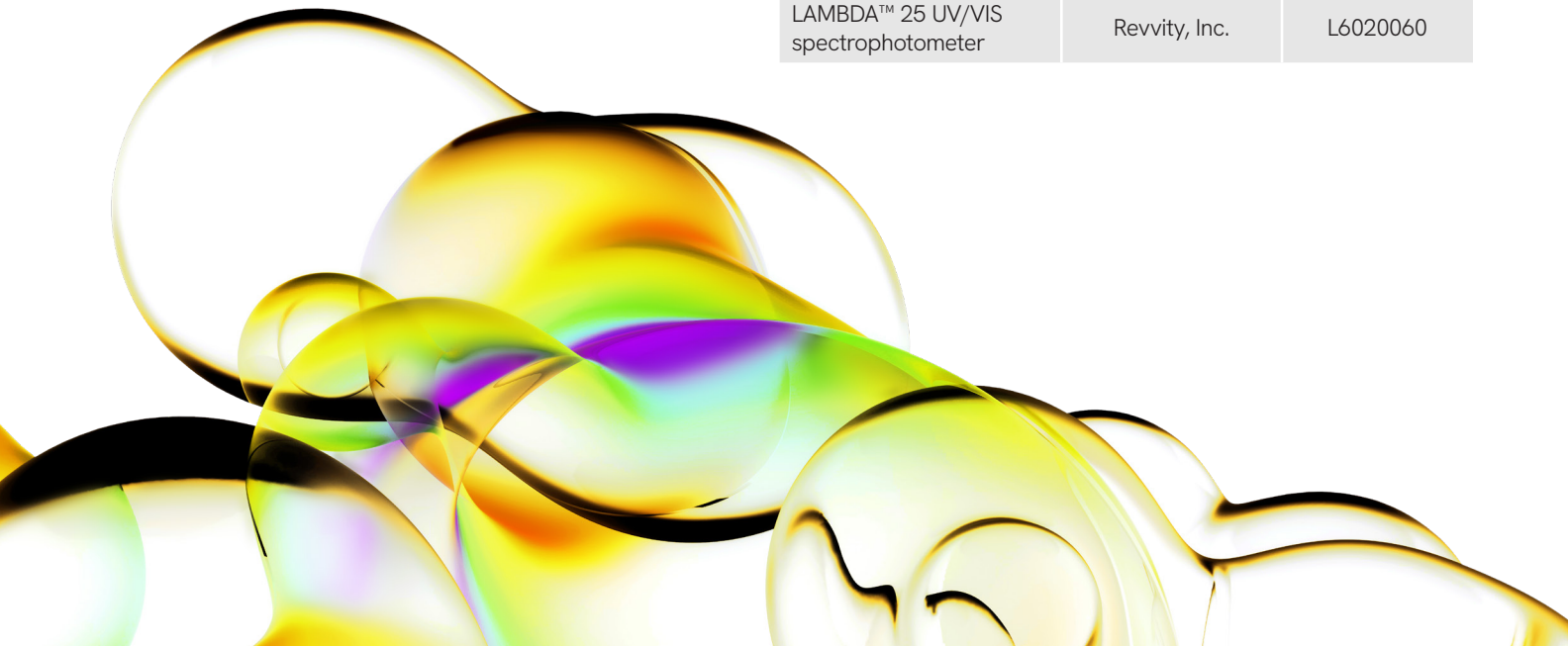
Antibody conjugation to AlphaLISA Acceptor beads detailed protocol.

