

Glycolmage®



Glycolmage® Sialic Acid Kit

Determination of sialic acid activity in milk and milk-derived samples

Cat.No 171011/ 171013

User Guide

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GlycoImage® Sialic acid Kit Contents

Lectin conjugate	1 vial (50 µL)
Enzyme conjugate	1 vial (20 µL)
Substrate	1 vial (28 mg)
Inhibition Control	1 vial (2 mL)
Assay Buffer (20x concentrate)	2 bottles (à 25 mL)
Detection Buffer („ready-to-use“)	1 bottle (15 mL)
Equilibration Buffer (2x concentrate)	1 bottle (25 mL)
Microplate	1
CD	1

Storage

Store the kit at 2°C to 8°C. Solutions contain 0.05% sodium azide as a preservative. The stability of the kit is at least 6 month.



For best results please maintain a constant temperature of 25°C (+/- 2°C) during incubation cycles and measurement. Avoid a direct exposure of the plate and the reagents to sun light. The Assay Buffer has to be kept at 2°C to 8°C during assay procedure.

Safety- and Product information

Handling of chemicals always requires wearing of safety clothes. For more information please read the material safety data sheets (MSDS). MSDS are provided on CD or at www.galab.de/technologies

The kit and the kit components are not for diagnostic, pharmaceutical or in-vivo use.

Instrumental Requirements

- microplate reader for measurements at 405nm
- pipettes/ multichannelpipettes (16-channel)/ multistepper

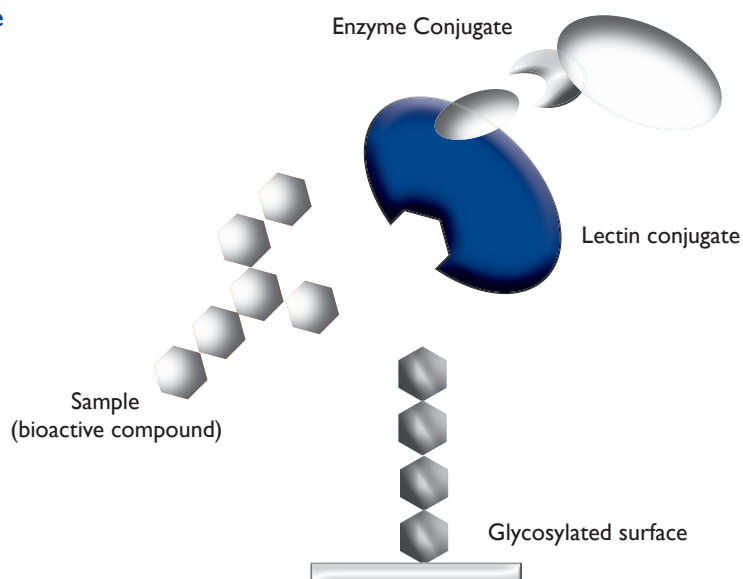
Field of application and principle

Glycolmage® Sialic Acid Kits are designed for the determination of conjugated, bioactive sialic acid in milk and milk-derived samples, including processed milk fractions like whey, caseinomacropeptide or buttermilk. The results are expressed as “SA = sialic acid activity”. The analytical kit allows a quick and direct comparison of up to 15 samples.

Acid oligosaccharides and sialylated glycoconjugates show prebiotic effects. Many other physiological effects are also described in the literature [1]. Due to these effects the compounds may derive important ingredients in dietary products, e.g. infant food, whey products and functional food. Unfortunately bound sialic acid shows a limited stability in view of conventional manufacturing steps like heating, drying or protein hydrolysis. The degradation of bound sialic acid results in a decrease of its biological (e.g. prebiotic) activity.

This can be monitored using the value of **SA = sialic acid activity**. The **Sialic acid activity needs to be preserved for high quality products**. The analysis using the Glycolmage® Sialic Acid Kit provides a fast and easy activity control of your products, raw materials or other samples of interest. Preservation of the sialic acid activity may require a modification of manufacturing steps, the choice of high value raw materials or improvement of storage conditions of the ingredients and products.

