VIROLOGY

Virus cultivation and assay 2

Virus assay: Quantitative virology

- Measurement of infectious units
- Measurement of virus particles and their components

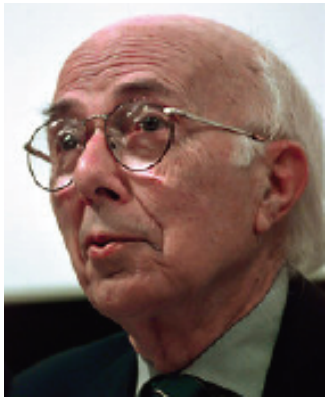
Measurement of infectious units

- *Plaque assay*
- *Immunoreactive focus assay*
- *Infectious center assay*
- *Transformation assay*
- *Endpoint dilution*

The third revolution in Animal Virology

1952 – Introduction of the Plaque Assay

R. Dulbecco & M.Vogt. Some problems of animal virology as studied by the plaque technique. *Cold Spring Harb Symp Quant Biol* 18: 273-279. 1953



PRODUCTION OF PLAQUES IN MONOLAYER TISSUE CULTURES BY SINGLE PARTICLES OF AN ANIMAL VIRUS

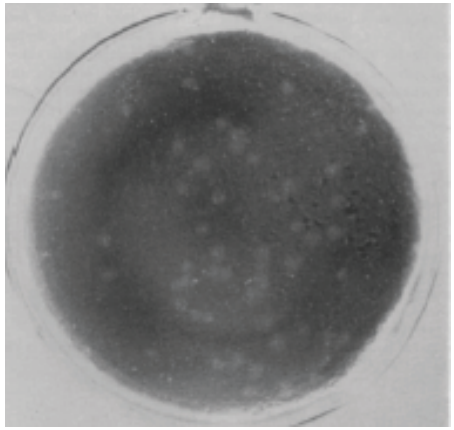
By Renato Dulbecco

California Institute of Technology, Pasadena, California

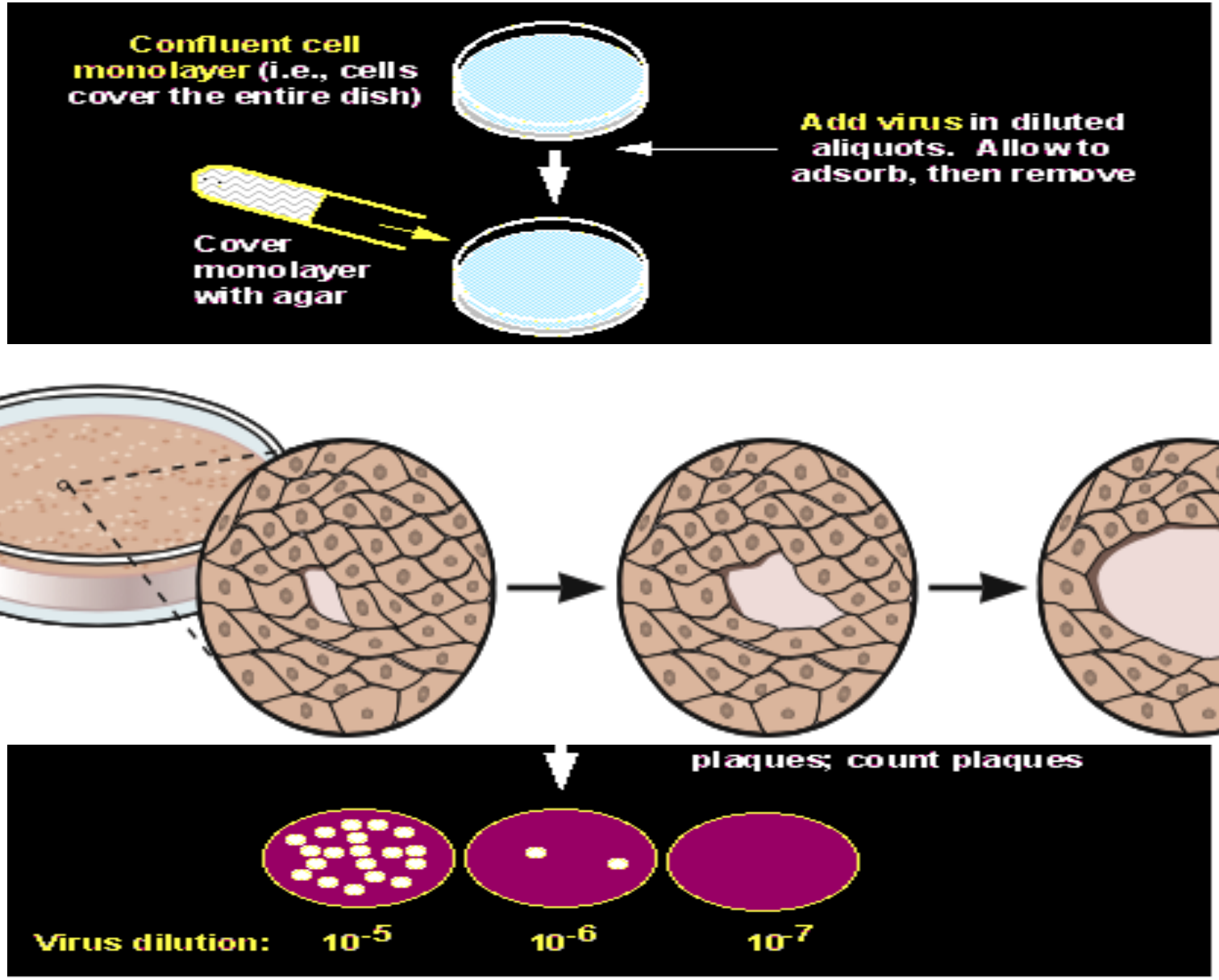
Read before the Academy, April 29, 1952

Research on the growth characteristics and genetic properties of animal viruses has stood greatly in need of improved quantitative techniques, such as those used in the related field of bacteriophage studies.

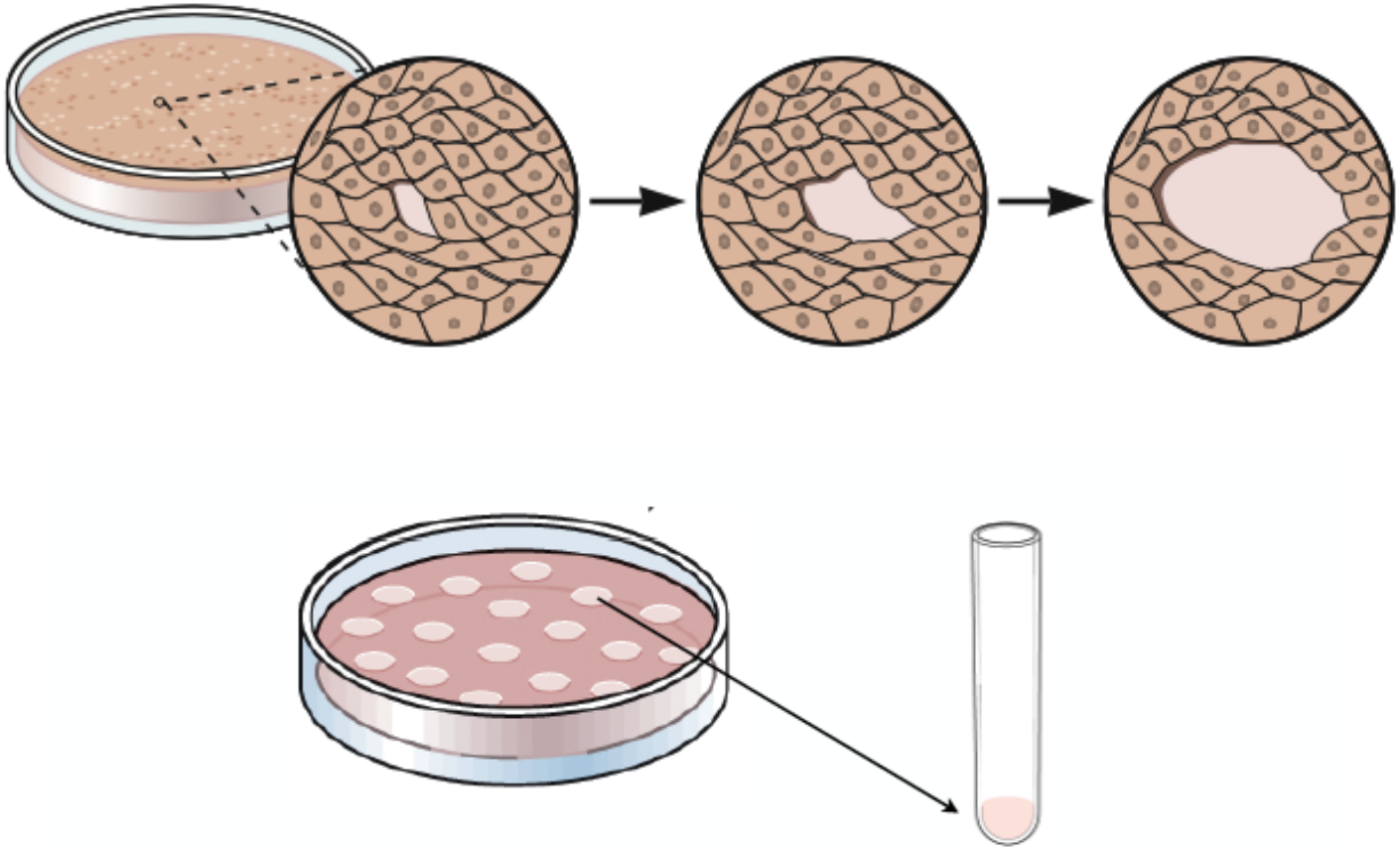
The requirements for a quantitative virus technique are as follows: (1) The use of a uniform type of host cell; (2) an accurate assay technique; (3) the isolation of the progeny of a single virus particle; and (4) the separate isolation of each of the virus particles produced by a single infected



Outline of Viral Plaque Assay



Plaque formation and purification

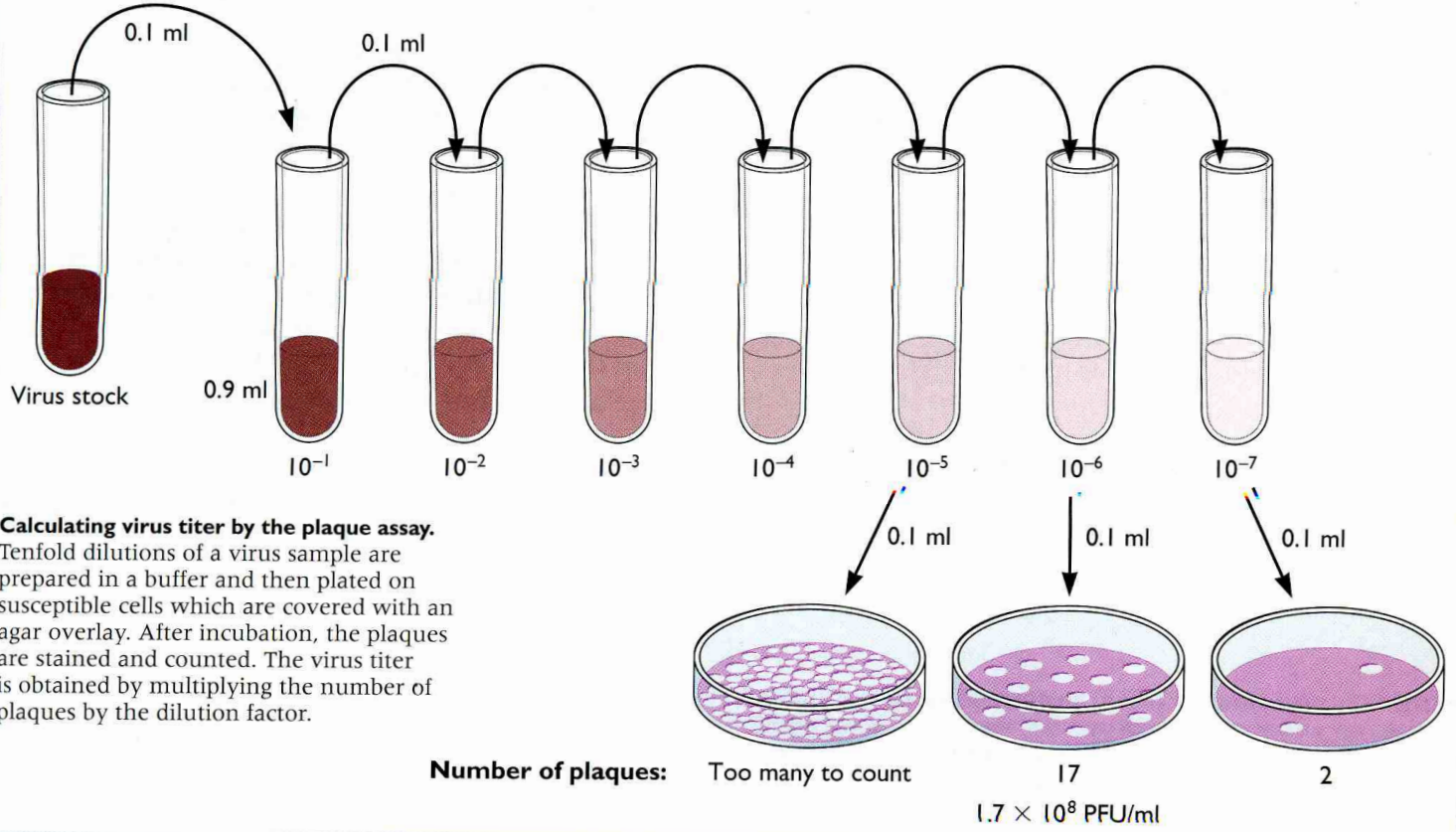


A method for obtaining clonal virus stocks. Usually it is performed three times consecutively

