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Combining Immuno-Affinity Chromatography and Filtration to Improve Specificity and Size Distribution of Exosome-Containing EV Populations

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Abstract

The enrichment of small extracellular vesicles of endosomal origin (exosomes) with average diameters of less than 150 nm is a prerequisite for the investigation of their exact biological functions. In this application note, an experimental setup is presented, which combines Fab TACS® immuno-affinity chromatography based on the Strep-tag® technology (IBA Lifesciences), with subsequent filtration using Minisart® High Flow syringe filters (Sartorius). We demonstrate that the average hydrodynamic diameter of $CD9^+$ or $CD81^+$ EVs enriched by affinity chromatography from HEK293 cell culture supernatant or plasma can be effectively reduced by eluate filtration. Thereby, EV populations with a small size distribution (30 – 150 nm) are created. Impurities caused by microvesicles, apoptotic bodies and lipoproteins that other enrichment techniques (e.g. differential and density gradient ultracentrifugation, ultrafiltration and size exclusion chromatography) struggle with, are avoided with our experimental setup.

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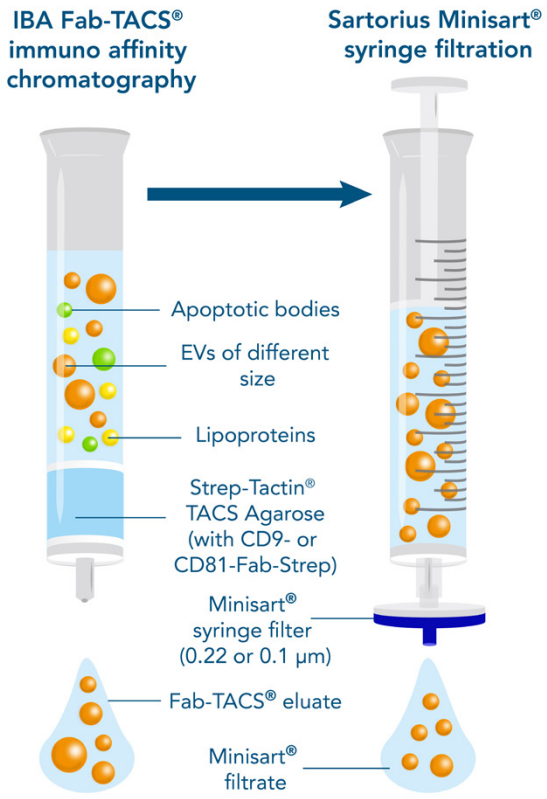
Introduction

Exosomes are small extracellular vesicles (EVs) of endosomal origin and are produced by almost every cell type. They are defined by their biogenesis, and are partially characterized by physiological features, such as the presence of specific surface markers (CD9, CD63 and CD81) or physical characteristics like size and density. EVs with diameters in the range of 30 – 150 nm are classified as small EVs (sEVs) and are of great interest since exosomes also have the same size range. In general, exosomes are derived from early endosomes, by inward budding and subsequent release of the resulting vesicles into the extracellular space (via fusion of a multivesicular body with the plasma membrane). Exosomes support intercellular communication by transferring signals to remote recipient cells. This communication is necessary to maintain cellular functions and tissue homeostasis in all multicellular organisms. Alongside direct cell-cell contact or the transfer of secreted molecules, exosomes constitute an additional important pathway in intercellular communication by serving as carriers for proteins, lipids, RNA and DNA. Because of these properties and their proven importance in numerous physiological and pathological processes, exosomes can be used as biomarkers for diagnosis and prognosis of various disease states. Furthermore, they can be used for therapies, due to their biocompatibility and capacity for signal transfer by the above-mentioned biomolecules. Due to their potential use in clinical applications, exosomes have attracted much attention concerning their roles in health and disease. [1–4]



The enrichment of exosomes is a critical step for investigating their physiological mechanisms and applications in biomedical science. Various techniques have been adopted to facilitate the enrichment of these vesicles. However, since size and density of exosomes overlap with other cellular components, their isolation and analysis can be challenging. Established enrichment techniques are ultracentrifugation, ultrafiltration, size exclusion chromatography (SEC), polymer-based precipitation, and immuno-affinity capture.[5] For most of these methods, the size of the EVs has an impact on separation and all of them come with different advantages and drawbacks. Thus, a combination of different methods leads to synergetic effects and can improve the enrichment process. In the past, filtration methods were combined with ultracentrifugation. Filtration membranes are used to remove cells and large EVs, and subsequent ultracentrifugation is utilized to separate exosomes from proteins.[6,7] However, this isolation procedure is time intensive. In addition, it only focuses on physical properties, such as size and density. The main disadvantage of procedures like this is the co-enrichment of non-exosomal material, such as protein aggregates, lipoproteins, macromolecules and microvesicles (MVs).

