Antibody Labeling kit

100-250 µg

INSTRUCTIONS

Last revised May 2020

Intructions for product no:

L1-F01-025	GlyCLICK® AlexaFluor488 - 250 µg IgG
L1-F02-025	GlyCLICK® AlexaFluor555 - 250 µg IgG
L1-F03-025	GlyCLICK® AlexaFluor647 - 250 µg IgG
L1-C01-025	GlyCLICK® DFO - 250 µg IgG
L1-A01-025	GlyCLICK® Biotin - 250 µg IgG

Product description

GlyCLICK is for site specific labeling of up to 250 µg of antibody. The conserved N-linked glycosylation site on the CH2 domain of each heavy chain of the Fc region is used by GlyCLICK for specific conjugation.

Immobilized GlycINATOR® removes all Fc N-glycans, including high-mannose, hybrid-type and bisected glycans to the inner GlcNAc. The subsequent azide activation at the GlcNAc is followed by a click reaction for attachment of a selected dibenzocyclooctyne (DIBO)- functionalized label molecule. The conjugation of the desired molecule occurs at the azide activated sites on the Fc region for incorporation of two labels per antibody (DOL=2), see Figure 1.

The conjugation procedure is performed by combining enzymatic steps and copper-free click chemistry to covalently link the label to the Fc domain of the IgG. All steps are performed under physiological conditions, thus maintaining the quality of the antibody. The site-specific conjugation on the Fc domain preserves the affinity of the antigen-binding sites. GlyCLICK is a versatile tool for conjugation of any IgG with a selection of labels and functional molecules. The antibody can be conjugated with for example a dye, an affinity tag or a chelator.

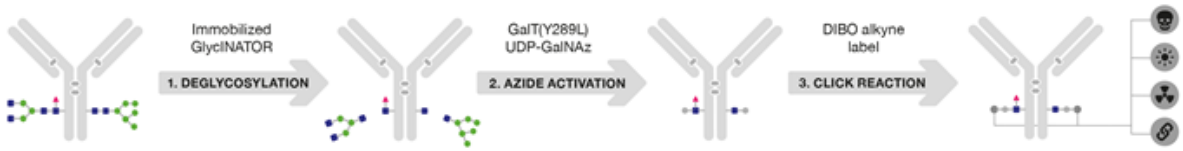

Figure 1. Schematic overview of the GlyCLICK technology. The labeling is performed in three steps:

1. Immobilized GlycINATOR® hydrolyzes the N-glycans on the Fc-part of the IgG to the inner GlcNAc.
2. Azide attachment on the GlcNAc using GalT(Y289L)* and UDP-GalNAz*.
3. The azide activated antibody reacts with a DIBO-alkyne label in a strain-promoted, copper-free click reaction to form a stable and homogenous antibody conjugate.

*GalT(Y289L) and UDP-GalNAz are components of SiteClick™ and are provided under an intellectual property license from Life Technologies Corporation. The trademark SiteClick™ is the property of Life Technologies Corporation.

Content and storage

GlyCLICK Antibody Labeling kit contains enzymes, reagents and material to label 100-250 µg antibody.

GlyCLICK Antibody Labeling kit is shipped cold and components should be stored at different temperatures upon arrival.

Table 1. Content and storage temperatures of GlyCLICK components.

Name	Amount	Store at
Antibody concentrator (small), 50K	1 piece	4 °C to 25 °C
Collection tube for small concentrator	2 pieces	4 °C to 25 °C
Desalting spin column, 40K, 0.5 ml	1 piece	4 °C to 8 °C
Immobilized GlycINATOR®, spin column	1 piece	4 °C to 8 °C
UDP-GalNAz (0.22 mg)	1 vial solid	4 °C to 8 °C Protect from light
20× TBS pH 7.4 (0.5 M)	1.8 mL	4 °C to 8 °C
Buffer additive	30 µL	4 °C to 8 °C Protect from light
β-1,4-galactosyltransferase (Y289L) (GalT)	25 µL	4 °C to 8 °C Protect from light
Antibody concentrator (large), 50K	2 pieces	4 °C to 25 °C
DIBO-modified label*	1 vial solid **or** 25 µL in DMSO	-25 °C to -5°C Protect from light 4 °C to 8 °C Protect from light

***Note: The label is provided in DMSO for products L1-F02-025 and L1-F03-025.**

Before you begin, briefly centrifuge tubes. Always wear suitable laboratory protective clothing and gloves when handling these reagents.

