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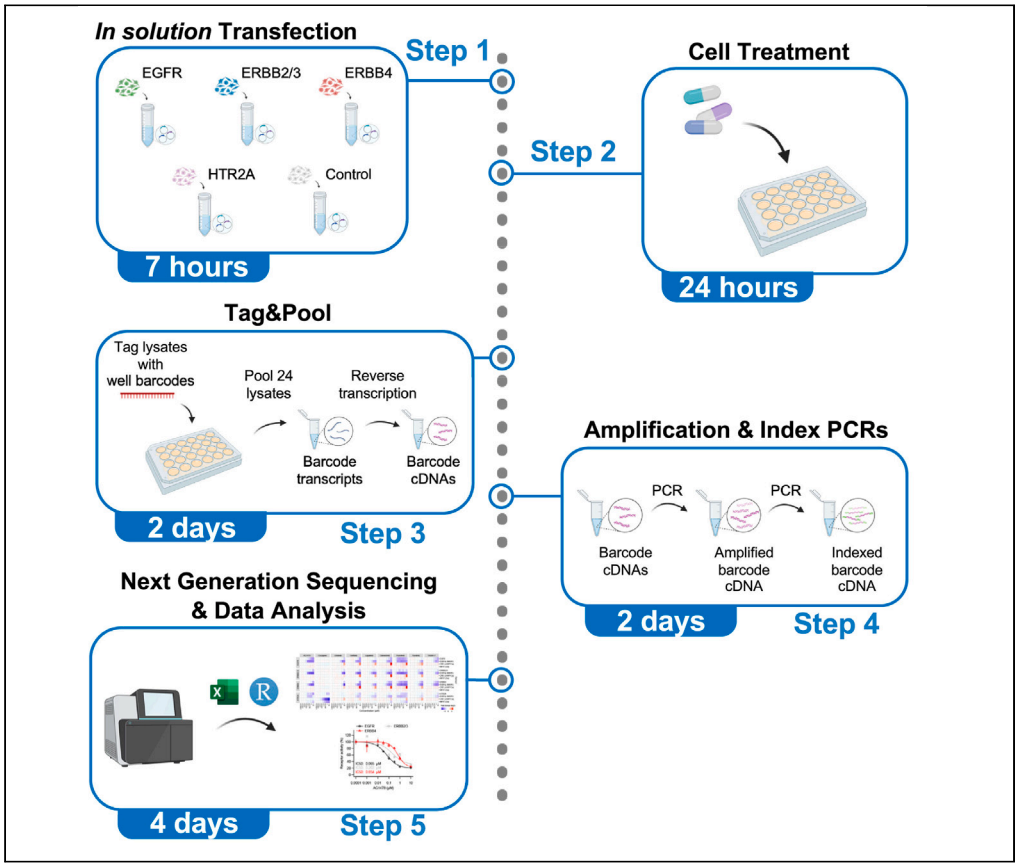
Protocol

Protocol for identifying properties of ERBB receptor antagonists using the barcoded ERBBprofiler assay



The ERBBprofiler assay measures compound effects on ERBB family receptors and key downstream signaling pathways that are implicated in cancer or other complex diseases. Here, we present a protocol for identifying properties of ERBB receptor antagonists using the barcoded ERBBprofiler assay. We describe steps for in-solution transfection, cell treatment, combined processing of samples, amplification and indexing of PCRs, sequencing, and data analysis. This approach allows for the simultaneous assessment of drug effects and cell-type-dependent effects.

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

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Highlights

Multi-level barcoded reporter assay for profiling of ERBB receptors and pathways

Monitors ERBB receptor, MAPK, cAMP, and calcium pathway activities in one well

Assesses on-target from off-target effects of ERBB receptor antagonists

Integrated analysis of cell-intrinsic effects using optional data normalization routes

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Protocol for identifying properties of ERBB receptor antagonists using the barcoded ERBBprofiler assay

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SUMMARY

The ERBBprofiler assay measures compound effects on ERBB family receptors and key downstream signaling pathways that are implicated in cancer or other complex diseases. Here, we present a protocol for identifying properties of ERBB receptor antagonists using the barcoded ERBBprofiler assay. We describe steps for in-solution transfection, cell treatment, combined processing of samples, amplification and indexing of PCRs, sequencing, and data analysis. This approach allows for the simultaneous assessment of drug effects and cell-type-dependent effects.

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

BEFORE YOU BEGIN

This protocol describes the experimental procedure for a standard ERBBprofiler assay and outlines the steps to analyze the resulting data using various normalization options. The ERBBprofiler uses the split TEV protein-protein interaction technique for monitoring the activity of ERBB receptors EGFR, ERBB2/ERBB3, and ERBB4 directly at the cell membrane and synthetic pathway reporters to simultaneously assess their downstream responses to key cellular pathways in living cells.[1] Major pathways monitored simultaneously are mitogen-activated protein kinase (MAPK) signaling, cAMP signaling, and calcium signaling (Figure 1A). The ERBBprofiler assay components are encoded on plasmids that are transiently expressed in PC12 cells (Figures 1B and 1C). All plasmids required for the ERBBprofiler assay are available through Addgene (refer to Table S1). Table S2 outlines the plasmid distribution for the ERBBprofiler experiment. Refer to the Supplemental Note for a more detailed description of the ERBBprofiler assay.

For data normalization, three routes are offered by using unique barcodes that each define cell number controls (so-called internal control barcodes that control for cell number and transfection, i.e., using barcodes from the MLPmin reporter), the barcoded readout of each ERBB receptor and downstream pathway reporters, and control cells that only contain barcoded pathway reporters and the MLPmin reporter as readouts but do not express a transfected ERBB receptor (referred to as 'receptor-free' control cells). By using these barcoded readouts, the following three normalization routes exist: (1) a route 'A' evaluating cell extrinsic effects through cell number by referencing to internal barcodes, (2) a route 'B' evaluating cell intrinsic effects by referencing to control cells, and (3) a route 'C' combining both extrinsic and intrinsic effects.