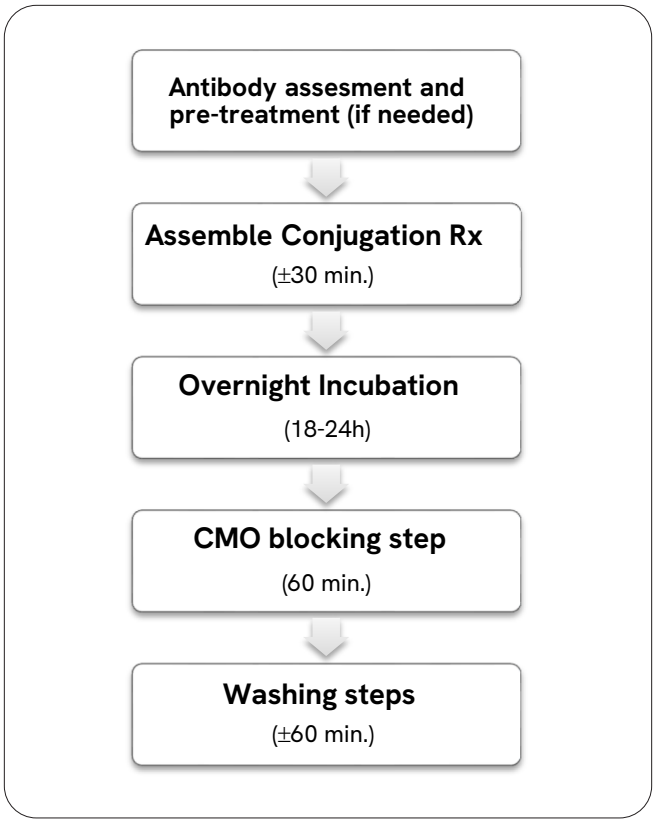
Recommended reagents and materials

*Equivalent sources can be used.

Item	Suggested source	Catalog #
Antibody	N/A. Specific to the target of interest	N/A
AlphaLISA™ Acceptor beads	Revvity, Inc.	6772001 (1 mg) 6772002 (5 mg) 6772003 (50 mg)
100 mM Hepes pH 7.4	To prepare	N/A
10% Tween-20 (Surfact-Amps 20)	Thermo Fisher Scientific Inc.	28320
Na cyanoborohydride powder	Sigma-Aldrich Co.	156159
Carboxymethoxylamine (CMO)	Sigma-Aldrich Co.	C13408
100 mM Tris-HCl pH 8.0	To prepare	N/A
800 mM NaOH	To prepare	N/A
Proclin™-300 or Kathon® CG/ICP II	Sigma-Aldrich Co.	48912-U / 48178-U
PBS 1X pH 7.4	Invitrogen (Life Technologies)	10010
1X PBS + 0.05% Proclin-300	To prepare	N/A
Bench centrifuge	Eppendorf AG	5145 R
Sonicator	Fisher	F60
Rotary shaker (Labquake®)	Barnstead Int.	400110
LAMBDA™ 25 UV/VIS spectrophotometer	Revvity, Inc.	L6020060



Procedure outline



Note: This procedure can be also used with AlphaScreen Donor and Acceptor beads. Donor beads should be handled under subdued light.

1. Antibody assessment and pre-treatment

A preliminary check of the antibody to be conjugated is mandatory. The User must check for the followings:

1.1 Antibody concentration

- To accurately determine the concentration of the antibody, please refer to the method described in ***Appendix 1***.
- The conjugation will perform best when the antibody concentration is at least 1 mg/mL (when conjugating 1-2 mg of beads) or 0.53 mg/mL (when conjugating 2.5 mg of beads or higher amounts). Lower concentrations of antibody yield lower coupling efficiency.
- If required, antibody solutions can be concentrated using an iCON Concentrator (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions (www.piercenet.com/files/2177as8.pdf) (or equivalent device).

1.2 Antibody formulation

- The antibodies must **not** be in any amine-based buffer, including Tris, glycine, bicine, tricine, etc.
- If buffer exchange is necessary, the buffer should be replaced by a neutral to slightly alkaline buffer, such as PBS or carbonate buffer pH 8.0. Although both buffers can be utilized, phosphate buffer will be used in the following protocols. See ***Appendix 2*** for buffer exchange protocol using Zeba™ Desalt Spin Columns.
- Ideally, antibody 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.
- Glycerol will significantly impact coupling efficiency. For that reason, extensive dialysis of the antibody is recommended prior to coupling if the antibody is stored in glycerol. Alternatively, sequential purification on two Zeba Desalt Spin Columns can be used (***Appendix 2***).

2. Bead conjugation reaction

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.

