Exploring the Diagnostic Potential of Extracellular Vesicles

By
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Chapter 1: Introduction

Extracellular Vesicles

Extracellular vesicles (EVs) are emerging as a powerful tool in diagnostics and therapeutic research. Scientists began actively searching for these vesicles in the 1960s, when, before that, there was evidence of EVs from happenstance. The 1980s marked the beginning of the research into understanding EVs after the release of two papers by Harding and Pan & Johnstone. Harding focused on the membrane trafficking of EVs, while Pan & Johnstone examined the elimination of transferrin receptors during the differentiation of erythrocytes and reticulocytes. Both publications reported that the translocation of the transferrin receptors is through vesicle release from multivesicular bodies after fusing with the cell membrane.[1]

EVs are classified based on their subcellular origin. They have a lipid bilayer and are enriched with transmembrane proteins. There are considered to be three major types of EVs – exosomes, ectosomes, and apoptotic bodies.[2]

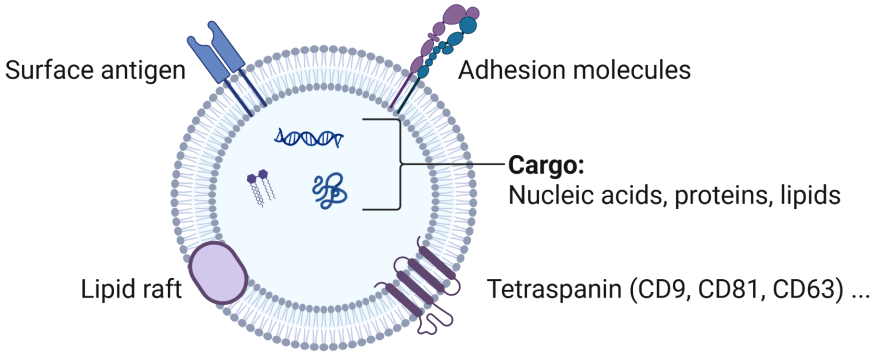


Figure 1a: EV structure (created by Biorender)

Exosomes, generally categorized as small EVs(sEVs), will be the focus of this study. They are formed when early endosomes internalize membrane components and mature into multivesicular endosomes (MVEs) or bodies. These MVEs have a membrane that buds inward to create intraluminal vesicles. Rab27, a regulator protein, traffics MVEs towards the plasma membrane of the cell by facilitating docking and fusion of the MVE to the plasma membrane, where the formed intraluminal vesicles are released from the cell into the extracellular space.[3,40] Exosomes vary in size from 50 - 200 nm, and their cargo consists of

protein, nucleic acids (miRNA, mRNA & other non-coding RNAs), and lipids from the endosome of origin.[4]

Ectosomes, also known as microvesicles, are formed when the plasma membrane buds outwards and pinches off. The process of membrane deformation, vesicle scission, and finally vesicle release is regulated and coordinated by actin filaments and ADP-Ribosylation Factor 6(ARF6). Ectosomes differ in size between 100 nm - 1000 nm in diameter.

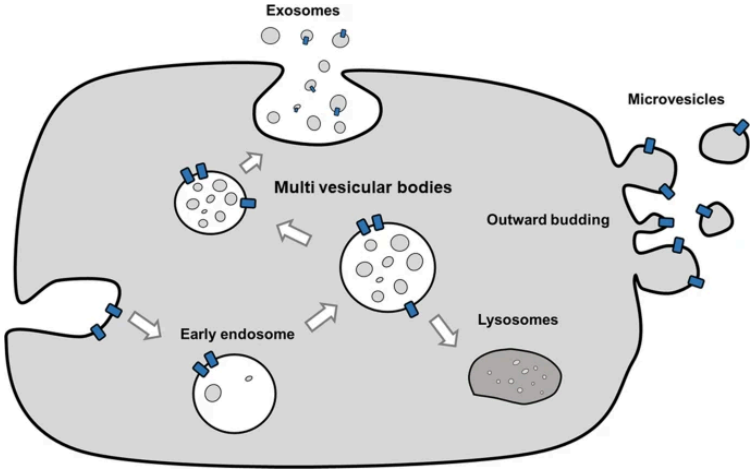


Figure 1b Production of Exosomes and Ectosomes (created in BioRender.com)

Apoptotic bodies are produced as a result of programmed cell death, either due to cell injury or ageing. These are released as blebs and range in size from 500 nm to 1000 nm. They possess signalling mechanisms that facilitate phagocytosis and cell removal.[5]

The main functions of extracellular vesicles include mediating intercellular communication, influencing the recipient cell behavior, enhancing of coagulation (haemostasis), and transporting cytokines such as interleukin-1β. They have also been implicated in autoimmune regulation as well as general signaling and regulation of the immune system. Additionally, EVs serve as cellular maintenance structures, removing unwanted materials from cells.

There are multiple theories regarding how EVs are taken up by recipient cells, these include direct binding via ligands and the surface-exposed receptors on the cells, phagocytosis, or macropinocytosis for the delivery of cargo.[6]

An interesting aspect of small extracellular vesicles, or exosomes, is their ability to carry RNA, an area that has gained heightened interest in the research community since the COVID-19 pandemic, due to its diagnostic potential. EVs

have been shown to carry multiple types of RNA derived from their cells of origin. Notably, microRNAs (miRNAs) regulate gene activity at the post-transcriptional level and are being increasingly studied as molecular biomarkers and mediators for disease[3].

RNA found in EVs typically ranges in size up to 700 nucleotides (nt), compared to cellular RNA, which ranges between 400 to 1200 nt.[6] EVs are also enriched in a variety of non-coding RNAs, including intact messenger RNAs, ribosomal RNAs, messenger RNA fragments, long non-coding RNA, miRNA, Piwi-interacting RNAs, and transfer RNA fragments.

EVs have been isolated from a variety of cell types – including lymphocytes, dendritic cells, macrophages, platelets, epithelial cells, and tumorigenic cells as well as from diverse biological fluids such as saliva, cerebrospinal fluid, plasma and serum, urine, nasal and bronchial lavage fluid, breast milk, amniotic fluid, and seminal fluid.

Importantly, EV content is dynamic and depends on the physiological state of the origin cell and its surrounding environment. The ubiquity of EVs and their potential for origin-specific cargo have made them a compelling research focus in biomedical research, spanning both therapeutic to diagnostic applications.

Extracellular Vesicle Isolation & Analysis

There is significant heterogeneity in approaches to isolating and enriching extracellular vesicles, and current efforts are focused on standardizing these methods.[41] EV isolation methods affect the purity, yield, and even their proteomic profiles. This variability complicates the reproducibility of EV studies. The most widely used EV isolation methods exploit their differences with other molecules based on vesicle size, density, shape, or surface markers. These methods include differential ultracentrifugation, density gradient centrifugation, filtration, size exclusion chromatography (SEC), precipitation, and immunoaffinity-based isolation.

EV Isolation Method	Description	Advantages and Disadvantages
Precipitation	Uses hydrophilic polymers such as Polyethylene glycol (PEG) to alter the solubility of extracellular vesicles, causing them to aggregate and precipitate at low centrifugal forces (1500 - 3000g). Cell debris and other larger contaminants are cleared by centrifugation before the addition of the polymer. Commercial kits such as ExoQuick are readily available for this process.	Advantages Preserves vesicle integrity by avoiding high-speed centrifugation. Fast, minimum technical skills, and is scalable for high-throughput use. Works in physiological pH, preserving the functionality of surface proteins. Disadvantages Polymer contaminants due to difficulty after elution may cause low purity Co-precipitation with non-EV contaminants such as lipoproteins and protein aggregates
Differential Ultracentrifugation	Stepwise centrifugation at increasing centrifugal forces. First at 300g to remove cells, then at 2000g to remove cell debris, and then at 10,000g to remove apoptotic bodies. Finally, the samples are centrifuged at > 100,000 g for sEV isolation	Advantages Validated method and widely recognized for processing large samples. Disadvantages Contamination from aggregated proteins that have co-sedimented after ultracentrifugation Bulky, Complex, and Expensive instrumentation. Requires high skills (labor-intensive) and specialized instruments.
Density Gradient Ultracentrifugation	Can be used to further isolate EVs after Differential centrifugation. Samples are separated on a rate based on their buoyant density in a sucrose or iodixanol density gradient tube.	Advantages Advantageous over Differential UC as it also eliminates particles that are isolated with EVs due to similar physical properties.

	Particles with a specific density remain suspended in the liquid layer with similar density after centrifugation. Exosomes equilibrate at densities between 1.5 to 1.19g/mL	Maintain vesicle structure and membrane topology. <u>Disadvantages</u> Complex process with a low throughput and it may be difficult to separate gradients for downstream applications.
Ultrafiltration	Uses a nanomembrane with a molecular weight cut-off to separate EVs by size. The membranes are usually 100 - 300 kDa for EV isolation.	<u>Advantages</u> Scalable method <u>Disadvantages</u> EVs may adhere to the nanomembrane and be lost, leading to a low yield. Similar-sized particles also get filtered with the EVs.
Size Exclusion Chromatography	Separates molecules based on their size by filtration through a porous gel matrix. Uses polymers to form a porous stationary phase in a chromatographic column. Particles are then separated based on their path length, which is influenced by the size of the particle. Larger particles are eluted and exit the SEC column earlier than smaller molecules.	<u>Advantages</u> EVs maintain their integrity and biological activity. <u>Disadvantages</u> Low purity (similar-sized particles, such as VLDL and chylomicrons, remain in the sample). Unfit for larger sample sizes.
Immunoprecipitation	Takes advantage of surface markers on EVs, specifically tetraspanins, to capture the target EVs using microtiter plates, affinity columns, or magnetic beads. The most prevalent method is using magnetic beads, where the EVs are captured with specific antibodies against known EV markers or	<u>Advantages</u> High EV purity and specificity Excellent for isolating EV subtypes based on their surface markers. <u>Disadvantages:</u> Expensive reagents, low yield due to the method being biomarker dependent (therefore underestimating the EV

	aptamers.	count). Strong antibody-antigen affinity makes elution of the EVs more difficult.

Table 1: EV Isolation Methods [3,7,8]

After isolation and enrichment, EV analysis to ensure sample quality, verify EV subtypes, and further characterize their composition is essential for downstream applications. EV tools for analysis range from morphology characterization, such as microscopy-based methods(Scanning Electron Microscopy, Atomic Force Microscopy, Transmission Electron Microscopy). Nanoparticle tracking analysis and Dynamic Light scattering (DLS) are important for size and size distribution determination, and methods such as western blotting, ELISA, and flow cytometry are useful for biochemical characterization. Other analysis tools may analyze their cargo depending on the type of cargo, e.g,. Liquid Chromatography-Mass Spectrometry (LC-MS) for proteome analysis. A summary of the most common methods used is discussed in Table 2 below.

Tool	**Description**	**Advantages & Disadvantages**
Nanoparticle Tracking Analysis (NTA)	Measure EV concentration and size distribution by tracking the Brownian displacements detected from the scattered light of a laser beam with a charge-coupled device (CCD) camera. The speed of motion informs the diffusion constant, which is then related to the diameter of an equivalent spherical molecule using the Stokes-Einstein equation.	<u>Advantages</u> Used for a wide range of detection capabilities, 10nm - 2000 nm. It may be used for both monodisperse and polydisperse samples. Requires a low concentration of particles for accurate results (10^6 - 10^10 particles/ml) <u>Disadvantages</u> NTA cannot be used to confirm the presence of EVs. Particles may be impurities of similar size.

Dynamic Light Scattering (DLS)	Takes advantage of Brownian motion as a monochromatic laser beam is applied to a suspension of sample, and the scattered beam is captured at variable angles by a detector. The size of the molecule is determined by measuring the random changes in the intensity of the scattered light as a function of time.	<u>Advantages</u> Measure a wide range of particle sizes, 1 nm to 6 μm. DLS is rapid and easy to use. <u>Disadvantages</u> Accurate in monodisperse suspensions and not in polydisperse suspensions (presence of larger particles influences results)
Microscopy	Microscopy is used for the morphology determination of EVs. TEM uses electrons transmitted through the sample to determine the internal structure of the sample, while SEM uses electrons that scatter after contacting the sample to determine the surface structure of the sample. AFM records surface information of a structure using a cantilever, which deforms as it contacts the surface of the sample based on the sample's morphology.	High resolution imaging of EVs (~ 1 nm) Costly to operate EVs may lose their original structure, and EVs may be immobilized first before imaging (by cryo-TEM may be used instead of TEM to prevent deformation).
Enzyme-Linked Immunosorbent Assay (ELISA)	Used for the detection and quantification of EVs by using antigen-antibody reactions. An antibody linked to an enzyme is used to detect a molecule and produces a detectable signal (often fluorescent) when a substrate is added. The signal may be measured to determine the concentration of the molecule.	High specificity of EVs captured Time-consuming, antibodies are expensive

Western Blotting	It may be used to determine the presence of surface proteins (such as tetraspanins) that are on extracellular vesicles.	Cannot identify if proteins are solely from EVs or if they are from other non-EV sources. Useful when EV samples are pure
Flow Cytometry	Used to interrogate quantitative information of surface markers on EVs by employing a laser beam of a specific wavelength, which disturbs a sample stream consisting of single particles arranged in a single file line (achieved using a sheath flow). The particles then scatter light and can be used to determine particle size. Fluorescence readout can be used to fluorescently label certain molecules and determine expression levels of that marker. For EVs, their membranes are labeled with a lipophilic dye for signal intensity or using a calibrated adapter to indirectly determine EV concentration. Acquires particle's phenotype, absolute number, and size.	High throughput, fast measurement of EVs. Subpopulations of EVs may be studied by conjugating monoclonal antibodies with the fluorochrome. Limited resolution and sensitivity for smaller particles. May underestimate EV count when their concentration is high due to the swarming effect.

Table 2: Common EV quantification and analysis tool [5, 9, 10,37, 38]

Two technologies that leverage the methodologies described above were employed for detecting EVs in the context of Breast Cancer Diagnostics. These platforms – Meso Scale Discovery (MSD) and Single Molecule Array – were selected for their multiplexing capabilities and their ultrasensitive protein detection, particularly targeting tetraspanins, which are abundant on extracellular vesicle surfaces.[5]

Both technologies offer limits of Detection (LODs) in the sub-femtomolar range, enabling the quantification of extremely low abundance EVs. This ultrasensitivity is critical,a s EVs are a small fraction of total particles in biological fluids compared to soluble proteins, lipoprotein,s and albumin.

Single Molecule Array Technology - An Ultrasensitive Digital ELISA

The single Molecule Array platform is a digital ELISA technology that can detect thousands of single molecules simultaneously at sub-femtomolar concentrations.[12] The technology utilizes arrays of femtoliter-sized reaction chambers to detect single enzyme-labeled molecules. It uses paramagnetic beads that are conjugated with an antibody to capture the analytes in a sample. A second detection antibody is added to complete the ELISA sandwich, with a streptavidin beta-galactosidase (SBG) enzyme as the label. When the target analyte is present, it binds to both the capture and the detector antibodies, forming an enzyme-labeled immunocomplex. The complexes are distributed into femtoliter wells on a chip, following a Poisson distribution, resulting in most wells having either one bead or no bead complex. A chemiluminescent substrate, resorufin-β-d-galactopyranoside (RGP), is added, and the chip is sealed to trap bead complexes and substrates into the individual wells. Beads with both the streptavidin enzyme and the substrate turn “on” as the two substances react, while wells without an enzyme, and therefore no analyte captured by both antibodies, stay “off”. Time-lapse fluorescent images are taken to analyse brightness in the “on” wells, which then inform the concentration values.[11, 12,13,14]

Since the analyte is in very low concentrations, with either 0 or 1 bead complex binding to an enzyme, it follows a Poisson distribution. The fluorescence signal detection is enhanced due to the low-volume wells used (50 fL), which accommodate only one bead per well. Imaging of these femtoliter wells allows for simultaneous analysis of data from thousands of single bead complexes. These data are used to calculate the fraction of bead complexes with at least one enzyme(f_{ON}) and the average intensity of the beads in an array, resulting in the calculation of the Average Enzyme per Bead (AEB) value.
When the f_{ON} is low, meaning most beads have either 0 or 1 enzyme per bead complex, the system assumes the digital mode. As analyte concentration

increases, and the Poisson distribution models more than one enzyme will be attached per bead, the system adapts the analog mode, measuring fluorescence intensity as opposed to counting. The number of beads and/or intensity value is then compared to known concentrations and determine the concentration of the unknown analyte in the sample.[15,16]

When the enzyme: bead ratio increases, each well may end up with bead complexes binding to more than one enzyme, but since the digital system treats wells as either ON or OFF, it cannot resolve how many analytes are present per well, leading to an underestimation of the actual concentration of the analyte.[16] This error occurs when more than ~10% of the beads are “ON”. To address this potential underestimation issue, a Poisson correction is made according to the Poisson probability mass function (equation), which informs the probability of a bead conjugate having a discrete number of enzymes.

$$P(k,\ \lambda)\ = \frac{(e^{-\lambda} * \lambda^{k})}{k!}$$

$$P(0)\ =\ fraction\ of\ OFF\ beads\ =>\ e^{-\lambda}$$
$$1\ -\ P(0)\ =\ fraction\ of\ ON\ beads$$
$$Average\ enzyme\ per\ bead\ (\lambda)\ =\ -\ ln\ (P(0))\ =\ -\ ln(fraction\ of\ OFF\ beads)$$

Equation 1

Therefore, counting the number of OFF beads ($f_{ON)}$ is used to calculate λ, which can then be utilized to estimate the actual number of enzymes/proteins. Single Molecular Array has an advantage over other digital ELISAs due to its limited Poisson noise achieved by using a high quantity of beads and arrays with tens of thousands of wells, reducing the statistical error. It is also more advantageous than other ELISAs due to its ability to operate in both digital mode, when the ratio of enzyme to bead is low, or in analog mode, when the ratio of enzyme to bead is higher than 1, which is determined by the Poisson probability distribution in equation 1. Finally, a calibration curve from known concentrations can be used with λ to obtain the concentration of the desired analyte.[15]

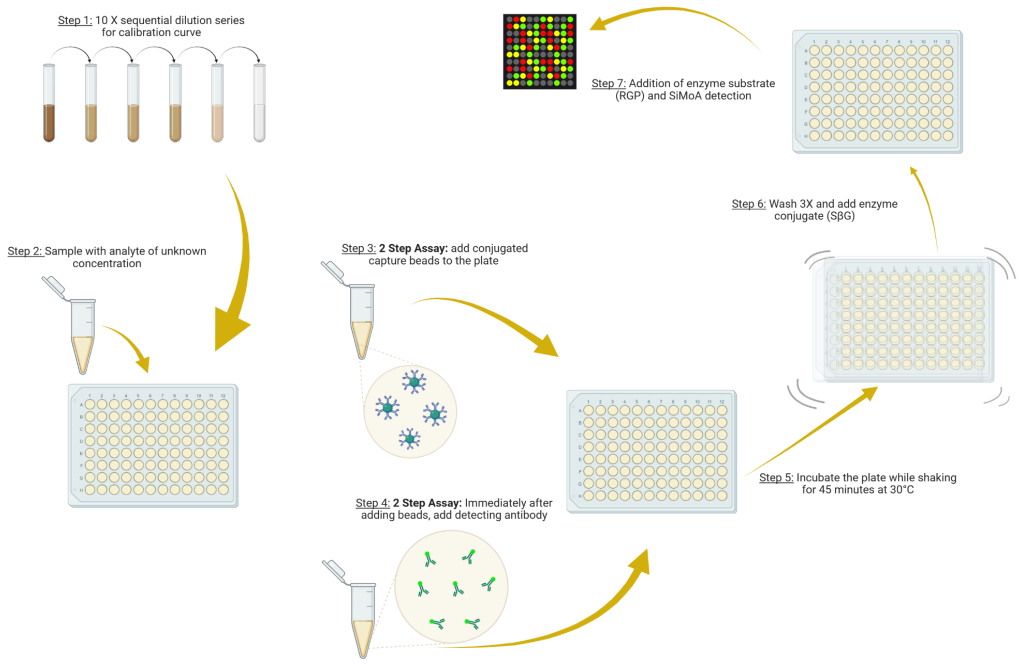


Figure 2: Single Molecule Array 2 Step Process for analyte detection and concentration measurements (Created in BioRender)

Meso Scale Discovery (electrochemiluminescence)

Electrochemiluminescence (ECL) occurs when a photon is emitted and its ground state is regenerated, allowing an ECL label to undergo multiple reaction cycles that can be recorded. Meso Scale Discovery(MSD) employs this principle in a sandwich immunoassay format, in which streptavidin is pre-coated on the assay plate and conjugated with a biotinylated capture antibody that binds the target analyte in the sample.

Following capture, a secondary detection antibody, conjugated to an electrochemiluminescent label (MSD GOLD SULFO-TAG), binds to the analyte to complete the immunocomplex. The Sulfo-tag, a ruthenium-based electrochemiluminescent molecule, emits light upon electrochemical stimulation. When a voltage is applied to the plate electrodes, light is emitted from the complex at the electrode surface.[18]

MSD, like Single Molecule Array, allows for an assay sensitivity in the fg/mL range. The intensity of the emitted light is proportional to the concentration of the analyte in the sample. Similarly to Single Molecular Array, a calibration curve from known concentrations (provided by the MSD manufacturer) is used to determine unknown analyte concentrations. In the R-Plex assay, which is specific for extracellular vesicle detection, the assay is optimized to ensure that both capture and detection antibodies bind to distinct copies of the target protein on the EV surface. This optimization prevents non-specific binding to free proteins in the sample.

Breast Cancer

Breast cancer had the highest incidence among all cancers in women in 157 of 185 countries in 2022, with 2.3 million women diagnosed and 670,000 deaths reported globally.[19] As of 2023, it accounts for 31% of female cancers. Early detection through mammography has contributed to a 43% decline in mortality in the U.S. since 1989, with death rates falling from 48 per 100,000 women in 1975 to 27 per 100,000 in 2019.[20,21] When detected early, breast cancer has a cure rate of approximately 90% compared to 50 - 80% when diagnosed at a more advanced stages.[21]

Clinically, the most common diagnostic methods for breast cancer include imaging modalities, such as mammography, positron emission tomography (PET), computed tomography, thermography, and ultrasound, as well as biopsies and histopathological analysis. Among these options, mammography remains the primary screening tool used to detect breast cancer before a palpable mass is present.[22] However, its sensitivity decreases with increasing breast density. This limitation is particularly significant in younger women(< 49 years), who are more likely to receive false positives and subsequently undergo unnecessary biopsies.[23]

Given the rising incidence of breast cancer, it is becoming more important to develop more sensitive, noninvasive, safe, and effective diagnostic methods. One promising avenue is the use of biomarker-specific biosensors, which enable the

early detection of cancer and monitoring of treatment response.[24] These biosensors rely on biological analytes such as proteins, nucleic acids and lipids. These analyte concentrations differ between cancerous and non-cancerous samples. Using electrochemical or optical transducers, biosensors convert changes in these biomolecular signals into quantifiable outputs.

Among the most promising biomarkers are extracellular vesicles (EVs), which, as previously discussed, can be isolated from various body fluids. EV cargo has been shown to reflect the physiological and pathological state of their parent cells, which has prompted research into their ability to delineate the presence and extent of disease. Due to the collection of EVs through liquid biopsies, they offer a noninvasive means of assessing systemic health in real time.[25]

In this study, serum-derived extracellular vesicles are explored for their utility in breast cancer screening. The detection of the sEVs is based on surface markers, tetraspanins, such as CD9, CD63, and CD81, which are commonly used due to their specificity to sEVs.[26] Part of the presented work involves comparing Meso Scale Discovery (MSD) and Single Molecule Array technologies for phenotyping breast cancer-derived sEVs. In both platforms, CD9, CD63 were used as capture antibodies, and each was tested against CD9, CD63, and CD81 detecting antibodies.

