ABICH S.r.l.
Biological and Chemical Analysis
Toxicology, Research and Services

AMES TEST

SPONSOR	**Chase Life Extension Foundation Ltd 64 Stapleford Cres, Browns Bay 0630 Auckland () - New Zealand**
SAMPLE	**818 Anti-aging Serum *Batch*: n.p.**
REPORT DATE	**07/28/2014**
REPORT N.	**REL/1326/2014/AMES/ELB**

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UNI EN ISO 9001:2008
Certificate N°. 501004992 – Rev. 03

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Preliminary

This report contains the experimental data compiled during the in vitro studies of the test product.
The test results are presented in a concise table format for easy interpretation.
The first part provides information regarding sponsor and test product identifications, assay type, entrusted laboratory, study initiation and completion dates and supervisory personnel.
The second part describes the study design, including materials and procedures.
The test results and conclusions are presented in the third and last part of the report.

Note:

The results reported in the present brochure refer only to the tested sample/samples and to the particular experimental conditions hereby described. This report or parts of it can be reproduced only with the experimenters' agreement.

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Summary

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1 PART ONE –GENERAL INFORMATION

1.1 Sponsor

Chase Life Extension Foundation Ltd
64 Stapleford Cres, Browns Bay
0630 Auckland () - New Zealand

1.2 Tested Material

Sample	Internal code	Description
818 Anti-aging Serum Batch: n.p.	4456/14-01	beige/yellow gel serum

1.3 Assay

- *Ames test to evaluate the genotoxic potential according to OECD 471*

1.4 Entrusted Laboratory

ABICH S.r.l.
Via 42 Martiri, 213/B – 28924
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1.5 Study Dates

Starting date: 07/04/2014
Ending date: 07/22/2014

1.6 Main investigator

Elza Severi – Tecnico di Laboratorio Microbiologico / Microbiological Laboratory Technician
ABICH S.r.l.

1.7 Scientific supervisor

Dr. Elena Bocchietto – Biologist, biotechnology specialist.
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2 PART TWO - STUDY DESIGN

2.1 Aim of the test

Aim of the test is to investigate the possible genotoxic activity exerted by the tested substance in strains of *Salmonella typhimurium*, with and without metabolic activation with S9.
Ames test allows to detect the induction of point mutations in nucleotidic bases, such as deletions, insertions, transversions and frameshift errors by using modified *Salmonella typhimurium* strains. These strains carry a defective gene in the histidine operone making them auxotrophe for this aminoacid (mutants His^- which require histidine in the culture medium for growth). The method guiding principle is based on the backmutation: phenomenon by which bacteria exposed to a mutagenic substance may change back and become again prototrophe concerning histidine (His^+), so revertant bacteria become histidine-independent.
The bacterial cells, in growth phase, are exposed to different concentrations of the test agent and mutagenic activity is determined by the capacity of the test substance to induce a significant increase in the number of reverted colonies (histidine-indipendent mutant, His^+) in comparison to the spontaneous reversions occurring in the control cultures.
Some chemical agents are not directly mutagen but become so following transformation and metabolic activation occurring in the organism by liver enzyme activity. In order to study this genotoxic effect, rat liver microsomial fraction (S9) has been added. S9 employ admits to identify indirect mutagen substances.

2.2 Assay procedures

2.2.1 Bacterial strains

In this assay TA 1535, TA100, TA 102, TA 1537 and TA98 strains have been used, their characteristics are explained in Table 1.
Each tester strains contains a different type of mutation in the histidine operon. TA 1535 and TA 100 strains are specific testers for mutagens causing base substitutions. The TA 102 strain is used for detecting mutagens that require an intact excision repair system. The sensitivity of TA 100 and TA 102 is greatly enhanced by the introduction of an R factor, pKM101, which confers ampicillin resistance. Furthermore the TA 102 strain contains the multicopy plasmid, pAQ1, which confers tetracycline resistance. The frameshift tester strains used are TA 1537 and TA 98. TA 98, like TA 100, is ampicillin resistant. All *S. typhimurium* strains carry, along with the defect in the histidine gene (His^-), a deep rough (rfa) character, a mutation that causes partial loss of the lipopolysaccharide barrier that coats the surface of the bacteria and increase permeability to large molecules. At the end in these strains, except TA 102 strain, there is a delection of a gene coding for the DNA excision repair system ($uvrB^-$), resulting in greatly increased sensitivity in detecting many mutagens. For technical reason, the delection excising the uvrB gene extends through the bio gene and, as a consequence, these bacteria also require biotin for growth.

