Study code: PCDL-0603

FINAL REPORT

Bacterial reverse mutation (Ames) test

of Pueraria mirifica, PE

Initiation of the study: February 17, 2006

Experimental period: from February 20 to March 10, 2006

Sponsor:
COFOPEX Ltd.
H-1022 Budapest,
Bimbó út 92.
Contact Person:
István Bara

Study to be performed at:
Pharmaceutical Control and
Development Laboratory Co. Ltd.
H-1149 Budapest, Mexikói út 9.
Contact Person:
János Horváth

This report consists of 23 pages plus 1 attachment.

2006

Staff in Charge

	Signature	Date
Director of the Laboratory:	István Financsek M.D., Ph.D.	03-04-2006
Study Director:	János Horváth M.Sc. food industry engineer, engineer of food safety	03.04.2006.
Deputy Study Director:	Andrea Mózes biologist	03-04-2006.
Quality Assurance Unit:	Anikó Kövér, M.Sc. bioengineer	April 3, 2006
Sponsor:	István Bara Managing Director COFOPEX Ltd.	03.04.2006
Monitoring Scientist:	Alexander G. Schauss Ph.D. (AIBMR Life Sciences)	April 19, 2006

Bacterial reverse mutation (Ames) test of Pueraria mirifica, PE

Study code: PCDL-0603

SUMMARY

General information:

A plate incorporating method was used in the test with 5 doses in triplicates on four Salmonella (TA98, TA100, TA1535, TA1537) and one E. coli (WP2 uvrA) tester strains, with and without S9 activation. After Cytotoxicity test a Definitive assay and a confirmatory Repeat assay were made. Doses of test article were up to 5000 μg/plate. Solvent (distilled water) and suitable Positive controls were used. The plates were counted after 3 days incubation.

Evaluation:

There were no revertants exceeding three times the background average either with or without metabolic activation, and there was no dose-related increase over the range tested, so this study gave negative result to Puraria mirifica, PE as test article.

The results of Definitive assay showed that the test article had no mutagenic effect to any strain used in this test. The results of the Repeat assay confirmed the negative results of the Definitive assay.

Bacterial reverse mutation (Ames) test of Pueraria mirifica, PE

Study code: PCDL-0603

Statement of Study Director

I hereby certify that this study report provides a true and complete record of the data generated and that the study was conducted in accordance with the Principles of Good Laboratory Practice as set forth in the following documents:

1. US Food and Drug Administration Title 21, Code of Federal Regulations, Part 58 Good Laboratory Practice Regulations for Nonclinical Laboratory Studies
2. Good Laboratory Practice Regulations (National GLP, Joint Decree 9/2001. (III.30) EüM-FVM)
3. OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98)17 as revised in 1997);

There were no significant deviations from the aforementioned regulations which affected the quality or integrity of the study or the interpretation of the results in the report. A few minor deviations from the Study Protocol are listed in 1.5 in the present Report.

Date: 03. 04. 2006. Signature:

János Horváth, M.Sc.

Study Director

Bacterial reverse mutation (Ames) test of Pueraria mirifica, PE

Statement of the Quality Assurance Unit

This study has been inspected and the report audited by the Quality Assurance Unit of PCDL in compliance with the Principles of Good Laboratory Practice. As far as it can be reasonably established, the methods described and the results incorporated int he report accurately reflect the raw data produced during this study.

Inspections concerning adherence to the protocol were performed:

Date of Inspection / Audit	Type or Phase of Inspection	Date of Report to the	
		Study Director	Management
February 16, 2006	Protocol audit	February 16, 2006	February 16, 2006
February 20, 2006	Preparation of inoculums.	February 21, 2006	February 22, 2006
February 21, 2006	Checking the density of the inoculums	February 21, 2006	February 22, 2006
February 21, 2006	Experimental procedure of the Cytotoxicity test.	February 22, 2006	February 22, 2006
February 21, 2006	Checking tester strains.	February 22, 2006	February 22, 2006
February 22, 2006	Evaluation of checking tester strains	February 22, 2006	February 22, 2006
February 23, 2006	Preparation of media	February 23, 2006	February 24, 2006
February 24, 2006	Counting procedure	February 24, 2006	February 24, 2006
February 28, 2006	Experimental procedure of the Definitive assay	February 28, 2006	February 28, 2006
February 28, 2006	Checking tester strains	February 28, 2006	February 28, 2006
March 01, 2006	Evaluation of checking tester strains.	March 01, 2006	March 02, 2006
March 07, 2006	Experimental procedure of the Repeat assay	March 08, 2006	March 08, 2006
March 30, 2006	Draft report audit	March 31, 2006	March 31, 2006

