bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

1 **Critical comparison of ultracentrifugation and a commercial kit for isolation of**
2 **exosomes derived from glioblastoma and breast cancer cell lines**

3 Frøydis Sved Skottvoll[a]*, Henriette Engen Berg[a]*, Kamilla Bjørseth[a], Kaja Lund[b], Norbert
4 Roos[c], Sara Bekhradnia[a], Bernd Thiede[c], Hanne Roberg-Larsen[a], Bo Nyström[a], Elsa
5 Lundanes[a], Steven Ray Wilson[a]**

6 [a]Department of Chemistry, University of Oslo, Post Box 1033, Blindern, NO-0315 Oslo, Norway

7 [b]Department of Microbiology, Unit Cell Signaling, Oslo University Hostpital, Gaustadalleen 34,

8 NO-0372 Oslo, Norway

9 [c]Department of Biosciences, University of Oslo, Post Box 1066, Blindern, NO-0316 Oslo, Norway

10

11 * These authors contributed equally to the work.

12 ** stevenw@kjemi.uio.no, +47 97010953

13

14 **Abbreviations:** ABC, ammonium bicarbonate; ACN, acetonitrile; AGC, automatic gain
15 control; BC, breast cancer; BSA, bovine serum albumin; dd, data-dependent; DLS, dynamic
16 light scattering; DTT, dithiothreitol; ECL, enhanced chemiluminescence; ER, endoplasmic
17 reticulum; EVs, extracellular vesicles; FA, formic acid; FBS, fetal bovine serum; FSG, fish
18 serum gelatine; GBM, glioblastoma multiforme; GO, gene ontology; IAM, 2-iodoacetamide;
19 ISEV, The International Society of Extracellular Vesicles; LC-MS/MS, liquid
20 chromatography tandem mass spectrometry; MP, mobile phase; MS, mass spectrometry;
21 MVBs, multivesicular bodies; nuclear magnetic resonance, NMR; PBS, phosphate buffered
22 saline; RPMI, Rosewell Park Memorial Institute; RT, room temperature; S/N, signal to noise
23 ratio; TEM, transmission electron microscopy; TFA, trifluoroacetic acid; UC,
24 ultracentrifugation; WB, Western blot

25

26 **Key words:** *Exosomes, Ultracentrifugation, LC-MS, Proteomics, Glioblastoma, Breast*
27 *cancer*

28

29

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

30 # ABSTRACT

31 Exosomes are small extracellular vesicles (around 30-100 nm in diameter) that are secreted
32 from cells and can be found in most body fluids. Exosomes can be a vital source of
33 biomarkers as they contain various substances (e.g. lipids, RNAs, metabolites and proteins)
34 that can reflect the cell of origin (e.g. cancer cells). For isolating exosomes present in
35 biological matrices, ultracentrifugation (UC)-based procedures are most common. Other
36 approaches exist, including commercial kits developed for easy and low sample volume
37 isolation. In this study, the performance of differential UC and an isolation kit from a major
38 vendor (Total Exosome Isolation Reagent from Thermo Fisher) were compared. Exosomes
39 were isolated from cell culture media of two different cell lines (patient derived cells from
40 glioblastoma multiforme and the breast cancer cell line MDA-MB-231). Transmission
41 electron microscopy (TEM), dynamic light scattering (DLS) and Western blot (WB) indicated
42 the presence of exosomes for both isolation methods. The isolation kit (using <10 % of the
43 sample volume than for UC) and UC contained similar amounts of protein determined by
44 bicinchoninic acid assay (BCA) with absorbance at 562 nm. Using WB, positive exosome
45 markers were identified in all samples. Using mass spectrometry (MS)-based proteomics,
46 additional exosome markers were identified. Regarding glioblastoma exosomes, the number
47 of proteins identified with MS was higher for the UC samples when injecting equal protein
48 amounts, opposite to the breast cancer exosomes. However, negative markers could also be
49 found using MS. Thus, we are cautious to use the term "exosome isolation" as impurities may
50 be present regarding both procedures. Notably, known potential biomarkers for both diseases
51 were detected in the samples using MS. In our opinion, the two isolation methods had rather
52 similar performance, although with some minor differences based on cell of origin.

53

54

55

56

57

58

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

59 # 1 Introduction

60 Exosomes are extracellular vesicles (EVs) with small membrane-bound bodies of 30-100 nm
61 sizes which are secreted from cells to the extracellular environment as a part of the endocytic
62 pathway [1]. Exosomes are formed by invagination of an endosome membrane to create
63 intraluminal vesicles inside the endosome (multivesicular bodies (MVBs)) and are secreted
64 when the endosomes fuse with the plasma membrane [2]. Exosomes commonly contain
65 proteins originating from the cellular cytosol and the plasma membrane (for both common
66 proteins and proteins specific to the cell type), nucleic acids (e.g. DNA, mRNA, microRNA
67 and non-coding RNA), lipids and metabolites [1, 3-8], and are believed to take part in e.g.
68 cell-cell communication, transfer of proteins/nucleic acids, coagulation and antigen
69 presentation [6, 9].
70
71 Cancer cells have been found to release more exosomes than stromal cells [10, 11] and are
72 linked to metastasis and tumor progression [7, 12, 13]. Hence, cancer exosomes, when e.g.
73 isolated from body fluids, may be a source of biomarkers for diagnosing cancers such as
74 breast cancer (BC) and glioblastoma multiforme (GBM). BC is the most dominant type of
75 cancer in women [14], with recurrent metastatic disease being responsible for the majority of
76 BC deaths [15]. GBM is the most frequent occurring form of malignant glioma (tumor
77 originating in glial cells in the brain) in adults and is a highly aggressive astrocytoma [16-18].
78 The diagnosis of both BC and GBM rely on highly invasive patient tissue biopsies at
79 relatively late stages [16, 19, 20]. Thus, a non-invasive disease monitoring e.g. for early
80 diagnosis and prognosis assessment using biomarkers in more accessible body fluids such as
81 blood (liquid biopsy) is desirable for both GBM and BC [16, 21, 22]. Hence, the isolation of
82 exosomes for cancer biomarker discovery has emerged [23-30].
83
84 The isolation of exosomes is mostly performed from body fluids (e.g. blood, urine, and saliva)
85 or cell culture media by centrifugation-based methods (e.g. sucrose gradient
86 ultracentrifugation and ultracentrifugation, UC) [31, 32]. In addition, other isolation protocols
87 and principles have been developed to overcome the drawbacks of ultracentrifugation such as
88 large amounts of starting volume needed and poor reproducibility [8, 33-39]. Moreover, there
89 is a great need for exosome isolation protocols to tailor towards the isolation of smaller
90 starting volumes (< µL) for e.g. miniaturized cell culture models like organoids and "organ on
91 a chip" [40, 41]. However, there is a lack of consensus between the laboratories as the
92 methods for rigorous isolation are still largely empirical.

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

93

94 The protein content of exosomes has been previously extensively characterized using Western
95 blotting, but also by liquid chromatography tandem mass spectrometry (LC-MS/MS) [42-44].
96 Tetraspanins (e.g. CD9, CD63 and CD81) have been used as positive exosome protein
97 markers for targeted analysis as they are particularly known to be enriched in exosomes
98 compared to cells [1, 45-48]. These positive exosome markers generally take part in exosome
99 biogenesis, and are hence expected to be present in, but are not specific to, exosomes [49]. A
100 large range of exosome markers (both positive and negative) for exosome characterization are
101 presented by The International Society of Extracellular Vesicles (ISEV) [45]. In addition,
102 larger protein databases covering occurring proteins in exosomes and other extracellular
103 vesicles are available [50-52]. ISEV also recommends using other characterization methods in
104 addition to WB and LC-MS/MS, to study the heterogeneity and morphology of vesicles
105 present in the isolated sample.
106
107 In the present study, we have compared two exosome isolation methods (UC and a Total
108 Exosome Isolation kit from Thermo Fisher). The methods were evaluated using
109 characterization techniques recommended by ISEV: WB, transmission electron microscopy
110 (TEM), dynamic light scattering (DLS), quantitative total protein analysis using UV-Vis
111 spectrophotometry and LC-MS/MS for untargeted proteomic analysis. Exosomes were
112 isolated from cell culture media from patient-derived GBM cells (T1018) and one BC cell line
113 (MDA-MB-231). The impact of different isolation techniques for these cell lines have not yet
114 been studied, and studies of exosome presence and purity are also limited for these cell lines.
115 We also present a critical view of the characterization methods used to detect exosomes and
116 evaluate the purity of the exosomes samples.

117 2 Material and Methods

118 Unless otherwise stated, water (commonly type 1 water purified by a Direct-Q® water
119 purification system from Millipore (Billerica, MA, USA)) was used as solvent. For detailed
120 information about chemicals, solutions and experimental methods used, see **Supplementary**
121 **Materials 1** (**SM-1**).
122

123 2.1 MDA MB-231 cell culturing

124 The BC cell line was purchased from American Type Culture Collection (ATCC, Sesto San
125 Giovanni, Milan, Italy) and is a triple-negative human metastatic breast carcinoma. The cells

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

126 were maintained in Rosewell Park Memorial Institute (RPMI) 1640 growth medium depleted
127 of phenol red (Sigma-Aldrich, St.Louis, MO, USA) supplemented with 10% exosome-
128 depleted fetal bovine serum (FBS) (System Biosciences, Palo Alto, CA, USA) and 1 %
129 penicillin/ streptomycin (Sigma- Aldrich). The cells were incubated in a humidifying
130 atmosphere at 5% CO_2 and at 37 °C. Prior to exosome isolation, 1-2.3 million cells (in T75-
131 T175 culturing flasks) were incubated for 6-7 days (always using a passage lower than 12).
132 The incubated cell culture medium was spun down at a RCF of 906 *g* (30 minutes at 23 °C).
133

134 **2.2 Glioblastoma cell culturing**

135 The glioblastoma (GBM) cell line (T1018) was obtained from the Langmoen Lab at Oslo
136 University Hospital, Norway. The cell line is derived from primary glioblastoma multiforme
137 tumours. The cells were maintained in DMEM/F12 medium (Gibco, Thermo Fisher
138 Scientific, Waltham, MA, USA). The medium was supplied with HEPES buffer (10mM) and
139 penicillin/ streptomycin from Lonza (Basel, Switzerland), B27 w/o Vit A from Thermo
140 Fisher, epidermal growth factor (20 ng/mL) and basic fibroblast growth factor (10 ng/ mL)
141 from R&D Systems (Minneapolis, MN, USA) and heparin (2,5 µg/mL) obtained from LEO
142 Pharma AS (Ballerup, Denmark). The cells were incubated in a humidifying atmosphere at 5
143 % CO_2 and 37 °C. Prior to exosome isolation, the incubated cell culture media was
144 centrifuges twice at a RCF of 453 g and 1811 *g* for 5 minutes each.
145

