VIRGIL Antiviral Course
3-6 October 2006

Micro-plaque assay of influenza virus sensitivity to neuraminidase inhibitors

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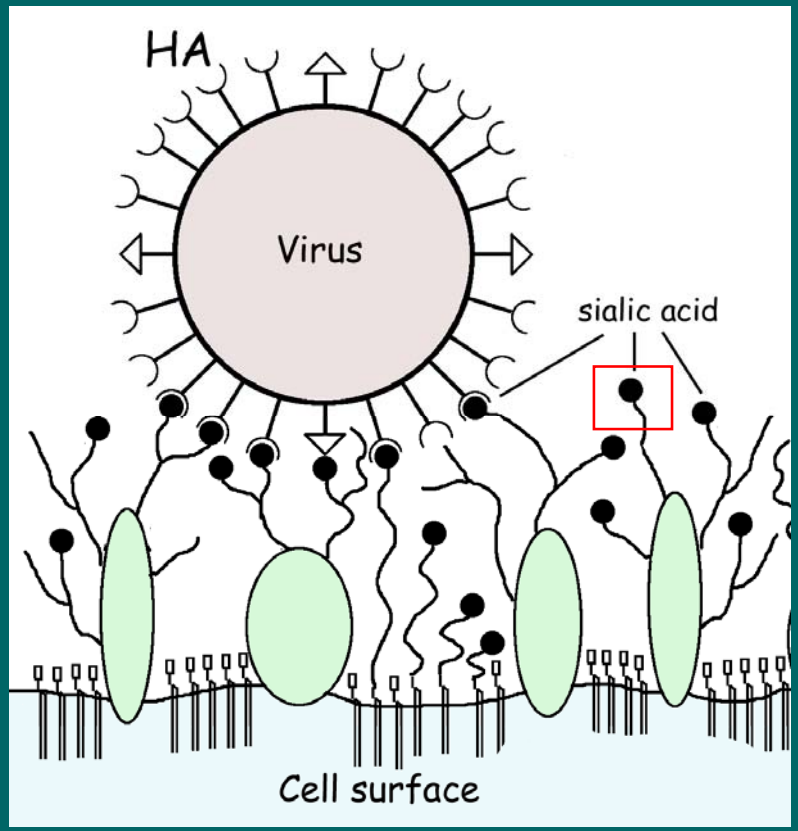
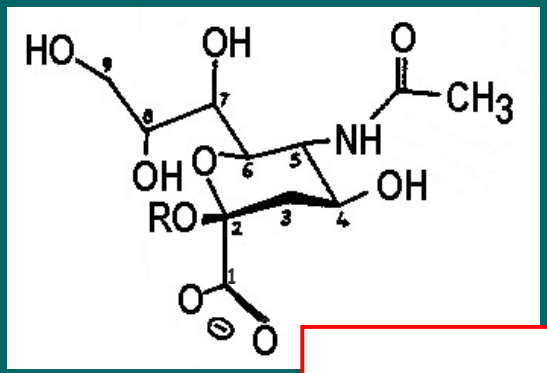
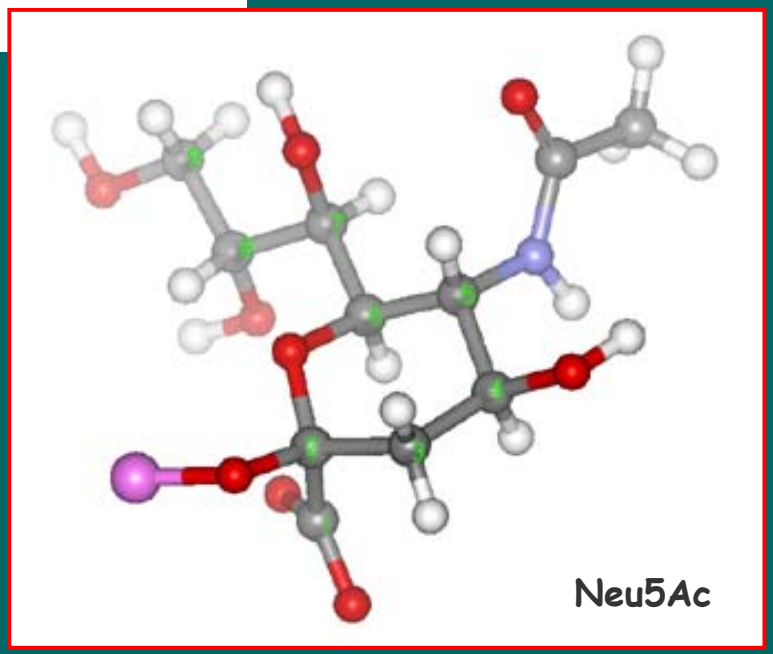
Influenza virus receptors: sialic acids

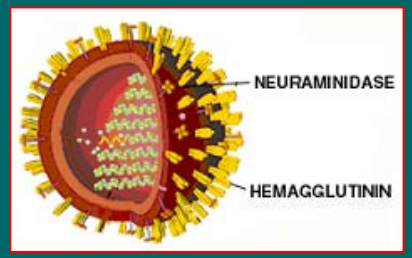


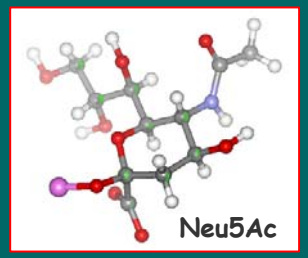
HA binds to sialic acid (Sia)





Functions of influenza virus neuraminidase (NA)

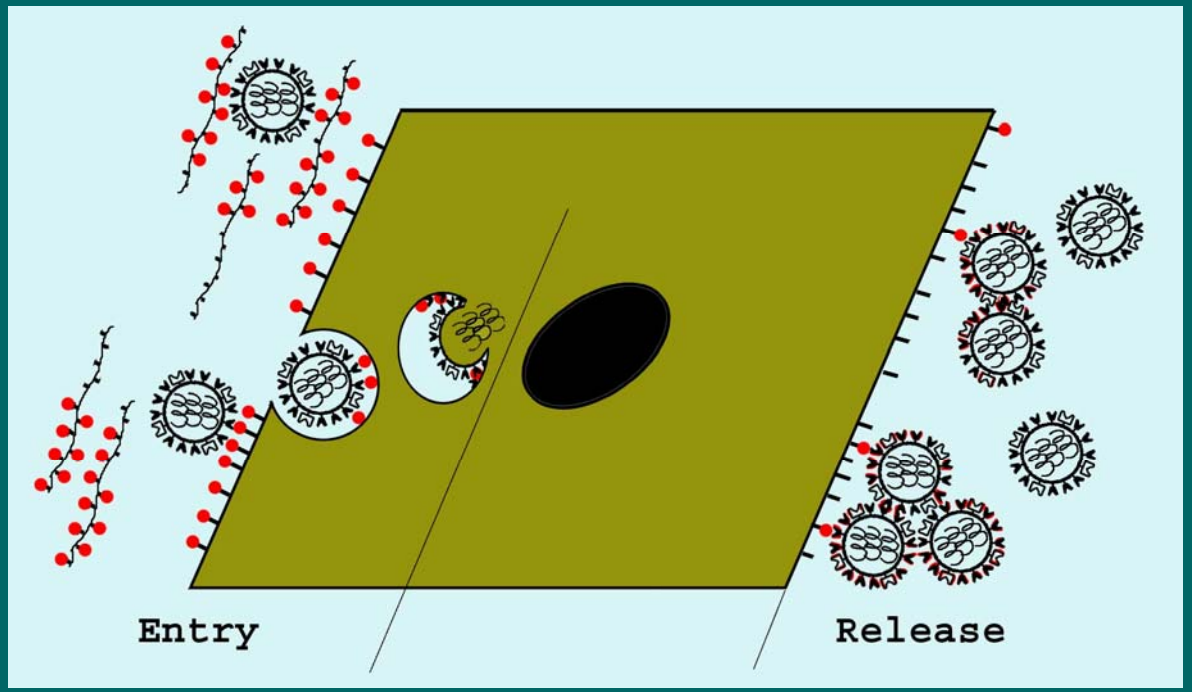




Early in infection:

Destroys mucin inhibitors and decoy receptors

Promotes virus entry into cell

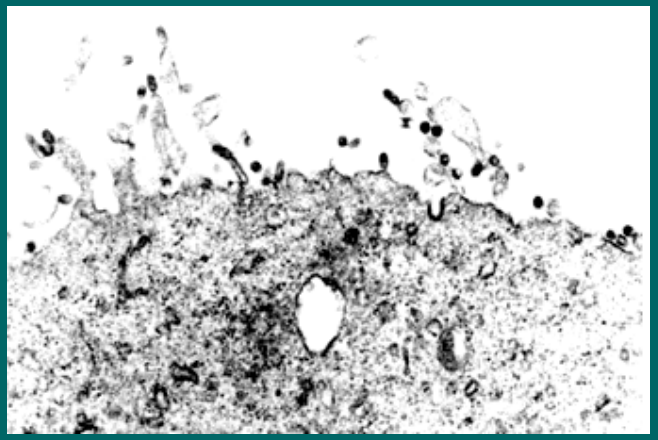


Late in infection:

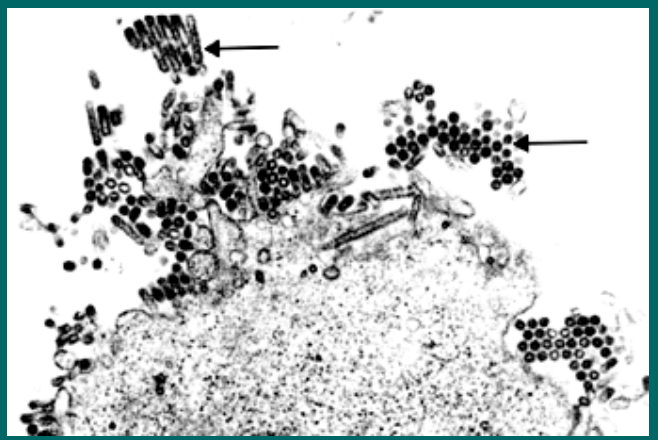
Removes receptors from virus progeny and cell surface

Promotes virus release and spread

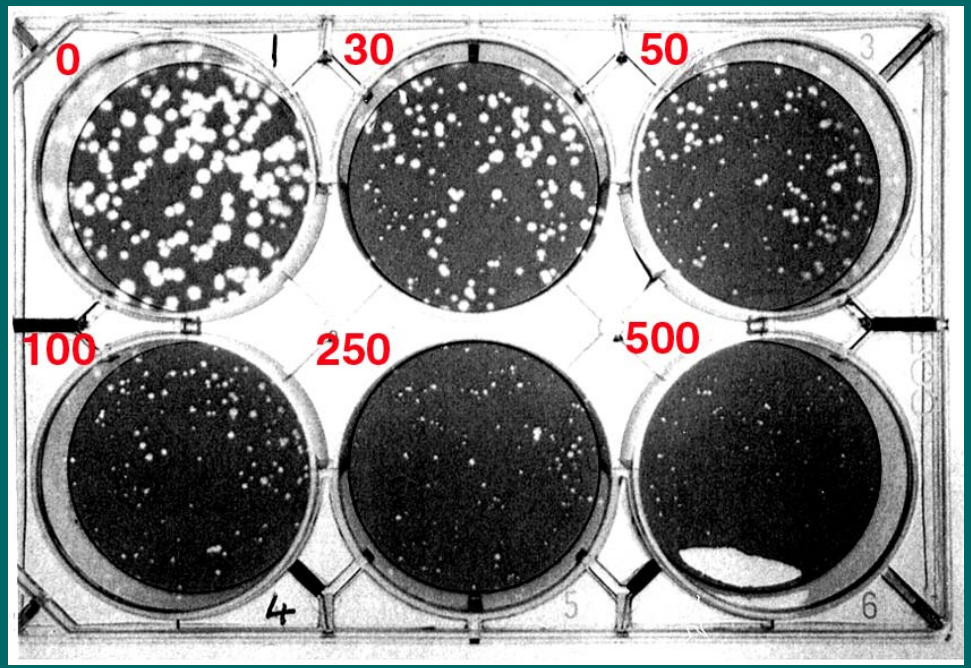
Inhibition of NA impaires virus release and spread



Normal virus release (top)
and release in the presence of
NA inhibitor (bottom)
From Gubareva et al., 2000



Plaque reduction assay



NA inhibitor decreases size of plaques produced by influenza virus

From Bantia et al., 1998

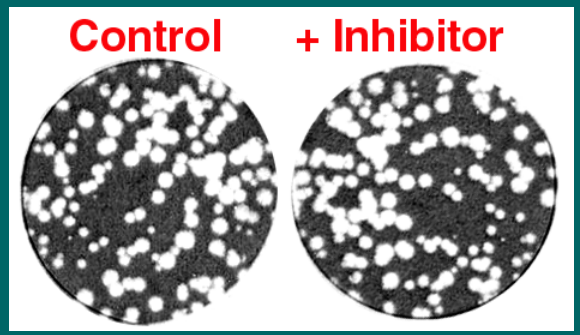
Pitfalls of plaque assays

1. Assays in MDCK cells do not correlate with virus sensitivity to NA inhibitors in vivo

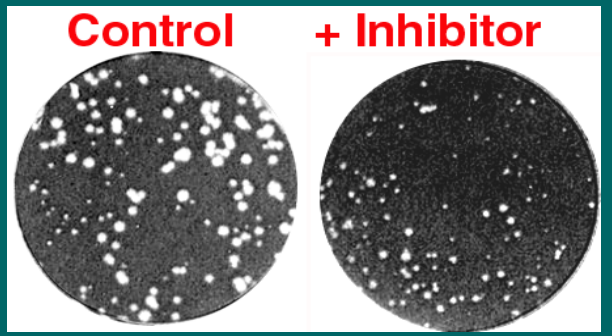
2. Plaque assays under agar overlays are cumbersome and cannot be performed in 96-well plates

New assay solves these problems

I. Viral sensitivity to NAI in MDCK cells do not correlate with sensitivity in vivo



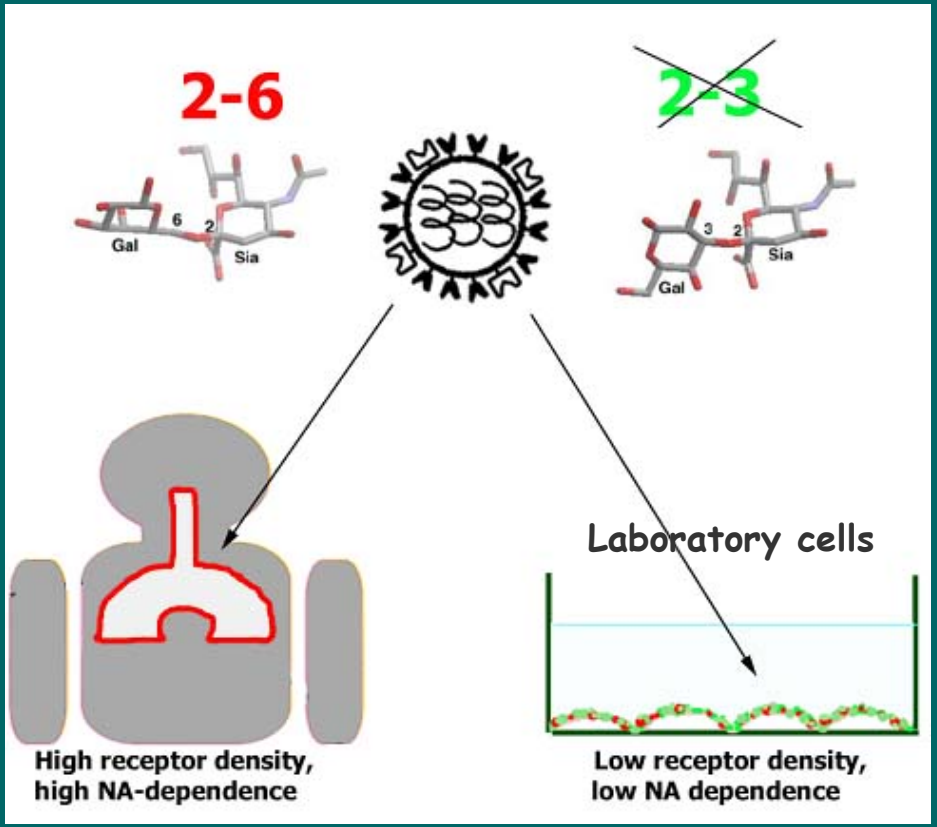
Viruses with drug-sensitive NA can be resistant



Viruses with drug-resistant mutations in HA and NA can display sensitivity

Laboratory cells do not mimic receptors in human airway epithelium

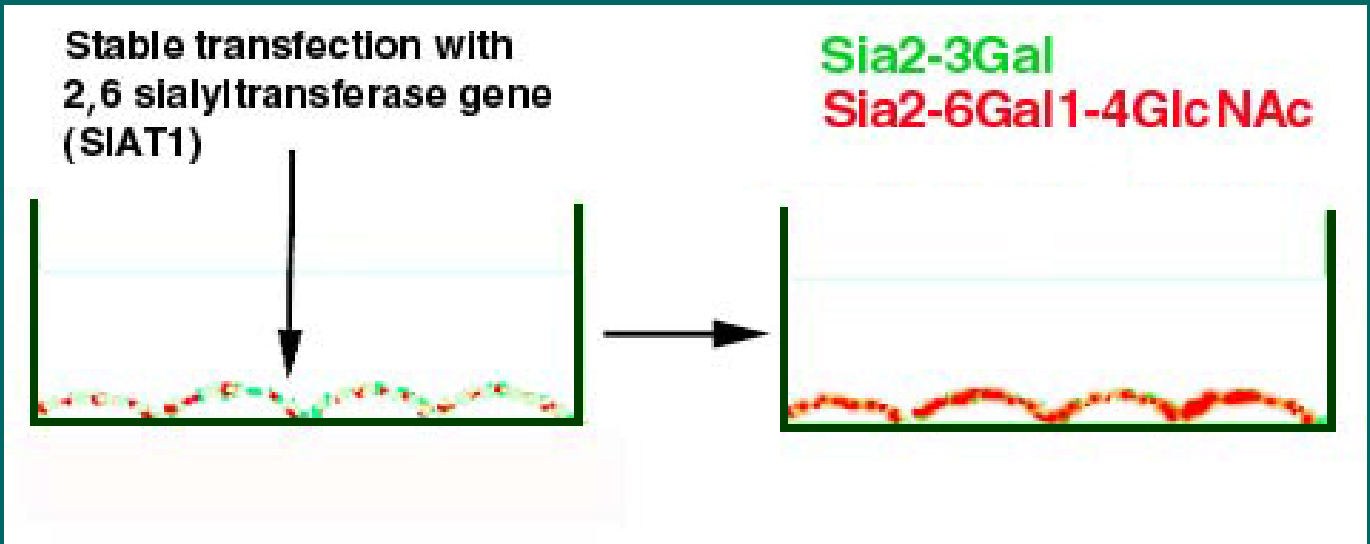
How to model influenza virus receptors of human airway tissues in a laboratory cell line?



