

- 01/2008:50600 – To each flask, add 5 ml of trypsin solution heated at 37 °C (a trypsin stock solution contains 4 mg/ml of *trypsin R* and 4 mg/ml of *sodium edetate R*; immediately before use, dilute 50 times with phosphate buffered saline). Swirl the capped flask to wash the cell monolayer. Remove the excess of trypsin solution.

5.6. ASSAY OF INTERFERONS

The following chapter is published for information.

1. INTRODUCTION

Monographs on human interferons generally contain a bioassay based on the inhibitory activity of the interferon on the cytopathic action of a virus on a cell line in culture. In most cases, however, the virus, cell line and the assay details are not specified, in order to allow the appropriate flexibility, where the monograph covers more than one sub-class of interferon.

The present text is intended to provide outline information for the analyst on how to design, optimise and validate such an assay once an appropriate combination of cell line and cytopathic virus has been identified. A detailed procedure for a particular cytopathic antiviral assay is described as an example of a suitable method, together with information on other virus-cell line combinations and guidance on how to adapt and validate the procedure for these other combinations.

2. ANTIVIRAL (CYTOPATHIC EFFECT REDUCTION) ASSAYS

The antiviral assay of human interferons is based on the induction of a cellular response in human cells, which prevents or reduces the cytopathic effect of an infectious virus. The potency of interferon is estimated by comparing its protective effect against a viral cytopathic effect with the same effect of the appropriate reference preparation calibrated in International Units.

3. INTERFERON ASSAY USING Hep2c CELLS AND INFECTIOUS ENCEPHALOMYOCARDITIS VIRUS

The antiviral assay of human interferons described is of the cytopathic effect reduction type. It uses human Hep2c cells infected by encephalomyocarditis virus (EMCV) to measure the potencies of different human interferon test preparations. This assay has been used in three World Health Organisation (WHO) international collaborative studies of candidate International Standards for human interferon alpha, human interferon beta and human interferon gamma and has repeatedly been demonstrated to be sensitive, reliable and reproducible for potency estimations of the different types of human interferon.

For the culture of mammalian cells, all procedures are carried out using standard operating procedures for the maintenance of such cell lines in culture. Volumes of reagents are indicated for cell cultures carried out in 75 cm² flasks. Other types of containers (flasks or plates) may be used but volumes must be adapted accordingly.

3.1. MAINTENANCE AND PREPARATION OF Hep2c CELLS

Hep2c cells are maintained and passaged in culture medium A.

Cells are stored as frozen stocks using standard operating procedures. Growing cells may be maintained in culture up to a permitted passage number of 30, after which new cultures are established from frozen stocks.

At the beginning of the assay procedure, harvest the cells from the flasks showing 90 per cent confluent monolayers using the trypsin-treatment procedure described below.

- Remove the culture medium from the flasks.

- Incubate the flasks for 5 min to 10 min at 37 °C. Microscopically or visually observe the cells for signs of detachment. When viewed microscopically, the cells appear rounded up or detached and free-floating. Shake the flask vigorously to detach all the cells, add approximately 5 ml of culture medium A. Shake vigorously to yield a suspension of single cells.
- To prepare the cell suspensions for the assay procedure, carefully disperse the cells by pipetting up and down to disrupt cell aggregates, count the cells and resuspend at a concentration of 6×10^5 cells/ml.

3.2. PROPAGATION OF ENCEPHALOMYOCARDITIS VIRUS

Encephalomyocarditis virus is propagated in mouse L-929 cells in order to produce a stock of progeny virus. L-929 cells are maintained by trypsin treatment and passage as described for Hep2c cells (*NOTE: it may be necessary to substitute neonatal calf serum with foetal bovine serum if the cells show poor growth*).

Take several flasks containing confluent cultures of L-929 cells. Pour off the medium from the flasks. Inoculate with 2 ml of the EMCV suspension appropriately diluted in culture medium B so that it contains approximately 2.5×10^8 plaque forming units (PFU) per millilitre. Each flask will contain 4.6×10^7 L-929 cells and therefore the multiplicity of infection (m.o.i.) will be approximately 10 PFU/cell. Carefully swirl the virus suspension over the entire cell monolayer and return the flasks to the incubator for approximately 1 h. Maintain the medium at pH 7.4 to 7.8.

