	Thermo Fisher Scientific	21041025
Dithiothreitol (DTT)	Sigma-Aldrich	DTT-RO
UltraPure 0.5 M EDTA, pH 8	Thermo Fisher Scientific	15575020
Fetal bovine serum (FBS)	Thermo Fisher Scientific	10270106
Formaldehyde	Sigma-Aldrich	F8775
Forskolin	Sigma-Aldrich	F3917
H_2O_2	Sigma-Aldrich	1.08600
HCl	Sigma-Aldrich	258148
HEPES (1 M)	Thermo Fisher Scientific	15630056
Lipofectamine 2000	Thermo Fisher Scientific	11668019
$Na_2HPO_4 \cdot 2\ H_2O$	Sigma-Aldrich	S9763
Na_2SO_3	Sigma-Aldrich	S0505
Nonidet P40 (substitute) (NP-40)	Santa Cruz	sc-29102
o-phenylenediamine	Sigma-Aldrich	P23938
Opti-MEM	Thermo Fisher Scientific	31985070
Penicillin-Streptomycin (5.000 U/mL)	Thermo Fisher Scientific	15140122
Poly-L-lysine solution (0.1% (w/v) in H_2O)	Sigma-Aldrich	P8920
Q5 High-Fidelity DNA Polymerase	NEB	M0491
Sodium-Deoxycholate	Sigma-Aldrich	30970
Sodium tetraborate decahydrate	Sigma-Aldrich	S9640
Triton X-100	Sigma-Aldrich	T8787
Tris/HCl	Sigma-Aldrich	10812846001
Trypsin-EDTA (0.25%), phenol red	Thermo Fisher Scientific	25200056
Tween 20	Sigma-Aldrich	P1379
Critical commercial assays		
AlphaScreen cAMP assay kit	PerkinElmer Life Sciences	6760635M
Experimental models: Cell lines		
CHO-K1 (passage #3 to max. #40)	ATCC	CCL-61
HEK293-T (passage #3 to max #40)	ATCC	CRL-3216
Recombinant DNA		
N-HA-GPCR-FLAG-C-tagged receptor in pcDps	See references[1–8]	N/A
Software and algorithms		
GraphPad Prism	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
Bio-Render	Bio-Render	https://biorender.com/
Other		
Maxi-Sorp flat bottom 96-well plates	Thermo Fisher Scientific	44-2404-21
OptiPlate-384, White Opaque 384-well Microplate	PerkinElmer Life Sciences	6007290
25 cm^2 cell culture flask	Greiner Bio-One	690175
75 cm^2 cell culture flask	Greiner Bio-One	658175
6-well plate	Greiner Bio-One	657160

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
48-well plate	Greiner Bio-One	655180
96-well plate	Greiner Bio-One	677165
Multimode multilabel plate reader able to detect PerkinElmer Alpha Technology	PerkinElmer Life Sciences	EnVision 2104 multilabel plate reader with barcode #444 mirror (cat#2101-4010), barcode #244 AlphaScreen 570/ 100 filter (cat #2100-5710) equipped with laser (excitation: 680 nm) by default)
Plate reader to measure absorbance at 492 nm and 620 nm	Tecan	Tecan-Reader Infinite M Nano
Orbital shaker	Heidolph	Heidolph Titramax 1000
Shaker with 37°C incubator hood	Edmund Bühler	Edmund Bühler shaker SM30 with incubator hood TH30
Overhead shaker	Heidolph	Heidolph Reax 2
Multi-dispensing multi-channel pipettes	Gilson/Brandt/Thermo	Pipetman G Multichannel P12x200G (12 channel) Tranferpette S-12, (0.5–10 µL) Finnpipette Novus (16 channel)
Aspiration system	Biovac	Biovac 106 with 2 l glass bottle

MATERIALS AND EQUIPMENT

Versene buffer

Reagent	Final concentration	Amount
EDTA 0.5 M, pH 8	0.5 mM	0.5 mL
D-PBS 1×	N/A	500 mL
Total	**N/A**	**500 mL**

Store in aliquots at 4°C for no longer than 6 months. Do not adjust final pH. It is about 7.3.

HBSS/ HEPES assay buffer

Reagent	Final concentration	Amount
10× HBSS buffer	1×	100 mL
HEPES (1 M)	20 mM	20 mL
ddH_2O	N/A	Add to 1,000 mL
Total	**N/A**	**1,000 mL**

Adjust pH to 7.4.
Store in aliquots at −20°C to avoid repeated freeze-thaw cycles and contamination. Do not store longer than 24 months.

LI buffer

Reagent	Final concentration	Amount
BSA	N/A	500 mg
Tween 20	20 mM	1.5 mL
IBMX (500 mM)	1 mM	1 mL
HEPES (1 M)	5 mM	2.5 mL
ddH_2O	N/A	Add to 500 mL
Total	**N/A**	**500 mL**

Adjust pH to 7.4 before the addition of BSA!
Store in aliquots at −20°C to avoid repeated freeze-thaw cycles. Do not store longer than 24 months. Thaw in the fridge overnight prior to the assay.

