May/16/2016

Heat treated Bovine Protein ELISA Kit Ver.2

For Laboratory Use Only.
Please read full descriptions in this manual before use.

http://www.miobs-e.com

I. Kit Components

Label	Name of component	Identification	Content	Quantity
A	Reagent A（10X Concentrate）	･･･Red dot label for Reagent A	100 mL	1bottle
B	Reagent B（10X Concentrate）	･･･Blue dot label for Reagent B	100 mL	1bottle
C	Reagent C（10X Concentrate）		100 mL	1bottle
D	Sample buffer（10X Concentrate）	･･･Red label	13 mL	1bottle
E	Positive control（High Concentration）	･･･Red cap	1.8 mL	1bottle
F	Positive control（Low Concentration）	･･･Blue cap	1.8 mL	1bottle
G	Negative control Ⅰ	･･･Yellow cap	1.8 mL	1bottle
H	Negative control Ⅱ	･･･Green cap	1.8 mL	1bottle
Ⅰ	Wash buffer（20X Concentrate）		50 mL	1bottle
J	Enzyme-conjugated Antibody		13 mL	1bottle
K	Enzyme Substrate（TMB Solution）		13 mL	1bottle
L	Stop Solution（1N Sulfuric Acid）		13 mL	1bottle
M	Antibody-coated Microplate Module		8 well X 3strips	4 packs
N	Plate Sealers		－	2 sheets
	Frame for mounting the microplate module		－	1 piece
	Microplate cover		－	1 piece

II. OTHER REQUIRED TOOLS AND EQUIPMENTS

1. Homogenizer / blender
   One possible to homogenize a sample.
2. Weighing balance
   One possible to weigh a 0.4–1.0 g sample.
3. Polypropylene centrifuge tube (50 mL)
4. Graduated cylinder, plastic pipette
5. Vortex mixer
6. Water bath
   One possible to keep boiling.
7. Centrifuge
   One possible to exert 3000×*g* or more centrifugal acceleration.
8. Filter paper (The recommended one is 5A filter paper made by ADVANTEC)
9. Micropipette (50 µL–1000 µL)
10. Polypropylene tube (1.5 mL)
11. Plate washer
    The washer specific for the present kit is recommended to be ready.
    ⚠ **Note: In case of using the other measuring reagent(s) and the washer in combination, to avoid a carryover of the used reagent, rinse thoroughly the washer before use.**
12. Plate reader Single wavelength : 450 nm
    Dual wavelength : dominant wavelength 450 nm, complementary wavelength 600-650 nm
13. Mask, disposable plastic gloves

III. PRECAUTIONS ON MEASUREMENT

1. All reagents should be equilibrated to 25 °C before use.
2. Assays should be performed at least in duplicate.
3. Reaction time for assay should be kept strictly.
4. On a primary reaction and a secondary reaction, plate seals attached to the kit should be necessary to use.

IV. ASSAY PROTOCOL

1. PROCEDURE OF REAGENT PREPARATION

1) M Antibody-coated Microplate Module should be equilibrated to 25 °C without being unpacked from aluminum pouch, before opening. Immediately use after opening.

2) The following reagents are used directly. Equilibrate to 25 °C before use.

E Positive control (High Concentration)

F Positive control (Low Concentration)

G Negative control I

H Negative control II

J Enzyme-conjugated Antibody

K Enzyme Substrate (TMB Solution)

L Stop Solution (1N Sulfuric Acid)

3) Preparation of extraction solution

Mix Reagent A (10X conc.), Reagent B (10X conc.), Reagent C (10X conc.) and distilled water at a ratio of 1:1:1:7. Necessary volumes should be prepared.

※ If a precipitation has occurred in Solution A, warm the solution and solubilize the precipitation thoroughly to use. Possible to keep at room temperature after the solubilization.

(Example: if assaying 24 samples)

Reagent A (10X conc.)	·····	50 mL
Reagent B (10X conc.)	·····	50 mL
Reagent C (10X conc.)	·····	50 mL
Distilled water	·····	350 mL
Extraction solution	·····	500 mL

4) Preparation of sample dilution solution

Dilute D Sample buffer (10X conc.) with distilled water 10-fold. Prepare a required volume.

