Immunofluorescence Assays

Fluorescent Antibody Test (FA)

Mechanism	Fluorescently labeled mAb to bind and illuminate a target Ag/Ab
Designed to Detect	A sample's Antigen **OR** Antibody
Reagent	mAb-FITC conjugate ~ *Ab tagged with a Fluorescein*
Compatible Sample Types	Serum or tissue section

Types

1. Direct (DFA)	Detection of sample's **antigen**
Sample	Unknown **Antigen** in blood
2. Indirect (IFA)	Detection of sample's **antibody**
Sample	Unknown **Antibody** in blood (Ag = known)
Reagent	Secondary mAb-FITC conjugate

Direct Fluorescent Antibody

Uses

Bovine Viral Diarrhea Virus (BVDV)	Detection of **live BVDV** in bovine blood

Direct Fluorescent Antibody (cont)

Rabies in Brain Necropsy	Detection of the Rabies **virus** in the brain tissue

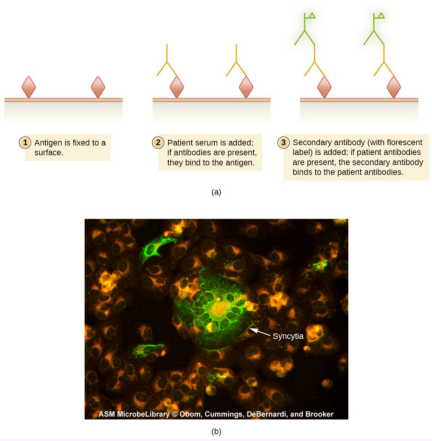
Sample	Ag from culture/slide
Known	mAb-FITC conjugate against antigen of interest
Detects (unknown reactant)	Antigen from sample
Reagent	mAb-FITC conjugate

Results

Positive Test	Fluorescence = Ag present
Negative Test	No fluorescence = No Ag

*Only ONE 'known' Ab is used in this test ~ so the known test component IS the Reagent

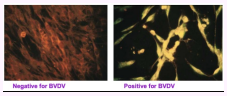
DFA Mechanism of Action



DFA Example ~ BVDV

1. Incubate patient serum (containing the virus) with a cultured cell line
~ Cell-line must be permissive to BVDV infection
2. Probe with mAb-FITC conj. that targets the viral Ag of BVDV

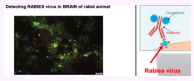
BVDV DFA Results



DFA ~ Rabies Brain Necropsy Dx

DFA is required for an official Rabies Dx
- An impression or tissue section of the euthanized animal's **Cerebellum, Hippocampus, and/or Brainstem** is collected
- mAb-FITC targeting the Rabies virus' antigen

Positive DFA of Rabies in the Brain



Indirect Fluorescent Antibody Test (IFA)

Uses

Porcine Reproductive and Respiratory Virus (PRRSV)	Detection of **PRRSV Antibody** in Porcine serum
Titers	Highest serial dilution of serum with Ab - that fluoresces

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Indirect Fluorescent Antibody Test (IFA) (cont)

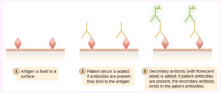
Dengue Fever/ChikV/Zika Virus

Sample	Serum
Known	Antigen
Detects (unknown reactant)	Antibody from serum (1ºAb)
Reagent	Anti-spp. Ab-FITC conjugate (2ºAb)

Results	
Positive Test	Fluorescence = Ab present
Negative Test	No fluorescence = No Ab

*This test uses TWO antibodies ~ a 1º and 2º antibody

IFA Mechanism



IFA Example ~ PRRSV

Detection of *Antibody* against PRRSV in Swine serum

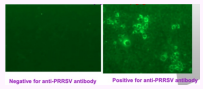
> **Known:** PRRSV infected cell line

> **Sample:** Porcine serum incubated with cell line

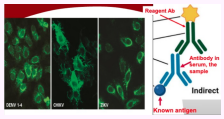
> **2ºAb Probe:** Anti-pig IgG conjugated with FITC