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Table 1

Salmonella strains features						
S.typhimurium *strain*	***Gene Affected***	**DNA Repair Mutation**	***LPS Barrier***	***Biotin Requirement***	***Plasmids***	***Mutational Event***
TA 1535	hisG	uvrB-	rfa-	bio-	-	base-pair substitution
TA 1537	hisC	uvrB-	rfa-	bio-	-	frameshift
TA 100	hisG	uvrB-	rfa-	bio-	pKM101	base-pair substitution
TA 98	hisD	uvrB-	rfa-	bio-	pKM101	frameshift
TA 102	hisG	uvrB	rfa-	bio-	pKM101 pAQ1	base-pair substitution

2.2.2 *Test description*

The study was performed using the plate incorporation assay with and without a 4% S9 Mix: liophilized Aroclor 1254 rat liver induced (Moltox) supplemented with different cofactors (glucose-6-phosphate and NADP).
The experiment was performed, setting up duplicate Petri dishes containing minimum medium on which an aliquot of further medium containing histidine and biotine, the *Salmonella typhimurium* suspension, the sample to be tested at different concentrations and, if the case of metabolic activation, a 4% S9 mix.
Dishes have been incubated for 48 hours at 37°C.

When the incubation time is over, a basal bacterial growth is achieved related to limited amount of histidine in the medium together with revertant colonies (histidine-independent). In particular, the number of revertant colonies is steady, different for each strain, due to the spontaneous mutation rate in bacteria and an increase in revertant colony number is observed proportional to the tested sample concentration and to its mutagen capability.

In each Ames test assay the following parameters must be considered:

- the **negative control** (or blank) represented by dishes used to detect so called spontaneous revertant: Salmonellae that spontaneously, without any induction by the sample, revert to a normal condition. These dishes contain mutagens solvents (water and another solvent) and test agent solvents (in this case water).

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- the **positive control** given by standard mutagen to check strains functionality.

Direct test:	**Mutagens without S9**
S. typhimurium TA 1535	sodium azide (NaN_3)
S. typhimurium TA 1537	*9-aminoacridine (9AA)*
S. typhimurium TA 100	*sodium azide (NaN_3)*
S. typhimurium TA 98	*4-nitroquinoline-N-oxide (NQNO)*
S. typhimurium TA 102	*4-nitroquinoline-N-oxide (NQNO)*

Indirect test:	**Mutagens with S9**
All strains	2-aminoanthracene (2AAn)

Mutagenesis test has been carried out with the following concentrations in dishes with and without S9:

- 5 mg/plate
- 1,6 mg/plate
- 0,512 mg/plate
- 0,164mg/plate
- 0,052mg/plate

Negative and positive control dosage level:

Water: 100µl/plate

Table 2

STRAINS		***POSITIVE CONTROLS***	***DOSAGE LEVELS***
S. typhimurium TA 1535	**WITHOUT S9**	sodium azide (NaN_3) in water	1,5 µg/plate
S. typhimurium TA 1537		9-aminoacridine (9AA) in water	75 µg/plate
S. typhimurium TA 100		sodium azide (NaN_3) in water	2,0 µg/plate
S. typhimurium TA 98		4-nitroquinoline-N-oxide (4NQO) in DMSO	0,5 µg/plate
S. typhimurium TA 102		4-nitroquinoline-N-oxide (4NQO) in DMSO	1,5 µg/plate

