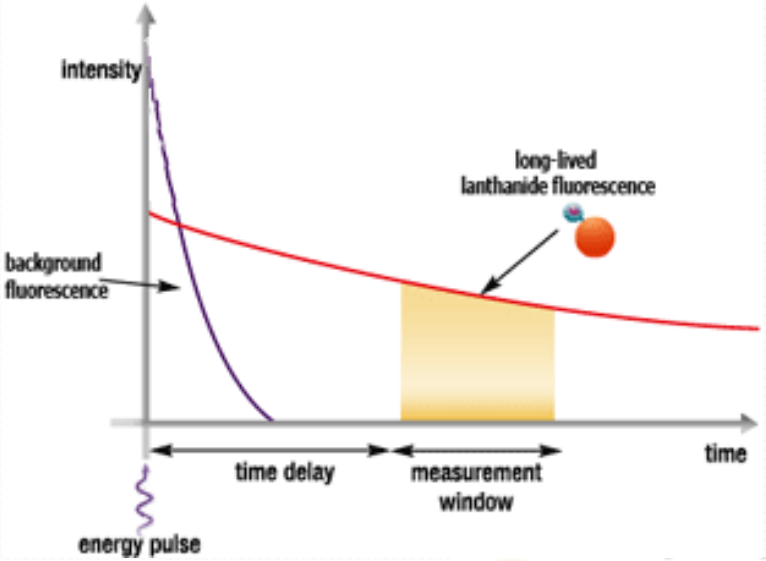
HTRF: One technology, many uses in a pre-clinical laboratory setting.

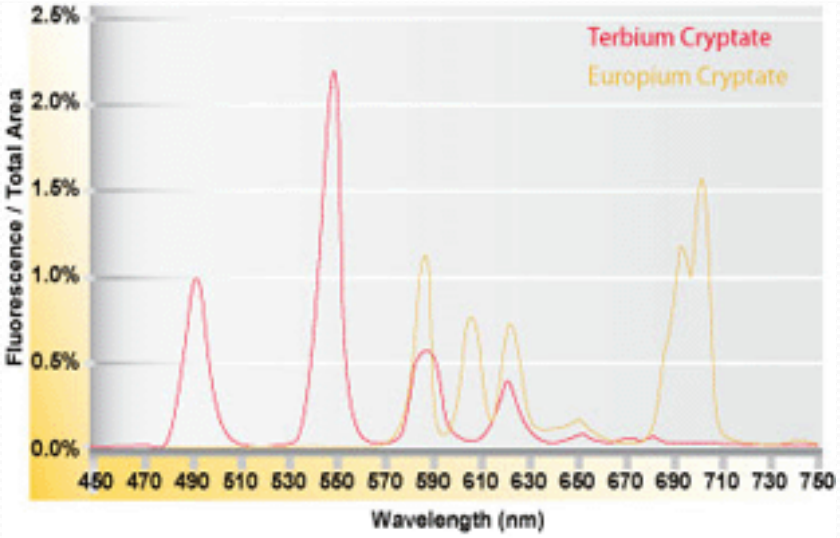
SLAS 2012

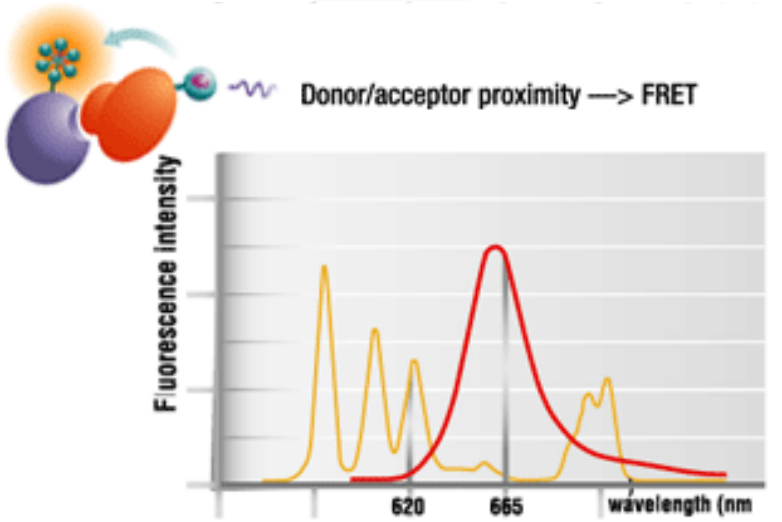
Outline of Discussion

1. Brief Review of HTRF Technology
2. Our Capabilities/Desires for the use of HTRF in drug development research.
3. Uses of HTRF Technology:
   - Binding Assay
     - Receptor Tyrosine Kinase ECD Dimer Competition Assay
     - Assay development discussion
   - Quantitation Assays
     - Cell line characterization
     - Serum samples
   - Cellular Activity Assay
     - pAkt and pERK evaluation
- Summary and Discussion of Additional Planned Uses of HTRF

Homogenous Time Resolved Fluorescence:







Benefits and Considerations of HTRF:

- Advantages:
  - Homogeneous: No plate binding, washing or detection development
  - Quick assay development.
  - Low matrix effects
  - Practical cost/well
  - Slow bleach rates of lanthinides allow multiple reads (kinetic runs)

- Disadvantages:
  - Requires a good set of reagents
  - Potential for “hook” effect

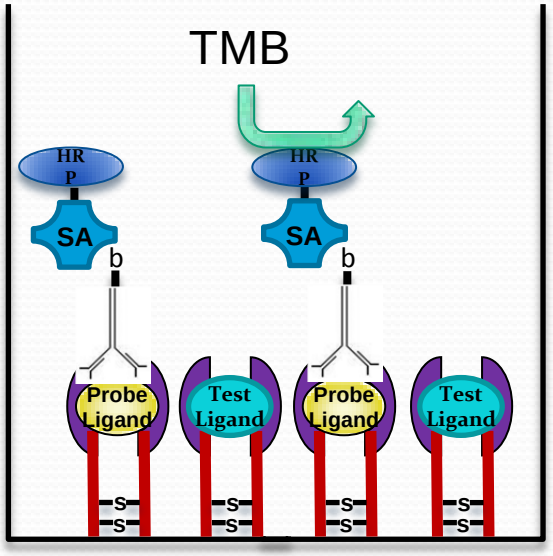
General Methods:

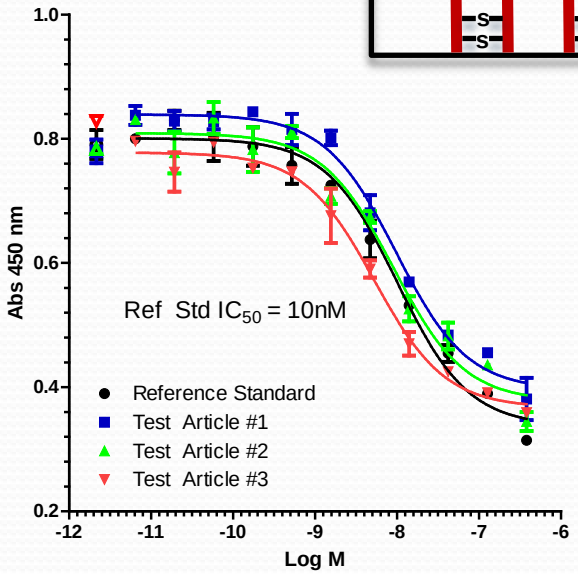
- Biotek Synergy 2 Instrument
  - Equipped with:
    - 330 ± 40 Excitation Filter
    - 620 ± 5 Emission Filter
    - 665 ± 4 Emission Filter
    - Xenon flash lamp as excitation source
  - Data Acquisition:
    - Gen5 software
    - Sequential Mode: 665nm emission then 620nm emis...
    - 100µs delay; 300µs integration
    - 10 – 20 reads/well
    - Gain set to highest expect signal source to maximize dynamic range

- Corning #3673 half-volume, 384-well white plates
  - Reaction volumes 20µl
- Data analysis:
  - $$\text{HTRF Ratio} = \frac{665_{em}}{620_{em}} \text{x} 10{,}000 \text{ or } \Delta F\% = \frac{(HTRF_{Test} - HTRF_{Control})}{HTRF_{Control}}$$