Cell line with high concentration of 6-linked sialic acids is required

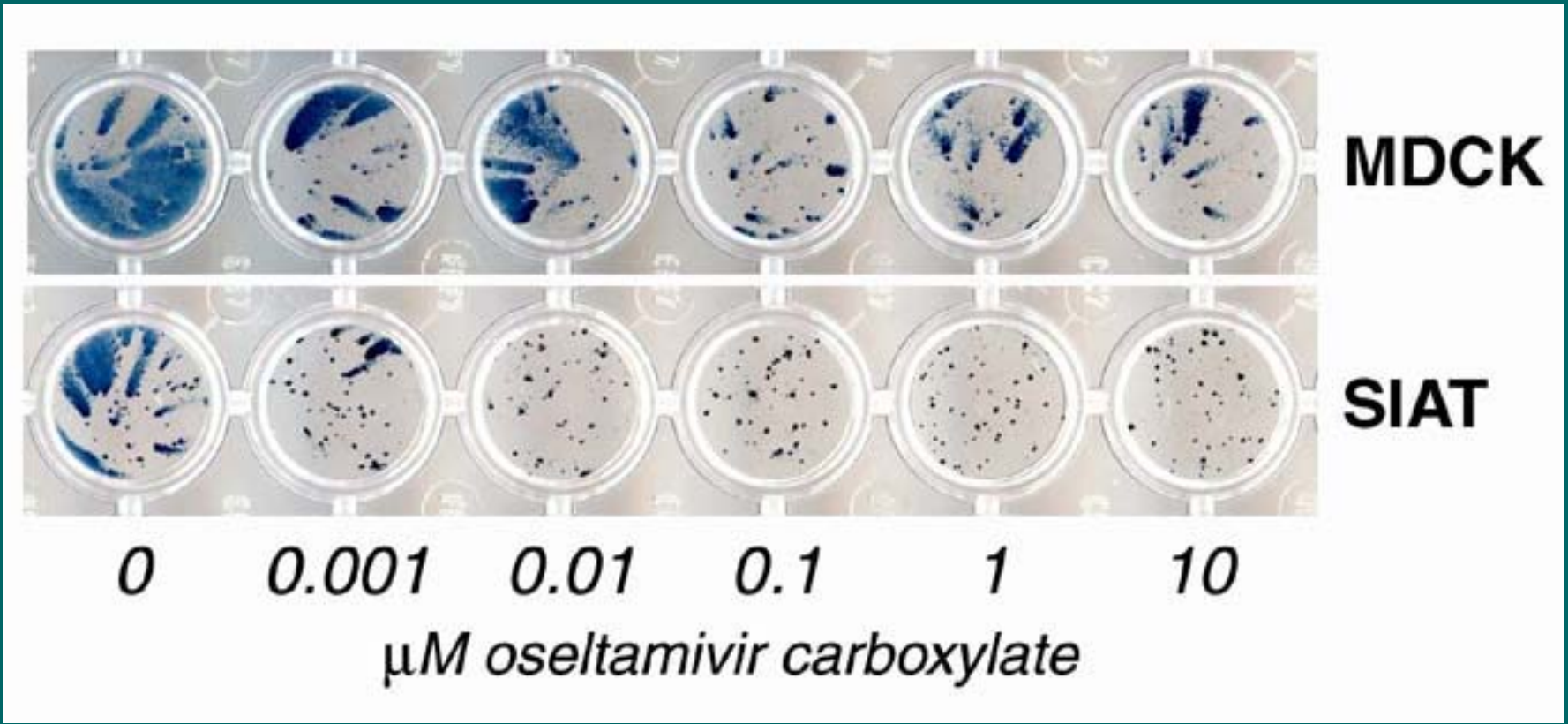
Preparation of a cell line for resistance assay: overexpression of SIAT1 in MDCK cells

SIAT1 (beta-galactoside a2,6-sialyltransferase) generates 6-linked sialic acid receptors recognized by human influenza viruses

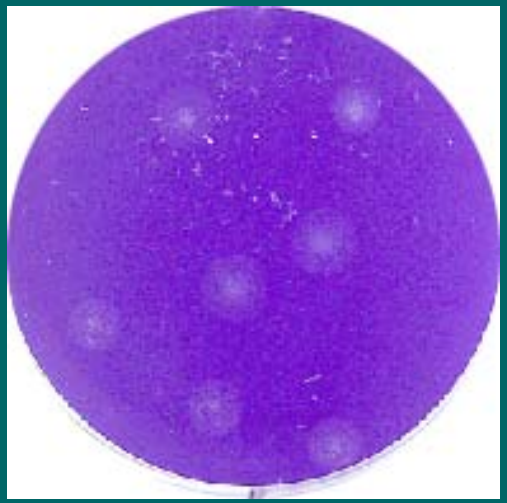


Influenza viruses are more sensitive to NA inhibitor in MDCK-SIAT1 cells than in MDCK cells:

A/Sydney/5/97 (H3N2)



Viral plaque assays



General cellular stain
detects destroyed cells

Immuno-staining
detects infected cells

Under liquid medium, the plaques are not localised and cannot be counted

Known overlays

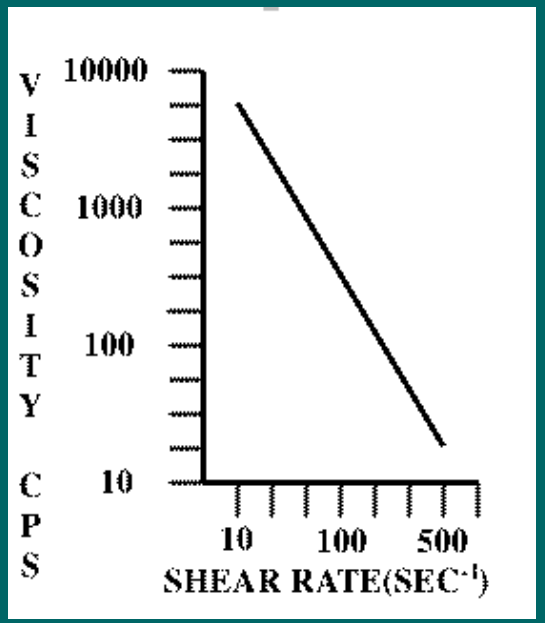
- Gels (agar, agarose)

  Time and labor consuming; heated agar can damage cells; cannot be used in 96-well plates

- "Semi-liquid" overlays (solutions of methylcellulose, tragacanth gum, etc)

  High viscousity --> particularly difficult to handle in microplate format

Our approach: Thixotropic gels



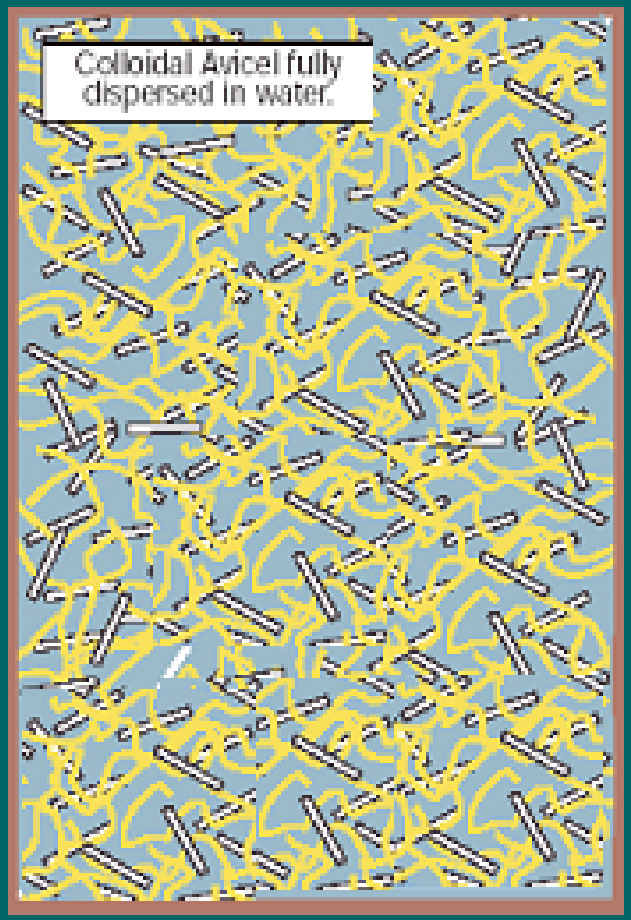
The viscosity decreases as shear rate increases

(Examples: yogurt, ketchup)

Avicel™ (FMC BioPolymer)

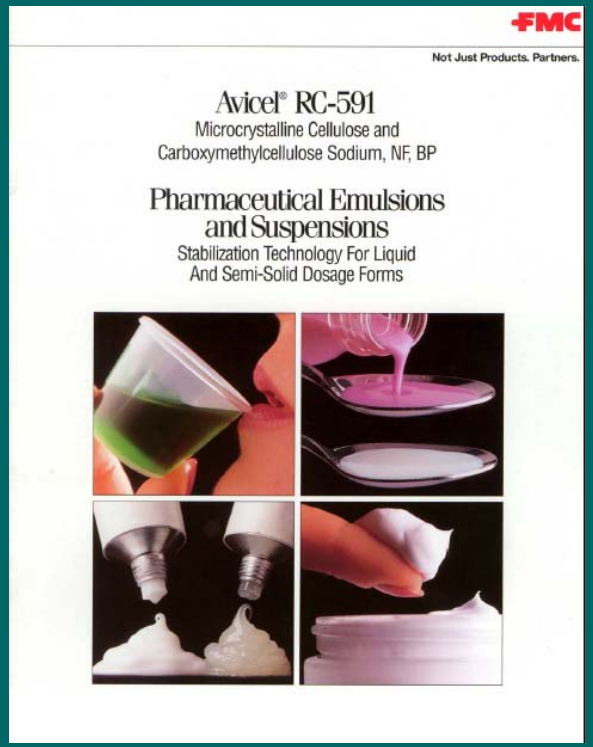
- Microcrystalline water insoluble cellulose

