



FACE[®] N-Linked Oligosaccharide Sequencing Kit

GK90300

TOOLS FOR GLYCOBIOLOGY

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FACE N-Linked Oligosaccharide Sequencing Kit (10 Reactions)

Please read the following protocol right through before starting your experiment

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Kit Contents

Code	Component	Quantity	Catalog #	Storage
	Oligo Profiling Gels	10 precast gels	GK60000	Room Temp
	Oligo Gel Reagent Pack	1 ea	WS0007	Room Temp on arrival
	5 ea Running Buffer,			Store Buffer at 4°C when
	1 ea 2x Loading Buffer (E1)			reconstituted
	1 ea Tracking Dye (E2)			
S1	Sialidase A (<i>A.ureafaciens</i>) / NANase III	1 tube	WS0050	4°C
S2	β (1-4)-Galactosidase (<i>S.pneumoniae</i>) / GALase III	1 tube	WS0051	4°C
S3	β -N-acetylhexosaminidase (Jack bean) / HEXase III	1 tube	WS0052	4°C
S4	α -Mannosidase (Jack bean) / MANase II	1 tube	WS0053	
S5	α -Mannosidase (<i>X.manihotis</i>) / MANase VI	1 tube	WS0054	4°C
S6	5X Enzyme reaction Buffer	1 tube	WS0055	
E3	OLIGO Ladder Standard	1 tube	WS0014	-70°C
E6	N-Linked Sequencing Control	1 tube	WS0064	-70°C
E7	Core Standard	1 tube	WS0065	-70°C

Additional Reagents and Equipment Required

- FACE Electrophoresis Gel Box
- FACE N-linked Oligosaccharide Profiling Kit
- A CAP Type I water supply (such as Milli-Q)
- Assorted pipeting devices including a 0-10 μ l capillary positive displacement pipette (e.g. Drummond Microdispenser, Fisher Cat# 21-176D)
- Centrifugal vacuum evaporator
- 37°C oven or water bath
- Longwave U.V. Transilluminator (lightbox) or FACE Imaging System
- 1.5 ml microcentrifuge tubes
- Microfuge and spin filters

Introduction

Complex carbohydrates are important components of all living things. In addition to providing energy and structural supports for cells, increasing evidence has shown that the carbohydrate moieties of glycoconjugates are often important as recognition determinants in receptor-ligand or cell-cell interactions, in the modulation of immunogenicity and protein folding, and in the regulation of protein bioactivity. Changes in the biological activity of glycoproteins often result from alterations in protein glycosylation either through variable site occupancy or changes in the structure of the oligosaccharide occupying a particular site. Sequencing of oligosaccharides is most easily accomplished by following the progressive degradation of oligosaccharides using exoglycosidases. This method is one of the most widely accepted and reliable methods for determining an oligosaccharide sequence. The FACE N-linked Oligosaccharide Sequencing System utilizes glycosidases with precisely defined monosaccharide and linkage specificity to determine the sequence of oligosaccharides.

In order to sequence an oligosaccharide, it is best to isolate it from other oligosaccharides in the mixture. Once an oligosaccharide has been isolated, the sequence can be determined through a series of enzymatic digestions. The FACE sequencing strategy, described in this manual, incorporates the general structural features of N-linked oligosaccharides, the pre-determined migration patterns of oligosaccharides of known sequence, and the degradation patterns resulting from digests with exoglycosidases of known specificity to elucidate the sequence of an unknown oligosaccharide.

Description of the FACE N-linked OLIGO Sequencing System

The FACE N-linked Oligosaccharide Sequencing System consists of five steps:

Step I) Purification of the fluorophore labeled N-linked oligosaccharide is accomplished using a FACE N-linked Oligosaccharide Profiling Gel.

Step II) Digestion of the purified oligosaccharide is performed using mixtures of exoglycosidases.

Step III) Separation of the enzyme digestion products is accomplished on an N-linked Oligosaccharide Sequencing Gel.

Step IV) Imaging of the sequencing gel is performed using a longwave U.V. transilluminator (lightbox) or the FACE Imaging System.

Step V) Evaluation of the image to determine the sequence of the oligosaccharide.

Step I) Purification of the Oligosaccharide

1 Release of N-linked oligosaccharides

In general, three different types of N-linked oligosaccharides can be isolated from glycoproteins – high-mannose, complex and hybrid. Complex types can be further subdivided into neutral and those containing sialic acid. Different oligosaccharide types are selectively released using appropriate release enzymes. We routinely use the two enzymes listed below to release the N-linked oligosaccharides from glycoproteins. The types of oligosaccharides released will depend on the enzymatic selectivity.

Enzyme	Selectivity	Glyko Cat #
N-Glycanase [®] (PNGase F)	High-mannose, hybrid and complex	GKE-5006
Endoglycosidase H (Endo H)	High-mannose and hybrid	GKE-5002

Following the release of the oligosaccharides from the glycoprotein, the mixture of oligosaccharides are labeled at the reducing end with a fluorescent tag and separated on an oligosaccharide profiling gel. This procedure is detailed in the FACE N-linked Oligosaccharide Profiling Kit (Glyko Cat GK90000).

If N-Glycanase is used as the release enzyme, the oligosaccharide profile will contain a mixture of oligomannose, hybrid and complex oligosaccharides. If Endo H is used as the release enzyme, the profile will contain only high-mannose-type or hybrid-type of oligosaccharides. Digestion by these enzymes (detected by FACE) is the first indicator of the types of oligosaccharides on the glycoprotein.

Additional endoglycosidases (e.g. Endo D and Endo F) are available commercially and all of these can be used to release different types of oligosaccharides from glycoproteins. N-linked type oligosaccharides can also be released using hydrazine, which is believed to be non-selective and may cause some degradation of the oligosaccharide.

2 Isolation of N-linked Oligosaccharides

FACE N-linked oligosaccharide sequencing begins with the isolation of a fluorophore labeled oligosaccharide from an oligosaccharide profiling gel. This is best accomplished by preparing an oligosaccharide preparative gel as described in the beginning of the protocol section of this manual (page 10).

Once the isolated oligosaccharides are eluted and dried, a small aliquot should be run on a single lane of a profiling gel to determine the extraction efficiency as shown for oligosaccharides isolated from alpha-1 acid glycoprotein (AAG) in Figure 1. In most cases 300 pmoles of purified oligosaccharide is required for each sequencing experiment when using the FACE Imaging System. When using a U.V. transilluminator (lightbox) to visualize the oligosaccharide, the amount of oligosaccharide required will depend on the spectral properties of the lightbox and filters used. In general, 500-700 pmoles of oligosaccharide may be required. Remember that some photobleaching of the fluorophore labeled oligosaccharide will inevitably occur during the excision from the gel so that accurate quantitation will no longer be possible on isolated oligosaccharides.

Any N-linked type oligosaccharide with a free reducing end isolated by methods other than FACE (e.g. Biogel P4, HPLC etc.) can be labeled with the fluorophore tag using the OLIGO Labeling Reagent Pack (Glyko Cat # GK50004) and sequenced.

Note: Prior to sequencing you should retain some of your released glycan pool to compare with the enzyme digests.

Step II) Digestions of the Purified Oligosaccharide:

Oligosaccharide sequencing is performed by setting up a series of enzyme digests. These digests result in a stepwise degradation of the oligosaccharide from an intact structure through steps that selectively remove the outermost monosaccharides down to one or two core structures. The FACE N-linked Oligosaccharide Sequencing Kit contains five highly purified exoglycosidases of the following specificity:

Enzyme Name	Specificity	Previous Name	Tube
Sialidase A (<i>A.ureafaciens</i>)	α 2- 3,6,8 linked sialic acid	NANase III	S1
β (1-4) Galactosidase (<i>S.pneumoniae</i>)	β 1-4 linked galactose	GALase III	S2
β -N-Acetylhexosaminidase* (Jack bean)	β 1-2,3,4,6 N-acetylglucosamine	HEXase III	S3
α -Mannosidase (Jack bean)	α 1-2,3,6 linked mannose	MANase II	S4
α -Mannosidase (<i>X.manihotis</i>)	α 1-6 linked mannose	MANase VI	S5

* This enzyme at the indicated concentration will not fully digest bisecting GlcNAc containing oligosaccharides.

The kit contains enough of these five enzymes to perform 10 complete oligosaccharide sequence determinations. In addition, the kit contains labeled electrophoresis size marker, control oligosaccharide and core oligosaccharide standards as well as 10 precast N-linked Sequencing Gels with electrophoresis buffer.

A typical sequencing experiment involves setting up 5 separate enzyme digests. Each of 5 tubes receives 50-100 pmoles of fluorophore labeled oligosaccharide, reaction buffer and one or more of the exoglycosidases supplied in the kit. The first tube of the sequencing experiment does not receive any of the enzymes and is used as a size marker for the location of the starting material on the gel. The second tube contains only sialidase, which will release sialic acid. The third tube contains sialidase and β -galactosidase, which will release both sialic acid and galactose. The fourth tube contains sialidase, β -galactosidase and β -N-acetylhexosaminidase which will release sialic acid, galactose and N-acetylglucosamine. The fifth tube contains all the above enzymes plus 2 α -mannosidases and therefore will degrade the oligosaccharide down to the $\text{Man}(\beta 1-4)\text{GlcNAc}(\beta 1-4)\text{GlcNAc}$ or $[\text{Man}(\beta 1-4)\text{GlcNAc}(\beta 1-4)[\text{Fuc}(\alpha 1-6)]\text{GlcNAc}$ oligosaccharide core structure. α -Mannosidase (*X.manihotis*) is added to the fifth tube after digestion by all other enzymes to complete the digestion of $\text{Man}(\alpha 1-6)$.

