GlykoPrep® Microfuge Method - Rapid N-Glycan Preparation with InstantPC™

Rapid N-Glycan Preparation with InstantPC (product code GP24NG-PC or GP96NG-PC) using a microfuge and PCR heat block

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REQUIRED REAGENTS/EQUIPMENT

Centrifuge (capable of 50–1000 x g) and rotor for 1.5/2.0-ml microcentrifuge tubes

Heater and heat block capable of 45-100°C that accommodates 0.2-ml PCR tubes

Vortexer

Glass, graduated cylinder, 25-ml

Vials, 1-ml, polypropylene for use with organic solvents

Ultrapure, deionized water (Milli-Q® or equivalent)

Acetonitrile (100%, HPLC-grade)

Pipettors & disposable tips (P5/P10, P200 and P1000)

Formic acid (HPLC-grade)

Nitrile gloves

Required Labware (per sample)

1 x Cartridge Adaptor A, ProZyme Product Code AM400 (16 ea), Part Number WS0337

1 x Cartridge Adaptor B, ProZyme Product Code AM400 (16 ea), Part Number WS0336

1 x 0.5-ml Microtube, screw cap, Sarstedt part number 72.730.711 or equivalent

GlykoPrep Cartridges (supplied with each Kit)

1 x 0.2-ml PCR tube, domed cap, Axygen® part number PCR-02D-C or equivalent

2 x 1.75-ml Microcentrifuge tube, flip top, E&K Scientific part number 290175 or equivalent

NOTE: Adaptors are reusable. Do not discard.

NOTE: Some brands of PCR tubes form a tight seal between the tube and Cartridge. Do not use these brands.

SAFETY & HANDLING

Some of the reagents in the GlykoPrep Kits are hazardous. Please refer to the Safety Data Sheets (SDS) posted on ProZyme's website under the component name or Product Code:

http://www.prozyme.com

General Laboratory Procedures

Use powder-free, nitrile gloves for all sample handling procedures. Ensure that all glass, plasticware and solvents are free of glycosidases and environmental carbohydrates.

INTRODUCTION

The GlykoPrep Sample Preparation Platform (GlykoPrep) dramatically streamlines glycoanalysis by facilitating optional protein purification, quantitative deglycosylation and separation of N-glycans, complete fluorescent labeling and efficient cleanup to reduce excess reagent peaks.

GlykoPrep is modular and can be integrated into any workflow, regardless of throughput or sample type. Components are available individually as a Purification Module (optional), Digestion Module and dye-specific Labeling & Cleanup Modules.

GlykoPrep is built on AssayMAP technology, performed using centrifugation to move liquid through the Cartridges (spin format). The Microfuge Method is useful for those interested in using the spin format to run only a handful of samples. Using the spin format with a 96-well microplate and microplate centrifuge, up to 192 samples can be processed simultaneously with 2 Kits. GlykoPrep-plus employs the Syringe Head on the Agilent AssayMAP Bravo Liquid Handling Workstation to move liquid through the Cartridges, for automated high-throughput.

Important general information for achieving success with the spin format, as well as special tips particular to individual Modules, may be found in the GlykoPrep Guidebook under Using Specific Kits and Modules:

http://www.prozyme.com/documents/TNGP100.pdf

USING THE KIT

Preparation of Samples

Sample Quantities

The quantitative binding for each Cartridge is:

AssayMAP PA50 Cartridge	125 µg of MAb or Fc-fusion protein
RX Cartridge	50 µg of most standard proteins
CU Cartridge	30 µg of N-glycans

The binding capacity for specific glycoproteins may need to be determined.

Cartridges are capable of binding more target, but will do so with increasing breakthrough, making the process non-quantitative.

Less than the maximum quantity may be processed, for example, when the sample is available only in limited amounts. The smallest amount of sample that will give good results depends on the sensitivity requirements of the analytical methods and the specific application (e.g., screening vs. QC release).

For quantitative loading, prepare an excess of 10% or more sample, and prepare replicates together. For example, for Digestion, samples should be denatured together and loaded individually.

Sample Denaturation

Prior to deglycosylation, the samples are denatured by pre-mixing with Denaturation Reagent. The suggested sample concentration prior to deglycosylation is 1–5 mg/ml, and sufficient reagents have been provided for the standard sample concentration range.