※This is used as a Blank for dilution and assay of samples.

(Example: if assaying 24 samples)

Sample buffer (10X conc.)	·····	3 mL
Distilled water	·····	27 mL
Sample dilution solution	·····	30 mL

5) Preparation of washing solution

Dilute I Wash buffer (20X conc.) with distilled water 20-fold. Prepare a required volume.

※ Use prepared washing solution for washing manipulation.

2. METHOD OF PREPARING ASSAY SOLUTION AND EXTRACTING

1) Grind a sample to homogeneity with a blender and the like.

2) Take 1 g of the homogenized sample into a polypropylene centrifuge tube (50 mL), add 19 mL of extraction solution (see “3) Preparation of extraction solution” on page 4) to it, and stir it with a vortex mixer for 30 seconds. Then allow to stand for 10 seconds. Repeat this stirring manipulation 3 times.

3) Heat it in a boiled water bath for 10 minutes. After heating, allow to cool in a water bath so that the temperature of the extracted sample becomes 25 °C.
4) Centrifuge at 3000×*g* for 10 minutes at room temperature, filtrate the supernatant, and make the obtained a filtrate as an extracted sample solution.
5) Designate the 20-fold diluted extracted sample solution with sample dilution solution (see "4) Preparation of sample dilution solution" on page 4) as assay solution.

(Preparation of assay solution)

Extracted sample solution	·····	50 µL
Sample dilution solution	·····	950 µL
Assay solution	·····	1,000 µL

3. WORKING PROCEDURE OF ASSAY

- All reagents should be equilibrated to 25 °C before use.
- Reaction temperature should be kept at 25 °C.
- Assays should be performed at least in duplicate.
- Give attention to that the pipetting volumes are even.

(Primary reaction)

1) Set an M Antibody-coated Microplate Module on a mounting frame (supplied).
2) To respective wells, dispense 100µL each of Blank (Sample dilution solution), E Positive control (High concentration), F Positive control (Low concentration), G Negative control I, H Negative control II, and Assay solution.
3) Seal the plate with an N Plate Sealers, additionally cover it with a microplate cover (supplied), and allow to stand for 1 hour with accuracy at 25 °C to react.

(Secondary reaction)

4) Remove the solution completely from each well, and wash with 300 µL of washing solution per well 6 times.
5) Dispense 100 µL each of J Enzyme-conjugated Antibody to respective wells.
6) Seal the plate with an N Plate Sealers, additionally cover it with a microplate cover, and allow to stand for 1 hour with accuracy at 25 °C to react.

(Enzyme reaction)

7) Remove the solution completely from each well, and wash with 300 µL of washing solution per well 6 times.
8) Dispense 100µL each of K Enzyme Substrate to respective wells.

9) Cover the plate with a microplate cover, and allow to stand for 20 minutes with accuracy at 25 °C under light shielding to react.
10) Dispense 100 µL each of L Stop Solution to respective wells, and stop the enzyme reaction.
11) Measure the absorbance at 450 nm and 600-650nm of respective wells by a microplate reader, and designate the subtraction of the absorbance value at 600-650 nm from the absorbance value at 450 nm of each well as the absorbance value of the well.

⚠ **Note: The absorbance must be measured within 30 minutes after stopping the enzyme reaction.**

12) Determine the contamination of heat-treated bovine protein based on the absorbance of F Positive control (Low Concentration). For the determination method, see “Method of determination” on page 6.

V. METHOD OF DETERMINATION

1. Calculate the average of dual-assayed absorbance.
2. The formation of study is limited to the case where the following conditions are met. If the conditions are not met, then restart the assay:
   (1) The absorbance (average) of Blank, G Negative control I, H Negative control II is 0.08 or below.
   (2) The absorbance (average) of Blank, G Negative control I, H Negative control II is lower than that of F Positive control (Low Concentration) or lower.
   (3) The absorbance (average) of E Positive control (High Concentration) is 0.6 or more and 1.6 or less.
3. If the absorbance (average) of assay solution is equal to that of F Positive control (Low Concentration) or more, determine as having the contamination of heat-treated bovine protein.

