



360 DIAGNOSTICS™

Serologic Methods Manual

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EVERY STEP OF THE WAY

I. Overview

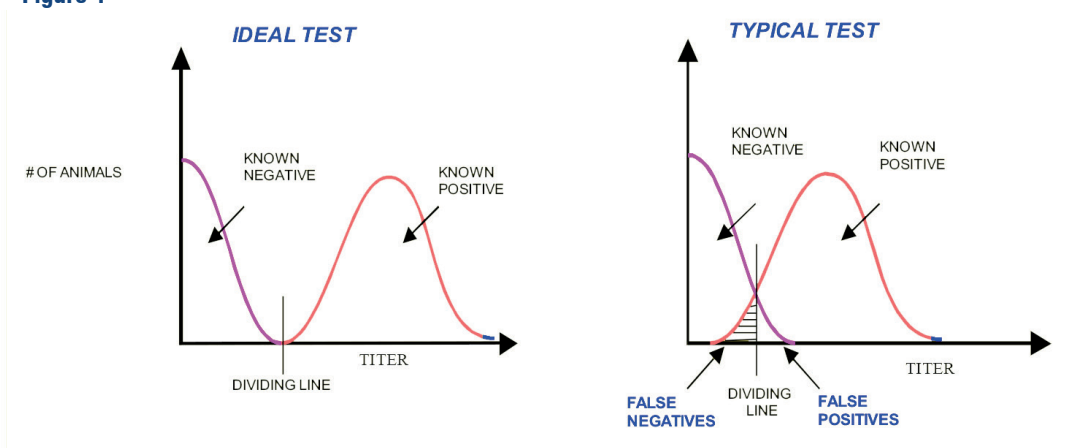
At Charles River Laboratories, ensuring the quality of animal models used in biomedical research is our highest priority. To accomplish this goal, we have developed a number of diagnostic testing strategies and methods to determine if animals have been exposed to adventitious infectious agents. Infections of immunocompetent animals are generally transient, yet serum antibody responses to infection often can be detected within days to weeks and persist throughout the life of the host. As immunoassays for antibodies to etiologic agents are rapid, inexpensive, specific and sensitive, serology is the primary diagnostic methodology by which laboratory animals are monitored for adventitious infections with viruses, mycoplasma, and other fastidious microorganisms. While serologic tests are designed to be sensitive and specific, false-positive and -negative results do occur (Figure 1). We strongly recommend that clients confirm new positive findings by both alternative diagnostic methods and testing additional animals. The enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent antibody test (IFA) are extremely sensitive. In the case of the ELISA, the test procedure and reading of results are amenable to automation.

The purpose of this manual is to outline the ELISA and IFA procedures used at Charles River. The following topics will be covered for each assay:

- Methodology overview
- Materials/reagent preparation
- Sample preparation
- Step-by-step assay procedure
- Results interpretation
- Troubleshooting

The information provided is intended to help our clients develop a rodent serologic testing program. We encourage clients to contact us with any comments or questions regarding this manual.

Figure 1



II. Enzyme-Linked Immunosorbent Assay (ELISA)

A. Methodology Overview

The indirect ELISA method is one of the assays most often used for detection of antibodies. Typically, microbial-specific antigen is immobilized on the surface of wells in microtiter plates made of specially-prepared polystyrene. Serum samples are incubated in the well, to which antibodies may bind. Unbound antibodies are removed by washing. Antibodies that have bound to the attached antigen are demonstrated by incubating first with an enzyme-conjugated anti-immunoglobulin, and then, following a wash to remove unbound conjugate, with a chromogenic enzyme substrate. A colored product develops at a rate proportional to the amount of antigen-specific antibody in the specimen. Color intensity can be assessed visually or spectrophotometrically (in absorbance units) with an ELISA reader.

Ideally, antibodies bind specifically to their corresponding antigen. In practice, however, they may bind nonspecifically to antigens. In addition to the antigen-coated well, we incubate each sample in an adjacent tissue control well to detect nonspecific binding. The tissue control does not contain any material from an infectious agent but is usually prepared from the host system in which the infectious agent is propagated. For example, we propagate MHV virus in mammalian NCTC cells. The tissue control for MHV is a cellular lysate of uninfected NCTC cells. In the case of *M. pulmonis*, however, the tissue control is another cross-reacting rodent mycoplasma, *M. arthritidis*. Wild-type baculovirus-infected insect cell lysate is used as the tissue control for recombinant antigens (e.g., NS-1, Hantaan).

