Qproteome GlycoArray Handbook

Qproteome GlycoArray Kit
Qproteome GlycoArray Analysis Software

For glycan structure analysis
of glycosylated proteins

QIAGEN

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Contents

Kit Contents

Qproteome GlycoArray Kit For 6 glycoprotein analyses.	Cat. no. 37702
Qproteome GlycoArrays	2 boxes of 3 slides
Blocking Buffer 10 x	1 x 175 ml
Wash Buffer 10 x	2 x 175 ml
Solution C	1 x 10 ml
Solution M	2 x 1.5 ml
Solution T	1 x 1.5 ml
Incubation Frames	7
Handbook	1
CD containing Qproteome GlycoArray Analysis Software and kit definition file	1

Storage and Stability

Qproteome GlycoArray Kits should be stored in the dark at 2–8°C. Under these conditions, Qproteome GlycoArrays will retain their activity for a minimum of 6 months. Packs of arrays should be resealed after each array is removed.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/ts/msds.asp where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany, Tel: +49-6131-19240

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of Qproteome GlycoArrays is tested against predetermined specifications to ensure consistent product quality.

Product Use Limitations

The Qproteome GlycoArray Kit is intended for research use only. Prior to using it for other purposes, the user must validate the system in compliance with the applicable law, directives, and regulations. All due care and attention should be exercised in the handling of the product.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish. Separate conditions apply to QIAGEN scientific instruments, service products and to products shipped on dry ice. Please inquire for more information.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see back cover).

Technical Assistance

At QIAGEN we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in molecular biology and the use of QIAGEN® products. If you have any questions or experience any difficulties regarding Qproteome kits or QIAGEN products in general, please do not hesitate to contact us.

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Introduction

Glycosylation is the most versatile and one of the most abundant of all co- and post-translational modifications. It results from the addition of sugar residues to a protein backbone to form a glycoprotein. Glycosylation affects stability of protein conformation, clearance rate, protection from proteolysis, and improves protein solubility. In addition, glycosylation plays an important role in many specific biological functions, including immune defense, fertilization, viral replication, parasitic infection, cell growth, inflammation, and cell-cell adhesion. A protein's pattern of glycosylation depends on many factors, including the type of cell producing the glycoprotein, nutrient concentrations, pH, cell density, and age. Different cell lines and different fermentation conditions can produce significantly different glycosylation patterns.

Glycosylation sites on glycoproteins commonly display micro-heterogeneity – they can be fully or partially occupied by structurally diverse oligosaccharides. Mammalian glyco-protein oligosaccharides are commonly built from a limited number of monosaccharides, but their structural diversity is vast, mainly because they often form complex branching patterns. Glycosylation is not template-driven, and is currently impossible to predict.

Most cell surface and secreted proteins are glycosylated, with either N-linked carbohydrates covalently attached through the side chain amide of asparagines, or O-linked carbohydrates covalently attached through the side-chain hydroxyl groups of serine or threonine. All N-linked glycans contain the pentasaccharide Man α 1-6 (Man α1-3) Man β1-4 GlcNAc β1-4 GlcNAc as a common core and can be classified into four main groups based on the residues added to the core: oligomannose (high mannose), hybrid, and complex. The complex-type glycans have "antennae" with N-acetylglucosamine residues at their reducing termini attached to the core. The number of antennae usually ranges from two (biantennary) to four (tetraantennary); pentaantennary structures are rare. The structural variation results from the various mono-saccharides, such as sialic acid and fucose, which can be found in the antennae and in the core.

Qproteome GlycoArrays provide researchers with a simple, rapid, kit-based method for determining the pattern and relative abundance of specific mammalian glycosylation epitopes in a glycosylated protein. The analysis can be performed on crude samples in growth media, eliminating the need for time-consuming purification and sample preparation steps. The technology consists of arrays of selected lectins, which are used to determine the glycosylation features of the analyzed glycoproteins.

Lectins are a family of carbohydrate-recognizing proteins that are classified into a number of specificity groups based on the monosaccharides for which they exhibit the highest affinity. The lectin arrays in this kit consist of a set of over 20 lectins with overlapping specificities, which have been characterized using a large dataset of carefully chosen, well-characterized glycoproteins. Binding of a glycoprotein to the array results in a characteristic fingerprint that is highly sensitive to changes in the protein's glycan composition (see Table 1).

The fingerprint itself, while being a sensitive tool for comparing differences among samples of the same protein, is not a quantitative readout of the glycan structures in each sample. Fingerprint signals must be deconvoluted to give a quantitative profile of the glycan structures.

Table 1. Glycosylation features Detected by Qproteome GlycoArray Analysis

Feature.	Quantification classification
N-glycans	
Bi-antennary	High/Medium/Low/Not detected
Tri/tetra antennary	High/Medium/Low/Not detected
High mannose	High/Medium/Low/Not detected
Sialic acid	High/Medium/Low/Not detected
Terminal GlcNAc	High/Medium/Low/Not detected
Terminal GalNAc	Detected/Not detected
Bisecting GlcNAc	Detected/Not detected
O-glycans	Detected/Not detected

Structural classification of glycans

Oligosaccharides can be attached to proteins in three common ways:

■ via an N-glycosidic bond to the R group of an Asn residue within the motif Asn-X-Ser/Thr

■ via an O-glycosidic bond to the R group of Ser or Thr

■ via a glycophosphatidylinositol (GPI) anchor, which secures some glycoproteins to the membrane through one of six amino acids.

Unlike O-linked glycans, all mature N-linked glycan structures have a common core of three mannose and two N-acetylglucosamine residues (Man3, GlcNac2) that typically extends with two to four outer arms, each consisting of two to three sugar residues (See Figure 1).

Typical Structure of an N-Linked Glycan
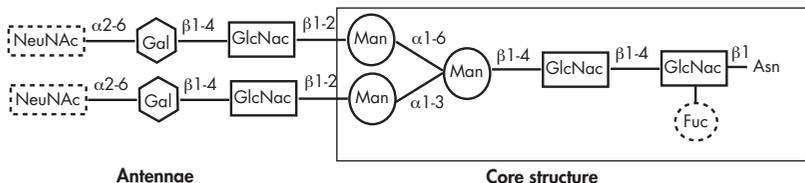
Figure 1 Basic structure of N-linked glycans. **Asn**; asparagine, **Fuc**; fucose, **GlcNac**; N-acetylglucosamine, **Man**; mannose, **Gal**; galactose, **NeuNAc**; N-acetylneuraminic acid.

N-linked glycans are classified into three main groups: oligomannose (high-mannose), complex types (bi-, tri-, and tetra- antennary), and hybrid type. In addition to these common linkages, there are about thirteen other monosaccharides and eight other amino acids that participate in forming around forty other types of glycopeptide bond, which include C-mannosylation and phosphoglycosylation. However, most of these bonds form minor groups of glycoproteins and not all of them are found in mammalian proteins.

In living cells, proteins are glycosylated by the actions of a series of glycosidases and glycosyltransferases that act sequentially on the growing glycan as it passes through the lumen of the endoplasmic reticulum (ER) and the Golgi apparatus. Since the various enzymatic reactions may not all reach completion, a variety of glycan structures could potentially be attached at each glycosylation site. Consequently, under a given set of conditions, a different population of glycosylation forms may be formed for a single type of protein. A protein with a defined glycan pattern is termed a glycoform. The number of glycoforms and their relative abundance within a cell are affected by the intrinsic structural properties of the individual protein, as well as the repertoire of glycosylation enzymes available (their type, concentration, kinetic characteristics, compartmentalization, etc.). Qproteome GlycoArray technology analyzes the proportion of various features within a glycoform population.

The properties of lectins used in Qproteome GlycoArrays

The plant lectins used in Qproteome GlycoArrays cover a broad range of specificities. The primary specificity groups of plant lectins include mannose/glucose, galactose/N-acetylgalactosamine, N-acetylglucosamine, fucose, and sialic acid. Primary specificity for other monosaccharides is very rare.

Some lectins have a pronounced preference for either the α- or the β-glycoside of a specific monosaccharide, whereas other lectins show no anomeric specificity. Moreover, the anomeric specificity may be affected by the nature of the non-sugar portion of the glycan. Individual lectins also vary considerably in their ability to react with isomers and derivatives of the sugar for which they are specific. For example, many lectins tolerate substitutions at the C-2 position of the sugar whereas an -OH group at C-3 or C-4 inhibits reaction with many lectins.

It should be emphasized, however, that the classification of lectins on the basis of their preferential binding to monosaccharides is useful to distinguish groups with differing gross specificities, but in many cases gives a misleading idea about the real specificity. The concentrations required for abolishing binding by mono- or disaccharides are usually high (association constants of 10^3–10^4 M^{-1}) and their specificity is relaxed, especially when compared to the typical inhibitory concentrations of oligosaccharides or complex glycans (association constants of 10^5–10^7 M^{-1}) whose binding specificity is tighter. This may be explained by the occurrence of extended lectin-binding sites that preferentially accommodate oligosaccharides. Many plant lectins react exclusively with oligosaccharides or complex glycans.

As well as generating data on lectin specificities, QIAGEN has carried out comparisons of its technology with results obtained by other glycoanalysis techniques. This has included validation with the following proteins that possess a wide range of glycan features.

Category	Subcategory	Protein
Molecular weight	High	Thyroglobulin
	Low	Epo, AsF
% Glycosylation	High	Thyroglobulin
	Low	hmLF
Glycosylation types	Biantennary	hmLF, Fetuin, ALP
	Tri/tetraantennary	tPA, Thyroglobulin, Epo
	High mannose	bmLF, tPA
	Terminal GlcNAc	hIgG
	Sialic acid	Fetuin, tPA
Sources	Human	hmLF, tPA, hIgG
	Bovine	Fetuin
	Porcine	Thyroglobulin
	Recombinant	Epo

Principle and procedure

Lectins with differing, defined glycan-binding specificities are spotted on the surface of an array. The array is probed with a glycoprotein, washed to reduce background, and visualized using a microarray scanner. Bound glycoproteins can be visualized by direct fluorescent labeling of the glycoprotein target(s), biotinylation of the target(s) followed by detection with fluorescent streptavidin, or by using a fluorescently labeled protein-specific antibody (see Figure 2). After scanning the array, fluorescent signals are evaluated using Qproteome GlycoArray Analysis Software.

