Laboratoire BIO-EC

Centre de Recherches Biologiques et d'Expérimentations Cutanées

Study 17E3754

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

According to the study plan D17-133-1

Tested products: P1 : Le Visage Emulsion Equilibrante ref. lab-00733.6
P2 : Suncream SPF 25 ref. lab-00591.16

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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)*	30th March 2017	
Date of the beginning of the technical phase of the study	05th April 2017	
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	2nd June 2017	

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AIM OF THE STUDY

The aim of this study is to evaluate the anti-oxidant activity of 2 products.

After a treatment during 4 days and UVA+B irradiation, this activity has been evaluated by:

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

MATERIAL & METHODS

1. Products

The sponsor has provided the following products:

Product	Reference	Batch	Aspect	Quantity
P1	Le Visage Emulsion Equilibrante	lab-00733.6 - 31.01.2017	Blue cream	1 vial
P2	Suncream SPF 25	lab-00591.16 1.7.2015	Beige cream	1 tube

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

2. Characteristic of the plasty

21 skin explants of an average diameter of 11 mm (±1mm) were prepared on an abdoplasty coming from a 43-year-old caucasian woman (reference: P1799-AB43). 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.

3. Explant distribution

The explants were distributed into 7 batches as follows:

Batches	Treatment	UV	Nbr explant	Sampling
T0	/	-	3	J0
T	/	-	3	Day 6
P1	Le Visage Emulsion Equilibrante	-	3	Day 6
P2	Suncream SPF 25	-	3	Day 6
UV	/	+	3	Day 6
P1UV	Le Visage Emulsion Equilibrante	+	3	Day 6
P2UV	Suncream SPF 25	+	3	Day 6

4. Products application

On day 0 (D0), D1, D2 and D5 the products 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 refreshing of the culture medium.
The culture medium (1 mL) was refreshed on D2 and D5.

5. Irradiation

On D5, the culture media of all the batches was replaced by HBSS (Hank's Balanced Saline Solution; 1 ml per explant).
The batches "UV", "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 the explants were put back in 2 mL of fresh BEM medium.

6. 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 D6, 3 explants from each batch were collected and processed in the same way than for day 0.
NB : Days of treatment, irradiation and sampling are likely to be modified to fit the schedule of the study based on working days.

7. Histological processing

After fixation for 24 hours in buffered formalin, the samples were dehydrated and impregnated in paraffin using a Leica TP 1020 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 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.

7.1. Viability control

The morphological study of epidermal and dermal structures will be realized on sections stained according to Masson's trichrom staining.
Concerned batches: *all, so 21 explants.*

7.1. Protein oxidized immunostaining

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 (Vector laboratories, Ref. SK-4600).
The immunostaining was assessed by microscopical observation.
Concerned batches: 3 explants / batches, 21 explants

7.2. Glutathione reductase immunostaining

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% and incubated 1 hour at room temperature using a Vectastain Kit Vector amplifier system avidin/biotin, and revealed by VIP (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 that could be used in the reports

General

D	Day
J	for “Jours”, the French word for day, used for the pictures. D and J are used interchangeably to indicate DAY
MED	Minimal Erythema Dose
SC	Stratum Corneum
EpiD	Epidermis
DEJ	Demo-Epidermal Junction
PD	Papillary Dermis
RD	Reticular Dermis
URD	Upper Reticular Dermis
LRD	Lower Reticular Dermis

Viability (general morphology)

G	Good
QG	Quite Good
SA	Slightly Altered
MA	Moderately altered
QCA	Quite Clearly Altered
CA	Clearly Altered
VCA	Very Clearly Altered

Intensity (staining evaluation)

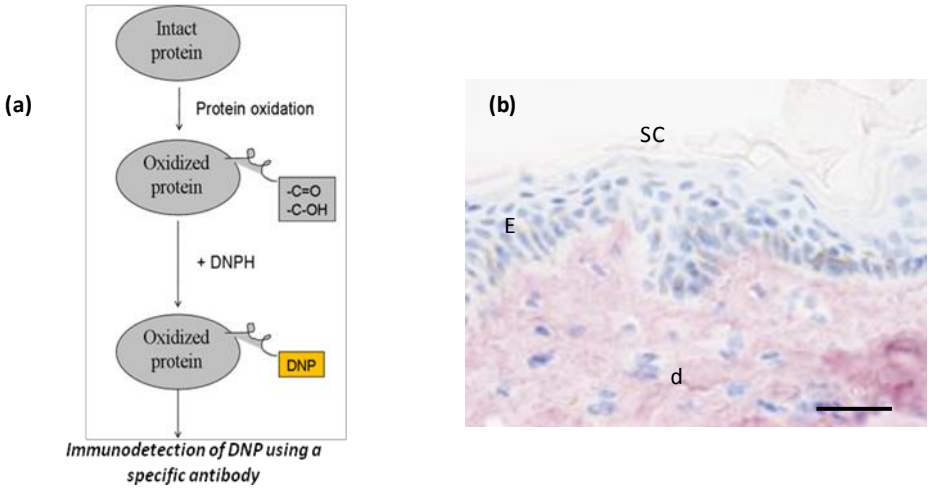
W	Weak
M	Moderate
QC	Quite Clear
C	Clear
VC	Very Clear

BACKGROUND

1. Oxidized protein

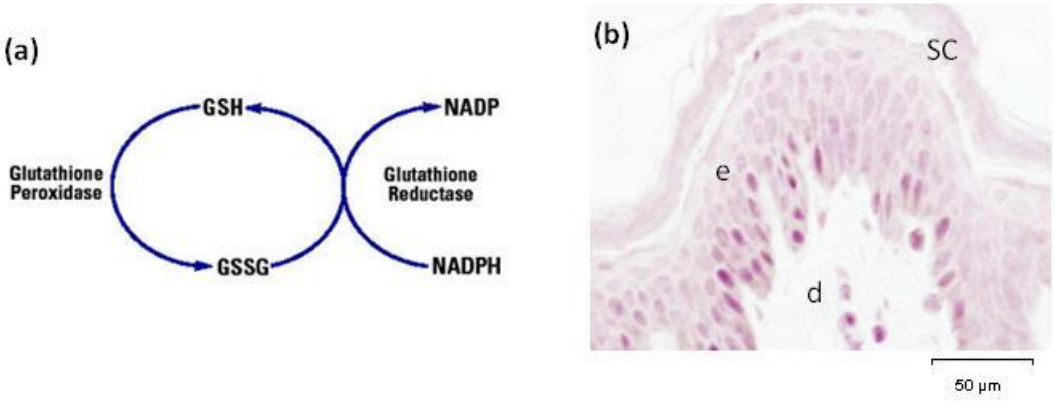
The staining of oxidized proteins was realized using the OxyBlotTM protein oxidation kit (Millipore, S7150) on frozen sections. This kit allows the immunoblot detection of carbonyl groups introduced into proteins by oxidative reactions with 2,4-dinitrophenylhydrazine (DNPH), which leads to the formation 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. 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).



RESULTS & DISCUSSION

1. Cell viability

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

Batch	Cell viability		Remarks
	Epidermis	Dermis (PD)	
T0	G	G	/
TJ6	QG	G	/
P1J6	QG	G	/
P2J6	QG	G	/
TUVJ6	MA	G	Numerous cells in the epidermis with a nucleus in early pycnosis
P1UVJ6	MA	G	Numerous cells in the epidermis with a nucleus in early pycnosis
P2UVJ6	QG	G	/

Abbreviation: PD= papillary dermis
Legend of cell viability: G= good, QG= quite good, SA=slightly altered, MA= moderately altered, QCA= quite clearly altered, CA= clearly altered, VCA=very clearly altered
Evaluation intensity: W= weak; M=moderate; QC=quite clear; C=Clear, VC=very clear

On the **batch T0** the cell viability is good in the epidermis and in the dermal cells of the papillary dermis.

On D6,
On the **batch TJ3** the epidermal and dermal viability is similar to the one observed on the batch T0.

Effect of product application on the cell viability, compared to the batch TJ6:

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

On the UV-irradiated batch (TUVJ6), the cell viability is moderately altered.

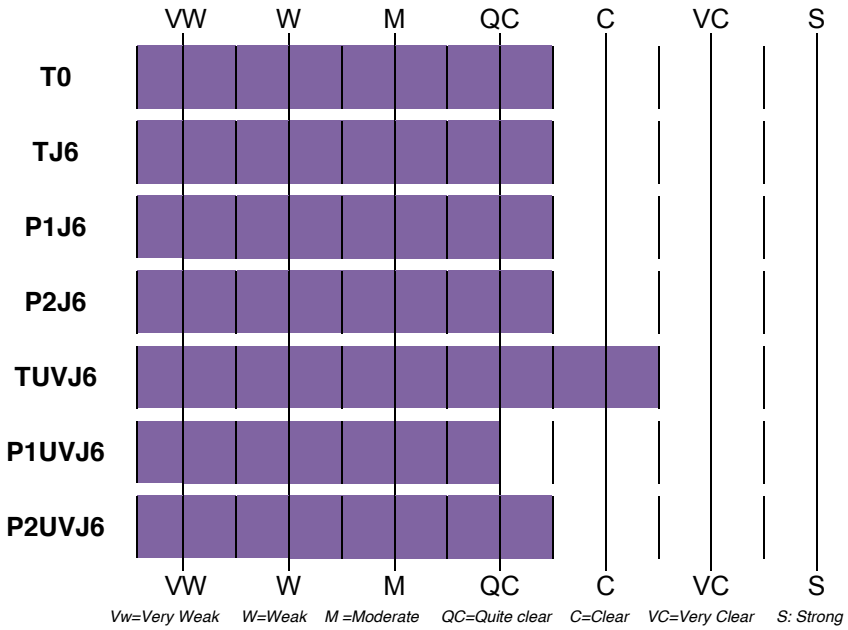
Effect of product application on the cell viability, compared to the batch TUVJ6:

- **The product P1** induces no visible modification
- **The product P2** prevents the formation of the UV-induced alteration.

2. Oxidized protein

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

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



On D6,
On the **batch TJ6**, the content of oxidized proteins is quite clear to clear in the papillary dermis.

Effect of product application on oxidized proteins content, compared to the batch TJ6:

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

The UV1 irradiation induces a moderate increase of oxidized proteins content in the papillary dermis (TUVJ6 *vs* TJ6).

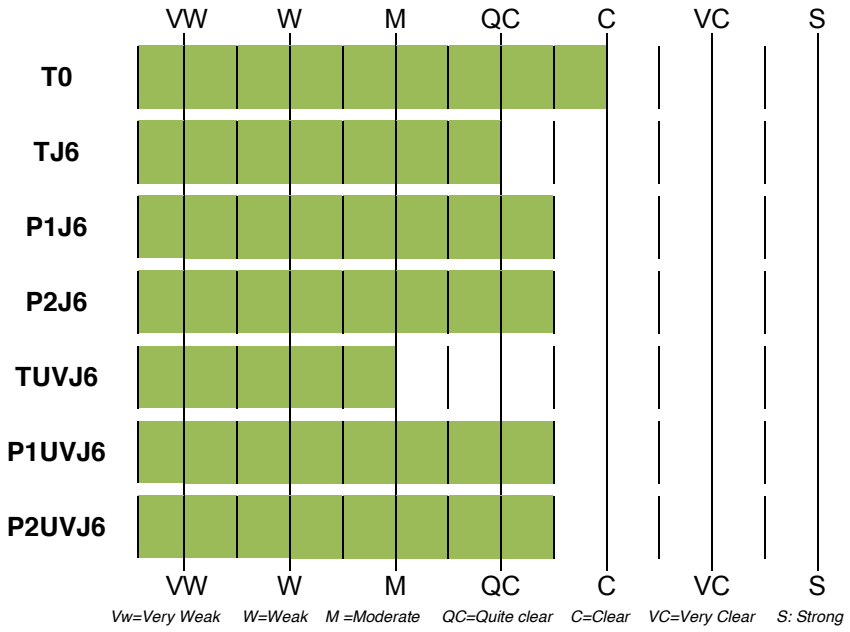
Effect of product application on oxidized proteins content, compared to the batch TUVJ6:

- **The product P1** induces a quite clear decrease
- **The product P2** induces a moderate decrease.

3. Glutathione reductase

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

The staining of gluthatione reductase in the epidermis of the other batches is shown here below:



On D6,
On the **batch TJ6**, the expression of gluthatione reductase is quite clear in the epidermis.

Effect of product application on gluthatione reductase expression, compared to the batch TJ6:

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

The UV1 irradiation induces a moderate decrease of gluthatione reductase expression in the epidermis (TUVJ6 *vs* TJ6).

Effect of product application on gluthatione reductase expression, compared to the batch TUVJ6:

- **The product P1** induces a quite clear increase
- **The product P2** induces a quite clear increase.

CONCLUSION

According to these experimental conditions, compared to the batches on day 6 without or with UV (TJ6 or TUVJ6):

vs T or TUV on day 6		**P1**	**P2**
Cell viability	*vs TJ6*	↔	↔
	vs TUVJ6	↔	Good protection
Oxidized proteins	*vs TJ6*	↔	↔
	vs TUVJ6	↘↘↘	↘↘
Glutathione reductase	*vs TJ6*	↗	↗
	vs TUVJ6	↗↗↗	↗↗↗

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

The product **Le Visage Emulsion Equilibrante** (P1) shows a **quite good anti-oxydant activity** by reducing the UV-induced oxidized proteins in the papillary dermis and inhibiting the UV-induced decrease of gluthatione reductase expression in the epidermis.

