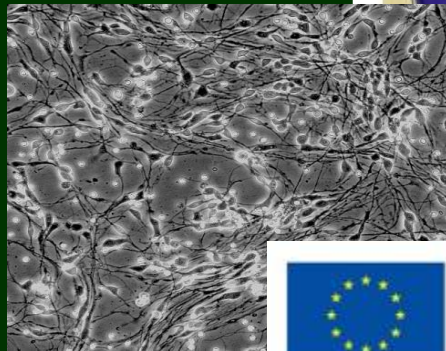
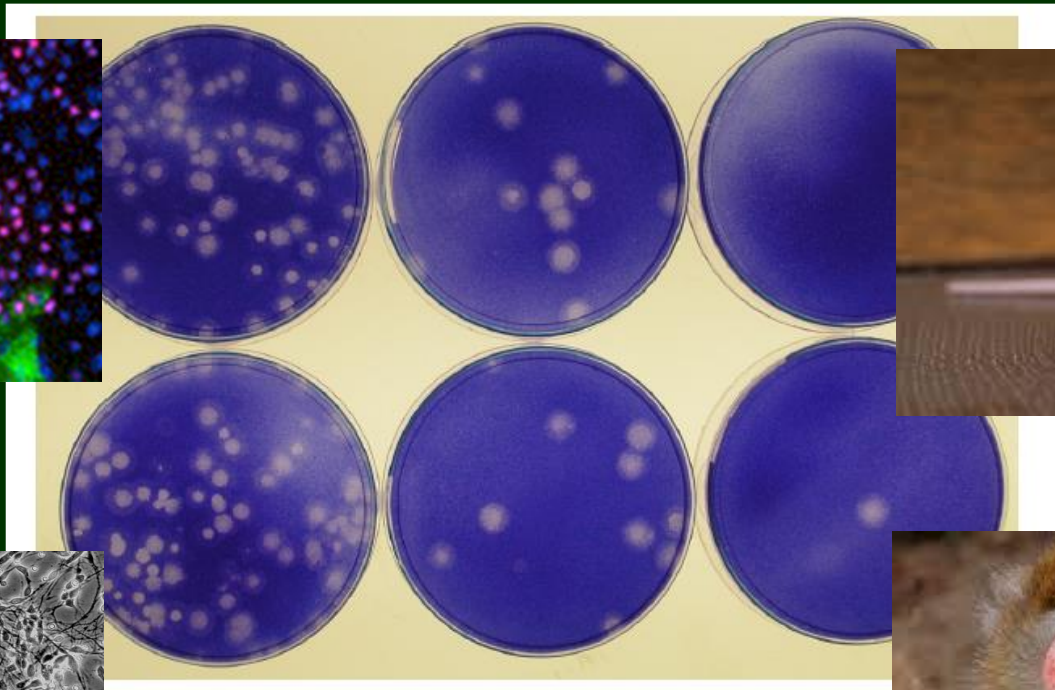
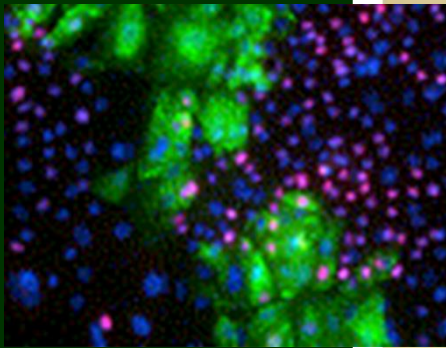


Téma 03_Kultivační virologické metody a animální modely



Financováno
Evropskou unií
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NÁRODNÍ
PLÁN OBNOVY

MŠMT
MINISTERSTVO ŠKOLSTVÍ,
MLÁDEŽE A TĚLOVÝCHOVY

Vzorky pro diagnostiku virových infekcí

- Respirační infekce – výtěry z krku nebo nosu, postnasální výplachy
- Gastrointestinální infekce – stolice
- Měchýřkovitá vyrážka – vezikulární tekutina, výtěr
- Hepatitida – krev (sérologie), stolice
- Infekce centrálního nervového systému – mozkomíšní mok
- AIDS - krev

Metody diagnostiky virových infekcí

- Přímá detekce (obvykle kultivace v buněčných kulturách – izolace viru; elektronová mikroskopie, imunofluorescence, PCR)
- Nepřímá – serologické metody – identifikace a kvantifikace specifických protilátek v séru pacienta

Oba způsoby přinášejí značné nevýhody – v prvním případě pracnost, nákladnost a časová náročnost (kromě PCR), ve druhém malá vypovídací hodnota a spolehlivost

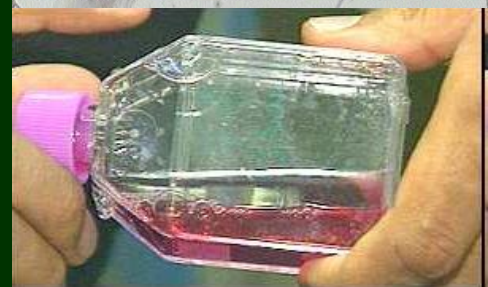
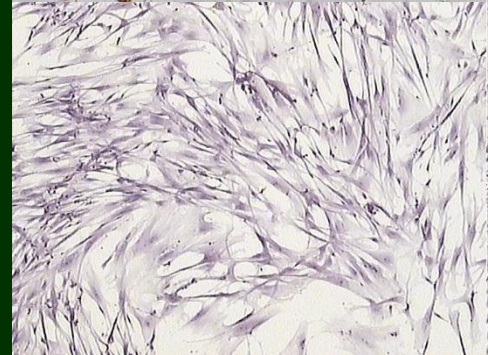
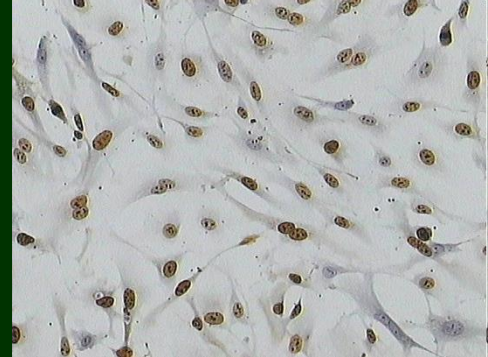
Metody pro izolaci viru a následnou diagnostiku

Kultivační metody

- Kultivace na buněčných (tkáňových) kulturách (TK) – použitelné jen pro některé viry
 - Klasická s cytopatickým efektem (dlouhá doba vyšetření až 4 týdny)
 - Zrychlená s průkazem virových antigenů imunohistochemicky (cca 2 – 4 dny)
- Kultivace na kuřecím embryu – použitelné jen pro některé viry
- Pokus na zvířeti – na konkrétním vhodném zvířeti lze kultivovat většinu virů

Buněčné kultury

- 1949 – poprvé pozorováno, že poliovirus se množí v kultuře buněk za produkce charakteristických histologických změn
- Práce za aseptických podmínek (přesto časté problémy s kontaminacemi bakteriemi, mykoplasmaty, kvasinkami či houbami)
- Buněčné kultury posloužily k objevu řady nových virů a virových čeledí (např. adenovirů, echovirů, rhinovirů apod.)
- Cenná pomůcka při diagnostice virových onemocnění či pro vývoj vakcín (dětská obrna, spalničky, zarděnky)
- HeLa je buněčná linie lidských epiteliálních buněk. Jedná se o nádorové buňky izolované v roce 1951 a pocházející z nádoru děložního hrdla Henrietty Lacksové (z prvních dvou písmen jména a příjmení této ženy pochází i název této buněčné linie).



Druhy kultur živočišných buněk

Primokultury (primární kultury)

Jedná se o buňky či tkáně čerstvě odebrané z organismu. Z celé populace vložené do živného média přežívá jen ta část, která se nejlépe přizpůsobí novým podmínkám. Buňky, které se přizpůsobily se začínají dělit. Tato kultura existuje zpravidla jen pár dní, protože poté se musí pasážovat.

Buněčné kmeny

Jedná se o buněčnou kulturu normálních diploidních buněk, které minimálně jedenkrát již prošly procesem pasážování. Tyto kmeny zpravidla zanikají po 40-50ti děleních z důvodu zkracování telomer.

Buněčné linie (permanentní buněčné linie)

Jsou charakteru nádorových buněk, plně adaptovány na podmínky in vitro a neomezeně se dělí. Jejich „nesmrtelnost“ je způsobena přítomností telomerázy. Jednou z nejznámějších buněčných linií je HeLa (Henrietta Lacks).

Růstová křivka buněčné kultury

Lag-fáze

Počet buněk v médiu nejprve lehce poklesne, což je způsobeno adaptací na kultivační prostředí. Po tomto úvodním „šoku“ začíná ovšem velikost populace rapidně růst.

Log-fáze (logaritmická resp. exponenciální fáze)

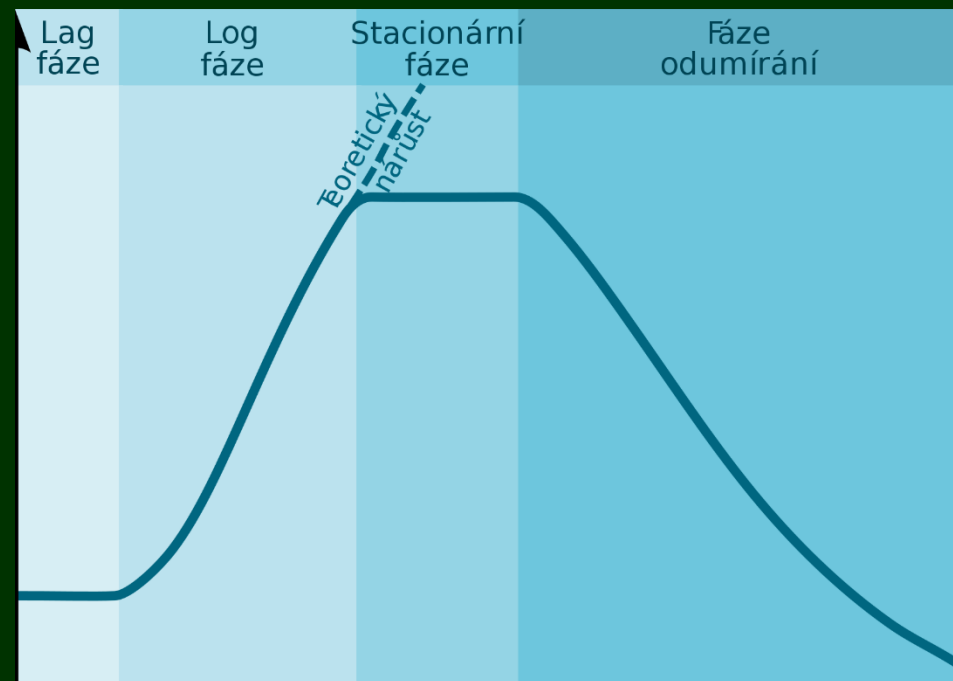
V této fázi roste počet buněk v kultuře exponenciálně a je možné zachytit vysoké procento buněk v mitóze (čehož lze využít pro karyotypizaci).

Stacionární fáze

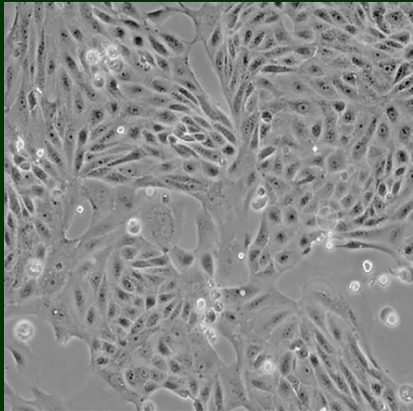
Buňky v kultuře se přestávají množit. To je způsobeno inhibičními mechanismy (např. kontaktní inhibice) a částečně také vyčerpáním živného média.

Fáze úbytku buněk

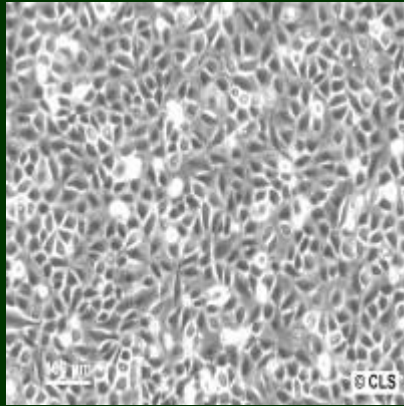
Dochází k ní z důvodu nedostatku živin, snížení pH (díky zvýšení CO₂), kumulace toxických produktů metabolismu atd.



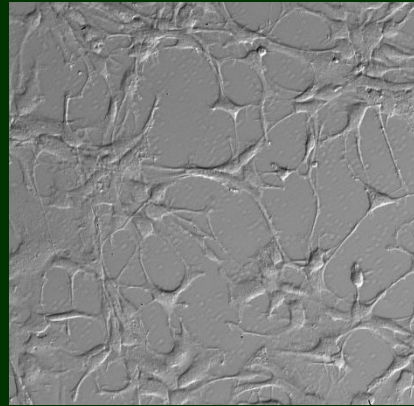
Buněčné kultury ve virologii



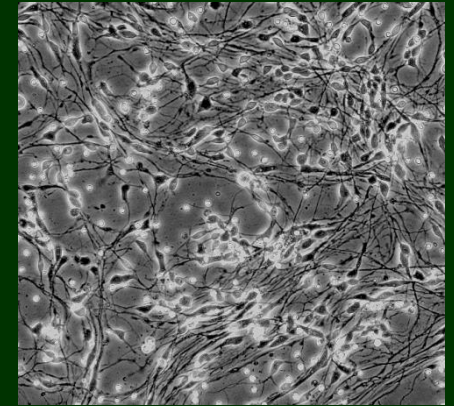
Madin-Darby Canine Kidney cells
(influenza)



Huh-7 cells
(hepatitis B,C)



Human astrocytes
(tick-borne encefalitis)



Human neurons
(neurotropic viruses)

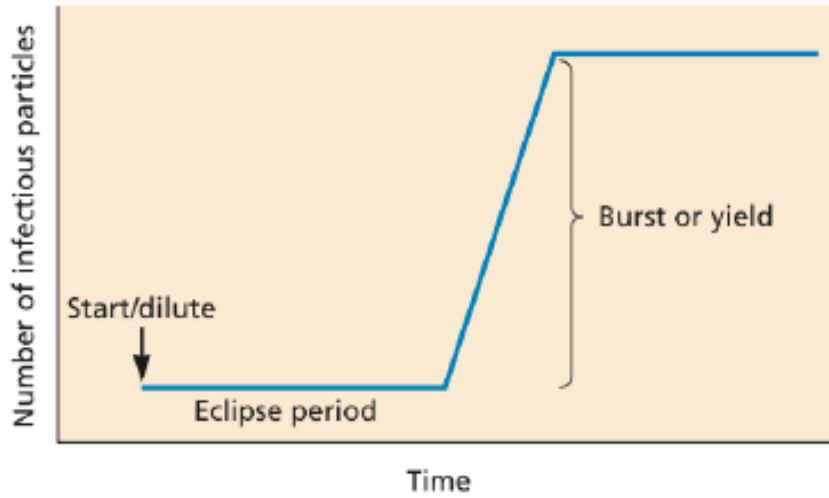
Imortalizované buněčné linie (nádorový původ, neomezená proliferace)

Primární buňky (omezená proliferace, vyšší nároky na kultivaci)

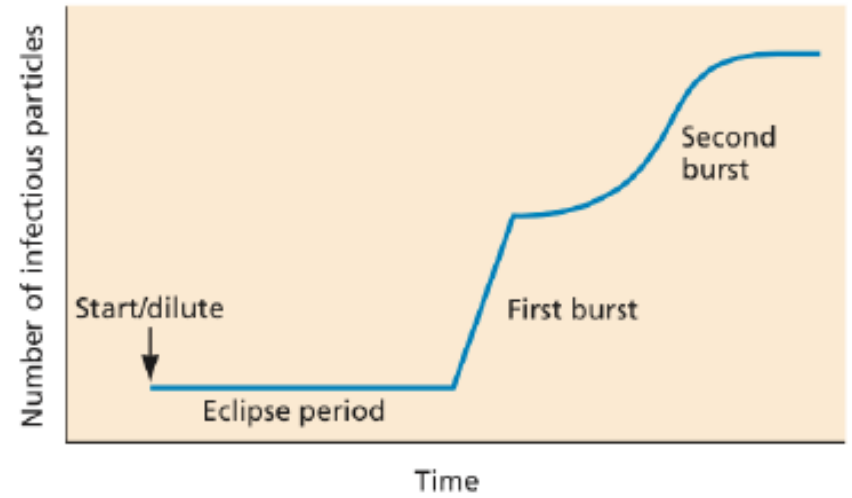
- získané od dárce
- diferencované z kmenových/primordiálních buněk



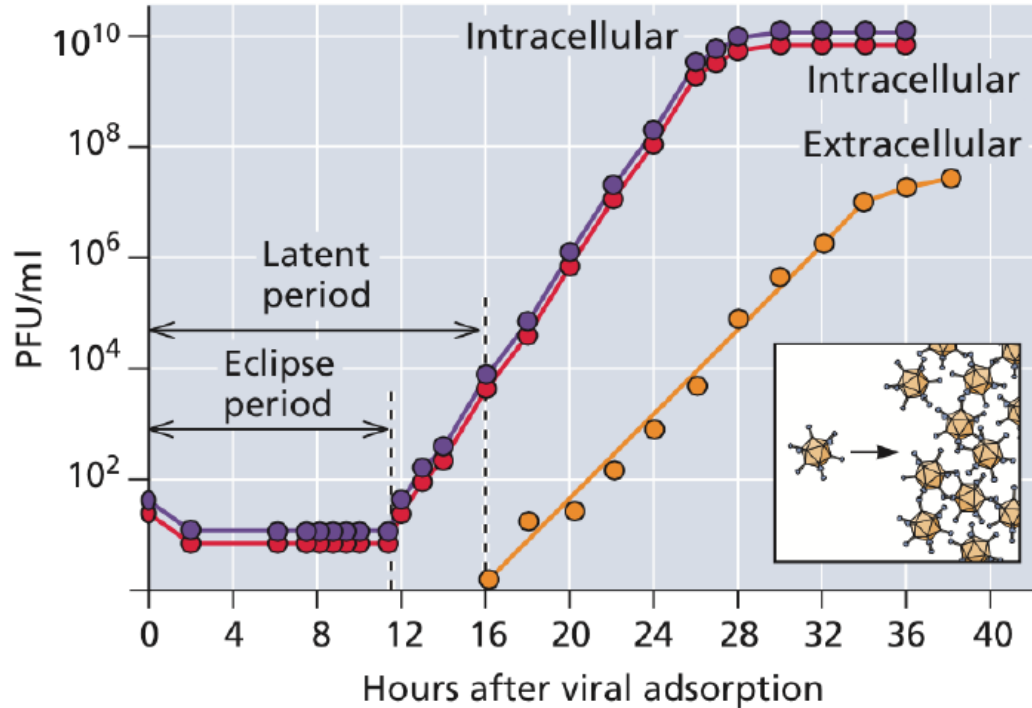
All cells infected



Few cells infected



Adenovirus type 5



Multiplicity of infection (MOI)

