Detection of MISEV recommended EV Protein-Markers using Automated Western Blotting

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The limited amount of material and the diverse methods for isolation of extracellular vesicle (EV) pose unique challenges to proper characterization of EV preparations. For EV protein content, the “Minimal Information for Studies of Extracellular Vesicles” (MISEV) guidelines recommends characterizing preparations for transmembrane-, cytosolic- and contaminating non-EV proteins.

Compliance with MISEV can mean a considerable effort to the individual laboratories due to lack of easy and robust analytical protocols. Here we present a simple method for isolation of EVs and automated protein separation and immunodetection of MISEV-recommended proteins.

Workflow

Intact EV Isolation by exoEasy

QIAGEN

Automated Western Blot by Jess

protein simple

Optimized Protein Assays

	MISEV category	Target	Catalogue #
EV	1a - Transmembrane	CD63	Ab68418[&]
EV	1b - Transmembrane	CD9	13403S *
EV	2a - Cytosolic	Alix	NBP1-49701
EV	2a - Cytosolic	Annexin V	MAB3991-SP
EV	2a - Cytosolic	Flotillin-1	Ab133497[&]
EV	2b - Cytosolic	ß-Actin	MAB8929
Non-EV	3a - Free Proteins	Albumin	MAB1455-SP
Non-EV	3a - HDL Particles	ApoA1	AF3664-SP
Non-EV	3a - HDL & (V)LDL	ApoE	NBP2-67565

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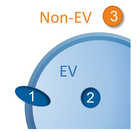
* Cell Signaling
[&] Abcam

◄ *Fig.1 A new and simple workflow for EV protein characterization*

We developed a new workflow for EV protein characterization using the intact EVs eluted from the exoEasy™ kit (QIAGEN) as input for automated western blotting (protein separation and immunodetection) on the ProteinSimple Simple Western Jess instrument. We utilized this workflow for detection of nine prominent targets in categories 1, 2 & 3 of the MISEV guidelines. Results and optimized conditions are presented in Fig. 3. A single isolation yields 250 µL EV eluate, which can be used for > 60 individual protein assays due to the low input requirements of the method (4 µL) or total protein detection (e.g. BCA assay), protein modification reactions (e.g. deglycosylation) as well as stored for later analysis (e.g. -20°C). Using capillary electrophoresis-based protein separation and chemiluminescence-immunodetection we received quantitative, size-based data for all 9 protein assays.



Results

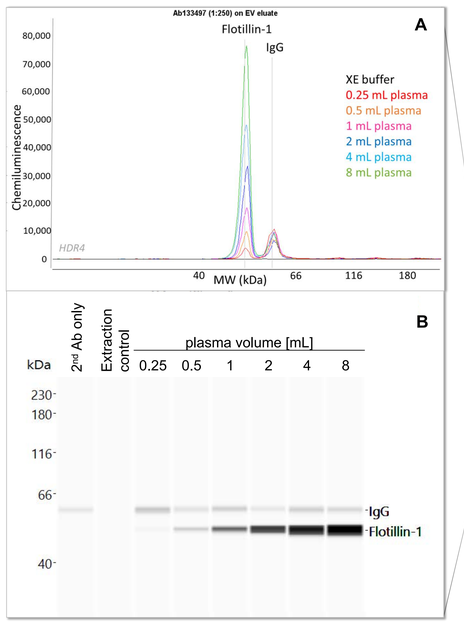


Fig.2: Immunodetected Flotillin-1 signal in 1:10 diluted human plasma derived EV eluate shown as [A] Electropherogram Data and [B] Lane View Image Data at expected MW of ca. 50 kDa. ***Flotillin-1 signal increases with input volume while background signal from plasma-contained antibodies (IgG) stays at the same level and can be excluded via 2nd-antibody-only-control.***

