SIRIM Berhad
Industrial Biotechnology Research Centre, Building 19
Tel: 03-55446953/6960
Fax:03-55446988

STUDY REPORT

EVALUATION OF NANO COLLOIDAL ARGENTUM IN THE BALB/c 3T3 NRU CYTOTOXICITY STUDY

Study No. J205/13

Report No. R205/13/B19/03

Sponsor:
Trumer Medicare Sdn Bhd.
No. 16-2, Jalan SS19/1G,
47500 Subang Jaya,
Selangor.

Sponsor Representative:
Mr. KC Yap

Test Facility:
Industrial Biotechnology Research Centre (IBRC),
Building 19, SIRIM Berhad.

Study Initiation Date:
21 June 2013

Experimental Start Date:
24 September 2013

Experimental End Date:
04 October 2013

Study Completion Date:
07 October 2013

SIRIM Berhad
(No. Syarikat 367474 - V)
1, Persiaran Dato' Menteri
Seksyen 2, Peti Surat 7035
40700 Shah Alam
MALAYSIA
Tel: 60-3-55446000
Hotline: 60-3-55103535
Faks: 60-3-55108095
Website : www.sirim.my

SIRIM Berhad
Industrial Biotechnology Research Centre, Building 19
Tel: 03-55446953/6960
Fax:03-55446988

STUDY REPORT

EVALUATION OF NANO COLLOIDAL ARGENTUM IN THE BALB/c 3T3 NRU CYTOTOXICITY STUDY

Study No. J205/13

Report No. R205/13/B19/03

Sponsor:
Trumer Medicare Sdn Bhd.
No. 16-2, Jalan SS19/1G,
47500 Subang Jaya,
Selangor.

Sponsor Representative:
Mr. KC Yap

Test Facility:
Industrial Biotechnology Research Centre (IBRC),
Building 19, SIRIM Berhad.

Study Initiation Date:
21 June 2013

Experimental Start Date:
24 September 2013

Experimental End Date:
04 October 2013

Study Completion Date:
07 October 2013

SIRIM Berhad
(No. Syarikat 367474 - V)
1, Persiaran Dato' Menteri
Seksyen 2, Peti Surat 7035
40700 Shah Alam
MALAYSIA
Tel: 60-3-55446000
Hotline: 60-3-55103535
Faks: 60-3-55108095
Website : www.sirim.my

STUDY REPORT

REPORT NO: R205/13/B19/03	PAGE: 2 of 13
This report is NOT a Quality Assurance Certificate NOR an Approval Permit. This report refers only to test item submitted by the customer to SIRIM Berhad and tested by SIRIM Berhad. This report shall not be reproduced, except in full and shall not be used for advertising purposes by any means or forms without written approval from President & Chief Executive of SIRIM Berhad.	

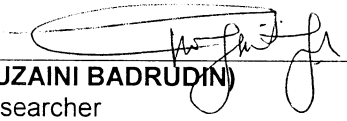
KEY PERSONNEL PARTICIPATING IN THIS STUDY

We, the undersigned, declare that the methods, results and data contained in this report faithfully reflect the procedures used and raw data collected throughout the study.

07 OCT 2013

(DR. NEELAM SHAHAB, *AMIC*)
Senior Principal Researcher
Industrial Biotechnology Research Centre

Date



07 OCT 2013

(SUZAINI BADRUDIN)
Researcher
Industrial Biotechnology Research Centre

Date

07 OCT 2013

(ANA SHARMILA SHAFIE)
Senior Assistant Researcher
Industrial Biotechnology Research Centre

Date

STUDY REPORT

SUMMARY

EVALUATION OF NANO COLLOIDAL IN THE BALB/c 3T3 NRU CYTOTOXICITY STUDY

The cytotoxicity potential of Nano Colloidal Argentum was determined in BALB/c 3T3 NRU cytotoxicity test according to Guidance Document on Using Cytotoxicity Tests to Estimate Starting Doses for Acute Oral Systemic Toxicity Tests – Environmental Health and Safety Monograph Series on Testing and Assessment No 129. Results from this study provide an estimate starting dose of Nano Colloidal Argentum for acute oral systemic toxicity test.

