

*Progress towards
a generic cellular S/T-kinase assay platform**

Yu Wang, Deanna Adams & Achim Brinker, GNF

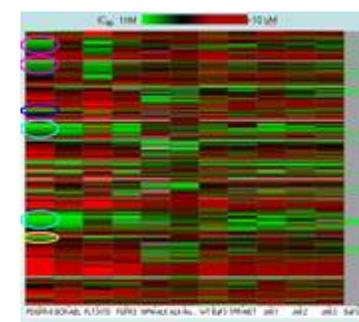
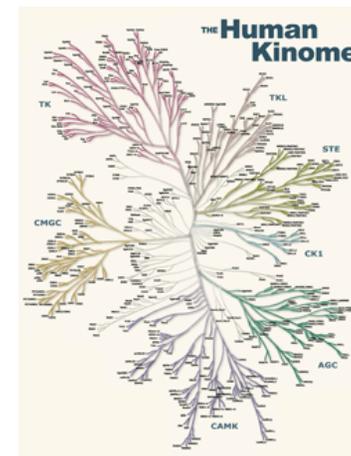
Emmanuel Claret & Gérard Mathis, Cisbio



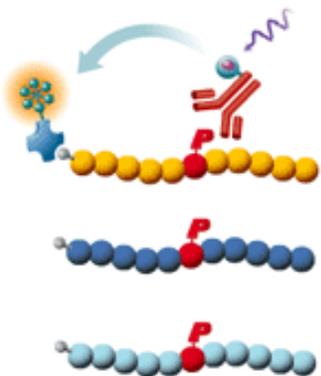
Genomics Institute of the
Novartis Research
Foundation

Specifications

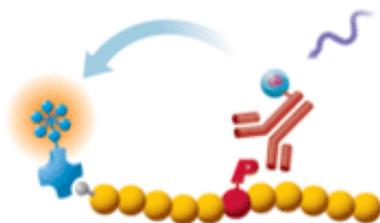
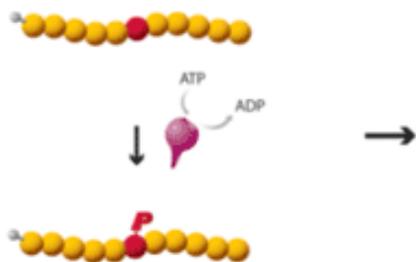
- broad coverage of STKs across the phylogenetic tree
- generic assay protocol
- automation and high-throughput friendly
- robust performance in high-density formats (384w/1536w)
- low compound interference
- engineered system is acceptable
 - overexpression of target (GPCRs)
 - generic cellular background (HEK293, CHO)
 - modifications of substrates (truncations, peptides, tagging)
 - modifications of target kinases (CA-mutants/ fusions, kinase domains)
- pathway characteristics are acceptable, not preferred
- potential to monitor endogenous kinases is desirable
- potential to induce STK activity by physiologic stimuli is desirable



HTRF® – KinEASE™: a biochemical assay platform



1 Antibody
3 Substrates
> 107 STKs

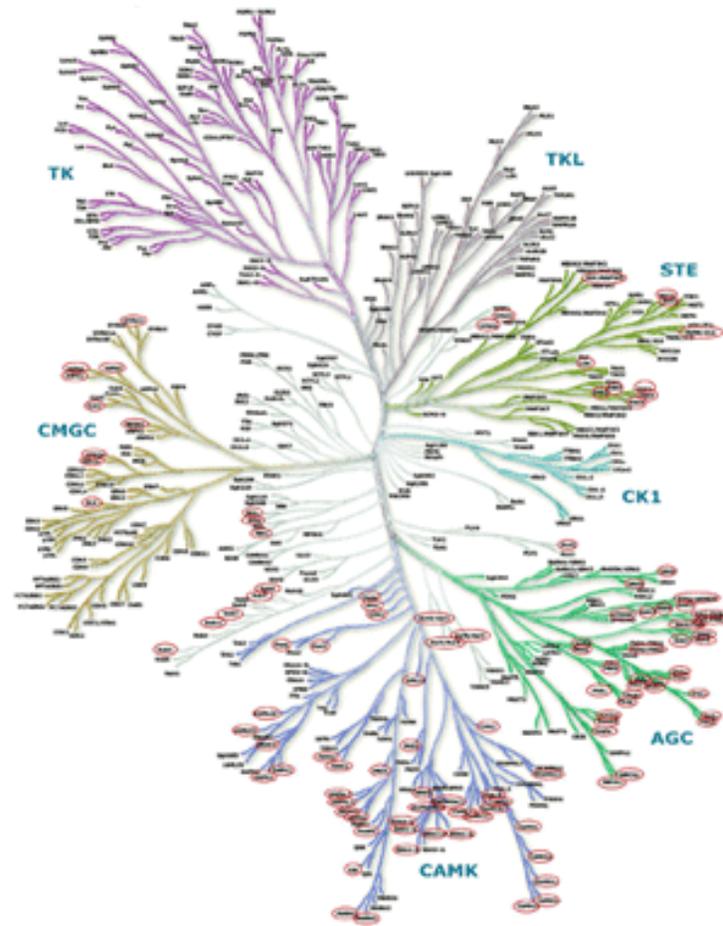


Step 1: Kinase reaction

- 20 µL Compound
 - 10 µL Biotin-STK substrate 1, 2 or 3
 - 10 µL Kinase
 - 10 µL ATP
- Incubate 30 min at 37°C

Step 2: Detection

- 50 µL Anti Eu(K)-STK antibody / Streptavidin-XL665
- Incubate 1h at RT then Read on an HTRF reader



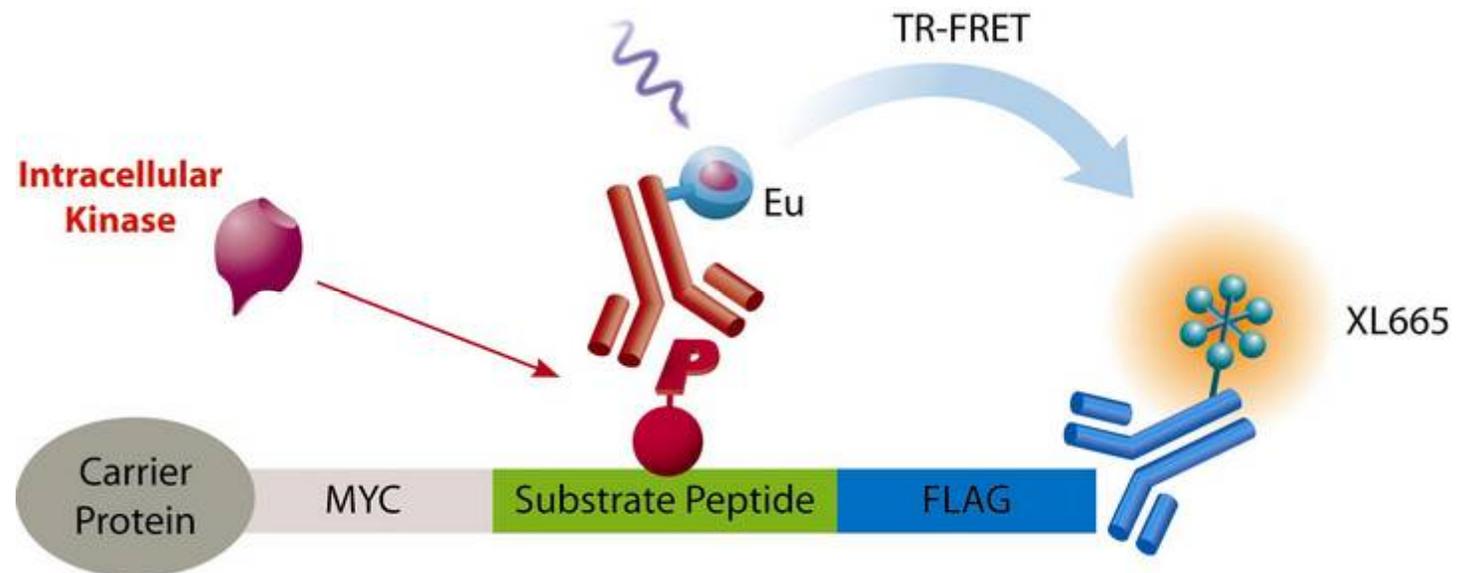
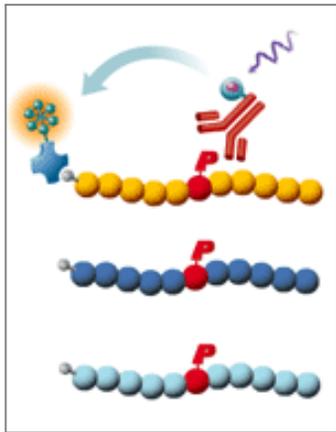
 KinEASE™ - Assay



Strategy

Translating traditional biochemical HTRF®-based kinase assays into a cellular format

- (transient) coexpression of kinase & peptide-carrier fusion construct in cells (HEK, HeLa, etc.)
- kinase inhibitor/compound treatment
- cell lysis & HTRF®-detection using combinations of phospho-specific and epitope-tag antibodies



Protocol

Day1

- 384w: reverse transfection (8,000 cells/well)
- 1536w: transient forward (bulk) transfection & cell seeding (2,000 cells/well)



48h

(stable adherent cells - o/n

stable suspension cells – immediate treatment)

Day3

- 1-2h compound treatment
- cell lysis & HTRF® detection

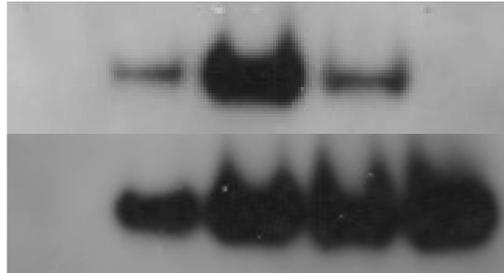
Short treatment → Low 'uninteresting positive' rate ?!