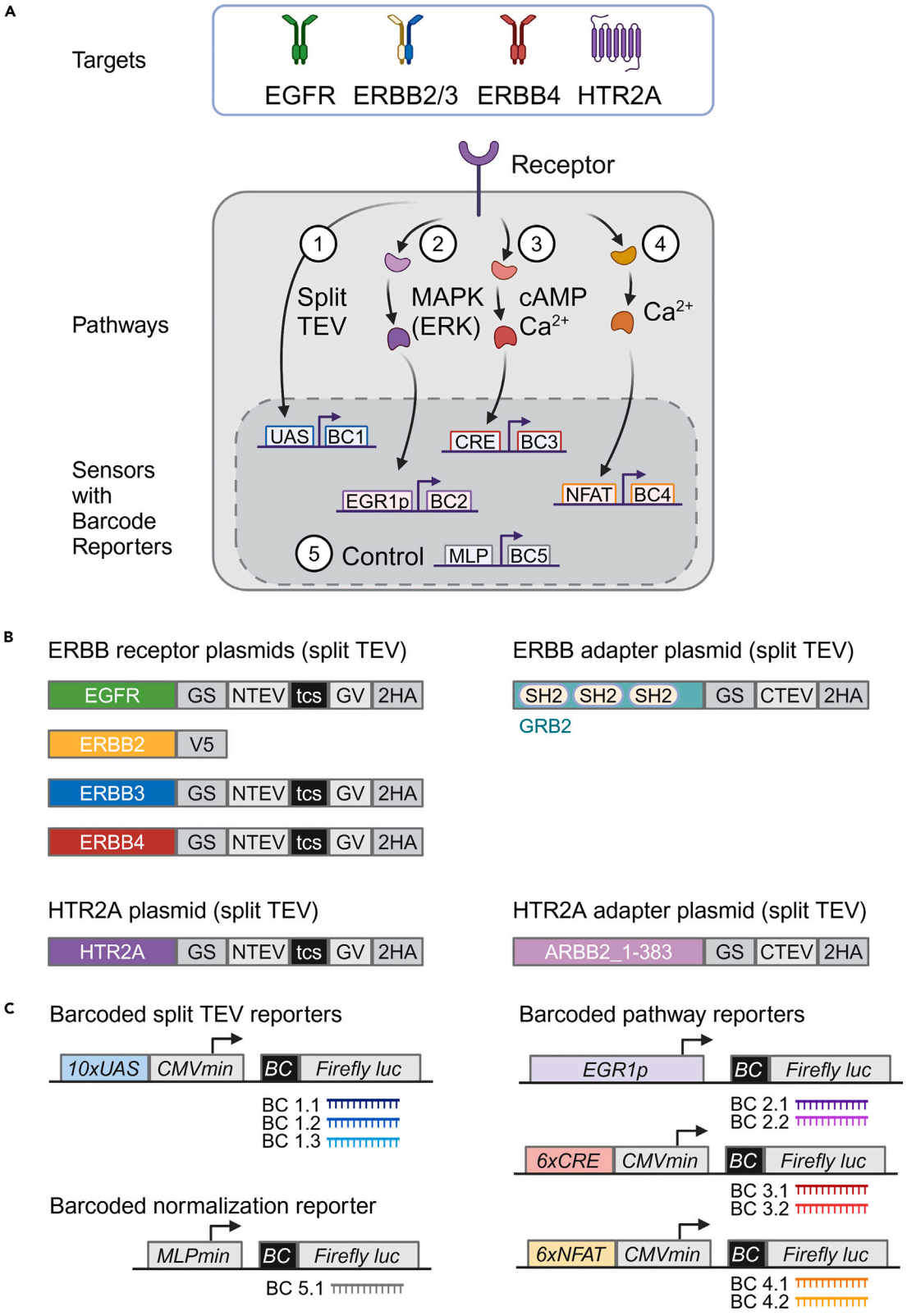

A
Targets
EGFR
ERBB2/3
ERBB4
HTR2A
Receptor
Pathways
1
Split TEV
2
MAPK (ERK)
3
cAMP Ca2+
4
Ca2+
Sensors with Barcode Reporters
UAS BC1
CRE BC3
EGR1p BC2
NFAT BC4
5 Control
MLP BC5
B
ERBB receptor plasmids (split TEV)
EGFR GS NTEV tcs GV 2HA
ERBB2 V5
ERBB3 GS NTEV tcs GV 2HA
ERBB4 GS NTEV tcs GV 2HA
ERBB adapter plasmid (split TEV)
SH2 SH2 SH2 GS CTEV 2HA
GRB2
HTR2A plasmid (split TEV)
HTR2A GS NTEV tcs GV 2HA
HTR2A adapter plasmid (split TEV)
ARBB2_1-383 GS CTEV 2HA
C
Barcoded split TEV reporters
10xUAS CMVmin BC Firefly luc
BC 1.1
BC 1.2
BC 1.3
Barcoded normalization reporter
MLPmin BC Firefly luc
BC 5.1
Barcoded pathway reporters
EGR1p BC Firefly luc
BC 2.1
BC 2.2
6xCRE CMVmin BC Firefly luc
BC 3.1
BC 3.2
6xNFAT CMVmin BC Firefly luc
BC 4.1
BC 4.2

Figure 1. Concept of the ERBBprofiler assay

(A) The activity of ERBB receptors and the serotonin receptor HTR2A are monitored by split TEV assays, while the activities of key downstream pathways are measured by synthetic barcode reporters. BC, barcode.

(B) Schematic representation of the split TEV assay plasmids for EGFR, ERBB2, ERBB3, ERBB4, HTR2A, and their adapter plasmids based on Growth Factor Receptor Bound Protein 2 (GRB2) (for ERBB receptors) and β-Arrestin-2 (ARBB2) (for HTR2A). The structure of the ERBB2 construct differs from that of the other ERBB constructs due to the analysis of ERBB2/ERBB3 heterodimers.

(C) Schematic representation of the barcoded split TEV reporter plasmids (10xUAS, 10× clustered upstream activating sequences; CMVmin, CMV minimal promoter), the barcoded pathway reporters for MAPK signaling (EGR1p, promoter of the EGR1 gene), cAMP and calcium signaling (6xCRE, 6x clustered cAMP responsive elements) linked to CMVmin, and calcium signaling (6xNFAT, 6× clustered response elements of the nuclear factor of activated T-cells) linked to CMVmin, and the barcoded normalization reporter harboring a adenovirus minimal major late promoter (MLPmin). Note that unique barcodes (BC) are expressed from each reporter plasmid. Parts of this figure were reused from Popovic et al.[1]

Note: This protocol describes normalization routes for profiling drug effects on ERBB family receptors expressed in PC12 cells. The HTR2A, a receptor of the G protein coupled receptor (GPCR) family was used as a control to assess compound selectivity amongst different target families. We selected MAPK, calcium, and cAMP signaling pathways for their significant association with ERBB receptors and HTR2A.

Design of experiment (DOE)

Timing: ~2 h

1. Consider that a run of the ERBBprofiler assay can be conducted within 10 days (Figure 2A).
2. Design the layout of the ERBBprofiler assay.
   a. Consider the number of compounds to be tested and materials and reagents (see below) required.
   b. Include positive stimulation controls by adding the stimulation mix, and possibly single stimuli, i.e., EGF, EGF-like domain (EGFld) and serotonin.
   c. Include reference ERBB antagonists, e.g., AG1478 and pyrotinib.

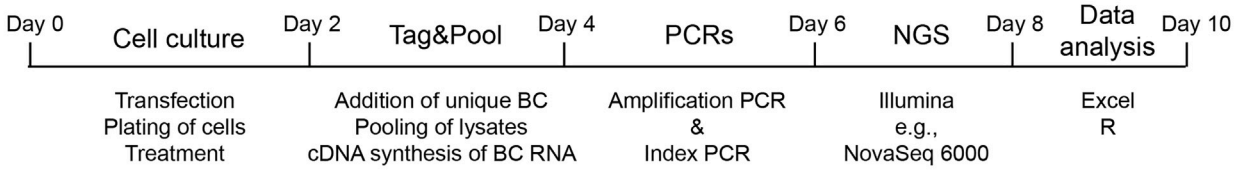
Note: At least three replicates are suggested per experimental condition. When assessing compound effects as dose-response, at least eight different concentrations at logarithmic intervals, or preferably at half-logarithmic intervals are suggested. For experiments in PC12 cells, we propose using compound concentrations for the reference compounds AG1478 and pyrotinib ranging from 0.0001 μM to 10 μM, i.e. 0.0001 μM, 0.001 μM, 0.01 μM, 0.031 μM, 0.1 μM, 0.316 μM, 1 μM, and 10 μM. Calculate amounts of needed material (i.e., plasmids and cells) and reagents (i.e., transfection reagents and supplements, media) per individual transfection (i.e., a cell batch), including 20% additional material and reagents. These numbers will depend on the total number of cell batches, cells per well, DNA per well and used wells.

⚠ CRITICAL: Keep the total amount of plasmid DNA the same across different transfection batches. This simplifies the calculation, but more importantly the handling of the transfection step, while keeping the transfection efficiency the same for each batch. If some batches lack certain plasmids, e.g., the receptor-free batch, compensate for the missing amount with a blank plasmid, e.g., pcDNA3.1.

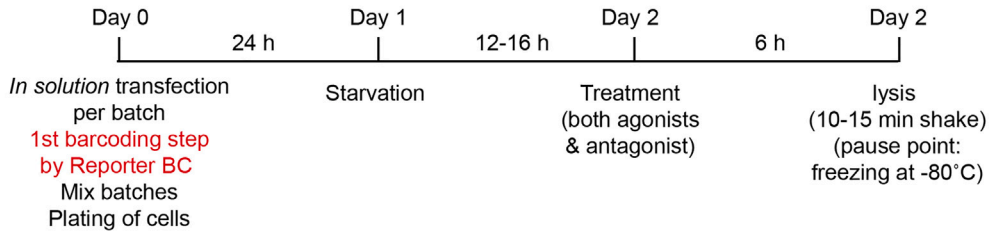
Example: The standard ERBBprofiler assay has five cell batches (EGFR, ERBB2/3, ERBB4, HTR2A, receptor-free) that are distributed in ten 24-well plates, totaling 240 samples.

   d. For each 24-well, use 250,000 PC12 cells, 33 ng of each receptor and adapter plasmid, and 27 ng of each barcode reporter plasmid (3 10xUAS plasmids, 2 6xCRE plasmids, 2 EGR1p plasmids, 2 6xNFAT plasmids, 1 MLP control plasmid), totaling to 336 ng per 24-well.
   e. Each batch requires 1,440,000 cells (240 samples x 250,000 cells/well = 60,000,000 cells; 60,000,000 cells/5 transfections = 12,000,000 cells; 12,000,000 cells x 1.2 = 14,400,000 cells).
   f. Extract the multiplication factor from the required number of cells (1,440,000 cells/250,000 cells = 57.6).