> **Unknown:** Antibody against PRRSV

Results of PRRSV IFA Test



IFAs Results for DENV 1-4/CHIKV/ZIKV



Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA

High *sensitivity* / Low *specificity*

Test Types that *Detect Antigen*	
>	**Direct ELISA**
or	*Sandwich ELISA*
or	*Antigen Capture ELISA*
or	*Antigen ELISA*
USES	
Heartworm Test (HWTM)	Using the Anti-HTWM-Ab-HRP

Test Types that *Detect Antibody*	
>	**Indirect ELISA**
or	*Antibody ELISA*
USES	
Titrations (titers)	Quantifies the amount of Ab present

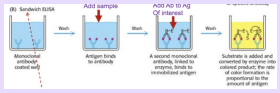
DIRECT (Ag Capture) ELISA

Sample	Serum from patient
Known	1º Capture Ab (coats wells in tray)
Detection of (unknown)	Antigen
Reagent + Substrate*	2º Detection Ab ~ *specific to disease conjugated to enzyme*
Positive Test	Color change = Ag present
Negative Test	No color change = No Ag

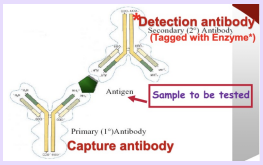
* substrate = activates enzyme

** The capture Ab and the detection Ab may be the same Ab > BUT ONLY the detection Ab will be tagged with the enzyme

Ag-Capture ELISA ~ MOA



Ag-Capture ELISA ~ MOA



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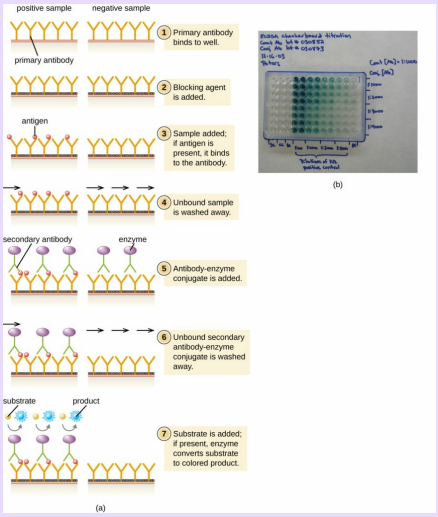
Ag-Capture ELISA ~ MOA



Ag Capture ELISA ~ HTWM Ag Detection

> **1º Capture Ab:** Ab that targets Ab

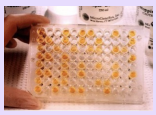
> **Sample with unknown:** Serum with HTWM Ag

> Wash slide

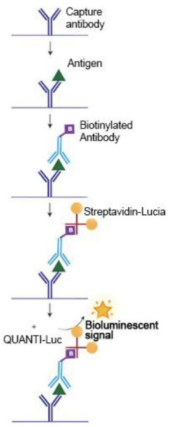
> **2º Detection Ab:** Anti-HTWM-Ab-HRP

> Add substrate to activate enzyme to show color change if bound

HTWM Ag-Capture ELISA Results



Ag-Capture ELISA ~ MOA



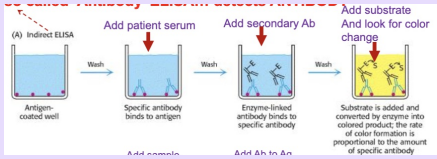
ANTIGEN CAPTURE ELISA

In this example, the Detection is the emission Of light instead of a Color change.

