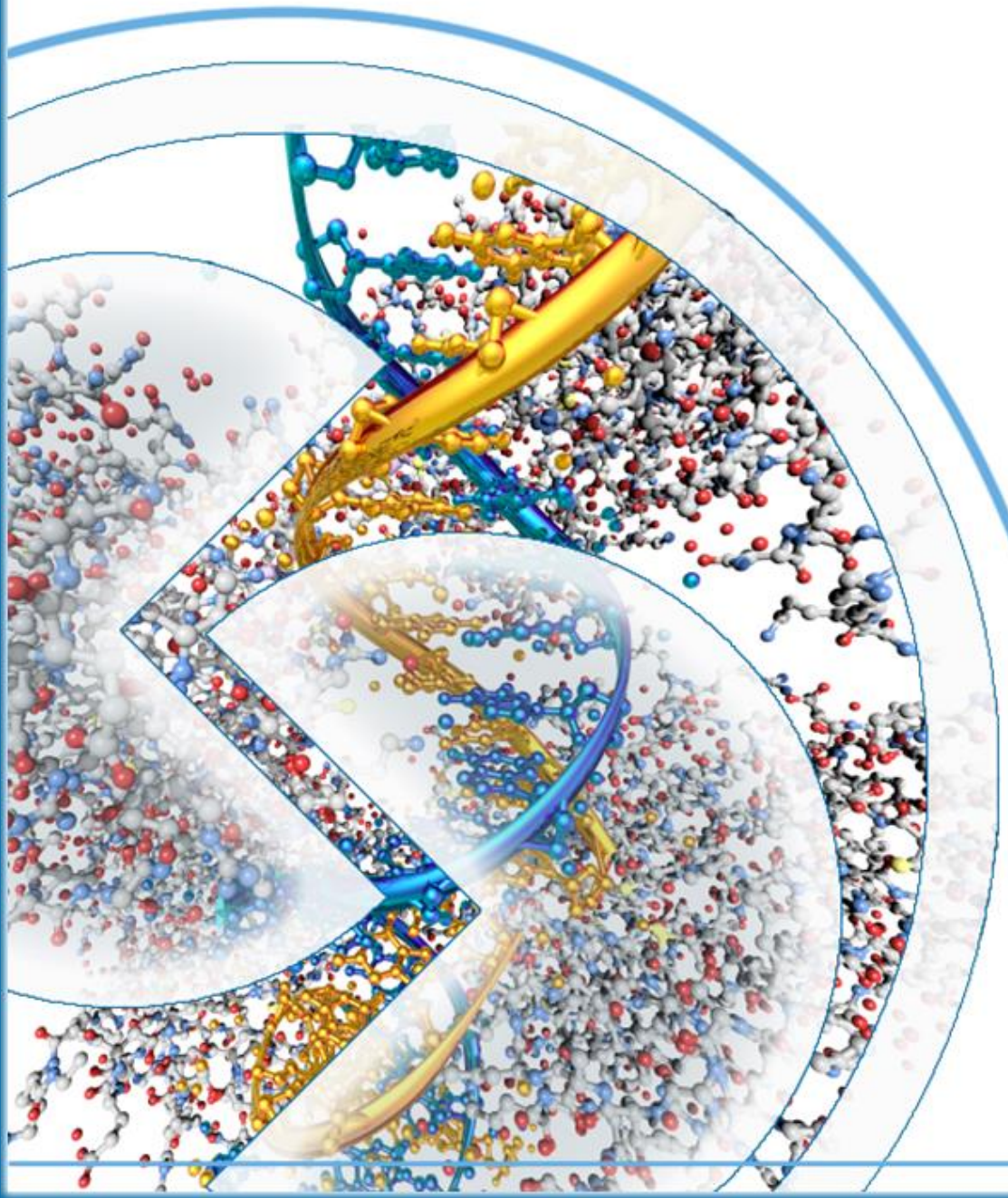
ChemDiv
The chemistry of cures℠



CASE STUDIES
HIGH THROUGHPUT SCREENING

6605 Nancy Ridge Drive, San Diego, CA 92121
Tel.: 858-794-4860, Fax: 858-794-4931
E-mail: chemdiv@chemdiv.com

Discovery™ outSource

Target to Lead
Lead to Candidate
Candidate to POC
POC to Market

SCREENING PLATFORMS IN CHEMDIV

ChemDiv
The chemistry of cures℠

- **Liquid Handling**
  - **Automated Work Stations (Beckman)**
  - **Assembly of Liquid handlers**
    - 96/384 tip heads: BiomekFX, BiomekNX (screening)
    - 1-8 tip head - Biomek2000 (concentration curves)

SCREENING PLATFORMS IN CHEMDIV

ChemDiv
The chemistry of cures℠

Readout

- **Plate readers**
  - Tecan M1000 infinite Pro, Tecan M200, VictorV3, VictorV3 light
- **FLIPR Tetra (Molecular Devices)**
- **High Content Screening Platform:**
  - ImageXpress Micro Widefield High Content Screening System (Molecular Devices)
- **Flow cytometry platform**
  - GUAVA EasyCyte Plus

ASSAY FORMATS

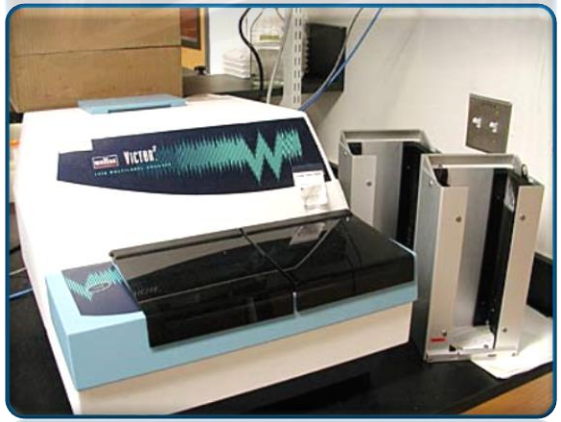
ChemDiv
The chemistry of cures℠

DETECTION FORMATS

- Fluorescence, including polarization, FRET, Alpha-screen etc.
- Luminescence
- Absorbance
- High Content Cell imaging
- Flow cytometry

ASSAY FORMATS

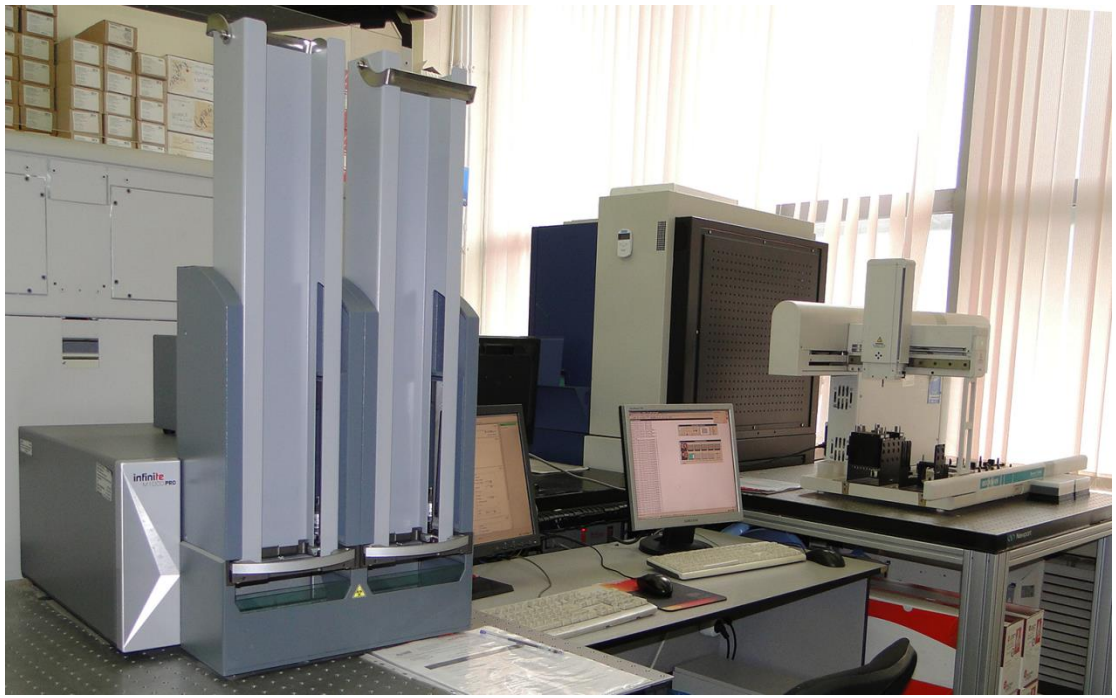
- Cells-based assays (FLIPR, LANCE, cytotox etc), including assays with primary cells culture (hepatocytes, PBMC etc)
- Homogeneous (mix and read)
- Heterogeneous (ELISA)



