Study Report

Virucidal Activity of Test Sample as per ASTM E1052 - 20: Standard Practice to Assess the Activity of Microbicides against Viruses in Suspension

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Test Items	Toothpaste
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1.0. Objective

This study is designed to measure the virucidal effectiveness of a test substance and determine its potential to inactivate the target virus, Influenza-A (H1N1), in suspension. The test follows the ASTM international test method designated E1052- "Standard Test Method to Assess the Activity of Microbicides against Viruses in Suspension."

2.0. Study Plan Summary

Table 1: Virucidal Assay Study Summary

S. No	Method	Virucidal Assay
1	Test Virus	Influenza-A (A/PR/8/34 (TC adapted)
2	Test Item	Toothpaste
3	Study Method	ASTM E1052
4	Cell Line	MDCK
5	Testing Concentration	1:80 dilution
6	Contact Time	20 seconds, 30 seconds, and 3 minutes
7	Temperature	37°C
8	Diluent	Sterile Water

3.0. Test Item Details

Table 2: Test Item

S. No	Field	Details
1	Test Item Name	Toothpaste
2	Batch Number	N/A
3	Manufacturing date	N/A
4	Expiry date	N/A

4.0. Materials

Table 3: Materials

S. No.	Item	Make	Catalogue Number
1	Antibiotic Antimycotic solution	Sigma Aldrich	A5955
2	Cell Culture Plate, 96-Well	Eppendorf	0030730011
3	Crystal violet solution	Sigma Aldrich	V5265
4	Dimethyl sulfoxide (DMSO)	Sigma Aldrich	D4540
5	Eagle's Minimum Essential Medium (EMEM) (with glutamine)	Sigma Aldrich	M4655
6	Fetal Bovine Serum (FBS)	Sigma Aldrich	F4135
7	Bovine Serum Albumin (Fraction V)	HiMedia	TC194
8	Formaldehyde solution (36 % in water)	Sigma Aldrich	47608
9	Ethanol (Cas No. 64-17-5)	Sigma Aldrich	277649
10	Phosphate Buffered Saline	Thermo scientific/Gibco	70011044
11	Influenza-A (A/PR/8/34 (TC adapted)	ATCC	VR-1469
12	Tissue Culture Grade Water (ultrapure distilled)	HiMedia	TCL019
13	TPCK-treated Trypsin	Sigma-Aldrich	T1426
14	MDCK	ATCC	CCL-34

5.0. Assay Procedures

5.1. Determination of the non-cytotoxic concentration of the Test Item

1. Test Item at 1:80 dilution (w/v) was prepared in sterile water and used in the assay.
2. The Test Item (90 µL) and EMEM (10 µL) were mixed and incubated at 37^0C for 20 sec, 30 sec, and 3 minutes.
3. Following the contact period, the test Item mixture was subjected to 1/10 dilution in infection medium (EMEM) and transferred 50 µL of each dilution onto 4 wells of a 96-well plate with 80-100% confluent cells.

Infection Medium: EMEM (high glucose) supplemented with 0.125% Bovine Serum Albumin (BSA) Fraction V, TPCK-treated Trypsin (1 µg/mL), and Antibiotic Antimycotic solution.

4. The plate was then incubated at 37° C in a CO_2 incubator (5%) for 1 hour.
5. Upon completion of incubation, the plate was supplemented with 150 µL test media (EMEM) and incubated at 37° C in a CO_2 incubator (5%) for 3 days.
6. After 3 days of incubation, the plate was fixed in 4% paraformaldehyde and stained with crystal violet to determine the test item's non-cytotoxic concentration.

5.2. Evaluation of the virucidal activity of the Test Item against Influenza-A (H1N1)

1. Test Item at 1:80 dilution was used in the assay.
2. Infection medium containing virus (Influenza-A, ATCC, USA) was prepared at approximately 106 TCID50/mL.