After adsorption of the EMCV, add approximately 40 ml of culture medium B to each flask and return the flasks to the incubator at 37 °C for about 30 h. Maintain the medium at pH 7.4 to 7.8 to obtain a maximum virus yield. Remove the culture fluid and store at approximately 4 °C.

Place the flasks at –20 °C to freeze the cell monolayer. Then thaw to room temperature. Add approximately 5 ml of culture medium and shake the flask to disrupt the cell walls. Transfer the contents of each flask to the container of culture fluid. Transfer the culture fluid containing the EMCV to 50 ml plastic centrifuge tubes and centrifuge at approximately 500 g for about 10 min to remove cell debris. Dispense the clarified culture fluid into glass screw-capped bottles, in quantities of 20 ml, 10 ml, 5 ml, 1 ml, 0.5 ml or 0.2 ml, as appropriate. Store at –70 °C. Larger volumes can be thawed, dispensed into smaller quantities and re-frozen when required. The EMCV stock will retain its original titre if stored permanently at approximately –70 °C, but repeated freeze-thaw cycles or storage at higher temperatures, e.g. at approximately –20 °C, results in progressive loss of titre.

3.3. ASSAY PROCEDURE

3.3.1. Determination of the dose-response range

Preparation of the solutions

Dilute the appropriate standard for interferon (for example a specific WHO sub-type interferon standard) in culture medium A, in 10-fold dose increments, to give doses covering the range of 1000 - 0.001 IU/ml. Carry out the assay procedure in 96-well microtitre plates. To each well add 100 µl of culture medium A. Add approximately 100 µl of each dilution of the reference preparation to each well except for those intended for virus controls. Using a multichannel pipette set at 100 µl, mix the contents of the wells.

Dispensing of the cell suspension

Pour the cell suspension of Hep2c cells, which has been adjusted to contain approximately 6×10^5 cells/ml of culture medium A, into a plastic Petri-dish. Dispense the cell suspension from the Petri-dish into each well of the microtitre plates, using a multichannel pipette set at 100 μ l.

Incubate the plates for about 24 h in an incubator set at 37 °C and 5 per cent CO₂.

Viral infection

At this stage, using an inverted microscope, check that the monolayers of Hep2c cells are confluent, that they show a relatively even distribution of cells, that they have correct morphology and that they are healthy.

Remove most of the culture medium from the wells by inverting the plate and shaking it and blotting on a paper towel (proceed in an identical way when discarding fluids from micro-titre plates as described later). Dilute the EMCV stock with fresh culture medium A to a titre of approximately 3×10^7 PFU/ml (*NOTE: each plate requires approximately 20 ml of diluted virus, plus 5 per cent to 10 per cent of extra volume*). Dispense the diluted suspension from a 9 cm sterile Petri-dish using a multichannel pipette set at 200 μ l to all wells including virus controls, but excluding cell controls. Add approximately 200 μ l of culture medium A without virus to each of the cell control wells.

Return the plates to the incubator set at 37 °C and 5 per cent CO₂ for approximately 24 h.

Staining

Examine the plates microscopically to check that the EMCV has caused a cytopathic effect (c.p.e.) in the virus controls. The time interval for maximum c.p.e. may vary from one assay to the next because of inherent variation of Hep2c cells to virus challenge over a given period of continuous cultivation.

Remove most of the culture medium from the wells by discarding into an appropriate decontaminating solution (sodium hypochlorite is suitable). Dispense *phosphate buffered saline pH 7.4 R* into each well. Discard the *phosphate buffered saline pH 7.4 R* into a decontaminating solution. Dispense into each well 150 μ l of staining solution. Stain the cells for approximately 30 min at room temperature. Discard the staining solution into a decontaminating solution. Dispense approximately 150 μ l of fixing solution. Fix for 10 min at room temperature. Discard the fixing solution into a decontaminating solution and wash the cell monolayers by immersing the assay plates in a plastic box containing running water. Discard the water and superficially dry the plates with paper towels. Dry the assay plates at 20 °C to 37 °C until all moisture has evaporated.