If quantitation is desired, pipetting less than 10 µl is not recommended; pipetting smaller volumes introduces variability, especially when samples are highly concentrated. If necessary, dilute the sample to within the 1-5 mg/ml range with Digestion Buffer before starting.

The Kit is useful for very dilute samples without requiring further concentration, by expanding this load step to multiple spins. See the GlykoPrep Guidebook section "Loading."

When performed in a single spin, the amount loaded to each RX Cartridge should be 10–100 µl. The recommended starting ratio of Denaturation Reagent to sample is 1:1 (v/v), as in Example 1 below. More Denaturation Reagent may be used for problematic glycoproteins, as shown in Example 2 (sample to Denaturation Reagent ratio of 9:1).

NOTE: The final denatured Sample must be at least 50% Denaturation Reagent.

Example 1:
Sample concentration 1 mg/ml
Sample amount needed: 50 µg

50 µl (50 µg) Sample + 50 µl Denaturation Reagent = 100 µl denatured sample

The current protocol employs a 5-minute, relatively gentle denaturation, but any custom denaturation may be performed and the subsequent protocol followed as described, as long as no SDS or other detergents are used. Please see the GlykoPrep Guidebook under Digestion Modules or contact us to discuss custom denaturation conditions for your glycoprotein.

Example 2:
Sample concentration 5 mg/ml
Sample amount needed: 50 µg

10 µl (50 µg) Sample + 90 µl Denaturation Reagent = 100 µl denatured sample

Digestion Time and Temperature

Time

The Digest procedure has been optimized to deliver deglycosylation of N-glycans in 15-60 minutes. The optimal incubation time will vary depending on the specific glycoprotein; those which have proven to be resistant to deglycosylation via conventional enzymatic methods may require longer incubation times (up to 60 minutes). For glycoproteins that are comparatively easy to deglycosylate, such as monoclonal antibodies, a 15-minute incubation is generally sufficient. The selected Incubation Time will be used in the Digestion Module.

NOTE: It is critical not to exceed a 60-minute incubation, as the Cartridge resin bed may dry out, yielding uncertain results.

Temperature

The PCR heat block is set to 45°C for the Digest procedure (deglycosylation step).

NOTE: Optimal temperatures for the Microfuge Method differ from those used with a microplate centrifuge.

USE OF ADAPTORS AND CONSUMABLES

0.5-ml Screwcap Microtube

GlykoPrep Cartridge

0.2-ml PCR Tube

1.75-ml Microcentrifuge Tube

Cartridge Adaptor A

Cartridge Adaptor B

Most Standard Operations

Basic Assembly: Nest the RX Cartridge into Cartridge Adaptor A, then into a 0.5-ml screw cap Microtube.

RX Cartridge (only)

Cartridge Adaptor A

0.5-ml screw-cap Microtube

Equilibrate, Prepare, Block, Wash, Load Sample (except Cleanup).

In this configuration, flow-through remains in contact with the tip of the RX Cartridge in the Microtube ("Tips Wet").

Operations with Special Requirements

"Tips Wet" Adaptor Assembly: Insert an RX Cartridge into a 0.2-ml PCR tube. Insert into a Cartridge Adaptor B and nest into a 1.75-ml, flip-top Microcentrifuge Tube.

RX Cartridge (only)

0.2-ml PCR tube

Cartridge Adaptor B

Flip-top, 1.75-ml Microcentrifuge Tube

Incubation Assembly: Perform all incubations (including N-Glycanase and Labeling incubations) in a 0.2-ml capped PCR tube. Note that the RX Cartridge will be nested in the uncapped PCR tube for the N-Glycanase Digestion step.

RX Cartridge (only)

0.2-ml PCR tube

OR

0.2-ml capped PCR tube

Load N-Glycanase and Elute (Digestion steps 7 & 9)

In this configuration, flow-through remains in contact with the tip of the RX Cartridge inside the 0.2-ml PCR tube ("Tips Wet").

- For N-Glycanase Digestion: when ready to Incubate, remove the RX Cartridge with the 0.2-ml PCR tube from the "Tips Wet" Assembly and place them on the PCR heat block. When the incubation is complete, return the RX Cartridge/PCR tube to the "Tips Wet" Assembly for elution of N-glycans.
- For Labeling Incubation: add Labeling Reagent directly to the 0.2-ml PCR tube with the N-glycans and perform incubation as instructed.

