Antibody Labeling kit

Version 17.1.3

100-250 µg

INSTRUCTIONS

Intructions for product no:

L1-F01-025	GlyCLICK™ AlexaFluor®488, labeling of up to 250 µg IgG
L1-C01-025	GlyCLICK™ DFO, labeling of up to 250 µg IgG
L1-A01-025	GlyCLICK™ Biotin, labeling of up to 250 µg IgG

Product description

GlyCLICK™ is for selective 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 selective conjugation.

Immobilized GlycINATOR® removes all Fc N-glycans, including high-mannose, hybrid-type and bisected glycans to the first GlcNAc. The subsequent azide activation at the GlcNAc is followed by a "click" reaction for selective attachment of a selected dibenzocyclooctyne (DIBO)- functionalized label molecule. The conjugation of the desired molecule occurs at 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.

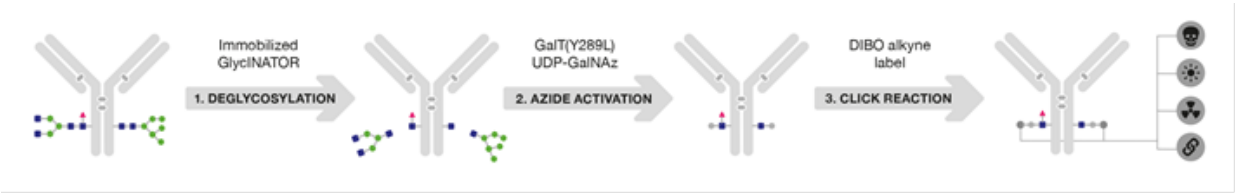


Figure 1. Schematic overview of the GlyCLICK™ technology. The labeling is performed in three steps:

1. Immobilized GlycINATOR® hydrolyzes the N-glycans on the Fc-part of the IgG to the first 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 on ice 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	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)	2 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 for reconstitution	1 vial solid	-25 °C to -5°C Protect from light

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 conjugation 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 de-glycosylated Ab is mixed with UDP-GalNAz, GalT, buffer additive and TBS buffer. **Incubation at 30 °C overnight.**

↓

DAY 2

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

↓

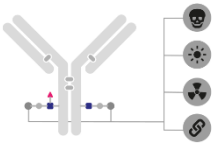
DIBO-label conjugation. Conjugation of chosen DIBO-label. The azide-activated Ab is mixed with DIBO-modified label. **Incubation at 25 °C overnight**.

↓

DAY 3

Purification of conjugated Ab. Removal of excess reagents using MWCO-filter. Centrifugation in 4 steps. **Approx. 1h**

↓



Detailed protocol for labeling of 100 - 250 µg of antibody

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, in a non-phosphate or Tris-based buffer, free of carrier proteins and/or azide. For adjusting the antibody buffer see Step 1.
- Centrifuge tubes: 1.5-2 ml and 15 ml.
- Dimethyl sulfoxide (DMSO) for reconstitution of DIBO-modified label.
- ddH_2O**. Note: if a chelating agent will be used as label (as DFO) it is important to use metal free water (trace analysis grade) throughout the protocol.**
- PBS buffer, optional for step 6.

Sodium azide must be avoided throughout the protocol!

If labeling is performed with DIBO-DFO the antibody must not be in contact with glass or metal.

Step 1. Antibody concentration and/or 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), start at Step 1.9.

Time required (concentration and buffer exchange): 1 hour

Materials from kit: Small antibody concentrator with 2 collection tubes

Concentration step

Wash of small concentrator

1.1. Add 500 µl of ddH_2O to the small antibody concentrator and cap the device as shown in Figure 2.

1.2. 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).

1.3. Discard the flow-through.

Concentrate Antibody with small concentrator

1.4. Add a sufficient volume of antibody solution to contain 100-250 µg of antibody to the small antibody concentrator. For example, if the antibody concentration is 1 mg/ml, add 250 µl.

1.5. 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).

