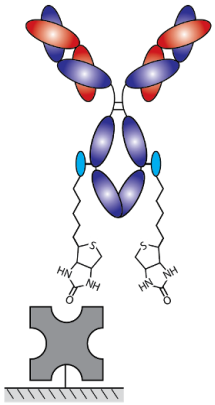

AlphaThera

oYo-Link™ Single-Biotin

Application Guide:

Antibody immobilization on 96-well plates

Catalog #AT4001

FOR TECHNICAL INQUIRIES AND ADVICE CONTACT

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AlphaThera oYo-Link™ Single-Biotin enables the site-specific labeling of **oYo-Link-compatible antibodies** with one biotin label per heavy chain. oYo-Link Single Biotin is an approximately 8 kDa molecule composed of an antibody-binding protein domain, which can become covalently photo-crosslinked to the Fc region of most antibodies, and a C-terminal biotin molecule that can strongly interact with Streptavidin-coated and other biotin-binding surfaces.

Before using oYo-Link Single-Biotin, carefully review the **Antibody Conjugation User Manual** to learn the antibody labeling protocol. The guide below provides protocols for performing antibody immobilization on biotin-binding plates and shares assay optimization advice.

Required Equipment and Reagents:

- Orbital plate shaker
- **AlphaThera LED PX Device** or **compatible 365 nm UV light source**
- Microplate reader
- oYo-Link Single-Biotin
- **Compatible antibody**
- Antigen solution
- Biotin-binding 96-well plate
- Phosphate buffered saline (PBS)
- Tween 20
- Bovine serum albumin (BSA) or other suitable blocking reagent

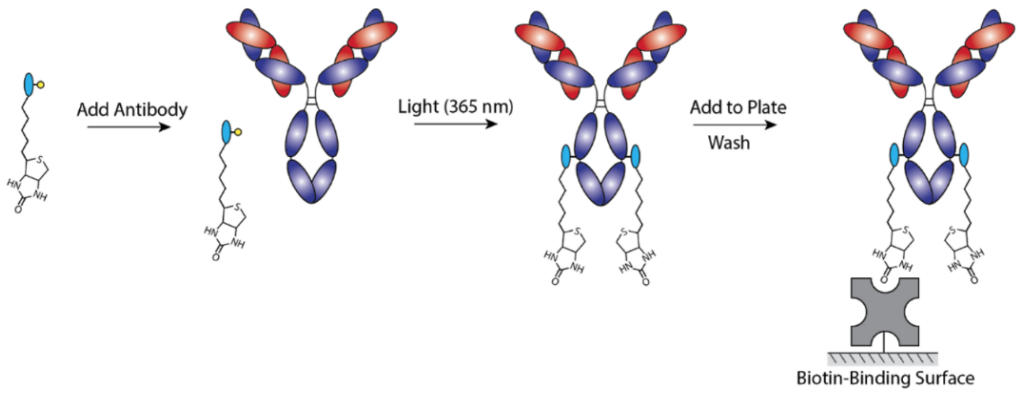
Material Preparation:

- PBST: PBS containing 0.05% (v/v) Tween 20 for washing buffer. Be careful of microbial growth in old buffer.
- PBS-BSA-T: PBST containing 0.25% (g/ml) BSA for blocking buffer. Store solution at 4°C.

Antibody Immobilization Strategy Options

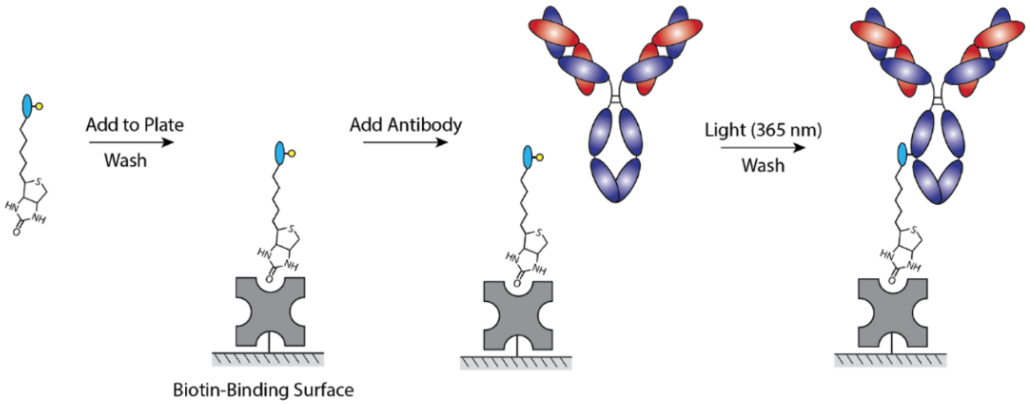
oYo-Link Single-Biotin can be used for the surface immobilization of antibodies using two different strategies:

Immobilization Strategy 1: Pre-label antibody with oYo-Link Single-Biotin and then incubate conjugated antibody on biotin-binding surface.



