CYGNUS TECHNOLOGIES
part of Maravai LifeSciences

Insulin Assay – *Ultra Sensitive*

Immunoenzymetric Assay for the *Ultra Sensitive* Measurement of Insulin
Catalog # F280

Intended Use

This kit is intended for use in quantitating very low concentrations of insulin, in the range of 25 to 1250pg/mL from bovine, human or porcine sources. The kit is for **Research and Manufacturing Use Only** and can be used to quantitate insulin in products produced by recombinant methods or in serum samples. The kit is not approved for diagnostic use in humans or animals.

Summary and Explanation

The manufacture of products by various biotechnological processes such as cell or tissue culture can result in residual impurities 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 impurity 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 which 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 *Cygnus Technologies* Insulin assay is designed to provide a simple to use, precise, and highly sensitive method to detect Insulin impurities to less than 25pg/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. If your application does not require detection of ultra low levels of insulin, you may consider using our standard Insulin ELISA kit, Catalog #F040. That kit has an analytical range of 0.25 to 20ng/mL, with a limit of quantitation of ~0.25ng/mL. The assay time for the F040 kit requires approximately 2 hours whereas the *Ultra Sensitive* Insulin kit, #F280 requires 4 hours.

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	F281
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, 25, 80, 200, 500, and 1250pg/mL. 1 mL/vial	F282
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 stopped absorbance at 405nm is greater than 0.4.
- 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 405 & 492nm. *(If your plate reader does not provide dual wavelength analysis you may read at just the 405nm wavelength.)*
- Pipettor - 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

Precautions

- For Research or Manufacturing use only.
- 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 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 polluted 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 2µg/mL may give absorbances less than the 1250pg/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. Such samples should be diluted until the dilution adjusted value remains constant.

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. Rat and mouse insulins have been reported to cross-react in the range of 50-70%. This kit can be used to quantitate rat and mouse insulin provided the laboratory has its own standards or can apply the appropriate cross reactivity correction factor.
- Before reporting Insulin 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.
- Certain sample matrices 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) or very high protein concentrations may give erroneous results. This assay will accurately quantitate insulin in human serum. Various types of plasma may result in under-recovery of insulin due to interference from anticoagulants. It is recommended to test each sample matrix for interference by diluting the 1250pg/mL standard 1 part to 4 parts of the matrix which does not contain any insulin. This diluted standard when assayed as an unknown should give a value of 200 to 300pg/mL. If this is not the case then standards may be made up in the actual sample matrix being tested. 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.
- 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.

- All standards, controls and samples should be assayed in duplicate. Samples which could contain very high levels of Insulin above the 1250pg/mL standard and in the "Hook" region of this assay, should also be assayed diluted.
- 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.
- 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 polluted. If this appears to be the case read 100µL of substrate against a water blank. If the absorbance is greater than 0.2 it may be necessary to obtain new substrate or the sensitivity of the assay may be compromised.

Assay Protocol

1. Pipette 100µL of standards, controls and samples into wells indicated on work list.

2. Pipette 100µL of anti-Insulin:Alkaline Phosphatase (#F281) into each well.

3. Cover & incubate on orbital shaker at 400-600 rpm for 3 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 PNPP substrate.

5. Pipette 100µL of PNPP substrate (#F008).

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

7. Read absorbance at 405/492nm.

Calculation of Results

The standards may be used to construct a standard curve with values reported in pg/mL or in µUnits/mL. The conversion factor for pg/mL to µUnits/mL is 0.03. 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.

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.

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 less than 1ng/mL may be greater than 10%.
- For optimal performance the absorbance of the substrate when blanked against water should be less than 0.4.
- It is recommended that each laboratory assay appropriate quality control samples in each run to ensure that all reagents and procedures are correct.

Example Data

Well #	Contents	Abs. at 405-490nm	Mean Abs.
A1	Zero Std	0.083	0.085
B1	Zero Std	0.087	
C1	25pg/mL	0.102	0.102
D1	25pg/mL	0.101	
E1	80pg/mL	0.153	0.152
F1	80pg/mL	0.150	
G1	200pg/mL	0.287	0.286
H1	200pg/mL	0.285	
A2	500pg/mL	0.633	0.632
B2	500pg/mL	0.631	
C2	1250pg/mL	1.595	1.622
D2	1250pg/mL	1.649	

Performance Characteristics

Sensitivity

The lower limit of detection (**LOD**) defined as that concentration corresponding to a signal two standard deviations above the mean of the zero standard is ~6pg/mL.

The lower limit of quantitation (**LOQ**) defined as that concentration where concentration coefficients of variation (CVs) are less than 20% is 25 pg/mL.

Precision

Both intra (n=12 replicates) and inter-assay (n=5 assays) precision were determined on 3 pools with low (48 pg/mL), medium (189.4 pg/mL), and high concentrations (932.1 pg/mL). The % CV is the standard deviation divided by the mean multiplied by 100.

Pool	Intra assay CV	Inter assay CV
Low	10.2%	6.3%
Medium	3.0%	3.5%
High	2.5%	1.7%

Recovery/ Interference Studies

Various buffer matrices were evaluated by adding known amounts of Insulin used to make the standards in this kit. These data serve as examples of certain buffers or buffer components which may or may not give matrix interference. Matrix interference can be either positive (false increase in insulin) or negative (false decrease in insulin). This assay has been designed to minimize matrix interference but it is strongly recommended that the user qualify that their sample matrices yield accurate recovery by performing a similar experiment. Such an experiment can be performed by diluting the 1250pg/mL standard provided with this kit into the sample matrix in question.

Sample Matrix Buffer	Average %Recovery Assayed/ added x100
Human Serum	97
Citrate/Sucrose/Arginine/Tween 80 with 1mg/mL Human IgG, pH 7.0	98
TRIS/Phosphate/Sucrose with 1mg/mL BSA, pH 7.5	87
TRIS buffer saline with 4mg/mL Human IgG, pH 7.2	70
TRIS buffer saline with 8mg/mL BSA	100

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 insulins have been reported to cross-react in the range of 50-70%. This kit can be used to quantitate rat and mouse insulin provided the laboratory has its own standards or can apply the appropriate cross-reactivity correction factor.

Cross Reactant	% Cross Reactivity
Bovine Insulin	100
Porcine Insulin	96
Human Insulin, natural	101
Human Insulin, recombinant	104

Hook Capacity

Very high concentrations of Insulin (up to 2μg/mL) were evaluated for the hook effect. At concentrations exceeding 2μg/mL, the apparent concentration of Insulin may read less than the 1250pg/mL standard. Samples yielding signals above the 1250pg/mL standard or suspected of having concentrations in excess of 2μg/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

- 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*:

www.cygnustechnologies.com
Cygnus Technologies, LLC
1523 Olde Waterford Way
Leland, NC 28451 USA
Tel: 910-454-9442
Fax: 910-454-9443
Email: techsupport@cygnustechnologies.com