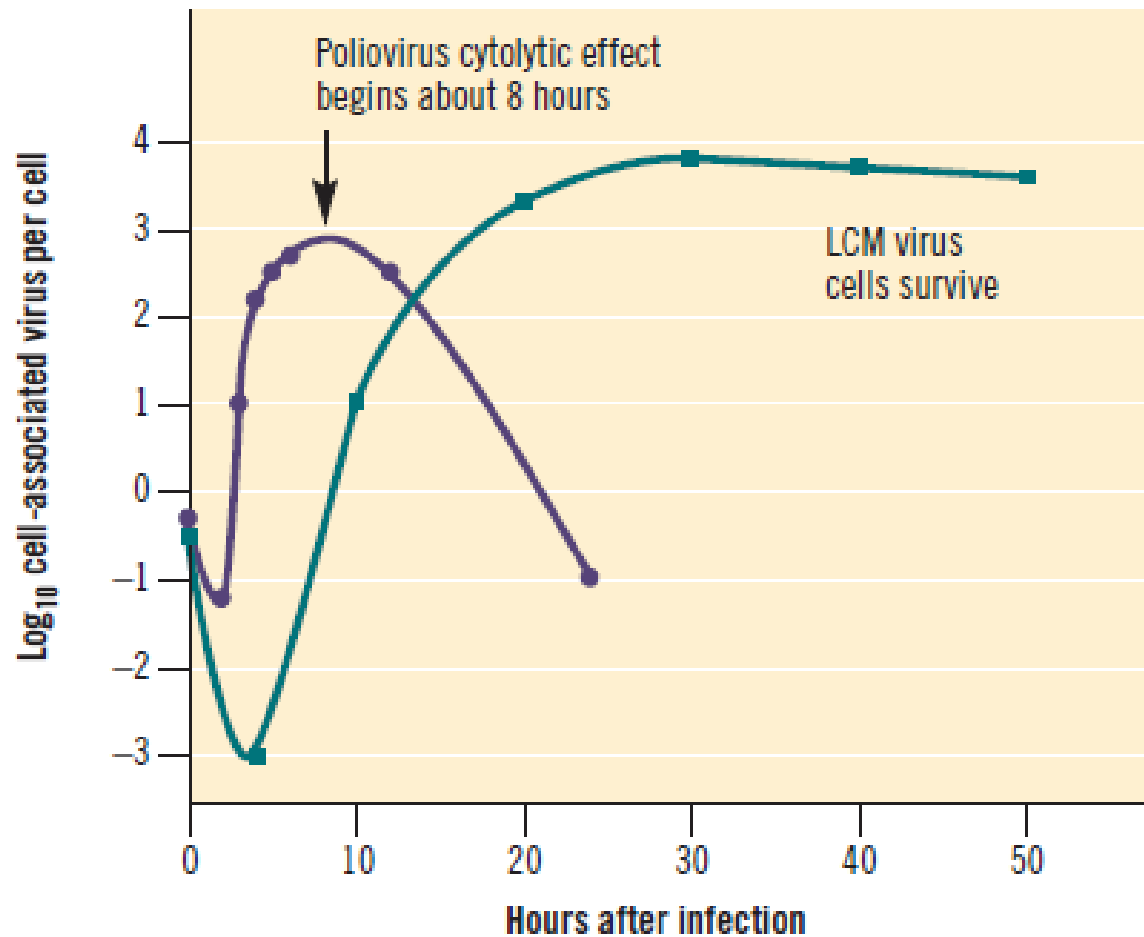
- Number of infectious particles ADDED per cell
- Not the number of infectious particles each cell *receives*
- Add 10^7 virus particles to 10^6 cells - MOI of 10 - each cell does NOT receive 10 virions
- Infection depends on the random collision of virions and cells
- When susceptible cells are mixed 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*

Efekt virové replikace na hostitelskou buňku

-Infekce probíhá ve **vnímavých (permissivních)** buňkách

-V **nepermisivních** buňkách – se nemůže uskutečnit některý ze stupňů morfogeneze viru

- Formy:
- a. **cytoidní produktivní** infekce – lytická
 - b. **produktivní** infekce
 - c. **perzistentní** infekce – virové částice se stále tvoří, buňka nebývá poškozena a dále se dělí
 - d. **trasfomace** – nádorová přeměna buňky
 - e. **abortivní infekce** – vznikají jen některé strukturní virové složky, netvoří se kompletní virové částice, chybí např. některý enzym



Replikace cytotolytického viru (poliovirus) a necytolytického viru (LCMV, Lymphocytic Choriomeningitis virus, Arenaviridae) v buněčné kultuře

- neobalené viry – obvykle cytotolytické
- obalené viry – nemusí být cytotolytické

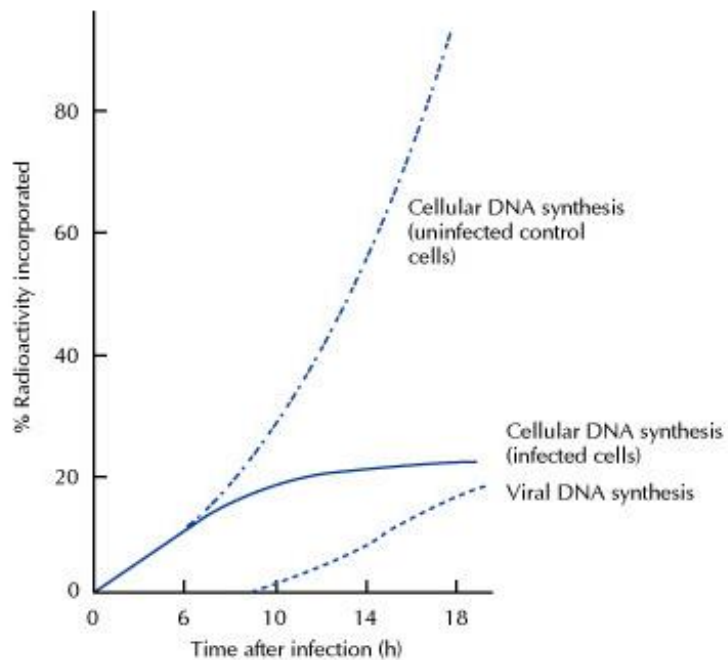
Změny v infikovaných buňkách

-**Cytopatický efekt** – soubor změn, které vznikají v buňce v důsledku proniknutí a rozmnožování viru a způsobují nevratné narušení jejich metabolických procesů

-**Inhibice syntézy buněčných makromolekul** (inhibice syntézy buněčné RNA, blokáda asociace ribozómů s buněčnou mRNA, inhibice syntézy buněčné DNA)

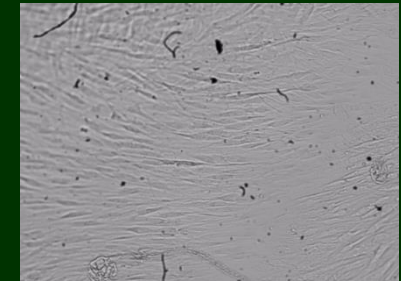
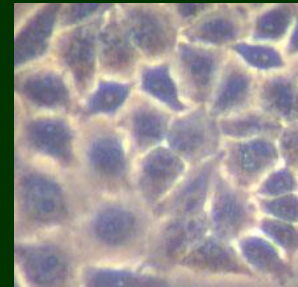
-**Přestavba membrán ER a jádra**, změna permeability membrán, únik iontů a makromolekul

-**Změny buněčných mikrofibril a mikrotubulů**

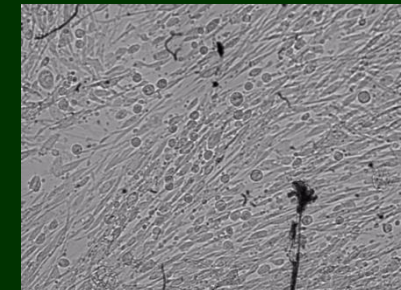
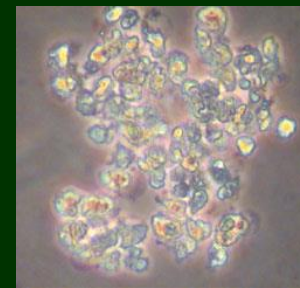


© Academic Press, 2000.

Neinfikovaná
buněčná
kultura



Infikovaná
buněčná
kultura



Virová infekce v buněčné kultuře

Normální TK – mikroskop.



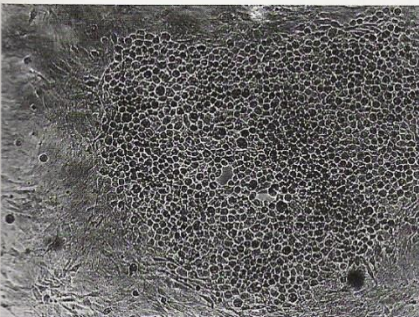
Množení viru – mikroskop.



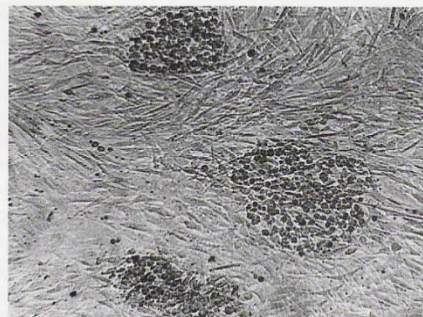
Syncytium



A

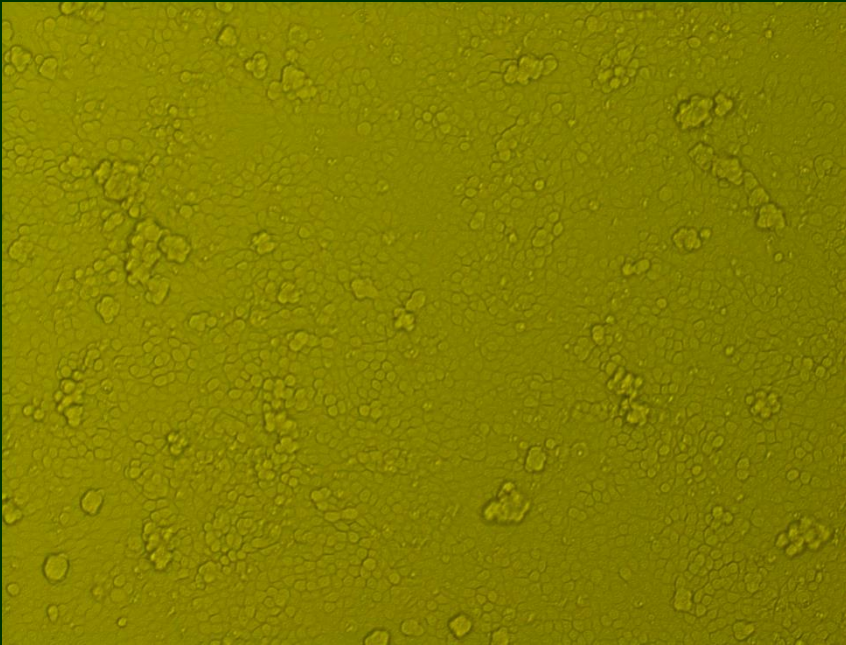


B



Nádorová transformace
buněk

Cytopathic effect



Mock-infected cells: (no CPE)



Tick borne encephalitis virus
infected cells: (strong CPE)

Cell lines for TBE: porcine kidney cells (PS), human neuroblastoma cells (UKF-NB-4)

Examples of cytopathic effects

Cytopathic effect(s)	Virus(es)
Morphological alterations	
Nuclear shrinking (pyknosis), proliferation of membrane	Picornaviruses
Proliferation of nuclear membrane	Alphaviruses, herpesviruses
Vacuoles in cytoplasm	Polyomaviruses, papillomaviruses
Syncytium formation (cell fusion)	Paramyxoviruses, coronaviruses
Margination and breaking of chromosomes	Herpesviruses
Rounding up and detachment of cultured cells	Herpesviruses, rhabdoviruses, adenoviruses, picornaviruses
Inclusion bodies	
Virions in nucleus	Adenoviruses
Virions in cytoplasm (Negri bodies)	Rabies virus
“Factories” in cytoplasm (Guarnieri bodies)	Poxviruses
Clumps of ribosomes in virions	Arenaviruses
Clumps of chromatin in nucleus	Herpesviruses

Kvantifikace cytopatického efektu jako diagnostická metoda

TCID₅₀ = Median Tissue Culture Infectious Dose

TCID₅₀ assay (for DENV, JEV, HSV-1, SINV, VSNJV and EMCV)

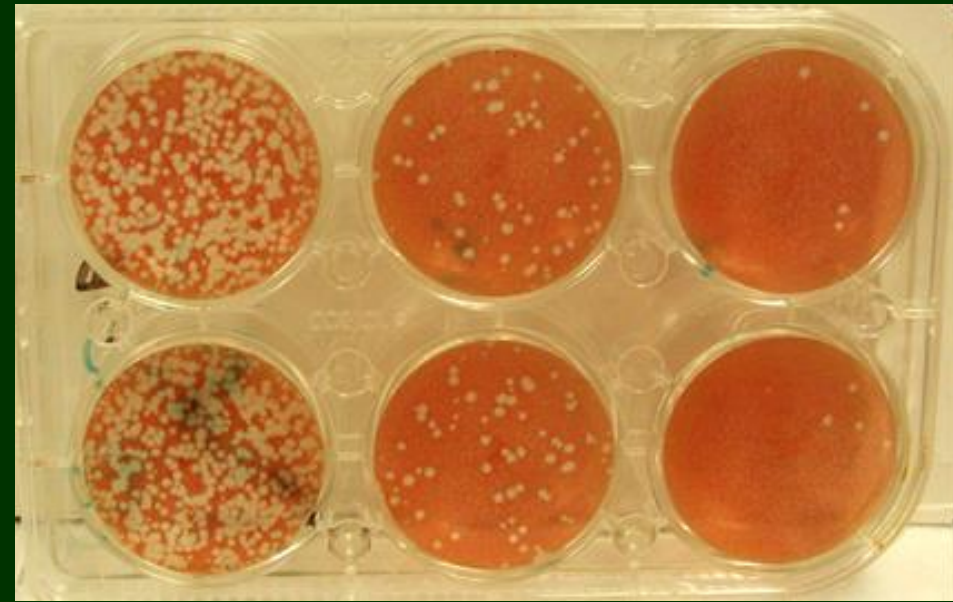
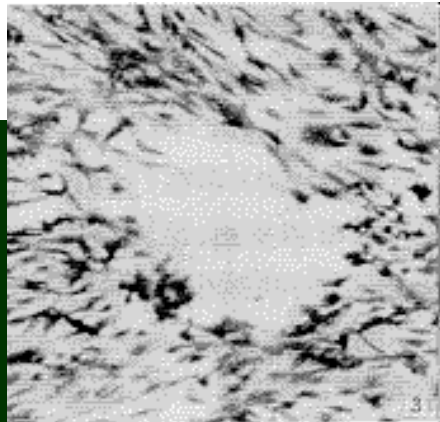
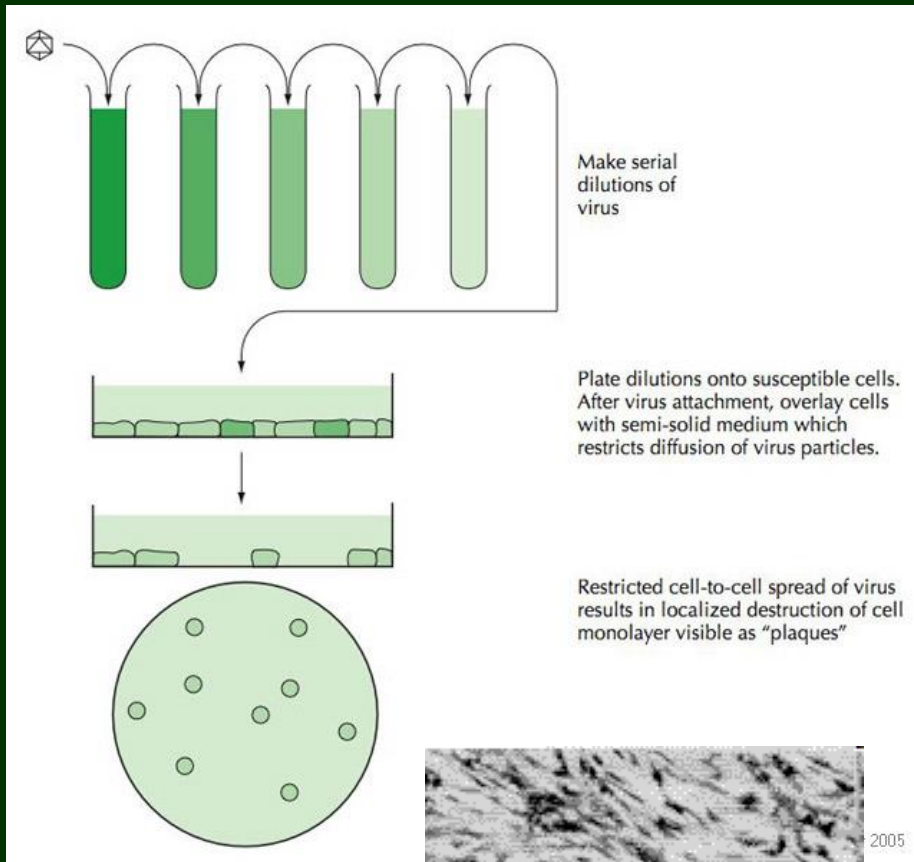
TCID₅₀ assay can be used to measure infectivity of viruses that cause strong CPE. TCID₅₀ represents a dilution of virus that makes 50% of the test wells show cell detachment (Figure 4). The infectivity titer is expressed as TCID₅₀/ml.

Virus dilution	CPE-positive / Total								Proportion positive	
	1	2	3	4	5	6	7	8		
2 ⁻¹	○	○	○	○	○	○	○	○	8/8	1.0
2 ⁻²	○	○	○	○	○	○	○	○	8/8	1.0
2 ⁻³	○	○	○	○	○	○	●	○	7/8	0.88
2 ⁻⁴	●	○	○	○	○	●	○	○	6/8	0.75
2 ⁻⁵	●	○	○	●	○	●	○	○	5/8	0.63
2 ⁻⁶	●	●	●	○	●	●	○	○	3/8	0.38
2 ⁻⁷	●	●	●	○	●	○	●	●	2/8	0.25
2 ⁻⁸	●	○	●	●	●	●	●	●	1/8	0.13
2 ⁻⁹	●	●	●	●	●	●	●	●	0/8	0
2 ⁻¹⁰	●	●	●	●	●	●	●	●	0/8	0
control	virus (-)	●	●	●	●	●	●	●	0/8	0
	virus (-)	●	●	●	●	●	●	●	0/8	0

Výpočet dle Reedovy-Muenchovy metody

Figure 4. Schematic presentation of a result of TCID₅₀ assay. White circles indicate the wells in which the cells have undergone cell death to detach from the plastic plate due to strong CPE. Blue circles indicate the wells in which the cells remain intact to be stained with crystal violet.

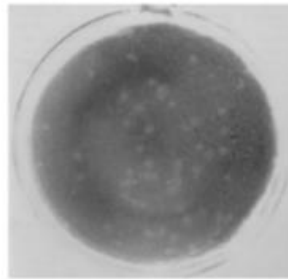
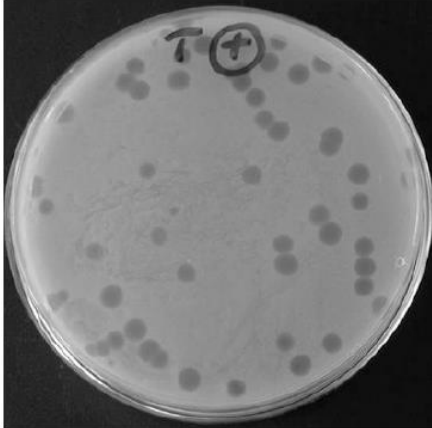
Plaková titrace – plaque assay



Plaková titrace. Virová suspenze ředěná určitým faktorem se přidá k jednovrstvě buněk. Buňky se barví v okamžiku, kdy virus infikuje jednu buňku, tu zlyzuje a nakazí okolní buňky za vzniku plaku. Každý plak je tedy výsledkem původně infekce jedné buňky jedním virem.