AKT

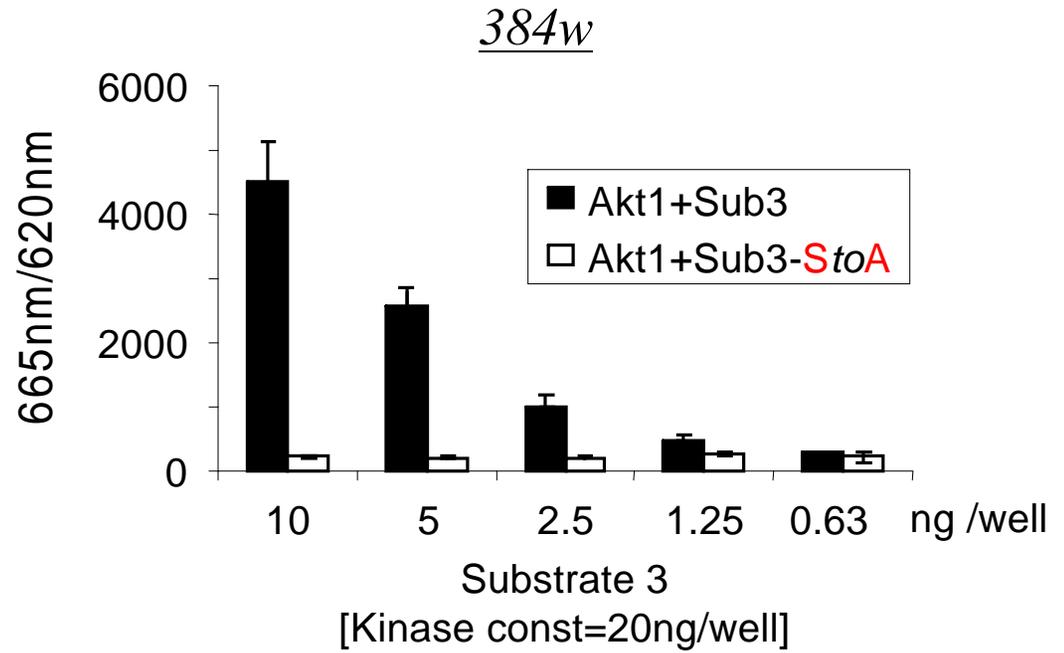
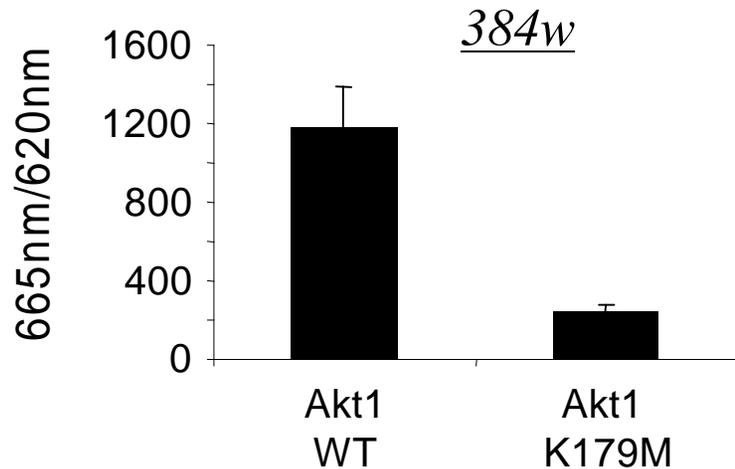
HEK293

Sub3
 Sub3 + AKT- WT
 Sub3 + AKT- **K179M**
 Sub3-**S/A** + AKT - WT

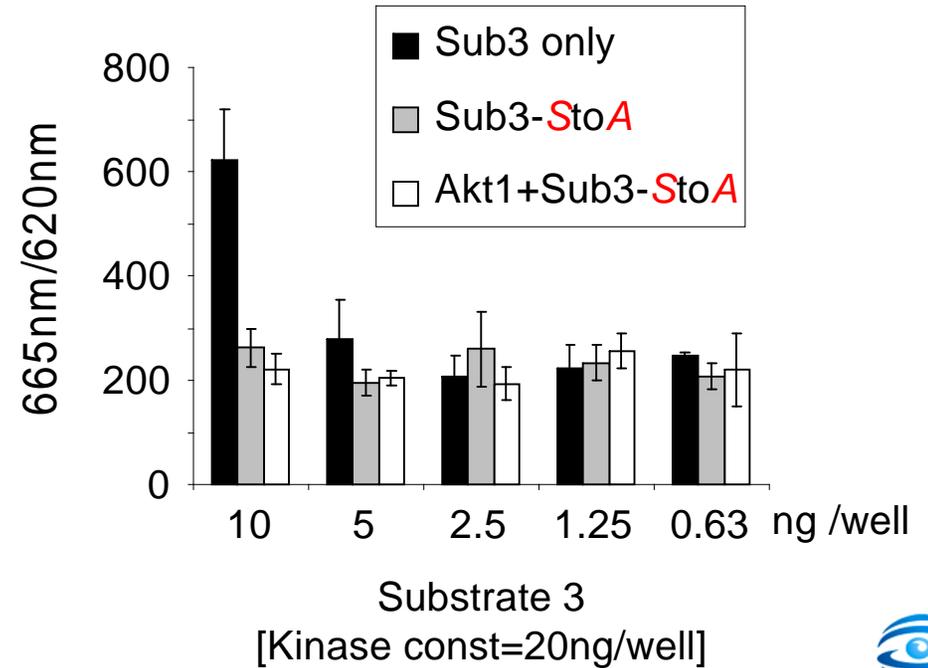


α -pSTK

α -Myc

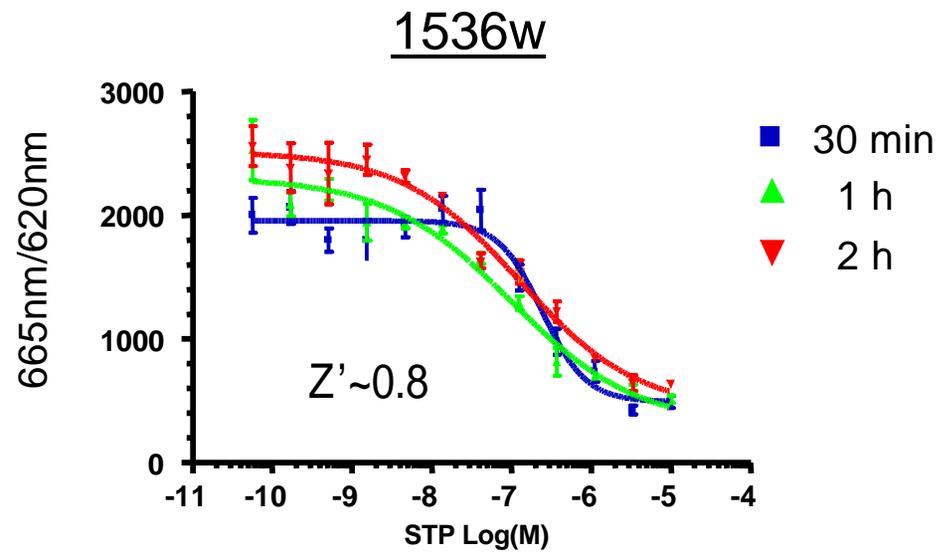
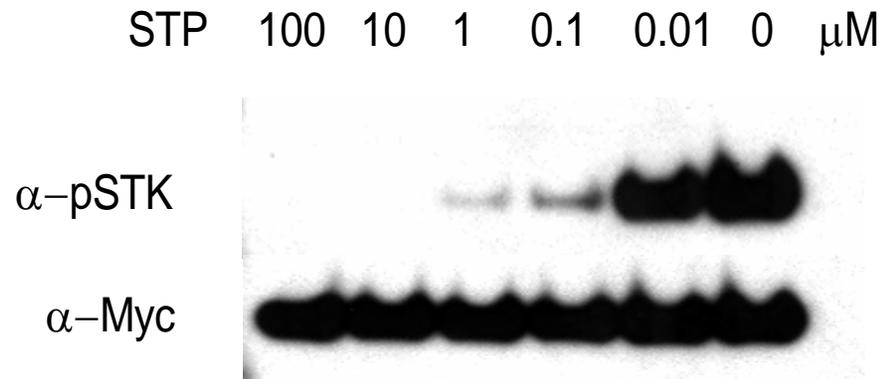


