Problems on this study (Comments from participants)

Reference Material (Lab #: 1, 3, 6, 15)

- Materials A and B were difficult to handle since they became very sticky and fused together and to other surfaces after autoclaving and were distorted in shape after autoclaving. Perhaps another method of sterilization would be satisfactory. If this problem cannot be avoided, then the materials need to be marked with this caution and they should be cut to shape aseptically after autoclaving.

Floating of materials (Lab #: 1, 3, 6, 10, 15)

- Both SRM A and SRM B floated in the culture media. A more dense reference material may therefore be more beneficial for use in the Direct Contact Tests.
- A negative control material was also needed for use in the control dishes of the Direct Contact Tests to provide a more reliable comparison with the test dishes. This was needed to cover the same area of tissue culture plastic as that covered by the test materials. This would then have irradiated the possibility of any "false" results indicating cytotoxicity which may have been due to a smaller surface area of tissue culture plastic been available to cells in the test dishes.

Cell counting (Lab #: 6, 10, 15)

- Some cells were scraped from the surface of the plate when the material was removed and some cells that were on or under the material were also removed. In addition the SRM's A and B floated and moved somewhat when the plate was transported and thus the contact site was variable.
- The procedure for doing cell counts should be standardized. It was left to the discretion of the investigator as to scraping, trypsinizing, or using a grid in microscope eyepiece to evaluate the culture well. The use of trypan blue to distinguish living from dead cells was also optional. Thus some investigators may have counted all cells, not just viable cells.
- If cell counts are to be done in the direct contact cultures, the material should be left in place until the cells are removed by trypsin or scraping so that loose cells and cells attached to the material are not lost upon removal of the material.
- I feel that viable cell counting dependent upon successful, consistent removal of cells from the culture plate will always prove problematic and it may be beneficial to use a quantitative measure of cell number/cell viability while the cells remain in the culture vessel. Indicators are now readily available which could achieve this task.
- As for the extract tests I generally found the viable cell counting procedure a very difficult method to use in order to gain reliable consistency and believe that other, more reliable and quantitative methods are available.

Cell growth (Lab #: 2, 6)

- The cell concentration was too high, because our L929 cells are growing very fast (more than one doubling per day). After 24 hrs the wells were full or more than full and after 72 hrs most of the cells were detached in the controls. If every participant in the round robin has a L929 strain which growths differently, the results will never been the same.
- In our laboratory the L929 cells grew better in RPMI. The testing laboratory should use the media they are accustomed to. There was also confusion in this study protocol as to whether MEM or DMEM was to be used.
- The use of heat inactivated or not heat inactivated serum needs to be specified.

PROTOCOL

The TC194/WG5 Validation Study for SRMs on Cytotoxicity Testing

1. Purpose

According to the agreement of TC194/WG5 meeting in York (April 1997), we decided to perform an inter-laboratory validation study, «round robin test», on the Japanese Standard Reference Materials (SRMs) using the protocol arranged in WG5.

The aim of this study is to assess the cytotoxicity of each SRM by using a protocol in compliance with ISO 10993-5.

2. SRMs

In this study, three kinds of unsterilized SRMs are distributed. They should be autoclaved after cutting (121°C, 20 min). The size of SRMs are 15 x 10 cm (thickness: approx. 0.3 mm). One sheet is sufficient for repeating both tests (extraction and direct contact) 4-5 times.

2.1.Negative SRM*:	High density polyethylene sheet (Lot No. 96001C)
2.2.Positive SRM-A (moderate):	Polyetherurethane film containing 0.1% zinc diethyldithiocarbamate (ZDEC) (Lot No. 96011A)
2.3.Positive SRM-B (weak):	Polyetherurethane film containing 0.25% zinc dibutyldithiocarbamate (ZDBC) (Lot No. 96011B)

*: Negative SRM is usable only use for extraction method but not for direct contact method because of the floating nature.

3. Test System

- **3.1. Extraction method**
- **3.2. Direct contact method**

4. Culture medium

Eagle's MEM containing Earle's salts, L-glutamine and non-essential amino acids, adjusted with 2.2 g/L of $NaHCO_3$ (Cat. No. 41500-034, GIBCO-BRL) are recommended. Antibiotics may be added if needed. MEM medium is supplemented with 10% FCS.

5. Cell Types

L-929 cell line is requested. It shall be checked for mycoplasma contamination.