BOX 2.5 METHODS

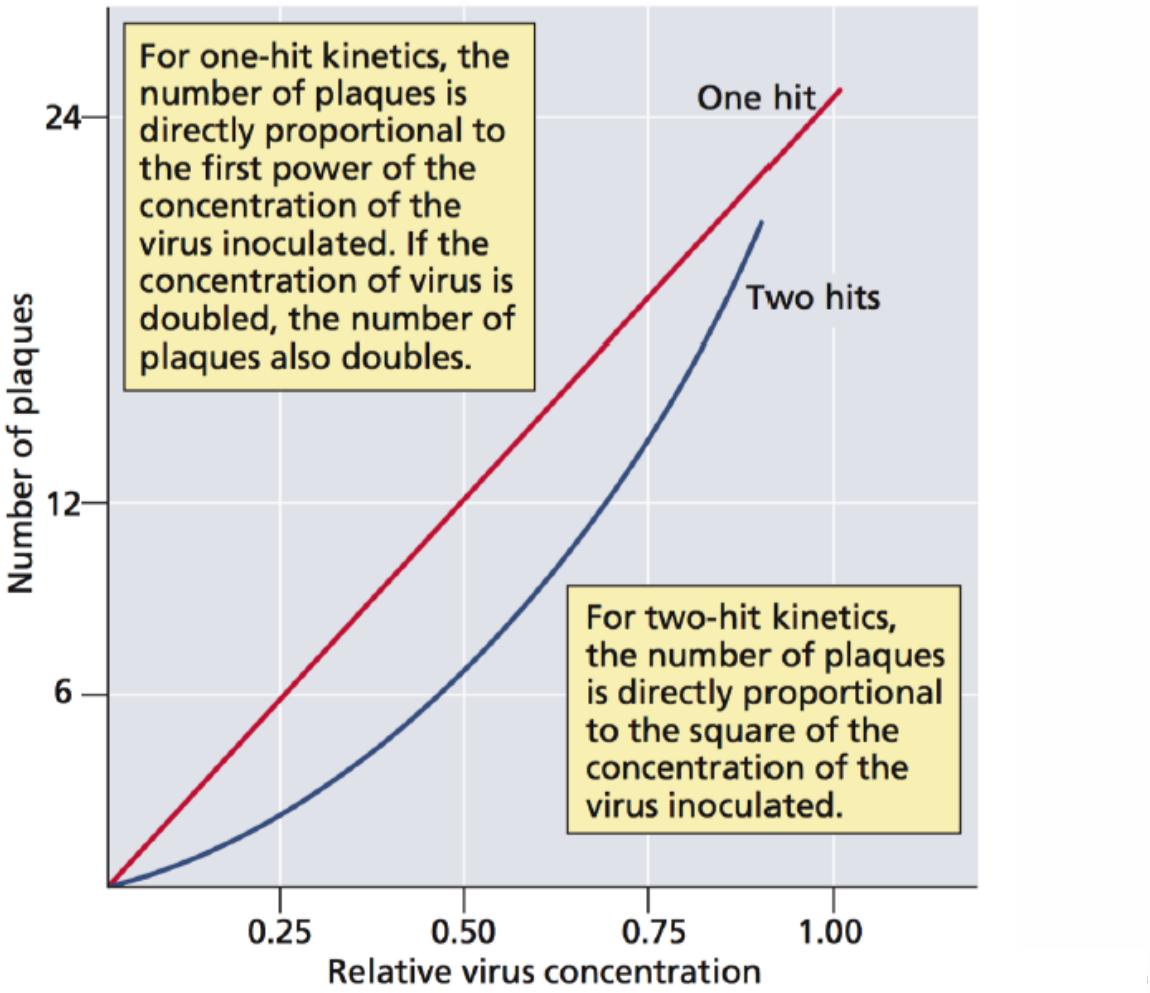
Calculating virus titer from the plaque assay

To calculate the titer of a virus in plaque-forming units per milliliter, 10-fold serial dilutions of a virus stock are prepared, and 0.1-ml aliquots are inoculated onto susceptible cell monolayers (see figure). After a suitable incubation period, the monolayers are stained and the plaques are counted. To minimize error in calculating the virus titer, only plates containing between 10 and 100 plaques are counted, depending on the area of the cell culture vessel. According to statistical principles, when 100 plaques are counted, the sample titer varies by ±10%. For accuracy, each dilution is plated in duplicate or triplicate (not shown in the figure). Plates with more than 100 plaques are generally not counted because the plaques may overlap, causing inaccuracies. In the example shown in the figure, 17 plaques are observed on the plate produced from the 10^{-6} dilution. Therefore, the 10^{-6} dilution tube contains 17 PFU per 0.1 ml, or 170 PFU per ml, and the titer of the virus stock is 170×10^6 or 1.7×10^8 PFU/ml.



Calculating virus titer by the plaque assay. Tenfold dilutions of a virus sample are prepared in a buffer and then plated on susceptible cells which are covered with an agar overlay. After incubation, the plaques are stained and counted. The virus titer is obtained by multiplying the number of plaques by the dilution factor.

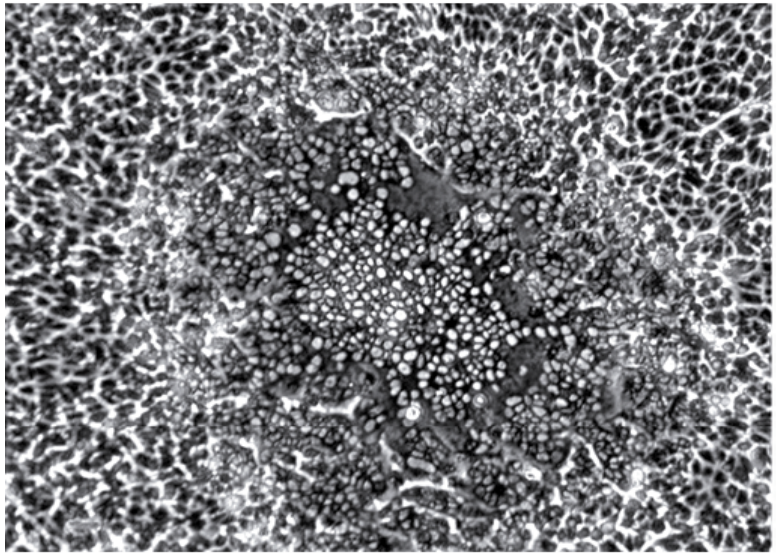
The dose-response curve of plaque assay

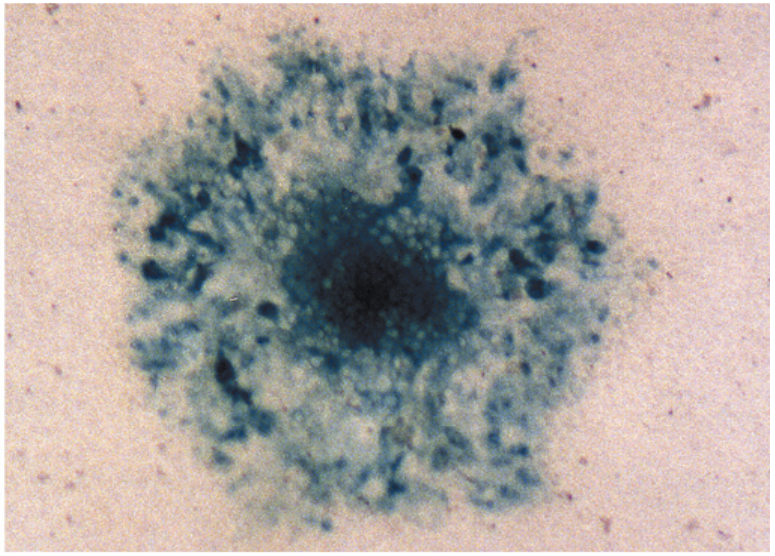


For the majority of animal viruses there is a linear relationship between the number of infectious particles the plaque count. One infectious particle is therefore sufficient to initiate infection, and the virus is said to infect cells with **one-hit kinetics**.

Plaques formed by different animal viruses

A





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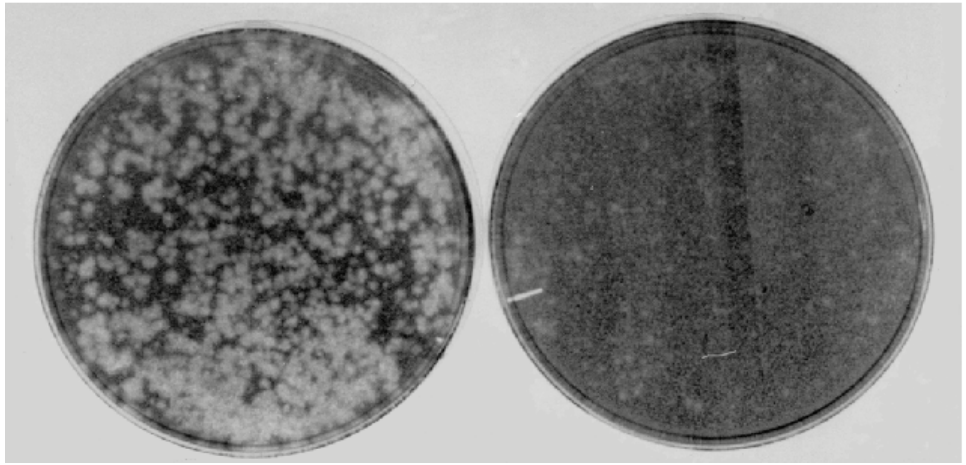

