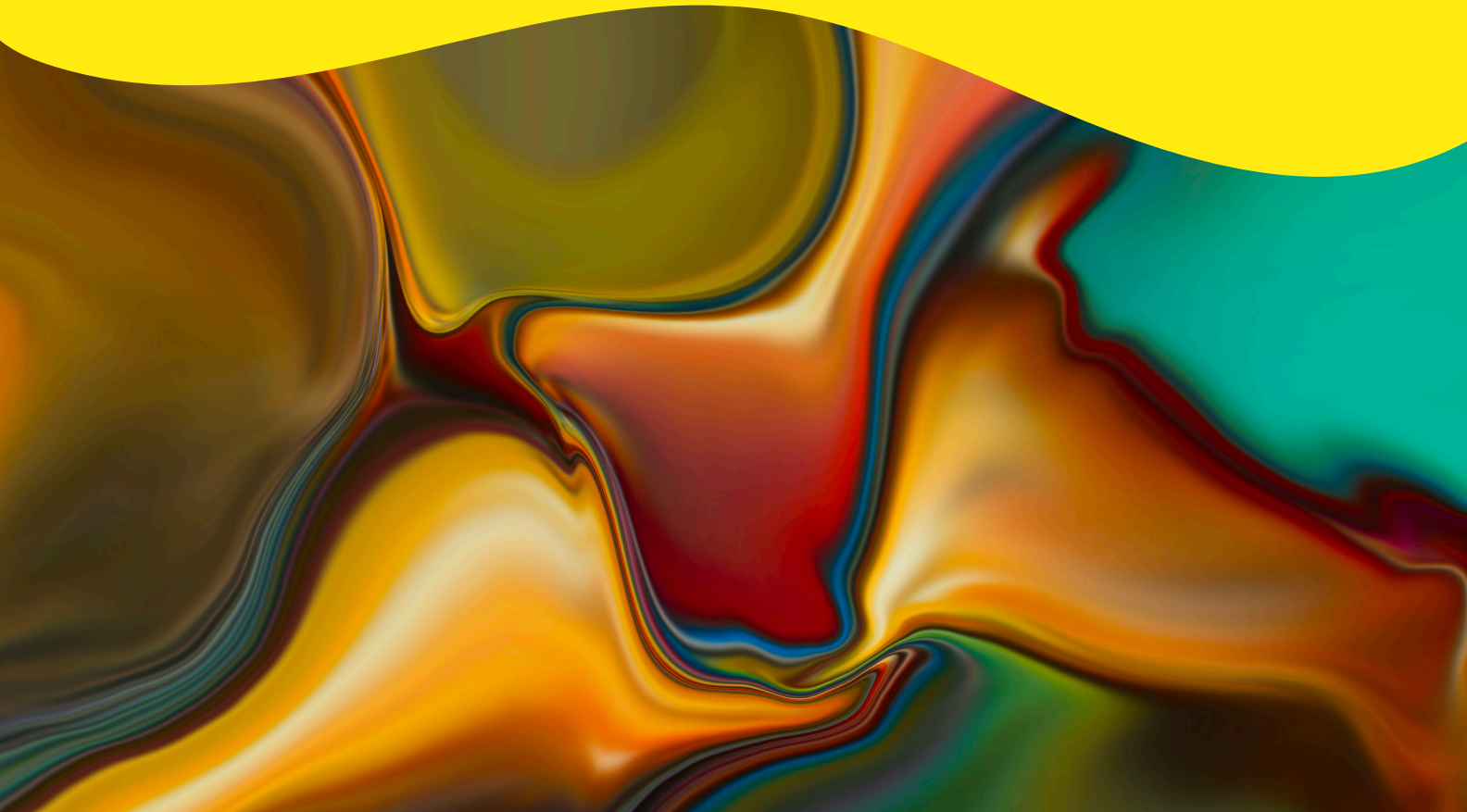
AlphaLISA immunogenicity assay development guide



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I. Intended use

The AlphaLISA™ technology is widely used to measure any biological events, such as biomarkers detection, protein-protein interactions, intracellular target detection and immunogenicity testing (referred in this guide).

II. Introduction and assay principle

Biological drug products often elicit an immune response in patients. Clinical consequences of the presence of Anti-Drug Antibodies (ADA) in serum can vary from mild to serious adverse events. Therefore, the presence of ADAs is a major safety and efficacy concern and should be evaluated and correlated with any pharmacological or toxicological observations. The development of rapid and sensitive assay platforms for the detection of anti-drug antibodies is an essential step of the drug development process.

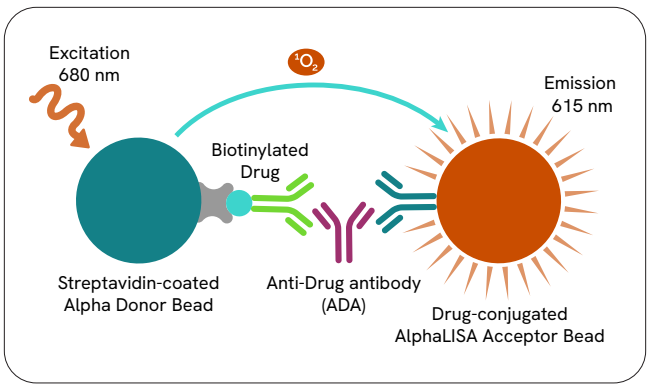


Figure 1: AlphaLISA ADA Detection Assay Configuration. *The drug is represented as an antibody in this picture, but could be any proteins.*

The AlphaLISA technology is a bead-based luminescent proximity assay technology, where specific signals are produced as a result of the close proximity of the Donor and Acceptor beads: laser excitation of the Donor beads provokes the release of singlet oxygen molecules that trigger a cascade of energy transfer in surrounding Acceptor beads, resulting in a time-delayed sharp peak of light emission at 615 nm.

AlphaLISA ADA detection assays are developed using a **bridging assay configuration**, benefiting from bivalent binding of anti-drug antibodies to biotinylated drug molecules captured by the Streptavidin (SA)-Donor beads and drug molecules immobilized on the Acceptor beads (an alternative assay configuration involving drug-conjugated Donor beads is presented in Section VI). Anti-drug antibodies present in serum will bridge the two drug molecules and, consequently, bring the two beads in close proximity. If free drug molecules are present in serum samples, an acid dissociation step is performed to dissociate the drug-ADA complex and improve the drug tolerance of the assay.

For more details about the AlphaLISA technology, please refer to the guide entitled *'A Practical Guide to Working with AlphaScreen'*.

This guide provides basic principles for AlphaLISA ADA detection assay development. General guidelines for the development and validation of ADA assays are described in the publications listed in the References section (Section VIII).

A few simple steps are needed to develop an AlphaLISA ADA bridging assay. The generic protocols presented in this guide are the starting point for successful development of AlphaLISA ADA assays. It is also important to note that some assays may require modified protocols based on the specific nature of the drug under investigation.