Plaque assay

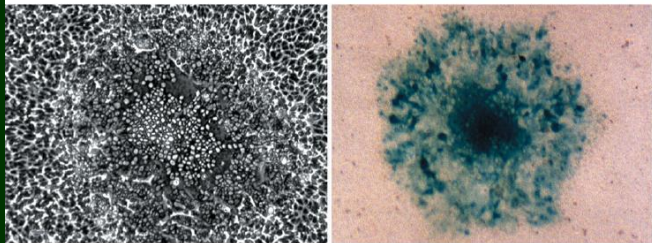
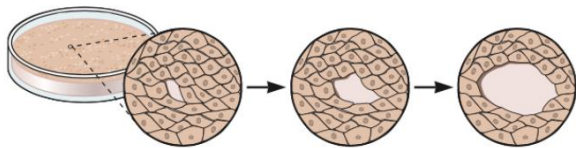
1930s: used to study multiplication of bacteriophages



Plaque assay



1952, Renato Dulbecco developed for animal viruses
Nobel Prize, 1975



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

BY RENATO DULBECCO

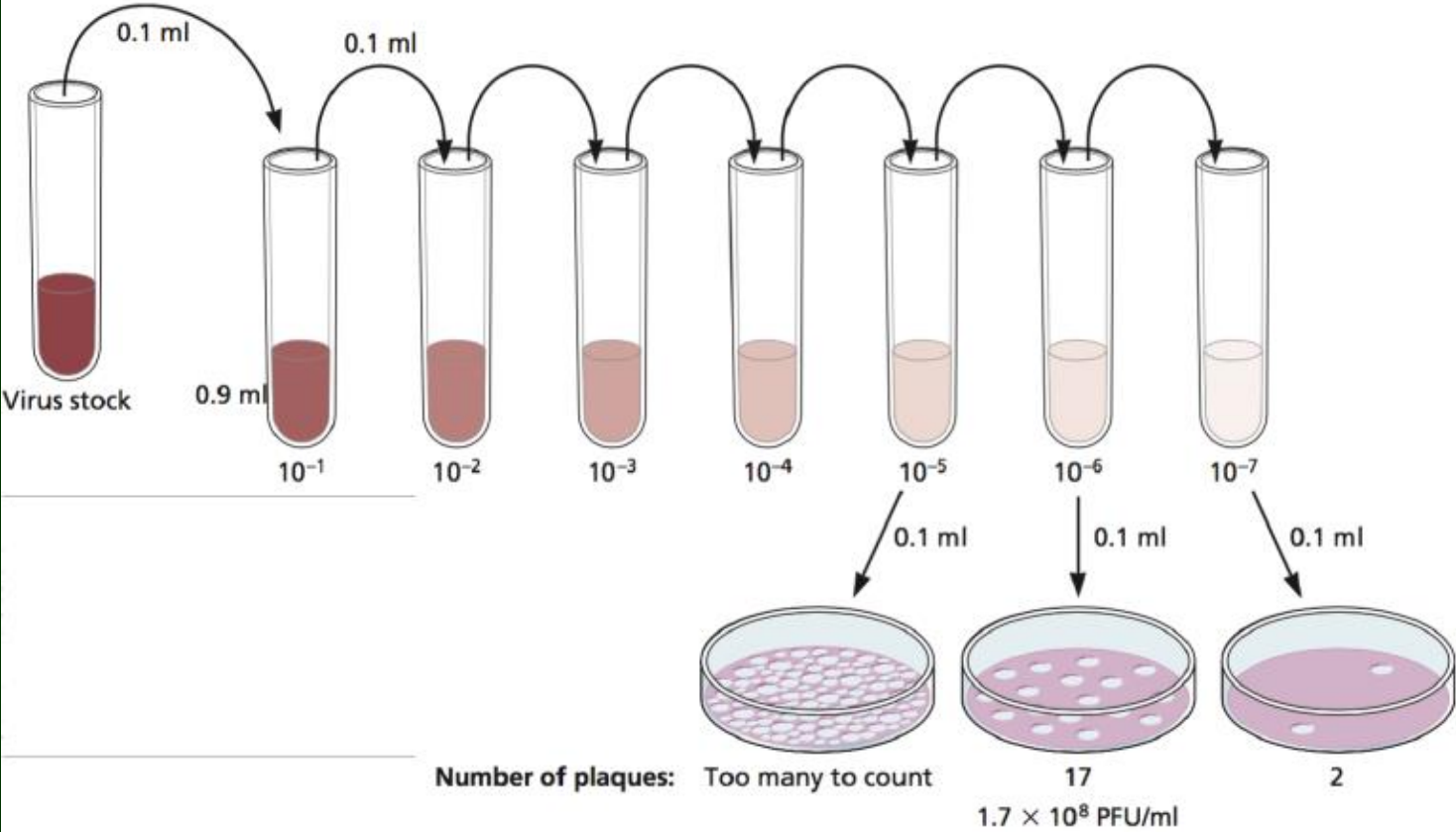
CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIFORNIA

Read before the Academy, April 20, 1952

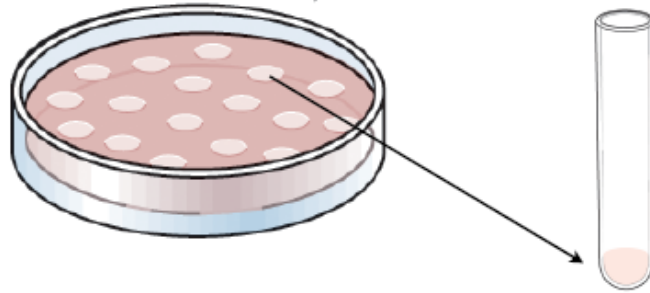
Research on the growth characteristics and genetic properties of animal viruses has stood greatly in need of improved quantitative techniques, such as those used in the related field of bacteriophage studies.

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

Plaque assay



Plaque purification



A method for producing clonal virus stocks
Usually done 3 times

Tato metoda se používá pro izolaci viru z klinického (nebo jiného) vzorku.

Particle-to-PFU ratio

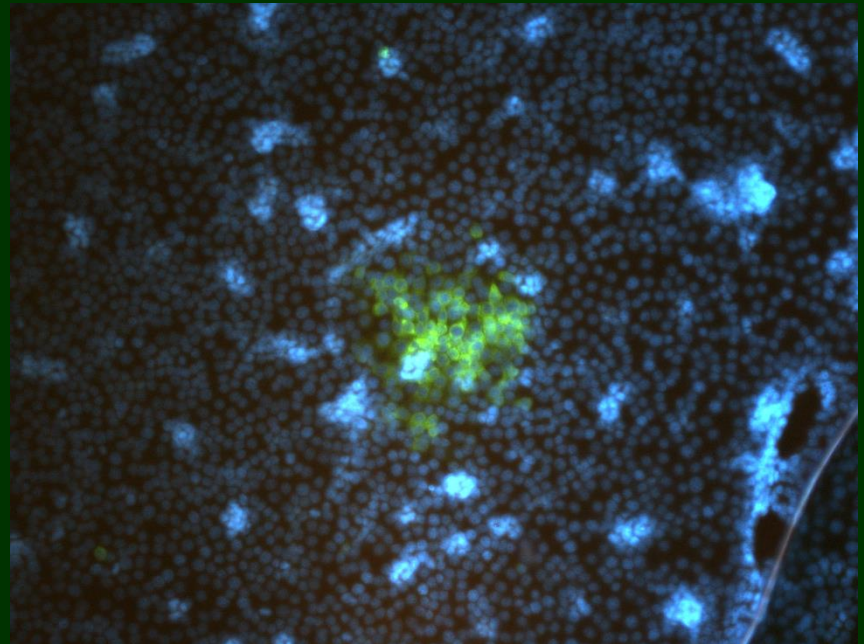
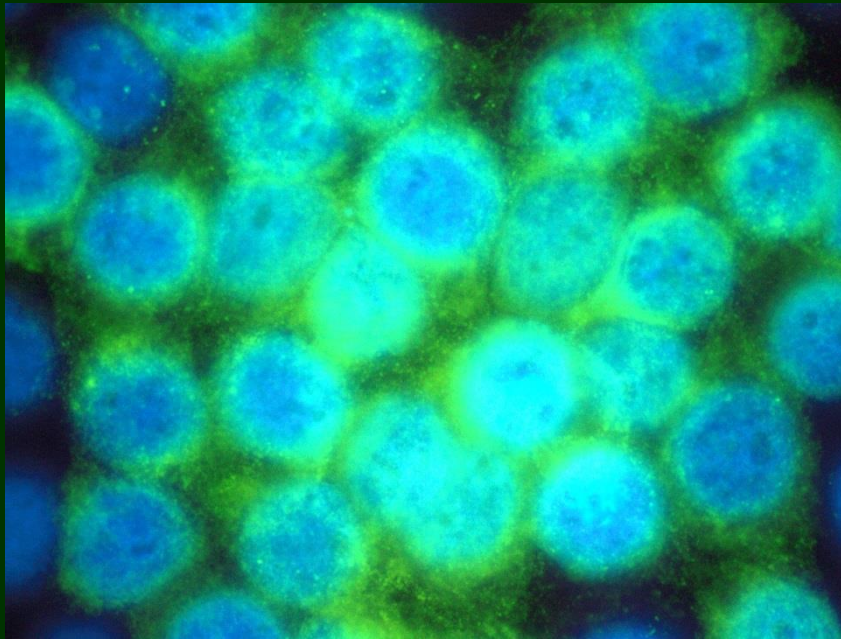
- # of *physical* particles \div # of *infectious* particles
- A single particle *can* initiate infection
- Not all viruses are successful
 - Damaged particles
 - Mutations
 - Complexity of infectious cycle
- Complicates study

Particle-to-PFU ratios of some animal viruses

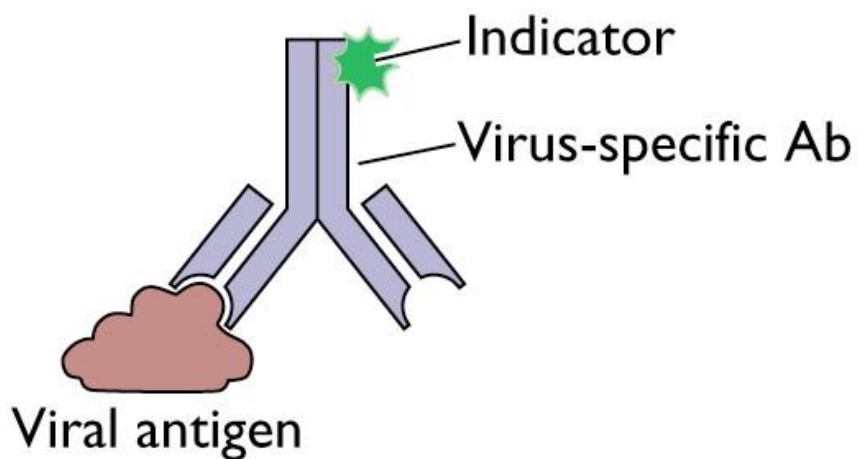
Virus	Particle/PFU ratio
<i>Papillomaviridae</i>	
Papillomavirus	10,000
<i>Picornaviridae</i>	
Poliovirus	30–1,000
<i>Herpesviridae</i>	
Herpes simplex virus	50–200
<i>Polyomaviridae</i>	
Polyomavirus	38–50
Simian virus 40	100–200
<i>Adenoviridae</i>	20–100
<i>Poxviridae</i>	1–100
<i>Orthomyxoviridae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

Imunofluorescenční barvení

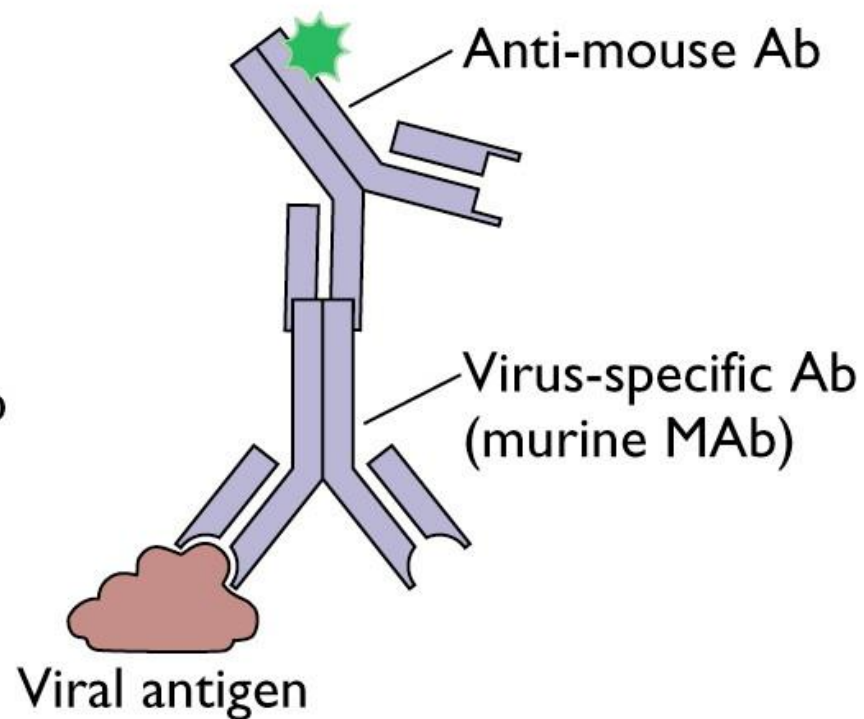
Kombinace kultivačních, imunologických a mikroskopických technik



Direct



Indirect



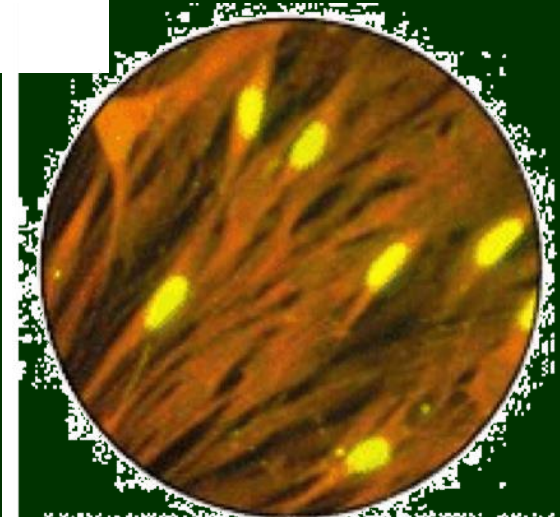
Fluorochrome Excitation/Emission/Cube/Application Data

Fluorochrome	Excitation Wavelength	Emission Wavelength	Applicable Excitation	Comment
Fluorochrome Antibody Method				
Allophycocyanin (APC)	650	660	IY	Antibody Labeling
7-Amino-4-Methylcoumarin-3-Acetic Acid (AMCA)	350	450	U	Antibody Labeling
BODIPY FL	503	512	B, IB	Antibody Labeling
Cascade Blue	376	425	U	Antibody Labeling
Fluorescein-Isothiocyanate (FITC)	490	520	IB	Antibody Labeling
Phycoerythrin B (PE-B)	545	576	G	Antibody Labeling
Phycoerythrin R (PE-R)	490, 565	578	G, IB	Antibody Labeling
Rhodamine B-Isothiocyanate (RITC)	570	595	G	Antibody Labeling
Texas Red	596	620	IY	Antibody Labeling
Tetramethylrhodamine-Isothiocyanate (TRITC)	541	572	IG	Antibody Labeling

Rychlé kultivační vyšetření

Při těchto rychlých technikách lze v TK nalézt virové antigeny již za 2-4 dny. Dobrým příkladem je CMV DEAFF (detection of early antigen fluorescent foci):

- Tkáňová kultura roste v mediu v plastové lahvi lahvi na krycím sklíčku.
- Po inokulaci se lahví pro urychlení adsorpce viru hodinu pomalu kýve a láhev se pak inkubuje 2-4 dny.
- Sklíčko se pak vyndá a imunofluorescenční metodou se v buňkách TK hledají časné virové antigeny CMV.



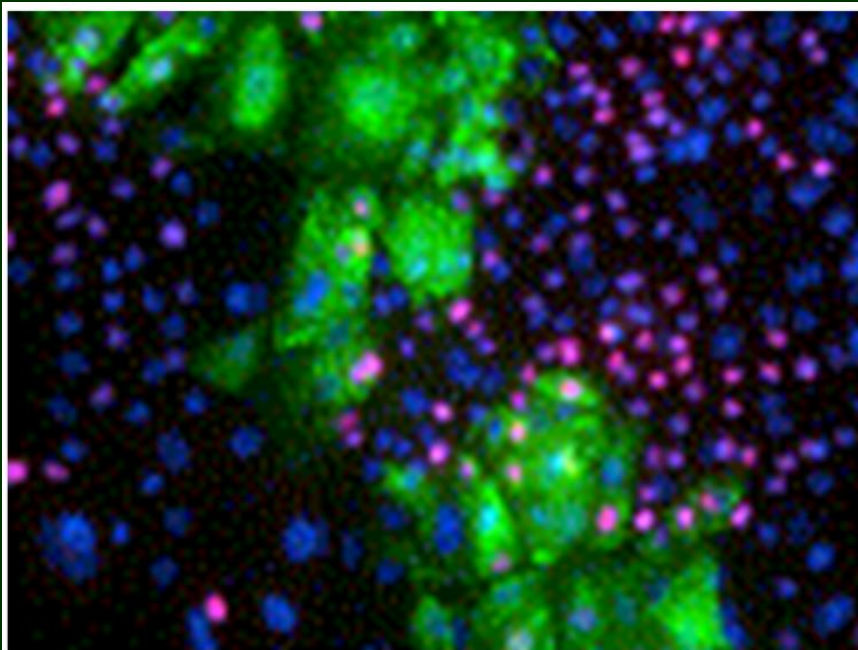


Figure 1. Immunofluorescence double-staining of CDV-infected canine footpad keratinocytes with an antibody against viral N-protein (green) and proliferation marker BrdU (red). Nuclei stained with DAPI (blue). Figure adopted from Engelhardt P, *et al.* [1].

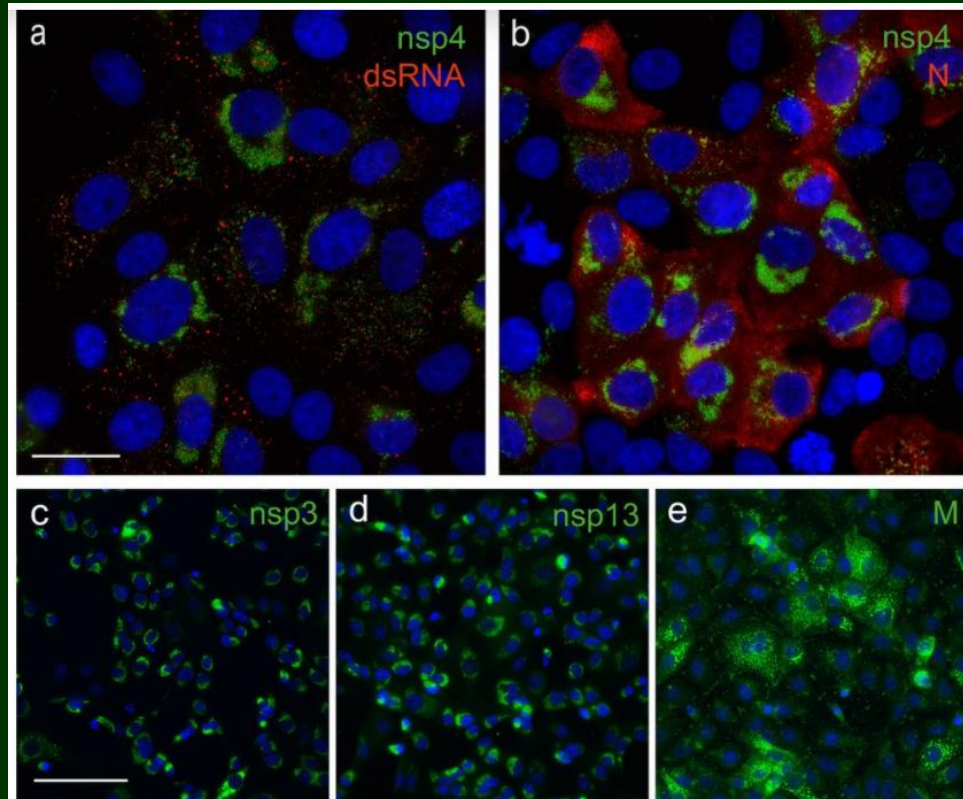
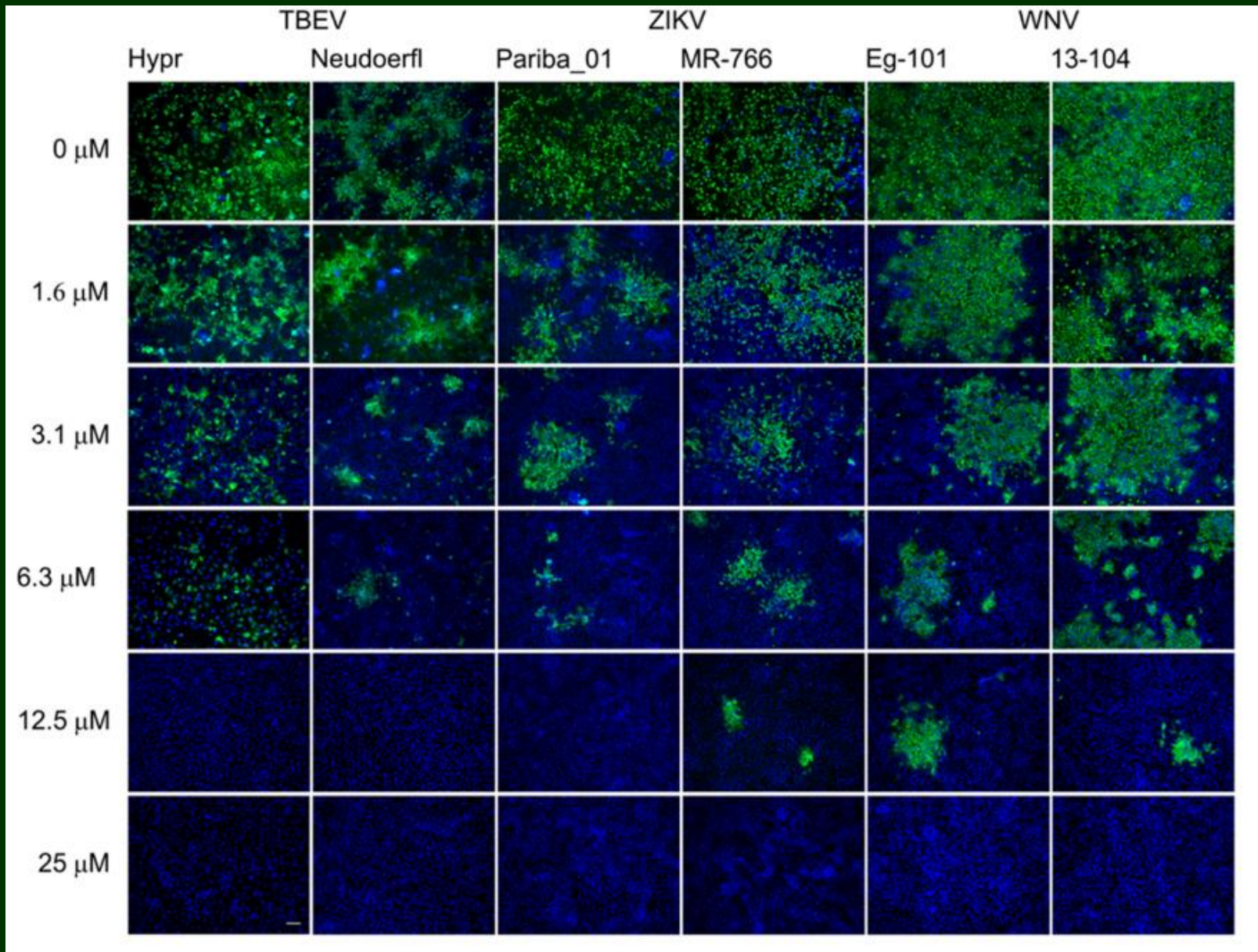


Fig. 3: Immunofluorescence microscopy of SARS-CoV-2 infected Vero E6 cells which were treated with rabbit SARS-CoV antisera. Nsps can be found near the nucleus, while the M protein is located at the Golgi apparatus. Anti-dsRNA antibodies were used to label replicative intermediates of viral RNA synthesis. Nuclei were stained with Hoechst. Scale bar is 25 μm for (a) and (b); 100 μm for (c), (d) and (e). Courtesy of Ogando *et al.*, *Journal of General Virology* (DOI 10.1099/jgv.0.001453).

