Harvesting Virus

- Take flask containing the virus to be harvested and scrape all the cells from the bottom of the flask using a cell scraper.
- Place the flask in a freezer until frozen and thaw at room temperature at least three times to release virus from the infected cells.
- Transfer contents of the flask into a 15 mL conical tube. Vortex moderately to mix and centrifuge at 1500 × g for 10 minutes to remove cell debris.
- Collect the FCV-containing supernatant into a sterile plastic centrifuge tube and filter through a sterile 0.22µ syringe filter.
- Aliquot ~100 ul of the virus suspension per cryovial and store at ≤-70°C. Each cryovial is for single use only.
- Virus stocks may be stored and used for up to 18 months.

SUB-CULTURING CRFK CELL LINE

- Make the required amount of Complete Growth Medium (CGM) containing 10% FBS and warm it to 37 °.

- Remove flasks that exhibit at least an 80% confluent monolayer from incubator and check cell morphology. Label new flasks with passage #, cell line name and date.

- Following equilibration of temperature of all media, remove spent medium from flask and wash with PBS 2-3 times.

- Dispense 3 mL of trypsin+EDTA flask and observe frequently for cell detachment.

- Dispense 9 ml of CGM and pipet the cell suspension up and down 3-5 times to break up any clumps.

- Transfer the required volume of cell suspension for the desired split ratio, then add CGM up to the working volume of the flask. For example, for a 1:3 split, add 3 ml cell suspension to each of three new flasks and then add 12 ml fresh CGM. For 24 well plates, add 200 µl of the cell suspension to 800 µl of CGM.

- Place the flasks into the incubator and do not disturb then for 2-3 hours to allow for cell adherence to the flask surface.

- Monolayers prepared this way can be used for making virus pools, for making plates for virus infectivity assays or for another passage for subsequent experiments.

- A maximum of 50 passes can be made before fresh cells should be started.

Virus Titration by TCID50 Method

- Prepare 24-well tissue culture plate with at least an 80% confluent monolayer of CRFK cells. This plate should be incubated at 37±1° C in 5% CO_2 for no more than 2-3 days. Ensure all wells have a healthy monolayer before use.
- To determine the titer of FCV, prepare a serial ten-fold dilution of FCV from 10^{-1} to 10^{-9} (or as required) in pre-warmed CGM with 2% FBS .
- Aspirate gently the used CGM from each well needed on the 24 well plate. This process must be performed quickly to minimize cell death due to lack of CGM on the monolayer.
- Add 1 mL of diluted FCV filtrate from each dilution to each of four wells. When done, cover that column with the tissue culture plate cover to prevent contamination. Work from most dilute to least dilute.
- Cover the plate, mix gently by hand by and incubate the 24-well tissue culture plate at 37±1° C in 5% CO_2 for 90±5 minutes to allow for FCV adsorption. Use a hematology rotator or rock the plate gently by hand every 10-15 minutes.
- Post-virus adsorption, remove CGM containing non-adsorbed FCV from highest to lowest dilutions. Wash the monolayer once with PBS if desired then add CGM with 2% FBS to each well in the same order.
- Continue incubation at 37±1° C in 5% CO_2 until about 75-100% of the monolayer shows virus-induced cell degeneration or cytopathic effects (CPE). Incubation of a minimum of 4 days is necessary, but may require up to 7 days.
- Check the cells for CPE after each 20±4 hours of incubation. If cytotoxicity (i.e., non viral-induced CPE) is observed, wash cells with PBS and replace growth media. Otherwise, do not replace growth media for the duration of the incubation. Check the plate/s daily.
- At the end of the incubation period, the infectious FCV will be scored microscopically (and recorded) by observing virus-specific CPE produced in four replicas of each dilution as either positive for CPE or the absence of CPE.

