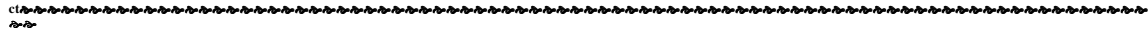




Insulin Assay-Ultra Sensitive

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 Of the Test

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 contaminants but it may still be necessary to determine trace contamination 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 contaminants. Efforts to reduce trace media contamination 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 contamination 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

<u>Component</u>	<u>Product #</u>
Mouse anti-Insulin	F281
alkaline phosphatase labeled	
Mouse monoclonal antibody conjugated to alkaline phosphatase in a protein matrix with preservative, 1x12mL	
Monoclonal Anti-Insulin coated microtiter strips	F042
12x8 well strips in a bag with desiccant	
Insulin Standards	F282
Bovine Insulin in a protein matrix with preservative. Standards at 0, 25,80,200,500 and 1250pg/mL. 1mL/vial	
PNPP Substrate	F008
p-nitrophenyl phosphate in a Diethanolamine buffer with preservative, 1x12mL	
Wash Concentrate (20X)	F004
Tris buffered saline with preservative, 1x50mL	

Storage & Stability

- * All reagents should be stored a 2°C to 8°C until the expiration date printed on the label. **Do not freeze!**
- * 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.

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 (150 - 200 rpm)

Sample Diluent (Recommend Cygnus Cat # I028)

Distilled water

1 liter wash bottle for diluted wash solution

Precautions

For research or manufacturing use only. At the concentrations used in this kit none of the reagents are believed to be harmful.

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, in a plastic wash/squirt bottle and label with kit lot and expiration date. 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 minus a substrate blank is greater than 0.10, evaluate plate washing procedure for proper performance.
2. When dilution of samples is required, dilution should be performed in a diluent validated 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 validated 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 contamination using this kit, each laboratory should validate that the kit and assay procedure utilized yield acceptable specificity, accuracy, and precision. A suggested protocol for this validation 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 (<6.0 and >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. Blank the instrument using the zero standard wells after assay completion.
- * 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 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 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.

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.

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.

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.

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).

3. Cover & incubate on rotator at ~ 180rpm for 3 hours at room temperature, 22°C ± 4°.

4. Dump the contents into waste or gently aspirate using a multi-channel pipetor. Blot and bang out residual liquid over low lint absorbance paper. Wash generously with diluted wash solution by flooding the wells with solution from a squirt bottle or by pipetting in ~350µL. Repeat for a total of 4 washes. Wipe off any liquid from the bottom outside of the wells as any residue can interfere in the reading step.

5. Pipette 100µL of PNPP substrate.

6. Incubate for 1 hour.

7. Read absorbance at 405/492nm blanking on the Zero standard.

Example Data

Well #	Contents	Abs. at 405nm	Mean Abs.	pg/mL Insulin
1A	Zero Std	0.083		
1B	Zero Std	0.087	0.082	
1C	25 pg/mL	0.102		
1D	25 pg/mL	0.101	0.102	
1E	80pg/mL	0.153		
1F	80pg/mL	0.150	0.152	
1G	200pg/mL	0.287		
1H	200pg/mL	0.285	0.291	
2A	500pg/mL	0.633		
2B	500pg/mL	0.631	0.632	
2C	1250pg/mL	1.595		
2D	1250pg/mL	1.649	1.622	
2E	sample 1	0.122		
2F	sample 1	0.119	0.120	45.7pg/mL
2G	sample 2	1.176		
2H	sample 2	1.151	1.163	902.4pg/mL

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 <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 validate 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

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

Technologies Customer Support:

Tel: 910-454-9442 Fax: 910-454-9443

Email: cygnustec@aol.com

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