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HUMAN HEALTH | ENVIRONMENTAL HEALTH

How to run Alpha assay:

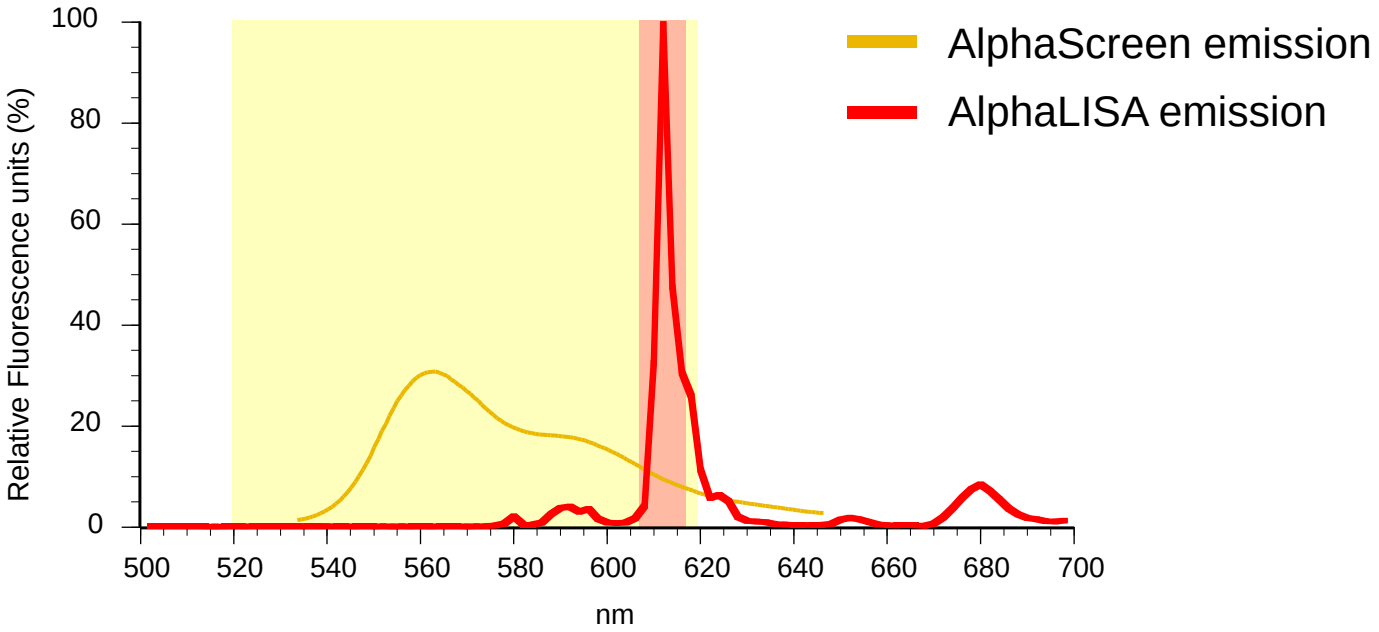
- How to setup an Alpha assay
- Make your own assay!

AlphaLISA kits - recommendations before starting the assay

- Samples:
  - Phenol red and hemoglobin: choose AlphaLISA beads instead of AlphaScreen beads
  - Pay attention to **RPMI** and **tissue homogenates**: they can contain biotin which will bind to streptavidin-coated beads
    - Biotin-free kits are available
    - Carefully plan order of addition (pre-incubate biotinylated antibody + streptavidin-beads)
  - 1% FBS or 0.1% BSA can be added to cell culture medium to enhance assay sensitivity
  - Serum samples: serum should not exceed 10% of final assay
  - If necessary, **dilute your samples with AlphaLISA buffer**

- Standard curve:
  - Prepare:
    - standard curve in the **same matrix** as samples (lysis buffer, serum without analyte) and
    - **standard curve in AlphaLISA buffer in parallel** to check interference from matrix.
  - If samples are diluted in AlphaLISA Buffer, prepare a standard curve in **diluted matrix** (example, serum diluted in AlphaLISA buffer)

