

## Azide activation kit

Version 17.1.3

100-250 µg

### INSTRUCTIONS

Instructions for product no:

L1-AZ1-025

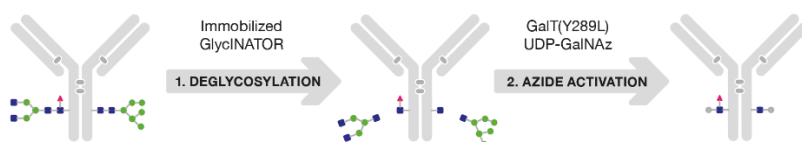
GlyCLICK™ Azide activation of up to 250 µg IgG

#### Product description

GlyCLICK™ Azide activation is for selective azide activation 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 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. strain promoted azide-alkyne cycloaddition 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.

## Content and storage

GlyCLICK™ Azide activation kit contains enzymes, reagents and material to azide activate 100-250 µg antibody.

GlyCLICK™ Azide activation kit is shipped on ice and components should be stored at different temperatures upon arrival.

**Table 1.** Content and storage temperatures of GlyCLICK™ components.

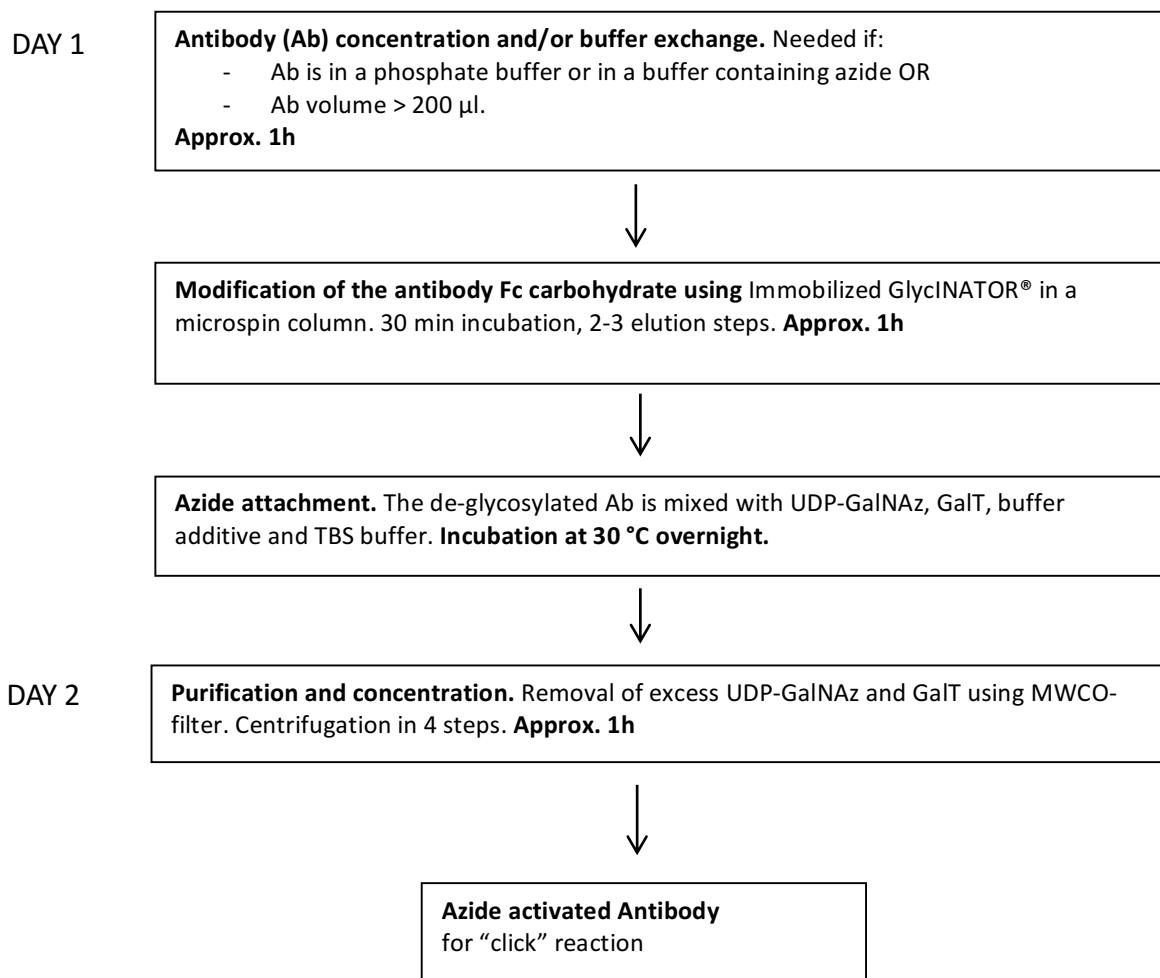
Name	Amount	Store at
Antibody concentrator (small), 50K	1 piece	4 °C to 25 °C
Collection tube for small concentrator	2 pieces	4 °C to 25 °C
Desalting Spin column, 40K	1 piece	4 °C to 8 °C
Immobilized GlycINATOR <sup>®</sup> , spin column	1 piece	4 °C to 8 °C
UDP-GalNAz (0.22 mg)	1 vial solid	4 °C to 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

