

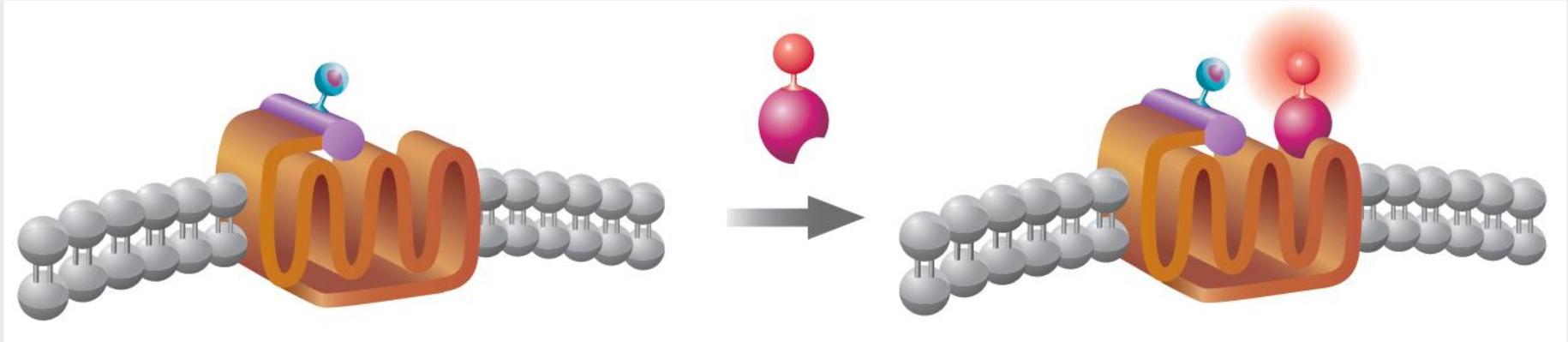
Evaluation of a Tag-lite binding assay for a class B receptor

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25th April 2013

5th Symposium HTRF in Drug Discovery

- **Introduction**
- Assay development
- Screening of cluster pool compounds
- Characterization of hits
- Summary
- Acknowledgements



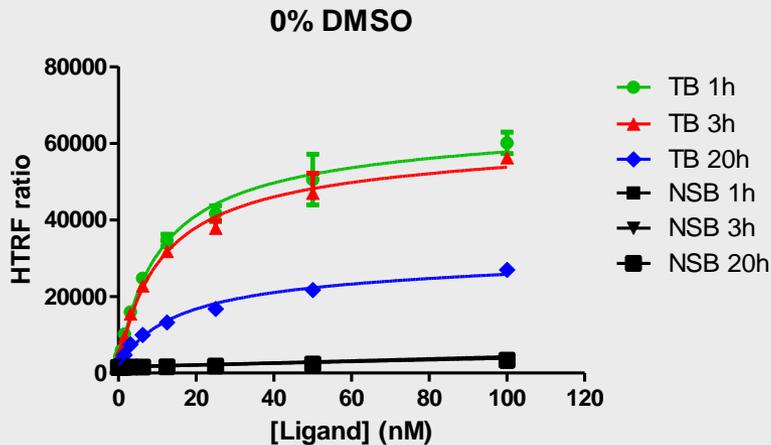
- FRET (HTRF signal) between terbium cryptate covalently attached to a SNAP-tag fused GPCR and a high-affinity red fluorescent labeled GPCR ligand

- Tag-lite binding assays for GPCRs seem to be a good alternative to radio-ligand binding assay
 - Binding assays can be useful for:
 - Determination of K_d
 - Determination of k_{off} - Target coverage
 - Allosteric modulator: Info on binding and efficacy
 - Orthogonal screening: Alternative assay format to confirm activity of hits identified in HTS
- Conduct in house validation of Tag-lite assays for two closely related GPCRs (with peptides as endogenous ligands)
- In house HTS (functional assay) to identify antagonists for one of the targets (GPCR1) several years ago
 - Some internal compounds are available

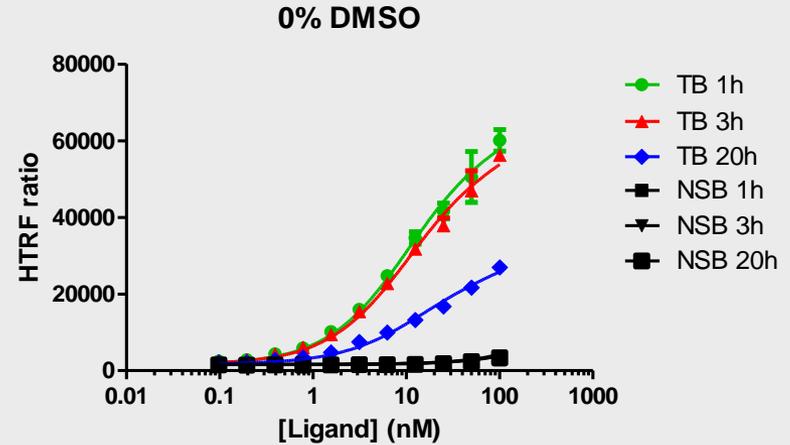
- How do $K_d/K_i/IC_{50}$ values for a variety of ligands compare with data reported in literature or generated in house?
- How robust are the Tag-lite assays and are they suitable for HTS/MTS:
 - DMSO tolerance
 - Signal stability over time
 - Stability of cells
 - Impact of incubation time on assay performance
- How do the Tag-lite assays perform in a semi-automatic screen of cluster pool compounds?

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GPCR1: K_d value determination for labeled ligand



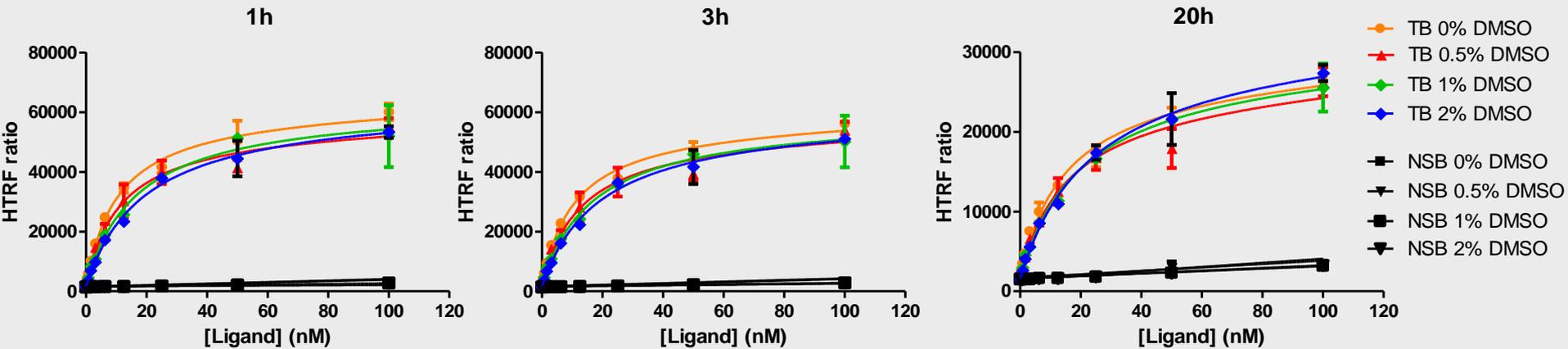
	K_d (nM)
1 h	10.34
3 h	10.53
20 h	14.38



TB = Total binding
NSB = Non specific binding

- Drop in signal over time probably due to possible receptor internalisation (ligand is an agonist?)
- Higher signals with laser excitation than with flashlamp (EnVision from PerkinElmer) but K_d value not affected
- Low unspecific binding

GPCR1: K_d value determination of ligand at different DMSO concentrations

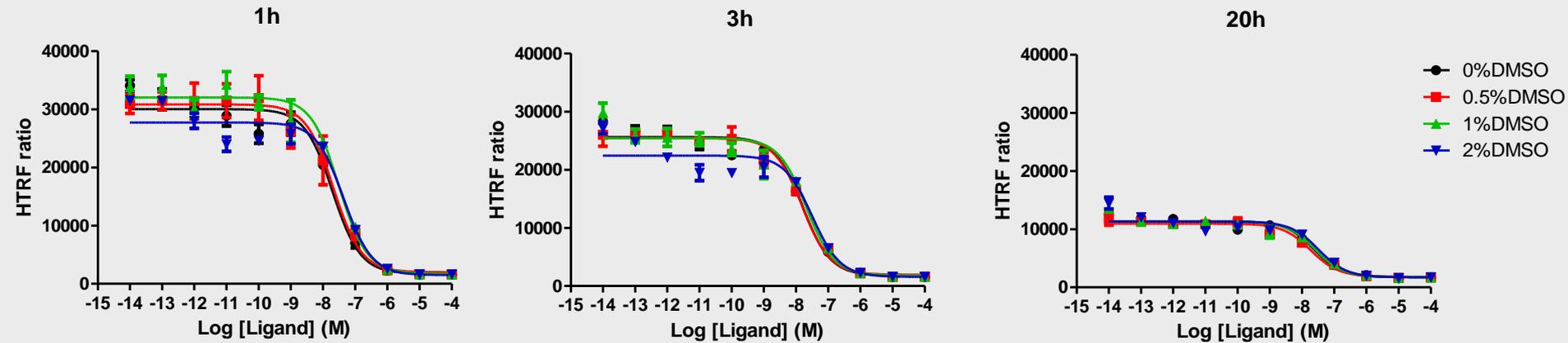


	K_d (nM) - 1h	K_d (nM) - 3h	K_d (nM) - 20h
0% DMSO	10.34	10.53	14.38
0.5% DMSO	10.94	12.26	14.65
1.0% DMSO	16.58	16.52	19.45
2.0% DMSO	19.10	18.99	23.32

