Insulin Rodent (Mouse/Rat) Chemiluminescence ELISA

For the quantitative determination of insulin in rodent serum, plasma, and tissue culture supernatants.

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

Catalog Number:	**80-INSMR-CH01, CH10**
Size:	**96 Wells, 10 x 96 Wells**
Version:	**September 13, 2017**

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INTENDED USE

The Insulin Rodent (Mouse/Rat) Chemiluminescence ELISA is designed for the quantitative determination of insulin in rat and mouse serum, plasma, and tissue culture supernatants. Specifically, Rat Serum, Rat Heparin Plasma, Rat EDTA Plasma, Mouse Serum, Mouse Heparin Plasma, and Tissue culture supernatants may be used in this assay.

PRINCIPLE OF THE ASSAY

The Insulin Rodent (Mouse/Rat) Chemiluminescence 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 at room temperature on a microplate shaker at 700-900 rpm. After the incubation is complete, the wells are washed with Wash Buffer and blotted dry. Chemiluminescent Substrate is added, and the microplate is read by a luminescence plate reader after 1 minute. The intensity of the light generated is directly proportional to the amount of insulin in the sample.

MATERIALS SUPPLIED

80-INSMR-CH01		
Component	**Quantity**	**Preparation**
Insulin Microplate (96 wells)	12 x 8 strips	Ready to use
Zero Standard	5 mL	Ready to use
Standards (A-G) (0.1, 0.25, 0.5, 1, 10, 100, 150 ng/mL)	1 mL each	Ready to use
Control Levels Low, Mid, High*	1 vial each	Lyophilized*
Conjugate Stock	0.9 mL	11X
Conjugate Buffer	9 mL	Ready to use
Wash Buffer Concentrate	40 mL	21X
Substrate A	6 mL	Ready to use
Substrate B	6 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-INSMR-CH10		
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) (0.1, 0.25, 0.5, 1, 10, 100, 150 ng/mL)	1 mL each	Ready to use
Control Levels Low, Mid, High*	1 vial each	Lyophilized*
Conjugate Stock	9 mL	11X
Conjugate Buffer	90 mL	Ready to use
Wash Buffer Concentrate	2 x 200 mL	21X
Substrate A	60 mL	Ready to use
Substrate B	60 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 5-100 µL (with disposable tips)
- Repeating or multi-channel pipette for dispensing up to 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 luminometer

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 infectious. 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 treated as potentially infectious.
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.

10. **An appendix has been included with examples of instrument settings for reading a chemiluminescent output.** Each lab should optimize their instrument settings according to the manufacturer's instructions. Please contact the technical services department of the manufacturer of the microplate reader for optimal instrument settings.

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, plasma, and tissue culture supernatant 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.

It is recommended to 1) thoroughly vortex each sample before use and 2) perform pipetting actions 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 freezing/thawing of the sample.

REAGENT PREPARATION

All reagents must be equilibrated to room temperature prior to preparation and subsequent use in the assay. Prepare reagents according to the number of plates or strips to be used. Store the remaining concentrates at 2-8ºC.

Controls (Levels Low, Mid, High) 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. 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.

Conjugate Stock (11X) is to be diluted with 10 parts Conjugate Buffer. Working Strength Conjugate is stable for 4 weeks at 2-8°C.

Number of plates	Amount of Concentrate	Amount of Conjugate Buffer
0.5 (6 strips)	0.45 mL	4.5 mL
1 (12 strips)	0.90 mL	9.0 mL
2	1.8 mL	18 mL
5	4.5 mL	45 mL
10	9.0 mL	90 mL

Wash Buffer Concentrate (21X) is to be diluted with 20 parts distilled water. Working Strength Wash Buffer is stable for 4 weeks at room temperature.

NOTE: All labs should account for plate washer void volume to prime the machine. The values below **do not** account for priming automated plate washers.

Number of plates	Amount of Concentrate (6 x 350 µL wash)	Amount of dH_2O (6 x 350 µL wash)
0.5 (6 strips)	10 mL	200 mL
1 (12 strips)	15 mL	300 mL
2	25 mL	500 mL
5	65 mL	1300 mL
10	130 mL	2600 mL

Substrates A & B are provided individually and should be combined in equal parts to create the Working Chemiluminescent Substrate prior to use. The Working Chemiluminescent Substrate is stable for 1 hour at room temperature.

Number of plates	Amount of Substrate A	Amount of Substrate B
0.5 (6 strips)	3 mL	3 mL
1 (12 strips)	6 mL	6 mL
2	12 mL	12 mL
5	30 mL	30 mL
10	60 mL	60 mL

QUALITY CONTROL

It is recommended that the Controls provided with the Insulin Rodent (Mouse/Rat) Chemiluminescence 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 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. A suggested plate layout is provided.

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

a. Alternatively, fill the wells with Working Strength Wash Buffer using a wash bottle equipped with a wash nozzle or manual washer. (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.) Soak the wells for 1 minute. Invert the microplate to discard the liquid and firmly tap the inverted microplate on absorbent paper towels. Repeat the wash and soak procedure 2 more times, for a total of 3 washes.