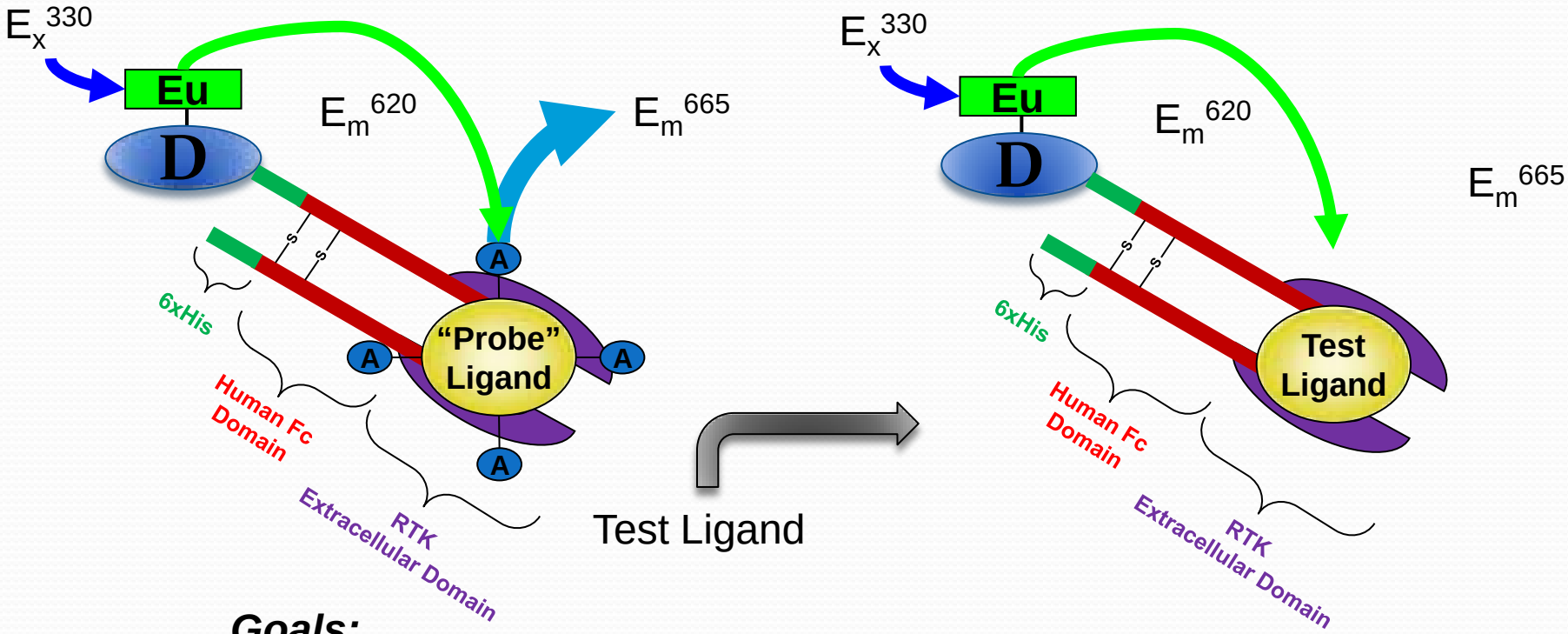
Plate Bound Competition Assay:

1. Coat o/n with RTK-ECD (18h)
2. Wash
3. Block (1h)
4. Perform binding with probe/test ligands (1h)
5. Wash
6. Label with biotinylated detection antibody (1h)
7. Wash
8. Label with streptavidin-HRP (0.5h)
9. Wash
10. Incubate with TMB (0.5h)
11. Read in plate reader





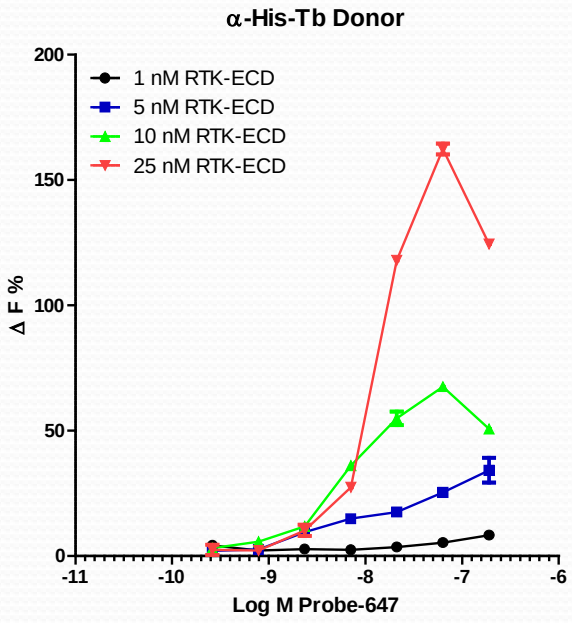
HTRF Binding Assays: Competition Format

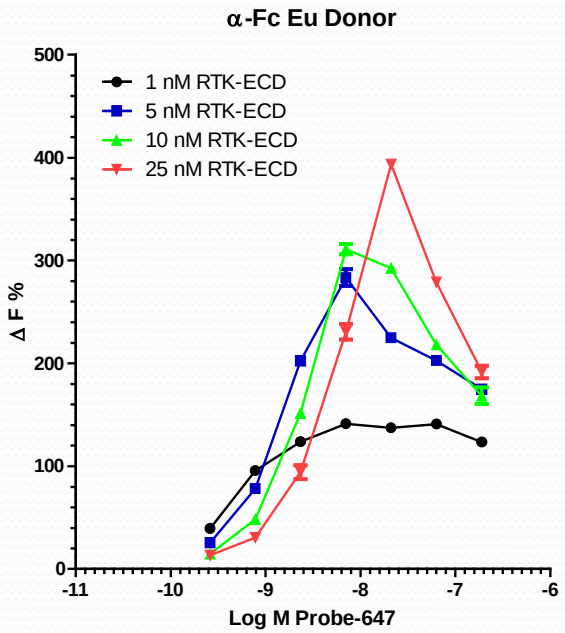


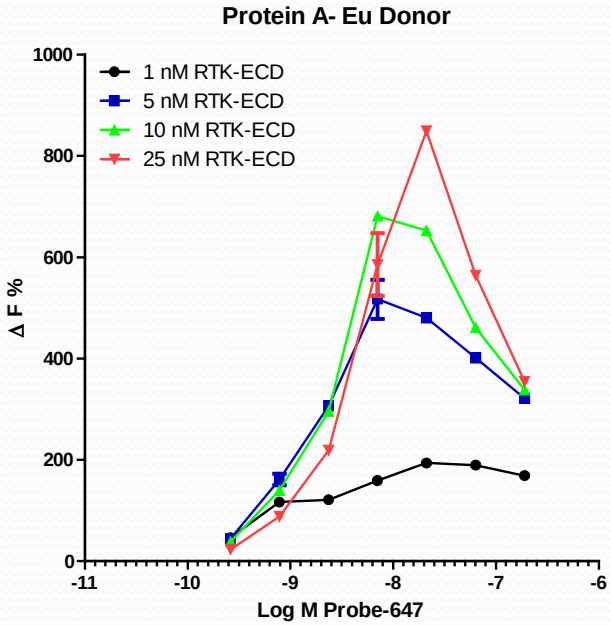
Goals:

1. True estimate of IC_{50} of test ligands
2. Non-radioactive
3. Low (<10%) ligand depletion conditions
4. Works within the pM – nM range
5. Highly reproducible
6. Measure dozens/hundreds of variants at a time

Identification of Donor Molecule:

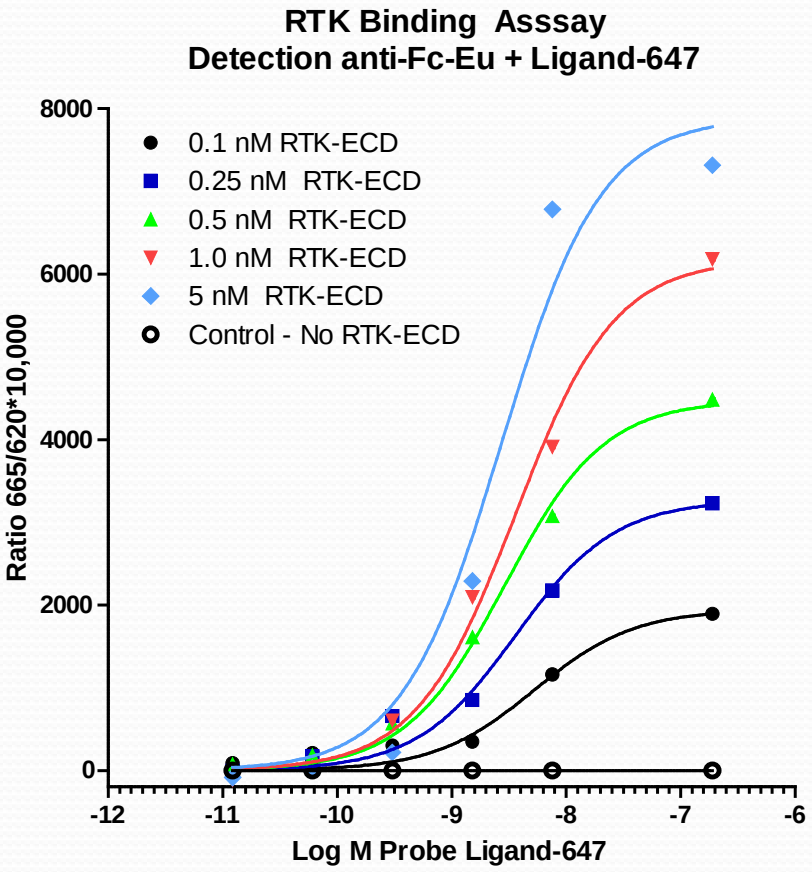


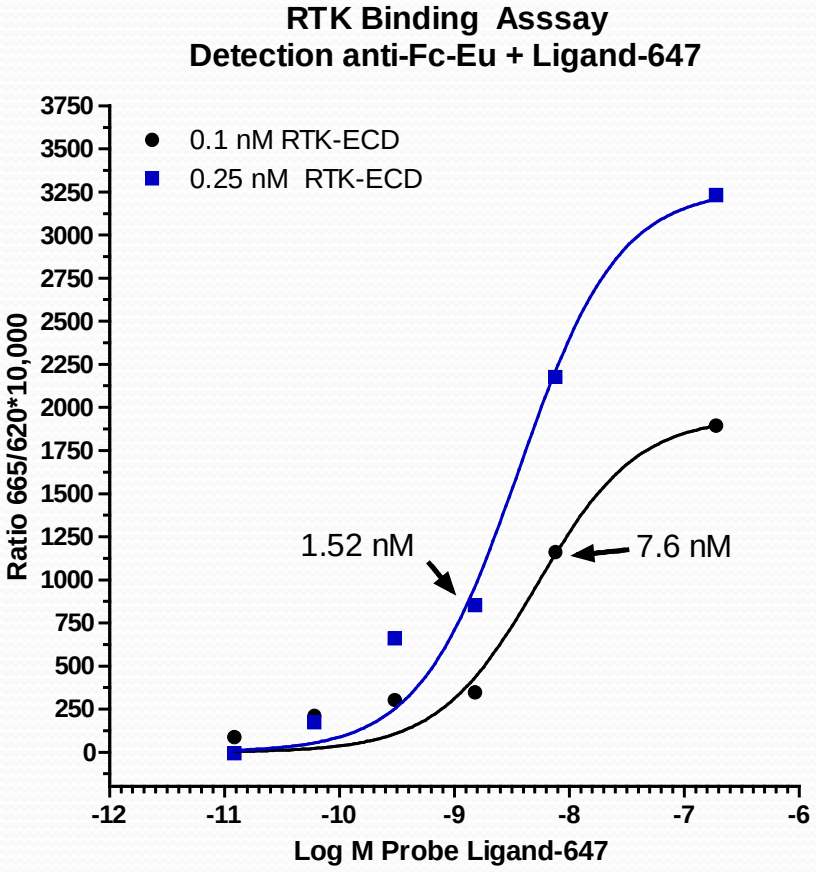




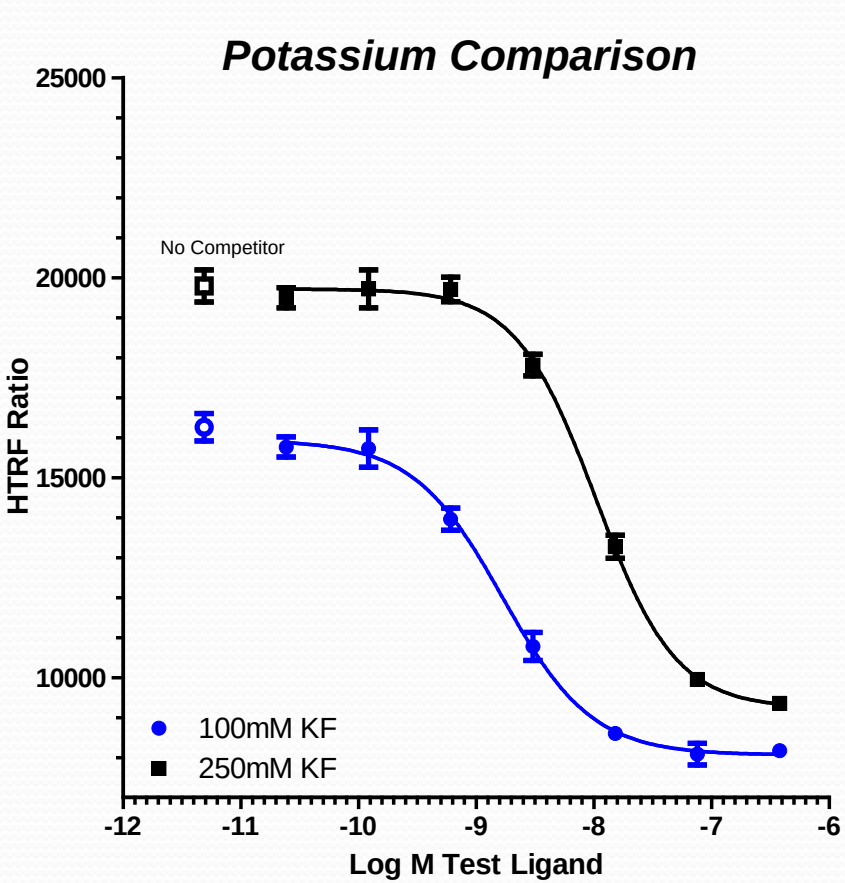
- Alexa-647 labeled Probe Ligand
- Donors at 1:400
- 2.0 h Incubation with all components

