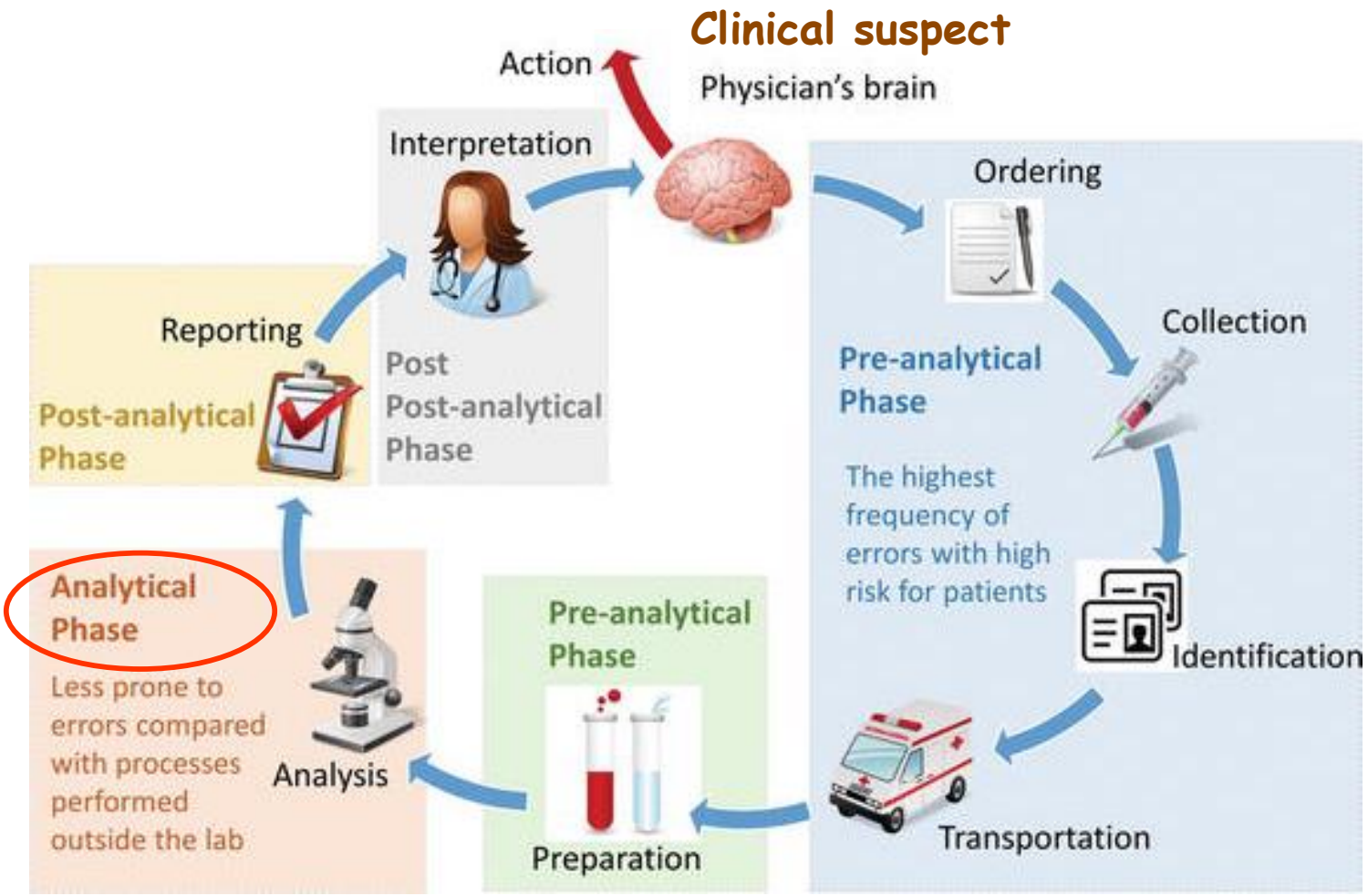
	List of the topics covered during Lab Medicine/ Microbiology course 2023-2024 (Guido Antonelli)
1	vaccination against infectious diseases
2	immune response to infection / vaccination
3	CHEMOTHERAPY/Antibiotics-antiviral agents, drug resistance and spread or resistant bacteria and virus
4	Clinical Microbiology - Diagnosis of infection (bacteria, viruses, fungi)
5	Respiratory tract infections
6	Bacteremia/fungemia and Catheter-Associated Infections
7	Chronic viral infections - Viral Hepatitis
8	Infections in the compromised host

Diagnosis

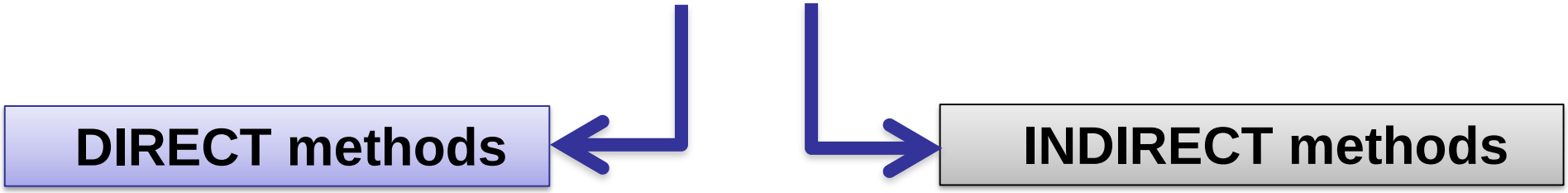


Virus
Bacteria
Fungi
Parasites

Microbial diagnosis

Analytical phase

Suspected infection



To detect microorganisms or their products in specimens collected from the patient

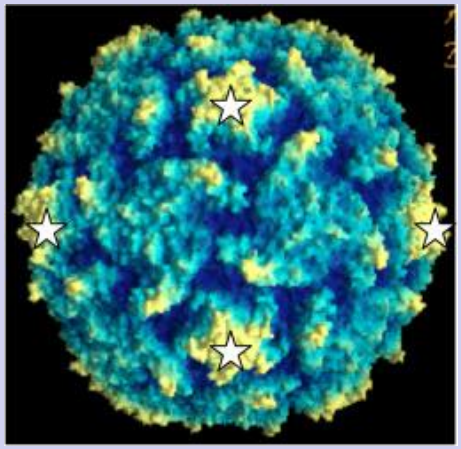
To detect evidence of the patient's immune response (production of antibodies) to infection

Blood, stool, urine, swab, etc.

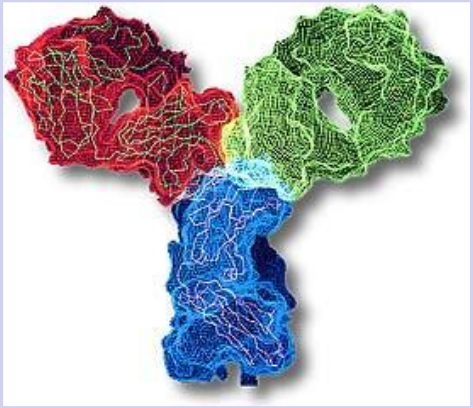
Serum

Laboratory diagnosis of VIRAL INFECTIONS

DIRECT:
Detection of virus, viral proteins (antigens), nucleic acids in different clinical samples
(nasal wash specimen, BAL, LCR, biopsy, etc)



INDIRECT:
Detection of virus-specific antibodies (serum)



Diagnosis
DIRECT DIAGNOSIS: more specific, sometimes time-consuming
INDIRECT DIAGNOSIS: less expensive, rapid, fully automatic preocedure

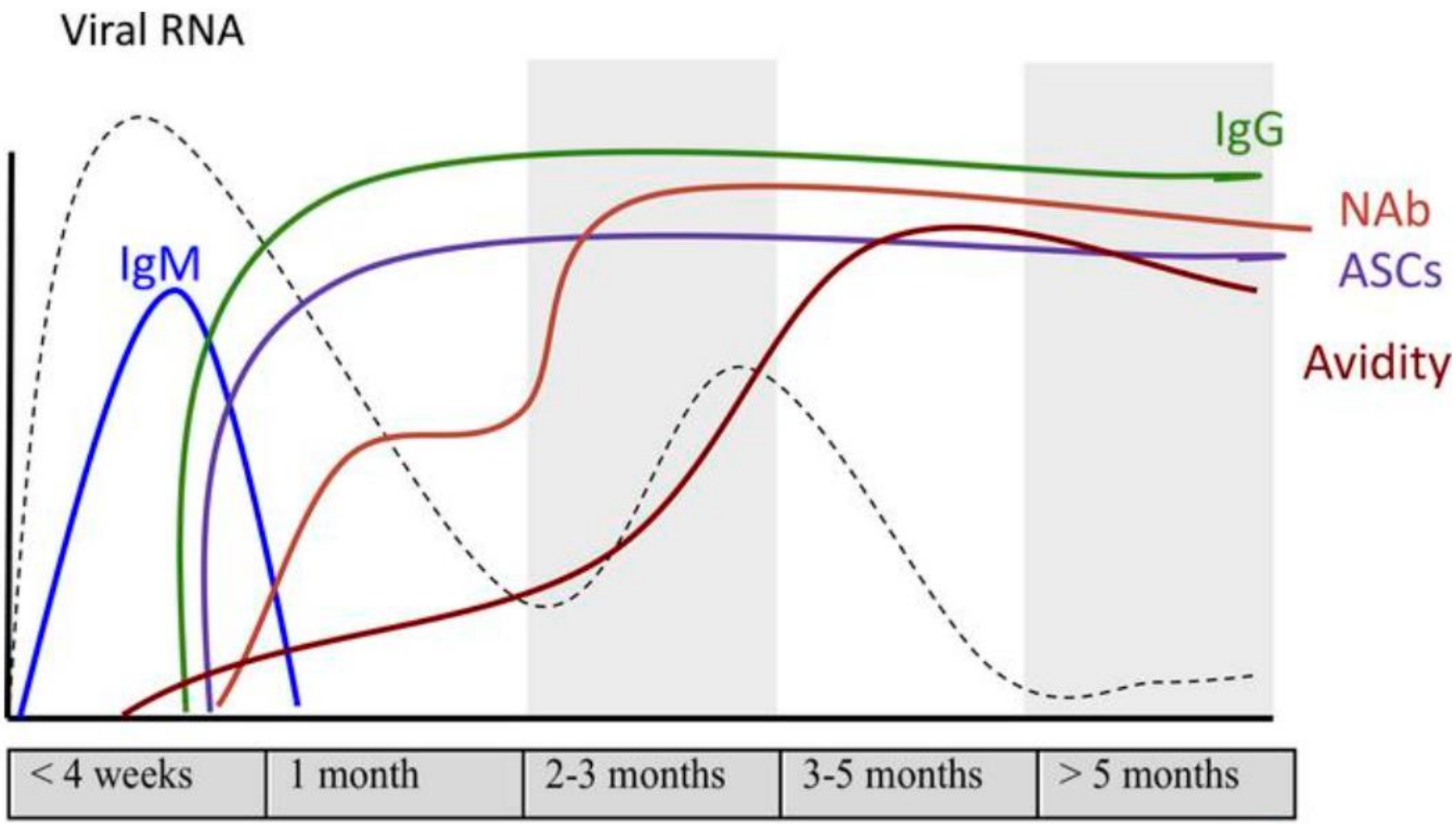
Indirect methods (serological methods)

- Etiological diagnosis
- Blood donors screening (HBV, HCV, HIV, HCMV)
- Solid organ/bone marrow donors screening
- Vaccine programs evaluation
- Prevalence and distribution of infectious diseases in the population

Indirect methods (serological methods)

INDIRECT DIAGNOSIS of infections aims to detect the specific immune response of the host towards the pathogen

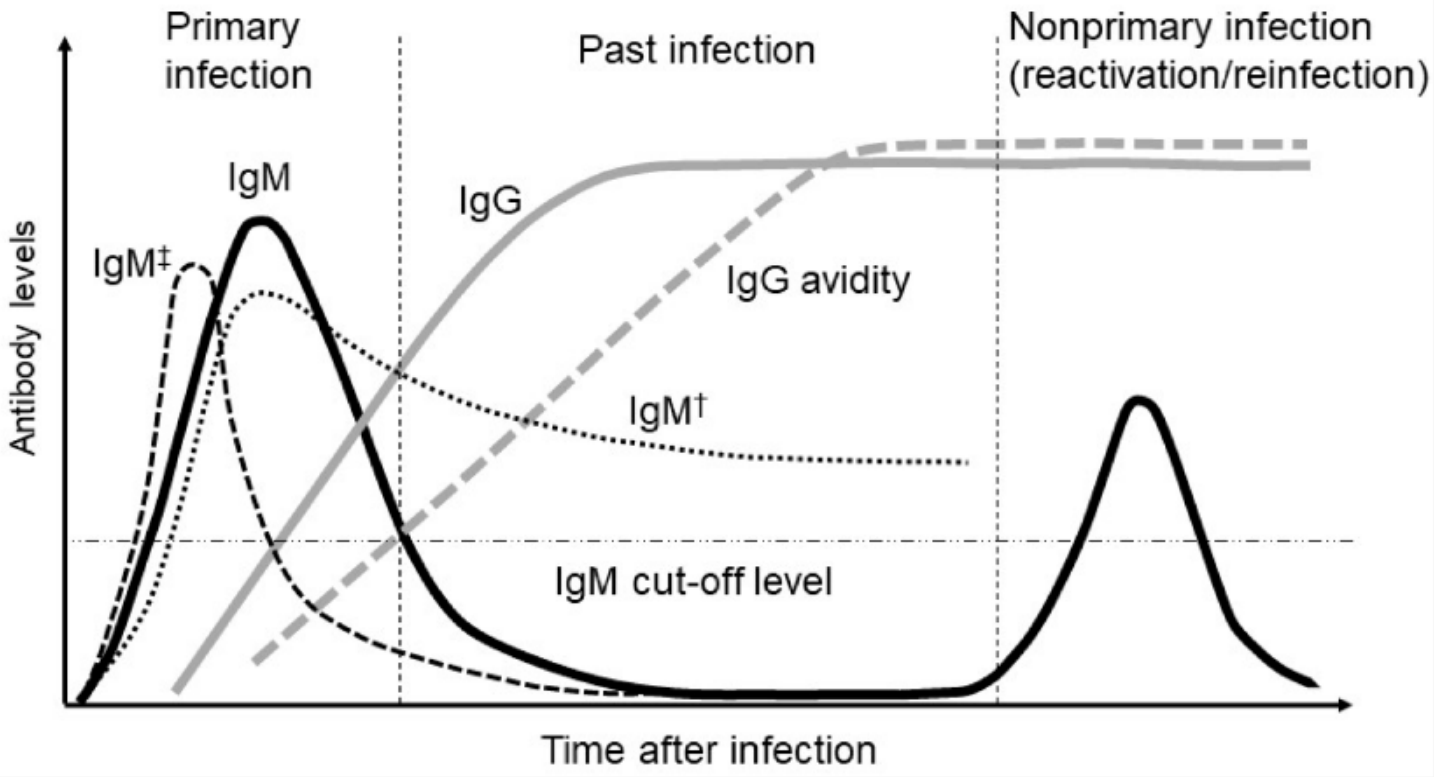
It is less timely than DIRECT diagnosis, as it can be performed only when the host immune response has developed