"Tips Free" Assembly:

CU Cartridge (only, no PCR tube)

Cartridge Adaptor B

Flip-top, 1.75-ml Microcentrifuge tube

For superior removal of free dye, the Cleanup procedure requires that the tip of the Cartridge not be in direct contact with the flow-through at the bottom of the microtube. This is accomplished by placing an adaptor on a 1.75-ml Microcentrifuge tube and then positioning the CU Cartridge on top.

Add the N-glycan Sample to the Sample Cup of the CU Cartridge in the "Tips Free" Assembly. Load the Assembly into the Microcentrifuge, balance and spin as instructed.

Keep the Cartridge in the "Tips Free" Assembly to Wash and Elute, but prior to eluting the labeled N-glycans from the CU Cartridge, transfer the Adaptor and CU Cartridge to a fresh 1.75-ml Microcentrifuge tube.

PROTOCOLS

Getting Started

Heater Setting

Turn on the heat block. Set to 45°C and allow to equilibrate for a minimum of 1 hour.

Centrifuge Settings

Convert the required RCF values (i.e., 50 x g, 100 x g, 300 x g and 1000 x g) to the microcentrifuge's RPM settings based on the radius of the rotor to be used.

Many microcentrifuges allow direct setting of RCF values.

_____ rpm = 50 x g

_____ rpm = 300 x g

_____ rpm = 1000 x g

Preparation of Reagents

Glycoprotein Samples

This protocol begins with purified Glycoprotein Samples (see page 5 for details). Glycoprotein Samples must not contain any particulates, as they will plug the top frit, or sit on the top of the resin bed and impede the flow. Spin samples to remove particulates before processing.

Digestion Buffer

May be prepared up to one week before use. Store at 2–8°C.

25x Digestion Buffer (supplied with the Kit)

Ultrapure water

Dilute one volume of 25x Digestion Buffer with twenty-four volumes of ultrapure water to obtain Digestion Buffer.

Prepare 100 µl of Digestion Buffer for each sample to be processed. For example, for 10 samples add 40 µl of 25x Digestion Buffer to 960 µl of ultrapure water to make 1 ml of Digestion Buffer.

Cap tightly and vortex on high for 10 seconds to mix.

Enzyme Solution

Prepare only on the day of use. Store at RT.

N-Glycanase (supplied with the Digestion Module)

Digestion Buffer (prepared previously)

0.6-ml microcentrifuge vial

Spin the N-Glycanase briefly to collect the contents in the base of the vial. Vortex the solution prior to dispensing.

In a separate vial, prepare a mixture of 2.5 µl of N-Glycanase and 7.5 µl of Digestion Buffer for each sample to be processed, plus 20% for overage.

For example, 10 samples would require 25 + 5 = 30 µl of N-Glycanase and 75 +15 = 90 µl of Digestion Buffer.

Digest

GlykoPrep Digestion Module (product codes GS24-RX and GS96-RX)

Reagents and other Supplies
Glycoprotein Samples
RX Cartridges (supplied with the Kit, 1 per sample)
0.5-ml Microtube, screw cap, 0.2-ml PCR tubes, Cartridge Adaptors A and B and flip-top, 1.75-ml Microcentrifuge tubes
Denaturation Reagent (supplied with the Kit)
Acetonitrile (100%, HPLC-grade), ~2 ml
Blocking Reagent (supplied with the 2- Kit)
Digestion Buffer (prepared previously)
Enzyme Solution (prepared previously)

Procedure

Denature

1.a Add Denaturation Reagent to each Sample as described in Sample Denaturation (page 5).

The Denaturation Reagent is viscous and needs to be mixed well.

1.b Pipet up and down to mix well.

1.c Allow to incubate at room temperature for at least 5 minutes.

Prepare

Proceed through the Prepare, Equilibrate and Load steps without interruption, as evaporation can lead to airlock.

2.a Prepare a Basic Assembly for each Sample by nesting an RX Cartridge into a 0.5-ml screw cap Microtube.

2.b Pipet 50 µl of 100% Acetonitrile into the Sample Cup of each RX Cartridge in the Basic Assemblies.