- Particles (~0,2 uM) form a network of weak hydrogen bonds that account for thixotropic properties of Avicel dispersions



- Low viscosity (~ 100-200 mPa.s at 1,5% Compare to 3000 mPa.s for 1,5% solution of methylcellulose)

Avicel™ (FMC BioPolymer)

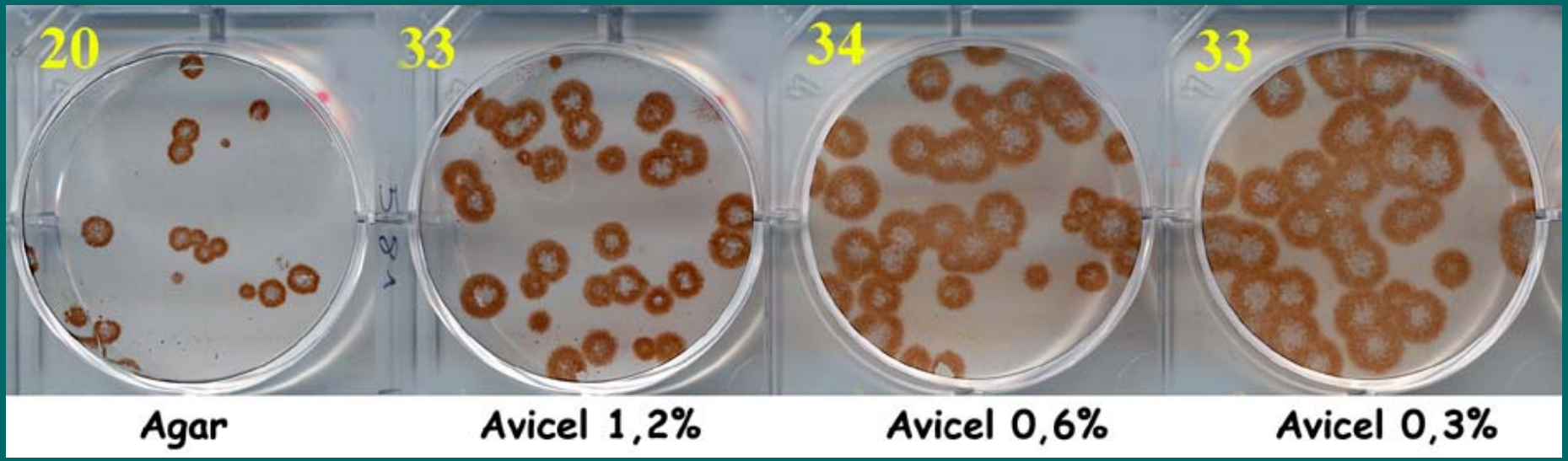


Standardised commercial product:

Widely used as vehicle for the preparation of pharmaceutical suspensions and emulsions

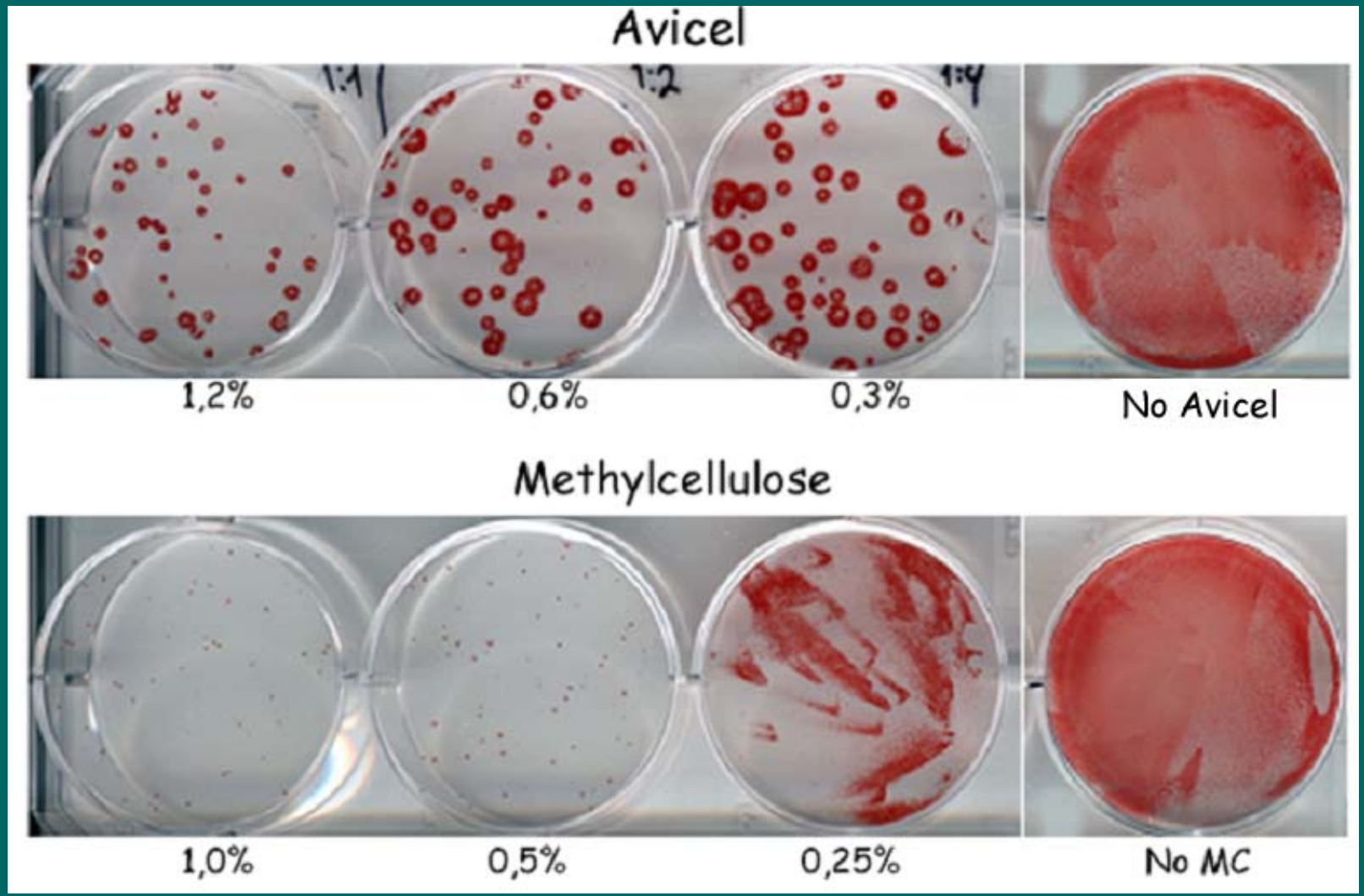
Plaque assays under Avicel vs agar

influenza virus A/Memphis/14/96 (H1N1)

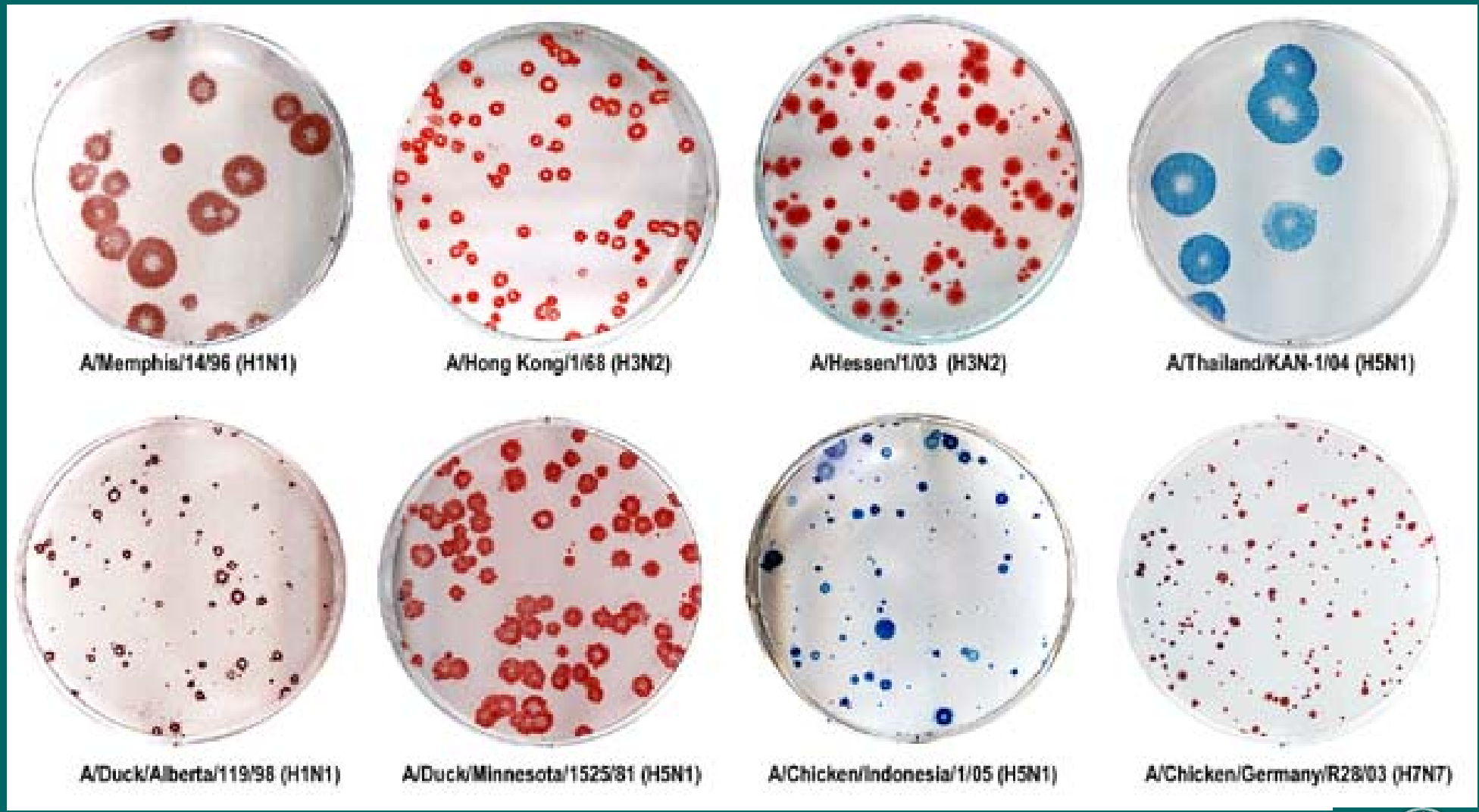


- Plaques are bigger; size can be controlled
- As low as 0.3% (!) of Avicel is still sufficient to localize plaques
- More plaques under Avicel than under agar

