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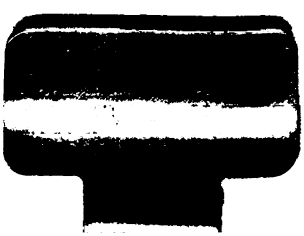
SERIE: ANIMAL HEALTH. SCIENTIFIC PUBLICATION No. 15



MANUAL OF RAPID DIAGNOSIS OF ANIMAL VIRUS DISEASES BY ENZYME IMMUNOASSAY METHODS

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PREFACE

In recent years, the best known immuno-enzymatic techniques such as ELISA (Enzyme-linked Immuno-sorbent Assay), have been developed to diagnose diseases in humans, as well as in animals and plants. Their high sensitivity, specificity, speed and economy convert them into essential instruments for diagnosis. Normally they are superior to many conventional serological techniques, and are applicable not only in virology, which has great advantages, but in microbiology in general, toxicology, and modern biotechnology.

This manual is a product of the presentations and practices developed in the Course for Veterinary Laboratory Technicians of Government Agencies in Latin American Countries, held in the City of Buenos Aires, Argentina, on September 19-20, 1985. This course was organized under the direction of the Animal Health Program of the Inter-American Institute for Cooperation on Agriculture (IICA), with the collaboration of the Department of Animal Health of the Center for Veterinary Research of the National Institute of Agricultural Technology (INTA) of Argentina and the Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech - VPI, Blacksburg, Virginia.

IICA's Animal Health Program is pleased to present this publication as part of its technical cooperation activities for the veterinary services of Latin American countries and other areas. Likewise, IICA expresses its deep appreciation to the authors of this manual, who generously shared their knowledge and experience to complete this publication, which we are sure will be of great use toward control and prevention of animal viral diseases.

Héctor Campos López

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Introduction

Infectious agents are generally diagnosed by isolating the etiologic agent in cultures despite the disadvantage of this procedure in those frequent cases in which a rapid diagnosis is required. Growing pure, virus-rich cultures is a time-consuming process, which means that, even if the clinic receives an accurate diagnosis, the information arrives too late for any effective change to be made in the treatment of animal patients. Culturing and identifying viruses often takes two to four weeks and longer. Besides, many viruses are difficult to cultivate in the tissue cultures usually available in laboratories. This is generally the case with such organisms as rotavirus, adenovirus, Norwalk virus and astrovirus.

In this situation, it becomes urgent to develop more rapid and more sensitive methods for the diagnosis of viral diseases.

One way to perform quick, accurate diagnoses is direct identification of the pathogens in the clinical sample. This is entirely feasible because most pathogens contain protein or polysaccharide antigens that can be readily distinguished from host cell antigens by enzyme immunoassay (EIA) procedures.

There are a number of EIA procedures by which the distinctive antigens of infectious agents can be detected. To be sucessful, however, the immunoassay must be sensitive, rapid, convenient and, moreover, specific.

Quick and reliable diagnostic methods require stable reagents that are available whenever needed. The EIA methods meet this requirement, one of those most frequently employed being the enzyme-linked immunosorbent assay, or ELISA. Enzyme immunoassays rely on enzyme-labeled immunoglobulins and proteins that retain their specific reactivity with antigens.

The usefulness of the enzyme immunoassay is based on the inherent magnification of the enzyme substrate system, since one single molecule of alkaline phosphatase or horseradish peroxidase - the enzymes employed in these tests - can react with up to 100,000 molecules of substrate per minute and, in the proper conditions, generate a visible product. This remarkable magnification effect allows for the measurement of very

minute quantities of antigen or antibody - often in the picogram range - by the simple measurement of an enzyme-substrate reaction.

The best substrates are those that in reaction generate a visible color that is easy to measure by instruments or with the naked eye.

Advantages of enzyme immunoassays

- Inherent magnification of enzyme substrate reaction ($x10^6$/10 min.)
- Stability of labeled reagents
- Results measured by visual interpretation or simple instruments
- Small reaction volumes
- Quantitative results
- Ability to control reactions
- Low biological hazard potential
- Low cost

Enzyme immunoassays are excellent rapid-diagnosis tools because they permit the detection of viruses difficult to culture in ordinary cell cultures like the pathogens of diarrheal and respiratory diseases.

EIAs can also be used to work with large numbers of samples, a particular advantage in studying the epidemiology of infectious diseases.

There are today many EIAs that can be performed in 20 minutes, which offers advantages in the treatment of infectious diseases.

Disadvantages of enzyme immunoassays

- Dependence on antigen-antibody kinetics

  Cross-reactivity
  Overspecificity
  Limitations in antibody affinity

- Nonspecific reactivity
- Variation in ability of antigens to bind to the solid phase.
- Loss of antibody affinity following conjugation with enzyme
- Variability of the enzyme-substrate reaction
- Sensitivity of the enzyme activity to inhibitors

- Presence of endogenous enzyme activity of the antigens to be detected
- Limitations in detection of the substrate or its breakdown products.

Methods for the performance of enzyme immunoassays

Direct (sandwich) method for the determination of antigen

1. In this method the antibody specific to the antigen (called the first antibody) is adsorbed to the solid phase. While several types of solid phases can be used, one of the more common of them is the plastic microtiter plate with 96 wells, allowing many reactions to be carried out at the same time. There are several commercial sources from which these solid phases may be obtained. One in considerable use is the Immulon II (Dynatech Laboratories, Alexandria, VA, USA).

   Antibodies can also be coated onto nitrocellulose paper and plastic beads as the solid phase instead of onto microtiter plates. In theory, the most efficient solid phases for this test are those that form covalent bonds through the carboxyl groups of the antibody molecules. Proteins are physically adsorbed to the plastic in noncovalent bonds.

2. Add the sample to the solid phase coated with the first antibody. In this step antigen present in the sample binds to the first antibody adsorbed to the solid phase. All excess antigen that does not react specifically with antibody is removed in the wash step that follows.

3. Wash in the presence of detergents.

4. Add enzyme-labeled (conjugated) antibody specific to the antigen. In this step the enzyme-labeled antibody binds to the antigen.

5. Wash in the presence of detergents.

   To remove all enzyme-labeled antibody that did not react specifically with the antigen.

6. Add the substrate.

   The enzyme coupled to the antibody reacts with the substrate to produce a result measurable by a change of color in the reactants. The reaction must be stopped at a predetermined time. This is done by adding a drop of sulfuric acid to the reaction. The change of color may be measured with a spectrophotometer or colorimeter specially designed

for microtiter plates, or by the naked eye. The change of color is proportional to the quantity of antigen present in the sample.

The direct method has the advantage of being simple, rapid, and minimally nonspecific in its reactivity. However, it requires the availability of enzyme-labeled antibodies specific to each virus or antigen to be measured.

