SiteClick™ Antibody Azido Modification Kit

Catalog No. S20026

Pub. No. MAN0017078 **Rev.** B.0

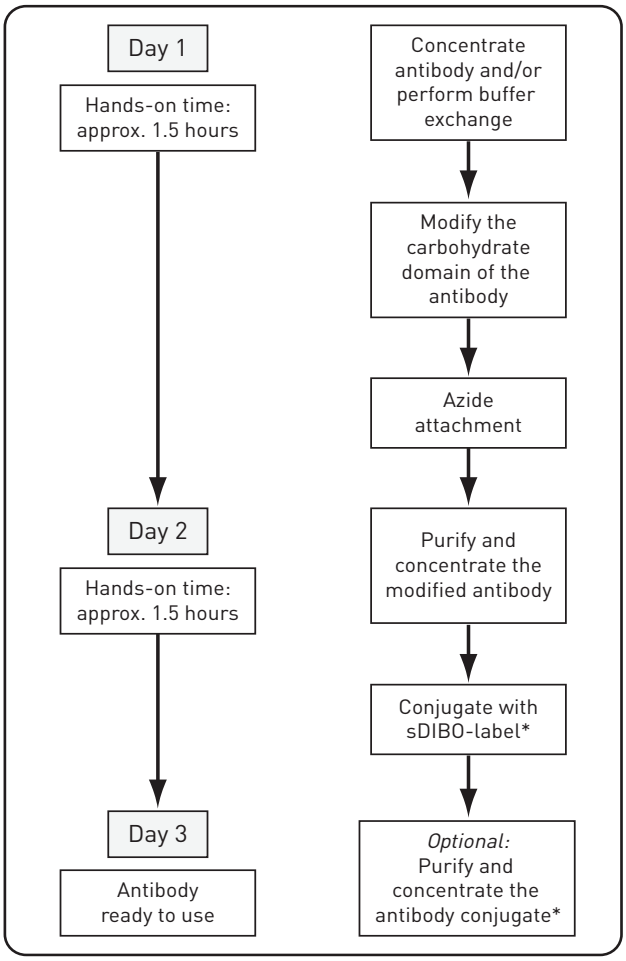
Product description

The SiteClick™ Antibody Azido Modification Kit allows you to specifically attach an azide moiety to the heavy chains of an unlabeled IgG antibody, ensuring that the antigen binding domains of the antibody remain unaltered for binding to your antigen target. The azide-modified antibody can then be covalently linked to SiteClick™ or Click-iT™ sDIBO Alkyne labels (available separately; see Table 2, page 2) in a copper-free click reaction without reducing the protein. This gives you the option to choose different fluorescent labels for your antibody, attach another molecule via streptavidin, or attach your own molecule via amine-reactive or amine-containing moieties depending on your assay.

Each SiteClick™ Antibody Azido Modification Kit contains sufficient reagents to perform one azido modification reaction starting with 100–250 µg of whole IgG from any host species produced in eukaryotic cells. The antibody concentrators provided in the kit are used to purify and concentrate the antibody at each step of the SiteClick™ antibody labeling workflow (Figure 1, page 2)

Table 1 Contents and storage

Material	Amount	Storage*
Antibody preparation buffer (Component A)	1.8 mL	• 2–8°C • DO NOT FREEZE
Antibody concentrator (small) (Component B)	each	
Collection tube (Component C)	each	
ß-Galactosidase (Component D)	12 µL	
UDP-GalNAz (Component E)	220 µg	
20X Tris pH 7.0 (1M) (Component F)	1.8 mL	
Buffer additive (Component G)	30 µL	
ß-1,4-galactosyltransferase (GalT) (Component H)	88 µL	
Antibody concentrator (large) (Component I)	2 each	
* When stored as directed, this kit is stable for at least 6 months.		



* Requires the use of the Click-iT™ sDIBO Alkyne for SiteClick™ Antibody Labeling kits (available separately).

