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Cell-based Receptor Tyrosine Kinase Screening Reloaded

Development of a new cellular assay system for a target protein so far resistent against HTS formats

Benjamin Bader

SBS-meeting Lille 2009

- Rationale for cell-based screening of RTKs
- Some project history
- The DiscoveRx workplan
- Assay development data
- Assay transfer, adaptation and optimization
- Summary and Outlook

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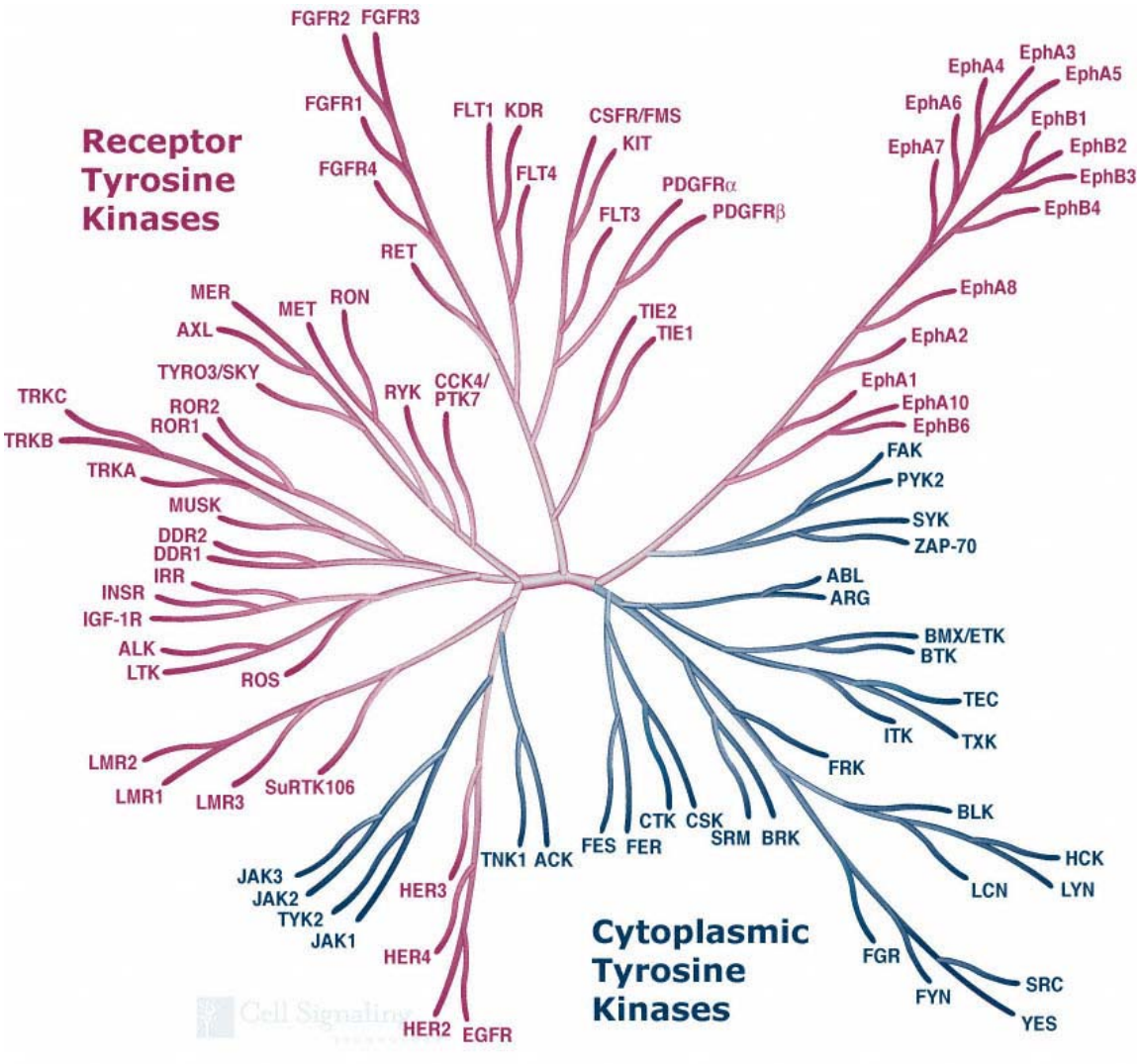
Receptor Tyrosine Kinases