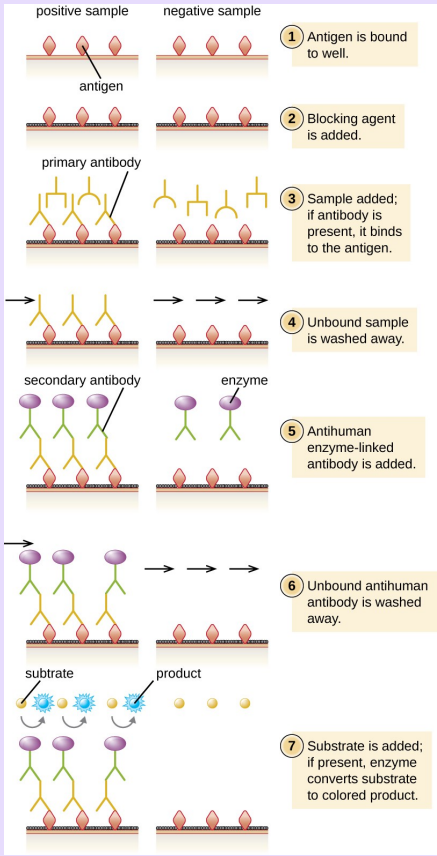
INDIRECT (Antibody) ELISA

Sample	Serum from patient
Known	Antigen (coating wells)
Detection of (unknown)	1º Ab in serum (spec. for Ag)
Reagent + Substrate*	Anti-Ab 2º Detection Antibody-HRP conj. ~ *Targets hosts own Ab (1º Ab from serum)
Positive Test	Color change = Ab present
Negative Test	No color change = No Ab

Ab ELISA ~ MOA



Ab ELISA ~ MOA



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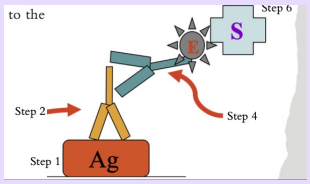
Ab ELISA Titration

> Coat ELISA wells with Ag (can get commercial Ag of interest)
> Add serial dilutions of patient's serum into wells
> wash off unbound Ab
> Add 2º Ab like Rabbit-Anti-Horse-Ig thats conjugated w enzyme, to the wells
> wash off unbound Ab
> add substrate

Ab ELISA ~ MOA



• Can run serum from multiple patients at once
• Can determine titer by running serial dilutions of the serum

Indirect ELISA Ab Titer

Microplate ELISA for antibody: colored wells indicate reactivity

Western Blot (WB)

WB

Higher *specificity* than ELISA

Designed to ID/Detect PROTEINS:	
1. Detection of *Antibody*	if patients serum contains Ab against a *specific* protein in a complex protein mixture
2. ID specific protein *antigen* in mix	Use of a known reagent Ab to the protein of interest

MOA	This is a three-stage primary binding test
Stage I	Electrophoresis of a protein mixture on gels so that each component is resolved into a single band
Stage II	Blotting of these protein bands to an immobilizing nitrocell-ulose membrane

WB (cont)

Stage III	Visualization of transferred Ag by either directly or indirectly probing the membrane with Ab's

WB Probing Methods	
Direct	Detection of the **Protein Antigen**
Indirect	Detection of the **Antibody**

WB MOA

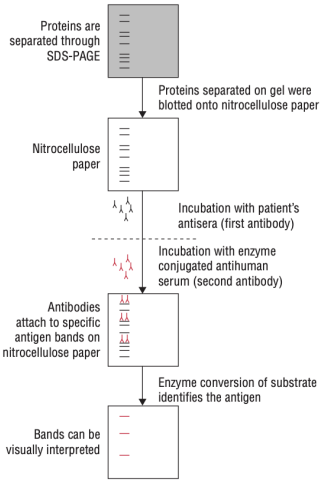


FIG. 14-16. Western blot test.

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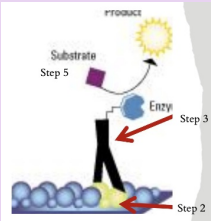
DIRECT WB



1. Separate out proteins by size and charge in the unknown antigen using gel electrophoresis
2.Transfer molecules to secondary matrix
3.Probe with enzyme-labeled known antibody to the antigen of interest
4.Wash
5. Add substrate and observe change (color or light)

DIRECT WB ~ Bovine Spongiform Encephalopathy (BSE)

Sample	Serum w Ag from pt. (separated by electroph.)
_____-_____-_____	Brain tissue
Known	BSE specific Ab-tagged with an enzyme (reagent)
Detects	BSE Prion protein **Antigen** (*Unknown*)

RESULTS

Positive Test	Banding that match the positive band pattern = Antigen present

DIRECT WB ~ Bovine Spongiform Encephalopathy (BSE) (cont)

