Measurement of Polyclonal Immunoglobulin Synthesis Using ELISA

The enzyme-linked immunosorbent assay (ELISA) is a simple, specific, highly reproducible, and nonradioactive method for measuring immunoglobulin levels in cell culture supernatants and other biologic fluids. It can be used to measure the common immunoglobulin classes (IgG, IgM, and IgA) as described in the basic protocol, as well as classes and subclasses found in low levels in these fluids (IgG4 and IgE), as detailed in the alternate protocols.

CAUTION: When working with human blood, cells, or infectious agents, biosafety practices must be followed (see Chapter 7 introduction).

MEASUREMENT OF IgG, IgM, AND IgA RESPONSES BY ELISA

The ELISA described in this protocol can be used for measuring immunoglobulins in serum or in culture supernatants (Fig. 7.12.1). In these assays, a first (capture) antibody is adsorbed on the well surfaces of microtiter plates. After blocking unbound sites, supernatants are incubated in separate wells, allowing the immunoglobulin of interest to bind to the capture antibody. Unbound supernatant components are removed by washing, and a second antibody conjugated to alkaline phosphatase (or horseradish peroxidase) directed against the immunoglobulin of interest is bound to that immunoglobulin. Excess conjugate is washed away, and substrate solution is added to each well. The amount of color developed by the interaction of the enzyme conjugate with substrate is directly proportional to the amount of immunoglobulin in the supernatant.

Materials

First (capture) antibody: 10 μg/ml goat anti-human IgM, IgG, or IgA (IgG fraction) in coating buffer
Wash buffer: 0.05% (v/v) Tween 20 in PBS (APPENDIX 2)
Blocking buffer: 5% (w/v) BSA in wash buffer (filter sterilize; store at 4°C)
Immunoglobulin standards (see reagents and solutions) or supernatant from mononuclear cell cultures (UNIT 7.13)
Diluent buffer: 1% (w/v) BSA in wash buffer (filter sterilize and store at 4°C)
Second antibody: affinity-purified, Fc-specific, alkaline phosphatase–conjugated goat anti-human IgM, IgG, or IgA antibody (Sigma or Jackson Immunoresearch)
1 mg/ml p-nitrophenyl phosphate (Sigma) in substrate buffer
3 M NaOH
96-well ELISA plates (e.g., Immulon 4, Dynatech)
Plate sealers (Dynatech #001-010-350) or plastic wrap
Multiwell scanning spectrophotometer

1. Add 100 μl of 10 μg/ml first antibody in coating buffer to the wells of a 96-well ELISA plate. Cover plate with plastic seal. Incubate 2 hr at 37°C (or overnight at 4°C).

These plates can usually be stored in a humidified chamber for as long as 1 month at 4°C; however, the stability of each antibody preparation must be verified and care must be taken to prevent evaporation.

2. Wash plate five times by successively filling wells with wash buffer and then discarding the wash buffer. Aspirate with a plastic tip, or strike plate sharply on a firm surface to remove residual fluid from the last wash.

Various ELISA plate washers are available for automation of the wash process;
however, washing is easily accomplished using a squeeze bottle containing wash buffer to fill the wells, followed by a flick of the plate over a sink to empty the wells.

3. Block unbound sites by adding 200 µl of blocking buffer to each well. Incubate 1 hr at room temperature and wash as in step 2.

4. Add 100 µl immunoglobulin standards or supernatant fluid (diluted to appropriate concentrations with diluent buffer) to wells and incubate 2 hr at room temperature (or overnight at 4°C).

   For measurement of immunoglobulin in supernatants from unstimulated mononuclear cell cultures, the supernatant fluid can be used undiluted. For mitogen-driven culture supernatants, dilutions of 1:10 or 1:20 or more will be necessary.

   The immunoglobulin standards can be diluted either in diluent buffer or in the basal medium used for the supernatant generation (UNIT 7.13). In general, the working range for the ELISA is between 100 ng/ml and 1 ng/ml, so that serial dilutions from 100 ng/ml can be made accordingly. The diluted standards should be performed in duplicate or triplicate. Unknown samples should be run in duplicate.

5. Wash plate as above (step 2).

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**Figure 7.12.1** Antibody-sandwich ELISA to detect polyclonal Ig response. Ag = antigen; Ab = antibody; E = enzyme.
6. Add 100 µl of alkaline phosphatase–linked goat anti-human IgM, IgG, or IgA antibody (second antibody) to each well. Incubate 2 hr at room temperature or overnight at 4°C.

