Final Report

EVALUATION OF A PROCESS FOR GENERATING BACTERIA DE NOVO FROM EUKARYOTIC CELLS

Study Number: R96AE40.510002

Test Article ID: RT-HCMV endothelial cells (Source: ATCC)

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CONCLUSION

Two runs involving the periodic reintroduction of an aerobic atmosphere during an anaerobic eukaryotic cell culture phase resulted in the isolation of bacteria, specifically *Bacillus licheniformis*. Four different colony morphologies were observed. Two runs in which an aerobic atmosphere was not periodically reintroduced during an anaerobic eukaryotic cell culture phase resulted in the isolation of no bacteria. All eukaryotic cell controls and media controls were negative. The isolation of bacteria from eukaryotic cells subjected to alternating anaerobic/aerobic cell culture conditions provides supporting evidence for the hypothesis of *de novo* evolution of bacteria from eukaryotic cells. On the other hand, the possibility of environmental contamination as the source of the bacterial isolates cannot be absolutely eliminated. Environmental contamination is unlikely due to the cGMP compliance procedures and practices employed in the performance of the sterility assays, which includes a stringent environmental and personnel monitoring program. Also, no tube, plate, or bottle inoculated with eukaryotic cell control samples or media control samples showed any microbial outgrowth. These negative results for all the numerous control samples tested minimized significantly the possibility of environmental contamination.

STUDY INFORMATION

Test Article:	RT-HCMV endothelial cells (Source: ATCC) were received by the Biotechnology Group on 05/16/95.
Testing Facility:	Biotechnology Group BioReliance Corporation (formerly Microbiological Associates, Inc.) Life Sciences Center 9900 Blackwell Road Rockville, Maryland 20850 USA
Schedule:	
Study Initiation:	02/23/96
Lab Initiation:	02/23/96
Lab Completion:	07/03/96
Study Director:	Anton F. Steuer, Ph.D.
Archives:	All raw data, records, protocol and a copy of the report will be maintained by the testing facility. Regulatory Affairs/Quality Assurance Department Microbiological Associates, Inc. 9900 Blackwell Road Rockville, Maryland 20850 USA

OBJECTIVE

The purpose of this study is to determine if bacteria can be isolated from cells maintained in anaerobic conditions as described below in the Procedures Section.

PROCEDURES

Procedures are described below for four separate experiments or runs. Each run followed a basic procedural design with variations as noted below. All cell culture manipulations were performed

with aseptic techniques in a biological safety cabinet.

Run #1 (Initiated on 02/23/96)

RT-HCMV endothelial cells were cultured in medium 199 supplemented with 100 ìg heparin/ml, 2 mM L-glutamine, and 10% heat inactivated fetal bovine serum (FBS). Twelve T-75 cm^2 flasks with vented filter caps at passage 3 were supplied by the MAI's Client Cell Culture Lab to the testing lab. Each flask contained approximately 1.96 x 10^7 viable (trypan blue excluding) cells/flask. Medium was aspirated from each of six flasks, and each flask was refed with 25 ml of nitrogen (N_2) flushed medium 199 and Ham's F12 (1:1 proportions) supplemented with 80 mM DMSO, 11 mM fructose, 25 mM succinic acid, 800 mg L-glutamic acid and 100 ìg heparin/ml. The inadvertent inclusion of heparin in the medium was a deviation from the protocol. All medium used was filtered using a Corning 0.22 ìm sterile filter unit. Prior to feeding, medium was flushed with sterile filtered N_2 gas for 15-20 minutes. N_2 flushing time was a deviation from the protocol, which states 10-15 minutes. One control flask (with no cells) containing N_2 flushed growth medium was used as a medium control flask. The seven flasks were transferred to the anaerobic chamber, and the chamber was sealed and purged with sterile filtered N_2 four times over approximately a two hour period for a total flush time of approximately one hour. The air in the chamber was analyzed for oxygen concentration with a Fyrite analyzer until the O_2 level read 0% for at least two consecutive Fyrite O_2 tests. The chamber was placed for approximately 72 hours in an incubator at 30°C ± 2°C.

Six control flasks were aspirated and refed with the same growth medium (25 ml/flask), which was not flushed with N_2. One flask (with no cells) containing medium only was included as a medium control flask. These seven control flasks were incubated aerobically for approximately 72 hours at 30°C ± 2°C in the same incubator that housed the experimental chamber.

After 72 hours incubation, the chamber and control flasks were removed. The O_2 concentration of the chamber was measured. The contents from each flask were vigorously mixed and aspirated several times with a 10 ml pipet. The contents from the experimental flasks were pooled, and samples collected for the sterility and mycoplasma assays. The pooled samples were then sterile filtered through a 0.22 ìm filter. The filtrate samples were submitted for sterility testing. The control cell flasks were scraped, pooled, snap frozen in an alcohol/dry ice bath, and thawed at 37°C ± 2°C. Aliquots of the thawed material were submitted for sterility and mycoplasma testing. The control material was then filtered through a 0.22 ìm filter, and the filtrate was submitted for sterility testing. Media from the two flasks without cells were also submitted for sterility testing. Figure 1 shows the sample and testing scheme for Run #1.

Run #2 (Initiated 03/18/96)

RT-HCMV endothelial cells were cultured in medium 199 supplemented with 100 ìg heparin/ml, 2 mM L-glutamine, and 10% heat inactivated fetal bovine serum (FBS). Prior to cell seeding, all cell culture flasks were pretreated for 15 minutes (followed by a 1x PBS wash) with

1% gelatin (2% gelatin solution from Sigma diluted with an equal volume of sterile tissue culture grade water). Twelve T-75 cm^2 flasks with standard (no filter) caps at passage 6 were supplied by the Client Cell Culture Lab to the testing lab. Each flask contained approximately 2.23 x 10^7 viable (trypan blue excluding) cells/flask. Medium was aspirated from each of six flasks, and each flask was washed with HBSS and refed with 25 ml of nitrogen (N_2) flushed medium 199 and Ham's F12 (1:1 proportions) supplemented with 80 mM DMSO, 11 mM fructose, 25 mM succinic acid, 800 mg L-glutamic acid and 100 ìg heparin/ml. The inadvertent inclusion of heparin in the medium was a deviation from the protocol. All medium used was filtered using a Corning 0.22 ìm sterile filter unit. Prior to feeding, medium was flushed with sterile filtered N_2 gas for 15-20 minutes. N_2 flushing time was a deviation from the protocol, which states 10-15 minutes. One control flask (with no cells) containing N_2 flushed growth medium was used as a medium control flask. The seven flasks were transferred to the anaerobic chamber, and the chamber was sealed and purged with sterile filtered N_2 four times over approximately a two hour period for a total flush time of approximately one hour. The air in the chamber was analyzed for oxygen concentration with a Fyrite analyzer until the O_2 level read 0% for at least two consecutive Fyrite O_2 tests. The tubing to the chamber was clampled off, and the chamber was placed for approximately 72 hours in an incubator at 36°C ± 2°C.

Six control flasks were aspirated and refed with the same growth medium (25 ml/flask), which was not flushed with N_2. One flask (with no cells) containing medium only was included as a medium control flask. These seven control flasks were incubated aerobically for approximately 72 hours at 36°C ± 2°C in the same incubator that housed the experimental chamber.

Approximately 24 and 48 hours after initiation of the experiment, the chamber was opened at room temperature, and the flask caps were sealed. The flasks were observed microscopically and observations were recorded. These observations were performed within 10 minutes. The flasks were then placed (not stacked) in a laminar flow hood (with fluorescent light on) for approximately 30 minutes and after 30 minutes the caps were loosened and the flasks remained in the hood for another 2-3 minutes. The flasks were transferred to the anaerobic chamber, which was sealed and flushed with N_2 until the effluent gas measured 0% oxygen. The tubing to the chamber was clamped off, and the chamber was placed back into the incubator. The control flasks were manipulated in the same manner and returned (not in the anaerobic chamber) to the incubator.

