Serologic Methods Manual

June 19, 2001

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Contents

Section 1: Introduction

CHARLES RIVER
LABORATORIES
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1.1 Introduction

Laboratory animals adventitiously (i.e., accidentally) infected with pathogenic bacteria, viruses, and parasites may not suitable for research. This is because adventitious infections may lead to clinical disease and pathological changes, especially in perinatal and immunodeficient animals. Although infections in post-weaning, immunocompetent animals are often subclinical, they can contaminate biological materials and produce other changes that cloud the interpretation of experimental results. Furthermore, some agents indigenous to laboratory animals are zoonotic. While causing asymptomatic infections of their natural hosts, such agents may cause disease in people.

Charles River Laboratories has pioneered large-scale production of rodents free from adventitious infection using cesarean derivation and barrier maintenance. To prevent infections in transit, these "virus antibody-free" (VAF) rodents are shipped in filtered crates. Investigators can maintain the VAF status by strict biosecurity that may include the use of barrier rooms, isolators or microisolation units. However, adventitious infections continue to occur at both breeder and customer facilities, despite the use of rigorous procedures. It is therefore essential to perform routine comprehensive health monitoring employing a variety of diagnostic methodologies.

Although barrier rooms have successfully excluded most pathogenic bacteria and parasites, viral contaminations continue to be prevalent. This is because viruses are small, shed in large amounts and highly infectious. Routine monitoring for virus exposure is accomplished by serologic testing for virus-specific antibodies formed as part of the immune response to infection. Serology is also commonly used to test mice and rats for antibodies to Mycoplasma pulmonis (the cause of murine respiratory and genital mycoplasmosis) and a few other non-viral agents (Table 1.1). This is the case because antibodies are persistent, and assays for their detection are rapid, sensitive and specific when carefully controlled.

AGENT	ABBRV.	FAMILY/ORDER	SUBFAM/GENUS	HOST SPECIES
Sendai virus	SEND	Parainfluenza	Para-1	M,R,GP,H
Simian virus 5	SV-5	Parainfluenza	Para-2	GP,H
Pneumonia virus of mice	PVM	Paramyxo	Pneumo	M,R,GP,H
Mouse hepatitis virus	MHV	Corona	Corona	M
Rat coronavirus/Sialodacryoadenitis virus	SDAV	Corona	Corona	R
Minute virus of mice	MVM	Parvo	Parvo	M
Rat parvovirus	RPV	Parvo	Parvo	R
Mouse parvovirus	MPV	Parvo	Parvo	M
Kilham rat virus	KRV	Parvo	Parvo	R
Toolan's H-1 virus	H-1	Parvo	Parvo	R
Theiler's murine encephalomyelitis virus	TMEV	Picorna	Cardio	M,R
Reovirus	REO	Reo	Reo	M,R,GP,H
Lymphocytic choriomeningitis virus	LCMV	Arena	Arena	M,R,GP,H
Hantaan virus	HTN	Bunya	Hanta	M,R
Seoul virus	SEO	Bunya	Hanta	M,R
Prospect Hill Virus	PHV	Bunya	Hanta	M
Ectromelia virus	ECTRO	Pox	Orthopox	M
Mouse adenovirus FL/K87	MAV	Adeno	Mastadeno	M,R
Guinea Pig adenovirus	GAV	Adeno	Mastadeno	GP
Mouse pneumonitis virus	K	Papova	Polyoma	M
Polyoma virus	POLY	Papova	Polyoma	M
Epizootic diarrhea of infant mice virus	EDIM	Reo	Rota	M
Mouse cytomegalovirus	MCMV	Herpes	Betaherpes	M
Mouse thymic virus	MTLV	Herpes	Gammaherpes	M
Lactate Dehydrogenase-elevating virus	LDV	Toga	Toga	M
Mycoplasma pulmonis	MPUL	Mycoplasmataceae	Mycoplasma	M,R
Encephalitozoon cuniculi	ECUN	Pleistophoridiae		M,R,GP,H
Cilia-Associated Respiratory Bacillus	CARB			M,R
Treponema paraluis-cuniculi	TREP			Rb
Clostridium piliforme	CPIL			M,R

Species: M = mouse, R = rat, GP = guinea pig, H = hamster, Rb = rabbit

Table 1.1

While serologic tests are designed to be sensitive and specific, false positive and negative results do occur (Figure 1.1). Some of the reasons for inaccurate results are described in the CRL Technical Bulletin (Serological Testing to Monitor Rodents for Viral and Mycoplasmal Infection - Fall 1990) on interpretation of serologic results. We strongly recommend that you confirm new positive findings by alternative diagnostic methods and by testing additional animals.

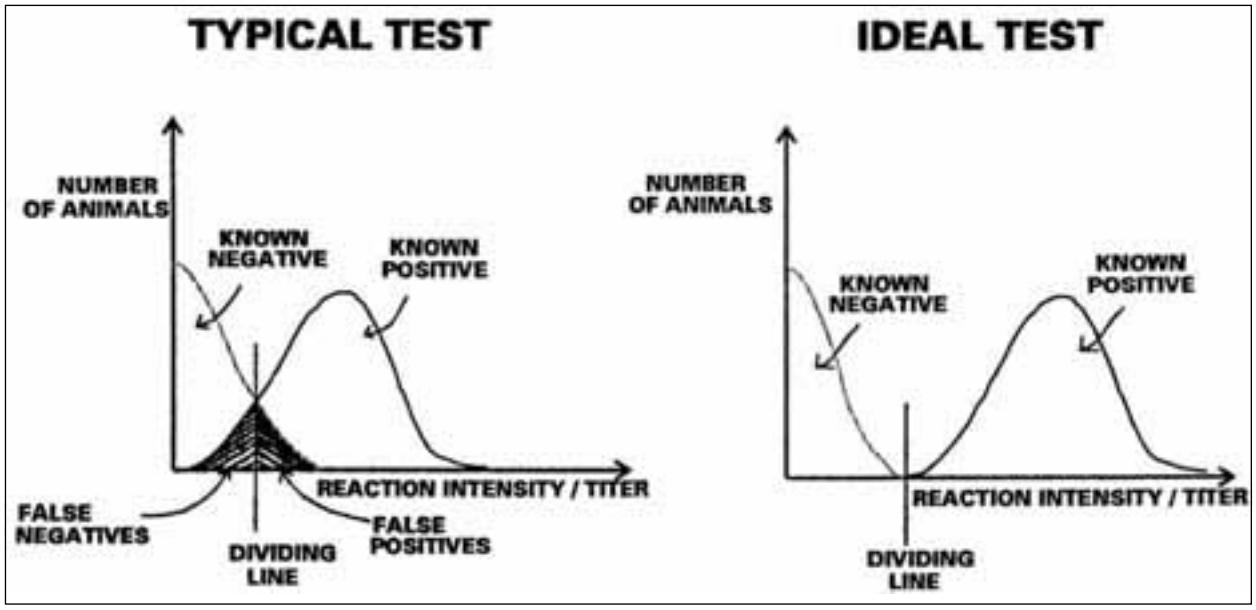


Figure 1.1

Traditional serologic tests, such as complement fixation (CF) and hemagglutination inhibition (HAI), have been supplanted by non-radioisotopic solid phase immunoassays, notably the enzyme-linked immunosorbent assay (ELISA) and the indirect fluorescent antibody test (IFA). The ELISA and 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 by the Serology Department at Charles River. The following topics will be covered for each assay:

1. Methodology Overview
2. Materials, including equipment and supplies.
3. Reagent Preparation
4. Sample Preparation
5. Step-by-Step Assay Procedure
6. Results Interpretation
7. Trouble Shooting

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

Section 2: Sample Processing and Results Reporting

2.1 Overview

The actual performance of assays is only one part of a successful testing process. Before assays are performed, specimens are collected and submitted along with the information required for processing. Once received, sample groups and individual specimens are uniquely identified and the tests to be performed on them recorded. They are arranged in an orderly manner, with their locations in each test plate or slide identified on the appropriate forms. Once assays are completed, results are recorded and reported. It is important to have a system for distributing and filing reports. Finally, specimens should be archived just in case additional testing is requested.

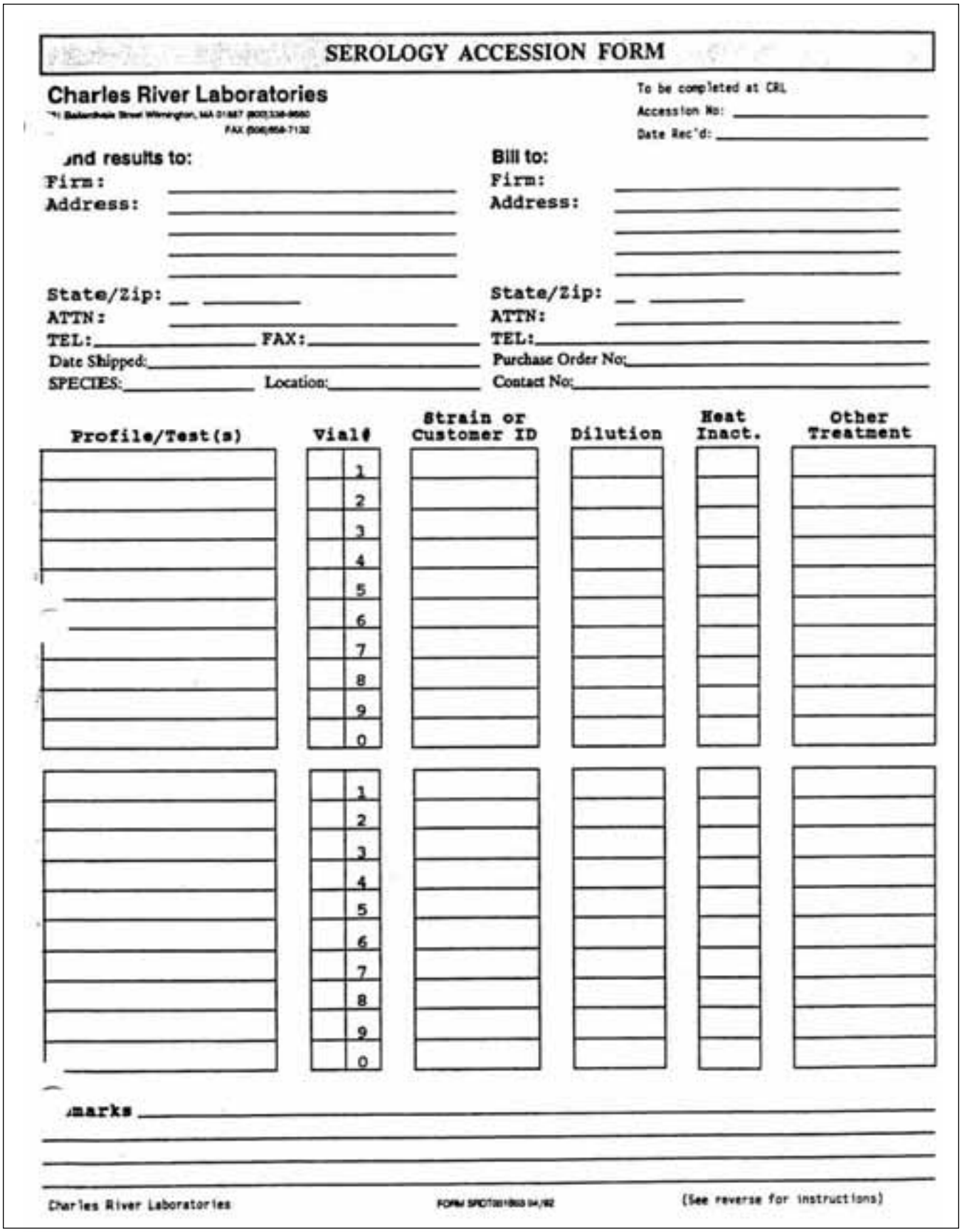
2.2 Charles River Sample Processing

2.2.1 Submission

In order to make initial sample processing simpler and more efficient, we request that customers submit samples diluted 1 part serum to 4 parts buffered saline in standard vials.