Ashley N. Nelson, … , Victoria K. Baxter, Diane E. Griffin

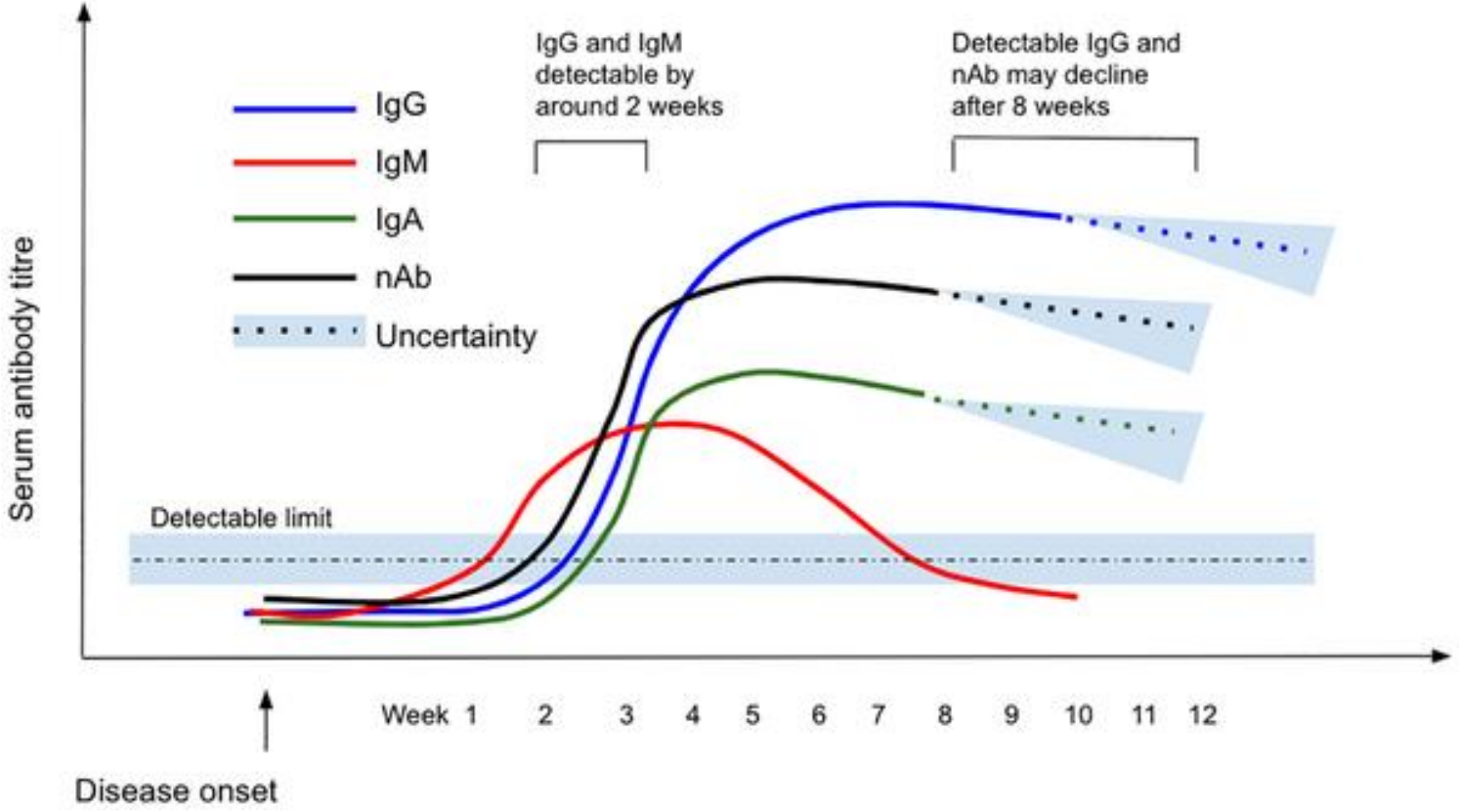
JCI Insight. 2020;5(3):e134992. https://doi.org/10.1172/jci.insight.134992.

Figure 2. Relative changes in cytomegalovirus (CMV) IgM (immunoglobulin M), IgG (immunoglobulin G), and IgG avidity levels over time following a primary CMV infection. Another pattern of IgM presentation represents the long-term persistence of IgM (†) and the rapid clearance of IgM (‡) as an atypical IgM response.



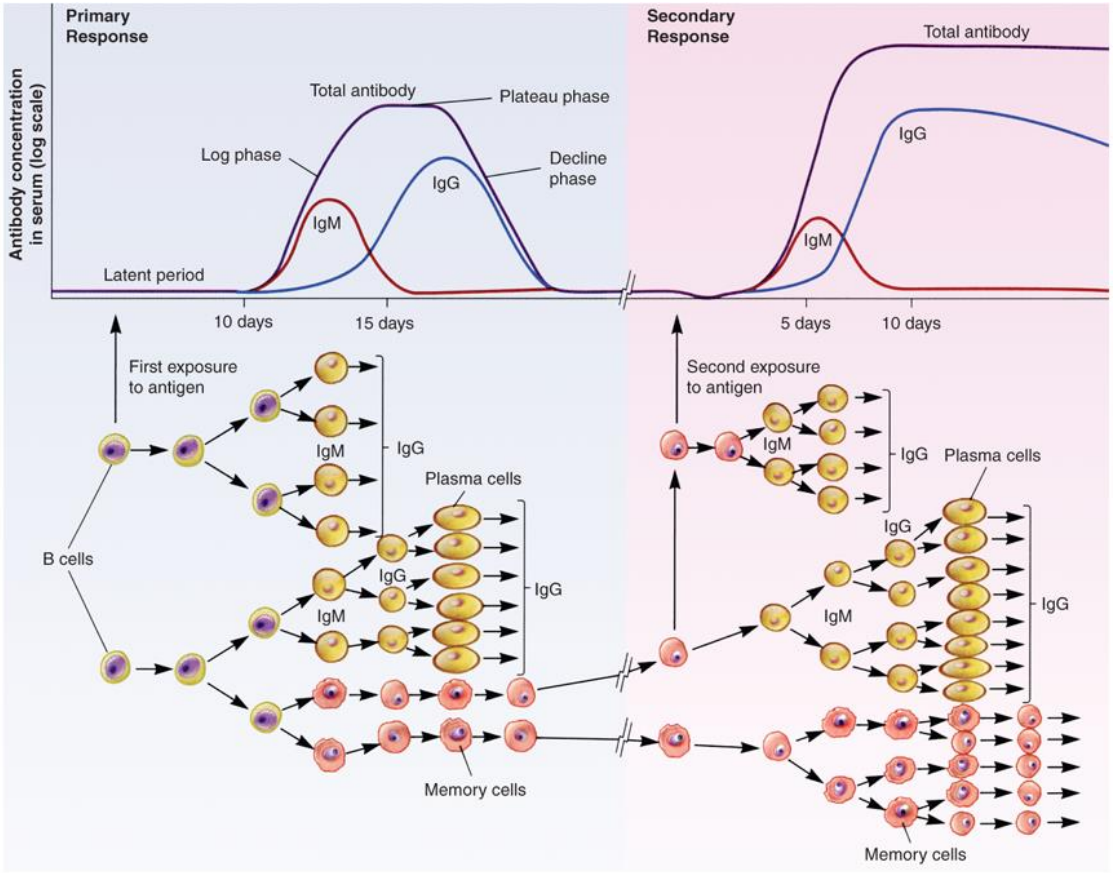
J. Clin. Med. **2022**, *11*(17), 5006; **https://doi.org/10.3390/jcm11175006**

Fig 3. Schematic showing the scale of IgG/IgM/IgA/Neutralising Ab response over time from disease onset.



Post N, Eddy D, Huntley C, van Schalkwyk MCI, Shrotri M, et al. (2020) Antibody response to SARS-CoV-2 infection in humans: A systematic review. PLOS ONE 15(12): e0244126. https://doi.org/10.1371/journal.pone.0244126
https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0244126

Maturation of the antibody response



	Primary	Secondary
Prevailing Ab class	IgM IgA (mucous membranes)	IgG (serum) IgA (mucous membranes)
Life	Weeks - months	Months- years- always
Concentration peak	7-20 days	10 days
Antigen affinity	low	high

Serological diagnosis

For the **IgG** result interpretation paired samples are required:

1) 5-10 days after symptoms onset — during ACUTE PHASE
2) 1-2 weeks after the first sample — during CONVALESCENCE

For the **IgM** assay, only one sample is required:

- IgM appear in the first days of infection
- peak after 7-10 days
- disappear in the following months

→ **Marker of ACUTE INFECTION**

- In some cases IgM reappear during recurrent infections and exacerbations
  (ex: HCV, CMV)
- Neonatal infection can be determined performing the IgM assay on cord blood samples

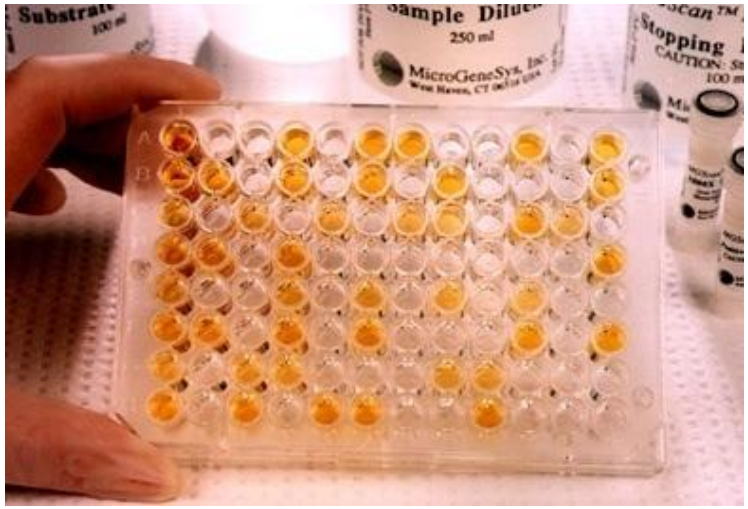
The IgM→IgG seroconversion or a 4-fold IgG titer-increase in the second sample is suggestive of acute viral infections

Indirect methods (serological methods)

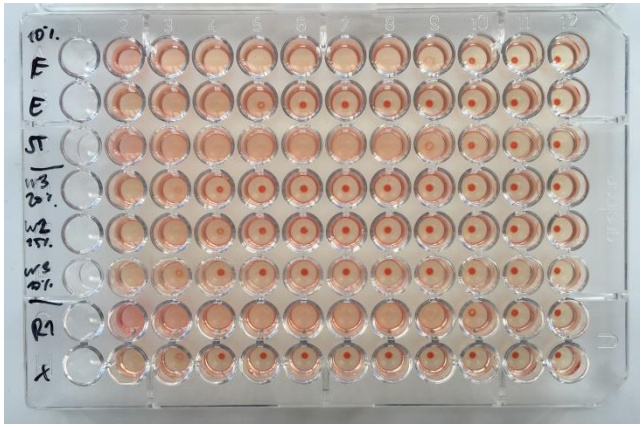
- Neutralization
- Complement fixation
- Hemagglutination inhibition
- ELISA and IFA
- Western blot

