





# 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**

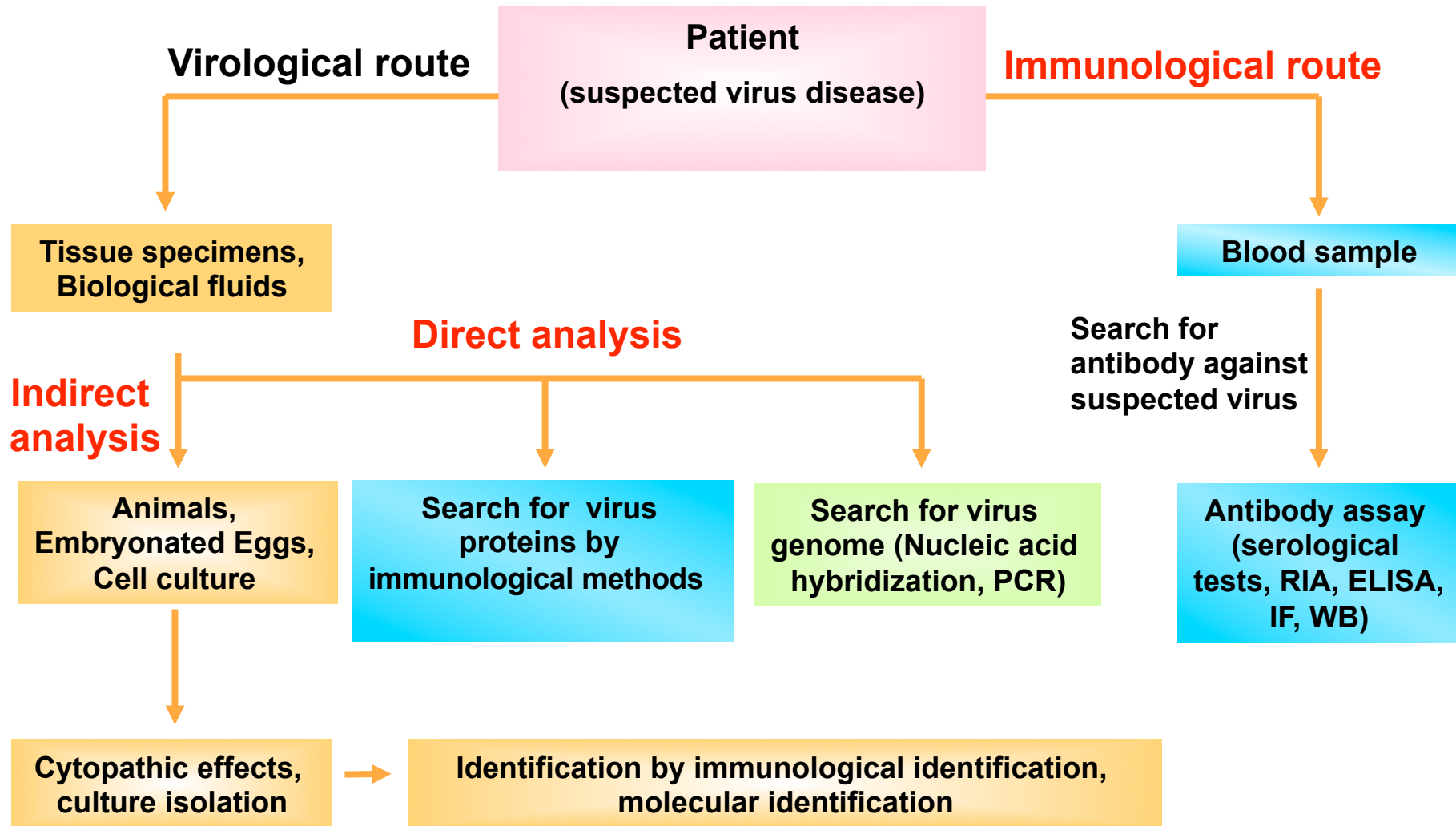
Diagnostic Strategies in Virology :  
**State of the art**

**1. DIRECT ANALYSIS**

**2. INDIRECT ANALYSIS**

**3. SEROLOGY**

# Diagnostic strategies for virus infections





# Diagnostic Strategies in Virology :

## State of the art

**1. DIRECT ANALYSIS**

**2. INDIRECT ANALYSIS**

**3. SEROLOGY**

# Diagnostic Strategies in Virology:

## **DIRECT ANALYSIS**

### **1. VIRAL ANTIGENS DETECTION**

Immunofluorescence, ...

### **2. ELECTRON MICROSCOPY**

Morphology and titer of viral particles

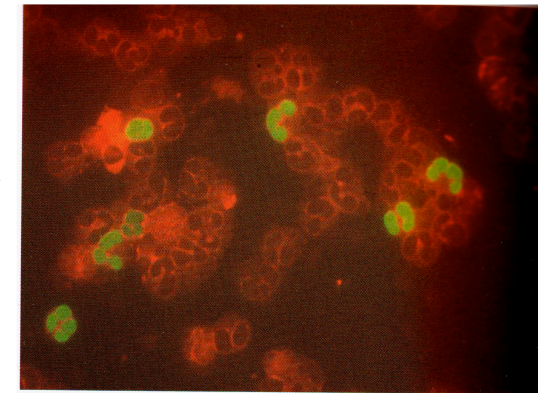
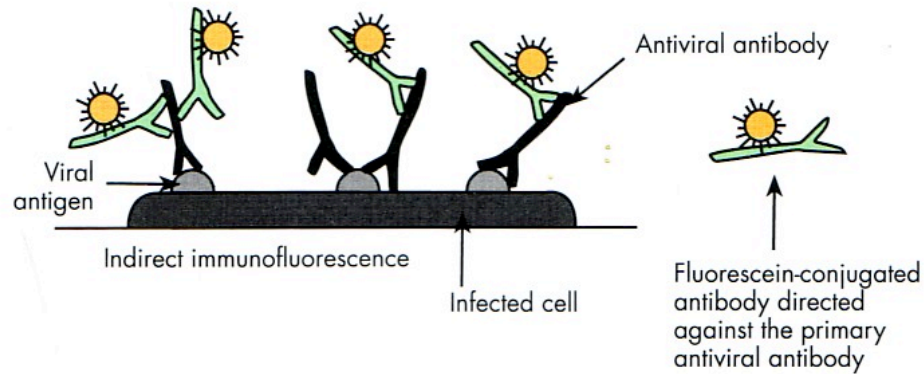
### **3. LIGHT MICROSCOPY**

Histology, inclusion bodies

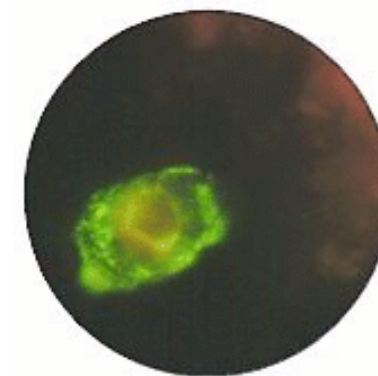
### **4. VIRAL NUCLEIC ACID DETECTION**

Hybridization with specific probes, PCR

# DIRECT ANALYSIS : VIRAL ANTIGENS DETECTION: IF



HCMV antigenemia in PBMCs

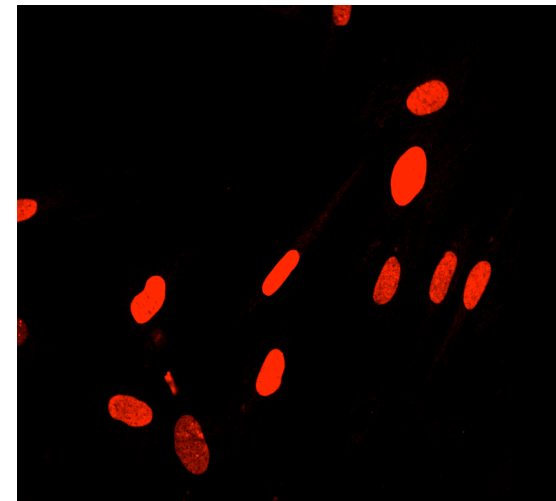
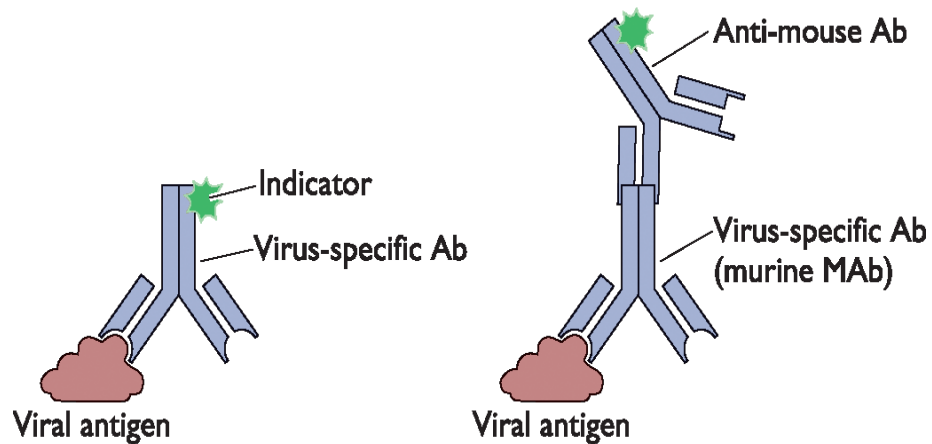


HSV: epithelial cell from  
a cutaneous lesion

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

# DIRECT ANALYSIS: VIRAL ANTIGENS DETECTION: IF

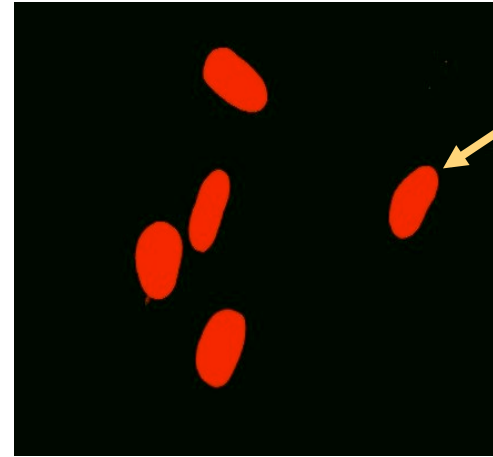
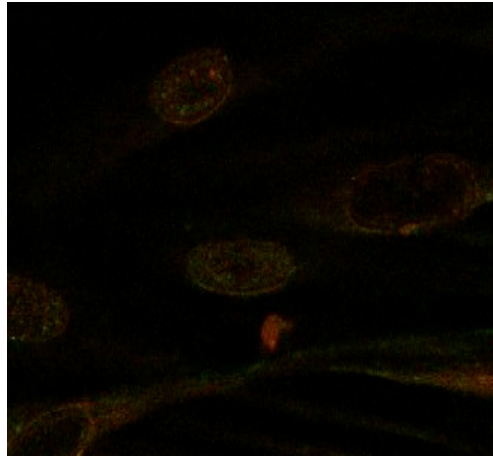
- Detection of viral antigens in infected tissues
- Requires diagnostic antibodies



**IF staining of HCMV IE  
proteins in infected HELF cells**

DIRECT ANALYSIS :  
VIRAL ANTIGENS DETECTION: IF

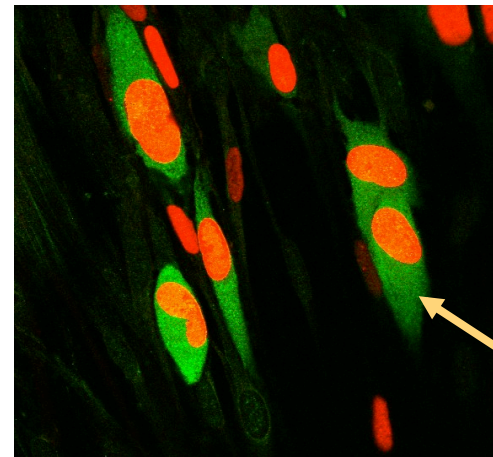
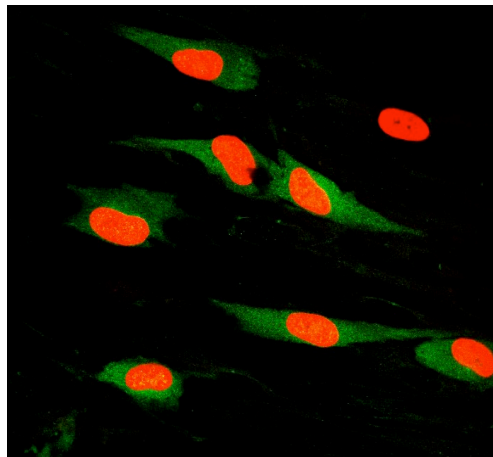
mock



IE1/2

24hpi

48 h.p.i.