6. Outline of Experimental Design and Methodology

6.1. Extraction Method

6.1.1. Extract condition

SRM shall be cut into a small pieces (10 mm x 50 mm) before extraction.

The extraction (6 cm^2/mL) of SRM is performed in MEM supplemented with 10% FCS at 37°C for 24h. The original extract is defined as 100% extract. A reagent control (blank) is prepared in parallel.

6.1.2. Concentrations

The following dilutions of original extract and original blank shall be studied: 1, 1/2, 1/4, 1/8, 1/16, 1/32 (or more lower doses by a factor 2).

6.1.3. Cell seeding and exposure

L-929 cells are seeded in 24 well plates (5.2 x 10^4 cells/0.5 mL/well). Triplicate wells are prepared for each concentration of the extract or of the blank. After 24 h incubation in a humidified atmosphere containing 5% CO_2 incubator at 37°C ± 2°C, culture medium is withdrawn and replaced with 0.5 mL of extract or the blank. Exposure period shall be both 24h and 72h.

6.1.4. Measurement of cytotoxicity

After 24h, or 72h incubation, with extract and blank, cell suspension is prepared by trypsin treatment, then viable cells are counted in each well using a hematocytometer. Staining with trypan blue, or a vital dye, is recommended for accurate counting.

6.1.5. Data analysis

The mean cell numbers in the control wells and the treated wells in each group is calculated. The relative survival (% of control) versus the concentration of SRM extracts is plotted. The IC_{50} is calculated from the plot.

6.1.6. Report

The report should include raw data and IC_{50} values obtained with each SRM independently.

6.2. Direct Contact Method

6.2.1. Cell seeding and treatment

Triplicate cultures are prepared for each positive SRM. L-929 are seeded in 35-mm dish (2.6 x 10^5 cells/2 mL/dish). Following 24 h culture in a humidified atmosphere containing 5% CO_2 incubator at 37°C±2, the culture medium is replaced with 2 mL fresh medium.

Test sample (approx. 1 cm^2) shall be placed onto the center of each dish. All cultures are incubated for both 24h and 72 h at 37°C.

6.2.2. Measurement of cytotoxicity

Following 24h or 72h incubation, the test sample shall be carefully removed. The cell morphology around and under each test sample is examined under the microscope. Then, viable cells are counted in each dish using a hemocytometer. Staining with trypan blue, or a vital dye, could be useful.

6.2.3. Data analysis

The mean cell number of the control dishes and the treated dishes in each group is calculated. The relative survival (% of control) is evaluated.

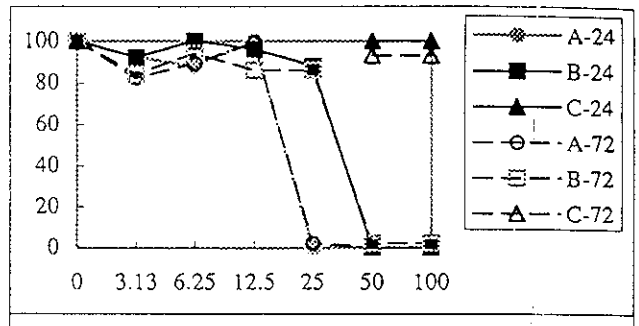
6.2.4. Report

The report should include raw data obtained with each SRM independently.

ORIGINAL DATA OF EXTRACTION METHOD

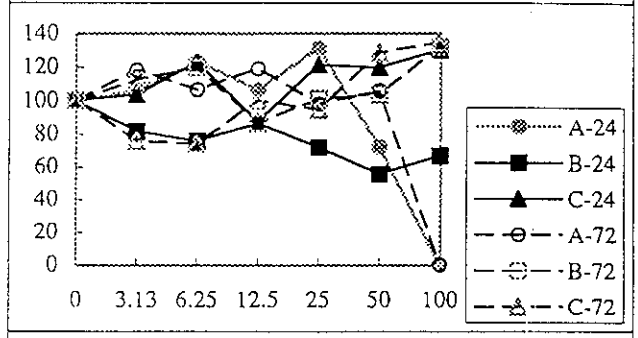
1. MDT

	A-24	B-24	C-24	A-72	B-72	C-72
0	100	100	100	100	100	100
3.125	92	92		82	84	
6.25	88	100		89	94	
12.5	100	96		99	86	
25	0	88		2	86	
50	0	0	100	0	2	93
100	0	0	100	0	2	93