Avicel vs. methylcellulose, MDCK-SIAT1 cells



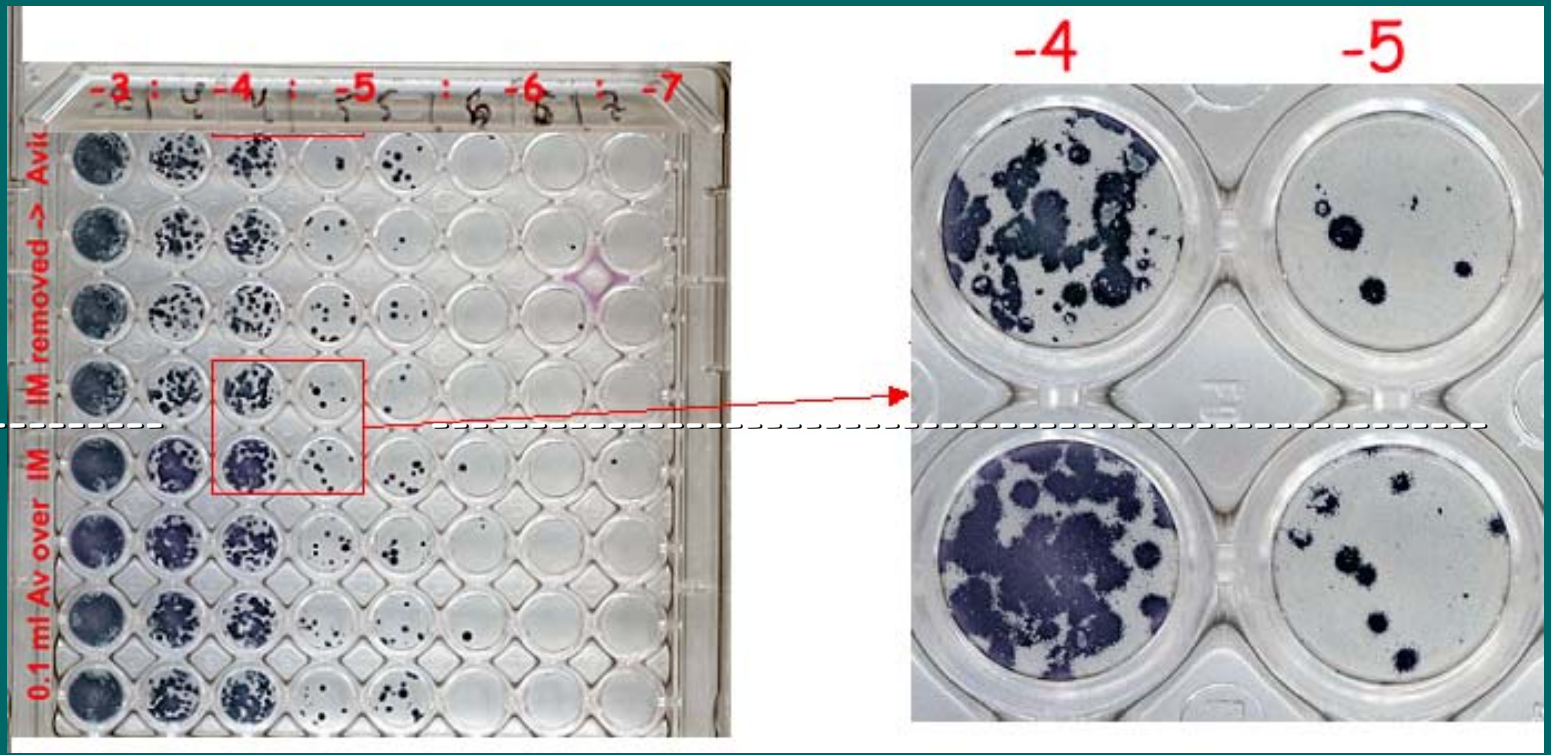
Plaque formation by different human and avian viruses



Assay variants in 96-well plate

Viral inoculum was removed before adding Avicel overlay

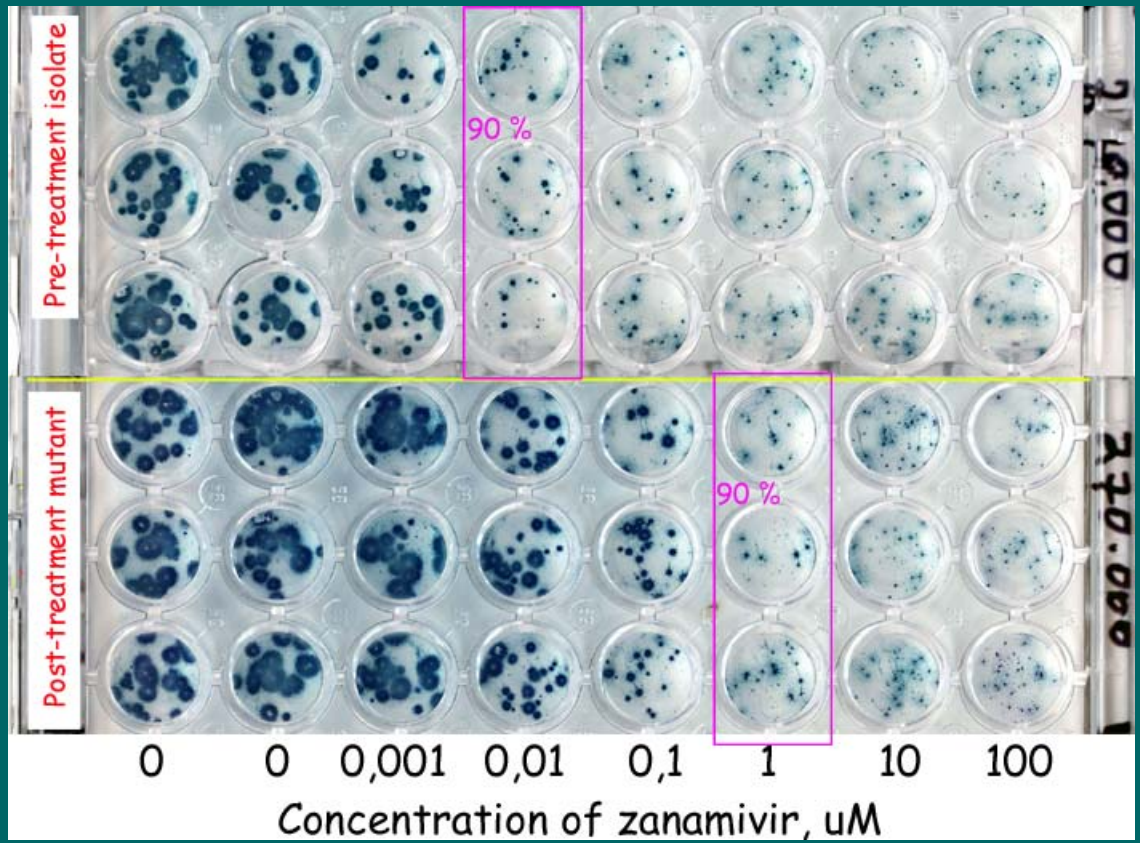
Avicel overlay was added w/o removing inoculum



No need to remove viral inoculum: easier to perform, lower chances of cross-contamination