Do not freeze Desalting Spin column, Immobilized GlycINATOR column or GalT enzyme!

GlyCLICK Antibody Labeling kit is for R&D use only.

Overview of the protocol for antibody labeling using GlyCLICK

DAY 1

Antibody (Ab) concentration and/or buffer exchange. Needed if:
- Ab is in a phosphate buffer or in a buffer containing azide, OR
- Ab volume > 200 µl.

Approx. 1h

↓

Modification of the antibody Fc carbohydrate using Immobilized GlycINATOR in a microspin column. 30 min incubation, 2-3 elution steps. **Approx. 1h**

↓

Azide attachment. The deglycosylated Ab is mixed with UDP-GalNAz, GalT, buffer additive and TBS buffer. **Incubation at 30 °C overnight.**

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DAY 2

Purification and concentration. Removal of excess UDP-GalNAz using MWCO-filter. Centrifugation in 4 steps. **Approx. 1h**

↓

DIBO-label conjugation. Conjugation of chosen DIBO-label. The azide-activated Ab is mixed with DIBO-modified label. **Incubation at 25 °C overnight**.

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DAY 3

Optional: Purification of conjugated Ab. Removal of excess DIBO-label reagent using MWCO-filter. Centrifugation in 4 steps. **Approx. 1h**

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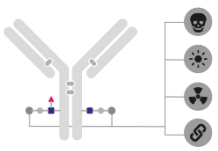

Equipment required

- Centrifuge with fixed angle rotor that can accommodate 1.5-2 ml centrifuge tubes.
- Centrifuge with swinging bucket rotor that can accommodate 17 mm × 100 mm centrifuge tubes.
- Incubator or water bath for 25 °C and 30 °C.
- End-over-end mixer.

Additional Materials required

- 100 to 250 µg of antibody, max volume of 200 µl in a non-phosphate or Tris-based buffer, free of carrier proteins and/or azide. 20x TBS, a desalting spin column (40K) for buffer exchange and a small concentrator (50K) is provided for convenience. For adjusting the antibody solution, please follow "Guidance for concentration and buffer exchange" below.
- Centrifuge tubes: 1.5-2 ml and 15 ml.
- Dimethyl sulfoxide (DMSO) for reconstitution of DIBO-modified label, if supplied as solid material.
- ddH_2O**. Note: if a chelating agent will be used as label (L1-C01-025) it is important to use metal free water (trace analysis grade) throughout the protocol.**
- PBS buffer, optional for purification of antibody conjugate

Sodium azide must be avoided throughout the protocol!

If labeling is performed with DIBO-DFO (L1-C01-025) the antibody must not be in contact with glass or metal.

Guidance for concentration and buffer exchange

The antibody concentration step **is required if:**

- The volume of the antibody is more than 200 µl.

If the sample volume is 100-200 µl but needs a buffer exchange (if it contains phosphate or azide), follow the instruction in section "Buffer exchange with Desalting column, 0.5 ml". It is advisable to start with more than 250 µg of antibody if concentration or buffer exchange of the sample is needed prior to "Step 1. Modification of the carbohydrate on antibody Fc domain, deglycosylation".

<u>Concentration step</u>

1. Add 500 µl of ddH_2O to the small antibody concentrator and cap the device as shown in Figure 2.
2. Centrifuge at 5000 × g for 6 minutes. Make sure that **the cap strap and one membrane panel of the concentrator face the center of the rotor** (Fig. 2).
3. Discard the flow-through.
4. Add the antibody solution to the small antibody concentrator.
5. Centrifuge at 5000 × g for 2-6 minutes. Make sure that **the cap strap and one membrane panel of the concentrator face the center of the rotor** (Fig. 2).
6. Discard the flow-through.

Note: If the antibody volume in the concentrator is more than 200 µl, centrifuge for an additional 2 minutes at 5000 × g, or until the appropriate volume is achieved.