The product **Suncream SPF 25** (P2) shows a **good anti-oxydant activity** by reducing the UV-induced oxidized proteins in the papillary dermis and inhibiting the UV-induced decrease of gluthatione reductase expression in the epidermis. In addition it prevents totally from the epidermal alterations induces by the UV.

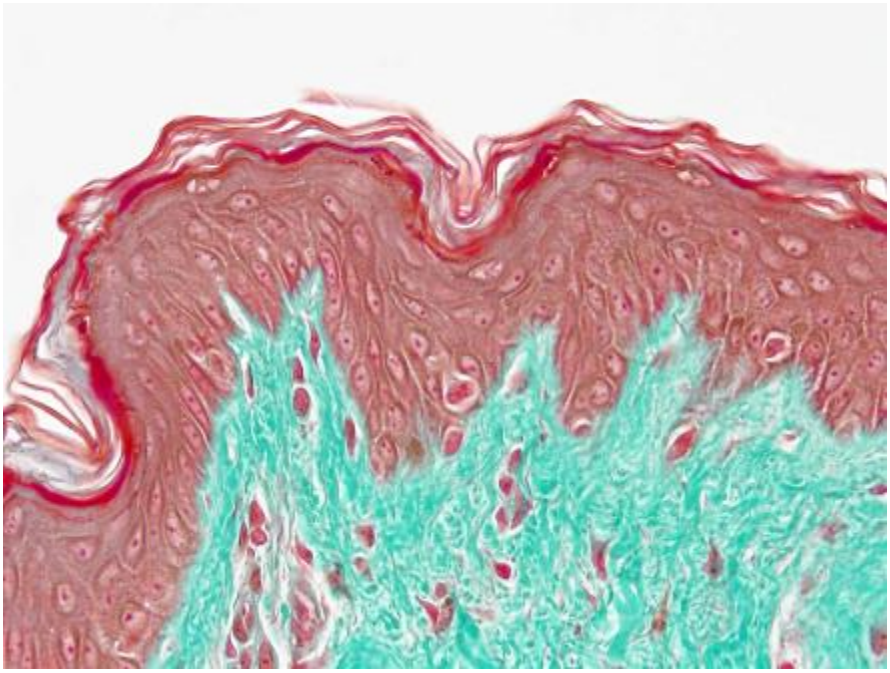
APPENDIXES

1. Glossary of histological terminology

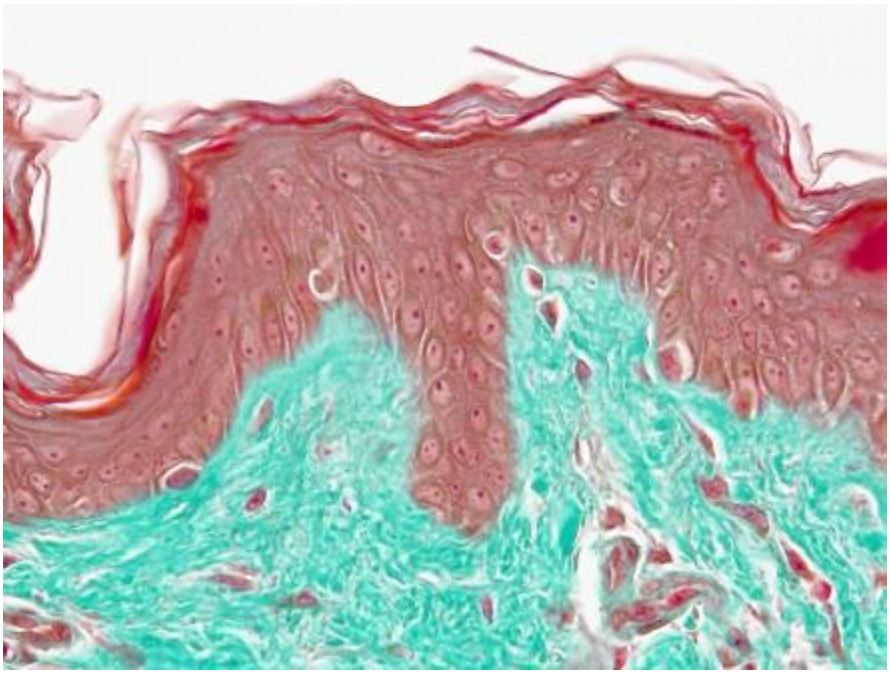
Parakeratosis	Abnormal keratinisation in the granular layer (the last of the epidermal living layers), characterized by the persistence of nuclei in the keratinized layers
Pyknotic nuclei	Condensation of the nucleus content, the nucleus is retracted and hyper stainable ⇒ *Nucleus degeneration leading to cellular necrosis*
Karyolitic nuclei	Dissolution of the components of the nucleus, which becomes less stainable then invisible ⇒ *Nucleus degeneration leading to cellular necrosis*
Cellular oedema	Swelling of the cell due to an accumulation or excess of liquid
Spongiosis	Inter cellular oedema without breaking of the desmosomal links.
Acantholysis	Inter cellular oedema with breaking of the desmosomal links.
Epidermal acanthosis	Increase in epidermal thickness due to the increase in the number of cellular layers (hyperplasic acanthosis) or an increase in the size of keratinocytes (hypertrophic acanthosis).
Diskeratosic cell	Cell with a pyknotic nucleus and acidophilic (red) cytoplasm: keratinocyte maturation defect or cell undergoing apoptosis.
Cleavage of the dermal-epidermal junction	Proteolysis of one of the components of the dermal-epidermal junction

2. General morphology

Blank batch on D0 (T0)



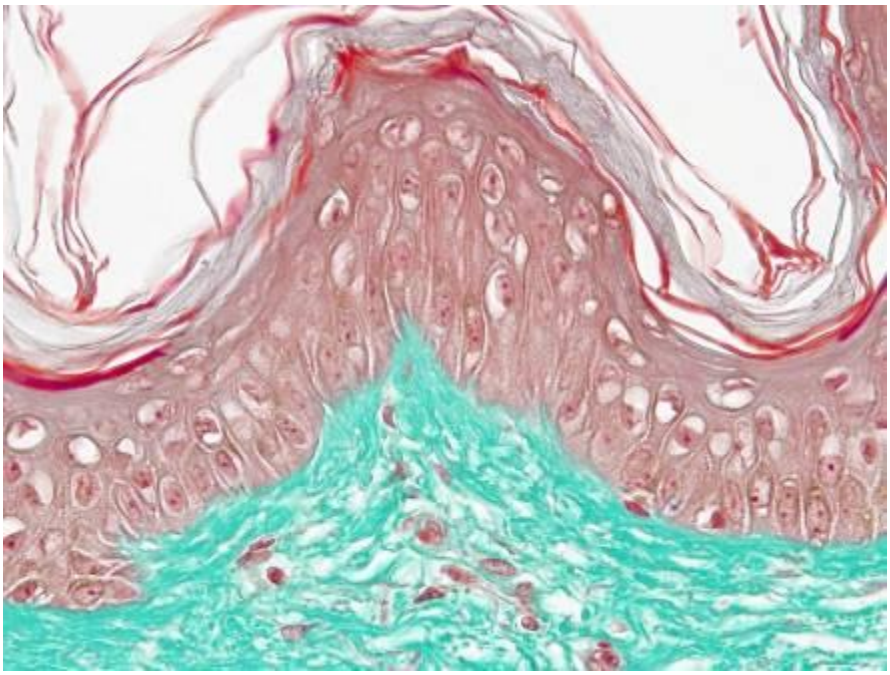
T0_5-5 17E3754 Trich 50 µm



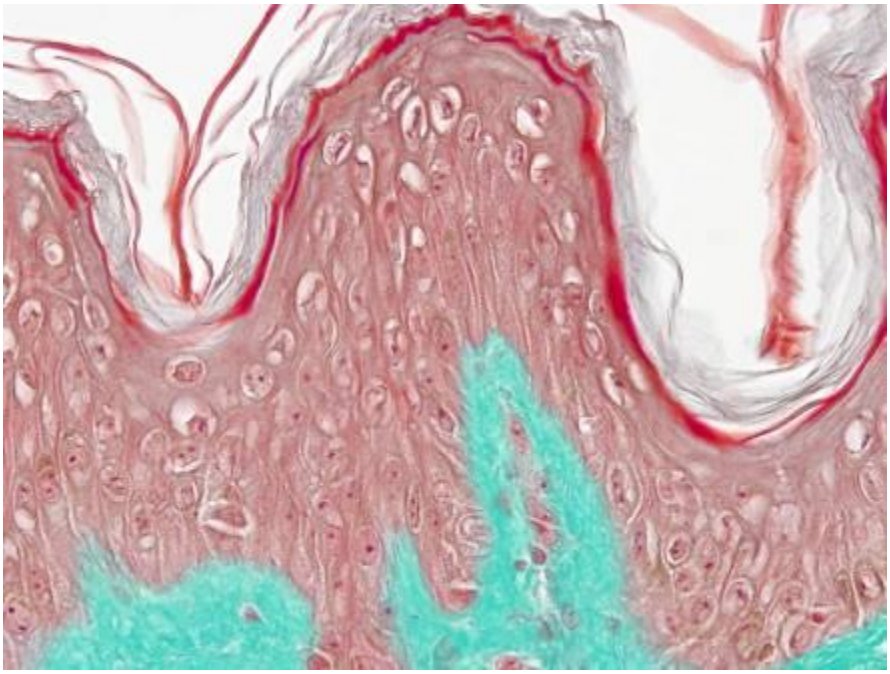
T0_3-3 17E3754 Trich 50 µm

General morphology

Blank batch on D6 (TJ6)



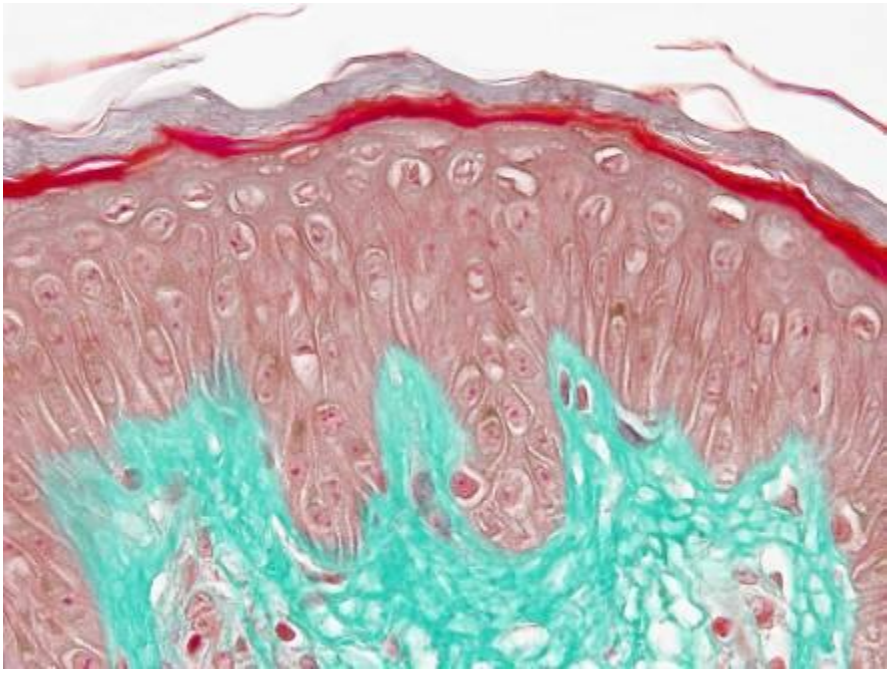
TJ6_4-31 17E3754 Trich 50 µm



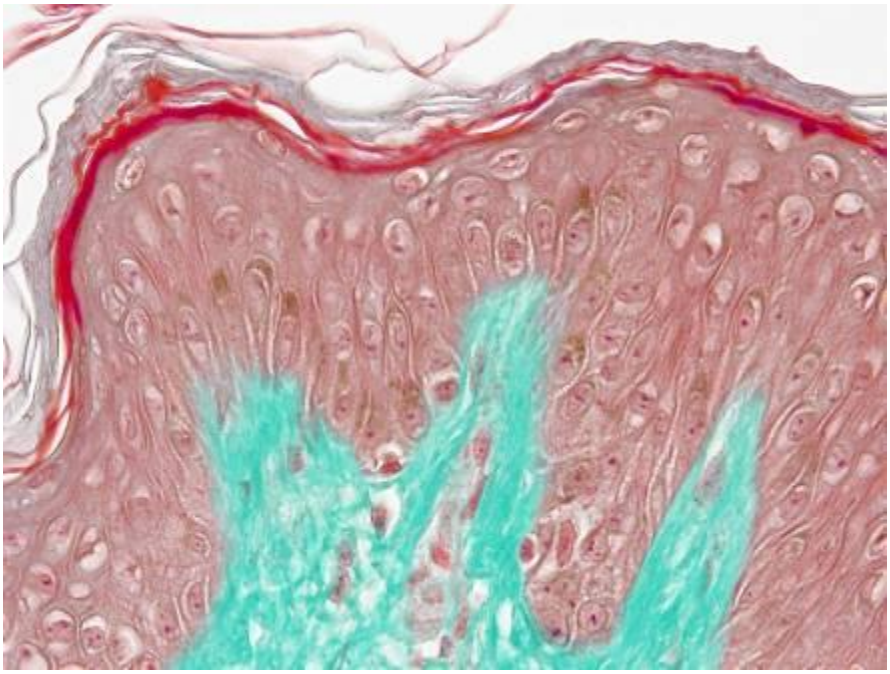
TJ6_5-32 17E3754 Trich 50 µm

General morphology

Batch P1 on D6 (P1J6)