7. Wash plate as in step 2. Add 100 µl of 1 mg/ml \( p \)-nitrophenyl phosphate in substrate buffer to each well.

8. Read absorbance at 405 to 410 nm on a multiwell scanning spectrophotometer.

9. Add 25 µl of 3 M NaOH to stop the reaction, if necessary.

   The reaction should be stopped as the color in the wells with the highest concentration of the standards reaches the threshold optical density of the ELISA reader.

10. Plot absorbance versus antibody concentration (of standards) on semilog paper. Use the linear range of curve for interpolation of the experimental values.

   There are many ways to derive antibody concentrations from known standards, including Rodbard analysis (Rodbard and Feldman, 1978) and least squares fit. The method used here is simple and can be done without a computer. For further discussion of data analysis, see key references.

**MEASUREMENT OF IgG SUBCLASS RESPONSES USING ELISA**

To detect some of the IgG subclasses, an ELISA with additional sensitivity (below 1 ng/ml) is necessary. In this assay, this is accomplished by using a biotinylated antibody to human IgG with enzyme-linked streptavidin, rather than antibody directly linked to enzyme. Since one immunoglobulin molecule can accommodate up to 12 molecules of biotin, this strategy theoretically increases sensitivity by a factor of 10 to 12.

**Additional Materials**

- Anti-subclass antibody (first antibody): 10 µg/ml IgG fraction of monoclonal antibodies directed against human IgG1, IgG2, IgG3, and IgG4, in PBS (APPENDIX 2)
- Biotinylated, Fc-specific, affinity-purified goat anti-human IgG (e.g., GIBCO/BRL, or Jackson Immunoresearch)
- Alkaline phosphatase–conjugated streptavidin

1. Add 100 µl of 10 µg/ml anti-subclass antibody (first antibody) in PBS to the wells of an ELISA plate and cover plate with plastic seal. Incubate overnight at 4°C.

   See note regarding storage in step 1 of basic protocol.

2. Wash plate and block unbound sites as described in steps 2 and 3 of basic protocol.

3. Add 100 µl subclass antibody standards or supernatant fluid (diluted to appropriate concentrations with diluent buffer) to wells. Incubate 2 hr at 37°C and then overnight at 4°C. Wash plate as before.

   Initially it is best to use undiluted supernatants for subclass measurements.

4. Add 100 µl biotinylated goat anti-human IgG (second antibody) to each well. Incubate 2 hr at room temperature or overnight at 4°C. Wash plate as in step 2 of basic protocol.

   Each anti-IgG preparation must be optimized. Generally, a concentration of 1 µg/ml will be optimal.

5. Add alkaline phosphatase–conjugated streptavidin, prepared in diluent buffer at manufacturer’s recommended dilution. Incubate 2 hr at room temperature.

6. Proceed as in steps 7 through 10 of the basic protocol.
MEASUREMENT OF IgE RESPONSES USING ELISA

The basic approach to the measurement of IgE in supernatants using an ELISA assay is similar to that used for the IgG subclasses in that a specific monoclonal first antibody is used to coat microtiter wells and a biotinylated second antibody is used to reveal the bound supernatant-derived IgE. Nevertheless, the ELISA assay for IgE is subject to more variability than the IgG subclass assays (Helm et al., 1986), and in general, more care must be taken in the generation of the supernatants and in the execution of the ELISA.

Additional Materials

1 µg/ml monoclonal anti-IgE antibody (Novo Biolabs) in coating buffer
Biotinylated, Fc-specific, affinity-purified goat anti-human IgE–F(ab′)2 fragment
or biotinylated, purified polyclonal or monoclonal anti-IgE in diluent buffer
Alkaline phosphatase–streptavidin in diluent buffer

1. Add 100 µl of 1 µg/ml anti-IgE first antibody in coating buffer to the wells of an ELISA plate and cover plate with plastic seal. Incubate overnight at 4°C.
   
   See note regarding storage in step 1 of basic protocol.

2. Wash plate and block unbound sites as described in steps 2 and 3 of the basic protocol.

3. Add 100 µl IgE standards or supernatant fluid to wells. Incubate 2 hr at 37°C and then overnight at 4°C. Wash plate as before.
   
   Initially it is best to use undiluted supernatants for the IgE measurements.

4. Add 100 µl biotinylated, purified polyclonal or monoclonal anti-IgE (second antibody) diluted to the appropriate concentration in diluent buffer. Incubate 6 hr at room temperature or overnight at 4°C. Wash plate as in step 2 of basic protocol.

5. Add alkaline phosphatase–conjugated streptavidin, prepared in diluent buffer at manufacturer’s recommended concentration. Incubate 2 hr at room temperature.