To overcome impurities caused by size- and density-based EV preparations, immuno-affinity purification techniques have been used to selectively capture exosomes from complex populations by exploiting surface markers like CD81 and CD9. [5,8] Exosome membranes contain large quantities of these surface protein markers and immuno-affinity chromatography is based on the interaction between these and special selection reagents. Exosomes can be enriched from complex biological mixtures like cell culture supernatants, tissues, and biological fluids by immuno-affinity chromatography. This method is rapid, easy and compatible with standard laboratory equipment, but lacks separation capabilities based on size of already mentioned methods, because it is prone to co-isolate unwanted particles, such as MVs, which carry the same surface markers. Furthermore, most immuno-affinity exosome enrichment reagents (mainly antibodies against the aforementioned surface markers) bind to the surface of isolated EVs, thereby potentially affecting their biological function. Fab-TACS® affinity chromatography allows the enrichment of label-free extracellular vesicles, but also cannot prevent the selection of larger, target-marker-positive EVs.

We aimed to develop a protocol combining reversible Fab-TACS® immuno-affinity chromatography with sieving effects of Minisart® High Flow PES syringe filters with pore sizes of 0.22 µm or 0.1 µm to generate a defined EV product and circumvent the drawbacks of these isolation techniques when used independently.

Methods

The herein presented enrichment of exosomes is based on Fab-TACS® affinity chromatography and subsequent eluate treatment with syringe filters of different pore sizes.

Sample Peparation

HEK293 cell culture supernatant was differentially centrifuged at 300 *g* and 3,000 *g* for 10 min, respectively, and filtered using a 0.45 µm Minisart® High Flow PES filter (Sartorius, 16537-K). 10 mL of filtrate was used to conduct the described experiments.

To prepare plasma, buffy coat (BC) was sedimented overnight at 4 °C and the established supernatant was centrifuged twice at 3,000 *g* for 10 min. Finally, the supernatant was filtered using a 0.45 µm Minisart® High Flow PES filter. 9 mL of pre-treated plasma was used for subsequent experiments.

All reagents were equilibrated to room temperature prior to use. Lyophilized Fab-Streps (IBA Lifesciences, human CD81 Fab-Strep, 6-8015-150 or human CD9 Fab-Strep, 6-8019-150) was dissolved in 1 mL PBS (Gibco™, 20012027) by carefully pipetting up and down. Further, 1 mM Biotin Elution Buffer was prepared by mixing 40 µL of a 100 mM Biotin stock solution (IBA Lifesciences, 6-6325-001) with 4 mL PBS. The Fab-Strep, Biotin Elution and PBS buffers were filtered using 0.2 µm Minisart® NML cellulose acetate filters (Sartorius S6534-FMOSK).

Enrichment and Filtration

The cap of a 1 mL Strep-Tactin® TACS Agarose Column (IBA Lifesciences, 6-6310-001) was removed, and the sealed end of the column was cut at the notch to allow the storage solution to drain. Afterwards, the column was washed by applying 5 mL PBS, before loading the Fab-Strep solution onto the column. After a short incubation (2 min), to allow Fab binding, the column was washed with 5 mL PBS. The pre-treated samples (9 mL of plasma or 10 mL of HEK293 cell culture supernatant, respectively) were applied in steps of 1 ml. After complete sample loading, the column was washed twice with 5 mL PBS to remove unwanted particles and proteins. 1 mL Biotin Elution Buffer was applied and incubated for 5 min to release bound EVs. To complete elution, 3 x 1 mL Biotin Elution Buffer were added.