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

This procedure is appropriate for an antibody solution of ≥ 1 mg/mL.

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 pipet 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), add:

- 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 with mild agitation (6 – 10 rpm) using a rotary shaker.

Blocking

- 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 minutes at 16,000 × g (or maximum speed) at 4 °C.
- Remove the supernatant with a micropipette and resuspend the bead pellet in 200 µL of 100 mM Tris-HCl pH 8.0.
- Briefly sonicate the bead solution (10 short pulses of 1 second 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 minutes at 16,000 × g (or maximum speed) at 4 °C, and then remove the supernatant.
- Repeat the washing step (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 second using a probe sonicator; 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.

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

This procedure is appropriate for a solution of antibody ≥ 0.53 mg/mL.

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 pipet 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), add:

- 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

- 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 minutes 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 100 mM Tris-HCl pH 8.0 (200 µL per mg of beads).
- Briefly sonicate the bead solution (10 short pulses of 1 second 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 minutes 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 second using a probe sonicator; the sonicator power should not exceed 20% of the maximal power). This step is recommended but not mandatory.

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.

2.3 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. # 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 centrifugations steps in a Sorvall RC-5B centrifuge (or equivalent) at 16,000 rpm for 40 min at 4 °C.

2. 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.

Appendix 1: Determination of antibody concentration

- 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 antibody concentration:
  (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 Ab = 214,400 M^{-1}/ 160,000 g/mol = 1.34 mg/mL)

Appendix 2: Buffer exchange using Zeba™ Desalt Spin Columns

Selection of column

The choice is based upon the initial volume of the sample. A combination of many columns (alike or different) can be used to accommodate the sample volume.

Column	Sample volume	Source and Catalog #
Zeba Desalt Spin Column, 0.5 mL	30 – 130 µL	Pierce # 89882 or 89883 (25 or 50 columns)
Zeba Desalt Spin Column, 2 mL	200 – 700 µL	Pierce # 89889 or 89890 (5 or 25 columns)
Zeba Desalt Spin Column, 5 mL	500 – 2000 µL	Pierce # 89891 or 89892 (5 or 25 columns)
Zeba Desalt Spin Column, 10 mL	700 – 4000 µL	Pierce # 89893 or 89894 (5 or 25 columns)

Column preparation

- Remove the column's bottom closure and loosen the cap (do not remove the cap). Place the column in an appropriate collection tube.
- Centrifuge the column (1 min at 1,500 x g at 4 °C) to remove the storage solution.
- Place a mark on the side of the column where the compacted resin is slanted upward when using fixed-angle rotors. Place the column in the centrifuge with the mark facing outward in all subsequent centrifugation steps. *(Note: resin will appear compacted after centrifugation.)*
- Add buffer (1X PBS pH 7.4) to the column

Column	Volume of 1X PBS
0.5 mL	300 µL
2 mL	1 mL
5 mL	2.5 mL
10 mL	5 mL

- Centrifuge to remove the buffer:
  - 0.5 mL columns: 1,500 x g for 1 min at 4 °C
  - 2 – 10 mL columns: 1,000 x g for 2 min at 4 °C
- Repeat the addition of buffer and centrifugation steps four additional times, discarding buffer from the collection tube after each cycle.

Buffer exchange

- Place the column in a new collection tube, remove the cap and slowly apply the sample to the center of the compact resin bed. 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 below.)
  - 0.5 mL columns: for samples < 70 µL, add 15 µL buffer
  - 2 mL columns: for samples < 350 µL, add 40 µL buffer
  - 5 mL columns: for samples < 750 µL, add 100 µL buffer
  - 10 mL columns: for samples < 1500 µL, add 200 µL buffer
- Centrifuge to collect the sample
  - 0.5 mL columns: 1,500 x g for 2 min at 4 °C
  - 2 – 10 mL columns: 1,000 x g for 2 min at 4 °C
- Discard column after use.

Determination of antibody concentration

- Measure the OD of the product at 450 nm and 280 nm on a spectrophotometer after pre-treatment, as described in Appendix 1. Use the buffer in which the antibody is diluted to make the blank and the dilution.

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