Smart Print Bio Vitality BATCH PVA3 004/24

In vitro mammalian micronucleus test using Chinese Hamster Ovary cells

ISO 10993-3 (2014), NF EN ISO 10993-3 (2014), ISO 10993-12 (2021), NF EN ISO 10993-12 (2021), ISO/TR 10993-33 (2015) & OECD n°487 (04 July 2023)

SPONSOR	**MMTech Projetos Tecnologicos Importaçao e Exportaçao LTDA** Rua Dr Procopio Toledo Malta, 62, Morada dos Deuses São Carlos/SP ZIP 13.562-291 Brazil
SPONSOR'S REPRESENTATIVE	**Marcelo DEL GUERRA**
STUDY NUMBER	**MNS-PH-24/0562**
TEST FACILITY	**LABORATOIRE ICARE - Site de Martillac - Biotox** **Technopôle Montesquieu** 14 Allée Jacques Latrille 33650 MARTILLAC FRANCE
STUDY DIRECTOR	**Alexandre PEROCHE**
STUDY COMPLETION DATE	**21 NOVEMBER 2024**

CONTENTS

STUDY DIRECTOR STATEMENT AND GLP

I, the undersigned, Alexandre PEROCHE, Study Director, certify that the study **MNS-PH-24/0562** was performed on the premises of Laboratoire ICARE - Site de Martillac.

I certify that the objectives laid down in the study plan were achieved and no undesirable event occurred to affect the quality or the integrity of the study. I consider the raw data to be valid. This report fully and accurately reflects the operating procedures used and raw data.

The entire work was planned and performed according to the principles of the Good Laboratory Practice (G.L.P.), as defined in the directive 2004/10/EC, the O.E.C.D. ruling relative to the mutual acceptance of data in the evaluation of chemical substances (C(81) 30 (final) appendices - May 12th, 1981; C (97) 186, November 26th, 1997), and in ENV/MC/CHEM (98)17 (see G.L.P. certificate in appendices).

It must be noted that no certificate of analysis of the test item or of the raw materials used in its manufacturing was provided by the Sponsor.

Deviation to the Principles of the Good Laboratory Practice:
The purity and homogeneity of the tested batch were not supplied by the Sponsor. The Sponsor stated that the purity and homogeneity are not applicable to the test item (solid state). These deviations to GLP are under the responsibility of the Sponsor and are considered as without impact on the conclusion of the study.

The lack of verification of the concentration and stability of the test item in the vehicle during this study is a deviation to the GLP but has no impact on the reliability of the results generated for the following reasons:
- The process used to prepare the extraction of the test item was suitable according to International standard ISO 10993-12. The standard specifies that the stability must be checked only if the extracts are kept for more than 24 hours, which is not the case during this test.
- Information concerning the preparation and the visual homogeneity of the test item in vehicle are described in this report.

Date: 21 November 2024

Alexandre PEROCHE
Study Director

QUALITY ASSURANCE ATTESTATION

The study n° **MNS-PH-24/0562** was submitted to the control of Quality Assurance in compliance with the principles of Good Laboratory Practice (G.L.P.).

Quality assurance inspections are carried out according to a process of verification of critical phases during the conduct of the study.
The Study and/or the test facility and/or the process are periodically inspected by the quality assurance according to the corresponding Standard Operating Procedures.
Dates of inspections related to the different process of the study are given below:

Process	Date of inspection*	Date of reporting to study director	Date of reporting to test facility manager
Test item reception	09.08.2024	12.08.2024	05.09.2024
Cell seeding	05.08.2024	06.08.2024	19.08.2024
Cell counting	05.08.2024	06.08.2024	19.08.2024
S9-mix preparation	04.09.2023	04.09.2023	08.09.2023
Test item preparation	06.08.2024	09.08.2024	02.09.2024
Micronucleus reading	04.07.2024	11.07.2024	12.07.2024
Harvest	03+04.07.2024	04.07.2024	08.07.2024

It must be noted that these process inspection dates are not specific to this study but result from our internal audit program carried out by the Quality Assurance Unit.

This report was inspected by the Quality Assurance unit at Laboratoire ICARE – Site de Martillac.
It is considered to be an accurate account of the raw data and the application of the operating procedures in use within the laboratory.
The following inspections have been carried out in relation with this study:

Types of QA inspections	Date of inspection	Date of reporting to study director	Date of reporting to test facility manager
Study plan	18.09.2024	18.09.2024	18.09.2024
Final report	19.11.2024	19.11.2024	21.11.2024

Date: 21 November 2024

For the Quality Assurance Unit

H. CALIOT

*dd.mm.yyyy

SUMMARY

Assay: *In vitro* genotoxicity assay through detection of micronuclei in Chinese Hamster Ovary (CHO) cells according to the OECD guideline n° 487 of 04th July, 2023 « *In vitro* mammalian cell micronucleus test » (Laboratoire ICARE - Site de Martillac SOP n° INT503).

Extracts performed in the absence and presence of fetal calf serum were tested for their ability to induce *in vitro* micronuclei in cultured CHO cells. This study was carried out in the absence and presence of metabolic activation.

For Assay 1 (short-term treatment), CHO were exposed to extracts of the medical device 4h in the absence of metabolic activation (culture medium with and without FCS) and 4h in the presence of metabolic activation (S9-mix 10% (v/v)) (culture medium with and without FCS).

For Assay 2 (long-term treatment), CHO were exposed to extract of the medical device between about 1.5 and 2 times the normal cell cycle in the absence of metabolic activation (culture medium with and without FCS).

For the two assays, positive and negative controls were carried out in parallel. Both assays' positive controls induced a statistically significant increase in the number of micronuclei in comparison with corresponding negative controls. The values of negative and positive controls do not show a significant difference with the historical experimental values of the laboratory. Negative controls and positive controls validate the two assays.

According to the criteria of conclusion of the study protocol and OECD 487, extracts obtained from Smart Print Bio Vitality BATCH PVA3 004/24 (Identification code: PH-24/0562) provided by MMTech Projetos Tecnologicos Importaçao e Exportaçao LTDA, are not considered clastogenic and aneugenic in the test system used (CHO cells) in the conditions of the assay.

REPORT

1. SCOPE

The purpose of this study is to assess « *in vitro* » the possible genotoxicity of a test item by detection of micronuclei in cultured mammalian cells, in compliance with OECD guideline n° 487.

The assay is performed in both the absence and the presence of an appropriate metabolic activation system (Rat liver microsome fraction) to also detect promutagens.

Chinese Hamster Ovary cells (CHO- K1) are exposed to the test item (two original extracts) performed with and without serum for 4 hours and between about 1.5 and 2 times the normal cell cycle in the absence of metabolic activation and to the test item (two original extract) performed with and without serum for 4 hours in the presence of metabolic activation. Cells are maintained in culture for a time equivalent about to 1.5 to 2 times their normal cell cycle, after the beginning of the treatment and before to be harvested, fixed and stained with Giemsa. Cytoplasm of cells is analysed microscopically (x1000) to identify and count micronuclei.

2. STANDARDS

The study was performed according to following standard:

- ISO 10993-3:2014, «Biological evaluation of Medical Devices Part. 3 – Tests for genotoxicity, carcinogenicity and reproductive toxicity ».
- NF EN ISO 10993-3:2014, «Biological evaluation of Medical Devices Part. 3 – Tests for genotoxicity, carcinogenicity and reproductive toxicity ». The European Standard EN ISO 10993-3: 2014 has the status of a French standard and fully reproduces the International Standard ISO 10993-3: 2014.
- ISO 10993-12:2021, « Biological evaluation of Medical Devices Part. 12 – Sample preparations and reference materials ».
- NF EN ISO 10993-12:2021, « Biological evaluation of Medical Devices Part. 12 – Sample preparations and reference materials ». The European Standard EN ISO 10993-12: 2021 has the status of a French standard and fully reproduces the International Standard ISO 10993-12: 2021.
- ISO/TR 10993-33:2015, «Biological evaluation of Medical Devices Part. 33 - Guidance on tests to evaluate genotoxicity - Supplement to ISO 10993-3».
- OECD Guideline n° 487 (04 July 2023): "In vitro mammalian cell micronucleus test" (adapted for medical devices).

3. DEVIATIONS TO STUDY PLAN

No deviation.

4. TIMETABLE FOR THE STUDY*

Study initiation date:	18.09.2024
Experimental starting date:	27.09.2024
Experimental completion date:	16.10.2024
Study completion date:	21.11.2024

**(dd.mm.yyyy)*

5. IDENTIFICATION AND CHARACTERIZATION

5.1. Item received

Name:	**Smart Print Bio Vitality BATCH PVA3 004/24**
Category:	**Medical device**
Container:	See photo in appendices
Quantity:	5 bags each containing 5 units / 50x50x1.2 MM
Documents for characterization:	Test item data sheet (certificate of analysis not provided by the sponsor)
Composition:	Amorphous Silica (<5%), silanized silica (>50%), Wetting and dispersing additive (<4%), Photoinitiator (<4%), Monomeric Base (UDMA and THFMA: >40%) and Pigments (<0.07%)
Physical properties:	
. Aspect:	See photo in appendices
. Sterility:	Not sterilized
. Stability:	Stable under storage conditions
. Purity and homogeneity:	NA (solid state)
****Storage conditions:**	Room temperature**, Keep away from light
***Expiry date:**	09.04.2026
***Production date:**	09.04.2024
***Reception date:**	16.09.2024
Identification code:	PH-24/0562

The medical device showed no visible defect upon receipt.

**(dd.mm.yyyy)*
*** (10°C to 30°C, condition used to store the test item in the ICARE laboratory.*

Information concerning the test item is the responsibility of the Sponsor.