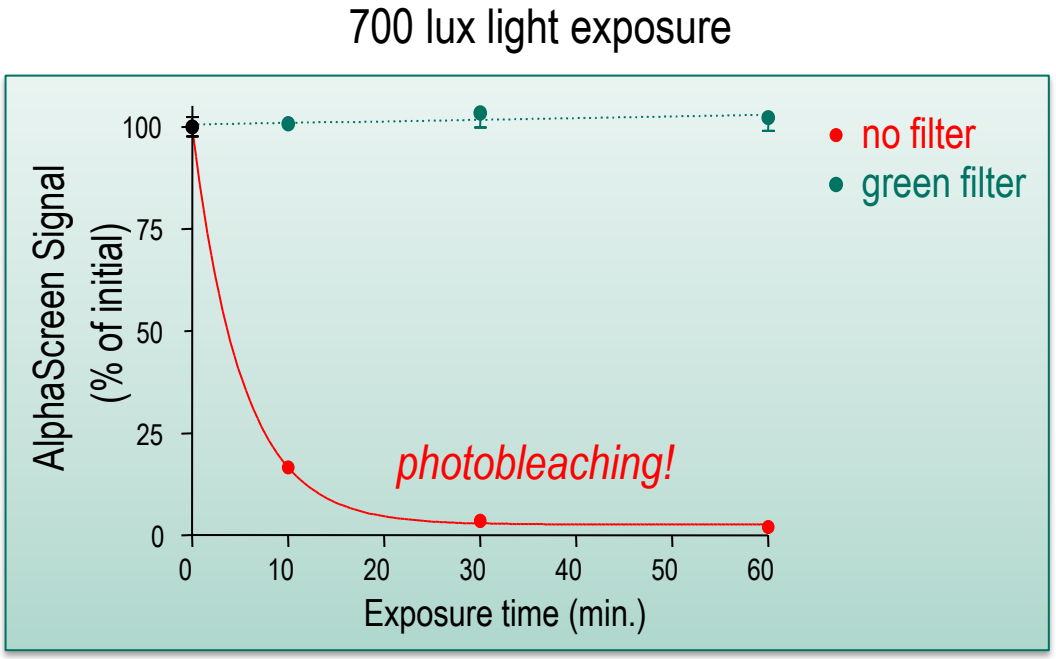
AlphaScreen & AlphaLISA



AlphaLISA beads contain europium:

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

Donor beads: effect of light exposure



Lighting condition	From (lux)	To (lux)	Mean value (lux)
Pitch Black	0	10	5
Very Dark	11	50	30
Dark Indoors	51	200	125
Dim Indoors	201	400	300
Normal Indoors	401	1000	700
Bright Indoors	1001	5000	3000
Dim Outdoors	5001	10,000	7500
Cloudy Outdoors	10,001	30,000	20,000
Direct Sunlight	30,001	100,000	65,000

- Switch off lights!
- Avoid direct day light (≤ 100 lux)
- Protect Donor beads vials with aluminum
- Cover plate (with another plate)
- Incubate plate in the dark (drawer)
- Use green filters

EnSpire multi-mode reader

Alpha

Absorbance

Fluorescence top & bottom

Ultra-sensitive luminescence

TRF

Label-free

Please select a protocol and press the 'Run' button above

	Valid	Technology	Name
		Abs	Protein Concentration Absorbance
		Abs	Spectrum Scan Absorbance
		Abs	Nucleotide Purity Absorbance
		Alpha	Alpha 96-well
		Alpha	Alpha 384-well Low Volume
		Alpha	Alpha 384-well
		Alpha	Performance IPA Alpha

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8	STD9	STD10	BL	BL
B	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8	STD9	STD10	BL	BL

Curve fitting UNK calculation

Source: Meas A (Alpha)

