VIROLOGY

Laboratory diagnosis of viral infections

Milestones of diagnostic virology

1929 Bedson S, Bland J. Complement-fixation with filterable virus and their antisera. *Br. J. Exp. Pathol.* → **Serology**

1948 Weller RH, Enders JF. Production of hemagglutinin by mumps and influenza A viruses in suspended cell tissue cultures. *Proc Soc Exp Biol Med* → **Virus Cultivation**

1956 Liu C. Rapid diagnosis of human influenza. *Proc Soc Exp Biol Med*

1975 Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* → **Mabs as diagnostic reagents**

1980 Gardner PS, McQuillin J.. *Rapid virus diagnosis:* application of immunofluorescence. London: Butterworths, 1980

1985 Saiki RK, Scharf S, Faloona F, et al. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis of sickle cell anemia. *Science* → **PCR**

Diagnostic Strategies in Virology :

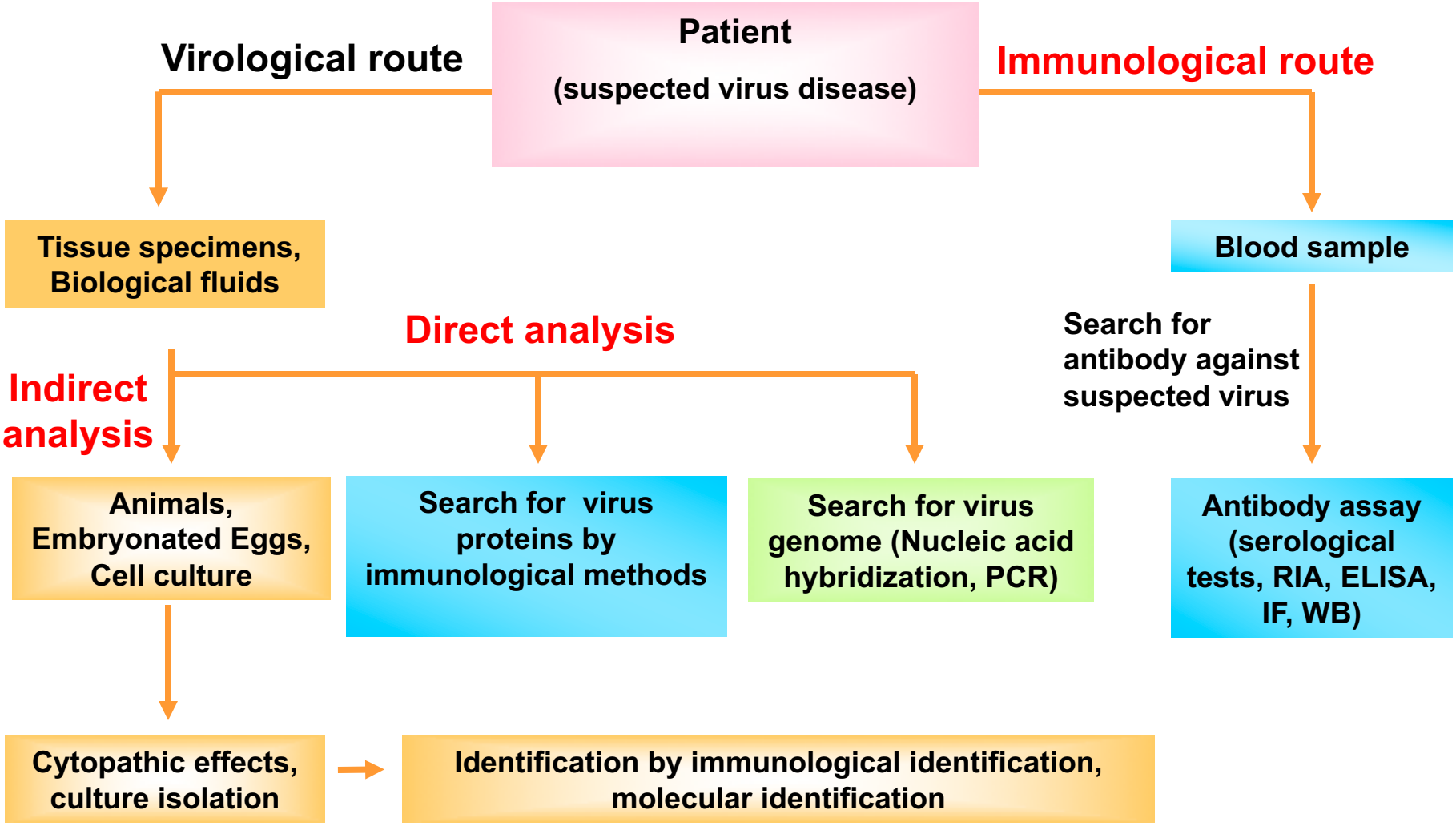
State of the art

1. DIRECT ANALYSIS

2. INDIRECT ANALYSIS

3. SEROLOGY

Diagnostic stategies for virus infections



Diagnostic Strategies in Virology :

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Diagnostic Strategies in Virology:
DIRECT ANALYSIS

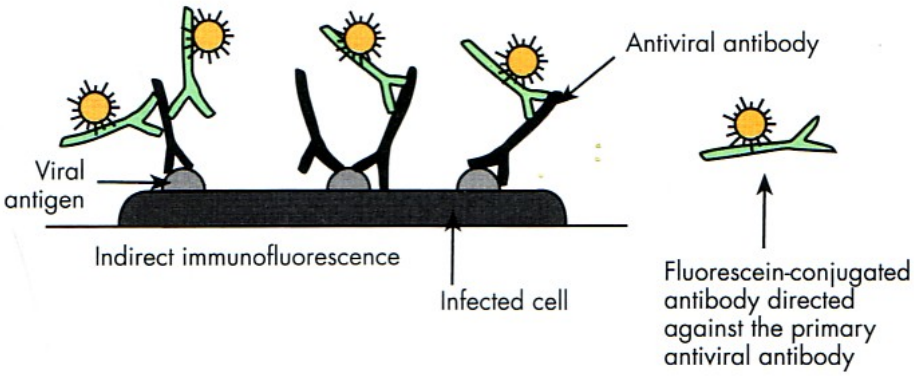
1. **VIRAL ANTIGENS DETECTION**
   Immunofluorescence, …

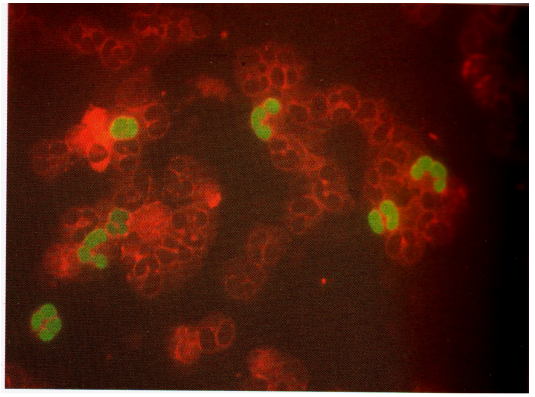
2. **ELECTRON MICROSCOPY**
   Morphology and titer of viral particles

3. **LIGHT MICROSCOPY**
   Histology, inclusion bodies

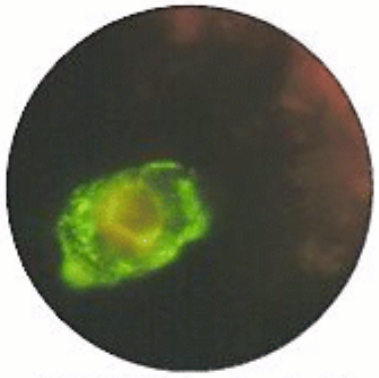
4. **VIRAL NUCLEIC ACID DETECTION**
   Hybridization with specific probes, PCR

DIRECT ANALYSIS :
VIRAL ANTIGENS DETECTION: IF





HCMV antigenemia in PBMCs

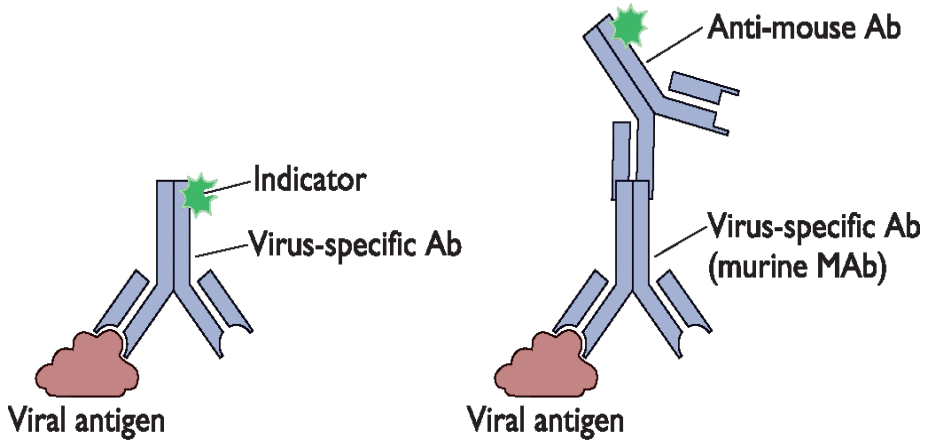


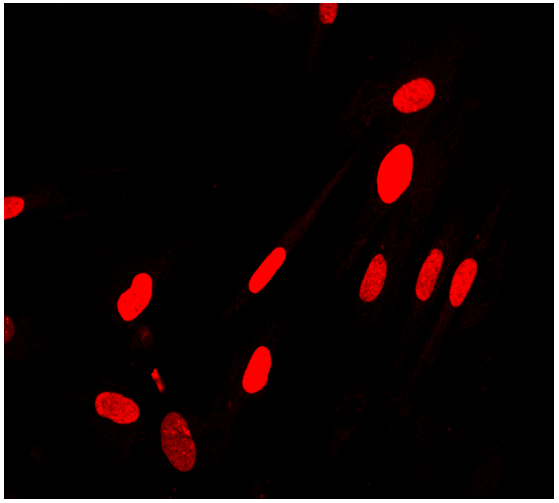
HSV: epithelial cell from a cutaneous lesion

PROs	CONs
Fast (3-4 hrs)	Required trained personnel
Sensitive	Sample dependent

DIRECT ANALYSIS:
VIRAL ANTIGENS DETECTION: IF

- Detection of viral antigens in infected tissues
- Requires diagnostic antibodies

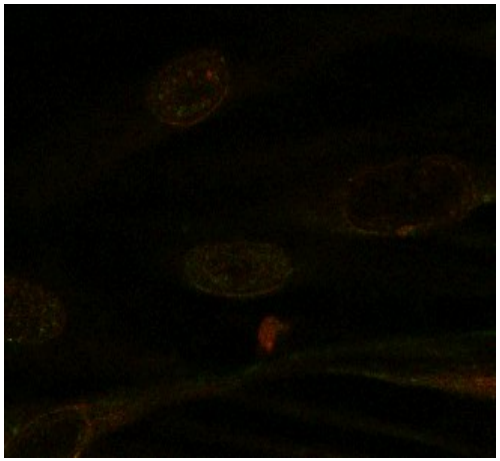


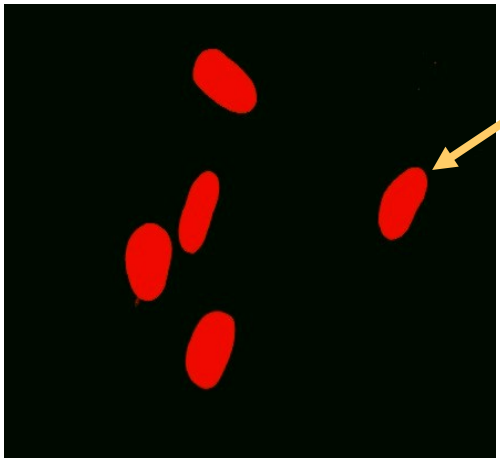


IF staining of HCMV IE proteins in infected HELF cells

DIRECT ANALYSIS :
VIRAL ANTIGENS DETECTION: IF

mock

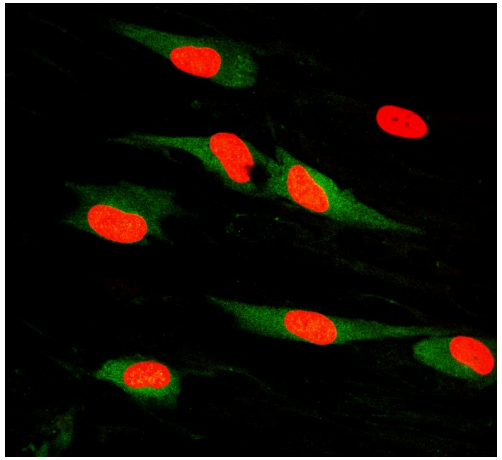


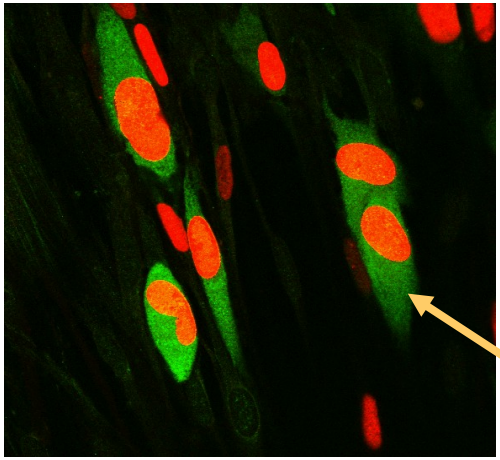


IE1/2

24hpi

48 h.p.i.