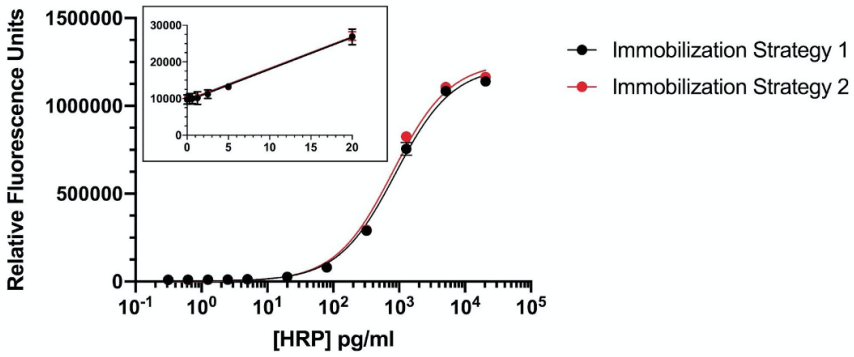
One advantage of Immobilization Strategy 1 is that it requires one less washing step than Strategy 2 (below).

Immobilization Strategy 2: Immobilize oYo-Link Single-Biotin to biotin-binding surface and then covalently cross-link antibody to the surface-tethered oYo-Link Single-Biotin.



The two immobilization strategies give similar results using a Horseradish peroxidase (HRP) immunoassay with a chemifluorescent substrate (10-acetyl-3,7-dihydroxyphenoxazine) to monitor antibody immobilization and antigen capture (**Figure 1**).

Figure 1. Comparison of oYo-Link Single-Biotin immobilization strategies. Both strategies applied an equivalent amount of oYo-Link Single-Biotin (~100 ng) and antibody (400 ng) per well. Data plotted for each assay are average ± one standard deviation for four technical replicates from one representative experiment.



Immobilization Strategy 1 Protocol

Part 1 - Conjugate antibodies with oYo-Link™ Single-Biotin

- Check your antibody and buffer compatibility before proceeding: https://alphathera.com/buffer-antibody-compatibilities
- Calculate the quantity of antibody needed for the experiment and perform the antibody labeling protocol. Use 1 µL oYo-Link Single-Biotin for every 1 µg of antibody. Conjugate the antibodies by photo-crosslinking the mixture under 365 nm UV light source for 2 hr on ice.

It is best to empirically determine the optimal amount of antibody required per well, for any given assay. To start, we recommend an "in well" antibody concentration of 2-4 µg/ml (200-400 ng/well in a full-format 96-well plate), which we find works well, but further adjustments may be necessary.

*Purifying away excess oYo-Link Single-Biotin is not necessary under typical circumstances. We find that removal of free oYo-Link Single Biotin does not further improve assay sensitivity when using optimal saturating levels of labeled antibody (see **Appendix C**).*

Part 2 - Prepare antibody-coated plates

- After the photo-crosslinking reaction is complete, dilute the labeled antibody solution to the desired concentration with PBS. Review the manufacturer's instructions for biotinylated protein immobilization with your biotin-binding plate. For full-format plates we rinse wells 3 times with 200 µL PBST before use. Then, 100 µl of the diluted labeled antibody solution is added to wells and incubated for 1 hr at room temperature with orbital shaking.

Orbital shaking (120+ rpm) is required for maximum immobilization during plate incubation steps.

- Following immobilization, wash wells 3 times with PBST to remove excess labeled antibody and excess free oYo-Link Single-Biotin.

If applicable: **Part 3 - Incubate with target antigen**

- Dilute antigen in blocking buffer and transfer to wells for incubation at room temperature for 1 hr with shaking.
- Wash wells 3 times with PBST to remove unbound antigen.
- Proceed with quantification of antigen capture.

Immobilization Strategy 2 Protocol

Part 1 - Coat plates with oYo-Link™ Single-Biotin

- Review the biotin-binding plate manufacturer's instructions for biotinylated protein immobilization with your biotin-binding plate. For full-format plates we recommend rinsing wells 3 times with 200 µL PBST before use.
- Next, aliquot 100 µL of 0.5 µg/mL oYo-Link Single-Biotin diluted in PBST into each well and incubate for 1 hr at room temperature with shaking to allow binding of oYo-Link Single-Biotin to the biotin-binding surface.