Fitting: 4PL

Weighted: Weighted

X-axis scale: Logarithmic

Y-axis scale: Logarithmic

Concentration unit: pg/ml

Advanced options

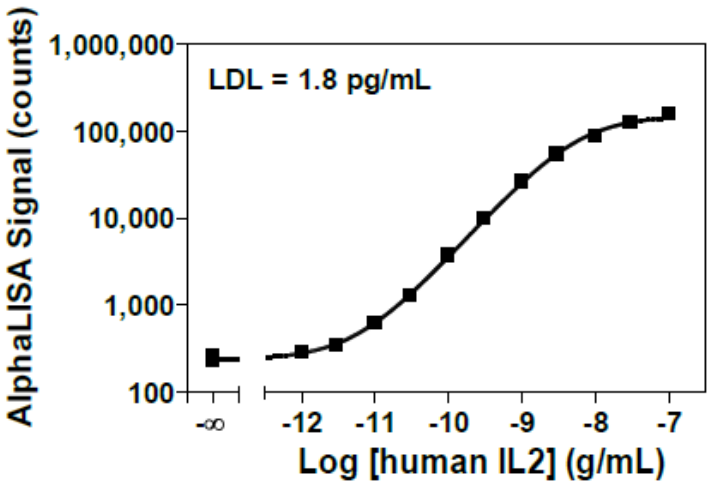
Concentration options

STD	Concentration
1	30000
2	10000
3	3000
4	1000
5	300
6	100
7	30
8	10
9	3
10	1

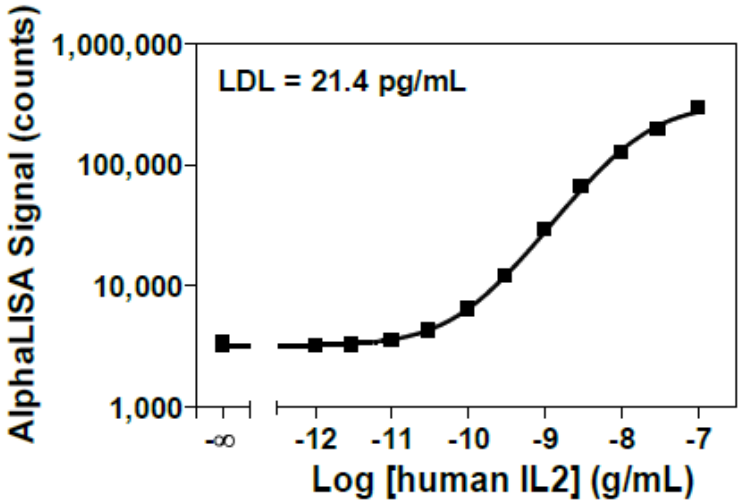
Data analysis

- Plot signal vs concentration of analyte (Log scale for either or both axes)
- Analyze data according to a nonlinear regression using the **4-Parameter Logistic** equation (Sigmoidal Dose-Response curve with variable slope)
- Add $1/Y^2$ weighting
- Values at maximal concentrations of analyte after the hook point must be **removed**
- **LDL (12 blk) = average + 3 x st.dev**.

St. curved in Alpha buffer



St. curve in diluted serum



Notice the matrix effect of serum

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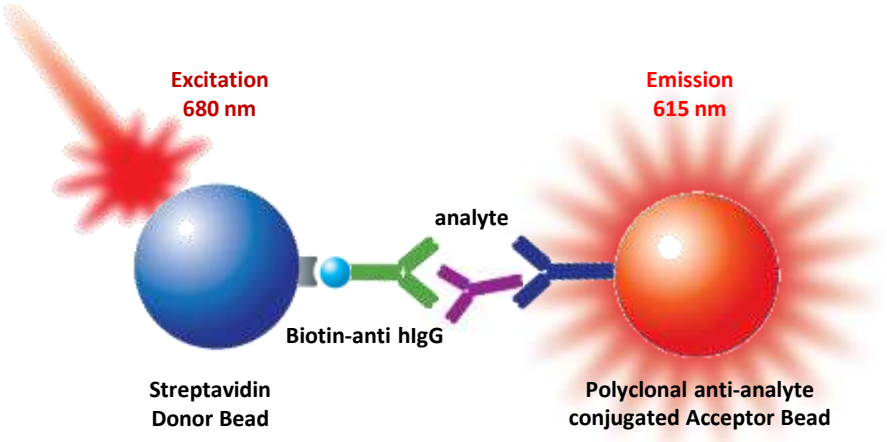
Make your own assay

Develop your own assay with Alpha Toolbox

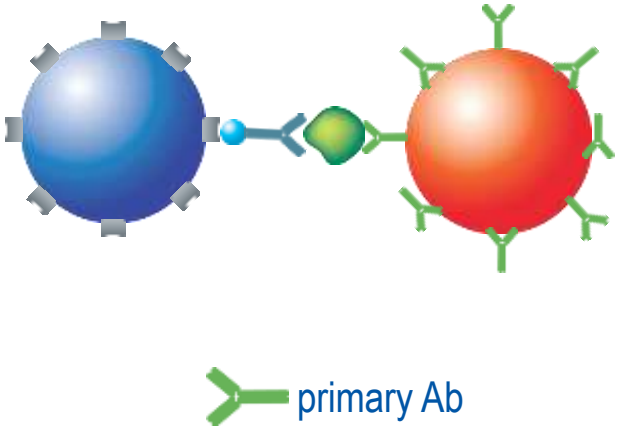
- Beads can be coated with antibodies or other binding molecules to develop virtually any immunoassay

Protein-protein interactions	Biomarkers	Pathway mapping	Cellular markers
	Biotherapeutics	Histone modifications	

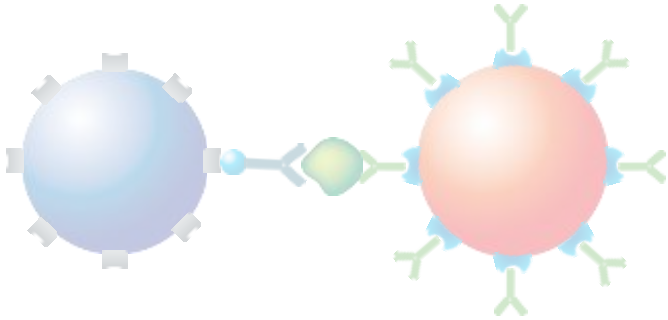
- Virtually any assay can be developed, as long as you can bring the beads together



Alpha immunoassay formats: conjugation vs. indirect binding



primary Ab



Protein A (or secondary IgG)