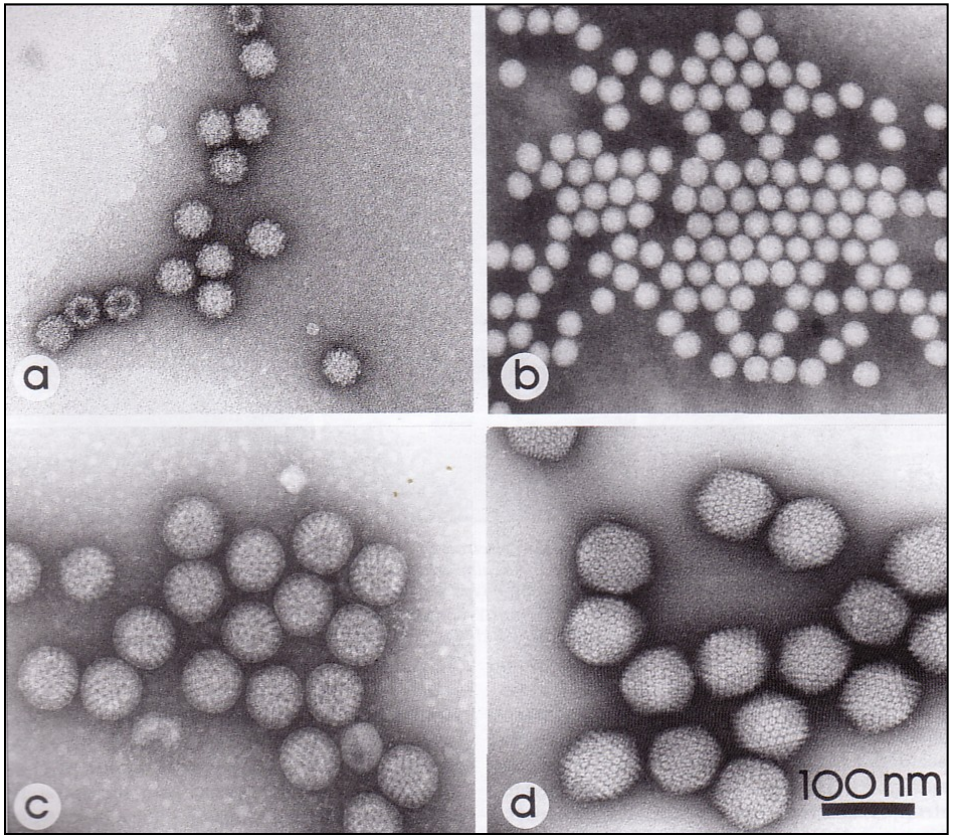
72hpi

UL72

Caposio et al., Virology 2004

DIRECT ANALYSIS :
ELECTRON MICROSCOPY

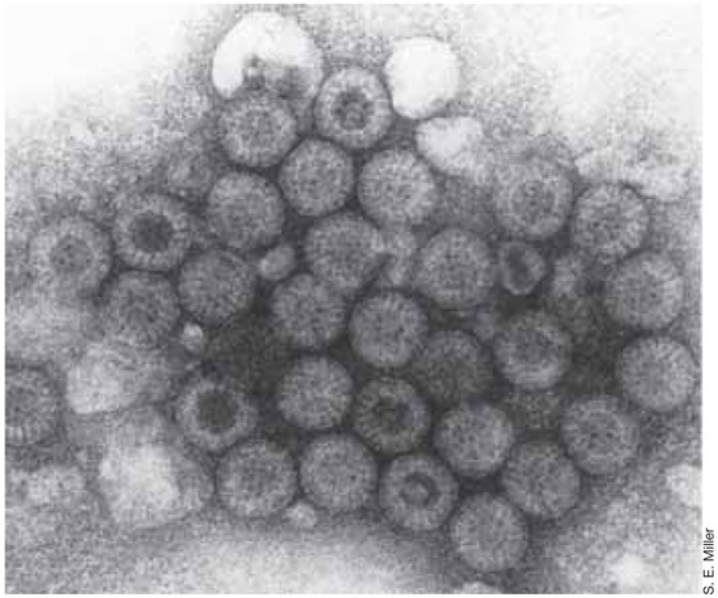
EM identifies viral particles in samples on the basis of their morphology



Four gastrointestinal viruses in feces samples:

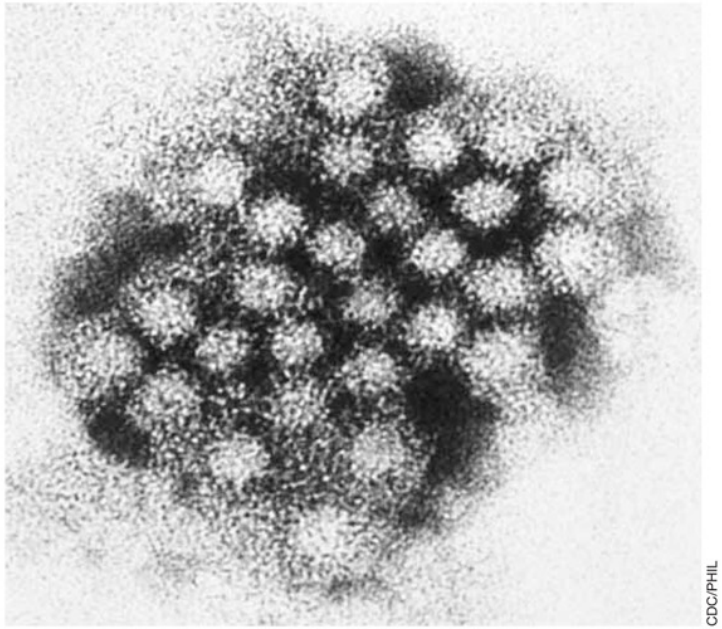
a) CALICIVIRUS
b) POLIOVIRUS
c) ROTAVIRUS
d) ADENOVIRUS

Magnification: x 150.000



(a)

ROTAVIRUS



(b)

NORWALK

DIRECT ANALYSIS :
ELECTRON MICROSCOPY

FECES	Rotavirus, Adenovirus Norwalk like viruses Astrovirus, Calicivirus
FLUIDS FROM BLISTERS	HSV VZV
CUTANEOUS WARTS	Papillomavirus, Pox (molluscum contagiosum)

DIRECT ANALYSIS :
ELECTRON MICROSCOPY

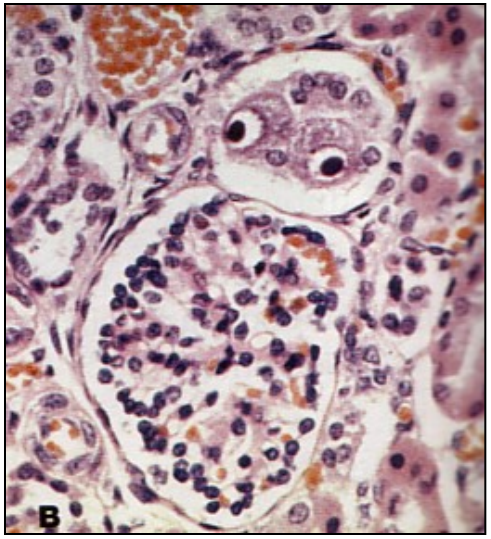
PROs	CONs
- A “catch all” technique	- Low sensitivity (10^5-10^6 virus/ml)
- Useful technique for the search of uncultivable virus	- High costs of acquisition and maintenance
- Independent from virus-specific reagents	- Required trained personnel

DIRECT ANALYSIS :
LIGHT MICROSCOPY

Search for **inclusion bodies** (virus materials) in histological samples.

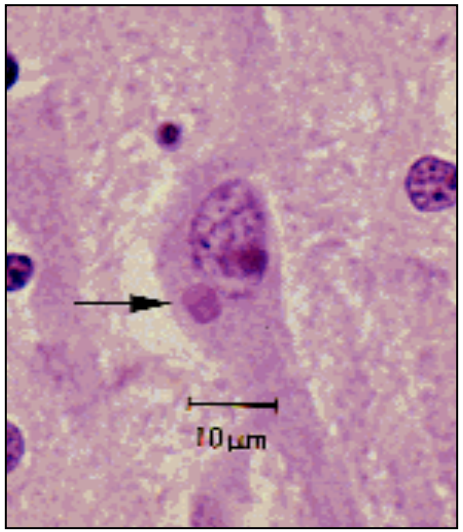
It is a low-specificity and low-sensitivity technique. However, it can be useful for diagnosis of few selected viral infections.

HCMV



Owl eyes (nuclear)

Rabies virus

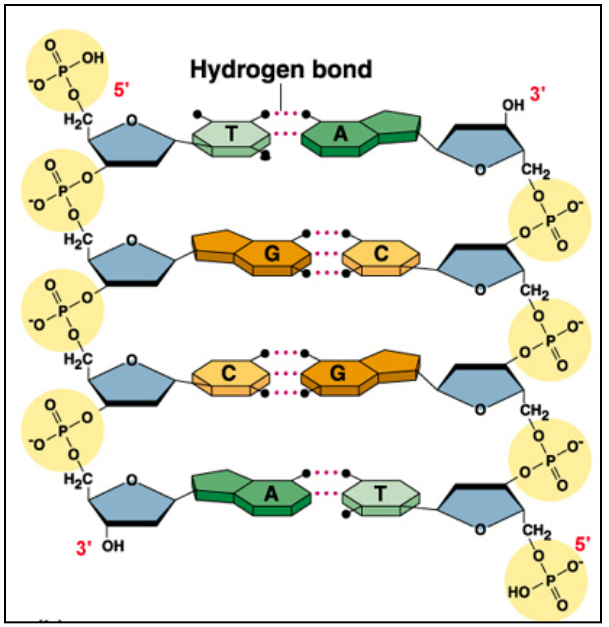


Negri's bodies (cytoplasmatic)

DIRECT ANALYSIS:
VIRAL NUCLEIC ACID DETECTION

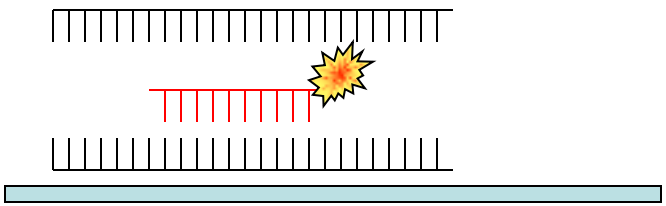
HYBRIDIZATION WITH SPECIFIC PROBES

NUCLEIC ACID AMPLIFICATION

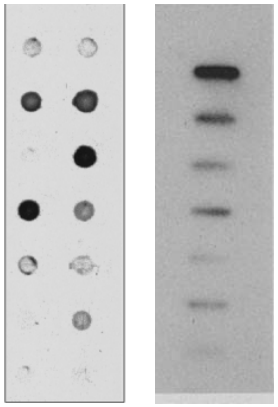


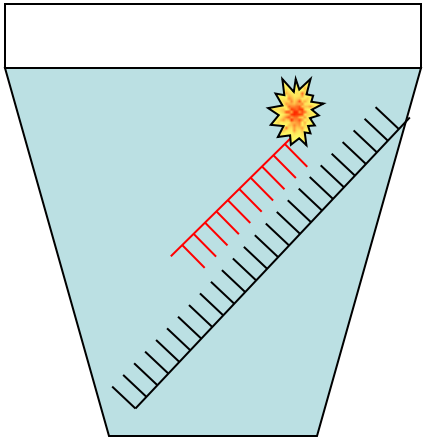
VIRAL NUCLEIC ACID DETECTION

HYBRIDIZATION WITH SPECIFIC PROBES



Solid phase hybridization (eg. dot blot, slot blot)



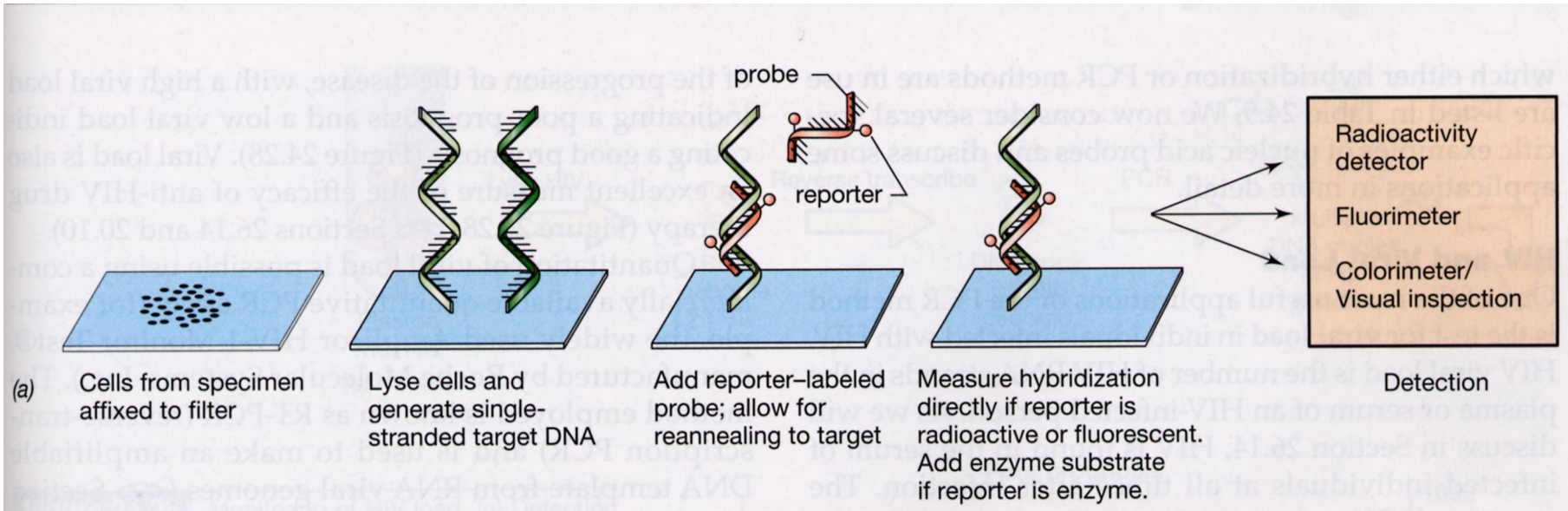


Liquid hybridization
(es. HPV hybrid capture system)

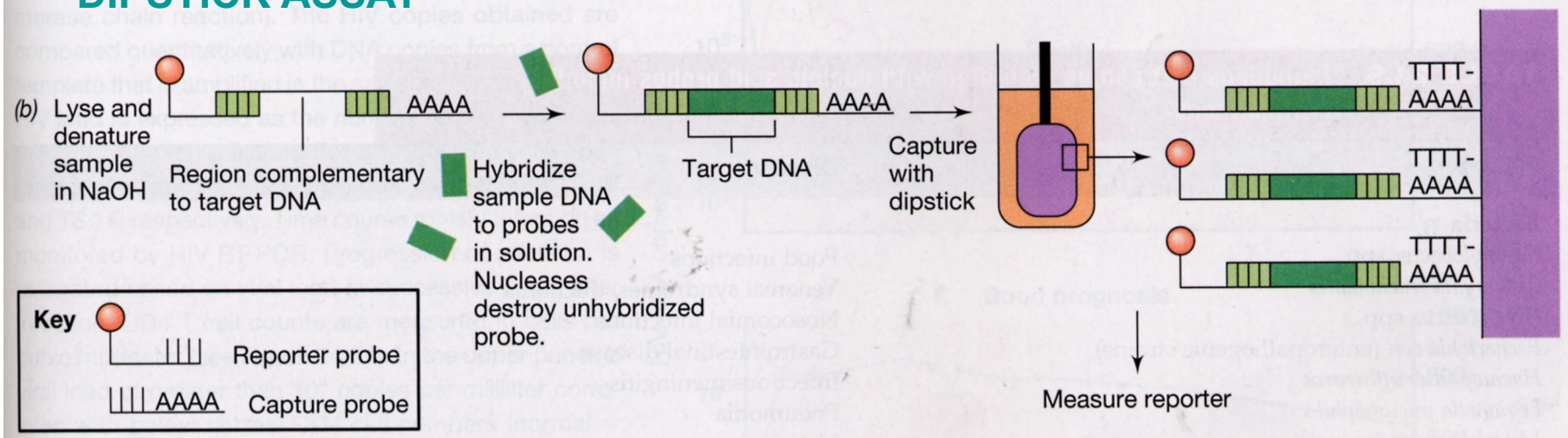
- **sensitivity**:
  - **10^4 DNA copies/μl** w/o target amplification
  - **500 DNA copies/μl** with target amplification
- **specificity** dependent from **stringecy** conditions (T° , Salts, pH)