7. Invert the small antibody concentrator into the collection tube as shown in Figure 2.
8. Centrifuge at 1000 × g for 3 minutes to collect the concentrated antibody. After collection, the amount of concentrated Ab should be approximately 150-200 µl in the collection tube.

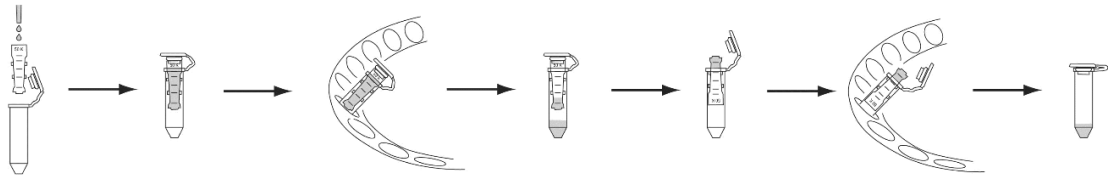

Figure 2. Antibody concentration step

If you need to buffer exchange the sample, follow the steps below.

Buffer exchange with Desalting Spin column, 0.5 ml

This step **is required if:**

- The antibody is in a phosphate-based buffer (e.g. PBS), and/or
- The antibody is in a buffer containing azide

1. Prepare 10 ml 1x TBS buffer by adding 500 µl 20× TBS to 9.5 ml ddH_2O in a 15 ml tube. Vortex briefly to mix.
2. Break off the bottom closure of the Desalting Spin column. Loosen the lid (**do not** remove the lid).
3. Place the column in a collection tube (1.5-2 ml) and centrifuge at 1500 × g for 1 min to remove the storage solution.
4. Discard the flow-through and place the column in the collection tube.
5. Add 300 µl 1x TBS buffer on top of the resin. Centrifuge the column at 1500 × g for 1 min and discard the flow-through.
6. Repeat step 5 **two more times**. Last spin for 2 minutes.
7. Blot the bottom of the column to remove excess liquid. Place the column in a new tube (1.5-2 ml).
8. Apply the antibody solution on top of the resin (100-200 µl).
9. Centrifuge at 1500 × g for 2 min and retain the flow-through containing the antibody in 1x TBS buffer.

Detailed protocol for labeling of 100 - 250 µg of antibody

Step 1. Modification of the carbohydrate on Antibody Fc domain, deglycosylation

The antibody solution should be in non-phoshate based buffer with no azide. Max 250 µg in 200 µl.

Time Required: 15 minutes hands-on, 30 minutes hands-off

Materials from kit: 1× TBS buffer (prepared from 20× TBS),
Spin column with Immobilized GlycINATOR®

- The lid and the cap of the spin column are used during the incubation
- Before the centrifugations, remove the bottom cap and slightly open the lid
- Let the Immobilized GlycINATOR column equilibrate to room temperature before use.

1.1 Prepare 10 ml 1x TBS buffer by adding 500 µl 20× TBS to 9.5 ml ddH_2O in a 15 ml tube, vortex briefly to mix.

1.2 Break off the bottom plastic cap of the GlycINATOR column (save the cap) and slightly open the lid. Place the column in a 1.5-2 ml collection tube.

1.3 Centrifuge the column at 200 × g for 1 min to remove the storage solution.

1.4 Discard the flow-through.

1.5 Place the column in the collection tube.

1.6 Add 300 µl 1× TBS buffer to the top of the resin. Centrifuge the column at 200 x g for 1 minute and discard the flow- through.

