Antibody Labeling kit

2 mg

INSTRUCTIONS

Last revised May 2020

Intructions for product no:

L1-F01-200	GlyCLICK® AlexaFluor488 - 2 mg IgG
L1-F02-200	GlyCLICK® AlexaFluor555 - 2 mg IgG
L1-F03-200	GlyCLICK® AlexaFluor647 - 2 mg IgG
L1-C01-200	GlyCLICK® DFO - 2 mg IgG
L1-A01-200	GlyCLICK® Biotin - 2 mg IgG

Product description

GlyCLICK is for site specific labeling of up to 2 mg 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. 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.

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 using e. g. copper free strain promoted azide-alkyne cycloaddition (SPAAC) for specific 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 two 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 modification on the Fc domain preserves the affinity of the antigen binding sites.

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.

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Content and storage

GlyCLICK Antibody Labeling kit contains enzymes, reagents and material to label up to 2 mg 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
Desalting Spin column, 0.5 ml, 40K	1 piece	4 °C to 8 °C
Antibody concentrator (incl 2 collection tubes), 0.5 ml, 50K	1 piece	4 °C to 25 °C
Desalting Spin column, 2 ml, 40K	2 pieces	4 °C to 8 °C
Immobilized GlycINATOR®, microspin column	1 piece	4 °C to 8 °C
UDP-GalNAz	1 vial solid	4 °C to 8 °C Protect from light
20× TBS pH 7.4 (0.5 M)	2 x 2 ml	4 °C to 8 °C
Buffer additive	1 x 50 µl	4 °C to 8 °C Protect from light
β-1,4-galactosyltransferase (Y289L) (GalT)	1 x 40 µl	4 °C to 8 °C Protect from light
DIBO-modified label	L1-F01-200: 1 vial solid L1-C01-200: 2 vials solid L1-A01-200: 2 vials solid L1-F02-200: 1 x 25 µl* L1-F03-200: 1 x 25 µl*	-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-F02-200 and L1-F03-200.**

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

Do not freeze Desalting Spin columns, 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

For conjugation of up to 2 mg IgG

DAY 1

Modification of the antibody Fc carbohydrate using Immobilized GlycINATOR in a spin column. 120 min incubation, 3 elution steps resulting in a final sample of 0.55 ml. **Approx. 2.5 h**

↓

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

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DAY 2

Removal of excess UDP-GalNAz using a 2 ml 40K desalting column. **Approx. 1 h**

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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 using a 2 ml 40K desalting column. **Approx. 1 h**

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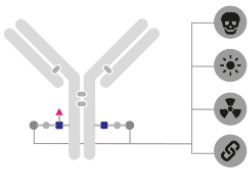

Equipment required

- Centrifuge with swinging bucket rotor that can accommodate 17 mm × 100 mm (15 ml) tubes
- Centrifuge for microcentrifuge tubes
- Incubator or water bath for 25°C and 30 °C.
- End-over-end mixer

Additional Materials required

- Antibody in 1x TBS, pH 7.4, free of carrier proteins and/or azide. 2 mg IgG in a maximum volume of 250 µl. 20x TBS buffer, a desalting spin column (40K) for buffer exchange and a 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 provided in solid form.
- ddH_2O**. Note: if a chelating agent will be used as label (L1-C01-200) it is important to use metal free water (trace analysis grade) throughout the protocol.**

Sodium azide must be avoided throughout the protocol! If labeling is performed with conjugation reagent with chelator, 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 is more than 250 µl

If the sample volume is 250 µl but needs a buffer exchange (if it contains phosphate or azide), concentrate the sample to <200 µl and then follow the instruction in section "Buffer exchange with Desalting column, 0.5 ml". It is advisable to start with more than 2 mg of antibody if you need to concentrate or buffer exchange the sample prior to "Step 1. Modification of the carbohydrate on antibody Fc domain, deglycosylation".

Concentration step

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 a sufficient volume of antibody solution to contain 2.5 mg of antibody 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 and the sample needs a buffer exchange, 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.

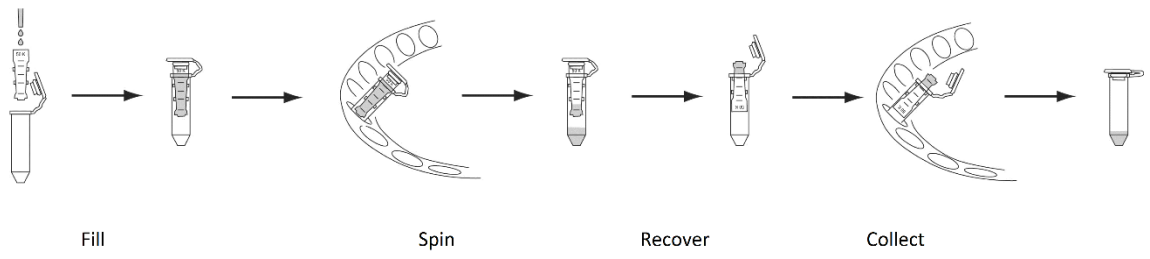

Figure 2. Antibody concentration step

If buffer exchange is needed, follow the steps below:

Buffer exchange with Desalting Spin column, 0.5 ml

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 collection 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 collect the flow-through containing the antibody in 1x TBS buffer.