Date: April 3, 2006 Signature:

Anikó Kövér, M.Sc.
bioengineer
Quality Assurance Unit at PCDL

Table of contents

1. General information

1.1. Title of the study

Bacterial reverse mutation (Ames) test of Pueraria mirifica, PE.

Initiation of the study: February 17, 2006

Experimental period: from February 20 to March 10, 2006

1.2. Objective of the study

The objective of this test was to evaluate the ability of the test article to induce mutagenic response in four strains of Salmonella typhimurium (TA98, TA100, TA1535, and TA1537) and one strain of Escherichia coli [WP2 (uvrA)]. The test article was plated in triplicates, at five concentrations both in the presence and absence of S9 metabolic activation. A cytotoxicity assessment was performed prior to the definitive study, and an independent repeat assay was conducted, to confirm the negative results.

1.3. Type of the study

Preclinical toxicology study in compliance with the principles of the

- Good Laboratory Practice Regulations for Nonclinical Laboratory Studies of the United States Food and Drug Administration, (21 CFR 58)
- Good Laboratory Practice Regulations (National GLP, Joint Decree 9/2001.(III.30) EüM-FVM) and
- OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98)17 as revised in 1997);

The study was set up according to the OECD GUIDELINE FOR TESTING OF CHEMICALS (Guideline No.: 471, adopted: 21st July 1997, Bacterial Reverse Mutation Test.)

1.4. Introduction

The reverse mutation assay (Ames test) is used to evaluate the mutagenic properties of test articles. The test uses histidine-dependent strains of S. typhimurium and tryptophan-dependent strain of E. coli. In the absence of an external histidine or tryptophan source, the cells cannot grow to form colonies. Colony growth is evident if a reversion of the mutation occurs, allowing the production of histidine or tryptophan to be resumed. Spontaneous reversions occur with each of the strains; mutagenic compounds cause an increase in the number of revertant colonies relative to the background level.

1.5. Deviations

There were two deviations in this study:

- In the Definitive assay the test article was formulated 55 minutes earlier before experimental procedures instead of maximum 30 minutes.
- In the Repeat assay the dose of 2-NF was 10 times lower than in the Definitive assay.

2. Test article

Name:	**Pueraria mirifica, PE**
Specification:	powder from root of Pueraria mirifica
Manufacturer:	Bio-Botanica, Inc.
	75 Commerce Drive, Hauppauge, NY 11788
Lot number:	031615
Identification number at PCDL:	2006/01812
Appearance:	Light beige, free-flowing powder
Package form:	plastic sac (sealed), 500g
Storage conditions:	cool, dry
Expiration date:	02.2007

For ingredients see Certificate of Analysis attached!

2.1. Microbiological analysis

The test article was subjected to microbial limit test, plate-count method, according to the USP29. Because there was no data on the stability of the test article (suspension in water) during autoclaving, it is was not possible to have sterile material. The low total plate count was not disturb the assay and the counting procedure.

2.2. Chemical analysis

Certificate of Analysis provided by the Sponsor is attached to this Final Report. Composition of the mixture and the analytical control are the Sponsor's responsibility.

2.3. Stability control of the test article

Stability control of the test article is the Sponsor's responsibility. The product itself was considered to be stable.

3. Test System

3.1. Test System Description

All Salmonella strains are histidine-dependent. Revertants was identified as colonies that grew in low levels of histidine. The E. coli strain is tryptophan-dependent. Revertants was identified as colonies that grew in low levels of tryptophan. Frameshift and base-pair substitution defects are represented to identify mutagens of both types. Additional genetic markers enhance sensitivity of the strains to certain types of mutagens. The DNA repair mutations (uvrB and uvrA) eliminate excision repair, a repair pathway for DNA damage from UV light and certain chemical mutagens. The uvrB mutation, presents in strains TA98, TA100, TA1535 and TA1537, and the uvrA mutation, presents in strain WP2 (uvrA), was indicated by sensitivity to UV light. The rfa mutation changes the properties of the bacterial cell wall, increasing the permeability of cells to certain types of chemicals. The rfa mutation, presented in all Salmonella strains, was indicated by sensitivity to crystal violet. The R factor plasmid (pKM101) presents in strains TA98 and TA100 makes them more responsive to a variety of mutagens. The plasmid carries an ampicillin resistance gene therefore ampicillin resistance indicated that the strains retain the plasmid.

