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Short Communication

Heat Shock Protein 60 (HSP60) detection by QCM Biosensor and Antibody Covered Gold Nanoparticles

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Heat Shock Protein 60 (HSP60) is a 60 kDa weighting chaperonin that is an evolutionary conserved protein occurring in a wide number of organisms. It can serve as a plasma or blood serum biomarker of serious pathologies including cancer. In this work, quartz crystal microbalance (QCM) biosensor was constructed as a tool for a simple measurement of HSP 60 level. The QCMs were covered with an antibody specific to HSP60 and the antibody modified gold nanoparticles were also prepared. The assay was based of sandwich forming with captured HSP60, presence of HSP60 caused decrease of QCM oscillation frequency. The described assay exerted good sensitivity to HSP60, limit of detection for the assay was equal to 83 pg/ml for a sample with volume 5 µl, one assay cycle was finished within 90 minutes. The assay well correlated to standard Enzyme-Linked ImmunoSorbent Assay (ELISA) with coefficient of determination 0.985 and was not sensitive to interference by selected proteins (HSP70, albumin, immunoglobulins). In a conclusion, the biosensor appears to be a reliable tool for the detection of HSP60, the achieved specifications make it promising for a routine application.

Keywords: bioassay; biomarker; biosensor; cancer; chaperonin; diagnosis; heat shock protein; immunoassay; sensor

1. INTRODUCTION

Cells have more mechanism how to prevent pathological consequences of stress conditions. Release of Heat Shock Proteins (HSP) is one of the significant regulation pathways. The heat shock proteins have a multiple effect but it can be commonly entitled as chaperones and major types HSP60, HSP70, HSP90 and HSP100 can be distinguished [1]. Other types like HSP27 [2,3], HSP40 [4], and HSP110 [5] exist as well. The HSP60 also known as 60 kDa chaperonin and it is an evolutionary very conserved protein occurring in wide number of organisms and having various functions in and outside cells [6-8]. For instance, mycorrhizal soil protein glomalin is structurally close to HSP60 [9-12]. In the medicine, HSP60 has a role as an emerging marker able to point at wide number of pathologies. Elevated

level of HSP60 is typical for patients with some types of cancer from which the colorectal [13,14], hepatocellular [15], and bladder [16] cancer can be mentioned as the most relevant. In a study, involvement of HSP60 in the α-synuclein amyloid fibril formation was also proved [17]. Distinguishing of the HSP60 origin can also help in the diagnosis of infectious diseases by revealing this isotype in tissue sample, plasma or serum of infected patients. Diagnosis of infection by chlamydia and chlamydia initiated ovarian cancer [18] can be mentioned. On the other hand, experiment on paracoccidioidomycosis recognition proved that false positivity can occur [19], specificity of this marker for infectious diseases is disputable for the reason.

Though the HSP60 is a relevant marker in clinical biochemistry which importance will probably grow in the next years, there is a limited number of methods for its detection. Especially methods suitable for point of care testing are missing. Currently, the immunoassays like Enzyme-Linked ImmunoSorbent Assay (ELISA) are predominant [20-24]. In this paper, a new piezoelectric biosensor is introduced a tool suitable for HSP60 measurement and being an alternative to the standard immunoassays like ELISA.

Biosensor is a device where a part of biological origin is tightly combined with physico-chemical transducer which is a device that can be also simply called a physical sensor [25-32]. Piezoelectric biosensors are based on a piezoelectric sensor allowing to record amount of material catch by surface of the sensor and the piezoelectric biosensors are actually a balance providing information about weight of an analyte attached to the biosensor surface. The piezoelectric biosensors work on piezoelectric principle where the sensor is a part of oscillation circuit and frequency of the oscillations is changing (decreasing) due to the binding of an analyte [26]. Quartz Crystal Microbalance (QCM) is probably the most important type of a piezoelectric device which is widely available in the current market as a material for electrotechnology; however, biosensor devices based on QCM became popular due to reliability, price, sensitivity as well as easy integration into final analytical device and many biosensor applications working on QCM has been developed [33-39]. The QCM sensors have been chosen for the aforementioned reasons as a platform for the detection of HSP60, it is expected that experiments on a biosensor for HSP60 detection will bring a method being an alternative to the standard immunoassays.