   Infection Medium: EMEM (high glucose) supplemented with 0.125% Bovine Serum Albumin (BSA) Fraction V, TPCK-treated Trypsin (1 µg/mL), and Antibiotic Antimycotic solution.
7. The Test Item (90 µL) and virus (10 µL) were mixed and incubated at 37^0 C for 20 sec, 30 sec, and 3 minutes.
3. Following the contact period, the Test Item-virus mixture was ten-fold diluted (1/10) in the Infection Medium.
4. The virus load in the Test Item-treated samples and controls was determined by a standard end-point dilution assay.
5. Virus-only control (without Test Item), cytotoxicity control (only Test Item), positive control (75 % Ethanol), neutralization control* and cell-only control (without virus) were maintained during the assay.

   *To validate the neutralization, add equal volumes of the neutralized test substance, the neutralizer (EMEM) alone, and the infective units of the test virus. Hold the mixtures for the exposure time at room temperature.

5.3. Standard end-point dilution assay and calculation of $TCID_{50}$/mL

1. Approximately 30,000 MDCK cells in a volume of 200 µL/well in EMEM (containing 10 % FBS) were plated into 96-well plates and incubated overnight (12–18 h) at 37° C.
2. Ten-fold serial dilutions of the test-material virus mixture and all the controls were prepared after incubation. The 50 µL of each dilution was transferred to 4 wells of a 96-well plate with 80-100% confluent cells.
3. The plate was then incubated at 37° C in a CO_2 incubator (5%) for 1 hour.
4. Upon completion of incubation, the plate was supplemented with 150 µL test media (EMEM) and incubated at 37° C in a CO_2 incubator (5%) for 3 days.
5. After 3 days of incubation, the plate was fixed in 4% paraformaldehyde and stained with crystal violet to determine the activity of the test item.
6. After fixation and staining, the plate was scored for the presence or absence of viral cytopathic effect (CPE).
7. The end-point titers (50% tissue culture infectious dose, $TCID_{50}$) of the samples were determined by the Spearman-Karber method.
8. The log reduction value (LRV) of the test item was determined compared to the virus-only control.
9. The theoretical Limit of detection (LOD)-Indicates the lowest possible detection of virus load by the assay - which is 0.7 Log_{10} $TCID_{50}$/mL
10. Log reduction value (LRV) was calculated using the below-mentioned relevant formula.
    a. For the Test Item without cytotoxicity and no CPE at 10^{-1} dilution,

       LRV= Log_{10} Virus titer in control - Log_{10} LOD of the test.

    b. For Test Items with CPE at 10^{-1} dilution and beyond,

       LRV= Log_{10} Virus titer in control – Log_{10} Virus titer in the test item.

    C. For the Test Item with cytotoxicity and no CPE beyond the cytotoxic concentration

       LRV= Log_{10} Virus titer in control – Log_{10} highest cytotoxic concentration - LOD of the test.
11. LRV reflects the Virucidal activity of the test item.

6.0. Results

Table 4: Cytotoxicity control well scoring for the test item

Log_{10} test item dilution	Cytotoxicity/non-cytotoxicity				Total no. of wells	Total no. of cytotoxic wells	Percentage (%) cytotoxicity
-1	NC	NC	NC	NC	4	0	0%
-2	NC	NC	NC	NC	4	0	0%
-3	NC	NC	NC	NC	4	0	0%
-4	NC	NC	NC	NC	4	0	0%
-5	NC	NC	NC	NC	4	0	0%
-6	NC	NC	NC	NC	4	0	0%

Key: CT-= cytotoxicity observed; NC = No cytotoxicity observed

Table 5: Cytotoxicity control well scoring for Positive Control

Log_{10} test item dilution	Cytotoxicity/non-cytotoxicity				Total no. of wells	Total no. of cytotoxic wells	Percentage (%) cytotoxicity
-1	NC	NC	NC	NC	4	0	0%
-2	NC	NC	NC	NC	4	0	0%
-3	NC	NC	NC	NC	4	0	0%
-4	NC	NC	NC	NC	4	0	0%
-5	NC	NC	NC	NC	4	0	0%
-6	NC	NC	NC	NC	4	0	0%