1.7 Repeat the steps in 1.6 **two more times**.

1.8 Re-insert the bottom cap at the bottom of the spin column.

1.9 Immediately add the antibody solution (100- 200 µl) to the column. Re-seal the column with the lid.

1.10 Beware; fully suspend the media manually and make sure it is flowing in the column.

1.11 Incubate the column by end-over-end mixing at room temperature for 30 minutes.

1.12 Remove the bottom cap and place the column in a clean micro centrifuge tube (1.5-2 ml). Loosen the lid.

1.13 Centrifuge the column at 1000 × g for 1 minute to collect the deglycosylated antibody sample.

For maximum recovery of the sample:

1.14 Attach the bottom cap. Add 50 µl 1x TBS and seal the column with the lid.

1.15 Invert the column a couple of times.

1.16 Remove the bottom cap and place the column in a clean micro centrifuge tube (1.5-2 ml). Loosen the lid.

1.17 Centrifuge the tube at 1000 × g for 1 minute to collect the deglycosylated antibody sample.

1.18 Repeat steps 1.14 to 1.17 once more.

1.19 Pool the collected deglycosylated antibody samples.

Step 2. Azide attachment

Time required: 5 minutes hands-on, followed by overnight incubation

Materials from kit: 1× TBS buffer (prepared from 20× TBS),
UDP-GalNAz,
GalT enzyme,
Buffer additive

2.1 Prepare the azide modification solution by adding the following components to the tube containing UDP-GalNAz.

Add to the UDP-GalNAz tube:

- 30 µL of buffer additive
- Deglycosylated Ab solutions (from step 1.13 and 1.19) and 1x TBS buffer to a total of 375 µl.

Mix the solution by carefully pipetting up and down.

2.2 Add the GalT enzyme, 25 µl. The final reaction volume should be 400 µl. Mix the solution by carefully pipetting up and down. Wrap the tube cap with Parafilm® or similar.

2.3 Incubate overnight, protected from light at 30 °C.

Step 3. Purification and concentration of azide activated Antibody

Time required: 1 hour

Materials from kit: 1× TBS buffer (prepared from 20x TBS),
Large antibody concentrator

- This step will remove excess of UDP-GalNAz

Wash antibody concentrator

3.1 Prepare 10 ml of 1× TBS by adding 500 µl of 20× TBS to 9.5 ml of ddH_2O in a 15 ml tube. Vortex briefly to mix.

3.2 Remove the conical collection tube from the large antibody concentrator as shown in Figure 4.

3.3 Add 2 ml of 1× TBS to the large antibody concentrator and centrifuge at 1200 × g for 10 minutes. **Make sure that one membrane panel of the concentrator faces the center of the rotor**.

3.4 Discard the flow-through.

Purify the Antibody

3.5 Add 1.6 ml of 1× TBS and 400 µl of the azide activated antibody from Step 2.3 to the large antibody concentrator (Fig. 3).

3.6 Centrifuge at 1200 × g for 6 minutes. **Make sure that one membrane panel of the concentrator faces the center of the rotor**.

3.7 Discard the flow-through.

3.8 Add 1× TBS to a total of 2 ml to the large antibody concentrator.

3.9 Centrifuge at 1200 × g for 10 minutes. **Make sure that one membrane panel of the concentrator faces the center of the rotor**.

3.10 Discard the flow-through.

3.11 Repeat steps 3.8 -3.10 **two more times**.

Note: If the antibody volume in the concentrator is more than ~200 µl, the volume in the concentrator can be reduced by additional centrifugation e.g. for an additional 5 minutes at 1200 × g or until the appropriate volume is achieved.