7. Principal steps (Fig. 1).

Indirect (antiglobulin sandwich ELISA) method for the detection of antigen

1. Adsorb first antibody.

2. Wash with detergents.

3. Add the sample to the solid phase coated with first antibody.

4. Wash with detergents.

5. Add unlabeled antibody specific to the antigen (called the second antibody).

6. Wash.

   To remove all excess of second antibody that has not combined specifically with antigen.

7. Add specific enzyme-labeled immunoglobulin directed at the second antibody.

8. Wash.

9. Add substrate.

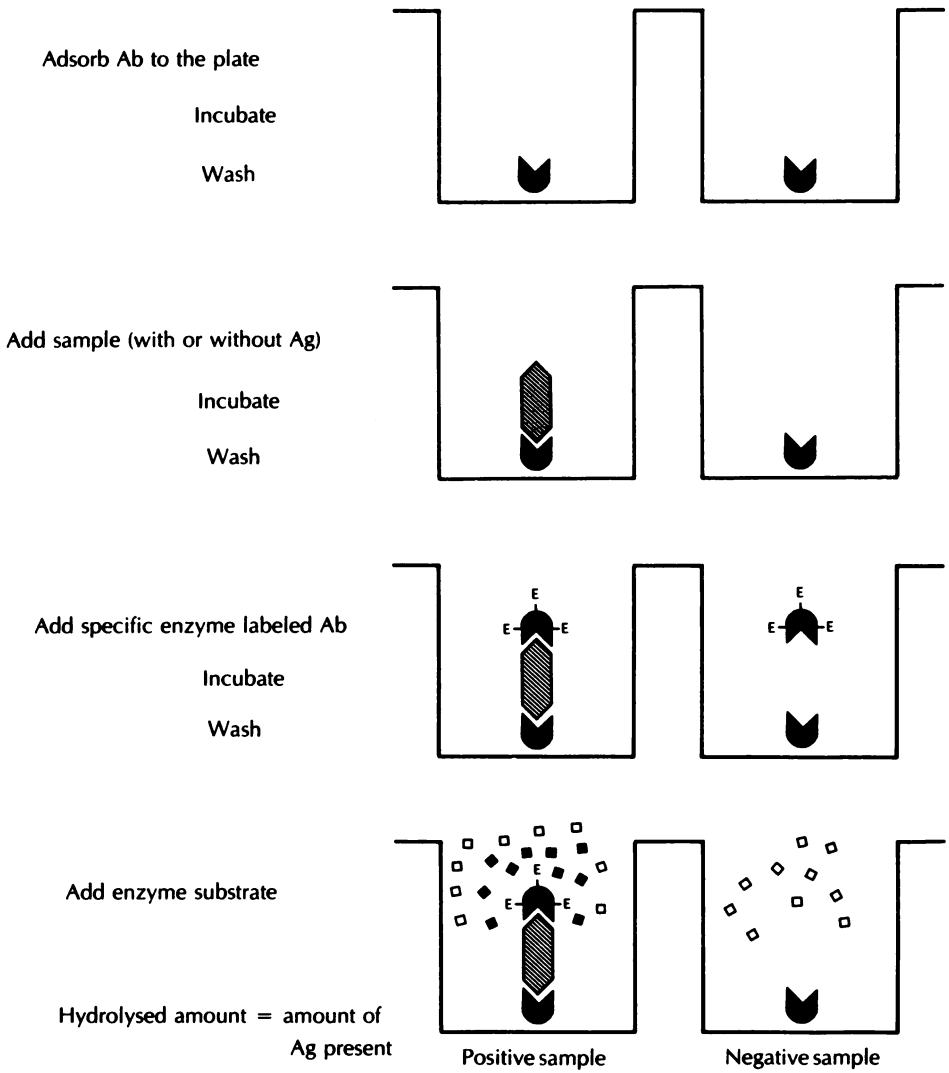
10. Stop reaction and measure change of color.

11. Principal steps (Fig. 2).

Staphylococcal protein A may be used instead of labeled immunoglobulin. Labeled protein A has the property of binding or adhering to the Fc portion of many animal globulins such as human, rat, guinea pig and rabbit, but not goat or sheep.

The indirect method has the advantage of detecting any viral antigen with a single labeled antibody provided that two conditions are satisfied:

a. That the labeled antibody does not react with the viral antigens to be detected, and



Adapted from Voller etal. Bull. Wld. Hlth. Org. (1976) 53, 55-56

Fig. 1 ELISA sandwich to detect Ag.