TB = Total binding
NSB = Non specific binding

- Assay is robust – however higher K_d values at higher DMSO concentrations (1&2%)
- Low unspecific binding
- Signal decrease over incubation time

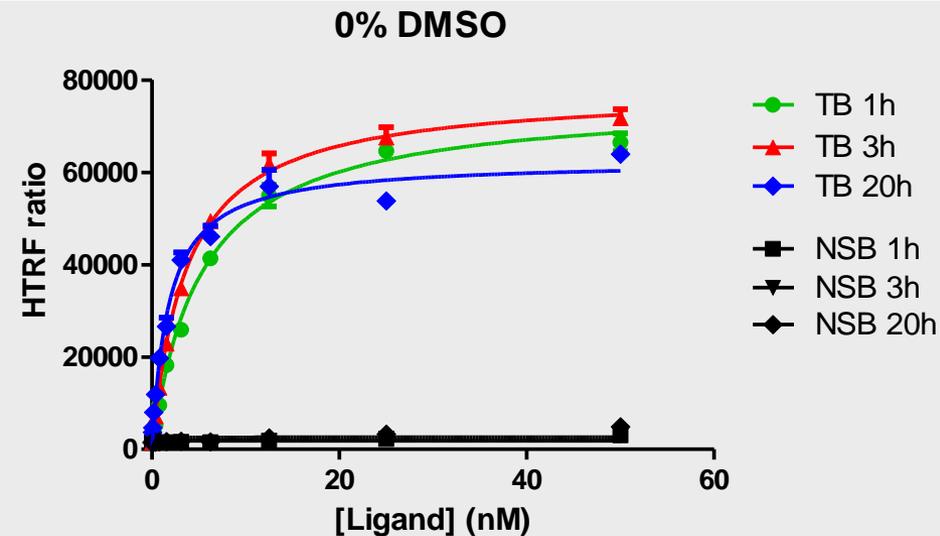
GPCR1: K_i value determination of endogenous ligand – DMSO sensitivity & signal stability over time



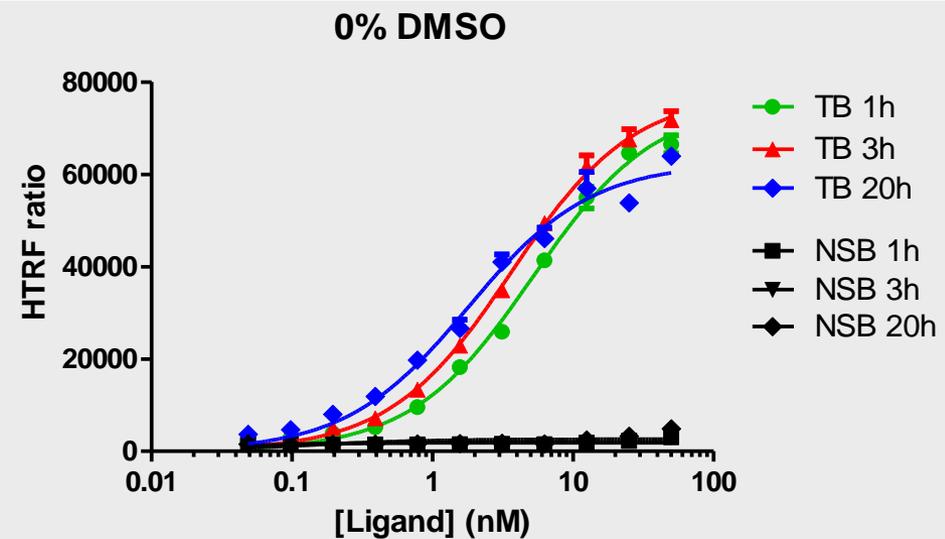
	K_i (nM) - 1h	K_i (nM) - 3h	K_i (nM) - 20h
0% DMSO	9.89	7.89	11.64
0.5% DMSO	10.65	7.83	9.36
1.0% DMSO	15.52	10.34	12.74
2.0% DMSO	22.20	16.01	16.27

- K_i values determined in line with published data in literature for ligand
- K_i values increase at higher DMSO concentrations (1&2% DMSO)
- Signal decrease over time

GPCR2: K_d value determination for labeled ligand



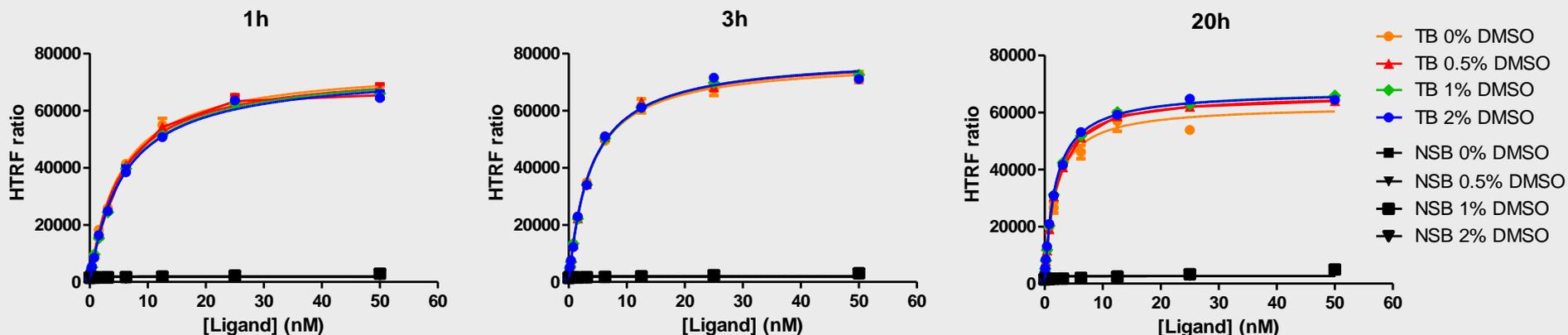
	K_d (nM)
1 h	5.21
3 h	3.65
20 h	1.80



TB = Total binding
NSB = Non specific binding

- No signal decrease over time - in contrast to GPCR1
- Low unspecific binding - like GPCR1

GPCR2: K_d value determination- DMSO sensitivity

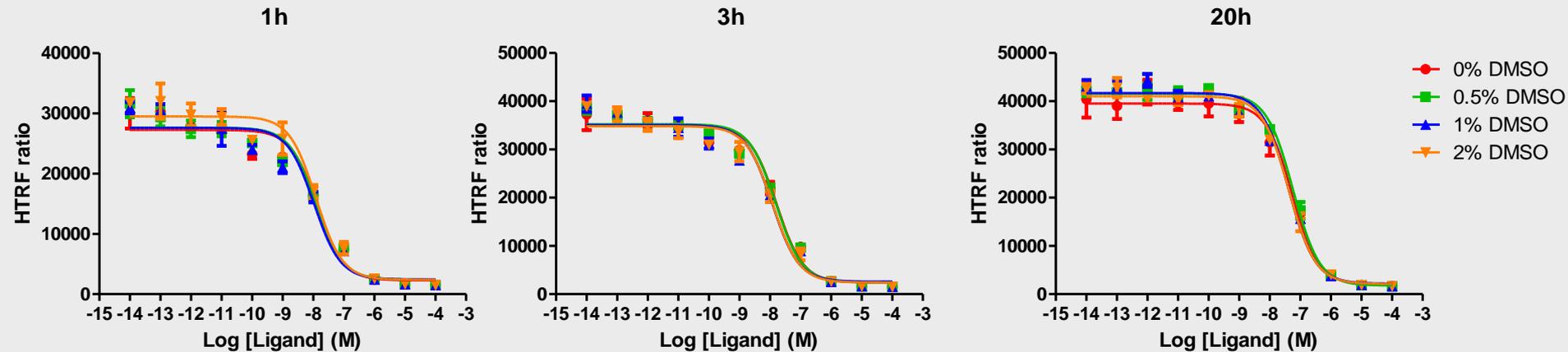


	K_d (nM) - 1h	K_d (nM) - 3h	K_d (nM) - 20h
0% DMSO	5.21	3.65	1.80
0.5% DMSO	5.30	3.75	1.85
1.0% DMSO	5.81	3.88	1.82
2.0% DMSO	5.72	3.80	1.77

TB = Total binding
NSB = Non specific binding

- Assay is robust up to 2% DMSO without increase of the K_d value
- Signal is more or less stable over 20h
- Low unspecific binding

GPCR2: K_i value determination of endogenous ligand – DMSO sensitivity & signal stability over time

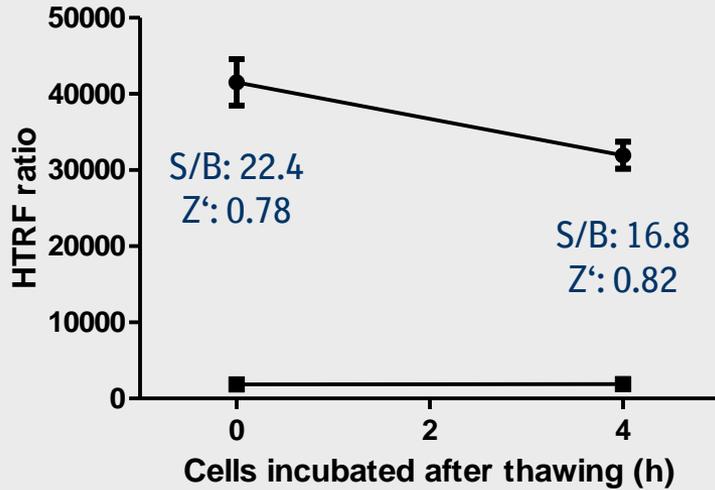


	K_i (nM) - 1h	K_i (nM) - 3h	K_i (nM) - 20h
0% DMSO	7.81	8.00	17.16
0.5% DMSO	7.17	7.65	17.93
1.0% DMSO	6.28	5.78	12.75
2.0% DMSO	7.27	6.03	13.09