Each eluate was divided into three aliquots of 1 mL, which were either retained without further treatment, or filtered through a 0.1 µm or 0.22 µm Minisart® High Flow PES syringe filter, respectively (Sartorius, 0.22 µm: 16532-K and 0.1 µm: 16537-K). To confirm that the tested TACS columns and syringe filters do not release particles that could influence concentration and size distribution measurements, the isolation process was also performed using 0.2-µm filtered PBS instead of EV sample. In addition, 1 mL aliquots of PBS were filtered through a 0.1 µm or 0.22 µm syringe filter. All three process controls were analyzed by nanoparticle tracking analysis and no particles were detected (data not shown).

NTA Measurement

To determine particle concentration and size distribution of the samples, nanoparticle tracking analysis (NTA) was performed using the NanoSight NS300 and the software package NTA 3.4 from Malvern (3 movies of 60 s at 25 frames per second, scattering mode, camera threshold: 13, detect threshold: 5, manual fixed focus). For NTA measurements, centrifuged and filtered HEK293 cell culture supernatants were diluted 20:1, whereas Fab-TACS® non-filtered and filtered eluates were diluted 5:1 with PBS. Plasma samples were diluted 400:1 and its eluates 50:1 using PBS as well.

SDS-PAGE and Western Blot

To study the protein composition of isolated EVs from cell culture supernatant, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analyses were performed. First, EVs were precipitated by mixing an aqueous solution of trichloroacetic acid (10 %, *w/v*) with the sample in a ratio of 1:1. After vortexing (30 s), the samples were allowed to precipitate on ice for 30 min. Subsequently, precipitates were centrifuged at 5,000 x *g* for 30 min. The obtained pellet was resuspended in 100 µL PBS and 200 µL 5x SDS loading buffer. The samples were incubated at 80 °C for 5 min and SDS-PAGE was performed at 85 V for 90 min on ice. Blotting to a nitrocellulose membrane was performed with the Trans-Blot® Turbo Transfer System of Bio-Rad (25 V and 1.7 A for 3 min). Then, the blots were blocked for 1 h in 3 % (*w/v*) BSA-PBS at room temperature. The primary antibodies were diluted 1:1,000 in 3 % (*w/v*) BSA-PBST and applied overnight at 4 °C on a shaker. The membranes were washed 3 x 5 min with PBST and then the secondary antibody with a dilution of 1:2,000 in 3 % (*w/v*) BSA-PBST was applied for 1 h at room temperature, while shaking. Finally, the blots were visualized using Western Lightning Plus-ECL reagent (Perkin Elmer) according to the manufacturer's protocol. The blots were analyzed with the Image Lab software (V.6.1) from Bio-Rad. Plasma samples were not analyzed.

Results and Discussion

Fab-TACS® Enables Specific Purification of EVs

We used two different sample types to test our protocol combining immuno-affinity chromatography and filtration: pre-treated HEK293 supernatant and plasma (see sample preparation).

Concerning the supernatant, the starting material contained 16.8 % particles between 30 – 150 nm (data not shown). For the non-filtered CD9- and CD81-specific eluates, proportions of 50 % and 51 % were determined, respectively (Figure 1 A). Using a feed of 10 mL cell culture supernatant, concentrations of 1.62×10^9 and 2.46×10^9 particles/mL in a range of 30 - 150 nm for the $CD9^+$ and $CD81^+$ eluates were measured, respectively. Considering a total volume of 4 mL for each eluate, amounts of 6.46×10^9 $CD9^+$ and 9.85×10^9 $CD81^+$ particles were collected (Figure 1 B). The particles of the pre-treated cell culture supernatant had a mean size (hydrodynamic diameter) of 191 nm (data not shown) and particles of the eluates had mean sizes of 161 nm ($CD9^+$ EVs) and 160 nm ($CD81^+$ EVs) (Figure 1 C). In summary, an increase of particle counts within the exosome typical size range was achieved through immuno-affinity chromatography. In addition, typical markers for exosomes, Hsp70 and syntenin-1, were detected in all samples (including filtered samples) by western blot analysis, confirming that the detected particles are EVs. Calnexin, as a negative marker for exosomes, was found only in the lysed cell culture (data not shown), but not in the centrifuged and further processed cell culture supernatant (Figure 2).

