Laboratoire BIO-EC

Centre de Recherches Biologiques et d'Expérimentations Cutanées

Study 18E4016

Assessment of anti-oxidant activity of two products on human living skin explants *ex vivo*

According to the study plan D17-654

Tested products P1 : Rivoli Creme de Jour Jeunesse II ref. Torstone
P2 : Sonnencreme SPF25

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Study director **L. Peno-Mazzarino**

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)*	19th January 2018	
Date of the beginning of the technical phase of the study	19th 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

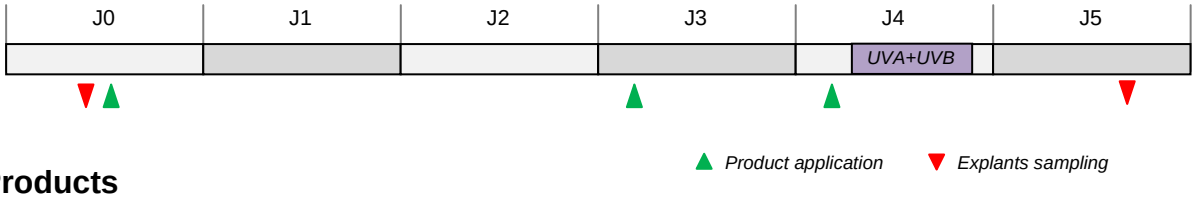
The aim of this study is to evaluate the anti-oxidant activity of two products.

After a treatment during 5 days and UVA+B irradiation, this activity has been evaluated by:

- Viability Control
- Immunostaining of peroxidized protein
- Immunostaining of glutathione reductase.

MATERIAL & METHODS

1. Study design



2. Products

The sponsor has provided the following products:

Product	Identification	Reference	Batch	Aspect	Quantity
P1	Rivoli Creme de jour Jeunesse II	Tortone	lab-01095.4 14.12.17	crème	1 tube
P2	Sonnencreme SPF25	Sonnencreme SPF25	lab-00061.1 30.10.2017	crème	1 tube

The products have been stored at room temperature within and after the duration of the study.

3. Characteristic of the plasty

21 skin explants of an average diameter of 12 mm (±1mm) were prepared on an abdoplasty coming from a 69-year-old caucasian woman (reference: P1930-AB69, 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.

4. Explant distribution

The explants were distributed into 7 batches as follows:

Batches	Treatment	UV	Nb explant	Sampling
T0	none	-	3	D0
T	none	-	3	D5
P1	Tested product 1	-	3	D5
P2	Tested product 2	-	3	D5
TUV	none	+	3	D5
P1UV	Tested product 1	+	3	D5
P2UV	Tested product 2	+	3	D5

5. Products application

On day 0 (D0), D3 and D4 the products P1 and P2 were topically applied on the basis of 2 mg per cm² (2µl per explant), and spread using a small spatula.
The control explants T did not receive any treatment except the renewing of the culture medium.
The culture medium was half renewed (1 mL) on D3.

6. Irradiation

On D4, the culture media of all the batches were replaced by HBSS (Hank's Balanced Saline Solution; 1 ml per explant).
The batches "TUV", "P1UV" and "P2UV" were irradiated by UVA+ UVB using a UV simulator Vibert Lourmat RMX 3W with a dose of 18 J/cm^2 of UVA and 0,6 J/cm^2 of UVB corresponding both to 4 MED (minimal erythemal dose).
The unirradiated batches were kept in HBSS in the dark.
At the end of the irradiation, all the explants were put back in 2 mL of BEM medium.

7. Sampling

On D0, the 3 explants from the batch T0 were collected and cut in two parts. One part was fixed in buffered formalin and the other one frozen at -80°C.
On D5, 3 explants from each batch were collected and processed in the same way than for day 0.
According to the dispositions in the study plan, the days of treatment were modified to fit the schedule of the study based on working days.

8. 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.

8.1. Cell viability control

The cell viability of epidermal and dermal structures were controlled on paraffin sections stained according to Masson's trichrom staining.
Concerned batches: *all, so 21 explants.*

8.1. 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 enhancement system and revealed by VIP, a substrate of peroxidase (Vector laboratories, ref. SK-4600).
The immunostaining was assessed by microscopical observation.
Concerned batches: 3 explants / batches, 21 explants

8.2. immunostaining of glutathione reductase

Glutathione reductase immunostaining was realized on paraffinized sections with a polyclonal anti-Glutathione reductase antibody (Abcam, ref. ab16801) diluted at 1:400 in PBS-BSA 0.3%-tween 20 at 0.05% and incubated 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.

Concerned batches: 3 explants / batches, 21 explants

ABBREVIATIONS

Listing of the abbreviations and symbols used in this report:

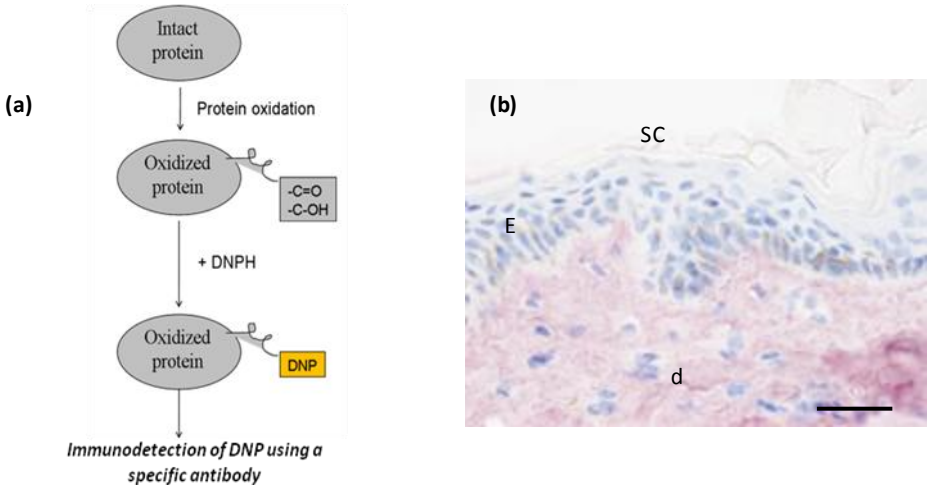
SD	Standard deviation
D	Day
J	for “Jours”, the French word for day, used for the pictures. D and J are used interchangeably to indicate DAY

BACKGROUND

1. Oxidized proteins

The staining of oxidized proteins was realized using the OxyBlotTM protein oxidation kit (Millipore, S7150) on frozen sections. This kit allows the immunoblot 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. 1**).

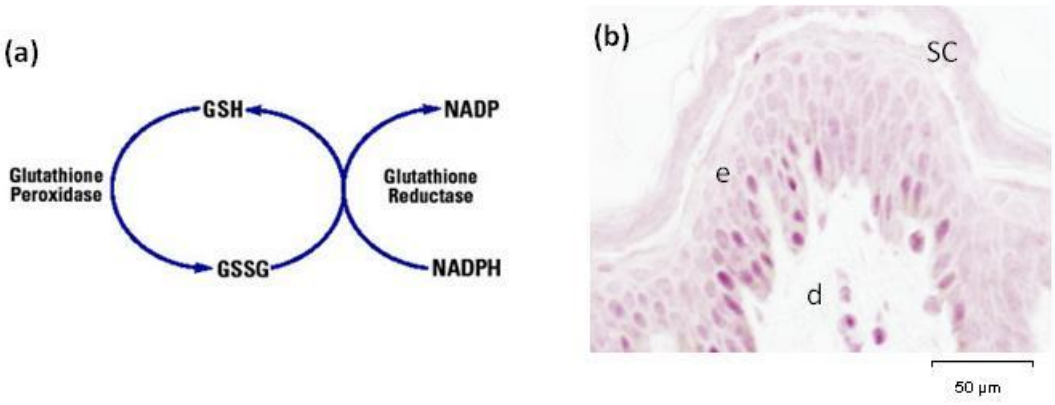
Figure 1. In (a) the different steps for oxidized protein revelation, and in (b) immunostaining of oxidized protein revealed by VIP. Abbreviations: d, dermis; E, epidermis; SC, stratum corneum. Scale bar: 50 µm.



2. Glutathione reductase

Glutathione reductase also known as glutathione-disulfide reductase is an enzyme that in humans is encoded by the GSR gene. Glutathione reductase catalyzes the reduction of glutathione disulfide (GSSG) to the sulfhydryl form glutathione (GSH), which is a critical molecule in resisting oxidative stress and maintaining the reducing environment of the cell (**Fig. 2**). This peptide is synthesized in a two-step process. The first step is performed by the gamma glutamylcysteine synthetase, the second step by the glutathione synthetase. The GSH acts as an anti-oxidant because of its thiol group. In the course of the process GSH is oxidized by reactive oxygen radicals and forms a dimer with another activated GSH via formation of a disulfidic bond (GSSG). GSH can be recovered in a reducing step by the glutathione reductase consuming NADPH GSH not only detoxifies ROS, but can also regenerate oxidized α-tocopherol and retinol (Aung-Htut et al., 2012. Biochem. 57:13–54). In addition it has been demonstrated that UV-A and UV-B irradiation reduced glutathione reductase activity in the skin (Shindo et al., 1994. J Invest Dermatol. 102:470-475).

Figure 2. (a) Glutathione reductase reaction. (b) Immunostaining of Glutathione reductase revealed by VIP. Abbreviations: d, dermis; e, epidermis; SC, stratum corneum.



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	/
TJ5	FG	G	/
P1J5	FG	G	/
P2J5	FG	G	/
TUVJ5	MA	Few altered cells	/
P1UVJ5	SA to MA	Few altered cells	/
P2UVJ5	FG	G	/

Legend of cell viability: G= good, QG= quite good, 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 cell viability is good in the epidermis and in the dermal cells of the papillary dermis.

On D5,

On the blank **batch TJ5,** the cell viability is fairly good in the epidermis and good in the dermal cells of the papillary dermis.

Effect of product application on the cell viability, compared to the batch TJ5:

- The product **P1** induces no visible modification
- The product **P2** induces no visible modification.

The UV irradiations (TUVJ5 vs TJ5) induce moderate epidermal alterations and also alterations on few dermal cells.

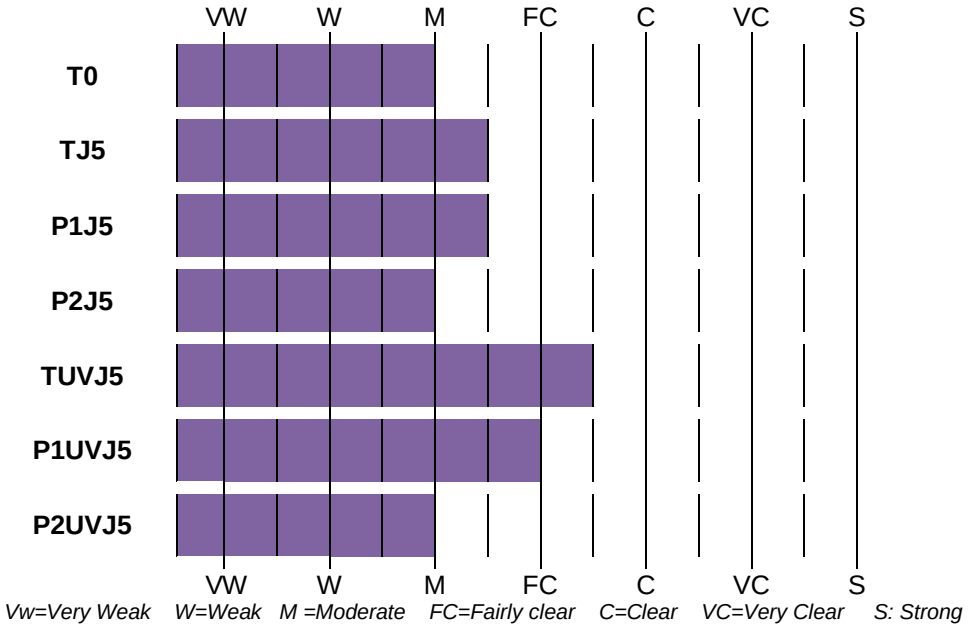
Effect of product application on the cell viability, compared to the batch TUVJ5:

- The product **P1** induces very slight decrease of epidermal alterations but no significant modification on dermal cells.
  - **So, the product P1 induces a partial protection against UV-induced epidermal alterations and no against dermal alterations**.
- The product **P2** induces a moderate decrease of epidermal alterations and a decrease of dermal alterations
  - **So, the product P2 induces a complete protection against UV-induced epidermal and dermal alterations.**

2. Oxidized proteins

On D0, on the blank batch T0, the staining of oxidized proteins is moderate in the papillary dermis.

The staining of oxidized proteins in the papillary dermis of all batches is shown here below:



On D5,
On the **batch TJ5**, the formation of oxidized proteins is moderate to fairly clear in the papillary dermis.

Effect of product application on oxidized proteins formation, compared to the batch TJ5:

- ➥ The product **P1** induces no visible modifications.
- ➥ The product **P2** induces a slight decrease.

The UV irradiations (TUVJ5 vs TJ5) induce a moderate increase of oxidized proteins formation.