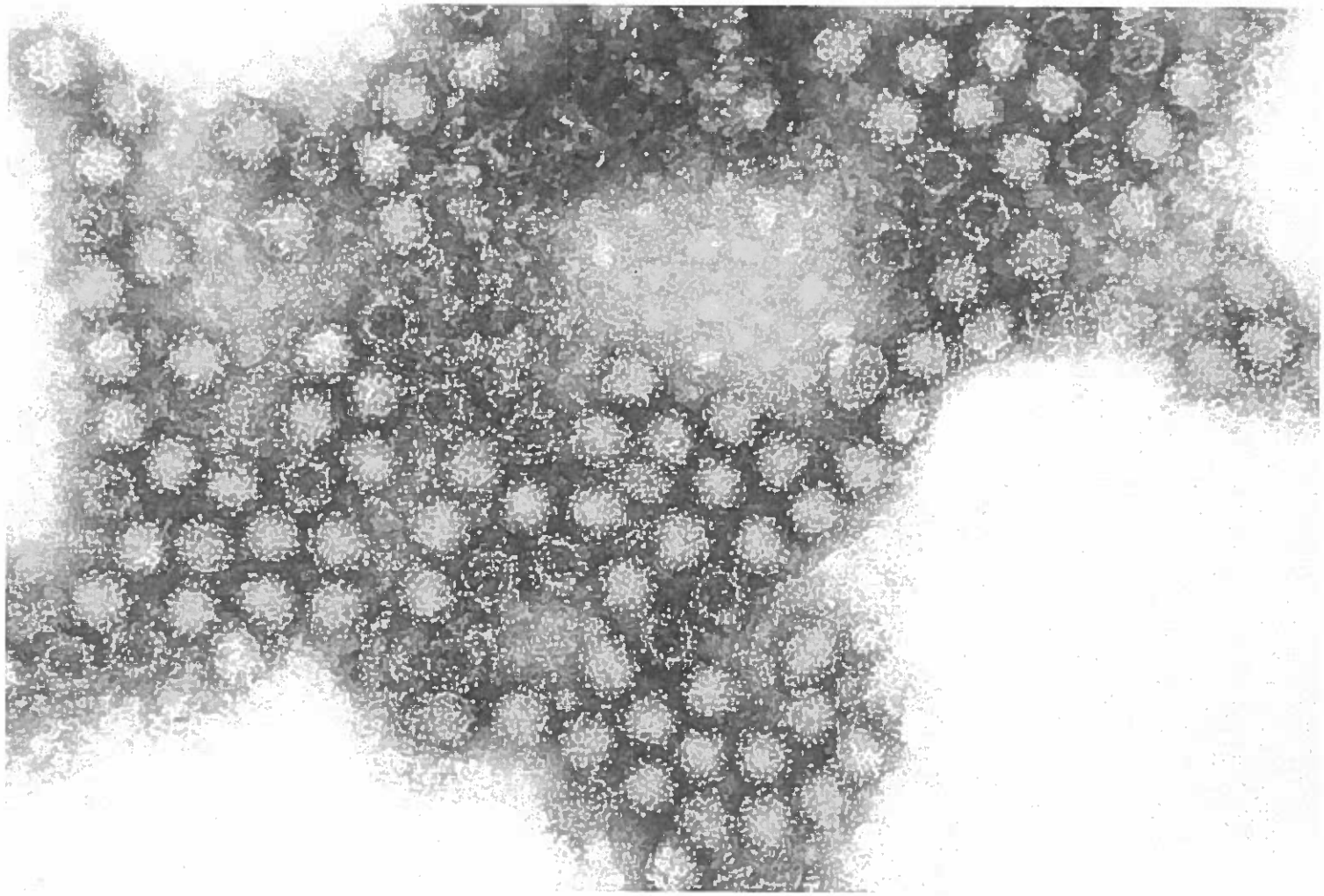
10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Positive	Positive	Positive	Negative	Negative	Negative
Positive	Positive	Negative	Positive	Positive	Negative
Positive	Positive	Positive	Negative	Negative	Negative
Positive	Positive	Positive	Positive	Negative	Negative