5.2. Test item: Extract of medical device

Refer to section 7 "Extracts preparation".

5.3. Extraction vehicles without and with serum

	Culture medium without FCS (polar extraction vehicle)	**Culture medium with FCS** (apolar extraction vehicle)
Cuture medium (Mc Coy's)**	GIBCO - 26600-023 – 2903191 – -.05.2025*	GIBCO - 26600-023 – 2903191 – -.05.2025*
Physical state	liquid	liquid
Colour	orange - red (pH 7.2)	orange - red (pH 7.2)
Stability	stable under normal conditions of storage and use	stable under normal conditions of storage and use
Fetal calf serum** (Supplier – Ref. – Batch – Expiry)	-	GIBCO - 10270-106 – 2440102 – -.08.2027*
Antibiotics** (Supplier – Ref. – Batch – Expiry)	GIBCO – 15240-096 – 2913072– 30.03.2025*	GIBCO – 15240-096 – 2913072– 30.03.2025*
Storage conditions	between 2 and 8 °C	between 2 and 8 °C
Safety precautions	standard laboratory conditions	standard laboratory conditions

**(dd.mm.yyyy)*
***(Supplier – Ref. – Batch – Exp.)*
Antibiotic composition: penicillin 10 000 U/mL, streptomycin 10 000 µg/mL, amphotericin B 25 µg/mL.

The culture medium with or without serum was chosen as an extraction vehicle because of its ability to support cellular growth as well as to extract polar or apolar substances. In addition, the extract can be tested at 100% (after serum supplementation for the serum-free extract).

5.4. Absolute negative control(s)

	Absolute negative control
Name (Supplier – Ref. – Batch – Expiry)	Mc Coy's GIBCO - 26600-023 – 2903191 – -.05.2025*
Physical state	liquid
Colour	orange - red (pH 7.2)
Stability	Stable under normal storage and handling
FCS (Supplier – Ref. – Batch – Expiry)	GIBCO - 10270-106 – 2440102 – --.08.2027*
Antibiotics** (Supplier – Ref. – Batch – Expiry)	GIBCO – 15240-096 – 2913072– 30.03.2025*
Storage conditions	between 2 °C and 8 °C
Safety precautions	Standard laboratory conditions

**(dd.mm.yyyy)*
*** Antibiotic composition: penicillin 10 000 U/mL, streptomycin 10 000 µg/mL, amphotericin B 25 µg/mL.*

5.5. Negative controls (Blanks)

Negative control 1 (Blank 1)	Extraction vehicle without FCS having undergone extraction conditions
Negative control 2 (Blank 2)	Extraction vehicle with FCS having undergone extraction conditions

5.6. Positive controls

	Without metabolic activation	
Name	**Mitomycin C**	**Colchicine**
CAS N°	[50-07-7]	[64-86-8]
Supplier – Ref. – Batch – Expiry	MERCK – M5353-0.2ML – 0000142310 – 30.09.2025*	MERCK – C9754 – SLCP6882 – --.10.2025*
Physical state	liquid	powder
Colour	purple	white
Solvent or vehicle	Mc Coy's	Mc Coy's
Storage conditions	- 20 °C	10°C to 30°C
Safety precautions	mutagenic agent	mutagenic agent
After solubilisation		
Visual aspect	homogenous, pink-blue solution	homogenous, orange-red solution
Stability	extemporaneous preparation	extemporaneous preparation

**(dd.mm.yyyy)*

	With metabolic activation
Name	**Cyclophosphamid monohydrate**
CAS N°	[6055-19-2]
Supplier – Ref. – Batch – Expiry	MERCK – 203960010 – A0444742 – 08.12.2027*
Physical state	powder
Colour	white
Solvent or vehicle	Mc Coy's
Storage conditions	between 2 °C and 8 °C
Safety precautions	mutagenic agent
After solubilisation	
Visual aspect	homogenous, pink solution
Stability	extemporaneous preparation

**(dd.mm.yyyy)*

6. TEST SYSTEM AND RATIONALE FOR THE CHOICE OF TEST SYSTEM

Chinese Hamster Ovary (CHO) cultures are recommended by the OECD guideline n° 487. Moreover, CHO are currently used in standard protocols for *in vitro* cytogenetic tests. CHO are tested for absence of mycoplasma and population doubling. Cultured cells are maintained according to Laboratoire ICARE - Site de Martillac SOP.

Cell type used	CHO-K1
Origin*	Sigma Aldrich (ECACC 85051005)
Caryotype	Stable
Chromosome modal number	20
Mycoplasma research	04.04.2024
Cell cycle	15.27h
Passage number	32 ** - 34 ***
Maintenance of cell cultures	Mc Coy's + 10 % FCS

** Criteria meet the requirements of OECD n° 487.*
***Assay 1*
****Assay 2*

7. EXTRACTS PREPARATION

Liquid extracts are prepared according to ISO 10993-3, NF EN ISO 10993-3, NF EN ISO 10993-12 and ISO 10993-12. Two extraction liquids and corresponding blanks are carried out according to the conditions described below.

All parts of the test item were used for the study.

No pre-treatment on the test item received, no filtration or no centrifugation and no pH adjustment on the extract obtained was performed.

The extract was used within less than 24 hours of recovery (stored at 5°C +/- 3°C from recovery to use).

	ASSAY n°1	**ASSAY n°2**
Test items	Extracts realized from PH-24/0562 o **Extract without S9**: culture medium without FCS o **Extract with S9:** culture medium without FCS o **Extract without S9:** culture medium with FCS o **Extract with S9:** culture medium with FCS	Extracts realized from PH-24/0562 o **Extract without S9**: culture medium without FCS o **Extract without S9**: culture medium with FCS
Extraction vehicles:	Culture medium (Mc Coy's) + 1 % (v/v) antibiotics Culture medium (Mc Coy's) supplemented with 10 % FCS (v/v) + 1 % (v/v) antibiotics	
Extraction performance	from 27.09.2024 at 14h40 to 30.09.2024 at 14h40 (with and without FCS, with and without S9) (72h)	from 04.10.2024 at 14h30 to 07.10.2024 at 14h30 (with and without FCS, without S9) (72h)
Contact extracts / test system	from 01.10.2024 at 09h30 to 01.10.2024 at 13h30 (with and without FCS, 4h with and without S9) (4h)	from 08.10.2024 at 09h20 to 09.10.2024 at 14h00 (with and without FCS, without S9) (28h40)
Extraction conditions:	* extraction temperature: 37° C ± 1°C * extraction time: 72 h ± 2 h * gentle agitation (8 oscillations/min) * area by test item: 50 cm² * number of test item used: 2 (without FCS with and without S9 assay 1), 2 (with FCS with and without S9 assay 1), 1 (without FCS without S9 assay 2) and 1 (with FCS without S9 assay 2)	

	* total area of test item used: 100 cm² (without FCS with and without S9 assay 1), 100 cm² (with FCS with and without S9 assay 1), 50 cm² (without FCS without S9 assay 2) and 50 cm² (with FCS without S9 assay 2) * extraction volume: 33.3 mL (without FCS with and without S9 assay 1), 33.3 mL (with FCS with and without S9 assay 1), 16.7 mL (without FCS without S9 assay 2) and 16.7 mL (with FCS without S9 assay 2) * adsorption volume: 0 mL (volume added at the start of the extraction) * ratio between the surface area of the test material and the volume of extraction vehicle: 3 cm²/mL (thickness > 0.5 mm)	
Aspect	Homogeneous limpid liquid	Homogeneous limpid liquid
Colour	Orange – red (no difference with extract vehicle)	Orange – red (no difference with extract vehicle)
Stability	24h (stored in fridge until its use)	24h (stored in fridge until its use)

The pH and osmolality of each extract (higher concentration tested) and respective blanks (extraction vehicles subjected to the conditions of the extraction in the absence of the test material) are reported in table 1 of the appendices. Measurement done using pH meter with a Mettler Toledo probe (reference: LE438) and a cryoscopic osmometer (löser type).

8. ASSAY CONDITIONS

8.1. Cell culture

Before exposure to test item, cells are seeded in 25 cm² culture flasks at the starting density of $10x10^3$ cells/cm² into 5 mL of complete culture medium (Mc Coy's supplemented with 10 % (v/v) Fetal Calf Serum (FCS)).

Cell cultures are incubated at 37°C in a humid atmosphere containing 5% (v/v) CO_2, for 24 hours. Two cultures are carried out for 100% extract (1 culture by dilutions of extract) and each control.

8.2. Exposure concentrations used

8.2.1. Controls

Absolute negative control	Culture medium	10 % FCS	n* = 2
Negative control 1 (Blank 1)	Extraction vehicle without FCS having undergone extraction conditions	100 %	n*=2
Negative control 2 (Blank 2)	Extraction vehicle with FCS having undergone extraction conditions	100%	n*=2
Positive control (without metabolic activation)	Mitomycin C (CAS n° [50-07-7])	0.30 µg/mL (Assay 1) 0.07 µg/mL (Assay 2)	n* = 2 n* = 2
Positive control (without metabolic activation)	Colchicine (CAS n° [64-86-8])	0.10 µg/mL (Assay 2)	n* = 2
Positive control (with metabolic activation)	Cyclophosphamid monohydrate (CAS n° [6055-19-2])	10 µg/mL (Assay 1)	n* = 2

** number of flask per assay*

8.2.2. Extracts of test material

Assay n°1 (short-term treatment):
Extract of medical device performed without FCS: the following concentrations of the extract are applied to the test system in the absence and presence of metabolic activation: 100, 40, 16 and 6.4 % (v/v) (n = 1 for each concentration and n=2 for 100%).

