Laboratoire BIO-EC

Centre de Recherches Biologiques et d'Expérimentations Cutanées

Study 18E4017

Assessment of the global anti-pollution protective activity of a product on human living skin explants

According to the study plan D17-732-3

Tested product P : Rivoli Crème de Jour Jeunesse II ref. Torstone

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End of the study

Chronological plan

Status	☐ BPL	☑ Non BPL
Date of the beginning of the study *(signature of the study plan by the study director)*	16th January 2018	
Date of the beginning of the technical phase of the study	17th January 2018	
Culture manager C. Durand	*Date*	*Signature*
Histology manager C. Delpy	*Date*	*Signature*
Expertise phase manager L. Peno-Mazzarino	*Date*	*Signature*
Partners and subcontracting	**None**	
Date of the end of the technical phase of the study	5th March 2018	

CONTENTS

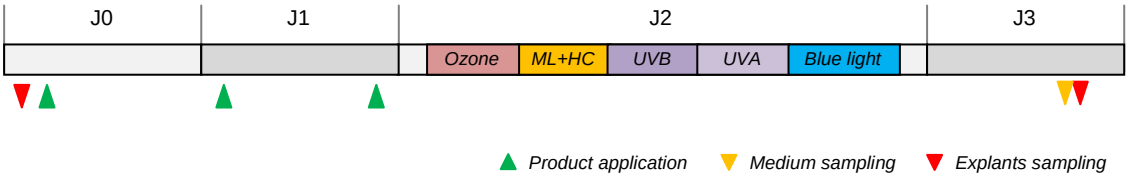
AIM OF THE STUDY

The aim of this study is to assess the protective effect of a cosmetic product against a global pollution induced by ozone, heavy metals, hydrocarbons, UV irradiations and blue light, on human living skin explants.

The activity has been evaluated by:
- Cell viability control
- Immunostaining of Nrf2
- Immunostaining of oxidized proteins
- Dosage of MDA

MATERIAL & METHODS

1. Study design



2. Product

The sponsor has provided the following product :

Product	Identification	Reference	Batch	Aspect	Quantity
P	Rivoli Creme de Jour Jeunesse II	Torstone	lab-01095.4 14.12.17	cream	1 vial

The product has been stored at room temperature before, within and after the duration of the study.

3. Pollutants

BIO-EC laboratory has provided the following products:

Product	Identification	Reference	Batch	Aspect
A	Solution ICP multielements standard V	1.10714.0500 (Merck)	HC309202	liquid
B	Benzene	12550 (Fluka)	128346521406P01	liquid
C	Xylene	95673 (Fluka)	135438840208115	liquid
D	Toluene	34866 (Sigma)	SHBD4964V	liquid

The solution A (ICP multi-element standard V) is a mixture of heavy metals supplemented with hydrocarbons (B+C+D). The composition and concentrations of each components of this pollutant mixture are indicated in the appendix.

4. Characteristic of the plasty

27 human skin explants of an average diameter of 12 mm (±1mm) were prepared on an abdoplasty coming from a 35-year-old caucasian woman (reference: P1928-AB35, phototype II). The explants were kept in survival in BEM culture medium (BIO-EC's Explants Medium) at 37°C in a humid, 5 %-CO_2 atmosphere.

5. Explant distribution

The explants were distributed into 7 batches as follows:

Batch	Denomination	Treatment	Nb of explants	Sampling
T0	Control plasty	none	3	D0
T	Blank batch	none	4	D3
P	Tested product	P	4	D3
ML1	Pollution (condition 1)	ozone (1h) + heavy metals and hydrocarbons (1.5 mL, 1h30) + UV A & B (1 MED) + blue light (2h)	4	D3
ML2	Pollution (condition 2)	ozone (2h) + heavy metals and hydrocarbons (3 mL, 1h30) + UV A & B (2 MED) + blue light (3h)	4	D3
PML1	Product + Pollution (condition 1)	P + ozone (1h) + heavy metals and hydrocarbons (1.5 mL, 1h30) + UV A & B (1 MED) + blue light (2h)	4	D3
PML2	Product + Pollution (condition 2)	P + ozone (2h) + heavy metals and hydrocarbons (3 mL, 1h30) + UV A & B (2 MED) + blue light (3h)	4	D3

6. Product application

On day 0 (D0) and D1 (morning and evening), the product P was topically applied on the basis of 2 µL per explant (2 mg/cm²), and spread using a small spatula.
The control explants T did not receive any treatment except the renewal of culture medium.
The culture medium was half renewed (1 mL per well) on D1 and completely (2 mL per well) on D2.

According to the study plan, the days of treatments, pollution induction and sampling were adjusted to fit the schedule of the working days.

7. Induction of global pollution

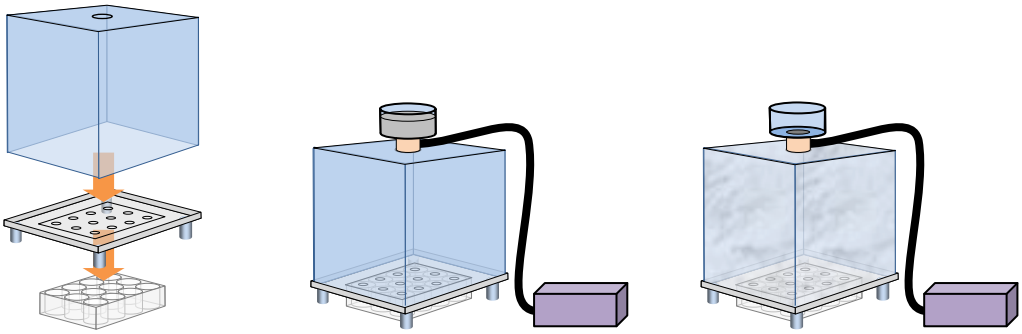
On D2, the explants of the concerned batches were exposed to successive stresses in this following order: ozone exposure, heavy metals and hydrocarbons exposure, UVA&B irradiations and blue light irradiation. Two different conditions (ML1 and ML2) were evaluated for each stress.

7.1. Ozone exposure

On D2, the explants of the batches “ML” were placed on the PolluBox® system with 900 µl per well of HBSS (Hank’s Balanced Saline Solution), and exposed by vaporization, to 5-10 ppm of ozone (Ozonator Ozone generator Mspa) for 1 hour for “ML1” batches and for 2h for “ML2” batches, as shown in the figure below.
The control explants T and P were kept in 1 ml of HBSS, during this time.

7.2. Heavy metals+ hydrocarbons exposure

On D2, the explants of the batches “ML” were placed on the PolluBox® system with 900 µl per well of HBSS, and exposed by spraying, to a mixture of hydrocarbons + heavy metals supplemented with NaCl 0.9% (150 µl of NaCl 0.9% per ml of pollutant solution) for 1.5 hour using 1,5 ml for “ML1” batches and for 1,5 hours using 3 ml for “ML2” batches, as shown in the figure below.
The control explants T and P were kept in 1 ml of HBSS, during this time.



7.3. UV irradiations

On D2, the explants of the batches “ML” were put in 1 ml of HBSS in 12-well plates and were irradiated by UVA + UVB using a UV simulator Vibert Lourmat RMX 3W with two different doses:
- UV intensity for “ML1” batches: 4,5 J/cm^2 of UVA and 0,15 J/cm^2 of UVB corresponding both to 1 MED (minimal erythemal dose);
- UV intensity for “ML2” batches: 9 J/cm^2 of UVA and 0,3 J/cm^2 of UVB corresponding both to 2 MED (minimal erythemal dose).
The unirradiated batches were kept in 1 ml of HBSS in the dark.

7.4. Blue light irradiation

On D2, the explants of the batches “ML” were put in 1 ml of HBSS in 12-well plates and irradiated with the Solarbox® with 2 different doses of blue light:
- Blue light Intensity for “ML1” batches: 42,5 J/cm² (2H)
- Blue light Intensity for “ML2” batches: 63,7 J/cm² (3H)
The unirradiated batches were kept in 1 ml of HBSS in the dark.

At the end of the last irradiation, all the explants were put back in 2 mL of fresh BEM medium.

8. Sampling

On D0, the 3 explants from the batch T0 were collected and cut in two parts. Half was fixed in buffered formalin and the half was frozen at -80°C.
On D3 (24 hours after global pollution exposure), 3 explants from each batch were collected and treated the same way than on D0; the fourth explant was kept frozen at -80°C.

The BEM culture media of all explants (2 ml) were harvested on D3 (24 hours after global pollution exposure) and frozen at -20°C for MDA dosage.

9. Histological processing

After fixation for 24 hours in buffered formalin, the samples were dehydrated and impregnated in paraffin using a Leica PEARL dehydration automat. The samples were embedded using a Leica EG 1160 embedding station.
5-µm-thick sections were made using a Leica RM 2125 Minot-type microtome, and the sections were mounted on Superfrost® histological glass slides.
The frozen samples were cut into 7-µm-thick sections using a Leica CM 3050 cryostat. Sections were then mounted on Superfrost® plus silanized glass slides.
The microscopical observations were realized using a Leica DMLB or Olympus BX43 microscope. Pictures were digitized with a numeric DP72 Olympus camera with CellD storing software.

9.1. Cell viability control

The cell viability of epidermal and dermal structures was observed on paraffinized sections after Masson's trichrom staining, Goldner variant.
The cellular viability was assessed by microscopical observation.

9.2. Immunostaining of Nrf2

Nrf2 immunostaining was realized on paraffinized sections with a monoclonal anti-Nrf2 antibody (Abcam, ref. ab76026, clone EP1809Y) diluted at 1:200 in PBS-BSA 0.3%-Tween 20 at 0.05% and incubated for 1 hour at room temperature using a Vectastain Kit Vector amplifier system avidin/biotin, and revealed by VIP, a substrate of peroxidase (Vector laboratories, Ref. SK-4600).
The immunostaining was performed using an automated slide processing system (Autostainer, Dako) and assessed by microscopical observation.

9.3. Immunostaining of oxidized proteins

Oxidized proteins immunostaining has been realized on frozen sections with an anti-DNP antibody (Kit Millipore, ref. S7150), diluted at 1:250 in PBS-BSA 0.3% and incubated for 1 hour at 37°C. The staining was enhanced with a biotin/streptavidin amplifier system and revealed by VIP, a substrate of peroxidase (Vector laboratories, Ref. SK-4600).
The immunostaining was assessed by microscopical observation.

10. Dosage of MDA

The malondialdehyde (MDA) assay was realized with an enhanced method of the TBARs assay. The MDA was assayed in HBSS medium by addition of TBARs solution (thiobarbituric acid, hydrochloric acid and tricholoroacetic acid) and placed in a water bath (80°c for 15 minutes). A lot of substances (like glucose…) which are not related with lipoperoxidation, react with thiobarbituric acid (ThioBarbituric Acid Reagents = TBARS), so to enhance the specificity of the assay, the MDA was extracted by a liquid/liquid extraction with butanol. The MDA in butanol was measured in spectrofluorimetry (excitation: 515 nm, emission: 550 nm) using a Tecan Infinite M200 Pro microplate reader.
Using this assay, MDA contained in the culture medium on day 3 was measured for the 4 explants per batch. The MDA concentration was expressed in nmol/L.

ABBREVIATIONS

Listing of the abbreviations and symbols used in this report:

SD	Standard deviation
D	Day
J	for “Jours”, the French word for day, used for the pictures. D and J are used interchangeably to indicate DAY

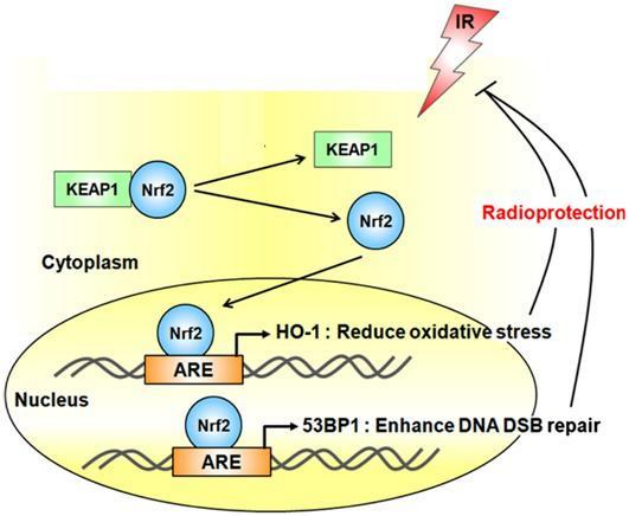
ns	Not significant
#	significant with $p<0.1$ (90%)
*	significant with $p<0.05$ (95%)
**	significant with $p<0.01$ (99%)

BACKGROUD

1. Nrf2

Nrf2 is a key transcription factor in the cellular response to oxidative stress. Human Nrf2 has a predicted molecular mass of 66 kDa and it is ubiquitously expressed in a wide range of tissues and cell types. Under oxidative stresses, including UV irradiation, Nrf2 is activated by phosphorylation and translocates from the cytoplasm to the nucleus (**Fig. 1**). So far different cytosolic kinase, including including protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), and ER-localized pancreatic endoplasmic reticulum kinase (PERK) have been shown to modify Nrf2 and are potentially involved in the dissociation of Nrf2 from its inhibitor, Keap1 (Pi et al., 2007. *Free Radic Biol Med*. 42: 1797–1806). Once in the nucleus, Nrf-2 binds to the DNA at the location of the Antioxidant Response Element (ARE) or also called hARE (Human Antioxidant Response Element) which is the master regulator of the total antioxidant system. Nrf2 plays a role in protecting human skin keratinocytes from UVA radiation-induced damage (Tia et al., 2011. *BioScience Trends*. 5:23-29).

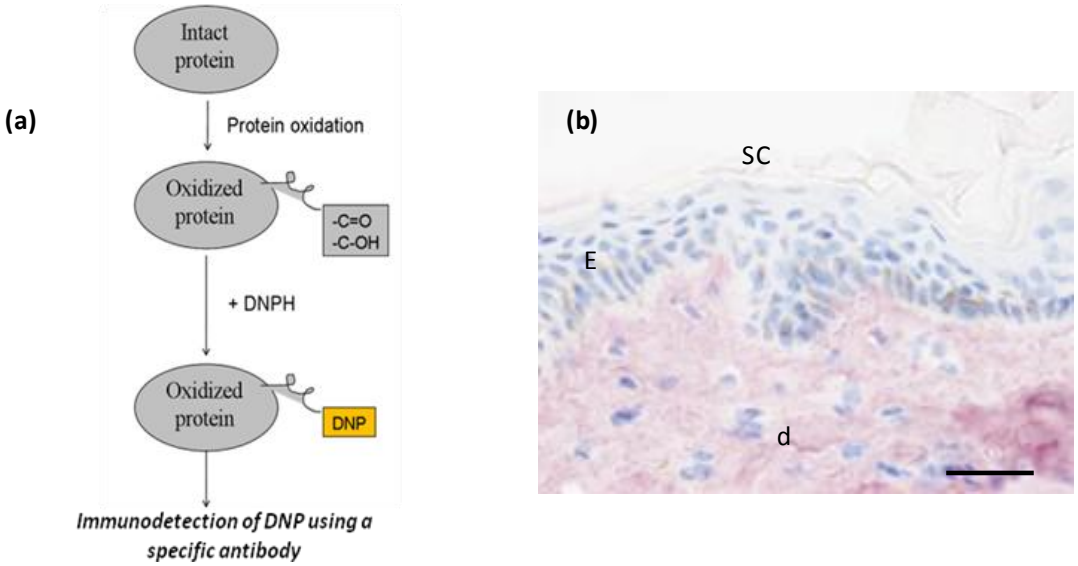
Figure 1. Schematic representation of Nrf2- activated response under oxidative stresses.



2. Oxidized proteins

The staining of oxidized proteins was realized using the OxyBlotTM protein oxidation kit (Millipore, S7150) on frozen sections. This kit allows the detection of carbonyl groups introduced into proteins by oxidative reactions with 2,4-dinitrophenylhydrazine (DNPH), which leads to the formation of a stable dinitrophenyl (DNP) hydrazone product, recognized successively by a specific antibody (**Fig. 2**).

Figure 2. In (a) the different steps for oxidized protein revelation, and in (b) immunostaining of oxidized protein revealed by VIP. Abbreviations: d, dermis; E, epidermis; SC, stratum corneum. Scale bar: 50 µm.



