BioVendor
Research
and Diagnostic Products

Mouse C-peptide ELISA Kit (U-Type)

Research Reagent

Cat. No.: RSHAKRCP031R

This kit is manufactured by Shibayagi Co., Ltd.
Use only the current version of Instruction Manual enclosed with the kit! For the detailed assay procedure, refer to **Key points for ELISA by movie** on our website: **http://www.shibayagi.co.jp/index-E.htm**

1. INTENDED USE

Mouse C-peptide ELISA Kit (U-type) is a sandwich ELISA system for quantitative measurement of mouse C-peptide. This is for research use only.

2. STORAGE AND EXPIRATION

When the complete kit is stored at 2-8°C, the kit is stable until the expiration date shown on the label on the box. Opened reagents should be used as soon as possible to avoid loss in optimal assay performance caused by storage environment.

3. INTRODUCTION

Insulin is first synthesized as a single chain polypeptide, proinsulin, then three disulfide bonds are formed, and finally divided into insulin and C-peptide through enzymatic splitting. Mouse C-peptide 1 is a single chain peptide composed of 29 amino acids, while C-peptide 2 is composed of 31 residues. C-peptide is secreted together with insulin. The role of C-peptide has been considered to keep the best configuration to form three disulfide bonds, and has no biological activity, however, recent studies revealed that C-peptide can bind, probably, a G-protein-coupling specific receptor present on the surface of endothelial cells, kidney microtubule cells and fibroblasts, resulting in activation of calcium-dependent

intracellular signaling, activation of Na+-K+-ATPase, and enhancement of NO synthesis. Administration of C-peptide to DM1 patients enhances blood circulation in the skeletal muscle and skin, and also minimizes kidney glomerular hyperfiltration, decreasing albumin excretion into urine, and also improves nervous function, indicating that C-peptide should be given together with insulin to DM1 patients. Important region to bind receptor has been reported to be C-terminal pentapeptide (27-31).

The biological half life of C-peptide is several times longer than that of insulin. Measurement of C-peptide is useful in estimation of pancreatic function for insulin synthesis and secretion. Urinary C-peptide concentration is well correlated to its blood level. C-peptide measurement is also useful in estimation of insulin secretion by cultured islet of Langerhans because very often insulin is added to the culture medium, and it is difficult to discriminate secreted insulin from added insulin. As Shibayagi's kit recognizes the common sequences between C-peptide 1 and 2, it can measure total amount of C-peptide.

4. ASSAY PRINCIPLE

In Shibayagi's Mouse C-Peptide ELISA Kit (U-type), standards or samples are incubated in monoclonal anti-C-peptide-coated wells to capture C-peptide. After 2 hours' incubation and washing, biotin-conjugated anti-C-peptide is added and incubated further for 2 hours to bind with captured C-peptide. Then HRP- (horse radish peroxidase) conjugated streptavidin is added, and incubated for 30 minutes. After washing, HRP-conjugated streptavidin remaining in wells are reacted with a chromogenic substrate reagent (TMB) for 30 minutes, and reaction is stopped by addition of acidic solution, and absorbance of yellow product is measured spectrophotometrically at 450 nm. The absorbance is proportional to C-peptide concentration. The standard curve is prepared by plotting absorbance against standard C-peptide concentrations. C-peptide concentrations in unknown samples are determined using this standard curve.

5. PRECAUTIONS

- For professional use only. Beginners are advised to use this kit under the guidance of experienced person.
- Do not drink, eat or smoke in the areas where assays are carried out.
- In treating assay samples of animal origin, be careful for possible biohazards.
- This kit contains components of animal origin. These materials should be handled as potentially infectious.