Assay Principle



The SA determination is based on a competitive lectin assay. The microplate-surface presents sialylated structures. They act as a ligand for the lectin conjugate. This lectin binding is inhibited by sialylated glycoconjugates which are present in the milk sample.

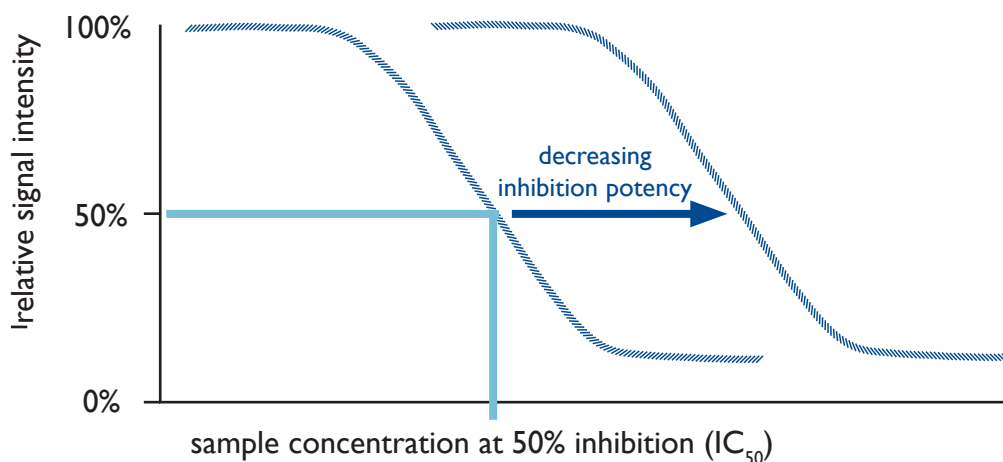
Two sialic acid recognising lectins are used in the Glycolmage kits: SNA and MAL. SNA is an agglutinin from elderberry bark (*Sambucus nigra*) which shows a specificity towards α 2,6-linked sialic acid. MAL (*Maackia amurensis* Leukoagglutinin) recognises glycostructures with α 2,3-linked sialic acid.

Assay results

As a first assay result an inhibition curve is generated. The degree of inhibition depends on the concentration of sialylated compounds and on their structure (type of linkage, multivalency). These characteristics together determine the physiological value of a milk sample.

They are expressed as “Sialic acid activity” (SA)

$$SA = \frac{1}{IC_{50}^{**}}$$



A high “Sialic acid activity” SA of a milk sample results from a high degree of complex sialylated structures. Thus the determination of SA allows the evaluation of the physiological quality of milk-derived samples. The Glycolmage assay kits provide a versatile tool to compare and evaluate milk products like whey, caseinomacropptide, buttermilk, formula or milk powder.

** IC50 = Sample concentration at 50 % inhibition of lectin binding. The assay kit provides a convenient calculation sheet where the SA is directly calculated from the IC50 values.

Preparation of kit reagents

*The kit offers the possibility for measurement of 16 samples per microplate (in triplicates).
The estimated time for running the protocol is 4-5 h. Preparations are calculated for **one** plate.*

Preparation of Buffer Solutions

- 1 The Assay Buffer** is a 20-fold concentrated solution.
Dilute the content of one bottle completely with 475 mL deionised water to a volume of 500mL.
The second bottle can be used, when working with an automated microplate washer.
Please keep the Assay Buffer at 2°C to 8°C even during the assay procedure.
- 2 The Equilibration Buffer** is 2-fold concentrated.
Dilute the content of one bottle completely with 25 mL deionised water to a volume of 50 mL.
- 3 The Detection Buffer** is „ready-to-use“.
- 4 Lectin Conjugate**
The lectin conjugate is 1000-fold concentrated.
(Dilute 10µL of the lectin conjugate with 9.990 µL Assay Buffer up to 10 mL)
- 5 Inhibition Control**
The Inhibition Control is „ready-to-use“.
- 6 Enzyme Conjugate**
The enzyme conjugate is 10.000-fold concentrated.
(In a first step, dilute 10µL of the enzyme conjugate with 990 µL Buffer Solution A up to 1mL (dilution 1), in a second step dilute 100µL of dilution 1 with 9,9mL Buffer Solution A up to 10 mL.)
- 7 Substrate**
Dissolve the whole content (28 mg) of one substrate vial in 10mL Detection Buffer ($\beta=2,8\text{mg/mL}$).
Prepare fresh solution **immediately** before measurement.