Direct conjugation

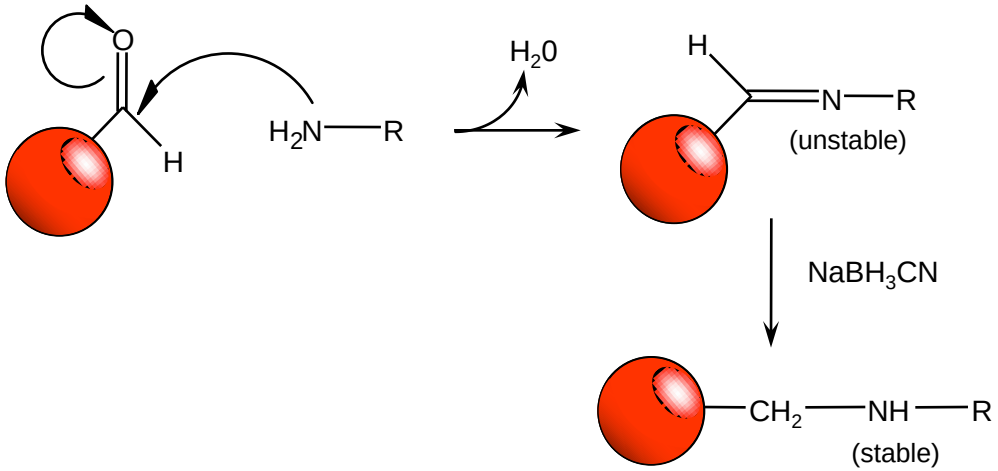
- Simple reaction (1 night incubation)
- Good control on binding events
- Random coupling (antibodies aligned randomly)
- Simple stoichiometry in Alpha assay

Indirect binding

- No custom bead conjugation required
- Save expensive primary Ab
- Antibodies aligned optimally
- **Additional equilibrium introduced**
- **Needs high affinity primary Ab + beads with selective secondary Ab or proteins A/G/L**

Antibody conjugation to "raw" Acceptor beads

Reductive amination:



- Monoclonal or purified polyclonal antibodies will perform best; **avoid anti-sera**
- Antibody concentration must be at least **1 mg/ml**
- **Antibody must not be in any amine-based buffer,** including TRIS, glycine, bicine, tricine, etc… Perform a buffer exchange in PBS pH 7.4
- Antibody solution **must not contain any protein (such BSA or gelatin) and glycerol**

Antibody assesment and pre-treatment (if needed)

↓

Wash raw beads
20 min.

↓

Assemble conjugation reaction in a vial
10 min.

↓

Overnight incubation
(18-24 h 37°C)

↓

Carboxymethoxylamine (CMO) blocking step
60 min. 37°C

↓

Washing steps
60 min.

Acceptor beads are preferred for conjugation

Many types of derivatized beads

Donor beads:

- Streptavidin
- Nickel chelate (His-tag)
- GSH
- Protein A
- Anti-FLAG
- Anti-DIG
- Anti-mouse IgG
- Anti-Rabbit IgG
- *Strep*-Tactin
- LCA (Lens Culinaris Agglutinin)

Acceptor beads:

Antibody capture

- Protein A
- Protein G
- Protein L
- Anti-human IgG1
- Anti-human IgG4
- Anti-human IgG
- Anti-rabbit IgG
- Anti-mouse IgG
- Anti-rat IgG
- Anti-goat IgG
- Anti-Mouse IgM
- Anti-Chicken IgY
- Anti-Sheep IgG
- Anti-bovine IgGA, IgG, IgG1, IgG2, IgGM

Anti-mouse:

- IgE
- IgM
- IgG1
- IgG2a
- IgG2b
- IgG3

(isotyping)

Fusion Tag detection

- Nickel chelate (His-tags)
- Anti-His
- Glutathione (GSH)
- Anti-GST
- Anti-c-myc
- Anti-FLAG
- Anti-DIG
- Anti- HA
- Anti-FITC
- Anti-V5
- Anti-GFP
- Anti-MBP
- LCA
- *Strep*-Tactin
- Streptavidin

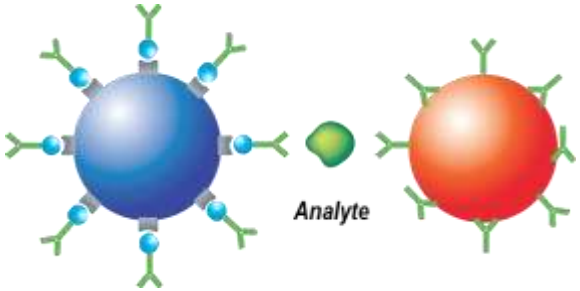
Streptavidin donor beads are generally preferred

