are usually done using factors of 2, 5 or 10, the former obviously giving a more precise titer. For routine use 10-fold dilutions are usually carried out.

When setting up such a dilution series consideration should be given to the final volume of diluent needed for the assay and thus aliquots of 0.9, 4.5, 9.0 ml are usually made in a series of sterile tubes or bottles. In order to conserve virus stocks it is usual for the first (lowest) dilution in the series to be achieved by the use of 0.1 ml stock suspension. With subsequent dilutions it is very important to use a new sterile pipette for each transfer and to thoroughly mix each virus dilution before further transfer. Use of the same pipette will transfer millions of virus particles along the series, resulting in a very large dilution error. Once diluted, virus should be assayed as soon as possible although, if necessary, some viruses will withstand storing at 4°C for a few hours before assay. The use of such a storage procedure should obviously be checked to determine if it is suitable for the virus under assay.

$TCID_{50}$

The $TCID_{50}$ is defined as that dilution of a virus required to infect 50% of a given batch of inoculated cell cultures. The assay relies on the presence and detection of cytocidal virus particles (i.e., those capable of causing CPE).

Host cells are grown in confluent healthy monolayers, usually in tubes or 96-well tissue culture grade plastic plates, to which aliquots of virus dilutions are added. The method becomes more accurate with increasing numbers of tubes or wells per dilution, but it is usual to use either 5 or 10 repetitions per dilution.

On incubation the virus replicates and progeny virions are released into the supernatant, these infecting healthy cells in the monolayer. The CPE is allowed to develop over a period of days (depending on the virus and cell type) at which time the cell monolayers are observed microscopically (they can be fixed and stained if necessary). Tubes are scored for the presence or absence of CPE.

It is thus a quantal assay in that each tube provides only one piece of information, i.e., is there CPE or not? The data is used to calculate the $TCID_{50}$ of the initial virus suspension by one of two ways – the Reed-Muench and the Spearman-Kärber methods (see below). The calculation does not tell us how many infectious units are present in the original virus suspension but what dilution of virus will give CPE in 50% of the cells inoculated.

Procedure

1. Seed tissue culture tubes/wells at a density of cells which will be confluent on the day of virus assay.
2. Make serial dilutions of virus suspension in appropriate diluent.
3. Remove tissue culture growth medium from healthy confluent monolayer and replace with appropriate dilution of virus. This would usually be 1 ml of virus dilution in a tissue culture tube and 0.1 ml in the well of a 96-well plate. Set up at least 5 tubes/wells per virus dilution.
4. Also include at least 5 control tubes/wells which contain diluent alone, i.e. no virus.
5. Incubate at appropriate temperature in either a closed or open incubator system and monitor the development of CPE. Record CPE after a designated time, having observed the cell control tubes/wells first.
6. CPE is usually graded on a 0–4 system; 0 (no CPE) 1 (less than 50% of cells showing CPE) 2 (about 50% of cells showing CPE) 3 (about 75% of cells showing CPE) 4 (the monolayer is totally destroyed or shows 100% CPE).
7. Calculate the $TCID_{50}$ counting all the tubes/wells with 1–4 CPE as being positive.

Table 2.1. Data used to calculate $TCID_{50}$ using Reed-Muench or Spearman-Kärber method

Log of virus dilution	*Infected test units*	*Cumulative infected (A)*	*Cumulative non-infected (B)*	*Ratio of A/(A + B)*	*Percent infected*
−5	5/5	9	0	9/9	100.0
−6	3/5	4	2	4/6	66.7
−7	1/5	1	6	1/7	14.3
−8	0/5	0	11	0/11	00.0

Calculation of $TCID_{50}$

The data shown in Table 2.1 will be used to demonstrate the calculation by either the Reed-Muench or Spearman-Kärber methods.

Reed-Muench method (Burleson et al 1992, Reed and Muench 1938)

The dilution in Table 2.1 that corresponds to the 50% end point obviously lies somewhere between the 10^{-6} (66.7% infected) and 10^{-7} (14.3% infected) dilutions. The proportionate distance between these two dilutions is calculated in the following manner:

$$\frac{(\%\text{ positive above }50\%) - 50\%}{(\%\text{ positive above }50\%) - (\%\text{ positive below }50\%)} = \text{Proportionate distance}$$

$$\text{i.e., } \frac{66.7\% - 50\%}{66.7\% - 14.3\%} = 0.3$$

Given that the log of the dilution above 50% is −6, the proportionate distance is 0.3 and the log of the dilution factor is −1 (i.e., serial 10 fold dilutions were used) the 50% end point is now calculated in the following way:

(log dilution above 50%) + (proportionate distance × log dilution factor) = log ID_{50}

$$(-6) + (0.3 \times -1.0) = -6.3$$

Therefore $ID_{50} = 10^{-6.3}$

This is the end point dilution, i.e., the dilution that will infect 50% of the test units inoculated.

The reciprocal of this number gives rise to the virus titer in terms of infectious doses per unit volume. If the inoculation of virus dilution was 0.1 ml the titer of the virus suspension would therefore be:

$$10^{6.3}\ TCID_{50}\ 0.1\ ml^{-1}$$
$$= 10 \times 10^{6.3}\ TCID\ ml^{-1}$$
$$= 10^{7.3}\ TCID_{50}\ ml^{-1}$$

Spearman Kärber method (Spearman, 1908; Kärber, 1931)

Again using the data from Table 2.1 the following formula is used to directly estimate the 50% end point:

Highest dilution giving 100% CPE $+\frac{1}{2}-\frac{\text{total number of test units showing CPE}}{\text{number of test units per dilution}} = TCID_{50}$

$$-5 + \tfrac{1}{2} - \tfrac{8}{5} = -6.3\ TCID_{50}$$

or $10^{-6.3}\ TCID_{50}$ unit volume^{-1}

The titer, given a volume of 0.1 ml, is therefore:

$$10^{6.3}\ TCID_{50}\ 0.1\ ml^{-1}$$
$$= 10 \times 10^{6.3}\ TCID\ ml^{-1}$$
$$= 10^{7.3}\ TCID_{50}\ ml^{-1}$$

The principle involved in the $TCID_{50}$ experiment is the same for either animal deaths (LD_{50}) or infection of a developing fertile hen's egg (EID_{50}).