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S. typhimurium TA 1535	WITH S9	2-aminoanthracene (2AAn) in DMSO	10 µg/plate
S. typhimurium TA 1537		2-aminoanthracene (2AAn) in DMSO	3,0 µg/plate
S. typhimurium TA 100		2-aminoanthracene (2AAn) in DMSO	3,0 µg/plate
S. typhimurium TA 98		2-aminoanthracene (2AAn) in DMSO	3,0 µg/plate
S. typhimurium TA 102		2-aminoanthracene (2AAn) in DMSO	10 µg/plate

2.2.3 Bacterial strains maintenance and check

The S. typhimurium strains were made from fresh bacterial culture derived from permanent cultures stored at -20°C in 9% DMSO. Fresh bacterial cultures were grown in Nutrient Broth (Oxoid) with ampicillin or tetracycline (for ampicillin or tetracycline resistent bacteria), were subcultivated on appropriate medium (Master plate) and stored at + 4°C for up to 2 months. The Master plates medium is particular for each strain: the TA 1535 and TA 1537 strains grow in Minimal medium with histidine and biotine, the TA 100 and TA 98 strains grow in Minimal medium with histidine, biotine and ampicillin. The TA 102 strain grows in Minimal medium with histidine, biotine, ampicillin and tetracycline.
The liquid culture, the permanent cultures and Master plates were checked with the following controls to confirm the tester strain genotype:

a) Check for histidine-requirement
Bacterial cultures are streaked on Minimal Medium (MM) + Bio plates and on MM + Bio + His plates. After 48h incubation at 37°C, bacterial growth should be observed on MM + Bio + His plates and shouldn't be observed on MM + Bio plates (negative control).

b) Check for the rfa mutation (Crystal violet sensitivity)
Culture (0,1ml) is added to 2ml of top agar and plated on Complete Medium plates. When the medium is solidified, a disc with 10µl of crystal violet solution (1 mg/ml) is put in the centre of agar surface. After 24h incubation at 37°C, a neat inhibition zone around the disc should be observed for all strains.

c) Check for the uvrB mutation (UV sensitivity)
Bacterial cultures are streaked on Complete Medium plates. Half plates are exposed to uvrB ray (15W germicidal lamp at 33cm distance) for 6 seconds (TA 1535 and TA 1537) or 8 seconds (TA 100, TA 98 and TA 102). After 24h incubation at 37°C all strains should grow on the un-irradiated side of the plate only. The strain TA 102 was used as a control and should grow in all the plate.

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d) Check for the R factor (ampicillin resistance)
Bacterial cultures are streaked on Minimal Medium + Bio + His + Amp plates. After 24h incubation at 37°C, bacterial growth should be observed for ampicillin resistance strains (TA 100, TA 98 and TA 102) only.

f) Check for the pAQ1 plasmid (tetracycline resistance)
Bacterial cultures were streaked on Minimal Medium + Bio + His + Amp + Tet plates. After 24h incubation at 37°C, bacterial growth should be observed for tetracycline resistance strain TA 102) only.

Culture media

Liquid growth medium
This medium was prepared by dissolving 25 grams of Nutrient Broth (Oxoid) and 5 grams of NaCl in one liter of deionized water and sterilized at 1 atmosfere at 121°C for 15 minutes.

Complete medium
This medium was prepared by dissolving 25g di Nutrient Broth (Oxoid), 5g di NaCl and 15g of agar in one liter of deionized water, è stato poi sterilizzato at 1 atmosfere at 121°C per 15 minuti.

Minimal Medium
The medium consisted of 15g of agar in 930ml of deionized water sterilized at 1 atmosfere at 121°C for 15 minutes. Thereafter, the temperature of the medium was brougth to about 60-65°C and 50ml glucose 40% sterile solution and 20ml of Vogel Bonner sterile solution 50X were added.
About 20ml of the medium was poured into each of sterile plastic Petri plates (9cm diameter).