Selecting a protocol

This handbook contains four protocols. The suitability of a protocol for a given experiment is dependent on the nature of the sample being analyzed and/or the desired detection method. Use the flowchart on page 12 to select the most suitable protocol.

Principle of GlycoArray Analysis
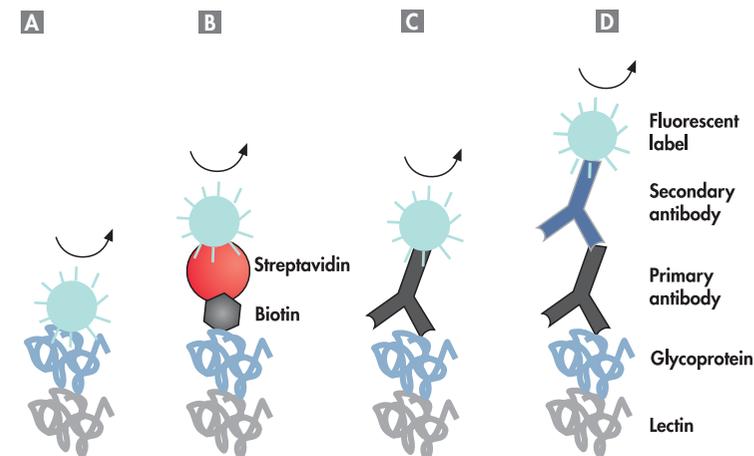
Figure 2 Detection of glycoproteins using A fluorescently labeled glycoprotein, B biotinylated glycoprotein and fluorescently labeled streptavidin, C fluorescently labeled protein-specific antibody, D protein-specific antibody and fluorescently labeled secondary antibody.

Reagents Required for Each Protocol

Protocol	Protein sample	Primary detection probe	Secondary detection probe
Fluorescently Labeled Protein Samples (see page 20)	Purified fluorescently labeled glycoprotein	–	–
Biotinylated Protein Samples Detected with Fluorescent Streptavidin (page 23)	Biotinylated glycoprotein	Fluorescently labeled streptavidin	–
Unlabeled Protein Samples Detected with a Fluorescently Labeled Primary Antibody (page 26)	Unlabeled glycoprotein	Fluorescently labeled protein-specific polyclonal rabbit antibody	–
Unlabeled Protein Samples Detected with a Fluorescently Labeled Secondary Antibody (page 29)	Unlabeled glycoprotein	Unlabeled protein-specific polyclonal rabbit antibody	Fluorescently labeled anti-rabbit polyclonal antibody

Is your protein purified?

No →

Yes →

Is your protein labeled?

Yes → Use protocol for fluorescently labeled protein (page 20) or biotin labeled protein (page 23)

No →

Do you have a specific antibody?

No → Nonpurified samples cannot be analyzed without a protein-specific antibody

Yes →

Do you have a specific antibody?

Yes →

No → Label your protein with a fluorescent dye or biotin → Use protocol for fluorescently labeled protein (page 20) or biotin labeled protein (page 23)

Is your specific antibody fluorescently labeled?

Yes →

No →

Do you have a labeled secondary antibody?

Yes → Use protocol for unlabeled protein and fluorescently labeled secondary antibody (page 29)

No → Label your primary antibody with a fluorescent dye →

Use protocol for unlabeled protein and fluorescently labeled primary antibody (page 26)

Protein Sample Requirements

The Qproteome GlycoArray Kit detects exposed glycans on the surface of glycoproteins. The sample to be analyzed may be a pure glycoprotein, or a glycoprotein in culture medium or in a protein mixture. The concentration of the glycoprotein of interest must be 0.2 µM and significantly (8–10 fold) higher than the rest of the proteins in the mixture combined.

Pure proteins

Protein concentration should be determined for the protein without posttranslational modifications. Concentration can be calculated by measuring the O.D. at 280 nm and using the protein's extinction coefficient (ε).

Protein concentration (M) = A_{280}/ε (M^{-1}cm^{-1})

A useful online tool for determining extinction coefficients can be found at http://ca.expasy.org/tools/protparam.html .

Note: glycoanalysis results may be altered in situations where the glycoprotein is in a complex with other proteins, or where its glycans are not completely exposed.

Fluorescently labeled proteins and probes

If the protein sample is fluorescently labeled make sure that the dye:protein ratio is 1–3:1 for FITC and 0.5–1:1 for Cy®3. Fluorescently labeled samples and antibody probes should be protected from light. All tubes or vessels containing these reagents should be wrapped in aluminum foil during storage, when used in the laboratory, and during incubations.

Protein mixtures or proteins in culture medium

Proteins in a mixture or in culture medium can only be analyzed with protein-specific antibodies using the either of the protocols on page 26 and page 29. Protein samples should be either free of proteases, or if proteases are present, inhibitors must be added to ensure full inhibition. For non-purified samples the medium or protein buffer must meet the following requirements.

Component	Concentration/value
Glucose concentration	<1 mM
Other monosaccharides	<25 mM
Tween® 20	<0.01%
Triton® X-100	<0.001%
Other detergents	May affect results
Salts	<0.5 M
pH range	7–8

Important Notes

Qproteome GlycoArray slides should be handled very carefully. Always wear gloves during slide handling and avoid any contact with the membrane-covered surface. After removing slides, reseal remaining unused slides in original packs and return to storage at 2–8°C.

Preparing the slides for use

■ Incubation Frames are placed onto Qproteome GlycoArray slides to form a chamber for the various incubation steps. To attach the frame peel off the backing paper and press the frame onto the array slide. The frame must be flush with the edges of the slide (see Figure 3). Make sure that all the blue spots are within the inner area of the frame. Note that the blue dye will wash off during the assay.

■ To record the sample ID on the slide, write only on the frosted part of the slide using either a Staedtler® Lumocolor® Fine Black 318-9 permanent marker or a pencil. Ink from other marker may stain the membrane during the incubation process.

Incubating slides

■ Each slide should be processed in a separate 9 cm diameter Petri dish to eliminate cross-contamination. When duplicate slides are processed, both duplicates can be placed together in a 15 cm Petri dish. The volume of wash and block solutions required for the relevant step depends on the size of the Petri dish used. Use 25 ml wash or blocking solution if using a 9 cm Petri dish (single slide) or 60 ml if using a 15 cm Petri dish (duplicate slides).

■ All incubations and wash steps should be carried out on a horizontal orbital shaker at 50 rpm. The sample or probe must be evenly distributed over the entire surface of the slide to obtain correct and reproducible results. This must be checked visually before and during the incubation.

■ During incubation, ensure that the membrane area of the slide is completely covered with sample or probe. The slide membrane must not dry out during incubation.

■ Keep the lid of the Petri dish on at all times to minimize evaporation.

■ In order to avoid slide drying, keep slides in the wash solution at the end of each wash step. Remove one slide at a time from the wash solution and probe it with the appropriate material (sample/control/antibody, etc.) Once the membrane dries out it is very hard to probe it with 450 µl of solution and the probing solution will not spread evenly on the slide.

■ Do not pour wash and block solutions directly on the slide.

Forming the Incubation Chamber
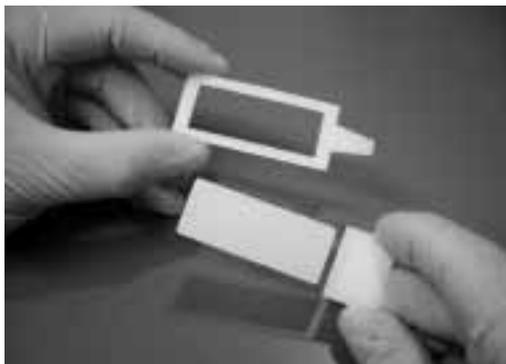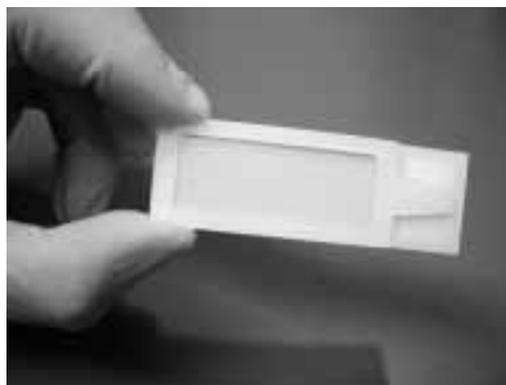

Figure 3 Forming the incubation chamber. The backing paper is peeled off the adhesive Incubation Frame, which is then aligned with the GlycoArray slide. The Incubation Frame is then gently pressed into place. The Incubation Frame must be flush with the edges of the slide.

Things to do Before Starting All Protocols

Preparation of reagents

Before use, prepare reagents according the following procedures. It is important that filtered, reverse osmosis (RO) grade water is used to produce all solutions used with the Qproteome GlycoArray Kit. This water must conform to the following specifications:

Resistivity   >18 MΩ (conductivity µs/cm = 1/resistivity MΩ).

Total organic carbon   <5 ppb

Alternatively, HPLC-grade water can be used.

Complete wash solution

1. **Bring all components to room temperature (15–25°C) before preparing complete wash solution.**
    Ensure that any precipitates in Wash Buffer 10x are completely dissolved before starting.

2. **Make up complete wash solution by adding the reagents in the exact order specified in Table 2.**
    At each step ensure that the solution is stirred until any precipitate formed is dissolved before the next reagent is added.

Table 2. Components of Complete Wash Solution

Reagent	Supplied in kit?	Complete wash solution final volume			
		250 ml	500 ml	1 liter	2 liters
Wash Buffer 10x	Yes	25 ml	50 ml	100 ml	200 ml
Water (filtered and RO- or HPLC-grade)	No	224.5 ml	449 ml	898 ml	1795 ml
Solution C	Yes	0.5 ml	1 ml	2 ml	4 ml
Solution M	Yes	125 µl	250 µl	500 µl	1 ml
Solution T*	Yes	62.5 µl	125 µl	250 µl	500 µl

* Filter solution with a 0.22 µM filter before adding Solution T. Stir gently after addition.

3. **Make up to the desired total volume with water.**

4. **Filter the solution using a 0.22 µm filter.**

5. **Add Solution T and stir the solution gently.**
    Complete wash solution should be stored at 2–8°C and used within 24 h.

Complete blocking solution

1. Bring all components to room temperature (15–25°C) before preparing complete blocking solution.

2. Make up complete blocking solution by adding the reagents in the exact order specified in Table 3.

   At each step ensure that the solution is stirred until any precipitate formed is dissolved before the next reagent is added. Complete blocking solution should be prepared freshly before each analysis.

