Chapter 1

Microplate Reader

GMDN Code	**37036**
ECRI Code	**16-979**
Denomination	**Photometric micro-plate reader**

The microplate reader also known as "Photometric micro-plate reader or ELISA reader" is a specialized spectrophotometer designed to read results of the ELISA test, a technique used to determine the presence of antibodies or specific antigens in samples. The technique is based on the detection of an antigen or antibodies captured on a solid surface using direct or secondary, labelled antibodies, producing a reaction whose product can be read by the spectrophotometer. The word ELISA is the acronym for "Enzyme-Linked Immunosorbent Assay". This chapter covers the use of microplate readers for ELISA testing. For additional information on the instrument principles of operation and maintenance, consult Chapter 11 discussing the spectrophotometer.

PHOTOGRAPH OF MICROPLATE READER

Photo courtesy of BioRad Laboratories

PURPOSE OF THE MICROPLATE READER

The microplate reader is used for reading the results of ELISA tests. This technique has a direct application in immunology and serology. Among other applications it confirms the presence of antibodies or antigens of an infectious agent in an organism, antibodies from a vaccine or auto-antibodies, for example in rheumatoid arthritis.

OPERATION PRINCIPLES

The microplate reader is a specialized spectrophotometer. Unlike the conventional spectrophotometer which facilitates readings on a wide range of wavelengths, the microplate reader has filters or diffraction gratings that limit the wavelength range to that used in ELISA, generally between 400 to 750 nm (nanometres). Some readers operate in the ultraviolet range and carry out analyses between 340 to 700 nm. The optical system exploited by many manufacturers uses optic fibres to supply light to the microplate wells containing the samples. The light beam, passing through the sample has a diameter ranging between 1 to 3 mm. A detection system detects the light coming from the sample, amplifies the signal and determines the sample's absorbance. A reading system converts it into data allowing the test result interpretation. Some microplate readers use double beam light systems.

Test samples are located in specially designed plates with a specific number of wells where the procedure or test is carried out. Plates of 8 columns by 12 rows with a total of 96 wells are common. There are also plates with a greater number of wells. For specialized applications, the current trend is to increase the number of wells (384-well plates) to reduce the amount of reagents and samples used and a greater throughput. The location of the optical sensors of the microplate reader varies depending on the manufacturers: these can be located above the sample plate, or directly underneath the plate's wells.

Nowadays microplate readers have controls regulated by microprocessors; connection interfaces to information systems; quality and process control programs, which by means of a computer, allow complete test automation.

Equipment required for ELISA testing

In order to perform the ELISA technique, the following equipment is required:

1. Microplate reader.
2. Microplate washer (Chapter 2).
3. Liquid dispensing system (multi-channel pipettes may be used).
4. Incubator to incubate the plates.

Figure 1 illustrates how this equipment is interrelated.

Mechanical phases of the ELISA technique

Using the equipment

When an ELISA test is conducted, it typically follows these steps:

1. A first washing of the plate may be done using the microplate washer.
2. Using a liquid dispenser or the multi-channel pipettes, wells are filled with the solution prepared to be used in the test.
3. The plate is placed in the incubator where at a controlled temperature, a series of reactions take place.

Stages 1, 2 and 3 can be repeated several times depending on the test, until the reagents added have completed their reactions.