3.2. Test System Justification

The five strains of bacteria that was used in this assay were among those recommended by OECD Guideline 471 for use in the Ames test. These five strains of S. typhimurium and E. coli have been shown to be reliably and reproducibly responsive between laboratories.

3.3. Source and Storage of Test System

The Salmonella and E. coli strains used in this study were obtained from Xenometrix GmbH (Gewerbestrasse 25, CH-4123 Allschwill), are maintained as frozen stocks.

3.4. Identification of Test System

Strains was identified by having certain characteristics (see Table 1). The strains also yield spontaneous revertant colony plate counts within the frequency ranges stated in the historical control data.

Table 1. Characteristics of Salmonella and E. coli strains

Strain	Gene Affected	DNA repair	LPS	Biotin requirement	Plasmids	Mutational event
TA98	*hisD*	*uvrB*	*rfa*	*bio-*	*pKM101*	frameshift
TA100	*hisG*	*uvrB*	*rfa*	*bio-*	*pKM101*	base-pair substitution
TA1535	*hisG*	*uvrB*	*rfa*	*bio-*	-	base-pair substitution
TA1537	*hisC*	*uvrB*	*rfa*	*bio-*	-	frameshift
WP2 (uvrA)	***trp***	***uvrA***	-	-	-	base-pair substitution

3.5. Preparation of Overnight Cell Cultures

Frozen stock cultures (stored at –80°C ± 5°C) were grown overnight at 37 ± 2 °C, with shaking, in nutrient broth until a cell density of about 10^9 cells/ml was obtained (determined by optical density). Cells were maintained at room temperature until use and during the test.

3.6. Control of Bias

In order to control bias, for each day of test system treatment, all test article doses, as well as controls, were plated against cells from a single flask.

4. Vehicle

Distilled water was used as vehicle without detergent (Tween 80). The vehicle did not affect the spontaneous mutation level and was recommended for use in this test.

4.1. Formulation of the test article

The amount of the test article (≈500mg) was weighted and suspended in sterile, distilled water not earlier than 30 min before experimental procedures. For dilution to final doses sterile distilled water was used. To enhance solubility and to obtain a homogenous suspension before treatment, vortexing was used.

5. Dose Levels

5.1. Doses Used in the Cytotoxicity Assessment

A cytotoxicity assessment was performed to determine the appropriate dose range for the definitive assay. Test doses (5, 10, 50, 100, 500, 1000 and 5000 µg/plate, along with negative controls, were plated against strain TA100, in duplicates, both with and without S9 activation, as described in Section 9, Experimental Procedures.

Toxicity is suggested by the absence of a confluent bacterial lawn, the presence of pinpoint colonies, and/or a substantial decrease or lack of revertant colonies.

5.2. Doses Used in the Definitive Assay

Five dose levels of test article were evaluated in the Definitive test in triplicates. The test concentrations for the non-cytotoxic test article were 50, 100, 500, 1000 and 5000 µg/plate.

5.3. Doses Used in the Independent Repeat Assay

As the Definitive assay yielded negative results, according to OECD guideline 471 an independent Repeat assay was performed. The above guideline suggests that study parameters should be modified. We have modified the concentration of S9 in S9/Cofactor Mix (from 4% up to 10%).

6. Metabolic Activation

6.1. S9 Fraction

Aroclor™ 1254-induced male Sprague-Dawley rat liver S9 (MOLTOX), was purchased from a commercial supplier (Trinova Biochem GmbH, Kerkrader Strasse 10. D-35394, Giessen).