- Be careful not to allow the reagent solutions of the kit to touch the skin, eyes and mucus membranes. Especially be careful for the reaction stopper because it is 1 M sulfuric acid. The reaction stopper and the substrate solution may cause skin/eyes irritation. In case of contact with these wash skin/eyes thoroughly with water and seek medical attention, when necessary.
- Avoid contact with the acidic Reaction stopper solution and Chromogenic substrate reagent containing hydrogen peroxide and tetramethylbenzidine (TMB). Wear gloves and eye and clothing protection when handling these reagents.
- The materials must not be pipetted by mouth.
- Unused samples and used tips should be rinsed in 1% formalin, 2% glutal aldehyde, or more than 0.1% sodium hypochlorite solution for more than 1 hour, or be treated by an autoclave before disposal.
- Dispose consumable materials and unused contents in accordance with applicable regional/national regulatory requirements.
- Use clean laboratory glassware.
- In order to avoid dryness of wells, contamination of foreign substances and evaporation of dispensed reagents, never forget to cover the well plate with a plate cover supplied, during incubation.
- ELISA can be easily affected by your laboratory environment. Room temperature should be at 20-25°C strictly. Avoid airstream velocity over 0.4 m/sec. ① (including wind from air conditioner), and humidity less than 30%. ①For airstream, refer to **[Assay circumstance]** on our web site.

6. REAGENTS SUPPLIED

Components	State	Amount
A. Anti-C-peptide-coated plate	Use after washing	96 wells/1 plate
B. Standard C-peptide solution (6000 pg/ml) (derived from mouse)	Concentrated. Use after dilution	500 µl/1 vial
C. Buffer solution	Ready for use.	60 ml/1 bottle
D. Biotin conjugated anti-C-peptide	Concentrated. Use after dilution.	100 µl/1 vial
E. HRP conjugated streptavidin	Concentrated. Use after dilution.	100 µl/1 vial
F. Chromogenic substrate reagent (TMB)	Ready for use.	12 ml/1 bottle
G. Reaction stopper (1M H_2SO_4) Be careful!	Ready for use.	12 ml/1 bottle
H. Concentrated washing buffer (10x)	Concentrated. Use after dilution.	100 ml/1 bottle
Plate seal	—	4 sheets
Instruction Manual	—	1 copy

7. EQUIPMENTS OR SUPPLIES REQUIRED BUT NOT SUPPLIED

USE AS A CHECK BOX

- Purified water (distilled water)
- Test tubes for preparation of standard solution series.
- Glassware for dilution of washing buffer (a graduated cylinder, a bottle)
- Pipettes (disposable tip type). One should be able to deliver 10 µl precisely, and another for 10-50 µl and 100-300 µl.
- Syringe-type repeating dispenser like Eppendorf multipette plus which can dispense 50 µl.
- Paper towel to remove washing buffer remaining in wells.
- A vortex-type mixer.
- A shaker for 96 well-plate (600-1200rpm)
- An automatic washer for 96 well-plate (if available), or a wash bottle with a jet nozzle. (refer to our web movie [Washing of microplate]).
- A 96 well-plate reader (450 nm ±10 nm, 620 nm: 600-650 nm)
- Software for data analysis, if available. Shibayagi is proposing the use of assay results calculation

- template for EXCEL. Please check our website (http://www.shibayagi.co.jp/en/tech_003.html).

8. PREPARATION OF REAGENTS

Bring all reagents of the kit to room temperature (20-25°C) before use.
Prepare reagent solutions in appropriate volume for your assay. Do not store the diluted reagents.

Concentrated reagents

(B) Standard C-peptide solution (6000 pg/ml)

Make a serial dilution of master standard (6000 pg/ml) solution to prepare each standard solution.

Volume of standard solution	Buffer solution	Concentration (pg/ml)
Original solution: 150 µl	150 µl	3000
3000 pg/ml solution: 150 µl	150 µl	1500
1500 pg/ml solution: 150 µl	150 µl	750
750 pg/ml solution: 150 µl	150 µl	375
375 pg/ml solution: 150 µl	150 µl	188
188 pg/ml solution: 150 µl	150 µl	93.8
93.8 pg/ml solution: 150 µl	150 µl	46.9
0 (Blank)	150 µl	0

(D) Biotin-conjugated anti-C-peptide

Prepare working solution by dilution of (D) with the buffer solution (C) to 1:100.
10 ml of the diluted solution is enough for 96 wells.

(E) HRP-conjugated streptavidin

Prepare working solution by dilution of (E) with the buffer solution (C) to 1:100.
10 ml of the diluted solution is enough for 96 wells.

(I) Concentrated washing buffer (10x)

Dilute 1 volume of the concentrated washing buffer (10x) to 10 volume with deionized water to prepare working solution. Example: 100 ml of concentrated washing buffer (10x) and 900ml of deionized water.

