Tracking disease outbreaks – detection by ELISA method- NEW

Introduction

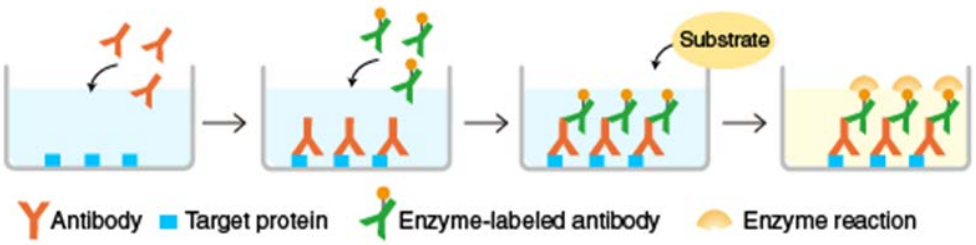
The task simulates the mode and variability of the spread of infectious agents in the population and the result of the test evaluation is the determination of the probable causative agent of the infectious disease. The simulation of the spread of a given agent is always performed in a group of 12 students, of which only one sample is native, i.e. positive. The ELISA-Immuno Explorer kit is designed for didactic purposes. It allows the determination of antigens and antibodies in unknown samples and the approximate concentration of agents in samples. This test allows for semi-quantitative analysis – i.e. determination of the approximate content of the antigen (in the case of manual evaluation) or its quantitative determination (using photometric determination of absorbance). To quantitatively determine the antigen content in the sample, students prepare a calibration series according to the instructions and process the resulting curve mathematically and determine the slope. At the same time, they read the values of antigen concentration in the sample from this graph. At the same time, the task serves as a demonstration of immunoanalytical determination of the substance on the principle of direct competition between the analyte and the detection affinity molecule. The instructions describe the individual steps of determination, supplemented by a practical example of the test.

Principle

ELISA-Immuno Explorer is a solid-phase immunoassay based on the principle of direct competition. An antigen is already bound to the surface of the wells of the microtiter plate, which competes ("competes") with the free antibody in the sample for a binding site. The excess antibody is removed during the subsequent washing of the wells with a washing solution. The amount of antibody captured is directly proportional to the concentration of the unknown agent in the sample. In order to determine the amount of agent captured, it is associated (conjugated) with enzyme HRP peroxidase. This enzyme reacts with a chromogenic substrate to change the colour of the substrate solution from clear to blue, depending on the amount of HRP peroxidase complex present in the well. (Fig. 1)

Washing: Between the individual steps of the test, it is necessary to remove unbound complexes from the wells. If the wells were not washed, the wells would be discolored at the end of the test even without the presence of the substances sought.

Substrate solution: Tetramethylbenzidine (TMB) dye based solution containing hydrogen peroxide. Thanks to an enzyme (peroxidase contained in the conjugate in this test), a catalytic reaction occurs in the substrate and a gradual change in the color of the solution to blue. If the peroxidase conjugate is not bound in the wells, the substrate will not be activated and the wells will remain without discoloration.



https://ruo.mbl.co.jp/bio/e/support/method/elisa.html

Fig. 1 ELISA method-indirect

Preparation of the calibration series: The set contains 1 standard – the student prepares the calibration series (see Fig. 2 according to the two-year dilution), according to which he determines the approximate content of the foreign agent in the samples. The sensitivity range is 1 to 1000 ng/ml.

