OXI-PROTEOME VIEW
STUDY REPORT

PROJECT:	**D03CR2021**
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** This study is totally performed under the responsibility of OXIPROTEOMICS.*
*** All the observations and numerical data collected throughout the study are reported in this document and annexed files. We certify that these data are in accordance with the obtained results.*

EXECUTIVE SUMMARY

The protective efficacy of MS beautilab's product (*POUR MOI SMOKE ALARM DROPS*) against fire-wood pollution-induced damage was evaluated on human skin explants (*ex-vivo*) through the protection of the skin proteome from oxidative damage (carbonylation). Abdominal skin explants were topically treated with the above-mentioned product before exposure (fire-wood pollution). The stress conditions (formaldehyde and particulate matter containing HAPs) have been selected from published data as components of wood smoke emissions [Burning Issues/Clean Air Revival (2001); Timothy V. Larson, US EPA(1993)].

Carbonylated proteins were labeled *in situ* with a specific fluorescent probe and visualized by epifluorescence microscopy. The semi-quantification of carbonyl content was performed by integrating the fluorescence intensity upon normalization with the evaluated area. As expected, a significant increase in oxidative damage (carbonylation) was observed upon fire-wood pollution exposure.

The treatment of skin explants with the product efficacy prevented the accumulation of oxidatively damaged proteins in the skin, both on stratum corneum, epidermis and dermis.

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STUDY DESCRIPTION

I. Purpose of Experiment

The aim of this study was the efficacy assessment of 1 product to protect the skin against the deleterious effects of fire-wood pollution.

II. Tested product

On October 12th, MS beautilab provided (1) formulation as described in Table 1. The product has been stored at room temperature in the dark until use following sponsor instructions.

Table 1. Tested product

Product	Galenic form	Batch
POUR MOI SMOKE ALARM DROPS	Liquid	1 (36961A/4810-1.07)

III. Experimental Procedures

A. Human organotypic skin explant culture

Skin explants were obtained with the informed consent from abdominal surgery of a 34 years old female Caucasian donor (phototype II, Ref BIOP-2021-10-13). The explants were kept alive by culturing on metal grids into standard 12-well plates in contact with DMEM medium, high glucose (4.5g/L), glutaMAX, supplemented with Fetal Bovine Serum (10%) and Penicillin-Streptomycin (1%) at 37 °C in 5% CO_2 humidified air.

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B. *Product application & Stress*

After reception, the explants were distributed in 3 experimental groups (n=3 *per* group; table 2), the culture media was renewed every 24 hours. At Day 1, the product was topically applied at 2 mg/cm^2. At day 2, 30µL of a solution containing formaldehyde 0.5% + PM_{10} at 200 µg/mL in H_20 was topically applied on explants as firewood pollution stress. The stress conditions (formaldehyde and particulate matter containing HAPs) have been selected from published data as components of wood smoke emissions [Burning Issues/Clean Air Revival (2001); Timothy V. Larson, US EPA(1993)]. The stress group received the treatment with pollution and the medium renewal only. The control group did not receive any treatment except for the medium renewal. The explants were incubated at 37 °C in 5% CO_2 humidified air for 6h.

Table 2. Experimental Groups

Batch	Description	Treatment	N° explants	Sampling
Control	**Control**	Not treated	3	Day_2
Stress	**Stress (Formaldehyde 0.5% +PM_{10})**	Fire-Wood Pollution (Formaldehyde 0.5% + PM_{10})	3	Day_2
Product + Stress	**Product + Stress (Formaldehyde 0.5% +PM_{10})**	Product + Fire-Wood Pollution (Form Formaldehyde 0.5% + PM_{10})	3	Day_2

C. *Sampling*

Upon 6h of exposure to the fire-wood pollution exposure, half of each explant was included in OCT for cryo-preservation the other half was snap-frozen in liquid nitrogen and stored at -80 °C.

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IV. Analyses

Explant sections of 5 µm of thickness were obtained using a cryostat (Leica) and fixed with a solution containing 95% Ethanol and 5% Acetic Acid. Carbonylated proteins were labeled using a specific fluorescent probe (λ_{Ex} = 647nm / λ_{Em} = 650nm) and the reagent DAPI (4',6-diamidino-2-phénylindole) was used for nuclear labeling. Fluorescent images were collected with an epi-fluorescent microscope (Evos M5000) and analyzed with ImageJ software (Schneider, 2012). Image comparisons of different conditions were achieved using identical conditions of acquisition (40x objective).

