Multiplexed Electrochemiluminescent Immunoassays for Characterizing Intact Extracellular Vesicles

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1 Abstract

Introduction: To support the growing field of extracellular vesicle (EV) research, there is a need for sensitive and reproducible assays that accurately characterize and quantify EVs from cell lines and human biofluids. We developed assays for intact EVs using an electrochemiluminescent (ECL) multiplexed immunoassay platform that enabled characterization of EVs bearing many surface markers in cell conditioned medium and human biofluids.

Methods: Multiplex sandwich immunoassays were run using MSD's flexible U-PLEX platform, which enables the self-assembly of antibody arrays in the wells of a multi-well plate at the time of use. Arrays were prepared with antibodies targeting three commonly expressed EV surface proteins: CD63, CD81, and CD9. EVs from each sample were captured on the plates, and detected with anti-CD63, -CD81, or -CD9 antibodies labeled with SULFO-TAG™, an ECL emitting label. Cell-line derived EVs were used to test analytical performance of the assays and compare the multiplex assays to singleplex assays where only one capture antibody was present in each well. Dilution linearity and spike recovery in serum, plasma, CSF, and urine were assessed to demonstrate the applicability of these assays to clinical samples. EVs from 19 cell lines and five human biofluids were screened using additional capture antibodies targeting 45 surface proteins suspected to be present on subsets of EVs.

Results: The EV assays yielded a linear response over three orders of magnitude in EV concentration and had sufficient sensitivity to measure EVs isolated from less than 1 µL of serum or plasma. Typical assay CVs were below 5% within the linear range and the multiplexed assay signals deviated by no more than 10% from singleplex assays. Spike recovery was typically between 80% and 120%. For 40 of the surface markers tested, we identified at least one cell model that secreted EVs bearing the surface marker. Twenty-two of these EV phenotypes were detected in at least one human biofluid.

Conclusion: We developed assays for various populations of EVs and demonstrated their analytical performance in commonly used sample types. The flexible multiplex format enables rapid development of assays for new EV subpopulations in cell lines and common human biofluids using minimal sample volume, and for some sample types, without sample purification. These capabilities may allow discovery of new EV populations with potential use as disease biomarkers as well as provide quantitative measurement of the EVs in human samples.

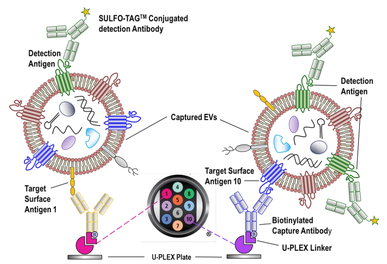
2 Methods

U-PLEX Multiplexed Immunoassays

Biotinylated capture antibodies are each coupled to one of ten unique U-PLEX Linkers, which self-assemble onto unique array elements (or "spots") on the U-PLEX plate. EVs presenting Target Surface Antigens recognized by the capture antibodies bind to the associated spots. Bound EVs are detected using detection antibodies conjugated with electrochemiluminescent labels that recognize Detection Antigen(s) on the EVs.

Electrochemiluminescence Technology

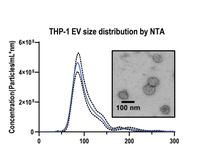
MSD's electrochemiluminescence detection technology uses SULFO-TAG labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® microplates.

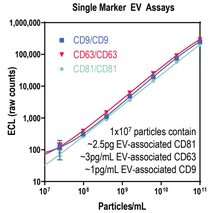


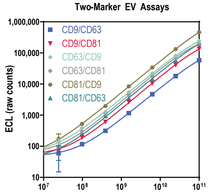
3 High Sensitivity and Wide Dynamic Range Singleplex Tetraspanin EV Assays

EVs were prepared from THP-1 conditioned medium by centrifugation, 0.2µm filtration then ultrafiltration and characterized by nanoparticle tracking analysis and TEM analysis. Nanoparticle tracking analysis (NTA) plot shows mean particle concentration ± 1 SEM. A dilution series of THP-1 EVs was prepared and assayed using single-marker immunoassays, which use the same capture and detection antibody, and two-marker assays, which use different capture and detection antibody targets, to measure CD9+ EVs, CD63+ EVs and CD81+EVs. All assays exhibit a high dynamic range (~3logs) and sensitivity.

