SHIBAYAGI	Dog Insulin ELISA Kit (TMB)	Research Reagent

Please, read this instruction carefully before use.

[Advantage]

(1) Dog Insulin Kit is a high speed EIA. (for 3-4 hours).

(2) Dog Insulin Kit can measure in serum or plasma* samples (10μl) of very small volume.

(3) Dog Insulin Kit ensures simple assay procedures.

*We recommend the use of heparin to obtain plasma.

[Reagents]

A:	Anti-Insulin-coated plate	96well(8x12)	x1
B:	Standard Dog insulin solution (240ng/ml)	25μl	x1
C:	Buffer solution	60ml	x1
D:	Biotin-conjugated anti-insulin	10μl	x1
E:	HRP-conjugated streptavidin	20μl	x1
F:	Chromogenic substrate reagent (TMB)	12ml	x1
H:	Reaction stopper (1M H_2SO_4)	12ml	x1
I:	Concentrated washing buffer (10x)	100ml	x1

[Preparation of reagents]

*Reagent solutions should be used immediately after preparation.

- Standard solutions of insulin
  Prepare standard solutions by dilution as shown by an example below.
- Biotin-conjugated anti-insulin
  Prepare by dilution of (D) with the buffer(C) to 1:4000.
- HRP-conjugated streptavidin
  Prepare by dilution of (E) with the buffer(C) to 1:2000.
- Chromogenic substrate solution (F)
  Use the original solution without dilution.
- Reaction stopper (H)

Use the original solution without dilution.

- Washing buffer

Prepare by dilution of concentrated buffer (I) with purified water to 1:10.

Preparation of standard insulin solutions (an example)

Insulin conc. (pg/ml)	12000	6000	3000	1500	750	375	188	0
Standard insulin (μl)	10*	100**	100**	100**	100**	100**	100**	0
Buffer solution (μl)	190	100	100	100	100	100	100	100

*Original standard insulin solution **One rank higher standard solution

As an example shown above, first prepare 10000pg/ml from attached original standard solution, and then, by serial dilution, prepare 5000pg/ml, 2500pg/ml, and so on.

For the zero standard, use buffer solution alone.

Please, avoid using NaF-containing blood sampling tube, because fluoride ion is a peroxidase inhibitor, and may reduce the coloration even after washing.

[Assay Procedure]

01) Rinse the anti-insulin coated plate (A) 4 times with washing buffer(I).

02) Pipette 100μl of Biotin-conjugated anti-insulin (D) into each well, and shake.

03) Pipette 10μl sample or standard Insulin solution (B) into each well and shake.

04) Incubate for 2hours at room temperature (20-25C).

05) Rinse the plate 4 times with washing buffer (I).

06) Pipette 100μl of HRP-conjugated streptavidin solution(E) into each well and shake.

07) Incubate for 30 minutes at room temperature (20-25C).

08) Rinse the plate 4 times with washing buffer (I).

09) Pipette 100μl of chromoqenic substrate reagent (F) into each well and shake.