We require that all submissions be preceded by a faxed accession form (Figure 2.1) on which the customer provides the name and address of the person to be given the results and the following information on each specimen:

- Species of origin
- A unique identification code
- Dilution and any treatment(s), e.g., heat inactivation.
- Tests to be performed. We refer to panels of assays commonly performed together as profiles. Profiles make it easier for customers to specify the tests they want done.



SEROLOGY ACCESSION FORM

Charles River Laboratories

To be completed at CRL
Accession No: _____
Date Rec'd: _____

nd results to:
Firm: _____
Address: _____

Bill to:
Firm: _____
Address: _____

State/Zip: __ _____
ATTN: _____
TEL: _____ FAX: _____
Date Shipped: _____
SPECIES: _____ Location: _____

State/Zip: __ _____
ATTN: _____
TEL: _____
Purchase Order No: _____
Contact No: _____

Profile/Test(s)	Vial#	Strain or Customer ID	Dilution	Heat Inact.	Other Treatment
	1				
	2				
	3				
	4				
	5				
	6				
	7				
	8				
	9				
	0				
	1				
	2				
	3				
	4				
	5				
	6				
	7				
	8				
	9				
	0				

marks _____

Charles River Laboratories FORM SRCT301/003 04/92 (See reverse for instructions)

Figure 2.1

2.2.2 Receipt

1. We record when a specimen group was received on the submission form and in our laboratory information management system (LIMS).
2. The LIMS assigns an accession order number to each group, e.g., 2000-001234.
3. Information from the submission form is transferred to LIMS and a serology log book (Figure 2.2)
4. Individual specimens within an accession are given sequential ID numbers generally starting with 1.
5. The information received and recorded is summarized in a setup sheet report generated from the LIMS (Figure 2.3).

CHARLES RIVER LABORATORIES, WILMINGTON, MA

SEROLOGY LOG BOOK

DATE: ____________

AREA/ SPECIES	TEST	H-1	KRV	SDA	MHV	PVM	Sendai	REO-3	GD-7	K	MVM	TV-3	MAD	ECTRO	POLY	LCM	ROTA	PPLO	E.CUN	CMV	HANT	MTV	CARB	B.FIL	#
	ELISA																								
	OTHER																								
	ELISA																								
	OTHER																								
	ELISA																								
	OTHER																								
	ELISA																								
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CRL FORM NALS SRDT-002 REV.04 3/24/04

Figure 2.2

LA0007 21:16:42 CRPS LABORATORY ACCESSION MANAGEMENT SYSTEM 7/22/93
(HEALTH MONITORING)
SEROLOGY SET-UP SHEET

ACCESSION NUMBER: 93-006225 DATE RECEIVED: 0/00/00
SCHEDULED DATE: 7/26/93
DUE DATE: 8/05/93

CUSTOMER NAME: CHARLES RIVER PORTAGE
CRL DIVISION PORTAGE AREA: P09 PURCHASE ORDER NO.:

PROTOCOL: HM 000044
PROTOCOL DESCRIPTION: SEROLOGY ONLY - AP - GUINEA PIG(FEMALE)

CUSTOMER ANIMAL SOURCE:

SPECIES: GUINEA PIG
NUMBER OF SAMPLES: 8

CRPS ID	CUSTOMER ID	CATALOG DESCRIPTION	DILUTION	TREATMENT	STRAIN	--- AGE --- MIN	MAX	UNIT	-WEIGHT- MIN	UNIT	SEX
001		GP ASSESSMENT	5		HARTLEY			RB			F
002		GP ASSESSMENT	5		HARTLEY			RB			F
003		GP ASSESSMENT	5		HARTLEY			RB			F
004		GP ASSESSMENT	5		HARTLEY			RB			F
005		GP ASSESSMENT	5		HARTLEY			RB			F
006		GP ASSESSMENT	5		HARTLEY			RB			F
007		GP ASSESSMENT	5		HARTLEY			RB			F
008		GP ASSESSMENT	5		HARTLEY			RB			F

COMMENTS:

END OF SET-UP

Figure 2.3

2.2.3 Organization of Samples for Testing

2.2.3.1 ELISA

ELISAs are performed in 96-well, polystyrene, flat-bottom microtiter plates. Sera specimens are assigned to sample plates based on the profile of tests to be performed. They are then arranged in racks and transferred to the appropriate sample plate. Specimen locations are recorded on a sample plate organization form (Figure 2.4) and entered into our ELISA computer program. An example of the report generated from the Charles River program is shown in Figure 2.5.
Each sample is transferred into a pair of wells containing diluent. Diluted samples are then transferred to corresponding wells in antigen-coated test plates (See Section 3).

Charles River Laboratories, Wilmington, MA
ELISA SAMPLE PLATE ORGANIZATION SHEET

DATE: __________ SPECIES: ____________________

PLATE: __________

	H	G	F	E	D	C	B	A	
	TC	AG	TC	AG	TC	AG	TC	AG	
									1
									2
									3
									4
									5
									6
									7
									8
									9
									10
									11
									12

CHECKED BY: __________

PLATE: __________

	H	G	F	E	D	C	B	A	
	TC	AG	TC	AG	TC	AG	TC	AG	
									1
									2
									3
									4
									5
									6
									7
									8
									9
									10
									11
									12

CHECKED BY: __________

PLATE: __________

	H	G	F	E	D	C	B	A	
	TC	AG	TC	AG	TC	AG	TC	AG	
									1
									2
									3
									4
									5
									6
									7
									8
									9
									10
									11
									12

CHECKED BY: __________

PLATE: __________

	H	G	F	E	D	C	B	A	
	TC	AG	TC	AG	TC	AG	TC	AG	
									1
									2
									3
									4
									5
									6
									7
									8
									9
									10
									11
									12

CHECKED BY: __________

CRL FORM NALS SRDT-003 Rev 03 3/24/94

Figure 2.4

SAMPLE PLATE IDENTIFICATION Date: 01-01-1980

Sample plate: 3

LOC	ACCSN#	CRP	LOC	ACCSN#	CRP	LOC	ACCSN#	C
1AB	88-3556	1	5AB	88-3661	13	9AB	88-3686	
1CD	88-3556	2	5CD	88-3672	1	9CD	88-3686	
1EF	88-3556	3	5EF	88-3716	1	9EF	88-3686	
1GH	88-3556	4	5GH	88-3716	2	9GH	88-3686	
2AB	88-3661	1	6AB	88-3720	2	10AB	88-3687	
2CD	88-3661	2	6CD	88-3720	3	10CD	88-3687	
2EF	88-3661	3	6EF	88-3720	4	10EF	88-3687	
2GH	88-3661	4	6GH	88-3720	5	10GH	88-3687	
3AB	88-3661	5	7AB	88-3685	1	11AB	88-3687	
3CD	88-3661	6	7CD	88-3685	2	11CD	88-3687	
3EF	88-3661	7	7EF	88-3685	3	11EF	-s437	
3GH	88-3661	8	7GH	88-3685	4	11GH	-s437	
4AB	88-3661	9	8AB	88-3685	5	12AB	CO-NTRL	00
4CD	88-3661	10	8CD	88-3685	6	12CD	CO-NTRL	00
4EF	88-3661	11	8EF	88-3686	1	12EF	CO-NTRL	00
4GH	88-3661	12	8GH	88-3686	2	12GH	CO-NTRL	00

Sample plate: 4

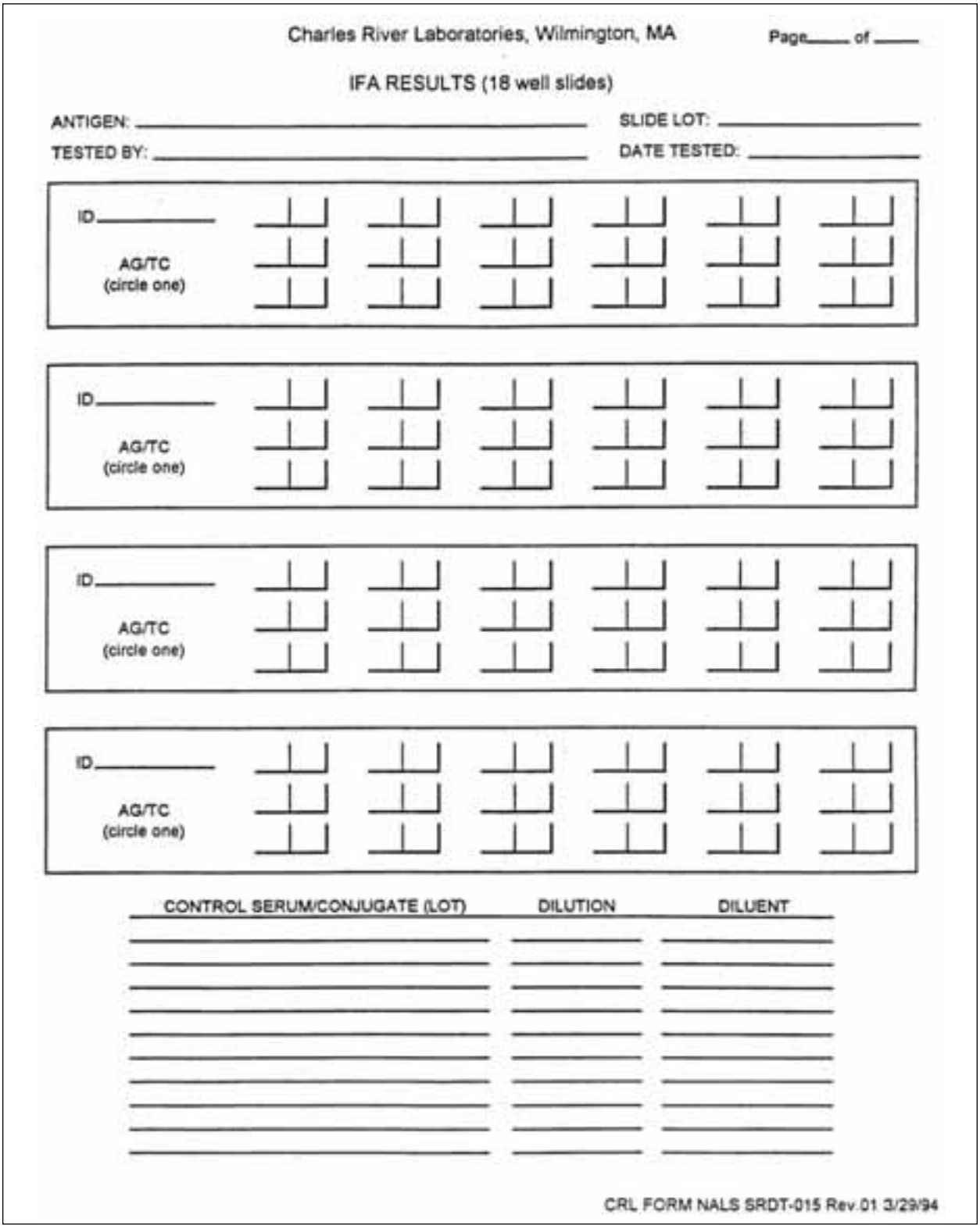
LOC	ACCSN#	CRP	LOC	ACCSN#	CRP	LOC	ACCSN#	C
1AB	88-3610	1	5AB	88-3610	17	9AB	88-3610	
1CD	88-3610	2	5CD	88-3610	18	9CD	88-3610	
1EF	88-3610	3	5EF	88-3610	19	9EF	88-3610	
1GH	88-3610	4	5GH	88-3610	20	9GH	88-3610	
2AB	88-3610	5	6AB	88-3610	21	10AB	88-3610	
2CD	88-3610	6	6CD	88-3610	22	10CD	88-3610	
2EF	88-3610	7	6EF	88-3610	23	10EF	88-3610	
2GH	88-3610	8	6GH	88-3610	24	10GH	88-3693	
3AB	88-3610	9	7AB	88-3610	25	11AB	88-3693	
3CD	88-3610	10	7CD	88-3610	26	11CD	88-3693	
3EF	88-3610	11	7EF	88-3610	27	11EF	88-3693	
3GH	88-3610	12	7GH	88-3610	28	11GH	88-3693	
4AB	88-3610	13	8AB	88-3610	29	12AB	CO-NTRL	00
4CD	88-3610	14	8CD	88-3610	30	12CD	CO-NTRL	00
4EF	88-3610	15	8EF	88-3610	31	12EF	CO-NTRL	00
4GH	88-3610	16	8GH	88-3610	32	12GH	CO-NTRL	00