Step III) Separation of the Enzyme Digestion Products

Once the incubations are complete, the reactions are dried in a centrifugal vacuum evaporator, loading buffer is added and aliquots of each reaction are loaded on an OLIGO profiling gel. An OLIGO Ladder Standard (E3) is added to the first lane of each gel as a size marker for determining the number of sugars released by each reaction step and an oligosaccharide core standard (E7) is added to the last lane.

Electrophoresis proceeds for approximately 1 hour after which time the gel is imaged and the positions of the digested products are determined.

Step IV) Imaging of the Sequencing Gel

Imaging of the sequencing gel is performed using a longwave U.V. transilluminator (lightbox) or the FACE Imaging System. Imaging is used to determine the location of oligosaccharide bands on the gel and the relative mobility of the bands to the OLIGO Ladder Standard. The migration pattern and the known specificity of the exoglycosidases used to digest the oligosaccharide in combination with

monosaccharide composition data will allow a determination of the oligosaccharide sequence to be made.

Step V) Evaluation of the Image of the Sequencing Gel

Experimentally determined migration values of N-linked type oligosaccharides: Migration values for oligosaccharides are defined by a DP number (**D**egree of **P**olymerization). DP units are used by carbohydrate chemists to describe the mobility of oligosaccharides relative to a mixture of glucose polymers. The OLIGO Ladder Standard (E3) in this kit contains such a mixture of glucose polymers and is used to determine DP numbers for oligosaccharides in the sample. For example, when the OLIGO Ladder Standard (E3) is loaded in the standard lane, the band corresponding to maltotetraose (glucose₄) has a DP of 4, the maltopentaose band (glucose₅) has a DP of 5, etc. When comparing the migration of an oligosaccharide in a sample lane with the glucose ladder in the standard lane, the oligosaccharide that migrates halfway between glucose₄ and glucose₅ bands is said to have a DP of 4.5. The actual DP number of an oligosaccharide in the sample will depend on the separation parameters described below:

Charge-based separation of oligosaccharides: The mobility of charged oligosaccharides on FACE gels is influenced by its charge to mass ratio as well as by its hydrodynamic volume. Oligosaccharides that contain sialic acid such as a tri-sialylated, triantennary complex oligosaccharide will migrate faster than smaller neutral oligosaccharides such as an Asialo biantennary oligosaccharide. This is because the carboxylic acid groups on the sialic acid residues increase the net negative charge on the molecule. Thus two oligosaccharides with the same number of monosaccharides but with different degrees of sialylation will separate on FACE profiling gels.

Size-based separation of oligosaccharides: The separation of neutral oligosaccharides (either native or sialylated oligosaccharides following the removal of sialic acid) is based on size. For example, oligomannose type structures (Man₃-GlcNAc₂ through Man₉-GlcNAc₂) that are commonly found on glycoproteins separate as a mannose ladder on FACE profiling gels. Each rung of the ladder represents an oligosaccharide containing one additional mannose residue.

Table I offers a general guideline as to the migration position of various oligosaccharides on FACE Sequencing Gels. Caution should be exercised when using these DP values to identify oligosaccharides in the sample because oligosaccharides with different structures may co-migrate (have the same DP

number). It is important to realize that no chromatography system including FACE can guarantee that oligosaccharides will not co-migrate. Although it is preferred to isolate one band containing one unique oligosaccharide prior to sequencing, it is possible to use the FACE system to sequence a mixture of oligosaccharides simultaneously as shown in Figure 2 for an isolated band from rgp120.

Table II offers rules for predicting monosaccharide sequence based on mobility shifts determined from DP numbers of known standards. Using these rules, the number of monosaccharides released by the action of each exoenzyme can be determined. An example of the interpretation of a FACE image of the N-linked Sequence Control is discussed in the following section and shown in Figure 3. Standards of various types of N-linked oligosaccharides were sequenced (Figure 4,5, and 6) to show the patterns expected. We recommend you use these examples to help interpret your image results.

Example of Sequencing an Oligosaccharide Isolated from Alpha-1 Acid Glycoprotein (AAG)

Purpose: The N-linked Sequencing Control (E6) in this kit contains 600 pmoles of the fluorescently labeled oligosaccharide for use in the sequencing part of this example. In order to help familiarize yourself with the principals and procedures involved in sequencing, we recommended that you use the sequencing control to perform the “practice” sequencing experiment.

Description of the method used to isolate oligosaccharides from alpha-1 acid glycoprotein: Using the reagents in the FACE N-linked Oligosaccharide Profiling Kit (Glyko Cat # 90000 / 90010), 100 µg of AAG was digested for 2 hours with N-Glycanase. The released oligosaccharides were labeled with the fluorescent tag and separated on an N-linked oligosaccharide profiling gel provided in the profiling kit. At least, twelve oligosaccharide bands were observed on the profiling gel and approximately 500 pmoles of each band was isolated from a preparative profiling gel by cutting out fluorescent bands. The oligosaccharides were eluted from each gel slice into 0.5 ml of H₂O overnight. The recovered oligosaccharides in the supernatant were dried in a centrifugal vacuum evaporator and resuspended into 20 µl of H₂O. 2 µl of each oligosaccharide solution was removed, combined with 2 µl of 2X Sample Loading Buffer (E1) and each oligosaccharide band was loaded onto separate lanes on the oligosaccharide profiling gel shown in Figure 1. This gel was used to determine the recovery and relative purity of the isolated material. The oligosaccharide corresponding to the band in lane 2 of Figure 1 was sequenced.

Oligosaccharide Sequencing: The isolated oligosaccharide was sequenced using a FACE N-linked Oligosaccharide Sequencing Kit. According to the protocols outlined in the kit starting on page 13, five reaction tubes were set up each containing 50 pmoles or 2 ml of the isolated oligosaccharide, reaction

buffer, and 1) without exoenzyme, 2) with sialidase, 3) same enzyme as 2 plus β -galactosidase, 4) same enzymes as 3 plus β -N-acetylhexosaminidase, and 5) same enzymes as 4 plus α -mannosidase.

The five reaction tubes were placed at 37°C for 16 hours (overnight). Following the incubation period the tubes were placed in a centrifugal vacuum evaporator and dried for 30 minutes. The dried pellet was resuspended in 4 ml of water and 4 ml of 2X Sample Loading Buffer (E1).

Because the sequence of the oligosaccharide is determined by analyzing the mobility shifts of the digestion products it is essential that all of the reactions be included on the same gel. Electrophoresis was performed at 20 mA per gel with a tank buffer temperature starting at 5°C and the run was terminated when the orange tracking dye just cleared the bottom of the gel (approximately 1 hour). The gel was imaged using a FACE Imaging System with the Acquire set at 0.5% saturation. An image of the resulting gel is shown in Figure 3.

Interpretation of sequencing results: A series of mobility rules generated through the sequence analysis of numerous known oligosaccharides are shown in Table II. These rules can be used to correlate the mobility shifts measured in the sequencing gel to the number of monosaccharides released sequentially from the oligosaccharide. For the current example (Figure 3, Lane 4), the sialidase digested oligosaccharide (Reaction 2) shifted 2 DP units upward from the undigested (Reaction 1). Since each sialic acid present on the oligosaccharide contributes an average of 1 DP unit shift upward (see Table II), two sialic acids are present. The actual DP units of these bands are shown in the band table below the image. The original oligosaccharide has a DP of 5.3; the asialo oligosaccharide has a DP of 7.6 for a difference of 2.3 DP. When you remove the sialic acid, the mobility of the band decreases. This is because the mobility of oligosaccharides on FACE gels is based on charge as well as size (see page 9). When two sialic acids are removed, two negative charges are also removed and the mobility of the oligosaccharide is reduced. As shown in lane 5, if the galactose residues are also removed, the resulting oligosaccharide displays an increase in mobility of 2 DP units from that in lane 4. The loss of 2 galactose is consistent with a di-sialylated bi-antennary structure. The analysis is continued for the remaining lanes with the release of 2 GlcNAc in Lane 6, and 1-2 mannose in Lane 7. In this analysis, α -Mannosidase (*X.manihotis*) was not added to complete the removal of Man(α 1-6). Therefore, two species are found in Lane 7. The fully digested core residue in Lane 7 co-migrates with the Man(β 1-4)GlcNAc(β 1-4)GlcNAc band in the Core Standard(E7) in Lane 8.