Background Phosphorylation



AKT

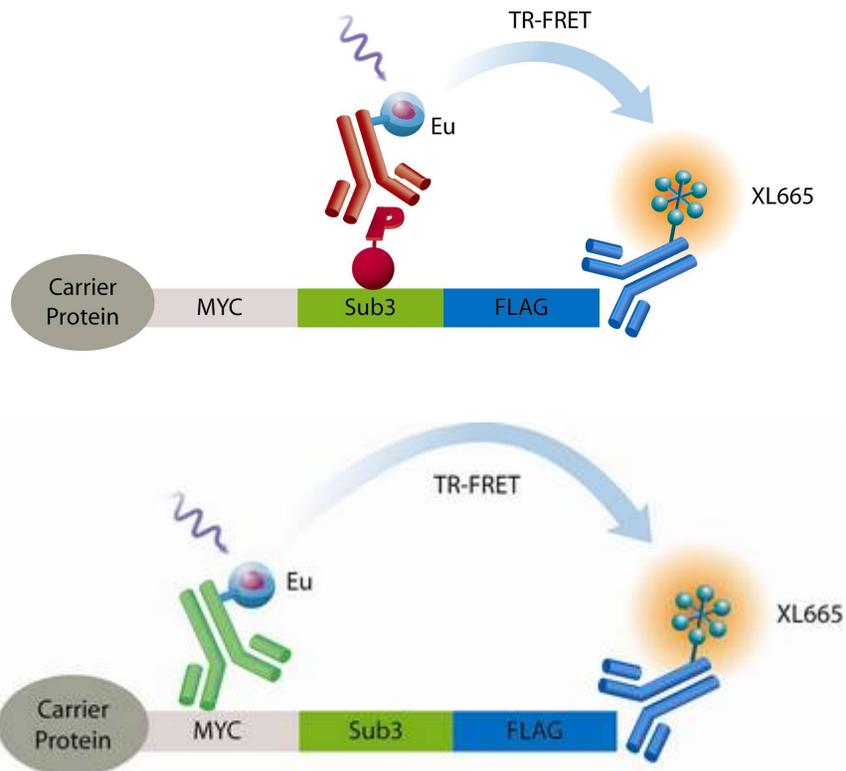
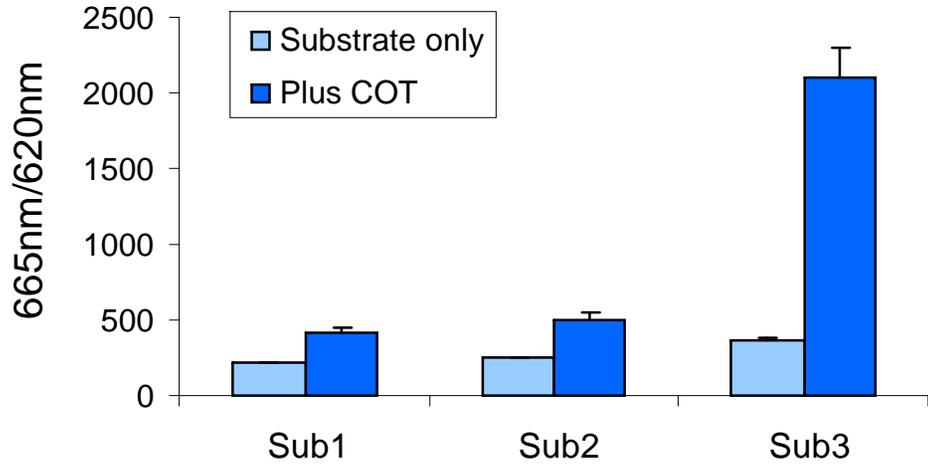
1h Staurosporine treatment, HEK293



	30min	1h	2h
EC50	2.430e-007	1.002e-007	1.268e-007

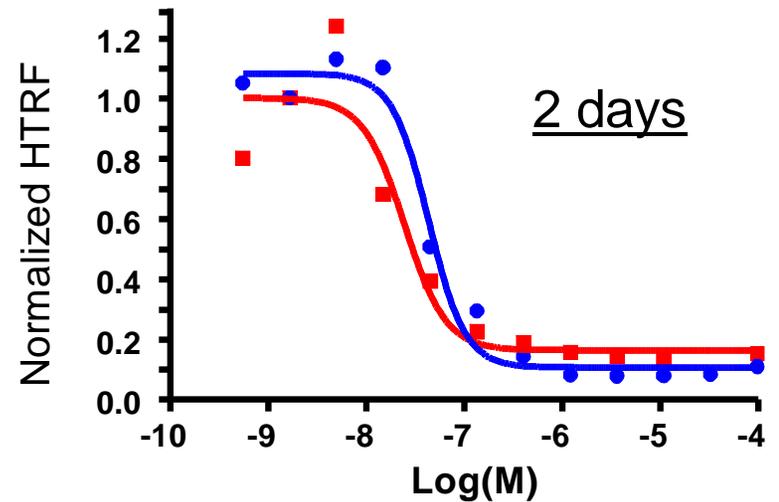
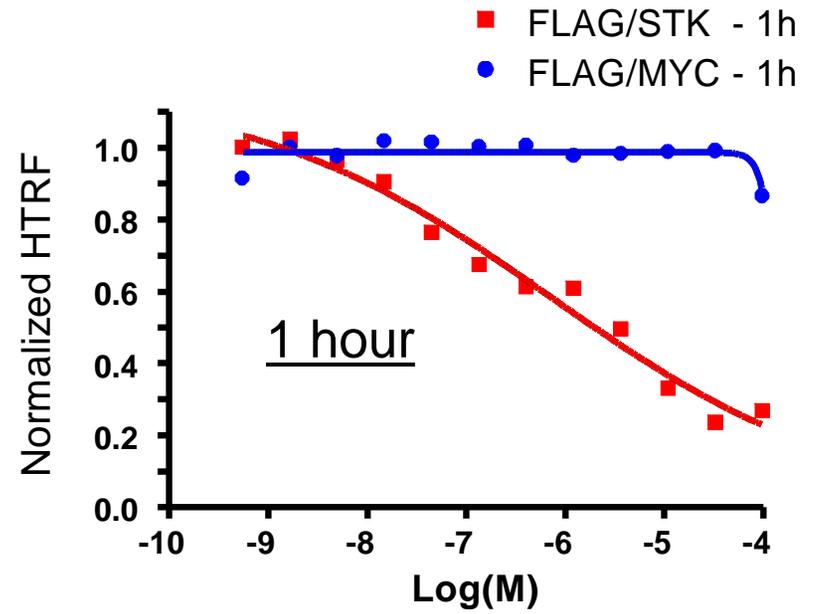


COT



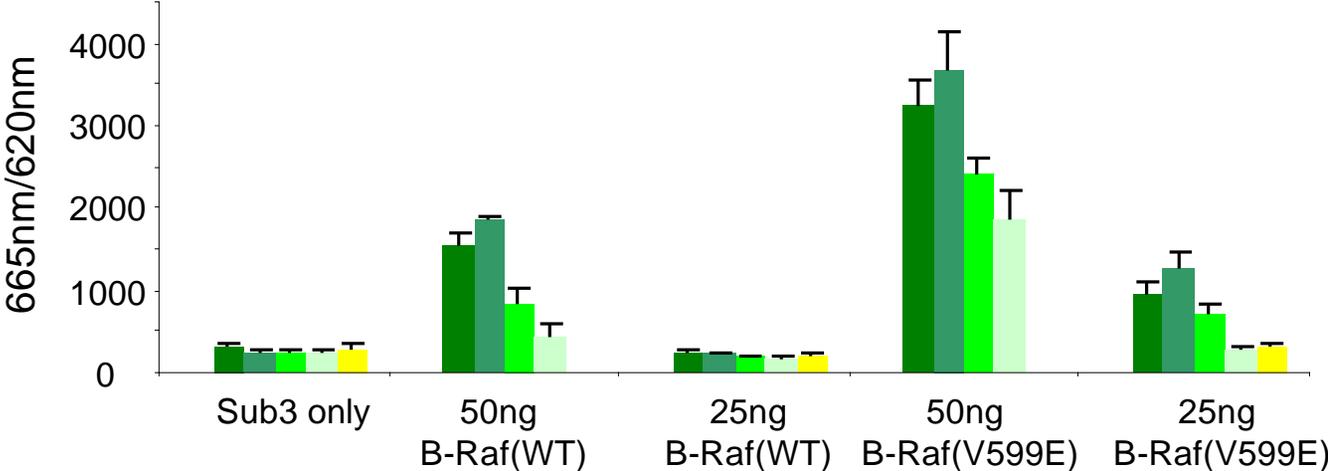
Toxicity – Control

Staurosporine, HEK293



Other kinases

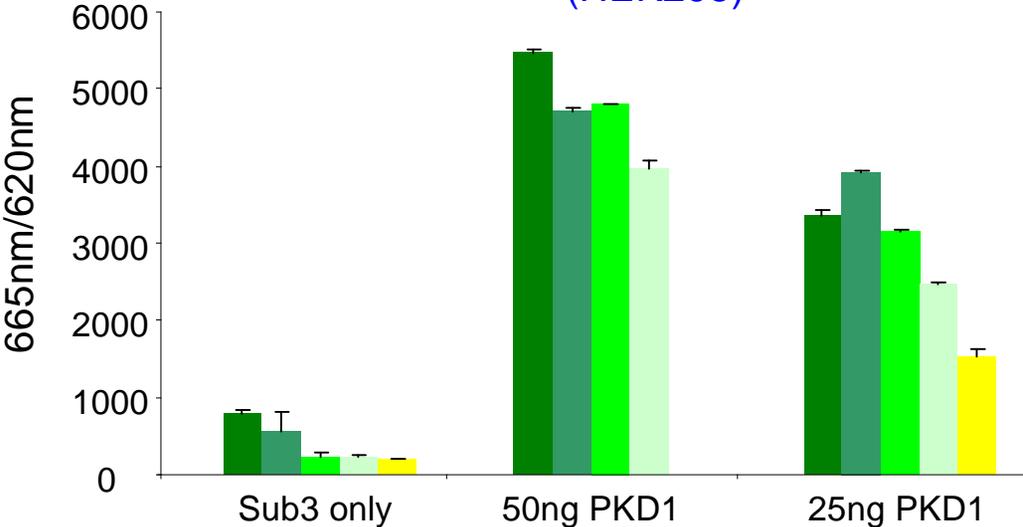
B-Raf (HEK293)



