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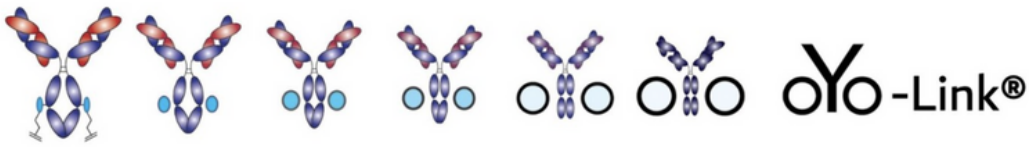
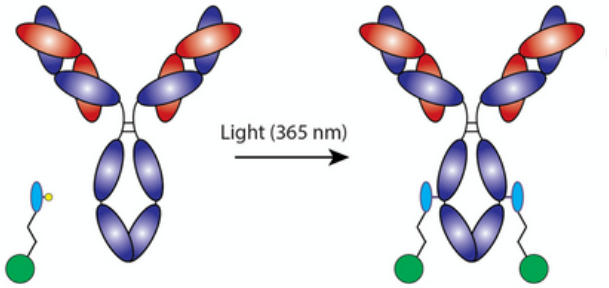
oYo-Link® CONJUGATION USER MANUAL



Antibody Conjugation User Manual

Site-specific Antibody Labeling



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alpha Thera

Version 6.0 May 2022

Table of Contents

Section 1: Introduction

1.1 oYo-Link® Description

AlphaThera's oYo-Link Antibody Labeling Reagents can be used to site-specifically and covalently label the Fc region of almost any "off-the-shelf" antibody with drugs, biotin, click chemistry tags (e.g. Azide, DBCO, Tetrazine), epitope tags, enzymes (e.g. MNase), oligonucleotides, and more. Labeling is simple, fast and site-specific. The entire procedure requires just two-steps and less than 30 seconds of hands-on time. AlphaThera's oYo-Link reagents consist of low molecular weight (~8 kDa), high-affinity antibody-binding domains that possess a photo-crosslinker within their Fc-binding site. Upon illumination with non-damaging 365 nm light, oYo-Link forms a covalent bond with the antibody. This procedure is referred to as Light-Activated Site-Specific Conjugation (LASIC[1]). Any label that is attached to oYo-Link will be covalently attached to the desired antibody.

Site-specific antibody labeling ensures that the label does not interfere with antigen binding. The oYo-Link site-specific antibody labeling system also avoids the need to optimize reaction conditions for each antibody. The same reaction conditions are used for all oYo-Link-compatible antibodies and results in a predictable number of labels per antibody. In contrast, the efficiency of non-site-specific labeling of antibody amino acid side chains (e.g. lysine) can vary dramatically from antibody to antibody. As a result, the reaction conditions may need to be optimized to achieve the desired results. A particularly notable advantage of the oYo-Link site-specific antibody labeling system is that antibody labeling can be conducted in nearly any buffer, including those containing amines (e.g. Tris) or storage proteins (e.g. Bovine Serum Albumin). Therefore, there is no need for time-consuming desalting and concentrating steps, avoiding the potential loss of precious antibodies. oYo-Link antibody labeling can be performed with as little as 1 µg of antibody at concentrations as low as 50 µg/mL.

Several oYo-Link products, e.g. oYo-Link Single-Biotin and oYo-Link His12 Tag, can also be used for the site-specific immobilization of antibodies onto surfaces. These products ensure the proper orientation of antibodies on streptavidin- or transition metal (e.g. Ni2+, Co2+)-coated surfaces, respectively. Proper antibody orientation maximizes the antigen binding density of the surface-immobilized antibodies, which can lead to an improvement in sensitivity, stability and longevity of capture antibodies. Although the magnitude of improvement is dependent on various parameters (i.e. antibody binding properties), uniform antibody orientation on a surface can lead to an increase in sensitivity in enzyme-linked immunosorbent assays (ELISA) by enabling the capture of more antigens per unit area.

1. Hui, J.Z., Tamsen, S., Song, Y., Tsourkas, A. (2015) LASIC: Light activated site-specific conjugation of native IgGs. Bioconjugate Chemistry, 26(8), 1456-1460. ***DOI: 10.1021/acs.bioconjchem.5b00275***

Section 2: Specifications

2.1 Required Equipment

- A long wavelength UV light source (~365 nm "black-light", one or more 6 – 10 W bulbs) is required for the photo-crosslinking of oYo-Link products to the desired antibodies. Compatible options are listed at: **https://alphathera.com/compatible-photocrosslinking-devices**.