Protocols

SECTION 1 ISOLATION OF OLIGOSACCHARIDES FOR SEQUENCING

I. Setting up a Preparative Oligosaccharide Gel

- 1 Release and label the N-linked oligosaccharides from a glycoprotein using a FACE N-linked Oligosaccharide Profiling kit. The amount of glycoprotein required for sequencing an oligosaccharide will depend on the degree of glycosylation and the relative abundance of the particular oligosaccharide of interest. Depending on the abundance, you may need to digest as much as 500 μg of glycoprotein in order to make sure enough sample is obtained. If you need to obtain a large amount of material (>50 nmoles of total oligosaccharides) it is better to prepare a series of reaction tubes and later pool the oligosaccharides than to scale up a single reaction since drying a large volume is relatively slow.
- 2 Profile the labeled oligosaccharides on a FACE N-linked Oligosaccharide Profiling gel. Image the gel. Use band finding. Set standard band G_4 of OLIGO Ladder Standard to 25 pmoles. Display the table and Portion of Lane Luminance to determine the relative amounts of each band present. Display the table Quantity to determine the picomolar amount of each band.
- 3 Based on the results of the profiling gel, you need to determine how much of the profiling mixture to load onto a preparative profiling gel. For example, if the oligosaccharide band you want to sequence contains 200 pmoles of oligosaccharides on the profiling gel, then you will need to isolate 2 to 3 times that amount for sequencing.
- 4 To purify oligosaccharides from a preparative gel, set up a second profiling gel and load three lanes with the same amount of the oligosaccharide mixture that you used in the original profiling gel. If the band you are interested in sequencing contains only 100 pmoles in the original profiling gel then you will need to load 6 lanes on the preparative gel or twice the amount in each lane. Isolate all bands of interest from the same preparative gel. Isolated oligosaccharides are stable for at least 6 months at -70°C .

II. Isolation of individual oligosaccharide bands from preparative profiling gel

CAUTION *U.V. protective eyewear and face shield should be worn. avoid prolonged exposure to U.V. light. turn off U.V. light as soon as possible.*

- 1 Following electrophoresis of the preparative gel, rinse the gel cassette with water, wipe dry, remove the tape, and carefully separate the glass plates with a broad spatula. Carefully remove the gel from the cassette and place directly on the longwave U.V. lightbox.
- 2 Visualize individual oligosaccharide bands by turning on the U.V. lightbox. Using a scalpel or razor blade, carefully excise the band, or bands, of interest. Turn off the U.V. light.
Note: This should be done as quickly as possible to minimize photobleaching.
- 3 Place the gel slices of each band in separate microcentrifuge tubes.
- 4 Soak the gel slices in a minimum volume of 100% ethanol for ½ hour.
- 5 Pipet off the ethanol and suspend the gel slices in a minimum volume of water, enough to cover the slices.
- 6 Vortex gently, place at 4°C overnight or all day in the dark (12-16 hours).
- 7 Remove the supernatant and place in a microcentrifuge tube.
- 8 Wash the gel slices, again, in a minimum volume of water. Place the gel slices at 4°C for 1 to 4 additional hours.
- 9 Remove the supernatant and pool with the first supernatant. Adjust pH to ~5.5 with 1 to 3 µl 1M phosphoric acid. Check with pH paper.
- 10 If any particulate material is visible (pieces of acrylamide etc.) you may need to filter the supernatant pool through a 0.65 micron filter (highly recommended).
- 11 Dry the samples in a centrifugal vacuum evaporator or freeze and lyophilize.

- 12 Add 22 μl of H_2O to the tube and vortex. If you dried down a large volume of supernatant ($>500 \mu\text{l}$) you may need to rinse the tube with a larger amount of H_2O in order to recover all of the oligosaccharide that may stick to the sides of the tube and bring it to the bottom of the tube (view with U.V. light). In this event, dry down a second time and resuspend the oligosaccharide pellet in 20 μl of H_2O .

III. Determining the recovery of oligosaccharides

We recommend before you proceed with sequencing that you run a fraction of the isolated oligosaccharide on a gel to determine both purity and recovery. (The combined effect of extraction efficiency and normal degree of photobleaching results in a recovery of about 60% of fluorescence.)

- 1 Add 2 μl of 2X Loading Buffer (El) to 2 μl of the isolated oligosaccharide.
- 2 Load the entire 4 μl onto one lane of a oligosaccharide profiling gel and proceed with electrophoresis as described on page 14.
- 3 Determine the amount of material in the oligosaccharide band. In order to have enough material to proceed with sequencing, you should have at least 100 pmoles of oligosaccharide in the band. If you do not have an imager, estimate the amounts relative to the G4 band in the OLIGO Ladder Standard (50 pm if loaded according to the N-linked Profiling kit, 25 pm if reconstituted and loaded according to Sequence Kit).
- 4 If the amount of material is less than 50 pmoles you may need to recover more oligosaccharide from a second preparative gel (and pool the supernatants together) or you may have to limit the number of sequencing reactions performed.

SECTION 2

SEQUENCING OF ISOLATED OLIGOSACCHARIDES

Note: We recommend performing a "practice" sequencing experiment with the N-link Seq Control (E6) to familiarize yourself with the procedures involved before proceeding with unknown samples. Dissolve E6 (600 pmos) in 12 μ l of water. Use 2 μ l in each "practice" reaction to obtain 100 pmoles in each tube.

- 1 Add 50-100 pmoles of each **fluorescently labeled** oligosaccharide to each numbered tube up to 8 μ l total volume. If greater than 8 μ l is required then dry the sample down and resuspend in a smaller volume of H₂O.
- 2 Follow the reaction scheme outlined below to complete the setup. The example is shown using 2 μ l of labeled oligosaccharide. Adjust your volumes appropriately. The amounts of enzymes in each reaction should be sufficient for \leq 100 pmoles of oligosaccharide.

		Reaction Number				
		1	2	3	4	5
Reagent						
	Oligosaccharide Solution, μ l	2	2	2	2	2
	H ₂ O, μ l	14	12	10	8	6
	Total Volume, μ l	16	14	12	10	8
S6	5X Reaction Buffer, μ l	4	4	4	4	4
S1	Sialidase A, μ l	-	2	2	2	2
S2	β (1-4)-galactosidase, μ l	-	-	2	2	2
S3	β -N-acetylhexosaminidase, μ l	-	-	-	2	2
S4	α -mannosidase (Jack bean) , μ l	-	-	-	-	2
	Total Volume, μ l	20	20	20	20	20

- 3 Incubate the reaction tubes at 37°C for 12 - 16 hours.
- 4 Add 1 μ l α -Mannosidase (*X.manihotis*) (S5) to tube 5 and incubate at 37°C for 30 mins. Do not add this enzyme at the same time as the α -Mannosidase from Jack bean (S4) as it will inhibit activity.
- 5 Dry the reaction tubes in a centrifugal vacuum evaporator.
- 6 Proceed with "Preparation of Standards and Samples for Electrophoresis" page 17.

SECTION 3

PREPARATION OF SAMPLE AND STANDARDS FOR ELECTROPHORESIS

Note: Sample Handling and Storage

- *Always avoid exposing labeled samples and dyes to light or excess heat.*
- *Labeled samples are stable when stored for 3 months at -70°C.*
- *Unused solutions of the dye and reducing agent can be stored for as long as 2 weeks at -70°C. Thaw immediately before use.*

I. Preparation of the OLIGO Ladder Standard (E3):

The OLIGO Ladder Standard (E3) consists of a mixture of glucose polymers ranging from Glucose₁ to greater than Glucose₂₀. An illustration of the OLIGO Ladder Standard as it should appear at the completion of electrophoresis is shown in Figure 1, Lane 1. When the standard is prepared as directed below and the recommended amount is loaded onto the gel, the band representing Glucose₄ contains **25 pmoles**. The intensity of the Glucose₄ band in the standard mixture is less than the adjacent bands. This allows for easy identification of this band even if the lower bands in the standard have run off the gel. Quantitation of the oligosaccharide bands in the samples is achieved by comparing the intensity of the Glucose₄ band with the intensity of the sample bands and it is therefore essential that the OLIGO Ladder Standard is present on each FACE OLIGO Sequencing Gel used. If you are using the FACE Imaging System, refer to the FACE Software Manual for a detailed description of the quantitation procedure, band finding, DP determination, etc.

- 1 Resuspend the OLIGO Ladder Standard (E3) in 100 µl of distilled H₂O.
- 2 Add 100 µl of 2X Loading Buffer (E1). After reconstitution, the OLIGO Ladder Standard should be stored at -70°C.
- 3 Load 4 µl directly onto the gel. This equals 25 pmoles maltotetraose

II. Preparation of the Core Standard (E7)

The Core Standard contains 600 pmoles each of Man(β1-4)GlcNAc(β1-4)GlcNAc and Man(β1-4)GlcNAc(β1-4)[Fuc(α1-6)]GlcNAc. These oligosaccharides are used to determine the presence or absence of core fucosylation.

- 1 Resuspend the Core Standard (E7) in 25 μl of H_2O .
- 2 Add 25 μl of 2X Loading Buffer (E1). After reconstitution, the Core Standard should be stored at -70°C .

III. Preparation of Samples from Reactions 1, 2, 3, 4, and 5

- 1 Resuspend the dried samples in 4 μl of H_2O .
- 2 Remove 2 μl from each and add 2 μl of 2x Loading Buffer (E1).

SECTION 4

FACE ELECTROPHORESIS

I. Preparation of 1.5 liter of FACE Oligo Gel Running Buffer

(1.5 liter of buffer is sufficient for one electrophoresis run of one or two gels.)

- 1 Remove one of the Oligo Profiling Gel Running Buffer packs from the kit.
- 2 Cut open one end of the packet and carefully pour the contents into a 2 liter graduated cylinder and add 1000 ml of distilled water.
- 3 Rinse buffer packet with 100 ml of water and add this rinse to the 1000 ml.
- 4 Bring up to a final volume of 1.5 liter with water.
- 5 Mix well. The buffer must be chilled to 4-6°C before use.