1.6. Discard the flow-through.

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

1.7. Invert the small antibody concentrator into the collection tube as shown in Figure 2.

1.8. 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.

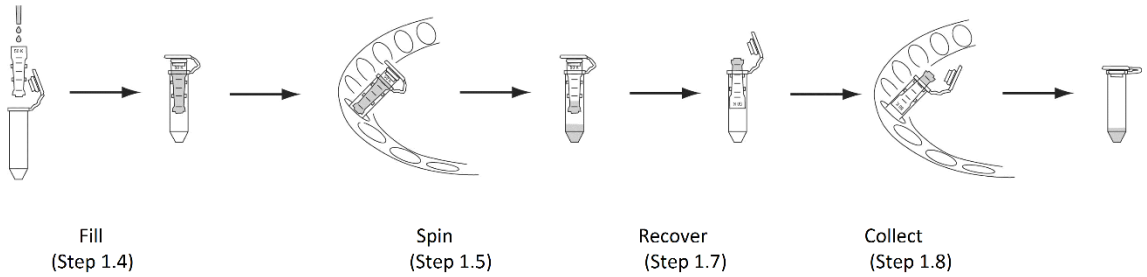


Figure 2. Antibody concentration step

Buffer exchange step

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

Materials from kit: 20x TBS buffer
Desalting spin column

Buffer exchange with Desalting spin column

1.9 Prepare 10 ml TBS buffer (1×) by adding 500 µl 20× TBS to 9.5 ml ddH_2O in a 15 ml tube. Vortex briefly to mix.

1.10 Break off the bottom closure of the Desalting Spin column. Loosen the lid (**do not** remove the lid).

1.11 Place the column in a collection tube (1.5-2 ml) and centrifuge at 1500 × g for 1 min to remove the storage solution.

1.12 Discard the flow-through and replace the column in the collection tube.

1.13 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.

1.14 Repeat step 1.13 **two more times**. Last spin for 2 minutes.

1.15 Blot the bottom of the column to remove excess liquid. Place the column in a new collection tube (1.5-2 ml).

1.16 Apply the antibody solution on top of the resin (100-200 µl).

1.17 Centrifuge at 1500 × g for 2 min and retain the flow-through containing the antibody in TBS buffer.

1.18 Use the left over 1× TBS buffer for Step 2, next section.

Step 2. Modification of the carbohydrate on Antibody Fc domain

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

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

2.2. Break of 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.

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

2.4. Discard the flow-through.

2.5. Replace the column in the collection tube.

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

2.7. Repeat the steps in 2.6 **two more times**.

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

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

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

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

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

2.13. Centrifuge the column at 1000 × g for 1 minute to elute the antibody sample.

For maximum recovery of the sample:

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

2.15. Invert the column a couple of times.

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

2.17. Centrifuge tube and centrifuge at 1000 × g for 1 minute to elute the antibody sample.

2.18. Repeat steps 2.14 to 2.17 once more.

2.19. Pool the eluates.

Step 3. 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

3.1. Prepare the azide modification solution by adding the following components to the tube containing UDP-GalNAz (Fig 3).

Add to the UDP-GalNAz tube:

- 30 µL of buffer additive
- Modified Ab solution (from step 2.13 and 2.19) and TBS buffer to a total of 375 µl.

Mix the solution by carefully pipetting up and down.

3.2. 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.