AlphaThera offers an easy-to-use LED Photo-crosslinking (LED PX) Device *(Catalog #: AT8001-D*) that uses two long-wavelength LED UV bulbs (365 nm, 9W) to enable efficient and site-specific labeling of antibodies with all oYo-Link products. For more detailed information, please visit: **www.alphathera.com/ledpx/leddevice**.

Germicidal UV lights in most biological hoods emit at a wavelength of 254 nm, which can damage proteins and is not suitable for conjugation. Thus, these UV lights are not appropriate for oYo-Link antibody conjugation.

2.2 Antibody Compatibility

- **oYo-Link is compatible with nearly all commonly used antibody isotypes**. Please check the oYo-Link Antibody Compatibility table in **Appendix Section 8.1** or on our website before proceeding to antibody photo-crosslinking.

2.3 Buffer Compatibility

- **oYo-Link is compatible with most commonly used buffers and solutions.**

- Please check the oYo-Link Buffer Compatibility table in **Appendix Section 8.1** or on our website before proceeding to antibody photo-crosslinking.

- In general, there is *no need* to purify the antibody before conjugation since oYo-Link reagents are compatible with all common buffers, including those containing storage proteins, cell culture medium, and serum. However, unusually high levels of azide (≥1%), glycerol (>50%), HEPES (>20mM) or other chemicals can potentially interfere with oYo-Link binding to antibodies and/or photo-crosslinking.

2.4 Working with oYo-Link® Azide, oYo-Link DBCO, oYo-Link® Tetrazine and oYo-Link® Thiol

- **It is required to perform the coupling reactions with the molecule of interest first,** via click-chemistry or maleimide coupling, **prior to photo-crosslinking** to an antibody. This is to avoid damaging the click moiety by UV illumination and to avoid diluting oYo-Link to maximize the reaction efficiency.
- A recommended protocol for copper-free click-chemistry is available in **Appendix Section 8.2**. A protocol for thiol-maleimide coupling is available in **Appendix Section 8.3**. See the Product Protocols on our website or contact support for further questions.

Section 3: Safety

WARNING: UV light poses potential health risks including injuries to the skin and eyes, please exercise caution when the LED PX device is running and avoid eye or skin exposure.

WARNING: Take extra caution when operating the LED PX Device to avoid electrical shock. Increased risk of electrical shock due to water exposure is possible. Be sure to carefully open the sample tray of the device to avoid splashing water onto the device walls or inner bulbs. Putting the ice in a bag is recommended.

oYo-Link products should be handled with care by trained personnel, taking all precautions to avoid unnecessary exposure. oYo-Link products do not meet hazard classification criteria based on evaluations made by our company under the USA's OSHA Hazard Communication Standard, 29 CFR 1910.1200. Please contact **support@alphathera.com** if you have further questions about product safety.

Section 4: Antibody Labeling Instructions

The following protocol is for the direct labeling of antibodies. If antibodies are to be immobilized on surfaces, please proceed to **Section 5.**

4.1 Applicable oYo-Link® products for Antibody Labeling

Product:	*Catalog #*
oYo-Link Azide; oYo-Link Azide Tamra	AT3002; AT3002-TAMRA
oYo-Link DBCO	AT3003
oYo-Link Thiol	AT3001

Product:	Catalog #
oYo-Link Tetrazine	AT3004
oYo-Link MNase	AT6001
oYo-Link Oligo Custom	AT1002
His12 Tag	AT2002
oYo-Link Antibody Drug-Conjugation: VcMMAE, DM1, VcMMAF	AT7001; AT7002; AT7003
oYo-Link Single Biotin	AT4001
oYo-Link Epitope Tags: DYKDDDK, V5, S, VSV-G, NWS, S1, AU1, AU5, HSV1, His12	AT9002- AT9010; AT2002

4.2 Sample Preparation

- If the oYo-Link product is shipped as a dried pellet: Reconstitute the oYo-Link reagents in ddH_2O following instructions on the product specification sheet delivered with the product or on the product webpage.

- Mix 1 µL oYo-Link reagent per 1 µg antibody. Pipette or vortex to mix. Any reconstituted oYo-Link that is not mixed with antibody can be stored for later use. Storage conditions are specific to each product. Please refer to the product specification sheet.

ℹ Antibodies can be conjugated in any clear and transparent microcentrifuge tube, PCR tube, glass container or microwell plate.

