CYGNUS TECHNOLOGIES
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Protein A Assay

Immunoenzymetric Assay for the Measurement of **Protein A** **Catalog # F400**

Intended Use

This kit is intended for use in quantitating Protein A. The kit is for **Research and Manufacturing Use Only** and is not intended for diagnostic use in humans or animals.

Summary and Explanation

Protein A immobilized on various chromatography media is commonly used to purify antibodies. Even when covalently attached, Protein A can leach off of the chromatography support and co-elute with the antibody. For applications such as the therapeutic use of the antibody, impurities with Protein A must be minimized to avoid any adverse patient effects. This immunoassay method provides sensitivity to detect Protein A impurities to less than 100pg/mL. Leached Protein A is typically bound to the product immunoglobulin present in the sample through its Fc region. This binding of Protein A to the product antibody can interfere in the accurate quantitation of Protein A in some immunoassays by inhibiting the ability of the anti-Protein A antibodies used in the assay to bind to the complexed Protein A. This inhibition can result in a significant underestimation of Protein A impurities. Such interference is highly variable from one product antibody to the next but is particularly common with many human IgGs.

There are several manufacturers of Protein A and Protein A chromatography supports. In addition to natural Protein A purified from *Staphylococcus aureus,* there are also various recombinant constructs of Protein A typically produced in *E.coli.* Some of these recombinant Protein A's are essentially identical to natural Protein A. However, there are other unnatural recombinant constructs of Protein A that have very significant structural differences when compared to natural Protein A. GE Healthcare sells one such unique construct of Protein A marketed as MabSelect SuRe™. Because of the very different structure of this protein the possibility exists that some antibodies for Protein A will recognize the various constructs of Protein A differently resulting in quantitation inaccuracies.

Such is the case with the two older Protein A kits manufactured by Cygnus Technologies, Cat #s F050 & F050H. These older kits recognize natural and as well as conserved recombinant Protein A with essentially the same structure as the purely natural Protein A. However, the F050 & F050H kits will underestimate the true concentration of the MabSelect SuRe™ ligand. For this reason, Cygnus has developed the F400 kit. This kit will cross react essentially 1:1 with MabSelect SuRe™ relative to natural and highly conserved recombinant Protein A. If you are using natural or structurally conserved, recombinant forms of Protein A and have successfully qualified the F050 or F050H kits you may continue to use those products as they will be manufactured indefinitely. If you are using a recombinant form of Protein A with very significant structural differences from natural Protein A such as MabSelect SuRe™ you should use the Cat # F400 kit. If you are evaluating our Protein A assays for the first time, we recommend use of Cat # F400 kit as it offers other robustness and procedural advantages over the older kits, in addition to its ability to more accurately quantitate unnatural forms of Protein A.

This kit, Cat # F400, is designed to detect all currently marketed constructs of Protein A. This kit will eliminate most product antibody inhibition and provide accurate quantitation through the use of a carefully qualified sample treatment step (See 'Limitations' section). This assay is designed to provide a simple to use, precise, and highly sensitive method to detect Protein A impurities to less than 100pg/mL in the presence of up to mg/mL quantities of humanized monoclonal antibodies. As such this kit can be uses as a tool to aid in optimal purification process development and in routine quality control of in-process streams as well as final product.

Principle of the Procedure

The Protein A assay is a two-site immunoenzymetric assay. Samples containing Protein A are first diluted with a sample denaturing buffer. This reagent dissociates the Protein A from the product antibody. Samples are then heated in a dry heating block or boiling water bath to denature and precipitate the product antibodies. After a centrifugation step to pellet the denatured product antibody, the samples are then reacted in microtiter strips coated with a polyclonal anti-Protein A capture antibody. A second anti-Protein A antibody labeled directly with Horse Radish Peroxidase (HRP) enzyme is simultaneously reacted forming a sandwich complex of solid phase antibody-Protein A-HRP labeled antibody. After a wash step to remove any unbound reactants, the strips are then reacted with tetramethylbenzidine (TMB) substrate. The amount of hydrolyzed substrate is read on a microtiter plate reader and will be directly proportional to the concentration of Protein A present. Accurate quantitation is achieved by comparing the signal of unknowns to Protein A standards assayed at the same time.