Running an m x n matrix of two-marker assays on a sample can be used to compare EV subpopulation abundance and relative expression of surface markers on a given captured EV subpopulation. To demonstrate this, four cell culture models were measured with all combinations of single-marker and multimarker tetraspanin assays (3x3 matrix). To compare EV population abundance, ECL signals from a sample captured using different antibodies but labeled with the same detector can be compared. For example, the data shows many CD9+ EVs from TT cells are captured by CD63, whereas very few are captured by CD81. From the same data set, relative abundance of each marker on a captured population of EVs can be estimated by comparing ECL across different detectors with the same capture. For example in the Expi293 sample, EVs captured with CD63 have low levels of CD63, abundant CD81 and moderate CD9. Finally, the abundance of EV populations in multiple samples can be compared using this data. For example, CD81+ EVs in each cell line follow, Expi293 > HCT-15 > BeWo > TT.





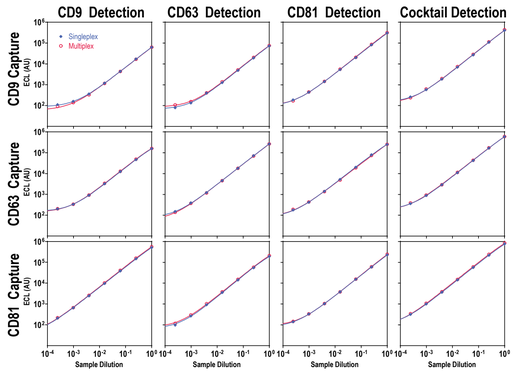


Four Cell Lines Assayed by Two-Marker EV Assays

Cell line	Capture Antibody	CD63	CD81	CD9	Detection Antibody
BeWo	CD63	7,039	14,319	12,731	CD63
	CD81	29,709	10,374	29,668	CD81
	CD9	78,407	85,704	66,220	CD9
TT	CD63	5,704	189	21,435	CD63
	CD81	293	56	280	CD81
	CD9	127,256	2,109	124,446	CD9
HCT-15	CD63	12,248	62,593	83,384	CD63
	CD81	119,232	55,215	256,598	CD81
	CD9	317,826	556,054	532,869	CD9
Expi293	CD63	2,679	31,688	12,161	CD63
	CD81	104,814	190,996	122,606	CD81
	CD9	57,770	171,455	26,891	CD9

4 Multiplex EV Assays Versus Singleplex EV Assays

To compare the performance of multiplex EV assays with singleplex assays, a dilution series of THP-1 cell derived EVs was assayed in both formats using all combinations of CD9, CD63 and CD81 capture and detector antibodies. Each capture was also combined with a cocktail of CD9, CD63 and CD81 detection antibodies. For all assays and across all dilutions the singleplex and multiplex assay signals were within 10% of each other. When the three detector antibodies are combined in a cocktail, the signal is nearly equivalent to the sum of the signals of the individual detectors, as would be expected. These results establish the ability to multiplex our tetraspanin EV assays and decrease sample consumption while maintaining the same level of accuracy and precision as obtained from the singleplex measurements.



5 Assay Performance in Human Biofluids

Dilution Linearity and Spike Recovery

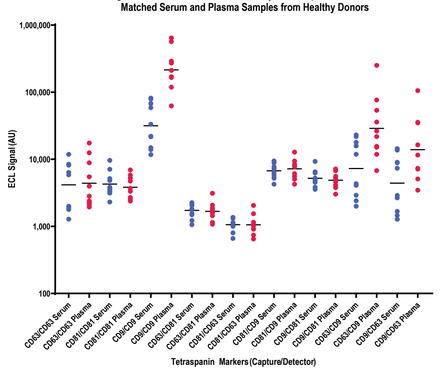
To show compatibility of tetraspinin assays in human matrices, dilution linearity and spike recovery experiments were performed on pooled human serum, plasma, CSF, and urine. Recommended dilutions were selected to yield near 100% dilution linearity. Spike recovery was performed by adding cell-culture derived EVs to the biofluid diluted at the recommended dilution. Spike levels were chosen to be approximately equivalent to the native signal level in the diluted biofluid. Signals from each assay were calibrated using a known EV standard, which allows the raw ECL signals to be converted to consistent units for each EV assay and provides for reliable plate-to-plate and day-to-day data comparison. The table showcases that the tetraspanin assays are compatible with all tested human matrices at listed dilutions.