3.3. Incubate overnight in darkness at 30 °C.

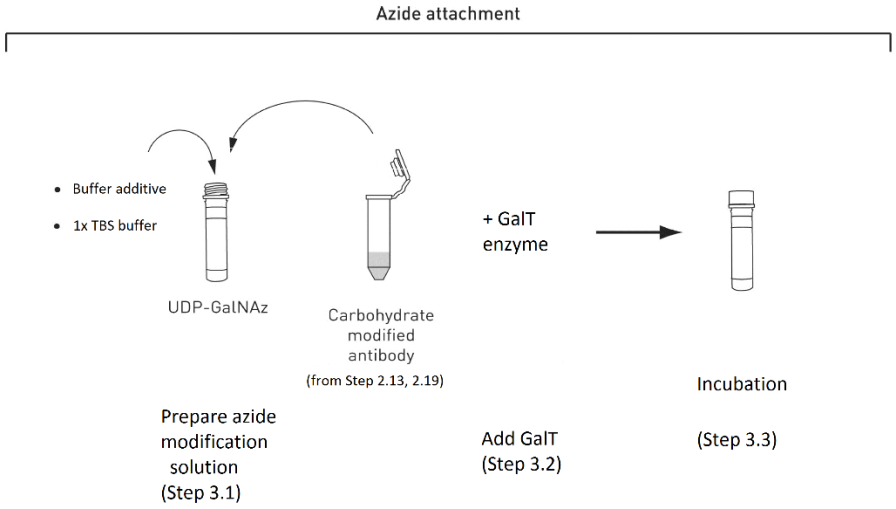


Figure 3. Azide attachment

Step 4. Purification and concentration of azide-modified Antibody

Time required: 1 hour

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

- This step will remove any excess of substrate UDP-GalNAz and GalT

Wash antibody concentrator

4.1 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.

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

4.3 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**.

4.4 Discard the flow-through.

Purify the Antibody

4.5 Add 1.6 ml of 1× TBS and 400 µl of the azide-modified antibody following Step 3.3 to the large antibody concentrator (Fig. 4).

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

4.7 Discard the flow-through.

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

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

4.10 Discard the flow-through.

4.11 Repeat steps 4.8 -4.10 **two times**.

Note: If the antibody volume in the concentrator is greater 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.

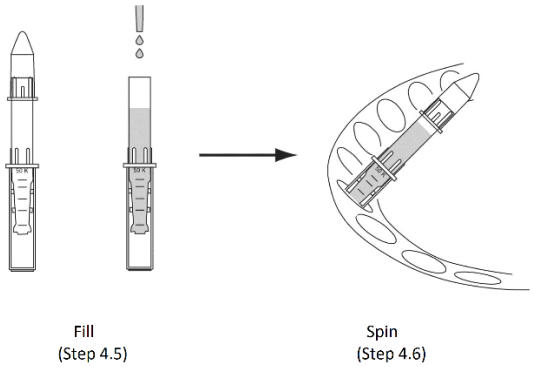


Figure 4. Purification and concentration of azide-modified antibody

Antibody collection

4.12 Invert the antibody concentrator into the conical collection tube as shown in Figure 5.

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

4.14 Transfer the antibody from the conical collection tube to a 1.5 ml centrifuge tube.

4.15 If Nanodrop is available, determine protein concentration.

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

4.17 Adjust the volume with 1xTBS buffer to 225 µl.

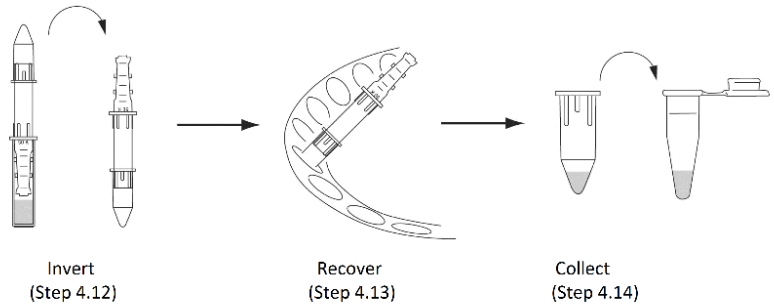


Figure 5. Collection of purified and concentrated azide-modified antibody

Step 5. Conjugation with DIBO-modified label

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

Materials from kit: DIBO-modified label

5.1 Reconstitute the DIBO-modified label in 27.5 µl DMSO.

5.2 Add 25 µL of DIBO-modified label to 225 µL azide-modified antibody in 1× TBS (from step 4.17) in the 1.5 ml centrifuge tube. Mix by carefully pipetting up and down.