ASSAY TYPES

ChemDiv
The chemistry of cures℠

- **Biochemical**
  - Enzymes
    - ✓ Kinases
    - ✓ Phosphatases
    - ✓ Proteases etc.
  - Protein-Protein interaction
  - DNA modifying enzymes
  - *other*
- **Cellular**
  - Receptors
    - ✓ GPCRs
    - ✓ Nuclear
  - Reporter assays
    - ✓ Nuclear factors
    - ✓ Splicing
  - Cell proliferation and cytotox
  - Cytokines expression
  - Cell imaging
  - *Other*



SOME TARGETS WE HAVE BEEN WORKING WITH

ChemDiv
The chemistry of cures℠

GPCRs
- Fatty acid receptors (GPR40, GPR119, GPR120, LPAR 1-3)
- Neuromediator receptors (GPR154, adenosinergic, 5HT 1A-1F, 5HT-4-7, Muscarinic, cGRP, others)
- Chemokine receptors (CXCRs)
- Peptide hormone receptors
- Protease-activated (PARs)
- Peptide hormone receptors (NPYR 1,2,5)

Kinases (ATP binding and non-ATP binding sites)
- Abl, Akt1-2, BRAF, CDLK1, GSK3B, EGFR, ErbB1[L858R], JAK2-3, PI3K, PDK1, IGF1R, VEGFRs, Pim1, PDK1, SGK1-3, others)

Ion channels (ligand- and potential-gated)
- TRPA1, 8, nAchR α7
- Na channels, Ca channels

Other targets
- Nucleases: APE1, Exo1, RAD51
- Deacetylases: HDAC1-11, SIRT1,2,6
- Pathways (Hh, WNT, Notch, CMA, Autophagy, Epigenetics)
- Arginase, PAI-1, PD-1/PD-L1 etc.
- Viral polymerases, proteases and others

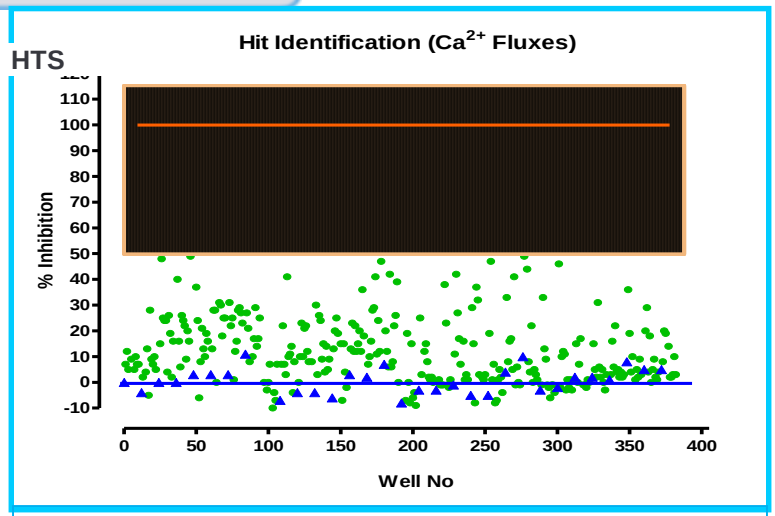
Cell lines
- Multiple cell lines with endogenously and exogenously expressed receptors

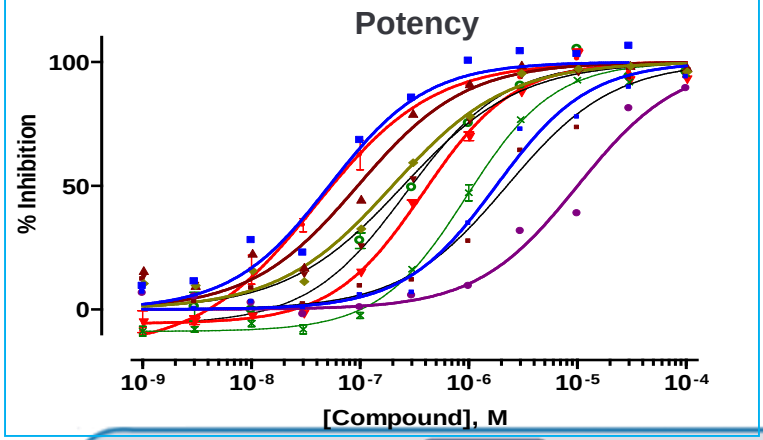
SCREENING FLOW :

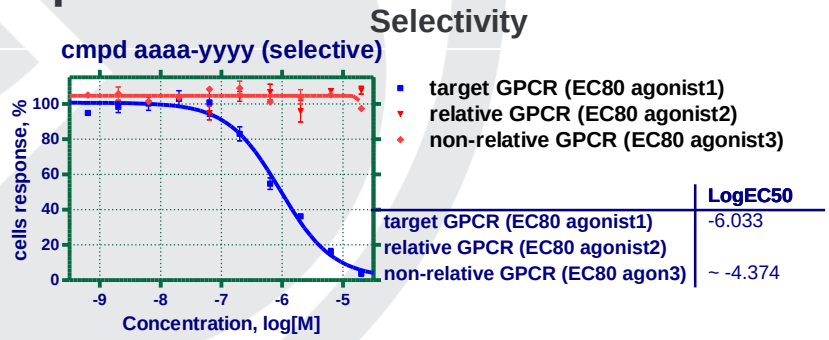
Screening/potency/specificity/cytotoxicity

ChemDiv
The chemistry of cures℠

1) Assay development and validation
2) HTS (single-points or duplicates)
   - Multiple target classes
   - Multiple readout technologies
3) Hit confirmation
   - In duplicates from fresh powder
4) Potency
   - Typically 10 concentration points in duplicates
5) Specificity and Selectivity
6) Secondary assays
7) Follow up
8) H2L optimization







	LogEC50
target GPCR (EC80 agonist1)	-6.033
relative GPCR (EC80 agonist2)	
non-relative GPCR (EC80 agon3)	~ -4.374

Discovery™ outSource

Target to Lead
Lead to Candidate
Candidate to POC
POC to Market

ChemDiv
The chemistry of cures℠

Cell Based Assays
Case studies

Discovery™ outSource
Target to Lead
Lead to Candidate
Candidate to POC
POC to Market

GPCRs: ASSAY DEVELOPMENT

ChemDiv
The chemistry of cures℠

- Selection of cell line with endogenous xxx expression and Ca^{2+} response to agonist
- Optimization
  - Agonist dose response curve
  - Optimum plate type, cell density and FBS concentration (minimal acceptable concentration), HEPES presence (helpful in some cases)
  - Optimum dye loading conditions: concentration/ T°C/time dependence/probenecid concentration (for cells like CHO)
  - Optimum FLIPR conditions: solutions addition height and speed, mixing parameters
  - DMSO tolerance
  - Reagents stability at RT
- Validation
  - NIH validation protocol
  - Statistics (Z'-factor; S/B; S/N; %CV)
  - Edge and drift effects

GPCRs: SCREENING

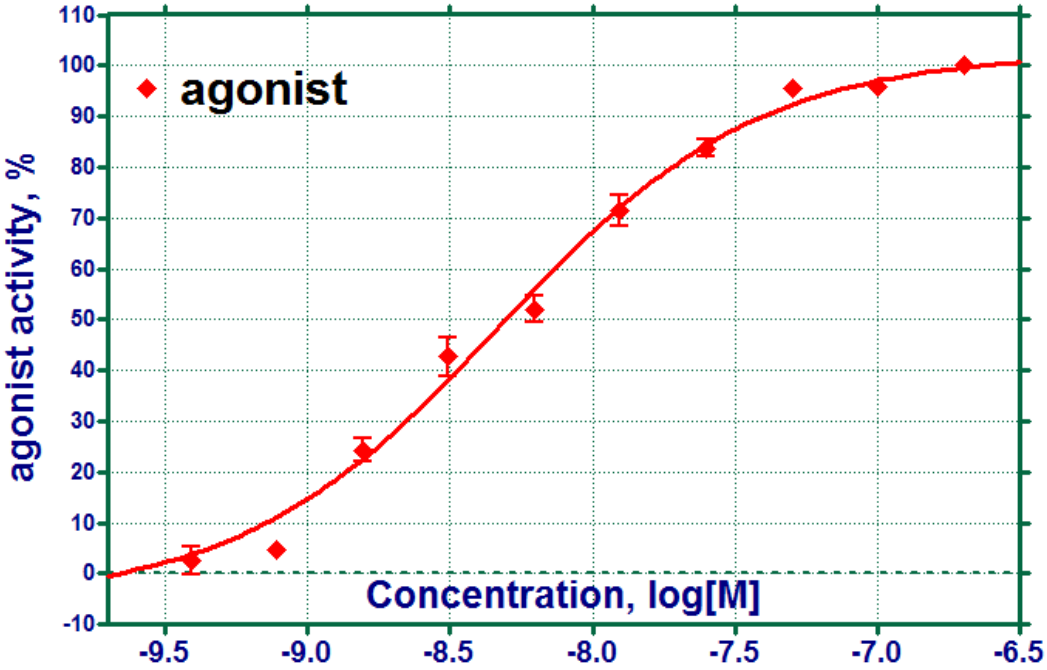
ChemDiv
The chemistry of cures℠

- **Primary screening**
  - Up to 10,000/day compounds at single concentration (typical, at 10μM)
- **Secondary screening**
  - Confirmation of cmpds which overcame the threshold in duplicate at same concentration
- **Determination of EC/IC50**
  - Concentration response curves (CRCs) at concentration range (typical, 10 concentration point with dilution step 3.16 from 30 μM to ~1nM)
- **Specificity and selectivity determination**
  - CRCs as in previous stage on cell lines with target and non-relative (for specificity) or relative (for selectivity) receptors

