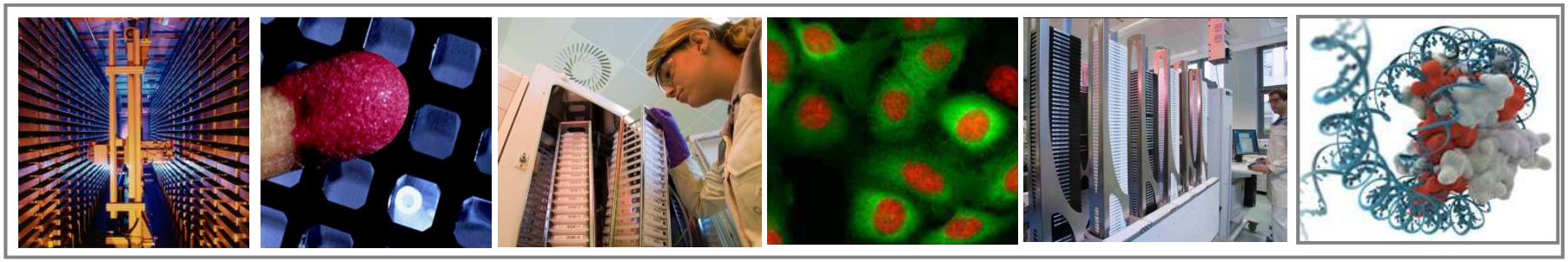


Cell-based kinase assays in HTS

Potential and limitations for primary and secondary screening

Benjamin Bader

HTRF-symposium Avignon 25. April 2013

- Introduction
- General considerations cell-based kinase assays
- EFC-assays for Tyr-Kinases
- TR-FRET systems: HTRF and Lanthascreen assays for the PI3K / Akt / mTOR pathway
- Summary & conclusions

Kinases as drug targets

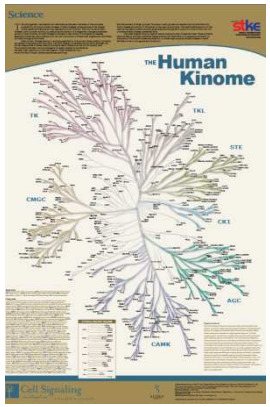
Registered kinase inhibitors

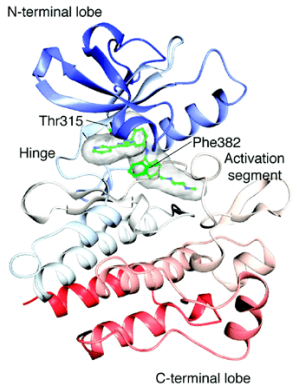
Compound	Kinase target	Cancer target	Company
Imatinib (Glivec, Gleevec, STI571)	ABL 1–2, PDGFR, KIT	CML, Ph+ B-ALL, MML, CEL, GIST	Novartis
Gefitinib (Iressa, ZD1839)	EGFR	NSCLC	AstraZeneca
Erlotinib (Tarceva, OSI-774)	EGFR	NSCLC, pancreatic cancer	OSI, Genentech Inc, Roche
Lapatinib (Tykerb, GW2016)	EGFR, ERBB2	Breast cancer	Glaxo SmithKline
Dasatinib (Sprycel, BM-354825)	ABL1–2, PDGFR, KIT, SRC	CML	Bristol Myers
Nilotinib (Tasigna, AMN107)	ABL1–2, PDGFR, KIT	CML	Novartis
Sunitinib (Sutent, SU11248)	VEGFR1–3, KIT, PDGFR, RET, CSF1R, FLT3	RCC, GIST	Pfizer
Sorafenib (Nexavar, Bay 43-9006)	VEGFR2, PDGFR, KIT, FLT3, BRAF	RCC	Onyx and Bayer Pharmaceuticals
Pazopanib (Votrient, GW-786034)	VEGFR1–3, PDGFR, KIT,	RCC	GlaxoSmithKline
Everolimus (Afinitor, Rad001)	mTOR	RCC	Novartis
Temsirolimus (Torisel, CCI-779)	mTOR	RCC	Wyeth

(Fabbro 2012)

recent additions

Vandetanib	VEGFR, EGFR, RET	thyroid	AstraZeneca
Vemurafenib	B-RafV600E	melanoma	Roche/Plexxikon
Regorafenib	multiKinase	colorectal	Bayer
Critozinib	ALK, ROS1	NSCLC	Pfizer
Bosutinib	BCR/Abl	CLL	Pfizer
Ruxolitinib	JAK1/2	Myelofibrose	Incyte/Novartis

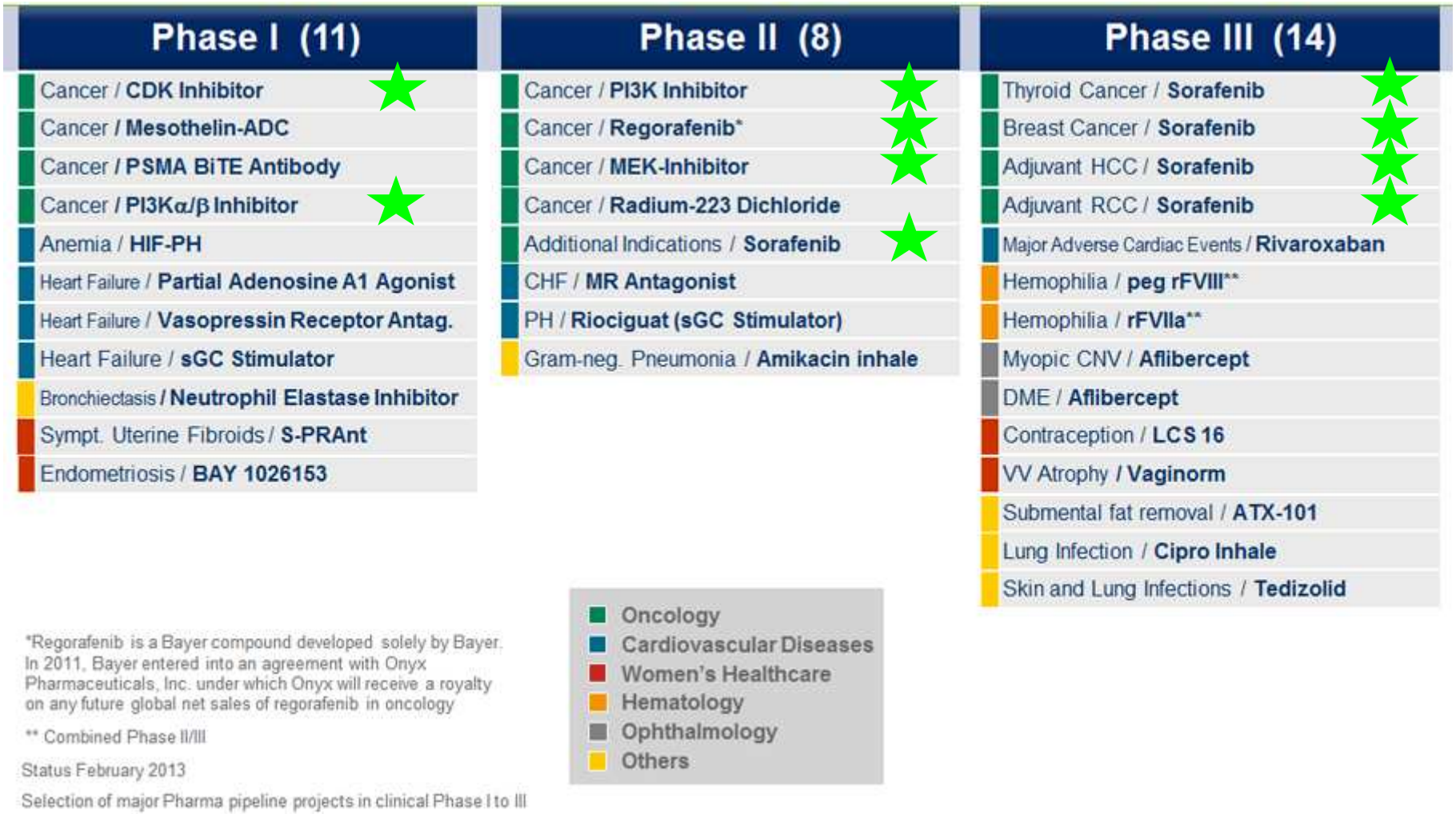




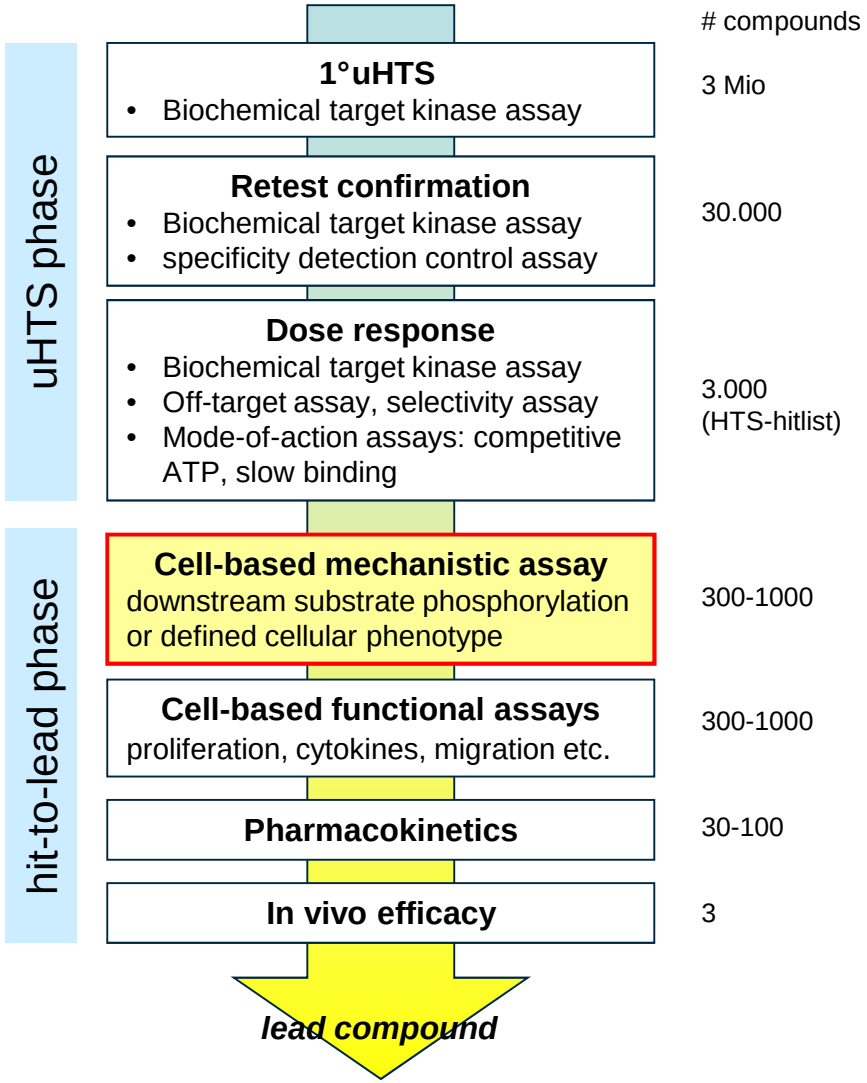
cAbl and Gleevec (Noble 2004)