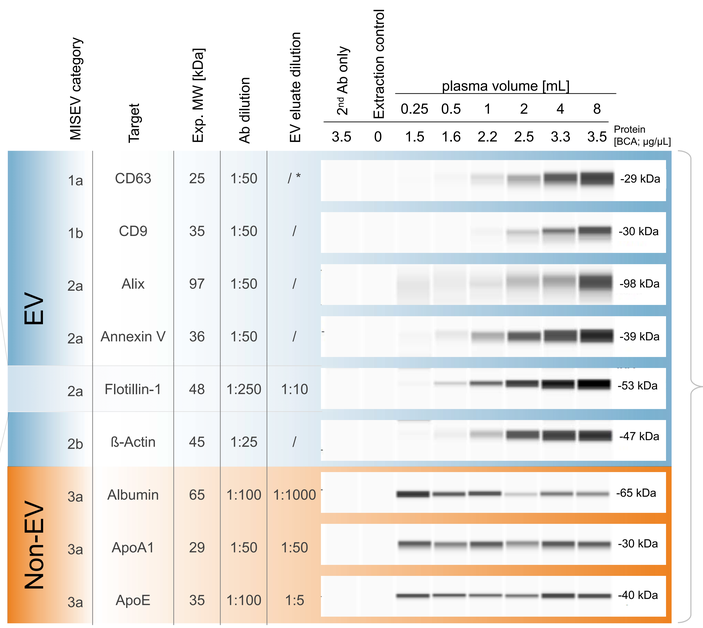


*Fig.3: Virtual Blot-Like Image (HDR4) overview of all immunodetected proteins in plasma-derived EV eluate ordered by MISEV classification and specified settings. For CD63 protein (marked with *) a deglycosylation reaction (PNGaseF, NEB) was essential to receive a band at the expected MW.* ***Increasing human plasma input lead to a dose-response in protein assay signal for immunodetected EV proteins (MISEV category 1-2), but not for contaminants (MISEV category 3).***

To demonstrate the functionality of the new workflow we initially performed a titration of human plasma input. All six EV-positive protein markers from two different categories (1 & 2) can be successfully detected in EV eluates and scale with plasma input while co-isolated IgG (Fig. 2) and other non-EV plasma constituents do not (Fig.3, cat. 3).

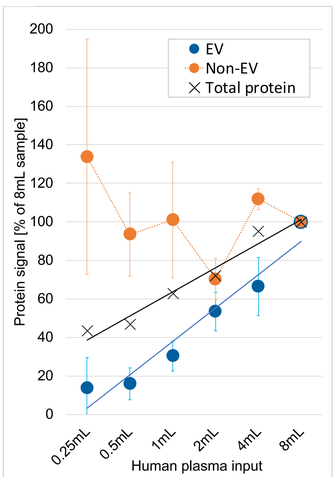


Fig.4: Relation of total protein quantity to immunodetected proteins. Average and SD of calculated peak area from six EV proteins (blue) and three Non-EV proteins (orange) compared to BCA measurement results - all displayed as percentage of maximum input sample. ***Increasing human plasma input lead to an increase of EV proteins similar to the increase in total protein - but not co-eluted non-EV proteins.***

EV proteins seem to scale with the measured total protein amount (Fig.4),demonstrating a useful range of detection and quantitative qualities of the new workflow using as little as 0.25mL human plasma.

To get a better estimation on the amount of contaminating protein, we looked at three different fractions from the same EV isolation (Fig.5).

EV proteins are not detectable in neat plasma but only when enriched by isolation (e.g. Flotillin-1). Non-EV proteins (e.g. ApoA1) are depleted >600-fold in the EV-eluate.

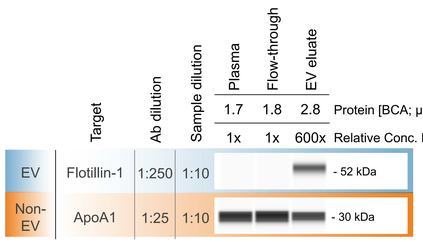


Fig.5: Comparison of Plasma Fractions. EV eluate from 3 mL plasma compared to the extraction's input (neat plasma; 1:50 diluted) and the unbound fraction found in the column flow-through (diluted 1:25 to a final 1:50). ***EV marker Flotillin-1 is not detectable in neat plasma but in EV eluate, while HDL-marker ApoA1 is reduced in EV eluate compared to plasma and FT fraction.***

Conclusions

- A simple workflow for general EV protein characterization
- Robust and quantitative: EV-signals scale with input volume and total protein amount isolated
- Signals from contaminating proteins do not scale with volume - and are efficiently reduced
- Optimized protein assays show MISEV-compliant protein characterization of exoEasy