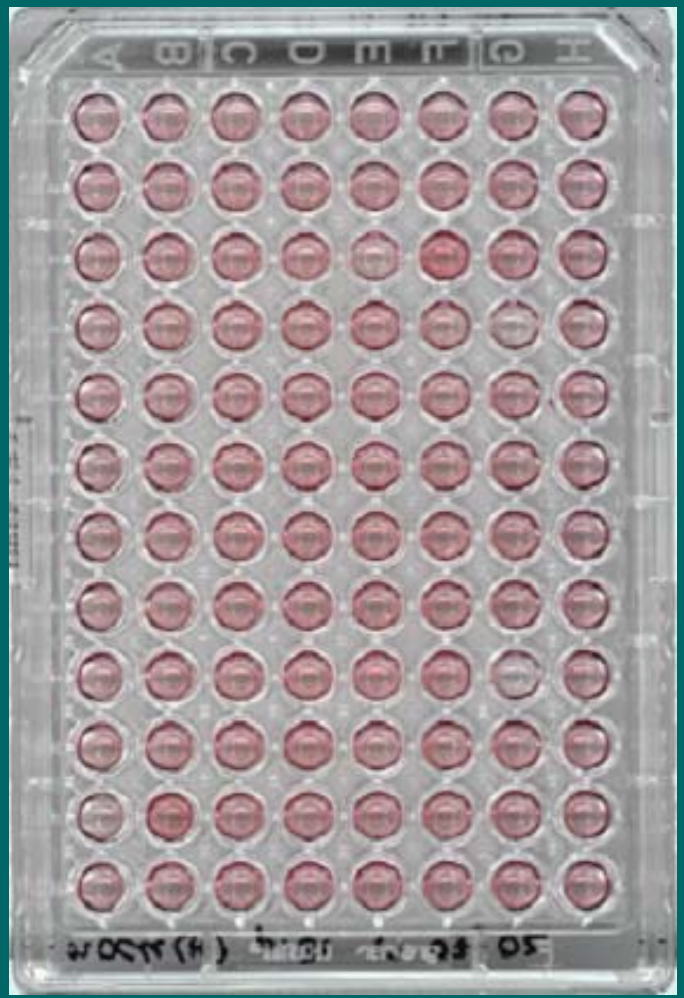
Detecting drug-resistant viruses

MDCK-SIAT1, 96-well format



The viruses were kindly provided by
Robert Webster

Step 1.
Seed MDCK-SIAT1 cells in 96-well plate



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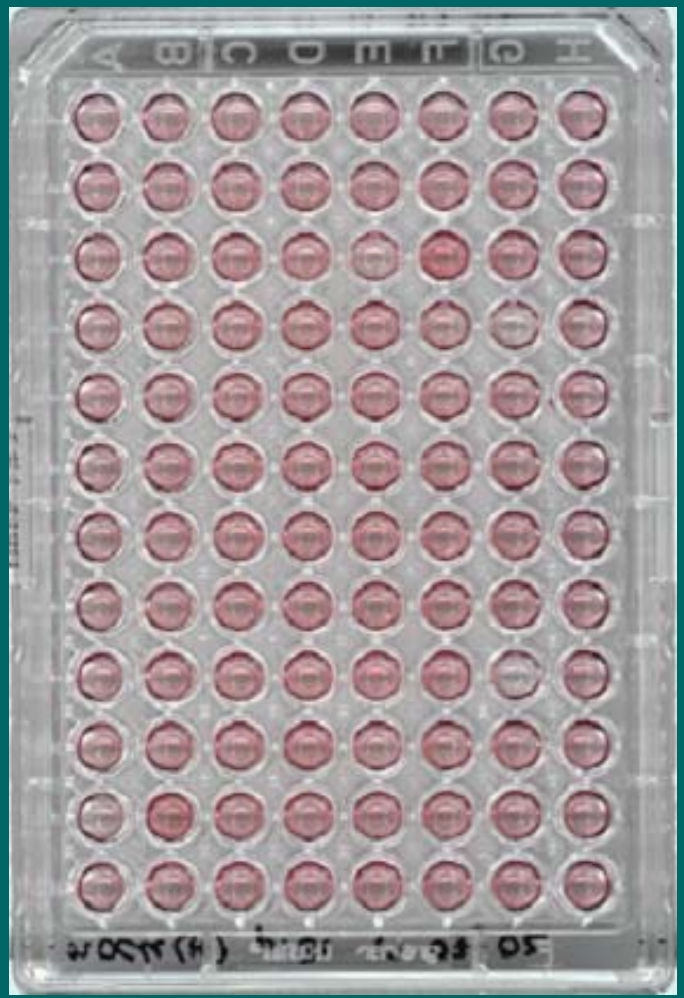
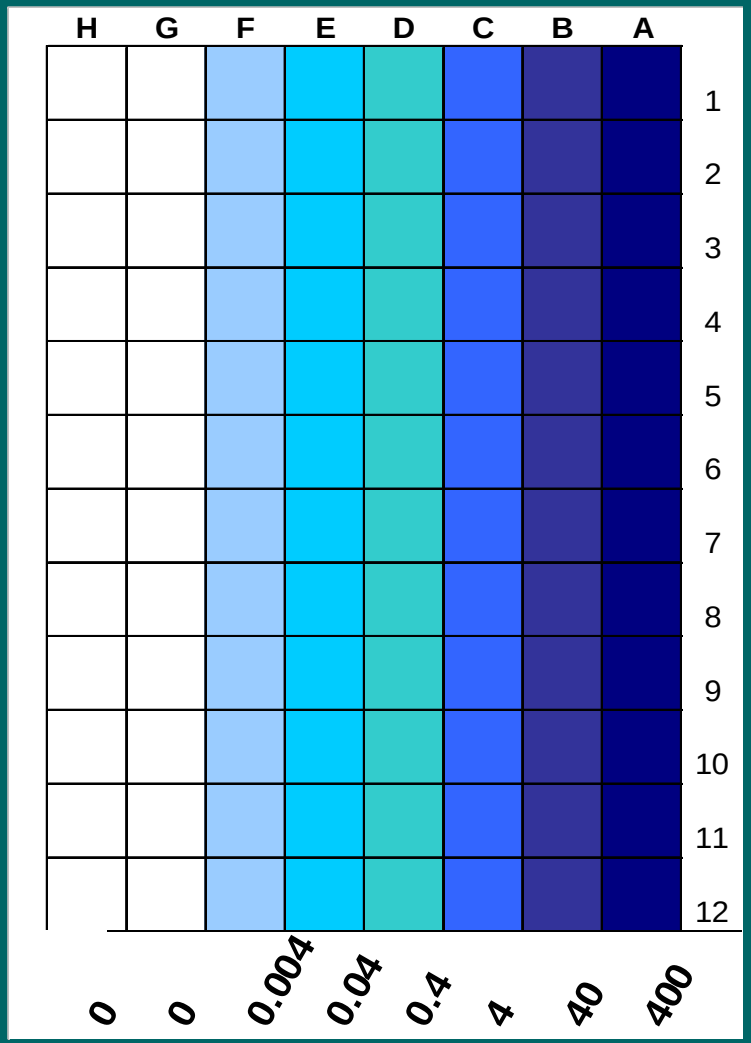
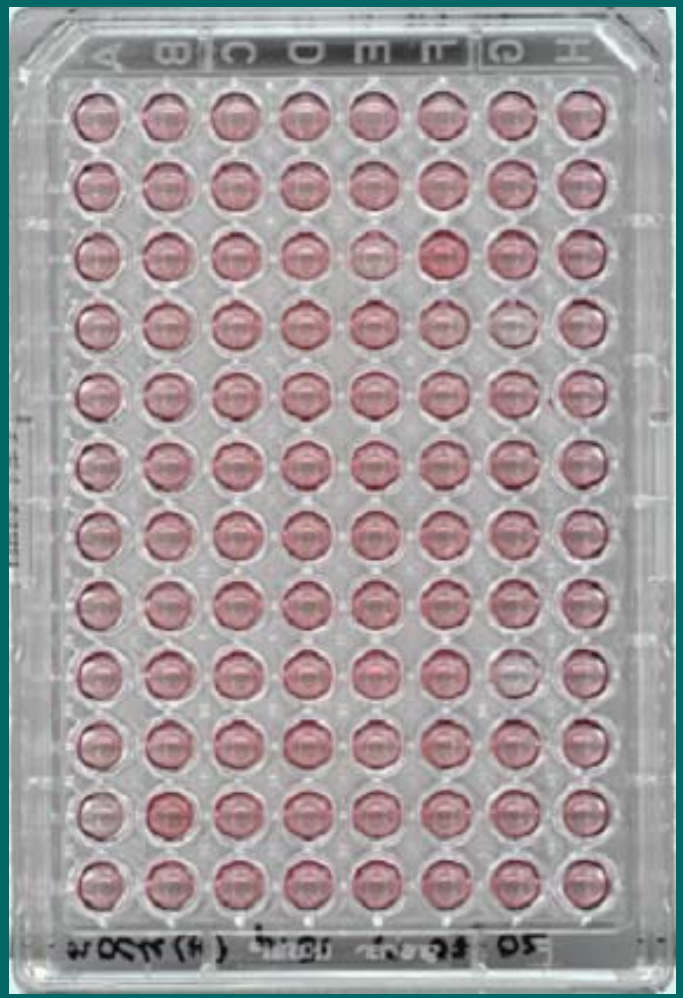
Step 2.
Wash the cells 3-4 times with serum-free medium