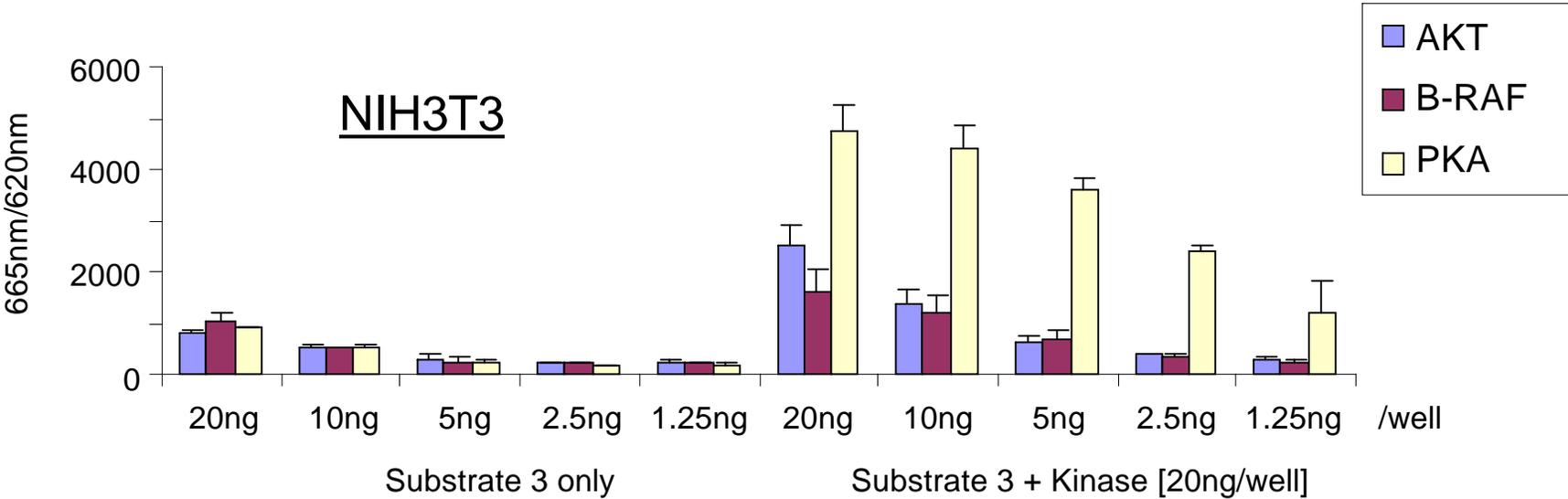
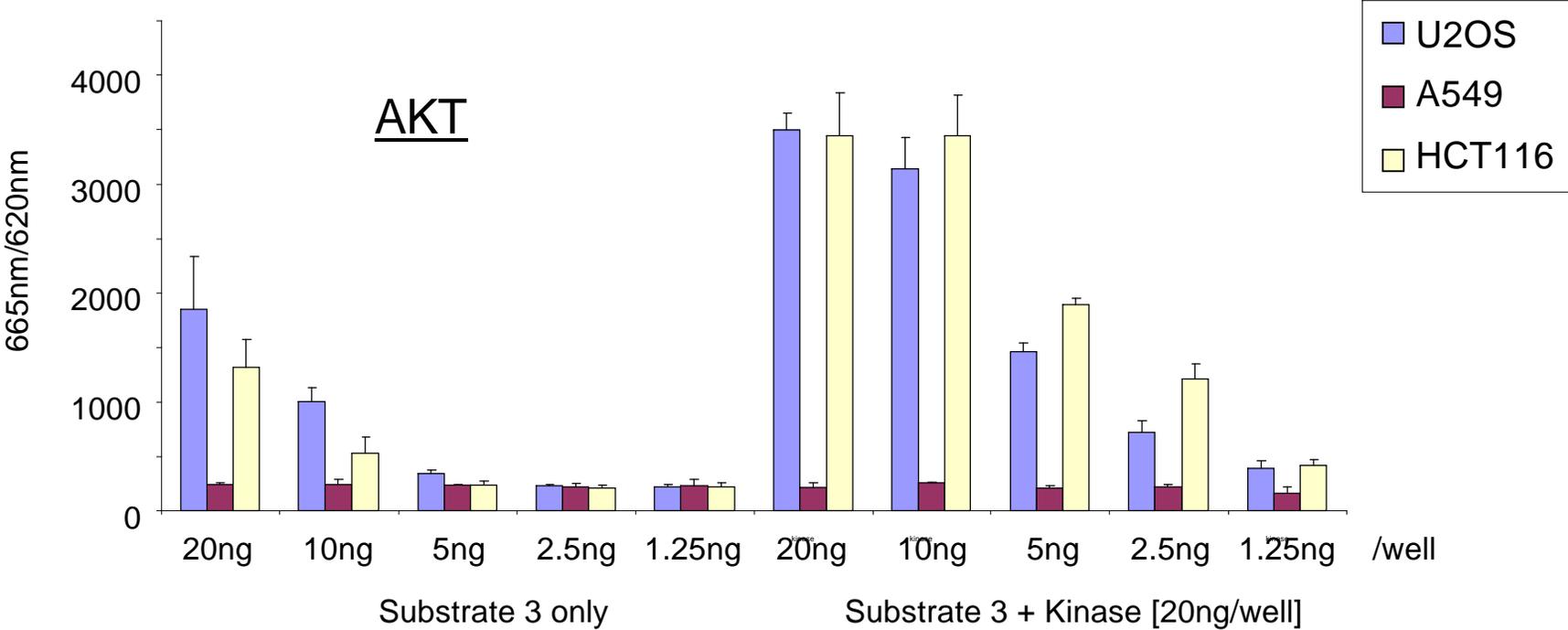
Substrate 3
(well)

- 25ng
- 12.5ng
- 6.25ng
- 3.12ng
- 1.56ng

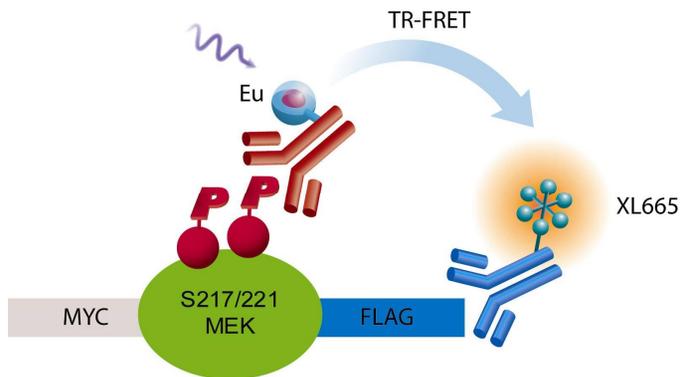
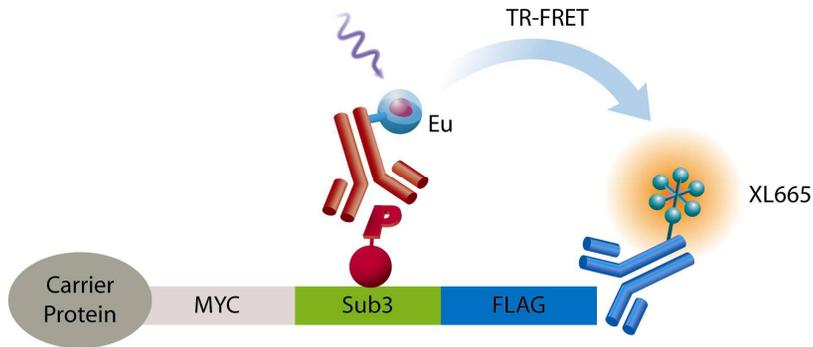
PKD1 (HEK293)



