

# Homogeneous assays for biomarker quantification and interaction studies



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Discovery & Analytical Solutions

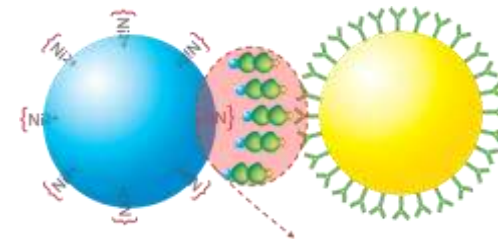
Teramo 14.12.2017



HUMAN HEALTH • ENVIRONMENTAL HEALTH

## ➤ Alpha technology

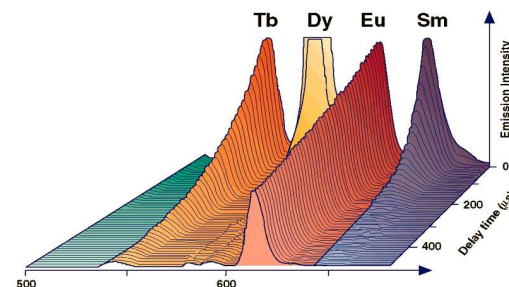
- biomarker quantification
- detection of phosphoproteins
- detection of protein:protein interactions



## *Detection of protein:protein interaction by "label-free"*

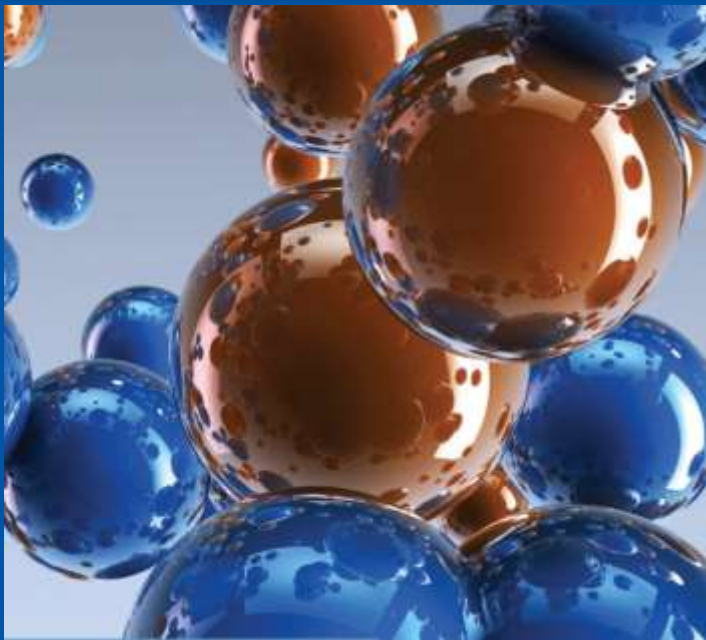
## ➤ LANCE (TR-FRET) technology

- biomarker quantification
- kinase assays



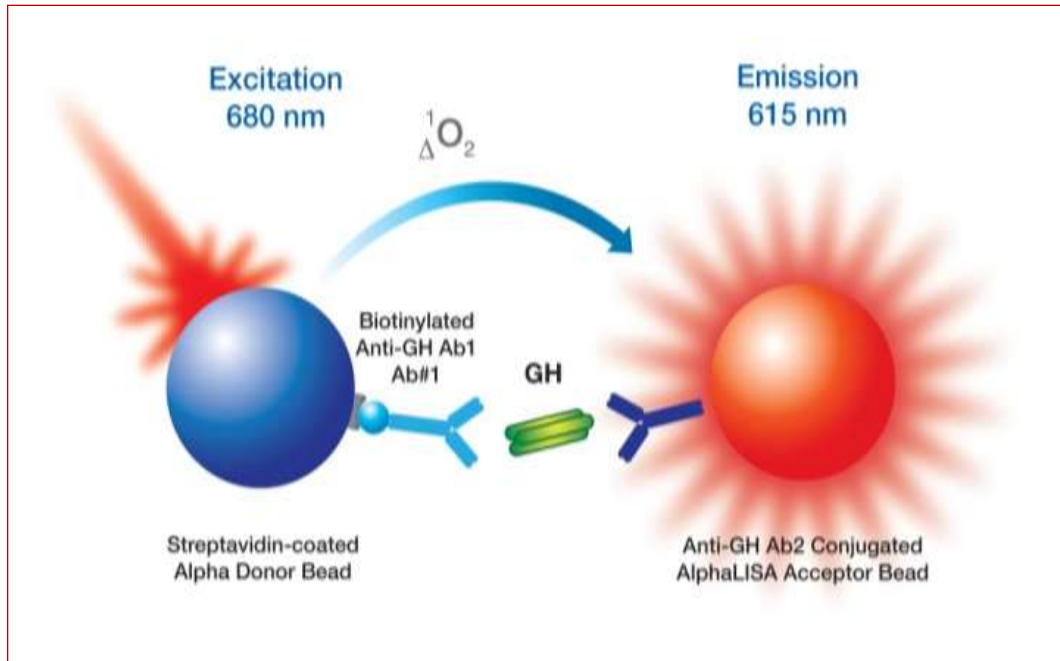
## ➤ How to setup your Alpha assay





## Alpha technology

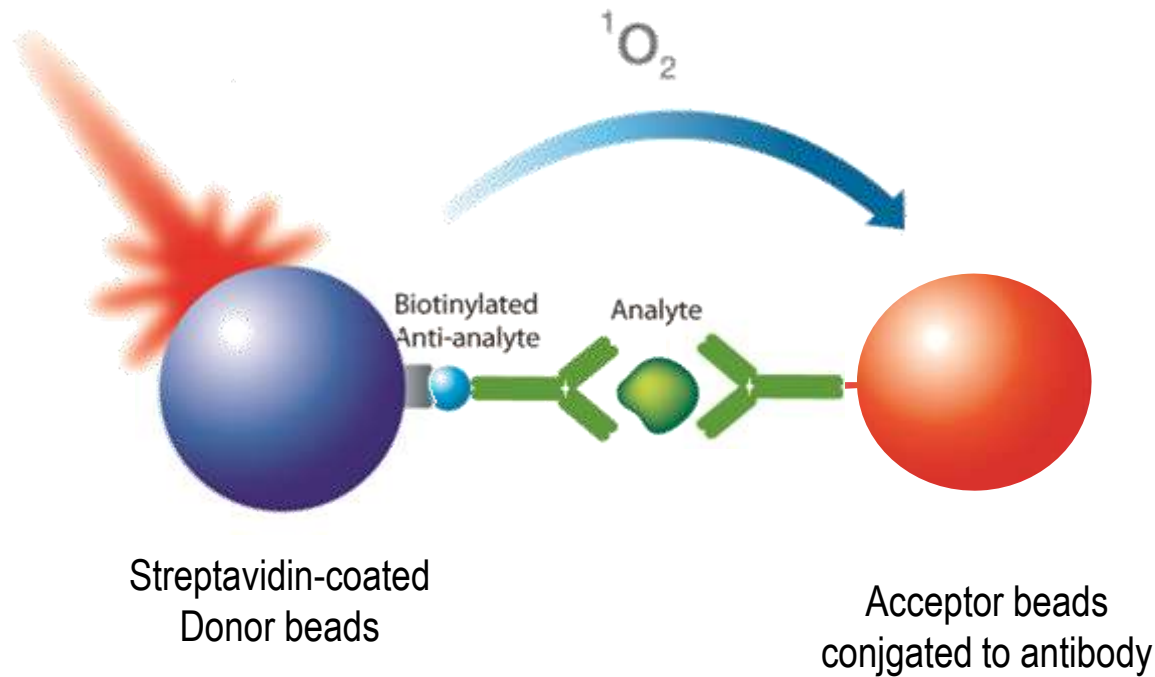
- assay principle
- AlphaLISA vs. ELISA



**A**mplified  
**L**uminescence  
**P**roximity  
**H**omogeneous  
**A**ssay

- High signal/background ratio; high dynamic range
- High sensitivity (**attomol**) with very low amount of sample (**5  $\mu$ l**)
- For small as well as big protein complexes, strong or weak affinity interactions
- For different matrix samples: serum, plasma, cell supernatant, CSF, cell lysates

# How does Alpha assay work?



- Laser excitation of phthalocyanine (680 nm)
- Singlet oxygen travels up to 200 nm (before decay)
- Excitation of thioxene → europium → signal (615 nm)

**Signal  
amplification**

Signal is proportional to analyte concentration



1



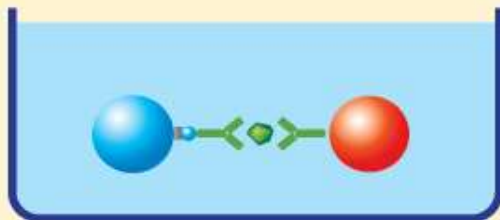
Add **analyte** to a microplate.

2



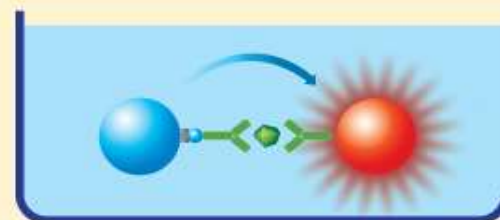
Add **biotinylated anti-analyte antibody** and anti-analyte antibody-conjugated **Acceptor beads**.  
**Incubate** for 60 minutes.

3



Add streptavidin-coated **Donor beads**.  
**Incubate** for 30 minutes.

4



Donor beads **release singlet oxygen**, activating Acceptor bead fluorescent **emission**.  
Molecules up to 200 nm in size can be measured: more than 20 times the size of TR-FRET assays.

## "ADD AND MIX" PROTOCOL

**NO** coating, **NO** washing:

- ✓ faster than ELISA
- ✓ more reproducible
- ✓ only 5 µl of sample
- ✓ cheaper
- ✓ more sensitive and wider dynamic range

## ► Beads

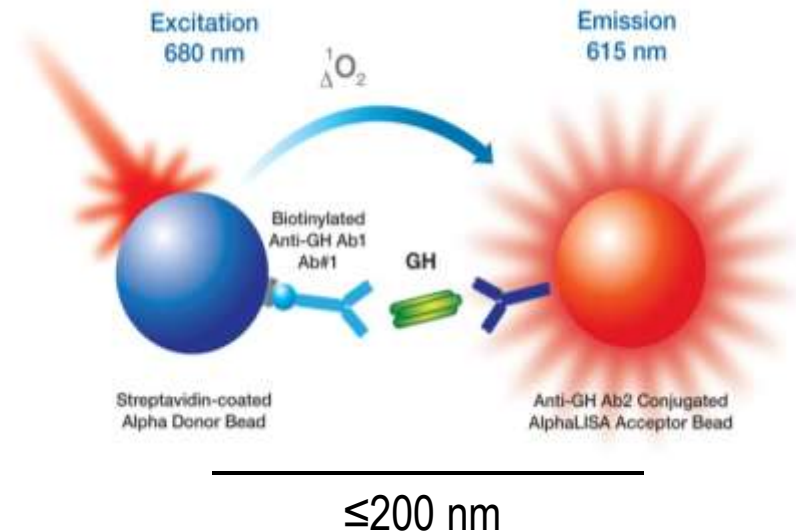
- Diameter 250 nm: **very stable colloid suspension**
- **Latex-based** and coated with a layer of hydrogel:
  - **no aggregation**
- 1 µg beads =  $2 \times 10^8$  beads
- heat stable (95°C)

## ► Detection

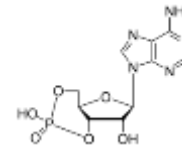
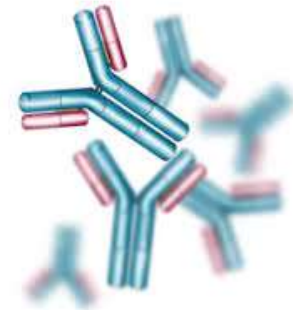
- $\lambda_{\text{ex}}$  680 nm: low compound interference
- $\lambda_{\text{em}}$  **615 nm: low background**
- time-resolved: low background
- detected on Alpha reader

## ► Flexibility

- **Distance (up to 200 nm)**
- Suitable for bulky binding partners
- Beads can be coated with antibodies or other binding molecules to develop virtually any immunoassay



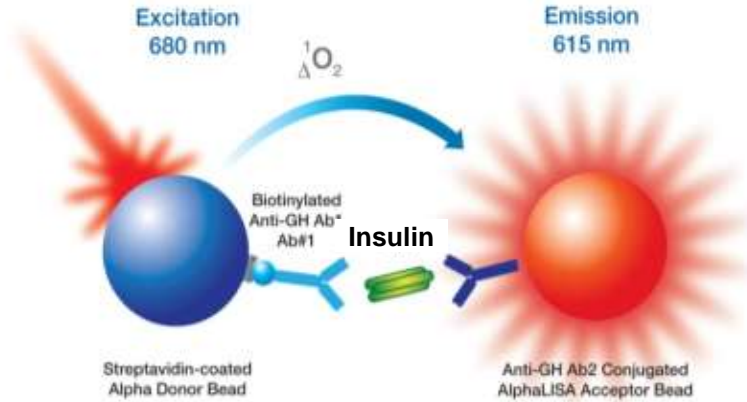
- ▶ Alpha can cover a wide range of sizes:
  - Giant particles (100MDa+) ex. M13 phages
  - Huge proteo-glycans (1MDa+) ex. chondroitine
  - Large proteins (150kDa+) ex. hIgG
  - Medium protein (50kDa+) ex. trimeric TNF $\alpha$
  - Small protein (5kDa+) ex. insulin
  - Large peptides (30aa+) ex. Ab42
  - Small peptides (5-30aa) ex. Angiotensin I
  - Small molecules ex. cAMP



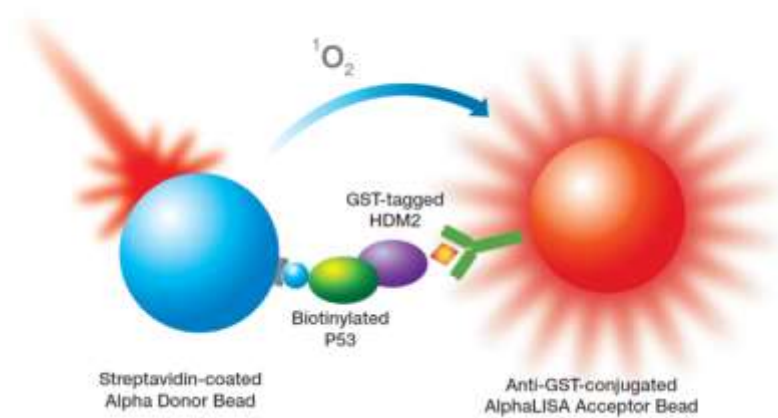


# Main applications: sensitive detection and quantification of...

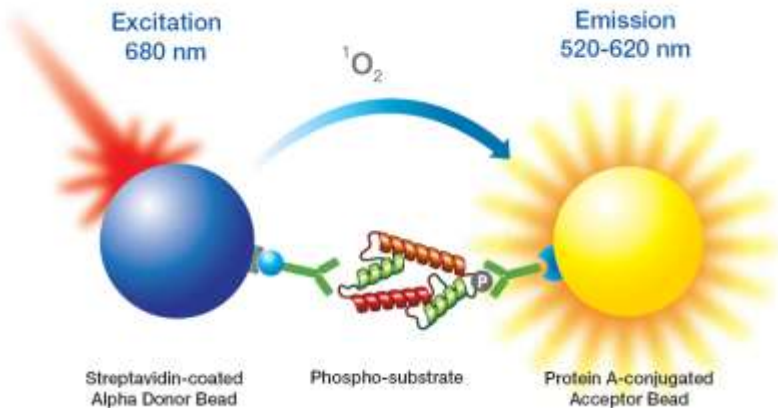
## biomarkers (immunoassay)



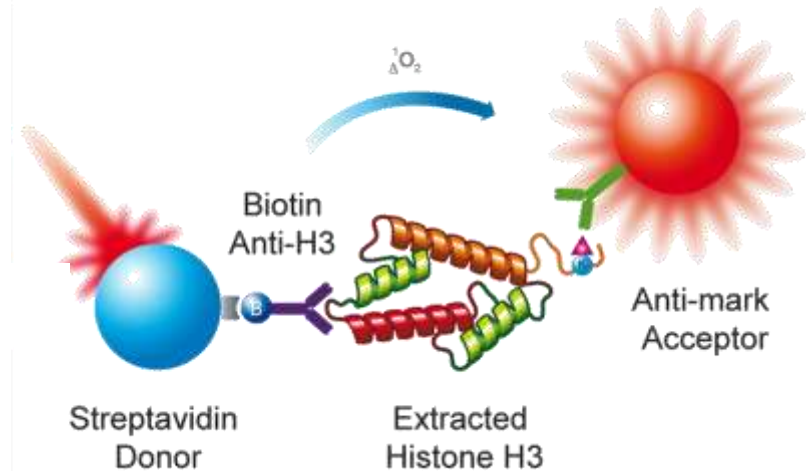
## biomolecular interactions



## protein phosphorylation



## epigenetic modifications

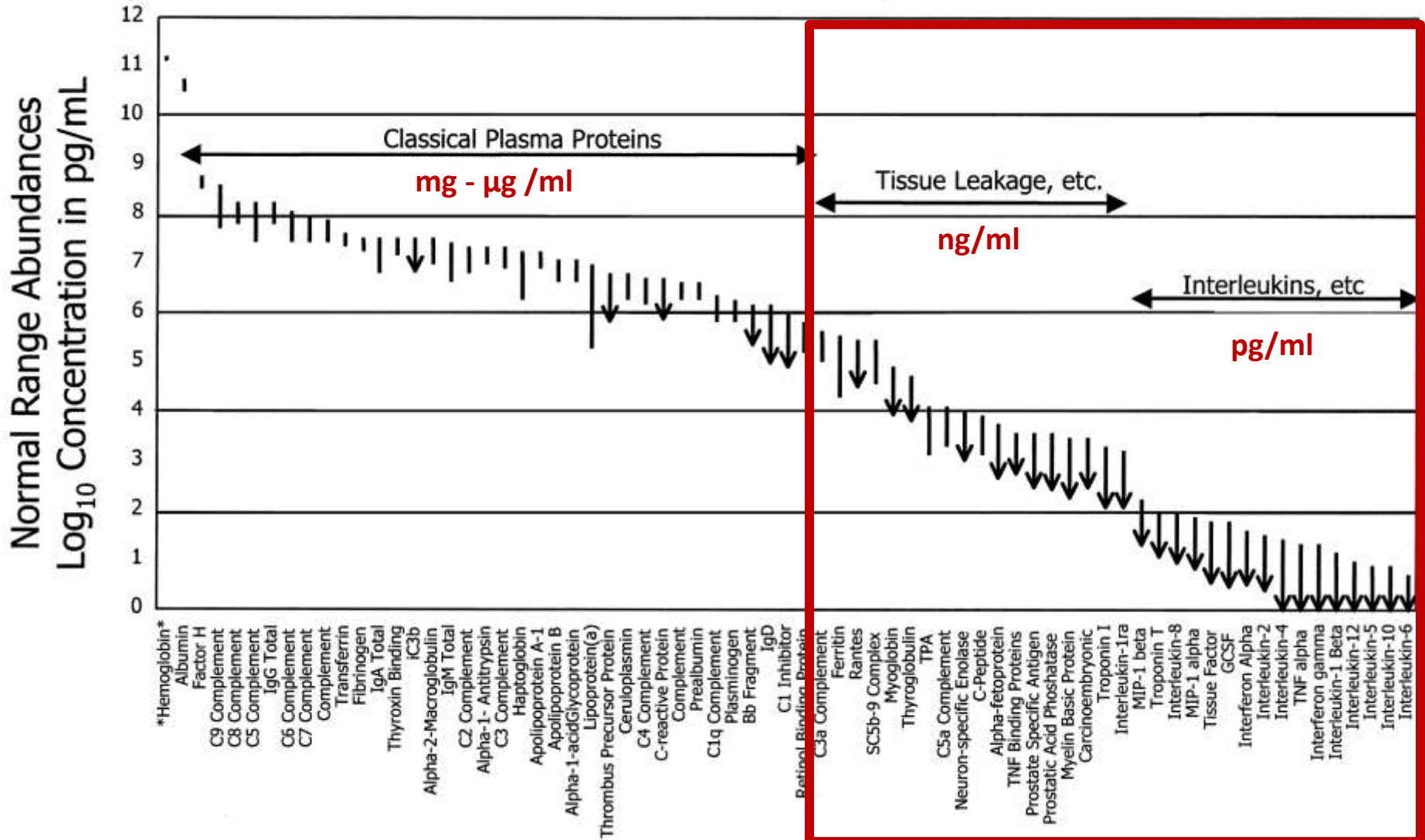


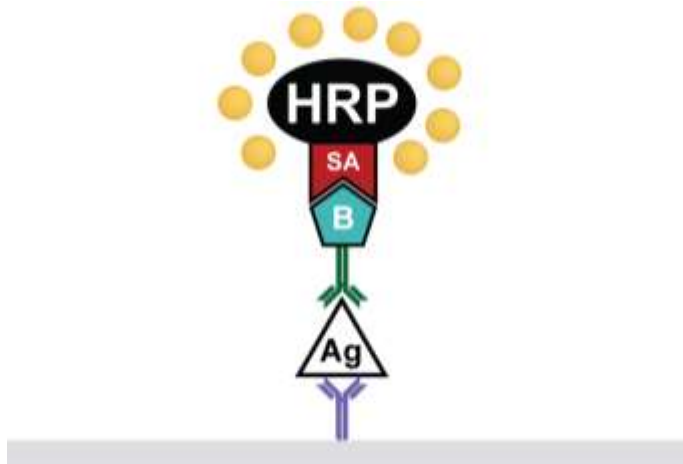


# Immunoassays for biomarker quantification

# Biomarkers abundance in plasma

## Reference intervals for 70 proteins in plasma





- Labor intensive
- Narrow dynamic range
- Limited sensitivity
- High sample volume
- Variability
- Difficult to automate
- Difficult to miniaturize