FLIPR:ASSAY DEVELOPMENT

agonist activity and concentration selection for antagonist screening

ChemDiv
The chemistry of cures℠



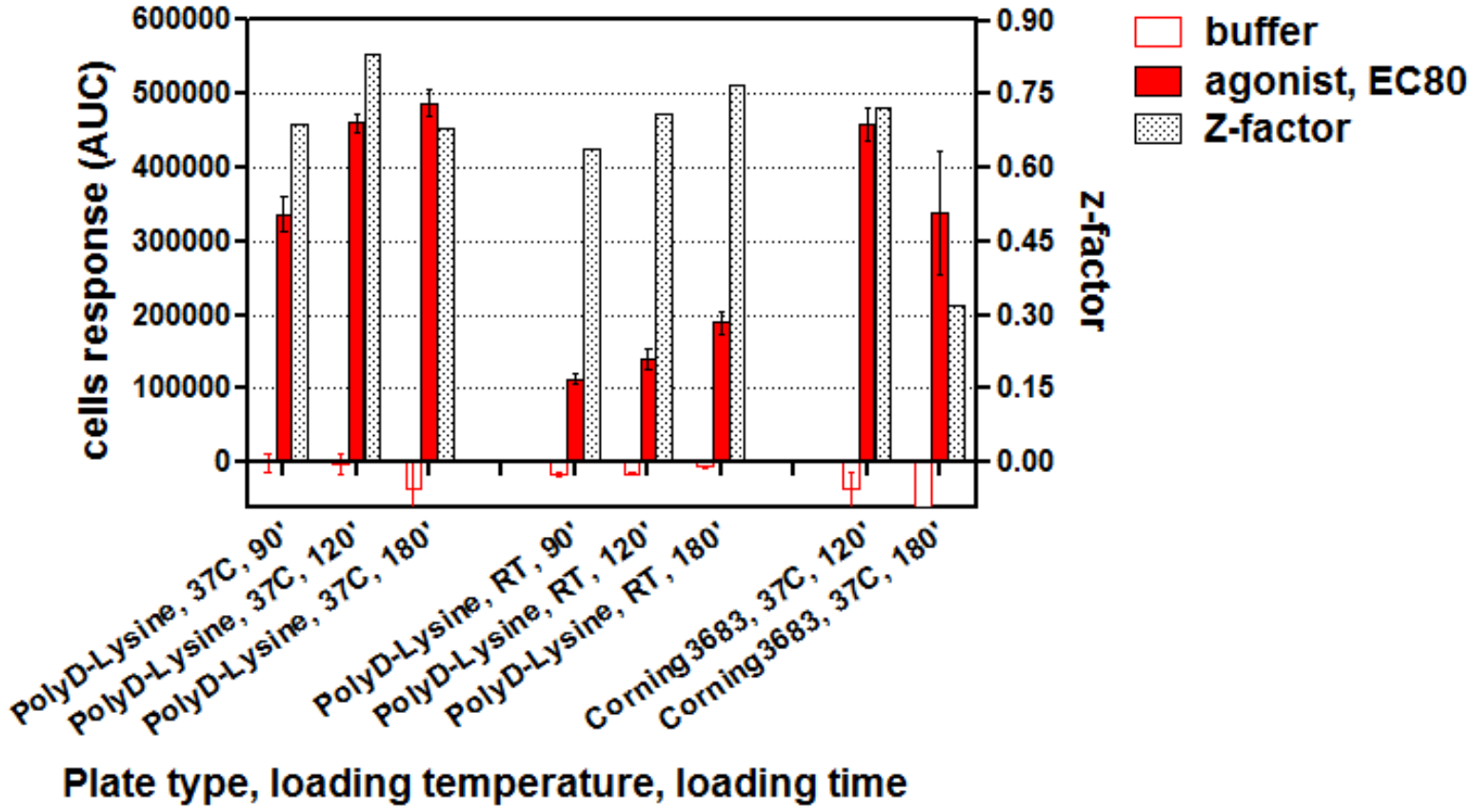
	LogEC50	HillSlope	EC50
agonist	-8.329	0.9547	4.685e-009