RESULTS & DISCUSSION

1. Cell viability

The cell viability of the different batches is shown here below:

Batch	Cell viability		Comments
	Epidermis	Dermis	
T0	G	G	-
TJ3	FG	G	-
PJ3	FG	G	-
ML1J3	VSA	G	-
PML1J3	FG	G	-
ML2J3	MA to FCA	G	-
PML2J3	MA	G	-
Morphology legend: G= good, FG= fairly good, VSA=very slightly altered, SA=slightly altered, MA= moderately altered, FCA= fairly clearly altered, CA= clearly altered, VCA=very clearly altered			

On D0,
On the blank batch (T0), the cellular viability of the epidermis and the dermis is good.

On D3,
On the blank batch TJ3, the cellular viability is fairly good in the epidermis and good in the dermis.

Effect of product application on the cellular viability, compared to the batch TJ3:

- The product **P** induces no modification of cell viability.

The global pollution ML1 induces very slight epidermal alterations but no modification of dermal cell viability, compared to TJ3.

Effect of product application on the cellular viability, compared to the batch **ML1J3**:

- The product **P** induces a very slight decrease of epidermal alterations
  - **So, the product P induces a complete protection against ML1-induced epidermal alterations.**

The global pollution ML2 induces moderate to fairly clear epidermal alterations but no modification of dermal cell viability, compared to TJ3.

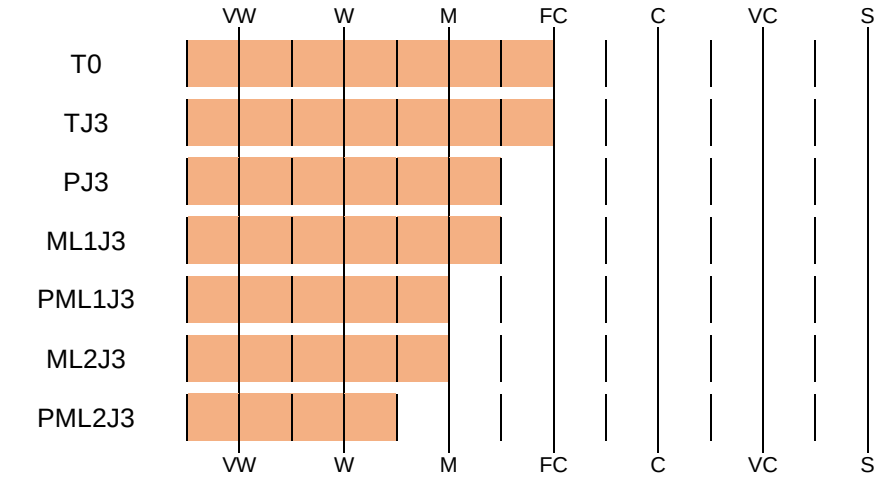
Effect of product application on the cellular viability, compared to the batch **ML2J3**:

- The product **P** induces a very slight decrease of epidermal alterations
  - **So, the product P induces a partial protection against ML2-induced epidermal alterations.**

2. NRF2

On day 0, on the blank batch T0, the staining of NRF2 is fairly clear in the epidermis.

The staining of NRF2 on all batches is shown here below:



VW: very weak, W: weak, M: moderate, FC: fairly clear, C: clear, VC: very clear, S: strong

On D3,
On the blank batch TJ3, the expression of NRF2 is fairly clear in the epidermis.

Effect of product application on NRF2 expression, compared to the batch TJ3:

- The product **P** induces a slight decrease.

The global pollution ML1 induces a slight decrease of NRF2 expression, compared to TJ3.

Effect of product application on NRF2 expression, compared to the batch **ML1J3**:

- The product **P** induces a slight decrease

The global pollution ML2 induces a moderate decrease of NRF2 expression, compared to TJ3.

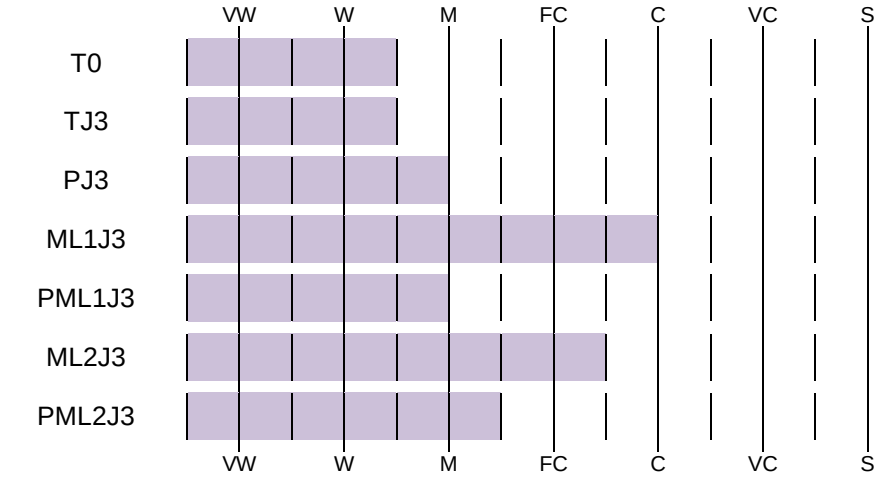
Effect of product application on NRF2 expression, compared to the batch **ML2J3**:

- The product **P** induces a slight decrease

3. Oxidized proteins

On day 0, on the blank batch T0, the staining of oxidized proteins is weak to moderate in the papillary dermis.

The staining of oxidized proteins on all batches is shown here below:



VW: very weak, W: weak, M: moderate, FC: fairly clear, C: clear, VC: very clear, S: strong

On D3,
On the blank batch TJ3, the formation of oxidized proteins is weak to moderate in the papillary dermis.

Effect of product application on oxidized proteins formation, compared to the batch TJ3:

- The product **P** induces a slight increase.

The global pollution ML1 induces a very clear increase of oxidized proteins formation, compared to TJ3.

Effect of product application on oxidized proteins formation, compared to the batch **ML1J3**:

- The product **P** induces a clear decrease
  - **So, the product P induces a almost complete protection against ML1-induced oxidized proteins.**

The global pollution ML2 induces a clear increase of oxidized proteins formation, compared to TJ3.

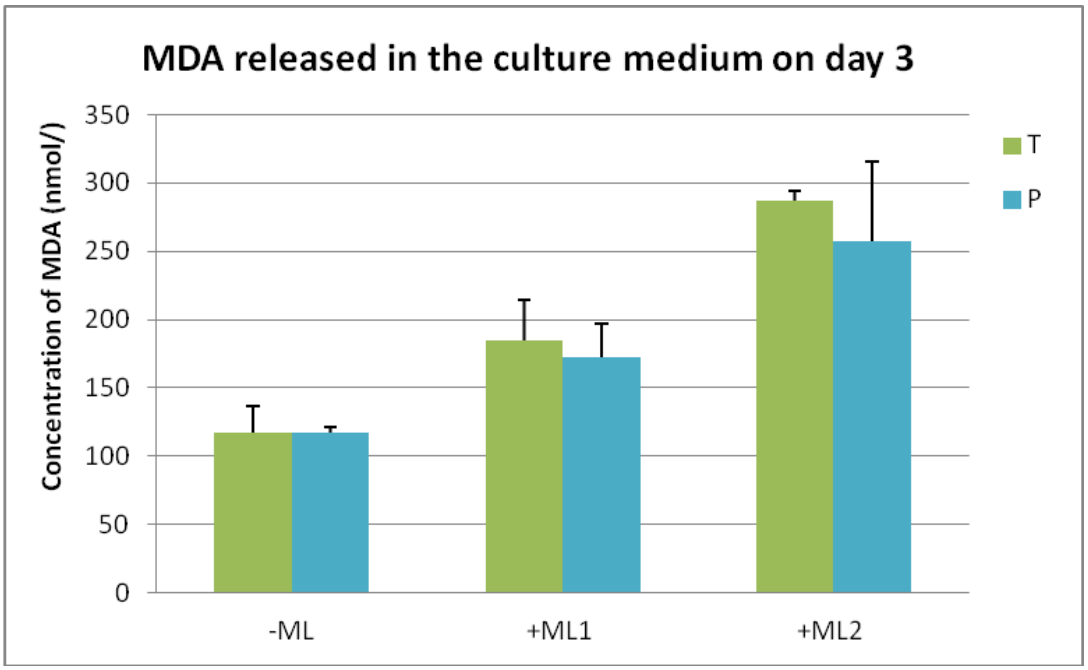
Effect of product application on oxidized proteins formation, compared to the batch **ML2J3**:

- The product **P** induces a moderate decrease
  - **So, the product P induces a partial protection against ML2-induced oxidized proteins.**

4. Dosage of MDA

The mean concentration of MDA released in BEM culture media on day 3 (in nmol/l) for all batches is shown hereblow:

	MDA (nmole/L)					
	TJ3	**PJ3**	**ML1J3**	**PML1J3**	**ML2J3**	**PML2J3**
Mean	**117,4**	**117,0**	**184,4**	**172,2**	**287,1**	**257,7**
SD	18,8	4,5	30,5	24,8	7,3	58,1



On day 3,

On the blank batch TJ3, the mean concentration of MDA released in BEM culture medium is 117,4 nmol/l.

Effect of product application on MDA concentration, compared to the batch TJ3:

- The product **P** induces a non significant decrease of 0,4%^{ns}.

The global pollution ML1 induces a significant increase of 57%* of MDA released in the culture medium, compared to TJ3.

Effect of product application on MDA concentration, compared to the batch **ML1J3**:

- The product **P** induces a non significant decrease of 7%^{ns}

The global pollution ML2 induces a significant increase of 144%** of MDA released in the culture medium, compared to TJ3.

Effect of product application on MDA concentration, compared to the batch **ML2J3**:

- The product **P** induces a non significant decrease of 10%^{ns}

Non-significant	ns	Significant	# : p<0.1 (90%)	* : p<0.05 (95%)	** : p<0.01 (99%)

5. Induction of MDA

If we consider that without ML, the product P induces slight variations of MDA concentration, it is possible to compare the induction of MDA after ML1 and ML2 application, for each explant.

The concentration of MDA induced after ML1 and ML2 application in the culture medium on D3 is calculated for each explant according to the following formula :

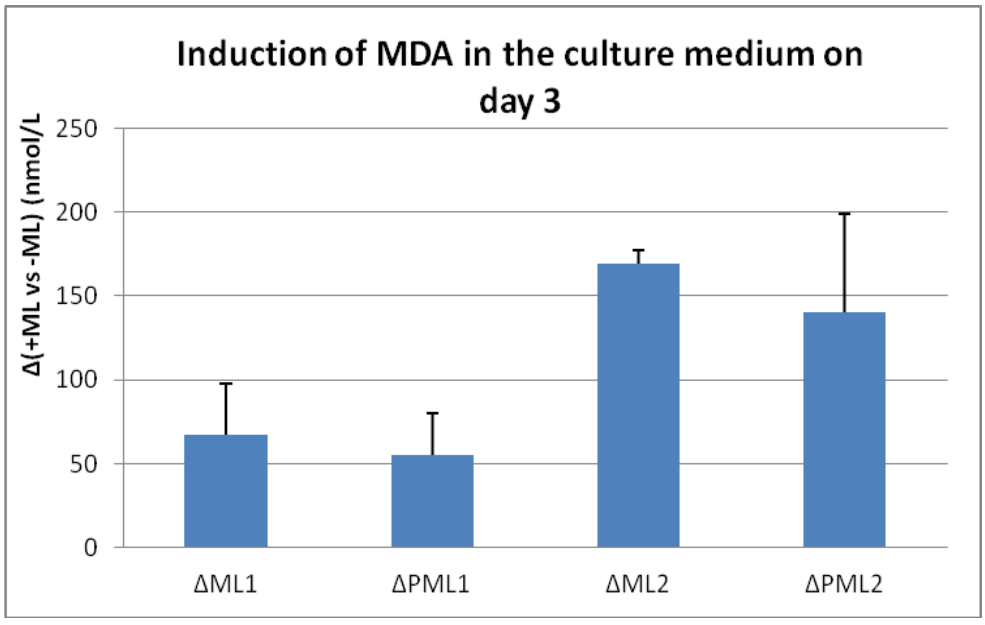
ΔML1**=** Δ(ML1J3-**meanTJ3**) => **meanTJ3=** 117,4 nmol/l.

So, for example, we obtained for ΔML1:
Explant A: 152,9 – 117,4 =35,5 nmol/l
Explant B: 163,8 – 117,4= 46,4 nmol/l
Explant C: 213,1 - 117,4 = 95,7 nmol/l
Explant D: 207,8 - 117,4 = 90,3 nmol/l

So a mean induction of 67 nmol/l of MDA released in the culture medium after ML1 application and 169,6 nmol/l after ML2 application.
The same calculation is performed for each batch (see page 43 in the appendix).

The mean concentration of MDA induced in the culture medium after ML1 and ML2 application of all batches is shown in the table below:

	Delta (+ML vs -ML)			
	ΔML1	**ΔPML1**	**ΔML2**	**ΔPML2**
Mean	**67,0**	**55,2**	**169,6**	**140,7**
SD	30,5	24,8	7,3	58,1



On day 3, the product **P** reduces non-significantly by 18%^{ns} the ML1-induced MDA increase in the culture medium (ΔPML1 vs ΔML1).

The product **P** reduces non-significantly by 17%^{ns} the ML2-induced MDA increase in the culture medium (ΔPML2 vs ΔML2).

Non-significant	ns	Significant	# : p<0.1 (90%)	* : p<0.05 (95%)	** : p<0.01 (99%)

CONCLUSION

According to the experimental conditions described above and compared to the blank batch (**TJ3**), to global pollution 1 treated batch on D3 (**ML1J3**) or global pollution 2 treated batch on D3 (**ML2J3**) :

variations vs TJ3 or ML1J3 or ML2J3		**Rivoli Creme de Jour Jeunesse II ref. Torstone (P)**
Cell viability	*vs TJ3*	↔
	vs ML1J3	(↘) epidermal alterations
	vs ML2J3	(↘) epidermal alterations
NRF2	*vs TJ3*	↘
	vs ML1J3	↘
	vs ML2J3	↘
Oxidized proteins	*vs TJ3*	↗
	vs ML1J3	↘↘↘↘
	vs ML2J3	↘↘
MDA	*vs TJ3*	-0,4%^{ns}
	vs ML1J3	-7%^{ns}
	vs ML2J3	-10%^{ns}
MDA induction	*vs ΔML1*	-18%^{ns}
	vs ΔML2	-17%^{ns}

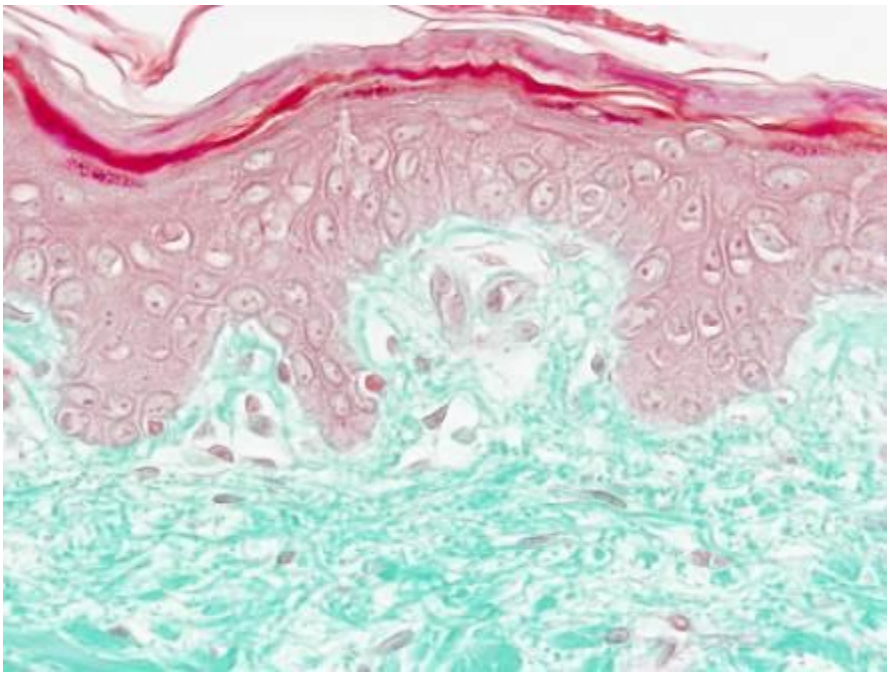
Decrease		**Increase**		
(↘)	Very slight	(↗)	↔	No variation
↘	Slight	↗		
↘↘	Moderate	↗↗	ns	Not significant
↘↘↘	Fairly clear	↗↗↗	#	significant with p<0.1 (90%)
↘↘↘↘	Clear	↗↗↗↗	*	significant with p<0.05 (95%)
↘↘↘↘↘	Very clear	↗↗↗↗↗	**	significant with p<0.01 (99%)

The product Rivoli Creme de Jour Jeunesse II ref. Torstone (P) exhibits a global anti-pollution activity characterized by a protection against pollution- induced epidermal alterations, a decrease of induced oxidized proteins, a decrease of NRF2, and a non significant reduction of induced lipid peroxidation (MDA).