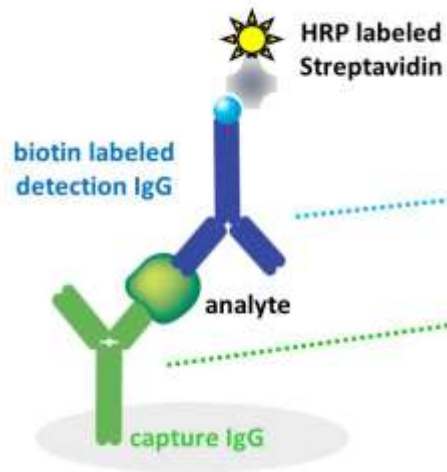
## Standard Immunoassay

(including heterogeneous electrochemiluminescence assays)



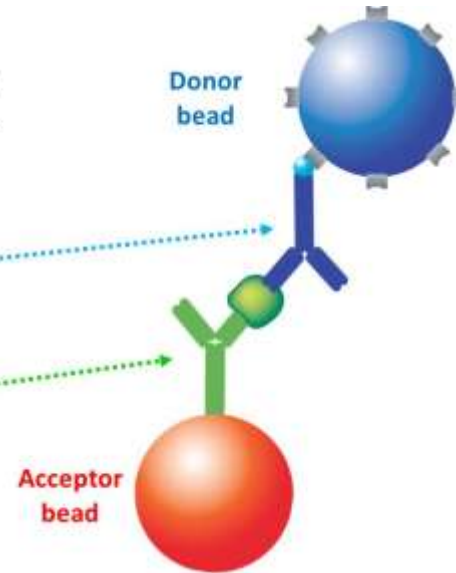
# ELISA vs AlphaLISA

ELISA



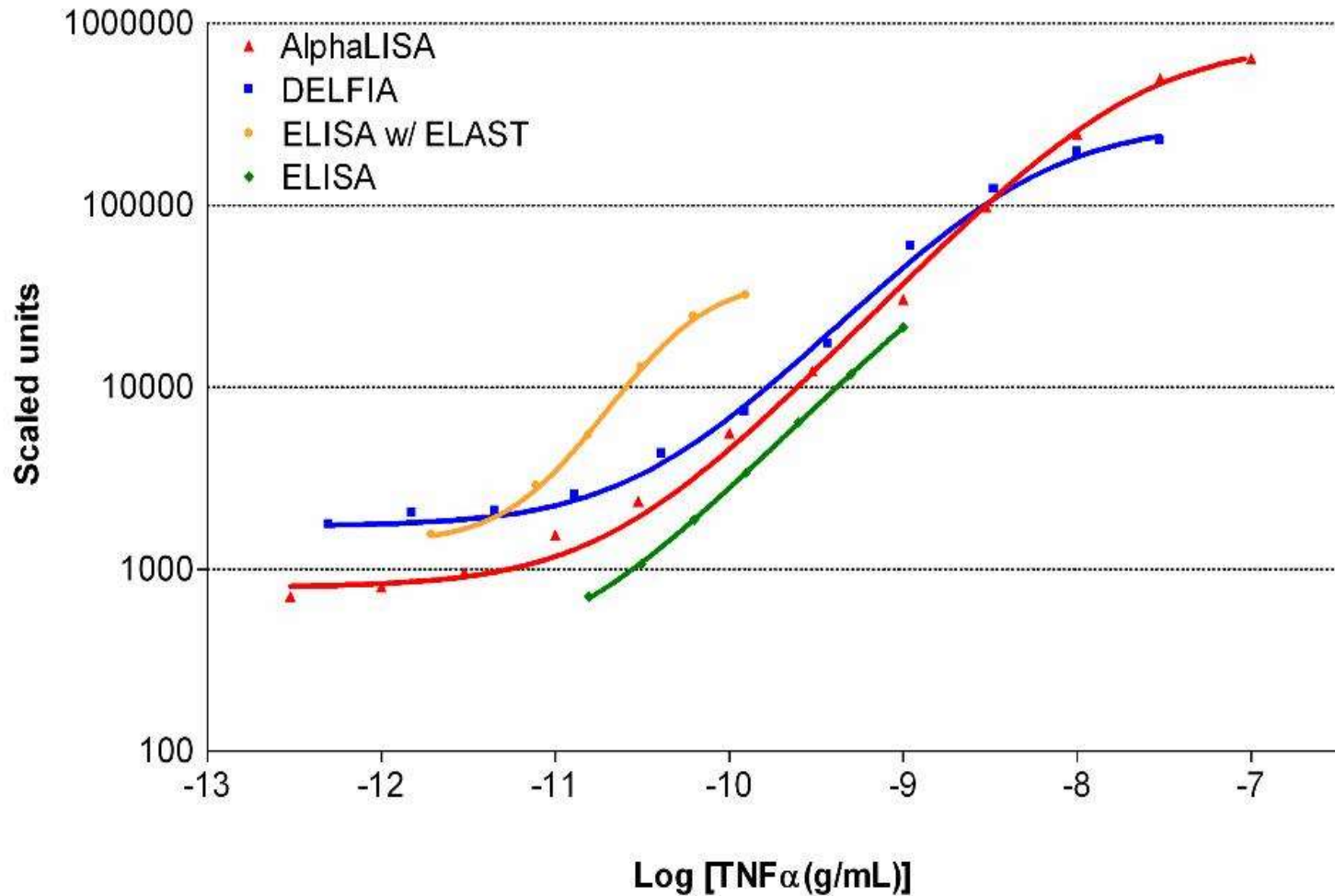
Heterogeneous assay

ALPHA



Homogeneous assay

## ELISA-Related Technologies

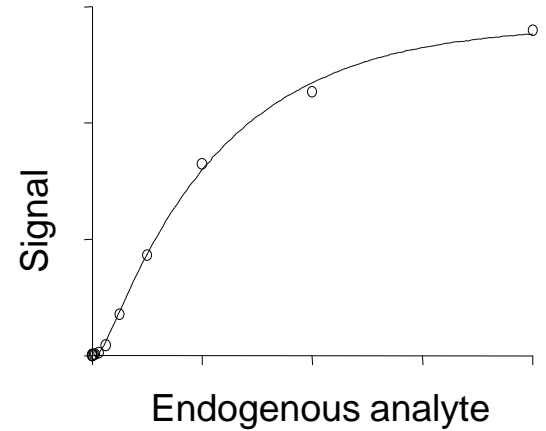
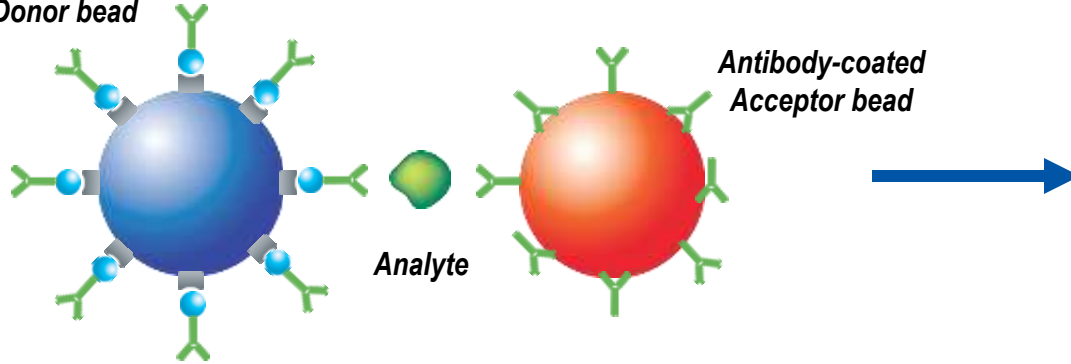




# Alpha immunoassay variants: direct and competition assays

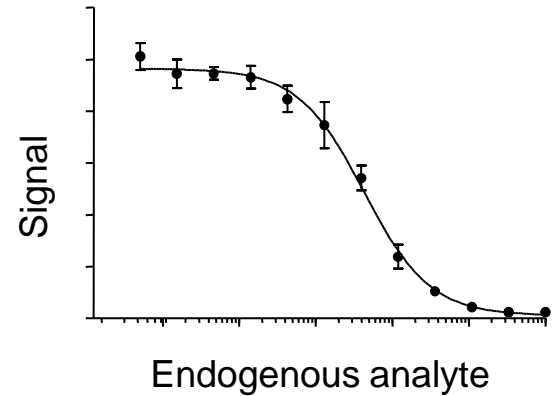
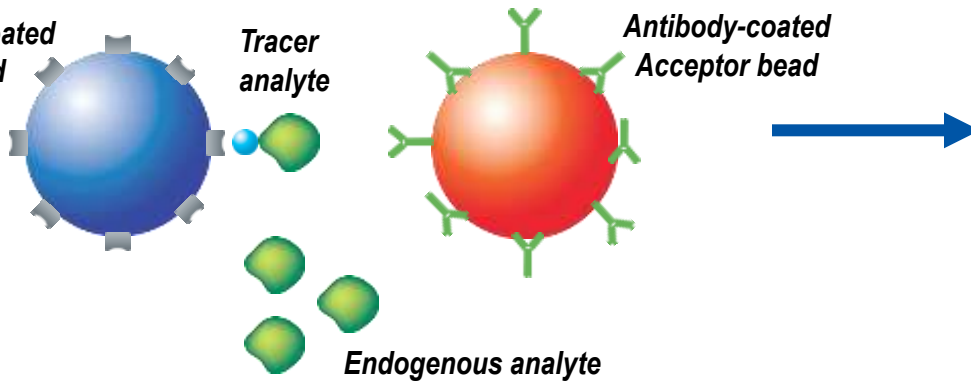
## Direct sandwich assay

*Biotinylated antibody captured on Streptavidin-coated Donor bead*



## Competition assay

*Streptavidin-coated Donor bead*




Alpha kits are ready to use.

## Components:

- Antibody-conjugated Acceptor beads
- Biotinylated antibody
- Streptavidin-Donor beads
- Buffer
- Lyophilized, standard analyte
- Detailed protocol with expected curve for standard analyte

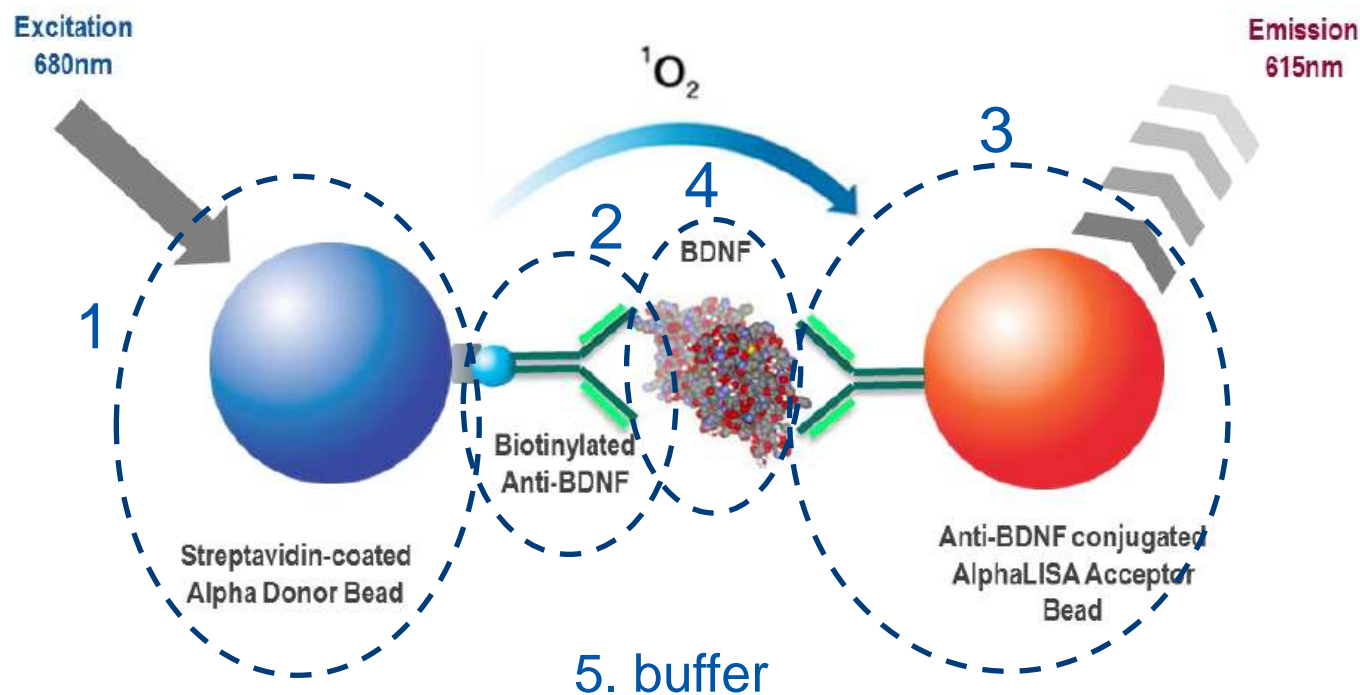


Kits are available for different applications: cancer, inflammation, cardiovascular, virology...

<p><b>Angiogenesis</b></p> <ul style="list-style-type: none"> <li>• ANGPT1</li> <li>• ANGPT2</li> <li>• ANGPTL3</li> <li>• ANGPTL4</li> <li>• EPO</li> <li>• TNF<math>\alpha</math></li> <li>• TGF-<math>\beta</math>1</li> <li>• VEGFA</li> <li>• VEGFC</li> <li>• VEGFD</li> </ul>	<p><b>Autophagy</b></p> <ul style="list-style-type: none"> <li>• p62</li> <li>• LC3B</li> </ul>	<p><b>Cardiovascular</b></p> <ul style="list-style-type: none"> <li>• Alpha-2 macroglobulin</li> <li>• Cardiac Troponin I</li> <li>• Cardiac Troponin T</li> <li>• D-dimer</li> <li>• ICAM-1</li> <li>• Fibrinogen</li> <li>• FLT1 (VEGFR1)</li> <li>• Myeloperoxidase</li> <li>• Myoglobin</li> <li>• PAI-1</li> <li>• PCSK9</li> <li>• Plasminogen</li> <li>• Renin/Prorenin</li> <li>• tPA</li> <li>• Transferrin</li> </ul>		<p><b>Cancer</b></p> <ul style="list-style-type: none"> <li>• A1AT</li> <li>• AFP</li> <li>• <math>\alpha</math>-tubulin</li> <li>• BCL-2</li> <li>• BMP-7</li> <li>• COL1A1</li> <li>• CA125</li> <li>• Caspase-3 active</li> <li>• Claudin 7</li> <li>• COX-2</li> <li>• CXCL11/I-TAC</li> <li>• EPO</li> <li>• ErbB2 (HER2)</li> <li>• E-cadherin</li> <li>• EGF</li> <li>• EGFR</li> <li>• FGF23</li> <li>• Fibronectin</li> <li>• HGFR/c-MET</li> <li>• Hyaluronic acid</li> <li>• IFN-<math>\beta</math></li> <li>• Laminin</li> <li>• MMP1</li> <li>• MMP2</li> <li>• MMP3</li> <li>• MMP7</li> <li>• MMP8</li> <li>• MMP9</li> <li>• MMP12</li> <li>• MMP13</li> <li>• Mucin-16</li> <li>• N-cadherin</li> <li>• Neprilysin</li> <li>• Nidogen</li> <li>• Oncostatin M</li> <li>• p53</li> <li>• PIP</li> <li>• collagen</li> <li>• PD-1</li> <li>• PD-L1</li> <li>• Perlecan</li> <li>• PSA</li> <li>• PTEN</li> <li>• SCF (KIT-ligand)</li> <li>• TFF3</li> <li>• TGF-<math>\beta</math>1</li> <li>• TIMP1</li> <li>• TNF<math>\alpha</math></li> <li>• VCAM-1</li> </ul>	
<p><b>Biologics &amp; bioprocess</b></p> <ul style="list-style-type: none"> <li>• Albumin</li> <li>• CHO HCP</li> <li>• IgA</li> <li>• IgE</li> <li>• IgG</li> <li>• IgG1</li> <li>• IgG2</li> <li>• IgG2a (mouse)</li> <li>• IgG2b (mouse)</li> <li>• IgG3</li> <li>• IgG4</li> <li>• IgM</li> <li>• PER.C6</li> <li>• Residual Protein A</li> <li>• Residual Host Cell DNA</li> <li>• Anti-PEG IgM</li> </ul> 		<p><b>Kidney</b></p> <ul style="list-style-type: none"> <li>• EPO</li> <li>• NGAL</li> </ul>	<p><b>Second Messenger</b></p> <ul style="list-style-type: none"> <li>• cAMP</li> <li>• cGMP</li> </ul>	<p><b>Metabolic</b></p> <ul style="list-style-type: none"> <li>• Adiponectin</li> <li>• Albumin</li> <li>• ApoA1</li> <li>• ApoC3</li> <li>• BMP-9</li> <li>• C-peptide</li> <li>• Corticosterone</li> <li>• Cortisol</li> <li>• Factor VIII</li> <li>• FGF21</li> <li>• Ghrelin</li> <li>• GLP-1</li> <li>• Glucagon</li> <li>• Growth Hormone</li> <li>• hCG</li> <li>• IGF1</li> <li>• IGF2</li> <li>• Insulin</li> <li>• Leptin</li> <li>• MANF</li> <li>• Osteoprotegerin</li> <li>• PAI-1</li> <li>• PCSK9</li> <li>• Prolactin</li> <li>• Progesterone</li> <li>• Somatostatin</li> <li>• Testosterone</li> <li>• TSH</li> <li>• Utrophin</li> </ul>	
<p><b>Inflammation</b></p> <ul style="list-style-type: none"> <li>• CCL2/MCP1</li> <li>• CCL3/MIP-1<math>\alpha</math></li> <li>• CCL4/MIP-1<math>\beta</math></li> <li>• CCL5/RANTES</li> <li>• CRP</li> <li>• CXCL1/GRO-<math>\alpha</math></li> <li>• CXCL4/PF4</li> <li>• CXCL9/MIG</li> <li>• CXCL10/IP-10</li> <li>• G-CSF</li> <li>• GM-CSF</li> <li>• IFN-<math>\alpha</math></li> <li>• IFN-<math>\gamma</math></li> <li>• IL1<math>\alpha</math></li> <li>• IL1<math>\beta</math></li> <li>• IL-1RA</li> <li>• IL2</li> <li>• IL3</li> <li>• IL4</li> <li>• IL5</li> <li>• IL6</li> <li>• IL7</li> <li>• IL8</li> <li>• IL10</li> <li>• IL11</li> <li>• IL12</li> <li>• IL13</li> <li>• IL15</li> </ul>		<p><b>Food testing</b></p> <ul style="list-style-type: none"> <li>• Aflatoxin</li> <li>• Beef troponin</li> <li>• Chloramphenicol</li> <li>• Soybean agglutinin</li> </ul>	<ul style="list-style-type: none"> <li>• IL17A</li> <li>• IL17F</li> <li>• IL18</li> <li>• IL22</li> <li>• IL23</li> <li>• IL28B</li> <li>• IL31</li> <li>• TNF<math>\alpha</math></li> </ul>	<p><b>Central Nervous System</b></p> <ul style="list-style-type: none"> <li>• Alpha-Synuclein</li> <li>• Amyloid <math>\beta</math> 1-15</li> <li>• Amyloid <math>\beta</math> 1-40</li> <li>• Amyloid <math>\beta</math> 1-42</li> <li>• Amyloid <math>\beta</math> 1-x</li> <li>• Amyloid <math>\beta</math> oligomers</li> <li>• ApoE</li> <li>• BDNF</li> <li>• <math>\beta</math>-NGF</li> <li>• Frataxin</li> <li>• GAD67</li> <li>• GDNF</li> <li>• sAPP<math>\alpha</math> (C-term specific)</li> <li>• Somatostatin</li> <li>• Tau</li> <li>• TDP-43</li> <li>• Total or oligomerized A<math>\beta</math> 42</li> <li>• Amyloid oligomers</li> </ul>	
<p><b>Binding</b></p> <ul style="list-style-type: none"> <li>• FCGR3A/CD16a (176Phe/F158)</li> <li>• FCGR3A/CD16a (176Val/V158)</li> <li>• PD-1/PD-L1</li> <li>• EGF/EGFR</li> </ul>		<p><b>Virology</b></p> <ul style="list-style-type: none"> <li>• HIV p24 (high sensitivity)</li> <li>• HIV p24</li> </ul>		<p><b>Agriculture</b></p> <ul style="list-style-type: none"> <li>• Cry1F</li> <li>• Cry2A</li> <li>• Cry1Ab/1Ac</li> </ul>	