EC80 selected for further assay development

FLIPR:ASSAY DEVELOPMENT

dye loading condition

ChemDiv
The chemistry of cures℠

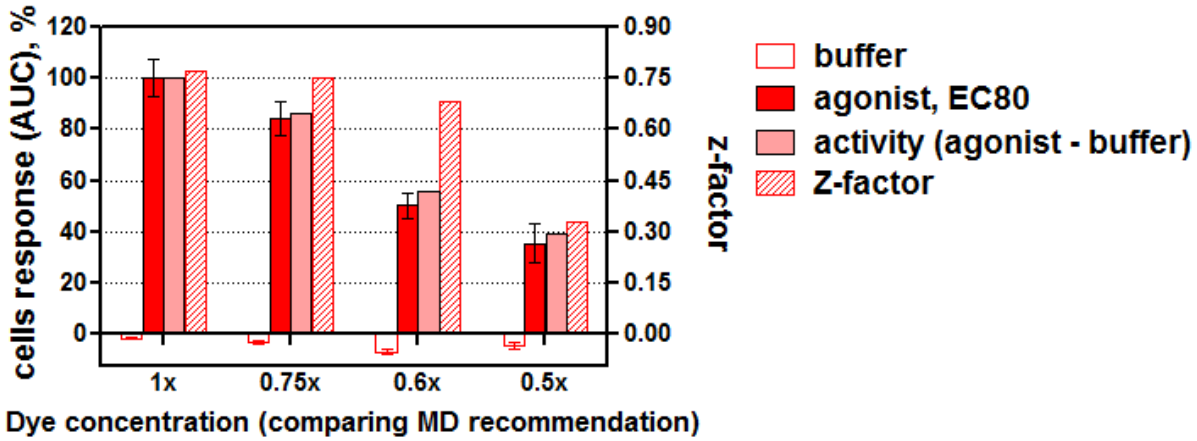


FLIPR:ASSAY DEVELOPMENT

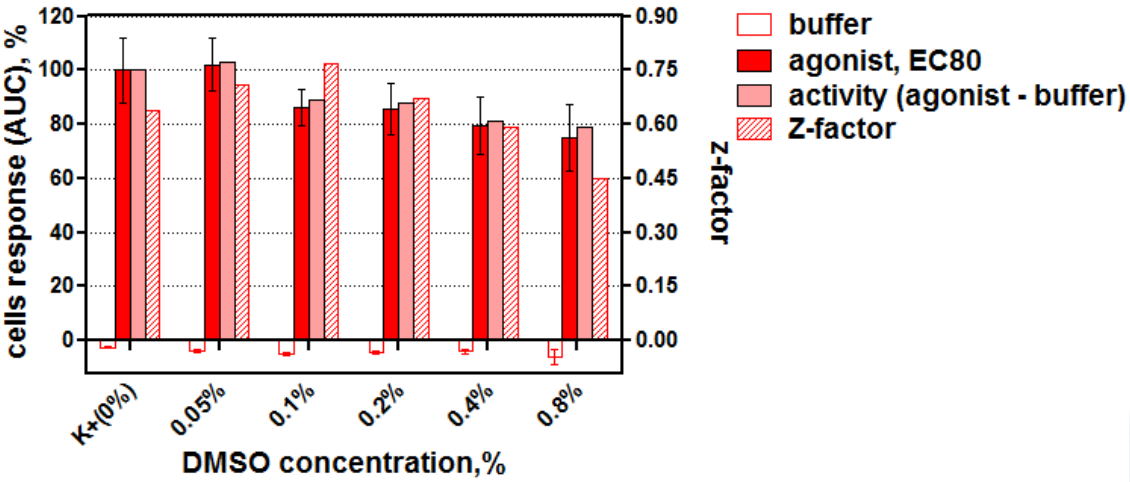
DMSO tolerance and dye concentration

ChemDiv
The chemistry of cures℠

Dye concentration

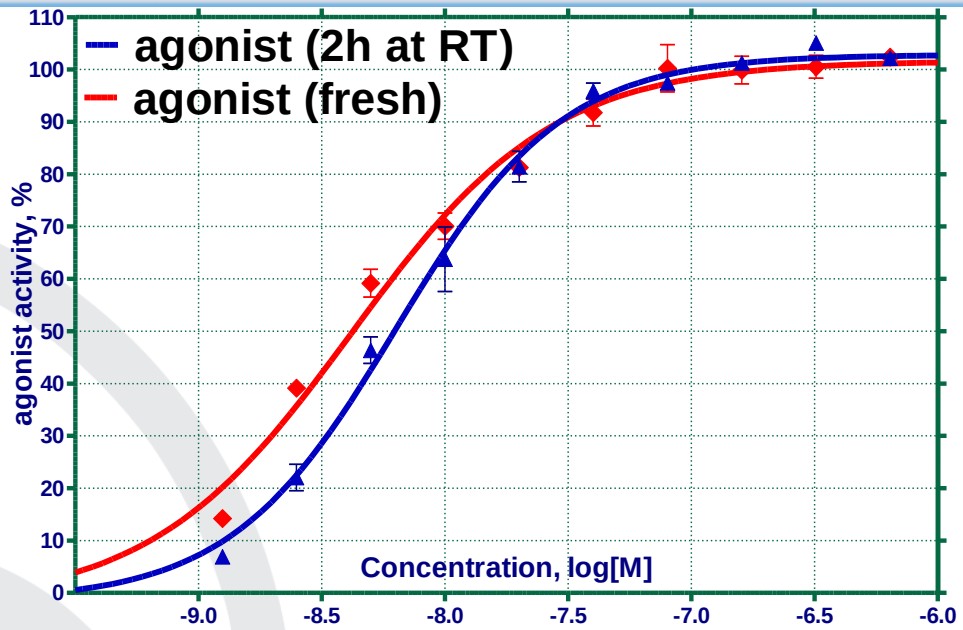


DMSO tolerance



FLIPR:ASSAY DEVELOPMENT

agonist stability



	LogEC50	HillSlope	EC50
agonist (fresh)	-8.379	1.066	4.179e-009
agonist (2h at RT)	-8.196	1.290	6.362e-009

- agonist is stable at EC80-EC90 during 2 hours and therefore may be used in HTS in this concentrations.
- For IDIP validation 40 nM and 4 nM of agonist could be used as high and medium concentration respectively

SUMMARY OF OPTIMAL ASSAY CONDITIONS

for CHO-xxx Cell Line

ChemDiv
The chemistry of cures℠

Steps to check		Conditions checked									
Cell incubation condition	Plate type	Corning, CellBind® Surface®			Poly-D-Lysine CELLCOAT® 384 well black µClear®						
	Medium	10% FBS	5% FBS	3% FBS	2% FBS	1% FBS	w/o FBS				
	cell concentration (cells/well)	6.000		8.000		12.000		16.000			
Cell-loading conditions	Loading temperature	RT			37 C						
	Loading time	1 hour		1.5 hours		2 hours		3 hours			
	Probenecid concentration	w/o probenecid		1.25 mM		2.5 mM		4 mM			
FLIPR condition	Addition height (µl)	24	25	26	27	28	30				
	Addition speed (µl/sec)	15			20						
	Pipetting	w/o pipetting		1 time		2 time					
DMSO tolerance, %		6.4	3.2	1.6	0.8	0.4	0.2	0.1	0.05	0.0025	0.00125
Acceptable conditions		*Chosen optimal conditions*									

SUMMARY OF OPTIMAL ASSAY CONDITIONS

for HEK293T-xxx Cell Line

ChemDiv
The chemistry of cures℠

Steps to check		Conditions checked							
Cell incubation condition	Plate type	Corning, CellBind® Surface®				Poly-D-Lysine CELLCOAT® 384 well black µClear®			
	Medium (with and w/o HEPES)	10% FBS	5% FBS	2% FBS with HEPES	2% FBS w/o HEPES	1% FBS with HEPES	1% FBS w/o HEPES	0% FBS with HEPES	0% FBS w/o HEPES
	cell concentration (cells/well)	10.000		15.000		20.000		30.000	
Cell-loading conditions	Loading temperature	RT				37 C			
	Loading time	1.5 hour			2 hours		3 hours		
	Dye concentration	1x		0.75x		0.6x		0.5x	
FLIPR condition	Addition height (µl)	35		40		45		50	
	Addition speed (µl/sec)	10		12		15		20	
	Pipetting	w/o pipetting		1 time		2 time			
	Pipetting height and volume (µl)	27 (25 µl)		32 (25 µl)		37 (25 µl)		42 (20 µl)	
	Pipetting speed (µl/sec)	15				20			
DMSO tolerance, %		1.6		0.8		0.4	0.2	0.1	0.05

Acceptable conditions | *Chosen optimal conditions*

INTRA-PLATE, INTER-PLATE AND INTER-DAY VALIDATION

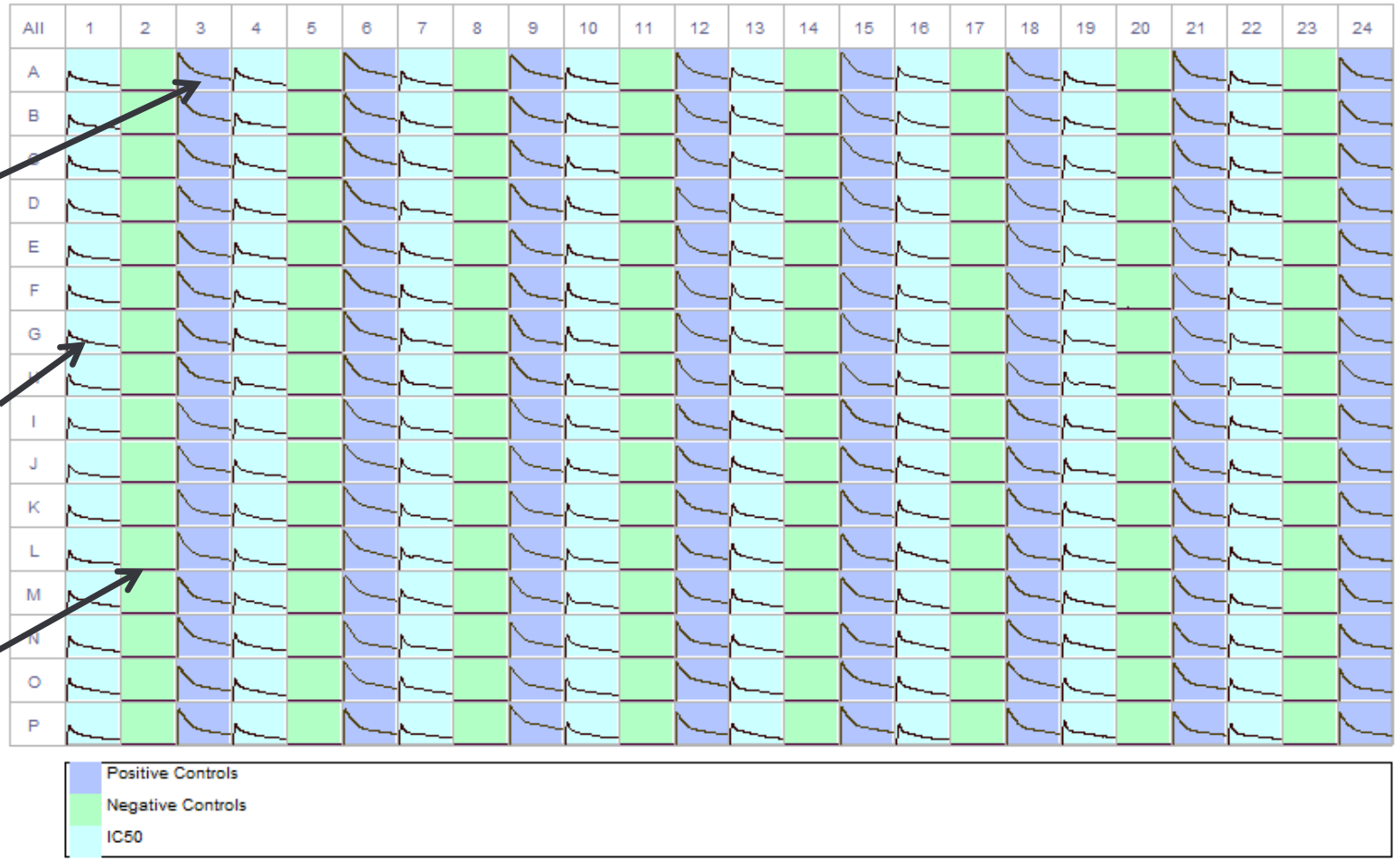
(3 plates / 2 or 3 days), single plate example

ChemDiv
The chemistry of cures℠

K+ (100% activation)

50% activation

K- (w/o agonist)