Protocol for labeling of up to 2 mg of antibody

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

The antibody solution should be in 1x TBS buffer pH 7.4, with no azide. Max 2 mg in 250 µl.

Time Required: 15 minutes hands-on, 120 minutes hands-off

- Lids and bottom caps are used during the incubation
- Before centrifugation remove the bottom cap and loosen the lid (do not remove it). Save the bottom cap.

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

1.1. Let the Immobilized GlycINATOR column equilibrate to room temperature before use.

1.2. Break off the bottom plastic cap of the GlycINATOR column and slightly open the lid. Place the column in a microcentrifuge collection tube. **Note:** Save the bottom cap.

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

1.4. Place the column in the collection tube.

1.5. 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.

1.6. Repeat the steps in 1.5 **two more times**.

1.7. Re-insert the bottom cap into the bottom of the spin column.

1.8. Adjust the antibody sample volume (**containing 2 mg antibody**) to 250 µl using 1x TBS and immediately add the antibody solution to the column.

1.9. Seal the column with the lid.

1.10. Fully suspend the resin manually and make sure the resin is flowing in the column.

1.11. Incubate the column by end-over-end mixing at room temperature for 120 minutes.

1.12. Remove the bottom cap and place the column in a clean microcentrifuge tube. Loosen the top lid.

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

1.14. Attach the bottom cap. Add 100 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 microcentrifuge tube. Loosen the lid.

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

1.18. Repeat steps 1.14 to 1.17 one more time.

1.19. Pool the collected deglycosylated antibody material and adjust the sample volume to 550 µl with 1x TBS buffer.

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. Add 7 µl Buffer additive to the pooled deglycosylated antibody from step 1.19.

2.2. Add the deglycosylated antibody-solution to the GalT vial.

2.3. Reconstitute the UDP-GalNAz in 40 µl 1x TBS and transfer the solution to the GalT vial.

2.4. Mix the sample solution by carefully pipetting up and down.

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

Step 3. Removal of excess UDP-GalNAz

Time required: 1 hour

Materials from kit: 1× TBS buffer (prepared from 20x TBS),
Desalting Spin column, 2 ml

3.1. Break off the bottom plastic cap of the column and slightly open the lid. Place the column in a 15 ml collection tube.

3.2. Centrifuge the column at 1000 × g for 2 minutes to remove the storage solution. Discard the flow-through.

3.3. Place the column in the collection tube.

3.4. Add 1 ml 1× TBS buffer on top of the resin. Centrifuge the column at 1000 x g for 2 minutes and discard the flow-through.

3.5. Repeat the steps in 3.4 **two more times.** The last centrifugation should be 3 minutes**.**

3.6. Place the column in a new 15 ml collection tube.

3.7. Apply the azide activated antibody sample (from step 2.5) on top of the resin.

3.8. Centrifuge the column at 1000 x g for 3 minutes and collect the flow-through that contains the azide activated antibody.

3.9. At this stage, the azide activated antibody can be stored at 2-8 °C protected from light for conjugation at a later time.

Step 4. Conjugation with DIBO-modified label

Time required: 10 minutes hands-on, followed by overnight incubation

Materials from kit: DIBO-modified label

4.1 If the label is provided in solid form, reconstitute the DIBO-modified label in 26 µl DMSO per vial. If provided in solution it is ready for use.

4.2 Transfer the azide activated antibody in 1× TBS (from step 3.9) to a 1.5 ml centrifuge tube and add all of the DIBO-modified label from step 4.1.
Mix by carefully pipetting up and down.

4.3 Seal the tube with Parafilm® or similar.

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

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

Purification of Antibody conjugate, Optional

Time required: 1 hour

- 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.

Materials from kit: 1× TBS buffer (prepared from 20x TBS) or other buffer of choice, for example PBS.
Desalting Spin column, 2 ml

1. Break off the bottom plastic cap of the column and slightly open the lid. Place the column in a 15 ml collection tube.
2. Centrifuge the column at 1000 × g for 2 minutes to remove the storage solution. Discard the flow-through.
3. Place the column in the collection tube.
4. Add 1 ml buffer on top of the resin. Centrifuge the column at 1000 x g for 2 minutes and discard the flow-through.
5. Repeat the steps in 4 **two more times.** The last centrifugation should be 3 minutes**.**
6. Place the column in a new 15 ml collection tube.
7. Apply the antibody conjugate sample (from step 4.4) on top of the resin.
8. Centrifuge the column at 1000 x g for 3 minutes and collect the flow-through that contains the antibody conjugate.
9. The antibody conjugate can now be stored protected from light at +4-8 °C. **DO NOT FREEZE!** If preferred, sodium azide or thimerosal can be added to a final conc. of 0.02% (w/v) for long time storage.

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.

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