Extract of medical device performed with FCS: the following concentrations of the extract are applied to the test system in the absence and presence of metabolic activation: 100, 40, 16 and 6.4 % (v/v) (n = 1 for each concentration and n=2 for 100%).

Assay n°2 (long-term treatment):
Extract of medical device performed without FCS: the following concentrations of the extract are applied to the test system in the absence of metabolic activation: 100, 40, 16, 6.4 and 2.56 % (v/v) (n = 1 for each concentration and n=2 for 100%).

Extract of medical device performed with FCS: the following concentrations of the extract are applied to the test system in the absence of metabolic activation: 100, 40, 16, 6.4 and 2.56 % (v/v) (n = 1 for each concentration and n=2 for 100%).

Cytotoxicity is assessed by the determination of the Relative Increase in Cell Count (RICC) with and without metabolic activation (cf. table 2a and 2b): this parameter evaluates the cytotoxicity of the different concentrations tested and allows the final selection of the concentrations to be tested in the micronucleus test.

If cytotoxicity observed, analysable concentrations should cover a range from the maximum to little or no toxicity. For micronuclei interpretation the highest concentration used should induce RICC reduction less than 60 %.

Note: the osmolality and pH of the highest concentration studied should be compatible with cell culture.

8.3. Exposure of test item (extracts)

Selected concentrations of test item extracts are placed in contact with the test system. Two independent tests are carried out.

According to OECD n° 487: "In the event that any of the above experimental conditions lead to a positive response, it may not be necessary to investigate any of the other treatment regimens."

- *Assay 1 (short-term treatment):*
  - Without metabolic activation: 4 hours exposure (expression time is about 1.5 to 2 normal cell cycle lengths after the beginning of the treatment).
  - With metabolic activation (S9-mix 7 % (v/v)): 4 hours exposure (expression time is about 1.5 to 2 normal cell cycle lengths after the beginning of the treatment).

- *Assay 2 (long-term treatment):*
  - Without metabolic activation: about 1.5 to 2 normal cell cycle lengths of exposition.

8.3.1. Without metabolic activation (Assay 1 and Assay 2)

24 hours after the seeding, the complete cell culture medium is removed and replaced by:

Extract without FCS 4h without S9:

	100%	40%	16%	6.4%
FCS	500µL	500µL	500µL	500µL
Extraction vehicle	-	2700µL	3780µL	4212µL
Extract	4500µL	1800µL	720µL	288µL

Extract with FCS 4h without S9:

	100%	40%	16%	6.4%
Extraction vehicle	-	3000µL	4200µL	4680µL
Extract	5000µL	2000µL	800µL	320µL

Extract without FCS about 1.5 to 2 normal cell cycle lengths *without S9:*

	100%	40%	16%	6.4%	2.56%
FCS	500µL	500µL	500µL	500µL	500µL
Extraction vehicle	-	2700µL	3780µL	4212µL	4384.8µL
Extract	4500µL	1800µL	720µL	288µL	115.2µL

Extract with FCS about 1.5 to 2 normal cell cycle lengths *without S9:*

	100%	40%	16%	6.4%	2.56%
Extraction vehicle	-	3000µL	4200µL	4680µL	4872µL
Extract	5000µL	2000µL	800µL	320µL	128µL

- **Short-term treatment (Assay 1):** After 4 hours incubation with the test item at 37° C in a humidified atmosphere containing 5 % (v/v) CO_2, the culture medium is discarded and the cells washed twice with culture medium. 5 mL of fresh complete culture medium are added and the cells incubated at 37° C in a humidified atmosphere containing 5 % (v/v) CO_2. Incubation time is about 1.5 to 2 normal cell cycle lengths after the beginning of the treatment.
- **Long-term treatment (Assay 2):** the cells are incubated with the test item about 1.5 to 2 normal cell cycle lengths at 37° C in a humidified atmosphere containing 5 % (v/v) CO_2.

8.3.2. With metabolic activation (Assay 1)

8.3.2.1. Preparation of S9 fraction

S9 (microsome fraction from the liver of Sprague Dawley rats treated with Phenobarbital/5,6-Benzoflavone is prepared in compliance with Matushima, et. al. and provided by MOLTOX™ (POB Box 1189 – 157 Industrial Park Dr – Boone, NC 28607 – USA). The S9 fraction (S9 Moltox - 11-105-5 - 4757 - expiry date: 24.07.2025) was previously validated on 22.12.2023 in the laboratory according to the Laboratoire ICARE - Site de Martillac SOP n° INT475.

8.3.2.2. Preparation of S9-mix

S9-mix composition is presented in the following table:

S9 fraction	7 % (v/v)
$MgCL_2$-$6H_2O$	8 mM
KCl	33 mM
Glucose-6-Phosphate Na_2	5 mM
NADP Na_2	4 mM
Phosphate buffer pH 7.4	0.1 M

8.3.2.3. Exposition

24 hours after the seeding, the complete culture medium is discarded, cells layer is washed with culture medium and incubated with reaction mixture composed by culture medium supplemented with 7 % (v/v) S9-mix.

Assay 1

Extract without FCS 4h with S9:

	100%	40%	16%	6.4%
S9 mix at 7%*	750µL	750µL	750µL	750µL
Extraction vehicle	-	2550µL	3570µL	3978µL
Extract	4250µL	1700µL	680µL	272µL

*: Final medium contains 1.1% S9.

Extract with FCS 4h with S9:

	100%	40%	16%	6.4%
S9 mix at 7%*	750µL	750µL	750µL	750µL
Extraction vehicle	-	2550µL	3570µL	3978µL
Extract	4250µL	1700µL	680µL	272µL

*: Final medium contains 1.1% S9.

Short-term treatment (Assay 1): After 4 hours incubation at 37° C in a humidified atmosphere containing 5 % (v/v) CO_2, the culture medium is discarded and the cells layer washed twice with culture medium. 5 mL of fresh complete culture medium are added and the cells layer incubated at 37° C in a humidified atmosphere containing 5 % (v/v) CO_2. Incubation time is about 1.5 to 2 normal cell cycle lengths after the beginning of the treatment.

8.4. Harvest and microscope slides preparation

At the end of the incubation period of about 1.5 to 2 normal cell cycle lengths, cells are harvested as follows:

- culture medium is removed
- cells layer washed once with PBS
- cells are detached (about 2 minutes at 37°C) using 0.5 mL trypsin (0.05 % (w/v) in Hank's balanced solution Ca^{2+} and Mg^{2+} free supplemented with 1 mM EDTA)
- then 4.5 mL of Mc Coy's supplemented with 5 % (v/v) Fetal Calf Serum (FCS) are added
- 200 µL of cell suspension and 200 µL of trypan blue solution at 0.2 % (w/v) in 0.15 M NaCl are added (incubation for 2 minutes).
- thereafter the living cells are counted using an haemocytometer (Malassez cell).
- the remaining cell suspension is centrifuged (200 g, 6 min)
- hypotonic shock (KCl 0.075 M) at 37° C for 3 minutes
- fixation (at least 1hour) using the Carnoy mixture (methanol: acetic acid, 3:1)
- spread on coded microscope slides
- stained using Giemsa stain.

Cells are analyzed under a microscope (magnification x1 000) for the detection of micronuclei.

9. EVALUATION CRITERIA

9.1. Relative Increase in Cell Count (RICC)

The RICC corresponds to the relative increase in the number of cells in exposed cultures versus increase in non-treated cultures, a ratio expressed as a percentage.

$$\text{RICC} = \frac{\text{Increase in number of cells in treated cultures (final – starting)}}{\text{Increase in number of cells in control cultures (final – starting)}} \times 100$$

RICC reduction = 100 - RICC

- "Starting" corresponding to the cell number before incubation (= pre-incubation control).
- For positive controls, RICC reduction must be ≤ 60%.
- The maximum concentration to be used for micronuclei interpretation is based on cytotoxicity, the highest concentration should aim to achieve 55 +/- 5 % cytotoxicity.

9.2. Detection of micronuclei (Assays n°1 and n°2)

Micronucleus frequency is analysed in at least 2 000 cells (1 000 cells per culture for the original extracts) for minimum 3 concentrations of the test item (inducing less than 60% of cytotoxicity) and controls. If the test item at the strongest concentration gives a negative result and there is no cytotoxic effect, lower doses may not be read.

A micronucleus will be taken into account if it collects the following conditions:

- micronucleus should be inside the cytoplasm of cell
- micronucleus is morphologically identical to but smaller than the main nucleus (diameter ≤ 1/3 of the diameter of main nucleus)
- micronucleus should not be retracted
- micronucleus should have round or oval shape
- micronucleus should not be linked or connected to the main nucleus
- micronucleus may touch main nucleus but twice membranes should be clearly distinct and intact
- it may have several micronuclei inside a cell.

The number of cells presenting one, or more, micronuclei is considered as a direct response and evaluated statistically using the χ^2 test (validated Excel™ spreadsheet).

The results of cultures treated with different concentrations of the test item and results of positive control are considered significant if $P < 0.05$ comparing to the corresponding negative control.

10. CRITERIA CONCLUSION

The test item will be considered as clastogen, and/or aneugen, *in vitro* with regards to the test system (CHO) if:

- at least one of the tested concentrations exhibits a statistically significant increase in number of micronuclei compared with the concurrent negative control,
- if there is a concentration-dependent increase in number of micronuclei,
- any of the results are outside the distribution of the historical negative control data.

A test item for which results do not meet the above criteria is considered non-clastogenic and non-aneugenic in the assay conditions.
Positive results indicate that the test substance induces micronuclei in culture of mammalian somatic cells. The positive control shall exhibit a statistically significant increase in number of micronuclei compared with the absolute negative control.

