INSTRUCTIONS

Last revised July 2019

Instructions for product no:

G3-OC6-002	Binding of up to 4 × 50 µg glycoprotein with mucin type O-glycosylation

Content and storage

GlycOCATCH™ box contains:

- 4 Microspin columns with GlycOCATCH resin (G1-OC6-001), each column includes sufficient material to bind 50 µg glycoprotein with mucin type O-glycosylation. The resin is supplied in 20% EtOH with no preservatives added.
- 1 vial of SialEXO® (200 units, G1-SM1-002) supplied lyophilized in TBS pH 7.6, with no preservatives added.
- 1 vial of OpeRATOR® (200 units, G1-OP1-002) supplied lyophilized in TBS pH 7.6, with no preservatives added.

GlycOCATCH box is shipped cold.
GlycOCATCH Microspin columns should be stored at +4-8°C upon arrival. **Do not freeze the columns!**
SialEXO and OpeRATOR vials should be stored at -20°C upon arrival. After reconstitution, the SialEXO and OpeRATOR are stable for at least 1 month at +4-8 °C.

GlycOCATCH box is for R&D use only.

Product Description

GlycOCATCH is a resin for affinity purification and selective enrichment of glycoproteins and glycopeptides with mucin type O-glycosylation. The affinity ligand is an engineered inactive version of the O-glycan dependent OpeRATOR protease. The GlycOCATCH agarose resin specifically binds glycoproteins and glycopeptides with mucin type O-glycosylation, selectively targeting O-glycan and not N-glycan sites. Asialylated O-glycoproteins with mucin type O-glycosylation are bound efficiently to the resin and the sialidase mix SialEXO, for removal of sialic acids, is included in the box.

In general, O-glycoproteins and O-glycopeptides with mucin type glycosylation bind to the resin at pH 5-8 and are recovered with 8 M urea (i.e. in a denatured state) in a 1 hour workflow. Alternatively, the OpeRATOR enzyme, an O-protease, can be used as an elution method. Bound O-glycoproteins are then eluted as peptides, with a size dependent on the positions of the O-glycan sites. At elution, the bound O-glycoproteins are digested N-terminally of the O-glycan sites, without the presence of a denaturing agent, and resulting peptides are recovered under native conditions.

SialEXO is a mix of sialidases for efficient removal of sialic acids on O-glycosylated and N-glycosylated proteins. SialEXO hydrolyzes glycoproteins under native conditions and displays a high activity in a broad pH range, 6.5 to 9. The mix is composed of two sialidases for highly efficient hydrolysis of α2-3, α2-6 or α2,8-linked sialic acids. The enzymes in SialEXO are derived from *A. muciniphila* and expressed in *E. coli*. The enzymes contain His-tags and the molecular weights are 43 kDa and 66 kDa, respectively.

OpeRATOR enzyme is an endoprotease digesting glycoproteins with mucin type O-glycosylation N-terminally of the Serine and Threonine glycosylation sites under native conditions. It is highly specific and digests the peptide backbone only in presence of mucin type O-glycans, generating distinct O-glycopeptides suitable for applications such as O-glycan profiling, O-glycan site determination, O-glycopeptide mapping and middle down approaches. OpeRATOR is active on O-glycan proteins with sialic acids but the enzymatic activity is higher if the sialic acids are removed. OpeRATOR enzyme is derived from *A. muciniphila* and expressed in *E. coli*. The enzyme contains a His-tag and the molecular weight is 42 kDa.

Binding Capacity and Unit Definitions

50 µl of GlycOCATCH resin binds ≥ 50 µg asialylated etanercept when incubated in 10 mM sodium phosphate, 150 mM NaCl, pH 7.4 at room temperature for 30 min.
One unit of SialEXO hydrolyzes sialic acids from ≥ 90% 1 µg glycoprotein (fetuin) when incubated in 20 mM Tris pH 6.8 at 37°C for 2h.
One unit of OpeRATOR digests ≥ 90% of 1 µg glycoprotein (TNFR) when incubated with one unit of SialEXO in 20 mM Tris pH 6.8 at 37°C for 2 h.

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Quality Control

All components in the GlycOCATCH box are tested to meet specifications and tested for absence of microbial contamination with blood agar plates, Sabouraud dextrose agar plates and fluid thioglycollate medium.

Additional Materials Required

- Binding buffer[1]: PBS (10-150 mM sodium phosphate, 150 mM NaCl, pH 7.4) or 50-150 mM Tris, 150 mM NaCl, pH 7-8.
- Wash buffer: Binding buffer with optional addition of up to 1 M NaCl and/or 0.5 M urea.
- Elution buffer for elution with urea: 8 M urea in PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4).
- Elution buffer for elution with OpeRATOR: 50 units of OpeRATOR in 100 µl PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4) for elution of one column.
- Collection tubes: Micro centrifuge tubes (1.5-2 ml).