Dried Blood Spots

Blood microsampling has become an important area of study due to advances in bioanalytical techniques. The need for blood microsampling methods, particularly self-administered ones, surged during the COVID-19 pandemic when contact reduction was paramount in preventing the spread of the disease. Specifically, dried blood spots (DBS) have gained popularity and are being widely studied as a method to replace larger blood volume collections. DBS is obtained from a needle prick as opposed to venipuncture (venous blood collection), and collected on specially treated paper cards made of cellulose or polymers that preserve their contents for a long period. Filter paper selection is based on the downstream analysis. Whatman 903 cards; FTA DMPK type-A, B, and C, and FTA Elute cards are commercially available cards that are routinely used for

newborn screening, pharmacokinetic studies, and for the collection and purification of DNA, respectively [27]

The practice of blood spotting dates back to 1913 when it was initially used for glucose analysis studies. The technique was later used in the 1960s to measure phenylalanine in newborns for phenylketonuria testing, and has gained traction since then. Recently, there has been widespread adoption of the sampling technique in epidemiological studies, metabolic screening, drug research and development (drug discovery, therapeutic drug monitoring), vaccine studies, and in diagnostics, due to its advantages over venous blood collection.

Venous blood collection is invasive, requiring a large volume of blood, while dried blood spot samples only take about 4 to 5 drops of whole blood. Venipuncture involves trained medical personnel(phlebotomist), and the collected blood can generally remain viable for only up to 6 hours. The sample is costly to store and requires cold chain shipping, which is not always available. These factors make DBS an attractive area of study. Additionally, DBS does not require a laboratory or other complex infrastructure. During transportation, DBS Samples are considered non-regulated infectious material and may be stored at room temperature during transport. Since DBS collection is not complex, it is useful for at-home collection (self-collection), which became practical during the height of the COVID-19 pandemic.

There have been studies reporting the variability in DBS analysis, with hematocrit being the most prominent source. Hematocrit refers to the percentage of red blood cells in a known volume of blood. This variability stems from biological differences (e.g., anemic vs. normal samples).[28] High hematocrit values increase the viscosity of the sample, causing a less homogenous spread of blood across the filter paper, which may lead to less reliability. Nevertheless, DBS remains promising, as it only requires small sample volumes, which is important in protein biomarker analysis, where target proteins are typically in low abundance. Such analysis has been done using immunoassays and Liquid chromatography-tandem mass spectroscopy (LC-MS/MS).

Minimal work has been published on quantifying small extracellular vesicles from Dried Blood Spots. Bæk et al's work, published in 2025, employed a reaction buffer (PBS with 0.05% Tween20®) and achieved a 40% EV recovery from DBS compared to EVs isolated directly from plasma.[29] As far as we know, no currently published work analyzes the RNA cargo of extracellular vesicles isolated from dried blood spots. However, published studies have demonstrated that miRNAs

remain relatively stable in DBS, which has promising implications for the integrity of miRNAs and other RNA cargo stored in sEVs.[30]

The Tripathi lab has previously published studies on DBS-based analyte extractions for DNA (for nucleic acid-based diagnostics), steroids, and opioids (used for neonatal abstinence syndrome detection).[31-34] In addition, the lab has developed several methods for sEV extraction, including optimization of the extraction buffer and the application of electric field-assisted recovery. Part of the work presented here aims to investigate sEV extraction using electric field-assisted methods, and also how we can optimize buffer extraction for higher yield and purity of EVs stored in Dried blood cards. There is also preliminary work on developing protocols for RNA recovery from DBS-recovered sEVs.

The objectives of the presented research are twofold. The first aim is to optimize the digital ELISA assay for screening of breast cancer using clinical serum samples of breast cancer-derived extracellular vesicles. We hypothesize that there is an increase in EV surface marker expression in breast cancer serum compared to normal serum. This objective will be realized by comparison studies of CD9 and CD63 tetraspanin expression on said samples. Chapter 2 of this manuscript has descriptive details on the methodology and results for the study. The second objective is to develop a methodology for improving the process for EV extraction from dried blood spots (DBS). Based on studies of similar molecules, we hypothesized that a stabilizing buffer coupled with the use of an electric field would lower the extraction time while maintaining the yield of EVs compared to other methods. For this second objective, DBS was soaked in buffers for one or two hours, and the concentrations of surfactants and sugars in the buffers were studied for maximum EV yield during soaking. An electric field of differing voltage was then applied to the spots with uniform electric field strength in either AC or DC mode, after which the EVs were isolated and characterized. Chapter 3 of this document has the full description of the methodology and results obtained from this study.

Chapter 2: Serum-Derived EVs for Breast Cancer Screening

Materials & Methods

In this study, breast cancer-free serum is compared to breast cancer-derived serum in both MSD and Single Molecule Array immunoassay platforms using the manufacturer-provided assays. Additionally, since the breast cancer samples had minimal data on the patient status, an additional breast cancer sample from the

MCF-7 cell line (purchased from Abcam, product #ab271144) was used as a positive control. Evaluations of the two systems include their lower limits of detection and quantification (LLOD, LLOQ, respectively), the range of detection, sensitivity (ability to obtain a true positive), and their specificity (ability to obtain a true negative). The comparison was done between plasma samples with a known history of breast cancer (n = 8) and normal plasma from a healthy volunteer (n = 8). Both breast cancer and normal plasma were from pooled human female subjects and were obtained from a commercial biobanking service. EV Isolation was accomplished by Ultracentrifugation, as described above, and stored in -80 °C until use. The breast cancer samples were spun down at 10,000g for 5 minutes after thawing. Anti-CD9, Anti-CD81, and Anti-CD63 antibodies were obtained from Abcam (product IDs ab236630, ab109201, and ab134045). Although MSD provides calibrators to use for creating a 4-fold serial dilution standard curve, an additional 10-fold calibration plasma and MCF-7 cell line series was done to allow for the direct comparison between the Meso Scale Discovery Assay and the Single Molecule Array assay. Three types of calibrator standards were created for this study to enable comparison between the two technologies: MSD provided standard calibrators, an MCF-7 cell line calibrator, and a normal plasma calibrator standard.

Control samples were made by spiking plasma with known concentrations of low, medium, and high concentrations as seen in the plate setup section. This additional control was added due to time constraints, as the breast cancer (BC) and healthy control (HC) samples required multiple plates for comparison.

Meso Scale Discovery

The MSD Assay was used as a commercial control platform. Samples were prepared as recommended by the manufacturer. The R-plex assays were performed according to manufacturer instructions (MSD R-Plex® kits; Meso Scale Discovery, LLC, Rockville, MD), [17] and the protocol can be found in Appendix A. The kits provide all necessary reagents (assay diluent, detection antibodies, and calibrators). All samples were assayed in technical duplicates, and the calibration

curve was established per manufacturer protocol, where a serial 4-fold dilution was done from the stock standards provided so that the first dilution in the series was 50 µL of calibrator in 150 µL of diluent. The negative control was established by only adding the sample diluent supplied with the kit. Plasma samples were run in duplicate as a 20X dilution. The plate layout for this study may be found in Appendix B.

Single Molecule Array

Anti-CD9, anti-CD63, and anti-CD81 assay beads were prepared in-house. Briefly, carboxylated paramagnetic beads from Quanterix were washed and then activated with 10 mM of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) at 4C for 30 minutes. EDC is used to activate the COO group on the bead surface and prepare it for binding to the antibody.

Next, they were conjugated to capture antibodies at a concentration of 0.3 mg/ml for 2 hours at 4C. After conjugation, the beads were washed and blocked with 1% BSA solution at room temperature for 1 hour. Before use, the beads were diluted to a final concentration of 5 x 10^6 beads/mL. Unconjugated “helper” beads from Quanterix were added at concentrations of 1.5 x 10^7 beads/mL.

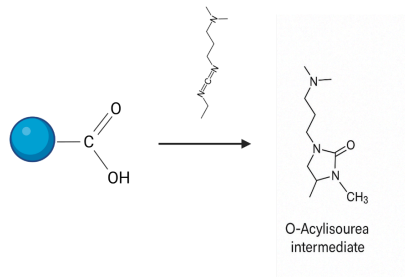


Figure 3: EDC action on carboxylated magnetic beads

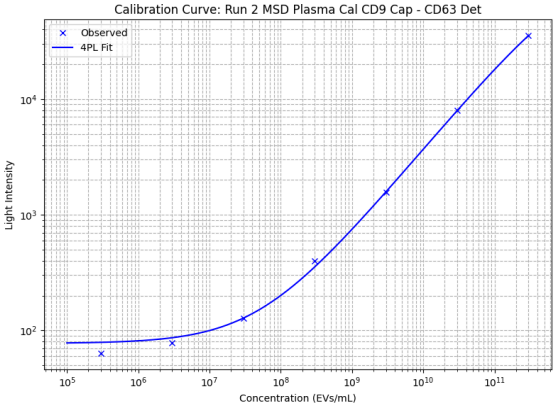
All EV assays were performed in a 2-step configuration. In the first step, samples were diluted 2X with EV sample diluent in a 96-well plate. Next, 25 µL of conjugated magnetic beads were added to each well, and immediately after, 20 µL of detector antibody at a concentration of 0.3 µg/mL was also added. The plate was incubated while shaking at a temperature of 30℃ for 45 minutes in a TriNEST™ Microplate Incubator Shaker by Perkin Elmer. For the second step, the resulting bead complex was washed in an Agilent Biotek 405 TS Microplate Washer, and the bead pellet was resuspended in 100 µL of 200 pM Streptavidin-ß-galactosidase (SBG) solution. The beads were incubated with the

SBG for 10 minutes at 30℃ and 800 rpm. After incubation, the pellet was placed back onto the microplate washer to remove excess SBG. After the final wash, the bead pellet was left to dry covered at room temperature for 10 minutes. Simultaneously, Resorufin ß-D-galactopyranoside (RGP) substrate was equilibrated to room temperature on a shaker for 30 minutes to ensure the substrate is fully dissolved. Once the pellet was dry and RGP fully dissolved, the sample plate and RGP bottle were both loaded onto the Single Molecule Array instrument for detection.

Calibration curves - MSD & Single Molecular Array

Normal EV calibration curves were prepared using EV isolated from pooled normal female plasma (BioIVT Cat#: HUMAN PLK2-9911-R). EVs were isolated from plasma via ultracentrifugation at the EV Core Facility, and the final concentration was determined using NTA. Calibration curves using EVs derived from the breast cancer cell line MCF7 were prepared using lyophilized small EVs obtained commercially from Hansa BioMed. In both cases, EVs were diluted with EV Sample Diluent to prepare a 10X dilution series. The same calibrator was run on both the MSD and the Single Molecular Array to obtain EV quantification.
Run 1 and Run 2 represent plates run on the first day and the following day, respectively. These calibration curves were used to determine the concentration of EVs in patient donors with a confirmed history of breast cancer.

A



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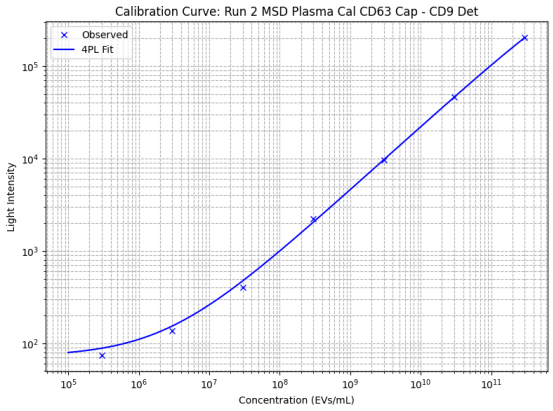

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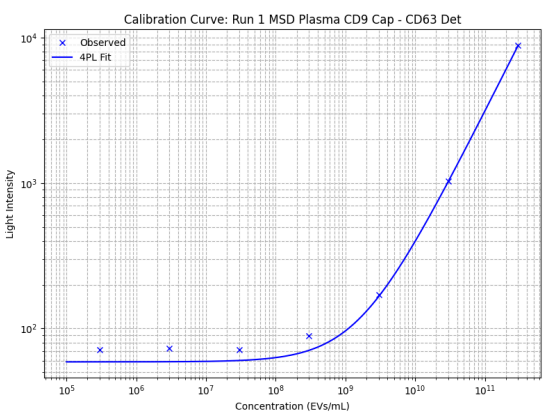

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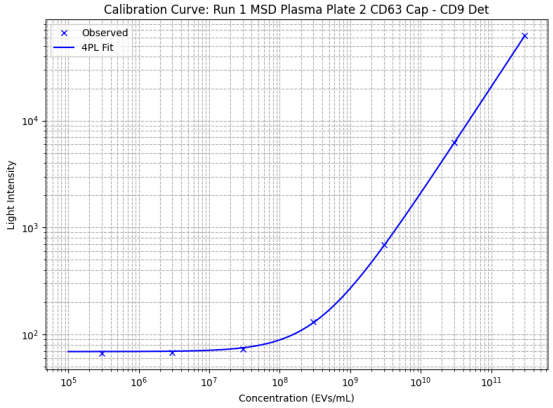


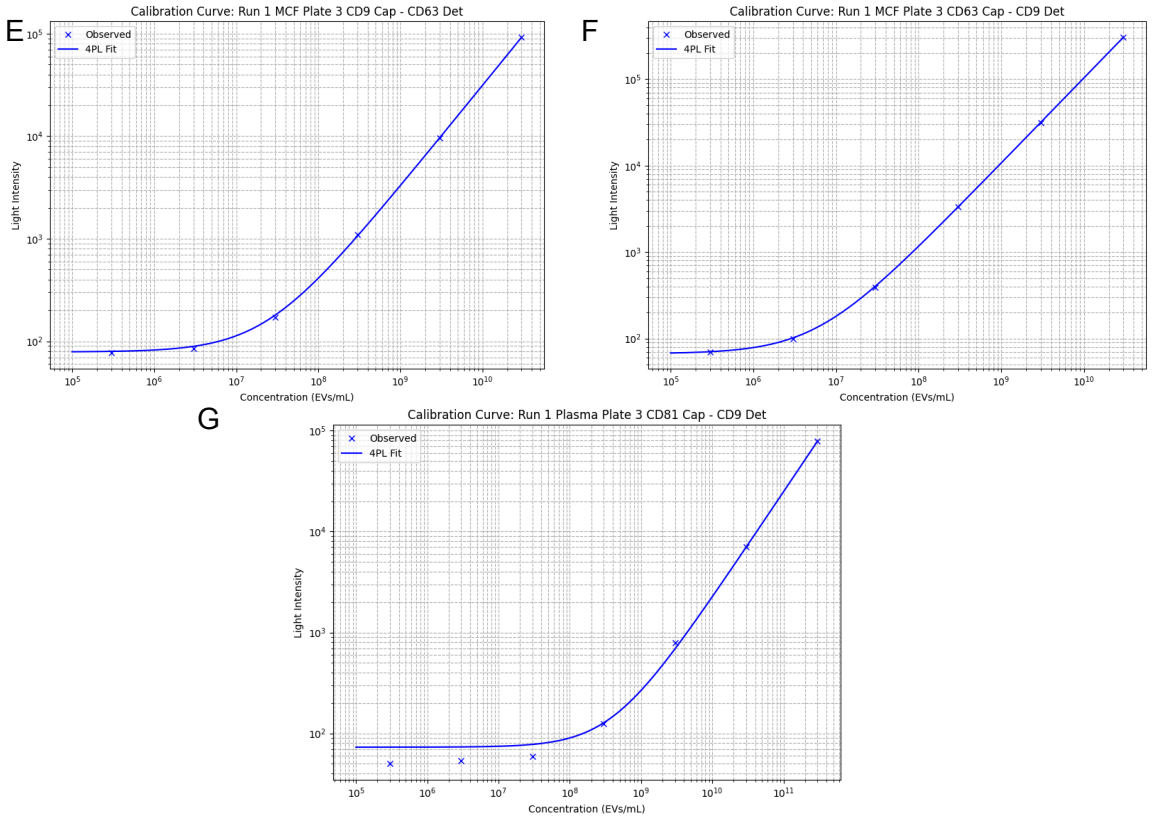
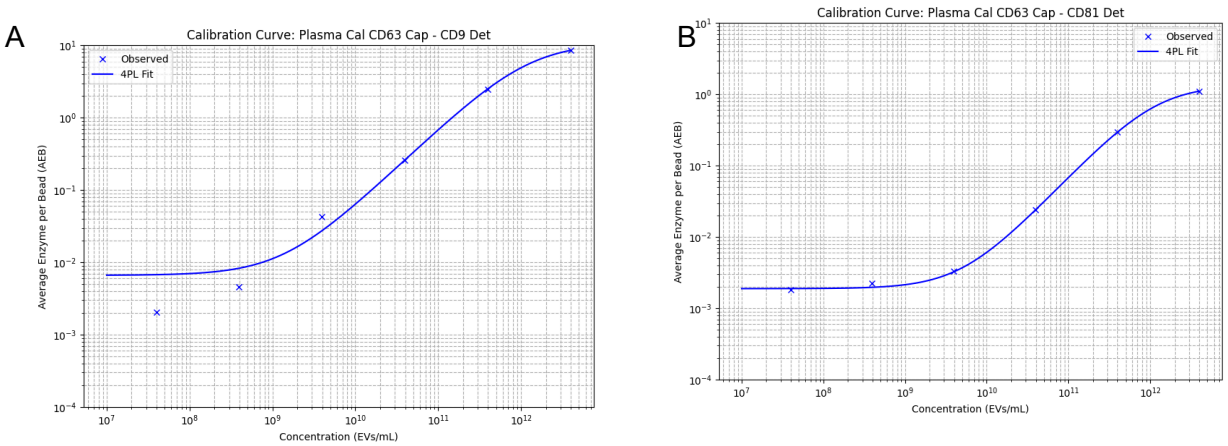


Figure 4: Meso Scale Discovery Calibration Curves used for Breast Cancer EV detection. Left side graphs showing calibration curves, with A & C showing Plasma series dilution with CD9 as the capture and CD63 as the detector. Figure 4C shows the MCF-7 cancer cell line EV series dilution, also with CD9 as the capture antibody and CD63 as the detecting antibody. On the right-hand side, Figures 4B and 4D show Plasma calibration curves with CD63 as the capture antibody and CD9 as the detecting antibody. The MCF-7 calibration curve is shown as the third curve (Figure F), also with CD63 as the capture antibody and CD9 as the detection antibody. The bottom calibration curve (Figure 4g) shows a third capture antibody – CD81 – used with CD9 as the detecting antibody.

For the single-molecule array technology, the calibration curves used for the breast cancer study are as shown below. The series dilution factor was also 1:10. The same samples were used in Single Molecule Array as in MSD above. Healthy controls did not have a history of cancer, while diseased samples had a confirmed previous history of breast cancer.



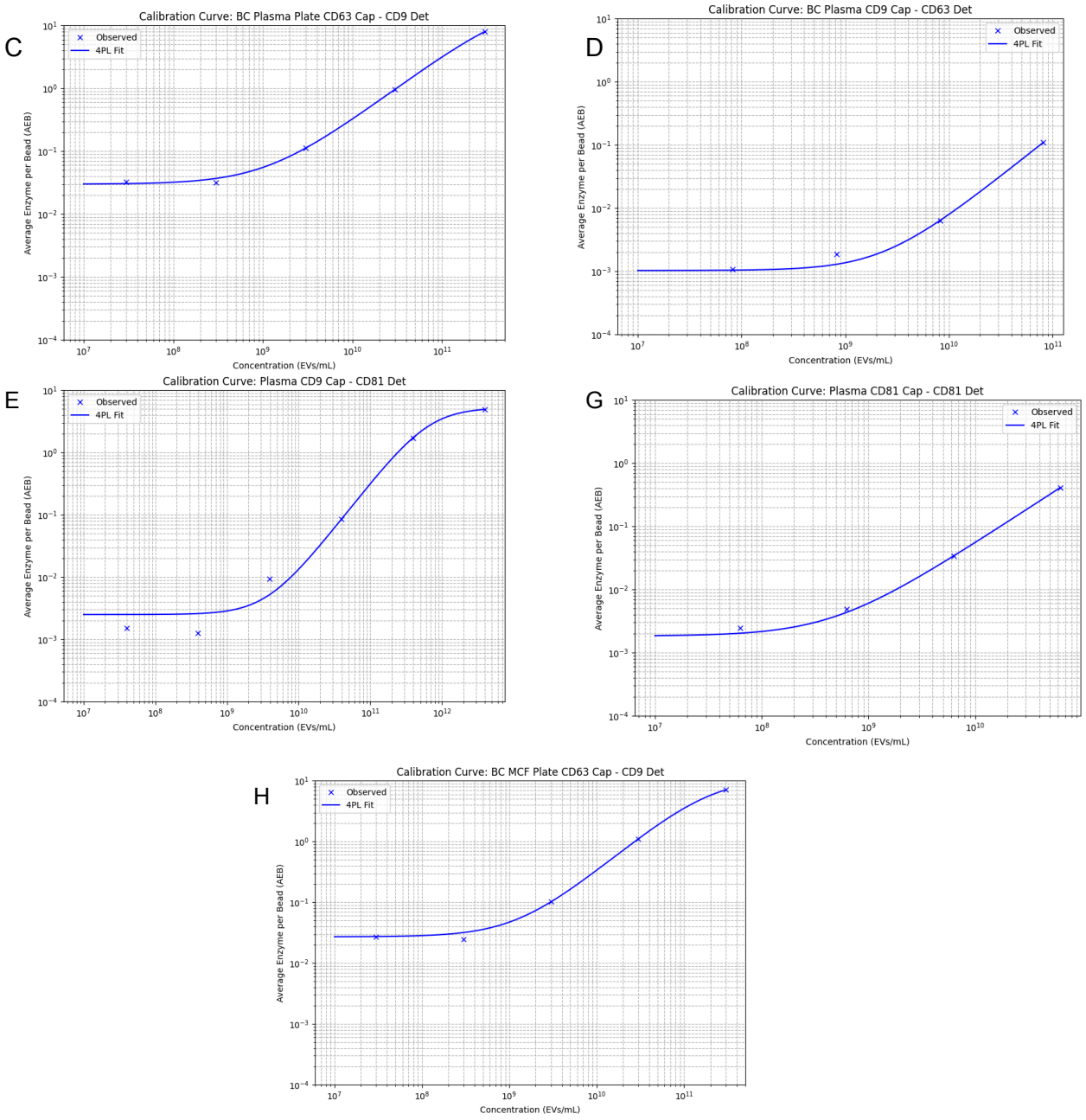


Figure 5 Single Molecule Array Calibration Curves used for Breast Cancer EV detection. Figure 5 A, B, and C all show the use of plasma series dilution for the calibration curve with CD63 as the capture antibody and CD9, CD81, and an additional CD9 detecting antibody. Figures 5D and 5E show a plasma curve with CD9 as the capture antibody and CD63 as the detecting antibody for 5D, while 5E has CD81 as the detecting antibody. Some data were using CD81 as both capture and detection antibody, and therefore, we created a calibration curve to address those data

points, shown here as Figure 5G. Figure 5H shows the MCF-7 cancer cell line with CD63 capture antibody and CD9 detection antibody.