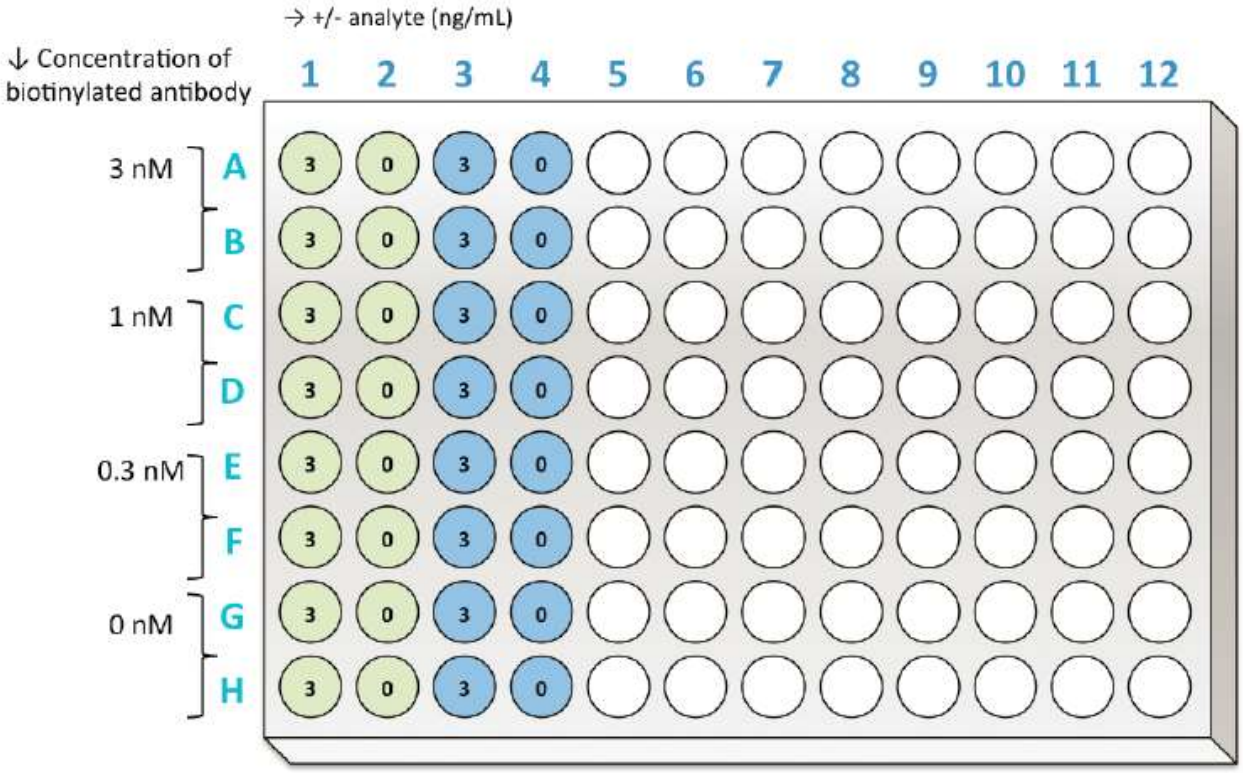
Bead choice: Protein A /G /L

- Protein A interacts strongly with:
  - Human IgG1, IgG2 and IgG4 and total IgG
  - Mouse IgG2A and IgG2B and total IgG
  - Rabbit total IgG
- Protein G binds to all subclasses of human and mouse IgG and to rat, goat, sheep, guinea pig, rabbit, cow, pig and horse antibodies
- Protein L binds to:
  - Total human IgG, IgM, IgA, IgE, IgD
  - Mouse IgG
  - Rat IgG
  - Binds poorly to mouse IgM and rabbit IgM
  - Does not bind to rabbit, sheep, goat and bovine IgG and IgM

