Mouse Ultrasensitive Insulin ELISA

For the quantitative determination of insulin in mouse serum and plasma.

Please read carefully due to Critical Changes, e.g., Calculation of Results.

For Research Use Only. Not For Use In Diagnostic Procedures.

Catalog Number:	80-INSMSU-E01, E10
Size:	96 Wells, 10 x 96 Wells
Version:	November 05, 2014

26-G Keewaydin Drive, Salem, NH 03079 | P: (800) 592-5726 | F: (603) 898-6854 | ts@alpco.com | www.alpco.com

INTENDED USE

The ALPCO Mouse Ultrasensitive Insulin ELISA is designed for the quantitative determination of insulin in mouse serum and plasma.

This kit is designed for use with either a 5 µL or 25 µL sample size. The five standards and the two controls appropriate for the assay will be determined by the sample size utilized.

PRINCIPLE OF THE ASSAY

The ALPCO Mouse Ultrasensitive Insulin ELISA is a sandwich type immunoassay. The 96-well microplate is coated with a monoclonal antibody specific for insulin. The standards, controls, and samples are added to the microplate wells with the conjugate. The microplate is then incubated on a microplate shaker at 700-900 rpm. After the first incubation is complete, the wells are washed with Wash Buffer and blotted dry. TMB Substrate is added, and the microplate is incubated a second time on a microplate shaker at 700-900 rpm. Once the second incubation is complete, Stop Solution is added, and the optical density (OD) is measured by a spectrophotometer at 450 nm. The intensity of the color generated is directly proportional to the amount of insulin in the sample.

MATERIALS SUPPLIED

80-INSMSU-E01		
Component	**Quantity**	**Preparation**
Insulin Microplate (96 wells)	12 x 8 strips	Ready to use
Zero Standard	5 mL	Ready to use
Standards (A-G)* (0.025, 0.09, 0.188, 0.5, 1.25, 3.75, 6.9 ng/mL)	1 mL each	Ready to use
Control Levels 1, 2, and 3*	1 vial each	Lyophilized
Conjugate Stock	0.9 mL	11X
Conjugate Buffer	9 mL	Ready to use
Wash Buffer Concentrate	40 mL	21X
TMB Substrate	12 mL	Ready to use
Stop Solution	12 mL	Ready to use
Plate Sealers	3	Ready to use

*Please refer to the Certificate of Analysis enclosed with each kit for more information.

80-INSMSU-E10		
Component	**Quantity**	**Preparation**
Insulin Microplate (96 wells)	10 x (12 x 8 strips)	Ready to use
Zero Standard	5 mL	Ready to use
Standards (A-G)* (0.025, 0.09, 0.188, 0.5, 1.25, 3.75, 6.9 ng/mL)	1 mL each	Ready to use
Control Levels 1, 2, and 3*	1 vial each	Lyophilized
Conjugate Stock	9 mL	11X
Conjugate Buffer	90 mL	Ready to use
Wash Buffer Concentrate	2 x 200 mL	21X
TMB Substrate	120 mL	Ready to use
Stop Solution	120 mL	Ready to use
Plate Sealers	20	Ready to use

*Please refer to the Certificate of Analysis enclosed with each kit for more information.

MATERIALS REQUIRED

- Precision pipettes for dispensing 5, 25, 75, and 100 µL (with disposable tips)
- Repeating or multi-channel pipette for dispensing 75 and 100 µL
- Volumetric containers and pipettes for reagent preparation
- Distilled or deionized water for reagent preparation
- Microplate washer or wash bottle
- Microplate shaker capable of 700-900 rpm
- Microplate reader with 450 nm filter
- Vortex for sample preparation

PRECAUTIONS

1. The human blood products incorporated into this kit have been tested for the presence of HIV (human Immunodeficiency virus), HBV (Hepatitis B virus), and HCV (Hepatitis C virus). Test methods for these viruses do not guarantee the absence of a virus; therefore, all reagents should be treated as potentially infections. Handling and disposal should be in accordance with all appropriate national and local regulations for the handling of potentially biohazardous materials.
2. All materials derived from animal sources are BSE negative. However, all materials should be kept from ruminating animals.
3. Avoid direct contact with skin.
4. This product is not for internal use.
5. Avoid eating, drinking, or smoking when using this product.
6. Do not pipette any reagents by mouth.
7. Reagents from this kit are lot-specific and must not be substituted.
8. Do not use reagents beyond the expiration date.
9. Variations to the test procedure are not recommended and may influence the test results.

STORAGE CONDITIONS

The kit should be stored at 2-8°C. The kit is stable until the expiration date on the box label.

SAMPLE HANDLING

Serum and plasma samples are appropriate for use in this assay. No dilution or treatment of the sample is required. However, if a sample has a greater concentration of insulin than the highest standard, the sample should be diluted in Zero Standard and the analysis should be repeated.