The values of negative and positive controls must not show a significant difference with the historical experimental values of the laboratory.
Note: Negative controls results should ideally be within the 95% control limits of distribution. If negative controls results are not included in the 99.74% control limits of distribution the impact on study results interpretation is done to determinate if the assay must be retested.

Equivocal or disputable results do not allow a clear positive response. Results should be clarified by further testing using modification of experimental conditions (concentration spacing and metabolic activation conditions).

11. RESULTS

pH and osmolality of the extracts of the test item received were determined. The data are presented in table 1 of the appendices.

The results obtained in the study defining the RICC of the culture of CHO in the presence of the original extracts and dilutions of the test item, with and without metabolic activation, are shown in table 2a and 2b in the appendices.

The raw results corresponding to the selected concentrations of the test material and to the various controls, as well as the percentage of cells with one, or more, micronuclei are reported in tables and figures (tables 3a, 3b, 4a, 4b and figures 1, 2, 3 and 4 of the appendices).

Historical data from the laboratory for absolute negative and positive controls are presented in the appendices:

- for tests in the absence of metabolic activation (-S9-mix): table 5 and figure 5,
- for the test performed in the presence of metabolic activation (+ S9-mix): table 5 and figure 6.

12. DISCUSSION OF RESULTS

12.1. Cytotoxicity

12.1.1. Doubling time

Short-term treatment (Assay 1):

Number of cells in flask 24 hours after seeding and before starting treatment is 745 000 cells per flask.

1.5 normal cell cycle lengths should induce around 2 235 000 cells per flask.

2 normal cell cycle lengths should induce around 2 980 000 cells per flask.

After treatment, rinsing and re-incubation absolute negative control contain 2 712 500 cells per flask (without S9).

This concentration of cells is compatible with the study (about 1.5 and 2 cell cycle lengths).

After treatment, rinsing and re-incubation absolute negative control contain 2 512 500 cells per flask (with S9).

This concentration of cells is compatible with the study (about 1.5 and 2 cell cycle lengths).

Long-term treatment (Assay 2):

Number of cells in flask 24 hours after seeding and before starting treatment is 750 000 cells per flask.

1.5 normal cell cycle lengths should induce around 2 250 000 cells per flask.

2 normal cell cycle lengths should induce around 3 000 000 cells per flask.

After long-term treatment absolute negative control contain 3 200 000 cells per flask.

This concentration of cells is compatible with the study (very slightly higher than 2 normal cell cycle lengths, it is accepted to be around 2 doubling times).

12.1.2. RICC

Assay n°1 (short-term treatment)

The highest concentration (100 %) extract **without serum** of the test material reduces the RICC by 39% **without metabolic activation**. This concentration is compatible with the study.

The 40% extract without serum of the test material reduces the RICC by 32% without metabolic activation. This concentration is compatible with the study.

The 16% extract without serum of the test material reduces the RICC by 20% without metabolic activation. This concentration is compatible with the study.

The 6.4% extract without serum of the test material reduces the RICC by 7% without metabolic activation. This concentration is compatible with the study.

The highest concentration (100 %) extract **with serum** of the test material reduces the RICC by 37% **without metabolic activation**. This concentration is compatible with the study.

The 40% extract with serum of the test material reduces the RICC by 26 % without metabolic activation. This concentration is compatible with the study.

The 16% extract with serum of the test material reduces the RICC by 16% without metabolic activation. This concentration is compatible with the study.

The 6.4% extract with serum of the test material reduces the RICC by 7% without metabolic activation. This concentration is compatible with the study.

The positive control (mitomycin C) **without metabolic activation** reduces the RICC by 55%. This concentration is compatible with the study.

Therefore, the extracts with and without serum at 100, 40 and 16 % were used to determine the genotoxic effects.

The highest concentration (100 %) extract **without serum** of the test material reduces the RICC by 10% **in the presence of metabolic activation**. This concentration is compatible with the study.

The 40% extract without serum of the test material reduces the RICC by 3% in the presence of metabolic activation. This concentration is compatible with the study.

The 16% extract without serum of the test material reduces the RICC by 1% in the presence of metabolic activation. This concentration is compatible with the study.

The 6.4% extract without serum of the test material does not reduce the RICC in the presence of metabolic activation. This concentration is compatible with the study.

The highest concentration (100 %) extract **with serum** of the test material reduces the RICC by 19% **in the presence of metabolic activation**. This concentration is compatible with the study.

The 40% extract with serum of the test material reduces the RICC by 12% in the presence of metabolic activation. This concentration is compatible with the study.

The 16% extract with serum of the test material reduces the RICC by 1% in the presence of metabolic activation. This concentration is compatible with the study.

The 6.4% extract with serum of the test material does not reduce the RICC in the presence of metabolic activation. This concentration is compatible with the study.

The positive control (cyclophosphamid) **with metabolic activation** reduces the RICC by 57%. This concentration is compatible with the study.

Therefore, the extracts with and without serum at 100, 40 and 16 % were used to determine the genotoxic effects.

Assay n°2 (long-term treatment):

The highest concentration (100 %) extract **without serum** of the test material reduces the RICC by 8% **without metabolic activation**. This concentration is compatible with the study.

The 40% extract without serum of the test material reduces the RICC by 2% without metabolic activation. This concentration is compatible with the study.

The 16% extract without serum of the test material does not reduce the RICC without metabolic activation. This concentration is compatible with the study.

The 6.4% extract without serum of the test material does not reduce the RICC without metabolic activation. This concentration is compatible with the study.

The 2.56% extract without serum of the test material does not reduce the RICC without metabolic activation. This concentration is compatible with the study.

The highest concentration (100 %) extract **with serum** of the test material reduces the RICC by 13% **without metabolic activation**. This concentration is compatible with the study.

The 40% extract with serum of the test material reduces the RICC by 6% without metabolic activation. This concentration is compatible with the study.

The 16% extract with serum of the test material reduces the RICC by 7% without metabolic activation. This concentration is compatible with the study.

The 6.4% extract with serum of the test material reduces the RICC by 7% without metabolic activation. This concentration is compatible with the study.

The 2.56% extract with serum of the test material reduces the RICC by 3% without metabolic activation. This concentration is compatible with the study.

The positive control mitomycin C **without metabolic activation** reduces the RICC by 49%. This concentration is compatible with the study.

The positive control colchicine **without metabolic activation** reduces the RICC by 54%. This concentration is compatible with the study.

Therefore, the extracts with and without serum at 100, 40 and 16 % were used to determine the genotoxic effects.

12.2. Genotoxicity

12.2.1. Absolute negative control

The percentage of cells with micronuclei is equal to 2.2 % for assay 1 and 1.9 % for assay 2 in the absence of metabolic activation and equal to 1.9 % for assay 1 in the presence of metabolic activation.

12.2.2. Positive controls

- *Without metabolic activation*: Mitomycin C positive control significantly increases the percentage of cells with micronuclei compared to absolute negative control ($P < 0.001$). This percentage is 18.3 % for assay 1 and 12.1 % for assay 2.
- *Without metabolic activation*: Colchicine positive control significantly increases the percentage of cells with micronuclei compared to absolute negative control ($P < 0.001$). This percentage is 9.7 % for assay 2.
- *With metabolic activation:* Cyclophosphamid positive control significantly increases the percentage of cells with micronuclei compared to absolute negative control ($P < 0.001$). This percentage is equal to 12.5 % for assay 1.

12.2.3. Negative controls

12.2.3.1. Negative control without FCS

- *Without metabolic activation: Extraction vehicle without serum induces the percentage of cells with* micronuclei compared to absolute negative control *to 2.1 % for assay 1 (short-term treatment) and 1.9 % for assay 2 (long-term treatment).*
- *With metabolic activation: Extraction vehicle without serum induces the percentage of cells with* micronuclei compared to absolute negative control *to 1.8 % for assay 1 (short-term treatment).*

12.2.3.2. Negative control with FCS

- *Without metabolic activation:* Extraction vehicle with serum induces the percentage of cells with micronuclei compared to absolute negative control to 2.1 % for assay 1 (short-term treatment) and 1.9 % for assay 2 (long-term treatment).
- *With metabolic activation: Extraction vehicle with serum induces the percentage of cells with* micronuclei compared to absolute negative control *to 2.1 % for assay 1 (short-term treatment).*

12.2.4. Test item

12.2.4.1. Extracts without FCS

- *Assay 1 (short-term treatment) without metabolic activation:*

  the 100% extract (test item) does not increase significantly the percentage of cells with micronuclei. This percentage is equal to 2.4%.

  the 40% extract (test item) does not increase significantly the percentage of cells with micronuclei. This percentage is equal to 2.0%.

  the 16% extract (test item) does not increase significantly the percentage of cells with micronuclei. This percentage is equal to 2.2%.

- *Assay 1 (short-term treatment) with metabolic activation:*

  the 100% extract (test item) does not increase significantly the percentage of cells with micronuclei. This percentage is equal to 2.4%.

  the 40% extract (test item) does not increase significantly the percentage of cells with micronuclei. This percentage is equal to 1.8%.

  the 16% extract (test item) does not increase significantly the percentage of cells with micronuclei. This percentage is equal to 1.8%.

- *Assay 2 (long-term treatment) without metabolic activation:*

  the 100% extract (test item) does not increase significantly the percentage of cells with micronuclei. This percentage is equal to 2.0%.

  the 40% extract (test item) does not increase significantly the percentage of cells with micronuclei. This percentage is equal to 1.8%.

  the 16% extract (test item) does not increase significantly the percentage of cells with micronuclei. This percentage is equal to 1.8%.