Titration of biotinylated antibody

The conc. of biotinylated antibody must be titrated to avoid Hook effect

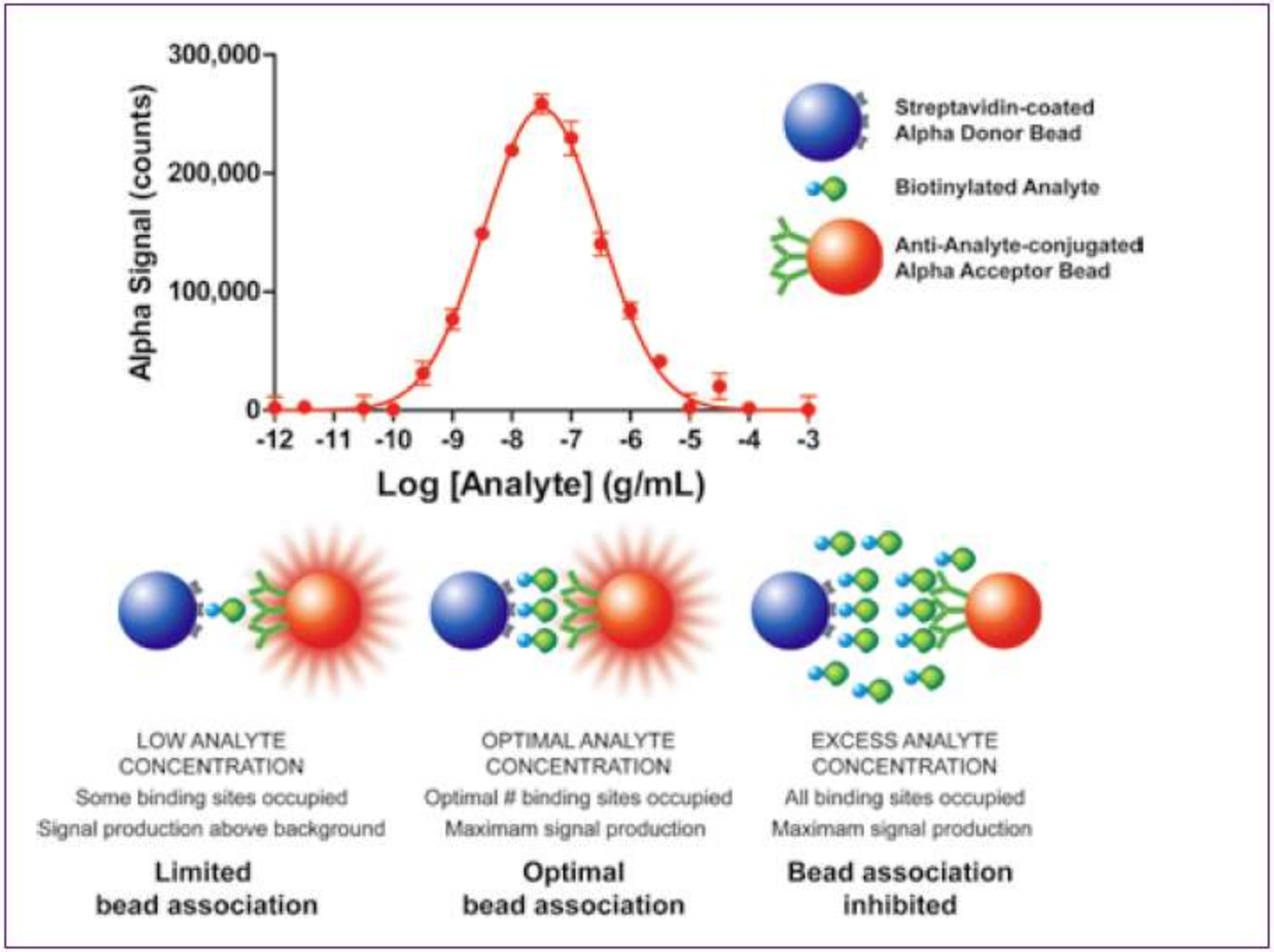




Biotinylated antibody A + antibody B-conjugated AlphaLISA Acceptor beads

Biotinylated antibody B + antibody A-conjugated AlphaLISA Acceptor beads

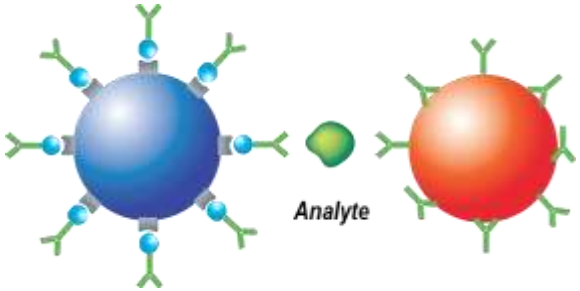
Hook effect in homogeneous assays

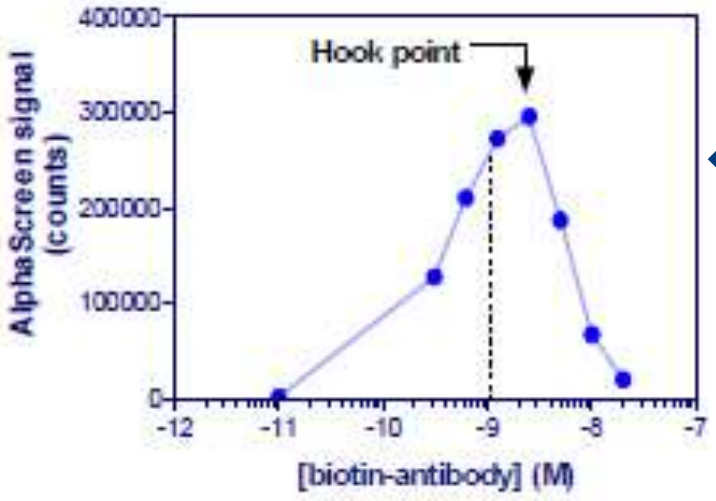


High analyte concentration can saturate the signal: dilute samples and find optimal antibody concentration!

Titration of biotinylated antibody

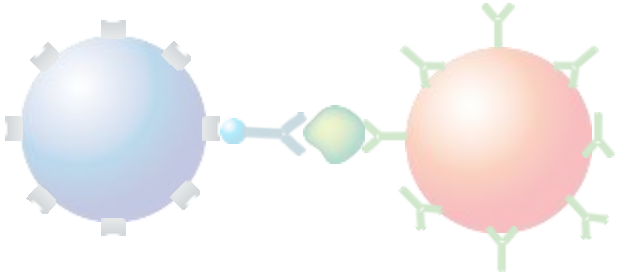
The conc. of biotinylated antibody must be titrated to avoid Hook effect



