Distribution profiling of circulating microRNAs in serum

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Supplemental methods:

Chemicals and biomaterials used in the study. All chemicals used to prepare the AF4 running buffer of 1X PBS (10 mM phosphate at pH 7.4, 137 mM NaCl, 2.7 mM KCl, and 1.0mM MgCl2), ethylene glycol, dimethyl sulfoxide, guanidine hydrochloride, RNA-grade glycogen, 2-propanol, and chloroform were purchased from Thermo Fisher (Pittsburgh, PA). All single proteins used as AF4 standards were purchased from Sigma-Aldrich (St. Luis, MO). Taq 5× master mix was purchased from New England Biolabs.

Protein and particle standards used in method optimization. Protein standards, as well as the pure HDL and LDL from Sigma, were prepared in solutions of 0.1 mg/mL for cytochrome C, albumin, transferrin, IgG, or thyroglobin. The 50-nm polystyrene beads were suspended at a concentration of 0.1 μM. Exosomes were prepared using an exosome precipitation kit (Invitrogen). In brief, whole serum was incubated with an exosome isolation reagent at a 5:1 v/v ratio for 20 minutes. The sample was then centrifuged at 4 degrees Celsius to precipitate the exosomes. The supernatant was removed, and the exosomes resuspended in 1× PBS to give a 2× concentrated solution. The exosomes were either run in the system as-is or preincubated with DiO (final concentration of 5μM) for 20 minutes at room temperature. All standards were analyzed using the same flow program but without the 5-min constant flow window.

LC-MS/MS Identification of proteins. Protein samples were subjected to tryptic digestion prior to LC-MS/MS analysis. Ammonium bicarbonate was added to reach a final concentration of ~50 mM. Samples were reduced and alkylated using the standard DTT/IAA reduction alkylation protocols. Trypsin was added to the samples, and the digestion proceeded overnight at 37 degrees Celsius. After digestion, samples were purified using a C18 ZipTip (Millipore), and eluted in 50% acetonitrile/0.1% trifluoroacetic acid. After elution, samples were dried and resuspended in 0.1% TFA. These samples were then subjected to nano-LC-MS/MS analysis using a Waters 2695 Separations Module interfaced with a Finnegan LTQ (Thermo). The parameters for the nano-LC-MS/MS separation can be found in Supplemental Methods.

The raw data was uploaded to the Protein Prospector search engine (provided online by the University of California, San Francisco) for peptide and protein identification. Spectral counting was conducted for relative protein quantitation using the number of identified peptides for each protein (keeping replicates). In addition, specific searches were conducted for lower-abundance proteins of interest.

MicroRNA Analysis. The six high-abundance strands (*hsa-miR*-16, *miR*-191, *let*-7a, *miR*-17, *miR*-155, and *miR*-375) were all analyzed from a single collection. The remaining two strands (hsa-*miR*-21, and *miR*-122) were analyzed from another single collection. Prior to reverse transcription, lyophilized miRNA pellets were reconstituted in either 31 μL (for the high abundance strands) or 16 uL (for the low abundance strands). In each RT reaction, 5 μL of sample was mixed with 3 μL of a reverse transcription master mix and 2 μL of a corresponding RT primer for reach miRNA strand (TaqMan reverse transcription probe). The master mix consisted of 1.1 μL nuclease-free water, 1 μL of a 10× buffer mix, 0.13 μL of RNAse inhibitor, 0.1 μL of a dNTP mix, and 0.67 μL reverse transcriptase (all components were provided in a TaqMan reverse transcription kit). After mixing, 5 μL of silicone oil was layered on top of the RT mixture, and reverse transcription conducted on a Perkin-Elmer 2400 GeneAmp PCR system. The RT reaction consisted of a 30-minute annealing step at 16°C, a 32-minute transcription step at 42°C, and a 5-minute denaturing step at 85°C.