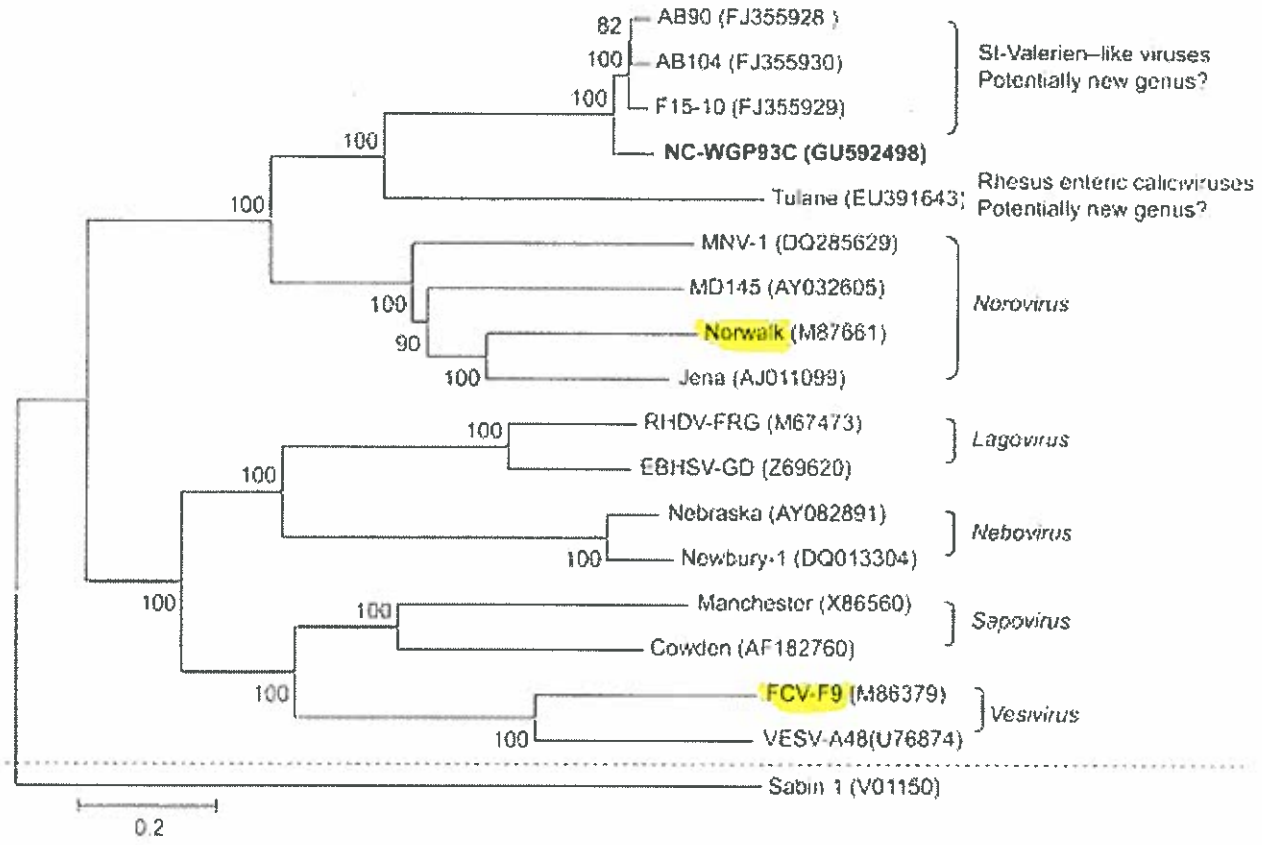
Reed & Muench Calculator

1. Enter the starting dilution	initial dilution:	0.1	<-Change!				
2. Enter the dilution factor	dilution factor:	10					
3. Enter the neutralizer volume	ml:	5					
4. Enter the volume tested per well	ml:	1					
This is your calculated dilution series:		1.00E-01	1.00E-02	1.00E-03	1.00E-04	1.00E-05	1.00E-06
5. Enter the total # of wells examined per dilution	total wells:	4	4	4	4	4	4
6. Enter the # of positive wells for each dilution	positive wells:	4	4	3	2	1	0
These values are calculated automatically:	negative wells:	0	0	1	2	3	4
	cum pos:	14	10	6	3	1	0
	cum neg:	0	0	1	3	6	10
	% infected:	100.00	100.00	85.71	50.00	14.29	0.00
	prop dist:	0	0	1	0	-2.5	0
7. This is your $TCID_{50}$	$TCID_{50}$:			1.00E-04			
8. This is your $TCID_{50}$/carrier	$TCID_{50}$/carrier:			5.00E+04			

Proposed Attachments to MLB Virology SOP

- Procedure for freezing cell line.
- Procedure for reviving cell line from freezing.
- Sub-culturing and care of cell line.
- Virus propagation, storage, and determination of concentration.
- OECD neutralization procedure.




82
100
100
100
100
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90
100
100
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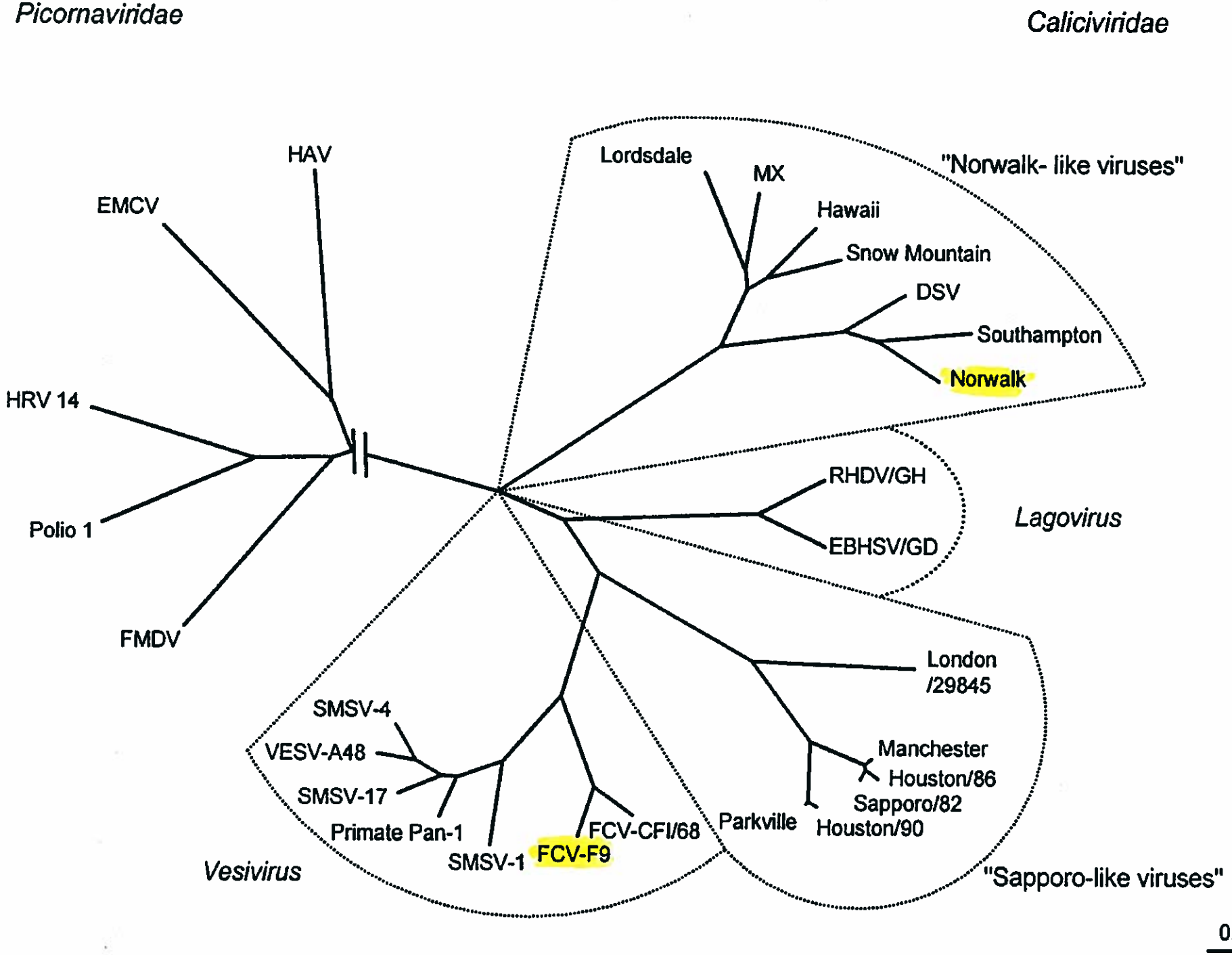
AB90 (FJ355928)
AB104 (FJ355930)
F15-10 (FJ355929)
NC-WGP93C (GU592498)
Tulane (EU391643)
MNV-1 (DQ285629)
MD145 (AY032605)
Norwalk (M87661)
Jena (AJ011099)
RHDV-FRG (M67473)
EBHSV-GD (Z69620)
Nebraska (AY082891)
Newbury-1 (DQ013304)
Manchester (X86560)
Cowden (AF182760)
FCV-F9 (M86379)
VESV-A48(U76874)
Sabin 1 (V01150)
0.2
St-Valerien–like viruses
Potentially new genus?
Rhesus enteric caliciviruses
Potentially new genus?
Norovirus
Lagovirus
Nebovirus
Sapovirus
Vesivirus


