

LABORATORY

LEMI

Technopole Bordeaux-Montesquieu
33650 MARTILLAC – FRANCE

Final report n° 2014-FQL-1

Evaluation of cytotoxicity (ISO 10993-5:2009)

Medical device:

ORAL ESSENTIALS - Lot N° 06/2014

- **Study director** : M.-F. HARMAND
- **Date reported** : 18 September 2014

Duplication of this report is only authorized as a complete photographic facsimile. It is composed of 15 pages.

SPONSOR

ORAL ESSENTIALS INC.

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CA 90212 U.S.A.

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Study Director Statement and GLP

Study reference: 2014-FQL-1

Test item: Solution of ORAL ESSENTIALS - Lot N° 06/2014 at 5 000 µg/mL

Test: Evaluation of cytotoxicity according to ISO 10993-5:2009 "Biological evaluation of medical devices - Tests for in vitro cytotoxicity" (LEMI SOP n° MB08/33).

I, the undersigned, **M.-F. HARMAND**, study director, certify that the study was performed in the LEMI test site, and according to Good Laboratory Practice (G.L.P.) from O.E.C.D. (C(97)186 - November 26th, 1997 which Member¹ States shall recognize the assurance by another Member State that test data have been generated in accordance with GLP principles) described in ENV/MC/CHEM(98)17 (OECD Principles on Good Laboratory Practice) and described in 2004/9/EC and 2004/10/EC European directives.

Information concerning the identity and characterization of the medical device are the responsibility of the sponsor.

I, certify that the objectives, laid down in the study plan and the associated experimental protocol, were achieved and no undesirable event occurred to affect the quality or the integrity of the study.

This report fully and accurately reflects the operating procedures used and raw data.

Date: 18 September 2014

M.-F. HARMAND
Study Director

A handwritten signature in black ink, appearing to read "M.-F. HARMAND", is written over a horizontal line. The signature is stylized and cursive.

¹ List of members available on www.oecd.org

Quality Assurance Attestation

I, the undersigned **Aurélië HORRIÈRE**, Quality Assurance unit of LEMI, attest that the study n° **2014-FQL-1** was submitted to the control of Quality Assurance in compliance with the principles of Good Laboratory Practice (G.L.P.).

⇒ Study plan n° **2014-FQL-1** was inspected in order to verify that it contains the necessary information in compliance with the principles of Good Laboratory Practice.

***Date of audit report inspection :** 01.09.2014

***Date of reporting to Study Director :** 01.09.2014

***Date of reporting to Test Facility Management :** 01.09.2014

⇒ Inspections of the experimental phases of the studies are carried out as a control process of the critical experimental phases of a type of study. Process inspections are intended to verify the repetitive experimental phases. They are made independently of the studies and are generally made in a random way.

Experimental phase: (Seeding - Incubation - Scoring)

***Date of audit report inspection :** 04-06.02.2014

***Date of reporting to Study Director :** 06.02.2014

***Date of reporting to Test Facility Management :** 06.02.2014

⇒ Test Facility inspections in LEMI allow to examine the procedures and the applied methods, to estimate the level of compliance with the principles of Good Laboratory Practice.

***Date of audit report inspection :** 05.09.2014

***Date of reporting to Test Facility Management :** 05.09.2014

⇒ This report **2014-FQL-1** has been audited by LEMI Quality Assurance unit. It is considered to be an accurate account of the raw data generated and the application of the operating procedures in use within the laboratory :

	<u>Draft</u>	<u>Final report</u>
*Date of audit report inspection :	NA	18.09.2014
*Date of reporting to Study Director :	NA	18.09.2014
*Date of reporting to Test Facility Management :	NA	18.09.2014

**(dd.mm.yyyy)*

Date : 18 September 2014

A. HORRIÈRE
Quality Assurance Manager



LEMI**SUMMARY**

Assay : Evaluation of cytotoxicity according to ISO 10993-5:2009 "Biological evaluation of medical devices - Tests for *in vitro* cytotoxicity" (LEMI SOP n° MB08/33).

TEST ITEM :

*Solution of ORAL ESSENTIALS - Lot N° 06/2014 at 5 000 µg/mL
of culture medium*

LEMI Code : FQL030914-S1

The solution at 5 000 µg/mL of culture medium prepared from ORAL ESSENTIALS - Lot N° 06/2014 (LEMI code: FQL040814) according to ISO 10993-12:2012, is tested to evaluate its ability to induce a cytotoxic effect in Balb/c 3T3 clone A31 cells by counting the living cells (Trypan blue exclusion test).

Controls (positive and negative) are run in parallel.