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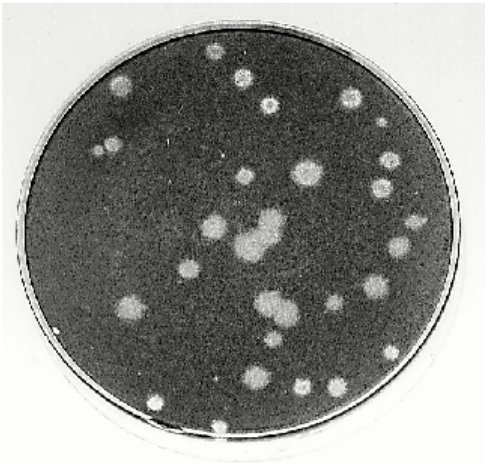
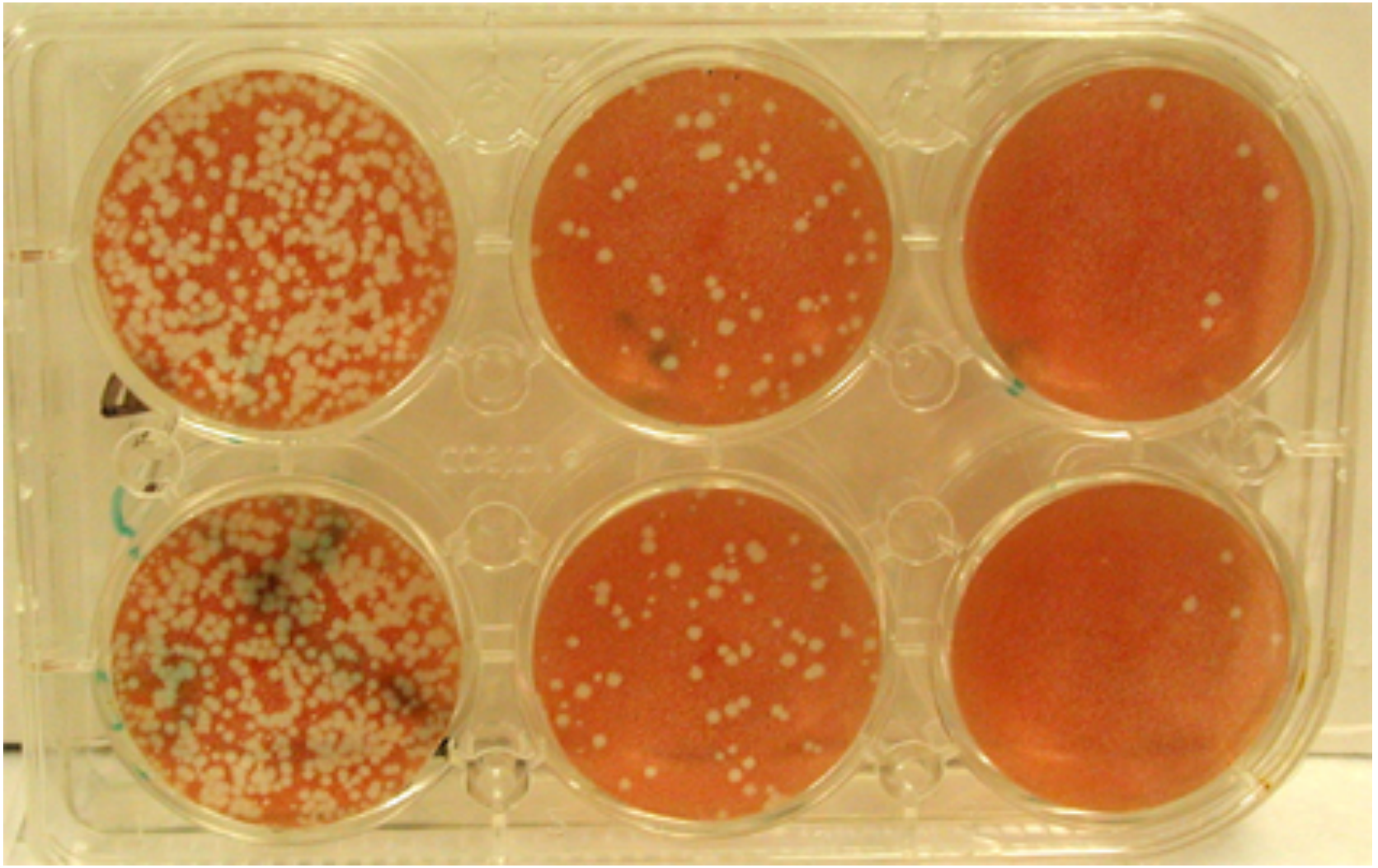
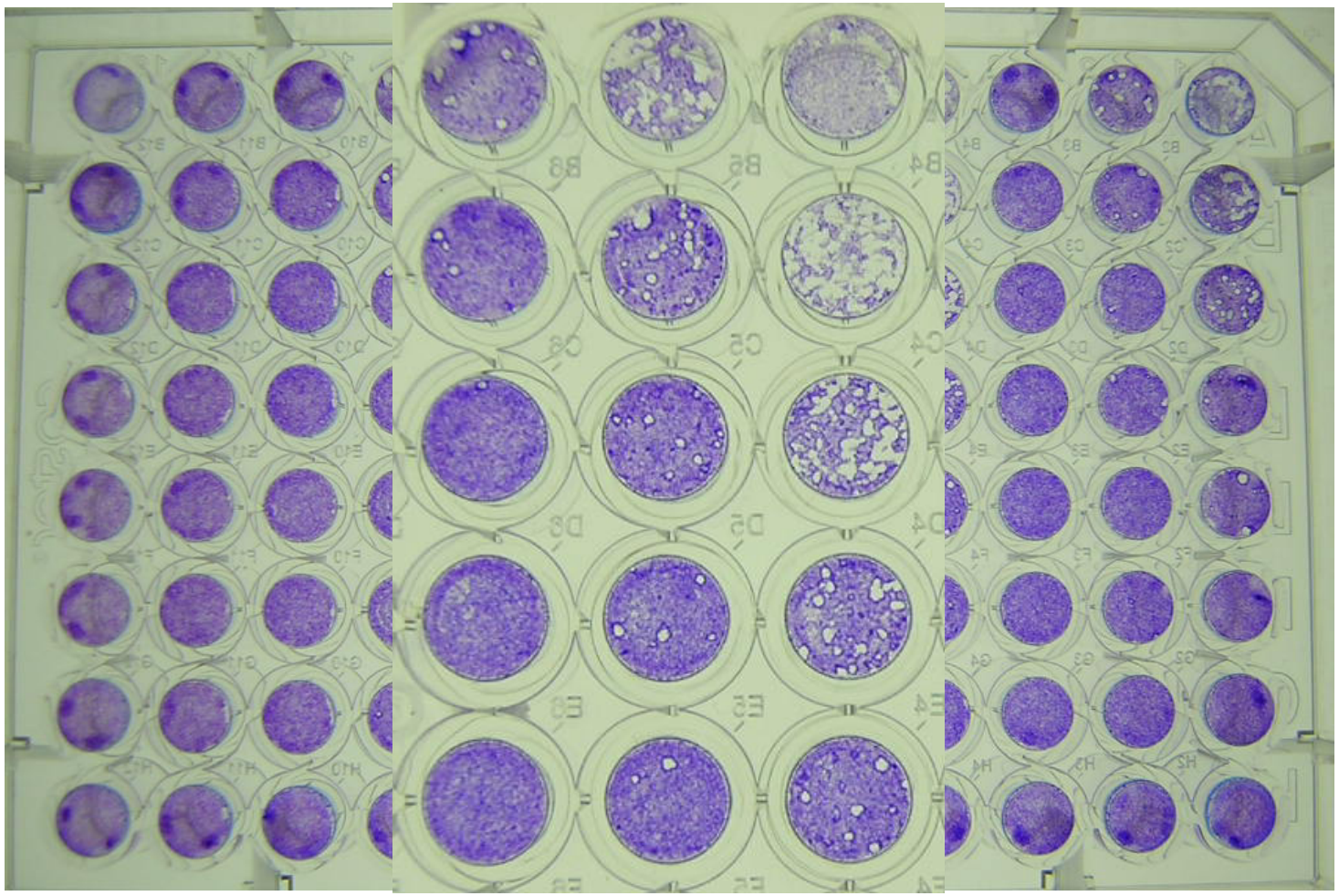