III. Materials

A. Alpha beads

Unconjugated Acceptor beads			
Component	**1 mg 6772001**	**5 mg 6772002**	**50 mg 6772003**
AlphaLISA unconjugated Acceptor beads stored in 0.1M Tris-HCl pH 8.0 containing 0.05% Proclin-300	50 µL (20 mg/mL)	250 µL (20 mg/mL)	5 x 500 µL (20 mg/mL)
Streptavidin-coated Donor beads			
Component	**1 mg 6760002S**	**5 mg 6760002**	**50 mg 6760002B**
Streptavidin-coated Donor beads stored in 25 mM Hepes pH 7.4, 100 mM NaCl, 0.05% Proclin-300	200 µL (5 mg/mL)	1 mL (5 mg/mL)	2 x 5 mL (5 mg/mL)
Unconjugated Donor beads (required for alternative assay configurations, see Section VI for details)			
Component	**1 mg 6762013**	**5 mg 6762011**	**50 mg 6762012**
Unconjugated Donor beads stored in 0.1M MES pH 6.0, 0.05% Proclin-300	50 µL (20 mg/mL)	5 x 50 µL (20 mg/mL)	5 x 500 µL (20 mg/mL)

**Please inquire for bulk format.*

Upon receiving the Alpha beads, ensure that they are on blue ice and that the ice packs are not completely thawed.

Notes before use

- For maximum recovery of contents, briefly spin the vials prior to removing the caps and resuspend the beads by pipetting up and down with a pipetman.
- The AlphaLISA Acceptor beads stock solution should be stored at +2 to +8 °C. Note that the beads may slightly sediment over several days. Therefore, it is advised to resuspend the beads solution by up and down pipetting prior to use.
- Alpha streptavidin-coated Donor beads (also referred in this guide as streptavidin Donor beads or SA-Donor beads) are light-sensitive. All assays using the streptavidin Donor beads should be performed under subdued laboratory lighting of less than 100 lux. Alternatively, green filters (e.g., Lee filters #090 or equivalent) can be applied to light fixtures. Any incubation of streptavidin Donor beads should be performed in the dark. Therefore, it is recommended to cover the plates during incubations, with a transparent TopSeal™ and an empty plate or an aluminum foil, a black TopSeal or a black plate lid.

B. Recommended additional reagents and materials

Item	Suggested source	Catalog number
Biotherapeutics molecule (drug)	to be supplied by the user	N/A
ADA positive control (ADA PC)	to be supplied by the user	N/A
ChromaLink™ Biotin (DMF Soluble)	SoluLink Inc.	B1001-105
Carboxymethylamine hemihydrochloride (CMO)	Sigma-Aldrich Co.	C13408
Sodium cyanoborohydride powder	Sigma-Aldrich Co.	152159
DMF	Sigma-Aldrich Co.	270547
Zeba™ desalting columns, 7K MWCO	Pierce (Thermo-Fisher Scientific Inc.)	89882 (0.5 mL) 89889 (2 mL) 89891 (5 mL) 89893 (10 mL)
Proclin™-300 (or Kathon® CG/ICP II)	Sigma-Aldrich Co.	48912-U (or 48178-U)
Tween®-20 detergent solution (Surfact-Amps 20)	Pierce (ThermoFisher Scientific Inc.)	28320
Dextran 500 – from Leuconostoc spp (MW ~500,000)	Sigma-Aldrich Co.	31392
Bovine γ globulin (from bovine blood)	Sigma-Aldrich Co.	G5009
Albumin from bovine serum, lyophilized powder, ≥98% (agarose gel electrophoresis)	Sigma-Aldrich Co.	A7030
Glycine	Sigma-Aldrich Co.	G2879
Sodium phosphate dibasic heptahydrate	Thermo-Fisher Scientific Inc.	S373-500
TRIS, Ultra Pure, 99.9% pure	MP Biomedicals	819638
PBS 1X pH 7.4	Invitrogen (Life Technologies)	10010
Microplates	Revvity, Inc.	see Section C
TopSeal™-A Self-Adhesive Sealing Film	Revvity, Inc.	6050195 (pack size of 100 pieces)
Aluminum foil to cover the plate during incubations	N/A	N/A
Single-channel pipettors*	N/A	N/A
Microcentrifuge	Eppendorf	5415R
Probe sonicator	Thermo-Fisher Scientific Inc.	F60 or Model 50 Sonic Dismembrator

** For lower volume additions (< 10 µL), we recommend a pipettor precision of ≤ 2%. For higher volume additions (25-1000 µL), a pipettor precision of ≤ 1% is recommended.*

C. Recommended microplates

Assay format	Microplate	Cat #*	Well volume	Recommended working volume
96	**½ AreaPlate-96™, white**	**6005560**	**180 µL**	**40-160 µL**
384	OptiPlate™-384, white	6007290	105 µL	24-90 µL
	AlphaPlate™-384, light gray	6005350	105 µL	24-90 µL
	ProxiPlate™-384	6008280	28 µL	10-20 µL

** For 50 plates; refer to Revvity, Inc. website for other quantities.*

The assay protocols included in this manual are for ½ AreaPlate-96 microplates.

Other plate types listed above may be used with the appropriate assay volumes.

D. Instruments

Assays must be read using an Alpha-compatible reader such as all EnVision™ Multilabel Plate Readers with an Alpha module or the EnSpire™ Multimode Plate Reader with Alpha technology. The normal Alpha settings should be used.

IV. Choosing and labeling reagents

A. Requirements for the drug molecule

1. Drug concentration

The drug concentration should be at least 0.5 mg/mL.

If required, antibody solutions can be concentrated using an iCON™ Concentrator (Thermo Fisher Scientific Inc.) or the Amicon® Ultra-0.5 Centrifugal Filter Unit with Ultracel-100 membrane (UFC510008) according to the manufacturer's instructions (or equivalent device).

To accurately determine the concentration of the drug, please refer to the following method:

- Measure the OD of the antibody solution at 450 nm and 280 nm on a spectrophotometer (scan)
- Use the 1X PBS, pH 7.4 buffer to prepare the blank and dilutions, if necessary
- The OD value at 280 nm must be within the linear limits of the device used (e.g. 0.200 and 1.800 on the spectrophotometer). Repeat dilutions as needed
- An A_{450} reading ≥ 0.1 possibly indicates protein aggregation or the presence of a precipitate in the antibody solution which can be removed by centrifugation. If necessary, centrifuge at 16,000 x g for 10 minutes at 4 °C. Transfer the supernatant and measure OD at 280 nm.
- Formula to calculate the concentration of an antibody:
  (OD280 x dilution factor) ÷ 1.34 mg/mL* = Antibody concentration (mg/mL)
  (considering that 1 unit OD280 = 0.75 mg/mL protein)
  (*The 1.34 mg/mL concentration comes from the following calculation:
  Ext. coeff. / molar mass of the Antibody = 214,400 M^{-1}/160,000 g/mol = 1.34 mg/mL)

2. Antibody formulation

For the development of an ADA bridging assay, the drug molecule needs to be labelled with biotin and conjugated to the Acceptor beads. These modifications occur through primary amine reactions. Therefore, any drug molecule that has at least one available primary amine could be used. To ensure successful modification, all drug molecules must have the following characteristics:

- The drug molecule sequence should contain free amine groups.
- The drugs must *not* be in any amine-based buffer (including Tris, glycine, bicine, tricine), sodium azide, borate, mercaptoethanol, glycerol. Glycerol will significantly impact bead coupling efficiency. For that reason, extensive dialysis of the antibody is recommended prior to coupling if the drug is stored in glycerol. Alternatively, sequential purification on two Zeba™ Desalt Spin Columns can be used.
- The drug should be soluble in slightly alkaline (pH 7-8) aqueous buffer (labeling conditions). If buffer exchange is necessary, the buffer should be replaced by a neutral to slightly alkaline buffer, such as PBS. Buffer exchange can be accomplished using Zeba™ Desalt Spin Columns following the recommended procedure from the manufacturer (www.piercenet.com) or alternatively appropriate Slide-A-Lyzer cassettes.
- Ideally, drug antibody/protein solutions should not contain any protein or peptide-based stabilizers (such as BSA or gelatin). It has been shown that BSA concentrations up to 0.1% in the antibody solution do not impact bead coupling. In the presence of higher concentrations of BSA, the conjugation process might result in lower coupling efficiency in some cases, and should be avoided if possible.
- If removal of protein stabilizers is necessary, we suggest the use of PhyTip® affinity columns (www.phynexus.com/technology/affinity-columns) on a liquid-handling system, such as the JANUS® Automated Workstation from Revvity Inc.