Nano Colloidal Argentum was soluble in Chemical Dilution Medium (CDM) at the highest stock concentration of 200,000 μg/mL. In this cytotoxicity study, BALB/c 3T3 cell line was treated with the extract at eight varying concentrations ranging from 0.02 μg/mL to 100,000 μg/mL. Cell viability was assessed using the Neutral Red Uptake method after a 48-hour treatment.

Results showed that the median inhibition concentration (IC_{50}) is between 10,000 μg/mL to 100,000 μg/mL. The calculated median lethal dose (LD50) would have been in the range of 3,251 mg/kg to 7,656 mg/kg. This value is beyond the intended limit test for acute oral toxicity which is 2,000 mg/kg.

In conclusion, the proposed starting dose of Nano Colloidal Argentum for acute oral toxicity test (Up-and-Down Procedure) is 2,000 mg/kg.

SUZAINI BINTI BADRUDIN
Researcher
Bioprocess Programme
Industrial Biotechnology Research Centre
SIRIM Berhad

STUDY REPORT

REPORT NO: R205/13/B19/03	PAGE: 4 of 13

CONTENTS

KEY PERSONNEL PARTICIPATING IN THIS STUDY 2

SUMMARY 3

CONTENTS 4

1.0 BACKGROUND 5

2.0 OBJECTIVE 5

3.0 TEST SYSTEM 5

4.0 MATERIAL
4.1 **Test Item** 5
4.2 **Cell Line** 6
4.3 **Reagents** 6
4.4 **Controls** 6

5.0 METHOD
5.1 **Maintenance and Preparation of Cell Line** 7
5.2 **Solubility Determination** 7
5.3 **Range Finder Test** 7
5.4 **Main Test** 7
5.5 **Controls** 8
5.6 **Neutral Red Uptake Assay** 8
5.7 **Interpretation of Result** 8

6.0 RESULT AND DISCUSSION
6.1 **Solubility Determination** 9
6.2 **Range Finder Test** 9
6.3 **Main Test** 9

7.0 CONCLUSION 9

8.0 RETENTION OF RECORDS AND TEST ITEM 9

9.0 REFERENCE 10

FIGURES
Figure 1. Range Finder Test 1: Concentration-response curve of Nano Colloidal Argentum 11
Figure 2. Range Finder Test 2: Concentration-response curve of Nano Colloidal Argentum 11

APPENDIX
A-1.1 Range Finder Test 1: NRU data of Nano Colloidal Argentum 12
A-1.2 Range Finder Test 1: Relative viability percentage Nano Colloidal Argentum 12
A-2.1 Range Finder Test 2: NRU data of Nano Collodial Argentum 13
A-2.2 Range Finder Test 2: Relative viability percentage Nano Collodial Argentum 13

SUZAINI BINTI BADRUDIN
Researcher
Bioprocess Programme
Industrial Biotechnology Research Centre
SIRIM Berhad

1.0 BACKGROUND

In 2002, the National Toxicology Program's Interagency Centre for the Evaluation of Alternative Toxicological Methods (NICEATM) and the European Centre for the Validation of Alternative Methods (ECVAM) initiated a collaborative, international, multi-laboratory validation study to independently evaluate the usefulness of 3T3 NRU basal cytotoxicity test for estimating starting doses in the acute oral rodent toxicity test. On March 25, 2008, NICEATM announced the availability of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Test Method Evaluation Report (Federal Register, Vol. 73, No. 58, pp 15757-15758). The report recommends the use of *in vitro* basal cytotoxicity neutral red uptake in a weight-of-evidence approach to determine starting doses for acute oral systemic toxicity tests with rodents.

2.0 OBJECTIVE

The objective of this study was to estimate the starting dose of Nano Colloidal Argentum for acute oral systemic toxicity study (Up-and-Down Procedure) using the BALB/c 3T3 NRU cytotoxicity test.