Form SRDT022801 1/86

Figure 2.5

2.2.3.2 IFA

The IFA is performed in Teflon-coated 12-18-well, glass microscope slides. The well to which sera specimens are to be added are recorded on the IFA results form (Figure 2.6) and the samples are then transferred into the appropriate slide wells for testing (see Section 4).


Charles River Laboratories, Wilmington, MA Page____ of ____

IFA RESULTS (18 well slides)

ANTIGEN: _____ SLIDE LOT: _____

TESTED BY: _____ DATE TESTED: _____

ID_____ AG/TC (circle one)

ID_____ AG/TC (circle one)

ID_____ AG/TC (circle one)

ID_____ AG/TC (circle one)

CONTROL SERUM/CONJUGATE (LOT)	DILUTION	DILUENT

CRL FORM NALS SRDT-015 Rev.01 3/29/94

Figure 2.6

2.2.3.3 HAI

The HAI is performed in v-bottom, polystyrene, 96 well microtiter plates. The sample locations are recorded on the HAI results form (Figure 2.7). Each specimen is serially diluted in two columns, one to which virus is added and the other a control column without virus (see Section 5).

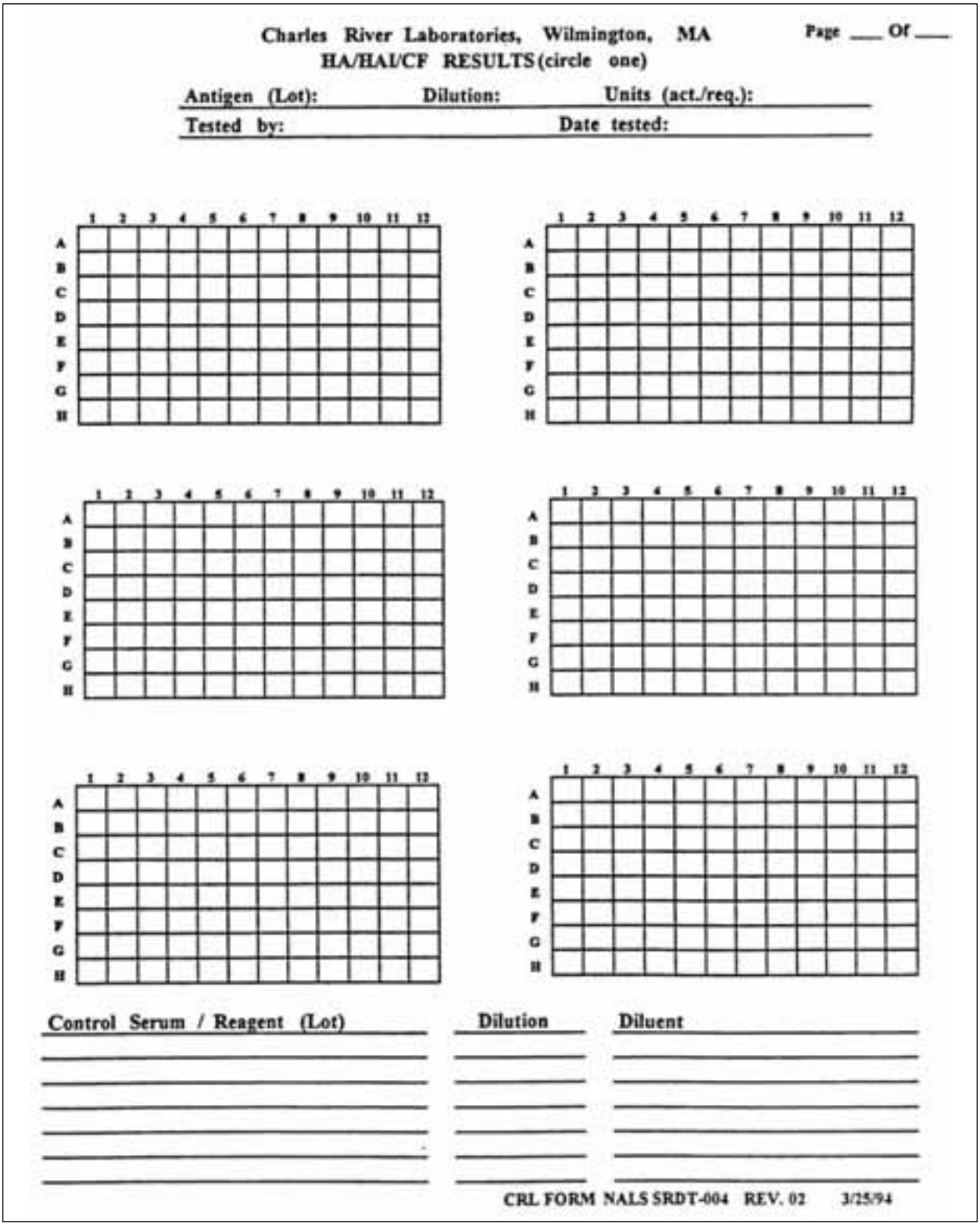


Figure 2.7

2.2.4 Recording Results

2.2.4.1 ELISA

Reactions are generally read spectrophotometrically with an ELISA plate reader. We have our reader connected to a PC equipped with specific ELISA software. However, there are a number of excellent commercial programs that can be used for this purpose. Absorbance values from the reader are automatically transferred to the PC where they are compiled into reports by accession. Alternatively, reports can be prepared manually from plate reader printouts (see Figure 2.8).

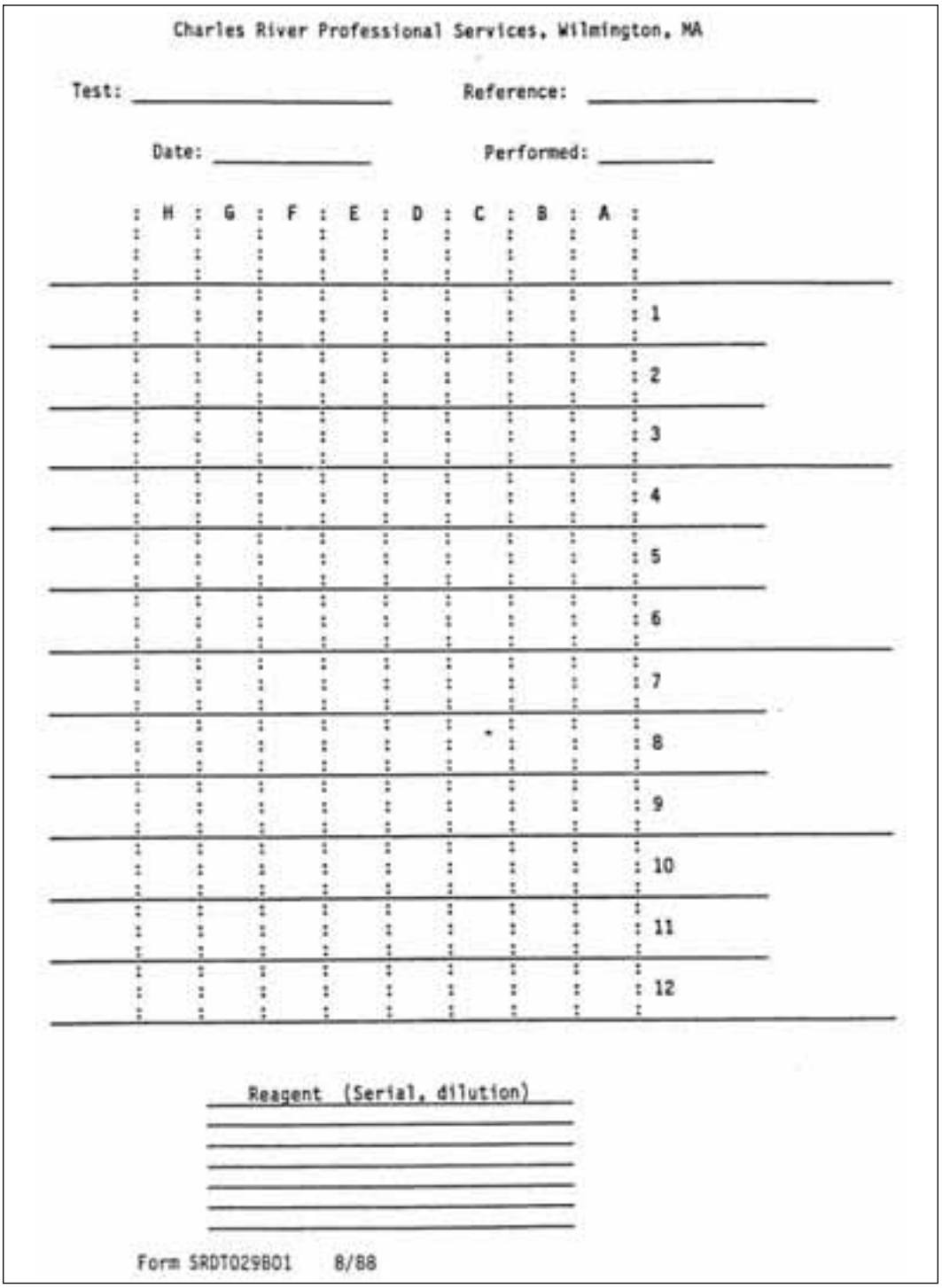


Figure 2.8

2.2.4.2 IFA

IFA slides are read with a fluorescence microscope and reactions scored on a result form.

2.2.4.3 HAI

HAI plates are read visually and the absence or presence of hemagglutination is recorded on a result form.

2.2.5 Reporting and Filing Results

We report results by group (i.e., accession number) with assays listed on one axis and sample ID's on the other, as shown in Figure 2.9. Also indicated on the report are the following:

- Accession Number
- Sponsor and contact person
- Sample information (source, sex, and strain)
- Report Distribution

The CRL Serology Department files all raw data by week. Final reports for internal CRL quality control are filed by facility, room and species. Commercial reports are filed by customer.