A positive result is recorded for a sample if color develops in the antigen well, but not in the tissue control well. Little or no color development in either the antigen or control well for the sample is recorded as a negative result. When a color reaction occurs in the tissue control well in addition to the antigen well, the result is recorded as a tissue-control reaction (TC). A TC result is considered nonspecific and does not indicate whether a sample is antibody-positive or -negative. In our Serology Department, we usually retest the sample by an alternative method and, if necessary, we test additional samples from the same source. A more detailed explanation of score calculation and interpretation is in Section II-E.

B. Materials

1. Equipment

a) Incubator at 35-40 °C

For the best sensitivity and reproducibility, test plates should reach the incubation temperature as quickly as possible. To accomplish this, we recommend using a mechanically-circulated hot air incubator rather than a convection incubator. Make sure to use an incubator with adequate shelf space to avoid stacking plates more than two high. We also recommend covering the plates with sealing tape and incubating them in a humidified chamber to prevent evaporation; we use a covered plastic tub with water-dampened paper towels.

b) Plate washer (optional but recommended)

Washing to remove unbound antibody is a crucial step in any solid-phase immunoassay. Although washing can be adequately performed without specialized equipment, a programmable 96-well plate washer offers several important advantages. These include consistency, speed and containment of potentially contaminated fluids aspirated from test plate wells.

c) Reader (optional but recommended)

While test results can be read visually, we recommend using an ELISA plate reader equipped with a light source and filter appropriate to the color produced by the substrate. We strongly recommend the use of Kirkegaard & Perry Laboratories' (KPL) 1-component substrate ABTS-H₂O₂ that requires a 405 nm filter. Most ELISA plate readers have at least one RS-232 serial port, which allows them to send results to a computer for analysis. We have found that using a computer to receive absorbance values and compile reports saves time and effort, and prevents errors.

d) Pipettors

Reagent and sample preparation and sample transfers require various pipettes that accurately dispense volumes of 10 to 1,000 μ L. For most purposes, the following pipettes are adequate:

Type	Volume (μ L)
Single-channel, adjustable volume	2-10
	10-100
	100-1,000
8- or 12-channel, adjustable volume	5-50
	50-300
Repeating pipette with 8- or 12-channel	50

2. Description of key reagents

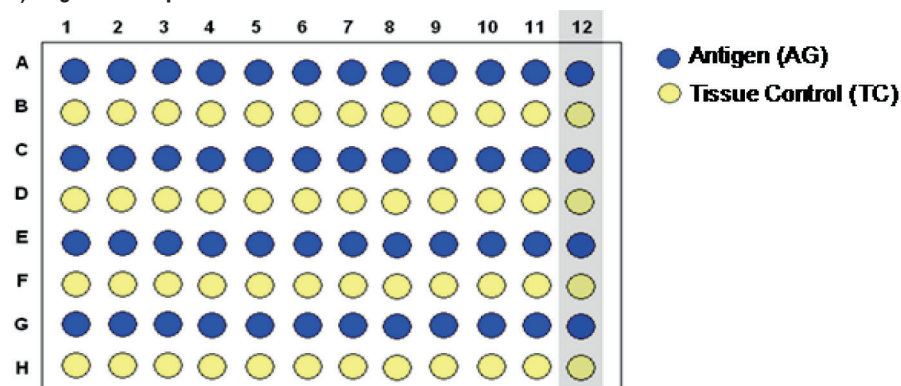
a) Antigen- and tissue-control-coated test plates

The pattern used to coat our ELISA plates is shown in Figure 2. Wells in rows A, C, E and G contain attached test-plate-specific antigen. The remaining wells (rows B, D, F and H) are coated with the corresponding tissue control. We routinely add the ELISA assay controls into column 12.

Our test plates (**intended for research use only**) are available in a 96-well format with removable 8-well strips. Plates with removable strips offer the flexibility of doing fewer than 48 tests at a time. For a list of available ELISA plates, go to http://www.criver.com/files/pdfs/research-models/rm_ld_m_elisa_reagent_item_list.aspx.