2. MATERIALS AND METHODS

2.1. Manufacturing of Biosensors

The prepared biosensors were based on standard commercially available QCMs. Photograph of the used QCM is given as figure 1. The used QCMs had following specifications - material: quartz; basic frequency of oscillations: 10 MHz; quartz disc diameter: 20 mm; quartz disc thickness: 166 µm; material of electrodes: gold on chromium interlayer; geometry of electrodes: two electrodes on opposite sites, circle shaped; diameter of electrode: 7 mm. The electrodes were purchased from company Krystaly (Hradec Kralove, Czech Republic).

Construction of biosensors was based on following steps performed under standard laboratory temperature 25 ºC; apart of drying, the particular steps were made in wet box to prevent premature desiccation – 1) washing of new QCMs in pure ethanol and drying; 2) 50 µl of cysteamine 50 mg/ml

was given per one electrode, let to incubate for five hours and then rinsed with deionized water; 3) water solved 5 % w/w glutardialdehyde was injected in an amount 50 µl per one electrode, let to react for five hours, finally rinsed with deionized water; 4) the previously activated and modified surface of electrode was covered by antibody specific to HSP60, a rabbit origin polyclonal isolated IgG isotype antibody with specificity to human and murine HSP60 was purchased from Sigma-Aldrich (Saint Louis, MO, USA). It was diluted 1:1000 by phosphate buffered saline pH 7.4 (PBS) and it was further applied in an amount 50 µl per one electrode, let to incubate for 24 hours and then rinsed with PSB; 5) bovine serum albumin 10 mg/ml solved in PBS was applied in an amount 50 µl per one electrode and let to incubate for 24 hours; 6) finished biosensors were washed by PBS pH 7.4 with 0.1 % w/w tween and then by PBS and finally stored in a fridge at 4 ºC until use.

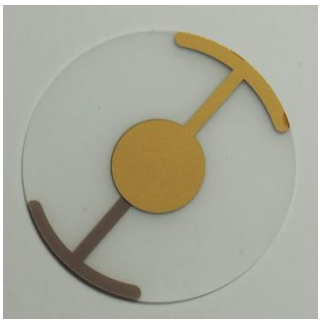

Figure 1. A 10 MHz QCM used for biosensor manufacturing.

2.2. Manufacturing of Antibody-Gold Nanoparticle Conjugate

Gold nanoparticles with declared size 100 nm were purchased from Sigma-Aldrich and modified in a similar way like the QCMs and under the same laboratory conditions. The procedure of making conjugate gold nanoparticle-antibody had following steps – 1) 500 mg of gold particles was mixed with 5 ml of cysteamine 50 mg/ml and let to incubate for five hours; 2) the suspension was centrifuged (9,000 ×g, 5 minutes), supernatant removed by gentle sucking out, the pellets were resuspended in 5 ml of deionized water, centrifuged again and supernatant removed again; 3) the particles were resuspended in 5 ml of 5 % w/w glutardialdehyde and let to incubate for five hours; 4) the suspension was centrifuged (9,000 ×g, 5 minutes), supernatant removed by gentle sucking out, the pellets were resuspended in 5 ml of deionized water, centrifuged again and supernatant removed again; 5) the particles were resuspended in 5 ml of rabbit origin polyclonal isolated IgG isotype antibody diluted 1:1000 (the same as in the previous subchapter) and let to incubate for five hours; 6) the suspension was centrifuged (9,000 ×g, 5 minutes), supernatant removed by gentle sucking out, the pellets were resuspended in 5 ml of PBS, centrifuged again and supernatant removed again; 7) the particles were resuspended in 5 ml of bovine serum albumin 10 mg/ml and let to incubate for five hours; 8) the suspension was centrifuged (9,000 ×g, 5 minutes), supernatant removed by gentle sucking out, the pellets were resuspended in 5 ml of PBS pH 7.4 with 0.1 % w/w tween, centrifuged again and supernatant removed again; 9) the suspension was centrifuged (9,000 ×g, 5 minutes), supernatant removed by gentle sucking out, the pellets were

resuspended in 5 ml of PBS, centrifuged again and supernatant removed again; 10) the particles were resuspended in 5 ml of PBS and stored in a fridge at 4 ºC until use.