DSK 8.29.94

Charles River Laboratories

251 Ballardvale Street, Wilmington, MA 01887
(508)658-6000 800-338-9680 Fax: 508-658-7132

SEROLOGY REPORT

8/29/94

TO: CHARLES RIVER PORTAGE
P.O. BOX 176
PORTAGE
MI 49081

ACCESSION NUMBER: 94-007289
PURCHASE ORDER NO.:
DATE SCHEDULED: 8/22/94
DATE RECEIVED: 8/25/94
DATE COMPLETED: 8/29/94

DIV: PORTAGE AREA: P01
SPECIES: MOUSE STRAIN: C3H

ATTN: CRPS

CRPS ID: / CUST ID:	001	002	003	004	005	006	007	008	SIG. TITER
METHOD AGENT									
ELISA SEND	-	-	-	-	-	-	-	-	
ELISA PVM	-	-	-	-	-	-	-	-	
ELISA MHV	-	-	-	-	-	-	-	-	
ELISA MVM	-	-	-	-	-	-	-	-	
IFA KRV	-	-	-	-	-	-	-	-	
ELISA GD-7	-	-	-	-	-	-	-	-	
ELISA REO-3	-	-	-	-	-	-	-	-	
ELISA MPUL	-	-	-	-	-	-	-	-	
ELISA EDIM	-	-	-	-	-	-	-	-	

COMMENTS:
ELISA/IFA Results: - = Negative; +/- = Equivocal; + = Moderate to strong positive; TC = Non-specific reaction with tissue control.

Figure 2.9

Section 3. Enzyme-Linked Immunosorbent Assay (ELISA)

3.1 Methodology Overview

The indirect ELISA method is the one most often used for detection of antibodies (Figure 3.1). Typically, antigen is immobilized on the surface of wells in microtiter plates made of specially prepared polystyrene or polyvinyl. The specimens are incubated in the well to which antibodies may bind. Unbound antibodies are removed by washing. Those that attach 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 antibodies from the specimen that have attached to a well. Color intensity can be assessed visually or spectrophotometrically (in absorbance units) with an ELISA reader.

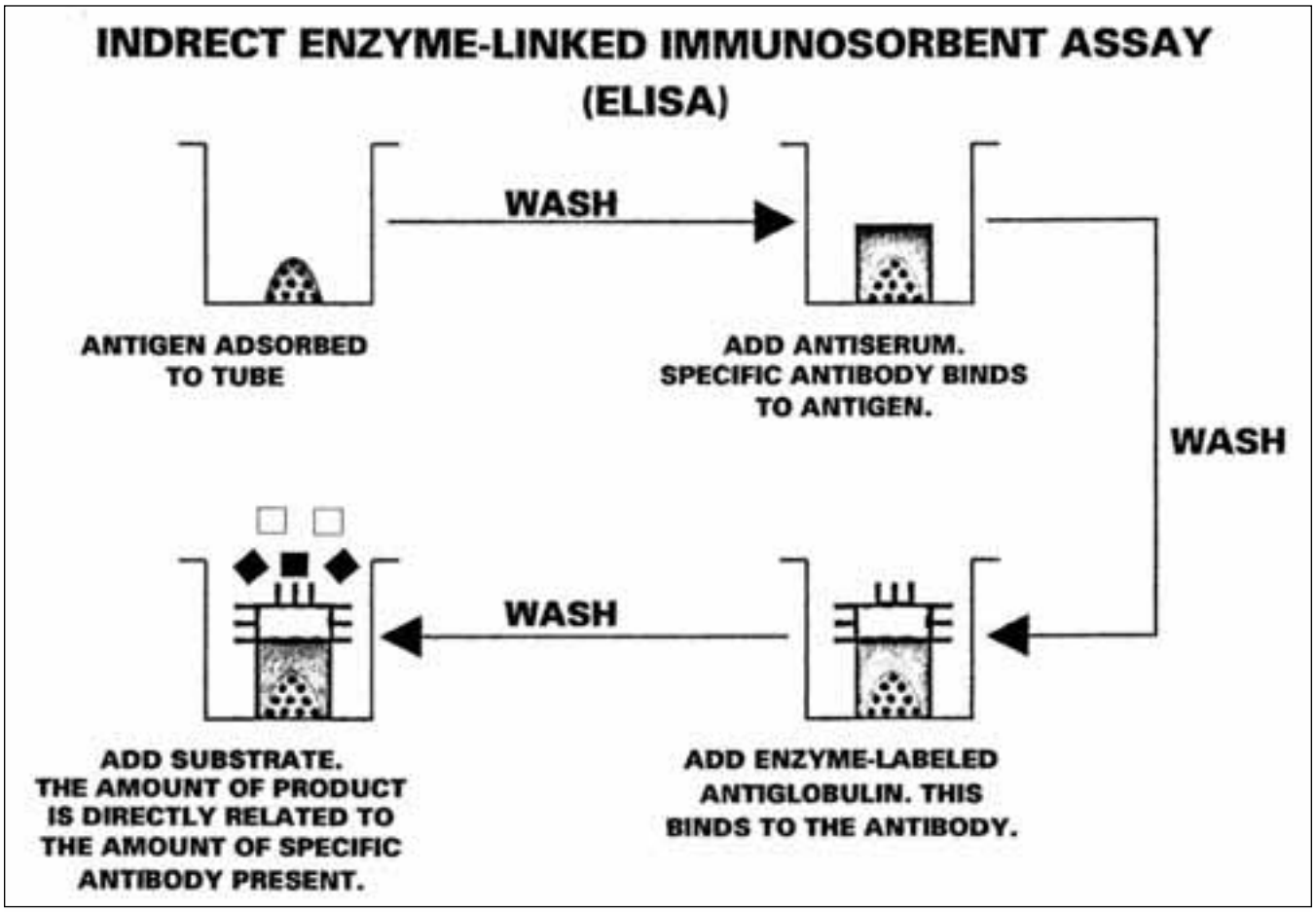


Figure 3.1

Ideally, attached antibodies are antigen-specific. In practice, however, they may bind non-specifically. We incubate each sample in a tissue control well to detect non-specific binding. The tissue control does not contain virus. It is usually prepared from the host system in which the virus is propagated. For example, we propagate Sendai virus in the allantoic sac of embryonated eggs. The tissue control for Sendai virus is allantoic fluid collected from un-infected eggs. In the case of M. pulmonis, however, the tissue control is another cross-reacting rodent mycoplasma, M. arthritidis.

A result is recorded as positive 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 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 (Figure 3.2). A TC result is considered non-specific and does not indicate whether a sample is antibody positive or negative. We usually retest the sample by an alternative method and if necessary, we test additional samples.

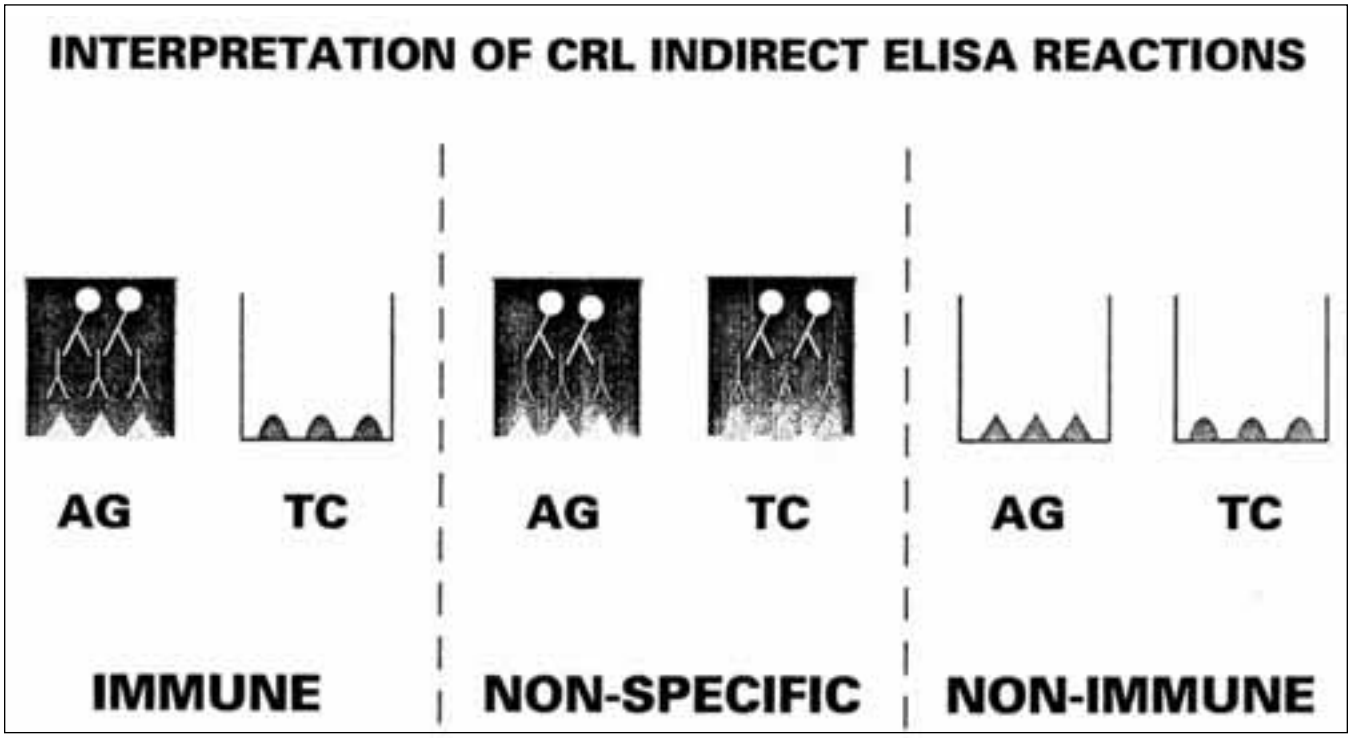


Figure 3.2

3.2 Materials

*3.2.1 Equipment**

1. **Incubator at 35-40º C:** For best sensitivity and reproducibility, you should bring test plates to the incubation temperature as quickly as possible. To accomplish this, we recommend that you use a mechanically circulated hot air incubator rather than a convection incubator. In addition, use an incubator with adequate shelf space to avoid stacking plates more than two high. Cover the plates or humidify the air to prevent evaporation.

2. **Plate Washer (Optional):** 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.

3. **Reader (Optional):** While you can read test results visually, we recommend that you use an ELISA plate reader equipped with a light source and filter appropriate to the color produced by your substrate. We will describe the preparation and use of the substrate ABTS-H_2O_2 that requires a 405nm filter. Most ELISA plate readers have RS-232 serial ports, which allow them to send results to a PC for analysis. We have found that using a computer to receive absorbance values and compile reports saves time and effort, and prevents errors.

4. **Pipettors:** Reagent and sample preparation and transfers require various pipetting devices that accurately dispense volumes of 10 to 1000 microliters. For most purposes that following pipettes are adequate:

TYPE	MICROLITERS
Single Channel, Adjustable Volume	2 - 10 10 - 100 100 - 1000
8 or 12 Channel, Adjustable Volume	5 - 50 50 - 300
Repeating Pipette with 8 or 12 channel	50

3.2.2 Description of Key Reagents

1. **Antigen- and Tissue Control-Coated Test Plates:** The pattern of how our ELISA plates are coated is shown in Figure 3.3. Wells in rows A, C, E, and G contain antigen. The remaining wells are coated with tissue control. Our test plates (INTENDED FOR RESEARCH USE ONLY) are available in a fixed 96-well format or filled with removable 8 or 16-well strips. Plates with removable strips offer you the flexibility of doing less than 48 tests at a time. As indicated in the Product Specification sheet (Figure 3.4), Charles River antigen-coated plates should be stored in a freezer at -10ºC or below. Some ELISA test plates (i.e., LCMV, SV-5, HANT, MPV and NS-1) must be stored at -60ºC or below.