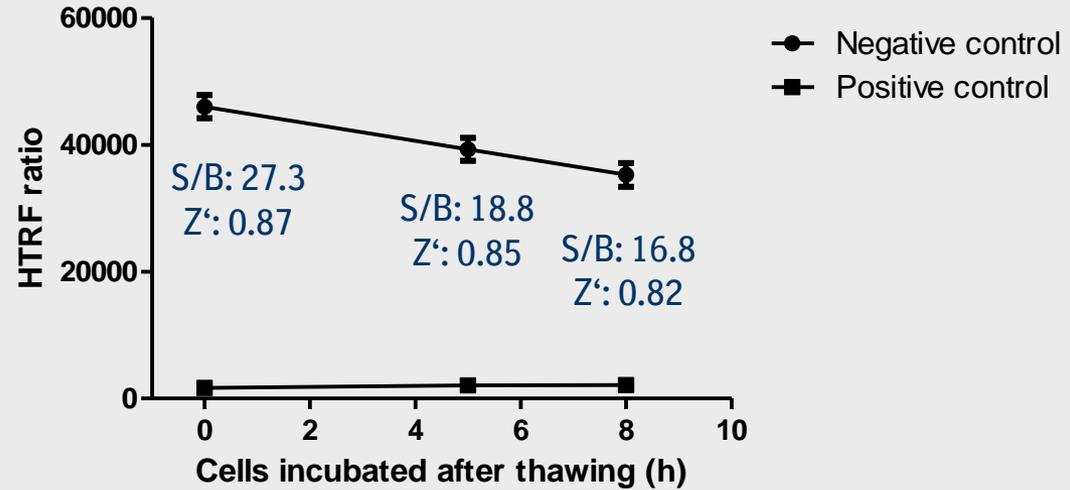
- K_i values determined are more or less in line with published data
- Marginal increase in K_i values after 20h incubation
- No impact of DMSO on K_i value
- Signal stable over time

Stability of GPCR1 & GPCR2 Tag-lite cell lines after thawing at RT

GPCR1

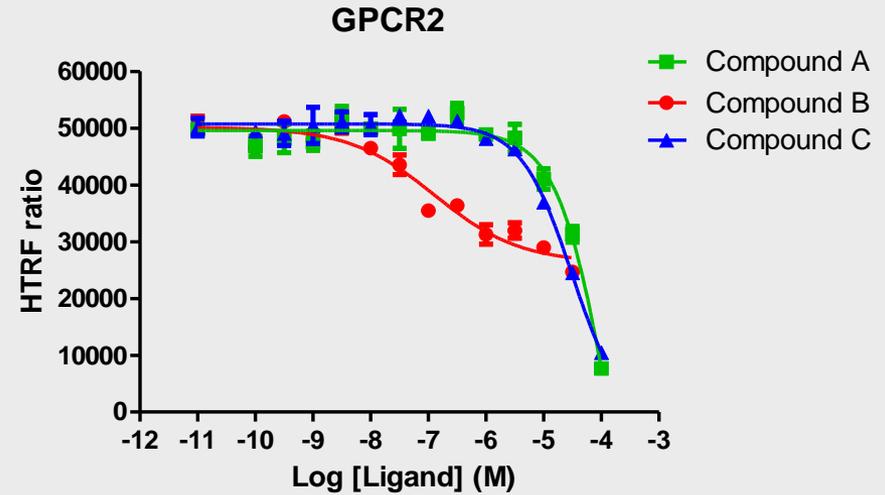
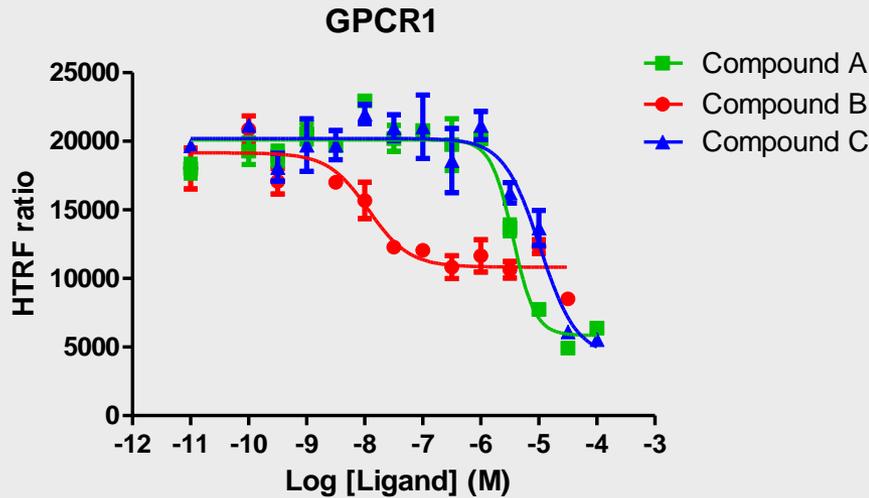


GPCR2



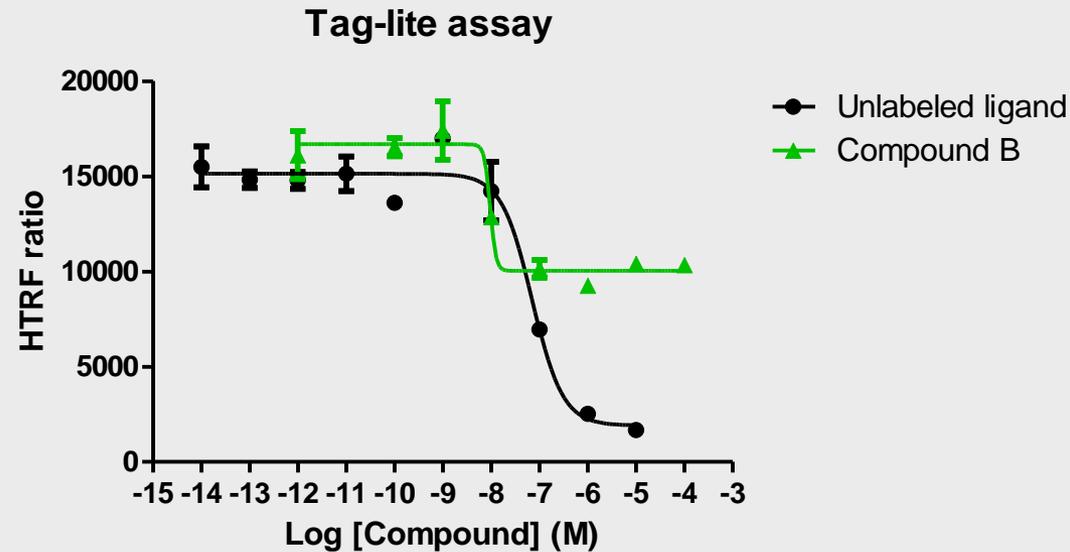
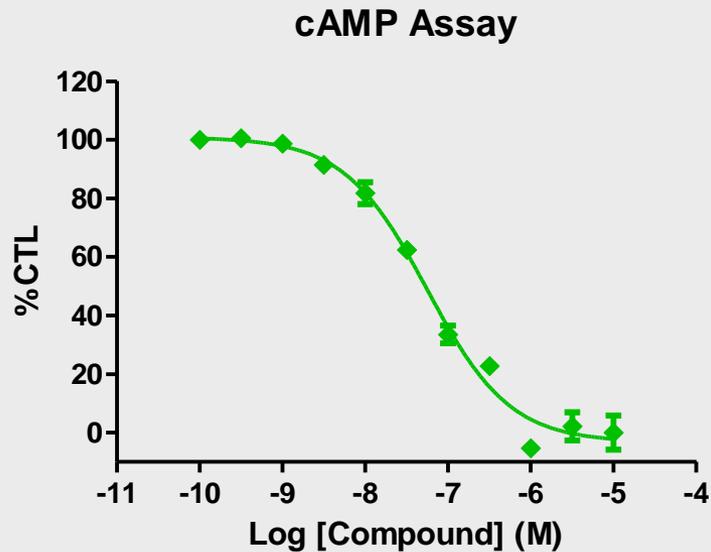
- Cells were thawed according to instruction from CisBio
- Cells suspension were stirred in a vessel at room temperature for the indicated hours before use in experiment
- Signal to background ratio drops over time for both Tag-lite cell lines

GPCR 1 & 2: IC₅₀ value determination of reference compounds



- IC₅₀ values in line with in house radio ligand binding data (Filter binding & SPA)
- Partial depression of signal by compounds B

IC₅₀ of compound B in cAMP assay and Tag-lite binding assay for GPCR1

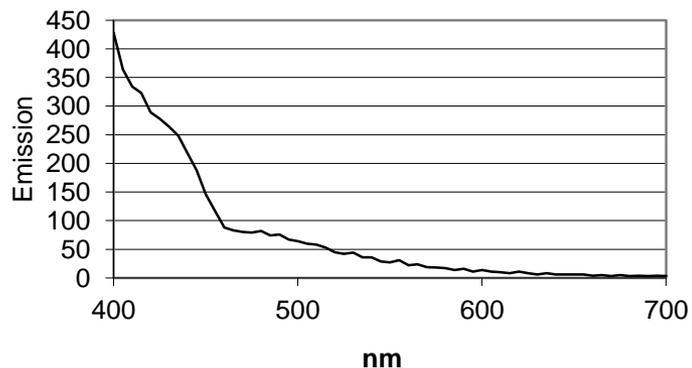


- Full depression of agonist induced signal in functional cAMP assay (not HTRF technology)
- Partial signal depression in Tag-lite binding assay
- Hypothesis:
 - (i) Assay artifact (auto-fluorescence)
 - (ii) Negative allosteric modulator with separate effects on efficacy and affinity of ligand

