culture of the diploid cells. If the vaccine virus is grown in a cell system other than simian or human, cells of that species, from a separate batch, are also inoculated. The cells are incubated at 36 ± 1 °C and observed for a period of 14 days. The virus seed lot or harvest passes the tests if none of the cell cultures shows evidence of the presence of any extraneous agents not attributable to accidental contamination. The test is not valid unless at least 80 per cent of the cell cultures remain viable.

Avian viruses (only required for virus propagated in avian tissues). Neutralise a sample equivalent to 100 human doses or 10 ml, whichever is the greater. Using 0.5 ml per egg, inoculate a group of fertilised SPF eggs, 9 to 11 days old, by the allantoic route and a second group, 5 to 7 days old, into the yolk sac. Incubate for 7 days. The virus seed lot or harvest complies with the test if the allantoic and yolk sac fluids show no sign of the presence of any haemagglutinating agent and if all embryos and chorio-allantoic membranes, examined for gross pathology, are normal. The test is not valid unless at least 80 per cent of the inoculated eggs survive for 7 days.

PRODUCTION CELL CULTURE: CONTROL CELLS

Examine the control cells microscopically for freedom from any virus causing cytopathic degeneration throughout the time of incubation of the inoculated production cell cultures or for not less than 14 days beyond the time of inoculation of the production vessels, whichever is the longer. The test is not valid unless at least 80 per cent of the control cell cultures survive to the end of the observation period.

At 14 days or at the time of the last virus harvest, whichever is the longer, carry out the tests described below.

Test for haemadsorbing viruses. Examine not fewer than 25 per cent of the control cultures for the presence of haemadsorbing viruses by the addition of guinea-pig red blood cells. If the guinea-pig red blood cells have been stored, they shall have been stored at 5 ± 3 °C for not more than 7 days. Read half of the cultures after incubation at 5 ± 3 °C for 30 min and the other half after incubation at 20 °C to 25 °C for 30 min. No evidence of haemadsorbing agents is found.

Tests in cell cultures for other extraneous agents. Pool the supernatant fluids from the control cells and examine for the presence of extraneous agents by inoculation of simian kidney and human cell cultures. If the vaccine virus is grown in a cell system other than simian or human, cells of that species, but from a separate batch, are also inoculated. In each cell system, at least 5 ml is tested. Incubate the inoculated cultures at a temperature of 36 ± 1 °C and observe for a period of 14 days. No evidence of extraneous agents is found.

If the production cell culture is maintained at a temperature different from 36 ± 1 °C, a supplementary test for extraneous agents is carried out at the production temperature using the same type of cells as used for growth of the virus.

Avian leucosis viruses (required only if the virus is propagated in avian tissues). Carry out a test for avian leucosis viruses using 5 ml of the supernatant fluid from the control cells.

CONTROL EGGS

Haemagglutinating agents. Examine 0.25 ml of the allantoic fluid from each egg for haemagglutinating agents by mixing directly with chicken red blood cells and after a passage in SPF eggs carried out as follows: inoculate a 5 ml sample of the pooled amniotic fluids from the control eggs in 0.5 ml volumes into the allantoic cavity and into the amniotic cavity of SPF eggs. The control eggs comply with the test if no evidence of the presence of haemagglutinating agents is found in either test.

Avian leucosis viruses. Use a 10 ml sample of the pooled amniotic fluids from the control eggs. Carry out amplification by five passages in leucosis-free chick-embryo cell cultures; carry out a test for avian leucosis using cells from the fifth passage. The control eggs comply with the test if no evidence of the presence of avian leucosis viruses is found.

Other extraneous agents. Inoculate 5 ml samples of the pooled amniotic fluids from the control eggs into human and simian cell cultures. Observe the cell cultures for 14 days. The control eggs comply with the test if no evidence of the presence of extraneous agents is found. The test is not valid unless 80 per cent of the inoculated cultures survive to the end of the observation period.

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2.6.17. TEST FOR ANTICOMPLEMENTARY ACTIVITY OF IMMUNOGLOBULIN

For the measurement of anticomplementary activity (ACA) of immunoglobulin, a defined amount of test material (10 mg of immunoglobulin) is incubated with a defined amount of guinea-pig complement (20 CH_{50}) and the remaining complement is titrated; the anticomplementary activity is expressed as the percentage consumption of complement relative to the complement control considered as 100 per cent.