Negative Test	Banding that match the negative band pattern = No Antigen

Direct WB *Required* for Dx of BSE! ~ Because:
- The BSE Ag that causes a disease is a normal brain protein in Bovine that is malfunctioning because it is folded incorrectly
- Since this is a normal protein in the Cow brain ~ There is NO IMMUNE RESPONSE that will generate

>>> THUS: We **have** to test for the Ag since the Ab will never be produced

Direct WB BSE Results

- 3 brain preps from 3 suspect cow with brain proteins separated
- Abnormal BSE-specific prion protein molecules can be detected using antibodies linked to an enzyme that results in a chemical reaction
- For this test a monoclonal antibody was made that recognizes BSE-specific abnormal prion protein >>> This antibody is a reagent antibody (tagged with an enzyme).

RESULTS
Cow #3 has BSE-specific prion proteins in its brain.

INDIRECT WB

USES

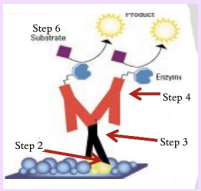
ELISA Dx Confirmation	Feline Immunodeficiency Virus (FIV)
	Human Immunodeficiency Virus (HIV)

Sample	Patient serum w Ab (separated by
Known	HIV Ag (from known HIV-infected cells)
Detects (unknown reactant)	Ab spec. to HIV Ag
Reagent	Anti-spp Ab conj. to enzyme

INTERPRETATION

Positive Test	Banding Pattern matches that of known positive = Ab present
Negative Test	Banding Pattern matches that of known negative = No Ab

INDIRECT WB MOA



1. Separate out proteins by size and charge in the known antigen using gel electrophoresis
2.Transfer molecules to secondary matrix
3.Probe with patient's serum antibody
4.Add enzyme-labeled antibody to patient's antibody
5.Wash
6. Add substrate and observe change (color or light)

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INDIRECT WB ~ FIV Confirmation

Source for Ag	Proteins from FIV-infected cells (separated out by electr-ophoresis)
Sample with unknown Ab	Cat's serum w/ Ab
Detection Reagent	Anti-cat Ab conju. w enzyme

Immunohistochemistry (IHC)

IHC Test

- Always detects antigen

- Horseradish peroxidase (brown color)

***- (-) Controls** = irrelevant Ab **OR** normal tissue section*

Sample	Thin tissue section
Known ~ 1º reagent	1º Reagent Antibody ~ probes Ag
Detects (unknown)	Antigen in the Tissue sect.
2º Detection reagent	2º Detection Ab conj. ~ spec. for 1º Ab

CONTROLS

Positive Control

1º Reagent	1º Reagent Ab ~ spec. for tissue Ag

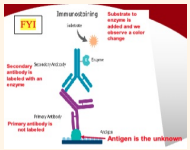
IHC Test (cont)

Positive Result	Brown in color = Ag is present

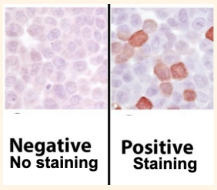
Negative Control

1º Reagent	1º Reagent Ab ~ spec. for tissue Ag **NOT IN SAMPLE**
Negative Result	No color change = No Ag

IHC MOA



IHC Microscopy Result



IHC Negative Control

USES

*Detection of mammary tumor Ag**	Use of an irrelevant rabbit mAb spec. to tumor Ag

*Detection of **Brucella Melitensis***

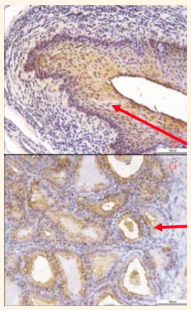
IHC ~ Brucella Melitensis

Sample	Histo section of a Goat's tissue from the Prepuce of the Penis and the Seminal Vesicular Gland
1º Reagent Ab	mAb spec. to *B. melitensis* Ag
2º Detection Ab	2º mAb spec. to 1º Ab

RESULTS

Positive Test	Observed brown color when compared to controls = **Ag present**
Negative Test	No color change from controls = **No Ag**

B. Melitensis IHC Results



Top: Mucosal epithelium of the Prepuce of the Penis
Bottom: Seminal Vesicular Gland epithelia

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