Note; The running buffer may be made in advance and stored at 4 °C

II. Set-up of FACE Electrophoresis Apparatus

FACE Electrophoresis of N-linked gels is best performed at a temperature range of 5-8°C. We recommend the use of a recirculating chiller, however satisfactory results can be obtained by chilling the buffer prior to the electrophoresis run.

CAUTION: *To prevent damage to the gel box, connect both quick disconnect fittings to gel box before turning on recirculator!*

- 1 Place the FACE electrophoresis tank containing a stir bar on a mechanical stirrer. If using a recirculating chiller connect it to the gel box cooling chamber with tubing and quick-connect fittings. Turn on the circulator and stirrer, set the coolant temperature to 5°C. Bleed all air out of system by tilting the gel box in the direction of the outlet.
- 2 Pour pre-cooled OLIGO Gel Running Buffer into the electrophoresis tank up to the level marked on the outside of the box. The temperature of the buffer can be monitored during the run using a thermometer inserted through the hole in the lid. The temperature will probably increase a few degrees during electrophoresis but should not exceed 10°C.

III. Loading and Running FACE OLIGO Profiling Gels

We recommend that you prepare your samples as follows:

E3	OLIGO Ladder Standard	Load 4 μ l of the standard in lane 1 when reconstituted as described on page 14.
E7	Core standard	Load 4 μ l in lane 8, when reconstituted as described on page 14.
	Sample	Load the entire 4 μ l from Reactions 1, 2, 3, 4, and 5 in lanes 3, 4, 5, 6, and 7, respectively, when prepared as described on page 15.
	Released Oligosaccharides	Load 4 μ l of the diluted mixture of released oligosaccharides from which the sequenced sample was isolated (a 2 - 4 fold dilution with 1:1 mixture of 2x Loading Buffer (El) and H ₂ O in lane 2.

- 1 Determine the number of gels required for the samples prepared. Each Oligo Profiling Gel contains 8 lanes. One gel will be required for each oligosaccharide sequenced. The FACE electrophoresis box will accept one or two gels.
- 2 Cut open 1 or 2 packages containing FACE Oligo Profiling Gels. Gently remove the comb(s) from the gel(s). To avoid distorting the wells, gently wiggle each comb to free the teeth from the gel, then lift up slowly until the comb is released.
- 3 It is essential that the wells of the gel are thoroughly rinsed out with Oligo Gel Running Buffer from the upper buffer reservoir prior to sample loading. This is best accomplished by using a syringe with a blunt needle (a Pasteur pipette is not recommended because of the possibility of breakage into the wells).
- 4 Place the gel cassette(s), one on each side of the center core unit of the gel box with the short glass plate against the gasket. Be sure the cassette is centered and that the cassette is resting on the "feet" at the bottom of the apparatus. If you are running only one gel place the buffer dam on the other side.
- 5 Place one wedge down each side of the cassette. Then push wedges down to obtain a seal between the inner short plate and the gasket. Repeat this procedure on the other side. Both sides should now be sealed against the gaskets.

- 6 Fill the upper buffer reservoir formed between the cassettes with approximately 100 ml of chilled Oligo Gel Running Buffer and check for leaks. Final buffer level should be just below the electrode supports. If a leak occurs first check that the apparatus was assembled properly, then try pushing down the wedge with slightly greater pressure. If the leak persists check the "Troubleshooting" section of this manual.
- 7 With the core unit containing the gels placed securely on the bench, load samples into the wells by underlaying the upper buffer. Use flat sequencing pipette tips (e.g. Sigma T-1656) to load by delivering the sample to the bottom of each well. Optimal resolution will be achieved by using 4 μ l of sample per lane.

Note: For the most reliable quantitation of oligosaccharide bands the use of a positive displacement pipette is recommended.

- 8 Place the core unit containing the loaded gels into the electrophoresis tank and place the lid on.
- 9 First connect the power cords to the electrophoresis tank then connect the power supply. Connect the positive (red) lead to the electrode marked with a red dot on the electrophoresis box. Connect the negative (black) lead to the electrode marked with a black dot.
- 10 A thermometer can be placed into the lower buffer chamber through the hole in the lid to monitor the temperature.

Note: The initial temperature of the lower buffer should be between 5 °C and 8 °C.

- 11 Turn on the power supply and select the proper current. FACE OLIGO Profiling Gels should be run at a constant current of 20 mA per gel (40 mA for 2 gels). Limits on the power supply should be set for 1000 volts and 60 watts. These run conditions will result in voltages of 100-400 V at the beginning of the run and may approach 800 V at the end of the run. If the initial voltage is significantly different check to be sure that the leads are connected properly and that the buffers are at the recommended levels.
- 12 Monitor electrophoresis by following the migration of the fast moving Orange Dye (E1). Generally, electrophoresis is complete when the orange dye just exits the bottom of the gel in approximately 1 hour and 5 minutes.
- 13 When the electrophoresis is complete, turn off the power supply. Disconnect the power cords from the power supply and the electrophoresis tank. Turn off the recirculating chiller if used.

Note: If using the FACE Imager begin warm-up now.

SECTION 5

PROCESSING OF FACE OLIGO PROFILING GELS

Following Step 13, above, carefully remove the gels from the electrophoresis tank

I. Gel imaging using the U.V. Transilluminator (lightbox)

CAUTION: U.V. protective eyewear or face shield should be worn. Avoid prolonged exposure to U.V. light.

- 1 Allow U.V. lightbox to "warm-up" for at least 2 minutes in order to get maximum intensity output. The lightbox must be long-wave U.V. (UVP Model TL-33 or equivalent) and have a peak output at approximately 360 nm; this is not the type of box typically used for ethidium stained DNA gels.
- 2 Optional: To increase sensitivity you may wish to peel the tape from the gel cassette, and disassemble the cassette by carefully prying open the two glass plates, removing the gel completely from the cassette and placing it directly on the U.V. lightbox.

Images of gels can be recorded using a Polaroid camera. The proper choice of light source, filters and film must be made. A filter must be fitted to the camera lens, which completely covers the glass of the lens, stray U.V. contacting the lens will cause it to fluoresce and subsequently lower the sensitivity of the film. A Kodak Wratten Number 8 filter is suggested and can be purchased from Kodak and Kodak distributors. A suitable filter will have no inherent fluorescence, peak transmission at approximately 500 nm and bandwidth of 80 nm FWHM.

A medium speed, medium resolution, Polaroid film is recommended. Use Polaroid 53 film for cameras that use single 4"x 5" sheet film; use Polaroid 553 film for cameras that use 8 sheet film cartridges.

Photographing Gels:

- 1 Photograph the gel using the lowest practical F setting on the lens with the gel filling as much of the frame as possible. In our experience, exposures at F5.6 using Polaroid 53 film have ranged from 5 to 40 seconds using above specified equipment. **KEEP EXPOSURE OF THE GEL TO A MINIMUM TO PREVENT BLEACHING.**
- 2 Develop film according to manufacturer's instructions.

II. Gel Imaging Using the FACE Imaging System

- 1 Turn on the FACE Imager and allow a warm-up period of at least 10 minutes before acquiring an image. Allow the computer to initialize the Imager.
- 2 Clean the glass plates completely with lint-free tissue. A little distilled H₂O should be used to clean any obvious residue on the plates before imaging. The gel cassettes should be relatively dry and free of dust before imaging.
- 3 Open the door to the imager and place the cassette containing the gel into the cassette holder. (see the "FACE Software Manual" for imaging instructions).

III. Gel Handling

After imaging, the gels can be processed in a number of ways depending on the needs of the investigator.

- If the gel is no longer needed it should be properly discarded.
- As long as the gel cassette is intact, it can be placed back in the electrophoresis apparatus and the run continued in order to improve the resolution of the oligosaccharide bands.
- Following imaging of the oligosaccharide gels, the glass plates can be separated and the gels dried on a flat bed gel drier between sheets of Teflon membrane at 80°C for 1 hour. After the gel is dry, carefully peel the Teflon sheets away from the gel. Gels dried in this manner can be stored indefinitely and re-imaged at any time.

SECTION 6 INTERPRETATION OF RESULTS

Interpretation of sequencing results

Some expertise is required to correctly identify N-linked oligosaccharides that are completely digested to core structures by the enzymes in this kit. The first step in elucidating structure is to distinguish between high mannose type and other types of N-linked oligosaccharides. High mannose sugars characteristically exhibit no digestion until α -mannosidase (Jack bean) is added. They then digest to core structures (Figure 5). A simple method of eliminating high mannose and hybrid structures from a labeled mixture of oligosaccharides is to treat the mixture with Endo H (see page 4). This will cleave off the labeled reducing terminal GlcNAc rendering all of the high mannose and hybrid structures invisible.

The number of antennae is the next parameter to be determined. Noting the DP shifts for galactose (tube 2 to 3) and GlcNAc (tube 3 to 4), the loss of each galactose should result in a DP shift of 1.0 and that of GlcNAc 0.75. Thus, a triantennary oligosaccharide should shift approximately 3 DP unit for galactose and 2.25 DP units for GlcNAc. Agreement between these two determinations unambiguously quantitates the number of antennae.