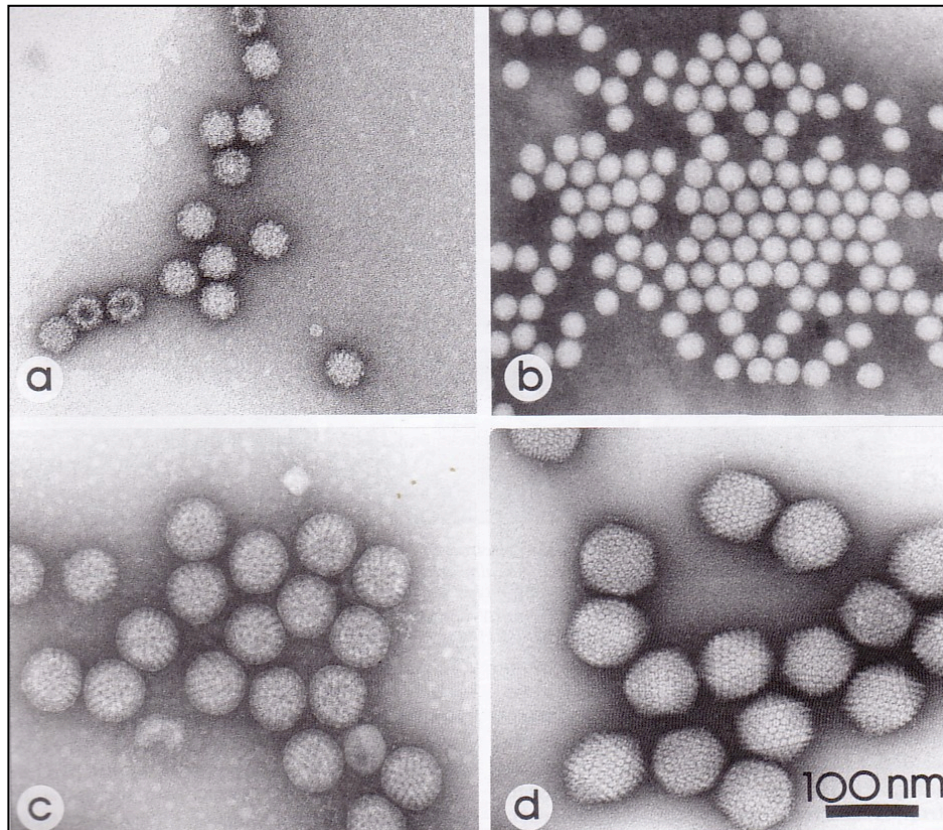


72hpi

UL72

# DIRECT ANALYSIS : ELECTRON MICROSCOPY

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

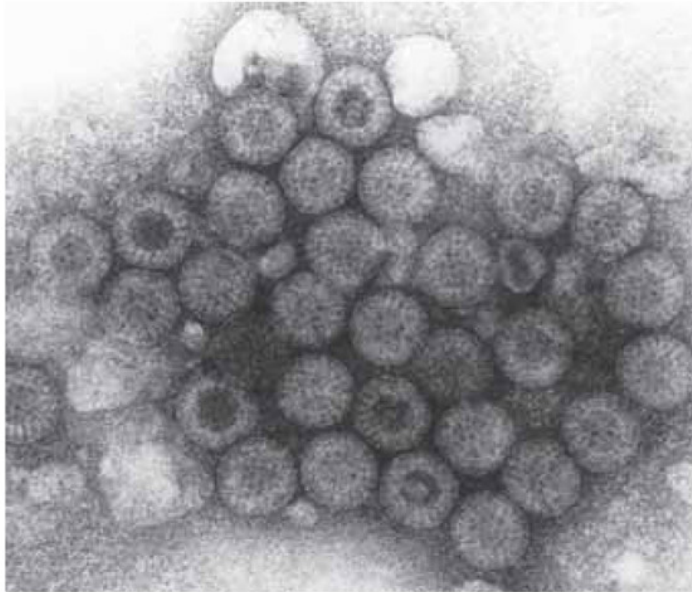


Four gastrointestinal viruses in feces samples:

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

*Magnification: x 150.000*

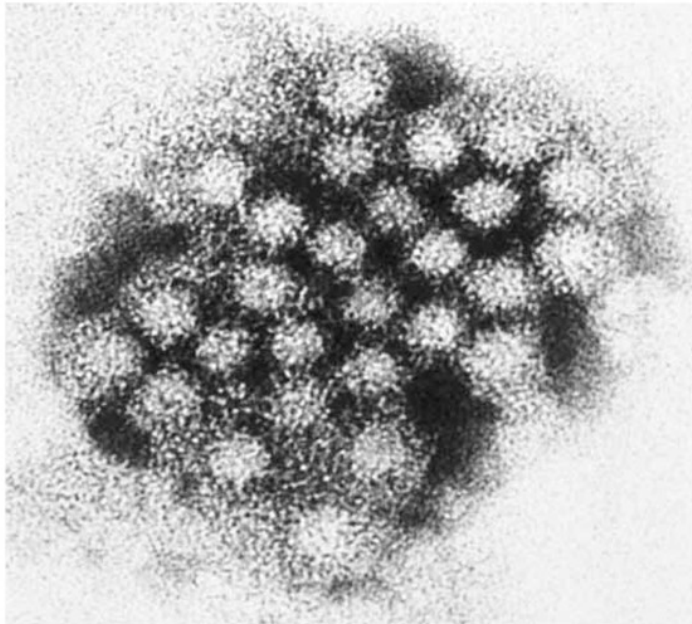




S. E. Miller

## ROTAVIRUS

(a)



CDC/PHIL

## NORWALK

(b)

# 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

- A “catch all” technique
- Useful technique for the search of uncultivable virus
- Independent from virus-specific reagents

## CONs

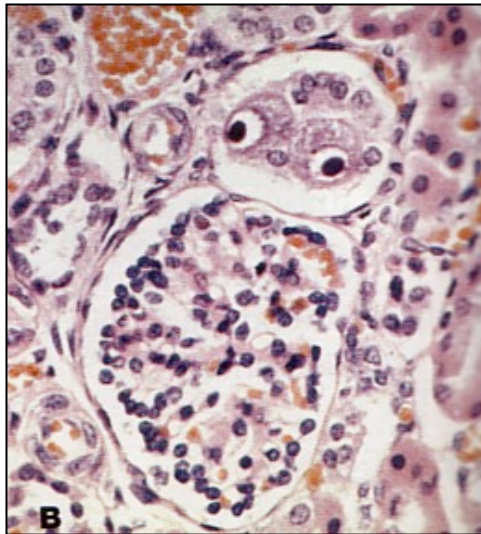
- Low sensitivity  
( $10^5$ - $10^6$  virus/ml)
- High costs of acquisition and maintenance
- Required trained personnel

# DIRECT ANALYSIS : LIGHT MICROSCOPY

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

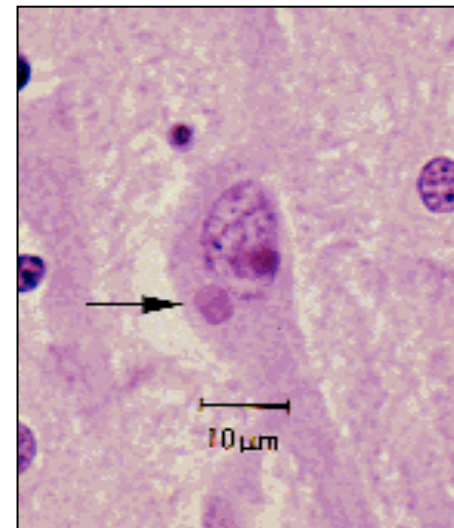
It is a low-specificity and low-sensitivity technique. However, it can be useful for diagnosis of few selected viral infections.

## HCMV



**Owl eyes** (nuclear)

## Rabies virus

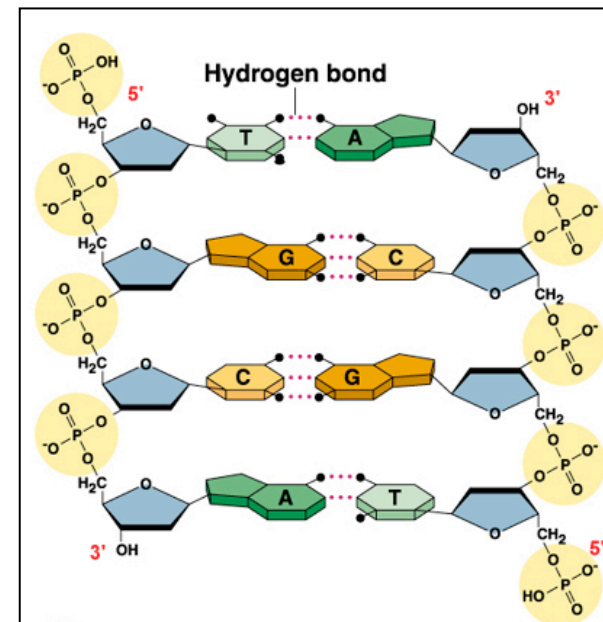


**Negri's bodies** (cytoplasmic)

# DIRECT ANALYSIS: VIRAL NUCLEIC ACID DETECTION

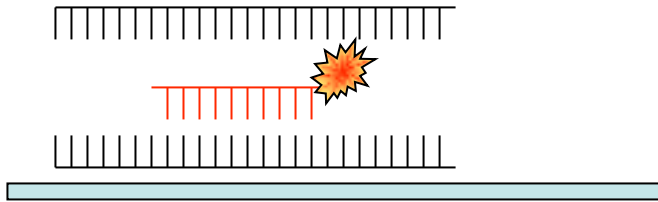
**HYBRIDIZATION WITH SPECIFIC PROBES**

**NUCLEIC ACID AMPLIFICATION**

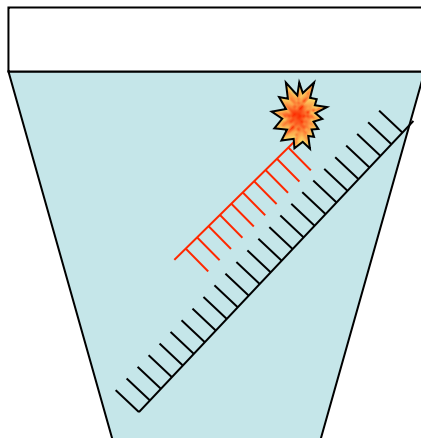
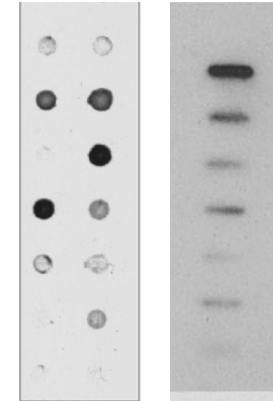