Substrate buffer for ELISA

Reagent	Final concentration	Amount
Citric acid	22.65 mM	2.38 g
$Na_2HPO_4 \cdot 2\ H_2O$	51.4 mM	4.575 g
ddH_2O	N/A	Add to 500 mL
Total	**N/A**	**500 mL**

Store at 4°C for no longer than 6 months.

Lysis buffer for total expression		
Reagent	Final concentration	Amount
Tris/ HCl	10 mM	N/A
NaCl	150 mM	N/A
DTT	1 mM	N/A
EDTA	1 mM	N/A
Sodium-Deoxycholate	0.2 mM	N/A
NP-40	1%	5 mL
ddH_2O	N/A	Add to 500 mL
Total	**N/A**	**500 mL**
Store at 4°C. Do not store longer than two weeks with detergents added.		

PBS-T for total expression		
Reagent	Final concentration	Amount
Triton-X100	0.1% (v/v) in D-PBS 1×	N/A
Store at 4°C for no longer than 6 months.		

Coating buffer for total expression		
Reagent	Final concentration	Amount
Sodium tetraborate/ HCl	0.1 M	N/A
ddH_2O	N/A	Add to 500 mL
Total	**N/A**	**500 mL**
pH 8.0. Store at 4°C for no longer than 6 months.		

⚠ CRITICAL: pH adjustment of all liquid recipes should be made before bringing the respective buffer to its final volume.

STEP-BY-STEP METHOD DETAILS

Day 1: Cell seeding

Timing: 1 h

Harvest the cells from the 75 cm^2 cell culture flask and seed them in 25 cm^2 cell culture flasks for transfection the following day.

1. Harvest the cells from the cell culture flask.
   a. Remove the medium.
   b. Wash the cells once with sterile D-PBS.
   c. Harvest the cells by adding trypsin-EDTA to cover the surface (e.g., 1 mL per 75 cm^2 flask) and incubate at room temperature (RT, 18°C–24°C) for 2–5 min.
   d. Stop trypsinization by adding the cell culture medium supplemented with 10% FBS and 1% penicillin/ streptomycin (growth medium) to the flask, resuspend the cells and transfer the cell suspension into a 15 mL tube. Use DMEM/ F12 for CHO-K1 cells and DMEM for HEK293-T cells.
2. Count cells and check viability following the previously described protocol.[9]
3. Seed cells in 25 cm^2 flasks: 0.9 × 10^6 cells for CHO-K1 and 1.6 × 10^6 for HEK293-T, respectively.
4. Incubate in a humidified CO_2 (5%) incubator at 37°C overnight (~16 h).

Note: Avoid the use of cells that exceed 40 passages, because we observed that thereafter cells may grow faster negatively affecting the transfection efficiency and the assay outcome.

Day 2: Transient transfection

Timing: 45 min + 5 h incubation

Transiently transfect cells in 25 cm^2 cell culture flasks to ensure homogenous transfection efficiency. Always include the transfection with an empty vector as a reference. If other transfection procedures/reagents shall be used, optimize transfection by transfecting a plasmid encoding a fluorescent protein (e.g., GFP) and subsequently analyze transfection efficiency, i.e., what percentage of cells is transfected, using a fluorescent microscope.

5. Ensure that the cells are 70%–80% confluent on the day of transfection.
6. Prepare transfection mix.
   a. In the first tube, add 3 μg (CHO-K1) or 4 μg (HEK293-T) of plasmid DNA, i.e., plasmid encoding for GPCR of interest or empty vector, to a final volume of 500 μL Opti-MEM.
   b. Mix well by inverting.
   c. Do not vortex because plasmids can linearize or nick due to shear stress.
   d. In the second tube, prepare the Lipofectamine 2000 dilution.
   e. Add 10 μL Lipofectamine 2000 to 500 μL Opti-MEM per transfected 25 cm^2 flask.
   f. Mix by inverting 3–4 times and incubate at RT for 3–5 min.
   g. Add the mixture of Opti-MEM and Lipofectamine 2000 (second tube) to the first tube containing Opti-MEM and plasmid DNA.
   h. Mix by inverting 3–4 times. Incubate at RT for 20 min.

Note: Tubes containing Lipofectamine 2000 (i.e., second tube and final transfection mix) should not be vortexed. Mix by inverting only. The amount of plasmid to be transfected was optimized in our lab for GPCRs cloned in the plasmid pcDps,[10] which contains an SV40 promoter upstream of the gene of interest. We observed the optimal transfection efficiency with the least cell toxicity for the indicated amounts of cells, Lipofectamine 2000 and the amount of plasmid. Transfection reagent manufacturers provide detailed guides for the optimization of transfection. Here, we state what works in our hands.

