SmartEnzymes™

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GlyCLICK®

DFO 250 µg

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Instructions for Use

GlyCLICK® DFO 250 µg (L1-C01-025)
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Site-specific Conjugation of IgG with DFO

GlyCLICK is a site-specific conjugation technology for IgG using Fc N-glycan remodeling and click chemistry. The technology generates stable and homogenous antibody conjugates from several species and subclasses. Fc N-glycan remodeling by deglycosylation of the antibody allows for site-specific conjugation using robust click chemistry, resulting in a degree of labeling (DOL) or drug-antibody ratio (DAR) of 2.

GlyCLICK DFO is available for site-specific labeling of 250 µg or 2 mg IgG with DFO. The conjugation 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 DFO 250 µg contains all reagents needed to conjugate 250 µg IgG. The conjugation is performed in four steps:

1. **Deglycosylation:** GlycINATOR Immobilized hydrolyzes the N-glycans on the Fc-part of the IgG to the inner GlcNAc.
2. **Azide Activation:** Azide attachment on the GlcNAc using GalT (Y289L)* and UDP-GalNAz*.
3. **Click Reaction:** The azide-activated antibody reacts with a DIBO-alkyne label in a strain-promoted, copper-free click reaction (SPAAC) to form a stable and homogenous antibody conjugate.
4. **Purification:** Excess DIBO-alkyne label is removed by using a MWCO filter.

YOU MIGHT ALSO BE INTERESTED IN

GlyCLICK® Fluorophore 488
Site-specific conjugation of IgG with Alexa Fluor® 488

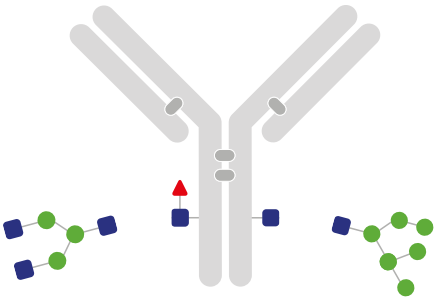
GlyCLICK® Fluorophore 555
Site-specific conjugation of IgG with Alexa Fluor® 555

GlyCLICK® Fluorophore 647
Site-specific conjugation of IgG with Alexa Fluor® 647

GlyCLICK® Azide Activation
Site-specific conjugation of IgG with azide-alkyne click chemistry

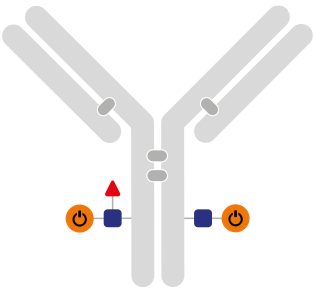
GlyCLICK® Biotin
Site-specific conjugation of IgG with biotin

* 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.



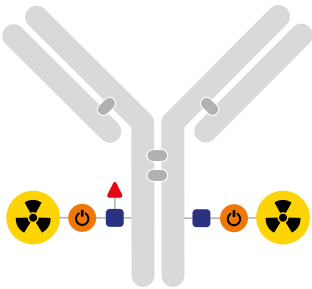
1. Deglycosylation

GlycINATOR Immobilized



2. Azide Activation

GalT + UDP-GalNAz



3. Click Reaction

DIBO-modified label: DFO

4. Purification

Figure 1. Schematic overview of the GlyCLICK technology for DFO conjugation.

Preparations

Important Information
Before you begin, briefly centrifuge tubes. Always wear suitable laboratory protective clothing and gloves when handling the reagents. **Keep in mind:** Sodium azide must be avoided throughout the protocol.

- Let the GlycINATOR Immobilized column equilibrate to room temperature before use.
- Use lids and bottom caps during the incubation.
- Before centrifugation, remove the bottom cap and loosen the lid (do *not* remove the lid).
- Since a chelating agent will be used as a label, it is important to use metal-free water (trace analysis grade) throughout the protocol. The antibody must not be in contact with glass or metal.