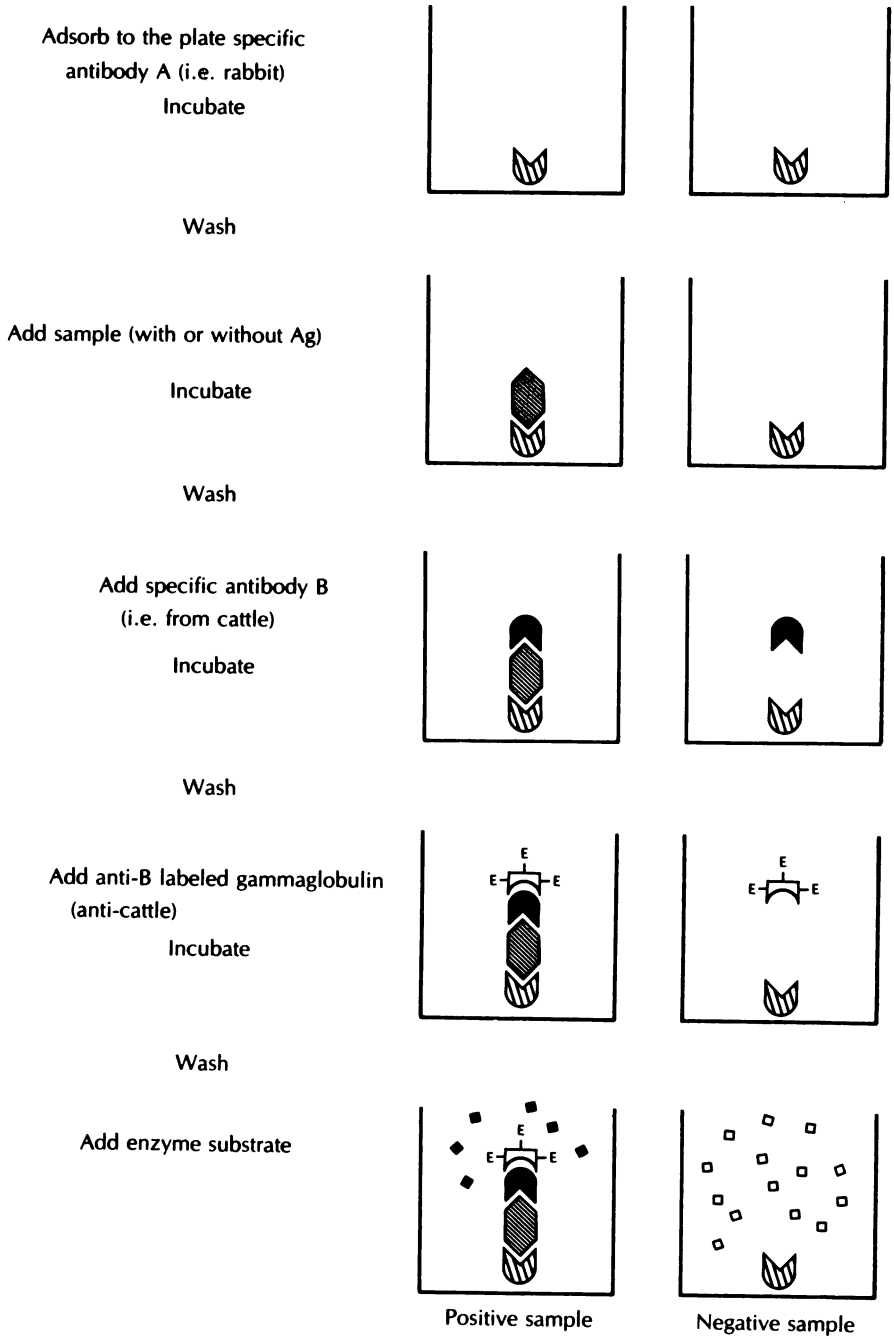


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Fig. 2 Antiglobulin sandwich method to detect Ag.

b. That the first and second antibodies are taken from different species.

The indirect method is often more sensitive than the direct, since several molecules of labeled antibody can react with one molecule of antibody specific to the antigen (the second antibody). However, this method requires an additional incubation step, and the first and second antibodies have to be prepared in different animal species to prevent the labeled antibodies from reacting with the first antibody.

Antibody determination

Indirect method

1. The antigen adheres to the solid phase.

2. Wash to remove all antigen that has not attached to the solid phase.

3. Add the problem sample. The antibody present will react with the antigen adsorbed to the solid phase. All excess antibody that does not react specifically with the antigen is removed by washing.

4. Wash.

5. Add antispecies immune globulin labeled with enzyme.

6. Wash.

7. Add substrate.

8. Stop reaction and read.

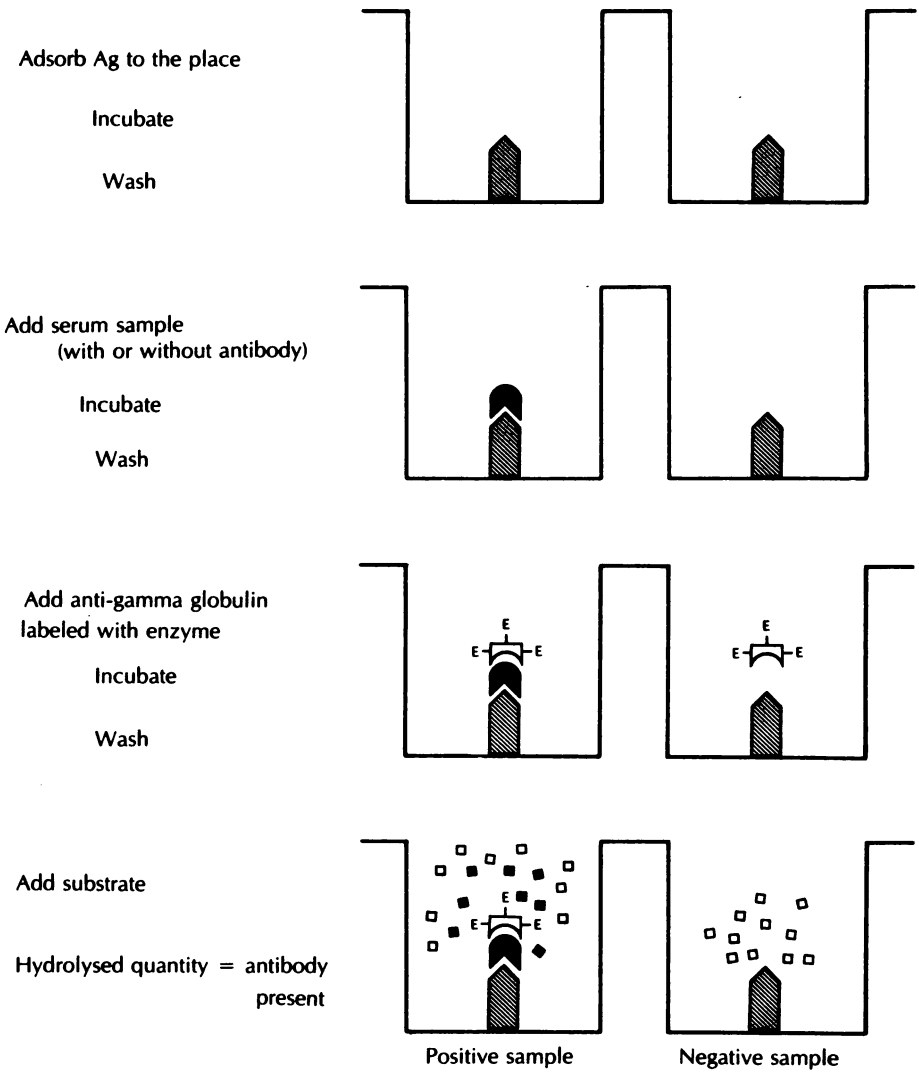
9. Principal steps (Fig. 3).

Reagent purity

Due to the high sensitivity of enzyme immunoassays, the reagents must be extremely specific and pure to keep down nonspecific responses and the level of background reactivity. The most useful reagents are those prepared with highly purified virus components. The same applies to the antibodies used. Antibodies of high homogeneity and specificity are monoclonal antibodies.

Preparation of reagents for the diagnosis of rotavirus

Rotaviruses are viral agents with a double strand of ribonucleic acid (RNA) measuring 65 nm and capable of producing infectious gastroenteritis in animals and man, and especially in infants.



Adapted from Voller etal. Bull.Wld.Hlth.Org.(1976)53,55-56

Fig. 3 Indirect ELISA for Antibody detection.

Because of the difficulty of culturing these viruses, diagnostic methods are needed that can detect them in feces or through their serologic response in blood serum. It is for this purpose that EIAs are so useful and rapid diagnostic method.