Other cellular backgrounds

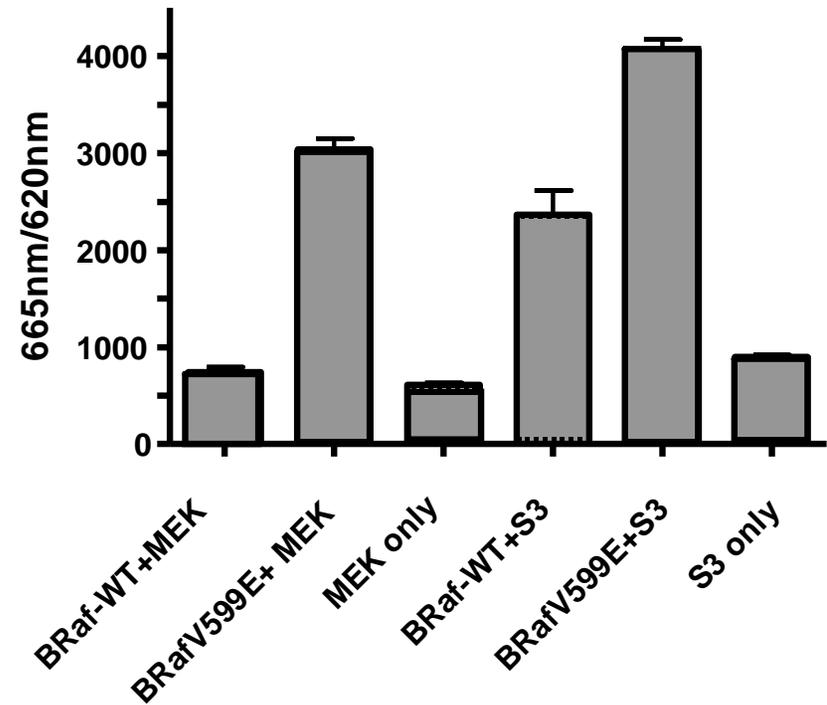
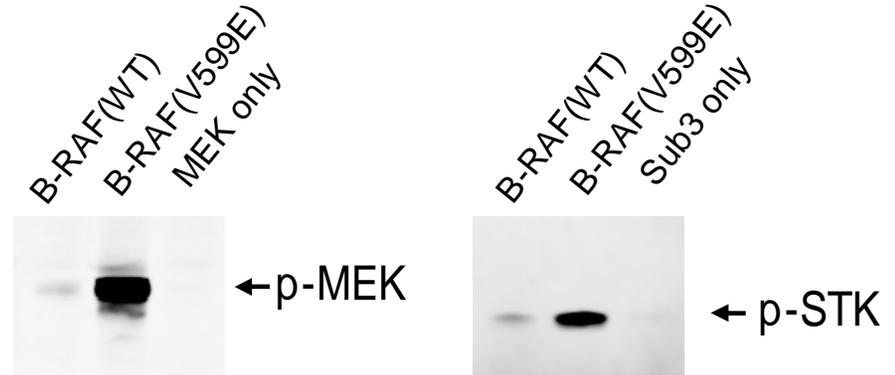


Full-length protein substrates – MEK-1



MEK-1

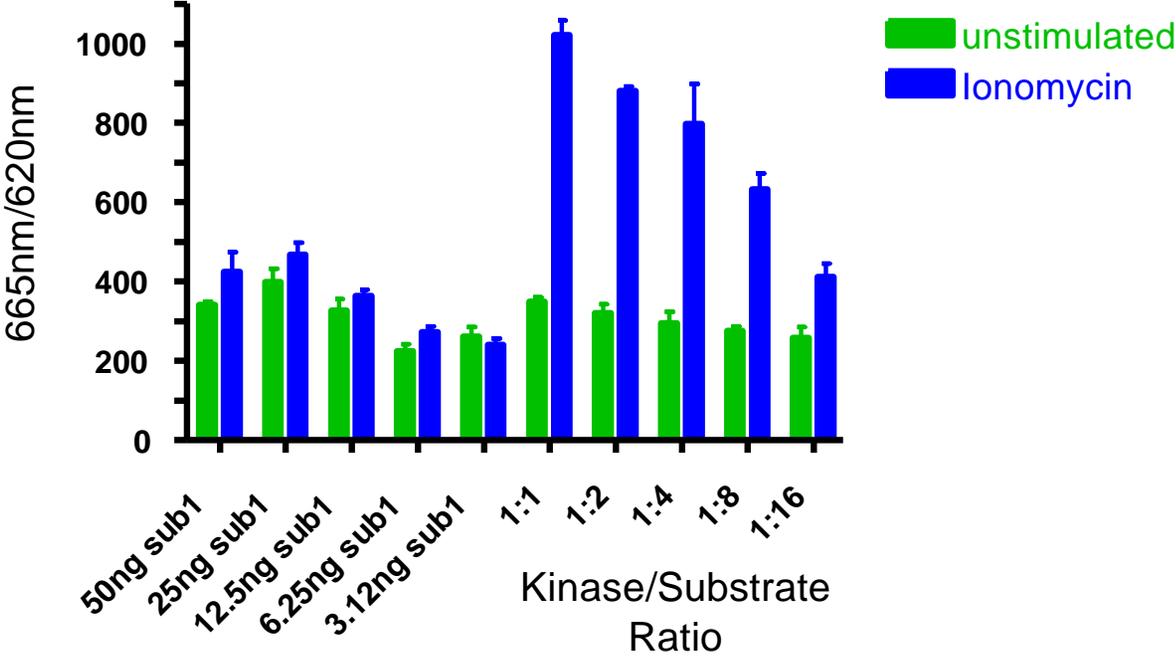
Substrate3



Inducible STK activity

CamK2 δ

384w , 16 hr serum free, 5min +/- 1 μ M Ionomycin



Panel development & progression



New assay

Substrate, pAb, PC kinase, cell line, stimulus



cDNA screen

384w; ~800 STK&Y-kinase clones;
reverse transfection; duplicates



Hit reconfirmation

sequence & activity (+/- kinase)



LMW inhibition - pilot

control cmpds; 384w or 1536w; *bulk* transfection



LMW inhibition – test screen

~76 reference kinase inhibitors; 1536w



HTS

- online screen >1M cmpds
 - focused libraries
 - secondary hit profiling



Panel expansion through cDNA screening

Focused cDNA library: ~800 STK&Y-kinase clones (25ng/well)

Screens run:

- 8 screens run in HEK293 cells
- KinEase™ 3 also screened in HCT116, U2OS

Screens in preparation:

Kinase™ 5, MEKtide, ATF2, ERK, stimulation, NLS-substrates

Hit distributions (HEK):

	<u>(>2x)</u>	<u>>10fold</u>	<u>5-10fold</u>	<u>2-5fold</u>	<u>(+/- kinase)</u>
Overall		1	10	28	
KinEASE™ 1		2	1	5	Cisbio pSTK Ab
KinEASE™ 2		0	4	8	
KinEASE™ 3		1	14	22	
Crosstide		0	0	0	
MBPtide		0	0	7	Other Abs
p38α-FL		0	0	0	
p38α-peptide		0	0	0	Cisbio Abs
CREBtide		0	0	2	



Confirmed activities

384-well plates, transient transfections

>10x

B-RAF(V599E)
COT
PKA

(3)

5-10x

AKT1
AKT2
B-RAF(WT)
CHEK2
MAP3K3/MEKK3
MAP3K11/MLK3
Pim-2
PKD1
PKD2
PRKX

(10)

2-5x

Aurora-A
BRSK2
CAMK2 δ^*
MEK1-CA
MKK3
MKK6
NEK6
NEK7
PAK4

(10)

(23)

* Ionomycin inducible



On biochemical KinEASE™ panel

384-well plates, transient transfection

>10x

B-RAF(V599E)
COT
PKA

(3)

5-10x

AKT1
AKT2
B-RAF(WT)
CHEK2
MAP3K3/MEKK3
MAP3K11/MLK3
Pim-2
PKD1
PKD2
PRKX

(10)

2-5x

Aurora-A
BRSK2
CAMK2 δ^*
MEK1-CA
MKK3
MKK6
NEK6
NEK7
PAK4

(10)

 Member of KinEASE™ panel

(13)

(23)