After 72 hours incubation, the chamber and control flasks were removed. The contents from each flask were scraped and vigorously mixed and aspirated several times with a 10 ml pipet for approximately two minutes and followed with vortexing for approximately two minutes. The contents from the experimental flasks were pooled, and samples were collected for the sterility and mycoplasma assays. The pooled samples were then sterile filtered through a 0.22 ìm filter, and the filtrate samples were submitted for sterility testing. The control cell flasks were treated in the same manner, and samples were submitted for sterility and mycoplasma testing. The

control material was filtered through a 0.22 ìm filter, and the filtrate was submitted for sterility testing. Media from the two flasks without cells were also submitted for sterility testing. Figure 2 shows the sample and testing scheme for Run #2.

Run #3 (Initiated 04/22/96)

RT-HCMV endothelial cells were cultured in medium 199 supplemented with 100 ìg heparin/ml, 2 mM L-glutamine, and 10% heat inactivated fetal bovine serum (FBS). Prior to cell seeding, all cell culture flasks were pretreated for 15 minutes (followed by a 1x PBS wash) with 1% gelatin (2% gelatin solution from Sigma diluted with an equal volume of sterile tissue culture grade water). Fourteen T-75 cm^2 flasks with standard caps at passage 6 were supplied by the Client Cell Culture Lab to the testing lab. Each flask contained approximately 3.11×10^7 viable (trypan blue excluding) cells/flask. Medium was aspirated from each of the seven flasks, and each flask was washed with HBSS and refed with 25 ml of nitrogen (N_2) flushed medium 199 and Ham's F12 (1:1 proportions) supplemented with 80 mM DMSO, 11 mM fructose, 25 mM succinic acid, 800 mg L-glutamic acid and 100 ìg heparin/ml. The inadvertent inclusion of heparin in the medium was a deviation from the protocol. All medium used was filtered using a Corning 0.22 ìm sterile filter unit. Prior to feeding, medium was flushed with sterile filtered N_2 gas for 15-20 minutes. N_2 flushing time was a deviation from the protocol, which states 10-15 minutes. One control flask (with no cells) containing N_2 flushed growth medium was used as a medium control flask. The seven flasks were transferred to the anaerobic chamber, and the chamber was sealed and purged with sterile filtered N_2 four times over a 1 1/2 hour period for a total flush time of approximately forty minutes. Total N_2 flushing time was a deviation from the protocol, which states a 2-3 hours total flushing time. The air in the chamber was analyzed for oxygen concentration with a Fyrite analyzer until the O_2 level read 0% for at least two consecutive Fyrite O_2 tests. The chamber was placed for approximately 72 hours in an incubator at 36°C ± 2°C.

Six control flasks were aspirated and refed with the same growth medium (25 ml/flask), which was not flushed with N_2. One flask (with no cells) containing medium only was included as a medium control flask. These seven control flasks were incubated aerobically for approximately 72 hours at 36°C ± 2°C in the same incubator that housed the experimental chamber.

Approximately 24 and 48 hours after initiation of the experiment, the chamber was opened at room temperature, and the flask caps were sealed. The flasks were observed microscopically and observations were recorded. These observations were performed within 10 minutes. The flasks were then placed (not stacked) in a laminar flow hood (with fluorescent light on) for approximately 30 minutes and after 30 minutes the caps were loosened and the flasks remained in the hood for another 2-3 minutes. The flasks were transferred to the anaerobic chamber, which was sealed and flushed with N_2 until the effluent gas measured 0% oxygen. The tubing to the chamber was clamped off, and the chamber was placed back into the incubator. The control

flasks were manipulated in the same manner and returned (not in the anaerobic chamber) to the incubator.

After 72 hours incubation, the chamber and control flasks were removed. The O_2 concentration of the chamber was measured. The contents from each flask were scraped and vigorously mixed and aspirated several times with a 10 ml pipet and followed with vortexing for approximately two minutes. The contents from the experimental flasks were pooled and submitted for sterility testing. The control cell flasks were treated in the same manner and submitted for sterility testing. Media from the two flasks without cells were also submitted for sterility testing. Figure 3 shows the sample and testing scheme for Run #3.

Run #4 (Initiated on 06/03/96)

RT-HCMV endothelial cells were cultured in medium 199 supplemented with 100 ìg heparin/ml, 2 mM L-glutamine, and 10% heat inactivated fetal bovine serum (FBS). Prior to cell seeding, all cell culture flasks were pretreated for 15 minutes (followed by a 1x PBS wash) with 1% gelatin (2% gelatin solution from Sigma diluted with an equal volume of sterile tissue culture grade water). Eight T-162 cm^2 flasks (NUNC) with standard caps at passage 6 were supplied by the Client Cell Culture Lab to the testing lab. Each flask contained approximately 5.6×10^7 viable (trypan blue excluding) cells/flask. Medium was aspirated from each of the four flasks, and each flask was refed with 25 ml of N_2 flushed medium 199 and Ham's F12 (1:1 proportions) supplemented with 80 mM DMSO, 11 mM fructose, 25 mM succinic acid, and 800 mg L-glutamic acid. All medium used was filtered using a Corning 0.22 ìm sterile filter unit. Prior to feeding, medium was flushed with sterile filtered N_2 for 15-20 minutes. The inadvertent inclusion of heparin in the medium was a deviation from the protocol. One control flask (with no cells) containing N_2 flushed growth medium was used as a medium control flask. The five flasks were transferred to the anaerobic chamber, and the chamber was sealed and purged with sterile filtered N_2 four times over a one hour and 16 minute period for a total flush time of approximately one hour. Total N_2 flushing time was a deviation from the protocol, whch states a 2-3 hour total flushing time. The air in the chamber was analyzed for oxygen concentration with a Fyrite analyzer until the O_2 level read 0% for at least two consecutive Fyrite O_2 tests. The chamber was placed for approximately 72 hours in an incubator at 36°C ± 2°C.

Four control flasks were aspirated and refed with the same growth medium (25 ml/flask), which was not flushed with N_2. One flask (with no cells) containing medium only was included as a medium control flask. These five control flasks were incubated aerobically for approximately 72 hours at 36°C ± 2°C in the same incubator that housed the experimental chamber.

After 72 hours incubation, the chamber and control flasks were removed. The O_2 concentration of the chamber was measured. The contents from each flask were scraped and vigorously mixed and aspirated several times with a 10 ml pipet and followed with vortexing for approximately two minutes. The contents from the experimental flasks were pooled, and submitted for sterility testing. Media from the two flasks without cells were also submitted for sterility testing. Figure 4 shows the sample and testing scheme for Run #4.

Sterility and Mycoplasma Tests - General Description of Procedures

Sterility Test (1514.510) - This test is designed to detect the presence of bacteria and fungus in samples by the direct inoculation of small sample volumes (0.5 ml/tube) into sterile fluid thioglycollate (THIO), soybean-casein digest (TSB) and peptone yeast glucose (PYG) broths and sabourand-dextrose agar (SAB) slants. The media are incubated at appropriate temperatures for 14 days and observed for evidence of growth according to SOP OPBT1602. Positive and negative controls are treated similarly. Positive controls are *Bacillus subtilis* (196 CFU/ml), *Bacteroides vulgatus* (198 CFU/ml), and *Candida albicans* (196 CFU/ml). Negative controls are uninoculated media and inoculated sterile peptamin. Samples are incubated at the following temperatures: Thio broth tubes at 30° to 35 °C, TSB broth tubes at 20° to 25°C, SAB slant tubes at 20° to 25°C and 36° ± 1°C, and PYG broth tubes anaerobically at 36° ± 1 °C. Sterility test (1514.510) meets or exceeds USP 23 and/or 21 CFR 610 requirements.