Key: CT-= cytotoxicity observed; NC = No cytotoxicity observed

Table 6: Log_{10} Reduction Values for Influenza-A (H1N1) testing

Sl. No.	Test item	Concentration	Contact Time	Log_{10} Reduction Value (LRV)	Equivalent Percent Kill
1	Virus only control	N/A	N/A	N/A	N/A
2	Cell only control	N/A	N/A	N/A	N/A
3	Ethanol (Positive control)	75%	20 sec	5.85	99.9999%
4	Ethanol (Positive control)	75%	30 sec	5.85	99.9999%
5	Ethanol (Positive control)	75%	3 min	5.85	99.9999%

Sl. No.	Test item	Concentration	Contact Time	Log_{10} Reduction Value (LRV)	Equivalent Percent Kill
6	Toothpaste (1:80 dilution)	**1:80**	**20 sec**	0.00	0%
7	Toothpaste (1:80 dilution)	**1:80**	**30 sec**	0.00	0%
8	Toothpaste (1:80 dilution)	**1:80**	**3 min**	1.75	98.22%

Table 7: Summary Results

S. No.	Compound	Sample Type	Concentration	Contact Time	Highest Non-cytotoxic concentration (Log_{10})	Cytotoxic concentration (Log_{10})	Test item VT (Log_{10} $TCID_{50}$/mL) or LOD	Neutralization control	VC Titer (Log_{10} $TCID_{50}$/mL)	Log_{10} Reduction Value LRV	Equivalent Percent Kill
1	Virus only control	Virus only control	N/A	N/A	N/A	N/A	N/A	N/A	6.55	N/A	N/A
2	Cell only	Cell only control	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
3	Ethanol (75%)	Positive control	75%	20 Seconds	1	N/A	0.70	None	N/A	5.85	99.9999%
4	Ethanol (75%)	Positive control	75%	30 Seconds	1	N/A	0.70	None	N/A	5.85	99.9999%
5	Ethanol (75%)	Positive control	75%	3 minutes	1	N/A	0.70	None	N/A	5.85	99.9999%
6	**Toothpaste**	**Test Item**	**1:80 dilution**	**20 Seconds**	**1**	**N/A**	**N/A**	**Pass**	**6.55**	**0.00**	**0.000%**
7	**Toothpaste**	**Test Item**	**1:80 dilution**	**30 Seconds**	**1**	**N/A**	**N/A**	**Pass**	**6.55**	**0.00**	**0.000%**
8	**Toothpaste**	**Test Item**	**1:80 dilution**	**3 minutes**	**1**	**N/A**	**N/A**	**Pass**	**4.80**	**1.75**	**98.22%**

KEY

Test item VT	Virus titer (VT)- virus titer of the test sample in log_{10} $TCID_{50}$ per mL
VC	Virus control-Virus titer of control (stock) used in the assay in log_{10} $TCID_{50}$ per mL
LRV	Log Reduction Value- reduction of virus in the test sample compared to the virus control in log_{10} $TCID_{50}$ per mL
Cytotoxicity	Indicates the dilution where 50-100% cytotoxicity was observed
LOD	Limit of detection (LOD)-Indicates the lowest possible detection of virus load by the assay - which is 5 $TCID_{50}$/mL or Log_{10} 0.7 $TCID_{50}$/mL
Passed	Comparable infectious virus particles were recovered from the neutralization control

7. Raw Data

7.1. Spearman-Karber Method Formula

Calculation of virus titer using the Spearman-Karber method:

$$\text{Log}_{10}\,50\%\ \text{endpoint dilution} = -(x_0 - d/2 + d\,\Sigma\, r_i/n_i)$$

x_0 = $\log_{10}$ of the reciprocal of the highest dilution (lowest concentration) at which all wells are positive.

d = $\log_{10}$ of the dilution factor.

n_i = number of wells used in each individual dilution

r_i = number of positive wells (out of n_i). Summation is started at dilution x_0.