B. Drug Biotinylation

1. **Prepare a 10 mg/mL (12.3 mM) solution of ChromaLink™ Biotin in DMF:**

   a) Add 100 µL DMF to the ChromaLink™ Biotin (1 mg) to prepare a 10 mg/mL solution

   b) This solution can be stored at -20 ˚C with desiccant for up to 2 months, but needs to be warmed up to room temperature before opening the vial.

2. **Prepare a 1/5 dilution of ChromaLink™ Biotin in PBS 1X pH 7.4 (2 mg/mL or 2.46 mM final):**

   a) In an Eppendorf tube, mix 400 µL PBS buffer with 100 µL ChromaLink™ Biotin (10 mg/mL) (or as required).

3. **Prepare the biotinylation reaction. A 30:1 molar ratio of biotin to drug is recommended for drug antibodies. Lower molar ratios (10:1, 20:1) could be tested if the drug has a lower molecular weight than antibodies.**

   a) In an Eppendorf tube, mix ChromaLink™ Biotin with the antibody to be biotinylated in a 30:1 ratio. Complete the reaction volume with PBS pH 7.4 to a final volume of 200 µL per 0.1 mg of antibody:

      1. Determine the amount / volume of drug antibody to be labeled:
         m_{Ab} = 0.1 mg c_{Ab} = 1.06 mg/mL
         $V_{Ab} = (m_{Ab} \div c_{Ab}) \times 1000 = (0.1 \text{ mg} \div 1.06 \text{ mg/mL}) \times 1000 = 94.3 \text{ µL}$
      2. Determine the total reaction volume:
         $V_{total} = m_{Ab} \times (2000 \text{ µL} / 1 \text{ mg}) = 0.1 \text{ mg} \times (2000 \text{ µL} / 1 \text{ mg}) = 200 \text{ µL}$
      3. Determine the volume of ChromaLink™ Biotin solution (at 2 mg/mL) to be added:
         $V_{Biotin} = V_{total} \times (7.62 \text{ µL} / 200 \text{ µL}) = 200 \text{ µL} \times (7.62 \text{ µL} / 200 \text{ µL}) = 7.62 \text{ µL}$
      4. Determine the volume of PBS buffer to be added:
         $V_{PBS} = V_{total} - V_{Ab} - V_{Biotin} = 200 \text{ µL} - 94.3 \text{ µL} - 7.62 \text{ µL} = 98 \text{ µL}$

5. Prepare the reaction mix in an Eppendorf tube in the order indicated in the following table:

		Volume (µL)	Volume (µL)
1	1 mg/mL (6.25 µM) antibody (V_{Ab})	100.0 (0.1 mg = 0.625 nmol)	94.3
2	PBS pH 7.4 (V_{PBS})	92.4	98
3	2 mg/mL (2.46 mM) ChromaLink Biotin (V_{Biotin})	7.6 (18.7 nmol)	7.6
	Total Volume (V_{total})	**200.00**	**200.00**

4. **Incubate for 2 hours in an incubator set to 21-23 °C.**

5. **Purify with a Zeba™ Desalt Spin Column (buffer exchange).**

6. **Remove the column's bottom closure and loosen cap (do not remove the cap). Place column in an appropriate collection tube.**

7. **Centrifuge column to remove storage solution. Always use the smallest column possible for the sample volume.**

Column size	Sample volume	Maximal volume	Collection tube	Centrifugation
0.5 mL	30-130 µL	260 µL	1.5-2 mL microtube	1,500 × g, 1 minute
2 x 0.5 mL	130-260 µL	52 µL	1.5-2 mL microtube	1,500 × g, 1 minute
2 mL	260-700 µL	1400 µL	15 mL conical tube	1,000 × g, 2 minutes
5 mL	500-2,000 µL	4000 µL	15 mL conical tube	1,000 × g, 2 minutes
10 mL	700-4,000 µL	no maximum	50 mL conical tube	1,000 × g, 2 minutes

8. **Place a mark on the side of the column where the compacted resin is slanted upward when using fixed-angle rotors. Place column in centrifuge with the mark facing outward in all subsequent centrifugation steps.**

(Note: resin will appear compacted after centrifugation.)

Approximate speed for different centrifuges:				
Centrifuge	**1,000 × *g***	**1,500 × *g***	**2,000 × *g***	**6,000 × *g***
Microcentrifuge (e.g. Biofuge® fresco, Sorvall® MC12V)	3,500-4,000 rpm	4,000-4,500 rpm	5,000-5,500 rpm	8,000-9,000 rpm
Beckman® GS-6R (G-3.8 rotor)	2,000 rpm	2,500 rpm	3,000 rpm	-

9. **Add PBS 1X pH 7.4 buffer to the column.**

Column size	Buffer volume
0.5 mL	300 µL
2 mL	1 mL
5 mL	2.5 mL
10 mL	5 mL

10. Centrifuge to remove buffer.

Column size	Centrifugation
0.5 mL	1,500 × g, 1 minute
2-10 mL	1,000 × g, 2 minutes

11. Repeat steps 9 and 10 four additional times, discarding buffer from the collection tube.

12. Place column in a new collection tube, remove cap and slowly apply sample to the center of the compact resin bed (refer to table below). To ensure maximal protein recovery from low-volume samples, apply a stacker of buffer to the resin bed after the sample has fully absorbed. The stacker is only applied if the sample volume is lower than indicated in the following table:

Column size	Sample volume	Stacker	
		For sample volume	Add buffer volume
0.5 mL	30-130 µL	< 70 µL	15 µL
2 mL	200-700 µL	< 350 µL	40 µL
5 mL	500-2,000 µL	< 750 µL	100 µL
10 mL	700-4,000 µL	< 1,500 µL	200 µL

13. Centrifuge to collect the sample and determine the volume of the biotinylated antibody (Ab-biotin).

Column size	Centrifugation
0.5 mL	1,500 × g, 2 minutes
2-10 mL	1,000 × g, 2 minutes

14. Discard column after use.

15. Determine the biotinylated antibody concentration and biotin/antibody labeling ratio:

a) Take the A_{280}, A_{354} and A_{450} readings with a spectrophotometer (using PBS as blank):
A_{280} = _____ A_{354} = _____ A_{450} = _____

b) Antibody concentration (c_{Ab} [mg/mL] and C_{Ab} [mM]):
$C_{Ab\text{-}biotin} = (A_{280} - (A_{354} \times 0.23)) \div 1.34 = (\_\_\_\_\_ - (\_\_\_\_\_ \times 0.23)) \div 1.34 = \_\_\_\_\_$ [mg/mL]
$C_{Ab\text{-}biotin} = (c_{Ab\text{-}biotin} \div 160{,}000) \times 10e6 = (\_\_\_\_\_ \div 160{,}000) \times 1 \times 10^6 = \_\_\_\_\_$ [µM]

c) Biotin concentration: $C_{Biotin} = (A_{354} \div 29) \times 1 \times 10^3 = (\_\_\_\_\_ \div 29) \times 1 \times 10^3 = \_\_\_\_\_$ [µM]