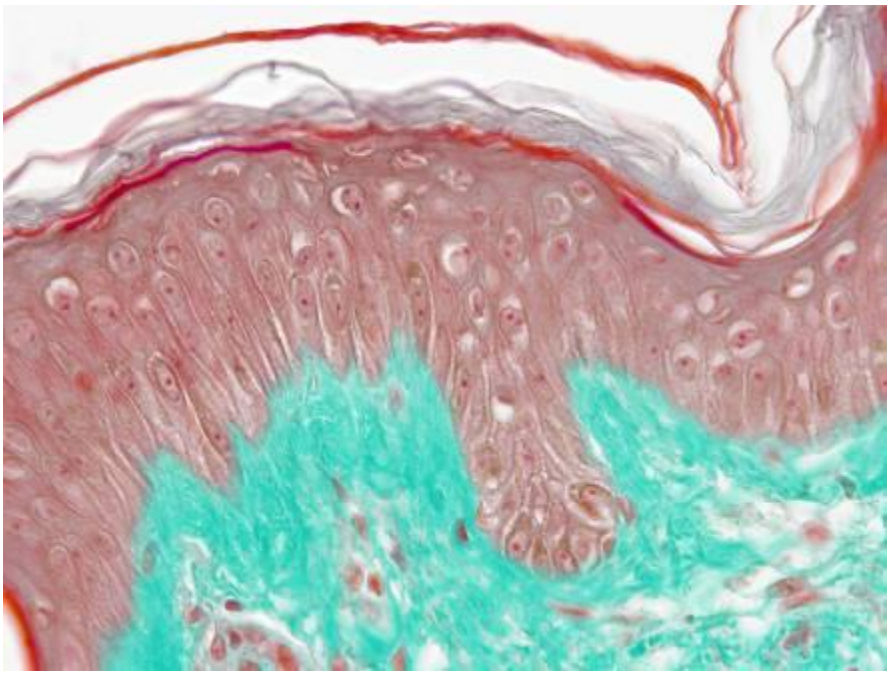
P1J6_7-43 17E3754 Trich 50 µm



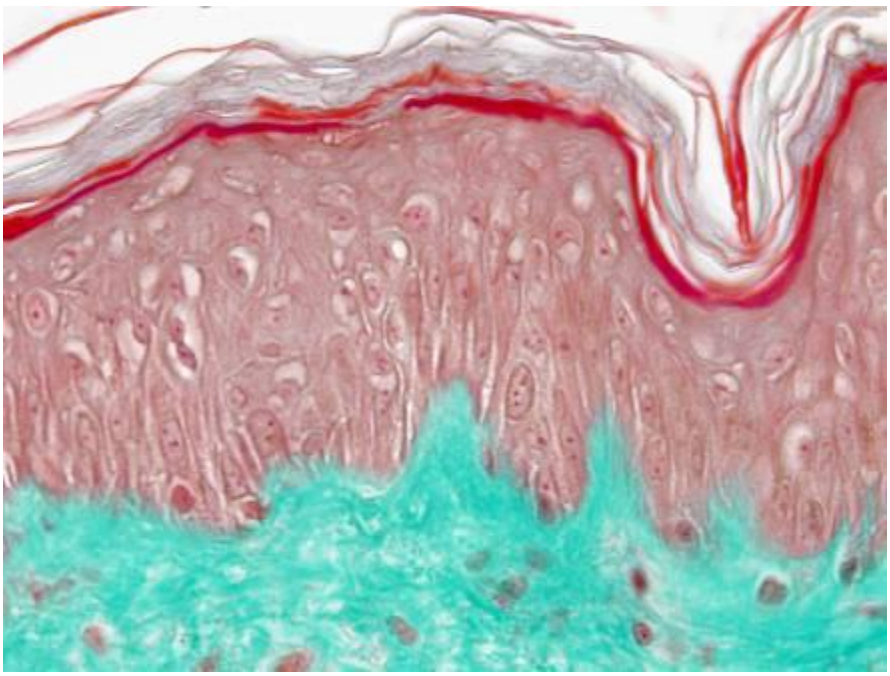
P1J6_8-44 17E3754 Trich 50 µm

General morphology

Batch P2 on D6 (P2J6)



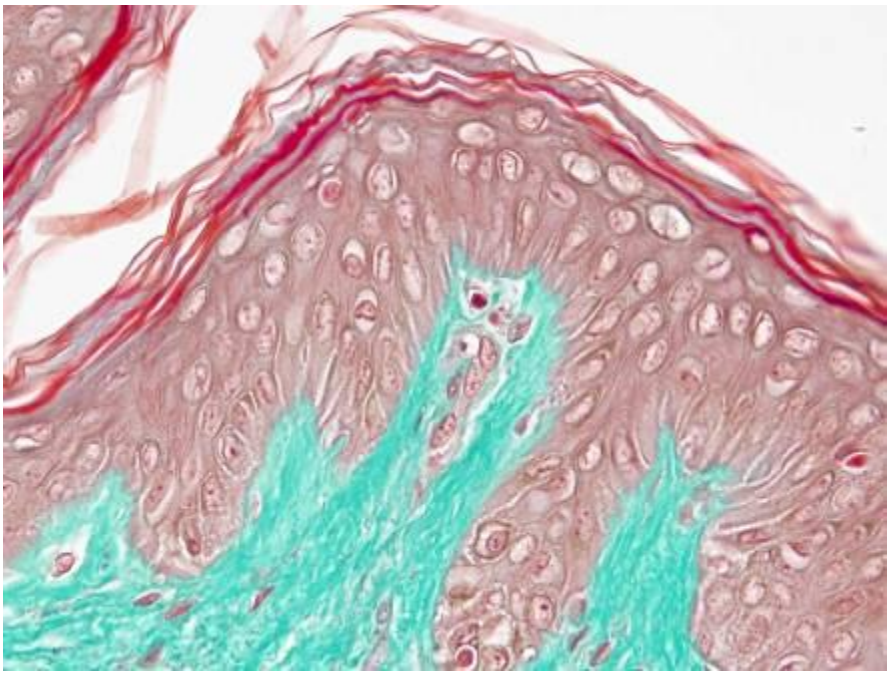
P2J6_4-49 17E3754 Trich 50 µm



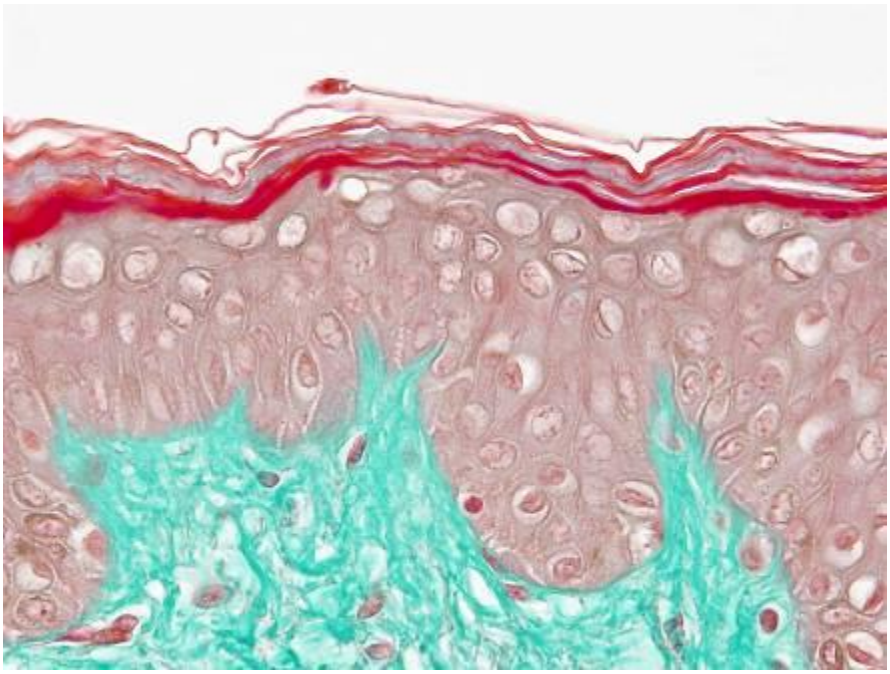
P2J6_7-52 17E3754 Trich 50 µm

General morphology

Batch TUV on D6 (TUVJ6)



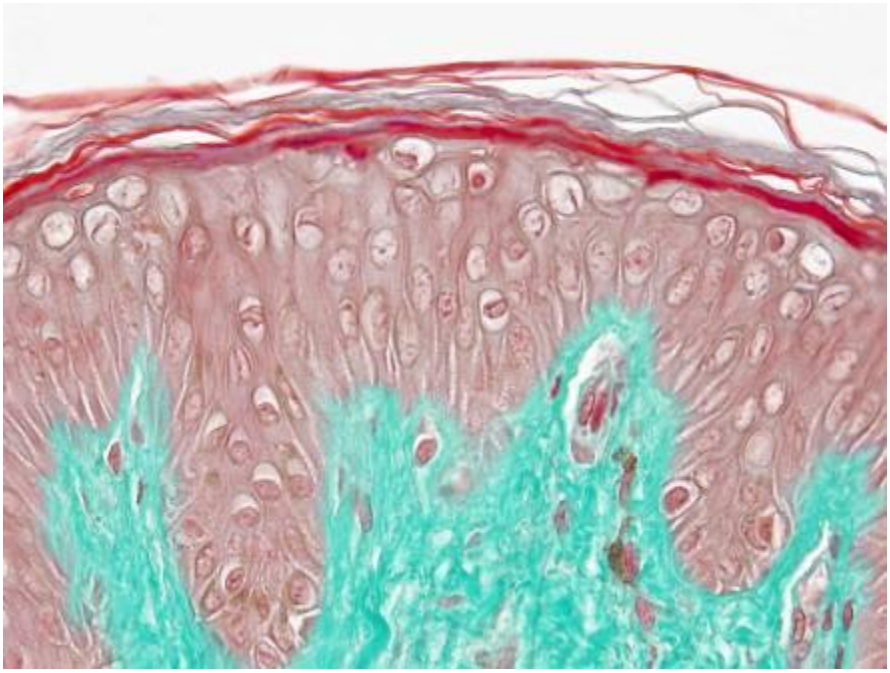
TUVJ6_9-63 17E3754 Trich 50 µm



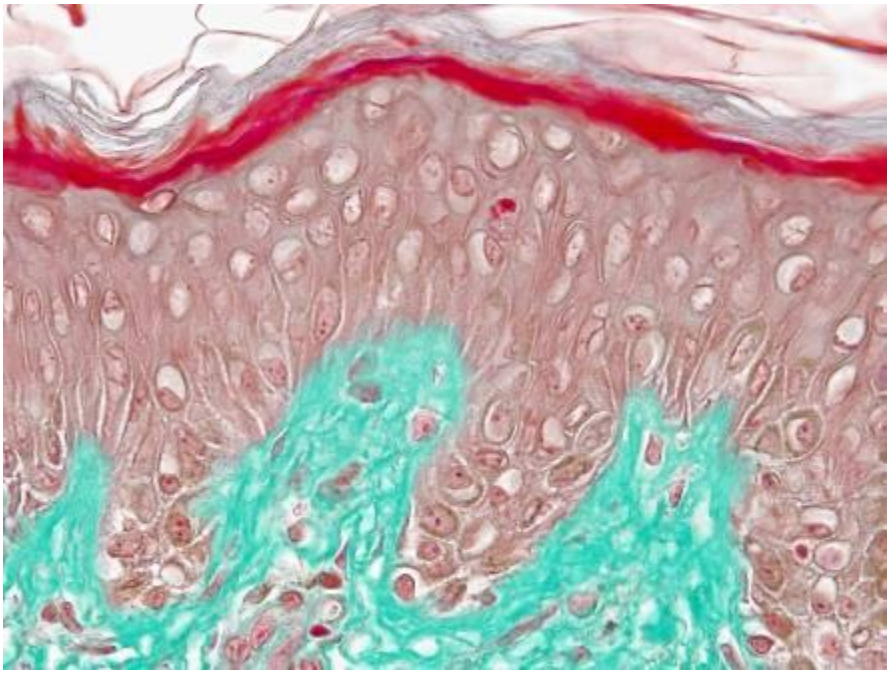
TUVJ6_7-61 17E3754 Trich 50 µm

General morphology

Batch P1UV on D6 (P1UVJ6)