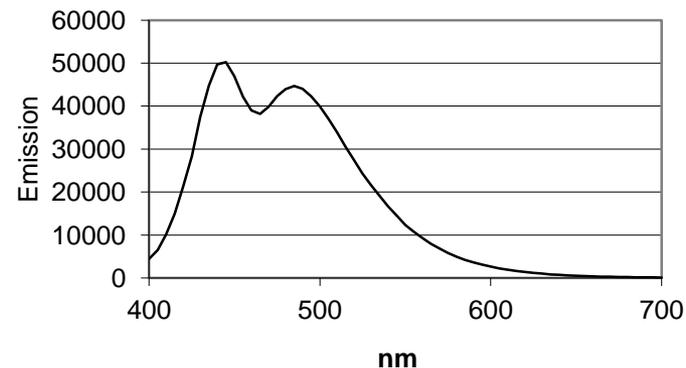
Fluorescence & Absorption spectrum of compound B

Excitation at 340 nm

Buffer

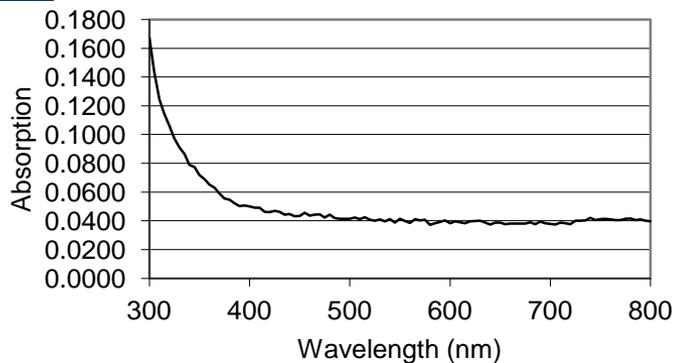


Compound B at 10^{-4} M in buffer

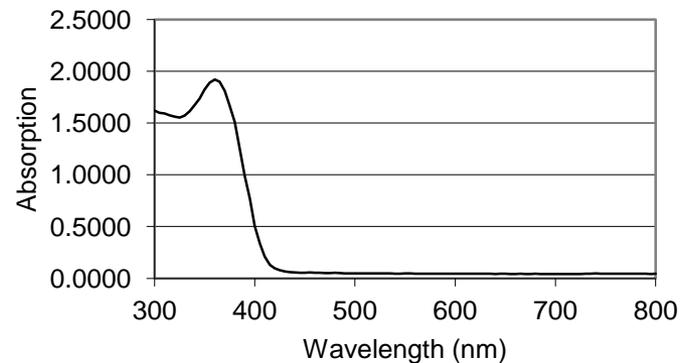


Absorption

Buffer

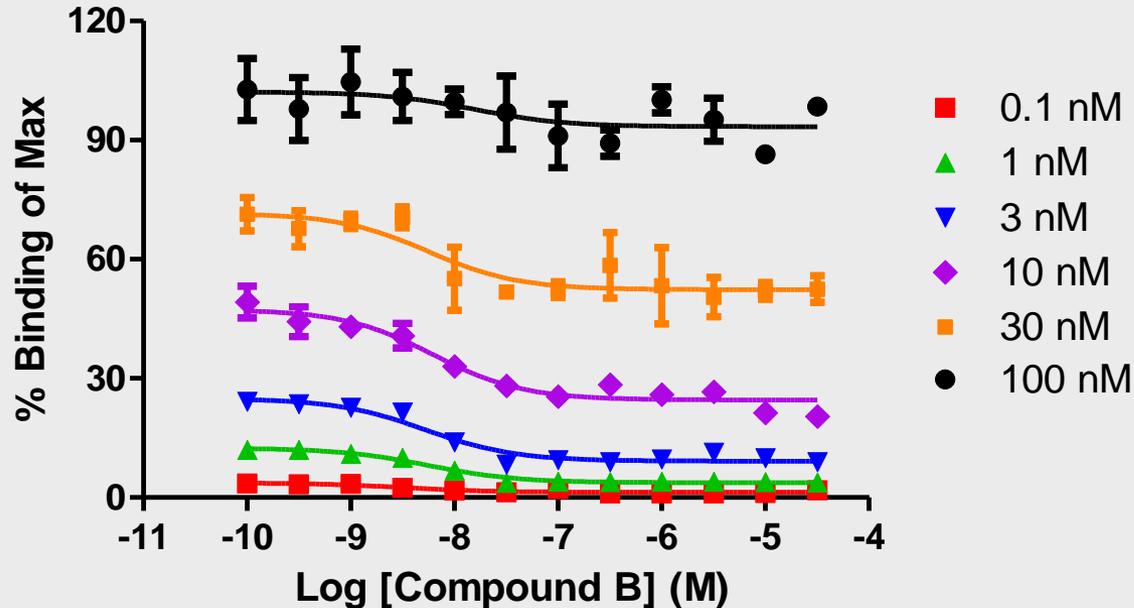


Compound B



- Apparently no auto-fluorescence interference

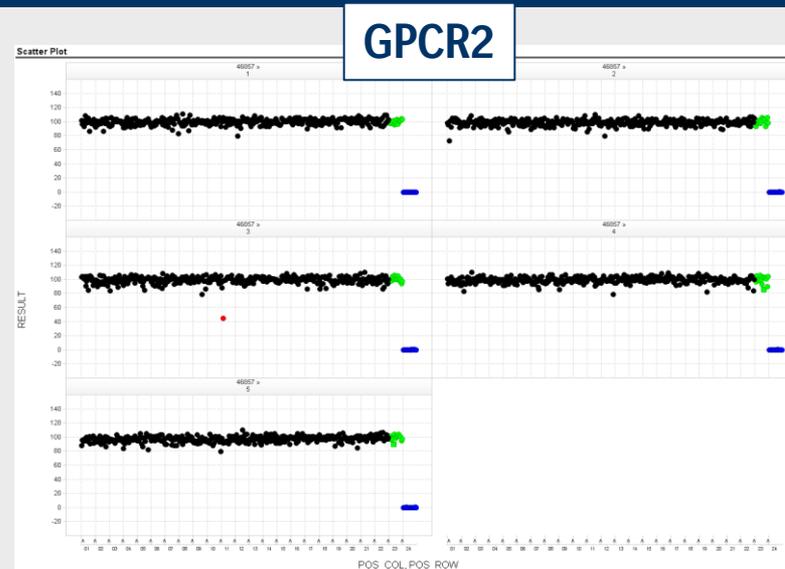
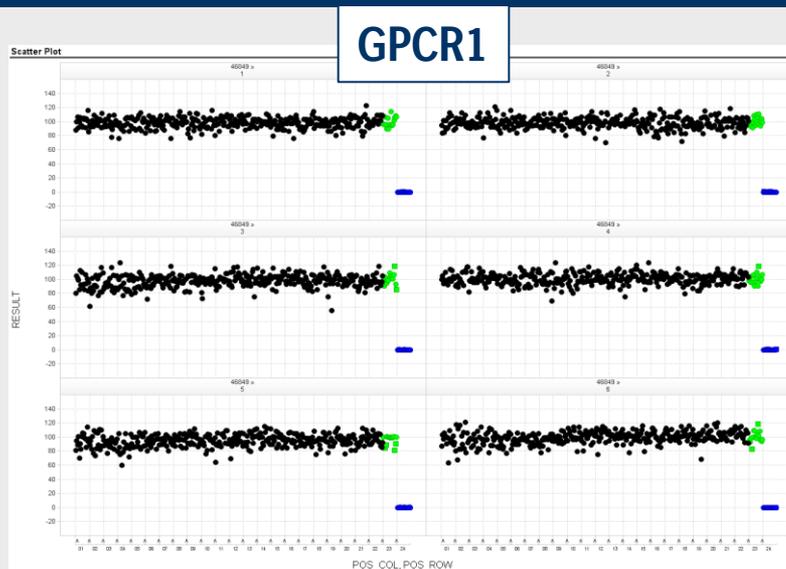
GPCR1: Displacement titration of compound B at different labeled ligand concentrations



- Value of the maximal displacement varies with different concentrations of labeled ligand suggesting that compound B might be a negative allosteric modulator

GPCR 1 & 2: Whole DMSO plates (1% DMSO)