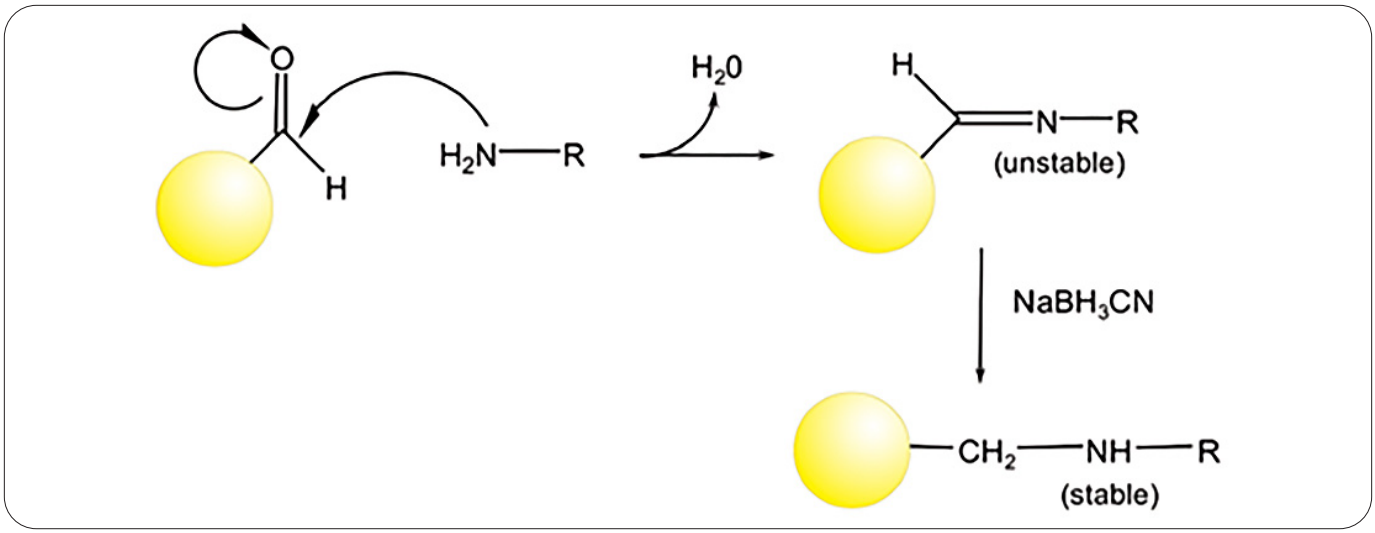
d) Molar substitution ratio: $MSR = C_{Biotin} / C_{Ab\text{-}biotin} = \_\_\_\_\_ / \_\_\_\_\_ = \_\_\_\_\_$ biotin/Ab

e) Antibody recovery: $R_{Ab} = (c_{Ab\text{-}biotin} \times V_{Ab\text{-}biotin} \times 100) \div (c_{Ab} \times V_{Ab})$
$= (\_\_\_\_\_ \times \_\_\_\_\_ \times 100) \div (\_\_\_\_\_ \times \_\_\_\_\_) = \_\_\_\_\_$ [%]

Note: you can refer to the SoluLink™ website (www.solulink.com) for calculations, and use the ChromaLink™ Biotin MSR Calculator that can be downloaded from the site (www.solulink.com/library#calcs).

C. Alphalisa bead conjugation with the drug

1. Bead conjugation chemistry: reductive amination



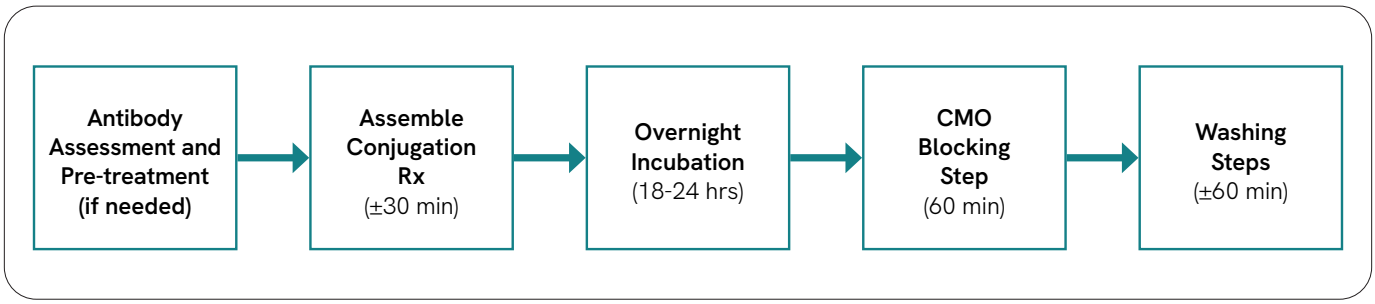
2. Preliminary notes

The ratio of antibody to mg of beads is an important parameter for successful assay development. Typical coupling weight ratios (amount of beads to amount of antibody) are either 10:1 or 50:1.

When preparing low amounts of beads (1-2 mg), a 10:1 ratio is recommended (i.e. 1 mg of Acceptor beads to 0.1 mg of antibody), while a ratio of 50:1 is used with bead amounts equal to or higher than 2.5 mg to minimize the antibody consumption (i.e. 5 mg of Acceptor beads to 0.1 mg of antibody).

Please note that the 50:1 protocol was also successfully used with 1 mg of beads, although smaller vials (0.5 mL Eppendorf tubes) are recommended due to the low volumes involved.

3. Procedure outline



4. Protocol for conjugating 1 mg AlphaLISA Acceptor beads (10:1 coupling ratio)

Bead washing

In a 1.5 mL Eppendorf tube, wash AlphaLISA Acceptor beads (50 µL at 20 mg/mL) once: add 50 µL PBS, centrifuge at 16,000 × g or maximum speed for 15 min and then discard the supernatant using a pipette tip. (Do not tilt the tube to prevent the pellet from coming off).

Conjugation

Prepare a fresh working $NaBH_3CN$ solution at 400 mM in water using $NaBH_3CN$ powder (25 mg in 1 mL H_2O).

In the Eppendorf tube containing 1 mg of AlphaLISA Acceptor bead pellet (prepared as described above), the following reagents are added in order to a final volume of 200 µL:

- 0.1 mg of antibody
- The appropriate volume of 100 mM Hepes, pH 7.4 to obtain a final reaction volume of 200 µL
- 1.25 µL of 10% Tween®-20
- 10 µL of a 400 mM solution of $NaBH^3CN$ in water
- Incubate for 18-24 hours at 37 ˚C using a rotary shaker (6-10 rpm).

Blocking

The blocking step is necessary to block unreacted aldehyde sites.

- Prepare a fresh 65 mg/mL solution of carboxymethoxylamine (CMO) in 800 mM NaOH.
- Add 10 µL of CMO solution to the reaction (to block unreacted sites).
- Incubate for 1 hr at 37 ˚C using a rotary shaker (6-10 rpm).