146 **2.3 Exosome isolation by ultracentrifugation**

147 For the BC and GM cells, 9-12 mL and 60 mL cull culture media were used for
148 centrifugation. Cell culture media was thawed on ice and centrifuged at a RCF of 1811 *g* (5
149 minutes). The supernatant was centrifuged at a RCF of 20 000 *g* (20 minutes at 20 °C) with an
150 Allegra 25R centrifuge (with TA-14-50 rotor) from Beckman Coulter (Brea, CA, USA) and
151 the supernatant was transferred to polycarbonate ultracentrifugation tubes (Beckman Coulter)
152 and diluted with phosphate buffered saline (PBS) until the tubes were full. The tubes were
153 centrifuged twice at a RCF of 100 000 *g* (90 minutes at 4 °C) with an L-80 ultracentrifuge (45
154 Ti rotor) from Beckman Coulter. The supernatant was removed (leaving suspension 1 cm
155 above the pellet) and the pellet was suspended with PBS between the centrifugations. Upon
156 centrifugation, the supernatant was discarded and the pellet was suspended in either PBS (3
157 mL for DLS- and 50-100 µL for TEM analysis) or the preferred lysis buffer (**Section 0 and**
158 **0**).
159

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

160 **2.4 Exosome isolation by isolation kit**

161 The isolation of exosomes with the use of the Total Exosome Isolation Reagent (from cell
162 culture media) was from Thermo Fisher (catalog nr. 4478359). The isolation was performed
163 according to the protocol of the supplier [53]. Starting volumes were ranging from 0.5-9 mL
164 cell culture media for the BC cells and 5-6 mL for the GBM cells. The samples were
165 centrifuged with the Allegra 25R centrifuge, and the pellet was suspended as with
166 ultracentrifugation (**Section 0**).
167

168 **2.5 Protein extraction and measurement with UV-Vis spectrophotometry**

169 Cell and exosome protein extracts were made by adding RIPA buffer (Thermo Fisher)
170 containing protease inhibitors (Protease Inhibitor Coctail Tablets, Roche, Basel, Switzerland)
171 and phosphatase inhibitors (PhosStop Tablets, Sigma-Aldrich). The BC exosomes were
172 extracted in 50 µL of the RIPA solution, and the BC cells in the 100 µL RIPA solution. The
173 GBM exosomes were extracted in 300 µL of the RIPA solution, and the cells in 1 mL RIPA
174 solution. The extracts were incubated while rotating for 30 minutes (4 ℃), and then snap
175 frozen (at -80 ℃). Extracted samples were thawed and centrifuged at a RCF of 20 570 *g* (30
176 minutes at 2℃) using a Heraeus Fresco 21 centrifuge or Eppendorf Centrifuge 5424R. The
177 pellet was discarded. The protein amount was determined using Pierce™ BCA protein Assay
178 Kit (Thermo Fisher), by measuring the absorbance at 562 nm. The BC protein measurements
179 were performed using a Wallac Victor2 1420 multilabel counter and Wallac 1420
180 Workstation software (version 3.00), both from Perkin Elmer (Waltham, MA, USA). The
181 GBM protein measurements were performed on a Nanodrop 2000 Spectrophotometer
182 (Thermo Fisher).
183

184 **2.6 Western blotting**

185 The samples containing 3-15 µg protein were diluted giving equal volume of water before
186 adding 5x loading buffer (see **SM-1** for solution preparation) to yield 1x. Prior to
187 electrophoresis, the samples were boiled for 3 minutes at 92 ℃. The samples with added
188 PageRuler™ Prestained protein ladder (Thermo Fisher) were loaded on to 4-12 % Bis-Tris
189 gels (Thermo Fisher) and run at 70-75 V for 1 hour. The voltage was increased to 110 V after
190 the first hour if the bromophenol blue lane was horizontally distributed. MOPS or MES was
191 used as SDS running buffers (Thermo Fisher). Proteins were transferred to a 45 µm
192 nitrocellulose membrane (Thermo Fisher) by semi-dry electroblotting (1 W, at 4 ℃ overnight)
193 with a transfer chamber from Bio-Rad (Hercules, CA, USA). The transferred proteins were

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

194 blocked with a blocking solution consisting of (5:95, *w/v*) non-fat dry milk (PanReac
195 AppliChem ITW reagents, Darmstadt, Germany) in tween-20/ TBS (0.05:99.95, *v/v*)
196 (Medicago, Uppsala, Sweden) for 1 hour on a mixing plate. The proteins were then stained
197 with primary antibodies (at 4 °C overnight) in blocking solution and with secondary
198 antibodies for 2 hours at room temperature with rocking in blocking solution. The antibodies
199 CD9 (10626D), CD63 (10628D) and CD81 (MA5-13548 and 10630D) were purchased from
200 Thermo Fisher. The antibody TSG101 (T5701) was purchased from Sigma-Aldrich, and anti-
201 flotillin-1 (610821) and anti-calnexin (610523) antibodies were purchased from BD
202 Biosciences (San Jose, CA, USA). The secondary antibodies (sc-2954 and sc-2955) were
203 purchased from Santa Cruz (Dallas, TX, USA). Next, the membranes were further washed
204 with tween-20 in TBS (0.05:99.95, *v/v*) for 30 minutes on a mixing plate. Protein bands were
205 visualized using an ECL- prime from GE Healthcare (Buckinghamshire, UK) and
206 Transparency films from Nobo (integrated part of ACCO Brands Corporation, Lake Zurich,
207 IL, USA). The bands were developed in a ChemidocTM touch imaging system (Bio-Rad).
208 The antibody actin (A2066, from Sigma) was used as a positive control (results not shown).
209

210 **2.7 Immunogold labelling and transmission electron microscopy**

211 One drop of 5-50 µL of the exosome samples was placed on clean Parafilm, and the formvar
212 coated copper grid (100 square mesh) was carefully placed to float on the drop with the
213 coated side facing the suspension. The material was adsorbed for 5-20 minutes, before rinsing
214 on two large drops of PBS for 5 minutes followed by incubation on a drop (8 µL) of the
215 primary anti-CD9 (PA5-11559) from Thermo Fisher (diluted 1+ 9 with fish serum gelatine
216 (FSG) in PBS (1:99, *v/v*)) for 20 minutes. Next the grids were again washed on two large
217 drops of PBS for 5 minutes and incubated on a drop (5 µL) of rabbit anti-mouse antibody
218 (Z0259, Dako Glostrup, Denmark) (diluted 1:200 with the FSG in PBS solution) for 25
219 minutes, before repeating the washing on two drops of PBS for 5 minutes. Prior to gold
220 labelling, the grid was incubated on one drop of the diluted protein A-gold solution (1:50 in
221 the FSG in PBS solution) (10 nm particle size) for 20 minutes, from Cell Microscopy Core
222 (CMC, University Medical Center Utrecht, Utrecht, The Netherlands). A final wash on 5
223 drops of PBS (2 minutes) followed by 5 drops of water (3 minutes) was performed before
224 negative staining with uranyl acetate/water (4:96, *w/v*) for 2 minutes. Excess fluid was
225 removed, and the grids were stored in a storage box at room temperature (RT) until use. The
226 samples were visualized with a JEM-1400Plus transmission electron microscope from JEOL
227 (Tokyo, Japan) and images were recorded at 80 kV.

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

228

229 **2.8 Dynamic light scattering**

230 The dynamic light scattering (DLS) experiments were conducted with the aid of an
231 ALV/CGS-8F multi-detector version compact goniometer system, with 8 fiber-optical
232 detection units, from ALV-GmbH., Langen, Germany. The beam from a Uniphase cylindrical
233 22 mW HeNe-laser, operating at a wavelength of 632.8 nm with vertically polarized light,
234 was focused on the sample cell (10-mm NMR tubes, Wilmad Glass Co., of highest quality)
235 through a temperature-controlled cylindrical quartz container (with 2 plane-parallel windows),
236 vat (the temperature constancy being controlled to within ± 0.01 ^{o}C with a heating/cooling
237 circulator), which is filled with a refractive index matching liquid (*cis*-decalin). The polymer
238 solutions were filtered in an atmosphere of filtered air through a 5 µm filter (Millipore)
239 directly into precleaned NMR tubes. The measurements were carried out at 25 ^{o}C. The
240 measurements revealed two relaxation modes, one fast and one slow mode. This suggests that
241 there is a coexistence between single entities and aggregates in the solution. The analyses of
242 the correlation function data are presented in **SM-1** (**SM-1.6 DLS**).

243

244 **2.9 Protein digestion**

245 *2.9.1 In-gel digestion*

246 Prior to in-gel digestion, the samples were prepared and run using the same procedures as
247 with gel electrophoresis (**Section 0**). Bovine serum albumin (BSA) was prepared in parallel.
248 The gel was covered by a fixation buffer (water/ methanol/ acetic acid, 40:50:10, *v/v/v*)
249 overnight (18 hours at 4°C), stained with Coomassie brilliant blue for 4 hours at RT and
250 destained overnight with water. The gel was cut to yield four fractions from each gel lane;
251 approximately 0-25 kDa, 25-70 kDa, 70-130 kDa (70-250 for BC samples) and 130-up kDa
252 (250-up kDa for BC samples). The lane containing BSA was cut in the mass range of 55-70
253 kDa. Each fraction was transferred to Protein LoBind tubes. The samples were further
254 reduced, alkylated and digested using the protocol of Shevchenko et al. [54], with trypsin
255 from Promega Biotech AB (Nacka, Sweden). The digested samples were concentrated to
256 dryness and kept at -20 °C until further use (not more than 7 days).

257

258 *2.9.2 In-solution digestion with peptide desalting*

259 The samples were concentrated, and the pellet was dissolved with 25 µL 6 M urea in 100 mM
260 ammonium bicarbonate (ABC). Then, the samples were reduced with 9.5 mM dithiothreitol

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

261 (DTT, 30 minutes at 30 °C) and alkylated with 25 mM 2-iodoacetamide (IAM, 60 minutes, at
262 RT and in the dark). The reduction with DTT was repeated for the BC samples, with 28 mM
263 DTT (30 minutes at 30 °C). The proteins in the BC exosomes were initially digested by
264 adding 0.1 µg Lys-C (120 minutes at 37 °C), before the samples were diluted to a final
265 concentration of 19 mM ABC. The trypsin digestion of the samples was performed with 1 µg
266 trypsin (16 hours at 37 °C) and the protease activity was terminated with formic acid,
267 FA/water (0.9:99.1, *v/v*). The desalting and concentrating of the samples were performed
268 using ZipTip® (silica particles with C_{18} resin) from Millipore. The ZipTip was wetted with
269 neat acetonitrile (ACN) and equilibrated with trifluoroacetic acid, TFA/water (0.1:99.9, *v/v*).
270 The peptide samples were then pipetted through the ZipTip. Washing the ZipTip was
271 performed with water/ methanol/ TFA (94.9:5:0.1, *v/v/v*) and the peptides were eluted with 5
272 µL water/ACN/TFA (29.9:70:0.1, *v/v/v*). The filtrate was concentrated to dryness at 30°C and
273 frozen at -20°C until further use (not more than 30 days).
274

275 **2.10 LC-MS/MS analysis**

276 Unless otherwise stated, the dilutions during sample preparations were performed using water
277 (HiPerSolv Chromanorm®) from VWR. A Q-Exactive™ equipped with a nanoFlex
278 nanospray ion source from Thermo Fisher was used for the LC-MS/MS analysis at the two
279 laboratories.