Reagents & Materials Provided

Component	Product #
Anti-Protein A:HRP Chicken antibody conjugated to horseradish peroxidase in a protein matrix with preservative. 1x12mL	**F401**
Polyclonal Anti-Protein A coated microtiter strips 12x8 well strips in a bag with desiccant	**F052A***
Protein A Standards Recombinant Protein A in a protein matrix with preservative. 7 standards at 0, 0.1, 0.25, 0.6, 1.5, 4, and 10 ng/mL. 1mL/vial	**F403**
Sample Denaturing Buffer Citrate buffer with detergent and preservative. 1x12mL	**F054R**
Stop Solution 0.5M sulfuric acid. 1x12mL	**F006**
TMB Substrate 3,3',5,5' Tetramethylbenzidine. 1x12mL	**F005**
Wash Concentrate (20X) Tris buffered saline with preservative. 1x50mL	**F004**
Sample Treatment Plate Skirted 96 well PCR plate with adhesive foil seal	**F402**

*All components can be purchased separately except # F052A and F054R.

Storage & Stability

- All reagents should be stored at 2°C to 8°C for stability until the expiration date printed on the kit.
- After prolonged storage, you may notice a salt precipitate and/or yellowing of the wash concentrate. These changes will not impact assay performance. To dissolve the precipitate, mix the wash concentrate thoroughly and dilute as directed in the 'Preparation of Reagents' section.
- Reconstituted wash solution is stable until the expiration date of the kit.

Materials & Equipment Required But Not Provided

- Microtiter plate reader spectrophotometer with dual wavelength capability at 450 & 650nm. *(If your plate reader does not provide dual wavelength analysis you may read at just the 450nm wavelength.)*
- Pipettors - 50µL and 100µL
- Repeating or multichannel pipettor - 100µL
- Microtiter plate rotator (400 - 600 rpm)
- Sample Diluent (recommended Cat # I028)
- Distilled water
- 1 liter wash bottle for diluted wash solution
- Microcentrifuge tubes
  If using Sample Treatment Plate method:
  96 well plate heating block (We recommend VWR Cat#13259-260)
  Bench top centrifuge capable of spinning 96 well plates
  If using Microfuge Tube Sample Treatment method:
  Boiling water bath or dry heating block
- Microcentrifuge

Precautions

- For Research or Manufacturing use only.
- Stop reagent is 0.5M H_2SO_4. Avoid contact with eyes, skin, and clothing.
- This kit should only be used by qualified technicians.

Preparation of Reagents

- Bring all reagents to room temperature.
- Dilute wash concentrate to 1 liter in distilled water, label with kit lot and expiration date, and store at 4°C.

Procedural Notes

1. Complete washing of the antibody coated plate to remove excess unreacted reagents is essential to good assay reproducibility and sensitivity. We advise against the use of automated or other manual operated vacuum aspiration devices for washing plates as these may result in lower specific absorbances, higher non-specific absorbance, and more variable precision. The manual wash procedure described below generally provides lower backgrounds, higher specific absorbance, and better precision. If duplicate CVs are poor or if the absorbance of the 0 standard is greater than 0.200, evaluate plate washing procedure for proper performance.

2. When dilution of samples is required, dilution should be performed in a diluent qualified to yield acceptable background and not impurities with Protein A. Sample dilution should be performed prior to the sample denaturation step for best results. The diluent should also give acceptable recovery when spiked with known quantities of Protein A. Sample Diluent, Cat #I028 has been qualified for use with this assay. This is the same diluent used to make the kit standards. As your sample is diluted in I028 its matrix begins to approach that of the standards thus reducing any inaccuracies caused by dilutional artifacts. Other prospective diluents must be tested for non-specific binding and acceptable spike & recovery as discussed below.

3. High Dose Hook Effect may be observed in samples with very high concentrations of Protein A. Samples greater than 20μg/mL may give absorbances less than the 10ng/mL standard. Hook effect is indicated when absorbance of the undiluted sample is less than the diluted samples. If a hook effect is possible, samples should be assayed over at least two dilutions.