# VIRAL NUCLEIC ACID DETECTION

## HYBRIDIZATION WITH SPECIFIC PROBES



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

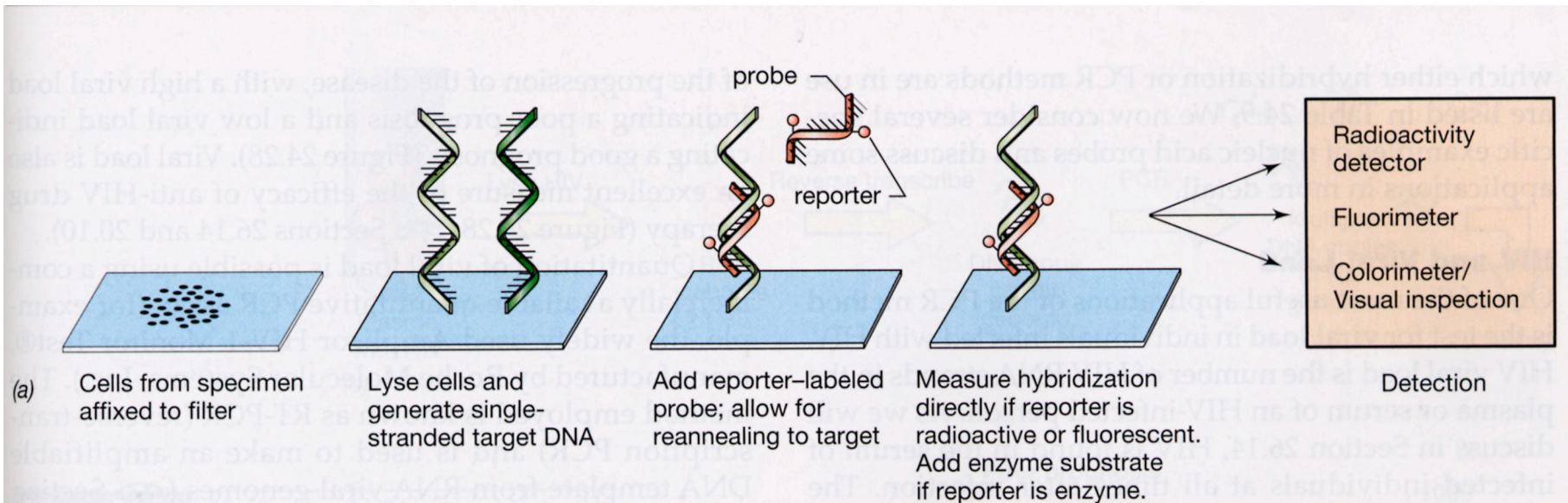


**Liquid hybridization**  
(es. HPV hybrid capture system)

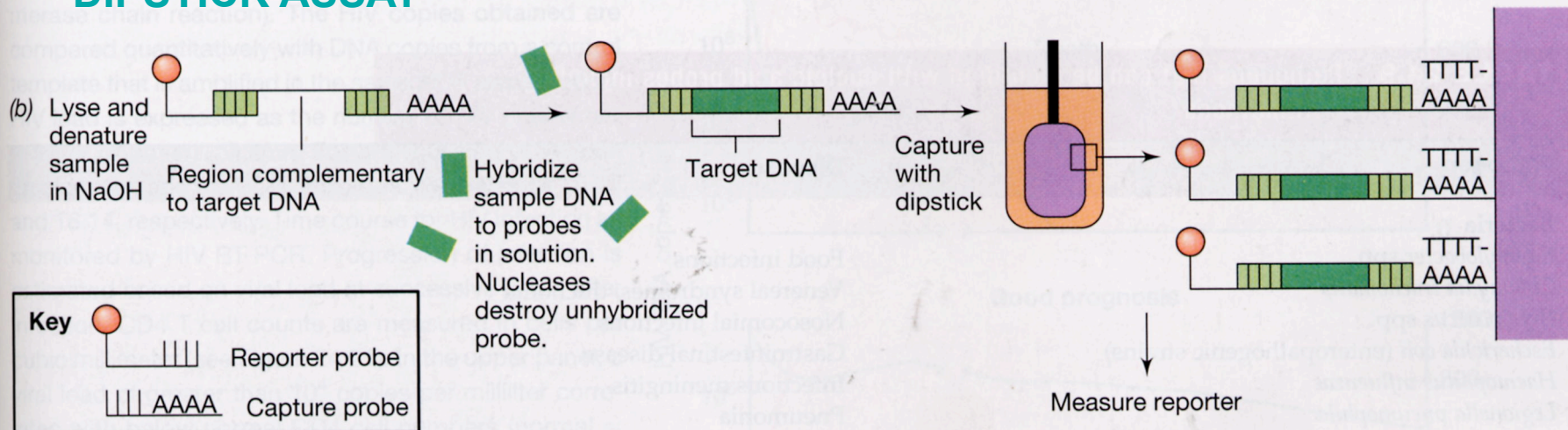
- **sensitivity:**  $10^4$  DNA copies/ $\mu$ l w/o target amplification  
 $500$  DNA copies/ $\mu$ l with target amplification
- **specificity** dependent from **stringency** conditions ( $T^\circ$ , Salts, pH)

# VIRAL NUCLEIC ACID DETECTION

## HYBRIDIZATION WITH SPECIFIC PROBES



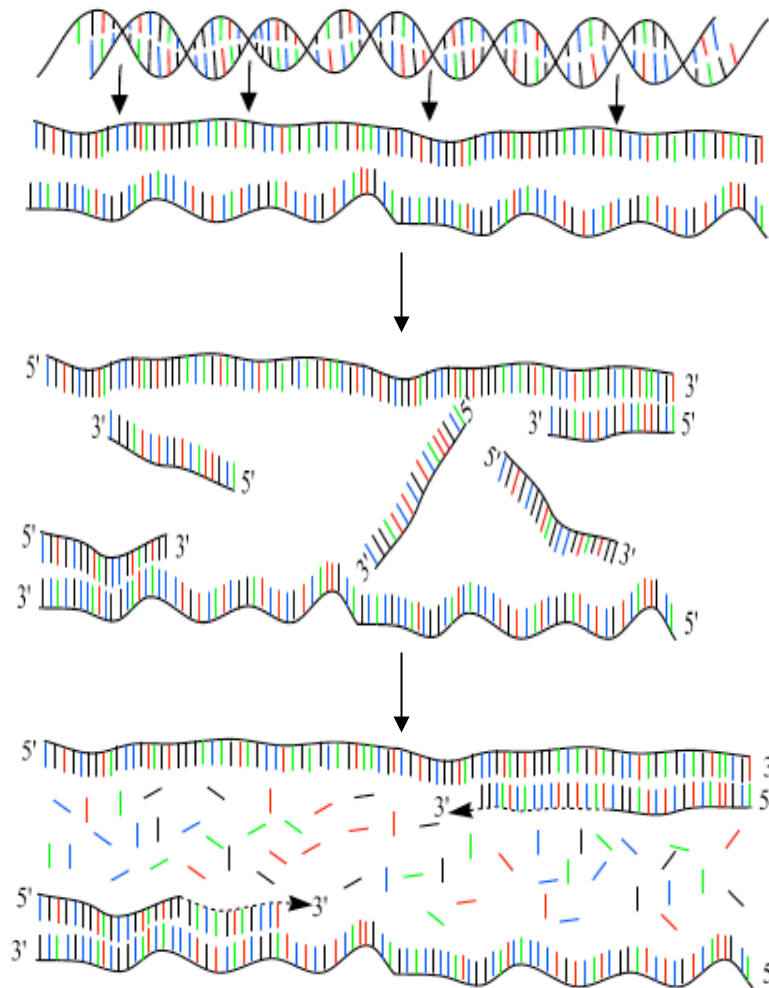
## DIPSTICK ASSAY





# VIRAL NUCLEIC ACID DETECTION

## TARGET AMPLIFICATION: PCR



I Phase: denaturation

1 min 94° C

II Phase: annealing

45 sec 54° C

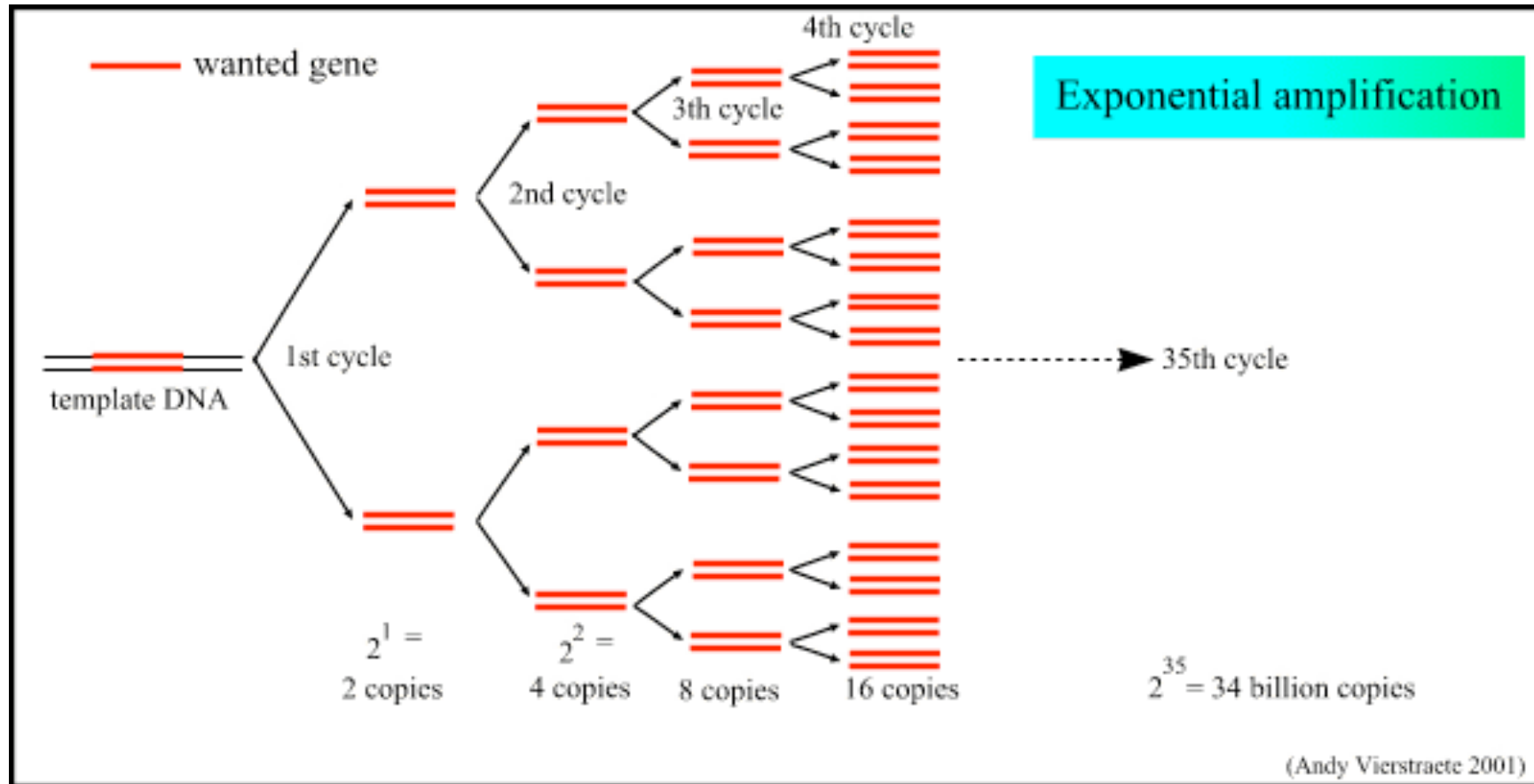
III Phase: extension

1 min 72° C

1 CYCLE

# VIRAL NUCLEIC ACID DETECTION

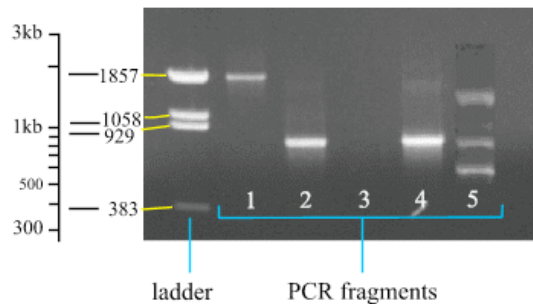
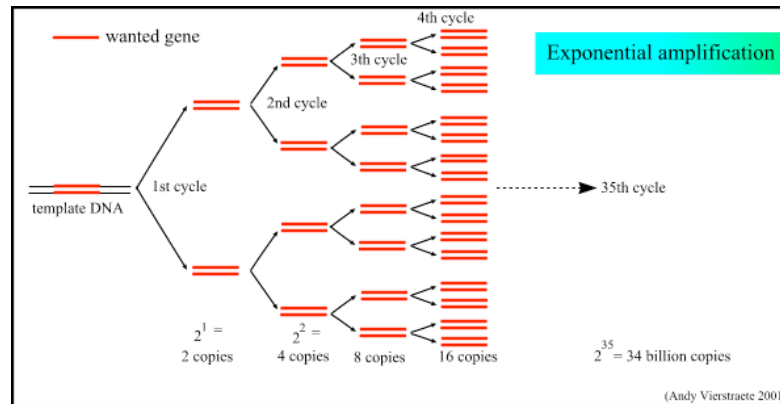
## TARGET AMPLIFICATION: PCR



# VIRAL NUCLEIC ACID DETECTION

## CONVENTIONAL PCR: VISUALIZATION AND IDENTIFICATION OF PRODUCTS

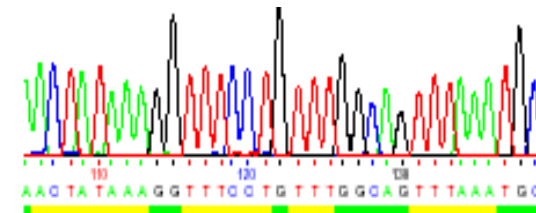
PCR



Agarose electrophoresis



Hybridization with  
specific probes



Sequencing



# VIRAL NUCLEIC ACID DETECTION

## CONVENTIONAL PCR

### PROs

- Extremely sensitive
- Easy to perform
- Fast

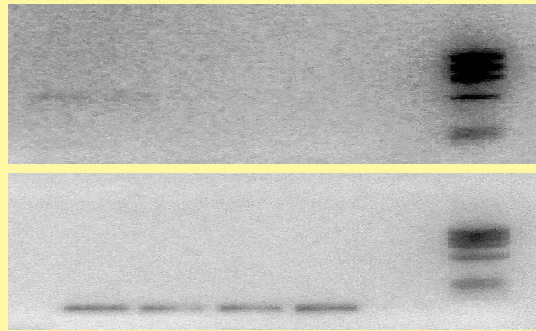
### CONs

- Susceptible to contaminations (false positives)
- Required trained personnel
- Qualitative