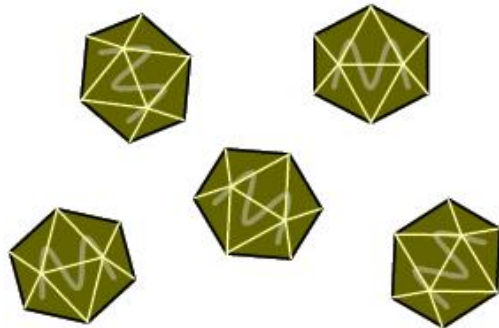
Imunofluorescenční barvení pro identifikaci viru v tkáňové kultuře

Imunofluorescenční barvení testování antivirotik v buněčné kultuře

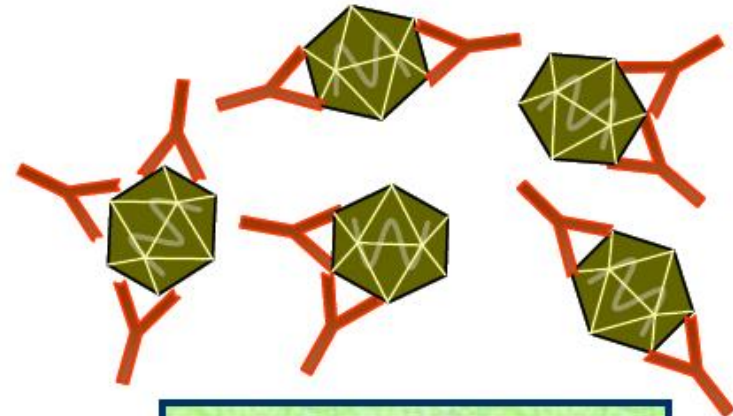
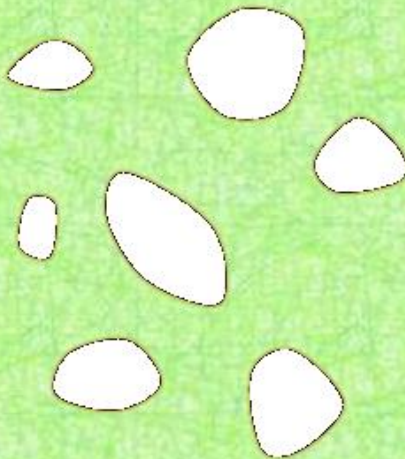
Koncentrace antivirotika



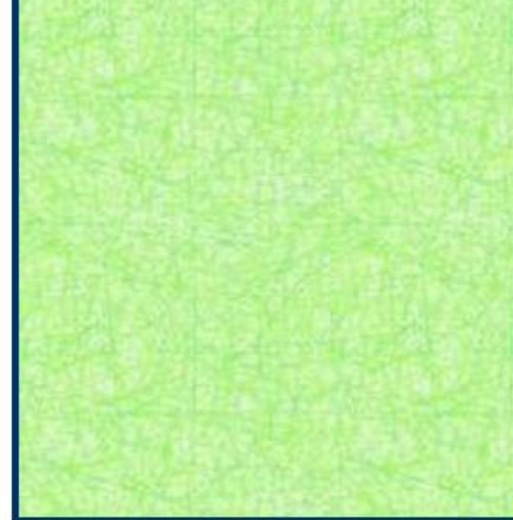
Virus-neutralizační test



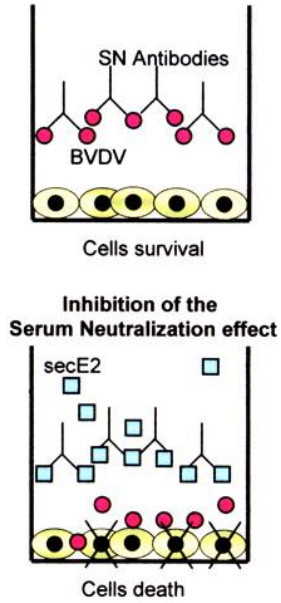
Tkáňová kultura
Množení viru –
makroskopicky po obarvení



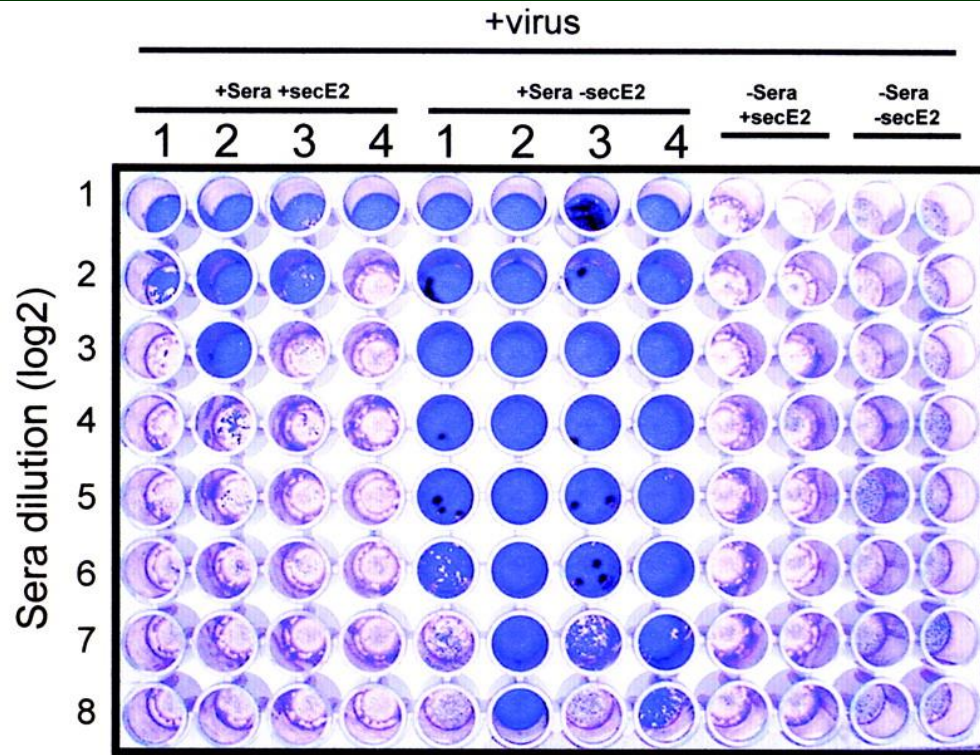
Tkáňová kultura
Virus neutralizován –
makroskopicky po obarvení



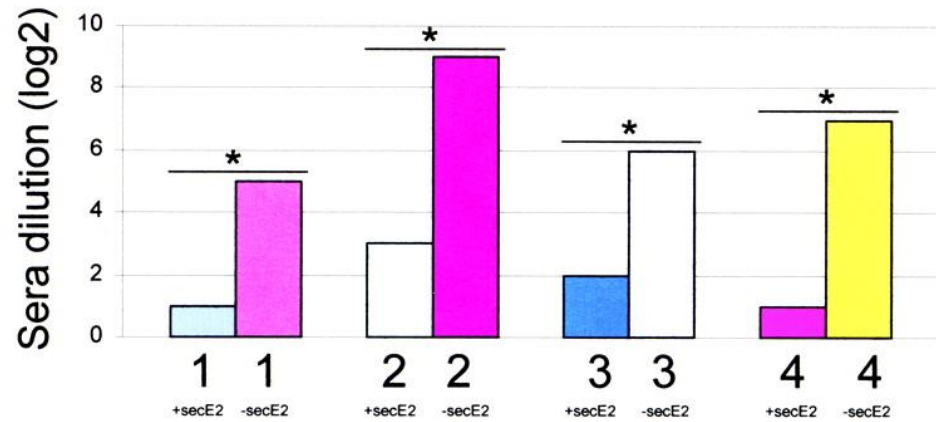
a) Serum Neutralization effect



b)

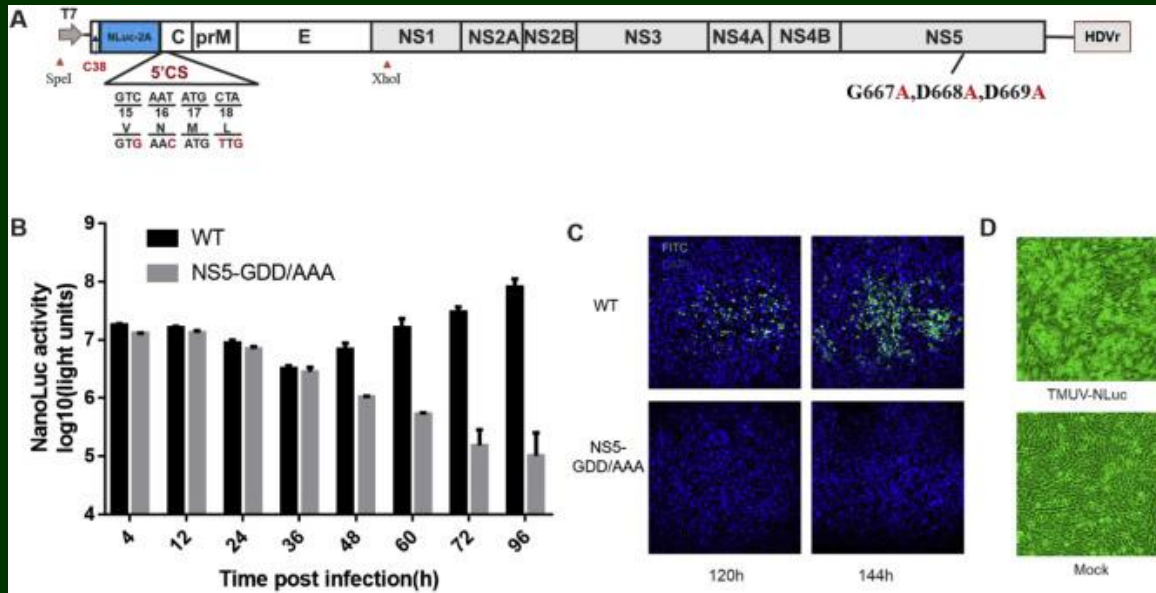


c)

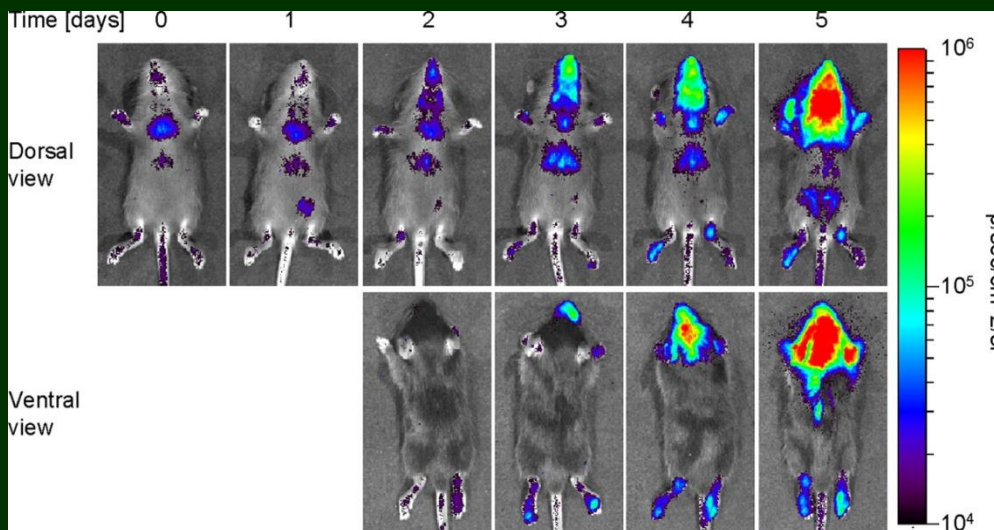


* = P < 0,009

Reportérové virové systémy



Reportérové proteiny:
 GFP
 Luciferáza
 Nano luciferáza (nLuc)
 mCherry
 Near Infra Red protein
 atd



Reportérové virové systémy: méně stabilní uspořádání

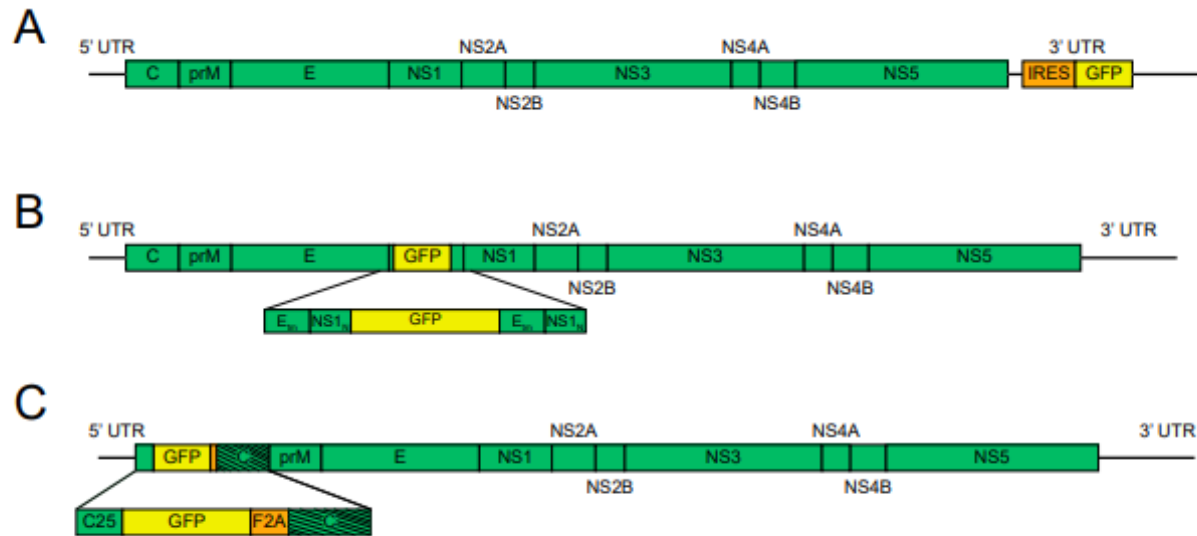


Figure 2. Flavivirus reporter schemes—less stable. (A). 3' untranslated region (UTR) reporter insertion. The reporter gene is inserted into a permissive site in the 3' UTR under the control of an internal ribosomal entry site (IRES). (B). E/NS1 reporter insertion. The reporter gene is placed at the junction between E and NS1, with a duplication of the N-terminus (N) of NS1 and the transmembrane (tm) domains of E. (C). 5' reporter insertion. The reporter gene is placed at the junction of the 5' UTR and the capsid gene. The first 25 amino acids of the capsid are duplicated (C25) and the reporter gene is followed by the foot and mouth disease virus 2A sequence (F2A) and codon scrambled capsid gene (represented by the slanted lines).