Washing

- Centrifuge for 15 min at 16,000 × g (or maximum speed) at 4 ˚C.
- Remove the supernatant with a micropipette and resuspend the bead pellet in 1 mL of PBS (200 µL per mg of beads).
- Briefly sonicate the bead solution (10 short pulses of 1 sec using a probe sonicator to ensure that the beads are not aggregated; the sonicator power should not exceed 20% of the maximal power).
- Centrifuge for 15 min at 16,000 × g (or maximum speed) at 4 ˚C, and then remove the supernatant.
- Repeat the washing steps (resuspend the pellet, sonicate and centrifuge) another time.
- After the last centrifugation, resuspend the beads at 5 mg/mL in storage buffer (200 µL of PBS + 0.05% Proclin®-300 as a preservative).
- Vortex, briefly spin down and sonicate the bead solution (10 short pulses of 1 sec using a probe sonicator to ensure that the beads are not aggregated; the sonicator power should not exceed 20% of the maximal power).

  The sonication steps are recommended but not mandatory. Note that a bath sonicator is not efficient and should not be used.

Storage

- Store the conjugated Acceptor bead solution at 4 ˚C in an opaque vial.
- *Important note*: always vortex conjugated AlphaLISA Acceptor beads before use, as beads tend to settle with time.

5. Protocol for conjugating 5 mg AlphaLISA Acceptor beads (50:1 coupling ratio)

Bead washing

In a 1.5 mL Eppendorf tube, wash AlphaLISA Acceptor beads (250 µL at 20 mg/mL) once: add 250 µL PBS, centrifuge at 16,000 × g or maximum speed for 15 min and then discard the supernatant using a pipette tip. (Do not tilt the tube to prevent the pellet from coming off).

Conjugation

Prepare a fresh working $NaBH_3CN$ solution at 400 mM in water using $NaBH_3CN$ powder (25 mg in 1 mL H_2O).

In the Eppendorf tube containing 5 mg of AlphaLISA Acceptor bead pellet (prepared as described above), the following reagents are added in order to a final volume of 200 µL:

- 0.1 mg of antibody
- The appropriate volume of 100 mM Hepes, pH 7.4 to obtain a final reaction volume of 200 µL
- 1.25 µL of 10% Tween®-20
- 10 µL of a 400 mM solution of $NaBH^3CN$ in water
- Incubate for 18-24 hours at 37 ˚C using a rotary shaker (6-10 rpm).

Blocking

The blocking step is necessary to block unreacted aldehyde sites.

- Prepare a fresh 65 mg/mL solution of carboxymethoxylamine (CMO) in 800 mM NaOH.
- Add 10 µL of CMO solution to the reaction (to block unreacted sites).
- Incubate for 1 hour at 37 ˚C using a rotary shaker (6-10 rpm).

Washing

- Centrifuge for 15 min at 16,000 × g (or maximum speed) at 4 ˚C.
- Remove the supernatant with a micropipette and resuspend the bead pellet in 1 mL of PBS (200 µL per mg of beads).
- Briefly sonicate the bead solution (10 short pulses of 1 sec using a probe sonicator to ensure that the beads are not aggregated; the sonicator power should not exceed 20% of the maximal power).
- Centrifuge for 15 min at 16,000 × g (or maximum speed) at 4 ˚C, and then remove the supernatant.
- Repeat the washing steps (resuspend the pellet, sonicate and centrifuge) another time.
- After the last centrifugation, resuspend the beads at 5 mg/mL in storage buffer (1 mL of PBS + 0.05% Proclin®-300 as a preservative).
- Vortex, briefly spin down and sonicate the bead solution (10 short pulses of 1 sec using a probe sonicator to ensure that the beads are not aggregated; the sonicator power should not exceed 20% of the maximal power).

  The sonication steps are recommended but not mandatory. Note that a bath sonicator is not efficient and should not be used.

Storage

- Store the conjugated Acceptor bead solution at 4 ˚C in an opaque vial.
- *Important note:* always vortex conjugated AlphaLISA Acceptor beads before use, as beads tend to settle with time.

6. **Protocol for conjugating large amounts of AlphaLISA Acceptor beads**

   If more than 5 mg of beads are to be conjugated, adapt the 5 mg protocol accordingly. In addition:

   - Use 50 mL 3118 Oak Ridge centrifuge tube from Nalgene® (Thermo Fisher Scientific, Cat. No. 3118-0050) instead of 1.5 mL Eppendorf tubes. The maximum volume in each tube should be ≤ 30 mL to allow proper centrifugation of the beads.
   - Perform all centrifugation steps in a Sorvall® RC-5B centrifuge (or equivalent) at 16,000 rpm for 40 min at 4 °C.

7. **Bead coupling optimization**

   Although the suggested coupling protocols presented in the previous sections work well for most conditions, the bead coupling process can be optimized further with the goal to increase the number of antibodies on beads, by following these recommendations:

   - Reduce the volume of reaction. Coupling efficiency will usually increase with the bead concentration. For instance, the bead concentration could be tested up to 100 mg/ mL (compared to 25 mg/mL in the protocol described in Section 5). Adding less volume of a more concentrated buffer could help to achieve this increased concentration.
   - Increase the antibody/beads ratio. Coupling efficiency will usually increase with the antibody concentration. However, the antibody stock solution should be concentrated enough to allow the addition of a minimal volume of antibody while maintaining the bead concentration as high as possible. Therefore, the 10:1 ratio, when performed at 25 mg/mL of beads should improve results. By increasing bead concentration to 75 mg/mL, while maintaining the ratio at 50:1, an antibody concentration of 1.7 mg/mL is required. By increasing simultaneously the ratio to 25:1, twice that concentration of antibody is needed, which lies close to the upper practical limit.
   - Try other buffers, such as 100 mM sodium phosphate, pH 8.0.

D. Positive control antibody characteristics

The positive control anti-drug antibody (ADA PC) should be selected specifically to target the drug molecule. Although it is preferable to use polyclonal antibodies to better mimic patient immunogenicity response, a monoclonal antibody could also be used if desired. The positive control antibody should be divalent and should ideally be in a purified form. Serum could also be used if the antibody titer is high enough (1/100 dilution or higher).

V. Standard procedures

A. Titration of biotinylated drug

One of the first development steps is the determination of the optimal concentration of the biotinylated drug. Indeed, the streptavidin Donor beads have a fixed capacity for capturing the biotinylated drug. Therefore, very low concentrations of biotinylated drug may not be sufficient to give acceptable signals while too high concentrations would lead to signal depletion due to the 'hooking effect' (as a result of saturation of the available binding sites on one or both of the Alpha beads). Usually, the biotinylated drug is titrated from 0.01 nM up to 10 nM final in the assay.

This section describes the method to be used to perform this titration.

A titration assay should be performed using fixed concentrations of the positive control antibody and up to 8 different concentrations of the biotinylated drug. Each concentration is tested in triplicate.

Reagent preparation *(typical preparation for Figure 2 example)*

Recommended assay buffer for ADA assays:

- 50 mM Tris-HCl, pH 7.6
- 150 mM NaCl
- 0.5% BSA
- 0.1% Tween-20
- 0.05% Bovine γ-globulin
- 10 mg/mL Dextran 500
  Adjust pH to 7.6

1. **Positive control antibody**

   Prepare a 1 µg/mL solution of the positive control antibody, as follows:

   - To 0.25 mL of assay buffer, add 2.5 µL of 100 µg/mL positive control antibody.