RTKs play important roles in fundamental cellular processes like

- proliferation,
- differentiation,
- migration,
- metabolism
- survival

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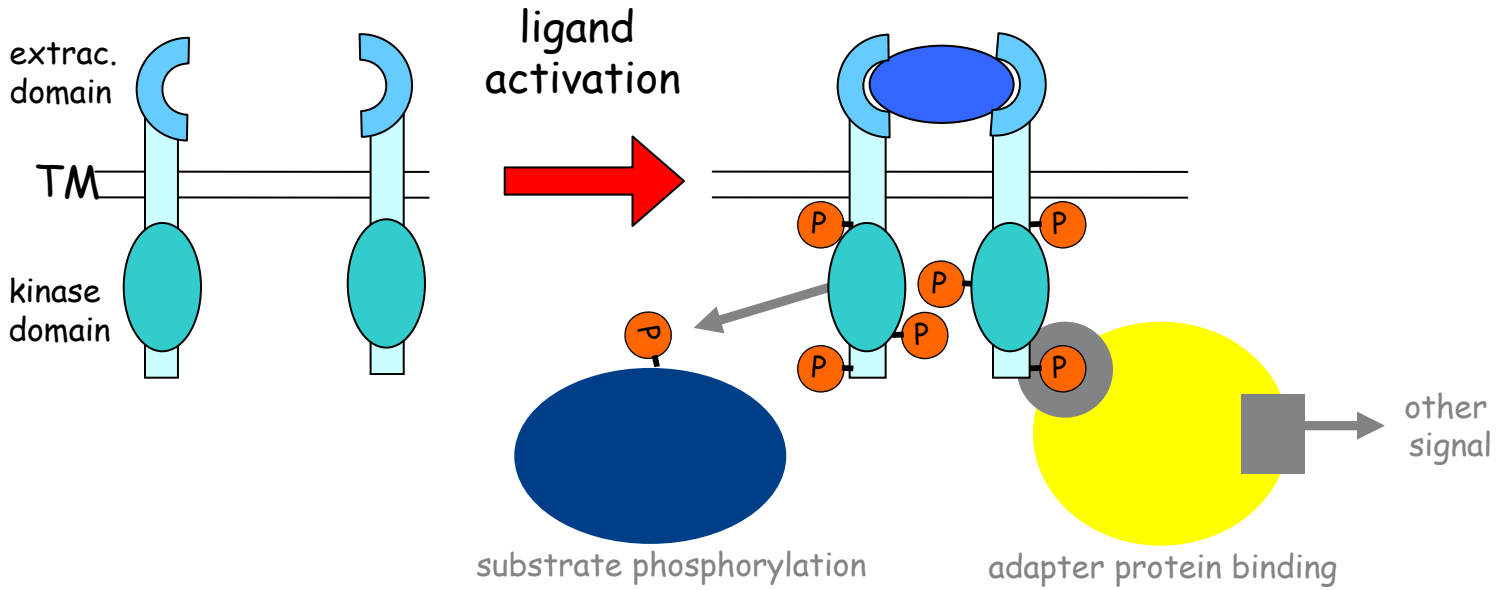
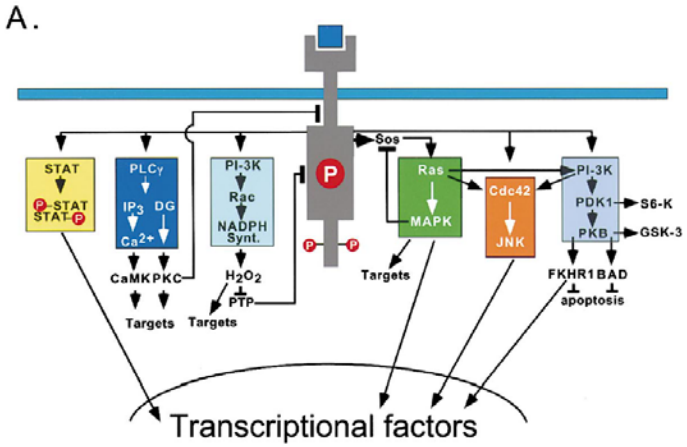
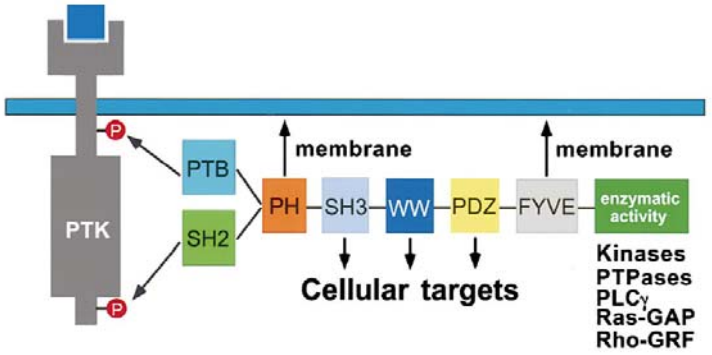
General scheme of activation and signal transduction of RTKs







Adapted from: Schlessinger, 2000, Cell 103, 211-225

Advantages of a cell-based RTK assay in comparison to enzymatic kinase assays

- ***full length protein***, not only kinase domain
- physiological ***membrane environment*** of RTK
- ***native substrate*** compared to peptides in enzyme assays
- the RTK is present in its ***inactive state*** when compounds are added
- possibility to find ***prodrugs*** which can be activated inside the cell
- ***inhibitors of ligand binding site*** can be found

Chance to find new inhibitors with different binding modes

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Project history

RTK target protein has been screened using enzymatic assays

- considerable amount of IC50 hits with activity in cellular assays
- but: no lead structure identified

Cellular ELISA assay has been set-up as secondary assay

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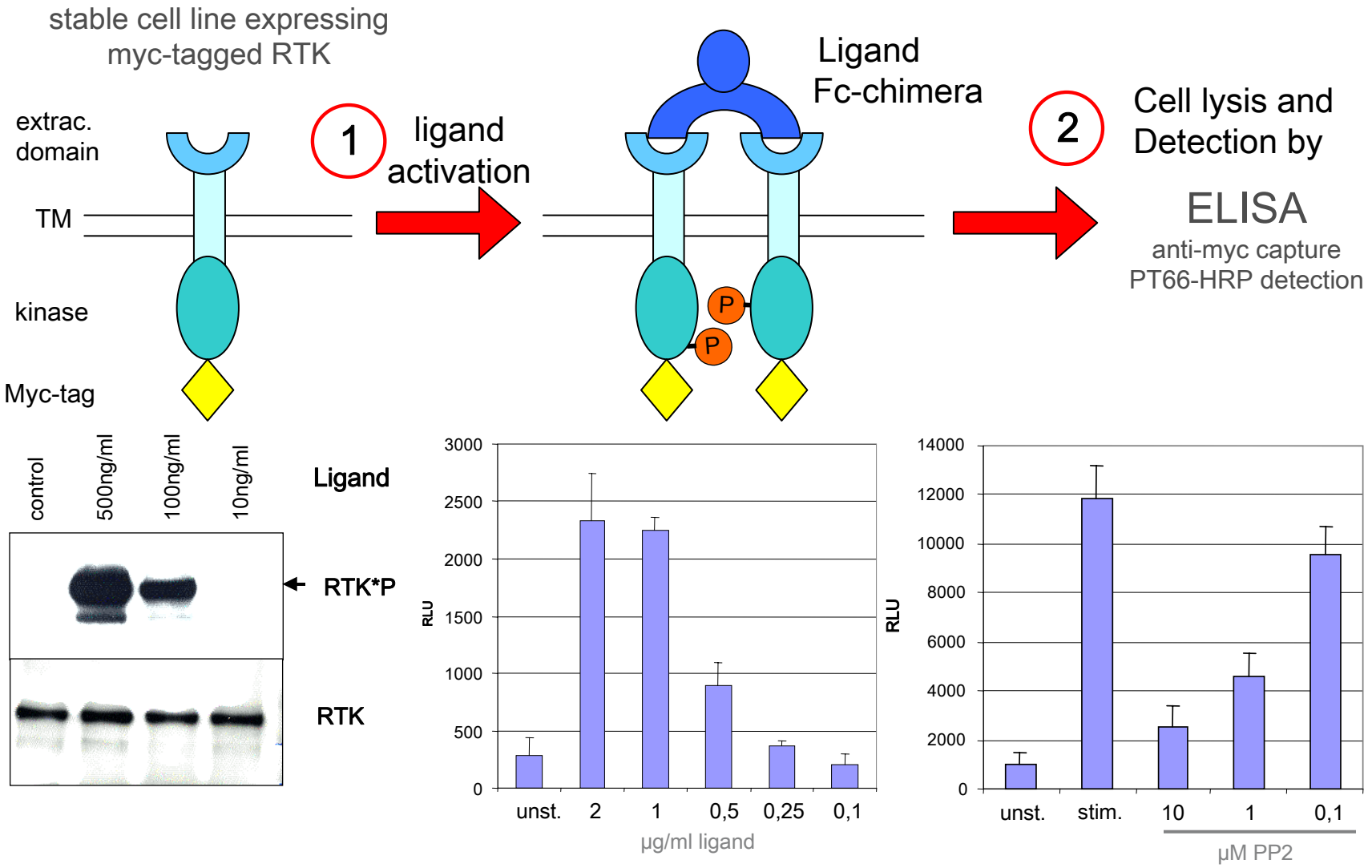
Cellular autophosphorylation ELISA in 384 well