6.2. S9/Cofactor Mix

The S9/cofactor mix contained: 4% S9, 8 mM magnesium chloride, 33 mM potassium chloride, 5 mM D-glucose-6-phosphate, and 4 mM nicotinamide adenine dinucleotide phosphate, in a 100 mM sodium phosphate buffer, pH 7.4. It was kept on ice during the experiment. The S9/cofactor mix contained 10% S9 in the Repeat assay.

6.3. Buffer

When S9 mix was not used in the test, sodium phosphate buffer pH 7.4 was added.

7. Tester Strain Media

7.1. Nutrient Broth

The broth used for the overnight cultures was 2.5% Oxoid Nutrient Broth #2.

7.2. Minimal glucose agar plates

The plates contained 1.5% agar and were supplemented with 2.0% glucose and 2.0% Vogel-Bonner buffer.

7.3. Top Agar

Top agar (0.6% agar and 0.6% NaCl) was supplemented with a 0.5 mM solution of histidine and biotin (for Salmonella) or tryptophan (for E. coli).

7.4. Other materials

All the materials were used in this study are listed below in Table 2. These materials were prepared in our laboratory.

Table 2: List. of media and solutions prepared

Name of media or solution	Lot No.
Oxoid Nutrient Broth #2	A004
Minimal glucose plate	A021, A024, A031
Top agar	A002
0.5mM Histidine/Biotin	A011
0.5mM Tryptophan	A013
S9/cofactor mix	A023, A029, A035
0.2M sodium phosphate buffer pH 7.4	A009
Sterile distilled water	A007
Sterile DMSO	A015
Histidine/Biotin plate (for control of strains)	A020, A025, A032
Tryptophan plate (for control of strain WP2)	A027, A034
Nutrient agar (for control of strains)	A003
0.1% Cristal violet (for control of strains)	A017
Ampicillin plate (for control of strains)	A022, A026, A033

8. Controls

8.1. Positive Controls (Table 3.)

Strains were tested with mutagens to demonstrate that the assay was working effectively and also to demonstrate that the metabolic activation system was operating.

Table 3.: Positive Controls

Strain	Positive Controls (without S9 activation)	Positive Controls (with S9 activation)
TA 98	2-Nitrofluorene (2NF) CAS# 607-57-8	Benzo(a)pyrene (BP) CAS# 50-32-8
TA100	Sodium-azide (NaN_3) CAS# 26628-22-8	2-Aminoanthracene (2AAn) CAS# 613-13-8
TA1535	Sodium-azide (NaN_3) CAS# 26628-22-8	2-Aminoanthracene (2AAn) CAS# 613-13-8
TA1537	9-Aminoacridine (9AA) CAS# 52417-22-8	Benzo(a)pyrene (BP) CAS# 50-32-8
WP2	Methyl-methansulfonate (MMS) CAS# 66-27-3	2-Aminoanthracene (2AAn) CAS# 613-13-8

Concentrations:

No activation: 2-Nitrofluorene 2.5 µg/plate (0.25 in the Repeat assay) TA98

Sodium-azide 1.5 µg/plate TA100, TA1535

9-Aminoacridine 10 µg/plate (50 µg/plate in the Repeat assay) TA1537

Methyl-methanesulfonate 2.5 µg/plate WP2

Activation: Benzo(a)pyrene 20 µg/plate TA98, TA1537

2-Aminoanthracene 10 µg/plate TA100, TA1535, WP2

9. Experimental Procedures for the Definitive Assay

In all cases the plate incorporation test procedure was used. For the Definitive assay, the following was added to each sterile culture tube containing 2.0 ml top agar: 0.1 ml of overnight cell culture, 0.1 ml of test or control article, and either 0.5 ml S9/cofactor or 0.5 ml phosphate buffer pH 7.4. The contents were vortexed, poured onto Minimal glucose plates, and evenly distributed. The agar was allowed to harden and the plates were inverted and incubated at 37 °C ± 2 °C for 72 ± 4 hours prior to scoring.

10. Experimental Procedures for Independent Repeat Assay

The Repeat assay was conducted as outlined above in Section 9, with the following modification: the S9 concentration in the S9/cofactor mix was increased to 10%. Concentration range was not modified.

11. Counting Procedure

Hand Counting: all of the plates for all concentrations were counted by hand (for all tester strains).