Any sample inoculated tube(s) which appear turbid should be plated out according to SOP OPBT0422 on the day turbidity is observed. Three blood agar plates should be streaked for each tube showing turbidity. One plate is incubated aerobically at 36° ± 1°C for at least 24 hours. The second plate is incubated anaerobically at 36° ± 1°C for at least 24 hours. The third plate is incubated aerobically at the same temperature as the broth tube which shows turbidity. A Gram stain is performed according to SOP SOPBT0409 for any outgrowths. Outgrowths are further identified for genus and species by an outside contractor.

Sterility Test (1514.510036) - This test is designed to detect bacteria and fungus in samples by the inoculation of larger sample volumes into bottles containing 100 ml of THIO broth or TSB broth. The bottles are incubated for 14 days at 30° to 35°C for THIO broth and at 20° to 25° for TSB broth and are observed for evidence of growth according to SOP OPBT1608. Positive and negative controls are treated similarly. Positive controls are *Bacillus subtilis* (99 CFU/ml), *Bacteroides vulgatus* (94 CFU/ml), and *Candida albicans* (99CFU/ml). Negative controls are uninoculated media and inoculated sterile peptamin.

Any sample inoculated bottle(s) which appears turbid is plated out according to SOP OPBT0422. Three blood agar plates are streaked for each bottle showing turbidity. One plate is incubated aerobically at 36° ± 1°C for at least 48 hours. The second plate is incubated anaerobically at 36° ± 1°C for at least 48 hours. The third plate is incubated aerobically at the same temperature and incubation period as the broth bottle which showed the turbidity. A Gram stain is performed according to SOP OPBT0409 for any outgrowth. Outgrowths will be identified for genus and species by an outside contractor.

MSA, S110, and BLD Plate Test - Samples are streaked onto Staph 100 (S110) agar, mannitol salt agar (MSA), or blood agar (BLD) plates and incubated at 36°C ± 1°C for 14-21 days. Positive controls are *Staphylococcus aureus* inoculated plates, and negative controls are uninoculated plates.

Mycoplasma (1514.102003) Test - The test is designed to detect agar-cultivable and non-cultivable mycoplasma in a large sample volume using two test systems: agar isolation and Hoechst stain. Positive controls for the agar isolation are *Mycoplasma pneumoniae* PI1428 (at 100 CFU or less per inoculum) and *Mycoplasma orale* JS (at 100 CFU or less per inoculum) . Positive controls for the Hoechst stain are *Mycoplasma hyorhinis* 1050 (at 100 CFU or less per inoculum) and *Mycoplasma orale* JS (at 100 CFU or less per inoculum). Negative control is sterile mycoplasma broth. For agar isolation, the sample and the positive and negative controls are inoculated into broth bottles and onto two types of agar plates. Half of the plates and bottles are incubated aerobically, and the remaining plates and bottles are incubated anaerobically. The broth bottles are observed every working day and subcultured on three separate occasions (days 3, 7, and 14) onto agar plates. These subcultured plates are incubated in the same atmosphere as the broth bottle from which the subculture was made. All plates are examined for the presence of mycoplasma colonies at least 14 days after inoculation. This procedure is performed according to SOP OPBT0336. For the Hoechst staining assay, the sample and the positive and negative controls are inoculated into a 24-well tray containing Vero (monkey kidney) cells. These cells are incubated for three to five days and stained using the Hoechst bisbenzamide stain and are evaluated microscopically by epifluorescence for the presence of mycoplasma. This procedure is performed according to SOP OPBT0334.

RESULTS

Run #1

The sterility (1514.510, 1514.510036, and MSA/S110 plates) and mycoplasma (1514.102003) test results were negative for all experimental cell and control cell samples. The assay negative controls showed no evidence of growth, and the assay positive controls showed the expected outgrowth. Tables 1 - 7 present the sterility testing results and Tables 8 and 9 present the mycoplasma results.

Run #2

The sterility (1514.510), MSA/blood agar plates, and mycoplasma (1514.102003) test results were negative for experimental cell and control cell samples. The assay negative controls showed no evidence of growth, and the assay positive controls showed the expected outgrowth. Tables 10 - 18 present the testing results.

On day 7 of the sterility (1514.510036) assay, one bottle of thioglycollate and one bottle of TSB inoculated with experimental cell sample (prefiltered) exhibited particulate cloudiness. Each bottle was streaked onto blood agar plates and incubated aerobically and anaerobically at 36° ± 1°C for 96 hours. Also, the TSB broth was streaked onto a blood agar plate and incubated aerobically at 20°C ± 25°C, and the THIO broth was streaked onto blood agar and incubated aerobically at 30-35°C for 96 hours. At 96 hours no growth was observed on any

of the plates streaked from the thioglycollate bottle. At 24 hours growth was observed on all blood agar plates streaked with the TSB broth. In addition, a sabouraud plate was streaked with the TSB broth, and there was outgrowth after 24 hours aerobic incubation at 36°C ± 1°C. Gram stain results indicated Gram positive rods. Four colony morphologies were observed:

1) white
2) clear mucoid
3) dull gray
4) dry white

These colonies were observed on the initial streaked blood plates. Colonies were restreaked to send out for identification by an outside company (Acculab, Inc.). All four colonies were identified as *Bacillus licheniformis*.

Run #3

The sterility (1514.510036) assay was performed on experimental cell and control cell samples. No filtration was performed. Bottles were negative (except the positive controls, see Tables 19 - 21) with the following exception. On day 14 of the 1514.510036 assay, all the bottles were placed in the refrigerator overnight. The next day (day 15) one of the thioglycollate bottles exhibited turbidity. Blood agar plates were streaked with a sample from the turbid solution and incubated aerobically for 72 hours at 36° ± 1°C. A clear mucoid colony was observed after 72 hours of incubation. The sample was sent to an outside lab for identification, and the isolate was identified as *Bacillus licheniformis*. **Note:** Prior to transfer to the refrigerator on day 14, the THIO and TSB bottles were streaked onto blood agar plates at day 11 of the 1514.510036 assay. This was performed since particulates were seen floating in the TSB bottles. After 72 hours incubation aerobically at 30°-35°C, 20°-25°C, 36°C ± 1°C and anaerobically at 36°C ± 1°C, no outgrowths were observed.

Run #4

The sterility (1514.510036) assay was performed on experimental cell and control cell samples. No filtration was performed. All bottles were negative (except for the positive controls, see Tables 22-24). Particulate cloudiness was observed for both experimental cell and control cell inoculated bottles, but upon streaking and incubating blood agar plates no outgrowth was observed. The cloudiness was attributed to cell debris from the original sample inoculation. The experimental cell material was incubated for an additional week beyond day 14. Bottles were then refrigerated for four days and examined. No turbidity was observed. Blood agar plates were streaked and incubated. No outgrowths were observed.

Runs 1, 2, 3, and 4 - Media Only (No Cells) Sterility Testing

All media from flasks (experimental and control with no cells) streaked onto MSA, S110 or blood agar plates were negative for outgrowths for all four runs.

APPROVAL

I accept responsibility for the conduct of this study which was performed in compliance with the U.S. FDA Good Laboratory Practice regulations (21 CFR 58) and in compliance with the Good Manufacturing Practices Regulations, Title 21 CFR 211 and 610.