7. Aspirate the medium from the 25 cm^2 flask cell culture flasks and add 1 mL Opti-MEM per 25 cm^2 flask.
8. Add the transfection mix (from step 6h) and incubate at 37°C with 5% CO_2 for 4–6 h.
9. After that incubation time, add 3 mL of growth medium per 25 cm^2 flask and leave the cells in a humidified CO_2 (5%) incubator at 37°C overnight (~16 h).

Day 3: Cell seeding in plates

Timing: 90 min cell harvest and seeding/ 6 h growing/ 30 min medium change

During this step, harvest the cells from the 25 cm^2 flasks and subsequently seed the cells in different plates, depending on the assay that will be performed: for cAMP measurements, seed cells in 96-well plates; for GPCR plasma membrane expression analyses, seed cells in 48-well plates and for sandwich ELISA to determine total expression levels of the receptor, seed cells in 6-well plates.

10. Check cell viability in the 25 cm^2 flask under the microscope before starting to harvest the cells.

Note: Transfection is always stressful for the cells, so potentially there will be more dead cells floating around than in the normal passaging process. However, the majority should still be

adherent. In addition, some receptors may cause cell death in the transfected cells, which can distort results.

11. For HEK293-T cells, coat plates with Poly-L-lysine (96-well plate: 50 µL/w, 48-well plate: 200 µL/w, 6-well plate: 500 µL/w), diluted 1:50 in D-PBS for at least 30 min at 37°C. Remove the Poly-L-lysine solution by aspiration before seeding the cells.
12. Harvest the cells and count them.
    a. Remove the medium.
    b. Wash once with sterile D-PBS.
    c. Add 1 mL of Versene per 25 cm^2 flask, gently move the flask back and forth until the whole area has been rinsed once.
    d. Subsequently, remove Versene immediately again before cells detach. Leave flasks for 2–5 min at RT to let cells detach.

Note: In contrast to trypsinization, harvest cells with Versene only after transfection. EDTA is sufficient to detach the cells. GPCRs expressed at the plasma membrane are not affected by Versene, while trypsin may cause digestion of the GPCR.

    e. Add respective growth medium to the 25 cm^2 flasks to harvest cells.

Note: If there are several flasks to count, it is recommended to directly count the first tube before harvesting the remaining flasks. This way, the harvesting volume can be adjusted to a countable cell number.

    f. Transfer the cell suspension to a 15 mL tube and count them using the method established in your lab.
    g. Typically, countable cell numbers are reached in a volume of around 6 mL.

Note: A detailed protocol for counting cells and assessment of cell viability has been previously published.[9]

13. Prepare a master mix for seeding the cells in growth medium. Cell numbers are listed in the following table.

	Cell line	
Plate format	CHO-K1	HEK293-T
96-well (cAMP measurement)	$2 \cdot 10^4$/w	$2.5 \cdot 10^4$/w
48-well (plasma membrane expression)	$0.8 \cdot 10^5$/w	$1.2 \cdot 10^5$/w
6-well (total expression)	$5 \cdot 10^5$/w	$8 \cdot 10^5$/w

Seed the cells in a total volume of 200 µL/w in 96-well plates, 500 µL/w in 48-well plates and 1 mL/w in 6-well plates.

Note: Prepare about 10% more cell suspension than needed, because cells are seeded with a multi-dispensing pipette.

14. Seed cells in the respective well format.
    a. Mix cell suspension again before seeding.
    b. Seed cells in triplicates for cAMP and GPCR plasma membrane expression measurements and one well for each construct for total expression level analyses.

Note: Avoid using the outer wells of the 96-well and 48-well plates but fill those wells with sterile H_2O.

c. Incubate the 96-well plates in a humidified incubator with 5% CO_2 at 37°C for approximately 6 h.

Note: After seeding, the cells should have time to attach to the plate for at least 5 h before changing the medium to serum-free, optimal are 6 h or more. We observed in our lab that the outer wells are more prone to evaporation compared to the interior wells. Cell densities in the outer wells were observed to be lower than in the interior wells which may hamper the results. A detailed analysis of this phenomenon has been previously published.[11]

d. Incubate the 6-well plates and 48-well plates in a humidified incubator with 5% CO_2 at 37°C until the following day.

Note: The medium of cells seeded for ELISA analyses is not exchanged.

15. For cAMP measurements:
    a. Remove the growth medium.
    b. Replace it with the respective serum-free cell culture medium.
    c. Starve cells overnight for 16 h at 37°C in a humidified incubator (5% CO_2).