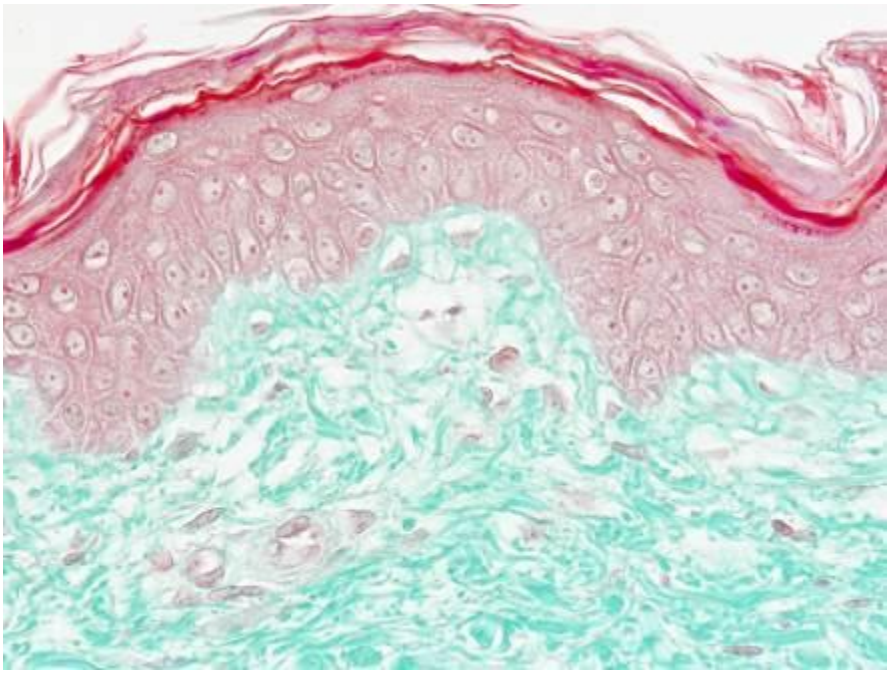
APPENDIXES

1. Cell viability

Blank batch on day 0 (T0)



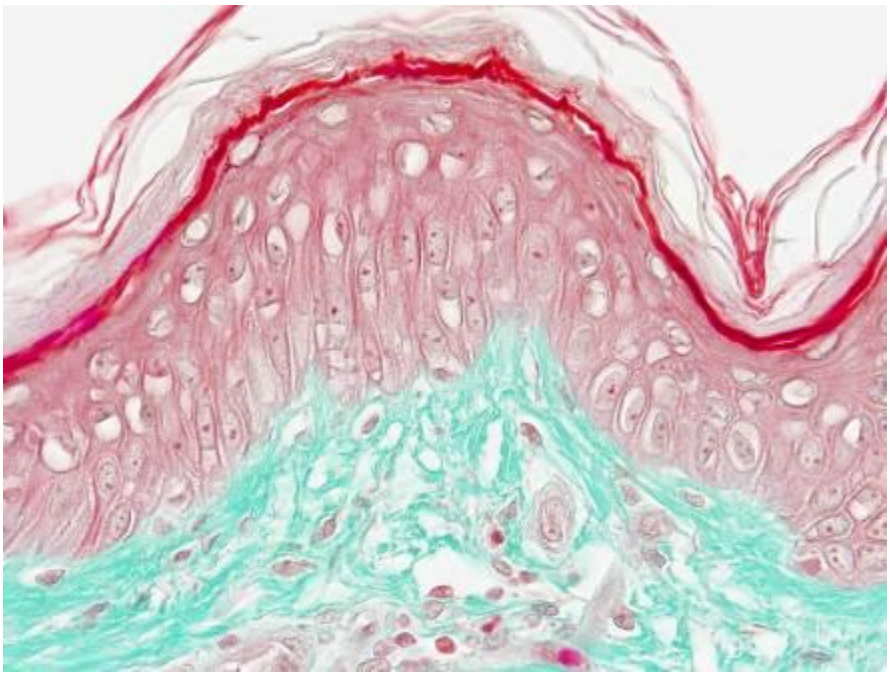
T0_10-7 18E4017 TCM 50 µm



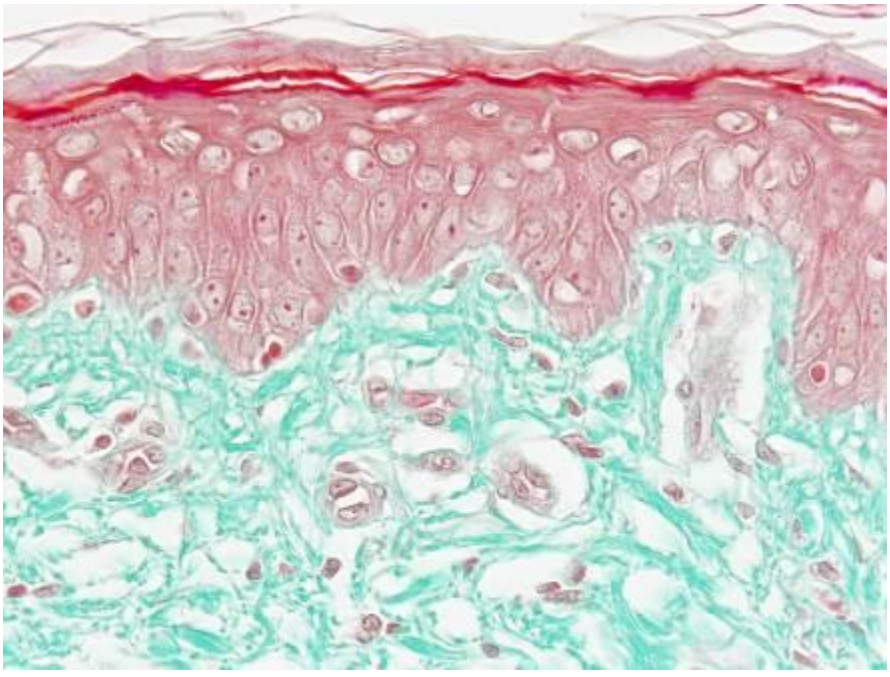
T0_14-9 18E4017 TCM 50 µm

Cell viability

Blank batch on day 3 (TJ3)



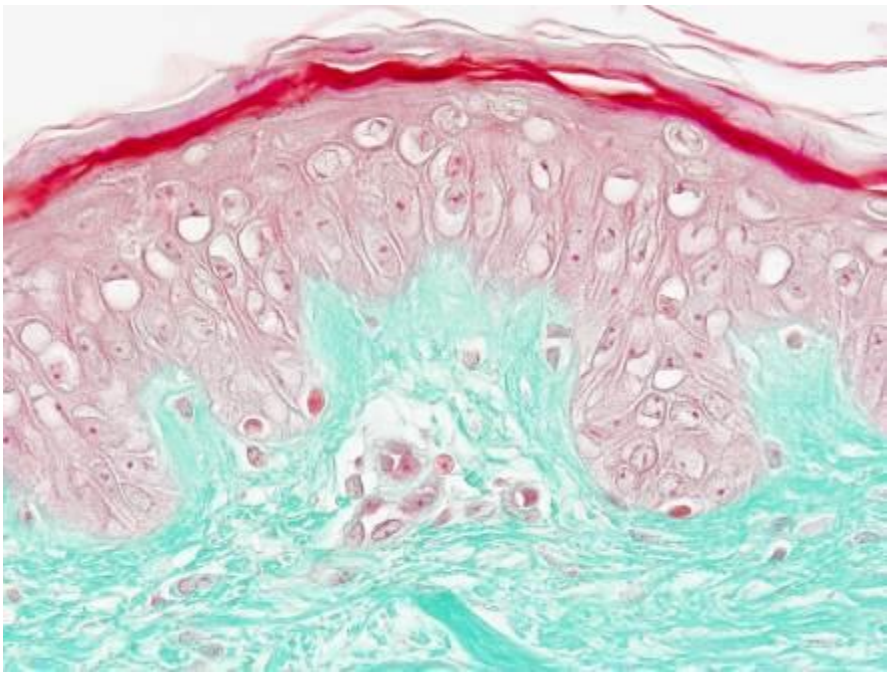
TJ3_1-13 18E4017 TCM 50 µm



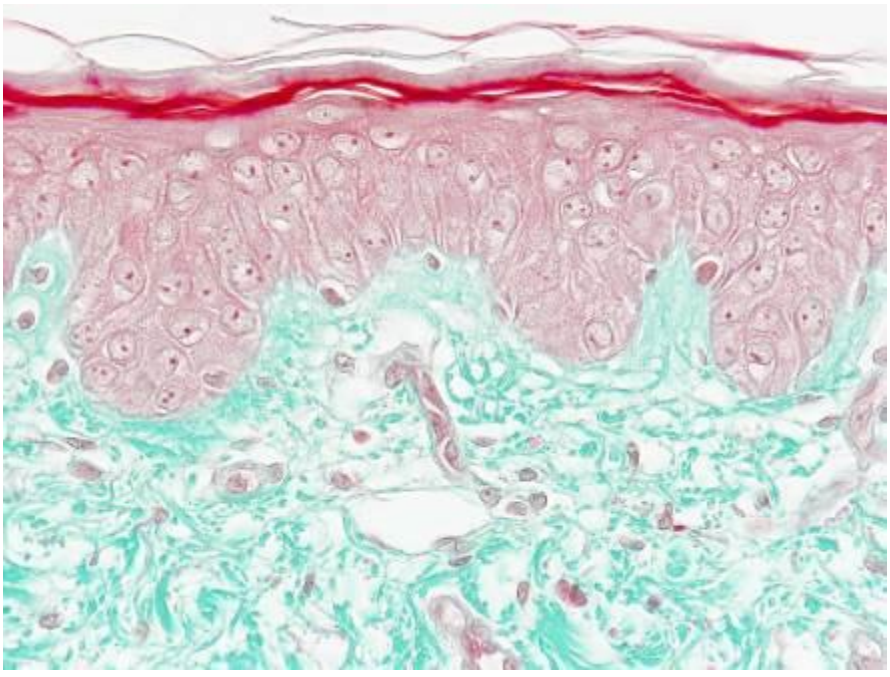
TJ3_3-15 18E4017 TCM 50 µm

Cell viability

Batch P on day 3 (PJ3)



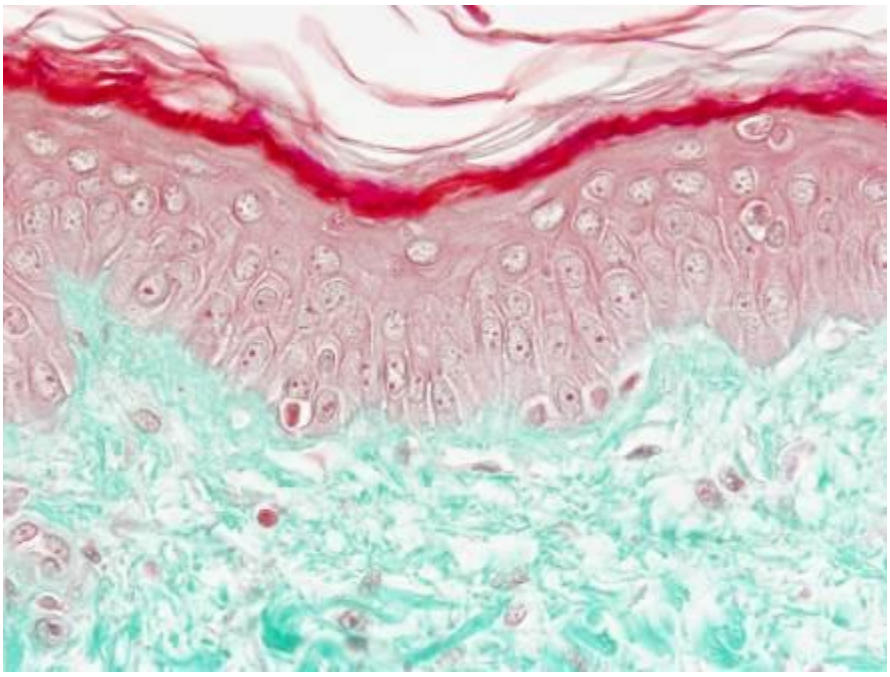
PJ3_3-25 18E4017 TCM 50 µm



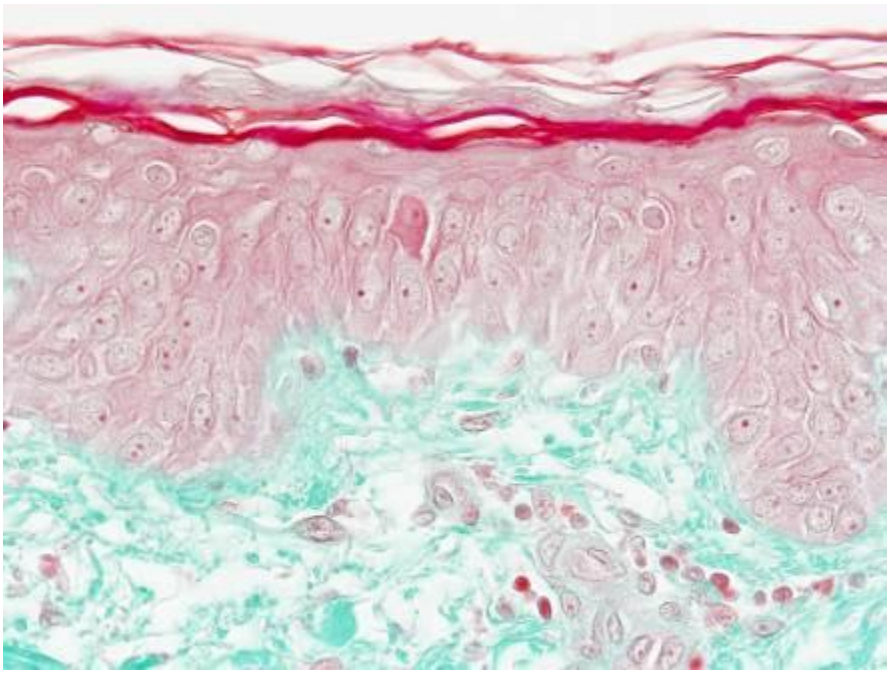
PJ3_15-34 18E4017 TCM 50 µm

Cell viability

Batch ML1 on day 3 (ML1J3)