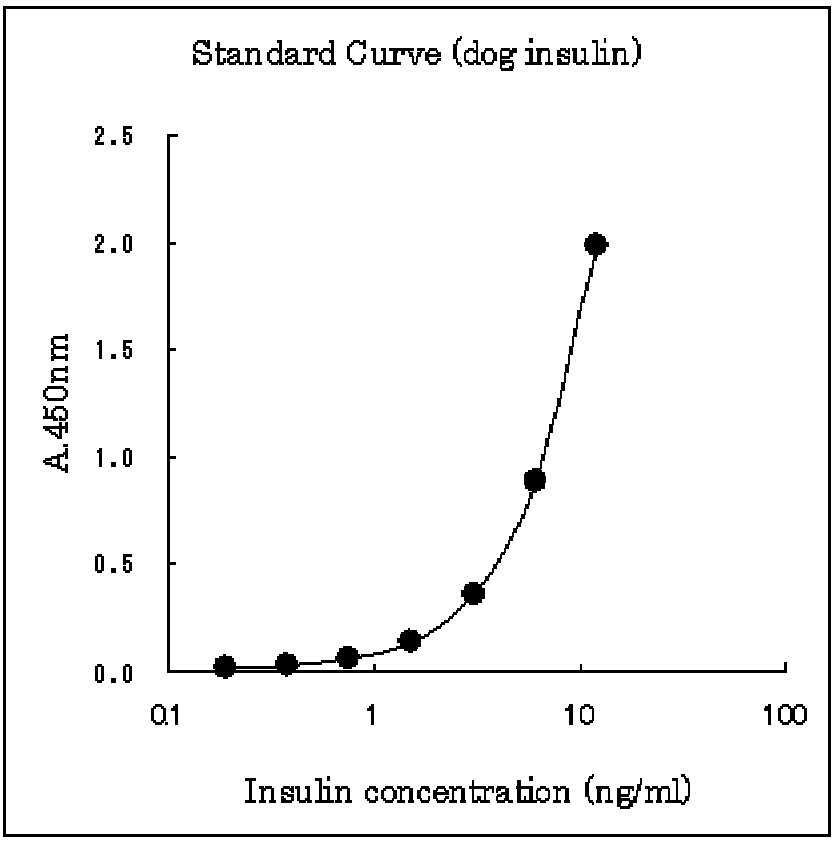
10) Incubate for 30 minutes at room temperature (20-25C).

11) Pipette 100μl of Reaction stopper (H) into each well and shake.

12) Measure each well's absorbance at 450 nm by the plate reader within 30 minutes.

[Calculation of Insulin Concentration]

1. Using hemi-logarithmic section paper, prepare a standard curve by plotting absorbance(Y-axis) against logarithm of insulin concentration (X-axis, ng/ml).
2.Using the standard curve, read the insulin concentration of a sample from their absorbance.
3. In case sample plasma is diluted, then multiply the concentration by sample dilution rate to obtain the insulin concentration of the original sample.



[Summary of Assay Procedure]

Antibody-coated plate
Washing
| +Biotin-conjugated anti-insulin 100µl, and shaking
| +sample or standard Insulin solution 10µl
Shaking , Reaction at room temp.(20 - 25C) for 2hr
|
Washing
| +HRP-avidin; 100µl
Shaking , Reaction at room temp.(20 - 25C) for 30mins
Washing
| + Chromogenic substrate solution; 100µl
Shaking , Reaction at room temp.(20 - 25C) for 30mins
| + Reaction stopper; 100µl
Shaking , Measurement of Absorbance(450nm)

[Precision and Reproducibility]

Precision (Within assay variation) : Average C.V.: 2.80 %

Reproducibility (Between assay variation) : Average C.V.: 4.17 %)

[Storage Condition]

2-8C, in a dark place. Do not freeze.

[Statements and Precautions]

01. This assay kit or its components should be used only for research works.
02. The reagent solutions of the kit should be used principally immediately after dilution. Otherwise, keep them in a dark place at 2-8℃, and use them within 5 days.
03. The reagents were prepared to give accurate results by their combination within the kit. So, do not combine the reagents in the kit of other lot number. Even the lot number is the same, do not mix the reagents with those that are preserved for some period.
04. Pipetting and dilution of the reagent solutions should be made accurately because these steps influence the assay precision.
05. Do not dry the assay plate to avoid denaturation of the coated antibody or antigen.
06. The reaction time should be counted from the onset of reagent pipetting.
07. Prepare the standard curve in every assay. (For kits with standard solution.)
08. Dilution of the assay sample must be carried out using the buffer solution attached to the kit.
09. Preservation condition for the kit or its components should be strictly kept.
10. ***Be careful not to allow the reagent solutions of the kit to contact with skin, mucus and eyes (wearing glasses for protection is recommended). Especially treat the stopping solution very carefully because it contains sulfuric acid.***
11. HRP-conjugated reagent solution, chromogenic substrate solution, and reaction stopper should be avoided from contacting with any metal.
12. ***In treating assay samples of animal origin, be careful for possible biohazards.***

Please, refer "All about Shibayagi's insulin kits" for further information.