The direct EIA is most used in the diagnosis of acute infectious gastroenteritis, whereas the indirect procedure is more commonly used in large-scale epidemiological studies of the disease (for the advantages and disadvantages of the two methods see the appropriate section of this manual).

Solid phase

As noted previously, there are several materials that can be used for the solid phase: gels, beads, sticks, filter papers, nitrocellulose paper, or plastic microtiter plates. The latter are the most common, especially those made of soft polyvinyl.

To avoid unspecific reactions in plate-reading spectrophotometers, it is advised to use only the inner 60 wells of 96-well plates, and to use not more than 100 µl of fluid per well even though each well can hold 250 µl. It is much better to adsorb (coat) the protein (whether antigen or antibody) on several plates at once.

The plates can be sealed with Parafilm or in plastic bags, and stored in a humid chamber at 4° C for up to four months with no perceptible loss of activity.

Washing

All EIAs in which proteins must be adsorbed on a solid phase require the removal of all unreacted reagents. This removal is accomplished by washes following each adsorption (incubation). Adding a nonionic detergent such as Tween 20 to the wash fluid.

It is advisable to remove the wash fluid with a hand-operated or automatic suction device, and not by just inverting the plates.

Enzymes

The enzymes most commonly used to label Ig or protein A are peroxidase and alkaline phosphatase. Peroxidase is inexpensive and catalyzes substrates that yield a dark, easily read color. Peroxidase conjugates are stable at 4° C, but their action is inhibited by bacteriostatic agents such as sodium azide.

Alkaline phosphatase, on the other hand, is not sensitive to bacteriostatic agents and is thus more stable under field conditions. The reaction between

this enzyme and the substrate is easily stopped by raising the pH above 12. However, the major disadvantage of this enzyme is that it is expensive and difficult to obtain, being extracted chiefly from calf intestine.

Substrates

The simplest substrate is one that when catalyzed by the enzyme, yields a visible reaction product. The color of this product is easily visible to the naked eye and can be quantitated with a spectrophotometer.

The substrate recommended for alkaline phosphatase is p-nitrophenylphosphate.

For peroxidase the recommended substrates are o-phenylenediamine, 5-aminosalicyclic acid, diaminobenzidine, and 4-chloro-1-naphthol.

Reagents

EIA are highly sensitive, and highly specific reagents must be employed which minimize nonspecific cross-reactivity. This must be done by preparing antibodies using purified viruses and antigens. This will ensure that the antibodies react chiefly with the viral antigen.

All these reagents must be heat-inactivated at 56° C for 30 minutes in order to eliminate interference from complement-mediated reactions.

Specific viral activity is defined as the difference in activity between wells containing antigen plus the specific antibody and those containing antigen plus nonimmune serum.

Protocol for direct rotavirus antigen assay (ELISA sandwich)

1. Adsorb in alternate rows of wells of the microtiter plates an appropriate dilution of goat antirotavirus (or IgG antirotavirus) serum and a dilution of goat serum (or IgG) containing no antirotavirus antibodies.

2. Incubate the plate at 4° C overnight. If the plate is not used the next day, it should be covered with Parafilm and stored at 4° C until used.

3. Wash the plate five times in phosphate-buffered saline (PBS) and Tween 20 (0.05-0.01%).

4. Add 50 ml of N-acetyl cysteine (adjusted to pH7) to each well. Add an equal quantity (50 µl) of specimen to two wells coated with goat antirotavirus serum and two wells coated with nonimmune (normal) goat serum. Include a weakly positive control and four negative controls in each test.

5. Incubate the plate for two hours at 37° C, or overnight at 4° C.

6. Wash plate(s) five times with PBS-Tween.

7. To all wells add the enzyme-labeled antirotavirus (goat or guinea pig) serum diluted with PBS-Tween containing 2% fetal calf serum (PBS-Tween-FCS).

8. Incubate the plate at 37° C for 1 hour.

9. Wash the plate five times with PBS-Tween.

10. Add appropriate substrate. Incubate the plate at 37° C or ambient temperature (AT) until the weakly positive control has visible color equivalent to an optical density (OD) of about 0.1. Calculate the specific activity by subtracting the mean activity of the sample in wells containing negative (control) serum from the mean activity of the wells coated with antirotavirus serum.

A sample is considered positive if its mean activity is greater than two standard deviations above the mean activity of the negative controls.

In other words, the specific activity is measured quantitatively by subtracting the activity found in the control wells from the activity found in the wells coated with antivirus antibodies.

The sample is also considered positive if its activity is greater than that of the weakly positive control. In visual qualitative determination the sample is considered positive if the color in the wells containing antirotavirus antibodies is more intense that that in the wells containing normal (nonimmune) and weakly positive serum.

Protocol for indirect rotavirus antigen assay (antiglobulin ELISA)

1. Proceed as in steps 1 to 6 of the direct assay for antigen.

2. Add unlabeled guinea-pig antirotavirus serum diluted in PBS-Tween-FCS.

3. Incubate plate at 37° C for one hour.

4. Wash plate five times with PBS-Tween.

5. Add to the wells enzyme-labeled antiguinea pig immunoglobulin prepared in goat or rabbit and diluted in PBS-Tween.

6. Incubate plate at 37° C for one hour.

7. Wash the plate five times with PBS-Tween.

8. Add substrate and interpret results as described in step 10 of the direct assay.

Protocol for indirect antibody assay

A. **Antigen(virus)-adsorption on plates**

1. Add 100 µl of antigen suitably diluted in PBS to each well.

2. Incubate at 4° C for 18 hours.

3. Wash five times in PBS-Tween.

4. Use plate immediately or store at 4° C until used (not longer than five months).

B. **Assay procedure**

1. Add 100 µl of diluted serum to wells in which antigen has been fixed.

2. Cover plate and incubate at 37° C for one hour.

3. Wash five times in PBS-Tween.

4. Add conjugated IgG against the species of the problem serum: 100 µl/well.

5. Incubate at AT for one hour.

6. Wash five times in PBS-Tween.

7. Add diluted substrate: 100 µl/well.

8. Incubate at AT for eight minutes.

9. Stop reaction by adding 100 µl of H_2SO_4-3N to each well.

10. Read with automatic spectrophotometer (Multiskan-Flow Lab) at an adequate wave length or by visual inspection.

Indirect assay protocol for antibody titration

I. Technique

1. Dissolve antigen in alkaline buffer.

2. Add 50 µl of the antigen solution to each well in the first 11 vertical rows and to the first four wells in the 12th vertical row of the microplate. The wells in row 12 are used as controls. The antigen solution must be freshly prepared.

3. Incubate the plate at 37° C for 2 hours in a humid atmosphere to prevent evaporation. The incubation time may be varied depending on the characteristics of the antigen. Plates incubated with antigen may also be frozen at -70° C.