Normalized data

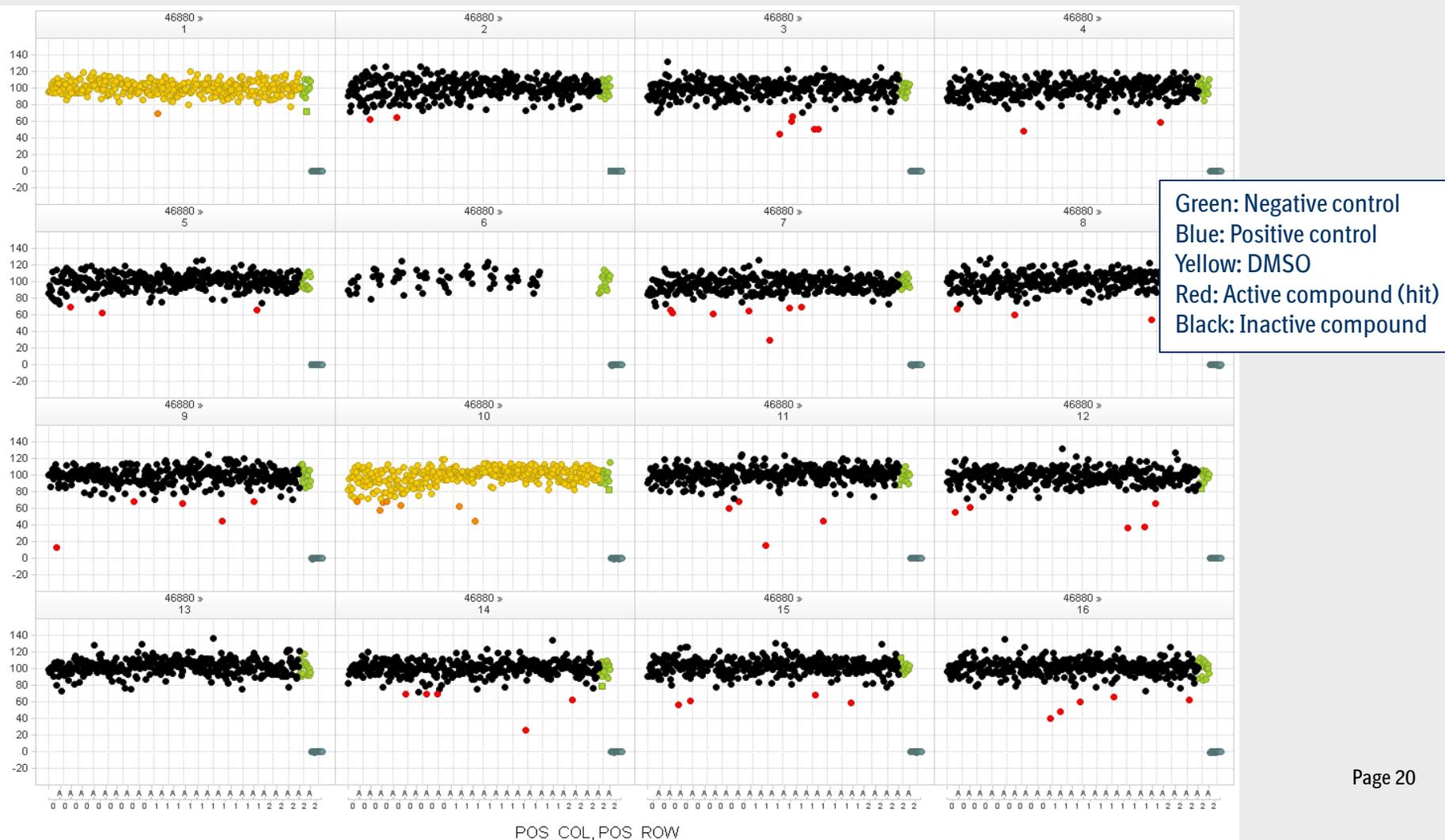


	GPCR1							GPCR2						
Plate No	Neg MW	Neg SD	Pos MW	Pos SD	DMSO CV (%)	S/B	Z'	Neg MW	Neg SD	Pos MW	Pos SD	DMSO CV (%)	S/B	Z'
1	100.0	7.2	0.0	0.1	7.4	20.7	0.78	100.0	2.7	0.0	0.1	4.2	24.5	0.92
2	100.0	6.1	0.0	0.1	7.9	20.8	0.81	100.0	3.8	0.0	0.1	4.3	25.0	0.88
3	100.0	5.1	0.0	0.1	8.9	20.0	0.84	100.0	3.3	0.0	0.2	5.3	24.3	0.90
4	100.0	6.0	0.0	0.1	7.6	20.1	0.82	100.0	4.3	0.0	0.1	4.2	23.9	0.87
5	100.0	0.8	0.0	0.2	8.9	19.4	0.97	100.0	2.8	0.0	0.1	4.2	24.0	0.91
6	100.0	5.5	0.0	0.1	8.6	19.1	0.83							

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- Characterization of hits
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GPCR1 cluster pool screen: Compound & DMSO plates (1% DMSO)

- 2 runs of cluster pool compounds with 36 plates (in total) at 5 $\mu\text{g/ml}$
- 4,990 compounds per run



GPCR1 cluster pool screen: Hit rates, S/B ratio & Z' values

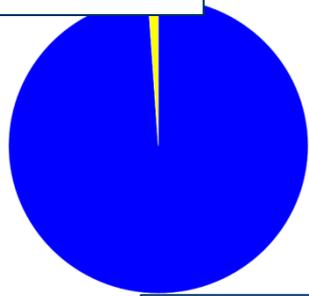


Boehringer
Ingelheim

125 Jahre mehr Gesundheit

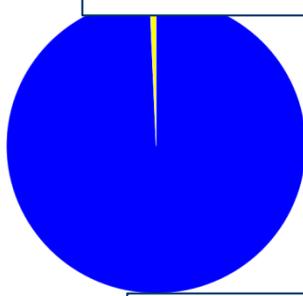
Hit Rates at a Cut Off of 70%CTL

54 Hits - 1.1%



4,936 - 98.9%

34 Hits - 0.7%

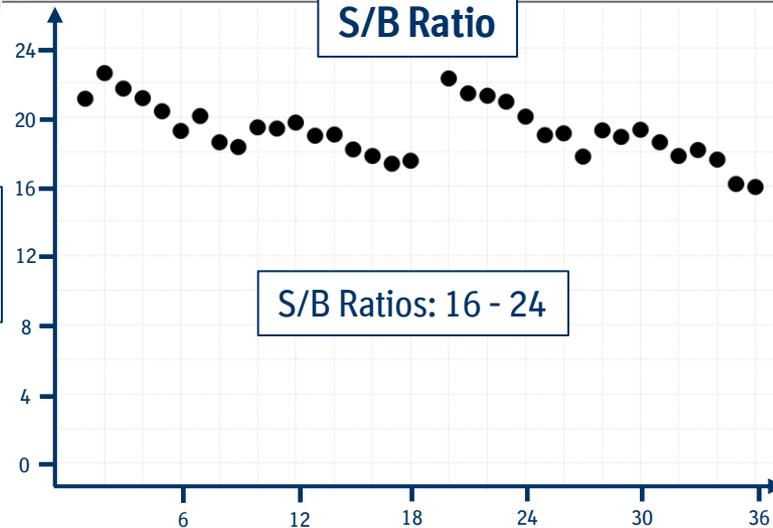


4,956 - 99.3%

Scatter Plot

S/B Ratio

S/B Ratio

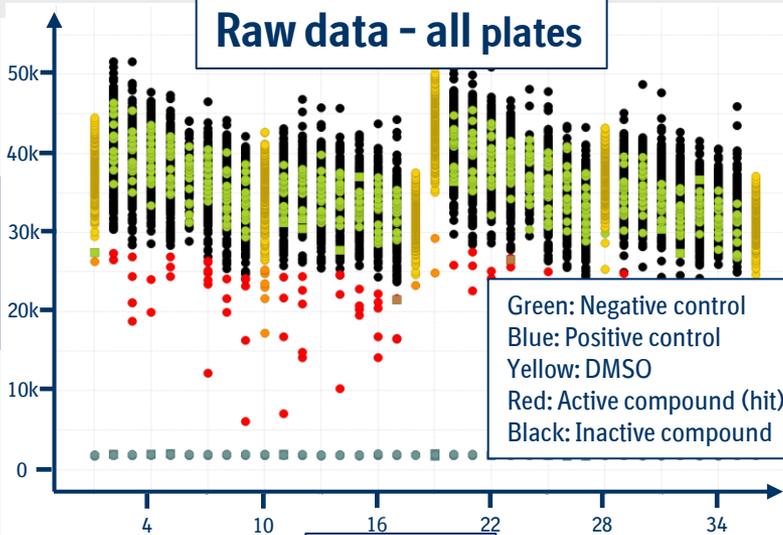


S/B Ratios: 16 - 24

Plate number

Raw data - all plates

HTRF Ratio



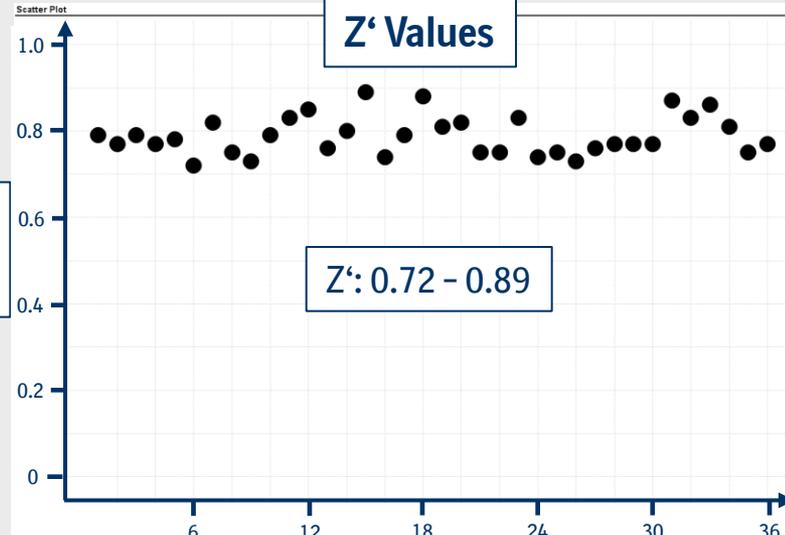
Green: Negative control
Blue: Positive control
Yellow: DMSO
Red: Active compound (hit)
Black: Inactive compound