Quality Control

- Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples greater than 0.5 ng/mL. CVs for samples less than 0.5 ng/mL may be greater than 10%.
- It is recommended that each laboratory assay appropriate quality control samples in each run to ensure that all reagents and procedures are correct. **You are strongly urged to make controls in your typical sample matrix using your product antibody. These controls can be aliquoted into single use vials and stored frozen for long-term stability.**

Limitations

- Before reporting Protein A impurities using this kit, each laboratory should qualify that the kit and assay procedure utilized yield acceptable specificity, accuracy, and precision. A suggested protocol for this qualification can be obtained by contacting our Technical Services Department or at our web site. In general the most critical qualification experiments involve spike & recovery and dilutional linearity/parallelism.
- The ability of anti-Protein A antibodies to immunologically bind to Protein A can be affected by the type of product immunoglobulin present in the sample. In particular, certain natural, serum derived human immunoglobulins when complexed to the Protein A can significantly reduce the binding of both the capture and HRP antibodies used in this kit. This interference is believed to be due to steric hindrances when the product antibody has been pre-bound to Protein A. Most mouse monoclonals, humanized monoclonals, and many human antibodies expressed in Chinese Hamster Ovary (CHO) cells or mouse hybridoma cell lines do not significantly inhibit and can be assayed at concentrations of 1mg/mL or greater. When detection sensitivity limits allow, simple dilution of the inhibitory product antibody in our recommended diluent Cat #I028 prior to sample denaturation will usually overcome the interference. Most antibodies we have tested show no significant interference when diluted in Cat # I028 diluent in the range of 0.1 to 1mg/mL
- Samples in concentrated strong acids can interfere in the assay by lowering the assay pH to below the optimal range of 7.0 to 7.5. The HRP labeled antibody is in a strong buffer designed to neutralize most samples back to the ideal assay pH range. If there is some doubt about the pH interference of your sample you may conduct a simple test prior to performing the assay by adding 1 part of the denatured sample to 2 parts of the HRP conjugate and testing for pH using paper pH indicator strips.

- Certain sample matrices and product antibodies may interfere in this assay. Although the assay is designed to minimize matrix interference, materials such as detergents in high concentration, extremes of pH (less than 6.0 and greater than 8.5), very high buffer molarity, or very high protein concentrations may give erroneous results. **For these reasons we recommend that you first establish acceptable recovery in your sample matrices by performing a spike recovery experiment.** This test can be very simply performed by diluting 1 part of the 10ng/mL standard supplied with the kit into 3 parts of your sample matrix which does not contain any or very low levels of Protein A. This diluted standard when assayed as an unknown should give a recovery value after correcting for any endogenous Protein A of ~2 to 3 ng/mL. Consult *Cygnus Technologies* Technical Service Department for advice on how to quantitate the assay in problematic matrices.

Calculation of Results

The standards may be used to construct a standard curve with values reported in ng/mL (See 'Limitations' Section). This data reduction may be performed through computer methods using curve fitting routines such as point-to-point, spline, or 4 parameter logistic fit. **Do not use linear regression analysis to interpolate values for samples as this may lead to significant inaccuracies!** Data may also be manually reduced by plotting the absorbance values of the standard on the y-axis versus concentration on the x-axis and drawing a smooth point-to-point line. Absorbances of samples are then interpolated from this standard curve.

Assay Protocol

- Bring all reagents to room temperature.

- Set-up plate spectrophotometer to read dual wavelength at 450nm for the test wavelength and 650nm for the reference wavelength. (A 630nm filter can be substituted for the 650nm if your instrument is so equipped.)

- All standards, controls and samples should be treated in exactly the same way. Assay all samples at least in duplicate.

- **Pipette the HRP labeled antibody (#F401) into the wells before adding the denatured samples. This will ensure that the sample is neutralized by the HRP antibody buffer solution before it is exposed to the microtiter strip coated antibody. Pipetting of the sample into the well before the HRP labeled antibody, can damage the antibody coating and result in lower and more variable absorbances.**

- Maintain a repetitive timing sequence from well to well for all assay steps to ensure that all incubation times are the same for each well. Accomplish all steps as rapidly as possible to avoid "end of run" sequential process time differences that could cause systematic inaccuracies (See 'Procedural Modifications' below.)

- Make a work list for each assay to identify the location of each standard, control, and sample.

- Thorough washing is essential to proper performance of this assay. Automated plate washing systems or other vacuum aspiration devices are not recommended. The manual method described in the assay protocol is preferred for best precision, sensitivity and accuracy. A more detailed discussion of this procedure can be obtained from our Technical Services Department or on our web site. In addition, a video demonstration of proper plate washing technique is available in the 'Technical Help' section of our website.