3.0 TEST SYSTEM

The Neutral Red Uptake (NRU) cytotoxicity assay procedure is based on the ability of viable cells to incorporate and bind neutral red (NR), a supravital dye. NR is a weak cationic dye that readily diffuses through the plasma membrane and concentrates in lysosomes where it electrostatically binds to the anionic lysosomal matrix. Alterations of the cell surface or the sensitive lysosomal membrane lead to lysosomal fragility and other adverse changes that gradually become irreversible. Thus, cell death and/or inhibition of cell growth decreases the amount of neutral red retained by the culture. Healthy proliferating mammalian cells, when properly maintained in culture, continuously divide and multiply over time. Toxicants, regardless of site or mechanism of action, will interfere with this process and result in a reduction of growth rate as reflected by cell number. Cytotoxicity is expressed as a concentration dependent reduction of the uptake of NR after chemical exposure, thus providing a sensitive, integrated signal of both cell integrity and growth inhibition.

4.0 MATERIAL

4.1 Test Item

4.1.1 Test Item: Nano Colloidal Argentum

4.1.2 Lot/Batch No.: Not provided

4.1.3 Date received: 21 June 2013

4.1.4 Physical Appearance: Liquid

4.1.5 Colour: Clear

SUZAINI BINTI BADRUDIN
Researcher
Bioprocess Programme
Industrial Biotechnology Research Centre
SIRIM Berhad

4.1 6 Physical Chemical Properties Data: Not provided

4.1.7 Quantity: Approximately 100 mL

4.1.8 Storage Condition: Refrigerated

4.1 9 pH: Not provided

4.1.10 Stability: Not provided

4.1.11 Expiration Date: Not provided

4.2 **Cell Line**

A murine fibroblast cell line, BALB/c 3T3 cells, clone A31, was obtained from American Type Culture Collection (ATCC), Manassas, USA (Catalog no. CCL-163).

4.3 **Reagents**

4.3.1 Culture Medium: DMEM (Thermo Scientific, buffered with sodium bicarbonate) supplemented with 10% (v/v) calf serum (Sigma), 100 IU/mL Penicillin and 100 mg/mL Streptomycin (PAA Lab).

4.3.2 Chemical Dilution Medium (CDM): DMEM (Thermo Scientific, buffered with sodium bicarbonate) supplemented with 100 IU/mL Penicillin and 100 mg/mL Streptomycin (PAA Lab).

4.3.3 Phosphate Buffered Saline, Calcium and Magnesium free (PBS): 8 mg/mL sodium chloride, 0.02 mg/mL potassium chloride, 0.2 mg/mL potassium dihydrogen orthophosphate and 1.15 mg/mL disodium hydrogen orthophosphate.

4.3.4 Trypsin-EDTA: 2 mg/mL trypsin (Sigma) and 0.3 mg/mL EDTA in PBS.

4.3.5 NR Stock Solution: 2.0 mg/mL NR (Sigma)

4.3.6 NR Medium: 50 μg/mL NR in CDM and culture medium

4.3.7 NR Desorb: 1% Glacial acetic acid and 50% ethanol

4.3.8 Dimethyl sulfoxide (DMSO)

4.3.9 Ethanol

4.4 **Controls**

4.4.1 Positive Control: Sodium lauryl sulphate (Calbiochem)

4.4.2 Vehicle Control: Solvent of preference from the solubility determination procedure (Item 5.2)

SUZAINI BINTI BADRUDIN
Researcher
Bioprocess Programme
Industrial Biotechnology Research Centre
SIRIM Berhad

5.0 METHOD

5.1 Maintenance and Preparation of Cell Line

BALB/c 3T3 cells were routinely grown as a monolayer in tissue culture grade flasks with culture medium at 37°C ± 1°C in a humidified atmosphere containing approximately 5% carbon dioxide. Cells were regularly examined under a phase contrast microscope and all observations were recorded.

When monolayer reached 50% to 80% confluence, cells were removed from flask by trypsinization. The cells were then seeded into 96-well tissue culture microtiter plates and incubated overnight to form a less than half (< 50%) confluent monolayer. This incubation period allows adequate cell recovery and adherence for progression to the exponential growth phase.

5.2 Solubility Determination

Solubility of the test items was determined in various solvents at a relatively high concentration, starting at 200 mg/mL, following a step-wise tiered and a sequence of mechanical procedures. The test item was considered dissolved based entirely on visual observation of achieving a clear solution without visible cloudiness or precipitate. Solvents used in the order of preference were CDM, DMSO, and ethanol. The hierarchy of mechanical procedures include vortexing the mixture at room temperature for 1-2 minutes, followed by waterbath sonication for up to 5 minutes, and finally warming the mixture in the carbon dioxide incubator for up to 60 minutes. If the test item did not dissolve, the volume of solvent was increased so as to decrease the test item concentration by a factor of 10 and the sequence of mixing procedures was repeated.