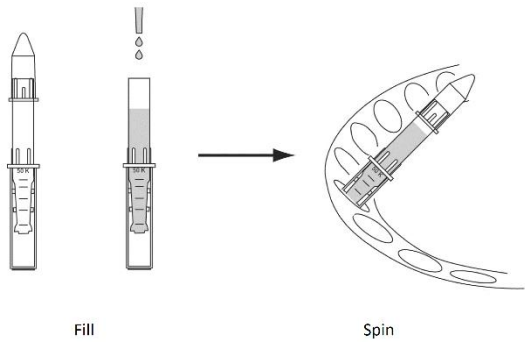

Figure 3. Use of large concentrator

Antibody collection

3.12 Invert the antibody concentrator into the conical collection tube as shown in Figure 4.

3.13 Centrifuge at 1000 × g for 3 minutes to collect the concentrated azide activated antibody.

3.14 Transfer the azide activated antibody from the conical collection tube to a 1.5 ml centrifuge tube.

3.15 If Nanodrop is available, determine protein concentration.

3.16 At this stage, the azide activated antibody can be stored at 2-8 °C for conjugation of label at a later time.

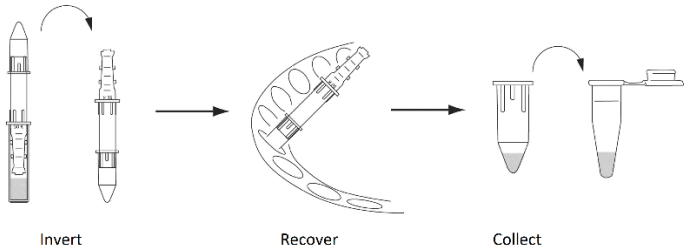

Figure 4. Collection of purified and concentrated antibody

Step 4. Conjugation with DIBO-modified label

Time required: 5 minutes hands-on, followed by overnight incubation

Materials from kit: DIBO-modified label

4.1 Adjust the volume of the azide activated antibody from step 3.16 with 1xTBS buffer to 225 µl.

4.2 If the label is provided in solid form, reconstitute the DIBO-modified label in 27.5 µl DMSO.

4.3 Add 25 µL of DIBO-modified label to 225 µL azide activated antibody in 1× TBS (from step 4.1). Mix by carefully pipetting up and down.

4.4 Seal the tube with Parafilm® or similar.

4.5 Incubate overnight, protected from light at 25 °C.

4.6 After the incubation, the antibody conjugate can be stored at +4-8 °C, protected from light, until needed. Optional is to purify the conjugate.

Purification of Antibody conjugate, Optional

Time required: 1 hour

Materials from kit: Large antibody concentrator

- This step is optional and dependent on following application.
- TBS or PBS may be used for the purification and collection of the conjugated antibody. 20× TBS is provided in the kit for convenience.

<u>Wash large antibody concentrator</u>

1. Remove the conical collection tube from a new large antibody concentrator as shown in Figure 3.
2. Add 2 ml of TBS or PBS to the large antibody concentrator and centrifuge at 1200 × g for 10 minutes. **Make sure that one membrane panel of the concentrator faces the center of the rotor** (Fig. 3).

3. Discard the flow-through.

Purify the conjugated Antibody

4. Add 1.6 ml of 1×TBS or PBS and the conjugated antibody from Step 4.5 to the large antibody concentrator.
5. Centrifuge at 1200 × g for 10 minutes. **Make sure that one membrane panel of the concentrator faces the center of the rotor** (Fig. 3).
6. Discard the flow-through.
7. Add 1× TBS or PBS to a total volume of 2 ml to the large antibody concentrator and centrifuge at 1200 × g for 10 minutes. **Make sure that one membrane panel of the concentrator faces the center of the rotor** (Fig. 3).
8. Discard the flow-through.
9. Repeat Steps 7 and 8 **at least two more times**.

Note: If antibody concentration of more than 2 mg/ml is desired, the volume in the concentrator can be reduced by prolonged centrifugation e.g. for an additional 5 minutes at 1200 × g or until the appropriate volume is achieved**.**

Antibody conjugate, collection and storage

10. Invert the antibody concentrator into the conical collection tube as shown in Figure 4.
11. Centrifuge at 1000 × g for 3 minutes to collect the antibody conjugate.
12. Transfer the antibody conjugate from the conical collection tube to a 1.5 ml micro centrifuge tube.
13. The antibody conjugate can now be stored protected from light at +4-8 °C. **DO NOT FREEZE!** Sodium azide or thimerosal can be added to a final conc. of 0.02% (w/v) for long time storage, if preferred.

References

1. Sjögren, J. et al., 2013. EndoS2 is a unique and conserved enzyme of serotype M49 group A Streptococcus that hydrolyses N-linked glycans on IgG and α1-acid glycoprotein. The Biochemical Journal, 455(1), pp.107–118.

2. Ramakrishnan, B. & Qasba, P.K., 2002. Structure-based design of beta 1,4-galactosyltransferase I (beta 4Gal-T1) with equally efficient N-acetylgalactosaminyltransferase activity: point mutation broadens beta 4Gal-T1 donor specificity. J Biol Chem, 277(23), pp.20833–20839.

GlyCLICK®
