Azide Activation kit

October 2019

2 mg

INSTRUCTIONS

Intructions for product no:

L1-AZ1-200	GlyCLICK® Azide activation of up to 2 mg IgG

Product description

GlyCLICK® Azide activation is for selective azide activation 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 selective antibody modification. The azide activation kit introduces GalNAz site-specifically on Fc for subsequent click reaction.

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 can be followed by a "click" reaction using e. g. copper free strain promoted azide-alkyne cycloaddition (SPAAC) for selective attachment of a label. The modification primes the antibody for conjugation of the desired molecule at activated sites on the Fc regions for incorporation of two labels per antibody (DOL=2), see Figure 1.

The modification procedure is performed by combining two enzymatic steps at 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.

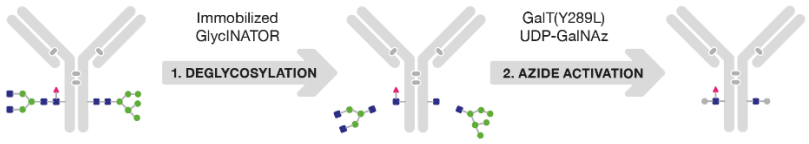

Figure 1. The modification is performed in two steps:

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*.

*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 Azide activation kit, contains enzymes, reagents and material to azide activate up to 2 mg antibody.

GlyCLICK Azide activation kit, is shipped cold and components should be stored at 4 °C to 8 °C upon arrival. **Note: Do NOT freeze!**

Table 1. Content and storage temperatures of GlyCLICK components.

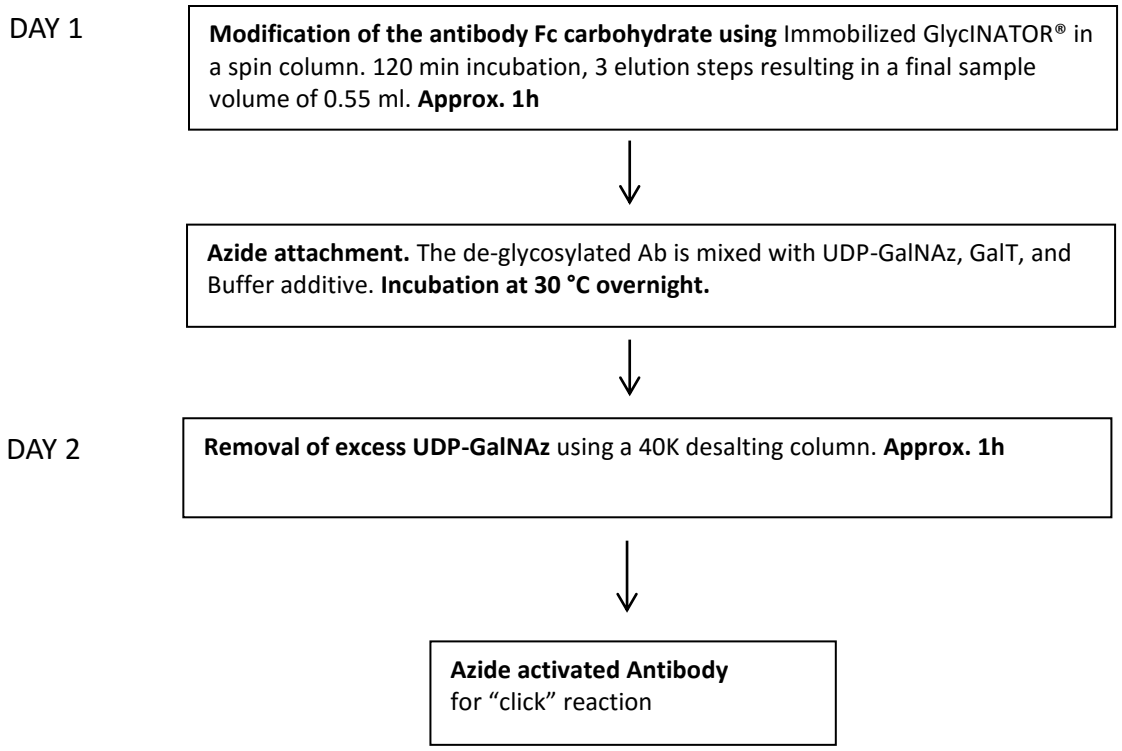
Name	Amount	Store at
Desalting Spin column, 0.5 ml, 40K	1 piece	4 °C to 8 °C
Antibody concentrator (incl 2 collection tubes), 0.5 ml, 50K	1 piece	4 °C to 25 °C
Desalting Spin column, 2 ml, 40K	1 piece	4 °C to 8 °C
Immobilized GlycINATOR®, microspin column	1 piece	4 °C to 8 °C
UDP-GalNAz	1 vial solid	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

GlyCLICK Azide activation kit is for R&D use only.

Before you begin, briefly centrifuge tubes.

Overview of the protocol for antibody azide activation using GlyCLICK®

For azide activation of 2 mg IgG



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 30 °C.
- End-over-end mixer

Additional Materials required

- Antibody, in TBS pH = 7.4, free of carrier proteins and/or azide, in a maximum volume of 0.25 ml, max 2 mg IgG. 20x TBS is provided for convenience. If buffer exchange is necessary, please follow guidance below. Desalting column (40K) for buffer exchange is provided for convenience. If concentration of the antibody sample is necessary, please follow guidance below. A concentrator (50K) is provided for convenience.
- Centrifuge tubes, 15 ml and microcentrifuge tubes.
- ddH_2O**. Note: if a chelating agent will be used as label 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

If your sample has a concentration of less than 8 mg/ml you need to concentrate your sample. Follow the steps below:

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 greater 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.

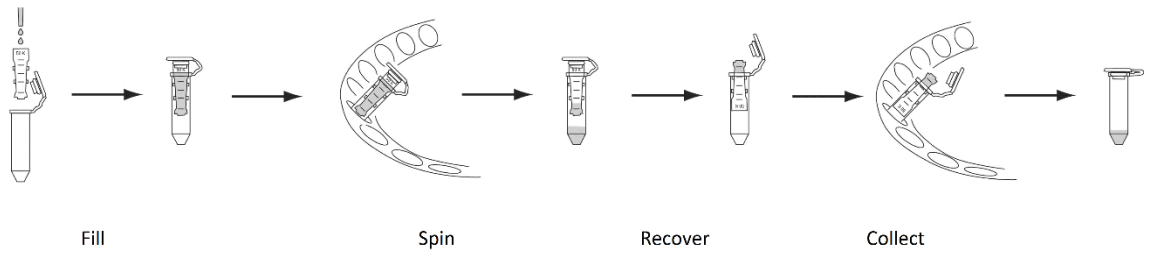


Figure 2. Antibody concentration step

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

Buffer exchange with Desalting Spin column, 0.5 ml

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. 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 replace 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 TBS buffer.

Protocol for modification of up to 2 mg antibody

Step 1. Modification of the carbohydrate on Antibody Fc domain

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 of 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 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 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 elute the 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 antibody sample.

1.18. Repeat steps 1.14 to 1.17 one more time.

1.19. Pool the collected 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 de-glycosylated antibody from step 1.19.

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

2.3. Reconstitute the UDP-GalNAz in 40 µl 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 of 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 times.** The last centrifugation should be 3 minutes**.**

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

3.7. Apply the 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-modified antibody.

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

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.