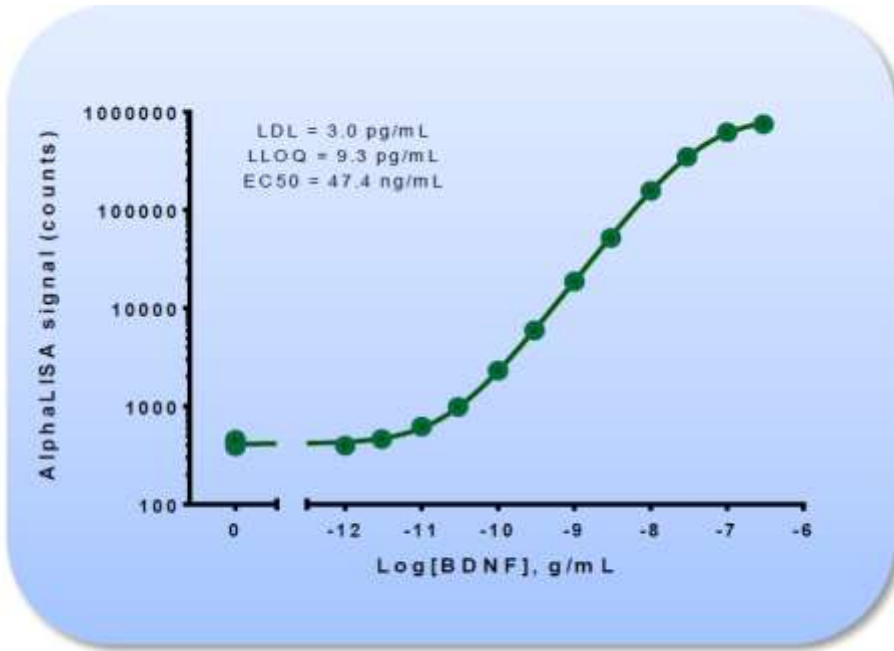
## Brain Derived Neurotrophic Factor

BDNF: neurotrophin acting within central and peripheral nervous systems; it generally promotes the survival of existing neurons. BDNF potentially plays a role in several diseases such as Alzheimer's, Parkinson's and Huntington's disease. The kit has been designed for the quantification of BDNF in serum and cell culture supernatants

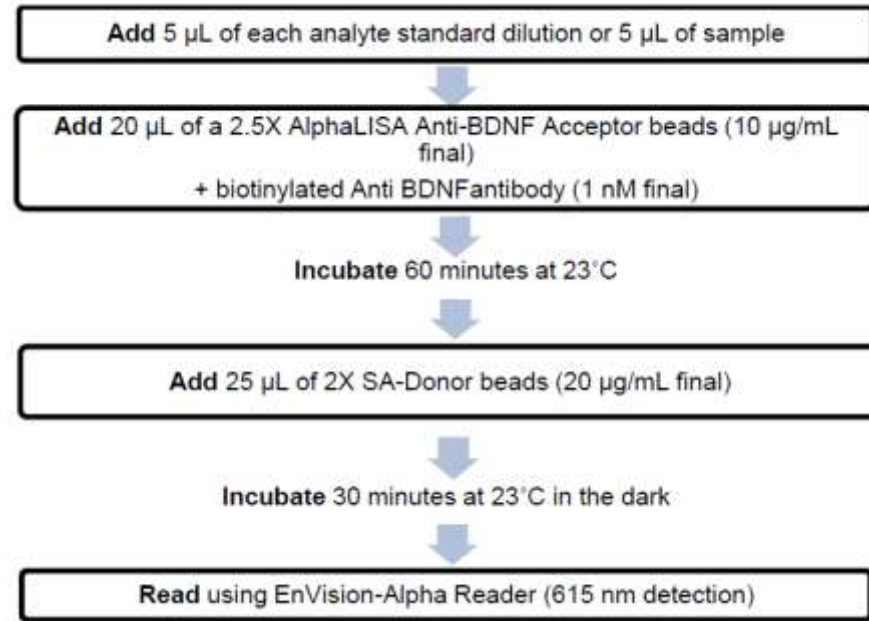


## Brain Derived Neurotrophic Factor

Range of detection with LDL



Protocol



Cross-reactivity for similar proteins:

Protein	% Cross-reactivity
Human Nerve Growth Factor	1.2
Human Neurotrophin 3	0.1
Human Neurotrophin 4	0.2

CV% precision (intra and inter-assay):

IAB	Neurobasal	DMEM/F12
6.6	8.8	7.9
12.0	9.7	8.9

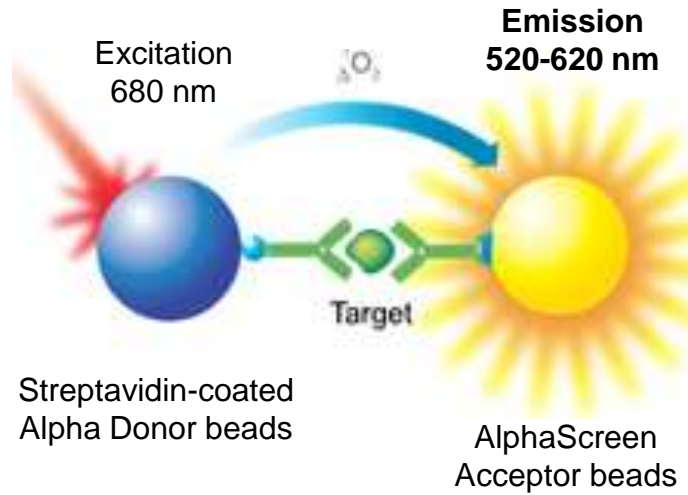
# How to perform Alpha assay: 2 - Alpha toolbox

		Donor Beads O <sub>2</sub>	Acceptor Beads				Control Beads
			AlphaLISA 615	AlphaPlex (Tb) 545	AlphaPlex (Sm) 645	AlphaScreen 520-620	
-	Unconjugated	6762011/2/3	6772001/2/3/4L	6782001/2/3	6792001/2/3	6762001/2/3/4	Omnibeads 6760626
Fusion Tag Detection	Streptavidin	6760002	AL125	AP125TB	AP125SM	6760002	TruHits Kit Eu, AL900
	Strep Tactin®	AS106	AL136				TruHits Kit Tb, AP900TB
	Anti-DIG	AS108	AL113			6760604	TruHits Kit Sm, AP900Sm
	Anti-FITC		AL127			6760605	TruHits Kit Eu, biotin-free AL901
	Nickel Chelate	AS101	AL108			6760619	
	Anti-6x His		AL128	AP128TB	AP128SM		
	Glutathione (GSH)	6765300/1/2/3	AL109	AP109TB			
	Anti-GST		AL110			6760603	
	Anti-c-myc		AL111			6760611	
	Anti-FLAG®	AS103	AL112	AP112TB	AP112SM	6760613	
	Anti-GFP		AL133				
	Anti-HA		AL170	AP170TB		6760612	
	Anti-HRP	AS109	AL171				
	LCA (Lens Culinaris Agglutinin)		AL140				
Anti-Maltose-Binding Protein (MBP)		AL134					
Anti-V5		Anti-V5					
Antibody Capture	Protein A	AS102	AL101	AP101TB	AP101SM	6760137	
	Protein G		AL102				
	Protein L		AL126				
	Anti-bovine IgA		AL169				
	Anti-chicken IgY		AL131				
	Anti-goat IgG		AL107				
	Anti-human IgA		AL262				
	Anti-human IgE		AL292				
	Anti-human IgG (Fc spec)		AL103	AP103TB	AP103SM		
	Anti-human IgG1		AL303, AL141				
	Anti-human IgG2 (isotyping)		AL308, AL154				
	Anti-human IgG4		AL304, AL142				
	Anti-human IgM		AL263				
	Anti-mouse IgG	AS104	AL105	AP105TB	AP105SM	6760606	
	Anti-mouse IgG1		AL522				
	Anti-mouse IgG2a		AL523				
	Anti-mouse IgG2 b		AL524				
	Anti-mouse IgG3		AL525				
	Anti-mouse IgE		AL526				
	Anti-mouse IgM		AL130				
Anti-rabbit IgG	AS105	AL104	AP104TB		6760607		
Anti-rat IgG	AS110	AL106					
Anti-sheep IgG		AL132					

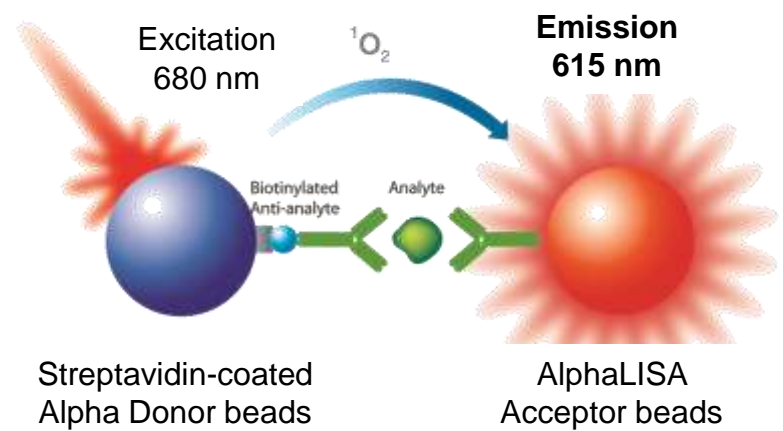


# AlphaScreen & AlphaLISA acceptor beads

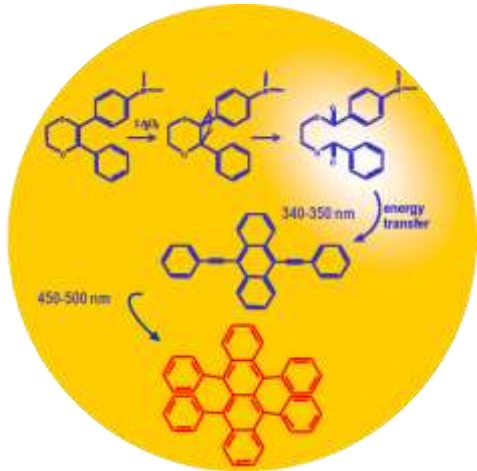
## AlphaScreen



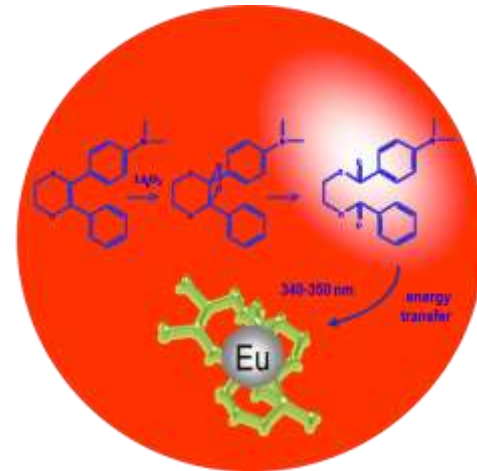
## AlphaLISA

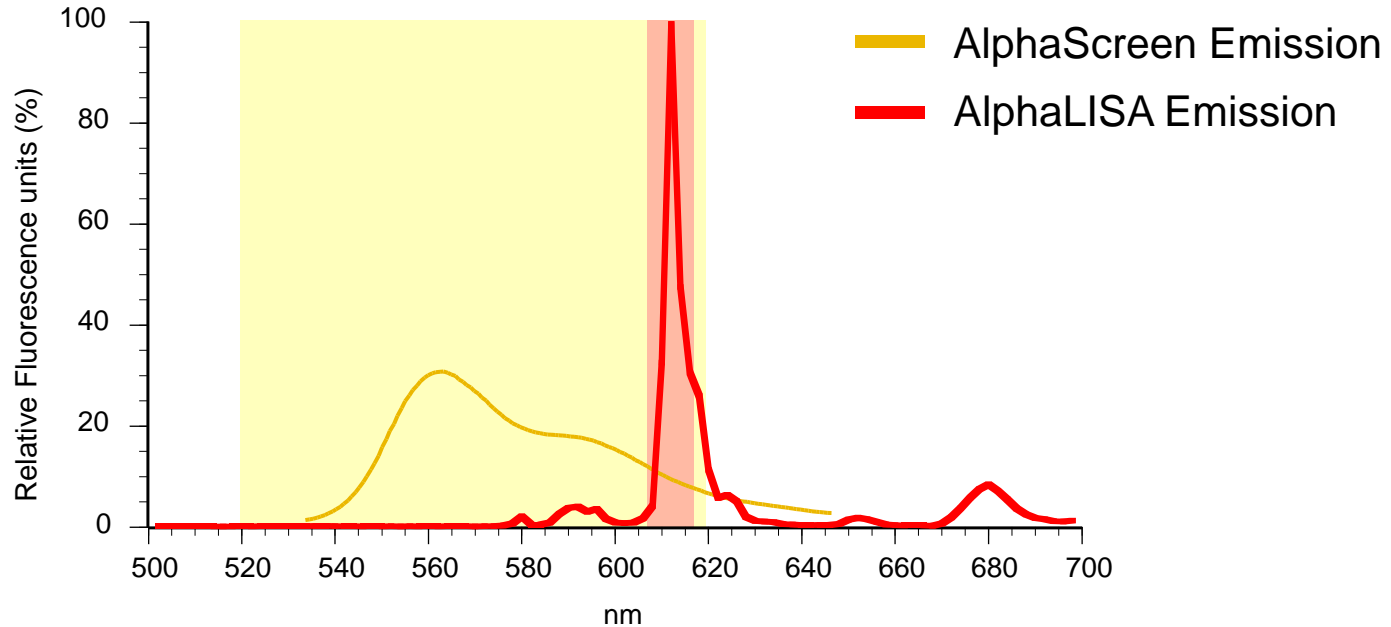


## rubrene



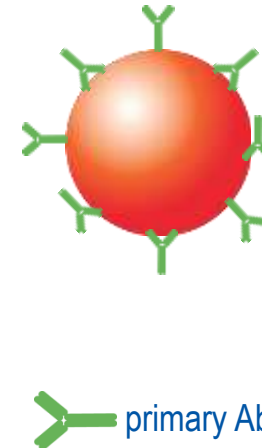
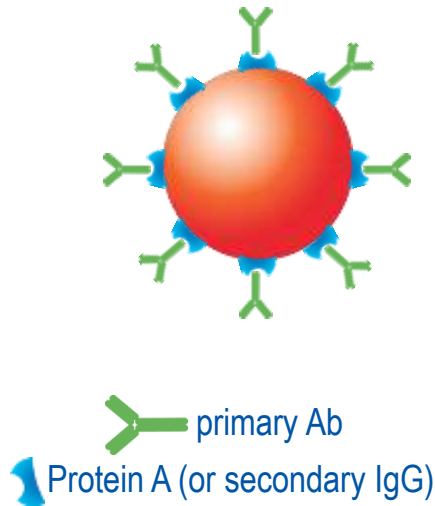
## europium





## Europium:

- highly intense and spectrally defined signal
- high signal/background ratio
- works in different samples including serum, plasma & cell culture supernatant



## Indirect (beads with ab / proteins)

- Antibodies aligned optimally
- **Additional equilibrium introduced**
- Secondary Ab or proteins A / G / L should be selective

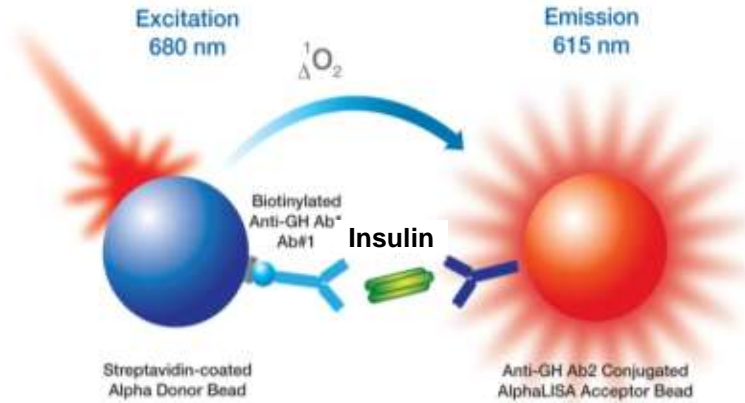
## Direct (unconjugated beads)

- Random coupling  
(antibodies aligned randomly)
- Good control on binding events
- Simple stoichiometry in Alpha assay

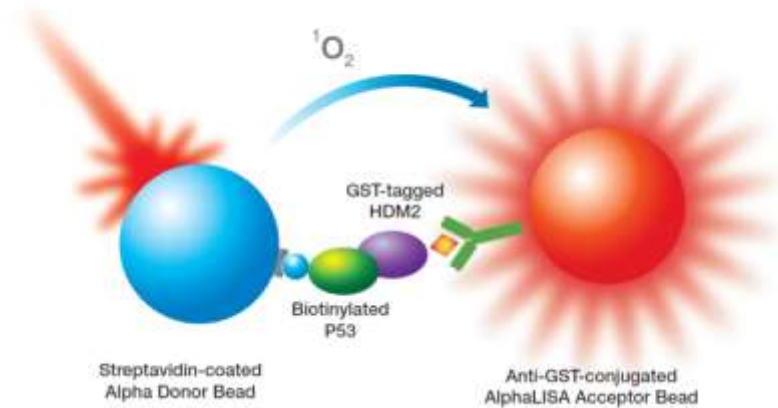


# Main applications: sensitive detection and quantification of...

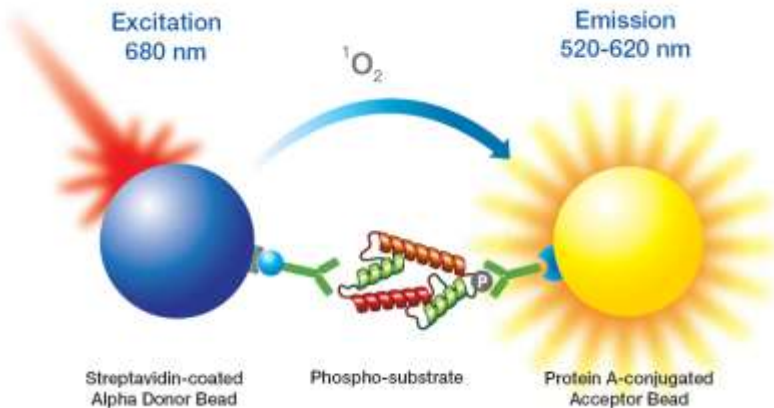
## biomarkers (immunoassay)