Figure 1: *NTA measurements of Fab-TACS® enriched exosomes using CD9 Fab-Streps or CD81 Fab-Streps from HEK293 supernatant samples (n = 3). Proportions of particles in exosomal size range (A), total particle amount (4 mL sample) within this size range (B) and mean particle size of all detected particles (C).*

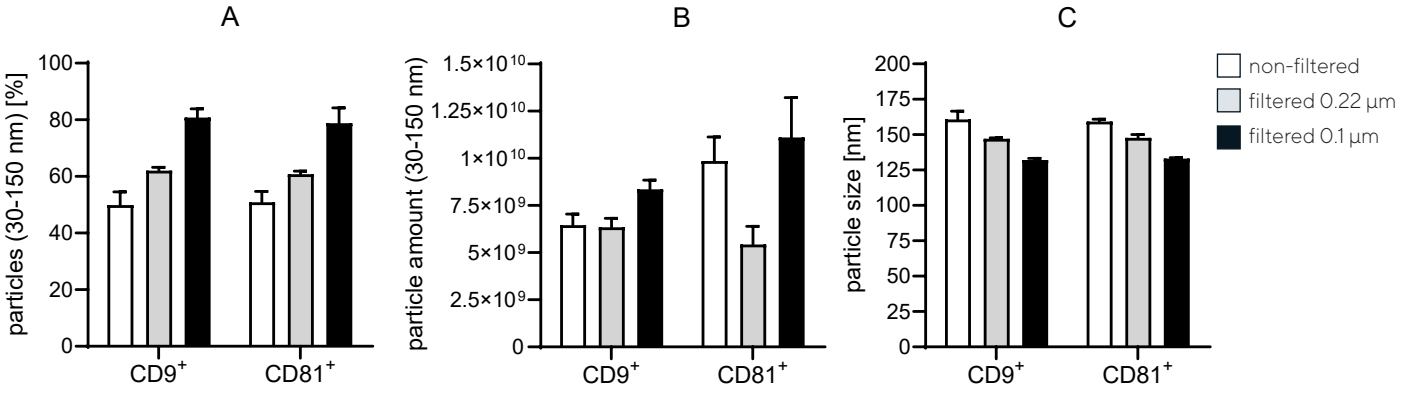


Figure 2: *Detection of EV-negative marker calnexin (90 kDa) and EV-positive markers Hsp70 (70 kDa) and syntenin-1 (32 kDa) by SDS-PAGE and western blot analysis.*