Images analyses and quantification

Images were collected using the full dynamic range of the fluorescence detector (from 0 to 65535, 16bit .TIFF format) and analyzed using ImageJ software. For each image, quantification of the carbonylation level (Oxi-Proteome) was performed independently for the total skin and for the different anatomical compartments (stratum corneum, epidermis, dermis) (Figure 2 and 3). The carbonylation rate was obtained by integrating the specific fluorescence signal normalized by the surface of the evaluation area. Three (3) images per condition were analyzed to obtain a mean value and a standard deviation. Finally, these values were normalized in relation to control (considered 100%). Statistical analyzes were performed using the "GraphPad" software (La Jolla, California, USA) and included in the annexed file (Annex1_OxiProteomeView-D03CR2021.xls).

A protection value (%) was obtained for the experimental groups according to the following formula. As references, the control group is considered at maximum efficiency (100%) and the stress group at minimum efficiency (at 0%):

$$\textbf{Protection}\ \%\ (\text{group } X) = \frac{\text{Oxidation level (Stress)} - \text{Oxidation level (group X)}}{\text{Oxidation level (Stress)} - \text{Oxidation level (Control)}} * 100$$

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Oxi-Proteome View

In situ detection of protein oxidation (carbonylation) was performed by epifluorescence microscopy (Figure 1). Carbonylated proteins are represented in red. The whole collection of images is presented in the annexed figure (page 10). Fire-wood pollution induced an increase in carbonylated proteins in stratum corneum, epidermis and dermis. The presence of the product prevented the accumulation of carbonylated proteins. The images in HD format are included in the attached folder (appendix-D03CR-imagesHD).

