Version 4 Last updated 7 September 2018

ab200011
Insulin Human
SimpleStep ELISA® Kit

For the quantitative measurement of Insulin in human serum and plasma (heparin).

This product is for research use only and is not intended for diagnostic use.

Copyright © 2018 Abcam. All rights reserved

Table of Contents

1. Overview 1
2. Protocol Summary 2
3. Precautions 3
4. Storage and Stability 3
5. Limitations 4
6. Materials Supplied 4
7. Materials Required, Not Supplied 5
8. Technical Hints 6
9. Reagent Preparation 7
10. Standard Preparation 8
11. Sample Preparation 9
12. Plate Preparation 10
13. Assay Procedure 11
14. Calculations 13
15. Typical Data 14
16. Calibration 15
17. Typical Sample Values 15
18. Assay Specificity 18
19. Species Reactivity 19
20. Troubleshooting 20
21. Notes 21

Technical Support 22

Copyright © 2018 Abcam. All rights reserved

1. Overview

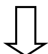
Abcam's Insulin in vitro SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of Insulin protein in human serum and plasma (heparin).

The SimpleStep ELISA® employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well. To perform the assay, samples or standards are added to the wells, followed by the antibody mix. After incubation, the wells are washed to remove unbound material. TMB substrate is added and during incubation is catalyzed by HRP, generating blue coloration. This reaction is then stopped by addition of Stop Solution completing any color change from blue to yellow. Signal is generated proportionally to the amount of bound analyte and the intensity is measured at 450 nm. Optionally, instead of the endpoint reading, development of TMB can be recorded kinetically at 600 nm.

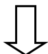
Insulin is a highly conserved, secreted hormone essential for glucose metabolism. Produced by pancreatic beta cells, proinsulin is proteolyzed into an A and a B chain, which form a 6 kDa mature protein. Basal levels of insulin are continuously delivered into the bloodstream, and additional levels are secreted proportional to food ingestion. Insulin secretion is highly regulated, and dysregulation of insulin production or sensitivity results in Type 1 diabetes mellitus or Type 2 diabetes mellitus, respectively.

2. Protocol Summary

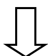
Remove appropriate number of antibody coated well strips. Equilibrate all reagents to room temperature. Prepare all reagents, samples, and standards as instructed.



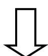
Add standard or sample to appropriate wells.



Add Antibody Cocktail to all wells. Incubate at room temperature.



Aspirate and wash each well. Add TMB Substrate to each well and incubate.



Add Stop Solution at a defined endpoint. Alternatively, record color development kinetically after TMB substrate addition.

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at +4°C immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage Condition
10X Human Insulin Capture Antibody	600 µL	4°C
10X Human Insulin Detector Antibody	600 µL	4°C
Human Insulin Lyophilized Recombinant Protein	2 Vials	4°C
Antibody Diluent 5BI	6 mL	4°C
10X Wash Buffer PT	20 mL	4°C
TMB Substrate	12 mL	4°C
Stop Solution	12 mL	4°C
Sample Diluent NS	50 mL	4°C
Pre-Coated 96 Well Microplate (12 x 8 well strips)	96 Wells	4°C
Plate Seal	1	4°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring absorbance at 450 or 600 nm.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- PBS (1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, 140 mM NaCl, 2.7 mM KCl, pH 7.4).
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.
- Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

8. Technical Hints

- This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Make sure you have the right type of plate for your detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on before starting the experiment.
- Complete removal of all solutions and buffers during wash steps is necessary to minimize background.
- As a guide, typical ranges of sample concentration for commonly used sample types are shown below in Sample Preparation.
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples.
- Incubate ELISA plates on a plate shaker during all incubation steps.
- When generating positive control samples, it is advisable to change pipette tips after each step.
- The provided Antibody Diluents and Sample Diluents contain protease inhibitor aprotinin. Additional protease inhibitors can be added if required.

- To avoid high background always add samples or standards to the well before the addition of the antibody cocktail.

9. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells. The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.
- Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the provided 10X formulations.

9.1 1X Wash Buffer PT

Prepare 1X Wash Buffer PT by diluting 10X Wash Buffer PT with deionized water. To make 50 mL 1X Wash Buffer PT combine 5 mL 10X Wash Buffer PT with 45 mL deionized water. Mix thoroughly and gently.

9.2 Antibody Cocktail

Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent 5BI. To make 3 mL of the Antibody Cocktail combine 300 µL 10X Capture Antibody and 300 µL 10X Detector Antibody with 2.4 mL Antibody Diluent 5BI. Mix thoroughly and gently.