Plate number

Scatter Plot

Z' Values

Z' Value

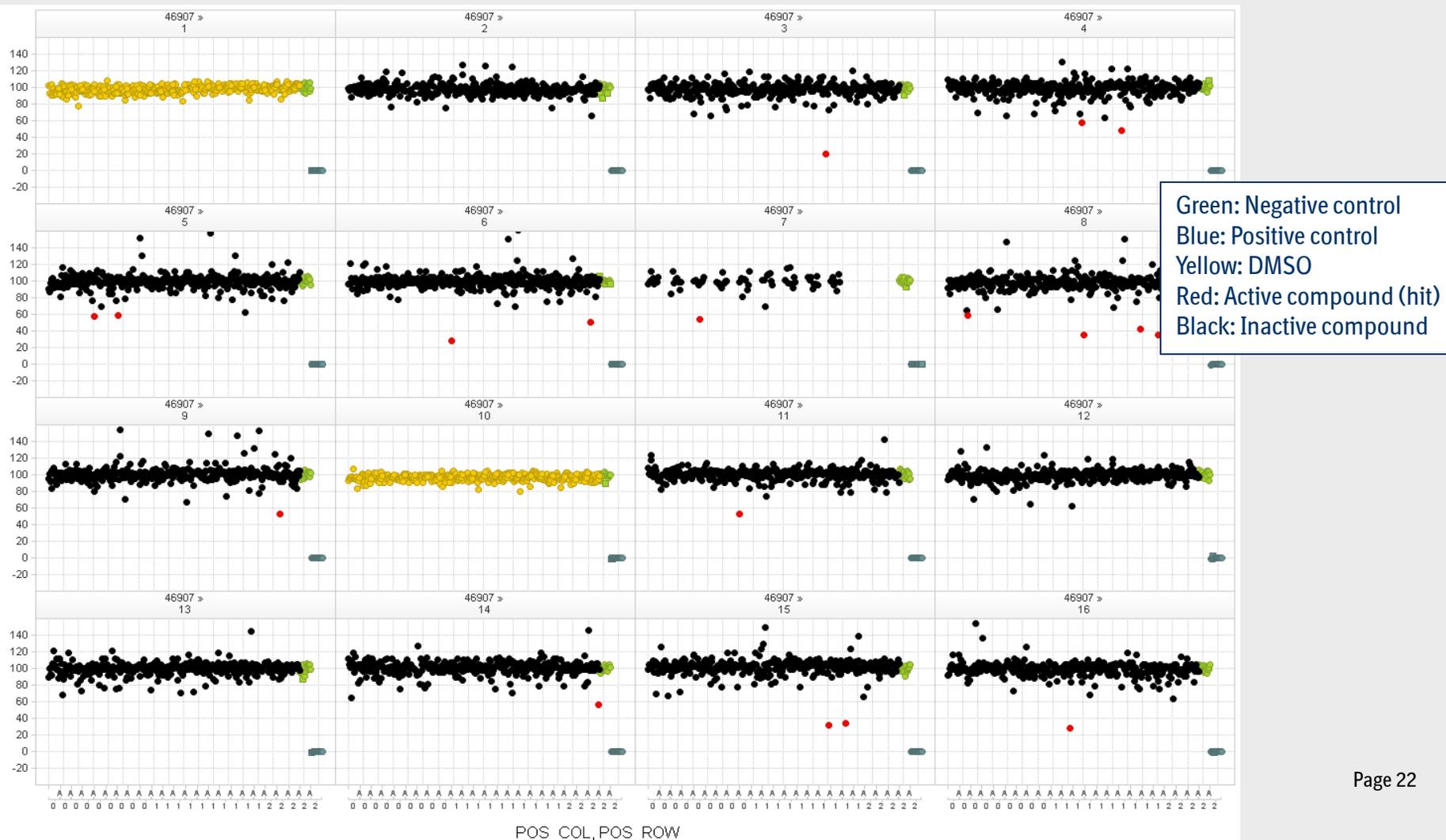


Z': 0.72 - 0.89

Plate number

GPCR2 cluster pool screen: Compound & DMSO plates (1% DMSO)

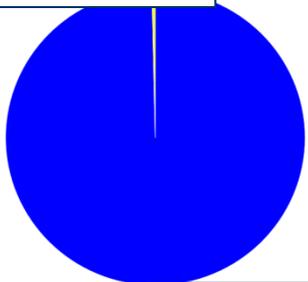
- 2 runs of cluster pool compounds with 36 plates (in total) at 5 µg/ml
- 4,990 compounds per run



GPCR2 cluster pool screen: Hit rates, S/B ratio & Z' values

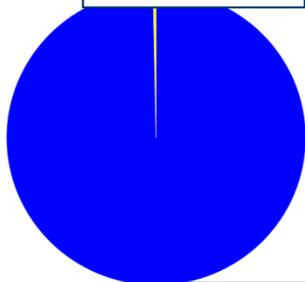
Hit Rates at a Cut Off of 70%CTL

18 Hits - 0.4%

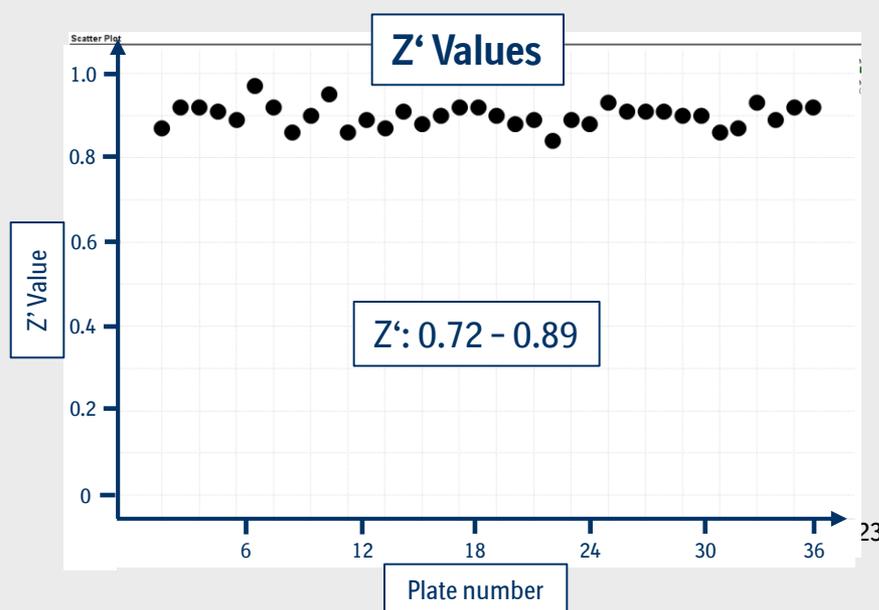
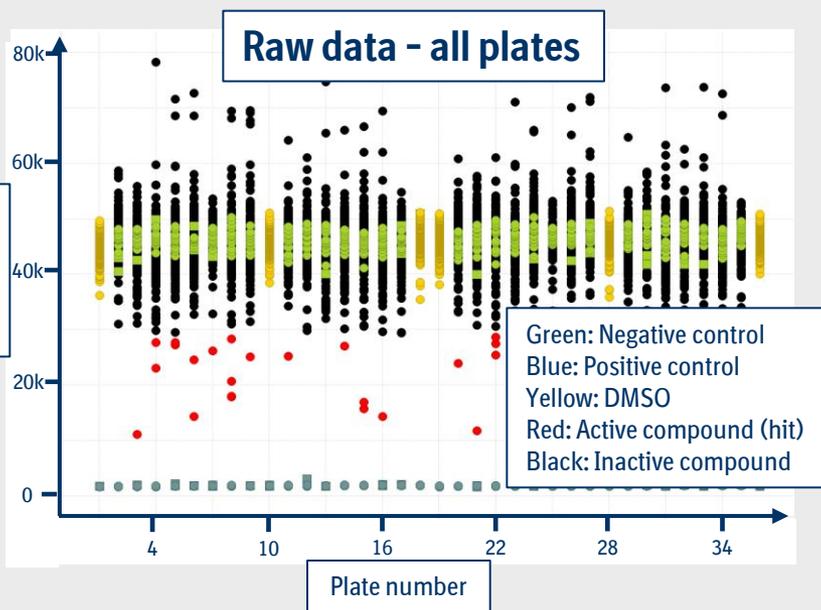
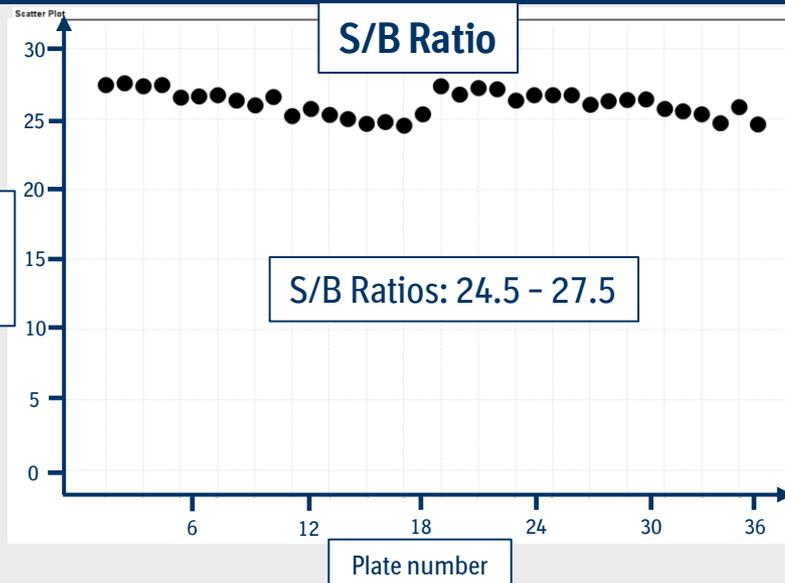