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Project history

RTK target protein has been screened using enzymatic assays

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Cellular ELISA assay has been set-up as secondary assay

- worked well in 384 well
- but: too complicated for full HTS
- and: ELISA in 1536 well ?!?!

Homogenous formats were developed (HTRF)

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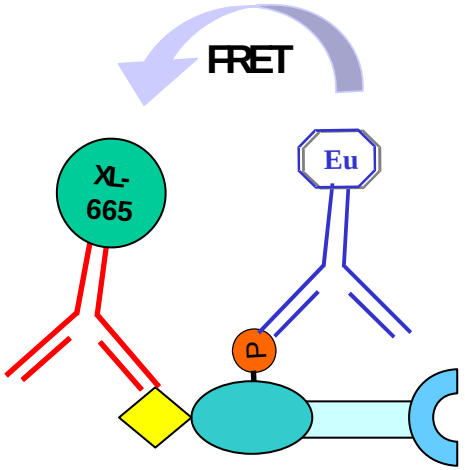
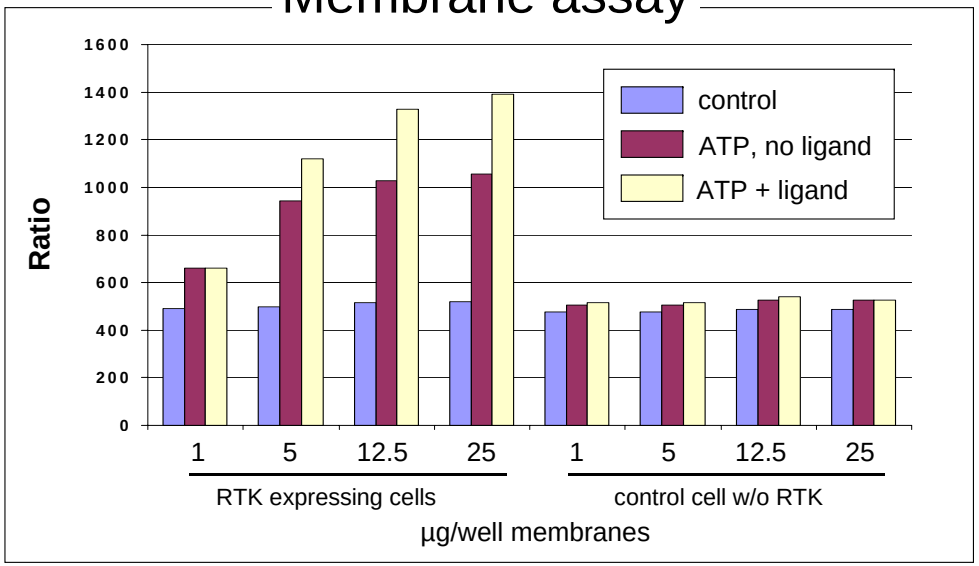
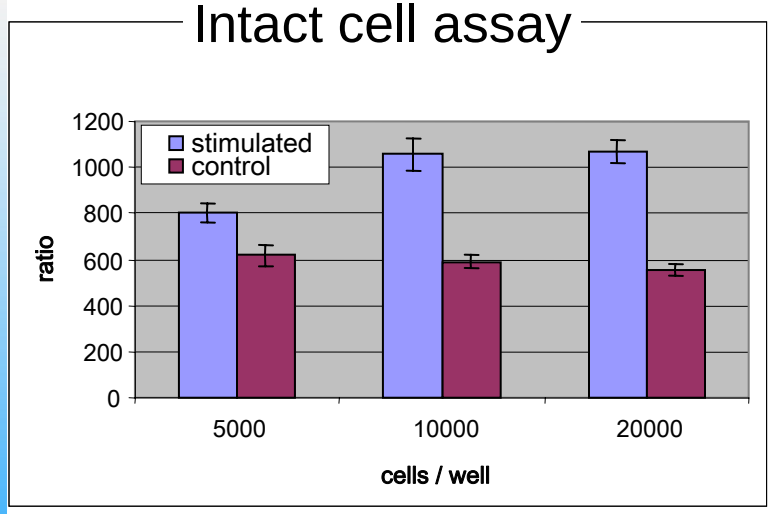
HTRF detection



Membrane assay



Intact cell assay



Membrane assay:

- Homogenous format possible
- Strong ligand independent signal

Intact cells assay:

- S/B too low for 384 well assay
- Too many cells needed
- protocol contains several wash steps

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Homogenous formats were developed (HTRF)

- Membranes feasible in 384 well
- but: ligand independent signal was not accepted by project team