Additional Materials Required

- IgG in 1×TBS, pH 7.4, free of carrier proteins and/or azide. 250 µg IgG in a maximum volume of 200 µl. To adjust the antibody solution, please follow "Guidance for Concentration and Buffer Exchange". 20×TBS, a desalting spin column (0.5 ml, 40K) for buffer exchange and a concentrator (0.5 ml, 50K) are provided for convenience.
- 1×TBS: 10 ml 1×TBS is prepared by adding 0.5 ml of 20×TBS to 9.5 ml of ddH_2O. Vortex briefly to mix.
- Centrifuge tubes: 1.5-2 ml and 15 ml.
- ddH_2O.
- Dimethyl sulfoxide (DMSO) for reconstitution of DFO.

Guidance for Concentration and Buffer Exchange

It is advisable to start with more IgG than 250 µg if concentration and/or buffer exchange of the sample is needed prior to "1. Deglycosylation: Modification of the N-glycan on the Antibody Fc Domain".

A. Concentration Step

This step **is required if:**

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

If the sample volume is 150-200 µl but needs a buffer exchange (if it contains phosphate or azide), concentrate the sample to < 150 µl and then follow the steps in section "B. Buffer Exchange".

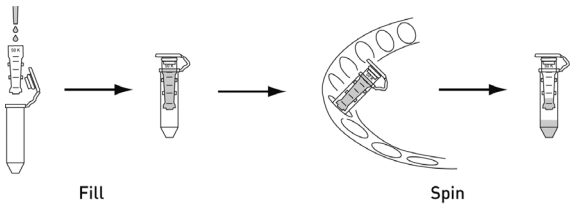


Figure 2. Antibody concentration step.

A.1 Add 500 µl of ddH_2O to the antibody concentrator (0.5 ml, 50 K) and cap the device as shown in Figure 2.

A.2 Centrifuge at 5000 × g for 6 min. **Make sure that the cap strap and one membrane panel of the concentrator face the center of the rotor** (Fig. 2).

A.3 Discard the flow-through.

A.4 Add the IgG solution to the antibody concentrator.

A.5 Centrifuge at 5000 × g for 2-6 min. **Make sure that the cap strap and one membrane panel of the concentrator face the center of the rotor** (Fig. 2).

Note: *If the IgG volume in the concentrator is more than 150 µl and the sample needs a buffer exchange, centrifuge for an additional 2 min at 5000 × g, or until the appropriate volume is achieved.*

A.6 Invert the antibody concentrator into a new collection tube as shown in Figure 2.

A.7 Centrifuge at 1000 × g for 3 min to collect the concentrated IgG. After collection, the volume of concentrated IgG should be approximately 100-150 µl in the collection tube.

B. Buffer Exchange

This step **is required if:**

- The IgG is in another buffer than 1x TBS,

and/or

- The IgG is in a buffer containing azide

B.1 Break off the bottom closure of the Desalting Spin column (0.5 ml, 40 K). Loosen the lid (do not remove the lid).

B.2 Place the column in a microcentrifuge tube and centrifuge at 700 × g for 1 min to remove the storage solution.

B.3 Discard the flow-through and place the column in the microcentrifuge tube.

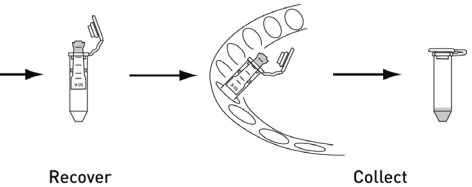
B.4 Add 300 µl of 1× TBS on top of the resin. Centrifuge at 700 × g for 1 min and discard the flow-through.

B.5 Perform step B.4 two additional times. The last time, centrifuge for 2 min.

B.6 Blot the bottom of the column to remove excess liquid. Place the column in a new microcentrifuge tube.

B.7 Apply the IgG solution (100-150 µl) on top of the resin.

B.8 Centrifuge at 700 × g for 2 min and collect the flow-through containing the antibody in 1× TBS.