The haemolytic unit of complement activity (CH_{50}) is the amount of complement that, in the given reaction conditions, will produce the lysis of 2.5×10^8 out of a total of 5×10^8 optimally sensitised red blood cells.

Magnesium and calcium stock solution. Dissolve 1.103 g of *calcium chloride R* and 5.083 g of *magnesium chloride R* in *water R* and dilute to 25 ml with the same solvent.

Barbital buffer stock solution. Dissolve 207.5 g of *sodium chloride R* and 25.48 g of *barbital sodium R* in 4000 ml of *water R* and adjust to pH 7.3 using *1 M hydrochloric acid*. Add 12.5 ml of magnesium and calcium stock solution and dilute to 5000 ml with *water R*. Filter through a membrane filter (pore size 0.22 µm). Store at 4 °C in glass containers.

Gelatin solution. Dissolve 12.5 g of *gelatin R* in about 800 ml of *water R* and heat to boiling in a water-bath. Cool to 20 °C and dilute to 10 litres with *water R*. Filter through a membrane filter (pore size: 0.22 µm). Store at 4 °C. Use clear solutions only.

Citrate solution. Dissolve 8.0 g of *sodium citrate R*, 4.2 g of *sodium chloride R* and 20.5 g of *glucose R* in 750 ml of *water R*. Adjust to pH 6.1 using a 100 g/l solution of *citric acid R* and dilute to 1000 ml with *water R*.

Gelatin barbital buffer solution. Add 4 volumes of gelatin solution to 1 volume of barbital buffer stock solution and mix. Adjust to pH 7.3, if necessary, using *1 M sodium hydroxide* or *1 M hydrochloric acid*. Maintain at 4 °C. Prepare fresh solutions daily.

Stabilised sheep blood. Collect one volume of sheep blood into one volume of citrate solution and mix. Store at 4 °C for not less than 7 days and not more than 28 days. (Stabilised sheep blood and sheep red blood cells are available from a number of commercial sources.)

Haemolysin. Antiserum against sheep red blood cells prepared in rabbits. (Such antisera are available from a number of commercial sources.)

Guinea-pig complement. Prepare a pool of serum from the blood of not fewer than ten guinea-pigs. Separate the serum from the clotted blood by centrifugation at about 4 °C. Store the serum in small amounts below – 70 °C.

METHOD

Preparation of standardised 5 per cent sheep red blood cell suspension. Separate sheep red blood cells by centrifuging an appropriate volume of stabilised sheep blood and wash the cells at least three times with gelatin barbital buffer solution and prepare as a 5 per cent *V/V* suspension in the same solution. Measure the cell density of the suspension as follows: add 0.2 ml to 2.8 ml of *water R* and centrifuge the lysed solution for 5 min at 1000 *g*; the cell density is suitable if the absorbance (*2.2.25*) of the supernatant liquid at 541 nm is 0.62 ± 0.01. Correct the cell density by adding gelatin barbital buffer solution according to the formula:

$$V_f = \frac{V_i \times A}{0.62}$$

V_f = final adjusted volume,

V_i = the initial volume,

A = absorbance of the original suspension at 541 nm.

The adjusted suspension contains about 1×10^9 cells/ml.

Haemolysin titration

Prepare haemolysin dilutions as shown in Table 2.6.17.-1.

Table 2.6.17.-1

Required dilution of haemolysin	Prepared using		
	Gelatin barbital buffer solution		Haemolysin
	Volume (millilitres)	Dilution (1: ...)	Volume (millilitres)
7.5	0.65	undiluted	0.1
10	0.90	undiluted	0.1
75	1.80	7.5	0.2
100	1.80	10	0.2
150	1.00	75	1.0
200	1.00	100	1.0
300	1.00	150	1.0
400	1.00	200	1.0
600	1.00	300	1.0
800	1.00	400	1.0
1200	1.00	600	1.0
1600	1.00	800	1.0
2400	1.00	1200	1.0
3200*	1.00	1600	1.0
4800*	1.00	2400	1.0

* discard 1.0 ml of the mixture.