5.3 Seal the tube with Parafilm® or similar.

5.4 Incubate overnight in darkness at 25 °C.

Step 6. Purification of Antibody conjugate

Time required: 1 hour

Materials from kit: Large antibody concentrator

- This step will remove excess DIBO-label that has not been bound to antibody.
- TBS or PBS may be used for the purification and collection of the modified antibody (Steps 6.2 – 6.12). 20× TBS is provided in the kit for convenience.

Wash large antibody concentrator

6.1. Remove the conical collection tube from a new large antibody concentrator as shown in Figure 6.

6.2. 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. 6).

6.3. Discard the flow-through.

Purify the conjugated Antibody

6.4. Add 1.6 ml of 1×TBS or PBS and the conjugated antibody from Step 5.4 to the large antibody concentrator.

6.5. Centrifuge at 1200 × g for 10 minutes. **Make sure that one membrane panel of the concentrator faces the center of the rotor** (Fig. 6).

6.6. Discard the flow-through.

6.7. Add 1× TBS or PBS to 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. 6).

6.8. Discard the flow-through.

6.9. Repeat Steps 6.7-6.8 **two 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.

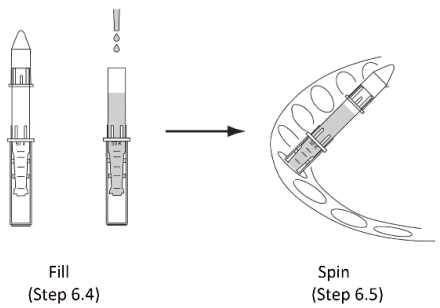


Figure 6. Purification and concentration of conjugated antibody

Antibody conjugate, collection and storage

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

6.11. Centrifuge at 1000 × g for 3 minutes to collect the antibody conjugate.

6.12. Transfer the antibody conjugate from the conical collection tube to a 1.5 ml micro centrifuge tube.

6.13. 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.

6.14. If Nanodrop is available, determine the protein concentration.

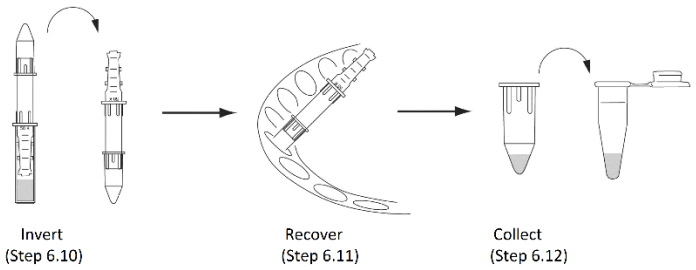


Figure 7. Collection of purified and concentrated conjugated antibody

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.

Patent and Disclaimer

GlycINATOR®
All rights reserved. Aspects of GlycINATOR® technology are encompassed by pending patent applications and by granted Patent in Singapore, Australia, Japan, China, New Zealand, USA and EPO, in the name of Genovis AB.
The trademark GlycINATOR® is the property of Genovis AB, and duly registered in EU, Australia, China, Japan, South Korea and USA.
The trademark GlyCLICK™ is the property of Genovis AB.
For research use only. Not intended for any animal or human therapeutic or diagnostic use.
All goods and services are sold subject to Genovis' General Terms and Conditions of Sale.

SiteClick™
This product is provided under an intellectual property license from Life Technologies Corporation. The transfer of this product is conditioned on the buyer using the purchased product solely in research conducted by the buyer, excluding contract research or any fee for service research, and the buyer must not (1) use this product or its components for (a) diagnostic, therapeutic or prophylactic purposes; (b) testing, analysis or screening services, or information in return for compensation on a per-test basis; or (c) manufacturing or quality assurance or quality control, and/or (2) sell or transfer this product or its components for resale, whether or not resold for use in research. For information on purchasing a license to this product for purposes other than as described above, contact Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, CA 92008 USA or outlicensing@lifetech.com.

The trademark SiteClick™ is the property of Life Technologies Corporation.

©2017 Genovis AB.