Many types of assays can be used for the serological diagnosis

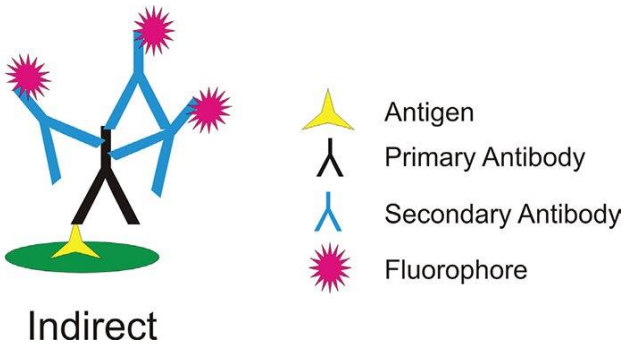
ELISA



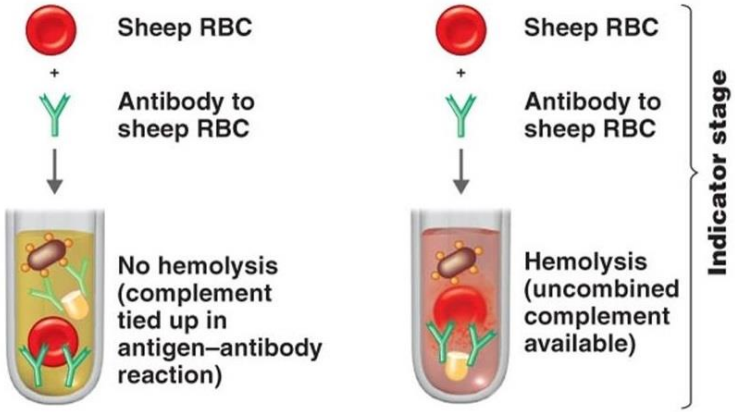
Hemagglutination assay



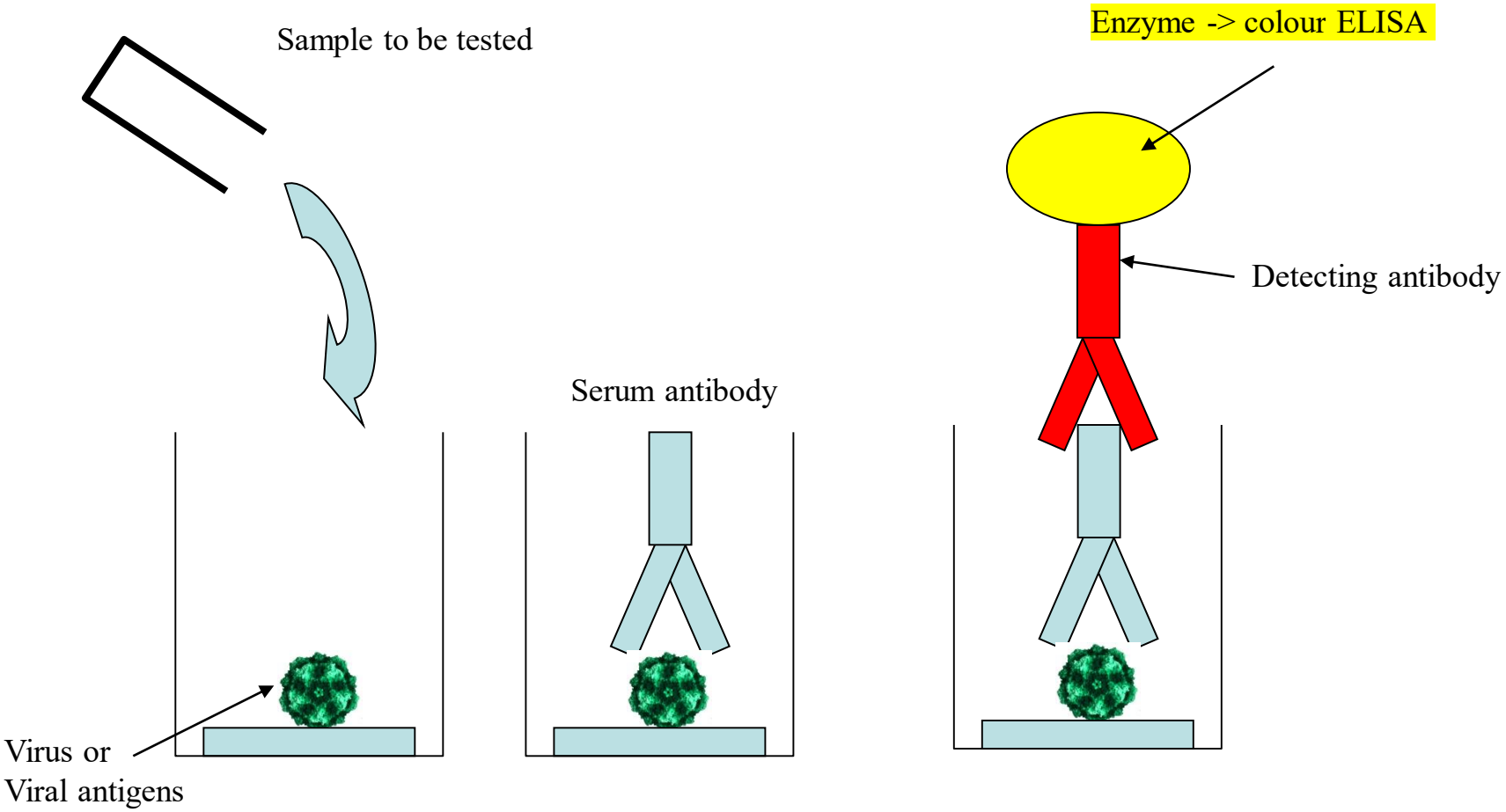
Immunofluorescence assay



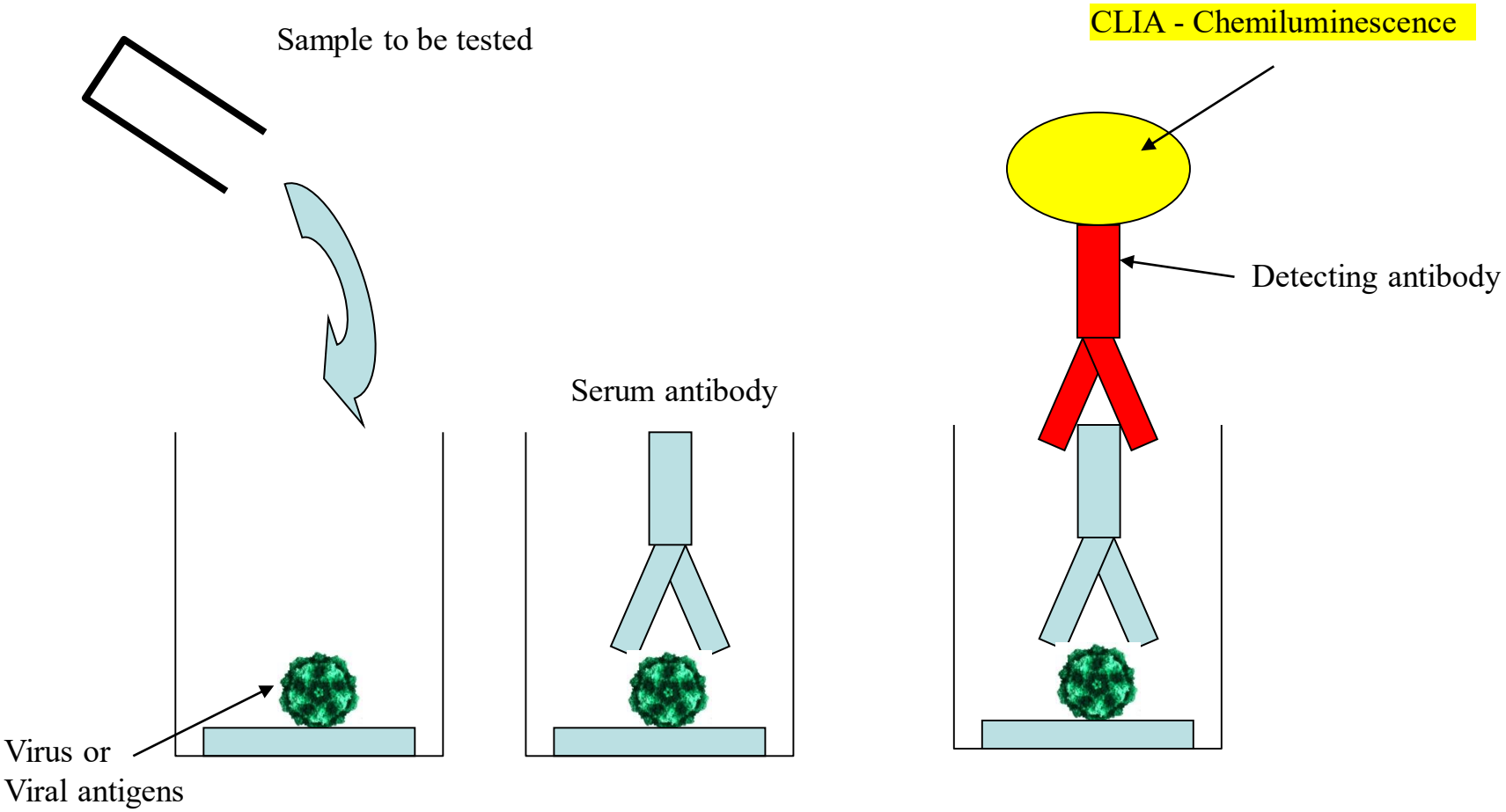
Complement fixation assay



Enzyme linked immunabsorbant assay (ELISA)



Enzyme linked immunabsorbant assay (ELISA)



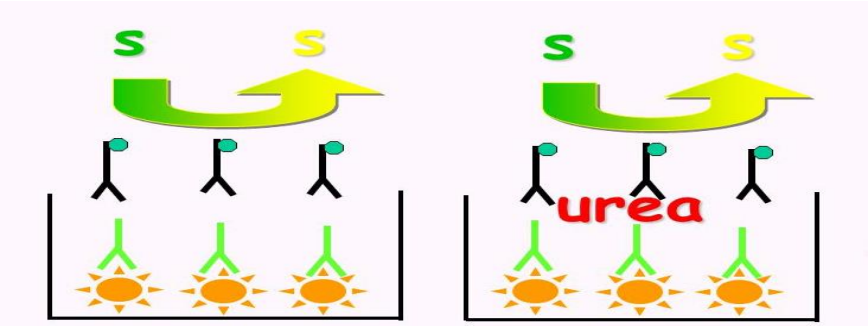
Automation

AVIDITY is the strength of the binding between the IgG antibody and its antigen.
Following antigenic challenge the IgG antibodies produced initially bind weakly to the antigen (**low avidity**).
As the immune response develops there is maturation of IgG antibody response and the avidity increases progressively over weeks or months (**high avidity**).

→ **The IgG avidity test helps in the discrimination between past and recently acquired infection.**

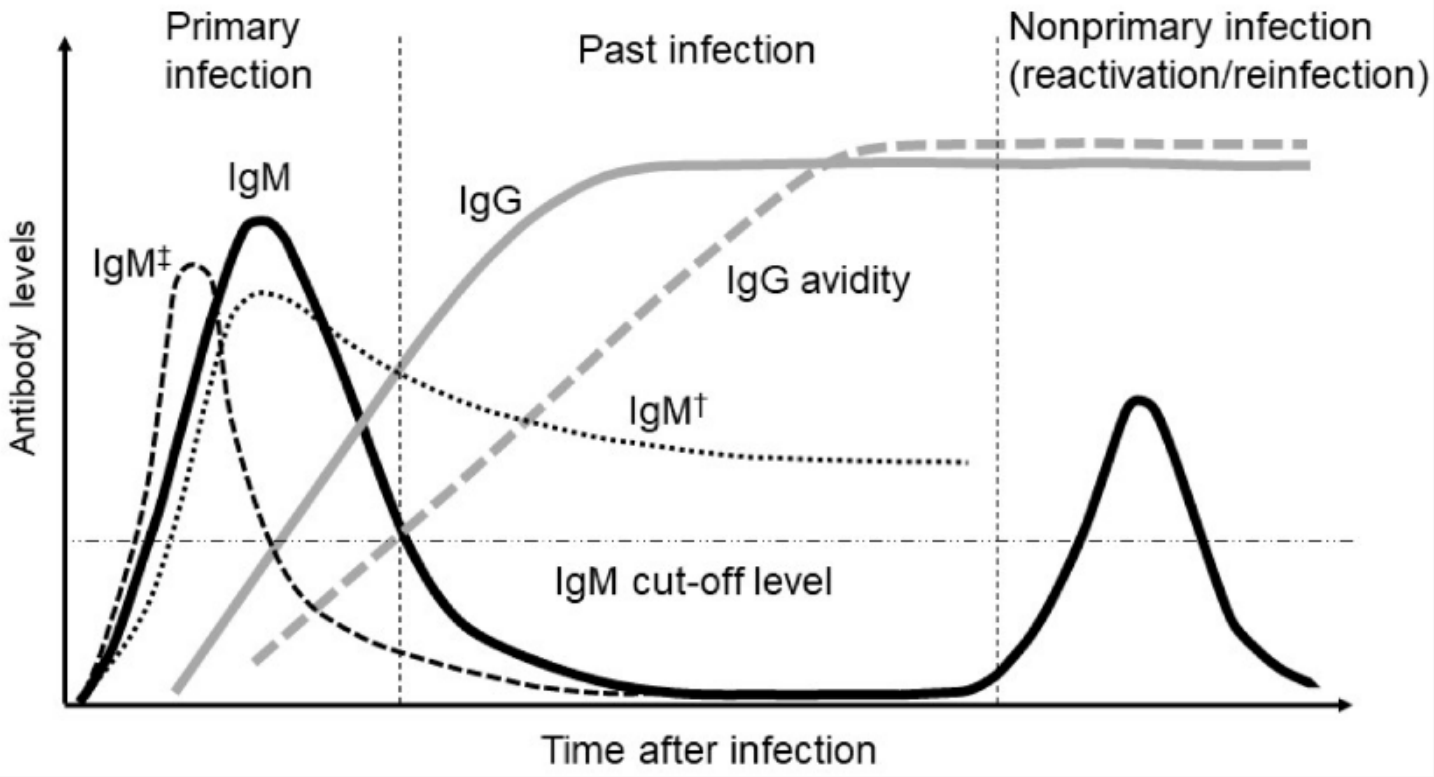
AVIDITY EIA ASSAY

The optical density of the sample is compared before and after the treatment with urea (denaturating agent)