Sample preparation

Samples have to be diluted in Assay Buffer. Prepare a dilution range of the samples as follows:

Caseinomakropeptid (CMP)

CMP-samples have to be diluted to a start concentration of 0,5%. Prepare at least 500 µL of this solution. Prepare five more dilution steps in a serial dilution of 1:2 (250µL buffer solution A + 250 µL sample).

Buttermilk

Buttermilk is a complex mixture of several glycoconjugates, containing oligosaccharides, glycoproteins and glycolipids. The start dilution for the determination of buttermilk is 1:8, this corresponds to a mass concentration of approximately 12 g/L. Make at least 500 µL of this first dilution. Prepare five more dilution steps in a serial dilution of 1:2 (250µL buffer solution A + 250 µL sample).

Milk powder/ Whey powder/ Formula

For determining milk-based powder the samples have to be dissolved at a concentration of 10 g/L. This concentration is only an approximate value and can vary in individual cases. Make at least 500 µL of this first dilution. Prepare five more dilution steps in a serial dilution of 1:2 (250µL buffer solution A + 250 µL sample).

For other sample types it can be required to remove milk fat by centrifugation

GlycoImage® Sialic Acid - Protocol

Sample application

Use the following scheme for the application of your samples to the microplate. This scheme corresponds to the „data interpretation sheet“.

One microplate provides the possibility to determine 4 samples in triplicates.

- Add 20µL of Assay Buffer to the wells of row A.**
- Add 20µL of the Inhibition Control to the wells of row B.**
- Add 20 µL of the samples to the wells.**

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	//		24
A																	
B																	
C																	
D																	
E																	
F																	
G																	
H																	
I																	
J																	
K																	
L																	
M																	
N																	
O																	
P																	

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	Sample 1	DILUTION 1										
D	Sample 1	DILUTION 2										
E	Sample 1	DILUTION 3										
F	Sample 1	DILUTION 4										
G	Sample 1	DILUTION 5										
H	Sample 1	DILUTION 6										

- After the application of buffer and samples, add 20 µL of the lectin conjugate to each well.**

Please determine, if it is necessary to centrifuge your plate !

GlycoImage® Sialic Acid - Protocol

Incubation und wash procedure

Incubate the microplate for 2h at room temperature (max. 25°C) in the dark. After incubation fridge the plate for 5 minutes and wash with cold Assay Buffer.

Therefor, empty the plate and pipette 50µL Assay Buffer in each well. Empty the plate again and drop it out onto a paper tissue. Repeat this procedure five times.

Enzyme conjugate

Add 20 µL of diluted enzyme conjugate to each well.

Incubation und wash procedure

Incubate the microplate for 2h at room temperature (max. 25°C) under light exclusion. After incubation fridge the plate for 5 minutes and wash with cold Assay Buffer.

Therefor, empty the plate and add 50µL Assay Buffer per well. Empty the plate again and drop it out onto a tissue. Repeat this procedure three times.

After the last cycle, wash the plate two times with the Equilibration Buffer.

Application of substrate and measurement

Immediately before measurement, add 20 µL substrate solution to each well.

The measurement occurs in two intervals.

interval 1: after 5 Minutes
interval 2: after 10 Minutes

wavelength: 405 nm

Glycolmage® Sialic Acid - Protocol

Data Interpretation

The analysis sheet is prepared for the determination in triplicates. The interpretation is based on the slope of the enzymatic reaction. This slope has to be calculated using the raw data. Some types of microplate reader are able to display the slope directly.