Determination of the number of sialic acid residues is somewhat problematic since charge, mass and linkage contribute to migration changes. For bi-antennary oligosaccharides, loss of the first sialic acid results in a 0.5-0.8 DP shift and loss of the second, a 1.6-1.8 DP shift. Triantennary oligosaccharides exhibit a <0.5, ~1.0 and ~1.5-2.0 DP shifts for the first, second and third sialic acid cleavages respectively. Partial digestions and / or the use of linkage-restricted sialidases (see below) can aid in this analysis. Antennae lacking sialic acid can be detected by digestion with β -galactosidase prior to sialidase treatment. These methods may be necessary in determining the extent of sialylation of tetraantennary oligosaccharides.

Polylactosamine is a repeating Gal-GlcNAc unit attached to one or more antennae. Although these structures will digest completely, they exhibit an unusually large DP shift when β -N-acetylhexosaminidase is added, since not only GlcNAc but also galactose is removed in tube 4. Two methods are recommended for polylactosamine analysis

- Test for polylactosamine by digestion with endo- β -galactosidase.
- Stepwise removal of each residue can be achieved by heat inactivating the β -galactosidase (10 min at 80°C) prior to addition of β -N-acetylhexosaminidase.

Core-fucose determination

The presence of a single fucose residue attached to the Man(β 1-4)GlcNAc₂ core is a common feature of N-linked type oligosaccharides.

The FACE N-linked oligosaccharide sequencing kit provides two methods to confirm the presence of core fucose. First, the Core Standard (E7) contains both non-fucosylated Man(β 1-4)GlcNAc₂ and fucose containing Man (β 1-4)GlcNAc(β 1-4)[Fuc(α 1-6)]GlcNAc core structures. These two standards migrate at different positions with the fucosylated core migrating at 0.6 DP units above the non-fucosylated core. By determining which standard co-migrates with the final digestion product from your sample (Reaction 5) you should be able to determine whether your sample contains core fucose.

If you want to confirm the presence of core fucose, we recommend that you enzymatically remove the core fucose using Fucosidase (Bovine kidney) Glyko Cat # GKX-5006 not included in this kit. This enzyme will cleave Man (β 1-4)GlcNAc(β 1-4)[Fuc(α 1-6)]GlcNAc core structure.

Oligosaccharides isolated from plant glycoproteins using Endoglycosidase A may contain Fucose(α 1-3) linked to the core. This structure will migrate between the fucosylated and non-fucosylated core standards and will therefore not be mistakenly assigned as either one.

There are several substitutions that will block digestion of one or more antennae using the enzymes supplied in the kit: N-linked structures containing branched or terminal fucose (Lewis structures), α 1-3 linked galactose, β 1-3 linked galactose, xylose, and phosphorylated or sulfated sugars will fail to digest down to core structures unless the appropriate exoglycosidases, not supplied in this kit, are added. We will discuss below how to deal with these structures.

- 1 The presence of bisecting GlcNAc will usually cause the incomplete digestion by β -N-acetylhexosaminidase (Figure 6). The species with all GlcNAc removed will show a GlcNAc DP shift \sim 0.5-0.75 greater than that of the galactose DP shift.
- 2 The presence of terminal α 1-3 galactose is often found in glycoproteins of murine cell line origin. Use α -galactosidase (Glyko Cat # GKX-5007) to remove α 1-3)galactose.
- 3 The presence of β 1-3 galactose instead of β 1,4 galactose will stall digestion after sialidase treatment. Use β -galactosidase (*X.manihotis*) (Glyko Cat # GK80120) to remove this.

- 4 Lewis structures containing (α 1-2), (α 1-3) and (α 1-4) Fucose linked to either galactose or GlcNAc will block digestion. These can be removed using either α -Fucosidase II or α -Fucosidase III (Glyko Cat # GK80170 and GK80180 respectively).
- 5 Phosphorylation of oligomannose and hybrid structures results in a greatly increased migration due to the negative charges. Some glycoproteins expressed in Chinese Hamster Ovary cells (CHO) have been found phosphorylated. Phosphate groups can be removed with Calf Intestine Alkaline Phosphatase (CIP), then proceeding with the standard protocols.
- 6 Sulfation also results in an increased mobility. There is no general enzymatic method of removing sulfate groups.

All sialic acids are removed by the sialidase supplied in the kit. Therefore, no information as to different sialic acid linkages and / or branching sialic acid structures is obtained. Digestion with linkage-restricted sialidases found in the Sialic Acid Linkage Kit (Glyko Cat # GK80010) can yield a great deal of information about the sialic acid structures.

The modifications discussed above often do not occur on all antennae of a polyantennary oligosaccharide. The modification will block digestion of the antenna on which it occurs, but not necessarily the free antennae. It is possible to enzymatically determine on which antennae the modifications occur.

Please call our Technical Service for more information.

TROUBLESHOOTING GUIDE

Sample not moving or moving slowly on gel

Leads may be reversed. Check leads to power supply and gel box. Check upper buffer level is above the top of the short glass plate. Check the lower buffer level, if the level of the buffer is above the orange gasket, remove 20-50 mls of buffer until the level is below the gasket.

Erratic voltage, voltage and / or current leak. (At the beginning of the run voltage is greater than 400V or readings are unstable)

Make sure that the electrical leads are not arcing due to condensation on the lid of the tank. Dry the area around the electrical posts on the inner box. Check that the leads to the power supply are connected securely. Verify that the power supply is operating properly.

Band distortion in gel

From time to time band distortions can occur when running O-linked gels. There may be various reasons including:

- Sample may be overloaded, use a maximum of 1/5th of the volume of the labeling reaction for each lane.
- Wells may have been torn when comb was removed. Remove comb slowly using a gentle back and forth rocking motion and lift vertically.
- “Smile effect” on gel can be a result of the gel not being cooled uniformly. Check that the cooling system is on and working properly, and is free of air bubbles in the electrophoresis box. Make sure that a stirring bar has been placed in the electrophoresis box and that the lower buffer is being mixed. Check that power supply is set for proper current level - see Section 4-III-11 for proper settings.
- “Fuzzy bands” may be due to too high current used. Check Section 4-III-11 for proper settings.

Upper buffer chamber leaks when cassettes are in place

Check that the plates are clean and not cracked or chipped, and that they are centered on the inner core assembly. Once the wedge has been brought into position against the glass, gently push down to engage the sealing gasket. Check that the gaskets are not cracked and that they are seated properly in place (do not grease gaskets). Pushing down with slightly greater pressure can stop most minor leaks.

If you are using a FACE Imaging system and would like us to take a look at your gel for troubleshooting purposes, please email a copy of the .raw or .IM2 file to us at thelab@glyko.com

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Figure 1 Isolation of Oligosaccharides from α -1 Acid Glycoprotein by FACE

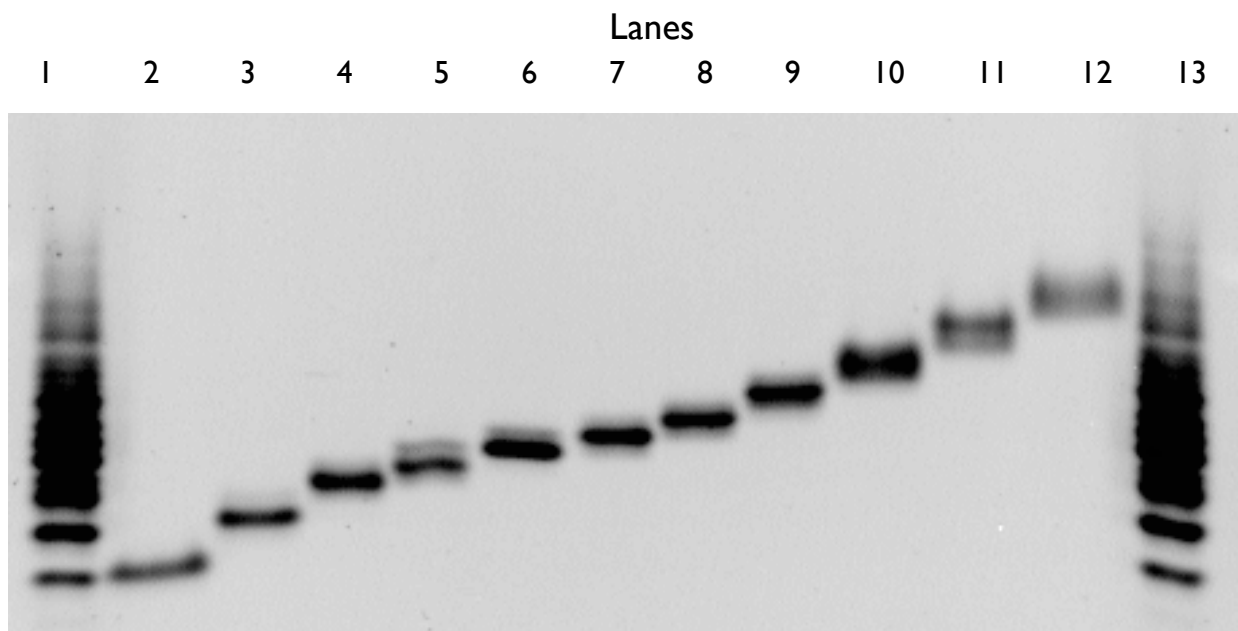
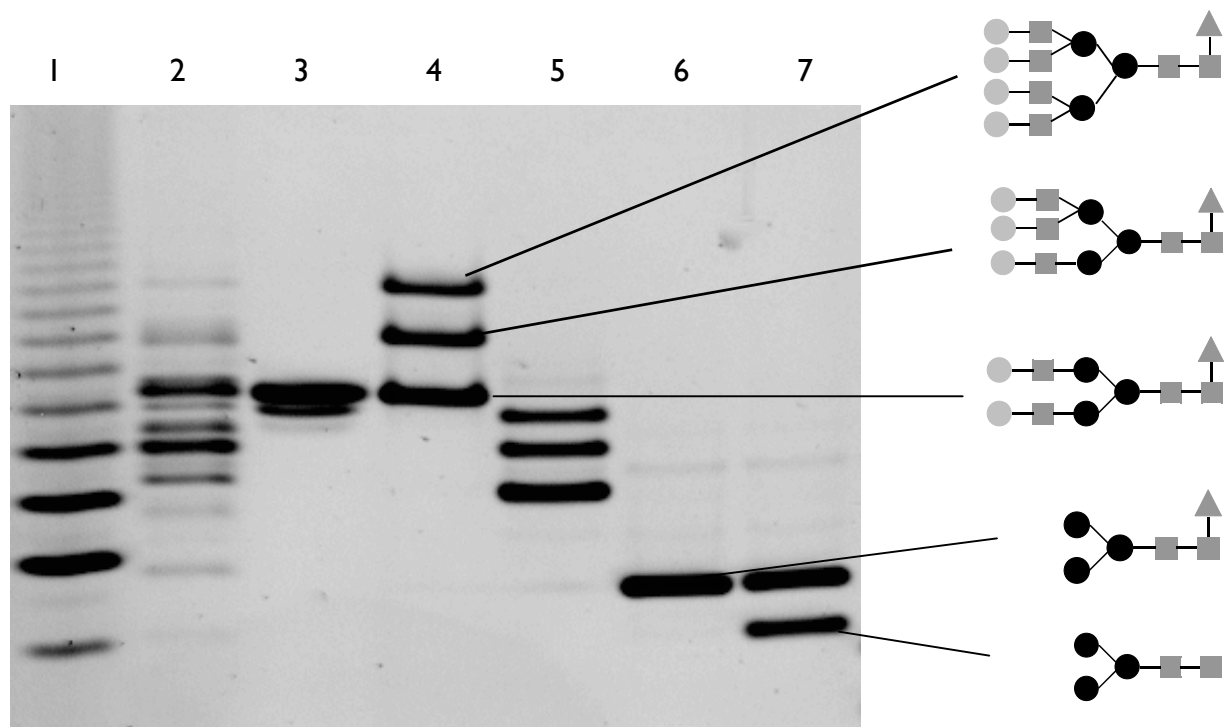