2.c Place the Basic Assemblies in the centrifuge and spin at 300 x g for 3 minutes.

RX Cartridge (only)

Cartridge Adaptor A

0.5-ml screw-cap Microtube

Equilibrate

3.a Pipet 150 µl of Denaturation Reagent into the Sample Cup of each RX Cartridge.

3.b Spin at 1000 x g for 2 minutes.

Load

4.a Load 100 µl of the denatured Samples into the Sample Cup of each RX Cartridge (see Sample Denaturation, page 5).

4.b Empty the flow-through by lifting each RX Cartridge and pouring out the liquid collected in the Microtube below. Dispose of the liquid as organic waste and return each RX Cartridge to its Microtube.

4.c Spin at 50 x g until all Sample Cups are empty (~15 minutes).

Block

5.a Pipet 50 µl of Blocking Reagent into the Sample Cup of each RX Cartridge.

5.b Empty the flow-through (as described in 4.b).

5.c Spin at 300 x g for 3 minutes.

Wash

6.a Pipet 50 µl of Digestion Buffer into the Sample Cup of each RX Cartridge.

6.b Empty the flow-through (as described in 4.b).

6.c Spin at 300 x g for 3 minutes.

Load N-Glycanase

7.a Prepare"Tips Wet" Adaptor Assemblies for each Sample by nesting PCR tubes into Cartridge Adaptor B and 1.75-ml Microcentrifuge tubes.

7.c Pipet 10 µl of Enzyme Solution into the Sample Cup of each RX Cartridge in the Basic Assemblies.

7.d Transfer the RX Cartridges into the corresponding "Tips Wet" Adaptor Assemblies. Dispose of Microtubes and flow-through from 6.

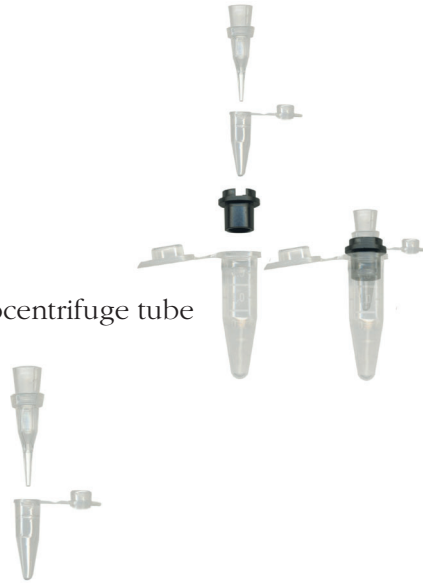
7.e Spin at 300 x g for 3 minutes; do not discard flow-through.

Incubate

8. Transfer the RX Cartridges/PCR tubes (Incubation Assembly) to the equilibrated 45°C PCR heat block and incubate for the chosen incubation time (see page 6).

Use the special sample loading technique to load samples in all protocols to prevent the introduction and entrapment of air bubbles in the neck of the Sample Cup. Use a pipet to remove trapped air bubbles.

Check that Sample Cups are empty before proceeding or yield will be reduced.



Elute

9.a Remove the Incubation Assemblies from the PCR heat block and reinsert into the Microcentrifuge tubes to form “Tips Wet” Adaptor Assemblies again.

9.b Pipet 20 µl of Digestion Buffer into the Sample Cup of each RX Cartridge.

9.c Spin at 300 x g for 3 minutes.

9.d Remove Cartridges from the PCR tubes. The eluted glycans are in the PCR tubes; DO NOT DISCARD.

Proceed immediately to Labeling.

Retain the RX Cartridges to recover the deglycosylated protein for further analysis (see Tips & Hints).

Labeling with InstantDye requires the availability of reactive glycosylamine ends, such as those resulting from rapid digestion with N-Glycanase. Glycosylamine ends spontaneously hydrolyze over time to reducing ends which are incompatible with InstantDye chemistry. To maximize labeling efficiency, Labeling should be performed immediately following collection of the glycans from the GlykoPrep Digestion Module.

Label

Reagents and other Supplies

N-Glycan Sample (eluted N-glycans with reactive glycosylamine ends in 0.2-ml PCR tubes)

InstantPC Labeling Reagent (prepared blow)

Preparation of Reagents

InstantPC Labeling Reagent

NOTE: The InstantDye™ is hygroscopic; minimize exposure to air and protect from exposure to light. Reconstituted dye may be resealed, repackaged with desiccant in a resealable bag, and frozen (-20°C) for storage up to 3 months and 10 freeze thaw cycles; return to RT before opening for use.