ℹ In general, the recommended molar ratio of antibody to oYo-Link product is 1:5 to ensure the maximum antibody conjugation efficiency; however a molar ratio of 1:3 will maintain the maximum conjugation efficiency while minimizing the free oYo-Link in the mixture.

4.3 Photo-crosslinking

- Place the oYo-Link-antibody mixture under a long-wavelength UV (~365 nm) light source. If using microcentrifuge tubes, place the capped tube sideways under the UV light to maximize exposure. To minimize evaporation, keep the sample tube or container capped/closed. It is required that samples be placed on ice or at 4°C during photo-crosslinking since some antibodies may be less stable at room temperature.

- UV illuminate the mixture for 2 hours to ensure maximum conjugation efficiency. Once complete, the antibody is ready for use.

ℹ To label compatible antibodies with oYo-Link products, a light source emitting at 365 nm (one or more 6-10W bulbs) is required. Use any compatible handheld or photo-crosslinking device or purchase AlphaThera's LED PX Device (*Catalog #: AT8001-D*). See **Appendix Section 8.4** for instructions on how to photo-crosslink with compatible handheld UV devices. See the **LED PX Device User Manual** for instructions on how to use the device.

ℹ Up to 1 mL of sample placed in a standard 1.5mL tube can be efficiently photo-crosslinked using a compatible light source and up to 150 µL for PCR tubes. Multiple tubes can be illuminated at any given time, and the maximum number of tubes will vary depending on the light source being used.

ℹ If desired, a shorter duration of UV exposure can be determined experimentally. See **Section 4.5** below for checking photo-crosslinking efficiency.

4.4 Optional: Quenching/Removing Free oYo-Link®

- It is generally not necessary to quench or remove unreacted oYo-Link following antibody labeling. In most immunoassays, there are washing steps that will remove the free oYo-Link. Furthermore, there is little to no cross-talk between multiple antibodies labeled with different oYo-Link products because all of these antibodies will have their Fc region blocked with the respective oYo-Link label.
- If quenching of free oYo-Link is necessary for a particular application, the following approaches can be tried:
  - 5-10% FBS can be added to the assay. FBS contains bovine IgG, which can bind to free oYo-Link.
  - FcRn blocking reagent can be added after antibody-oYo-Link conjugation. The addition of >1µL of FcRn blockade per 1µg Ab-oYo-Link can efficiently quench free oYo-Link.

- For removal of free oYo-Link:
    - A dialysis membrane with a 100kDa MW cutoff, such as Repligen's Micro Float-A-Lyzer Dialysis Device can be used after antibody conjugation.
    - If none of the above options work, a centrifugal filter with a 100kDa MW cutoff can be used. The conjugated antibody will remain on top of the membrane, while the free oYo-Link will pass through. **However**, the antibody has a high risk of getting stuck in the membrane, and antibody recovery may be low.

4.5 Optional: Check the Photo-crosslinking Efficiency Using SDS-PAGE

- In general, load 1 µg of unconjugated antibody and 1 µg of conjugated antibody into two different wells of a gel for SDS-PAGE. Check your protein gel and staining system's recommendations for protein loading, which may deviate from 1 µg. Under reducing conditions, the oYo-Link-conjugated IgG heavy chain will appear as a higher molecular weight band (i.e. shifted upward) compared to the unconjugated IgG heavy chain, as shown in **Figure 1 below.**

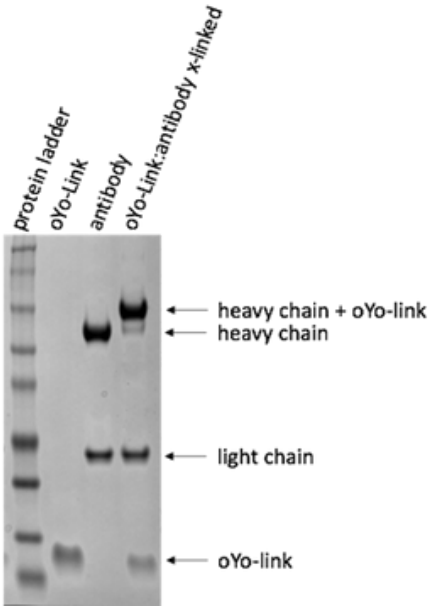

Figure 1: Photo-crosslinking efficiency shown by SDS-PAGE

Samples containing 1 µL oYo-Link, 1 µg unconjugated antibody, and a cross-linked sample containing both oYo-Link and antibody were reduced and run on a 4-12% Tris gel. An upward shift of the conjugated antibody heavy chain is observed. Note that each unreacted oYo-Link product will differ by size, migration, and staining intensity with Coomassie. Some oYo-Link products may require more than 1µL for visualization. Protein ladder bands shown range from 10-110 kDa (protein ladder; Novex Sharp Pre-Stained Protein Standard).