- human kinome: 518 protein kinases + 20 lipid kinases (Manning 2002)
- currently, ~150 kinase targeted drugs are in clincal development (Fabbro 2012)
- most registered kinase drugs target Tyrosine kinases, with more Ser/Thr kinase targetd drugs in the pipeline
- most kinase drugs target the ATP-pocket
- main indication: oncology

Kinase Inhibitors in the BAYER Development Pipeline



Features of a typical early kinase drug discovery project



compounds

3 Mio

30.000

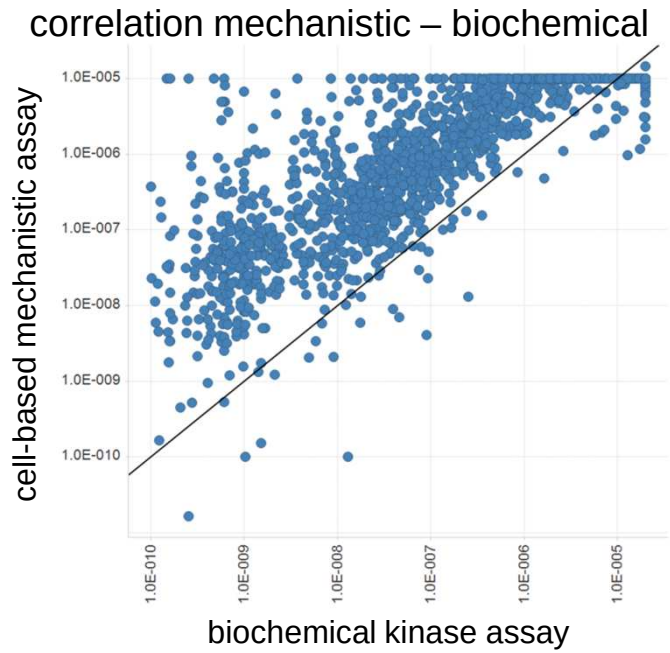
3.000
(HTS-hitlist)

300-1000

300-1000

30-100

3



- specific cell-based kinase assays are essential for hit profiling during hit-to-lead phase
- integration of cell-based kinase assays as early as possible, best case even before hitlist delivery

Requirements for efficient assay support in HTS and Hit-to-lead phase @ BAYER

Key objectives for every assay

(except HCA staining procedures)

- assay-ready plates
- 1536 well format, at least 384 well
- homogenous addition only
- endpoint assays
- frozen cells
- short
- robust

automated preparation of assay-ready plates

cells and reagent dispensing into 1536 well plates → addition-only

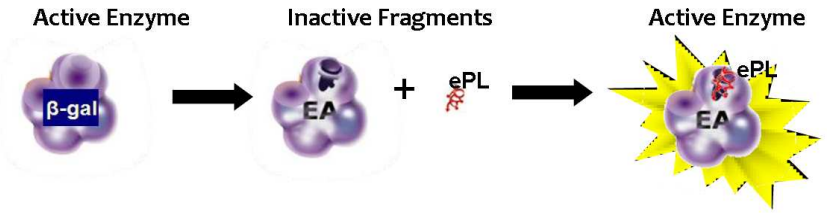
endpoint detection with imaging-based multimode-readers

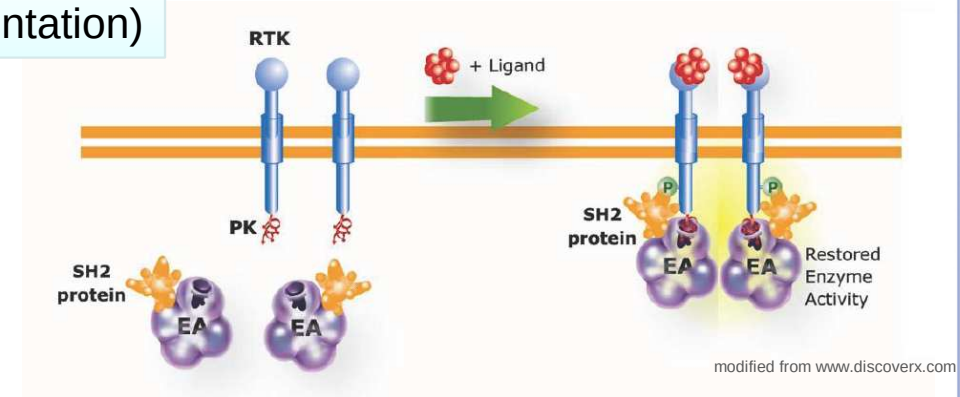
General options for cell-based kinase assays

Assay technology	Provider	comments	critical tools
Western Blot	various	heterogenous low throughput	phospho-antibody
Incell Western	various	high content imaging	phospho-antibody
ELISA-type	various	heterogenous	antibody pair
Luminex	Millipore	heterogenous	antibody pair
Surefire / ALPHAscreen	PerkinElmer	homogenous	antibody pair
HTRF	Cisbio	homogenous	antibody pair
Lanthascreen	LifeSciences	homogenous	phospho-antibody recombinant cell-line
EFC - Enzyme Fragment Complementation	DiscoveRx	homogenous	antibody-free recombinant cell-line
Survival assay	Advanced Cellular Dynamics / Carna	Tyr-kinase activity restores survival of Ba/F3 cells	antibody-free recombinant cell-line
Pathway reporter assays	various	downstream gene activation	antibody-free recombinant cell-line

EFC-assays for Tyr-Kinases (DiscoveRx)

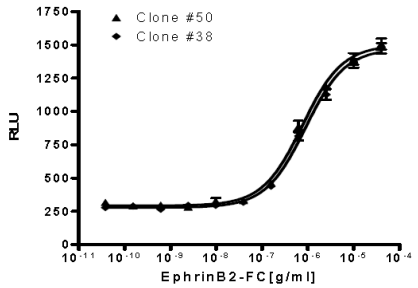
Technology EFC (enzyme fragment complementation)



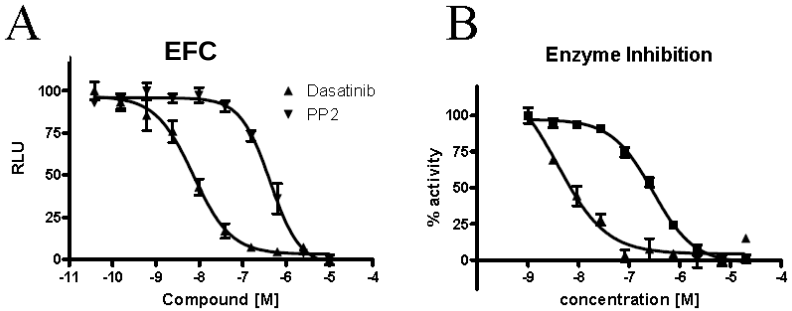


EphB4 cellular kinase activity assayed using an enzymatic protein interaction system
(Wehrman et al. 2013 ADT)