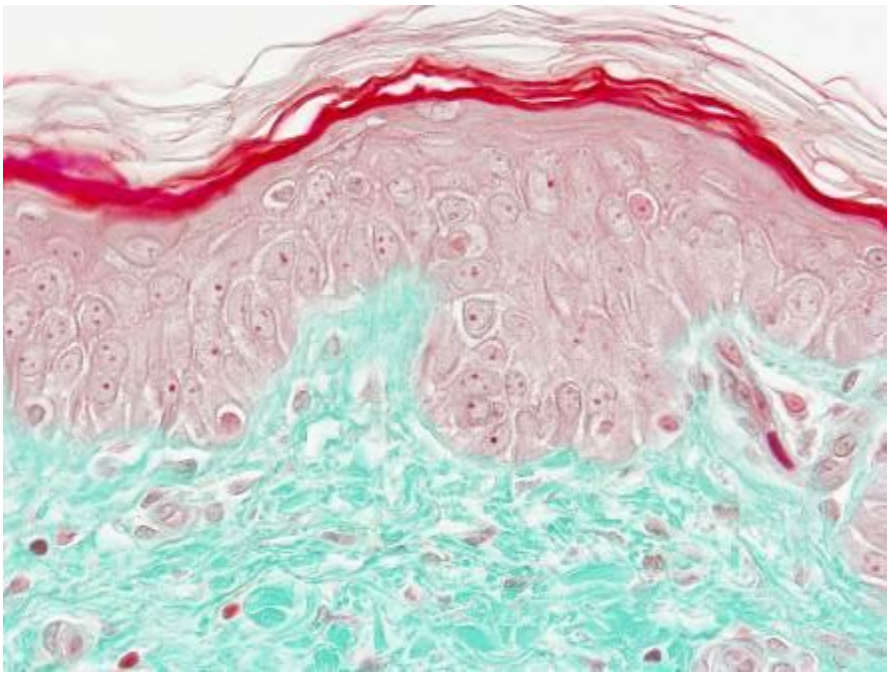
ML1J3_3-39 18E4017 TCM 50 µm



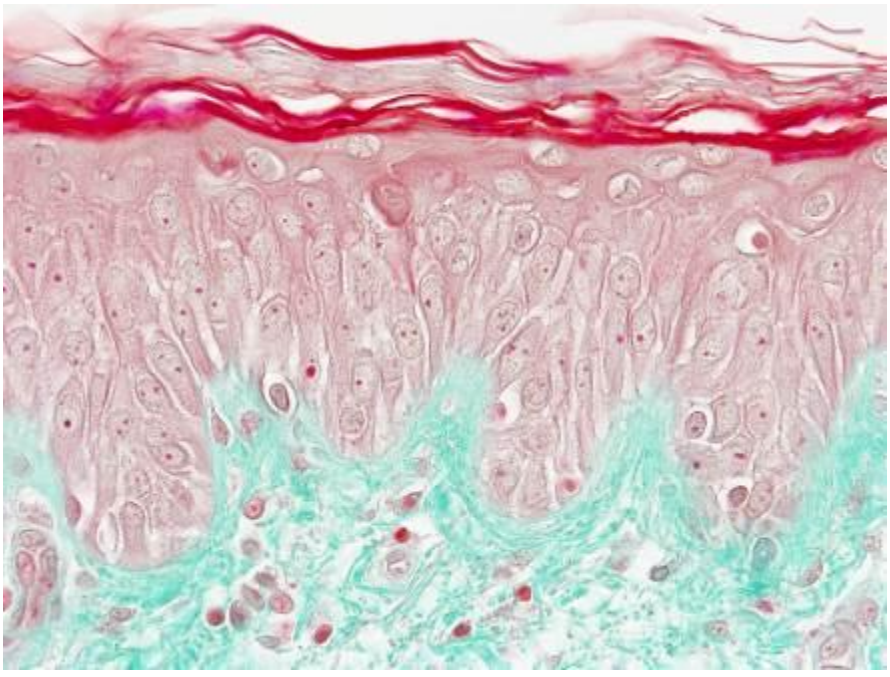
ML1J3_14-46 18E4017 TCM 50 µm

Cell viability

Batch PML1 on day 3 (PML1J3)



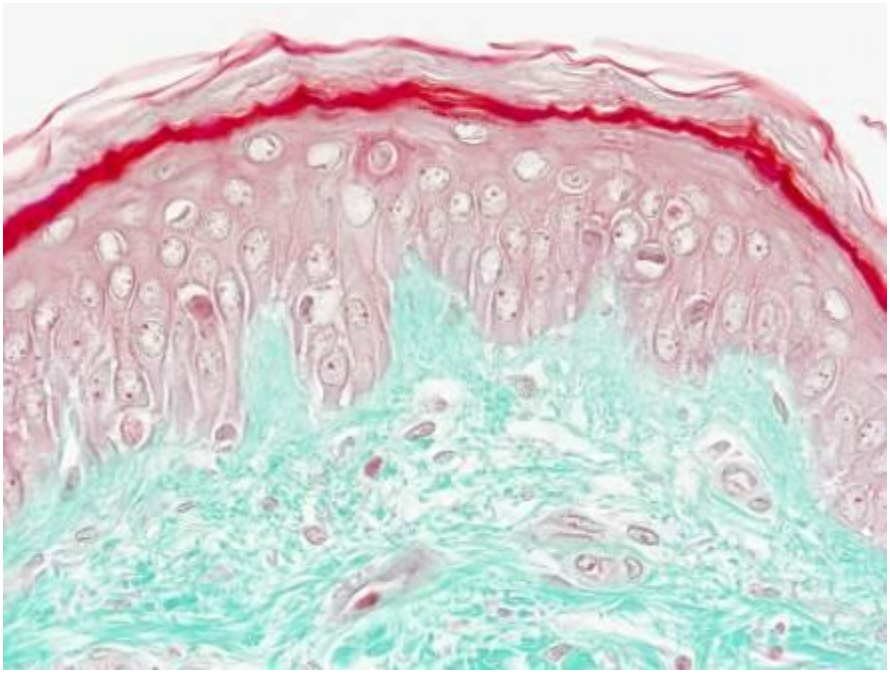
PML1J3_3-61 18E4017 TCM 50 µm



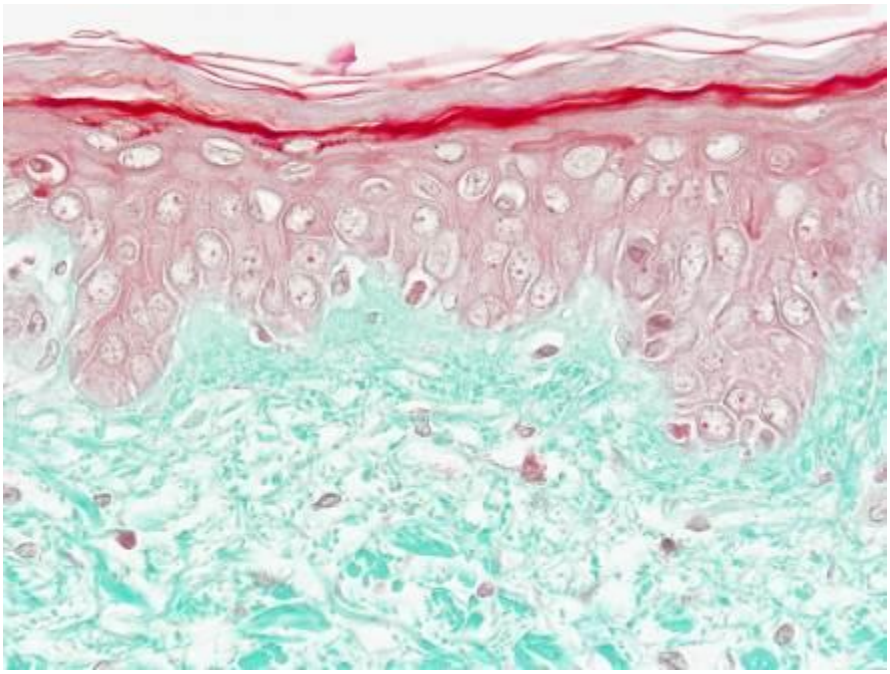
PML1J3_6-63 18E4017 TCM 50 µm

Cell viability

Batch ML2 on day 3 (ML2J3)



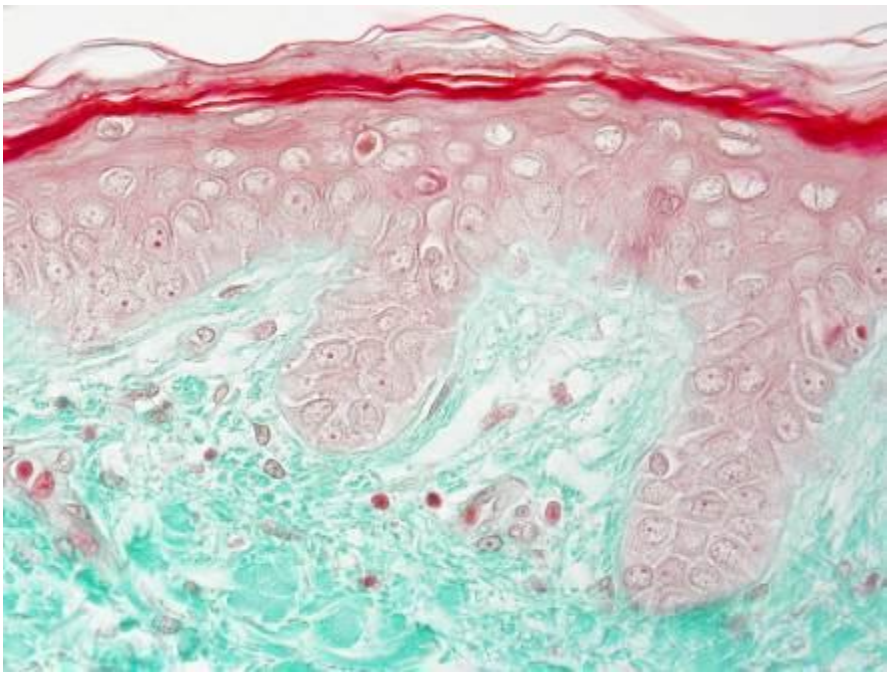
ML2J3_6-49 18E4017 TCM 50 µm



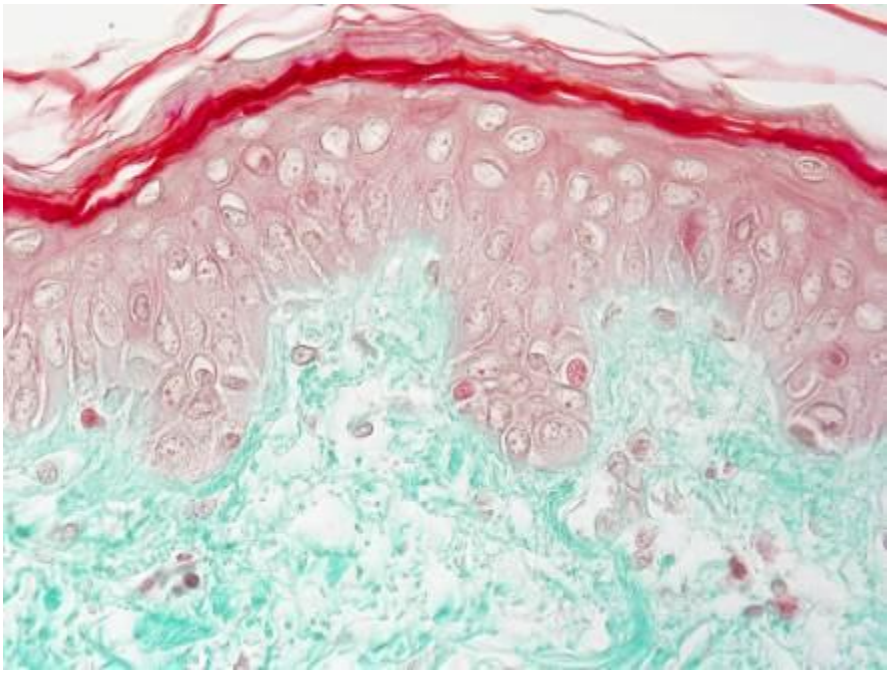
ML2J3_7-50 18E4017 TCM 50 µm

Cell viability

Batch PML2 on day 3 (PML2J3)



PML2J3_4-73 18E4017 TCM 50 µm



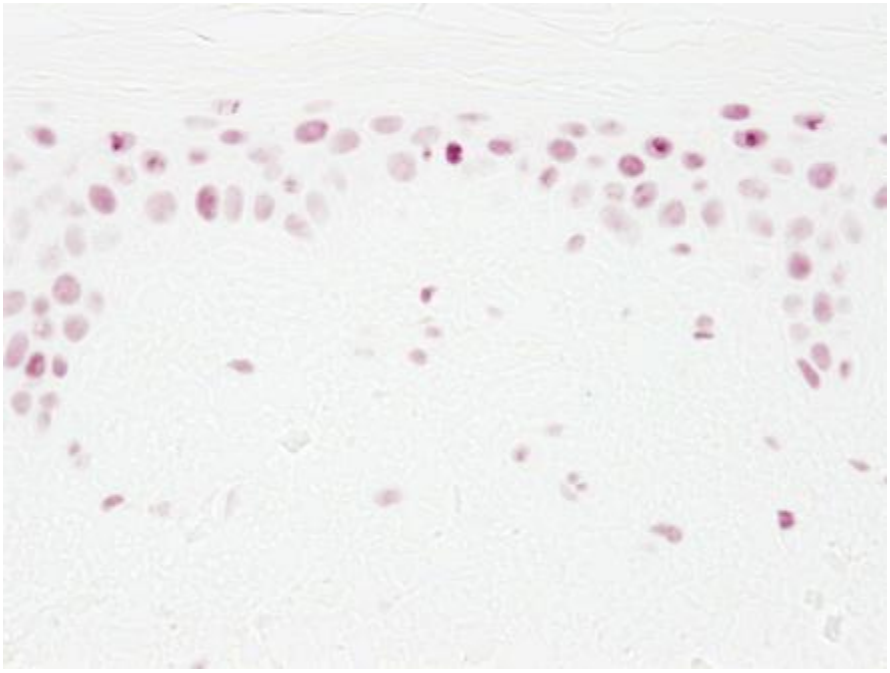
PML2J3_6-75 18E4017 TCM 50 µm

2. NRF2

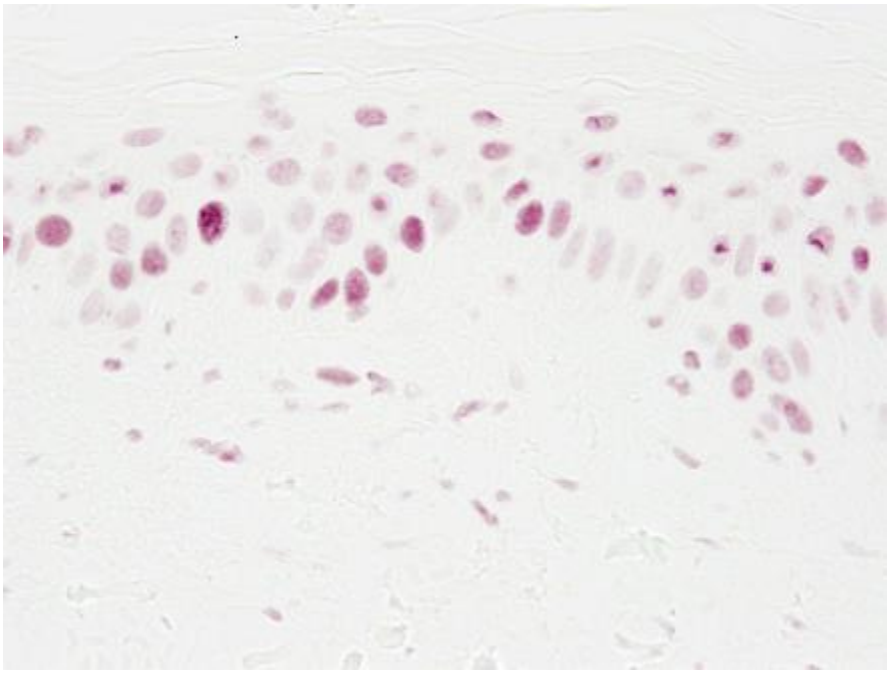
Negative control without primary antibody

NRF2

Blank batch on day 0 (T0)