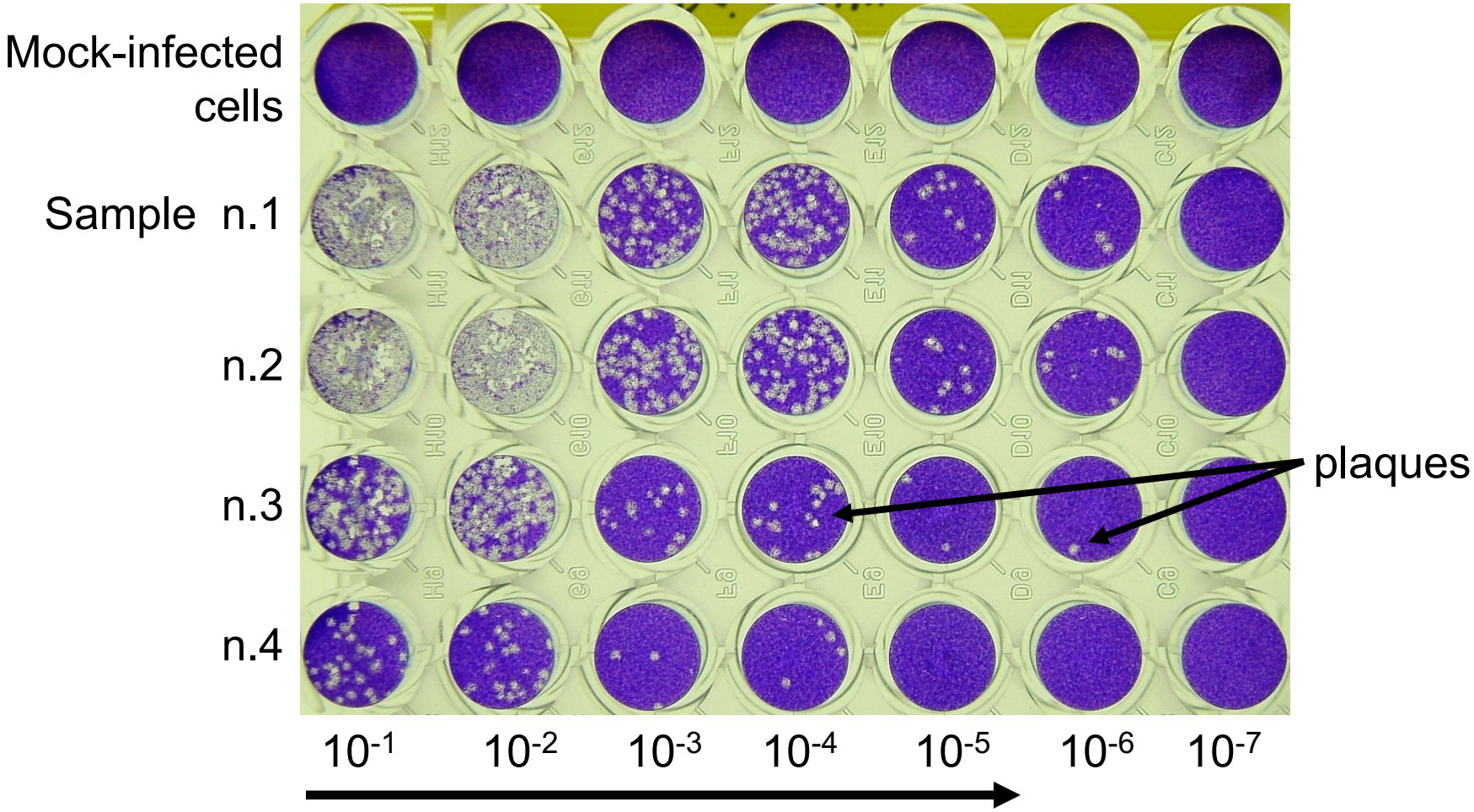
An example of Plaque Assay





An example of Plaque Assay: MCMV

An example of Plaque Assay: HSV1

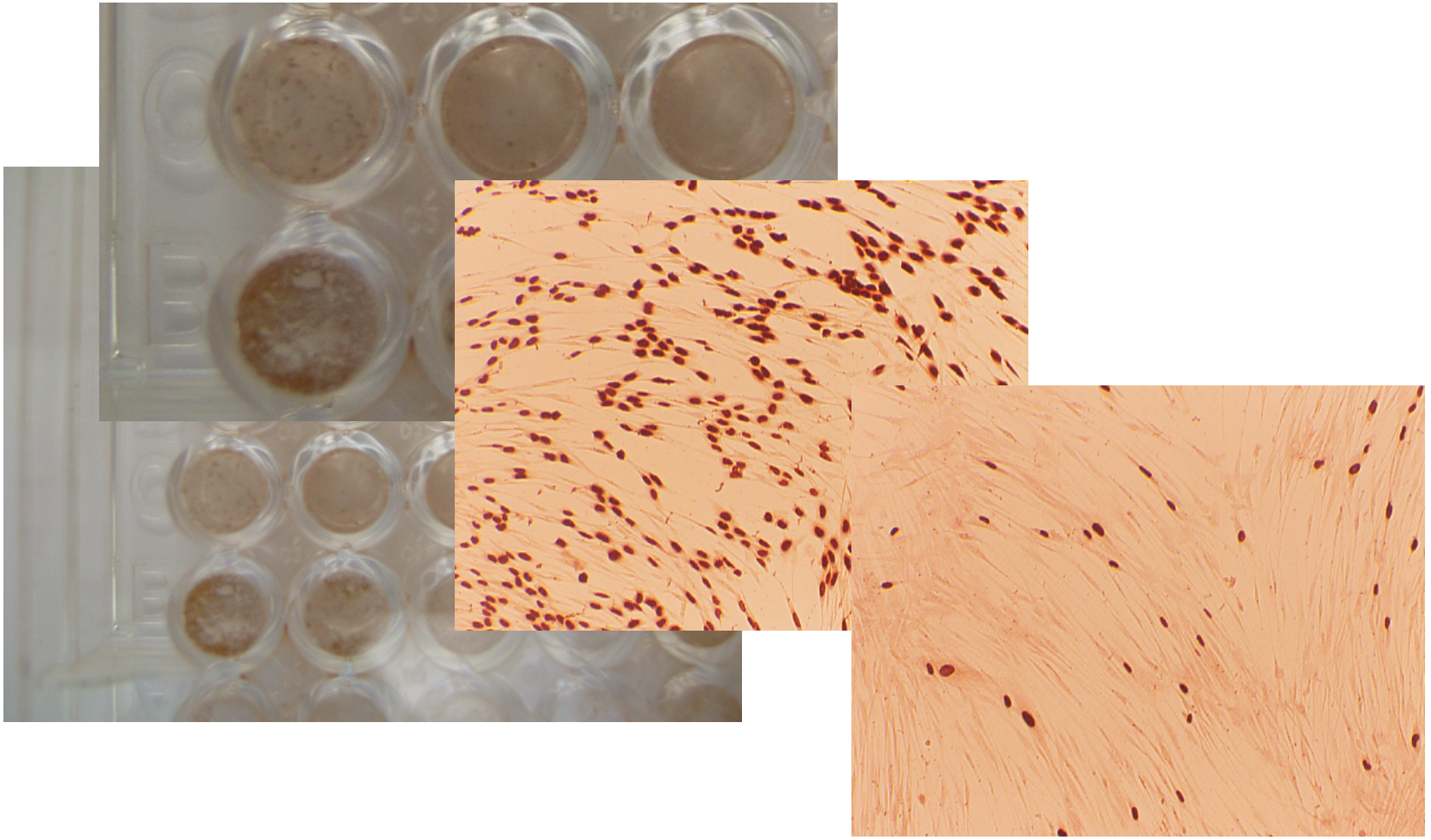


Number of plaques in the original sample = plaques : dV

d = dilution, V = volume of diluted virus added to the well

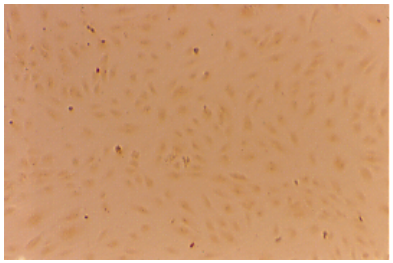
Which is the HSV-1 #1 titer? (V= 0.1 ml)

Titration of HCMV infectivity by quantitative IE proteins IPA staining (48 hpi):
an example of immunoreactive focus assay

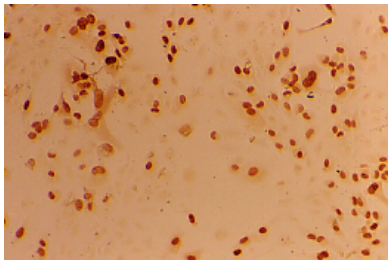


Immunoperoxidase staining of HCMV IE proteins at 48 hpi in infected-HUVEC:

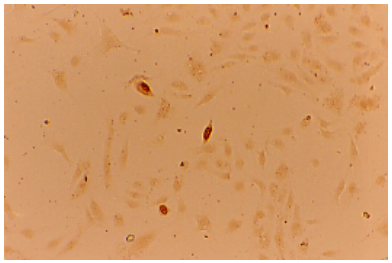
as a quantitative assessment of the extent of virus replication



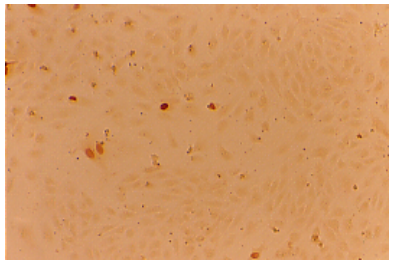
mock



VR1814



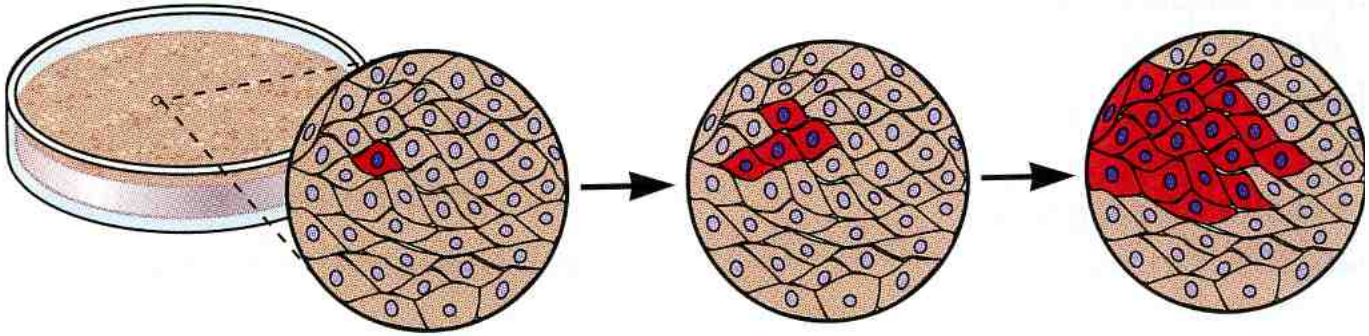
VR1814 + AS602868, 1μM



VR1814 + AS602868, 10μM

Caposio et al., Antivir. Res. 2007

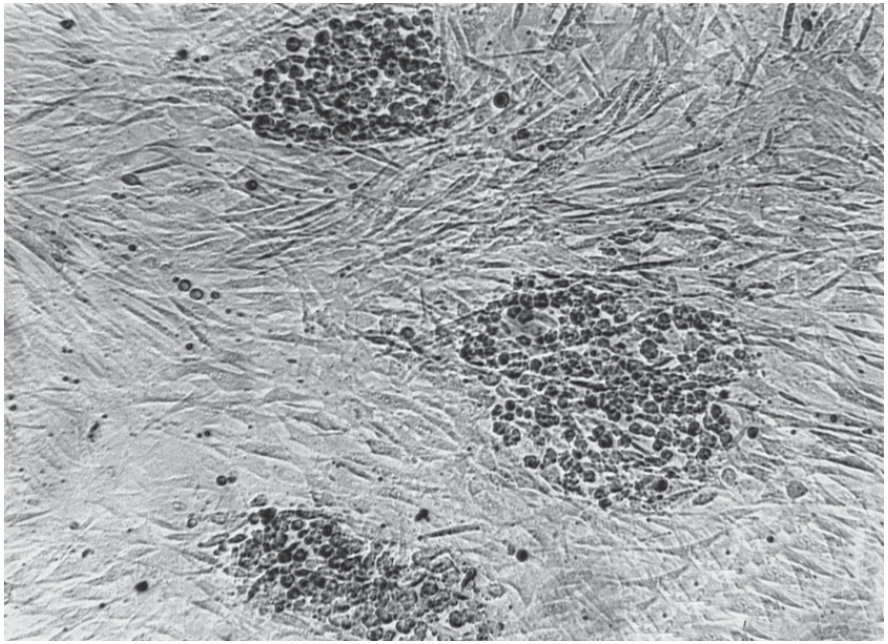
Infectious center assay:
a quantitative assessment of the ability of virus to spread to an indicator culture



The **infectious center assay** allows one to determine the fraction of cells within a culture that are infected with virus. In this case, the infected cells are suspended, counted, and plated onto monolayers of susceptible cells (indicator cells), which are then overlaid with methylcellulose.

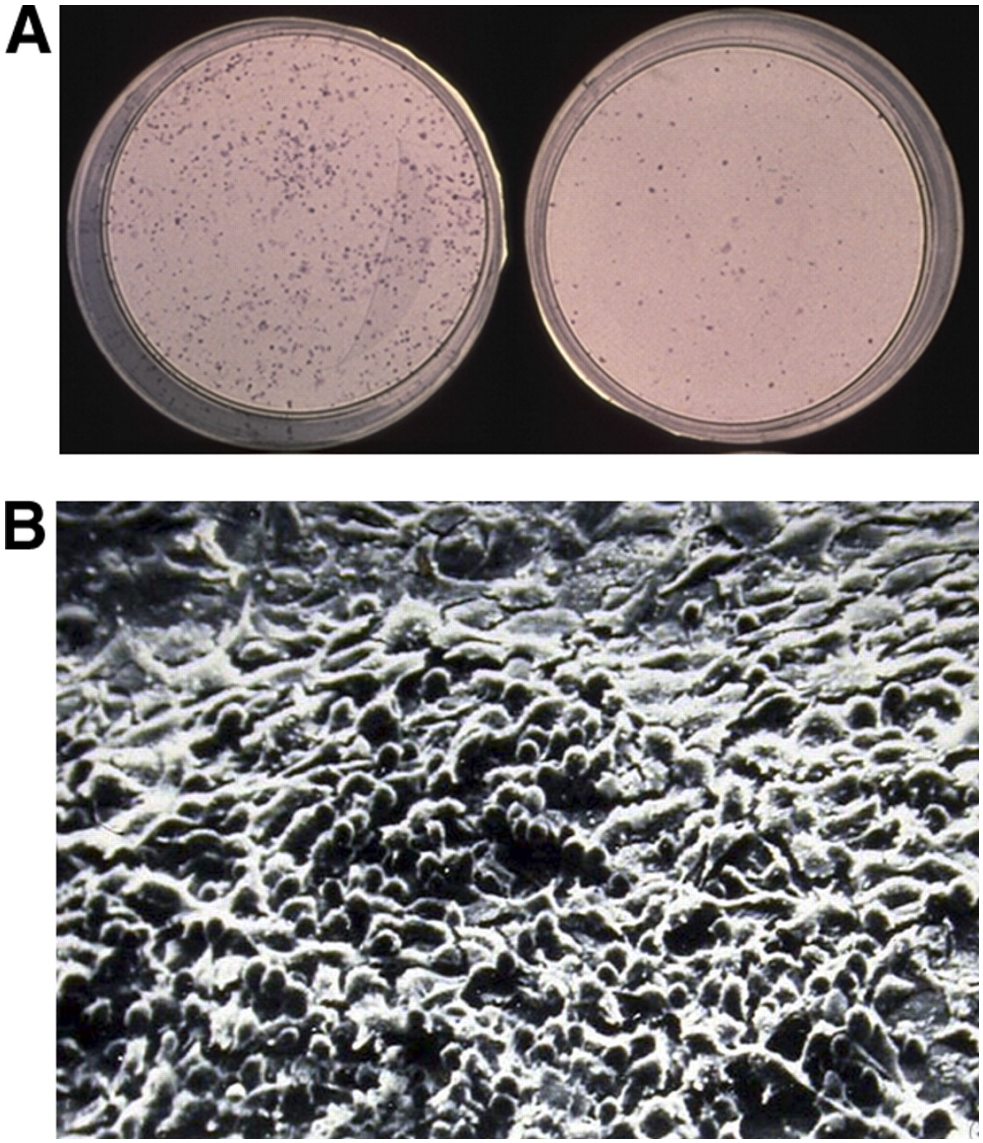
The number of plaques observed represents the number of infected cells in the original culture that harbored virus and infected the underlying indicator monolayer upon co-culture, thus allowing the quantitative evaluation of the **percentages of infected cells in the original culture able to spread virus** to the indicator culture.