Picornaviridae
Caliciviridae
HAV
EMCV
HRV 14
Polio 1
FMDV
Lordsdale
MX
Hawaii
Snow Mountain
DSV
Southampton
Norwalk
"Norwalk- like viruses"
RHDV/GH
EBHSV/GD
Lagovirus
London /29845
Manchester
Houston/86
Sapporo/82
Houston/90
Parkville
"Sapporo-like viruses"
SMSV-4
VESV-A48
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Vesivirus
0.2
0.1

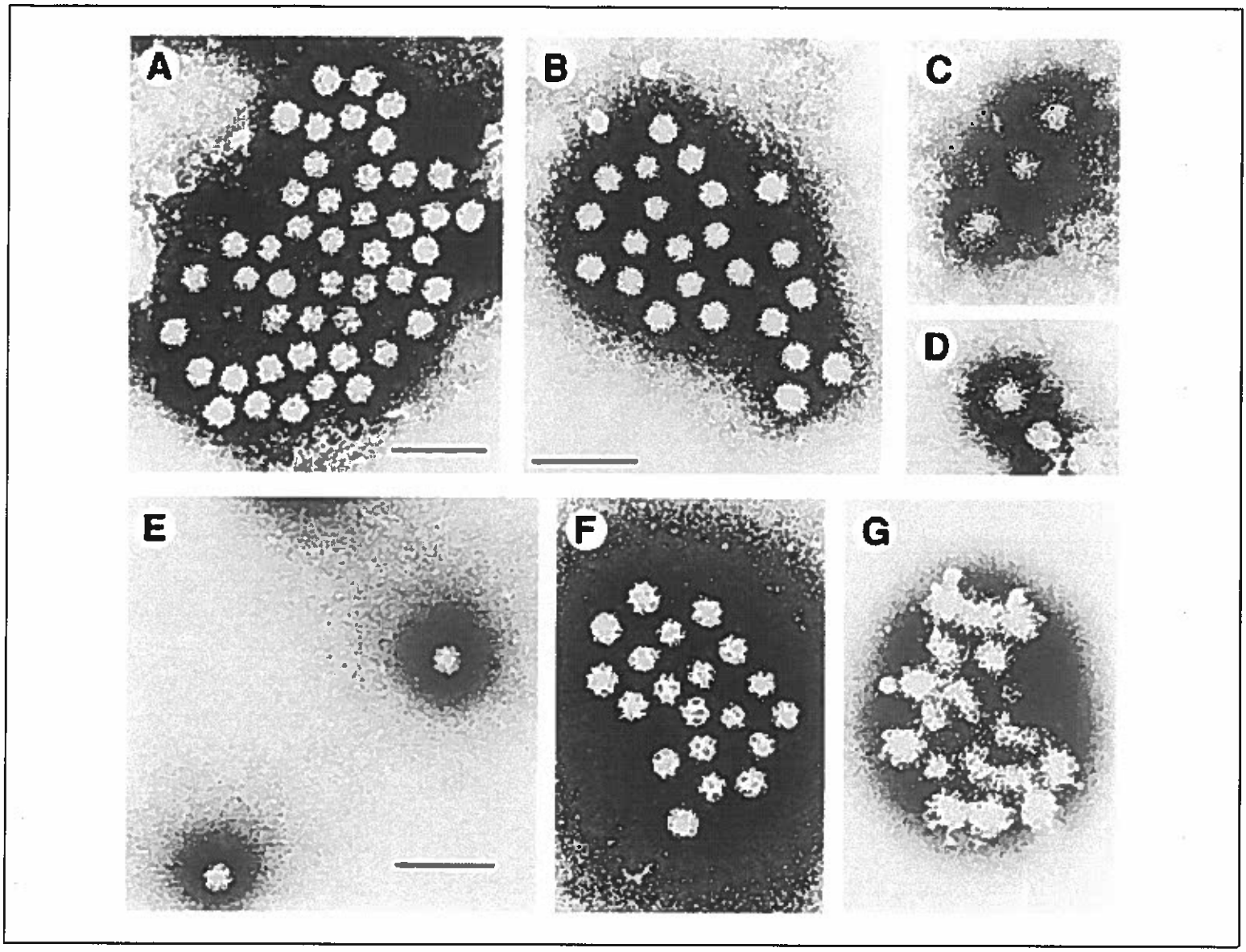
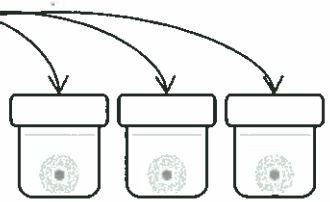


Figure 28.1 Norwalk virus (NV) (genus *Norovirus*) and Sapporo virus (genus *Sapovirus*) in stool material. **A:** NV particles in a stool filtrate visualized by immune electron microscopy (IEM). (From ref. (162), with permission.) **B:** An aggregate observed after incubation of NV stool filtrate with a 1:5 dilution of volunteer's prechallenge serum. Particles have a light coating of antibody molecules. The amount of antibody present on this aggregate was given a rating of 1 to 2 to 2+ and the serum was given an overall rating of 1 to 2+ on a scale of 1 to 4. (From ref.162, with permission.) **C** and **D:** Three single particles (**C**) and one individual particle (**D**) observed after incubation of the NV stool filtrate with a 1:5 dilution of the same volunteer's postchallenge convalescent serum. Particles are heavily coated with antibody molecules. At high antibody levels (antibody excess), large aggregates may not be seen. The amount of antibody was given a rating of 4+ and the serum was given an overall rating of 4+ on a scale of 1 to 4. (From ref. 162, with permission.) **E:** Sapporo virus particles in stool material visualized by direct EM. The distinct, hollow cup-like structures are apparent. (From ref. 253, with permission.) **F:** Sapporo virus particles after incubation with guinea pig hyperimmune serum. The amount of antibody was given a 1+ rating. (From ref. 253, with permission.) **G:** An aggregate observed after incubation of Sapporo virus stool filtrate with guinea pig hyperimmune serum. The amount of antibody was given a rating of 4+ on a scale of 1 to 4. (From ref. 253, with permission.)