4. Wash the plates once or twice in PBS-Tween.

5. Add 50 µl of blocking solution to all wells. Incubate at 37° C for 1/2 to 1 hour in a humid atmosphere. The purpose of this step is to saturate with protein the parts of the solid phase that have not combined with antigen. This will prevent the nonspecific adsorption of antibodies later on.

6. Wash five times in PBS-Tween.

7. Add 50 µl of PBS-Tween to all wells on the plate.

8. Add 50 µl of the sample to the first well in the vertical row (11 samples per plate may be titrated: one row for each sample). Row 12 is the control row, to which 50 µl must be added of:

   - a negative serum to the first and fith wells (wells A and E on the microplate).

9. Make double dilutions in all rows except number 12. Remove 50 µl from the last well of each of the 11 rows.

10. Incubate at 37° C for 30 minutes in a humid atmosphere.

11. Wash five times in PBS-Tween.

12. Add 50 µl of a standard dilution of enzyme-labeled anti-antibody to all wells in the first 11 rows. this antibody must be diluted in PBS-Tween containing 1% bovine serum albumin

(BSA). Add 50 µl of the labeled antibody to the following wells in the 12th row:

- the first three wells (A, B and C)
- the fifth, sixth and seventh wells (E, F and G).

13. Incubate in a humid atmosphere at 37°C for 30 minutes.

14. Wash five times in PBS-Tween.

15. Add 50 µl of substrate to all wells on the plate.

16. Incubate in a humid atmosphere at 37° C for 30 minutes.

17. Add 10 µl of 8M H_2SO_4 to stop the reaction.

18. Measure the reactions.

    A reaction may be considered positive if it produces a color more intense than that shown by the negative control (well A, row 12).

19. Controls

    Except for well B (positive control), all the wells in row 12 should show a color less intense than that of well A. It is possible, though not common, for a sample to contain enzymes that react with the substrate. A proper control for this possibility is not included in this protocol. If there is any suspicion of this problem, the serum must be reacted with the antigen (steps 1-10) after which labeled antibody must not be added (step 11). Proceed to steps 12 to 17. The reaction should be negative.

II. Reagents

1. **Alkaline buffer** (to dissolve the antigen)

Sodium carbonate	Na_2CO_3	1.59 g
Sodium bicarbonate	$NaHCO_3$	2.93 g
Distilled water		1 liter
	Adjust pH to 9.6.	

2. PBS-Tween

Sodium chloride	NaCL	8 g
Potassium phospate	KH_2PO_4	0.2 g
Sodium phosphate	Na_2HPO_4	0.9 g
Potassium choloride	KCL	0.2g

Add 1 liter of distilled water and adjust pH to 7.4 with NaOH-1N. Add 0.5 ml of Tween 40 (polyoxyethylene sorbitan monopalmitate) or Tween 80 (polyoxyethylene sorbitan monooleate).

3. **Blocking solution**

PBS-Tween	100 ml
Bovine serum albumin (BSA)	2 g

4. **Substrate**

o-phenylenediamine	10 mg
absolute methanol	1 mg

Mix well and then add the mixture to:

Distilled water	100 ml
Hydrogen peroxide H_2O_2 (3%)	100 ml

Immunodot

The method is demostrated with a microfiltration apparatus obtained from Biorad Laboratories. Apparatus from other companies should give similar results.

I. **Technique**

1. Assemble the microfiltration apparatus in accordance with the manufacturer's instructions (Bio-Dot microfiltration apparatus, Bio-Rad Laboratories). The following points are of special importance:

   a. Always wear gloves when handling nitrocellulose sheets (NC).

   b. Wet NCs with Tris-buffered saline (TBS).

   c. Tighten the screws of the apparatus before and after using the vacuum pump.

2. Switch on the vacuum pump and wash the wells once in TBS.

3. Switch off the vacuum pump before adding the antigen(s) to the wells. Add antigen to wells. The quantity of antigen to be added depends on the type of antigen and its ability to bind to NC. Adding too much antigen to a well can result in obstruction of the NC.

4. Allow the antigen to penetrate the NC during a period of incubation of the apparatus (30-60 minutes) at ambient temperature.

5. Turn on the vacuum pump for 1 minute. This will pass air through the wells and dry the NC. Switch off the vacuum pump.

6. Add the "blocking buffer." The buffer components will saturate with proteins the sites on the NC that did not bind to antigen. this prevents the nonspecific adsorption of immunoglobulins to the NC during the steps that follow.

7. Incubate the apparatus at ambient temperature (30-60 minutes).

   Repeat step 5; do not switch off the vacuum pump.

8. Wash all wells once with TBS-Tween 20. Switch off the vacuum pump.

9. Add the samples of serum to be titrated. They must be diluted in the blocking buffer. Incubate the apparatus at ambient temperature.

10. Repeat step 5; do not switch off the vacuum pump.

11. Wash the wells five times in TBS-Tween. Switch off the vacuum pump.

12. Add a standard quantity of enzyme-conjugated secondary antibodies diluted in blocking buffer. We recommend horseradish peroxidase as the enzyme of choice. Incubate the apparatus at ambient temperature.

13. Repeat step 5; do not switch off the vacuum pump.

14. Repeat step 11.

15. Remove NC from the apparatus with gloves.

16. Place NC in contact with substrate. Observe the reaction while keeping the NC in constant motion in the substrate solution.

17. Remove NC from substrate and wash in distilled water. Make a record of your results because the observed reaction gradually fades.

II. Reagents

All reagents are obtainable from Sigma Chemicals, St. Louis, MO, U.S.A.

1. TBS: Tris-buffered saline

   20 mM Tris (hydroxymethyl) aminomethane..2.42 g
   500 mM NaCL.....9.22 g

Distilled water.....1 liter
Adjust pH to 7.5 with HCl.

2. **TBS-Tween**

0.3 ml polyoxyethylene sorbitan monolaureate (Tween 20)
99.7 ml TBS

3. **Blocking buffer**

2 g bovine serum albumin (BSA)
100 ml TBS
(TBS-Tween can ocassionally be used in lieu of TBS.)

4. Nitrocellulose paper (NC). Obtained from Bio Rad Laboratories, Richmond, CA, USA.

5. **Substrate**

o-phenylenediamine	10 mg
absolute methanol	1 mg

Mix well and then add the mixture to:

Distilled water	100 ml
Hydrogen peroxide H_2O_2 (3%)	100 ml

Method for SDS polyacrylamide gel electrophoresis (SDS-PAGE)

This technique can be used for:

I. Determination of the molecular weight of proteins.

II. The detection of antigens via enzyme immunoassay (Western blot).

I. **SDS electrophoresis of protein in polyacrylamide gels**

1. Glass plates must be entirely clean and free from oils and other substances.

2. Separate the two plates with 1.5-mm thick teflon spacers.

3. Secure the spacers and glass plates firmly in place as a sandwich with clamps.

4. Seal the bottom of the sandwich (in this demostration a rubber sheet will be used).

5. Mix the acrylamide, bisacrylamide and buffer in a bottle and remove entrapped air from the mixture by connecting the bottle to a vacuum pump. Gloves must be worn because unpolymerized acrylamide is a neurotoxin.