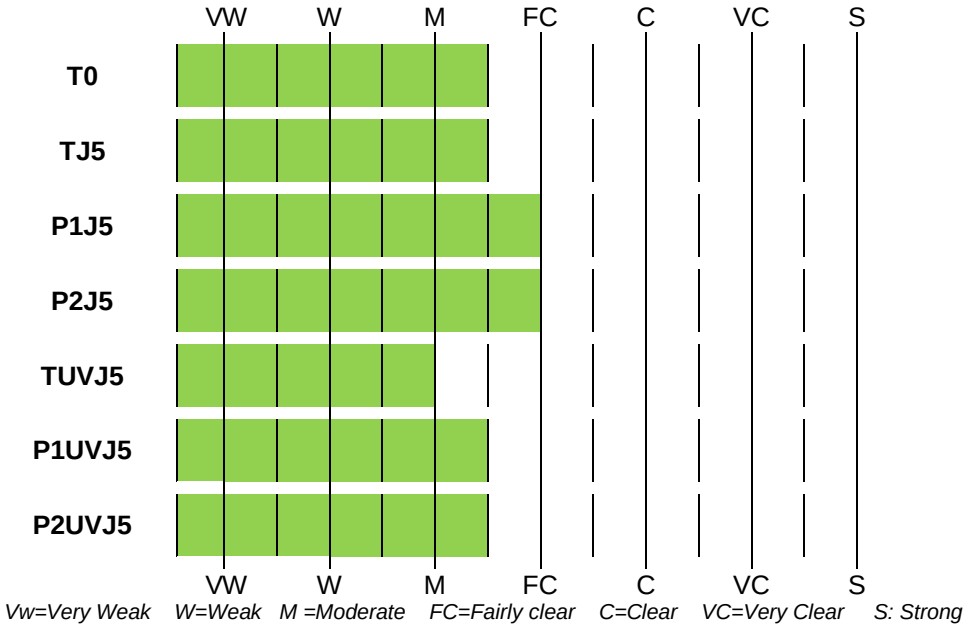
Effect of product application on oxidized proteins formation, compared to the batch TUVJ5:

- ➥ The product **P1** induces a slight decrease
  - **So, the product P1 induces a partial protection against UV-induced oxidized proteins.**
- ➥ The product **P2** induces a fairly clear decrease.
  - **So, the product P2 induces a complete protection against UV-induced oxidized proteins.**

3. Glutathione reductase

On D0, on the blank batch T0, the staining of glutathione reductase is moderate to fairly clear in the epidermis.

The staining of glutathione reductase in the epidermis of all batches is shown here below:



On D5,
On the **batch TJ5**, the expression of glutathione reductase is moderate to fairly clear in the epidermis.

Effect of product application on glutathione reductase expression, compared to the batch TJ5:

- ➥ The product **P1** induces a slight increase.
- ➥ The product **P2** induces a slight increase.

The UV irradiations (TUVJ5 vs TJ5) induce a slight decrease of glutathione reductase expression.

Effect of product application glutathione reductase expression, compared to the batch TUVJ5:

- ➥ The product **P1** induces a slight increase
  - **So, the product P1 induces a complete protection against UV-induced glutathione reductase decrease.**
- ➥ The product **P2** induces a slight increase
  - **So, the product P2 induces a complete protection against UV-induced glutathione reductase decrease.**

CONCLUSION

According to these experimental conditions, compared to the batches on day 5 without or with UVA & B 4 MED (TJ5 or TUVJ5):

vs T or TUV on day 5		**Rivoli Creme de Jour Jeunesse II ref. Torstone (P1)**	**Sonnencreme SPF25 (P2)**
Cell viability	*vs TJ5*	↔	↔
	vs TUVJ5	(↘) epidermal alterations ↔ dermal alterations	↘↘ epidermal alterations ↘ dermal alterations
Oxidized proteins	*vs TJ5*	↔	↘
	vs TUVJ5	↘	↘↘↘
Glutathione reductase	*vs TJ5*	↗	↗
	vs TUVJ5	↗	↗

Decrease		**Increase**		
(↘)	Very Slight	(↗)	↔	No variation
↘	Slight	↗		
↘↘	Moderate	↗↗	ns	non-significant
↘↘↘	Fairly clear	↗↗↗	#	significant with p<0.1 (90%)
↘↘↘↘	Clear	↗↗↗↗	*	significant with p<0.05 (95%)
↘↘↘↘↘	Very clear	↗↗↗↗↗	**	significant with p<0.01 (99%)

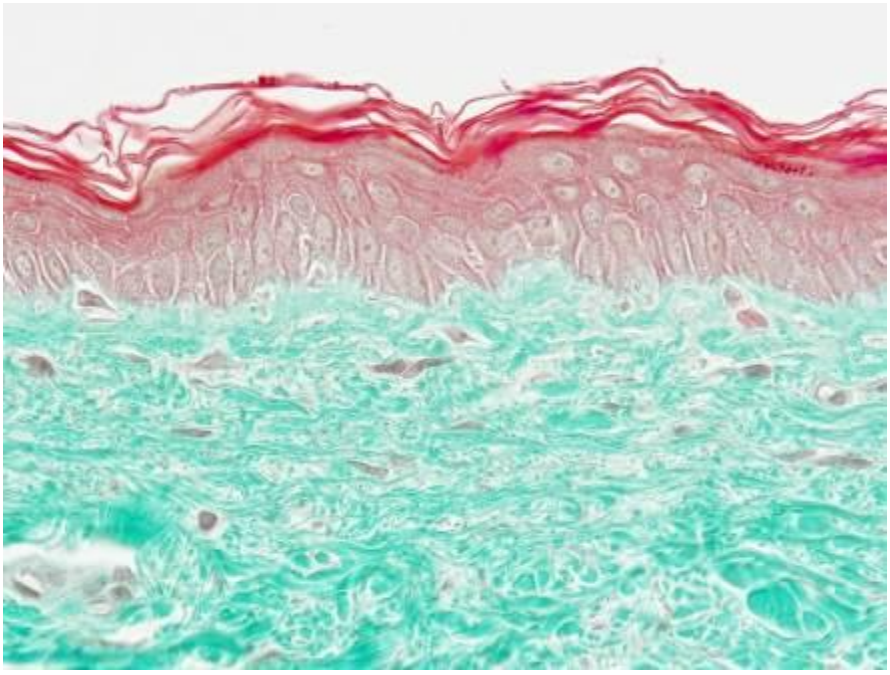
The product Sonnencreme SPF25 (P2) exhibits the best anti-oxidant activity by completely preventing the UV-induced epidermal and dermal alterations, by completely preventing the UV-induced glutathione reductase reduction and by completely blocking the UV-induced oxidized proteins.

The product Rivoli Creme de Jour Jeunesse II ref. Torstone (P1) exhibits a fairly good anti-oxidant activity by partially preventing the UV-induced epidermal alterations, by completely preventing the UV-induced glutathione reductase reduction and by partially blocking the UV-induced oxidized proteins.

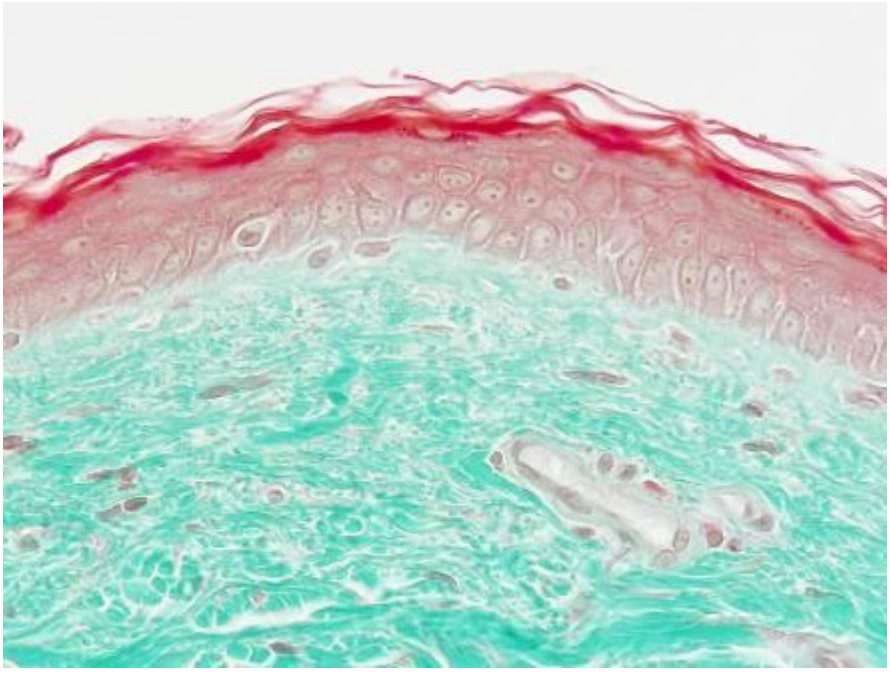
APPENDIXES

1. Cell viability

Blank batch on day 0 (T0)



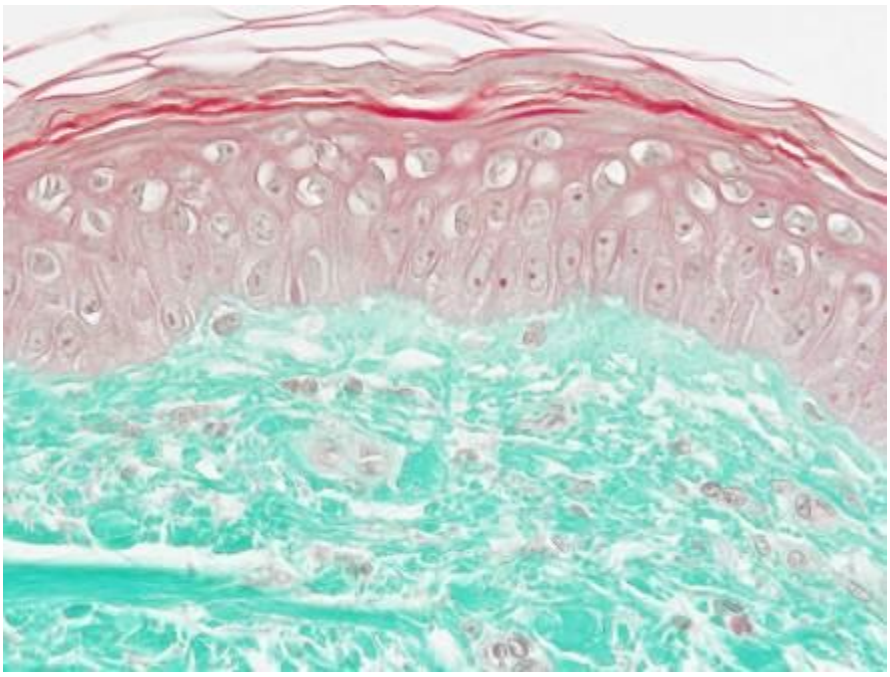
T0_1-1 17E4016 TCM 50 µm



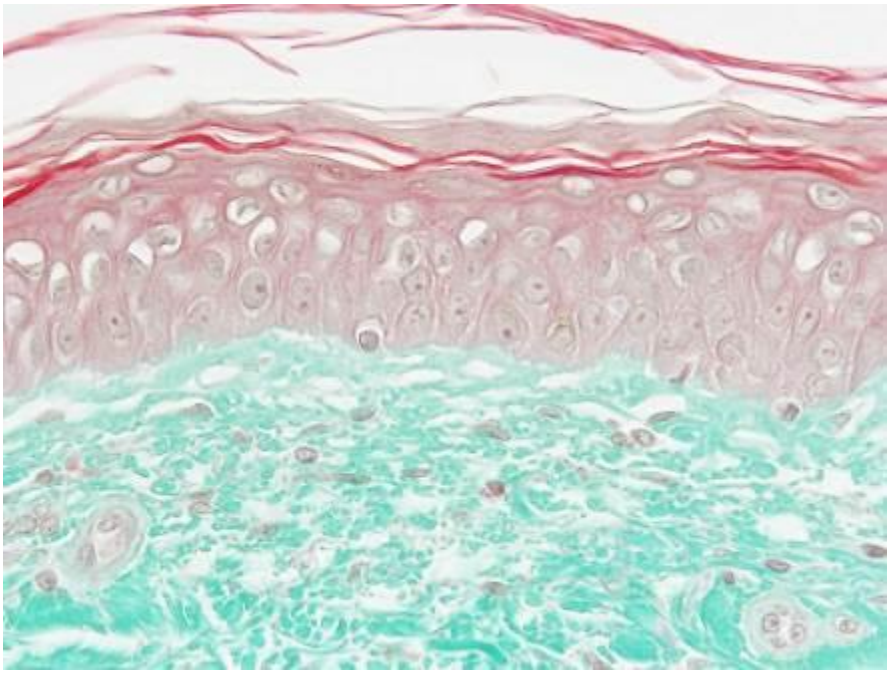
T0_4-4 17E4016 TCM 50 µm

Cell viability

Blank batch on day 5 (TJ5)