* SEE APPENDIX A FOR LIST OF EQUIPMENT MANUFACTURERS

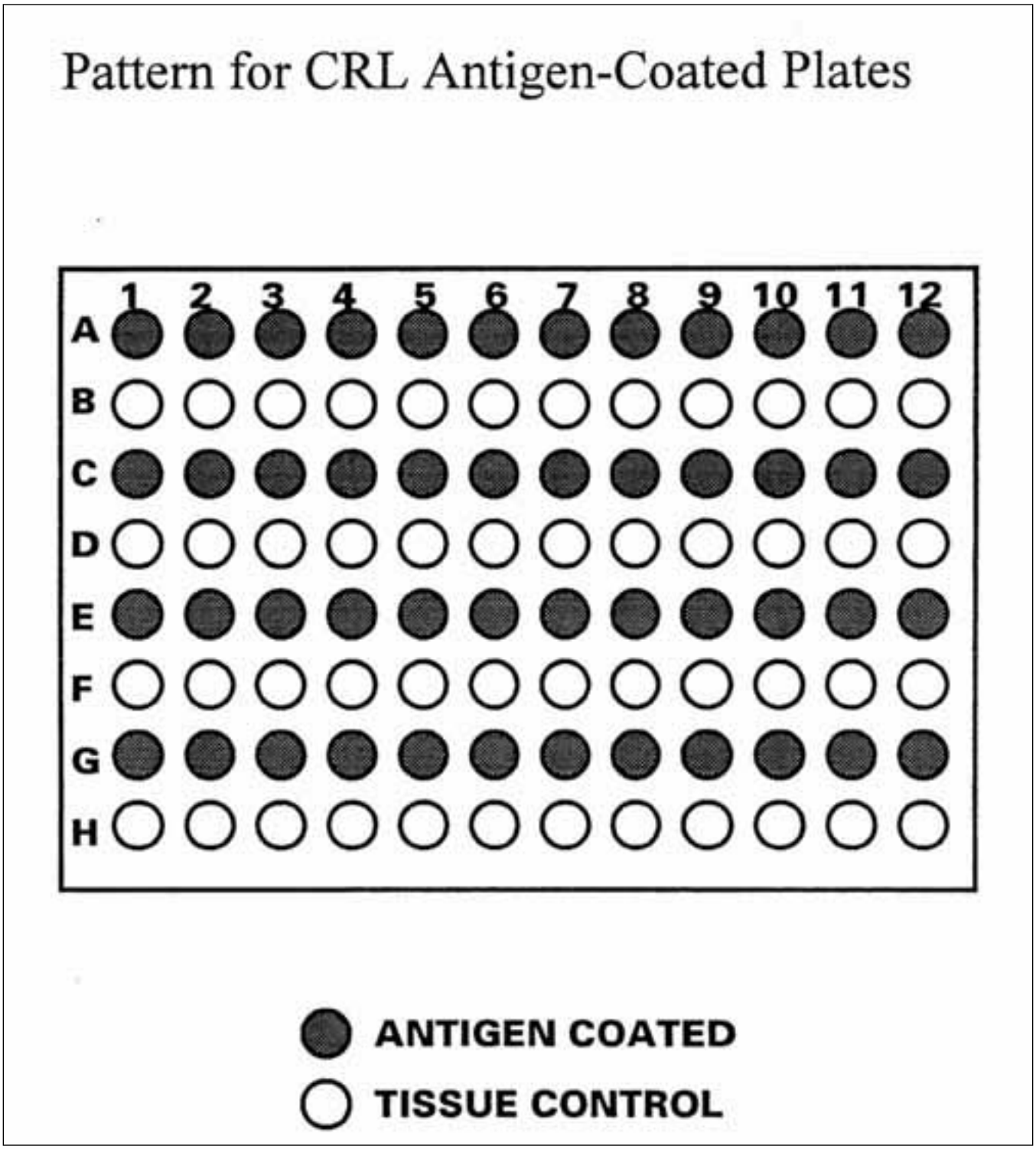

Pattern for CRL Antigen-Coated Plates
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
ANTIGEN COATED
TISSUE CONTROL

Figure 3.3

CHARLES RIVER LABORATORIES

251 Ballardvale Street
Wilmington, MA 01887
Telephone: (978) 658-6000

RESEARCH PRODUCT SPECIFICATIONS

ITEM NO: **PL-001**

NAME: **SENDAI VIRUS ANTIGEN-COATED 96-WELL MICROTITER PLATES**

LOT (BATCH): _____ PRODUCTION DATE: _____

DESCRIPTION: Partially-purified antigen and tissue control (an extract of the cell or tissue type in which the agent was propagated) were adsorbed to microtiter plate wells according to the conditions listed on the attached Antigen Purity Quality Control Results sheet. All wells in rows A, C, E, and G are coated with antigen; wells in rows B, D, F, and H are coated with tissue control.

FORM/STORAGE: Ready to use. Store at -20°C or below. Expiration date is 12 months from production date. Storage at -70°C will increase shelf life.

ANTIGEN PURITY: A plate from this research batch was evaluated with a panel of standard mouse and rat control sera by the indirect method of the enzyme-linked immunosorbent assay (ELISA) (Voller et al., Lab. Res. methods Biol. Med. 5:59-82, 1982). Briefly, 50 microliters of standard serum, diluted no less then 1/50 in phosphate-buffered saline (PBS) with 5% bovine serum or in BLOTTO (5% non-fat dry milk in PBS, Johnson et al., Gene. Anal. Techn. 1:3-8, 1984), were added to each of the appropriate antigen wells and adjacent, tissue control wells; the plate was covered and incubated for 40 minutes at 37°C. Following several washes with 0.9% saline containing 0.05% Tween 20, 50 microliters of horseradish peroxidase-conjugated, affinity-purified goat anti-rodent IgG were added to each well. After a 40 minute incubation at 37°C, the plate was again washed. One hundred microliters of 0.4mM ABTS-2.0mM H_2O_2 chromogenic substrate were added to each well and the plate was incubated at room temperature for 40 minutes. To stop the substrate-enzyme reaction, 25 microliters of 1% sodium dodecyl sulfate (SDS) in water were added per well. The reaction intensities at 405nm were determined with an ELISA reader. The net absorbance values (antigen-tissue control) were converted to scores by dividing by 0.13. A net score of 3 or above is considered positive. As the results of the attached Antigen Purity quality control report show, positive results occurred only with appropriate immune sera.

Note: FOR RESEARCH USE ONLY. NOT FOR DIAGNOSTIC USE. Nothing on this sheet is to be construed as a recommendation to use this research product in violation of any patents. The information presented above and on the attached quality control sheet is believed to be accurate. However, said information and product are offered without warranty or guarantee since the ultimate conditions of use and the variability of how the materials are treated are beyond our control. No claims beyond replacement of unacceptable material or refund of purchase price shall be allowed.

CRL FORM NALS DRRP-002 REV. 0, December 9, 1994

Figure 3.4

2. **High and Low 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 2X and therefore must be diluted with an equal volume of serum diluent when used. Non-immune control sera are pre-diluted 5-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. It is best to divide sera into small volume aliquots that you will use up when performing a test run.

3. **Enzyme-Conjugated Anti-immunoglobulin:** The two enzymes most often used in the ELISA are alkaline phosphatase and horseradish peroxidase (HRP). The quality and concentration of the enzyme-conjugate profoundly affect 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 IgG anti-rodent IgG with recommended working dilutions for use with our test plates.

4. **Chromogenic Substrate:** There are a variety of substrates that can be purchased ready-to-use including the HRP substrate ABTS-H2O2 that we use. Instructions for preparing this substrate are given in the next section.

5. **Stop Solution:** The two types of stop solutions that we have used are 0.12% hydrofluoric acid (HF) and 1% Sodium Dodecyl Sulfate (SDS). As hydrofluoric acid is caustic and corrosive, we recommend using SDS.

3.2.3 Preparation of Buffers, Diluents and Solutions

1. **BLOTTO Serum Diluent -** Bovine Lacto Transfer Technique Optimizer
(Johnson et al, Gen. Anal. Techn. 1:3-8, 1984)
The following recipe is for preparing 100 ml of BLOTTO.

COMPONENTS	AMOUNT
Non-fat Dry Milk	5 grams
1M Tris Buffer - pH 8	5 ml
Proclin	50 microliters
Anti-foam A	33 microliters
DI H_20	95 ml
NaCl	0.9 grams

a. Mixing Instructions

Make 50 Mm Tris Buffered saline mixing DI H20, 1M Tris Buffer and NaCl. Add Proclin, 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-micron nylon screen from TETKO in Elmsford, NY. Rinse nylon screen between filtrations.

b. Addition of 20% Fetal Bovine Serum (FBS)

Add 20 ml Fetal Bovine Serum to 80 ml BLOTTO 5%. FBS is added to block false positive reactions caused by antibodies to fetal bovine constituants that may be in the antigen preparation.

c. Storage:

This solution is NON-STERILE. Store at 4 degrees Celsius and use within two days.

2. Conjugate Diluent - 15% Bovine Serum and 0.9% NaCl in 0.01M Tris-HCl, pH 7.2-7.4.
The following recipe is for preparing 100 ml.

COMPONENTS	AMOUNT
1.0M Tris-HCl Stock Solution	1 ml
NaCl	0.9 grams
Gentamicin sulfate (50 mg/ml)	0.1 ml
Bovine Serum, Fetal or Colostrum-Free	15 ml
Deionized (DI) H_2O	qs 100 ml

a. Mixing Instructions:
Prepare 100 ml 1.0 M Tris-HCl Stock Solution by dissolving 12 grams of Tris in 60 ml of DI H_2O. Adjust pH to 7.2 with concentrated HCl and bring volume to 100 ml. Filter sterilize (0.2 micron) and store in a refrigerator for up to 6 months. To prepare the diluent, add the indicated amounts of each component in the order shown to 60 ml of DI water. Adjust to the final volume to 100 ml with DI water and stir until the NaCl is dissolved.

b. Storage:
Store in a refrigerator for up to 1 month if filtered (0.2 micron). If not filtered, store in a refrigerator and use within two days. Note: PBS can be used in place of 0.01M Tris-saline.

3. Substrate - 0.4 mM ABTS, 2 mM H2O2
The following recipe is for preparing 100 ml.

COMPONENTS	AMOUNT
0.05 M Citrate buffer, pH 4.0	100 ml
0.04 M ABTS Stock Solution	1 ml
0.5 M H_2O_2	0.9 ml

a. Mixing Instructions:
Prepare 1000 ml of 0.05M citrate buffer by dissolving 9.6 grams of citric acid monohydrate in 900 ml of DI H_2O. Adjust pH to 4.0 with 5N NaOH and bring volume to 1 liter. Filter sterilize (0.2 micron) and store in a refrigerator for up to 6 months. To prepare the ABTS stock solution, dissolve 2.2 grams of ABTS in 100 ml of DI H_2O. Filter sterilize and divide into 10 ml aliquots. Store in a refrigerator for up to 6 months. Protect from light. Prepare 0.5 M H_2O_2 immediately prior to making the Working Substrate by adding 0.5 ml of 30% H_2O_2 to 7.5 ml DI water. Prepare the Working Substrate by adding the specified amounts of stock ABTS solution and 0.5M H_2O_2 to the citrate buffer.

b. Storage
Store the Working Substrate in a refrigerator; protect from light and use within 24 hours.