Table 3. Components of Complete Blocking Solution

Reagent	Supplied in kit?	For 50 ml
Blocking Buffer 10x	Yes	5 ml
Water (filtered and RO- or HPLC-grade)	No	to 50 ml
Solution C	Yes	100 µl
Solution M	Yes	25 µl
Solution T*	Yes	12.5 µl

* Filter solution with a 0.22 µM filter before adding Solution T. Stir gently after addition.

Protein sample preparation

A total of 500 µl of a 0.2 µM protein solution is required for each slide. This solution must contain 25 µl of complete blocking solution, which means that the protein stock solution must have a concentration of at least 0.21 µM (see Table 4 below).

Table 4. Preparation of a Protein Sample for One Slide

Component	Volume
Protein sample	Varies
Complete blocking solution (prepared as described in Table 3)	25 µl
Complete wash solution (prepared as described in Table 2)	to final volume of 500 µl

Fluorescently labeled streptavidin preparation

The optimal working concentration for fluorescently labeled streptavidin has been determined to be 5 µg/ml. Prepare streptavidin as described in Table 5 below.

Table 5. Preparation of Fluorescently Labeled Streptavidin for Two Slides

Component	Volume
Fluorescently labeled streptavidin	Varies, final working conc. should be 5 µg/ml
Complete blocking solution (prepared as described in Table 3)	50 µl
Complete wash solution (prepared as described in Table 2)	to final volume of 1000 µl

Primary antibody preparation

The optimal working concentration for numerous polyclonal rabbit antibodies has been determined to be 60 nM (unlabeled and FITC-labeled antibodies) or 6 nM (Cy3-labeled antibodies). Prepare a primary antibody solution as described in Table 6 below. The primary antibody must be polyclonal and directed against the entire protein.

Table 6. Preparation of Primary Antibody Solution for Two Slides

Component	Volume
Antibody	Varies, final working conc. should be: Unlabeled antibodies 60 nM* FITC-labeled antibodies 60 nM Cy3-labeled antibodies 6 nM
Complete blocking solution (prepared as described in Table 3)	50 µl
Complete wash solution (prepared as described in Table 2)	to final volume of 1000 µl

* This concentration was found to be suitable for a large set of commercial rabbit polyclonal antibodies.

Secondary antibody preparation

When using rabbit primary antibodies, we recommend the FITC-labeled anti-rabbit antibody from Jackson (cat. no. 111-095-045) at a final concentration of 1.5 µg/ml or Cy3-labeled anti-rabbit antibody from Jackson (cat. no. 111-165-045) at a final concentration of 0.3 µg/ml.

Table 7. Preparation of Labeled Secondary Antibody for Two Slides

Component	Volume
Antibody	Varies, final working conc. should be: FITC-labeled antibodies 1.5 µg/ml Cy3-labeled antibodies 0.3 µg/ml
Complete blocking solution (prepared as described in Table 3)	50 µl
Complete wash solution (prepared as described in Table 2)	to final volume of 1000 µl

Negative control slides

To determine background binding levels of the probe to the array, negative control slides are required for experiments using antibody probes (see protocols on pages 26 and 29). Control slides should be incubated with buffer or media without protein during the sample incubation period.

Experiments using labeled proteins (see protocols on pages 20 and 23) do not require control slides.

Table 8. Preparation of a Negative Control Solution for One Slide

Component	Volume
Buffer or media without protein	Same volume as used in corresponding protein experiment
Complete blocking solution (prepared as described in Table 3)	25 µl
Complete wash solution (prepared as described in Table 2)	to final volume of 500 µl

Protocol: Glycoanalysis Using Purified Fluorescently Labeled Protein Samples

This protocol is used for analysis of the glycan makeup of glycoproteins that have been fluorescently labeled (e.g., using FITC or Cy3). If performing this protocol for the first time, ensure that you have read the "Important Notes" on page 14.

The dye:protein ratio should be 1–3:1 for FITC and 0.5–1:1 for Cy3. Fluorescently labeled proteins should be protected from light. All tubes or vessels containing labeled proteins should be wrapped in aluminum foil for storage, when used in the laboratory, and during incubations.

The table below shows the required volumes of protein, water, and complete wash and blocking solutions required for processing single and duplicate slides.

Solution	Single slide	Duplicate slides
Fluorescently labeled protein sample, 0.2 µM, prepared as described in Table 4, page 17	450 µl	900 µl
Complete wash solution (prepared as described in Table 2, page 16) at room temperature (15–25°C)	175 ml	400 ml
Complete blocking solution (prepared as described in Table 3, page 17) at room temperature (15–25°C)	25 ml	60 ml
RO- or HPLC-grade water	25 ml	60 ml

Equipment and reagents to be supplied by the user

- Fluorescently labeled protein sample, 0.2 µM, prepared as described in Table 4, page 17
- Petri dishes (2 x 9 cm diameter for single slides, 2 x 15 cm diameter for duplicate incubations)
- RO- or HPLC grade water
- Orbital shaker

Procedure

1. **Stick an Incubation Frame onto each Qproteome GlycoArray slide to be processed. The Incubation Frame must be flush with edges of the slide (see Figure 3, page 14).**

   Qproteome GlycoArray slides should be handled very carefully. Always wear gloves during slide handling and avoid any contact with the membrane-covered surface.

2. **Place the slide(s) membrane side up in a Petri dish.**

   Use a 9 cm diameter dish for single slides. Duplicate slides can be blocked side-by-side in a 15 cm Petri dish.

3. **Add 25 ml (single slide) or 60 ml (duplicate slides) complete blocking solution to the Petri dish. Incubate on an orbital shaker set to rotate at 50 rpm for 60 min at room temperature (15–25°C).**

4. **Discard blocking solution.**

5. **Wash slides by adding 25 ml (single slide) or 60 ml (duplicate slides) complete wash solution to the Petri dish. Incubate on an orbital shaker set to rotate at 50 rpm for 5 min at room temperature (15–25°C). Discard wash solution. Repeat wash step twice (a total of three wash steps).**

6. **After the third wash step, leave slides submerged in wash solution to prevent them drying out.**

7. **Take a single slide from the Petri dish and remove wash solution by pressing a paper towel to the back and edges of the slide.**

   Do not touch the membrane. Ensure that the membrane is fully covered. Avoid formation of bubbles on the membrane.

8. **Place the slide in a clean Petri dish and pipet 450 µl fluorescently labeled protein sample onto the membrane.**

   Do not touch the membrane.

9. **Incubate in the dark on an orbital shaker set to rotate at 50 rpm for 60 min at room temperature (15–25°C).**

   During incubation, ensure that the membrane area of the slide is completely covered with sample. The slide membrane must not dry out during incubation. Keep the lid of the Petri dish on at all times to minimize evaporation. The Petri dish should be covered with aluminum foil to exclude light.

10. **Wash slides in the dark by adding 25 ml (single slide) or 60 ml (duplicate slides) complete wash solution to the Petri dish, placing on an orbital shaker set to rotate at 50 rpm for 5 min at room temperature (15–25°C), and discarding wash solution. Repeat wash step twice (a total of three wash steps).**

11. **After the third wash step, carefully peel the Incubation Frame from each slide.**

12. **Wash slides in the dark for 1 min with 25 ml (single slide) or 60 ml (duplicate slides) RO- or HPLC-grade water.**

13. **Dry slide(s) using one of the methods described on page 33.**

14. **Scan and analyze slide(s) (see page 34).**

Protocol: Glycoanalysis Using Biotinylated Protein Samples Detected with Fluorescent Streptavidin

This protocol is used for analysis of the glycan makeup of biotin-labeled glycoproteins using fluorescently labeled streptavidin. If performing this protocol for the first time, ensure that you have read the "Important Notes" on page 14.

The table below shows the required volumes of water, sample, detection reagents, and complete wash and blocking solutions required for processing single and duplicate slides.

Solution	Single slide	Duplicate slides
Biotinylated protein sample, 0.2 µM, prepared as described in Table 4, page 17	450 µl	900 µl
Fluorescently labeled streptavidin, 5 µg/ml* prepared as described in Table 5, page 18	450 µl	900 µl
Complete wash solution (prepared as described in Table 2, page 16) at room temperature (15–25°C)	250 ml	600 ml
Complete blocking solution (prepared as described in Table 3, page 17) at room temperature (15–25°C)	30 ml	65 ml
RO- or HPLC-grade water	25 ml	60 ml

* Recommended working concentration.

Equipment and reagents to be supplied by the user

- Biotinylated glycoprotein sample, 0.2 µM, prepared as described in Table 4, page 17
- Fluorescently labeled streptavidin, 5 µg/ml, prepared as described in Table 5, page 18
- Petri dishes (2 x 9 cm diameter for single slides, 2 x 15 cm diameter for duplicate incubations)
- RO- or HPLC grade water
- Orbital shaker

Procedure

1. **Stick an Incubation Frame onto each Qproteome GlycoArray slide that will be processed. The Incubation Frame must be flush with edges of the slide.**

   Qproteome GlycoArray slides should be handled very carefully. Always wear gloves during slide handling and avoid any contact with the membrane-covered surface.

2. **Place the slide(s) membrane side up in a Petri dish.**

   Use a 9 cm diameter dish for single slides. Duplicate slides can be blocked side-by-side in a 15 cm Petri dish.

3. **Add 25 ml (single slide) or 60 ml (duplicate slides) complete blocking solution to the Petri dish. Incubate on an orbital shaker set to rotate at 50 rpm for 60 min at room temperature (15–25°C).**

4. **Discard blocking solution.**

5. **Wash slides by adding 25 ml (single slide) or 60 ml (duplicate slides) complete wash solution to the Petri dish. Incubate on an orbital shaker set to rotate at 50 rpm for 5 min at room temperature (15–25°C). Discard wash solution. Repeat wash step twice (a total of three wash steps).**

6. **After the third wash step, leave slides submerged in wash solution to prevent them drying out.**

7. **Take a single slide from the Petri dish and remove wash solution by pressing a paper towel to the back and edges of the slide.**

   Do not touch the membrane. Ensure that the membrane is fully covered. Avoid for mation of bubbles on the membrane.

8. **Place the slide in a clean Petri dish and pipet 450 µl biotinylated protein sample onto the membrane.**

   Do not touch the membrane. Ensure that the membrane is fully covered. Avoid for mation of bubbles on the membrane.

