

## Seven Important Points for Excellent Assay Results with Shibayagi's ELISA kits

Ver. 7

### **[Manual procedure for kit operation is not always same]**

Many kit makers are providing various ELISA kits, however, operative procedure is not always the same, e.g. mixing, reaction temperature, washing apparatus, washing method, and washing repetition, etc. Even in the same maker's products some modifications of the procedure have been made. Therefore, we recommend observing the recommended operative procedure in a preliminary test prior to your final assays.

### **[About Shibayagi's high sensitive assay kits]**

- ◆ Mouse Insulin ELISA KIT (U), (S)
- ◆ Rat Insulin ELISA KIT (U-E)
- ◆ Mouse Leptin ELISA KIT
- ◆ Mouse C-peptide ELISA KIT
- ◆ Rat C-peptide ELISA KIT
- ◆ Rat GH ELISA KIT
- ◆ GLP-1 (Active) ELISA KIT
- ◆ Rat LH ELISA KIT (S-type)

In the high sensitive assay kits listed above, operator's skill and sample conditions could influence the assay results. Before purchase, please read the precautions and procedure on our website or in the manuals to confirm if your sampling conditions fit to your intended experiments.

### **Point 1: How to store assay kits?**

- Check temperature of your refrigerator periodically. Do not put kits near the outlet of cold air stream. Direct air stream is often too cool and may freeze kits.
- If kits are kept below 2 °C, the constituents of the concentrated washing buffer will form crystals which look like glass or plastic fragments. In such case, place the bottle outside or in an incubator to get it back to room temperature to dissolve the crystals and use after stirring well. Refer to ELISA by MOVIE [Precaution to the safety]  
[http://www.shibayagi.co.jp/en/movie\\_001.html](http://www.shibayagi.co.jp/en/movie_001.html)

### **Point 2: How to treat assay samples?**

- Factors of assay samples affecting assay results.
  - ◎ Hemolysis  
Influence of hemoglobin on the assay results may be different according to assay kits. Confirm the influence before purchase. To minimize hemolysis, we recommend using anticoagulant-coated sampling tubing or syringe (heparin or EDTA·2Na). However, as heparin is not allowed with some of our kits, check before purchase. You can find the influence of heparin in the instruction paper of the kit. Vacuum blood collecting tube might often cause hemolysis and we don't recommend using this (check the influence beforehand).
  - ◎ Enzyme inhibitors  
Sodium azide (NaN<sub>3</sub>), generally used as antiseptic, and sodium fluoride (NaF), used as anticoagulant, may act as peroxidase inhibitors in the assay system, so avoid using sample tubes coated with these agents.

- ◎ Serum separating tubes  
When sampling, do not use sample tubes for human (serum separating tubes). Serum separation accelerator in tube might influence assay system.
- ◎ Chyle  
Turbidity of sample owing to the presence of chyle may sometimes influence assay. We can tell you the turbidity influencing the kit as formazin turbidity unit on request. Ask us before purchase.
- ◎ pH  
Sample pH should be adjusted to pH 6.5~7.5. Especially, be careful in measurement of C-peptide in culture medium. Serum, plasma, or culture medium has a tendency to become alkaline by losing carbon dioxide during storage.
- ◎ Sample dilution  
Some kits need minimum dilution of serum or plasma, while others can measure serum or plasma without dilution. Confirm the necessity of dilution in the instruction of each kit on our website. Also refer to the standard dilution rate. If the contents of the substance to be assayed extend over a wide range, decide the dilution rate for your experimental samples through preliminary test.
- ◎ Samples containing insoluble materials, highly viscous samples, high protein samples, high salt concentration (>0.5M) samples  
These factors may influence assay results. We recommend checking the influence by serial dilution test.
- ◎ Contamination with organic solvents (like ether after deep ether anesthesia) and detergent  
These substances influence antigen-antibody reaction. Especially, when high sensitive kit is used avoid ether anesthesia.
- ◎ Anticoagulants  
Some kits listed below will not work well with heparin-treated plasma. Check the instruction paper before purchase.
  - ◆ Mouse anti-dsDNA ELISA KIT
- ◎ Addition of enzyme inhibitor  
Presence of enzyme inhibitor is indispensable in blood sampling for GLP-1. Read instruction paper on our website for GLP-1 (Active) ELISAKIT before purchase. Check below in case of using aprotinin as a protease inhibitor and heparin as an anticoagulant:  
Check if fibrin might appear when thawing samples. Fibrin might influence the assay. Especially be careful when assaying low molecular substances such as GLP-1.
- ◎ Highly viscous standard solution and samples for Apo B48 ELISA KIT  
In measuring human Apo B48, get used to treat highly viscous solution with a pipette by proper training, and be careful for bubble formation in pipetting and mixing.
- ◎ Culture medium samples  
Before assay, check whether any interfering substance is present in the culture media or not. Prepare a test standard curve by using original culture medium, and compare the curve with that obtained with assay buffer. If a good correlation is observed, you can use supernatant of the culture media as assay samples. Usually, concentrations of a substance to be measured differ widely from culture to culture due to experimental

schemes. Therefore, it is recommended to measure a sample with several dilution rates in order not to miss the standard curve area.