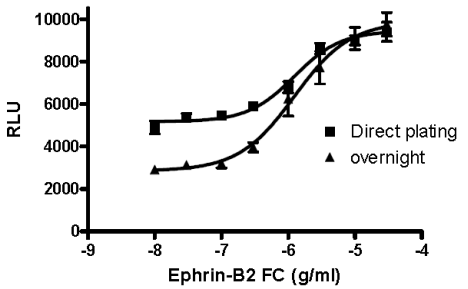
Agonist stimulation



Validation with kinase inhibitors

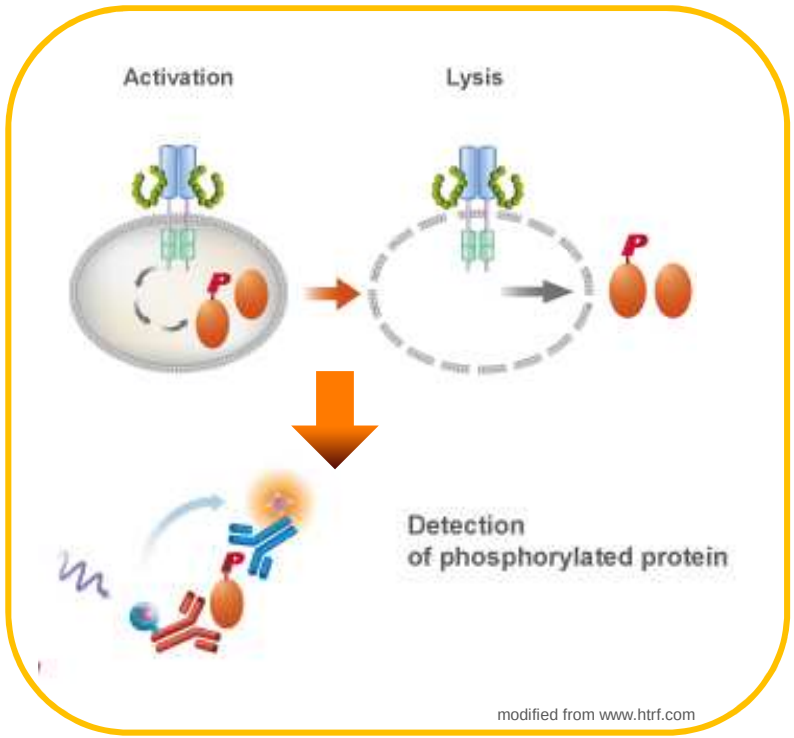


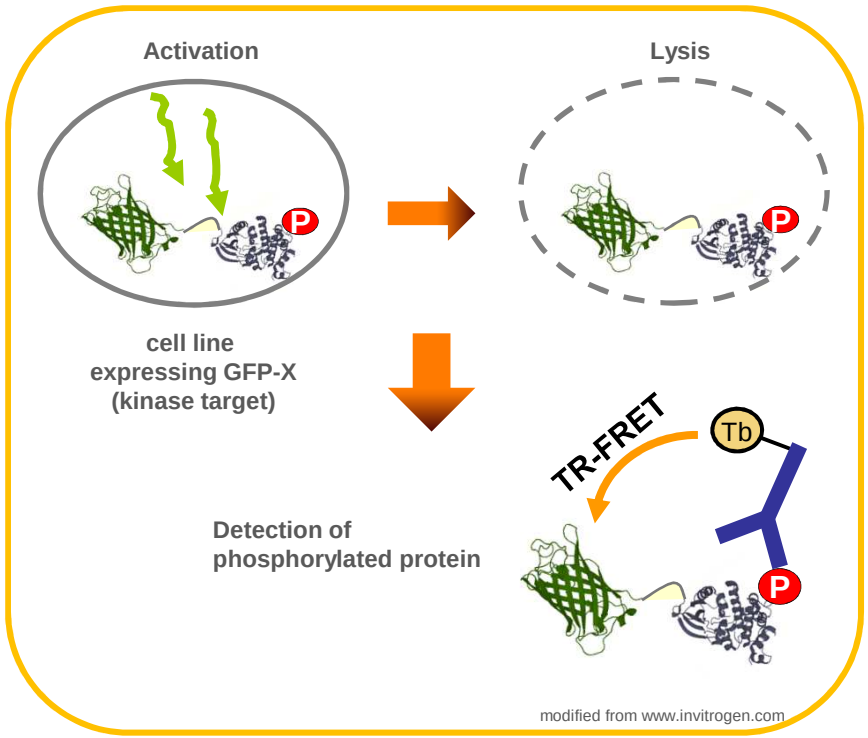
Frozen cell and direct assay format



- EFC-kinase technology is suitable for miniaturized frozen cell assays
- limitations: largely restricted to Tyrosine kinases, requires recombinant cells

TR-FRET systems: HTRF and Lanthascreen





HTRF

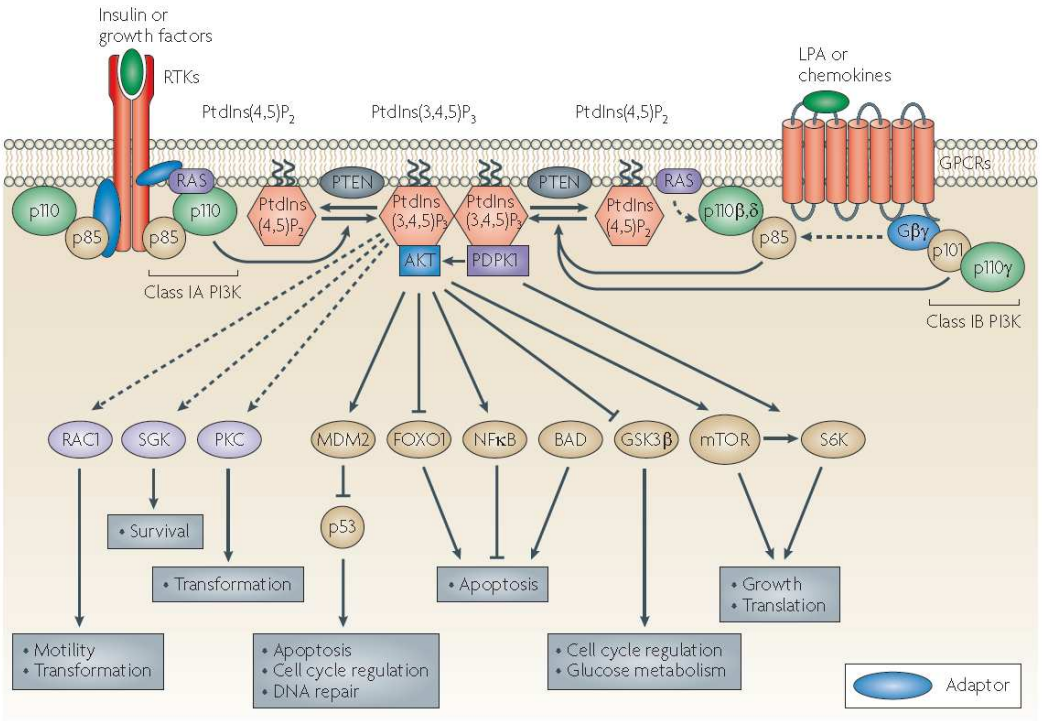
- endogenous phospho-protein
- any cell line expressing protein
- antibody pair required
- HTRF-detection at 620/665 nm

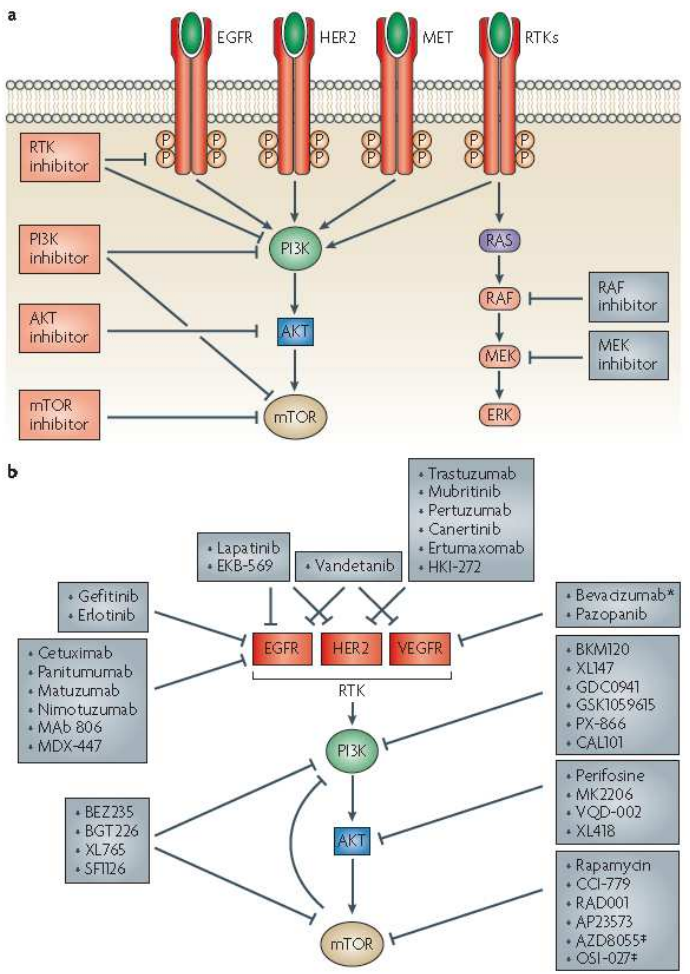
Lanthascreen

- overexpressed GFP-phospho-protein
- stable cell line or BacMam transient
- only phospho antibody required
- Lanthascreen detection at 490/520

HTRF and Lanthascreen assays for the PI3K / Akt / mTOR pathway

Introducing the PI3K / Akt / mTOR pathway

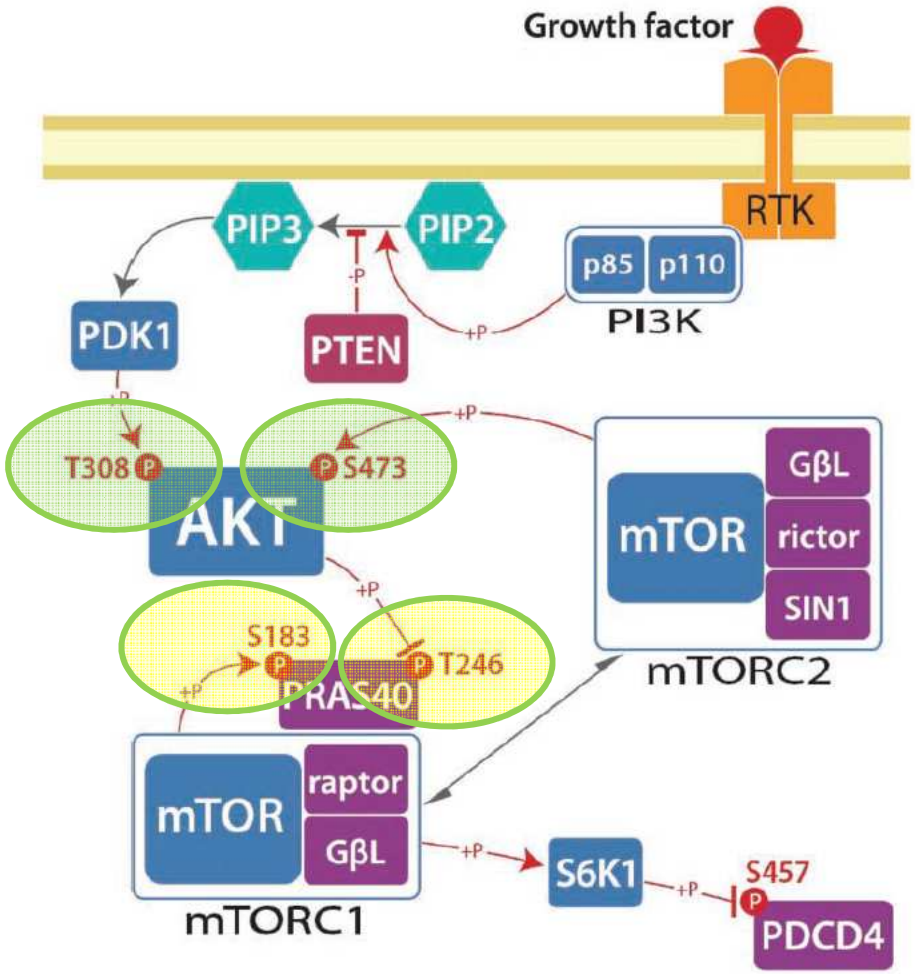




- PI3K / Akt pathway is central to cancer formation
- Chemotherapeutic approaches against multiple targets are in the pipelines