Note: We observed that activation of $G\alpha_{i/o}$-coupled receptors is more reliably detectable when cells are serum-starved overnight. The measurement window is increased as compared to cells grown in a medium containing 10% FBS. On the other hand, serum starvation did not affect the relative expression of the GPCRs we analyzed so far. Thus, the media of cells seeded for relative expression analyses is not changed. After seeding, the cells should have time to attach to the plate for at least 5 h before changing the medium to serum-free.

Day 4: ELISA to determine N-terminal HA-tagged GPCR plasma membrane expression

Timing: 4 h

During this step, the plasma membrane expression of the overexpressed unstimulated GPCRs is determined. The protocol is adapted from Schöneberg et al.[12]

16. Carefully aspirate the medium from the cells.
17. Add 500 µL 4% formaldehyde in PBS per well and fix the cells under light orbital shaking (0.16 rcf) for 20 min at RT.
18. Wash cells twice with 500 µL PBS per well and dry by tapping the inverted plate on a paper towel.
19. Block the wells by adding 500 µL cell culture medium with 10% FBS for 1 h at 37°C under light orbital shaking (0.16 rcf).

Note: All wells of the plate should contain 500 µL medium.

20. Dilute the Anti-HA-peroxidase antibody 1:1000 to a final concentration of 50 mU/mL in cell culture medium with 10% FBS.
21. Remove the blocking medium and add 100 µL of the prepared antibody solution per well.
22. Incubate for 1 h at RT under light orbital shaking (0.16 rcf). All wells of the plate should contain 100 µL medium.
23. Wash cells three times with 500 µL PBS per well for 5 min each under moderate orbital shaking (approximately 0.3 rcf).
24. Remove all residual PBS and ensure that wells are dry.

25. Prepare the detection solutions (starting solution and stopping reagent).
    a. The starting reagent contains 10 mg o-phenylenediamine, 25 mL substrate buffer and 40 µL H_2O_2.

⚠ CRITICAL: o-phenylenediamine is highly carcinogenic. It is highly recommended to work under a flow bench from this step on.

    b. The H_2O_2 should be added just before starting the detection.
    c. The stopping reagent contains 0.12 M Na_2SO_3 diluted in 1 M HCl.
26. Add 200 µL starting solution per well and incubate at RT for 2–20 min under moderate shaking. Stop the reaction by addition of 100 µL stopping reagent per well.

Note: The time indicated in this step is only an approximate indication because it is dependent on the respective GPCR. It is highly recommended to observe the change in color by placing the plate on a white surface. The observed color should not be too dark yellow, i.e., exceed the linear range of detection, because this may skew the results, and differences in plasma membrane expression will be difficult to detect. The addition of the stopping reagent will further darken the solution. In some cases, dilution of the final solution/well or transfer of less than 200 µL may improve the results.

27. Transfer 200 µL of each well to a non-sterile 96-well plate and measure the absorbance in absorbance units (measurement wavelength: 492 nm, reference wavelength: 620 nm) in a suitable plate reader.

Note: These analyses will only yield information about relative but not absolute expression levels.

Day 4: Stimulation of cells with ligand dilutions

Timing: 3 h

In this step, stimulate the cells transiently overexpressing a $G\alpha_s$- or $G\alpha_{i/o}$-coupled GPCR to subsequently (day 5) determine the intracellular cAMP levels. Stimulate $G\alpha_s$-coupled receptors in presence of 3-isobutyl-1-methylxanthine (IBMX), a nonspecific inhibitor of phosphodiesterases, alone. Stimulate $G\alpha_{i/o}$-coupled receptors in presence of IBMX and forskolin, an unspecific activator of adenylyl cyclases.

⚠ CRITICAL: IBMX is also an antagonist for adenosine receptors, which thus may skew results for these GPCRs. Thus, the performance of the experiments in absence of IBMX may serve as a control.[13]

28. Preparation of ligand dilutions:
    a. Prepare HBSS/ HEPES with 1 mM IBMX.
    b. For $G\alpha_{i/o}$-coupled receptors: Prepare HBSS/ HEPES containing 1 mM IBMX and 2 µM forskolin.
    c. Prepare serial ligand dilutions in a deep well plate. Dilute ligands in HBSS/ HEPES + IBMX ($G\alpha_s$) or HBSS/ HEPES + IBMX + forskolin ($G\alpha_{i/o}$).

⚠ CRITICAL: In case the agonist stock is not dissolved in H_2O or buffer, a vehicle, i.e., solvent control has to be included. The concentration of solvent should be the same in all dilutions of agonist matched to the highest solvent concentration used in the assay. Ethanol or DMSO may receptor-specific affect the assay performance.

29. Stimulation of cells in 96-well plate:
    a. Remove the medium from the cells.
    b. Add 50 µL HBSS/ HEPES + IBMX per well and incubate for 5 min at RT.
    c. Discard HBSS/ HEPES + IBMX from plates.
    d. Add 50 µL of ligand dilutions in HBSS/ HEPES + IBMX ($G\alpha_s$) or HBSS/ HEPES + IBMX + forskolin ($G\alpha_{i/o}$) per well and incubate plates at 37°C for 15 min. Use a multi-channel pipette for better workflow.
    e. Place plates on ice and gently aspirate medium twice.