2.3. HSP60 Measurement by Biosensor and Nanoparticle Conjugate

The biosensors were tested in an assay consisting from following steps: 1) biosensor was placed into holder with conductive springs, connected with ICM Level Oscillator 10.000 MHz by ICM (Oklahoma City, OK, USA) and then with frequency counter TF930 (Thurlby Thandar Instruments Limited, Huntingdon, UK); 2) oscillation frequency f_1 was measured for each biosensor; 3) a sample sized 5 µl was applied on electrode with 45 µl of PBS and washed out by PBS after 30 minutes; 4) a batch of modified nanoparticles was given per one electrode in an amount 50 µl, the suspension of nanoparticles was intensively shaken before application; 5) the suspension was let on the electrode for 30 minutes; 6) the electrodes were rinsed with PBS and let to dry; 7) oscillation frequency f_2 was measured; 8) outputting signal Δf was calculated according equation: $\Delta f = f_1 - f_2$. General principle of the assay can be learned from figure 2.

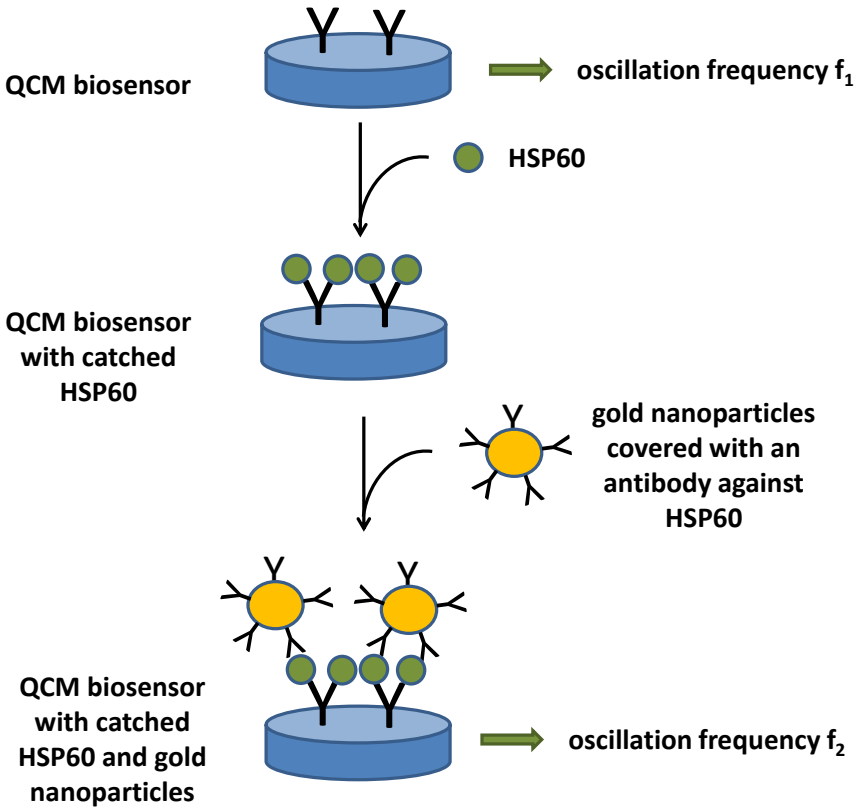

Figure 2. General principle of the QCM biosensor assay.

2.4. ELISA for Validation of the Biosensor

Samples tested by the biosensors were also measured by ELISA in order to validate the biosensor assay and to compare the methods mutually. Human HSP60 ELISA Kit (AbCam, Cambridge, United

Kingdom) was chosen for the purpose. The kit was performed in compliance with protocol provided by the manufacturer. The same samples with volume 5 µl mixed with 45 µl of PBS was used like in the biosensor assay.