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

**Do not freeze any of the components of the kit!**

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

## Overview of the protocol for antibody azide activation using GlyCLICK™



## Detailed protocol for modification 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.
- ddH<sub>2</sub>O. **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 DIBO-DFO, or other conjugation reagent with chelator, 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  $\mu\text{l}$ . If the sample volume is 100-200  $\mu\text{l}$ , 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

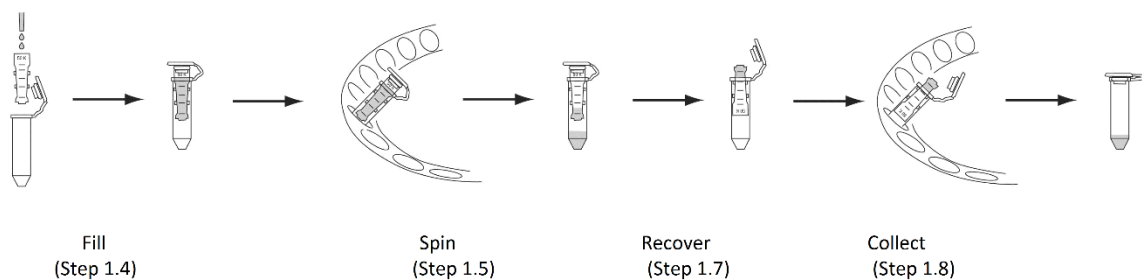
- 1.1. Add 500  $\mu\text{l}$  of ddH<sub>2</sub>O to the small antibody concentrator and cap the device as shown in Figure 2.
- 1.2. Centrifuge at 5000  $\times g$  for 6 minutes. Make sure that **the cap strap and one membrane panel of the concentrator face the center of the rotor** (Fig. 2).
- 1.3. Discard the flow-through.

##### Concentrate Antibody with small concentrator

- 1.4. Add a sufficient volume of antibody solution to contain 100-250  $\mu\text{g}$  of antibody to the small antibody concentrator. For example, if the antibody concentration is 1 mg/ml add 250  $\mu\text{l}$ .
- 1.5. Centrifuge at 5000  $\times g$  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  $\mu\text{l}$ , centrifuge for an additional 2 minutes at 5000  $\times g$ , 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  $\times g$  for 3 minutes to collect the concentrated antibody. After collection, the amount of concentrated Ab should be approximately 150-200  $\mu\text{l}$  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 (1x) by adding 500  $\mu$ l 20x TBS to 9.5 ml ddH<sub>2</sub>O 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  $\times$  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  $\mu$ l 1x TBS buffer on top of the resin. Centrifuge the column at 1500  $\times$  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 tube (1.5-2 ml).
- 1.16 Apply the antibody solution on top of the resin (100-200  $\mu$ l).
- 1.17 Centrifuge at 1500  $\times$  g for 2 min and retain the flow-through containing the antibody in TBS buffer.
- 1.18 Use the left over 1x 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:** 1x TBS buffer (prepared from 20x TBS),  
Spin column with Immobilized GlycINATOR<sup>®</sup>