4972 - 99.6%

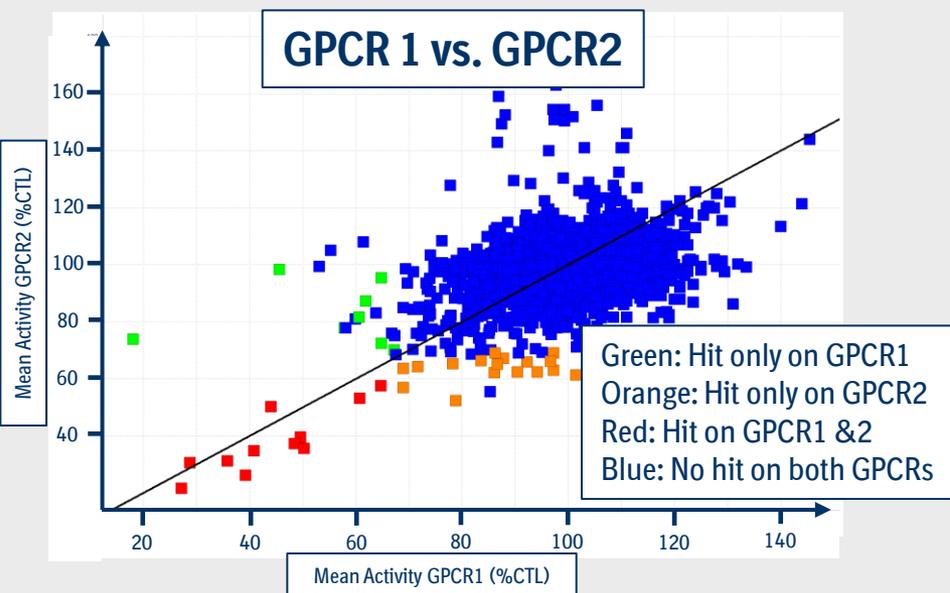
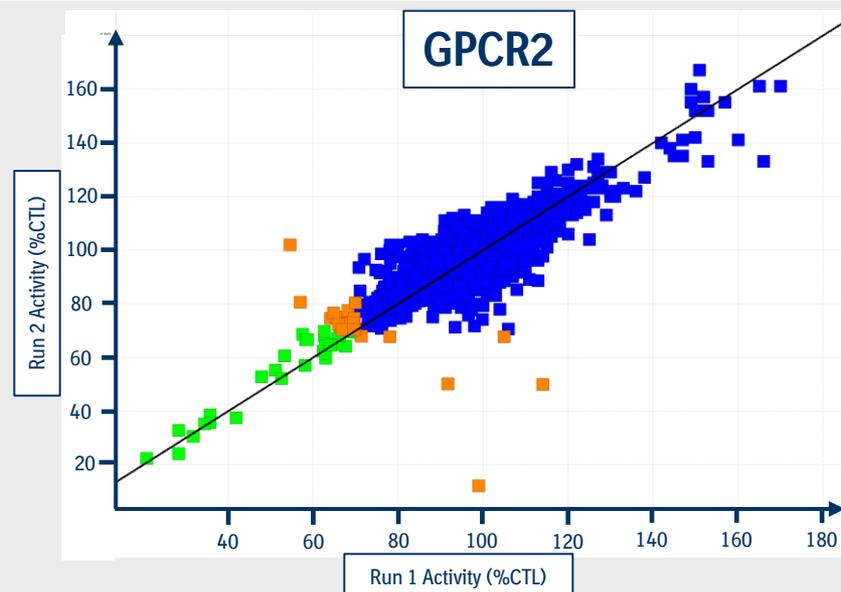
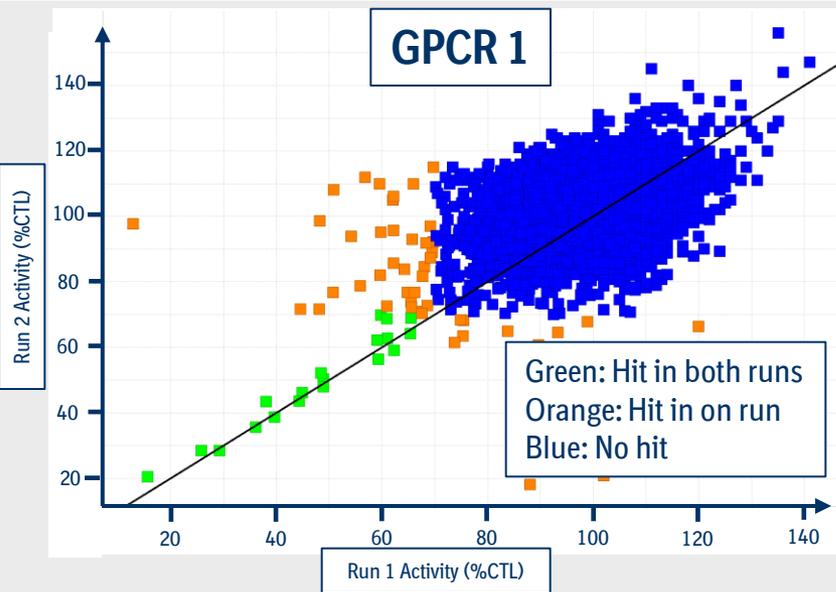
16 Hits - 0.3%



4974 - 99.7%



GPCR1 & 2: Correlations of both screens

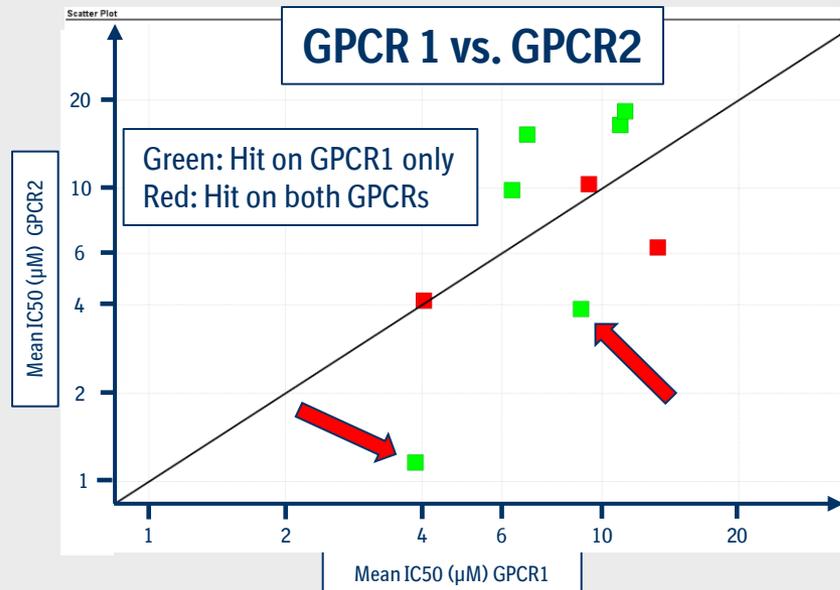
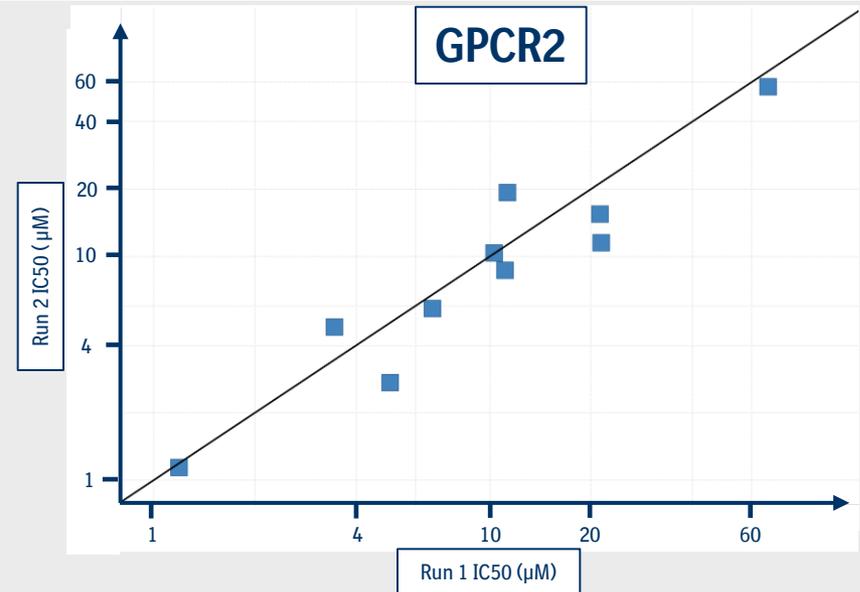
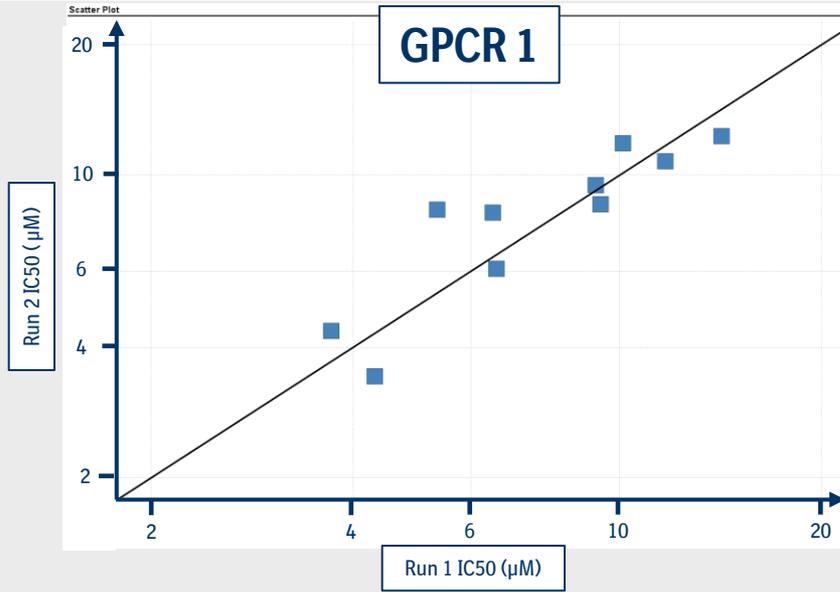


19 confirmed hits on GPCR1
↓
Minus 8 hits with auto-fluorescence
↓
11 confirmed hits on GPCR1

3 of these 11 compounds were also identified as hits in both runs of the GPCR2 test screen