280 *2.10.1 LC-MS/MS analysis at laboratory 1*

281 The precolumn (50 µm ID x 20-50 mm) and analytical column (50 µm ID x 150 mm) were
282 packed with C_{18}- Accucore particles (2.6 µm beads, 80 Å pore size) from Thermo Fisher,
283 using the developed method as described in our previous study [55]. An EASY-nLC 1000
284 pump (with autosampler) from Thermo Fisher was applied throughout the experiments. The
285 solvents used for peptide separation was of FA/ water (0.1:99.9, *v/v*) (mobile phase A, MP A)
286 and of ACN/ FA (0.1:99.9, *v/v*) (mobile phase B, MP B). Prior to LC-MS analyses, the
287 samples were thawed and dissolved in 10-15 µL FA/ water (0.1:99.9, *v/v*). 10 µL of the
288 sample was injected with a flowrate of 20 µL/min. Trapping of the analytes in the pre-column
289 was performed with 100 % MP A at a maximum flow rate restricted not to reach above 500
290 bar (12 µL). A 120- minute linear gradient elution from 3-15 % MP B with a flow rate of 130
291 nL/min was set for the exosome samples, starting at 3 % MP B for 3 minutes before the
292 percentage of MP B was increased to 15 % in 120 minutes. The MP B was increased to 50 %
293 for 5 minutes before the percentage of MP B increased to 80 % for 2 minutes (flow rate was
294 also increased to 173 nL/ min) and kept at 80 % MP B for another 15 minutes. The eluting

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

295 peptides were ionized at 1.8 kV at 250 °C for the BC exosome samples and at 275 °C for the
296 GBM exosome samples. The MS was operated in data-dependent (dd) positive mode to
297 automatically switch between MS and MS/MS acquisition. Survey full scan MS spectra (with
298 a mass filter of *m/z* 350 to 1850) were acquired with a resolution of 70 000, automatic gain
299 control (AGC) of $1x10^6$ and a maximum injection time of 120 ms. When dd/MS/MS, the
300 resolving power was set to 17 500, the AGC to $1 x10^5$ and the maximum injection time to 60
301 ms. Charges of 1, 7 or ≥ 8 were excluded and dynamic exclusion was set to 70.0 seconds. The
302 method allowed sequential isolation of up to the ten most intense ions depending on signal
303 intensity (intensity threshold $2.0 x10^4$), with isolation window of *m/z* 1.8.
304

305 *2.10.2 LC-MS/MS analysis at laboratory 2*

306 The analytical column applied was an Acclaim PepMap 100 column (C18, 3 µm beads, 100
307 Å, 75 µm ID) of 500 mm bed length and an Ultimate 3000 nano ultra-HPLC system from
308 Dionex (Sunnyvale, CA, USA) was connected to the LC-MS system. The MP A was set to
309 FA/water (0.1:99.9, *v/v*) and the MP B was set to be ACN/ FA/ water (90:0.1:9.9, *v/v/v*). A
310 207- minute linear gradient elution from 4-35 % MP B with a flow rate of 300 nL/ min was
311 set for the exosome samples. The percentage of MP B was increased to 50 % in 20 minutes
312 and 80 % MP B in 2 minutes. The MS was operated in data-dependent (dd) positive mode.
313 Survey full scan MS spectra (with a mass filter of *m/z* 400 to 1700) were acquired with a
314 resolution of 70 000, automatic gain control (AGC) of $3x10^6$ and maximum injection time of
315 100 milliseconds. When in dd/MS/MS the resolving power was set to 35 000 and the
316 maximum injection time to 120 milliseconds. The dynamic exclusion was set to 60.0 seconds.
317 The method allowed sequential isolation of up to the ten most intense ions depending on
318 signal intensity (intensity threshold $1.7x10^4$) isolation window was *m/z* 2 without offset.
319

320 *2.10.3 Data processing and protein identification*

321 Proteome Discoverer (version 1.4.0.228) was used to identify the peptides and proteins. The
322 proteins were identified using both SEQUEST and MASCOT algorithms. All searches were
323 performed setting the digestion enzyme to trypsin with maximum one missed cleavage,
324 fragment ion mass tolerance of 0.10 Da and a precursor mass tolerance of 10.0 ppm. Signal to
325 noise (S/N) threshold was set to 1.5 and the minimum ion count to 1. Carbamidomethylation
326 of cysteine was specified as static modification. Oxidation of methionine, acetylation of the
327 N-terminus and deamidation of glutamine and asparagine were specified as dynamic

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

328 modifications. The proteins were identified with high peptide confidence filter. Proteins
329 identified as keratin or trypsin were removed from the list, in addition to proteins found in the
330 blank samples.

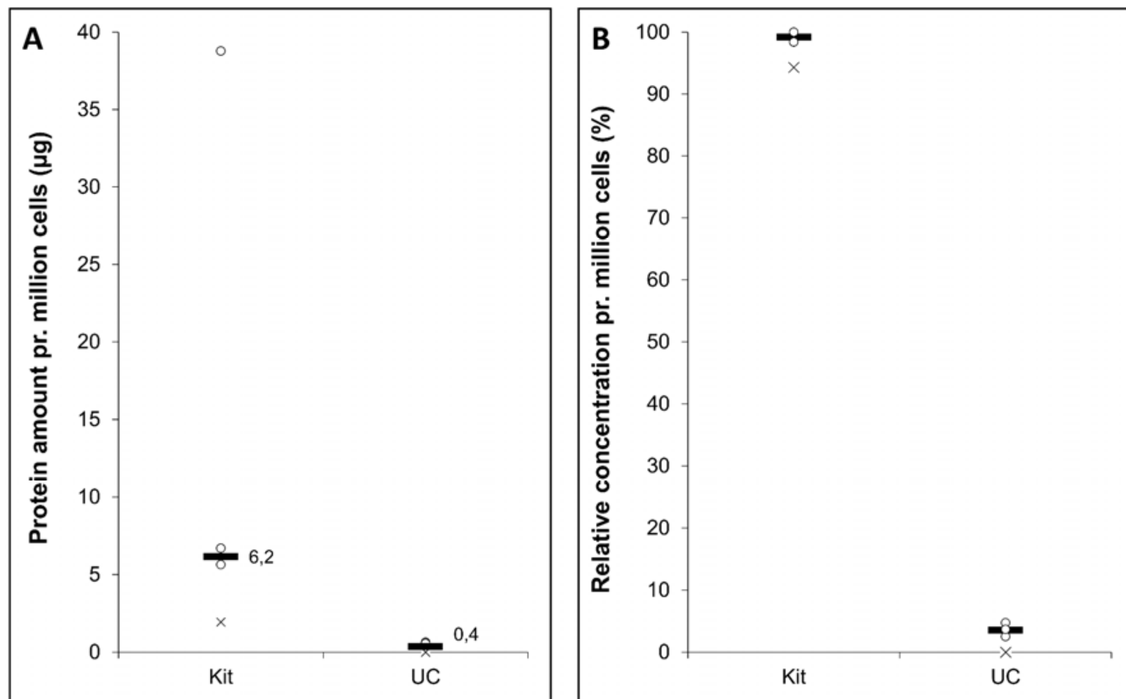
331 3 Results and Discussion

332 For comparison of the two exosome isolation methods (UC and Total Exosome Isolation kit)
333 using the cell lines T1018 (GBM) and MDA-MB-231 (BC), common characterization
334 methods were investigated and evaluated for their ability to prove the presence of exosomes
335 and/or determine the purity. The standard techniques TEM for morphological analysis and
336 WB for exosome marker analysis were performed in addition to total protein determination,
337 DLS and LC-MS/MS. Comprehensive proteome analysis using LC-MS/MS was applied to
338 complement WB for detecting exosome protein markers (hereby referred to as exosome
339 markers) and inclusion of additional positive and negative markers for a more complete
340 investigation.
341

342 3.1 Similar protein yields in kit and UC samples

343 The protein amount in the BC (**Figure 1A**) and GBM (**Figure 1B**) exosomes per million cells
344 was measured using UV-Vis spectrophotometry (i.e. absorbance at 562 nm). The total protein
345 amount measured for exosomes isolated by the kit was 15-28 times higher than for exosomes
346 isolated by UC. Elevated protein amounts in exosomes isolated by kit compared to UC was
347 also observed in a previous study by Van Deun et al. (UC compared to an identical isolation
348 kit for MCF7 derived exosomes) [56]. However, increased absorbance (indicating possible
349 protein contaminations) was measured in the kit blanks (i.e. isolation of cell culture medium
350 grown w/o cells with isolation kit), in comparison to UC blank (i.e. cell culture medium
351 grown w/o cells isolated by UC) where the absorbance was below the limit of quantification.
352 When correcting for the blank (subtracting the protein amount measured in blank samples
353 from the protein amount in exosome samples), the measured protein amount for exosomes
354 isolated by the kit and UC was similar.

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.