The solution at 5 000 µg/mL of culture medium prepared from ORAL ESSENTIALS - Lot N° 06/2014 (LEMI code: FQL040814), provided by ORAL ESSENTIALS INC., is not cytotoxic.



REPORT



1. Principle of the study

The purpose of this study is to assess the cytotoxicity of a test item (solution at 5 000 µg/mL of culture medium) in an established cell line. This study will determine the *in vitro* biological response of mammalian cells using appropriate biological parameters.

A monolayer of Balb/c 3T3 clones A31 cells (Mouse embryo fibroblasts) is exposed to the test item (solution at 5 000 µg/mL) and its dilutions for 24 hours. At the end of the incubation period, the cytotoxicity is evaluated by counting the living cells (Trypan blue exclusion test).

2. Standards

This study is performed according to:

- ISO 10993-1:2009, « Biological evaluation of Medical Devices Part. 1 – Evaluation and testing within a risk management process » ;
- ISO 10993-5:2009, « Biological evaluation of Medical Devices Part. 5 – Test for *in vitro* cytotoxicity » ;
- ISO 10993-12:2012, « Biological evaluation of Medical Devices Part. 12 – Sample preparations and reference materials ».

3. Study time-table

*Study initiation date :	01.09.2014
*Experimental starting date :	02.09.2014
*Experimental completion date :	04.09.2014
*Study completion date :	18.09.2014

*(dd.mm.yyyy)

4. Test item identification and characterization

4.1. Medical device received

Name :	ORAL ESSENTIALS - Lot N° 06/2014
Container :	Glass bottle
Quantity :	2 bottles
Composition :	Dead sea salt, aloe vera extract, peppermint essential oil wintergreen essential oil, Xylitol
Physical properties :	
. Aspect :	colorless transparent liquid
. Sterility :	yes
. Stability :	Stable under the storage conditions
Storage conditions :	Room temperature
*Expiration date :	-. 2015
*Reception date :	04.08.2014
LEMI Code :	FQL040814
The medical device showed no visible defect upon receipt at LEMI.	

*(dd.mm.yyyy)



4.2. Part of the medical device to be tested: NA

Name :	-
Quantity :	-
Composition :	-
Physical properties :	
. Aspect :	-
. Sterility :	-
. Stability :	-
Storage conditions :	-
*Expiration date :	-

*(dd.mm.yyyy)

4.3. Solvent

Culture medium supplemented with 10 % (v/v) Fetal Calf Serum (FCS).

Culture medium	DMEM
Supplier - Ref. - Batch :	GIBCO - 31966-021 - 1543712
Physical aspect :	liquid
Colour :	pink (pH : 7.30)
Stability :	stable at room temperature
FCS (Supplier - Ref. - Batch) :	GIBCO - 10270-098 - 41F2324K
Antibiotics (Supplier - Ref. - Batch) :	NA
Storage conditions :	between 2° C and 8° C
Security precautions :	standard laboratory conditions

4.4. Negative control

Culture medium supplemented with 10 % (v/v) FCS.

Culture medium	DMEM
Supplier - Ref. - Batch :	GIBCO - 31966-021 - 1543712
Physical aspect :	liquid
Colour :	pink (pH : 7.30)
Stability :	stable at room temperature
FCS (Supplier - Ref. - Batch) :	GIBCO - 10270-098 - 41F2324K
Antibiotics (Supplier - Ref. - Batch) :	NA
Storage conditions :	between 2° C and 8° C
Security precautions :	standard laboratory conditions



4.5. Positive control

Name :	Phenol
CAS N° :	[108-95-2]
Supplier – Ref. – Batch :	SIGMA - P5566 - BCBL7721V
Physical aspect :	powder
Colour :	white
Stability :	stable under the storage conditions
Storage conditions :	between 2 and 8°C
Security precautions :	irritant for eyes, skin and respiratory tracts
Solvent :	Culture medium (DMEM) supplemented with 10% (v/v) FCS
Concentration :	0.64 mg/mL of solvent
After solubilisation	
. Aspect	homogeneous pink solution
. Stability	extemporaneous preparation

4.6. Test item : Solution at 5 000 µg/mL of culture medium

Refer to section 6. « Preparation of the test item ».

5. Test system

Balb/c 3T3 clone A31 (ATCC CCL 163) (77th passage) tested free from mycoplasma (LEMI SOP n° MB05/02).