New option: DiscoveRx

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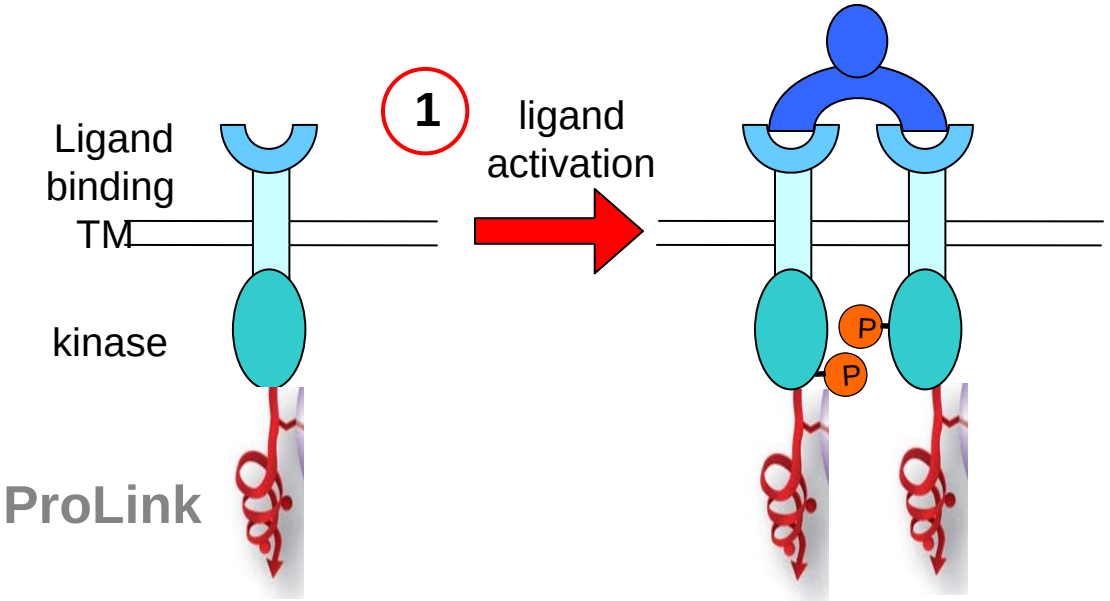
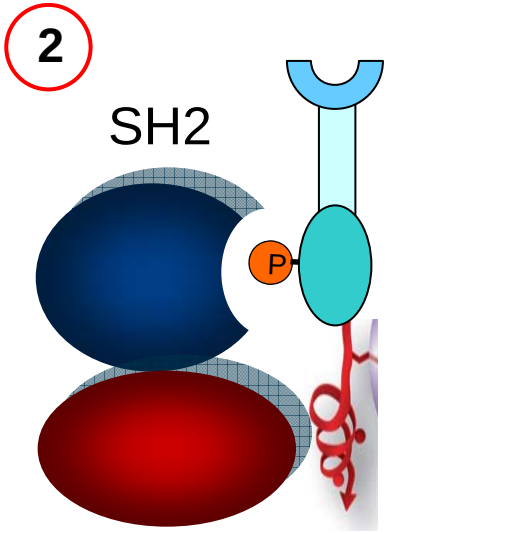
Concept for RTK activation assay (DiscoveRx):





needs one cell expressing both:

- RTK tagged with ProLink peptide
- pTyrosine binding partner for RTK as EA-fusion: e.g. SH2-domain

chemiluminescent signal

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The DiscoveRx workplan

Aim: build a PathHunter protein-protein interaction assay for monitoring autophosphorylation of the target RTK through the interaction of an SH2 binding domain

Milestone 1

cloning of vectors
protein expression in CHO-K1

Milestone 2

retroviral co-infections of
RTK + SH2-domain (3 pairs)
2 week selection and functional test

Milestone 3

Show ligand-mediated auto-phosphorylation of RTK in Western Blot

Milestone 4A-C

stable cell line generation of one RTK + SH2 pair:
clonal stability, functional tests, reference compounds, DMSO-tolerance, HTS-compatibility (%CV, S/B, cells/well), viability, Mycoplasm test

Milestone 5

reproduce data at BSP Screening Berlin

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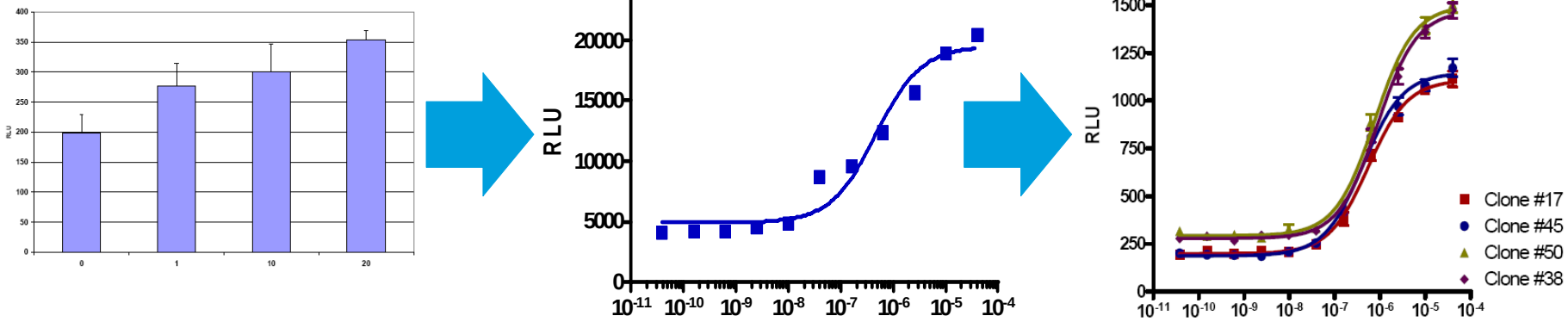
The data – Milestone 2

Aim: Find suitable RTK + SH2 pair and show functionality in the EFC-assay

- First of all: this was the hardest part
- More than 3 different SH2-domains were tested
- Other cell backgrounds were tested