Storage and stability

(A) Anti-C-peptide-coated plate

If seal is not removed, put the strip back in a plastic bag with zip-seal originally used for well-plate container and store at 2-8°C. The strip will be stable until expiration date.

(B) Standard C-peptide solution (6000 pg/ml)

Standard solutions prepared above should be used as soon as possible, and should not be stored.

The rest of original standard: if stored tightly closed at 2-8°C, it is stable until expiration date.

(C) Buffer solution & (F) Chromogenic substrate reagent

If not opened, store at 2-8°C. It maintains stability until expiration date. Once opened, we recommend using as soon as possible to avoid influence by environmental condition.

(D) Biotin-conjugated anti-C-peptide & (E) HRP-conjugated streptavidin

Unused working solution (already diluted) should be disposed.

The rest of the undiluted solution: if stored tightly closed at 2-8°C, it is stable until expiration date.

(H) Reaction stopper (1 M H_2SO_4)

Close the stopper tightly and store at 2-8°C. It maintains stability until expiration date.

(I) Concentrated washing buffer (10x)

The rest of undiluted buffer: if stored tightly closed at 2-8°C, it is stable until expiration date.

Dispose any unused diluted buffer.

9. TECHNICAL TIPS

- In manual operation, proficiency in pipetting technique is recommended.
- The reagents are prepared to give accurate results only when used in combination within the same box. Therefore, do not combine the reagents from kits with different lot numbers. Even if the lot number is the same, it is best not to mix the reagents with those that have been preserved for some period.
- Be careful to avoid any contamination of assay samples and reagents. We recommend the use of disposal pipette tips, and 1 tip for 1 well.
- Optimally, the reagent solutions of the kit should be used immediately after reconstitution. Otherwise, store them in a dark place at 2-8 ^{o}C.
- Time the reaction from the pipetting of the reagent to the first well.
- Prepare a standard curve for each assay.
- Dilution of the assay sample must be carried out using the buffer solution provided in the kit.
- The chromogenic sucstrate reagent (TMB) should be almost colorless before use. It turns blue during reaction, and gives yellowish color after addition of reaction stopper. Greenish color means incomplete mixing.
- To avoid denaturation of the coated antibody, do not let the plate go dry.
- As the anti-C-peptide-coated plate is module type of 8wells x 12 strips, each strip can be separated by cutting the cover sheet with a knife and used independently.
- When ELISA has to be done under the airstream velocity of over 0.4 m/sec. and the humidity of less than 30%, completely close each well in addition to cover the well plate with a plate cover in each step of incubation.
  Ex.) Cover the well plate with parafilm, and put the plate cover on it. Or place the well plate with the plate cover in an incubator, or in a styrofoam box. Take the best way depending on situation of each laboratory. For more details, watch our web movie [Assay circumstance].

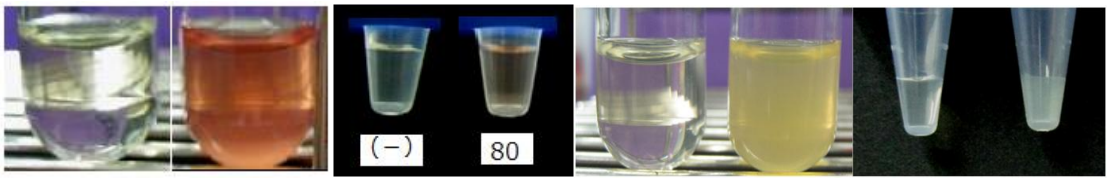
10. PREPARATION OF SAMPLES

This kit is intended to measure C-peptide in mouse serum or plasma. The necessary sample volume for the standard procedure is 10 µl.

Samples should be immediately assayed or stored below –35 °C until assay. Defrosted samples should be mixed thoroughly for best results. Do not repeat freeze and thaw cycles of samples. It may cause improper results.

Hemolytic and hyperlipemic serum samples are not suitable.

* To avoid influence of blood (high lipid or hemolysis, etc.), if your original samples have heavy chyle or hemolysis as the pictures below, do not use them for assay. Abnormal value might be obtained with hemolysis above 80mg/dL with this kit.



Normal	Hemolysis 80mg/dL	Normal	Hemolysis 80mg/dL	Normal	Chyle Highly lipid sample	Normal	Chyle Highly lipid sample

Sample's pH should be between 6.5-7.5. If presence of interfering substance is suspected, examine by dilution test at more than 2 points. Dilution of a sample should be made in a test tube using buffer solution prior to adding them to wells (e.g. sample 10 µl + buffer 40 µl). Turbid samples or those containing insoluble materials should be centrifuged before testing to remove any particulate matter.