9. **Repeat steps 7 and 8 for all slides.**

10. **Incubate slides in the dark on an orbital shaker set to rotate at 50 rpm for 60 min at room temperature (15–25°C).**

    During incubation, ensure that the membrane area of the slides is completely covered with sample. The slide membrane must not dry out during incubation. Keep the lids of Petri dishes on at all times to minimize evaporation. Petri dishes should be covered with aluminum foil to exclude light.

11. **Wash slides by adding 25 ml (single slide) or 60 ml (duplicate slides) complete wash solution to the Petri dish. Incubate on an orbital shaker set to rotate at 50 rpm for 5 min at room temperature (15–25°C). Discard wash solution. Repeat wash step twice (a total of three wash steps).**

12. After the third wash-step, keep all slides submerged in wash solution to prevent the membranes drying out.

13. Remove a single slide from the Petri dish and remove wash solution by pressing a paper towel to the back and edges of the slide.

14. Place the slide in a clean Petri dish and pipet 450 µl of fluorescently labeled streptavidin (5 µg/ml) onto the membrane. Close the lid of the Petri dish.

   Do not touch the membrane. Ensure that the membrane is fully covered. Avoid formation of bubbles on the membrane.

15. Repeat steps 12 and 13 for all slides.

16. Incubate slides in the dark on an orbital shaker set to rotate at 50 rpm for 20 min at room temperature (15–25°C).

   During incubation, ensure that the membrane area of the slides is completely covered with sample. The slide membrane must not dry out during incubation. Keep the lids of Petri dishes on at all times to minimize evaporation. Petri dishes should be covered with aluminum foil to exclude light.

17. Wash slides in the dark by adding 25 ml (single slide) or 60 ml (duplicate slides) complete wash solution to the Petri dish, placing on an orbital shaker set to rotate at 50 rpm for 5 min at room temperature (15–25°C), and discarding wash solution. Repeat wash step twice (a total of three wash steps).

18. After the third wash step, carefully peel the Incubation Frame from each slide.

19. Wash slides in the dark for 1 min with 25 ml (single slide) or 60 ml (duplicate slides) RO- or HPLC-grade water.

20. Dry slide(s) using one of the methods described on page 33.

21. Scan and analyze slide(s) (see page 34).

Protocol: Glycoanalysis Using Unlabeled Protein Samples Detected with a Fluorescently Labeled Antibody

This protocol is used for analysis of the glycan makeup of unlabeled glycoproteins for which a fluorescently labeled polyclonal antibody is available. If performing this protocol for the first time, ensure that you have read the "Important Notes " on page 14.

To determine background binding levels of the probe to the array, negative control slides are required for experiments using labeled antibody probes. Negative control slides should be incubated with buffer or media without protein during the sample incubation period. One control slide is sufficient for several sample slides provided that the same antibody and antibody concentration are used for detection on all slides.

The table below shows the required volumes of water, sample, detection reagents, and complete wash and blocking solutions required for processing single and duplicate slides.

Solution	Single slide + control	Duplicate slides + control
Protein sample, 0.2 µM, prepared as as described in Table 4, page 17	450 µl	900 µl
Control sample, 0.2 µM, prepared as as described in Table 8, page 19	450 µl	900 µl
Fluorescently labeled rabbit polyclonal antibody prepared as described in Table 6, page 18*	1000 µl	2000 µl
Complete wash solution (prepared as described in Table 2, page 16 at room temperature (15–25°C)	500 ml	1200 ml
Complete blocking solution (prepared as described in Table 3,page 17 at room temperature (15–25°C)	55 ml	130 ml
RO- or HPLC-grade water	50 ml	120 ml

* See page 18 for recommended working concentrations.

Equipment and reagents to be supplied by the user

- Glycoprotein sample, 0.2 μM, prepared as described in Table 4, page 17
- Protein-specific fluorescently labeled antibody, prepared as described in Table 6, page 18
- Petri dishes (6 x 9 cm diameter for single slides, 6 x 15 cm diameter for duplicate incubations)
- RO- or HPLC grade water
- Orbital shaker

Procedure

1. **Stick an Incubation Frame onto each Qproteome GlycoArray slide that will be processed. The Incubation Frame must be flush with edges of the slide.**

   Qproteome GlycoArray slides should be handled very carefully. Always wear gloves during slide handling and avoid any contact with the membrane-covered surface.

2. **Place the slide(s) membrane side up in a Petri dish.**

   Use a 9 cm diameter dish for single slides. Duplicate slides can be blocked side-by-side in a 15 cm Petri dish.

3. **Add 25 ml (single slide) or 60 ml (duplicate slides) complete blocking solution to the Petri dish. Incubate on an orbital shaker set to rotate at 50 rpm for 60 min at room temperature (15–25°C).**

4. **Discard blocking solution.**

5. **Wash slides by adding 25 ml (single slide) or 60 ml (duplicate slides) complete wash solution to the Petri dish. Incubate on an orbital shaker set to rotate at 50 rpm for 5 min at room temperature (15–25°C). Discard wash solution. Repeat wash step twice (a total of three wash steps).**

6. **After the third wash step, leave slides submerged in wash solution to prevent them drying out.**

7. **Remove a single slide from the Petri dish and remove wash solution by pressing a paper towel to the back and edges of the slide.**

8a. **Sample slides: Place the slide in a clean Petri dish and pipet 450 μl protein sample onto the membrane.**

   Do not touch the membrane. Ensure that the membrane is fully covered. Avoid formation of bubbles on the membrane.

8b. **Control slides: Place the slide in a clean Petri dish and pipet 450 μl negative control solution onto the membrane.**

   Do not touch the membrane. Ensure that the membrane is fully covered. Avoid formation of bubbles on the membrane.

9. **Incubate slides in the dark on an orbital shaker set to rotate at 50 rpm for 60 min at room temperature (15–25°C).**

   During incubation, ensure that the membrane area of the slides is completely covered with sample. The slide membrane must not dry out during incubation. Keep the lids of Petri dishes on at all times to minimize evaporation. Petri dishes can be covered with aluminum foil to exclude light.

10. **Wash slides separately by adding 25 ml complete wash solution to a 9 cm diaMeter Petri dish. Incubate in the dark on an orbital shaker set to rotate at 50 rpm for 5 min at room temperature (15–25°C). Discard wash solution. Repeat wash step twice (a total of three wash steps).**

11. **After the third wash-step incubation, keep all slides submerged in wash solution to prevent the membranes drying out.**

12. **Remove a single slide from the Petri dish and remove wash solution by pressing a paper towel to the back and edges of the slide.**

13. **Place the slide in a clean Petri dish and pipet 450 µl of fluorescently labeled antiody working solution onto the membrane.**

    Do not touch the membrane. Ensure that the membrane is fully covered. Avoid formation of bubbles on the membrane.

14. **Repeat steps 12 and 13 for all slides.**

15. **Incubate slides in the dark on an orbital shaker set to rotate at 50 rpm for 40 min at room temperature (15–25°C).**

    During incubation, ensure that the membrane area of the slides is completely covered with sample. The slide membrane must not dry out during incubation. Keep the lids of Petri dishes on at all times to minimize evaporation. Petri dishes should be covered with aluminum foil to exclude light.

16. **Wash slides in the dark by adding 25 ml (single slide) or 60 ml (duplicate slides) complete wash solution to the Petri dish, placing on an orbital shaker set to rotate at 50 rpm for 5 min at room temperature (15–25°C), and discarding wash solution. Repeat wash step twice (a total of three wash steps).**

17. **After the third wash step, carefully peel the incubation frame from each slide.**

18. **Wash slides in the dark for 1 min with 25 ml (single slide) or 60 ml (duplicate slides) RO- or HPLC-grade water.**

19. **Dry slide(s) using one of the methods described on page 33.**

20. **Scan and analyze slide(s) (see page 34).**

Protocol: Glycoanalysis Using Unlabeled Protein Samples Detected with a Fluorescently Labeled Secondary Antibody

This protocol is used for analysis of the glycan makeup of unlabeled glycoproteins for which a polyclonal antibody is available. This primary antibody is in turn detected using a fluorescently labeled secondary antibody. If performing this protocol for the first time, ensure that you have read the "Important Notes" on page 14.

To determine background binding levels of the probe to the array, negative control slides are required for experiments using labeled antibody probes. Control slides should be incubated with buffer or media without protein during the sample incubation period. One control slide is sufficient for several sample slides provided that the same antibody and antibody concentration are used for detection on all slides.

The table below shows the required volumes of water, sample, detection reagents, and complete wash and blocking solutions required for processing single and duplicate slides.

Solution	Single slide + control	Duplicate slides + control
Protein sample, 0.2 µM, prepared as as described in Table 4, page 17	450 µl	900 µl
Control sample, 0.2 µM, prepared as as described in Table 8, page 19	450 µl	900 µl
Protein-specific rabbit polyclonal antibody prepared as described in Table 6, page 18*	1000 µl	2000 µl
Fluorescently labeled anti-rabbit antibody prepared as described in Table 7, page 19*	1000 µl	2000 µl
Complete wash solution (prepared as described in Table 2, page 16 at room temperature (15–25°C)	500 ml	1200 ml
Complete blocking solution (prepared as described in Table 3, page 17 at room temperature (15–25°C)	55 ml	130 ml
RO- or HPLC-grade water	50 ml	120 ml

* See pages 18 and 19 for recommended working concentrations.

Equipment and reagents to be supplied by the user

- Glycoprotein sample, 0.2 μM, prepared as described in Table 4, page 17
- Protein-specific rabbit polyclonal primary antibody, prepared as described in Table 6, page 18
- Fluorescently labeled anti-rabbit polyclonal antibody, prepared as described in Table 7, page 19
- Petri dishes (8 x 9 cm diameter for single slides, 8 x 15 cm diameter for duplicate incubations)
- RO- or HPLC grade water
- Orbital shaker

Procedure

1. **Stick an Incubation Frame onto each Qproteome GlycoArray slide that will be processed. The Incubation Frame must be flush with edges of the slide.**

   Qproteome GlycoArray slides should be handled very carefully. Always wear gloves during slide handling and avoid any contact with the membrane-covered surface.

2. **Place the slide(s) membrane side up in a Petri dish.**

   Use a 9 cm diameter dish for single slides. Duplicate slides can be blocked side-by-side in a 15 cm Petri dish.