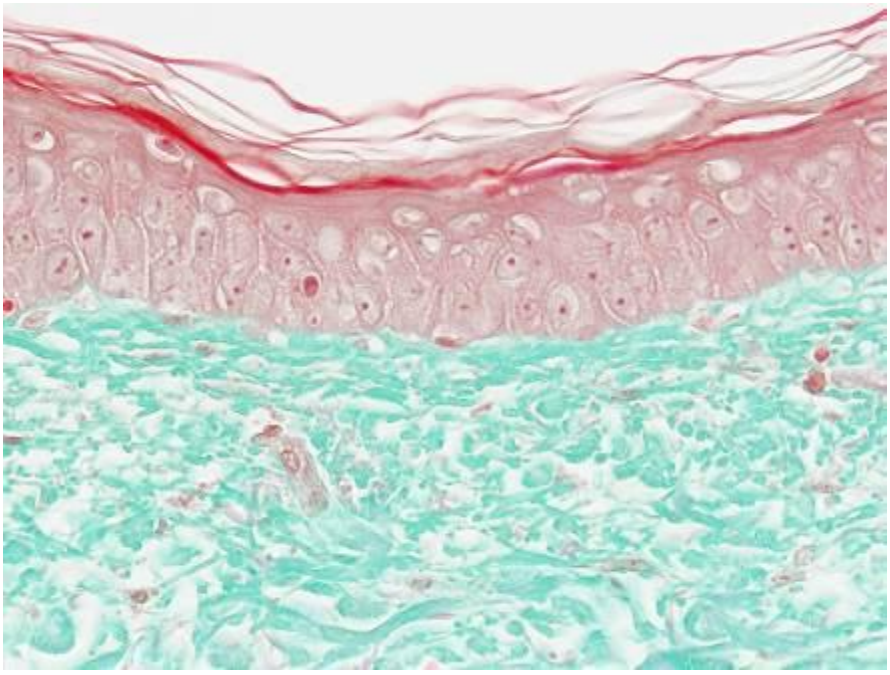
TJ5_5-12 17E4016 TCM 50 µm



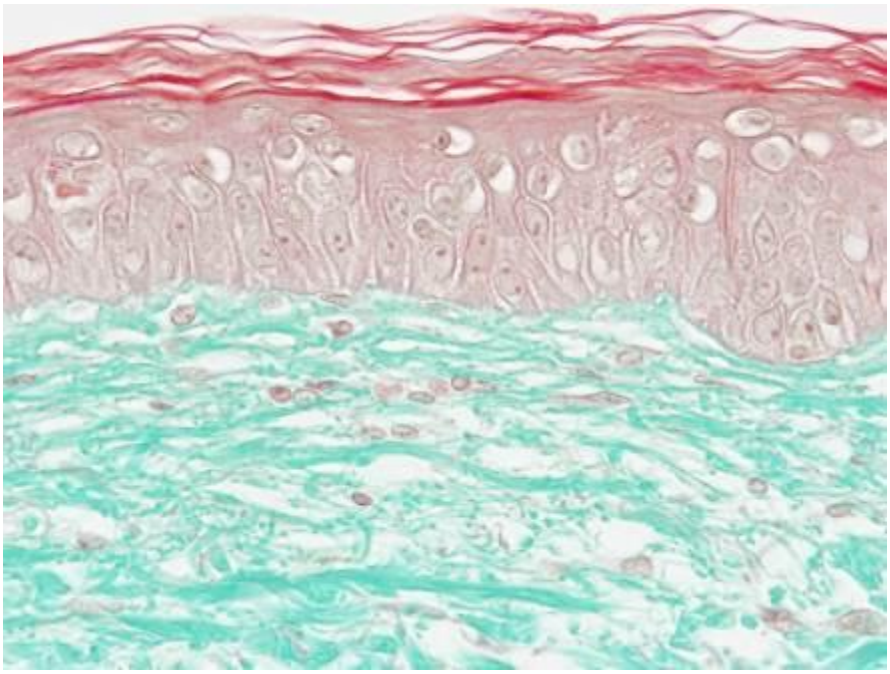
TJ5_7-14 17E4016 TCM 50 µm

Cell viability

Batch P1 on day 5 (P1J5)



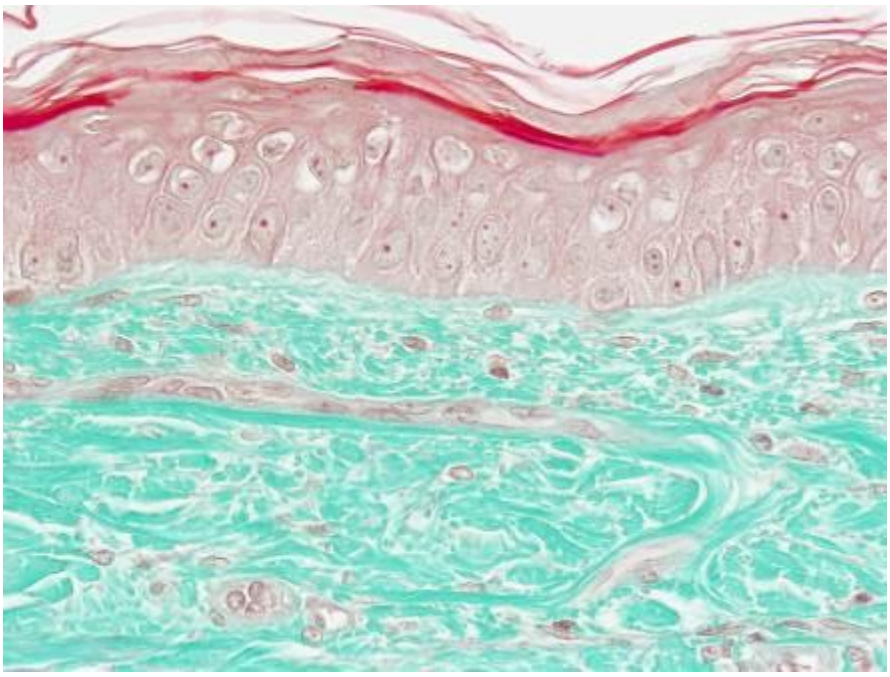
P1J5_4-21 17E4016 TCM 50 µm



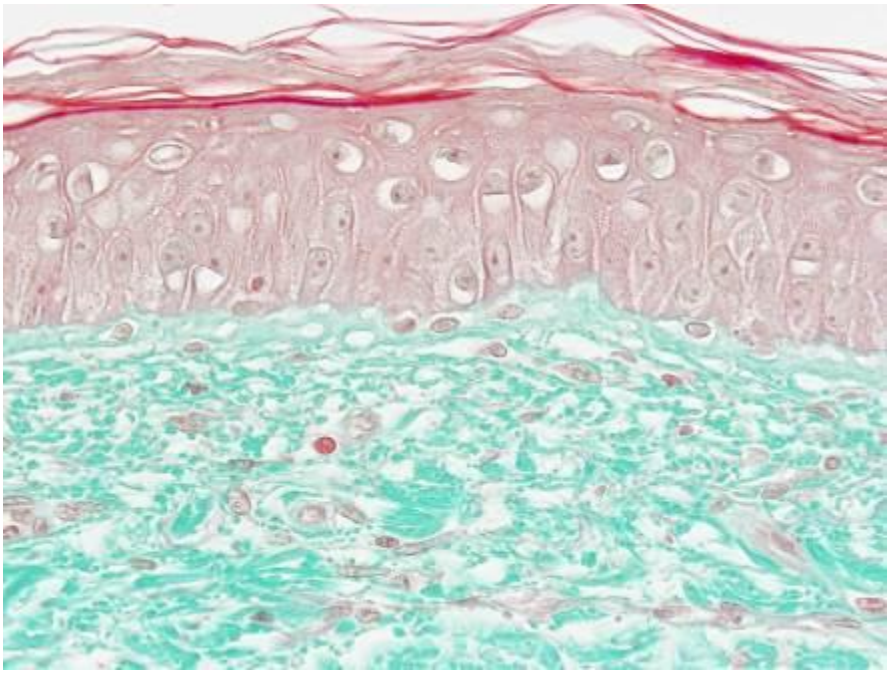
P1J5_5-22 17E4016 TCM 50 µm

Cell viability

Batch P2 on day 5 (P2J5)



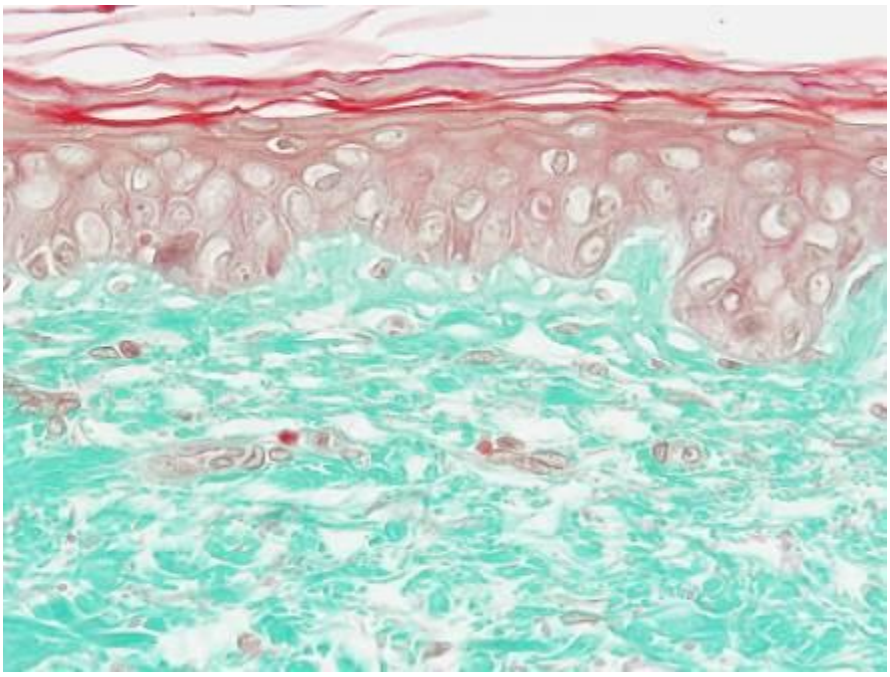
P2J5_6-30 17E4016 TCM 50 µm



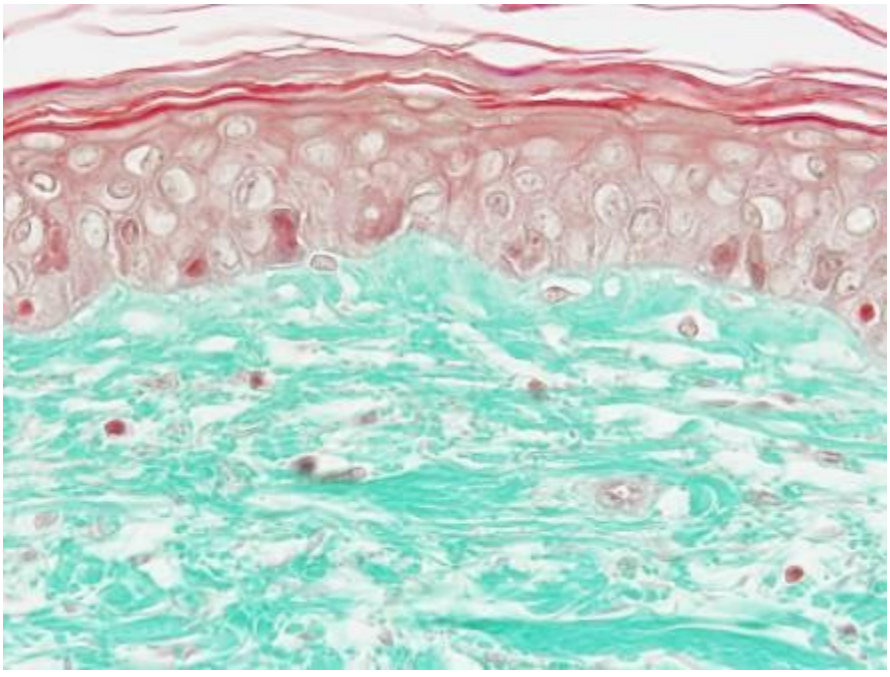
P2J5_14-36 17E4016 TCM 50 µm

Cell viability

Batch UV on day 5 (TUVJ5)



