

Plaque reduction assay in MDCK-SIAT1 cells under Avicel overlays.

How to cite MDCK-SIAT1 cells:

M.Matrosovich, T. Matrosovich, J.Carr, N.A.Roberts, and H.-D.Klenk. (2003). Overexpression of the alpha-2,6-sialyltransferase in MDCK cells increases influenza virus sensitivity to neuraminidase inhibitors. *J. Virol.* 77:8418-8425.

How to cite the plaque assay under Avicel overlays:

M.Matrosovich, T.Matrosovich, W.Garten, H.-D.Klenk. (2006). New low-viscosity overlay medium for viral plaque assays. *Virology Journal* 3:63.

Part I. Infection.

I-1. Media for serial passaging, plating and infection of MDCK-SIAT1 cells

Reagents

1. MDCK-SIAT1 cells (ECACC number 05071502; see more details in ref.1 above).
2. Dulbecco's Modified Eagle Medium (DMEM, 1X), Liquid (High Glucose) (Invitrogen GIBCO; Cat. # 21969-035)
 - With Sodium Pyruvate, 4500 mg/l
 - Without L-Glutamine
 - With pyridoxine
3. Double strength DMEM or MEM medium (2xDMEM or 2xMEM)
4. Fetal Bovine Serum mycoplasma free. (Heat-treat: 56°, 30 min, store at -20oC in aliquots)
5. L-Glutamine-200 mM, (100x) liquid (Invitrogen GIBCO; Cat. # 25030-081)
6. Antibiotic G-418 Sulfate (Promega, Cat. # V7982)
7. Penicillin-Streptomycin, liquid (100x) (Invitrogen GIBCO; Cat. # 15070-063)
8. Trypsin-EDTA (1x) (Gibco-Invitrogen, Cat. # 253000-054)
9. Bovine serum albumin, 30% solution (Sigma Cat. # A-0336)
10. Trypsin TPCK-treated (Sigma Cat. # T-1426)
11. Microcrystalline cellulose Avicel RC-581, FMC BioPolymer (Technical and other information on Avicels is available at <http://www.fmcbiopolymer.com>).

[*Comment:* Initially, we used Avicel that was provided to us by the company as free samples for testing. To get samples or to purchase larger amounts of Avicel, please contact one of the representatives of the company which are listed at <http://www.fmcbiopolymer.com/FMCBiopolymer/OurLocations/tabid/535/Default.aspx>]

12. Stock solutions of antiviral drug, 100x concentration in water or PBS. In the case of neuraminidase inhibitors, we use 10 mM water solutions of zanamivir and oseltamivir carboxylate.

Preparation of Antibiotic G-418 Sulfate

1. Dissolve 1g of Antibiotic G-418 Sulfate in 20 ml double-distilled sterile water (to 50 mg/ml)
2. Sterilize the solution by filtration through 0.2 micron filter, store at -20°C or -70°C in 1 ml aliquots
3. Add G-418 stock to the final concentration of 1 mg/ml (that is 1/50: for 100 ml add 2.0 ml G-418 stock).
[Comment: Alternatively, ready-to-use solution of G-418 can be purchased and aliquoted]

Preparation of 2.5% Avicel stock solution

1. Disperse 2,5 g of Avicel powder in 100 ml distilled water using standard magnetic stirrer. One hour stirring at room temperature is sufficient to obtain homogeneous suspension. Make sure that you have no obvious clumps in the Avicel suspension.
2. Autoclave at 121°C for 20 min to sterilise the suspension. One can prepare a larger volume of Avicel suspension and then aliquot into smaller aliquots before autoclaving.
3. Store at room temperature.

[Comment: Typically, 2,5% Avicel suspensions in water are stable. However, always mix them before use, just to make sure that suspension is homogeneous when you take an aliquot. This is even more important for working dilutions of Avicel, which may be less stable. Thus, you may see precipitation of Avicel in its mixtures with culture media over time. This is normal, just do not forget to mix before use. Mixing is easy (either shake by hand or vortex).]

Medium for serial passaging of cells

1x DMEM + 10% FBS + L-Glutamine (2 mM) + G-418 (1 mg/ml)

It is advisable to prepare fresh medium and store it at 4°C for no longer than 1 month. Passage MDCK-SIAT-1 cells in this medium at split ratio 1/12-1/16.

Medium for plating cells for experiments

1x DMEM + 10% FBS + L-Glutamine (2 mM) + Pen/Strep (1/100)

[Comment: Presence of G-418 in the growth medium during serial passaging of cells is needed to maintain selective pressure and to ensure that SIAT1-gene is preserved during passaging. However, when we plate cells for “terminal” experiments (such as virus infection), we do this one last passage without G-418. We use pen/strep instead. We also use pen/strep in the infection medium (see below).]

Medium for washing cells before infection

To remove fetal serum that was present in the growth medium, we wash cells 3 times with **Minimal Essential Medium (MEM)** before infection. Other non-expensive basal medium, balanced salt solutions or PBS supplemented with Ca^{2+} and Mg^{2+} can also be used.

Medium for infection (IM)

1x DMEM + L-Glutamine (1/100) + Pen/Strep (1/100) + 0.1% BSA

Avicel-containing overlay medium

1. Mix 2.5% Avicel stock in water with equal volume of 2x media. We use (2x MEM + 4 mM glutamine + pen/strep (1/50) plus 0.2%BSA).
2. Add 1 mg/ml TPCK-trypsin stock in water to final concentration 2 ug/ml (1/500). If needed, warm the overlay before adding to cell culture (both room temperature and 37o can be used).