# VIRAL NUCLEIC ACID DETECTION

## PCR: Evolution of methods

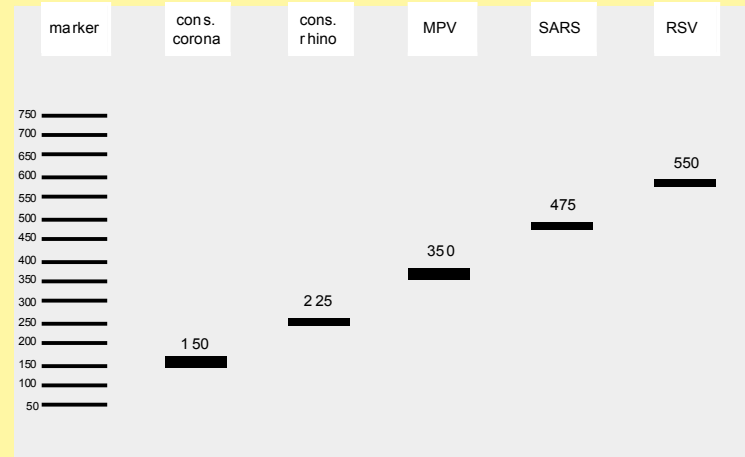
### NESTED PCR



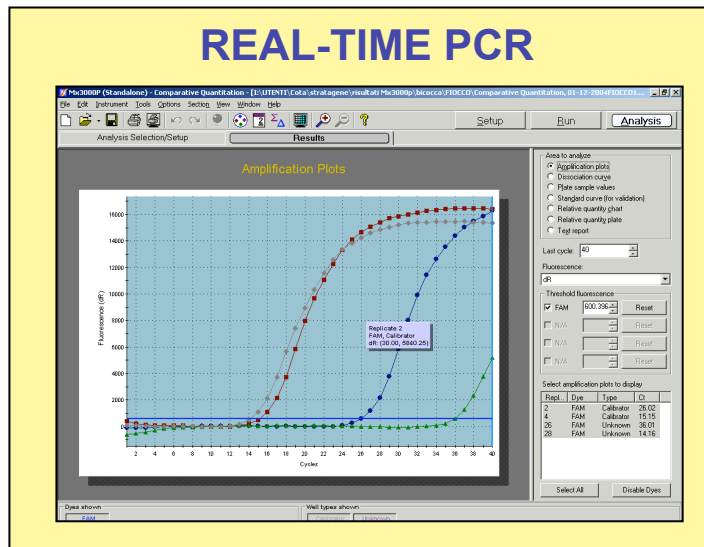
1° primers set

2° primers set  
(inside the 1° amplified)

### MULTIPLEX PCR



### REAL-TIME PCR



- LCR
- NASBA
- ...

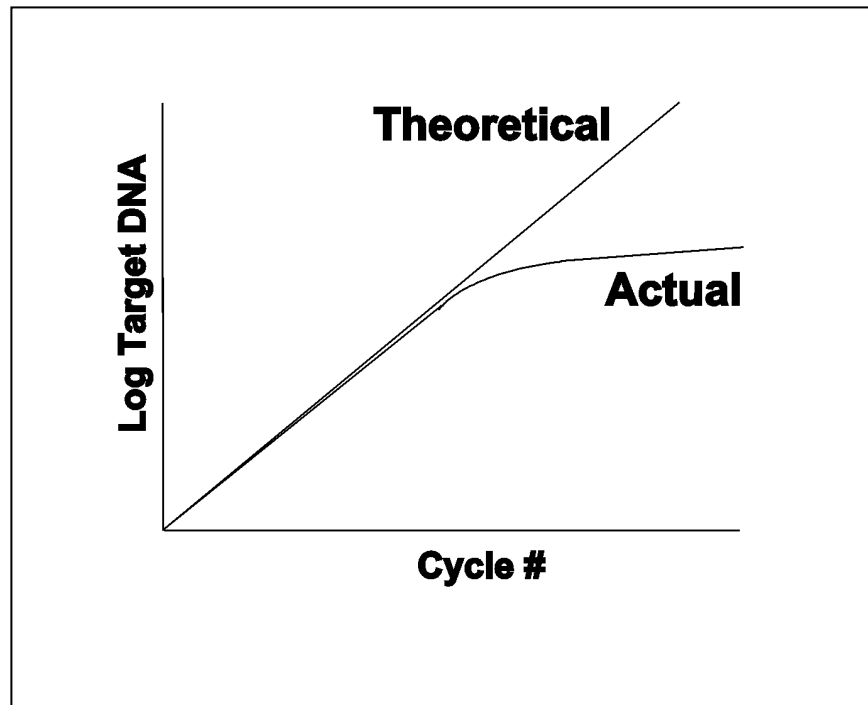
## VIRAL NUCLEIC ACID DETECTION

# 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 quantifying a gene characteristic for that particular virus.

# VIRAL NUCLEIC ACID DETECTION

## QUANTITATIVE REAL-TIME PCR (qPCR)

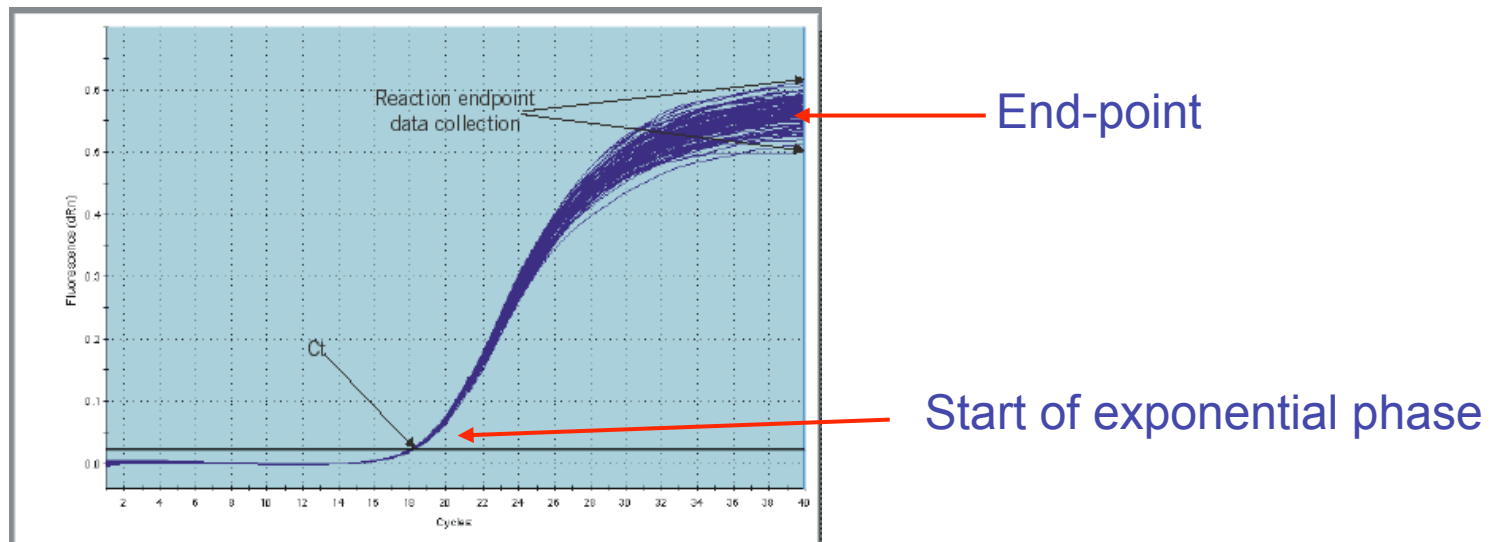


- **Theoretically**, a direct relationship exists between the final amount of the PCR product and the amount of initial target; the amount of product doubles at each cycle of the PCR rxn.
- **In practice**, with the progression of PCR cycles, some components of the mixture are exhausted and inhibitors of the PCR itself appear, so that the reaction reaches a plateau.

# VIRAL NUCLEIC ACID DETECTION

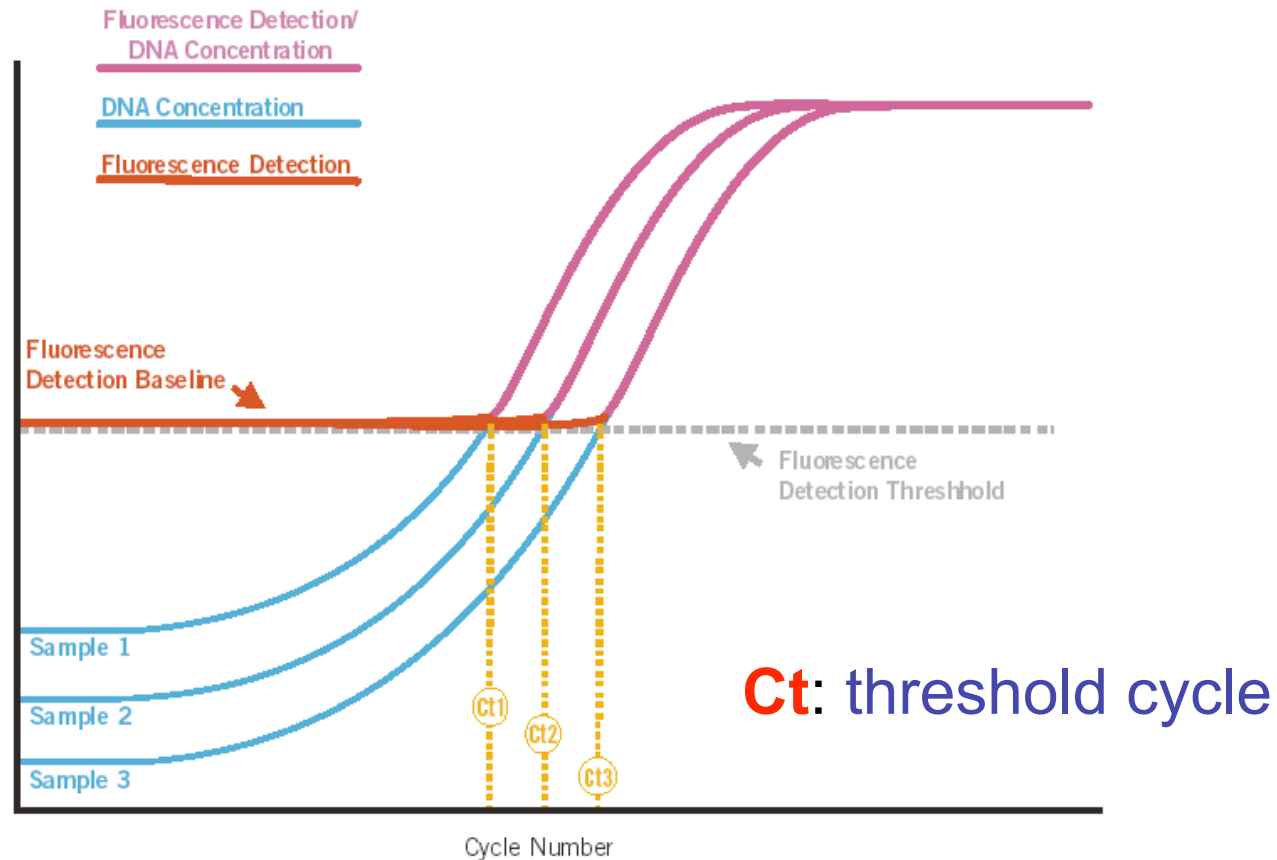
## Differences between conventional PCR and qPCR

- **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



# VIRAL NUCLEIC ACID DETECTION

## Principle of quantitative real-time PCR

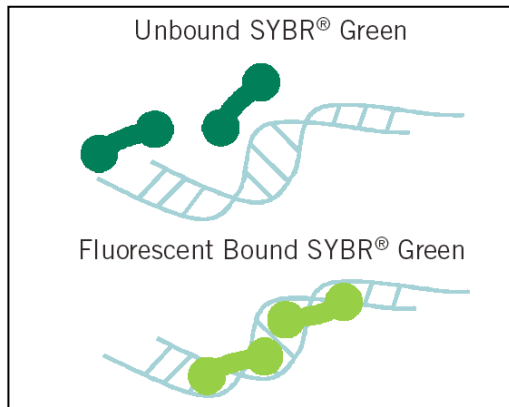


The **Ct** value is inversely proportional to the number of initial copies of target viral nucleic acid