The Vogel Bonner solution 50X was prepared with:

- 10g/l $MgSO_4 \cdot 7H_2O$
- 100g/l citric acid $\cdot H_2O$
- 500 g/l K_2PO_4 anhydrous
- 175 g/l $NaNH_4HPO_4 \cdot 4H_2O$.

Top agar
This superficial medium was prepared with 6g of agar and 5g of NaCl dissolved in one litre of deionized water and sterilized at 1 atmosfere at 121°C for 15 minutes.
For each 100ml of the top agar was added 10ml of 0,5mM Histidine/Biotine solution.

2.2.4 Preparation of the bacterial culture

Bacterial cell suspension were prepared by ionculating one colonie of the Master culture in 25ml liquid growth medium. The liquid culture was developed for about 16 hours a 37°C in a shaking thermostatic incubator (overnight culture).

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2.2.5 Preparation of the S9 mix

The S9 Mix contains an lyophilized homogenate of liver enzymes reconstituted with sterile water, prepared from adult male Sprague Dawley rats liver induced with Aroclor 1254 (Moltox). The ability of the S9 Mix to metabolically activate an indirect mutagen (2-aminoanthracene) was assayed by the Ames test.
The S9 Mix was prepared immediately before use.

S9 Mix composition (50ml):

- 2 ml Rat liver S9 (4%)
- 1ml $MgCl_2$ 0,4M and KCl 1,65M
- 0,25 ml glucose-6-phosphate 1M
- 2 ml NADP 0,1M
- 25 ml buffer sodium phosphate 0,2M pH 7.4
- 19,75 ml sterile deionized water

2.2.6 Plate incorporation test with and without metabolic activation

The test substance or positive or negative control solutions was put into sterile test tube containing 2ml of soft top agar kept liquid in a thermostatic bath at 45°C. A suspension of *Salmonella* strains in stationary growth phase (0,1ml) was rapidly added. For the test with metabolic activation 0,5ml of S9 Mix was also added, instead for the text without S9 Mix 0,5ml of a physiologic solution (PBS) was added. The test tubes were shaken rapidly and the contends poured onto plates containing solid growth minimal medium. The plates were incubated at 37°C for 48 hours. Two plates per dose per Salmonella strain were prepared both for the test with and without metabolic activation. The revertant colonies per plate were counted as UFC (Colony forming units) after 48 hours incubation.

2.2.7 Test results evaluation

For the test to be considered valid, the following criteria must be met:

a) The sterility check must prove negative for bacterial growth.

b) The growth of all the strains must be inhibited by crystal violet; the growth of strains TA 1535 and TA 1537 must be inhibited by ampicillin, while the growth of strains TA 100, TA 98 and TA 102 must not. The growth of all strains, except TA 102 must not inhibited by tetracycline.

c) The frequency of spontaneous for reversions for each strain must fall within both the range reported in the literature and the historical range for our laboratory.

d) The activity of the S9 Mix will be confirmed by its capability to activate the positive control (which requires a metabolic transformation in order to exert its mutagenic effect).

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The test substance is considered to have elicitated a positive response when:

- the number of reverted colonies increases when compared with the number of revertants in the negative controls in a dose-response related way or in a reproducible way at one or more concentrations in at least one strain with or without metabolic activation. Statistical methods may be used to decide the significance of the increase.

Data evaluation

The mean and the standard deviation will be calculated for reversions read in each dosage group and they will be compared with the spontaneous revertants (in the negative controls). The test was performed in triplicate