12.2.4.2. Extracts with FCS

- *Assay 1 (short-term treatment) without metabolic activation:*

  the 100% extract (test item) does not increase significantly the percentage of cells with micronuclei. This percentage is equal to 2.4%.

  the 40% extract (test item) does not increase significantly the percentage of cells with micronuclei. This percentage is equal to 1.8%.

  the 16% extract (test item) does not increase significantly the percentage of cells with micronuclei. This percentage is equal to 1.9%.

- *Assay 1 (short-term treatment) with metabolic activation:*

  the 100% extract (test item) does not increase significantly the percentage of cells with micronuclei. This percentage is equal to 2.1%.

  the 40% extract (test item) does not increase significantly the percentage of cells with micronuclei. This percentage is equal to 1.9%.

  the 16% extract (test item) does not increase significantly the percentage of cells with micronuclei. This percentage is equal to 1.9%.

- *Assay 2 (long-term treatment) without metabolic activation*

the 100% extract (test item) does not increase significantly the percentage of cells with micronuclei. This percentage is equal to 1.8%.

the 40% extract (test item) does not increase significantly the percentage of cells with micronuclei. This percentage is equal to 1.8%.

the 16% extract (test item) does not increase significantly the percentage of cells with micronuclei. This percentage is equal to 1.9%.

No concentration exhibits a statistically significant increase compared with the concurrent negative control. The test item is considered non-clastogenic and non-aneugenic in this test system (CHO).

13. CONCLUSION

The values of absolute negative and positive controls do not show a significant difference with the historical experimental values of the laboratory.

Negative controls and positive controls validate all the assays.

According to the criteria of conclusion of the study protocol and OECD n°487, extracts obtained from Smart Print Bio Vitality BATCH PVA3 004/24 provided by MMTech Projetos Tecnologicos Importaçao e Exportaçao LTDA are not considered clastogenic and aneugenic in the test system used (CHO) and in the conditions of the assay.

14. ARCHIVES

The protocol and amendments to the protocol (if any), the original data, correspondence, and final report of this short-term study are kept in a room 'salle archives" at Laboratoire ICARE - Site de Martillac for a 10-year period.
At the end of this period, the study archives will be either returned to the Sponsor of the study or destroyed.

As this test is considered as a short-term study, no archive will be performed for the test item. After the end of the study, test item from its initial container will be destroyed at the sponsor's request.

Remarks :

a) The assay report above applies only to the sample of the test item submitted to the assay. Extrapolation of these results to another batch of production is the responsibility of the manufacturer.
b) This report must not be duplicated, even partially, without the approval of Laboratoire ICARE - Site de Martillac.

APPENDICES

pH and osmolality

Serie	Assay	pH	Osmolality (mosm/Kg H_2O)
Absolute negative control	Short term without S9	7.8	300
	Short term with S9	7.6	306
	Long term without S9	7.5	299
Without FCS			
Negative control (blank 1)	Short term without S9	7.9	297
	Short term with S9	7.6	304
	Long term without S9	7.6	301
Extract obtained from Smart Print Bio Vitality BATCH PVA3 004/24	Short term without S9	7.9	303
	Short term with S9	7.7	310
	Long term without S9	7.7	304
With FCS			
Negative control (blank 2)	Short term without S9	7.9	299
	Short term with S9	7.6	306
	Long term without S9	7.6	301
Extract obtained from Smart Print Bio Vitality BATCH PVA3 004/24	Short term without S9	7.9	306
	Short term with S9	7.6	314
	Long term without S9	7.6	309

Table 1: pH and osmolality

Relative Increase in Cell Count - Extract without FCS

Serie		Assay	Concentration	Cells number	RICC reduction (%)***
Without metabolic activation					
Pre-incubation control**		1*	-	745 000	-
		2**	-	750 000	-
Absolute negative control		1*	-	2 712 500	-
		2**	-	3 200 000	-
Positive control	**(mitomycin C)**	1*	0.30 µg/mL	1 637 500	**55%**
		2**	0.07 µg/mL	1 987 500	**49%**
	(colchicine)	2**	0.10 µg/mL	1 887 500	**54%**
Negative control	**(blank 1: EV without FCS)**	1*	-	2 662 500	**3%**
		2**	-	3 200 000	**0%**
Extract obtained from Smart Print Bio Vitality BATCH PVA3 004/24		1*	6.4%	2 525 000	**7%**
			16%	2 275 000	**20%**
			40%	2 050 000	**32%**
			100%	1 912 500	**39%**
		2**	2,56%	3 250 000	-
			6.4%	3 200 000	**0%**
			16%	3 225 000	-
			40%	3 150 000	**2%**
			100%	3 000 000	**8%**
With metabolic activation (S9-mix)					
Pre-incubation control**		1*	-	745 000	-
Absolute negative control		1*	-	2 512 500	-
Positive control	**(cyclophosphamid)**	1*	10µg/mL	1 500 000	**57%**
Negative control	**(blank 1: EV without FCS)**	1*	-	2 450 000	**4%**
Extract obtained from Smart Print Bio Vitality BATCH PVA3 004/24		1*	6.4%	2 475 000	-
			16%	2 425 000	**1%**
			40%	2 400 000	**3%**
			100%	2 275 000	**10%**

Table 2a: RICC reduction Extract without FCS

** 1 : Assay 1 (short treatment, 4 hours without metabolic activation, 4 hours treatment with S9-mix)*

*** 2 : Assay 2 (continuous treatment of about 1.5 to 2 times the cell cycle without metabolic activation)*

**** RICC reduction % (extract): 100 – RICC*
RICC: Relative increase in the number of cells in exposed cultures versus increase in non-treated cultures, a ratio expressed as a percentage.
RICC (extract): [Increase in number of cells in treated cultures (final – starting)/ Increase in number of cells in negative control (final – starting)] x 100
RICC (positive control): [Increase in number of cells in positive control cultures (final – starting)/ Increase in number of cells in absolute negative control cultures (final – starting)] x 100
"-": no reduction in RICC
***** Pre-incubation control (initial number of cells): number of cells before incubation*

Relative Increase in Cell Count - Extract with FCS

Serie		Assay	Concentration	Cells number	RICC reduction (%)***
Without metabolic activation					
Pre-incubation control**		1*	-	745 000	-
		2**	-	750 000	-
Absolute negative control		1*	-	2 712 500	-
		2**	-	3 200 000	-
Positive control	**(mitomycin C)**	1*	0.30 µg/mL	1 637 500	**55%**
		2**	0.07 µg/mL	1 987 500	**49%**
	(colchicine)	2**	0.10 µg/mL	1 887 500	**54%**
Negative control	**(blank 2: EV with FCS)**	1*	-	2 687 500	**1%**
		2**	-	3 162 500	**2%**
Extract obtained from Smart Print Bio Vitality BATCH PVA3 004/24		1*	6.4%	2 550 000	**7%**
			16%	2 375 000	**16%**
			40%	2 175 000	**26%**
			100%	1 975 000	**37%**
		2**	2,56%	3 100 000	**3%**
			6.4%	3 000 000	**7%**
			16%	3 000 000	**7%**
			40%	3 025 000	**6%**
			100%	2 850 000	**13%**
With metabolic activation (S9-mix)					
Pre-incubation control**		1*	-	745 000	-
Absolute negative control		1*	-	2 512 500	-
Positive control	**(cyclophosphamid)**	1*	10µg/mL	1 500 000	**57%**
Negative control	**(blank 2: EV with FCS)**	1*	-	2 462 500	**3%**
Extract obtained from Smart Print Bio Vitality BATCH PVA3 004/24		1*	6.4%	2 475 000	-
			16%	2 450 000	**1%**
			40%	2 250 000	**12%**
			100%	2 137 500	**19%**

Table 2b: RICC reduction Extract with FCS

** 1 : Assay 1 (short treatment, 4 hours without metabolic activation, 4 hours treatment with S9-mix)*

*** 2 : Assay 2 (continuous treatment of about 1.5 to 2 times the cell cycle without metabolic activation)*

**** RICC reduction % (extract): 100 – RICC*
RICC: Relative increase in the number of cells in exposed cultures versus increase in non-treated cultures, a ratio expressed as a percentage.
RICC (extract): [Increase in number of cells in treated cultures (final – starting)/ Increase in number of cells in negative control (final – starting)] x 100
RICC (positive control): [Increase in number of cells in positive control cultures (final – starting)/ Increase in number of cells in absolute negative control cultures (final – starting)] x 100
"-": no reduction in RICC
***** Pre-incubation control (initial number of cells): number of cells before incubation*

Micronuclei in CHO: Extract without FCS

Serie		Assay	Concentration	Slide number	Total Cells observed	Number of cells with micronucleus				
						0 Micronucleus	1 Micronucleus	2 Micronucleus	3 Micronucleus	> 3 Micronucleus
Without metabolic activation										
Absolute negative control		1*	-	1	1010	987	22	1	0	0
				2	1016	994	21	1	0	0
		2**	-	101	1008	987	21	0	0	0
				102	1000	983	17	0	0	0
Positive control	(mitomycin C)	1*	0.30 µg/mL	49	1034	866	138	26	4	0
				50	1000	796	161	34	7	2
		2**	0.07 µg/mL	151	1026	912	99	14	1	0
				152	1022	888	116	18	0	0
	(colchicine)	2**	0.10 µg/mL	153	1008	913	87	6	1	1
				154	1011	910	92	8	1	0
Negative control	(blank 1: EV without FCS)	1*	-	3	1003	983	19	1	0	0
				4	1002	980	20	2	0	0
		2**	-	103	1010	990	20	0	0	0
				104	1003	985	18	0	0	0
Extract obtained from Smart Print Bio Vitality BATCH PVA3 004/24		1*	16%	22	1010	988	19	3	0	0
				22	1019	997	22	0	0	0
			40%	23	1007	987	20	0	0	0
				23	1013	993	18	2	0	0
			100%	24	1024	994	27	3	0	0
				25	1008	989	19	0	0	0
		2**	16%	123	1007	988	19	0	0	0
				123	1002	984	17	1	0	0
			40%	124	1004	987	17	0	0	0
				124	1001	981	18	2	0	0
			100%	125	1006	983	22	1	0	0
				126	1008	990	18	0	0	0
With metabolic activation (S9-mix)										
Absolute negative control		1*	-	51	1002	982	20	0	0	0
				52	1009	991	18	0	0	0
Positive control	(cyclophosphamid)	1*	10µg/mL	99	1074	926	107	24	16	1
				100	1020	906	89	19	6	0
Negative control	(blank 1: EV without FCS)	1*	-	53	1000	982	18	0	0	0
				54	1006	987	19	0	0	0
Extract obtained from Smart Print Bio Vitality BATCH PVA3 004/24		1*	16%	72	1009	990	18	1	0	0
				72	1003	985	18	0	0	0
			40%	73	1003	985	17	1	0	0
				73	1011	993	18	0	0	0
			100%	74	1001	978	23	0	0	0
				75	1019	994	23	2	0	0

