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Catalog No. 55-81-10, 55-81-50

INTENDED USE

Protein Detector™ ELISA Kits offer a convenient starting point for the development of microwell ELISA procedures for antigen or antibody detection. The kits provide pretested components and suggested protocols to facilitate the development of enzyme immunoassay procedures. Protein Detector ELISA Kits are available for the detection of mouse, rabbit or human primary antibodies.

INTRODUCTION

ELISA (Enzyme-Linked Immunosorbent Assay) has proven highly useful for the detection of antigen and/or antibody (1, 2). Protein Detector ELISA Kits offer the advantages of affinity purified antibody along with the convenience and improved reproducibility of pretested components. In the indirect procedure, antigens or antibodies are immobilized to the solid phase, then reacted with primary antibody. The alkaline phosphatase conjugated secondary antibody is reacted with the antigen antibody complex. Although elementary in principle, great effort is normally required to optimize this multi-step process to achieve a reliable assay procedure. The Protein Detector ELISA Kit substantially reduces the effort needed to develop a sensitive and reproducible assay.

MATERIALS AND EQUIPMENT

Kit Component	Product Code	Volume
Coating Buffer Concentrate	50-84-01	25 mL
BSA Diluent/Blocking Solution Concentrate	50-61-01	100 mL
Wash Solution Concentrate	50-63-03	100 mL x 2
50% Glycerol	50-83-00	5 mL
BluePhos Phosphatase Substrate System:		
BluePhos A	50-88-05	100 mL
BluePhos B	50-88-06	100 mL
BluePhos Stop Solution Concentrate	50-89-01	100 mL
Phosphatase-Labeled Antibody:		
For kit 55-81-10		
AP Anti-Human IgG (H+L)	05-10-06	0.1 mg
For kit 55-81-50		
AP Anti-Rabbit IgG (H+L)	05-15-06	0.1 mg
and AP Anti-Mouse IgG (H+L)	05-18-06	0.1 mg

Store reagents at 2 - 8°C. Kit reagents are stable for a minimum of 1 year from date of receipt when stored at 2 - 8°C. Sufficient reagents are provided for 2,000 tests (approximately 20 ELISA plates).

Materials not provided:

- Human, mouse, or rabbit primary antibodies
- Unlabeled Affinity Purified Antibody for use as a capture antibody (see KPL catalog)
- Microwell plates designed for immunoassay procedures (Tissue culture plates are not recommended.)
- Wash delivery system: 500 mL squeeze bottle or automatic plate washer
- ELISA Microplate reader with 630 filter
- Reagent quality water
- Pipettes, graduated cylinders, glass and plastic ware
- Multi-channel pipettes and reagent reservoirs
- Gloves
- Pipette tips

PRINCIPLE

Hybridoma Screening

This assay is a convenient means of analyzing Hybridoma cultures for the presence of antibodies of desired specificity. With the appropriate choice of labeled antibody, this assay will identify antigen specific antibodies of the human, mouse or rabbit.

Detection of Antigen or Antibody

- **Direct ELISA:** The simplest method for detection of antigen; an excellent means for demonstrating the principles of ELISA and for troubleshooting an assay. The antigen is applied to the plate and then the conjugate is reacted with it.

- **Indirect Antibody ELISA:** Capable of detecting either antigen or antibody, depending on which is defined as the unknown. It is most frequently used for the detection of specific antibodies to provide information on the immune status of an animal. It is also the procedure used to screen tissue culture fluids for monoclonal antibodies.

- **Antibody Sandwich ELISA:** an ultra sensitive method for the detection of antigen. It requires the use of highly purified antibodies for both the capture antibody and the detection antibody (enzyme conjugate). This is the preferred method when the amount of unknown antigen is too dilute to be detected when directly adsorbed onto the microplate surface, or when the presence of unknown constituents interfere with antigen detection.

DEFINING ASSAY CONDITIONS

Hybridoma Screening

The suggested procedure for monoclonal antibody screening is the Indirect Antibody ELISA (3). See page 12.