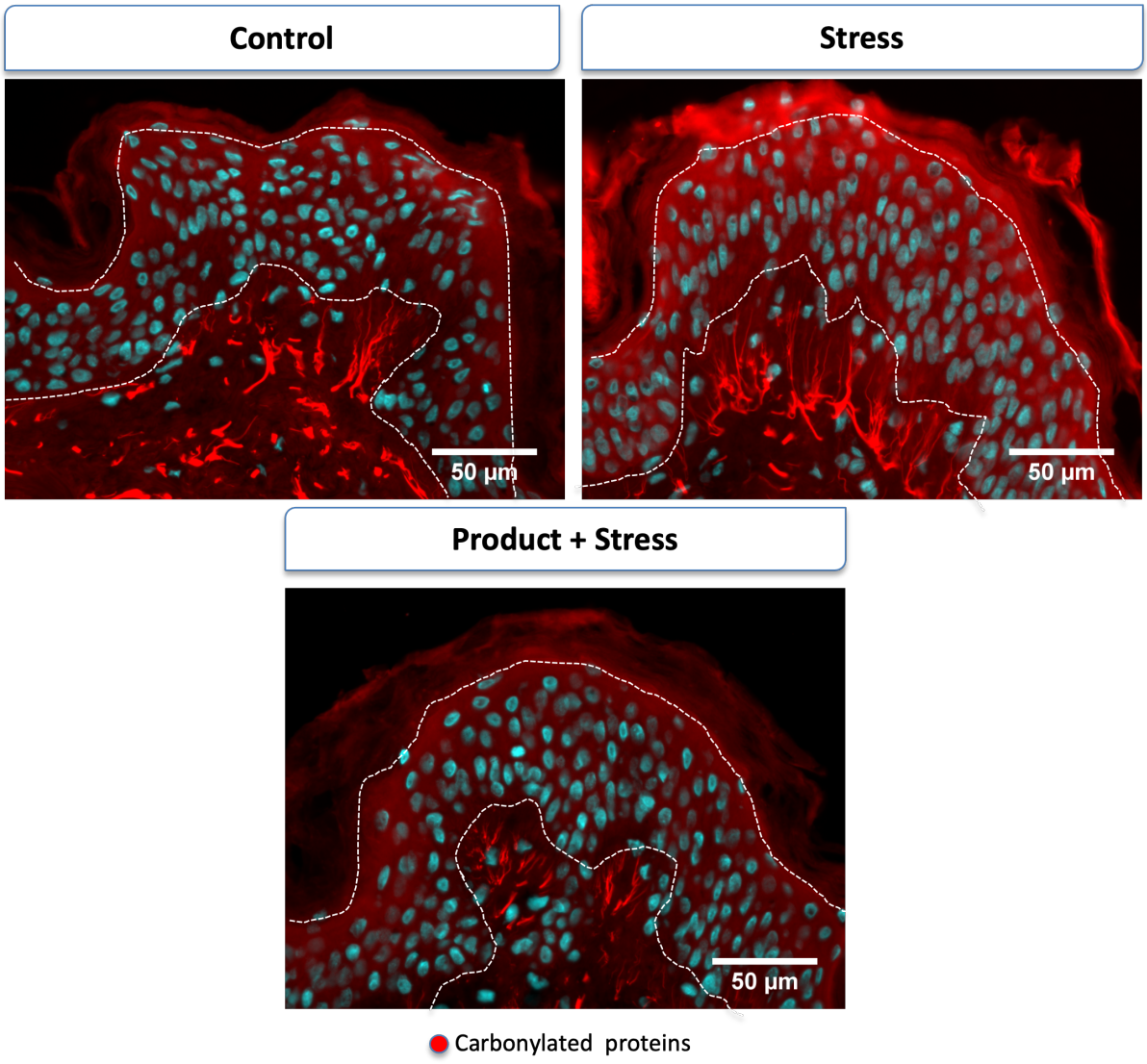

Figure 1. In situ visualization of oxidized proteins (red). Representative images for carbonylated proteins were obtained by using a functionalized fluorescent probe (λ_{Ex} 647nm / λ_{Em} 650 nm) while nuclear labeling is visualized in cyan (DAPI). White dotted lines separate the anatomical compartments of skin.

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Quantification

Three (3) images per experimental group were used for the quantification of carbonylated proteins by integrating the specific fluorescence signal in the whole image (including stratum corneum, epidermis and dermis; figure 2) and for each anatomical compartment independently (Figure 3). Densitometry analysis of *in situ* carbonylation signal was achieved using Image J software (NIH). All raw data are presented in attached file (Annex1_OxiProteomeView-D03CR2021.xls).

In the whole skin, the product showed a significant protection against protein carbonylation upon fire wood pollution (on *stratum corneum, epidermis, dermis*) :

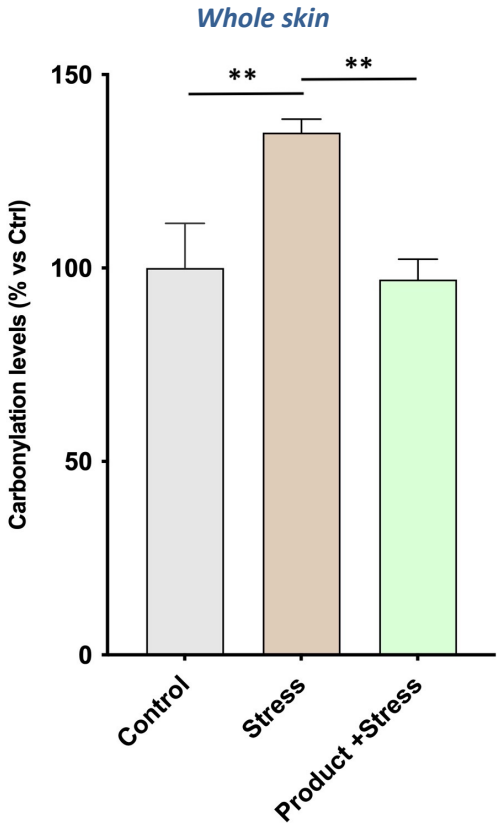

Figure 2. Oxidation levels on the whole skin. *In situ* oxidation is reported as bar graph (MEAN +/- SD).
*Statistics: ANOVA analysis with Dunnett's multiple comparison post-hoc test; **p<0.01*