Liu 2009 Nat. Rev Drug Discovery

Assaying the PI3K / Akt / mTOR pathway



(modified from Carlson 2009)

Lanthascreen study goals:

- Evaluate BacMam Lanthascreen technology
- GFP-Akt @ two sites (pT308 and pS473)
- GFP-PRAS40 @ two sites (S183 and T246)
- test different cell backgrounds
- validate with reference inhibitors

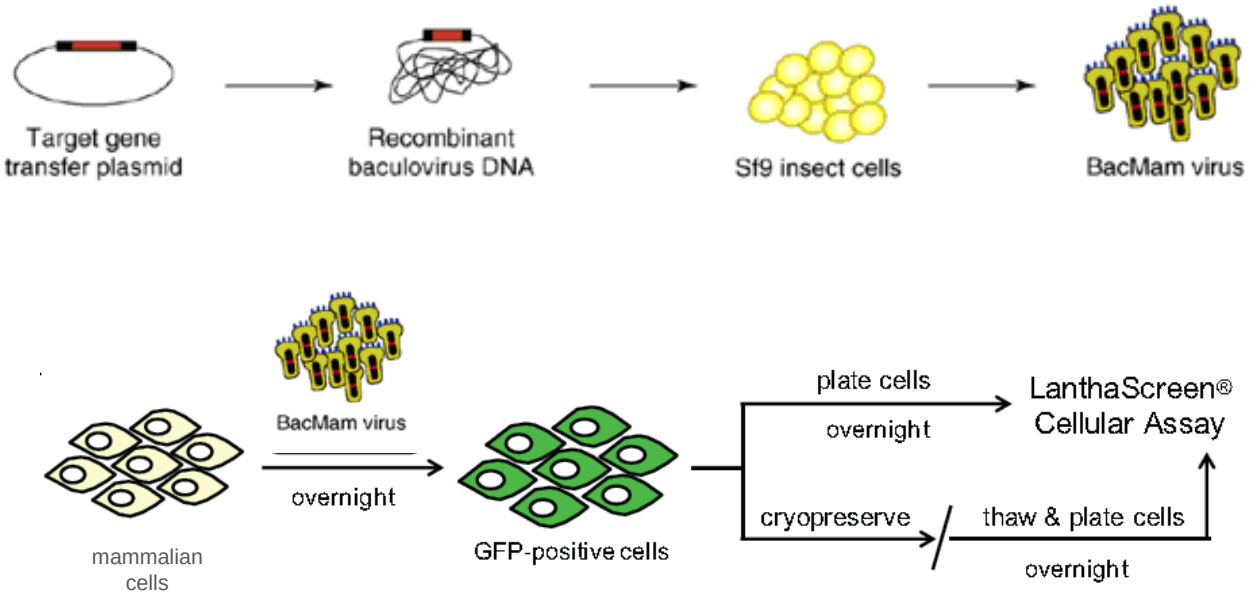
HTRF study goals:

- set up cell-based mTOR kinase activity assay for HTS
- identify optimal cancer cell background
- validate with reference inhibitors

→ validate HTS-compatibility with both formats using a miniaturized 384 well focussed screen

Lanthascreen Assay Development

BacMam technology combined with Lanthascreen Cellular assay



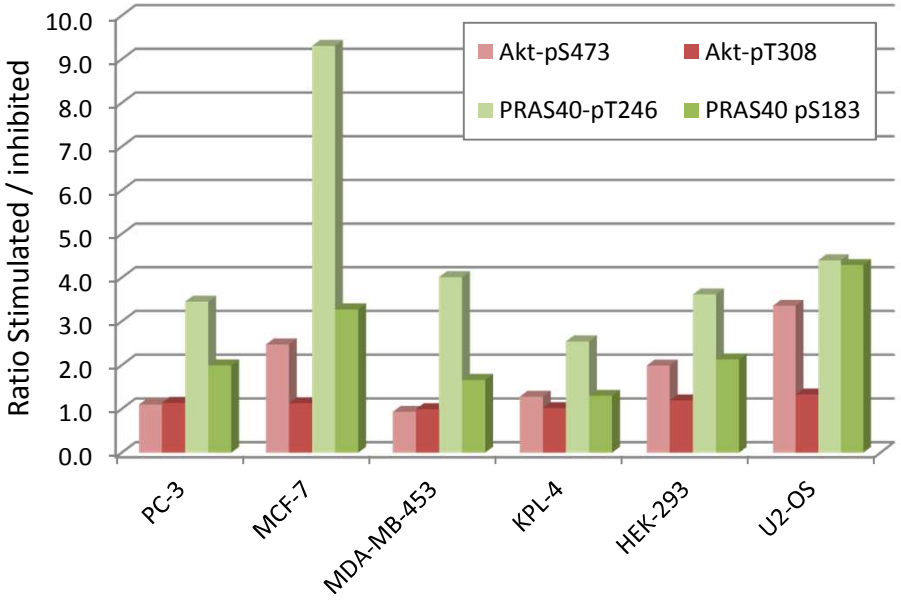
(modified from Kost 2007, Carlson 2010)

Lanthascreen – cellular background

Lanthascreen study goals:

- Evaluate BacMam Lanthascreen technology
- GFP-Akt @ two sites (pT308 and pS473)
- GFP-PRAS40 @ two sites (S183 and T246)
- test different cell backgrounds
- validate with reference inhibitors

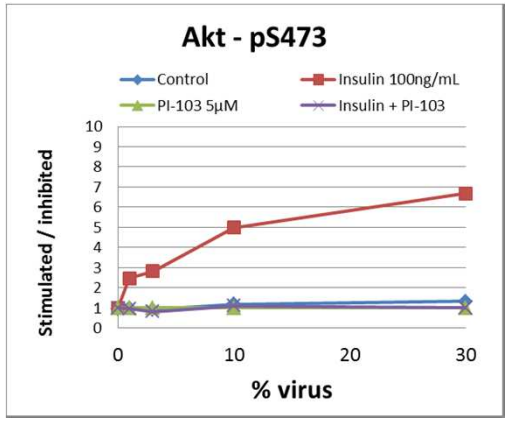
Cell line	Type	PI3K Pathway mutation
PC-3	Human prostate cancer	PTEN negative
MCF-7	Human breast cancer	PI-3-Kinase mutation E545K
MDA-MB-453		PI-3-Kinase mutation H1047R
KPL-4		
HEK-293	Human embryonal kidney	WT
U2-OS	Human osteosarcoma	WT

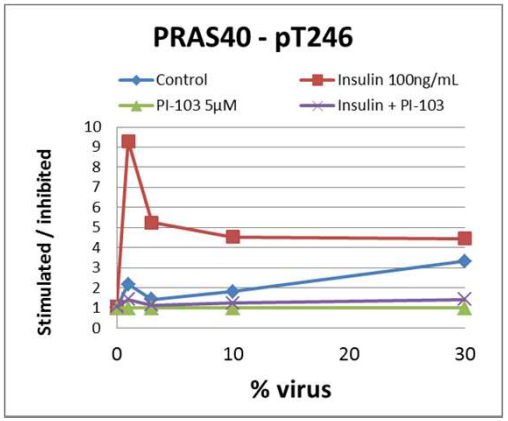


→ MCF7 PRAS40-pT246 selected for further optimization work

Lanthascreen – virus efficiency

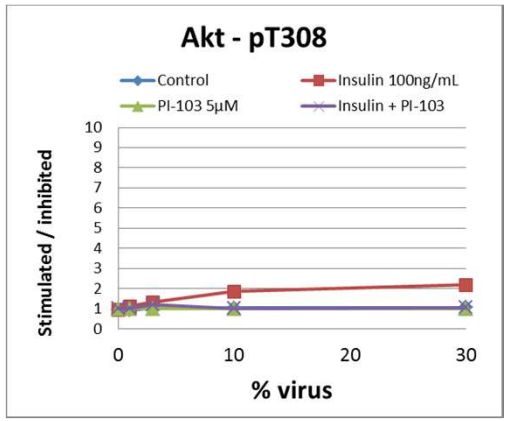
30% virus
S/B_{max} = 7

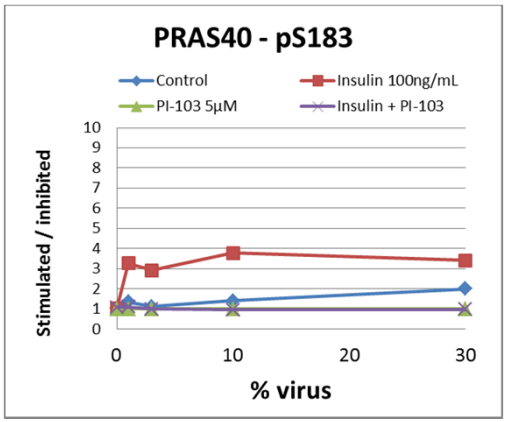




1% virus
S/B_{max} = 9

10% virus
S/B_{max} = 2



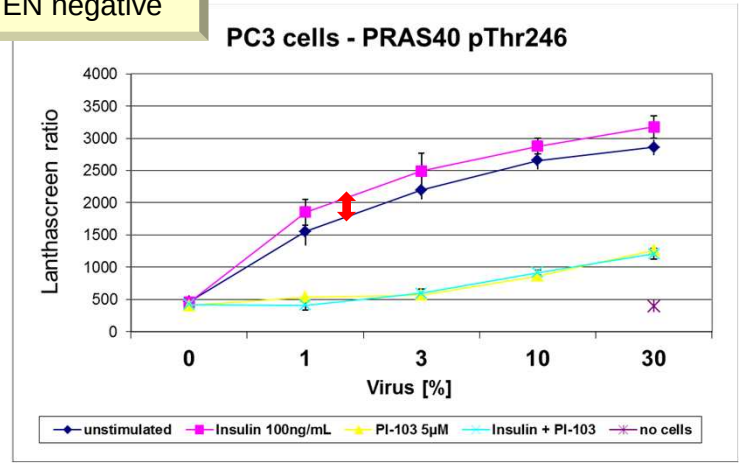


1% virus
S/B_{max} = 3

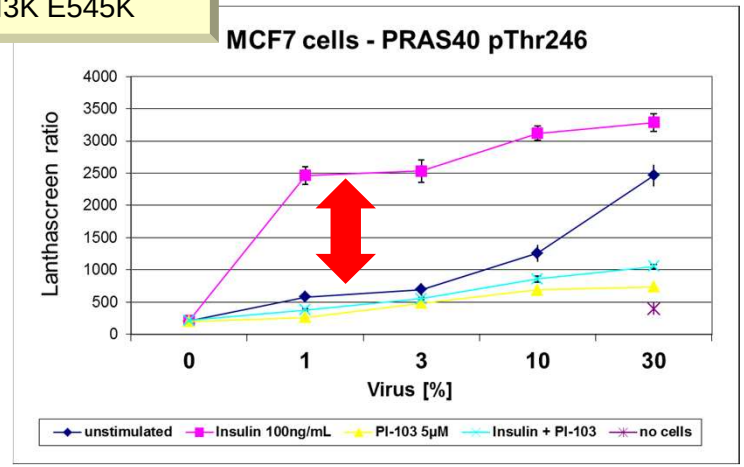
- different virus efficiency: PRAS40 with highly efficient expression (S/B_{max} reached at 1%)
- differences between phosphosites → antibody quality (?)