Section 5: Antibody Surface Immobilization Instructions

This protocol should be followed when immobilizing antibodies on surfaces. The protocol below is for 96-well plates, but can be adjusted accordingly for other plates, beads, nanoparticles or surfaces.

oYo-Link Single Biotin Detailed Immobilization Guide: For a detailed protocol on how to immobilize antibodies in a 96-well plate with oYo-Link Single Biotin, please refer to the **oYo-Link Single Biotin Application Guide** that can be found here: **www.alphathera.com/oyolink-singlebiotin-antibody-immobilization-user-manual**

If direct labeling of antibodies is desired and not surface immobilization, please refer to **Section 4.**

5.1 Applicable oYo-Link® products for Surface Immobilization

Table 1. oYo-Link surface immobilization products, dilutions and applicable plates

Product (Catalog #):	*Dilution of Stock solution in PBS (Working solution concentration)*	*Applicable microwell plate surface chemistry*
oYo-Link Single-Biotin (*AT4001*)	1:540 (0.5 µg/mL)	Streptavidin or NeutrAvidin coated plates
oYo-Link His12 Tag (*AT2002*)	1:100 (2.7 µg/mL)	Nickel, copper, or cobalt coated plates

5.2 Preparation of oYo-Link® Solution

- Prepare the oYo-Link Stock solution by reconstituting the oYo-Link reagents in ddH_2O following instructions on the product specification sheet delivered with the product or on the product webpage.

- Prepare a Working solution by diluting the needed oYo-Link Stock solution in PBS (Phosphate Buffered Saline) as indicated in **Table 1** above.

ℹ It is not necessary to dilute the entire Stock solution at a given time.

ℹ The Stock solution can be stored for later use. Storage of the Working solution is not recommended. Refer to the product specification sheet for storage condition guidelines.

5.3 Preparation of Microwell Plate

- Add 100 µL of the diluted oYo-Link Working solution to each needed well in a 96-well plate and incubate for 1 hour at room temperature with shaking. Only microwell plates with appropriate surface chemistries for attachment of the corresponding oYo-Link reagent should be utilized. See **Table 1** for applicable plates.

- Remove the oYo-Link solution and wash each well 3 times with 200 µL PBST (PBS with 0.05% Tween 20).

- Add 100 µL of any oYo-Link-compatible antibody (1-3 µg/mL) per well. The antibody can be diluted in any oYo-Link-compatible buffer. See **Appendix Section 8.1** for antibody and buffer compatibilities.

5.4 Photo-crosslinking

- Place the uncovered microwell plate on (bagged) ice under a long-wavelength UV (~365 nm) light source and UV illuminate for 2 hours to ensure maximum conjugation efficiency. Photo-crosslinking can be performed at 4ºC with equal efficiency.

To label compatible antibodies with oYo-Link products, a light source emitting at 365 nm (one or more 6-10W bulbs) is required. Use **any compatible handheld or photo-crosslinking device** or AlphaThera's **LEDPX Device** (*Catalog #: AT8001-D)*. See **Appendix Section 8.4** for instructions on how to photo-crosslink with compatible handheld UV devices. See the **LEDPX Device User Manual** for instructions on how to use AlphaThera's photo-crosslinking device.

- Remove the antibody solution and wash each well 3 times with 200 µL PBST.

- The plate is now ready for your assay.

Section 6: Troubleshooting

Problem	*Possible Reasons / Solutions*
No antibody cross-linking observed	If no cross-linking of antibody is observed by SDS-PAGE (**Section 4.5**), then: • Check the oYo-Link antibody compatibility table (**Appendix Section 8.1**) to confirm use of a compatible antibody. • Check to make sure the UV photo-crosslinking device is working properly. A longer UV exposure time can be tested, particularly when using a system with power below the recommended range. • There may be a variation in the Fc region for this particular antibody, or the subclass may be mis-identified. oYo-Link photo-crosslinking efficiency can be confirmed by labeling human IgG1 and checking the efficiency by SDS-PAGE. oYo-Link should result in the near complete labeling of human IgG1 heavy chains. If human IgG1 is labeled efficiently, but not your antibody, consider selecting a different antibody for your assay.
Antibody is cross-linked but not working as expected	If cross-linking is observed, but your assay is not working, then: Your assay may require additional optimization or use of a different antibody. Please contact our technical support for assistance by emailing **support@alphathera.com**.