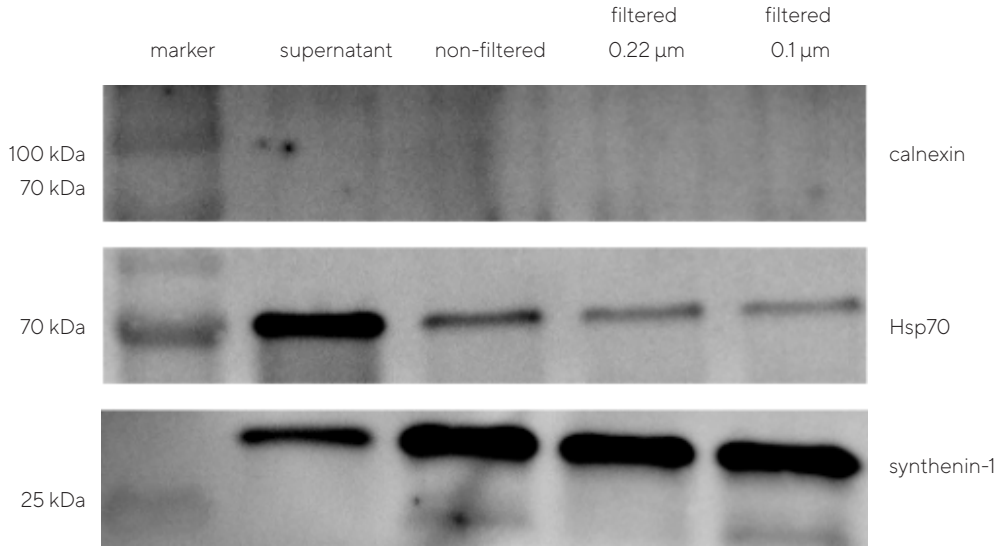
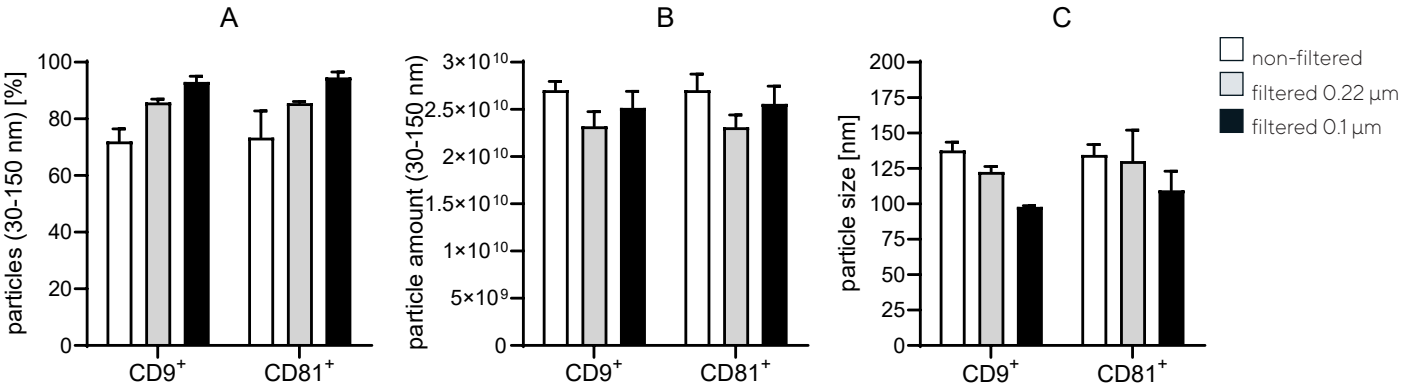


Figure 3: *NTA measurements of Fab-TACS® enriched exosomes using CD9 Fab-Streps or CD81 Fab-Streps from BC plasma samples (n = 3). Proportions of particles in exosomal size range (A), total particle amount (4 ml sample) within this size range (B) and mean particle size of all detected particles (C).*



According to the enrichment of EVs from cell culture supernatant, the same procedure was conducted using plasma. In comparison to supernatant, 70 % of the particles in the non-filtered CD9⁺ and CD81⁺ eluates were already within the exosomal size range of 30 – 150 nm, assuming that the pre-treated plasma contained more small particles than the supernatant used in this study (Figures 1 A and 3 A). After filtration, the proportion of particles in the desired EV size range increased to 93 % by CD9⁺ enrichment and up to 95 % with CD81⁺ selection. Furthermore, total particle amounts were in the same range for the CD9⁺ and CD81⁺ EV isolation: Yields of approx. 2.5 x 10^{10} particles demonstrate the high binding capacity of Strep-Tactin® TACS Agarose columns (Figure 3 B).

Minisart® Syringe Filtration Increases the Proportion of Small EVs

The eluates following CD9- and CD81-specific immuno-affinity chromatography of HEK293 and plasma samples were filtered in parallel runs using Minisart® High Flow PES syringe filters, with pore sizes of 0.22 µm or 0.1 µm.

For the non-filtered CD9⁺ HEK eluate a total particle amount of 6.46 x 10^9 for 4 mL was determined. Amounts of 6.34 x 10^9 (0.22 µm) and 8.36 x 10^9 (0.1 µm) were measured after eluate filtration. The treatment with a 0.22 µm filter did not change the particle amount for the CD9⁺ selection to a detectable extent, whereas 0.1 µm filtration led to an increased number of detected particles with regard to the non-filtered eluate (Figure 1 B). Under certain conditions decreasing pore sizes lead apparently to higher particle amounts. Considering the sieving effect, this tendency seems to be contradictory. However, NTA is based on light scattering and is a bulk measurement, which might explain this observation (see explanation of NTA effect below). Nevertheless, an enrichment of smaller particles through filtration was determined by mean particle sizes: For the CD9⁺ samples from HEK supernatant, hydrodynamic diameters of 161 nm (non-filtered), 147 nm (0.22 µm filtered) and 132 nm (0.1 µm filtered) were detected (Figure 1 C). This is in line with the sieving effect of the Minisart® filters and confirms the enrichment of particles in the exosomal size range (Figure 1 A).