Step 3.
Add 10-fold serial dilutions of the drug, 50 ul/well





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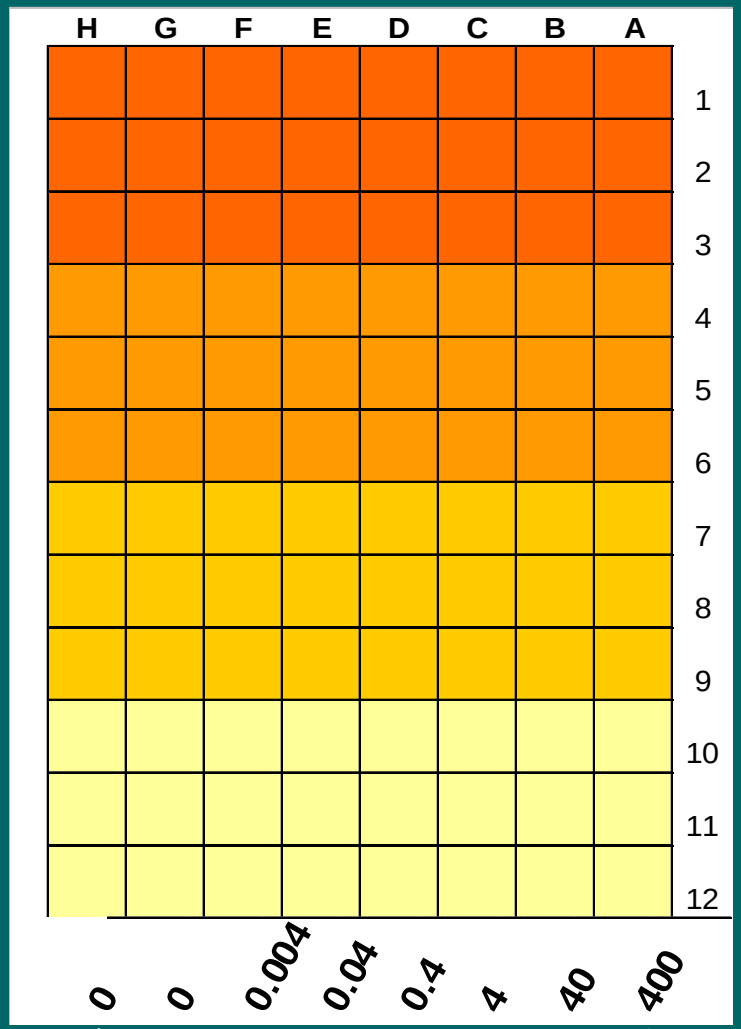
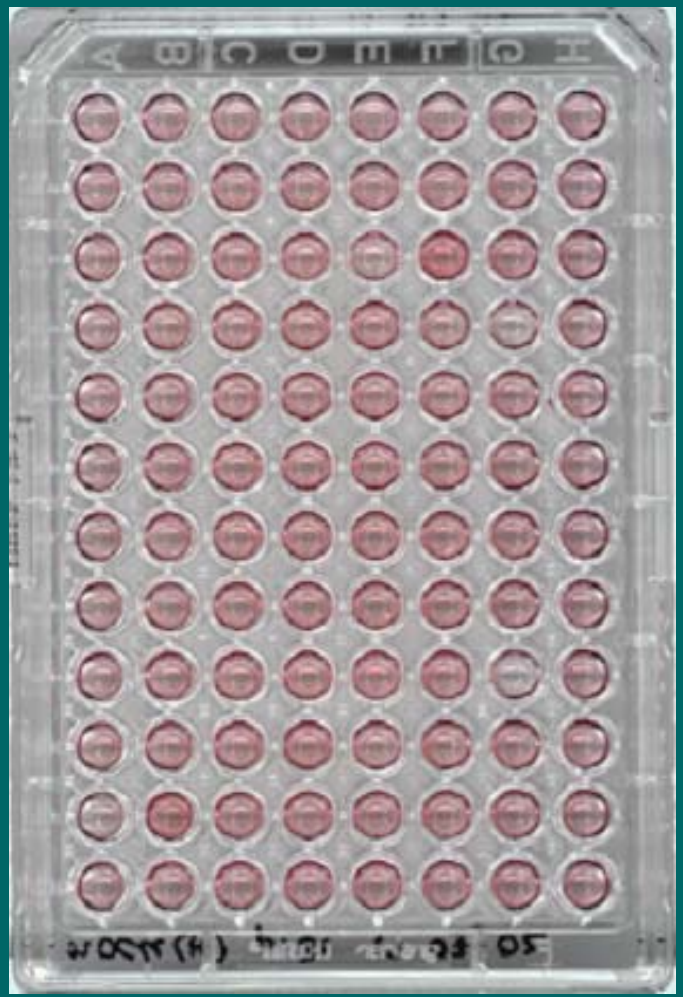
Step 4.
Add 3-fold serial dilutions of the virus, 50 ul/well





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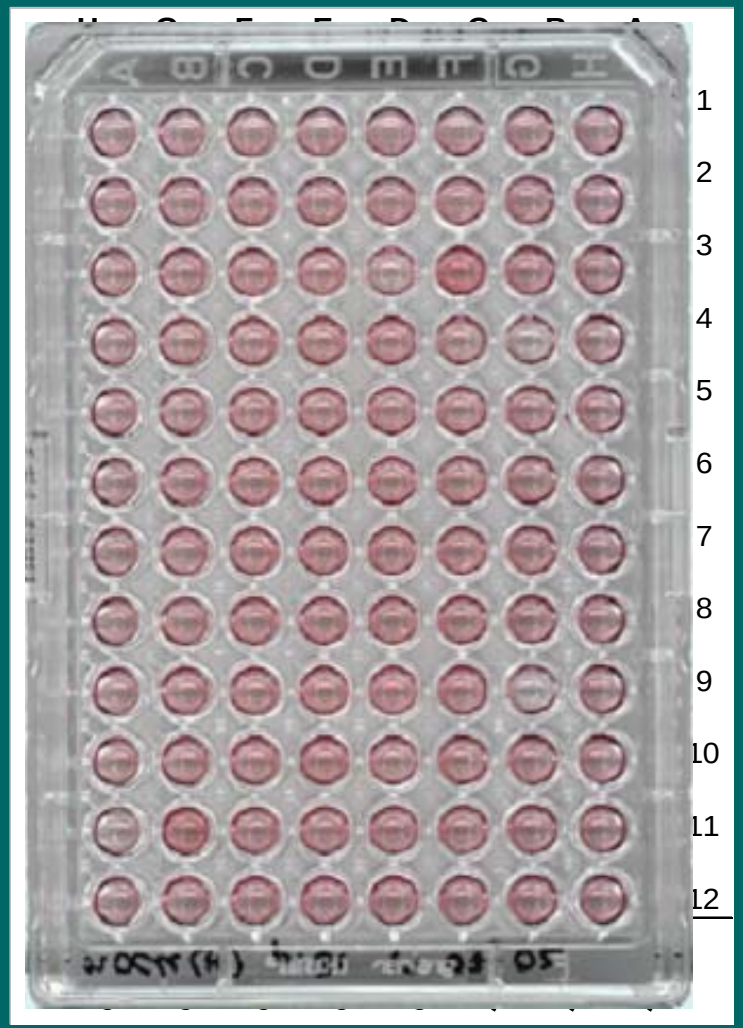
Step 5.
Mix, incubate 1-2 h for initiation of infection



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Step 6.
Add Avicel overlay medium, 100 ul/well