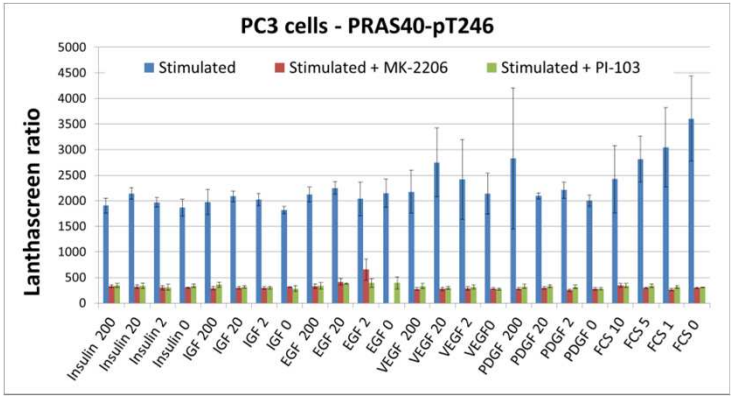
Lanthascreen – cellular background

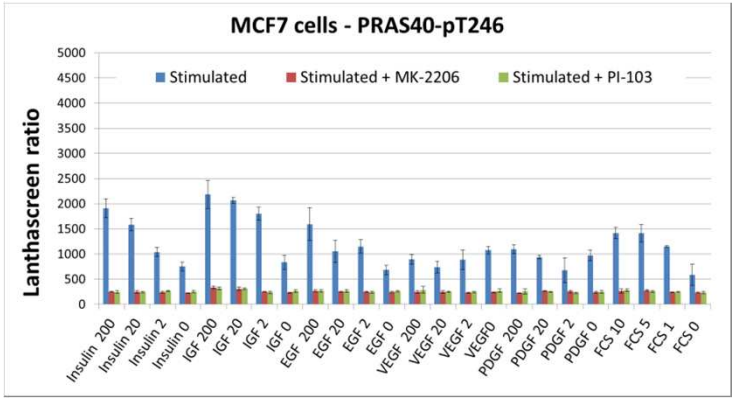
PTEN negative



PI3K E545K





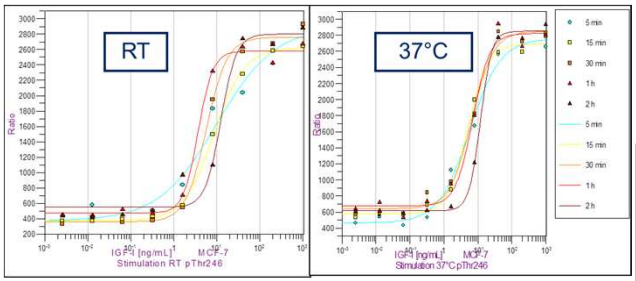


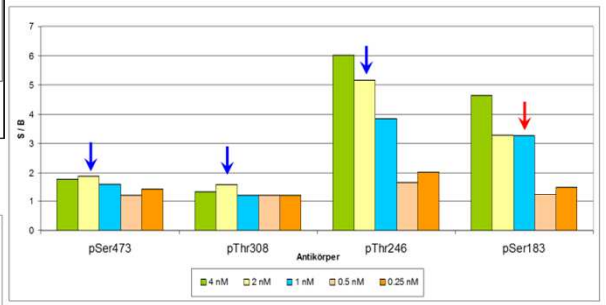
- PC3-cells - in contrast to MCF7 - have fully stimulated pathway

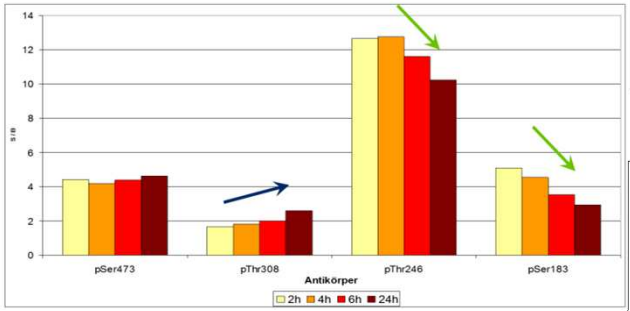
cave → different pathway mutations have different impact on basal activation

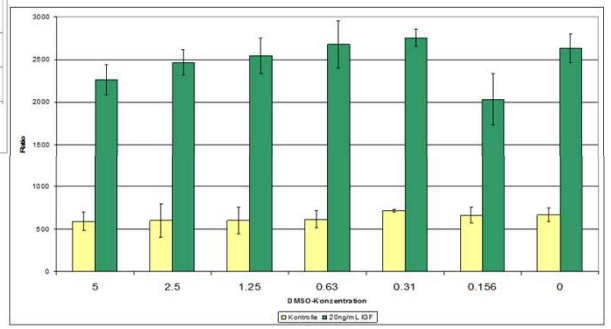
Lanthascreen - optimization

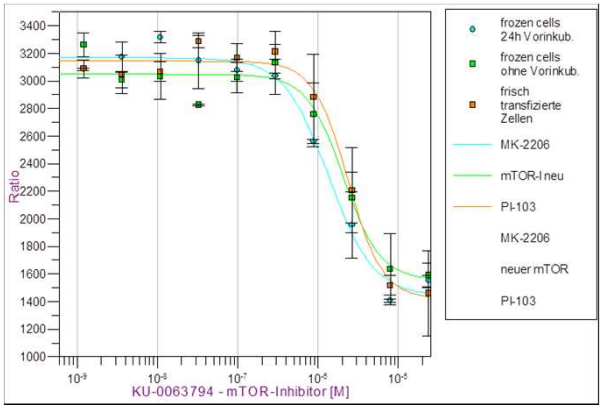
- stimulation time
- stimulation temperature
- antibody concentration
- time cell lysis → read
- DMSO sensitivity
- Volume 10 µl → 5 µl
- frozen cells











Lanthascreen - optimized assay protocol

day 1: seed cells into of MCF7 cells T-flask (frozen or continuous culture)

day 2: transduce MCF7 cells with 1% PRAS40 virus in T-flask

day 3: harvest transduced cells and prepare cell suspension in medium + 1% FCS

dispense 3 µl (5000 cells/well) into assay-ready MTP containing 50 nl cpd.

↓ 2 h @ 37℃

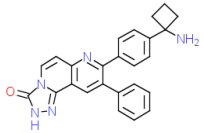
add 1 µl lysis/detection mix

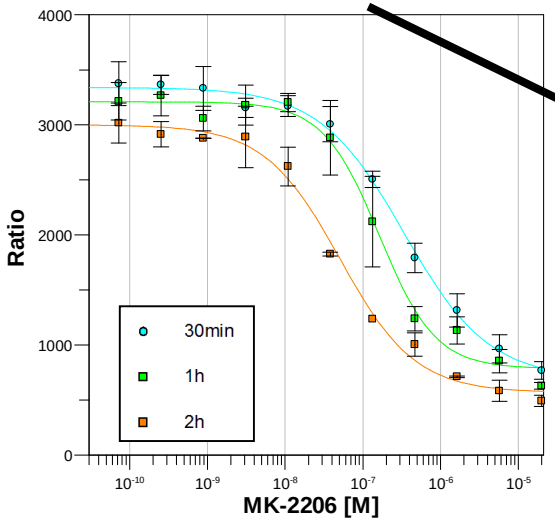
↓ 2 h @ RT

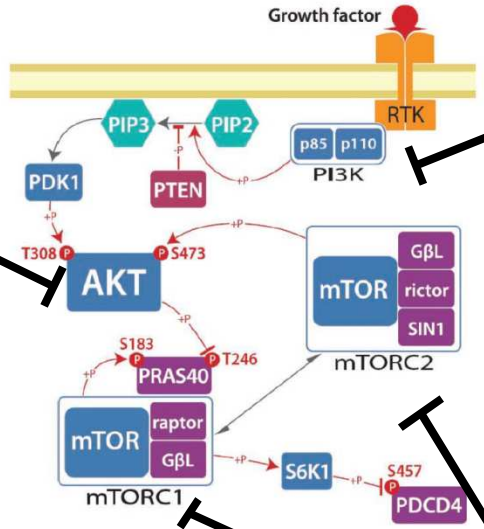
read TR-FRET

Lanthascreen - inhibitor validation PRAS40-pT246

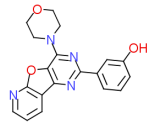
MK-2206
allosteric Akt inhibitor

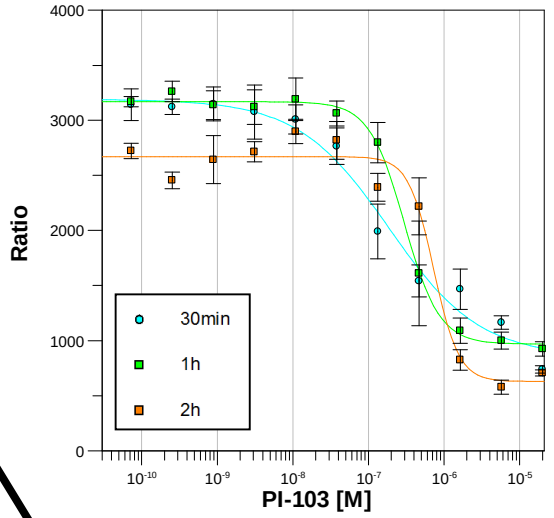




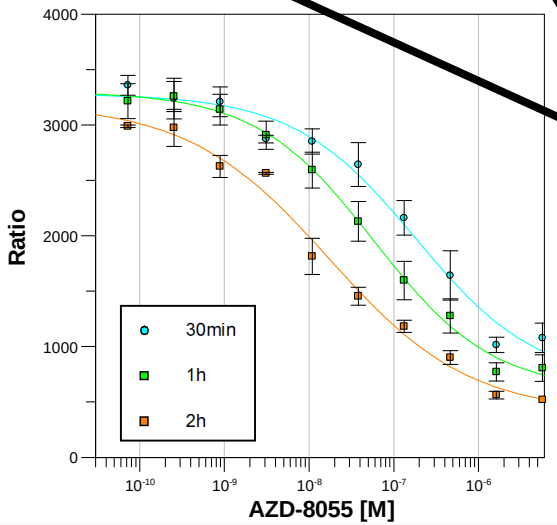


PI-103
PI3K kinase inhibitor

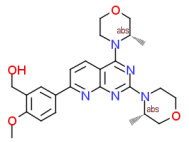




- potency of pathway inhibitors depends on incubation time
- Akt and mTOR inhibitors improved IC50 after 2 h