⚠ CRITICAL: This is important because the cells have to be absolutely dry before being lysed in the following step in an exact volume. Tilting the plates on ice facilitates the complete removal of the assay buffer.

    f. Add 50 µL of LI buffer per well and freeze plates at −20°C for at least 30 min or overnight.

Day 4: Sandwich-ELISA – Cell harvest and coating of a 96-well with anti-FLAG-antibody

Timing: 2 h

Determine the total protein expression levels of the N-terminally HA- and C-terminally FLAG-tagged receptor using a Sandwich-ELISA with Anti-HA and Anti-FLAG monoclonal antibodies.

30. Harvest cells from the 6-well plate and solubilize protein.
    a. Remove the medium from the 6-well plate and add 1 mL H_2O per well.
    b. Use a cell scraper to harvest the cells.
       Option: You can either freeze the plates at this step (−20°C) or continue with the protocol. When frozen, resuspend cells by pipetting up and down.
    c. Transfer the H_2O/cell suspension to a 2 mL tube.
    d. Centrifuge for 5 min at top speed at RT.
    e. Discard supernatant using a pipette.
    f. Gently resuspend the cell pellet in 150 µL lysis buffer using a pipette (Do not vortex!).
    g. Solubilize for 1 h at RT and then overnight at 4°C under light shaking.
31. Prepare plate for immobilization of FLAG-tagged receptor protein.
    a. Coat 96-well plate (Maxi-Sorp for antibody adsorption) with Anti-FLAG-M2 monoclonal antibody (10 µg/mL in coating buffer, which is equivalent to a 1:100 dilution).
    b. Add 100 µL/w of the 10 µg/mL Anti-FLAG-M2 monoclonal antibody solution.
       Attention: Each sample from the 6-well plate will be added to the plate in triplicates the following day!
    c. Shake for 1 h at RT and subsequently at 4°C overnight.

Day 5: Sandwich-ELISA – Measurement to determine total receptor protein expression levels

Timing: 4 h

32. Aspirate antibody dilution from 96-well Maxi-Sorp plate.
33. Wash plate 3× for 5 min with 200 µL PBS-T at RT, and tap dry between washing cycles.
34. Block with 200 µL cell culture medium containing 10% FBS for 1 h at 37°C under light orbital shaking (0.16 rcf).
35. Remove the medium from the plate and repeat step 33.
36. In the meantime, centrifuge solubilized cells (from step 30g) for 10 min at top speed (minimum 13.000 rcf) at RT.
37. Transfer supernatant (~ 130 µL) to a new tube and add 300 µL PBS-T. Discard pellet.

38. Transfer 100 μL of the diluted supernatant to a 96-well Maxi Sorp plate (after completion of step 31).
    a. Each sample is measured in triplicates, i.e., transfer 3 × 100 μL from one tube containing the solubilized protein to separate wells of the plate.
    b. The plate is then incubated for 1 h at 37°C under light shaking (0.16 rcf) to enable adsorption of FLAG-tagged receptor protein solubilsate to the immobilized Anti-FLAG antibody.
39. Remove the supernatant from the plate and repeat step 33.
40. Dilute the antibody (Anti-HA-peroxidase) 1:1000 to a final concentration of 50 mU/mL in PBS-T.
    a. Add 100 μL per well.
    b. Also, fill the surrounding wells with liquid.
    c. Incubate under light shaking (0.16 rcf) for 1 h at RT.
41. Remove the antibody solution from the plate and repeat step 33.
42. Perform steps 25–26. Use 150 μL of starting reagent and 50 μL stopping reagent per well.
43. Measure the absorbance (measurement wavelength: 492 nm, reference wavelength: 620 nm) in a suitable plate reader.

Note: These analyses will only yield information about relative but not absolute expression levels.

Day 5: Measurement of intracellular cAMP levels

Timing: 2.5 h

In this step, the cAMP content in the lysate of the ligand-stimulated GPCR-expressing cells is determined.

44. Thaw the 96-well plates from step 29f while shaking them moderately (approximately 0.3 rcf) on an orbital shaker for 30 min at RT to homogenously lyse the cells and distribute the cAMP in LI buffer.
45. Prepare the cAMP standard curve as described in the protocol provided by the manufacturer https://resources.perkinelmer.com/lab-solutions/resources/docs/MAN_AlphaScreen_cAMP_6760635DMR.pdf.

Note: It is possible to freeze the cAMP standard curve and store it at −20°C. For acceptor and donor bead solution, always calculate for 10% more points to compensate for dead volumes. Seal and spin down the 384-well plate briefly, not faster than 400 rcf otherwise the plate might break, after the addition of acceptor/donor beads.