Figure 2

b) High and low positive immune and nonimmune control sera



It is essential to test standard positive and negative control sera in addition to samples to verify assay sensitivity and specificity. Results for control sera are also helpful when troubleshooting. NOTE: Our immune control sera are supplied at 2X and therefore must be diluted with an equal volume of serum diluent when used. Nonimmune control sera are pre-diluted five-fold in PBS. Do not repeatedly freeze and thaw control sera or leave them at room or refrigeration temperature for more than 24 hours. We recommend dividing the sera into small-volume, single-use aliquots that can be thawed and used at the time the assay is performed. For a list of available ELISA control sera, go to: http://www.criver.com/files/pdfs/research-models/rm_ld_m_elisa_reagent_item_list.aspx.

c) Enzyme-conjugated anti-immunoglobulin

The quality and concentration of the horseradish peroxidase (HRP) enzyme-conjugate profoundly affects the accuracy of results. Therefore, the use of an affinity-purified conjugate, optimized for your ELISA system, is crucial. We provide pre-titrated, affinity-purified HRP-conjugated species-specific IgG with recommended working dilutions for use with our test plates. For a list of available conjugates, go to:

http://www.criver.com/files/pdfs/research-models/rm_ld_m_elisa_reagent_item_list.aspx.

d) Chromogenic substrate

There are a variety of substrates that can be purchased ready to use. We have optimized our assays using KPL 1-component substrate ABTS-H₂O₂ (catalog # 50-66-06) and recommend that you use it in your assays.

e) Stop solution

The stop solution that we recommend is 1% sodium dodecyl sulfate (SDS).

3. Preparation of buffers, diluents and solutions

a) 1.0 M Tris-HCl stock solution

Components	Amount
Tris base	12 g
DI H ₂ O	qs to 100 mL
HCl	various

(1) Mixing instructions

Dissolve 12 grams of Tris base in 60 mL of DI H₂O. Adjust the pH to 8.7 with concentrated HCl and bring the total volume to 100 mL with deionized water (DI). Filter sterilize (0.2 μ) and store in refrigerator for up to 6 months.

(2) Storage

Store at 2-8 °C for up to 6 months.

b) Bovine lacto transfer technique optimizer (BLOTTO) serum diluent

(Johnson et al., *Gen. Anal. Techn.* 1:3-8, 1984).

The following formulation is for preparing 100 mL of BLOTTO:

Components	Amount
Nonfat dry milk	5 g
1 M Tris buffer pH 8	5 mL
Proclin-300®	50 μ L
Anti-foam A	33 μ L
NaCl	0.9 g
DI H ₂ O	95 mL

(1) Mixing instructions

Make 50 mM Tris buffered saline by mixing DI H₂O, 1 M Tris buffer and NaCl. Add Proclin-300®, Anti-foam A and the milk powder. Mix with a magnetic stir bar until the milk is dissolved. Remove large undissolved milk particles by coarse filtration through a screen. We use a 60 μ nylon screen from Sefar America, Depew, NY. Rinse nylon screen between filtrations.

(2) Addition of fetal bovine serum (FBS)

Add 20 mL FBS to 80 mL BLOTTO. FBS is added to block false-positive reactions caused by antibodies to fetal bovine constituents that may be in the antigen preparation. It is important to filter-sterilize the FBS (0.2 μ) prior to use.

(3) Storage

This solution is NON-STERILE. Store at 2-8 °C and use within two weeks.

c Conjugate diluent – 15% FBS and 0.9% NaCl in 0.01 M Tris-HCl, pH 7.2-7.4

The following formulation is for preparing 100 mL.

NOTE: FBS should be filter-sterilized (0.2 μ) prior to use.

Components	Amount
1.0 M Tris-HCl stock solution	1 mL
NaCl	0.9 g
Gentamicin sulfate (50 mg/mL)	0.1 mL
FBS	15 mL
DI H ₂ O	qs or 100 mL

(1) Mixing instructions

Prepare 100 mL 1.0 M Tris-HCl stock solution by dissolving 12 grams of Tris in 60 mL of DI H₂O. Adjust pH to 7.2 with concentrated HCl and bring volume to 100 mL and filter-sterilize (0.2 μ). To prepare the diluent, add the indicated amounts of each component in the order shown to 60 mL of DI H₂O. Adjust to the final volume to 100 mL with DI H₂O and stir until the NaCl is dissolved.

(2) Storage

Store in at 2-8 °C for up to six months.

NOTE: PBS, pH 7.4, can be used in place of 0.01 M Tris-saline.

d) Stop solution – 1% SDS

The following formulation is for preparing 100 mL of 1% SDS:

Components	Amount
SDS	1 g
DI H ₂ O	100 mL

(1) Mixing instructions

Prepare 1% SDS by dissolving 1 gram of SDS into 100 mL of DI H₂O.

(2) Storage

Store 1% SDS at 2-8 °C and use within seven days.

e) Wash Solution – 0.9% NaCl, 0.05% Tween 20

The following formulation is for preparing 1,000 mL.

Components	Amount
NaCl	9 g
10% Tween 20	5 mL
DI H ₂ O	1,000 mL

(1) Mixing instructions

Prepare 10% Tween 20 by mixing 10 mL of Tween with 90 mL of DI H₂O. Store at room temperature. Add the specified amount of NaCl and 10% Tween 20 to the DI H₂O and mix until the salt is dissolved.