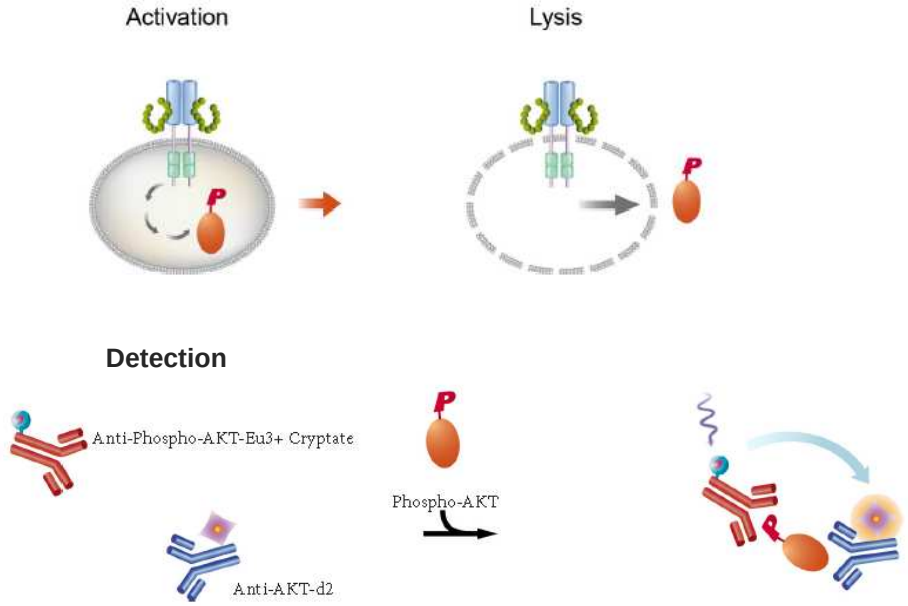
AZD-8055
mTOR kinase inhibitor



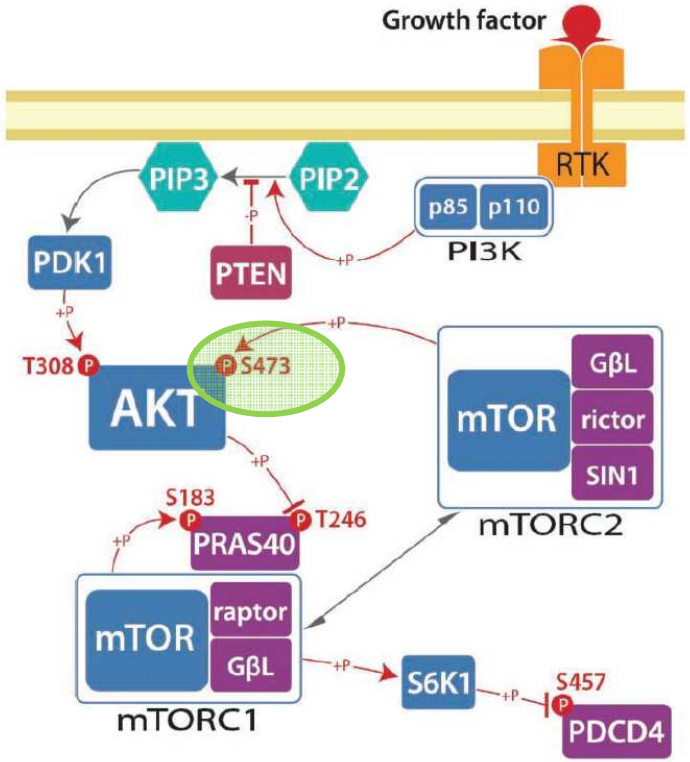
HTRF – assay

HTRF study goals:

- set up cell-based mTOR kinase activity assay for HTS using pAkt Ser473 readout
- identify optimal cancer cell background
- validate with reference inhibitors

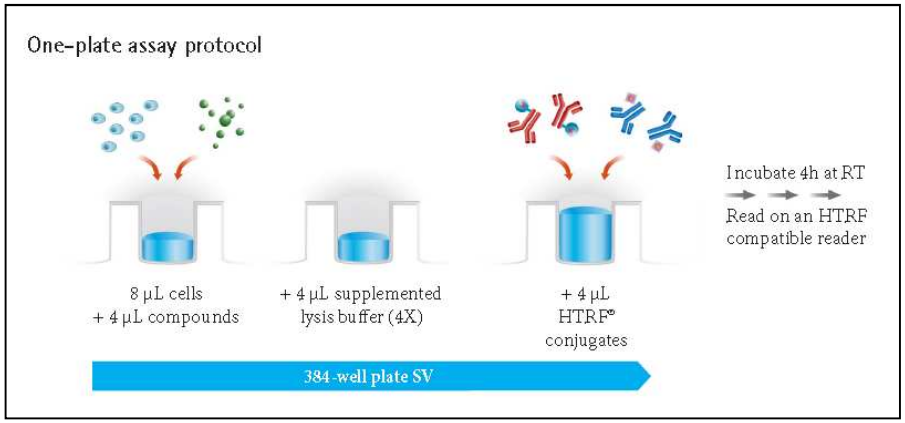


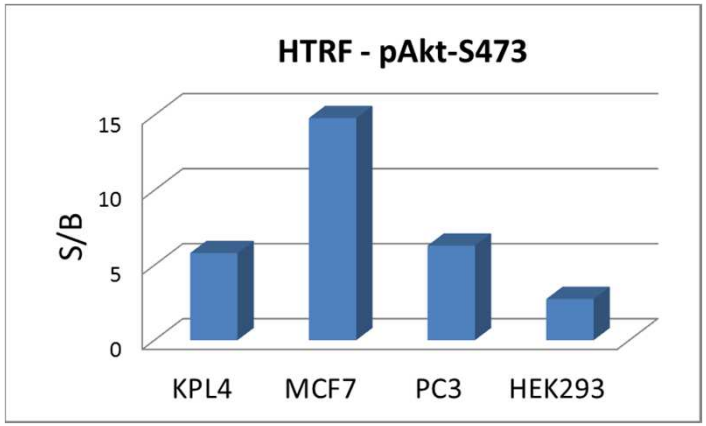
(from Cisbio product insert)

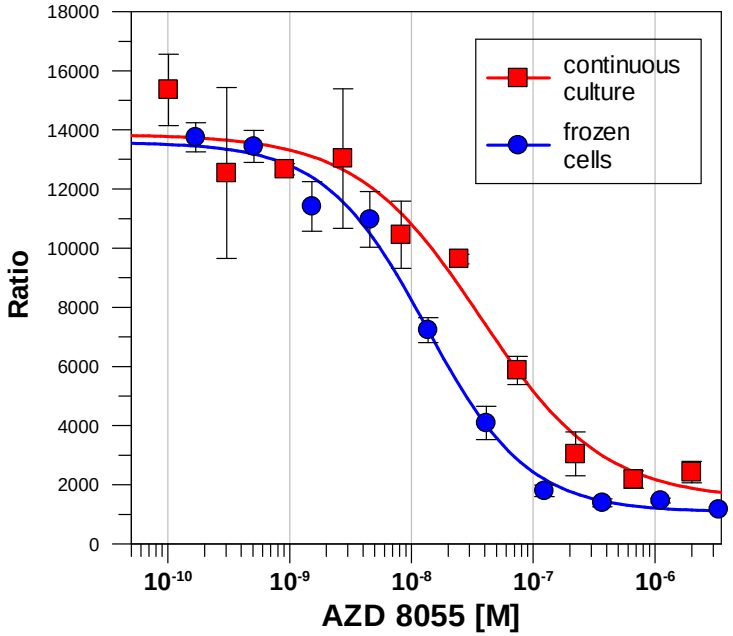


(modified from Carlson 2009)