6. Add SDS, TEMED and ammonioum persulfate to the mixture (make sure that the ammonium persulfate has been freshly made up).

7. Immediately fill 3/4 of the sandwich with this mixture.

8. Add distilled water to cover the acrylamide mixture to seal it off from the ambient air. Incubate 30 to 60 minutes. This allows polymerization of the acrylamide and formation of the "separator gel".

9. Remove the distilled water from atop the polyacrylamide.

10. Add the mixture needed to make the stacking or concentrator gel. This mixture uses a low percentage of acrylamide-bisacrylamide (see formula).

    To prepare it follow steps 5 and 6.

11. Add the mixture to the sandwich to cover the previously set polyacrylamide gel.

12. Insert the "comb" (a well-forming device) in the sandwich and cover with distilled water the points on the stacking gel mixture that are in contact with air.

13. Remove the comb once the acrylamide has polymerized. Rinse the wells with buffer using a pipette.

14. Dissolve the sample in buffer #1. Apply the sample to the wells. A total volume of 200 µl (with 100-200 mg of protein) can be placed in each well.

15. Apply buffer #2 over the samples in the wells.

16. Insert the sandwich with the samples in the lower compartment of the electrophoresis apparatus. The lower compartment is connected to the anode and must contain buffer #2.

17. Insert the upper compartment in the electrophoresis apparaturs.

18. Fill the upper compartment with buffer #2 until the electrode (cathode) is covered.

19. Switch on the electrophoresis apparatus.

    20 mA/gel for the first hour
    30 mA/gel for the second hour
    30 mA/gel from the third hour onward

20. Switch off the electric power when the bromophenol blue (the stain in buffer #1) nears the lower part of the gel. Remove the gel from the sandwich and cut the concentrator gel off the separator gel. Incubate separator gel in a dish with Coomassie blue (a stain for proteins) for 1-18 hours at ambient temperature.

21. Destain the gel with destaining solution.

II. Western Blot

A. Technique

1. Follow the SDS-PAGE procedure described above through step 19. When the electrophoresis has been completed cut out the stacking gel and cut off the part of the separator gel that contains the migration band of the bromophenol blue.

2. Place the separator gel in the transfer buffer for one hour.

3. Wet the nitrocellulose sheet in the transfer buffer (wear gloves).

4. Fill the chamber in the transfer apparatus with transfer buffer chilled to 4° C.

   Place the cooling apparatus in the chamber.

5. Assemble the transfer device in the following manner:

   a. porous fiber layer
   b. filter paper
   c. separator gel
   d. nitrocellulose sheet. Remove all air bubbles trapped between the gel and the sheet.
   e. filter paper
   f. porous fiber layer.

Important: All components must be wetted in the transfer buffer before the device is assembled.

6. Place the device in the chamber so that the nitrocellulose sheet is between the gel and the anode.

7. Switch on power for 2 or 3 hours between 90 and 95 volts.

8. Switch off power and remove the nitrocellulose sheet (wear gloves).

9. With a pencil, mark on the NC sheet the approximate positions of the wells using the comb for reference.

10. Cut the NC sheet in strips corresponding to the rows of wells.

11. Place the strips in polyethylene bags.

12. Add the blocking buffer; heat-seal the bags.

13. Agitate the bags for 1 hour.

14. Open the bags, add the first antibody diluted in blocking buffer, seal the bags, and agitate for 1/2 to 1 hour.

15. Remove the strips and wash them well in several volumes of TBS-Tween.

16. Place the strips in bags, add the labeled antibody diluted in blocking buffer, seal the bags, and agitate for 1 hour.

17. Remove the strips and repeat step 15.

18. Prepare the substrate. Add the strips to the substrate and agitate until reactions appear.

19. Wash strips in distilled water and dry them with filter paper.

B. Instruments and Reagents for Methods I & II

The techniques have been demonstrated using LKB and Bio-Rad equipment. Instruments manufactured by other firms should yield similar results. Most of the reagents were obtained from Sigma Chemical, St. Louis, MO, USA.

1. **TBS**

   20 mM Tris (hydroxymethyl) aminomethane
   500 mM NaCl
   Adjust pH to 7.5 with HCl

2. Blocking buffer

2 g bovine albumin (BSA)
100 ml TBS

3. TBS-Tween

0.3 ml polyoxyethylene sorbitan monolaureate (Tween 20)
99.7 ml TBS

4. Buffer #1

25 mM Tris, pH 6.8 (adjust with HCl)
1% sodium dodecyl sulfate (SDS, Bio-Rad Laboratories)
0.05% bromophenol blue
50% glycerol

5. Buffer #2

25 mM Tris
192 mM glycine
0.1% SDS
The pH will be about 8.3 (do not adjust it).

6. Transfer buffer (for Western blot)

25 mM Tris
192 mM glycine
20% methanol
Do not adjust pH.

7. Gels

7.1 Separator gel (15%)

14.7% acrylamide
0.3% bisacrylamide
0.1% SDS
0.781 M Tris, pH 8.8
0.065% ammonium persulfate
0.065% N,N,N',N'-tetramethyl-ethylene diamine (TEMED, Eastman Kodak Company)

7.2 Stacking (concentrator) gel (5.9%)

5.7% acrylamide
0.2% bisacrylamide
0.1% SDS
0.143 M Tris pH 6.8
0.0573% amonium persulfate
0.1145% TEMED

8. **Gel stains**

   0.3% Coomassie R blue
   10% acetic acid
   45% ethanol
   45% water

9. **Destaining solution**

   10% acetic acid
   25% ethanol
   65% water

10. **Enzyme = peroxidase**

11. **Substrate**

    a. 4-chloro-1-naphthol: 3 g/ml of methanol
    b. 0.018% H_2O_2 in TBS

    Mix 1 part (a) with 5 parts (b).

Production, maintenance and preservation of monoclonal antibodies

I. Production of monoclonal antibodies

The proven method uses Sp2/0 tumor cells from BALB/c mice. Hence, to cultivate hybridomas in the peritoneal cavity of the mouse, cells must be used that are taken from BALB/c mice for fusion with Sp2/0 cells.

A. Immunization

Inject antigens into the mouse or mice. Inject the antigen ip (with or without adjuvant). Repeat the injection 2 or 3 times every 14 to 21 days. Administer the antigen for the last time without adjuvant 4 days before the fusion.