Figure 2 FACE Sequencing of Complex Oligosaccharides from rgp120 with Exoenzymes.



E3	N-Gly Digest	Reactions				
		1	2	3	4	5
	Sialidase A	-	+	+	+	+
	$\beta(1-4)$ -galactosidase	-	-	+	+	+
	β -N-acetylhexosaminidase	-	-	-	+	+
	α -fucosidase	-	-	-	-	+

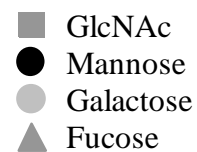
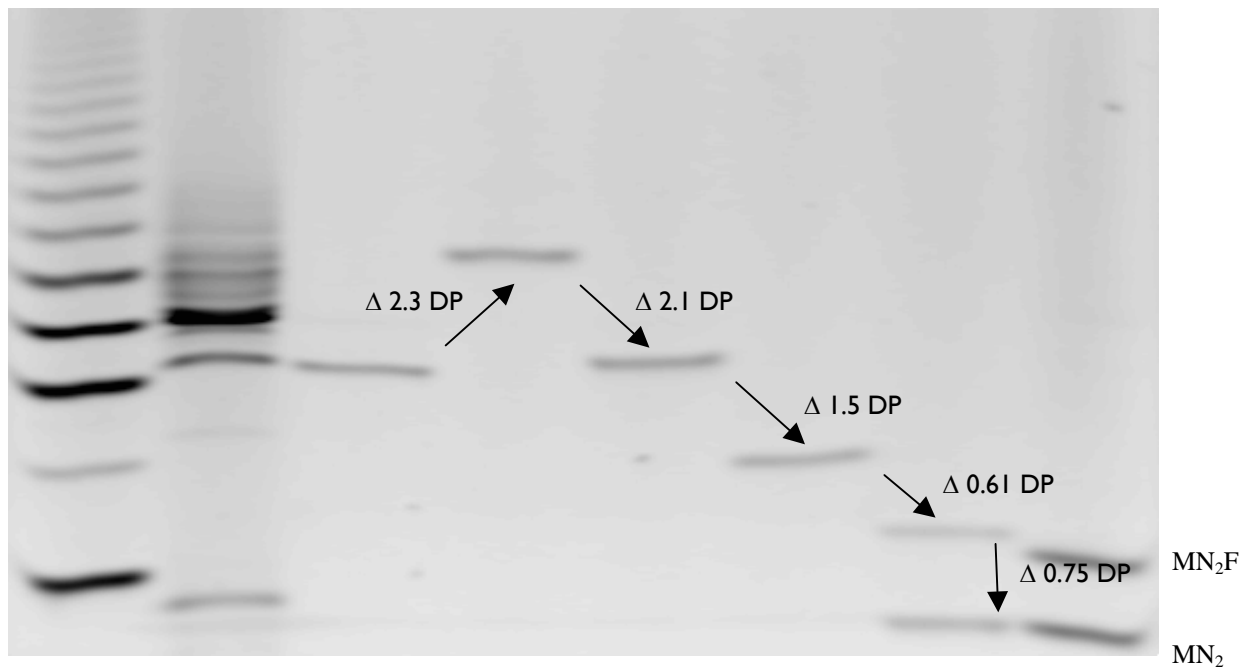
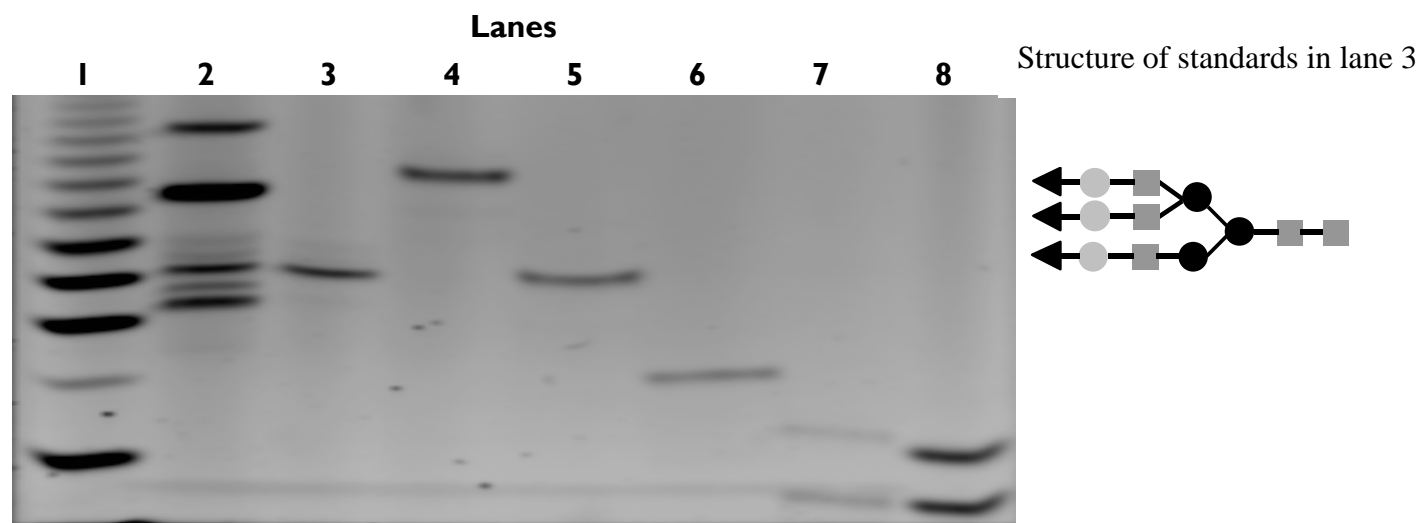
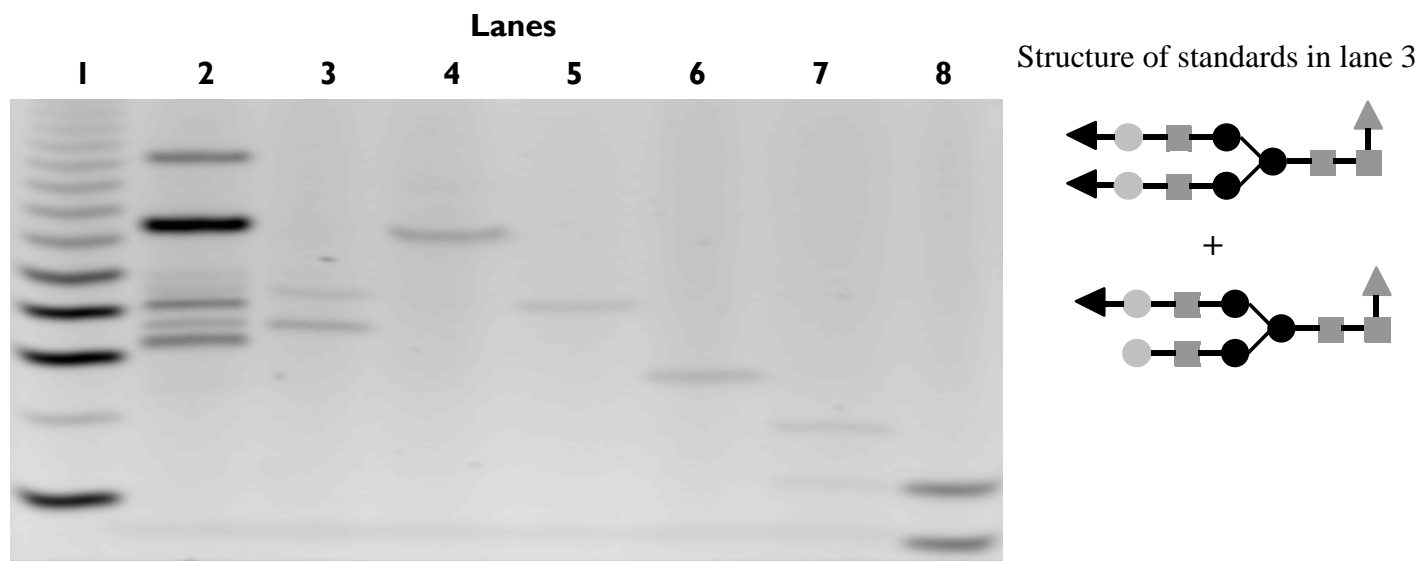