Table 3a: Micronuclei in CHO – Extract without serum

** 1 : Assay 1 (short treatment, 4 hours without metabolic activation, 4 hours treatment with S9-mix)*

*** 2 : Assay 2 (continuous treatment of about 1.5 to 2 times the cell cycle without metabolic activation)*

Number of micronuclei in CHO: Extract without FCS

Serie		Assay	Concentration	Total cells observed	Total number of cells with micronucleus	Cells with micronucleus (%)	χ^2	P***
Without metabolic activation								
Absolute negative control		1*	-	2026	45	2.2%	-	-
		2**	-	2008	38	1.9%	-	-
Positive control	(mitomycin C)	1*	0.30 µg/mL	2034	372	18.3%	**232.68**	< 0.001
		2**	0.07 µg/mL	2048	248	12.1%	**140.66**	*< 0.001*
	(colchicine)	2**	0.10 µg/mL	2019	196	9.7%	**100.17**	*< 0.001*
Negative control	(blank 1: EV without FCS)	1*	-	2005	42	2.1%	**0.07**	*NS****
		2**	-	2013	38	1.9%	**0.00**	*NS****
Extract obtained from Smart Print Bio Vitality BATCH PVA3 004/24		1*	16%	2029	44	2.2%	**0.03**	*NS****
			40%	2020	40	2.0%	**0.06**	*NS****
			100%	2032	49	2.4%	**0.44**	*NS****
		2**	16%	2009	37	1.8%	**0.01**	*NS****
			40%	2005	37	1.8%	**0.01**	*NS****
			100%	2014	41	2.0%	**0.11**	*NS****
With metabolic activation (S9-mix)								
Absolute negative control		1*	-	2011	38	1.9%	-	-
Positive control	(cyclophosphamid)	1*	10µg/mL	2094	262	12.5%	**148.26**	< 0.001
Negative control	(blank 1: EV without FCS)	1*	-	2006	37	1.8%	**0.01**	*NS****
Extract obtained from Smart Print Bio Vitality BATCH PVA3 004/24		1*	16%	2012	37	1.8%	**0.00**	*NS****
			40%	2014	36	1.8%	**0.02**	*NS****
			100%	2020	48	2.4%	**1.32**	*NS****

Table 3b: Number of micronuclei in CHO – Extract without serum

** 1 : Assay 1 (short treatment, 4 hours without metabolic activation, 4 hours treatment with S9-mix)*

*** 2 : Assay 2 (continuous treatment of about 1.5 to 2 times the cell cycle without metabolic activation)*

**** P : Statistical significance*

***** NS : not statistically significant, P > 0.05*

Percent of cells with micronuclei: Extract without FCS

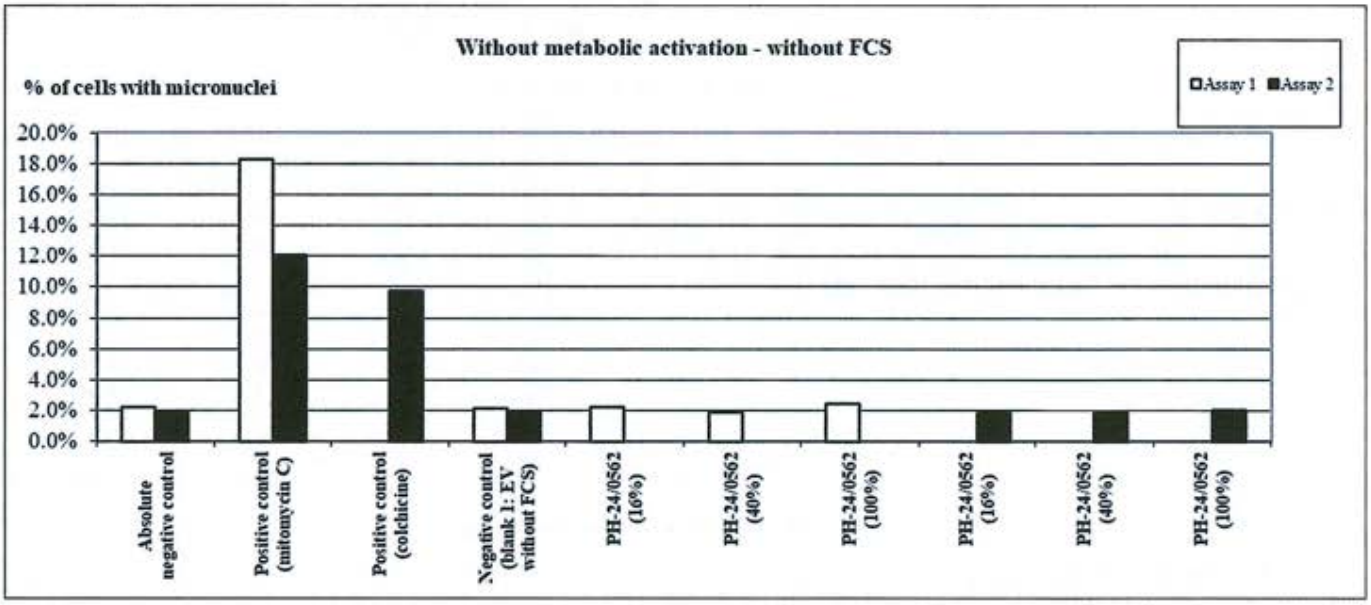

Figure 1: Percent cells with micronuclei – Assays 1 and 2
Extract without FCS (without metabolic activation)

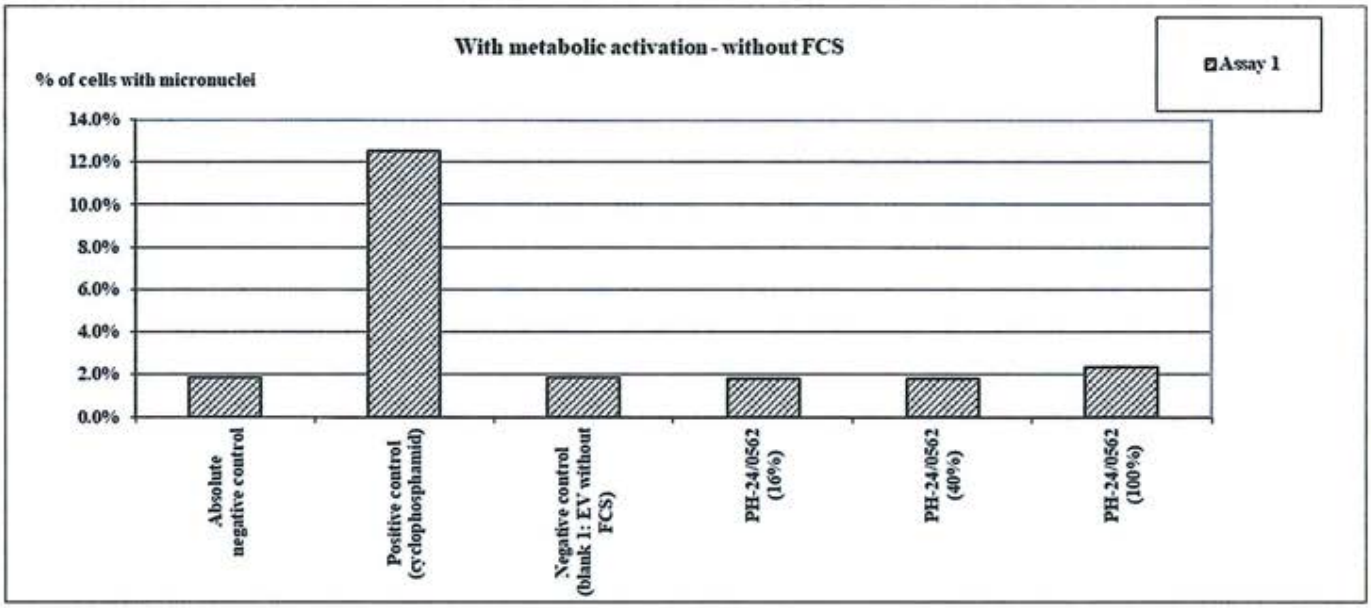

Figure 2: Percent cells with micronuclei – Assay 1
Extract without FCS (with metabolic activation)