A Overall timing of the ERBBprofiler assay



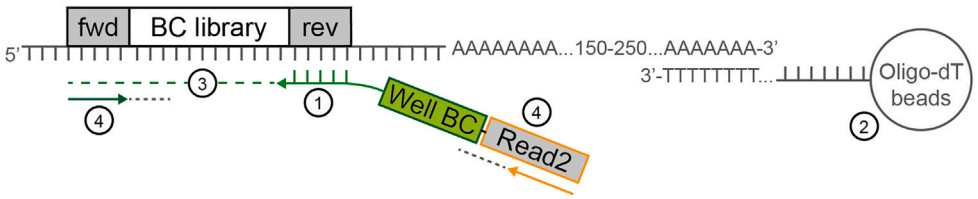
B Cell culture



C Tag&Pool

Adding unique BC to each lysate (2nd barcoding step by Well BC) → Pooling of 24 lysates → Reverse transcription (process time for two 24-well plates: ~ 3-4 h, for four 24-well plates: ~ 4-5 h)

D



E PCRs

Amplification PCR → Index PCR (3rd barcoding step by Index BC) → Purification of PCRs → Pooling of PCRs

F

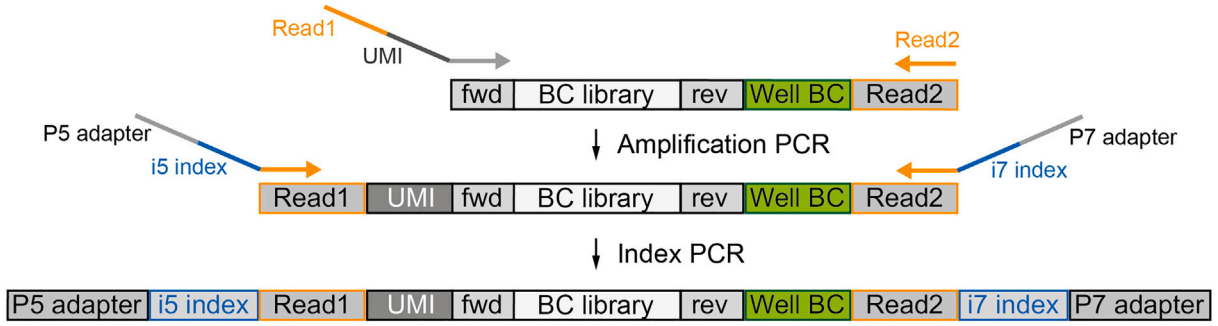


Figure 2. Experimental outline of the ERBBprofiler assay

(A) The ERBBprofiler assay can be conducted within ten days.

(B) The experimental steps of in solution transfection and the cell treatment are conducted within two days.

(C) 24 lysates are labeled with a well barcode (Well BC) and pooled within the Tag&Pool process.

(D) Unique well barcodes enable the pooling of samples at the lysate stage in the Tag&Pool process. First, barcode (BC) cassette-specific primers with a well BC (green) and an NGS adapter (gray/purple) were added into lysates of each well. This well BC primer annealed to the reverse (rev) region that was present in mRNA transcripts containing the BC library (step 1). Second, lysates were pooled and barcoded mRNAs from 24 different wells were purified with oligo-dT beads in one reaction (step 2). Third, cDNA was synthesized per pool using the well BC primer (step 3). Fourth, the first PCR (amplification PCR) was conducted using a forward primer annealing to the forward (fwd) region and the well BC primer as reverse primer (step 4).

(E) A series of an amplification PCR and an index PCR are needed to prepare a library of pooled PCRs, which is subjected to next-generation sequencing.

(F) Schematic visualization of amplification and index PCRs. UMI, unique molecular identifier. Panels B and D of this figure were reused from Popovic et al.[1]

Optional: For ease of calculation, round up the number of cells. In this case, it can be rounded up to 15,000,000 cells/transfection.

g. Multiply all plasmid DNA amounts with the multiplication factor of their respective batch (57.6 × 33 ng = 1900.8 ng for receptor and adapter plasmids respectively; 57.6 × 27 ng = 1555.2 ng for each barcode reporter plasmid; 57.6 × 336 ng = 19,353.6 ng for each transfection batch).

h. Determine volumes of Opti-MEM and Lipofectamine 3000 reagents (both Thermo Fisher Scientific) needed for individual transfection batches.

Note: We use 100 µL/well Opti-MEM per 24-well plate (57.6 × 100 µL = 5760 µL Opti-MEM; 5760 µL × 1.2 dead volume = 6912 µL Opti-MEM/transfection); and 2 µL P3000 (19,353.6 ng = 19.3536 µg; 19.3536 × 2 µL = 38.7072 µL P3000/transfection) and 3 µL Lipofectamine 3000 per 1 µg of DNA (19,353.6 ng = 19.3536 µg; 19.3536 × 3 µL = 58.0608 µL Lipofectamine 3000/transfection).

i. For ease of handling, mix P3000 and Lipofectamine 3000 of all transfection batches together.

⚠ CRITICAL: Keep in mind that in the transfection protocol, half of the Opti-MEM volume is mixed with P3000 and DNA plasmids, and the other half with Lipofectamine 3000 before mixing them together. In the example case, the calculation looked like this:

5 batches x 6912 µL Opti-MEM = 34,560 µL Opti-MEM; 34,560 µL Opti-MEM/2 = 17,280 µL Opti-MEM for P3000 + DNA and 17,280 µL Opti-MEM for Lipofectamine 3000.
5 batches x 38.7072 µL P3000 = 193.536 µL P3000; 193.536 µL P3000 × 1.2 dead volume = 232.2432 µL P3000.
5 batches x 58.0608 µL Lipofectamine 3000 = 290.304 µL Lipofectamine 3000; 290.304 µL Lipofectamine 3000 × 1.2 dead volume = 348.3648 µL P3000.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
DMEM, low glucose, pyruvate, no glutamine, no phenol red	Thermo Fisher Scientific	Cat# 11880028
Horse serum	Thermo Fisher Scientific	Cat# 16050122
Fetal bovine serum, qualified, heat inactivated, Brazil	Thermo Fisher Scientific	Cat# 10500064
GlutaMAX supplement	Thermo Fisher Scientific	Cat# 35050038
AG-1478 (Tyrphostin AG-1478)	Selleckchem	Cat# 2728; CAS: 153436-53-4
Clozapine	Sigma-Aldrich	Cat# C6305; CAS: 5786-21-0