Section 7: FAQ and General Questions

7.1 What antibodies can I label using oYo-Link® reagents?

Nearly all species and subclasses of antibody can be labeled with oYo-Link reagents. Please see our antibody-compatibility table, **Appendix Section 8.1** or visit our website. Note: oYo-Link mIgG1 only labels mouse IgG1 and is not compatible with other antibodies. Currently, oYo-Link mIgG1 is not available for all products - please see the antibody compatibility table.

7.2 Can oYo-Link® be used to label antibodies in the presence of albumin and/or Tris?

Yes, since oYo-Link specifically bind to the heavy chain of IgG, it works in the presence of both albumin and Tris. oYo-Link is compatible with all common buffers. The full buffer compatibility table is shown in **Appendix Section 8.1.** oYo-Link reagents are compatible with all common buffers; however, if there is concern about the compatibility of a particular buffer, SDS-PAGE can be used to confirm photo-crosslinking efficiency in the presence of the buffer in question (see **Section 4.5**) or you can contact support@alphathera.com.

7.3 If an oYo-Link® product has accidentally been left at room temperature for a week, will it still work?

With the exception of oYo-Link MNase, oYo-Link DBCO and oYo-Link Thiol, all oYo-Link products are shipped as white or clear pellets and are very stable. While we recommend cold storage, they will not exhibit any loss of activity if left at room temperature for a week. oYo-Link MNase and oYo-Link DBCO must be shipped with cold packs and stored cold (-20C) immediately upon arrival, otherwise there will be a loss in activity. oYo-Link Thiol ships as a liquid with cold packs and should be stored at 4C upon arrival.

7.4 Does AlphaThera offer a conjugation service?

Yes. If you are interested in our conjugation service, please contact support@alphathera.com. For custom conjugation services, you will need to send us your antibody. The conjugation and purification fee depends on the size and specifications of each order. Purification is not required for most applications.

7.5 How many oYo-Link® labels will be conjugated to each antibody?

For most subclasses and species of antibody, oYo-Link will result in the conjugation of 2 labels per antibody (maximum), one label per heavy chain. For example, ~95% of human IgG and Rabbit IgG will have two labels per antibody. However, there are a few antibodies such as mouse IgG2b and goat IgG, where the conjugation is slightly less efficient. In this case, 60 to 80% of the antibody will be labeled and have a mixture of 0, 1 and 2 labels per antibody. See the antibody conjugation efficiency in **Appendix 8.1.**

7.6 How do I know if oYo-Link® was successfully conjugated to my antibody?

Antibody conjugation can be checked on SDS-PAGE gel. An example is present in the supporting data on each product page. Human IgG1 should exhibit near complete labeling of the heavy chains when checked by SDS-PAGE (see **Section 4.5**).

7.7 What is the lowest amount of antibody that can be labeled by oYo-Link® reagents?

We recommend working with at least 1 µg of antibody; however, even lower amounts of antibody can be labeled by individually optimizing reaction conditions.

7.8 Can antibodies that are highly dilute be labeled by oYo-Link® reagents?

Yes, antibodies can be efficiently labeled even if diluted to concentrations as low as 50 µg/mL.

7.9 Do I need to remove free oYo-Link® after conjugating to antibody?

For most immunoassay applications, purification is not necessary. In our standard recommended protocol, the molar ratio of oYo-Link-to-antibody is 5:1. These conditions ensure that the antibody conjugation efficiency reaches its maximum; however there will be a slight excess remaining in your antibody mixture. This is usually removed during immunoassay washing steps.

However, if your application requires high antibody-conjugate purity, please review **Section 4.4** and contact support@alphathera.com if needed.

7.10 Does 365nm UV illumination damage the antibody and its binding affinity?

The conjugated and unconjugated antibody binding affinities have been tested via both ELISA and cell binding assays and do not show any difference.