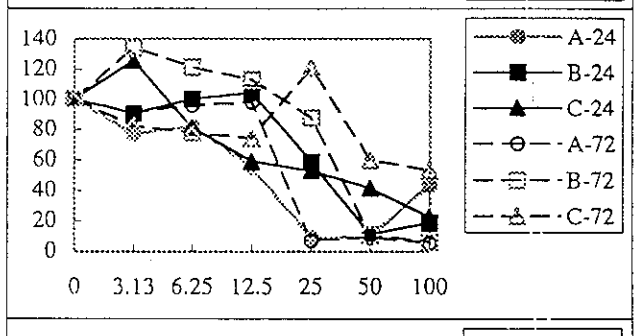
2. ITV2

	A-24	B-24	C-24	A-72	B-72	C-72
0	100	100	100	100	100	100
3.125	106	81.4	103.2	118.1	112.3	75.4
6.25	122.9	75.7	122.6	106.5	119.5	73.9
12.5	106	85.9	87.1	118.9	86.2	100
25	131.3	71.8	121	98.1	100.9	94.4
50	72.3	55.7	119.4	105.8	103.9	128.4
100	0	66.7	129.8	0	131.8	134.7



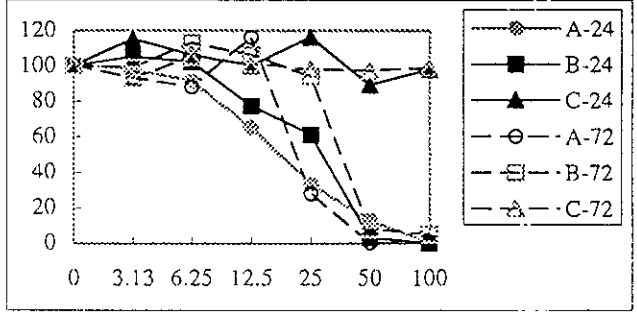
3. BECTON

	A-24	B-24	C-24	A-72	B-72	C-72
0	100	100	100	100	100	100
3.125	76.5	90.6	125.4	90.9	133.6	82
6.25	81.6	99.8	81	96	121.3	77.5
12.5	54.8	104.1	58.7	97.9	112.8	73.9
25	8.6	58.5	52.4	6.8	87.5	121
50	9.1	10.8	41.3	8.8	10.5	59.7
100	43	18.4	22.2	4.8	5.7	52.9



4. GAMBRO

	A-24	B-24	C-24	A-72	B-72	C-72
0	100	100	100	100	100	100
3.125	98	105	115	93	99	93
6.25	91	102	106	88	113	105
12.5	65	77	100	116	106	101
25	33	61	116	28	94	98
50	13	3	89	0	8	97
100	0	0	98	0	5	99

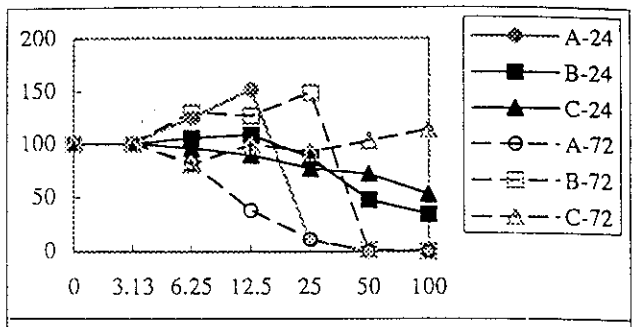


5. CORDIS CO.
Not tested

6. FDA

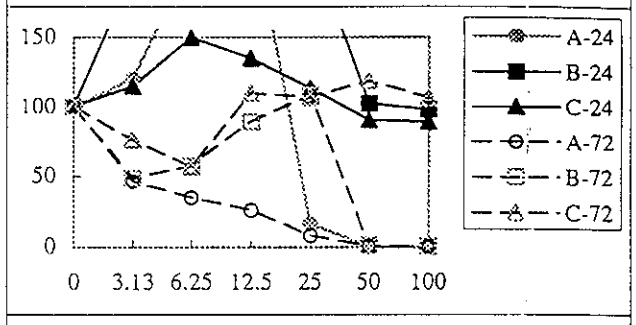
If 3.13% dose was defined as 100%

*	A-24	B-24	C-24	A-72	B-72	C-72
0	100	100	100	100	100	100
3.125	100	100	100	100	100	100
6.25	124.7	105.3	95.91	82.21	129.8	81
12.5	151.9	108.5	89.55	37.91	127	99.35
25	9.957	86.97	76.82	11.06	148.6	92.51
50	0	47.87	72.27	0	1	102.8
100	0	34.31	53.18	0	0	113.4