2. **Drug-conjugated Acceptor beads**

   Prepare a 80 µg/mL Acceptor beads solution to get a 20 µg/mL final con centration in the well, as follows:

   - To 1.5 mL of assay buffer, add 24 µL of the 5 mg/mL drug-conjugated Acceptor beads solution.

3. **Streptavidin Donor beads**

   Prepare a 40 µg/mL solution of SA-Donor beads to get a 20 µg/mL concentration in the well, as follows:

   - To 2.6 mL of assay buffer, add 21 µL of the 5 mg/mL SA-Donor beads solution. Prepare in subdued light conditions as SA-Donor beads are light sensitive.

4. **Biotinylated drug preparation**

   Dilute the biotinylated drug in assay buffer as follows:

Sample	Buffer vol.	Vol. of biotin-drug	Biotin-drug final concentration
1	285 µL	15 µL of stock at 1 µM	10 nM
2	200 µL	100 µL of sample 1	3.33 nM
3	200 µL	100 µL of sample 2	1.11 nM
4	200 µL	100 µL of sample 3	0.37 nM
5	200 µL	100 µL of sample 4	0.123 nM
6	200 µL	100 µL of sample 5	0.041 nM
7	200 µL	100 µL of sample 6	0.013 nM
8	200 µL	—	blank

Assay protocol

The assay is performed in a ½ AreaPlate-96 microplate as follows:

- Add 5 µL of the positive control antibody at 1 µg/mL diluted in assay buffer or 5 µL of assay buffer alone for the negative control
- Add 20 µL of 0.05-50 nM biotinylated drug (0.01-10 nM final concentrations in the well) diluted in assay buffer

  (Note that this addition step is normally used to perform the acid dissociation; however, the biotinylated drug titration experiment does not require any acid treatment)

- Add 25 µL of drug-conjugated AlphaLISA Acceptor beads at 80 µg/mL (20 µg/mL final in the well)
- Add a TopSeal to the plate, cover with an empty plate, and incubate at 23 °C for 60 min
- Add 50 µL of streptavidin Donor beads at 40 µg/mL (20 µg/mL final concentration in the well)
- Add a TopSeal to the plate, cover with an empty plate, and incubate the plate at 23 °C for 60 min
- Read the plate using an Alpha-compatible reader.

Typical results

As seen in Figure 2, the optimal concentration of biotinylated drug is 0.1 nM in this particular case, producing an assay window of 130. However, it is always preferable to use a concentration slightly above the optimal concentration (0.3 nM in this example) if the signal is strong enough. Indeed, higher concentrations of biotinylated drug will help achieve better drug tolerance.

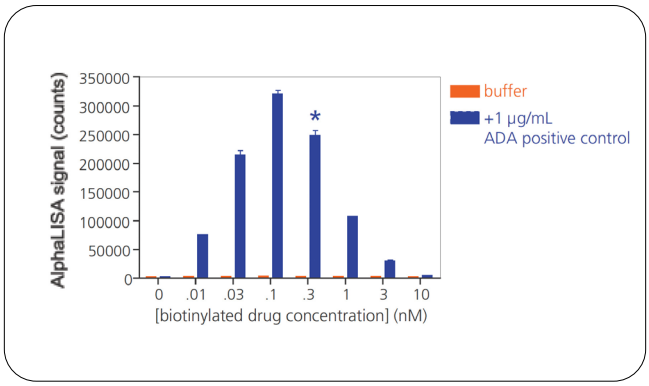


Figure 2: Typical biotinylated drug titration data.

A lack of specific signal at this point could be caused by:

a) Poor bead conjugation

In this case, ensure that free amine groups are available on the drug molecule, that the drug solution does not contain any interfering compounds (BSA, glycerol, amine-based buffer), and make sure to follow the conjugation protocol carefully.

If the drug is an antibody, bead conjugation efficiency could be confirmed by performing an AlphaLISA assay using the antibody-conjugated beads, SA-Donor and biotin-anti-species IgG.

b) Poor drug biotinylation

To fix the issue, follow the same recommendations as above and, in addition, ensure that the biotin reagents contain a spacer such as in the Chromalink-biotin reagents.

c) Positive control antibody not suited for the bridging assay set-up

For this latter case, try using another positive control antibody: polyclonal antibodies may give better performance.

B. Determination of assay sensitivity and testing matrix interference

The Food and Drug Administration (FDA) recommends a minimal sensitivity of 250-500 ng/mL for ADA assays. For determination of assay sensitivity, the positive control antibody is titrated using the optimal concentration of biotinylated drug determined in the previous experiment.

This experiment could be performed in assay buffer or in neat serum to determine at the same time the matrix effect and the possible impact of serum on signal. Our recommendation would be to perform both curves in the same experiment. The sensitivity is usually defined as the lowest level at which a positive sample can be reproducibly detected at or above the cut-point. The cut-point is usually calculated from a subset of drug-naive serum samples as described in References 2, 4, 6 and 7).

Reagent preparation *(typical preparation for Figure 3 example, see Page 17)*

1. **Acid solution: 500 mM Glycine-HCl, pH 3.0**

   Prepare a solution of 500 mM Glycine-HCl from the powder and adjust pH to 3.0 with concentrated HCl (12.1M).

2. **Neutralization buffer**

   The neutralization buffer is prepared by mixing the assay buffer with 1M Tris-HCl pH 9.5 at a 83:17 (vol:vol) ratio.

   For 2.0 mL of neutralization buffer:

   - Add 340 µL of 1M Tris-HCl pH 9.5 to 1660 µL of assay buffer

3. **Mix of biotinylated drug and drug-conjugated Acceptor beads**

   Prepare a 1.2 nM solution of biotinylated drug to get a 0.3 nM final concentration in the well, and a 80 µg/mL drug-conjugated Acceptor beads solution to get a 20 µg/mL final concentration in the well, as follows:

   - To 2.0 mL of neutralization buffer, add 2.4 µL of 1 µM biotinylated drug solution, then add 32 µL of the 5 mg/mL conjugated Acceptor beads.

4. **Streptavidin Donor beads**

   Prepare a 40 µg/mL solution of SA-Donor beads to get a 20 µg/mL concentration in the well, as follows:

   - To 3.5 mL of assay buffer, add 28 µL of the 5 mg/mL SA-Donor beads. Prepare in subdued light conditions as SA-Donor beads are light sensitive.

5. **Standard curve preparation**

   The positive control antibody (ADA PC) is spiked into the assay buffer or in the serum (diluents) as follows:

Sample	Diluent vol.	Vol. of ADA PC	ADA PC final concentration
1	270 µL	30 µL of stock at 100 µg/mL	10 µg/mL
2	200 µL	100 µL of sample 1	3.33 µg/mL
3	200 µL	100 µL of sample 2	1.11 µg/mL
4	200 µL	100 µL of sample 3	0.37 µg/mL
5	200 µL	100 µL of sample 4	0.123 µg/mL
6	200 µL	100 µL of sample 5	0.041 µg/mL
7	200 µL	100 µL of sample 6	0.0137 µg/mL
8	200 µL	100 µL of sample 7	0.00456 µg/mL
9	200 µL	100 µL of sample 8	0.00152 µg/mL
10	200 µL	—	blank

Assay protocol

The assay is performed in a ½ AreaPlate-96 microplate as follows:

- Add 5 µL of the positive control antibody at different dilutions (from 1 ng/mL to 10 µg/mL diluted in assay buffer or in serum).
- Add 20 µL of 500 mM Glycine-HCl, pH 3.0
- Add 25 µL of the mix of drug-conjugated AlphaLISA Acceptor bead s at 80 µg/mL (20 µg/mL final in the wells) and biotinylated drug at the selected concentration prepared in the neutralization buffer.
- Add a TopSeal to the plate, cover with an empty plate, and incubate at 23 ˚C for 60 min
- Add 50 µL of SA-Donor beads at 40 µg/mL (20 µg/mL final concentration in the wells)
- Add a TopSeal to the plate, cover with an empty plate, and incubate a t 23 ˚C for 60 min
- Read the plate using an Alpha-compatible reader.