Detection of Antigen or Antibody

After carefully studying the ELISA procedures on pages 10 - 13, select the one that meets your requirements. The experimental conditions recommended in these procedures are adequate for many applications, and serve as a starting point for assay development. Variables such as reaction time, reagent concentration and temperature can be adapted to meet individual needs.

Each step of the procedure should be systematically evaluated to establish the conditions that yield the most sensitive assay. For example, the two dimensional serial dilution experiment described on page 9 provides a means for determining optimal reagent concentrations. This experiment, together with the appropriate controls, is also useful in diagnosing problems with the procedure, enabling the researcher to pinpoint the component(s) that cause erroneous results or high background.

After deciding on the experiment design plan and plate layout, assemble the needed materials and prepare an appropriate amount of the necessary reagents. Before use it is recommended to prewarm all solutions to room temperature and mix. For a more detailed description of each step in an ELISA, see Review of Assay Conditions page 6 - 7.

REVIEW OF ASSAY CONDITIONS

Plate Coating

A number of coating conditions, (antigen or antibody concentrations, pH, ionic strength, temperature and incubation time) affect the efficiency of binding. In addition, the amount of protein that binds has been shown to be inversely proportional to molecular weight. The Coating Solution supplied with the kit contains an optimized phosphate buffered saline solution that is satisfactory for binding most antigens and all antibodies to the plate. Plates should be specifically formulated for ELISA; polystyrene plates made for tissue culture often produce erratic backgrounds and are not recommended.

In the first step, antigen or antibody diluted in Coating Buffer is added to the plate wells and incubated at room temperature. It is important to use the purest antigen/antibody preparations possible. In general, a concentration of 1 - 10 ug of protein per mL of coating solution will give adequate surface saturation in approximately one hour at room temperature. For convenience, overnight coating at 2 - 8°C also provides satisfactory binding.

Following coating, the plate is blocked by adding BSA Diluent/Blocking Solution to the emptied wells and incubated for five minutes. This solution contains 1% bovine serum albumin (BSA) in phosphate buffered saline. It reduces nonspecific binding by blocking any unreacted sites on the plate surface and protects adsorbed protein from surface denaturation. Plates can be stored at this point in the refrigerator after covering to prevent evaporation. When required, plates should equilibrate to room temperature before proceeding.

Plate Washing

The plate is washed after addition of sample and conjugate. This helps to reduce background color by removing unbound reactant from the wells. An adequate washing procedure should be uniform from well-to-well with no carryover or residual liquid in the wells. Between washes, plate wells should be emptied. This can be accomplished manually by tapping the plate upside down to remove residual moisture. The Wash Solution in the kit contains 0.02% Tween, a wetting agent that minimizes nonspecific attachment of reactants to the solid phase. If the procedure is interrupted at any point, fill the wells with Wash Solution so that the plate does not dry out.

Phosphatase Labeled Antibody

The alkaline phosphatase in the secondary antibody conjugate serves as a detector which, when reacted with the substrate, demonstrates the presence of unknown in the sample. **CARE SHOULD BE TAKEN DURING THE PROCEDURE TO AVOID CONTACT WITH EDTA AS IT WILL INACTIVATE THE ENZYME**. All KPL antibodies are affinity purified and conjugated to the enzyme with the highest specific activity.

Substrate

The chromagenic substrate provides a sensitive detection method for the enzyme in the conjugate. The BluePhos Phosphatase Substrate produces a deep purple color over a period of time. The use of Stop Solution is an optional step that halts further color development in the wells, maintaining a desired level of color intensity for visual observation.

Controls and Samples

Every assay should include appropriate controls to verify performance of the test system, define the test background and establish that the material measured is the limiting component of the assay system. It is recommended to test all additions to the procedure for nonspecific reactivity. Quantitative ELISA procedures should be restricted to the comparison of an unknown with known standard reference material. The following controls should be performed in every microplate processed:

- **Background Controls:** Wells that are reacted with all reagents except the sample. This allows the cause of specific background to be more easily ascertained. Acceptable levels of background should be subtracted from test results to assure accurate assay to assay comparisons.
- **Negative Controls:** Wells containing known negative reference samples.
- **Threshold Controls:** Wells containing known low positive reference samples to define the cutoff value of a positive.
- **Positive Controls:** Wells containing known high positive reference samples that define the maximum linear signal of the assay.