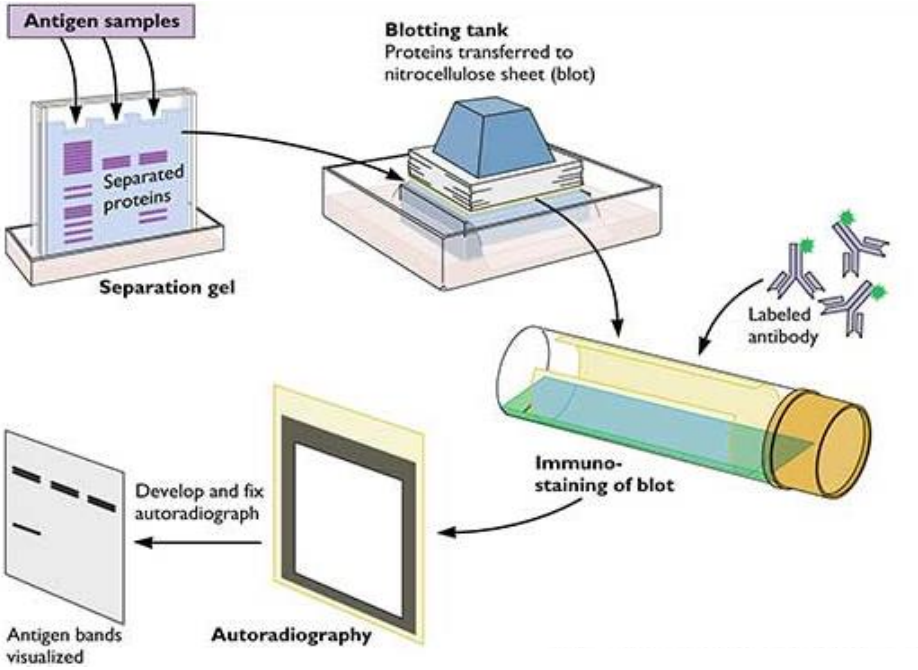
«Young» IgG, with low avidity, are dissociated after urea treatment and removed with washing
The % of residual IgG is lower when the amount of low-avidity IgG is higher

Figure 2. Relative changes in cytomegalovirus (CMV) IgM (immunoglobulin M), IgG (immunoglobulin G), and IgG avidity levels over time following a primary CMV infection. Another pattern of IgM presentation represents the long-term persistence of IgM (†) and the rapid clearance of IgM (‡) as an atypical IgM response.



J. Clin. Med. **2022**, *11*(17), 5006; **https://doi.org/10.3390/jcm11175006**

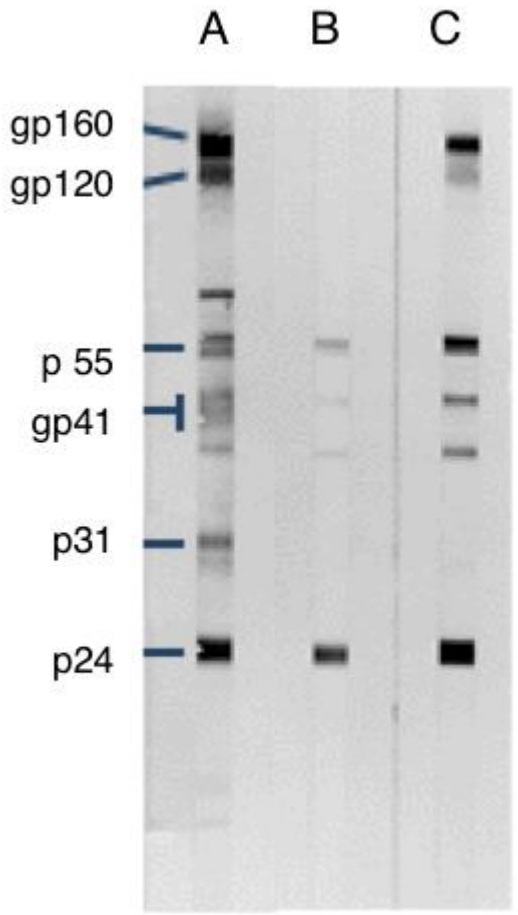
IMMUNOBLOT



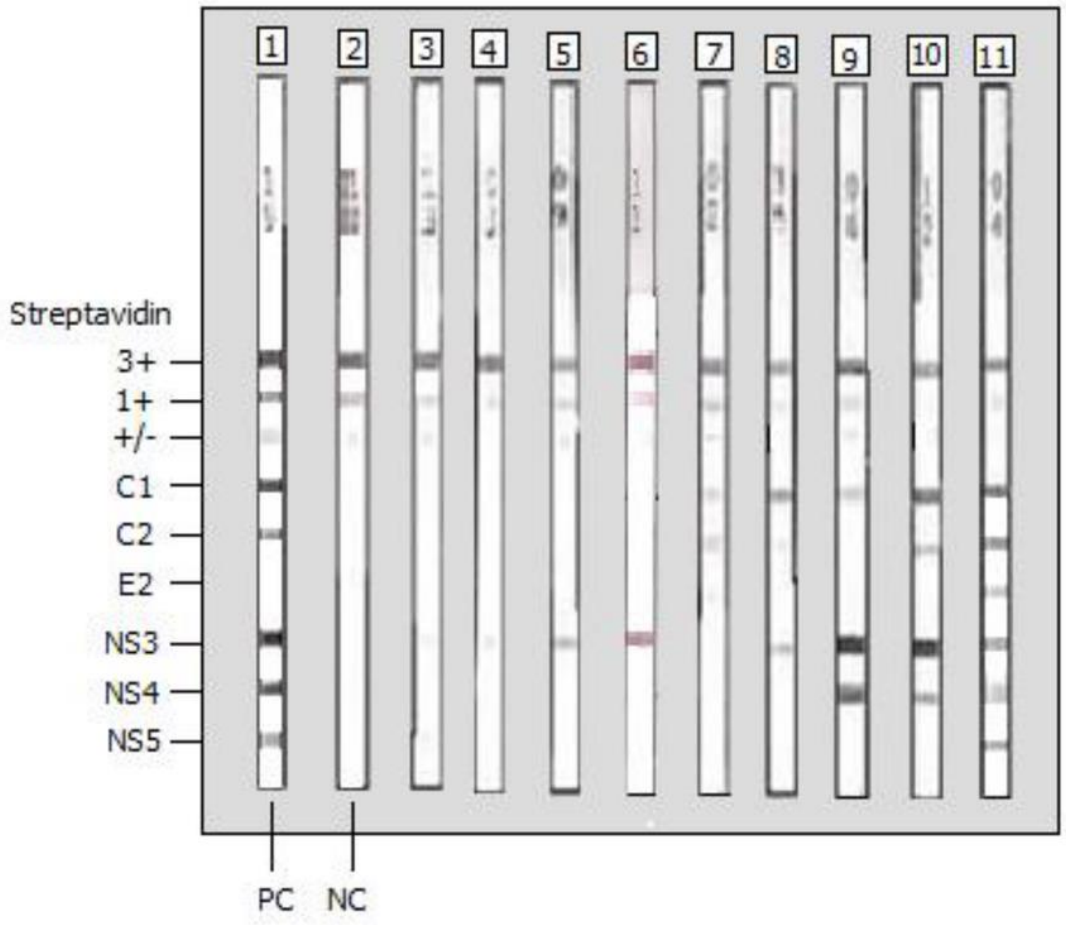
It is used as CONFIRMATORY test (more expensive)

ALLOWS THE DETECTION OF THE SPECIFIC Ab RESPONSE FOR EACH ANTIGEN

HIV IMMUNOBLOT

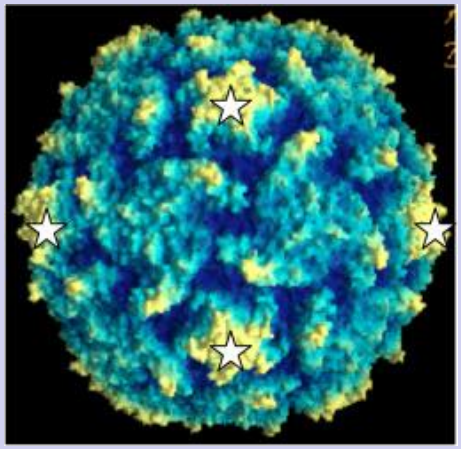


HCV Immunoblot

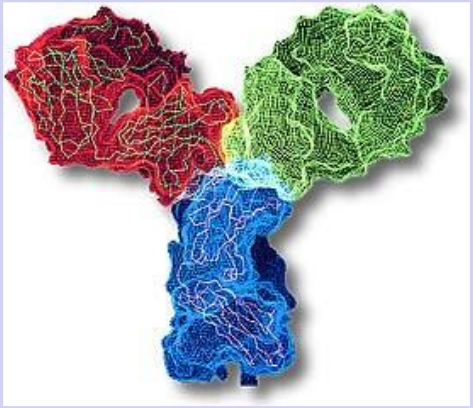


Laboratory diagnosis of VIRAL INFECTIONS

DIRECT:
Detection of virus, viral proteins (antigens), nucleic acids in different clinical samples
(nasal wash specimen, BAL, LCR, biopsy, etc)



INDIRECT:
Detection of virus-specific antibodies (serum)



PATHOGENESIS

Often the target organ is not the first choice for sampling
For DIRECT diagnosis of viral infection, the sampling must be performed taking into account:

- **the pathogen suspected to be responsible of the infection**
- **the localization of the suspected virus**

Virus	Entry	Diffusion	Target organ	Egress
Poliovirus Hepatitis A	Alimentary tract	Blood	SNC Liver	Stools
Measles Rubella	Pharynx Respiratory tract	Blood	Skin	Respiratory tract
HSV1 (acute infection)	Respiratory tract Skin, mucous membranes	Nerves, leucocytes	Many	Respiratory tract Skin
HSV2	Genital tract	Nerves	Genital tract	Genital tract
Hepatitis B, C	Skin lesions	Blood	Liver	Blood

Direct virological diagnosis

- Virus isolation
- Antigen Detection
- Nucleic Acid Detection
- Electron Microscopy
- Demonstration of the presence of viruses in biological samples

Direct virological diagnosis

- Virus isolation 
- Antigen Detection
- Nucleic Acid Detection
- Electron Microscopy
- Demonstration of the presence of viruses in biological samples

Samples for viral isolation

Virus	Sample
Influenza A and B	Respiratory secretions
RSV	Respiratory secretions
Rhinovirus	Respiratory secretions
Parainfluenza	Respiratory secretions
Measles	Respiratory secretions, conjunctival swab, lymphocytes
Rubella	Respiratory secretions, conjunctival swab, urine
Mumps	Cerebrospinal fluid, saliva
HIV	Lymphocytes, plasma
HSV	Cerebrospinal fluid, ocular fluids or scraping
CMV	Urine, leucocytes, liquor, amniotic fluid
EBV	Saliva, B lymphocytes
Rotavirus	Stools or rectal swabs
Enterovirus, poliovirus, echovirus, enteric adenovirus, coxsackievirus A and B, hepatitis A	Cerebrospinal fluid, stools or rectal swabs, throat swab