12. Data Provided

Experimental data are presented as the number of revertant colonies per plate. For the Cytotoxicity analysis, individual plate counts and the mean number of revertant colonies are provided in tabular form. For the Definitive and Repeat assays, individual plate counts, the mean number of revertant colonies are presented in tabular form for the test article and positive and negative controls. There was no significance to make graphs of the results.

13. Evaluation and Interpretation of Results

13.1. Criteria for a Valid Assay

The study is considered valid, because the following criteria were met:

- Tester strains TA98, TA100, TA1535, TA1537, and WP2 (uvrA) exhibited sensitivity to UV light.
- All Salmonella tester strains exhibited sensitivity to crystal violet.
- Tester strains TA98 and TA100 exhibited resistance to ampicillin.
-Tester strains exhibited a characteristic number of spontaneous revertant colonies when plated.
- Tester strains exhibited at least a three-fold increase in mutagen-induced revertant colonies (two-fold for strain TA100) when plated with positive control chemicals.

13.2. Statistical Analysis of the Data

There was no test article related increase in average number of revertant colonies relative to the negative control, dose-related increase has not occurred, so it was not reasonable to make statistical (regression) analysis.

13.3. Determining Mutagenicity

A test article is considered positive if the background average were below six colonies, the average number of revertants for any test article dose exceeds 20 colonies/plate.

A positive result would have indicated that the test article induces mutations in Salmonella typhimurium or Escherichia coli cells.

The negative results obtained in this study indicate that under the test conditions, the test article did not produce mutations in test cells.

14. Records Maintained

The data obtained in the course of the study are collected in a Study File. The Study Protocol, all data generated during and as a result of the study, the documents and all information in connection with the study, a control sample of the test article, and the Final Report will be stored at least for 15 years in the Archives of the PCDL then will be offered to the Sponsor.

15. Schedule of the study

Cytotoxicity Assessment	February 20-24, 2006
Definitive Assay	February 27-March 3, 2006
Repeat Assay	March 6-10, 2006

16. Results

16.1 Cytotoxicity test

Table 4. Results of Cytotoxicity test

Test article	Dose [μg/plate)	S. typhimurium TA 100 Revertant colonies per plate [**Mean** ± S.D.]	
		without activation	with 4% S9 activation
Pueraria mirifica, PE	0*	197 199 204 [**200** ± 3.6]	161 164 142 [**156** ± 11.9]
	5	175 186 [**181** ± 7.8]	151 168 [**160** ± 12.0]
	10	195 182 [**189** ± 9.2]	174 180 [**177** ± 4.2]
	50	186 187 [**187** ± 0.7]	185 156 [**171** ± 20.5]
	100	182 178 [**180** ± 2.8]	164 163 [**164** ± 0.7]
	500	198 177 [**188** ± 14.8]	172 157 [**165** ± 10.6]
	1000	210 225 [**218** ± 10.6]	156 161 [**159** ± 3.5]
	5000	178 213 [**196** ± 24.7]	185 171 [**178** ± 9.9]
Historical negative*	-	60-220	

* Negative (solvent) control: spontaneous revertant number.

Comments on Cytotoxicity test:

There was normal backround lawn with no significant reduction (more than 50%) in the number of revertant colonies at any doses.

Since there was no cytotoxic effect and no precipitation (that could show insolubility), the limit dose of 5000 μg/plate was used as the highest dose.