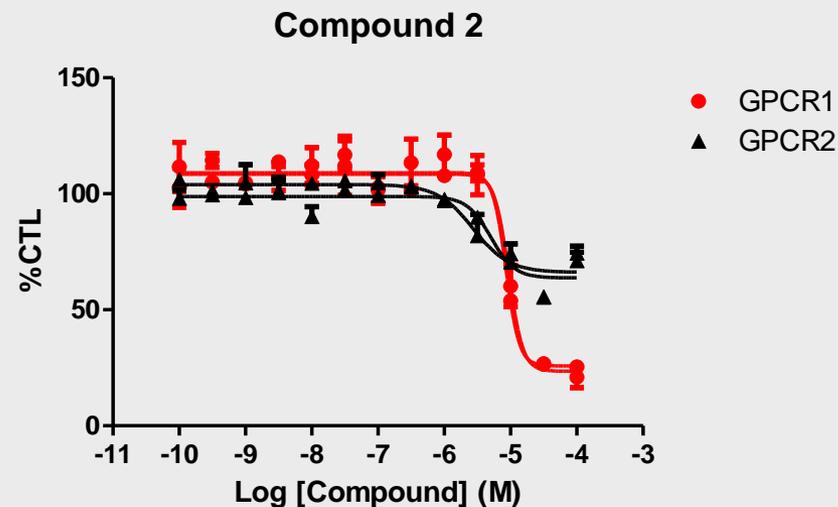
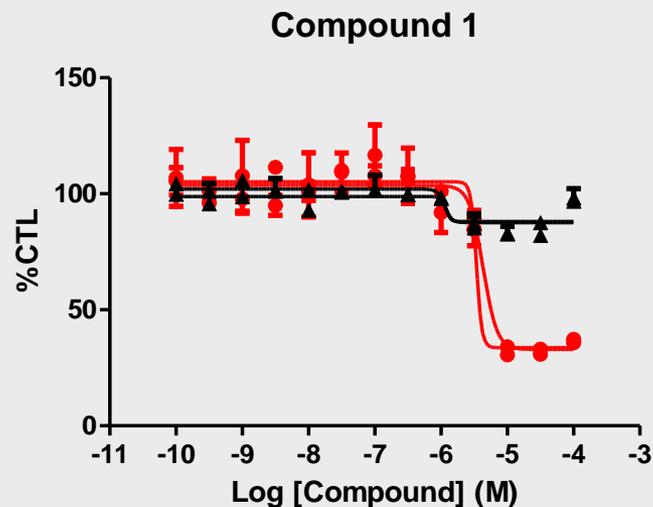
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Correlations of IC50 value determination for 11 confirmed hit compounds of GPCR1



- For both GPCRs good correlation of IC50 values
- IC50 values were in the range between 1 und 15 μM - poor selectivity
- 2 compounds that were identified as hits on GPCR1 but not on GPCR2 are more potent on GPCR2 than on GPCR1

IC₅₀ curves of compounds in Tag-lite binding assays that seem to be more potent on GPCR2 than on GPCR1



	GPCR1	GPCR2
Mean Activity Screen (%CTL)	45.6	98.5
Mean IC50 (M)	3.9 E-06	1.1 E-06

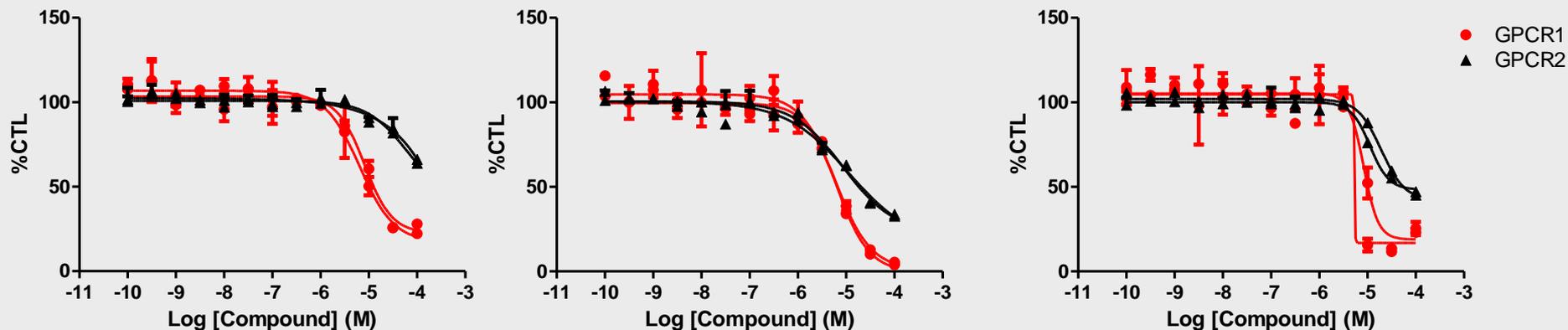
	GPCR1	GPCR2
Mean Activity Screen (%CTL)	60.1	81.7
Mean IC50 (M)	8.8 E-06	3.9 E-06

IC50 curves of other GPCR1 hit compounds

Compound 3

Compound 4

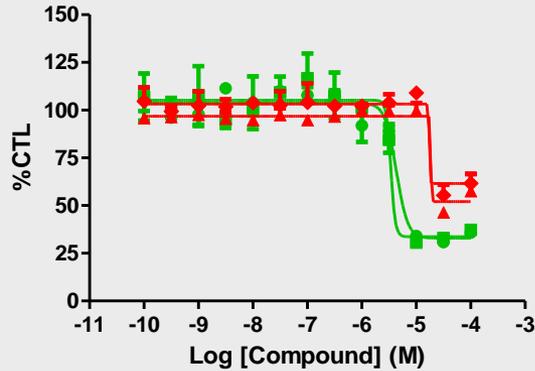
Compound 5



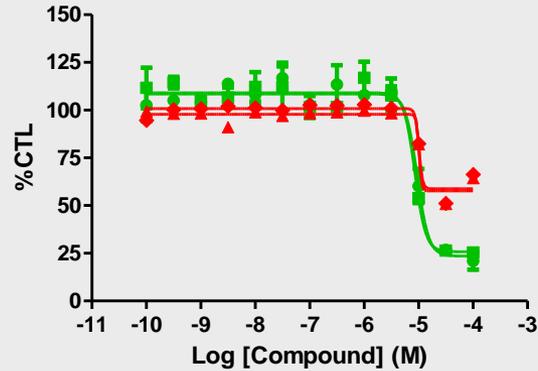
	Compound 3		Compound 4		Compound 5	
	GPCR1	GPCR2	GPCR1	GPCR2	GPCR1	GPCR2
Mean Activity Screen (%CTL)	64.9	95.5	57.9	77.9	18.1	73.9
Mean IC50 (M)	7.4 E-06	7.9 E-05	6.2 E-06	9.9 E-06	6.9 E-06	1.5 E-05

IC50 curves of compounds 1-5 in GPCR1 Tag-lite binding and GPCR1 cAMP assay

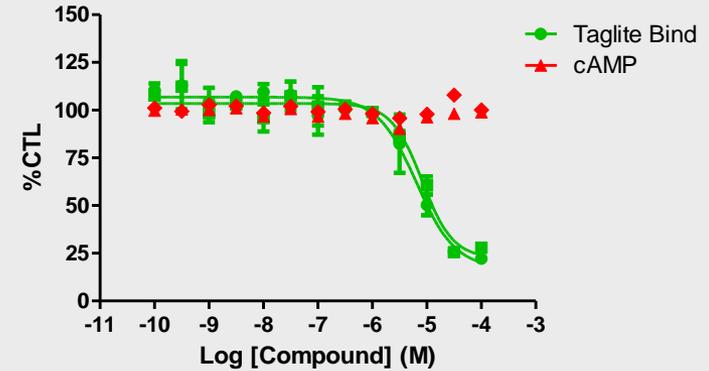
Compound 1



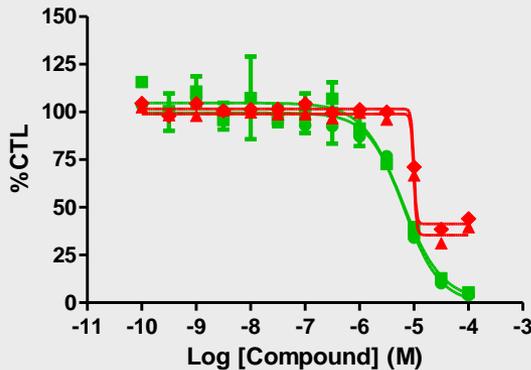
Compound 2



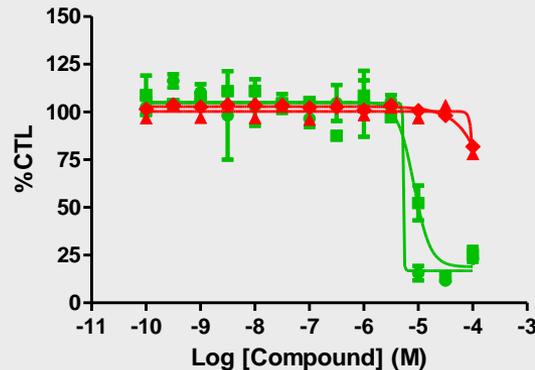
Compound 3



Compound 4



Compound 5



- Some of the compounds show also activity in the GPCR1 functional assay
- Partial inhibition in functional assays

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- K_d & K_i & IC_{50} values for various ligands fit well with published or in house generated data
- Signal reduction of GPCR1 over time makes it difficult to use the Tag-lite binding assay for k_{off} determination/target coverage
- Good to very good performance in mini-screens
- Tag-lite binding assays are suitable for HTS or MTS

- HTS team 1 in BC:
 - Claudia Karg



- A-Team in BC:
 - Helga Bronner
 - Michael Karnath

Back up