OECD Neutralization Assay Flow Chart

Treatment 1a

Add 1 carrier inoculated with *Test Suspension B* to each vial containing 950 µl PBST.

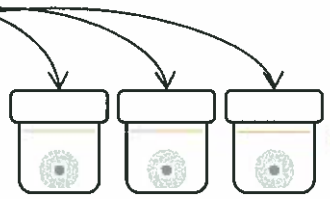


Titer Control (with SL)

Vortex for 30±2 sec. and hold for 10 min. at 20-25°C. Proceed to vortexing/titration.

Treatment 2

Add 1 carrier inoculated with *Test Suspension B* to each vial containing 950 µl neutralizer.

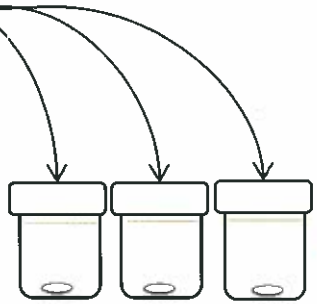


Neutralizer Toxicity Control

Vortex for 30±2 sec. and hold for 10 min. at 20-25°C. Proceed to vortexing/titration.

Treatment 3a

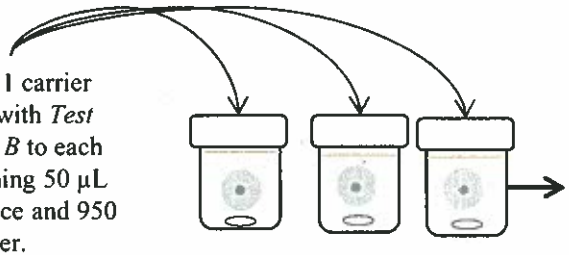
Add 50 µL of test substance to each vial, then add 950 µl neutralizer to each vial and briefly swirl.



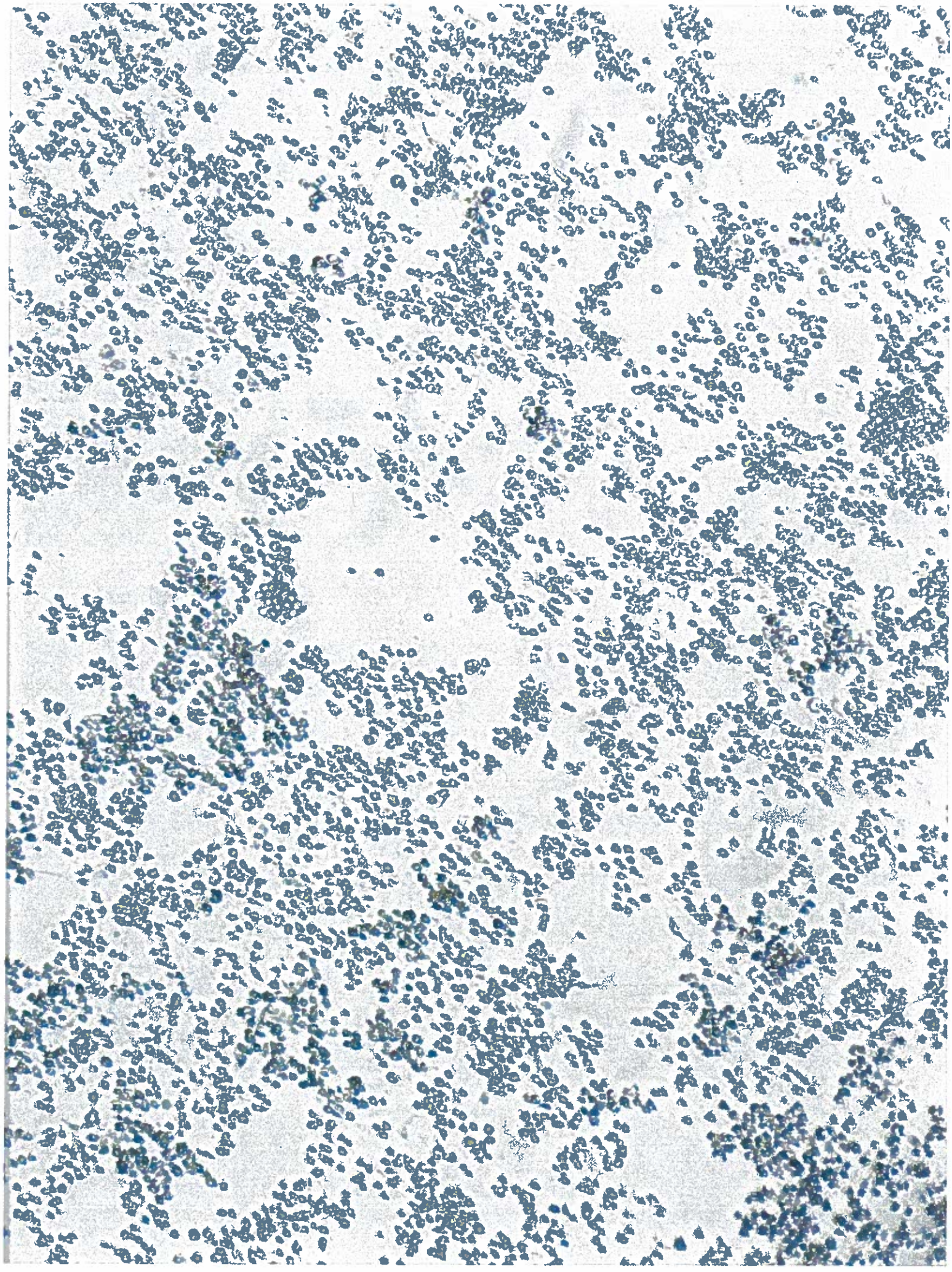
Neutralizer Effectiveness (with SL)