* Ionomycin inducible



Substrate comparison

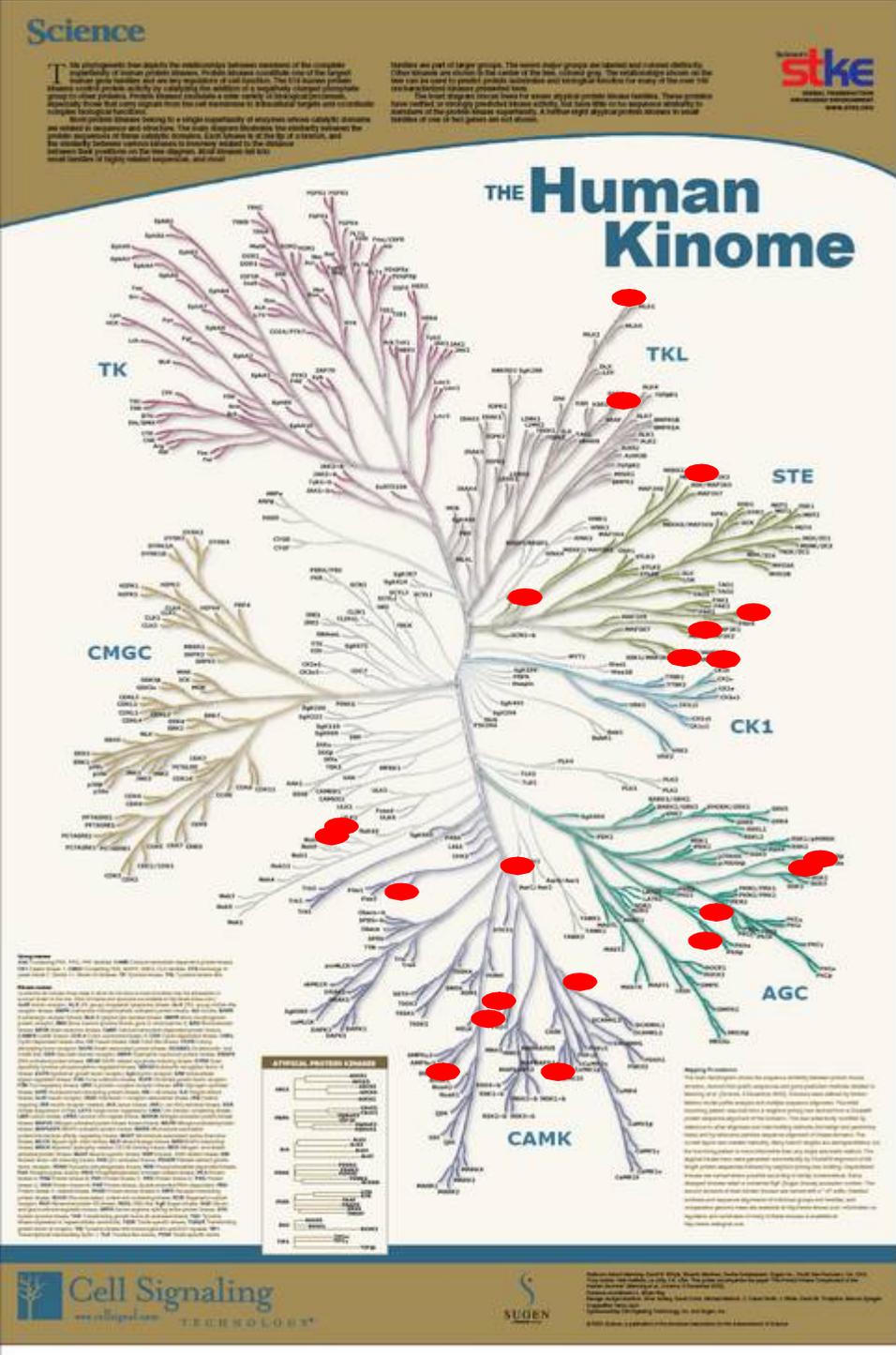
KinEASE™ substrate

	<u>cellular</u>	<u>biochem</u>	
AKT1	3	3	✓
AKT2	3	3	✓
Aurora -A	2	2	✓
BRSK2	3	1	
CAMK1/2 δ	1	1	✓
CHK2	3	1	
NEK6	3	3	✓
NEK7	3	3	✓
PAK4	3	2	
Pim -2	3	3	✓
PKA	2	2	✓
PKD2	3	1	
PRKX	2	2	✓

9/13

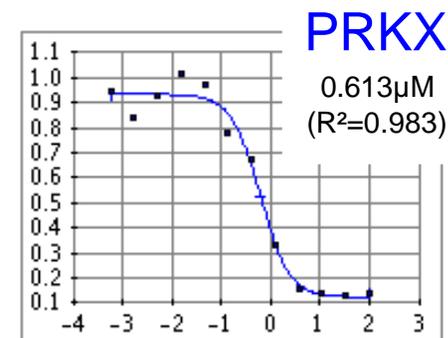
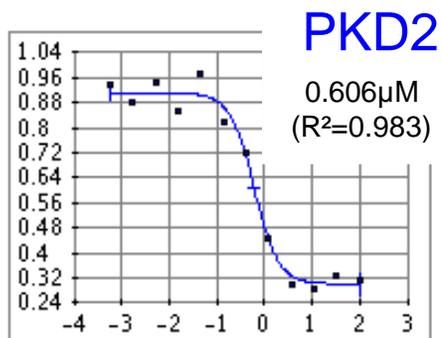
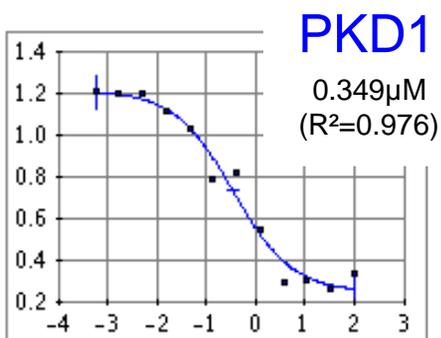
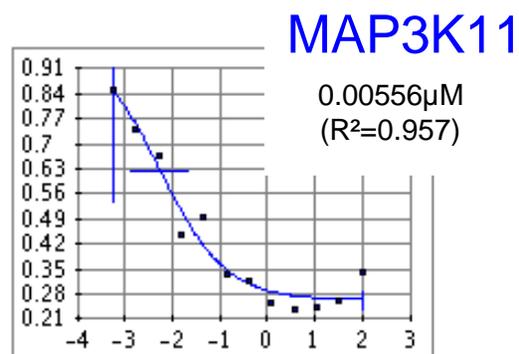
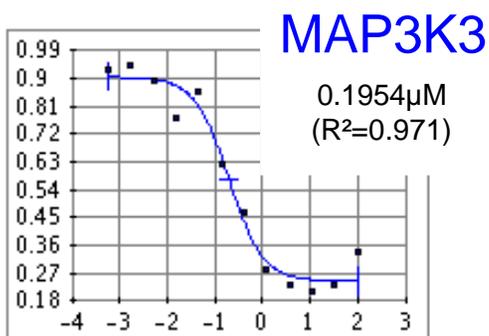
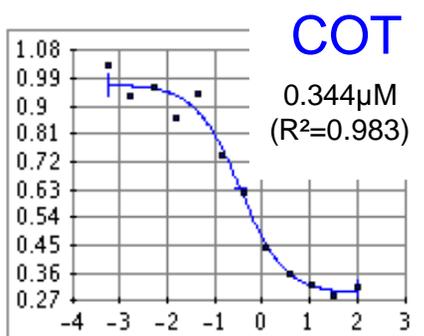
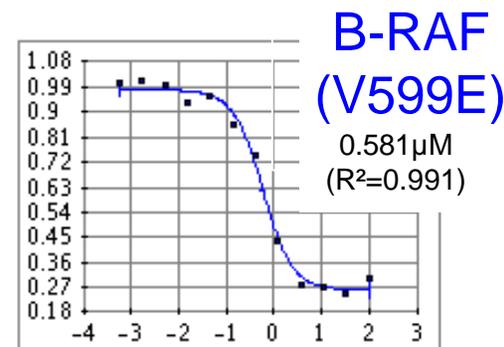
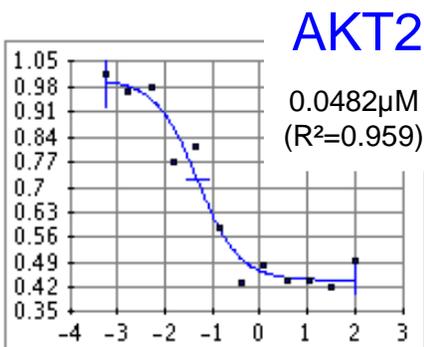
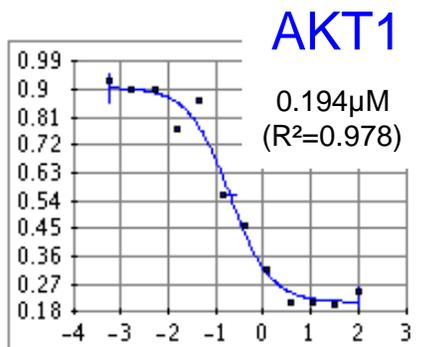
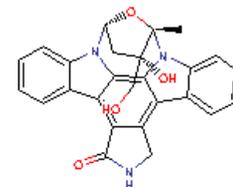


Phylogenetic distribution



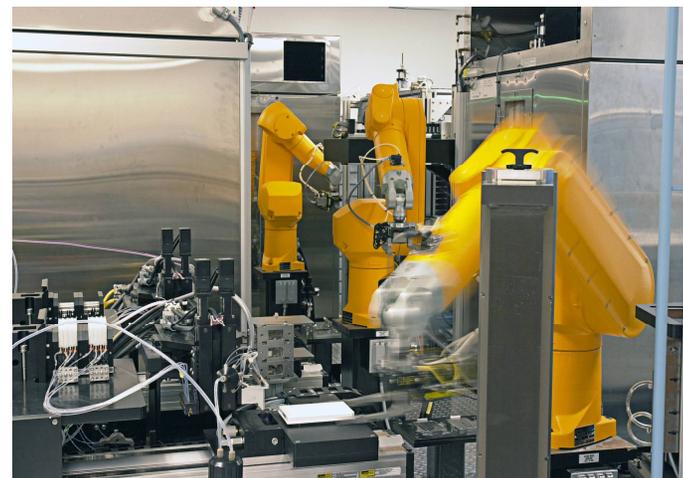
Towards HT-screening & profiling

1536w

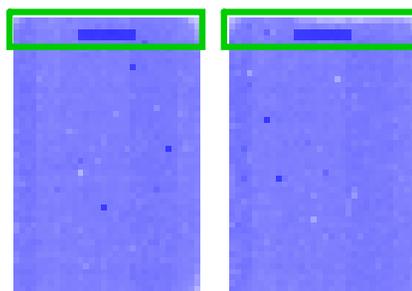


AKT1 – online validation

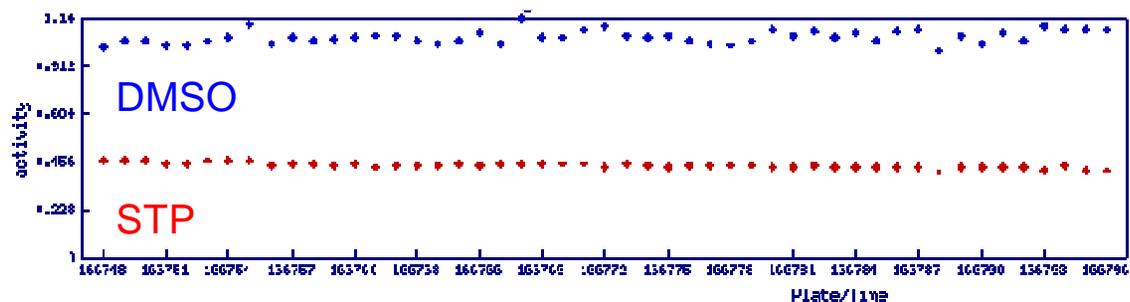
- 15 plates in triplicates ~21k cmpds, 10 μ M
- transient bulk transfection
- $CV_{STP} = 3\%$; $CV_{DMSO} = 8\%$; $Z' \sim 0.5$