If control group defined as 100%

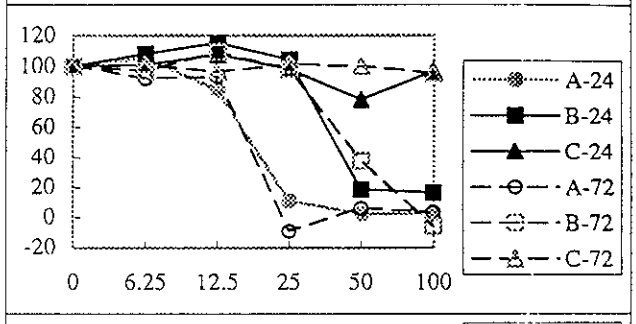
	A-24	B-24	C-24	A-72	B-72	C-72
0	100	100	100	100	100	100
3.125	120.3	195.8	114.6	45.85	48.02	75.8
6.25	204.3	280.9	149.6	34.48	57.04	56.17
12.5	240.4	279.5	134.9	25.39	89.09	110
25	15.44	219.5	113.4	7.697	108.3	106.5
50	0	102.3	90.34	0	0.733	118.9
100	0	97.73	88.64	0	0	106.2



7. NIHSJ

counted by CV

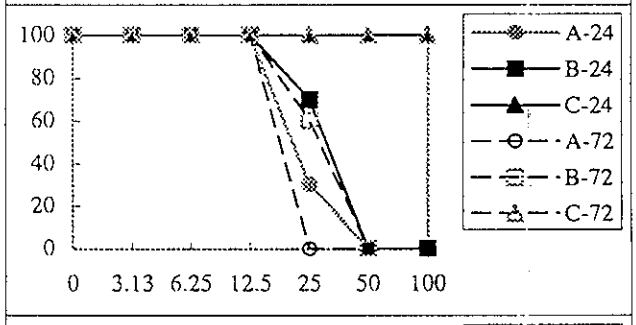
	A-24	B-24	C-24	A-72	B-72	C-72
0	100	100	100	100	100	100
6.25	105.1	107.8	100.7	92	96.7	100.7
12.5	84.6	114.9	107.3	93.2	109.3	96.1
25	11	104.3	98	-9.1	98	101.1
50	2.2	18.4	78.1	5.7	37.7	100
100	2.2	16.3	96.7	3.4	-6	95.4



8. NAMSA

no cell count (by observation) NA=0

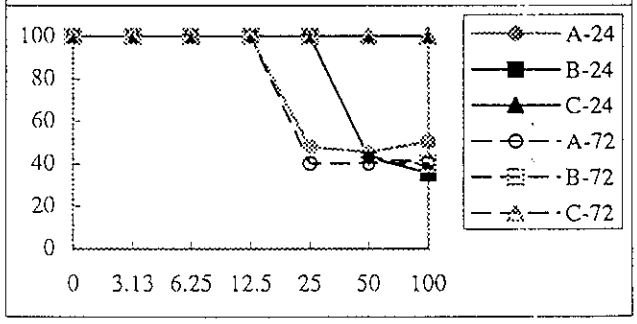
	A-24	B-24	C-24	A-72	B-72	C-72
0	100	100	100	100	100	100
3.125	100	100	100	100	100	100
6.25	100	100	100	100	100	100
12.5	100	100	100	100	100	100
25	30	70	100	0	60	100
50	0	0	100	0	0	100
100	0	0	100	0		100



9. 3M

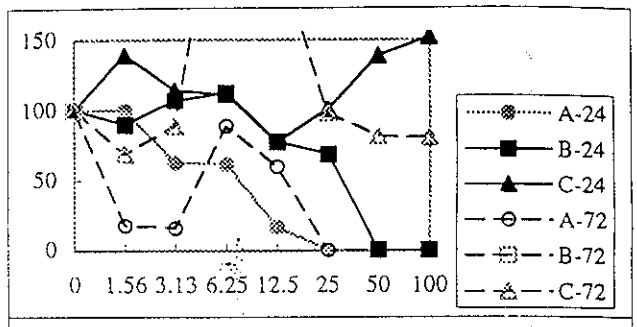
no cell count (by observation)