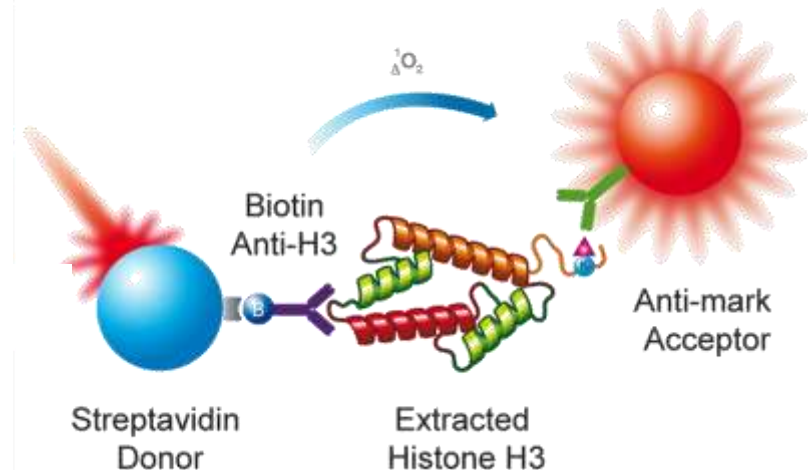
## biomolecular interactions



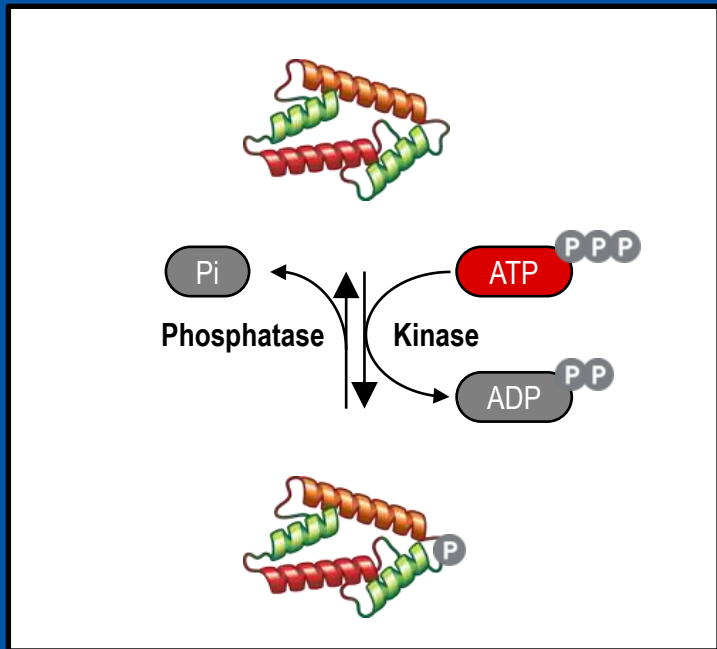
## protein phosphorylation



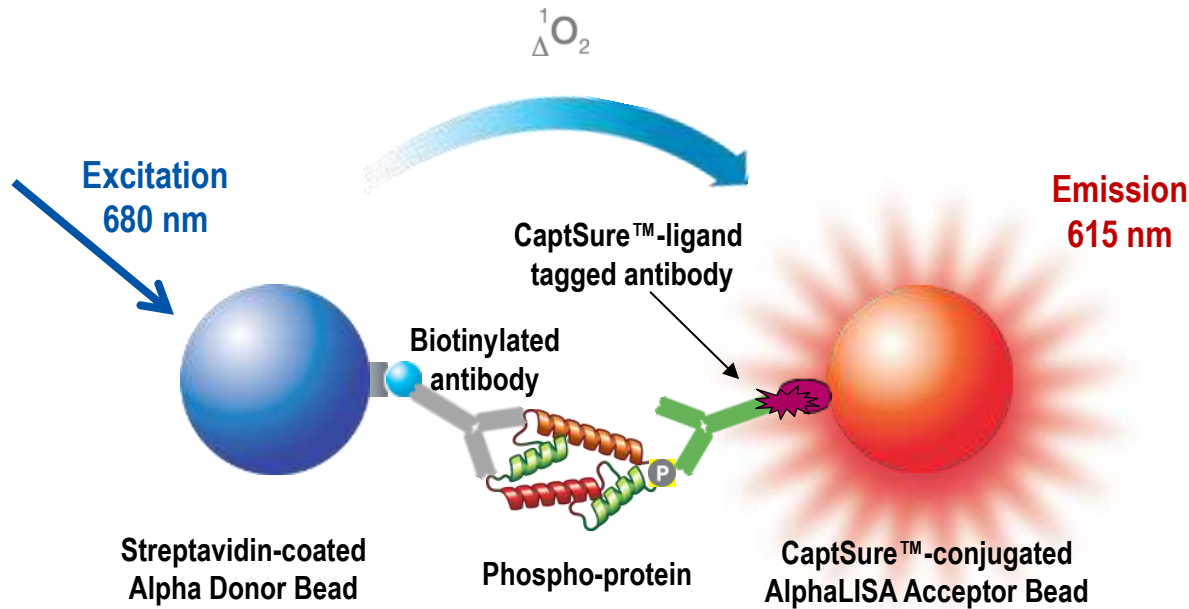
## epigenetic modifications



## Detection of phospho-proteins by Alpha

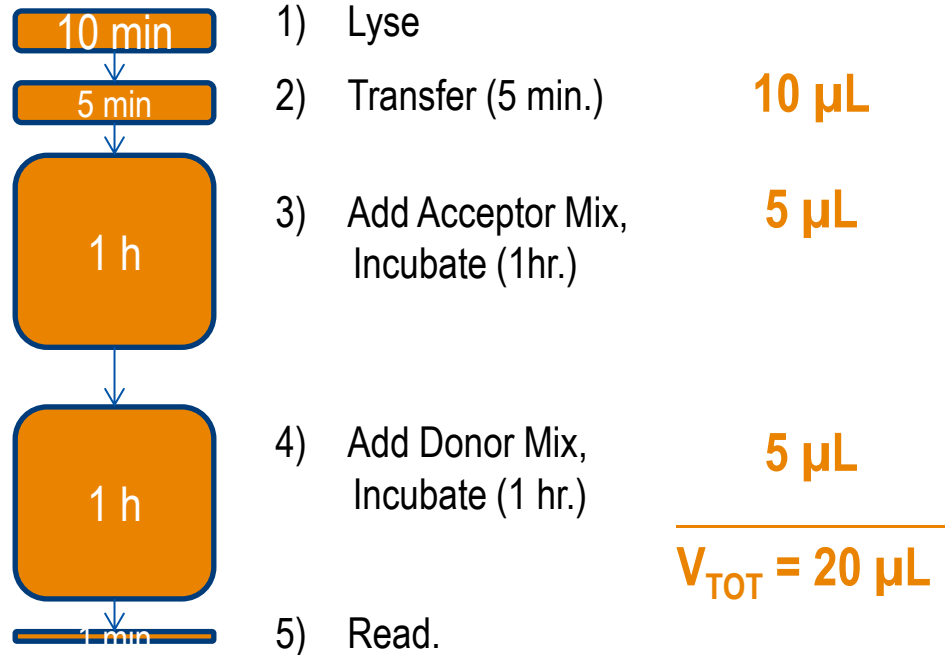






- **Highly sensitive:** over-expressed and endogenous receptors, only 10 µl lysate
- Receptor-mediated signaling modulation : GPCRs, Receptors Tyr Kinases, Cytokine Receptors...
- Intracellular kinase signaling cascade modulation: monitor a single target or multiple targets
- Use in many different cell lines, incl. **primary cells**
- Tissue extract analysis
- **It is possible to start assay from cells lysis**

## AlphaLISA SureFire ULTRA

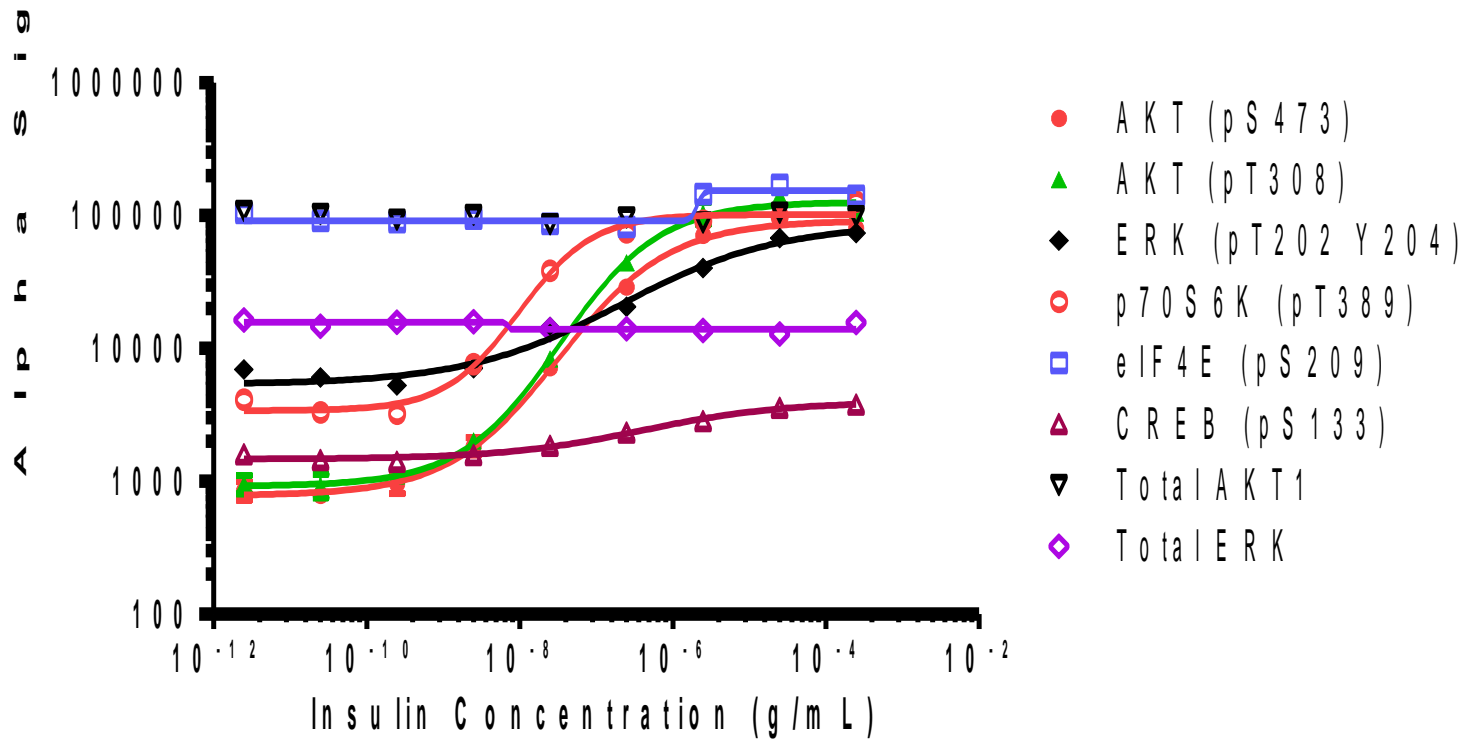


Total Assay Time: ~**2.5hrs.**

[video](#)

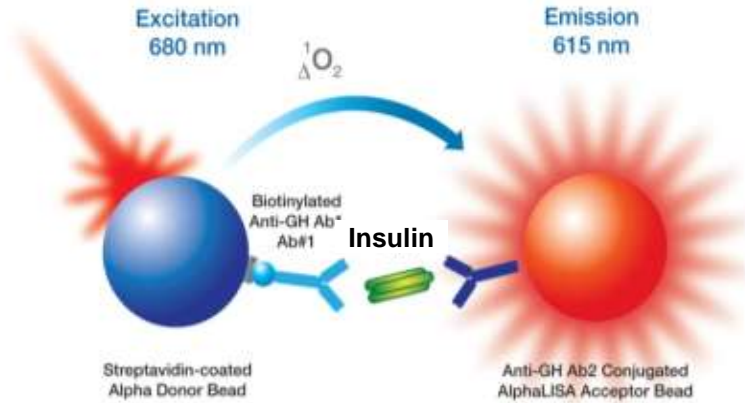
Faster results, easy pipetting

- **MCF-7 cells** were plated overnight (200k/ml) in MEM + 10% FCS
- serum starved for 2 h
- **stimulated** for 30' with increasing concentrations of insulin
- **lysed in 100  $\mu$ l SureFire Ultra** lysis buffer
- **10  $\mu$ l aliquots** were assayed using AlphaLISA SureFire ULTRA kits:

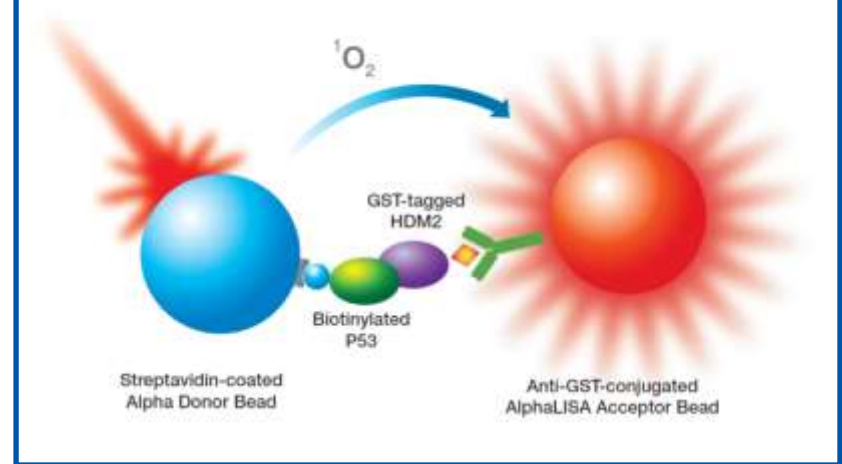


# Main applications: sensitive detection and quantification of...

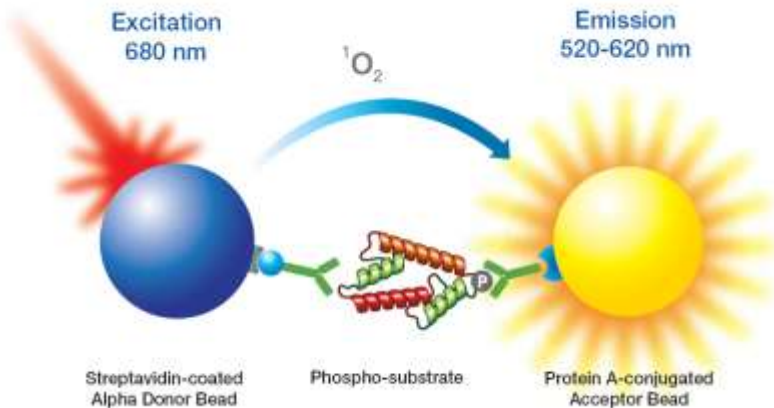
## biomarkers (immunoassay)



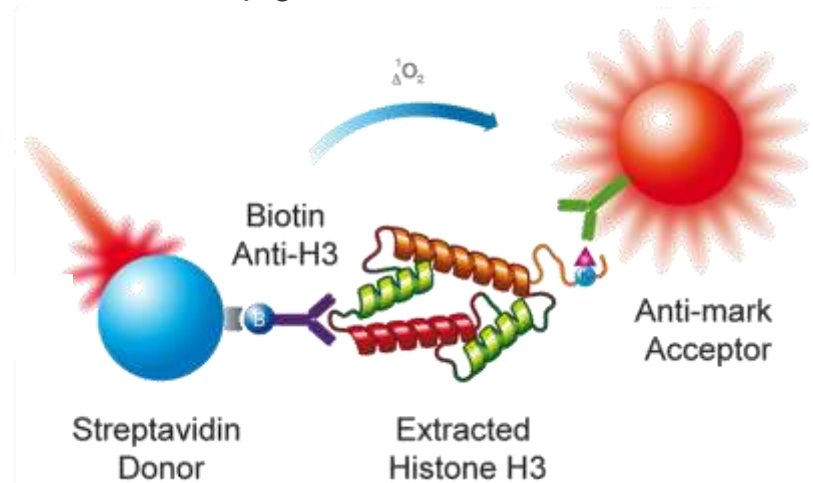
## biomolecular interactions



## protein phosphorylation



## epigenetic modifications





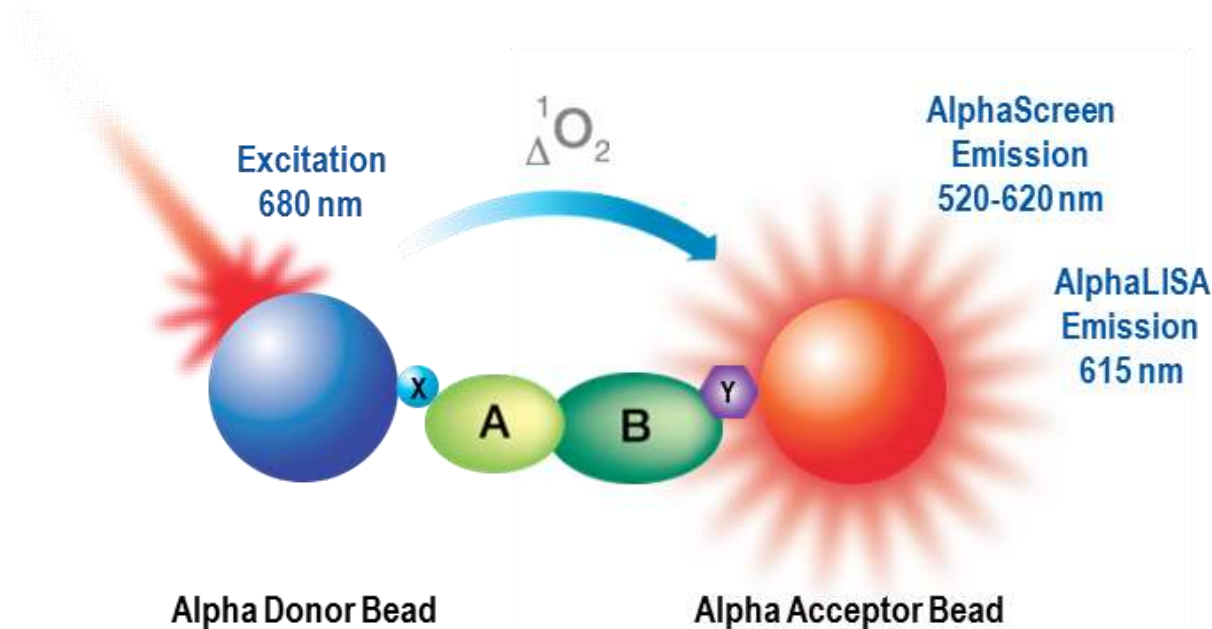
## Detection of protein-protein interactions using Alpha

Study of strong & weak interactions

Kits

Customized assays

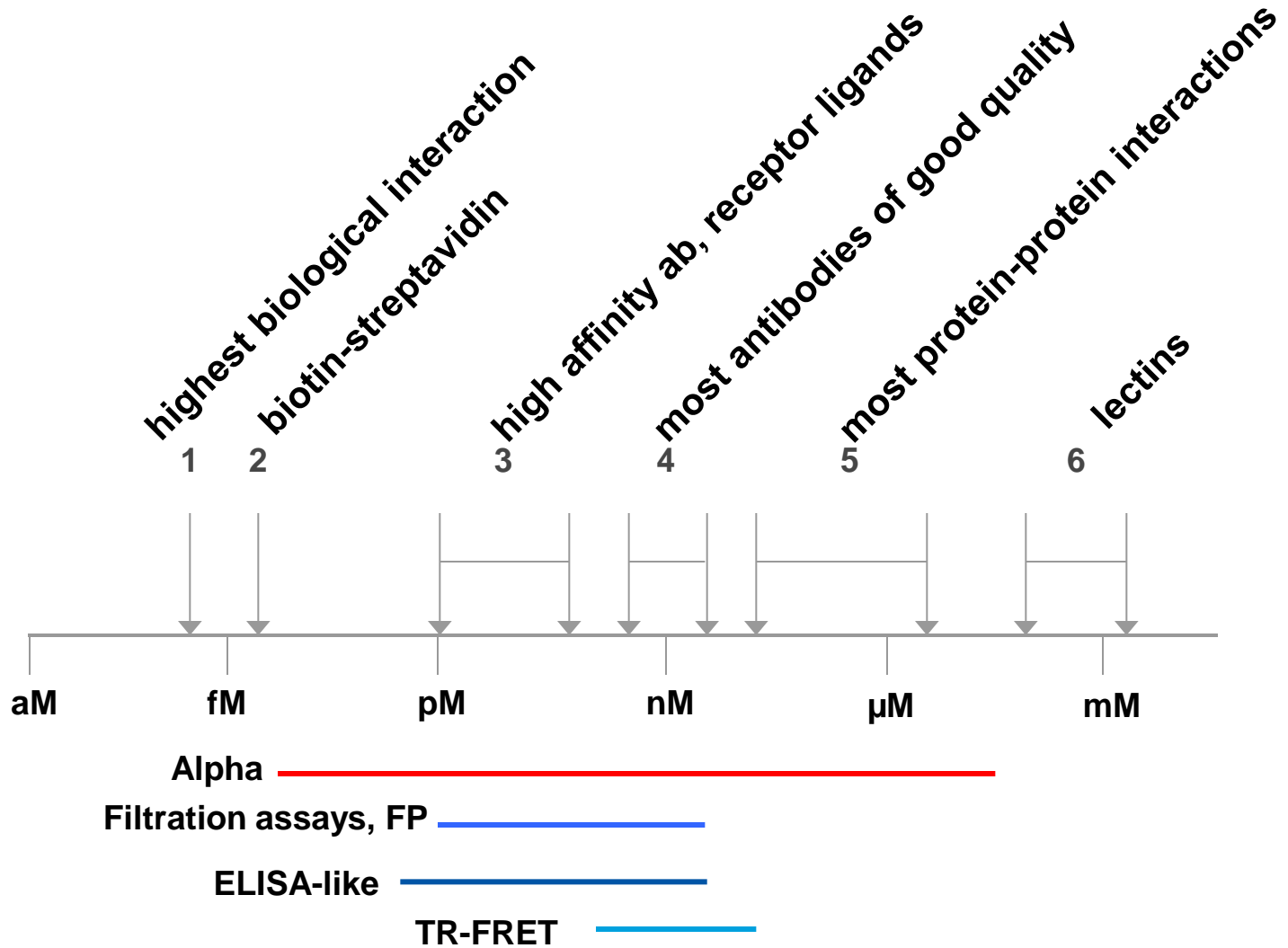
Virtually any assay can be developed, as long as you can bring the beads together



- ▶ **A** and **B** can be: proteins, peptides, antibody, oligonucleotides, polysaccharides, small molecules.
- ▶ **X** can be streptavidin, antibody, binding protein, chelate, small molecule, oligonucleotide.
- ▶ **Y** can be antibody, binding protein, chelate, oligonucleotide, small molecule.