7.2. Calculations

7.2.1 Virus Control

Table 8: Calculation of Virus Titer - Virus Control

Log_{10} Virus dilution	**Positive/Negative wells**				**Total no. of wells**	**No. of positive wells**
-1	+	+	+	+	4	4
-2	+	+	+	+	4	4
-3	+	+	+	+	4	4
-4	+	+	+	+	4	4
-5	+	-	+	+	4	3
-6	-	-	-	-	4	0

Key: + = Virus recovered; - = Virus not recovered

x_0=4; d=1; n_i=4; r_i=7

Log_{10} of 50% = - [4 - ½ + 1 (7/4)] = -5.25

Virus titer per 50 µL= $10^{5.25}$

Antilog of Virus titer per 50 µL (5.25) = 177827.94

Virus titer per mL= 3556558.82

Log_{10} Virus titer per mL= 6.55

7.2.2 Positive Control

Table 9: Calculation of Virus Titer – Positive Control

Log_{10} Virus dilution	**Positive/Negative wells**				**Total no. of wells**	**No. of positive wells**
-1	-	-	-	-	4	0
-2	-	-	-	-	4	0
-3	-	-	-	-	4	0
-4	-	-	-	-	4	0
-5	-	-	-	-	4	0
-6	-	-	-	-	4	0

Key: + = Virus recovered; - = Virus not recovered

x_0=0; d=1; n_i=4; r_i=0

Log_{10} of 50% = - [0 - ½ + 1(0 /4)] = ND

Virus titer per 50 µL= ND

Antilog of Virus titer per 50 µL = ND

Virus titer per mL= ND

Log_{10} Virus titer per mL= ND

[ND = Not Determined]

For samples where no cytotoxicity is observed in any well and no CPE is seen at the 10^{-1} dilution, the following formula is used:

Log_{10} Reduction Value (LRV) = [(Log_{10} $TCID_{50}$/mL (Virus Control) - Log_{10} $TCID_{50}$/mL (Limit of Detection)]

Log_{10} Reduction Value (LRV) of Positive Control = [(6.55) -(0.70)] = 5.85

7.2.3 Test Item

Table 10: Calculation of Virus Titer – Test Item (20 seconds)

Log_{10} Virus dilution	**Positive/Negative wells**				**Total no. of wells**	**No. of positive wells**
-1	+	+	+	+	4	4
-2	+	+	+	+	4	4
-3	+	+	+	+	4	4
-4	+	+	+	+	4	4
-5	+	+	+	+	4	4

Log_{10} Virus dilution	Positive/Negative wells				Total no. of wells	No. of positive wells
-6	-	-	-	-	4	0

Key: + = Virus recovered; - = Virus not recovered, CT=Cytotoxicity

x_0=4; d=1; n_i=4; r_i=7

Log_{10} of 50% = - [4 - ½ + 1(7/4)] = -5.25

Virus titer per 50 µL= $10^{5.25}$

Antilog of Virus titer per 50 µL = 177827.94

Virus titer per mL= 3556558.82

Log_{10} Virus titer per mL= 6.55

For samples where cytotoxicity is not observed, and CPE is seen, the following formula is used:

Log_{10} Reduction Value (LRV) = [(Log_{10} $TCID_{50}$/mL (Virus Control) - Log_{10} $TCID_{50}$/mL (test item)

Log_{10} Reduction Value (LRV) of Test Item = [(6.55) -(6.55)]**= 0.0**

Table 11: Calculation of Virus Titer – Test Item (30 seconds)

Log_{10} Virus dilution	Positive/Negative wells				Total no. of wells	No. of positive wells
-1	+	+	+	+	4	4
-2	+	+	+	+	4	4
-3	+	+	+	+	4	4
-4	+	+	+	+	4	4
-5	+	+	+	+	4	4
-6	-	-	-	-	4	0

