CYGNUS TECHNOLOGIES
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Insulin

Immunoenzymetric Assay for the Measurement of Insulin
Catalog # F040

Intended Use

This kit is intended for use in quantitating insulin from bovine, human or porcine sources. The kit is for **Research and Manufacturing Use Only** and is not approved for diagnostic use in humans or animals. For applications requiring lower limits of detection, *Cygnus Technologies LLC* manufactures an *Ultra Sensitive* Insulin ELISA kit, Catalog #F280. That kit has standards in the range of 25-1250pg/mL with a limit of quantitation of <25pg/mL. As such that kit can be used for testing insulin levels in serum from fasting individuals.

Summary and Explanation

The manufacture of products by various biotechnological processes such as cell or tissue culture can result in residual contamination of the desired product by components used in the culture media. The use of so called serum free defined media greatly reduces the number of potential impurities but it may still be necessary to determine trace impurities levels of the proteins and growth factors used in these media. Most commercial formulations of serum free media contain significant amounts of albumin and transferrin either of bovine or human origin, and insulin from various species. When the intended product may be used as a therapeutic agent in humans or animals the product should be highly purified to avoid potential health risks or other problems that might result from trace impurities. Efforts to reduce trace media impurities to the lowest levels practical require a highly sensitive and reliable analytical method. The Insulin assay is designed to provide a simple to use, precise, and highly sensitive method to detect Insulin impurities to less than 250 pg/mL. As such this kit can be used 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

This insulin assay is a two-site immunoenzymetric assay. Samples containing insulin are reacted simultaneously in microtiter strips coated with an affinity purified capture antibody. A second anti-Insulin antibody labeled alkaline phosphatase is reacted forming a sandwich complex of solid phase antibody- Insulin-Alkaline phosphatase labeled antibody. After a wash step to remove any unbound reactants, the strips are then reacted with p-nitrophenyl phosphate (PNPP) substrate. The amount of hydrolyzed substrate is read on a microtiter plate reader and will be directly proportional to the concentration of Insulin present. Accurate quantitation is achieved by comparing the signal of unknowns to Insulin standards assayed at the same time.

Reagents & Materials Provided

Component	Product #
Anti-Insulin:Alkaline Phosphatase Mouse monoclonal antibody conjugated to alkaline phosphatase in a protein matrix with preservative. 1x12mL	**F041**
Monoclonal Anti-Insulin coated microtiter strips 12x8 well strips in a bag with desiccant	**F042***
Insulin Standards Human Recombinant Insulin in a protein matrix with preservative. Standards at 0, 0.25, 1, 2, 4, 10,and 20 ng/mL. 1mL/vial	**F043**
PNPP Substrate p-nitrophenyl phosphate in a Diethanolamine buffer with preservative. 1x12mL	**F008**
Wash Concentrate (20X) Tris buffered saline with preservative. 1x50mL	**F004**

*All components can be purchased separately except # F042.

Storage & Stability

- All reagents should be stored at 2°C to 8°C for stability until the expiration date printed on the kit.
- The substrate reagent should not be used if its absorbance at 405nm is greater than 0.4.
- Reconstituted wash solution is stable until the expiration date of 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.

Materials & Equipment Required But Not Provided

- Microtiter plate reader spectrophotometer with dual wavelength capability at 405 & 492nm. *(If your plate reader does not provide dual wavelength analysis you may read at just the 405nm wavelength.)*
- Pipettors - 25µL 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

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.

Precautions

- For Research or Manufacturing use only.
- At the concentrations used in this kit, none of the other reagents are believed to be harmful.
- This kit should only be used by qualified technicians.

Procedural Notes

1. Complete washing of the plates 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 0.300, 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 contaminated with Insulin. The diluent should also give acceptable recovery when spiked with known quantities of Insulin. Alternatively, *Cygnus* sells a diluent qualified for this assay, Sample Diluent product number I028.