[*Comment:* Although we typically use 1.25% overlay, one can decrease Avicel concentration if needed. We have tested working suspensions with Avicel concentrations down to 0,3%. Lower Avicel concentrations result in bigger plaques.]

I-2. Assay protocol

1. **One day before infection**, plate MDCK-SIAT1 cells in 96-well plates, 0.1 ml of cell suspension per well. Plate cell using split ratio about 1:3 to ensure that cells are 95-100% confluent on the next day.

On the day of infection:

2. Prepare 10-fold 4x-dilutions of the neuraminidase inhibitor in infection medium (IM), concentration range from 0.004 uM to 400 uM.

		Drug concentration, uM (50 ul per well)								
		0	0	0.004	0.04	0.40	4	40	400	
Virus Dilution 1										1
										2
										3
Virus Dilution 3										4
										5
										6
Virus Dilution 9										7
										8
										9
Virus Dilution 27										10
										11
										12
		H	G	F	E	D	C	B	A	

3. Prepare four 3-fold virus dilutions in IM. The lowest dilution should contain about 100 PFU of the virus in 50 ul (thus, the highest dilution will contain about 3 PFU in 50 ul).

4. Wash 1-day-old nearly or fully confluent cells 2 times with 0.1 ml and once with 0.2 ml of warm MEM (or another washing medium – see above). Use 8-channel suction manifold connected to the vacuum and 8-channel dispenser (Eppendorf).
5. Remove the last wash, add drug dilutions in IM as shown on the scheme, 50 ul per well. Use Eppendorf dispenser with 2.5 ml syringe do dispense the drug; start from filling wells with the IM containing no drug (columns G and H), continue using increasing drug concentrations. Remove washing medium and fill the wells stepwise to ensure that cells do not get dry during filling.
6. After filling the plate with the drug dilutions, add 50 ul of virus dilutions per well as indicated on the scheme. Use Eppendorf dispenser with 2.5 ml syringe, start from the highest dilution (rows 9-12).
7. Mix the plate by gently tapping with hand, or my using 96-well plate mixer operating at low speed. Incubate in CO₂ incubator at 35o for one-two hours.
8. Addition of Avicel. Take the plate from incubator. Do not remove viral inoculum. Add 0.1 ml per well of the overlay medium: [MEM-glutamine-antibiotics-0.1%BSA-2 ug/ml TPCK-trypsin-1.25% Avicel RC-581]. Add overlay at either room temperature or 35o. Use Eppendorf dispenser (Multipette) with 2.5 ml syringe and dispense Avicel fast in order to help its mixing with the inoculum.
9. After filling the plate with the overlay, mix the plate either by tapping by hand or on 96-well plate mixer. Do not worry if it looks like you cannot ensure complete homogeneity in the wells, this is not necessary.
10. Incubate the plate for 20 h to 48 h in CO₂ incubator, depending on how fast the virus grow. For influenza A viruses we use 20-24 h, for influenza B – 40-48 h.

[*Comment:* – make sure that the plate is not shaken during incubation, as this will destroy localisation of plaques. Shaking during the first 1-2 h after application of the overlay has no effect]

Part II. Fixation and immuno-staining (one-two days post infection):

Fixation.

1. Shake the plate gently to reduce the viscosity of the overlay and to facilitate its removal. Remove the overlay by suction. Add 0.1 ml fixing solution (we normally

use cold 4% paraformaldehyde in MEM; 10% formalin in PBS(v/v) is also fine) and shake the plate to ensure mixing of the fixing solution with what is left from overlay.

2. Incubate with fixing solution for 30 min at 4 oC.
3. Remove fixing solution; wash plates 2-3 times with 0.1 ml/well PBS (3-5 min incubation). Plates can be either stored under PBS at 4 oC or immediately processed further.

[*Comment.* We usually do not wash after removing the overlay. However, it is important to make sure that after addition of the fixing solution it is homogeneously distributed in the wells. That is, you should not have big remnants of the overlay left on cells, which would hinder the cells from fixing solution. If you have problems with that, wash once-twice with PBS before adding fixing solution.]

Immuno-staining.

4. Add 0.1 ml/well of 0.5% Triton-X-100 in PBS (prepared from 10% Triton-X-100 stock), incubate 10 min at room t.
5. Remove T-X-100, add 0.05 ml/well monoclonal antibody against influenza A virus NP (Centers for Disease Control and Prevention, Atlanta, GA, USA), 1/1000 in ELISA buffer (**EB**: 10% horse serum, 0.1% tween-80 in PBS). Incubate 1 h at room temperature. (Alternatively, incubate at 4oC overnight)
6. Wash 3 times with PBS-0.05% tween-80 (washing buffer, **WB**), 0.1 ml/well. (Incubate 3-5 min with each portion of washing buffer.
7. Add 0.05 ml/well of peroxidase-labeled anti-mouse antibody (DAKO), 1/2000 in EB. Incubate 1 h at room temperature.
8. Wash 3-4 times with WB, 0.1 ml/well. (Incubate 3-5 min with each portion of washing buffer).
9. Add substrate, (True Blue peroxidase substrate, KPL, No.50-78-02), 40-50 ul/well. Incubate for 20-40 min. Monitor development of staining and stop the reaction with substrate before high background develops. To stop the reaction, wash the plate 2 times with distilled water. Dry the plate and store it in a dark place.
10. Scan the plates on a flat-bed scanner and analyse images at optimal magnification.