10. Standard Preparation

- Prepare serially diluted standards immediately prior to use.
- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.
- The following section describes the preparation of a standard curve for duplicate measurements (recommended).

10.1 Reconstitute the human Insulin protein standard sample by adding 100 µL water by pipette. Mix thoroughly and gently. Hold at room temperature for 10 minutes and mix gently. This is the 1,002 pmol/L **Stock Standard** Solution.

10.2 Label eight tubes, standards 1-8.

10.3 Add 390 µL Sample Diluent NS into **Standard #1** and 150 µL Sample Diluent NS into **Standard #2-8.**

10.4 To prepare **Standard #1**, add 60 µL of the **Stock Standard** into tube #1 and mix gently.

10.5 To prepare **Standard #2**, add 150 µL of the **Standard #1** into tube #2 and mix gently.

10.6 Repeat for tubes #3 through #8.

10.7 Using the table below as a guide, prepare subsequent serial dilutions. Standard #8 contains no protein and is the Blank control.

Standard #	Volume to dilute (µL)	Volume Diluent N (µL)	Human Insulin (pmol/L)
1	Stock	60	133.6
2	150 µL Standard #1	150	66.8
3	150 µL Standard #2	150	33.4
4	150 µL Standard #3	150	16.7
5	150 µL Standard #4	150	8.4
6	150 µL Standard #5	150	4.2

7	150 µL Standard #6	150	2.1
8 (Blank)	N/A	150	0

11. Sample Preparation

TYPICAL SAMPLE DYNAMIC RANGE	
Sample Type	**Range (%)**
Human Serum	3 – 50
Human Plasma – Heparin	3 – 50

11.1 Plasma:

Collect plasma using heparin. Centrifuge samples at 2,000 x *g* for 10 minutes. Dilute samples into Sample Diluent NS and assay. Store un-diluted plasma samples at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

11.2 Serum:

Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2,000 x *g* for 10 minutes and collect serum. Dilute samples into Sample Diluent NS and assay. Store un-diluted serum at -20°C or below. Avoid repeated freeze-thaw cycles.

12. Plate Preparation

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused plate strips should be immediately returned to the foil pouch containing the desiccant pack, resealed and stored at 4°C.
- For each assay performed, a minimum of two wells must be used as the zero control.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Differences in well absorbance or "edge effects" have not been observed with this assay.

13. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.

13.1 Prepare all reagents, working standards, and samples as directed in the previous sections.

13.2 Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4°C storage.

13.3 Add 50 µL of all sample or standard to appropriate wells.

13.4 Add 50 µL of the Antibody Cocktail to each well.

13.5 Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.

13.6 Wash each well with 3 x 350 µL 1X Wash Buffer PT. Wash by aspirating or decanting from wells then dispensing 350 µL 1X Wash Buffer PT into each well. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and blot it against clean paper towels to remove excess liquid.

13.7 Add 100 µL of TMB Substrate to each well and incubate for 10 minutes in the dark on a plate shaker set to 400 rpm.

13.8 Add 100 µL of Stop Solution to each well. Shake plate on a plate shaker for 1 minute to mix. Record the OD at 450 nm. This is an endpoint reading.

Alternative to 13.7 – 13.8: Instead of the endpoint reading at 450 nm, record the development of TMB Substrate kinetically. Immediately after addition of TMB Development Solution begin recording the blue color development with elapsed time in the microplate reader prepared with the following settings:

Mode:	Kinetic
Wavelength:	600 nm
Time:	Up to 15 min
Interval:	20 sec – 1 min
Shaking:	Shake between readings

Note that an endpoint reading can also be recorded at the completion of the kinetic read by adding 100 µL Stop Solution to each well and recording the OD at 450 nm.

13.9 Analyze the data as described below.

14. Calculations

14.1 Calculate the average absorbance value for the blank control (zero) standards. Subtract the average blank control standard absorbance value from all other absorbance values.

14.2 Create a standard curve by plotting the average blank control subtracted absorbance value for each standard concentration (y axis) against the target protein concentration (x axis) of the standard. Use graphing software to draw the best smooth curve through these points to construct the standard curve.