Storage and stability

C-peptide in samples will be inactivated if stored at 2-8ºC. If it is necessary to store sample in refrigerator (2-8ºC), add aprotinin at final concentration of 100-500 KIU/ml. (KIU: kallikrein inhibitor unit).

If you have to store assay samples for a longer period, snap-freeze samples and keep them below –35°C. Avoid repeated freeze-thaw cycles.

11. ASSAY PROCEDURE

Remove the cover sheet of the anti-C-peptide-coated plate after bringing up to 20-25°C.

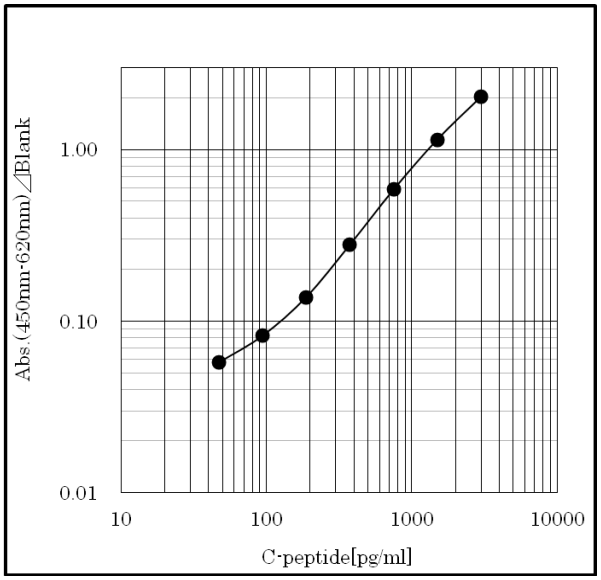
1. Wash the anti-C-peptide-coated plate (A) by filling the wells with washing buffer and discard 3 times(*②), then strike the plate upside-down onto several sheets of paper towel to remove residual buffer in the wells.
2. Pipette 40 µl of buffer solution (C) and 10 µl of samples to the designated sample wells.
3. Pipette 50 µl of standard solution to the wells designated for standards.
4. Shake the plate gently on a plate shaker (*③).
5. Stick a plate seal (*④) on the plate and incubate for 2 hours at 20-25°C.
6. Discard the reaction mixture and rinse wells as step (1).
7. Pipette 50 µl of Biotin-conjugated anti-C-peptide to all wells, and shake as step (4).
8. Stick a plate seal (*④) on the plate and incubate the plate for 2 hours at 20-25°C.
9. Discard the reaction mixture and rinse wells as step (1).
10. Pipette 50 µl of HRP-conjugated streptavidin to all wells, and shake as step (4).
11. Stick a plate seal (*④) on the plate and incubate the plate for 30 minutes at 20-25°C.
12. Discard the reaction mixture and rinse wells as step (1).
13. Pipette 50 µl of Chromogenic substrate reagent to wells, and shake as step (4).
14. Stick a plate seal (*④) on the plate and incubate the plate for 30 minutes at 20-25°C.
15. Add 50 µl of the reaction stopper to all wells and shake as step (4).
16. Measure the absorbance of each well at 450 nm (reference wavelength, 620*nm) using a plate reader within 30 minutes.

*Refer to the page 7-8 for notes of *②, *③ and *④.

12. CALCULATIONS

1. Prepare a standard curve by plotting standard concentration on X-axis and absorbance on Y-axis. (Refer to our web site for more detailed explanation about standard curve. Shibayagi is offering a convenient Excel template. http://www.shibayagi.co.jp/en/tech_003.html)
2. Using the standard curve, read the C-peptide concentration of a sample at its absorbance*, and multiply the assay value by dilution factor if the sample has been diluted. Though the assay range is wide enough, in case the absorbance of some samples is higher than that of the highest standard, please repeat the assay after proper dilution of samples with the buffer solution.

* We recommend the use of 3rd order regression curve for log-log plot, or 4 parameters method for log-normal plot in computer calculation.
Physiological or pathological situation of animals should be judged comprehensively taking other examination results into consideration.