A concentration of 0.5 µg/mL oYo-Link Single-Biotin is optimal for the biotin-binding plates listed in ***Table 1****. The optimal amount of oYo-Link Single-Biotin for other biotin-binding plates should be determined empirically (see* ***Appendix A****).*

Orbital shaking (120+ rpm) is required for maximum immobilization during plate incubation steps

- Discard plate contents and wash wells 3 times with PBST.

Part 2 - Conjugate antibody to oYo-Link™ Single-Biotin

- Dilute antibody in PBS to the total required volume and concentration (**Table 1**)

Table 1. Recommended final "in well" concentration of antibody for commercial biotin-binding plates

Plate (Supplier, catalog #)	*D-biotin binding capacity per well*	*Recommended Antibody Concentration in well*
PierceTM Streptavidin Coated Plates (Thermo Fisher, 15124)	5 pmol	2 µg/mL
PierceTM NeutrAvidinTM Coated Plates (Thermo Fisher, 15123)	15 pmol	2 µg/mL
NuncTM ImmobilizerTM Streptavidin Plates (Fisher Scientific, 436014)	20 pmol	3 µg/mL
PierceTM Streptavidin Coated High Capacity Plates Thermo Fisher, 15500)	125 pmol	2 µg/mL

Table 2 (continued): Antibody concentration recommendations are based on saturating assay conditions using anti-Horseradish peroxidase (HRP) antibody (LSBio, LS-C147582). D-biotin binding capacity differences reflect capture of small biotinylated ligands. The four plates give roughly similar performance when immobilizing oYo-Link Single-Biotin for immunoassays using either immobilization strategy (see ***Appendix B***).

*The optimal amount of antibody for other biotin-binding plates should be determined empirically (**Appendix A**).*

- Add 100 µL diluted antibody to each well.
- Conjugate antibodies to the immobilized oYo-Link Single Biotin by photo-crosslinking wells under 365 nm UV light source for 2 hr at room temperature

Photo-crosslinking can also be performed at 4ºC with equal efficiency. Covering the plate with a clear plastic cover to prevent sample evaporation is acceptable but not required.

- Wash wells 3 times with PBST to remove excess antibody.

If applicable:

Part 3 - Incubate with target antigen

- Dilute antigen in blocking buffer and transfer 100 µL to wells for incubation at room temperature for 1 hr with shaking.
- Wash wells 3 times with PBST to remove unbound antigen.
- Proceed with quantification of antigen capture.

Optimization of Conditions for Immobilization:

For best results with oYo-Link Single-Biotin, the optimal conditions for the application should be identified. The information shared next in the **Appendix** is provided to help guide users seeking to optimize the use of oYo-Link Single-Biotin for a new application.

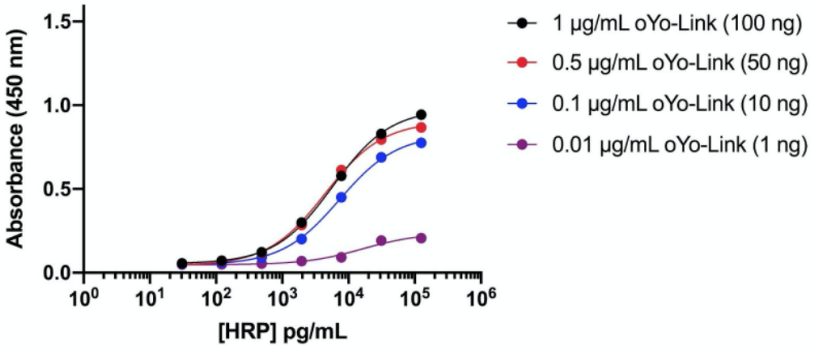
APPENDIX

APPENDIX A - Identifying optimal conditions for a new application

In the following steps, the optimal amounts of oYo-Link Single-Biotin and antibody can be identified for an uncharacterized biotin-binding plate using Immobilization Strategy 2. Data is presented from optimization of the 5 pmol biotin-binding capacity plate (**Table 1**). A Horseradish peroxidase (HRP) immunoassay with a chromogenic substrate (3,3',5,5'-tetramethyl- benzidine) was used to monitor antibody immobilization and antigen capture.

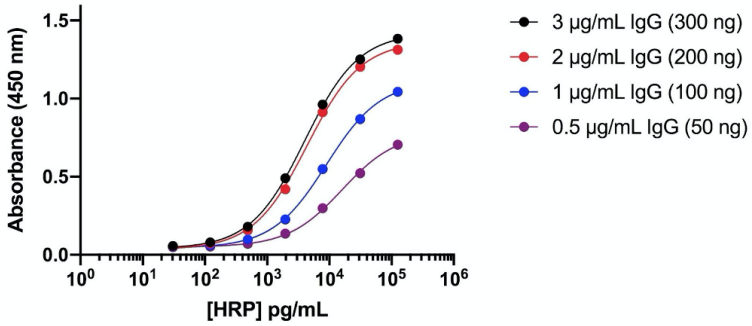
1.Vary oYo-Link Single-Biotin while using a constant, saturating amount of antibody

Figure 2. 3 µg/mL antibody (LSBio, LS-C147582) was used in wells for each assay while oYo-Link Single-Biotin was varied (0.125 - 12.5 pmol). Data are single replicates from one representative experiment.