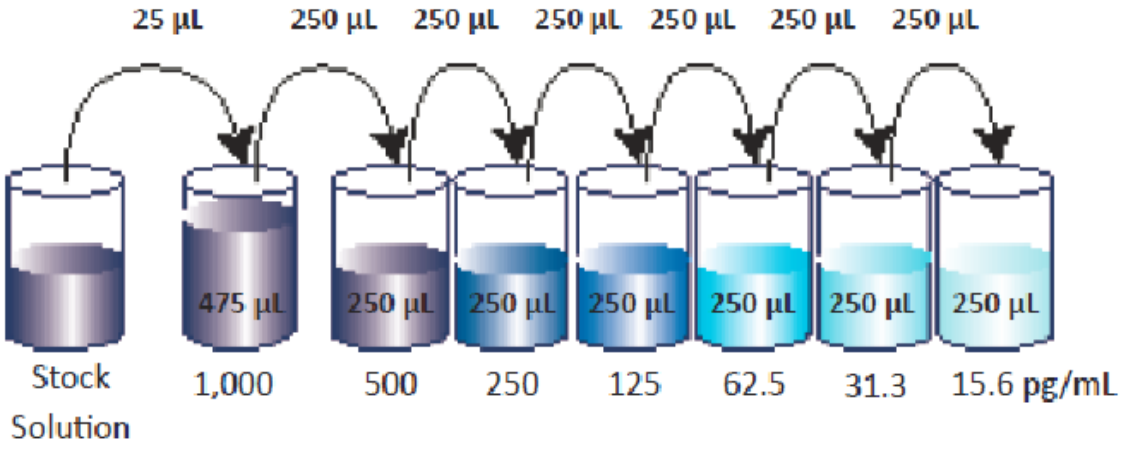


Fig.2 Two dilution of stock solution

Application: The resulting concentration of the sample can be evaluated using special laboratory equipment – ELISA reader/spectrophotometer.

Calibration: The student creates a calibration of the absorbance and concentration values from the measured absorbance values and the given concentration values.

Reagents:

ELISA strips (12 wells) coated with STRIPS polymer

A set of reagents containing:

Test sample – unknown (0.75 ml)

positive control (antigen, 0.50 ml),

negative control (PBS 0.50 ml)

primary antibody 1.5 ml (contains anti –rabbit polyclonal antibody)

secondary antibody with HRP (horseradish peroxidasa) enzyme(1,5 ml)

conjugate TMB chromogen substrate(tetramethylbenzidin), r.t.u., (1,5 ml)

WASH buffer aprox. 60 ml

PBS (phosphate buffer.)

stock solution c= 1000 ng/ml

add.: * r.t.u. – „ready to use“ (it is not necessary to dilute)

Equipement:

ELISA reader: SpectraMax M2 Multi-Mode Microplate Reader Unit2 - AV

Utilities:

distilled water to dilute the washing solution (ordinary water can also be used, but depending on the quality, it may slightly discolor the background of the reaction)

Measuring cylinder for the preparation of the washing solution

Micropipettes

Glass pipettes

Pasteur pipettes 1 ml with 0.25 ml scale 10 pcs

Microtubes

Pipette Tips

beaker or tub of water

Waste container

Eppendorf test tubes

gauze/filter paper/cellulose wadding to dry the strips after washing

watch/stopwatch for measuring incubation times,

permanent fix

Laboratory procedure:

1. Buffers preparation

1.1. Take the kit ingredients out on the table and let them adjust to room temperature (approx. 10 minutes). Remove the dimple strips from the bag only after they have been brought to room temperature to avoid condensation. Mix all the ingredients thoroughly by turning the vials several times. If you are performing the test on a color table, it is advisable to underlay the strips with white paper for a better overview when dripping into the wells and assessing the color in the wells.

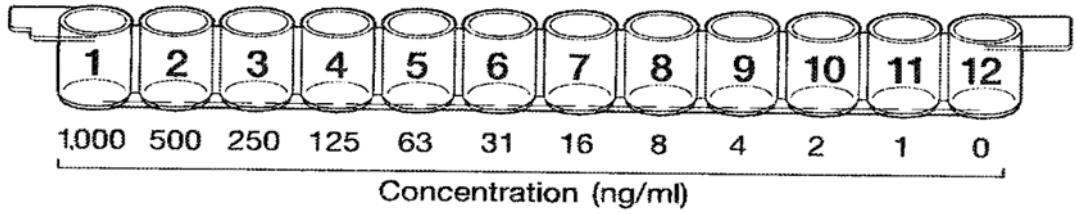
1.2. Prepare the working concentration of the washing solution by diluting it 10 times in a suitable volume of water using a measuring cylinder (60 ml of washing solution + 540 ml of H2O). Record the procedure used to prepare the washing solution in the protocol. Pour the washing solution into a suitable beaker/tray so that it can be easily sucked in with the dropper during washing. The unused washing solution diluted to the working concentration can be stored for a maximum of 2 weeks at a temperature of +2 to +10 °C. In the event that the diluted washing solution is ready to use.