Figure 1 SiteClick™ antibody azido modification and antibody labeling workflow. The SiteClick™ Antibody Azido Modification Kit is designed to be used with the SiteClick™ or Click-iT™ sDIBO Alkyne for SiteClick™ Antibody Labeling kits (available separately; see Table 2) for a complete antibody labeling workflow.

Table 2 Click-iT™ sDIBO Alkynes for SiteClick™ Antibody Labeling. The Click-iT™ sDIBO Alkyne labels (available separately) are used in conjunction with the SiteClick™ Antibody Azido Modification Kit or with engineered antibodies containing azido moieties to create high-quality antibody conjugates.

Product	Catalog No.
Click-iT™ Alexa Fluor™ 488 sDIBO Alkyne	C20027
Click-iT™ Alexa Fluor™ 555 sDIBO Alkyne	C20028
Click-iT™ Alexa Fluor™ 647 sDIBO Alkyne	C20029
Click-iT™ Biotin sDIBO Alkyne	C20030
Click-iT™ Amine sDIBO Alkyne	C20031
Click-iT™ SDP Ester sDIBO Alkyne	C20032
Click-iT™ pHrodo™ iFL Red sDIBO Alkyne	C20034
SiteClick™ pHrodo™ Deep Red sDIBO Alkyne	S10914

Before you begin

Equipment required

- Centrifuge with fixed angle rotor that can accommodate 1.5-mL centrifuge tubes
- Centrifuge with swinging bucket rotor that can accommodate 17 mm × 100 mm centrifuge tubes

Materials required but not provided

- 100–250 µg of whole IgG antibody produced in eukaryotic cells, preferably at a concentration of 2–20 mg/mL in a Tris-based buffer, free of carrier proteins and/or azide
- Centrifuge tubes: 1.5-mL and 15-mL
- Distilled water (dH_2O)
- PBS or TBS
- SiteClick™ or Click-iT™ sDIBO Alkyne label (sDIBO-dye, sDIBO-biotin, or sDIBO-chelator) (Table 2, page 2)

Caution

- **IMPORTANT!** Sodium azide must be avoided throughout the protocol.
- β-Galactosidase (Component D) may cause an allergic skin reaction, and it may cause allergy or asthma symptoms or breathing difficulties, if inhaled. Read the Safety Data Sheet (SDS), available at **thermofisher.com**, before handling this reagent.
- Dispose of the reagents in compliance with all pertaining local regulations. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Always wear suitable laboratory protective clothing and gloves when handling these reagents.

Step 1. Concentrate antibody and/or perform buffer exchange (optional)

Time required: 1 hour

This antibody concentration and buffer exchange step is required if:

- Your antibody concentration is less than 2 mg/mL, and/or
- Your antibody is in a phosphate-based buffer (e.g. PBS), and/or
- Your antibody is in a buffer containing azide.

Before you begin, briefly centrifuge the tubes containing enzymes, substrates, or dyes to ensure all material is at the bottom of the tubes.

Wash the antibody concentrator

1.1 Add 500 µL of dH_2O to the small antibody concentrator (Component B) and cap the device as shown in Figure 2 (page 4).

1.2 Centrifuge at 5000 × *g* for 6 minutes, ensuring that the cap strap and one membrane panel of the concentrator faces the center of the rotor.