4. Stop - 1% Sodium Dodecyl Sulfate (SDS)
The following recipe is for preparing 100 ml of 1% SDS

COMPONENTS	AMOUNT
SDS	1 gram
DI H_2O	99 ml

a. Mixing Instructions:
Prepare 1% SDS by dissolving 1 gram of SDS into 100ml of DI H_2O. Filter sterilize into a sterile container.

b. Storage:
Store 1% SDS in the refrigerator and use within 7 days.

5. Wash Solution - 0.9% NaCl, 0.05% Tween 20
The following recipe is for preparing 1000 ml.

COMPONENTS	AMOUNT
NaCl	9 grams
10% Tween 20	5 ml
DI H_2O	1000 ml

a. Mixing Instructions
Prepare 10% Tween-20 by mixing 10 ml of Tween with 90 ml of DI H_2O. Filter sterilize and store a room temperature in a sterile container. Add the specified amount of NaCl and 10% Tween-20 to the DI H_2O and mix until the salt is dissolved.

b. Storage
Use the same day.

6. Working Dilution of Conjugate

COMPONENTS	AMOUNT
Reconstituted Conjugate Stock	Variable
Conjugate Diluent or Serum Diluent	Final Volume

a. Mixing Instructions
Commercial conjugate is supplied in the lyophilized form. Reconstitute the conjugate with half the manufacturer recommended volume of DI water. After the conjugate has dissolved completely double the volume with glycerol to remain a liquid at the typical storage temperature of -20ºC. The allow the conjugate to be used without repeated freeze-thaw cycles. Storage of the stock below -20ºC may result in freezing which is to be avoided. Dilute the CRL-supplied stock conjugate as indicated on the Product Specification Sheet.

b. Storage
Refrigerate the diluted conjugate and use within 5 days.

3.3 Sample Preparation

3.3.1 Collection and Storage

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.

1. Collect blood by your customary method and allow it to clot at room temperature. Centrifuge the clotted blood at 2,000 to 3,000 RPM for approximately 15 minutes and transfer the serum to a separate vial. If you have sterile PBS, pH 7.0 to 7.4, dilute the specimen by mixing one part serum with four parts PBS.

2. Alternatively, the same final serum dilution of 1:4 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.

3. It is best to store serum specimens in sturdy, leak-proof plastic vials at - 10ºC or below. If they cannot be frozen, refrigerate at 4ºC. Specimens refrigerated for more than 24 hours should be protected from the growth of bacteria and fungi by adding an antibacterial agent such as Proclin.

3.3.2 Recommendation

We recommend that you do not heat inactive serum samples, as we have observed that this contributes to non-specific background radiation.

3.3.3 Preparing Sample Plate

1. While samples can be diluted directly in the test plate, we recommend that you make dilutions in separate 96-well microtiter plates.

2. First arrange your sample vials (containing serum diluted 5 fold with PBS) in racks in the order that they will appear on the test plates (Figure 3.5)

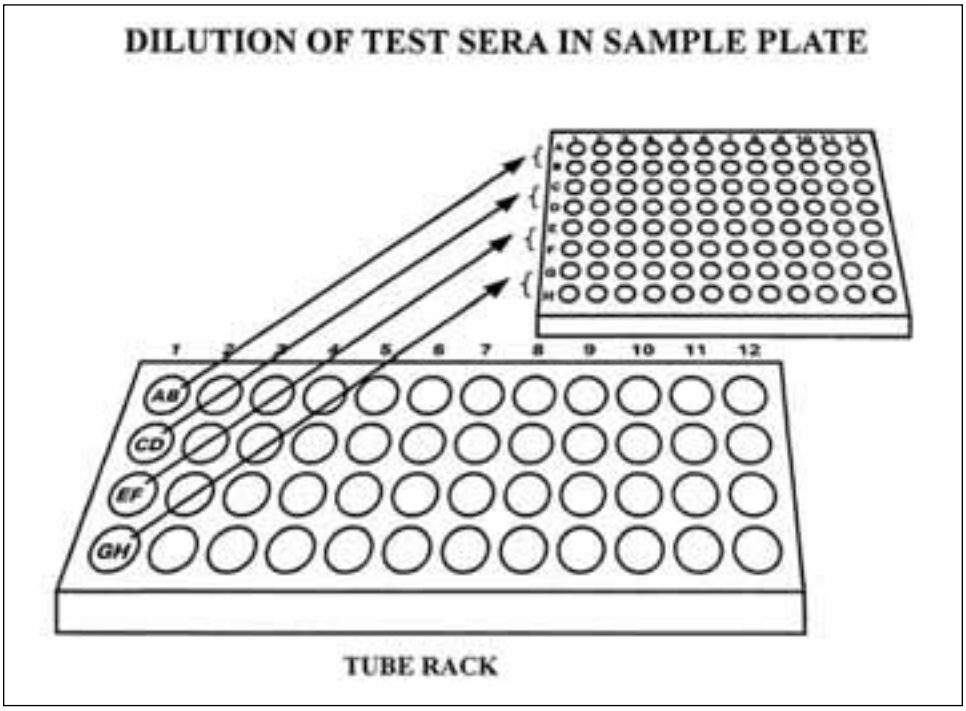


Figure 3.5

3. The dilution at which serum samples are tested is 1:60.

   a. To prepare enough diluted serum to perform 4 tests, add 220 microliters of BLOTTO diluent to all wells except those reserved for the positive control antisera. We usually reserve wells 12AB and 12CD for the high and low positive controls, respectively. Then add 20 microliters of sample to each of two adjacent wells corresponding to antigen and tissue control wells on the test plates.

   b. Alternatively you may prefer to dilute serum 1:30 in order to double the number of test plates that can be filled from a single sample plate from 4 to 8. To do this, add 225 microliters of BLOTTO diluent to all wells except those reserved for the positive control antisera. Then add 45 microliters of sample to each of two adjacent wells corresponding to antigen and tissue control wells on the test plates.

   c. We recommend that you also dilute the non-immune control serum in the sample plate. We typically use wells 12EF for the non-immune control serum and leave 12GH as a diluent control.

4. Cover the sample plates (with an another plate) to prevent evaporation. Refrigerate and use within seven days.

3.4 Testing

3.4.1 Transfer

Transfer Diluted Sera From a Sample to Corresponding Wells in Test Plates (Figure 3.6).

1. 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. If the sample plate contains sera diluted 1:60, transfer 50 microliters per well.

3. Alternatively, when the sample plate sera are 2X, i.e., diluted 1:30, first add 25 microliters of BLOTTO diluent or PBS to all wells of the test plates. Then transfer 25 microliters of sample per well.

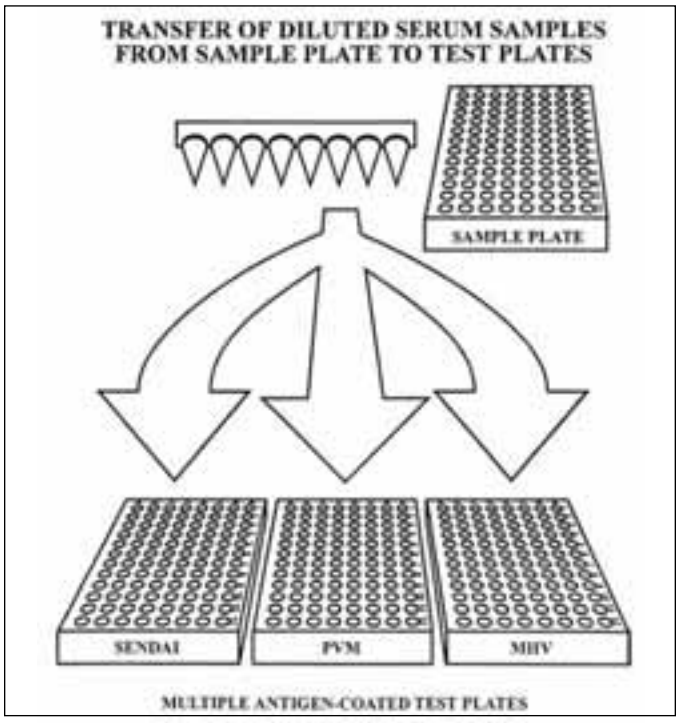


Figure 3.6

3.4.2 Add Immune Control Sera

Add immune control sera to the reserved test plate wells.
See SMJ Memo -Appendix B

1. Control sera may be added to any plate location, although we routinely, as noted, reserve wells in column 12 for this purpose.

2. Each run should include the high and low positive controls (which we add to wells 12AB and 12CD, respectively).
   a. NOTE: The CRL positive control sera are supplied at 2X concentration.
   b. Therefore, if you haven't already done so, add 25 microliters of BLOTTO diluent to each positive control well; then add 25 microliters of control serum per well.

3.4.3 Incubate Test Plates

Incubate Test Plates at 35-40ºC for 40 minutes.

1. Be sure that all plates are covered.

2. Do Not stack the plates more than two high.

3.4.4 Wash the Plates

1. If you are using an automatic 96-well washer, we recommend 3-5 fill-aspiration cycles. Overfill the well with 350-400 microliters if your washer has this capability. A soak time between cycles does not appear to be necessary.

2. To wash without a plate washer, do the following:

   a. Prepare a wide waste container with paper towels in the bottom to prevent splattering.

   b. To expel the well contents into the waste container, invert and rapidly flick the plate.

   c. Using a repeating pipette with a multi-channel manifold, fill all wells with wash solution.

   d. Repeat steps b and c five to six times.

   e. Expel the well contents as in step b. Bang the plates against several layers of dry paper towels to remove the last traces of wash fluid.

3.4.5 Add Diluted Conjugate

Add 50 microliters of diluted conjugate to all wells and incubate at 35-40ºC for 40 minutes.

3.4.6 Wash and Add Substrate

Wash and add 100 microliters of ABTS-H_2O_2 substrate per well. Incubate at room temperature for 40 minutes.

3.4.7 Add SDS Stop

Add 25 microliters of SDS Stop per well.

3.4.8 Read the Results

1. The product of the reaction between ABTS-H2O2 and HRP is GREEN. The rate of color development is proportional to the amount of HRP-conjugate bound to the well.

2. The addition of Stop dramatically slows, but does not completely prevent, further color development. Therefore, Read the Test Plates Immediately!!

3. 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.

4. When visually reading color development, score the reactions as follows:

SCORE	COLOR DEVELOPMENT
0	NONE: I.E., EQUIVALENT TO DILUENT CONTROL
1	SLIGHT
2	MODERATE
3	INTENSE

3.5 Results Interpretation

3.5.1 Charles River Scoring System

1. We transmit our optical density (OD) readings from the ELISA reader to a PC where they are converted to scores by dividing by 0.13. In comparison to the 3 decimal absorbance values, integer scores are easier to read and interpret. The denominator of 0.13 divides absorbances of 0.13 to 1.3 into scores of 1 to 10.