INTRA-PLATE, INTER-PLATE AND INTER-DAY VALIDATION

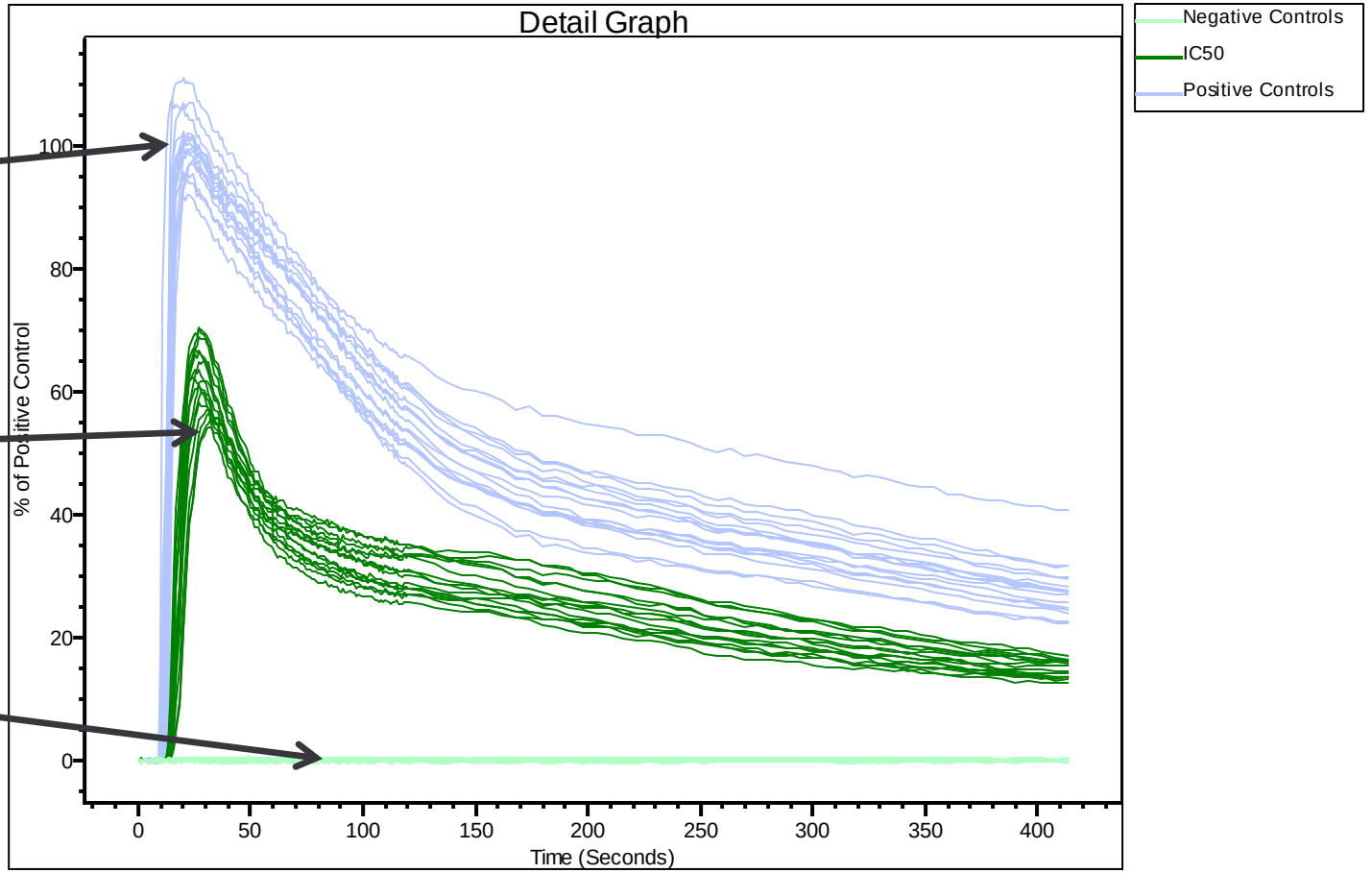
(3 plates / 2 or 3 days), single plate example

ChemDiv
The chemistry of cures℠

K+ (100% activation)

50% activation

K- (w/o agonist)



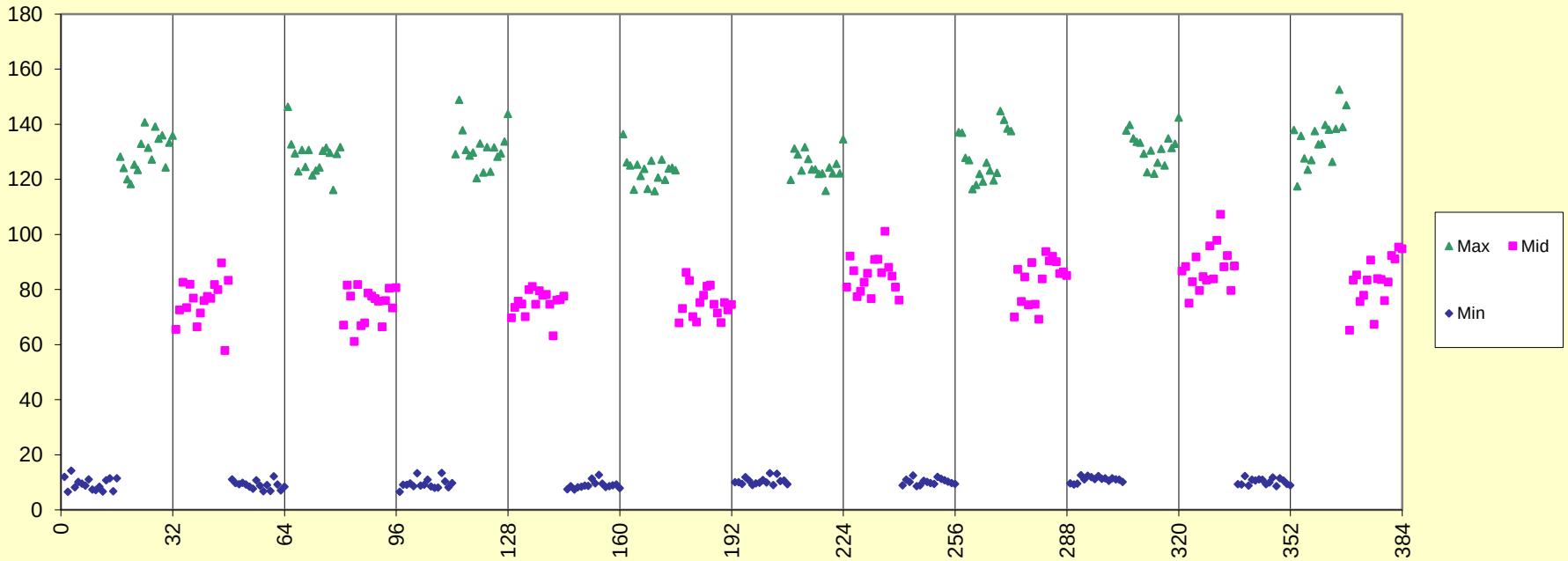
Discovery™ outSource

Target to Lead
Lead to Candidate
Candidate to POC
POC to Market

INTRA-PLATE, INTER-PLATE AND INTER-DAY VALIDATION

(3 plates / 2 or 3 days), single plate example

ChemDiv
The chemistry of cures℠



Discovery™ outSource

Target to Lead
Lead to Candidate
Candidate to POC
POC to Market

VALIDATION TEST CHECKLIST

Intra-Plate Tests		Meets Criterion?
1	Check for drift and edge effects in all plates (you must check manually)	
2	All max (HI) signal CV's < 20%	Yes
3	All mid signal (unnormalized) CV's < 20%	Yes
4	All normalized mid signal (mid %) SD's < 20	Yes
5	All min (LO) SD's < Min(max (HI) SD, mid SD)	Yes
6	All SW's >2	Yes
7	All Z' Factors > 0.4 (and < 1; must pass one of 6 or 7)	Yes

Inter-Plate Tests		
1	All within-day fold shifts < 2	Yes
2	All Average (between-)Day fold shifts < 2	Yes

ChemDiv
The chemistry of cures℠

GPCRs

GPCR-X Agonists Screening Case Study

HTS CAMPAIGN (agonist screening)

ChemDiv
The chemistry of cures℠

- Goal: Search for GPCR-X novel agonists
- Assay: Calcium Mobilization Assay (FLIPR platform)