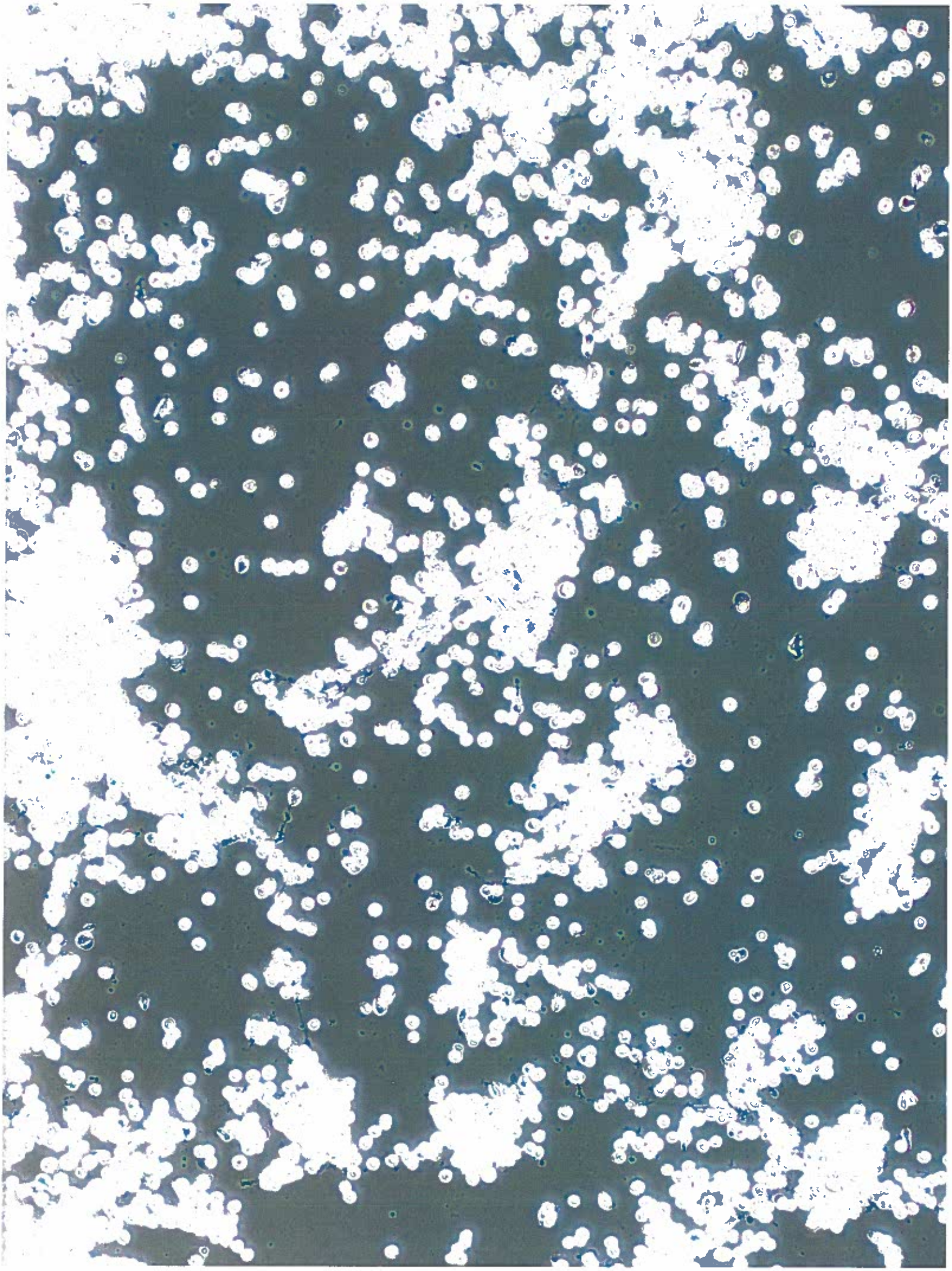
Wait 10 sec.