2. A $Score_{ANTIGEN}$ of 0 or 1 is considered NEGATIVE, regardless of the $Score_{TISSUE}$.

3. A result is considered non-specific and recorded as TC when both $Score_{ANTIGEN}$ and $Score_{TISSUE}$ are > 2.

4. The net score = $Score_{AG}$ -- $Score_{TC}$. Provided that the $Score_{TC}$ is < 2 (i.e., OD < 0.26), net scores are interpreted as follows:

NET SCORE	ABS_{405}	INTERPRETATION
0,1	0.13	NEGATIVE
2	0.26	BORDERLINE
≥ 3	≥ 0.39	POSITIVE

3.5.2 Visual Scoring

(see previous section)

ANTIGEN	TISSUE	INTERPRETATION
0	0-4	NEGATIVE
1	0	BORDERLINE
2,3	0	POSITIVE
>1	>1	NON-SPECIFIC

3.6 Troubleshooting

OBSERVATION	CAUSE	
	COMPONENT	PROBLEM
NO COLOR IN HIGH AND LOW POSITIVE SERUM CONTROL WELLS	CONTROL SERA	- Not Added. - Incorrect specificity. - Diluted improperly (i.e., too dilute). - Inactivated by improper storage or repeated freeze-thaws.
	CONJUGATE	- Incorrect specificity. - Too dilute. - Inactivated by improper storage or repeated freeze-thaws.
	SUBSTRATE	- Insufficient H2O2 added . - Degredation of stock solution. - Citrate buffer pH incorrect. - ABTS not added.
	ANTIGEN	- Too dilute or of low potency. - Incorrect agent. - Degraded due to improper storage.
	READER	- At wrong wavelength. - Bulb burned out. - Out of calibration.
WEAK COLOR DEVELOPMENT IN POSITIVE CONTROL WELLS	COMPONENTS LISTED ABOVE	Problems listed above.
	INCUBATION	- Temperature too low or exceedingly high. - Time too short.
EXCESSIVE BACKGROUND	WASHER	- Too few fill-aspirate cycles. - Wash incompletely aspirated after each fill. - Fill volume low.
	SERUM SAMPLE	- 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.
	CONJUGATE	- Dilution too low. - Poor quality (try another lot or different vendor)
	SUBSTRATE	- Activated non-specifically prior to being added to plate or by contaminants in plate.
	ANTIGEN	- Binds antibody in serum or conjugate non-specifically because "sticky" or used at too low a dilution. - Activates substrate.
	INCUBATION	- Time too long - Temperature too high

Section 4. Indirect Fluorescent Antibody Test (IFA)

4.1 Methodology Overview

The steps of the IFA are very similar to those of the ELISA (Figure 4.1). 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.

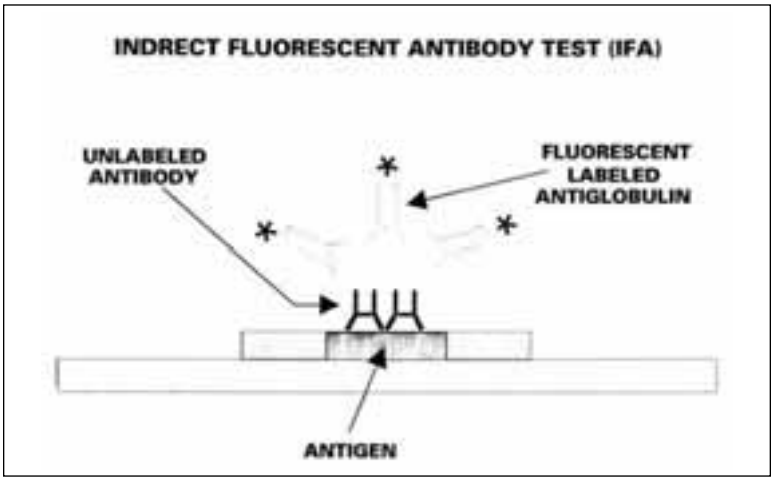


Figure 4.1

Epi-illumination is recommended. Epi-illuminated fluorescence is 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 (Figure 4.2).

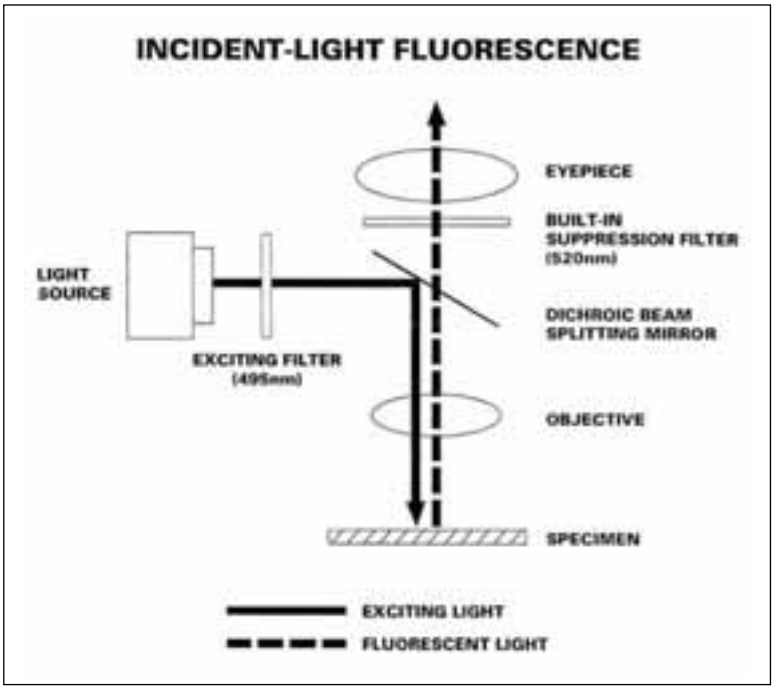


Figure 4.2

In the IFA, the morphology and location of fluorescence can be evaluated to differentiate specific from non-specific 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 non-specific reaction. Fluorescence may be mostly nuclear or cytoplasmic depending on the virus (Table 4.1). Nuclear fluorescence is characteristic of DNA viruses (e.g., the rodent parvoviruses MVM, KRV, and H-1).

Location of Fluorescence in CRL IFA

AGENT	Fluorescence		
	Nuclear	Cytoplasmic	Both
SENDAI		✓	
SV-5		✓	
PVM		✓	
MHV		✓	
MVM			✓
KRV			✓
H-1			✓
REO-3		✓	
GD-7		✓	
MAD	✓		
LCMV		✓	
ECTRO		✓	
POLY	✓		
EDIM		✓	
MCMV	✓		
MPUL[1]		✓	
ECUN[2]	-	-	-

[1]Mycoplasma attaches to outer cell membrane
[2]E. cuniculi wells contain only protozoan cells

Table 4.1

4.2 Materials

4.2.1 Equipment

1. 2-10 microliter single-channel, adjustable pipette for adding sample

2. 2-5 ml repeating pipettes for dispensing FITC-labeled Anti-immunoglobulin

3. Humidified Chamber

4. Incubator at 35-40ºC

5. Slide Washing Reservoirs

6. Coverslips

7. Fluorescence Microscope: For fluorescein isothiocyanate (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 250-500X.

4.2.2 Description of Key Reagents

1. 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. As indicated in the Product Specification sheets (Figure 4.3), Charles River antigen-coated slides should be stored in a freezer at -10ºC or below, preferably at -70ºC. Slides stored at -70ºC remain antigenic for at least one year.

2. 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 5-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. It is best to divide sera into small volume aliquots that you will use up when performing a test run.

3. FITC-Labeled Anti-immunoglobulin: Charles River Laboratories does supply this however, there are many commercial sources of affinity-purified FITC-labeled IgG anti-rodent IgG. It has been our experience that these conjugates work fine at the dilutions recommended by the manufacturer (generally 1:25-50).

4. Coverslip Mounting Medium - Tris-Buffered Glycerol, pH 8.7: Mounting medium for FITC is buffered to a basic pH to enhance fluorescence.

4.2.3 Preparations of Buffers, Diluents and Solutions

1. **BLOTTO Serum Diluent** (Refer to ELISA for Preparation Instructions)

2. **Mounting Medium:** - 0.1M Tris- Buffered Glycerol, pH 8.7.
   The following recipe is for preparing 20 ml.

COMPONENTS	AMOUNT
1.0M Tris-HC1 Stock Solution, pH 8.7	2 ml
Glycerol	18 ml

   a. Mixing Instructions
   Prepare 100 ml 1.0 M Tris-HCl Stock Solution by dissolving 12 grams of Tris in 60 ml of DI water. Adjust pH to 8.7 with concentrated HCl and bring volume to 100 ml. Filter sterilize (0.2 micron) and store in refrigerator for up to 6 months. Add 2 ml of 1.0 M Tris-HCl Stock Solution to 18 ml of glycerol mix thoroughly.

   b. Storage
   Store at room temperature and use within one month.

3. **Conjugate in PBS**.

COMPONENTS	AMOUNT
Reconstituted FITC Conjugate Stock	Variable
PBS	Final Volume

   a. 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 -10oC or below. Thaw a vial of FITC conjugate and prepare the recommended working dilution in PBS.

   b. Storage
   Refrigerate the working dilution and use within 5 days.

4.3 Sample Preparation

4.3.1 Collection and Storage

Same as for Elisa (Section 3)

4.3.2 Sample Preparation

No other sample preparation required.

4.4 Testing

(Figure 4.4)