3. High Dose Hook Effect may be observed in samples with very high concentrations of Insulin. Samples greater than 6 µg/mL may give absorbances less than the 20 ng/mL standard. If a hook effect is possible, samples should also be assayed diluted. If the absorbance of the undiluted sample is less than the diluted samples this may be indicative of the hook effect.

4. If the substrate has a distinct yellow color prior to performing the assay it may have been contaminated. If this appears to be the case read 200µL of substrate against a water blank. If the absorbance is greater than 0.4 it may be necessary to obtain new substrate or the sensitivity of the assay may be compromised. The PNPP substrate is very sensitive to environmental impurities. Do not leave bottle open or at room temperature for longer than is needed. Only remove as much reagent as is needed for your assay run and do not return any unused substrate back into the substrate bottle. Additional substrate can be purchased separately as Cat # F008.

Limitations

- The antibodies used in this kit cross-react 100% with insulin from human (natural and recombinant) bovine, and porcine. Cross reactivity with insulin from other species has not been extensively investigated.
- Before reporting Insulin contamination 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.
- Certain sample matrices and recombinant product themselves may interfere in this assay. Although the assay is designed to minimize matrix interference, materials such as detergents in high concentration, extremes of pH (<6.0 and >8.5), very high buffer molarity, or very high protein concentrations may give erroneous results. It is recommended to test the sample matrix for interference by diluting the 20ng/mL standard 1 part to 4 parts of your sample matrix which does not contain any Insulin. This diluted standard when assayed as an unknown should give a value of 3 to 5ng/mL. Consult *Cygnus Technologies* Technical Service Department for advice on how to quantitate the assay in problematic matrices.

Assay Protocol

- Bring all reagents to room temperature.
- Set-up plate spectrophotometer to read dual wavelength at 405nm for the test wavelength and 492nm for the reference wavelength. Blank the instrument using the zero standard wells after assay completion.
- All standards, controls and samples should be assayed in duplicate.
- Maintain a repetitive timing sequence from well to well for all assay steps to insure that all incubation times are the same for each well.
- Make a work list for each assay to identify the location of each standard control and sample.
- If the substrate has a distinct yellow color prior to the assay it may have been contaminated. If this appears to be the case read 200µL of substrate against a water blank. If the absorbance is greater than 0.4 it may be necessary to obtain new substrate or the sensitivity of the assay may be compromised.
- 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 web site.
- 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 60-minute substrate incubation step, as this may result in higher backgrounds and worse precision.**

Assay Protocol

1. Pipette 25µL of standards, controls and samples into wells indicated on work list.

2. Pipette 100µL of anti-Insulin:Alkaline Phosphatase (#F041) into each well.

3. Cover & incubate on orbital shaker at 400-600rpm for 1 hour at room temperature, 24°C ± 4°.

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 PNPP substrate (#F008).

6. Incubate for 1 hour at room temperature, 24°C ± 4°. DO NOT SHAKE.

7. Read absorbance at 405/492nm

Procedural Modifications

- The assay is very robust such that assay variables like incubation times, sample size, and other sequential incubation schemes can be altered to manipulate assay performance for more sensitivity, increased upper analytical range, or reduced sample matrix interference. Increasing incubation time for the PNPP substrate step will in general increase absorbances proportionately for all wells. For example, doubling the substrate step time from 60 minutes to 120 minutes will double all ODs. Before modifying the protocol from what is recommended, users are advised to contact our technical services for input on the best way to achieve your desired goals. If more sensitivity is required, we recommend the use of the *Ultra-Sensitive* Insulin ELISA kit, #F280. The limit of quantitation for the *Ultra-Sensitive* Insulin ELISA kit is 25pg/mL.

Quality Control

- Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples greater than 1ng/mL. CVs for samples < 1ng/mL may be greater than 10%.
- For optimal performance the absorbance of the substrate when blanked against water should be < 0.4.
- It is recommended that each laboratory assay appropriate quality control samples in each run to insure that all reagents and procedures are correct.