VIRUS ISOLATION

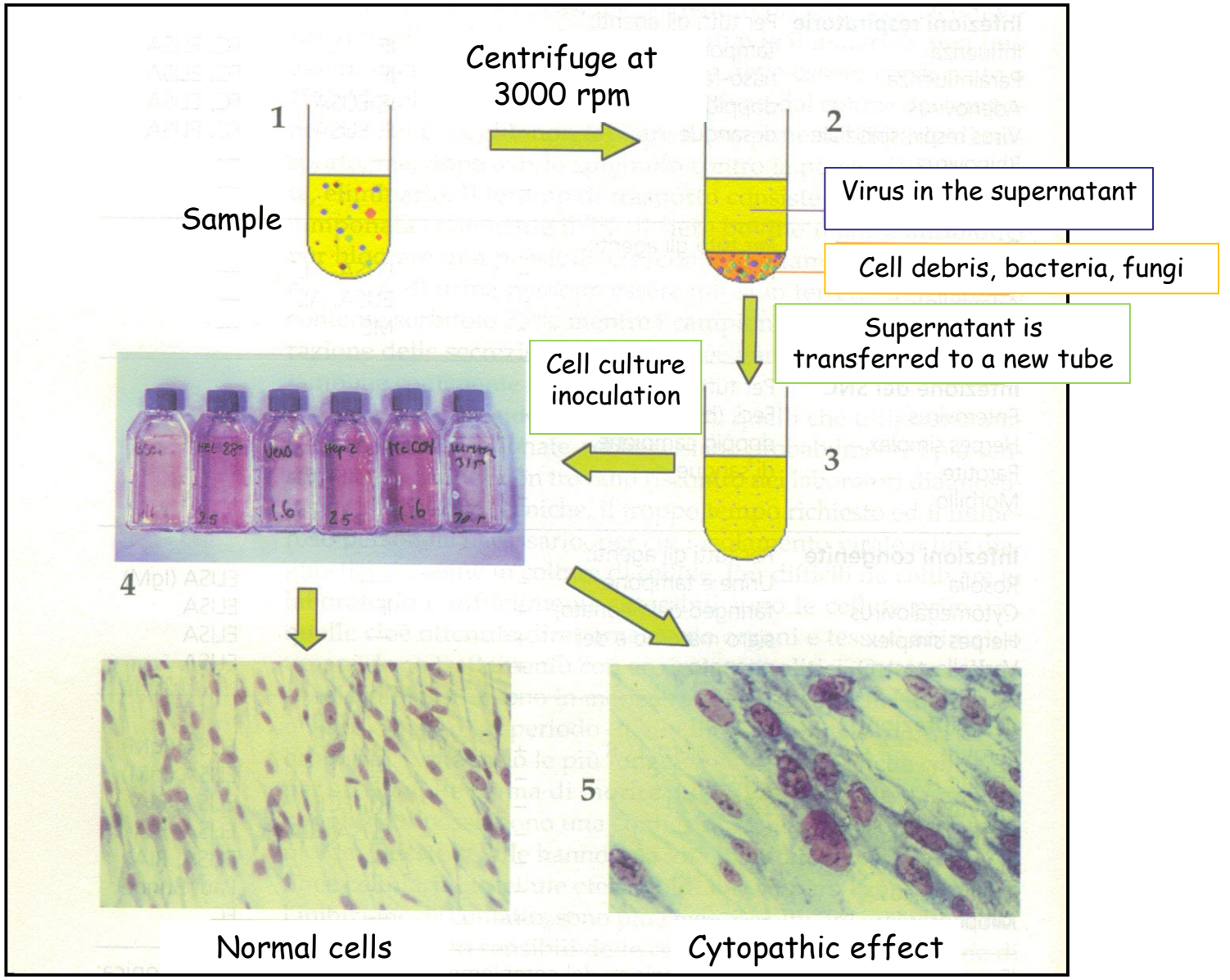
Virus isolation is commonly carried out *in vitro* by **CELL CULTURE**

Different types of cell lines are available, which allow the reproduction of different viruses.

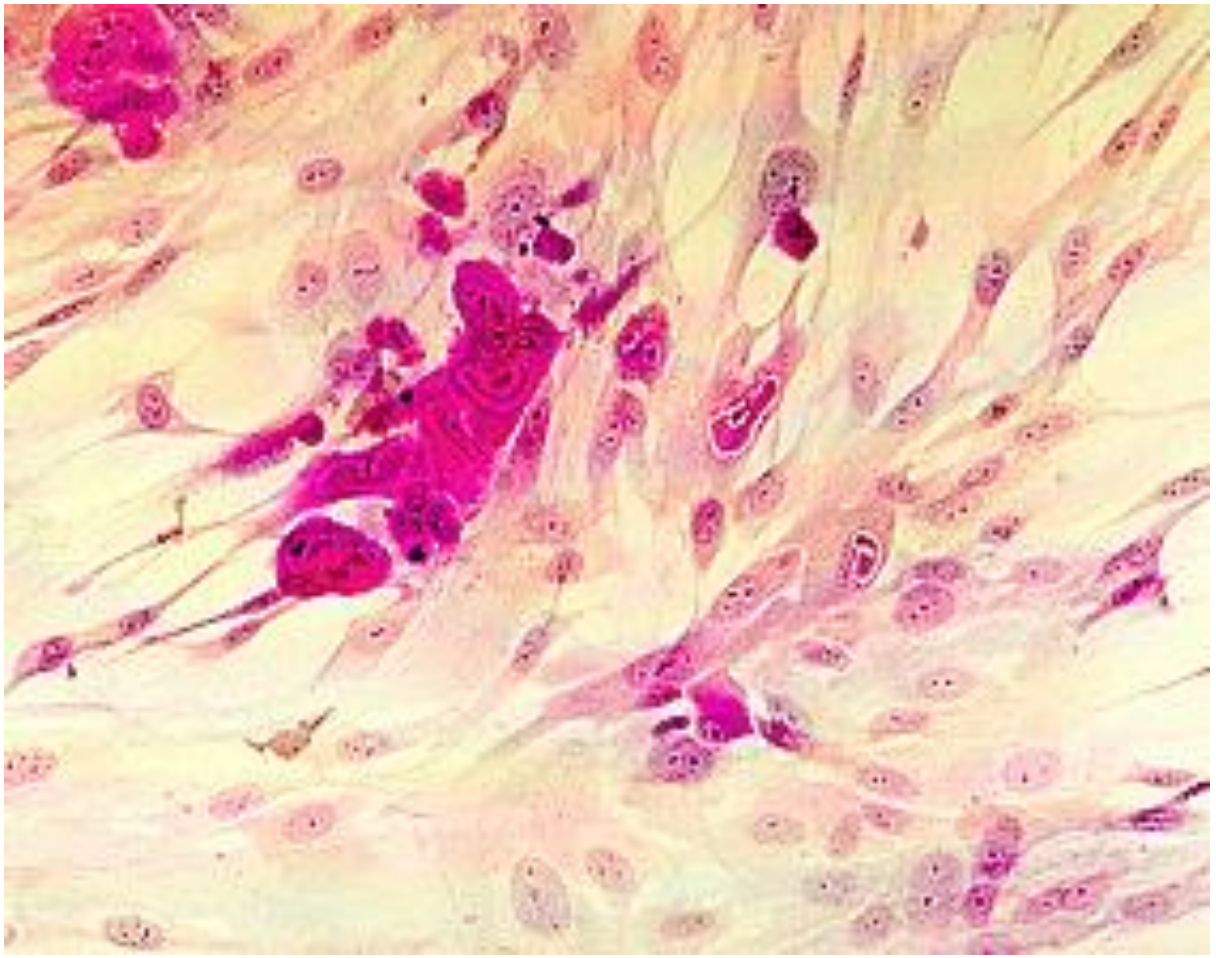
Cells can be derived from solid tissues through the digestion of the extracellular matrix by enzymes such as trypsin, and then are cultured in flasks containing growth medium. Most cells derived from solid tissues require a surface to grow in adherence
→ **Monolayer cell cultures**
Some cells naturally live in **suspension** such as cells that exist in the bloodstream.

PRIMARY CULTURES
Directly derived from tissues and composed of diploid cells. Their use is limited due to the possible presence of latent viruses in the animal tissue which could produce false isolations.

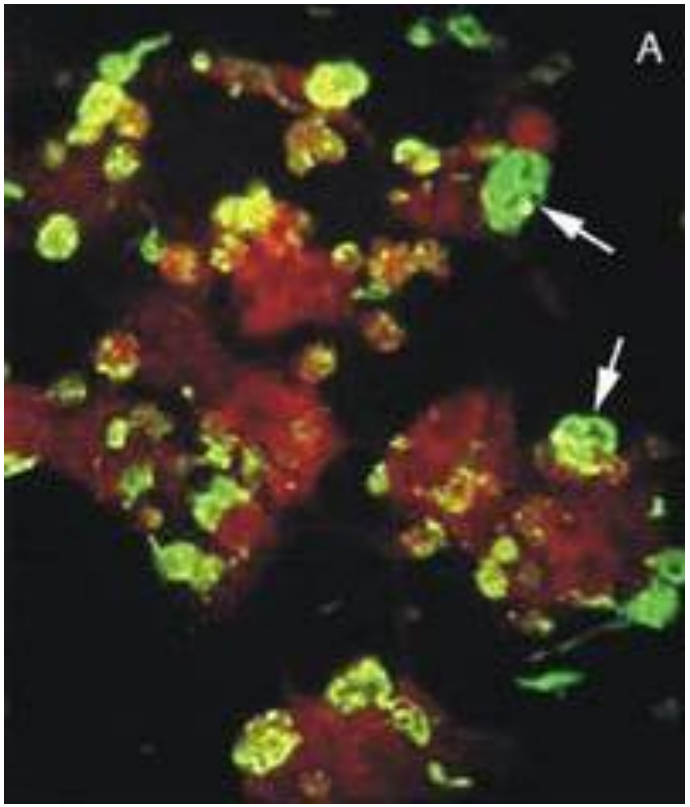
CYTOPATHIC EFFECT (CPE)



CPE caused by CMV in human fibroblasts



Viral antigen detection
after virus isolation



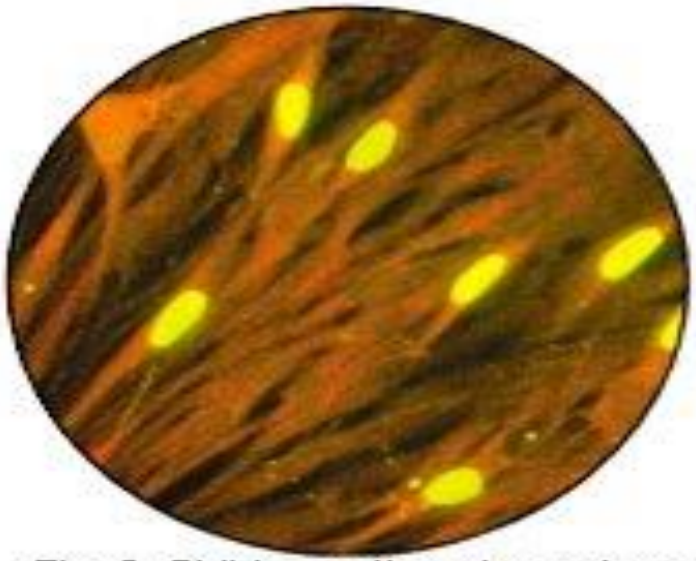
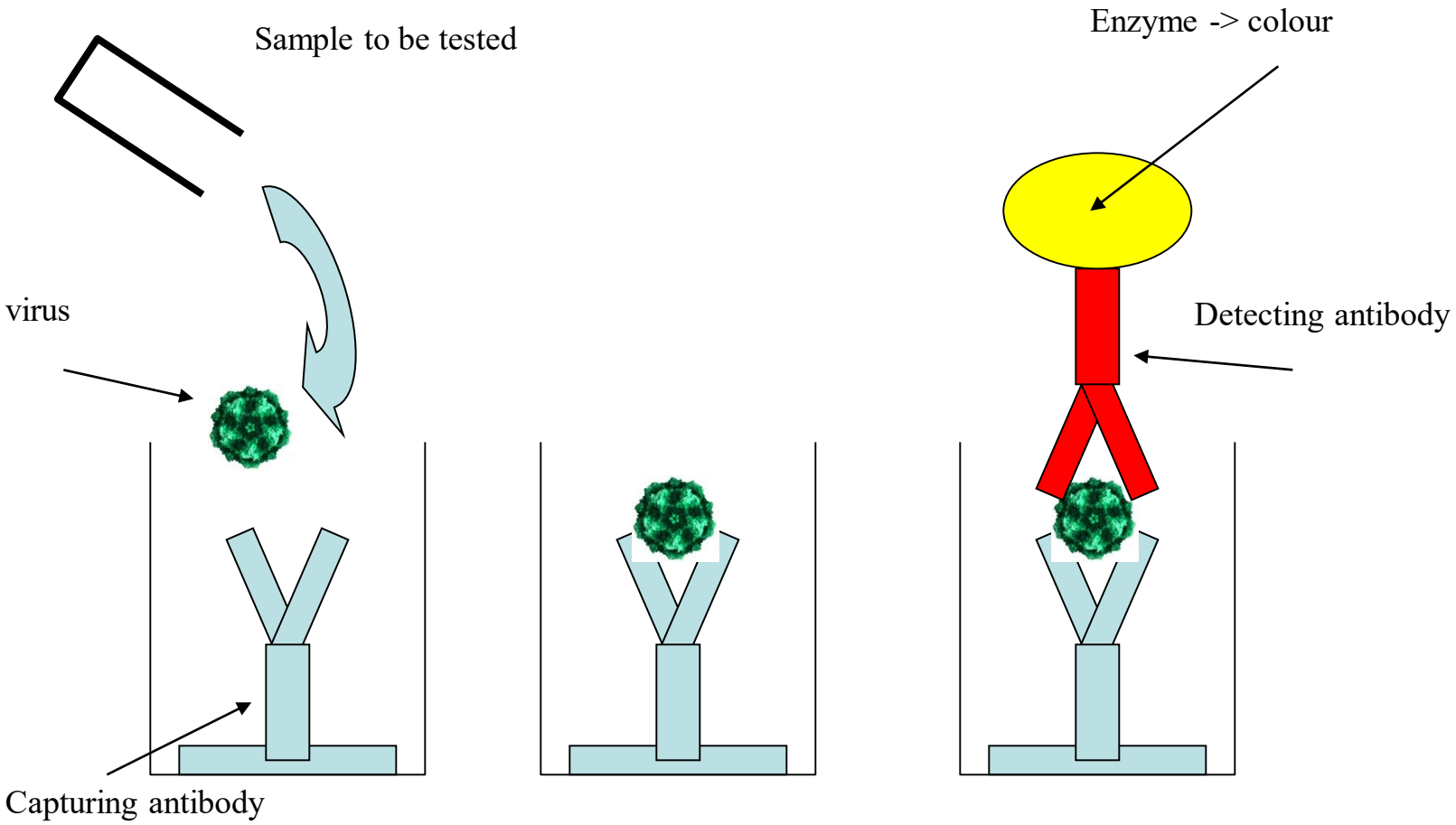


Fig. 2, CMV centrifugation culture fixed and stained 16 hrs after inoculation showing viral proteins in nuclei of infected human fibroblast cells