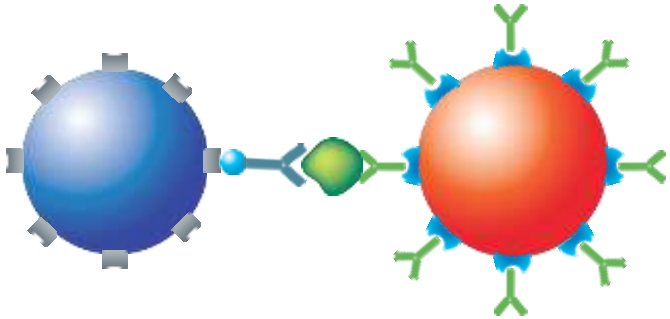


Choose antibody conc. giving strong Alpha signal, without Hook effect

Alpha immunoassay formats: conjugation vs. indirect binding



primary Ab



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Indirect binding

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Can I use streptavidin acceptor beads, in the example above?

Check beads compatibility (antibody cross-reactivity):

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ACCEPTOR BEADS	DONOR BEADS: Streptavidin (6760002)	Anti-FLAG (AS103)	Anti-Mouse (AS104)	Anti-Rabbit (AS105)	Streptactin (AS106)	Ni chelate (AS101)	Glutathione (6765300)	Protein A (AS102)	Anti-DNP (AS111)	Anti-HRP (AS109)	Anti-Rat (AS110)	Anti-DIG (AS108)	Anti-GFP (AS112)
Streptavidin (AL125)													
Protein L (AL126)													
Anti-FITC (AL127)													
Anti-His (AL128)													
Anti-V5 (AL129)													
Anti-Mouse IgM (AL130)													
Anti-Chicken IgY (AL131)													
Anti-Sheep IgG (AL132)													
Anti-GFP (AL133)													
Anti-MBP (AL134)													
Streptactin (Al136)													
Protein A (AL101)													
Protein G (AL102)													
Anti-Human IgG (AL103)													
Anti-Rabbit IgG (AL104)													
Anti-Mouse IgG (AL105)													
Anti-Rat IgG (AL106)													
Anti-Goat IgG (AL107)													
Ni chelate (AL108)													
Glutathione (AL109)													
Anti-GST (AL110)													
Anti-cMyc (AL111)													
Anti-FLAG (AL112)													
Anti-Digoxigenin (AL113)													
Anti-DNP (AL173)													
Anti-HRP (AL171)													
Anti-Protein C tag (AL172)													
Anti-HA (AL170)													
Anti-Mouse IgG (AL164)													
Anti-Human IgG1 (AL153)													
Anti-Human IgG2 (AL154)													
Anti-Human IgG4 (AL156)													
Anti-Mouse IgE (AL161)													
Anti-Mouse IgG1 (AL157)													
Anti-Mouse IgG2a (AL158)													
Anti-Mouse IgG2b (AL159)													
Anti-Mouse IgG3 (AL160)													
Anti-Mouse IgM (AL162)													
LCA (AL140)													

Add 10 µL Antibody #1 (0, 0.3 nM, 1 nM, and 3 nM final concentrations in 50 µL reaction)

⇩

Add 10 µL Antibody #2 (0, 0.3 nM, 1 nM, and 3 nM final concentrations in 50 µL reaction)

⇩

Add 5 µL analyte (5 ng/mL final concentration in 50 µL reaction, or 1X AlphaLISA ImmunoAssay Buffer alone – refer to plate map)

⇩

Incubate at 23°C for 60 minutes

⇩

Add 12.5 µL of AlphaLISA Acceptor beads (10 µg/mL final concentration in 50 µL reaction)

⇩

Incubate at 23°C for 60 minutes

⇩

Add 12.5 µL Alpha Donor beads (final concentration 40 µg/mL in 50 µL reaction)