CHO RTK/Shc1
S/B = 1.7

U2OS RTK/Shc1
S/B = 4

U2OS RTK/PTPN6
S/B = 5-6

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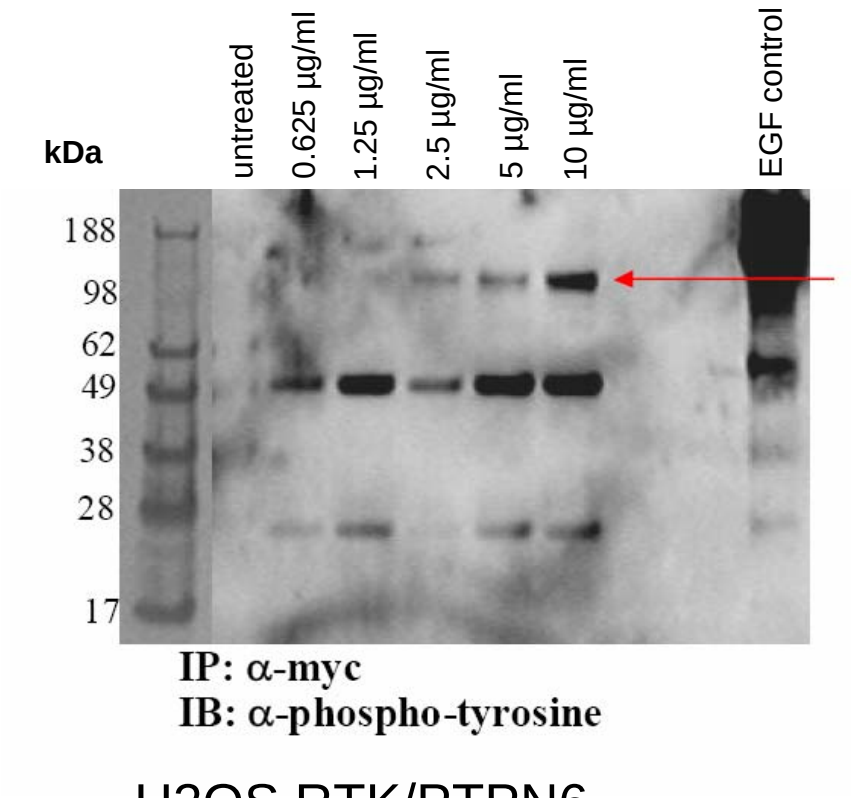
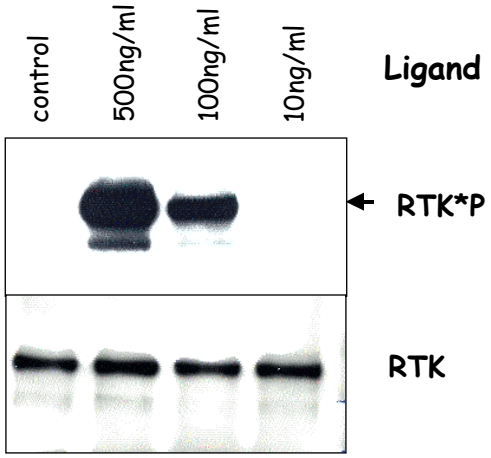
The data – Milestone 3

Aim: Show ligand-mediated auto-phosphorylation of RTK in Western Blot



Phospho-RTK-PK
Expected size: 108 kDa

U2OS RTK/PTPN6



BSP data in CHO-cells

Data in line with expected outcome

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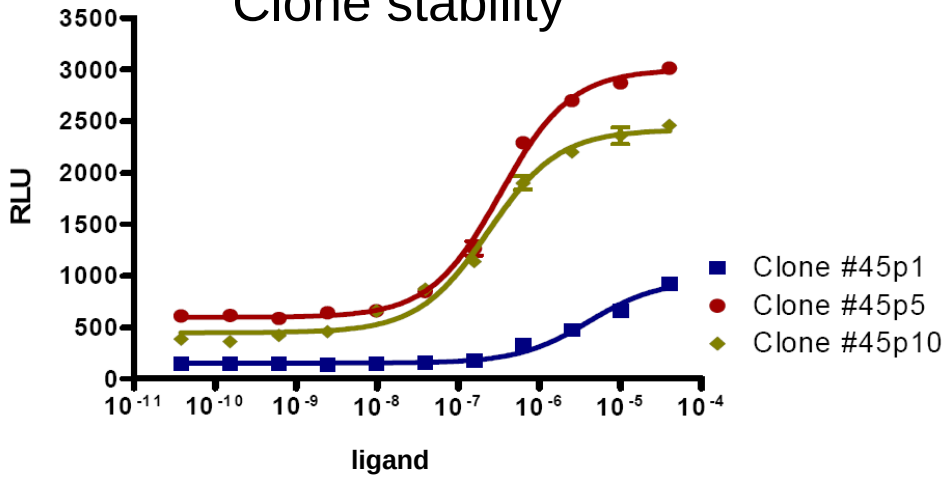
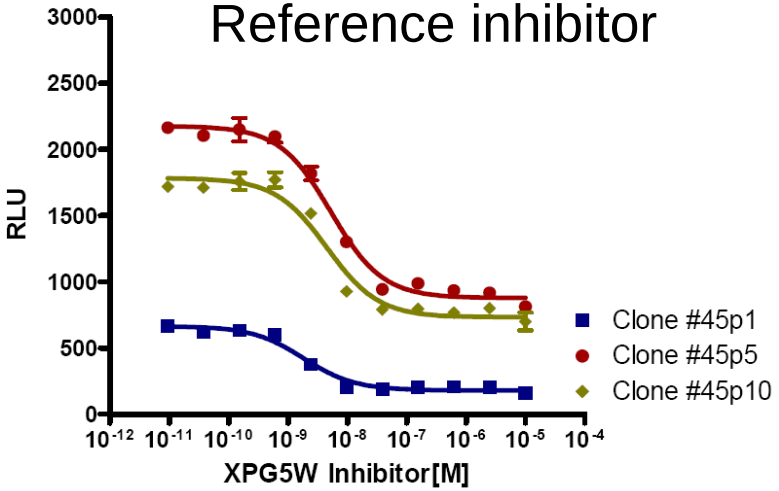
The data – Milestone 4