16.2 Definitive assay

Table 5. Results of Definitive assay, without activation

Test article	Dose [μg/plate)	Revertant colonies per plate [Mean ± S.D.]				
		TA98	TA100	TA1535	TA1537	WP2 uvrA
Pueraria mirifica, PE	0*	36 35 41 [**37** ± 3.2]	180 169 188 [**179** ± 9.5]	37 42 43 [**41** ± 3.2]	7 8 11 [**9** ± 2.1]	94 83 90 [**89** ± 5.6]
	50	43 36 43 [**41** ± 4.0]	173 164 203 [**180** ± 20.4]	33 37 35 [**35** ± 2.0]	9 13 12 [**11** ± 2.1]	95 83 86 [**88** ± 6.2]
	100	42 34 45 [**40** ± 5.7]	159 182 165 [**169** ± 11.9]	29 38 36 [**34** ± 4.7]	7 12 10 [**10** ± 2.5]	81 89 80 [**83** ± 4.9]
	500	32 37 39 [**36** ± 3.6]	153 189 157 [**166** ± 19.7]	39 27 36 [**34** ± 6.2]	11 8 7 [**9** ± 2.1]	95 80 85 [**87** ± 7.6]
	1000	41 33 42 [**39** ± 4.9]	188 173 163 [**175** ± 12.6]	38 32 32 [**34** ± 3.5]	11 7 10 [**9** ± 2.1]	81 77 78 [**79** ± 2.1]
	5000	36 46 40 [**41** ± 5.0]	175 156 201 [**177** ± 22.6]	35 33 39 [**36** ± 3.1]	10 7 9 [**9** ± 1.5]	85 81 90 [**85** ± 4.5]
Positive control	**	715 826 622 [**721** ± 102.1]	800 928 882 [**870** ± 64.8]	680 606 632 [**639** ± 37.5]	24 28 33 [**28** ± 4.5]	616 668 678 [**654** ± 33.3]
Historical negative***	-	10-50	60-220	5-50	1-25	19-110

* Negative (solvent) control: spontaneous revertant number.

** Dose of suitable mutagen material, see 8.1.

*** The historical negative ranges (refers to all of assays) were formed by our lab experiences and a reference literature (Invittox Protocol no. 30, 1992.)

Table 6. Results of Definitive assay, with 4% S9 activation

Test article	Dose [μg/plate)	Revertant colonies per plate [**Mean** ± S.D.]				
		TA98	TA100	TA1535	TA1537	WP2 uvrA
Pueraria mirifica, PE	0*	37 43 40 [**40** ± 3.0]	184 192 205 [**194** ± 10.6]	28 33 30 [**30** ± 2.5]	11 10 8 [**10** ± 1.5]	111 100 93 [**101** ± 9.1]
	50	36 42 45 [**41** ± 4.6]	194 216 182 [**197** ± 17.2]	31 32 29 [**31** ± 1.5]	7 7 9 [**8** ± 1.2]	99 91 112 [**101** ± 10.6]
	100	33 43 41 [**39** ± 5.3]	171 179 216 [**189** ± 24.0]	28 24 29 [**27** ± 2.6]	6 9 10 [**8** ± 2.1]	112 88 98 [**99** ± 12.1]
	500	38 34 42 [**38** ± 4.0]	173 207 175 [**185** ± 19.1]	22 26 25 [**24** ± 2.1]	11 9 8 [**9** ± 1.5]	88 89 109 [**95** ± 11.8]
	1000	41 45 39 [**42** ± 3.1]	188 162 184 [**178** ± 14.0]	27 25 31 [**28** ± 3.1]	6 8 9 [**8** ± 1.5]	100 87 91 [**93** ± 6.7]
	5000	42 37 44 [**41** ± 3.6]	199 165 185 [**183** ± 17.1]	28 28 22 [**26** ± 3.5]	9 8 10 [**9** ± 1.0]	109 94 94 [**99** ± 8.7]
Positive control	**	320 360 420 [**367** ± 50.3]	732 676 744 [**717** ± 36.3]	288 261 305 [**285** ± 22.2]	82 77 91 [**83** ± 7.1]	491 511 449 [**484** ± 31.6]
Historical negative	-	10-50	60-220	5-50	1-25	19-110

* Negative (solvent) control: spontaneous revertant number.

** Dose of suitable mutagen material, see 8.1.

Comments on Definitive test:

The backround lawn was normal at every dose. The positive and negative (solvent) control values were appropriate for the respective strains, but the positive control without S9 (10 μg 9AA /plate) at TA1537 was too close to the criteria, so the dose was increased to 50 μg 9AA / plate in the Repeat assay.