# VIRAL NUCLEIC ACID DETECTION

## qPCR fluorescent probes

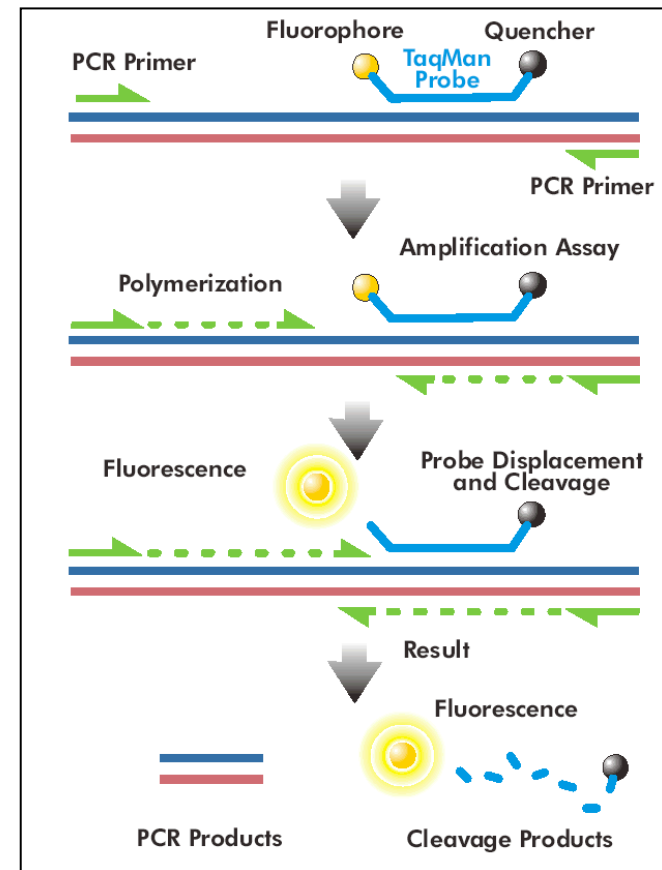
### SYBR Green



**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.

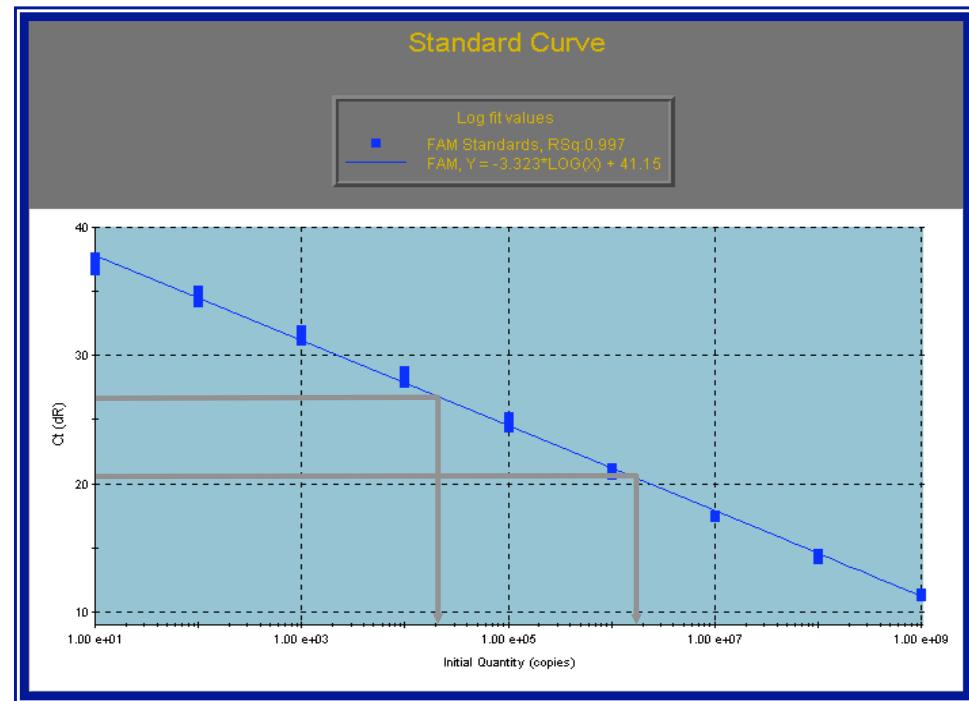
### Gene-specific probes (TaqMan)



# VIRAL NUCLEIC 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 NUCLEIC ACID DETECTION

## qPCR: an example of absolute quantification

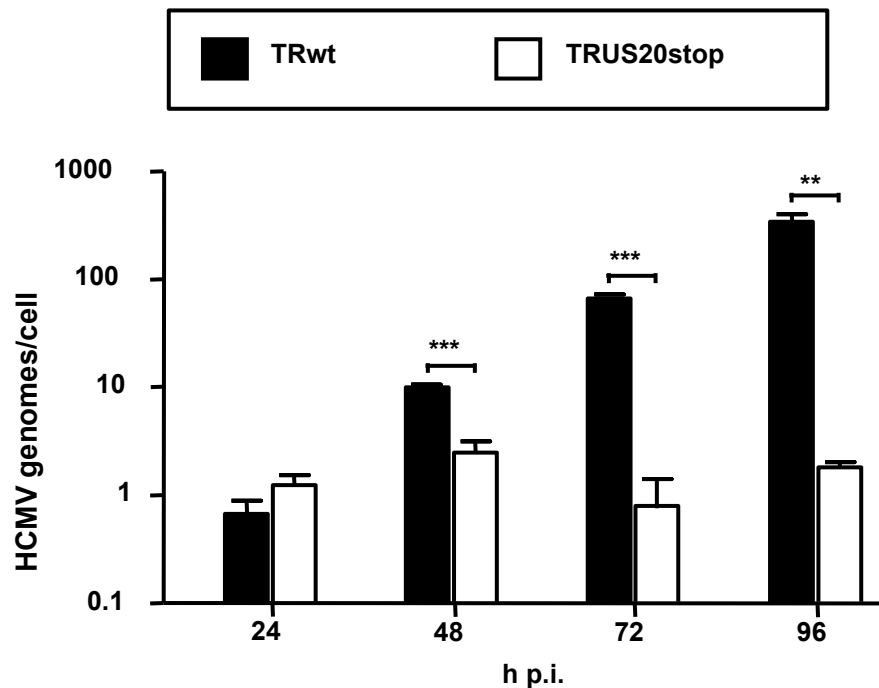


Figure 6. Lack of viral DNA synthesis in endothelial cells infected with a US20-mutant virus. HMVECs were infected with TRwt or TRUS20stop (MOI 1 pfu/cell) and at the indicated times p.i. total genomic DNA was isolated to quantify viral DNA levels by qPCR. The data shown are the mean values of two independent experiments  $\pm$  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 NUCLEIC ACID DETECTION

## qPCR: PROs

- Extremely sensitive
- More reliable results  
(qPCR can be monitored continuously)
- 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

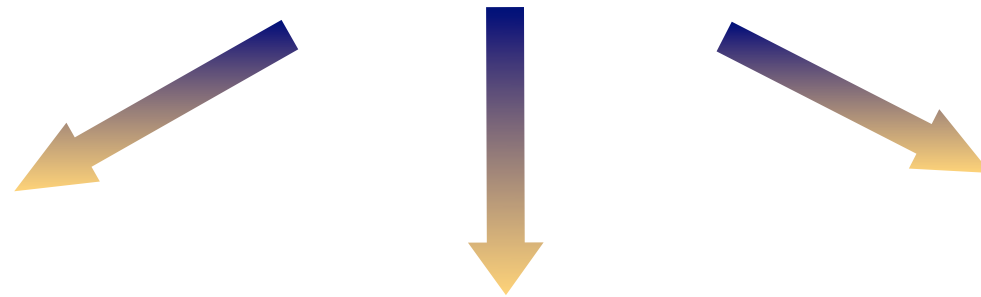
CAM lesions, hemoagglutination,...

### 3. LAB ANIMALS

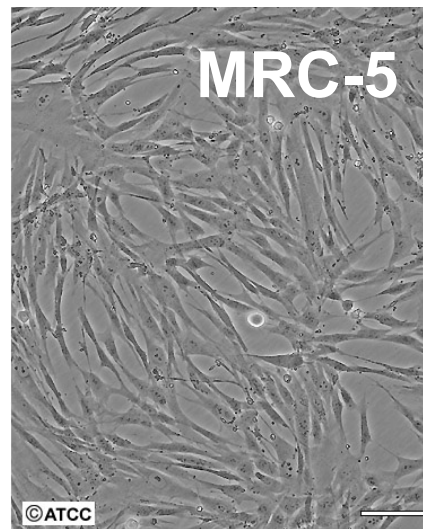
Disease, death

# Diagnostic methods in Virology: CELL CULTURES

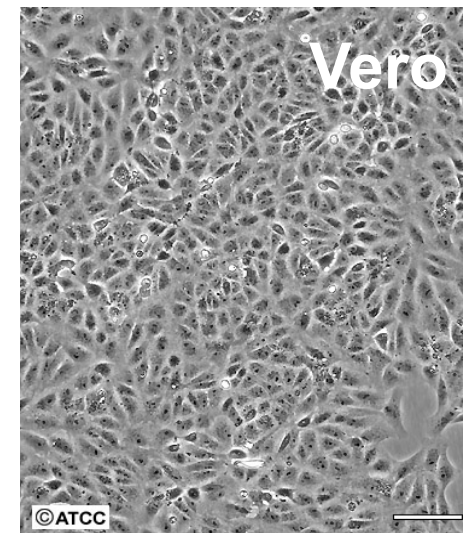
The most widely used method for virus isolation and growth



Primary cell cultures



Diploid cell strains



Continuous cell lines

## Diagnostic methods in Virology: **CELL CULTURES**

Effects of productive viral replication in cell culture:

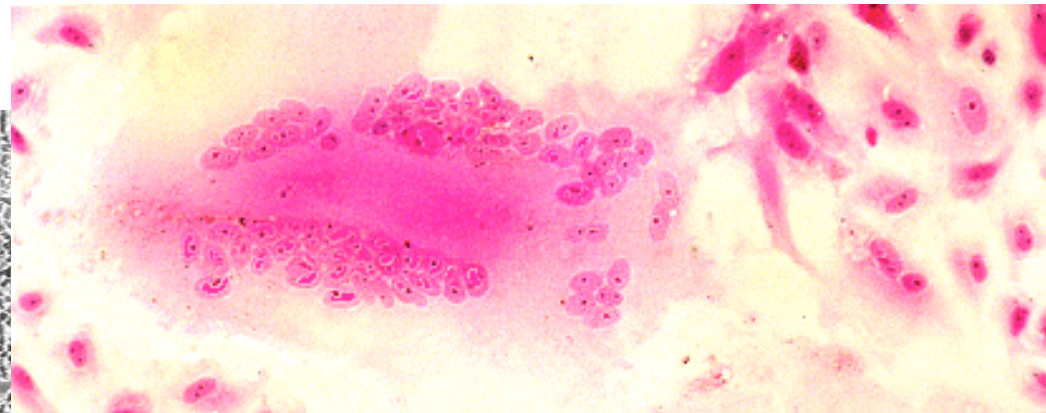
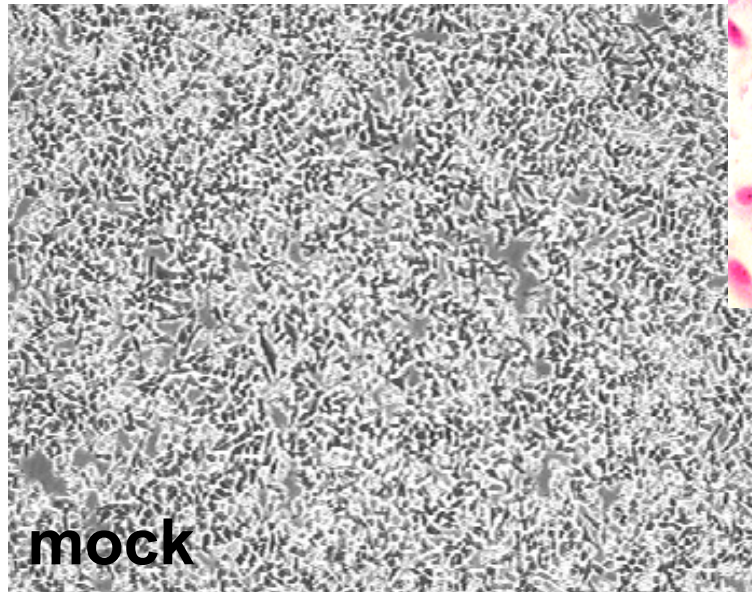
- **Cytopathic effect(s) (CPE)**
- **Syncytia (cell fusion)**
- **Hemadsorption**

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

**Syncytia formation: measles virus** (giant multinuclear cells)



# Diagnostic methods in Virology:

## CELL CULTURES

### **Hemoadsorption: mumps virus**

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





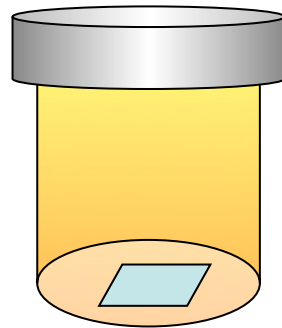
## Diagnostic methods in Virology:

### **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 virus cannot replicate in cell culture:
  - Gastrointestinal viruses
  - HBV
  - Parvovirus
  - HPV