Insert the calculated data into the „raw data“ sheet. Based on these data, the Sialic Acid Activity (SA) is calculated.

Slope (calculated from raw data)

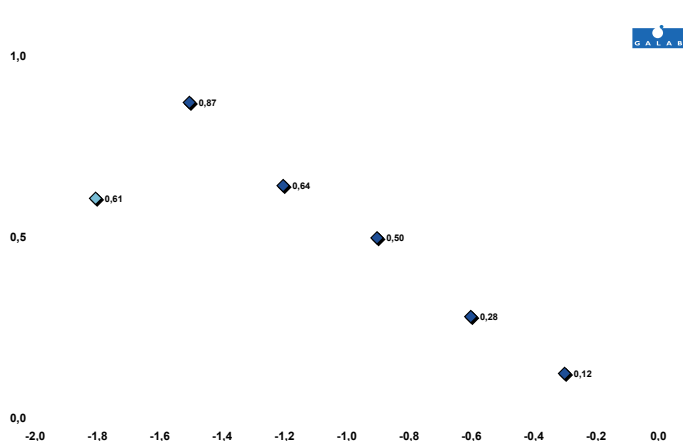
	1	2	3	4	5	6	7	8	9	10	11	12
A	0,0154	0,0168	0,0155	0,0154	0,0168	0,0155	0,0154	0,0168	0,0155	0,0154	0,0168	0,0155
B	0,0003	0,0003	0,0004	0,0003	0,0003	0,0004	0,0003	0,0003	0,0004	0,0003	0,0003	0,0004
C	0,0019	0,0025	0,0024	0,0019	0,0025	0,0024	0,0019	0,0025	0,0024	0,0019	0,0025	0,0024
D	0,0049	0,0043	0,0049	0,0049	0,0043	0,0049	0,0049	0,0043	0,0049	0,0049	0,0043	0,0049
E	0,0075	0,0084	0,0083	0,0075	0,0084	0,0083	0,0075	0,0084	0,0083	0,0075	0,0084	0,0083
F	0,01	0,0112	0,0097	0,01	0,0112	0,0097	0,01	0,0112	0,0097	0,01	0,0112	0,0097
G	0,0134	0,016	0,0122	0,0134	0,016	0,0122	0,0134	0,016	0,0122	0,0134	0,016	0,0122
H	0,015	0,012	0,015	0,013	0,013	0,013	0,013	0,010	0,008	0,010	0,011	0,009

... up to no. 24

... up to
line 'P'

The „data interpretation“ sheet shows your calculated values. In this sheet, you have to insert the sample concentrations. Further, you have to define the linear range of each sample in the table (the chart is intended as a support).

sample no.	1	2	3	4
sample	sample 1	sample 2	sample 3	sample 4
positive control (fr. row A)	0,016	0,016	0,016	0,016
blank (fr. row B)	0,000	0,000	0,000	0,000
sample concentration (*):				
0,50	0,002	0,002	0,002	0,002
0,25	0,005	0,005	0,005	0,005
0,13	0,008	0,008	0,008	0,008
0,06	0,010	0,010	0,010	0,010
0,03	0,014	0,014	0,014	0,014
0,02	0,014	0,013	0,010	0,010
-0,30	0,12	0,12	0,12	0,12
-0,60	0,28	0,28	0,28	0,28
-0,90	0,50	0,50	0,50	0,50
-1,20	0,64	0,64	0,64	0,64
-1,51	0,87	0,87	0,87	0,87
-1,81	0,86	0,82	0,65	0,61
slope	-0,64	-0,64	-0,64	-0,64
intercept	-0,10	-0,10	-0,10	-0,10
regression	0,99	0,99	0,99	0,99
Sialic Acid Activity	8,7	8,7	8,7	8,7