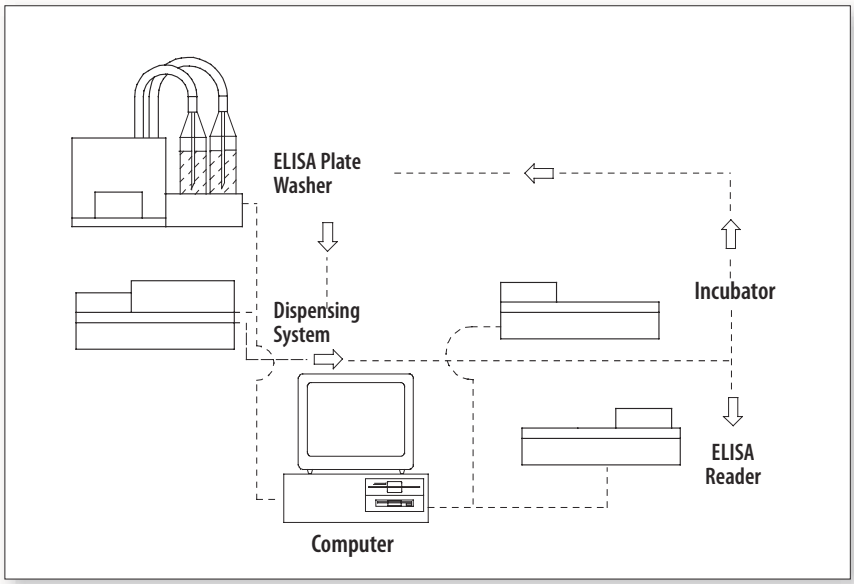
Finally, when all the incubation steps have been completed, the plate is transferred to the microplate reader. The reading of the plate is done and a diagnosis can be deduced.

Biochemical phases of the ELISA technique[1]

The ELISA technique from a biochemical point of view:

1. The plate wells are coated with antibodies or antigens.
2. Samples, controls and standards are added to the wells and incubated at temperatures ranging between room temperature and 37 °C for a determined period of time, according to the test's characteristics. During the incubation, the sample's antigen binds to the antibody coated to the plate; or the antibody in the sample binds to the antigen coated on the plate, according to their presence and quantity in the sample analyzed.
3. After incubation, the unbound antigen or antibodies are washed and removed from the plate by the microplate washer using an appropriate washing buffer.
4. Next, a secondary antibody, called the conjugate, is added. This harbours an enzyme which will react with a substrate to produce a change of colour at a later step.
5. Then begins a second period of incubation during which this conjugate will bind to the antigen-antibody complex in the wells.
6. After the incubation, a new washing cycle is done to remove unbound conjugate from the wells.
7. A substrate is added. The enzyme reacts with the substrate and causes the solution to change in colour. This will indicate how much antigen-antibody complex is present at the end of the test.
8. Once the incubation time is completed, a reagent is added to stop the enzyme-substrate reaction and to prevent further changes in colour. This reagent is generally a diluted acid.
9. Finally, the plate in is read by the microplate. The resulting values are used to determine the specific amounts or the presence of antigens or antibodies in the sample.

Figure 1. Equipment used in ELISA tests



Note: Some of the wells are used for standards and controls. Standards allow the cut-off points to be defined. The standards and controls are of known quantities and are used for measuring the success of the test, evaluating data against known concentrations for each control. The process described above is common, although there are many ELISA tests with test-specific variants.

[1] More detailed explanations must be consulted in specialized literature.

INSTALLATION REQUIREMENTS

In order for the microplate reader to operate correctly, the following points need to be respected:

1. A clean, dust free environment.
2. A stable work table away from equipment that vibrates (centrifuges, agitators). It should be of a suitable size so that there is working space at the side of the microplate reader. The required complementary equipment for conducting the technique described above is: washer, incubator, dispenser and computer with its peripheral attachments.
3. An electrical supply source, which complies with the country's norms and standards. In the countries of the Americas for example, 110 V and 60 Hertz frequencies are generally used, whereas other regions of the World use 220-240V, 50/60HZ.

Calibration of the microplate reader

The calibration of a microplate reader is a specialized process which must be executed by a technician or trained engineer following the instructions provided by each manufacturer. In order to do the calibration, it is necessary to have a set of grey filters mounted on a plate of equal geometric size to those used in the analyses. Manufacturers provide these calibration plates for any wavelength the equipment uses.