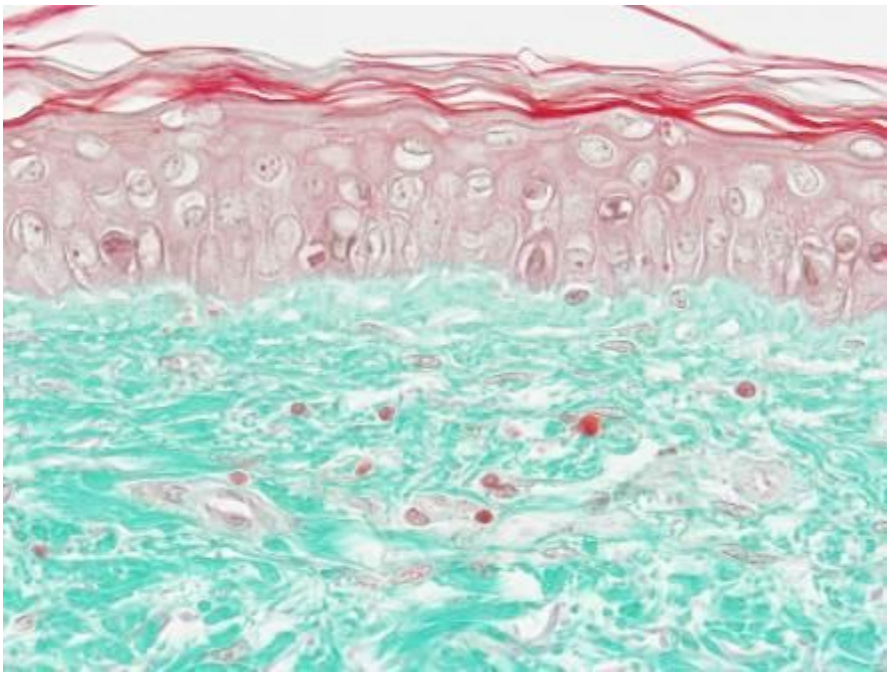
TUVJ5_3-37 17E4016 TCM 50 µm



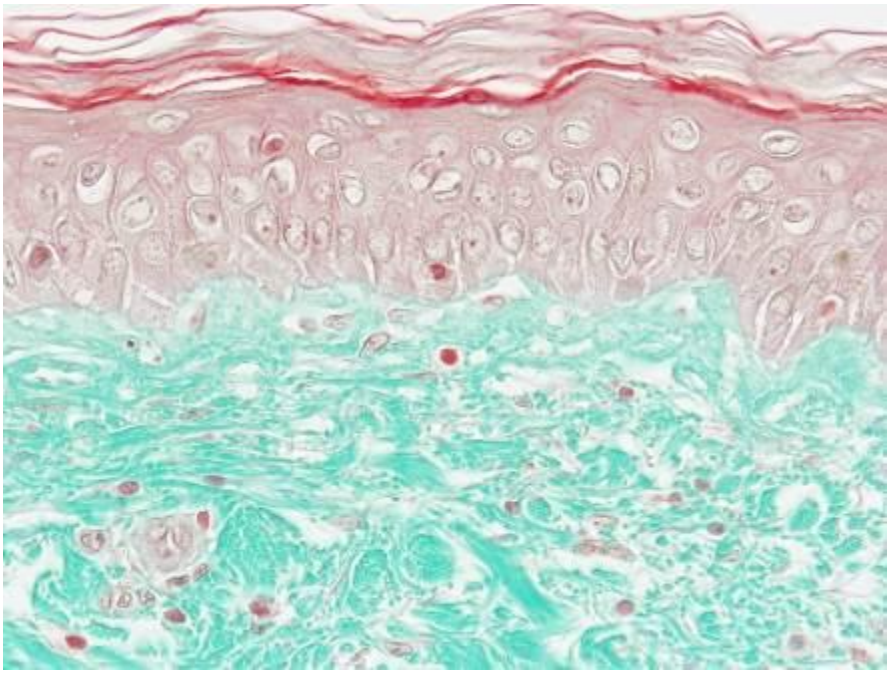
TUVJ5_5-39 17E4016 TCM 50 µm

Cell viability

Batch P1UV on day 5 (P1UVJ5)



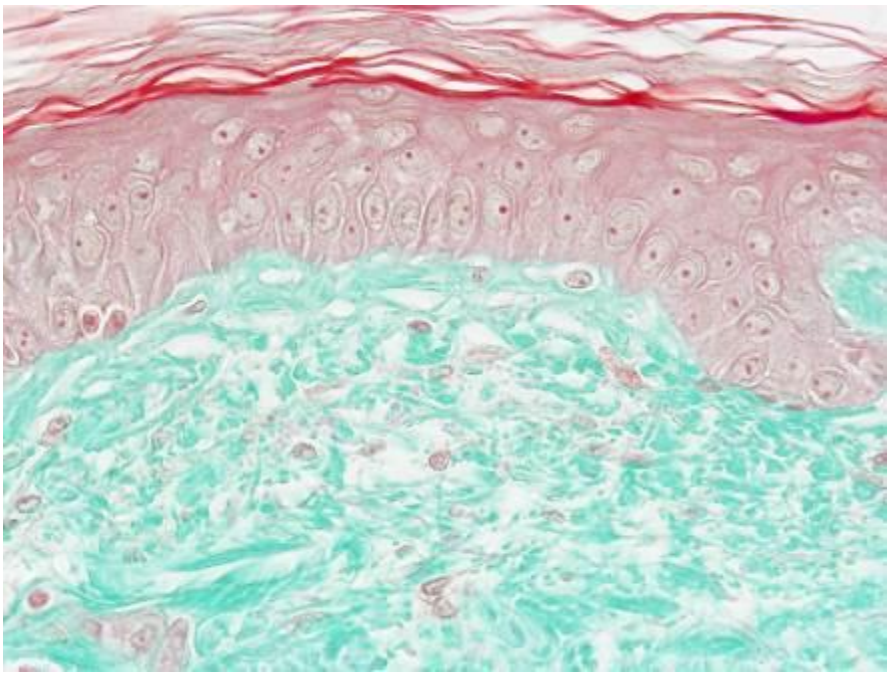
P1UVJ5_4-49 17E4016 TCM 50 µm



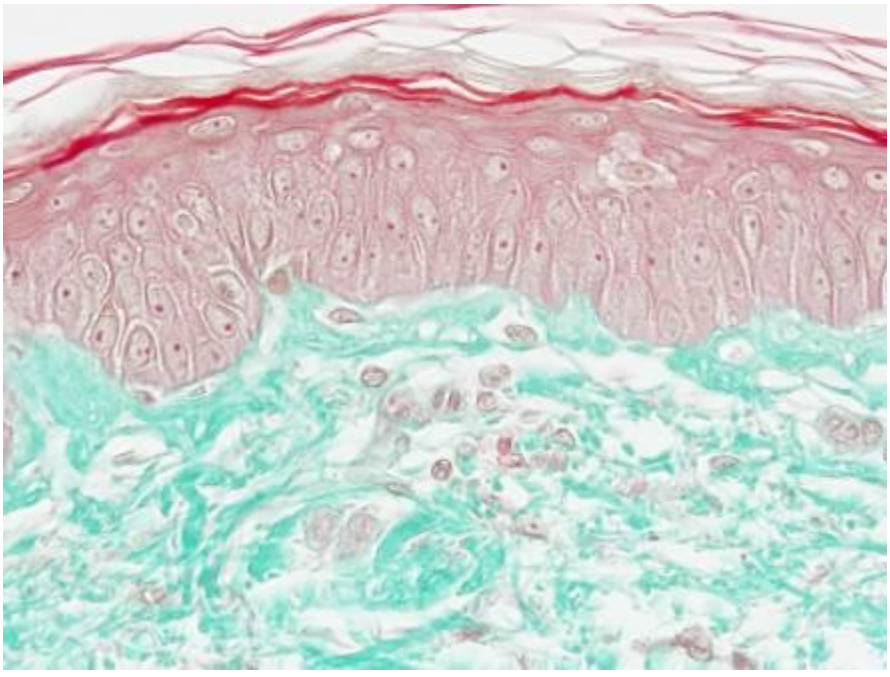
P1UVJ5_10-53 17E4016 TCM 50 µm

Cell viability

Batch P2UV on day 5 (P2UVJ5)



P2UVJ5_5-58 17E4016 TCM 50 µm



P2UVJ5_6-59 17E4016 TCM 50 µm

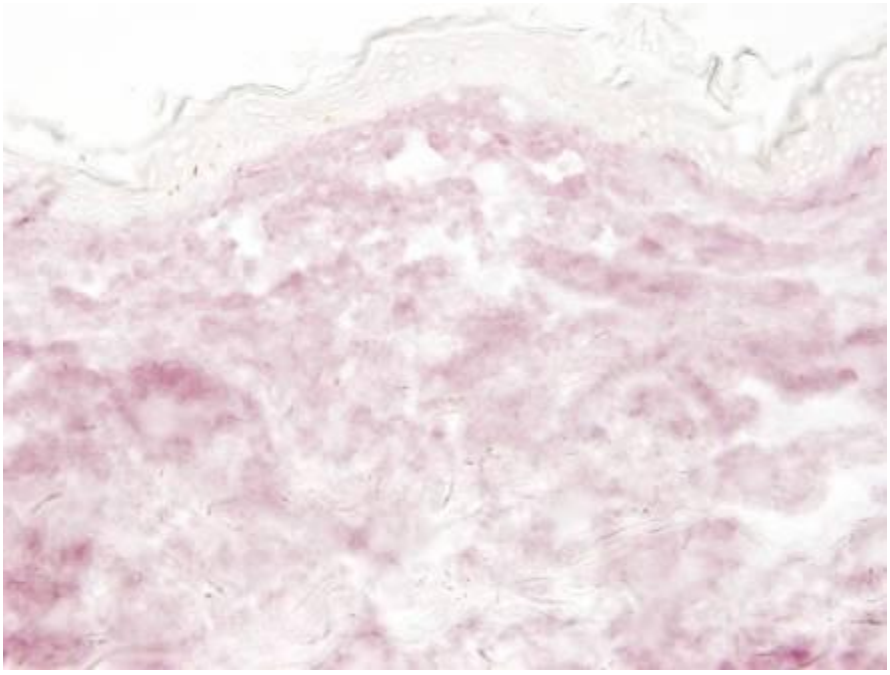
2. Oxidized proteins

Negative control without anti-DNP antibody

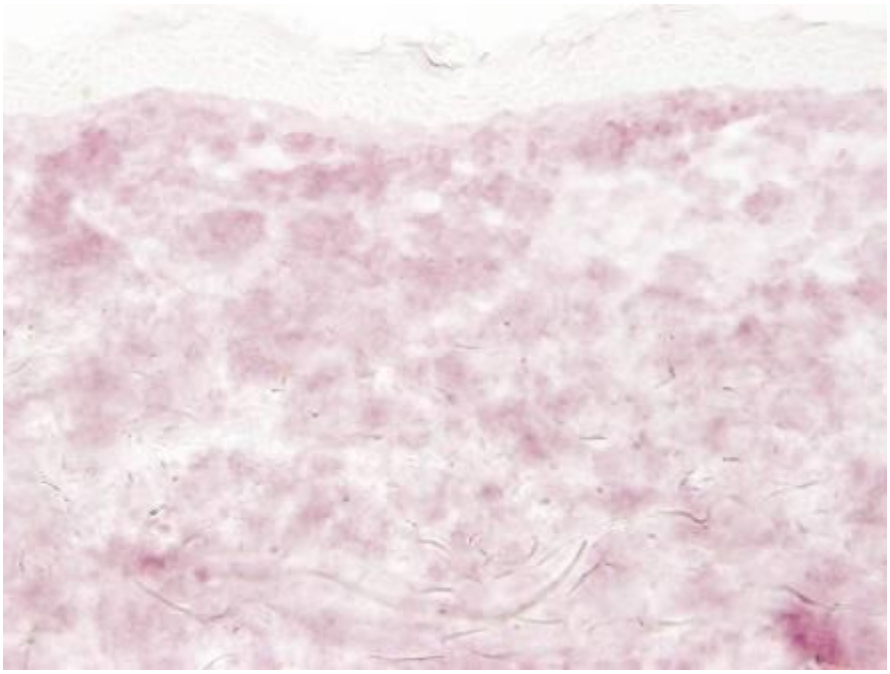
T1_1-64 18E4016 Protéines oxydées 100 µm

Oxidized proteins

Blank batch on day 0 (T0)



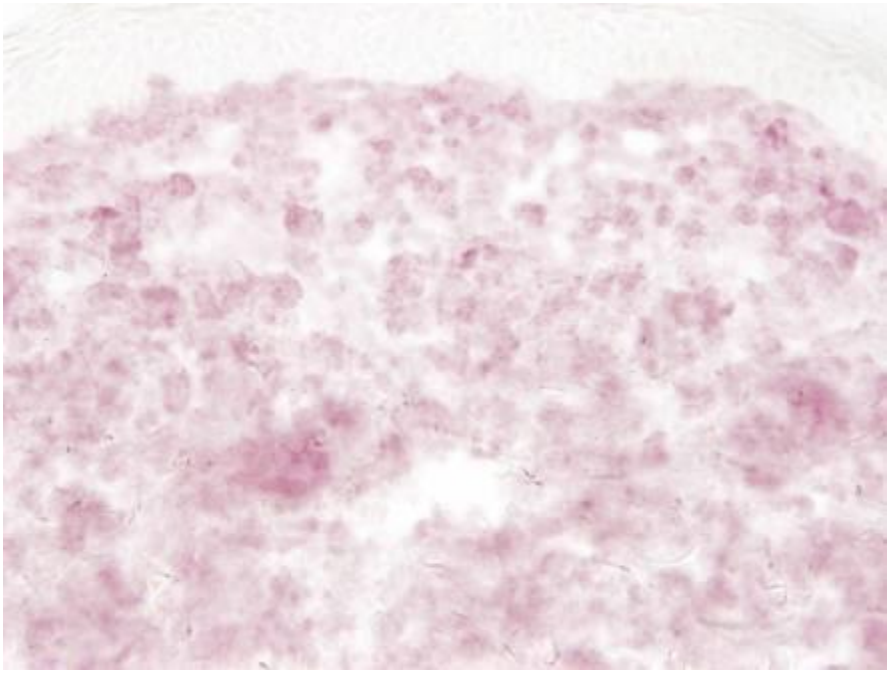
T0_1-1 18E4016 Protéines oxydées 100 µm



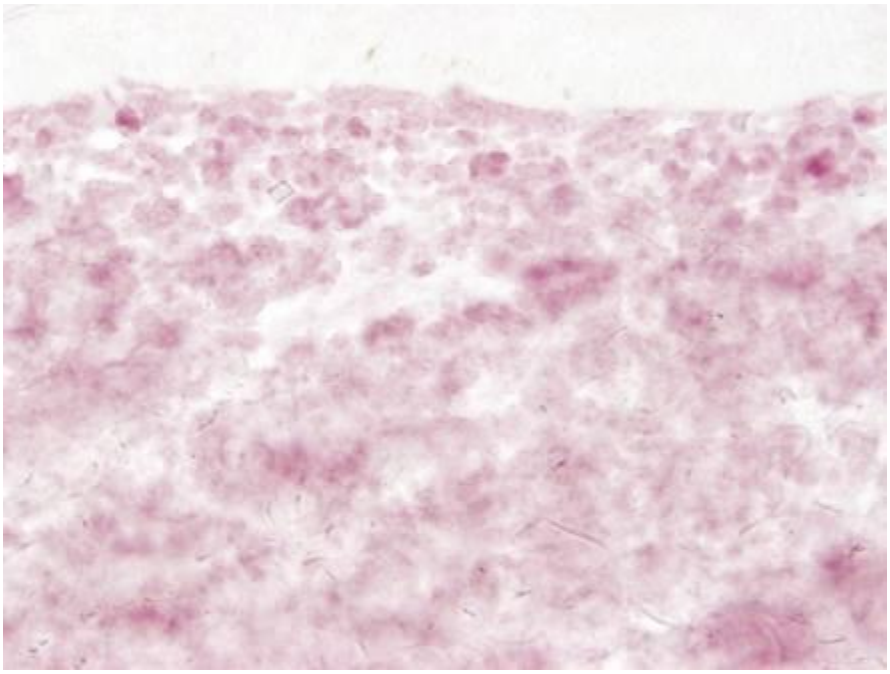
T0_5-5 18E4016 Protéines oxydées 100 µm

Oxidized proteins

Blank batch on day 5 (TJ5)