5.3 Range Finder Test

An initial cytotoxicity test was performed to determine starting concentration for the main test. Test item was assayed at eight concentrations. The test item solution (from 5.2) was diluted in the suitable solvent at log dilutions.

Culture medium from the 96-well tissue culture microtiter plate with less than half confluent monolayer was replaced with the test item solution, each concentration in replicates of six. Vehicle controls were included in the assay. Empty wells (without cells) were filled with culture medium to act as the blank reference.

The plate was incubated for approximately 48 hours. NRU assay was carried out and the data was used to plot a concentration-response curve. Relative cell viability was presented as mean optical density of the replicate values (at least three) at each concentration against the mean optical density of vehicle control.

5.4 Main Test

Main cytotoxicity test was supposed to be performed to determine the test item concentration producing a 50% inhibition of cellular viability (IC_{50}). From the range finder test, an estimated IC_{50} value from the concentration-response curve was to be used as a central concentration. The relevant concentration range was to be determined around the estimated IC_{50} value with several points of a graded effect, with a minimum of two points, one on each side of the value while avoiding too many non-cytotoxic and/or 100%-cytotoxic concentrations.

SUZAINI BINTI BADRUDIN
Researcher
Bioprocess Programme
Industrial Biotechnology Research Centre
SIRIM Berhad

Similar to the Range Finder Test, monolayer was supposed to be treated with the varying concentrations of test item solution and incubated for approximately 48 hours before performing the NRU assay.

5.5 **Controls**

5.5.1 Positive control: Sodium lauryl sulphate was individually assayed as a complete concentration-response curve of eight concentrations in all experiments. Treatment and assessment on a separate 96-well plate was simultaneously carried out following the same schedule and procedures as the test item plates. All test data were accepted only when the positive control meets these two criteria:

(i) the IC_{50} value falls between 29.5 μg/mL and 53.5 μg/mL, and
(ii) the dose-response shows an R^2 value (coefficient of determination) of more than 0.85 for the Hill model fit.

5.5.2 Vehicle control: Solvent of preference from the solubility determination procedure (from 5.2) was used as the vehicle control, included in Column 2 and Column 11 on each test plate. All test data were accepted only when the mean of vehicle control in the two columns do not differ by more than 15% from the mean of all vehicle controls.

5.6 **Neutral Red Uptake Assay**

After incubation, the medium was removed and the cells carefully rinsed with PBS. NR medium was added to all wells and the microtiter plate incubated for approximately 3 hours. The NR medium was then removed and the cells were again carefully rinsed with PBS. NR Desorb solution was added to all wells and the plate was rapidly shaken on a microtiter plate shaker for 10 minutes to 30 minutes. Optical density was measured at 570 nm ± 10 nm (OD_{570}) in a microplate spectrophotometer using blanks as reference.

5.7 **Interpretation of Result**

The OD_{570} data was transferred to a Microsoft Excel spreadsheet and the relative cell viability was presented as a percentage of each optical density value against mean optical density of vehicle control. The calculated values with its equivalent test item concentrations was supposed to be applied to a Hill function analysis using GraphPad Prism® version 5.04 for Windows software. The concentration corresponding to the IC_{50} was to be calculated as follows:

$$\log IC_{50} = \log EC_{50} - [\log ((Top-Bottom)/(Y-Bottom) -1) / HillSlope]$$

where
IC_{50} is the concentration producing 50% toxicity;
EC_{50} is the concentration producing a response midway between the Top and Bottom responses;
Top is the maximum response (100% viability, maximum survival);
Bottom is the minimum response (0% viability, maximum toxicity);
Y=50 (i.e. 50% viability); and
HillSlope describes the slope of the response.