Transformation Assay



CEFs transformed by RSV

Cell transformation by RSV. (A) The RSV focus assay of transformed cells in a chick embryo fibroblast monolayer as described by Temin and Rubin (1958) showing a 1:100 and 1:1000 dilution of the virus stock.



Weiss R A , Vogt P K J Exp Med 2011;208:2351-2355

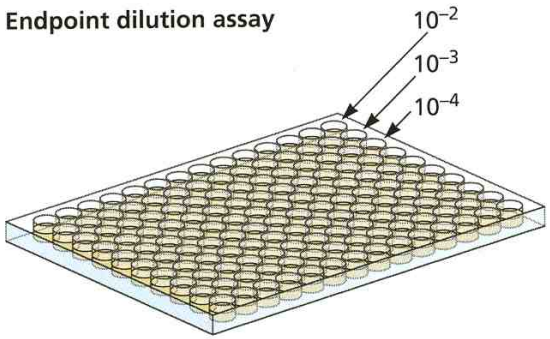
Virus assay: Endpoint Methods

- How to quantitate viruses that cannot be adapted to either a plaque or a focus assay?
- The infectious dose 50 concept (ID_{50})
- Tissue culture infective dose 50 ($TCID_{50}$)
- Egg infectious dose 50 (EID_{50})
- Lethal dose 50 (LD_{50})

BOX 2.6

METHODS

End-point dilution assays



Virus dilution	Cytopathic effect									
10^{-2}	+	+	+	+	+	+	+	+	+	+
10^{-3}	+	+	+	+	+	+	+	+	+	+
10^{-4}	+	+	−	+	+	+	+	+	+	+
10^{-5}	−	+	+	−	+	−	−	+	−	+
10^{-6}	−	−	−	−	−	−	+	−	−	−
10^{-7}	−	−	−	−	−	−	−	−	−	−

End-point dilution assays are usually carried out in multiwell plastic plates (see the figure). In the example shown in the first table, 10 monolayer cell cultures were infected with each virus dilution. After the incubation period, plates that displayed cytopathic effect were scored +. Fifty percent of the cell cultures displayed cytopathic effect at the 10^{-5} dilution, and therefore the virus stock contains 10^5 $TCID_{50}$ units.

In most cases, the 50% end point does not fall on a dilution tested as shown in the example; for this reason, various statistical procedures have been developed to calculate the end point of the titration. In one popular method, the dilution containing the ID_{50} is identified by interpolation between the dilutions on either side of this value. The assumption is made that the location of the 50% end point varies linearly with the log of the dilution. Because the number of test units used at each dilution is usually small, the accuracy of this method is relatively low. For example, if six test units are used at each 10-fold dilution, differences in virus titer of only 50-fold or more can be detected reliably. The method is illustrated in the second example, in which the lethality of poliovirus in mice is the end point. Eight mice were inoculated per dilution. In the method of Reed and Muench, the results are pooled, as shown in the table, which equalizes chance variations (another way to achieve the same result would be to utilize greater numbers of animals at each dilution). The interpolated value of the 50% end point, which in this case falls between the 5th and 6th dilutions, is calculated to be $10^{-6.5}$. The virus sample therefore contains $10^{6.5}$ LD_{50}s. The LD_{50} may also be calculated as the concentration of the stock virus in PFU per milliliter (1×10^9) times the 50% end-point titer. In the example shown, the LD_{50} is 3×10^2 PFU.

Reed LJ, Muench H. 1938. A simple method of estimating fifty per cent endpoints. *Am J Hyg* **27**:493–497.

Dilution	Alive	Dead	Total alive	Total dead	Mortality ratio	Mortality (%)
10^{-2}	0	8	0	40	0/40	100
10^{-3}	0	8	0	32	0/32	100
10^{-4}	1	7	1	24	1/25	96
10^{-5}	0	8	1	17	1/18	94
10^{-6}	2	6	3	9	3/12	75
10^{-7}	5	3	8	3	8/11	27

Endpoint Method: Data for ID_{50}

Logs of virus dilution	Infected test units	Cumulative infected (A)	Cumulative uninfected (B)	Ratio of A/(A+B)	Percent infected
-4					
-5					
-6					
-7					

Endpoint Method: Data for ID_{50}

Logs of virus dilution	Infected test units	Cumulative infected (A)	Cumulative uninfected (B)	Ratio of A/(A+B)	Percent infected
-4	10/10				
-5	7/10				
-6	4/10				
-7	0/10				

Endpoint Method: Data for ID_{50}

Logs of virus dilution	Infected test units	Cumulative infected (A)	Cumulative uninfected (B)	Ratio of A/(A+B)	Percent infected
-4	10/10	21			
-5	7/10	11			
-6	4/10	4			
-7	0/10	0			

Endpoint Method: Data for ID_{50}

Logs of virus dilution	Infected test units	Cumulative infected (A)	Cumulative uninfected (B)	Ratio of A/(A+B)	Percent infected
-4	10/10	21	0		
-5	7/10	11	3		
-6	4/10	4	9		
-7	0/10	0	19		

Endpoint Method: Data for ID_{50}

Logs of virus dilution	Infected test units	Cumulative infected (A)	Cumulative uninfected (B)	Ratio of A/(A+B)	Percent infected
-4	10/10	21	0	21/21	
-5	7/10	11	3	11/14	
-6	4/10	4	9	4/13	
-7	0/10	0	19	0/19	

Endpoint Method: Data for ID_{50}

Logs of virus dilution	Infected test units	Cumulative infected (A)	Cumulative uninfected (B)	Ratio of A/(A+B)	Percent infected
-4	10/10	21	0	21/21	100
-5	7/10	11	3	11/14	78.5
-6	4/10	4	9	4/13	30.7
-7	0/10	0	19	0/19	0.00

Endpoint Method: calculation of $TCID_{50}$

- $I = h \dfrac{\text{(\% positive above 50\%)-50\%}}{\text{(\% positive above 50\%)-(\%positive below 50\%)}}$

h= dilution factor (10)

- I =(78.5% – 50%)/(78.5%-30.7%) =0.8
- 50% endpoint titer $=10^{[\text{log dilution} > 50\% - (I \times \log h)]}$
- **$ID_{50} = 10^{-5 -(0.8 \times 1.0)} = 10^{-5.8}$**

Particles vs. Infectious Particles (particle to-PFU-ratio)

- ✓ # of physical particles : # of infectious particles
- ✓ A single particle *can* initiate infection
- ✓ Not all viruses are successful:
  - Damaged particles
  - Mutations
  - Complexity of infectious study
- ✓ Complicates study

Particles vs. Infectious Particles

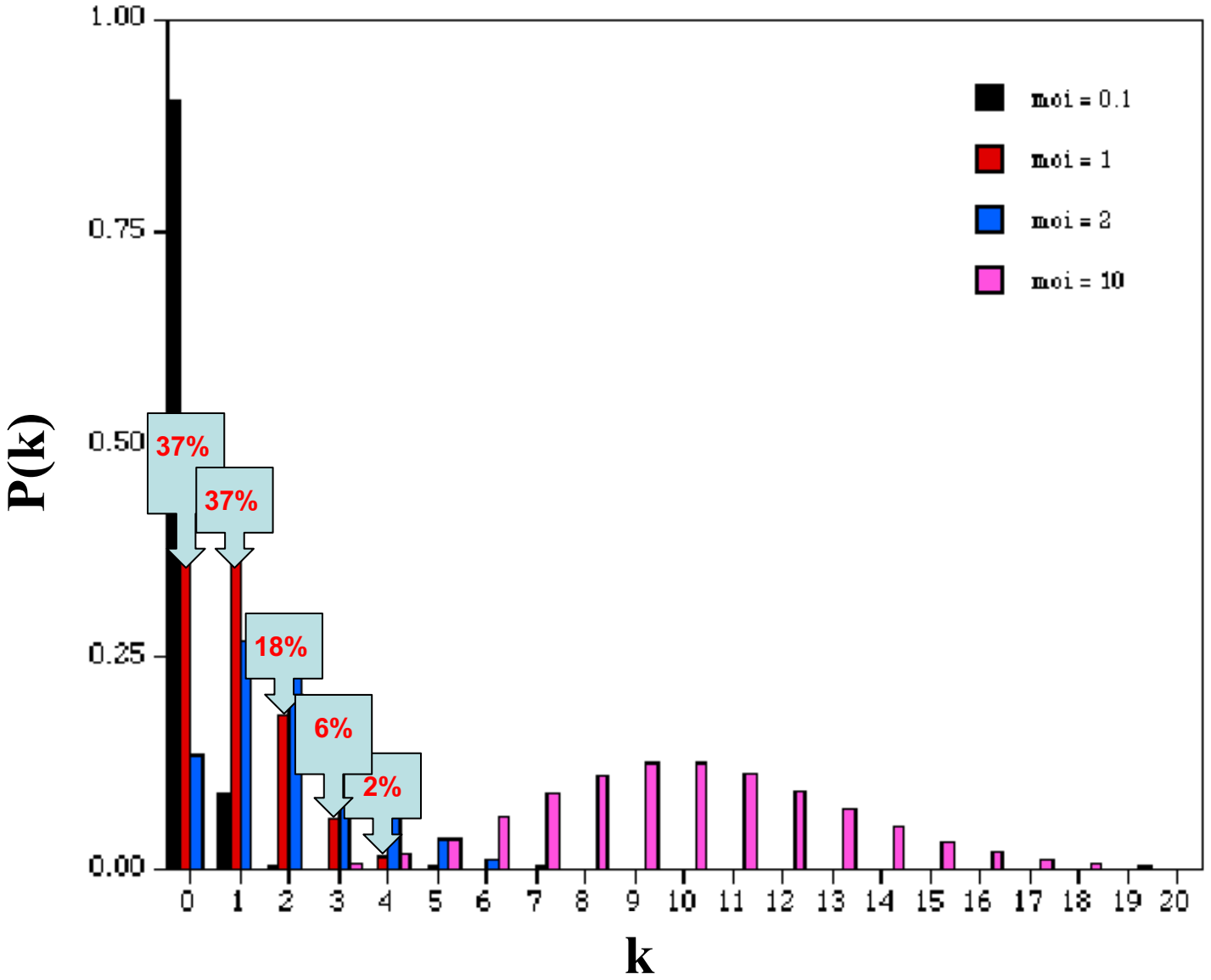
Table 2.2 Particle-to-PFU ratios of some animal viruses

Virus	Particle/PFU ratio
Adenoviridae	20–100
Alphaviridae	
Semliki Forest virus	1–2
Herpesviridae	
Herpes simplex virus	50–200
Orthomyxoviridae	
Influenza virus	20–50
Papillomaviridae	
Papillomavirus	10,000
Picornaviridae	
Poliovirus	30–1,000
Polyomaviridae	
Polyomavirus	38–50
Simian virus 40	100–200
Poxviridae	1–100
Reoviridae	
Reovirus	10

The Multiplicity of Infection (MOI)

- ✓ Number of infectious particles ADDED per cell
- ✓ Not the number of infectious particles each cell receives
- ✓ Adding 10^7 virus particles to 10^6 cells – MOI of 10 –each cell does NOT receive 10 virions
- ✓ Infection depends on the random collision of virions and cells
- ✓ When susceptible cells are mixed with virus, some cells are uninfected, some receive one, two, three or more particles
- ✓ The distribution of virus particles per cell is best described by the *Poisson distribution*

The Poisson distribution: values of P(k) for various values of MOI and k



The Multiplicity of Infection (MOI)

- $P(k)=m^k e^{-m}/k$
  - **m** = multiplicity of infection (MOI);
  - **K** = number of virus infecting a cell;
  - **P(k)** = fraction of cells infected by **k** virus
  - **m** is calculated from the proportion of uninfected cells P(0)
  - If k is made 0 then, $P(0)=e^{-m}$ and **m**= -ln P(0)

MOI (m)	1	3	5	10
% uninfected cells	0.37	0.05	0.01	0.00

Then, the MOI required to infect 99% of the cells will be:

P(0)=1%=0.01 m= -ln P(0) = - ln(0.01) = **4.6 PFU/cell**

The Multiplicity of Infection (MOI)

Examples:

If 10^6 cells are infected at MOI of 10:
45 cells are uninfected
450 cells receive 1 particle
The rest receive > 1 particle

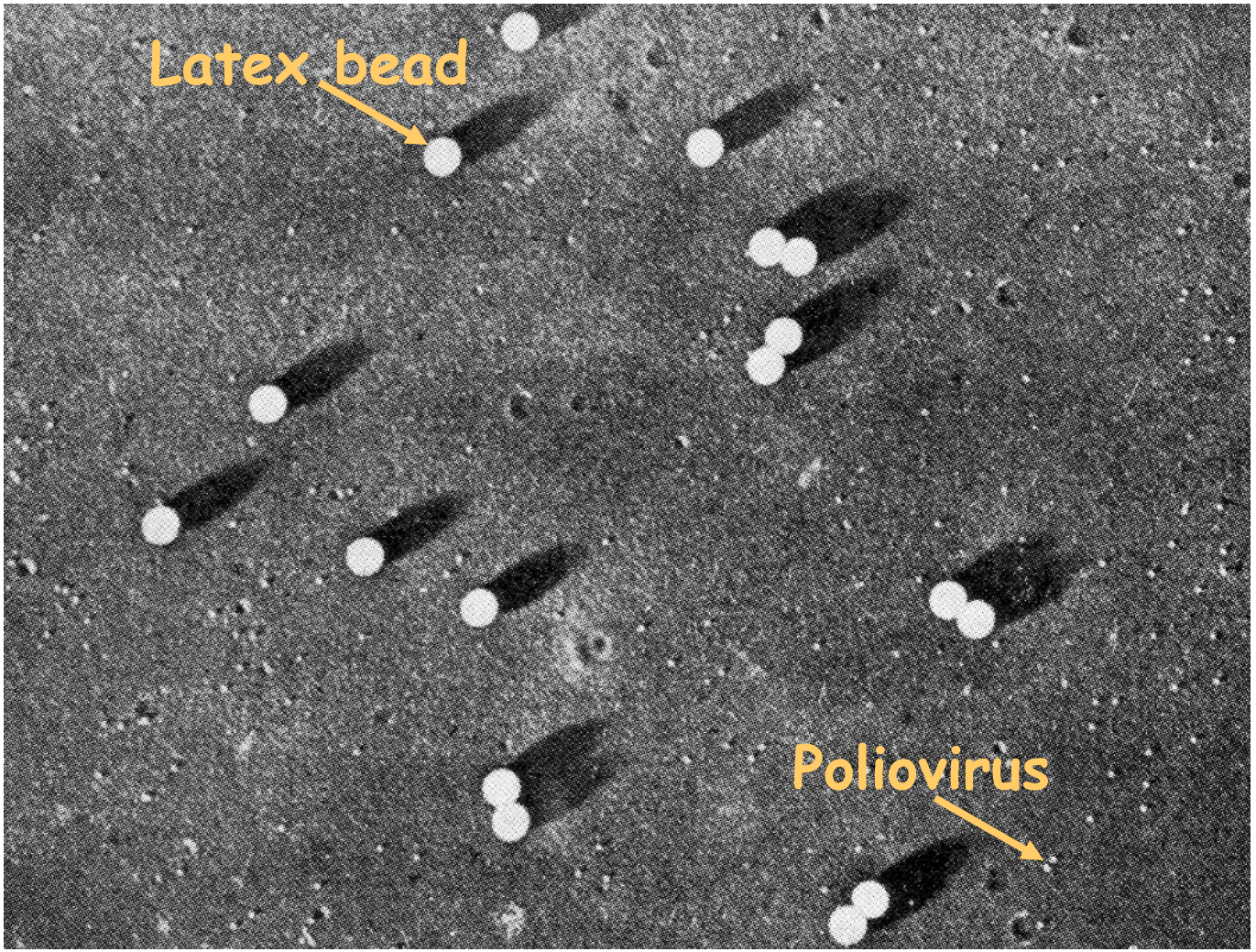
If 10^6 cells are infected at MOI of 1:
37% of the cells are uninfected
37% of the cells receive 1 particle
26% receive > 1 particle

If 10^6 cells are infected at MOI of 0.01:
99.9% of the cells are unifected
0.099% of the cells receive 1 particle (10^4 cells)
0.0001% receive > 1 particle (100 cells)

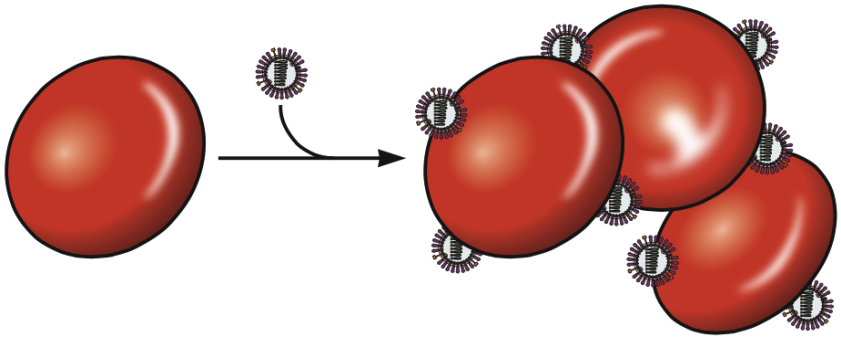
Measurement of virus particles and their components

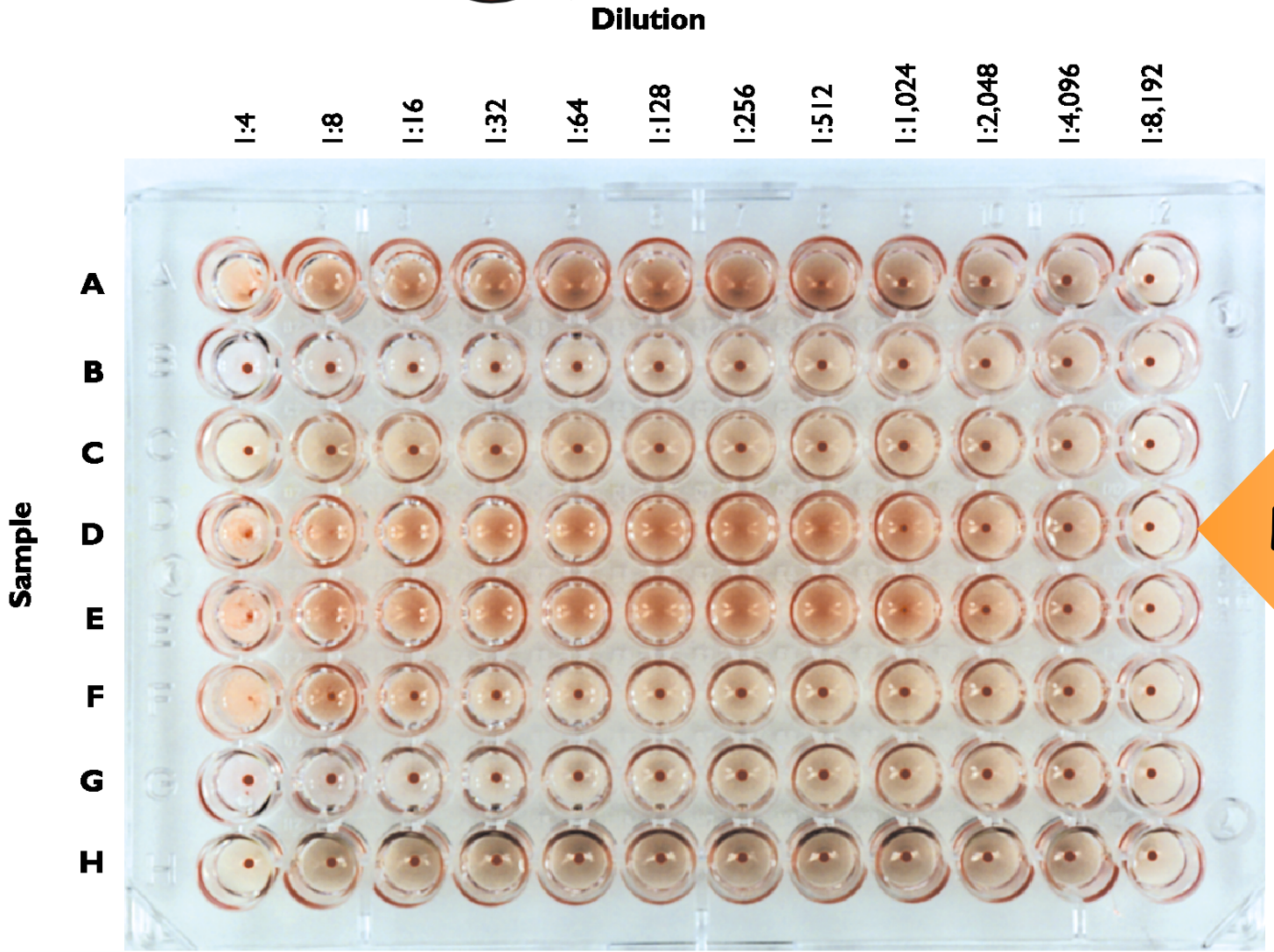
- E.M. particle counts
- Hemagglutination
- Viral enzyme activity
- Serological methods
- Nucleic Acid detection:
  - PCR
  - DNA microrrays
  - High-throughput sequencing

Direct Particle count by EM



Hemagglutination



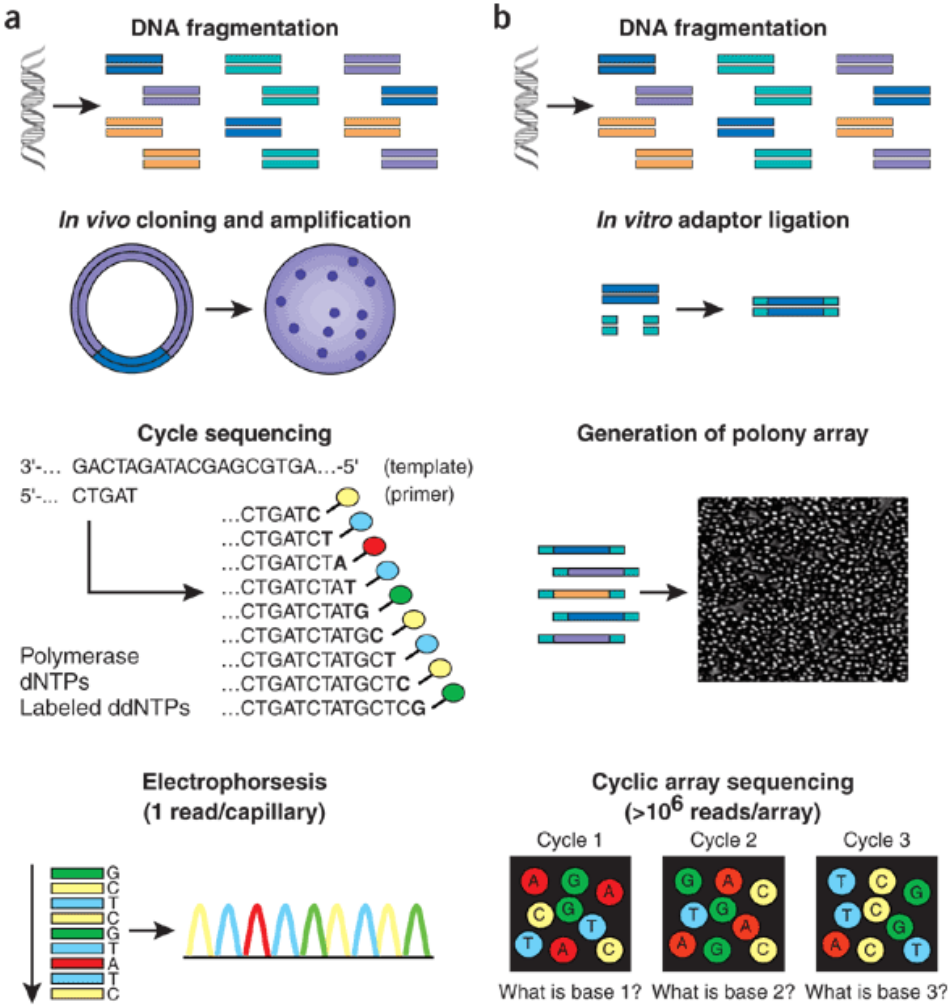


Serological methods in Virology

- Virus neutralization
- Hemagglutination inhibition
- Complement fixation
- Immunostaining
- Immunoprecipitation and immunoblotting
- ELISA

Deep, high-throughput sequencing (NGS)

- ✓ Metagenomics
- ✓ Identification of new viruses in environmental samples
- ✓ Identification of new pathogens
- ✓ Used to study the **virome**



Can techniques of genetic engineering facilitate the cultivation and assay of viruses?