3. **Add 25 ml (single slide) or 60 ml (duplicate slides) complete blocking solution to the Petri dish. Incubate on an orbital shaker set to rotate at 50 rpm for 60 min at room temperature (15–25°C).**

4. **Discard blocking solution.**

5. **Wash slides by adding 25 ml (single slide) or 60 ml (duplicate slides) complete wash solution to the Petri dish. Incubate on an orbital shaker set to rotate at 50 rpm for 5 min at room temperature (15–25°C). Discard wash solution. Repeat wash step twice (a total of three wash steps).**

6. **After the third wash step, leave slides submerged in wash solution to prevent them drying out.**

7. **Remove a single slide from the Petri dish and remove wash solution by pressing a paper towel to the back and edges of the slide.**

8a. **Sample slides: Place the slide in a clean Petri dish and pipet 450 μl protein sample onto the membrane.**

   Do not touch the membrane. Ensure that the membrane is fully covered. Avoid formation of bubbles on the membrane.

8b. **Control slides: Place the slide in a clean Petri dish and pipet 450 µl negative control solution onto the membrane.**

Do not touch the membrane. Ensure that the membrane is fully covered. Avoid formation of bubbles on the membrane.

9. **Incubate slides in the dark on an orbital shaker set to rotate at 50 rpm for 60 min at room temperature (15–25°C).**

During incubation, ensure that the membrane area of the slides is completely covered with sample. The slide membrane must not dry out during incubation. Keep the lids of Petri dishes on at all times to minimize evaporation. Petri dishes can be covered with aluminum foil to exclude light.

10. **Wash slides separately by adding 25 ml complete wash solution to a 9 cm diameter Petri dish. Incubate in the dark on an orbital shaker set to rotate at 50 rpm for 5 min at room temperature (15–25°C). Discard wash solution. Repeat wash step twice (a total of three wash steps).**

11. **After the third wash-step incubation, keep all slides submerged in wash solution to prevent the membranes drying out.**

12. **Take a single slide and remove wash solution by pressing a paper towel to the back and edges of the slide.**

13. **Place the slide in a clean Petri dish and pipet 450 µl of primary antibody working solution onto the membrane.**

Do not touch the membrane. Ensure that the membrane is fully covered. Avoid formation of bubbles on the membrane.

14. **Repeat steps 12 and 13 for all slides.**

15. **Incubate slides in the dark on an orbital shaker set to rotate at 50 rpm for 40 min at room temperature (15–25°C).**

During incubation, ensure that the membrane area of the slides is completely covered with sample. The slide membrane must not dry out during incubation. Keep the lids of Petri dishes on at all times to minimize evaporation. Petri dishes can be covered with aluminum foil to exclude light.

16. **Wash slides by adding 25 ml (single slide) or 60 ml (duplicate slides) complete wash solution to the Petri dish. Incubate in the dark on an orbital shaker set to rotate at 50 rpm for 5 min at room temperature (15–25°C). Discard wash solution. Repeat wash step twice (a total of three wash steps).**

17. **After the third wash-step incubation, keep all slides submerged in wash solution to prevent the membranes drying out.**

18. **Take a single slide and remove wash solution by pressing a paper towel to the back and edges of the slide.**

19. **Place the slide in a clean Petri dish and pipet 450 µl of fluorescently labeled secondary antibody working solution onto the membrane.**

    Do not touch the membrane. Ensure that the membrane is fully covered. Avoid formation of bubbles on the membrane.

20. **Repeat steps 18 and 19 for all slides.**

21. **Incubate slides in the dark on an orbital shaker set to rotate at 50 rpm for 30 min at room temperature (15–25°C).**

    During incubation, ensure that the membrane area of the slides is completely covered with sample. The slide membrane must not dry out during incubation. Keep the lids of Petri dishes on at all times to minimize evaporation. Petri dishes should be covered with aluminum foil to exclude light.

22. **Wash slides in the dark by adding 25 ml (single slide) or 60 ml (duplicate slides) complete wash solution to the Petri dish, placing on an orbital shaker set to rotate at 50 rpm for 5 min at room temperature (15–25°C), and discarding wash solution. Repeat wash step twice (a total of three wash steps).**

23. **After the third wash step, carefully peel the incubation frame from each slide.**

24. **Wash slides in the dark for 1 min with 25 ml (single slide) or 60 ml (duplicate slides) RO- or HPLC-grade water.**

25. **Dry slide(s) using one of the methods described on page 33.**

26. **Scan and analyze slide(s) (see page 34).**

Protocol: Drying Slides after Processing

To avoid nonspecific background signals, slides must be dried before scanning using one of the protocols below.

Using a centrifuge

1. Remove slide(s) from final water wash.
2. Gently wipe the back of the slide(s) with a laboratory wipe.
3. Centrifuge at 200 x g for 5–10 min (or until slides are dry) in a Coplin jar or a centrifuge slide carrier.
4. Air dry in the dark until membrane is completely white.

If no centrifuge is available, slides can be dried manually.

Manual procedure

1. Remove slide(s) from final water wash.
2. Gently wipe the back of the slide(s) with a laboratory wipe.
3. Press a laboratory wipe to the sides of the membrane. Do not touch the central region of the membrane!
4. Air dry in the dark until membrane is completely white.

Scanning Slides

Following sample processing and drying, slides should be scanned using a confocal laser slide scanner with adjustable laser power and photomultiplier tube (PMT).

Compatible scanners

Slides can be scanned using any confocal scanner that meets the following specifications:

Resolution:	20 µm
Slide type:	Standard microscope slides (75 x 25 mm)
Digital resolution:	16 bit
Output file format:	TIFF
Laser/filter:	Compatible with label used

Recommended scanners

QIAGEN has tested the scanners listed below and found them to be compatible with Qproteome GlycoArrays. Scanners from other manufacturers meeting the specifications above can also be used.

Manufacturer	Model
Axon*	GenePix® 4200
Perkin Elmer	ScanArray® 4000, 5000, Express HT
Tecan	LS 200/300/400
Agilent	Agilent microarray scanner

* All models with adjustable laser power and PMT.

Important points before starting

The laser and filter set must be selected according to the type of dye used for labeling of the probe or the sample. Since only one dye is used for the assay, a single laser and filter set should be selected.

■ Set the scanning resolution to 20 µm.

■ Slides should be scanned according to the procedure in the respective scanner manual.

■ The scanning area must include the complete arrayed membrane area but should not include the membrane borders or the glass area surrounding the membrane.

■ Slides should be placed in the scanner and scanned so that the area closest to the slide label (frosted area) is at the bottom of the output image.

■ Sample and control slides should be scanned using the same scanning conditions.

Define scanning conditions using a sample slide (not a control slide).

Note: spot intensity values should be determined using the image analysis software supplied with your scanner.

- Scan your image so that the highest density obtained is ≤55,000 for lectin (analysis) spots (see Figure 4 for information on spot positions).

- Do not allow any lectin (analysis) spots to be saturated. Positive control spots can be saturated if necessary.

- Background density (outside arrayed spots) should be ≤10,000.

- In extreme cases, where the above two guidelines cannot be met, reduce the scanning conditions so that the background is ≤10,000.

- If a control slide was used in the experiment, ensure that the analysis spots (not positive control spots) are not saturated under the scanning conditions for the sample slide.

- The output file should be saved in 16 bit uncompressed ".TIFF" format. Before saving ensure that:

    - Scanned image is at 20 μM resolution

    - Saved image is for the fluorophore only and not the ratio image (i.e., the image is scanned with one laser and not a composite image of successive scans with different lasers)

Slide layout and positive control spots

The slide is printed with lectins specific for different glycosylation unit. The lectins are printed in 2 duplicate blocks each comprsing 12 squares of 3 x 3 spots. Each lectin is printed in 3 replicate spots in each block.

The slide contains a number of fluorescently labeled protein spots that serve as alignment aids during analysis (circled spots in Figure 4). The image of the scanned slide should present bright spots of the alignment aid protein and of the lectins that identified a glycan moiety in the sample and interacted with it. Figure 4 shows a typical processed slide.

Qproteome GlycoArray Layout
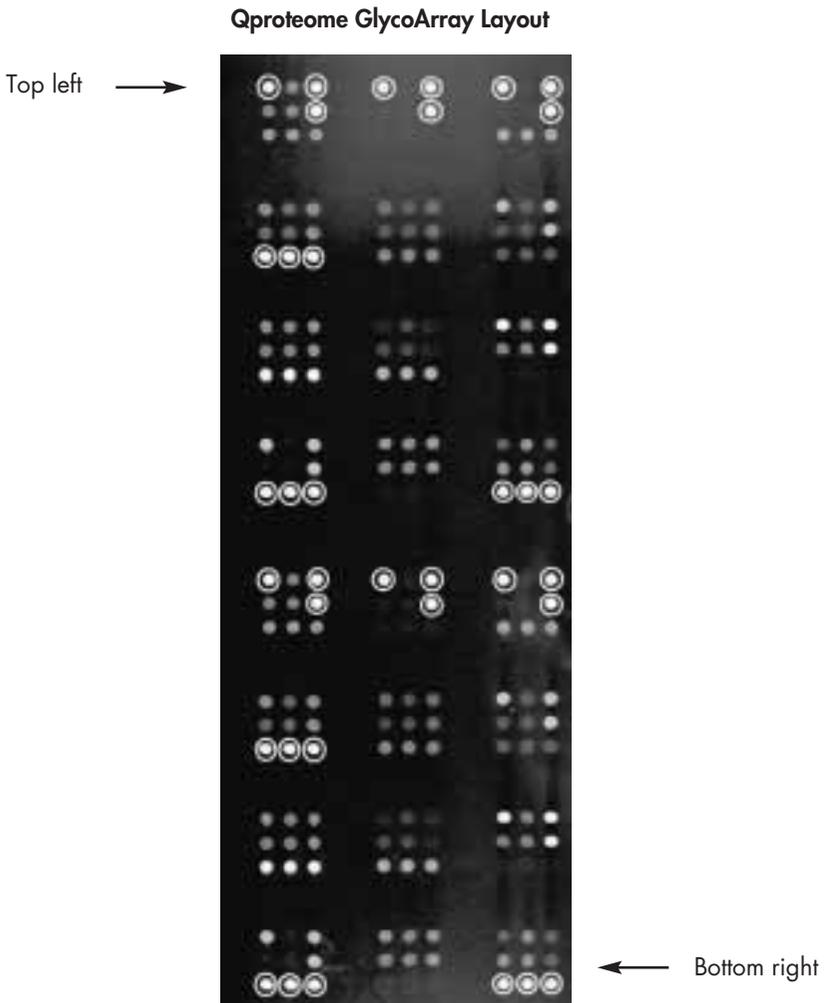
Figure 4 Layout of analysis and positive control spots (circled) on Qproteome GlycoArrays.