Detailed Protocol

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

Sample preparation

- Prepare the protein sample solution to be affinity purified in 100-300 µl binding buffer[2] / column. The sample solution can contain up to 0.5 M urea.

  Please note: If the sample has been treated with proteases (like trypsin) relevant inhibitors must be added before applying the sample on the column.

Affinity Purification on the GlycOCATCH™ column

- Reconstitute SialEXO in 20 µl ddH_2O to a concentration of 10 units / µl.
- If elution will be performed with OpeRATOR (see below Elution Option 2): reconstitute OpeRATOR in 20 µl ddH_2O to a concentration of 10 units / µl.

Equilibration

1. Break off the bottom seal (save the cap) of the column and loosen the lid.
2. Place the column in a 1.5-2 ml collection tube.
3. Centrifuge the column at 200×g for 1 min to remove the storage solution.
4. Equilibrate the column by adding 300 µl binding buffer.
5. Centrifuge the column at 200×g for 1 min.
6. Repeat steps 4 and 5 two times.
7. Seal the spin column with the bottom cap.

Desialylation and Binding

8. Add 50 units of SialEXO to the mucin type O-glycoprotein sample solution for one column[3].
9. Add the prepared protein sample solution (100-300 µl) to the column.
10. Seal the column with the top lid.
11. Take care to fully suspend the media, mix by inversion and **make sure the resin is flowing in the column**.
12. Incubate the column by end-over-end mixing at room temperature for 30 min - 2 h[4]. **A good mixing is important for optimal performance.**
13. Remove the bottom cap and place the column in a 1.5-2 ml collection tube. Loosen the top lid.
14. Centrifuge the column at 1000xg for 1 min to collect the flow through (FT).
15. Seal the spin column with the bottom cap.

Washing

16. Wash the column by adding 300 µl wash buffer. Mix by inversion of the column.
17. Remove the bottom cap and place the column in a 1.5-2 ml collection tube. Loosen the top lid.
18. Centrifuge the column at 200×g for 1 min.
19. Seal the spin column with the bottom cap.
20. Repeat washing steps 16-19 at least 3-5 times[5].
21. Seal the spin column with the bottom cap.

Elution of O-glycoproteins / O-glycopeptides

Option 1- with urea

1. Add 50 µl elution buffer (8 M urea in PBS) to the column.
2. Seal the column and mix by tapping the tube and incubate at RT for 5 min.
3. Remove the bottom cap and place the column in a 1.5-2 ml collection tube. Loosen the top lid.
4. Centrifuge the column at 1000×g for 1 min to collect the eluate.
5. Repeat elution steps 1 to 4 once more. Use the same collection tube.

Option 2 – with OpeRATOR

1. Add 50 units of OpeRATOR in 100 µl PBS for one column.
2. Take care to fully suspend the media, mix by inversion and **make sure the resin is flowing in the column**.
3. Incubate the column by end-over-end mixing at 37°C overnight. **A good mixing is important for optimal performance.**
4. Remove the bottom cap and place the column in a 1.5-2 ml collection tube. Loosen the top lid.
5. Centrifuge the column at 1000×g for 1 min to collect the eluate.

After elution of the O-glycan containing fraction, the samples can be further processed with trypsin (or other suitable protease) and prepared for LC-MS analysis.

After elution with OpeRATOR, long peptides can be generated depending on the position of the O-glycans and the distance between O-glycan sites. Further proteolytic digestion with trypsin (or other suitable protease) may be needed to obtain shorter peptides prior to LC-MS analysis.

Notes

1. Optimal binding is obtained in sodium phosphate (10-150 mM) and Tris (50-150 mM) buffers at pH 6.0-8.0. Sodium chloride up to 1 M and/or 0.5 M urea can be added without affecting the performance of the column/purification. Optimal buffer conditions may need to be tested for the glycoprotein solution to be purified.
2. The volume should be at least 100 µl / column and can be increased up to 300 µl / column.
3. Sialic acids need to be removed for optimal performance of GlycOCATCH. Optionally, desialylation of glycoproteins with SialEXO can be performed before affinity purification on the GlycOCATCH column by adding 1 unit of SialEXO / µg of glycoprotein and incubate at 37°C for 2 h.
4. Incubation time may need optimization depending on sample composition and glycoproteins.
5. Extended washing, up to ten or more times, may decrease unspecific binding.