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 Working Chemiluminescent Substrate (see *Reagent Preparation*) into each well.
7. Place the microplate in a microplate reader capable of reading the luminosity of the wells. The microplate should be analyzed at 1 minute after the addition of the Chemiluminescent Substrate, and no more than 20 minutes. The plate should be read using a 1 second integration time.

CALCULATION OF RESULTS

Construct a standard curve from the standards. 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 with $1/y^2$ weighting is recommended for data analysis.

The Insulin Rodent (Mouse/Rat) Chemiluminescence 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 4 or 5 parameter logistic (pl) fit, as these models optimize the accuracy and precision across a greater range.

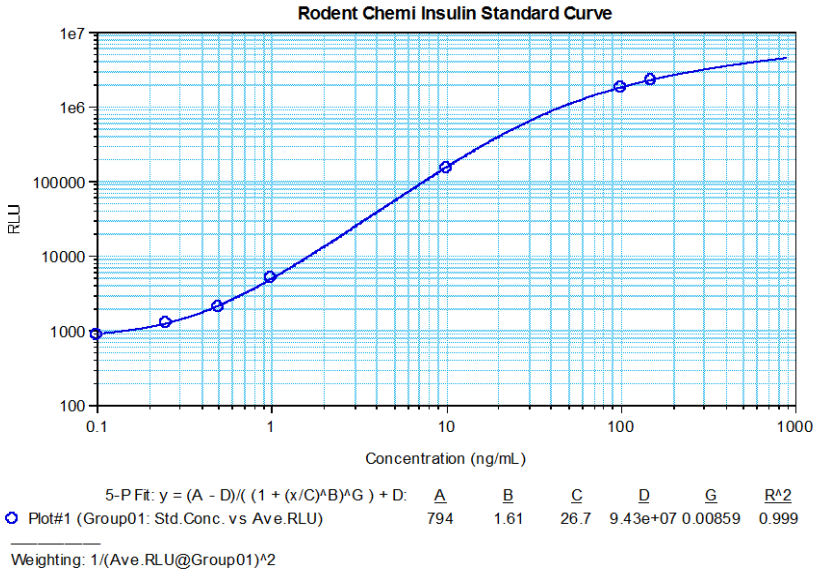
A 5 pl curve fit was used with $1/y^2$ weighting to maximize the accuracy and precision of samples with low concentrations. However, the accuracy and precision of all models are limited at the lowest and highest ends of the detectable range due to the influence of individual laboratory conditions. As 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 in place of data obtained with the assay. A standard curve must be performed with each assay run and plate tested. A 5-parameter curve fit with $1/y^2$ weighting is used for data analysis.

Standard	Conc. (ng/mL)	RLU
A	0.1	869
B	0.25	1277
C	0.5	2051
D	1	5044
E	10	151255
F	100	1801897
G	150	2265011
Zero	0	809



ASSAY DEVELOPMENT TIME

It is recommended to read the plate between 1-20 minutes after the addition of the substrate to the wells. Concentration values have been shown to have ≤ 5.1% CV from 1-30 minutes.

	Concentration [ng/mL]						
	1 minute	5 minutes	10 minutes	20 minutes	30 minutes	Average [ng/mL]	CV(%)
Sample 1	0.275	0.253	0.283	0.277	0.255	0.269	5.1
Sample 2	0.297	0.271	0.281	0.293	0.287	0.286	3.6
Sample 3	1.236	1.184	1.200	1.174	1.188	1.196	2.0
Sample 4	3.966	4.077	4.045	4.040	4.200	4.066	2.1
Sample 5	6.010	6.269	6.151	6.258	6.433	6.224	2.5
Sample 6	38.43	38.44	37.86	38.02	37.39	38.03	1.2
Sample 7	142.8	152.1	149.6	150.2	151.2	149.2	2.5

PERFORMANCE CHARACTERISTICS

Sensitivity

LOD (Limit of Detection) is defined as the highest value expected to see in a series of results on a sample that contains no analyte. The LOD of the assay is 0.089 ng/mL.

LOQ (Limit of Quantitation) is defined as the lowest concentration which meets precision and accuracy within 80 – 120% of the nominal concentration. The LOQ of the assay is 0.15 ng/mL.

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 16 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.

	Sample 1	Sample 2	Sample 3
Mean	0.233 ng/mL	4.005 ng/mL	124.9 ng/mL
CV (%)	11.4	7.4	11.1
n	16	16	16

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 2 assays of 32 measurements of a single sample. The table below shows the results of 3 samples that span the range of the assay.