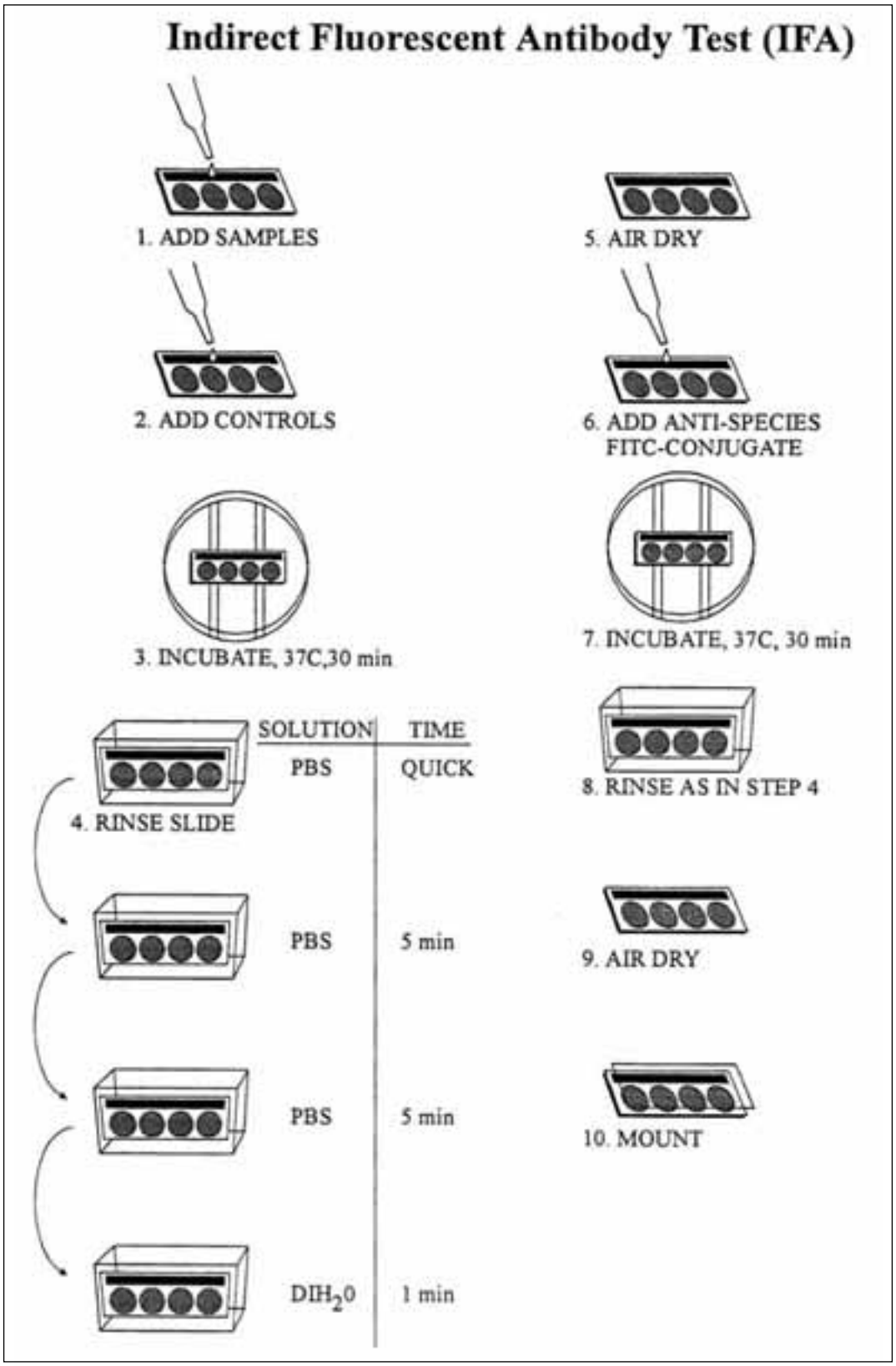


Figure 4.4

4.4.1 Prepare Slides

1. Remove the appropriate type and number of slides from the freezer.
2. Allow them to warm to room temperature.

4.4.2 Organize Test Specimens

1. Retrieve and arrange your samples.
2. On the IFA results form, record the way in which the samples and controls are arranged (Figure 2.6).

4.4.3 Place Specimines and Controls on Slides and Incubate

1. Add five microliters of BLOTTO diluent to all wells except those reserved for positive control sera.
2. Using the IFA Form as a guide, add the test specimens and negative controls to the appropriate wells. The volume of specimen or control per well is 5 microliters. Spread each sample over the whole well being careful not to scrape the surface.
3. Add 10 microliters of positive control serum to the appropriate well(s).
4. Incubate the slides at 35-40oC in a humidified chamber for 30 minutes.

4.4.4 Wash

1. Fill each well of the slide with PBS and aspirate off using a pipette. This initial quick rinse step helps prevent cross-contamination
2. Fill two separate wash reservoirs with PBS and a third with DI water.
3. Incubate the slides in the first PBS-filled reservoir for 5 minutes.
4. Move the slide to the next wash reservoir and incubate for another 5 minutes.
5. Transfer the slides to the DI water wash reservoir and incubate for one minute.
6. Air dry. DO NOT BLOT.

4.4.5 Add Conjugate and Incubate

1. Using a repeating Pipetter, add 10 microliters of diluted FITC conjugate to all wells.
2. Incubate the slides at 35-40oC in a humidified chamber for 30 minutes.

4.4.6 Wash and Cover with Mounting Medium

1. Wash, beginning with step D -2
2. 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 get on the microscope objective.
3. You may read the slides right away or store them at -20oC for up to one week without deterioration of the fluorescence.

4.4.7 Read Results

1. Examine the slides with the fluorescence microscope at a magnification of 250-500X. FITC fluorescence is yellow-green in color.
2. After examination of the positive and negative controls, score the results as follows:

SCORE	FLUORESCENCE
-	Minimal, like non-immune control
1	Very dim, granular
2	Moderate granular
3	Bright granular
4	Glaring granular
TC	Diffuse

4.5 Results Interpretation

Correct reading of IFA reactions takes practice. Bright granular fluorescence is typical of a specific antibody-viral antigen reactions, whereas diffuse fluorescence suggests a non-specific TC reaction (Figure 4.5). A TC reaction is also suggested 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 (Table 4.1).

4.6 Troubleshooting

OBSERVATION	CAUSE	
	COMPONENT	**PROBLEM**
NO OR WEAK FLUORESCENCE IN POSITIVE CONTROL WELL	CONTROL SERA	- Not Added. - Incorrect Specificity. - Too dilute. - Inactivated by improper storage or repeated freeze-thaws.
	CONJUGATE	- Incorrect Conjugate Used. - Too dilute. - Inactive due to improper storage or repeated freeze-thaws.
	SLIDE	- Percentage of infected cells too low. - No cells or scraped off during procedures. - Wrong virus.
	FLUORESCENCE MICROSCOPE	- Wrong light source. - Incorrect exciting/suppression filter. - Not aligned properly.
EXCESSIVE BACKGROUND	SERUM SAMPLES	- Improperly collected and/or stored. - Bacterially contaminated. - Dilution too low . - BLOTTO blocking diluent not used. - Source animals parentally immunized or have autoimmune disease.
	CONJUGATE	- Dilution too low. - Poor quality, not affinity purified.
	SLIDE	Cells not spread out. This increases background and reduces specific fluorescence
		Bacterially contaminated during preparation.

Appendices


CHARLES RIVER
LABORATORIES
Contributing to the Search for Healthier Lives™

Appendix A

A.1 Equipment List for Serology Testing

A.1.1 Pipettors, Single and Multi Channel

COMPANY	ADDRESS
Eppendorf	University Research Park 545 Science Drive Madison, WI 53711-1082 (800) 421 – 9988
Rainin	5400 Hollis St. Emeryville, CA 94608-2508 (510) 654-9142
Finnpette	Labsystems 8 East Forge Pkwy Franklin, MA 02038 (800) LAB-PROD
Hamilton	4970 Energy Way Reno, NV 89502 (800) 648- 5950

A.1.2 Plate Washers/Readers

COMPANY	ADDRESS
Biotek	Biotek Instrument Co. Highland Park Winooski, VT 05404-0998
Dynex Technologies	14340 Sullyfield Circle Chantilly, VA 20151 (800) 336-4543

Appendix B

B.1 Reagent Suppliers for Serology Testing

COMPANY	ADDRESS
Pierce	P.O. Box 117 Rockford, IL 61105 (800) 325-3010
Kirkegaard & Perry	2 Cessna Ct. Gaithersburg, MD 20879 (800) 638-3167
Sigma	P.O. Box 14508 St. Louis, MO 63178 (800) 325-3010 www.sigma-aldrich.com
Fisher	711 Forbes Ave. Pittsburgh, PA 15219-4785 (800) 766-7000
Jackson ImmunoResearch	872 West Baltimore Pike P.O. Box 9 West Grove, PA 19309 (800) 367-5296 www.jacksonimmuno.com

Appendix C

C.1 Parvovirus NS-1 and MPV-VP2 ELISA Testing

We are happy to report an improvement in performance for each of the parvovirus NS-1 and MPV-VP2 parvovirus ELISAs. **It is important that you please read this notice before performing the parvovirus assays.**

C.1.1 PARVO-NS-1

We have continued to use a recombinant form of the highly conserved NS-1 (non-structural protein 1) for the antigen in this test. Recently, however, we have adopted a new production system that enables us to produce much larger amounts of NS-1 protein more efficiently. The histidine tag on the protein facilitates purification by metal chelating chromatography.

For you, the good news is twofold. First, these improvements allow for a single NS-1 plate that can be used for both rat and mouse serum testing. Note that for the NS-1 assay, positive control sera will now be called either mouse anti-NS-1 or rat anti-NS-1, as indicated in the attached Table 1.

Second, you can now perform the NS-1 assay with the same volumes of reagents and the same incubation times as our standard ELISA. The 100 microliter volumes and 60-minute incubations with serum and conjugate are no longer necessary. The NS-1 procedure is listed below, and is summarized in the attached Table 2.

1. If possible, store the NS-1 plates at -70ºC. Warm the plates to room temperature just before use.

2. Dilute serum samples to 1:60 in BLOTTO.

3. Add 50µl of diluted sample and 50µl of diluted control to antigen (AG) and tissue control (TC) wells. Cover the plate tightly and incubate for 40 minutes at 35 - 40ºC. (We strongly advise that the plates be placed on a solid, non-perforated surface in your incubator to minimize air flow in and around the wells of the plate).

4. Wash the plates twice as much as you normally do.

5. Add 50µl of conjugate solution to all wells. Cover the plate tightly and incubate for 40 minutes at 35 - 40ºC.

6. Wash the plate twice as much as you normally do.

7. Add 100µl of substrate solution to all wells. Incubate for 40 minutes at room temperature and add stop solution as normal.

C.1.2 MPV

Our MPV antigen is purified recombinant VP2 protein that has been produced in a bacterial vector. See the attached Table 1 for plate and control serum information. It should be noted that the procedure for the MPV-VP2 ELISA is slightly different from that for the NS-1 ELISA. The MPV-VP2 ELISA procedure is described below and summarized in the attached Table 2.

1. If possible, store the plates at -70ºC. Warm the plates to room temperature just before use.
2. Dilute serum samples to 1:60 in BLOTTO.
3. Add 100µl of diluted sample and 100µl of diluted control to antigen (AG) and tissue control (TC) wells. Cover the plate tightly and incubate for 60 minutes at 35 - 40ºC. (We strongly advise that the plates be placed on a solid, non-perforated surface in your incubator to minimize air flow in and around the wells of the plate).
4. Wash the plates twice as much as you normally do.
5. Add 100µl of conjugate solution to all wells. Cover the plate tightly and incubate for 60 minutes at 35 - 40ºC.
6. Wash the plate twice as much as you normally do.
7. Add 100µl of substrate solution to all wells. Incubate for 40 minutes at room temperature and add stop solution as normal.

It should be noted that in certain instances we have observed that repeated freezing and thawing of NS-1 and MPV positive control antisera could result in a substantial loss of titer. To reduce this likelihood, we have, for your convenience, divided these controls into several aliquots of 100µl. Thus, for each NS-1 or MPV control you order, you will receive 5 x 100µl vials, rather than 1 x 500µl
vial.

PLEASE NOTE: In all of our ELISA we are using 1% sodium dodecyl sulfate (SDS) as a stop solution in place of 0.12% hydrofluoric acid, which is a highly corrosive chemical compound. Twenty-five microliters of a 1% SDS works as well as 0.12% hydrofluoric acid, but is much less harmful. SDS can be readily obtained from most chemical suppliers (for example: Sigma, St. Louis, MO; product number L4509).

ITEM	ITEM #	SPECIES
NS-1 ELISA plate	PL-039	mouse, rat
Mouse anti-NS-1 control serum	CL-537	mouse
Rat anti-NS-1 control serum	CL-637	rat
MPV ELISA plate	PL-036	mouse
Mouse anti-MPV	CL-536	mouse

Table C.1 — Rodent Parvovirus ELISA Reagent Ordering Information

	VOLUME IN µl / TIME IN MINUTES		
REAGENT	NS-1	MPV	STANDARD
Serum	50 / 40	100 / 60	50 / 40
Conjugate	50 / 40	100 / 60	50 / 40
Substrate	100 / 40	100 / 40	100 / 40

Table C.2 — Rodent Parvovirus ELISA Reagent Volumes and Incubation Times

The serum and conjugate incubations are at 35 - 40ºC. The substrate incubation is at room temperature.