Add 1.0 ml of 5 per cent sheep red blood cell suspension to each tube of the haemolysin dilution series, starting at the 1:75 dilution, and mix. Incubate at 37 °C for 30 min.

Transfer 0.2 ml of each of these incubated mixtures to new tubes and add 1.10 ml of gelatin barbital buffer solution and 0.2 ml of diluted guinea-pig complement (for example, 1:150). Perform this in duplicate.

As the unhaemolysed cell control, prepare three tubes with 1.4 ml of gelatin barbital buffer solution and 0.1 ml of 5 per cent sheep red blood cell suspension.

As the fully haemolysed control, prepare three tubes with 1.4 ml of *water R* and 0.1 ml of 5 per cent sheep red cell suspension.

Incubate all tubes at 37 °C for 60 min and centrifuge at 1000 *g* for 5 min. Measure the absorbance (*2.2.25*) of the supernatants at 541 nm and calculate the percentage degree of haemolysis in each tube using the expression:

$$\frac{A_a - A_1}{A_b - A_1} \times 100$$

A_a = absorbance of tubes with haemolysin dilution,

A_b = mean absorbance of the three tubes with full haemolysis,

A_1 = mean absorbance of the three tubes with no haemolysis.

Plot the percentage degree of haemolysis as the ordinate against the corresponding reciprocal value of the haemolysin dilution as the abscissa on linear graph paper. Determine the optimal dilution of the haemolysin from the graph by inspection. Select a dilution such that further increase in the amount of haemolysin does not cause appreciable change in the degree of haemolysis. This dilution is defined as one minimal haemolytic unit (1 MHU) in 1.0 ml. The optimal haemolytic haemolysin dilution for preparation of sensitised sheep red blood cells contains 2 MHU/ml.

The haemolysin titration is not valid unless the maximum degree of haemolysis is 50 per cent to 70 per cent. If the maximum degree of haemolysis is not in this range, repeat the titration with more or less diluted complement solution.

Preparation of optimised sensitised sheep red blood cells (haemolytic system)

Prepare an appropriate volume of diluted haemolysin containing 2 MHU/ml and an equal volume of standardised 5 per cent sheep red blood cell suspension. Add the haemolysin dilution to the standardised cell suspension and mix. Incubate at 37 °C for 15 min, store at 2 °C to 8 °C and use within 6 h.

Titration of complement

Prepare an appropriate dilution of complement (for example, 1:250) with gelatin barbital buffer solution and perform the titration in duplicate as shown in Table 2.6.17.-2.

Add 0.2 ml of sensitised sheep red blood cells to each tube, mix well and incubate at 37 °C for 60 min. Cool the tubes in an ice-bath and centrifuge at 1000 *g* for 5 min. Measure the absorbance of the supernatant liquid at 541 nm and calculate the degree of haemolysis (*Y*) using the expression:

$$\frac{A_c - A_1}{A_b - A_1}$$

A_c = absorbance of tubes 1 to 12,

A_b = mean absorbance of tubes with 100 per cent haemolysis,

A_1 = mean absorbance of cell controls with 0 per cent haemolysis.

Plot $Y/(1-Y)$ as the abscissa against the amount of diluted complement in millilitres as the ordinate on log–log graph paper. Fit the best line to the points and determine the ordinate for the 50 per cent haemolytic complement dose where $Y/(1-Y) = 1.0$. Calculate the activity in haemolytic units (CH_{50}/ml) from the expression:

$$\frac{C_d}{C_a \times 5}$$

C_d = reciprocal value of the complement dilution,

C_a = volume of diluted complement in millilitres resulting in 50 per cent haemolysis,

5 = scaling factor to take account of the number of red blood cells.

The test is not valid unless the plot is a straight line between 15 per cent and 85 per cent haemolysis and the slope is 0.15 to 0.40, and preferably 0.18 to 0.30.