46. Transfer 5 μL lysate from each well of the 96-well plate to a 384-well white opaque Opti-Plate (see Figure 2 for an example plate map using a 12-channel transfer pipette, 0.5–10 μL).
47. Add triplicates of each concentration of the standard curve (see Figure 2). Seal and spin down (not faster than 400 rcf otherwise the plate might break).
48. From now on, work in a dark room with only dim green light.
    a. The assay is performed as described in the manufacturer's protocol: https://resources.perkinelmer.com/lab-solutions/resources/docs/MAN_AlphaScreen_cAMP_6760635DMR.pdf.
    b. If possible, use an automatic multi-channel pipette for better workflow and reproducibility.
    c. Measure the 384-well plate using a plate reader able to detect the AlphaScreen technology (excitation 680 nm; emission 520–620 nm).

EXPECTED OUTCOMES

The described protocol will reveal information about:

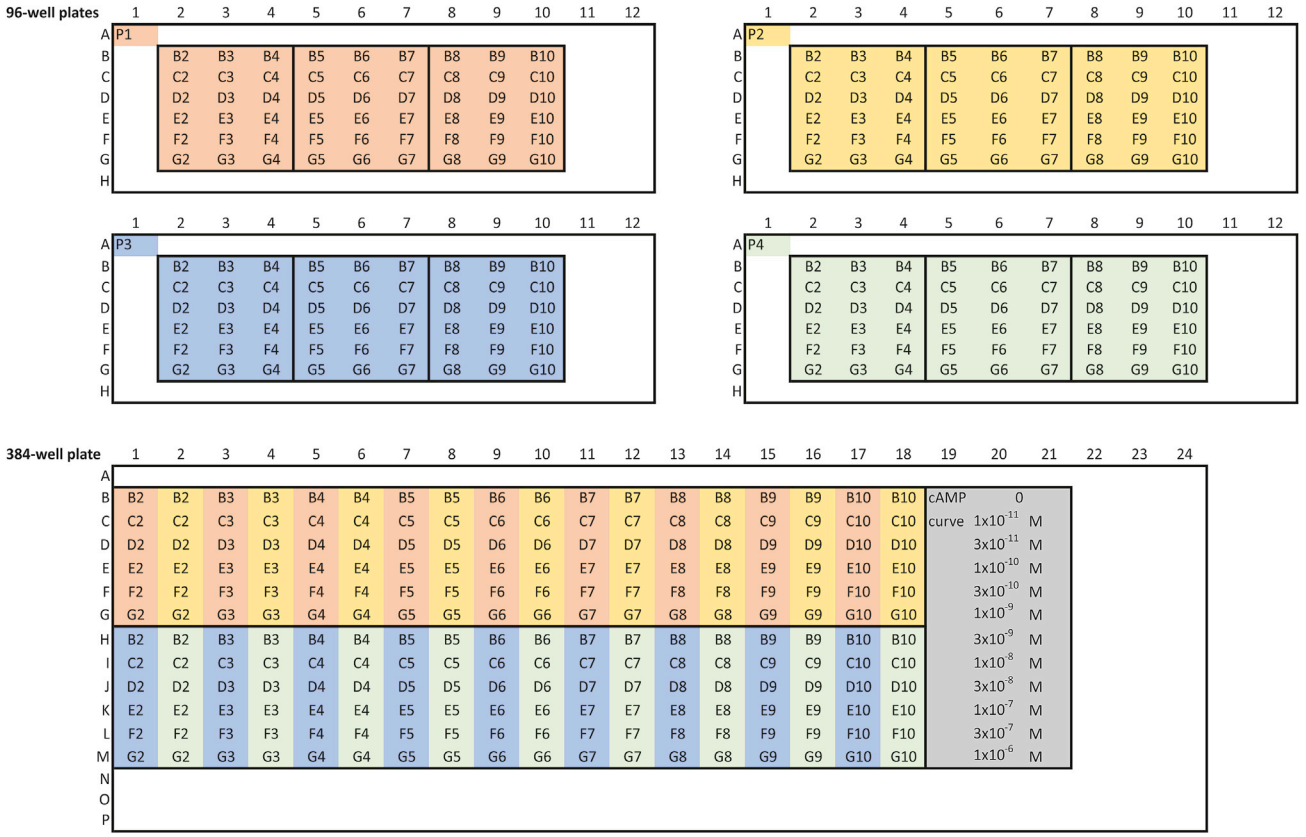

Figure 2. Example for transfer of lysate from 96-well plates to a 384-well Opti plate for cAMP measurement

The GPCR protein expression level only as a relative quantity compared to a control GPCR with known relative expression levels (sandwich-ELISA). As a control for first-time evaluation of the relative expression of a GPCR, we have not worked with so far, we include HA-GPCR-FLAG constructs encoding melanocortin receptor 4,[8] P2Y12 receptor,[14] β2-adrenergic receptor,[9] vasopressin receptor 2[15] or muscarinic M3 receptor,[16] which we have previously found well-expressed at the plasma membrane in our assay setup. For comparison of mutant GPCRs, we always include the wildtype receptor in the analyses and for comparison of GPCR orthologs, we include the human receptor in the analyses.