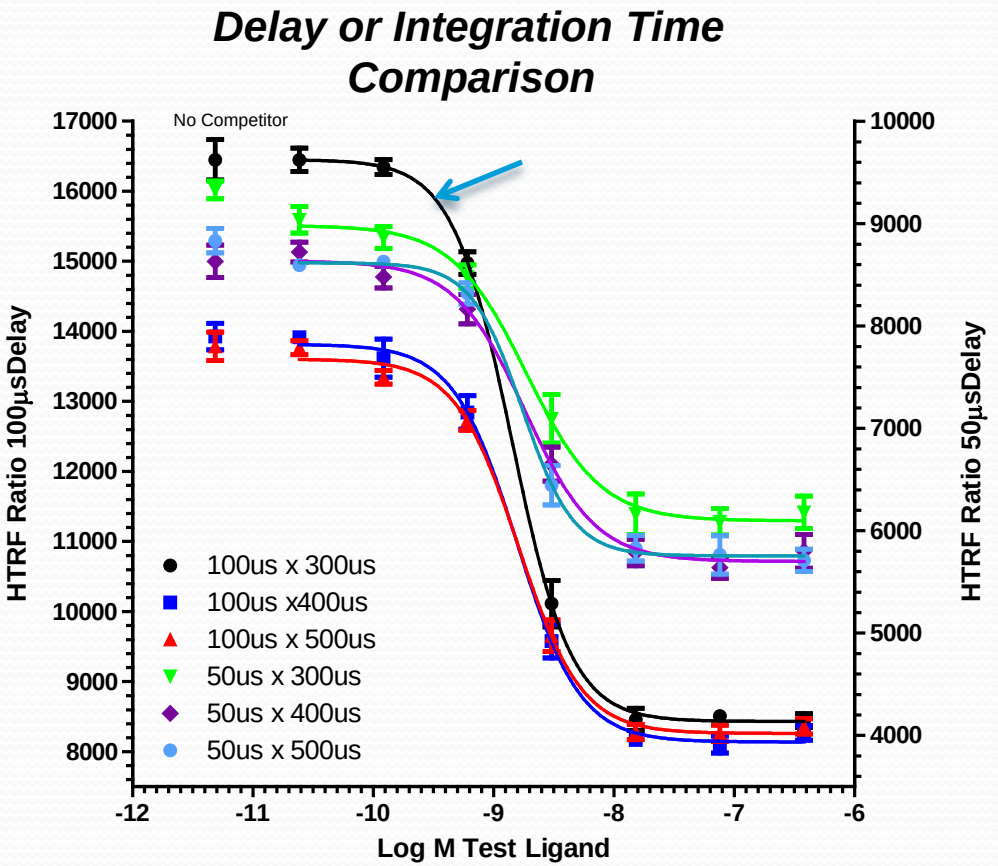
Determining Practical Concentrations of RTK-ECD and Probe Ligand:





Effects of KF and Read Times on Assay Performance:

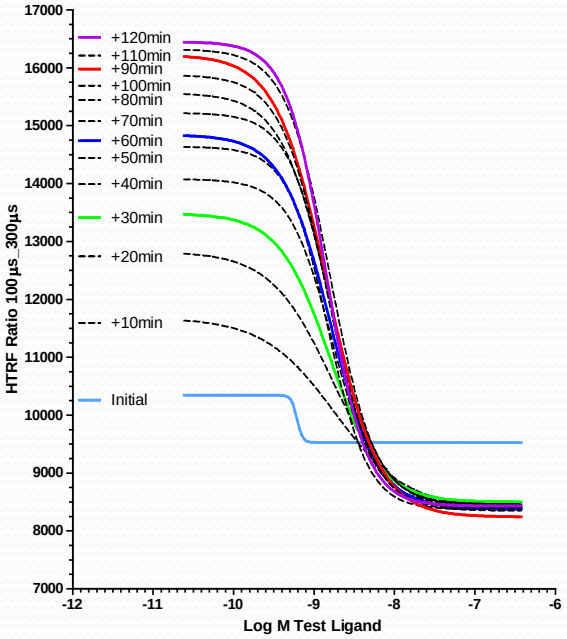




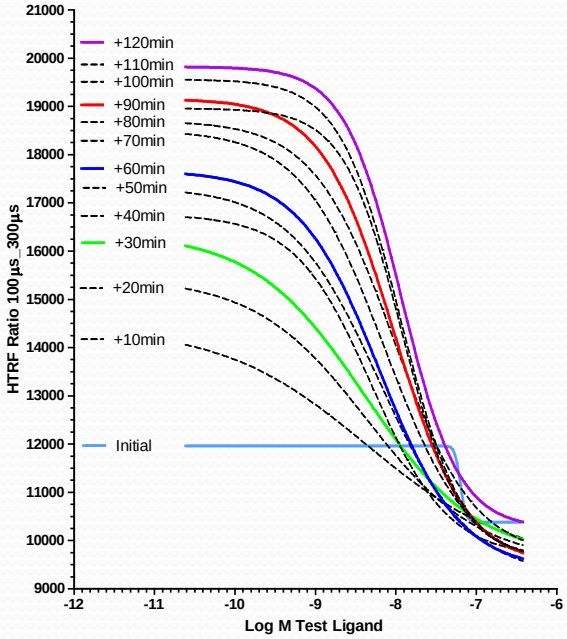
- *biotinylated Probe Ligand (7.6nM) + 0.1nM RTK Dimer
- Strept-D2 + anti-Fc-Eu (1:400)
- 2.0 h Incubation with all components

Time Course of Binding Equilibrium

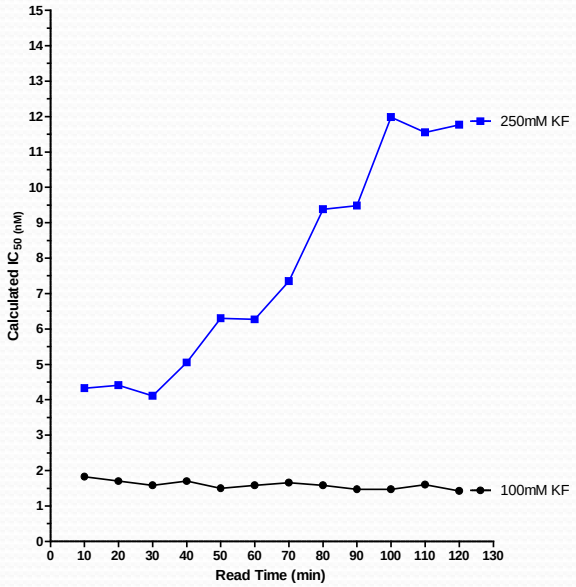
Time Course 100mM KF



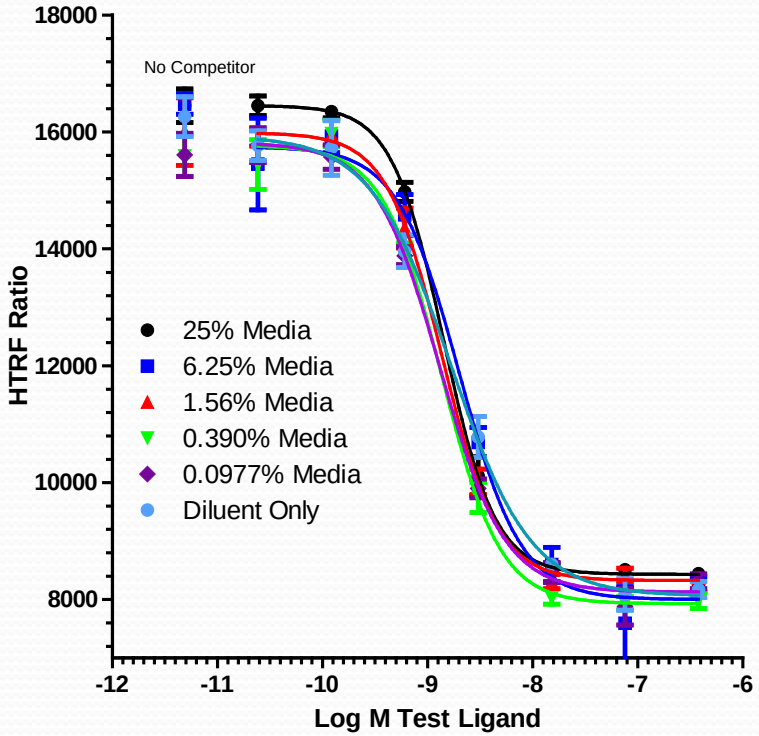
Time Course 250mM KF



Calculated IC_{50}s



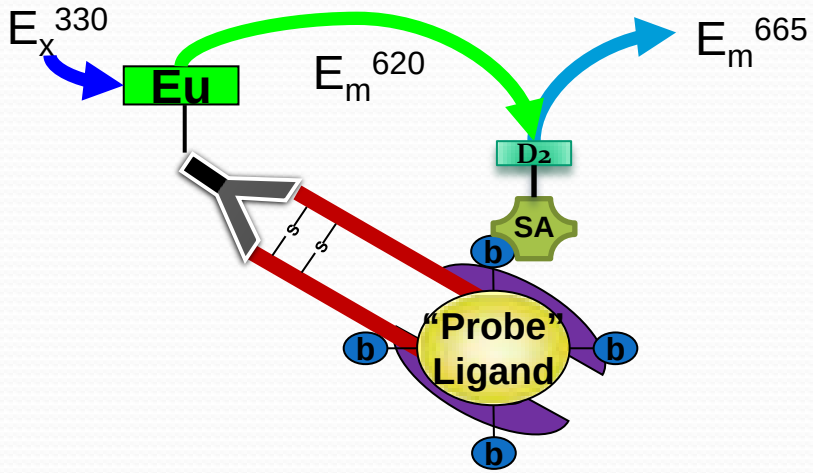
Effect of Matrix Concentrations on Assay Performance:



Column1	25% Media	6.25% Media	1.56% Media	0.390% Media	0.0977% Media	Diluent Only	Mean	Standard Dev
Bottom	8431	8000	8324	7928	8128	8063	**8145.7**	**194.4**
Top	16450	15745	15984	15770	15816	15934	**15949.8**	**262.2**
Log IC50	-8.847	-8.697	-8.861	-8.867	-8.882	-8.775	**-8.8**	**0.07**
HillSlope	-1.762	-1.464	-1.639	-1.546	-1.415	-1.141	**-1.5**	**0.21**
IC50	1.4E-09	2.008E-09	1.4E-09	1.357E-09	1.31E-09	1.7E-09	**1.53E-09**	**2.70E-10**
Span	8019	7745	7660	7842	7688	7871	**7804.2**	**134.0**

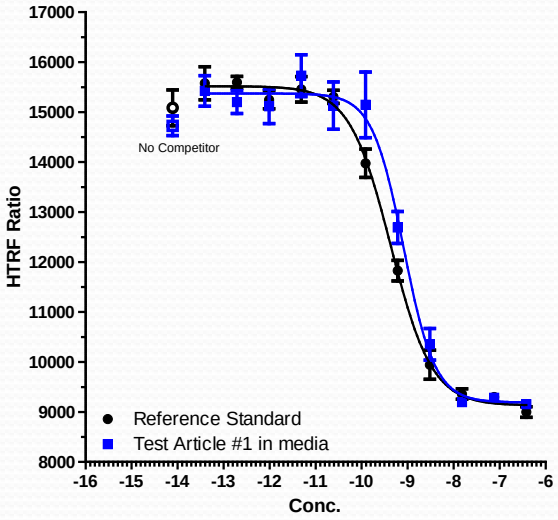
Summary of HTRF RTK-ECD Competition Assay Procedure:

1. RTK-ECD at 4x (5µl/well)
2. Reference Standard/Test Article in diluent + Probe Ligand at 2x (10µl/well)
3. Detection Reagents in diluent + KF at 4x (5µl/well)
   - StreptAv-D2 and anti-Fc-Eu
4. Read with HTRF settings

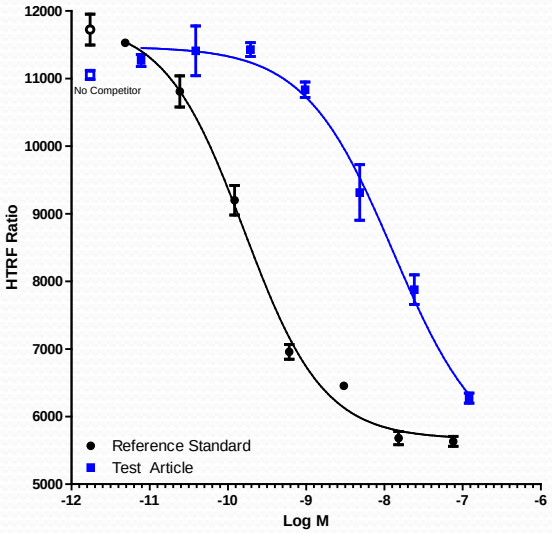


HTRF Competition Assay in Practice:

Two Related Molecules



Two Related Molecules



Same molecule, different production parameters

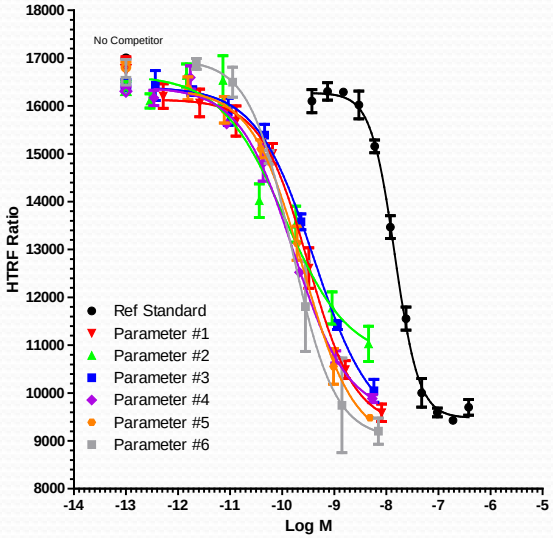


Plate Bound Assay

Reagent	Cost	Amt	cost/well
RTK-ECD	$200	50 ug	**$0.20**
anti-Probe Abs	$380	50 ug	**$0.02**
Probe Ligand	$295	10 ug	**$0.06**
Strept-Av-HRP	$695	0.5 ml	**$0.01**
High Bind Plates	$242	100 each	**$0.03**
TMB	$135	600 ml	**$0.02**
Total			**$0.34**

HTRF Assay

Reagent	Cost	Amt	cost/well
RTK-ECD	$200	50 ug	**$0.02**
Strept-D2	$793	1 ml	**$0.04**
anti-Fc-EU	$790	1 ml	**$0.04**
White Plates	$900	100 each	**$0.09**
Total			**$0.19**

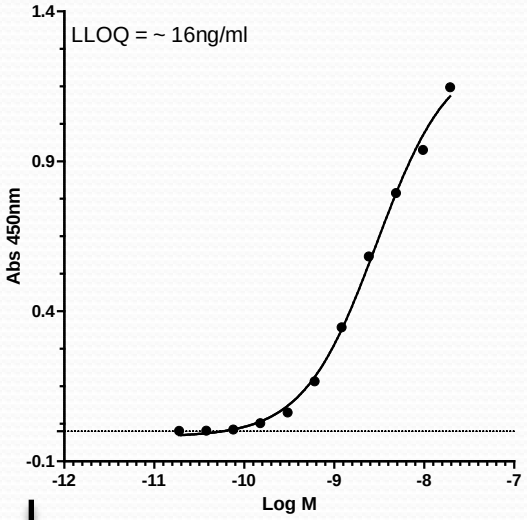
HTRF Quantiation Assays:

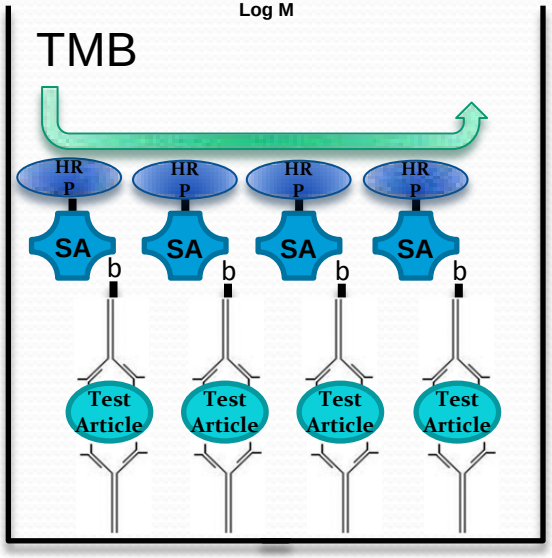
- Applications:
  - Evaluating production amounts in conditioned media.
    - Both transient and clonal cell lines
  - Identifying best producing cell clone in cells under selection
  - Evaluating drug product present in biological test samples.