7.11 For oYo-Link® click-chemistry products, should I perform the click reaction before conjugation?

I**t is required to perform the click chemistry coupling reactions with the molecule of interest first, prior to photo-crosslinking to an antibody.** This is to avoid UV illumination damage to the click moiety and to avoid diluting oYo-Link prior to the reaction to maximize the reaction efficiency.

oYo-Link Oligo FAQs

7.12 Does the oYo-Link® Oligo price include oligo synthesis cost?

Yes, the cost of the oligonucleotide is included in our price. Please fill in the form with your oligo sequence. All oligos are synthesized by Integrated DNA Technologies. The oligos are HPLC or PAGE grade.

7.13 Does UV illumination for 2 hours damage the oligo?

No, the antibody-oYo-Link Oligo conjugation is performed under 365nm wavelength, which is safe for the oligo. No degradation or mutation has been observed.

7.14 What is the length of oYo-Link® Oligo that AlphaThera can provide?

Our standard pricing applies to oYo-Link oligos up to a length of 80 nt. The antibody conjugation efficiency is >90% for oligos of all lengths. Information can be found here: **https://alphathera.com/oligo/p/custom**. oYo-Link Oligos longer than 80 nt can also be made, but need to be special ordered. Please contact sales@alphathera.com.

7.15 How long will it take to receive my oYo-Link® Oligo once I place the order?

It usually takes 2-3 weeks from the time of processing the order until the product ships out.

7.16 Are oYo-Link® Oligos attached to the antibody at the 5'or 3' end?

oYo-Link oligos can be attached to antibodies at either the 5' or 3' end. You can specify your preference at the time of ordering. The conjugation efficiency is the same.

7.17 Can I have oYo-Link Oligo prepared with a fluorescent label?

Yes. If the oligo 5' end is attached to oYo-Link, then the fluorescent dye can be attached at the 3' end or vice versa. This will be a special order. Please contact sales@alphathera.com.

7.18 How stable is oYo-Link Oligo?

oYo-Link Oligo is very stable. Heating up to 75°C for 5 mins and cooling down will not affect the antibody conjugation efficiency.

7.19 How should I store the conjugated Antibody-oYo-Link Oligo?

Please store the antibody-oYo-Link Oligo conjugate according to the antibody vendor's recommendation.

7.20 Can oYo-Link Oligo be cleaved from the antibody following conjugation?

One strategy to cleave oYo-Link Oligo from the antibody is to insert a restriction enzyme sequence into oligo sequence. Please note that most digestion enzymes work for dsDNA. oYo-Link Oligo is not UV cleavable.

7.21 Can I order oYo-Link Oligo with an RNA sequence?

Since RNA oligos are prone to degradation and unstable, we are not currently offering this option; however, oYo-Link oligos can be made with U's in the oligo sequence, but it will be on a DNA backbone.

oYo-Link ADC FAQs

7.22 How many cells are suitable to seed on a 96-well plate?

Typically, 2500-5000 cells per well is suitable for most cell lines, although this may need to be adjusted for some cell lines.

7.23 Do I need to remove free oYo-Link® drugs after antibody conjugations?

No, it is not necessary to remove unconjugated oYo-Link® drugs prior to adding the oYo-Link®-Antibody sample to cells. Unconjugated oYo-Link® drugs exhibit little to no background cell killing.

7.24 What is the concentration range of antibody-oYo-Link® drug for cell killing assays?

Most antibody drug conjugates will exhibit an EC50 between 10 pM and 10 nM, 72 to 96 hours after adding the ADC. If weak cell killing is observed at an ADC concentration of 10 nM, then extend the incubation time or increase the ADC concentration up to 200 nM. If this doesn't result in significant cell killing, it usually indicates that the ADC is not able to effectively kill the selected cell line. You may consider testing alternative cell lines that may be more sensitive to the ADC or a different antibody.

7.25 When should antibody-oYo-Link® drugs be added to cells and how long before cytolysis is observed?

Antibody-oYo-Link® drugs can be added 24 hours after seeding cells. Cell cytolysis is typically apparent between 72 and 96 hours after adding the ADCs to cells.

7.26 What are the suggested negative control groups in a cell killing assay?

The recommended control groups are antibody alone, oYo-Link® drug alone, and isotype control antibody-oYo-Link® drug conjugate.