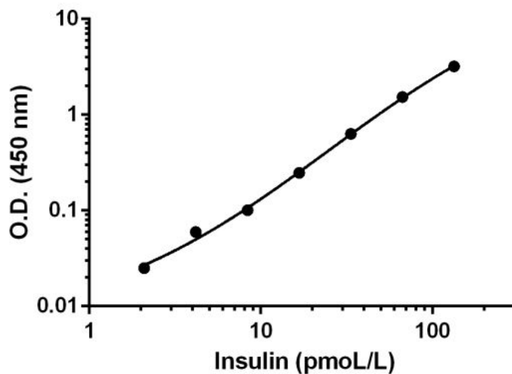
ΔNote: *Most microplate reader software or graphing software will plot these values and fit a curve to the data. A four parameter curve fit (4PL) is often the best choice; however, other algorithms (e.g. linear, semi-log, log/log, 4 parameter logistic) can also be tested to determine if it provides a better curve fit to the standard values.*

14.3 Determine the concentration of the target protein in the sample by interpolating the blank control subtracted absorbance values against the standard curve. Multiply the resulting value by the appropriate sample dilution factor, if used, to obtain the concentration of target protein in the sample.

14.4 Samples generating absorbance values greater than that of the highest standard should be further diluted and reanalyzed. Similarly, samples which measure at an absorbance values less than that of the lowest standard should be retested in a less dilute form.

15. Typical Data

Typical standard curve – data provided **for demonstration purposes only**. A new standard curve must be generated for each assay performed.



Standard Curve Measurements			
Conc. (pmol/L)	O.D. 450 nm		Mean O.D.
	1	2	
0	0.114	0.128	0.121
2.1	0.139	0.134	0.136
4.2	0.173	0.160	0.167
8.4	0.224	0.221	0.222
16.8	0.365	0.371	0.368
33.4	0.759	0.75	0.754
66.8	1.673	1.632	1.652
133.6	3.313	3.328	3.320

Figure 1. Example of Insulin standard curve. The Insulin standard curve was prepared as described in Section 10. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.

16. Calibration

This immunoassay is calibrated against a highly purified human Insulin. The NIBSC/WHO unclassified purified human Insulin preparation 83/500 was evaluated in this kit.

The dose response curve of the unclassified standard Insulin parallels the SimpleStep standard curve. To convert sample values obtained with the SimpleStep human Insulin kit to approximate NIBSC 83/500 units, use the equation below.

NIBSC (83/500) approximate value (IU/mL) = 0.291×10^{-6} x SimpleStep human Insulin value (pmol/L)

A molecular weight of 5807.67 Da was used to convert insulin from picograms to picomoles. The conversion factor of 0.172 was used to convert values from pg/mL to pmol/L.

17. Typical Sample Values

SENSITIVITY –

The calculated minimal detectable dose (MDD) is 1.1 pmol/L. The MDD was determined by calculating the mean of zero standard replicates (n=25) and adding 2 standard deviations then extrapolating the corresponding concentration.

RECOVERY –

Three concentrations of Insulin were spiked in duplicate to the indicated biological matrix to evaluate signal recovery in the working range of the assay.

Sample Type	Average % Recovery	Range (%)
25% Human Serum	106	103-111
25% Human Plasma - Heparin	112	108-120

Linearity of Dilution

Linearity of dilution is determined based on interpolated values from the standard curve. Linearity of dilution defines a sample concentration interval in which interpolated target concentrations are directly proportional to sample dilution.

Native Insulin was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS.

Dilution Factor	Interpolated value	12.5 % Human Serum	12.5% Human Plasma (Heparin)
Undiluted	pmol/L	31.3	36.5
	% Expected value	**100**	**100**
2	pmol/L	13.3	18.8
	% Expected value	**85**	**103**
4	pmol/L	5.96	9.1
	% Expected value	**76**	**100**
8	pmol/L	3.27	4.5
	% Expected value	**83**	**98**
16	pmol/L	2.32	**NL**
	% Expected value	**118**	**NL**

NL - Non Linear

PRECISION –

Mean coefficient of variations of interpolated values from 3 concentrations of Insulin within the working range of the assay.

	Intra-Assay	Inter-Assay
n=	5	3
CV (%)	8.6	4.9

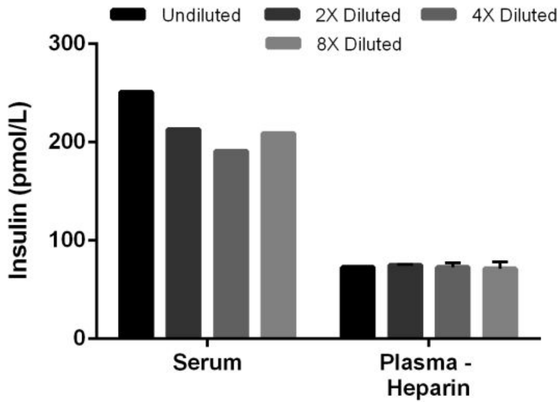


Figure 2. Titration of human serum and human plasma within the working range of the assay. Data shown for serum is from an individual donor with high endogenous insulin levels. Data shown for plasma is from a pooled (n=50) sample. Background-subtracted data values (mean +/- SD, n = 3) are graphed.

