# *Protocol for enzymatic desialylation of native apo-transferrin*

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## Abstract

This protocol describes the method used for desialylation of native apo-transferrin. Desialylation is done by incubation of immobilized neuraminidase enzyme in the buffered solution of native apo-transferrin for 48 hours. After incubation, the desialylated sample is collected, washed out using a suitable buffer and concentrated by centrifugal filtration. The percentage recovery of the obtained desialylated transferrin is calculated from the absorbance at 280 nm.

## Keywords

transferrin, desialylation, neuraminidase, enzyme

## 1.1 Materials and instrumentation

a) GlycoCleave® Immobilized Enzymes for Glycoanalysis (GALAB Technologies GmbH, cat. no. 132011 or 132012) suspension of immobilized neuraminidase (sialidase, N-acetylneuraminyl hydrolase, E.C. 3.2.1.18, from Vibrio cholerae culture filtrates) [1]
b) Human apo-Transferrin protein (Biorbyt Ltd, cat. no. orb80927) [2]
c) Sodium acetate trihydrate (Kemika, cat. no. 14481)
d) Calcium chloride, dehydrated (Fluka, cat. no. 21079)
e) Sodium chloride, cryst. (Kemika, cat. no. 14175)
f) Hydrogen chloride, 37 % (Carlo Erba Reagents, cat. no. 403871)
g) Sodium hydroxide, pellets 2 – 5 mm (Kemika, cat. no. 1452506)
h) Merck Millipore Ltd. Amicon® Ultra – 0.5 mL Centrifugal Filters (Ultracel® – 30 K, cat. no. UFC503096) [3]
i) Eppendorf® Centrifuge 5424
j) Incubator INCU-Line®, IL 53 (VWR)
k) Tube Revolver / Rotator (Thermo Scientific, cat.no. 88881001)
l) Water, double distilled in an all-glass apparatus

## 1.2 Glycocleave protocol and separation.

### 1.2.1 Re-suspend the immobilized enzyme slurry.

Take the GlycoCleave Neuraminidase kit (GALAB Technologies GmbH) and gently re-suspend the immobilized enzyme slurry by turning the bottle upside down several times. **Do not shake the enzyme suspension.**

### 1.2.2 Add slurry into the 1.5 mL centrifuge tube.

Immediately after the re-suspension add 400 µL of the slurry into the centrifuge tube. Neuraminidase beads should consist roughly half (200 µL) of the total volume, the rest of the volume is suspension (storage) buffer. **GlycoCleave Neuraminidase kit bottle should be stored at 2-8 °C**.

### 1.2.3 Prepare working and storage buffers.

a) **Working buffer** is prepared by dissolving the appropriate mass of NaOAc and $CaCl_2$ in redistilled water. The final solution consists of 0.2 mol / L sodium acetate and 0.001 mol / L calcium chloride; pH is to be adjusted to 5.5.
b) **Storage buffer** is prepared by dissolving the appropriate mass of NaOAc, $CaCl_2$ and NaCl in redistilled water. The final solution consists of 0.05 mol / L sodium acetate, 0.009 mol / L calcium chloride and 0.15 mol / L sodium chloride; pH is to be adjusted to 5.5.

The prepared solutions should be filtered using 0.2 µm filter and stored at 2-8 °C. The solutions should be allowed to reach room temperature before use.

### 1.2.4 Prepare apo-transferrin solution.

Prepare apo-transferrin solution $\gamma$ = 6.25 mg / mL in working buffer. For each centrifuge tube (containing 200 µL beads) prepare 800 µL of the apo-

transferrin solution (corresponding to 5 mg protein). For larger batches use multiple centrifuge tubes. **After dissolving transferrin in the buffer check the pH value and adjust to pH=5.5 if necessary** by adding small volume of HCl or NaOH solution ($c \leq 0.5$ mol / L). Since working buffer consists of 0.2 mol / L acetate buffer, significant change in pH is not to be expected during the dissolution of transferrin. However, value of pH = 5.5 is crucial for successful reaction and should always be verified.