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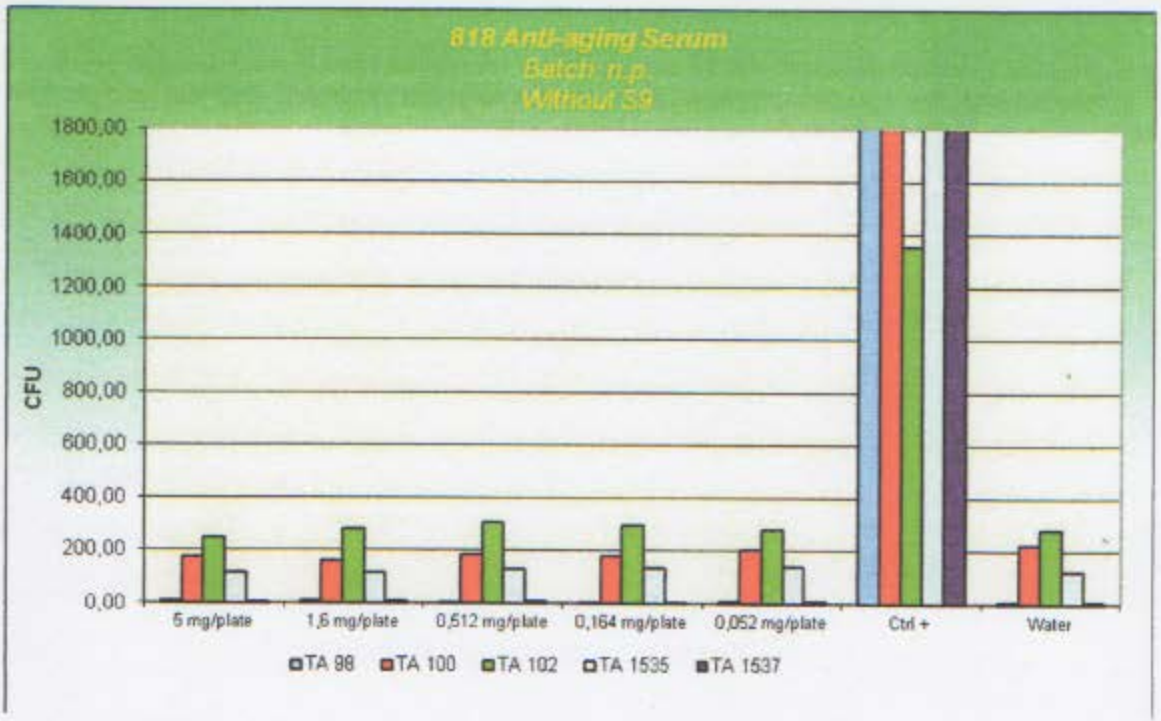
3 PART THREE - RESULTS AND CONCLUSIONS

3.1 Results

The following tables and graphs reports the mean values as number of colonies (UFC) for revertant obtained in assay calculating 3 replicates for each dilution.

Graph 1



Ctrl + : *Positive Control (see table 2)*
Water : *Negative control with water*

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Table 3

818 Anti-aging Serum *Batch*: n.p. *Without S9*										
mg/plate	TA1537		TA1535		TA100		TA98		TA102	
	Mean	SD	*Mean*	SD	*Mean*	SD	*Mean*	SD	*Mean*	SD
5,0	5,3	1,2	115,0	14,9	172,7	11,7	6,7	3,1	245,7	58,5
1,6	6,0	0,0	116,7	3,1	158,7	33,9	8,0	1,0	281,3	53,2
0,512	6,7	2,1	128,7	3,1	184,3	15,9	4,3	1,2	306,0	58,0
0,164	2,3	0,6	132,0	2,0	179,0	26,0	4,7	2,1	293,0	53,8
0,052	5,7	1,5	137,3	3,1	201,7	37,5	6,7	1,5	279,7	59,2
-	---	---	---	---	---	---	---	---	---	---
Ctrl +*	4207,0	5065,0	10001,0	0,0	10001,0	0,0	10001,0	0,0	1355,7	24,6
Water	9,0	1,0	123,3	21,6	223,7	92,8	7,7	2,1	282,0	19,7