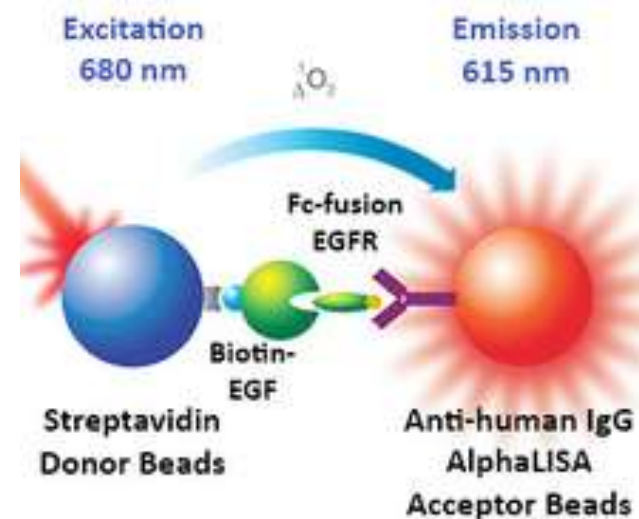
# Alpha working range: affinities



Weak interactions are preserved in Alpha assays

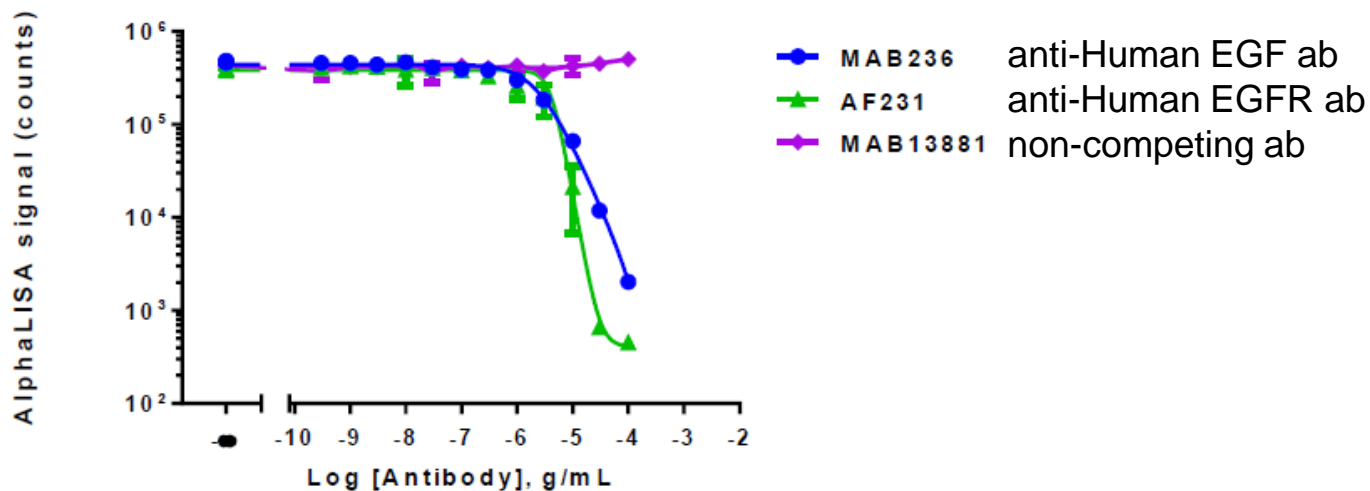
## EGF/EGFR binding kit:

- designed for the detection of EGF:EGFR binding;
- can facilitate the design and development of therapeutics which competitively inhibit the EGF/EGFR interaction.

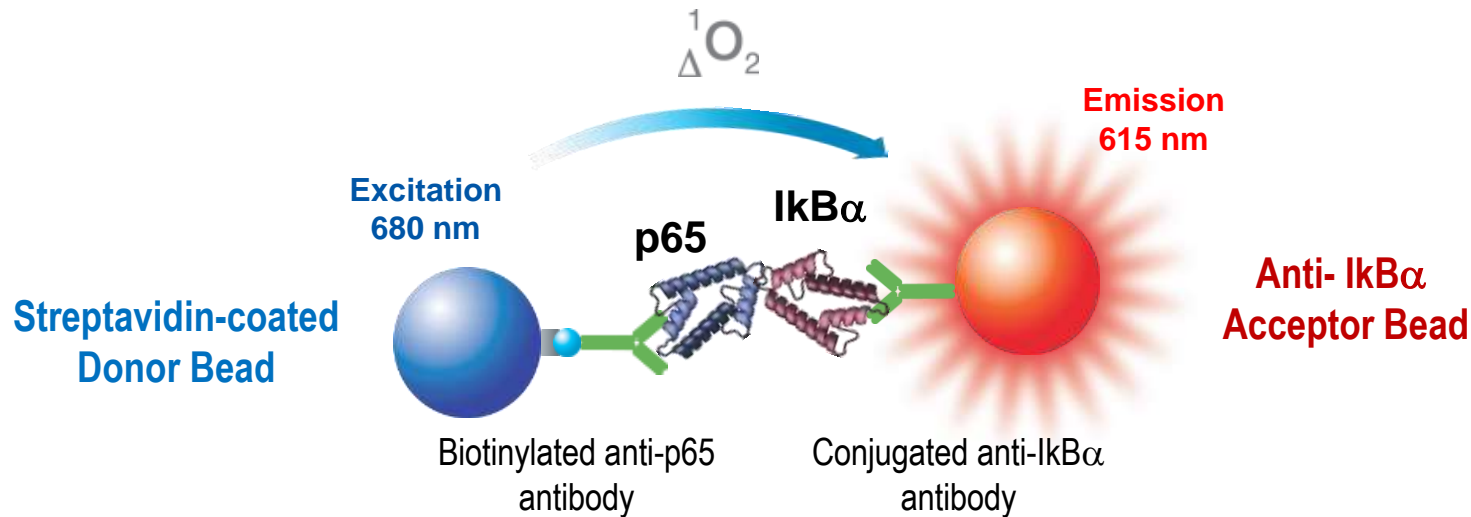


## Antibody inhibitory curve

### Sample Results:



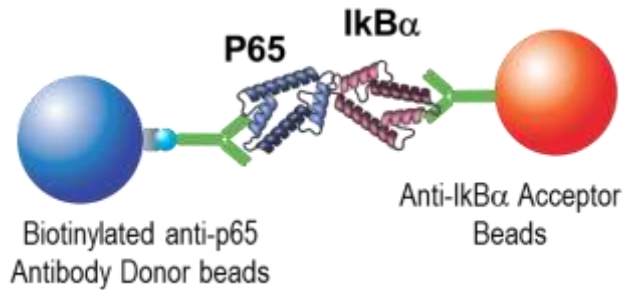
Aim: develop assay to detect NF- $\kappa$ B p65 / I $\kappa$ B $\alpha$  complex in Hela cell lysate



- ▶ Tools: antibodies
  - ab  $\alpha$ -P65 ab was biotinylated
  - ab  $\alpha$ I $\kappa$ B $\alpha$  was conjugated to bead
- ▶ Assay setup & optimization steps:
  - antibodies selection (5 for p65 vs. 5 for I $\kappa$ B $\alpha$ )
  - lysis buffer selection
  - titration of number of cells/well

# Assay setup: antibody selection and cell lysis buffer

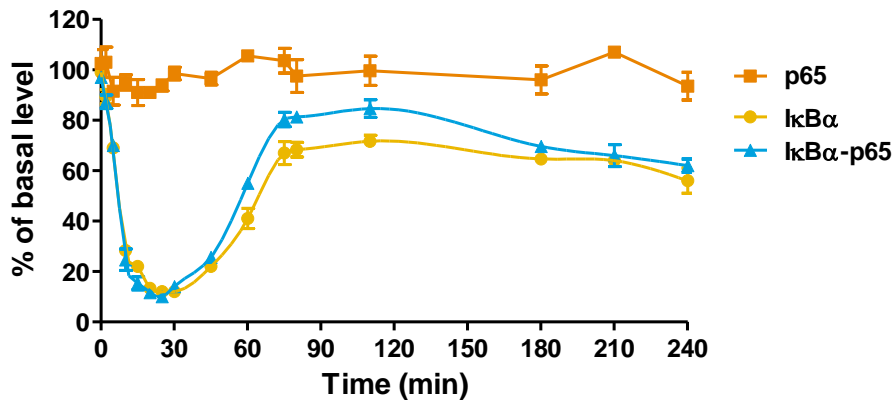
S/B ratio: Alpha signal in cell lysates (25000 cells) vs. lysis buffer alone



		Alpha Lysis	M-PER	Pierce IP	CST Lysis	average
AB1	b-Ab1	31	35	13	19	25
AB1	b-Ab2	10	23	9	10	13
AB1	b-Ab3	70	134	45	53	76
AB1	b-Ab4	28	41	10	21	25
AB1	b-Ab5	7	10	3	4	6
AB2	b-Ab1	87	84	89	174	109
AB2	b-Ab2	26	48	30	37	35
AB2	b-Ab3	44	149	54	193	110
AB2	b-Ab4	80	159	46	132	104
AB2	b-Ab5	23	19	7	9	15
AB3	b-Ab1	5	5	3	5	5
AB3	b-Ab2	6	6	6	5	6
AB3	b-Ab3	44	50	22	38	38
AB3	b-Ab4	8	7	4	6	6
AB3	b-Ab5	1	2	1	2	2
AB4	b-Ab1	3	3	2	3	3
AB4	b-Ab2	2	5	4	2	3
AB4	b-Ab3	4	8	3	6	5
AB4	b-Ab4	2	2	2	2	2
AB4	b-Ab5	1	1	1	1	1
AB5	b-Ab1	63	70	44	60	59
AB5	b-Ab2	13	19	10	12	13
AB5	b-Ab3	41	71	30	53	49
AB5	b-Ab4	19	34	11	22	21
AB5	b-Ab5	11	9	4	5	7

## Time-course TNF $\alpha$ (10 ng/mL)

10 000 cells/well  
(AlphaLISA lysis buffer)

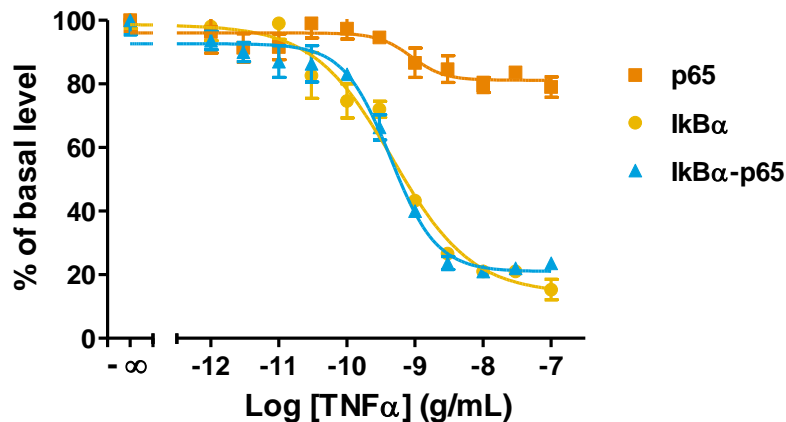


30' TNF $\alpha$  stimulation cause decrease of:

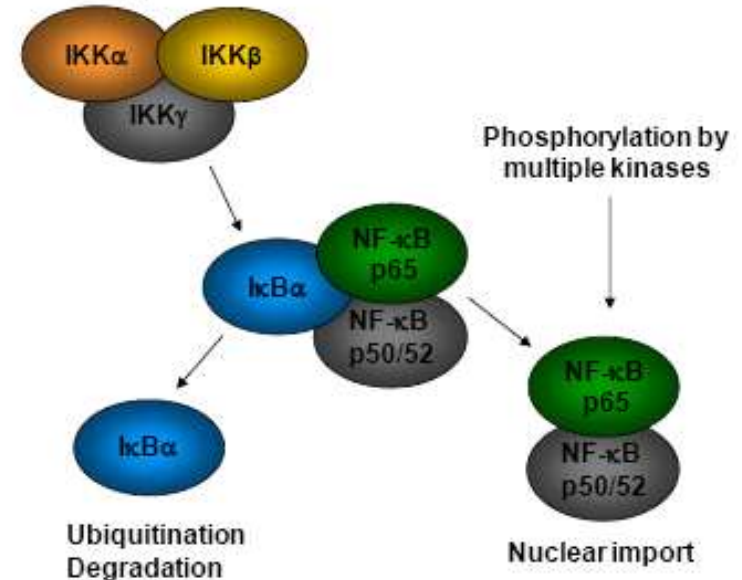
- I $\kappa$ B $\alpha$  signal
- p65 / I $\kappa$ B $\alpha$  complex

## Dose-response curve for TNF $\alpha$ (30' stimulation)

without medium, 10 000 cells/well  
(AlphaLISA lysis buffer)

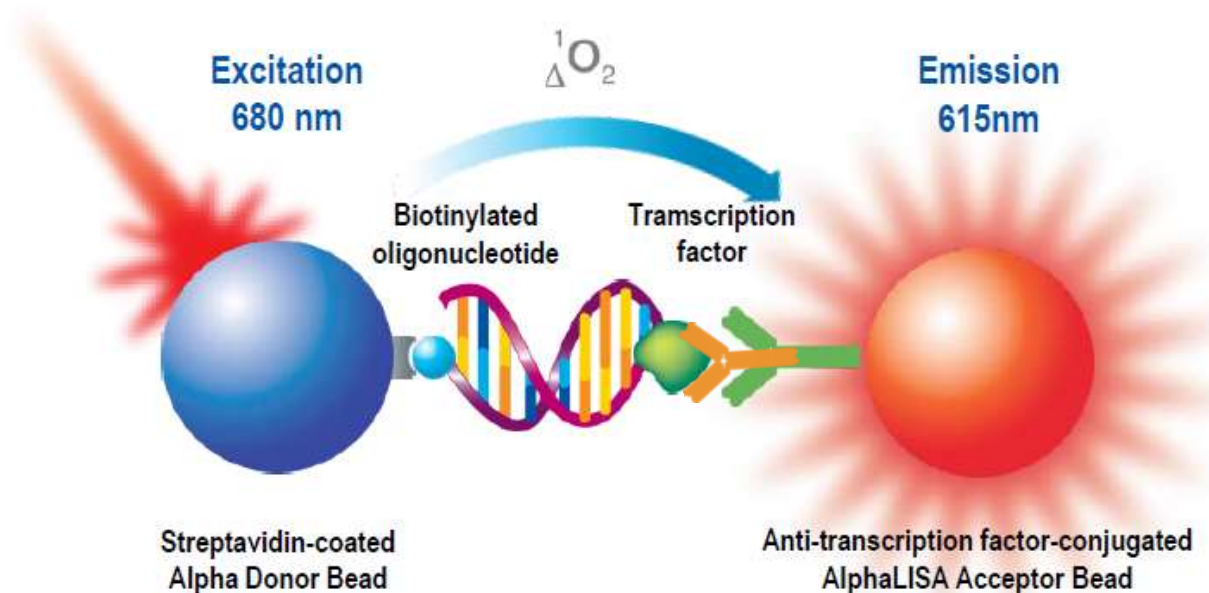


Inflammatory mediators  
Inflammatory cytokines



# Monitoring DNA-protein interaction in nuclear extracts

AIM: replace a radioactivity-based assays for Sp1 and HNF1 transcription factors using Alpha technology



Oligonucleotide	Sequence (5'- to 3')
Sp1-Consensus	ATTCGATCGGGGCGGGGCGAGC
Biotin-Sp1-Consensus	Biotin-TEG-ATTCGATCGGGGCGGGGCGAGC
Sp1-Mutated	ATTCGATCGGTTTCGGGGCGAGC
Biotin-Sp1-Mutated	Biotin-TEG-ATTCGATCGGTTTCGGGGCGAGC
HNF1-Consensus	TATTATGGTGGAGCTAATAAGTTGCAAGTCCCT
Biotin-HNF1-Consensus	Biotin-TEG-TATTATGGTGGAGCTAATAAGTTGCAAGTCCCT
HNF1-Mutated	TATTATGGTGGAGCCAATAAGTTGCAAGTCCCT
Biotin-HNF1-Mutated	Biotin-TEG-TATTATGGTGGAGCCAATAAGTTGCAAGTCCCT
Non-Specific Competitor	GATCGAACTGACCGCTTGCGGCCCGT



HUMAN HEALTH

ENVIRONMENTAL HEALTH

MAKE AN EPIC  
DIFFERENCE  
IN YOUR RESEARCH



HUMAN HEALTH | ENVIRONMENTAL HEALTH

**Epic<sup>®</sup> label-free technology  
for interaction studies**



# Label-free module on EnSpire

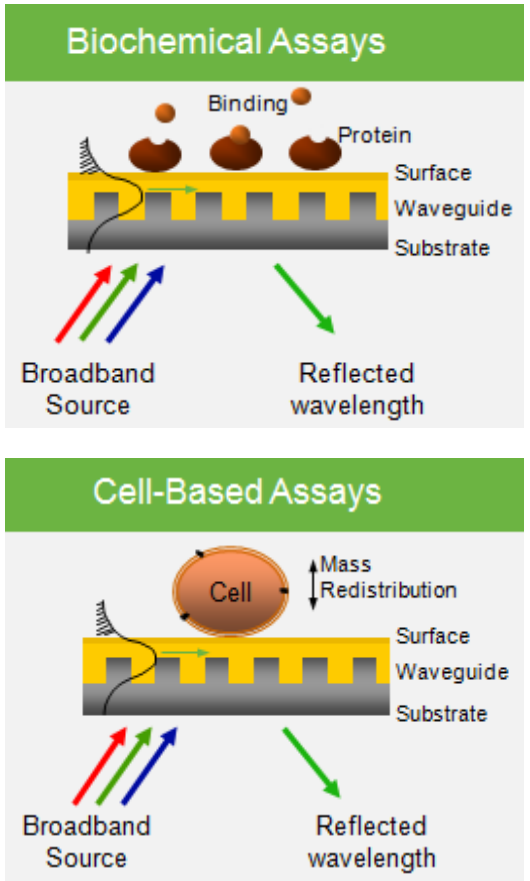
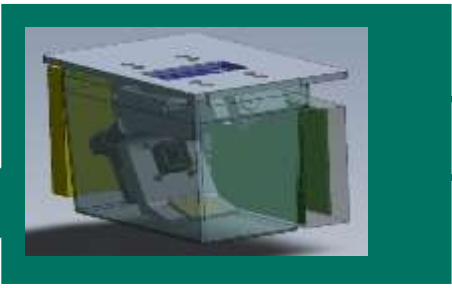
## Difficulties with labeling-based technologies:

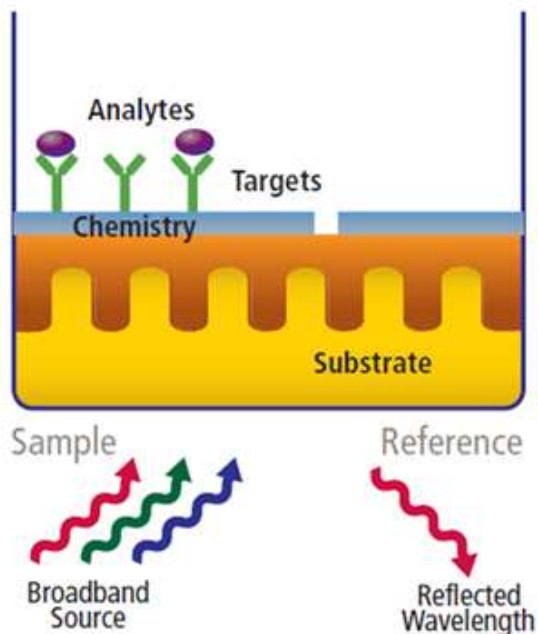
- Cost of label (including safety arrangements for radioactive labels)
- Photo-bleaching/-toxic effects
- Engineering of cell lines
- Fluorescent/radioactive tags can interfere with biochemistry

**EnSpire  
multi-mode reader**



**Label-free module**





## Biochemical LF protocol

Protein immobilization  
on LFB plate

Incubate 60 min or O/N  
Wash plate 4x

Buffer equilibration

Equilibrate for 2/3 hr

Baseline measurement  
(5-10 min)