Calibration plates are equipped with at least three pre-established optic density values within the measurement ranges; low, medium, and high value. In order to perform the calibration, follow this process:

1. Place the calibration plate on the equipment.
2. Carry out a complete reading with the calibration plate. Verify if there are differences in the readings obtained from well to well. If this is the case, invert the plate (180°) and repeat the reading to rule out that differences are attributed to the plate itself. In general, it is accepted that the instrument does not need further calibration if the plate results are as expected at two wavelengths.
3. Verify if the reader requires calibration. If so, proceed with the calibration following the routine outlined by the manufacturer, verifying that the reading's linearity is maintained as rigorously as possible.
4. If the instrument does not have a calibration plate, verify it by placing a coloured solution in the wells of a plate and immediately carry out a complete reading. Then invert the plate 180° and read the plate again. If both readings display identical, average values in each row, the reader is calibrated.
5. Verify that the reader is calibrated, column by column. Place a clean, empty plate and carry out a reading. If there is no difference between each of the average reading of the first to the last column, it can be assumed that the reader is calibrated.

ROUTINE MAINTENANCE

Maintenance described next focuses exclusively on the microplate reader. The maintenance of the microplate washer is described in Chapter 2.

Basic maintenance

Frequency: Daily

1. Review that optical sensors of each channel are clean. If dirt is detected, clean the surface of the windows of the light emitters and the sensors with a small brush.
2. Confirm that the lighting system is clean.
3. Verify that the reader's calibration is adequate. When the daily operations begin, let the reader warm up for 30 minutes. Next, do a blank reading and then read a full plate of substrate. The readings must be identical. If not, invert the plate and repeat the reading in order to determine if the deviation originated in the plate or the reader.
4. Examine the automatic drawer sliding system. It must be smooth and constant.

Preventive maintenance

Frequency: Quarterly

1. Verify the stability of the lamp. Use the calibration plate, conducting readings with intervals of 30 minutes with the same plate. Compare readings. There must be no differences.
2. Clean the detectors' optical systems and the lighting systems.
3. Clean the plate drawer.
4. Verify the alignment of each well with the light emission and detection systems.

TROUBLESHOOTING TABLE		
PROBLEM	**PROBABLE CAUSE**	**SOLUTION**
The reader gives a reading that does not make sense.	The illumination lamp is out of service.	Replace the lamp with one with the same characteristics as the original.
The reader's readings vary from row to row.	Dirty optical sensors.	Clean the sensors.
	The illumination system's lenses or parts are dirty.	Clean the lighting system's lenses.
	Lack of calibration in one or more channels.	Verify the calibration of each one of the channels.
The reader displays high absorbance values.	Reagents expired and/or incorrectly prepared.	Check to see if the TMB is colourless and the preparation adequate.
	Contamination with other samples.	Repeat the test verifying the labelling, the washer and how the pipette was used.
	Incorrect wavelength filter.	Verify the recommended wavelength for the test. Adjust if it is incorrect.
	Insufficient or inefficient washing.	Verify the washing method used. Use an appropriate quality control test.
	Very long incubation time or very high temperature.	Check incubation times and temperatures.
	Incorrect sample dilution.	Check process for sample dilution.
	Some reagent was omitted.	Verify that the test has been carried out according to the established procedure.
The reader displays low absorbance values.	Very short incubation time and very low temperature.	Check temperatures and incubation times.
	The reagents were not at room temperature.	Check that the reagents are stable at room temperature.
	Excessive washing of the plate.	Adjust the washing process to what the test manufacturers indicate.
	Incorrect wavelength filter.	Verify the wavelength selected. Use wavelength recommended for the test.
	Expired or incorrectly prepared reagents.	Check the used reagents. Test the dilutions.
	A reagent was omitted.	Verify that the test was done according to the established procedure.
	The plate displays scratches at the bottom of the wells.	Prepare a new plate and repeat the test.
	Incorrectly selected or dirty plate.	Verify the type of plate used. Prepare a new plate and repeat the test.
	The plate wells have dried up.	Change the manner in which the plate is washed.
	The plate is incorrectly placed or is seated unevenly in the reader.	Check the placement of the plate. Repeat the reading.
	Humidity or fingerprints on the outer part of the bottom of the plate.	Verify that the plate under the bottom of the wells is clean.
	Residual quantities of washing buffer in the wells before adding the substrate.	Confirm that the washing buffer is completely removed.
	The substrate tablets do not dissolve completely.	Verify that the tablets dissolve correctly.
	The substrate tablet has been contaminated by humidity or metal clips or is not complete.	Test the integrity and handling of substrate tablets.
	The position of the blank well could have been changed and an incorrect quantity has been subtracted at each reading.	Verify that the plate set-up is correct.
The reader displays unexpected variation in the optical density readings.	The reader's lamp is unstable.	Replace the lamp with one that has similar characteristics as the original.
The reader displays a gradual increase or decrease from column to column.	Inappropriate calibration of the plate's advance motor.	Calibrate the advance so that at each step the wells remain exactly aligned with the lighting system.
The optical density readings are very low compared to the operator's optical evaluation criteria.	The reading is being carried out with a different wavelength than required for the test.	Verify the wavelength used when conducting the reading. If this is the problem, adjust the wavelength and repeat the reading. Verify that the recommended wavelength filter has been selected.