Examples of Quantitation Assays:

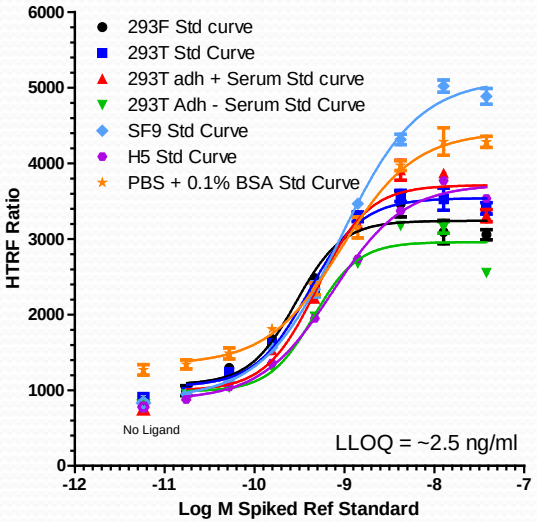
Sandwich Format

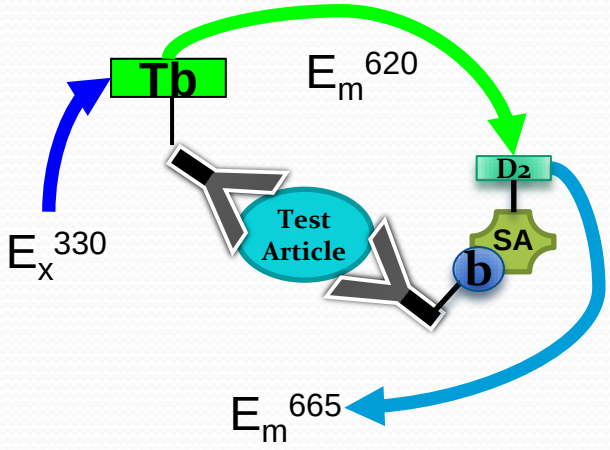
Sandwich ELISA



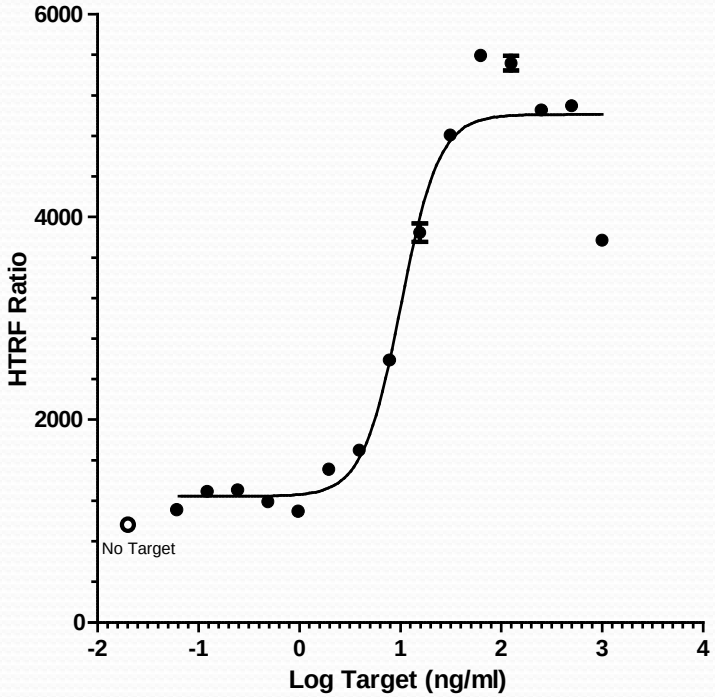


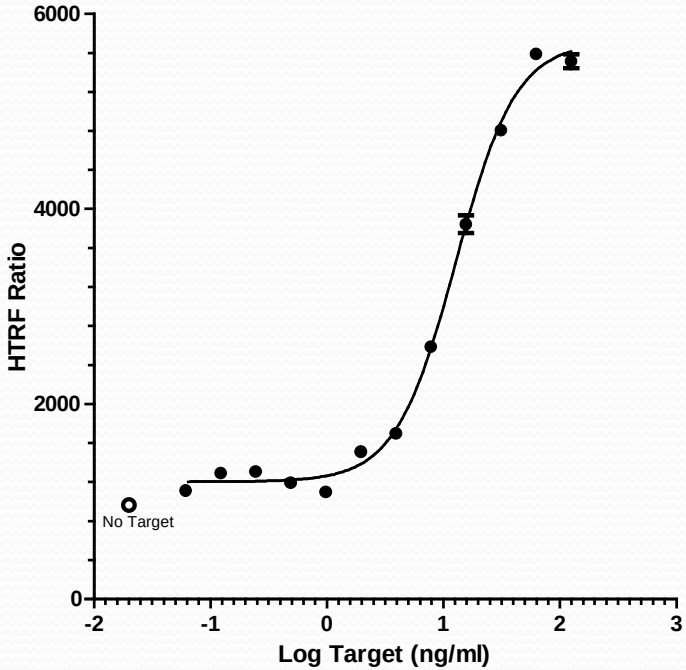
Sandwich HTRF





Considerations with Quantitation:

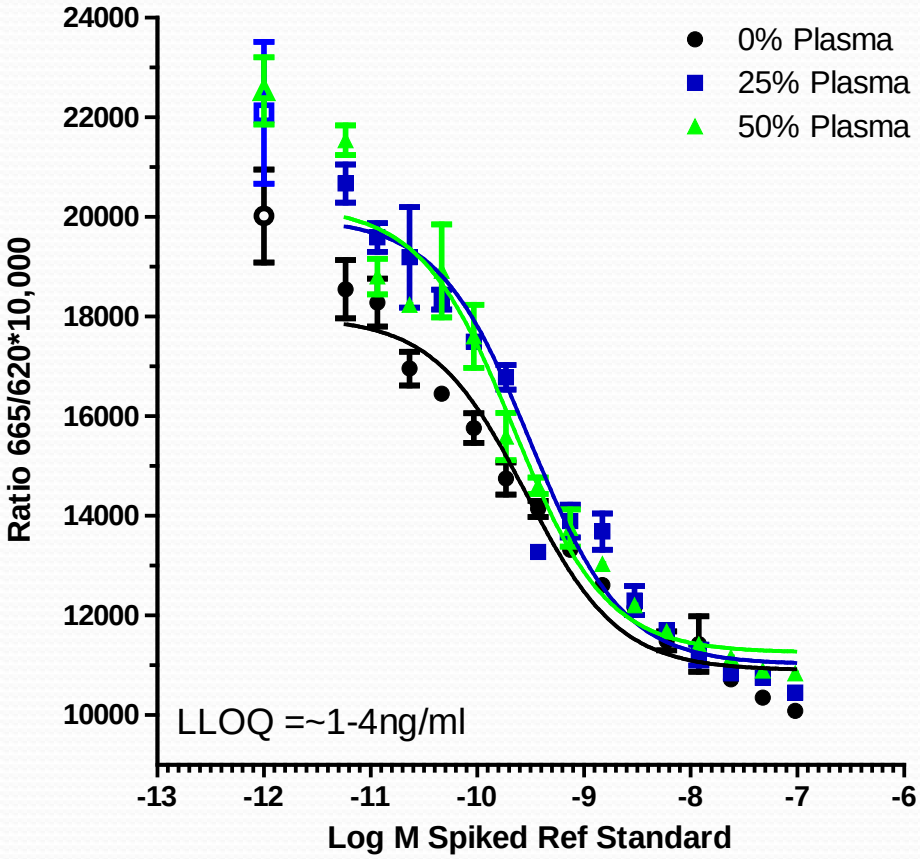


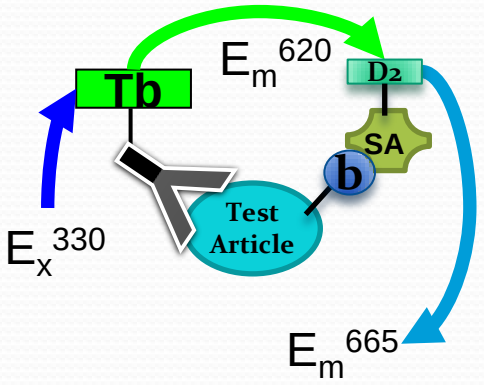


Multiple dilutions of a test sample must be tested.