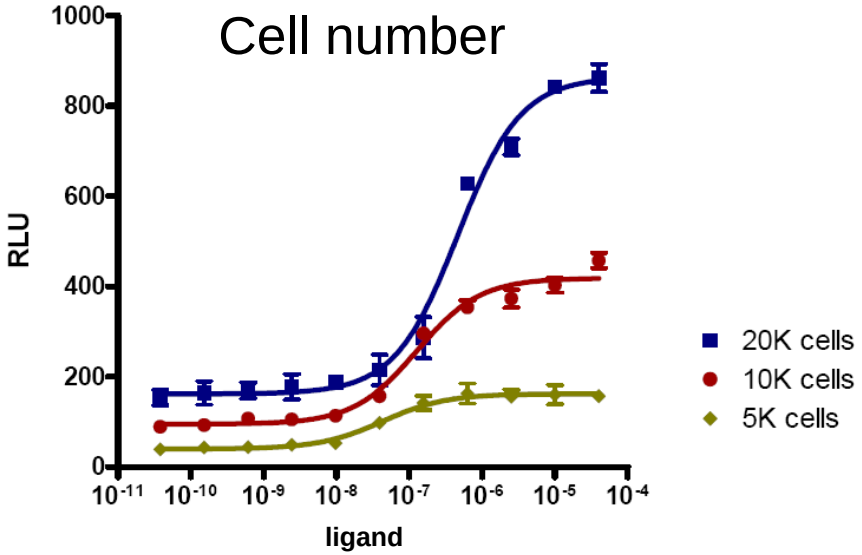
Clone stability



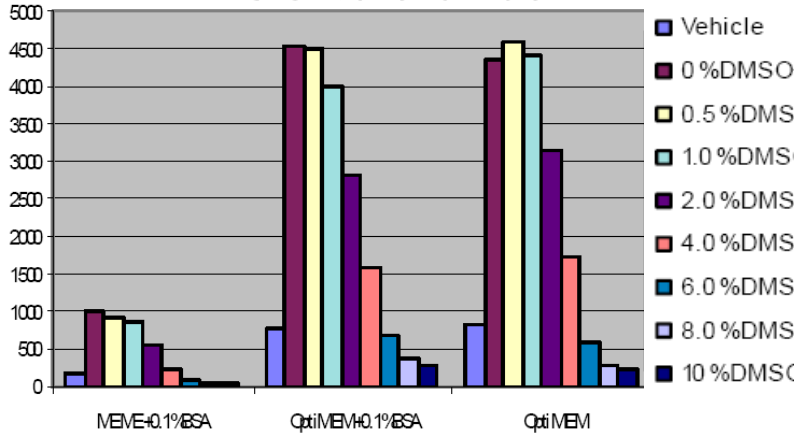
Reference inhibitor



Cell number



DMSO tolerance



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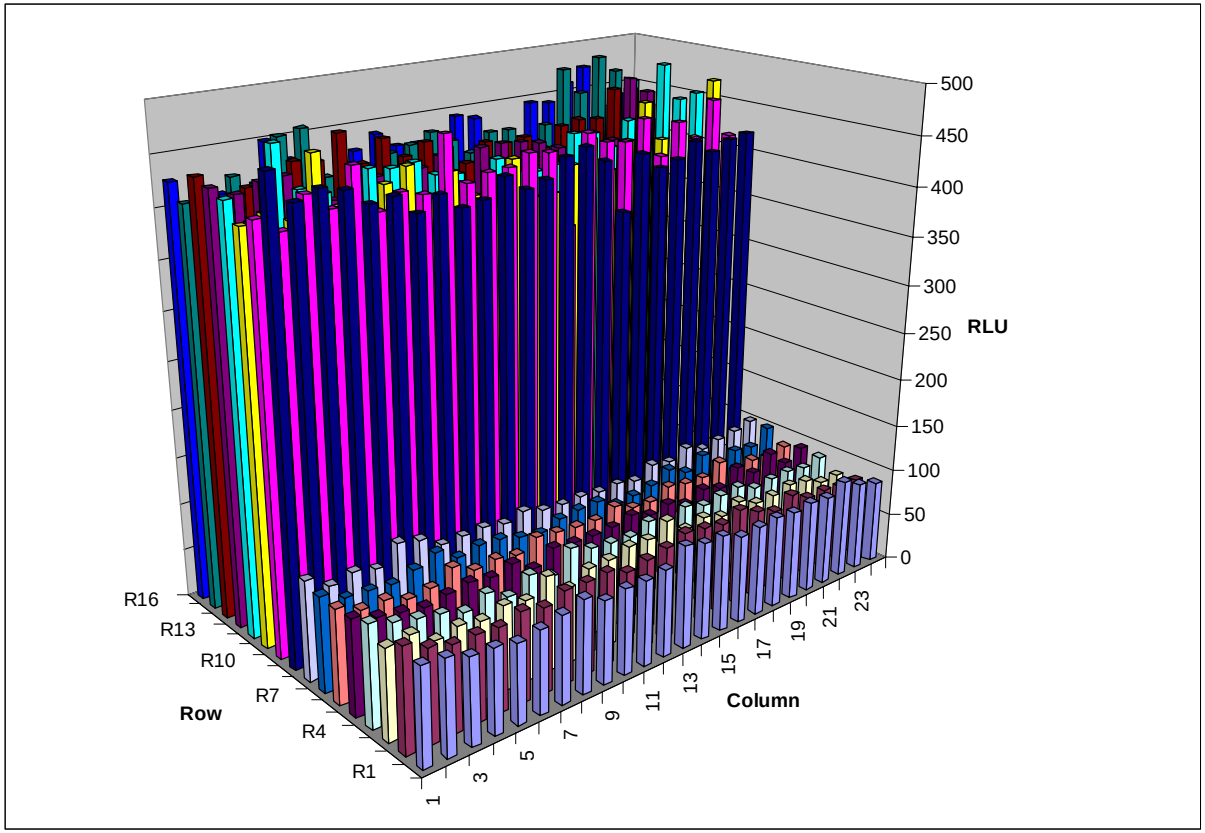
The data – Milestone 4 cont.

Aim: show Z‘-factor > 0.55 for whole plate 384 well assay with 10.000 cells



	mean	SD	%CV
high	424.7	22.3	5%
low	89.7	6.5	7%

S/B	**4.7**
Z'F	**0.74**

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Assay transfer, adaptation, optimization

1. Does the DiscoveRx protocol work?
2. Can we miniaturize ?
3. Can we use frozen cells ?

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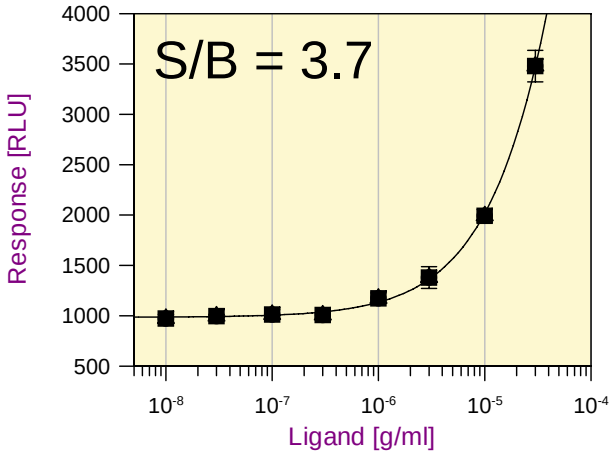
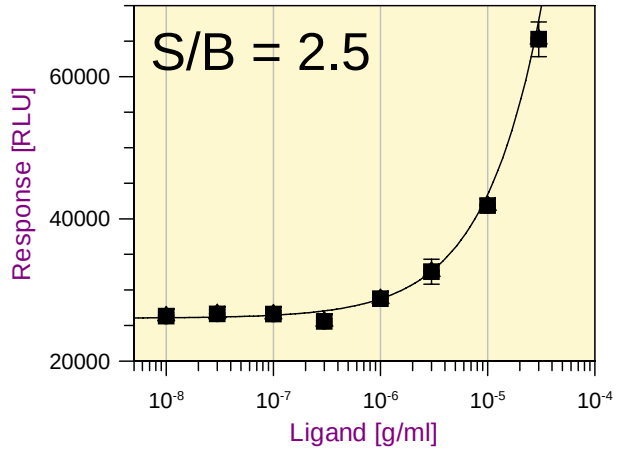
1. Does the DiscoveRx protocol work?