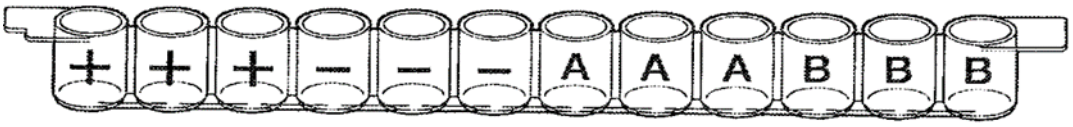
2.: Tracking disease outbreaks – detection by ELISA method

2.1. Preparation of calibration curve 1. strip

Prepare a sample breakdown for the protocol. Two strips according to fig3.

Strip n.1:





Strip n.2:

Fig 3 -Dilution process

Use PBS as a negative control (-). As positive (+) – antigen solution. It is advisable to add samples in triplets – 3 wells. Three wells are used to minimize lab error. The recommended dilution method for the calibration series is given in the sample application diagram (see Fig.3)

2.2. Simulate the spread of an infectious disease in the population:

2.2.1. Transfer the entire contents of your serum sample quantitatively to another student's sample using a Pasteur pipette.

2.2.2. Stir and remove 50 ml of sample

2.2.3. Repeat 1 more time with another student, mark the resulting mixed serum as your sample (e.g. A)

2.3. Preparation of the 1st and 2nd strips for antigen detection

2.3.1. Drop 2 drops (50 µL) of the standards and samples into the wells according to the schedule. (See. Fig 3):

3 wells with a positive control(+), 3 wells with a negative control(-) and 3 wells with an unknown student sample. (student A and B)

After each sample, rinse the pipette thoroughly with water or replace it with a clean one. Attention!!! Do not touch the walls or bottom of the wells with the pipette to avoid crossing the lay! Stop 5 minutes.

2.3.2. Pour the contents of the wells onto paper towels and carefully pat the upside-down strip upside down. Wash: Fill all wells (not to the brim) with Wash buffer and pour again. Repeat this wash 1 more time.

ATTENTION!!!

Avoid overflowing the solution out of the wells. Then tap the washing solution out of the wells again. Wash a total of 2 times and tap out the washing solution at the end so that the wells remain empty before the next step. You can wipe off the drops on the surface of the strip by tapping it on a piece of gauze or filter paper

2.3.3. Drip 50 µl of primary antibody into all wells. Hitchhike for 5 minutes, empty the holes and wash again 2 times. point 2.2.2.

2.3.4. Transfer (50 µL) of secondary antibody to all wells. Track for 5 minutes, empty all the holes and repeat the wash again (point 2.2.2.) 3 times.

2.3.5. Drip 100 µl of chromogen TMB (SUB) substrate solution in 3 drops (0.1 mL TMB/well). Track the time since the substrate was added to the

first hole. Incubate for 5 minutes (± 1 min.) at room temperature. In wells with low concentrations could be less color.

2.3.6. Check the resulting coloration of the holes. In wells with calibration standards, you should observe a decrease in coloration intensity depending on the antigen concentration. In a standard sample of 1000 ng/ml (the highest antigen concentration), there should be a maximum blue coloration. According to the scheme, assign the appropriate concentration value (intensely colored to uncolored) to each standard. By comparing the intensity of coloration of standards and samples, assign ratings to individual samples.

2.3.7. Perform concentration measurements in the wells of the strip, both the calibration series and your sample of unknown concentration. Perform the measurement using the end-point method at λ = 650nm in the ELISA reader. Enter the resulting absorbance values in the table:

Koncentrace antigenu *ng/ml*	Absorbance *nm*

2.3.8. Construct a calibration curve of the dependence of antigen concentration on absorbance. (concentration should be given in ng/ml)

Follow the instructions in Chart 1

Evaluation of the test:

A/ Antigen samples after the process of spreading an infectious agent:

- Write down the positive or negative nature of your sample

- draw the calibration curve of concentration on absorbance

- Reading values – read the concentration of mixed samples using the calibration curve.

B/ Write down the results:

The concentration of antigen in a pooled serum sample is:

The causative agent of the infectious agent is the student:

Control questions:

• What is the importance of antigen detection of an infectious agent in relation to the patient and why is the causative agent of the disease determined?

• Why is it necessary to use both primary and secondary antibodies?

• What is the function of the enzyme in this reaction?