Key: + = Virus recovered; - = Virus not recovered, CT=Cytotoxicity

x_0=4; d=1; n_i=4; r_i=7

Log_{10} of 50% = - [4 - ½ + 1(7/4)] = -5.25

Virus titer per 50 µL= $10^{5.25}$

Antilog of Virus titer per 50 µL = 177827.94

Virus titer per mL= 3556558.82

Log_{10} Virus titer per mL= 6.55

For samples where cytotoxicity is not observed, and CPE is seen, the following formula is used:

Log_{10} Reduction Value (LRV) = [(Log_{10} $TCID_{50}$/mL (Virus Control) - Log_{10} $TCID_{50}$/mL (test item)

Log_{10} Reduction Value (LRV) of Test Item = [(6.55) -(6.55)]**= 0.0**

Table 12: Calculation of Virus Titer – Test Item (3 minutes)

Log_{10} Virus dilution	**Positive/Negative wells**				**Total no. of wells**	**No. of positive wells**
-1	+	+	+	+	4	4
-2	+	+	+	+	4	4
-3	+	+	+	+	4	4
-4	-	-	-	-	4	0
-5	-	-	-	-	4	0
-6	-	-	-	-	4	0

Key: + = Virus recovered; - = Virus not recovered, CT=Cytotoxicity

x_0=3; d=1; n_i=4; r_i=4

Log_{10} of 50% = - [3 - ½ + 1(4/4)] = -3.5

Virus titer per 50 µL= $10^{3.5}$

Antilog of Virus titer per 50 µL = 3162.28

Virus titer per mL= 63245.55

Log_{10} Virus titer per mL= 4.80

For samples where cytotoxicity is not observed, and CPE is seen, the following formula is used:

Log_{10} Reduction Value (LRV) = [(Log_{10} $TCID_{50}$/mL (Virus Control) - Log_{10} $TCID_{50}$/mL (test item)

Log_{10} Reduction Value (LRV) of Test Item = [(6.55) -(4.80)]**= 1.75**

8.0. Conclusion

1. The virus recovery titer was 6.55 log_{10} $TCID_{50}$/mL.
2. **Toothpaste (1:80 dilution)** at 20- and 30-second exposure showed a log_{10} Reduction of 0.
3. **Toothpaste (1:80 dilution)** at 3 minutes exposure showed a log_{10} Reduction of 1.75, equivalent to 98.22% against Influenza-A (H1N1) virus.

Test item	**Contact Time**	**Log_{10} Reduction Value (LRV)**	**Equivalent Percent Kill**
Toothpaste (1:80 dilution) (Test Item)	20 Seconds	0	0%

Test item	Contact Time	Log_{10} Reduction Value (LRV)	Equivalent Percent Kill
Toothpaste (1:80 dilution) (Test Item)	30 Seconds	0	0%
Toothpaste (1:80 dilution) (Test Item)	3 minutes	1.75	98.22%

9.0. Summary

This study evaluated the virucidal effectiveness of a 1:80 toothpaste dilution against Influenza-A (H1N1) using the ASTM E1052 method to assess the activity of microbicides against viruses in suspension. The virus recovery titer was 6.55 log_{10} $TCID_{50}$/mL. The test item (toothpaste) showed no reduction in viral titer after 20- or 30-second exposure. However, a 3-minute exposure achieved a log_{10} Reduction of 1.75, equivalent to a 98.22% against Influenza-A (H1N1) virus. These findings demonstrate that the test item (toothpaste) exhibits measurable virucidal activity with extended contact time against Influenza-A (H1N1) virus.

10.0. References

1. ASTM E1052-20, Standard Practice to Assess the Activity of Microbicides against Viruses in Suspension, ASTM International, West Conshohocken, PA, 2020, www.astm.org
2. Fadilah, N. Q., Jittmittraphap, A., Leaungwutiwong, P., Pripdeevech, P., Dhanushka, D., Mahidol, C., ... & Kittakoop, P. (2022). Virucidal Activity of Essential Oils From Citrus x aurantium L. Against Influenza A Virus H 1 N 1: Limonene as a Potential Household Disinfectant Against Virus. Natural Product Communications, 17(1), 1934578X211072713.
3. Ghaffari, H., Tavakoli, A., Moradi, A., Tabarraei, A., Bokharaei-Salim, F., Zahmatkeshan, M., & Ataei-Pirkooh, A. (2019). Inhibition of H1N1 influenza virus infection by zinc oxide nanoparticles: another emerging application of nanomedicine. Journal of biomedical science, 26(1), 1-10.