Direct virological diagnosis

- Virus isolation
- Antigen Detection 
- Nucleic Acid Detection
- Electron Microscopy
- Demonstration of the presence of viruses in biological samples

Enzyme linked immunabsorbant assay (ELISA)



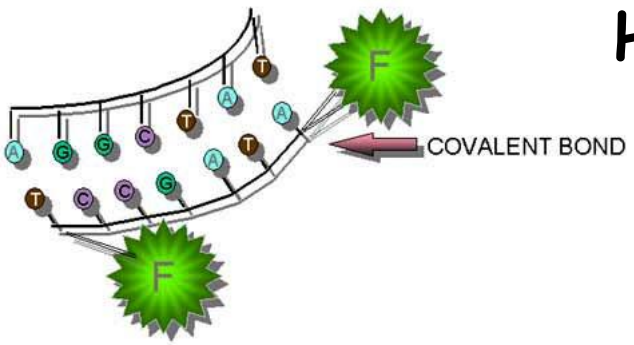
Detection of viral antigens: specimens and detected virus

Specimens	Virus
Swabs or nasopharyngeal aspirates, nasopharyngeal washes, bronchoalveolar aspirates	RSV, influenza A and B virus, parainfluenza virus 1-3, adenovirus
Skin or mucosal scraping	Measles, HSV, VZV
Conjunctival scraping	HSV, adenovirus
Stool	Rotavirus, enteric adenoviruses
Blood	CMV (pp56 antigen), HBV (HBsAg), HIV (p24 antigen)

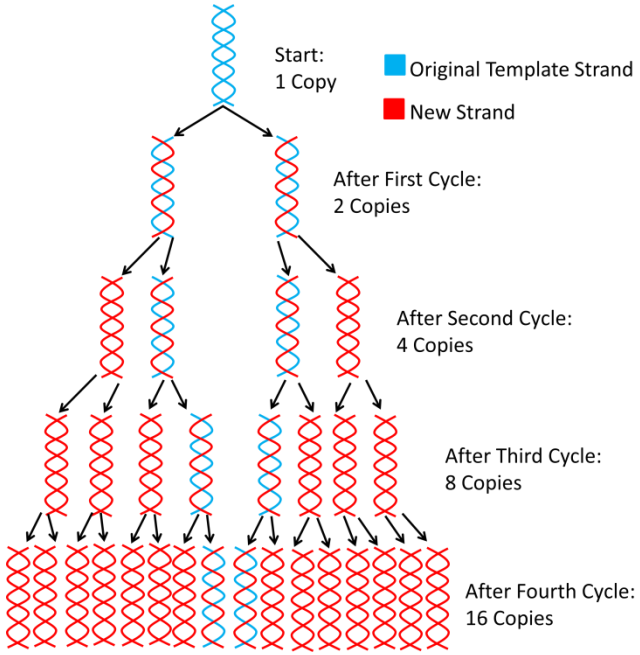
Direct virological diagnosis

- Virus isolation
- Antigen Detection
- Nucleic Acid Detection 
- Electron Microscopy
- Demonstration of the presence of viruses in biological samples

MOLECULAR METHODS



Hybridization with specific probes

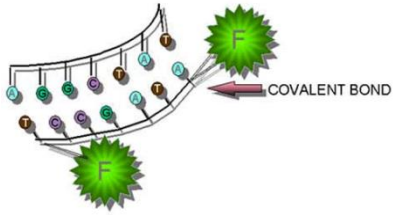


Polymerase chain reaction (PCR)

Allows the exponential amplification of a target sequence

Hybridization with specific probes

Organisms can be identified using nucleic acids probes that match specific gene sequences



Molecular probe: single-stranded DNA or RNA molecules used to detect a complementary sequence by hybridizing to it

The probe can be labelled with enzymes, chemiluminescent or fluorescent molecules, radioisotopes, and allows the detection of the «probe-target» hybridization through automatic systems

Three types of hybridization:

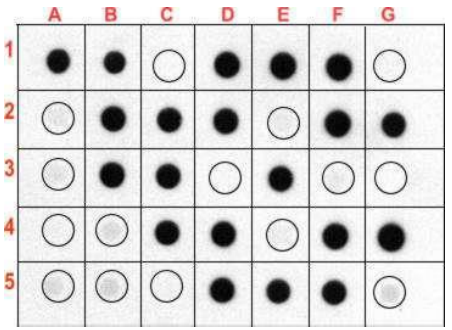
- Solid phase: the probe is adsorbed on a substrate (dot-blot, northern-blot, southern-blot)
- Liquid phase: the probe is suspended in a liquid solution (more rapid than solid phase)
- *in situ*: using probes labelled with fluorophores

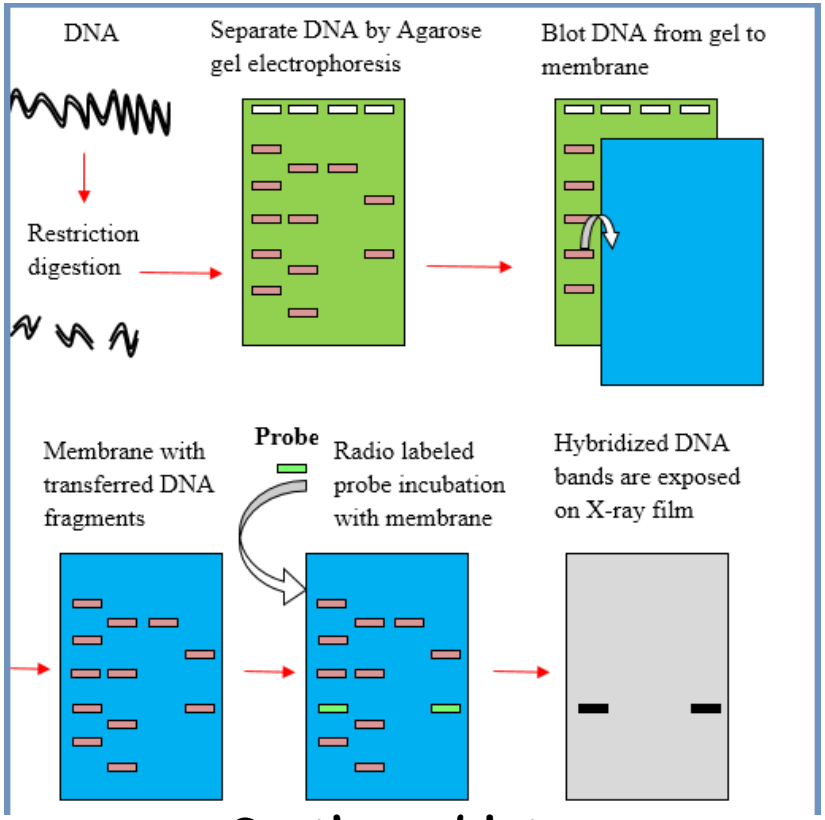
The hybridization can be applied to different samples:
purified DNA preparations (southern, liquid phase), clinical samples (*in situ*).
High specificity
Good sensitivity (but lower than amplification methods): allow the detection of 10^4-10^6 copies of the target sequence

Hybridization with specific probes

The nucleic acid probe is labelled with a dye and hybridized to the extracted viral nucleic acid that has been denatured (to make single-stranded) and immobilized onto a nitrocellulose membrane.
The labelled probe can be visualized by chemiluminescent methods, depending on the label used.

Dot blot





Southern blot

Different DNA fragments with different molecular weight can be separated by agarose gel electrophoresis, denaturated and then trasferred onto a membrane for the probe hybridization and detection.

In **Northern blot** RNA molecules separated by electrophoresis are used

Molecular methods for the diagnosis of viral infections

Classical techniques

- Dot-blot
- Southern-blot
- Northern-blot

Newer techniques

- PCR
- bDNA
- TMA
- Real-time PCR

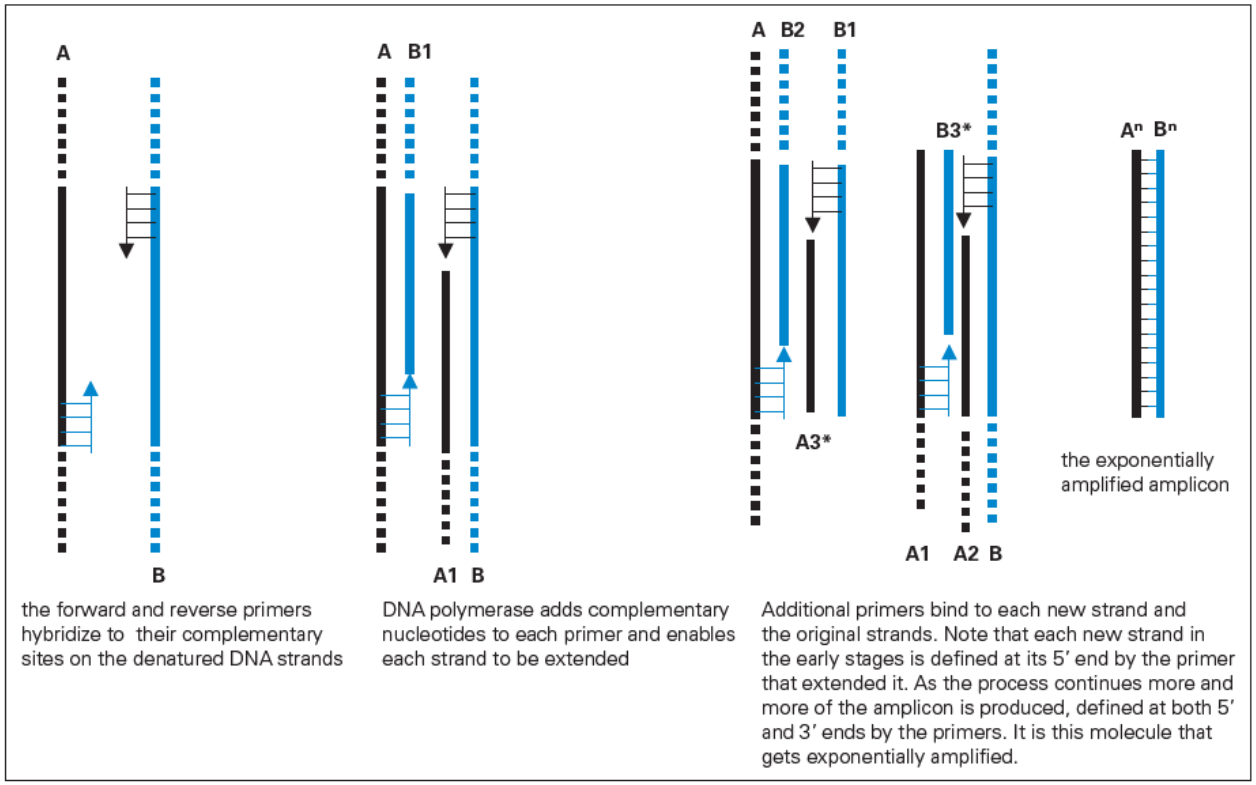
Real-time PCR

Polymerase Chain Reaction (PCR)

PCR can be used to amplify a specific DNA sequence to produce millions of copies within a few hours

1. **Nucleic acids extraction** from clinical samples

2. **Amplification** using primers, nucleotides and the enzyme Taq Polymerase



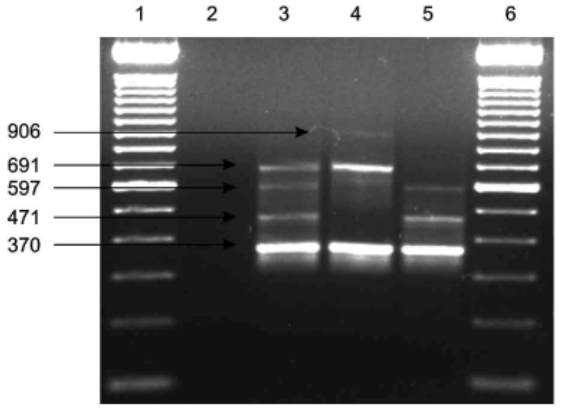
DENATURATION 95°C
ANNEALING 56-65 °C
ELONGATION 72 °C

QUALITATIVE AND QUANTITATIVE METHODS

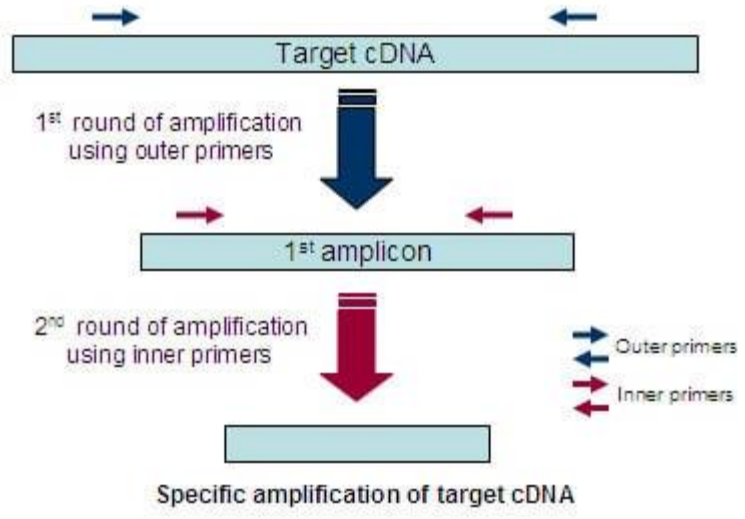
Polymerase Chain Reaction (PCR)

Multiplex-PCR: more than one primer couple is used, allowing the simultaneous detection of many sequences and thus of many pathogens in the same sample

- costs and time reduction
- allows tests to be grouped into disease syndromes (ex. Respiratory infections, sexually acquired infections)



Representative Multiplex-PCR amplification patterns of clinical isolate of *Listeria monocytogenes* analyzed by 2% agarose gel electrophoresis. Lanes 1, 6: molecular weight marker (100 bp, Invitrogen); 2: Multiplex-PCR negative control; 3: *L. monocytogenes* isolated (370, 471, 597, 691 bp); 4: positive control 1, ATCC 7644 (INCQS 00266) (370, 691, 906 bp; Serovars: 1/2c and 3c); 5: positive control 2, ATCC 19117 (INCQS 00327) (370, 471, 597 bp; Serovars: 4b, 4d and 4e).