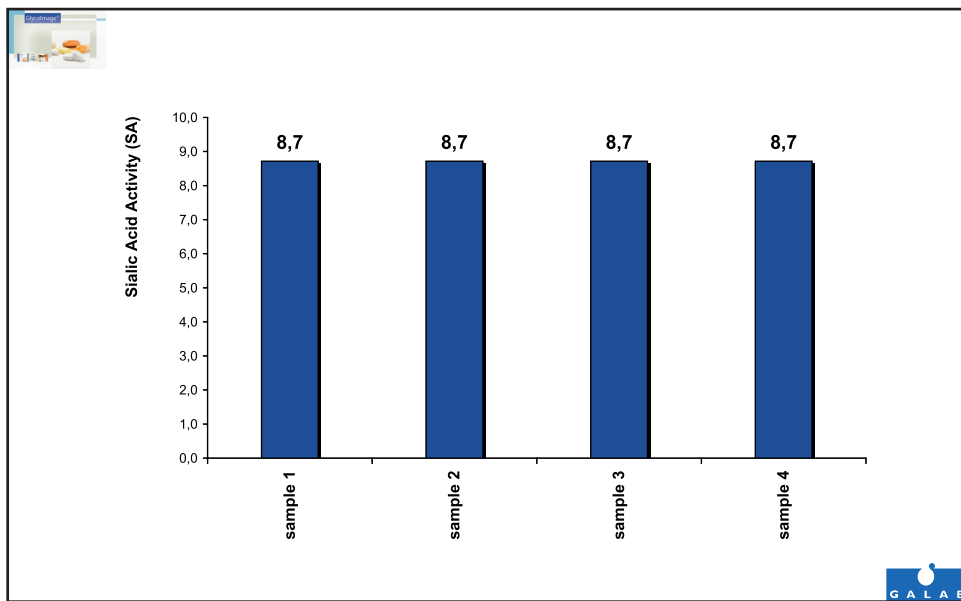


(* please insert)

Glycolmage® Sialic Acid - Protocol

Data interpretation occurs automatically after the insert of raw data and definition of the linear range. Within the defined range, the sample concentration is calculated, which leads to an inhibition (signal reduction) of 50%. The reciprocal value of this concentration, the Sialic Acid Activity, is then calculated and shown in the „results“ chart.

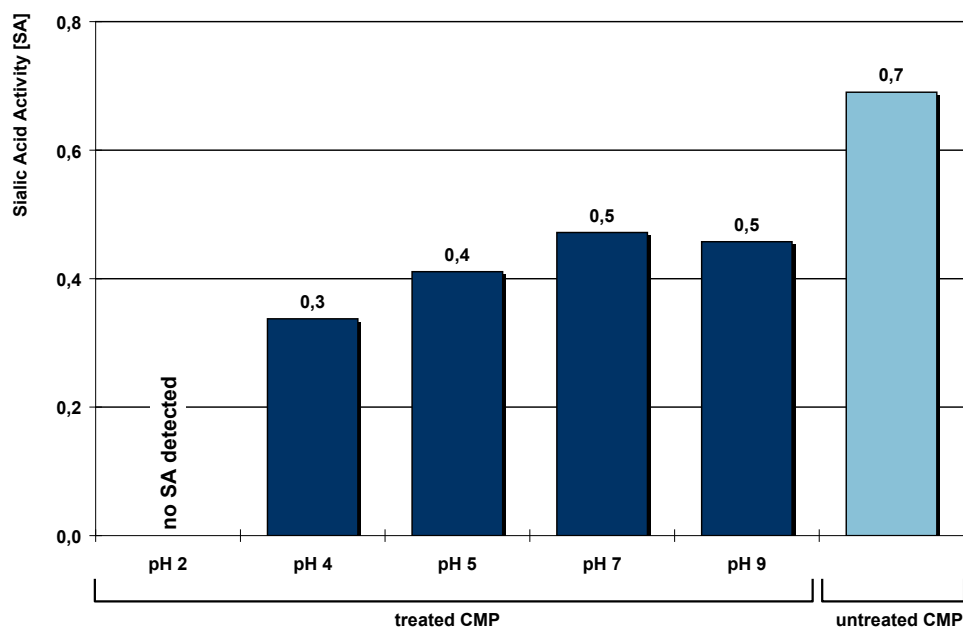
Further, the user can calculate normed results under inclusion of the reference sample.