◎ Storage of samples for insulin assay

Protect insulin from enzymatic inactivation by adding aprotinin (trasylol).

Avoid storing the samples in a refrigerator for a long time without adding an inhibitor of insulin degradation enzyme.

The samples should be stored below  $-35^{\circ}\text{C}$ , if possible, in an ultra-low temperature ( $-80^{\circ}\text{C}$ ) freezer.

◎ If you want to measure frozen-stored samples, confirm beforehand that freezing-thawing treatment does not influence the assay value of the target substance.

\* Never forget to mix enough after thawing using a vortex-type mixer.

● Quality control (positive control) samples

Prepare control samples by storing aliquots of serum, plasma or culture medium containing fixed amount of the substance to be measured through preliminary assay.

Measure these control samples together with other samples in every assay to confirm the reproducibility and successful working of the assay.

**Point 3: Pipetting with a tip-exchanging type pipette**

**Pipetting of samples and standard solutions.**

● Choice of pipettes including those with 8 / 12 channels

With Shibayagi's ELISA kits volumes to be delivered are mostly 5, 10, 50, and 100  $\mu\text{l}$ /well. In the use of variable pipette, delivery of 5  $\mu\text{l}$  and 10  $\mu\text{l}$  should be made by a pipette with the maximum volume of 10  $\mu\text{l}$ , and 50  $\mu\text{l}$  delivery with maximum volume of 100  $\mu\text{l}$ . Never use maximum 100  $\mu\text{l}$  or 200  $\mu\text{l}$  pipette for 5, and 10  $\mu\text{l}$  deliveries. Use the appropriate tips provided by the pipette makers, exclusively.

We don't recommend using 8 or 12 channel pipettes when operating ELISA (sandwich) kits especially for standards and samples. Dispensing with these multi-channel pipettes requires proficiency and appropriate conditions like even liquid level of samples, or low and nearly the same viscosity of samples. We usually recommend using multi-dispensers for example, Eppendorf Multipette plus, when dispensing same solution into all of 96 wells. Well-maintained auto pipettes are also recommended.

● Bubble formation and measures for double pushing type pipette

The material of pipette tip is highly water repellent, however, is wet more or less when water solution is filled and dispensed. If you simply fill a solution and dispense, you will deliver less than your intension by the volume used for wetting of the tip

One pipetting method recommended by pipette maker, is "pre-wetting method". The procedure of the method is: first push the plunger to the first stop, and fill up the solution to the tip. Then push the plunger again to the first stop to blow the solution out to the original container. By this action, the tip is pre-wetted. After this, you can fill the solution to the tip and dispense it into a well by pushing the plunger all the way to the second stop (this is called blow-out).

But if you blowout, you will often make a bubble because excessive air is also sending out. In order not to form bubble, it is better to stop the plunger at the first stop. This will not influence the dispensed volume.

◎ When blown out, keep a pipette's tip away from well surface at a distance of a bubble formed.

It would be good for a bubble to touch the wall of wells.

- ⊙ When dispensing, do not scratch or touch strongly the bottom and wall of the well because the antibody is coated.
- ⊙ When dispensing push the plunger at a constant speed.
- ⊙ After dispensing, touch the end of the tip very slightly to the upper part of the wall in order to remove the sample solution remaining on the outer surface of the tip (this is called 'touch and go'), and then change the tip to new one.
- ⊙ A small bubble less than 1mm in diameter will not influence the reaction so much, and can be left as it is. However, several big bubbles may interfere with the reaction. Break them by touching a tip or a needle to the surface of the bubble.

Refer to ELISA by MOVIE [Handling of pipette]

[http://www.shibayagi.co.jp/en/movie\\_004.html](http://www.shibayagi.co.jp/en/movie_004.html)

#### **Point 4: Get all components of the kit to room temperature**

● Before starting assay, take out the kit from the refrigerator, and put it back to room temperature. Especially, in winter, the surface of the laboratory table stayed cool for rather long time after air conditioner is started. Under such situation, put a sheet of Styrofoam on the table, and place the components on it. It will take several hours until the components reach to room temperature (20~25°C).

● The seal of the 96 well-plate should be peeled off at room temperature. At low temperature, the seal will not be removed easily and some parts may be left.

Refer to ELISA by MOVIE [Temperature adaptation]

[http://www.shibayagi.co.jp/en/movie\\_003.html](http://www.shibayagi.co.jp/en/movie_003.html)

#### **Point 5: Dilution of the assay reagents**

● Preparation of the standard solutions

An example of dilution for serial standard solutions is shown in each instruction paper of the kit. Some describe serial dilution that is easy and safe, and other describe different rate of dilution. You can choose any procedure.