Site-specific Conjugation of IgG with DFO

1. Deglycosylation: Modification of the N-glycan on the Antibody Fc Domain

Make sure that the antibody solution is 250 µg IgG in 100-200 µl of 1× TBS (pH 7.4, without azide).

Time required: 15 min hands-on, 30 min hands-off.

Materials from kit:

- 1× TBS (prepared from 20× TBS)
- Spin column with GlycINATOR Immobilized

1.1 Let the GlycINATOR Immobilized column equilibrate to room temperature before use. Break off the bottom cap of the GlycINATOR Immobilized column (save the cap) and place the column in a microcentrifuge tube. Loosen the lid.

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

1.3 Place the column in the microcentrifuge tube.

1.4 Add 300 µl 1× TBS on top of the resin. Centrifuge at 200 × g for 1 min and discard the flow-through.

1.5 Perform step 1.4 two additional times.

1.6 Insert the bottom cap.

1.7 Immediately add the antibody solution (100-200 µl) to the column.

1.8 Seal the column with the lid.

1.9 Fully suspend the media, mix by inversion and make sure there is a flow in the column.

1.10 Incubate the column with end-over-end mixing at room temperature for 30 min.

1.11 Remove the bottom cap and place the column in a new microcentrifuge tube. Loosen the lid.

1.12 Centrifuge at 1000 × g for 1 min to collect the processed material.

1.13 Insert the bottom cap. Add 50 µl 1× TBS and seal the column with the lid.

1.14 Invert the column a couple of times.

1.15 Remove the bottom cap and place the column in a new microcentrifuge tube. Loosen the lid.

1.16 Centrifuge at 1000 × g for 1 min to collect the processed material.

1.17 Repeat steps 1.13 to 1.16.

1.18 Pool the collected fractions, including the sample from step 1.12.

2. Azide Activation

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

Materials from kit:

- 1× TBS (prepared from 20× TBS)
- UDP-GalNAz
- GalT enzyme
- Buffer additive

2.1 Add 30 µl Buffer additive to the tube containing UDP-GalNAz.

2.2 Add the pooled fractions from step 1.18, and add 1× TBS to achieve a total volume of 375 µl.

2.3 Mix the solution by carefully pipetting up and down.

2.4 Add 25 µl of the GalT enzyme. The final reaction volume should be 400 µl. Mix the solution by carefully pipetting up and down. Seal the vial with the lid and wrap the lid with Parafilm® or similar.

2.5 Incubate overnight protected from light at 30°C.

3. Removal of Excess UDP-GalNAz

Time required: 1 h

Materials from kit:

- 1× TBS (prepared from 20× TBS)
- Antibody concentrator (2 ml, 50 K)

3.1 Remove the conical collection tube from the antibody concentrator as shown in Figure 3.

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

3.3 Discard the flow-through.

3.4 Add 1.6 ml of 1× TBS and 400 µl of the azide-activated antibody from step 2.5 to the antibody concentrator (Fig. 3).

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

3.6 Discard the flow-through.

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

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

3.9 Discard the flow-through.

3.10 Perform steps 3.7-3.9 two additional 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 min at 1200 × g or until the appropriate volume is achieved.*

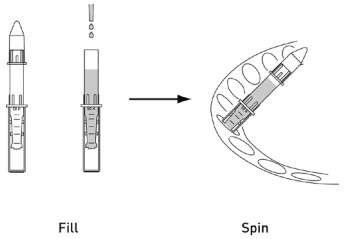


Figure 3. Use of the Antibody concentrator (2 ml, 50 K).

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

3.12 Centrifuge at 1000 × g for 3 min to collect the concentrated azide-activated antibody.

3.13 Transfer the azide-activated antibody from the conical collection tube to a microcentrifuge tube.

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

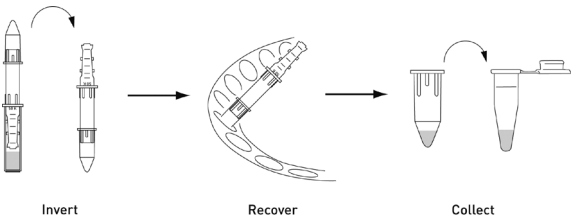


Figure 4. Collection of processed antibody material.