6. Proceed as in steps 7 through 10 of the basic protocol.

BIOTINYLATION OF IMMUNOGLOBULIN

Additional Materials

Affinity-purified polyclonal antibodies or monoclonal antibodies, precipitated twice with ammonium sulfate (UNIT 2.7)
0.1 M NaHCO3, pH 8.0
1 mg/ml biotin N-hydroxysuccinimide ester (Pierce) in dimethylsulfoxide (DMSO; store at −70°C)
Phosphate-buffered saline (PBS; APPENDIX 2)
Additional reagents and equipment for protein dialysis (APPENDIX 3)

1. Dilute affinity-purified polyclonal antibodies, or monoclonal antibodies precipitated twice with ammonium sulfate, in 0.1 M NaHCO3, pH 8.0, to a final concentration of 1 mg/ml.

2. Dialyze overnight at 4°C against 0.1 M NaHCO3.

3. Add 200 µl of 1 mg/ml biotin N-hydroxysuccinimide ester in DMSO. Incubate 4 hr at room temperature.

4. Dialyze overnight at 4°C against PBS.
REAGENTS AND SOLUTIONS

Coating buffer

A: 8.4 g NaHCO₃/100 ml H₂O (1 M)
B: 10.6 g Na₂CO₃/100 ml H₂O (1 M)
Mix 45.3 ml A with 18.2 ml B and add H₂O to 1 liter. Adjust to pH 9.6 as needed with A or B. Store at room temperature.

Immunoglobulin standards

Standards containing known concentrations of IgG, IgA, IgM can be purchased commercially as part of radial immunodiffusion kits (ICN).
IgG subclass standards can be purchased as part of a kit from ICN or obtained from the World Health Organization (WHO).
Standards for IgE are commercially available from Pharmacia.

Monoclonal and polyclonal antibodies

Cell lines producing antibodies to the IgE and IgG subclasses are available from ATCC (see APPENDIX 5) as follows: IgG1, CRL 1755; IgG2, CRL 1788; IgG3, CRL 1774; IgG4, CRL 1776; IgE, HB 121.
All labeled and unlabeled polyclonal antibodies are commercially available. Affinity-purified antibodies have given the best results.

Substrate buffer

1.69 g NaHCO₃
2.51 g Na₂CO₃
0.41 g MgCl₂
H₂O to 1950 ml
Adjust pH to 8.6 with concentrated NaOH or HCl and add H₂O to 2 liters.

COMMENTARY

Background Information

The ELISA is the method of choice for measuring immunoglobulins in culture systems because of its sensitivity (equivalent to that of radioimmunoassays), simplicity, and speed. An added advantage is that it does not require the use of radioactive isotopes.
The ELISA technique was first described by Engvall and Perlmann (1971, 1972). Subsequently, it has been extensively discussed in books detailing the development, use, and limitations of ELISA technology (key references).
Alkaline phosphatase–conjugated antibodies are particularly useful for measuring antibodies produced in vitro because color development is easily apparent without the addition of acids or bases to stop the reaction. Other enzyme-antibody conjugates have been used successfully to measure human antibodies, including horseradish peroxidase, glucose oxidase, and urease. A complete discussion of these enzymes can be found in Voller and Bidwell (1987), Winston et al. (1989), and Fuller et al. (1989). The addition of an amplification step using of biotinylated second antibody followed by enzyme-labeled streptavidin results in 10-fold greater sensitivity.

Critical Parameters

Reagents used in these protocols must be of high quality and purity. Each reagent, if imperfect, is capable of modifying the data. Even the microtiter plates can vary between manufacturers (or between different lots from the same manufacturer), so care should be taken to optimize and standardize all components of the experiment.
The specificity and the sensitivity of the assay are clearly related to the enzyme-conjugated antibody. Each antibody used in the assays must be checked for isotype specificity, and the other reagents must be used in a manner to prevent nonspecific binding. This is particularly important when using IgE and IgG subclass antibodies (Hussain et al., 1986) where in vitro levels of immunoglobulin are relatively low and the assay’s sensitivity depends on low background. A blocking step is added in each procedure to reduce nonspecific binding and is essential for detecting antibodies found in low
levels. Incubation times can be increased or incubation temperatures raised to 37°C for increasing assay sensitivity.

**Anticipated Results**

ELISAs for IgG, IgM, and IgA have a detection range of 500 ng/ml to 1 ng/ml, whereas those for IgE and the IgG subclasses range from 100 ng/ml to 100 pg/ml when optimized.

**Time Considerations**

The procedures vary with each antibody being measured, but for assays where sensitivity is of concern (as with in vitro IgE antibody production), 2 days are required; most of this time is needed for incubations.

**Literature Cited**


**Key References**


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