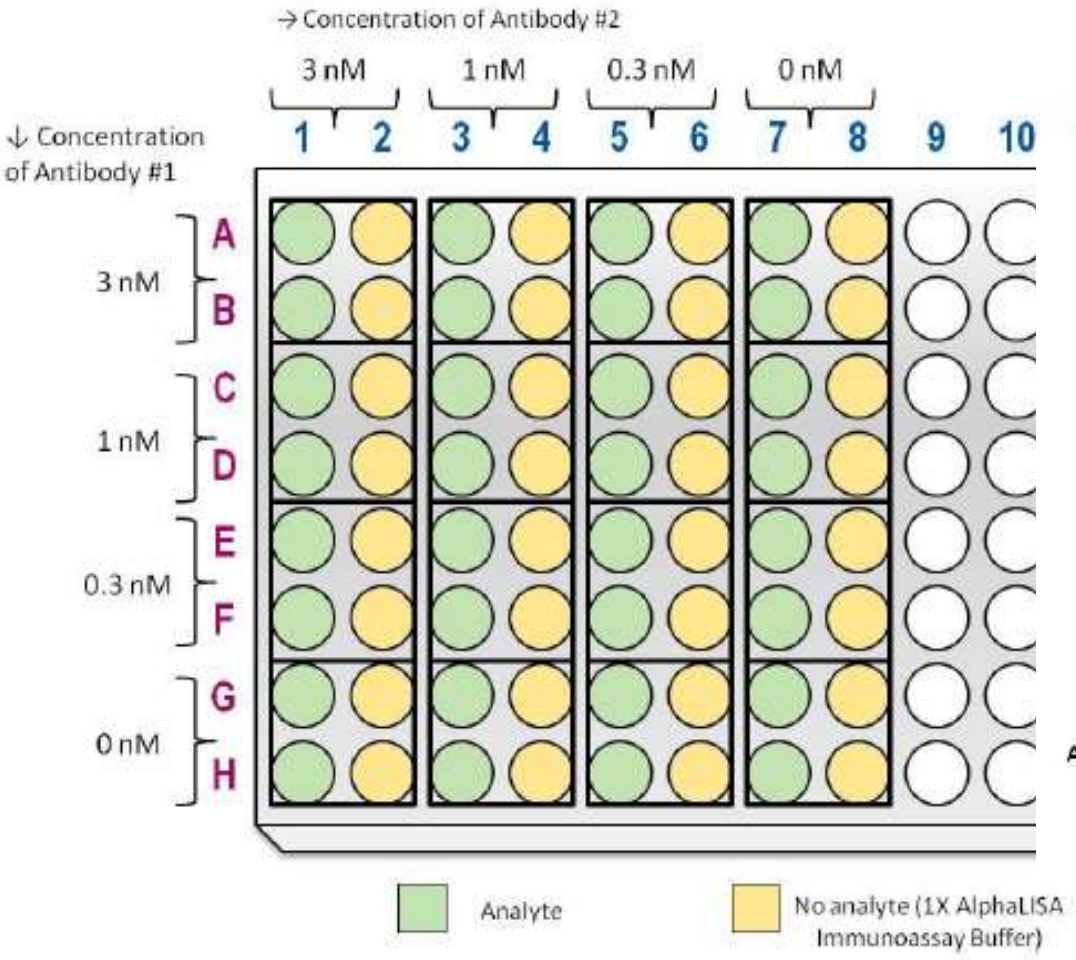
⇩

Incubate in dark at 23°C for 60 minutes

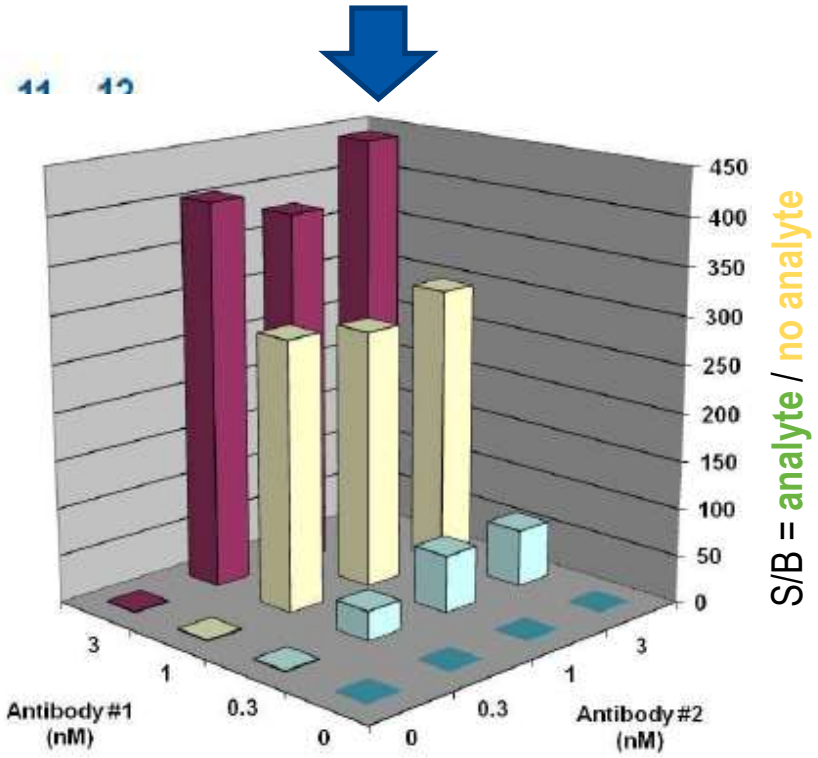
⇩

Read

Plate map for antibodies cross-titration



Choose antibodies conc. giving strong Alpha signal, without Hook effect



More optimizations

- Bead concentration: 10 - 40 μg/ml, keeping the ratio antibody/bead constant
- Incubation time: 30' – overnight
- Assay volume and sample volume
- Assay buffers
  - Tris, HEPES, PBS at different pH values
  - Mild detergents like Tween-20, CHAPS and Triton X-100 (0.01% - 1%)
  - Protein blockers, such as casein or BSA, at a concentration between 0.01 to 1%
  - Dextran 500: for some serum or plasma samples, it is important to add Dextran 500 at 1 mg/mL in order to prevent non-specific bead aggregation

More optimizations (2): order of addition