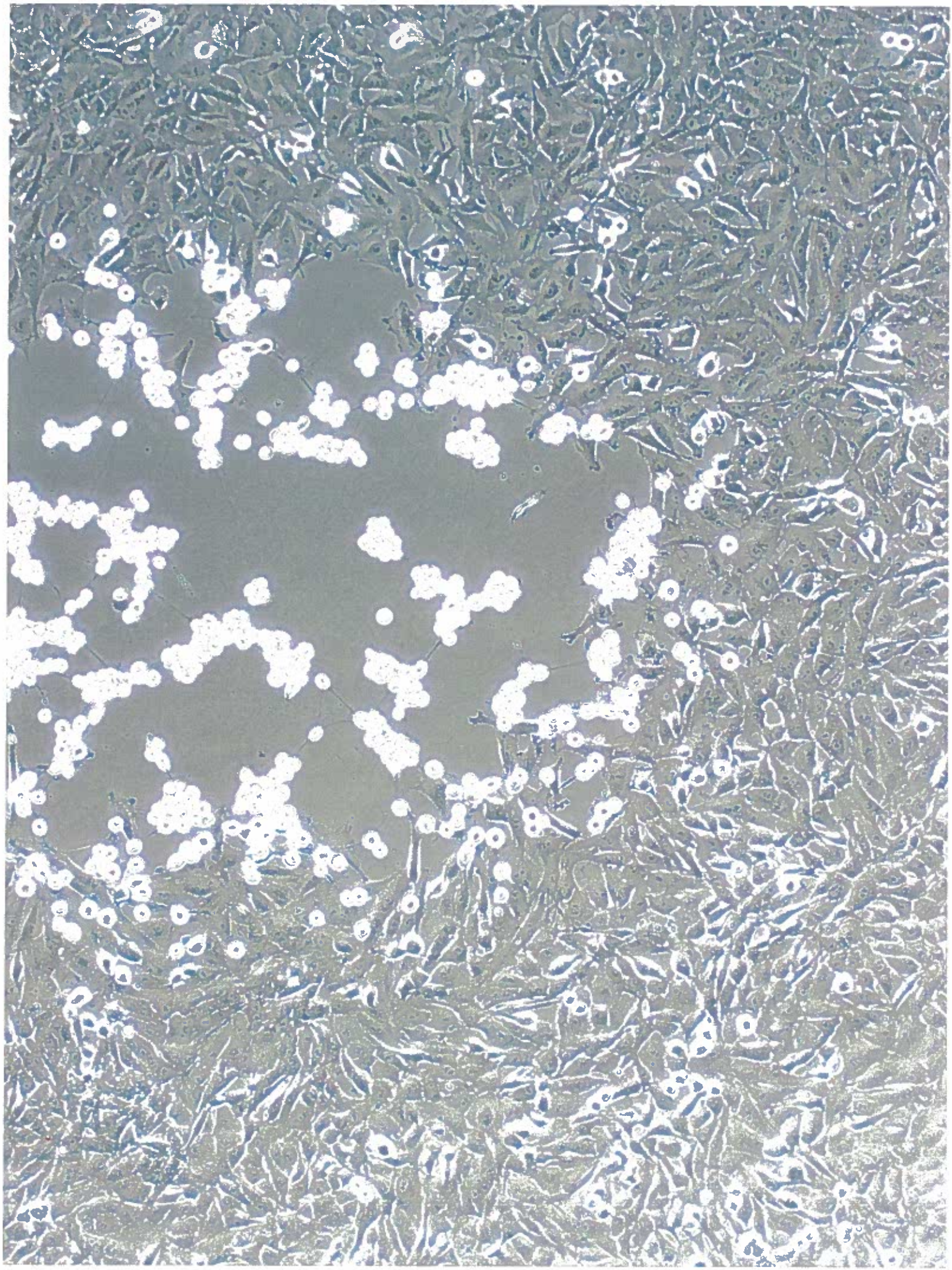
Gently add 1 carrier inoculated with *Test Suspension B* to each vial containing 50 µL test substance and 950 µl neutralizer.