355

356 **Figure 1: Measured protein amount pr. million cells in exosome samples from GBM-**
357 **and BC cells isolated by kit and UC (n ≥ 2). A**) Measured absolute protein amount (µg) for
358 the BC exosome sample. **B**) The measured relative protein amount (due to low regression
359 coefficients, %) for the GBM exosome samples. Each replicate is depicted as circles, and the
360 median depicted as a line. The X-mark shows the measured protein amount in the blank
361 sample (isolated cell culture medium). The protein amounts were measured by UV-Vis
362 spectroscopy (absorption at λ= 562 nm) after reaction with BCA kit reagents.
363

364 ## 3.2 TEM and DLS detected vesicles in the expected size range for exosomes

365 Morphological analysis of the exosome samples was performed using TEM and immunogold
366 labelling of CD9. In addition, the hydrodynamic particle size distribution was measured using
367 DLS analysis. Clusters of vesicles in the samples isolated with both kit and UC were observed
368 in the micrographs (**Figure 2**). Vesicle structures similar to that described in literature were
369 observed [6, 57, 58]. The DLS experiments disclosed the coexistence of two populations of
370 moieties, single entities and clusters, both with a narrow size distribution.
371

372 ### *3.2.1 GBM exosomes*

373 No CD9-labelling was observed for the vesicle structures observed in the GBM exosomes
374 (**Figure 2A-I** and **2A-III**) and could not confirm the presence of a membrane enclosing the
375 vesicles. The UC sample presented more distinct double membranes in the expected size
376 range for exosomes compared to the kit sample. The blank samples for both isolation methods
377 did not display membrane structures and seemed pure (**Figure 2A-II** and **2A-IV**). The high

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

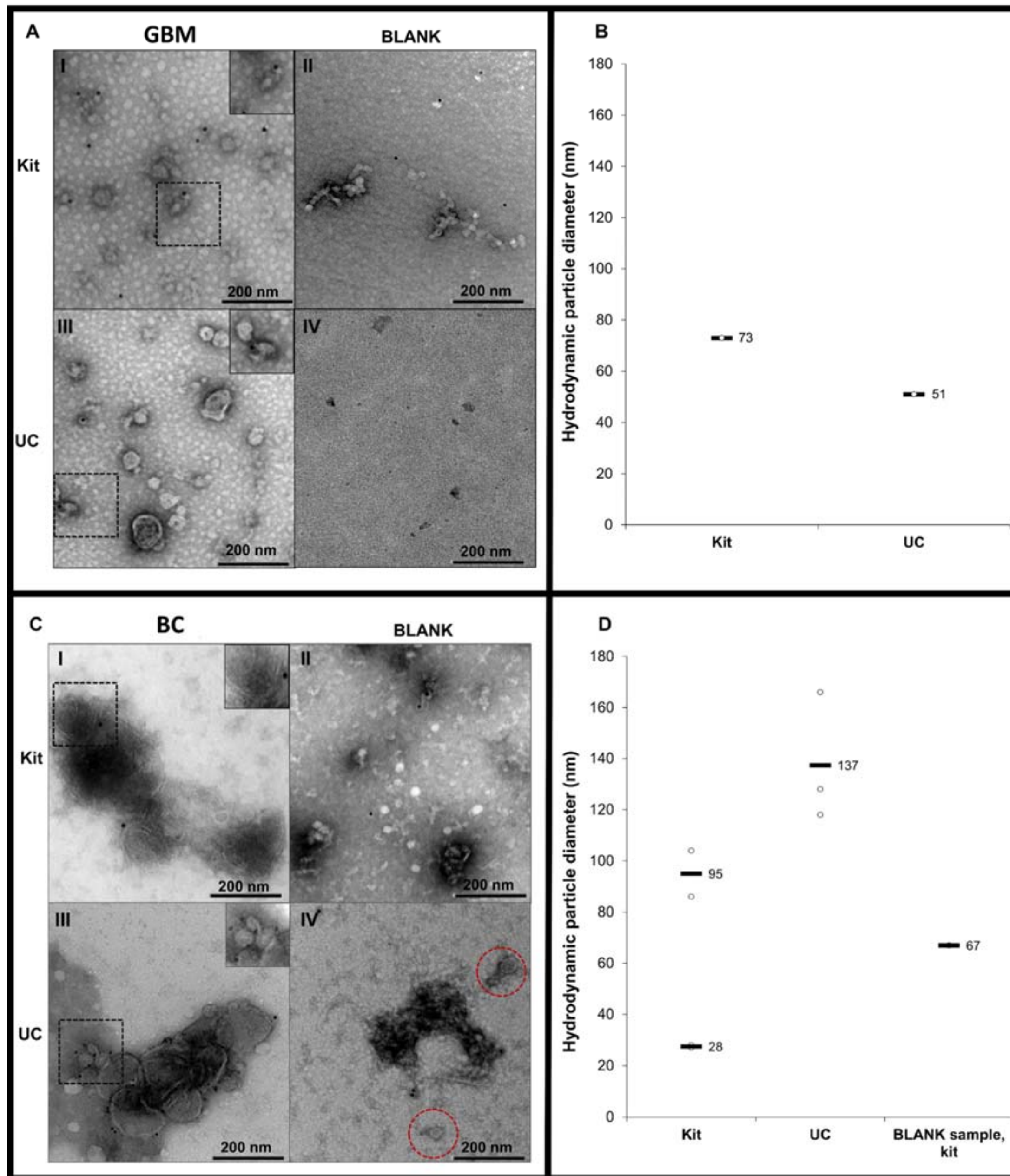
378 purity was further confirmed by DLS analysis of the UC blank (**Figure 2B**). The DLS-
379 analysis of the exosome samples exhibited particles of similar sizes of 51 and 73 nm (mean)
380 with both isolation methods (**Figure 2B**). Thus, both isolation methods isolated comparable
381 exosome populations.
382

383 *3.2.2 BC exosomes*

384 Several of the BC vesicle structures were CD9-labelled (**Figure 2B-I** and **B-III**). CD9-
385 labelled vesicles have also been observed in a previous study of the same cell line [59].
386 Notably, the blank samples displayed contamination (**Figure 2C-II and C-IV**), e.g. exosome-
387 resembling vesicles were found in the UC blank (red dashed circles). However, no
388 contaminations were found in the UC blank using DLS, while the kit blank displayed 67 nm
389 (mean) contaminations (**Figure 2D**). The DLS analysis also presented two distinct particle
390 diameters in exosome samples from kit (28 and 95 nm, mean values) while only one was
391 presented using UC (137 nm, mean value), indicating differences in the particle sizes isolated
392 with the two isolation methods.
393

394 The sizes observed with DLS correlates well with other studies (30– 250 nm), also displaying
395 a heterogeneity of particle sizes in exosome samples [13, 56, 60-64]. In conclusion, the
396 isolated samples did show structures resembling to those of EVs, but some blank samples
397 were not entirely pure from vesicles or particles. Observations made with TEM are not
398 necessarily detectable with DLS because TEM measure on dry samples, whereas DLS probes
399 on solution or suspension of particles. In addition, the micrographs taken with TEM display a
400 narrow section of the grid, which again represent a small part of the isolated sample.
401

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.



402

403 **Figure 2: Transmission electron micrographs and hydrodynamic particle size (nm)**
404 **distribution by DLS analysis of exosome samples from GBM- and BC cells isolated by**
405 **kit and UC.** The TEM-samples were immunogold labelled with anti-CD9, with gold particles
406 depicted as 10 nm black dots. Images were taken with a magnification of 400 000, and the
407 dashed areas were additionally zoomed. **A**) Micrographs of CD9-labelled GBM exosome
408 samples. **I** depict the micrograph from a kit sample, **II** the kit blank, **III** the UC sample, and
409 **IV** the UC blank. **B**) DLS analysis of GBM exosome samples isolated by kit and UC (n = 1).
410 No particles were detected in the blank sample isolated with UC (n = 1). DLS analysis of the
411 kit blank was not performed. **C**) Micrographs of CD9-labelled BC exosome samples. **I** depicts
412 the micrograph from the kit sample, **II** the kit blank, **III** a UC sample, and **IV** the UC blank.
413 **D**) DLS analysis of BC exosome samples isolated by kit (n = 2) and UC (n = 3), including the
414 kit blank (n = 1). No particles were detected in the blank sample isolated with UC.

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

3.3 Western blot analyses indicated the presence of exosomes, but detected impurities in the GBM exosome samples

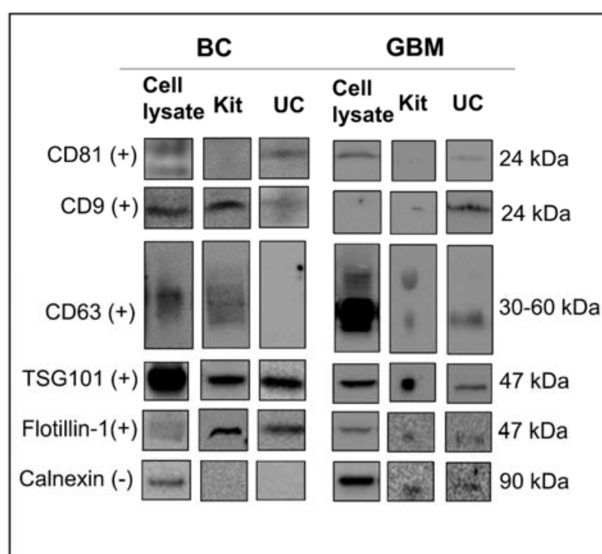
According to ISEV, at least three markers should be included for characterization of exosomes, originating from both transmembrane proteins (e.g. tetraspanins), cytosolic proteins (e.g. TSG101 or annexins) and negative markers (e.g. calnexin) [45]. In the present study, WB was performed using antibodies for a selection of positive exosome markers (the tetraspanins CD81, CD9 and CD63, TSG101 and flotillin-1). Calnexin was selected as a negative marker for purity evaluation as recommended by ISEV. This protein is located at the endoplasmic reticulum (ER) and have been absent in exosome samples in some studies [45, 60]. Hence, the presence of calnexin is assumed to signalize ER-contamination (which enables possibilities of contaminations from other cellular organelles).

3.3.1 GBM exosomes

For the GBM cells and exosomes, all exosome markers (both positive and negative) were found in samples from both kit- and UC isolation except for CD81, which was only found in UC samples (**Figure 3**). The WB-bands were more apparent for most positive markers for exosomes isolated by UC (even with higher protein amount loaded for the kit samples), which is also in accordance with Van Deun's study [56].

3.3.2 BC exosomes

For the BC exosomes, inconsistency for several positive exosome markers were observed between the kit and UC samples (**Figure 3**). The positive marker CD63 was only detected in kit samples, while CD81 was only detected in samples from UC (similar to GBM exosomes). The proteins TSG101, flotillin-1 and CD9 (barely visible in the UC samples) were detected for both isolation methods. Although, a study by Harris et al. did not detect TSG101 using WB on BC exosomes isolated by UC [13]. The proteins CD9, CD81 and flotillin-1 were detected in other WB-studies on UC samples from the same cell line [23, 65, 66].



bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

448 The reason for the absence of some tetraspanins in kit and others in UC samples could be due
449 to protein concentrations below detection limits or poor antibody quality. Several antibodies
450 for CD63 and CD81 (from different companies) were tested on the BC exosomes before a
451 signal was obtained, which could indicate poor antibody quality. On the other hand, the WB
452 was performed under reducing conditions. When the epitope binds to cysteine-conserved
453 protein domains (i.e. tetraspanins), performing WB under non-reducing conditions is more
454 commonly selected. The stronger signals for the kit samples from BC could be due to the
455 higher loaded protein amount. Nevertheless, the presence of positive markers indicates the
456 presence of exosomes in the isolates obtained using both methods. The absence of calnexin in
457 BC exosomes from both isolation methods indicate that the samples do not contain
458 contamination from the ER. However, only one negative marker is insufficient to exclude cell
459 organelle impurities. Further
460 investigation by implementing more
461 comprehensive methods like LC-
462 MS/MS was considered to be
463 beneficial.

Figure 3: Western blot of common protein exosome markers. The protein markers CD81, CD9, CD63, TSG101, flotillin-1 (positive markers, +) and calnexin (negative marker, -) were targeted in cell lysates and exosome samples isolated with kit and by UC (n ≥ 2). For the BC exosomes, 15 μg protein was loaded for kit samples and 3 μg for UC samples. For the GBM exosomes, ~14 μg was loaded for kit samples and ~8 μg for UC samples. Uncropped Western blots are presented in **Supplementary Materials 2 (SM-2)**.