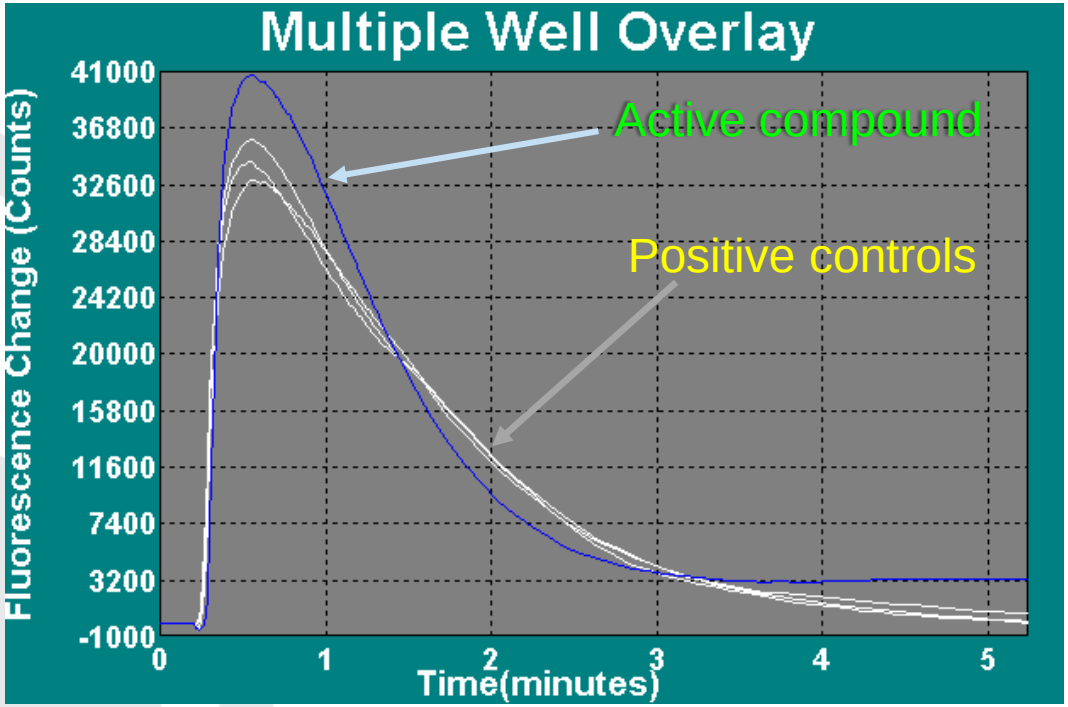
- HTS-1 campaign (diverse library) and HTS-2 campaign (GPCR biased library)
  - *Hit Criteria:*

  1)Agonistic activity - more than 50% of positive control

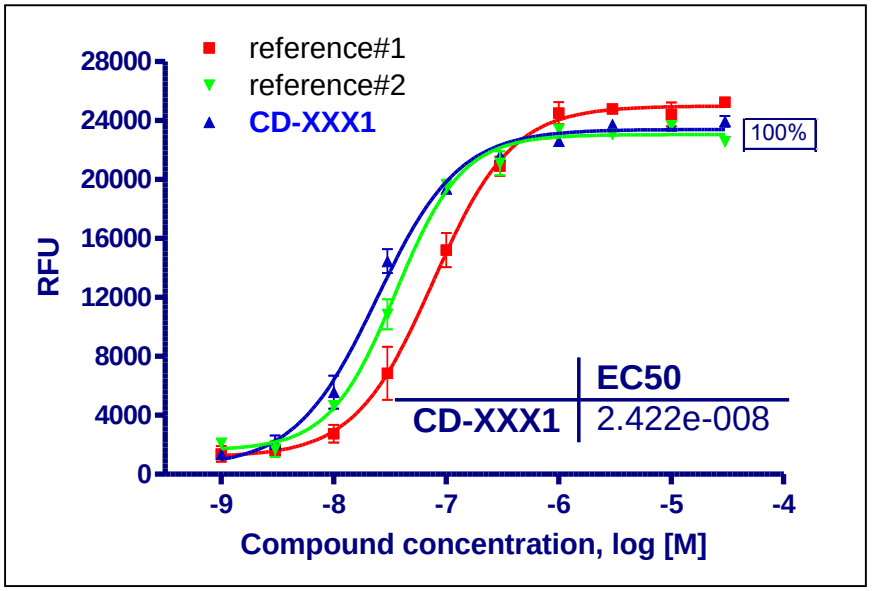
  2)Maximal calcium spike at less than 60 sec
- Re-screen at 10μM in duplicates
- Counter screen
  - *Naïve CHO cell line*
- 127 compounds were confirmed (Hit rate - 0.39%)
  - *Potency profiling with EC_{50}<10μM*
- 3 Series selected for follow-up MedChem campaign

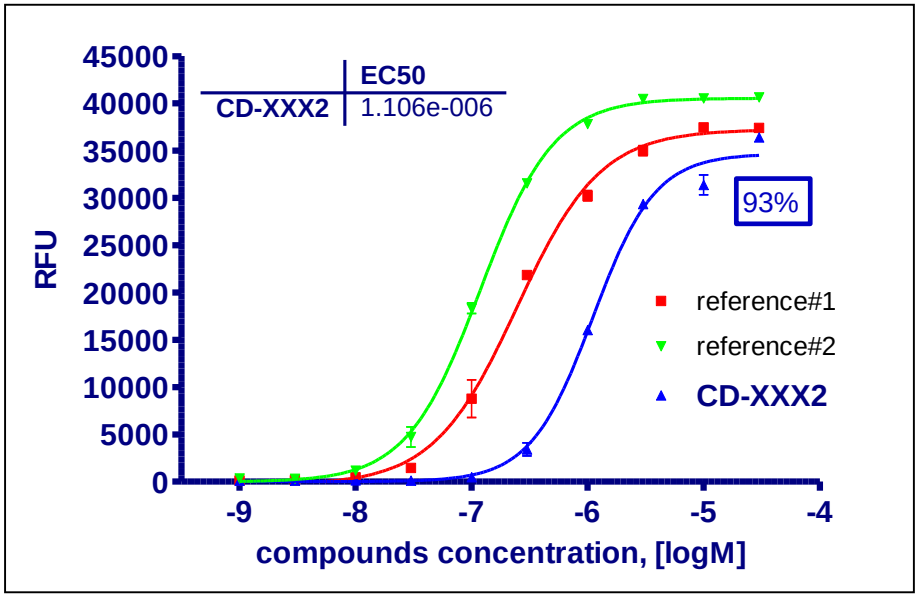
EXAMPLES OF FLIPR CURVES

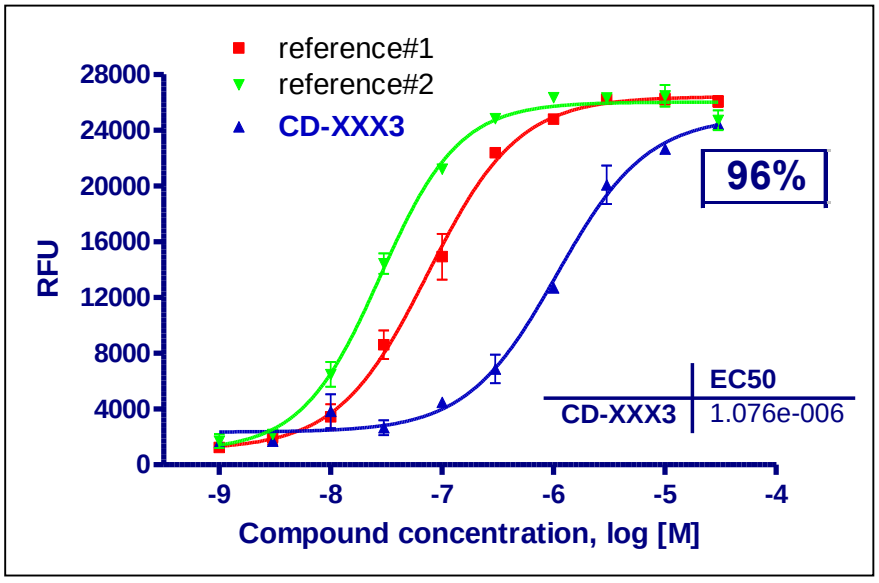
represent cellular Ca $^{2+}$ response to agonists and compounds tested (agonist screening)



Confirmation of Hits (Examples)







- Novel hits from 5 different series were identified

ChemDiv
The chemistry of cures℠

GPCRs

GPCR-X Antagonists Screening Case Study

Discovery™ outSource
Target to Lead
Lead to Candidate
Candidate to POC
POC to Market

HTS SUMMARY
(antagonist screening)

ChemDiv
The chemistry of cures℠

Primary screening
hit criteria: INH% > 50% - 3*SD(reference) and
ACT% < 20% + 3*SD(buffer) at 10μM

85000

↓

Rescreening
hit criteria: INH% > 50% - 3*SD(reference) in each replicate and
ACT% < 20% + 3*SD(buffer) in each replicate at 10μM

7839

↓

Counterscreening
hit criteria: INH% < 10% + 3*SD(non-relative GPCR**) at 10μM**

2621

↓

Selectivity panel
hit criteria: INH%(target GPCR**)>50% at 20μM with significant selectivity to** relative GPCR **and** non-relative GPCR