Mouse C-peptide assay standard curve
Absorbance may change due to assay environment.

13. PERFORMANCE CHARACTERISTICS

- Assay range

The assay range of the kit is 46.9 pg/ml ~ 3,000 pg/ml.
The effective assay range by standard assay procedure (dilution rate: 5x) is 234.5 pg/ml ~ 15,000 pg/ml.

- Specificity

The antibodies used in this kit are specific to mouse C-peptide. Cross-reactivity against rat C-peptide is 89% and human C-peptide is 85% when tested at 3000 pg/ml.

- Precision of assay

Within assay variation (2 samples, 8 replicates assay), Mean CV is less than 5%.

- Reproducibility

Between assay variation (3 samples, 4 days, 4 replicates assay), Mean CV is less than 5%

- Recovery test

Standard C-peptide was added in 3 concentrations to 2 serum samples and were assayed.
The recoveries were 94 ~104%

- Dilution test

2 serum samples were serially diluted by 3 steps.
The dilution curves showed linearity with $R^2 = 1.0$.

14. TROUBLE SHOOTING

- Low absorbance in all wells

Possible explanations:
1) The standard or samples might not be added.
2) Reagents necessary for coloration such as Biotin-conjugated anti-C-peptide, HRP-conjugated streptavidin, or Chromogenic substrate reagent might not be added.
3) Wrong reagents related to coloration might have been added. Wrong dilution of biotin-conjugated anti- C-peptide or HRP-conjugated streptavidin.
4) Contamination of enzyme inhibitor(s).
5) Influence of the temperature under which the kits had been stored.
6) Excessive hard washing of the well plate.

7) Addition of chromogenic substrate reagent soon after taking out from a refrigerator might cause poor coloration owing to low temperature.

- Blank OD was higher that that of the lowest standard concentration (46.9 pg/ml).

Possible explanations: Improper or inadequate washing. (Change washing frequency from 3 times to 4-6 times at the constant stroke after the reaction with HRP-conjugated streptavidin.)

- High coefficient of variation (CV)

Possible explanation:
1) Improper or inadequate washing.
2) Improper mixing of standard or samples.
3) Pipetting at irregular intervals.

- Q-1: Can I divide the plate to use it for the other testing?
  A-1: Yes, cut off the clear seal on the plate with cutter along strip. Put the residual plate, which is still the seal on, in a refrigerator soon

- Q-2: I found there contains liquid in 96 well-plate when I opened the box. What is it?
  A-2: When we manufacture 96 well-plate, we insert preservation stabilizer in wells.

For detailed FAQS and explanations, refer to **"Trouble shooting and Important Points in Shibayagi's ELISA kits**" on our website (http://www.shibayagi.co.jp/en/tech_004.html).

15. REFERENCES

Please, refer to [User's Publication] on our website.
Summary of assay procedure: Use as a check box
***First, read this instruction manual carefully and start your assay after confirmation of details.**
For more details, watch our web movie [ELISA by MOVIE] on our website.
Bring the well-plate and all reagents back to 20-25°C for 2 hours.
Washing buffer concentrate must be diluted to 10 times by purified water that returned to 20-25°C.

Standard C-peptide solution dilution example 1:

Concentration (pg/ml)	3000	1500	750	375	188	93.8	46.9	0
Std. C-peptide solution (µl)	150	150*	150*	150*	150*	150*	150*	0
Buffer solution (µl)	150	150	150	150	150	150	150	150

*One rank higher standard.

Standard C-peptide solution dilution example 2:

Concentration (pg/ml)	3000	1500	600	300	150	60	30	0
Std. C-peptide solution (µl)	150	150*	150*	150*	150*	150*	150*	0
Buffer solution (µl)	150	150	225	150	150	225	150	150

*One rank higher standard.