Reportérové virové systémy: stabilnější upořádání

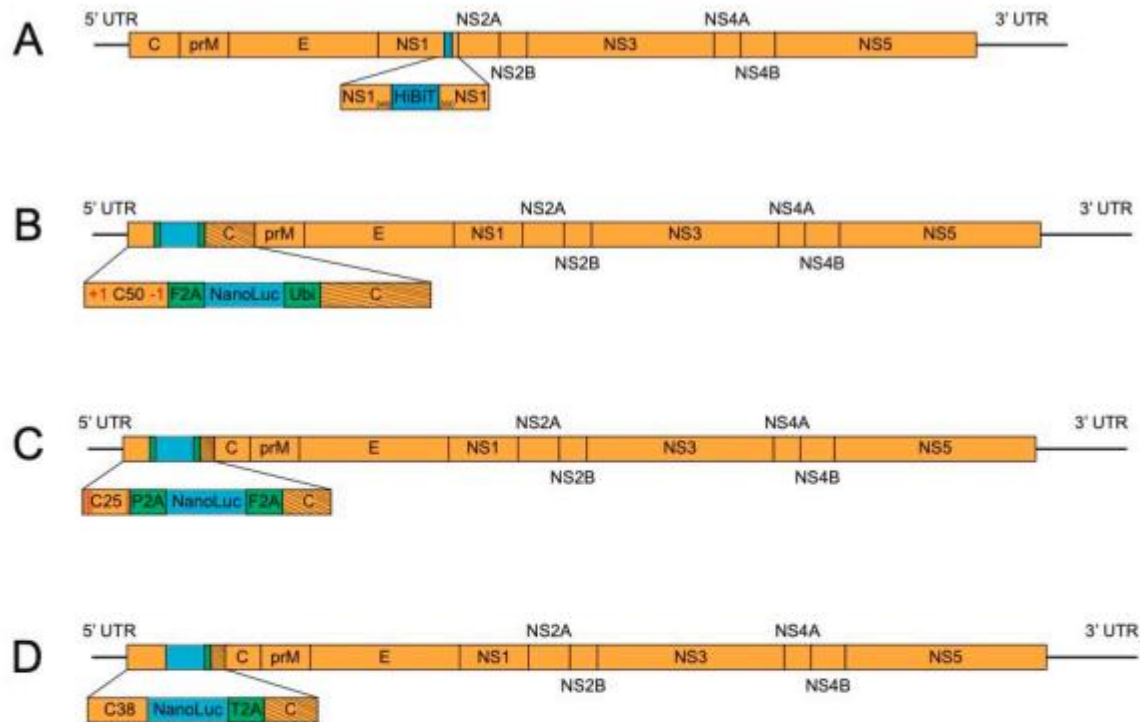
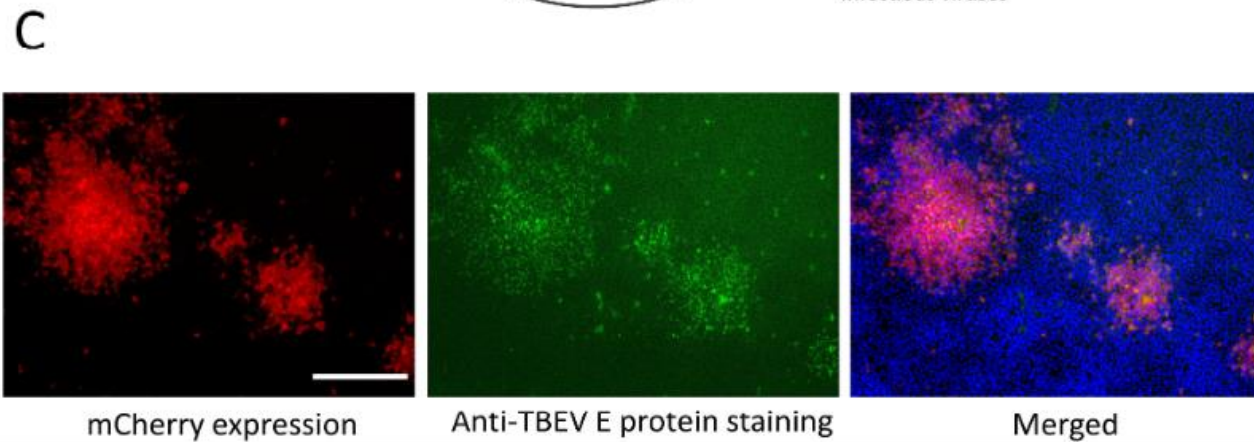
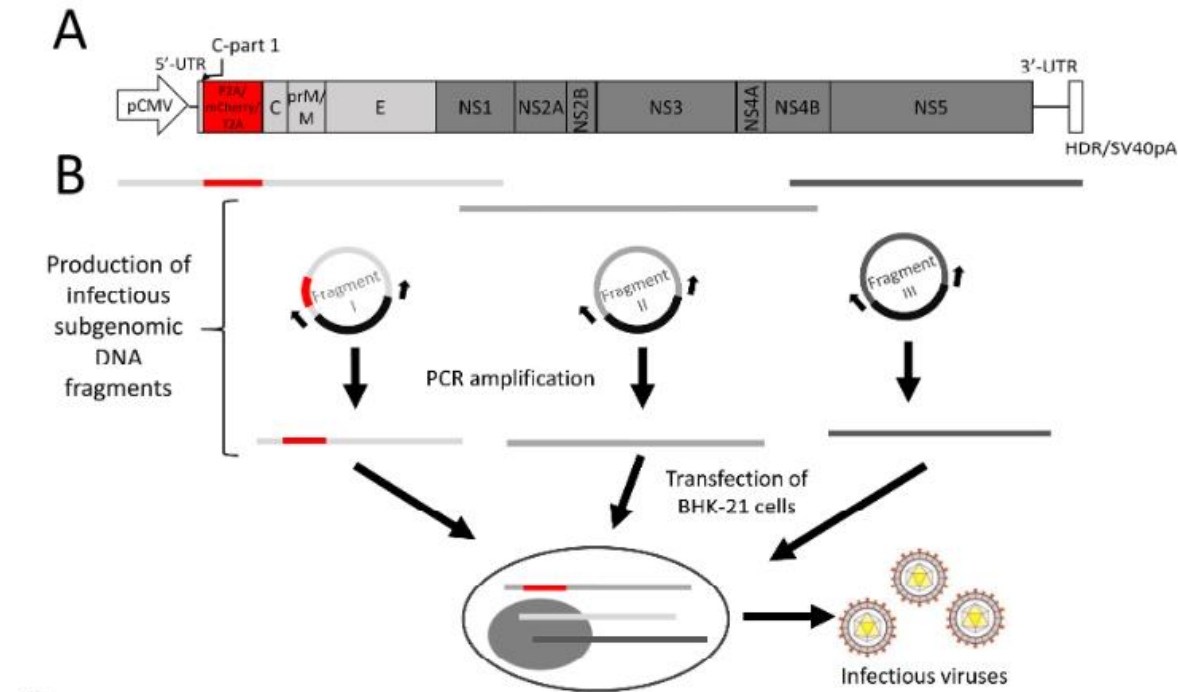
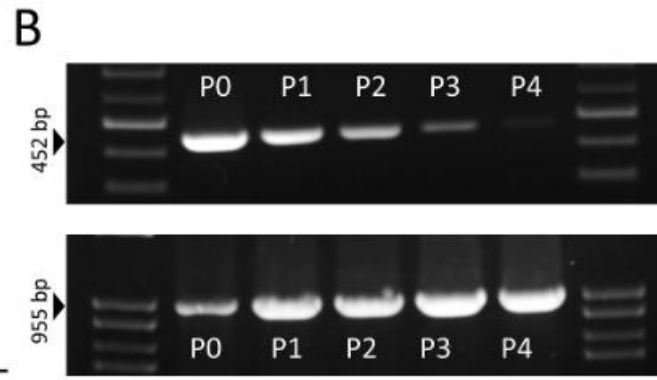
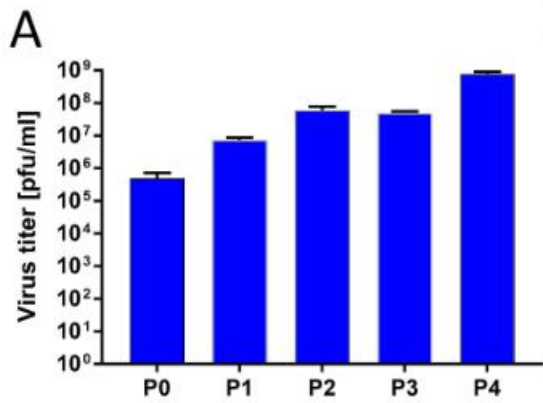


Figure 3. Flavivirus reporter schemes—stable. **(A).** NS1 insertion of split luciferase. The small subunit of split NanoLuc (HiBit) is inserted in NS1 at amino acid 349. **(B).** C50 with frameshift mutation. The reporter gene is engineered after a duplication of 50 capsid amino acids (C50) and flanked by F2A and the ubiquitin sequence (Ubi). C50 contains a +1-frameshift mutation after the fourth codon, which is restored at the end of C50. Slanted lines indicate codon scrambling. **(C).** Recombination-dependent lethal mutations. The reporter gene is inserted at the 5' end of the genome after a 25 amino acid capsid duplication (C25) and flanked by different 2A sequences (F2A and porcine teschovirus-1 2A, P2A). The C25 region contains four amino acid mutations, denoted by red lines. Slanted lines in the capsid gene represent codon scrambled sequence. **(D).** Lengthened capsid duplication. The reporter gene is located at the beginning of the capsid gene, with a duplication of 38 amino acids. Slanted lines in the capsid gene correspond to the 38 codons that have been scrambled (T2A, thosea asigna virus 2A).

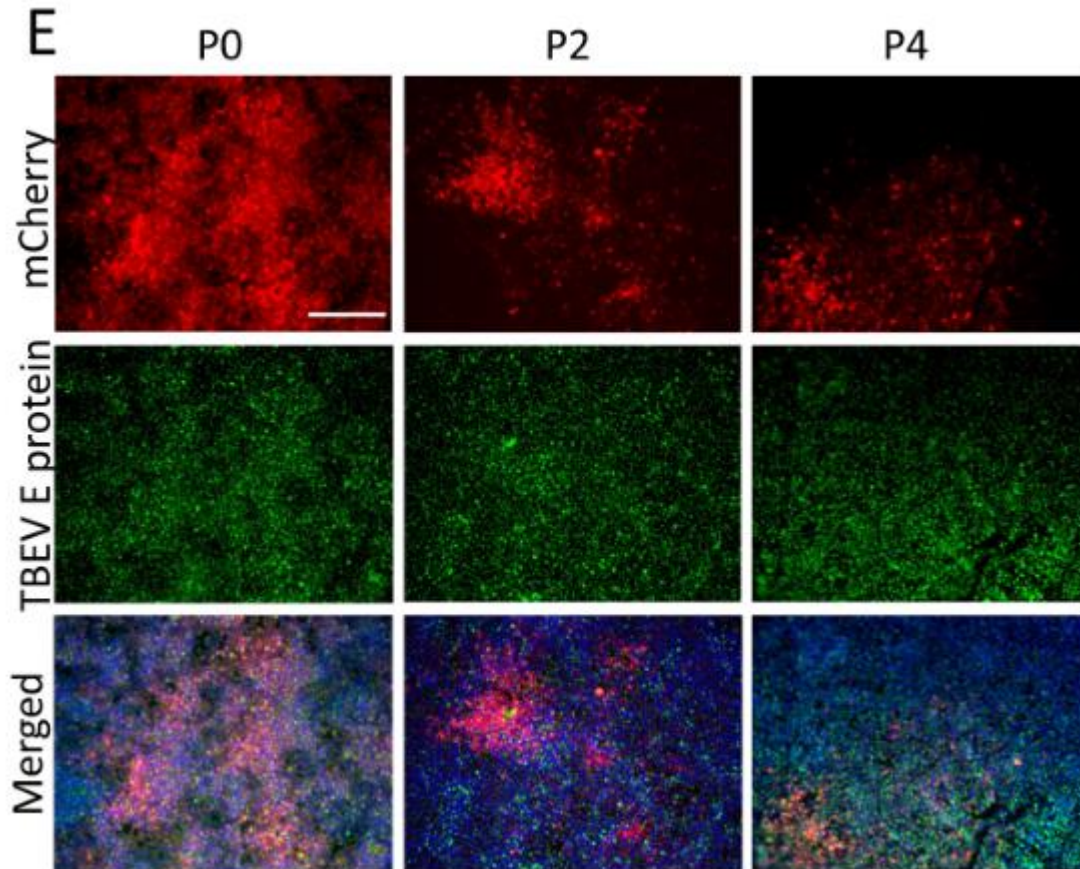
Reportérové virové systémy: mCherry-TBEV



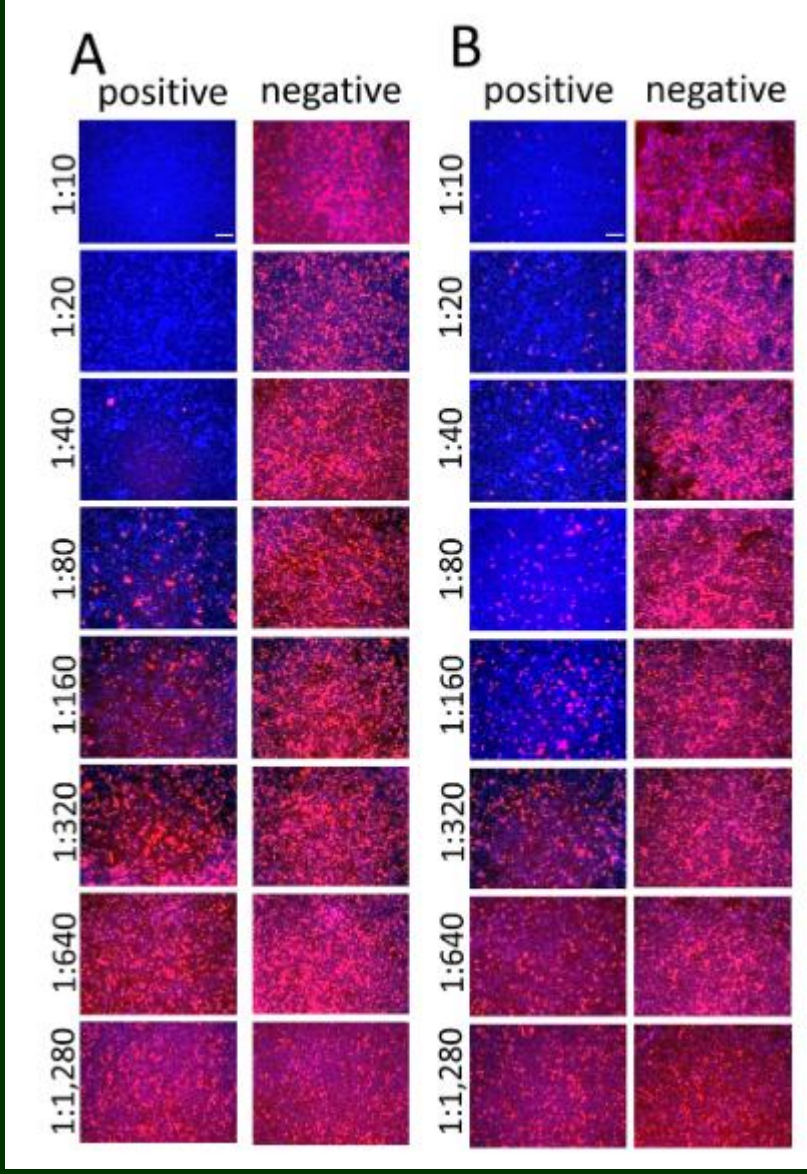


mCherry-TBEV

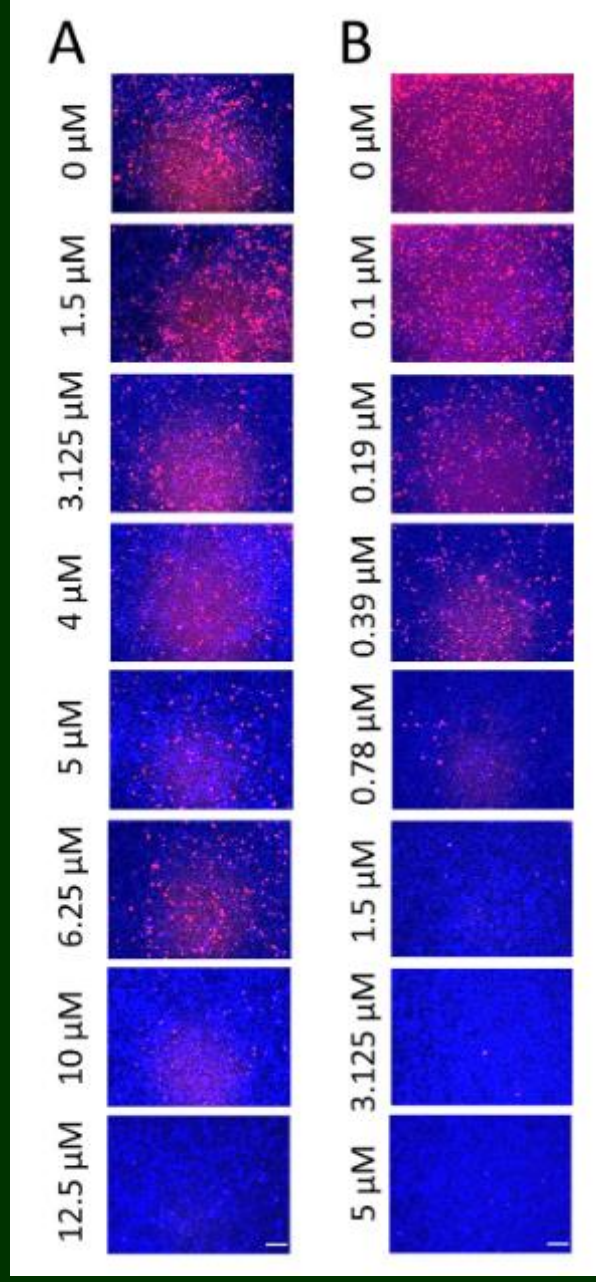
TBEV - wt



Stabilitní studie



Využití mCherry-TBEV pro virus neutralizační test



Využití mCherry-TBEV pro testování antivirotik

Table 1. List of currently available US FDA-approved antiretroviral drugs

Generic name	Abbreviation	Brand name (US) ^a
Nucleoside reverse transcriptase inhibitors (NRTIs)		
Abacavir	ABC	Ziagen [®]
Didanosine	ddI	Videx [®]
Emtricitabine	FTC	Emtriva [®]
Lamivudine	3TC	Epivir [®]
Stavudine	d4T	Zerit [®]
Tenofovir	TDF	Viread [®]
Zidovudine	AZT, ZDV	Retrovir [®]
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)		
Delavirdine	DLV	Rescriptor [®]
Efavirenz	EFV	Sustiva [®]
Etravirine	ETR	Intelence [®]
Nevirapine	NVP	Viramune [®]
Nevirapine extended release	NVP XR	Viramune [®] XR [™]
Rilpivirine	RPV	Edurant [®]
Protease inhibitors (PIs)		
Atazanavir	ATV	Reyataz [®]
Darunavir	DRV	Prezista [®]
Fosamprenavir	FPV	Lexiva [®]
Indinavir	IDV	Crixivan [®]
Lopinavir/ritonavir	LPV/r	Kaletra [®]
Nelfinavir	NFV	Viracept [®]
Ritonavir	RTV, /r	Norvir [®]
Saquinavir hard gel caps	SQV	Invirase [®]
Tipranavir	TPV	Aptivus [®]
Integrase inhibitors (INIs)		
Raltegravir	RAL	Isentress [®]
CCR5 antagonist		
Maraviroc	MVC	Selzentry [®]
Fusion inhibitor		
Enfuvirtide (T20)	ENF	Fuzeon [®]

Vyšetření rezistence viru na léčiva (antivirotika)

Příklad: HIV-1

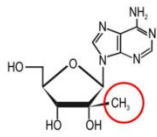
- v dnešní době drtivá převaha mol-biol metod
- kultivačně hlavně v rámci základního výzkumu

	Discriminatory mutations					Thymidine analogue mutations (TAMs)					MDR mutations	
	184	65	74	115	41	67	70	210	215	219	69	151
<i>Cons</i>	M	K	L	Y	M	D	K	L	T	K	T	Q
3TC	<u>V</u>	R									Ins	M
FTC	<u>V</u>	R									Ins	M
ABC	V	<u>R</u>	<u>V</u>	F	L			W	YF		Ins	<u>M</u>
ddI	V	<u>R</u>	<u>V</u>		L			W	YF		Ins	<u>M</u>
TDF	***	<u>R</u>	*	F	L		R	W	YF		Ins	M
d4T	***	R			L	N	R	W	<u>YF</u>	QE	<u>Ins</u>	<u>M</u>
ZDV	***	***	*		L	N	R	W	<u>YF</u>	QE	<u>Ins</u>	<u>M</u>

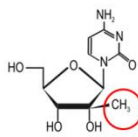
Fig. 2. Summary of nucleoside/nucleotide reverse transcriptase inhibitor drug-resistance mutations. Mutations are represented by their numeric position and amino acid letter code. The amino acid of the consensus wild-type sequence is represented by letters in the top row, while the amino acids of the mutations are below. Bold and underlining indicates mutations with high-level phenotypic and/or clinical resistance; bold indicates moderate phenotypic and/or clinical resistance; plain text indicates low-level resistance. Asterisks (***) represent increased susceptibility to the drug if the mutation is present. Refer to table 1 for a full list of drug name abbreviations and definitions. Adapted from the Stanford University HIV Drug Resistance Database.¹⁴⁰ **Cons** = consensus wild-type; **Ins** = insertion; **MDR** = multi-drug resistance.