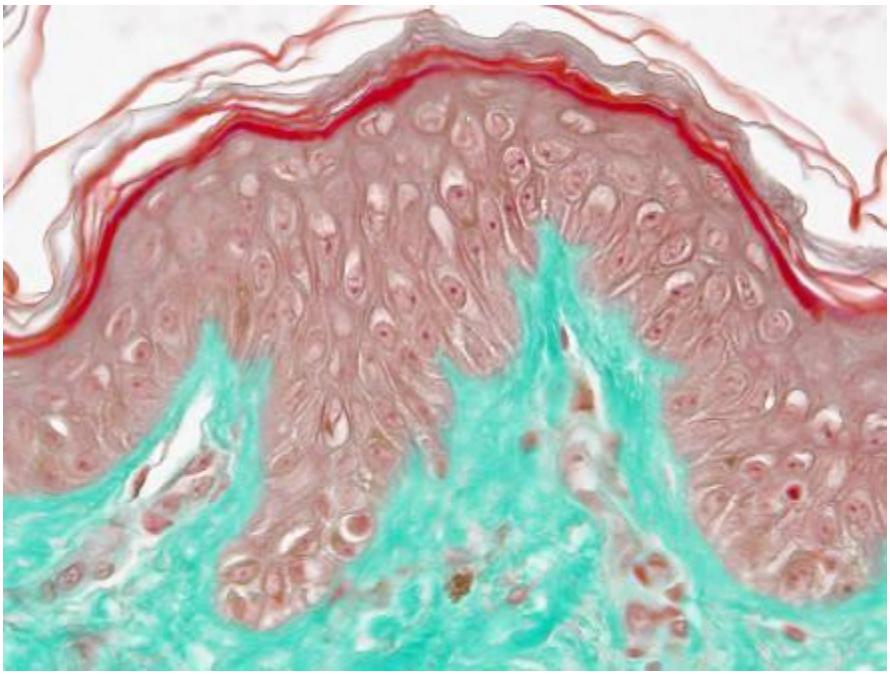
P1UVJ6_6-69 17E3754 Trich 50 µm



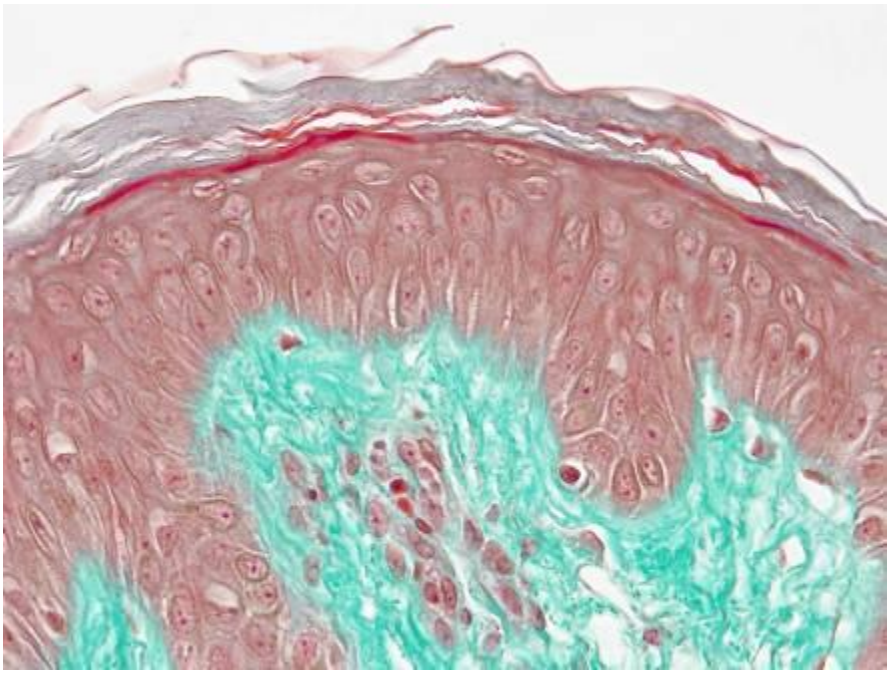
P1UVJ6_1-64 17E3754 Trich 50 µm

General morphology

Batch P2UV on D6 (P2UVJ6)



P2UVJ6_2-20 17E3754 Trich 50 µm



P2UVJ6_8-26 17E3754 Trich 50 µm

3. Oxidized proteins

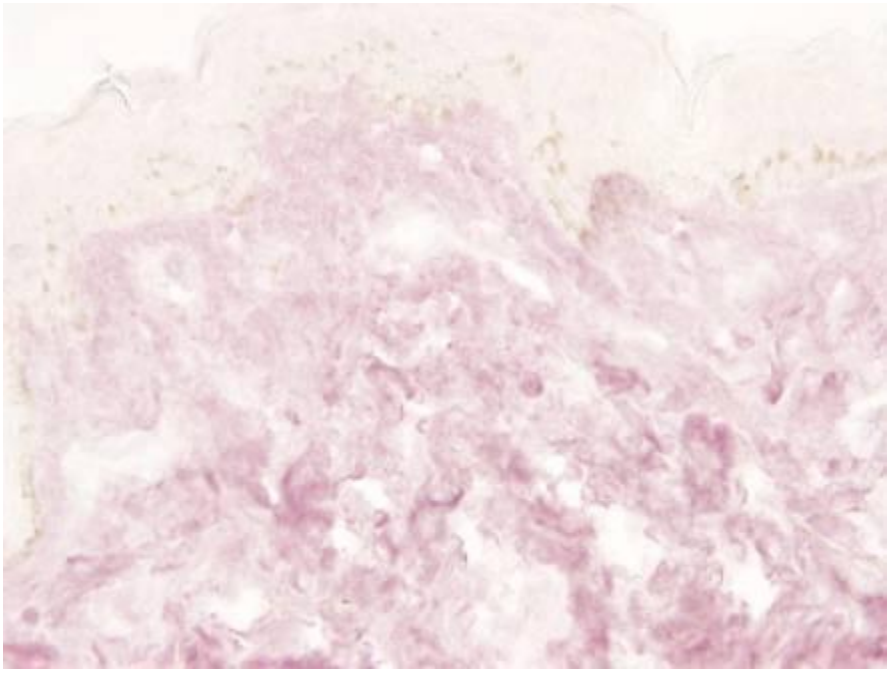
Negative control without DNPH

Oxidized proteins

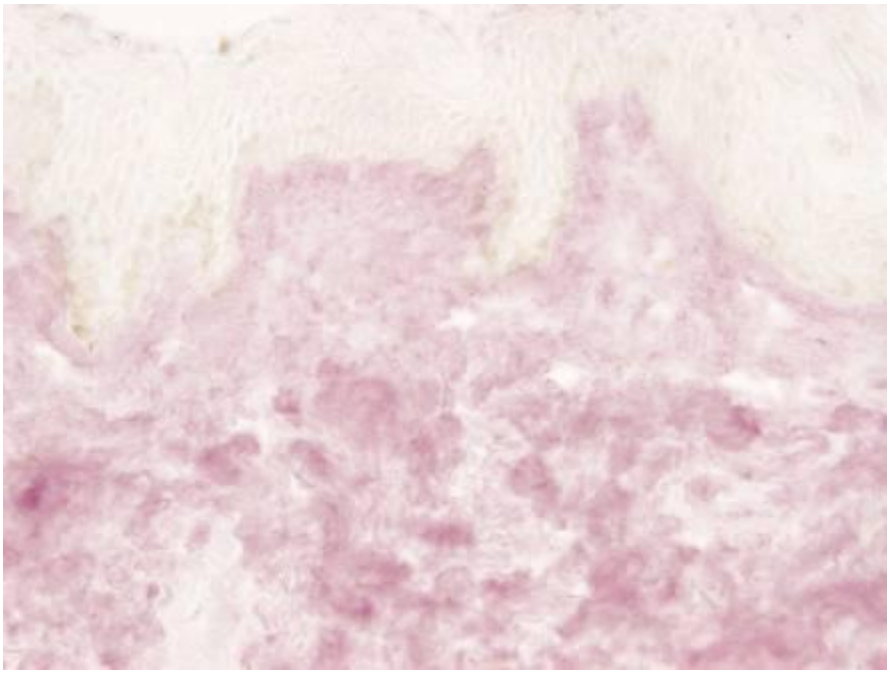
Negative control without the antibody anti-DNP

Oxidized proteins

Blank batch on D0 (T0)



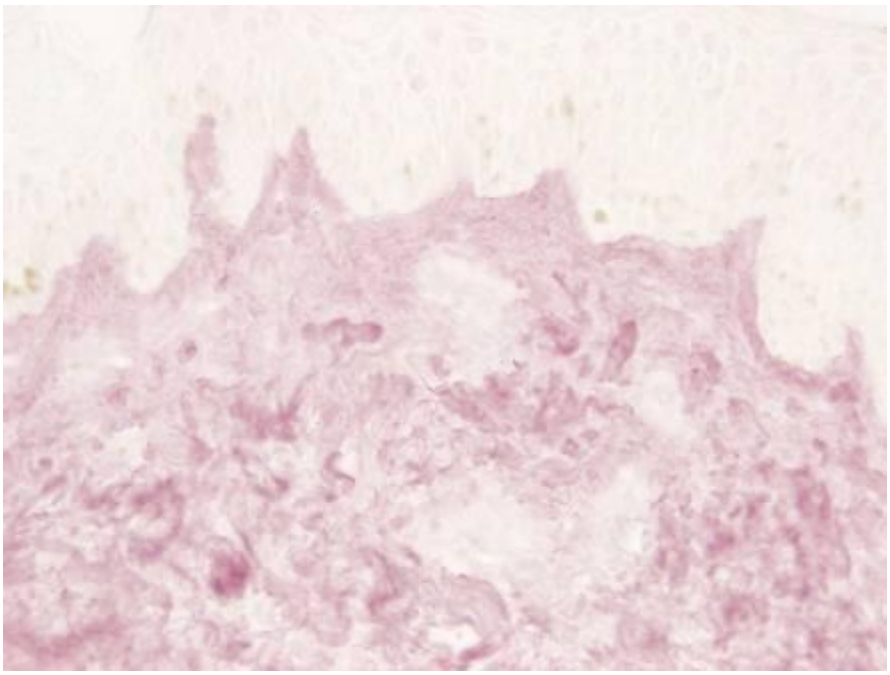
T0_1-2 17E3754 Prot Oxydées 100 µm



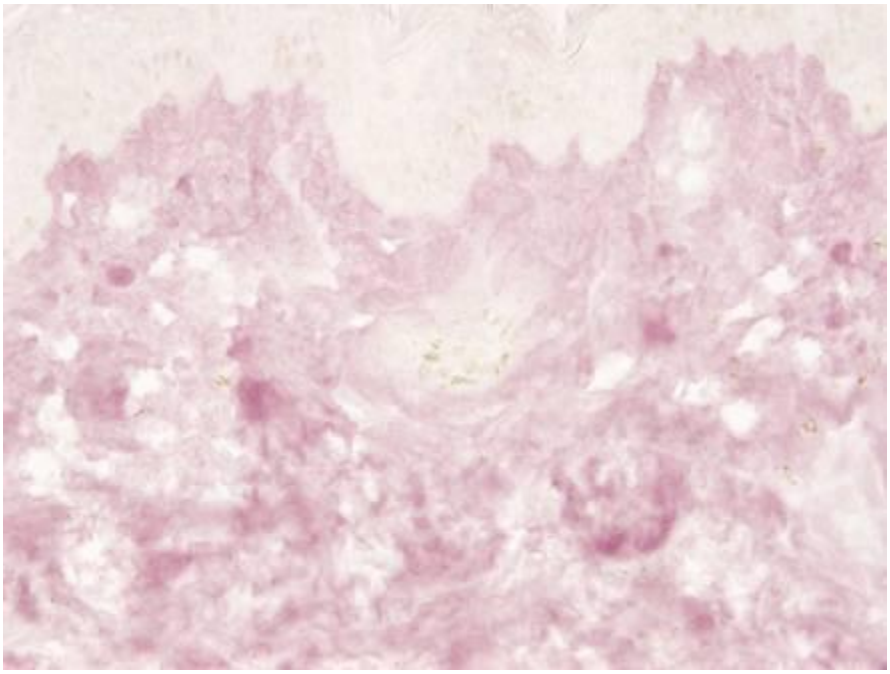
T0_9-10 17E3754 Prot Oxydées 100 µm

Oxidized proteins

Blank batch on D6 (TJ6)