Both controls and samples should be diluted in BSA Diluent/Blocking Solution to keep background to a minimum. Ideally, all tests should be performed in duplicate. The material to be measured should be the limiting component of the system determined empirically by the Assay Optimization procedure (page 9).

PREPARATION OF REAGENTS

Mix all solutions the day of use. Volumes given are sufficient for **ONE** 96-well microwell plate. Larger volumes may be prepared for multiple plate detection by increasing the volumes stated below accordingly, (i.e. multiply volumes by 2 for two plates, by 3 for three plates, etc.)

1. **1X Coating Solution:** Dilute the Coating Solution Concentrate 1/10 with reagent quality water (i.e. mix 1 mL of Coating Solution Concentrate + 9 mL reagent quality water). Note: If crystals appear in 10X concentrate, warm to room temperature or 37°C with mixing to redissolve.
2. **1X BSA Diluent/Blocking Solution:**

For Hybridoma Screening: Dilute BSA Diluent/Blocking Solution Concentrate 1/5 with reagent quality water (i.e. for blocking, mix 3 mL of BSA Diluent/Blocking Solution Concentrate + 12 mL reagent quality water; for antibody diluent, mix 0.5 mL BSA Diluent/Blocking Solution Concentrate + 4.5 mL reagent quality water).

For Other Assays: Dilute BSA Diluent/Blocking Solution Concentrate 1/10 with reagent quality water (i.e. mix 5 mL of BSA Diluent/Blocking Solution Concentrate + 45 mL reagent quality water). Note: If crystals appear in 10X concentrate, warm to room temperature or 37°C with mixing to redissolve.

3. **1X Wash Solution:** Dilute Wash Solution Concentrate 1/20 with reagent quality water (i.e. mix 15 mL of Wash Solution Concentrate + 285 mL reagent quality water). Diluted Wash Solution is stable for at least 6 months at room temperature. A minimum of 87 mL of the 1X solution is required per plate for the direct and hybridoma screening. 173 mL of the 1X solution is required for indirect or capture assays.
4. **50% Glycerol Solution:** Ready to use.
5. **Secondary Antibody Solution:** Rehydrate phosphatase-labeled antibody with 1 mL of 50% glycerol. The resulting 0.1 mg/mL conjugate solution may be stored at 2 - 8°C or -20°C for at least 1 year. Dilute to desired concentration with 1X BSA Diluent/Blocking Solution (from step 2). For most assays a concentration of 0.1 to 2 ug/mL is sufficient.
6. **Enzyme Substrate:** Prepare immediately before use. Mix 5 mL of BluePhos Solution A and 5 mL of BluePhos Solution B.
7. **Phosphatase Stop Solution:** Dilute BluePhos Stop Solution Concentrate 1/10 with reagent quality water (i.e. mix 1 mL BluePhos Stop Solution Concentrate + 9 mL reagent quality water).
8. **Samples:** Dilute samples in 1X Coating Buffer to keep background to a minimum.
9. **Controls:** See Review of Assay Conditions (page 6 - 7) for suggestions of appropriate controls.

ASSAY OPTIMIZATION

Optimization of an ELISA to fit a specific need involves the manipulation of three variables: reagent concentration, temperature and length of incubation. The use of the 96-well matrix on a microwell plate is an excellent method of simultaneously surveying many variations in reagent concentration and incubation time. The 96-well format does not allow easy testing of various incubation temperatures. From our experience, it is sufficient to run most assays at room temperature.

A serial dilution in one or two dimensions provides a means for determining optimal reagent concentrations and incubation times in the immunoassay procedure. As illustrated below, the assay can be designed to serially dilute the first reagent across the plate. In a subsequent step a second reagent is serially diluted down the plate. Each well represents a different concentration of the two reagents, permitting choice of the well representing the optimal combination of both reagents.