Micronuclei in CHO: Extract with FCS

Serie		Assay	Concentration	Slide number	Total Cells observed	Number of cells with micronucleus				
						0 Micronucleus	1 Micronucleus	2 Micronucleus	3 Micronucleus	> 3 Micronucleus
Without metabolic activation										
Absolute negative control		1*	-	1	1010	987	22	1	0	0
				2	1016	994	21	1	0	0
		2**	-	101	1008	987	21	0	0	0
				102	1000	983	17	0	0	0
Positive control	(mitomycin C)	1*	0.30 µg/mL	49	1034	866	138	26	4	0
				50	1000	796	161	34	7	2
		2**	0.07 µg/mL	151	1026	912	99	14	1	0
				152	1022	888	116	18	0	0
	(colchicine)	2**	0.10 µg/mL	153	1008	913	87	6	1	1
				154	1011	910	92	8	1	0
Negative control	(blank 2: EV with FCS)	1*	-	26	1009	991	18	0	0	0
				27	1018	994	24	0	0	0
		2**	-	127	1004	985	19	0	0	0
				128	1006	987	18	1	0	0
Extract obtained from Smart Print Bio Vitality BATCH PVA3 004/24		1*	16%	45	1002	984	18	0	0	0
				45	1013	992	21	0	0	0
			40%	46	1008	990	17	1	0	0
				46	1005	986	19	0	0	0
			100%	47	1002	980	21	1	0	0
				48	1018	992	24	2	0	0
		2**	16%	147	1013	994	19	0	0	0
				147	1012	992	20	0	0	0
			40%	148	1010	992	18	0	0	0
				148	1001	983	17	1	0	0
			100%	149	1010	991	17	2	0	0
				150	1013	995	17	1	0	0
With metabolic activation (S9-mix)										
Absolute negative control		1*	-	51	1002	982	20	0	0	0
				52	1009	991	18	0	0	0
Positive control	(cyclophosphamid)	1*	10µg/mL	99	1074	926	107	24	16	1
				100	1020	906	89	19	6	0
Negative control	(blank 2: EV with FCS)	1*	-	76	1001	983	18	0	0	0
				77	1012	988	23	1	0	0
Extract obtained from Smart Print Bio Vitality BATCH PVA3 004/24		1*	16%	95	999	981	18	0	0	0
				95	1011	991	20	0	0	0
			40%	96	1004	985	19	0	0	0
				96	1013	994	18	1	0	0
			100%	97	1013	993	20	0	0	0
				98	1011	988	23	0	0	0

Table 4a: Micronuclei in CHO – Extract with serum

** 1 : Assay 1 (short treatment, 4 hours without metabolic activation, 4 hours treatment with S9-mix)*

*** 2 : Assay 2 (continuous treatment of about 1.5 to 2 times the cell cycle without metabolic activation)*

Number of micronuclei in CHO: Extract with FCS

Serie		Assay	Concentration	Total cells observed	Total number of cells with micronucleu	Cells with micronucleus (%)	χ^2	P***
Without metabolic activation								
Absolute negative control		1*	-	2026	45	2.2%	-	-
		2**	-	2008	38	1.9%	-	-
Positive control	**(mitomycin C)**	1*	0.30 µg/mL	2034	372	18.3%	**232.68**	< 0.001
		2**	0.07 µg/mL	2048	248	12.1%	**140.66**	*< 0.001*
	(colchicine)	2**	0.10 µg/mL	2019	196	9.7%	**100.17**	*< 0.001*
Negative control	**(blank 2: EV with FCS)**	1*	-	2027	42	2.1%	**0.10**	*NS****
		2**	-	2010	38	1.9%	**0.00**	*NS****
Extract obtained from Smart Print Bio Vitality BATCH PVA3 004/24		1*	16%	2015	39	1.9%	**0.09**	*NS****
			40%	2013	37	1.8%	**0.28**	*NS****
			100%	2020	48	2.4%	**0.41**	*NS****
		2**	16%	2025	39	1.9%	**0.01**	*NS****
			40%	2011	36	1.8%	**0.05**	*NS****
			100%	2023	37	1.8%	**0.02**	*NS****
With metabolic activation (S9-mix)								
Absolute negative control		1*	-	2011	38	1.9%	-	-
Positive control	**(cyclophosphamid)**	1*	10µg/mL	2094	262	12.5%	**148.26**	< 0.001
Negative control	**(blank 2: EV with FCS)**	1*	-	2013	42	2.1%	**0.19**	*NS****
Extract obtained from Smart Print Bio Vitality BATCH PVA3 004/24		1*	16%	2010	38	1.9%	**0.19**	*NS****
			40%	2017	38	1.9%	**0.20**	*NS****
			100%	2024	43	2.1%	**0.01**	*NS****

Table 4b: Number of micronuclei in CHO – Extract with serum

** 1 : Assay 1 (short treatment, 4 hours without metabolic activation, 4 hours treatment with S9-mix)*

*** 2 : Assay 2 (continuous treatment of about 1.5 to 2 times the cell cycle without metabolic activation)*

**** P : Statistical significance*

***** NS : not statistically significant, P > 0.05*

Percent of cells with micronuclei

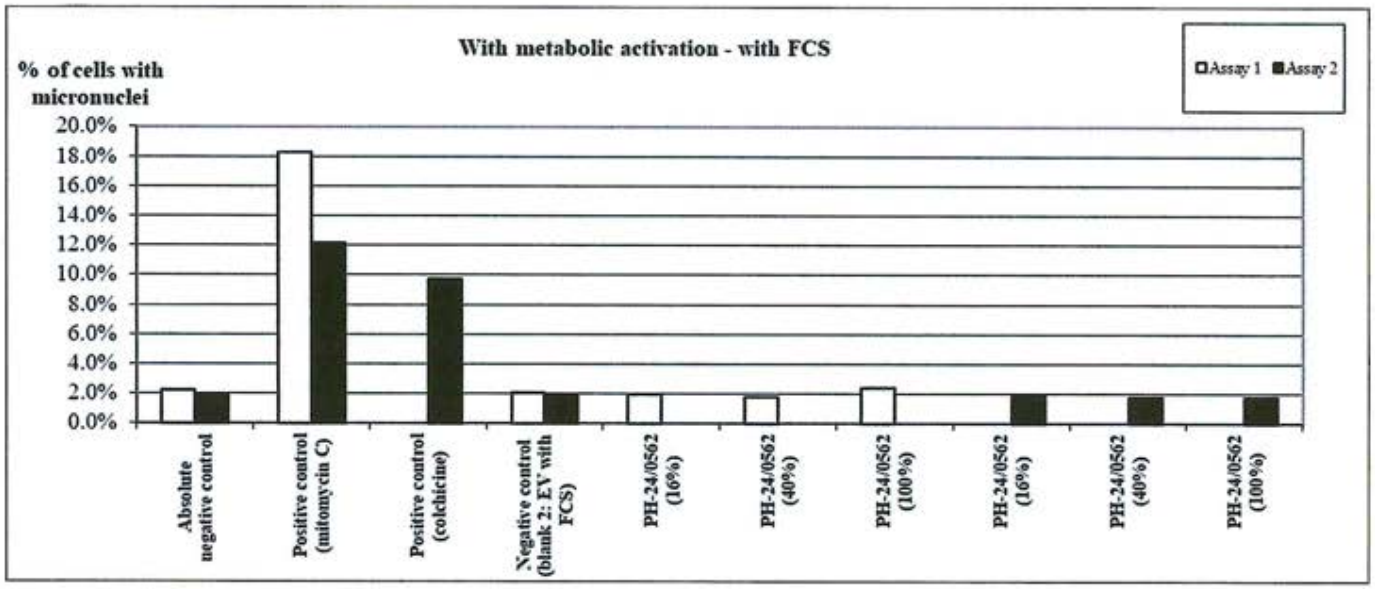

Figure 3: Percent cells with micronuclei – Assays 1 and 2
Extract with FCS (without metabolic activation)

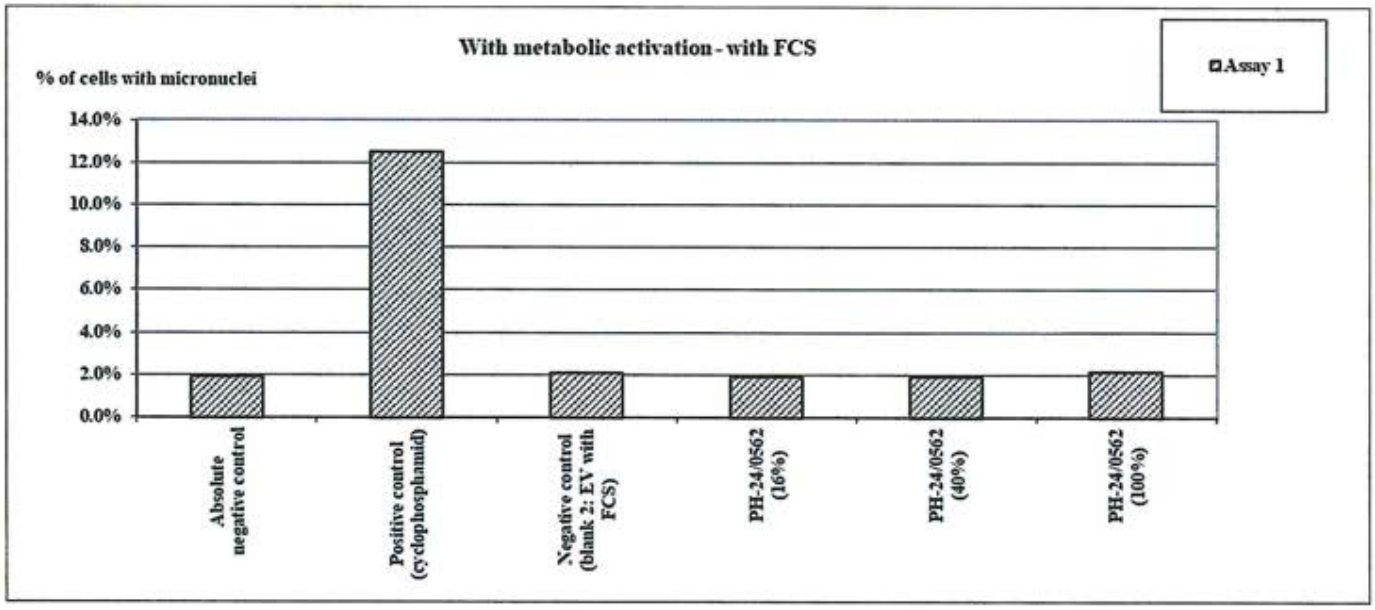

Figure 4: Percent cells with micronuclei – Assay 1
Extract with FCS (with metabolic activation)