Genetically engineered cell lines to render them:

- more suitable hosts for viral replication
- more convenient substrates for rapidly detecting virus-infected cells

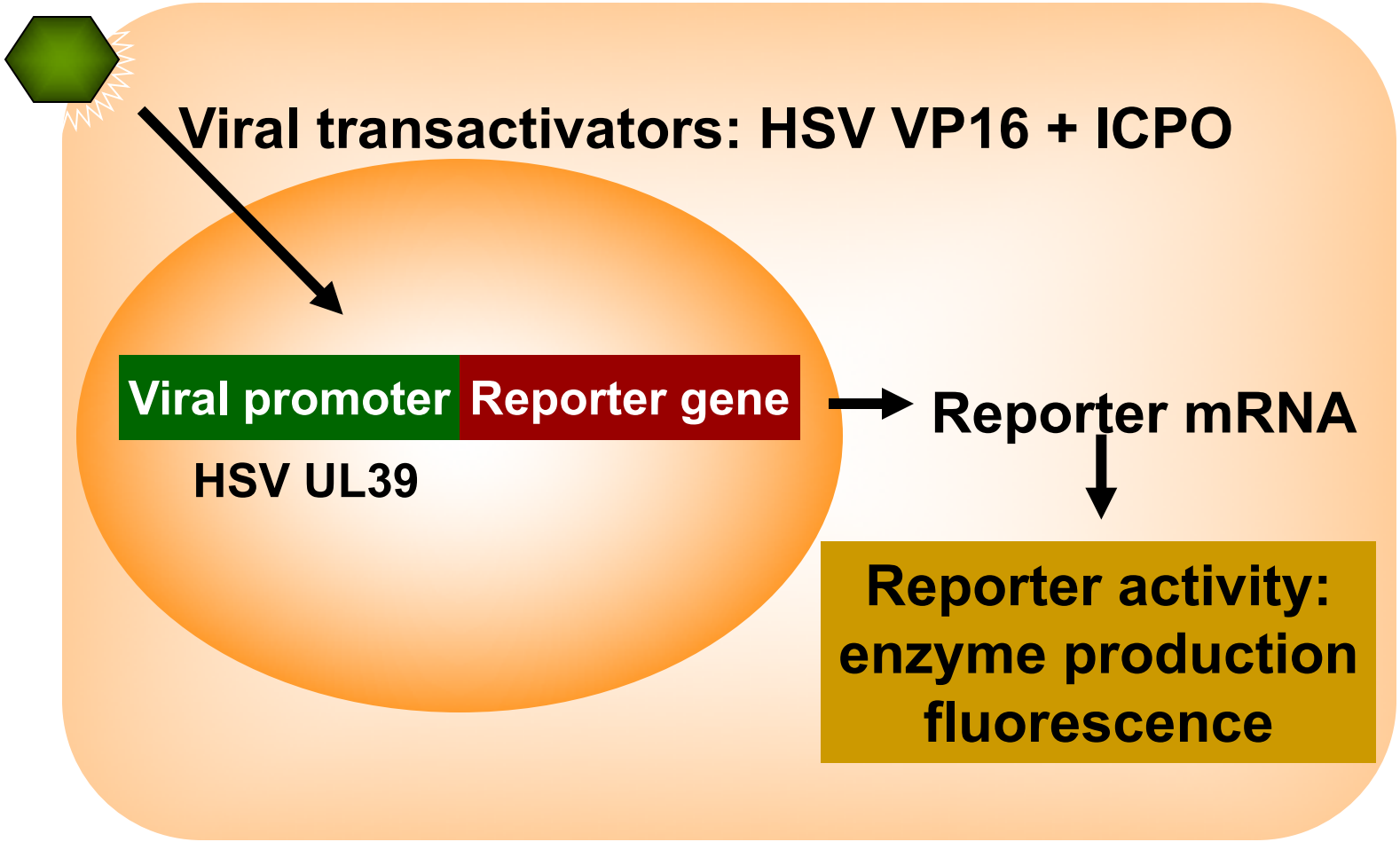
Genetically modified cells in Virology

- to modify susceptibility and permissivity
  - expression of virus receptors
- to support replication of mutant viruses
- to increase lifespan of primary or diploid cell lines
  - hTERT immortalization
- to facilitate virus detection
  - indicator cell lines

Genetically engineered cell lines to detect Herpesviruses

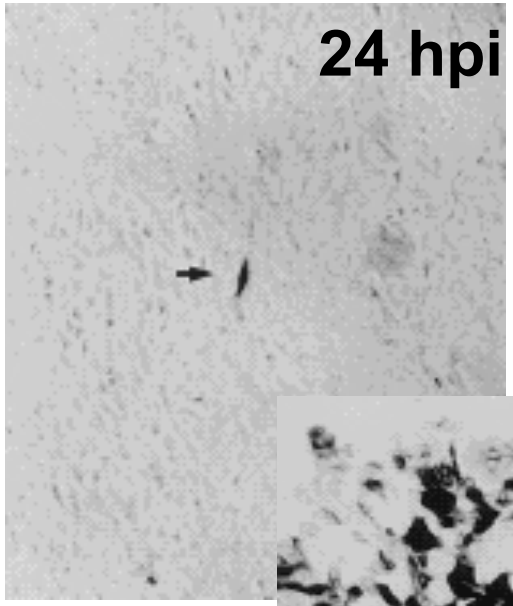
- to perform simple, rapid, sensitive and specific assay for virus detection in clinical specimens
- to perform rapid antiviral drug susceptibility testing

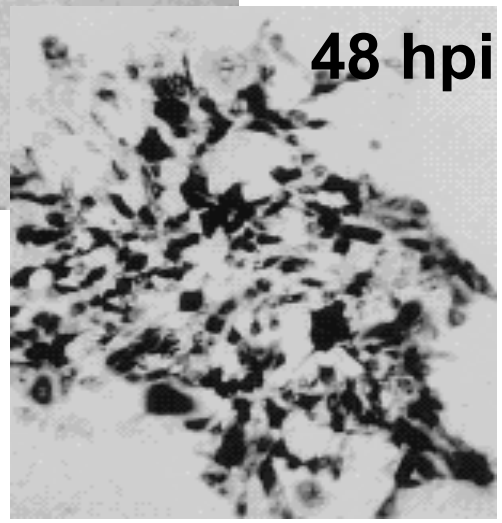
Genetically engineered cell lines to detect Herpes simplex viruses

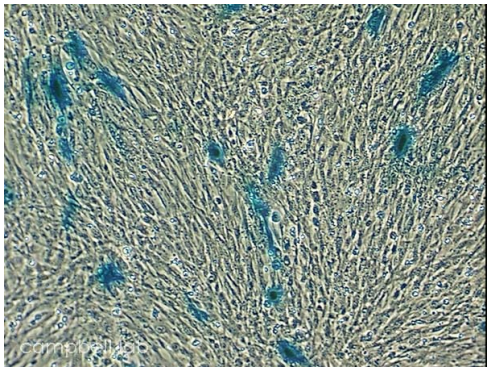


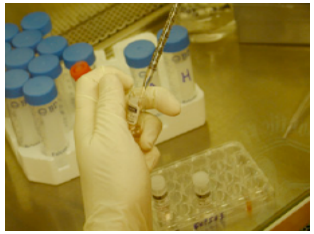
An engineered BHKICP6LacZ cell line to detect HSV-1 infection

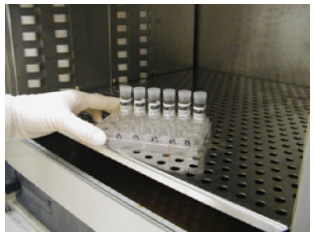
(ELVIS® HSV Test System)

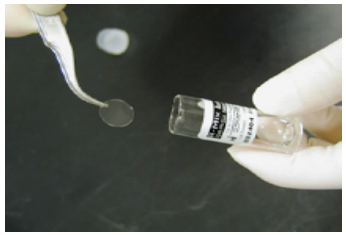












DIAGNOSTIC HYBRIDS
A Quidel Company

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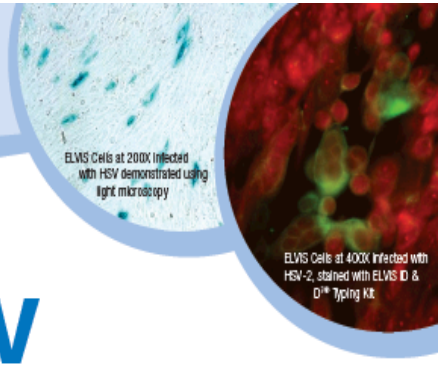


ELVIS®HSV

Sensitivity of a Ten-Day Cell Culture in Less Than One Day

- Overnight HSV detection system for culture confirmation of positive OR negative HSV-1 and HSV-2 infections.
- No fluorescence required for non-typing assay.
- Typing can be done in a single vial through sequential staining applications.
- Uses patented engineered BHK cells which produce a detectable enzyme when infected and ONLY when infected with Herpes simplex.

For Rapid Identification and Typing of Herpes Simplex Virus — *17-hour turnaround time for reporting both positive and negative results for HSV*

For More Information, Contact Us | dhlusa.com | (866) 344-3477

ELVIS®HSV

Flowchart of ELVIS Procedure

Step 1: Clinical Specimen

Pre-incubate ELVIS cell culture, 2 to 16 hours, 35° C to 37° C

Process Specimen

Remove Culture Maintenance Medium from Cell Culture

Add Replacement Medium

Inoculate Cells with Prepared Specimen Material

Step 2: Virus Amplification

Centrifuge and incubate cells @ 700xg, 60 min.

Incubate Cultures at 35° C to 37° C for 17 to 24 hours

Step 3: Color Development

Remove the Replacement Medium from the Culture

Add Solution 1 to Fix the Cell Monolayers (1 to 10 min.)

Remove the Solution 1 Cell Fixative

Immediately add Solution 2 and incubate at 35° C to 37° C for 1 hr.

Step 4: Examination for Result

Are Blue-Stained Cells Present?

Yes → Specimen Positive for HSV. Proceed to typing if required

No → Specimen Negative for HSV

Plates: Remove liquid from Plates and add Mounting Fluid. or Shell-vials: Place coverslip over Mounting Fluid on slide

Are Fluorescent Cells Present?

Yes → Specimen Positive for HSV Type 2

No → Remove liquid, rinse w/ 1 X PBS and add Solution 3. Incubate at 35° to 37° C for 15 min.

Remove liquid and add Mounting Fluid

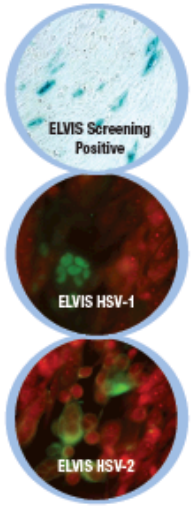
Are Fluorescent Cells Present?

Yes → Specimen Positive for HSV Type 1

No → Type result not reportable; re-examine procedures

ELVIS Product Codes

Catalog Code	Description
55-2406	24 Well Plate/6 fill
55-2412	24 Well Plate/12 fill
55-2418	24 Well Plate/18 fill
55-2424	24 Well Plate/24 fill
55-0101	1 Vial
55-0102	1 Shell Vial with Coverslip
SK-ELVIS-100	ELVIS ID Staining Kit (100)
SK-ELVIS-200	ELVIS ID Staining Kit (200)
SK-ELVIS-500	ELVIS ID Staining Kit (500)
SK-ELVIS-1000	ELVIS ID Staining Kit (1000)
SKT-ELVIS-60.V2	ELVIS ID & D® Typing / Staining Kit (60)
SKT-ELVIS-300.V2	ELVIS ID & D® Typing / Staining Kit (300)
10-220100	ELVIS Replacement Medium (100-mL)
10-220500	ELVIS Replacement Medium (500-mL)



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MKT00558v2011MAY3

Genetically engineered cell lines that facilitate Herpesvirus detection: critical issues?