After RT, the samples underwent quantitative PCR (qPCR). On the qPCR plate, 1 μL of the RT product was mixed with 9 μL of qPCR master mix for a final volume of 10μL. As an overlay, 5 μL of silicone oil was added to the top of each sample to limit evaporative loss. The master mix consisted of 4.9 μL of nuclease-free water, 1 μL of ethylene glycol, 0.1 μL of DMSO, 0.5 μL of 25 mM magnesium chloride, 2 μL of Taq 5× master mix, and 0.5 μL of TaqMan microRNA Assay 20× qPCR reagent (containing miRNA RT product specific forward and reverse PCR primers, and also a RT product specific TaqMan fluorescent probe). Each sample was plated in triplicate, as were any standards corresponding to the samples analyzed (high- versus low-abundance). The qPCR analysis was conducted on a Bio-Rad CFX real-time instrument, with an initial activation step at 95 °C for 90s followed by a initial annealing

step at 59°C for 50s, then followed by a 40-cycle PCR with 30s denaturation at 95 °C and 70s annealing/extension at 53 °C for each cycle. *Cel-miR*-67 was used as an exogenous standard to account for sample loss during extraction, and miRNA levels were normalized and quantified using a standard calibration curve.

ELISA for exosome detection. The total amount of proteins in each of the 6 collected fractions added into the well of the microtiter plate (Thermo, Microfluor 2 coated, flat bottom) were around 22 ng and diluted up to 50 µl with 1×PBS. The ELISA plate was incubated overnight at 4 °C to let the proteins be adsorbed onto the bottom of the well. Then, the protein solution was discarded, and the plate was washed with 200 µl 1×PBS for two times (all washing buffers used in our assay were 1×PBS), before 200 µl of the blocking buffer containing 5% non-fat milk in 1×PBS was added for each well. After 2-hr incubation at room temperature with gentle shaking, the blocking buffer was dumped and the wells were washed twice. Next, 100 µl of the primary antibody (mouse anti-human CD63, Catalog #ab8219, Abcam, Cambridge, MA) in 1 : 5000 dilution with 1×PBS was added to the wells, followed with another 2-hr incubation at room temperature. Following 4 washes, 100 µl of the secondary antibody, HRP conjugated rabbit anti mouse IgG (Catalog # ab97046, Abcam) in 1 : 25000 dilution was added and incubated for 1 hour at room temperature with gentle shaking. The plate was washed 4 times before 30 µl of the Perice ECL substrate (Thermo Fisher) was added, and incubated for 5 minutes. The resulted chemiluminescence was detected. Two repeats were done at the same plate. For the standard curve, two repeats of human CD63 (Sino Biology) with gradient concentrations were added in the same plate. The blank contained only 1×PBS in the adsorption step.

Table S1. MicroRNA strand information.

Strand	Sequence	Rationale to be included in our study
cel-miR-67	5'-cgcucauucugccgguuguuaug-3'	As internal standard for correction of extraction efficiency
hsa-let-7a	5'-ugagguaguagguuguauaguu-3'	Reported as potential BC markers, upregulated in references shown in Table 1 and in *miRCancer*; an exosomal miRNA in Arroyo, 2011
hsa-miR-16	5'-uagcagcacguaaauauuggcg-3'	Reported in *miRNAdola* as circulating miRNA; in *miRCancer* as a potential BC marker; in Arroyo, 2011 as protein-bound miRNA
hsa-miR-191	5'-caacggaaucccaaaagcagcug-3'	Reported in *miRNAdola* as a circulating, exosomal miRNA; in Elyakin, 2010 as potential BC marker
hsa-miR-17	5'-caaagugcuuacagugcagguag-3'	Reported in *miRNAdola* as circulating in BC; in Vickers, 2011 as HDL-bound miRNA
hsa-miR-155	5'-uuaaugcuaaucgugauaggggu-3'	Reported in *miRNAdola* as an exosomal miRNA, and in references shown in Table 1 as potential BC markers
hsa-miR-375	5'-uuuguucguucggcucgcguga-3'	Reported by our collaborator, Dr. Emily Wang, as a potential marker for prediction of clinical outcome of BC patients (Ref. 18); in *miRNAdola* as exosomal miRNA; in Vicker 2011 as HDL-bound miRNA
hsa-miR-21	5'-uagcuuaucagacugauguuga-3'	Reported as potential BC markers, upregulated in references shown in Table 1; in *miRNAdola* as a circulating miRNA; in Arroyo, 2011 as protein-bound miRNA
hsa-miR-122	5'-uggagugugacaaugguguuug-3'	Discovered by our collaborators as a potential BC marker locating mainly in exosomes (manuscript in revision)