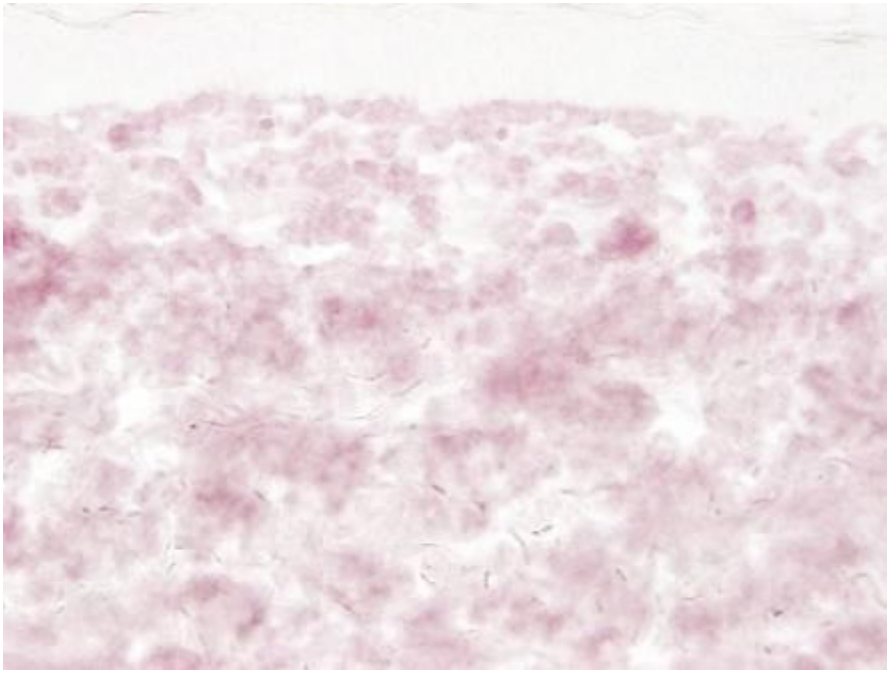
TJ5_2-11 18E4016 Protéines oxydées 100 µm



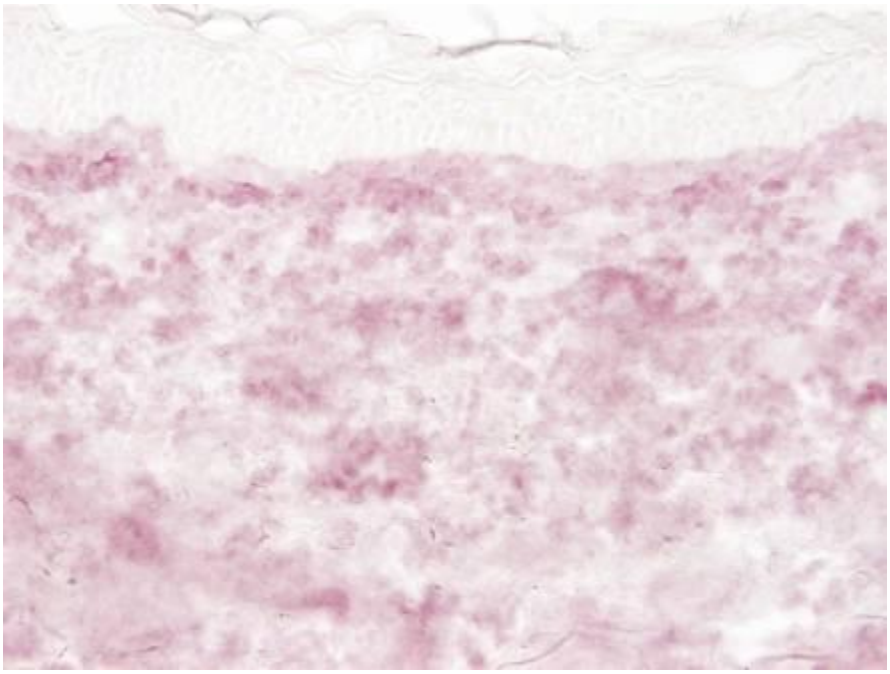
TJ5_7-16 18E4016 Protéines oxydées 100 µm

Oxidized proteins

Batch P1 on day 5 (P1J5)



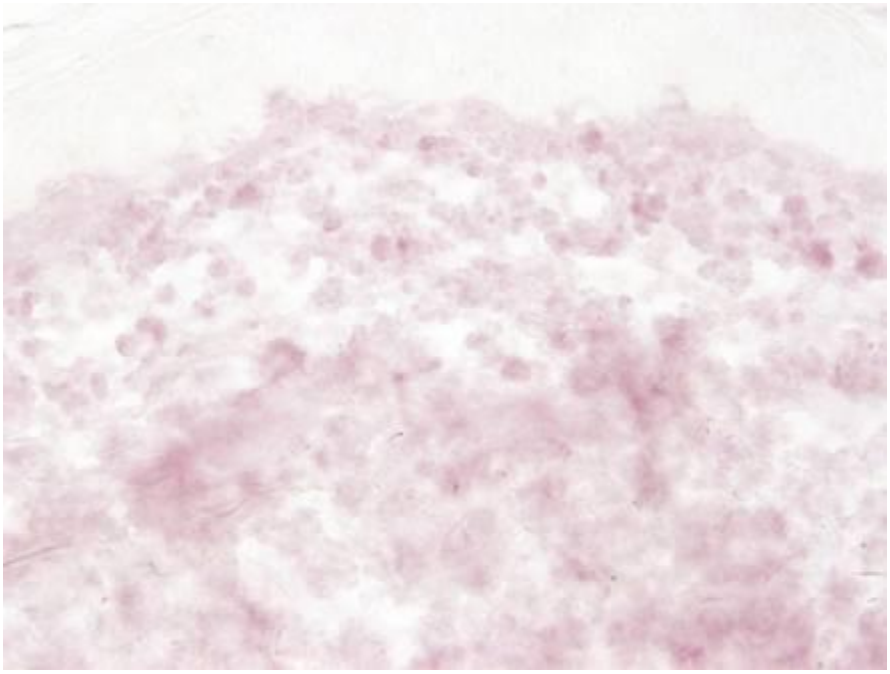
P1J5_1-19 18E4016 Protéines oxydées 100 µm



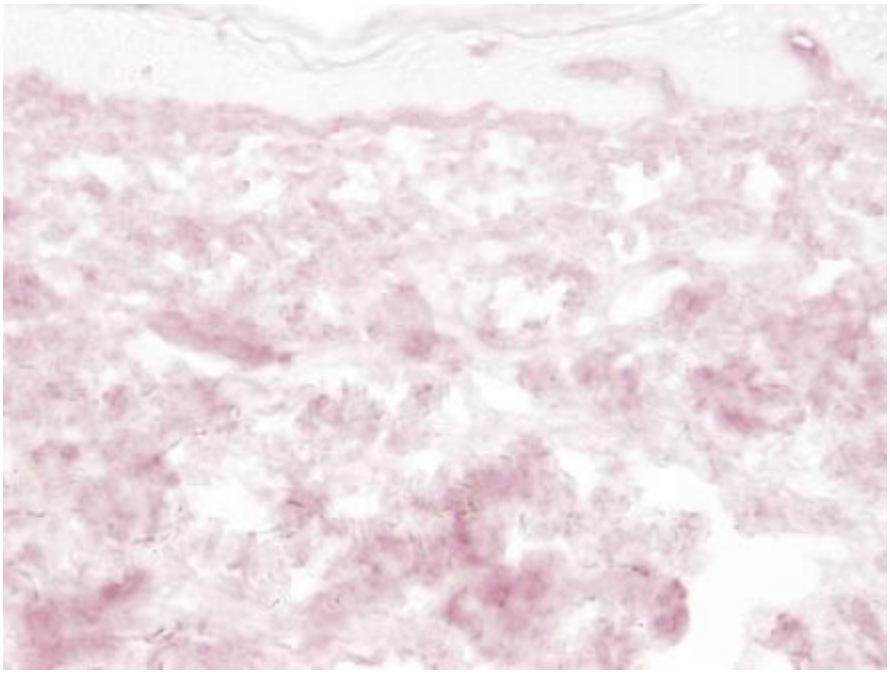
P1J5_9-27 18E4016 Protéines oxydées 100 µm

Oxidized proteins

Batch P2 on day 5 (P2J5)



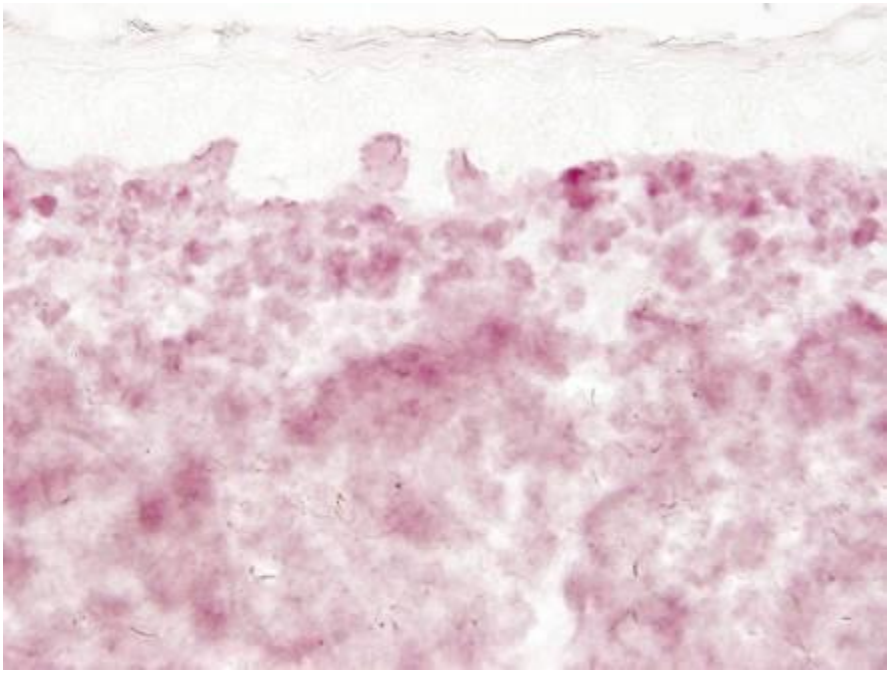
P2J5_2-29 18E4016 Protéines oxydées 100 µm



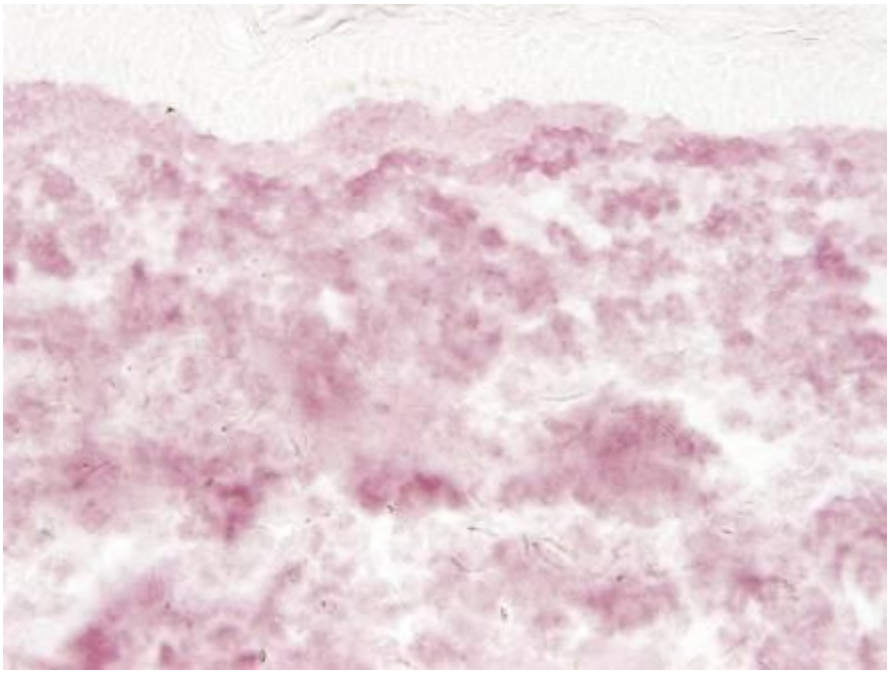
P2J5_9-36 18E4016 Protéines oxydées 100 µm