B. Fusion

1. Kill the mouse in a CO_2 chamber.

2. Remove the spleen aseptically and place it in a Petri dish with 5 ml serum-free RPMI-1640 culture medium.

3. Remove the lymphocytes from the spleen with tweezers or syringe needles (18-G).

4. Transfer the suspension of lymphocytes from the Petri dish to centrifuge tubes.

5. Wash the lymphocytes 3 times in serum-free RPMI-1640 (at 380-400 g for 10 minutes).

6. Suspend the lymphocytes in 1-3 ml serum-free RPMI-1640 and estimate the number of dead cells with trypan blue. The cells should be 85% viable.

7. Prepare a suspension of Sp2/0 cells. Wash (400 g for 10 minutes) once or twice in serum-free RPMI-1640. Suspend the cells in 1-3 ml serum free medium and estimate their viability (should be over 90%).

8. Combine Sp2/0 cells and lymphocytes in a proportion of 5 (lymphocytes): 1 (sp2/0). Centrifuge the cell mixture at 400 g for 10 minutes. The optimal number of cells in the mixture is 3×10^8.

9. Remove supernatant, loose pellet by tapping tube and place the tube with the cell pellet in water bath at 37° C.

10. Slowly add 1 ml of PEG (50%) **preheated** to 37° C with a Pasteur pipette, stirring carefully and continuously. Adding this volume of PEG should take 1 minute. **Important**: Avoid aspirating cells into the pipette in this operation.

11. Add 1 ml of serum-free RPMI-1640 evenly during 1 minute. Agitate continuously.

12. Repeat step #11.

13. Add 7 ml of serum-free RPMI-1640 preheated to 37° C during 2-3 minutes. Agitate continuously.

14. Centrifuge at 350 g for 10 minutes.

15. Remove supernatant.

16. Add 10 ml of RPMI-1640 + 10% horse serum. The cells can be suspended by agitating the tube and stirring them with a Pasteur pippete **without aspiration**. This procedure is easier if the 10 ml of culture medium are added 2 ml at a time.

17. Estimate the number of cells and adjust the volume to obtain 10^7 cells/ml.

18. Distribute the cells on culture microplates (96 wells), about 0.1 ml, or 10^6 cells, in each well. This number is obtained by placing two drops in a well with a Pasteur pipette. To some wells add (a) Sp2/0 cells (to verify that later on the HAT medium does not permit the survival of these cells), and (b) hybridomas obtained in

an earlier fusion (to ensure that the culture media used permit the proliferation of hybridomas).

19. Incubate the cells for one hour (5% CO_2, 37° C.)

20. Inspect the wells under the microscope. Incubate again for about 12-18 hours. Inspect the cells again. The Sp2/0 cells may have started proliferating. Viability should approximate 90%.

21. Add (with a Pasteur pipette) two drops of HAT culture medium 24 hours after the fusion. If viability does not approximate 90%, wait another 10-24 hours before adding the HAT medium. In general, with good viability good results are obtained with the following protocol.

Day	Operation
0	Fusion.
1	Add 2 drops of HAT medium.
2	*Remove half of medium, add 2 drops of HAT medium.
3	Repeat step 2.
4	Repeat step 2.
7 & 10	Repeat step 2.
14 & 17	Repeat procedure, but substituting HT for HAT.

Changes may be made in this protocol on the basis of **daily** inspection of the wells.

*The removal of culture medium must be effected with extreme care to avoid removing cells.

Typically, the first hybridoma colonies are seen between day 7 and day 14. Theoretically, the supernatant contains the antibodies secreted by the hybridomas, and may be analyzed for them between days 9 and 14. Depending on the efficiency of the fusion, this process may sometimes take 21 to 28 days.

C. Detection of Antibodies

1. Once it has been determined that one or more hybridomas have grown sufficiently, remove 100 µl of supernatant and analyze it by a technique suitable for determining the presence of specific antibodies. A capability for the analysis of hundreds of supernatants in a short time (6 hours) must be available. We recommend that the procedure used posses the sensitivity to

detect antibodies in small quantities. We have used the following procedures with good results:

a. ELISA on microplates.
b. Immunodot assay.
c. Fluorescent antibodies.

2. Once a positive reaction is identified in the supernatant of a well, proceed to clone.

D. Cloning

1. Remove the cells from the positive well and transfer them to a larger well (1 ml; 24-well plate). Suspend them in 1 ml of serum-free RPMI-1640. Remove half of the culture medium every 2 or 3 days and add a similar volume of fresh medium.

2. When the cells have proliferated to a suitable number in the well, cloning is started. The following points are of utmost importance:

   a. The day before the cloning is to be done, prepare cultures of the spleens of normal mice on 96-well plates. Follow steps B.1-5, but suspend the cells in RPMI-1640 + 10% serum. Adjust the number of cells to 5 X 10^6 cells/ml and add 0.1 ml to each well of the 96-well plate. incubate until the cloning day. These cultures are used as feeder layers. Feeder layers may be replaced with CR-EGGS (Collaborative Research Inc., Lexington, MA, USA), which appears to be more effective than feeder layers, although more expensive as well.

   b. Never clone all the cells in the selected well. Keep one third of the cells in that well as a reserve against possible failure of the procedure.

3. Dilute the hybridomas from the well as follows:

   a. 10 cells/ml (1 cell/0.1 ml/well)
   b. 5 cells/ml (0.5 cells/0.1 ml/well)
   c. 1 cell/ml (0.1 cell/0.1 ml/well)

   Place 0.1 ml of dilution (a) in each of the first 24 wells of the microplate containing feeder layers, 0.1 ml of dilution (b) in the next 36 wells, and 0.1 ml of dilution (c) in the remaining wells.

Replace half of the medium (RPMI-1640 + 10% serum) every 2-3 days with fresh medium. The colonies must be visible in 7 days, and the supernatant must contain enough antibodies for analysis in 10-14 days.

4. Analyze for the presence of antibodies and select positive wells.

5. To ensure that cloning has actually taken place (cells have grown from one single hybrid cell), it is recommended that another cloning be done from the positive well. In general, selecting positive wells obtained from dilution (c) (see step D.3.c) heightens the probability that the colony was grown from a single cell.

6. The positive well is transferred to larger wells and finally to culture bottles (see step D.1). Avoid overdiluting the hybridomas when making the transfers.

7. As the cells proliferate the supernatant containing the monoclonal antibodies is harvested.

8. The hybridoma cells can be injected into the peritoneal cavities of BALB/c mice to obtain high monoclonal antibody titers. Inject 3 x 10^6 to 6 x 10^6 hybrid cells, suspended in **serum-free** RPMI-1640, into each mouse. The ascites containing antibodies can be withdrawn 7 to 10 days following this inoculation.