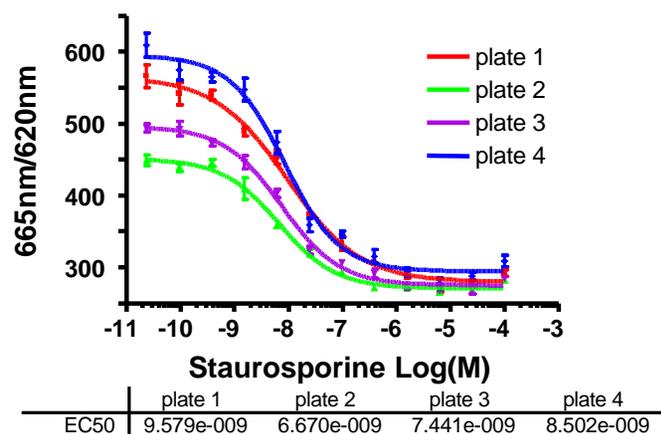
Internal Controls
1% DMSO, 1 μ M STP



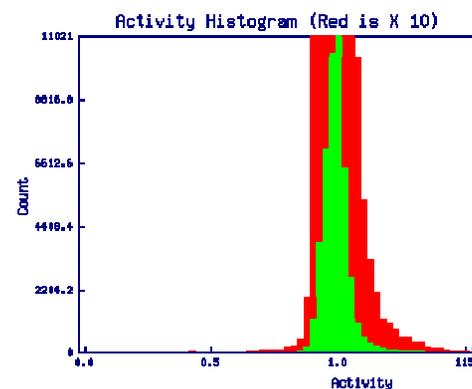
Controls across screen



Control curves

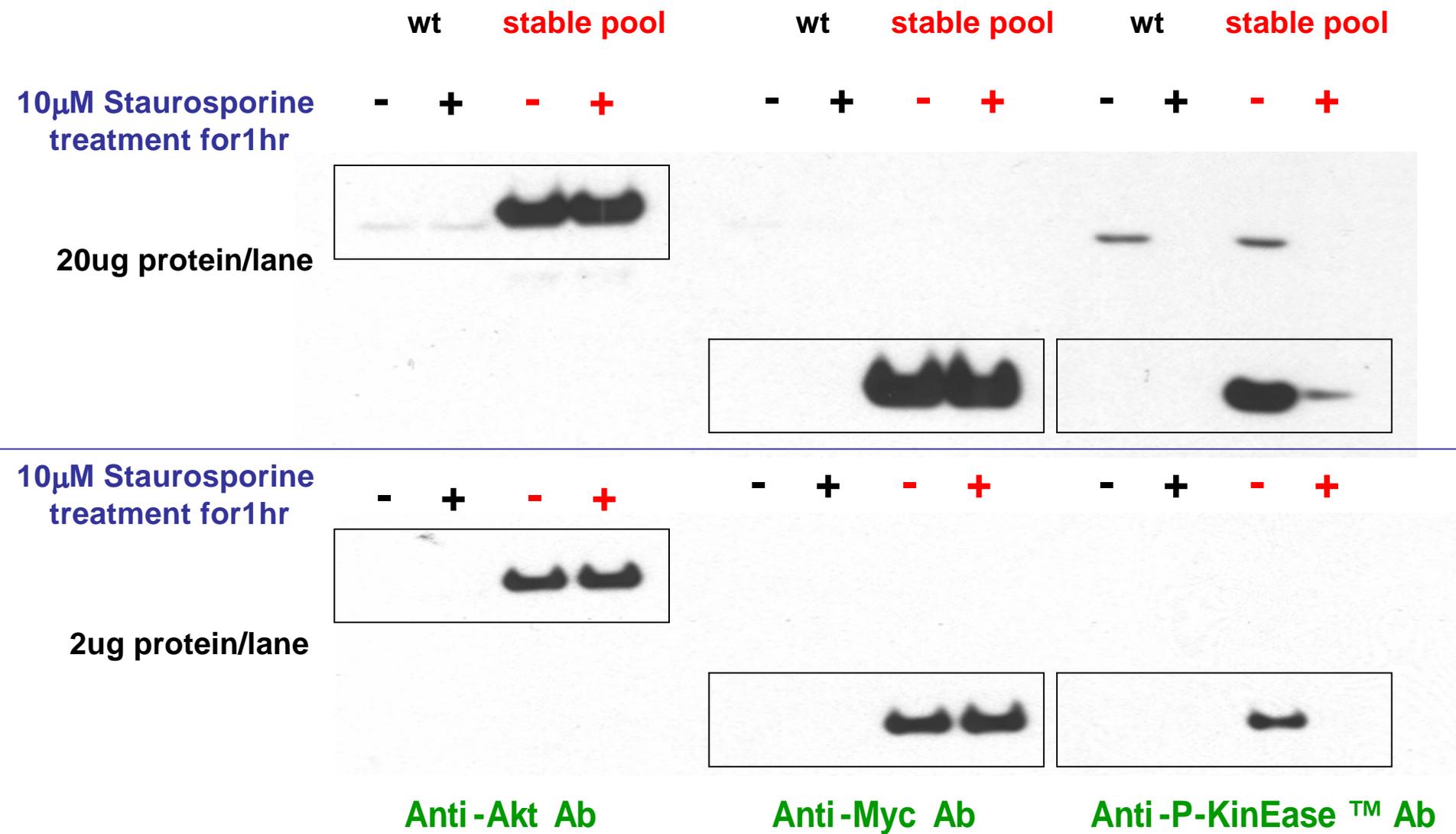


Hit distribution



Towards stable cell lines

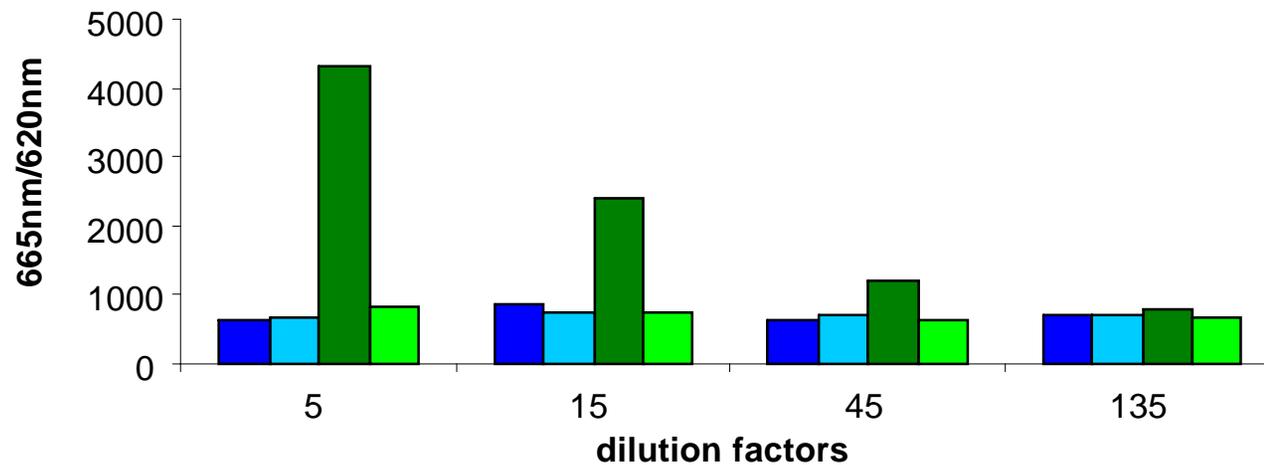
AKT & Substrate3, HEK293



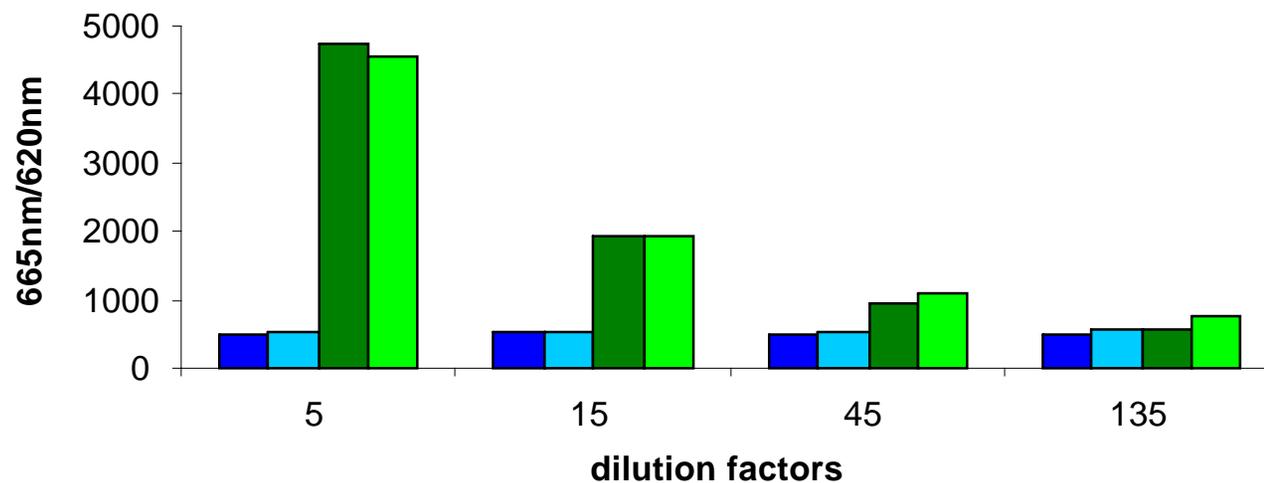
Towards stable cell lines

HTRF® of wt or stable pool lysates by **XL-FLAG** and **Eu-KinEase™** Abs

■ wt, no treatment ■ wt, staurosporine ■ stable pool, no treatment ■ stable pool, staurosporine