VIRAL NUCLEIC ACID DETECTION

HYBRIDIZATION WITH SPECIFIC PROBES

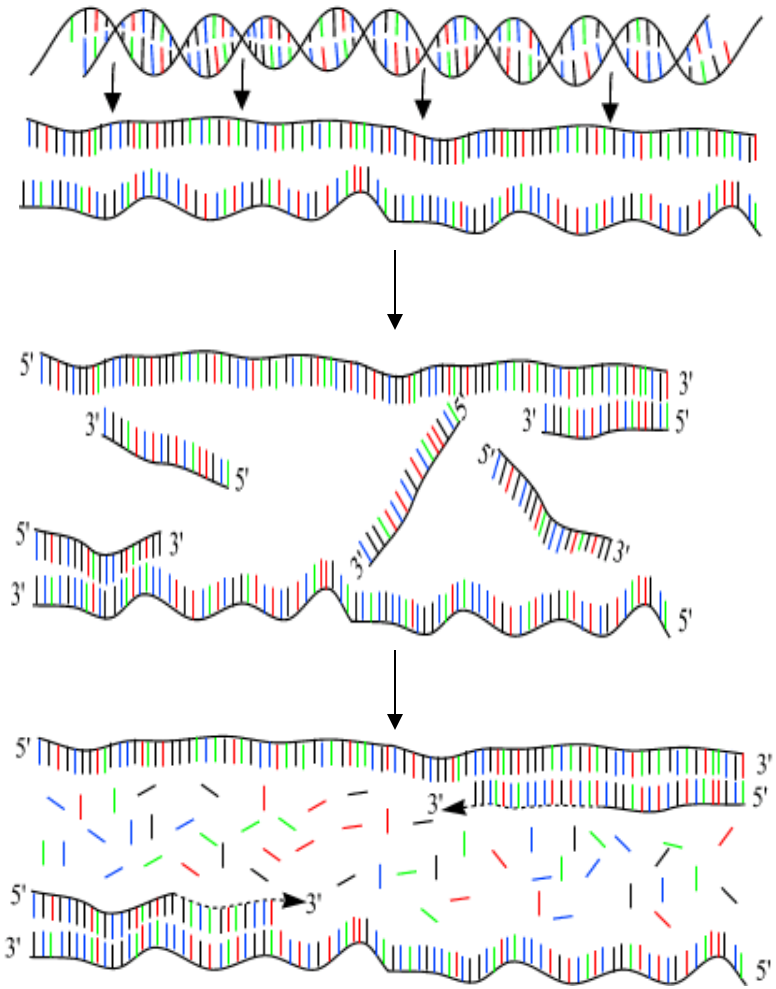


DIPSTICK ASSAY



VIRAL NUCLEIC ACID DETECTON

TARGET AMPLIFICATION: PCR



I Phase: denaturation

1 min 94° C

II Phase: annealing

45 sec 54° C

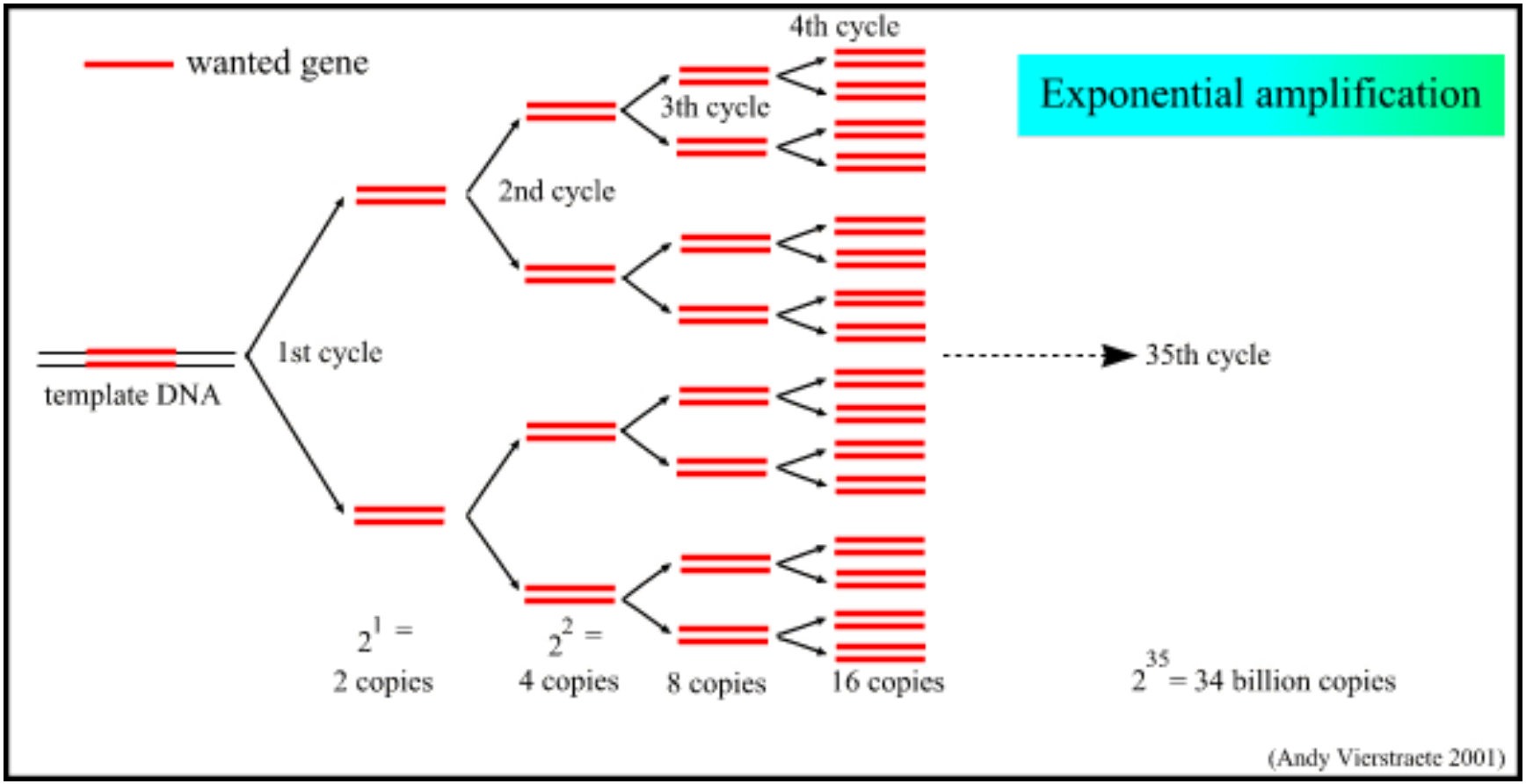
III Phase: extension

1 min 72° C

1 CYCLE

VIRAL NUCLEIC ACID DETECTON

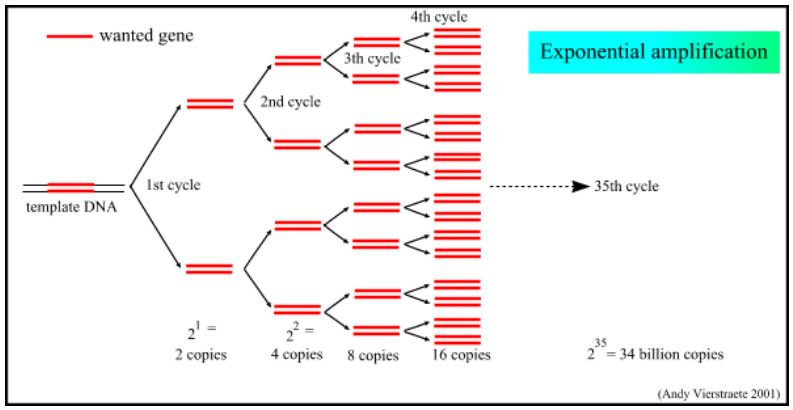
TARGET AMPLIFICATION: PCR

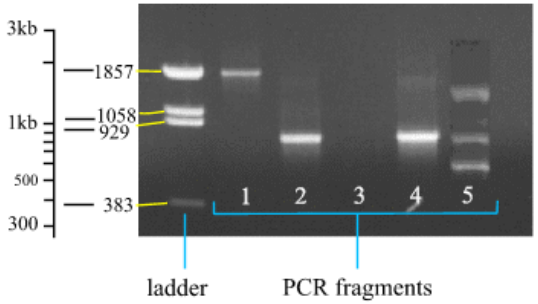


VIRAL NUCLEIC ACID DETECTON

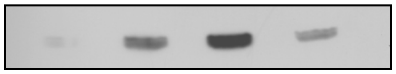
CONVENTIONAL PCR:
VISUALIZATION AND IDENTIFICATION OF PRODUCTS

PCR

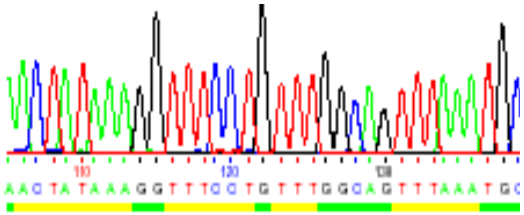




Agarose electrophoresis



Hybridzation with specific probes



Sequencing

VIRAL NUCLEIC ACID DETECTON

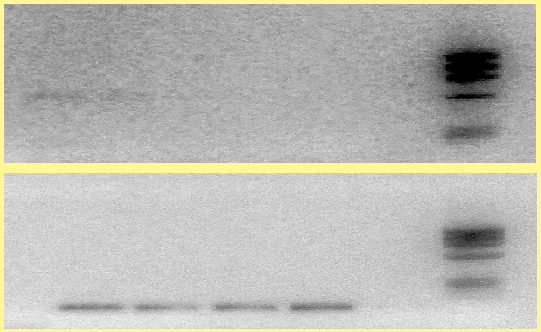
CONVENTIONAL PCR

PROs	CONs
- Extremely sensitive	- Susceptible to contaminations (false positives)
- Easy to perform	- Required trained personnel
- Fast	- Qualitative

VIRAL NUCLEIC ACID DETECTON

PCR: Evolution of methods

NESTED PCR

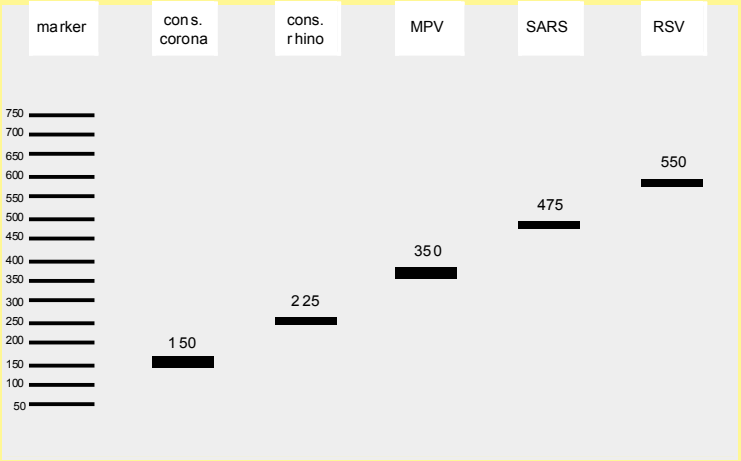


1° primers set

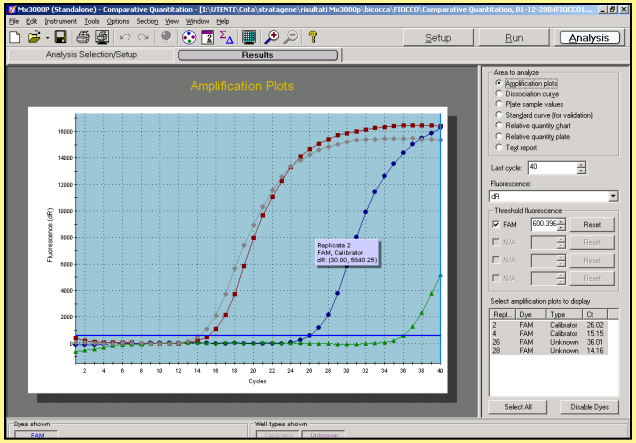
2° primers set

(inside the 1° amplified)

MULTIPLEX PCR



REAL-TIME PCR



- **LCR**
- **NASBA**
- **…**

VIRAL NUCLEIC ACID DETECTON

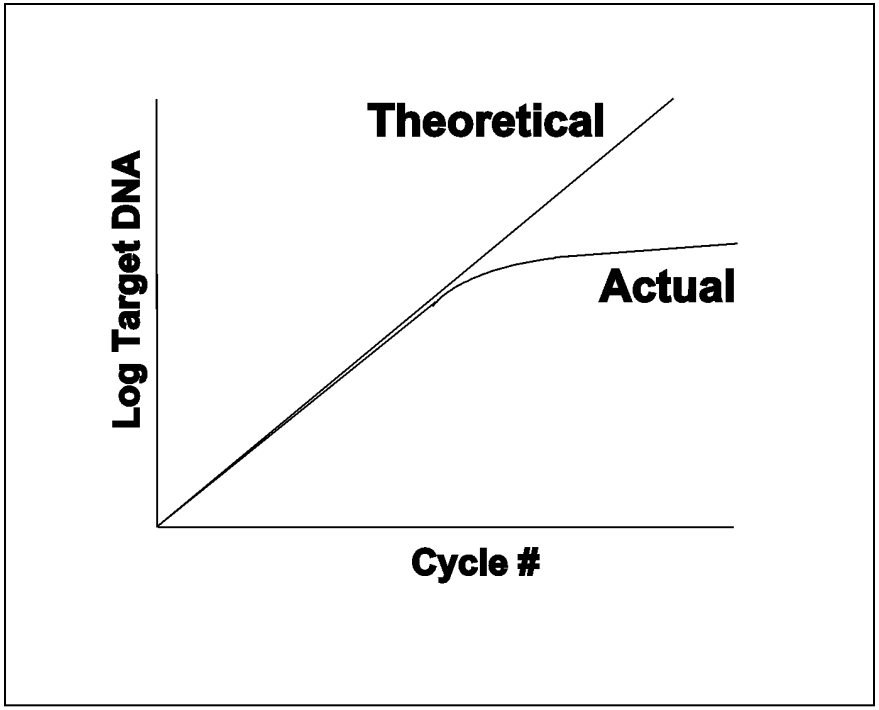
QUANTITATIVE REAL-TIME PCR (qPCR)

- **Definition**:

- Accumulation of a specific DNA (or RNA) is monitored during qPCR process by adding fluorescent probes to the PCR rxn mix.
- Probe fluorescence increase upon binding to DNA.
- As the target DNA is amplified, the level of fluorescence increase proportionally.
- Thus by monitoring the rate of fluorescence increase in the PCR rxn, it is possible to accurately determine the amount of target DNA present in the original sample.
- qPCR can be used to assess the abundance of a virus in a sample by quantiying a gene characteristic for that particular virus.

VIRAL NUCLEIC ACID DETECTON

QUANTITATIVE REAL-TIME PCR (qPCR)



- **Theoretically,** a direct relationship exists between the final amount of the PCR product and the amount of initial target; the amount of product doubles at each cycle of the PCR rxn.

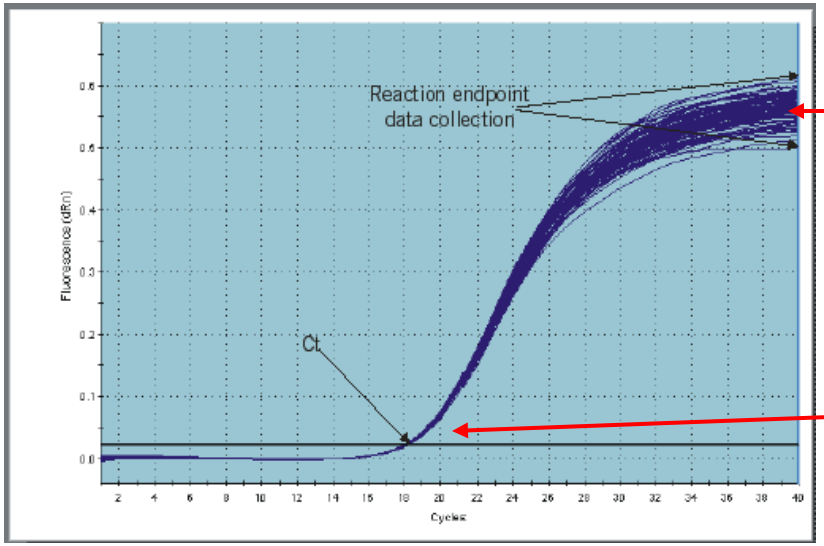
- **In pratice,** with the progression of PCR cycles, some components of the mixture are exhausted and inhibitors of the PCR itself appear, so that the reaction reaches a plateau.