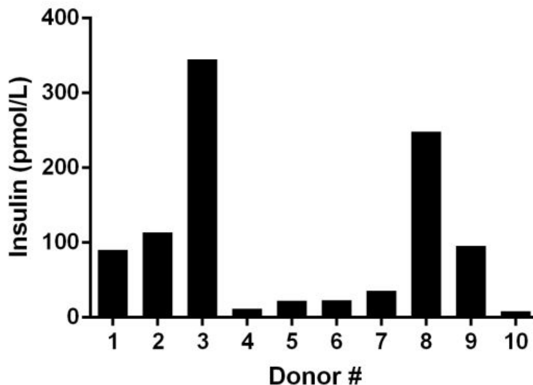


Figure 3. Insulin levels in individual healthy donors. Ten individual healthy donors were evaluated for the presence of Insulin in serum using this assay. The range was from 11.8 – 345.1 pmol/L, with an average of 99.4 pmol/L. Health history and dietary status of donors were unknown.

18. Assay Specificity

This kit recognizes both native and recombinant human Insulin protein in serum and heparin plasma samples only.

This kit cross reacts with the Proinsulin form <18%.

Cell and tissue extract samples have not been tested with this kit.

CROSS REACTIVTY –

The following proteins were prepared at 50 ng/mL and assayed for cross reactivity. No cross-reactivity was observed.

- Insulin Receptor

INTERFERENCES –

The following protein were prepared at 50 ng/mL and assayed for assay interference in the presence of 500 pg/mL Insulin. No interference was observed.

- Insulin Receptor

19. Species Reactivity

This kit recognizes human Insulin protein.

Other species reactivity was determined by measuring 50% serum samples of various species, interpolating the protein concentrations from the human standard curve, and expressing the interpolated concentrations as a percentage of the protein concentration in human serum assayed at the same dilution.

Reactivity < 5% was determined for the following species:

- Guinea Pig
- Sheep
- Dog
- Goat

This kit is not suitable for use in the following species: mouse, rabbit, bovine, pig, hamster and rat.

Please contact our Technical Support team for more information.

20. Troubleshooting

Problem	Reason	Solution
Poor standard curve	Inaccurate Pipetting	Check pipettes
	Improper standard dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing.
Low signal	Incubation times too brief	Ensure sufficient incubation times; increase to 2 or 3 hour standard/sample incubation.
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation.
	Incubation times with TMB too brief	Ensure sufficient incubation time until blue color develops prior addition of Stop solution.
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions.
	Contaminated wash buffer	Prepare fresh wash buffer.
Low sensitivity	Improper storage of the ELISA kit	Store your reconstituted standards at -80°C, all other assay components 4°C. Keep TMB substrate solution protected from light.
Precipitate in Diluent	Precipitation and/or coagulation of components within the Diluent.	Precipitate can be removed by gently warming the Diluent to 37°C.

21. Notes

Technical Support

Copyright © 2018 Abcam, All Rights Reserved. The Abcam logo is a registered trademark. All information / detail is correct at time of going to print.

Austria
wissenschaftlicherdienst@abcam.com | 019-288-259
France
supportscientifique@abcam.com | 01.46.94.62.96
Germany
wissenschaftlicherdienst@abcam.com | 030-896-779-154
Spain
soportecientifico@abcam.com | 91-114-65-60

Switzerland
technical@abcam.com
Deutsch: 043-501-64-24 | Français: 061-500-05-30
UK, EU and ROW
technical@abcam.com | +44(0)1223-696000

Canada
ca.technical@abcam.com | 877-749-8807
US and Latin America
us.technical@abcam.com | 888-772-2226

Asia Pacific
hk.technical@abcam.com | (852) 2603-6823
China
cn.technical@abcam.com | +86-21-5110-5938 | 400-628-6880
Japan
technical@abcam.co.jp | +81-(0)3-6231-0940
Singapore
sg.technical@abcam.com | 800 188-5244

Australia
au.technical@abcam.com | +61-(0)3-8652-1450
New Zealand
nz.technical@abcam.com | +64-(0)9-909-7829