Figure S1. Optimization of AF4 flow profile using exosome isolates. (A) constant flow rates of 3.0 mL/min cross and 0.3 mL/min detector flow. (B) Post-AF4 collection (cross-flow turned off). (C-E) Rampdown of cross-flow from 3.0 mL/min to zero cross flow over 30 minutes (C), 20 minutes (D), and 15 minutes (E). Absorbance detection for all samples was measured at 280 nm. All isolates were prepared from healthy human male pooled serum.

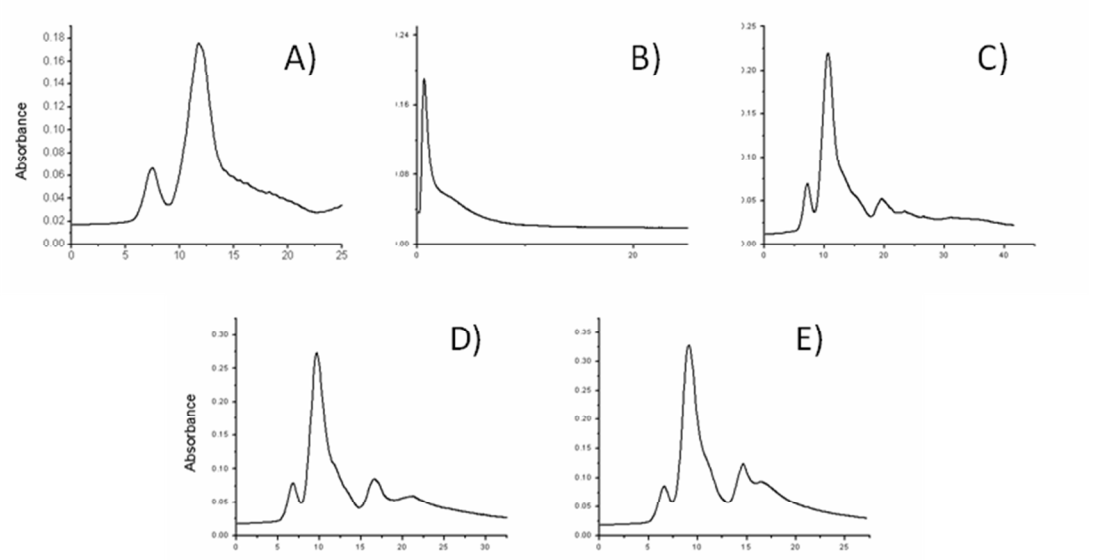

Figure S2. (A) AF4 of protein and nanoparticle standards. (B) AF4 of exosome isolates and lipoprotein complex standards. All samples were detected via absorbance at 280 nm. Exosome isolates were prepared from healthy human male pooled serum.