The GPCR protein expression level at the plasma membrane in intact cells as a relative quantity compared to a control GPCR with known expression levels (ELISA).

Potency (EC_{50}) and efficacy (E_{max}) with which GPCRs are activated by an agonist.

Basal activity, i.e., in absence of ligand, of a GPCR as compared to an empty vector control.

Previously published data following this protocol includes data for hydroxycarboxylic acid receptors and GPR84,[1–3] trace amine-associated receptors[4–6] and melanocortin receptors.[7,8]

QUANTIFICATION AND STATISTICAL ANALYSIS

Data analyses are done using GraphPad Prism. For total and surface expression, the absorbance of the empty vector has to be subtracted from the value measured for the GPCR of interest (Figure 3A). If applicable e.g., for comparison of certain receptor variants, mutants, orthologs or paralogs, it is

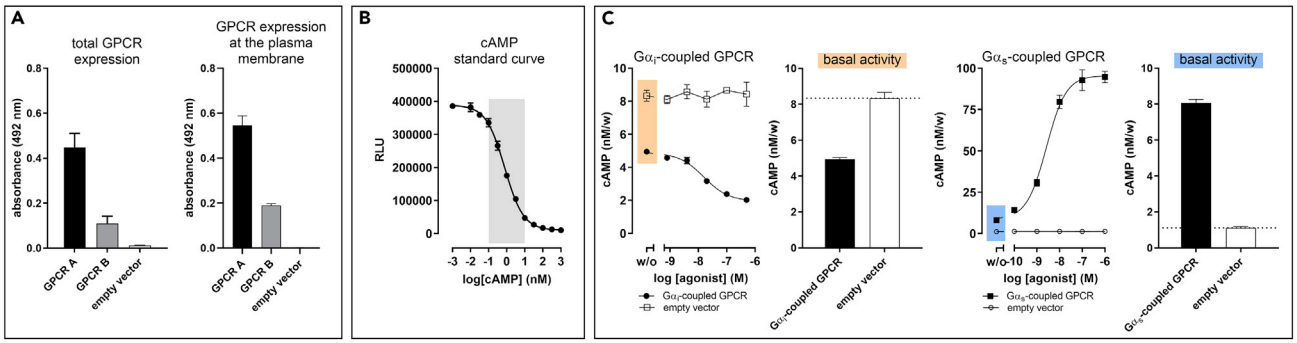

Figure 3. Example data for total and surface expression of GPCRs and cAMP-stimulation or inhibition

(A) Total and plasma membrane protein expression levels of selected GPCRs as compared to the negative control (empty vector) as absorbance at 492 nm.

(B) cAMP standard curve as measured, with the linear range highlighted in gray.

(C) Example date for a $G\alpha_{i/o}$- and $G\alpha_s$-coupled receptor as compared to a respective negative control (empty vector). Basal activity is highlighted in orange and blue, respectively.

(A–C) Data are shown as mean ± S.D. of three technical replicates, each.

possible to present data as a percentage of a reference GPCR (e.g., the wild type). Determination of the measured cAMP concentrations is performed using the interpolate unknowns from the sigmoidal curve of the cAMP standard curve: https://www.graphpad.com/guides/prism/7/curve-fitting/index.htm?reg_interpolating_from_a_standard_.htm (Figure 3B). Measured cAMP values should be in the linear range of the cAMP standard curve (Figure 3B). Subsequently, the antilogarithm of the resulting value has to be taken to yield the cAMP concentration in nM. Example data for a $G\alpha_{i/o}$- and a $G\alpha_s$-coupled receptor is depicted in Figure 3C compared to an empty vector control. In orange and blue the respective basal activity is shown.

LIMITATIONS

This assay setup is optimized for transiently overexpressed GPCRs in CHO-K1 and HEK293-T cells. However, it is possible to measure the activation of $G\alpha_{i/o}$- or $G\alpha_s$-coupled receptors at endogenous expression levels. Adjustments regarding cell numbers, incubation time, forskolin concentration (for $G\alpha_{i/o}$-coupled receptors), and volume of LI buffer need to be made. Determination of plasma membrane expression or total expression levels of endogenously expressed GPCRs is only possible if reliable antibodies targeting extra- and intracellular epitopes, respectively, are available.

TROUBLESHOOTING

Problem 1

No PCR product (before you begin: cloning your GPCR of interest with N-terminal HA- and C-terminal FLAG tag).

Potential solution

A detailed troubleshooting guide is available online here: https://international.neb.com/tools-and-resources/troubleshooting-guides/pcr-troubleshooting-guide.