Oxidized proteins

Batch TUV on day 5 (TUVJ5)



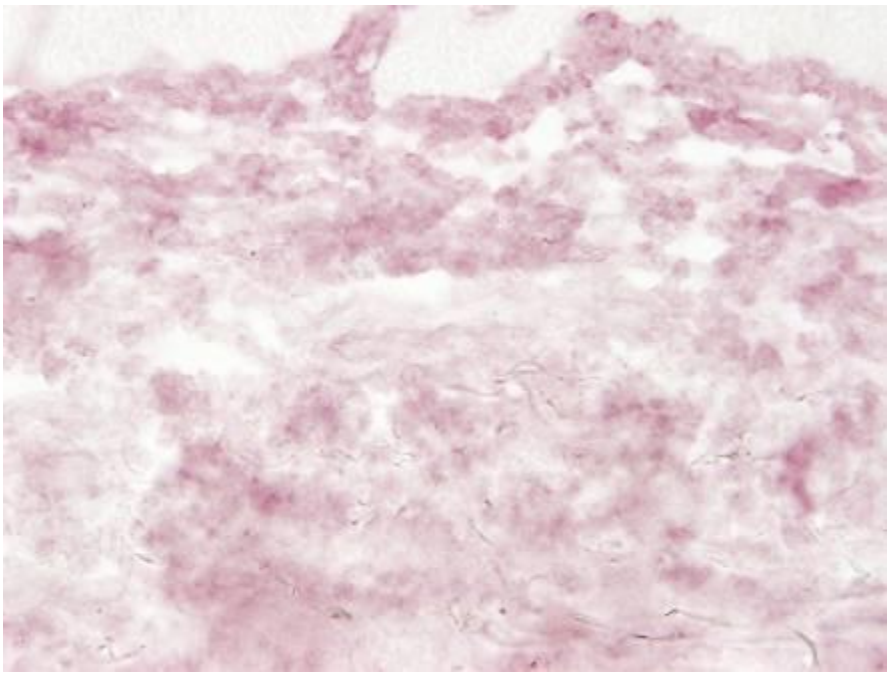
TUVJ5_1-37 18E4016 Protéines oxydées 100 µm



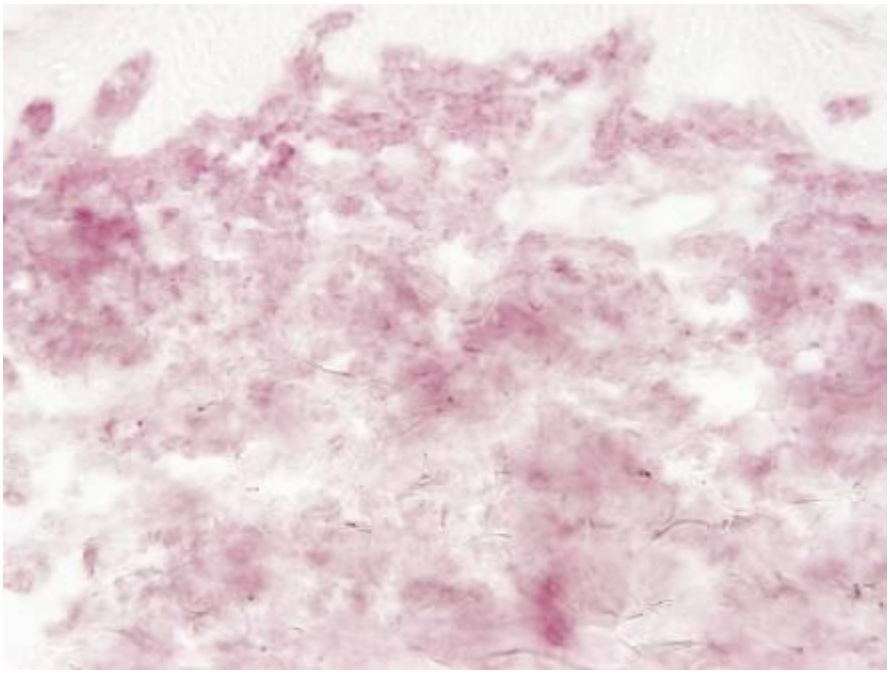
TUVJ5_7-43 18E4016 Protéines oxydées 100 µm

Oxidized proteins

Batch P1UV on day 5 (P1UVJ5)



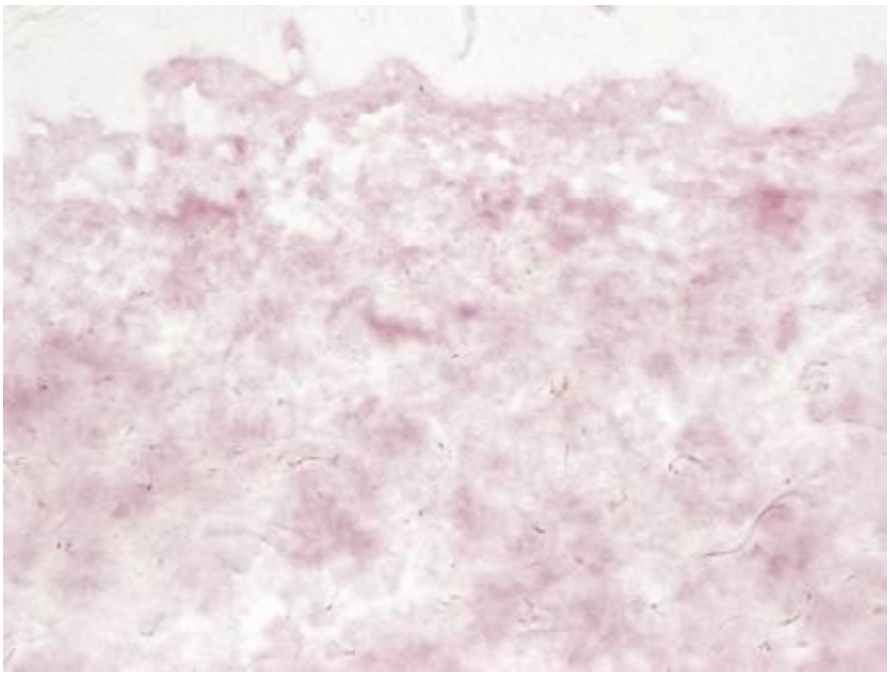
P1UVJ5_5-50 18E4016 Protéines oxydées 100 µm



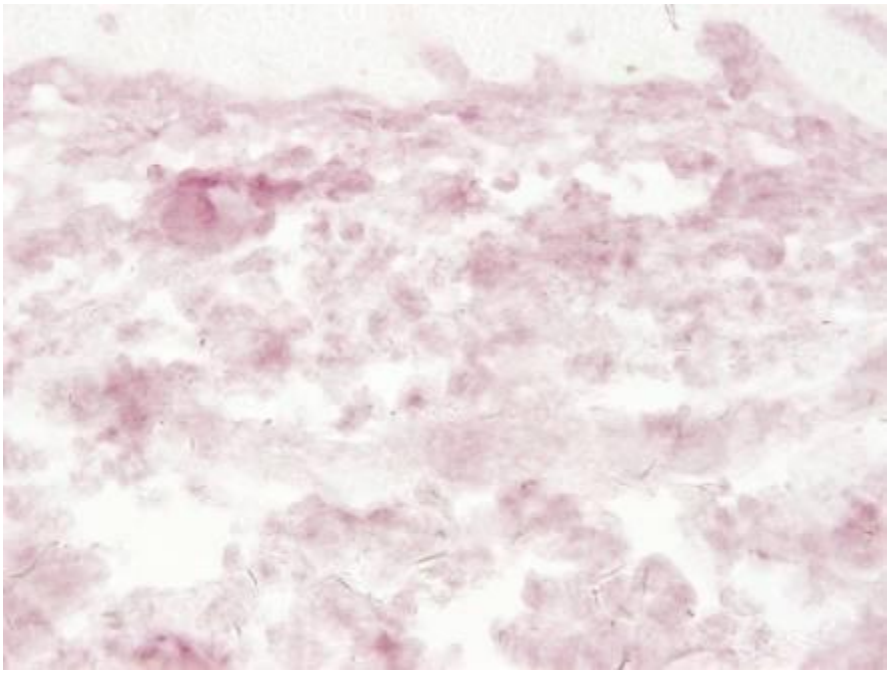
P1UVJ5_6-51 18E4016 Protéines oxydées 100 µm

Oxidized proteins

Batch P2UV on day 5 (P2UVJ5)



P2UVJ5_5-59 18E4016 Protéines oxydées 100 µm



P2UVJ5_9-63 18E4016 Protéines oxydées 100 µm

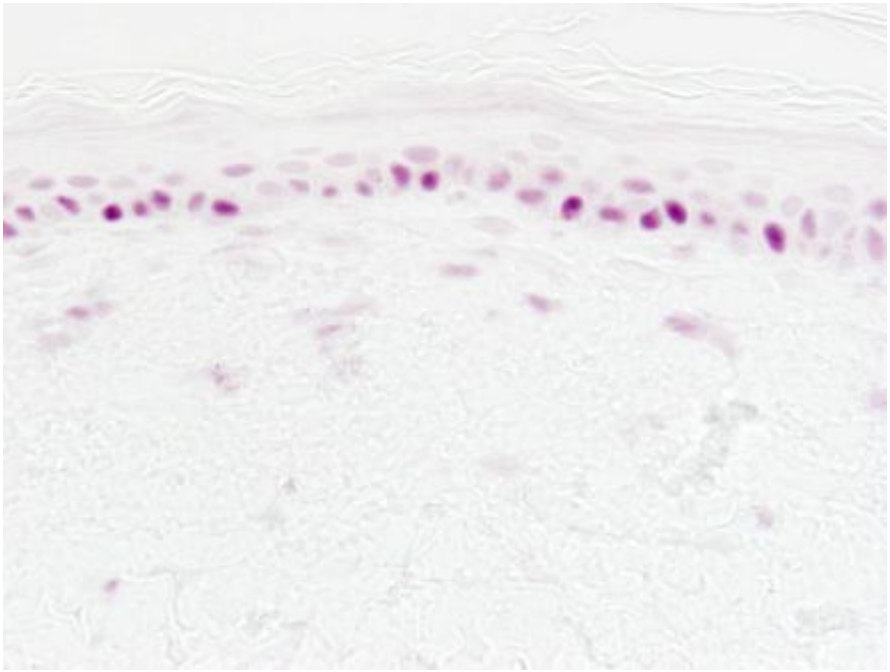
3. Gluthatione Reductase

Negative control without primary antibody

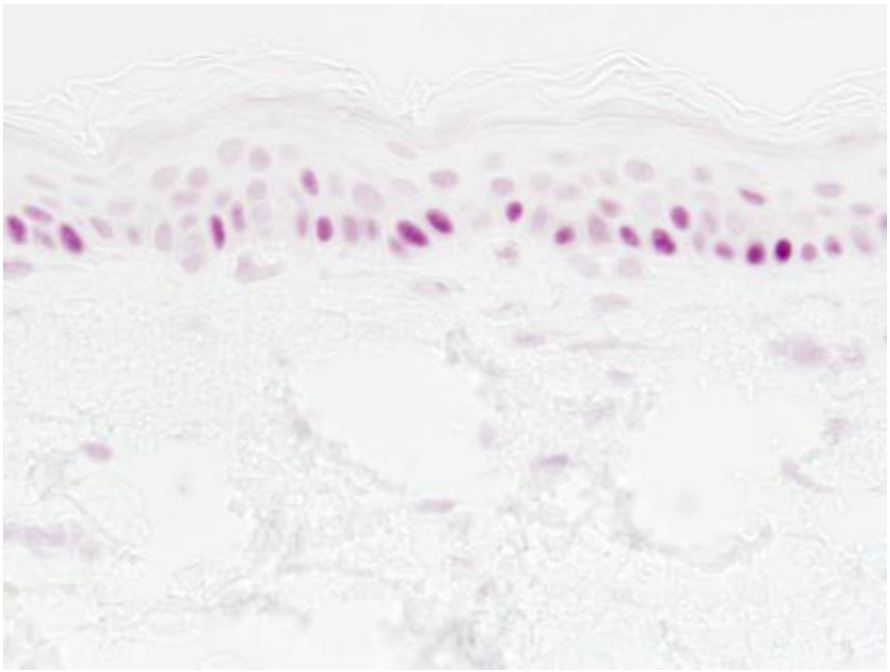
Tem_3-66 18E4016 Glutathion reductase 50 µm