VIRAL NULEIC ACID DETECTION

Differences between conventional PCR and qPCR

- **Conventional PCR:** analysis of the amplified products occurs at the plateau phase end-point)

- **Quantitative Real time-PCR:** analysis of the amplified products occurs during the exponential phase of rxn

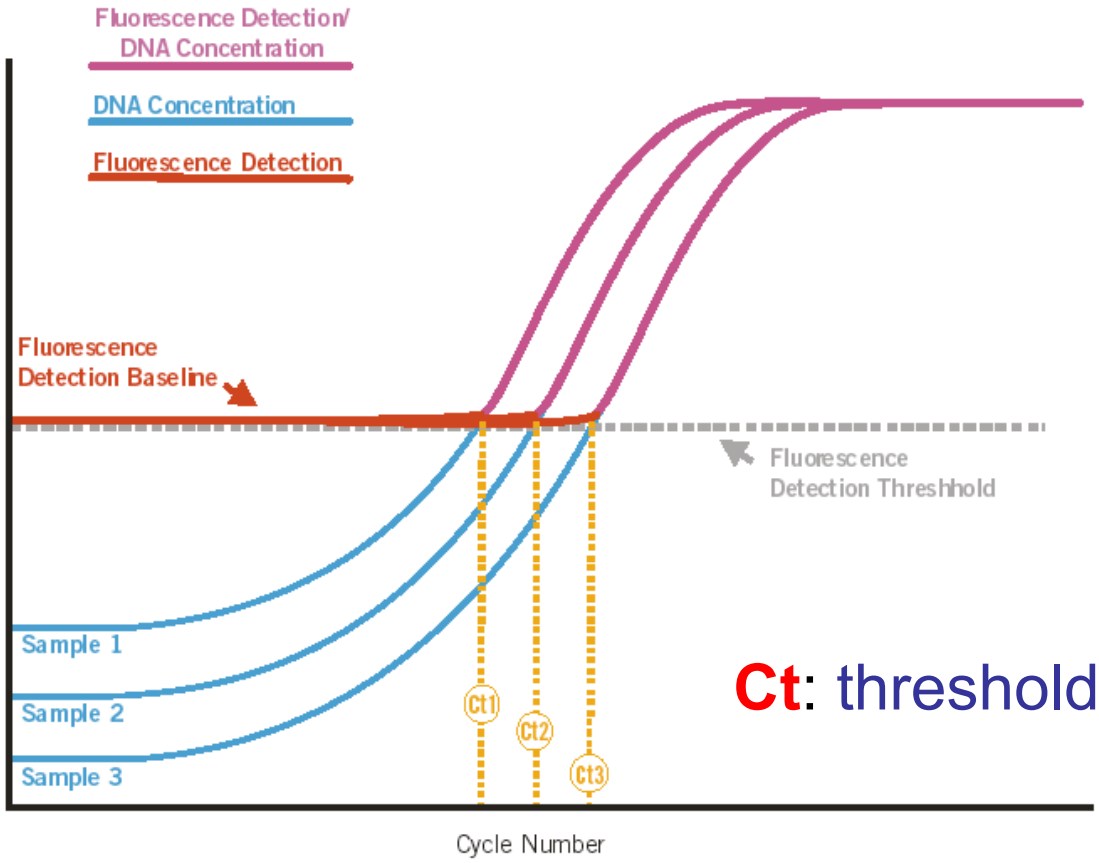


End-point

Start of exponential phase

VIRAL NULEIC ACID DETECTION

Principle of quantitative real-time PCR



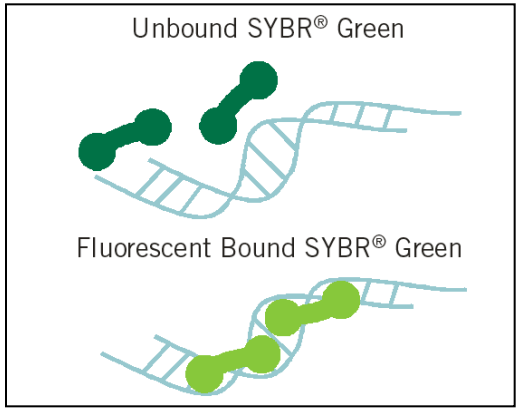
Ct: threshold cycle

The **Ct** value is inversely proportional to the number of initial copies of target viral nucleic acid

VIRAL NULEIC ACID DETECTION

qPCR fluorescent probes

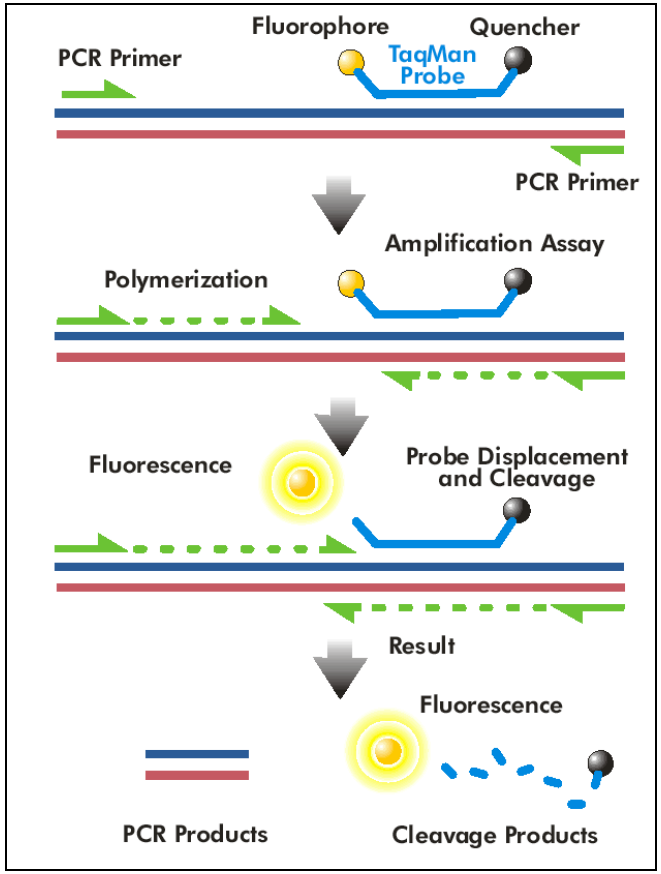
SYBR Green



SYBR Green binds nonspecifically to dsDNA, but does not bind to ssDNA or RNA. SYBR Green added to PCR mix becomes fluorescent only when bound, thus indicating that dsDNA is present, in this case due to the amplification process.

Gene-specific fluorescent probes are made by attaching a fluorescent dye to a short DNA probe that matches the target sequence being amplified. The dye fluoresces only when dsDNA of the correct sequence accumulates.

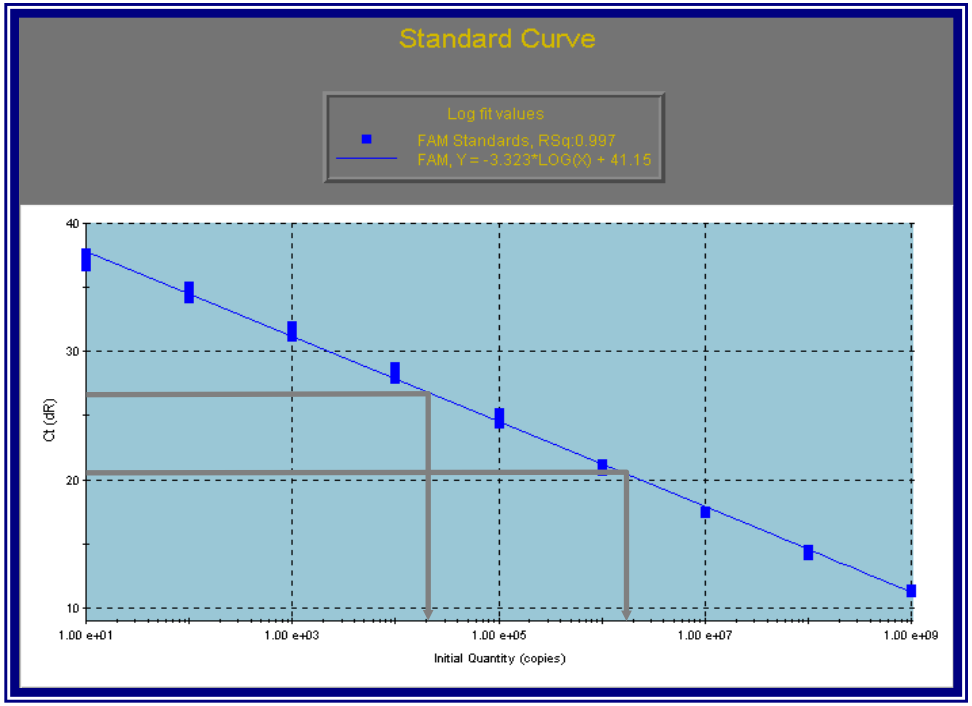
Gene-specific probes (TaqMan)



VIRAL NULEIC ACID DETECTION

qPCR: absolute quantification

- Based on a series of samples with known amounts of target DNA (standard curve)
- The amplification efficiency of “unknowns” and “standards” should be always identical
- The standard curve for a specific target should be included in each qPCR determination



VIRAL NULEIC ACID DETECTION

qPCR: an example of absolute quantification

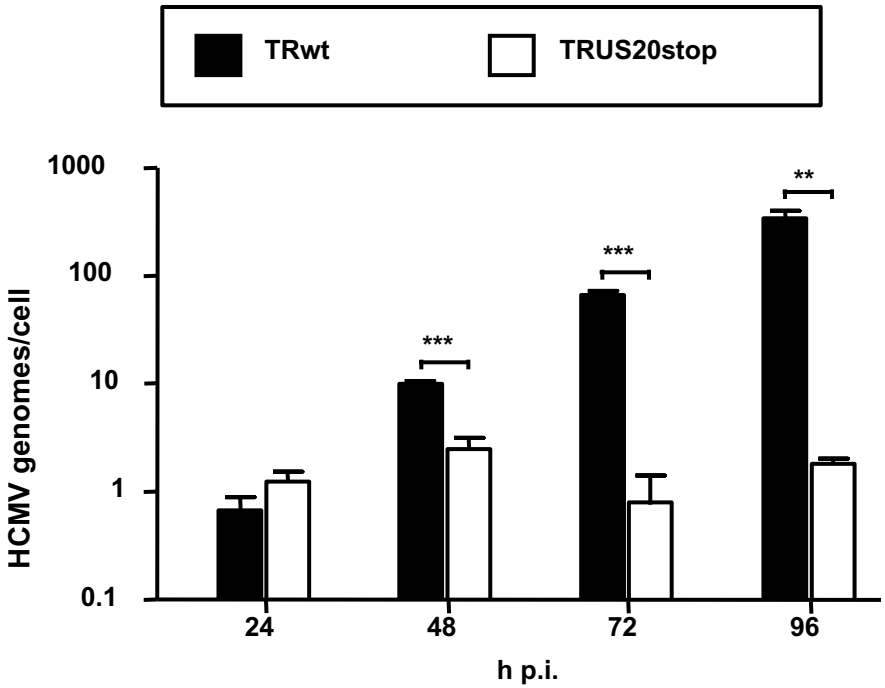


Figure 6. Lack of viral DNA synthesis in endothelial cells infected with a US20-mutant virus. HMVECs were infected with TRwt or TRUS20stop (MOI 1 pfu/cell) and at the indicated times p.i. total genomic DNA was isolated to quantify viral DNA levels by qPCR. The data shown are the mean values of two independent experiments ± SD. **, $p<0.001$, ***, $p<0.0001$ compared to the amount of viral DNA measured in cells infected with TRwt.

Cavaletto N., Luganini A., and Gribaudo G. J. Virol. 89, 2015.

VIRAL NULEIC ACID DETECTION

qPCR: PROs

- Extremely sensitive
- More reliable results
  (qPCR can be monitored continuosly)
- Precise quantification of target sequences
- No post-PCR visualization or identification methods to confirm amplification →
  - faster results
  - less chance of cross-contamination

Diagnostic Strategies in Virology :

State of the art

1. DIRECT ANALYSIS

2. INDIRECT ANALYSIS

3. SEROLOGY

Diagnostic Strategies in Virology:
INDIRECT ANALYSIS

1. **CELL CULTURES**
   CPE, hemadsorption, immunofluorescence

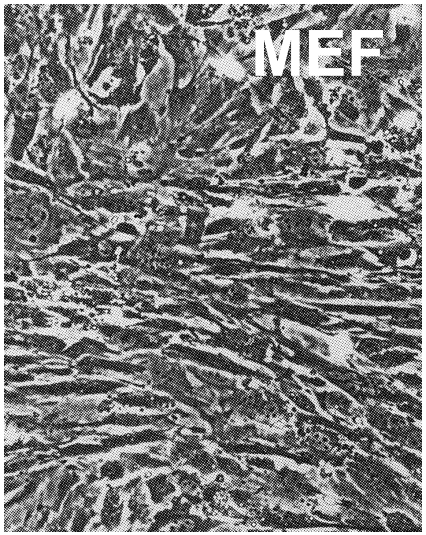
2. **EMBRYONATED EGGS**
   CAM lesions, hemoagglutination,…

3. **LAB ANIMALS**
   Disease, death

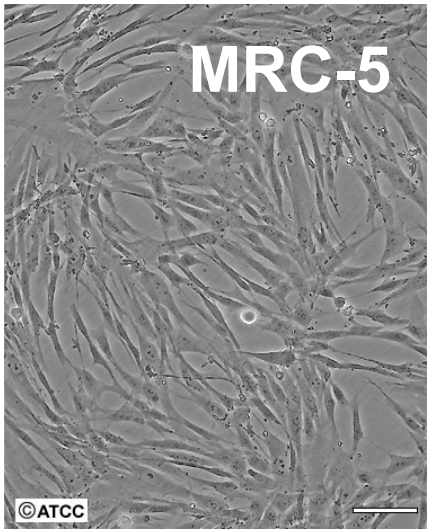
Diagnostic methods in Virology:

CELL CULTURES

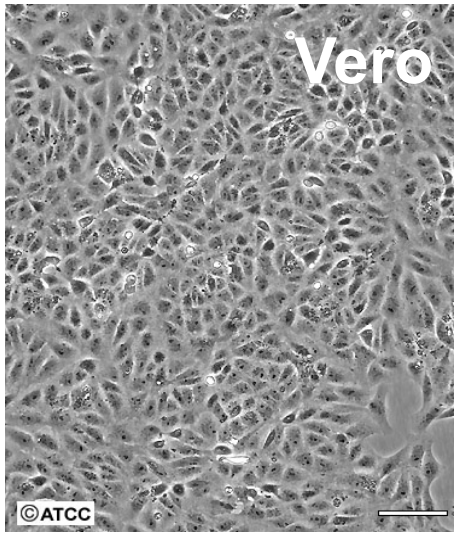
The most widely used method for virus isolation and growth