In order to reduce potential time-associated sample drift, it is recommended to thoroughly vortex each sample before use and perform each pipetting action without pausing.

Samples can be stored at 2-8°C for 24 hours prior to analysis in this assay. For longer periods, storage at ≤ -20°C is recommended. Avoid repeated freeze/thaw cycles.

REAGENT PREPARATION

All reagents must be equilibrated to room temperature prior to preparation and subsequent use in the assay.

Conjugate Stock is to be diluted with 10 parts Conjugate Buffer. For example, to prepare enough Working Strength Conjugate for one complete microplate, dilute 0.9 mL of Conjugate Stock (11X) with 9 mL of Conjugate Buffer.

Wash Buffer Concentrate is to be diluted with 20 parts distilled water. For example, to prepare Working Strength Wash Buffer, dilute 20 mL of Wash Buffer Concentrate (21X) with 400 mL of deionized water. Working Strength Wash Buffer is stable for 4 weeks at room temperature (18-25°C).

Controls (Levels 1, 2, & 3) are provided in a lyophilized form. Please refer to the Certificate of Analysis provided with each kit for the appropriate volume of deionized water for reconstitution. Close the vial with the rubber stopper and cap, gently swirl the vial, and allow it to stand for 30 minutes prior to use. The contents of the vial should be in solution with no visible particulates. The reconstituted controls are stable for 7 days stored at 2-8°C. If desired, the controls can be stored at ≤ -20°C in aliquots for up to 6 months. The controls should not be repeatedly frozen and thawed.

QUALITY CONTROL

It is recommended that the Controls provided with the ALPCO Mouse Ultrasensitive Insulin ELISA be included in every assay. The concentration ranges of the controls are provided on the Certificate of Analysis provided with each kit; however, it is recommended that each laboratory establishes its own acceptable ranges.

ASSAY PROCEDURE

All reagents and microplate strips should be equilibrated to room temperature (18-25°C) prior to use. Gently mix all reagents before use. A standard curve must be performed with each assay run and with each microplate if more than one is used at a time. All standards, controls, and samples should be run in duplicate.

1. The microplate should be equilibrated to room temperature prior to opening the foil pouch. Designate enough microplate strips for duplicate determinations of the standards, controls, and samples. The remaining microplate strips should be stored at 2-8°C in the tightly sealed foil pouch containing the desiccant.

2. **Pipette 5 or 25 µL** of each standard, control, and sample into their respective wells. See *Reagent Preparation* and Certificate of Analysis for control reconstitution instructions.

5 µL sample size -Use standards 0.188, 0.5, 1.25, 3.75, & 6.9 ng/mL (C-G) and Control Levels 2 & 3.

25 µL sample size -Use standards 0.025, 0.09, 0.188, 0.5, & 1.25 ng/mL (A-E) and Control Levels 1 & 2.

3. **Pipette 75 µL** of Working Strength Conjugate (see *Reagent Preparation*) into each well.

4. Cover microplate with a plate sealer and **incubate for 2 hours** at room temperature, shaking at 700-900 rpm on a microplate shaker.

5. Decant the contents of the wells and **wash the microplate 6 times** with 350 µL of Working Strength Wash Buffer per well (see *Reagent Preparation*) using a microplate washer. Alternatively, fill the wells with Working Strength Wash Buffer using a wash bottle equipped with a wash nozzle. (It is not recommended to use a multichannel pipette. Wash buffer must be dispensed with adequate and equal force in order to properly wash the wells.) Between washes, invert the microplate to discard the liquid and firmly tap the inverted microplate on absorbent paper towels. After the final wash, (automated or manual), remove any residual Wash Buffer and bubbles from the wells by inverting and firmly tapping the microplate on absorbent paper towels.

6. **Pipette 100 µL** of TMB Substrate into each well.

7. Cover microplate with a plate sealer and **incubate for 30 minutes** at room temperature, shaking at 700-900 rpm on a microplate shaker.

8. **Pipette 100 µL** of Stop Solution into each well and gently shake the microplate to mix the contents. Remove any bubbles before proceeding with the next step.

9. Place the microplate in a microplate reader capable of reading the absorbance at 450 nm. The microplate should be analyzed immediately after the addition of the Stop Solution, and no longer than 30 minutes after.

CALCULATION OF RESULTS

Construct a standard curve from the standards. Plot the zero standard as part of the curve. It is recommended to use a software program to calculate the standard curve and to determine the concentration of the samples. A 5 parameter curve is recommended for data analysis.

The ALPCO Mouse Ultrasensitive Insulin ELISA is a ligand binding assay, with responses exhibiting a sigmoidal relationship to the analyte concentration. Currently accepted reference models for such curves use a 5 parameter logistic (pl) fit, as these models optimize the accuracy and precision across a greater range. Although cubic spline and other models are acceptable methods, they generally show less intra-assay accuracy and precision at the low and high ends of the range.