	A-24	B-24	C-24	A-72	B-72	C-72
0	100	100	100	100	100	100
3.125	100	100	100	100	100	100
6.25	100	100	100	100	100	100
12.5	100	100	100	100	100	100
25	47.5	100	100	40	100	100
50	45	42.5	100	40	42.5	100
100	50	35	100	40	40	100



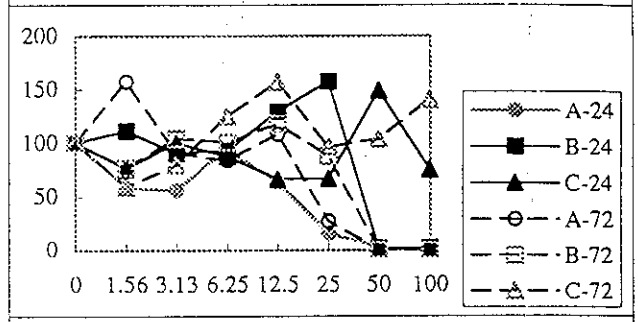
10. Univ.Newcastle

	A-24	B-24	C-24	A-72	B-72	C-72
0	100	100	100	100	100	100
1.563	99.6	89.6	138.9	17.3		68.8
3.125	62.3	107.1	114.5	15.7		88.8
6.25	61.8	112.7	111.8	88.9		288.2
12.5	16	77.6	77.2	60		213.9
25	0	68.9	101	0		97.9
50	0	0	138.2	0		81.6
100	0	0	152	0		81



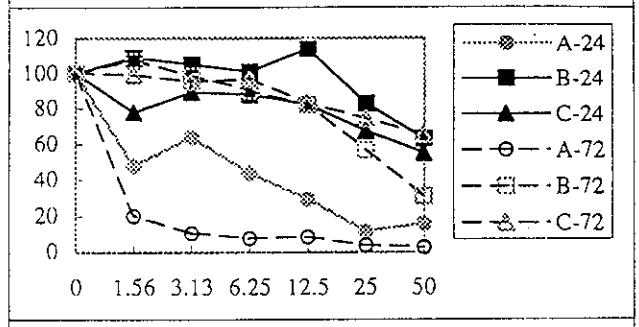
*

	A-24	B-24	C-24	A-72	B-72	C-72
0	100	100	100	100	100	100
1.563	57.5	111.3	75.8	157.1	76.5	59
3.125	55.9	89.7	100	89.7	103.9	79.2
6.25	94.2	92.9	87.1	83.4	100.5	124.8
12.5	63.1	129.4	66.2	108.2	118.1	156.4
25	15.3	157.1	66.9	26.9	87.3	96.6
50	2.4	1.2	148.8	0.7	0	103.4
100	0	1.7	75	0	0	140.7



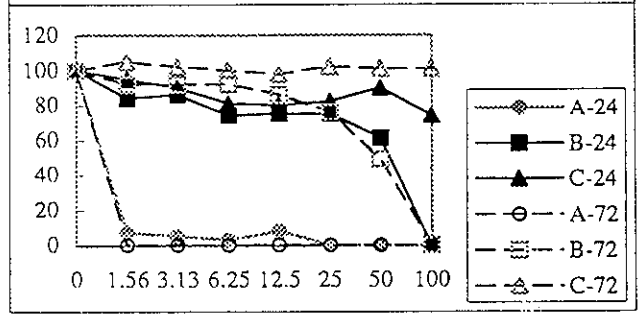
11. RCC

	A-24	B-24	C-24	A-72	B-72	C-72
0	100	100	100	100	100	100
1.563	47.5	108.8	77.6	19.9	106.8	98.9
3.125	63.5	104.8	88.8	10.4	98.3	95.3
6.25	43.5	100.7	87.6	7.4	90	96.4
12.5	28.9	113.8	81.9	8.1	81.9	82.7
25	11.5	82.6	66.9	3.7	56.2	73.9
50	15.6	62.5	54.4	2.4	30.9	64.9