(2) Storage

Store at room temperature and use within five days.

f) Working dilution of conjugate

(1) Mixing instructions

Freezing the Charles River-supplied conjugate stock solution should be avoided; therefore do not store the stock solution below -30 °C. Dilute the Charles River-supplied conjugate stock as indicated on the Product Specification Sheet.

(2) Storage

Store the diluted conjugate at 2-8 °C and use within 1 month. We recommend making multiple small-volume aliquots for use. This decreases the chance of microbial contamination of a large-volume single vessel. Conjugate showing visible signs of contamination (turbidity) should not be used.

C. Sample Preparation

1. Collection and storage

- a) Careful preparation and proper storage of serum samples are essential to obtain meaningful results. Tests on specimens of poor quality often yield results that are difficult to interpret.
- b) Collect blood following your standard protocols and allow it to clot at room temperature for a minimum of 30 minutes.
 - (1) For samples collected in Microtainer® or equivalent serum separator tubes, centrifuge the samples at 6,000-15,000 g for a minimum of two minutes to separate the serum.
 - (2) For samples collected in 16 x 100 mm glass tubes or equivalent, centrifuge at 2,000 g for 10-15 minutes.
- c) Transfer the separated serum to a new vial. If sterile PBS, pH 7.0 to 7.4, is available, dilute the specimen by mixing one part serum with four parts PBS.
- d) Alternatively, the same final serum dilution of ~1/5 may be achieved by adding PBS directly to the blood: Mix one part blood with two parts PBS, allow the blood to clot, and separate the serum from the clot by centrifugation as described above.
- e) It is best to store serum specimens in sturdy, leak-proof plastic vials at -10 °C or below. If they cannot be frozen, refrigerate at 2-8 °C. Specimens refrigerated for more than 24 hours should be protected from the growth of bacteria and fungi by adding an antimicrobial agent such as Proclin-300®.

2. Heat inactivation of samples

We recommend that you do **NOT** heat inactive serum samples, as we have observed that this contributes to nonspecific background color development.

3. Preparing sample plate

- a) First arrange your sample vials (containing serum diluted five-fold with PBS) in racks in the order that they will appear on the test plates.
- b) While samples can be diluted directly in the test plate, we recommend that you make dilutions in separate 96-well low-protein binding microtiter plates.
- c) Serum samples are tested at a dilution of 1/60.
 - (1) To prepare enough diluted serum to perform four tests, add 220 μ L of BLOTTO diluent to all wells, except those reserved for the assay controls (we usually reserve wells 12A, B and 12C, D for the high and low positive controls, respectively). Next, add 20 μ L of sample to each of two adjacent wells corresponding to antigen and tissue control wells on the test plates.
 - (2) We typically use wells 12E, F for the non-immune control serum and leave 12G, H as the diluent control. The non-immune control sera should be diluted in the test plate (Step D).
 - (3) Cover the sample plates with sealing tape to prevent evaporation.
 - (4) Refrigerate and use within seven days.

D. Testing

1. Transfer diluted sera to test plates

50 μ L of 1/60 diluted sera can be transferred from a sample plate into test plates with a single-channel pipette or multi-channel pipette. However, a 96-well transfer device is most efficient for this purpose

2. Add assay controls to the reserved test plate wells.

- a) Assay controls may be added to any plate location, although, as noted above, we routinely reserve wells in column 12 for this purpose.
- b) Each test plate should include the high and low positive controls.
NOTE: The Charles River positive control sera are supplied at 2X concentration.
- c) For 2X controls, if you haven't already done so, add 25 μ L of BLOTTO diluent to each positive control well, then add 25 μ L of control serum per well.

3. Incubate the test plates at 35-40 °C for 40 minutes.

- a) Be sure that all plates are completely covered to minimize evaporation. Incubate in a covered, humidified chamber if possible.
- b) Do not stack the plates more than two high.

4. Wash the test plates.

- a) If you are using an automatic 96-well washer, we recommend 3-5 fill-aspiration cycles. Overfill the well with 350-400 μL if your washer has this capability (aspiration of overflow volume). A soak time between cycles is not necessary.
- b) To wash without a plate washer:
 - (1) Prepare a wide waste container with paper towels in the bottom to prevent splattering.
 - (2) To expel the well contents into the waste container, invert and rapidly flick the plate.
 - (3) Using a repeating pipette with a multi-channel manifold, fill all wells with wash solution ($\sim 300 \mu\text{L}$).
 - (4) Repeat steps b-2 and b-3 five to six times.
 - (5) Expel the well contents as in step b-2. Invert and tap the plates against several layers of dry paper towels to remove the last traces of wash fluid.