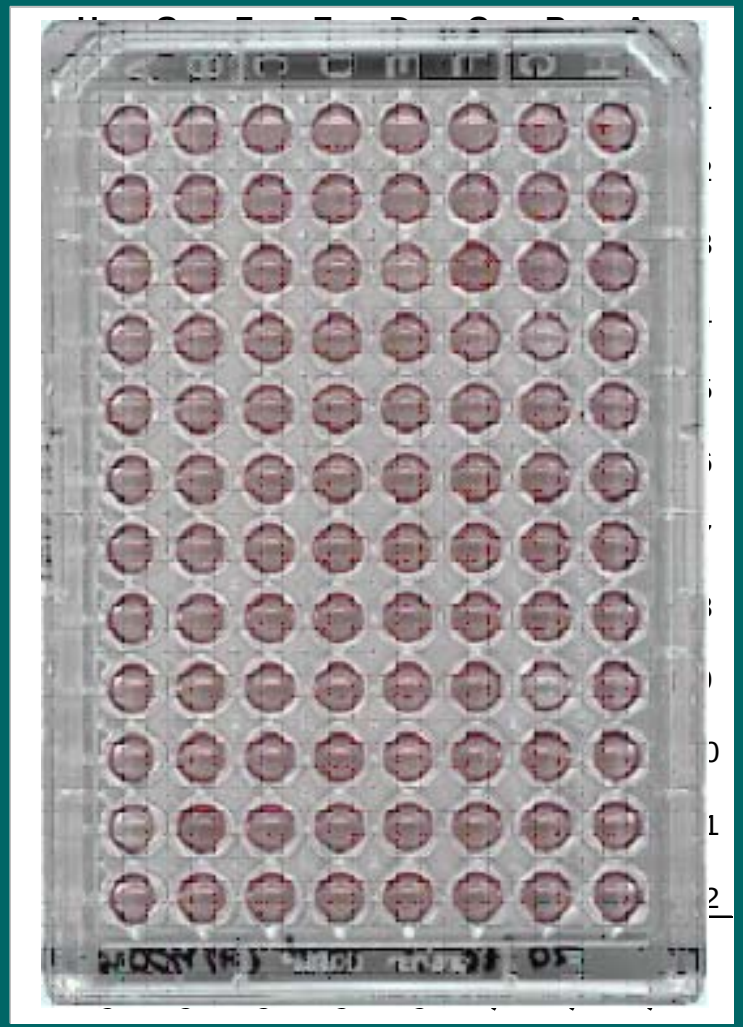
VIRUS

Dilution 1

Dilution 3

Dilution 9

Dilution 27



Drug

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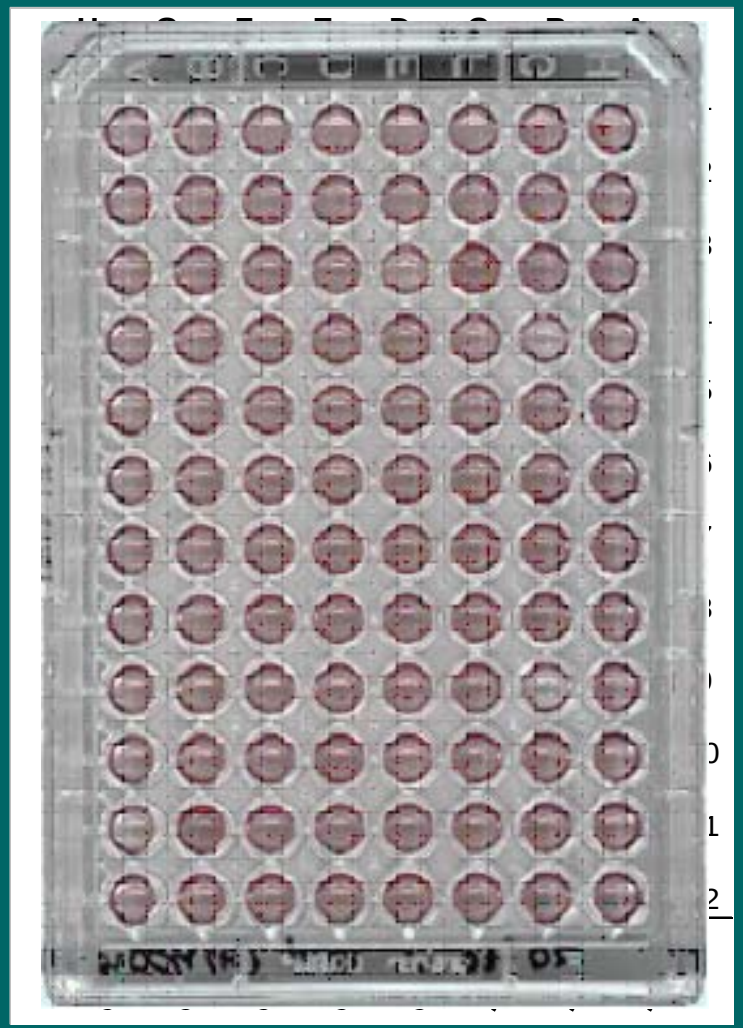
Step 7.
Incubate for 20-48 h to allow formation of plaques



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Step 8.
Fix and immunostain to visualise plaques:

- remove overlay medium, incubate with 4% paraformaldehyde, 30 min at 4 oC
- permeabilize the cells with 0.5% Triton-X-100, 10 min
- incubate with primary antibodies (anti-NP-A or -NP-B), 1 h
- incubate with HRP-labeled secondary antibodies, 1 h
- incubate with precipitate-forming peroxidase substrate, 30 min
- let dry and analyse

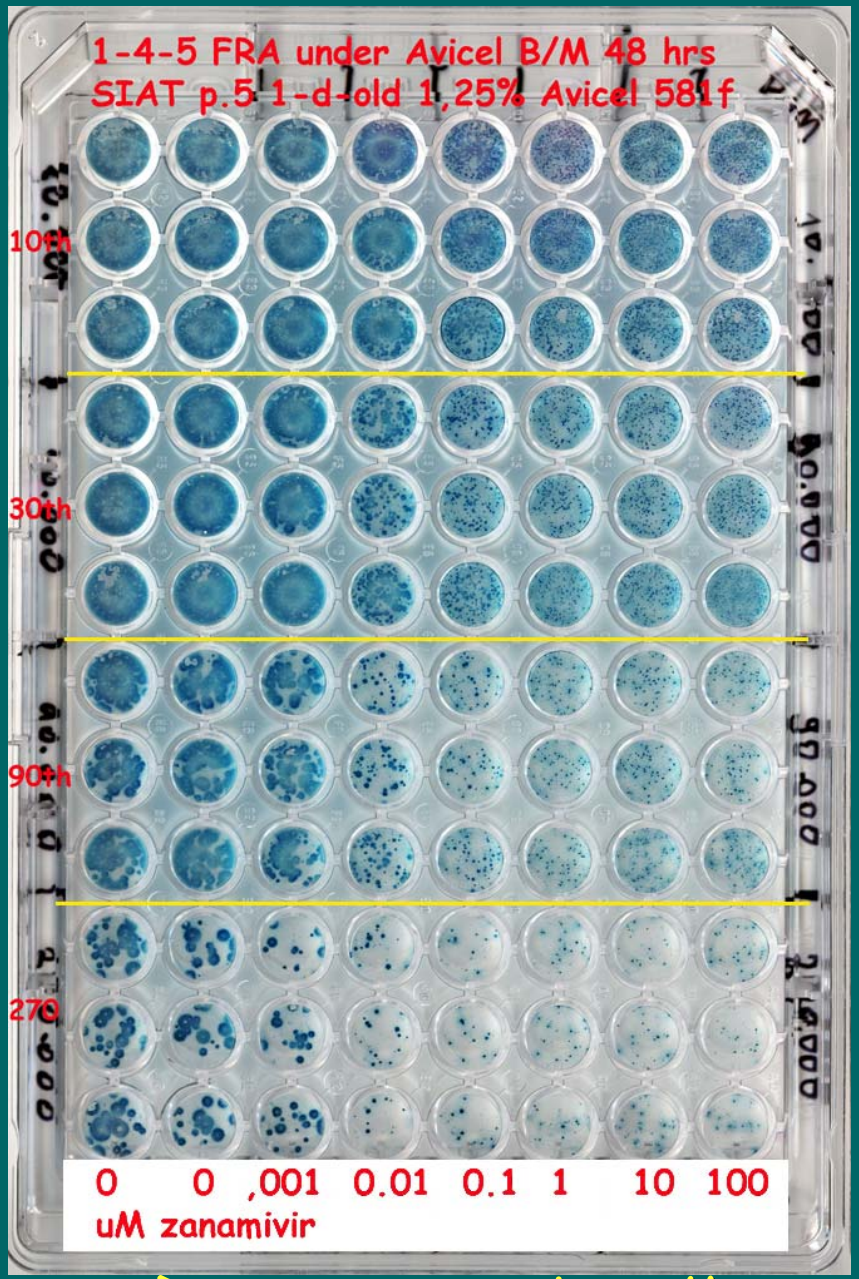
VIRUS

Dilution 1

Dilution 3

Dilution 9

Dilution 27



Drug concentration, uM

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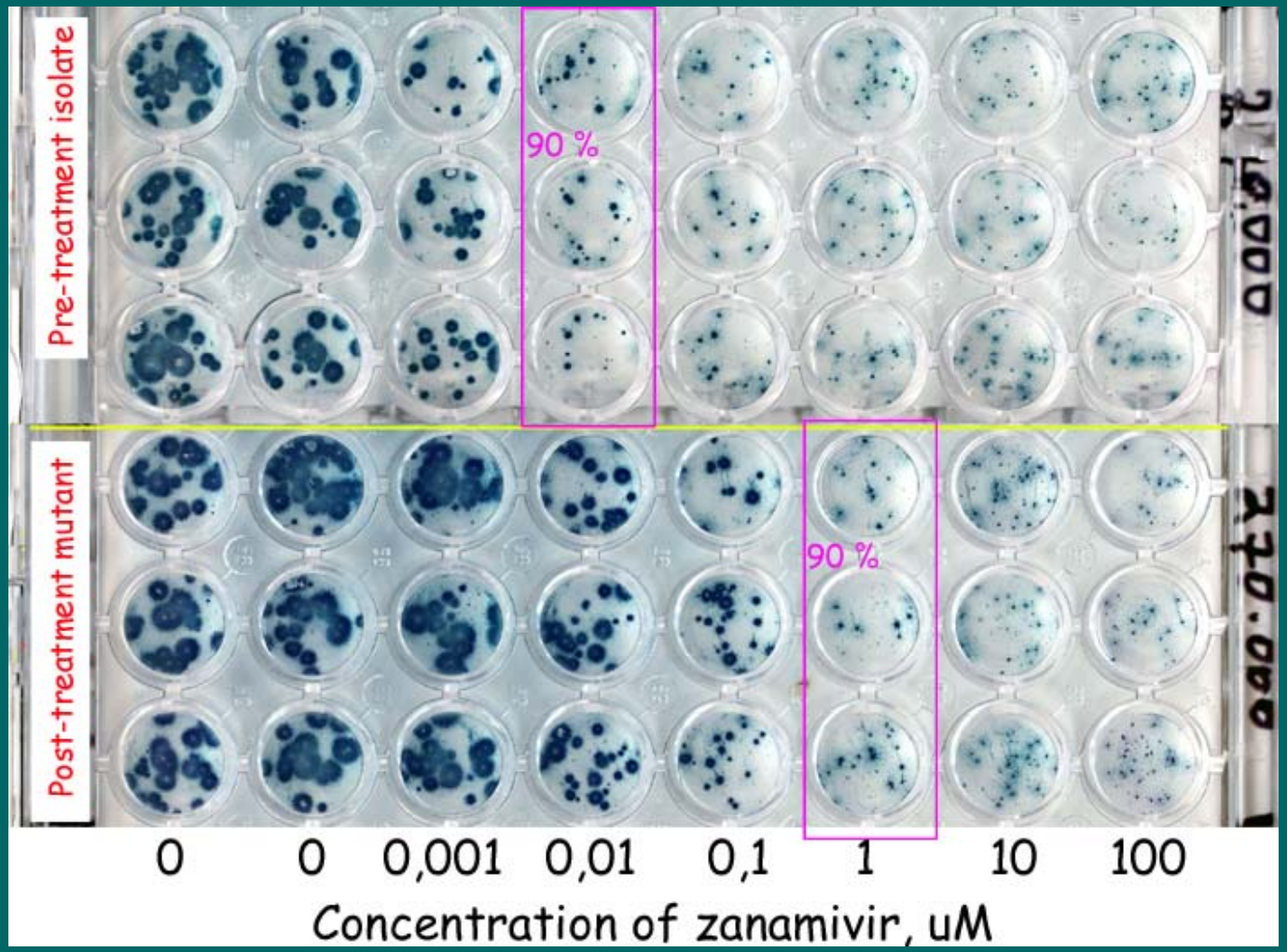
Post-treatment virus is NAI-resistent

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