Historical controls (2007 to 2023) - percentage of cells with micronuclei

Absolute negatives controls and positives controls	Without metabolic activation (-S9-mix)		
	Negative control	Positive control (Mitomycin)	Positive control (Colchicin)
Assay (short term)	*n* = 52 **2.01 ± 0.46** *Distribution 95%* min : 1.08 - max : 2.93 *Distribution 99.7%* min : 0.61 - max : 3.4	*n* = 52 **20.48 ± 3.05** *Distribution 95%* min : 14.39 - max : 26.58 *Distribution 99.7%* min : 11.34 - max : 29.63	-
Assay (long term)	*n* = 52 **2.01 ± 0.48** *Distribution 95%* min : 1.06 - max : 2.97 *Distribution 99.7%* min : 0.58 - max : 3.44	*n* = 50 **15.08 ± 2.78** *Distribution 95%* min : 9.51 - max : 20.64 *Distribution 99.7%* min : 6.73 - max : 23.42	*n* = 51 **16.79 ± 4.83** *Distribution 95%* min : 7.12 - max : 26.45 *Distribution 99.7%* min : 2.29 - max : 31.29

Absolute negatives controls and positives controls	With metabolic activation (+S9-mix)	
	Negative control	Positive control (Cyclophosphamid)
Assay (short term)	*n* = 52 **2.02 ± 0.43** *Distribution 95%* min : 1.15 - max : 2.89 *Distribution 99.7%* min : 0.72 - max : 3.33	*n* = 52 **21.48 ± 5.53** *Distribution 95%* min : 10.41 - max : 32.54 *Distribution 99.7%* min : 4.88 - max : 38.08
Assay (long term)	-	-

Table 5: Historical controls

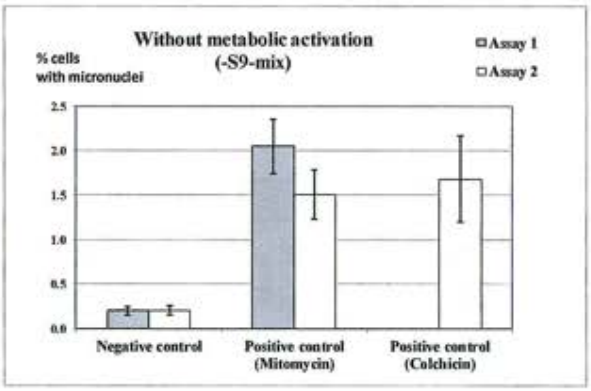

Figure 5: Historical controls percent of cells with micronuclei (-S9-mix)

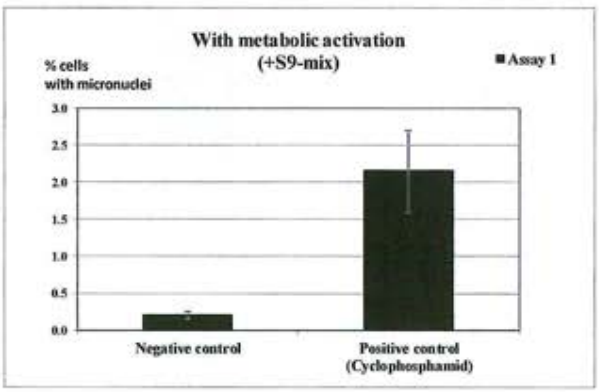

Figure 6: Historical controls percent of cells with micronuclei (+S9-mix)

Photo of medical device

TRINOVABIOCHEM

MOLTOX®
Molecular Toxicology, Inc.

POST MITOCHONDRIAL SUPERNATANT (S9)
QUALITY CONTROL & PRODUCTION CERTIFICATE

Animal Information
SPECIES: Rat
STRAIN: Sprague Dawley
SEX: Male
AGE: 5 – 6 weeks
WEIGHT: 175 – 199 g
TISSUE: Liver

Part Number Information
LOT NO.: 4757
PART NO.: 11-105
VOLUME: 5 mL
BUFFER: 0.15 M KCl
STORAGE: At or below -70°C

PREP: July 24, 2023
EXPIRY: July 24, 2025
INDUCING AGENT:
Phenobarbital-5.6
Benzoflavone

REFERENCE: Matsushima, et al., *In Vitro Metabolic Activation in Mutagenesis Testing* (F.J. de Serres, ed.), Elsevier, 1976, p 85

For Research Purposes Only

BIOCHEMISTRY:
- PROTEIN: 38.6 mg/ml

Assayed according to the method of Lowry et al., *JBC* 193:265, 1951 using bovine serum albumin as the standard.

- ALKOXYRESORUFIN-O-DEALKYLASE ACTIVITIES

Activity	P450	Fold - Induction
BROD	2B1, 2B2	87
EROD	1A1, 1A2	82.2
MROD	1A1, 1A2	16.6
PROD	2B1, 2B2	45.6

Assays for ethoxyresorufin-O-deethylase (EROD), pentoxy-, benzyl- and methoxyresorufin-O-dealkylases (PROD, BROD, & PROD, MROD) were conducted using a modification of the methods of Burke, et al., *Biochem Pharm* 34:3337, 1985. Fold-inductions were calculated as the ratio of the sample vs. uninduced specific activities (SA's). Control SA's (pmoles/min/ mg protein) were 103.4, 92.8, 47.1, & 33.6 for BROD, EROD, MROD and PROD, respectively.

BIOASSAY:
- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS

Samples of S-9 were assayed for the presence of contaminating microorganisms by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-biotin) media. Duplicate plates were read after 40 - 48 h incubation at 35 ± 2°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION

No. His+ Revertants	
TA98	TA1535
191.2	1484

The ability of the sample to activate ethidium bromide (EtBr) and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lesca, et al., *Mutation Res* 129: 299, 1984. Data were expressed as revertants per µg EtBr or per mg CPA.

Dilutions of the sample S9, ranging from 0.2 – 10% in S9 mix, were tested for their ability to activate benzo(α)pyrene (BP) and 2-aminoanthracene (2-AA) to metabolites mutagenic to TA100. Assays were conducted as described by Maron & Ames, (*Mutat Res* 113: 173, 1983.)

	µl S9 per plate/number *his*+ revertants per plate					
Promutagen	0	1	5	10	20	50
BP (5 µg)	139	143	221	275	397	571
AA (2.5 µg)	83	142	501	1051	1918	1537

Approved: 07/28/2023

MOLECULAR TOXICOLOGY, INC. www.moltox.com (828) 264-9099

TRINOVA BIOCHEM GmbH
Rathenau Str. 2
35394 Giessen
GERMANY

fon +49 (0) 641 94393-0
fax +49 (0) 641 94393-22
info@trinova.de
www.trinova.de

ansm
Agence nationale de sécurité du médicament et des produits de santé

RÉPUBLIQUE FRANÇAISE

Direction de l'inspection

EVALUATION DE LA CONFORMITE AUX BONNES PRATIQUES DE LABORATOIRE

selon la directive 2004/9/CE (ESSAIS DE SECURITE)
STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICES
according to Directive 2004/9/CE (SAFETY TESTS)

Nom et adresse de l'installation d'essai :
Name and location of the test facility

Laboratoire ICARE -Site de Martillac
Technopôle Montesquieu
14 Allée Jacques Latrille
33650 MARTILLAC

Objet de l'inspection : *Purpose of the inspection*
état des lieux : ☒ *Test facility inspection*
vérification d'étude(s) : ☒ *Study audit*

Date(s) d'inspection : *Date(s) of the inspection*
10 au 13 janvier 2023

Degré de conformité aux B.P.L. * : *Status*
Conforme

Degré de conformité aux BPL valant pour les études achevées entre le 24 septembre 2021 et le 13 janvier 2023
Level of compliance with GLP for studies performed between

Catégorie(s) d'éléments d'essai : *Types of Test items :*
Dispositifs médicaux
Medical devices

Domaine d'activité : *Areas of expertise*
Cytotoxicité, sensibilisation cutanée, irritations et corrosions cutanées, oculaires et des muqueuses, réactivité intradermique, hémocompatibilité, pyrogénicité, toxicité systémique aiguë, subchronique et subaiguë et toxicité chronique, essais d'implantation et génotoxicité..

Catégorie OCDE (appendice à l'annexe III de C(89)87(Final)/révisée dans C(95)8(Final))
OECD category

X	2	3	X	X	X	X	X	X

Commentaires éventuels : néant.
Observations (if applicable)

Fait à Saint-Denis (France), le : 24 février 2023
Date of the statement

Frederique MARCHAL
Signature numérique de Frederique MARCHAL
Date: 2023.02.24 15:57:15 +01'00'

Cheffe du pôle Inspection des essais et des vigilances

* Conforme: conformité aux B.P.L. (*in conformity with GLP*);
Non-Conforme :absence de conformité aux B.P.L. (*not in conformity with GLP*).

Code : DOC_524_v01

143/147 boulevard Anatole France - F-93285 Saint-Denis Cedex - Tél. +33 (0)1 55 87 30 00 - www.ansm.sante.fr