Section 8: Appendix

8.1 Antibody & Buffer Compatibility Tables

Species	Ig Subclass	Crosslinking Efficiency
Human	IgG1	+++
	IgG2	+++
	IgG3	+++
	IgG4	+++
	IgA	-
	IgM	-
Mouse	IgG1 *(Separate Product)*	++ *
	IgG2a	+++
	IgG2b	++
	IgG2c	+++
	IgG3	+++
Rat	IgG1	+
	IgG2a	-
	IgG2b	-
	IgG2b kappa	+
	IgG2c	+++
Goat	IgG	++
Rabbit	IgG	+++
Sheep	IgG	++
Cow/Bovine	IgG	++
Dog	IgG	++
Pig/Porcine	IgG	+++
Guinea Pig	IgG	+++
Armenian Hamster	IgG	+
Chicken	IgY	-
Donkey	IgG	+++
Horse/Equine	IgG	+++
Monkey	IgG	+++

+++: Efficient Crosslinking, ++: Moderate Crosslinking, +: Low Crosslinking, -: No Crosslinking

* Labeling mouse IgG1 requires the use of oYo-Link mIgG1. **oYo-Link mIgG1 is available as a separate product** with the following labels: Oligo Custom, Single-Biotin, DBCO, Azide, Tetrazine, Thiol, VcMMAE, DM1, and VcMMAF. **oYo-Link mIgG1 should only be used to label mouse IgG1.** It is not compatible with other antibodies

Buffer Category	Specific Chemical	Compatibility
pH		4-10
Buffer	Tris buffer	✓
	PBS buffer	✓
	HEPES buffer (<20 mM)	✓
	Borate buffer	✓
	MOPS buffer	✓
	MES buffer (50-500 mM)	Reduces photo-crosslinking efficiency by 50%
Salts	Sodium citrate	✓
	Sodium Azide (<1%)	✓
Chelating reagent	EDTA	✓
Buffer additives	Trehalose	✓
	Sugars	✓
	L-Arginine	✓
	Proclin	✓
	Thimerosal	✓
	Merthiolate	✓
	Glycine	✓
	Glycerol (=<50%)	✓
	Gelatin (0.5%)	✓
Storage protein	BSA	✓
Serum	Human	✓
	Bovine	✓
Ascitic Fluid		✓
Cell culture medium	DMEM, RPMI-1640	✓
Hybridoma supernatant		✓

8.2 Click chemistry reactions with oYo-Link Azide, oYo-Link DBCO or oYo-Link Tetrazine

It is required to perform the click chemistry coupling reactions with the molecule of interest first, prior to photo-crosslinking to an antibody. This is to avoid UV illumination damage to the click moiety and to avoid diluting oYo-Link prior to the reaction to maximize the reaction efficiency.

ⓘ Copper-free click chemistry reactions are recommended because copper can affect oYo-Link photo-crosslinking efficiency.

Applicable Products:

Product:	*Catalog #*
oYo-Link Azide	AT3002
oYo-Link DBCO	AT3003
oYo-Link Tetrazine	AT3004

8.2.1 Copper-Free Click Chemistry Procedure

- Mix oYo-Link with the labeled molecule of interest in PBS (pH 7.3) at the indicated molar ratio below:
  - **oYo-Link Azide:** Mix oYo-Link Azide with a 1.5-fold molar excess of the DBCO-labeled molecule of interest.
  - **oYo-Link DBCO:** Mix oYo-Link DBCO with a 1.5-fold molar excess of the Azide-labeled molecule of interest.
  - **oYo-Link Tetrazine**: Mix oYo-Link Tetrazine with a 2.5-fold molar excess of the TCO (trans-cyclooctene)-labeled molecule of interest.

The reaction should be kept at the highest concentration possible to maximize the reaction efficiency. oYo-Link concentration is referenced on the Product Specification Sheet shipped with your product or on the product page under the Specifications Tab.

- Proceed to antibody labeling protocol **(Section 4)**.
- Incubate for 2 hr at 37°C or incubate at 4°C overnight.

For some DBCO/Azide/TCO-labeled molecules, longer reaction times may be required. In some cases, the reaction time can be up to 48 hr.

DBCO/Azide/TCO-labeled molecules of interest include peptides, proteins and oligonucleotides. For peptides or proteins, the linker length between the tag and the peptide or protein may affect the click reaction efficiency. For oligonucleotides, we recommend a C6 or PEG4 or longer linker between the tag and the oligonucleotide.

Typically, no further purification is required at this stage. However, if purification is required, please follow the purification protocol of your choice, keeping in mind that oYo-Link has a molecular weight of ~8 kDa.

8.3 oYo-Link® Thiol reactions via Thiol-Maleimide coupling

It is required that coupling reactions with a maleimide-labeled molecule of interest be performed first, prior to photo-crosslinking to an antibody. This is to avoid diluting oYo-Link Thiol prior to the reaction with the maleimide-labeled molecule, to maximize the reaction efficiency.