SUZAINI BINTI BADRUDIN
Researcher
Bioprocess Programme
Industrial Biotechnology Research Centre
SIRIM Berhad

A mean IC_{50} from two main tests was to be calculated as the final result and applied to the following regression formula for estimation of a median lethal dose or LD50 in mg/kg:

$$\log LD50\ (mg/kg) = 0.372 \log IC_{50}\ (\mu g/mL) + 2.024$$

Starting dose for the Up-and-Down Procedure (UDP) method is the next dose lower than the estimated LD50 in the default dose progression. The default dose progression for UDP is 1.75, 5.5, 17.5, 55, 175, 550 and 2,000 mg/kg for the 2,000 mg/kg limit test.

6.0 RESULT AND DISCUSSION

6.1 Solubility Determination

The test item was soluble in CDM at the highest stock concentration of 200,000 µg/mL. CDM was used as the vehicle control.

6.2 Range Finder Test

Range Finder Tests were carried out at the highest treatment concentration of 100,000 µg/mL. The concentration-response curves are presented in Figure 1 and Figure 2. Individual NRU data and the calculated relative viability percentages are presented in the Appendix (A-1.1, A-1.2, A-2.1 and A-2.2).

6.3 Main Test

Data from the Range Finder Tests suggested Main Test at 10,000 µg/mL to 100,000 µg/mL, with the former demonstrating a non-cytotoxic and the latter a 100%-cytotoxic concentrations. The calculated LD50 would have been in the range of 3,251 mg/kg to 7,656 mg/kg. Taking into account that the highest default dose progression for UDP in this limit test is 2,000 mg/kg, the Main Test was not carried out. The starting dose for acute oral toxicity test is proposed at 2,000 mg/kg.

7.0 CONCLUSION

Under the condition of this study, the median inhibition concentration (IC_{50}) level of Nano Colloidal Argentum predicted an LD50 value of more than 2,000 mg/kg body weight. Therefore, the proposed starting dose for acute oral toxicity test according to the Up-and-Down Procedure is 2,000 mg/kg.

8.0 RETENTION OF RECORDS AND TEST ITEM

Two original, signed final reports were prepared. One report will be forwarded to the Sponsor. The other report, together with all generated raw data, is maintained at the Industrial Biotechnology Research Centre Archives. IBRC will maintain these records for a period of ten years.

SUZAINI BINTI BADRUDIN
Researcher
Bioprocess Programme
Industrial Biotechnology Research Centre
SIRIM Berhad

9.0 REFERENCE

9.1 Federal Register, Vol. 73, No. 58, 25 March 2008, pp 15757-15758. Availability of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Test Method Evaluation Report: In Vitro Cytotoxicity Test Methods for Estimating Starting Doses for Acute Oral Systemic Toxicity Tests and the Final Background Review Document for In Vitro Cytotoxicity Test Methods for Estimating Acute Oral Systemic Toxicity.

9.2 ICCVAM Test Method Evaluation Report Appendix C: Recommended Test Method Protocols (November 2006)

9.3 OECD (2010). Guidance Document on Using Cytotoxicity Tests to Estimate Starting Doses for Acute Oral Systemic Toxicity Tests. Environmental Health and Safety Monograph Series on Testing and Assessment No 129.

SUZAINI BINTI BADRUDIN
Researcher
Bioprocess Programme
Industrial Biotechnology Research Centre
SIRIM Berhad

STUDY REPORT

REPORT NO: R205/13/B19/03	PAGE: 11 of 13

Figure 1. Range Finder Test: Concentration-response curve of Nano Colloidal Argentum