16.3 Repeat assay

Table 7. Results of Repeat assay, without activation

Test article	Dose [µg/plate)	Revertant colonies per plate [**Mean** ± S.D.]				
		TA98	TA100	TA1535	TA1537	WP2 uvrA
Pueraria mirifica, PE	0 [a)]	41 40 33 [**38** ± 4.4]	199 208 185 [**197** ± 11.6]	41 46 39 [**42** ± 3.6]	10 9 15 [**11** ± 3.2]	83 89 92 [**88** ± 4.6]
	50	38 44 42 [**41** ± 3.1]	208 197 194 [**200** ± 7.4]	43 35 39 [**39** ± 4.0]	8 10 9 [**9** ± 1.0]	90 85 82 [**86** ± 4.0]
	100	38 36 46 [**40** ± 5.3]	196 215 199 [**203** ± 10.2]	46 39 37 [**41** ± 4.7]	14 7 8 [**10** ± 3.8]	92 98 76 [**89** ± 11.4]
	500	38 43 34 [**38** ± 4.5]	205 193 208 [**202** ± 7.9]	39 45 39 [**41** ± 3.5]	11 13 10 [**11** ± 1.5]	82 89 92 [**88** ± 5.1]
	1000	37 45 37 [**40** ± 4.6]	201 187 214 [**201** ± 13.5]	44 34 40 [**39** ± 5.0]	9 13 12 [**11** ± 2.1]	91 83 74 [**83** ± 8.5]
	5000	41 35 33 [**36** ± 4.2]	196 204 215 [**205** ± 9.5]	40 40 48 [**43** ± 4.6]	8 7 13 [**9** ± 3.2]	97 80 88 [**88** ± 8.5]
Positive control	[b)]	152 118 119[c)] [**130** ± 19.3]	1244 1134 1080 [**1153** ± 83.6]	888 928 974 [**930** ± 43.0]	1063 807 881 [**917** ± 131.7]	618 571 644 [**611** ± 37.0]
Historical negative	-	10-50	60-220	5-50	1-25	19-110

[a)] Negative (solvent) control: spontaneous revertant number.

[b)] Dose of suitable mutagen material, see 8.1.

[c)] Dose of 2-NF was 10 times lower, than in the Definitive assay. As the revertant number are at least three times higher then spontaneus number, the assay remained valid.

Table 8. Results of Repeat assay, with 10% S9 activation

Test article	Dose [μg/plate)	Revertant colonies per plate [**Mean** ± S.D.]				
		TA98	TA100	TA1535	TA1537	WP2 uvrA
Pueraria mirifica, PE	0*	40 34 37 [**37** ± 3.0]	179 169 186 [**178** ± 8.5]	29 37 30 [**32** ± 4.4]	6 8 6 [7 ± 1.2]	73 81 89 [**81** ± 8.0]
	50	43 49 41 [**44** ± 4.2]	157 184 172 [**171** ± 13.5]	27 28 26 [**27** ± 1.0]	9 7 6 [7 ± 1.5]	90 84 78 [**84** ± 6.0]
	100	41 47 36 [**41** ± 5.5]	167 168 181 [**172** ± 7.8]	24 28 27 [**26** ± 2.1]	8 9 8 [**8** ± 0.6]	78 82 86 [**82** ± 4.0]
	500	37 46 46 [**43** ± 5.2]	166 181 192 [**180** ± 13.1]	26 24 31 [**27** ± 3.6]	5 10 8 [**8** ± 2.5]	86 72 90 [**83** ± 9.5]
	1000	43 40 40 [**41** ± 1.7]	184 165 175 [**175** ± 9.5]	29 26 31 [**29** ± 2.5]	12 7 9 [**9** ± 2.5]	84 90 77 [**84** ± 6.5]
	5000	38 43 43 [**41** ± 2.9]	192 164 184 [**180** ± 14.4]	26 28 32 [**29** ± 3.1]	8 4 10 [7 ± 3.1]	80 86 78 [**81** ± 4.2]
Positive control	**	446 451 401 [**433** ± 27.5]	2936 2808 3344 [**3029** ± 279.9]	698 676 632 [**669** ± 33.6]	171 142 163 [**159** ± 15.0]	426 383 358 [**389** ± 34.4]
Historical negative	-	10-50	60-220	5-50	1-25	19-110

* Negative (solvent) control: spontaneous revertant number.

** Dose of suitable mutagen material, see 8.1.

Comments on Repeat assay:

The backround lawn was normal at every dose. The positive and negative (solvent) control values were appropriate for the respective strains. The test show similar results to those of the Definitive assay.

17. Conclusion

There were no revertants exceeding three times the background average either with or without metabolic activation, and there was no dose-related increase over the range tested, so this study gave negative result to Puraria mirifica, PE as test article.