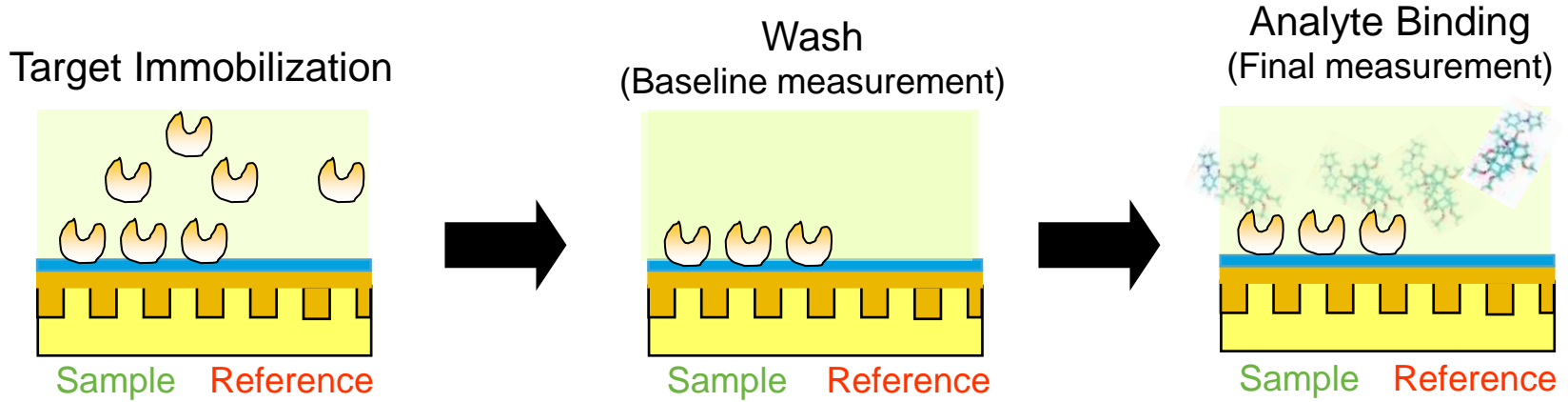
Add ligands (serial dilutions in buffer)

Final measurement  
(30-60 min)

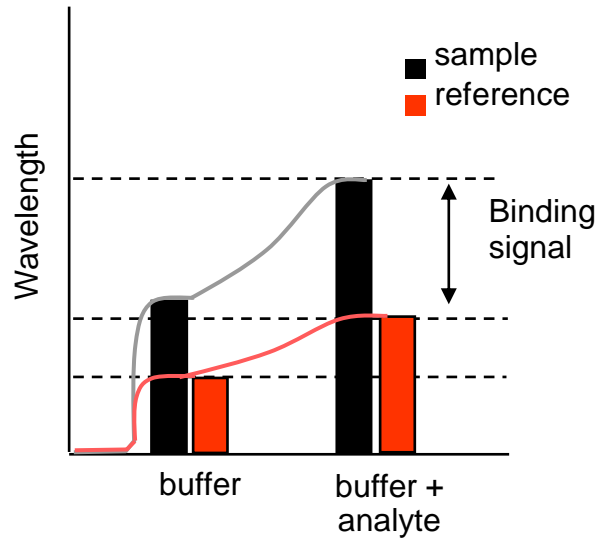
Data analysis  
to obtain  $K_d$

Read times vary based  
on ligand properties

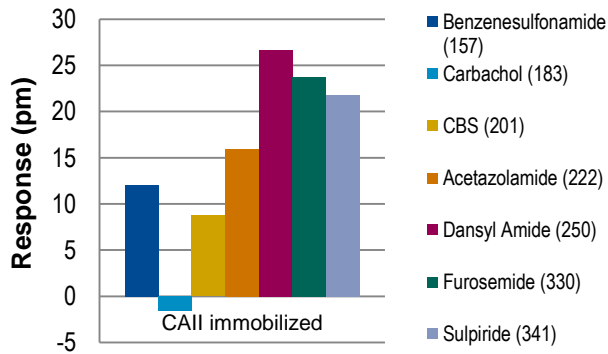
# "Sample" & "Reference" sensors in biochemical LF plates



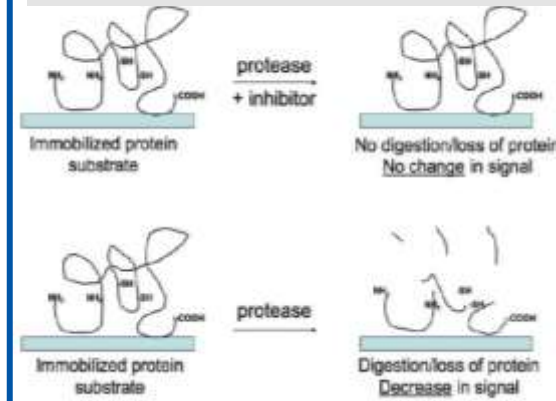
## Binding data



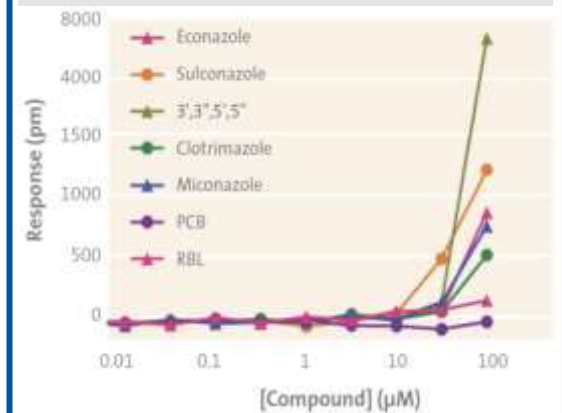
## Small molecules



## Protease functional assays



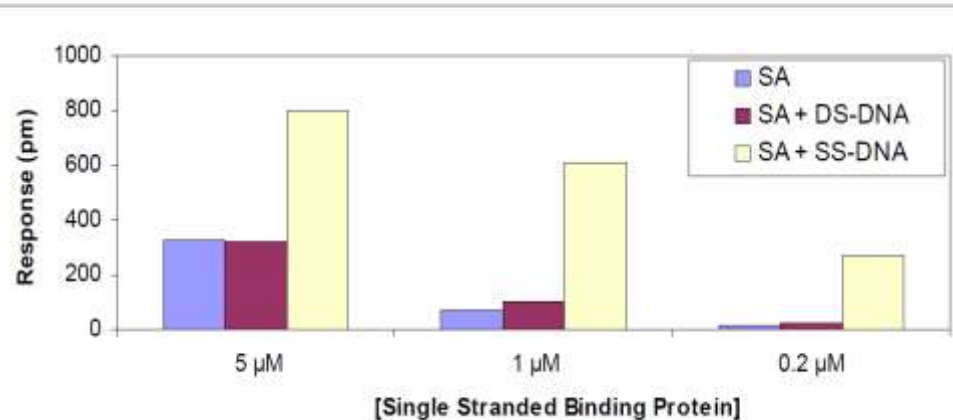
## Aggregation



## Antibody-receptor binding

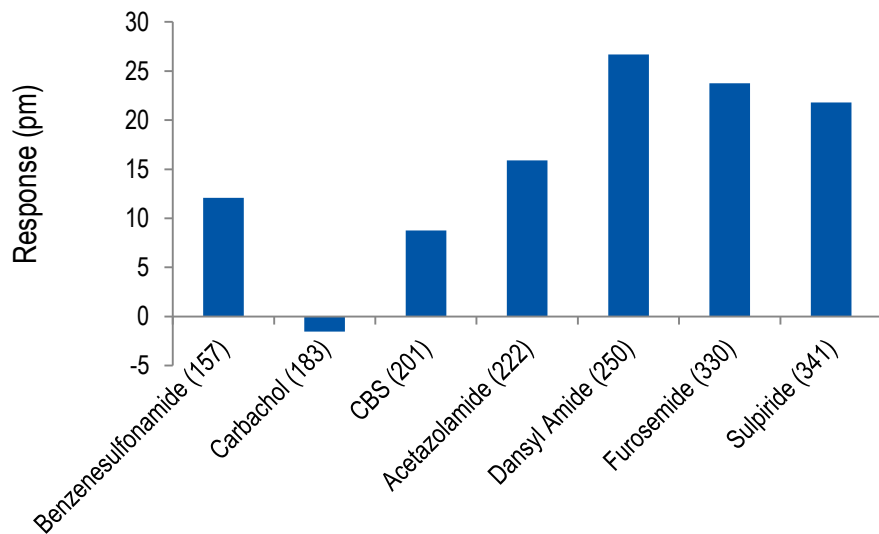


## DNA-Protein binding



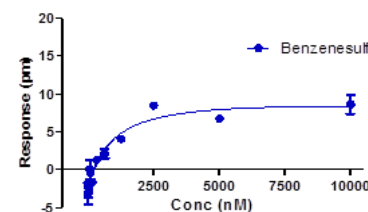
# Small molecule biochemical DMR: binding data

Carbonic Anhydrase II binding to 6 small molecule inhibitors (high-sensitivity biochemical plate)

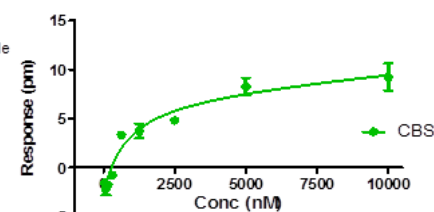


## Dose-response curves

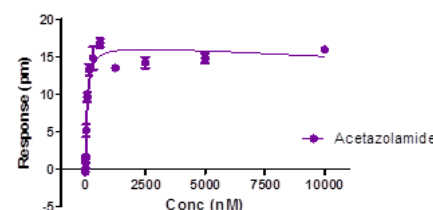
Benzenesulfonamide (157 Da),  $K_D = 983$  nM



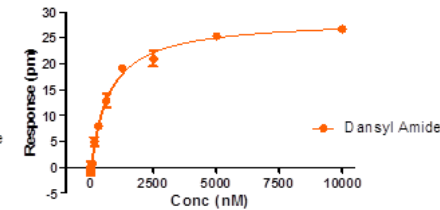
CBS (201 Da),  $K_D = 790$  nM



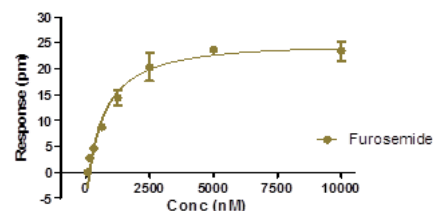
Acetazolamide (222 Da),  $K_D = 55$  nM



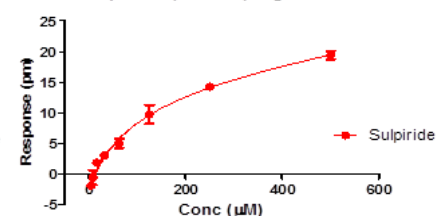
Dansyl Amide (250 Da),  $K_D = 701$  nM



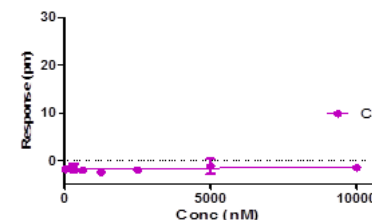
Furosemide (330 Da),  $K_D = 639$  nM



Sulpiride (341 Da),  $K_D = 96$  nM



Carbachol (182 Da), No Binding

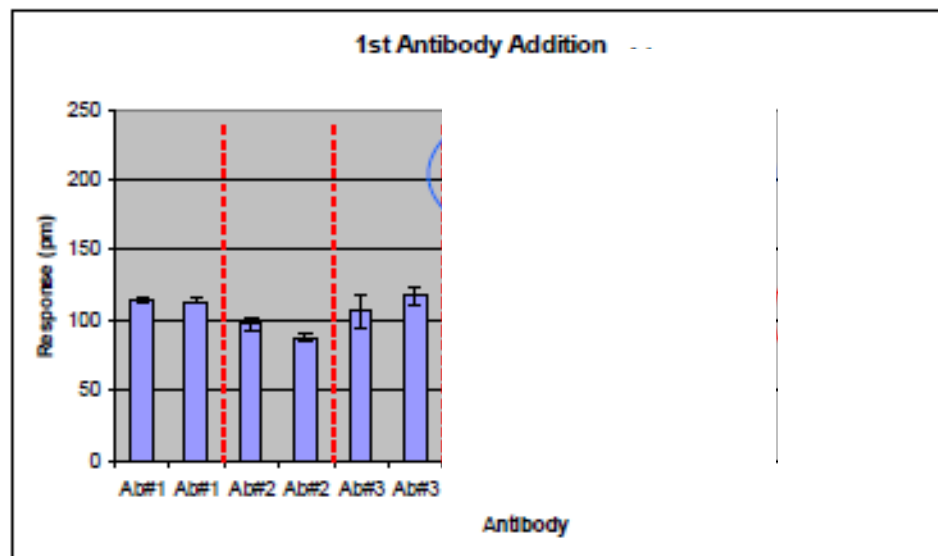


Target	Ligand	MW (Da)	DMR $K_d$ (nM)	Biacore $K_d$ (nM)*
Carbonic anhydrase (30 kDa)	Benzenesulfonamide	157	<b>983</b>	848
	CBS	201	<b>790</b>	893
	Acetazolamide	222	<b>55</b>	19
	Dansylamide	250	<b>701</b>	760
	Furosemide	330	<b>639</b>	513
	(±)-Sulpiride	341	<b>96**</b>	186**

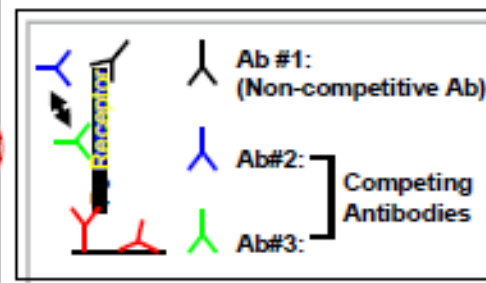
\*Myszka, D.G. Analysis of small-molecule interactions using Biacore S51 technology. Anal. Biochem. 329, 316-323 (2004) - \*\* Concentration values:  $\mu$ M

Binding observed for 157 - 341 Daltons compounds

Biological question: do antibodies #1 #2 and #3 bind to the same epitope of the colony stimulating factor 1 receptor?



Data generated with  
Roche Pharma



Analyte is M-CSF receptor

- Each antibody binds to the Receptor when added individually.

Take the following equation for calculation:

$$\text{immobilization}[pm] = \frac{\text{average}(\text{protein}[fm]) - \text{average}(\text{buffer}[fm])}{1000}$$

Typical immobilization levels on pre-activated Label-free plates are ~1500 – 2500 pm for many proteins and ~ 300 – 500 pm for many peptides. On user-activated plates immobilization rates are usually much higher and can go up to 4500 pm.

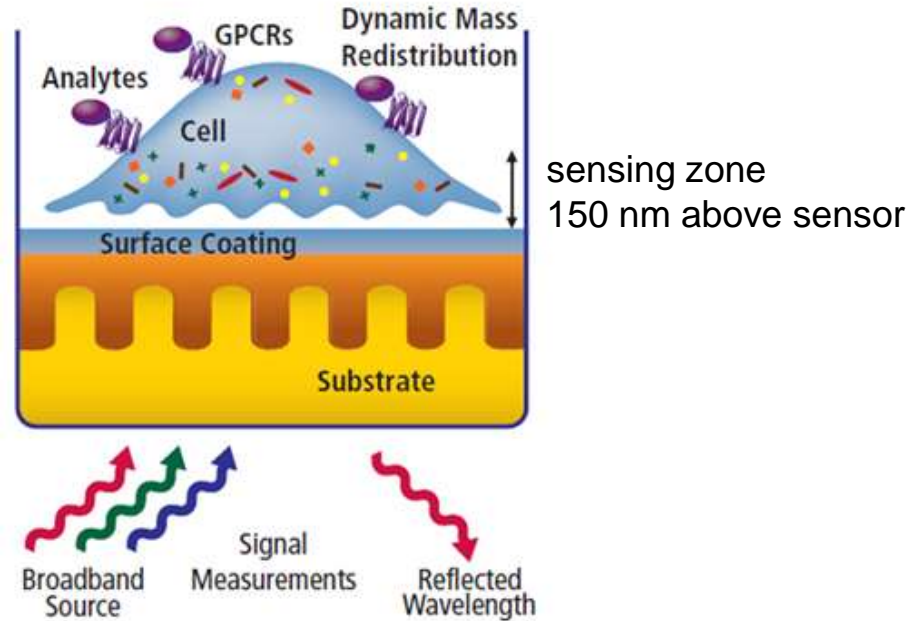
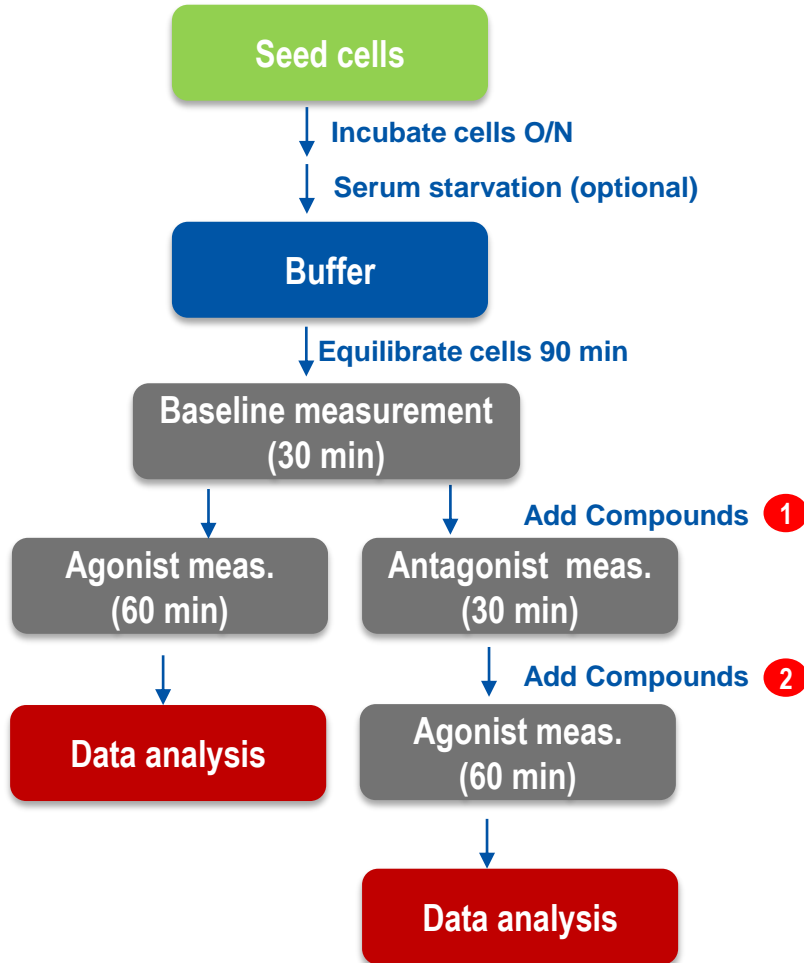
$$\text{Max theoretical binding signal (pm)} = \text{Target Immobilization Level} \times \frac{\text{Binder MW}}{\text{Target MW}} \times \text{stoichiometry}$$

$$\begin{aligned} \text{Max theoretical binding signal (pm)} &= 2500 \text{ pm} \times \frac{300 \text{ Da}}{30000 \text{ Da}} \times 1:1 \\ &= 25 \text{ pm} \end{aligned}$$

**Figure 8-2:** Calculation to determine the maximum theoretical binding ( $R_{\max}$ ) that can be expected for an immobilized target of 30 kDa which binds its binder of 300 Da. In this case the immobilization level resulted in 2500 pm and the stoichiometry is known as 1:1. The maximum theoretical binding that can be expected is 25 pm shift.



## Cell-based LF protocol

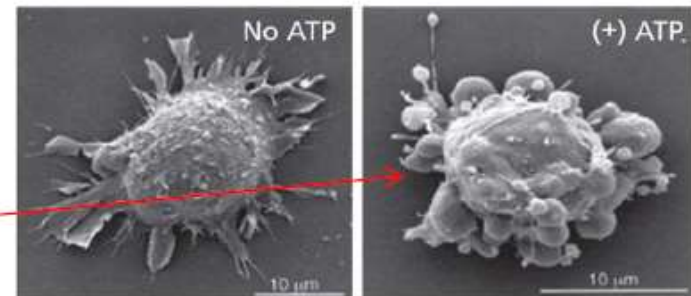
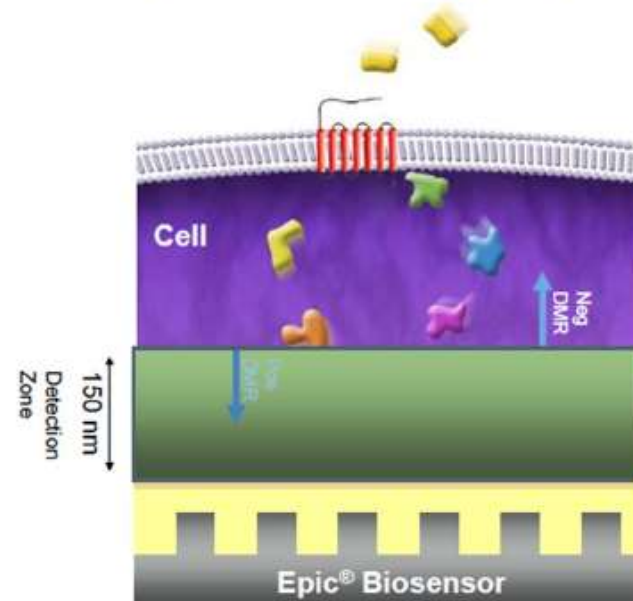


In the sensing zone mass can be added or removed (+/- **DMR** = e.g. relocation of intracellular components)

- ▶ Cellular response to ligand stimulation is the result of a series of biochemical interactions within a cell
- ▶ Label-free measures mass movements (mostly proteins) within a specific detection zone (~150nm)
- ▶ The mass movement is referred to as **dynamic mass redistribution (DMR)**, which is an integrated measure of cellular response

Ability to measure these types of “whole cell” responses!