6. Preparation of the test item

The test item is prepared as described below:

Identification of the solution :	FQL030914-S1
Solvent :	Culture medium (DMEM) supplemented with 10% (v/v) FCS
Solution :	5 000 µg/mL
Dilutions :	1 500 – 500 and 150 µg/mL
Aspect :	pink – red homogenous transparent
Stability :	prepared extemporaneously
pH :	7.6 (solution at 5 000 µg/mL)
Osmolality :	339 mOsm/kg H ₂ O (solution at 5 000 µg/mL)



7. Assay conditions

7.1. Cell culture

Cells are seeded in multiwell plates (24 wells, 15.5 mm in diameter) at the starting density of 35 000 cells/cm²; culture medium is DMEM supplemented with 10 % (v/v) FCS. No antibiotics are used. Cultures are incubated at 37° C in a humidified atmosphere containing 5 % (v/v) CO₂, for 24 hours.

7.2. Exposure concentrations used

Test item :	solutions	5 000 µg/mL	n* = 4
		1 500 µg/mL	n = 4
		500 µg/mL	n = 4
		150 µg/mL	n = 4
Negative control :	DMEM + 10 % FCS	100 %	n = 4
Positive control :	Phenol	0.64 mg/mL	n = 4

* number of culture wells

7.3. Test

Cultures are examined with a microscope to verify that cells constitute a subconfluent monolayer and that their morphology is not altered. Culture medium is withdrawn and replaced with the solution at 5 000 µg/mL and its dilutions (1 500, 500 and 150 µg/mL), negative and positive controls supplemented with 10 % (v/v) FCS.

Wells are incubated at 37° C in a humidified atmosphere containing 5 % (v/v) CO₂, for a 24 hour period.

7.4. Cytotoxicity evaluation

7.4.1. Qualitative evaluation

Photos are made (x 320) showing the cell layer in contact with negative control, positive control and the solution at 5 000 µg/mL, to observe morphology and cell density of the cultures.

7.4.2. Quantitative evaluation

At the end of the incubation period, culture medium is removed. Cells are detached (2 minutes) using 250 µL trypsin (OC070714-1) (0.05 % (w/v) in Hank's balanced solution Ca⁺⁺ and Mg⁺⁺ free supplemented with 1 mM EDTA). Then 250 µL of a Trypan blue (AP260814-1) solution at 0.2 % (w/v) in 0.15 M NaCl + 10 % (v/v) FCS are added (incubation for 2 minutes).

Thereafter living cells (uncoloured) are counted using an haemocytometer (Malassez cell) (LEMI SOP n° MB08/23).

7.5. Expression of results

Results are expressed as number of cells per cm² (cell density).

8. Evaluation and conclusion criteria

8.1. For positive control

A statistically significant inhibition of cell growth superior to 30 % compared to the negative control must be observed.

8.2. For test item

Qualitative evaluation:

Morphology and cell density of the cell layer in contact with the solution at 5 000 µg/mL are compared to the negative control and the positive control.

Quantitative evaluation:

The inhibition of cell growth is used to evaluate the cytotoxic effect of the test item.

Statistical analysis is performed using the Student's *t* test. P values less than 0.05 are considered statistically significant. However, the biological relevance of the results shall be considered first.

A test item is considered as cytotoxic if the inhibition of the cell growth is superior to 30 %.

9. Deviation

No deviation.

10. Results

- Qualitative results are presented in photos 1 to 3, in the annex.
- Quantitative results are presented in table 1 (mean ± standard deviation), in the annex.

11. Interpretation of results

11.1. Qualitative evaluation

Photos (x 320) show quite obviously the lack of cytotoxic effect.

- Photo 1 shows the cell layer of negative control (non-cytotoxic).
- Photo 2 shows the cell layer of the positive control (cytotoxic).
- Photo 3 shows the cell layer in contact with the solution at 5 000 µg/mL: cell morphology and cell density are comparable to those of negative control.



11.2. Quantitative evaluation

- Comparison of the results obtained for :
 - the solution at 5 000 µg/mL (pH 7.6 - osmolality 339 mOsm/kg H₂O) and dilutions,
 - negative control (pH 7.7 - osmolality 337 mOsm/kg H₂O) and dilutions,does not show any inhibition of cell growth statistically significant superior to 30 %.

According to the evaluation criteria of the cytotoxicity (see 8.2.), the solution at 5 000 µg/mL prepared from the test material is not cytotoxic.

- Positive control induces a 71 % (P < 0.001) inhibition on cell growth which validates the study.

12. Conclusion

The test item (solution at 5 000 µg/mL) prepared from ORAL ESSENTIALS - Lot N° 06/2014 (LEMI code : FQL040814), provided by ORAL ESSENTIALS INC., is not cytotoxic in the framework of ISO 10993-5:2009 "Biological evaluation of medical devices - Tests for *in vitro* cytotoxicity".