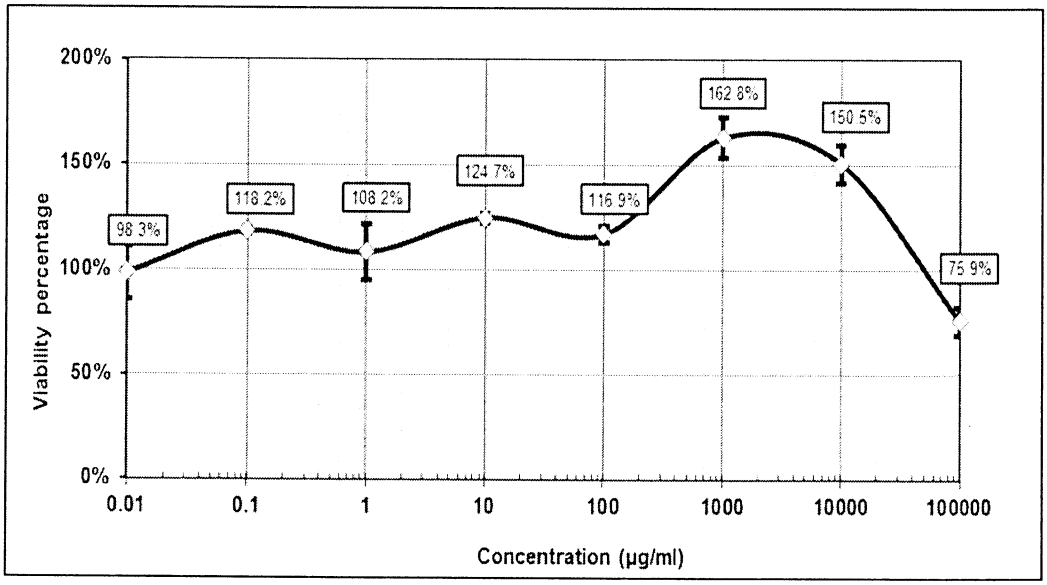
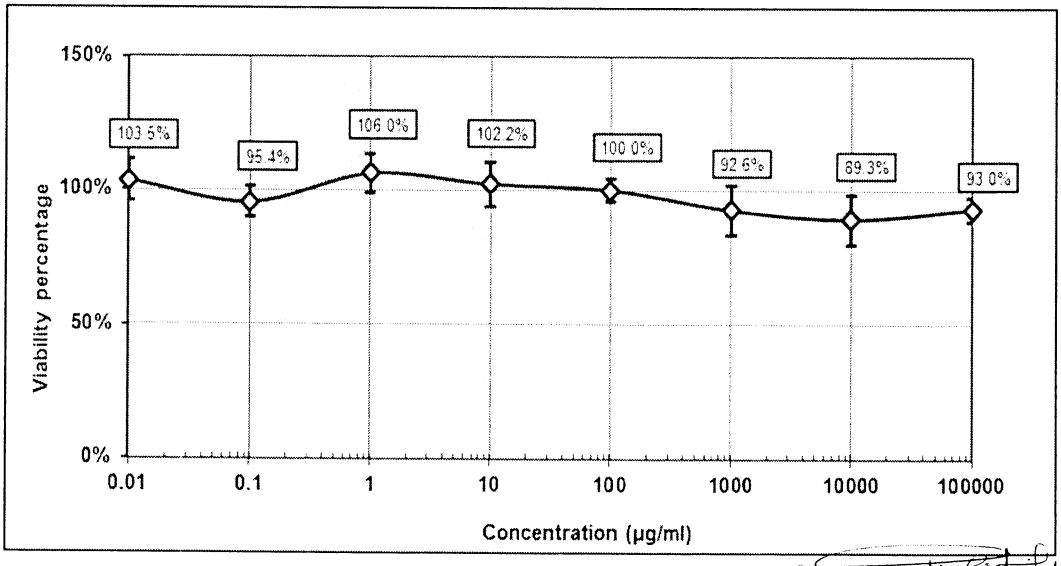


Figure 2. Range Finder Test 2: Concentration-response curve of Nano Colloidal Argentum



SUZAINI BINTI BADRUDIN
Researcher
Bioprocess Programme
Industrial Biotechnology Research Centre
SIRIM Berhad

APPENDIX
Range Finder Test

A-1.1 Range Finder Test 1: NRU data of Nano Colloidal Argentum
Corrected optical density values (OD_{570} values minus mean OD_{570} of blank) and vehicle controls

Vehicle Control	Nano Colloidal Argentum (µg/mL)								Vehicle Control
	100000	10000	1000	100	10	1	0.1	0.01	
0.160	*0.080	*0.135	*0.179	0.180	0.198	0.165	*0.138	0.140	*0.086
*0.205	*0.028	*0.206	0.240	0.190	0.204	*0.232	0.189	0.148	0.116
0.194	0.109	0.252	0.276	*0.265	*0.224	*0.216	*0.214	*0.223	0.128
*0.200	0.128	0.224	0.262	*0.284	*0.238	0.198	0.187	0.155	0.171
0.192	0.117	0.235	0.252	0.184	0.197	*0.240	*0.256	*0.215	*0.208
*0.223	0.132	0.253	0.274	0.195	0.250	0.157	0.192	0.187	*0.099