Sample Type	Assay	Recommended Dilution	Spike Recovery at Recommended dilution	Linear Range of Assay (80%-120% dilution linearity)
Human Serum	CD9/CD9	10-fold	96%	Neat to 1024-fold
	CD63/CD63	10-fold	77%	Neat to 256-fold
	CD81/CD81	4-fold	90%	Neat to 256-fold
Human Plasma	CD9/CD9	50-fold	118%	16 to 1024-fold
	CD63/CD63	20-fold	82%	8 to 32 fold
	CD81/CD81	10-fold	104%	Neat to 256-fold
Human CSF	CD9/CD9	10-fold	96%	4 to 256-fold
	CD63/CD63	10-fold	104%	4 to 256-fold
	CD81/CD81	10-fold	102%	Neat to 256-fold
Human Urine	CD9/CD9	4-fold	94%	Neat to 256-fold
	CD63/CD63	4-fold	94%	Neat to 8-fold
	CD81/CD81	4-fold	87%	Neat to 4 fold

Assaying EVs from Normal Human Biofluids

Single and two-marker EV assays were performed on SEC-purified EVs from matched human serum and plasma from ten healthy donors. Assays that included the marker CD81 as either capture or detector show excellent agreement between matched serum and plasma. This is likely because CD81 is not present on platelet-derived EVs, thus, it is less sensitive to variations in phlebotomy and sample handling that affect EV secretion by platelets. Conversely, CD63 and CD9 are both abundant on platelet-derived EVs which are often elevated in plasma relative to serum, depending on post-phlebotomy sample-handling. Each assay was run in triplicate on EVs purified from 10uL of serum or plasma. These data suggest that serum may be a preferable matrix for analyzing non-platelet derived EVs but that the use of CD81 as one of the targeted surface markers for EV assays may mitigate platelet related variability.

Single Marker and Two-Marker EV Assays in SEC-purified EVs from Matched Serum and Plasma Samples from Healthy Donors



6 Beyond the Tetraspanins: Assaying EV Subpopulations with Additional Specific Surface Markers

Capture antibodies were selected for 45 cell surface markers shown in the table and screened for their ability to capture EVs. Each panel also contains negative control IgG1 antibody. For each target, multiple capture antibody clones were arrayed on MSD® U-PLEX plates and used to capture EVs from conditioned medium of multiple cell lines known to express the target marker. Captured EVs were detected with CD9, CD81, and CD63 antibodies. Markers with similar expected signal levels were grouped together to minimize false positives due to potential cross-reactivity. CD4 is shown here as an example of the capture antibody selection process.

Five EV Capture Panels

Panel 1	Panel 2	Panel 3	Panel 4	Panel 5
CD81	E-Cadherin	Endoglin	CD3	Nectin-4
CD15	EGFR	PSGL-1	MET	FasL
IgG1	IgG1	IgG1	IgG1	IgG1
CD9	EphA2	SCFR/Kit	CD5	TNF-RII
EpCAM	CA-50	sFAS (FASR)	CD8	CD27
CD54 (ICAM-1)	CA-125	FAP-a	CD19	IL-6R
CD31 (PECAM)	CEA	CD4	CD56 (NCAM)	Flk-1 (VEGFR1)
CD276 (B7-H3)	CD50 (ICAM-3)	CA-19-9	CD171 (L1CAM)	Osteoactivin
P-Selectin	Mesothelin	Thrombo-modulin	DPPIV	TNF-RI
CD63	E-Selectin	sTfR-1 (TFRC)	CA-15-3	CD144 (VE-Cadherin)