13. Archives

Original data, correspondence, and assay report are to be kept by LEMI in a dedicated store room, for a period of ten years. At the end of this period, the assay archives will be either returned to the sponsor of the study or destroyed.

The test item is archived as a general rule 10 years, in its container of origin, whatever is its nature. However:

- the test item shall be sent back to the sponsor, at his request,
- if the sample presents a health or environmental risk, the storage conditions of the test item shall be charged to the sponsor.

Remarks:

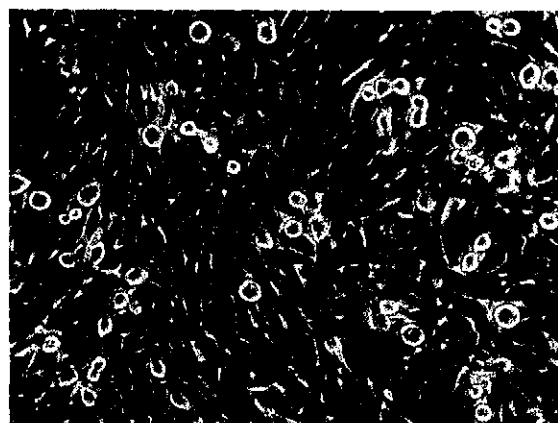
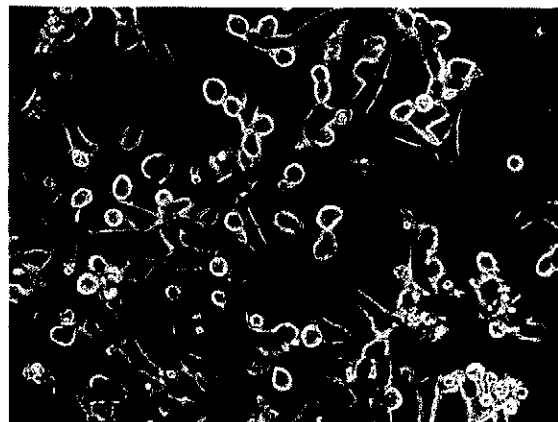
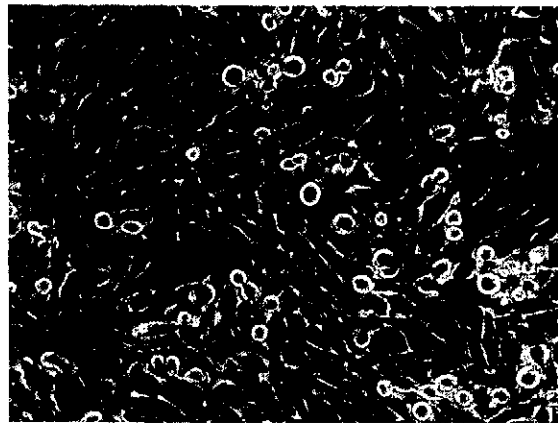
- a) The assay report above applies only to the sample of the test item submitted to the assay. Extrapolation of these results to another batch of production is the responsibility of the manufacturer.
- b) This report is not to be partially duplicated without the approval of LEMI.



TECHNICAL ANNEX



Qualitative results



1 : Negative control	<u>1</u>
2 : Positive control	<u>2</u>
3 : FQL030914-S1 – solution at 5 000 µg/mL	<u>3</u>



Quantitative results

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SERIES	150 µg/mL		500 µg/mL		1500 µg/mL		5000 µg/mL	
	Cellules/cm ²	%	Cellules/cm ²	%	Cellules/cm ²	%	Cellules/cm ²	%
Solution of ORAL ESSENTIALS - Lot N° 06/2014 LEMI code : FQL030914-S1	282 500 ± 2 105	101 *	288 905 ± 4 370	103 *	282 030 ± 13 350	101 *	274 530 ± 12 135	98 *
		NS		NS		NS		NS
Negative control (culture medium)	280 000 ± 8 585	100						
Positive control (phenol 0.64 mg/mL)	81 720 ± 4 960	29 *						
		P < 0.001						

Table n° 1

* Versus negative control

NS: not statistically significant (P ≥ 0.05)

A test item (solution at 5 000 µg/mL) is considered as cytotoxic if the inhibition of the cell growth is superior to 30%.

The test item (solution at 5 000 µg/mL) prepared from ORAL ESSENTIALS - Lot N° 06/2014 (LEMI code: FQL040814), provided by ORAL ESSENTIALS INC., is not cytotoxic².

Date : 18 September 2014

M.-F. HARMAND
Study Director

² Accreditation of COFRAC testifies to the competence of the laboratories for only the assays covered by the accreditation.