11.0. Annexures

Below are the virucidal plate images, which are summarised as follows,

1. Virus-only control and test item at 20- and 30-second incubation showed positive wells up to 10^{-5} dilution (Figure 1 and Figure 2).
2. The Test item at 3 minutes of incubation showed positive wells up to 10^{-3} dilution (Figure 3).
3. Cytotoxicity and cell-only controls showed no change/positive wells (Figure 4).

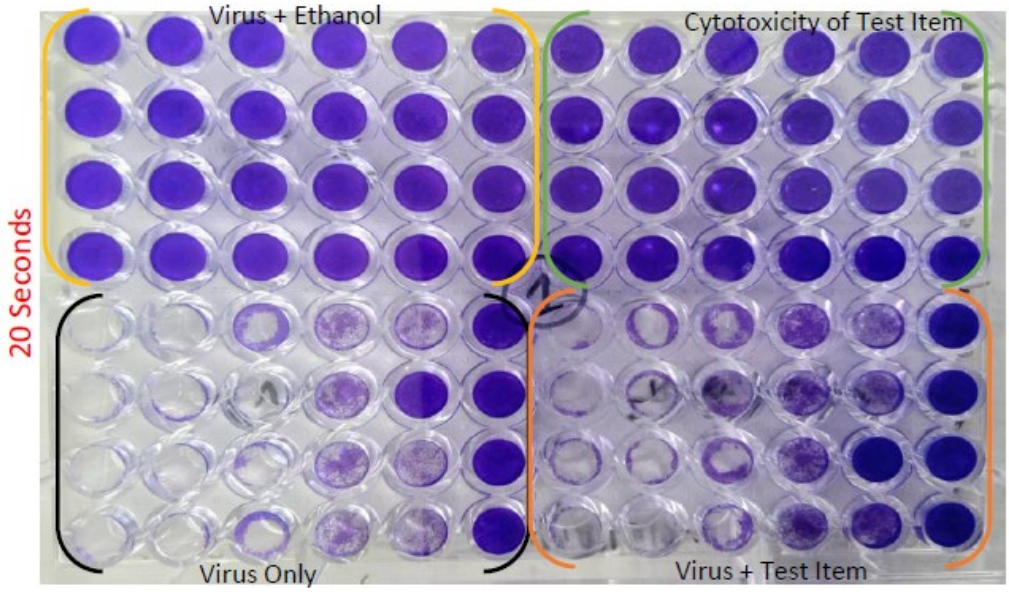

Figure 1: Plate map: Virus + Ethanol; Cytotoxicity of test item; Virus-only control and virus + test item at 20 seconds