1.3 Discard the flow-through.

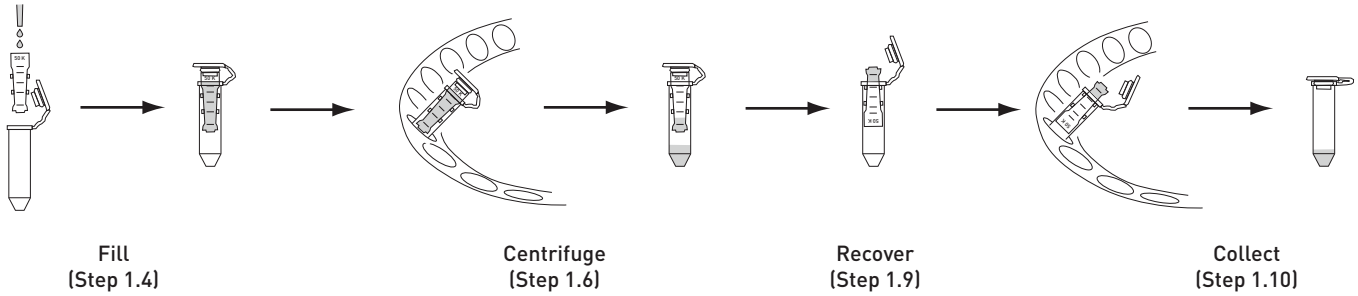


Figure 2 Antibody concentration and/or buffer exchange

Concentrate antibody and/or perform buffer exchange

1.4 Add a sufficient volume of antibody solution to contain 100–300 µg of antibody to the small antibody concentrator.

1.5 Dilute the added antibody to 500 µL using antibody preparation buffer (Component A).

1.6 Centrifuge at 5000 × *g* for 6 minutes, ensuring that the cap strap and one membrane panel of the concentrator faces the center of the rotor.

1.7 Discard the flow-through.

1.8 Add 450 µL of antibody preparation buffer (Component A) to the small antibody concentrator (Component B) and centrifuge at 5000 × *g* for 6 minutes, ensuring that the cap strap and one membrane panel of the concentrator faces the center of the rotor.

Note: If antibody volume in concentrator is greater than 50 µL following Step 1.8, centrifuge at 5000 × *g* for an additional 3 minutes or until the appropriate volume is achieved.

1.9 Invert the small antibody concentrator (Component B) into the collection tube (Component C) as shown in Figure 2.

1.10 Centrifuge for 3 minutes at 1000 × *g* to collect the concentrated antibody. Following collection, you should have approximately 50 µL of concentrated antibody in the collection tube.