4. Conjugation with DFO

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

Materials from kit:

- DIBO-modified label: DFO

4.1 Adjust the volume of the azide activated antibody from step 3.14 with 1x TBS buffer to 225 µl.

4.2 Reconstitute the DIBO-DFO in 27.5 µl DMSO

4.3 Add 25 µl of DIBO-DFO to 225 µl of 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. **DO NOT FREEZE!** If preferred, sodium azide or thimerosal can be added to a final concentration of 0.02% (w/v) for long time storage.

5. Removal of Excess DFO[1]

Time required: 1 h.

Materials from kit:

- Antibody concentrator (2 ml, 50K)
- 1× TBS buffer (prepared from 20× TBS) or other buffer of choice, for example PBS

5.1 Remove the conical collection tube from a new antibody concentrator as shown in Figure 3.

5.2 Add 2 ml of 1× TBS or PBS to the antibody concentrator and centrifuge at 1200 × g for 10 min. **Make sure that one membrane panel of the concentrator faces the center of the rotor** (Fig. 3).

5.3 Discard the flow-through.

5.4 Add 1.6 ml of 1× TBS or PBS and the conjugated antibody from step 4.6 to the antibody concentrator.

5.5 Centrifuge at 1200 × g for 10 min. **Make sure that one membrane panel of the concentrator faces the center of the rotor** (Fig. 3).

5.6 Discard the flow-through.

5.7 Add 1× TBS or PBS to a total volume of 2 ml to the antibody concentrator and centrifuge at 1200 × g for 10 min. **Make sure that one membrane panel of the concentrator faces the center of the rotor** (Fig. 3).

5.8 Discard the flow-through.

5.9 Perform steps 5.7-5.8 at least two additional times.

Note: If an 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 min at 1200 × g or until the appropriate volume is achieved.

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

5.11 Centrifuge at 1000 × g for 3 min to collect the antibody conjugate.

5.12 Transfer the antibody conjugate from the conical collection tube to a 1.5 ml microcentrifuge tube.

5.13 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 concentration of 0.02% (w/v) for long time storage.

1. This step is optional and dependent on your application.

CONTENT AND STORAGE

GlyCLICK DFO 250 µg contains several components. The product is shipped cold, and the components should be stored at different temperatures upon arrival (see Table 1).

GlyCLICK DFO is for R&D use only.

Table 1. Content and Storage Temperatures of GlyCLICK Components

Name	Amount	Store at
Antibody concentrator, 0.5 ml, 50K	1 piece	4-25 °C
Collection tube for antibody concentrator, 0.5 ml, 50K	2 pieces	4-25 °C
Desalting Spin column, 0.5 ml, 40K	1 piece	4-8 °C
GlycINATOR Immobilized, Microspin column	1 piece	4- 8 °C
UDP-GalNAz	1 vial solid	4-8 °C Protect from light
20× TBS pH 7.4 (0.5 M)	1 × 1.8 ml	4-8 °C
Buffer additive	1 × 30 µl	4-8 °C Protect from light
β-1,4-galactosyltransferase GalT (Y289L)	1 × 25 µl	4-8 °C Protect from light
Antibody concentrator, 2 ml, 50 K	2 pieces	4-25 °C
DIBO-modified label: DFO	1 vial solid	(-25)-(-5)°C Protect from light

USA & Canada
Genovis Inc.
10919 Technology Place Suite C, San Diego, CA 92127, USA
Phone: 1-855-782-0084 (toll free)
Fax: 1-858-524-3006

EMEA & Asia
Genovis AB
Box 4, SE-244 21 Kävlinge, Sweden
Phone: +46 46 10 12 30
Fax: +46 46 12 80 20

support@genovis.com
www.genovis.com

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