Table 2.6.17.-2

Tube Number	Volume of diluted complement in millilitres (for example 1:250)	Volume of gelatin barbital buffer solution in millilitres
1	0.1	1.2
2	0.2	1.1
3	0.3	1.0
4	0.4	0.9
5	0.5	0.8
6	0.6	0.7
7	0.7	0.6
8	0.8	0.5
9	0.9	0.4
10	1.0	0.3
11	1.1	0.2
12	1.2	0.1
Three tubes as cell control at 0 per cent haemolysis	–	1.3
Three tubes at 100 per cent haemolysis	–	1.3 ml of water

Test for anticomplementary activity

Prepare a complement dilution having 100 CH_{50}/ml by diluting titrated guinea-pig complement with gelatin barbital buffer solution. If necessary, adjust the immunoglobulin to be examined to pH 7. Prepare incubation mixtures as follows for an immunoglobulin containing 50 mg/ml:

Table 2.6.17.-3

	Immunoglobulin to be examined	Complement control (in duplicate)
Immunoglobulin (50 mg/ml)	0.2 ml	–
Gelatin barbital buffer	0.6 ml	0.8 ml
Complement	0.2 ml	0.2 ml

Carry out the test on the immunoglobulin to be examined and prepare ACA negative and positive controls using *human immunoglobulin BRP,* as indicated in the leaflet accompanying the reference preparation. Higher or lower volumes of sample and of gelatin barbital buffer solution are added if the immunoglobulin concentration varies from 50 mg/ml; for example, 0.47 ml of gelatin barbital buffer solution is added to 0.33 ml of immunoglobulin containing 30 mg/ml to give 0.8 ml. Close the tubes and incubate at 37 °C for 60 min. Add 0.2 ml of each incubation mixture to 9.8 ml of gelatin barbital buffer solution to dilute the complement. Perform complement titrations as described above on each tube to determine the remaining complement activity (Table 2.6.17.-2). Calculate the anticomplementary activity of the preparation to be examined relative to the complement control considered as 100 per cent, from the expression:

$$\frac{a-b}{a} \times 100$$

a = mean complement activity (CH_{50}/ml) of complement control,

b = complement activity (CH_{50}/ml) of tested sample.

The test is not valid unless:

– the anticomplementary activities found for ACA negative control and ACA positive control are within the limits stated in the leaflet accompanying the reference preparation,

– the complement activity of the complement control (a) is in the range 80 to 120 CH_{50}/ml.

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2.6.18. TEST FOR NEUROVIRULENCE OF LIVE VIRUS VACCINES

For each test, use not fewer than ten monkeys that are seronegative for the virus to be tested. For each monkey, inject not more than 0.5 ml of the material to be examined into the thalamic region of each hemisphere, unless otherwise prescribed. The total amount of virus inoculated in each monkey must be not less than the amount contained in the recommended single human dose of the vaccine. As a check against the introduction of wild neurovirulent virus, keep a group of not fewer than four control monkeys as cage-mates or in the immediate vicinity of the inoculated monkeys. Observe the inoculated monkeys for 17 to 21 days for symptoms of paralysis and other evidence of neurological involvement; observe the control monkeys for the same period plus 10 days. Animals that die within 48 h of injection are considered to have died from non-specific causes and may be replaced. The test is not valid if: more than 20 per cent of the inoculated monkeys die from nonspecific causes; serum samples taken from the control monkeys at the time of inoculation of the test animals and 10 days after the latter are euthanised show evidence of infection by wild virus of the type to be tested or by measles virus. At the end of the observation period, carry out autopsy and histopathological examinations of appropriate areas of the brain for evidence of central nervous system involvement. The material complies with the test if there is no unexpected clinical or histopathological evidence of involvement of the central nervous system attributable to the inoculated virus.

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2.6.19. TEST FOR NEUROVIRULENCE OF POLIOMYELITIS VACCINE (ORAL)

Monkeys used in the neurovirulence test comply with the requirements given in the monograph on *Poliomyelitis vaccine oral (0215)* and weigh not less than 1.5 kg. The pathogenicity for *Macaca* or *Cercopithecus monkeys* is tested in comparison with that of a reference virus preparation for neurovirulence testing by inoculation into the lumbar region of the central nervous system after sedation with a suitable substance, for example, ketamine hydrochloride. A sample of serum taken before the injection shall be shown not to contain neutralising antibody at a dilution of 1:4 when tested against not more than 1000 $CCID_{50}$ of each of the three types of poliovirus.