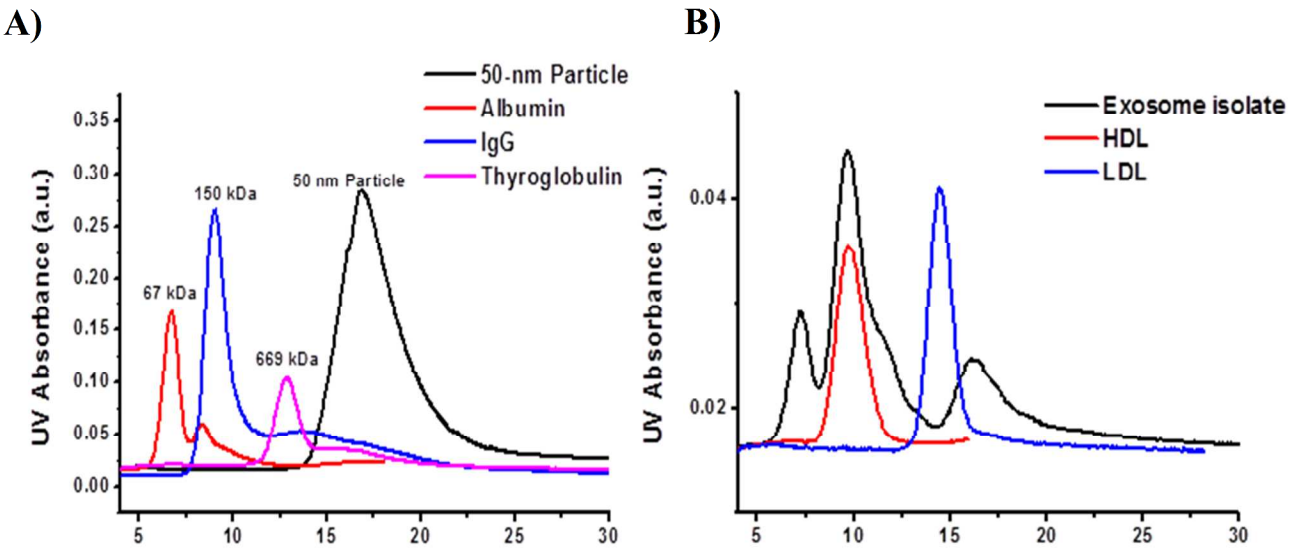

Figure S3. Addition of a 5-minute constant flow region at the start of the AF4 separation allows for improved resolution of analytes in the exosome isolate. The AF4 flow profile for this method included 5 minutes with a cross-flow of 3.0 mL/min and 0.3 mL/min detector flow, followed by a 15 minute rampdown of the cross-flow from 3.0 mL/min to zero flow. Absorbance detection was conducted at 280 nm.

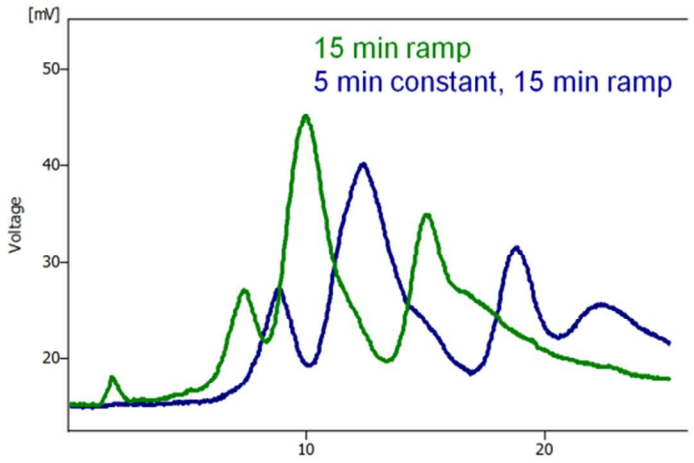

Figure S4. Absorbance (A) and DiO-stained fluorescence (B) fractograms of healthy serum samples. Black – Control 1, Red – Control 2. Absorbance (C) and DiO-stained fluorescence (D) fractograms of serum samples from BC patients. Black – Case #1, Red – Case #2. All absorbance measurements were taken at 280 nm. All fluorescence fractograms were measured at an excitation of 485 nm and an emission of 510 nm. Samples were fractionated using the optimized AF4 fractionation protocol.