464

465 **3.4 LC-MS/MS studies**
466 **confirmed and complemented the**
467 **WB study**

468 LC-MS/MS was performed to confirm the WB observations, using in-house packed nano
469 separation columns [55]. One positive marker (annexin A2) and one negative marker
470 (serine/threonine-protein kinase 26) was added to complement the WB study. The detection
471 of the selected exosome markers using LC-MS/MS is presented in **Table 1**, with
472 chromatograms and MS/MS spectra of a CD9 signature peptide (BC, **Figure 4A**) and
473 calnexin signature peptide (GBM, **Figure 4B**). Similar to the WB analysis, the same
474 tetraspanins including calnexin observed in kit and UC samples were detected for the GBM
475 exosomes using LC-MS/MS. However, the tetraspanins detected from kit samples were only
476 found in one replicate, indicating low concentrations and high detection uncertainty. Flotillin-
477 1 was also detected, but only for UC samples with LC-MS/MS. TSG101 was identified in
478 neither kit nor UC (GBM exosomes) using LC-MS/MS in contradiction to WB.
479

480 **Table 1: A selection of common protein markers (from LC-MS analyses) with a specific**
481 **exosome related function.** The exosome markers were identified in exosome samples from

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

482 MDA-MB-231 and glioblastoma isolated with kit and by UC. In addition, proteins identified
483 in blank samples are shown.

Protein marker	Exosome related function	GBM				BC			
		UC	UC blank	Kit	Kit blank	UC	UC blank	Kit	Kit blank
CD81	Membrane protein	x	-	-	-	x	-	-	-
CD9	Membrane protein	x	-	x	-	x	x	x	-
CD63	Membrane protein	x	-	x	-	x	-	-	-
TSG101	Exosome biogenesis	-	-	-	-	x	-	-	-
Annexin A2	Exosome biogenesis	x	-	x	-	x	-	x	-
Flotillin-1	Exosome biogenesis	-	-	-	-	x	-	-	-
Calnexin	Negative	x	-	x	-	-	-	-	-
Serine/ threonine-protein kinase 26	Negative	-	-	-	-	-	-	-	-
Actin	General marker	x	x	x	x	x	-	x	x

484

485 For the BC exosomes, the LC-MS/MS analysis is contradictory to the trend observed in the
486 WB analysis where kit samples provided more pronounced signals for positive markers than
487 the UC samples. Using LC-MS/MS, several positive markers were absent in the kit samples
488 when injecting similar amounts of protein as with UC samples. The reason for the difference
489 to WB could hence be partially due to higher protein amount loaded onto the gel for kit
490 samples. Calnexin was, similar to the WB, not detected in the BC samples. Annexin A2
491 (positive marker) was found in all samples and serine/threonine-protein kinase 26 (second
492 negative marker) was not detected in any samples.

493

494 Other negative markers from peroxisomes (PMP70), mitochondria (prohibitin-1, hexokinase-
495 2 and mitochondrial phosphoenolpyruvate carboxykinase [GTP]), Golgi apparatus (GM130,
496 translocation protein SEC62, translocation protein SEC63 and protein disulfide-isomerase
497 TMX3), nucleus (Bcl-2-associated transcription factor 1 and c) and ER (calreticulin), as used
498 in other studies [56, 67], were not detected in any BC samples in our study (only translocation

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

protein SEC63 and protein disulfide-isomerase TMX3 detected in GBM samples). The absence of several negative markers in the BC exosomes supports the low cell organelle contamination observed with WB and LC-MS/MS.

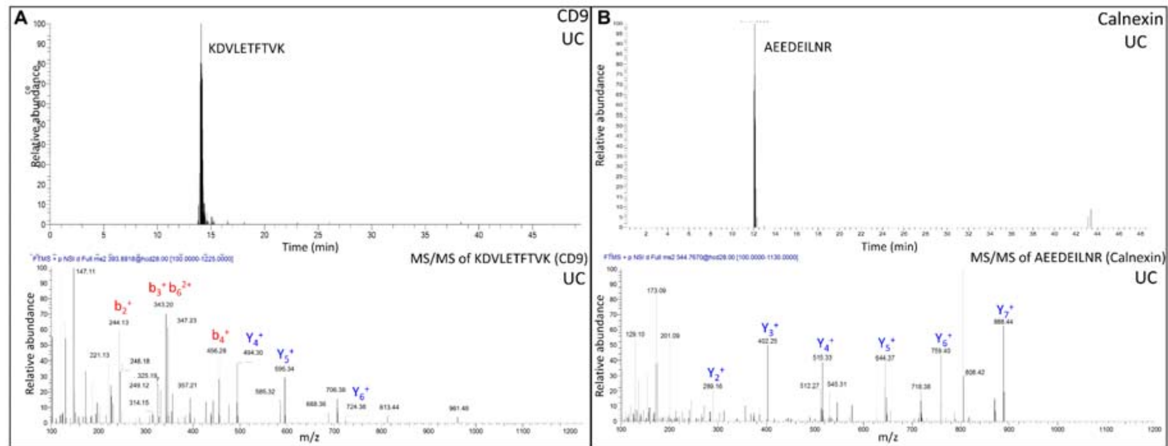


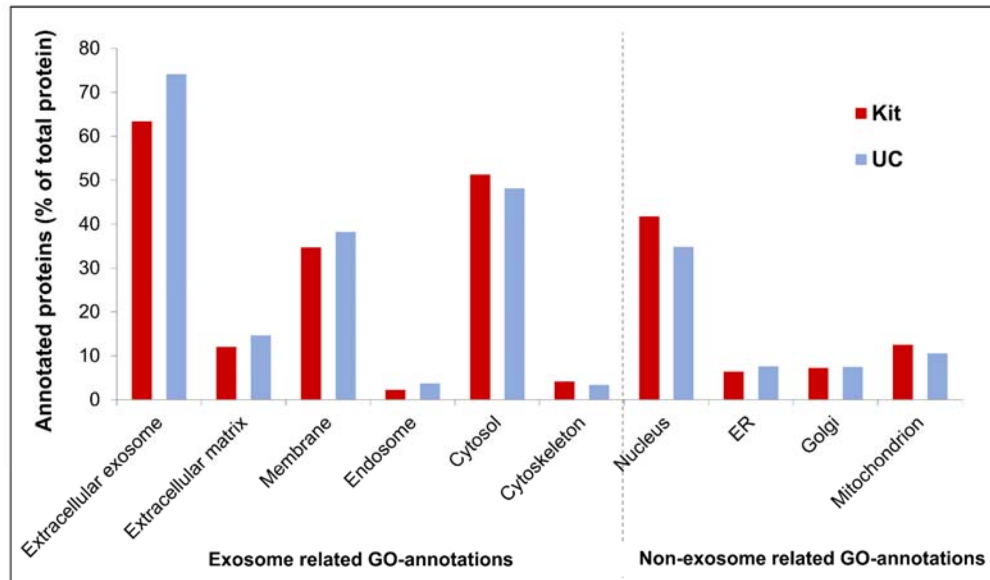
Figure 4: Chromatograms and MS/MS spectrums from LC-MS/MS analysis of GBM- and BC exosome peptides. A) Chromatogram with corresponding MS/MS spectrum for the CD9 signature peptide KDVLETFTVK (*m/z*=393.89, z=3) in BC exosomes isolated by UC. **C)** Chromatogram with corresponding MS/MS spectrum for the calnexin signature peptide AEEDEILNR (*m/z*=544.77, z=2) from GBM exosomes isolated by UC.

3.5 GO annotations reveal proteins annotated to cell organelles in BC exosomes

However, the possibility of contaminants being present in BC samples cannot be excluded. Other general proteins related to e.g. the nucleus, Golgi apparatus, mitochondrion and ER was indeed identified in the BC exosomes using LC-MS/MS (**Figure 5**). The proteins identified in BC exosomes were classified based on their gene ontology (GO) annotations to different cellular localizations, where one protein can be annotated to several cellular localizations. Out of the 668 proteins identified from the UC and 814 from the kit, 615 and 749 DAVID ID`s were annotated to selected cellular localizations. From them, both isolation methods generated samples enriched in exosome related proteins. Several proteins were annotated to the cellular organelles mitochondria (11-13 %), ER (6-8 %) and Golgi apparatus (7 %). Interestingly, 35-42 % of the proteins were also annotated to the nucleus (e.g. histones), which seems to imply impure exosome samples [45]. On the other hand, a high percentage (20-40 %) of other proteins related to the nucleus has also been found in exosome samples from other studies [68, 69]. The presence of cell organelle annotated proteins could point toward cellular impurities in the samples, which would not have been discovered by targeted protein characterization methods (e.g. WB). However, there is not sufficient knowledge on

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

525 whether cell organelle proteins derive exclusively from cell impurities, or if they occur
526 naturally in EVs. To summarize, from our point of view, complete information about
527 exosome purity cannot be obtained by any of the common characterization techniques used
528 today.



529

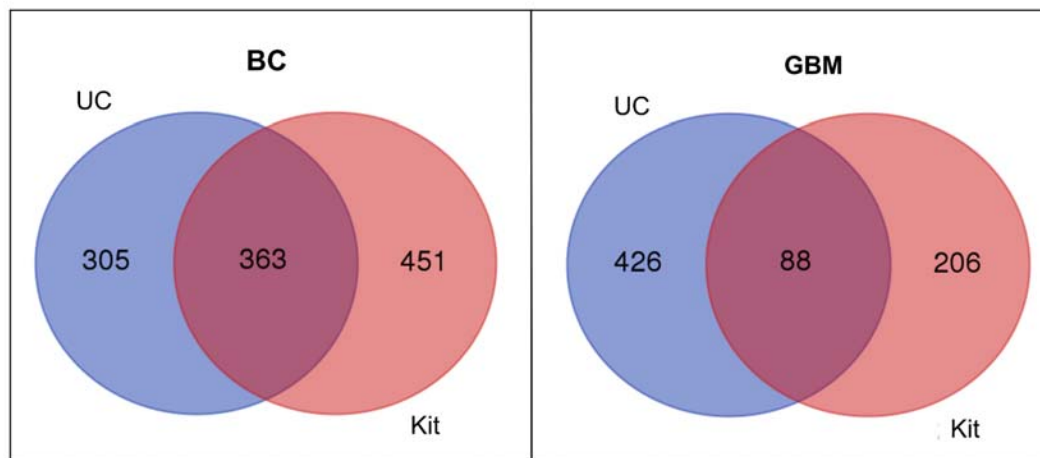
530 **Figure 5: GO annotation of proteins in BC exosomes to different cellular locations.** The
531 identified proteins classified by their cellular location (GO annotations) grouped based on
532 their positive/ negative relevance towards exosomes. The annotated proteins (% of total
533 proteins) and their cellular location, with proteins annotated from the kit samples are shown in
534 red (from 749 DAVID ID's), while proteins annotated from the UC samples are shown in
535 blue (from 615 DAVID ID's).
536