- The protocol specifies use of an approved orbital microtiter plate shaker for the immunological steps. These can be purchased from most laboratory supply companies. If you do not have such a device, it is possible to incubate the plate without shaking however it will be necessary to extend the immunological incubation step in the plate by about one hour in order to achieve comparable results to the shaking protocol. **Do not shake during the 30-minute substrate incubation step, as this may result in higher backgrounds and worse precision.**

Assay Protocol

1. Pipette 100µL of anti-Protein A:HRP (#F401) into each well of the antibody coated microtiter plate, Cat #F052A.

2. Pi
pette 50µL of supernatant from the denatured standards, controls and samples into wells indicated on work list.

3. Cover & incubate on orbital shaker at 400-600 rpm for 2 hours at room temperature, 24°C ± 4°C.

4. Dump contents of wells into waste. Blot and gently but firmly tap over absorbent paper to remove most of the residual liquid. Overly aggressive banging of the plate or use of vacuum aspiration devices in an attempt to remove all residual liquid is not necessary and may cause variable dissociation of antibody bound material resulting in lower ODs and worse precision. Fill wells generously to overflowing with diluted wash solution using a squirt bottle or by pipetting in ~350µL. Dump and tap again. Repeat for a total of 4 washes. Wipe off any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step. Do not allow wash solution to remain in wells for longer than a few seconds. Do not allow wells to dry before adding substrate.

5. Pipette 100µL of TMB substrate (#F005).

6. Incubate at room temperature for 30 minutes. DO NOT SHAKE.

7. Pipette 100µL of Stop Solution (#F006).

8. Read absorbance at 450/650nm blanking on the Zero standard.

Procedural Modifications

- The assay is very robust such that assay variables like incubation times, and sample size can be altered to manipulate assay performance for more sensitivity, increased upper analytical range, or reduced sample matrix interference. Before modifying the protocol from what is recommended users are advised to contact Technical Service for input on the best way to achieve your desired goals.
- Samples containing Protein A greater than 10ng/mL should be diluted in an appropriate diluent. (See Procedural Note # 2.) Be sure to multiply diluted sample concentrations by the dilution factor when calculating the results.

Because of the multiple steps involved in sample treatment it is possible to introduce systematic effects as function of pipetting and incubation timing differences. These effects will manifest as what can be called “end of run” or “front to back” effects. In such cases what is typically seen are that samples assayed at the front of the plate give higher values than those at the end of the plate. Provided all the steps are performed quickly, end of run effects are insignificant for runs involving no more than 6 strips. When an entire plate of 12 strips is performed as a single assay, samples run at the end of the plate can show significant differences in apparent Protein A concentration due to timing differences. If you are performing runs of more than 6 strips we suggest using the Sample Treatment Plate method. If microcentrifuge tubes are used end of run effects can usually be overcome in multiple strip runs by modifying the assay protocol for Steps 1 & 2 in the Assay Protocol section below. Rather than adding the HRP conjugate to the coated wells followed by addition of the denatured samples, we suggest pipetting 50µL of denatured samples into an uncoated “template” microtiter plate (not provided with the kit) in the same positions as the samples will be put into the coated plate provided with the kit. After addition of all samples to the uncoated, template plate, pipette 100µL of the HRP conjugated antibody into all wells using a multichannel pipettor. Mix the contents on a microtiter plate shaker. Using a multichannel pipettor, transfer 100uL of the mixture from each uncoated template well into the corresponding well of the anti-Protein A microtiter plate, Cat #F052. Proceed with the assay as described in the Assay Protocol from Step 3 on.

Sample Treatment

Failure to completely dissociate and remove the product antibody from the sample during the sample treatment step can result in under-recovery of Protein A. The usual cause of poor Protein A recovery is due to small amounts of residual sample antibody remaining in the supernatant after the centrifugation step. Product antibody can re-associate with the Protein A during the assay protocol and cause under recovery. Careful adherence to the procedure below should ensure full recovery of Protein A.