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Erlotinib hydrochloride	Sigma-Aldrich	Cat# SML2156; CAS: 183319-69-9
Gefitinib	Sigma-Aldrich	Cat# SML1657; CAS: 184475-35-2
Lapatinib (GW-572016) Ditosylate	Selleckchem	Cat# S1028; CAS: 388082-77-7
Osimertinib (AZD9291)	Selleckchem	Cat# S7297; CAS:1421373-65-0
Poziotinib (HM781-36B)	Selleckchem	Cat# S7358; CAS: 1092364-38-9
Pyrotinib	MedChemExpress	Cat# HY-104065; CAS: 1269662-73-8
TAS6417 (Zipalertinib)	Selleckchem	Cat# S8814; CAS: 1661854-97-2
hEGF	Sigma-Aldrich	Cat# E9644; CAS: 62253-63-8
Heregulin-β1 (EGF domain) human (EGF-like domain)	Sigma-Aldrich	Cat# H7660
Serotonin hydrochloride	Tocris Bioscience	Cat# 3547; CAS: 153-98-0
Lipofectamine 3000 transfection reagent	Thermo Fisher Scientific	Cat# L3000015
Buffer RLT	QIAGEN	Cat# 79216
Poly-L-lysine hydrobromide	Sigma-Aldrich	Cat# P1274
Sodium hydroxide	Sigma-Aldrich	Cat# 367176
Tris hydrochloride solution	Sigma-Aldrich	Cat# T3038
NEBNext Q5 Hot Start HiFi PCR master mix	New England Biolabs	Cat# M0543L
Hoechst 33342 solution (20 mM)	Thermo Fisher Scientific	Cat# 62249
Critical commercial assays		
High-Capacity cDNA Reverse Transcription Kit	Thermo Fisher Scientific	Cat# 4368813
NucleoSpin Gel and PCR Clean-up, Mini kit for gel extraction and PCR clean up	Macherey-Nagel	Cat# 740609.50
NovaSeq 6000 SP Reagent Kit v1.5 (100 cycles)	Illumina	Cat# 20028401
NovaSeq 6000 SP Reagent Kit v1.5 (200 cycles)	Illumina	Cat# 20040719
Qubit dsDNA Assay Kits	Thermo Fisher Scientific	Cat# Q32851
KAPA Library Quantification Kit	Roche	Cat# KR0405
Deposited data		
Analyzed data	This paper	Mendeley Data: https://www.doi.org/10.17632/8j3rcgs7jv.1
Data of barcoded ERBBprofiler assays	This paper	Mendeley Data: https://www.doi.org/10.17632/8j3rcgs7jv.1
DRC R script	This paper	Mendeley Data: https://www.doi.org/10.17632/8j3rcgs7jv.1
Heatmap R script	This paper	Mendeley Data: https://www.doi.org/10.17632/8j3rcgs7jv.1
Experimental models: Cell lines		
Rattus norvegicus: PC12 Tet-Off cells	Clontech	Cat# 631134; RRID: CVCL_V361
Oligonucleotides		
See Table S3	This paper	Mendeley Data: https://www.doi.org/10.17632/8j3rcgs7jv.1
Recombinant DNA		
See Table S1	This paper	Mendeley Data: https://www.doi.org/10.17632/8j3rcgs7jv.1
Software and algorithms		
Adobe Illustrator CS6	Adobe, Inc.	https://adobe.com/products/illustrator
BioRender	BioRender.com (2024)	https://app.biorender.com/biorender-templates
R version 4.2.3 or higher	R Core Team (2023)	https://www.R-project.org/
RStudio2023.03.0 + 386	RStudio Team (2023)	http://www.rstudio.com/
Drc	Ritz et al.[2]	http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0146021
ggplot2	Wickham[3]	https://ggplot2.tidyverse.org
Reshape	Wickham[4]	http://www.jstatsoft.org/v21/i12/paper
Tidyr	Wickham et al.[5]	https://CRAN.R-project.org/package=tidyr
Tidyverse	Wickham et al.[6]	https://doi.org/10.21105/joss.01686
Fiji	Schindelin et al.[7]	https://imagej.net/software/fiji/downloads

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
ZEN Microscopy Software	Zeiss	https://www.zeiss.com/microscopy/en/products/software/zeiss-zen.html
Other		
DELFIA Plateshake	PerkinElmer	Cat# 1296-001
M-PVA OdT2 magnetic beads	PerkinElmer	Cat# CMG-231
HulaMixer sample mixer	Thermo Fisher Scientific	Cat# 15920D
Nunc 14 mL round-bottom tube	Thermo Fisher Scientific	Cat# 150268
NovaSeq 6000 system	Illumina	https://emea.illumina.com/systems/sequencing-platforms/novaseq.html
EasyEights EasySep magnet	STEMCELL Technologies	Cat# 18103
LifeSep magnetic separation stand for 1.5 mL tubes	Sigma-Aldrich	Cat# Z740155
NEBNext magnetic separation rack for PCR tubes	New England Biolabs	Cat# S1515S
Falcon 24-well clear flat bottom TC-treated multiwell cell culture Plate, with lid, sterile, 50/Case	Corning	Cat# 353047
Zeiss Axio Observer Z1	Zeiss	https://www.zeiss.com/microscopy/en/products/light-microscopes/widefield-microscopes/axio-observer-for-life-science-research.html

MATERIALS AND EQUIPMENT

Introduction of plasmids carrying receptors and barcodes can be performed by any transfection method. Results can be sequenced using any sequencing kit.

Tag&Pool lysis buffer			
Reagent	Stock concentration	Final concentration	Amount
Tris/HCl, pH 7.5	1 M	100 mM	50 mL
LiCl	5 M	500 mM	50 mL
EDTA	100 mM	10 mM	50 mL
DTT	-	5 mM	385.63 g
LiDS	-	1%	5 g
H_2O	-	-	~325 mL
Total	-	-	**500 mL**

The Tag&Pool lysis buffer should be aliquoted in 50 mL tubes and can be stored at −20°C for up to 1 year. We have not observed any performance decrease within 5 freeze-thaw cycles.

Wash buffer A			
Reagent	Stock concentration	Final concentration	Amount
Tris/HCl, pH 7.5	1 M	10 mM	5 mL
LiDS	-	0.1%	500 mg
LiCl	5 M	150 mM	15 mL
EDTA	100 mM	1 mM	5 mL
H_2O	-	-	~475 mL
Total	-	-	**500 mL**

The wash buffer A can be stored in 500 mL bottle at 4°C for up to 1 year.

Wash buffer B			
Reagent	Stock concentration	Final concentration	Amount
Tris/HCl, pH 7.5	1 M	10 mM	5 mL
LiCl	5 M	150 mM	15 mL
EDTA	100 mM	1 mM	5 mL
H_2O	-	-	475 mL
Total	-	-	**500 mL**

The wash buffer B can be stored in 500 mL bottle at 4°C for up to 1 year.

STEP-BY-STEP METHOD DETAILS

In solution co-transfection of ERBB receptors and respective barcodes

Timing: ~7 h

ERBB receptors together with their unique set of barcodes are transiently introduced into separate batches of PC12 cells, mixed together and plated into 24-well plates.

1. Mix your DNA plasmids in a 15 mL tube (using amounts calculated in the example of "Design of Experiment" Step 2d), so that each tube contains a single receptor with its own unique set of barcodes.

   Optional: With larger number of plasmids, perform the first step on a separate day than the rest of the protocol for a better time distribution. Tubes can be stored at 4°C for up to one week.

   Note: The experimental steps of the *in solution* transfection and the cell treatment take two days to complete (Figure 2B)

2. Collect PC12 cells in PC12 transfection medium (DMEM 1 g/L glucose, 10% horse serum, 5% fetal bovine serum, 1% GlutaMAX) to a known cell number (using cell number calculated in the example of "Design of Experiment" Step 2b).

   Note: The cell number per mL should not be too low, so that each transfection batch has about 2–3 mL.

3. Mix each DNA mix with Lipofectamine 3000 transfection mix following the manufacturer's instructions (using volumes calculated in the example of "Design of Experiment" Step 2f).
4. Add the calculated number of cells to plasmid DNA mixes and place the transfection batches into the incubator at 37°C and 5% CO_2 for 2 h.

   ⚠ CRITICAL: Tubes should be slightly open to allow for the CO_2 flow and titled at 45° angle to enlarge the contact area between cells and the transfection mixture.

5. Centrifuge cells for 5 min at 200 × *g* and remove the whole supernatant, before resuspending cells in 5 mL PC12 maintenance medium (DMEM 1 g/L glucose, 10% horse serum, 5% fetal bovine serum, 1% GlutaMAX, 1% penicillin/streptomycin).

   ⚠ CRITICAL: If any transfection mixture is left, it can lead to cross-transfection among batches when these are mixed. Slowly tilt the tube to allow the liquid to flow away from cells and aspirate it when on the edge of the tube to avoid aspirating the cells.

   Optional: Conduct an additional washing step with maintenance medium to avoid cross-transfection (c.f. step 5).

6. Mix all cell batches together in a 250 mL cell culture container.
   a. Shake lightly in circular movements to reach the homogenous mixture of the batches.
   b. Plate cells onto the 0.02 mg/mL poly-L-lysine (PLL) coated 24-well plates for 24 h.

   Note: PLL coating is necessary for PC12 cells to adhere to the plate surface. Coating is done with volume enough to cover the surface of the whole well for 30 min at 20°C–25°C. Remove PLL and wash plates twice with double distilled H_2O. After the second wash, plates can be

used directly or stored at 4°C. PLL can be re-used 2 more times (total 3 coatings) and should be stored at 4°C. If using other cell lines, plates do not have to be PLL coated.

Cell treatment

Timing: ~24 h

Cell division cycles are synchronized with serum starvation before treating them with inhibitors for 6 h and lysing them.

7. Replace the maintenance medium with the 250 µL/well starvation medium (DMEM 1 g/L glucose, 1% fetal bovine serum, 1% GlutaMAX, 1% penicillin/streptomycin) for 12–16 h.