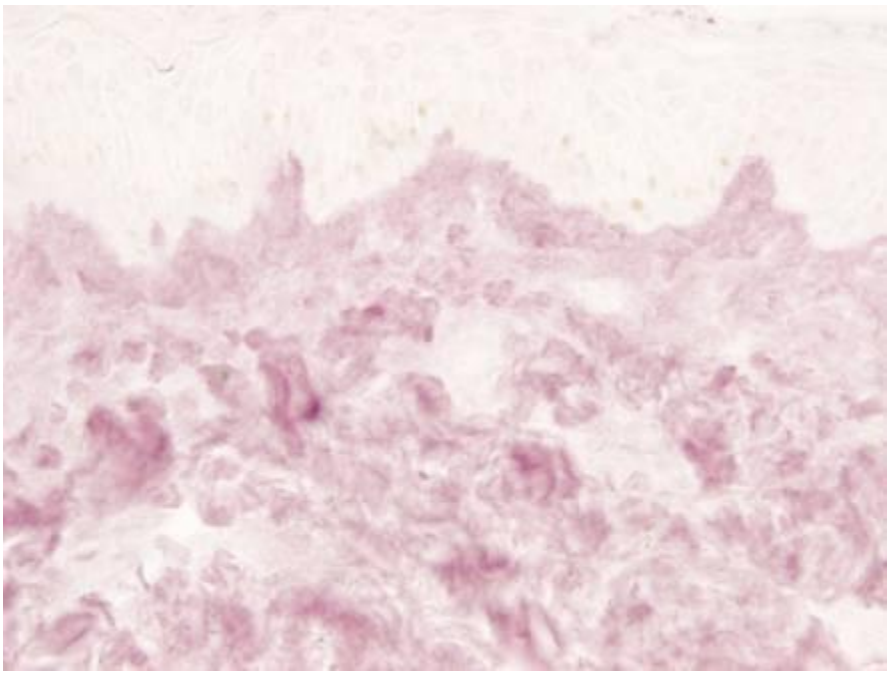
TJ6_2-12 17E3754 Prot Oxydées 100 µm



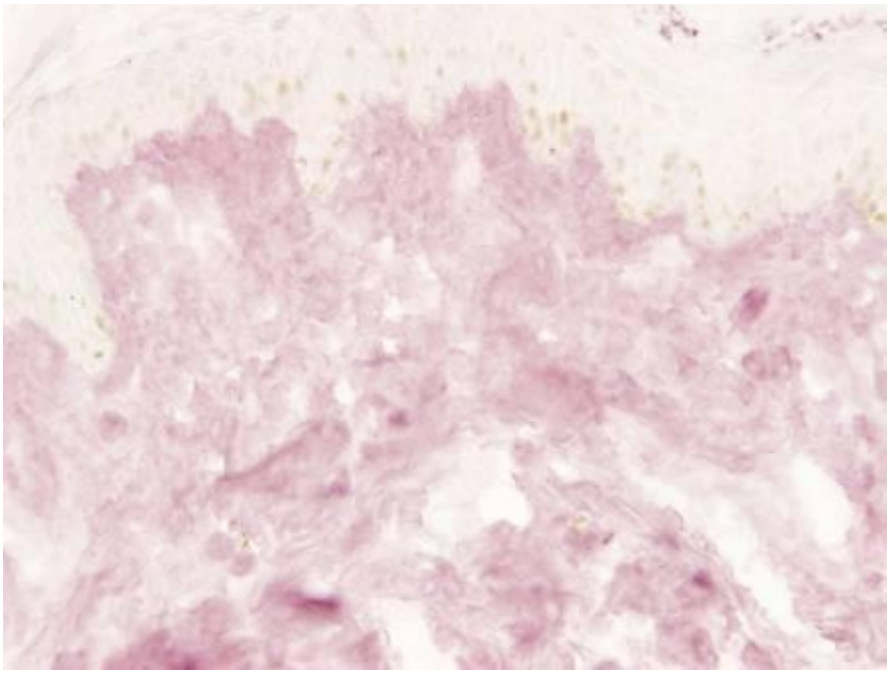
TJ6_4-14 17E3754 Prot Oxydées 100 µm

Oxidized proteins

Batch P1 on D6 (P1J6)



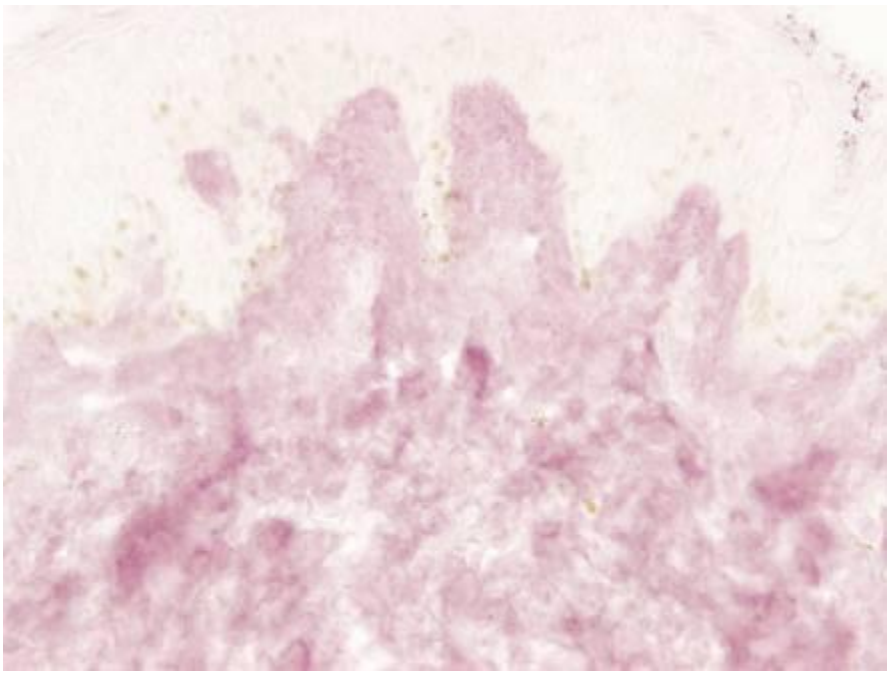
P1J6_1-20 17E3754 Prot Oxydées 100 µm



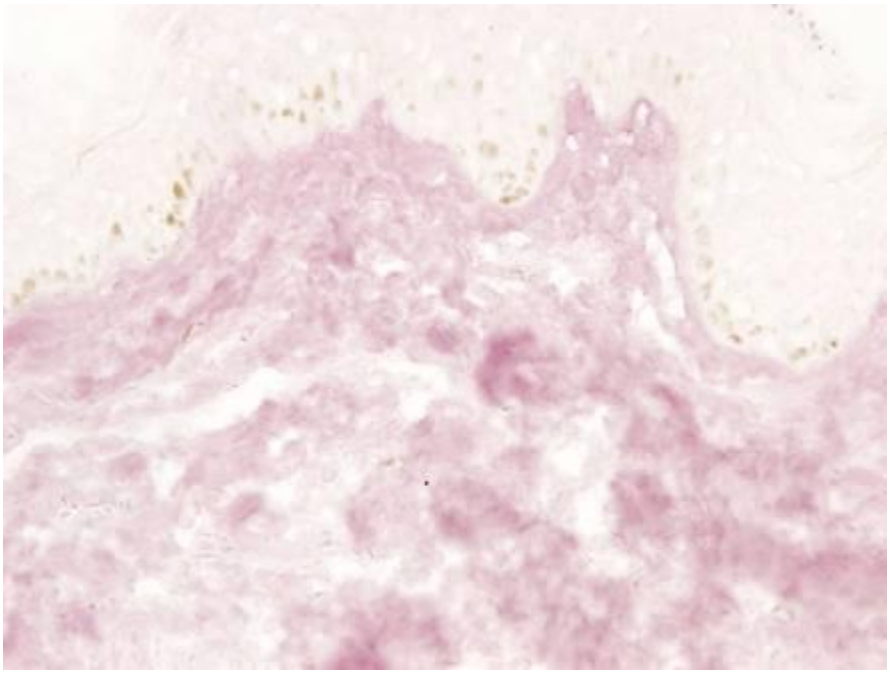
P1J6_4-23 17E3754 Prot Oxydées 100 µm

Oxidized proteins

Batch P2 on D6 (P2J6)



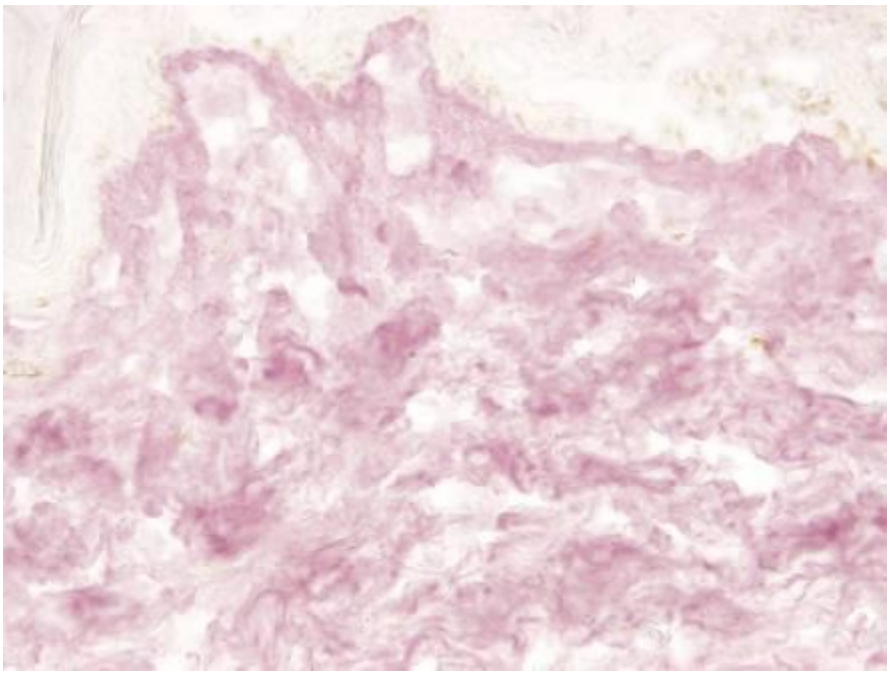
P2J6_12-67 17E3754 Prot Oxydées 100 µm



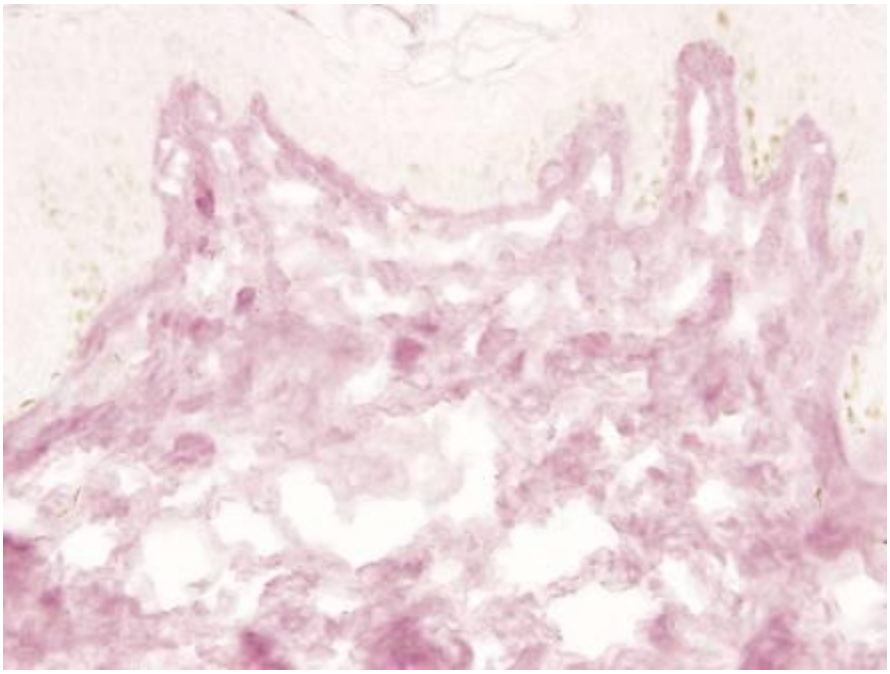
P2J6_6-34 17E3754 Prot Oxydées 100 µm

Oxidized proteins

Batch TUV on D6 (TUVJ6)