## Cell Assays Measure DMR from Protein Movements within the Cell



J. Brown GSK Neuroscience

## ENDPOINT ASSAY

- ▶ You know exactly what you see but you don't know whether anything else is happening...

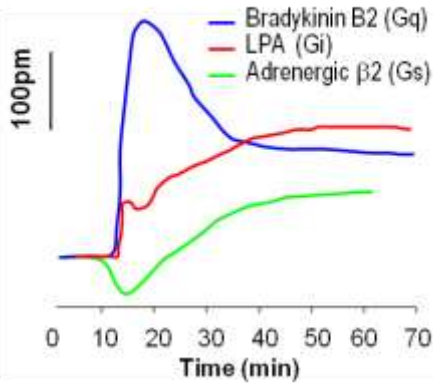
## DMR ASSAY

- ▶ You see „everything“ but you don't know what you see

from E. Kostenis, Institute for Pharmaceutical Biology, University of Bonn, Germany

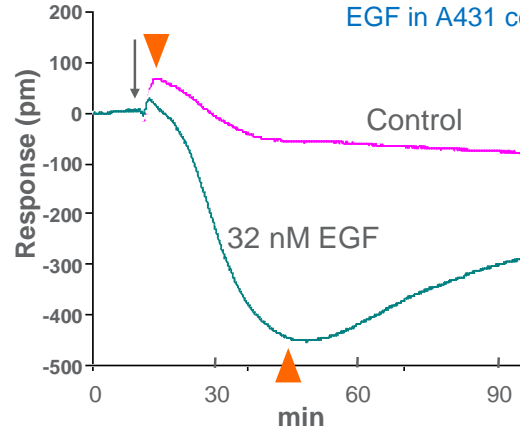
## GPCR

Endogenous GPCRs in A431



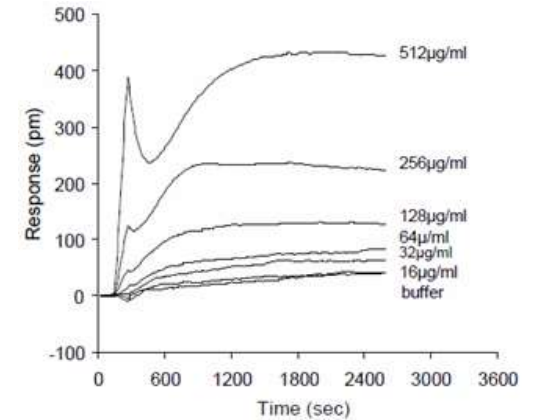
## Receptor Tyrosine Kinases

EGF in A431 cells



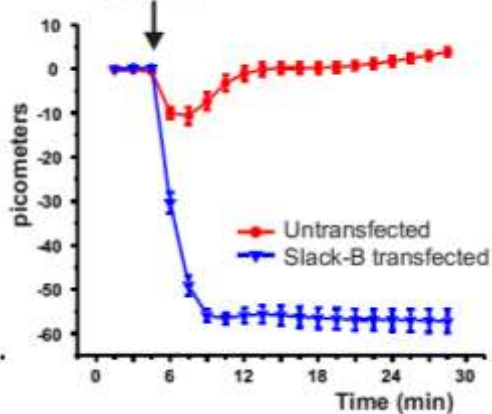
## Toll-Like Receptors

dose-dependant activation of TLR3 in A549



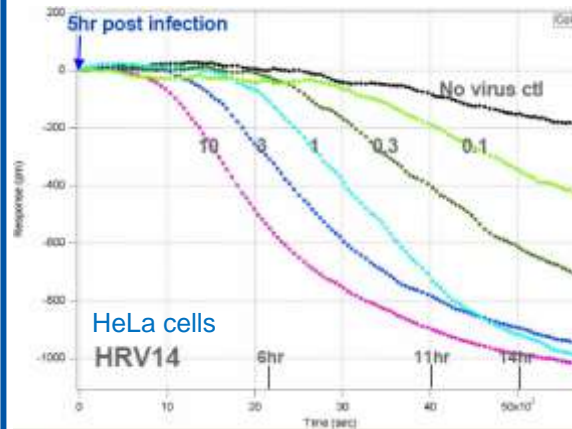
## Ion Channels

Bithionol



Courtesy of Len Kaczmarek, Yale University

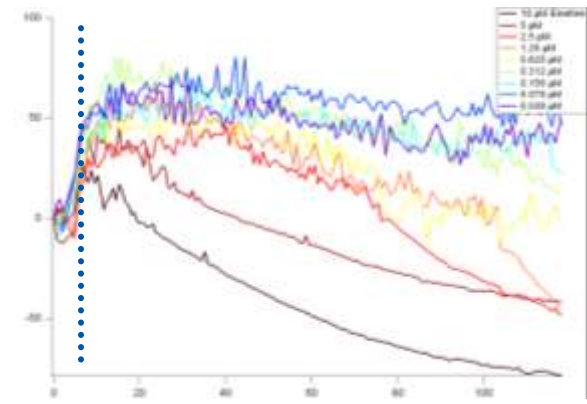
## Viral Infection



Courtesy of Moonsoo Jin, Cornell University

## Cytotoxicity

Emetine-induced toxicity on Cardiomyocytes





# LANCE homogeneous assay

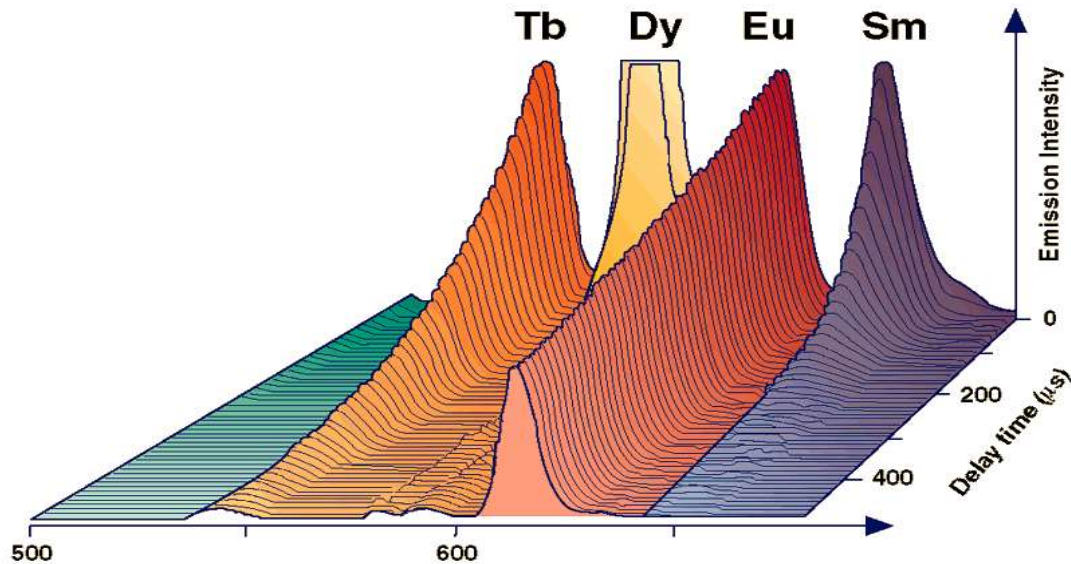
Lanthanide chelate excite



# Lanthanide "fluorescence" decay lifetime

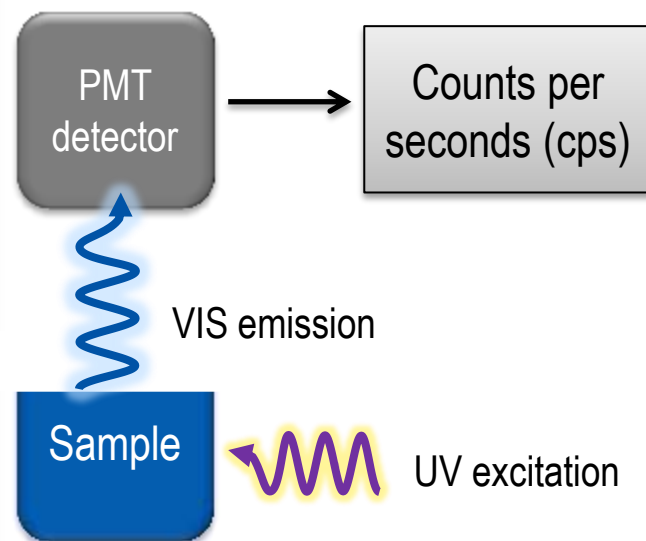
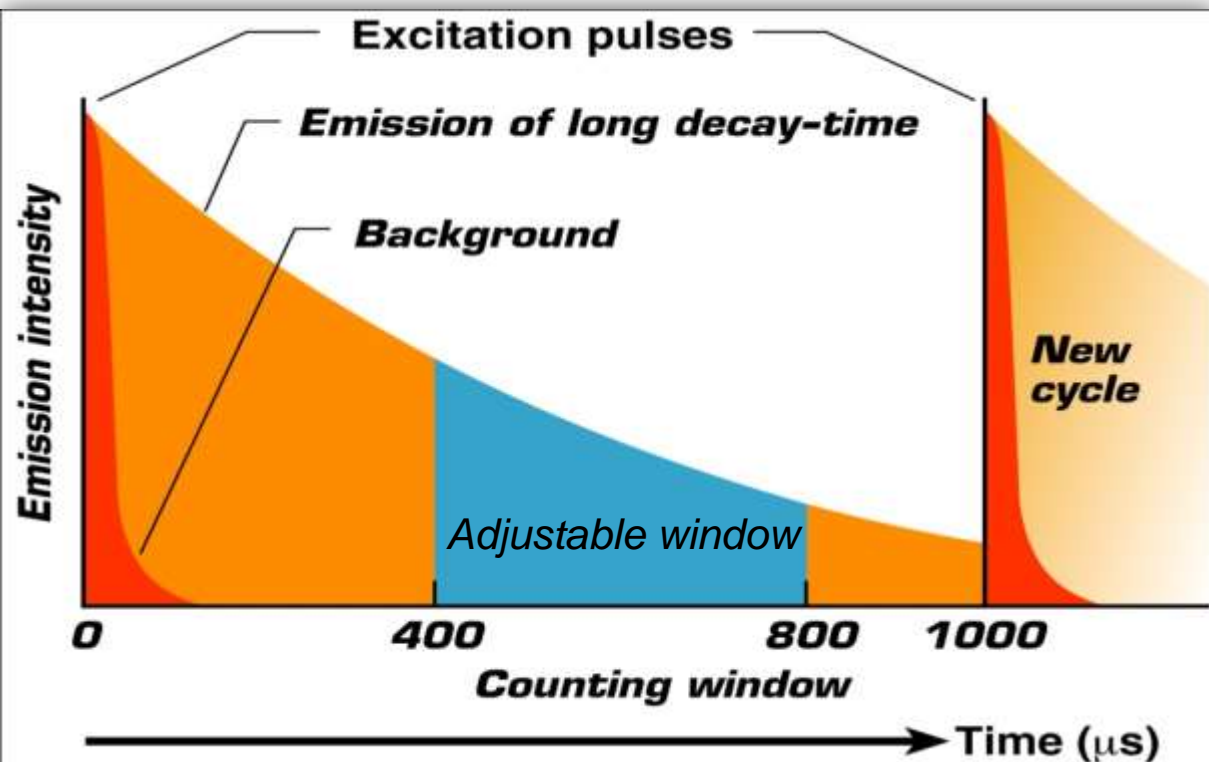
- ▶ Lanthanides have long "fluorescence" lifetime ( $\mu\text{s}$  to  $\text{ms}$ )
- ▶ Surrounding chelate structure improves excitation

Group	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	1 H																	2 He
2	3 Li	4 Be											5 B	6 C	7 N	8 O	9 F	10 Ne
3	11 Na	12 Mg											13 Al	14 Si	15 P	16 S	17 Cl	18 Ar
4	19 K	20 Ca	21 Sc	22 Ti	23 V	24 Cr	25 Mn	26 Fe	27 Co	28 Ni	29 Cu	30 Zn	31 Ga	32 Ge	33 As	34 Se	35 Br	36 Kr
5	37 Rb	38 Sr	39 Y	40 Zr	41 Nb	42 Mo	43 Tc	44 Ru	45 Rh	46 Pd	47 Ag	48 Cd	49 In	50 Sn	51 Sb	52 Te	53 I	54 Xe
6	55 Cs	56 Ba	* 71 Lu	* 72 Hf	* 73 Ta	* 74 W	* 75 Re	* 76 Os	* 77 Ir	* 78 Pt	* 79 Au	* 80 Hg	* 81 Tl	* 82 Pb	* 83 Bi	* 84 Po	* 85 At	* 86 Rn
7	87 Fr	88 Ra	* 103 Lr	* 104 Rf	* 105 Db	* 106 Sg	* 107 Bh	* 108 Hs	* 109 Mt	* 110 Ds	* 111 Rg	* 112 Cn	* 113 Uut	* 114 Fl	* 115 Uup	* 116 Lv	* 117 Uus	* 118 Uuo
			* 57 La	* 58 Ce	* 59 Pr	* 60 Nd	* 61 Pm	* 62 Sm	* 63 Eu	* 64 Gd	* 65 Tb	* 66 Dy	* 67 Ho	* 68 Er	* 69 Tm	* 70 Yb		
			* 89 Ac	* 90 Th	* 91 Pa	* 92 U	* 93 Np	* 94 Pu	* 95 Am	* 96 Cm	* 97 Bk	* 98 Cf	* 99 Es	* 100 Fm	* 101 Md	* 102 No		



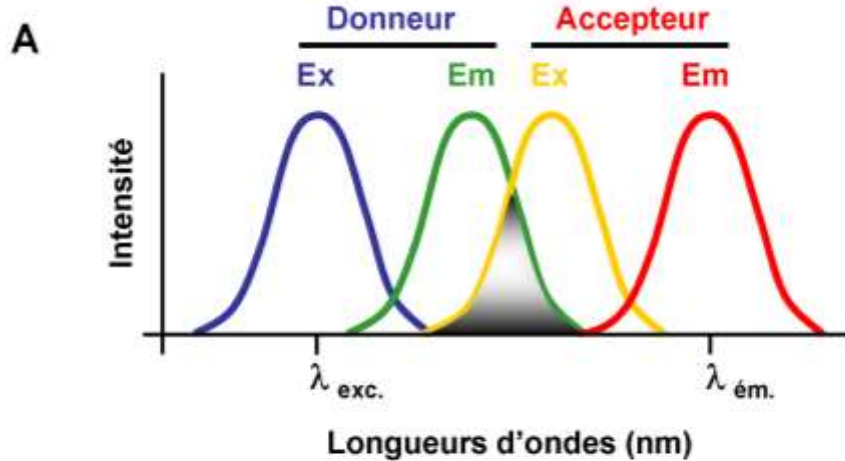
# Time-resolved fluorescence (TRF) principle

- ▶ Low background noise → High signal/background ratio and sensitivity!
- ▶ Signals are normalized to counts per second: results become independent of actual measurement time → enables comparison of different measurements

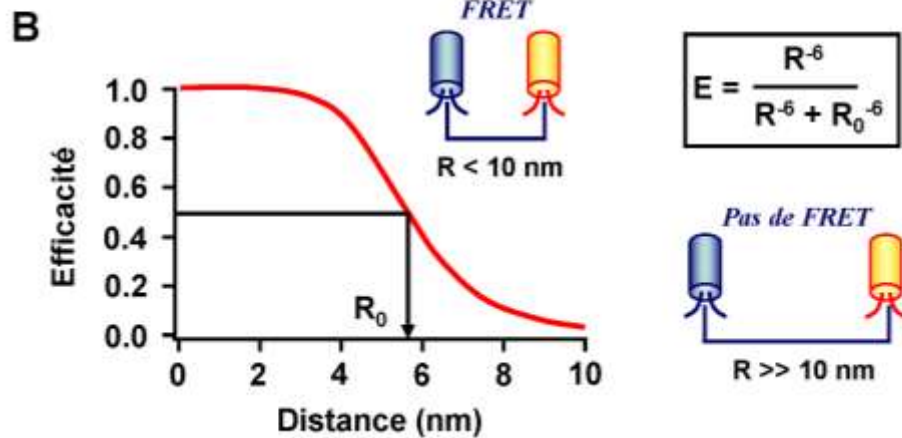




# FRET: Förster Resonance Energy Transfer

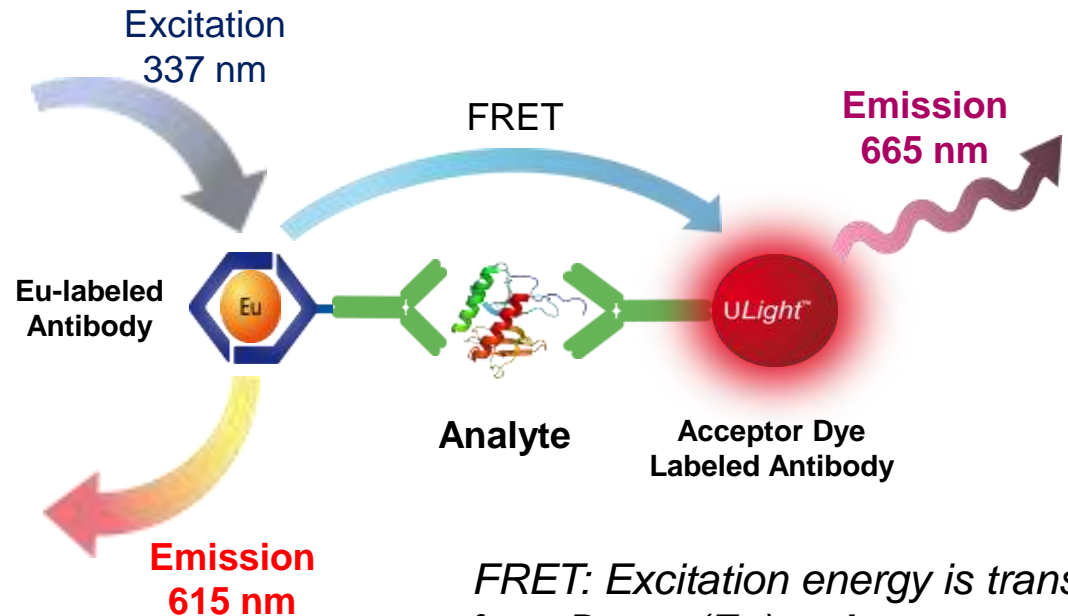
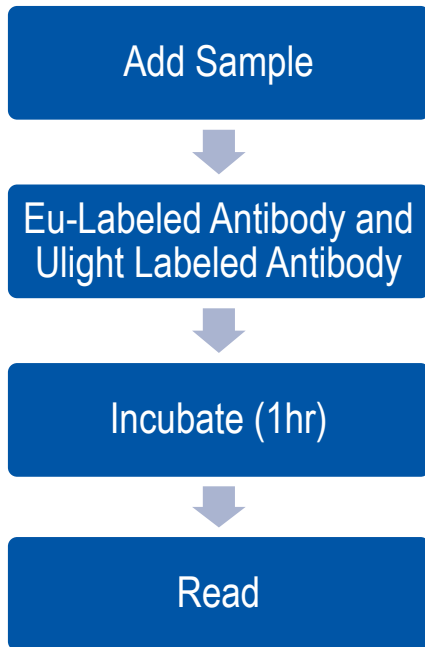


FRET: a donor chromophore, initially in its electronic excited state, may transfer energy to an acceptor chromophore through dipole–dipole coupling.



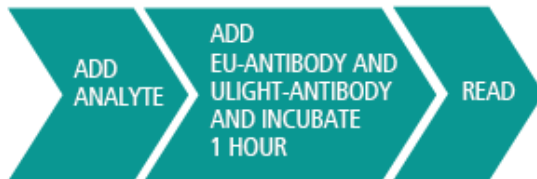
The efficiency of this energy transfer is inversely proportional to the **sixth power of the distance** between donor and acceptor, making FRET extremely sensitive to small changes in distance.