⚠ CRITICAL: Make sure that the starvation period for PC12 cells does not exceed 16 h. We noticed that when starving PC12 cells longer than 16 h, the signal-to-noise ratio reduces significantly.

Note: Different cell lines grow at different rates. For any cell line, we recommend a minimum of 12 h starvation period.

8. Per compound to be tested, prepare 1400 µL of a 20 µM solution in PC12 starving medium containing the stimulation mix (60 ng/mL EGF, 20 ng/mL EGFld, and 2 µM serotonin) and make 3.16-fold ($10^{0.5}$-fold) serial dilutions.

⚠ CRITICAL: Compounds will be added as 2× concentrated solution to the cells.

Note: We suggest using half-logarithmic dilutions of compounds to be tested, covering five orders of magnitude typically ranging from 0.0001 µM to 10 µM. However, users may adapt this to their needs.

9. Add 250 µL of the different compound solutions to each well and incubate for 6 h.

Note: The duration of compound treatment was optimized for the usage of ERBB receptors and HTR2A in PC12 cells. If a user wishes to use the ERBBprofiler assay in other cell types, the duration of compound treatment should be assessed first to enable an optimal measurement window. Testing these conditions can be done using a live cell luminometer readout, as all barcode reporters are linked to a firefly luciferase gene.

10. Aspirate all the treatment solution and immediately lyse cells in 400 µL/well of Tag&Pool lysis buffer at 20°C–25°C and shake for 10 min at 200 rpm.

⚠ CRITICAL: Insufficient buffer volumes for cell lysis may result in viscous samples, leading to significant sample losses, increased pipetting time during the Tag&Pool step, and ultimately, unreliable results.

Alternatives: The required volume of Tag&Pool lysis buffer varies depending on the cell line, with a positive correlation between the volume and the amount of genetic material. To prevent spillage between wells when using larger volumes, it is recommended to use adhesive cover film. Shaking can be performed up to 400 rpm with the use of an adhesive cover film.

Alternatives: To isolate RNA and to proceed with a single processing of samples (i.e., without Tag&Pool), lyse cells in RLT buffer by following the supplier's instructions and then proceed to

step 18. It's important to note that the Tag&Pool step for combined processing of samples reduces the sample number by a factor of 24, which also reduces hands-on time.

Pause point: Cell lysates can be stored at −80°C up to 6 months.

Tag&Pool for combined processing of samples

Timing: 2–3 days

The first level barcodes from individual wells are tagged with the second level barcodes. All the lysates from a single 24-well plate are then pooled together.

⚠ CRITICAL: Viscous samples may increase the processing time by an additional 1–3 h, which may result in critical sample losses. To have more fluid samples, increase the amount of lysis buffer as described in the CRITICAL section of step 10. Be aware that higher volumes of lysis buffer may require the usage of adhesive cover film, as described in the first Alternatives section of step 10.

Note: As a rough guide for Tag&Pool, it takes approximately 3–4 h to process two 24-well plates (2 pools) and 4–5 h to process four 24-well plates (4 pools) (Figure 2C). The Tag&Pool process uses oligos that encode a unique well barcode each, which binds to synthetic barcoded reporters for tagging single lysates for pooling (Figure 2D). The time required will depend on how many plates are processed at a time, and the pooling factor which refers to the number of wells that are combined into a single pool. As standard practice, we are combining 24 wells into a single pool.

11. Thaw cell lysates and second level barcodes at 20°C–25°C.

Note: This will last about 45 min.

12. Add second level barcode oligos containing well barcodes (see Table S3) to the lysates at a final concentration of 0.125 μM for annealing at 65°C for 15 min.

⚠ CRITICAL: To track each well in a pool, they should have a unique second level barcode.

13. Leave samples to cool down.
    a. Put at 20°C–25°C for about 10–15 min.
    b. Place samples for 5 min on ice, before placing it back to 20°C–25°C.

⚠ CRITICAL: Cooling down the samples to 4°C (i.e., placing the samples on ice) is highly recommended to prevent crosstalk between second level barcodes when pooling. It is important to wait until the samples have completely cooled down before proceeding to the next step.

14. Pool 24 lysates from one 24-well plate together and 20 μL of M-PVA OdT2 beads in a polypropylene tube with a round bottom and rotate them on mini-rotator for about 10 min until the beads are homogenously mixed.

⚠ CRITICAL: It is recommended to use a range between 10 μL and 20 μL of M-PVA OdT2 beads per pool. Using more than 20 μL of M-PVA OdT2 beads per pool will lead to samples becoming too large and clogging in step 16. Using less than 10 μL of M-PVA OdT2 beads per pool of 24 lysates will result into sample loss and unreliable results. Therefore, we strongly suggest using a

range between 10–20 μL, as this volume of beads proved to work well for 24 lysates of a 24-well plate that are combined into a single pool.

Note: We regularly use 5 s for one spin with 3 s vibrating at 5° angle (total cycle lasting 8 s).

Note: Tubes that are reduced in width at the bottom will lead to beads being too far from the magnetic stand and will restrain washing steps, if using the same magnetic stand as ours. However, there are magnetic stands from other suppliers that might be better suited for tubes that are reduced in width at the bottom.

15. Perform 5 serial washes to reduce the amount of lithium salts.
    a. Place tubes on a magnetic tube holder for about 3–5 min, until all the beads are on the wall of the tube.
        i. Gently remove all the liquid with a pipette, before taking the tube off the tube holder.
        ii. For the 1st wash, resuspend magnetic beads in 600 μL wash buffer A and place in 1.5 mL Eppendorf tube.
    b. Place tubes on a 1.5 mL magnetic stand separator.
        i. For the 2nd wash, wash one more time with 600 μL wash buffer A.
        ii. For the 3rd and 4th wash, wash twice with 300 μL wash buffer B.
        iii. For the 5th wash, use 100 μL of 1× High-Capacity reaction buffer (Thermo Fisher Scientific) and place the sample in PCR tube on 0.2 mL magnetic rack separator.

⚠ CRITICAL: If the viscosity of the samples is too high, the beads will not adhere to the magnets, making it difficult to separate them from the liquid.

16. Remove all the High-Capacity reaction buffer and resuspend the beads in 20 μL of cDNA High-Capacity cDNA Reverse Transcription mix (Thermo Fisher Scientific).
17. Perform cDNA synthesis at 25°C for 25 min.

Pause point: The cDNA product can directly be stored at −20°C for ∼ 1 year.

Amplification and index PCRs

Timing: ∼2 days

Illumina adapter sequences and the third level barcodes (indexes) are attached to samples.

Note: We use Illumina sequencing kits. If using other sequencing kits, adapter sequences will differ.

18. Amplify cDNA using forward primer containing the Read1 Illumina adapter sequence and a unique molecular identifier (UMI) sequence in combination with a Read2 reverse primer for 30 PCR cycles (Figures 2E and 2F).

Pause point: Amplification PCRs can be stored in TE buffer at −20°C for ∼ 1 year.

19. Take out 1 μL of the amplified product and dilute 1:100 in distilled H_2O, add a unique combination of 5′ and 3′ index primers to each pool (3rd level barcoding) and run PCR for 10 cycles.

Note: The index forward primer contains Illumina P5 adapter sequence, i5 index and the beginning of the Read1 Illumina adapter sequence. The index reverse primer contains the Illumina P7 adapter sequence, i7 index and the end of the Read2 Illumina adapter sequence.

Optional: Perform agarose gel electrophoresis after each PCR run as a control point.

Pause point: Index PCRs can be stored in distilled H_2O at −20°C for ∼ 1 year.

20. Purify index PCR products following the Macherey-Nagel Mini kit according to the manufacturer's instructions (Thermo Fisher Scientific) and measure their concentration using highly efficient assays such as Qubit Assay or KAPA Library Quantification Kit.

Optional: If agarose gel electrophoresis was performed at the previous step, and it showed similar amounts of all the pools, partial combining of pools (3–4 pools into one purification column in a 1:1 ratio) could already be done at this step to ease handling. Be aware that the final combined pool should ideally consist of equimolar ratios of individual pools and represents the library that will be subjected to next generation sequencing (NGS).

Pause point: The library (PCR pools) can be stored in TE buffer at −20°C for ∼ 1 year.

Next generation sequencing

Timing: up to 48 h

Samples are combined into a barcode library. The barcode library undergoes serial dilution and is sequenced to acquire results readout.