Low reproducibility.	Sample homogeneity.	Mix the reagents before use. Allow these to equilibrate to room temperature.
	Incorrect pipetting procedure.	Ensure pipette's tips are changed between samples and that excessive liquid inside is removed.
	Reader not calibrated.	Check the calibration. Use an appropriate quality control set.
	Reading without sufficient warming time of the instrument.	Wait until the reader has warmed up to its operating temperature.
	Expired reagents.	Verify the expiry dates of the reagents.
	Insufficient or inefficient washing.	Remove the buffer from the washer. Check that the wells are filled and aspirated in a uniform manner when washed.
The blank sample shows high absorbance.	Contaminated substrate.	Check that TMB is colourless and its preparation.
The data are not transferred from the reader to the microprocessor.	The reader and the microprocessor have differently defined codes.	Verify selected codes.
	Different (Baud) information transfer rates.	Confirm transfer rates selected.
	Incorrect configuration selected for the reception/ transmission plugs.	Review the configuration of the plugs. The configuration must follow parameters defined by the manufacturer.
Misaligned light beam.	The reader was transferred or moved without using the necessary precautions.	Call the specialized service technician.
	The light source – lamp – has been changed and the replacement has not been installed or aligned correctly.	Verify its assembly and alignment.
Incorrect identification of the sample.	The plate was incorrectly loaded.	Check the samples' identification process. Repeat the reading carrying out the adjustments.
	Incorrect identification of the sample registered in the reader.	Check the samples' identification process. Repeat the reading carrying out the adjustments.
Computer fails to indicate the error codes.	The programme which controls the activation of alarms and warning messages is defective or is not validated by the manufacturer.	Call the specialized service technician.
The reader demonstrates failure in detecting errors.	Various components of the system display failure, such as the liquid level detection system.	Call the specialized service technician.

BASIC DEFINITIONS

Chemiluminescence. Emission of light or luminescence resulting directly from a chemical reaction at environmental temperatures.

ELISA (Enzyme-Linked Immunosorbent Assay). Biochemical technique used mainly in Immunology to detect the presence of an antibody or an antigen in a sample.

ELISA plate. Consumable standardized to carry out the ELISA technique. Generally, plates have 96 wells in a typical configuration of 8 rows by 12 columns. There are also ELISA plates with 384 wells or up to 1536 wells for specialized high throughput testing in centres with high demand.

Microplate washer. Equipment used for washing plates during specific stages of an ELISA test with the aim of removing unbound components during reactions. Microplate washers use special buffers in the washing process.

Enzyme. Protein that accelerates (catalyses) chemical reactions.

Fluorophore. Molecules absorbing light at a determined wavelength and emitting it at a higher wavelength.

Microplate reader. The name given to spectrophotometers with the capacity to read microplates.

TMB. Tetramethylbenzidine, a substrate for the horseradish peroxidase (HRP) enzyme.