Mix & read protocol: quick, no washing steps



*FRET: Excitation energy is transferred from Donor (Eu) to Acceptor molecule, if proximal (<10 nm)*

## LANCE TR-FRET Assay



# Available LANCE *Ultra* immunoassay kits

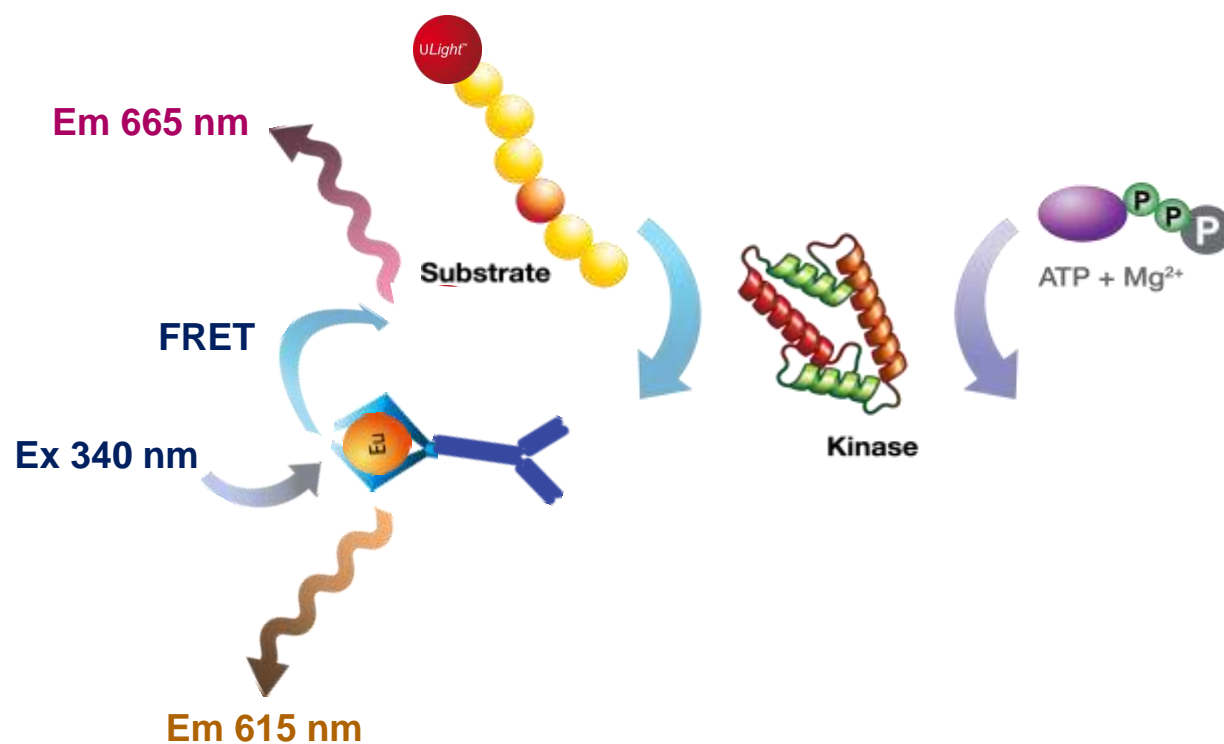
Autophagy	Biologics	Cancer	Cardiovascular	CNS	Inflammation	Metabolic	Animal health
<ul style="list-style-type: none"> <li>• LC3B</li> </ul>	<ul style="list-style-type: none"> <li>• Human IgE</li> <li>• Human IgG</li> <li>• Human IgM</li> <li>• Human Kappa Light Chain</li> <li>• Mouse IgG</li> <li>• Rabbit IgG</li> <li>• CHO-HCP</li> <li>• CHO-P</li> <li>• E.coli HCP</li> <li>• PER.C6 HCP</li> <li>• Residual Protein A</li> <li>• Residual DNA</li> </ul>	<ul style="list-style-type: none"> <li>• BCL2</li> <li>• COL1A1</li> <li>• COX-2</li> <li>• E-cadherin</li> <li>• EGF</li> <li>• EGFR</li> <li>• ErbB2 (HER2)</li> <li>• Fibronectin</li> <li>• MANF</li> <li>• MMP-3</li> <li>• MMP-7</li> <li>• MMP-8</li> <li>• MMP-12</li> <li>• Nidogen-1</li> <li>• Oncostatin M</li> <li>• PD-1</li> <li>• PD-L1</li> <li>• Perlecan</li> <li>• PIP collagen</li> <li>• PTEN</li> <li>• VEGF</li> <li>• VEGF (mouse)</li> </ul>	<ul style="list-style-type: none"> <li>• Fibrinogen</li> <li>• tPA</li> <li>• Transferrin</li> </ul>	<ul style="list-style-type: none"> <li>• A<math>\beta</math> (1-40)</li> <li>• BDNF</li> <li>• Frataxin</li> <li>• GAD67</li> <li>• Tau</li> <li>• TDP-43</li> </ul>	<ul style="list-style-type: none"> <li>• CCL2/MCP-1<math>\alpha</math></li> <li>• CCL2 (mouse)</li> <li>• CCL3/MIP1<math>\alpha</math></li> <li>• CCL4</li> <li>• CCL5</li> <li>• CRP</li> <li>• CXCL1/GRO-<math>\alpha</math></li> <li>• CXCL9</li> <li>• CXCL10</li> <li>• IFN<math>\alpha</math></li> <li>• IFN<math>\gamma</math></li> <li>• IFN<math>\gamma</math> (mouse)</li> <li>• IL-1<math>\alpha</math></li> <li>• IL-1<math>\beta</math></li> <li>• IL-1<math>\beta</math> (mouse)</li> <li>• IL-1RA</li> <li>• IL-2</li> <li>• IL-4</li> <li>• IL-5</li> <li>• IL-6</li> <li>• IL-6 (mouse)</li> <li>• IL-8</li> <li>• IL-11</li> <li>• IL-17A</li> <li>• IL-17A (mouse)</li> <li>• IL-23A</li> <li>• IL-28B</li> <li>• IL-31</li> <li>• TNF<math>\alpha</math></li> <li>• TNF<math>\alpha</math> (mouse)</li> </ul>	<ul style="list-style-type: none"> <li>• Adiponectin</li> <li>• Albumin</li> <li>• ApoA1</li> <li>• Apo-CIII</li> <li>• ApoE</li> <li>• C-peptide (mouse/rat)</li> <li>• hCG</li> <li>• FSH</li> <li>• GLP-1</li> <li>• Glucagon</li> <li>• IGF-1</li> <li>• Insulin</li> <li>• Leptin</li> <li>• Leptin (mouse)</li> <li>• PAI-1</li> <li>• PCSK9</li> <li>• TSH</li> </ul>	<ul style="list-style-type: none"> <li>• IgG (bovine)</li> </ul>

Example of data obtained for inflammation biomarker in App note # 012987

	Donor Fluorophores	Acceptor Fluorophores	
		ULight	APC (SureLight)
Labeling Reagents	LANCE Eu-W1024-ITC labeling reagent (AD0096)		
	LANCE Eu-W1284-iodoacetamide labeling reagent (AD0107)		
	LANCE Eu-W8044-DTA labeling reagent (AD0020)		
	Custom-Labeling Service	Custom-Labeling Service	Custom-Labeling Service
Fusion Tag Detection	<b>Streptavidin</b> , Eu-labeled ( Eu-W1024: AD0062, AD0063 and W8044: AD0060, AD0061)	<b>Streptavidin</b> , <i>ULight</i> -labeled (TRF0102-D/M/R)	<b>Streptavidin</b> , APC-labeled (CR130-100/150, AD0201, AD0202)
	Anti- <b>GST</b> , Eu-labeled (AD0252, AD0253, AD0254)	Anti- <b>GST</b> , <i>ULight</i> -labeled (TRF0104-D/M/R)	Anti- <b>GST</b> , APC-labeled (AD0059G)
	Anti- <b>c-myc</b> , Eu-labeled (AD0206, AD0114, AD0115)	Anti- <b>c-myc</b> , <i>ULight</i> -labeled (TRF504-D/M/R)	Anti- <b>c-myc</b> , APC-labeled (AD0059B)
	Anti- <b>FLAG</b> <sup>®</sup> , Eu-labeled (AD0273, AD0274, AD0275)	Anti- <b>FLAG</b> <sup>®</sup> , <i>ULight</i> -labeled (TRF0059-D/M/R)	Anti- <b>FLAG</b> <sup>®</sup> , APC-labeled (AD0059F)
	Anti- <b>6x His</b> Ab, Eu-labeled (AD0205, AD0110, AD0111)	Anti- <b>6x His</b> , <i>ULight</i> -labeled (TRF0105-D/M/R)	Anti- <b>6x His</b> , APC-labeled (AD0059H)
	Anti- <b>HA</b> , Eu-labeled (AD0084, AD0085)		
	<b>Biotin</b> , Eu-labeled (CR91-100)		
	Anti- <b>FITC</b> , Eu-labeled (AD0270, AD0271, AD0272)	Anti- <b>FITC</b> , <i>ULight</i> -labeled (TRF0503-D/M/R)	Wheat Germ Agglutinin ( <b>WGA</b> ), APC-labeled (AD0059W)
Antibody Capture		<b>Protein A</b> , <i>ULight</i> -labeled (TRF0103-D/M)	
	<b>Protein G</b> , Eu-labeled (AD0211, AD0070, AD0071)		
	Anti- <b>human IgG</b> , Eu-labeled (AD0212, AD0074, AD0075)	Anti- <b>human IgG</b> , <i>ULight</i> -labeled (TRF500-D/M/R)	
	Anti- <b>mouse IgG</b> , Eu-labeled (AD0076, AD0077)	Anti- <b>mouse IgG</b> , <i>ULight</i> -labeled (TRF501-D/M/R)	Anti- <b>mouse IgG</b> , APC-labeled (AD0059M)
	Anti- <b>rabbit IgG</b> , Eu-labeled (AD0082, AD0083)	Anti- <b>rabbit IgG</b> , <i>ULight</i> -labeled (TRF502-D/M/R)	Anti- <b>rabbit IgG</b> , APC-labeled (AD0059R)

- Proteins are often expressed with tags (GST, etc.) to facilitate purification
- Flexibility in assay development
- More Toolbox products available “on demand”

- Quantify kinase activity *in vitro* by detection of phospho-substrate accumulation
- Substrate directly labelled with ULight
- Mix & read, no washing steps



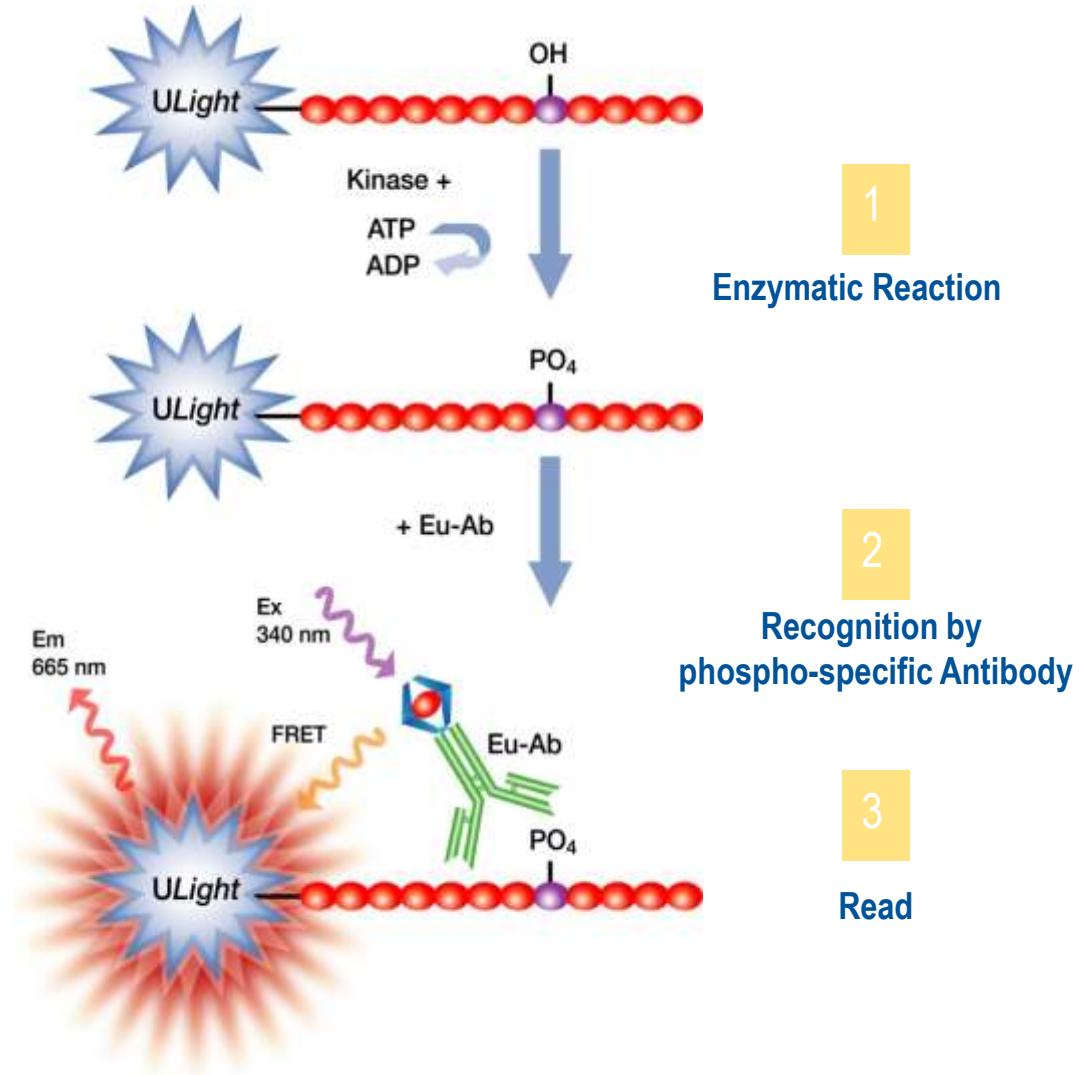
Add enzyme + compound + ATP +  
ULight-peptide

Incubate

Eu-labeled Antibody

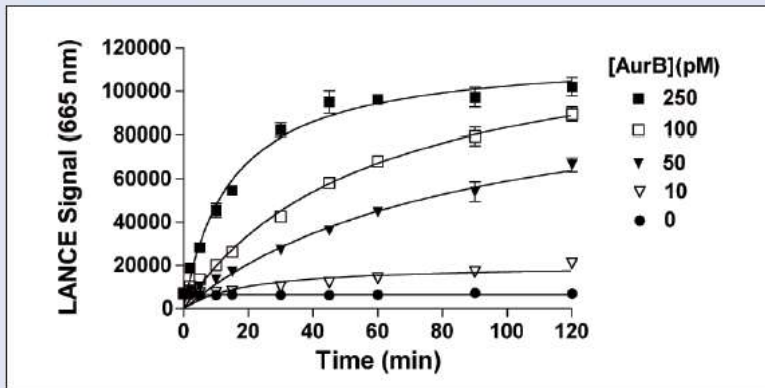
Incubate 1 hr

Read



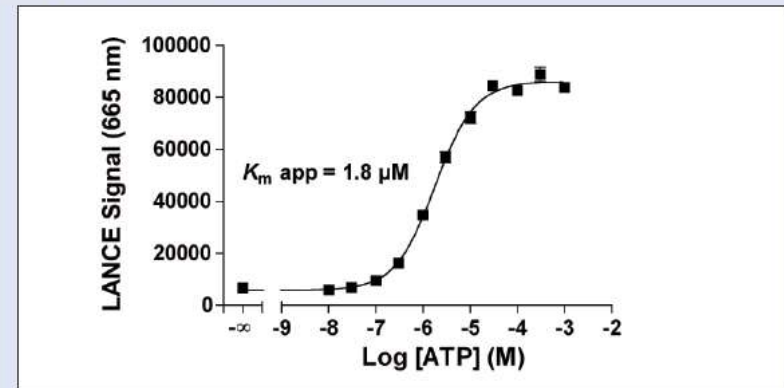
This strategy allowed testing 83 human Tyr kinases against 6 substrates

## Experiment 1: Enzymatic Time Course



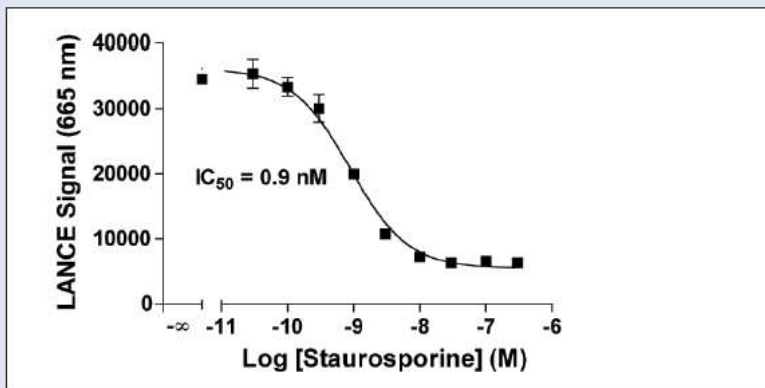
Aurora B enzyme was incubated at concentrations ranging from 10 to 250 pM with 50 nM *ULight*-Histone H3 (Thr3/Ser10) Peptide and 20 μM ATP. Kinase reactions were terminated after 0 to 120 min by the addition of EDTA.

## Experiment 2: ATP Titration



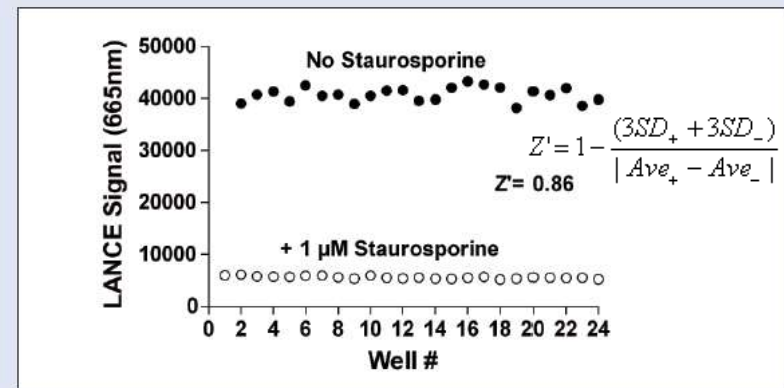
Serial dilutions of ATP ranging from 10 nM to 1 mM were added to 100 pM Aurora B and 50 nM *ULight*-Histone H3 (Thr3/Ser10) Peptide. Kinase reactions were terminated after 60 min by the addition of EDTA.

## Experiment 3: Enzyme Inhibition Curve



Serial dilutions of staurosporine ranging from 0.03 nM to 0.3 μM (final concentrations in 2% DMSO) were incubated with 100 pM Aurora B, 50 nM *ULight*-Histone H3 (Thr3/Ser10) Peptide and 2 μM ATP. Kinase reactions were terminated after 60 min by the addition of EDTA.

## Experiment 4: Z'-factor Determination

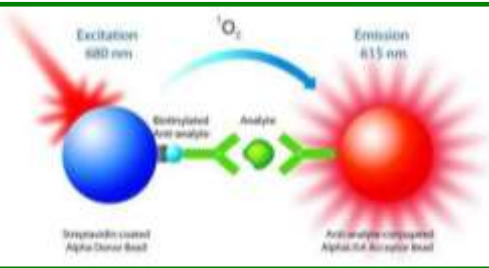


Aurora B enzyme at 100 pM was incubated with 50 nM *ULight*-Histone H3 (Thr3/Ser10) Peptide and 2 μM ATP with or without 1 μM staurosporine (final concentrations in 2% DMSO). Kinase reactions were terminated after 60 min by the addition of EDTA.

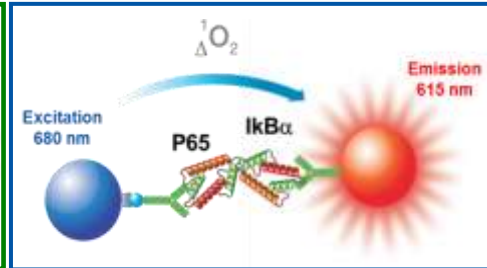


Alpha & LANCE technologies provide you the flexibility and versatility to replace traditional ELISAs and immunoblots, as sensitive and simple alternative enabling the detection and characterization of proteins and their interactions

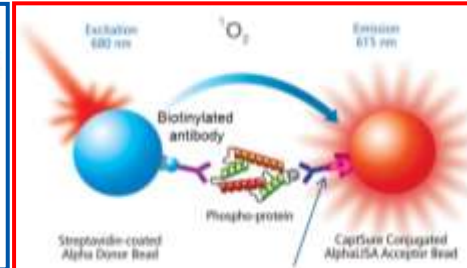
*immunoassays*



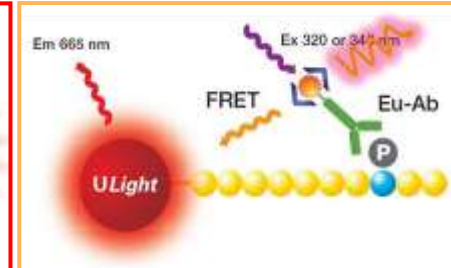
*protein:protein interaction*



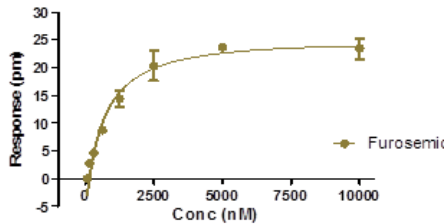
*phospho-protein detection*



*kinase assays*



Furosemide (330 Da),  $K_D = 639$  nM



"Label-free" is a convenient approach to detect protein:protein interactions, allowing  $K_D$  measurement in biochemical assays or detecting compounds able to elicit cellular responses

Thank you