The results of Definitive assay showed that the test article had no mutagenic effect to any strain used in this test. The results of the Repeat assay confirmed the negative results of the Definitive assay.

03.04.2006

János Horváth, M.Sc.

Study Director

18. References

- Maron, D. and Ames B. (1983) Mutation Res. 113, p.173-215.
- Good Laboratory Practices (GLP) Regulations for non-clinical laboratory studies by the U.S. Food and Drug Administration (21 CFR 58, 21 CFR 820), current versions as of April 2003.
- International Conference on Harmonisation (ICH) Tripartite Guideline: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals, S2A, adopted April 24, 1996.
- International Conference on Harmonisation (ICH) Tripartite Guideline: Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals, S2B, adopted November 21, 1997.
- Organisation for Economic Cooperation and Development (OECD) Section 4 of the OECD Guidelines for the Testing of Chemicals: Bacteria Reverse Mutation Test, Guideline 471, adopted 21 July 1997.
- Invittox Protocol no. 30, The Ames Test: Critical Assessment, January 1992. http/embryo.ib.amwaw.edu.pl/invittox/prot/30.htm

Botanical Extracts
Tinctures
Nutraceuticals
Cosmetic Intermediates
Phytochemicals
Spray Drying
Product Development

BIO-BOTANICA® INC

Code: 4523
Lot #: 031615
Analysis Date: 02/02/06

Pueraria mirifica, PE

Certificate of Analysis

Product Description:

MANUFACTURER: Bio-Botanica, Inc.
PLANT: *Pueraria mirifica* Family: Fabaceae
PART USED: Root
ACTIVE CONSTITUENTS: Miroestrol and Isoflavonoids
APPEARANCE: Free-flowing Powder
COLOR: Light Beige
AROMA: Characteristic, Aromatic
TASTE: Aromatic, Characteristic, Slightly Sweet
METHOD OF ASSAY: HPLC
METHOD OF MANUFACTURING: Extraction by *Bio-Chelation®
SOLVENT FOR EXTRACTION: Aqueous Alcohol
EXCIPIENT: Maltodextrin, Capsul, Colloidal Silicon Dioxide
ADDITIVE: None
SOLUBILITY: Miscible with Water
RECOMMENDED STORAGE: Store in a cool dry place, away from excessive heat, light or freezing temperatures.
SHELF LIFE: 12 Months – from the date of analysis

Specification:	Analysis:
MOISTURE: ≤7.0% (2 hrs@ 105°C)	**MOISTURE:** 2.29%
FOREIGN ORGANIC MATTER: ≤0.02 %	**FOREIGN ORGANIC MATTER:** N/A
BULK DENSITY: 0.25-0.60 g/CC	**BULK DENSITY:** 0.46
ASH: ≤10.00 % (2 hrs@ 600°C)	**ASH:** 6.00%
MESH SIZE: ≥95.00% through #40 mesh	**MESH SIZE:** 95.60%
ASSAY: Each 100 g contains: ≥ 20 mg Miroestrol and ≥ 20 mg Isoflavonoids (Including: Daidzin, 3-11 mg; Puerarin, 12-30 mg; Genistin, 0.5-2 mg; Daidzein, 1.1-3.6 mg; Genistein, 0.2-2 mg)	**ASSAY:** Each 100 g contains: Miroestrol, 34.14 mg; Daidzin, 11.71 mg; Puerarin, 23.72 mg; Genistin, 1.27 mg; Daidzein, 1.67 mg; Genistein, 1.39 mg.

Microbial:	Analysis:
TOTAL PLATE COUNT: ≤ 5000 C.F.U.@72hrs@ 37°C	**TOTAL PLATE COUNT:** 600
YEAST & MOLD COUNT: ≤ 500 C.F.U./gm	**YEAST&MOLD COUNT:** 60
STAPHYLOCOCCUS AUREUS: Negative	**STAPHYLOCOCCUS AUREUS:** Neg.
SALMONELLA: Negative	**SALMONELLA:** Neg.
E. COLI: Negative	**E. COLI:** Neg.

*Bio-Chelation® is a registered trademark of Bio-Botanica, Inc.. Bio-Chelation® is a proprietary cold extraction process.

Written by: _____

Quality Control: _____

Approved by Vice President of Research & Development _____

BIO-CHELATED

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