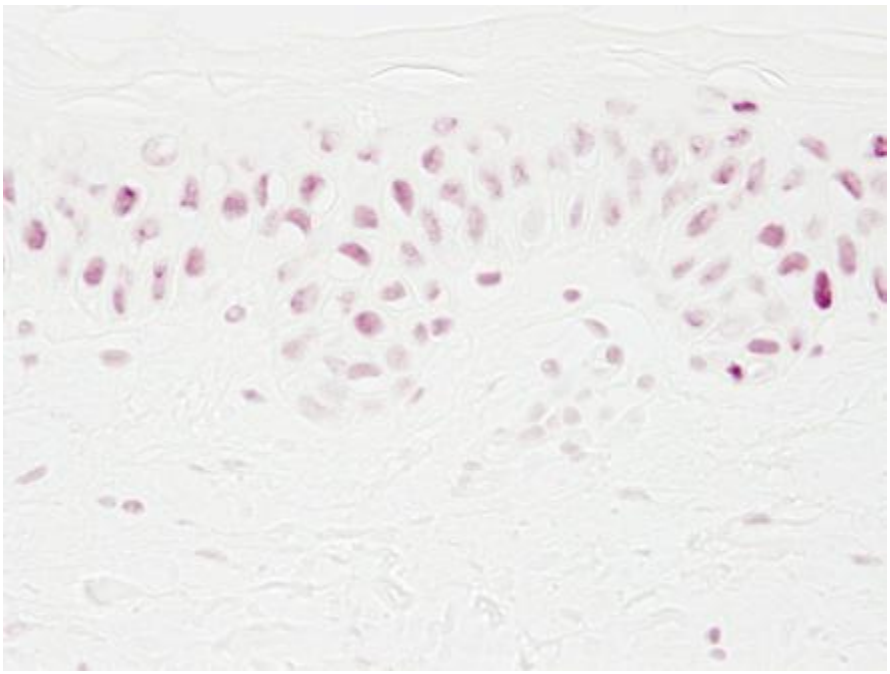
T0_2-2 18E4017 NRF2 50 µm



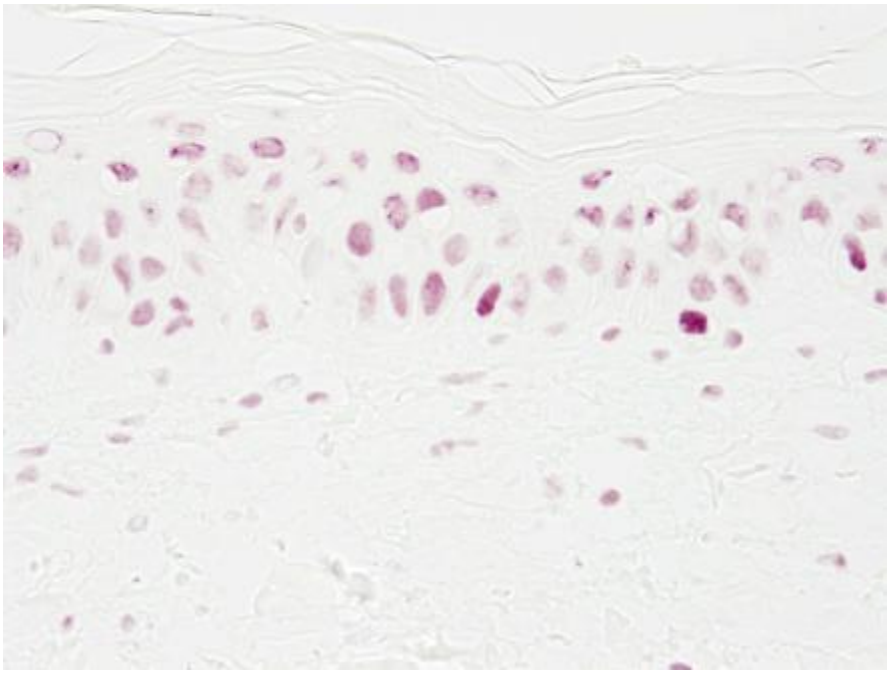
T0_12-8 18E4017 NRF2 50 µm

NRF2

Blank batch on day 3 (TJ3)



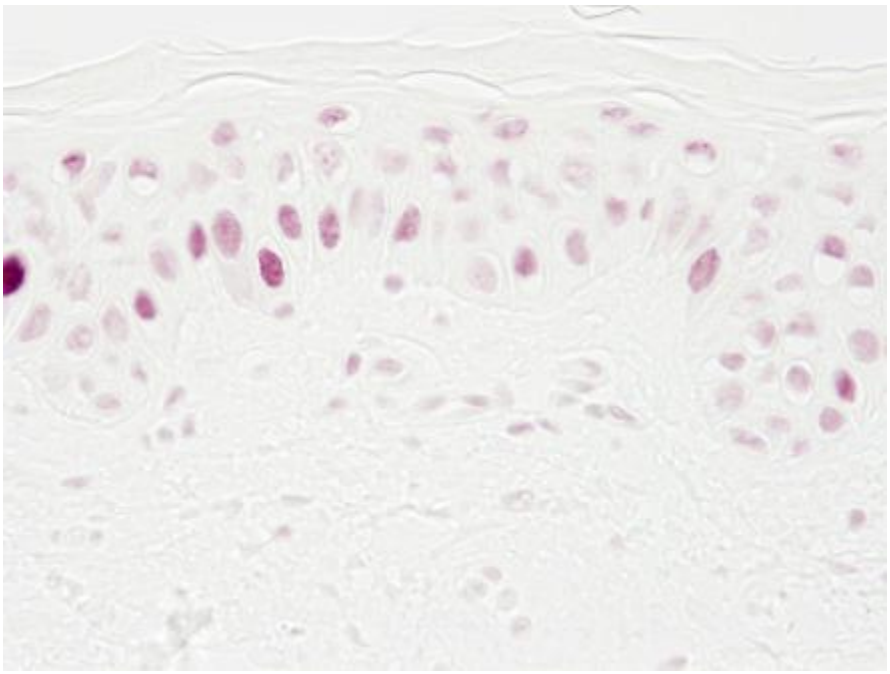
TJ3_7-13 18E4017 NRF2 50 µm



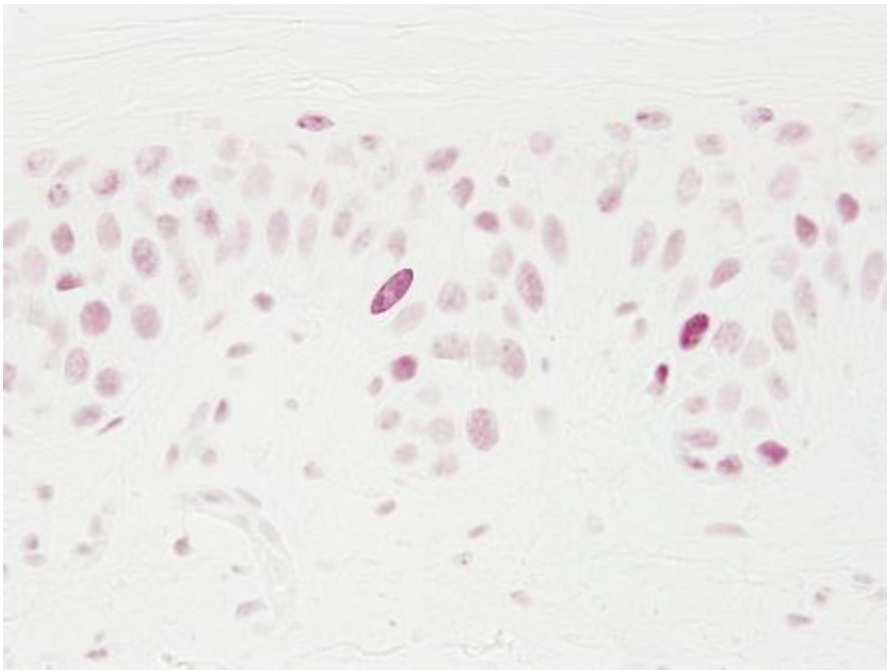
TJ3_9-15 18E4017 NRF2 50 µm

NRF2

Batch P on day 3 (PJ3)



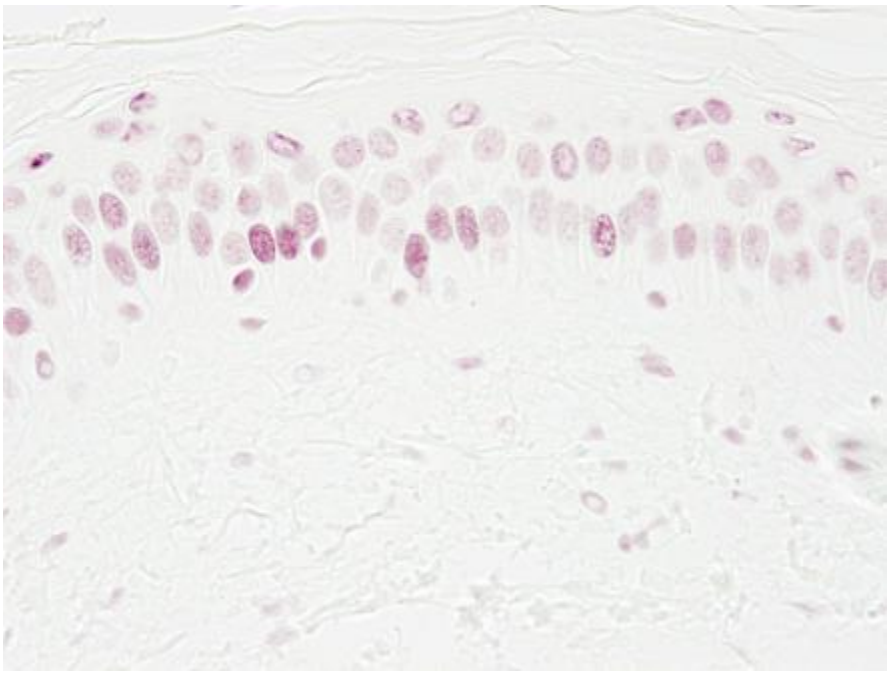
PJ3_1-19 18E4017 NRF2 50 µm



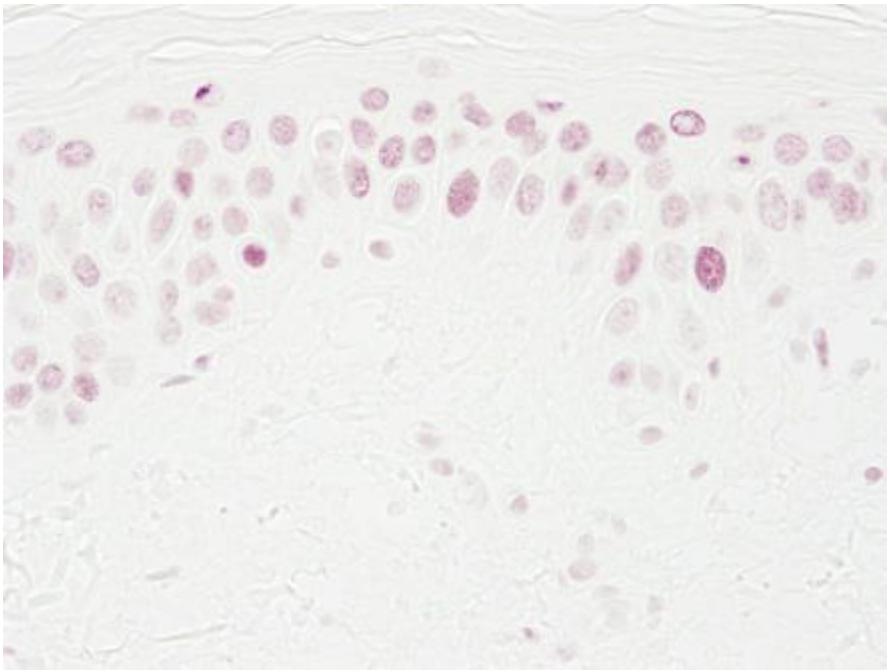
PJ3_8-24 18E4017 NRF2 50 µm

NRF2

Batch ML1 on day 3 (ML1J3)



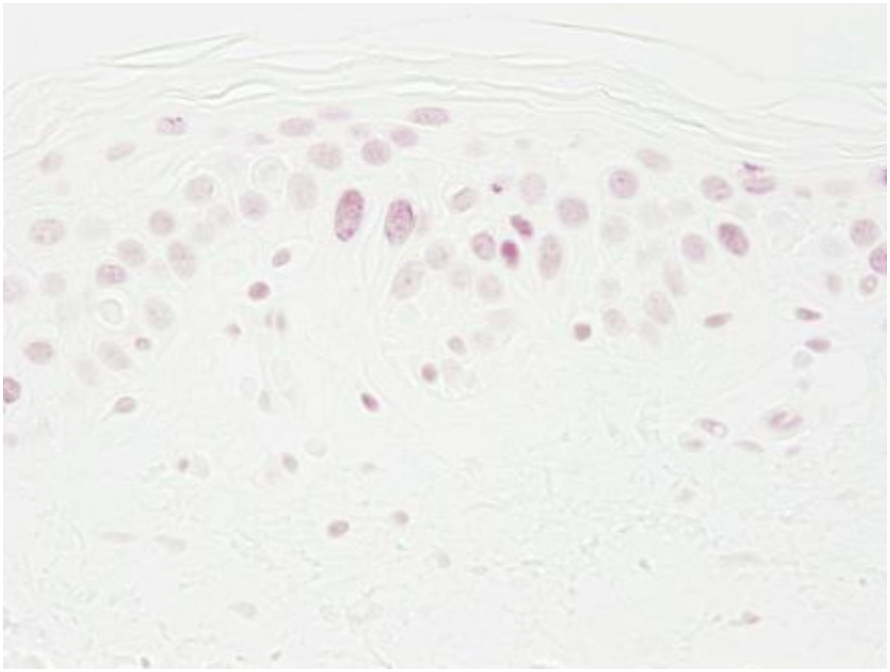
ML1J3_7-33 18E4017 NRF2 50 µm



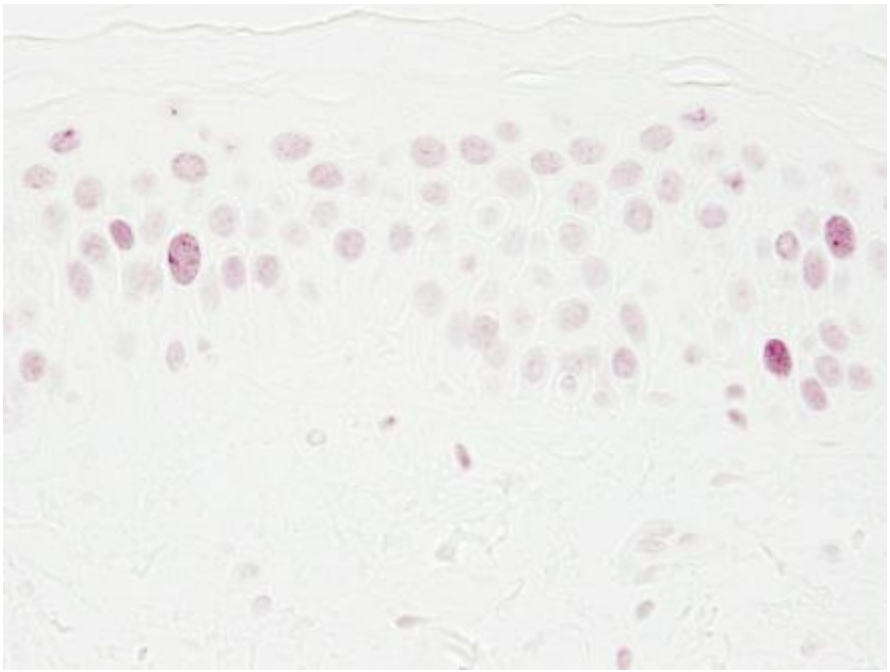
ML1J3_12-35 18E4017 NRF2 50 µm

NRF2

Batch PML1 on day 3 (PML1J3)