625

↓

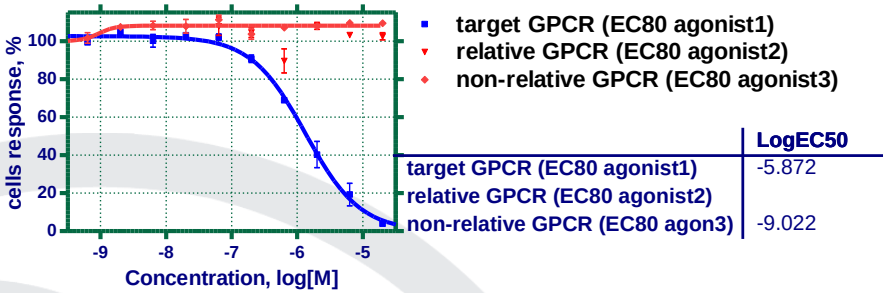
Target GPCR selective hits confirmed

69

HITS POTENCY PROFILING
(antagonist screening)

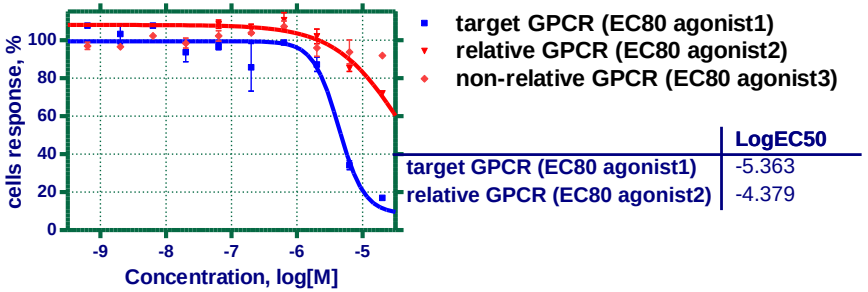
ChemDiv
The chemistry of cures℠

cmpd aaaa-xxxx (selective)



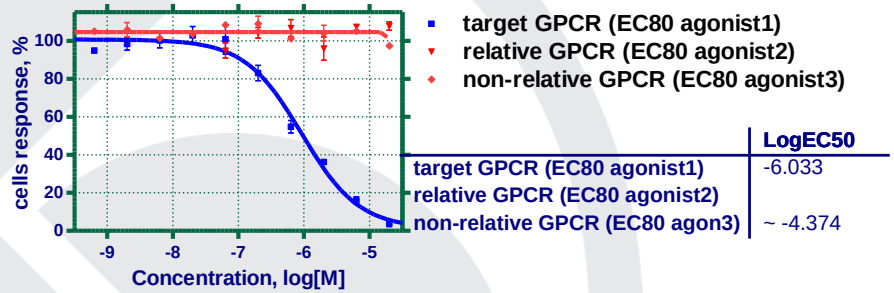
	LogEC50
target GPCR (EC80 agonist1)	-5.872
relative GPCR (EC80 agonist2)	
non-relative GPCR (EC80 agon3)	-9.022

cmpd cccc-rrrr (low selectivity)



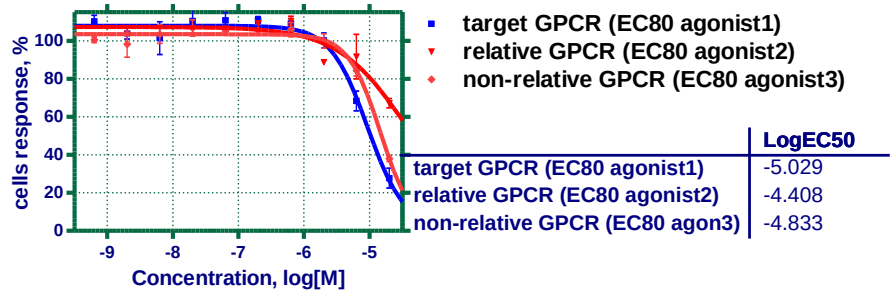
	LogEC50
target GPCR (EC80 agonist1)	-5.363
relative GPCR (EC80 agonist2)	-4.379

cmpd aaaa-yyyy (selective)



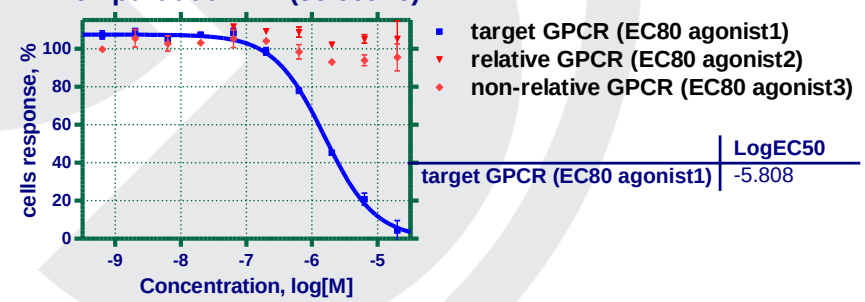
	LogEC50
target GPCR (EC80 agonist1)	-6.033
relative GPCR (EC80 agonist2)	
non-relative GPCR (EC80 agon3)	~ -4.374

cmpd dddd-ttttt (non-specific)



	LogEC50
target GPCR (EC80 agonist1)	-5.029
relative GPCR (EC80 agonist2)	-4.408
non-relative GPCR (EC80 agon3)	-4.833

cmpd bbbb-zzzz (selective)



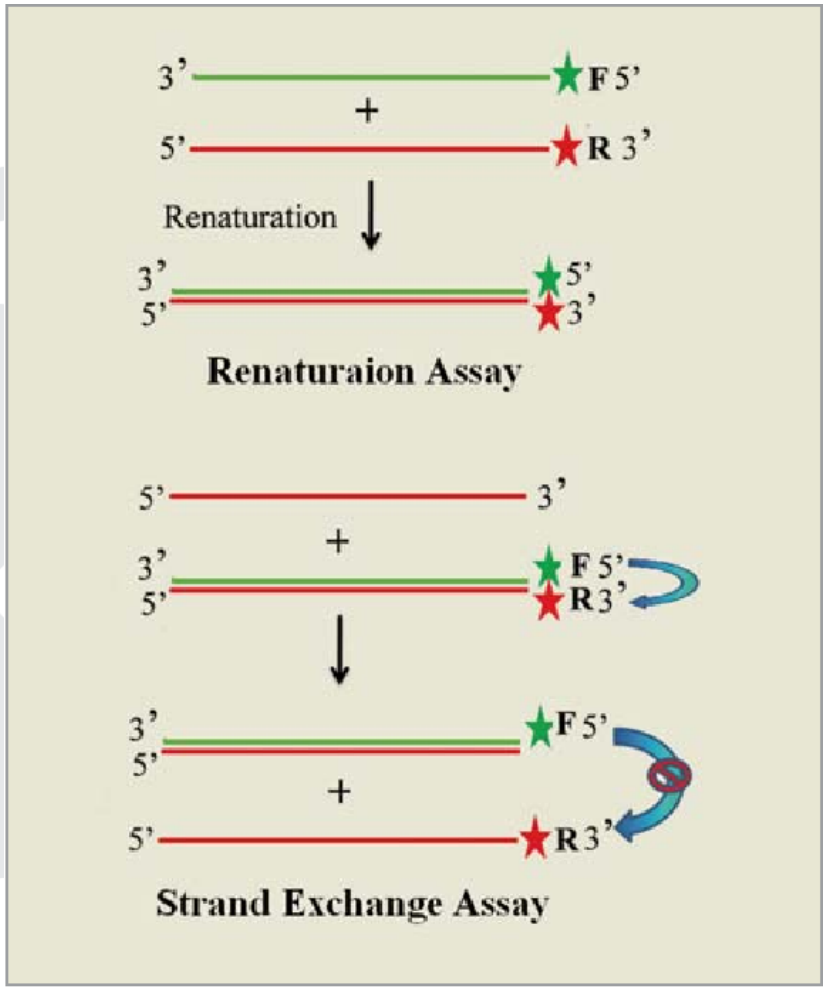
	LogEC50
target GPCR (EC80 agonist1)	-5.808

ChemDiv
The chemistry of cures℠

Enzymatic assay Case Study

Discovery™ outSource
Target to Lead
Lead to Candidate
Candidate to POC
POC to Market

Strand exchange assay successfully used in HTS

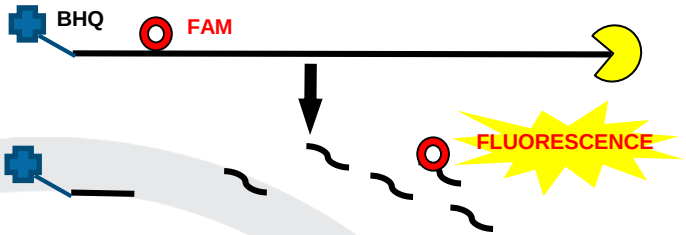


Enzymatic DNA repair assays

Schematic representation of renaturation and strand exchange assay monitored by FRET. Fluoresciene (F) and Rhodamine (R) used as a FRET pair in these assays. FRET in case of renaturation as both the dyes are juxtaposed after annealing and loss of FRET in case of strand exchange as F and R were separated as result of strand exchange was monitored.