Primary cell cultures



Diploid cell strains



Continuos cell lines

Diagnostic methods in Virology:

CELL CULTURES

Effects of productive viral replication in cell culture:

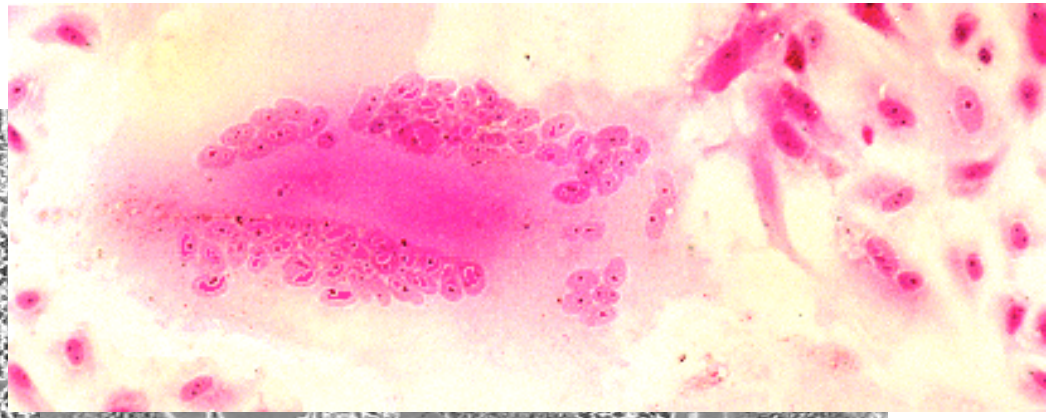
- **Cytopathic effect(s) (CPE)**
- **Syncitia (cell fusion)**
- **Hemadsorption**

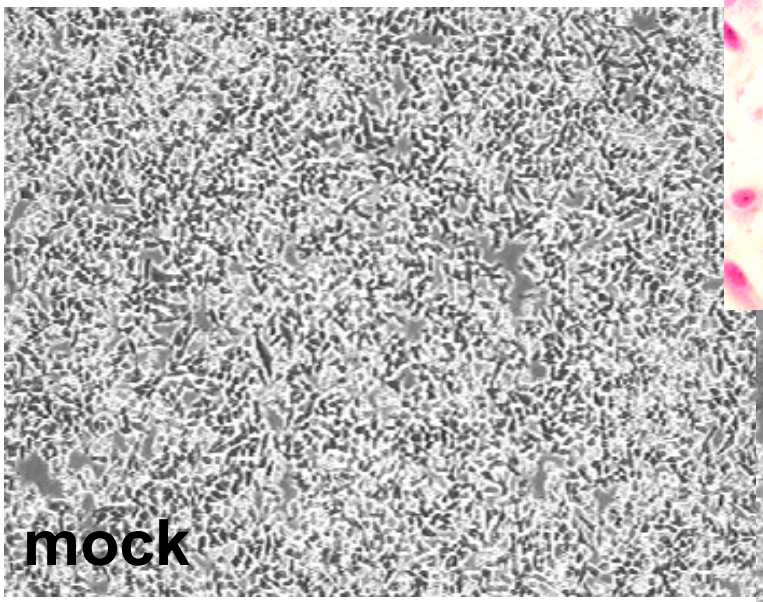
Identification of a specific virus grown in infected cell cultures can be performed by neutralization of infectivity, hemadsorption inhibition, and immunofluorescence

Diagnostic methods in Virology:

CELL CULTURES

Syncitia formation: measle virus (giant multinuclear cells)



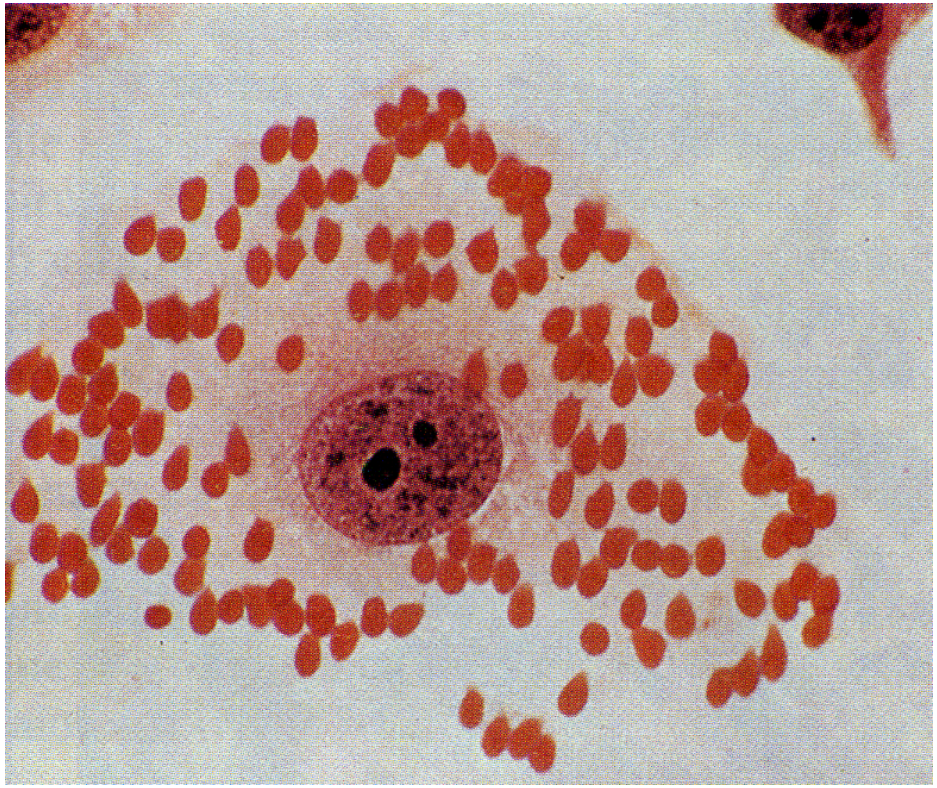


Diagnostic methods in Virology:

CELL CULTURES

Hemoadsorption: mumps virus

(red blood cells specifically adsorpt to mumps virus-infected cells)



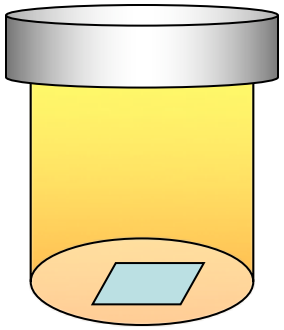
Diagnostic methods in Virology:

CELL CULTURES: TROUBLES

- They may require long incubation times (eg. HCMV)
- Low sensitivity. Sample dependent.
- Susceptible to bacteria and fungi contamination
- Susceptible to toxic compounds in samples
- Some human viruses cannot replicate in cell culture:
    - Gastrointestinal viruses
    - HBV
    - Parvovirus
    - HPV

Diagnostic methods in Virology:

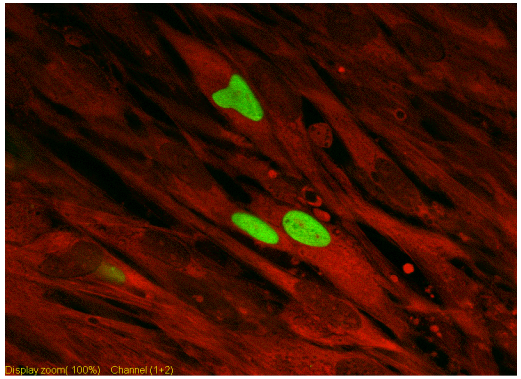
CELL CULTURES: A rapid method



1. Cells seeding on a coverslip contained in a shell vial

2. Sample inoculum and incubation

3. Immunofluorescence for viral antigens detection



Eg. HCMV viremia (24 h p.i.)

Diagnostic Strategies in Virology :

State of the art

1. DIRECT ANALYSIS

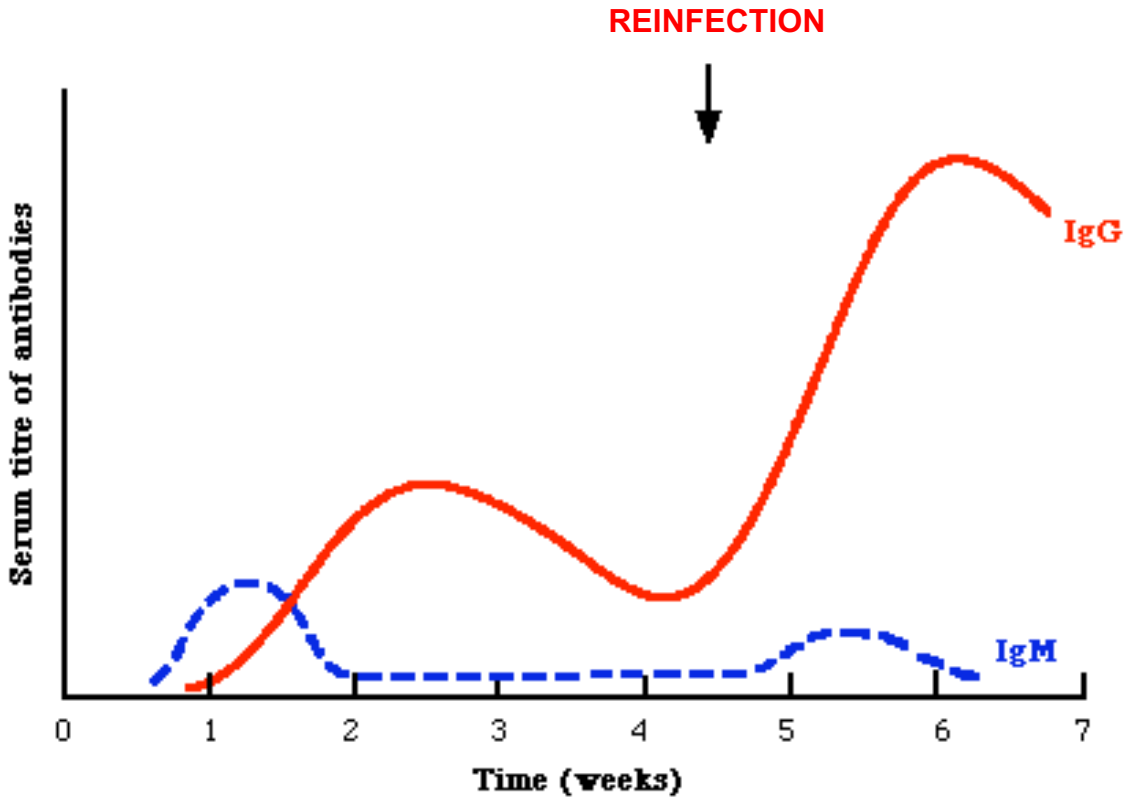
2. INDIRECT ANALYSIS

3. SEROLOGY

Diagnostic methods in Virology:

SEROLOGY

Typical serological profile resulting from an acute infection



Diagnostic methods in Virology:

SEROLOGY

CRITERIA FOR PRIMARY INFECTION DIAGNOSIS

- **At least a 4-fold incresase of IgG titer in acute serum compared to convalescent serum**
- **IgM detection**
- **Seroconversion**

CRITERIA FOR DIAGNOSIS OF REINFECTION/REACTIVATION

- **A strong increase of IgG titer**
- **Absence or low IgM titer**

However, it is often difficult to discriminate primary infections from reinfections /reactivations

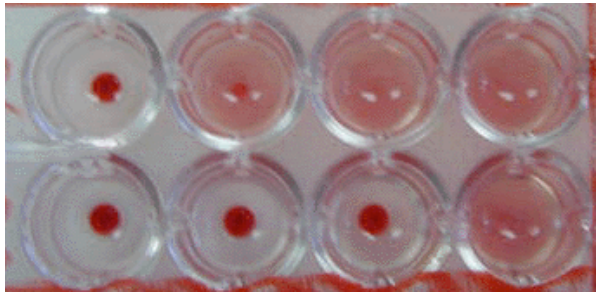
In some cases is crucial: eg. Rubella in pregnancy

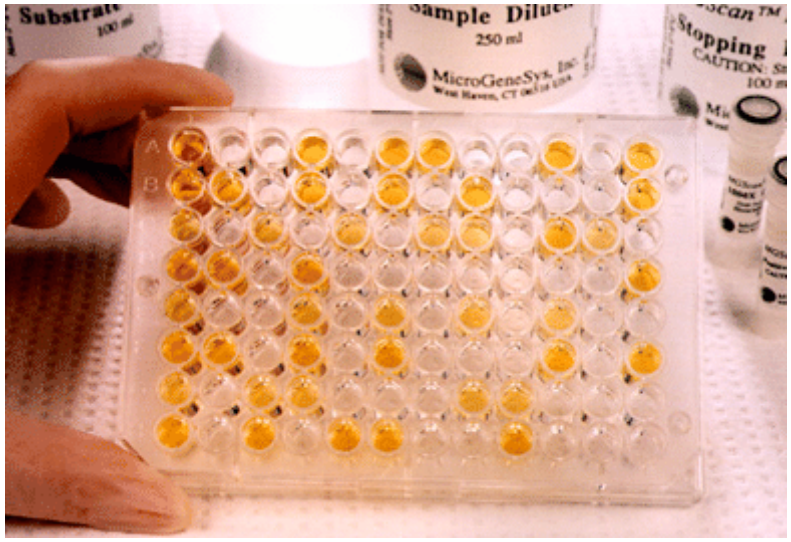
Diagnostic methods in Virology:

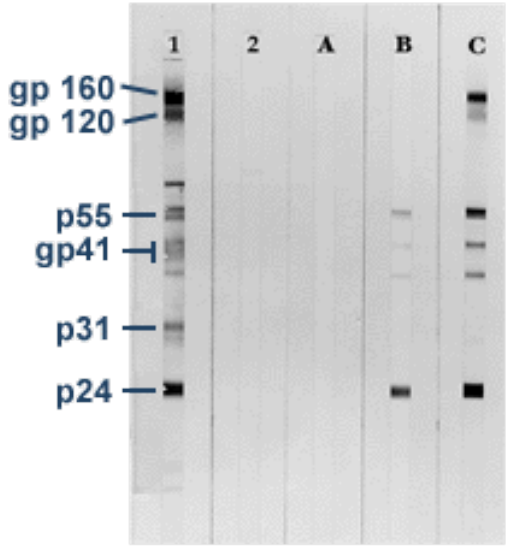
SEROLOGY

TECHNIQUES

- **Enzyme immunoassays (ELISA)**
- **Radioimmunoassays (RIA)**
- **Complement fixation**
- **Western Blot**
- …