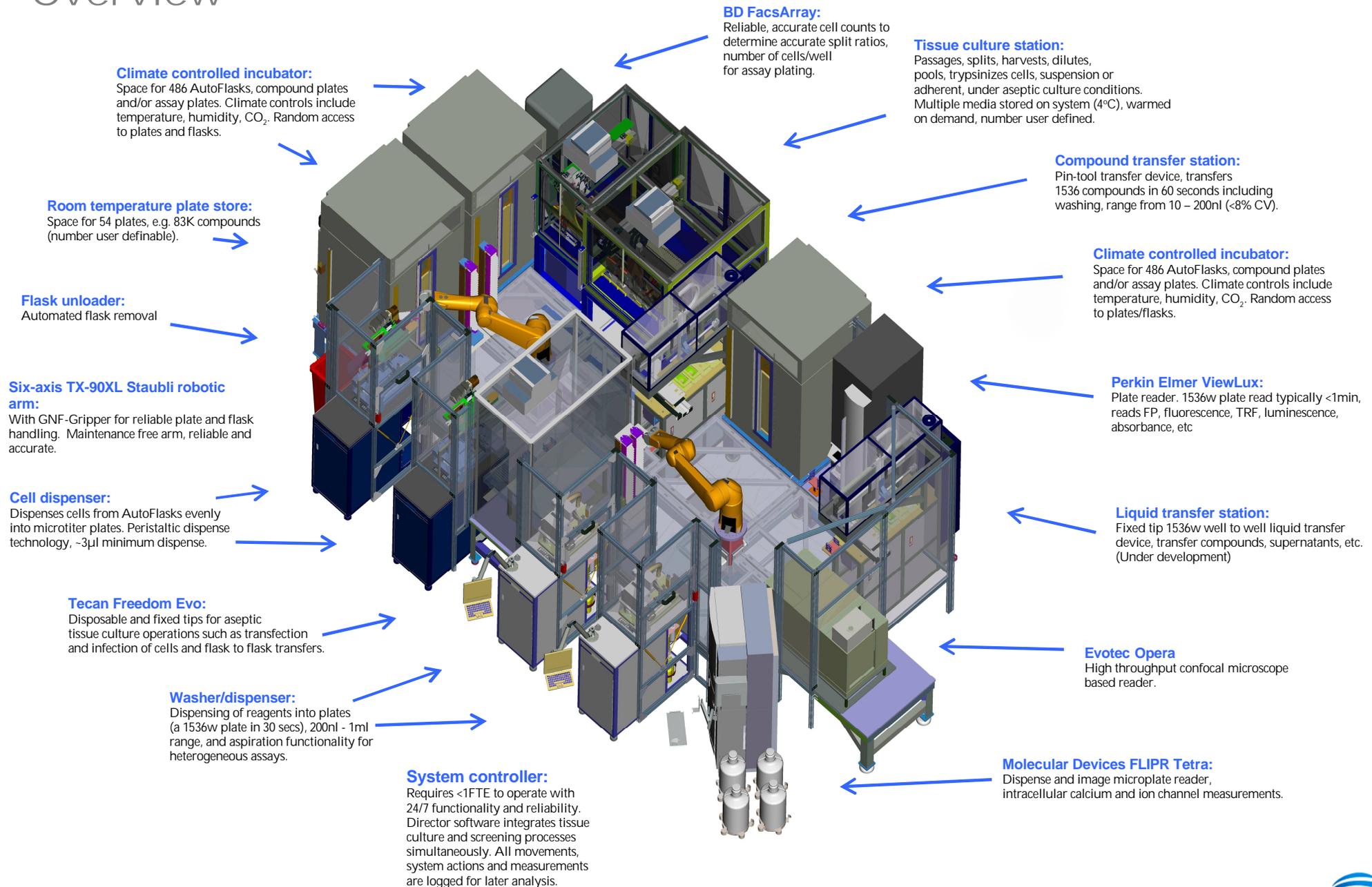


HTRF® of wt or stable pool lysates by **XL-FLAG** and **Eu-Myc** Abs



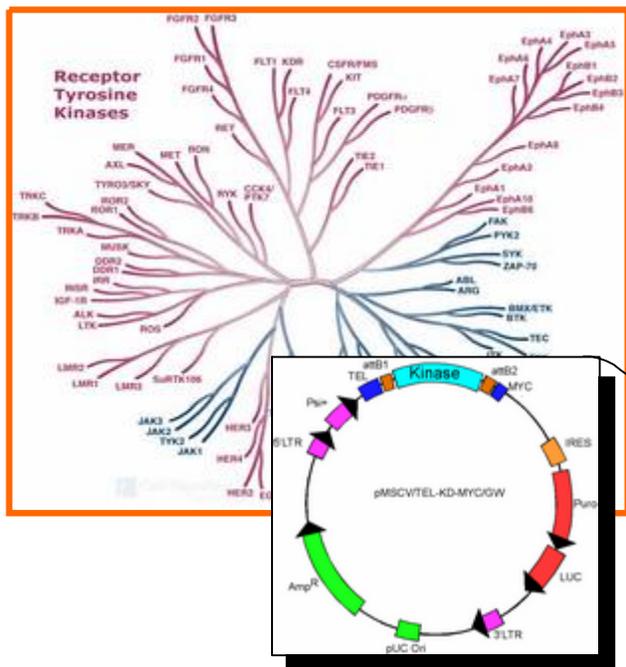
Towards automated profiling – GNF's ACP Gen2

Overview



Automated profiling in the YK - space

Kinase family

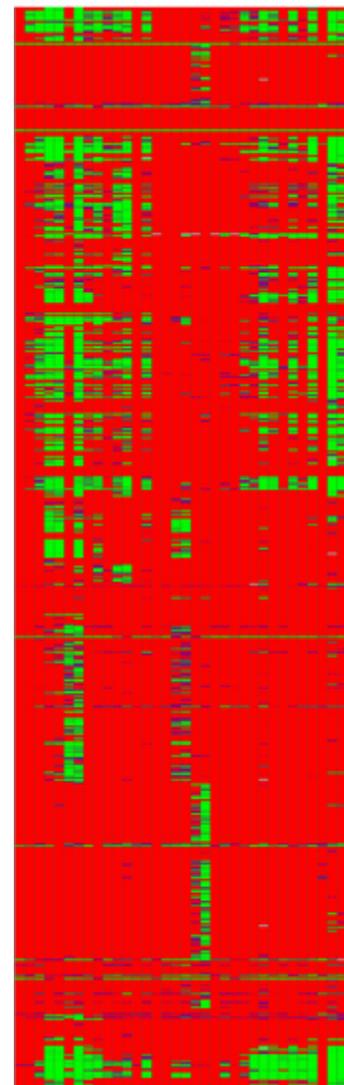


ACP Automated cellular profiling system

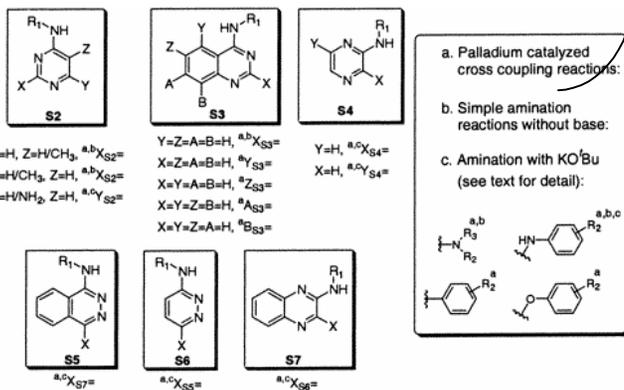
- 100+ cell lines
- 5,000 compounds
- 2-week turnaround



Panel profile



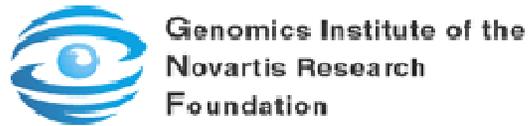
Kinase -targeted library



Melnick (2006) PNAS



Thank you!!



Yu Wang
Deanna Adams

Dan Sipes
Avi Spier

Jeremy Caldwell

Peter Schultz

Emmanuel Claret
Michel Fink

Krista Steger
Ron Bedell
Mark Schmeizl

G rard Mathis