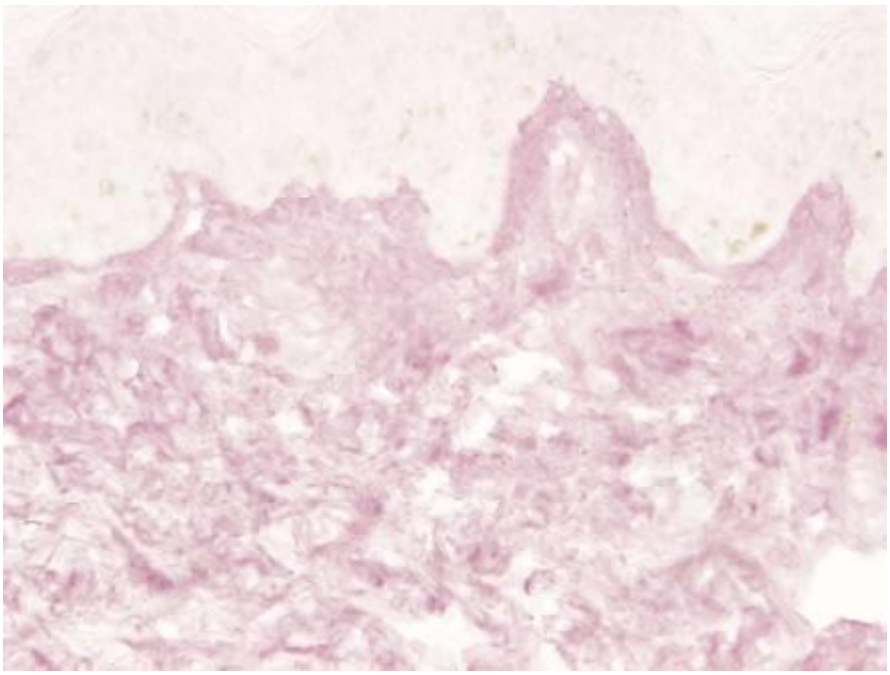
TUVJ6_2-39 17E3754 Prot Oxydées 100 µm



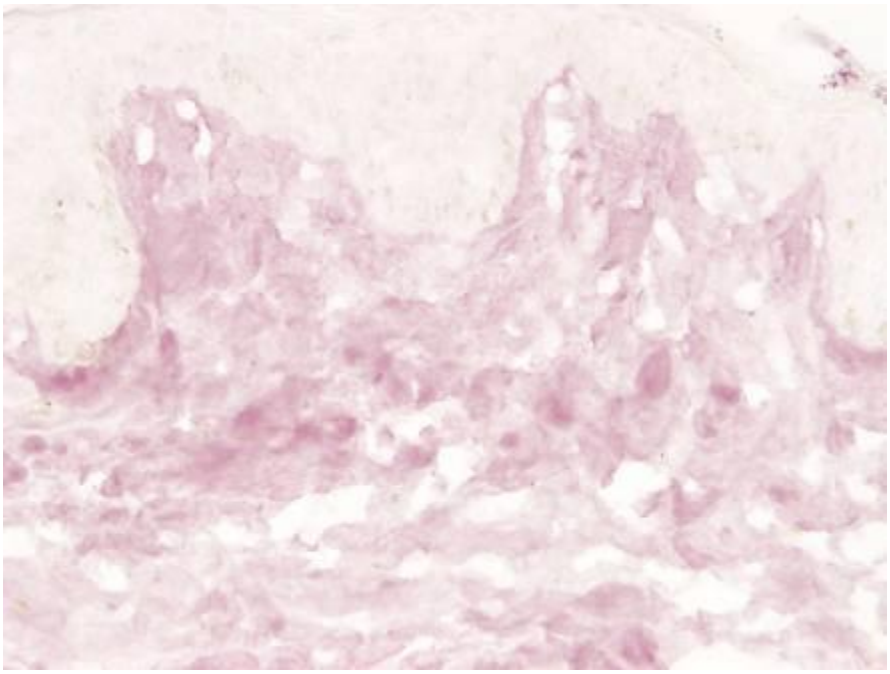
TUVJ6_3-40 17E3754 Prot Oxydées 100 µm

Oxidized proteins

Batch P1UV on D6 (P1UVJ6)



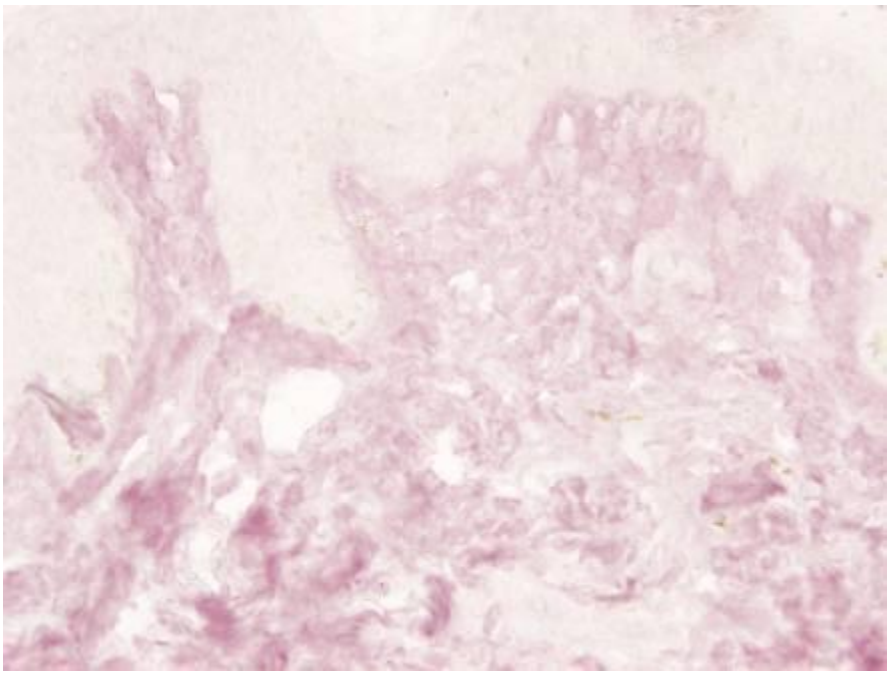
P1UVJ6_8-54 17E3754 Prot Oxydées 100 µm



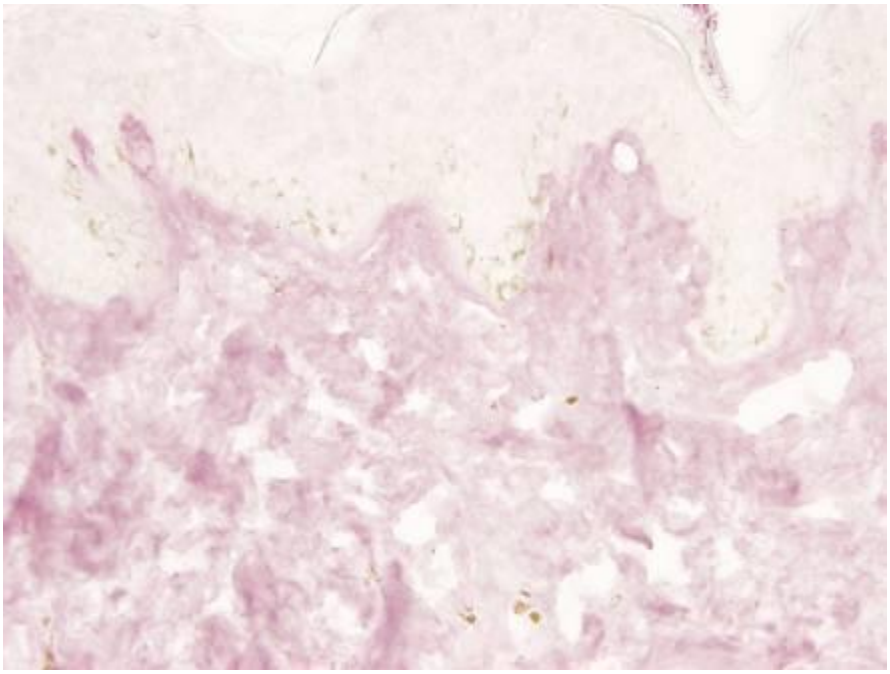
P1UVJ6_6-52 17E3754 Prot Oxydées 100 µm

Oxidized proteins

Batch P2UV on D6 (P2UVJ6)



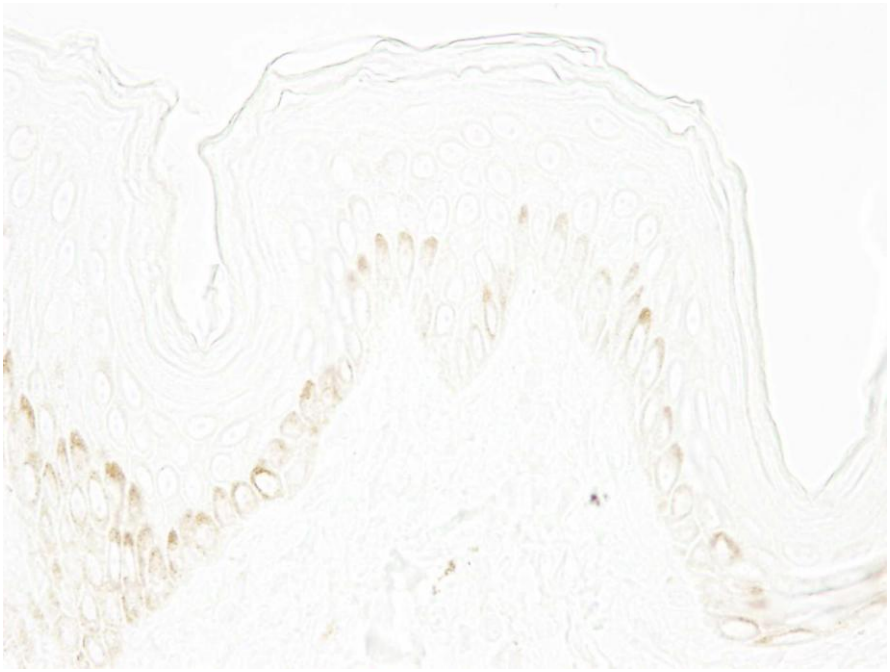
P2UVJ6_8-63 17E3754 Prot Oxydées 100 µm



P2UVJ6_5-60 17E3754 Prot Oxydées 100 µm

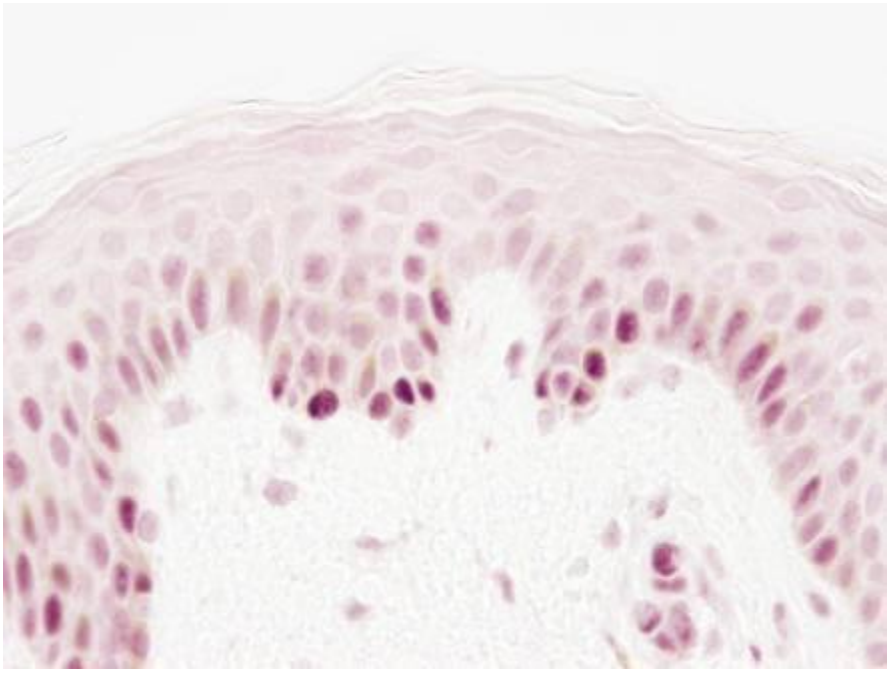
4. Gluthatione Reductase

Negative control without the antibody anti-gulthatione reductase

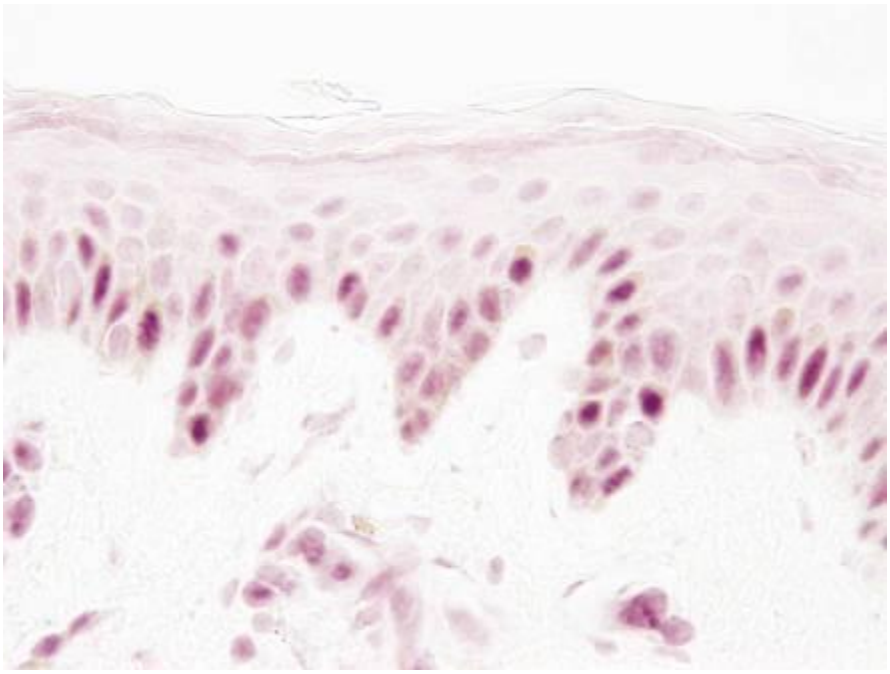


Gluthatione Reductase

Blank batch on D0 (T0)