When comparing the MCF-7 calibration curve to the Plasma curve using CD63 Capture antibody and CD9 detector antibody, there was a significant difference between the signals (AEBs) obtained from the curves for concentrations above the LLOD. This 10X difference suggests an increased expression of CD63 in EVs from the MCF-7 cell line compared to normal plasma. The LLOD is also at a lower concentration in the MCF-7 cell line compared to normal samples.

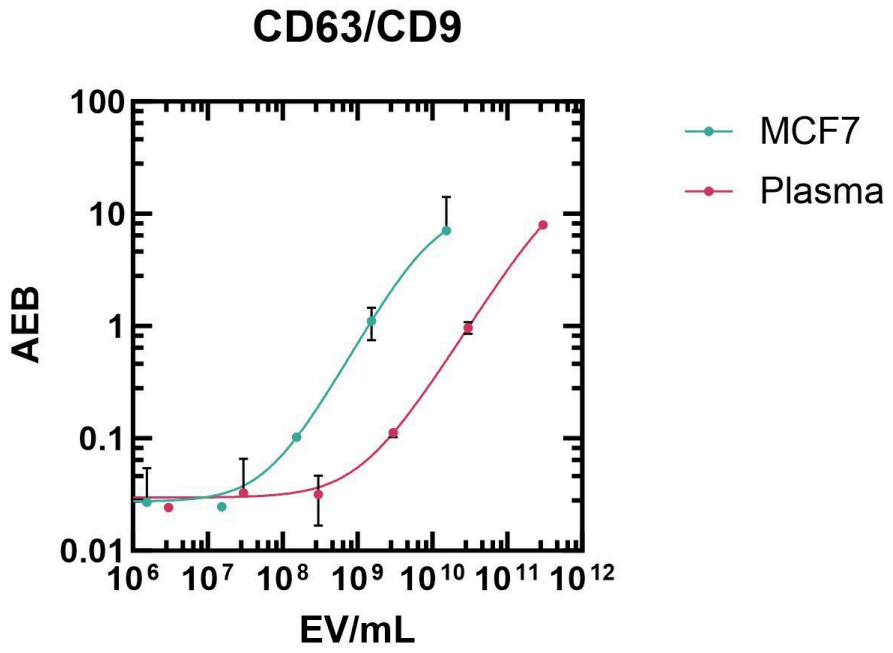


Figure 6: MCF-7 Breast Cancer Cell Line Calibration Curve versus the Plasma Calibration Curve using CD63 Capture and CD9 Detector

Comparison in the detection of Breast Cancer sEVs between Single Molecule Array and Meso Scale Discovery

Based on the preliminary results shown in Figure 7, CD63 demonstrates promise as a capture antibody for breast cancer EV detection across both technologies. There are some outliers, which may be attributed to known variability in EV concentration between individuals and even within the same person, depending on factors such as sample collection timing, fasting state, stres,s and physical exertion. Reported normal EV concentration is on average ~10^{10} EVs/ml.

There is some difference when CD63 is used in Single Molecule Array technology, showing higher EV concentration compared to healthy controls. A similar observation is made on the MSD, with the differences between breast cancer samples and normal controls having significance, as revealed by the p-values.

Contrastingly, using CD9 as the capture antibody shows less distinction between cancerous samples and noncancerous samples on the single-molecule array platform. It does better in MSD, with a significant p-value. These differences highlight the importance of platform-specific optimization when developing assays. Further studies are needed to identify the reason behind the differences. One hypothesis sis that detection in the MSD instrument may be diffusion limited due to the samples being analyzed from a plate as opposed to the single molecule array, where each bead is segregated into a well.

It is important to note that a limited sample size (n=8) may have affected the ability to detect statistically significant differences, particularly when compared to findings from other studies that reported an increase in CD9 and CD63 in EVs originating from cancer cells. While breast cancer samples were from individuals with previously confirmed diagnoses, we did not have access to the clinical metadata to assess the disease's status or treatment stage during sample collection.

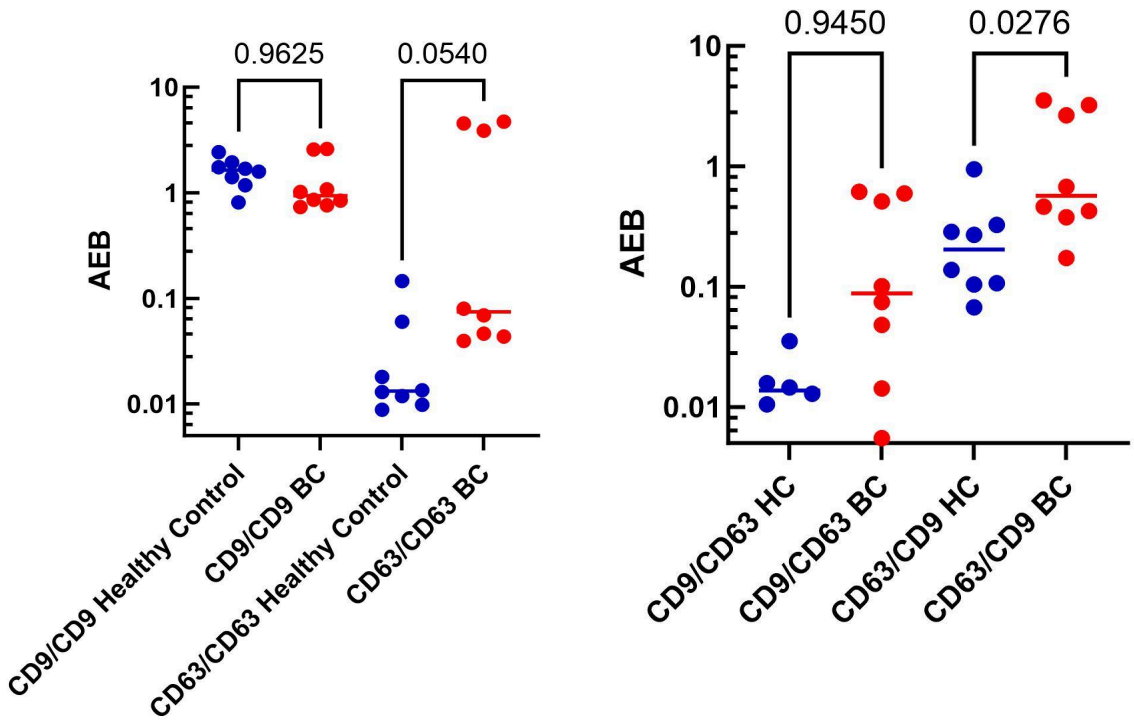


Figure 7A shows single-molecule array results for homogeneous capture detector pairing. CD9, as a capture and detection antibody, shows minimal differences between healthy control and breast cancer samples, while CD63, as the capture antibody and detecting antibody, shows a difference in signal between healthy control and breast cancer samples. Similarly, Figure 7B shows the single molecule array results when using a heterogeneous capture detector antibody pair. CD63 as the capture shows a significant difference between breast cancer samples and healthy controls, with breast cancer samples having higher signals compared to normal samples.

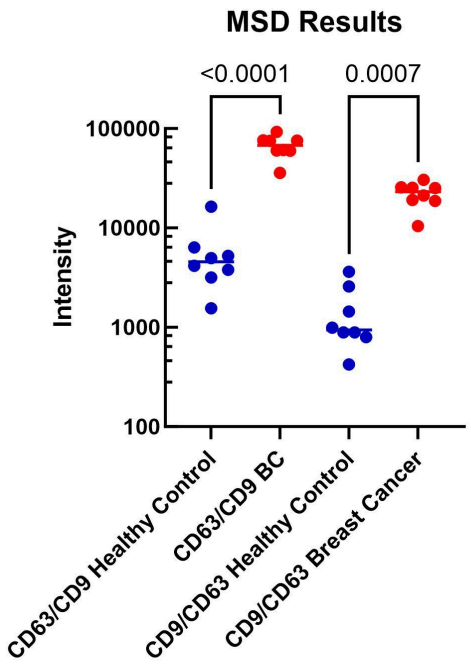


Figure 7C shows the MSD results for using CD63 and CD9 as capture antibodies, both of which show a notable difference in intensity signals and therefore in concentration between normal controls and breast cancer samples.

Chapter 3: Extracting EVs from Dried Blood Spots for Downstream RNA and Proteomic Analysis

EV Extraction from Dried Blood Spots

Preparation of Dried Blood Spots

Single human donor normal whole blood in a vacutainer® (K2 EDTA, Thomas Scientific) was purchased from Research Blood Components LLC. Dried blood spots were prepared by pipetting 100 µL of whole blood within 5 hours of receiving the blood from the supplier, onto 3 of the 5 circles on a protein saver card (Whatman 903 Protein Saver Card; Cytiva; Marlborough, MA, USA). The protocol for blood spotting was developed according to the previous methodology established and published in the Tripathi Lab group. Once arrived, the blood is gently mixed periodically during spotting, ensuring no bubbles are formed. All

experimentation using whole blood is done under a biosafety cabinet. The cards are left open in the biosafety cabinet overnight to dry. For more precision between spots, spotting is completed when the whole blood is a lighter color. Plasma is created from the remaining blood by centrifugation at 2000 g for 10 minutes. All cards are folded on the following day and bagged with a package of desiccant to prevent the dried blood spots from absorbing water during storage. Blood spots were stored at -20 °C due to the anticipated long storage times; it has been shown that more genes are expressed in frozen blood spots compared to those stored in 4 °C and room temperature, and that there is lower result variation in immunoassays.[35,36] See Appendix C for the completed protocol for preparing dried blood spots.

There is minimal work accomplished and no current gold standard for the Extraction of EVs from DBS in the literature, to the best of our knowledge. For this study, EVs were extracted from DBS samples via thermal extraction, electro-extraction, and soaking in buffers and optimizing yield and effort applied. Furthermore, we aimed to assess EV cargo when stored in DBS, with particular interest in Total RNA yield, due to the emergence of EV RNA as important markers for disease diagnosis and prognosis.

DBS Derived EV Extraction: Methodologies & Results

Buffer EV Extraction

The first part of this study focused on evaluating buffer requirements for effective EV extraction from DBS. First, we aimed to evaluate the effects of buffer formulations on EV extraction from DBS. We investigated a variety of formulations, as shown in Table 3 based on previous work identifying storage conditions that preserved and stabilized EVs.[43] These included 1X PBS + 1% Tween 20, Tris-EDTA, a HEPES-based buffer, and Tris-EDTA with PBS. Phosphate-buffered saline (PBS) was used as a control due to its established role in EV extraction.

Additionally, we compared incubation times with each of the buffers (1 hour vs 2 hours). Briefly, the procedure involved cutting out DBS from the Whatman protein saver cards

using a 6 mm biopsy punch, wetting the paper with 500 µL of extraction buffer, and soaking for either 1 or 2 hours. The paper was then removed, and the remaining solution was centrifuged at 10,000g for 5 minutes, which is standard for the removal of cell debris. The final volume was measured, and EV quantification was performed using the Single Molecule Array Technology. For that, 1:1 sample and diluent were added to the well plate, with a 1:1:1 ratio of CD9: CD63: CD81 – each at 0.1ug/ml (pan-tetraspanin) beads used for both capture and detection. 200pM of SBG was added during the 2-step Single Molecule Array Protocol. For each of the experiments done, a plasma sample from the same donor (if available) was used as a positive control to assess the difference between EV yield in plasma versus in dried blood spots. These samples were collected before spotting by centrifuging the blood at 2000g for 10 minutes at 4C. The obtained sample is then aliquoted and stored in the -80 °C freezer.

Extraction Buffer	Buffer Volume	Blood Spot Volume	Time	Final Liquid Volume
1X PBS	500 uL	100 uL	1 hr	370 ul
1X PBS (2% Tween 20)	500 uL	100 uL	1 hr	390 ul
Tris EDTA (10mM Tris + 1 mM EDTA)	500 uL	100 uL	1 hr	400 ul
HEPES + Tween 20 based Sample Diluent	500 uL	100 uL	1 hr	390 ul
1X PBS	500 uL	100 uL	2 hr	380 ul
1X PBS (2% Tween 20)	500 uL	100 uL	2 hr	365 ul
Tris EDTA (10mM Tris + 1 mM EDTA)	500 uL	100 uL	2 hr	375 ul
HEPES + Tween 20 based Sample Diluent	500 uL	100 uL	2 hr	370 ul

Table 3: Initial Experimental setup for buffer extraction assessment

Buffer Extraction comparison (Tris EDTA Elimination)

Initial results showed that Tris EDTA was not as successful in maintaining EV integrity and therefore should not be used as an extraction buffer. This is because of EDTA which chelates cations like calcium and magnesium, essentially disrupting the membrane EVs. Both the internally developed HEPE-based buffer and the PBS with 2% Tween showed promising results, as seen in the AEB concentrations below. There was also no significant difference between the 1-hour incubation time and the 2-hour incubation time. Therefore, we opted to optimize this method by continuing further experiments with PBS

Tween and HEPES-based sample diluent.[43,44] The hypothesis for why these two work well is because of the presence of Tween 20, which is a surfactant known to help maintain EV integrity by reducing surface tension, improving solubilization of membrane-bound vesicles. HEPES-based buffers, most commonly used in cell culture media, have great buffering capacity, therefore maintaining the pH of the sample, which is crucial for the integrity of EVs.

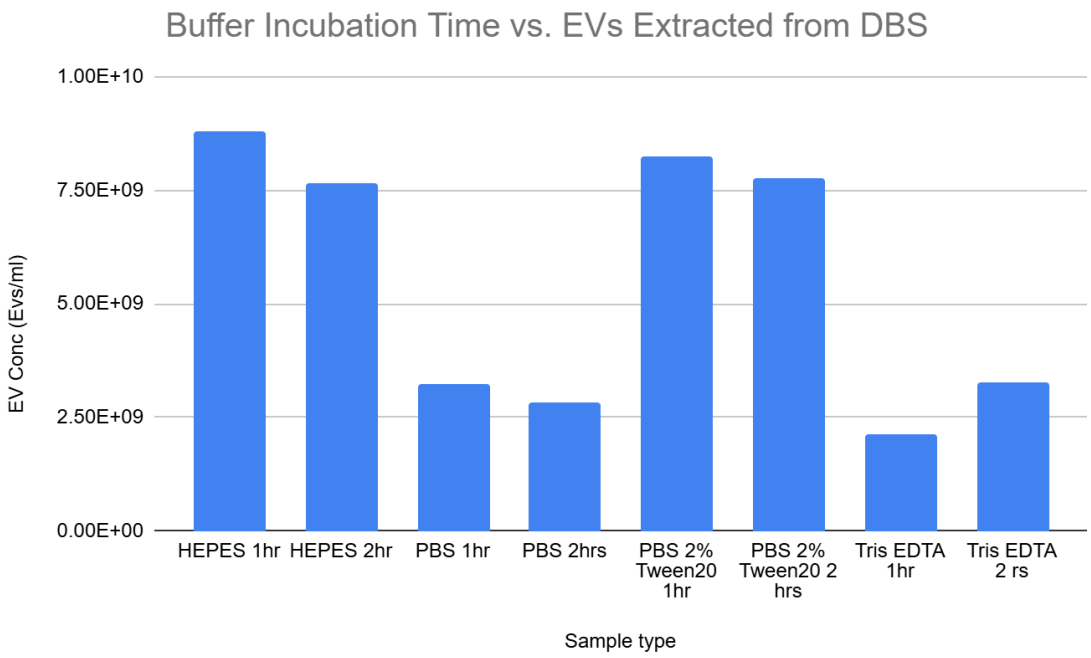


Figure 8 Single Molecule Array EV Detection based on Buffer Extraction Type and the Length of time Blood spots are incubated for.

Further experiments were conducted to compare an in-house HEPEs-based buffer with PBS containing 0.05% Tween 20. Bæk et al recently published a paper showing a 40% yield of EV compared to plasma extraction. We aimed to use PBS Tween 0.05% as a control group and compare that to our original HEPEs-based formulation.[29] Baek's protocol also incorporated a spin column filter during soaking to further remove the cell debris and other large contaminants. For this, a 2-micron filter was employed per the methodology published initially by Bæk et al. The full protocol is listed in Appendix D. To improve EV yield, especially due to the differences in dried blood spots spread over the protein card, two dried blood spots were soaked per sample instead of one.

Briefly, dried spots were wet with 60 µL of the buffer and placed in a spin column, and then centrifuged over a collection tube containing 60 µL of buffer. The spin columns were incubated at room temperature for an hour and centrifuged at 20,000g for 5 minutes. The DBS is discarded, and 50 - 60 µL of the sample is obtained from the resulting supernatant, taking care not to disturb the separated solution.
EVs were then isolated using either the ExoQuick precipitation method or immunoaffinity pulldown using CD9 beads.

For ExoQuick, directions from the manufacturer were followed, where the PEG solution was added to the sample and incubated at 30 °C for 4 minutes without mixing. Afterwards, the samples were centrifuged at low force (1500g) for another 30 minutes while being refrigerated at 4C. The supernatant is then removed, and the remaining sample is re-centrifuged for 5 minutes at 1500g. The second supernatant is then removed, taking care not to disturb the pellet formed at the bottom of the tube. The pellet is then resuspended in a buffer (HEPEs-based) and used immediately. Results from only ExoQUick isolation were not favorable, as AEBs observed in the Single Molecule Array technology were very low, even with this isolation method. The immunoprecipitation process involved aliquoting 100 µL of the sample and adding CD9 beads stock solution. An end-over-end mixer was used to incubate overnight while mixing at 4 °C, which has been shown to improve pull-down efficiency.[39] The following day, the beads are pelleted with a magnet, and the supernatant is removed. The beads are washed with PBS three times and then resuspended in an elution buffer that constitutes 0.1M Glycine HCl at a pH of 2.5. The elution buffer is incubated while mixing at room temperature for 45 minutes before being pelleted. To neutralize and stop any further reactions, a 1M Sodium Hydroxide solution is added, and the eluent is transferred to a clean Eppendorf for use.
For the single-molecule detection step, a 1:1 ratio (50 µL each) of the supernatant and sample diluent was added to a 96-well plate, and the 2-step assay was performed with similar concentrations of beads, detector, and SBG as described in previous sections. To get a direct comparison of results with Baek et al's methodology for the in-house HEPEs buffer, a 0.05% Tween PBS solution was used to elute the EVs from the spots.

Electro-DBS

The Tripathi group has published on dried blood spots in multiple works. The most related to this work is Lee et al's paper on nucleic acid extraction from dried blood spots using electric current and Fariha's work on electric field-assisted DBS sample preparation for steroid analysis. There is also a lot of work happening on using extracellular vesicles for diagnosis and prognosis. Combining these two fields, we thought that we could explore using electricity to extract EVs from dried blood spots to reduce the time required for extraction and to hopefully improve yield and purity compared to other extraction methods. For this work, a 96-well plate was previously used in Ramisa's work for high-throughput sample preparation of DBS for Neonatal Abstinence Syndrome Detection. The plate is fitted with a copper FR4 0.62, 3.20X 4.9" PCB and the screws used for that were used as electrodes for the bottom part of the device. The top electrodes were made by drilling a copper FR4 0.62, 3.20X4.9" PCB, and stainless steel PEM spacers were press fit onto it. Electrical wires were soldered on both sides (top and bottom PCB electrodes), and insulated with 4" wide tape. Before use, the plate was sterilized using ethanol and bleach and left to air dry. More details on the device may be found in the associated paper.[32]

For feasibility, a DC charge from 0V - 40V was applied to a dried blood spot soaked in HEPEs-based buffer while the spot was in the setup for 5 minutes. Afterwards, the dried blood spot paper was removed, and the sample was collected, centrifuged at 10,000g for 5 minutes, and the supernatant collected for single-molecule array detection of EVs using the 2-step assay. CD9 conjugated beads were used, and a pan tetraspanin solution of equal concentrations of CD9, CD81, and CD63 was used for detection. The results were promising, however suspiciously high, and we thought other non-EV contaminants may be detected since there is no EV isolation step performed before using the Single Molecule Array technology. We were also not able to replicate these results.

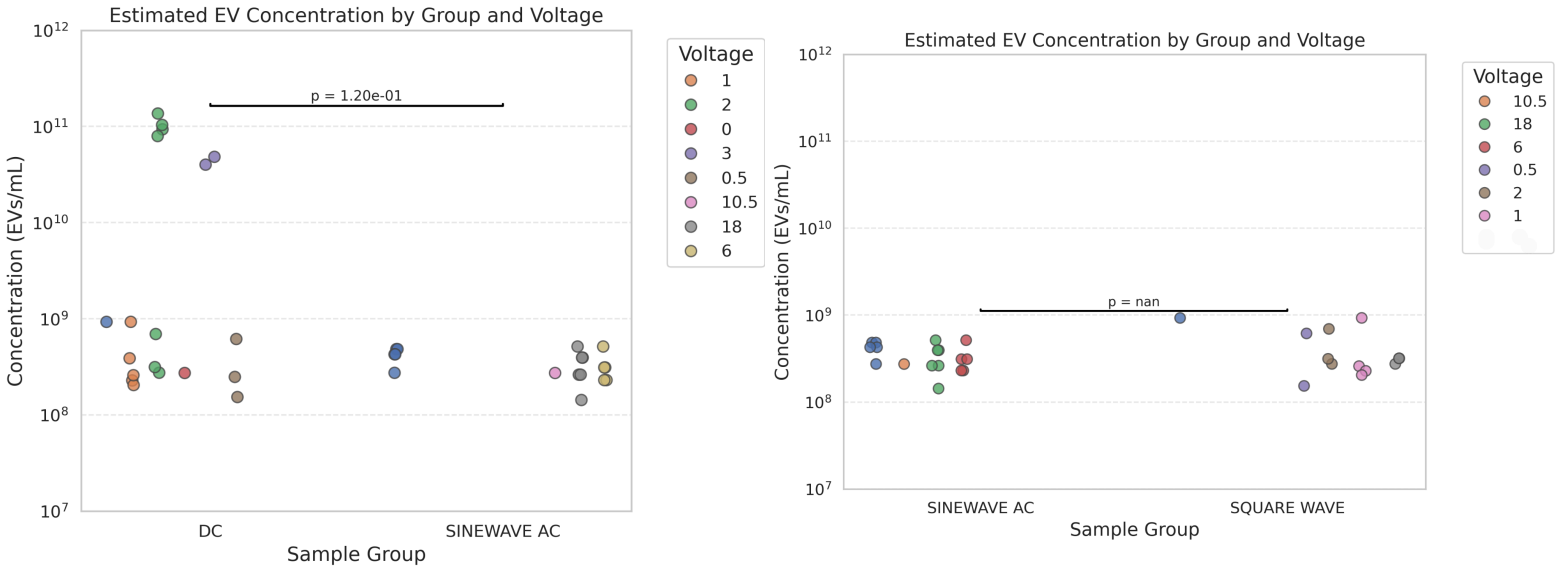


Figure 9: Electro-DBS Comparison between AC Current and DC Current, with Figure 9A showing a comparison between DC and sine wave at different voltages and Figure 9B showing the comparison between the use of a 1 kHz sine wave versus a 1 kHz Square wave for different valutages. Both p values were not significant.

For electro-DBS, a COMSOL Multiphysics 2D model was created per well for the 96-well plate used. As shown in Figure 9, the electric field remained constant across the whole sample when 0.5V, 1V, 2V, 3V, and 5V were applied to the sample. The model is based on a rectangular shape with three components, top and bottom electrode are assumed to be made out of copper with a width of 3.51 mm and a height of 8mm (based on McAllister product specifications), and the central rectangular shape was assumed to be aqueous with a length of 14.1 mm, which is the standard well plate height.