Note: The following part of the protocol is valid only if using Illumina NovaSeq 6000 Reagent Kits. If using other sequencing kits and sequencing instruments, please follow their instructions.

21. Pool all the individual pools at equimolar ratio into one barcode library.

Note: Equimolarity of barcodes is achieved by comparing the concentrations of all (combined) pools to each other and adjusting them for equal molarity across all (combined) pools.

Example: 3 (combined) pools have concentrations of 10 ng/μL, 20 ng/μL and 30 ng/μL. Mix 1 μL of the highest concentrated (combined) pool (30 ng/30 ng/μL = 1 μL), 1.5 μL of the second highest concentrated (combined) pool (30 ng/20 ng/μL = 1.5 μL), and 3 μL of the lowest concentrated (combined) pool (30 ng/10 ng/μL = 3 μL).

⚠ CRITICAL: Make sure that all the lysates have the same number of barcodes, and all pools have the same number of lysates. If partial pool combining was performed, make sure that all combined pools have the same number of pools.

22. Measure again the concentration of the barcode library using KAPA Library Quantification Kit.

⚠ CRITICAL: You should have at least 20 μL with a concentration of 1 nM. If the volume is less than 20 μL and the concentration higher than 1 nM, dilute the library with TE buffer to 1 nM, having at least 20 μL.

23. Perform serial dilution of the library.
    a. Mix 20 μL of the library with a concentration of 1 nM with 20 μL of freshly prepared 0.2 M NaOH.
        i. Vortex 5 s, spin down for 2 s and leave for 6 min at 20°C–25°C.

Note: The concentration of the library is now 500 pM.

b. Add 20 μL of freshly prepared 0.2 M Tris-HCl pH 7.
   i. Vortex 5 s and spin down for 2 s.

Note: The concentration of the library is now 333.33 pM.

c. Add 940 μL of 4°C Hybridization Buffer (HT1, part of the NextSeq Kit).

Note: The concentration is 20 pM now.

⚠ CRITICAL: From now until loading the library onto Reagent Cartridge, keep the library on ice.

d. Mix 450 μL of 20 pM library with 550 μL of 4°C HT1 by pipetting and spin down for 2 s.

Note: The concentration is 9 pM now.

e. Mix 666 μL of 9 pM library with 334 μL of HT1.

Note: The concentration is 6 pM now.

24. Load 2 mL of HT1 buffer and 1 mL of the 6 pM library into reservoir in Reagent Cartridge.
    a. Mix by pipetting in Reagent Cartridge.

Note: The final concentration is 2 pM.

25. Perform next generation sequencing following the manufacturer's instructions (e.g., NovaSeq 6000, Illumina).

EXPECTED OUTCOMES

Cells treated with ERBB receptor family antagonists should show a reduced activity of affected barcode reporters. Likewise, the positive controls containing the stimulation mix and the single stimulations should result in a strong activation of EGFR, ERBB2/3, ERBB4, HTR2A, EGR1p, CRE, and NFAT reporters. From a sequencing run, expect to have 100,000 to 150,000 reads per well that were receiving the stimulation mix. However, the number of reads obtained from compound treated wells will heavily depend on the application of antagonists that will cause a reduction of reads. The number of reads for an activated barcode reporter can be several 1000 reads. By contrast, baseline readings for a non-stimulated barcode reporter should be above 200 reads. However, if a barcode is constantly showing less than 150–200 reads over all wells, it will produce unreliable data. When computing the IC_{50} values of compounds in dose response assays, active compounds should produce clear dose-response curves across the concentrations tested, as exemplarily shown for the reference compound AG1478 (Figure 3). Exact details on data analysis are provided in the Quantification and statistical analysis section.

QUANTIFICATION AND STATISTICAL ANALYSIS

When evaluating compound effects, analysis of ERBBprofiler assay data can either include or exclude cell intrinsic effects on MAPK, cAMP, and calcium pathway activities. It should be noted that only endogenous signaling pathways could be modulated by cell intrinsic effects, e.g., by substantial expression of an ERBB receptor such as EGFR in PC12 cells, whereas split TEV assay activities are not affected as they rely only on a synthetic pathway. To integrate this information from our ERBB antagonist data set, we developed normalization routes that are based on a batch-internal cell number control (monitored by the MLP control reporter that is present in each cell batch) and/or an additional 'receptor-free' control batch (contains no transfected ERBB receptor nor HTR2A, but pathway sensors including the MLP sensor control were transfected) that controlled for cell type dependent effects

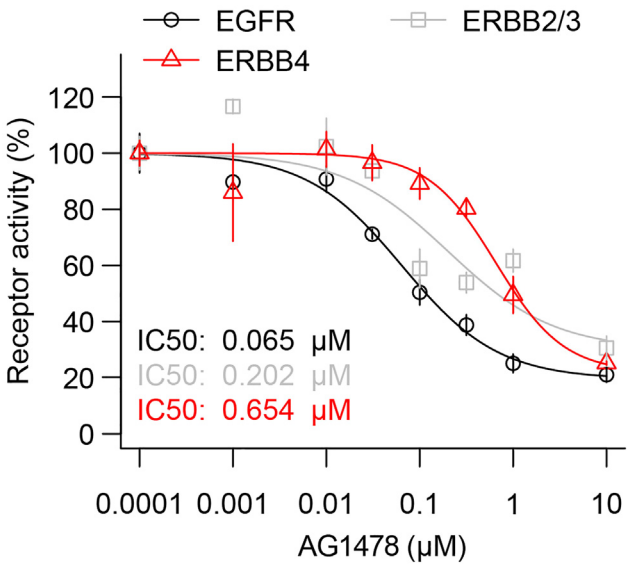


Figure 3. Exemplary dose response curves for the reference compound AG1478 on ERBB receptor inhibition

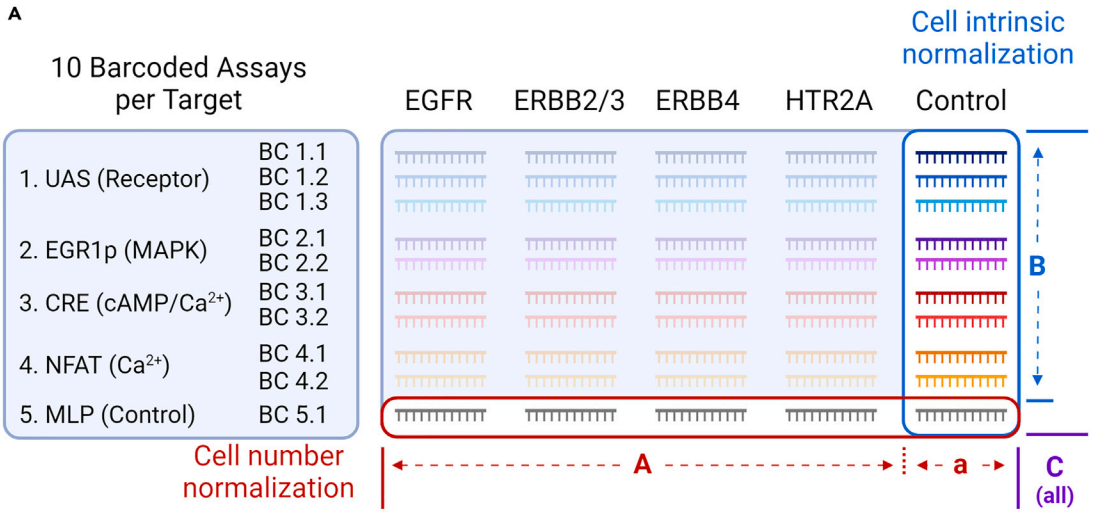
The normalized ERBBprofiler data for compound AG1478 was used to generate dose response curves for the inhibition of EGFR, ERBB2/ERBB3, and ERBB4. Error bars represent SEM, n = 3. This figure was reused from Popovic et al.[1]