Step 2. Modify the carbohydrate domain of the antibody

Time required: 5 minutes hands-on, then 6 hours incubation

Add ß-galactosidase

2.1 Add 10 µL of β-galactosidase (Component D) to the antibody collected in Step 1.10, as shown in Figure 3.

2.2 Wrap the tube cap with Parafilm™ laboratory film or similar, then incubate for 6 hours to overnight at 37°C.

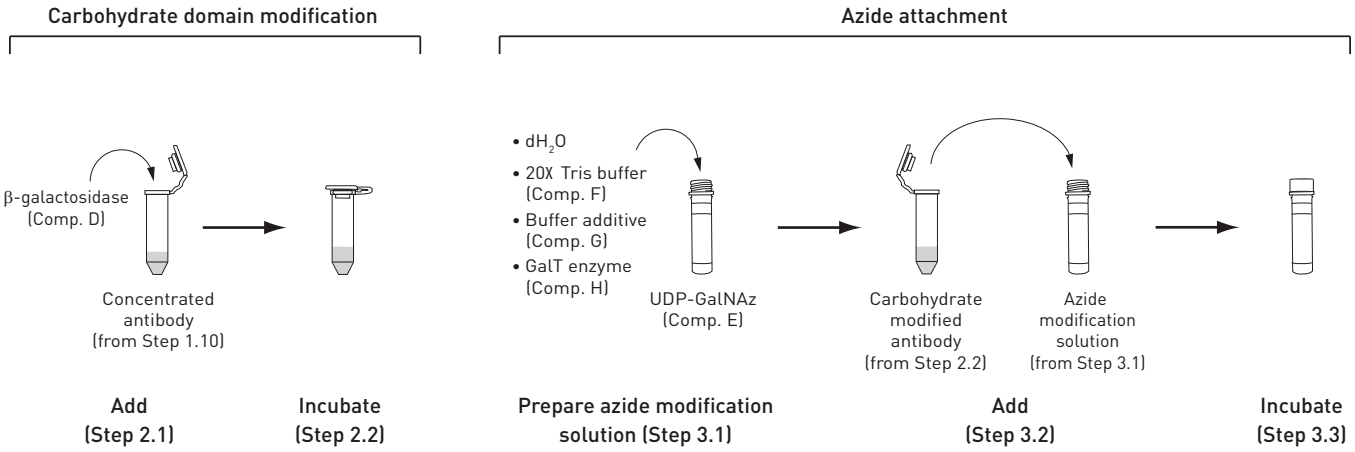


Figure 3 Modification of antibody carbohydrate domain and azide attachment

Step 3. Azide attachment

Time required: 5 minutes hands-on, then overnight incubation

Add GalT enzyme

3.1 Prepare the azide modification solution by adding the following components to the tube containing UDP-GalNAz (Component E), as shown in Figure 3:

- 75 µL of dH_2O
- 12.5 µL of 20X Tris buffer, pH 7.0 (Component F)
- 25 µL of buffer additive (Component G)
- 80 µL of GalT enzyme (Component H)

3.2 Vortex the reaction components and then add the modified antibody from Step 2.2 to the tube.

3.3 Briefly centrifuge the tube, wrap the tube cap with Parafilm™ laboratory film or similar, then incubate overnight at 30°C.

Step 4. Purify and concentrate the azide-modified antibody

Time required: 1 hour

- This step removes any excess substrate UDP-GalNAz.
- You may also use TBS or other phosphate free buffers for purification and collection of the modified antibody (Steps 4.2–4.12). 20X Tris, pH 7.0 is provided for your convenience.

4.1 Prepare 10 mL of 1X Tris, pH 7.0 by adding 500 µL of 20X Tris, pH 7.0 (Component F) to 9.5 mL of dH_2O in a 15-mL conical tube. Vortex briefly to mix.

Wash the antibody concentrator

4.2 Remove the conical collection tube from the large antibody concentrator (Component I).

4.3 Add 1 mL of 1X Tris, pH 7.0 (or TBS) to the large antibody concentrator (Component I) as shown in Figure 4.

4.4 Centrifuge at 1200 × *g* for 10 minutes, ensuring that one membrane panel of the concentrator faces the center of the rotor. Discard the flow-through.

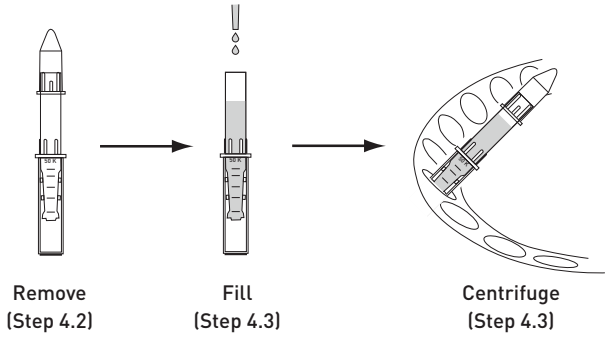


Figure 4 Wash the antibody concentrator

Purify the antibody

4.5 Add 1.6 mL of 1X Tris pH 7.0 (or TBS) and 250 µL of the azide-modified antibody from Step 3.3 to the large antibody concentrator (Component I) as shown in Figure 5.

4.6 Centrifuge at 1200 × *g* for 6 minutes, ensuring one membrane panel of the concentrator faces the center of the rotor. Discard the flow-through.

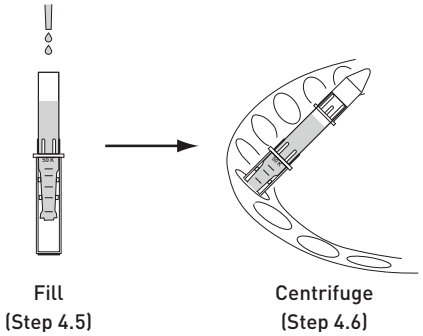


Figure 5 Purification and concentration of azide-modifed antibody

4.7 Add 1X Tris pH 7.0 (or TBS) to a total volume of 2 mL to the large antibody concentrator (Component I) and centrifuge at 1200 × *g* for 10 minutes, ensuring that one membrane panel of the concentrator faces the center of the rotor.