537 3.6 The isolation method and cell line influence the number of cancer related proteins 538 identified

539 The total number of proteins identified in the GBM and BC exosome samples using LC-
540 MS/MS (performed in two different laboratories) is presented in the Venn diagrams in **Figure**
541 **6** (see **SM-3** for a list of all identified proteins). For the GBM exosomes, the number of
542 identified proteins reflects the findings in both WB and LC-MS/MS exosome marker
543 investigations. UC-isolated exosomes provided more unique proteins than the kit-isolated
544 exosomes (75 % higher number of identified proteins than the kit). An increased number of
545 potential biomarkers for GBM (e.g. heat shock proteins 70 kDa and 90 kDa [70-72],
546 chondroitin sulfate proteoglycan 4 [70, 73], CD44 [70, 73, 74] and CD276 [75]) were also
547 identified in the UC samples compared to the kit samples using LC-MS/MS. The

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

548 identification of relevant biomarkers is of great interest for further studies on exosomes.
549 However, the identified biomarkers cannot exclusively be related to exosomes due to the
550 presence of negative exosomes markers indicating cellular contaminations.
551
552 For the BC exosomes, the tables are turned, and the kit-isolated exosomes provided 12 %
553 higher number of identified proteins than UC-isolated exosomes. However, there was no
554 correlation between the injected protein amount or the starting volume used for isolation, and
555 the number of identified proteins in kit- vs UC samples for BC exosomes (result not shown).
556 Thus, the reason for why the protein number is changed between the two cell lines and
557 isolation methods is unknown. The number of identified biomarkers related to the triple
558 negative breast cancer (e.g. histone H4 [76], heat shock 90 kDa α and β protein [77],
559 calmodulin and epithermal growth factor receptor [78]) in the UC samples compared to kit
560 samples were comparable.
561
562 When comparing cell lines, the number of identified proteins was lower in GBM exosomes
563 than BC exosomes, but the number for GBM exosomes is comparable to another LC-MS/MS
564 study on GBM exosomes [79].



565
566 **Figure 6: Venn Diagram presenting the number of proteins identified by LC-MS/MS in**
567 **GBM- and BC exosome samples isolated with kit and by UC.** The numbers are the total
568 number of unique proteins identified when trypsin, keratin related proteins and the proteins
569 identified in blank samples were disregarded. One signature peptide was selected as
570 requirement for positive identifications during database search. Equal amounts of protein
571 were injected for both kit and UC (~ 1.5 µg protein for GBM exosomes (n= 6) and ~2- 5 µg
572 protein for BC exosomes (n= 3)). A list of all proteins identified is presented in
573 Supplementary Materials 3 (SM-3).
574

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

575 **3.7 Choosing the proper exosome isolation method is not straight forward**

576 A complete comparison of the characteristics of exosome isolation methods is given in **Table**
577 **2**. For all exosome samples, the kit and UC samples display similarities and differences.
578

579 *3.7.1 GBM exosomes*

580 For the GBM exosomes, one of the positive markers detected in the UC samples (CD81) was
581 not found in kit samples by WB. In TEM, double membrane structures were more eminent in
582 the UC samples, but the existence of double membranes cannot be excluded by looking at the
583 micrographs from the kit samples. The largest differences between kit and UC for the GBM
584 exosomes were found in the LC-MS/MS studies recoding positive markers and general
585 protein number. All tetraspanins investigated were detected in the UC samples in several
586 replicates. For the kit, CD81 was not found, and the detected tetraspanins (CD63 and CD9)
587 were only found in one replicate each. A larger number of proteins and biomarker candidates
588 were also identified in the UC samples compared to kit samples. However, the negative
589 marker calnexin was detected in more replicates for the UC than the kit using LC-MS/MS. In
590 total, from **Table 2**, UC appear to be the method of choice for isolation of GBM (T1018)
591 exosomes.
592

593 *3.7.2 BC exosomes*

594 For the BC exosomes, there was a slight difference in favor of the kit method regarding
595 positive markers in WB and the number of identified proteins (LC-MS/MS). However, using
596 LC-MS/MS, more relevant protein markers were found in the UC samples in contrary to the
597 WB study. In addition, TEM presented slightly more defined double membrane structures
598 with more labeling using UC. However, the micrograph displays an extremely small part of
599 the whole sample. The isolation methods also performed similarly regarding biomarker
600 identifications. Based on the above, there is no obvious reason for choosing one method over
601 the other. However, tendencies towards different characteristics of the isolated exosomes
602 from kit and UC have been observed.
603

604 **Table 2: Comparison of characteristics of exosome isolated from GBM and BC cell**
605 **culture medium.** In the table, UC and kit was compared for their ability to isolate pure
606 exosomes based on the characterization techniques used. Increasing number of + signs
607 indicate positive relation regarding indications of exosomes and/or high purity of exosomes.

Characterization method	GBM		BC	
	UC	Kit	UC	Kit

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Protein amount measured (corrected for blank)	+	++	+	++
TEM	++	+	+++	++
DLS	+++	+++	+	++
WB (positive markers)	++	+	+	++
WB (negative marker)	+	+	+++	+++
LC-MS/MS (positive markers)	++	+	+++	+
LC-MS/MS (negative markers)	+	++	+++	+++
LC-MS/MS number of proteins identified	+++	+	+	++
LC-MS/MS relevant biomarkers	++	+	++	++

608

609 # 4 Conclusions

610 The observations in our study support the perception of exosome isolation being highly
611 dependent on the isolation protocol used, general differences in the behavior of
612 cells/exosomes between cell lines and the characterization methods and conditions applied.
613 The knowledge about potentially natural occurring proteins in exosomes is insufficient and a
614 selection of expected negative markers cannot exclusively be used to determine the purity of
615 exosome samples. Hence, the application area (e.g. proving presence of exosomes, determine
616 exosome purity, biomarker discovery, or lower the dynamic range of proteins in body fluids
617 like blood) and sample volume available for the exosome isolation should be stronger
618 determent factors when selecting the proper isolation method. Thus, the characterization
619 methods used in this study are not able to distinguish exosomes from cellular contaminations
620 and other vesicles. Untargeted proteome analyses using LC-MS/MS did provide more
621 extensive and versatile information on the protein content of the samples than most
622 commonly used targeted WB of few proteins. Consequently, we suggest that LC-MS/MS
623 should be implemented to a higher extend regarding exosome characterization. Considering
624 our findings, it is important to state that the term "exosome enrichment" is more appropriate
625 than "exosome isolation".

626 # 5 References
627

628 [1] C. Théry, L. Zitvogel, S. Amigorena, Exosomes: composition, biogenesis and function,
629 Nature Reviews Immunology 2 (2002) 569.
630 [2] T. Lener, M. Gimona, L. Aigner, V. Borger, E. Buzas, G. Camussi, N. Chaput, D. Chatterjee,
631 F.A. Court, H.A. del Portillo, L. O'Driscoll, S. Fais, J.M. Falcon-Perez, U. Felderhoff-Mueser, L.
632 Fraile, Y.S. Gho, A. Gorgens, R.C. Gupta, A. Hendrix, D.M. Hermann, A.F. Hill, F. Hochberg,
633 P.A. Horn, D. de Kleijn, L. Kordelas, B.W. Kramer, E.M. Kramer-Albers, S. Laner-Plamberger, S.
634 Laitinen, T. Leonardi, M.J. Lorenowicz, S.K. Lim, J. Lotvall, C.A. Maguire, A. Marcilla, I.

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

635 Nazarenko, T. Ochiya, T. Patel, S. Pedersen, G. Pocsfalvi, S. Pluchino, P. Quesenberry, I.G.
636 Reischl, F.J. Rivera, R. Sanzenbacher, K. Schallmoser, I. Slaper-Cortenbach, D. Strunk, T. Tonn,
637 P. Vader, B.W.M. van Balkom, M. Wauben, S. El Andaloussi, C. Thery, E. Rohde, B. Giebel,
638 Applying extracellular vesicles based therapeutics in clinical trials - an ISEV position paper, J.
639 Extracell. Vesicles 4 (2015) 31.
640 [3] H. Zhao, L. Yang, J. Baddour, A. Achreja, V. Bernard, T. Moss, J.C. Marini, T. Tudawe, E.G.
641 Seviour, F.A. San Lucas, H. Alvarez, S. Gupta, S.N. Maiti, L. Cooper, D. Peehl, P.T. Ram, A.
642 Maitra, D. Nagrath, Tumor microenvironment derived exosomes pleiotropically modulate
643 cancer cell metabolism, eLife 5 (2016) e10250.
644 [4] T. Skotland, K. Sandvig, A. Llorente, Lipids in exosomes: Current knowledge and the way
645 forward, Progress in Lipid Research 66 (2017) 30-41.
646 [5] A. Beach, H.-G. Zhang, M.Z. Ratajczak, S.S. Kakar, Exosomes: an overview of biogenesis,
647 composition and role in ovarian cancer, Journal of Ovarian Research 7 (2014) 14-14.
648 [6] P. Li, M. Kaslan, S.H. Lee, J. Yao, Z. Gao, Progress in Exosome Isolation Techniques,
649 Theranostics 7(3) (2017) 789-804.
650 [7] A. Becker, B.K. Thakur, J.M. Weiss, H.S. Kim, H. Peinado, D. Lyden, Extracellular Vesicles in
651 Cancer: Cell-to-Cell Mediators of Metastasis, Cancer Cell 30(6) (2016) 836-848.
652 [8] G. Pocsfalvi, C. Stanly, I. Fiume, K. Vekey, Chromatography and its hyphenation to mass
653 spectrometry for extracellular vesicle analysis, J. Chromatogr. A 1439 (2016) 26-41.
654 [9] M. Farahani, C. Rubbi, L. Liu, J.R. Slupsky, N. Kalakonda, CLL exosomes modulate the
655 transcriptome and behaviour of recipient stromal cells and are selectively enriched in miR-
656 202-3p, PloS one 10(10) (2015) e0141429.
657 [10] M. Logozzi, A. De Milito, L. Lugini, M. Borghi, L. Calabro, M. Spada, M. Perdicchio, M.L.
658 Marino, C. Federici, E. Iessi, D. Brambilla, G. Venturi, F. Lozupone, M. Santinami, V. Huber, M.
659 Maio, L. Rivoltini, S. Fais, High Levels of Exosomes Expressing CD63 and Caveolin-1 in Plasma
660 of Melanoma Patients, Plos One 4(4) (2009) 10.
661 [11] I. Parolini, C. Federici, C. Raggi, L. Lugini, S. Palleschi, A. De Milito, C. Coscia, E. Iessi, M.
662 Logozzi, A. Molinari, M. Colone, M. Tatti, M. Sargiacomo, S. Fais, Microenvironmental pH Is a
663 Key Factor for Exosome Traffic in Tumor Cells, J. Biol. Chem. 284(49) (2009) 34211-34222.
664 [12] H. Peinado, M. Alečković, S. Lavotshkin, I. Matei, B. Costa-Silva, G. Moreno-Bueno, M.
665 Hergueta-Redondo, C. Williams, G. García-Santos, C.M. Ghajar, Melanoma exosomes
666 educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET,
667 Nature medicine 18(6) (2012) 883-891.
668 [13] D.A. Harris, S.H. Patel, M. Gucek, A. Hendrix, W. Westbroek, J.W. Taraska, Exosomes
669 released from breast cancer carcinomas stimulate cell movement, PloS one 10(3) (2015)
670 e0117495.
671 [14] C.E. DeSantis, C.C. Lin, A.B. Mariotto, R.L. Siegel, K.D. Stein, J.L. Kramer, R. Alteri, A.S.
672 Robbins, A. Jemal, Cancer treatment and survivorship statistics, 2014, CA: A Cancer Journal
673 for Clinicians 64(4) (2014) 252-271.
674 [15] A.J. Redig, S.S. McAllister, Breast cancer as a systemic disease: a view of metastasis,
675 Journal of Internal Medicine 274(2) (2013) 113-126.
676 [16] M. Touat, A. Duran-Pena, A. Alentorn, L. Lacroix, C. Massard, A. Idbaih, Emerging
677 circulating biomarkers in glioblastoma: promises and challenges, Expert Rev. Mol. Diagn.
678 15(10) (2015) 1311-1323.
679 [17] J.R. Molina, Y. Hayashi, C. Stephens, M.M. Georgescu, Invasive Glioblastoma Cells
680 Acquire Stemness and Increased Akt Activation, Neoplasia 12(6) (2010) 453-U37.