Glutathione reductase

Blank batch on day 0 (T0)



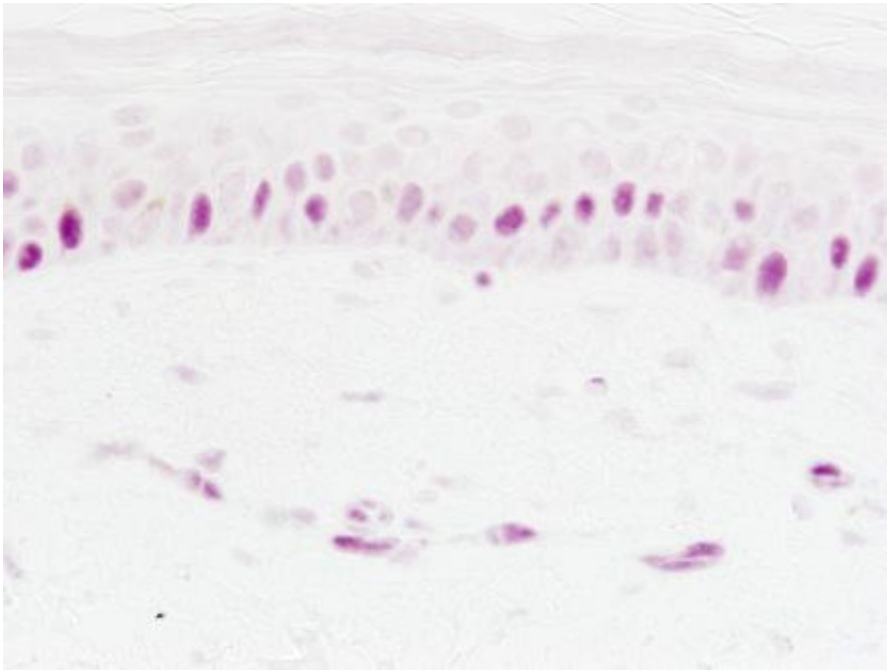
T0_2-2 18E4016 Glutathion reductase 50 µm



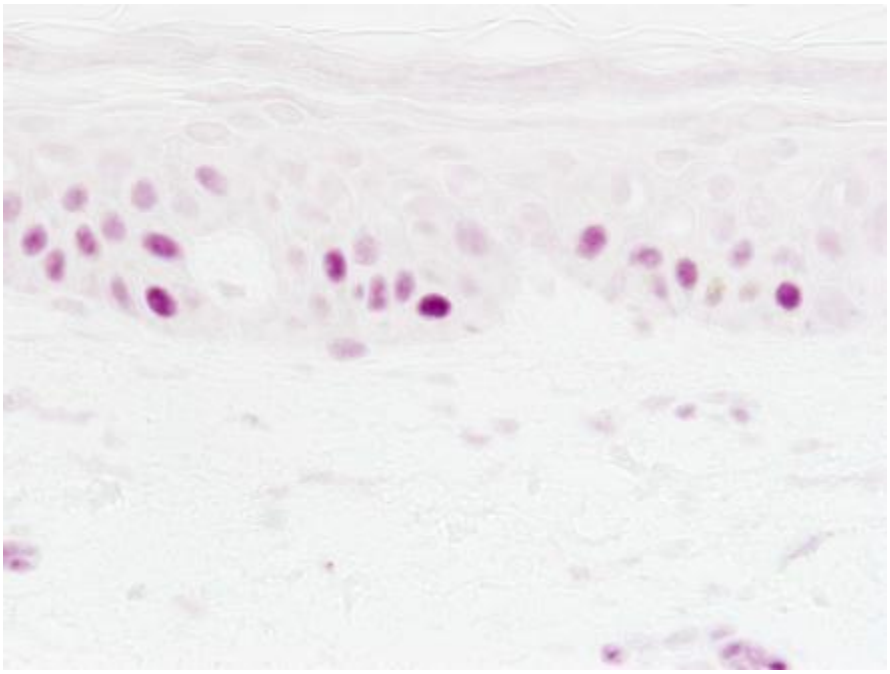
T0_3-3 18E4016 Glutathion reductase 50 µm

Glutathione reductase

Blank batch on day 5 (TJ5)



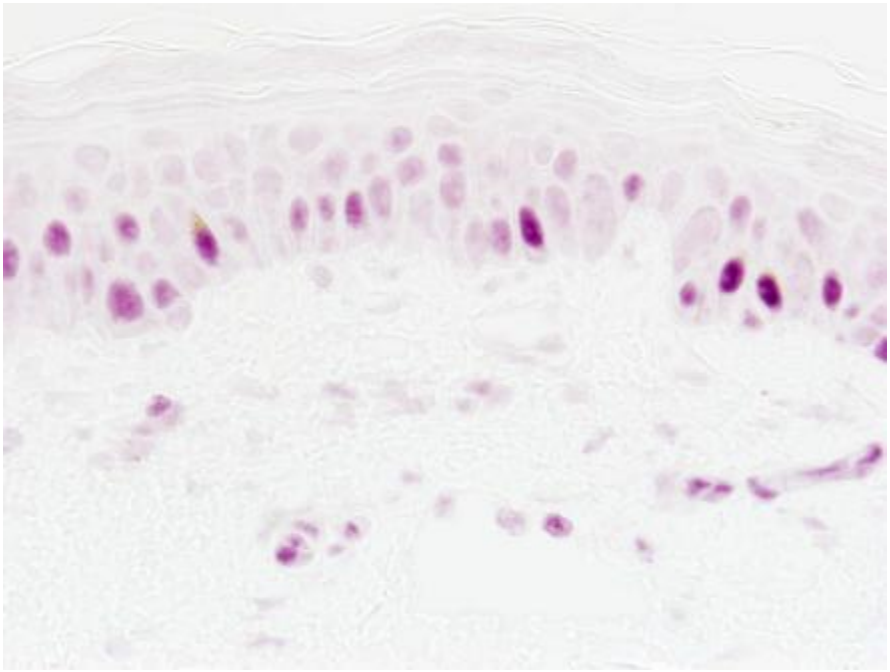
TJ5_9-15 18E4016 Glutathion reductase 50 µm



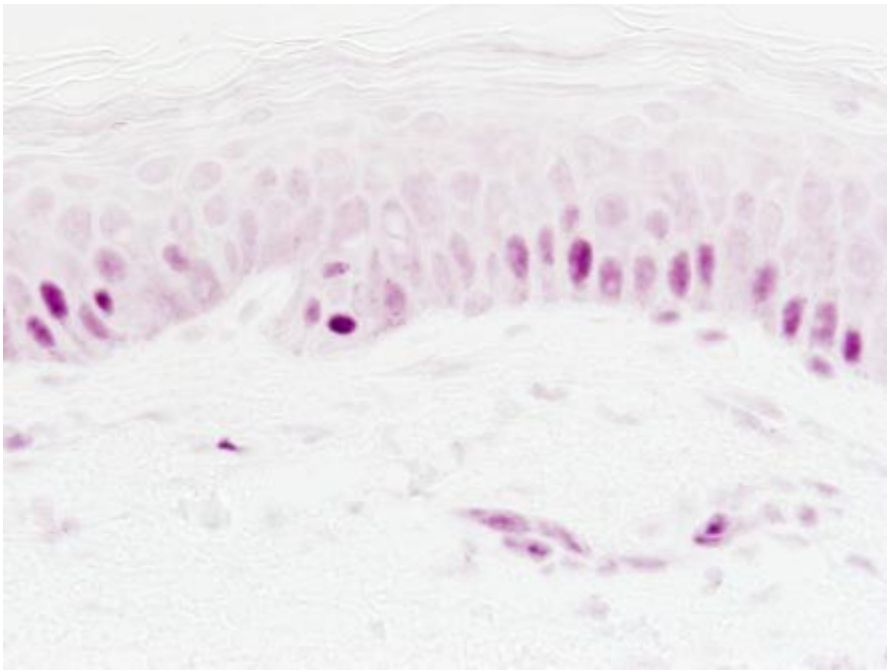
TJ5_10-16 18E4016 Glutathion reductase 50 µm

Glutathione reductase

Batch P1 on day 5 (P1J5)



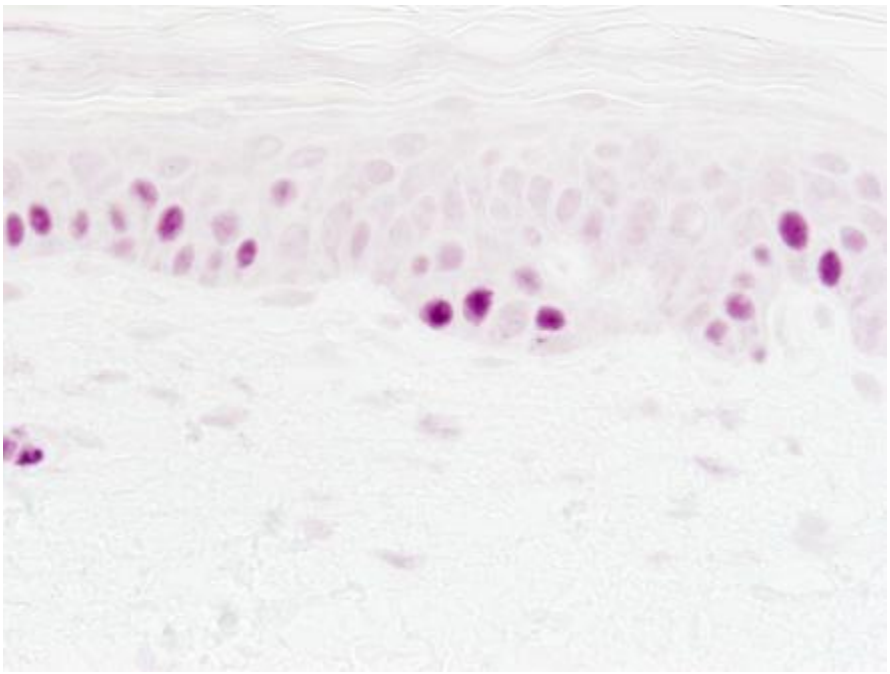
P1J5_4-21 18E4016 Glutathion reductase 50 µm



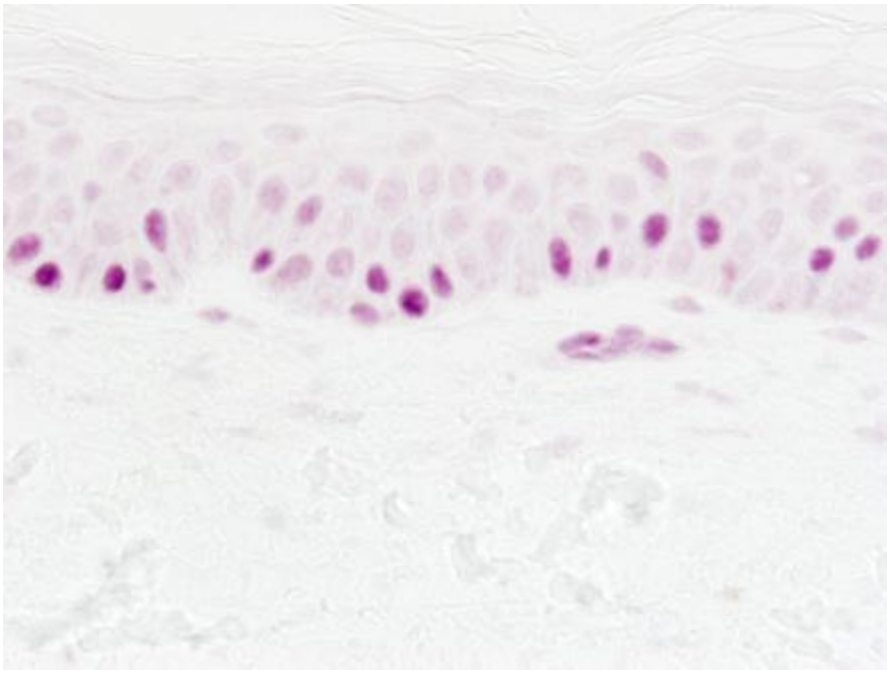
P1J5_8-24 18E4016 Glutathion reductase 50 µm

Glutathione reductase

Batch P2 on day 5 (P2J5)