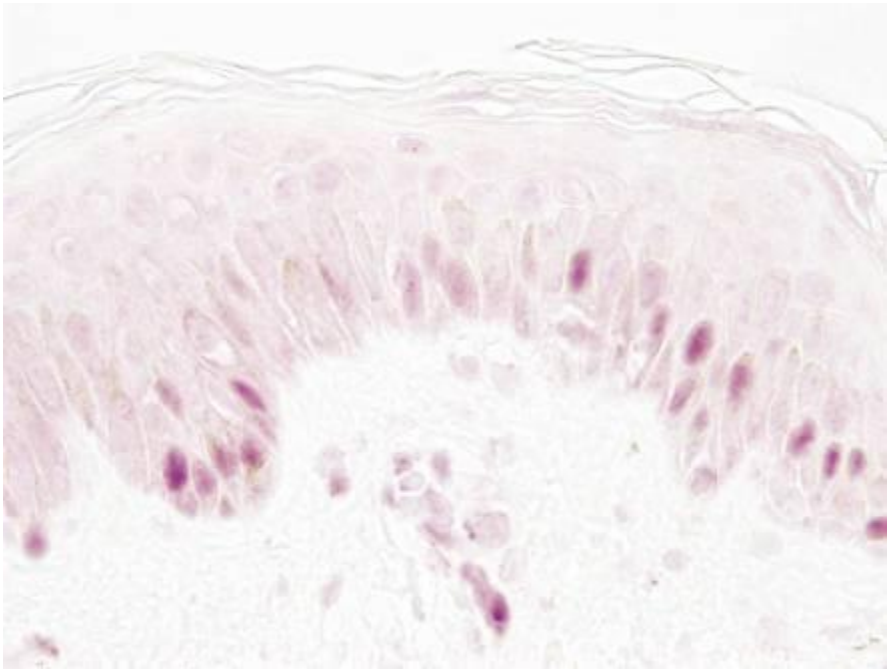
T0_3-3 17E3754 Gluth Reduct 50 µm



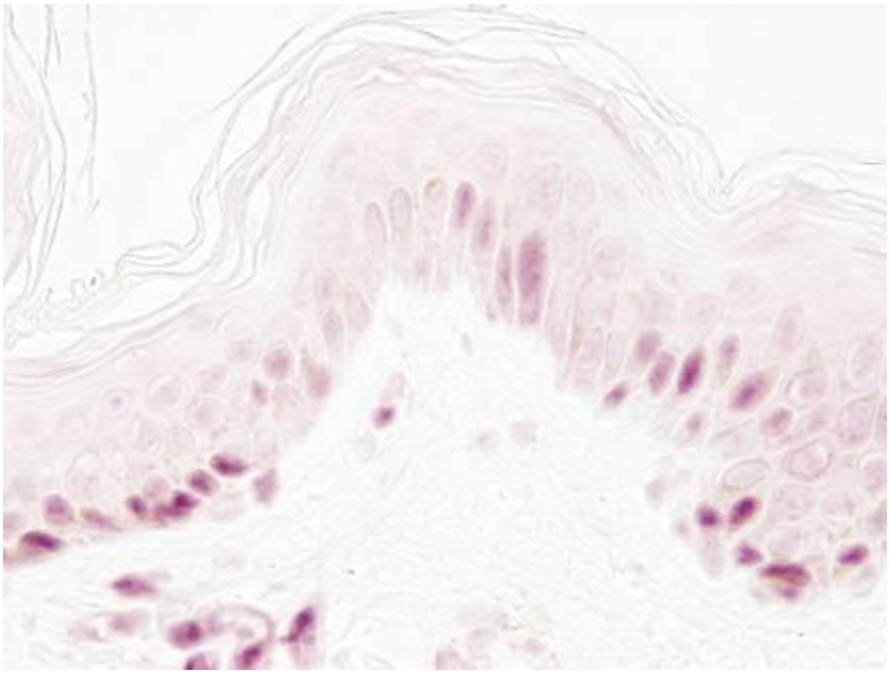
T0_8-8 17E3754 Gluth Reduct 50 µm

Gluthatione Reductase

Blank batch on D6 (TJ6)



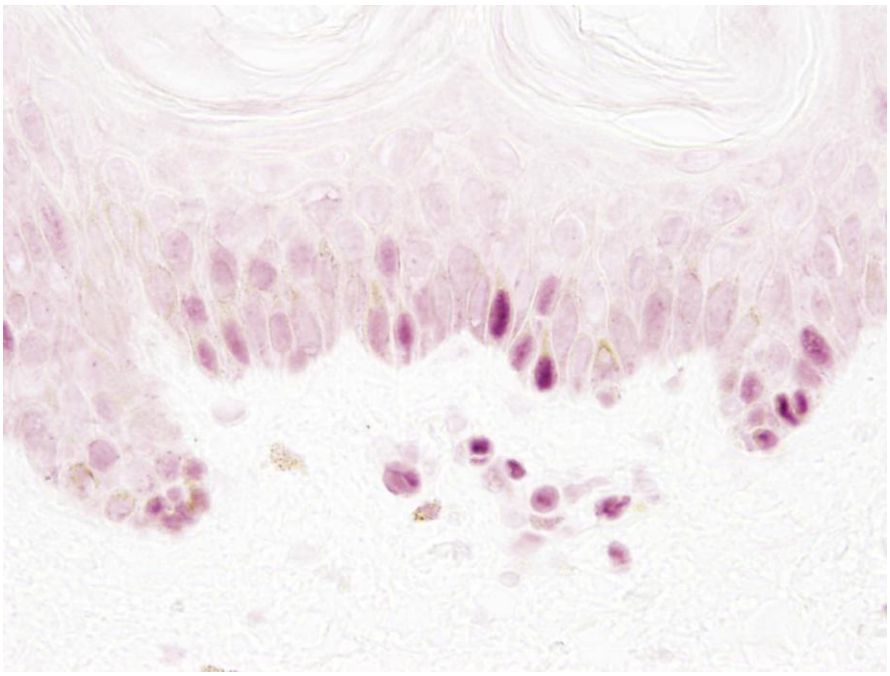
TJ6_9-18 17E3754 Gluth Reduct 50 µm



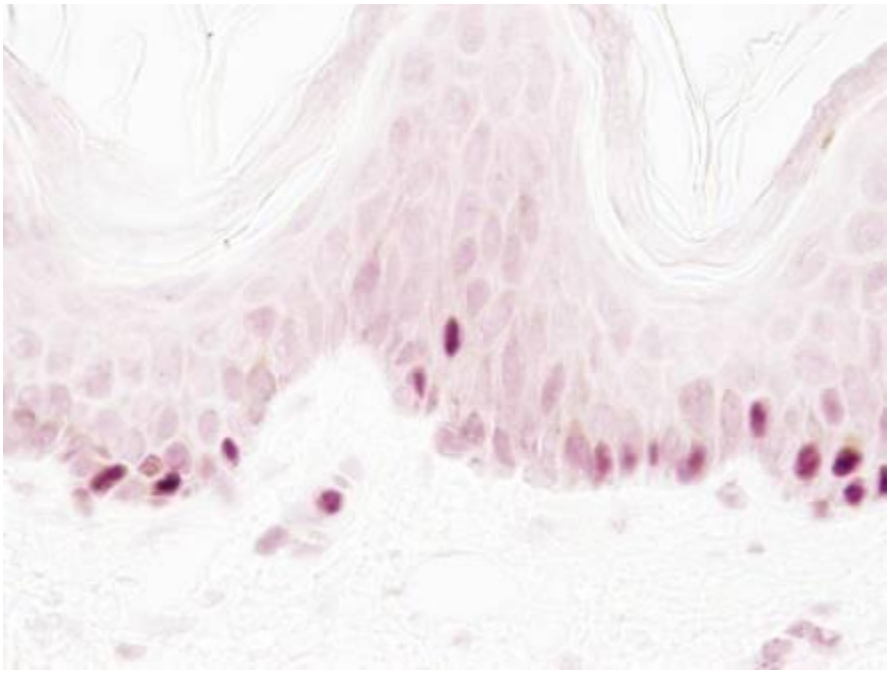
TJ6_10-19 17E3754 Gluth Reduct 50 µm

Gluthatione Reductase

Batch P1 on D6 (P1J6)



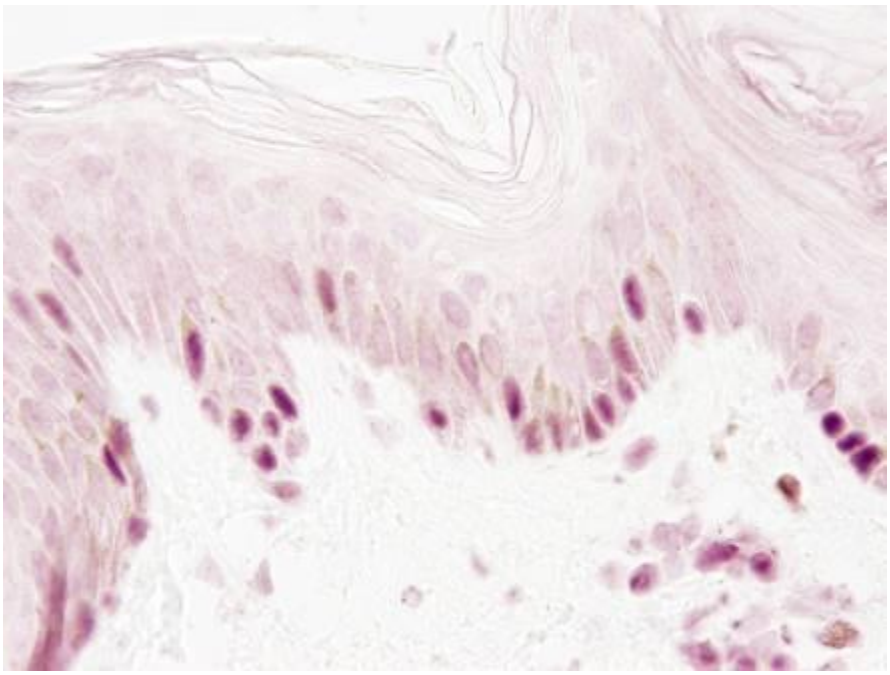
P1J6_2-21 17E3754 Gluth Reduct 50 µm



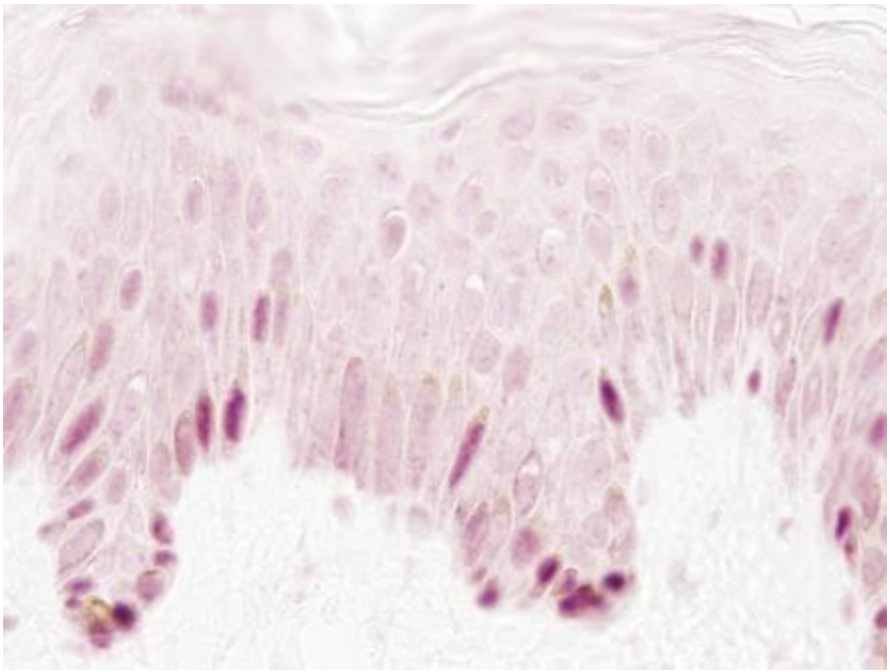
P1J6_6-25 17E3754 Gluth Reduct 50 µm

Gluthatione Reductase

Batch P2 on D6 (P2J6)