5. Add diluted conjugate.

Add 50 μL of diluted conjugate to all wells and incubate at 35-40 $^{\circ}\text{C}$, as above, for 40 minutes.

6. Wash and add substrate and incubate.

- a) Wash the test plates as in step 4.
- b) Add 100 μL of ABTS- H_2O_2 substrate per well.
- c) Incubate at room temperature for 40 minutes.

7. Add SDS-stop solution (optional).

- a) Add 25 μL of SDS stop solution per well.

8. Read the test plate.

- a) The product of the reaction between ABTS- H_2O_2 and HRP is **green**. The rate of color development is proportional to the amount of HRP-conjugate bound to the well.
- b) The addition of stop solution dramatically slows, but does not completely prevent, further color development. It is strongly recommended to read the test plates immediately.
- c) When using an ELISA reader, measure absorbance values at 405 nm. If you are reading at dual wavelengths, set the second filter to 620 nm.

E. Results Interpretation: Charles River Scoring System

We transmit our optical density (OD) readings from the ELISA reader to a computer, where they are converted to scores by dividing by 0.13. In comparison to the 3-decimal-place absorbance values, integer scores are easier to read and interpret. This factor converts blanked absorbance values into scores between 0 and ~20.

Value	Formula
Blanked OD	Raw OD – Blank plate OD average
Score	Blanked OD/0.13
Net Score	Antigen score – Tissue control score

The scoring interpretation is given below.

Score ^A			Interpretation
Net	TC	AG	
N/A	N/A	≤ 1	Negative (-)
≤ 1	≤ 1	N/A	Negative (-)
2	≤ 1	N/A	Borderline or equivocal (+/-)
≤ 3	≤ 1	N/A	Positive (+)
N/A	≤ 2	≤ 3	Nonspecific (TC)

^A Score: TC = Tissue control, Net = Antigen score - TC score

A result with a high tissue score can still be interpreted as negative, provided that the antigen score is less than 2.5. A result is considered nonspecific and recorded as TC when both Score_{AG} and Score_{TC} are > 2.

We strongly recommend that you confirm new ELISA positive findings by alternative diagnostic methods (IFA and/or Western blot), alternate methodologies (PCR), and by testing the same and/or additional animals. Alternatively, suspect serum samples can be shipped to Charles River Serology for confirmation testing.

F. Troubleshooting

The following table describes common ELISA issues and their probable cause

Observation	Possible Cause	
	Component	Problem
No color in high and low positive control wells	Control sera	<ul style="list-style-type: none"> Not added Incorrect specificity Diluted improperly (i.e., too dilute) Inactivated by improper storage or repeated freeze-thaws
	Conjugate	<ul style="list-style-type: none"> Incorrect specificity Too dilute Inactivated by improper storage or repeated freeze-thaws
	Substrate	<ul style="list-style-type: none"> Alternate ABTS substrate used other than recommended KPL catalog #50-66-06
	Antigen	<ul style="list-style-type: none"> Too dilute or of low potency Incorrect agent Degraded due to improper storage
	Reader	<ul style="list-style-type: none"> At wrong wavelength Bulb burned out Out of calibration
Weak color development in high and low positive control wells	Components listed above	<ul style="list-style-type: none"> Plate read in wrong orientation Problems listed above
	Incubation	<ul style="list-style-type: none"> Temperature too low or exceedingly high Time too short
Excessive background	Washer	<ul style="list-style-type: none"> Too few fill-aspirate cycles Wash incompletely aspirated after each fill Fill volume low
	Serum sample	<ul style="list-style-type: none"> Sticky due to improper collection and storage or bacterial contamination Dilution too low or not diluted in BLOTTO serum diluent From animal immunized or used to grow tumor cells; animal with autoimmune disease Older-age animal used
	Conjugate	<ul style="list-style-type: none"> Dilution too low Poor quality (try another lot or different vendor)
	Substrate	<ul style="list-style-type: none"> Activated nonspecifically prior to being added to plate or by contaminants in plate
	Antigen	<ul style="list-style-type: none"> Binds antibody in serum or conjugate nonspecifically because “sticky” or used at too low a dilution
	Incubation	<ul style="list-style-type: none"> Time too long Temperature too high Plates were not completely covered to prevent evaporation of well contents No humidity to prevent evaporation of well contents