Add 150 μ l of 0.1 M sodium hydroxide to each well. Elute the stain by gentle agitation of the plates or by hitting them against the palm of the hand. Make sure that the stain is evenly distributed in all wells before making spectrophotometric readings.

Read the absorbance at 610 nm to 620 nm, using a microtitre plate reader, taking as a blank a well or a column of wells containing no cells and approximately 150 μ l of 0.1 M sodium hydroxide.

Estimate the concentrations of interferon standard that give the maximum and minimum reduction of cytopathic effect. This is the dose response corresponding to the working range of the assay.

3.3.2. Assay procedure

Carry out the assay as described above, using:

- as test solutions, the substance to be examined, diluted in two-fold increments with culture medium A to give nominal concentrations covering the working range of the assay,
- as reference solutions, the appropriate standard for interferon (for example, a specific WHO sub-type interferon) in culture medium A, diluted in two-fold increments to give nominal concentrations covering the working range of the assay.

3.3.3. Data analysis

Results of the cytopathic effect reduction assay generally fit a sigmoidal dose-response curve, when the interferon concentration (the log of the reciprocal of the interferon dilution) is plotted versus stain absorbance.

Plot the interferon concentration (log reciprocal of dilution) versus the stain absorbance for the interferon reference preparation and for the interferon test solutions. Using the linear portion of the curve, calculate the concentration of interferon in the sample by comparing the responses for test and reference solutions, using the usual statistical methods for a parallel line assay.

4. VALIDATION OF OTHER PROCEDURES**4.1. CHOICE OF CELL LINE AND VIRUS**

A number of other combinations of cell line and virus have been used in anti-viral assays for interferons. For example, EMCV has been used in combination with the A549 epithelial lung carcinoma cell line, Semliki Forest virus or Sindbis virus have been used with human fibroblasts, and vesicular stomatitis virus has been used with either human diploid fibroblasts, the human amnion WISH cell line or the Madin-Darby bovine kidney cell line. In each case the choice of the cell line/virus combination is usually based on that which gives the most sensitive response to the interferon preparation to be assayed, and gives parallel responses when comparing the test preparation and interferon standard.

4.2. CHOICE OF RESPONSE

The staining procedure described above measures remaining viable cells. A number of other responses have been used, including methyl violet or crystal violet staining, or the thiazolyl blue (MTT) conversion procedure. In each case, the method is selected on the basis of producing a suitably linear and sensitive relationship between response colour and viable cell count.

4.3. STATISTICAL VALIDATION

As with all parallel line bioassays, the assay must satisfy the usual statistical criteria of linearity of response, parallelism and variance.

4.4. VALIDATION OF ASSAY LAYOUT

As with all microtitre plate assay procedures, attention must be given to validating the assay layout. In particular, bias due to non-random pipetting order or plate edge effects must be investigated and eliminated, by randomising the assay layout, or by avoiding the use of edge wells.

REAGENTS AND CULTURE MEDIA

Culture medium A (10 per cent neonatal calf serum)

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|---|--------|
| RPMI 1640 culture medium, supplemented with antibiotics if necessary (penicillin 10 000 IU/ml; streptomycin 10 ng/ml) | 450 ml |
| L-Glutamine, 200 mM, sterile | 5 ml |
| Neonatal calf serum | 50 ml |

Culture medium B (2 per cent foetal bovine serum)

| | |
|---|--------|
| RPMI 1640 culture medium, supplemented with antibiotics if necessary (penicillin 10 000 IU/ml; streptomycin 10 ng/ml) | 490 ml |
| L-Glutamine, 200 mM, sterile | 5 ml |
| Foetal bovine serum | 10 ml |

Staining solution

| | |
|---------------------------|---------|
| Naphthalene black | 0.5 g |
| Acetic acid, glacial | 90 ml |
| Sodium acetate, anhydrous | 8.2 g |
| Water to produce | 1000 ml |

Fixing solution

| | |
|---------------------------|---------|
| Formaldehyde, 40 per cent | 100 ml |
| Acetic acid, glacial | 90 ml |
| Sodium acetate, anhydrous | 8.2 g |
| Water to produce | 1000 ml |