________________________ ___________* see Page 52 of 53 for Approval Signature and Date

Anton F. Steuer, Ph.D. Date
Study Director

FIGURE 1

SAMPLES GENERATED FOR STERILITY AND MYCOPLASMA TESTS, RUN #1

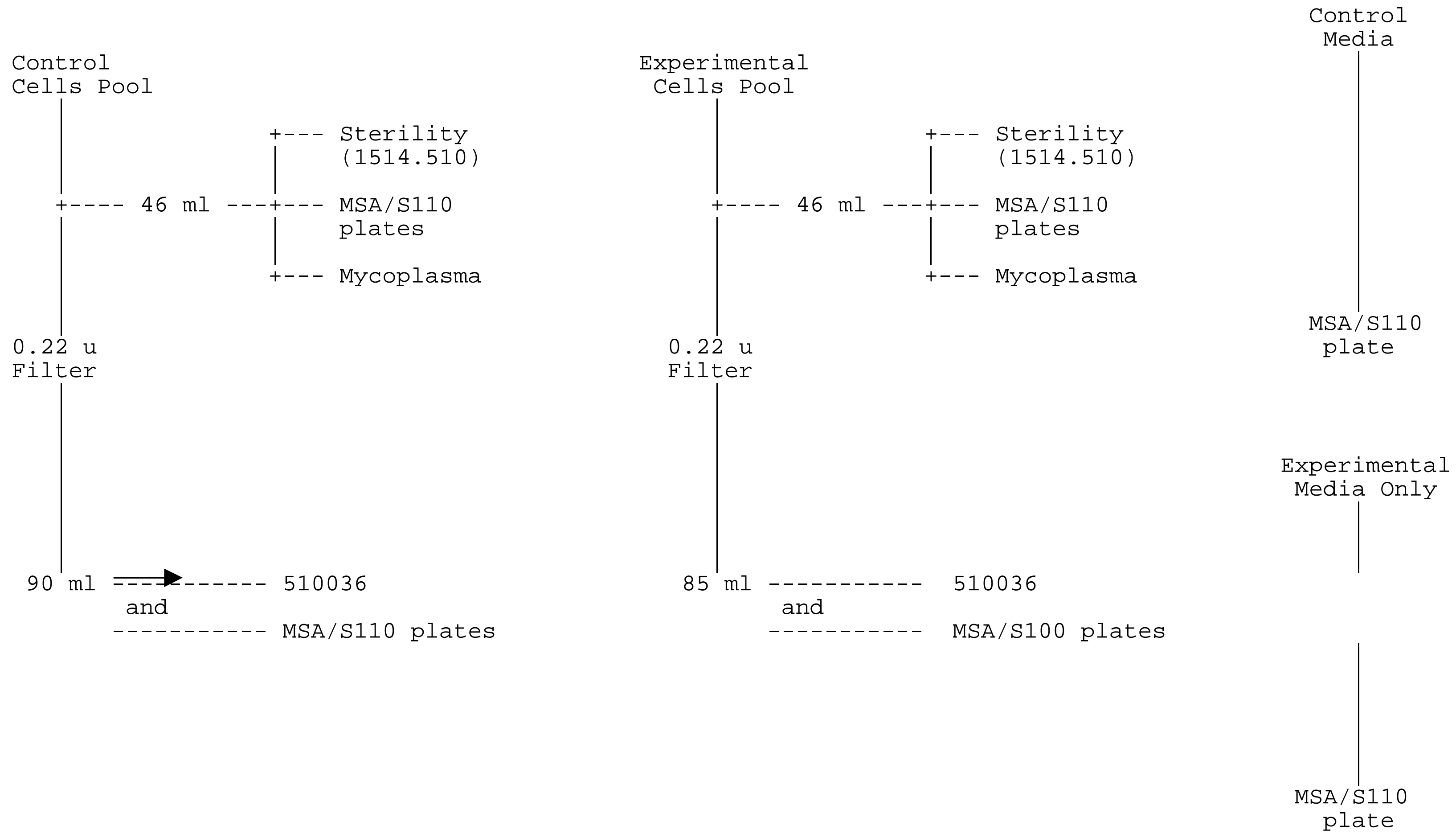
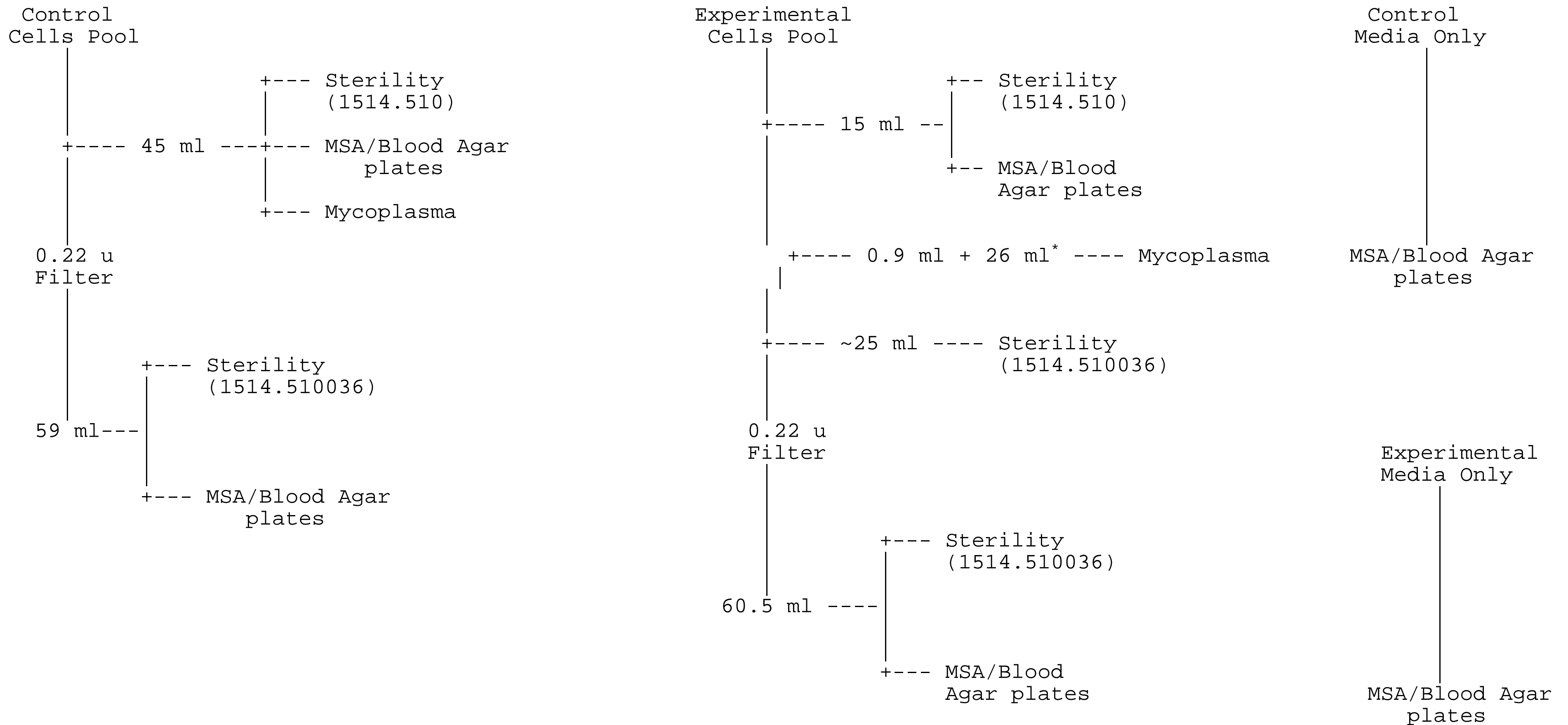