Application - CMP

pH-stability of Caseinomacropeptide

This application shows an example for the determination of the Sialic Acid Activity of CMP. The influence of processing parameters like pH-value and temperature on the bound sialic acid is demonstrated.



The chart shows the SA of CMP samples, which were heated (120°C) at different pH values.

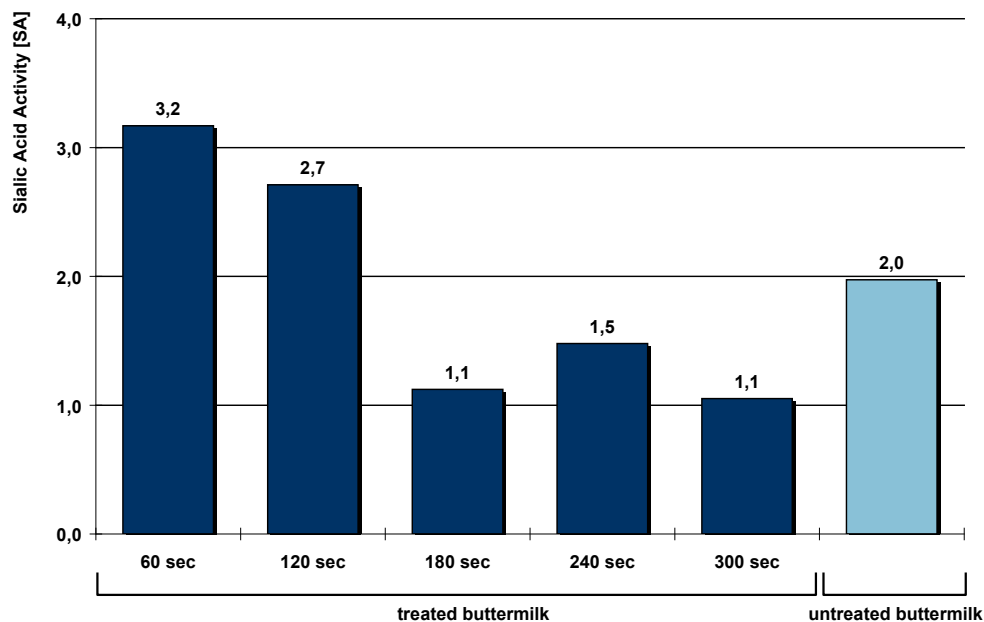
Results show the SA in dependence to the pH-value of heated samples. All samples were heated for 2 minutes at 120°C. After treating at pH 2 there was no SA detected. This demonstrates the hydrolysis of sialic acids. Even after treatment at pH 4, there is a reduction of SA detected. An alkaline pH (herein: pH 9) seems not to have great influence on SA. The optimal pH in combination with heat-treatment for preservation of the sialic acid activity is the neutral milieu. The highest SA was determined at pH 7.

Application - Buttermilk

The influence of temperature on buttermilk

This application shows the determination of SA in heated buttermilk. The influence of high temperature at different heating times on the SA of buttermilk is assayed.

The following chart represents the results of a heat-treatment at 140°C.



The chart presents the SA of buttermilk. The samples were determined in their native state and after heating for different times at 140°C.

The chart shows the SA of buttermilk samples and a reference sample (untreated buttermilk). A short-time heating (up to 2 minutes) of the buttermilk leads to an increase of SA. This can be explained by an aggregation of glycoconjugates. Clustered conjugates show multivalencies, which induce a stronger inhibition. After a longer heat-treatment (> 2 minutes) a rapid decrease of SA was detected.

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