III. Indirect Fluorescent Antibody Test (IFA)

A. Methodology Overview

The steps of the IFA are very similar to those of the ELISA. Virus-infected cells and uninfected cells are fixed to wells on a glass slide. The fixative is usually cold acetone, which permeabilizes the cell membrane, making the intracellular viral antigens more accessible to antibodies. As with the ELISA, unbound antibodies are removed by washing. Instead of the enzyme conjugate and substrate indicator system used in the ELISA, the binding of primary antibodies to the slide wells in the IFA is demonstrated with a fluorescent dye-conjugated anti-immunoglobulin. After washing to remove unbound conjugate, slides are covered with buffered glycerol and examined with a fluorescence microscope. Epi-illumination is recommended due to the fluorescence being much brighter than transmitted-light darkfield fluorescence, resulting in a clearer, crisper image. Fluorescence microscopes have a light source with an exciter filter to exclude all but the appropriate wavelengths and a reflector/barrier filter combination to reflect the light onto the slide, so fluorescence may be observed.

In the IFA, the morphology and location of fluorescence can be evaluated to differentiate specific from nonspecific reactions. This is not true for most other serologic tests and is a major advantage of the IFA. Bright, granular fluorescence is typical of a specific antibody-viral antigen reaction. By contrast, diffuse fluorescence suggests a nonspecific reaction. Fluorescence may be mostly nuclear or cytoplasmic, depending on the virus (see table below). Nuclear fluorescence is characteristic of DNA viruses (e.g., the rodent parvoviruses MVM, MPV, RPV, KRV, RMV and H-1). However some minimal cytoplasmic fluorescence may be observed.

Rodent						Simian		
Agent	Fluorescence		Agent	Fluorescence		Agent	Fluorescence	
	Nuclear	Cytoplasmic		Nuclear	Cytoplasmic		Nuclear	Cytoplasmic
ECTRO		+	PIV-3		+	SIV		+
EDIM		+	POLY	+		SRV-1		+
GDVII		+	PVM		+	SRV-2		+
HANT		+	REO		+	STLV (HTLV)		+
H-1	+	+	RMV	+	+	B-Virus (HSVm)		+
KRV	+	+	RPV	+	+	Measles		+
K-Virus		+	SEND		+	SCMV	+	
LCMV		+	SV-5		+	SFV-1	+	
MAV	+	+	RTV		+	SV-40	+	
MCMV		+	MPUL ¹	+	+	SVV		+
MHV		+	MARTH ¹	+	+	MRV		+
MNV		+	CARB ²	—	—	SA-11 (ROTA)		+
MPV	+	+	CPIL ²	—	—	Hepatitis-A		+
MVM	+	+	ECUN ²	—	—			
PCAR ²	+		<i>T. gondii</i> ²	—	—			
PHV		+	MTLV		+			

¹ Mycoplasma attaches to the host cell membrane.

² IFA slide wells contain only microorganisms.

B. Materials

1. Equipment

- a) 2- to 10- μ L single-channel, adjustable pipette for adding sample
- b) 2- to 5-mL repeating pipettes for dispensing fluorescein (FITC)-labeled anti-immunoglobulin
- c) Humidified chamber
- d) Incubator at 35-40 °C
- e) Slide washing reservoirs
- f) Coverslips
- g) Fluorescence microscope

For FITC, the microscope should have a mercury or xenon light source with a 495 nm exciting filter and a 520 nm suppression filter. We typically examine slides at magnifications of 100-400X.

2. Description of key reagents

a) Antigen-coated slides

For most viruses, we have both virus-infected and uninfected control cells in the same slide well. These slides (INTENDED FOR RESEARCH USE ONLY) are produced by infecting cells grown on the slide with near-endpoint dilutions of virus. Slides are incubated for 1-3 days and then are acetone-fixed. For a list of available IFA slides, go to: http://www.criver.com/files/pdfs/research-models/rm_ld_m_ifa_reagents_item_list.aspx

b) Positive immune and nonimmune control sera

It is essential that you test standard positive and negative control sera along with your samples to verify assay sensitivity and specificity. Results for control sera are also helpful when troubleshooting. **NOTE:** Our immune control sera are supplied at their working dilutions. Non-immune control sera are pre-diluted five-fold in PBS. You should not repeatedly freeze and thaw control sera or leave them at room or refrigeration temperature for more than 24 hours. We recommend dividing the sera into small-volume, single-use aliquots that can be thawed and used at the time the assay is performed. For a list of available IFA controls, go to: http://www.criver.com/files/pdfs/research-models/rm_ld_m_ifa_reagents_item_list.aspx.