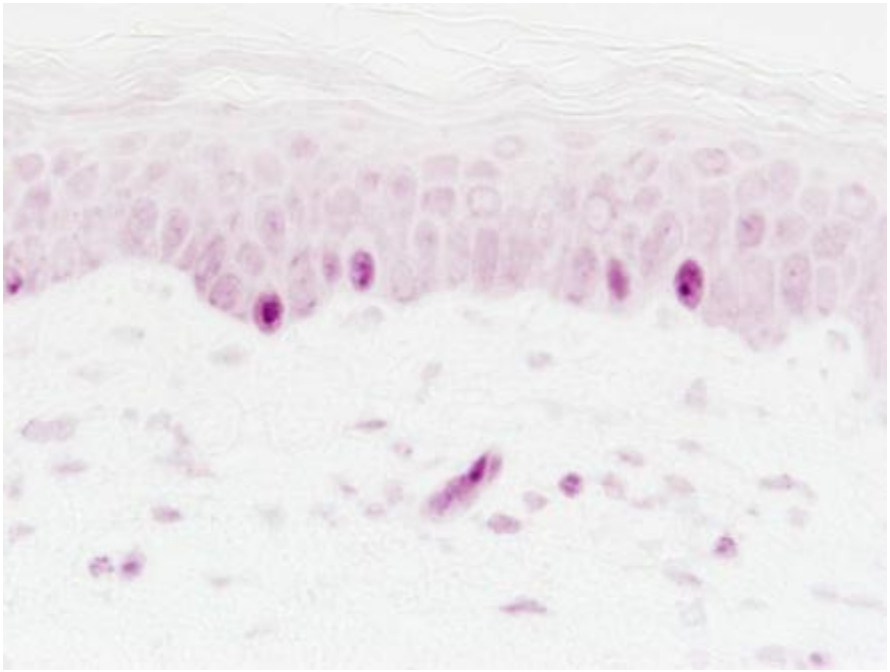
P2J5_5-32 18E4016 Glutathion reductase 50 µm



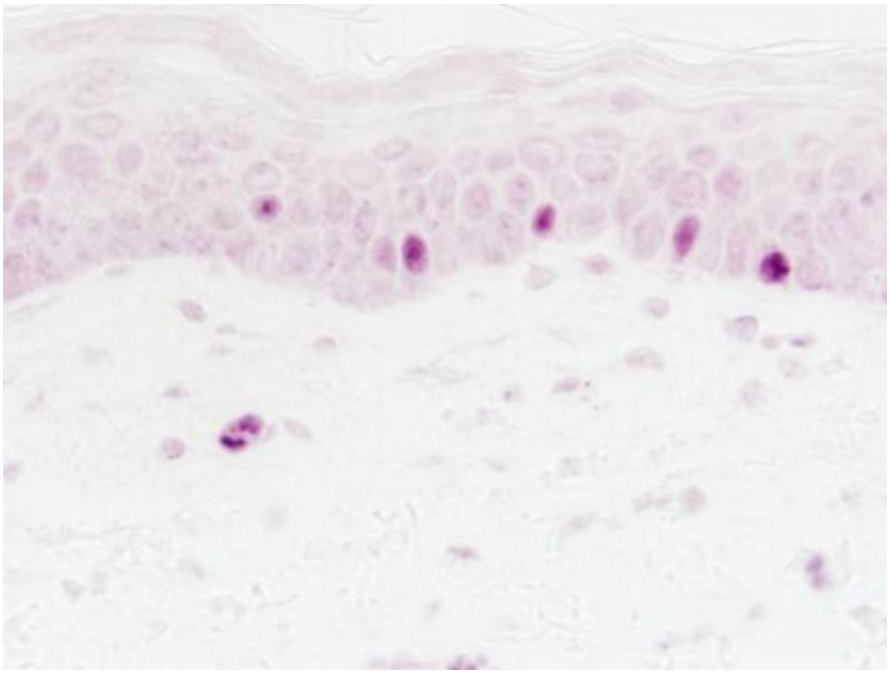
P2J5_6-33 18E4016 Glutathion reductase 50 µm

Glutathione reductase

Batch UV on day 5 (TUVJ5)



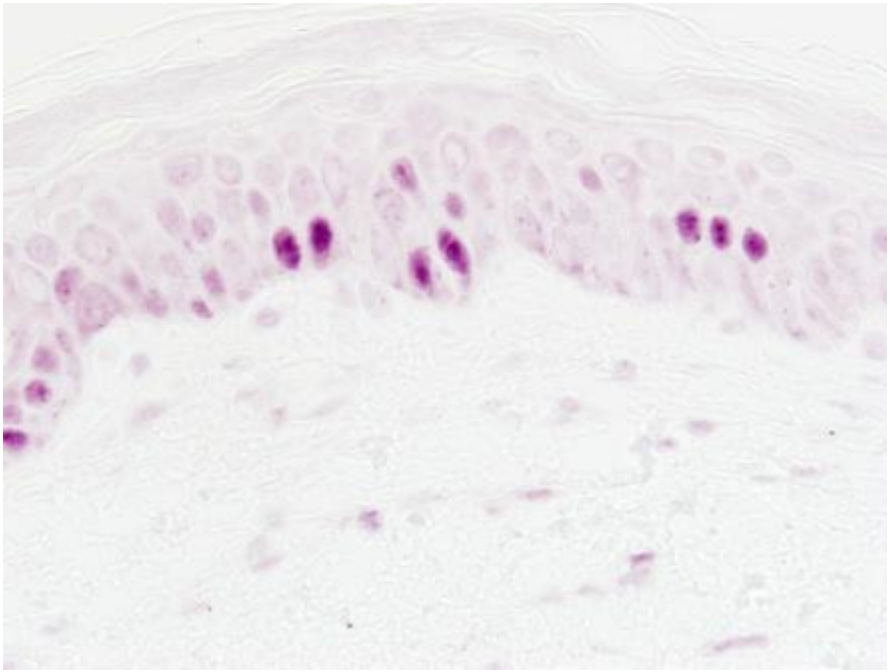
TUVJ5_4-40 18E4016 Glutathion reductase 50 µm



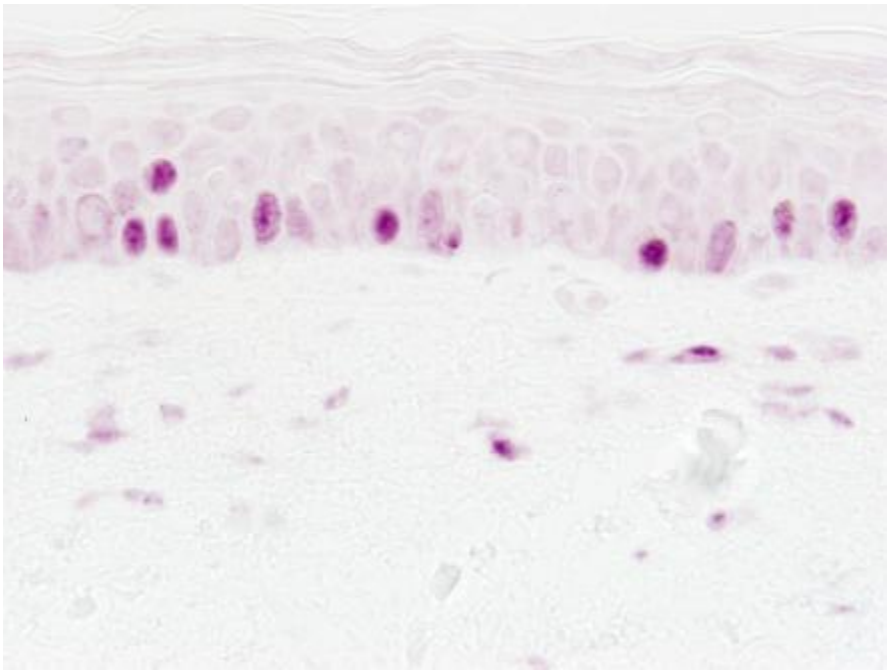
TUVJ5_6-41 18E4016 Glutathion reductase 50 µm

Glutathione reductase

Batch P1UV on day 5 (P1UVJ5)



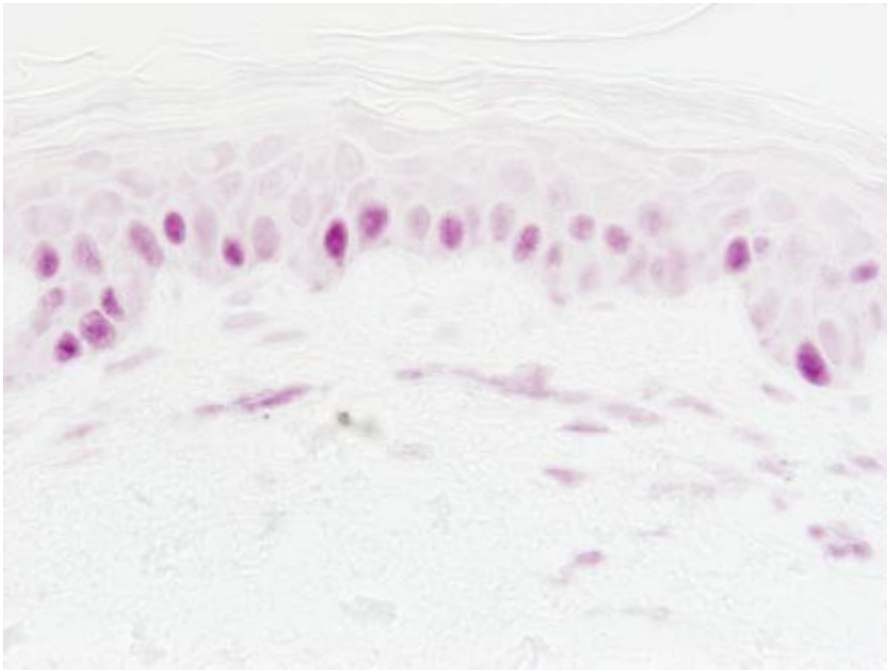
P1UVJ5_2-47 18E4016 Glutathion reductase 50 µm



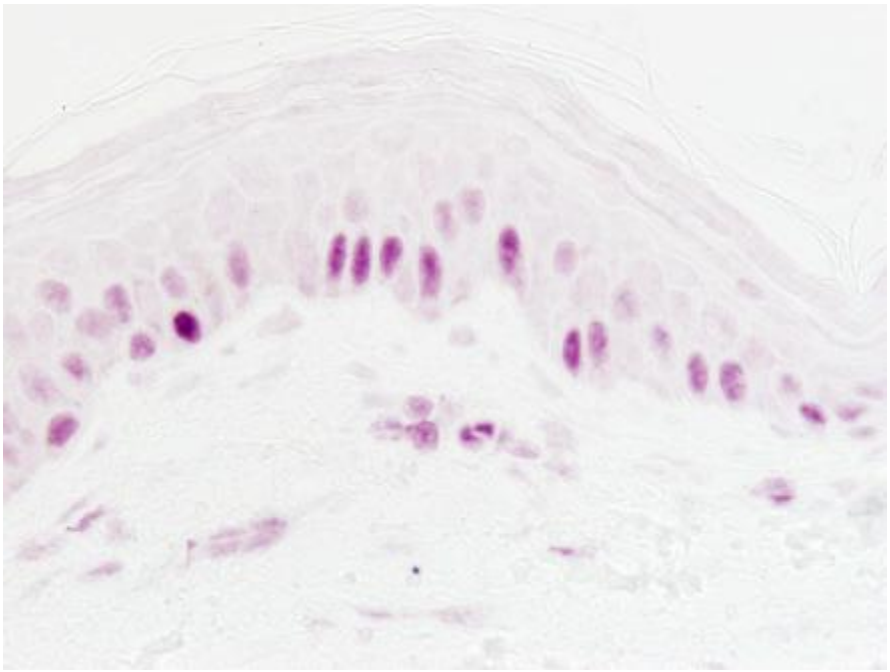
P1UVJ5_6-49 18E4016 Glutathion reductase 50 µm

Glutathione reductase

Batch P2UV on day 5 (P2UVJ5)



P2UVJ5_11-61 18E4016 Glutathion reductase 50 µm



P2UVJ5_12-62 18E4016 Glutathion reductase 50 µm

DEVIATIONS

None.

ATTESTATIONS

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 products

P1 : Rivoli Creme de Jour Jeunesse II ref. Torstone
P2 : Sonnencreme SPF25

Model

Human living skin explants.
Topical treatment (2 mg/cm^2) with the products P1 and P2 on D0, D3 and D4.
On D4, the explants of the batches “TUV”, “P1UV” and “P2UV” were irradiated by UVA+ UVB with a dose of 18 J/cm^2 of UVA and 0,6 J/cm^2 of UVB corresponding both to 4 MED (minimal erythemal dose).
Sampling of skin explants on D5.
Evaluated parameters: cell viability, immunostainings of oxidized proteins and gluthatione reductase.

Conclusion

According to these experimental conditions, compared to the batches on day 5 without or with UV (TJ5 or TUVJ5):

vs T or TUV on day 5		**Rivoli Creme de Jour Jeunesse II ref. Torstone (P1)**	**Sonnencreme SPF25 (P2)**
Cell viability	*vs TJ5*	↔	↔
	vs TUVJ5	(↘) epidermal alterations ↔ dermal alterations	↘↘ epidermal alterations ↘ dermal alterations
Oxidized proteins	*vs TJ5*	↔	↘
	vs TUVJ5	↘	↘↘↘
Glutathione reductase	*vs TJ5*	↗	↗
	vs TUVJ5	↗	↗

Decrease		**Increase**	↔	No variation
(↘)	Very Slight	(↗)		
↘	Slight	↗		
↘↘	Moderate	↗↗	ns	non-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 Sonnencreme SPF25 (P2) exhibits the best anti-oxidant activity by completely preventing the UV-induced epidermal and dermal alterations, by completely preventing the UV-induced glutathione reductase reduction and by completely blocking the UV-induced oxidized proteins.

The product Rivoli Creme de Jour Jeunesse II ref. Torstone (P1) exhibits a fairly good anti-oxidant activity by partially preventing the UV-induced epidermal alterations, by completely preventing the UV-induced glutathione reductase reduction and by partially blocking the UV-induced oxidized proteins.