** see table 2*

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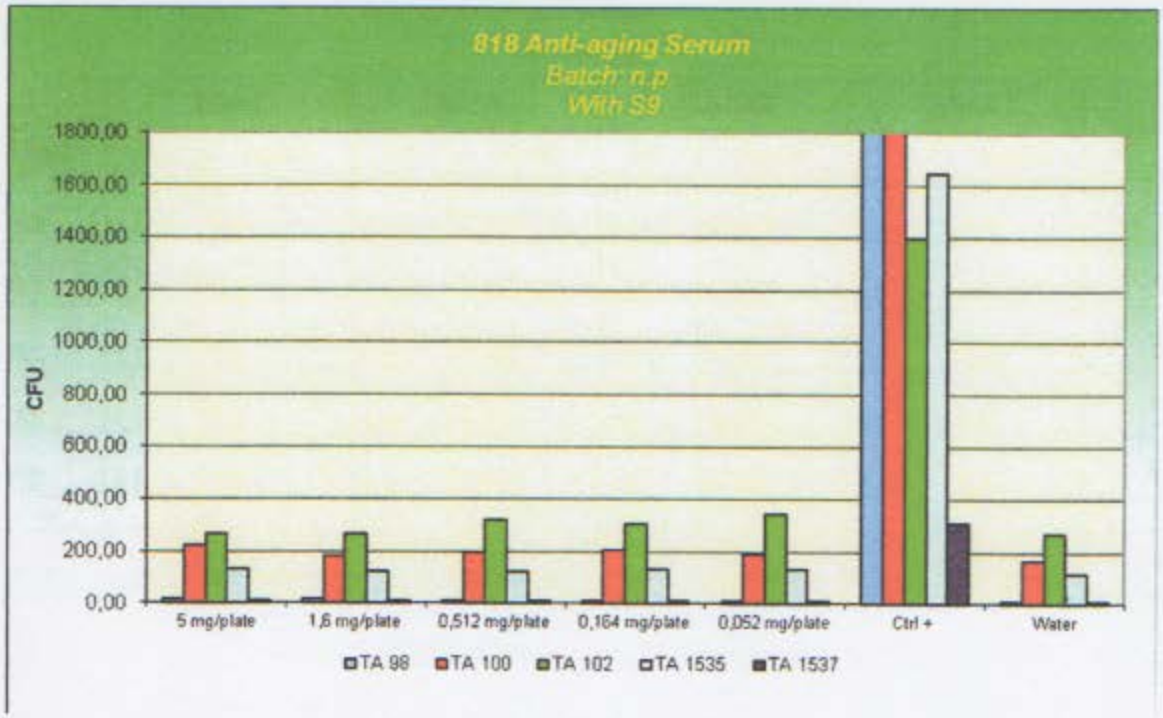
Graph 2



Ctrl + : *Positive Control (see table 2)*
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Table 4

818 Anti-aging Serum *Batch*: n.p. *With S9*										
mg/plate	TA1537		TA1535		TA100		TA98		TA102	
	Mean	SD	*Mean*	SD	*Mean*	SD	*Mean*	SD	*Mean*	SD
5,0	7,7	2,1	131,3	13,3	222,3	71,2	12,7	0,6	262,0	63,3
1,6	9,0	1,6	123,0	17,6	180,0	23,6	12,7	0,6	264,0	59,3
0,512	9,7	2,9	122,7	10,4	197,3	14,2	10,0	1,7	317,7	71,4
0,164	7,3	1,9	133,7	5,5	203,7	46,3	10,3	2,5	301,7	57,5
0,052	8,3	1,2	130,0	2,0	192,7	17,0	8,7	4,0	345,0	105,3
-	---	---	---	---	---	---	---	---	---	---
Ctrl +*	306,7	72,0	1646,7	185,8	10001,0	0,0	10001,0	0,0	1398,7	23,4
Water	10,3	1,7	116,0	4,4	168,3	17,4	11,0	1,7	268,7	44,0
DMSO	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0

* Ctrl+: *Positive Control with 2-aminoanthracene*

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3.1 Conclusions

On the basis of the results from this investigation, the product named :

818 Anti-aging Serum
***Batch*: n.p.**

Did not show any evidence of mutagenicity

Date: 07/28/2014

The Scientific Supervisor

Dr. Elena Bocchietto

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