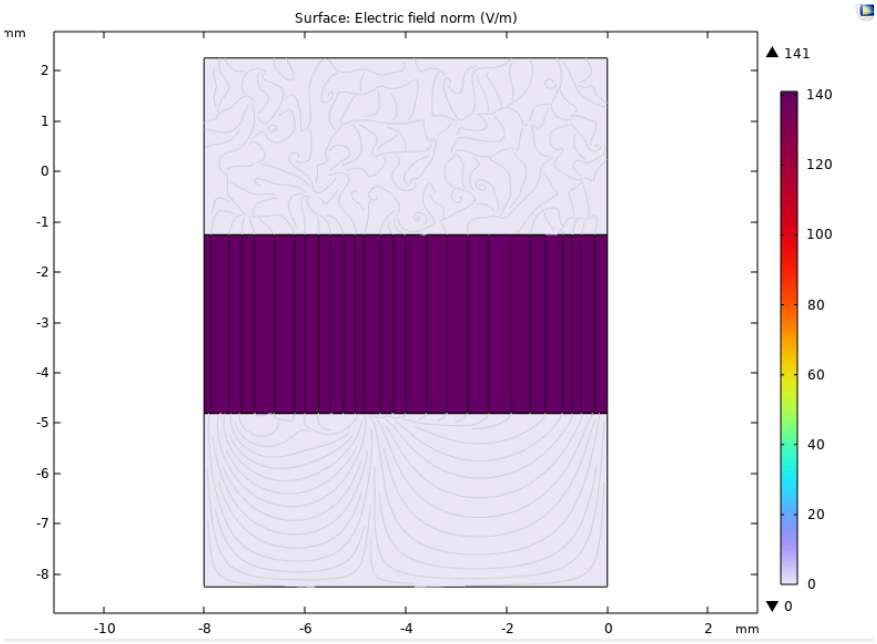


Figure 10: 2D Electric field strength from DC Current Application

Lysis and RNA Extraction from Dried Blood Spot Derived EVs

One of the main curiosities of EVs for dried blood spots is whether they can maintain an intact cargo once extracted. This may be revolutionary in diagnostics and prognosis, as DBS has low sample collection and storage requirements compared to venous blood collection. For each of the above extraction studies, when there was some evidence of EVs, we prepared and investigated the samples for cargo identification, specifically to identify if there was intact RNA from the extracted samples to be used for downstream processing.

Methodology:

EVs were extracted per the procedures previously mentioned, either by Eletro DBS or by soaking in a buffer for 1 hour. Samples were then centrifuged for 5 minutes at 20,000g, papers removed from the sample, and the remaining sample re-centrifuged for 10,000g for 30 minutes. Final volume was measured, and either Exoquick or Immunopulldown was performed to isolate the EVs. Matched donor plasma was used as a control, thawed at room temperature, and centrifuged at 4 °C at 10,000g for 30 minutes as well.

During Exoquick, instead of resuspending the final beads with supernatant, a lysing solution was added, and the sample was incubated on ice for 45 minutes with 30 seconds of vortexing every 15 minutes. A control sample is maintained, where the sample is resuspended in HEPES buffer instead. Two methods of lysis were used: CHAPS 2% + Triton 0.5% solution. The solution is then added to an end-over-end mixer at 4C and incubated for 45 minutes.

Afterwards, the Zymo Midiprep RNA isolation kit (Zymo Research product R1056) is used, with instructions as provided by the manufacturer (see Appendix E). Important to note is the use of a DNase to eliminate non-RNA molecules from the sample, which eliminates non-RNA nucleic acids from the sample. To confirm the presence of RNA, a NanoDrop spectrophotometer was used. It utilizes a UV-Visible spectrophotometer that measures concentration and purity of nano molecules such as RNA and DNA using light absorbance values.

Sample (in 50ul of NFW)	NanoDrop Reading	NanoDrop Reading	Average (ng/uL)
50 uL	1.2	1.9	1.55
100 uL	2.1	2.3	2.2
1 mL	8.6	9.4	9

Table 4: Initial NanoDrop results, prompting further exploration into RNA yield. This study only used plasma and was for a quick study to confirm any nucleic acid presence.

Sample name	Nanodrop #1	Nanodrop #2	Nanodrop #3	Avg (ng/uL)
HEPEs 1	4.3	6.8	7.7	6.37
HEPEs 2	10.3	10.1	10.6	10.33
PBS tween 1 2%	0.7	-0.1		0.3
PBS tween 2 2%	1.5	0.9	0.6	1
PBS tween 2% control	2.28	3.08	2.5	2.62
Hepes control	2.27	1.54	1.09	1.63

Table 5: NanoDrop results after Exoquick treatment (better yield, and better 260/280 (purity results). However, further work was needed to increase purity. Both HEPEs and PBS Tween 2% were lysed in 2% CHAPS solution. The controls were in the HEPE buffer.)

Optimization of the buffer for extracting EVs showed that isolation with Exoquick via precipitation and following up with CD9 stock (15 ulµL immunoaffinity pulldown is the most promising method. There is also evidence that there is better yield when using a filter during DBS soaking versus not using a filter, both for HEPE buffer and PBS Tween 20 buffer. This is most likely due to removing impurities of larger sizes. Below are the results obtained with both filtering and using a combination of Exoquick and Immunoaffinity pull-down for isolation.

Sample name	Nanodrop #1	Nanodrop #2	Nanodrop #3	Avg (ng/uL)
HEPES	2.8	2.6	2.6	2.7 ng/uL
PBS Tween 0.05%	2.3	4.0	3.7	3.3 ng/uL
PBS Tween 0.05% Boek's method	2.1	2.6	1.4	2.0 ng/uL
HEPEs Control	0.7	0.6	0.4	0.6
PBS Tween 0.05%	1.7	1.9	0.9	1.5

Table 6: Optimized Buffer Extraction from PBS Tween 0.05% and HEPEs-based solution

Nanoparticle Tracking Analysis shows that the 'PJ' sample, which exclusively followed Baek et al's method, had more impurities of larger volumes, even after performing Exoquick precipitation for EV Isolation. P3 (PBS Tween20 0.05%) and HEPES-based solutions both had 2X blood spots, and both utilized Exoquick and Immunoaffinity pull down using CD9 beads for EV Isolation and concentration. HEPEs do better than P3 in this regard, but more work needs to be done to replicate results and to further optimize this work.

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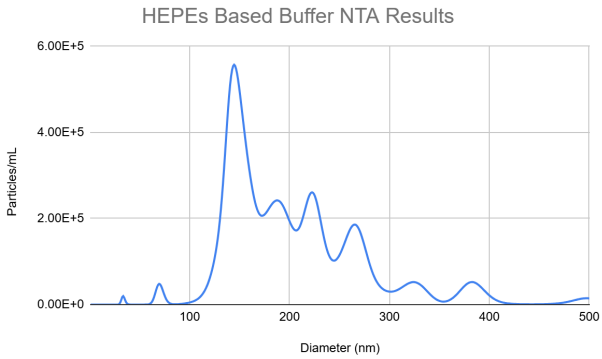

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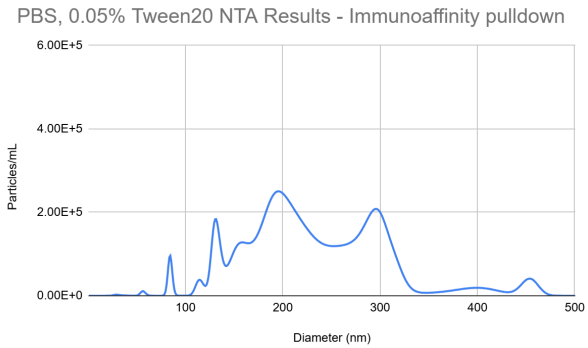

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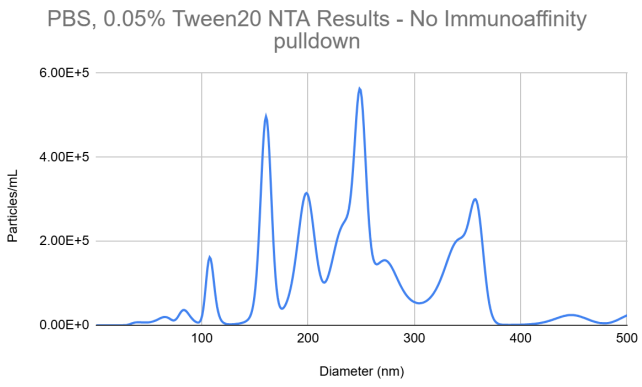


Figure 11: NTA results assessing EV purity when using PBS with Tween 20 compared to a HEPES-based buffer

Sample	EV Concentration	Mode Diameter	Mean Diameter
HEPES-based buffer	1.71E+09 +/- 3.40E+08	183.2 +/- 23.1	204.1 +/-5.8
PBS Tween 0.05% (no pulldown) - PJ	1.88E+09 +/- 2.19E+08	193.3 +/- 50.2	252.8 +/- 12.5
PBS Tween 0.05% with pull down) P3	1.38E+09+/- 2.53E+08	197.8 +/- 21.4	235.7 +/- 4

Table 7: Nanoparticle Tracking Analysis Results for buffer elution of EVs from Dried Blood Spots

Chapter 4: Conclusions, and Future Directions

Breast Cancer Screening with sEVs

Breast Cancer screenings using liquid biopsies are an important area of study due to their early diagnostic capabilities. Currently, there is limited validation of methods for EV isolation, and therefore, sEV yield and purity is variable across studies. sEVs are notoriously heterogeneous, and there are limitations in differentiating between tumor sEVs and non-tumor sEVs. Although sEVs show promise in preclinical research, there is a gap between bench work and clinical translational research for EVs, with hardly any FDA-approved tools.

Due to variability in sEVs, future work should rely on multimodal EV analysis, combining surface markers, internal cargo analysis, imaging, and machine learning to provide a more holistic tool for breast cancer diagnosis. Combining sEV analysis with other liquid biopsy approaches may also increase its benefits in diagnostics.[46]

From the presented work, a larger patient sample may have been more indicative of the differences between the two technologies compared, especially if we can obtain patient-matched and clinical metadata to track EV expression over time. Knowledge of breast cancer subtypes may also help determine if there is a difference in sEV expression based on that aspect. Screening using EV surface markers specific for breast cancer, such as CD105, VEGF, and HER2, in combination with the markers used in this study, may provide more specificity for breast cancer screening [45] Finally, MCF-7 is a luminal A breast cancer cell line. A validation study comparing MCF-7, normal plasma,

and other breast cancer cell line types, luminal B, and triple negative breast cancer will be impactful in confirming the increased concentration of extracellular vesicles in these breast cancer types.

EV Extraction from Dried Blood Spots & Proteomics

Decentralized biosampling shows promise in the future of biomedical research. In diagnostics, the method may be used in resource limited settings, during rapidly infectious epidemics and pandemics such as COVID-19 and Ebola where no contact medical attention is necessary and for prognosis of disease.

Although the findings presented here are preliminary, using electro-DBS may save time, a difference between five minutes and an hour of incubation, and produce similar sEV purity and yield compared to buffer extraction. Improvements to our methodology may be done by understanding the difference in EV stability and viability when the dried blood spot is stored for a length of time. For our study, Dried blood spots were stored up to five months in -20 °C. There are also no studies on how EVs fare when spotted in room temperature versus in -20 °C, which may be an important avenue considering one of the main advantages of DBS is its ability to be stored at ambient temperature. Furthermore, more work is needed to assess the effect of hematocrit on EV purity and yield, especially on dried blood and microsampling processes.

For the work presented here, a future endeavor would be to investigate the effect of the protein saver card used to store DBS, as the type of treatment done on the saver cards has been reported to affect the analyte stability when spotted. This will help determine if there is any lysis caused by the cards.[27] It would also be beneficial to study the differences in EV yield and expression between a healthy sample and a clinical sample, such as in breast cancer, and assess the prognostic capabilities of sEVs.

Additional work is needed to study EV cargo when stored in DBS. We attempted to perform NGS Library prep, but after studying the quality of the cDNA produced using the Agilent 2100 Bioanalyzer, it was determined that more work is needed before proceeding to sequencing. Such work includes treatment with proteinase K, which is known to cleave peptide bonds and purify the RNA sample. We did not use RNAse before lysis of the sample due to a limited number of floating RNA fragments, however, this may be useful in future studies to ensure any RNA obtained is from the EV cargo.

Appendix A - MSD Protocol

Reagent Preparation

Refer to the Best Practices section (page 9) before beginning the protocol.

Important: Upon first thaw, aliquot diluents into suitable volumes before refreezing.

To prepare supplemental reagents such as MSD Wash Buffer, please refer to the Components section (page 6).

Coat Plate

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 200 µL of biotinylated capture antibody to 5.8 mL of Diluent 100. Mix by vortexing.
- ❑ Add 50 µL of the above solution to each well of the MSD GOLD Small-Spot Streptavidin plate. Tap the plate gently on all sides. Seal the plate with an adhesive plate seal and incubate with vigorous shaking (~700 RPM) at room temperature for 1 hour.

Prepare Calibrator Standards

MSD supplies calibrator for R-PLEX EV Antibody Sets at the working concentration for the highest standard (Calibrator Standard 1). We recommend a 7-point calibration curve with 4-fold serial dilution steps and a zero calibrator blank. Thaw the stock calibrator and keep on ice, then add to assay diluent (Diluent 52) to make the calibration curve solutions.

To prepare 7 calibrator solutions plus a zero calibrator for up to 3 replicates (see Figure 2), perform the following.

- ❑ Human EV Calibrator 1 is used without dilution for Calibrator Standard 1. Vortex briefly and spin down in minicentrifuge before opening tube.
- ❑ Prepare the next calibrator (Calibrator Standard 2) by adding 50 µL of Calibrator Standard 1 to 150 µL of assay diluent. Vortex briefly to mix.
- ❑ Repeat 4-fold serial dilutions (50 µL previous standard into 150 µL diluent) to generate Calibrator Standards 3 through Calibrator Standard 7.
- ❑ Use assay diluent as the blank (Calibrator Standard 8).

Notes:

- Dilution volumes can be adjusted for fewer replicates.
- For the Calibrator Standard 1 concentration, refer to the product-specific datasheet supplied with the R-PLEX Antibody Set. The datasheet is also available at www.mesoscale.com/R-PLEX-documents.

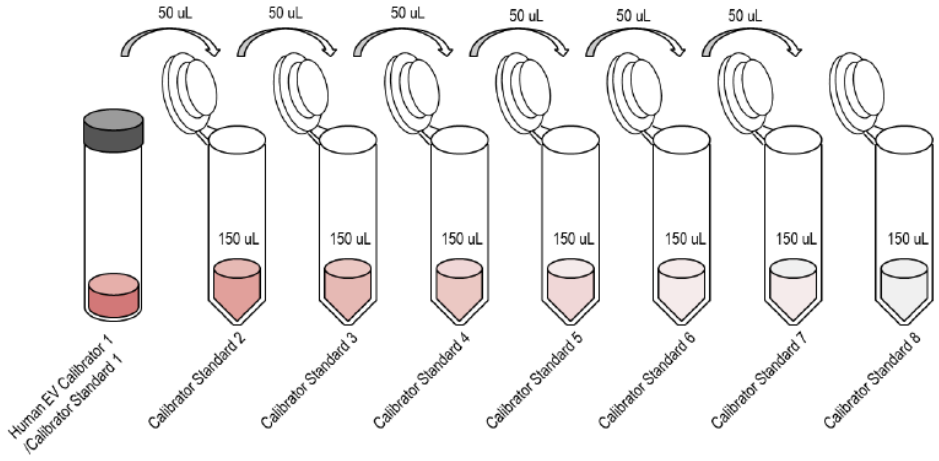


Figure 2. Dilution schema for Calibrator Standards for R-PLEX EV singleplex assays.

Prepare Detection Antibody Solution

The detection antibody is provided as a 100X stock solution. The working solution is 1X. Prepare the detection antibody solution in Diluent 53 immediately prior to use.

For one plate, combine:

- ❑ 30 µL of the supplied 100X detection antibody
- ❑ 2.97 mL of antibody diluent (Diluent 53)

Dilute Samples

Depending on the sample set under investigation, a dilution may be necessary. Suggested dilution factors are provided in the R-PLEX product-specific datasheet. Assay diluent should be used for sample dilution. The dilution factor for a given sample type should be optimized. Additional assay diluent may be necessary for samples that are diluted greater than 10-fold.

Prepare MSD GOLD Read Buffer B

MSD provides MSD GOLD Read Buffer B at the working concentration of the assay; do not dilute. Equilibrate the Read Buffer to room temperature before use. To avoid bubbles, do not vortex.

Assay Protocol

Note: Before beginning STEP 1, prepare the plate as described on page 10.

STEP 1: **Wash and add Samples and Calibrators**

- ❑ Prior to adding samples, remove coating solution and wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 25 µL of assay diluent to each well. Tap the plate gently on all sides to ensure even coating of the plate surface.
- ❑ Add 25 µL of the prepared Calibrator Standard or sample to each well. Seal the plate with an adhesive plate seal. Incubate at room temperature with vigorous shaking (~700 rpm) for at least 1 hour. Longer incubation times can improve EV assay sensitivities for dilute samples.

STEP 2: **Wash and Add Detection Antibody Solution**

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 25 µL of detection antibody solution to each well. Seal the plate with an adhesive plate seal. Incubate at room temperature with vigorous shaking (~700 rpm) for 1 hour.

STEP 3: **Wash and Read**

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 150 µL of MSD GOLD Read Buffer B to each well and promptly analyze the plate on an MSD instrument. Incubation in Read Buffer is not required before reading the plate.

Appendix B

	1	2		4	5	6	7	8	9		11	12
A	MSD Cal 1	MSD Cal 1	BC 1	BC 1	HC 1	HC 1	BC 1	BC 1	HC 1	HC 1	Plasma Cal A	Plasma Cal A
B	MSD Cal 2	MSD Cal 2	BC 2	BC 2	HC 2	HC 2	BC 2	BC 2	HC 2	HC 2	Plasma Cal B	Plasma Cal B
C	MSD Cal 3	MSD Cal 3	BC 3	BC 3	HC 3	HC 3	BC 3	BC 3	HC 3	HC 3	Plasma Cal C	Plasma Cal C
D	MSD Cal 4	MSD Cal 4	BC 4	BC 4	HC 4	HC 4	BC 4	BC 4	HC 4	HC 4	Plasma Cal D	Plasma Cal D
E	MSD Cal 5	MSD Cal 5	BC 5	BC 5	HC 5	HC 5	BC 5	BC 5	HC 5	HC 5	Plasma Cal E	Plasma Cal E
F	MSD Cal 6	MSD Cal 6	BC 6	BC 6	HC 6	HC 6	BC 6	BC 6	HC 6	HC 6	Plasma Cal F	Plasma Cal F
G	MSD Cal 7	MSD Cal 7	BC 7	BC 7	HC 7	HC 7	BC 7	BC 7	HC 7	HC 7	Plasma Cal G	Plasma Cal G
H	MSD Cal blank	MSD Cal blank	BC 8	BC 8	HC 8	HC 8	BC 8	BC 8	HC 8	HC 8	Plasma Cal H	Plasma Cal H
	CD63		CD63		CD63		CD9		CD9		CD63	
	CD9 Capture											
	Plate 1											

	1	2	3	4	5	6	7	8	9		11	12
A	MSD Cal 1	MSD Cal 1	BC 1	BC 1	HC 1	HC 1	BC 1	BC 1	HC 1	HC 1	Plasma Cal A	Plasma Cal A
B	MSD Cal 2	MSD Cal 2	BC 2	BC 2	HC 2	HC 2	BC 2	BC 2	HC 2	HC 2	Plasma Cal B	Plasma Cal B
C	MSD Cal 3	MSD Cal 3	BC 3	BC 3	HC 3	HC 3	BC 3	BC 3	HC 3	HC 3	Plasma Cal C	Plasma Cal C
D	MSD Cal 4	MSD Cal 4	BC 4	BC 4	HC 4	HC 4	BC 4	BC 4	HC 4	HC 4	Plasma Cal D	Plasma Cal D
E	MSD Cal 5	MSD Cal 5	BC 5	BC 5	HC 5	HC 5	BC 5	BC 5	HC 5	HC 5	Plasma Cal E	Plasma Cal E
F	MSD Cal 6	MSD Cal 6	BC 6	BC 6	HC 6	HC 6	BC 6	BC 6	HC 6	HC 6	Plasma Cal F	Plasma Cal F
G	MSD Cal 7	MSD Cal 7	BC 7	BC 7	HC 7	HC 7	BC 7	BC 7	HC 7	HC 7	Plasma Cal G	Plasma Cal G
H	MSD Cal blank	MSD Cal blank	BC 8	BC 8	HC 8	HC 8	BC 8	BC 8	HC 8	HC 8	Plasma Cal H	Plasma Cal H
	CD9		CD9		CD9		CD63		CD63		CD9	
	CD63 Capture											
	Plate 2											

	1	2	3	4	5	6	7	8	9		11	12
A	MCF7 Cal 1	MCF7 Cal 1	MCF7 Cal 1	MCF7 Cal 1	Plasma Cal A	Plasma Cal A	Plasma Cal A	Plasma Cal A	MSD Cal 1	MSD Cal 1	MSD Cal 1	MSD Cal 1
B	MCF7 Cal 2	MCF7 Cal 2	MCF7 Cal 2	MCF7 Cal 2	Plasma Cal B	Plasma Cal B	Plasma Cal B	Plasma Cal B	MSD Cal 2	MSD Cal 2	MSD Cal 2	MSD Cal 2
C	MCF7 Cal 3	MCF7 Cal 3	MCF7 Cal 3	MCF7 Cal 3	Plasma Cal C	Plasma Cal C	Plasma Cal C	Plasma Cal C	MSD Cal 3	MSD Cal 3	MSD Cal 3	MSD Cal 3
D	MCF7 Cal 4	MCF7 Cal 4	MCF7 Cal 4	MCF7 Cal 4	Plasma Cal D	Plasma Cal D	Plasma Cal D	Plasma Cal D	MSD Cal 4	MSD Cal 4	MSD Cal 4	MSD Cal 4
E	MCF7 Cal 5	MCF7 Cal 5	MCF7 Cal 5	MCF7 Cal 5	Plasma Cal E	Plasma Cal E	Plasma Cal E	Plasma Cal E	MSD Cal 5	MSD Cal 5	MSD Cal 5	MSD Cal 5
F	MCF7 Cal 6	MCF7 Cal 6	MCF7 Cal 6	MCF7 Cal 6	Plasma Cal F	Plasma Cal F	Plasma Cal F	Plasma Cal F	MSD Cal 6	MSD Cal 6	MSD Cal 6	MSD Cal 6
G	MCF7 Cal 7	MCF7 Cal 7	MCF7 Cal 7	MCF7 Cal 7	Plasma Cal G	Plasma Cal G	Plasma Cal G	Plasma Cal G	MSD Cal 7	MSD Cal 7	MSD Cal 7	MSD Cal 7
H	MCF7 Cal 8	MCF7 Cal 8	MCF7 Cal 8	MCF7 Cal 8	Plasma Cal H	Plasma Cal H	Plasma Cal H	Plasma Cal H	MSD Cal blank	MSD Cal blank	MSD Cal blank	MSD Cal blank
	CD9/CD63		CD63/CD9		CD81/CD9		CD81/CD81		CD81/CD9		CD81/CD81	
	Plate 3											

Ordering Information

Product Description	Catalog No.	Size
Quick-RNA™ Midiprep Kit	R1056	25 preps.