Analyzing GlycoArray Data

Required specifications for computer workstation

The workstation used to run the sample analysis software must conform to the following specifications:

Operating system: Microsoft® Windows® 2000 or Windows XP

Memory: Minimum 256 MB

Free disk space: Minimum 100 MB

To perform comparisons of samples, Microsoft Excel™ is required.

Software Installation

Reinstallation of Qproteome GlycoArray Analysis Software on a computer on which an existing version is installed.

1. **Uninstall software by clicking on: Start > Settings > Control Panel > Add/Remove Programs. Select "Qproteome GlycoArray Analysis Software" and click on "Change/Remove". In the install wizard window select the "Remove" option.**

2. **Reinstall software according to the steps below.**

Installing Qproteome GlycoArray Analysis Software

1. **Insert the CD provided with the kit into the CD drive. Make sure you have administrative rights on the workstation. Locate the setup.exe file located in the root folder of the CD drive and double click on it.**

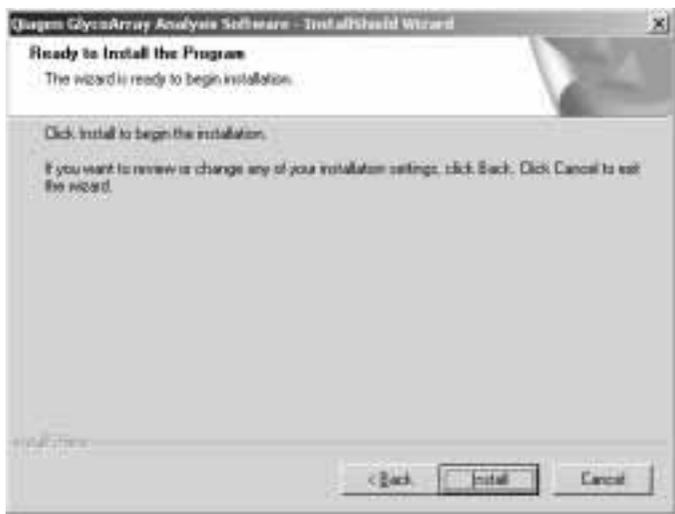   The following screen will be displayed.
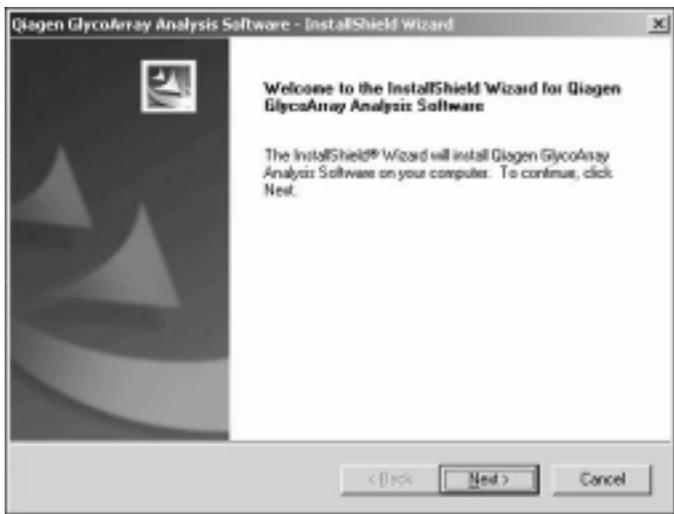
2. **Click "Next".**

The following screen will be displayed. You can change the installation folder location, however we recommended using the default destination.
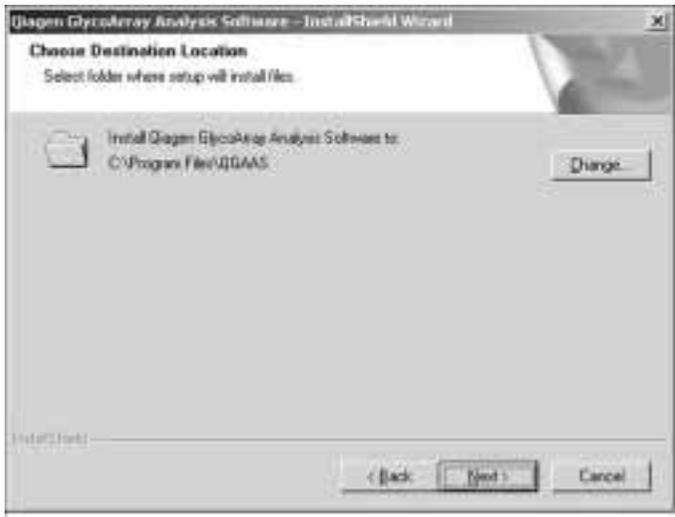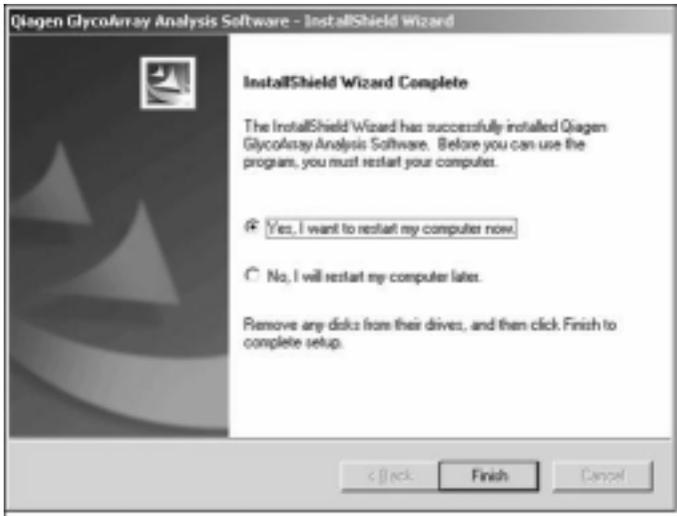

3. **Click "Next" when ready.**

The following screen will be displayed.

4. Click "Install" to start the installation.

5. Once installation is completed, restart the computer. After restart, the software is ready to run. Use the same user name (with administrative rights) in order to log in to the station.

6. The software can be started by clicking the QGAS icon on the desktop or by choosing Start > Programs > Qiagen > Qproteome GlycoArray Analysis Software.

Running GlycoArray Analysis Software

1. **Start the analysis software by selecting Start >Programs >Qiagen > Qproteome GlycoArray Analysis Software.**

   You will see the following screen.

2. **Select the kit definition file by clicking on** **.**

   This file contains information specific to the kit, therefore it is important that you load it from the CD supplied with the kit from which the slides were taken. If the software version supplied in the kit is higher than that installed on the computer, uninstall software from the computer and install the latest version supplied in the kit (see "Software Installation", page 37).

3. **Select the relevant Detection Method option using the following table.**

Assay procedure performed	Detection method option
Fluorescently labeled glycoprotein	Labeled sample
Biotin labeled glycoprotein	Labeled sample
Unlabelled glycoprotein with a fluorescently labeled primary antibody	Labeled probe
Unlabelled glycoprotein with a fluorescently labeled secondary antibody	Labeled probe

4. Select Image Analysis option. If slides were scanned using a microarray scanner at 20 μm/pixel and saved as 16-bit TIFF format select "Automatic". Image analysis will be performed without user intervention. If scanning was not performed according to recommendations, select the "Semi Automatic" option.

5. Select the slide images (.TIFF files) produced by your scanner by clicking on 🗁 for each file. The order of the duplicates is not important, but for quality control reasons, it is recommended to record the order of the duplicate slides.

6. After defining the assay type, analysis options, and images to be analyzed, click the ▨ button.

    If image analysis "Automatic" option was used, analysis will be conducted auto matically and the results screen will appear. If the "Semi Automatic" option was used, the software will prompt for user intervention for each of the slides that need to be analyzed. The following screen will appear.
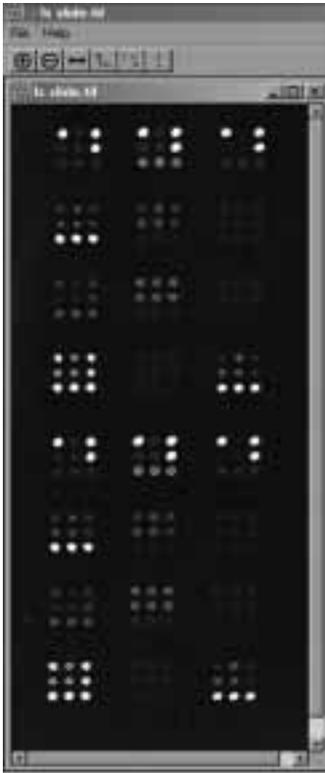

Zoom in or out using the ⊕ and ⊖ buttons respectively.

If positive control spots are not aligned as described on page 36, flip image left to right using the ↦ button.

To perform image analysis the user must define the top left and bottom right corners of the array on the image. This is done by clicking on the ▨ button and selecting the top left spot in the image (see page 36). Drag the circle drawn on the screen so that it is centered on the spot in the top left corner of the image (this is better done in zoom mode).