Another variation of the serial dilution assay involves diluting a single reagent across the plate at an appropriate step and varying the incubation times down the plate. In this instance, a single row of wells could be chosen representing the optimal balance between reagent concentration and length of incubation.

Performing a One-or-Two-Dimensional Serial Dilution on Microwell Plates:

The following procedure is suggested for performing two-fold serial dilutions on a microwell plate. It is best performed with an 8- or 12-channel multi-well pipette.

1. Add 100 uL diluent to the wells in columns 2 - 12.
2. Add 200 uL of diluted reagent to the wells in column 1.
3. Transfer 100 uL from column 1 to column 2. Mix carefully by filling and emptying pipette 3 - 5 times.
4. Repeat step 3 across the plate. Discard the final 100 uL from the wells in column 11 after mixing. Column 12 serves as a control.

The above procedure is repeated down the plate (rows A - G) in the appropriate step using a second reagent. In this instance row H serves as a control row. The control wells are important in verifying the functionality of the assay (See Results and Discussion, page 14).

PROCEDURES

Volumes are recommended for detection of 96-well plates. These volumes may be scaled to accommodate plates with differing numbers of wells.

Hybridoma Screening

Coating Solution: Dilute antigen in IX Coating Buffer to appropriate antigen concentration (see Assay Optimization, page 9).
Primary Antibody Solution: Culture fluids containing monoclonal antibody.
Secondary Antibody Solution: Dilute 0.025 mL phosphatase-labeled secondary antibody in 5.0 mL 1X BSA Diluent/Blocking Solution.

Apply Antigen

1. Adsorb antigen onto plate.
2. Incubate 1 hour at room temperature.
3. Empty plate and tap out residual liquid.

Block Plate

1. Add 150 uL 1X BSA Diluent/Blocking Solution to each well.
2. Incubate 5 - 15 minutes, empty plate and tap out residual liquid.

React Culture Fluids Containing Monoclonal Antibodies

1. Add 50 uL culture fluids containing monoclonal antibodies to each well.
2. React 1 hour at room temperature.
3. Empty plate, tap out residual liquid.

Wash Plate

1. Fill each well with 1X Wash Solution.
2. Invert plate to empty, tap out residual liquid.
3. Repeat for a total of 3 washes.

Add Secondary Antibody Solution

1. Add 50 uL Secondary Antibody Solution to each well.
2. React 1 hour at room temperature.
3. Empty plate, tap out residual liquid and wash as above.

React Substrate

1. Dispense 50 uL Substrate Solution into each well.
2. If desired, after sufficient color development, add 50 uL Stop Solution to each well.
3. Read plate with plate reader at 630 nm.

Direct ELISA

Coating Solution: Dilute antigen in 1X Coating Buffer to appropriate concentration (see Assay Optimization, page 9). A concentration of 1 - 10 ug/mL is usually sufficient.
Antibody Conjugate Solution: Dilute antibody conjugate in 1X BSA Diluent/Blocking Solution to appropriate concentration (see Assay Optimization, page 9).

Apply Antigen
1. Add 100 uL antigen diluted in 1X Coating Solution to appropriate wells.
2. Incubate 1 hour at room temperature.
3. Empty plate and tap out residual liquid.

Block Plate
1. Add 300 uL 1X BSA Diluent/Blocking Solution to each well.
2. React 5 minutes, empty plate and tap out residual liquid.

Add Antibody Conjugate Solution
1. Add 100 uL Antibody Conjugate Solution to each well.
2. Incubate 1 hour at room temperature.
3. Empty plate, tap out residual liquid.

Wash Plate
1. Fill each well with 1X Wash Solution.
2. Invert plate to empty, tap out residual liquid.
3. Repeat for a total of 3 washes.

React Substrate
1. Dispense 100 uL Substrate Solution into each well.
2. If desired, after sufficient color development, add 100 uL Stop Solution to each well.
3. Read plate with plate reader at 630 nm.