2. Use a constant, saturating amount of oYo-Link Single-Biotin and vary antibody

Figure 3. 1 µg/mL oYo-Link Single-Biotin (100 ng, 12.5 pmol) was used in wells for each assay while antibody (IgG) was varied (0.33 - 2 pmol). Data are single replicates from one representative experiment.



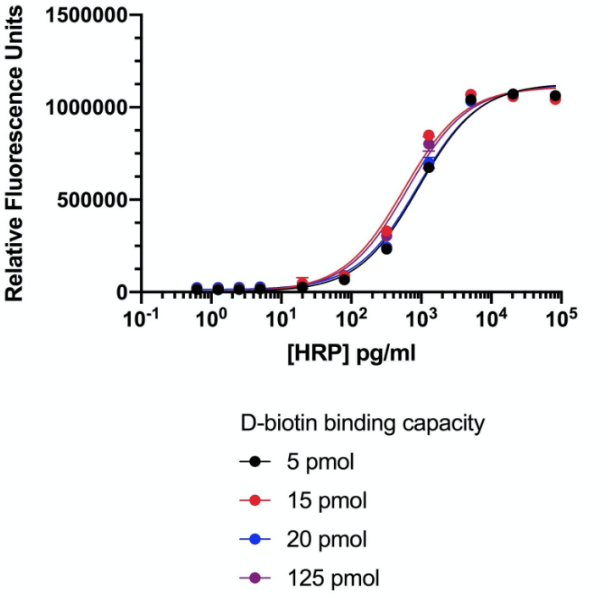
3. Proceed with optimal oYo-Link Single-Biotin and antibody concentrations for the application

APPENDIX B - Comparisons for antibody immobilization and antigen capture on plates with different biotin-binding capacities

Commercial Streptavidin-coated plates can vary by D-biotin binding capacity and coating technique. The performance of four different biotin-binding plates (**Table 1**) were examined using an oYo-Link Single-Biotin immunoassay for capturing Horseradish peroxidase (HRP). First, the saturating amounts of oYo-Link Single-Biotin and antibody were identified (as described in ***Appendix A***). Then, the immunoassay performance was assessed using saturating conditions for each plate (**Figure 4**).

The plate with the lowest biotin-binding capacity (5 pmol) was slightly less sensitive for this application compared to the other three plates. Plates with greater D-biotin binding capacities did not markedly improve immunoassay performance. Outcomes may vary for other plate manufacturers and applications.

Figure 4. D-biotin binding plates of varying binding capacities (5-125 pmol; **Table 1**) were evaluated for performance of the oYo-Link Single-Biotin immunoassay for Horseradish peroxidase (HRP). Immobilization Strategy 1 was used to pre-label antibody with oYo-Link Single-Biotin. 400 ng antibody per well (4 µg/ml) was used for all plates. Antibody immobilization and antigen capture was quantified with a chemifluorescent substrate (10-acetyl-3,7-dihydroxyphenoxazine). Data for each plate are average ± one standard deviation for four technical replicates from a single experiment.



APPENDIX C - Whether to purify excess oYo-Link Single-Biotin from labeled antibody reaction

When using Immobilization Strategy 1 the labeled antibody solution will contain some unreacted oYo-Link Single-Biotin. If excess oYo-Link Single-Biotin is of concern, the labeled antibody solution can be purified. A brief incubation of the labeled antibody solution with IgG Sepharose extracts free oYo-Link to produce an oYo-Link-depleted solution of labeled antibody. For a detailed protocol for this purification process please contact our technical support team.

Purification does not improve performance and is not required for the many assays that are insensitive to excess oYo-Link Single-Biotin, such as the Horseradish peroxidase immunoassay from this guide (**Figure 5**).

Figure 5. Purification does not improve the limit of detection of the oYo-Link Single-Biotin immunoassay for Horseradish peroxidase (HRP). Immobilization strategy 1 was used to pre-label antibody with oYo-Link Single-Biotin. The labeled antibody solution was processed through IgG-Sepharose resin to bind to and remove free oYo-Link Single-Biotin. 400 ng antibody per well (4 µg/ml) was used for each assay. Antibody immobilization and antigen capture was quantified with a chemifluorescent substrate (10-acetyl-3,7-dihydroxyphenoxazine). Data plotted for each assay are the average ± one standard deviation of two technical replicates from one representative experiment.