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Reference report

This is an assay report for dog plasma insulin levels following intravenous glucose tolerance tests using normal, IDDM, and NIDDM animals, showing the usefulness of SHIBAYAGI's assay kit.

(Translated into English by K. Wakabayashi, Ph. D.)

Evaluation of an Insulin Assay Kit By Using Anti-Canine Insulin Antibody

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Abstract: Assay performance and clinical application of a highly sensitive ELISA kit for dog insulin, Lbis Insulin Kit (Shibayagi) in which anti-canine antibody was used, were evaluated using IVGTT (intravenous glucose tolerance test) samples obtained from normal, IDDM, and NIDDM dogs. The high sensitivity and the high reproducibility of the kit were confirmed, and differences among the changes in blood insulin levels of these animals were clearly observed. These results indicate the validity of the assay kit.

Introduction

Recently, owing to improvement in the environmental condition of pet animals, occurrence of diabetes has been increasing. Formerly, most of the dogs suspected to be diabetic were IDDM (insulin dependent diabetes mellitus) type. But now we very often encounter NIDDM dogs due to the advance of the diagnosis, clarification of obesity, insulin resistance and abnormal glucose tolerance in the luteal phase, and improvement of the knowledge of the pet owners.

We perform IVGTT (intravenous glucose tolerance test) for the diagnosis and the decision of treatment strategy (insulin therapy or regimen). In this case, we make it a role not only to measure blood glucose and estimate its half-life, but also to measure blood insulin simultaneously to find out insulin resistance and possibility of getting out of insulin therapy from the data concerning to capability of insulin secretion and to insulin secretory responsiveness. However, the assay systems for blood insulin used in diagnosis centers are ELISA with anti-human insulin antibody, and owing to the low cross-reactivity, the assay values obtained are too low, making the evaluation of the insulin response very difficult. The kits recently became available as laboratory animal insulin assay kits are for rat and mouse, and also with low cross-reactivity to dog insulin.

A new highly sensitive assay kit for dog insulin was developed and announced by Shibayagi Co., Ltd., Shibukawa, Japan in which they use anti-canine insulin antibody. We present here a trial report of the kit for its assay performance and usefulness for clinical application.

Materials and Methods

1. Animals

Three IDDM dogs induced by streptozotocin treatment in the Department of Internal Medicine of the School of Veterinary Medicine, 2 NIDDM dogs and 1 NIDDM dog diagnosed to be NIDDM related to estrous state in the Animal Hospital.

2. Blood sampling

IVGTT was carried out on these dogs with a dose of 0.5g/kg of glucose. Blood samples were obtained before and 5, 10, 15, 25, 35, 45, 60 and 90 minutes after the injection. The samples were added with heparin Na, and plasma samples were separated for glucose and insulin assays. From the decay curves of plasma glucose plotted against time on the semi-logarithmic paper, the half-lives were calculated.

3. Insulin assay kit

We used Lbis Insulin Kit for Dog (Shibayagi). This is an ELISA kit using monoclonal antibodies with characteristics of 1) rapid assay (3-4hrs), 2) small volume of sample (10 μl of serum or plasma), 3) simple procedure without any pretreatment of samples.

4. Basic validity tests

The assay standard curve, assay precision (within assay variability, n=8), reproducibility (between assay variability, n=5) were examined, and a recovery test was carried out.

5. Assay of the IVGTT samples

The plasma samples obtained by IVGTT were assayed for insulin as follows.

The antibody-coated plates were rinsed 4 times with a washing buffer, and 100 μl of biotin-conjugated anti-insulin solution were placed in each well, then 10 μl of standard solution or sample was added and shaken. After 2 hours' standing at room temperature, the plates were washed 4 times with the washing buffer, then 100 μl of HRP-conjugated streptavidin solution was added to each well and shaken, and allowed to react for 30 minutes at room temperature. The plates were then rinsed 4 times with the washing buffer, and 100 μl of chromogenic substrate reagent was added and shaken. After 30 minutes' standing, the reaction was stopped by adding 100 μl of stopping solution, and the absorbance of each well was measured at 450nm in a plate reader within 30 minutes.