Individual Kit Components	Catalog No.	Amount
ZR RNA Buffer	R1020-1-100	100 ml
RNA Pre-Wash Buffer	R1020-2-50 R1020-2-100	50 ml 100 ml
RNA Wash Buffer (concentrate)	R1003-3-6 R1003-3-12	6 ml 12 ml
Zymo-Spin™ V-E Columns w/ Reservoir	C1029-25	25
Collection Tubes	C1001-50	50
DNase/RNase-Free Water	W1001-6 W1001-10	6 ml 10 ml

Appendix C

Protocol for preparing dry blood spots

Notes:

- Using Cytiva whatman 903™ Protein Saver cards (pcard)
- The blood (pcard) arrives in a glass tube, it will need homogenizing as it separates: mix gentle (no bubbles)
- After preparation, there is some DNA loss over time
- Desiccant: blue crystals turn pink when they absorb water (prevents the paper getting soggy)

******ALL BLOOD SPOTTING MUST BE DONE IN THE BIOSAFETY CABINET******

Working in a biosafety cabinet:

When using BioSafety Cabinet, make sure everything is ethanol wiped before it enters the hood

1) Mix the blood using a P1000 (1 mL Pipette); gently, not introducing bubbles
2) Take 200 uL (using a P200, or other appropriate pipette) and drop into the center of a circle on the protein saver card to make a spot
    a) Corresponding to the volumes of blood we want to test. We said 200 ul on the excel sheet so make 200 ul spots. It may also be a good idea to make a few spots of different volumes so we can test those as well without getting new blood
        i) Ie. Make a couple 100 ul, 50 ul, 75 ul, 150 ul, 250 ul
3) Repeat 4 times (there are 5 total circles on each card); re-mixing the blood using the method in step 1 if you notice any separation!
4) Leave the completed card open in the hood to dry
5) Get a new protein saver card and repeat the process in steps 2-4. Ensure to periodically re-mix the blood to avoid separation.
    a) To be safe: Mix blood after every 1-2 cards
6) Repeat step 5 until you have used all of the blood sample (10 mL)
    a) 10,000 uL of sample, 200 uL per spot → approximately 50 spots (10 cards)
    b) Stop making more spots if (towards the end) the spots are lighter coloured.
7) Leave all the cards out and open **overnight in the cabinet.**
    a) Leave the fan on, close the hood, and leave a note with your name/info.
8) Return in the morning. The DBSs should be dry.
9) Fold the cards, put 2 cards per plastic bag and put a package of desiccant into the bag and seal
    a) Place the bags into a styrofoam box in the -20C
10) Biosafety cabinet clean up:

a) Next day, clean surfaces with ethanol (+ bleach if any blood spills). Close hood, turn off fan, turn on UV light for 10 min. Turn off UV lights when 10 minutes are done.

Material Locations:

- Protein Saver Cards: Bench E table top
- Desiccant Packs: Underneath Bench A in gray bucket
- 

Appendix D

Supplementary Material

1 S1: Detailed protocol

1. Wash hands for 30 sec with soap and warm water. Rinse hands thoroughly for 30 sec with warm water and dry completely. Decontaminate the finger with an ethanol swap. Place the lancet on the finger gently on the top of the lancet. Press down firmly on the lancet to pierce the fingertip.

2. Massage whole blood droplets out of the fingertip by applying firm pressure to the finger. Repeated firm pressure strokes from the base to the tip of the finger will accelerate blood flow. Saturate each 12.5 mm dotted circle completely so that the blood migrates outward and slightly beyond each dotted circle. Typically, 2-3 drops of whole blood placed in the center of the dotted circle are sufficient to saturate each 12.5 mm collection area. A slight excess of whole blood is preferable because the 12.5 mm disc punch excises precise 12.5 mm disc regions, allowing well-calibrated sample collection volumes if each 12.5 mm dotted circle is wetted completely.

3. After filling each of the four 12.5 mm dotted circle collection regions with whole blood, fold the top flap of the card inside the bottom flap and allow the collected blood spots to dry for 60 min at room temperature. The drying process removes water from the collected samples and stabilizes the collected samples. After the 60 min drying period, progress immediately to step 4. Alternatively, the cards can be placed inside a sealed plastic bag and stored at room temperature for future use.

4. The dried whole blood spots can be excised using a disc punch; for these experiments a 12.5 mm disc punch was used. Align the punch with the dotted circle at each collection location and press the disc punch firmly to excise the collection matrix containing the whole blood sample. For the EV Array analysis only a single 12.5 mm disc is required, which allows the remaining three discs to be saved and stored for future use. The disc punch should be rinsed with water and dried between each card to prevent sample cross contamination.

5. After the 12.5 mm disc containing the whole blood sample is excised with the disc punch, pick up the disc with fine-tipped forceps and wet each side of the 12.5 mm disc with 60 µl of reaction buffer (PBS with 0.05% Tween20®).

6. Apply additional 60 µl of reaction buffer into a collection tube and place a filter unit/spin column on top of the tube. Using fine-tipped forceps place the wetted disc into the filter unit orifice. Close the lid on the collection tube and incubate for 1 h at room temperature.

7. Place the filter unit-collection tube assembly in a high-speed microfuge and centrifuge at 20,000 x g for 5 min to elute the whole blood components and EVs off the collection disc and into the 60 µl of reaction buffer at the bottom. A properly eluted sample will appear as two layers in the bottom of the tube.

8. Harvest the eluted EVs in the top layer (approximately 60 µl) and transfer to another tube for either further analysis or storage (-80 °C). Make sure not touch the bottom layer as the sample then will be contaminated with cell remnants.

RNA Purification Made Simple™

Quick-RNA™ Midiprep Kit

RNA from any sample

Highlights

- Spin-column purification of RNA (up to 1 mg) from cells and tissue.
- High-quality RNA is ready for any downstream application.

Catalog Numbers:
R1056

Scan with your smart-phone camera to view the online protocol/video.

Specifications

- **Sample Sources –** Cells (animal, gram(-) bacteria), soft and easy-to-lyse tissue, and enzymatic reactions (e.g., DNase I treated, Proteinase K treated). Not compatible with whole blood[1], urine[1], or samples in DNA/RNA Shield™[2].
- **Size –** Total RNA including small/microRNAs (≥ 17 nt).
- **Purity –** A_{260}/A_{280} & A_{260}/A_{230} > 1.8. RNA is ready for Next-Gen Sequencing, RT/qPCR, etc.
- **Binding Capacity – Zymo-Spin™ V-E Column** yield up to 1 mg RNA.
- **Elution Volume –** ≥ 200 µl **DNase/RNase-Free Water.**
- **Equipment Needed (user provided) –** Vacuum manifold, microcentrifuge, vortex.

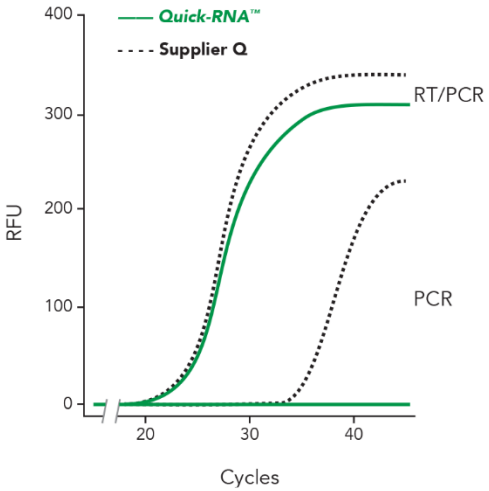
1 For RNA purification from whole-blood and urine, see the Quick-RNA Miniprep Plus Kit (R1057, R1058) or the Quick-RNA MagBead Kit (R2132, R2133).
2 For RNA purification from samples in DNA/RNA Shield™, see the Quick-RNA Microprep kit (R1050, R1051), the Quick-RNA Miniprep Kit (R1054, R1055), the Quick-RNA Miniprep Plus Kit (R1057, R1058), the Quick-RNA MagBead Kit (R2132, R2133), or the Quick-RNA 96 Kit (R1052, R1053).

Product Description

The **Quick-RNA™ Midiprep Kit** provides a quick method for the isolation of high-quality RNA of up to 1 mg from cells (animal, buccal, buffy coat) and soft, easy-to-lyse tissue.

The procedure uses unique spin-column technology that results in high-quality RNA. Simply add the provided **ZR RNA Buffer** to the sample and bind RNA directly on the **Zymo-Spin™ V-E Column**. Then wash and elute. RNA is ready for Next-Gen Sequencing, RT/qPCR, hybridization, etc.

High-Quality RNA



RNA isolated with the ***Quick*-RNA™ Kits** is DNA-free. Samples isolated with Supplier Q's kit are provided for comparison. Total RNA was isolated from 10^6 human epithelial cells. Each amplification curve represents an average of three independent isolation experiments.

Protocol

The protocol consists of: (I) Buffer Preparation, (II) Sample Preparation and (III) Total RNA Purification.

(I) Buffer Preparation

- ✓ Add 24 ml 100% ethanol (26 ml 95% ethanol) to the 6 ml **RNA Wash Buffer** concentrate.

(II) Sample Preparation

✓ Perform all steps at room temperature and centrifugation at ≤ 500 x g for 30 seconds, unless specified.

Cells

a. **To pellet cells**: Centrifuge liquid sample at ≤ 500 x g for 1 minute and remove the supernatant. Then resuspend the cell pellet in **ZR RNA Buffer** (see table below).

b. **Adherent cells**: Remove liquid media from the culture container. Then add **ZR RNA Buffer** directly to the monolayer (see table below). Remove cells from the culture surface by scraping, pipetting, scraping, etc.

c. **Cells in suspension**: Add ≥ 3 volumes **ZR RNA Buffer** to 1 volume of liquid sample and mix well.

Mammalian	Add ZR RNA Buffer[1]
10^3 - 10^8	≥ 6 ml

To remove particulate debris, centrifuge and transfer the supernatant into a new nuclease-free tube (not provided). Then proceed to purification, page 6.

Tissue

≤ 100 mg low yield tissue (or ≤ 50 mg high yield tissue) can be mechanically homogenized in ≥ 6 ml **ZR RNA Buffer** with a mortar/pestle, dounce, syringe, tissue grinder, or bead beating (recommended). To remove particulate debris from homogenate, centrifuge and transfer the supernatant into a new nuclease-free tube (not provided). Proceed to purification, page 6.

Recommended: Use ZR BashingBead Lysis Tubes (#S6003; sold separately) and a high-speed homogenizer (e.g., MP Bio FastPrep-24, Bertin Precellys) for 30-60 seconds.

1 If the sample lysate is turbid, the volume of **ZR RNA Buffer** can be increased for complete lysis of the sample (sample lysate should appear clear with no particulate debris).

(III) Total RNA Purification

✓ Perform all steps at room temperature and centrifugation at 10,000-16,000 x g for 30 seconds, unless specified.

1. Add 1 volume[1] ethanol (95-100%) to 1 volume sample lysed in **ZR RNA Buffer** (1:1) and mix well.

   Example: Add 6 ml ethanol to 6 ml mixture (sample lysed in **ZR RNA Buffer**).

2. Transfer the mixture into a **Zymo-Spin™ V-E Column**[2] **with reservoir** mounted on a vacuum manifold and start vacuum[3].

3. Remove the reservoir and transfer the column into a **Collection Tube**. Then centrifuge the column.

4. Add 400 µl **RNA Pre-Wash Buffer** to the column and centrifuge. Discard the flow-through.

5. Add 400 µl **RNA Wash Buffer** to the column and centrifuge. Discard the flow-through.

6. Add 400 µl **RNA Wash Buffer** and centrifuge the column for 2 minutes to ensure complete removal of the wash buffer. Then carefully, transfer the column into a nuclease-free tube (not provided).

7. Add ≥ 200 µl **DNase/RNase-Free Water** directly to the column matrix and centrifuge.

   The eluted RNA[4] can be used immediately or stored frozen.

---

1 For total RNA without small/micro RNAs (17-200 nt), proceed directly to step 2.
2 To process samples > 400 µl, columns may be reloaded.
3 Set vacuum source at ≥ 500 mm Hg.
4 Eluted RNA can be DNase I treated using DNase I Set (cat. #E1010). For the protocol, see the RNA Clean & Concentrator-100 (cat. #R1019).

Complete Your Workflow

✓ For tough-to-lyse samples, use ZR BashingBead Lysis Tubes:

ZR BashingBead Lysis Tubes	
2.0 mm beads #S6003	Plant/animal tissue
0.1 + 0.5 mm beads #S6012	Microbes
0.1 + 2.0 mm beads #S6014	Microbes in tissue/insects

✓ For isolation of RNA from any sample:

Quick-RNA kits	
Miniprep Plus #R1057/R1058	≤ 10^7 cells, ≤ 50 mg tissue
MagBeads #R2132/R2133	Automatable (Tecan, Hamilton, Kingfisher, etc.)

✓ For clean-up (purification) and concentration of any RNA sample. (e.g., from the aqueous phase of TRIzol® extractions) or from any enzymatic reaction (e.g., DNase I treated RNA):

RNA Clean & Concentrator kits	
Microprep #R1013-R1014	DNase I Set included
MagBeads #R1082	Automatable (Tecan, Hamilton, Kingfisher, etc.)

✓ For NGS:

Zymo-Seq RiboFree Total RNA Library Prep kit	
#R3000	12 preps
#R3003	96 preps

Appendix F

```
# -*- coding: utf-8 -*-
"""ScM Thesis Lujuo - Code for data analysis

Automatically generated by Colab.

Original file is located at
    https://colab.research.google.com/drive/1jE7dgu9zeLciQayJPn_6Fmdvk8QijyPD

MSD Results Comparing CD9 and CD63 for Capture (Healthy vs Breast Cancer Samples)

# Libraries
"""

# --- Install Required Libraries ---
!pip install matplotlib scipy pandas --quiet

# --- Import Libraries ---
import numpy as np
import pandas as pd
import matplotlib.pyplot as plt
import seaborn as sns
from scipy.optimize import curve_fit
from scipy.optimize import root_scalar
from google.colab import files
from scipy.stats import ttest_ind
import io
import warnings
warnings.filterwarnings("ignore", category=RuntimeWarning)

"""# Data Import
The section below imports data files from a Google Sheet
"""

# Import data from Google Sheets

from google.colab import auth
auth.authenticate_user()

import gspread
from google.auth import default
creds, _ = default()

gc = gspread.authorize(creds)

# Enter worksheets names, add depending on number
spreadsheet_name = "SIMOA Calibration Curves + Analysis"
spreadsheet_id = "13mMg1OTbTTx4uE_9iF57PtXZl2P5MPLDgPIRcZZCFMs"

MSD_BreastCancer_worksheet_name = "MSD Breast Cancer Results"
SIMOA_BreastCancer_worksheet_name = "SIMOA Breast Cancer Results"
```

```
SIMOA_DriedBlood_worksheet_name = "SIMOA Dried Blood Spot Results"

# Open the spreadsheet and worksheets
MSD_BreastCancer_worksheet =
gc.open_by_key(spreadsheet_id).worksheet(MSD_BreastCancer_worksheet_name)
SIMOA_BreastCancer_worksheet =
gc.open_by_key(spreadsheet_id).worksheet(SIMOA_BreastCancer_worksheet_name)
SIMOA_DriedBlood_worksheet =
gc.open_by_key(spreadsheet_id).worksheet(SIMOA_DriedBlood_worksheet_name)

# Get all values from the worksheets as a list of lists
MSD_BreastCancer_data = MSD_BreastCancer_worksheet.get_all_values()
SIMOA_BreastCancer_data = SIMOA_BreastCancer_worksheet.get_all_values()
SIMOA_DriedBlood_data = SIMOA_DriedBlood_worksheet.get_all_values()

# Convert the data to a Pandas DataFrame
MSD_BreastCancer_df = pd.DataFrame(MSD_BreastCancer_data[1:], columns =
MSD_BreastCancer_data[0])
SIMOA_BreastCancer_df = pd.DataFrame(SIMOA_BreastCancer_data[1:], columns =
SIMOA_BreastCancer_data[0])
SIMOA_DriedBlood_df = pd.DataFrame(SIMOA_DriedBlood_data[1:], columns =
SIMOA_DriedBlood_data[0])

# IMPORTANT FOR EXTRAPOLATION
# Convert intensity column to numeric
MSD_BreastCancer_df["Intensity"] = pd.to_numeric(MSD_BreastCancer_df["Intensity"])
SIMOA_BreastCancer_df["Intensity"] =
pd.to_numeric(SIMOA_BreastCancer_df["Intensity"])
SIMOA_DriedBlood_df["Intensity"] = pd.to_numeric(SIMOA_DriedBlood_df["Intensity"])


display(MSD_BreastCancer_df.head())
display(SIMOA_BreastCancer_df.head())
display(SIMOA_DriedBlood_df.head())

"""# Calibration Curves

## MSD Curves
"""

# --- Define the 4PL Function (safe) ---
def four_param_logistic(x, A, B, C, D):
    x = np.clip(x, 1e-10, None)  # avoid division by zero
    return D + (A - D) / (1 + (x / C) ** B)

# --- Define MSD Calibration Curves ---
MSD_calibration_curves = {
    "Run 2 MSD Plasma Cal CD9 Cap - CD63 Det": (
        np.array([1e-2, 3e5, 3e6, 3e7, 3e8, 3e9, 3e10, 3e11]),
        np.array([71.5, 63, 78.5, 128, 399, 1562, 7966, 35431.5])
```

```
    ),
    "Run 2 MSD Plasma Cal CD63 Cap - CD9 Det": (
        np.array([1e-2, 3e5, 3e6, 3e7, 3e8, 3e9, 3e10, 3e11]),
        np.array([68, 73.5, 137, 403, 2209, 9658, 46333.5, 204123])
    ),
    "Run 1 MSD Plasma CD9 Cap - CD63 Det": (
        np.array([1e-2, 3e5, 3e6, 3e7, 3e8, 3e9, 3e10, 3e11]),
        np.array([0, 71, 73, 71, 89, 171, 1028, 8868])
    ),
    "Run 1 MSD Plasma Plate 2 CD63 Cap - CD9 Det": (
        np.array([1e-2, 3e5, 3e6, 3e7, 3e8, 3e9, 3e10, 3e11]),
        np.array([65.5, 66.5, 68, 73.5, 131, 688, 6220.5, 62368.5])
    ),
    "Run 1 MCF Plate 3 CD9 Cap - CD63 Det": (
        np.array([1e-2, 3e4, 3e5, 3e6, 3e7, 3e8, 3e9, 3e10]),
        np.array([75, 73, 77, 85, 173, 1095, 9600, 92074])
    ),
    "Run 1 MCF Plate 3 CD63 Cap - CD9 Det": (
        np.array([1e-2, 3e4, 3e5, 3e6, 3e7, 3e8, 3e9, 3e10]),
        np.array([66, 66, 69.5, 100, 388, 3351, 31646.5, 305763.5])
    ),
    "Run 1 Plasma Plate 3 CD81 Cap - CD9 Det": (
        np.array([1e-2, 3e5, 3e6, 3e7, 3e8, 3e9, 3e10, 3e11]),
        np.array([50, 50, 54, 59.5, 125, 791.5, 7016.5, 78786])
    ),
}

# --- Fit and Plot Each Curve ---
MSD_fitted_params = {}

for label, (concs, intensities) in MSD_calibration_curves.items():
    try:
        #initial guess
        p0 = [min(intensities), 1.0, 1e8, max(intensities)]
        params, _ = curve_fit(
            four_param_logistic,
            concs,
            intensities,
            p0=p0,
            maxfev=20000
        )
        MSD_fitted_params[label] = params

        # Plot from LLOD
        LLOD = 1e5 # change this based on plot results
        x_fit = np.logspace(np.log10(LLOD), np.log10(max(concs)), 500)
        y_fit = four_param_logistic(x_fit, *params)


        plt.figure(figsize=(8, 6))
```

```
        mask = concs > LLOD
        plt.semilogx(concs[mask], intensities[mask], 'bx', label='Observed')
        plt.semilogx(x_fit, y_fit, 'b-',label='4PL Fit')
        plt.xlabel("Concentration (EVs/mL)")
        plt.ylabel("Light Intensity")
        plt.title(f"Calibration Curve: {label}")
        plt.grid(True, which="both", ls="--")
        plt.legend()

        plt.yscale("log")
        plt.tight_layout()
        plt.show()

        # Print Equation
        print(f"\n{label} 4PL Equation:")
        print(f"Y = {D:.2f} + ({A:.2f} - {D:.2f}) / (1 + (X / {C:.2e})^{B:.2f})")

    except Exception as e:
        print(f"\n❌ Error fitting curve for {label}: {e}")

"""## Single Molecule Array Curves"""

def four_param_logistic(x, A, B, C, D):
    x = np.clip(x, 1e-10, None)  # avoid division by zero
    return D + (A - D) / (1 + (x / C) ** B)

# --- Define SIMOA Calibration Curves ---
SIMOA_calibration_curves = {
    "Plasma Cal CD63 Cap - CD81 Det": (
        np.array([3.96e6, 3.96e7, 3.96e8, 3.96e9, 3.96e10, 3.96e11,3.96e12]),
        np.array([0.001601, 1.83E-03, 2.26E-03, 3.34E-03, 2.41E-02, 0.294045,
1.108726])
    ),
    "Plasma Cal CD63 Cap - CD9 Det": (
        np.array([3.96e6, 3.96e7, 3.96e8, 3.96e9, 3.96e10, 3.96e11,3.96e12]),
        np.array([0.001903, 0.002033,0.004573, 0.04314,0.257798, 2.500593,
8.549505])
    ),
    "Plasma CD9 Cap - CD81 Det": (
        np.array([3.96e6, 3.96e7, 3.96e8, 3.96e9, 3.96e10, 3.96e11,3.96e12]),
        np.array([0.000912, 0.001519, 0.001264, 0.00945, 0.084853, 1.728944,
4.941015])
    ),
    "Plasma CD63 Cap - CD63 Det": (
        np.array([3.96e6, 3.96e7, 3.96e8, 3.96e9, 3.96e10, 3.96e11,3.96e12]),
        np.array([0, 0.000635, 0.000688, 0.000841, 0.000454,0.001647, 0.038805])
    ),
    "HC CD63 Cap - CD9 Det": (
        np.array([0.00E+00, 3.00E+06, 1.50E+07, 7.49E+07, 3.74E+08, 1.87E+09]),
        np.array([0.002488, 0.015148, 0.062322, 0.375754, 2.178114, 8.995719])
```

```
        x_fit = np.logspace(np.log10(LLOD), np.log10(max(concs)), 500)
        y_fit = four_param_logistic(x_fit, *params)

        plt.figure(figsize=(8, 6))
        mask = concs > LLOD
        plt.semilogx(concs[mask], AEB[mask], 'bx', label='Observed')
        plt.semilogx(x_fit, y_fit, 'b-',label='4PL Fit')
        plt.yscale("log")
        plt.ylim(0.0001,100)

        plt.xlabel("Concentration (EVs/mL)")
        plt.ylabel("Average Enzyme per Bead (AEB)")
        plt.title(f"Calibration Curve: {label}")
        plt.grid(True, which="both", ls="--")


        plt.tight_layout()
        plt.show()

        # Print Equation
        #print(f"\n{label} 4PL Equation:")
        #print(f"Y = {D:.2f} + ({A:.2f} - {D:.2f}) / (1 + (X / {C:.2e})^{B:.2f})")

    except Exception as e:
        print(f"\n❌ Error fitting curve for {label}: {e}")

"""# Interpolate"""

#  --- Interpolate Unknown Concentrations ---

def estimate_concentration(row, fitted_params):
    label = row["Calibration_Curve"]
    intensity = row["Intensity"]

    if label in fitted_params and not pd.isnull(intensity):
        A, B, C, D = fitted_params[label]
        try:
            result = root_scalar(lambda x: four_param_logistic(x, A, B, C, D) -
intensity,
                                 bracket=[1e3, 1e13], method='brentq')
            return result.root if result.converged else np.nan
        except:
            return np.nan
    else:
        return np.nan

# Interpolate for each dataset
MSD_BreastCancer_df["Estimated_Concentration_MSD_Evaluation"] =
MSD_BreastCancer_df.apply(estimate_concentration, axis=1, fitted_params =
MSD_fitted_params)
```

```
SIMOA_BreastCancer_df["Estimated_Concentration_SIMOA_Evaluation"] =
SIMOA_BreastCancer_df.apply(estimate_concentration, axis=1, fitted_params =
SIMOA_fitted_params)
SIMOA_DriedBlood_df["Estimated_Concentration_SIMOA_DB_Evaluation"] =
SIMOA_DriedBlood_df.apply(estimate_concentration, axis=1, fitted_params =
SIMOA_fitted_params)

"""# Plots"""

sns.set_theme(style="whitegrid", font_scale=1.2, rc={"lines.linewidth": 2.5})
plt.rcParams['axes.labelsize'] = 14
plt.rcParams['xtick.labelsize'] = 12
plt.rcParams['ytick.labelsize'] = 12
plt.rcParams['legend.fontsize'] = 12
plt.rcParams['figure.figsize'] = (8, 6)
plt.rcParams['figure.dpi'] = 300

"""## MSD BC Plots"""

#CD9 MSD
# Add capture type from Calibration_Curve
MSD_BreastCancer_df["Capture"] = MSD_BreastCancer_df["Calibration_Curve"].apply(
    lambda x: "CD63 Cap" if "CD63 Cap" in x else ("CD9 Cap" if "CD9 Cap" in x else
"Other")
)

# Average replicates with Capture info
avg_df = MSD_BreastCancer_df.groupby(["Sample_Group", "Sample_ID", "Capture"]).agg({
    "Estimated_Concentration_MSD_Evaluation": "mean"
}).reset_index()

# Color by HC vs BC
group_colors = {"HC": "#1f77b4", "BC": "#d62728"}

# Filter for CD9 Cap
cd9_df = avg_df[
    (avg_df["Capture"] == "CD9 Cap") &
    (~avg_df["Estimated_Concentration_MSD_Evaluation"].isna())
]

# Group statistics
groups = ["BC", "HC"]  # force consistent order
means = []
stds = []

# Mann–Whitney U test
u_stat, p_val = mannwhitneyu(bc_vals, hc_vals, alternative='two-sided')

# Significance label
def get_significance_label(p):
```

```
    if p < 0.001:
        return '***'
    elif p < 0.01:
        return '**'
    elif p < 0.05:
        return '*'
    else:
        return 'ns'

sig_label = get_significance_label(p_val)

for group in groups:
    group_data = cd9_df[cd9_df['Sample_Group'] ==
group]['Estimated_Concentration_MSD_Evaluation']
    means.append(np.mean(group_data))
    stds.append(np.std(group_data))

# Calculate y-axis limits for error bars
all_yerr_lower = [max(mean - std, 1e9) for mean, std in zip(means, stds)]
all_yerr_upper = [mean + std for mean, std in zip(means, stds)]
ymin = max(min(all_yerr_lower) * 0.8, 1e9)
ymax = max(all_yerr_upper) * 1.2

# Plotting
plt.figure(figsize=(8, 6))
sns.stripplot(data=cd9_df,
              x="Sample_Group",
              y="Estimated_Concentration_MSD_Evaluation",
              hue="Sample_Group",
              palette=group_colors,
              jitter=0.15,
              dodge=False,
              size=10,
              linewidth=1.5,
              marker="x")

# Add error bars using numeric x-axis
for i, (mean_val, std_val, group) in enumerate(zip(means, stds, groups)):
    lower = max(mean_val - std_val, 1e9)
    upper = mean_val + std_val
    plt.errorbar(i,
                 mean_val,
                 yerr=[[mean_val - lower], [upper - mean_val]],
                 fmt='o',
                 color='blue' if group == 'HC' else 'red',
                 capsize=5)

from scipy.stats import mannwhitneyu

# Extract group values
```

```
bc_vals = cd9_df[cd9_df["Sample_Group"] ==
"BC"]["Estimated_Concentration_MSD_Evaluation"]
hc_vals = cd9_df[cd9_df["Sample_Group"] ==
"HC"]["Estimated_Concentration_MSD_Evaluation"]

# Run Mann–Whitney U test (non-parametric, two-sided)
u_stat, p_val = mannwhitneyu(bc_vals, hc_vals, alternative='two-sided')


# Annotate p-value on the side
plt.text(1.1, ymin * 2, f"p = {p_val:.2e} ({sig_label})", ha='left', va='center',
fontsize=12)

# Final formatting
plt.title("CD9 Capture: Estimated_Concentration_MSD_Evaluation", fontsize=16)
plt.xlabel("Group", fontsize=14)
plt.ylabel("Concentration (EVs/mL)", fontsize=14)
plt.yscale("log")
plt.ylim(ymin, ymax)
plt.legend([], [], frameon=False)
plt.grid(axis="y", linestyle="--", alpha=0.4)
plt.tight_layout()
plt.savefig("figure_name_cd9_with_pval.png", dpi=300)
plt.show()

#CD9
for group in ['BC', 'HC']:
    values = cd9_df[cd9_df['Sample_Group'] ==
group]['Estimated_Concentration_MSD_Evaluation']
    print(f"{group}: n = {len(values)}, mean = {np.mean(values):.2e}, std =
{np.std(values):.2e}")

#CD9
plt.figure(figsize=(8, 6))
sns.boxplot(data=cd9_df, x='Sample_Group',
y='Estimated_Concentration_MSD_Evaluation',
            showfliers=False, linewidth=1.5, width=0.5, palette=group_colors)
sns.stripplot(data=cd9_df, x='Sample_Group',
y='Estimated_Concentration_MSD_Evaluation',
              hue='Sample_Group', dodge=False, size=7, linewidth=1, marker='x',
palette=group_colors)
plt.yscale("log")
plt.legend([],[], frameon=False)
plt.title("CD9 Cap: MSD Evaluation", fontsize=14)
plt.tight_layout()

# Get the y-axis limits after plotting
ymin, ymax = plt.gca().get_ylim()
x_pos = 1.1
y_pos = ymin * 2
```

```
# --- CD63 Cap: Average & Std Dev ---
cd63_stats =
cd63_df.groupby("Sample_Group")["Estimated_Concentration_MSD_Evaluation"].agg(['mean
', 'std', 'count'])
print("CD63 Cap Concentration Stats by Group:")
print(cd63_stats)

# --- CD9 Cap: Average & Std Dev ---
cd9_stats =
cd9_df.groupby("Sample_Group")["Estimated_Concentration_MSD_Evaluation"].agg(['mean'
, 'std', 'count'])
print("\nCD9 Cap Concentration Stats by Group:")
print(cd9_stats)

print(avg_df["Sample_ID"].value_counts())

"""## SIMOA BC Curve"""

# Add capture type
SIMOA_BreastCancer_df["Capture"] = SIMOA_BreastCancer_df["Sample_ID"].apply(
    lambda x: "CD63 Cap" if "CD63Cap" in x else ("CD9 Cap" if "CD9Cap" in x else
"Other")
)

# Filter for CD63 Cap
cd63_df = SIMOA_BreastCancer_df[
    (SIMOA_BreastCancer_df["Capture"] == "CD63 Cap") &
    (~SIMOA_BreastCancer_df["Estimated_Concentration_SIMOA_Evaluation"].isna())
]

# Plot settings
group_colors = {"HC": "#1f77b4", "BC": "#d62728"}

# CD63 Plot
plt.figure(figsize=(8, 6))
sns.stripplot(data=cd63_df,
              x="Sample_Group",
              y="Estimated_Concentration_SIMOA_Evaluation",
              hue="Sample_Group",
              palette=group_colors,
              jitter=0.15,
              dodge=False,
              size=10,
              linewidth=1.5,
              marker="x")
plt.title("CD63 Capture: SIMOA Replicates", fontsize=14)
plt.xlabel("Group")
plt.ylabel("Concentration (EVs/mL)")
plt.yscale("log")
plt.legend([],[], frameon=False)
```

```
plt.grid(axis="y", linestyle="--", alpha=0.4)
plt.tight_layout()
plt.show()

# Filter for CD63 Cap
cd63_df = SIMOA_BreastCancer_df[
    (SIMOA_BreastCancer_df["Capture"] == "CD63 Cap") &
    (~SIMOA_BreastCancer_df["Estimated_Concentration_SIMOA_Evaluation"].isna())
]

# Create the box plot
plt.figure(figsize=(8, 6))
sns.boxplot(data=cd63_df, x='Sample_Group',
y='Estimated_Concentration_SIMOA_Evaluation',
            showfliers=False, linewidth=1.5, width=0.5, palette=group_colors)
sns.stripplot(data=cd63_df, x='Sample_Group',
y='Estimated_Concentration_SIMOA_Evaluation',
              hue='Sample_Group', dodge=False, size=7, linewidth=1, marker='x',
palette=group_colors)
plt.yscale("log")
plt.legend([],[], frameon=False)
plt.title("CD63 Cap: SIMOA Evaluation", fontsize=14)
plt.tight_layout()

from scipy.stats import mannwhitneyu

# Get the BC and HC values
bc_vals = cd63_df[cd63_df["Sample_Group"] ==
"BC"]["Estimated_Concentration_SIMOA_Evaluation"]
hc_vals = cd63_df[cd63_df["Sample_Group"] ==
"HC"]["Estimated_Concentration_SIMOA_Evaluation"]

# Run Mann–Whitney U test
u_stat, p_val = mannwhitneyu(bc_vals, hc_vals, alternative='two-sided')

# Define significance label function
def get_significance_label(p):
    if p < 0.001:
        return '***'
    elif p < 0.01:
        return '**'
    elif p < 0.05:
        return '*'
    else:
        return 'ns'

sig_label = get_significance_label(p_val)

# Add p-value with label to the plot (adjust position for log scale)
plt.text(1.1, 1e10, f"p = {p_val:.2e} ({sig_label})", ha='left', va='center',
```

```
fontsize=12)
plt.show()

# Add capture type
SIMOA_BreastCancer_df["Capture"] = SIMOA_BreastCancer_df["Sample_ID"].apply(
    lambda x: "CD63 Cap" if "CD63Cap" in x else ("CD9 Cap" if "CD9Cap" in x else
"Other")
)

# Filter for CD9 Cap
cd9_df = SIMOA_BreastCancer_df[
    (SIMOA_BreastCancer_df["Capture"] == "CD9 Cap") &
    (~SIMOA_BreastCancer_df["Estimated_Concentration_SIMOA_Evaluation"].isna())
]

# Plot settings
group_colors = {"HC": "#1f77b4", "BC": "#d62728"}

# CD9 Plot
plt.figure(figsize=(8, 6))
sns.stripplot(data=cd9_df,
              x="Sample_Group",
              y="Estimated_Concentration_SIMOA_Evaluation",
              hue="Sample_Group",
              palette=group_colors,
              jitter=0.15,
              dodge=False,
              size=10,
              linewidth=1.5,
              marker="x")
plt.title("CD9 Capture: SIMOA Replicates", fontsize=14)
plt.xlabel("Group")
plt.ylabel("Concentration (EVs/mL)")
plt.yscale("log")
plt.legend([],[], frameon=False)
plt.grid(axis="y", linestyle="--", alpha=0.4)
plt.tight_layout()
plt.show()

# Create the box plot
plt.figure(figsize=(8, 6))
sns.boxplot(data=cd9_df, x='Sample_Group',
y='Estimated_Concentration_SIMOA_Evaluation',
            showfliers=False, linewidth=1.5, width=0.5, palette=group_colors)
sns.stripplot(data=cd9_df, x='Sample_Group',
y='Estimated_Concentration_SIMOA_Evaluation',
              hue='Sample_Group', dodge=False, size=7, linewidth=1, marker='x',
palette=group_colors)
plt.yscale("log")
plt.legend([],[], frameon=False)
```

```
plt.title("CD9 Cap: SIMOA Evaluation", fontsize=14)
plt.tight_layout()


# Get the BC and HC values
bc_vals = cd9_df[cd9_df["Sample_Group"] ==
"BC"]["Estimated_Concentration_SIMOA_Evaluation"]
hc_vals = cd9_df[cd9_df["Sample_Group"] ==
"HC"]["Estimated_Concentration_SIMOA_Evaluation"]

# Run Mann–Whitney U test
u_stat, p_val = mannwhitneyu(bc_vals, hc_vals, alternative='two-sided')

# Define significance label function
def get_significance_label(p):
    if p < 0.001:
        return '***'
    elif p < 0.01:
        return '**'
    elif p < 0.05:
        return '*'
    else:
        return 'ns'

sig_label = get_significance_label(p_val)

plt.text(1.1, 1e10, f"p = {p_val:.2e} ({sig_label})", ha='left', va='center',
fontsize=12)
plt.show()

"""## SIMOA Dried Blood Spots"""

# Add capture type from Calibration_Curve
SIMOA_DriedBlood_df["Capture"] = SIMOA_DriedBlood_df["Calibration_Curve"].apply(
    lambda x: "CD63 Cap" if "CD63 Cap" in x else ("CD9 Cap" if "CD9 Cap" in x else
"Other")
)

# Average replicates with Capture info
avg_df = SIMOA_DriedBlood_df.groupby(["Sample_Group", "Sample_ID", "Capture"]).agg({
    "Estimated_Concentration_SIMOA_DB_Evaluation": "mean"
}).reset_index()

avg_df = pd.merge(avg_df, SIMOA_DriedBlood_df[['Sample_ID', 'Voltage']],
on='Sample_ID', how='left')

filtered_df = avg_df[(avg_df["Sample_Group"] == "SINEWAVE AC") |
(avg_df["Sample_Group"] == "DC") &
(~avg_df["Estimated_Concentration_SIMOA_DB_Evaluation"].isna())]
# Extract values
```

```
dc_vals = filtered_df[filtered_df["Sample_Group"] ==
"DC"]["Estimated_Concentration_SIMOA_DB_Evaluation"]
sine_vals = filtered_df[filtered_df["Sample_Group"] == "SINEWAVE
AC"]["Estimated_Concentration_SIMOA_DB_Evaluation"]

# Mann–Whitney U test
u_stat, p_val = mannwhitneyu(dc_vals, sine_vals, alternative='two-sided')

# Plot
plt.figure(figsize=(8, 6))
sns.boxplot(data=filtered_df, x="Sample_Group",
y="Estimated_Concentration_SIMOA_DB_Evaluation", showfliers=False)
sns.stripplot(data=filtered_df, x="Sample_Group",
y="Estimated_Concentration_SIMOA_DB_Evaluation",
              hue="Voltage", dodge=True, jitter=0.15, size=6, alpha=0.7)

plt.yscale("log")
plt.ylim(bottom=1e7, top=1e12)
plt.title("Estimated EV Concentration by Group and Voltage", fontsize=14)
plt.xlabel("Sample Group")
plt.ylabel("Concentration (EVs/mL)")
plt.grid(axis="y", linestyle="--", alpha=0.4)

# --- Add significance bar and p-value ---
x1, x2 = 0, 1  # positions for "DC" and "SINEWAVE AC"
y_max = max(filtered_df["Estimated_Concentration_SIMOA_DB_Evaluation"]) * 1.2
bar_height = y_max * 1.1

plt.plot([x1, x1, x2, x2], [bar_height, y_max, y_max, bar_height], lw=1.5,
c='black')
plt.text((x1 + x2) * 0.5, bar_height * 1.01, f"p = {p_val:.2e}", ha='center',
va='bottom', fontsize=10)

# Legend
plt.legend(title="Voltage", bbox_to_anchor=(1.05, 1), loc='upper left')
plt.tight_layout()
plt.show()

from scipy.stats import mannwhitneyu
import matplotlib.pyplot as plt
import seaborn as sns
import pandas as pd
import numpy as np

# --- Add capture type from Calibration_Curve ---
SIMOA_DriedBlood_df["Capture"] = SIMOA_DriedBlood_df["Calibration_Curve"].apply(
    lambda x: "CD63 Cap" if "CD63 Cap" in x else ("CD9 Cap" if "CD9 Cap" in x else
"Other")
)
```

```
# --- Average replicates with capture info ---
avg_df = SIMOA_DriedBlood_df.groupby(["Sample_Group", "Sample_ID", "Capture"]).agg({
    "Estimated_Concentration_SIMOA_DB_Evaluation": "mean"
}).reset_index()

# --- Merge back Voltage column ---
avg_df = pd.merge(avg_df, SIMOA_DriedBlood_df[['Sample_ID', 'Voltage']],
on='Sample_ID', how='left')

# --- Filter relevant data (DC & SINEWAVE AC) ---
filtered_df = avg_df[
    ((avg_df["Sample_Group"] == "SINEWAVE AC") | (avg_df["Sample_Group"] == "SQUARE
WAVE")) &
    (~avg_df["Estimated_Concentration_SIMOA_DB_Evaluation"].isna())
]

# --- Mann–Whitney U Test ---
dc_vals = filtered_df[filtered_df["Sample_Group"] ==
"DC"]["Estimated_Concentration_SIMOA_DB_Evaluation"]
sine_vals = filtered_df[filtered_df["Sample_Group"] == "SINEWAVE
AC"]["Estimated_Concentration_SIMOA_DB_Evaluation"]
u_stat, p_val = mannwhitneyu(dc_vals, sine_vals, alternative='two-sided')

plt.figure(figsize=(8, 6))

# Stripplot with Voltage hue
sns.stripplot(
    data=filtered_df,
    x="Sample_Group",
    y="Estimated_Concentration_SIMOA_DB_Evaluation",
    hue="Voltage",
    jitter=0.15,
    dodge=True,
    size=8,
    linewidth=1,
    alpha=0.8
)

plt.yscale("log")
plt.ylim(1e7, 1e12)
plt.title("Estimated EV Concentration by Group and Voltage", fontsize=14)
plt.xlabel("Sample Group")
plt.ylabel("Concentration (EVs/mL)")
plt.grid(axis="y", linestyle="--", alpha=0.4)

# --- p-value annotation bar ---
x1, x2 = 0, 1
y_max = filtered_df["Estimated_Concentration_SIMOA_DB_Evaluation"].max() * 1.2
bar_height = y_max * 1.05
plt.plot([x1, x1, x2, x2], [bar_height, y_max, y_max, bar_height], lw=1.5,
```

```
c='black')
plt.text((x1 + x2) / 2, bar_height * 1.01, f"p = {p_val:.2e}", ha='center',
va='bottom', fontsize=10)


# --- Legend and layout ---
sorted_voltage = sorted(filtered_df["Voltage"].dropna().unique())
plt.legend(title="sorted_Voltage", bbox_to_anchor=(1.05, 1), loc='upper left')
plt.tight_layout()
plt.show()
```