Once the top left spot is marked click on the ▨ button and repeat procedure for the spot at the bottom right corner of the image (see page 36). Once the top-left and bottom-right spots are defined, click on the ❗ button. The software will proceed with image analysis.
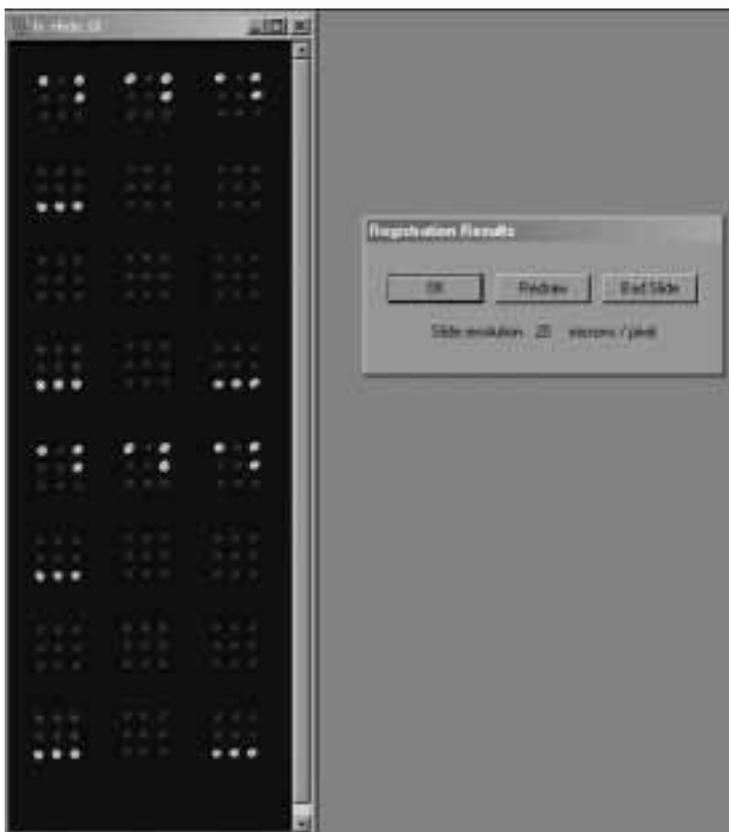
7. **Check that the virtual grid (red circles) is aligned properly on the spots. If the image is misaligned, perform image analysis again by clicking on "Redraw" and care fully positioning the top-left and bottom-right spots. If image alignment is correct click on "OK" Image analysis will be completed.**

Note that this procedure must be performed for all slides in the experiment.

Once the image analysis step is complete, the software analyzes the fluorescence images to produce a glycan "fingerprint". The fingerprint is interpreted by the soft ware to determine the glycan features present (interpretation table). A quality report is produced.

Once analysis is complete, the results screen will automatically appear. The time required for analysis depends on computer speed, but it is generally less than one minute per slide. If the results screen does not appear as expected, please refer to the Troubleshooting Guide on page 50.

GlycoArray Fingerprint Analysis Screen

The results can be printed or exported to Microsoft Excel: when clicking on the "Export to Excel" button the following window will appear.

Select "Glyco Analysis report" to save the calculated results; quality monitors source file data, and interpretation table.

Select "Sample/Control report" in order to compare sample and control signals generated in labeled probe experiments (required for troubleshooting and calibration).

Select the file name you want to save to, and click "Save". Files are saved in CSV format.

The saved file (in .CSV format) contains all the data about the experiment (file names, etc.), fingerprint, interpretation, and quality report.

Interpreting the Glycoprotein Fingerprint

The fingerprint is a histogram where each bar represents the calculated signals of a single lectin arrayed on the slide in several replicates. The lectins in the fingerprint are grouped by their specificity, and presented in a group-number format; the group-number combination is constant for each lectin and does not change.

The fingerprint itself provides valuable data for sample analysis. It is particularly useful for comparative analysis of several samples, to show differences in glycosylation. The fingerprint interpretation is performed by deconvoluting the fingerprint. Detection of each feature is based on deconvolution of signals from several lectins with overlapping and/or complimentary specificities. The fingerprint deconvolution is performed by proprietary algorithms that use rule-based methods that have been calibrated using a wide set of standard proteins. Each lectin group specifically recognizes one or two glycans/epitopes, although there are interdependencies between these groups. The following guidelines can be used to interpret the fingerprint.

Complex

The lectins in this group recognize the glycan structure of the N-complex type. The different reactivities of the lectins within this group allows the deconvolution of bi- and tri-/tetraantennary structures. Some of the lectins of this group are sensitive to different antennae termini as they bind large parts of the glycan structure.

Mannose

This group consists of lectins that bind specifically to the core mannose structure of the N-glycan type. These lectins will bind high mannose structures as well as biantennary complex structures. Binding to biantennary structures is enhanced in the presence of truncated antenna structures.

Gal/GalNAc

These lectins are specific for the terminal galactose/N-acetyl galactose amine residue at the end of the antenna structure of N- and O- glycans. Their binding is inhibited in the presence of acidic residues (e.g., sialic acid).

Beta Gal

These lectins specifically bind the terminal (non-sialylated) beta-galactose subunits at the end of N-linked antennae. Their binding is inhibited in the presence of acidic residues (e.g., sialic acid).

Terminal GlcNAc

This lectin specifically recognizes the truncated antennary structure by binding to the N-acetyl glucosamine residue.
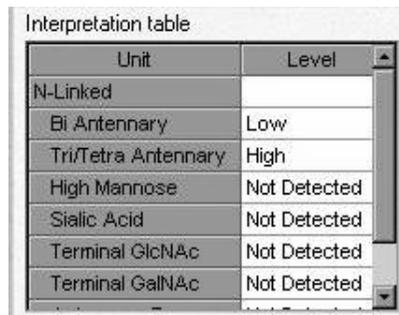
Sialic acid

The sialic acid lectins react with charged sialic acid residues. Different lectins recognize different linkage of this residue (2–3 and 2–6). In general, high levels of sialic acid will result in a fingerprint with high signals from the sialic acid specific lectins and low signals from the Gal Beta, GlcNAc and GalNAc specific lectins.

Relative levels

The interpretation table (bottom left part of the screen, see Figure 5) provides an esti-mation of the relative abundance of specific glycan structures in the tested sample. The estimations correspond to the absolute figures given in Table 9.

Typical Interpretation Table Result

Figure 5 An interpretation table result after image processing using the Qproteome GlycoArray Analysis Software.

Table 9. Interpreting Analysis Results

Interpretation	Values*
Not Detected	Less than 10%
Low	11–30%
Medium	31–70%
High	71–100%
Detected	More than 11%

* If an epitope's relative abundance lies close to an interpretation boundary (±5%) or if the protein's conformation affects its binding to the lectin probes, the interpretation may be mis-categorized by up to one level.

Evaluating the Quality Control Report

The quality control report summarizes the technical quality of the processed slides in the assay. Every slide in the experiment is tested for 4 parameters:

■ Lack of signals on slide

■ Scanning

■ Process quality

■ Nonspecific binding

The reproducibility between duplicate slides is also measured (where applicable).

The process quality report is performed during the process of fingerprint generation. Since each lectin spot is arrayed in 6 replicates on the slide, a bad replicate spot will be excluded from the calculation. In the same manner, in the case of duplicate slides, only the slide with a good score will be included in the calculation.

For a detailed explanation and troubleshooting of quality control parameters, refer to the table below.

Quality Control Report Parameters

Possible explanation	Solution and recommendations
Monitor warning — Low scanning quality	
a) Scanning conditions are too high	Reduce laser power, PMT gain settings, or both.
b) Poor blocking/Sample and/or probe concentrations are too high	Compare background signals to background signals for other samples. If problem persists contact your local QIAGEN Technical Support Department.
Monitor warning — Low process quality	
a) Poor blocking	Ensure blocking solution is correctly prepared and that the membrane was blocked for 60 min.
b) Slides dried out during the process	Ensure that there is sufficient solution to cover membrane. When processing multiple slides, leave all slides not being handled in complete wash solution. Keep Petri dish lids closed during incubation.
c) Insufficient washing	Ensure wash solution is correctly prepared and that the membrane was washed as described in the relevant protocol.

d) Samples or probes did not evenly cover the arrayed area	Ensure that samples and probes evenly cover the array area. Avoid introduction of bubbles.

Monitor warning — Nonspecific binding

a) Sample concentration too high	Reduce sample concentration.
b) Sample is contaminated	Remove contaminant before processing.
c) Non-specific binding of probe	Use a different, more specific antibody.
d) Sample buffer contains components that bind with the lectins	Exchange or pretreat buffer to remove lectin binding components before processing.

Monitor warning — Low duplicate reproducibility

a) Wrong slide order (incorrect files were uploaded)	Check slide order, and amend where necessary.
b) Poor processing of one of the slides	Visually inspect each image.
c) Duplicates were not scanned using the same scanning conditions	Re-scan slides using appropriate conditions.

Monitor warning — Low signals

a) Scanning parameters were too low	Increase scanning amplification.
b) No glycosylation on sample	Check level of glycosylation by SDS-PAGE.
c) Insufficient binding by antibody (if relevant)	Use a different antibody with higher affinity.
d) Poor sample or antibody labeling	Repeat sample or probe labeling procedure.

Monitor warning — Consider desialylation or calibration

Sample and control signal are too close to each other	Calibrate sample or antibody concentration according to the procedure described on page 55.
In cases where charged molecules impede lectin binding to the glycan feature, consider desialylation according to the procedure described on page 56.

Comparing Samples

For a valid comparison, the following criteria must be met:

- Samples must be processed using the same protocol and solutions
- Dye:protein ratio of the sample or the probe must be similar
- Slides must have been scanned using the same scanning parameters (laser power and PMT gain settings)
- Results for all samples must be generated and exported to Microsoft Excel using the same Qproteome GlycoArray Analysis Software version.

The fingerprints of the various samples can be compared by clicking on the compare files button.

Once the "Compare Files" button is clicked the following window will appear.
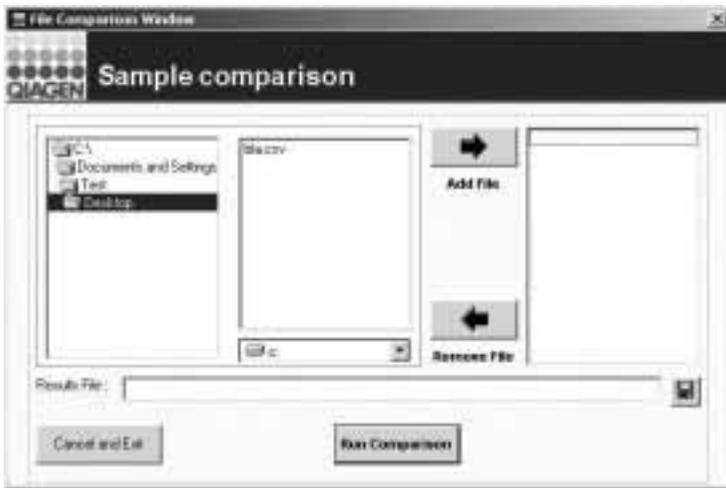
Select the drive and directory in which the slides are located. Browse using the left-most window to the directory in which the *.csv files were saved.

Select the *.csv file and click on the add file button from the middle window (alternatively double click on the file name). Make sure that all the files required for comparison are in listed in the rightmost window.

Click on 🖫 in order to define save path and saved filename. Click on "Save".

Click on "Run Comparison". An Excel file will be generated containing the following:

■ "Compare Data": tab contains the data used for comparison.