FIGURE 2

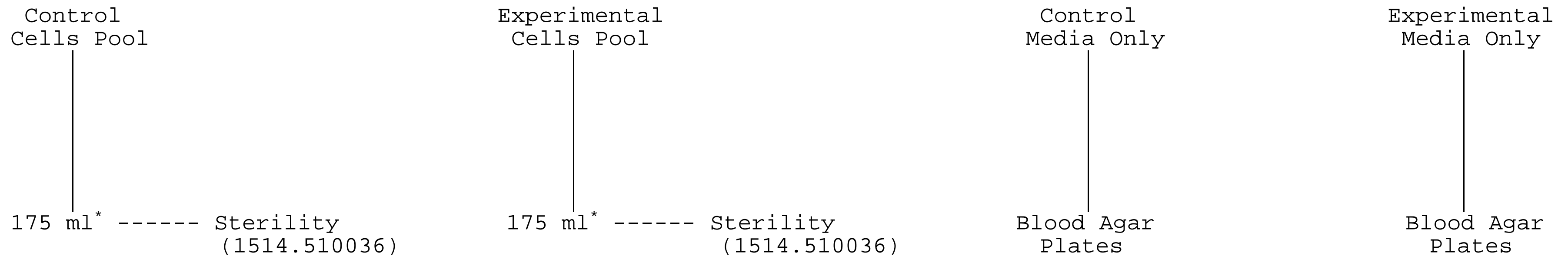
SAMPLES GENERATED FOR STERILITY AND MYCOPLASMA TESTS, RUN #2



* Mycoplasma sample inadvertently used for 510036 assay. Remaining prefiltered sample was diluted up with media and used for mycoplasma test.

FIGURE 3

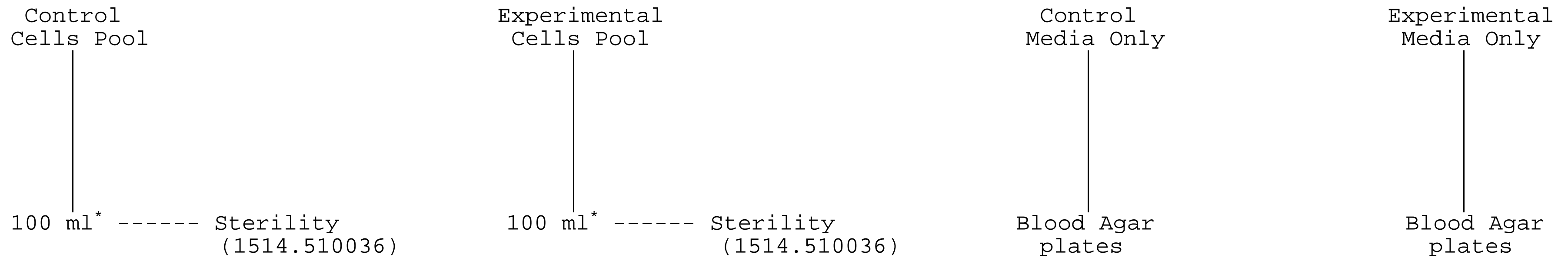
SAMPLES GENERATED FOR STERILITY TESTS - RUN #3



* No filtration was performed.

FIGURE 4

SAMPLES GENERATED FOR STERILITY TESTS - RUN #4



* No filtration was performed.

TABLE 1

STERILITY TEST (1514.510), RUN #1
ASSAY CONTROL OBSERVATIONS - TURBIDITY READINGS*

{PRIVATE }MEDIA	INOCULATIONS	OBSERVATIONS		
		DAY 4	DAY 7	DAY 14
THIO	*Bacillus subtilis*	0/2	2/2	2/2
	Candida albicans	0/2	2/2	2/2
	Bacteroides vulgatus	0/2	2/2	2/2
	Inoculated Negative	0/2	0/2	0/2
	Uninoculated Negative	0/2	0/2	0/2
TSB	*Bacillus subtilis*	0/2	2/2	2/2
	Candida albicans	0/2	2/2	2/2
	Inoculated Negative	0/2	0/2	0/2
	Uninoculated Negative	0/2	0/2	0/2
SAB:36±1°C	*Candida albicans*	0/2	2/2	2/2
	Inoculated Negative	0/2	0/2	0/2
	Uninoculated Negative	0/2	0/2	0/2
SAB:20-25°C	*Candida albicans*	0/2	2/2	2/2
	Inoculated Negative	0/2	0/2	0/2
	Uninoculated Negative	0/2	0/2	0/2
PYG	*Bacteroides vulgatus*	0/2	2/2	2/2
	Inoculated Negative	0/2	0/2	0/2
	Uninoculated Negative	0/2	0/2	0/2

* Number turbid/number of tubes inoculated

TABLE 2

STERILITY TEST (1514.510) - RUN #1
TURBIDITY READING (0.5 ML/TUBE INOCULATION)
EXPERIMENTAL CELLS

{PRIVATE } MEDIA	TURBIDITY READINGS*		
	DAY 4	DAY 7	DAY 14
THIO	0/2	0/2	0/2
TSB	0/2	0/2	0/2
SAB: 20-25°C	0/2	0/2	0/2
SAB: 36±1°C	0/2	0/2	0/2
PYG	0/2	0/2	0/2

* Number turbid/number of tubes inoculated

TABLE 3

STERILITY TEST (1514.510), RUN #1
TURBIDITY READINGS
(0.5 ML/TUBE INOCULATION
CONTROL CELLS

{PRIVATE } MEDIA	TURBIDITY READINGS*		
	DAY 4	DAY 7	DAY 14
THIO	0/2	0/2	0/2
TSB	0/2	0/2	0/2
SAB: 20-25°C	0/2	0/2	0/2

SAB: 36±1°C	0/2	0/2	0/2
PYG	0/2	0/2	0/2

* Number turbid/number of tubes inoculated

TABLE 4

STERILITY TEST (1514.510036), RUN #1
ASSAY CONTROL OBSERVATIONS - TURBIDITY READINGS*

{PRIVATE } MEDIA	INOCULATIONS	OBSERVATIONS		
		DAY 4	DAY 7	DAY 14
THIO 30-35°C	*Bacillus subtilis*	0/2	2/2	2/2
	Candida albicans	0/2	2/2	2/2
	Bacteroides vulgatus	0/2	2/2	2/2
	Inoculated Negative	0/2	0/2	0/2
	Uninoculated Negative	0/2	0/2	0/2
TSB 20-25°C	*Bacillus subtilis*	0/2	2/2	2/2
	Candida albicans	0/2	2/2	2/2
	Inoculated Negative	0/2	0/2	0/2
	Uninoculated Negative	0/2	0/2	0/2

* Number turbid/number of tubes inoculated

TABLE 5

STERILITY TEST (1514.510036), RUN #1
TURBIDITY READINGS*
EXPERIMENTAL CELLS

{PRIVATE }MEDIA INOCULATIONS	DAY 4	DAY 7	DAY 14
Tryptic Soy Broth @ 20-25°C	0/4	0/4	0/4
Fluid Thioglycollate broth @ 30 - 35°C	0/4	0/4	0/4

* Number turbid/Number inoculated

TABLE 6

STERILITY TEST (1514.510036), RUN #1
TURBIDITY READINGS*
CONTROL CELLS

{PRIVATE }MEDIA INOCULATIONS	DAY 4	DAY 7	DAY 14
Tryptic Soy Broth @ 20-25°C	0/4	0/4	0/4
Fluid Thioglycollate broth @ 30 - 35°C	0/4	0/4	0/4