The effects of eluate filtration following selection for CD81⁺ from cell culture supernatant samples were similar: Total amounts of 9.85 x 10^9 (non-filtered), 5.43 x 10^9 (0.22 µm) and 1.11 x 10^{10} particles (0.1 µm) were determined (Figure 1 B). After 0.1 µm filtration the highest particle amount was measured, which was in keeping with the results from CD9⁺ selection. However, a pronounced particle reduction after 0.22 µm filtration was detected for the CD81⁺ eluate. Together with the strong increase after 0.1 µm filtration, it might be concluded that the CD81⁺ eluate contained more large particles after the 0.22 µm filtration, which cause the NTA effect (see below). These particles are more strongly retained by filters with small pore sizes, when compared to filters with larger pores. Mean sizes of these particles were 160 nm for the non-filtered, 148 nm for the 0.22 µm-filtered and 133 nm for the 0.1 µm-filtered sample (Figure 1 C). Thus, as for CD9⁺ selection the filtration of CD81⁺ eluates led to an enrichment of exosome-sized particles.

Like the observed effect in cell culture supernatant, we observed a reduction in particle size by filtration for the plasma samples. The particles of the non-filtered eluate were 138 nm in size after CD9⁺ enrichment and could be reduced to 122 nm (0.22 µm filtration) and 98 nm (0.1 µm filtration). After CD81⁺ enrichment the size was reduced from 135 nm to 130 nm (0.22 µm filtration) and 110 nm (0.1 µm filtration) (Figure 3 C).

The reduction of mean particle sizes was also reflected through the proportions of particles in the exosomal size range. The proportions were 50 % (non-filtered), 62 % (0.22 µm) and 81 % (0.1 µm) for CD9⁺ samples and 51 % (non-filtered), 61 % (0.22 µm) and 78 % (0.1 µm) for CD81⁺ samples (Figure 1 A). Thus, with decreasing pore size,

particles between 30 – 150 nm are more strongly enriched, and therefore probably the proportion of exosomes within the eluted EV population is also increased. The same tendencies concerning size proportions were observed for the enrichment of EVs from plasma (Figure 3 A). In particular, the proportion of particles up to 150 nm was generally higher when the starting material was derived from plasma. Thus, it was easier to refine this sample type to a level where the exosomal size range represented > 90 % of the particle population.

NTA Effect

NTA is based on analysis of scattered light of a bulk of particles. Larger particles have a much higher intensity than smaller particles (data not shown). Therefore, larger particles tend to mask the presence of smaller particles, leading to an underestimation of the concentration of smaller particles in a sample. The use of filters with suitable pore sizes has a positive effect on particle size homogeneity. This is beneficial for particle concentration determination by light scattering methods like NTA. In summary, a more homogenous sample concerning particle sizes will yield more exact results regarding particle concentration.[10] Filtration with 0.1 µm filters resulted in improved homogeneity of the samples and an elevated confidence in the particle concentration, especially in the size range of interest (30 – 150 nm).

Conclusion

The combination of Fab-TACS® immuno-affinity chromatography and Minisart® High Flow PES syringe filtration with pore sizes of 0.22 µm or 0.1 µm enables selective purification of EVs with specific markers such as CD9 and CD81. Regarding size, the proportion of particles with exosomal sizes of 30 – 150 nm can be significantly increased through the filtration step. Larger particles, such as MVs, are depleted to a considerable extent. The process product of CD9 or CD81 immuno-affinity chromatography and 0.1 µm syringe filtration is an EV population which encompasses some important exosome characteristics, like typical surface markers and small hydrodynamic diameters.

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Abbreviations

HEK	human embryonic kidney
RNA	ribonucleic acid
DNA	deoxyribonucleic acid
Fab	antigen-binding fragment
Fab-TACS®	Fab traceless affinity cell selection
PES	polyethersulfone
PBS	phosphate buffered saline
NTA	nanoparticle tracking analysis
BSA	bovine serum albumin
PBST	phosphate buffered saline with 0.01 % (v/v) Tween 20

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