■ "Charts" tab contains 2 graphs: one for the raw intensity and one for the normalized data.

If a manual comparison is performed, make sure the signals from the same lectin are compared for both samples

Once this has been done, epitopes can be ranked between samples by looking for trends in specific lectins (e.g., higher sialic acid level, lower antennarity, etc.)

In case the above comparison conditions were not met, the comparison should be based on the "Normalized to average" data in the "Charts" worksheet in Excel. This comparison may not be as accurate.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or molecular biology applications (see back page for contact information).

	Comments and suggestions
Processing slides	
a) Solutions are turbid	Solutions were prepared without proper stirring. Add each solution in the given order while stirring. Do not add the next ingredient before last one is dissolved.
	Water quality is low. Use RO- or HPLC-grade water filtered with 0.22 micron filter.
b) Insufficient sample to cover the slides	Sample was incorrectly prepared. Make sure that you have 450 µl sample/control per slide before starting analysis.
c) There are bubbles on the arrayed area	The sample was not placed properly on the slide. Try to remove the bubbles gently with a pipet tip without touching the membrane.
d) Membrane was damaged	If the edges of the membrane are scratched, continue the experiment. Otherwise, start again with a new slide.
Scanning	
a) No signals seen	Scanning conditions are too low. The slide is arrayed with fluorescently labeled positive control spots. Change your scanning conditions until you see them.
	Refer to section "Autofluorescence" on page 54.
b) High background or signals are saturated	Scanning conditions are too high. Reduce scanning conditions. The optimum is to have no saturated spots (except for positive control spots). The positive control spots should have a relatively lhigh signal (see page 34).
c) There are bubble shaped areas with high or no fluorescence	Membrane was not covered completely during incubation or bubbles were created on the membrane. Repeat the experiment. Make sure the entire surface of the membrane is covered.

Comments and suggestions

d) Error message "Increase scanning parameters"	If scanning parameters conform to the guidelines on page 34, ignore this message. Otherwise increase scanning parameters according to the guidelines on page 34.
	If the error message is obtained during the analysis of a comparative experiment in which several samples were run in parallel and scanning was defined according to the sample with the lowest scanning parameters fulfilling the scanning guidelines, ignore this error message.

Software installation

a) Installation fails	Operating system is not Windows 2000/XP. Use a computer with Windows 2000/XP.
	You do not have administrator rights on the machine. Contact your system administrator
b) Software does not start	Restart your computer and try again.
c) Installation was not successfully completed	Uninstall the software, reboot computer, re-install software, and reboot computer again. Refer to page 37 for installation protocol.
d) Error message "This Kit Definition File requires software version X.X or higher" or "Kit Definition File Structure is invalid"	Software version is not compatible with Kit Definition File. Uninstall software from computer. Install Software and use Kit Definition File provided with the kit. Refer to page 37 for installation protocol.

Analysis

a) Software cannot load image file	Image not saved in *.TIFF format. Rescan slides at 20 μm/pixel and save them in uncompressed 16-bit .TIFF format.

b) Image analysis fails — grid cannot be set	No spots found on the slide. Perform "Semi Automatic" image analysis. Change scanning conditions.
	Signal of positive control spots is too low. Change scanning conditions. The minimum positive control spot signal should be at least 2000 times above background.
	Scanning resolution is incorrect. Change scanning resolution to 20 microns.
	Scanned slide area is smaller than the actual arrayed area. Check slide area.
	If the above steps do not solve the problem, con tact QIAGEN Technical Service.
c) The software does not display the results	Anti-virus software blocks scripting. Unblock scripting on your computer. Reinstall the software.

Low signal warning during analysis

a) No sample on slide/Sample or probe concentration is too low/Poor sample or probe labeling/No glycosylation of the sample	Scan an empty slide and compare pattern for finding autofluorescence. See the auto-fluorescence section on page 54. Check labeling by SDS-PAGE. Consider higher sample or probe concentration.
b) Scanning conditions too low	Consider changing scanning conditions.
c) Laser and filter not as required	Use appropriate laser and filter.
d) Probe specificity too low	Use a probe with a higher specificity.
e) Sample glycans are heavily sialylated or have high levels of charged molecules (e.g., sulfate, phosphate)	Consider sample desialylation (see page 56), dephosphorylation, or desulfatation.

Results not as expected

a) Experiment parameters were not set correctly	Ensure that parameters were set correctly.
b) Slides were scanned upside down	Ensure slides are scanned with the membrane side uppermost.
c) Sample is heavily sialylated or ends of antennae are capped by sulfate or phosphate	Consider sample desialylation (see spage 56), dephosphorylation, or desulfation.

Comments and suggestions

d) Glycans are not recognized by the system

Sample was produced in insect or plant system. This system is calibrated for glycans from mam malian sources.

e) Glycans, or parts of glycans are not exposed on the surface of the protein

In some cases, the 3D conformation of the protein will not allow detection of the core structure of the glycan.

f) Sample contains structures that are not detected by the kit (e.g., hybrids)

GlycoArrays are designed to detect and deconvolute the mammalian glycan structures described in Table 1.

g) All the results in the interpretation are (–1)

The software has indicated that no significant signals were found on the slide. See comments and suggestions in "Low signal warning during analysis" section on page 52.

Appendix A: Optimizing the Analysis Procedure

Autofluorescence

When abnormally high scanning conditions are required to see lectin signals, it is advisable to check that these signals come from actual binding of the sample to the array. Some of the arrayed lectins possess autofluorescence properties.

In order to verify that the signals are not a result of autofluorescence, follow the following procedure.

1. **Record the scanning conditions (laser power, PMT gain, focus) used to scan the sample slide.**

2. **Export the fingerprint of the sample slide (without any controls used) to an Excel file.**

3 **Scan an unprocessed slide (from the kit) using the scanning conditions recorded in step 1.**

4. **Use the software to analyze the image and export the result to Excel.**

5. **Compare the results.**

   The absolute (not normalized) intensities from the sample slide should be signifi cantly higher than those obtained from the unprocessed slide.

   The pattern of lectin binding should change dramatically.

   If it is still suspected that signals arise from autofluorescence, follow the guidelines in the troubleshooting guide to make sure the sample and/or probes are active and labeled correctly.

Calibration of sample concentration

The recommended sample concentration is 0.2 μM. However for some proteins, the optimal concentration may be significantly lower, especially when using an antibody as a probe for detection. Under these conditions it is possible to reduce sample con centration by simple calibration.

The optimal sample concentration is that for which the interaction of the arrayed-lectins with the sample is in the linear range of the assay.

Check concentration by running the sample at several concentrations (0.05–0.2 μM). The following parameters should be considered for selecting the optimal concentration:

■ The obtained glycosylation interpretation should be the same as that obtained for samples at 0.2 μM.

■ The response of each lectin versus the concentration of the sample should be linear.

■ Quality monitors are "OK".

Optimizing primary antibody probe concentration

A final concentration of 60 nM has been found to be suitable for a large number of unlabeled and FITC-labeled rabbit polyclonal antibodies tested in optimization trials. A final concentration of 6 nM has been found to be suitable for a number of Cy3-labeled rabbit polyclonal antibodies. In cases where information regarding the primary antibody concentration is not available, or when the antibody is from a different species, the following optimization procedure can be used.

Procedure

1. **Perform the assay with one sample slide and one control slide using the antibody dilution recommended for western blot analysis.**

2. **Use the Qproteome GlycoArray Analysis Software in "Labeled Sample" mode to analyze each slide individually.**

3. **Save the results for each slide, noting whether it is a sample or control.**

4. **Calculate the sample:control signal ratio and sample minus control signal for each lectin.**

    If most of the lectins have a sample:control ratio that is close to 1, perform another experiment with lower antibody concentrations (twofold dilutions).

    If the sample:control ratio for most lectins is higher than 2, and the sample signal minus control signal for reactive lectins is lower than 1500, perform another experiment with higher antibody concentrations (twofold increase).

    If the sample:control signal ratio for most reactive lectins is higher than 2 and the sample signal minus control signal for reactive lectins is higher than 1500, no further calibration is needed.

Appendix B: Recommended Method for Sample Desialylation

Materials and equipment to be supplied by user

- Neuraminidase 10 U/µl (e.g., Roche, cat. no. 269611)
- Protease inhibitor (e.g., PI cocktail Set I, Calbiochem, Cat. no. 539131). Dissolve 1 tablet in 1 ml RO- or HPLC-grade water to obtain 100x stock solution.
- Lockable 1.5 ml tubes
- PBS buffer

Procedure

1. Dilute your protein sample to a concentration of 0.433 µM using PBS.
2. Dilute 1 µl of neuraminidase 1:20 in PBS to a final concentration of 0.5mU/µl.
3. Add 1.2 µl of neuraminidase prepared in step 2 to 233 µl of the protein sample prepared in step 1.
4. Add 2.3 µl 100x protease inhibitor cocktail.
5. Incubate at 37°C in a lockable 1.5 ml tube for 16–18 h.

Sample preparation for glycoanalysis

6. Add 238.5 µl of complete wash solution (prepared according to page XX) to the desialylated sample.
7. Add 25 µl of complete blocking solution (prepared according to page XX) to the diluted desialylated sample.

    Sample is now at a concentration of 0.2 µM in a volume of 500 µl.

Notes

Ordering Information

Product	Contents	Cat. No.
Qproteome GlycoArray Kit	For 6 glycoprotein analyses: 6 lectin-based arrays, Incubation Frames, Buffers, CD containing analysis software and kit definition file	37702
Related products		
Qproteome Total Glycoprotein Kit	For 6 total glycoprotein preps: Buffers, Lectin Spin Columns (6), Detergent Solution, Protease Inhibitor Solution, Collection Tubes (6 x 2 ml)	37541
Qproteome Mannose Glycoprotein Kit	For 6 mannose glycoprotein preps: Buffers, Lectin Spin Columns (6), Detergent Solution, Protease Inhibitor Solution, Collection Tubes (6 x 2 ml)	37551
Qproteome Sialic Glycoprotein Kit	For 6 sialic glycoprotein preps: Buffers, Lectin Spin Columns (6), Detergent Solution, Protease Inhibitor Solution, Collection Tubes (6 x 2 ml)	37561
Qproteome O-Glycan Glycoprotein Kit	For 6 O-Glycan glycoprotein preps: Buffers, Lectin Spin Columns (6), Detergent Solution, Protease Inhibitor Solution, Collection Tubes (6 x 2 ml)	37571

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