4.8 Discard the flow-through and repeat Step 4.7 two more times.

Note: If the antibody volume in the concentrator is greater than ~100 µL or an antibody concentration of more than ~2.0 mg/mL is desired, you can reduce the volume in the concentrator by additional centrifugation (e.g., at 1200 × *g* for an additional 5 minutes or until the appropriate volume is achieved).

Collect the azide-modified antibody

4.9 Invert the antibody concentrator into the conical collection tube as shown in Figure 6.

4.10 Centrifuge at 1000 × *g* for 3 minutes to collect the concentrated antibody.

4.11 Transfer the antibody from the conical collection tube to a 1.5-mL centrifuge tube.

4.12 Determine the antibody concentration by measuring OD_{280} (with OD_{280} at 1.4 = 1 mg/mL). Expected concentration is ~1–5 mg/mL.

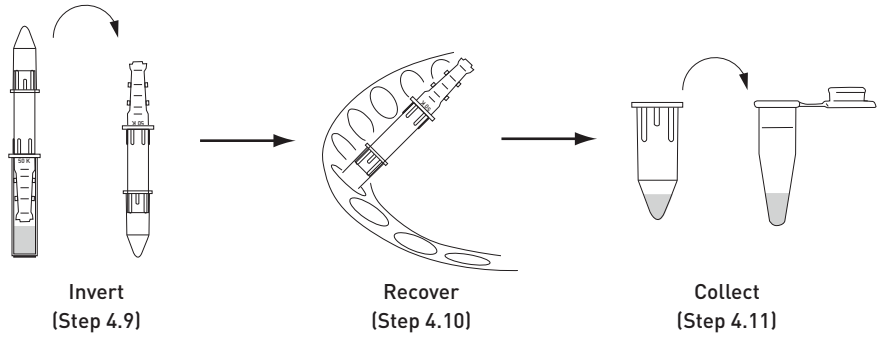


Figure 6 Collection of purified and concentrated azide-modifed antibody

Store the azide-modified antibody

At this point, you can store the azide-modified antibody at 2–8°C until needed. Do not freeze the azide-modified antibody.

IMPORTANT! If you wish to perform a click reaction to conjugate your azide-modified antibody to a SiteClick™ or Click-iT™ sDIBO Alkyne label, do not add sodium azide to your modified antibody. Sodium azide must be avoided throughout the protocol.

Step 5. Attach sDIBO-modified label to azide-modified antibody

Time required: 5 minutes hands-on, then overnight incubation

This section provides instructions to covalently link the azide-modified antibody to a SiteClick™ or Click-iT™ sDIBO Alkyne label in a copper-free click reaction.

Materials required but not provided

- Azide-modified antibody (from Step 4.11) in a Tris-based buffer, free of carrier proteins and/or azide
- SiteClick™ or Click-iT™ sDIBO Alkyne label (available separately; see Table 2, page 2)
- Anhydrous DMSO (only required to dissolve Click-iT™ SDP Ester sDIBO Alkyne; included in Cat. No. C20032)
- Distilled water (dH_2O)
- PBS or TBS
- 1.5-mL centrifuge tubes

Caution

- **IMPORTANT!** Sodium azide must be avoided throughout the protocol.
- DMSO is known to facilitate the entry of organic molecules into tissues. Handle reagents containing DMSO using equipment and practices appropriate for the hazards posed by such materials.
- Read the Safety Data Sheet (SDS), available at **thermofisher.com**, before handling the reagents.
- Dispose of the reagents in compliance with all pertaining local regulations. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Always wear suitable laboratory protective clothing and gloves when handling these reagents.