Problem 2

Your cell culture is contaminated (before you begin: cell culture of CHO-K1 and HEK293-T cells; day 1: Cell seeding; day 2: Transient transfection; day 3: Cell seeding in plates).

Potential solution

A detailed troubleshooting guide is available here[17]:

Problem 3

No GPCR expression is detected (day 4: ELISA to determine N-terminal HA-tagged GPCR plasma membrane expression; day 5: Sandwich-ELISA – measurement to determine total receptor protein expression levels).

Potential solution

- The antibody does not work, maybe due to too many freeze-thaw cycles. Properly aliquot the antibody and use a new aliquot for the next experiment.
- If the GPCR that serves as a positive control gives a signal, a reason for an absent GPCR plasma membrane expression could be a cleaved signal peptide, which only 5%–10% of GPCR contain.[18] This would be accompanied by cleavage of the N-terminal HA-tag and thus result in no signal although the receptor may still be expressed at the plasma membrane.
- The N-terminal and C-terminal epitopes themselves may affect GPCR expression and trafficking.[18]
- If it is a mutant receptor, disturbed trafficking to the plasma membrane could be the cause for the absence of a signal.
- If total expression levels are low, the receptor mutant may cause disturbed folding and subsequent degradation of the GPCR.

Problem 4

The observed color exceeds the linear range of detection (day 4: ELISA to determine N-terminal HA-tagged GPCR plasma membrane expression; day 5: Sandwich-ELISA – measurement to determine total receptor protein expression levels).

Potential solution

- Dilution of the final solution/well or transfer of less than 200 μL may improve the results.
- Use shorter incubation time with detection solution next time.

Problem 5

The cAMP values fall out of the lowest or highest limit of the linear part of the cAMP standard curve (day 5: Measurement of intracellular cAMP levels).

Potential solution

- This means that cAMP values are either too low or too high, adjustments of cell number used in the assay, incubation time, amount of LI buffer added to the 96-well plate, and forskolin concentration (only for $G\alpha_{i/o}$) should be made. If the measured cAMP concentration is too high, samples may be diluted in LI buffer and measured again. The EC_{50} value of forskolin is cell line-specific. Thus, it may be necessary to determine the forskolin potency for the cell line you use.[19]
- The non-selective phosphodiesterase inhibitor IBMX may induce unspecific signals, especially on adenosine receptors, and evaluation of the assay sensitivity in the absence and presence of different concentrations of IBMX may be useful.[19]

Problem 6

No increase or decrease in cAMP is detected or there is also a ligand-dependent increase or decrease in cells transfected with an empty vector (day 5: Measurement of intracellular cAMP levels).

Potential solution

- No ligand-dependent differences in cAMP levels may be caused by hampered expression of the GPCR, which can be verified with the ELISA analyses performed in parallel. Another reason is that the tested ligands are no agonists at the receptor. Other potential ligands may be tested and concentrations, as well as incubation time of the assay reaction, may be increased.
- In addition, the properties of the ligand itself may hamper the observed results in cAMP assays. Because some GPCRs may reside intracellularly and signal from there, non-permeable ligands will not result in a change in cAMP levels.
- The ligands need to be carefully chosen and cross-validated. The specificity of the ligands to your GPCR of interest has to be ensured because off-target effects by activation or inhibition of other endogenously expressed GPCRs affect the measured cAMP levels.
- Always include a negative control (empty vector) to check for unspecific/non-receptor-mediated effects of the tested ligands.
- Signals detected in the empty vector control may be due to the activity of the tested ligands on endogenously in your chosen cell line expressed GPCRs.
- The N- terminal HA-tag as well as the C-terminal FLAG-tag may impact receptor expression, trafficking and signaling.
- Always include a positive control, i.e., a receptor that is known to couple to $G\alpha_s$ or $G\alpha_{i/o}$, which is properly expressed at the plasma membrane e.g., β2-adrenergic receptor stimulated with isoproterenol to make sure the assay itself works.
- More information about assay optimization and troubleshooting can be found online here: https://resources.perkinelmer.com/lab-solutions/resources/docs/MAN_AlphaScreen_cAMP_6760635DMR.pdf and is published here.[20]

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, PD Dr. Claudia Stäubert (Claudia.Staeubert@medizin.uni-leipzig.de).

Materials availability

This study did not generate unique reagents.

Data and code availability

This study did not generate or analyze code or data sets.

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AUTHOR CONTRIBUTIONS

Methodology, Validation, Formal Analysis, A.-D.L., P.K., C.S.; Writing – Original Draft, A.-D.L.; Writing – Review & Editing, A.-D.L., P.K., C.S.; Visualization, A.-D.L., C.S.; Supervision, C.S.; Project Administration, C.S.; Funding Acquisition, A.-D.L., C.S. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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