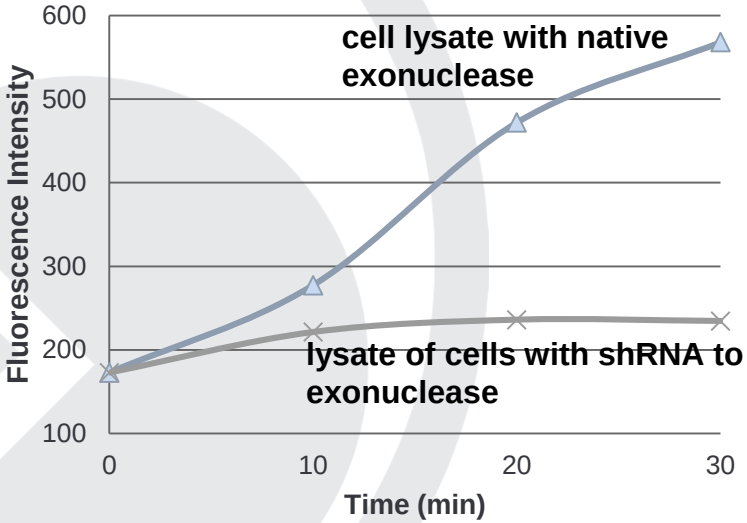
Also fluorophore-quencher pair may be used in this assay (like FAM-BHQ1)

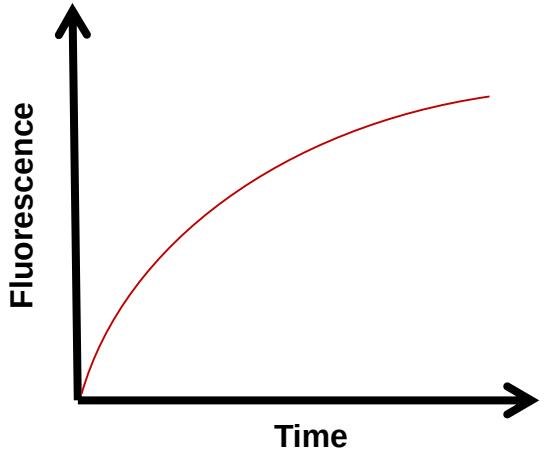
High throughput approach to measure exonuclease activity



ssDNA+exonuclease $\xrightarrow{ATP}$ nucleotides+signal

ADP





HTS assay validation: plate uniformity and signal variability assessment (one of the assay)

ChemDiv
The chemistry of cures℠

Impact of Midpoint Percent Activity Change on Ratio of EC50/IC50/Ki

Mid % data

	Plate 1	Plate 2	Plate 3	Day Ave.
Day 1	75.61	79.47	78.24	77.77
Day 2	70.72	74.82	75.00	73.51

Mid % difference within Days

	Plate 1 - Plate 2	Plate 1 - Plate 3	Plate 2 - Plate 3
Day 1	-3.86	-2.63	1.23
Day 2	-4.10	-4.28	-0.18

Mid % difference between Days

Day 1 - Day 2
4.26

Typical Value for Slope of dose-response curve? 1

Ratio EC50/IC50/Ki within Days (larger over smaller)

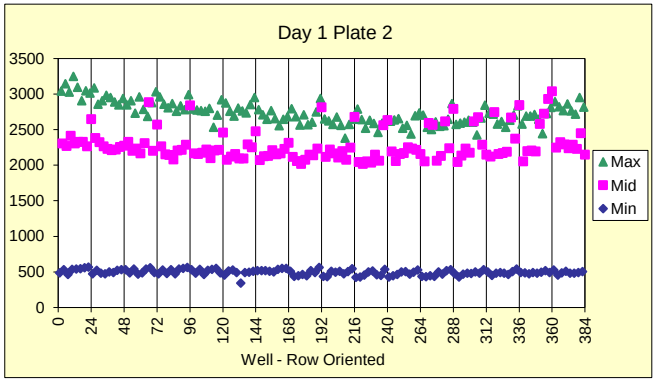
	Plate 1 - Plate 2	Plate 1 - Plate 3	Plate 2 - Plate 3	
Day 1	1.2	1.2	1.1	Meets Criterion
Day 2	1.2	1.2	1.0	Meets Criterion

Ratio EC50/IC50/Ki between Days (larger over smaller)

Day 1 - Day 2	
1.3	Meets Criterion

Validation Checklist

Intra-Plate Tests		Meets Criterion ?
1	Check for drift and edge effects in all plates (you must check manually)	
2	All max (HI) signal CV's < 20%	Yes
3	All mid signal (unnormalized) CV's < 20%	Yes
4	All normalized mid signal (mid %) SD's < 20	Yes
5	All min (LO) SD's < Min(max (HI) SD, mid SD)	Yes
6	All SW's >2	Yes
7	All Z' Factors > 0.4 (and < 1 ; must pass one of 6 or 7)	Yes
Inter-Plate Tests		
1	All within-day fold shifts < 2	Yes
2	All Average (between-)Day fold shifts < 2	Yes

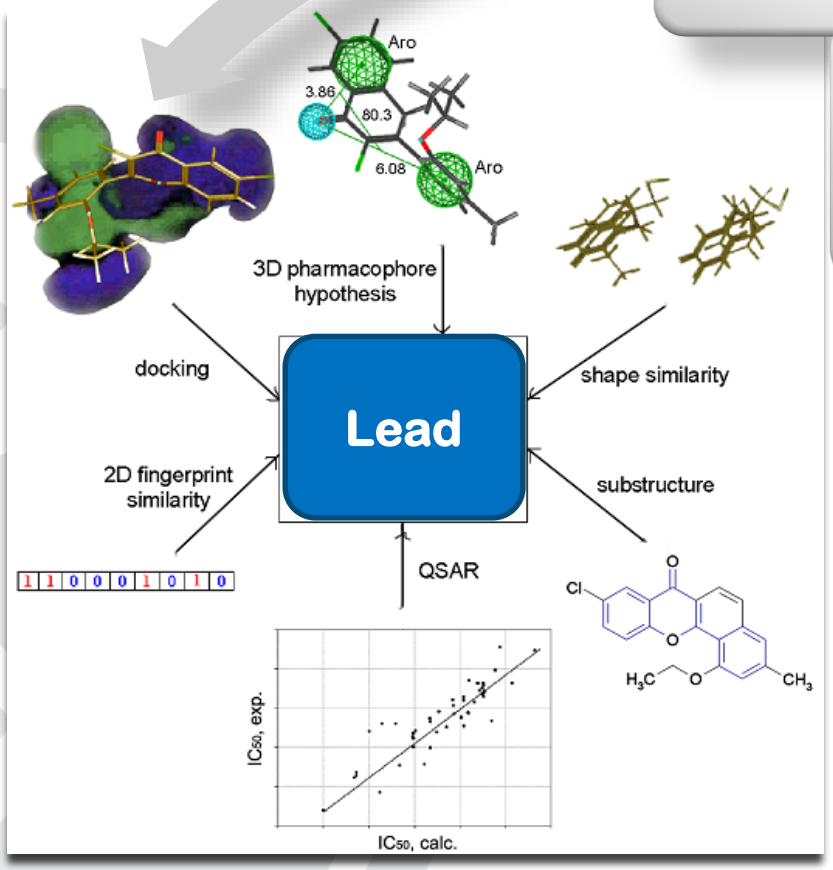


Conclusions:

1) All assay parameters meets with criteria
2) Mean Z-factor in assay validation is 0.73, minimum is 0.67 (should be not less then 0.4)
3) No any significant trends effects was observed,
4) Some edge effect observed (see example of one of the plate above) but isn't crucial in this case (not exceed 5% of mean value) and anyway Z-factor still significantly higher then acceptable level without excluding of edge wells
5) Totally, assay may be used for searching inhibitor in HTS

AFTER FINDING HITS

ChemDiv
The chemistry of cures℠



Quick establishment of Hit Series vs. Singletons

In house synthesis of all selected compounds allows for Quick Hit Clusterization and SAR

Chemistry On Demand ™
15,000 pre-developed Chemical Libraries allows for fast Hit explosion synthesis

Fast re-synthesis, scale up Iterative design, and novel chemistry series / IP

Discovery™ outSource

Target to Lead
Lead to Candidate
Candidate to POC
POC to Market

FOLLOW UP OPTIONS

ChemDiv
The chemistry of cures℠

- **Hit Confirmation, CRCs, hit characterization**
  - Hit Material (in desired amount)
  - Hits Re-synthesis, Scale-up synthesis, synthetic protocols
- **Specificity/Selectivity profiling**
- **Mechanism of action determination**
- **MedChem optimization in H2L program**
  - Supply of Analogs (for the same templates) from 1.5M+ library
  - Rapid expansion on identified active templates (focused libraries)
- **Preclinical Development**
  - Physiochemical profiling
  - Biochemical profiling
  - Cell-based tox profiling
  - DMPK
  - Bioavailability
  - ADME/Tox optimization

ChemDiv
The chemistry of cures℠

Thank You!