Steps	Volumes (384-well)
Step 1: Plate Cells & Incubate Overnight @ 37ºC	Add 20 µL of cells in each well at a preferred density of ~10,000 cells per well. Cells should be seeded in MEME +0.1% BSA.
Step 2: Treat Cells @ RT	Add 5 µL of 5X concentrations of ligand made up in MEME (3-fold serial dilutions, highest final concentration = 40 µg/mL). Treat for 1 hour at room temperature.
Step 3: Add CL Mix @ RT	Add 12 µL of CL mix. Incubate at room temperature and in the dark for 60 minutes.
Step 4: Read Samples	Samples can be read on any standard luminescence plate reader.

PE ViewLux



BMG Pherastar



Yes, but…

…EC50 of ligand quite high, no saturation

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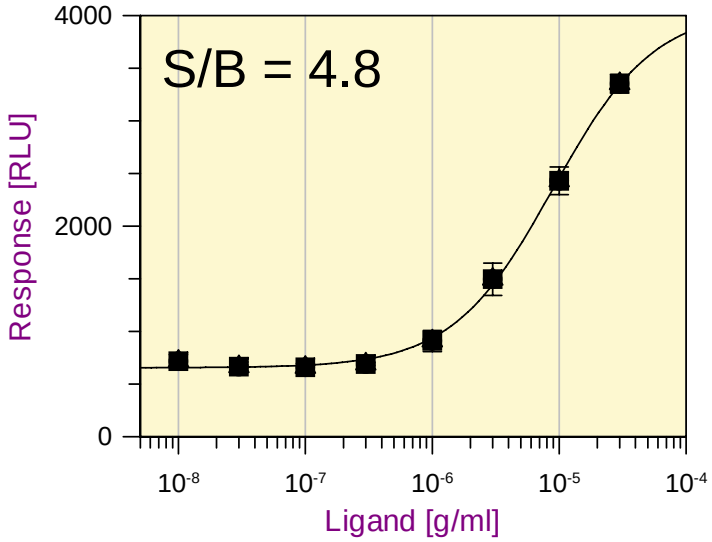
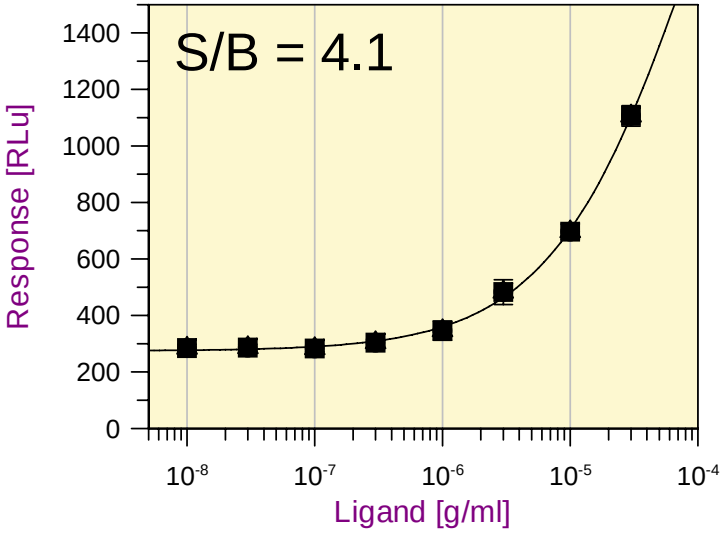
2. Can we miniaturize ?

1. 5 µl cells (5000) into 384 SV tissue culture plates, ON 37°C
2. 2 µl Ligand stimulation, 60 min RT
3. 3 µl detection

Standard protocol, 10000 cells



Small volume (5000 cells)