3. RESULTS AND DISCUSSION

In the first round, the prepared biosensors were tested for the standard solution of HSP60 and concentration of HSP60 in the tested samples corresponded with the expected concentrations in human plasma. Expected HSP60 level in the human plasma was determined by Lewthwaite and coworkers for a civil servants (126 men and 103 women) to be in a range 13 – 1,589 ng/ml (interquartile range), 20 % of tested people had level above 1 µg/ml and median of the population was equal to 110 ng/ml [40]. In a common sense, concentration of HSP60 plasma above 1 µg/ml can be considered as a warning one, it can reach level up to 1 mg/ml is some individuals but levels under 1 ng/ml can be expected as well [41,42]. In this experiment, samples with concentration of HSP60: 100 µg/ml – 10 µg/ml – 1 µg/ml – 100 ng/ml – 10 ng/ml – 1 ng/ml – 100 pg/ml – 10 pg/ml – 0 pg/ml (blank assay, PBS only) served for the biosensor characterization. The concentration range should cover all expected spectrum of HSP60 levels in humans. Calibration of the biosensors is graphically depicted in figure 3. The assay of HSP60 exerted limit of detection 83 pg/ml for a sample sized 5 µl and an assay lasting approximately 90 minutes (time including drying and sample manipulation). The measured signal quite well correlated with the concentration of HSP60 because coefficient of determination for the calibration plot was equal to 0.983.

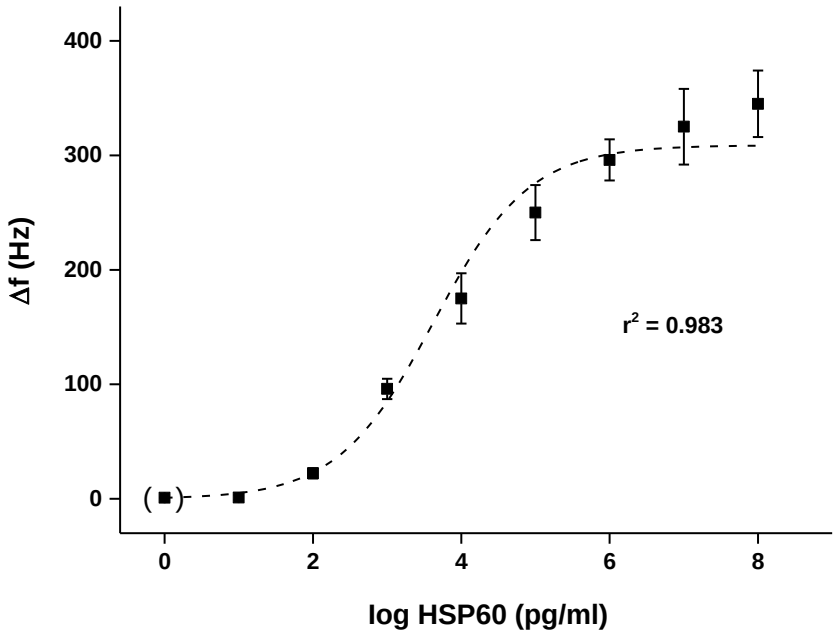

Figure 3. Calibration of QCM biosensor for HSP60 assay. Decimal logarithm used for HSP60 concentration expression. The point in brackets was obtained by blank assay. Error bars indicate standard deviation for measurement of a sample by five biosensors.

The same samples which were measured by biosensor were also tested by ELISA method and the results were mutually compared as a validation experiment (Figure 4). The two methods had comparable sensitivity to HSP60. Limit of detection for ELISA was 173 pg/ml, this limit of detection is approximately two times worse compared with the biosensor assay. Coefficient of determination for the validation extrapolation was equal to 0.985 which can be interpreted as a good relationship and confirms applicability of the HSP60 assay by biosensor.