Precautions & related info

□	Anti-C-peptide-coated plate	
□	↓Washing 3 times(*②)	*⑥
□	Diluted samples (e.g. buffer (C) 40 µl + sample 10µl), or Standards 50 µl	*⑦ [Handling of pipetting]
□	↓Shaking(*③), Incubation for 2 hours at 20-25°C. (Standing(*④))	*⑧ [Assay circumstance]
□	Dilute Biotin conjugated anti-C-peptide to 100x with buffer returnedto 20-25°C.	This should be prepared during incubation.
□	↓Washing 3 times(*②)	*⑥
□	Biotin-conjugated anti-C-peptide solution 50 µl	*⑦ [Handling of pipetting]
□	↓Shaking(*③), Incubation for 2 hours at 20-25°C. (Standing(*④))	*⑧ [Assay circumstance]
□	Dilute HRP-conjugated streptavidin to 100x with buffer returned to 20-25°C.	This should be prepared during incubation.
□	↓Washing 3 times(*②)	*⑥
□	HRP conjugated streptavidin 50 µl	*⑦ [Handling of pipetting]
□	↓Shaking(*③), Incubation for 30 minutes at 20-25°C. (Standing(*④))	*⑧ [Assay circumstance]
□	↓Washing 3 times(*②)	*⑥
□	Chromogenic substrate reagent (TMB) 50 µl	After dispense, the color turns to blue depending on the concentration.
□	↓Shaking(*③), Incubation for 30 minutes at 20-25°C. (Standing(*④))	*⑧ [Assay circumstance]
□	Reaction stopper (1M H_2SO_4) 50 µl	After dispense, the color turns to yellow depending on the concentration.
□	↓Shaking(*③)	Immediately shake.
□	Measurement of absorbance (450 nm, Ref 620 nm(*⑤))	Ref. wave cancels the dirt in the back of plate.

*②After dispensing wash buffer to wells, lightly shake the plate on your palm for 10 sec and remove the buffer. Guideline of washing volume: 300 µl/well for an automatic washer and for a pipette if the washing buffer is added by pipette. In case of washing by using 8 channel pipette, sometimes the back ground tends to be high. If so, change washing frequency from 3 times to 4-6 times at the constant stroke after the reaction with HRP conjugated streptavidin.

Standard of plate-washing pressure: 5-25 ml/min. (Adjust it depending on the nozzle's diameter.) Refer to our web movie **[Washing of microplate]**.

*③Guideline of shaking: 600-1,200 rpm for 10 seconds x 3 times.

*④Put a plate cover on the plate during the reaction after shaking.

*⑤600-650 nm can be used as reference wavelength.

*⑥After removal of wash buffer, immediately dispense the next reagent.

*⑦Refer to our web movie [Handling of pipetting].

*⑧Refer to our web movie [Assay circumstance].

Worksheet example

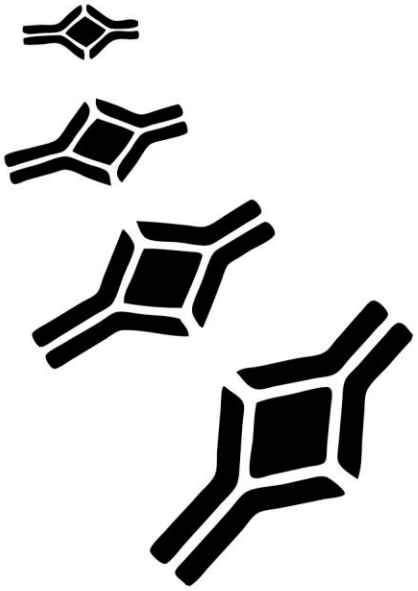
	Strip 1&2	**Strip 3&4**	**Strip 5&6**	**Strip 7&8**	**Strip 9&10**	**Strip 11&12**
A	**3000 pg/ml**	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
B	**1500 pg/ml**	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
C	**750 pg/ml**	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
D	**375 pg/ml**	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
E	**188 pg/ml**	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
F	**93.8 pg/ml**	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
G	**46.9 pg/ml**	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39
H	0	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40

Assay worksheet

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Storage condition
Store the kit at 2-8°C (Do not freeze).

Term of validity
6 months from production (Expiration date is indicated on the container.)



Distributed by:
BioVendor – Laboratorní medicína a.s.
Karasek 1767/1, 621 00 Brno, Czech Republic
Phone: +420-549-124-185, Fax: +420-549-211-460
E-mail: info@biovendor.com, sales@biovendor.com
Web: www.biovendor.com

Manufactured by:
Shibayagi Co., Ltd.
1062-1 Ishihara, Shibukawa
Gunma, Japan 377-0007

BioVendor
Research
and Diagnostic Products

There are BioVendor branches and distributors near you. To find the office closest to you, visit **www.biovendor.com/contact**