	Sample 1	Sample 2	Sample 3
Mean	0.223 ng/mL	3.794 ng/mL	126.9 ng/mL
CV (%)	13.2	8.3	9.5
n	32	32	32

Linearity
The linearity of the assay was determined by preparing dilutions of the aforementioned validated samples in the Zero Standard. The expected values were compared to the obtained values to determine a percent recovery. The average recoveries fall within 80-120% of their corresponding expected values. The range of recovery for 27 samples is as follows:

Dilution	Average % Recovery	Min (%)	Max (%)
1:2	99	90	108
1:4	102	86	121
1:8	103	70	122

Spike and Recovery
The spike and recovery of the assay was determined by adding various known amounts of insulin to samples. The expected values were compared to the observed values to determine a percent recovery. All samples tested (serum, plasma, and tissue culture supernatants) recover within 80-120% of expected values. The range of recovery for 29 samples is as follows:

Spike Level	Mean % Recovery	Min (%)	Max (%)
Low	95	77	112
Mid	89	77	98
High	90	76	115

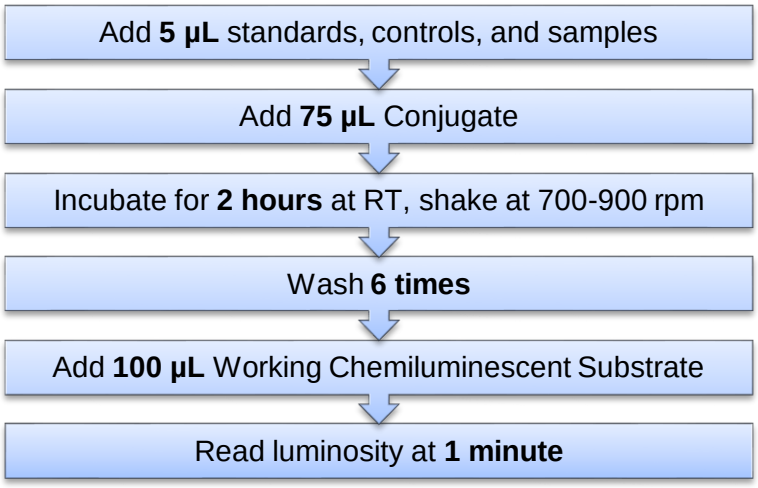
Specificity
The table below indicates the analyte and the percent cross-reactivity observed in the assay.

Analyte	% Cross-reactivity
Human insulin	120
Human C-peptide	< 0.01
Human proinsulin	0.18
Humalog	115
Novolog	140
Humulin R	200
Humulin N	229
Lantus	83.8
Porcine insulin	113
Mouse C-peptide 1	Not detected
Mouse C-peptide 2	<0.01
Rat C-peptide 1	<0.01
Rat C-peptide 2	<0.01
Human IGF-1	<0.01
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 1,536 ng/mL.

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

SHORT ASSAY PROTOCOL



Incubation Time = 2 hours, 1 minute

SUGGESTED PLATE LAYOUT

Below is a suggested plate layout for running standards, controls, and up to 36 samples in duplicate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std A	Std A	Ctrl Low	Ctrl Low	6	6	14	14	22	22	30	30
B	Std B	Std B	Ctrl Mid	Ctrl Mid	7	7	15	15	23	23	31	31
C	Std C	Std C	Ctrl High	Ctrl High	8	8	16	16	24	24	32	32
D	Std D	Std D	1	1	9	9	17	17	25	25	33	33
E	Std E	Std E	2	2	10	10	18	18	26	26	34	34
F	Std F	Std F	3	3	11	11	19	19	27	27	35	35
G	Std G	Std G	4	4	12	12	20	20	28	28	36	36
H	Zero	Zero	5	5	13	13	21	21	29	29	37	37

Std= Standard
Ctrl = Control
Numbered wells = Samples

APPENDIX

Instrument settings: Please contact the microplate reader manufacturer's technical services department for additional assistance. These instrument settings are to be used as a guideline. It is optional to shake the plates before reading for ≤ 3 seconds.

Molecular Devices Spectramax L
Read Mode: Luminescence
Integration Time: 1 second (1000 ms)
Sensitivity:
PMT: MaxRange
Target Calibration Wavelength: 470 nm
Automix: Classic: 30 mm/s
Automix before read: Off
Plate Type: 96 well standard
Injection and Delay: Off
Injection wells: None
Dark Adapt: Off
AutoRead: Off

Molecular Devices Spectramax M5
Read Mode: Luminescence (LUM)
Read Type: Endpoint
Wavelength: All
Plate Type: 96 well standard opaque
Read Area: Variable based on experiment
PMT and Optics: Integration Time 1000 ms
Shake: Off
More Settings: Calibrate (on); Carriage Speed (Normal); Read Order (Column); Setting Time (off)

Biotek Synergy2
Detection Method: Luminescence
Read Type: Endpoint
Integration: 0:01.00 (MM:SS.ss) (1 second)
Emission: Hole
Optics Position: Top
Sensitivity: 150
Top Probe Vertical Offset: 1.00mm

Tecan Infinite 200
Plate: Corning 96 Flat Bottom Black Polystyrol
Mode: Luminescence
Attenuation: NONE
Integration Time: 1000 ms
Settle Time: 0 ms