KPL Protein Detector™
ELISA Kit

HRP, ABTS System

Catalog Number	*System*
5110-0009 (54-62-10)	*HRP Anti-Human IgG (H+L)*
5110-0010 (54-62-15)	*HRP Anti-Rabbit IgG (H+L)*
5110-0011 (54-62-18)	*HRP Anti-Mouse IgG (H+L)*

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5110-0009 (54-62-10), 5110-0010 (54-62-15), 5110-0011 (54-62-18)

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INTENDED USE

KPL 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. KPL 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]. KPL Protein Detector ELISA Kits offer the advantages of affinity purified antibody and 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 horseradish peroxidase conjugated secondary antibody is reacted with the antigen antibody complex. The addition of KPL ABTS Peroxidase Substrate, which turns blue-green in the presence of HRP, indicates a positive reaction. Ideally, the rate and degree of substrate color change caused by the HRP conjugate provides a measure of the unknown. Although elementary in principle, great effort is normally required to optimize this multi-step process to achieve a reliable assay procedure. The KPL Protein Detector ELISA Kit substantially reduces the effort needed to develop a sensitive and reproducible assay.

MATERIALS AND EQUIPMENT

KIT COMPONENTS	CAT. NO.	VOLUME
KPL Coating Buffer	5150-0015 (50-84-01)	25 mL
KPL BSA Diluent/Blocking Solution	5140-0007 (50-61-01)	100 mL
KPL 20X Wash Solution	5150-0009 (50-63-01)	400 mL
KPL 50% Glycerol	5290-0004 (50-83-00)	5 mL
KPL ABTS HRP Substrate	5120-0034 (50-64-00)	100 mL
KPL Peroxidase Solution B	5120-0037 (50-65-00)	100 mL
KPL HRP Stop Solution	5150-0016 (50-85-00)	25 mL
KPL HRP Labeled Antibody:		
HRP, Anti-Mouse IgG (H+L)	5220-0286 (04-18-06)	0.1 mg
HRP, Anti-Human IgG (H+L)	5220-0277 (04-10-06)	0.1 mg
HRP, anti-Rabbit IgG (H+L)	5220-0283 (04-15-06)	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 approximately 20 ELISA plates.

Materials not Provided:

- Human, mouse or rabbit primary antibodies
- KPL Unlabeled Affinity Purified Antibody for use as a capture antibody
- Microwell plates designed for immunoassay procedures (tissue culture plates are not recommended)
- Absorbent towels, paper or cloth
- Wash delivery system: 500 mL squeeze bottle or automatic plate washer
- ELISA microplate reader with 405 filter
- Reagent quality water
- Pipettes, graduated cylinders, glass and plastic ware
- Multichannel pipettes and reagent reservoirs
- Gloves
- Pipette tips

PRINCIPLE

Hybridoma Screening

The suggested 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.

- 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 ultrasensitive 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 detectable 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 pages 9-10.

Detection of Antigen or Antibody

After carefully studying the ELISA procedures on pages 8-11, 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 experimental plan and plate layout, assemble the needed materials and prepare an appropriate amount the necessary reagents. Before use it is advisable 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 pages 5-6.

REVIEW OF ASSAY CONDITIONS

Plate Coating

A number of coating conditions, i.e. 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 KPL 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 KPL 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 µg 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 KPL 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 KPL 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 KPL Wash Solution so that the plate does not dry out.

Peroxidase Labeled Antibody

The horseradish peroxidase 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 sodium azide as it will inactivate the enzyme. All SeraCare KPL antibodies are affinity purified and conjugated to enzymes with the highest specific activity.

Substrate

The chromogenic substrate provides a sensitive detection method for the enzyme conjugate. Generally, the color produced is proportional to the amount of unknown in the sample. The KPL ABTS HRP Substrate produces an intense blue-green reaction product which may be visually detectable in minutes. Color develops quickly for 1 - 2 hours, after which it reaches a plateau for several hours. The use of KPL 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 advisable 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 maximal linear signal of the assay.

Both controls and samples should be diluted in KPL 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 (pages 7-8).

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 doubling, tripling, etc. the volumes stated below.

1. **1X KPL Coating Solution**: Dilute the KPL Coating Solution Concentrate 1/10 with reagent quality water (i.e. mix 1 mL of KPL Coating Solution Concentrate + 9 mL reagent quality water).
   NOTE: If crystals appear in 10X concentrate, warm to room temperature or 37ºC with mixing to redissolve.

2. **1X BSA Diluent/Blocking Solution:**
   For Hybridoma Screening: Dilute KPL BSA Diluent/Blocking Solution Concentrate 1/5 with reagent quality water (i.e. mix 2 mL of KPL BSA Diluent/Blocking Solution Concentrate + 8 mL reagent quality water).

   For Other Assays: Dilute KPL BSA Diluent/Blocking Solution Concentrate 1/10 with reagent quality water (i.e. mix 5 mL of KPL 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 KPL Wash Solution**: Dilute KPL Wash Solution Concentrate 1/20 with reagent quality water (i.e. mix 15 mL of KPL Wash Solution Concentrate + 285 mL reagent quality water). Diluted KPL Wash Solution is stable for at least 6 months at room temperature.

4. **KPL 50% Glycerol Solution**: Ready to use.

5. **Secondary Antibody Solution**: Rehydrate peroxidase-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 KPL BSA Diluent/Blocking Solution (from step 2). For most assays a concentration of 0.1 - 2.0 µg/mL is sufficient.