InstantPC Dye (30 mg, supplied with the kit)

Dye Solvent (supplied with the kit)

Add 150 µl of Dye Solvent directly into the InstantPC Dye vial.

Replace the cap and vortex the vial to ensure the dye is completely dissolved.

Sufficient for 24 samples; prepare 4 sets for an entire 96-ct kit.

Tightly recap unused Dye Solvent and return to the foil pouch with dessicant.

Procedure

Label

1. Pipet 5 µl of InstantPC Labeling Reagent into each well of N-glycan eluate in the 0.2-ml PCR tubes and pipet up and down several times to mix. Allow the samples to incubate at room temperature for 5 minutes.

Glycans are now labeled and ready for Cleanup.

NOTE: A precipitate may form in the labeling reaction over time. This precipitate is removed by Cleanup, though it may require longer spin times. To avoid this, proceed directly to Cleanup.

Cleanup

GlykoPrep Cleanup Module (product code GS24-CU and GS96-CU)

This entire section is performed with the CU Cartridges "Tips Free."

Reagents and other Supplies

N-Glycan Samples from InstantPC Labeling (in PCR tubes)

CU Cartridges (supplied with the Kit, 1 per N-glycan sample)

Cartridge Adaptor B and flip-top, 1.75-ml Microcentrifuge tubes, 2 per sample

Acetonitrile (100%)

Formic acid (HPLC-grade)

Dimethyl sulfoxide (HPLC-grade)

Ultrapure water

Volumetric pipettes, 5-ml

DO NOT use standard air-displacement pipettes to measure Acetonitrile.

Preparation of Reagents

Load and Wash Solution: 1% Formic acid in Acetonitrile

To make 30 ml (enough for a full 24-well Kit) of 1% Formic acid in Acetonitrile (v/v), add 300 µl of Formic acid to a glass, graduated cylinder. Bring the volume up to 30 ml with 100% Acetonitrile. Transfer to a glass storage vessel, cap tightly and swirl gently to mix. Scale as necessary (prepare 450 µl per sample).

Elute Solution: 10% Acetonitrile in water

To make 10 ml (enough for a full 24-well Kit) of 10% Acetonitrile in water (v/v), add 1.0 ml of Acetonitrile to a glass, graduated cylinder. Bring the volume up to 10 ml with ultrapure water. Transfer to a glass storage vessel, cap tightly and swirl gently to mix. Scale as necessary (prepare 250 µl per sample).

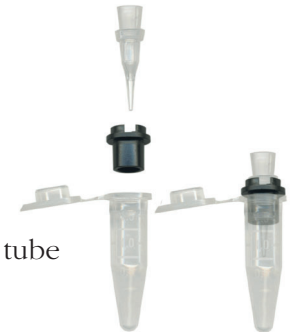
Procedure

Prepare one “Tips Free” Assembly per Sample Replicate by nesting a CU Cartridge into a Cartridge Adaptor B and a 1.75-ml Microcentrifuge tube.

CU Cartridge

Cartridge Adaptor B

Flip-top, 1.75-ml Microcentrifuge tube



Load

1.a Add 180 µl of Load Solution (1% Formic acid in Acetonitrile) to each Sample in the PCR tubes. Pipet up and down to mix.

1.b Transfer each Sample into the Sample Cup of a CU Cartridge in a “Tips Free” Assembly.

1.c Spin at 50 x g for 10 minutes or until the Sample Cup of each CU Cartridge is empty.

Transfer the mixture as quickly as possible because high organic solutions have low viscosity and may drip from the pipette tip; each sample may be pipetted in multiple rounds in order to achieve a quantitative transfer.

Wash

2.a Pipet 200 µl of Wash Solution (1% Formic acid in Acetonitrile) into the Sample Cup of each CU Cartridge in the “Tips Free” Assembly.

2.b Spin at 300 x g for 3 minutes.

Elute

3.a Transfer each CU Cartridge with its Adaptor to the new 1.75-ml Microcentrifuge Microcentrifuge tube.