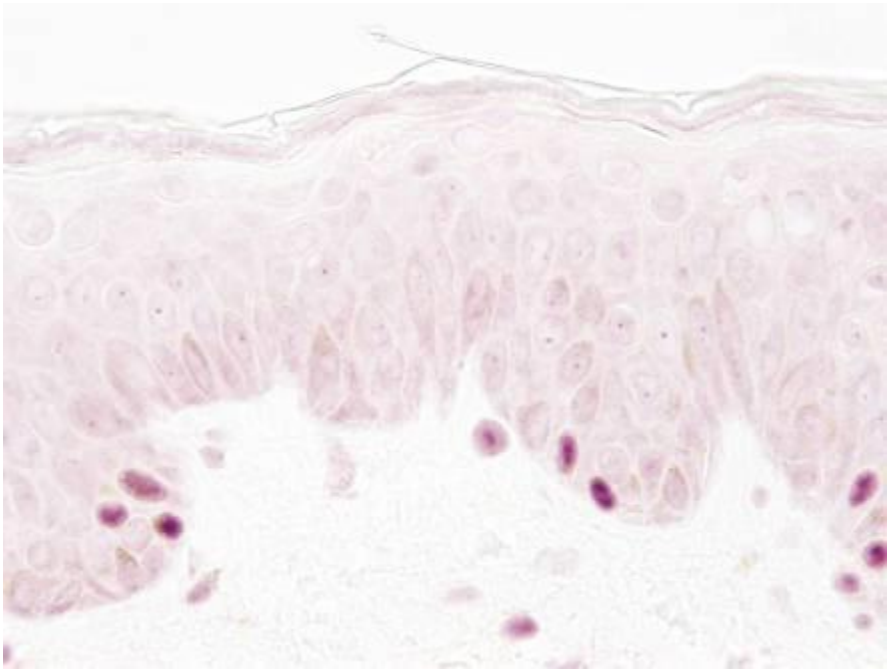
P2J6_5-33 17E3754 Gluth Reduct 50 µm



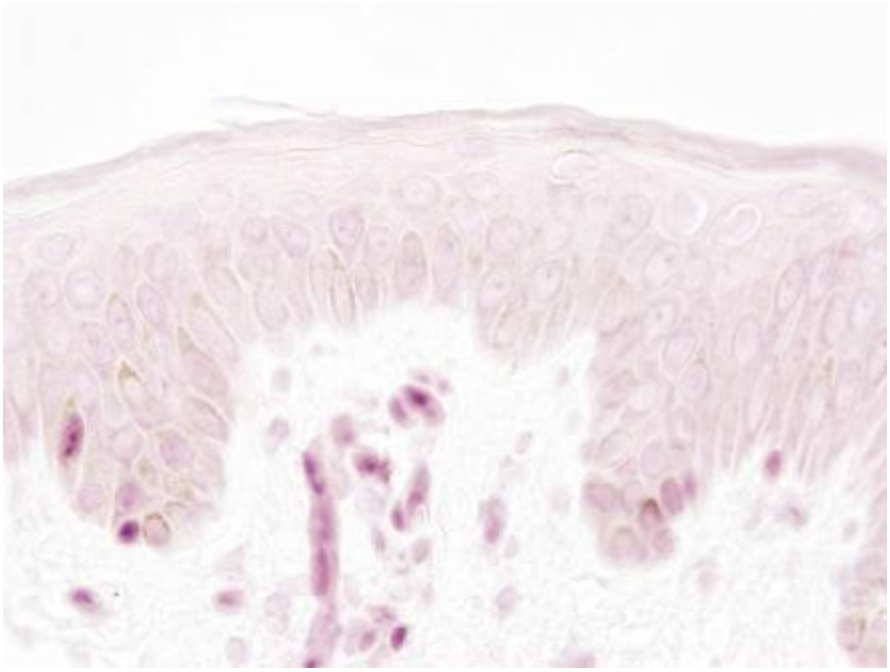
P2J6_6-34 17E3754 Gluth Reduct 50 µm

Gluthatione Reductase

Batch TUV on D6 (TUVJ6)



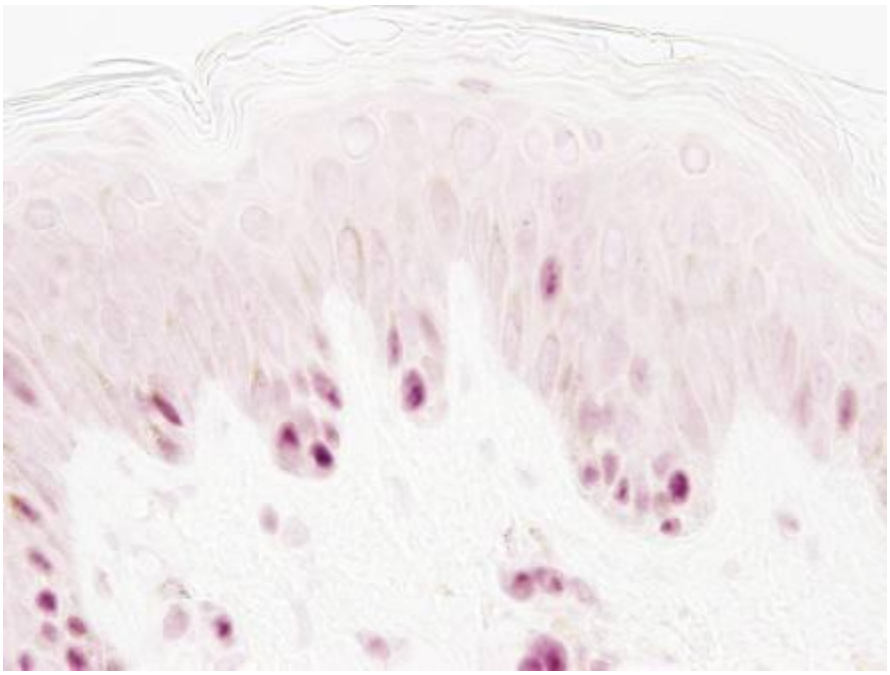
TUVJ6_10-72 17E3754 Gluth Reduct 50 µm



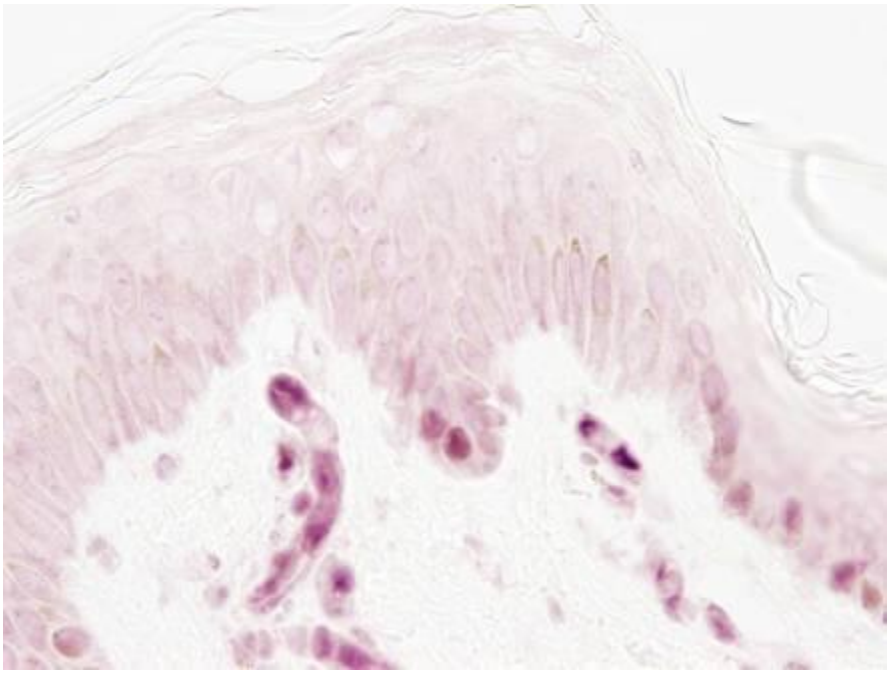
TUVJ6_7-69 17E3754 Gluth Reduct 50 µm

Gluthatione Reductase

Batch P1UV on D6 (P1UVJ6)



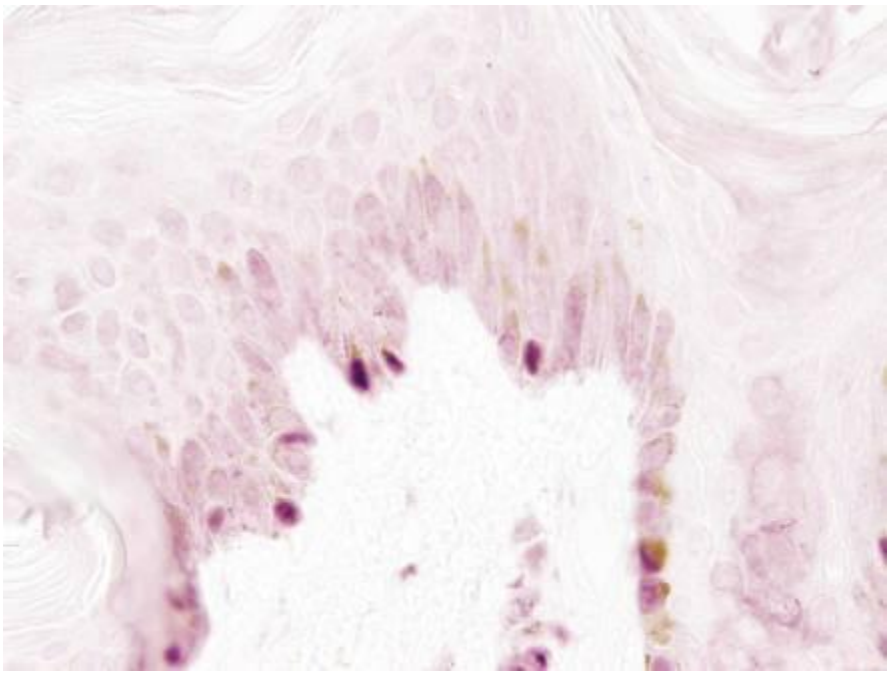
P1UVJ6_8-55 17E3754 Gluth Reduct 50 µm



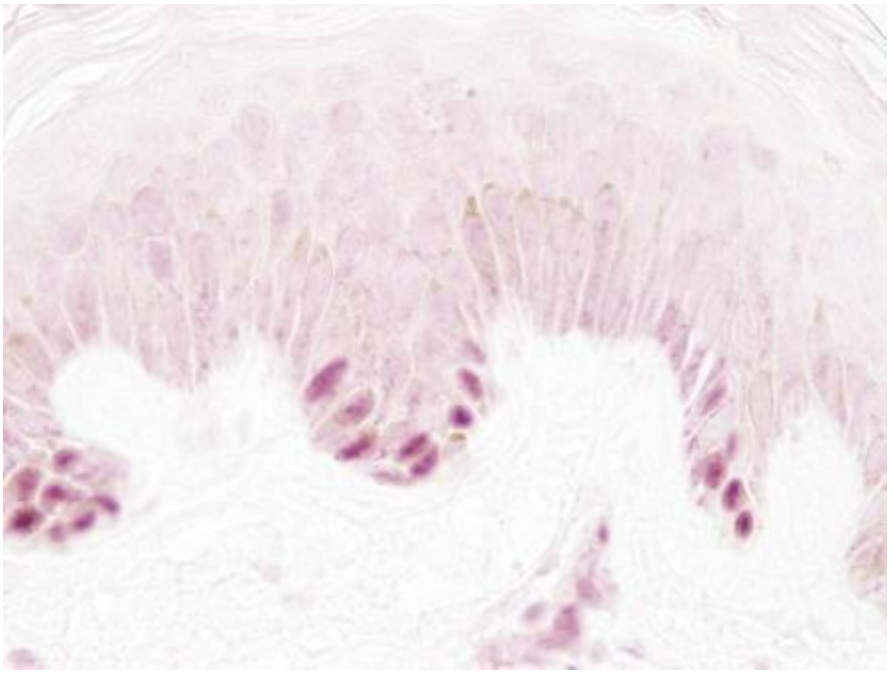
P1UVJ6_7-54 17E3754 Gluth Reduct 50 µm

Gluthatione Reductase

Batch P2UV on D6 (P2UVJ6)



P2UVJ6_6-62 17E3754 Gluth Reduct 50 µm



P2UVJ6_4-60 17E3754 Gluth Reduct 50 µm

DEVIATIONS

None.

ATTESTATIONS

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 reports to the study director	Dates of reports to the director
Internal	21/06/2012	Histology laboratory	03/07/2012	03/07/2012
Internal	12/04/2016	Management of tested prodcuts	Writing	Writing
Internal	12/04/2016	Management of reagent and antibody	Writing	Writing

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 delivering the study report to the client.

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

The fluorescent immunostained slides will be kept during one month at minus 20°C.

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 one year 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 : Le Visage Emulsion Equilibrante ref. lab-00733.6
P2 : Suncream SPF 25 ref. lab-00591.16

Model

Human living skin explants.
Topical treatment (2 mg/explants) with the products on D0, D1, D2 and D5.
On D5, the explants of the batches "UV", "P1UV" and "P2UV" were irradiated by UV-A+ UV-B with a dose of 18 J/cm^2 of UV A and 0,6 J/cm^2 of UV-B corresponding both to 4 MED (minimal erythemal dose).
Sampling of skin explants on D6.
Evaluated parameters: cell viability, oxidized proteins content, gluthatione reductase expression.

Results/ conclusion

According to these experimental conditions, compared to the batches on day 6 without or with UV (TJ6 or TUVJ6):

vs T or TUV on day 6		**P1**	**P2**
Cell viability	*vs TJ6*	↔	↔
	vs TUVJ6	↔	Good protection
Oxidized protein	*vs TJ6*	↔	↔
	vs TUVJ6	↘↘↘	↘↘
Glutathione reductase	*vs TJ6*	↗	↗
	vs TUVJ6	↗↗↗	↗↗↗

Decrease		**Increase**			
↘	Slight	↗		#	significant with p<0.1 (90%)
↘↘	Moderate	↗↗		*	significant with p<0.05 (95%)
↘↘↘	Quite clear	↗↗↗		**	significant with p<0.01 (99%)
↘↘↘↘	Clear	↗↗↗↗		ns	non-significant
↘↘↘↘↘	Very clear	↗↗↗↗↗			

The product **Le Visage Emulsion Equilibrante** (P1) shows a **quite good anti-oxydant activity** by reducing the UV-induced oxidized proteins in the papillary dermis and inhibiting the UV-induced decrease of gluthatione reductase expression in the epidermis.

The product **Suncream SPF 25** (P2) shows a **good anti-oxydant activity** by reducing the UV-induced oxidized proteins in the papillary dermis and inhibiting the UV-induced decrease of gluthatione reductase expression in the epidermis. In addition it prevents totally from the epidermal alterations induces by the UV.