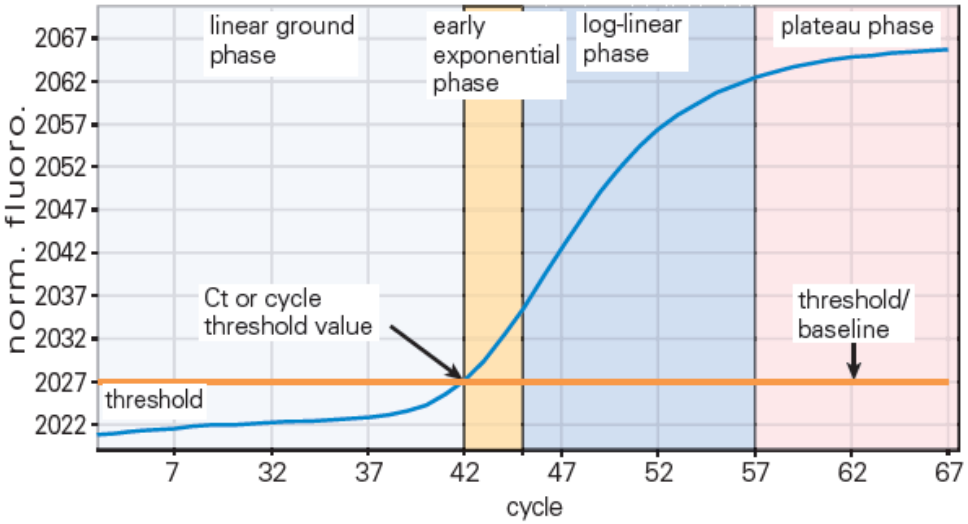


Nested-PCR: higher specificity (and/or sensitivity) of the reaction.

Two consecutive amplifications:
The DNA amplified in the first reaction is used as template DNA in the second reaction
For the second reaction primers mapping inside the first amplification product are used

Real Time PCR

The amplification process can be monitored in real time.
The amount of fluorescence detected during the reaction is directly proportional to the amount of amplicon produced.
By including a set of prequantified DNA standards, co-amplified during the reaction, the copy number of nucleic acid in the original sample can be estimated



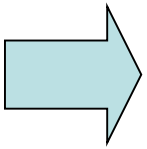
- ✓ The detection is performed during the exponential phase, when no factor limiting the reaction is present
- ✓ Sensitive
- ✓ Wide dinamic range (10^1–10^7; no sample dilutions required)
- ✓ Limited possibilities of contamination (no post-amplification handling)

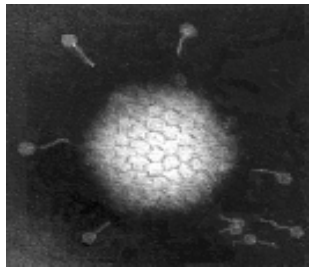
Direct virological diagnosis

- Virus isolation
- Antigen Detection
- Nucleic Acid Detection
- Electron Microscopy
- Demonstration of the presence of viruses in biological samples

Direct methods

- Electron microscopy (EM)





SAMPLING for the DIRECT diagnosis

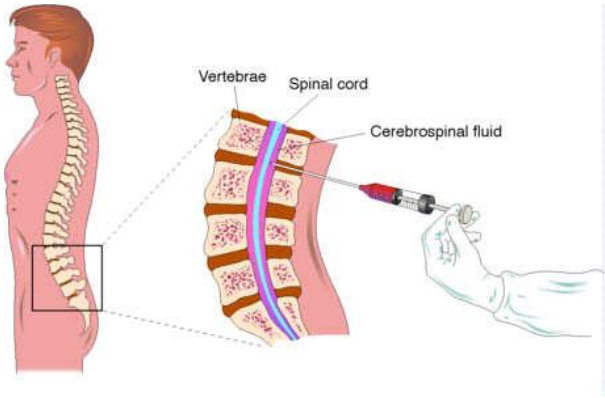
NORMALLY STERILE sites

BLOOD

Plasma liquid part of the non coagulated blood
(the type of anticoagulant is important)

Serum liquid part of the coagulated blood

PBL for CMV, EBV, HIV
obtained by
- red blood cells lysis
- density gradient



CEREBROSPINAL FLUID

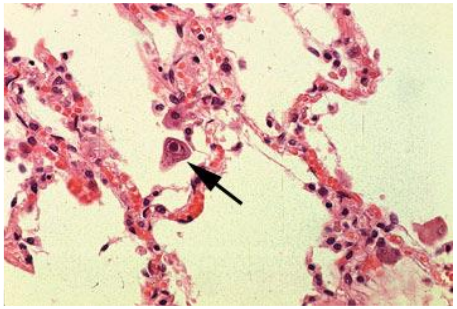
SAMPLING for the DIRECT diagnosis

POLYMICROBIC samples: handling is generally needed

SWABS in transport medium
(PBS, 20% FCS or BSA, antibiotics, antimycotic)

URINE 1:2 dilution
filtration, centrif. at 15000 rpm

STOOLS 1:5 / 10 dilution
centrif. at low speed
centrif. supernatant at high speed



BAL dilution, mucus removal

BIOPSY homogenization, centrif.

IMMUNOFLUORESCENCE

One of the most sensitive methods

Used:

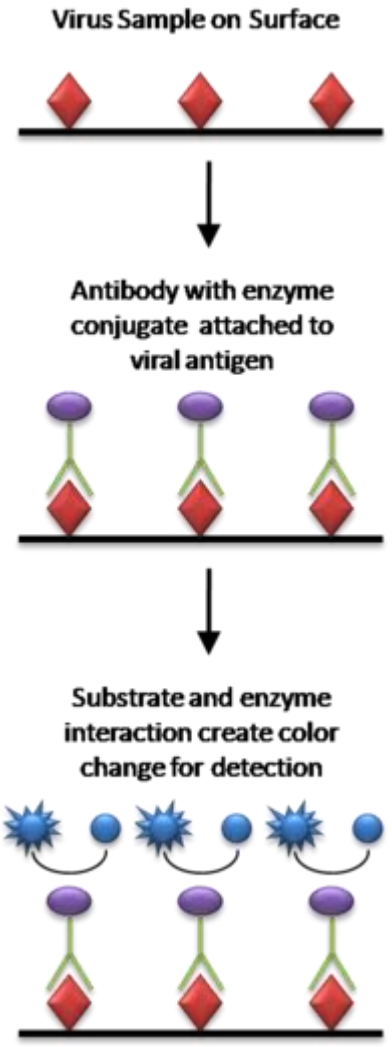
- to detect serum antibodies (using known antigens)
- to detect antigens (using commercially available antibodies)

- for the direct analysis of the specimens
- for cell analysis after viral isolation

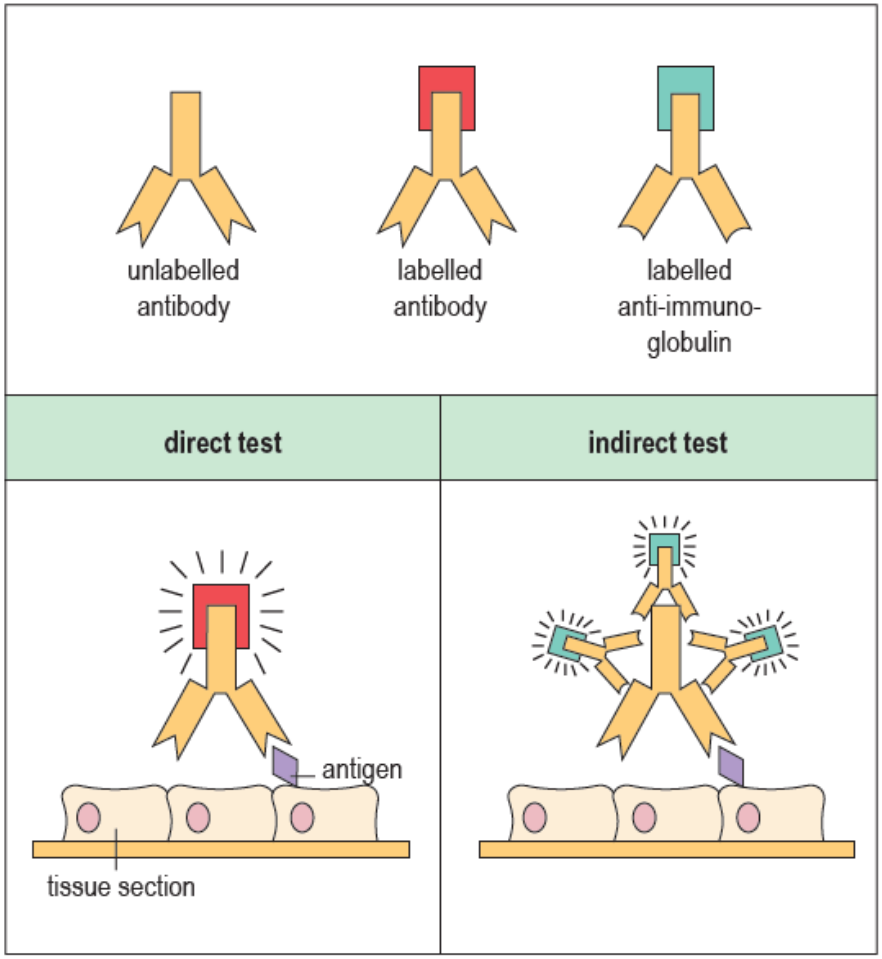
Allow to:

· observe the cell localization of an antigen (cytoplasm, membrane, nucleus)

· determine the presence of multiple antigens or markers in the same cell using different fluorescent dyes



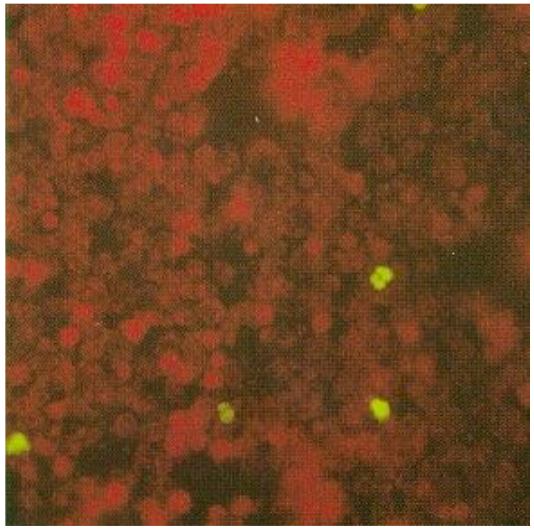
IMMUNOFLUORESCENCE



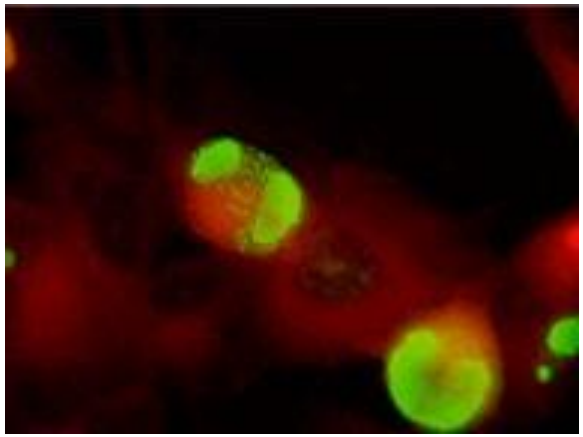
DIRECT AND INDIRECT ASSAYS

Figure 32.8 The fluorescent antibody test for detection and identification of microbial (or tissue) antigens or antibodies directed against them. In the direct test, antibody labelled with a fluorescent dye is applied to a tissue section bearing the antigen, unbound antibody is washed away, and the bound antibody showing the presence and location of the antigen is visualized by fluorescence microscopy. In the indirect test, antigen is revealed by successive treatments with unlabelled antigen-specific antibody and then fluorescent-labelled anti-immunoglobulin, which amplifies the signal (thus if the first antibody is human, the labelled antibody will be an anti-human Ig).