3.b Pipet 50 µl of Elute Solution (10 % Acetonitrile) into the Sample Cup of each CU Cartridge.

3.c Spin at 150 x g for 5 minutes.

3.d Spin at 1000 x g for 1 minute.

NOTE: Up to 200 µl of Elute Solution may be used if more dilute glycans are desired.

Used CU Cartridges may be discarded. Adaptors are reusable, DO NOT DISCARD.

The 1.75-ml Microcentrifuge tubes now contain the InstantPC-labeled N-Glycans with free dye and buffer salts removed; DO NOT DISCARD.

Mix the eluate by pipette action or vortexing prior to analysis to ensure homogeneity. N-Glycan Samples are now ready to be analyzed. If not analyzed immediately, store sealed at -20°C in the dark.

ANALYSIS OF LABELED GLYCANS

NOTE: Injection of 1 µl InstantPC-glycans in InstantPC Eluent (10% (v/v) Acetonitrile) is recommended for UHPLC. For larger injection volumes (>1 µl) of InstantPC-glycans, do not use ACN alone to dilute the sample. Use 1 part sample in eluent to 3 parts 50:50 ACN:DMF, for a final concentration of 22.5% aqueous buffer, 37.5% DMF, 40.0% ACN.

Use standard techniques, such as Hydrophilic Interaction Liquid Chromatography (HILLC), to analyze the eluted, labeled glycans [1].

For suggested HILIC methods, MS conditions and FAQs, please visit the main GP24NG-PC and GP96NG-PC GlykoPrep InstantPC manuals:

www.prozyme/GlykoPrep/InstantPC-Analysis

REFERENCES

1. Kimzey et al. Development of an Instant Glycan Labeling Dye for High Throughput Analysis by Mass Spectrometry. Poster presented at ASMS 2015, St. Louis MO.

prozyme.com/posters/instantpc

Optimizing Excitation/Emission Wavelengths

The Optimal excitation/emission wavelengths for InstantPC Dye conjugated to an N-glycan are:

Excitation: 285 nm
Emission: 345 nm

Recovery of the Deglycosylated Protein from the Digestion (RX) Cartridge

Often, the deglycosylated protein is analyzed to evaluate the completeness of deglycosylation using such electrophoretic methods as SDS-PAGE or microfluidic lab-on-a-chip technology. Please contact us for guidelines for eluting your glycoprotein from the RX Cartridge.

Calculating the Mass of Glycans Labeled with InstantPC

Mass added to glycan with a free reducing end:

$$Mass_{Glycan\ (free\ reducing\ end)} + C_{14}N_3O_2H_{19} = Mass_{InstantPC\text{-}Labeled\ Glycan}$$

Mass added by $C_{14}N_3O_2H_{19}$ (Da):

Monoisotopic:	261.14773
Average:	261.3

Mass added to glycosylamine:

$$Mass_{Glycan\ (glycosylamine)} + C_{14}N_2O_3H_{18} = Mass_{InstantPC\text{-}Labeled\ Glycan}$$

Mass added by $C_{14}N_2O_3H_{18}$ (Da):

Monoisotopic:	262.13174
Average:	262.3

TECHNICAL ASSISTANCE

ProZyme is committed to developing rapid, automatable methods for glycan analysis. Call us to discuss products in development.

If you have any questions or experience difficulties regarding any aspect of our products, please contact us:

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ProZyme values customer opinions and considers customers an important source for information regarding advanced or specialized uses of our products. We encourage you to contact us. We welcome your suggestions about product performance or new applications and techniques.

TRADEMARKS AND TRADENAMES

PATENTS

www.prozyme.com/patents

The AssayMAP technology is licensed from Agilent Technologies.

OTHER PROZYME PRODUCTS & KITS

A wide variety of glycoanalysis products are available from ProZyme. A complete listing is accessible on our website by clicking on GlykoPrep® Rapid Sample Preparation Platform:

www.prozyme.com

ORDERING INFORMATION

For North American destinations: telephone orders may be placed between 8:00 am and 5:00 pm Pacific Time. Telefax or e-mail orders may be sent or messages recorded anytime.

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A list of ProZyme's distributors, with contact information, may be found at:

www.prozyme.com/distributors

If there is no distributor in your area, instructions for placing an international order may be found at:

www.prozyme.com/pages/ordering

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