Indirect Antibody ELISA

Coating Solution: Dilute antigen in 1X Coating Buffer to appropriate concentration (see Assay Optimization, page 9). A concentration of 1 - 10 ug/mL is usually sufficient.
Primary/Secondary Antibody Solution: Dilute antibody in 1X BSA Diluent/Blocking Solution to appropriate concentration (See Assay Optimization, page 9).

Apply Antigen
1. Add 100 uL Coating Solution to appropriate wells.
2. Incubate one hour at room temperature.
3. Empty plate, tap out residual liquid.

Block Plate
1. Add 300 uL 1X BSA Diluent/Blocking Solution to each well.
2. Incubate 5 - 15 minutes, empty plate and tap out residual liquid.

React Primary Antibody
1. Add 100 uL Primary Antibody Solution to each well.
2. React 1 hour at room temperature.
3. Empty plate, tap out residual liquid.

Wash Plate
1. Fill each well with 1X Wash Solution.
2. Invert plate to empty, tap out residual liquid.
3. Repeat for a total of 3 washes.

Add Secondary Antibody Solution
1. Add 100 uL Secondary Antibody Solution to each well.
2. React 1 hour at room temperature.
3. Empty plate, tap out residual liquid and wash as above.

React Substrate
1. Dispense 100 uL Substrate Solution into each well.
2. If desired, after sufficient color development, add 100 uL Stop Solution to each well.
3. Read plate with plate reader at 630 nm.

Antibody Sandwich(Capture) ELISA

Coating Solution: Dilute antigen in 1X Coating Buffer to appropriate concentration (see Assay Optimization, page 9). A concentration of 1 - 10 ug/mL is usually sufficient.
Antigen Sample: Dilute antigen sample in 1X BS Diluent/Blocking Solution.
Secondary Antibody Solution: Dilute antibody in 1X BSA Diluent/Blocking Solution to appropriate concentration (See Assay Optimization, page .9).

Apply Capture Antibody
1. Add 100 uL Coating Solution to appropriate wells.
2. Incubate 1 hour at room temperature.
3. Empty plate, tap out residual liquid.

Block Plate
1. Add 300 uL BSA Diluent/Blocking Solution to each well.
2. Incubate 5 - 15 minutes, empty plate and tap out residual liquid.

React Sample Antigen
1. Add 100 uL Antigen Sample to each well.
2. React at room temperature for 1 hour to overnight.
3. Empty plate, tap out residual liquid.

Wash Plate
1. Fill each well with 1X Wash Solution.
2. Invert plate to empty, tap out residual liquid.
3. Repeat for a total of 3 washes.

Add Secondary Antibody Solution
1. Add 100 uL Secondary Antibody Solution to each well.
2. React 1 hour, room temperature.
3. Empty plate, tap out residual liquid and wash as above.

Read Substrate
1. Dispense 100 uL Substrate Solution into each well.
2. If desired, after sufficient color development, add 100 uL Stop Solution to each well.
3. Read plate with plate reader at 630 nm.

RESULTS AND DISCUSSION

The immunoassay is a sensitive method for comparing samples. Therefore, it must be interpreted in the context of appropriate controls. In an ideal assay the intensity of color development is proportional to the amount of bound conjugate which is proportional to the amount of unknown in the test solution. The accurate interpretation of test results is dependent on the results of standards and controls included in each run. They verify the functionality of the assay and provide the basis for comparison of test results. Duplicates ensure a measure of result reliability. Well to well variations of 10% are common; however, the results of any test where there is a significant difference between duplicates should be regarded as suspect. A more detailed discussion of controls and samples may be found in Review of Assay Conditions.

There are a number of ways of interpreting test results, depending on the type of information required by the researcher. The preferred method is to present the results of an unknown sample as a multiple of the reference positive. Accurate quantitation becomes possible by assaying a range of concentrations of the reference standard along with the unknown samples. Results can then be analyzed from a standard curve.