Structure-activity relationships TBEV

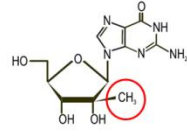
2'-C-modification



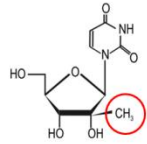
2'-C-methyladenosine



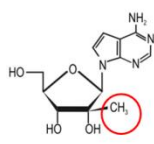
2'-C-methylcytidine



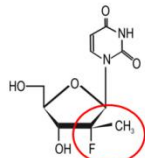
2'-C-methylguanosine



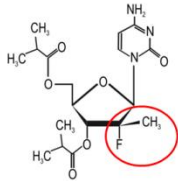
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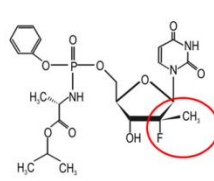
7-deaza-2'-C-methyladenosine



PSI 6206

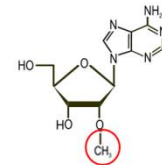


mericitabine

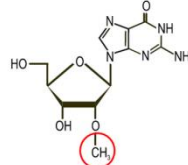


sofosbuvir

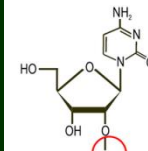
2'-O-modification



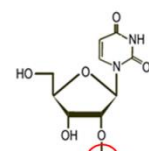
2'-O-methyladenosine



2'-O-methylguanosine

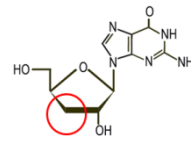


2'-O-methylcytidine

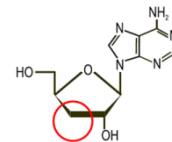


2'-O-methyluridine

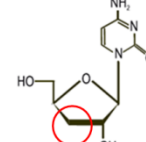
3'-C-modification



3'-deoxyguanosine

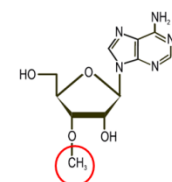


3'-deoxyadenosine

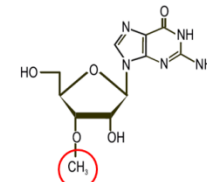


3'-deoxycytidine

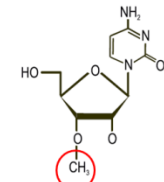
3'-O-modification



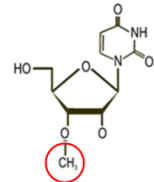
3'-O-methyladenosine



3'-O-methylguanosine

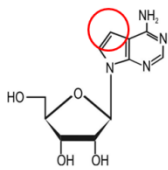


3'-O-methylcytidine

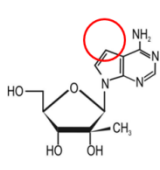


3'-O-methyluridine

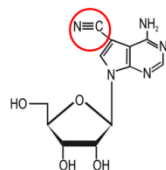
Modification of heterobase



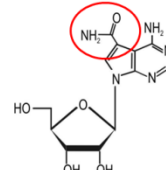
tubercidin



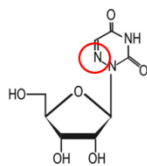
7-deaza-2'-C-methyladenosine



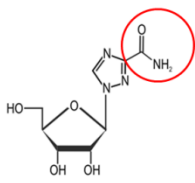
toyocamycin



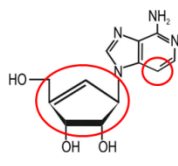
sangivamycin



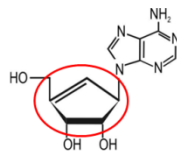
6-azauridine



ribavirin

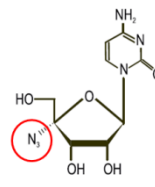


3-deazaneplanocin A

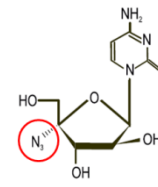


neplanocin A

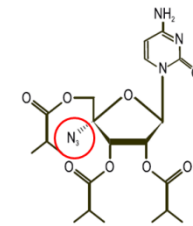
4'-C-modification



4'-azidocytidine

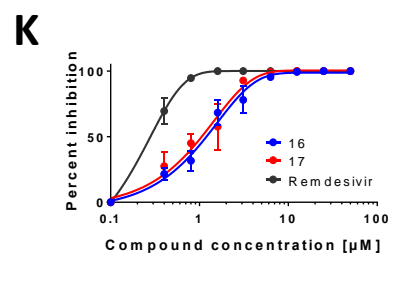
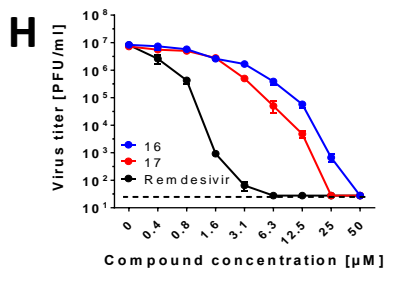
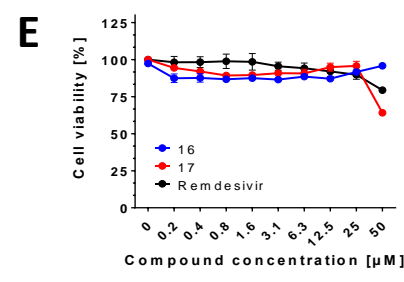
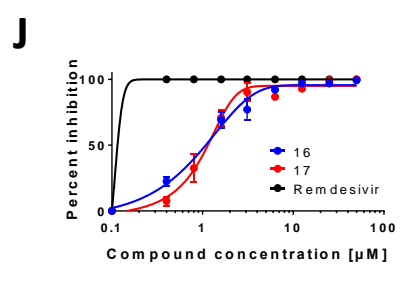
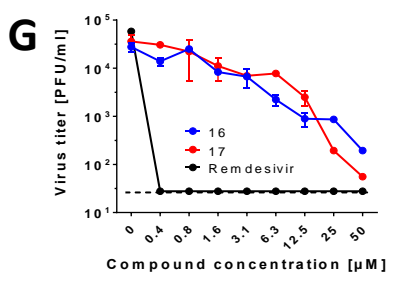
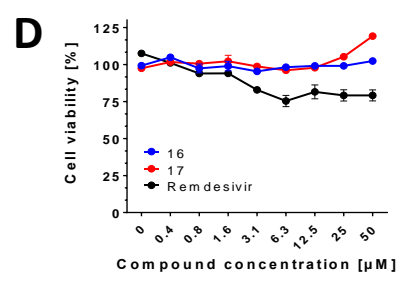
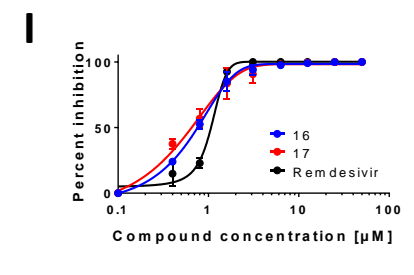
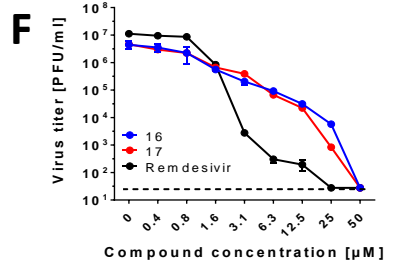
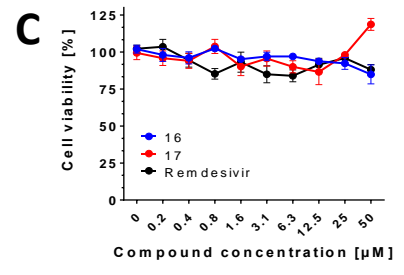
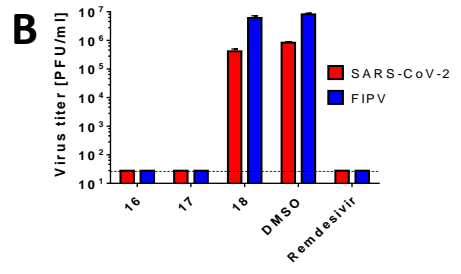
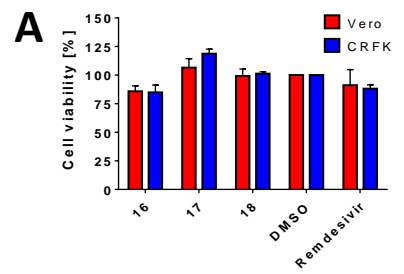


RO-9187

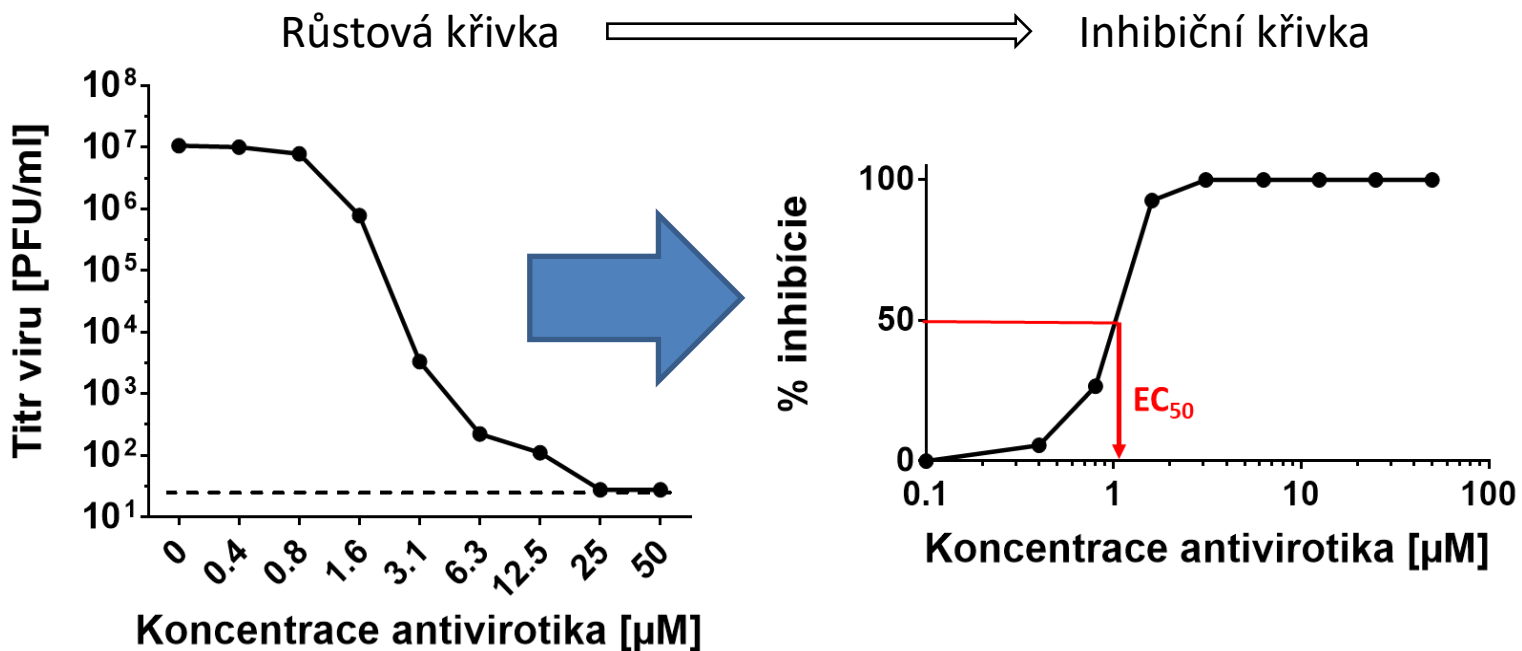


balapiravir

Analýza toxicity a antivirové aktivity



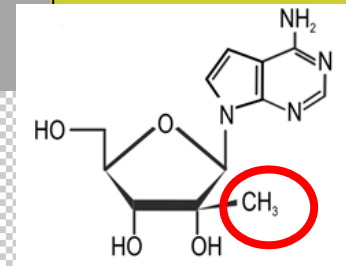
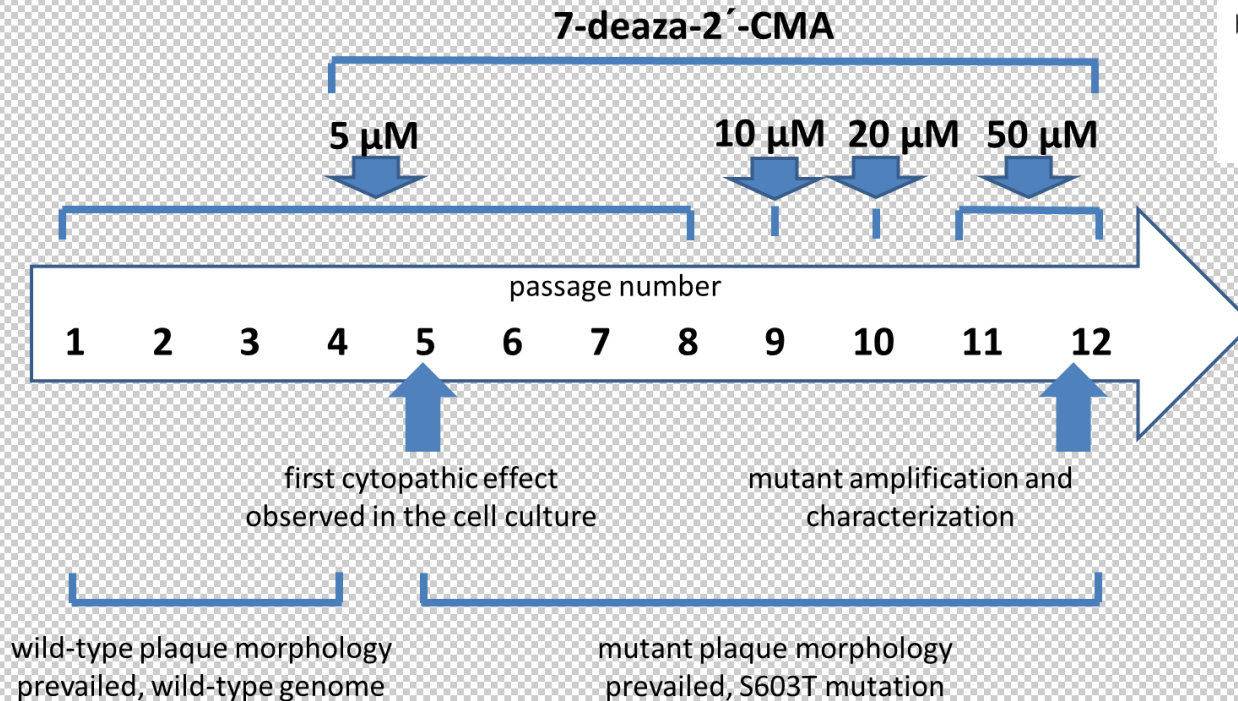
Výpočet hodnoty EC50 nebo IC50 (efektivní/inhibiční koncentrace, která způsobí pokles titru na polovinu – 50%)



Podobně počítáme CC50 (cytotoxická koncentrace, která snižuje viabilitu hostitelských buněk na polovinu)

TBEV resistance to 2'-C-methylated nucleosides

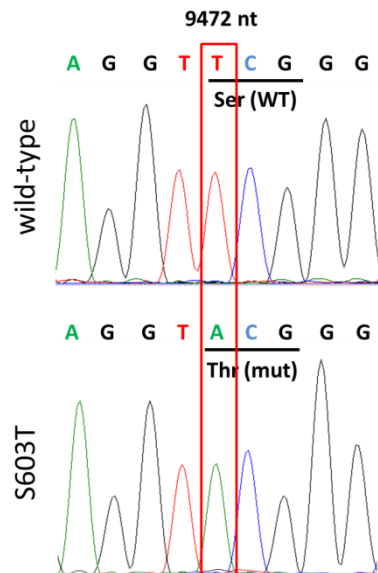
Selection strategy to isolate drug-resistant TBEV strain



Eyer et al. 2017; Journal of Virology

Resistance-conferring mutation S603T in the NS5 coding region

Mutation: T9471A (S603T)



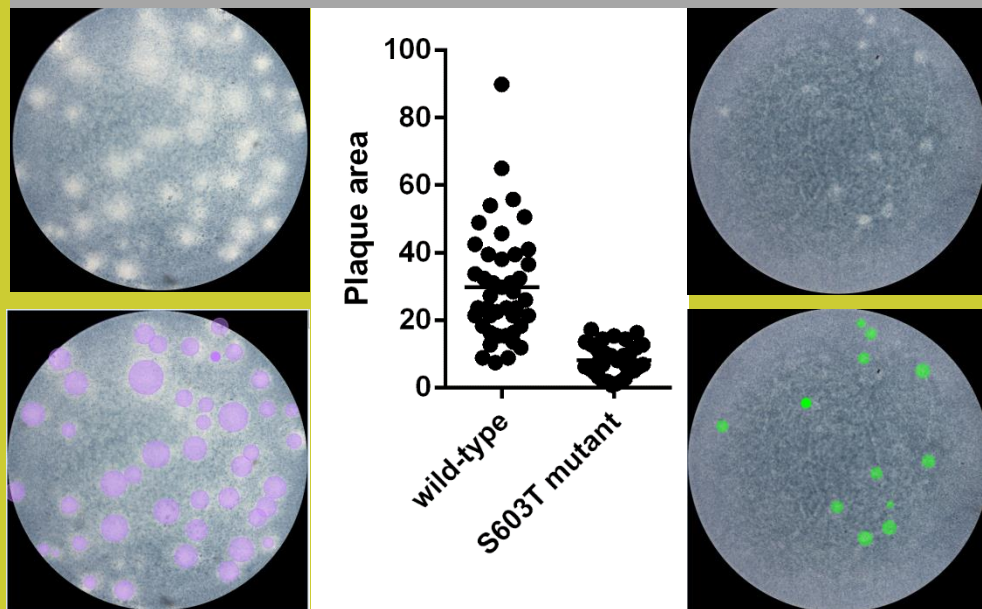
9472 nt

passage number	WT	1	2	3	4	5	6	7	8	9	10	11	12
	AAAGAGGTTTCGGGCCAGGTTGTGA	AAAGAGGTTTCGGGCCAGGTTGTGA	AAAGAGGTTTCGGGCCAGGTTGTGA	AAAGAGGTTTCGGGCCAGGTTGTGA	AAAGAGGTAACGGGCCAGGTTGTGA	AAAGAGGTAACGGGCCAGGTTGTGA	AAAGAGGTAACGGGCCAGGTTGTGA	AAAGAGGTAACGGGCCAGGTTGTGA	AAAGAGGTAACGGGCCAGGTTGTGA	AAAGAGGTAACGGGCCAGGTTGTGA	AAAGAGGTAACGGGCCAGGTTGTGA	AAAGAGGTAACGGGCCAGGTTGTGA	AAAGAGGTAACGGGCCAGGTTGTGA

Detailed description: This table shows the sequence evolution at position 9472 nt over 12 passages. The WT sequence is AAAGAGGTTTCGGGCCAGGTTGTGA. Passages 1-3 show the WT sequence. From passage 4 onwards, the sequence has mutated to AAAGAGGTAACGGGCCAGGTTGTGA, where the T at position 9472 nt has been replaced by an A. The mutation is stable across all 12 passages.

In vitro properties of the S603T mutant

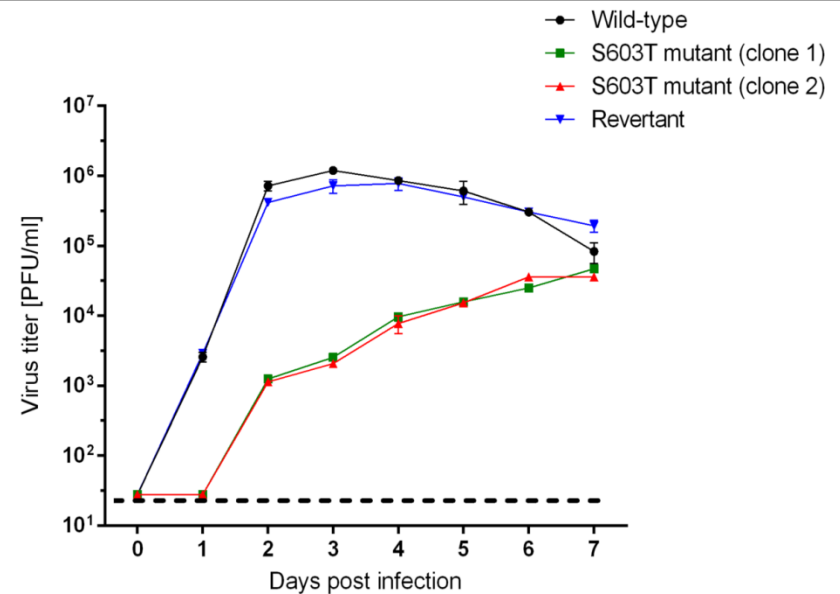
Plaque morphology of S603T mutant and wild-type