c) FITC-labeled anti-immunoglobulin

Charles River does supply this component; however, there are many commercial sources of affinity-purified species-specific FITC-labeled IgG. It has been our experience that these conjugates work well at the dilutions recommended by the manufacturer (generally 1:25-50). For a list of available IFA conjugates, go to: http://www.criver.com/files/pdfs/research-models/rm_ld_m_ifa_reagents_item_list.aspx

d) Coverslip mounting medium: Tris-buffered glycerol, pH 8.7

Mounting medium for FITC is buffered to a basic pH to enhance fluorescence.

e) Wash solutions: PBS pH 7.4 and DI H₂O

3. Preparation of buffers, diluents and solutions

a) BLOTTO serum diluent:

Refer to ELISA section for preparation

b) Mounting medium: 0.1 M Tris-buffered glycerol, pH 8.7.

The following formulation is for preparing 20 mL:

Components	Amount
1.0 M Tris-HCl stock solution	2 mL
Glycerol	18 mL

(1) Mixing instructions

Add 2 mL of 1.0 M Tris-HCl stock solution (refer to ELISA section for preparation) to 18 mL of glycerol and mix thoroughly.

(2) Storage

Store at room temperature and use within one month.

c) Diluted conjugate

Components	Amount
Reconstituted FITC conjugate stock	Variable
PBS	Variable

(1) Mixing instructions

Commercial conjugates are generally supplied in the lyophilized form. Follow the manufacturer's instructions to reconstitute the conjugate. Divide into small aliquots and store frozen at -10 °C or below. Thaw a vial of FITC conjugate and prepare the recommended working dilution in PBS.

(2) Storage

Store at 2-8 °C and use within five days.

d) Wash buffer (PBS, pH 7.4): Sigma (P3813-10 PAK or equivalent)

(1) Mixing instructions

Measure out and dispense one liter of filtered DI H₂O into a 1-L bottle. Open a PBS packet and carefully dispense the entire contents into the bottle. Mix the solution until all components are dissolved.

(2) Storage

Store at room temperature and use within six months.

C. Sample Preparation, Collection and Storage

Collection and storage is the same as for ELISA (Section 2.C). No other sample preparation is necessary.

D. Testing

1. Prepare slides.

- a) Remove the appropriate type and number of slides from the freezer.
- b) Allow them to warm to room temperature.

2. Organize test samples.

- a) Retrieve and arrange your samples.
- b) Record the well locations of samples and controls used, as well as appropriate lot information.

3. Place samples and controls on slides and incubate.

- a) Add 5 μ L of BLOTTO diluent to all wells except those reserved for positive control sera.
- b) Add the test samples and negative controls to the appropriate wells. The volume of sample or control per well is 5 μ L (final dilution 1/10). Spread each sample over the whole well, being careful not to scrape the surface.
- c) Add 10 μ L of positive control serum to the appropriate well(s).
- d) Incubate the slides at 35-40 °C in a humidified chamber for 30 minutes.

4. Wash.

- a) Gently aspirate the serum samples from the individual wells. Carefully rinse the slides in PBS by gentle agitation. This initial quick-rinse step helps prevent cross-contamination
- b) Fill three separate wash reservoirs with PBS and a fourth with DI H₂O.
- c) Incubate the slides in the second PBS-filled reservoir for 2-5 minutes.
- d) Move the slide to the next wash reservoir and incubate for another 2-5 minutes.
- e) Transfer the slides to the DI water wash reservoir and rinse.
- f) Air dry. DO NOT BLOT.

5. Add conjugate and incubate.

- a) Using a repeating pipette, add 10 μ L of diluted FITC conjugate to all wells.
- b) Incubate the slides at 35-40 °C in a humidified chamber for 30 minutes.

6. Wash and cover with mounting medium.

- a) Wash the slides as in Step 4.
- b) Add a drop of mounting medium and place a coverslip on each slide. Avoid trapping air bubbles. Avoid using too much mounting medium, as the coverslip will float and the excess medium may spread to the microscope objective.

7. Read results.

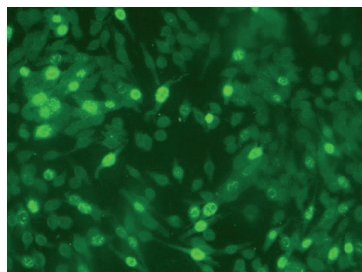
- a) It is recommended to read the slides immediately to verify that the assay is valid (positive and negative controls are acceptable). Samples may be read immediately or the slides may be stored at -20 °C for up to one week without deterioration of the fluorescence.
- b) Examine the slides with the fluorescence microscope at a magnification of 100-400X. FITC fluorescence is yellow-green in color.
- c) After examination of the positive and negative controls, score the results as follows:

Flourescence	Score
Minimal, comparable to nonimmune control	–
Very dim, granular	1
Moderate granular	2
Bright granular	3
Glaring granular	4
Diffuse	TC

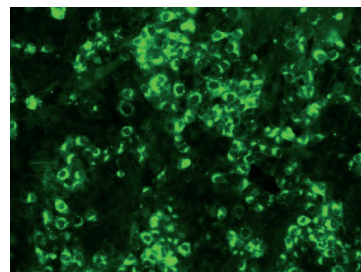
E. Results Interpretation

Correct reading of IFA reactions takes practice. Bright granular fluorescence is typical of specific antibody-viral antigen reactions, whereas diffuse fluorescence suggests a nonspecific tissue reaction (TC). A TC reaction is also probable when the percentage of fluorescing cells or the location of the fluorescence is markedly different from that observed in the positive control well. In the case of certain DNA viruses, such as the rodent parvoviruses MVM, KRV and H-1, strong nuclear fluorescence is characteristic, while other viruses show predominantly cytoplasmic fluorescence (see table on page 12).

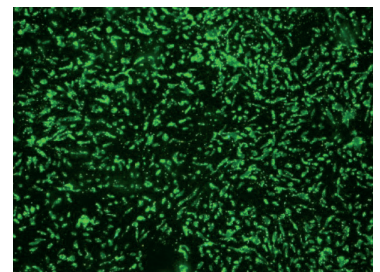
Examples of IFA Staining



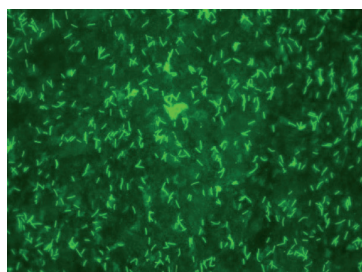
Nuclear: MVM



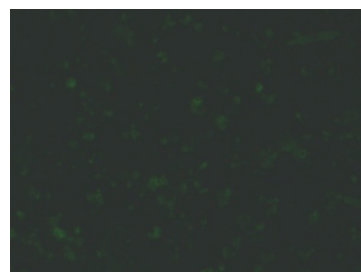
Cytoplasmic: Hantaan



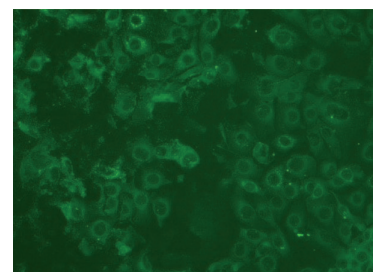
Mycoplasma
M. pulmonis attached to host cell membrane



Bacterial: *C. pil*



Negative serum (MAD IFA slide)



TC/Nonspecific (EDIM IFA slide)

F. Troubleshooting

The following table describes common IFA issues and their probable cause.

Observation	Possible Cause	
	Component	Problem
No or weak fluorescence in positive control well	Control sera	<ul style="list-style-type: none"> • Not added • Incorrect specificity • Diluted improperly (i.e., too dilute) • Inactivated by improper storage or repeated freeze-thaws
	Conjugate	<ul style="list-style-type: none"> • Incorrect specificity • Too dilute • Inactivated by improper storage or repeated freeze-thaws
	Slide	<ul style="list-style-type: none"> • Percentage of infected cells too low • No cells or cells scraped off during procedures • Wrong virus
	Fluorescence	<ul style="list-style-type: none"> • Wrong light source
	Microscope	<ul style="list-style-type: none"> • Incorrect exciting/suppression filter • Not aligned properly
Excessive background	Serum samples	<ul style="list-style-type: none"> • Dilution too low • BLOTTO blocking diluent not used • Source animals parentally immunized or have autoimmune disease
	Conjugate	<ul style="list-style-type: none"> • Dilution too low • Poor quality, not affinity purified
	Slide	<ul style="list-style-type: none"> • Cells not spread out. This increases background and reduces specific fluorescence. • Bacterially contaminated during preparation • No humidity to prevent evaporation of well contents

IV. Appendices

A. Equipment List for Serology Testing

1. Pipettes, single- and multi-channel

Company	Web Address
Eppendorf	www.eppendorf.com
Rainin	www.shoprainin.com
Hamilton	www.hamiltoncompany.com

2. Plate washers/readers

Company	Web Address
BioTek	www.biotek.com

B. Reagent Suppliers for Serology Testing

Company	Web Address
Kirkegaard & Perry	www.kpl.com
Sigma-Aldrich	www.sigma-aldrich.com
Fisher Scientific	www.fishersci.com