Typical results

Typical ADA PC dose-response curves are displayed in Figure 3. In this specific case, the signal increased linearly up to the maximal positive control concentration tested (10 µg/mL). The sensitivity of the assay, determined by calculating the sample concentration that is reproducibly at or above the cut-point, was calculated to be 3 ng/mL in neat serum.

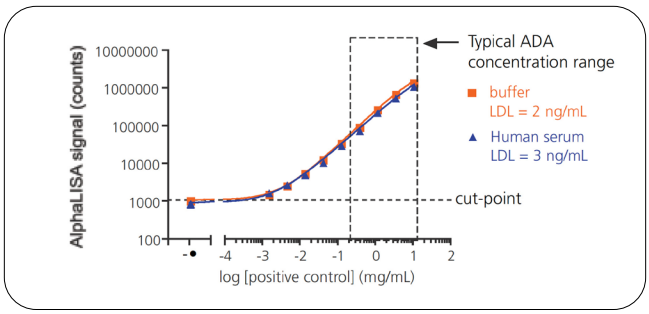


Figure 3: Typical dose-response curves for an ADA detection assay, with ADA dilutions prepared either in assay buffer or in naive human serum.

It is noteworthy that the sensitivity is dependent on the nature of the positive control antibody used for the assay. Low affinity antibodies will likely result in low assay sensitivity. The matrix effect was negligible in this example; however, some serum samples may show higher interference. The interference could be reduced by diluting the sample before the analysis or by reducing the sample volume.

C. Determination of free drug tolerance of the assay

Samples containing circulating drug could generate some assay interference, as the result of the competition between the circulating drug and the labeled drug of the assay system for binding to anti-drug antibodies. Therefore, evaluation of the free drug tolerance is an important step in immunogenicity tests to determine this potential interference.

Assessing drug tolerance is done using neat serum that is spiked with the positive control antibody and increasing concentrations of the unlabeled drug. High levels of unlabeled drug will eventually hamper the ADA detection.

Reagent preparation *(typical preparation for Figure 4 example, see Page 18)*

The samples are usually prepared in neat serum by mixing a 2x concentration of the positive control antibody with a 2x concentration of the drug. They should be incubated for 1 hour before analysis to allow for binding of the antibody to the drug molecule. The following conditions may be used for this experiment.

1. **Acid solution: 500 mM Glycine-HCl, pH 3.0**

   Prepare a solution of 500 mM Glycine-HCl from the powder and adjust pH to 3.0 with HCl.

2. **Neutralization buffer**

   The neutralization buffer is prepared by mixing the assay buffer with 1M Tris-HCl pH 9.5 at a 83:17 (vol:vol) ratio.

   For 1.0 mL of neutralization buffer:

   - Add 170 µL of 1M Tris-HCl pH 9.5 to 830 µL of assay buffer

3. **Mix of biotinylated drug and drug-conjugated Acceptor beads**

   Prepare a 1.2 nM solution of biotinylated drug to get a 0.3 nM final concentration in the well, and a 80 µg/mL Acceptor beads solution to get a 20 µg/mL final concentration in the well, as follows:

   - To 1.0 mL of neutralization buffer, add 1.2 µL of 1 µM biotinylated drug solution, then add 16 µL of the 5 mg/mL Acceptor beads.

4. **Streptavidin Donor beads**

   Prepare a 40 µg/mL solution of SA-Donor beads to get a 20 µg/mL concentration in the well.

   - To 2.0 mL of assay buffer, add 16 µL of the 5 mg/mL SA-Donor beads. Prepare in subdued light conditions as SA-Donor beads are light sensitive.

5. Sample preparation

The free drug is spiked in serum as follows:

Sample	Serum vol.	Vol. of drug	Drug 2x concentration
1	160 µL	40 µL of stock at 1 mg/mL	200 µg/mL
2	100 µL	100 µL of sample 1	100 µg/mL
3	100 µL	100 µL of sample 2	50 µg/mL
4	100 µL	100 µL of sample 3	25 µg/mL
5	100 µL	100 µL of sample 4	12.5 µg/mL
6	100 µL	100 µL of sample 5	6.25 µg/mL
7	100 µL	100 µL of sample 6	3.12 µg/mL
8	100 µL	100 µL of sample 7	1.56 µg/mL
9	100 µL	100 µL of sample 8	0.78 µg/mL
10	100 µL	100 µL of sample 9	0.39 µg/mL
11	100 µL	100 µL of sample 10	0.19 µg/mL
12	100 µL	-	blank

The positive control antibody is prepared at 500 ng/mL (2x) by spiking the serum as follows:

(Note that the drug tolerance could also be determined using other positive control antibody concentrations.)

- To 1.5 mL of serum, add 3.75 µL of the 100 µg/mL of the positive control antibody.

Final samples are prepared by adding 100 µL of the positive control antibody dilutions (2x) to each of the 2x concentrated drug dilutions. The mix (1x) is then incubated at RT for at least 1 hour.

Assay protocol

The assay is performed in a ½ AreaPlate-96 microplate as follows:

- Add 5 µL of the sample mix (positive control antibody at 250 ng/mL and unlabeled drug dilutions)
- Add 20 µL of 500 mM Glycine-HCl, pH 3.0
- Add a TopSeal to the plate, cover with an empty plate, and incubate at 23 °C for 60 min
- Add 25 µL of the mix of drug-conjugated AlphaLISA Acceptor beads at 80 µg/mL (20 µg/mL final in the well) and biotinylated drug at the selected concentration prepared in the neutralization buffer
- Add a TopSeal to the plate, cover with an empty plate, and incubate at 23 °C for 60 min
- Add 50 µL of SA-Donor beads at 40 µg/mL (20 µg/mL final concentration in each well)
- Add a TopSeal to the plate, cover with an empty plate, and incubate at 23 °C in the dark for 60 min
- Read the plate using an Alpha-compatible reader.

Typical results

Typical results are presented in Figure 4. In this example, as expected, high concentrations of the drug reduced the signal level. The drug tolerance was determined to be 12.5 µg/mL in neat serum using the positive control antibody at 250 ng/mL, by calculating the highest drug concentration allowing the detection of samples at or above the cut-point.

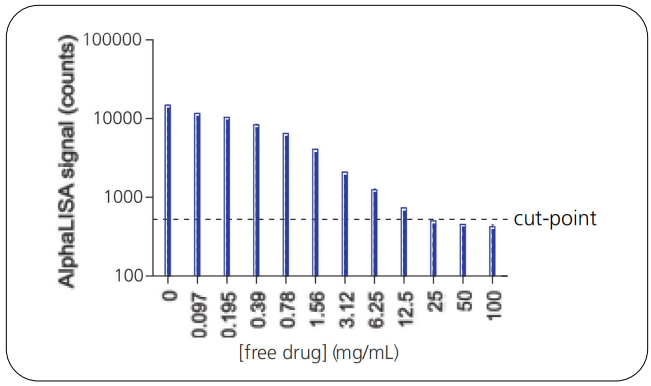


Figure 4: Typical data from free drug tolerance experiment.