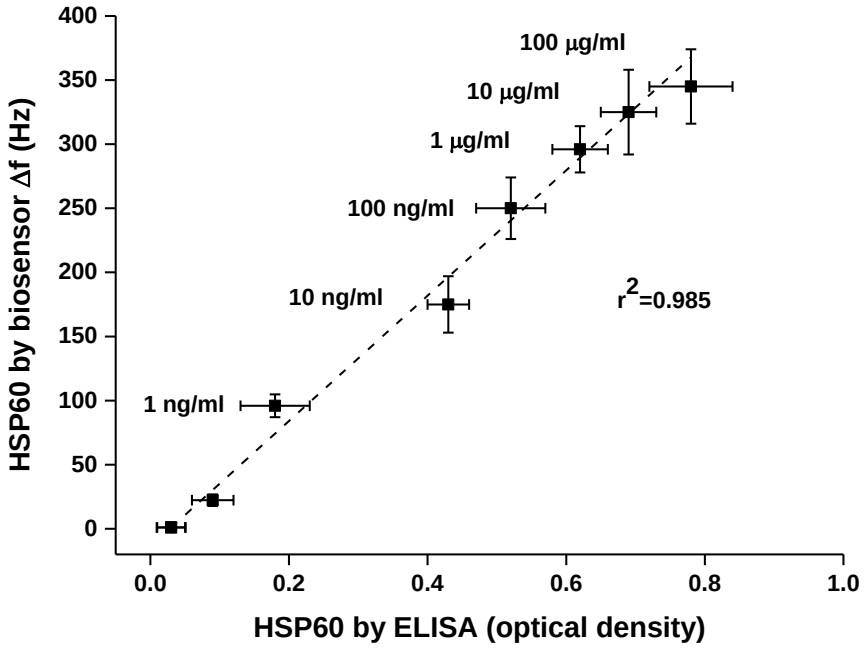

Figure 4. Comparison between biosensor and ELISA measurements of HSP60 containing samples. The point in brackets was obtained by blank assay. Error bars indicate standard deviation for five repeats of measurement (a sample assayed by five biosensors or five wells of ELISA microplate).

Interferences caused by compounds similar to HSP60 or compounds that can be expected in a sample beside the HSP60 would be a serious problem. A group of compounds that would be considered as an interferent was tested. Namely HSP70, human serum albumin, IgG from human serum and PBS pH 7.4 as a matrix were tested and compared with the signal provided by HSP60. Following concentration of the molecules were chosen: HSP70 100 μg/ml, albumin 100 mg/ml, IgG 100 mg/ml and standard PBS pH 7.4. The selected concentrations were above expected levels in clinical samples therefore neglecting of interference should be valid for such concentration [39,43-45]. Results from the interference testing are depicted in figure 5. According to ANOVA test, the signal provided by HSP60 was significantly differing to the signals from the other compounds (HSP70, albumin, IgG and PBS) on probability level 0.01. The signal caused by HSP70, albumin and IgG were insignificant by ANOVA test on probability level P 0.05. The results can be interpreted that way the assay does not suffer to false positivity due to interference by the tested compounds.

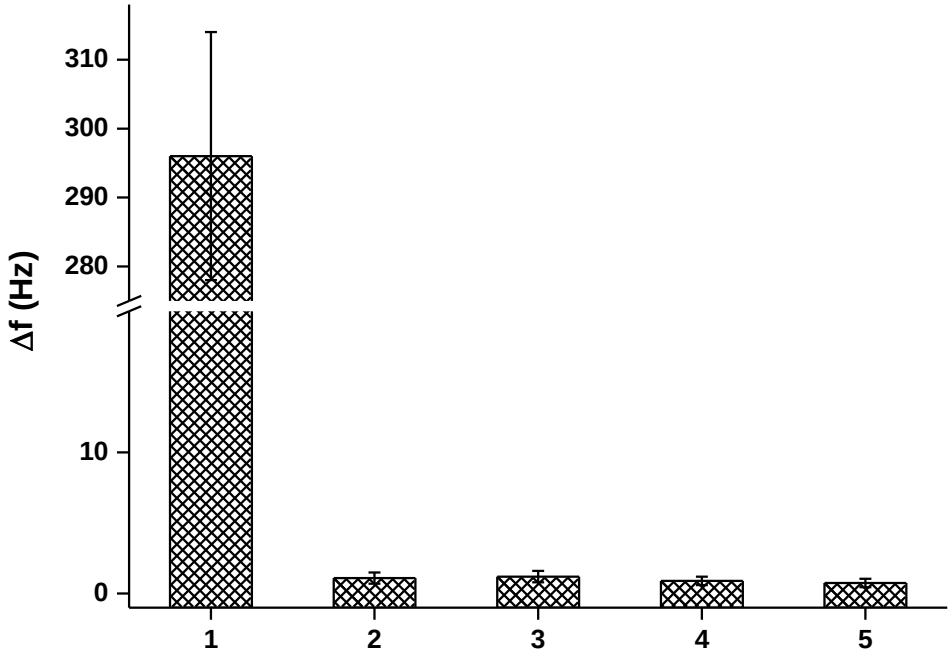

Figure 5. Testing of interferences caused by HSP70 100 µg/ml (column 2), albumin 100 mg/ml (column 3), IgG 100 mg/ml (column 4) and PBS (column 5) while column 1 is a signal for HSP60 1 µg/ml. Error bars indicate standard deviation for five repeats of measurement (a sample measured by five biosensors).