Appendix G - Nanoparticle Tracking Analysis Raw Data

h3							p3							PJ						
Bin centre (nm)	Concentration (p	Concentration (p	Concentration (p	Concentration	% Particles < 200nm diameter		Bin centre (nm)	Concentration (p	Concentration (p	Concentration (p	Concentration	% Particles < 200nm diameter		Bin centre (nm)	Concentration (p	Concentration (p	Concentration (p	Concentration	% Particles < 200nm diameter	
0.5	0	0	0	0	57.07575812		0.5	0	0	0	0	37.82810999		0.5	0	0	0	0	29.61576432	
1.5	0	0	0	0			1.5	0	0	0	0			1.5	0	0	0	0		
2.5	0	0	0	0	Mean Diameter	andard Deviation	2.5	0	0	0	0	Mean Diameter	andard Deviation	2.5	0	0	0	0	Mean Diameter	andard Deviation
3.5	0	0	0	0	204.1	5.8	3.5	0	0	0	0	235.7	4	3.5	0	0	0	0	252.8	12.5
4.5	0	0	0	0			4.5	0	0	0	0			4.5	0	0	0	0		
5.5	0	0	0	0	Mode Diameter	andard Deviation	5.5	0	0	0	0	Mode Diameter	andard Deviation	5.5	0	0	0	0	Mode Diameter	andard Deviation
6.5	0	0	0	0	183.2	23.1	6.5	0	0	0	0	193.3	50.2	6.5	0	0	0	0	197.8	21.4
7.5	0	0	0	0			7.5	0	0	0	0			7.5	0	0	0	0		
8.5	0	0	0	0	Concentration	andard Deviation	8.5	0	0	0	0	Concentration	andard Deviation	8.5	0	0	0	0	Concentration	andard Deviation
9.5	0	0	0	0	4.27E+07	8.50E+06	9.5	0	0	0	0	3.44E+07	5.47E+06	9.5	0	0	0	0	4.71E+07	6.33E+06
10.5	0	0	0	0	True Concentration		10.5	0	0	0	0	True Concentration		10.5	0	0	0	0	True Concentration	
11.5	0	0	0	0	1.71E+09		11.5	0	0	0	0	1.38E+09		11.5	0	0	0	0	1.88E+09	
12.5	0	0	0	0			12.5	0	0	0	0			12.5	0	0	0	0		
13.5	0	0	0	0			13.5	0	0	0	0			13.5	0	0	0	0		
14.5	0	0	0	0			14.5	0	2	0	0			14.5	0	0	0	0		
15.5	0	0	0	0			15.5	0	6	0	2			15.5	0	0	0	0		
16.5	0	0	0	0			16.5	0	18	0	6			16.5	0	0	0	0		
17.5	0	0	0	0			17.5	0	43	0	14			17.5	0	0	0	0		
18.5	0	0	0	0			18.5	0	94	0	31			18.5	0	0	0	0		
19.5	0	0	0	0			19.5	0	184	0	62			19.5	0	0	0	0		
20.5	0	0	0	0			20.5	4	330	0	111			20.5	0	0	0	0		
21.5	0	0	0	0			21.5	27	544	0	190			21.5	0	0	0	0		
22.5	0	0	0	0			22.5	120	833	0	317			22.5	0	0	0	0		
23.5	0	0	0	0			23.5	389	1193	0	527			23.5	1	0	0	0		
24.5	0	0	0	0			24.5	965	1608	0	858			24.5	9	0	0	3		
25.5	0	0	0	0			25.5	1882	2053	0	1312			25.5	43	0	0	14		
26.5	3	0	0	1			26.5	2962	2493	0	1818			26.5	153	0	0	51		
27.5	59	0	0	20			27.5	3849	2894	0	2247			27.5	433	0	0	144		
28.5	587	0	0	196			28.5	4222	3223	0	2482			28.5	1018	0	0	339		
29.5	3522	0	0	1174			29.5	3990	3458	0	2483			29.5	2047	0	0	682		
30.5	13187	0	0	4396			30.5	3311	3585	0	2299			30.5	3612	0	0	1204		
31.5	31940	0	0	10647			31.5	2454	3604	0	2019			31.5	5710	0	0	1903		
32.5	51955	0	0	17318			32.5	1651	3522	0	1725			32.5	8230	0	0	2743		
33.5	58938	0	0	19646			33.5	1025	3356	0	1460			33.5	10973	0	0	3658		
34.5	48468	0	0	16156			34.5	595	3125	0	1240			34.5	13701	0	0	4567		
35.5	30079	0	0	10026			35.5	327	2850	0	1059			35.5	16188	0	0	5396		
36.5	14696	0	0	4899			36.5	172	2551	0	908			36.5	18263	0	0	6088		
37.5	5911	0	0	1970			37.5	88	2247	0	778			37.5	19831	0	0	6610		
38.5	2053	0	0	684			38.5	44	1950	0	665			38.5	20870	0	0	6957		
39.5	647	0	0	216			39.5	22	1670	0	564			39.5	21426	0	0	7142		
40.5	195	0	0	65			40.5	11	1415	0	475			40.5	21585	0	0	7195		
41.5	60	0	0	20			41.5	5	1188	0	398			41.5	21452	0	0	7151		
42.5	19	0	0	6			42.5	3	989	0	331			42.5	21140	0	0	7047		
43.5	7	0	0	2			43.5	1	819	0	273			43.5	20748	0	0	6916		
44.5	3	0	0	1			44.5	0	674	0	225			44.5	20365	0	0	6788		
45.5	2	0	0	0			45.5	0	553	0	185			45.5	20060	0	0	6687		
46.5	1	0	0	0			46.5	0	453	6	153			46.5	19892	0	0	6631		
47.5	0	0	0	0			47.5	0	371	39	137			47.5	19904	0	0	6635		
48.5	0	0	0	0			48.5	0	304	188	164			48.5	20134	0	0	6711		
49.5	1	0	0	0			49.5	0	249	715	321			49.5	20614	0	0	6871		
50.5	2	0	0	0			50.5	0	205	2180	795			50.5	21374	0	0	7125		
51.5	3	0	0	1			51.5	0	169	5395	1855			51.5	22444	0	0	7481		
52.5	7	0	0	2			52.5	0	140	10963	3701			52.5	23851	0	0	7950		
53.5	15	0	0	5			53.5	0	117	18501	6206			53.5	25623	0	0	8541		
54.5	37	0	0	13			54.5	0	99	26219	8772			54.5	27781	0	0	9260		
55.5	94	0	0	31			55.5	0	84	31535	10540			55.5	30337	0	0	10112		
56.5	234	0	0	78			56.5	0	72	32529	10867			56.5	33284	0	0	11095		
57.5	573	0	0	191			57.5	0	62	29072	9711			57.5	36594	0	0	12198		
58.5	1347	0	0	449			58.5	0	54	22740	7598			58.5	40200	0	0	13400		
59.5	3001	0	0	1000			59.5	0	48	15725	5258			59.5	43995	0	0	14665		
60.5	6265	0	0	2089			60.5	0	42	9710	3251			60.5	47823	0	0	15941		
61.5	12171	0	1	4057			61.5	0	38	5408	1815			61.5	51478	0	0	17159		
62.5	21884	0	2	7295			62.5	0	35	2745	926			62.5	54717	0	0	18239		
63.5	36322	0	2	12108			63.5	0	32	1283	438			63.5	57274	0	0	19091		
64.5	55582	0	3	18529			64.5	0	30	558	196			64.5	58890	0	0	19630		
65.5	78446	0	5	26150			65.5	0	28	228	85			65.5	59348	4	0	19784		
66.5	102252	0	7	34086			66.5	0	27	89	39			66.5	58506	14	0	19507		
67.5	123377	0	9	41129			67.5	0	26	33	20			67.5	56329	47	0	18792		
68.5	138206	0	13	46073			68.5	0	25	12	12			68.5	52898	143	0	17680		
69.5	144230	0	18	48083			69.5	0	25	4	10			69.5	48413	394	0	16269		
70.5	140760	0	24	46928			70.5	0	25	2	9			70.5	43158	979	0	14712		
71.5	128997	0	34	43010			71.5	0	25	0	9			71.5	37473	2206	0	13226		
72.5	111485	0	46	37177			72.5	2	25	0	9			72.5	31698	4529	0	12076		
73.5	91267	0	62	30443			73.5	8	26	0	11			73.5	26142	8516	0	11552		
74.5	71093	0	84	23726			74.5	41	26	0	22			74.5	21041	14738	0	11926		
75.5	52933	0	113	17682			75.5	191	27	0	73			75.5	16554	23591	0	13382		
76.5	37844	0	151	12665			76.5	825	28	0	284			76.5	12752	35098	0	15950		
77.5	26100	0	201	8767			77.5	3181	29	0	1070			77.5	9639	48764	0	19468		

78.5	17445	0	264	5903	78.5	10641	31	0	3557	78.5	7168	63568	0	23579
79.5	11352	0	346	3899	79.5	30150	32	0	10061	79.5	5258	78112	0	27790
80.5	7226	0	448	2558	80.5	71044	34	0	23693	80.5	3815	90886	0	31567
81.5	4521	0	577	1699	81.5	137425	36	0	45820	81.5	2748	100576	0	34441
82.5	2793	0	735	1176	82.5	216466	38	0	72168	82.5	1970	106314	0	36095
83.5	1712	0	930	880	83.5	276716	41	0	92252	83.5	1412	107798	0	36403
84.5	1046	0	1165	737	84.5	287385	43	0	95809	84.5	1014	105277	0	35431
85.5	641	0	1447	696	85.5	243756	46	0	81267	85.5	733	99423	0	33385
86.5	396	0	1781	726	86.5	170408	49	0	56819	86.5	534	91144	0	30559
87.5	247	0	2174	807	87.5	99465	52	0	33172	87.5	394	81409	0	27268
88.5	157	0	2630	929	88.5	49274	55	0	16443	88.5	296	71099	0	23798
89.5	102	0	3156	1086	89.5	21129	58	0	7062	89.5	226	60924	2	20384
90.5	68	0	3757	1275	90.5	8023	62	0	2695	90.5	176	51389	7	17191
91.5	47	0	4438	1495	91.5	2768	66	0	944	91.5	140	42802	22	14321
92.5	34	0	5206	1746	92.5	892	69	0	320	92.5	114	35305	66	11828
93.5	25	0	6065	2030	93.5	277	73	0	117	93.5	95	28922	188	9735
94.5	20	0	7023	2348	94.5	85	77	0	54	94.5	82	23592	520	8065
95.5	16	0	8087	2701	95.5	27	81	2	37	95.5	72	19210	1367	6883
96.5	14	0	9268	3094	96.5	9	86	4	33	96.5	65	15649	3396	6370
97.5	12	0	10576	3530	97.5	3	90	12	35	97.5	60	12782	7912	6918
98.5	12	0	12027	4013	98.5	1	94	32	42	98.5	57	10488	17179	9241
99.5	12	0	13638	4550	99.5	0	99	81	60	99.5	55	8660	34580	14431
100.5	13	0	15432	5148	100.5	0	104	202	102	100.5	54	7207	64263	23841
101.5	14	0	17437	5817	101.5	0	109	479	196	101.5	55	6054	109950	38686
102.5	16	0	19687	6568	102.5	0	115	1079	398	102.5	56	5139	172919	59371
103.5	20	0	22222	7414	103.5	0	121	2299	806	103.5	58	4414	249880	84784
104.5	25	0	25090	8372	104.5	0	127	4605	1577	104.5	60	3838	332047	111982
105.5	33	0	28348	9460	105.5	0	134	8642	2926	105.5	63	3382	406500	136648
106.5	45	0	32062	10702	106.5	0	143	15154	5099	106.5	67	3022	459805	154298
107.5	63	0	36308	12124	107.5	0	153	24785	8313	107.5	71	2738	482405	161738
108.5	90	0	41171	13754	108.5	0	164	37780	12648	108.5	75	2517	471643	158078
109.5	131	0	46745	15625	109.5	2	178	53676	17952	109.5	80	2347	432058	144828
110.5	194	0	53134	17776	110.5	4	194	71140	23780	110.5	84	2221	373108	125138
111.5	290	0	60447	20246	111.5	10	214	88104	29443	111.5	89	2131	305737	102652
112.5	438	0	68795	23078	112.5	25	239	102196	34153	112.5	93	2074	239385	80517
113.5	664	0	78287	26317	113.5	61	269	111358	37229	113.5	96	2046	180385	60842
114.5	1008	0	89020	30009	114.5	152	307	114392	38283	114.5	99	2045	131768	44638
115.5	1530	0	101073	34201	115.5	373	354	111225	37317	115.5	102	2070	93984	32052
116.5	2316	0	114495	38937	116.5	896	415	102821	34711	116.5	104	2121	65911	22712
117.5	3490	0	129289	44260	117.5	2077	492	90809	31126	117.5	106	2197	45753	16018
118.5	5226	0	145403	50210	118.5	4610	592	77015	27406	118.5	107	2300	31630	11346
119.5	7766	0	162713	56827	119.5	9722	721	63057	24500	119.5	108	2431	21899	8146
120.5	11442	0	181016	64153	120.5	19369	889	50119	23459	120.5	110	2593	15259	5987
121.5	16695	0	200018	72238	121.5	36289	1109	38887	25428	121.5	112	2788	10745	4549
122.5	24099	1	219340	81147	122.5	63729	1398	29619	31582	122.5	115	3020	7673	3603
123.5	34391	3	238519	90971	123.5	104691	1779	22269	42913	123.5	118	3293	5571	2994
124.5	48475	7	257026	101836	124.5	160708	2282	16618	59869	124.5	123	3610	4120	2618
125.5	67435	17	274297	113916	125.5	230522	2946	12372	81946	125.5	131	3978	3107	2405
126.5	92515	40	289755	127437	126.5	309246	3822	9236	107434	126.5	140	4402	2390	2311
127.5	125067	90	302858	142672	127.5	388632	4973	6945	133517	127.5	154	4887	1876	2306
128.5	166466	198	313135	159933	128.5	458625	6481	5284	156797	128.5	172	5440	1501	2371
129.5	217983	418	320221	179541	129.5	509788	8445	4083	174105	129.5	197	6069	1224	2496
130.5	280602	847	323888	201779	130.5	535693	10983	3215	183297	130.5	231	6778	1015	2675
131.5	354815	1651	324064	226843	131.5	534366	14236	2588	183730	131.5	278	7576	855	2903
132.5	440405	3084	320835	254775	132.5	508332	18361	2135	176276	132.5	342	8469	731	3181
133.5	536249	5526	314436	285403	133.5	463433	23533	1808	162925	133.5	433	9462	633	3509
134.5	640187	9491	305233	318304	134.5	407028	29937	1575	146180	134.5	563	10561	554	3893
135.5	749004	15636	293687	352776	135.5	346267	37757	1411	128479	135.5	750	11769	490	4336
136.5	858550	24727	280320	387866	136.5	286914	47164	1302	111793	136.5	1023	13089	436	4849
137.5	964005	37575	265678	422419	137.5	232842	58304	1237	97461	137.5	1427	14520	392	5446
138.5	1060284	54940	250294	455173	138.5	186097	71276	1210	86194	138.5	2031	16061	354	6149
139.5	1142525	77413	234663	484867	139.5	147272	86121	1216	78203	139.5	2944	17705	321	6990
140.5	1206576	105306	219219	510367	140.5	115999	102804	1255	73353	140.5	4331	19446	292	8023
141.5	1249433	138551	204321	530769	141.5	91383	121201	1329	71304	141.5	6450	21270	267	9329
142.5	1269519	176666	190255	545480	142.5	72330	141098	1439	71623	142.5	9692	23164	245	11034
143.5	1266796	218754	177229	554260	143.5	57757	162188	1593	73846	143.5	14640	25110	226	13325
144.5	1242681	263577	165385	557214	144.5	46700	184078	1796	77525	144.5	22155	27087	208	16484
145.5	1199795	309656	154805	554752	145.5	38354	206313	2060	82242	145.5	33468	29073	192	20911
146.5	1141606	355400	145527	547511	146.5	32081	228389	2397	87622	146.5	50280	31042	178	27167
147.5	1072030	399235	137555	536273	147.5	27385	249785	2823	93331	147.5	74852	32971	165	35996
148.5	995039	439716	130867	521874	148.5	23894	269989	3358	99081	148.5	110029	34836	154	48340
149.5	914347	475608	125428	505128	149.5	21332	288529	4025	104628	149.5	159149	36615	144	65303
150.5	833177	505940	121198	486772	150.5	19495	304991	4851	109779	150.5	225769	38289	135	88064
151.5	754138	530022	118134	467431	151.5	18240	319046	5867	114384	151.5	313136	39842	126	117701
152.5	679180	547444	116199	447608	152.5	17465	330458	7108	118343	152.5	423390	41266	119	154925
153.5	609630	558053	115361	427681	153.5	17100	339087	8611	121599	153.5	556569	42556	113	199746
154.5	546267	561924	115598	407930	154.5	17104	344895	10416	124138	154.5	709575	43715	107	251132
155.5	489422	559324	116898	388548	155.5	17452	347933	12565	125984	155.5	875429	44751	102	306760
156.5	439086	550679	119254	369673	156.5	18139	348331	15097	127189	156.5	1043124	45678	98	362967
157.5	395011	536541	122672	351408	157.5	19172	346284	18052	127836	157.5	1198413	46519	94	415009

158.5	356792	517561	127159	333837				158.5	20569	342032	21464	128022				158.5	1325586	47298	91	457658			
159.5	323942	494459	132730	317044				159.5	22360	335849	25364	127857				159.5	1410020	48047	88	486052			
160.5	295942	468002	139396	301113				160.5	24583	328025	29774	127460				160.5	1440969	48802	86	496619			
161.5	272275	438979	147167	286140				161.5	27284	318850	34710	126948				161.5	1413835	49601	85	487840			
162.5	252453	408176	156046	272225				162.5	30518	308608	40180	126435				162.5	1331271	50487	84	460614			
163.5	236032	376353	166022	259469				163.5	34344	297564	46184	126031				163.5	1202728	51507	84	418106			
164.5	222615	344225	177070	247970				164.5	38823	285961	52714	125833				164.5	1042588	52710	84	365127			
165.5	211857	312439	189144	237813				165.5	44022	274015	59755	125931				165.5	867378	54151	84	307205			
166.5	203463	281558	202174	229065				166.5	50005	261914	67286	126401				166.5	692877	55891	86	249618			
167.5	197184	252051	216061	221766				167.5	56834	249815	75279	127309				167.5	531802	57995	88	196628			
168.5	192812	224287	230681	215926				168.5	64563	237851	83704	128706				168.5	392530	60538	90	151053			
169.5	190173	198530	245876	211526				169.5	73241	226127	92527	130632				169.5	278931	63606	94	114210			
170.5	189125	174949	261462	208512				170.5	82903	214726	101709	133113				170.5	191064	67297	98	86153			
171.5	189550	153621	277229	206800				171.5	93571	203710	111208	136163				171.5	126346	71725	104	66058			
172.5	191347	134547	292946	206280				172.5	105250	193124	120977	139784				172.5	80793	77022	110	52642			
173.5	194431	117665	308366	206820				173.5	117926	182999	130966	143964				173.5	50051	83346	118	44505			
174.5	198720	102864	323235	208273				174.5	131569	173353	141118	148680				174.5	30101	90877	127	40368			
175.5	204137	90000	337301	210479				175.5	146125	164194	151368	153896				175.5	17613	99828	138	39193			
176.5	210600	78909	350322	213277				176.5	161526	155523	161643	159564				176.5	10051	110446	152	40216			
177.5	218019	69416	362072	216502				177.5	177680	147335	171863	165626				177.5	5608	123011	168	42929			
178.5	226290	61349	372354	219997				178.5	194483	139621	181936	172013				178.5	3068	137844	187	47033			
179.5	235289	54540	381004	223611				179.5	211813	132366	191762	178647				179.5	1651	155298	210	52386			
180.5	244872	48833	387896	227200				180.5	229539	125557	201232	185443				180.5	876	175758	237	58957			
181.5	254870	44090	392947	230636				181.5	247519	119176	210232	192309				181.5	460	199629	271	66786			
182.5	265090	40185	396117	233797				182.5	265605	113206	218643	199151				182.5	240	227317	311	75956			
183.5	275312	37014	397410	236578				183.5	283648	107629	226344	205874				183.5	124	259207	360	86564			
184.5	285296	34484	396869	238883				184.5	301495	102428	233220	212381				184.5	65	295626	420	98703			
185.5	294785	32524	394577	240629				185.5	318999	97585	239160	218581				185.5	34	336796	493	112441			
186.5	303510	31076	390646	241744				186.5	336019	93083	244070	224390				186.5	18	382785	583	127795			
187.5	311205	30095	385216	242172				187.5	352419	88906	247870	229732				187.5	9	433440	693	144714			
188.5	317610	29552	378445	241869				188.5	368076	85038	250502	234539				188.5	5	488327	830	163054			
189.5	322488	29432	370506	240809				189.5	382877	81465	251933	238758				189.5	3	546675	1000	182559			
190.5	325634	29732	361578	238981				190.5	396723	78173	252156	242350				190.5	2	607331	1211	202848			
191.5	326888	30460	351841	236396				191.5	409529	75147	251193	245290				191.5	0	668747	1473	223407			
192.5	326140	31642	341472	233085				192.5	421224	72377	249091	247564				192.5	0	729003	1801	243601			
193.5	323342	33313	330640	229098				193.5	431755	69850	245924	249176				193.5	0	785872	2210	262694			
194.5	318504	35528	319505	224512				194.5	441082	67556	241786	250142				194.5	0	836937	2722	279887			
195.5	311703	38355	308210	219422				195.5	449179	65485	236792	250485				195.5	0	879757	3363	294373			
196.5	303070	41880	296886	213945				196.5	456036	63628	231068	250244				196.5	0	912054	4165	305406			
197.5	292791	46210	285647	208216				197.5	461655	61976	224747	249459				197.5	0	931923	5168	312364			
198.5	281093	51470	274592	202385				198.5	466050	60523	217968	248180				198.5	0	938021	6421	314814			
199.5	268230	57808	263804	196614				199.5	469246	59260	210865	246457				199.5	0	929714	7984	312566			
200.5	254479	65393	253350	191074				200.5	471277	58183	203569	244343				200.5	0	907160	9928	305696			
201.5	240117	74414	243284	185939				201.5	472183	57286	196199	241889				201.5	0	871311	12340	294551			
202.5	225419	85079	233649	181382				202.5	472012	56565	188864	239147				202.5	0	823834	15322	279719			
203.5	210640	97606	224473	177573				203.5	470814	56015	181658	236163				203.5	0	766953	18991	261981			
204.5	196013	112220	215777	174670				204.5	468644	55635	174664	232981				204.5	0	703250	23486	242245			
205.5	181740	129138	207573	172817				205.5	465557	55421	167945	229641				205.5	0	635442	28962	221468			
206.5	167989	148555	199866	172137				206.5	461606	55372	161556	226178				206.5	0	566160	35591	200584			
207.5	154894	170627	192653	172725				207.5	456848	55487	155534	222623				207.5	0	497772	43565	180446			
208.5	142553	195448	185928	174643				208.5	451333	55766	149908	219002				208.5	0	432251	53085	161779			
209.5	131036	223024	179681	177913				209.5	445112	56208	144694	215338				209.5	0	371103	64361	145155			
210.5	120381	253249	173897	182509				210.5	438232	56815	139900	211649				210.5	0	315347	77604	130984			
211.5	110605	285885	168561	188350				211.5	430736	57588	135527	207950				211.5	0	265552	93018	119523			
212.5	101700	320538	163656	195298				212.5	422666	58528	131568	204254				212.5	0	221893	110785	110893			
213.5	93648	356650	159163	203154				213.5	414060	59637	128013	200570				213.5	0	184233	131059	105097			
214.5	86415	393496	155062	211658				214.5	404954	60918	124846	196906				214.5	0	152211	153945	102052			
215.5	79961	430200	151333	220498				215.5	395381	62372	122050	193268				215.5	0	125323	179493	101606			
216.5	74239	465758	147957	229318				216.5	385374	64004	119603	189660				216.5	0	102987	207678	103555			
217.5	69201	499087	144913	237734				217.5	374965	65814	117485	186088				217.5	1	84599	238393	107664			
218.5	64800	529070	142183	245351				218.5	364185	67806	115671	182554				218.5	2	69576	271437	113672			
219.5	60988	554630	139747	251788				219.5	353068	69981	114136	179062				219.5	3	57375	306514	121298			
220.5	57723	574793	137586	256701				220.5	341647	72342	112857	175615				220.5	6	47514	343231	130250			
221.5	54963	588754	135683	259800				221.5	329957	74890	111807	172218				221.5	11	39571	381104	140229			
222.5	52674	595937	134019	260877				222.5	318037	77625	110961	168874				222.5	21	33190	419570	150927			
223.5	50824	596034	132578	259812				223.5	305927	80548	110295	165590				223.5	38	28074	458002	162038			
224.5	49388	589028	131342	256586				224.5	293670	83657	109782	162369				224.5	71	23978	495732	173260			
225.5	48346	575193	130297	251279				225.5	281311	86950	109399	159220				225.5	134	20702	532078	184305			
226.5	47684	555074	129426	244061				226.5	268898	90423	109122	156148				226.5	250	18088	566365	194901			
227.5	47391	529447	128714	235184				227.5	256482	94073	108928	153161				227.5	464	16008	597955	204809			
228.5	47464	499260	128148	224957				228.5	244113	97893	108795	150267				228.5	856	14362	626276	213831			
229.5	47904	465574	127712	213730				229.5	231843	101876	108704	147474				229.5	1562	13072	650835	221823			
230.5	48719	429493	127394	201868				230.5	219723	106011	108635	144790				230.5	2813	12075	671244	228711			
231.5	49922	392100	127179	189733				231.5	207806	110289	108573	142223				231.5	4986	11327	687228	234514			
232.5	51531	354406	127056	177664				232.5	196139	114696	108503	139779				232.5	8677	10792	698629	239366			
233.5	53572	317307	127013	165964				233.5	184768	119219	108413	137467				233.5	14793	10444	705411	243549			
234.5	56075	281553	127037	154888				234.5	173736	123841	108295	135291				234.5	24650	10267	707649	247522			
235.5	59077	247735	127118	144643				235.5	163082	128545	108141	133256				235.5	40071	10251	705521	251947			
236.5	62621	216283	127244	135383				236.5	152839	133312	107948	131366				236.5	63422	10392	699294	257703			
237.5	66756	187473	127407	127212				237.5	143036	138121	107714	129624				237.5	97570	10692	689306	265856			