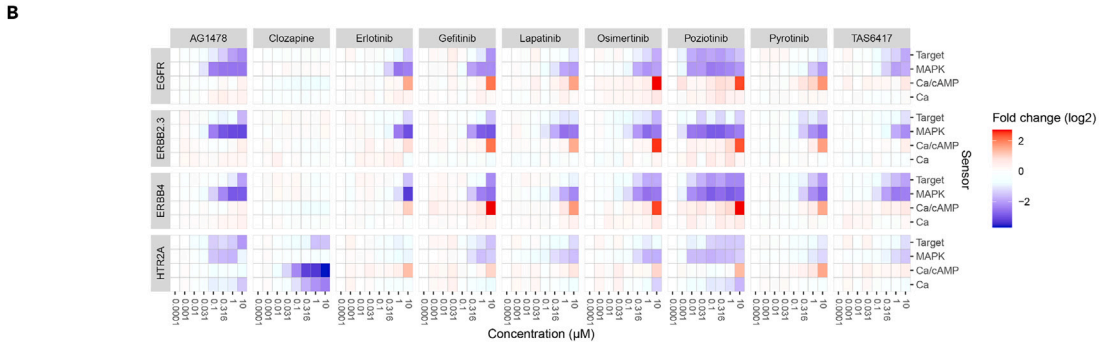
(Figure 4A). The main difference between these two control entities was that either the intrinsic cell effect of PC12 cells was included (when normalizing by cell number only using the MLP sensor) or removed when normalizing to a cell type dependent background ('receptor-free' control batch). By applying combinatorial normalization strategies with the two control entities, three different normalization routes were identified: (1) route A, a cell number normalization using the MLP sensor barcodes; (2) route B, a cell type dependent normalization for eliminating baseline effects using barcodes from receptor and pathway sensors of the 'receptor-free' control batch; and (3) route C, a normalization for eliminating cell type dependent baseline effects and additionally controlling for cell number effects using MLP sensor barcode controls. Route C combines normalization routes A and B, thus doubly normalizing to cell number effects using MLP sensor barcodes.

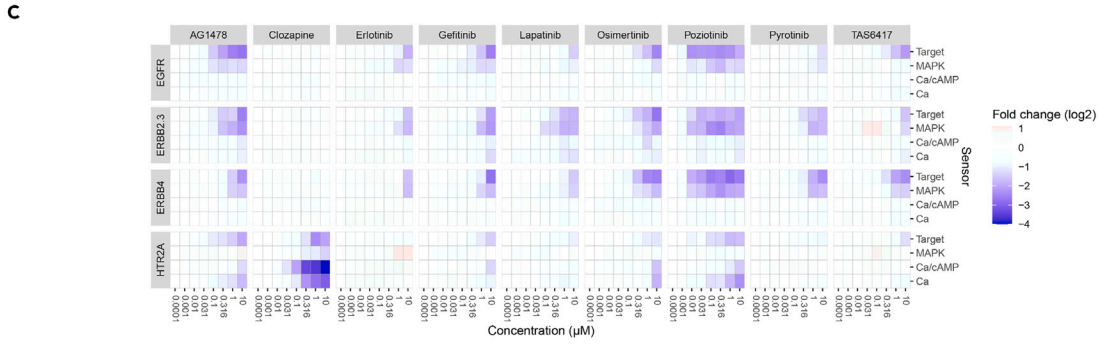
Cell number normalization

Cell number normalization route (route A; Table S4) assesses cell extrinsic effects, i.e., compound and stimulation effects. This route uses MLP sensor control barcodes in receptor cell batches to normalize for cell number, toxicity, and transfection effects, e.g., differences in expression of the transfected plasmids (Figure 4B). It can be performed by normalizing raw reads of UAS, EGR1p, CRE, and NFAT reporters to the raw reads of MLP barcode reporters of the same cell batch (see main route 'A' in Figure 4A). Alternatively, cell number normalization can be performed by normalizing raw reads of the reporters to a single MLP barcode from the control cell batch (minor route 'a'; Table S5). These two variations of route A should produce similar results, unless an overexpressed receptor does not significantly change the cell viability, thus also serving as an additional validation control of the whole assay. After the cell number normalization, technical replicates are averaged, followed by averaging biological replicates.

> ***Note:*** The advantage of normalization route A is that it retains cell intrinsic effects, and thus enables users to control the activities of cellular signaling pathways. In our recent study, this cell normalization route allowed the identification of CRE sensor activation at high doses of most ERBB antagonists and indicated stronger EGR1p sensor responses, which could otherwise be masked (c.f. normalization route A vs. normalization route B, which controls for cell intrinsic effects, see below).[1] Notably, normalization route A represents the most appropriate analysis for potential


A
10 Barcoded Assays per Target
1. UAS (Receptor)
BC 1.1
BC 1.2
BC 1.3
2. EGR1p (MAPK)
BC 2.1
BC 2.2
3. CRE (cAMP/Ca2+)
BC 3.1
BC 3.2
4. NFAT (Ca2+)
BC 4.1
BC 4.2
5. MLP (Control)
BC 5.1
EGFR
ERBB2/3
ERBB4
HTR2A
Control
Cell intrinsic normalization
B
Cell number normalization
A
a
C (all)


B
AG1478
Clozapine
Erlotinib
Gefitinib
Lapatinib
Osimertinib
Poziotinib
Pyrotinib
TAS6417
EGFR
ERBB2,3
ERBB4
HTR2A
Target
MAPK
Ca/cAMP
Ca
Sensor
Fold change (log2)
Concentration (μM)


C
AG1478
Clozapine
Erlotinib
Gefitinib
Lapatinib
Osimertinib
Poziotinib
Pyrotinib
TAS6417
EGFR
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HTR2A
Target
MAPK
Ca/cAMP
Ca
Sensor
Fold change (log2)
Concentration (μM)

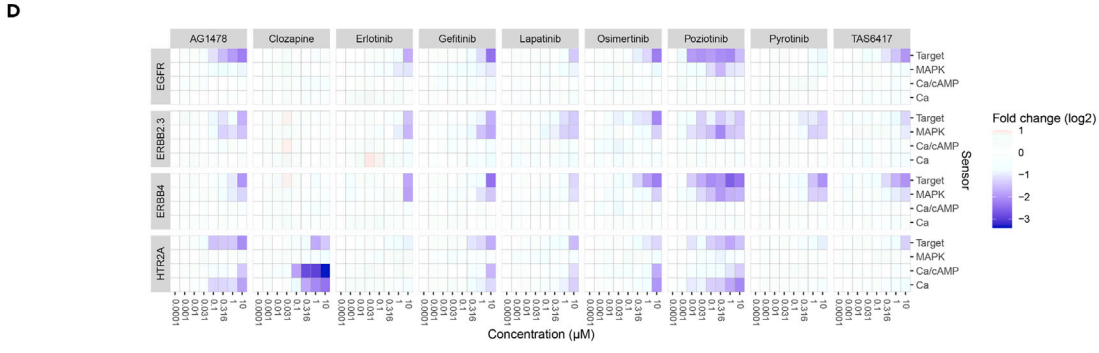
D
AG1478
Clozapine
Erlotinib
Gefitinib
Lapatinib
Osimertinib
Poziotinib
Pyrotinib
TAS6417
EGFR
ERBB2,3
ERBB4
HTR2A
Target
MAPK
Ca/cAMP
Ca
Sensor
Fold change (log2)
Concentration (μM)

Figure 4. Normalization strategies for ERBBprofiler assays

(A) Graphical visualization of the 50 barcodes used per well, with 10 barcodes used per target batch and control batch. MLP sensor barcodes are depicted in gray, receptor and pathway sensor barcodes are shown in color. Three normalization routes were used. (1) A cell number normalization using the MLP sensor barcodes of each target batch, using MLP sensor barcodes covered by route 'A'; (2) a cell type dependent normalization for eliminating baseline effects using barcodes from receptor and pathway sensors of the 'receptor-free' control batch, using receptor and pathway sensor barcodes covered by route 'B'; and (3) a normalization route 'C' combining both the cell intrinsic normalization using the MLP sensor barcodes of each target batch covered by route 'A' and eliminating cell type dependent baseline effects in each experimental condition using receptor, pathway, and MLP sensor barcodes covered by route 'B'.

(B) Heatmap using normalization route A. Note that this is the same analysis as displayed in Popovic et al., Figure 3A.

(C) Heatmap using normalization route B. (D) Heatmap using normalization route C. Panel B of this figure was reused from Popovic et al.[1]

future experiments that may be conducted in patient-derived cells to identify patient and cell type dependent pathway effects. Furthermore, normalization route A makes the control batch without receptor condition obsolete, thus reducing the number of required cells, experimental conditions, and handling during both profiling and validation experiments. Due to the mentioned reasons, this was the preferred normalization route in our recent study.