VI. Additional optimization

A. Assay set-up

The approach described in the previous sections involves the use of streptavidin coated Donor beads and biotinylated drug. However, it is also possible to use drug conjugated Donor beads (ADA binding to drug conjugated Donor beads and drug conjugated Acceptor beads). One of the advantages of this specific set-up is that there are fewer reagent addition steps, which makes the assay faster and simpler. However, the sensitivity of this set-up could be lower compared to the original protocol. Ideally, the best assay configuration should be evaluated based on the specific assay requirements.

The conjugation of the drug to the Donor beads should be performed according to the protocol presented in Section C for the Acceptor beads, with the exception that an additional precaution should be taken to avoid light exposition of the Donor beads.

The following protocol is recommended for this alternative set-up:

In a ½ AreaPlate-96 microplate:

- Add 5 µL of the positive control antibody at different dilutions (from 1 ng/mL to 100 µg/mL, diluted in assay buffer or in serum)
- Add 20 µL of 500 mM Glycine-HCl, pH 3.0
- Add a TopSeal to the plate, cover with an empty plate, and incubate at 23 °C for 60 min
- Add 25 µL of the mix of drug-conjugated AlphaLISA Acceptor beads at 40 µg/mL (20 µg/mL final in the well) and Drug-conjugated Donor beads at 40 µg/mL (20 µg/mL final concentration in each well) prepared in neutralization buffer
- Add a TopSeal to the plate, cover with an empty plate, and incubate at 23 °C in the dark for 60 min
- Read the plate using an Alpha-compatible reader.

B. Assay buffer

Although the recommended assay buffer has been shown to be optimal for most of the ADA assays developed, other buffers could be used in case the performance of the assay has to be improved. The following parameters can be tested:

- Buffer type: Tris-HEPES at different pH values (pH 7 to 8)
- The presence of non-ionic detergent, such as Tween-20 or Triton X 100 at a concentration varying between 0.01 and 1%
- The presence of protein blockers, such as casein, BSA or bovine γ-globulin at a concentration varying between 0.01 and 1%
- The presence of salt, like NaCl, at a concentration between 50 and 500 mM.

C. Assay volume and plate format

Most AlphaLISA ADA detection assays will perform well in 100 µL final volume in ½ AreaPlate-96 microplates. However, the volume can be adjusted to fulfill specific user requirements. Alpha assays are very flexible and can be performed in low or high volume in almost all plate formats. However, the proportion of reagents should be kept constant according to the recommended protocol.

It is recommended that the serum sample volume represents no more than 10% of the total final volume in the well in order to reduce matrix interference. Different dilutions of the serum could be tested (up to 1/50).

It is also possible to consider a transfer assay where the sample dilution and the acid dissociation are performed in one plate followed by the transfer of a fixed volume to a second plate for the Alpha detection assay. Most often, any changes in the assay volume will not affect the performance of the assay.

D. Incubation time

The performance of ADA assays could be improved by testing different incubation times after addition of the drug conjugated Acceptor beads. A time course from 1 to 18 hours could be performed for this incubation period. The incubation time after addition of Donor beads is often optimal after 1 hour due to faster kinetic of the streptavidinbiotin interaction.

Noteworthy, the signals of most assays are stable and the plates can be read after an overnight incubation without any significant impact on the performance.

E. Order of addition

For the majority of assays, the order of addition described in Section V gives satisfactory results. However, this sequence of addition steps can be modified to achieve better assay performance.
It could be beneficial to add the biotin-drug molecule and the conjugated Acceptor beads separately. In this case, an incubation time of 30-60 min is necessary after each addition step.

F. Acid dissociation alternatives

The acid dissociation protocol presented in this guide involves Glycine-HCl as the acid solution.
This treatment helps improving the free drug tolerance due to dissociation of immune complexes between the drug and the ADA molecules. Alternative acid treatments have been successfully reported such as the use of 300-600 mM acetic acid. Importantly, when the acid treatment differs from the one described in this guide, the neutralization buffer will have to be adjusted accordingly. This adjustment should ensure that the pH of the assay is restored to a neutral pH for the final steps of the detection.

If no acid treatments are used, it is possible to increase the free drug tolerance by using a longer incubation time after addition of the drug-conjugated Acceptor beads.

If the free drug tolerance is not an issue for the assay or if the drug levels in the samples are below a certain threshold (for instance 100 ng/mL), the acid dissociation step may be skipped and replaced by the addition of 20 µL of PBS. In those instances, the neutralization buffer is also replaced by the assay buffer.

VII. Assay performance parameters

A. Background level

Background signals are usually below 1,000 counts. Higher background levels may indicate drug aggregation. Non-specific interaction between the two beads could also be caused by the sample matrix. The assay buffer composition is critical to achieve the lowest background possible (see Section VI.B.). It could also be valuable to test another assay set-up (see Section VI.A.) or to consider using black ½ AreaPlate microplates (Revvity Part No. 6005540).

B. Intra-assay variability

The intra-assay variability is usually in the range of 5-15% when using recommended pipetting procedures.

C. Inter-assay variability

The inter-assay variability is usually in the range of 5-15%. Several parameters should be carefully controlled to minimize the inter-assay variability including temperature, time of incubation, light intensity, consistent protocol and pipetting techniques.

VIII. References

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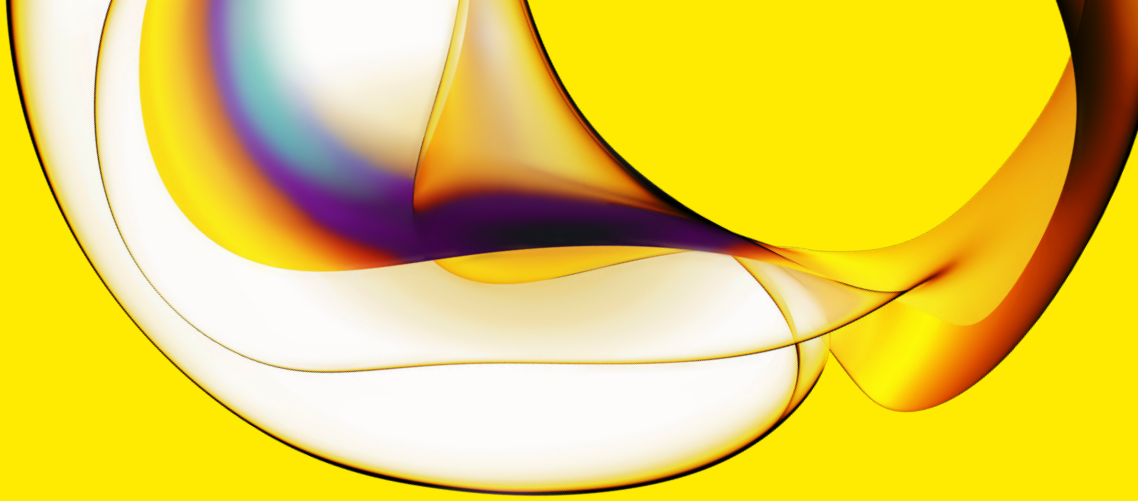

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Waltham, MA 02451 USA
www.revvity.com

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