In the example below, a 5 pl curve fit was used to maximize the accuracy and precision of samples with low concentrations. However, the accuracy and precision of all models are limited at the lowest and highest ends of the detectable range due to the influence of individual laboratory conditions. As a result, caution should always be used when interpreting results where the analyte response becomes non-linear.[1]

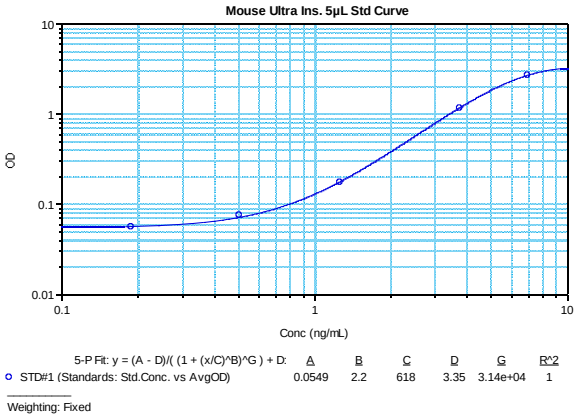
Extrapolating sample concentration values outside the range of the standard concentration values is not recommended.

TYPICAL STANDARD CURVE

The following results are provided for demonstration purposes only and cannot be used instead of data obtained with the assay. A standard curve must be performed with each assay run and plate tested.

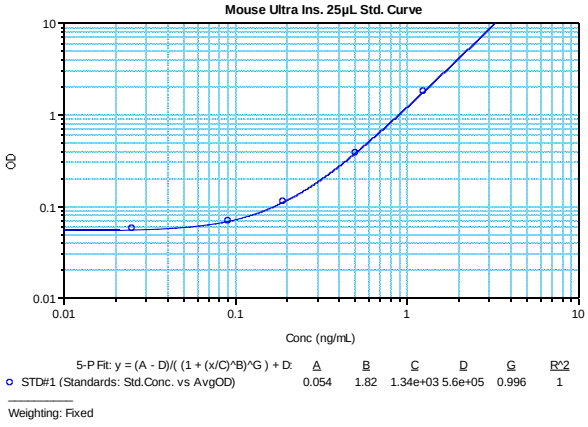
5 µL Sample Size Assay

Standard	Conc. (ng/mL)	OD
Zero	0	0.051
C	0.188	0.057
D	0.5	0.077
E	1.25	0.175
F	3.75	1.187
G	6.9	2.691



25 µL Sample Size Assay

Standard	Conc. (ng/mL)	OD
Zero	0	0.046
A	0.025	0.058
B	0.09	0.070
C	0.188	0.116
D	0.5	0.384
E	1.25	1.807



PERFORMANCE CHARACTERISTICS

Sensitivity

The analytical sensitivity was determined by calculating the mean + 2 standard deviations for 20 replicates of the Zero Standard. The sensitivity of the assay is 0.115 ng/mL (5 µL sample) and 0.019 ng/mL (25 µL sample).

Precision: Within run (intra-assay) variation

The within run precision is expressed as the percentage coefficient of variation (CV %). This was determined based on the mean and standard deviation of 20 replicates of a sample run in a single assay. The table below shows the results of 3 samples that span the range of the assay.

5 µL sample	**Sample 1**	**Sample 2**	**Sample 3**
Mean	0.44 ng/mL	1.41 ng/mL	4.24 ng/mL
Std. Dev.	0.041 ng/mL	0.064 ng/mL	0.209 ng/mL
CV %	9.30	4.53	4.93
n	20	20	20

25 µL sample	**Sample 1**	**Sample 2**	**Sample 3**
Mean	0.21 ng/mL	0.41 ng/mL	0.54 ng/mL
Std. Dev.	0.014 ng/mL	0.013 ng/mL	0.021 ng/mL
CV %	6.60	3.10	3.90
n	20	20	20

Precision: Between run (inter-assay) variation

The between run precision is expressed as the percentage coefficient of variation (CV %). This was determined based on the mean and standard deviation across 10 assays of duplicate measurements of a single sample. The table below shows the results of 3 samples that span the range of the assay.

5 µL sample	**Sample 1**	**Sample 2**	**Sample 3**
Mean	1.085 ng/mL	1.892 ng/mL	5.166 ng/mL
Std. Dev.	0.119 ng/mL	0.218 ng/mL	0.484 ng/mL
CV %	10.97	11.49	9.37
n	10	10	10

25 µL sample	**Sample 1**	**Sample 2**	**Sample 3**
Mean	0.214 ng/mL	0.416 ng/mL	0.558 ng/mL
Std. Dev.	0.012 ng/mL	0.023 ng/mL	0.032 ng/mL
CV %	5.83	5.55	5.7
n	10	10	10

Linearity

The linearity of the assay was determined by preparing dilutions of a sample with high insulin concentrations with the Zero Standard. The expected values were compared to the obtained values to determine a percent recovery. The average recovery was 107% (5 µL sample) and 82% (25 µL sample).