The heating step will typically result in a denatured protein precipitate containing the product antibody. The Protein A will be in the supernatant. Samples containing a high product antibody concentration (greater than 4mg/mL) may yield a very large precipitate pellet making it difficult to recover sufficient supernatant for the assay. In such cases it is best to dilute the sample prior to denaturation in a neutral pH buffer (Cat #I028). Be sure to correct the assay result for any dilution factor.

Using the Sample Treatment Plate (STP)

1. Prepare initial sample dilutions as required prior to sample treatment. Transfer to the appropriate wells of the STP, Cat #F402.

2. All subsequent dilutions to be assayed can be made in the STP using Sample Diluent, Cat. # I028. Ensure final volume in every well is 100µL.

3. Add 100µL of the kit standards and controls to the wells.

4. Add 50µL of Sample Denaturing Buffer, Cat #F054R to each well. Mix by pipetting up and down ~10 times. Use fresh tips for each addition.

5. Seal the STP with the adhesive foil. Be sure that the seal is firm over all wells.

6. Place STP in a preheated block and place this device into a qualified 80°C dry heating block for 15 minutes.

7. Remove the STP and allow to cool for 5 minutes.

8. Centrifuge the STP for 5-10 minutes at ~3,000 x g (acceptable range is 2,000-4,000 x g) in a swing bucket rotor with a microtiter plate adapter.

Using Microcentrifuge Tubes

1. Process all samples including the standards and controls by adding 1 part of sample denaturing buffer (Cat. # F054R) to 2 parts of sample into a microfuge vial. (For example: Pipette 100µL of F054R into a microfuge tube containing 200µL of sample. These volumes will provide for at least triplicate analysis of your samples). **Mix thoroughly by vortexing.**

2. Make a small pin or needle hole in the cap of each microfuge tube to allow for venting of heated, expanded air inside the tube.

3. Place the tubes in the preheated block or flotation device and place this device into a qualified 100°C dry heating block or boiling water bath for 5 to 10 minutes. While 5 minutes is adequate for most samples we have seen some samples where a 10-minute heat step improves recovery.

4. Remove the tubes, allow to cool for 5 minutes, and then centrifuge at 6000 to 15,000 x g for 5 minutes in a microcentrifuge or other adapted centrifuge. If your centrifuge is capable of rates of centrifugation higher than 6000x g a higher speed can yield a more tightly packed pellet less subject to re-suspension. Make certain your centrifuge is very well balanced. If you feel or hear any vibrations as the centrifuge accelerates or decelerates your rotor is unbalanced. A poorly balanced centrifuge will result in some of the pelleted product antibody being re-suspended. This re-suspended antibody is a frequent cause for under-recovery of Protein A. For more dilute samples a pellet may not be visible after centrifugation. Therefore, always orient the centrifuge tubes in the same way so you will know where the pellet will be. In this way you can avoid disruption of the pellet when removing the test sample. Avoid any delays in removing the supernatant for testing. Handle the tubes carefully to avoid bumping or vibrations that might re-suspend some of the pellet.

If you continue to have poor recovery after carefully following the procedures above it may be necessary to further dilute your sample prior to assay using Sample Diluent Cat #I028. Dilution to the range of 1.0 to 0.1 mg/mL is usually sufficient to obtain acceptable recovery. Contact our very experienced Technical Service Department if you have any problems with recovery. If you have concerns about your technique, a video demonstration on 'How to Denature Samples for the Protein A Kit' is available in the 'Technical Help' section of our web site.

Example Data

Well #	Contents	Abs. at 450nm-650nm	Mean Abs.
A1	0ng/mL	0.053	0.057
B1	0ng/mL	0.061	
C1	0.1ng/mL	0.084	0.083
D1	0.1ng/mL	0.081	
E1	0.25ng/mL	0.142	0.141
F1	0.25ng/mL	0.139	
G1	0.6ng/mL	0.239	0.244
H1	0.6ng/mL	0.249	
A2	1.5ng/mL	0.486	0.480
B2	1.5ng/mL	0.473	
C2	4.0ng/mL	1.285	1.306
D2	4.0ng/mL	1.327	
E2	10ng/mL	2.696	2.669
F2	10ng/mL	2.642	

Performance Characteristics

Cygnus Technologies has qualified this assay by conventional criteria as indicated below. A more detailed copy of this "Qualification Summary" report can be obtained by request or by accessing our Web site at www.cygnustechnologies.com. This qualification is generic in nature and is intended to supplement but not replace certain user and product specific qualification and qualification that should be performed by each laboratory. At a minimum each laboratory is urged to perform a spike and recovery study in their sample types. In addition, any of your samples types containing Protein A within or above the analytical range of this assay should be evaluated for dilutional linearity to ensure that the assay is accurate and does not suffer from "Hook Effect". Each laboratory and technician should also demonstrate competency in the assay by performing a precision study similar to that described below. A more detailed discussion of recommended user qualification protocols can be obtained by contacting our Technical Services Department or on-line at our web site.