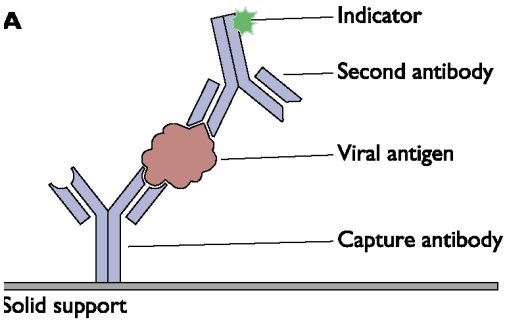


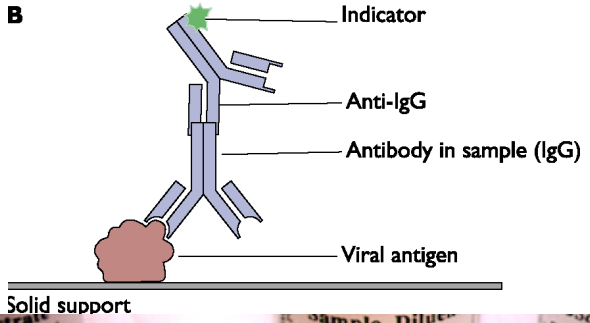


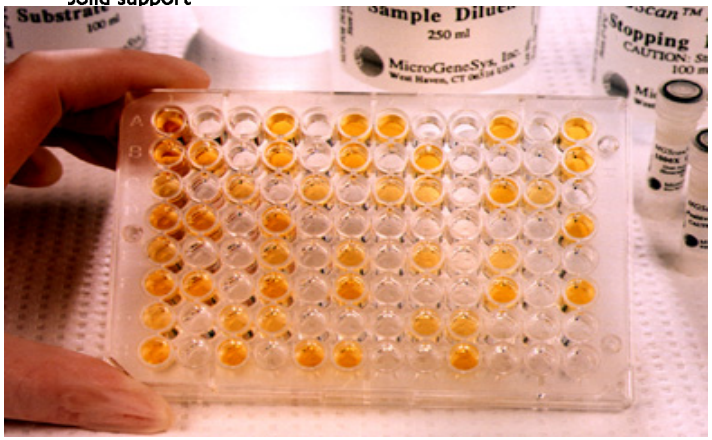


Diagnostic methods in Virology:

SEROLOGY: ELISA



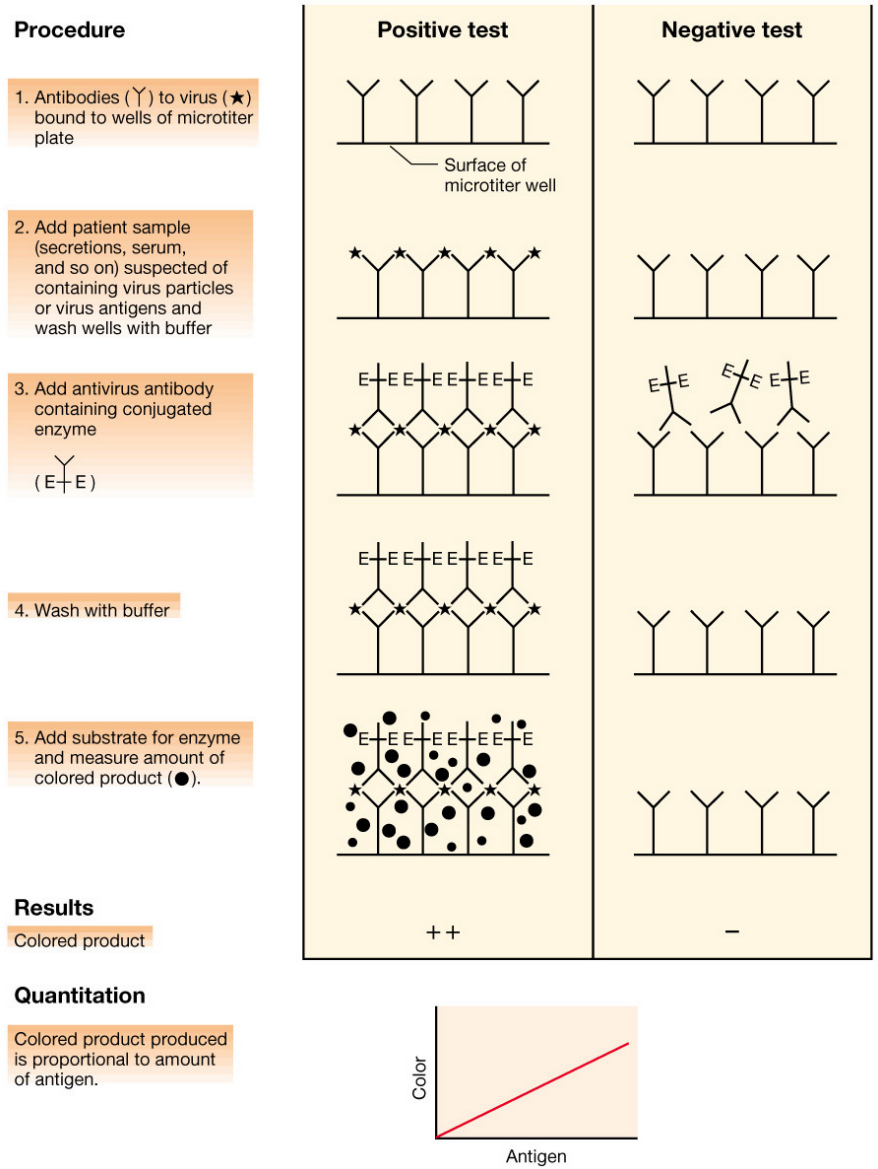




- A very sensitive immunological assays widely used in diagnostic virology. An ELISA can detect as little as 0.01 nanograms of antigen or antibody.
- Speed (typically few hours)
- Low cost
- Lack of hazardous wastes
- Long shelf life
- High specificity
- High sensitivity
- These features make ELISA tests particularly useful as immunodiagnostic tools.

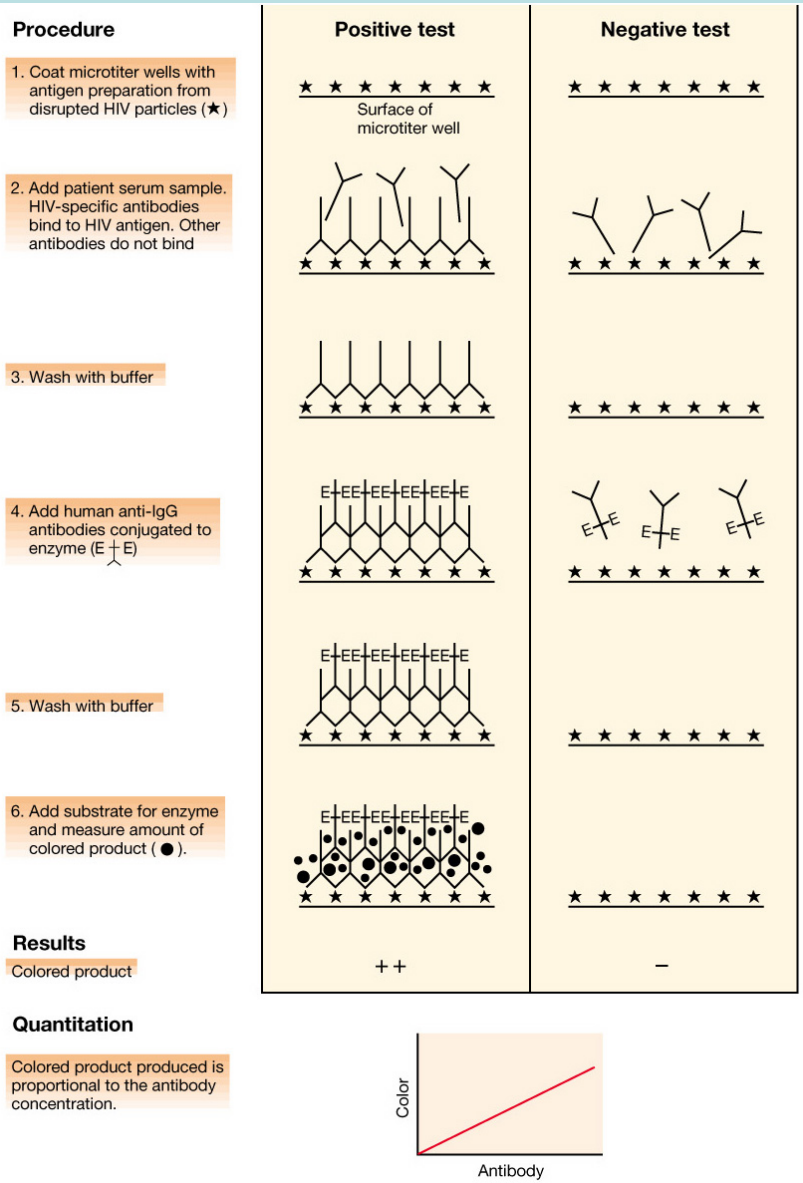
Diagnostic methods in Virology:

SEROLOGY: A direct ELISA to detect viral antigens



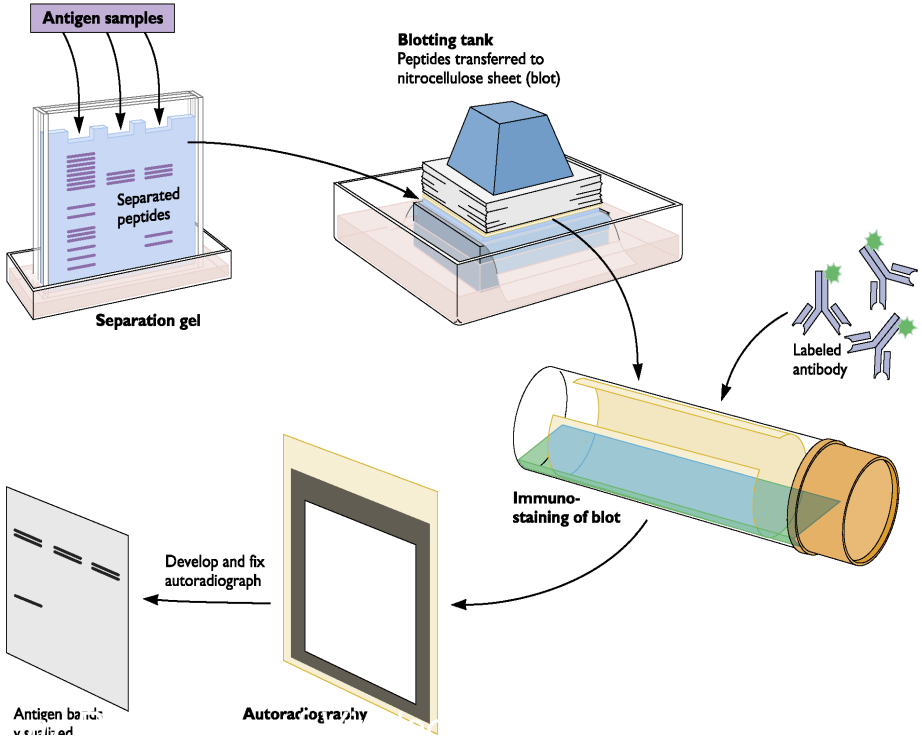
Diagnostic methods in Virology:

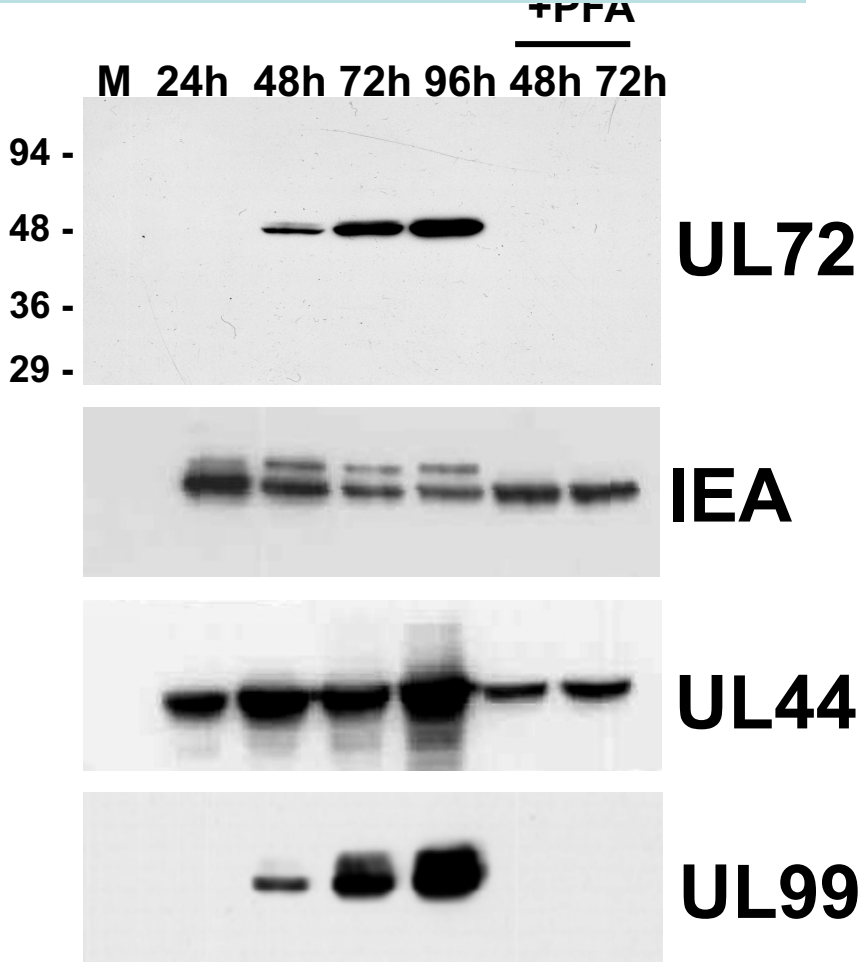
SEROLOGY: An indirect ELISA to detect antibodies



Diagnostic methods in Virology:

SEROLOGY: WESTERN BLOT

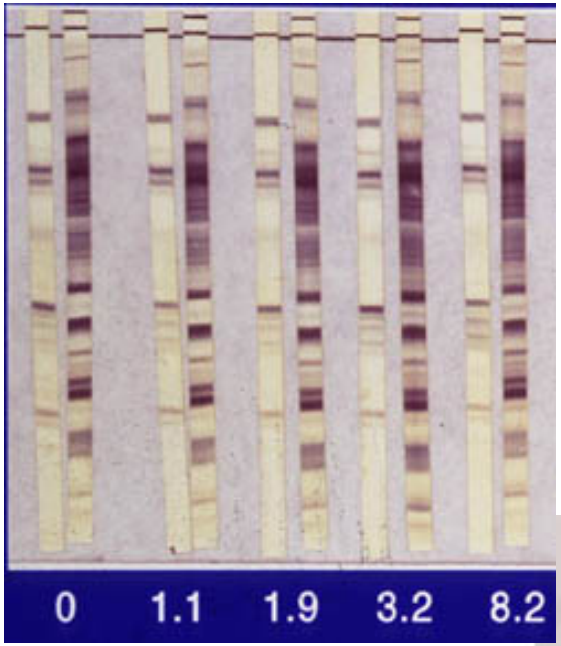


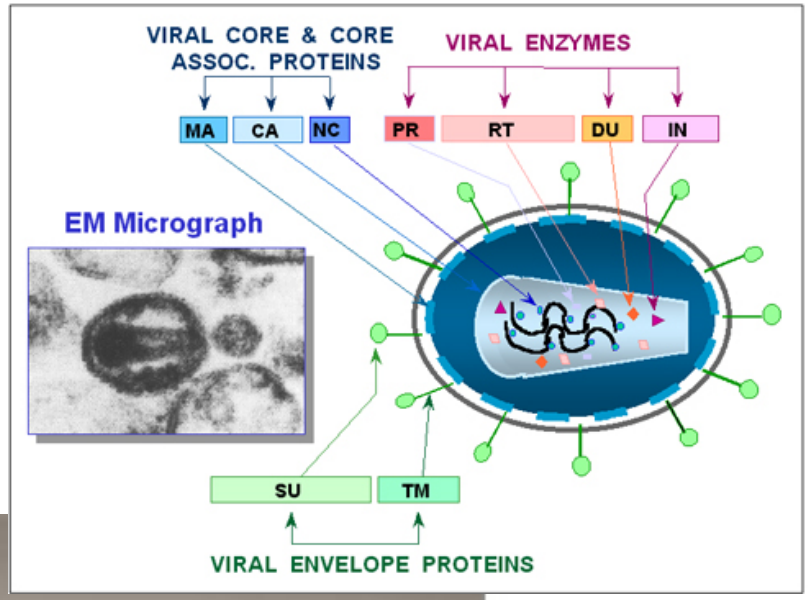


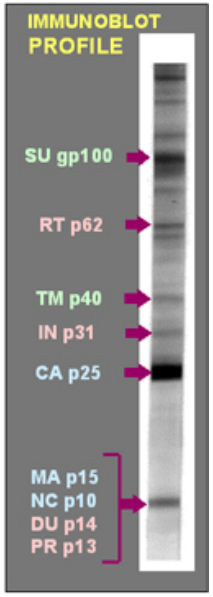
Caposio et al., Virology, 2004

Diagnostic methods in Virology:

SEROLOGY: WESTERN BLOT







Diagnostic methods in Virology:

SEROLOGY

USELFULNESS OF SEROLOGICAL RESULTS

- Useful when the onset of symptoms onset correlates with the appearance of specific antibodies
  - HAV
  - Rubella

- Useful for virus infections in which symptoms arise months or years after infection
  - HIV
  - Rabies

In these infections, detection of specific antibodies is sufficient for conclusive diagnosis

- Only retrospective value in case of viruses that produce clinical symptoms before the appearance of specific antibodies

- Respiratory viruses
- Gastroenteritis viruses

- Useful to establish the virological state of donors and recipients in transplants
  - HCMV

Diagnostic methods in Virology:

SEROLOGY: TROUBLES

- ✓ Long time to obtain acute and convalescent sera
- ✓ Local infections (eg. HSV-2) may not induce a significant antibody response
- ✓ Cross-reactivity between related viruses (eg. HSV / VZV, Japanese B encephalitis / dengue)
- ✓ Absence of or a reduced antibody response in immunocompromised individuals
- ✓ Patients with infectious mononucleosis, or with connective tissue diseases (SLE) or recipients of blood transfusions may give false positive results

Diagnostic methods in Virology:

THE MOLECULAR REVOLUTION

The development of molecular techniques has brought a revolution in the diagnosis of virus infections, thus enabling:

✓a considerable increase in the assay sensitivity

✓shortening response times

✓widening the range of the identified viruses

The revolution has led to a transition from conventional methods based on cell cultures to new molecular techniques

Diagnostic methods in Virology:

THE MOLECULAR REVOLUTION

However, the molecular revolution has led to the need to:

- ✓lower costs and automate new tests
- ✓redefine the clinical relevance of the new tests
- ✓identify new standards of sensitivity and specificity
- ✓redefine the clinical interpretive criteria

Diagnostic methods in Virology:

AN EXAMPLE FROM THE REAL WORLD:

DIAGNOSIS OF HCMV INFECTIONS

- **Serology (first-line)**

  ELISA assay to search for specific IgG/IgM

- **Direct detection of virus antigens**

  Search for virus antigen by IF (pp65) on PBMCs - **ANTIGENEMIA**

- **Indirect virus rapid isolation**

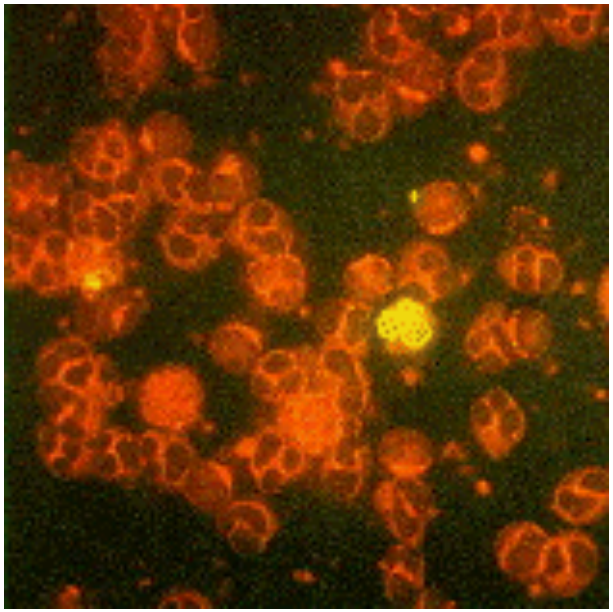
  Infection of Human Foreskin Fibroblasts (HFF) and then infectious virus identification by IF (IE1) at 24 h p.i. - **VIREMIA**

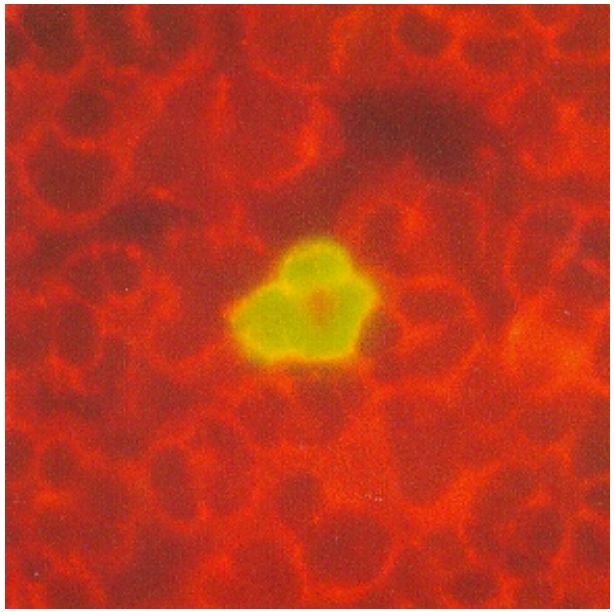
- **Quantification of virus DNA/RNA (monitoring patients)**

  qPCR - n. of viral genomes – **DNAEMIA**

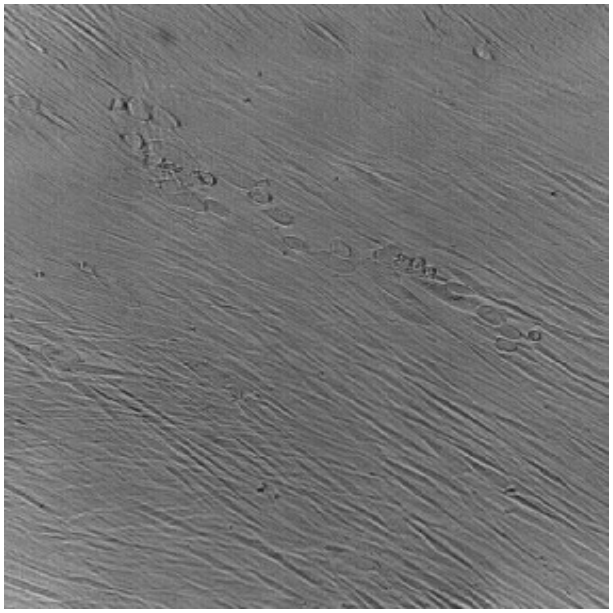
  RT-real time PCR - **RNAEMIA**

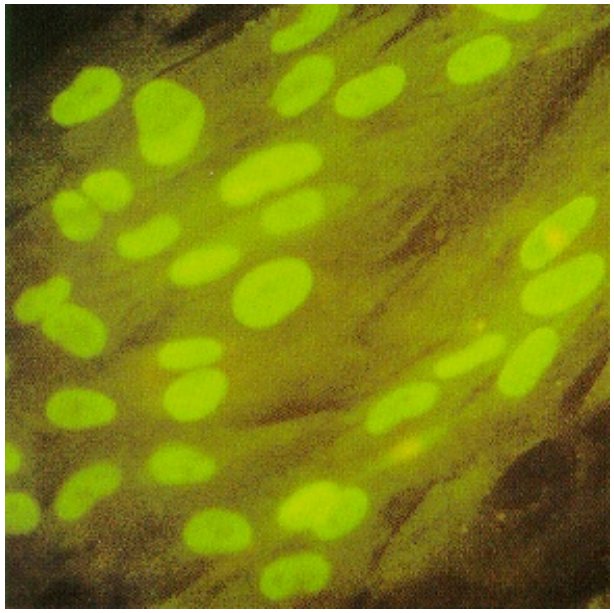
HCMV antigenemia





HCMV viremia

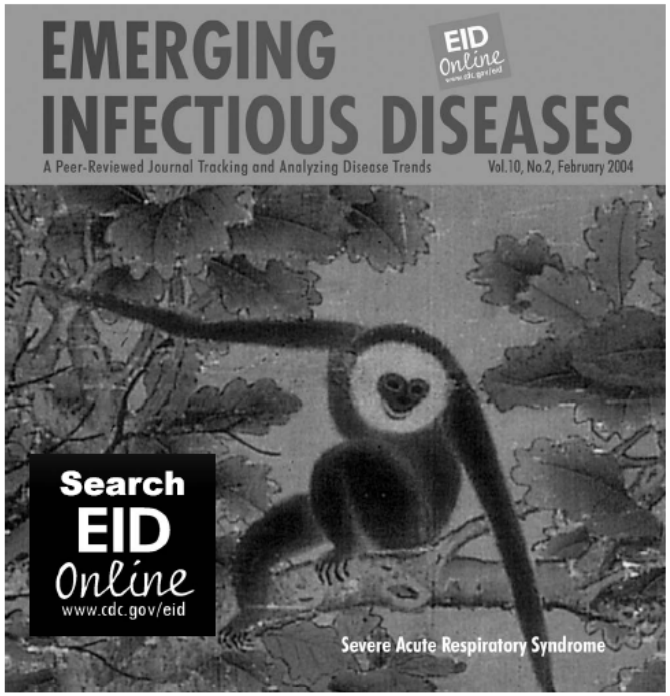




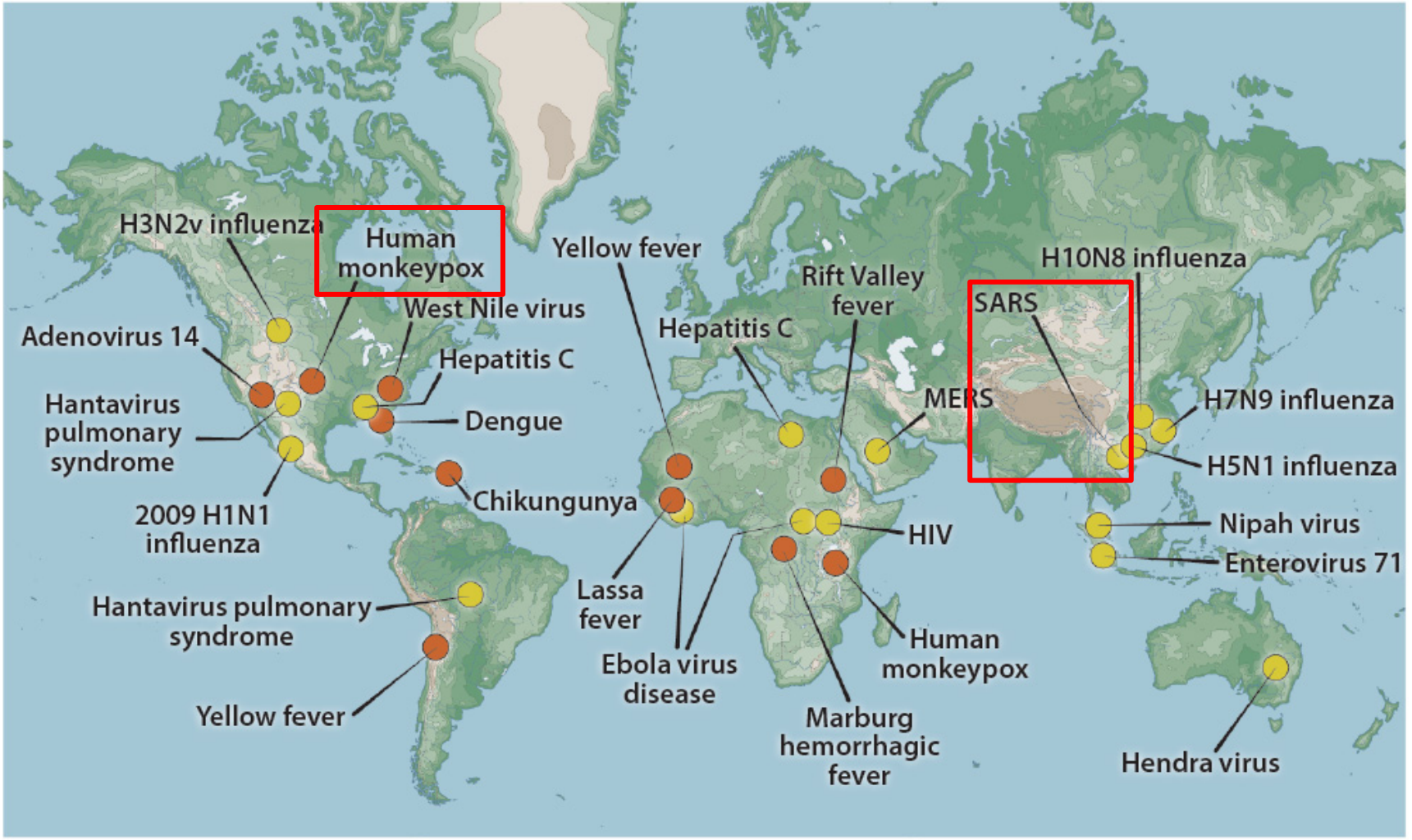
Diagnostic methods in Virology:

THE MOLECULAR REVOLUTION

There will still be room for conventional methods ?



Mapping emerging viral diseases (Marston et al., Science Translational Medicine, 2014)



Newly emerging

Reemerging

Developments facilitating spread

- Commercial air travel
- Global trade
- Urbanization
- Unchecked population growth
- Climate change

Advances facilitating control

- Genome sequencing to identify emerging viruses
- Global communication networks
- Rapid diagnostics
- New approaches to vaccine and therapeutic design

2003 – USA

MONKEYPOX

Rare disease outbreak in Midwest

Four people in the Midwest have confirmed cases of the monkeypox virus and dozens of others have suspected cases, health officials say. Officials suspect they caught the illness from exposure to pet prairie dogs.

Prairie dog

Origin – This outbreak is the first to be reported in the Western Hemisphere. It is usually found in remote villages in Central and West Africa.

The virus – Related to the smallpox virus; being vaccinated against smallpox provides protection against it. The death rate among those with monkeypox ranges from 1 to 10 percent with the highest rates among young children. The largest outbreak was in the Congo where 511 suspect cases were identified between 1996 to 1997.

Transmission and symptoms – Transmitted to people from squirrels and primates through bite or contact with the animal's blood; a preliminary investigation shows the virus was transmitted to humans through "close contact" with the infected prairie dogs. Symptoms include rashes, fevers and chills in infected persons.