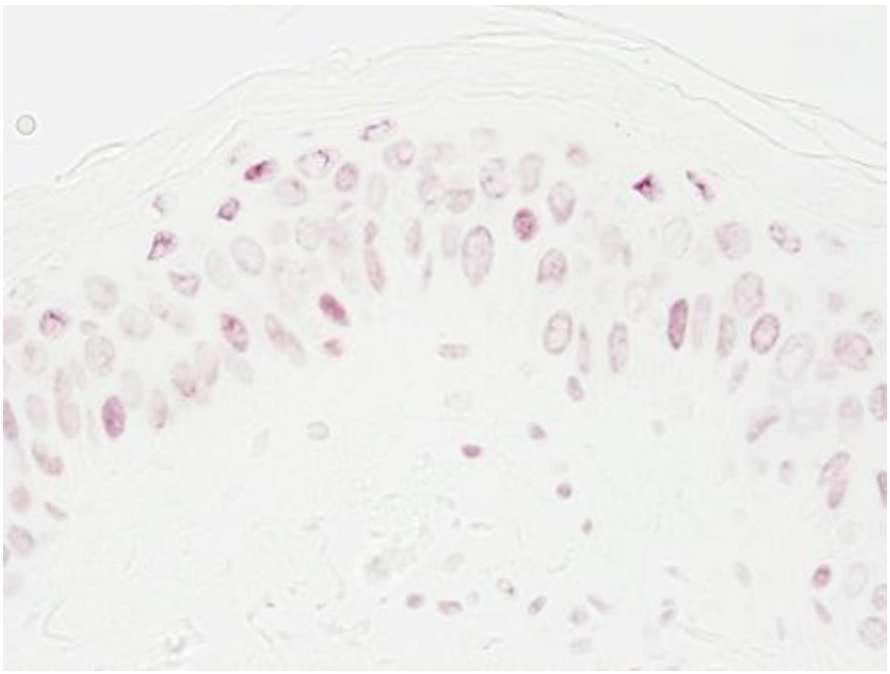
PML1J3_1-46 18E4017 NRF2 50 µm



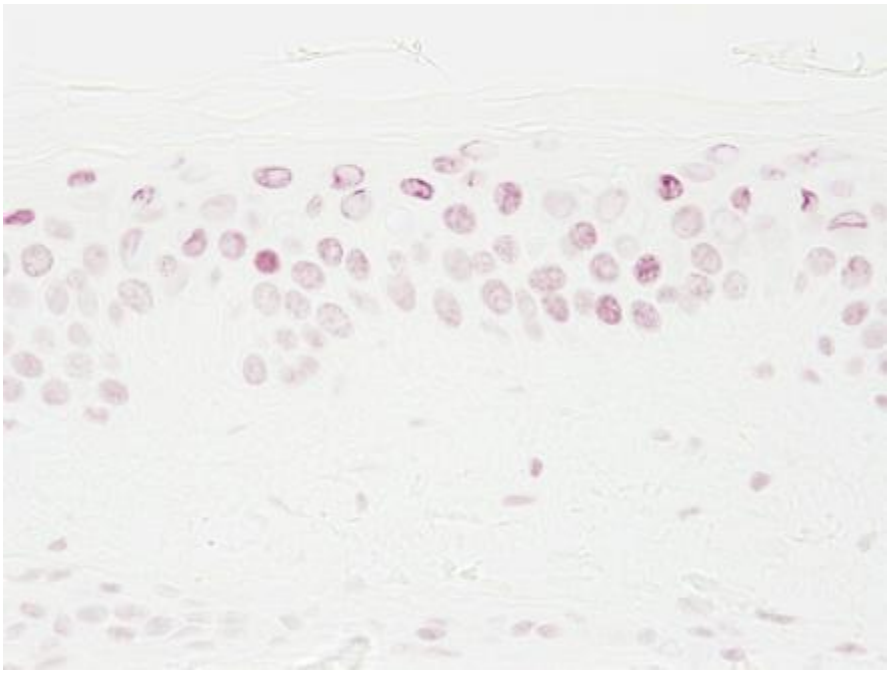
PML1J3_3-47 18E4017 NRF2 50 µm

NRF2

Batch ML2 on day 3 (ML2J3)



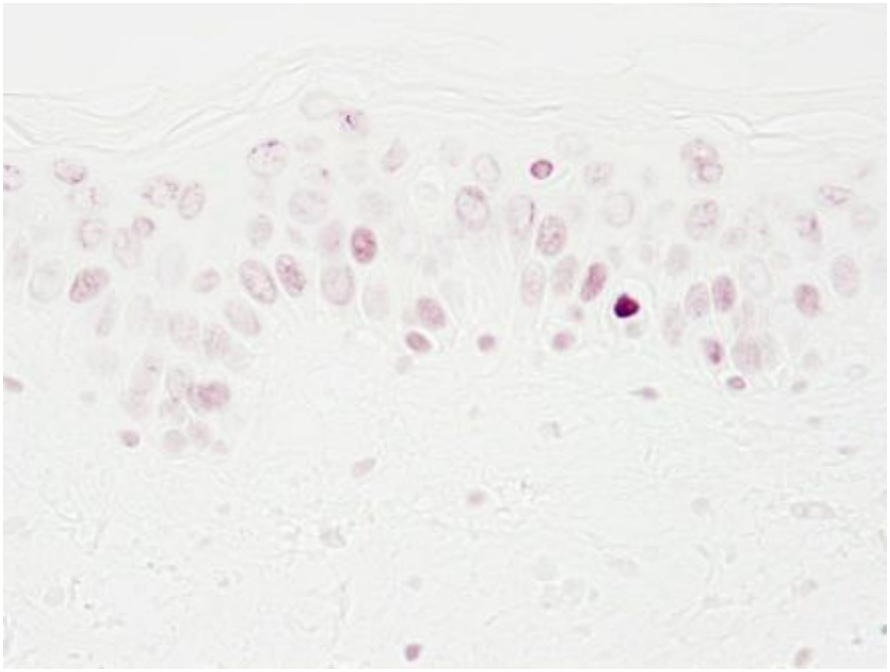
ML2J3_1-37 18E4017 NRF2 50 µm



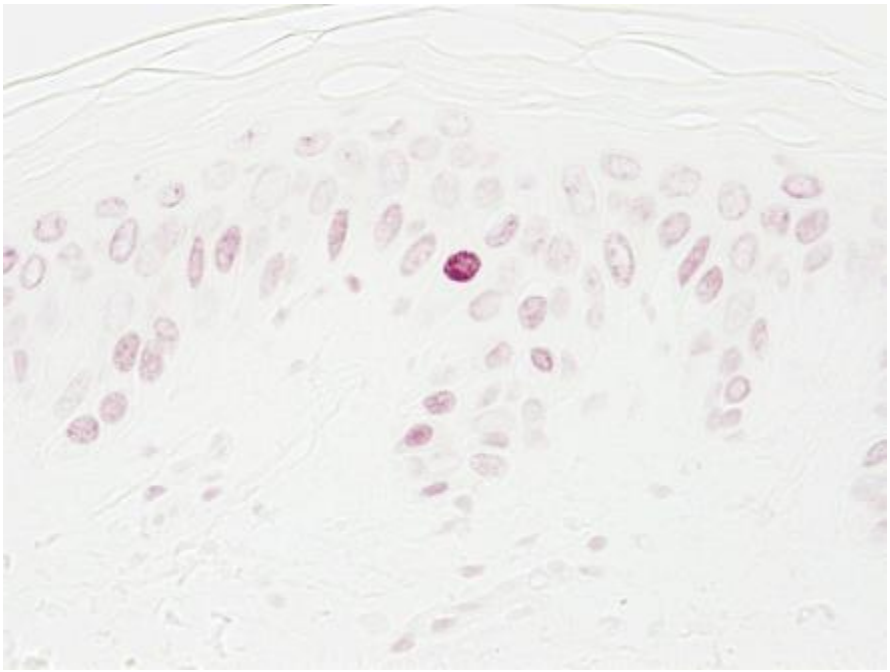
ML2J3_4-39 18E4017 NRF2 50 µm

NRF2

Batch PML2 on day 3 (PML2J3)



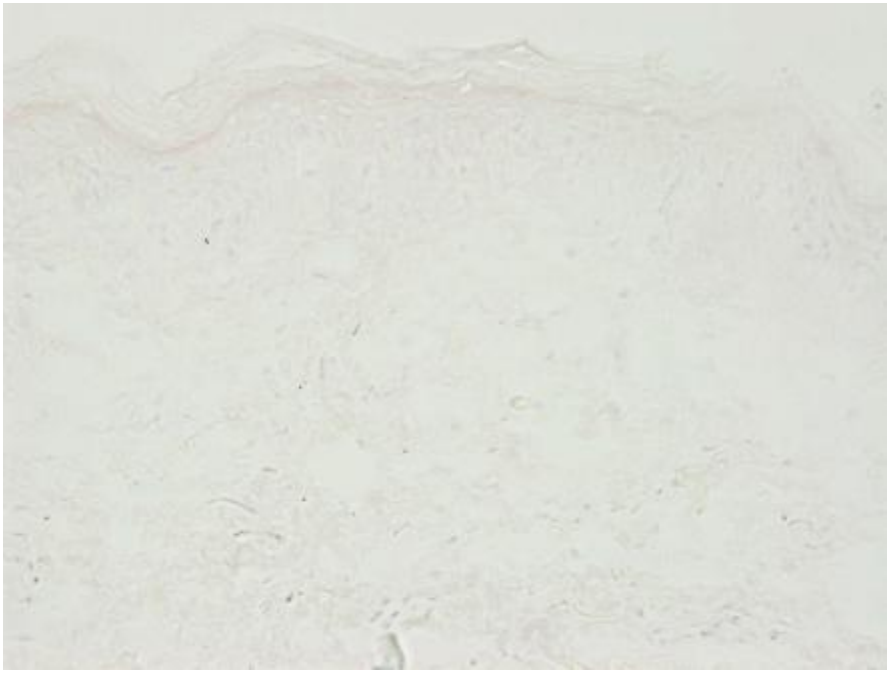
PML2J3_3-55 18E4017 NRF2 50 µm



PML2J3_5-57 18E4017 NRF2 50 µm

3. Oxidized proteins

Negative control without DNPH



T1_2-86 18E4017 Protéines oxydées 100 µm

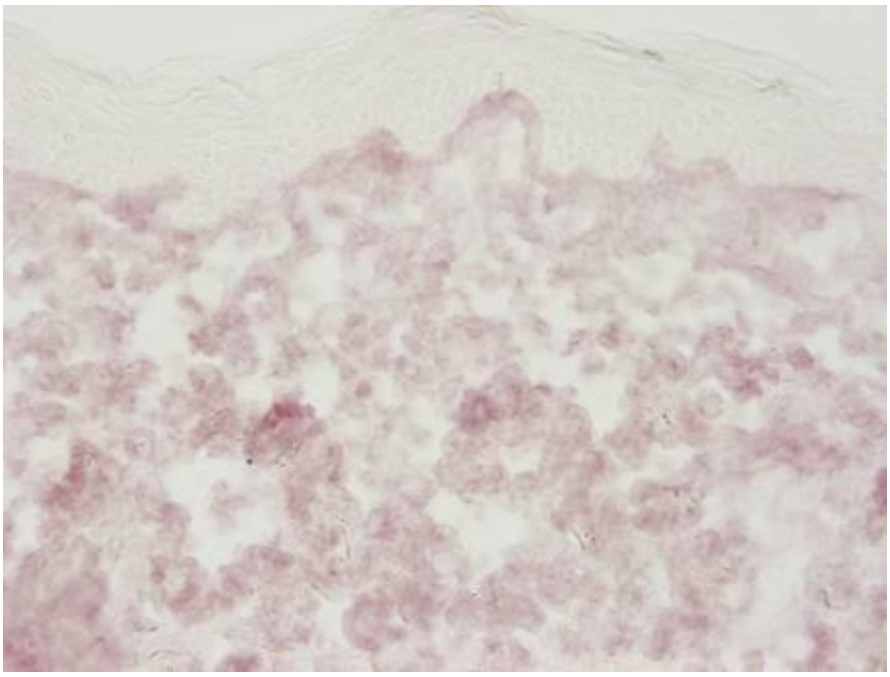
Oxidized proteins

Negative control without anti-DNP antibody

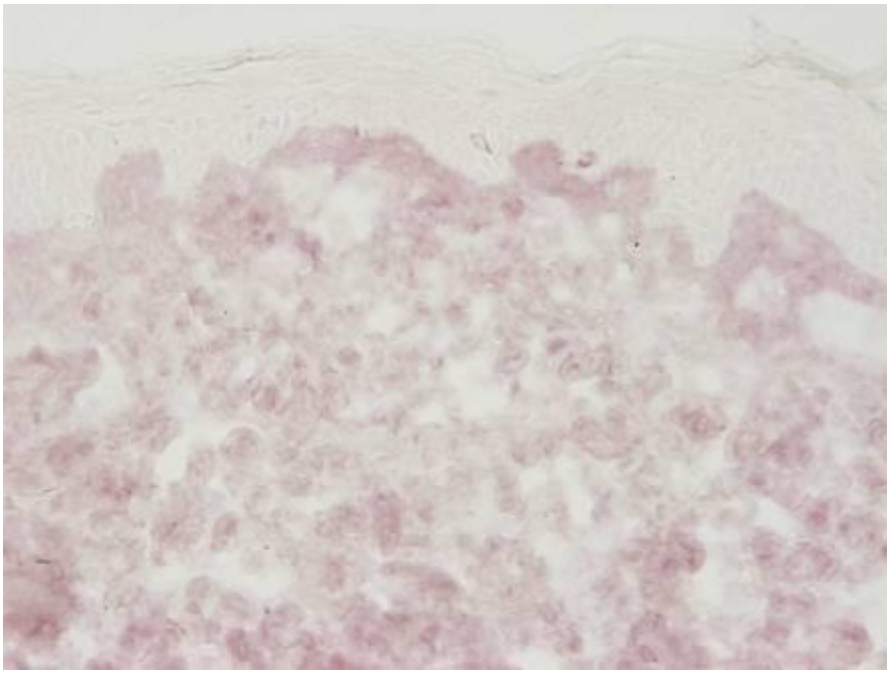
T2_1-88 18E4017 Protéines oxydées 100 µm

Oxidized proteins

Blank batch on day 0 (T0)



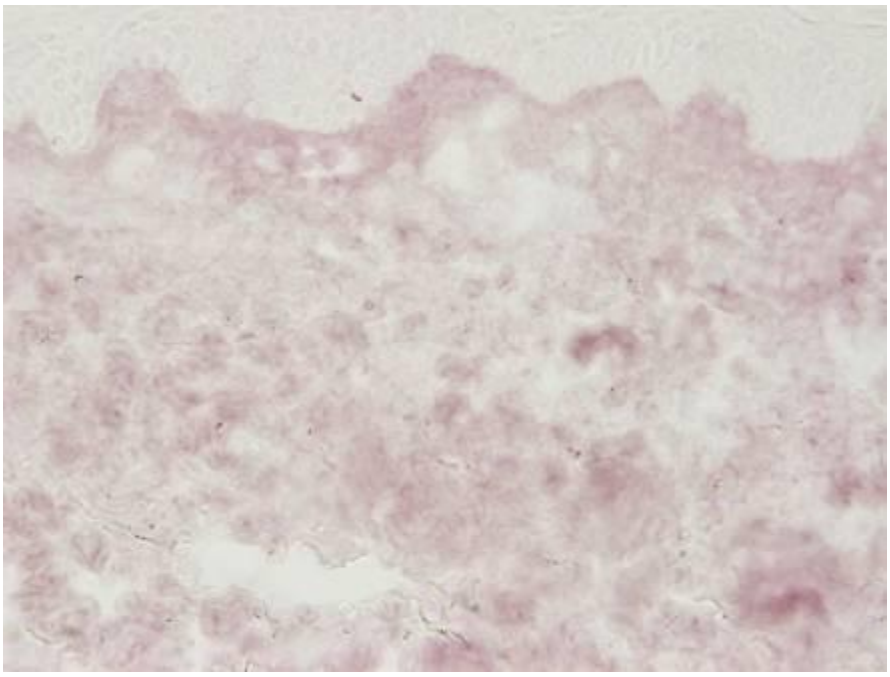
T0_9-9 18E4017 Protéines oxydées 100 µm



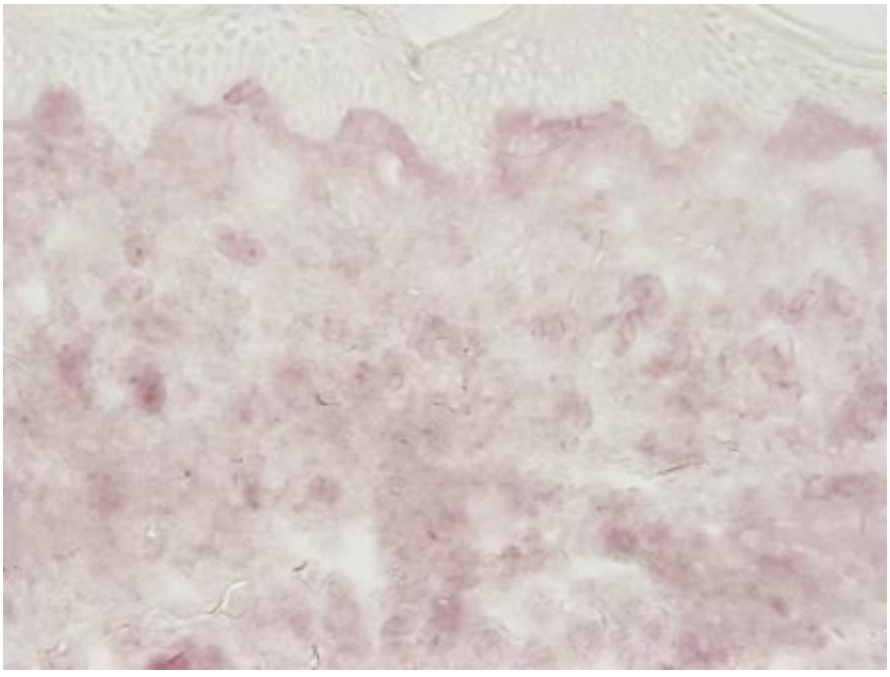
T0_10-10 18E4017 Protéines oxydées 100 µm