Insulin concentration was calculated from a standard curve using a semi-logarithmic paper obtained by plotting the absorbance against the logarithm of the standard insulin concentration.

6. Assay of the low level samples

Samples contained insulin at very low levels out of the ordinary rage of the standard curve were treated as follows.

One hundred μl of the sample was mixed with the same volume of the assay buffer, then 50 μl of the mixture was added to the well. The assay value obtained was divided by a correction factor 1.8 (=2.5x110 μl/150 μl) for the insulin

concentration in the original sample. We also examined the correlation between the modified method and ordinary method (n=13).

7. Examination for the correlation with the assay using anti-human insulin antibody

In the human insulin assay kit, the results are expressed as the potency, i.e. μU/ml. In the present dog insulin assay kit, the assay values are expressed as ng/ml. We examined the correlation of the assay values between two assay methods and calculated an equation (n=104).

Results

1. Basic performance of the kit

As shown in Fig. 1, this assay kit was reliable even at high concentration and showed small variation. Precision and reproducibility tests showed only small variation even at low concentrations and excellent reproducibility as shown in Table 1. The recovery test gave satisfactory results (Table 2). The recovery rate of very low concentration was about 90%.

2. Assay results of IVGTT samples

Fig. 2 – 4 show the results of IVGTT tests in IDDM, NIDDM and normal dogs. In normal dogs, there was no problem in the insulin secretion and responsiveness. In IDDM dogs, the amount of insulin secretion and the responsiveness to the glucose were considerably low compared with those of normal dogs. In NIDDM dogs, the amount of insulin secretion was low, but not so much as IDDM dogs. The insulin secretion response showed some delay compared with that in normal dogs.

3. Assay results of low level samples

The modified procedure for low-level samples proved to be useful in measuring insulin levels that cannot be measured by the ordinary procedure. The correlation coefficient obtained between two methods was high (R^2 = 0.9217) as shown in Table 3 and Fig. 5.

4. Correlation with the assay method using anti-insulin antibody

The correlation between these two assays was high with R^2 = 0.9276. The conversion equation obtained between two assays was Y(ng/ml) = 0.0719X(μU/ml) + 0.0502 as shown in Fig. 6

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Discussion

As stated in the introduction, we carry out IVGTT for the diagnosis of diabetes, decision of the treatment strategy, and understanding of the diabetic states. However, the presently available kits with anti-human insulin antibody give unreliable assay values at lower levels, making it difficult to the diagnosis of NIDDM that shows small changes in insulin response.

As was indicated in the present examination, Lbis Insulin Kit for Dog could measure low blood levels of insulin, it proved to be useful in the classification of diabetes types and diagnosis because it shows clearly the differences in insulin secretion and responsiveness among normal, IDDM and NIDDM.

Chances to encounter NIDDM seem to be increasing from now. Then, proper diagnosis, understanding of the diabetic states, and the proper treatments based on them will be necessary. The highly sensitive insulin assay kit will play an important role for the first step, namely, proper diagnosis.

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Tables and Figures
(As to figures, only attached tables are shown..)

Table 1 Precision and reproducibility
Precision test (within assay variation) (n=8)

Sample	Mean(ng/ml)	S.D.	CV(%)
1	0.872	0.0244	2.8
2	0.538	0.0184	3.42
3	0.24	0.0161	6.73

Reproducibility test (between assay variation) (n=5)

Sample	Mean(ng/ml)	S.D.	CV(%)
1	1.523	0.0494	3.25
2	0.903	0.0377	4.17
3	0.476	0.0227	4.77
4	0.219	0.0099	4.53