Long term stability of the biosensors and modified gold nanoparticles was tested (figure 6). The biosensors and gold nanoparticles were kept in a fridge at 4 ºC until use. The biosensors were performed for only one time since there are expected to serve as a disposable device. In a total five biosensors and doses of modified gold particles were used for each five days. As can be seen in the graphical presentation, the drop of signal was minimal (signal after 60 days represents approximately 95 % of the initial signal) and the drop of signal was not significant (ANOVA, P = 0.05) when compared the intervals with the signal provided by the new biosensors and modified nanoparticles.

Survey of analytical specifications of the prepared biosensor is given in table 1. Comparing the experimental data with the current literature is not easy because similar type of biosensor for HSP60 has not been developed yet. On the other hand, some other types of biosensors respective biosensors for structurally similar analytes are known. For instance a flow cytometric bioassay for HSP70 [46], immunosensor based on indium tin oxide modified gold nanoparticles and impedance spectroscopy and cyclic voltammetry for HSP70 [47] and electrochemical impedance and cyclic voltammetry based biosensor using porous graphene electrodes exerting limit of detection for HSP70 equal to 0.02 ng/ml [48] can be mentioned. Considering the current trends, the here presented biosensor is a competitive analytical device with expected practical impact in the future.

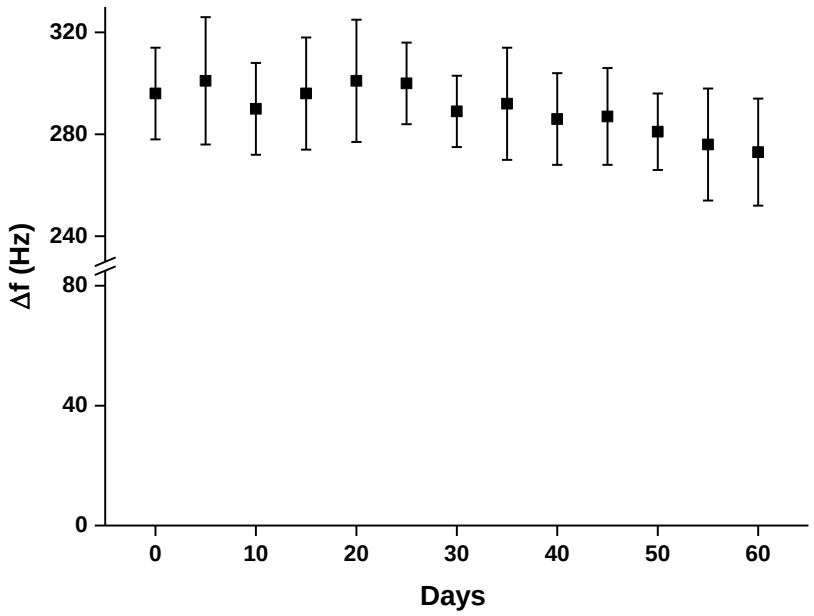

Figure 6. Long term stability of the tested biosensors and modified gold nanoparticles. Error bars indicate standard deviation for five repeats of measurement (a sample measured by five biosensors).

Table 1. Specifications of biosensor analytical parameters

Specification	*Value*
limit of detection	83 pg/ml
volume of applied samples	5 µl
coefficient of determination for calibration	0.983
coefficient of determination for validation: biosensor vs. ELISA	0.985
interference by HSP70 100 µg/ml, albumin 100 mg/ml, IgG 100 mg/ml	not significant
time per one assay	approximately 90 minutes

4. CONCLUSION

The QCM biosensor tested in this work appears to be a reliable tool for the detection of HSP60, the achieved specifications make it promising for a routine application. The limit of detection is low enough to cover even threshold levels of HSP60, the range of calibration is also adequate to measure all expected concentrations in the biological samples. Low volume of sample is another advantage of the assay. Small size of the biosensor, simple assay protocol and potency to mass produce the biosensor are also pros of it. Next development of final analytical devices based on QCM are expected and the biosensor for HSP60 would be a promising application.

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