Screening Antibodies for CD4+ EV capture

Sample	Sample Type	Negative Control IgG1	CD4 Clone A	CD4 Clone B	CD4 Clone C	CD4 Clone D
DPBS	Negative Control	175	89	193	126	172
RPMI+10%FBS	Negative Control	162	66	184	115	129
Jurkat E6.1	Positive Cell Line	195	1575	868	1514	9015
HL60	Positive Cell Line	191	1446	738	1252	10914
THP-1	Positive Cell Line	353	2201	1452	3611	22357
U-937	Positive Cell Line	178	167	223	216	934
MOLT-4	Positive Cell Line	198	209	192	199	770
HDLM-2	Positive Cell Line	217	131	220	175	460
Primary PBMCs	Positive Primary	237	538	489	765	4784
GA10 clone 20	Negative Cell Line	204	83	161	114	160
Ramos	Negative Cell Line	173	64	172	102	90
HUVEC	Negative Cell Line	179	53	185	115	161
PANC-1	Negative Cell Line	178	108	208	135	180
HaCat	Negative Cell Line	186	582	182	117	160
HCT-15	Negative Cell Line	204	366	778	206	227
H-2228	Negative Cell Line	195	87	182	136	148

7 Cell Lines Secrete EVs with Surface Markers Indicative of Lineage

The five assay panels above were used to assess secretion of EV subpopulations in nineteen cell lines. Conditioned media were clarified by centrifugation to remove dead cells, debris and very large EVs but no other purification was needed. Captured EVs were detected with a cocktail of CD9, CD63 and CD81 detection antibodies to maximize signals. Each assay panel contained one spot that was coated with a negative control antibody, used to assess the non-specific binding of EVs from each sample to the plate surface. Assays were also run without sample (Blanks) to assess the assay background. The blank-subtracted ECL values from each specific capture spot were divided by the ECL value produced by the negative control spot in the same well to yield a normalized signal representing the EVs specifically captured by each antibody. All samples were run in triplicate. Epithelial cell lines were observed to secrete EVs expressing typical epithelial markers, e.g. EpCAM, EGFR, EphA2, E-Cadherin, while monocytic cells secreted EVs bearing CD50, PSGL-1, CD31 and CD4. Endothelial cells secreted EVs expressing endothelial markers PECAM, endoglin, and thrombomodulin.