# Diagnostic methods in Virology:

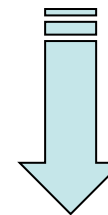
## CELL CULTURES: A rapid method



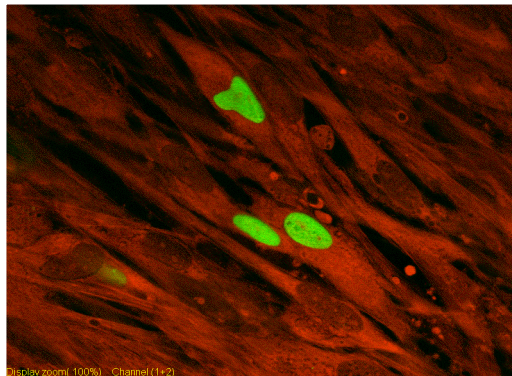
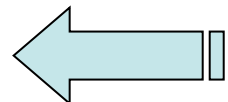
1. Cells seeding on a coverslip contained in a shell vial



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

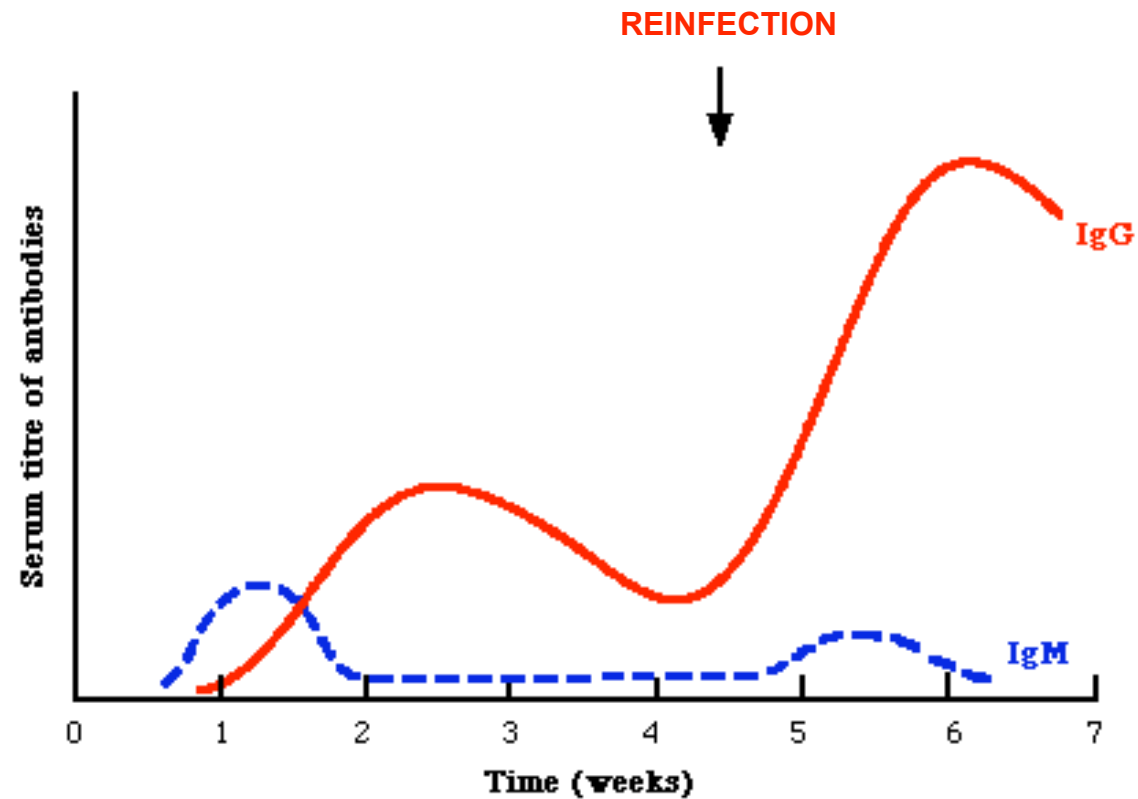
1. DIRECT ANALYSIS

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 increase 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

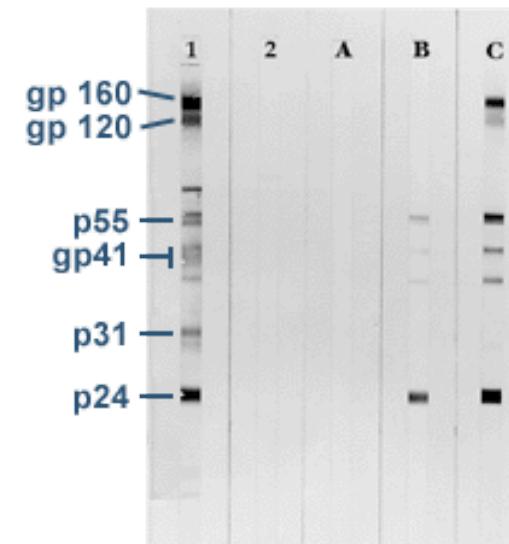
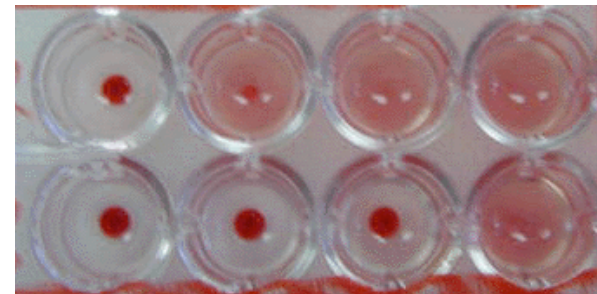
In some cases is crucial: eg. Rubella in pregnancy

# Diagnostic methods in Virology:

## SEROLOGY

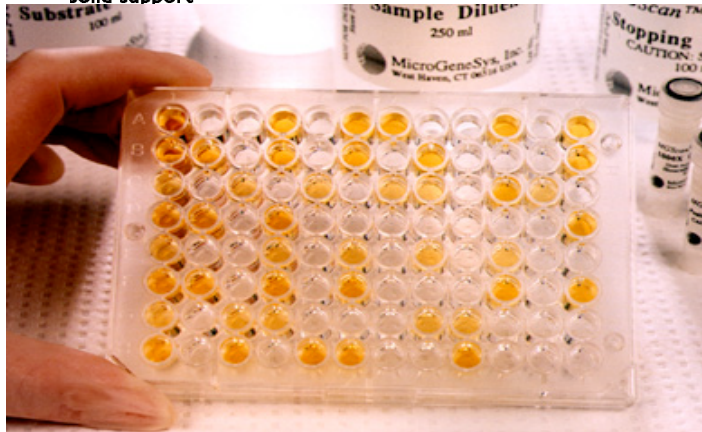
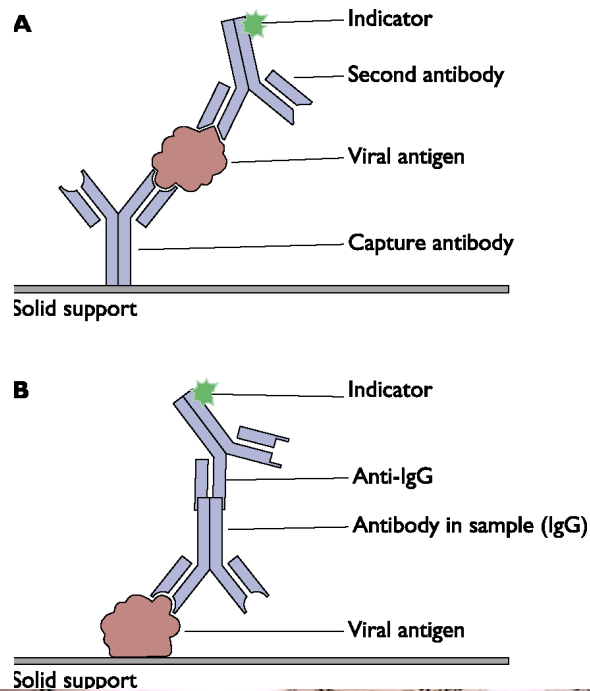
### TECHNIQUES

- 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 waste

- 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

### Procedure

1. Antibodies (Y) to virus (★) bound to wells of microtiter plate

2. Add patient sample (secretions, serum, and so on) suspected of containing virus particles or virus antigens and wash wells with buffer

3. Add antivirus antibody containing conjugated enzyme



4. Wash with buffer

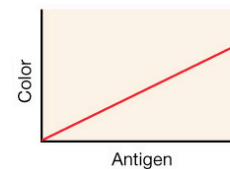
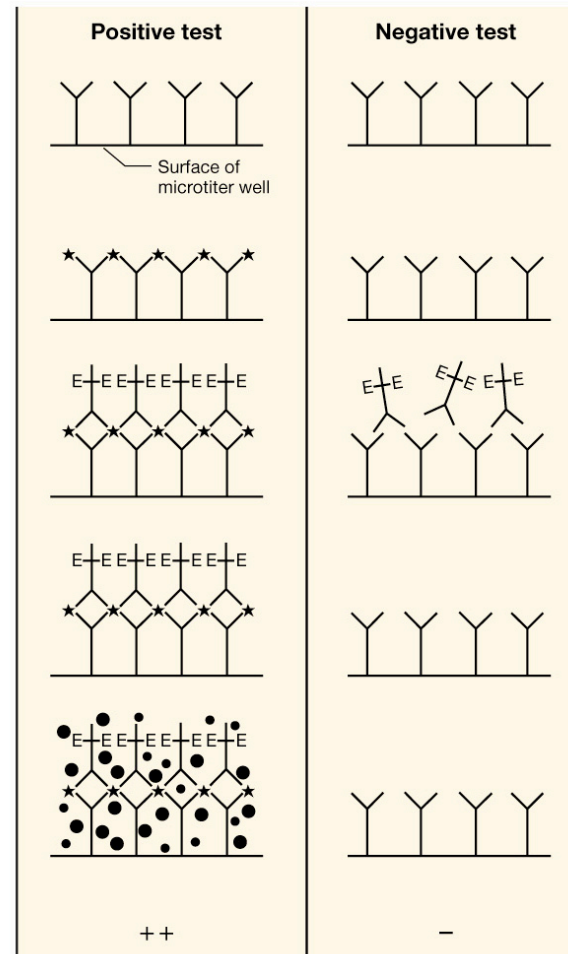
5. Add substrate for enzyme and measure amount of colored product (●).

### Results

Colored product

### Quantitation

Colored product produced is proportional to amount of antigen.





# Diagnostic methods in Virology:

## SEROLOGY: An indirect ELISA to detect antibodies

### Procedure

1. Coat microtiter wells with antigen preparation from disrupted HIV particles (★)

2. Add patient serum sample. HIV-specific antibodies bind to HIV antigen. Other antibodies do not bind

3. Wash with buffer

4. Add human anti-IgG antibodies conjugated to enzyme (E + E)

5. Wash with buffer

6. Add substrate for enzyme and measure amount of colored product (●).

### Results

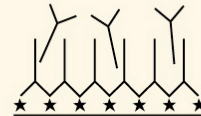
Colored product

### Quantitation

Colored product produced is proportional to the antibody concentration.

### Positive test

★ ★ ★ ★ ★ ★ ★  
Surface of microtiter well



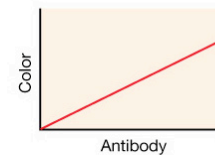
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### Negative test