HTRF – cell lines

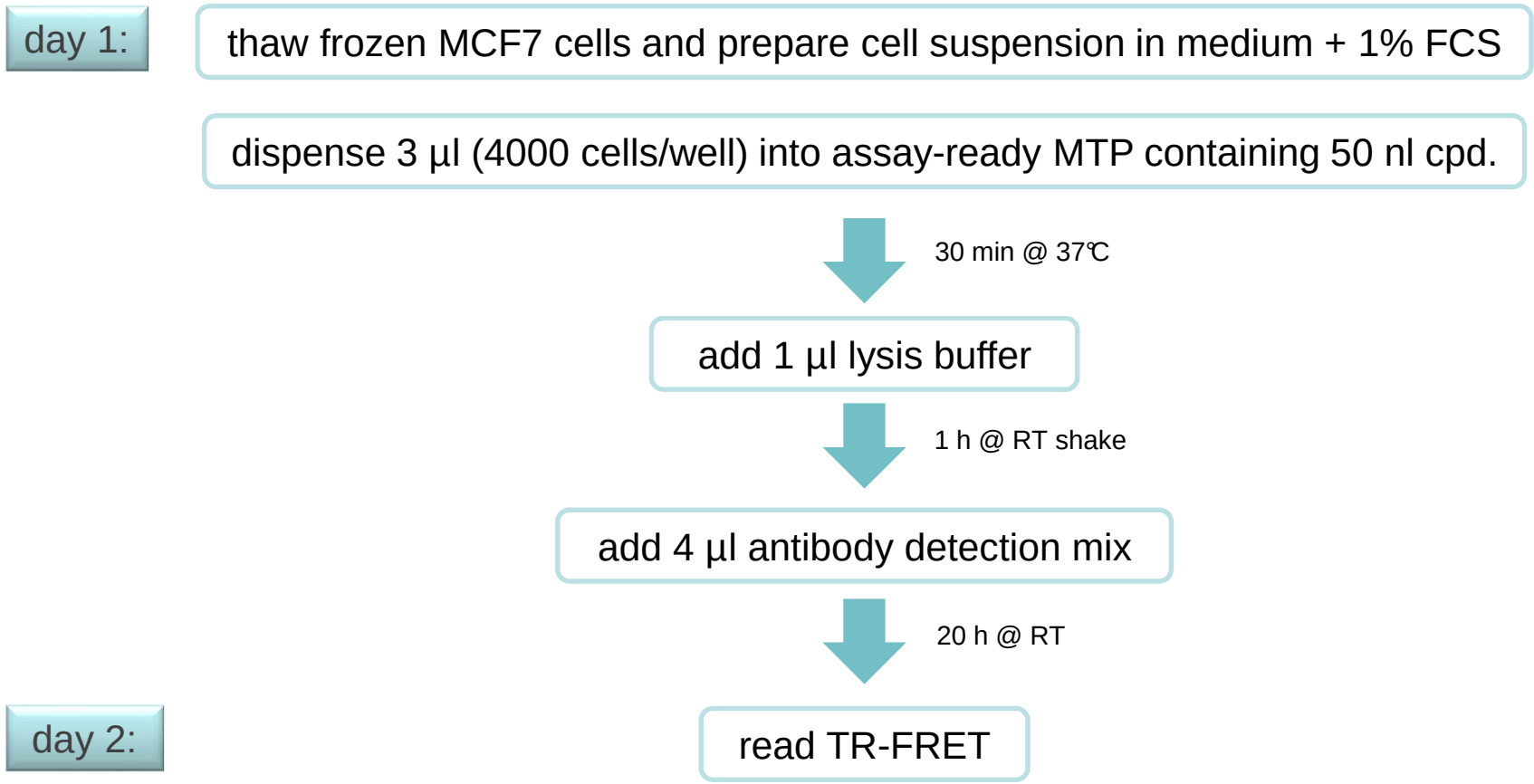




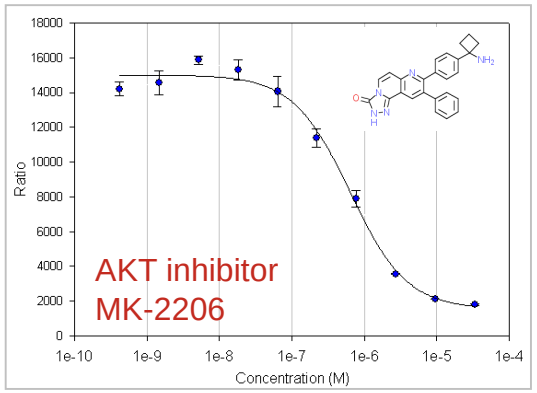


- MCF7 cells strongest S/B
- frozen cell assay
- suspension cell format
- miniaturized to 1536 well

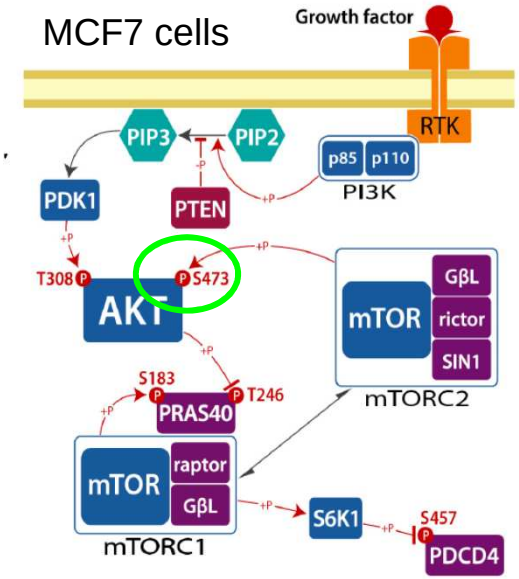
HTRF - optimized assay protocol

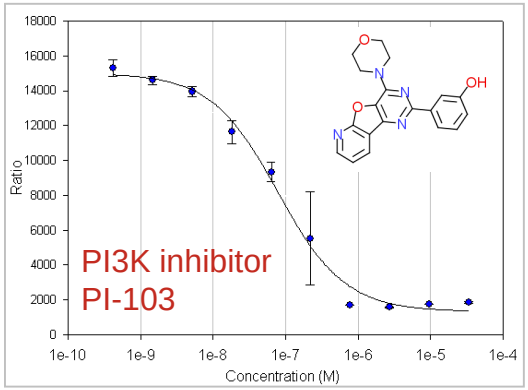


HTRF – pathway inhibitor validation

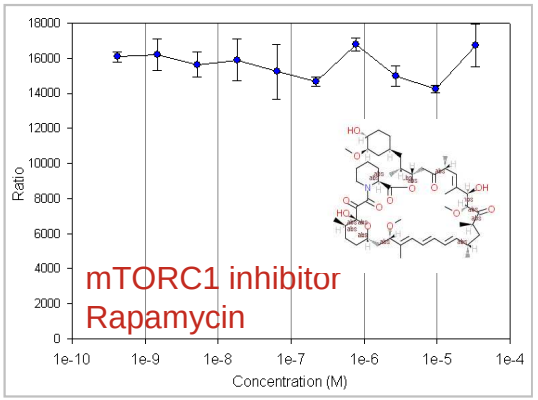


IC50 = 635nM

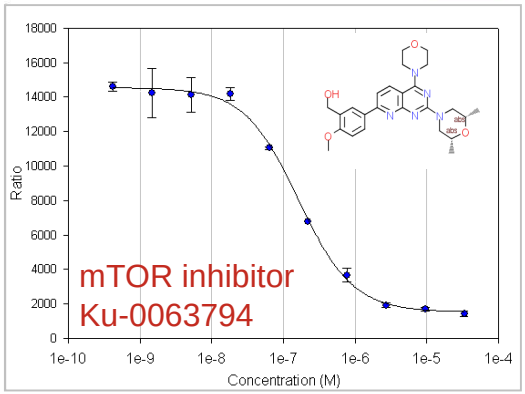




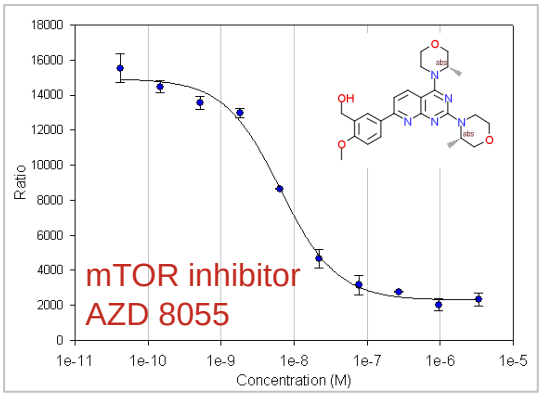
IC50 = 79nM



no effect



IC50 = 163nM



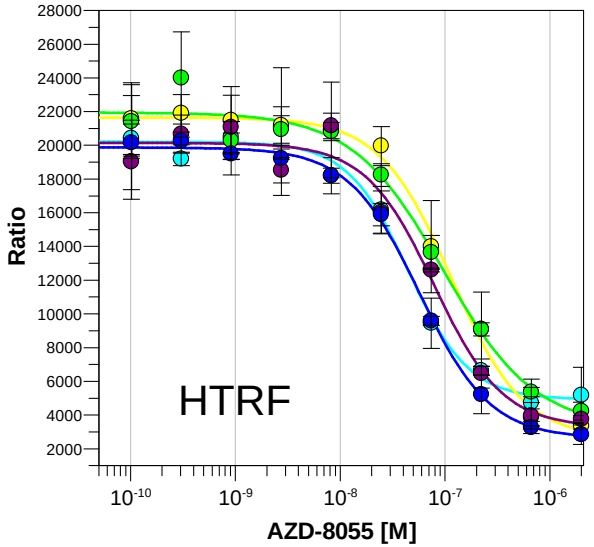
IC50 = 7nM

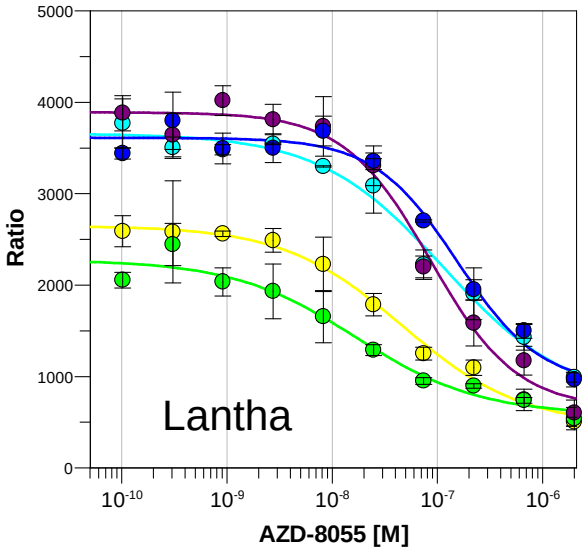
Focussed medium throughput screen:

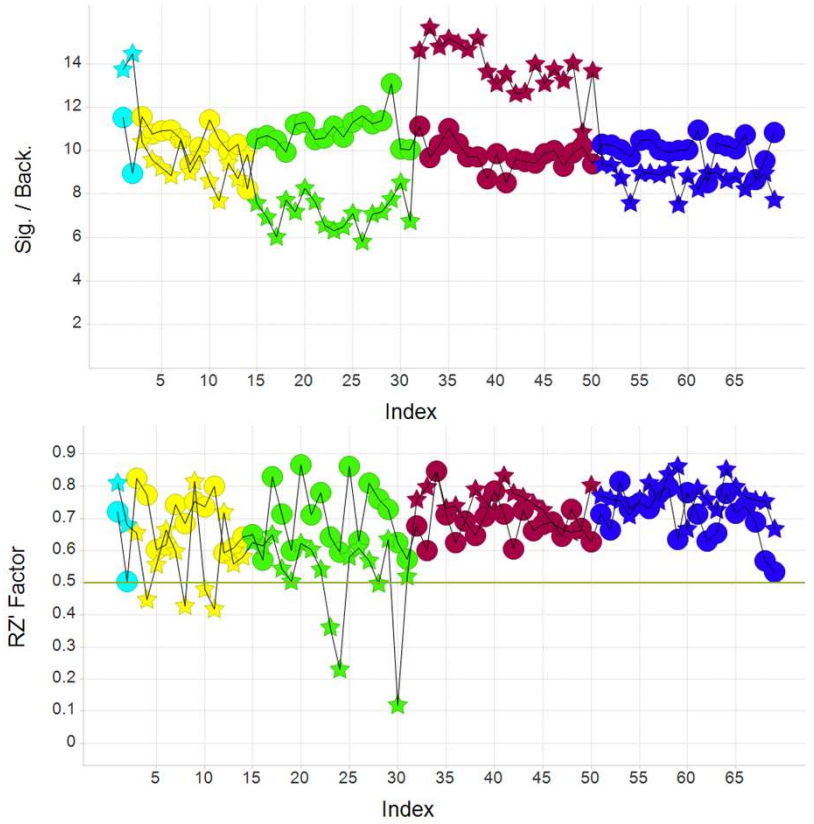
- BacMAM Lantha PRAS40-pT246
- HTRF Akt-pS473

- 24.300 compounds, kinase targeted library
- 384 well single format
- 69 MTPs
- 5 parallel assay runs (Lantha and HTRF)

Focussed medium throughput screen







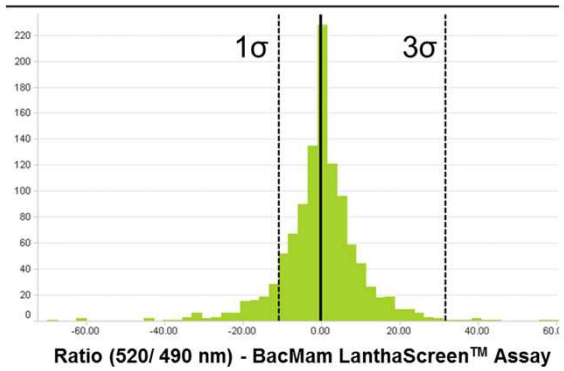
shape by assay:
- HTRF
- Lanthascreen

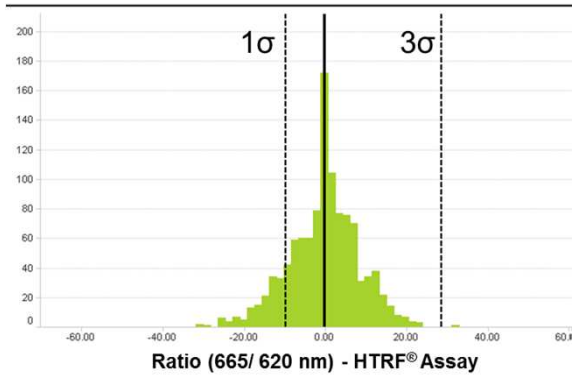
color by run
- Run 1
- Run 2
- Run 3
- Run 4
- Run 5

- Similar sensitivity of both assays aginst mTORi
- HTRF: stable and robust assay performance
- Lanthascreen: day-to-day variability in S/B

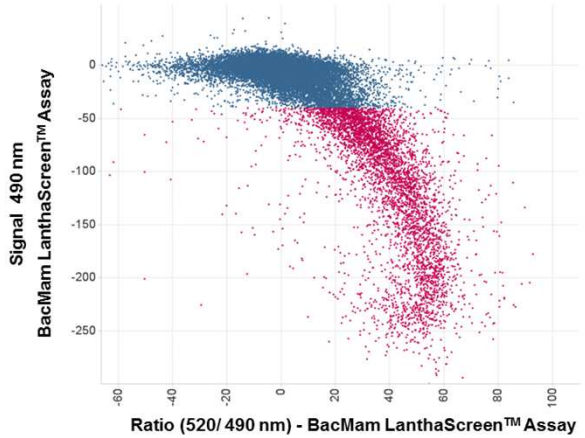
Focussed medium throughput screen

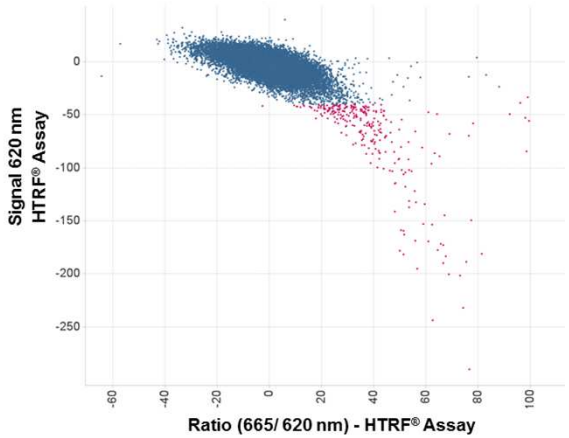
Distribution neutral controls:

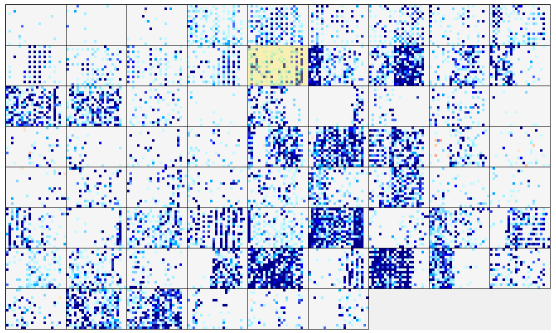


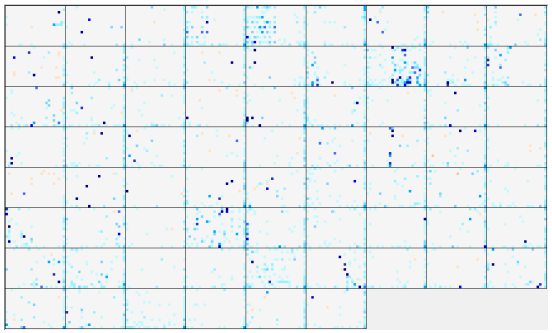


Distribution compounds / donor interference:



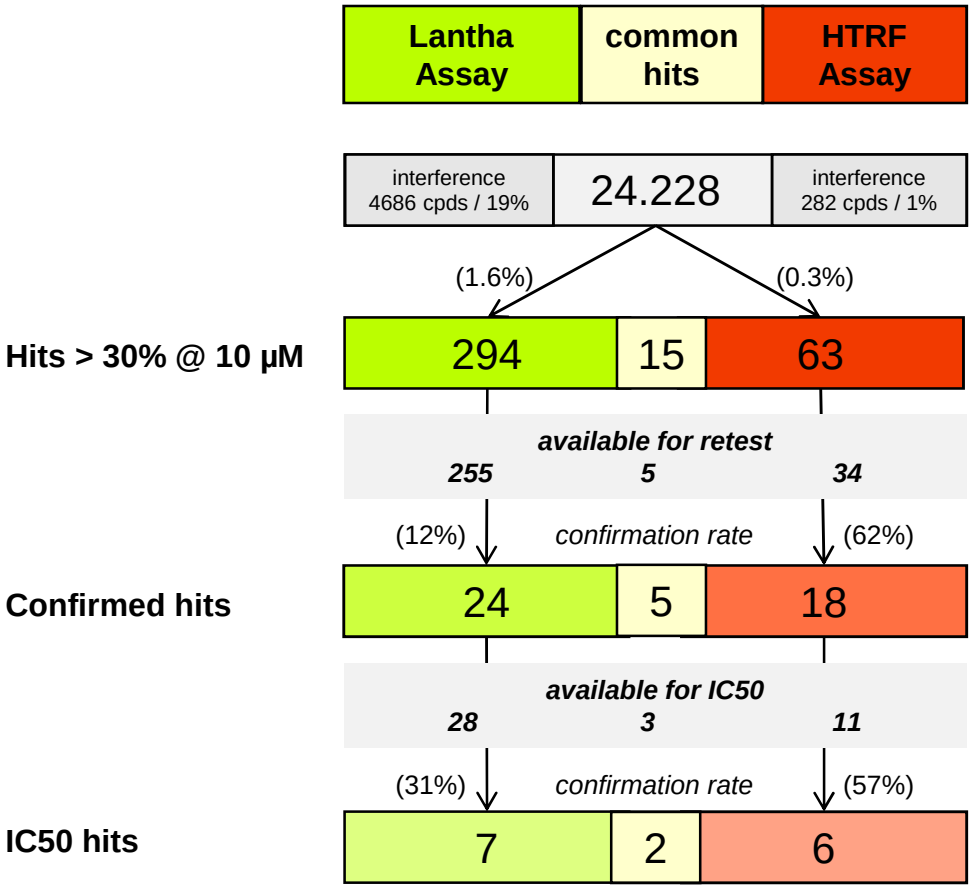






- Neutral controls: similar distribution for both assays
- Compounds: strong interference in BacMAM donor channel

Focussed medium throughput screen



	Lantha	HTRF
Interference	high (19%)	low (1%)
Hit rate	high (1.6%)	low (0.3%)
Confirmation rate	low (12%)	high (62%)

Summary & Conclusions

- Kinases remain interesting target class in pharmaceutical research
- Specific and efficient cell-based kinase assays are essential in pharma research projects
- Homogenous assay systems fit best to Bayers lead discovery platform
- Positive experience at Bayer with EFC-technology, HTRF and Lanthascreen
- EFC-technology interesting for Tyr-Kinase uHTS
- Lanthascreen technology positioned for secondary testing
- HTRF positioned for uHTS and secondary testing

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Thank you!