Table 2 Recovery test

Samples	Added (ng/ml)	Assay value (ng/ml)	Recovered (ng/ml)	Recovery (%)
1	0	0.291	-	-
	0.25	0.516	0.224	89.7
	0.5	0.765	0.474	94.7
	1	1.262	0.970	97
	2	2.243	1.951	97.6
2	0	0.182	-	-
	0.25	0.416	0.234	93.8
	0.5	0.641	0.459	91.8
	1	1.189	1.007	100.7
	2	2.253	2.071	103.6

Table 3 Assay results of modified procedure for low level

Dog	Time (min)	Ordinary method (ng/ml)	Modified procedure (ng/ml)	Value before correction (ng/ml)

95-33	Pre	low	low	low
	5	0.054	0.0682	0.1227
	10	0.0234	0.0495	0.0891
	15	0.0409	0.0597	0.1075
	25	0.058	0.0732	0.1318
	35	low	0.0427	0.0769
	45	low	0.0376	0.0677
	60	low	0.0478	0.0861
	90	low	0.0086	0.0115
95-33	Pre	0.2201	0.2288	0.4118
	5	0.2420	0.2848	0.5127
	10	0.2074	0.2818	0.5073
	15	0.2451	0.2997	0.5395
	25	0.2389	0.2728	0.4911
	35	0.2481	0.3041	0.5474
	45	0.2570	0.3218	0.5792
	60	0.2283	0.3519	0.5686
	90	0.2482	0.3880	0.6984

Figure 1 table Standard curve

STD (ng.ml)	A450	Average	S.D.	CV (%)
0	0.014 0.016	0.015	0.0014	9.4
0.1875	0.023 0.025	0.024	0.0014	5.9
0.375	0.032 0.035	0.034	0.0021	6.3
0.75	0.065 0.067	0.066	0.0014	2.1
1.5	0.145 0.150	0.148	0.0028	1.9
3	0.355 0.363	0.359	0.0057	1.6
6	0.874 0.894	0.884	0.0141	1.6
12	1.957 2.013	1.985	0.0396	2.0

Figure 2 table IVGTT (intra venous glucose tolerance test) in normal dog

Time (min)	Glucose (mg/dl)	Log(Glucose)	IRI (ng/ml)
0	79		0.642
5	375	2.57403127	6.287
10	312	2.49415459	4.852
15	268	2.42813479	3.156
25	186	2.26951294	1.875
35	135	2.13033377	0.773
45	103	2.01283722	0.625
60	84	1.92427924	0.581

90	87	1.93951925	0.469

C0 442.650766
C1/2 221.325383
T1/2 20.30725813
B.W. 14.5 kg

Figure 3 table IVGTT in IDDM dog

Time (min)	Glucose (mg/dl)	Log(Glucose)	IRI (ng/ml)
0	163		0.216
5	342	2.53402611	0.947
10	304	2.48287358	1.235
15	286	2.45636603	0.346
25	262	2.41830129	0.395
35	236	2.372912	0.402
45	221	2.34439227	0.313
60	201	2.30319606	0.376
90	174	2.24054925	0.419

C0 349.2265416
C1/2 174.6132708
T1/2 60.06488689
B.W. 7.1 kg

Figure 4 table IVGTT in NIDDM dog

Time (min)	Glucose (mg/dl)	Log(Glucose)	IRI (ng/ml)
0	94		0.433
5	226	2.35410844	2.871
10	222	2.34635297	4.016
15	208	2.31806333	3.824
25	182	2.26007139	2.682
35	151	2.17897695	1.793
45	131	2.1172713	0.882
60	91	1.95904139	0.621
90	89	1.94939001	0.525

C0 252.5895361
C1/2 126.2947681
T1/2 48.40011695
B.W. 7.7 kg

Figure 5 table Correlation between ordinary method and modified procedure
Y = 1.2455X +0.0081 (X: ordinary method)

(Y: modified procedure)

$R^2 = 0.9217$

Figure 6 table Correlation between the dog assay kit and human assay kit

$Y = 0.072X + 0.0416$ (X: human assay kit, μU/ml)

(Y: dog assay kit. ng/ml)

$R^2 = 0.9276$