Spike and Recovery

The spike and recovery of the assay was determined by adding various known amounts of insulin to a sample. This spiked sample was evaluated in the assay and the measured concentration was compared to the expected concentration (endogenous + spiked). The range of recovery was 87-116 % with an average of 100% (5 µL sample) and 79-106% with an average of 96% (25 µL sample).

Specificity

The table below indicates the analyte and the percent cross-reactivity observed in the assay.

Analyte	**% Cross-reactivity**
Human insulin	147.0
Human C-peptide	<0.01
Human proinsulin (intact)	0.27
Humalog	153
Novolog	180
Humulin R	253
Humulin N	305
Lantus	109
Porcine insulin	147
Porcine C-peptide	Not detected
Mouse C-peptide 1	<0.01
Mouse C-peptide 2	<0.01
Rat C-peptide 1	<0.01
Rat C-peptide 2	<0.01
Human IGF-1	<0.01

Analyte	% Cross-reactivity
Human IGF-2	<0.01
Mouse IGF-1	<0.01
Mouse IGF-2	<0.01

Hook Effect
No high dose hook effect was observed with insulin concentrations up to 2,662 ng/mL (5 µL sample) and up to 2,048 ng/mL (25 µL sample).

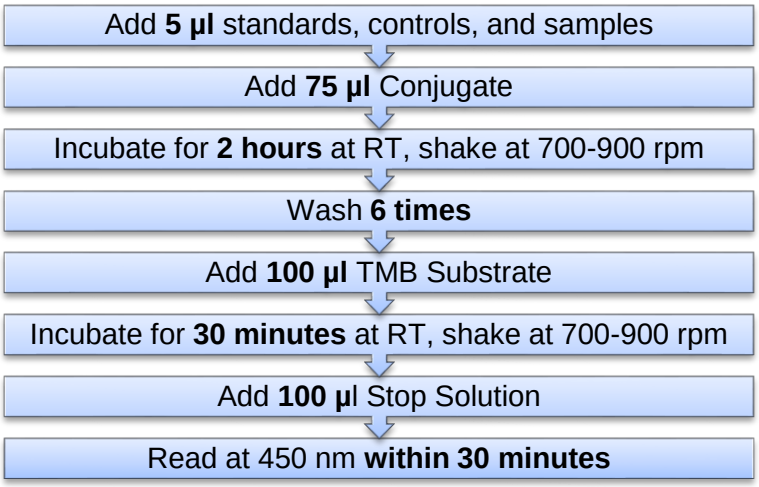
REFERENCES

1. Finlay JWA, Dillard RF. Appropriate Calibration Curve Fitting in Ligand Binding Assays. *AAPS Journal*. 2007; 9(2): E260-E267.

80-INSMSU-E01, E10

5 µL sample size

Standards 0.188, 0.5, 1.25, 3.75, & 6.9 ng/mL (C-G) and Control Levels 2 & 3.

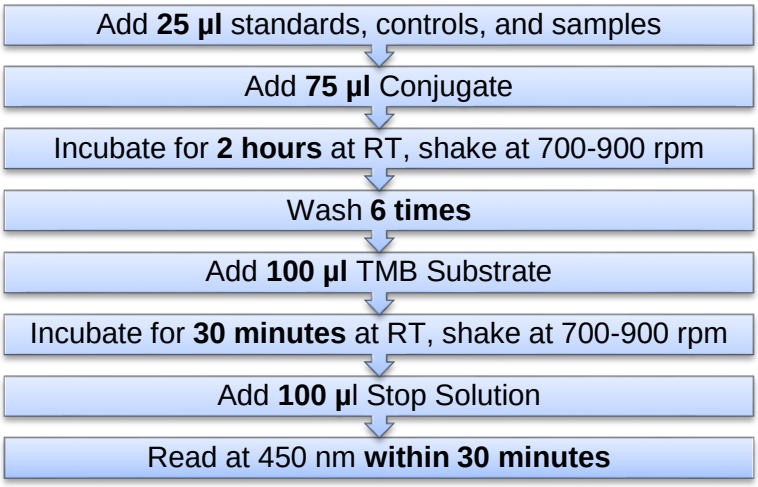


Total Time = 2 hours, 30 minutes

80-INSMSU-E01, E10

25 µL sample size

Standards 0.025, 0.09, 0.188, 0.5, & 1.25 ng/mL (A-E) and Control Levels 1 & 2.



Total Time = 2 hours, 30 minutes