* Number turbid/Number inoculated

TABLE 7
MSA AND S110 AGAR PLATE RESULTS* - RUN #1
DAY OF INCUBATION

{PRIVATE }	1		2		3		4		7		8		9		10		11		14	
	MSA	S110	MSA	S110	MSA	S110	MSA	S110	MSA	S110	MSA	S110	MSA	S110	MSA	S110	MSA	S110	MSA	S110
Prefiltered Control Cells	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Postfiltered Control Cells	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20
Prefiltered Exper. Cells	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Postfiltered Exper. Cells	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20
S. aureus Assay Positive Control	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2
Aerobic Control Media Only	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
Anaerobic Exper. Media Only	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
Assay Negative Control	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2

* Number of plates exhibiting outgrowth/Number of plates inoculated

TABLE 8, RUN #1
MYCOPLASMA TEST (1514.102003) RESULTS
CONTROL CELLS

{PRIVATE } Inoculum	Number Positive/Number Tested												
			Direct Plates[b]				Subculture Plates[b]						Vero Cell Assay
	Broth[a]		A Agar		B Agar		Sub I		Sub II		Sub III		
	Aer[c]	Anaer[d]	Aer	Anaer	Aer	Anaer	Aer	Anaer	Aer	Anaer	Aer	Anaer	
Neg. Control	0/1	0/1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/2
Pos. Control M. hyorhinis	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2/2
Pos. Control M. orale	1/1[e]	1/1[e]	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	2/2
Pos. Control M. pneumoniae	1/1[e]	1/1[e]	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	NA
Sample	0/1	0/1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

[a] Number of bottles with pH change (color shift)/number of bottles inoculated with 10 ml each.

[b] Number of plates with mycoplasma colonies/number of plates inoculated with 0.2 ml each.

[c] Aer: Aerobic Incubation

[d] Anaer: Anaerobic Incubation

[e] All of the colorshift subculture plates (three/bottle on each of type A and type B agar) inoculated from this bottle contained mycoplasma colonies.

TABLE 9, RUN #1
MYCOPLASMA (1514.102003) TEST RESULTS
EXPERIMENTAL CELLS

{PRIVATE } Inoculum	Number Positive/Number Tested												
			Direct Plates[b]				Subculture Plates[b]						Vero Cell Assay
	Broth[a]		A Agar		B Agar		Sub I		Sub II		Sub III		
	Aer[c]	Anaer[d]	Aer	Anaer	Aer	Anaer	Aer	Anaer	Aer	Anaer	Aer	Anaer	
Neg. Control	0/1	0/1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/2
Pos. Control M. hyorhinis	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2/2
Pos. Control M. orale	1/1[e]	1/1[e]	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	2/2
Pos. Control M. pneumoniae	1/1[e]	1/1[e]	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	NA
Sample	0/1	0/1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

[a] Number of bottles with pH change (color shift)/number of bottles inoculated with 10 ml each.

[b] Number of plates with mycoplasma colonies/number of plates inoculated with 0.2 ml each.

[c] Aer: Aerobic Incubation

[d] Anaer: Anaerobic Incubation

[e] All of the colorshift subculture plates (three/bottle on each of type A and type B agar) inoculated from this bottle contained mycoplasma colonies.

TABLE 10

STERILITY TEST (1514.510), RUN #2
ASSAY CONTROL OBSERVATIONS - TURBIDITY READINGS*

{PRIVATE }MEDIA	INOCULATIONS	OBSERVATIONS		
		DAY 5	DAY 7	DAY 14
THIO	*Bacillus subtilis*	2/2	2/2	2/2
	Candida albicans	2/2	2/2	2/2
	Bacteroides vulgatus	2/2	2/2	2/2
	Inoculated Negative	0/2	0/2	0/2
	Uninoculated Negative	0/2	0/2	0/2
TSB	*Bacillus subtilis*	2/2	2/2	2/2
	Candida albicans	2/2	2/2	2/2
	Inoculated Negative	0/2	0/2	0/2
	Uninoculated Negative	0/2	0/2	0/2
SAB:36±1°C	*Candida albicans*	2/2	2/2	2/2
	Inoculated Negative	0/2	0/2	0/2
	Uninoculated Negative	0/2	0/2	0/2
SAB:20-25°C	*Candida albicans*	2/2	2/2	2/2
	Inoculated Negative	0/2	0/2	0/2
	Uninoculated Negative	0/2	0/2	0/2
PYG	*Bacteroides vulgatus*	2/2	2/2	2/2
	Inoculated Negative	0/2	0/2	0/2
	Uninoculated Negative	0/2	0/2	0/2

* Number turbid/number of tubes inoculated

TABLE 11

STERILITY TEST (1514.510) - RUN #2
TURBIDITY READINGS* (0.5 ML/TUBE INOCULATION)
EXPERIMENTAL CELLS

{PRIVATE } MEDIA	TURBIDITY READINGS*		
	DAY 5	DAY 7	DAY 14
THIO	0/2	0/2	0/2
TSB	0/2	0/2	0/2
SAB: 20-25°C	0/2	0/2	0/2
SAB: 36±1°C	0/2	0/2	0/2
PYG	0/2	0/2	0/2

* Number turbid/number of tubes inoculated

TABLE 12

STERILITY TEST (1514.510), RUN #2
TURBIDITY READINGS*
(0.5 ML/TUBE INOCULATION)
CONTROL CELLS

{PRIVATE } MEDIA	TURBIDITY READINGS*		
	DAY 5	DAY 7	DAY 14
THIO	0/2	0/2	0/2
TSB	0/2	0/2	0/2
SAB: 20-25°C	0/2	0/2	0/2
SAB: 36±1°C	0/2	0/2	0/2

PYG	0/2	0/2	0/2

* Number turbid/number of tubes inoculated

TABLE 13

STERILITY TEST (1514.510036), RUN #2
ASSAY CONTROL OBSERVATIONS - TURBIDITY READINGS*

{PRIVATE } MEDIA	INOCULATIONS	OBSERVATIONS		
		DAY 5	DAY 7	DAY 14
THIO 30-35°C	*Bacillus subtilis*	2/2	2/2	2/2
	Candida albicans	2/2	2/2	2/2
	Bacteroides vulgatus	2/2	2/2	2/2
	Inoculated Negative	0/2	0/2	0/2
	Uninoculated Negative	0/2	0/2	0/2
TSB 20-25°C	*Bacillus subtilis*	2/2	2/2	2/2
	Candida albicans	2/2	2/2	2/2
	Inoculated Negative	0/2	0/2	0/2
	Uninoculated Negative	0/2	0/2	0/2

* Number turbid/number of tubes inoculated

TABLE 14

STERILITY TEST (1514.510036), RUN #2
TURBIDITY READINGS*
EXPERIMENTAL CELLS

{PRIVATE }MEDIA INOCULATIONS	DAY 5	DAY 7	DAY 14
Tryptic Soy Broth @ 20-25°C	0/4	0/4[1]	0/4
Fluid Thioglycollate broth @ 30 - 35°C	0/4	0/4[1]	0/4

* Number turbid/Number inoculated

[1] Particulate cloudiness noted in one bottle

TABLE 15

STERILITY TEST (1514.510036), RUN #2
TURBIDITY READINGS*
CONTROL CELLS

{PRIVATE }MEDIA INOCULATIONS	DAY 5	DAY 7	DAY 14
Tryptic Soy Broth @ 20-25°C	0/4	0/4	0/4
Fluid Thioglycollate broth @ 30 - 35°C	0/4	0/4	0/4

* Number turbid/Number inoculated

TABLE 16
MSA AND BLD AGAR PLATE RESULTS* - RUN #2
DAY OF INCUBATION

{PRIVATE }	1		4		5		6		7		8	
	MSA	BLD	MSA	BLD	MSA	BLD	MSA	BLD	MSA	BLD	MSA	BLD
Prefiltered Control Cells	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Postfiltered Control Cells	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20
Prefiltered Exper. Cells	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Postfiltered Exper. Cells	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20
S. aureus Assay Positive Control	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2
Aerobic Control Media Only	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
Anaerobic Exper. Media Only	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
Assay Negative Control	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2

{PRIVATE }	11		12		13		14		15‡	18‡	20‡	21‡
	MSA	BLD	MSA	BLD	MSA	BLD	MSA	BLD	MSA	MSA	MSA	MSA
Prefiltered Control Cells	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Postfiltered Control Cells	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20
Prefiltered Exper. Cells	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Postfiltered Exper. Cells	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20
S. aureus Assay Positive Control	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2
Aerobic Control Media Only	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2

Anaerobic Exper. Media Only	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
Assay Negative Control	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2

[*] Number of plates exhibiting outgrowth/Number of plates inoculated
[‡] At sponsor's request MSA plates were incubated for an additional week.