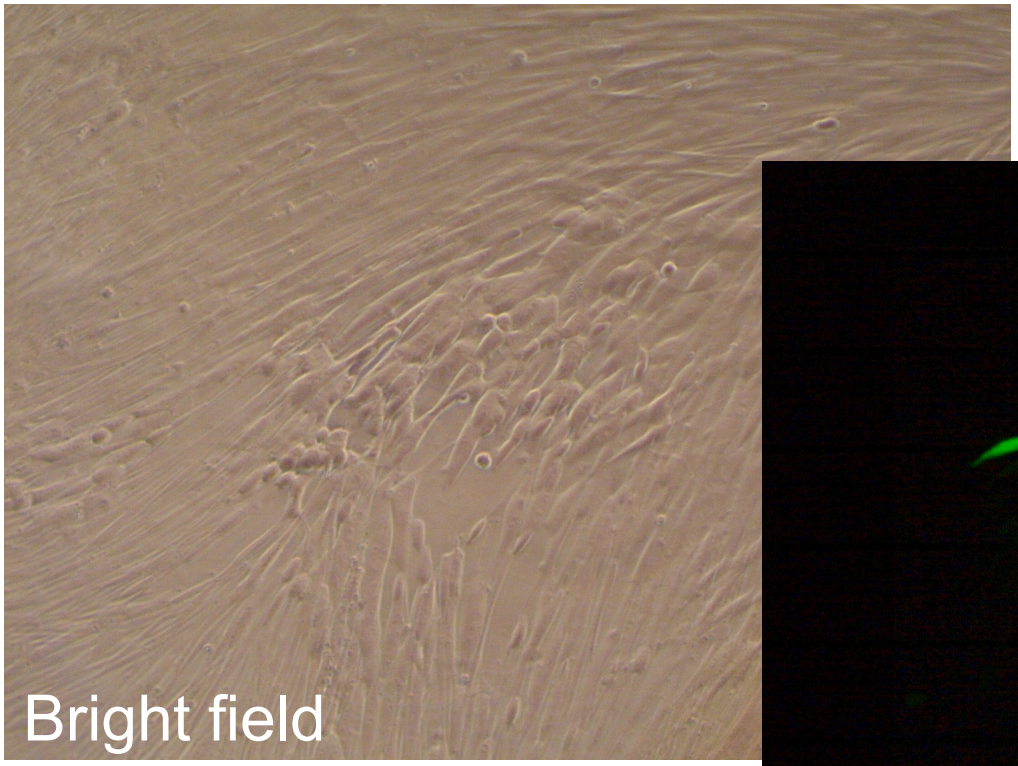
- the viral promoter
- the cell type
- the reporter gene

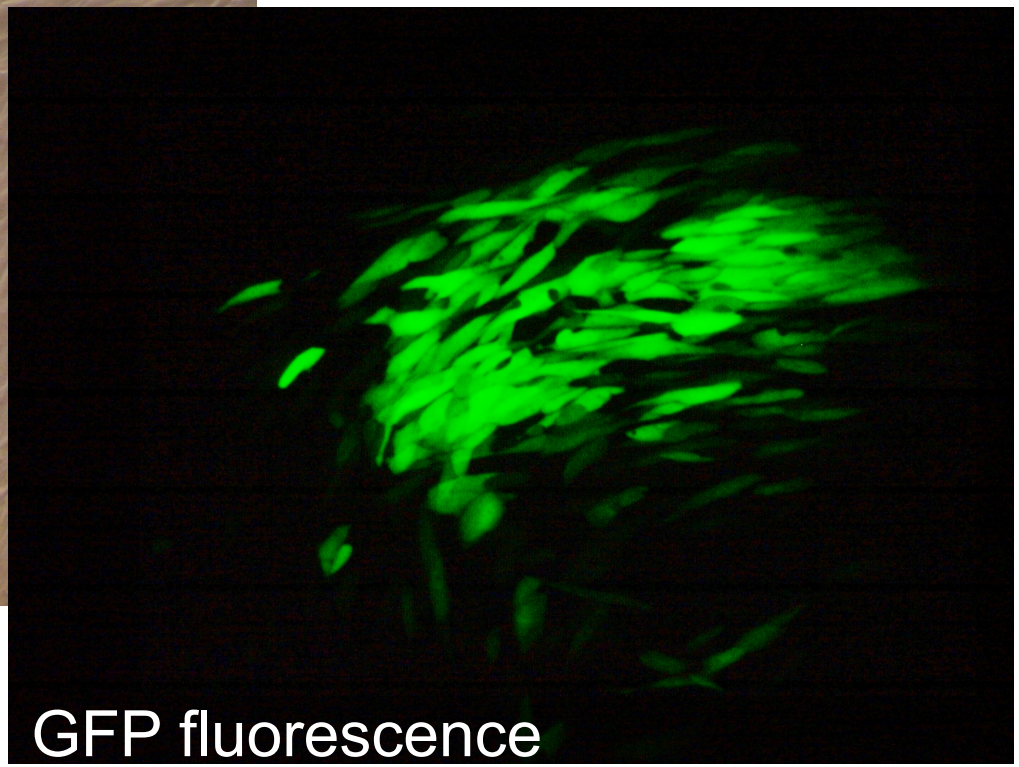
Genetically modified viruses

Aims?

- to characterize the functions of viral genes
- to facilitate virus detection
    - indicator virus strains

Plaque produced on HELFs by infection with a HCMV-GFP virus



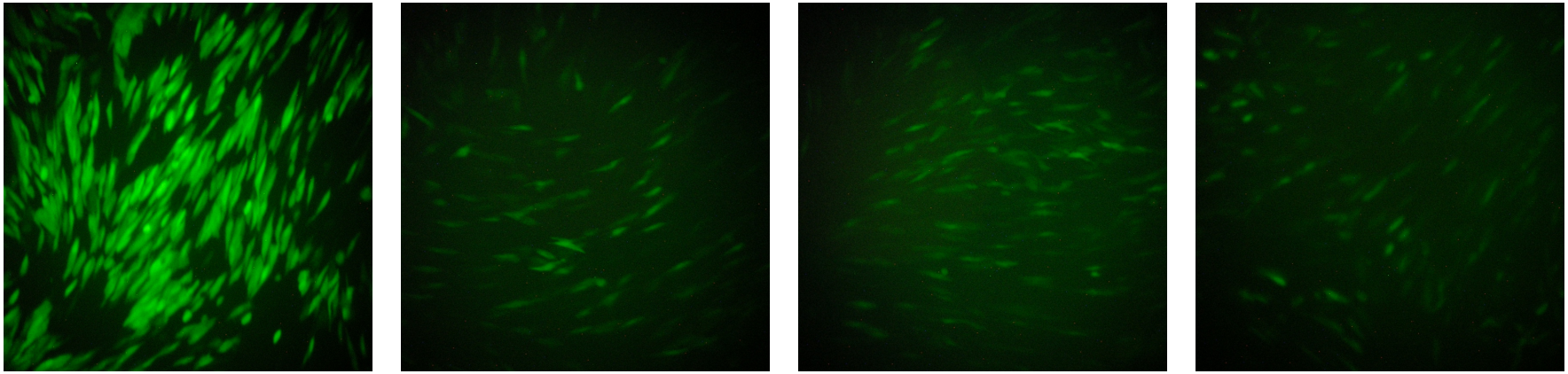


GFP-based HCMV assays

Readout of GFP signals:

- GFP fluorescence microscopy
- Flow cytometry
- Automated fluorometry

HCMV-GFP as a tool for antiviral screening assays



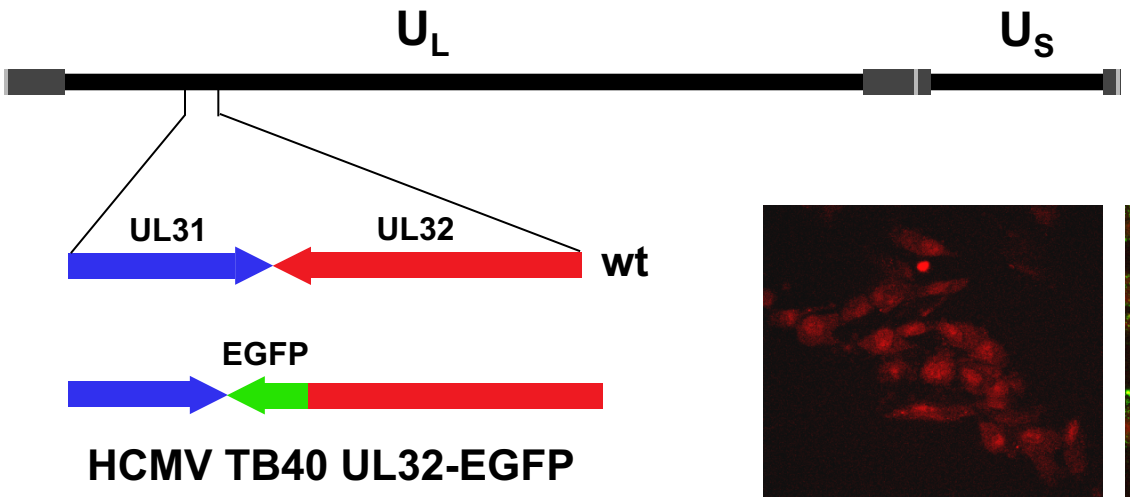
RCMV288 +GCV +PFA +AS602868

GFP-based antiviral assays: **advantages**

- Faster than Plaque Reduction Assay (PRA)
- Easier to perform than PRA
- Reliable as PRA
- Adaptable for both screening (HTS) and confirmation tests

CpG ODNs do not interfere with HCMV attachment

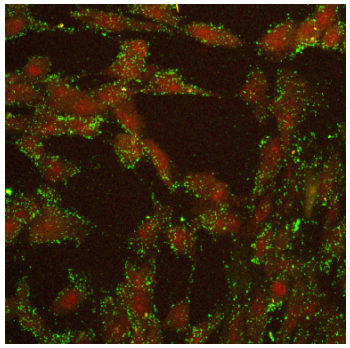
(Luganini et al., AAC 2008)



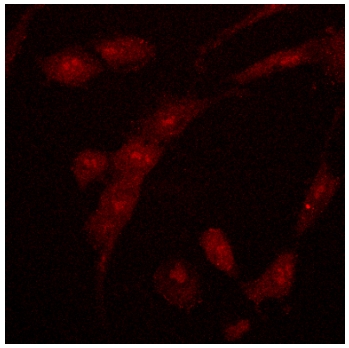
HCMV TB40 UL32-EGFP

(Laib Sampaio et al.,J. Virol. 2005)

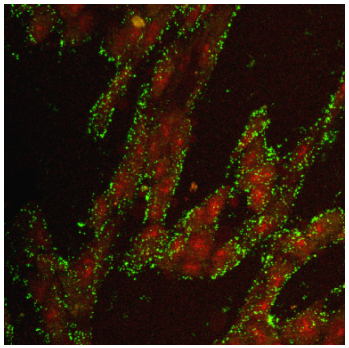
Mock-infection



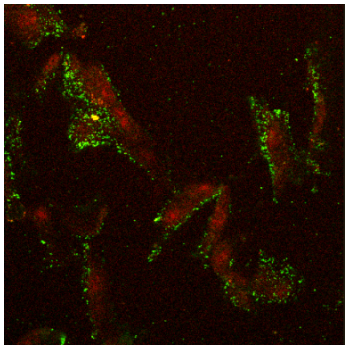
Control infection



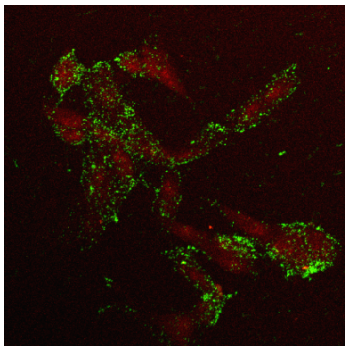
Heparin



CpG 2006



ODN 2137



CpG 2007