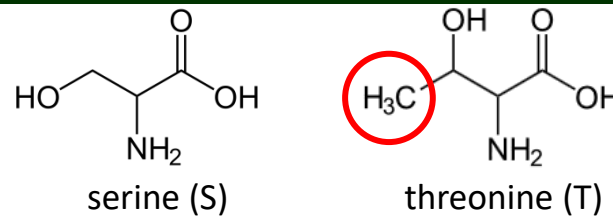
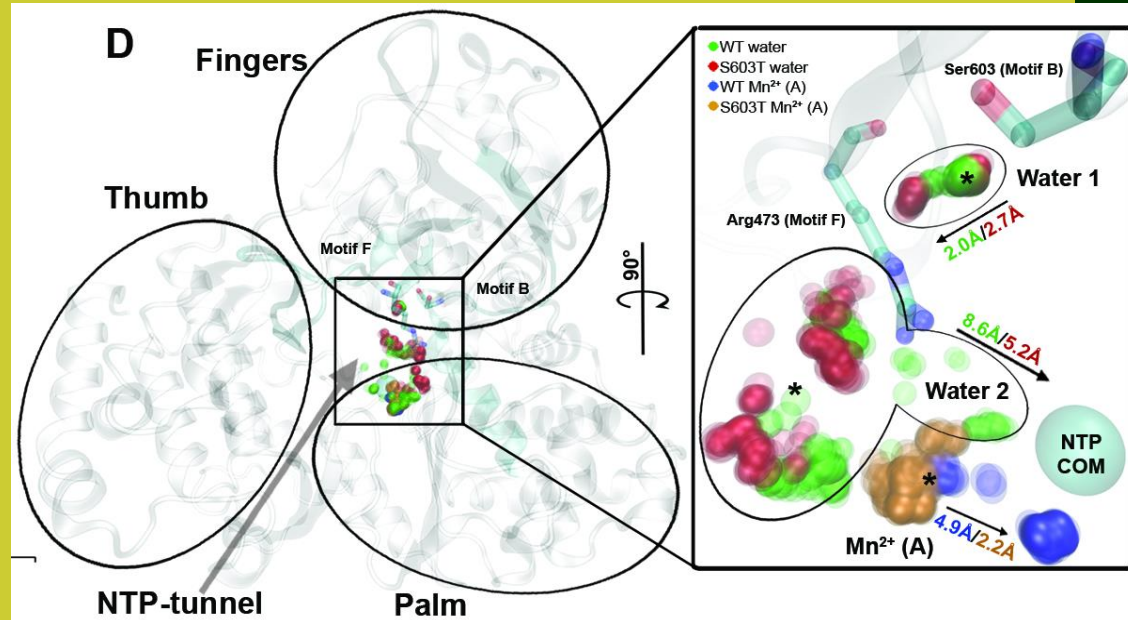
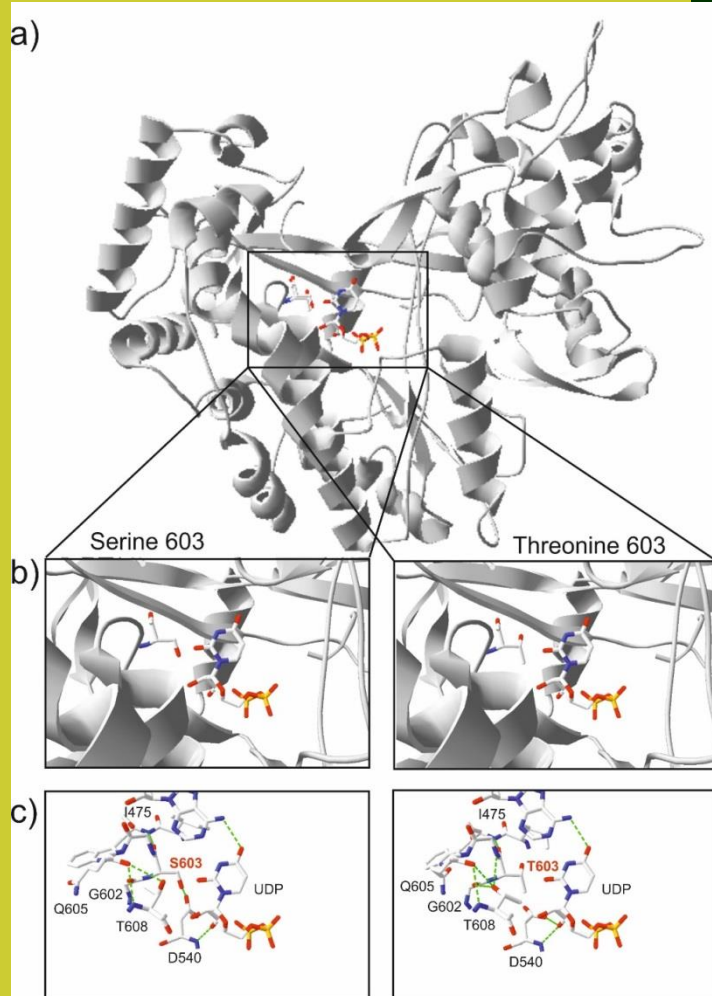
WT

S603T

Growth kinetics of wild-type, S603T mutant and revertant



Interaction of 7-deaza-2'-CMA with TBEV polymerase active site



Eyer et al. 2017; Journal of Virology

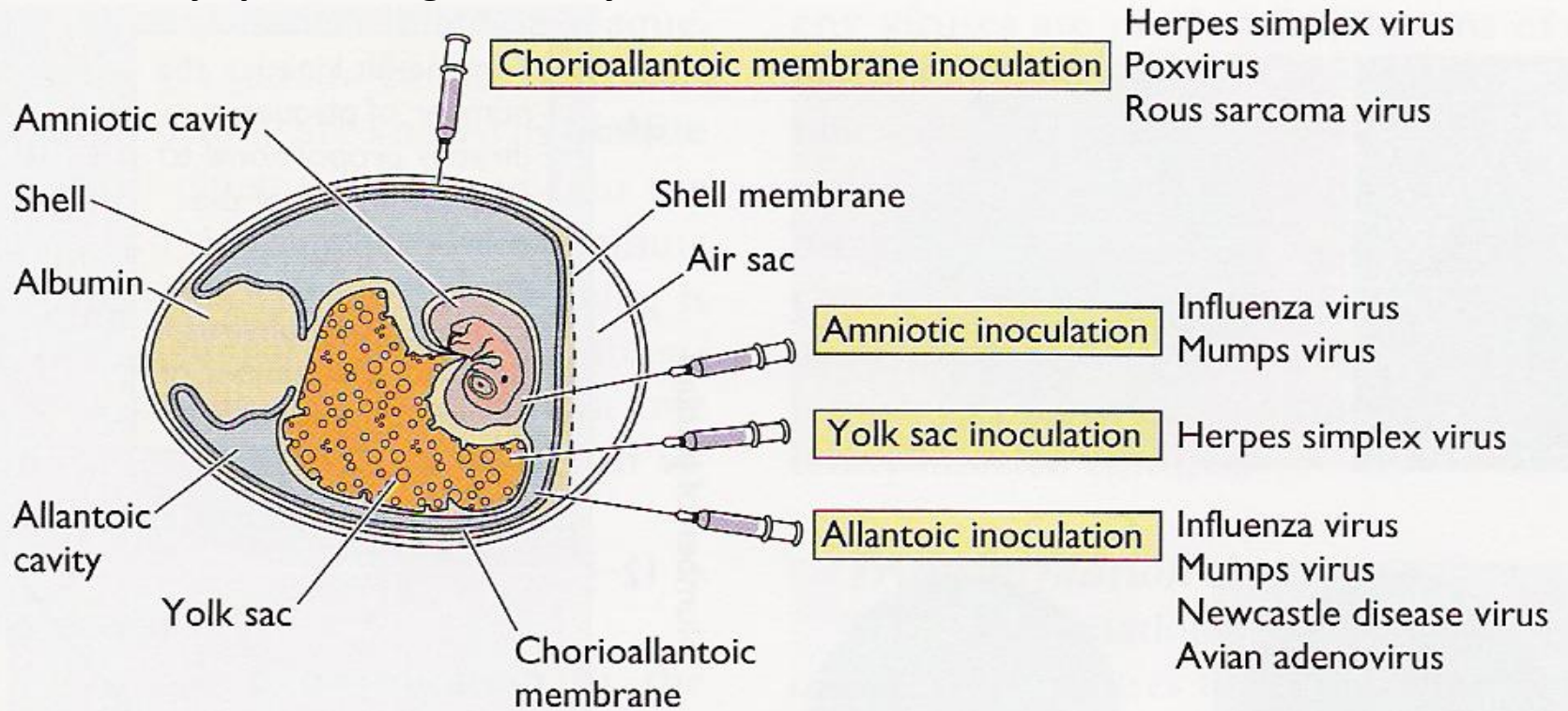
In vitro studie - závěr

- Excellent tools for simple questions with limited variables
- Cells are easily manipulated
- Cost efficient
- Scalable

In vitro studies have given us a lot BUT...

- Focus on one specific aspect of the disease process only
- Cannot predict relevant phenotypes associated with pathogenesis

Kuřecí embrya pro virologické účely



Produkce velkého množství viru pro
přípravu vakcín - chřipka



Laboratorní zvířata

- Laboratorní myši, potkani, morčata, primáti
- Nutno pracovat na základě zvláštního povolení, pouze pracovníci se zvláštní kvalifikací
- Zvláštními právními předpisy a vyhláškami stanoveno, jaké mají mít osvětlení, hladina hluku, denní režim, potrava (i zastoupení jednotlivých výživových látek), velikost chovných nádob, způsob nakládání...
- Zákon č. 246/1992 Sb., na ochranu zvířat proti týrání
- Experimenty na zvířatech mohou provádět pouze pracovníci se zvláštní kvalifikací, kterou dokládá Osvědčení o odborné způsobilosti k navrhování pokusů a projektů pokusů podle §15d odst. 3 zákona č. 246/1992 Sb.
- Každý experiment na zvířatech musí být ošetřen žádostí o schválení projektu pokusu, která je posouzena příslušným státním orgánem (AVČR, MZe, MZd atd.). a který vydá rozhodnutí o schválení a oprávnění vykonávat pokusy na zvířatech.





Species

Mouse

Commonly Studied Virus Families

Poxviridae, Herpesviridae, Adenoviridae, Hepadnaviridae, Parvoviridae, Retroviridae, Arenaviridae, Bunyaviridae, Orthomyxoviridae, Paramyxoviridae, Filoviridae, Rhabdoviridae, Reoviridae, Coronaviridae, Arteriviridae, Togaviridae, Flaviviridae, Caliciviridae, Picornaviridae



Guinea Pig

Herpesviridae, Arenaviridae, Orthomyxoviridae, Paramyxoviridae, Filoviridae



Hamster

Arenaviridae, Bunyaviridae, Filoviridae, Paramyxoviridae, Flaviviridae



Woodchuck

Hepadnaviridae



Prairie Dog

Poxviridae



Cotton Rat

Adenoviridae, Orthomyxoviridae, Paramyxoviridae



Ground Squirrel

Hepadnaviridae, Rhabdoviridae, Togaviridae



Ferret

Orthomyxoviridae, Paramyxoviridae, Coronaviridae



Chicken

Poxviridae, Retroviridae, Orthomyxoviridae



Macaque

Poxviridae, Herpesviridae, Adenoviridae, Papillomaviridae, Polyomaviridae, Hepadnaviridae, Retroviridae, Arenaviridae, Bunyaviridae, Orthomyxoviridae, Paramyxoviridae, Filoviridae, Rhabdoviridae, Reoviridae, Coronaviridae, Arteriviridae, Togaviridae, Flaviviridae, Caliciviridae, Picornaviridae



Pig

Poxviridae, Herpesviridae, Parvoviridae, Circoviridae, Orthomyxoviridae, Paramyxoviridae, Filoviridae, Rhabdoviridae, Reoviridae, Coronaviridae, Arteriviridae, Flaviviridae, Caliciviridae, Picornaviridae



Ruminant

Poxviridae, Herpesviridae, Papillomaviridae, Retroviridae, Bunyaviridae, Paramyxoviridae, Reoviridae, Flaviviridae, Picornaviridae

The most important species of laboratory animals

- **Mouse** – most frequently used. Pharmacology, genetics of mammals, virology, models of human diseases (mutant strains, transgenic and knock-out mice)
- **Rat** – physiology of cognitive processes, behaviour, models of diabetes
- **Rabbit** – serology, insulin quantification, pyrogens quantification, tests of irritable effect of chemical substances on the cornea
- **Cat** – study of CNS and respiratory system
- **Dog** – e.g. beagle, use in electrophysiology, neurophysiology
- **Guinea-pig** – in microbiology and serology, physiology of the auditory system
- **Hamster** - genetics
- **Pig** – training of surgical techniques, temporary covering of burns with porcine skin
- **Primates** – rhesus monkey, baboon, chimpanzee – use in neurology, virology, behaviour
- **Frog** – physiology of blood circulation, electrophysiology
- **Fish, molluscs, insects...**

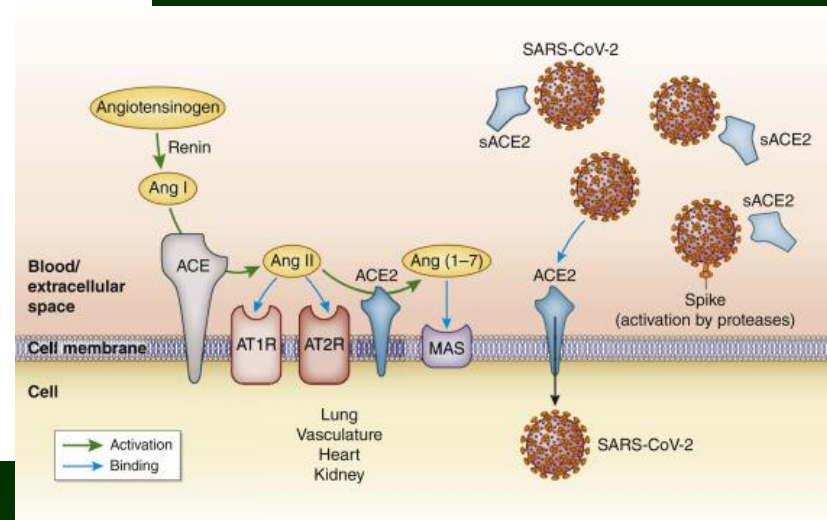
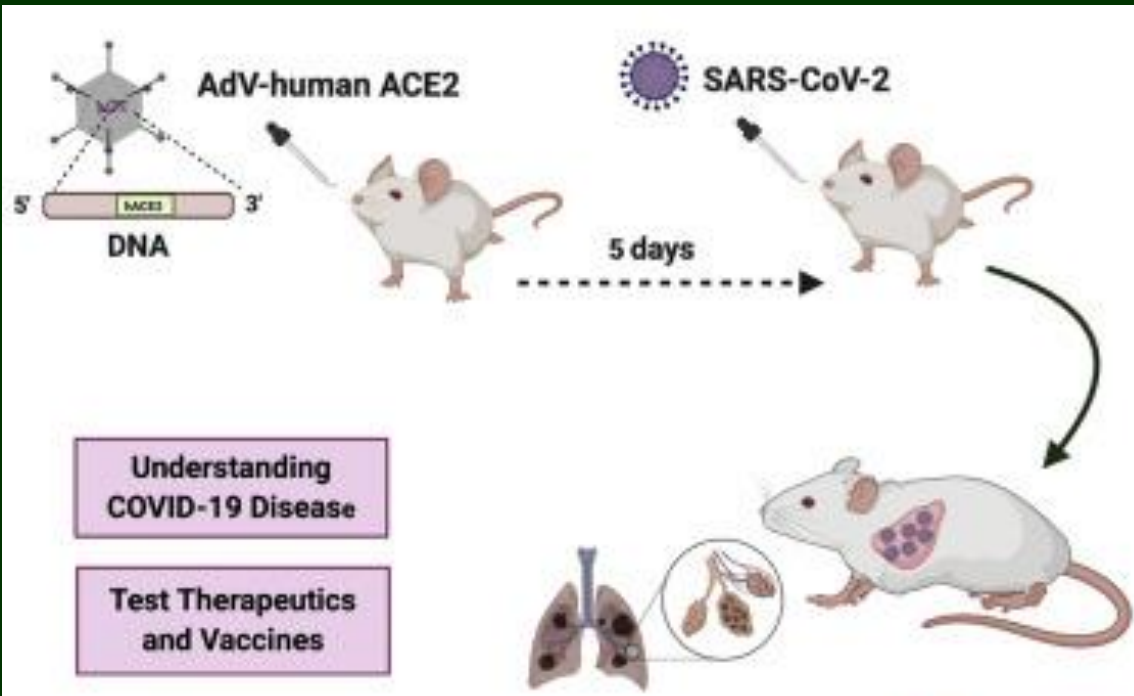
Hlodavci - myši, potkani, morčata

Některé kmeny hlodavců, zejména laboratorních myší, jsou **vysoce citlivé** na určité skupiny virů (např. **BALB/c** je vysoce vnímavá na virus **klíšťové encefalitidy**, **virus západonilské horečky** nebo některé **herpetické viry**). Kmeny **Black** jsou vnímavé na virus **vztekliny**.

Některé viry však **nepůsobí na většině myších kmenů viditelné projevy infekce**, nebo se v myších vůbec nereplikují (např. virus Zika, dengue nebo SARS-CoV-2). Zvolit sající, mladé nebo imunokomprimované myši (např. **AG129** neexprimující INF-a/b and INF-c receptory, **IFNAR^{-/-}** postrádající pouze IFNa/b receptory), nebo kompletně imunosuprimované myši nebo použít **virové kmeny adaptované na hlodavce** (např. kmen viru žluté zimnice Jimenez adaptovaný na křečky).

K dalším využívaným zvířecím modelům ve virologii patří **fretka** (pro studium viru chřipky) a v případě, že je třeba co nejdříve napodobit fyziologické podmínky lidského hostitele, jsou využíváni i **primáti**. Pro tvorbu vakcín se v minulosti hojně používala **kuřecí embrya**.

Myší model pro práci se SARS-CoV-2



GENETICS OF LABORATORY ANIMALS

1. **Isogenic** = genetically defined strains
(isogenicity= genetic uniformity of all individuals)
2. **Non-isogenic** = genetically undefined strains
3. **Genetically semi-defined strains**

Isogenic strains

Inbred strains

- obtained by close breeding for more than 20 generations (brother + sister or offspring + one of the parents)
- homozygosity higher than 98 % (Degree of homozygosity is expressed as a coefficient of inbreeding)
- features: isogenicity, phenotype uniformity (low variability of reactivity), usually low fertility, disposition to diseases
- advantages of use in experiment: homogenous statistical set, lower number of individuals is sufficient
- disadvantages of use in experiment: a risk, that the findings are strain-specific and are not valid for other strains, problematic generalization of the results

Coisogenic strains (mutant strains)

- differ from the original strain only in one gene, in which a mutation occurred

Congenic strains

- strains originated by cross-breeding of two strains and following back-cross-breeding with one of the original strains (at least 10 times, selection of some specific feature)
- presence of specific genes of one strain on genetical background of the second strain

Isogenic strains

Recombinant-inbred strains

- crossing of 2 strains, the hybrids give origin of new lines which are then crossed brother x sister, what leads to establishing of a new strain

Rekombinant-congenic strains

- crossing of 2 strains followed with 3 back-crosses to one of the original strains and inbreeding with crossing brother x sister (at least 14 times)

Consomic strains

- a complete chromosome of one strain is transferred on the background of the second strain with back-crosses (similarly as for individual genes in congenic strains, but the process is more complicated)

Non-isogenic strains

Outbred lines

- genetically heterogeneous population without crossing with individuals coming from different, in the frame of the population close crossbreeding is avoided so that the coefficient of inbreeding remains as low as possible
- features: some level of phenotype variability (higher variability of reactivity), higher fertility and resistance to diseases
- advantages of use in experiment: cheaper and easier production, the findings have more general validity
- disadvantages of use in experiment: less homogeneous set, higher number of animals is necessary

Genetically heterogeneous lines

- originate by crossing of several inbred strains followed with breeding according to principles of outbred population

Outbred selected lines

- in an outbred population given phenotype feature is selected

Animal models of diseases

Mutant animals

Transgenic animals

Knock-out animals

GNOTOBIOLOGY OF LABORATORY ANIMALS

Conventional animals

- undefined microflora
- open breeding facility complying basic hygienic conditions

SPF animals = specified pathogen free

- microflora of the animals certainly does not contain specified pathogens.
- barrier breeding facility

Gnotobiotic animals

- breeding isolators

1. Axenic animals = germ free

- without any microbes
- pups obtained with sterile hysterectomy or hysterotomy into sterile atmosphere of the isolator

2. Associated animals

- derived from axenic animals colonising them artificially with one or more species of microorganisms
- monoxenic, dixenic, polyxenic

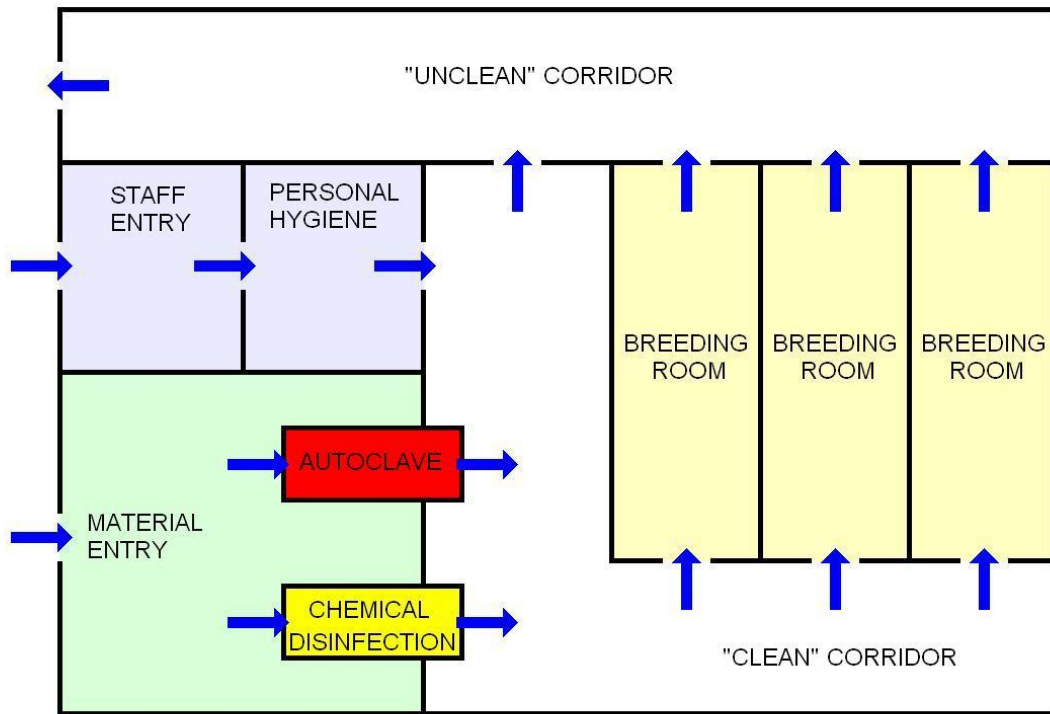
SYSTEMS OF BREEDING OF LABORATORY ANIMALS

Open – communication without the barrier

Barrier – the space with the animals is separated from external environment and movements of animals, people and material are controlled to eliminate possible introduction of alien factors from the external environment (infection) – sterilization of coming water, food, sawdust used for bedding, perfect personal hygiene of the personal.