Applicable Product:

Product:	*Catalog #*
oYo-Link Thiol	AT3001

8.3.1 Thiol-Maleimide Coupling Procedure:

- oYo-Link Thiol (33 uM concentration) is shipped in reduced form in H2O (pH4). Directly mix it with a 5-fold molar excess of the maleimide-labeled molecule of interest. The reaction should be kept at the highest concentration possible to maximize the reaction efficiency.
- Incubate for 2 hrs at RT or 37°C or incubate at 4°C overnight.
- Proceed to antibody labeling protocol (**Section 4)**.

ℹ Maleimide-tagged molecules of interest include peptides, proteins and oligonucleotides. For peptides/ proteins, the linker length between Maleimide and the peptide/protein may affect the reaction efficiency.

ℹ For some Maleimide-labeled molecules, longer reaction times may be required.

ℹ For some cases, oYo-Link Thiol needs to be concentrated by spin filter (3kDa) before reaction to maleimide-labeled molecules.

ℹ Typically, no further purification is required prior to photo-crosslinking with an antibody. However, if purification is required, please follow the purification protocol of your choice, keeping in mind that oYo-Link has a molecular weight of ~8 kDa.

8.4 Compatible Photo-Crosslinking Devices Instructions

A wide range of light sources that emit at 365 nm (6-10W per bulb) are compatible with oYo-Link products and can be used as alternatives to the **AlphaThera LEDPX Device**. See a list of Compatible Devices at https://alphathera.com/compatible-photocrosslinking-devices.

8.4.1 Assure Compatibility of Your Device

Please refer to the **Compatible Device List** on our website to determine if your handheld or crosslinking machine has been confirmed to be compatible with oYo-Link conjugations. The general requirements are that the device must emit at a wavelength of 365 nm and should operate between 6-10 W per bulb. Devices operating up to 20W may also be compatible, check with our support team to confirm.

Germicidal UV lights in most biological hoods emit at a wavelength of 254 nm, which can damage proteins and is not suitable for conjugation. Therefore, using these UV lights for oYo-Link antibody conjugation is not recommended.

If you have any questions concerning compatibility of your device or a device that is not listed, please contact us at **support@alphathera.com**

8.4.2 Setup of Compatible Photo-Crosslinking Device

The light source should be stably positioned 3 to 10 cm away from the sample. If you do not have a light stand or box, the light source can be positioned using a laboratory stand with clamps or on the edges of an ice bin/bucket. The height of the ice in the bin/bucket can be adjusted to bring the sample tube to within 3 to 10 cm of the light source. The capped sample tube should be placed on the ice in a horizontal position to maximize exposure (**Figure 2**).

Proceed to **Section 4.3** to complete your conjugation.

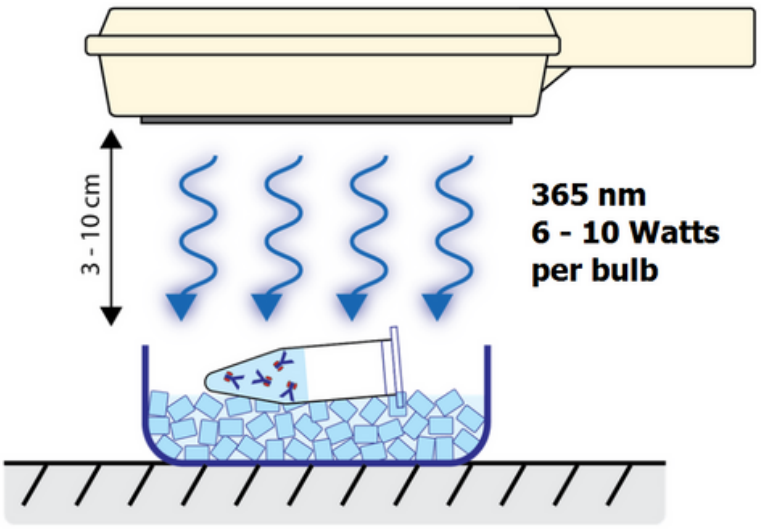

Figure 2. Compatible Handheld Photo-Crosslinking Device Setup. Ensure that the device is positioned between 3-10cm above your sample. Use a clamp or rest the device on the edges of your ice bin.

TECHNICAL SUPPORT

For technical inquiries, get in touch with our technical support team at **support@alphathera.com**

DISCLAIMER