Note
Cell passage number = 23
Positive control: IC_{50} = 34.2 µg/mL and R^2 = 0.9881
Vehicle control: Difference between Column 11 and the mean = 13.8%
Data marked * omitted from relative cell viability calculation

A-1.2 Range Finder Test 1: Relative viability percentage of Nano Colloidal Argentum
Percentage of OD_{570} against mean vehicle control, together with the calculated mean and standard deviation

Nano Colloidal Argentum (µg/mL)							
100000	10000	1000	100	10	1	0.1	0.01
-	-	-	112.4%	123.6%	103.0%	-	87.4%
-	-	149.8%	118.6%	127.4%	-	118.0%	92.4%
68.1%	157.3%	172.3%	-	-	-	-	-
79.9%	139.9%	163.6%	-	-	123.6%	116.8%	96.8%
73.0%	146.7%	157.3%	114.9%	123.0%	-	-	-
82.4%	158.0%	171.1%	121.7%	-	98.0%	119.9%	116.8%

Mean	75.9%	150.5%	162.8%	116.9%	124.7%	108.2%	118.2%	98.3%
s.d.	6.5%	8.8%	9.5%	4.1%	2.4%	13.6%	1.6%	12.9%

SUZAINI BINTI BADRUDIN
Researcher
Bioprocess Programme
Industrial Biotechnology Research Centre
SIRIM Berhad

STUDY REPORT

APPENDIX
Range Finder Test

A-2.1 Range Finder Test 2: NRU data of Nano Colloidal Argentum
Corrected optical density values (OD_{570} values minus mean OD_{570} of blank) and vehicle controls

Vehicle Control	Nano Colloidal Argentum (µg/mL)								Vehicle Control
	100000	10000	1000	100	10	1	0.1	0.01	
0.147	0.156	0.156	0.138	0.161	0.170	0.182	0.170	0.160	0.193
0.153	0.153	0.137	0.159	0.173	0.170	0.176	0.158	0.178	0.203
0.159	0.168	0.157	0.183	0.178	0.179	0.169	0.161	0.170	0.203
0.151	0.171	0.165	0.153	0.183	0.188	0.201	0.180	0.185	0.198
0.137	0.164	0.135	0.156	0.169	0.196	0.189	*0.203	0.182	0.196
0.151	0.153	0.176	0.172	0.173	0.157	*0.149	0.156	0.199	0.184

Note
Cell passage number = 25
Positive control: IC_{50} = 37.2 µg/mL and R^2 = 0.9630
Vehicle control: Difference between Column 2 and the mean = 13.5%
Data marked * omitted from relative cell viability calculation

A-2.2 Range Finder Test 2: Relative viability percentage of Nano Colloidal Argentum
Percentage of OD_{570} against mean vehicle control, together with the calculated mean and standard deviation

Nano Colloidal Argentum (µg/mL)							
100000	10000	1000	100	10	1	0.1	0.01
90.2%	90.2%	79.9%	93.1%	98.3%	105.2%	98.3%	92.5%
88.5%	79.3%	92.0%	100.0%	98.3%	101.8%	91.4%	102.9%
97.2%	90.8%	105.8%	102.9%	103.5%	97.7%	93.1%	98.3%
98.9%	95.4%	88.5%	105.8%	108.7%	116.2%	104.1%	107.0%
94.9%	78.1%	90.2%	97.7%	113.3%	109.3%	-	105.2%
88.5%	101.8%	99.5%	100.0%	90.8%	-	90.2%	115.1%

Mean	93.0%	89.3%	92.6%	100.0%	102.2%	106.0%	95.4%	103.5%
s.d.	4.5%	9.2%	9.0%	4.4%	8.1%	7.1%	5.7%	7.7%

SUZAINI BINTI BADRUDIN
Researcher
Bioprocess Programme
Industrial Biotechnology Research Centre
SIRIM Berhad