Examples of Quantitation Assays:

Competition Format





Cellular Activity Assay:

tAkt/pAkt-Ser^{473} Cell Based Detection Kit

Activate 5-30 min

Fix 20 min

Wash (3 x 5min)

Quench 20 min

Wash (3 x 5min)

Block 1h

Wash (3 x 5min)

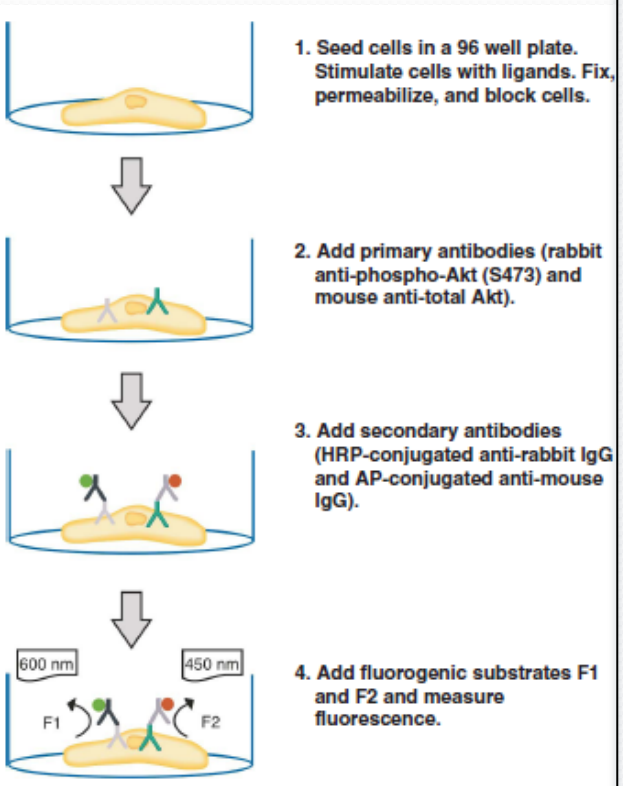
Label 1º 16h

Wash (3 x 5min)

Label 2º 2h

Wash (4 x 5min)

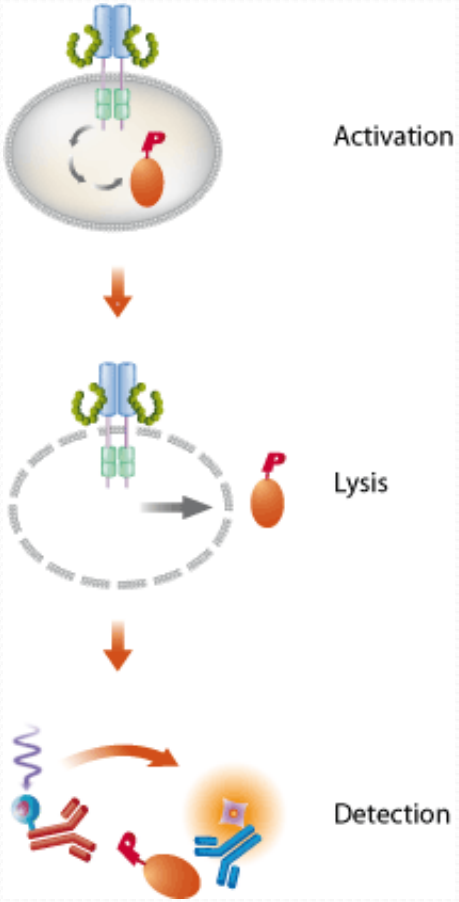
Develop ~30min



1. Seed cells in a 96 well plate. Stimulate cells with ligands. Fix, permeabilize, and block cells.
2. Add primary antibodies (rabbit anti-phospho-Akt (S473) and mouse anti-total Akt).
3. Add secondary antibodies (HRP-conjugated anti-rabbit IgG and AP-conjugated anti-mouse IgG).
4. Add fluorogenic substrates F1 and F2 and measure fluorescence.

~$4.70/well

pAkt-Ser^{473} or Cellul'ERK-Cisbio



Activation 5-30 min

Lysis

30 min

Freeze Lysates

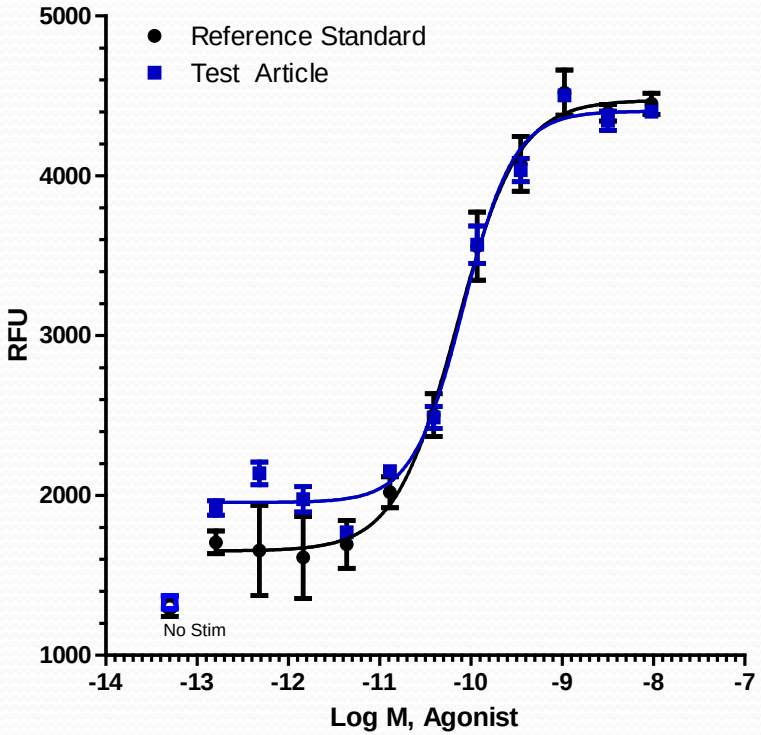
Detection

~3h

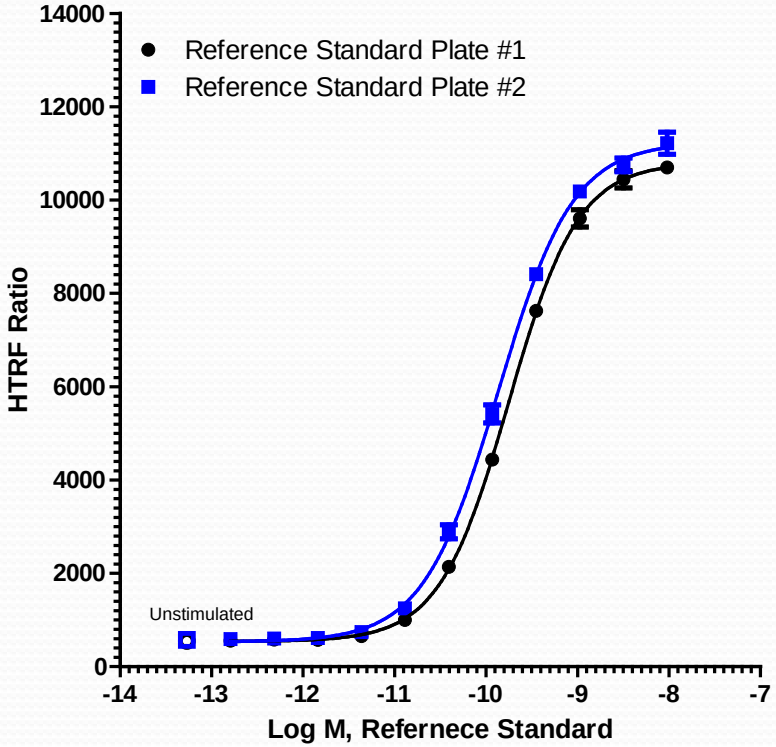
~$0.70/well

Comparison of Assay Performance:

pAkt-Ser^{473} Cell Based

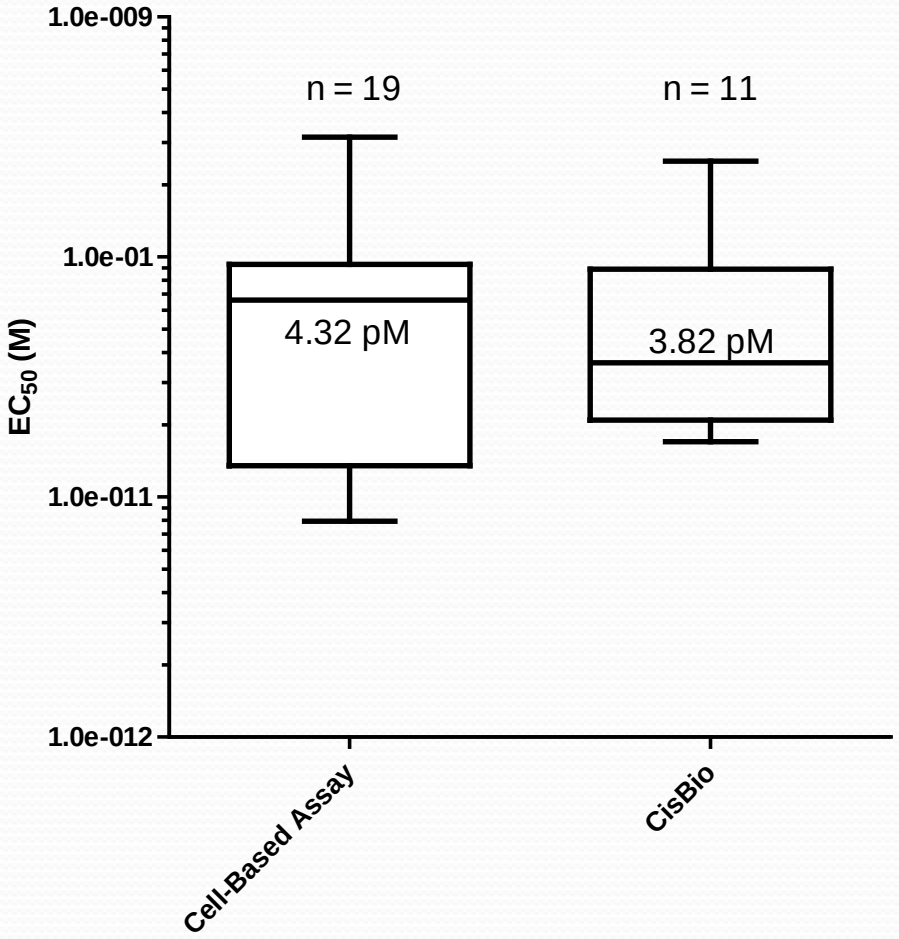


pAkt-Ser^{473} Cisbio



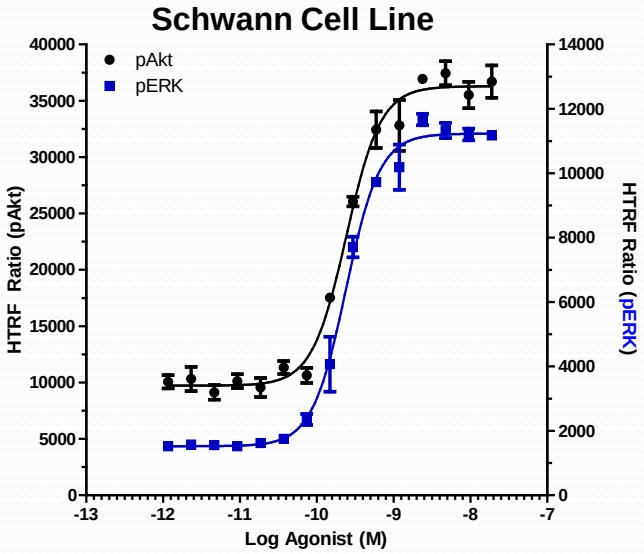
- Both Assays went through standard optimization parameters
  - Cell number
  - Stimulation time
  - Lysis volume (Cisbio)

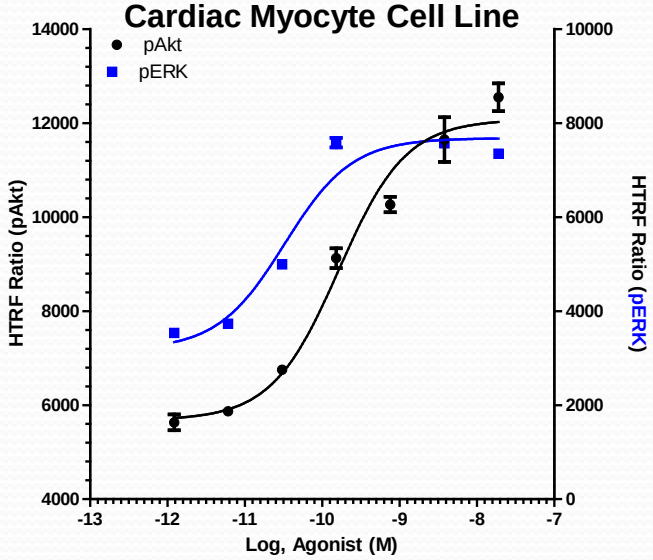
Comparison of Assay Performance: pAkt-Ser^{473}

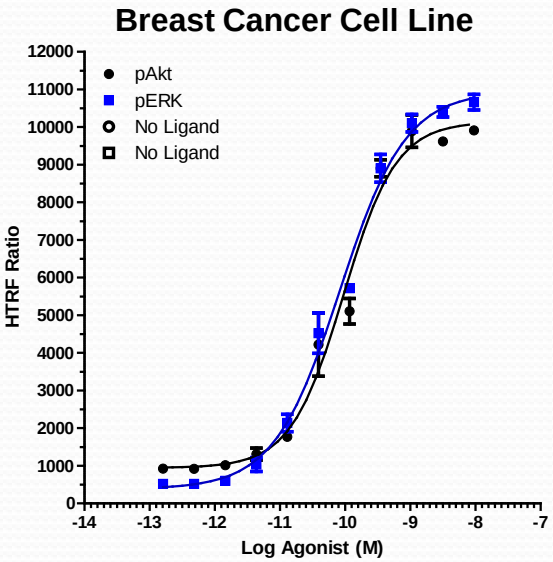


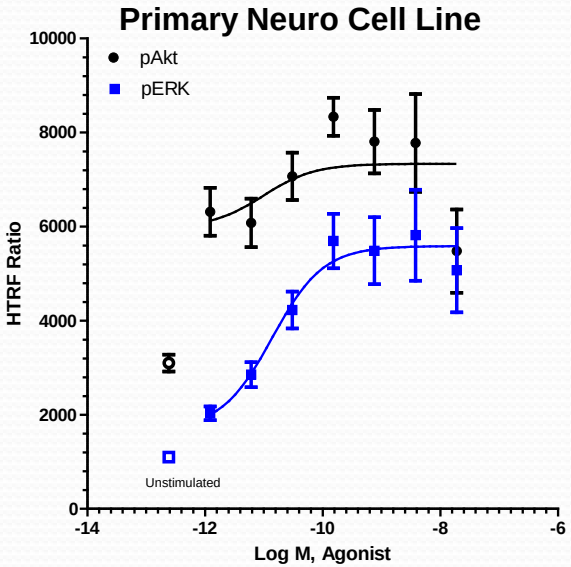
Activity Assays:

Comparison of pAkt-Ser^{473} and Cellul'ERK in Multiple Cell Lines









Summary

- HTRF is a robust methodology with several key benefits:
  - Time savings
    - Day-to-day
    - Assay development
  - Cost savings
    - Reagent
    - Personnel time
  - Equal if not better assay performance than standard assay formats
  - Flexible conditions
- Other uses:
  - Screening for novel molecules
  - Multiplexed assay
    - Using two acceptor assay systems
  - Moving assays into qualification/GXP studies
  - ATA Assay???