VIRIDIS BioPharma

Report prepared for
American Biotech Laboratory

Viricidal Activity of ASAP
against Hepatitis B Virus
&
Cytotoxicity of ASAP
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4 Test Procedure for Cytotoxicity

Cytotoxicity:-

Cytotoxicity test was carried out to evaluate toxic effects of ASAP in an *in vitro* system using two models Vero cells (African Green Monkey Cell line) and Hep2 (Human epithelial cell)

4.1 Aim

This Assay Method describes an *in vitro* test method for assaying cytotoxicity of ASAP solution.

4.2 Principle

Two freshly prepared cell cultures; Vero & Hep2 were inoculated with ASAP solutions and incubated under CO_2 environment. Cytopathic effects, if any are observed under inverted microscope. Cell culture with the addition of Phosphate Buffer Saline (PBS) served as reference control.

4.3 Equipment

1. Incubator having 5% CO_2 environment
2. Inverted microscope
3. Syringe
4. Microtitre wells
5. Autoclave

4.4 Materials

1. Fetal Calf serum
2. Vero cell line (African Green Monkey Cell line)
3. Hep2 (Human epithelial cell)
4. Minimum essential medium

   9.61 g MEM 16 with Earle's salts

   2.2 g sodium bicarbonate ($NaHCO_3$)17

   Dissolve reagents in above two materials in 900 ml deionized water (DW).

   Add 5.0 g lactalbumin hydrolysate or edamin 18 to 10 ml DW, and heat to 60° ± 2°C until dissolved. Add to above 900ml solution with constant mixing.

4.5 Procedure

Cells are prepared from healthy, confluent Vero cells and Hep2 cells that are maintained by passing every 3 to 4 days. One day prior to test initiation, using a self-refilling repetitive syringe, cells suspended in Growth Medium are dispensed into wells. Incubate at 37° ± 2°C in a 5% CO_2 incubator for 72 ± 12 hr.

100 µl of each substance to be tested was introduced into wells in triplicates. 100µl of PBS served as positive control. The cell lines were reincubated at 37° ± 2°C in a 5% CO_2 incubator for 72 ± 12 hr. Every 24 hrs wells were examined under high power of an inverted microscop to check for cytopathic effect (CPE).

Schematic Representation

Cultivate the susceptible cell lines (Vero, Hep2) in Minimum essential medium containing 10% Fetal Calf serum

Monolayer of a susceptible cell line

Inoculate 0.1 ml of the sample in the required number of wells.

Inoculate sterile 0.1 ml PBS into one of the wells as control

Incubate at 37^0C, under 5% CO_2 for 72 hrs

Observre under high power for cytotoxicity (Cytopathic effect)

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Cytotoxicity Test Results

Sample Source American Biotech Lab

Sample Description ASAP 10 ppm (Lot # 02198)
ASAP 14 ppm + 1.5% H_2O_2 (No lot #)
ASAP 22 ppm (Lot # 02193)

Date of Analysis 5th May 2003

Sample	***Vero cell line***	***Hep2 cell line***
ASAP-10	**No CPE**	**No CPE**
ASAP-14 + 1.5% H_2O_2	**CPE - positive**	**CPE - positive**
ASAP-22	**No CPE**	**No CPE**
PBS; (Control)	**No CPE**	**No CPE**

CPE - Cytopathic Effect

Quality Assurance

4.6 Conclusion:

Distinct cytopathic effect were observed in cell lines with ASAP-14. In fact, on addition of 100µl to the wells total bleaching of the vital indicator occurred. Cytopathic effects were noted as follows:

1. Rounding of cells
2. Granulation of cell cytoplasm
3. Detachment of cell monolayer from well surface.

The above can be easily seen in the attached photomicrographs (Pg. 22 & 23).
Cell lines treated with ASAP-10 & 22 were indistinguishable from the control indicating no cytotoxicity. ASAP 14 ppm displayed cytotoxicity which is likely due to H_2O_2.