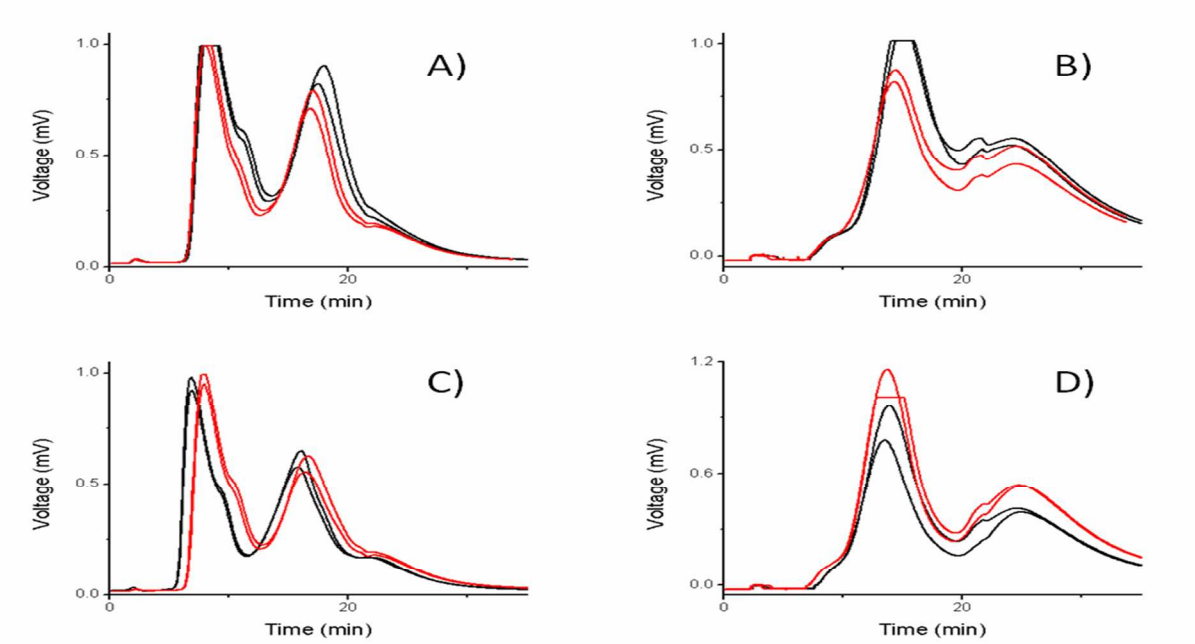

Figure S5. Recovery of hsa-miR-16 from pure serum or AF4 fractions.

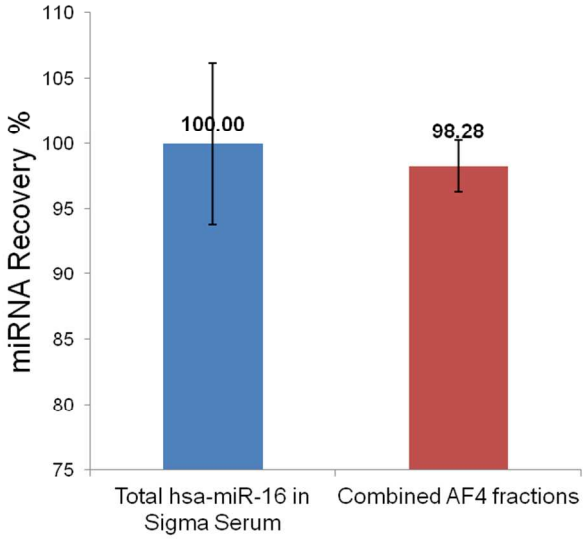

Figure S6. RT-qPCR analysis of each sample for each fraction. A- control 1, B- control 2, C –case 2. The calculated number of copies for each is normalized based on the number of copies of cel-mir-67 present in each sample. The Y-axis is the Log value of the copy number of the miRNA.