● Vortex reagents before dilution trying not to make bubbles.

● When mixing, do not put the tube while the mixer is moving. Bubbles will be formed. Put the tube on the mixer standing still, and then make the switch on.

Refer to ELISA by MOVIE [Dilution of condensed reagents]

[http://www.shibayagi.co.jp/en/movie\\_005.html](http://www.shibayagi.co.jp/en/movie_005.html)

#### **Point 6: 96 well-plate**

● Standard solutions and samples should be assayed in duplicates or more.

● Delivery of standard solutions should be started from the blank wells, and then directed from the lowest to higher standard wells.

● After dispensing samples or reagent solutions, never forget to mix the well enough.

● Do not form bubbles while mixing.

## Point 7: Washing of the plate

### Manual washing

●The first washing after the first reaction with standards or samples: Shake off the reaction mixture from the plate on a sink.

Then add washing solution using a multi-delivery pipette set at 250~300 $\mu$ l to avoid carry-over caused by flowing out the buffer to other wells. Then shake off the buffer.

●Insufficient washing might be caused if there is not a certain strength of water stream to fill wash buffer in wells. When absorbance of the blank wells are higher than those of the lowest standard wells, (this may often happens when using multi-channel pipettes with 8 or 12 tips), it will be lowered by increasing the repetition of washing without changing the flow-rate after the reaction with biotin-labeled antibody, however, increase the repetition of washing must be max. 2 times of that indicated in the instruction manual.

●After filling wash buffer to each well, lightly agitate the plate on palm for approx. 10 seconds and discard the buffer.

●If the tip of the nozzle of washing bottle is too thin, the jet stream of the washing buffer becomes too strong, and may remove the coated antibody, resulting in weak coloration or big absorbance variation. In such case cut off the tip of the nozzle a little to make the stream milder. Mild and more repeated washing will minimize absorbance and their variation in blank wells.

●Do not make wells dry until dispensing reagent solutions. Dried well will cause an increase of blank absorbance.

●Bubbles remaining after washing can be removed by overflowing the washing buffer.

### Washing with a plate washer

●Input the name of plate as [Nunc].

●The washing mode, if possible, should be set as “plate mode”.

●If the washing power is set too strong, well-coated capture antibody and bound substance may be removed, and final coloration may be poor. Check the absorbance of the highest standard well if it is not lower than expected.

The standard setting for stream strength is about 5~25 ml/min though it may be different depending on the diameter of the nozzle,

●If the washing power is set too weak, higher blank absorbance may be resulted. Non-specific adsorption of biotin-labeled antibody and HRP-labeled avidin is very crucial for the blank absorbance. So, even in the case of machine washing, if the blank absorbance is high, it will be lowered by increasing the repetition of washing without changing the flow-rate after the reaction with biotin-labeled antibody, however, increase the repetition of washing must be max. 2 times of that indicated in the instruction manual.

●Please, use your plate washer under enough maintenance such as cleaning of every tubing and nozzle.

Refer to ELISA by MOVIE [Washing of microplate]

[http://www.shibayagi.co.jp/en/movie\\_006.html](http://www.shibayagi.co.jp/en/movie_006.html)

### Edge effect and measures

#### Edge effect

In a well-plate, those wells locating at the edge of the plate are liable to be influenced by outside temperature source, and the binding and chemical reactions may proceed more (or less) quickly than in other wells located inside. As the blank wells are usually located at the edge, their absorbance may sometimes higher than that of the lowest standard, and in duplicate assay, absorbance of outside wells are higher than those of inside wells (if the outside temperature is

higher than inside). Edge effect also causes the poor sensitivity and big variation of the results.

### Measures

- ◎ Dilute the concentrated washing buffer to 10x using purified water of room temperature.
- ◎ Warm up every kit component to room temperature. Take out the assay kits 1-2 hours before starting assay.
- ◎ If the incubation is carried out in an incubator, check the uniformity of the temperature of the incubator before using. If the incubation is made in an open air, choose where to place the well-plate. Never place near heater or outlet of air-conditioner. Also, avoid heating the plate with your own hand by holding tightly for a long period, especially while dispensing HRP-containing reagent solution (HRP-antibody or HRP-avidin).
- ◎ Washing step is also important. Please, refer to the section "Point 7".

### To avoid dryness of well during reaction:

- Stick a plate seal on plate while incubation after dispensing reagents to wells.  
Refer to ELISA by MOVIE [Assay circumstance]  
[http://www.shibayagi.co.jp/en/movie\\_008.html](http://www.shibayagi.co.jp/en/movie_008.html)

● We recommend using an air-type incubator (22-25 °C) to stabilize the reaction temperature throughout the year.

### ELISA by MOVIE

You can check key points of ELISA easily by movie. Please watch ELISA by MOVIE prior to assay.

<http://www.shibayagi.co.jp/en/movie.html>

If you need further information, please don't hesitate to contact us.

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