Oxidized proteins

Blank batch on day 3 (TJ3)



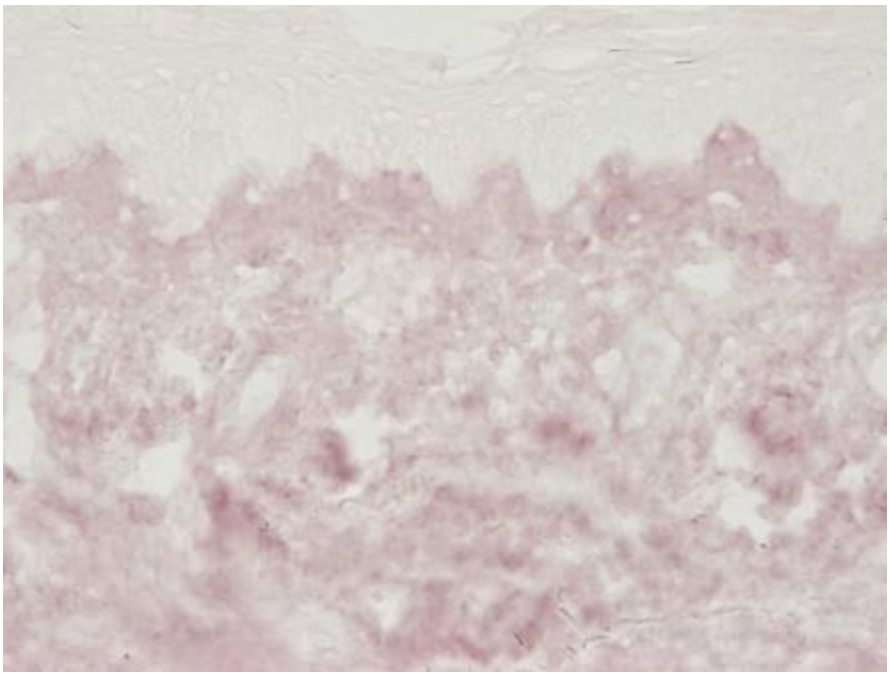
TJ3_5-17 18E4017 Protéines oxydées 100 µm



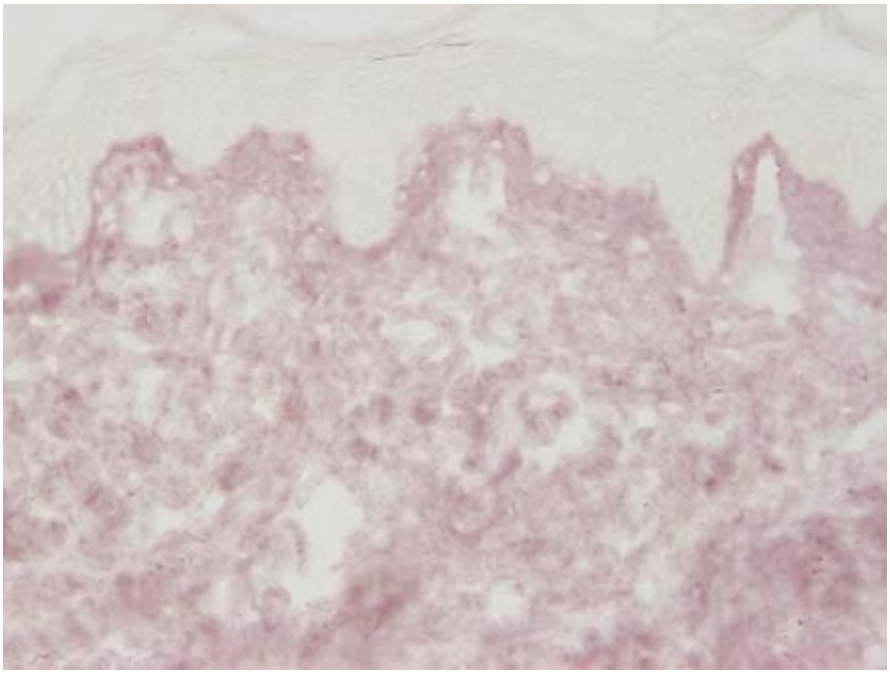
TJ3_7-19 18E4017 Protéines oxydées 100 µm

Oxidized proteins

Batch P on day 3 (PJ3)



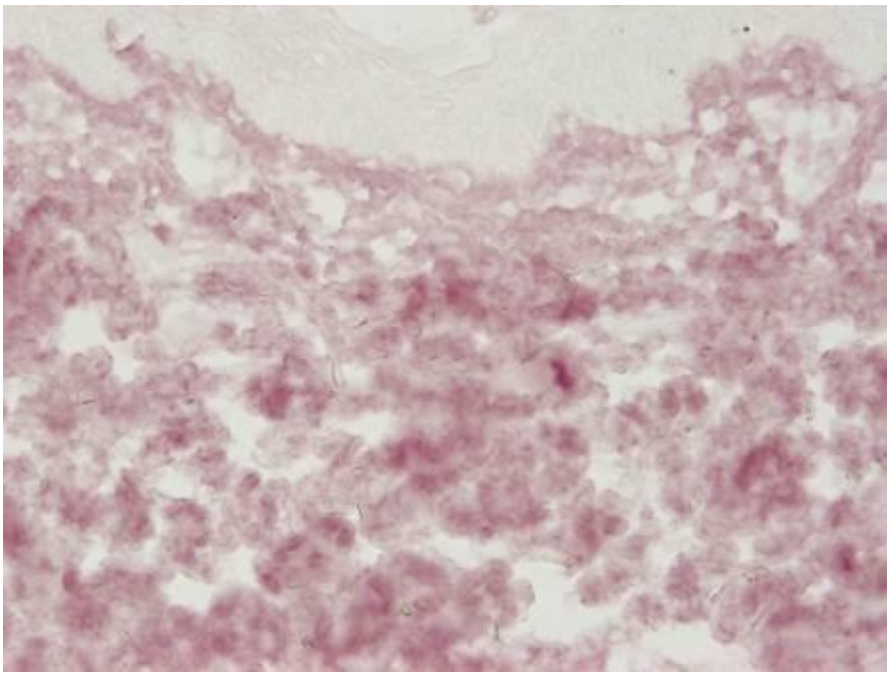
PJ3_3-27 18E4017 Protéines oxydées 100 µm



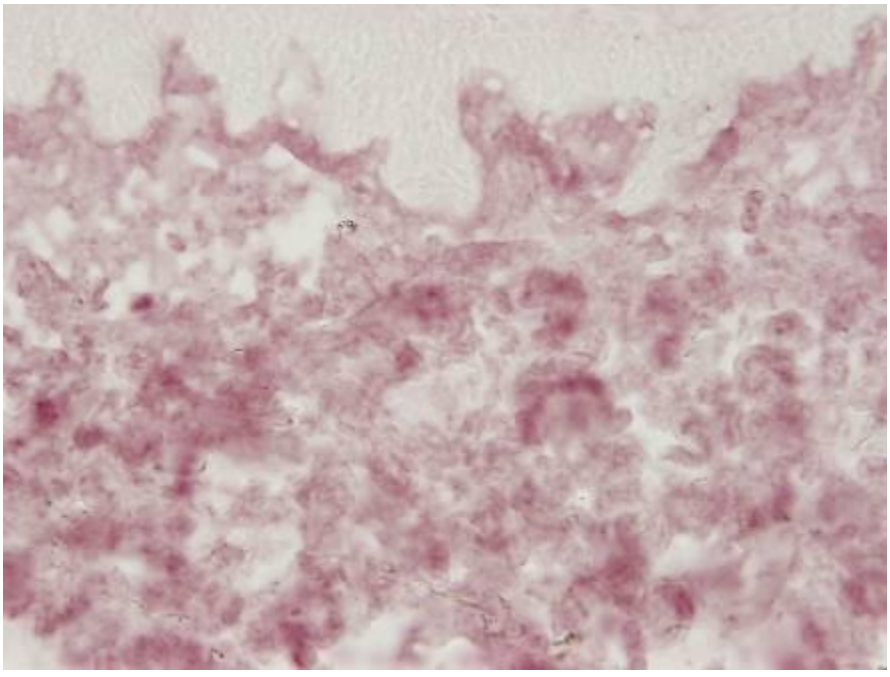
PJ3_10-34 18E4017 Protéines oxydées 100 µm

Oxidized proteins

Batch ML1 on day 3 (ML1J3)



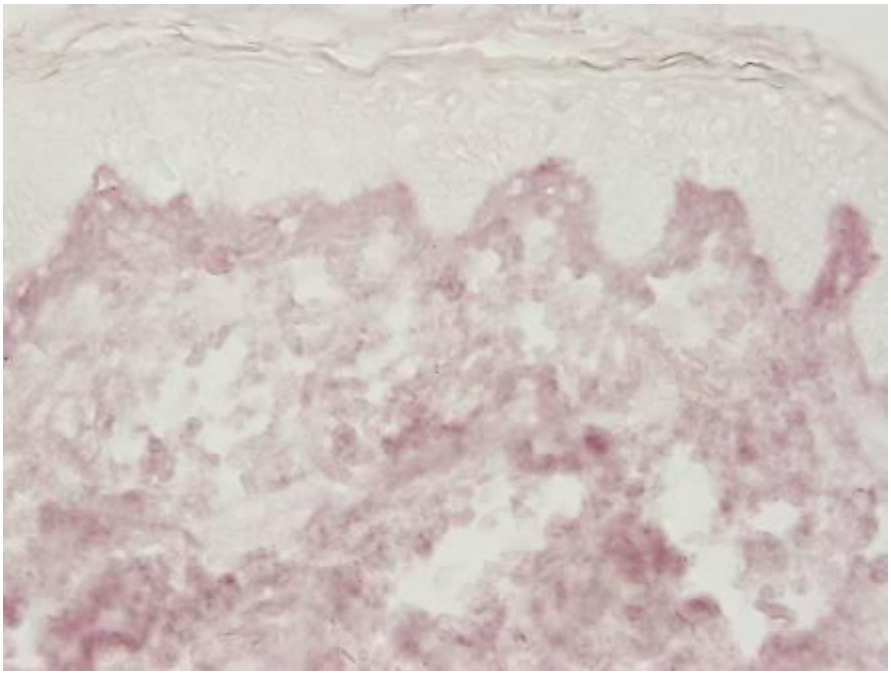
ML1J3_6-42 18E4017 Protéines oxydées 100 µm



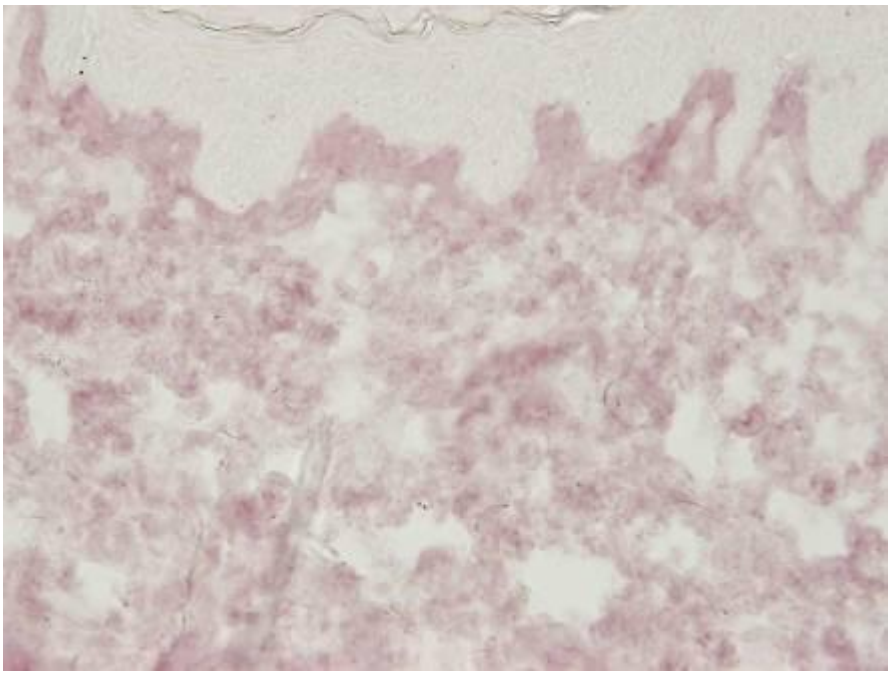
ML1J3_7-43 18E4017 Protéines oxydées 100 µm

Oxidized proteins

Batch PML1 on day 3 (PML1J3)



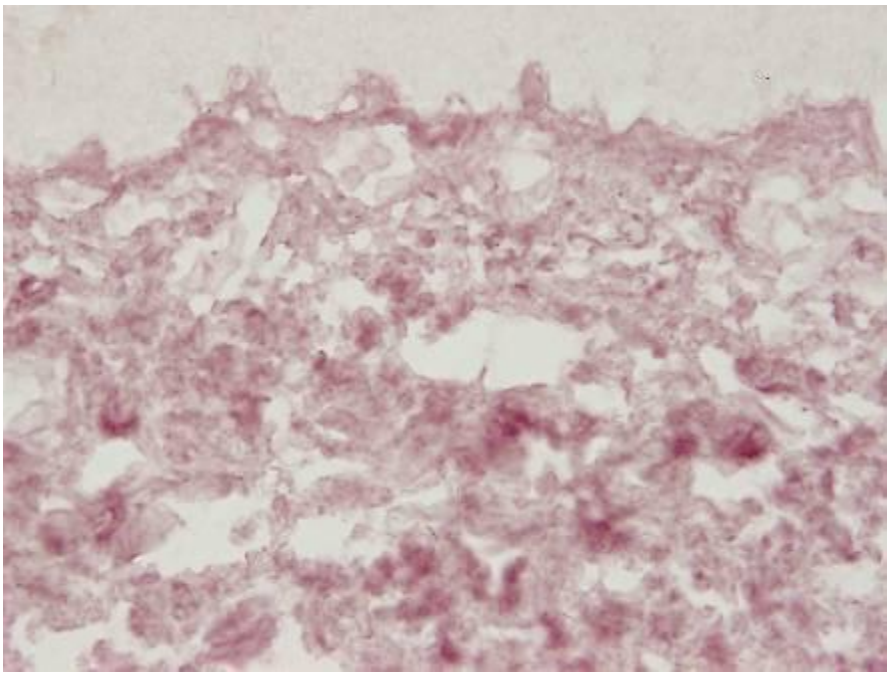
PML1J3_5-65 18E4017 Protéines oxydées 100 µm