VI. FLOW CHART FOR ASSAY

(Assay preparation) □ Prepare Blank, Positive control (High Concentration), Positive control (Low Concentration), Negative control I, Negative control II, and assay solution.

↓

(Primary reaction) □ Set an Antibody-coated Microplate Module on the mounting frame.

↓

□ Dispense Blank, Positive control (High Concentration), Positive control (Low concentration), Negative control I, Negative control II, and assay solution (100 µL/well) **(see X. ELISA format)**

↓

□ Seal the plate with a plate seal.

↓

□ Reaction (25 °C for 1 hour)

↓

(Secondary reaction) □ Washing (300 µL/well, 6 times)

↓

□ Dispense Enzyme-conjugated Antibody (100 µL/well)

↓

□ Seal the plate with a plate seal.

↓

□ Reaction (25 °C for 1 hour)

↓

(Enzyme reaction) □ Washing (300 µL/well, 6 times)

↓

□ Dispense Enzyme Substrate (100 µL/well)

↓

□ Reaction (25 °C for 20 minutes)

*** React under light shielding**

↓

(Reaction stopping) □ Dispense Stop Solution (100 µL/well)

↓

(Measurement □ Measure the absorbance (dominant wavelength: 450 nm, complementary wavelength: 600-650 nm)

*** Measure the absorbance within 30 minutes after stopping reaction.**

VII. ELISA FORMAT

(Plate preparation example)

	1 2	3 4	5 6	7 8	9 10	11 12
A	Blank	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
B	Positive control (High Concentration)	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
C	Positive control (Low Concentration)	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
D	Negative control I	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39
E	Negative control II	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40
F	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33	Sample 41
G	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34	Sample 42
H	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35	Sample 43

VIII. CONDITIONS OF STUDY FORMATION

(1) The absorbance of Blank, and the absorbance of Negative control I and II $\leq$ 0.08

(2) The absorbance of Blank, and the absorbance of Negative control I and II < the absorbance of Positive control (Low Concentration)

(3) 0.6 $\leq$ the absorbance of Positive control (High Concentration) $\leq$ 1.6

* For the conditions of study formation, use all the average of the absorbance at least in duplicate.

IX. CRETERIA OF DETERMINATION

[Positive] The absorbance of sample ≥ The absorbance of Positive control (Low Concentration)

[Negative] The absorbance of sample < The absorbance of Positive control (Low Concentration)

* For the determination as positive or negative, use all the average of the absorbance at least in duplicate.

X. ASSAY EXAMPLE

	1	2	Average
Blank	0.030	0.036	0.033
Positive control (High Concentration)	1.208	1.212	1.210
Positive control solution (Low Concentration)	0.123	0.127	0.125
Negative control I	0.022	0.028	0.025
Negative control II	0.037	0.035	0.036

* Condition for study formation

Average of the absorbance of Positive control (Low Concentration) = 0.125

(1) Average of the absorbance of Blank : $0.033 \leq 0.08$ ･･･Clear
Average of the absorbance of Negative control I : $0.025 \leq 0.08$ ･･･Clear
Average of the absorbance of Negative control II : $0.036 \leq 0.08$ ･･･Clear

(2) Average of the absorbance of Blank : $0.033 < 0.125$ ･･･Clear
Average of the absorbance of Negative control I : $0.025 < 0.125$ ･･･Clear
Average of the absorbance of Negative control II : $0.036 < 0.125$ ･･･Clear

(3) Average of the absorbance of Positive control (High Concentration) : $0.6 \leq 1.210 \leq 1.6$ ･･･Clear

Assay of samples

	1	2	Average	Determination
Sample 1	0.097	0.098	0.098	-
Sample 2	0.334	0.336	0.335	+

* Determination criteria as positive

The average of the absorbance of Positive control (Low concentration) = 0.125
The average of the absorbance of Sample 1 < 0.125 ･･･Negative
The average of the absorbance of Sample 2 ≥ 0.125 ･･･Positive