6. **Enzyme Substrate**: Prepare immediately before use. Mix 5 mL KPL ABTS Peroxidase Substrate Solution with 5 mL Peroxidase Solution B.

7. **KPL HRP Stop Solution**: Dilute KPL HRP Stop Solution 1/5 with reagent quality water (i.e. mix 2 mL HRP Stop Solution + 8 mL reagent quality water).
   NOTE: If solution is stored at 2-8°C, it will crystallize into a solid. Warm to room temperature with mixing to dissolve.

8. **Samples**: Dilute samples in 1X KPL Coating Buffer to keep background to a minimum.

9. **Controls**: See Review of Assay Conditions (pages 5-6) 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 µL diluent to the wells in columns 2 - 12.
2. Add 200 µL of diluted reagent to the wells in column 1.
3. Transfer 100 µL 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 µL 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 11).

PROCEDURES

Hybridoma Screening

Coating Solution: Dilute antigen in 1X Coating Buffer to appropriate antigen concentration (see Assay Optimization pages 7-8).

Primary Antibody Solution: Culture fluids containing monoclonal antibody.

Secondary Antibody Solution: Dilute 0.025 mL peroxidase-labeled secondary antibody in 5.0 mL 1X KPL 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 µL 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 µL 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 3 - 5 times.

Add Secondary Antibody Solution

1. Add 50 µL Secondary Antibody Solution to each well.
2. React 1 hour at room temperature.
3. Empty plate, tap out residual liquid and wash as above.
4. Give final 5 minute soak in Wash Solution; tap residual liquid from plate.

React Substrate

1. Dispense 50 uL Substrate Solution into each well.
2. If desired, after sufficient color development, add 50 µL Stop Solution to each well.
3. Read plate with plate reader at 405 nm.

Direct ELISA

Coating Solution: Dilute antigen in 1X KPL Coating Buffer to appropriate concentration (see Assay Optimization, pages 7-8). A concentration of 1 - 10 µg/mL is usually sufficient.

Antibody Conjugate Solution: Dilute antibody conjugate in 1X KPL BSA Diluent/Blocking Solution to appropriate concentration (see Assay Optimization, page 9).

Apply Antigen

1. Add 100 µL 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 µL 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 µL 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 3 - 5 times.
4. Give final 5 minute soak in Wash Solution; tap residual liquid from plate.

React Substrate

1. Dispense 100 µL Substrate Solution into each well.
2. If desired, after sufficient color development, add 100 µL Stop Solution to each well.
3. Read plate with plate reader at 405 nm.

Indirect Antibody ELISA

Coating Solution: Dilute antigen in 1X KPL Coating Buffer to appropriate concentration (see Assay Optimization, pages 7-8). A concentration of 1 - 10 µg/mL is usually sufficient.

Primary/Secondary Antibody Solution: Dilute antibody in 1X KPL BSA Diluent/Blocking Solution to appropriate concentration (See Assay Optimization, pages 7-8).

Apply Antigen

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

Block Plate
1. Add 300 µL 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 µL 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 KPL Wash Solution.
2. Invert plate to empty, tap out residual liquid.
3. Repeat 3 - 5 times.

Add Secondary Antibody Solution
1. Add 100 µL Secondary Antibody Solution to each well.
2. React 1 hour at room temperature.
3. Empty plate, tap out residual liquid and wash as above.
4. Give final 5 minute soak in Wash Solution; tap residual liquid from plate.

React Substrate
1. Dispense 100 µL Substrate Solution into each well.
2. If desired, after sufficient color development, add 100 µL KPL Stop Solution to each well.
3. Read plate with plate reader at 405 nm.

Antibody Sandwich(Capture) ELISA
Coating Solution: Dilute antigen in 1X KPL Coating Buffer to appropriate concentration (see Assay Optimization, pages 7-8). A concentration of 1 - 10 µg/mL is usually sufficient.

Antigen Sample: Dilute antigen sample in 1X KPL BSA Diluent/Blocking Solution.

Secondary Antibody Solution: Dilute antibody in 1X KPL BSA Diluent/Blocking Solution to appropriate concentration (See Assay Optimization, pages 7-8).

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

Block Plate
1. Add 300 µL KPL 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 µL 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 KPL Wash Solution.
2. Invert plate to empty, tap out residual liquid.
3. Repeat 3 - 5 times.

Add Secondary Antibody Solution

1. Add 100 µL Secondary Antibody Solution to each well.
2. React 1 hour, room temperature.
3. Empty plate, tap out residual liquid and wash as above.
4. Give final 5 minute soak in Wash Solution; tap residual liquid from plate.

Read Substrate

1. Dispense 100 µL Substrate Solution into each well.
2. If desired, after sufficient color development, add 100 µL Stop Solution to each well.
3. Read plate with plate reader at 405 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 effect 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	• Little to no color; absorbance readings of approximately 0.2 or less are acceptable.
Threshold Controls	• Moderately blue-green
High Positive Controls	• Deep blue-green

TROUBLESHOOTING

To increase specific signal:

1. Increase concentration of peroxidase conjugate or increase time/temperature of incubation.
2. Incubate substrate for a longer period of time before stopping.
3. Increase concentration of antigen used to sensitize plate or increase time of incubation.
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 peroxidase 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.

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NOTES:

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