Figure 3 FACE Image of N-Linked Sequencing Control (Sialylated, Galactosylated Bi-antennary Oligosaccharide)



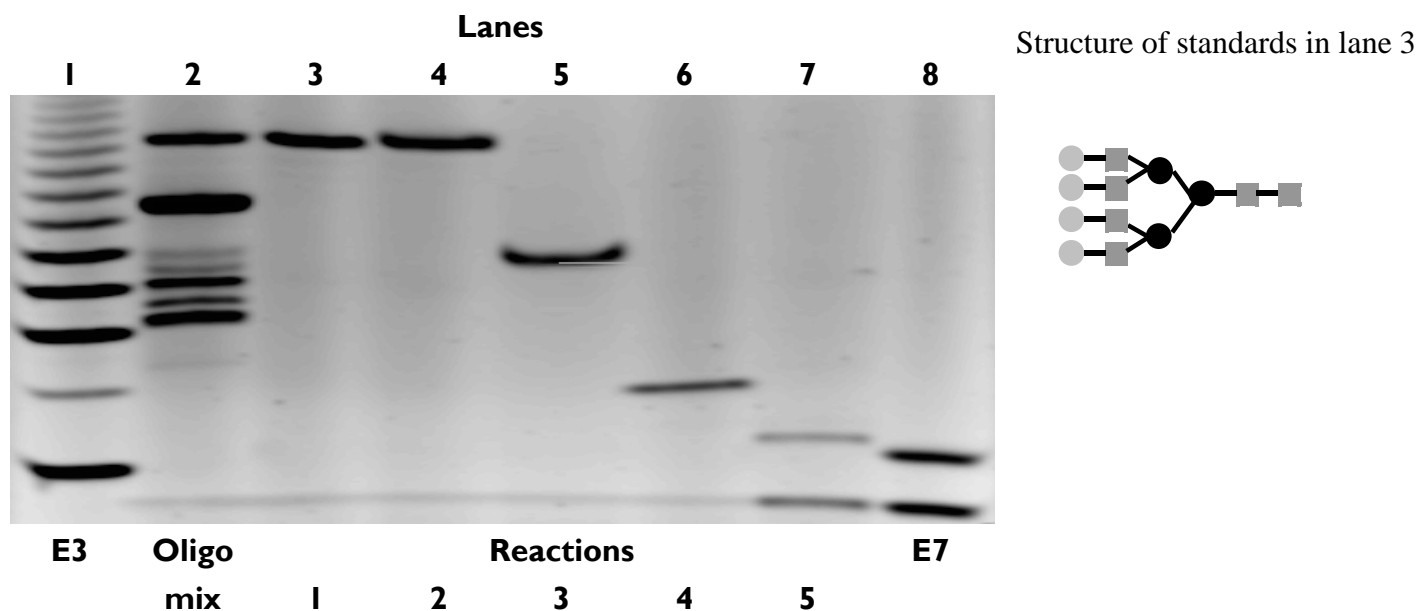
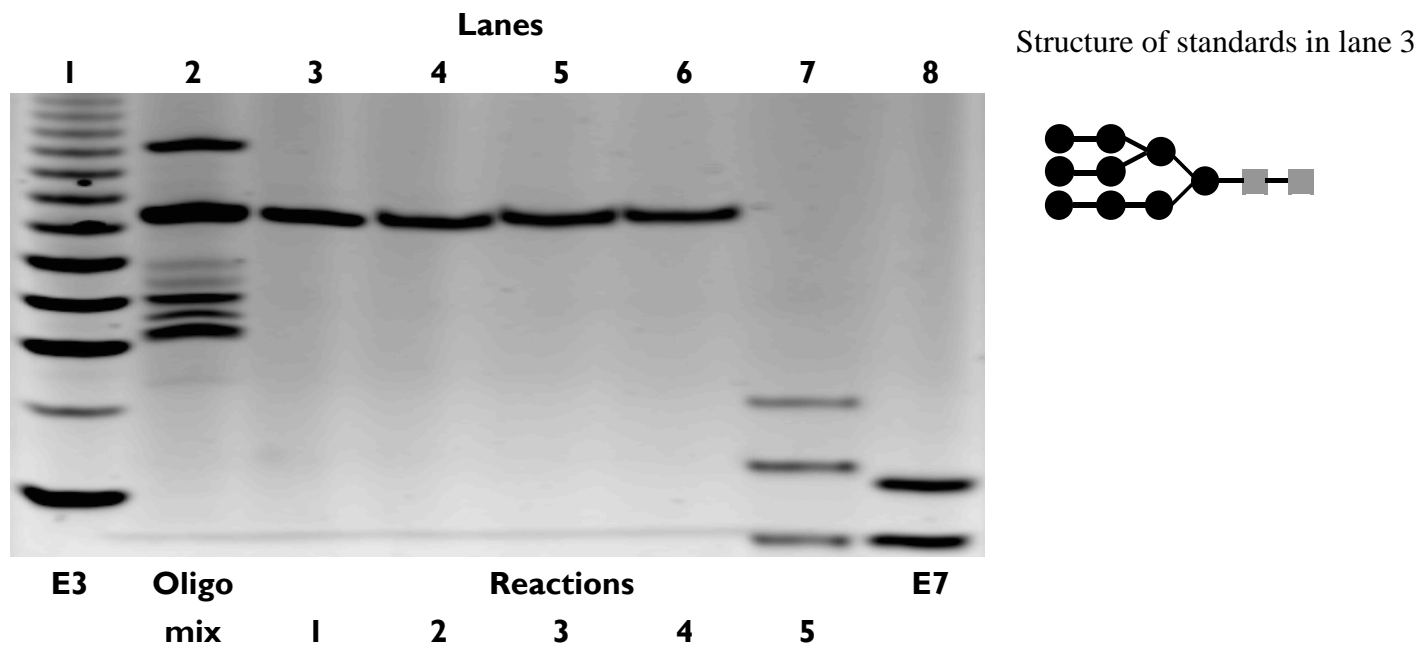
		DP						
	Oligos	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8
G9	9.00							
G8	8.00	7.92						
		7.28		7.56				
		6.96						
G7	7.00	6.54						
		6.15						
		6.00						
G6	6.00							
G5	5.00				5.46			
		5.29	5.25					
G4	4.00					3.95		
							3.34	
G3	3.00							3.20
							2.59	2.57

Figure 4 FACE Sequence Analysis of N-Linked Oligosaccharide Standards – Sialylated Complex Types



- GlcNAc
- Mannose
- Galactose
- ◄ Sialic Acid
- ▲ Fucose

Figure 5 FACE Sequence Analysis of N-Linked Oligosaccharide Standards – High Mannose and Complex Types



- GlcNAc
- Mannose
- Galactose
- ▲ Sialic Acid
- ▲ Fucose

Figure 6 FACE Sequence Analysis of N-Linked Oligosaccharide Standards – Hybrid and Bisecting GlcNAc Types