Cell intrinsic normalization

Cell intrinsic normalization uses the pathway sensors of the control cell batch to exclude cell type dependent effects. It can be based solely on the pathway sensors (route B; Table S6) (Figure 4C). This route indirectly already has the cell intrinsic normalization over the MLP barcode from the control batch, since all the changes in the control batch cell number are also reflected in pathway sensors. The cell intrinsic normalization can also be achieved (route C, Table S7) by using MLP barcode normalized counts across all cell batches to achieve maximal normalization (Figure 4D). Averaging technical and biological replicates are then performed as in the cell number normalization.

Note: In our example data set, most prominent differences between the normalization routes A vs. B and A vs. C were the absence of CRE sensor activation at high compound doses and a weaker MAPK inhibiting effect in the heatmaps based on normalization routes B and C. Both effects can be explained by the cell type dependent normalization, i.e., high CRE and EGR1p sensor activities in the primary measurement were nullified by high CRE and EGR1p sensor readings in the control batch. As PC12 cells endogenously express EGFR, experimental conditions with EGF treatment will yield activated MAPK signaling, thus leading to increased EGR1p sensor activity in both receptor and control batches. In turn, normalization routes B and C only show a reduced activity of the MAPK signaling regulated compound effects, as the endogenous MAPK signaling is already strongly affected by the compound treatment. Because normalization routes B and C yielded similar results in our previously reported assay, it confirmed the consistency of treatments across all experimental conditions, excluding substantial toxicity effects (note that cells were treated for 6 h only).

Significance of antagonists' effects is calculated as Z′ factor between the effects on the lowest and highest compound concentration, by the following equation:

$$1 - \frac{3(\sigma_H+\sigma_L)}{|\mu_H - \mu_L|}$$

Where μ is the mean and σ is the standard deviation of the highest (H) and lowest (L) concentration.

Alternatives: V factor is a variation of Z′ factor, which takes every concentration into account.

$$1 - \frac{6(\text{mean }(\sigma))}{|\mu_H - \mu_L|}$$

Where σ are the standard deviations of every concentration, and μ is the mean.

For building heatmaps, normalize data so that every compound highest concentration is put to 1, then transform all the data to log2. Highest concentration should now be 0, and activation and inhibition of the same magnitude will give the same absolute value (e.g., 2× inhibition, 50%, will be −1; 2× activation, 200%, will be 1). Generate heatmaps in R using the *ggplot2* package and script provided at 10.17632/8j3rcgs7jv.1.

The readings of the smallest concentration of each compound are set proportionally to the maximum of 100%. Generate dose response curves in R using the *drc* package and the script provided at 10.17632/8j3rcgs7jv.1. Relative IC_{50} values are automatically calculated by the script. If the script assumes that the curve goes beyond 100% and 0%, top and bottom values can be manually capped in LL.4 (fixed = c(hill slope, bottom plateau, top plateau, EC/IC_{50} value).

⚠ CRITICAL: Try to avoid manually changing any of the parameters in the LL.4 model, especially hill slope and EC/IC_{50} values.

Note: The script will always fit a curve and generate EC/IC_{50} values, even if there is no real effect. If data points are equally scattered within 100% +/− 15%, use model LL.3.

LIMITATIONS

The ERBBprofiler assay enables monitoring the activities of EGFR, ERBB2/3, ERBB4, and their key downstream pathways in living cells. ERBB2 and ERBB3 homodimers remain potential additions to an extended ERBBprofiler assay. Pathway sensors were incorporated to monitor three central downstream pathways of ERBB receptors: cAMP signaling, Ca^{2+} signaling, and the ERK branch of MAPK signaling. Notably, we omitted other pathways regulated by ERBB receptors, such as AKT signaling. Future adaptations may explore the inclusion of pathway sensors for AKT signaling and other relevant pathways.

The complexity of the ERBBprofiler assay depends on the quantity of barcoded assays per well. For this implementation, the ERBBprofiler assay comprised 50 distinct assays per well, conducted in 24-well formats. Examination of the assay's robustness in smaller well formats, like 48-well and 96-well configurations for high-throughput screening, remains unexplored. Thus, the present form of the ERBBprofiler assay is restricted to a medium throughput format utilizing 24-well plates. Further extension of complexity per well, by introducing more assays for receptors and pathways, could potentially impact the assay's performance in the 24-well format. Introducing additional sensors per well may necessitate a greater number of cells per well for the effective sequencing of barcoded reporters.

Finally, assays employing transient transfections exhibit diminished robustness and reproducibility compared to those with stably integrated assay components. Consequently, future developments that will concentrate on the establishment of cell lines with stably integrated receptors, may substantially improve the robustness and reproducibility of the ERBBprofiler assay.

TROUBLESHOOTING

Problem 1

Some barcodes consistently produce far fewer reads than others. For example, two barcodes are driven by the same sensor, i.e., an oligomerized transcription factor binding site element (10xUAS, 6xCRE, 6xNFAT) or EGR1p, and one of the two barcode reporters produces 10 times fewer reads than the other. The problem arises due to the low quality of plasmid DNA (related to before you begin section).

Potential solution

It is recommended to control the quality of the plasmid DNA. Use analytical digests on agarose gels and determine the concentration as well as the purity of the plasmids by determining the 260/280

ratio. As all plasmids in this assay are transfected, the user should make sure to use high quality and optimally purified plasmid DNA only. The concentration of the plasmid DNA should be measured before each experiment. Avoid freeze-thaw cycles of plasmid DNA, i.e., by storing each plasmid in multiple aliquots.

Problem 2

Cross-contamination during the transfection (related to Step 5).

Potential solution

Cross-contamination is caused by having some transfection mixture before mixing all cell batches together. Include an extra washing step to completely remove all the transfection mixture before mixing cell batches.

Problem 3

Viscous lysates after cell lysis (related to Step 10).

Potential solution

Add more lysis buffer to make the samples more fluid.

Problem 4

Most of the barcode reads correspond to certain experimental conditions. The problem often arises due to the high stimuli concentration (related to Step 8).

Potential solution

If a compound is highly responsive leading to the strong activation of a given reporter, it may result in consuming large number of reads, which could skew the result of the other conditions and potentially the measurement of further activations of a given receptor or a certain pathway. In such cases, lower stimuli concentrations should be used, or less receptor-expressing plasmid should be transfected.

Problem 5

No or very low barcode reads. The problem arises due to the insufficient transfection efficiency (related to in solution co-transfection of ERBB receptors and respective barcodes section).

Potential solution

Transfect an extra cell batch transfected with a plasmid harboring a fluorescent protein, such as EGFP, and plate it on a separate plate. EGFP transfection can be quantified using fluorescent microscopy. Stain the nuclei of all cells using Hoechst 33342. Count the total number of cells in the blue fluorescent channel and the number of green cells from the green channel. Specifically, at least 3 images from 3 different random locations within a single well should be taken. The imaging should be done from at least 3 different wells (at least 9 images in total). Calculate the transfection efficiency by dividing the number of green cells by the number of total cells. Alternatively, the transfection efficiency can be determined using a fluorescence plate reader. Do not proceed with the experiment if the transfection efficiency is lower than 30%. Ensure that cells are not clumping during the transfection step, as this can decrease transfection efficiency. This issue may also occur if the transfection reagents are getting too old.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Michael Wehr (michael.wehr@med.uni-muenchen.de).

Technical contact
Questions about the technical specifics of performing the protocol should be directed to and will be answered by the technical contact, Michael Wehr (michael.wehr@med.uni-muenchen.de).

Materials availability
Plasmids used for the ERBBprofiler assay are available from Addgene.

Data and code availability
Data used for multiparametric assays and validation assays are available at Mendeley Data (https://doi.org/10.17632/8j3rcgs7jv.1).

All original code has been deposited at Mendeley Data and is publicly available as of the date of publication. DOIs are listed in the key resources table.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

The Supplemental Note is available at Mendeley Data (https://doi.org/10.17632/8j3rcgs7jv.1).

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.102987.

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AUTHOR CONTRIBUTIONS
Conceptualization, L.P. and M.C.W.; data curation, L.P. and M.C.W.; formal analysis, L.P.; methodology, L.P. and M.C.W.; investigation, L.P. and M.C.W.; visualization, L.P. and M.C.W.; resources, M.J.R. and M.C.W.; supervision, M.C.W.; writing – original draft, L.P. and M.C.W.; writing – review and editing, all authors.

DECLARATION OF INTERESTS
M.J.R. and M.C.W. are employees and shareholders of Systasy Bioscience GmbH.

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