Add SiteClick™ or Click-iT™ sDIBO Alkyne to azide-modified antibody

5.1 Add 11 µL of SiteClick™ or Click-iT™ sDIBO Alkyne label to 100 µg azide-modified antibody in 100 µL of 1X Tris pH 7.0 (or TBS) in the 1.5-mL centrifuge tube.

Note: The Click-iT™ SDP Ester sDIBO Alkyne for SiteClick™ Antibody Labeling (Cat. No. C20032) is supplied lyophilized as a solid powder. Before use, resuspend the Click-iT™ SDP Ester sDIBO Alkyne in 25 µL of anhydrous DMSO, which is included in the kit.

Other SiteClick™ or Click-iT™ sDIBO Alkynes for SiteClick™ Antibody Labeling are supplied as 25-µL solutions in DMSO and do not need to be resuspended.

5.2 Vortex the reaction mixture, briefly centrifuge, and incubate overnight at 25°C.

Note: Following incubation, you can store the antibody conjugate at 2–8°C until needed (see "Store the antibody conjugate", page 10) or purify it of the excess unconjugated antibody (Step 6, optional).

Step 6. Purify and concentrate the antibody conjugate *(optional)*

Time required: 1 hour

Note: For SiteClick™ pHrodo™ Deep Red (Cat. Nos. S10914) purification, proceed to "Step 7. Purify and concentrate the SiteClick™ pHrodo™ Deep Red antibody conjugate", page 10.

- The purification step removes any excess Click-iT™ or SiteClick™ sDIBO alkyne label that has not been conjugated with the antibody. This removal can be achieved by size exclusion chromatography or centrifugal filtration. For convenience, antibody concentrator has been included in Click-iT™ sDIBO Alkyne products.
- You can use TBS or PBS for the purification and collection of the modified antibody (Steps 6.2–6.7)

Materials required but not provided

- Antibody conjugate (from Step 5.2)
- Antibody concentrator, large (included in the Click-iT™ sDIBO Alkyne for SiteClick™ Antibody Labeling kits)

  Note: The antibody concentrator included in the Click-iT™ sDIBO Alkyne kits (Component B; Component C in Cat. No. C20032) is identical to the large antibody concentrator (Component I) supplied with the SiteClick™ Antibody Azido Modification Kit.
- Distilled water (dH_2O)
- PBS or TBS
- 1.5-mL centrifuge tubes

Wash the antibody concentrator

6.1 Remove the conical collection tube from the large antibody concentrator.

6.2 Add 1X Tris, TBS, or PBS to a total volume of 2 mL to the large antibody concentrator (Component I) as shown in Figure 4 (page 6).

6.3 Centrifuge at 1200 × *g* for 10 minutes, ensuring that one membrane panel of the concentrator faces the center of the rotor. Discard the flow-through.

Purify the antibody conjugate

6.4 Add 1.6 mL of 1X Tris, TBS , or PBS and 200 µL of the sDIBO-modified antibody (from Step 5.2) to the large antibody concentrator.

6.2 Centrifuge at 1200 × *g* for 10 minutes, ensuring that one membrane panel of the concentrator faces the center of the rotor. Discard the flow-through.

6.3 Add 1.8 mL of 1X Tris, TBS , or PBS to the large antibody concentrator (Component I), then centrifuge at 1200 × *g* for 10 minutes, ensuring that one membrane panel of the concentrator faces the center of the rotor.

6.4 Discard the flow-through and repeat Step 6.3 two more times.

Note: If an antibody concentration of more than ~0.5 mg/mL is desired, you can reduce the volume in the concentrator by additional centrifugation (e.g., at 1200 × *g* for an additional 5 minutes or until the appropriate volume is achieved).