Surface Marker	Source of EVs (Cell Line) MCF-7	HCT116	HCT-15	Expi293	Caltu-3	H2228	PANC-1	GA-10	Ramos	KU-812	THP-1	HL60	Jurkat E6.1	MOLT-4	Hecat	TT	HUVEC	MC-04	HT-1376
CD81	703.8	871.1	728.7	1532.3	207.9	186.9	389.4	67.2	173.7	256.8	752	335.6	845.9	258.6	104.9	11.9	266.1	500.4	613.9
CD15	791.6	1.4	246.1	170.3	15.9	1.4	120.8	0.1	0.2	0.4	323.9	1103.8	3.1	0.9	2	350.1	0.3	1.1	32.4
CD9	599.8	830.2	815.2	813.5	333.6	305.9	377.6	236.8	275.7	268.3	513.4	255.8	355	429.3	922.2	870.8	539.2	609.7	653.2
EpCAM	214.7	245	504	8.9	74.2	7.9	5.4	0.1	0	0.8	1.6	0	0	0.4	24.5	127.9	0.5	3.2	13
CD54	2.9	3.3	89.8	2	23.6	23.9	101.3	0.3	26.2	15.8	187.1	5.2	1.2	0.7	1.6	2	1.8	11.2	1.8
CD31	1.6	1.6	2	1.8	0.6	0.6	0.9	0.4	0.1	2.1	310	9.9	164.5	8.3	1.3	1.6	201.8	132.4	1.3
CD276	199.7	36.3	41.4	160.5	33.5	19.3	50.7	0	0	0	65.9	0	0	0	67.4	28.4	29.5	36.6	23.7
CD63	215.1	331.7	275.6	615.1	153.5	472.3	286.8	33.3	101.3	180.5	541.9	292.3	89.4	18.6	139	379.7	137.9	271.3	342.6
E-Cadherin	23.3	6.3	191.8	1.9	6.4	0.3	0.2	0	0	2.4	2	0	0	NT	17.8	3.8	0	0	11.9
EGFR	2.5	40.1	141.8	65.6	18	21.8	52.6	0	0.2	0	1.9	0.2	0.9	NT	103.3	27.6	1.3	3	70
EphA2	17.1	291.3	174.8	81.9	7.7	6	143.2	0.1	0	0.1	3.5	0.3	1	NT	1.5	4.6	37	142.2	38.3
CA50	0.7	0.5	1.5	0.8	312.6	11.3	0.4	0	0	0.7	0.1	0.5	NT	0.4	10.5	0	0.3	3	
CA125	1	2	2.5	1.5	0.8	3.5	1.2	0	0	0.1	NT	26.2	1.6	0.2	0.7	16.7			
CEA	8.8	1.5	2.6	0.7	17.5	7.3	0.6	0.4	0.4	0.5	1.4	0.8	0.4	NT	193.3	394	0.4	0.7	0.8
CD50	0.5	0.1	0.7	0.4	1.1	0.3	2.9	34.4	111.4	301.1	52.4	211.6	NT	0.4	8.2	0.2	0.5	0.2	
E-Selectin	2.9	8.1	15.6	5.5	0.9	0.1	0.5	0	0	0	4.2	0.1	0	NT	2.7	5.3	1.4	6.2	1.6
Endoglin	0.6	0.6	0.8	0.7	0.9	5.4	7	0.3	0.3	4.9	181	1.9	0.5	NT	0.1	1.4	443	1019.2	4.4
PSGL-1	0.3	0.3	0.9	0.6	0.3	0.2	0.1	0.5	11.1	34.1	288.1	19.4	28.9	NT	0.1	1.1	0.1	0	0.3
SCFR/ Kit	0.4	0.2	4.3	44.7	0.5	0	1.3	0	-0.5	85.6	3.2	0.7	0.2	NT	0	116.7	0.2	4	0
sFAS	3.4	34.2	128.7	154.1	2.4	9.1	3.1	0.3	1.9	0	3.2	4.9	31.4	NT	3.7	9.6	2.3	4.7	5.3
FAP-a	0.4	0.3	0.9	0.6	0.3	0.2	0.1	0	0	0.3	0.1	0.4	0.3	NT	0.4	145.9	0.4	0	0.4
CD4	0.6	0.3	1.1	1.5	0.9	0.2	0.3	0	0	26.5	362.5	109.5	92	NT	0	2.4	0.2	0	0.4
CA19.9	0.3	0.1	0.4	0.5	87.5	3.5	0.1	0.1	0.1	0.3	1.1	0.2	0	NT	0.3	4.5	0.4	0.1	0.9
Thrombomodulin	7.5	0.4	0.6	12.7	0.7	27.1	0.2	0	0	0.8	107	0.1	0	NT	42.7	16.7	11.5	7.6	36.2
sTfR-1	61.1	13.6	117.5	148.7	8.8	45	82.1	12.3	53.4	286	35.5	83.4	32.3	NT	49.3	64.8	6.5	41.8	95.1
CD3	0.4	0	3.6	0.8	0.4	0	0	0	0	0.8	0	364.5	0.7	0	0	1	0	0.2	0
MET	0.1	2.7	6.8	18.4	0.3	0	0	0	0	0.5	0	0	0	NT	0.3	2	0	0	0.7
CD5	1	0.3	1.3	0.3	0.9	0.1	1.3	0.1	0.7	0.3	0.7	0.5	196.1	23.4	0.4	1.4	0.7	0.6	0.4
CD8	1.2	1.9	5.1	1.7	0.7	0.3	0.6	0	0.3	0	2.9	0.2	0.2	16.8	1.8	2.4	0.5	1	0.7
CD19	0.5	0.2	0.7	0.6	0.2	0.1	0.2	32.3	120.7	0.2	3.8	0.1	0.3	0.2	0.1	0.3	0	0.2	0.1
CD56	0.8	0.1	0.8	33.1	0.5	0	0.4	0	0.5	0	1.8	0.3	0.2	0.5	0.3	25.9	0.5	0.8	0.1
CD171	24.5	2.1	28.6	1.9	1.3	0.5	15.3	0.1	0.5	0.1	35.4	0.2	0.3	1.3	1.7	3.3	0.1	1.7	1.7
DPPIV	1.6	4.6	5.4	8.5	1.4	74.1	0.7	0.1	0.3	0.1	2.4	0.3	0.3	1.2	2.6	1.5	0.9	10.2	1.1
CA15.3	1.2	0.1	1.3	0.6	0.4	2.4	2.9	0	0	0.5	0.7	0	0.1	0.7	0	23.6	0	0	16.4
TNF-RI	0.4	0.1	1.1	0.4	0.3	0	0	0	0	0.6	1.2	0	NT	0.4	0	3.1	0.1	0.4	0.3
IL-6R	1.1	1.1	2.8	5.8	0.7	0.4	1.2	0.4	0	0.6	9.1	2.2	0.4	NT	0.6	2.6	0.2	1	0.7
TNF-RI	1.6	1.4	5	17.7	0.7	0.4	0.9	0	0	1	98.3	3.2	4.3	NT	1.2	1.4	0.5	0.9	4
Flt-1	0.6	0.2	5.8	7.8	0.4	0	0.2	0.2	0.1	0.1	2.8	0.4	6.1	NT	0.1	2.5	1.2	4.4	0
CD144	0.7	0.2	1	0.4	0.8	0	0	0	0	0	0.1	0.6	4.1	10.1	0.2	0.1	0.2	16.7	