Viral antigen detection in clinical specimens

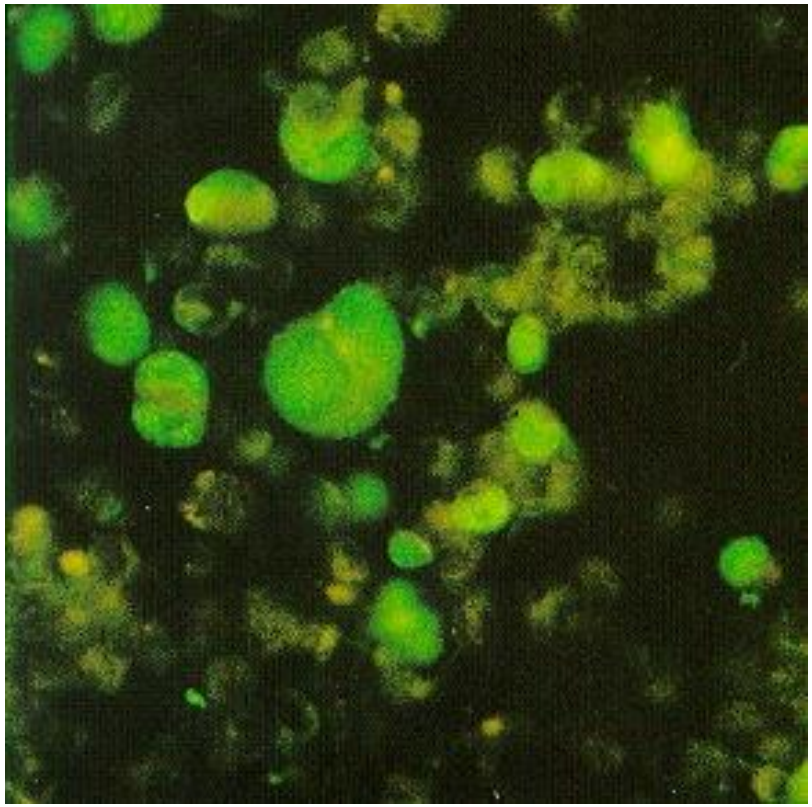


CMV Antigenemia (pp65)
(after staining with Evans Blue)



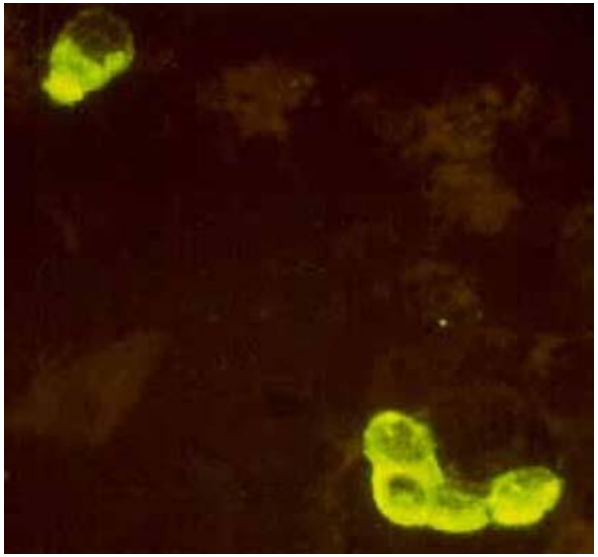
- for PBL samples
- semiquantitative

Viral antigen detection in clinical specimens

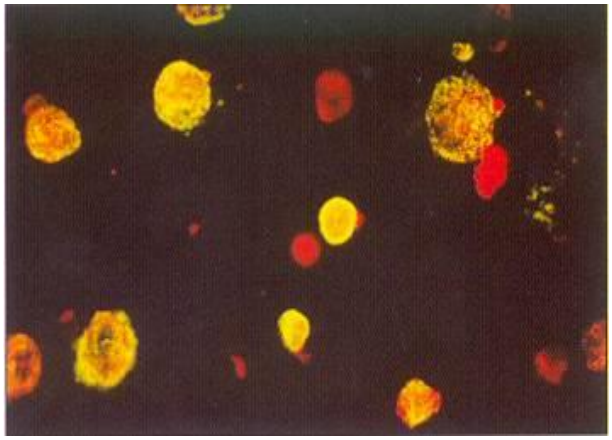


IF HCMV
amniotic fluid

Viral antigen detection in clinical specimens



RSV in smear from nasopharyngeal swab (without contrast staining)



HSV1 in epithelial cells

Direct virological diagnosis

- Virus isolation
- Antigen Detection
- Nucleic Acid Detection 
- Electron Microscopy
- Demonstration of the presence of viruses in biological samples

Qualitative PCR: diagnostic applications

- Diagnosis of perinatal infections
- Diagnosis of meningitis, encephalitis, retinitis
- Diagnosis of infection during the "window period"

Quantitative PCR: diagnostic applications

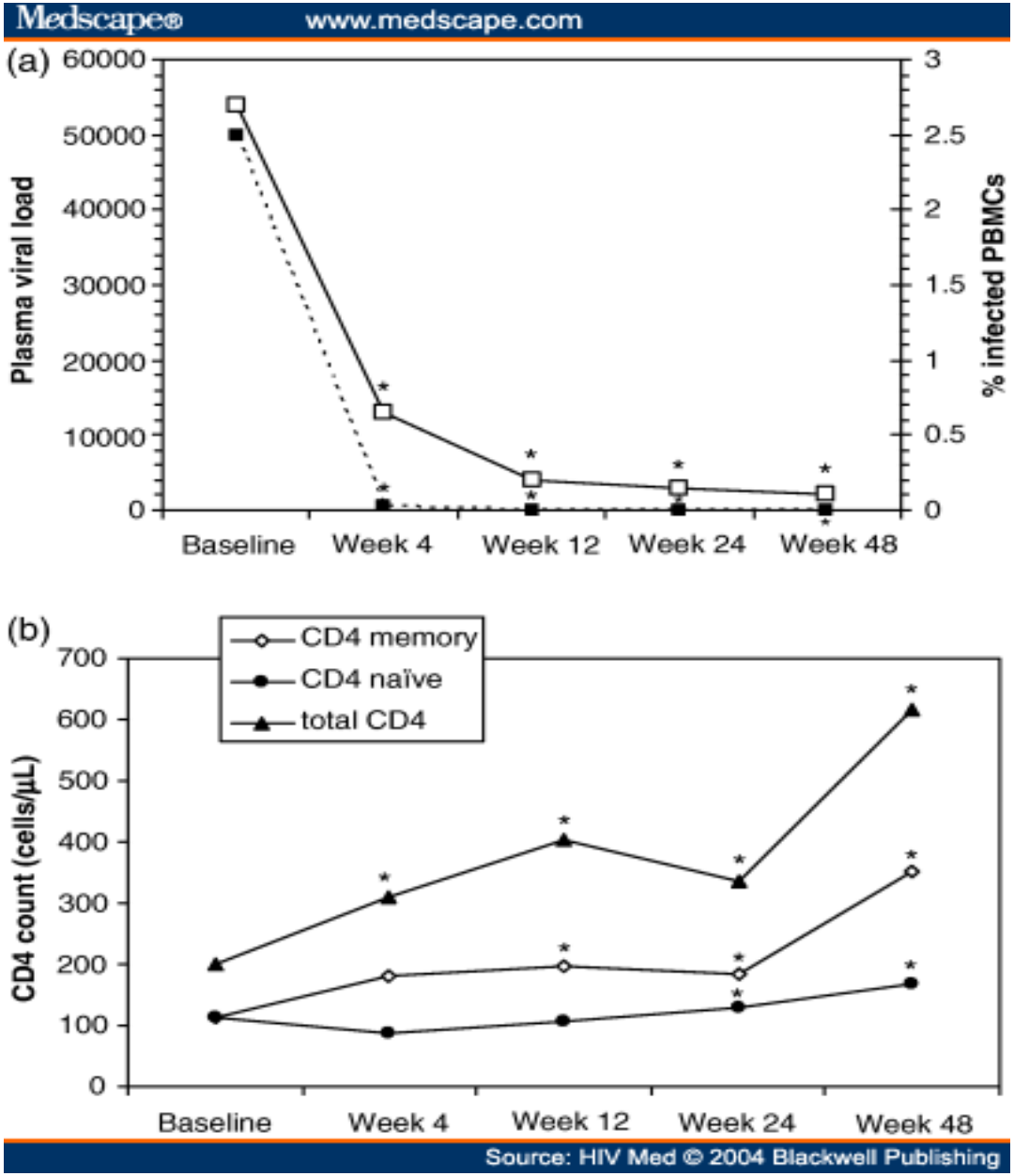
- Prognosis for infection
- Monitoring response to therapy

Molecular tests for virological diagnosis

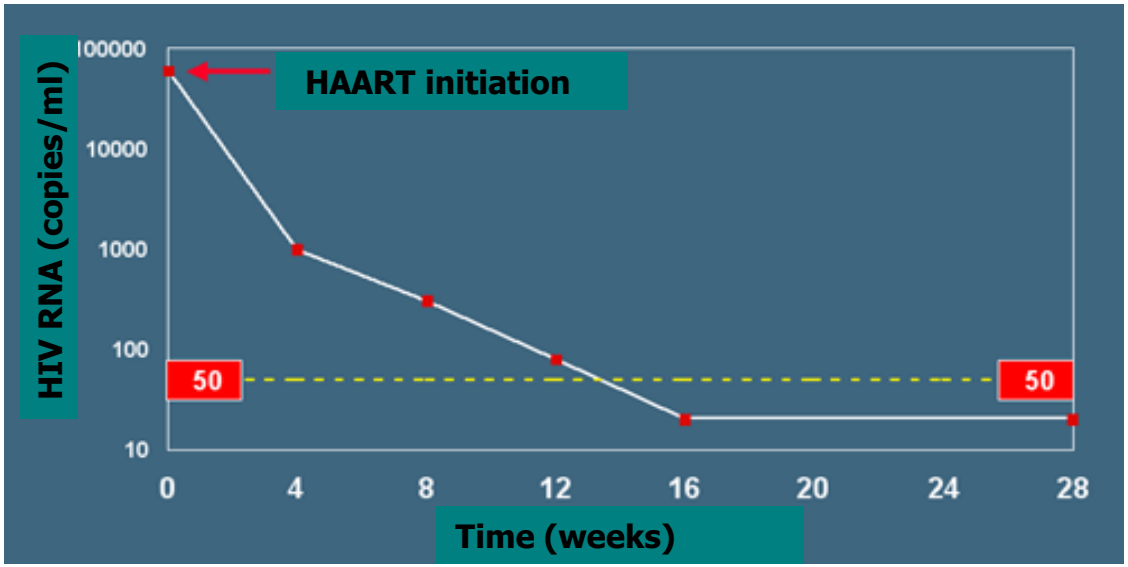
Virus	Specimen	Application
HIV (qualitative) (quantitative)	Lymphocytes Plasma	Early diagnosis, perinatal diagnosis, Monitoring of infection and treatment
HSV	CSF, swab corneal scraping, ocular fluid	Encephalitis, retinitis, meningitis
VZV	CSF, swab corneal scraping, ocular fluid	Encephalitis, retinitis, meningitis
CMV	Leukocytes, plasma, blood, CSF, swab corneal scraping, ocular fluid, amniotic fluid	Diagnosis of systemic infection in transplants, encephalitis, myelitis, retinitis, congenital infection
JC	CSF	Progressive multifocal leukoencephalopathy
BK	Urine	Hemorrhagic cystitis
HPV	Vaginal, penile and anal swabs	Diagnosis of infection and determination of low- and high-risk types
HCV (qualitative) (quantitative)	Serum Serum	Diagnosis of infection Monitoring of infection and treatment
HBV (qualitative) (quantitative)	Serum Serum	Diagnosis of infection Monitoring of infection and treatment

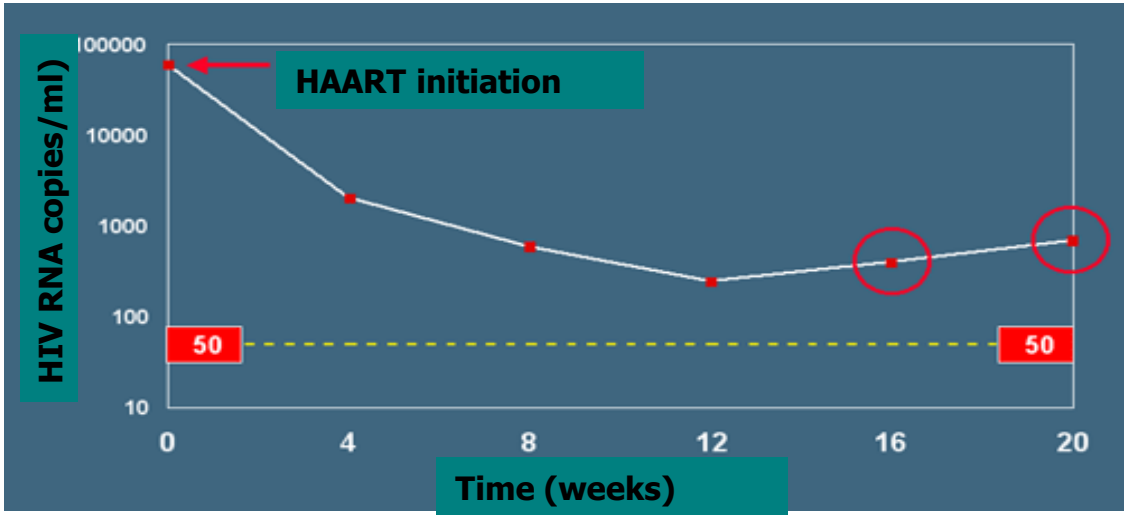
Decline of plasma viral load and percentage of infected PBMC following HAART

Kinetics of CD4 change



Effect of antiretroviral therapy on viral load





Diagnosis

DIRECT DIAGNOSIS: more specific, sometimes time-consuming
INDIRECT DIAGNOSIS: less expensive, rapid, fully automatic preocedure

The choice depends upon: Diagnosis or monitoring

DISEASE STAGE

The virus or the antibodies must be present in the sample

DIAGNOSTIC VALUE (Ab titer)

The Ab positivity must be significant in clinical terms

TYPE OF TEST

Screening, suspected case or confirmed case?