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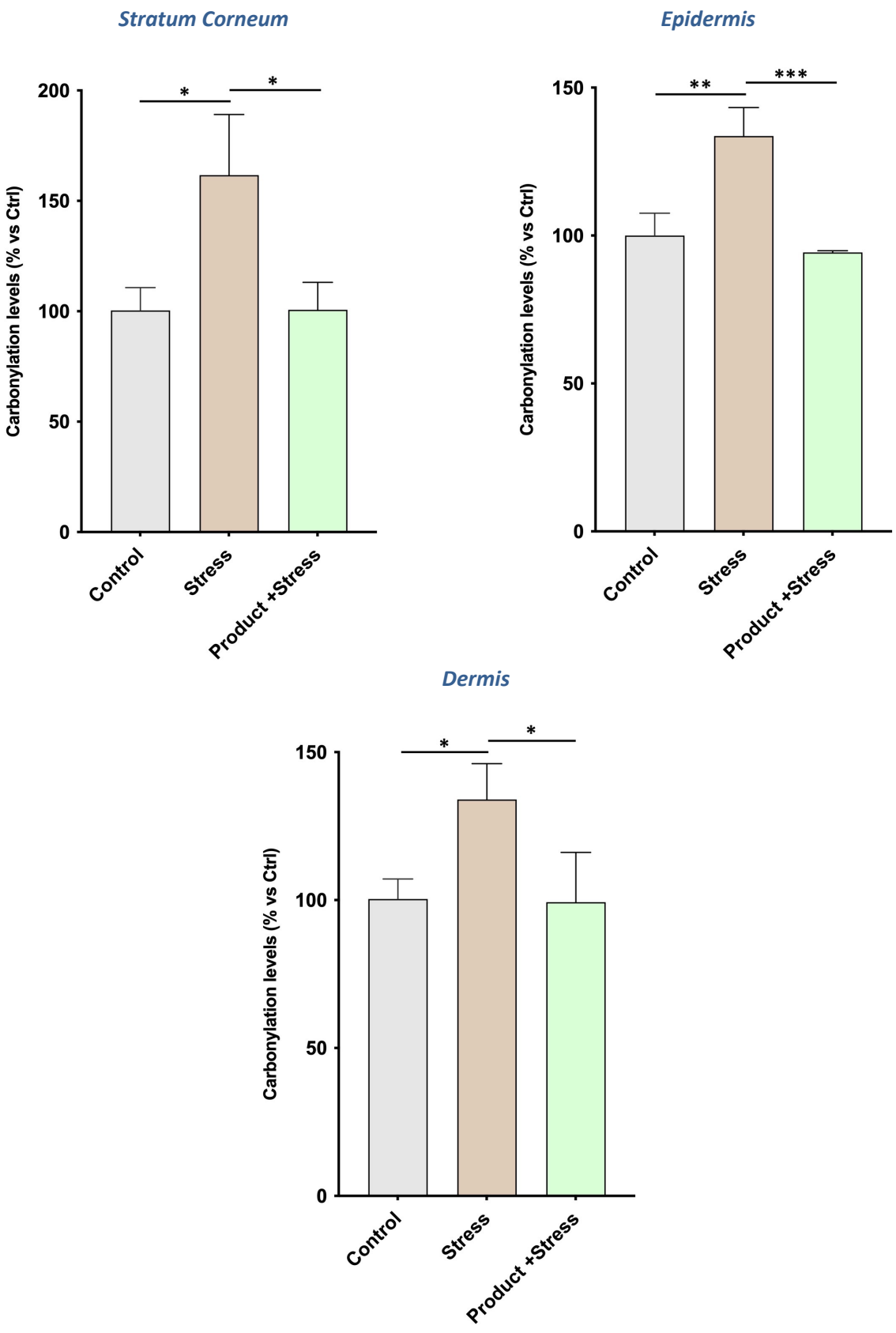

Figure 3. Oxidation levels by compartment. *In situ* oxidation is reported as bar graph (MEAN +/- SD) *per* skin compartment analysis (*stratum corneum, epidermis; dermis*).
*Statistics: ANOVA analysis with Dunnett's multiple comparison post-hoc test; *p<0.05, **p<0.01, ***p<0.001*

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CONCLUSIONS

In this study, the efficacy of protection from fire-wood pollution-induced protein oxidative damage of 1 formulated product was assessed *ex vivo* (on human skin explants).

A significant increase of carbonylation was observed in skin explants upon exposure to fire-wood pollution (stress versus control).