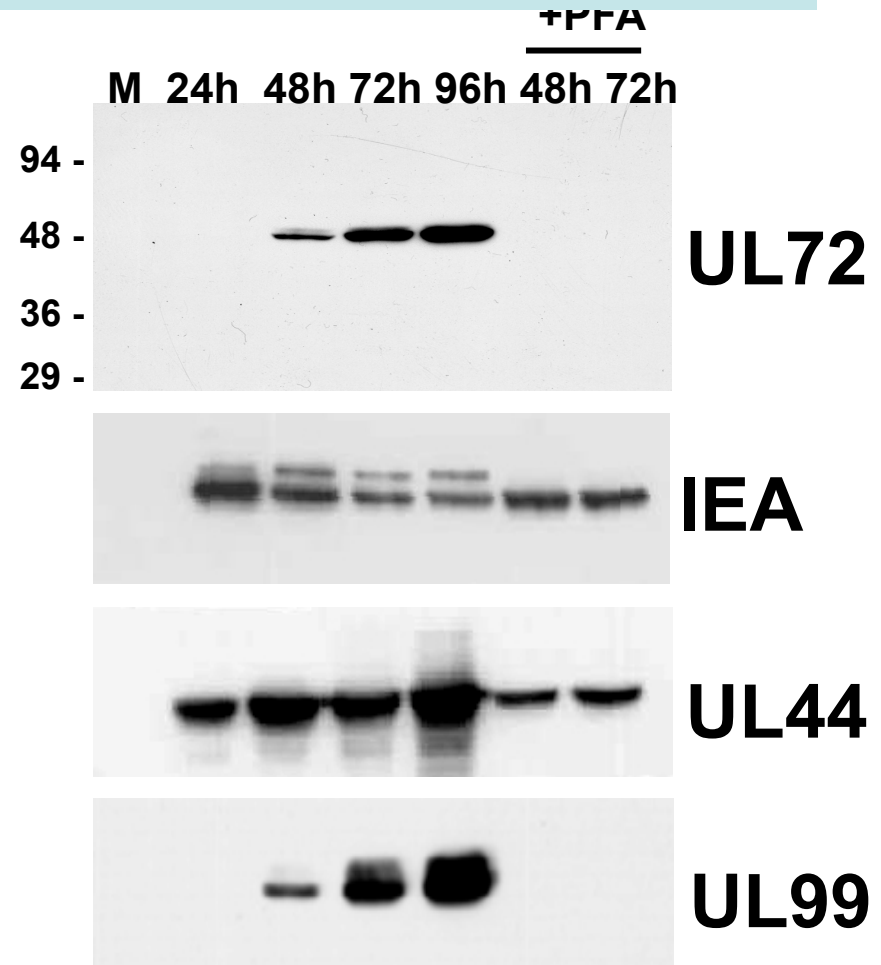
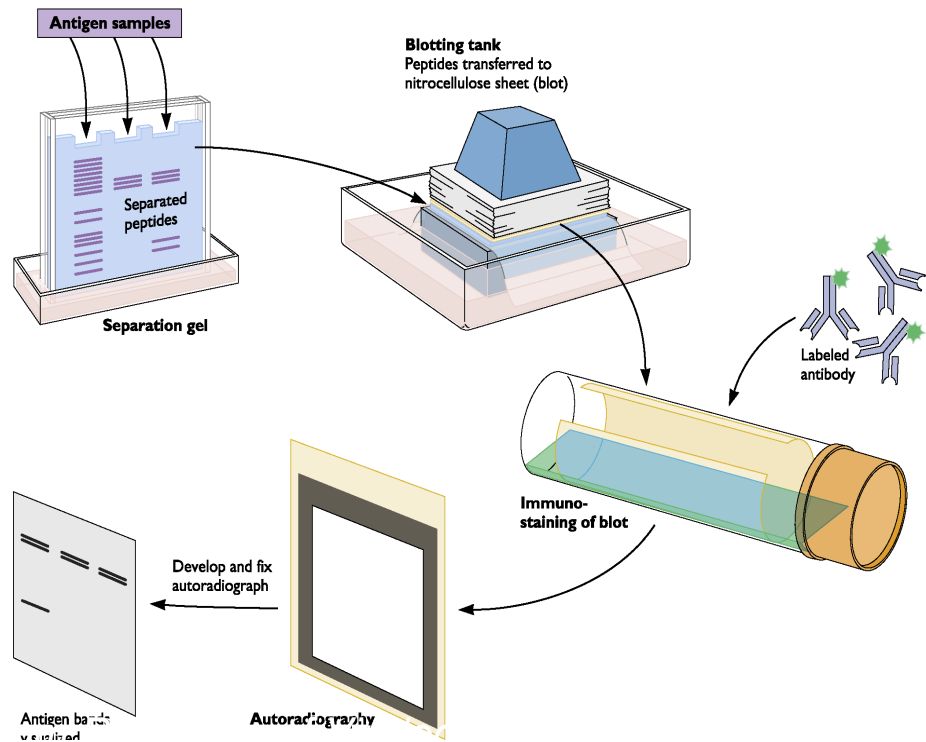
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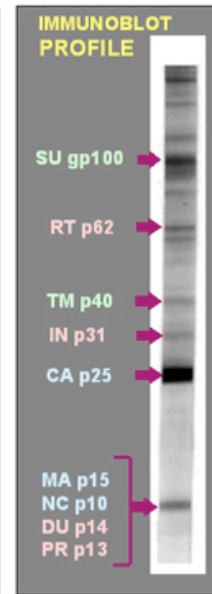
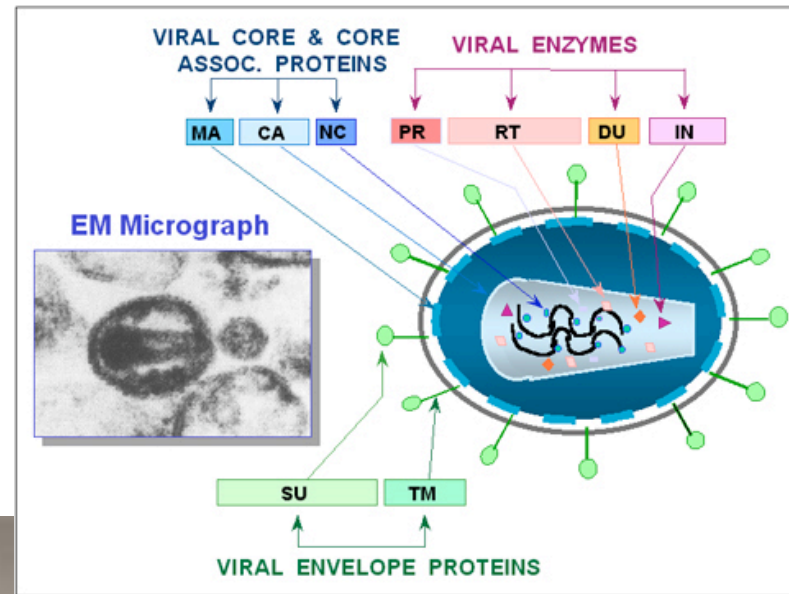
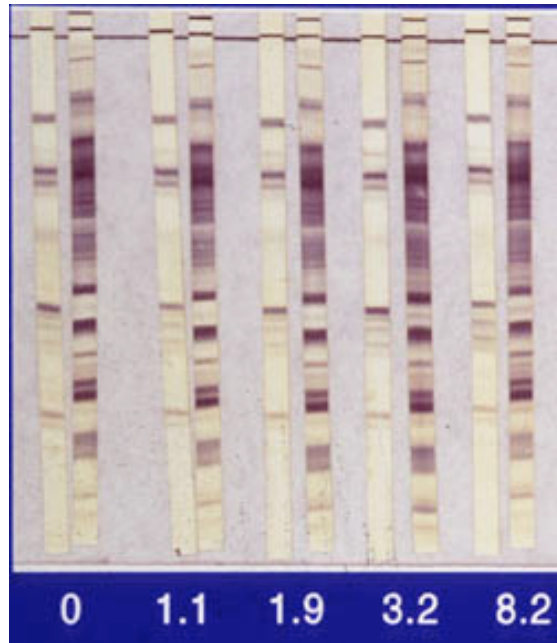
# Diagnostic methods in Virology: SEROLOGY: WESTERN BLOT



Caposio et al., Virology, 2004

# Diagnostic methods in Virology:

## SEROLOGY: WESTERN BLOT



# Diagnostic methods in Virology:

## SEROLOGY

### USEFULNESS 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**

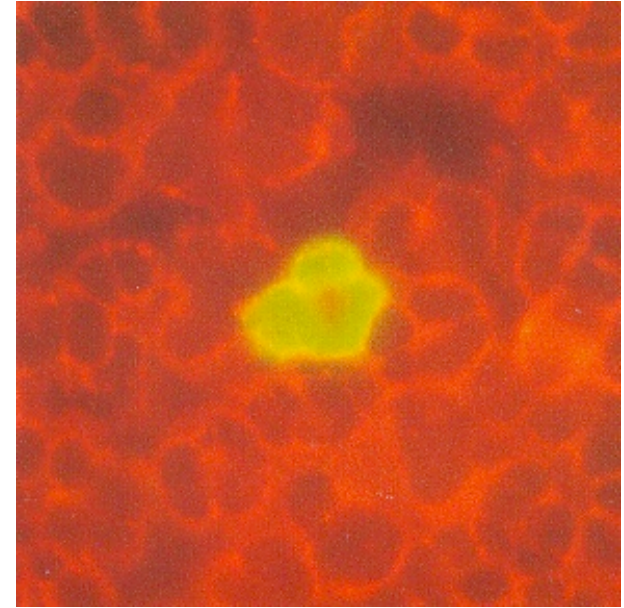
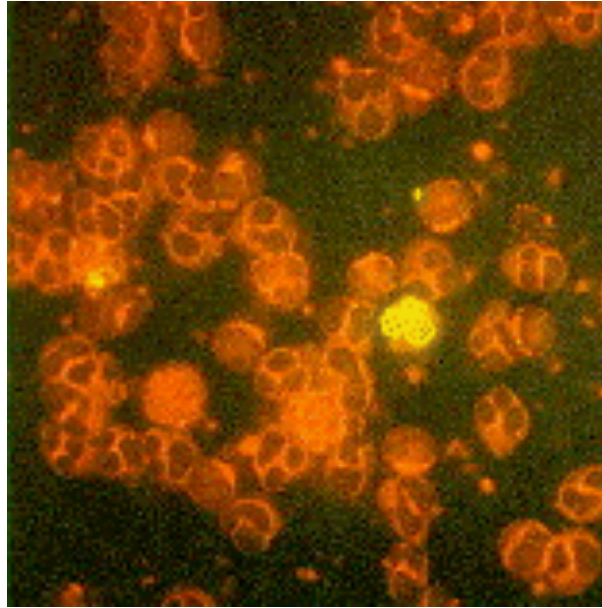
Infection of Human Foreskin Fibroblasts (HFF) and then infectious virus identification by IF (IE1) at 24 h p.i. - **VIREMIA**

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

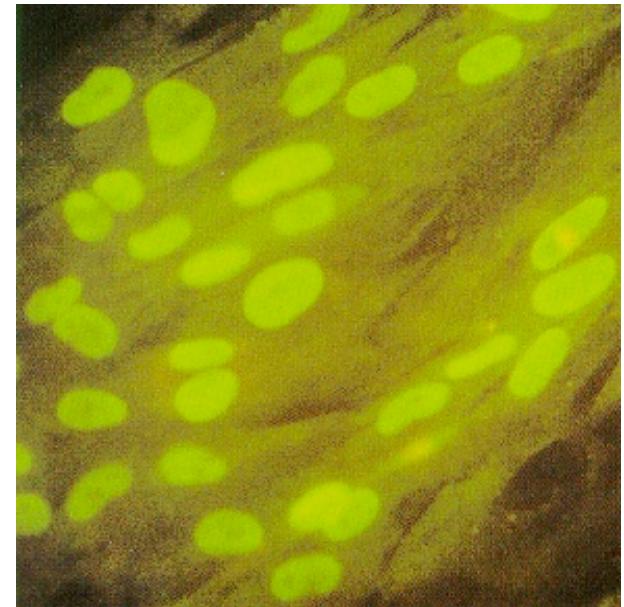
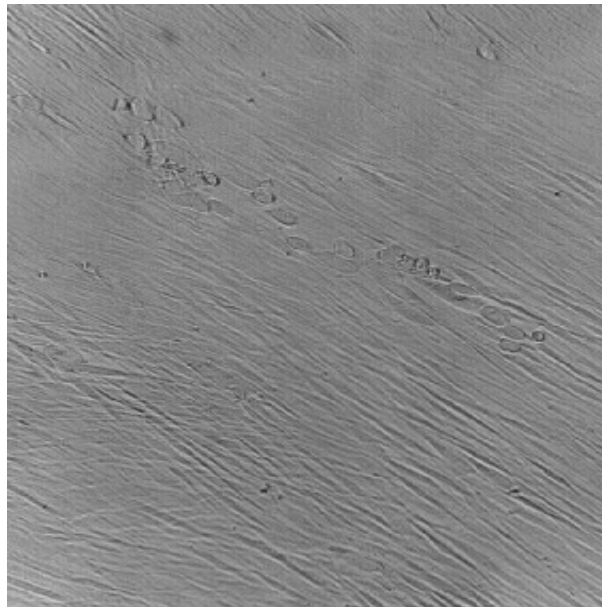
qPCR - n. of viral genomes – **DNAEMIA**  
RT-real time PCR - **RNAEMIA**