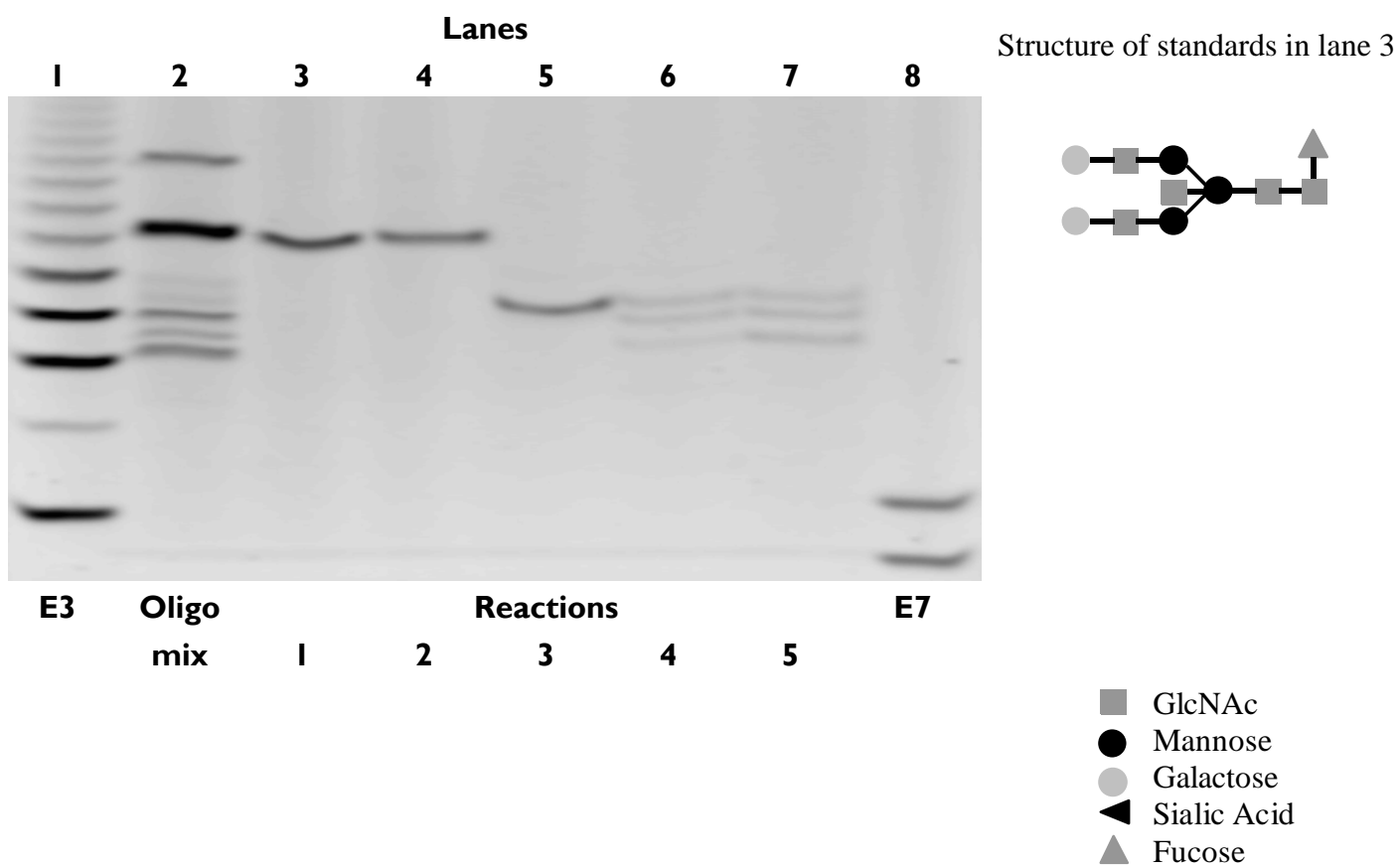
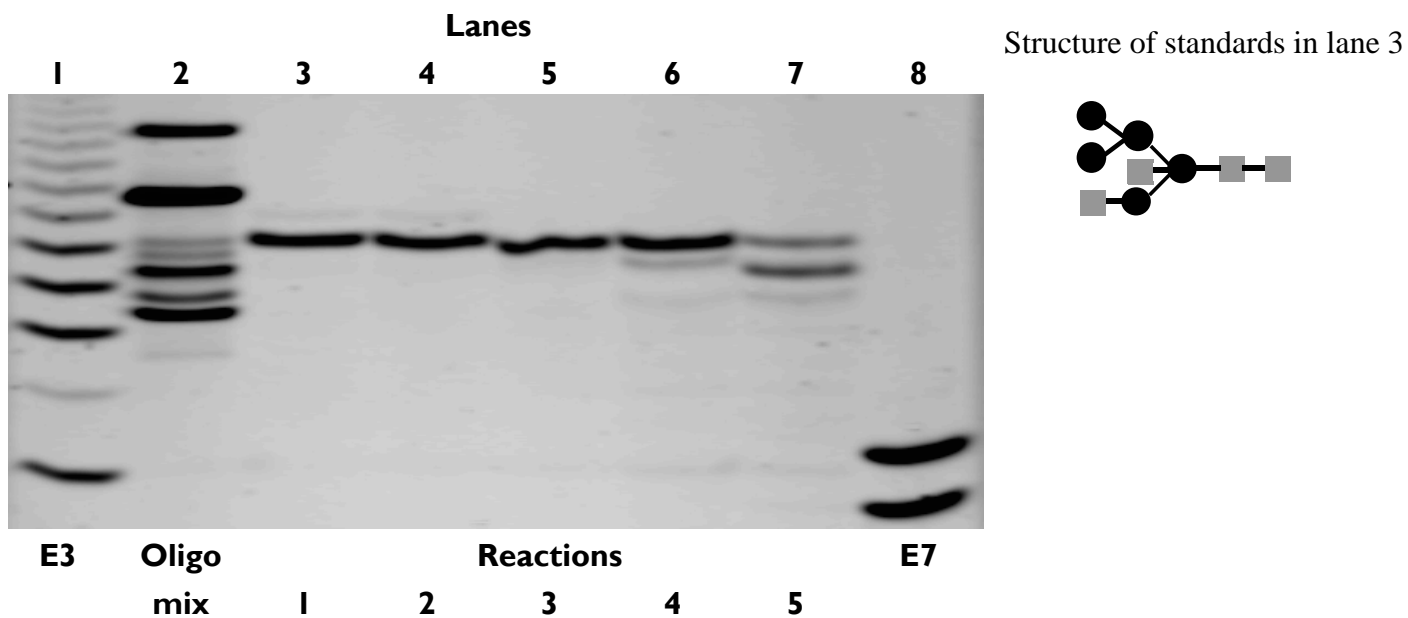


Table 1 Mobility of ANTS Labeled N-Linked Oligosaccharide Standards

Description	Abbrev	DP	+ / -
Complex			
Di-sialylated, galactosylated biantennary (2 α 2-6)	A2	5.4	0.3
Di-sialylated, galactosylated biantennary, core fucosylated (2 α 2-6)	A2F	5.7	0.2
Di-sialylated, galactosylated biantennary, core fucosylated (2 α 2-3)	A2F	6.2	
Tri-sialylated, galactosylated triantennary (2 α 2-6)	A3	6.2	0.3
Tri-sialylated, galactosylated triantennary (2 α 2-3)	A3	6.7*	0.4
Tri-sialylated, galactosylated triantennary core fucosylated (3 α 2-3)	A3F	7.0*	
Tetra-sialylated, galactosylated tetraantennary (isomers)	A4	7.6,7.2,6.7,6.2*	
Asialo-, galactosylated biantennary	NA2	7.8	0.2
Asialo-, galactosylated biantennary, core fucosylated	NA2F	8.5	0.3
Asialo-, galactosylated biantennary, bisecting GlcNAc, core fucosylated	NA2FB	8.5	0.3
Asialo-, galactosylated triantennary	NA3	9.6	0.3
Asialo-, galactosylated triantennary, core fucosylated	NA3F	10.5	0.5
Asialo-, galactosylated tetraantennary	NA4	11.5	0.4
Asialo-, galactosylated tetraantennary, core fucosylated	NA4F	12.1	0.1
Asialo-, agalacto biantennary	NGA2	5.6	0.5
Asialo-, agalacto biantennary, core fucosylated	NGA2F	6.2	0.2
Asialo-, agalacto triantennary	NGA3	6.4	0.4
Asialo-, agalacto triantennary, core fucosylated	NGA3F	7.1*	
Asialo-, agalacto tetraantennary	NGA4	7.3	0.3
Asialo-, agalacto tetraantennary, core fucosylated	NGA4F	7.9	
High Mannose & Hybrid			
Oligomannose 9	MAN-9	8.8	0.3
Oligomannose 8	MAN-8	8.1	0.2
Oligomannose 7	MAN-7	7.4	0.4
Oligomannose 6	MAN-6	6.5	0.2
Oligomannose 5	MAN-5	5.7	0.3
Hybrid type with bisecting GlcNAc		7.0	0.3
Core			
Conserved trimannosyl core	M3N2	4.2	0.2
Conserved trimannosyl core, core-fucosylated	M3N2F	4.8	0.1
Man(α 1-6)Man(β 1-4)GlcNAc(β 1-4)GlcNAc		3.3	0.1
Man(α 1-6)Man(β 1-4)GlcNAc(β 1-4)[Fuc(α 1-6)]GlcNAc		4.0*	
Man(β 1-4)GlcNAc(β 1-4)GlcNAc		2.5	0.1
Man(β 1-4)GlcNAc(β 1-4)[Fuc(α 1-6)]GlcNAc		3.2	0.2

* Average of less than 6 values

Table 2 FACE Rules for Predicting Monosaccharide Sequence Based on Mobility Shifts

Monosaccharide	Change in DP* Units of Oligosaccharide
Sialic Acid (NeuAc)	Increases by an average of 1 DP
Galactose (Gal)	Decreases by 1 DP
N-acetyl glucosamine(GlcNAc)	Decreases by an average of 0.75 DP
Mannose (Man)	Decreases by an average of 0.75 DP
Fucose (Fuc)	Decreases by 0.6 DP
Bisecting GlcNAc	Decreases by 0.5 DP

* DP Units / Glucose Units are measured relative to a ladder of glucose polymers.

ProZyme, Inc.

1933 Davis Street, Ste 207, San Leandro, CA 94577-1258 USA

Tel: 1(510) 638-6900 Fax: 1(510) 638-6919

E.mail: glyko@prozyme.com

www.prozyme.com/glyko