Calculation of Results

The standards may be used to construct a standard curve with values reported in ng/mL. This data reduction may be performed through computer methods using curve fitting routines such as point-to-point, cubic 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.

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. 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 Insulin within or above the analytical range of this assay should be evaluated for dilutional linearity to insure that the assay is accurate and does not suffer from "Hook Effect" or negative sample interferences. 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 shows both intra (n=10 replicates) and inter-assay (n=5 assays) coefficients of variation (%CVs). 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	0.80	6.7	5	0.81	8.4
12	8.11	5.8	5	8.00	5.9

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 125 pg/mL. The lower limit of quantitation (**LOQ**) is defined as the lowest standard and is thus ~250pg/mL.

Recovery/ Interference Studies

Various buffer matrices have been evaluated by spiking known amounts of Insulin. 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 (<5.0 and >8.5) or salt concentration as well as certain detergents can cause under-recovery. In some cases very high concentrations of the product protein may also cause a negative interference in this assay. Each user should qualify that their sample matrices and product itself yield accurate recovery in the protocol of their choice. This experiment can be performed by spiking the 20 ng/mL standard provided with this kit, into the sample in question. For example, we suggest adding 1 part of the 20 ng/mL standard to 4 parts of the test sample. This yields an added spike of 4 ng/mL. Any endogenous Insulin from the sample itself determined prior to spiking and corrected for by the 20% 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%. Should you have any problems achieving adequate spike and recovery data you are strongly urged to contact our Technical Services Department for recommendations on how to overcome sample matrix interference.

Specificity/Cross-Reactivity

The antibodies used in this kit substantially cross-react (~100%) with insulin from human (natural and recombinant), bovine, and porcine. Cross reactivity with insulin from other species has not been extensively investigated. Rat and mouse insulin have been reported to cross-react in the range of 50 to 70%. This kit might be used to quantitate rat and mouse insulin provided the laboratory has qualified the kit for such an application and has its own standards or can apply the appropriate cross reactivity correction factor.

Hook Capacity

Insulin concentrations up to 6μg/mL were evaluated for the hook effect. At concentrations exceeding 6μg/mL the apparent concentration of Insulin may read less than the 20 ng/mL standard. Samples yielding signals above the 20 ng/mL standard or suspected of having concentrations in excess of 6 μg/mL should be assayed diluted.

Example Data

Contents	Abs. at 405-490nm	Mean Abs.	ng/mL Insulin
Zero Std	0.099	0.095	N/A
Zero Std	0.097		
Zero Std	0.089		
0.25 ng/mL	0.136	0.139	N/A
0.25 ng/mL	0.143		
0.25 ng/mL	0.139		
1ng/mL	0.278	0.277	N/A
1ng/mL	0.273		
1ng/mL	0.280		
2ng/mL	0.421	0.435	N/A
2ng/mL	0.437		
2ng/mL	0.446		
4ng/mL	0.639	0.665	N/A
4ng/mL	0.671		
4ng/mL	0.686		
10ng/mL	1.020	1.045	N/A
10ng/mL	1.066		
10ng/mL	1.050		
20ng/mL	1.341	1.321	N/A
20ng/mL	1.256		
20ng/mL	1.367		
sample 1	0.650	0.637	3.8ng
sample 1	0.631		
sample 1	0.631		
sample 2	1.054	1.051	10.4ng
sample 2	1.014		
sample 2	1.084		

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

- Extraction and PCR amplification of CHO Host Cell DNA for use with user supplied master mix:

  Cat # D555W, DNA Extraction Kit in 96 deep well plate
  Cat # D555T, DNA Extraction Kit in microfuge tubes

- Residual CHO Host Cell DNA extraction and detection using PicoGreen® dye:

  Cat # D550W, DNA Extraction Kit in 96 deep well plate
  Cat # D550T, DNA Extraction Kit in microfuge tubes

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

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