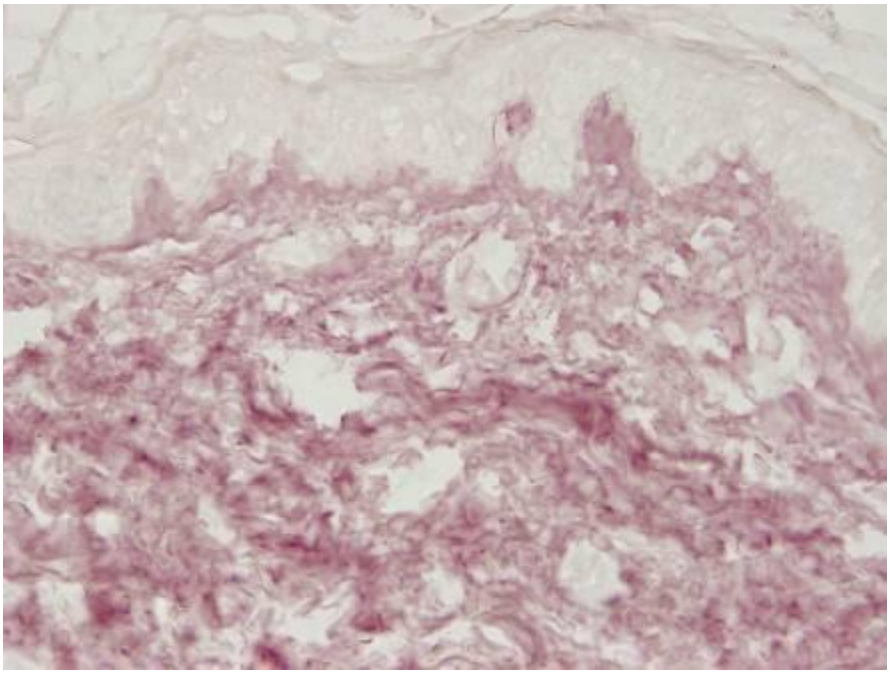
PML1J3_6-66 18E4017 Protéines oxydées 100 µm

Oxidized proteins

Batch ML2 on day 3 (ML2J3)



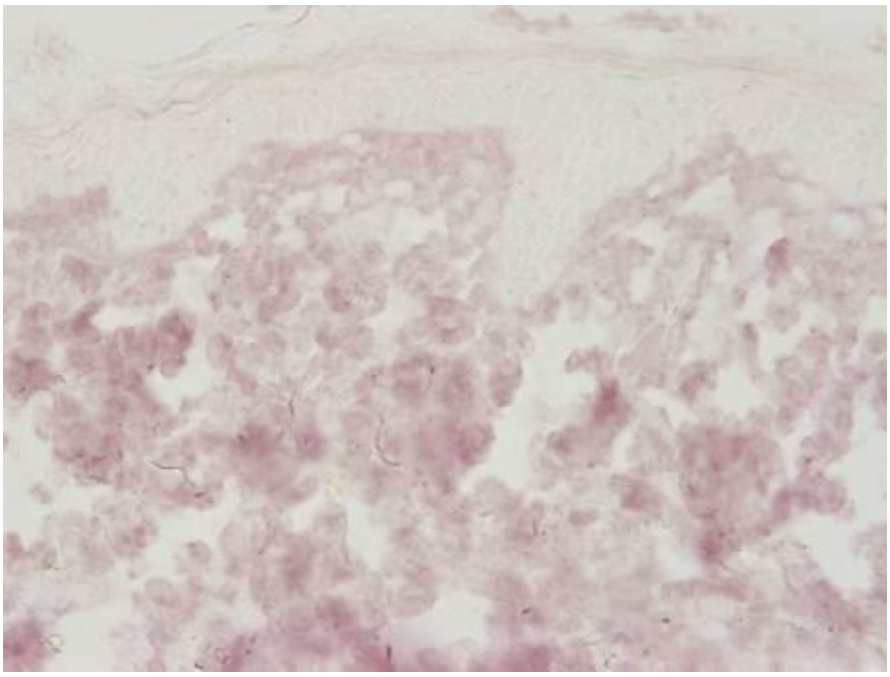
ML2J3_8-56 18E4017 Protéines oxydées 100 µm



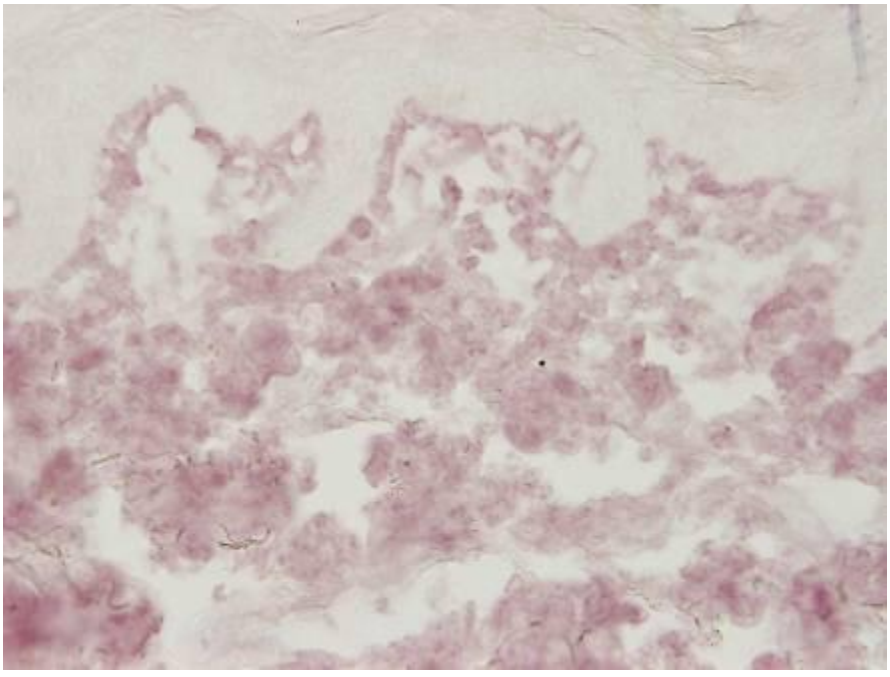
ML2J3_10-58 18E4017 Protéines oxydées 100 µm

Oxidized proteins

Batch PML2 on day 3 (PML2J3)



PML2J3_1-73 18E4017 Protéines oxydées 100 µm



PML2J3_6-78 18E4017 Protéines oxydées 100 µm

4. Raw data of MDA dosage

Concentration of MDA in BEM culture medium on day 3 (in nmol/l)

	MDA (nmole/L)					
	TJ3	**PJ3**	**ML1J3**	**PML1J3**	**ML2J3**	**PML2J3**
A	131,4	112,2	152,9	147,8	292,4	228,6
B	127,5	114,3	163,8	201,6	674,2	324,5
C	120,9	119,9	213,1	183,3	278,7	219,9
D	90,0	121,6	207,8	156,1	290,1	590,8
Mean	**117,4**	**117,0**	**184,4**	**172,2**	**287,1**	**257,7**
SD	18,8	4,5	30,5	24,8	7,3	58,1

The values in red were considered as aberrant values and so were excluded from the mean calculation.

Statistical analysis

Student t-test gives the probability "*p*" for two batches to be significantly different. The difference between two batches is significant if $p<0.1$(#; limit of significancy), so a probability of 90% for two batches to be significantly different or $p<0.05$(*), so a probability of 95% for two batches to be significantly different or $p<0.01$(**), so a probability of 99% for two batches to be significantly different.

p	**TJ3**	**PJ3**	**ML1J3**	**PML1J3**	**ML2J3**	**PML2J3**
TJ3		0,964	**0,013***	**0,014***	**0,000****	**0,044***
PJ3	0,964		**0,020***	**0,019***	**0,000****	0,052#
ML1J3	**0,013***	**0,020***		0,558	**0,005****	0,146
PML1J3	**0,014***	**0,019***	0,558		**0,001****	0,112
ML2J3	**0,000****	**0,000****	**0,005****	**0,001****		0,474
PML2J3	**0,044***	0,052#	0,146	0,112	0,474	

6. Raw data of induction of MDA

MDA concentration after ML induction on D3 for each batch (in nmol/l) using the following formulas:
ΔML1= Δ(ML1J3-**meanTJ3**)
ΔPML1 =Δ(PML1J3-**meanPJ3**)
ΔML2= Δ(ML2J3-**meanTJ3**)
ΔPML2 =Δ(PML2J3-**meanPJ3**)

	Delta (+ML vs -ML)			
	ΔML1	**ΔPML1**	**ΔML2**	**ΔPML2**
A	35,5	30,9	175,0	111,6
B	46,4	84,6	-117,4	207,6
C	95,7	66,3	161,2	102,9
D	90,3	39,1	172,6	-117,0
Mean	**67,0**	**55,2**	**169,6**	**140,7**
SD	30,5	24,8	7,3	58,1

The values in red were considered as aberrant values and so were excluded from the mean calculation.

Statistical analysis: Student t-test
Student t-test gives the probability "*p*" for two batches to be significantly different. The difference between two batches is significant if $p<0.1$(#; *limit of significancy*), so a probability of 90% for two batches to be significantly different or $p<0.05$(*), so a probability of 95% for two batches to be significantly different or $p<0.01$(**), so a probability of 99% for two batches to be significantly different

p	**ΔML1**	**ΔPML1**	**ΔML2**	**ΔPML2**
ΔML1		0,573	**0,005****	0,144
ΔPML1	0,573		**0,001****	0,112
ΔML2	**0,005****	**0,001****		0,480
ΔPML2	0,144	0,112	0,480	

5. Composition of heavy metals and hydrocarbons solution

Final concentrations applied on the explants of each compound of ICP multi-element standard V Certi Pur ® solution (Merck; reference 1.10714.0500; batch HC309202) supplemented with hydrocarbons :

Heavy metals

Al	0.01mg/mL
AS	0.01mg/mL
B	0.001mg/mL
Ba	0.001mg/mL
Be	0.0005mg/mL
Ca	0.005mg/mL
Cd	0.001mg/mL
Cr	0.001mg/mL
Cu	0.001mg/mL
Fe	0.001mg/mL
Hg	0.0025mg/mL
K	0.0495mg/mL
Li	0.001mg/mL
Mg	0.0005mg/mL
Mn	0.0005mg/mL
Na	0.01mg/mL
Ni	0.0025mg/mL
P	0 .005mg/mLg
Pb	0.01mg/mL
Sc	0.0005mg/mL
Sa	0.01mg/mL
Sr	0.0005mg/mL
Te	0.01mg/mL
Ti	0.001mg/mL
Y	0.0005mg/mL
Zn	0.001mg/mL

Hydrocarbons :

Benzene 1µL/ml
Xylene 1µL/mL
Toluene 1 µl/ml

DEVIATIONS

None

CERTIFICATE OF QUALITY CONTROL

This study was conducted in the spirit of the Good Laboratory Practices (Arrêté du 10 Août 2004), as well as in compliance with the validated procedures and SOP of Laboratoire BIO-EC.
The audits performed ensure that all the steps of the study are controlled. The dates and steps inspected during the various audits are presented in the table below:

Type of audit	Date	Controlled stages	Dates of diffusion at the study director	Dates of diffusion at the director
Internal	21/06/2012	Histology laboratory	03/07/2012	03/07/2012
Internal	12/04/2016	Reception, storage and destruction of product	17/05/2016	17/05/2016
Internal	13/04/2016	Reception, storage and destruction of reagents and antibodies	17/05/2016	17/05/2016
Internal	19/04/2016	Human ressources	23/05/2016	23/05/2016
Internal	23/05/2016	Reception, storage and destruction of plasty	02/06/2016	02/06/2016
Internal	07/06/2016	Study environment and waste management	13/06/2016	13/06/2016
Study (16E3520)	30/06/2016 01/07/2016	Explants treatment	27/07/2016	28/07/2016
Study (16E3520)	30/06/2016 01/07/2016	Freezing explants	27/07/2016	28/07/2016
Study (16E3520)	11/07/2016 12/07/2016	Tissues fixation and placing on cassette	27/07/2016	28/07/2016
Study (16E3520)	15/07/2016	Embedding tissues in paraffin blocks	27/07/2016	28/07/2016
Study (16E3520)	18/07/2016	Tissues sectioning	27/07/2016	28/07/2016
Internal	18/07/2016	Quality documentation	30/08/2016	30/08/2016
Study (16E3520)	25/07/2016	Microscopic examination of paraffin sections	27/07/2016	28/07/2016
Internal	13/09/2016	Equipment	26/09/2016	26/09/2016
Study (16E3606)	29-30/06/2016	Freezing explants	20/10/2016	20/10/2016
Study (16E3606)	17/10/2016	Tissues sectioning	20/10/2016	20/10/2016
Internal	24/11/2016	Archiving	05/12/2016	05/12/2016

This report has been reviewed by the quality assurance officer, certifying that the methods and the operating procedures were fully respected.
This report has also been reviewed by the study director, certifying that the observations and the results are clearly indicated and accurately show the raw data of the study.
The test facility director has reviewed that the responsibility of the quality assurance has been taken in accordance with the spirit of good laboratory practices.

Study Director **L. Peno-Mazzarino**
Date and signature :

Quality Assurance Officer **M. Daniel**
Date and signature :

Test Facility Director **E. Lati**
Date and signature :

ARCHIVAGE OF THE STUDY REPORT

- Raw data filing

The raw data are :

- Microscopic observations
- Image analysis results
- Assays results
- Biometrological results using devices

All these raw data are kept in a paper file and a backup is saved when it is possible (depending on the used device).

- Products, samples, blocs and slides filing

The products entrusted to BIO-EC are preserved one year after using the tested product.

The blocs, the stained and immunostained slides revealed by alkaline phosphatase and peroxidase are kept at BIO-EC's for fifteen years.

The frozen blocs will stay in possession of BIO-EC for two years at minus 80°C. If the culture media are harvested during the study, they will be stored for two years at minus 80°C.
After that, and without any other instructions from the client, they will all be destroyed.

- Final report filing

The paper file is archived and kept for 20 years

The study report (raw data, images, preliminary reports, final report) and all the computer data are saved thanks to a double internal backup (KERTEL BOX2CLOUD, RAID 1) and by an automated and daily external system, Backupia (KERTEL Group).

Our computer system is protected by the anti-viruses Microsoft Security Essential, F-Secure and McAfee Saas.

STUDY SUMMARY

Tested product

P : Rivoli Crème de Jour Jeunesse II ref. Torstone

Model

Human living skin explants.
Topical treatment (2 µl/explant) with the product P on D0 and D1 (morning and evening).
On D2, exposure to global pollution using 2 conditions :
- ML1 : ozone (1h) + heavy metals and hydrocarbons (1.5 mL for 1,5h) using Pollubox® system + UV A & B (1 MED) and blue light (2h) using Solarbox® system.
- ML2 : ozone (2h) + heavy metals and hydrocarbons (3 mL for 1,5h) using Pollubox® system + UV A & B (2 MED) + blue light (3h) using Solarbox® system.
Sampling of skin explants and BEM culture media on D3.
Evaluated parameters: cell viability, Nrf2 and oxidized proteins immunostainings and MDA dosage.

Conclusion

According to the experimental conditions described above and compared to the blank batch (**TJ3**), to global pollution 1- treated batch on D3 (**ML1J3**) or global pollution 2- treated batch on D3 (**ML2J3**) :

variations vs TJ3 or ML1J3 or ML2J3		**Rivoli Creme de Jour Jeunesse II ref. Torstone (P)**
Cell viability	*vs TJ3*	↔
	vs ML1J3	(↘) epidermal alterations
	vs ML2J3	(↘) epidermal alterations
NRF2	*vs TJ3*	↘
	vs ML1J3	↘
	vs ML2J3	↘
Oxidized proteins	*vs TJ3*	↗
	vs ML1J3	↘↘↘↘
	vs ML2J3	↘↘
MDA	*vs TJ3*	-0,4%^{ns}
	vs ML1J3	-7%^{ns}
	vs ML2J3	-10%^{ns}
MDA induction	*vs ΔML1*	-18%^{ns}
	vs ΔML2	-17%^{ns}

Decrease		**Increase**	↔	No variation
(↘)	Very slight	(↗)		
↘	Slight	↗		
↘↘	Moderate	↗↗	ns	Not significant
↘↘↘	Fairly clear	↗↗↗	#	significant with p<0.1 (90%)
↘↘↘↘	Clear	↗↗↗↗	*	significant with p<0.05 (95%)
↘↘↘↘↘	Very clear	↗↗↗↗↗	**	significant with p<0.01 (99%)

The product Rivoli Creme de Jour Jeunesse II ref. Torstone (P) exhibits a global anti-pollution activity characterized by a protection against pollution- induced epidermal alterations, a decrease of induced oxidized proteins, a decrease of NRF2, and a non significant reduction of induced lipid peroxidation (MDA).