E. Preservation

Cloned and noncloned hybridomas and Sp2/0 cells can be preserved in liquid nitrogen. This procedure is highly useful because it is not recommendable to keep hybridomas in culture very long.

1. Use bottle-cultured hybridomas. What is needed is 10 ml of dense culture with a vitality of close to 100%. Centrifuge the cells (360-400 g) for 10 minutes.

2. Suspended the pellet in 1 ml of RPMI-1640 with 10% serum.

3. Prepare 1 ml of serum with 10% DMSO.

4. Add the serum with the DMSO very slowly in an ice bath with continuous agitation.

5. Transfer the cells suspended in the culture medium with the DMSO to two special plastic tubes by means of a pipette. Seal the tubes. It is important that this be done over ice and that the pipetting be done very gently to avoid destroying the cells. Never keep the cells in these conditions for more than 5 minutes. Proceed to step #6 as quickly as possible.

6. Keep the tubes in liquid nitrogen vapor for 16-24 hours. Submerge in the liquid nitrogen.

F. **Recovery of hybridomas from liquid nitrogen**

1. Remove 1 to 3 tubes from the liquid nitrogen.

2. Immediately submerge the tube 2/3 in water at 37° C with continuous agitation until the contents are thawed. This procedure should not take longer than 2 to 3 minutes.

3. Wash the tube in ethanol (70-95%) and dry it with sterilized blotting paper.

4. Deposit the contents of the tube in a sterile tube. **Do not use pipettes.**

5. Add 5 ml of RPMI-1640 + 10% serum, drop by drop with continuous agitation. Add another 5 ml somewhat faster; steps 4 and 5 must be completed in less than 10 minutes. Once the 10 ml of culture medium have been added in step #5, the tubes may be left at ambient temperature for 30-40 minutes.

6. Centrifuge (350g for 10 minutes) and suspend the pellet in 1 ml of RPMI -1640 + 10% serum. The cells must be suspended by agitation. Do not use pipettes.

7. Pour the cell suspension into a 2-ml well (24-well culture plate). Do not use pippetes.

8. Replace half of the culture medium in 4 hours and in 24 hours. The cells may be pipetted in 24 hours.

Note: The use of feeder layers (or culture medium with CR-EGGS) in the wells is recommended if the cells are of low vitality.

Reagents

1. Serum-free RPMI-1640 Culture Medium

a. 1 x RPMI-1640 (with sodium bicarbonate)....100 ml
b. 1 M Hepes (N2 Hydroxyethyl piperazine -N' ethanesulfonic acid)..... 1 ml
   dissolve 23.83 g in 100 ml distilled water*
c. Penicillin-streptomycin (5,000 units - 5,000 meq)..... 1 ml
d. 200 mM L-glutamine..... 1 ml
e. 100 x MEM nonessential amino acids..... 1 ml
f. Sodium pyruvate
   Dissolve 0.5 g in 50 ml distilled water*.. 0.5 ml

*Sterilize by filtration.

2. RPMI-1640 Culture Medium + Serum

a. Serum-free RPMI-1640.....90 ml
b. Horse serum heat-inactivated at 56° C for 30 minutes

3. Vital stain

a. RPMI-1640 without serum.....0.85ml
b. Trypan blue (0.4% solution in physiological saline).....0.1 ml
c. Cell suspension.....0.05ml

4. 50% Polyethylene Glycol (PEG) solution

Keep the container of PEG away from moisture. Prepare solution on day on which fusion is to be performed. Dissolve 2 g of PEG of 50-56° C. Keep at 37° C to prevent solidification. Add 2 ml of serum-free RPMI-1640 sterilize by filtration and keep mixture at 37° C.

5. Preparation of HT and HAT Culture Media

HT medium

a. 100 x HT

- Dissolve 68.4 mg hypoxanthine in 50 ml distilled water at 70-80° C. Add 19.4 mg thymidine and stir until dissolved.

b. 50 x HT

- Mix 25 ml 100 x HT with 25 ml distilled water. Sterilize with 0.22 um filter. Divide solution into 2-ml portions and freeze at -20° C.

c. **HT for use in cultures**

- Mix 2 ml of 50 x HT with 100 ml RPMI-1640 with 10% horse serum.

HAT Medium (50x)

a. **Aminopterin solution**

- Dissolve 1.1 mg aminopterin in 2.5 ml of distilled water. If it does not go in solution add a few drops of IN-NaOH then add distilled water up to 6.25 ml.

b. **Mix: 25 ml 100 x HT** with 2.5 ml aminopterin solution and 22.5 ml distilled water.

Sterilize with filter (0.22 um), divide into 2-ml portions and freeze portions at -20°C.

c. **HAT to be used in cultures**

- Mix 2 ml of 50 x HAT with 100 ml of RPMI-1640 containing 10% horse serum.

Commercial sources of equipment

Bio-Rad Laboratories
P.O. Box 708
220 Maple Avenue
Rockville Center, NY 11571
U.S.A.

Immunodot. (from Bio Rad)

Bio Dot Apparatus
Catalog #170-6550

SDS-PAGE electrophoresis

Protein II Vertical Electrophoresis System
Catalog #165-1801
Combs and Spacers:
Catalog #165-1802

Power Source

Bio Rad Model 3000/300
120 V 60 Hz - Catalog #165-0550
220 V 50 Hz - Catalog #165-0551

Western Blot

Transbolt Electrophoretic Transfer Cell
Catalog #170-3910

Power Source

Bio Rad Model 250/2.5 220V 50 Hz
Catlog #165-4754

Microplate washer

Microwash II
Skatron Inc.
P.O. box 530
Sterling, VA 22170-0530
U.S.A.

Phone: (703) 450-6670

Microplate reader

Titertek Multiskan MC (Multichromatic) 110 V/60 Hz
Catalog #78-530-00

Antigen distributor

Titertek Autodrop 110 V/60 Hz
Catalog #78-511-00

Flow Laboratories, Inc.
7655 Old Springhouse Road
McLean, VA 22102
U.S.A.
Phone: (703) 893-5925

Ready-to-use enzyme immunoassay (ELISA) kits

1. Pitman-Moore, Inc.
   International Division
   P.O. box 344
   Washington Crossing, NJ 08560
   Phone: (609) 737-3700

2. Med-Tech
   P.O. Box 338
   Elwood, KS 66024
   Phone: (913) 365-9076

Editor's Note: As it was noted out on page 20, the equipment and reagents enumerate in this publication are those used in the course demonstrations in Buenos Aires, Argentina. Mention of commercial firms or brand names of these products do not imply IICA's approval or recommendation of similar products.

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