### 1.2.5 Wash out neuraminidase beads with working buffer.

The centrifuge tube containing about 200 µL of enzyme beads and 200 µL of storage buffer (from section 1.2.2) is centrifuged for 1 minute (1200 RCF). Supernatant containing storage buffer is to be removed using a micropipette. Good practice is not to fully remove the supernatant to prevent loss of neuraminidase beads. Washing neuraminidase beads with the working buffer is done by adding 800 µL of working buffer to the beads. Centrifuge tube should be gently turned upside down several times in order to resuspend the beads. **Do not shake the enzyme suspension.** Tube is the centrifuged for 1 minute (1200 RCF) and supernatant is removed. Wash neuraminidase beads with working buffer at least 3 times.

### 1.2.6 Add transferrin solution to the centrifuge tube.

Once the centrifuge tube containing 200 µL of enzyme beads has been washed at least 3 times with working buffer, remove the remaining supernatant and add approximately 800 µL of the apo-transferrin $\gamma$ = 6.25 mg / mL solution (from section 1.2.4). For batches larger than 5 mg protein use multiple centrifuge tubes prepared as described.

### 1.2.7 Add centrifuge tube to the incubator.

Add centrifuge tube(s) containing enzyme beads and apo-transferrin solution to the incubator. **Incubator should be preheated to 37 °C**. Attach the centrifuge tube(s) to the tube revolver rotating at 10 rpm and **incubate for at least 24 hours or longer** and then remove from the incubator. Very high degree of apo-transferrin desialylation can be obtained in roughly 48 h.

### 1.2.8 Collect desyalized transferrin solution.

After incubation, the tube(s) containing enzyme beads and apo-transferrin solution should be centrifuged for 1 minute (1200 RCF). Collect the supernatant using a micropipette into another 1.5 mL tube and centrifuge again for 1 minute (1200 RCF) in order to remove any remaining beads. Collect and store the supernatant containing the desialylated apo-transferrin. If possible, filter the solution using 4mm 0.2 µm syringe filter. The solution should be stored at 2-8 °C if not immediately processed further (step 1.2.10).

### 1.2.9 Wash out neuraminidase beads with storage buffer.

Wash the enzyme beads at least 3 times with storage buffer by adding 800 µL (per tube) of storage buffer each time, centrifuge the tube for 1 minute (1200 RCF) and remove the supernatant. Gently turn the centrifuge tube upside down several times before each centrifuge cycle. It is good practice to also save the supernatant after the first wash out since it can contain an appreciable amount of desialylated transferrin. After the 3 wash out cycles add 800 µL of the

storage buffer to the enzyme beads and store at 2-8 °C. It is advisable to keep a record of the neuraminidase beads usage because they can inactivate when often used or improperly stored. Additionally, **keep separate beads for separate proteins to avoid contamination.**

### 1.2.10 Wash and concantrate protein using centrifugal concentrator.

Add 0.5 mL of desialylated transferrin solution to an Amicon® Ultra MWCO 30 kDa centrifugal concentrator. Centrifuge for 5 minutes (12000 RCF). It is a good practice to temporarily save the filtrate so you can recover your sample in case of accidental membrane leakage. After each centrifuge cycle remove the filtrate and add the additional 0.5 mL of the protein solution (or slightly less depending on the volume of the concentrate).

Once all the protein solution has been concentrated, wash the sample at least 3 times with a suitable buffer by adding 0.5 mL of the buffer for each centrifuge cycle. If FPLC chromatofocusing is to be performed afterwards it is likely best to wash out the protein with the starting buffer in your chromatofocusing experiment.

### 1.2.11 Determine protein concentration and recovery.

After protein samples are concentrated and washed with the appropriate buffer, transfer the concentrate into a 0.5 mL tube. While transferring the protein with a micropipette try to determine the volume of the concentrate as precisely as possible.

Measure the sample absorbance at 280 nm and determine the protein concentration using the measured molar absorption coefficient. [4] Use the pure washing buffer from step 1.2.10 for baseline. If possible, use quartz cuvettes for measurements. Calculate the approximate recovery percentage from the determined protein concentration and the volume of protein concentrate.

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