Collect the purified antibody conjugate

6.5 Invert the antibody concentrator into the conical collection tube as shown in Figure 7.

6.6 Centrifuge at 1000 × *g* for 3 minutes to collect the concentrated antibody.

6.7 Transfer the purified antibody conjugate from the conical collection tube to a new 1.5-mL centrifuge tube.

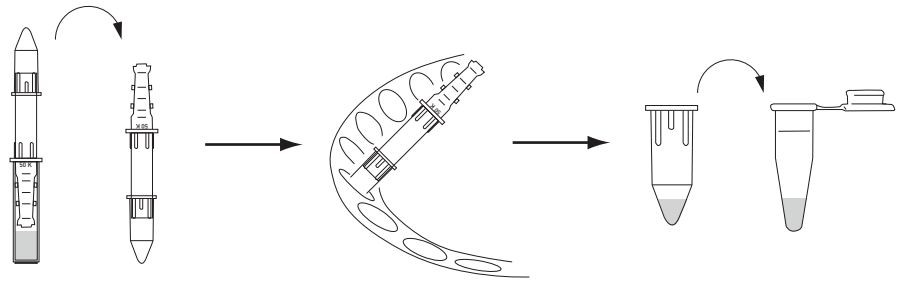


Figure 7 Optional purification and concentration of the labeled antibody conjugate

Store the antibody conjugate

Store the antibody conjugate at 2–8°C until needed. DO NOT FREEZE.

You can add sodium azide or thimerosal at this stage to a final concentration of 0.02% (w/v) for long term storage, if preferred.

Step 7. Purify and concentrate the SiteClick™ pHrodo™ Deep Red antibody conjugate *(optional)*

The following protocol describes the purification step that removes any excess SiteClick™ pHrodo™ Deep Red label that has not been conjugated with the antibody.

The pHrodo™ Deep Red dye removal column (Component C) contains a ready-to-use resin that is designed for rapid removal of pHrodo™ Deep Red dye with exceptional antibody recovery. Removal of the free dye after a labeling reaction is essential for the accurate determination of dye-to-antibody ratios. For optimal antibody recovery and dye removal, ensure that the appropriate amount of sample and buffer are used.

Materials required

- Antibody conjugate (from Step 5.2)

The following required materials are included in the SiteClick™ pHrodo™ Deep Red sDIBO Alkyne for SiteClick™ Antibody Labeling kits; Cat. No. S10914):

- pHrodo™ Deep Red dye removal column
- PBS exchange buffer
- Wash/collection vials

Procedural guidelines

- Do not reuse the purification resin
- Limit organic solvents to ≤10% of the volume

Prepare the spin column

7.1 Following the overnight reaction in step 5.2, loosen the cap on a spin column, twist the tab off of the bottom of the column, then place it into a wash vial.

Note: The wash vial included in Cat. No. S10914 does not have a cap.

7.2 Centrifuge the column-tube assembly at 1,000 × *g* for 2 minutes to remove the storage buffer and pack the column.

Note: If using a fixed angle rotor, place a mark on the side of the column facing away from the rotor center. For all subsequent centrifugation steps, place the column in the microcentrifuge with the mark facing away from the rotor center.

IMPORTANT! Improper orientation of the column during centrifugation can result in reduced dye removal.

7.3 Discard the flow-through, then place the column back into the wash vial.

7.4 Add 250 µL of the supplied PBS exchange buffer (Component B) or the desired buffer to equilibrate the column at 1,000 × *g* for 2 minutes. Discard the flow-through.

Purify the antibody conjugate

7.5 Transfer the packed and equilibrated column into a fresh collection vial.

7.6 Carefully drip the entire reaction mixture onto the column.

7.7 Centrifuge the column tube assembly at 1,000 × *g* for 2 minutes to collect the sample. Discard the column.

Store the antibody conjugate

Store the antibody conjugate at 2–8°C until needed. DO NOT FREEZE.

You can add sodium azide or thimerosal at this stage to a final concentration of 0.02% (w/v) for long term storage, if preferred.