TABLE 17, RUN #2
MYCOPLASMA (1514.102003) TEST RESULTS
EXPERIMENTAL CELLS

{PRIVATE } Inoculum	Number Positive/Number Tested												
			Direct Plates[b]				Subculture Plates[b]						Vero Cell Assay
	Broth[a]		A Agar		B Agar		Sub I		Sub II		Sub III		
	Aer[c]	Anaer[d]	Aer	Anaer	Aer	Anaer	Aer	Anaer	Aer	Anaer	Aer	Anaer	
Neg. Control	0/1	0/1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/2
Pos. Control M. hyorhinis	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2/2
Pos. Control M. orale	1/1[e]	1/1[e]	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	2/2
Pos. Control M. pneumoniae	1/1[e]	1/1[e]	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	NA
Sample	0/1	0/1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

[a] Number of bottles with pH change (color shift)/number of bottles inoculated with 10 ml each.

[b] Number of plates with mycoplasma colonies/number of plates inoculated with 0.2 ml each.

[c] Aer: Aerobic Incubation

[d] Anaer: Anaerobic Incubation

[e] All of the colorshift subculture plates (three/bottle on each of type A and type B agar) inoculated from this bottle contained mycoplasma colonies.

TABLE 18, RUN #2
MYCOPLASMA (1514.102003) TEST RESULTS
EXPERIMENTAL CELLS

{PRIVATE } Inoculum	Number Positive/Number Tested												
			Direct Plates[b]				Subculture Plates[b]						Vero Cell Assay
	Broth[a]		A Agar		B Agar		Sub I		Sub II		Sub III		
	Aer[c]	Anaer[d]	Aer	Anaer	Aer	Anaer	Aer	Anaer	Aer	Anaer	Aer	Anaer	
Neg. Control	0/1	0/1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/2
Pos. Control M. hyorhinis	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2/2
Pos. Control M. orale	1/1[e]	1/1[e]	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	2/2
Pos. Control M. pneumoniae	1/1[e]	1/1[e]	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	NA
Sample	0/1	0/1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

[a] Number of bottles with pH change (color shift)/number of bottles inoculated with 10 ml each.

[b] Number of plates with mycoplasma colonies/number of plates inoculated with 0.2 ml each.

[c] Aer: Aerobic Incubation

[d] Anaer: Anaerobic Incubation

[e] All of the colorshift subculture plates (three/bottle on each of type A and type B agar) inoculated from this bottle contained mycoplasma colonies.

TABLE 19

STERILITY TEST (1514.510036), RUN #3
ASSAY CONTROL OBSERVATIONS - TURBIDITY READINGS*

{PRIVATE } MEDIA	INOCULATIONS	OBSERVATIONS		
		DAY 4	DAY 7	DAY 14[1]
THIO 30-35°C	*Bacillus subtilis*	1/2	2/2	2/2
	Candida albicans	2/2	2/2	2/2
	Bacteroides vulgatus	0/2	0/2	2/2
	Inoculated Negative	0/2	0/2	0/2
	Uninoculated Negative	0/2	0/2	0/2
TSB 20-25°C	*Bacillus subtilis*	2/2	2/2	2/2
	Candida albicans	0/2	0/2	2/2
	Inoculated Negative	0/2	0/2	0/2
	Uninoculated Negative	0/2	0/2	0/2

* Number turbid/number of tubes inoculated

[1] Bottles transferred to refrigerator on day 14

TABLE 20

STERILITY TEST (1514.510036), RUN #3
TURBIDITY READINGS*
EXPERIMENTAL CELLS

{PRIVATE }MEDIA INOCULATIONS	DAY 4	DAY 7	DAY 14[1]
Tryptic Soy Broth @ 20-25°C	0/4	0/4	0/4
Fluid Thioglycollate broth @ 30 - 35°C	0/4	0/4	0/4

* Number turbid/Number inoculated
[1] Bottles transferred to refrigerator on day 14.
On day 15, one of the THIO bottles exhibited turbidity.

TABLE 21

STERILITY TEST (1514.510036), RUN #3
TURBIDITY READINGS*
CONTROL CELLS

{PRIVATE }MEDIA INOCULATIONS	DAY 4	DAY 7	DAY 14[1]
Tryptic Soy Broth @ 20-25°C	0/4	0/4	0/4
Fluid Thioglycollate broth @ 30 - 35°C	0/4	0/4	0/4

* Number turbid/Number inoculated
[1] Bottles transferred to refrigerator on day 14.

TABLE 22

STERILITY TEST (1514.510036), RUN #4
ASSAY CONTROL OBSERVATIONS - TURBIDITY READINGS*

{PRIVATE } MEDIA	INOCULATIONS	OBSERVATIONS		
		DAY 4	DAY 7	DAY 14
THIO 30-35°C	*Bacillus subtilis*	0/2	2/2	2/2
	Candida albicans	2/2	2/2	2/2
	Bacteroides vulgatus	0/2	2/2	2/2
	Inoculated Negative	0/2	0/2	0/2
	Uninoculated Negative	0/2	0/2	0/2
TSB 20-25°C	*Bacillus subtilis*	0/2	2/2	2/2
	Candida albicans	2/2	2/2	2/2
	Inoculated Negative	0/2	0/2	0/2
	Uninoculated Negative	0/2	0/2	0/2

* Number turbid/number of tubes inoculated

TABLE 23

STERILITY TEST (1514.510036), RUN #4
TURBIDITY READINGS*
EXPERIMENTAL CELLS

{PRIVATE }MEDIA INOCULATIONS	DAY 4	DAY 7	DAY 14	DAY 21
Tryptic Soy Broth @ 20-25°C	0/4	0/4[1]	0/4[1]	0/4
Fluid Thioglycollate broth @ 30 - 35°C	0/4	0/4[1]	0/4[1]	0/4

* Number turbid/Number inoculated
[1] Particulate cloudiness observed

TABLE 24

STERILITY TEST (1514.510036), RUN #4
TURBIDITY READINGS*
CONTROL CELLS

{PRIVATE }MEDIA INOCULATIONS	DAY 4	DAY 7	DAY 14
Tryptic Soy Broth @ 20-25°C	0/4	0/4[1]	0/4[1]
Fluid Thioglycollate broth @ 30 - 35°C	0/4	0/4[1]	0/4

* Number turbid/Number inoculated
[1] Particulate cloudiness observed

QA STATEMENT - *see Page 53 of 53 for Quality Assurance Statement

DATE: March 22, 1996

TEST ARTICLE I.D.: RT-HCMV endothelial cells

MA STUDY NO.: R96AE40.510002

PROTOCOL NO.: 1514.510002

PROTOCOL TITLE: EVALUATION OF A PROCESS FOR GENERATING BACTERIA DE NOVO FROM EUKARYOTIC CELLS

SECTION TO BE AMENDED: 6.1

AMENDMENT: Add the following: Prior to cell seeding all cell culture flasks will be pretreated for 15 minutes (followed by 1X PBS wash) with 1% gelatin (2% gelatin solution from Sigma diluted with an equal volume of sterile TC grade water).