SOURCES: World Health Organization; Associated Press

AP

Emerging Infectious Diseases • www.cdc.gov/eid • Vol. 10, No. 3, March 2004

Monkeypox Transmission and Pathogenesis in Prairie Dogs

Jeannette Guarner,* Bill J. Johnson,† Christopher D. Paddock,* Wun-Ju Shieh,* Cynthia S. Goldsmith,* Mary G. Reynolds,* Inger K. Damon,* Russell L. Regnery,* Sherif R. Zaki,* and the Veterinary Monkeypox Virus Working Group[1]

During May and June 2003, the first cluster of human monkeypox cases in the United States was reported. Most patients with this febrile vesicular rash illness presumably acquired the infection from prairie dogs. Monkeypox virus was demonstrated by using polymerase chain reaction in two prairie dogs in which pathologic studies showed necrotizing bronchopneumonia, conjunctivitis, and tongue ulceration. Immunohistochemical assays for orthopoxviruses demonstrated abundant viral antigens in surface epithelial cells of lesions in conjunctivae and tongue, with lower amounts in adjacent macrophages, fibroblasts, and connective tissues. Viral antigens in the lung were abundant in bronchial epithelial cells, macrophages, and fibroblasts. Virus isolation and electron microscopy demonstrated active viral replication in lungs and tongue. These findings indicate that both respiratory and direct mucocutaneous exposures are potentially important routes of transmission of monkeypox virus between rodents and to humans. Prairie dogs offer insights into transmission, pathogenesis, and new vaccine and treatment trials because they are susceptible to severe monkeypox infection.

MONKEYPOX

Pet prairie dogs linked to 15 states

Nine people have contracted the monkeypox virus and at least 50 more possible cases have been reported, health officials say. The investigation has expanded to more states where buyers may have possibly purchased infected prairie dogs since April 15.

Confirmed or suspected cases of monkeypox

Linked with possibly infected pet prairie dogs

Facts about the virus

Cause Monkeypox virus, which is related to the smallpox virus

Symptoms 12 days after exposure there is enlargement of lymph nodes, fever, headache, rash

Recovery Typically lasts two to four weeks; the mortality rate is one to ten percent

SOURCE: Centers for Disease Control and Prevention AP

Monkeypox outbreak-CDC final report, July 30, 2003

- 72 suspected cases of monkeypox investigated, primarily in Wisconsin, Indiana, and Illinois

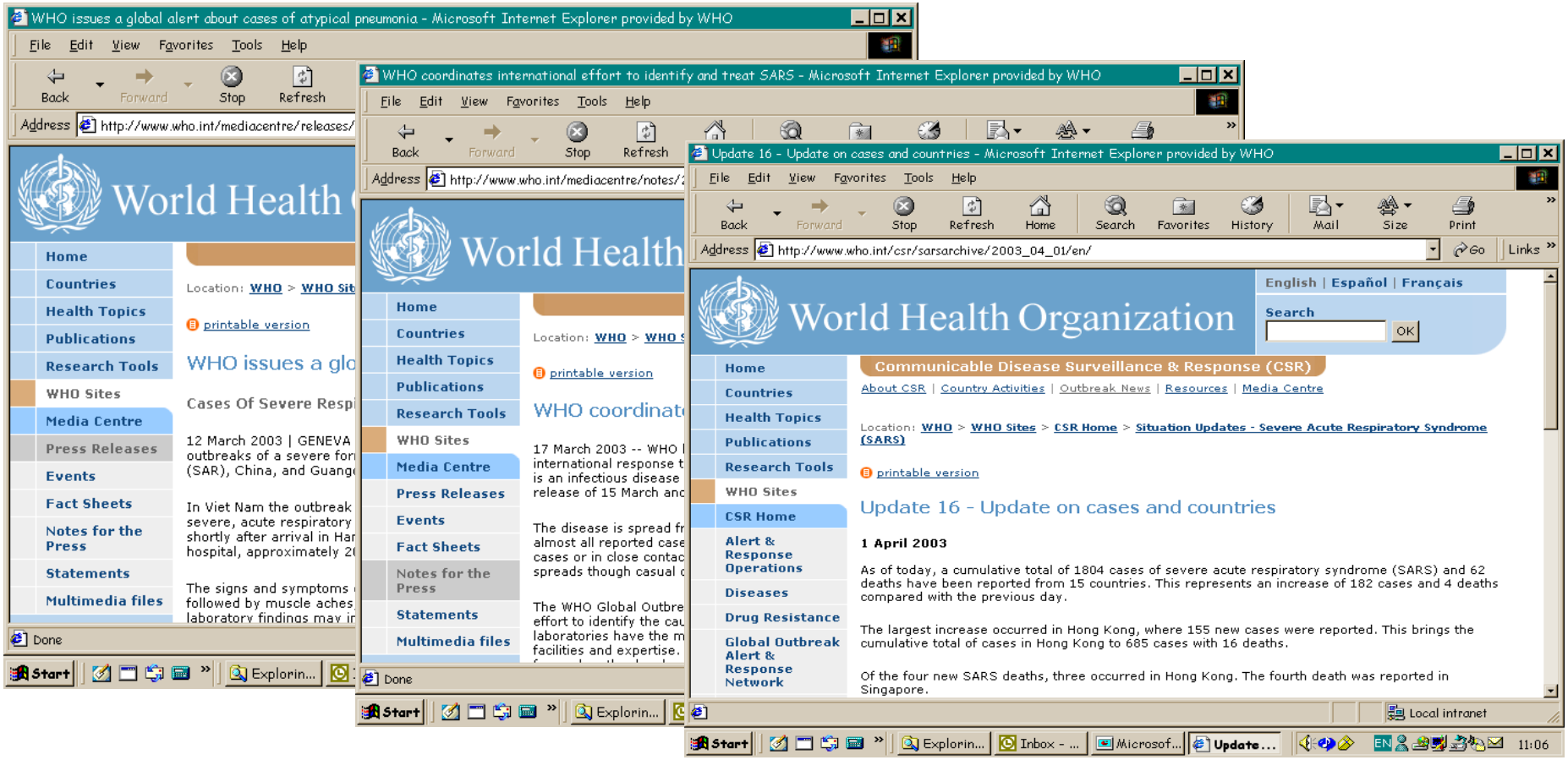
- In 37 cases, monkeypox infections was confirmed by laboratory testing

(electron microscopy images consistent with poxvirus, several polymerase chain reaction–based assays, serologic tests, immunohistochemistry, and gene sequencing)

Aetiology of SARS:

Story of an unprecedent success of international collaboration - 2003

www.who.int/csr/sars



Etiology establishment

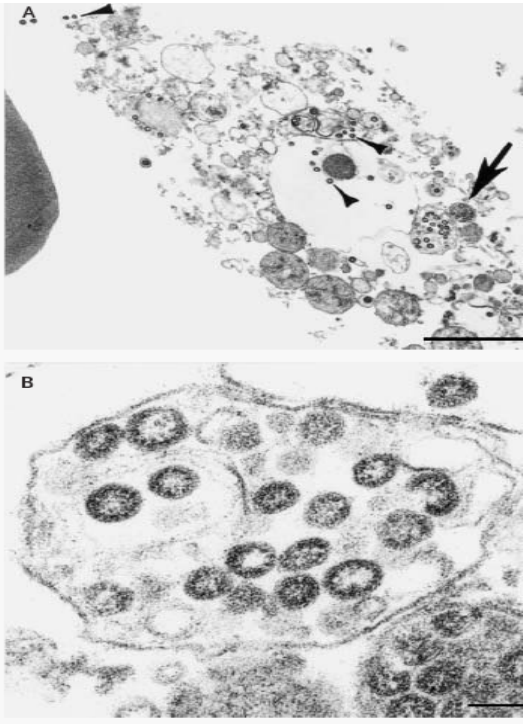


Figure 5. Ultrastructural Characteristics of a Coronavir Infected Cell in Bronchioaveolar-Lavage Fluid from a Patient with SARS, Showing Numerous Intracellular and Extracellular Particles.

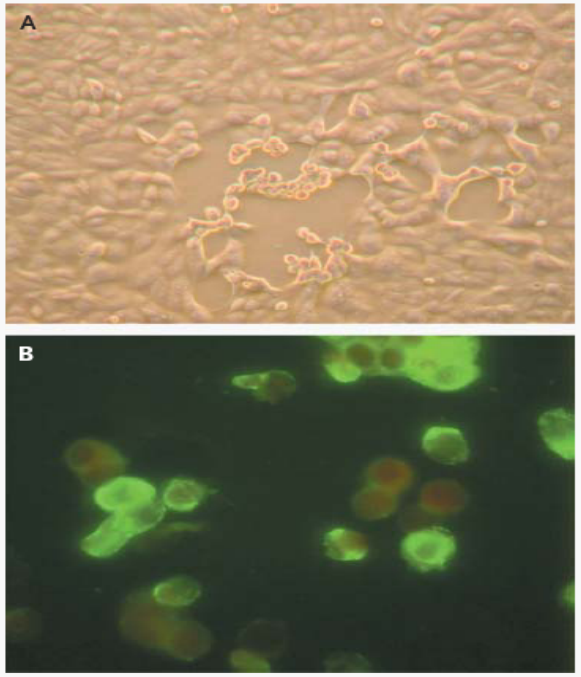
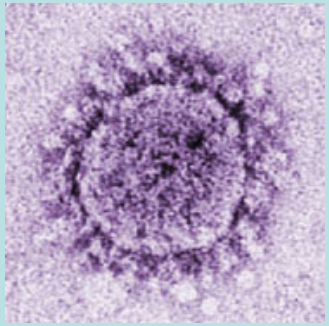


Figure 1. Vero E6 cells Inoculated with Oropharyngeal Specimens from Patients with SARS.

Ksiatzek TG: N.Engl. J Med. 2003

Strategy leading to the discovery or a new coronavirus

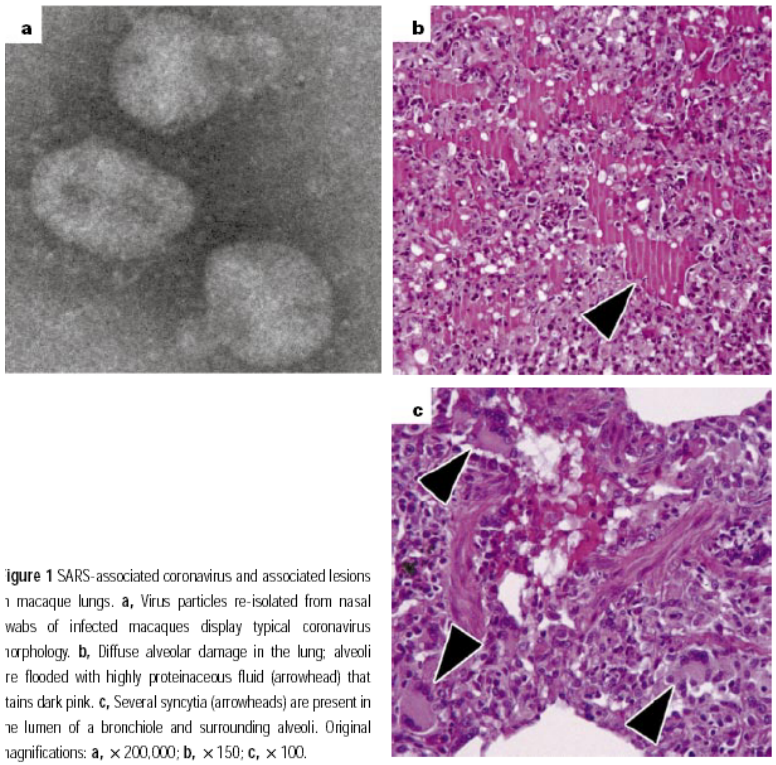
- EM on lung biopsy
- Virus isolation
- Consensus/ low stringency PCR
- Random primer RT-PCR/differential display
- Array technology



Koch's postulates fulfilled for SARS virus

NATURE | VOL 423 | 15 MAY 2003 |

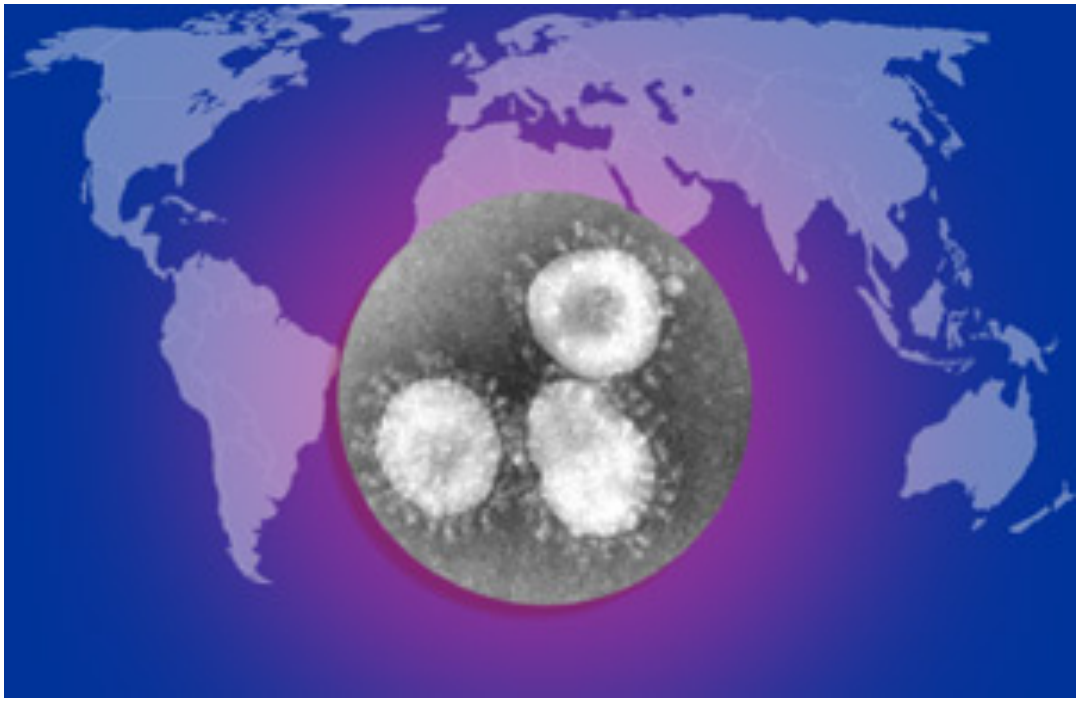
Ron A. M. Fouchier*, Thijs Kuiken*, Martin Schutten*, Geert van Amerongen*, Gerard J. J. van Doornum*, Bernadette G. van den Hoogen*, Malik Peiris†, Wilina Lim‡, Klaus Stöhr§, Albert D. M. E. Osterhaus*



igure 1 SARS-associated coronavirus and associated lesions n macaque lungs. **a,** Virus particles re-isolated from nasal wabs of infected macaques display typical coronavirus norphology. **b,** Diffuse alveolar damage in the lung; alveoli re flooded with highly proteinaceous fluid (arrowhead) that tains dark pink. **c,** Several syncytia (arrowheads) are present in ne lumen of a bronchiole and surrounding alveoli. Original nagnifications: **a,** × 200,000; **b,** × 150; **c,** × 100.

Six criteria are required to establish a virus as the cause of a disease (Koch/Rivers)

1. isolation of virus from diseased hosts,
2. cultivation in host cells,
3. proof of filterability
4. production of comparable disease in the original host species or a related one,
5. re-isolation of the virus,
6. detection of a specific immune response to the virus



A novel coronavirus is associated with SARS

Conclusive WHO announcement of April 16, 2003

Diagnostic methods in Virology:

SO, A FINAL COMMENT….

- *New methods for conventional viruses*
- *Conventional methods for new viruses*