8 Screening Human Biofluids for EVs

Human biofluids were screened for EVs with the same five assay panels. The table shows the ratio between the background subtracted signal on each specific capture spot and the negative control spot in the same well for the 23 markers that were detectable in one or more of the tested sample types. The abundant EV populations in human biofluids mostly expressed markers typically present on leukocytes, endothelial cells, and platelets. Populations expressing typical epithelial markers were notably low or undetectable in normal human biofluids.

Surface Marker	Source of EVs (Sample Type) Urine	CSF	PBMC CCM 1	PBMC CCM 2	Plasma	Precipitated Plasma EVs	Serum	Precipitated Serum EVs
CD81	41.8	799.1	48.3	112.8	41.2	32.2	278.1	152.6
CD15	22.8	3.3	112.5	3.6	3.8	4.7	6.3	6.3
CD9	295.6	448.4	211.2	377.9	243.4	230.4	342.5	115.5
CD54/ ICAM-1	0.5	3.1	5.7	4.2	1.6	2.8	1.9	3.8
CD31/ PECAM	0.4	3	42.8	140.6	226.1	276.8	181.4	47.5
CD276/ B7-H3	0	13.6	1.1	0.2	1.7	1.4	0.3	1.2
P-Selectin	0	0.7	14.4	14.6	205.6	502.8	49	27.7
CD63	281.5	214.2	331.6	83.2	115.9	110	249.3	60.3
CEA	10	1	0.9	0.6	1.8	1.9	4.2	0.8
CD50 (ICAM-3)	0.1	12.1	9.6	25.5	2.9	9.8	7.9	2.9
Endoglin	0.3	2.3	0.5	3.5	7.1	20.8	9.1	1.5
PSGL-1	0.2	1.5	22.1	15.5	3.6	16.9	12.4	2.9
sFAS (FASR)	0.4	22.4	2.3	2.8	1.5	2.8	0.9	0.7
CD4	0.1	2.3	27.4	20.3	3.6	7	11.8	1.3
Thrombo-modulin	0	0.4	0.8	0.6	6.2	9.8	3.5	1
sTfR-1 (TFRC)	0.1	1.4	2.6	0.3	1.5	15.1	0.6	0.5
CD3	0.6	0.2	10.9	8	18.9	6.1	2	1
CD5	0.5	0.1	7.3	6.6	14.4	2.9	1.8	3.8
CD8	0.3	0.3	87.3	24.4	27.6	6.6	14.1	11
CD19	0.2	0.1	1.1	8	57.6	3.5	3.6	5.2
DPPIV	10.5	0.1	0.9	1	22.2	1.4	0.4	2.2
CA15.3	56.7	0.1	0.3	0.4	5.5	0.8	0.2	1.2
TNF-RI	0.2	5.2	0.9	2.3	3.2	1.4	0.8	0.7

9 Conclusion

We developed a set of assays and techniques for measuring various populations of EVs in culture medium and human biofluids. We demonstrated that the assays have excellent analytical performance in commonly used sample types. The assays could be formatted to detect EVs based on the presence of a single surface marker, or require the presence of two different surface markers on the same EV. The assays enable EV characterization and quantitation in complex samples with minimal sample preparation. The flexible multiplex format minimizes sample consumption and enables large screening experiments and rapid development of assays for new EV subpopulations. These capabilities may allow discovery of new EV populations with potential use as disease biomarkers as well as provide quantitative measurement of the EVs in human samples.

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200-EV-719