One of the most troubling problems in interpreting an ELISA test is the presence of background color. There is an acceptable level of background (see below) and as long as it is subtracted from the test result it will not have an adverse affect on results. Excessive background color may occur when test components of the assay cross-react; for example, the enzyme-labeled second antibody may recognize antigenic determinants in the material used to block the plate. In addition, false positives may result when the enzyme-labeled antibody reacts with the coating antigen.

The use of a plate reader is recommended and will facilitate evaluation of test results. For a valid assay the control wells should appear as follows:

Negative Controls	• Colorless
Background Controls	• Light yellow; absorbance readings of approximately 0.2 or less are acceptable.
Threshold Controls	• Pale yellow – light purple
High Positive Controls	• Intense purple

TROUBLESHOOTING

To increase specific signal:

1. Increase concentration of antigen used to sensitize plate or increase time of incubation.
2. Increase concentration of phosphatase conjugate or increase time/temperature of incubation.
3. Incubate substrate for a longer period of time before stopping.
4. Use purified antigen.
5. Use shorter incubation time or greater dilution of BSA Diluent/Blocking Solution to test for antigen displacement at blocking step.
6. Use fewer, more gentle plate washes to check Wash Procedure.

To reduce non-specific signal:

1. Decrease concentration of antigen used to sensitize plate or decrease time of incubation.
2. Decrease concentration of phosphatase conjugate or decrease time/temperature of incubation.
3. Incubate substrate for a shorter period of time before stopping.
4. Increase number of washes. Allow wash solution more time to elute unbound conjugate.
5. Make certain well is filled with BSA Diluent/Blocking Solution during blocking step.
6. Reduce cross-reactivity of conjugate to plate coating solution by adding low concentration of coating antigen or capture antibody to diluted conjugate.
7. If random background appears, check for washer malfunction. Refer to instrument manual for assistance.

RELATED PRODUCTS

Product	Size	Catalog No.
Protein Detector ELISA Kits		
HRP Anti-Human, ABTS System	20 plates	54-62-10
HRP Anti-Rabbit, ABTS System	20 plates	54-62-15
HRP Anti-Mouse, ABTS System	20 plates	54-62-18
Protein Detector Western Blot Kits		
(***include anti-mouse and anti-rabbit conjugates)***		
AP, BCIP/NBT System	2500 cm^2	55-11-50
HRP, TMB System	2500 cm^2	54-11-50
HRP, LumiGLO Chemiluminescent System	2500 cm^2	54-12-50
BluePhos Microwell Substrate and Stop Solution		
BluePhos 2-Component Microwell Substrate Kit	600 mL	50-88-00
BluePhos 2-Component Microwell Substrate Kit	2700 mL	50-88-01
BluePhos 2-Component Microwell Substrate Kit	50 mL	50-88-02
BluePhos 10X Concentrate Stop Kit	200 mL	50-89-00

REORDERING INFORMATION

The components of the Protein Detector ELISA Kit, AP System may be reordered using the following information:

Product	Size	Catalog No.
Coating Solution Concentrate Kit	50 mL	50-84-00
BSA Diluent/Blocking Solution Concentrate Kit	200 mL	50-61-00
Wash Solution Concentrate Kit	800 mL	50-63-00
BluePhos 2-Component Microwell Substrate Kit	600 mL	50-88-00
AP Anti-Human IgG (H+L)	1.0 mg	075-1006
AP Anti-Rabbit IgG (H+L)	1.0 mg	075-1506
AP Anti-Mouse IgG (H+L)	1.0 mg	075-1806

The recommendations of this bulletin are provided solely for the benefit of users who need practical guidance on immunoassay procedures. Because experimental conditions for use of the suggested products are beyond the control Kirkegaard & Perry Laboratories, it is impossible for Kirkegaard & Perry laboratories to implicitly guarantee the performance of the mentioned products for any and all assay procedures. Users who need additional information are encouraged to call Technical Services 800-638-3167 (USA) or 301-948-7755 for assistance.