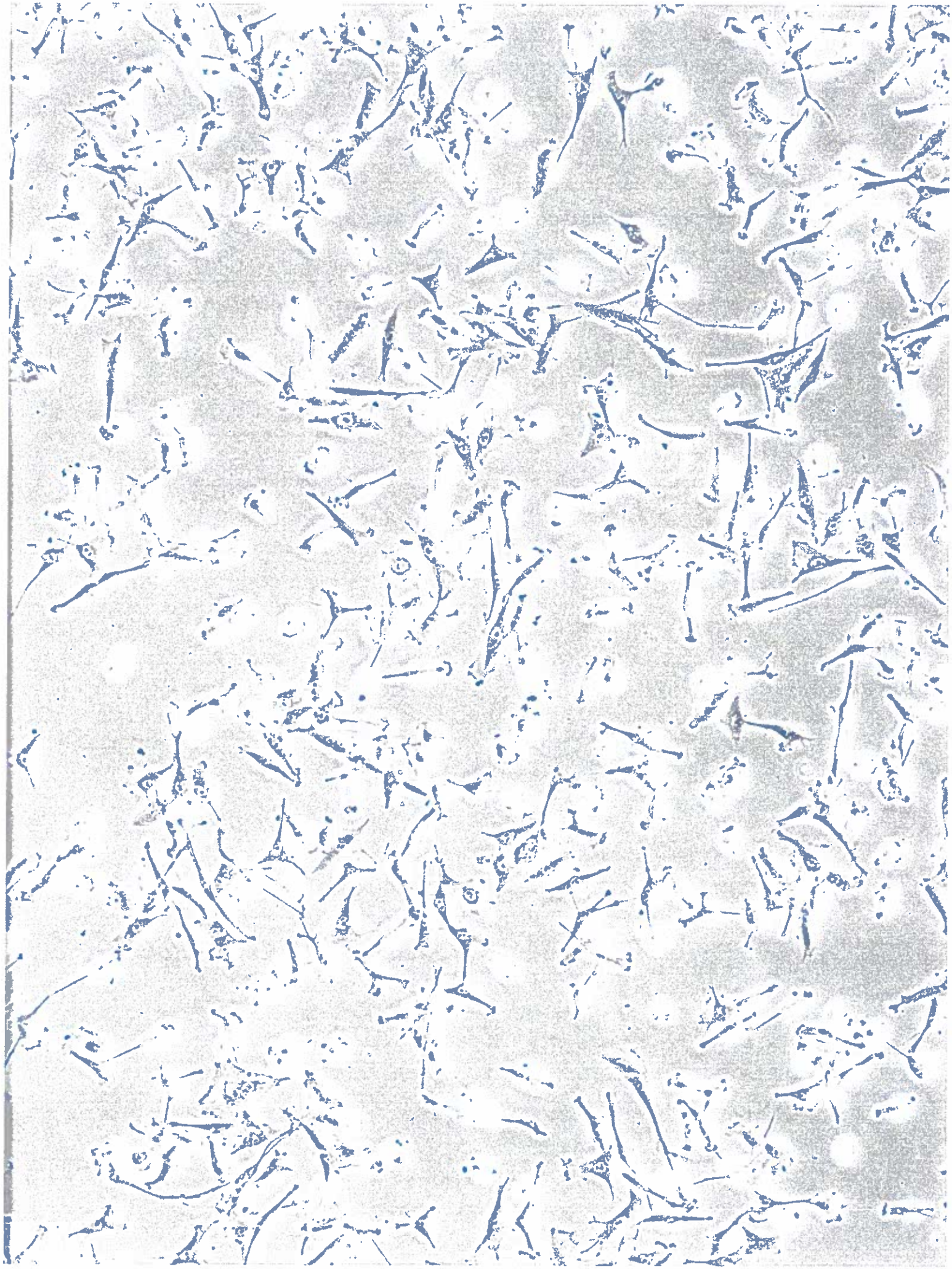


Vortex for 30±2 sec. and hold for 10 min. at 20-25°C. Proceed to vortexing/titration.









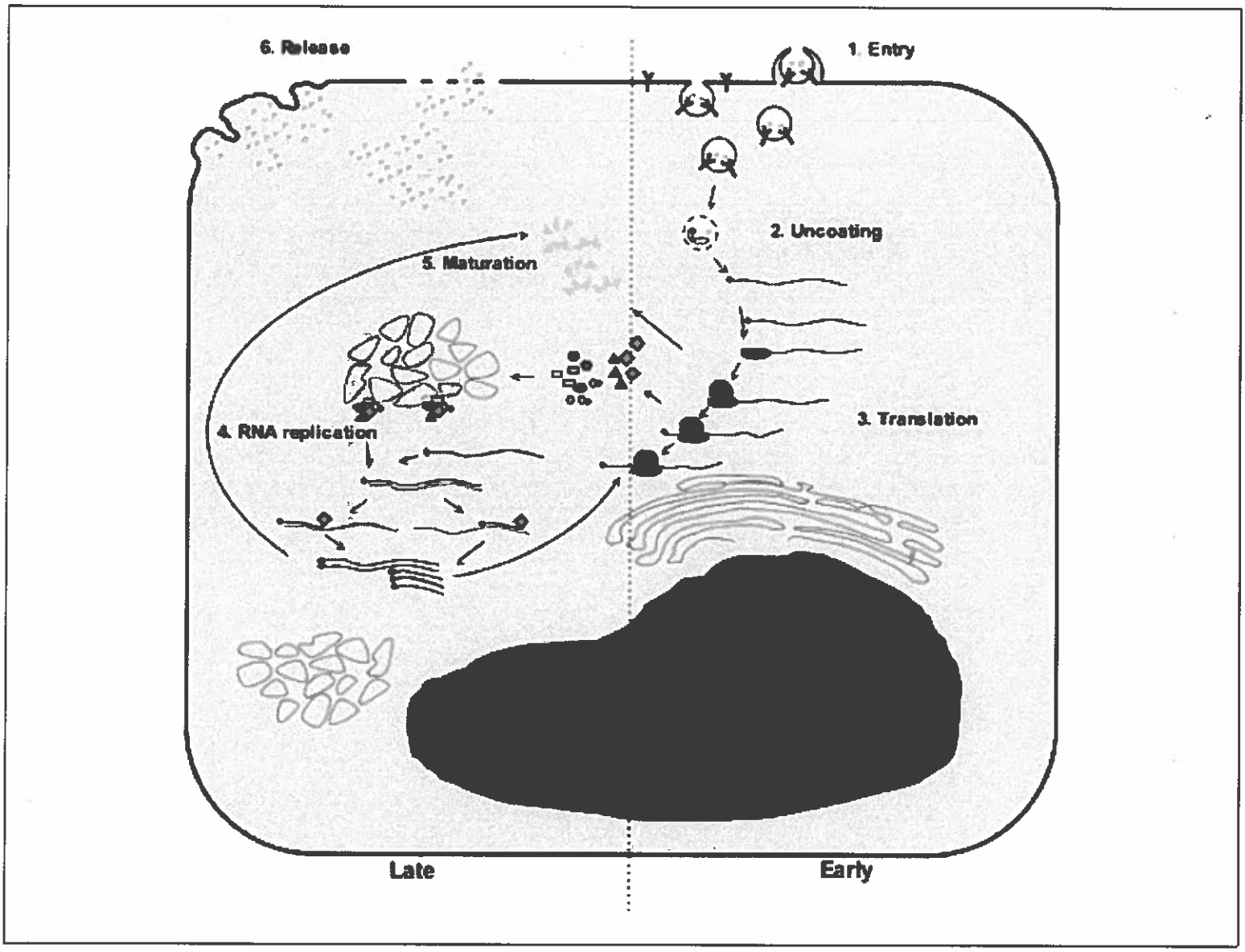


Figure 28.6 Schematic diagram of the proposed replication strategy of the caliciviruses. Consistent with other positive-strand RNA viruses, the replication cycle of a calicivirus involves the following stages: (1) Entry; (2) Uncoating; (3) Translation; (4) RNA replication; (5) Maturation; and (6) Release, as reviewed in the text. (Courtesy of S. Sosnovtsev.)