HCMV  
antigenemia



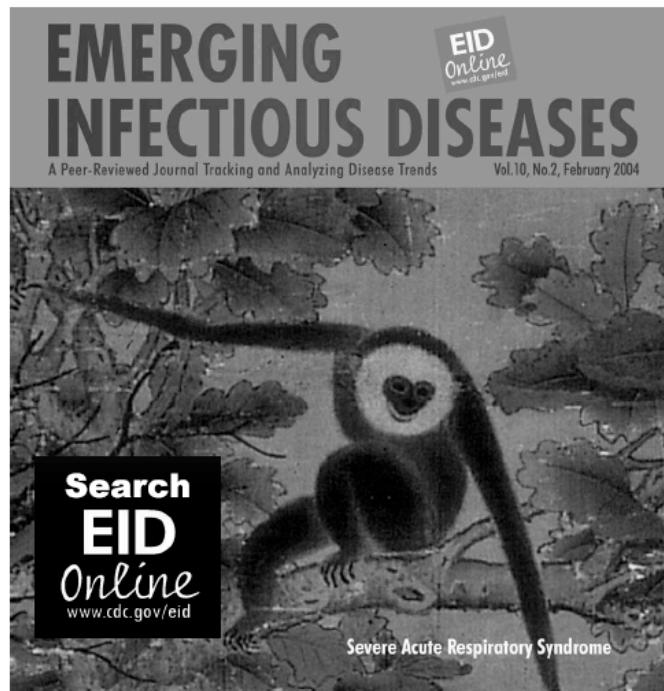
HCMV  
viremia



# Diagnostic methods in Virology:

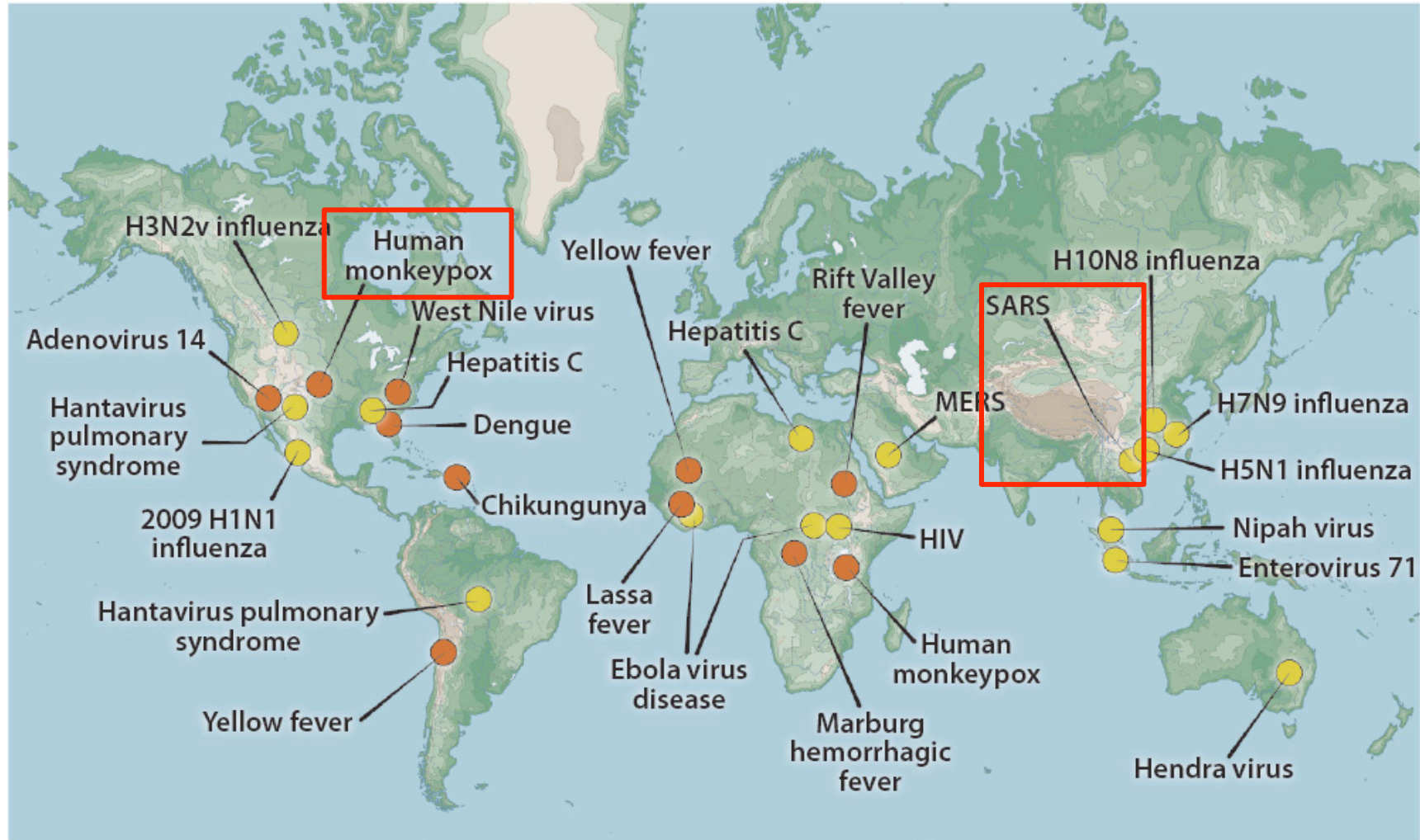
## THE MOLECULAR REVOLUTION

There will still be room for conventional methods ?





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



<p>● Newly emerging</p>	<p><b>Developments facilitating spread</b></p> <ul style="list-style-type: none"> <li>• Commercial air travel</li> <li>• Global trade</li> <li>• Urbanization</li> <li>• Unchecked population growth</li> <li>• Climate change</li> </ul>	<p><b>Advances facilitating control</b></p> <ul style="list-style-type: none"> <li>• Genome sequencing to identify emerging viruses</li> <li>• Global communication networks</li> <li>• Rapid diagnostics</li> <li>• New approaches to vaccine and therapeutic design</li> </ul>
<p>● Reemerging</p>		



## 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.



# 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<sup>1</sup>

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

## Monkeypox outbreak-CDC final report, July 30, 2003

- ❖ 72 suspected cases of monkeypox investigated, primarily in Wisconsin, Indiana, and Illinois
- ❖ In 37 cases, monkeypox infection 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:

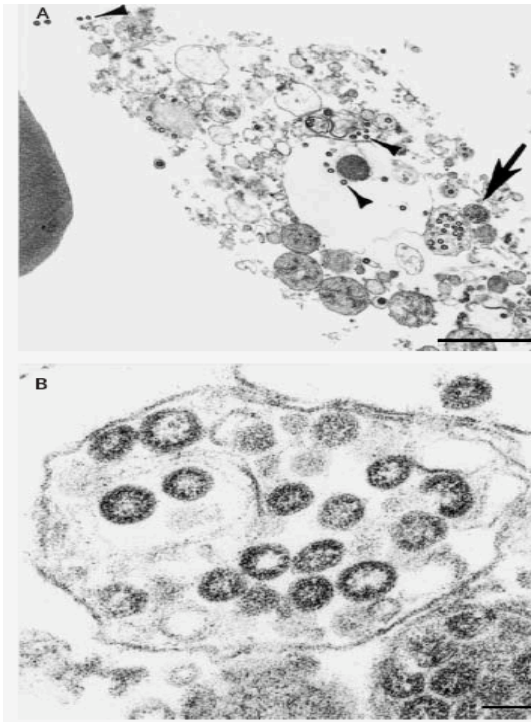
## Story of an unprecedented success of international collaboration - 2003

[www.who.int/csr/sars](http://www.who.int/csr/sars)

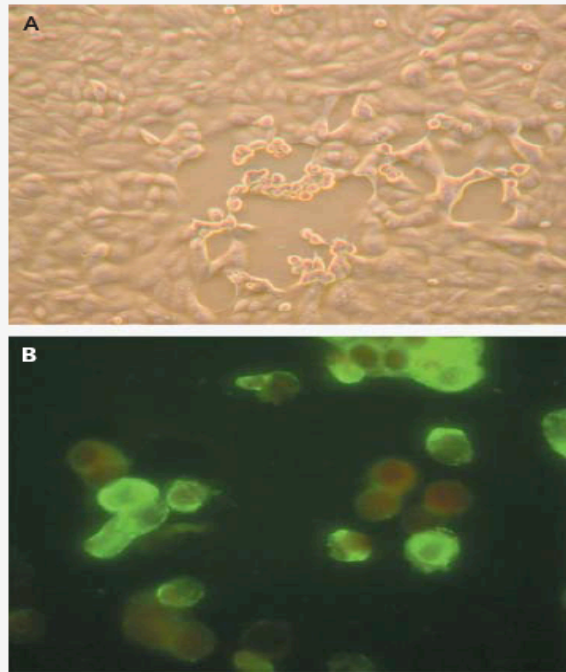




# Etiology establishment



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

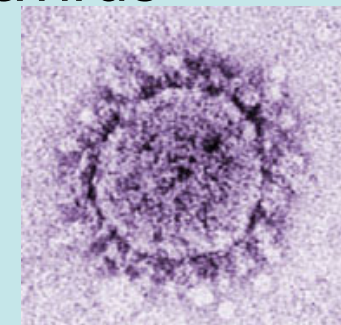


**Figure 1.** Vero E6 cells Inoculated with Oropharyngeal Specimens from Patients with SARS.

Ksiazek TG: N.Engl. J Med. 2003

## Strategy leading to the discovery of a new coronavirus

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





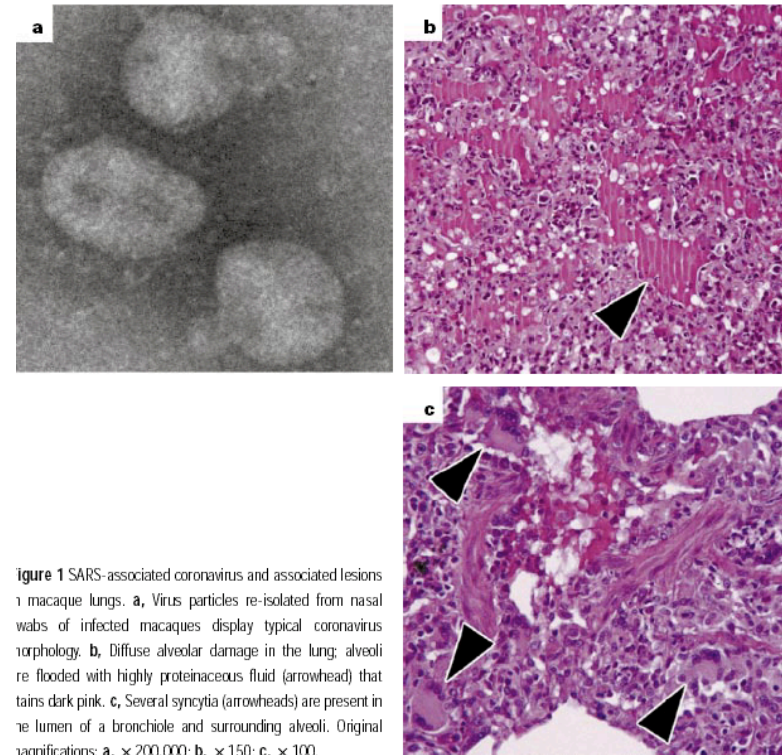
# Koch's postulates fulfilled for SARS virus

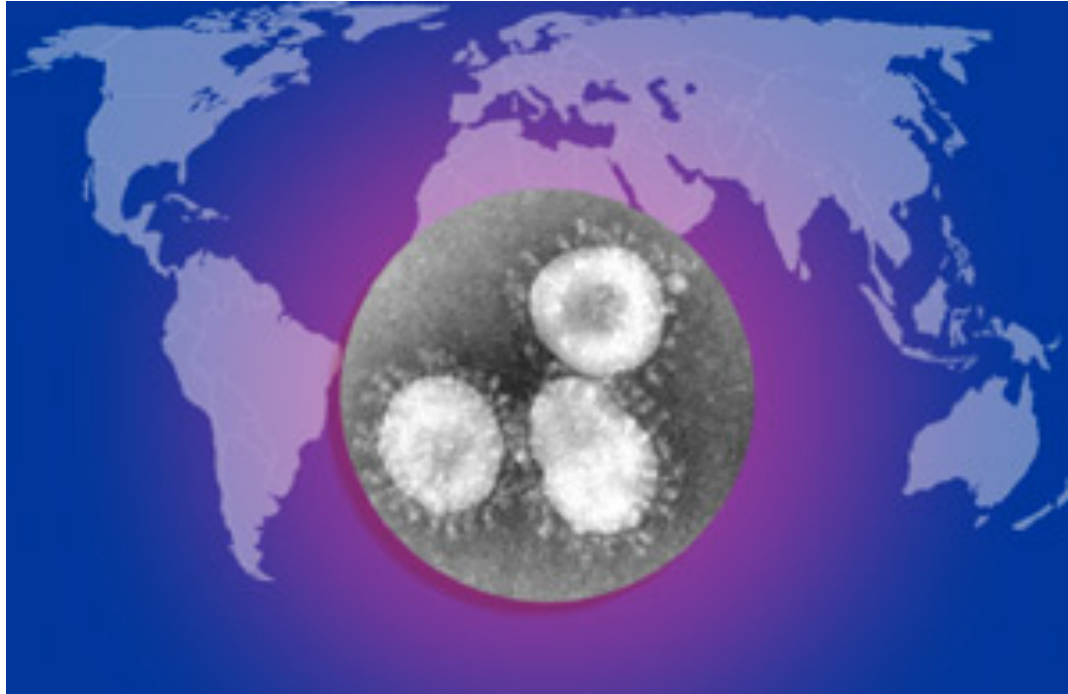
Ron A. M. Fouchier\*, Thijs Kuiken\*, Martin Schutten\*, Geert van Amerongen\*, Gerard J. J. van Doornum\*, Bernadette G. van den Hoogen\*, Malik Peiris†, Wilina Lim‡, Klaus Stöhr§, Albert D. M. E. Osterhaus\*

## 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

NATURE | VOL 423 | 15 MAY 2003 |





**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*