The product “POUR MOI SMOKE ALARM DROPS” significantly protected the skin from oxidative damage (carbonylation) :

- 99 % of efficacy on stratum corneum
- 100% of efficacy on epidermis and dermis
- for an efficient (100%) global protection of the skin (stratum corneum, epidermis, dermis)

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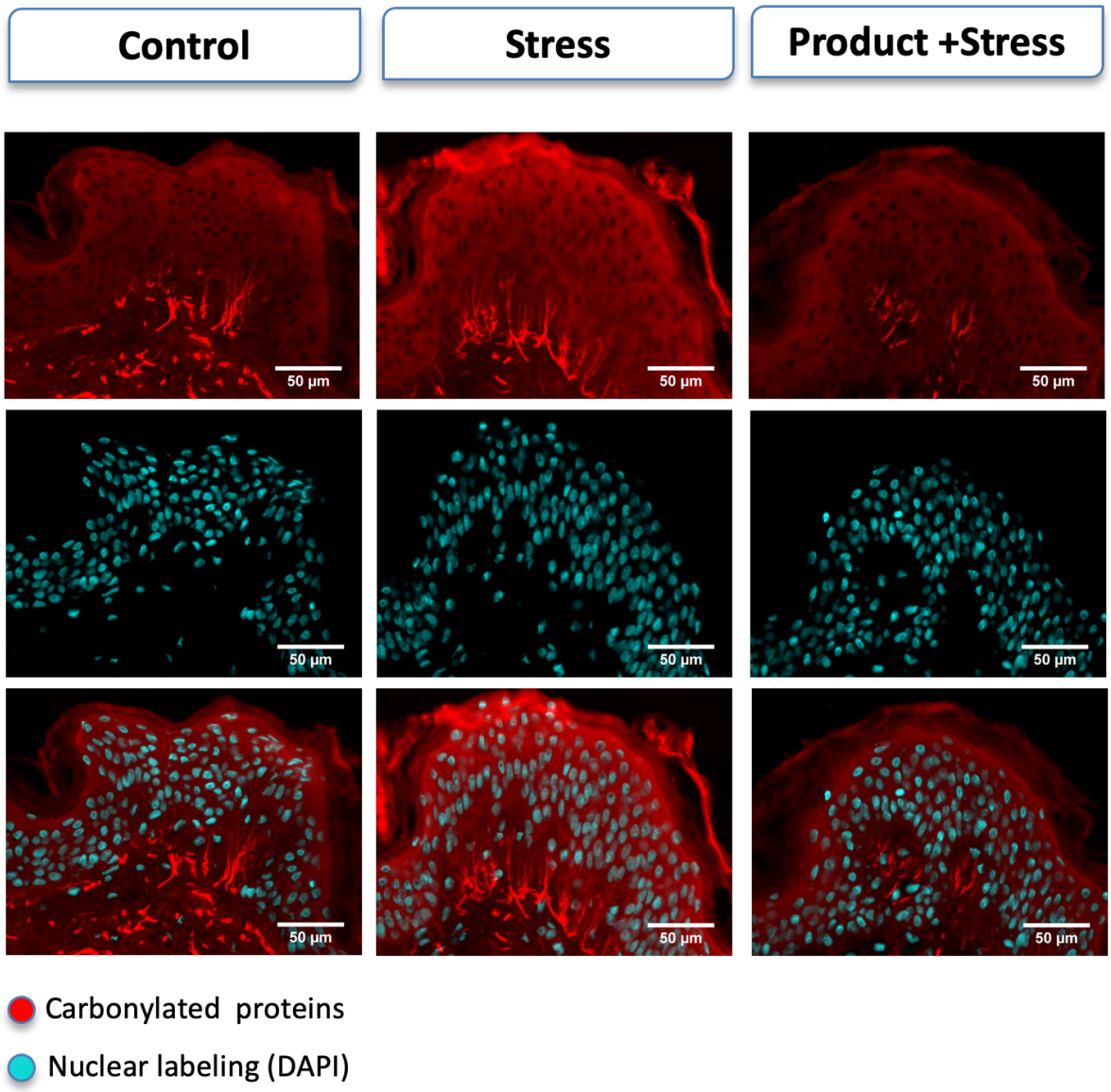
In the figure, the visualization of the *in-situ* oxidation level (carbonylation) is presented as a range of specific signal intensity (in red), followed by the images of nuclear labeling (in cyan) and their superposition. All images (in HD) are included in the attached file (Appendix-D03CR-imageHD).



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