REASON FOR AMENDMENT: Sponsor request

SECTION TO BE AMENDED: 6.2

AMENDMENT: A 72 hour incubation will be performed at 36°C ± 2°C instead of 30°C ± 2°C.

REASON FOR AMENDMENT: Sponsor request

SECTION TO BE AMENDED: 6.2

AMENDMENT: Approximately 24 and 48 hours after initiation of the experiment, the chamber will be opened at room temperature, and the flask caps will be sealed. The flasks will be observed microscopically and observations will be recorded. These observations will be performed within 10 minutes. The flasks will then be placed (not stacked) in a laminar flow hood (with fluorescent light on) for approximately 30 minutes and after 30 minutes the caps will be loosened and the flasks will remain in the hood for another 2-3 minutes. The flasks will be transferred to the anaerobic chamber, which will be sealed and flushed with nitrogen gas for approximately 1-1 1/2 hours until the effluent gas measures 0% oxygen. The tubing to the chamber will then be clamped off. The chamber will be placed back into the incubator. The control flasks will also be manipulated in the same manner and returned (not in the anaerobic chamber) to the incubator.

REASON FOR AMENDMENT: Sponsor request

R96AE40.510002

SECTION TO BE AMENDED: 6.2

AMENDMENT: Standard caps (no filter) will be used instead of vented caps.

REASON FOR AMENDMENT: Sponsor request

SECTION TO BE AMENDED: 6.3

AMENDMENT: The cell cultures will be vortexed vigorously for approximately two minutes after scraping and pooling. Control flasks will be treated in the same manner.

REASON FOR AMENDMENT: Sponsor request

SECTION TO BE AMENDED: 6.2

AMENDMENT: Cell cultures will be washed thoroughly one time with F12 medium (no supplements) prior to the addition of medium 199 and Ham's F12 (1:1 proportions) supplemented with 80 mM DMSO, 11 mM fructose, 35 mM succinate, and 800 mg glutamate/l.

REASON FOR THE AMENDMENT: Sponsor request

SECTION TO BE AMENDED: 6.3

AMENDMENT: Blood agar plates can be used instead of S110 plates.

REASON FOR THE AMENDMENT: Sponsor request

APPROVAL:

SPONSOR REPRESENTATIVE/INVESTIGATOR DATE

STUDY DIRECTOR DATE

DATE: April 22, 1996

TEST ARTICLE I.D.: RT-HCMV endothelial cells

MA STUDY NO.: R96AE40.510002

PROTOCOL NO.: 1514.510002

PROTOCOL TITLE: EVALUATION OF A PROCESS FOR GENERATING BACTERIA DE NOVO FROM EUKARYOTIC CELLS

SECTION TO BE AMENDED: 6.3.

AMENDMENT: Replace Section 6.3 with the following:

After undergoing the conditions described in Section 6.2, cell cultures will be removed by scraping. The harvested cells will be pooled and vortexed vigorously for approximately two minutes. Control flasks will be treated in the same manner. The samples will be submitted to the sterility lab for testing according to protocol 1514.510036. All cell culture manipulations will be performed with aseptic technique in a biological safety cabinet.

REASON FOR AMENDMENT: Sponsor request.

APPROVAL:

______________________ __________

SPONSOR REPRESENTATIVE/INVESTIGATOR DATE

______________________ __________

STUDY DIRECTOR DATE

DATE: June 3, 1996

TEST ARTICLE I.D.: RT-HCMV endothelial cells

MA STUDY NO.: R96AE40.510002

PROTOCOL NO.: 1514.510002

PROTOCOL TITLE: EVALUATION OF A PROCESS FOR GENERATING BACTERIA DE NOVO FROM EUKARYOTIC CELLS

SECTION TO BE AMENDED: 6.0

AMENDMENT: In the first sentence change three times to four times.

REASON FOR AMENDMENT: Sponsor requested four experiments instead of three.

SECTION TO BE AMENDED: 6.1

AMENDMENT: Four control and four experimental T-162 cm^2 (NUNC) flasks will be used for the fourth experiment.

REASON FOR AMENDMENT: Sponsor request

SECTION TO BE AMENDED: 6.2 (Protocol Amendment #1)

AMENDMENT: For experiment #4 delete Section 6.2 of Protocol Amendment #1 and replace with Section 6.2 of original protocol with the exception of keeping the incubator temperature at 36°C ± 2°C instead of 30°C ± 2°C.

REASON FOR AMENDMENT: Sponsor request

APPROVAL:

SPONSOR REPRESENTATIVE/INVESTIGATOR DATE

STUDY DIRECTOR DATE

DATE: December 23, 1996

TEST ARTICLE I.D.: RT-HCMV endothelial cells (Source: ATCC)

MA STUDY NO.: R96AE40.510002

PROTOCOL NO.: 1514.510002

PROTOCOL TITLE: EVALUATION OF A PROCESS FOR GENERATING BACTERIA DE NOVO FROM EUKARYOTIC CELLS

SECTION TO BE AMENDED: 6.3

AMENDMENT: Delete "(see protocol amendment to 1514.510)" from the fourth sentence in the paragraph.

REASON FOR AMENDMENT: Typographical error.

APPROVAL:

STUDY DIRECTOR DATE

DATE: December 23, 1996

TEST ARTICLE I.D.: RT-HCMV endothelial cells (Source: ATCC)

MA STUDY NO.: R96AE40.510002

PROTOCOL NO.: 1514.510002

PROTOCOL TITLE: EVALUATION OF A PROCESS FOR GENERATING BACTERIA DE NOVO FROM EUKARYOTIC CELLS

CLARIFICATION ON ORIGINAL PROTOCOL AND PROTOCOL AMENDMENTS NUMBERS 1, 2, AND 3

1. Original Protocol was followed for run #1.

2. Prtoocol Amendment #1 was in effect for runs # 2, #3, and #4 with the exceptions noted in Protocol Amendments #2 and #3.

3. Protocol Amendment #2 was in effect for run #3.

4. Protocol Amendment #3 was in effect for run #4.

APPROVAL

I accept responsibility for the conduct of this study which was performed in compliance with the U.S. FDA Good Laboratory Practice regulations (21 CFR 58) and in compliance with the Good Manufacturing Practices Regulations, Title 21 CFR 211 and 610.

Anton F. Steuer

Anton F. Steuer, Ph.D.
Study Director

12/24/96
Date

QUALITY ASSURANCE STATEMENT

Study Title: EVALUATION OF A PROCESS FOR GENERATING BACTERIA DE NOVO FROM EUKARYOTIC CELLS

Study Number: R96AE40.510002

Study Director: Anton F. Steuer, Ph.D.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Compliance Programme, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 23 FEB 96, TO STUDY DIR 23 FEB 96, TO MGMT 01 MAR 96
PHASE: Protocol Review

INSPECT ON 11 MAR 96, TO STUDY DIR 11 MAR 96, TO MGMT 17 SEP 96
PHASE: Observation for evidence of growth

INSPECT ON 04 APR 96, TO STUDY DIR 04 APR 96, TO MGMT 24 DEC 96
PHASE: Observation of broth/agar

INSPECT ON 04 NOV 96-07 NOV 96, TO STUDY DIR 08 NOV 96. TO MGMT 24 DEC 96
PHASE: Final Report

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Ed Warburton for L. St. John — 12/24/96

Loretta St. John
QUALITY ASSURANCE — DATE

MICROBIOLOGICAL ASSOCIATES, INC.