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

681 [18] M. Preusser, S. de Ribaupierre, A. Wohrer, S.C. Erridge, M. Hegi, M. Weller, R. Stupp,
682 Current Concepts and Management of Glioblastoma, Ann. Neurol. 70(1) (2011) 9-21.
683 [19] Z.N. Jan Chrastina, Milan Brazdil, Marketa Hermanova, Glioblastoma multiforme in a
684 patient with isolated hemimegalencephaly, Journal of Neurological Surgery Reports 76(1)
685 (2015) 160-163.
686 [20] H.L. Shao, J. Chung, K. Lee, L. Balaj, C. Min, B.S. Carter, F.H. Hochberg, X.O. Breakefield,
687 H. Lee, R. Weissleder, Chip-based analysis of exosomal mRNA mediating drug resistance in
688 glioblastoma, Nat. Commun. 6 (2015) 9.
689 [21] M.G. Best, N. Sol, S. Zijl, J.C. Reijneveld, P. Wesseling, T. Wurdinger, Liquid biopsies in
690 patients with diffuse glioma, Acta Neuropathol. 129(6) (2015) 849-865.
691 [22] M. Cristofanilli, G.T. Budd, M.J. Ellis, A. Stopeck, J. Matera, M.C. Miller, J.M. Reuben,
692 G.V. Doyle, W.J. Allard, L.W. Terstappen, Circulating tumor cells, disease progression, and
693 survival in metastatic breast cancer, New England Journal of Medicine 351(8) (2004) 781-
694 791.
695 [23] S.A. Melo, L.B. Luecke, C. Kahlert, A.F. Fernandez, S.T. Gammon, J. Kaye, V.S. LeBleu, E.A.
696 Mittendorf, J. Weitz, N. Rahbari, Glypican-1 identifies cancer exosomes and detects early
697 pancreatic cancer., Nature 523(7559) (2015) 177-182.
698 [24] D.D. Taylor, C. Gercel-Taylor, MicroRNA signatures of tumor-derived exosomes as
699 diagnostic biomarkers of ovarian cancer, Gynecologic Oncology 110(1) (2008) 13-21.
700 [25] H. Roberg-Larsen, K. Lund, K.E. Seterdal, S. Solheim, T. Vehus, N. Solberg, S. Krauss, E.
701 Lundanes, S.R. Wilson, Mass spectrometric detection of 27-hydroxycholesterol in breast
702 cancer exosomes, The Journal of steroid biochemistry and molecular biology 169 (2017) 22-
703 28.
704 [26] J. Skog, T. Würdinger, S. Van Rijn, D.H. Meijer, L. Gainche, W.T. Curry, B.S. Carter, A.M.
705 Krichevsky, X.O. Breakefield, Glioblastoma microvesicles transport RNA and proteins that
706 promote tumour growth and provide diagnostic biomarkers, Nature cell biology 10(12)
707 (2008) 1470-1476.
708 [27] F.H. Hochberg, N.A. Atai, D. Gonda, M.S. Hughes, B. Mawejje, L. Balaj, R.S. Carter,
709 Glioma diagnostics and biomarkers: an ongoing challenge in the field of medicine and
710 science, Expert Rev. Mol. Diagn. 14(4) (2014) 439-452.
711 [28] L. Saadatpour, E. Fadaee, S. Fadaei, R.N. Mansour, M. Mohammadi, S.M. Mousavi, M.
712 Goodarzi, J. Verdi, H. Mirzaei, Glioblastoma: exosome and microRNA as novel diagnosis
713 biomarkers, Cancer Gene Ther. 23(12) (2016) 415-418.
714 [29] A. Mondal, D.K. Singh, S. Panda, A. Shiras, Extracellular Vesicles As Modulators of Tumor
715 Microenvironment and Disease Progression in Glioma, Front. Oncol. 7 (2017) 8.
716 [30] S. Keller, J. Ridinger, A.-K. Rupp, J.W. Janssen, P. Altevogt, Body fluid derived exosomes
717 as a novel template for clinical diagnostics, Journal of translational medicine 9(1) (2011) 86.
718 [31] C. Théry, S. Amigorena, G. Raposo, A. Clayton, Isolation and characterization of
719 exosomes from cell culture supernatants and biological fluids, Current Protocols in Cell
720 Biology (2006) 3.22. 1-3.22. 29.
721 [32] R. Cantin, J. Diou, D. Bélanger, A.M. Tremblay, C. Gilbert, Discrimination between
722 exosomes and HIV-1: purification of both vesicles from cell-free supernatants, Journal of
723 Immunological Methods 338(1) (2008) 21-30.
724 [33] D.W. Greening, R. Xu, H. Ji, B.J. Tauro, R.J. Simpson, A protocol for exosome isolation
725 and characterization: evaluation of ultracentrifugation, density-gradient separation, and
726 immunoaffinity capture methods, Proteomic Profiling: Methods and Protocols (2015) 179-
727 209.

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

728 [34] M.A. Rider, S.N. Hurwitz, D.G. Meckes Jr, ExtraPEG: a polyethylene glycol-based method
729 for enrichment of extracellular vesicles, Scientific reports 6 (2016) 23978.
730 [35] A.N. Böing, E. Van Der Pol, A.E. Grootemaat, F.A. Coumans, A. Sturk, R. Nieuwland,
731 Single-step isolation of extracellular vesicles by size-exclusion chromatography, Journal of
732 Extracellular Vesicles 3 (2014) DOI: 10.3402/jev.v3.23430.
733 [36] A. Gámez-Valero, M. Monguió-Tortajada, L. Carreras-Planella, Size-Exclusion
734 Chromatography-based isolation minimally alters Extracellular Vesicles' characteristics
735 compared to precipitating agents, Scientific Reports 6 (2016) DOI: 10.1038/srep33641.
736 [37] R. Grant, E. Ansa-Addo, D. Stratton, S. Antwi-Baffour, S. Jorfi, S. Kholia, L. Krige, S. Lange,
737 J. Inal, A filtration-based protocol to isolate human plasma membrane-derived vesicles and
738 exosomes from blood plasma, Journal of Immunological Methods 371(1) (2011) 143-151.
739 [38] A. Clayton, J. Court, H. Navabi, M. Adams, M.D. Mason, J.A. Hobot, G.R. Newman, B.
740 Jasani, Analysis of antigen presenting cell derived exosomes, based on immuno-magnetic
741 isolation and flow cytometry, Journal of Immunological Methods 247(1) (2001) 163-174.
742 [39] J.S. Yang, J.C. Lee, S.K. Byeon, K.H. Rha, M.H. Moon, Size dependent lipidomic analysis of
743 urinary exosomes from patients with prostate cancer by flow field-flow fractionation and
744 nanoflow liquid chromatography-tandem mass spectrometry, Analytical Chemistry 89 (2017)
745 2488-2496.
746 [40] B.J. Tauro, D.W. Greening, R.A. Mathias, S. Mathivanan, H. Ji, R.J. Simpson, Two distinct
747 populations of exosomes are released from LIM1863 colon carcinoma cell-derived
748 organoids, Molecular & Cellular Proteomics 12(3) (2013) 587-598.
749 [41] S.N. Bhatia, D.E. Ingber, Microfluidic organs-on-chips, Nature Biotechnology 32(8)
750 (2014) 760-772.
751 [42] A. Abramowicz, P. Widlak, M. Pietrowska, Proteomic analysis of exosomal cargo: the
752 challenge of high purity vesicle isolation, Mol. Biosyst. 12(5) (2016) 1407-1419.
753 [43] M. He, Y. Zeng, Microfluidic Exosome Analysis toward Liquid Biopsy for Cancer, Jala
754 21(4) (2016) 599-608.
755 [44] S. Kreimer, A.M. Belov, I. Ghiran, S.K. Murthy, D.A. Frank, A.R. Ivanov, Mass-
756 Spectrometry-Based Molecular Characterization of Extracellular Vesicles: Lipidomics and
757 Proteomics, Journal of Proteome Research 14(6) (2015) 2367-2384.
758 [45] J. Lötvall, A.F. Hill, F. Hochberg, E.I. Buzás, D. Di Vizio, C. Gardiner, Y.S. Gho, I.V.
759 Kurochkin, S. Mathivanan, P. Quesenberry, S. Sahoo, H. Tahara, M.H. Wauben, K.W. Witwer,
760 C. Théry, Minimal experimental requirements for definition of extracellular vesicles and their
761 functions: a position statement from the International Society for Extracellular Vesicles, J.
762 Extracell. Vesicles 3 (2014) 10.3402/jev.v3.26913.
763 [46] Z. Andreu, M. Yáñez-Mó, Tetraspanins in Extracellular Vesicle Formation and Function,
764 Frontiers in Immunology 5 (2014) 442.
765 [47] J.M. Escola, M.J. Kleijmeer, W. Stoorvogel, J.M. Griffith, O. Yoshie, H.J. Geuze, Selective
766 enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and
767 on exosomes secreted by human B-lymphocytes, J. Biol. Chem. 273(32) (1998) 20121-20127.
768 [48] M.J. Kleijmeer, W. Stoorvogel, J.M. Griffith, O. Yoshie, H.J. Geuze, Selective enrichment
769 of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes
770 secreted by human B-lymphocytes, The Journal of Biological Chemistry 273(32) (1998)
771 20121-20127.
772 [49] N.P. Hessvik, A. Llorente, Current knowledge on exosome biogenesis and release,
773 Cellular and Molecular Life Sciences 75(2) (2018) 193-208.