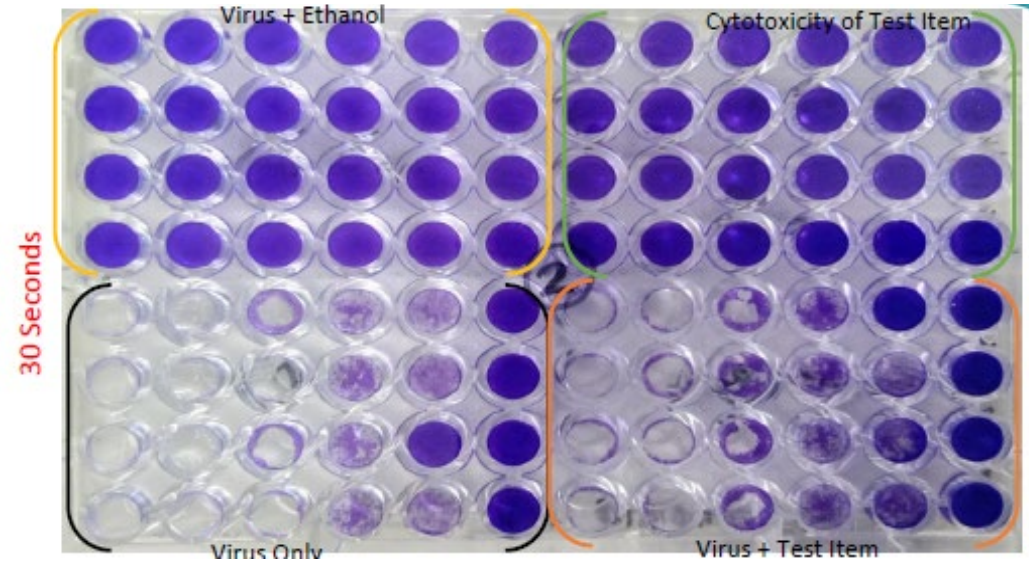

Figure 2: Plate map: Plate map: Virus + Ethanol; Cytotoxicity of test item; Virus-only control and virus + test item at 30 seconds

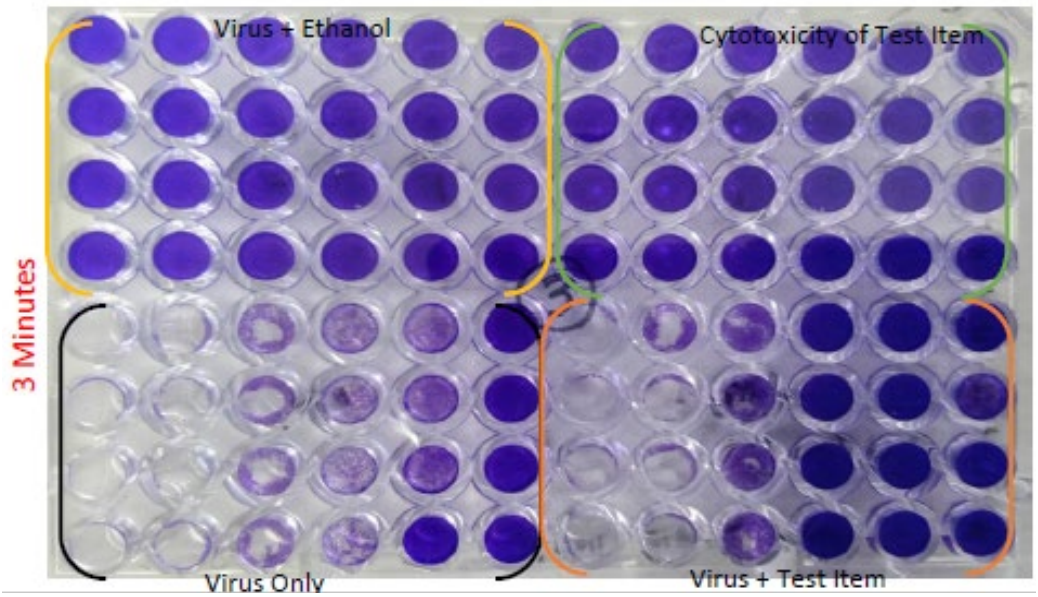

Figure 3: Plate map: Virus + Ethanol; Cytotoxicity of test item; Virus-only control and virus + test item at 3 minutes

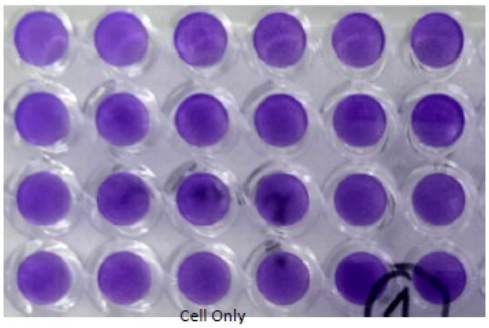

Figure 4: Cell only control