12. LEMI

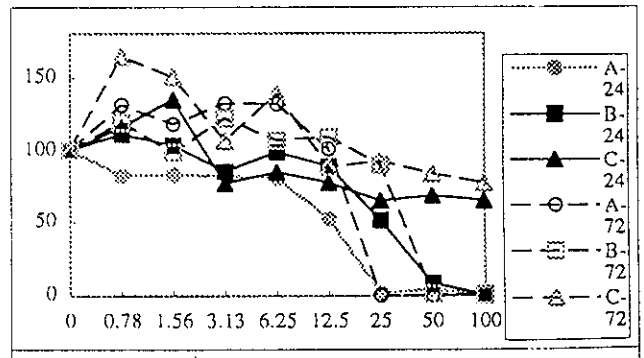
	A-24	B-24	C-24	A-72	B-72	C-72
0	100	100	100	100	100	100
1.563	7	84	95	0	92	105
3.125	5	86	90	0	92	102
6.25	3	74	81	0	92	100
12.5	8	75	80	0	86	98
25	0	75	82	0	76	102
50	0	61	90	0	49	101
100	0	0	74	0	0	101



13. Univ.Vienna
Not tested

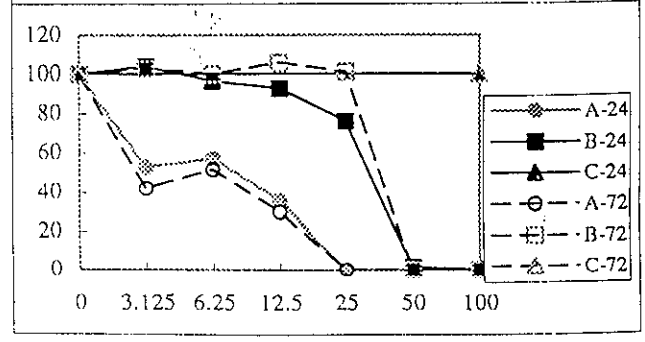
14. FDSC

	A-24	B-24	C-24	A-72	B-72	C-72
0	100	100	100	100	100	100
0.781	82.3	111.0	116.0	131.1	119.6	164.3
1.563	82.9	103.3	134.8	118.0	98.4	150.5
3.125	81.8	85.1	76.8	132.0	122.1	106.5
6.25	80.7	98.3	84.5	131.8	106.7	138.7
12.5	51.9	88.4	77.3	100.7	109.0	88.2
25	1.1	50.8	64.6	0.0	89.4	91.9
50	3.9	7.7	68.0	0.0	0.7	83.2
100	0.6	0.6	64.6	0.0	0.0	76.7



15. TGA

	A-24	B-24	C-24	A-72	B-72	C-72
0	100	100	100	100	100	100
3.125	52.5	103.5		41.7	102.7	
6.25	57.0	96.3		51.3	100.0	
12.5	35.8	92.5		29.3	105.7	
25	0.1	75.8		0.0	101.0	
50	0.0	1.3		0.0	0.0	
100	0.0	0.0	100.0	0.0	0.0	100.0



Dear Participants

I am now able to send a final protocol and Standard Reference Materials (SRMs) for "TC194/WG5 Validation Study for SRMs on Cytotoxicity Test" to the participating laboratories. The time schedule was delayed one month. Therefore, deadline of report is the end of November, 1997.

1. Management
   The study is being planned by Management Members, Drs. M-F Harmond (WG5: Convener), A. Nakamura (sponsorship), and myself (N. Tanaka) based on the agreement of WG5 meeting in York.

2. Project Leader
   For conducting the TC194/WG5 validation study, there will be a Project Leader who will be responsible for the project. If you have any comments and questions, please feel free to ask by Fax or E-mail.

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3. The Time Schedule of the Study
   Shipping of SRMs: July 8, 1997
   Deadline of Report: **November 30, 1997**

4. Protocol
   All participants should be follow this protocol. If the participant can't partly follow for some reason, it should be reported on the detail of modification.

5. Certification of the Data
   This study may or may not perform under GLP, however, the test results should be carefully checked by GLP spirit. Upon completion of the final report, all raw data and reports shall be achieved by the laboratory conducted.

6. Summarizing the Data
   All data will be sent to the Project Leader, then they will be circulated and summarized by the management members.

7. Future Work
   According to the data of present study, the "Optional Study" could be performed in a second step in order to validate the protocols of each laboratory with regards to the negative and positive controls, validated by extract test and direct contact test.

8. Sponsor
   The study is a partly supported by the Research Project (Project leader: Dr. A. Nakamura, NIHS) granted by the MHW (Ministry of Wealth and Welfare, Japan). Therefore, all participants will be received a set of SRMs with no charge,

Finally, I hope that this validation study will be successful and fruitful for the international harmonization of biological testing on the medical devices.
Yours sincerely,

Noriho Tanaka, PhD
Project Leader
TC194/WG5 Validation Study

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