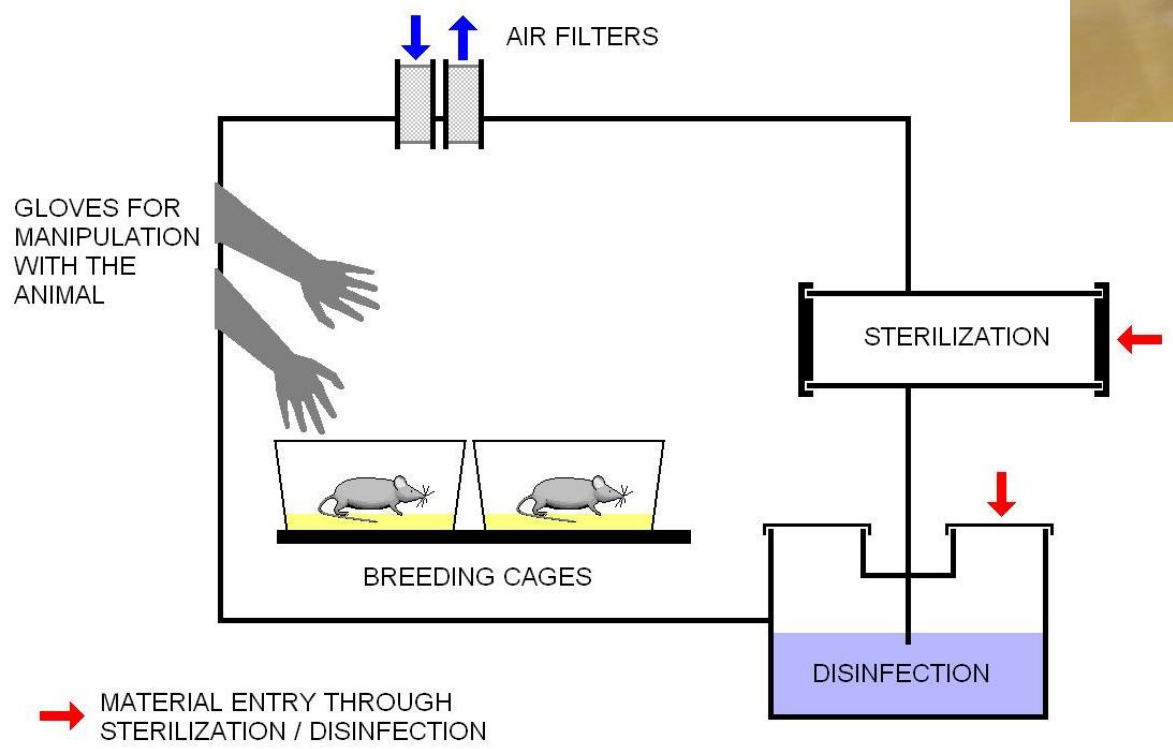
Isolator – the space for the animals is permanently separated by a barrier from external environment as well as from people who manipulate with the animals.

Scheme of barrier facility



Scheme of isolator

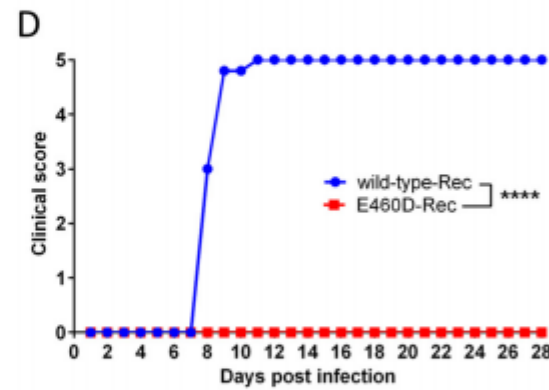
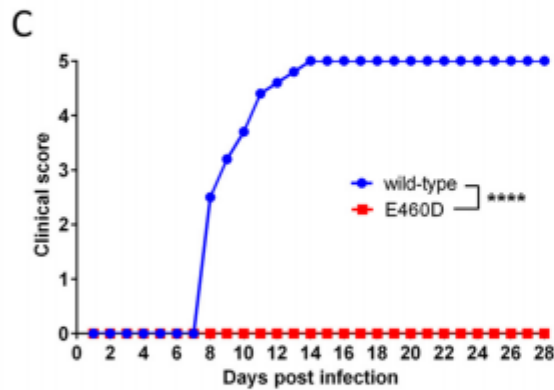
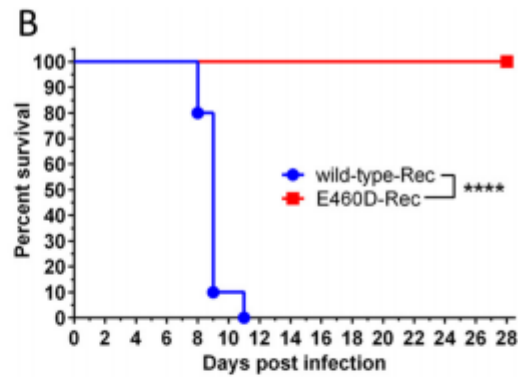
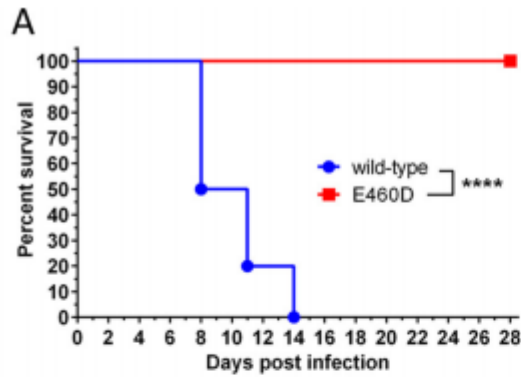
- Isolators:
- overpressure
 - underpressure



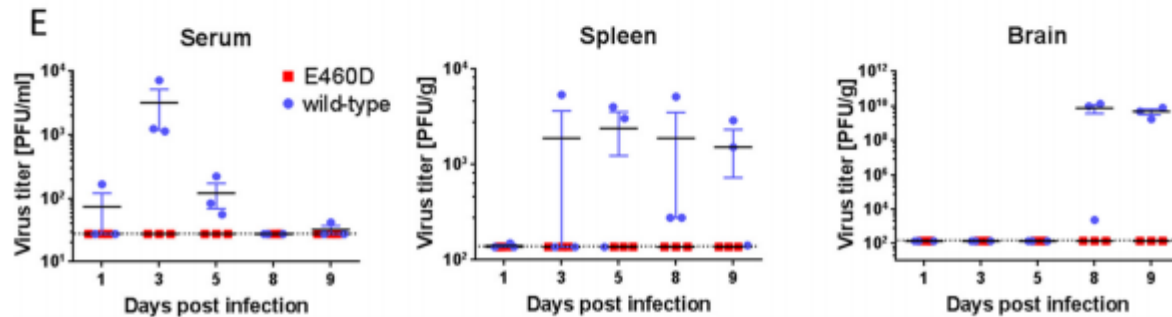
Virulence can be Quantitated

- Meantime to death
- Meantime to appearance of symptoms
- Measurement of fever, weight loss
 - Influenza infection
- Measurement of pathological lesions
 - Poliovirus infection
- Reduction in blood CD4+ lymphocytes
 - HIV-1 infection

Přežívání myši po infekci



Klinické skóre myši po infekci



Titř viru v orgánech

Histopathological changes

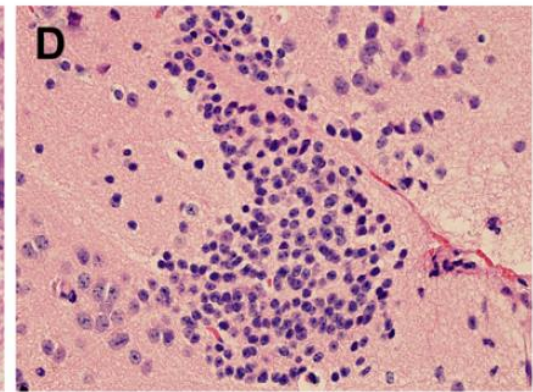
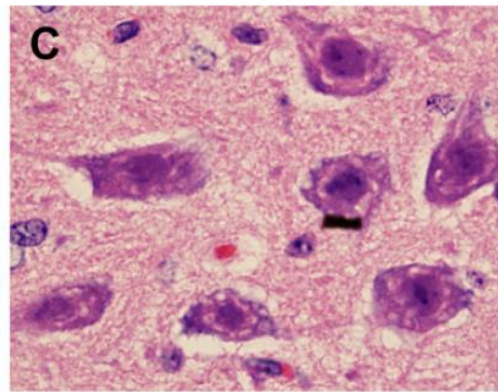
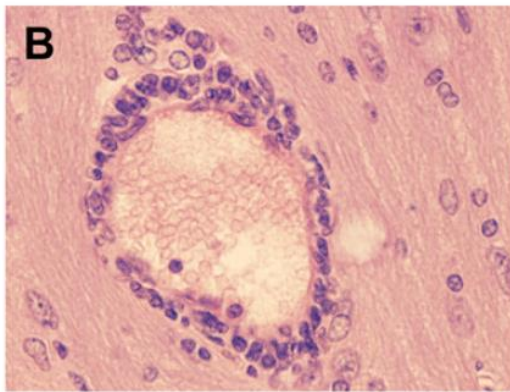
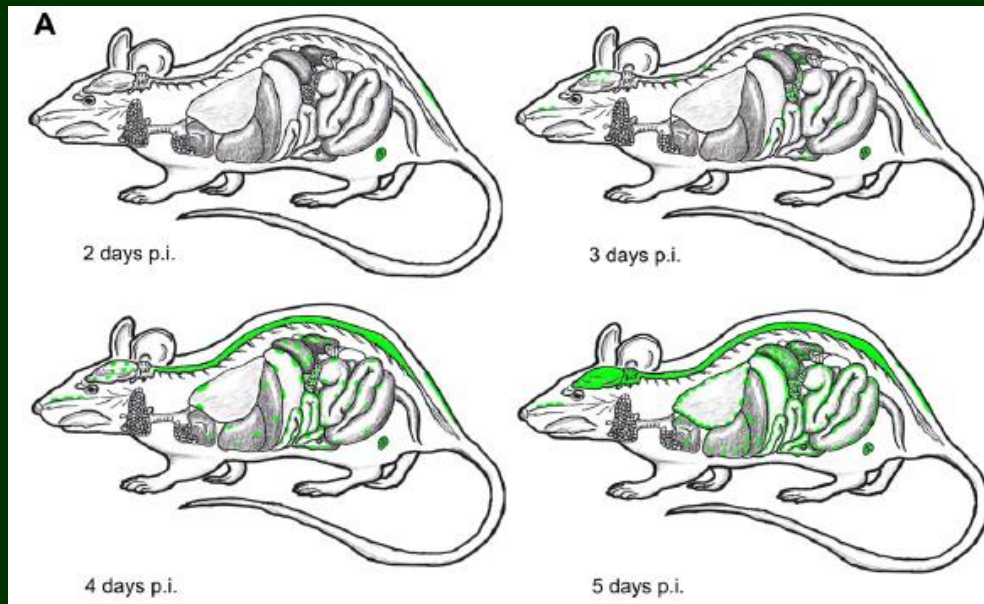


Figure 2 (A) Distribution of TBEV in mice after subcutaneous inoculation. (B–D) Some of the main histological hallmarks of TBE in brains of subcutaneously inoculated mice: perivascular lymphocyte infiltrates (B), acute neuronal necrosis (C), proliferation of microglial cells (D). (Figure by DR).

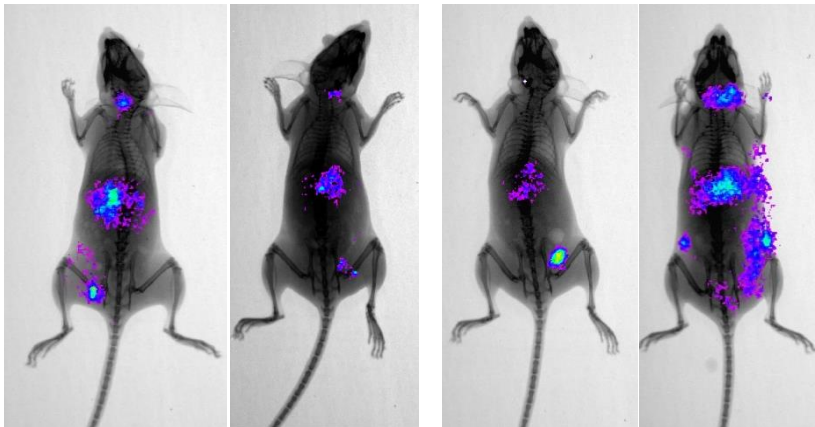
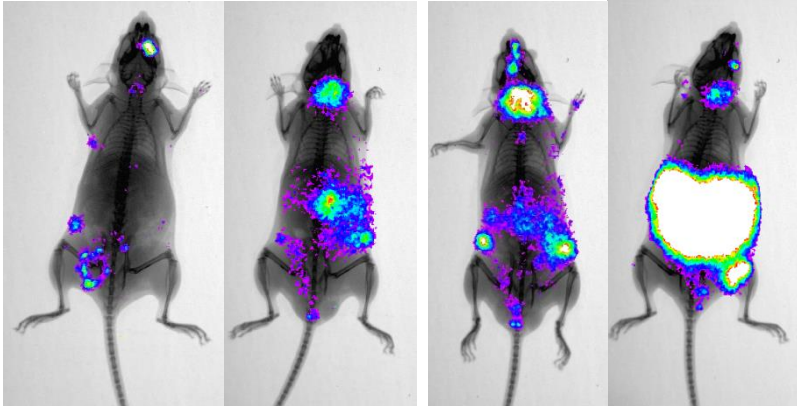
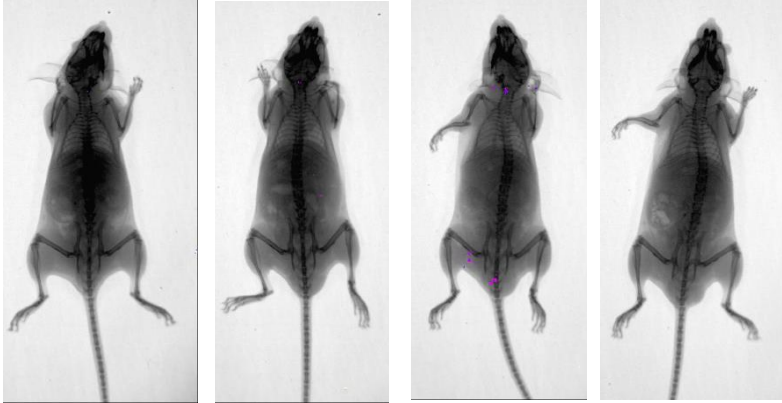
Days post infection

1

4

5

8



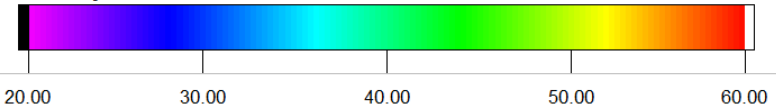
Monitoring the course of infection
using *in vivo* imaging system

Non-infected

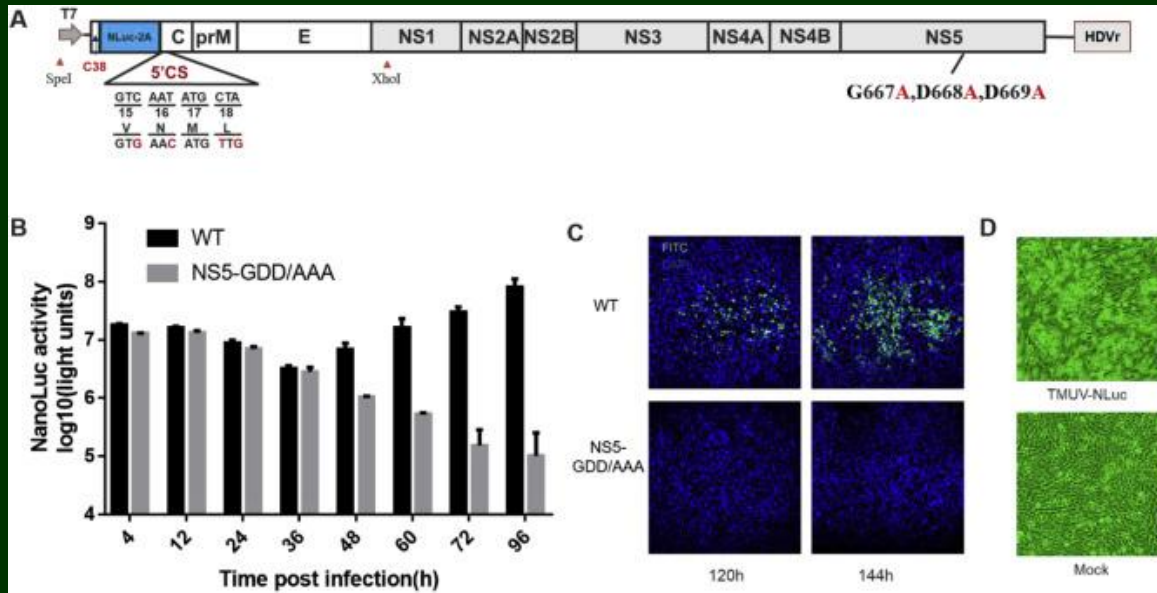
TBEV infected, non-treated

TBEV infected, treated with
7-deaza-2'-CMA (50 mg/kg)

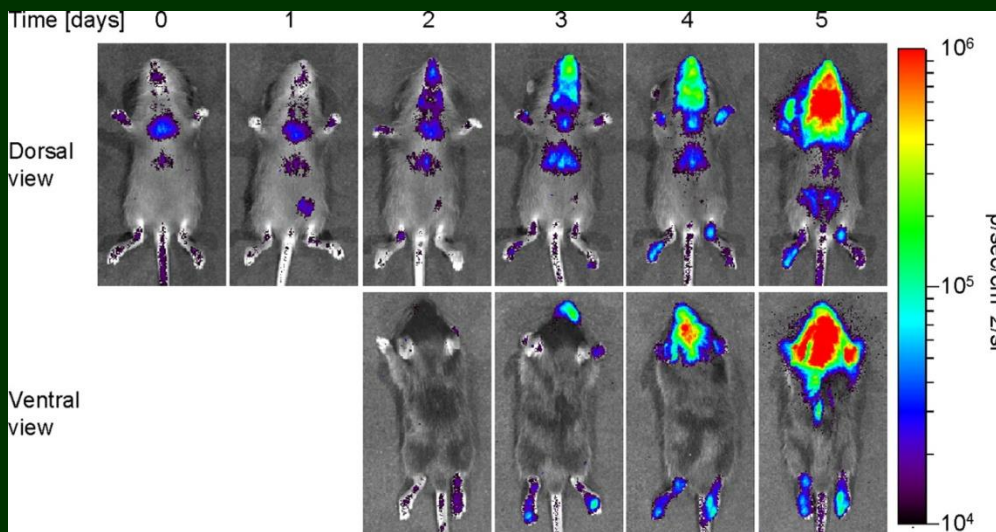
Overlay



Reportérové virové systémy



Reportérové proteiny:
 GFP
 Luciferáza
 Nano luciferáza (nLuc)
 mCherry
 Near Infra Red protein
 atd



Benefits and Problems of Using Animal Model

Benefits

- Show human-like clinical signs and pathology of disease
- Suitable for transmission experiments, vaccine research
- Helps to understand some certain mechanism of disease and therapeutic agent observed in animal model.

Problems

- Practical considerations
- Absence of small animal model
- Never mimics exactly what happens in people