- 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 (1x) by adding 500  $\mu$ l 20x TBS to 9.5 ml ddH<sub>2</sub>O in a 15 ml tube. Vortex briefly to mix.
  - 2.2. Break of the bottom plastic cap of the GlycINATOR<sup>®</sup> 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  $\times$  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  $\mu$ l 1x TBS buffer on top of the resin. Centrifuge the column at 200  $\times$  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  $\mu$ l) 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

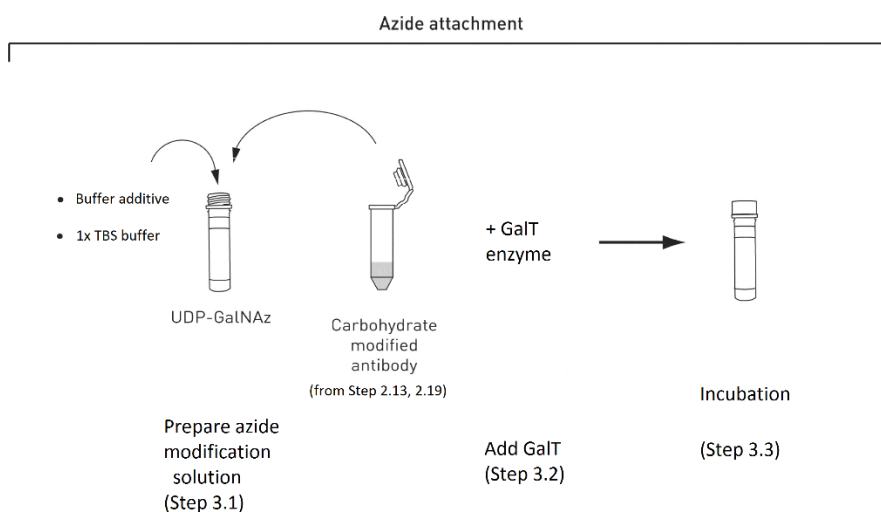
- 3.1. Prepare the azide modification solution by adding the following components to the tube containing UDP-GalNAz (Fig 3).

**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<sup>®</sup> 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 20× TBS),  
Large antibody concentrator

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

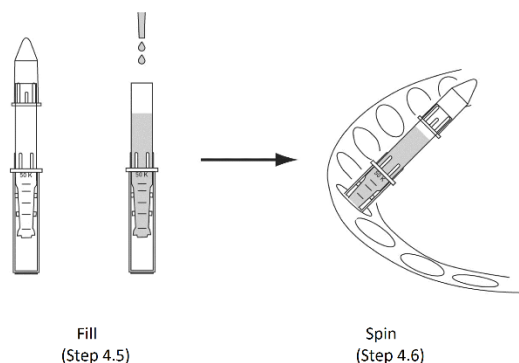
Wash antibody concentrator

- 4.1 Prepare 10 ml of 1× TBS by adding 500 µl of 20× TBS to 9.5 ml of ddH<sub>2</sub>O 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 flow-through.
- 4.11 Repeat steps 4.8 -4.10 **two times**.

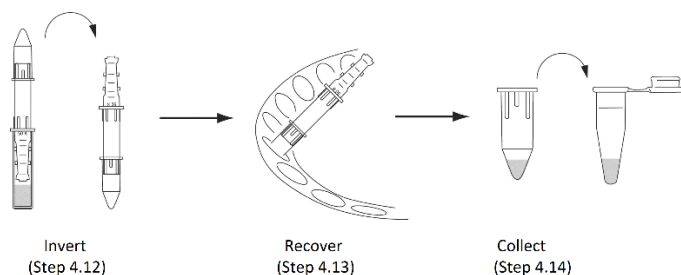
**Note:** If the antibody volume in the concentrator is greater than ~200 µl, the volume in the concentrator can be reduced by additional centrifugation e.g. for an additional 5 minutes at 1200 × g or until the appropriate volume is achieved.



**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  $1000 \times g$  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.



**Figure 5.** Collection of purified and concentrated azide-modified 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  $\alpha$ 1-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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