Yes, we can…

- less signal, but similar S/B
- again EC50 of ligand too high, no saturation

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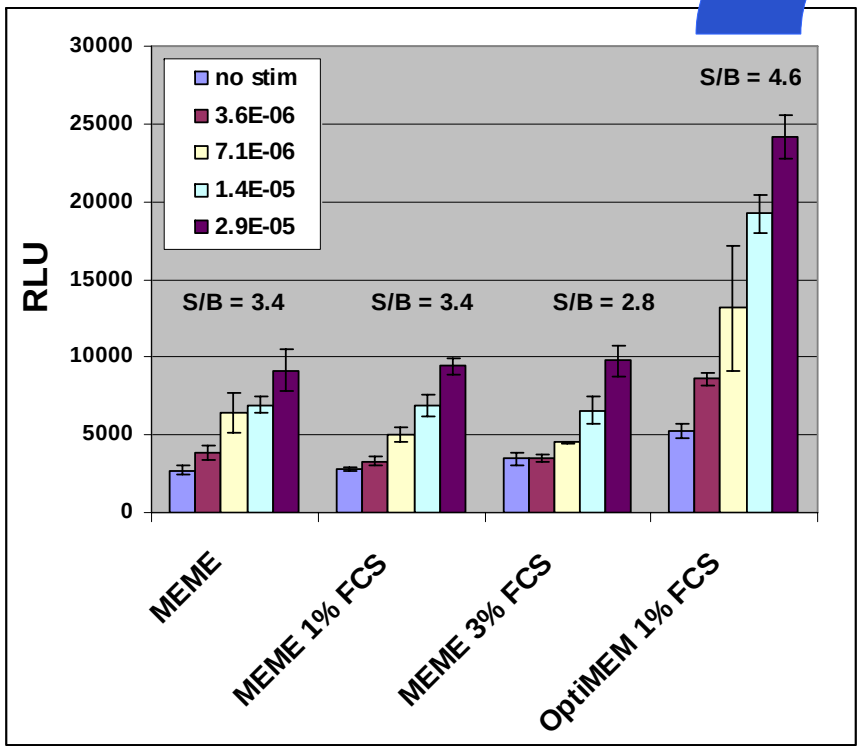
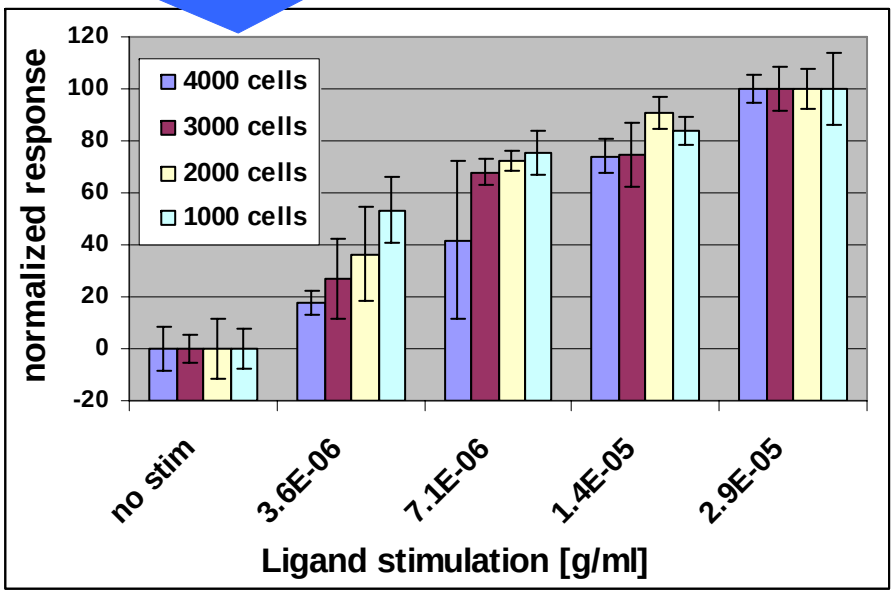
3. Can we use frozen cells ?

1. Thaw frozen cells and dilute in MEME + 0 / 1 / 3 % FCS or OptiMEM + 1% FCS
2. 5 µl cells (4000) into 384 SV tissue culture plates, ON 37°C
3. 2 µl Ligand stimulation, 60 min RT
4. 3 µl detection





cell #	4000	3000	2000	1000
S/B	4.6	4.5	3.9	3.8
Z'F	0.7	0.6	0.6	0.4
EC50 (µg/ml)	10	5.7	4.7	3.9

Yes, we can…

- FCS has no impact
- OptiMEM is enhancing signal and S/B
- Less cells in OptiMEM improves EC50

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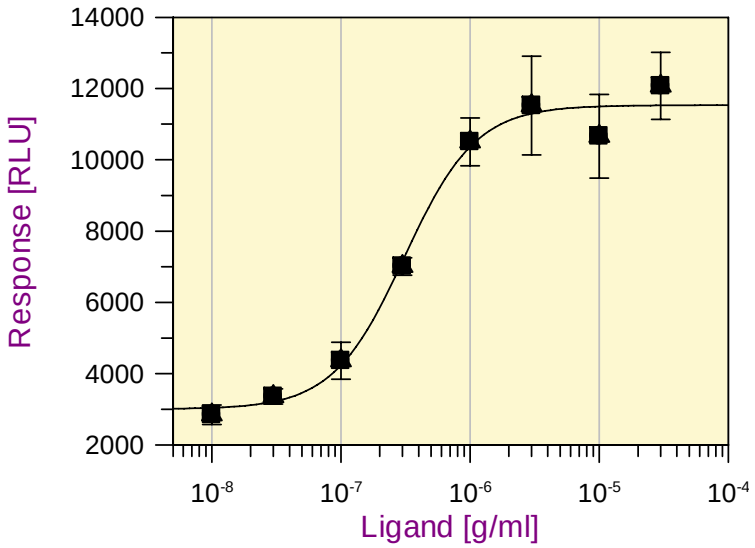
4. Optimized assay using OptiMEM

OptiMEM without additives optimal for this assay

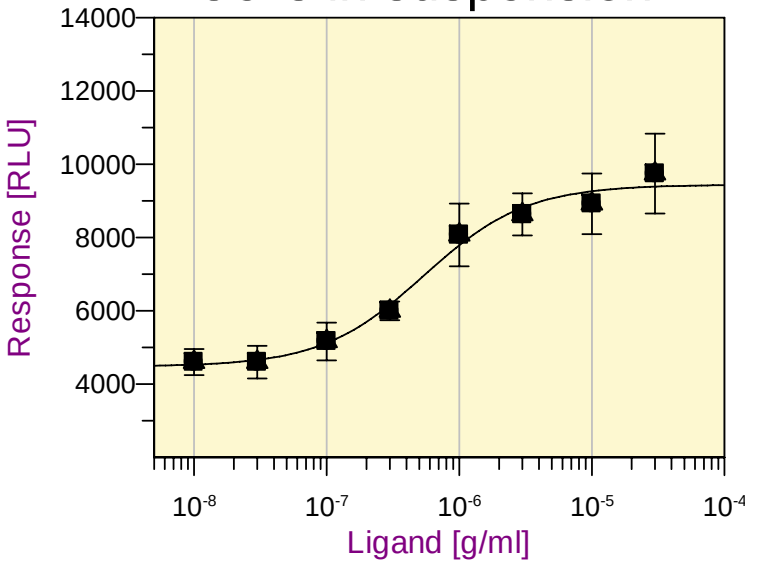
Cells adherent



S/B = 4.2

EC50 = 0.3 µg/ml

Cells in suspension



S/B = 2.2

EC50 = 0.5 µg/ml

- EC50 as expected
- Adherent cells with superior S/B

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Summary and Outlook

- A cell-based, ligand inducible RTK-SH2 interaction assay was developed by DiscoveRx in an assay development project with Bayer Schering Pharma AG.
- The assay meets the requirements in terms of S/B, Z‘-factor and IC50s of reference inhibitors
- The assay could be successfully miniaturized and applied to frozen cells
- Further work will aim at testing robustness in automation and 1536 well format

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