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

774 [50] S. Mathivanan, C.J. Fahner, G.E. Reid, R.J. Simpson, ExoCarta 2012: database of
775 exosomal proteins, RNA and lipids, Nucleic Acids Research 40(D1) (2012) D1241-D1244.
776 [51] H. Kalra, R.J. Simpson, H. Ji, E. Aikawa, P. Altevogt, P. Askenase, V.C. Bond, F.E. Borras, X.
777 Breakefield, V. Budnik, E. Buzas, G. Camussi, A. Clayton, E. Cocucci, J.M. Falcon-Perez, S.
778 Gabrielsson, Y.S. Gho, D. Gupta, H.C. Harsha, A. Hendrix, A.F. Hill, J.M. Inal, G. Jenster, E.M.
779 Kramer-Albers, S.K. Lim, A. Llorente, J. Lotvall, A. Marcilla, L. Mincheva-Nilsson, I. Nazarenko,
780 R. Nieuwland, E.N.M. Hoen, A. Pandey, T. Patel, M.G. Piper, S. Pluchino, T.S.K. Prasad, L.
781 Rajendran, G. Raposo, M. Record, G.E. Reid, F. Sanchez-Madrid, R.M. Schiffelers, P. Siljander,
782 A. Stensballe, W. Stoorvogel, D. Taylor, C. Thery, H. Valadi, B.W.M. van Balkom, J. Vazquez,
783 M. Vidal, M.H.M. Wauben, M. Yanez-Mo, M. Zoeller, S. Mathivanan, Vesiclepedia: A
784 Compendium for Extracellular Vesicles with Continuous Community Annotation, PLoS. Biol.
785 10(12) (2012) 5.
786 [52] D.K. Kim, B. Kang, O.Y. Kim, D.S. Choi, J. Lee, S.R. Kim, G. Go, Y.J. Yoon, J.H. Kim, S.C.
787 Jang, K.S. Park, E.J. Choi, K.P. Kim, D.M. Desiderio, Y.K. Kim, J. Lotvall, D. Hwang, Y.S. Gho,
788 EVpedia: an integrated database of high-throughput data for systemic analyses of
789 extracellular vesicles, J Extracell Vesicles 2 (2013).
790 [53] ThermoFisher, Total Exosome Isolation Reagent (from cell culture media).
791 https://www.thermofisher.com/order/catalog/product/4478359?SID=srch-srp-4478359).
792 [54] A. Shevchenko, H. Tomas, J. Havli, J.V. Olsen, M. Mann, In-gel digestion for mass
793 spectrometric characterization of proteins and proteomes, Nature Protocols 1 (2007) 2856.
794 [55] H.S. Berg, K.E. Seterdal, T. Smetop, R. Rozenvalds, O.K. Brandtzaeg, T. Vehus, E.
795 Lundanes, S.R. Wilson, Self-packed core shell nano liquid chromatography columns and
796 silica-based monolithic trap columns for targeted proteomics, J. Chromatogr. A 1498 (2017)
797 111-119.
798 [56] J. Van Deun, P. Mestdagh, R. Sormunen, V. Cocquyt, K. Vermaelen, J. Vandesompele, M.
799 Bracke, O. De Wever, A. Hendrix, The impact of disparate isolation methods for extracellular
800 vesicles on downstream RNA profiling, J. Extracell. Vesicles 3(1) (2014) 24858.
801 [57] B.-T. Pan, K. Teng, C. Wu, M. Adam, R.M. Johnstone, Electron microscopic evidence for
802 externalization of the transferrin receptor in vesicular form in sheep reticulocytes, The
803 Journal of Cell Biology 101(3) (1985) 942-948.
804 [58] B.-T. Pan, R.M. Johnstone, Fate of the transferrin receptor during maturation of sheep
805 reticulocytes in vitro: selective externalization of the receptor, Cell 33(3) (1983) 967-978.
806 [59] S.A. Melo, H. Sugimoto, J.T. O'Connell, N. Kato, A. Villanueva, A. Vidal, L. Qiu, E. Vitkin,
807 L.T. Perelman, C.A. Melo, Cancer exosomes perform cell-independent microRNA biogenesis
808 and promote tumorigenesis, Cancer Cell 26(5) (2014) 707-721.
809 [60] R.J. Lobb, M. Becker, S.W. Wen, C.S.F. Wong, A.P. Wiegmans, A. Leimgruber, A. Moller,
810 Optimized exosome isolation protocol for cell culture supernatant and human plasma, J.
811 Extracell. Vesicles 4 (2015) 11.
812 [61] P. Jenjaroenpun, Y. Kremenska, V.M. Nair, M. Kremenskoy, B. Joseph, I.V. Kurochkin,
813 Characterization of RNA in exosomes secreted by human breast cancer cell lines using next-
814 generation sequencing, PeerJ 1 (2013) e201.
815 [62] G. Palazzolo, N.N. Albanese, G. Di Cara, D. Gygax, M.L. Vittorelli, I. Pucci-Minafra,
816 Proteomic analysis of exosome-like vesicles derived from breast cancer cells, Anticancer
817 Research 32(3) (2012) 847-860.
818 [63] ThermoFisherScientific, Boldly go above and beyond.
819 https://tools.thermofisher.com/content/sfs/brochures/Exosomes-brochure.pdf, 2016
820 (accessed COL12585 0916.).

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

821 [64] F. Liu, O. Vermesh, V. Mani, T.J. Ge, S.J. Madsen, A. Sabour, E.-C. Hsu, G. Gowrishankar,
822 M. Kanada, J.V. Jokerst, R.G. Sierra, E. Chang, K. Lau, K. Sridhar, A. Bermudez, S.J. Pitteri, T.
823 Stoyanova, R. Sinclair, V.S. Nair, S.S. Gambhir, U. Demirci, The Exosome Total Isolation Chip,
824 ACS Nano 11(11) (2017) 10712-10723.
825 [65] V. Ciravolo, V. Huber, G.C. Ghedini, E. Venturelli, F. Bianchi, M. Campiglio, D. Morelli, A.
826 Villa, P.D. Mina, S. Menard, Potential role of HER2-overexpressing exosomes in countering
827 trastuzumab-based therapy, Journal of Cellular Physiology 227(2) (2012) 658-667.
828 [66] J.N. Higginbotham, M.D. Beckler, J.D. Gephart, J.L. Franklin, G. Bogatcheva, G.-J.
829 Kremers, D.W. Piston, G.D. Ayers, R.E. McConnell, M.J. Tyska, Amphiregulin exosomes
830 increase cancer cell invasion, Current Biology 21(9) (2011) 779-786.
831 [67] D.J. Clark, W.E. Fondrie, Z. Liao, P.I. Hanson, A. Fulton, L. Mao, A.J. Yang, Redefining the
832 breast cancer exosome proteome by tandem mass tag quantitative proteomics and
833 multivariate cluster analysis, Analytical Chemistry 87(20) (2015) 10462-10469.
834 [68] K. Sandvig, A. Llorente, Proteomic analysis of microvesicles released by the human
835 prostate cancer cell line PC-3, Molecular & Cellular Proteomics 11(7) (2012) M111. 012914.
836 [69] S. Mathivanan, J.W. Lim, B.J. Tauro, H. Ji, R.L. Moritz, R.J. Simpson, Proteomics analysis
837 of A33 immunoaffinity-purified exosomes released from the human colon tumor cell line
838 LIM1215 reveals a tissue-specific protein signature, Molecular & Cellular Proteomics 9(2)
839 (2010) 197-208.
840 [70] J.S. Redzic, T.H. Ung, M.W. Graner, Glioblastoma extracellular vesicles: reservoirs of
841 potential biomarkers, Pharmacogenomics and personalized medicine 7 (2014) 65-77.
842 [71] D. Thuringer, A. Hammann, N. Benikhlef, E. Fourmaux, A. Bouchot, G. Wettstein, E.
843 Solary, C. Garrido, Transactivation of the epidermal growth factor receptor by heat shock
844 protein 90 via Toll-like receptor 4 contributes to the migration of glioblastoma cells, The
845 Journal of biological chemistry 286(5) (2011) 3418-28.
846 [72] M.W. Graner, R.I. Cumming, D.D. Bigner, The Heat Shock Response and
847 Chaperones/Heat Shock Proteins in Brain Tumors: Surface Expression, Release, and Possible
848 Immune Consequences, The Journal of Neuroscience 27(42) (2007) 11214-11227.
849 [73] A. Wade, A.E. Robinson, J.R. Engler, C. Petritsch, C.D. James, J.J. Phillips, Proteoglycans
850 and their roles in brain cancer, The FEBS journal 280(10) (2013) 2399-2417.
851 [74] K.L. Mooney, W. Choy, S. Sidhu, P. Pelargos, T.T. Bui, B. Voth, N. Barnette, I. Yang, The
852 role of CD44 in glioblastoma multiforme, Journal of Clinical Neuroscience 34 (2016) 1-5.
853 [75] J. Kraan, P. van den Broek, C. Verhoef, D.J. Grunhagen, W. Taal, J.W. Gratama, S. Sleijfer,
854 Endothelial CD276 (B7-H3) expression is increased in human malignancies and distinguishes
855 between normal and tumour-derived circulating endothelial cells, British journal of cancer
856 111(1) (2014) 149-56.
857 [76] V.P. Tryndyak, O. Kovalchuk, I.P. Pogribny, Loss of DNA methylation and histone H4
858 lysine 20 trimethylation in human breast cancer cells is associated with aberrant expression
859 of DNA methyltransferase 1, Suv4-20h2 histone methyltransferase and methyl-binding
860 proteins, Cancer Biology & Therapy 5(1) (2006) 65-70.
861 [77] J. Beliakoff, L. Whitesell, Hsp90: an emerging target for breast cancer therapy, Anti-
862 Cancer Drugs 15(7) (2004) 651-662.
863 [78] J.T. Price, T. Tiganis, A. Agarwal, D. Djakiew, E.W. Thompson, Epidermal growth factor
864 promotes MDA-MB-231 breast cancer cell migration through a phosphatidylinositol 3′-
865 kinase and phospholipase C-dependent mechanism, Cancer Research 59(21) (1999) 5475-
866 5478.

bioRxiv preprint doi: https://doi.org/10.1101/274910; this version posted March 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

867 [79] J. de Vrij, S.L.N. Maas, K.M.C. Kwappenberg, R. Schnoor, A. Kleijn, L. Dekker, T.M. Luider,
868 L.D. de Witte, M. Litjens, M.E. van Strien, E.M. Hol, J. Kroonen, P.A. Robe, M.L. Lamfers,
869 M.W. Schilham, M.L.D. Broekman, Glioblastoma-derived extracellular vesicles modify the
870 phenotype of monocytic cells, International Journal of Cancer 137(7) (2015) 1630-1642.
871