Precision

The data below show both intra (n=12 replicates) and inter-assay (n=10 assays) coefficients of variation (%CVs) for 3 control samples in the low, middle, and upper range of the standards. Each laboratory is encouraged to establish precision with its protocol using a similar study.

Intra-assay			Inter-assay		
# of tests	**Mean ng/mL**	**%CV**	**# of assays**	**Mean ng/mL**	**%CV**
12	0.258	8.3	10	0.253	10.0
12	1.546	2.0	10	1.496	4.6
12	4.373	5.1	10	4.046	6.1

Sensitivity

The lower limit of detection (**LOD**) is defined as that concentration corresponding to a signal two standard deviations above the mean of the zero standard. The LOD is ~50 pg/mL. The lower limit of quantitation (**LOQ**) is defined as the first dosed standard at 100pg/mL.

Spike & Recovery/ Interference Studies

Each user should qualify that their sample matrices and product itself yield accurate recovery. This experiment can be performed by spiking the 10ng/mL standard provided with this kit, into the sample in question. For example, we suggest adding 1 part of the 10 ng/mL standard to 3 parts of the test sample. This yields an added spike of 2.5 ng/mL. Any endogenous Protein A from the sample itself determined prior to spiking and corrected for by the 25% dilution of that sample should be subtracted from the value determined for the spiked sample. The added spike and recovery should be within allowable limits, e.g. 80% to 120%.

Various buffer matrices have been evaluated by spiking known amounts of Protein A. Because this assay is designed to minimize matrix interference most of these buffers yielded acceptable recovery (defined as between 80-120%). In general extremes in pH (less than 5.0 and greater than 8.5) or salt concentration as well as certain detergents can cause under-recovery. In some cases high concentrations of the product antibody may also cause a negative interference. Serum derived polyclonal human and rabbit IgGs have been reported to partially inhibit the ability of some kit anti-Protein A antibodies to bind to Protein A resulting in potential under-recovery of true Protein A levels. While this kit has been designed to overcome such interference your product antibodies should be evaluated for any negative inhibition before reporting results as described in the "Limitations" section. If you encounter product antibody interference contact *Cygnus Technologies* Technical Services Department for advice on how to solve this problem. When detection sensitivity limits allow, simple dilution of the inhibitory product antibody in our recommended diluent Cat #I028 will usually overcome the interference. Most antibodies we have tested show no significant interference in the range of 0.1 to 1 mg/mL.

Specificity/Cross-Reactivity

This kit will detect natural *Staphylococcal* and various commercially available recombinant Protein A constructs. On a molar basis, 4 different constructs of Protein A commercially available at the time of the qualification of this assay (August 2007) reacted essentially equally with recovery between 90 to 100%. However, you may want to test your source of Protein A for recovery to ensure accurate quantitation by this kit. If recovery is unacceptable contact our Technical Services Department for guidance. Substitution of standards made with your source of Protein A in place of the kit standards may be a solution.

Hook Capacity

Very high concentrations of Protein A were evaluated for the hook effect. At concentrations exceeding 20,000 ng/mL, the apparent concentration of Protein A may read less than the 10ng/mL standard. Samples yielding signals above the 10ng/mL standard or suspected of having concentrations in excess of 20,000 ng/mL should be assayed diluted.

Ordering Information/ Customer Service

Cygnus Technologies also offers kits for the extraction and detection of CHO Host Cell DNA. The following kits are available:

- Residual Host Cell DNA extraction:

  Cat # D100W, DNA Extraction Kit in 96 deep well plate
  Cat # D100T, DNA Extraction Kit in microfuge tubes

To place an order or to obtain additional product information contact *Cygnus Technologies*:

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