In the first step of SiteClick™ conjugation, terminal galactose residues on the N-linked sugars in the Fc region of the antibody are removed by β-Galactosidase. The azide-containing sugar, GalNAz, is then added to the modified carbohydrate domain of the antibody via the β-1,4-galactosyltransferase (Gal-T)-catalyzed reaction targeting the terminal GlcNAc residues. This specific targeting maintains the integrity of the antigen binding site on the antibody. Finally, the antibody (now containing an azide moiety) is conjugated to the sDIBO-modified label in a copper-free click reaction with simple overnight incubation (Figure 8).

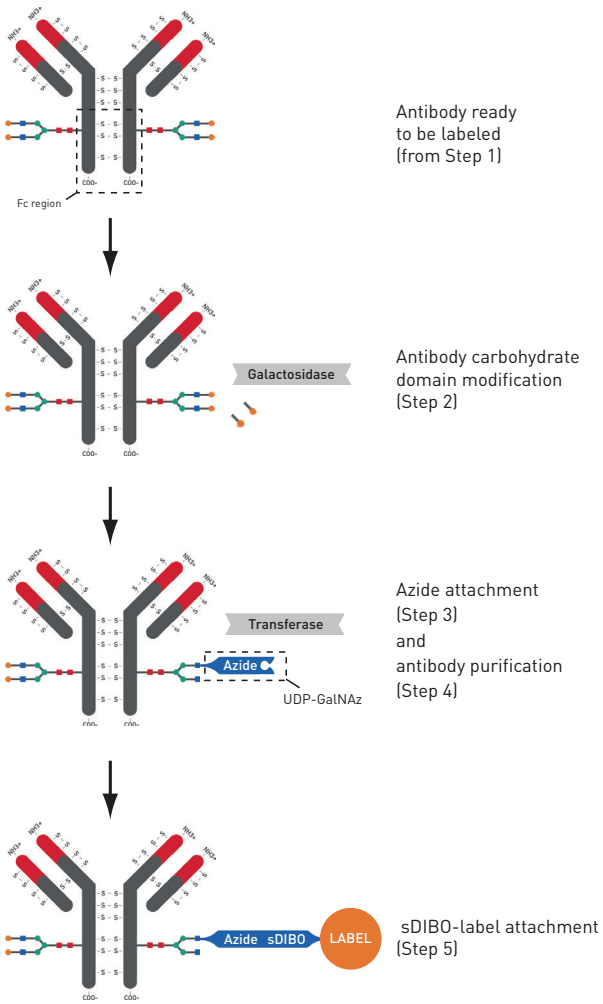


Figure 8 SiteClick™ conjugation reaction

Ordering information

Cat. No.	Product name	Unit size
S20026	SiteClick™ Antibody Azido Modification Kit	1 kit
Related products		
C20027	Click-iT™ Alexa Fluor™ 488 sDIBO Alkyne for SiteClick™ Antibody Labeling	1 kit
C20028	Click-iT™ Alexa Fluor™ 555 sDIBO Alkyne for SiteClick™ Antibody Labeling	1 kit
C20029	Click-iT™ Alexa Fluor™ 647 sDIBO Alkyne for SiteClick™ Antibody Labeling	1 kit
C20030	Click-iT™ Biotin sDIBO Alkyne for SiteClick™ Antibody Labeling	1 kit
C20031	Click-iT™ Amine sDIBO Alkyne for SiteClick™ Antibody Labeling	1 kit
C20032	Click-iT™ SDP Ester sDIBO Alkyne for SiteClick™ Antibody Labeling	1 kit
C20034	Click-iT™ pHrodo™ iFL Red sDIBO Alkyne for SiteClick™ Antibody Labeling	1 kit
S10914	SiteClick™ pHrodo™ Deep Red sDIBO Alkyne for SiteClick™ Antibody Labeling	1 kit
S20033	SiteClick™ Biotin Antibody Labeling Kit	1 kit

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  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

    Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

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Revision history: Pub. No. MAN0018872

Revision	Date	Description
B.0	07 October 2020	Add protocol for SiteClick™ pHrodo™ Deep Red purification.
A.0	11 May 2017	New User Guide

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