238.5	71537	161445	127597	120193				238.5	133695	142950	107442	128029				238.5	145670	11160	675950	277593										
239.5	77024	138223	127805	114351				239.5	124833	147777	107136	126582				239.5	210753	11808	659652	294071										
240.5	83281	117738	128022	109680				240.5	116462	152578	106802	125281				240.5	295103	12657	640860	316207										
241.5	90375	99850	128242	106156				241.5	108588	157328	106449	124122				241.5	399459	13731	620021	344404										
242.5	98377	84373	128457	103736				242.5	101213	162003	106087	123101				242.5	522203	15065	597574	378281										
243.5	107354	71090	128663	102369				243.5	94331	166578	105730	122213				243.5	658727	16699	573933	416453										
244.5	117371	59773	128852	101999				244.5	87936	171027	105390	121451				244.5	801242	18682	549485	456470										
245.5	128487	50191	129021	102566				245.5	82015	175325	105082	120807				245.5	939221	21071	524579	494957										
246.5	140748	42122	129165	104012				246.5	76555	179448	104823	120275				246.5	1060553	23933	499524	528004										
247.5	154187	35358	129281	106275				247.5	71538	183372	104628	119846				247.5	1153273	27347	474587	551736										
248.5	168813	29709	129367	109296				248.5	66946	187075	104515	119512				248.5	1207531	31399	449996	562975										
249.5	184610	25007	129419	113012				249.5	62758	190535	104500	119264				249.5	1217367	36184	425938	559829										
250.5	201529	21102	129435	117355				250.5	58954	193732	104601	119096				250.5	1181810	41806	402561	542059										
251.5	219482	17864	129415	122254				251.5	55514	196647	104835	118998				251.5	1105041	48372	379983	511132										
252.5	238338	15183	129358	127626				252.5	52415	199263	105221	118966				252.5	995565	55993	358287	469948										
253.5	257919	12963	129262	133381				253.5	49638	201565	105775	118993				253.5	864630	64775	337534	422313										
254.5	277995	11127	129126	139416				254.5	47164	203540	106515	119073				254.5	724307	74813	317757	372292										
255.5	298289	9607	128951	145616				255.5	44975	205177	107461	119204				255.5	585677	86187	298975	323613										
256.5	318475	8348	128736	151853				256.5	43052	206467	108629	119383				256.5	457508	98951	281187	279215										
257.5	338182	7306	128481	157989				257.5	41381	207402	110039	119608				257.5	345585	113124	264382	241031										
258.5	357008	6442	128184	163878				258.5	39948	207980	111710	119879				258.5	252689	128681	248538	209969										
259.5	374527	5725	127846	169366				259.5	38741	208196	113661	120199				259.5	179058	145546	233626	186076										
260.5	390306	5131	127465	174301				260.5	37749	208050	115913	120571				260.5	123120	163583	219610	168771										
261.5	403921	4639	127042	178534				261.5	36963	207546	118487	120999				261.5	82259	182596	206452	157102										
262.5	414974	4233	126573	181926				262.5	36376	206686	121405	121489				262.5	53480	202326	194112	149973										
263.5	423113	3898	126057	184356				263.5	35984	205477	124689	122050				263.5	33887	222456	182547	146296										
264.5	428051	3625	125493	185723				264.5	35783	203927	128363	122691				264.5	20961	242617	171715	145098										
265.5	429580	3403	124878	185954				265.5	35772	202046	132450	123422				265.5	12679	262405	161575	145553										
266.5	427585	3227	124209	185007				266.5	35952	199845	136974	124257				266.5	7513	281393	152087	146998										
267.5	422048	3090	123483	182874				267.5	36325	197339	141960	125208				267.5	4370	299152	143211	148911										
268.5	413057	2988	122697	179581				268.5	36897	194541	147431	126290				268.5	2499	315273	134911	150894										
269.5	400801	2918	121846	175188				269.5	37674	191468	153412	127518				269.5	1409	329384	127152	152648										
270.5	385559	2878	120926	169788				270.5	38665	188138	159924	128909				270.5	784	341172	119899	153952										
271.5	367692	2866	119934	163497				271.5	39881	184569	166987	130479				271.5	432	350396	113123	154650										
272.5	347625	2881	118864	156457				272.5	41337	180780	174617	132245				272.5	236	356898	106793	154642										
273.5	325825	2923	117711	148820				273.5	43047	176791	182826	134221				273.5	128	360610	100883	153874										
274.5	302785	2992	116472	140749				274.5	45029	172624	191619	136424				274.5	69	361549	95367	152328										
275.5	278996	3090	115140	132409				275.5	47304	168299	200995	138866				275.5	37	359820	90221	150026										
276.5	254935	3218	113713	123955				276.5	49895	163837	210943	141558				276.5	20	355598	85424	147014										
277.5	231045	3378	112185	115536				277.5	52826	159260	221438	144508				277.5	11	349120	80954	143362										
278.5	207719	3573	110553	107282				278.5	56123	154589	232442	147718				278.5	6	340670	76793	139156										
279.5	185293	3806	108813	99304				279.5	59816	149844	243901	151187				279.5	3	330560	72923	134495										
280.5	164038	4082	106964	91695				280.5	63933	145046	255740	154907				280.5	2	319116	69327	129482										
281.5	144162	4407	105001	84523				281.5	68507	140216	267864	158862				281.5	1	306665	65989	124218										
282.5	125806	4785	102926	77839				282.5	73568	135371	280150	163029				282.5	0	293522	62895	118806										
283.5	109052	5223	100736	71670				283.5	79146	130530	292451	167376				283.5	0	279979	60031	113337										
284.5	93927	5730	98434	66030				284.5	85272	125712	304591	171858				284.5	0	266301	57384	107895										
285.5	80415	6315	96020	60917				285.5	91972	120931	316367	176423				285.5	0	252717	54943	102553										
286.5	68461	6987	93498	56315				286.5	99269	116203	327546	181006				286.5	0	239424	52695	97373										
287.5	57980	7757	90872	52203				287.5	107180	111543	337872	185532				287.5	0	226581	50631	92404										
288.5	48869	8638	88147	48551				288.5	115717	106963	347066	189915				288.5	0	214317	48740	87686										
289.5	41012	9644	85330	45328				289.5	124880	102475	354835	194063				289.5	0	202726	47014	83246										
290.5	34286	10789	82428	42501				290.5	134658	98089	360877	197875				290.5	0	191876	45442	79106										
291.5	28567	12089	79451	40035				291.5	145030	93814	364898	201247				291.5	0	181813	44019	75277										
292.5	23734	13563	76407	37901				292.5	155956	89659	366616	204077				292.5	0	172561	42735	71765										
293.5	19673	15227	73309	36069				293.5	167380	85630	365783	206264				293.5	0	164128	41583	68571										
294.5	16278	17100	70166	34515				294.5	179227	81732	362197	207719				294.5	0	156513	40558	65690										
295.5	13452	19203	66993	33216				295.5	191402	77970	355718	208363				295.5	0	149702	39653	63118										
296.5	11110	21554	63800	32155				296.5	203787	74348	346282	208139				296.5	0	143677	38862	60846										
297.5	9175	24172	60603	31316				297.5	216246	70867	333916	207010				297.5	0	138417	38180	58866										
298.5	7581	27075	57413	30690				298.5	228618	67529	318743	204964				298.5	0	133898	37603	57167										
299.5	6270	30280	54244	30265				299.5	240727	64335	300989	202017				299.5	0	130097	37124	55741										
300.5	5196	33801	51110	30035				300.5	252379	61283	280977	198213				300.5	0	126993	36741	54578										
301.5	4315	37648	48022	29995				301.5	263366	58374	259122	193621				301.5	0	124566	36449	53672										
302.5	3594	41830	44994	30139				302.5	273474	55605	235912	188330				302.5	0	122802	36243	53015										
303.5	3004	46348	42036	30463				303.5	282487	52974	211888	182450				303.5	0	121688	36121	52603										
304.5	2522	51201	39159	30960				304.5	290195	50478	187614	176096				304.5	0	121218	36079	52432										
305.5	2126	56377	36372	31625				305.5	296396	48115	163653	169388				305.5	0	121391	36113	52501										
306.5	1803	61862	33685	32450				306.5	300911	45880	140533	162441				306.5	0	122209	36219	52809										
307.5	1537	67631	31105	33424				307.5	303588	43770	118718	155359				307.5	0	123682	36394	53359										
308.5	1319	73650	28637	34535				308.5	304307	41781	98592	148227				308.5	0	125825	36633	54153										
309.5	1140	79880	26287	35769				309.5	302988	39908	80436	141111				309.5	0	128657	36933	55197										
310.5	992	86270	24058	37107				310.5	299598	38147	64423	134056				310.5	0	132204	37289	56498										
311.5	871	92763	21953	38529				311.5	294150	36495	50619	127088				311.5	0	136496	37697	58064										
312.5	770	99292	19973	40012				312.5	286707	34945	38993	120215				312.5	0	141569	38151	59906										
313.5	688	105785	18118	41530				313.5	277380	33495	29428	113434				313.5	0	147462	38645	62036										
314.5	619	112162	16387	43056				314.5	266327	32139	21745	106737				314.5	0	154220	39174	64465										
315.5	563	118340	14779	44561				315.5	253743	30873	15722	100113				315.5	0	161888	39730	67206										
316.5	517	124231	13290	46013				316.5	239862	29693	11115	93557				316.5	0	170514	40308	70274										
317.5	480	129748	11917	47381				317.5	224939	28595	7679	87071				317.5	0	180146	40897	73681										

318.5	450	134802	10655	48636	318.5	209249	27575	5182	80669	318.5	0	190831	41491	77441
319.5	426	139311	9501	49746	319.5	193071	26629	3413	74371	319.5	0	202610	42081	81564
320.5	408	143194	8448	50684	320.5	176684	25754	2193	68210	320.5	0	215518	42655	86058
321.5	395	146381	7492	51423	321.5	160353	24945	1374	62224	321.5	1	229582	43206	90930
322.5	386	148808	6626	51940	322.5	144323	24201	839	56454	322.5	2	244812	43722	96178
323.5	382	150424	5845	52217	323.5	128813	23517	499	50943	323.5	3	261202	44193	101799
324.5	382	151192	5143	52239	324.5	114011	22890	289	45730	324.5	5	278724	44609	107779
325.5	386	151088	4514	51996	325.5	100067	22319	163	40849	325.5	8	297324	44959	114097
326.5	395	150102	3953	51483	326.5	87096	21799	89	36328	326.5	14	316915	45233	120721
327.5	407	148242	3452	50700	327.5	75178	21330	48	32185	327.5	24	337377	45422	127608
328.5	425	145529	3009	49654	328.5	64354	20908	25	28429	328.5	42	358549	45516	134702
329.5	447	142001	2616	48355	329.5	54636	20532	12	25060	329.5	71	380229	45508	141936
330.5	476	137710	2270	46819	330.5	46009	20199	6	22072	330.5	120	402168	45392	149227
331.5	511	132721	1965	45066	331.5	38433	19909	3	19448	331.5	203	424077	45161	156480
332.5	554	127110	1698	43121	332.5	31851	19658	1	17170	332.5	340	445621	44811	163591
333.5	606	120963	1464	41011	333.5	26190	19447	0	15212	333.5	565	466428	44342	170445
334.5	668	114372	1260	38767	334.5	21370	19273	0	13548	334.5	929	486094	43751	176925
335.5	742	107435	1082	36420	335.5	17307	19136	0	12148	335.5	1513	504194	43040	182915
336.5	831	100253	928	34004	336.5	13914	19034	0	10982	336.5	2432	520288	42213	188311
337.5	937	92924	795	31552	337.5	11106	18966	0	10024	337.5	3860	533944	41274	193026
338.5	1063	85547	679	29096	338.5	8804	18932	0	9245	338.5	6039	544746	40229	197005
339.5	1213	78213	580	26669	339.5	6933	18931	0	8621	339.5	9302	552318	39088	200236
340.5	1392	71009	494	24298	340.5	5425	18962	0	8129	340.5	14098	556334	37858	202763
341.5	1606	64011	421	22013	341.5	4218	19025	0	7748	341.5	21002	556541	36550	204698
342.5	1860	57289	358	19836	342.5	3261	19120	0	7460	342.5	30727	552771	35177	206225
343.5	2163	50899	304	17788	343.5	2508	19246	0	7251	343.5	44114	544953	33750	207606
344.5	2524	44886	258	15889	344.5	1918	19402	0	7107	344.5	62106	533123	32281	209170
345.5	2953	39286	218	14152	345.5	1460	19589	0	7017	345.5	85681	517423	30784	211296
346.5	3463	34123	185	12590	346.5	1107	19807	0	6971	346.5	115761	498103	29270	214378
347.5	4067	29408	156	11211	347.5	835	20055	0	6964	347.5	153080	475512	27752	218781
348.5	4783	25146	132	10020	348.5	628	20334	0	6987	348.5	198027	450086	26241	224785
349.5	5629	21329	111	9023	349.5	471	20643	0	7038	349.5	250484	422332	24748	232521
350.5	6626	17945	94	8222	350.5	352	20983	0	7112	350.5	309670	392805	23282	241919
351.5	7796	14974	79	7617	351.5	263	21353	0	7205	351.5	374042	362090	21852	252661
352.5	9167	12390	67	7208	352.5	196	21755	0	7317	352.5	441269	330774	20466	264170
353.5	10764	10165	56	6995	353.5	146	22187	0	7444	353.5	508309	299426	19129	275621
354.5	12619	8268	47	6978	354.5	108	22650	0	7586	354.5	571596	268579	17848	286007
355.5	14761	6666	40	7156	355.5	81	23144	0	7741	355.5	627344	238707	16626	294225
356.5	17224	5327	34	7528	356.5	60	23669	0	7910	356.5	671912	210216	15465	299198
357.5	20039	4219	28	8095	357.5	45	24225	0	8090	357.5	702201	183435	14369	300001
358.5	23236	3310	24	8857	358.5	33	24812	0	8282	358.5	716016	158609	13337	295987
359.5	26845	2574	20	9813	359.5	25	25431	0	8485	359.5	712333	135904	12371	286869
360.5	30890	1982	17	10963	360.5	19	26081	0	8700	360.5	691424	115405	11469	272766
361.5	35392	1512	14	12306	361.5	14	26762	0	8925	361.5	654825	97131	10630	254195
362.5	40362	1143	12	13839	362.5	11	27473	0	9161	362.5	605143	81035	9852	232010
363.5	45804	855	10	15556	363.5	8	28214	0	9407	363.5	545747	67026	9133	207302
364.5	51711	633	8	17451	364.5	6	28985	0	9664	364.5	480380	54971	8471	181274
365.5	58065	465	7	19512	365.5	5	29786	0	9930	365.5	412776	44712	7863	155117
366.5	64833	337	6	21726	366.5	4	30615	0	10206	366.5	346312	36075	7306	129898
367.5	71968	243	5	24072	367.5	3	31471	0	10491	367.5	283756	28878	6797	106477
368.5	79407	173	4	26528	368.5	2	32353	0	10785	368.5	227121	22942	6333	85465
369.5	87074	121	4	29066	369.5	2	33261	0	11088	369.5	177635	18093	5911	67213
370.5	94876	85	3	31655	370.5	1	34193	0	11398	370.5	135798	14168	5529	51832
371.5	102711	58	3	34257	371.5	1	35146	0	11716	371.5	101507	11020	5182	39236
372.5	110461	40	2	36834	372.5	0	36120	0	12040	372.5	74214	8516	4870	29200
373.5	118005	27	2	39345	373.5	0	37112	0	12371	373.5	53093	6542	4589	21408
374.5	125214	18	2	41745	374.5	0	38119	0	12707	374.5	37181	4996	4337	15504
375.5	131961	12	1	43991	375.5	0	39140	0	13047	375.5	25498	3795	4111	11135
376.5	138119	8	1	46043	376.5	0	40171	0	13390	376.5	17131	2868	3911	7970
377.5	143572	5	0	47859	377.5	0	41209	0	13736	377.5	11282	2158	3733	5724
378.5	148213	3	0	49406	378.5	0	42251	0	14084	378.5	7286	1617	3577	4160
379.5	151952	2	0	50652	379.5	0	43293	0	14431	379.5	4616	1207	3440	3088
380.5	154718	1	0	51573	380.5	0	44331	0	14777	380.5	2871	898	3323	2364
381.5	156460	0	0	52154	381.5	0	45361	0	15121	381.5	1753	666	3222	1881
382.5	157150	0	0	52384	382.5	0	46380	0	15460	382.5	1052	493	3138	1561
383.5	156785	0	0	52262	383.5	0	47381	0	15794	383.5	621	364	3070	1352
384.5	155384	0	0	51795	384.5	0	48361	0	16120	384.5	360	269	3016	1215
385.5	152990	0	0	50997	385.5	0	49315	0	16438	385.5	206	198	2977	1127
386.5	149664	0	0	49888	386.5	0	50237	0	16746	386.5	116	146	2952	1071
387.5	145487	0	0	48496	387.5	0	51121	0	17041	387.5	64	108	2941	1038
388.5	140553	0	0	46851	388.5	0	51964	0	17321	388.5	35	79	2944	1019
389.5	134966	0	0	44989	389.5	0	52759	0	17586	389.5	19	59	2960	1013
390.5	128840	0	0	42947	390.5	0	53501	0	17834	390.5	10	43	2991	1015
391.5	122290	0	0	40764	391.5	0	54184	0	18062	391.5	5	32	3035	1024
392.5	115432	0	0	38477	392.5	0	54804	0	18268	392.5	3	24	3095	1041
393.5	108376	0	0	36125	393.5	0	55354	0	18452	393.5	1	18	3170	1063
394.5	101230	0	0	33743	394.5	0	55831	0	18610	394.5	0	13	3261	1092
395.5	94089	0	0	31363	395.5	0	56228	0	18743	395.5	0	10	3370	1127
396.5	87041	0	0	29014	396.5	0	56542	0	18848	396.5	0	8	3497	1168
397.5	80161	0	0	26720	397.5	0	56769	0	18923	397.5	0	6	3644	1217

398.5	73513	0	0	24504				398.5	0	56904	0	18968				398.5	0	4	3812	1272						
399.5	67149	0	0	22383				399.5	0	56945	0	18982				399.5	0	3	4003	1335						
400.5	61107	0	0	20369				400.5	1	56888	0	18963				400.5	0	3	4219	1407						
401.5	55418	0	0	18473				401.5	1	56732	0	18911				401.5	0	2	4463	1488						
402.5	50099	0	0	16700				402.5	2	56474	0	18825				402.5	0	2	4737	1580						
403.5	45160	0	0	15053				403.5	2	56114	0	18705				403.5	0	1	5043	1682						
404.5	40602	0	0	13534				404.5	3	55651	0	18551				404.5	0	1	5385	1795						
405.5	36420	0	0	12140				405.5	4	55085	0	18363				405.5	0	0	5766	1922						
406.5	32603	0	0	10868				406.5	5	54418	0	18141				406.5	0	0	6190	2064						
407.5	29136	0	0	9712				407.5	6	53651	0	17886				407.5	0	0	6661	2220						
408.5	26002	0	0	8667				408.5	8	52786	0	17598				408.5	0	0	7182	2394						
409.5	23180	0	0	7727				409.5	11	51827	0	17280				409.5	0	0	7759	2586						
410.5	20649	0	0	6883				410.5	15	50778	0	16931				410.5	0	0	8396	2799						
411.5	18386	0	0	6129				411.5	20	49642	0	16554				411.5	0	0	9098	3033						
412.5	16368	0	0	5456				412.5	27	48426	0	16151				412.5	0	0	9870	3290						
413.5	14575	0	0	4858				413.5	36	47133	0	15723				413.5	0	0	10719	3573						
414.5	12985	0	0	4328				414.5	49	45772	0	15274				414.5	0	0	11649	3883						
415.5	11578	0	0	3859				415.5	66	44348	0	14805				415.5	0	0	12665	4222						
416.5	10335	0	0	3445				416.5	89	42868	0	14319				416.5	0	0	13775	4592						
417.5	9239	0	0	3080				417.5	121	41340	0	13820				417.5	0	0	14981	4994						
418.5	8274	0	0	2758				418.5	164	39771	0	13312				418.5	0	0	16291	5430						
419.5	7425	0	0	2475				419.5	222	38170	0	12797				419.5	0	0	17707	5902						
420.5	6678	0	0	2226				420.5	300	36543	0	12281				420.5	0	0	19235	6412						
421.5	6023	0	0	2008				421.5	405	34900	0	11768				421.5	0	0	20876	6959						
422.5	5448	0	0	1816				422.5	544	33247	0	11264				422.5	0	0	22633	7544						
423.5	4943	0	0	1648				423.5	730	31593	0	10774				423.5	0	0	24507	8169						
424.5	4501	0	0	1500				424.5	974	29945	0	10306				424.5	0	0	26497	8832						
425.5	4113	0	0	1371				425.5	1296	28310	0	9868				425.5	0	0	28601	9534						
426.5	3774	0	0	1258				426.5	1714	26695	0	9470				426.5	0	0	30814	10272						
427.5	3477	0	0	1159				427.5	2256	25106	0	9121				427.5	0	0	33131	11044						
428.5	3218	0	0	1073				428.5	2951	23550	0	8834				428.5	0	0	35543	11848						
429.5	2991	0	0	997				429.5	3836	22033	0	8623				429.5	0	0	38039	12680						
430.5	2794	0	0	931				430.5	4953	20558	0	8504				430.5	0	0	40606	13536						
431.5	2623	0	0	874				431.5	6349	19130	0	8493				431.5	0	0	43229	14410						
432.5	2475	0	0	825				432.5	8075	17754	0	8610				432.5	0	0	45888	15296						
433.5	2347	0	0	782				433.5	10189	16432	0	8873				433.5	0	0	48564	16188						
434.5	2238	0	0	746				434.5	12747	15167	0	9305				434.5	0	0	51234	17078						
435.5	2145	0	0	715				435.5	15808	13961	0	9923				435.5	0	0	53872	17958						
436.5	2068	0	0	689				436.5	19424	12816	0	10747				436.5	0	0	56453	18818						
437.5	2005	0	0	668				437.5	23641	11732	0	11791				437.5	0	0	58948	19650						
438.5	1954	0	0	651				438.5	28493	10710	0	13068				438.5	0	0	61329	20443						
439.5	1916	0	0	639				439.5	33994	9751	0	14582				439.5	0	0	63566	21189						
440.5	1889	0	0	630				440.5	40137	8852	0	16330				440.5	0	0	65631	21877						
441.5	1873	0	0	624				441.5	46885	8014	0	18299				441.5	0	1	67495	22499						
442.5	1868	0	0	623				442.5	54170	7235	0	20468				442.5	0	1	69132	23044						
443.5	1874	0	0	625				443.5	61891	6513	0	22801				443.5	0	2	70517	23506						
444.5	1889	0	0	630				444.5	69907	5847	0	25251				444.5	0	2	71628	23877						
445.5	1916	0	0	639				445.5	78048	5234	0	27761				445.5	0	2	72448	24150						
446.5	1953	0	0	651				446.5	86108	4672	0	30260				446.5	0	3	72960	24321						
447.5	2001	0	0	667				447.5	93862	4159	0	32674				447.5	0	4	73154	24386						
448.5	2061	0	0	687				448.5	101070	3692	0	34921				448.5	0	5	73024	24343						
449.5	2134	0	0	711				449.5	107488	3268	0	36919				449.5	0	6	72569	24192						
450.5	2219	0	0	740				450.5	112886	2884	0	38590				450.5	0	8	71791	23933						
451.5	2319	0	0	773				451.5	117055	2539	0	39865				451.5	0	10	70698	23569						
452.5	2435	0	0	812				452.5	119827	2228	0	40685				452.5	0	13	69304	23106						
453.5	2567	0	0	856				453.5	121079	1950	0	41010				453.5	0	17	67625	22547						
454.5	2717	0	0	906				454.5	120750	1702	0	40817				454.5	0	21	65683	21901						
455.5	2888	0	0	963				455.5	118838	1482	0	40106				455.5	0	28	63500	21176						
456.5	3080	0	0	1027				456.5	115405	1286	0	38897				456.5	0	36	61106	20381						
457.5	3297	0	0	1099				457.5	110576	1113	0	37230				457.5	0	46	58529	19525						
458.5	3539	0	0	1180				458.5	104524	960	0	35161				458.5	0	60	55801	18620						
459.5	3811	0	0	1270				459.5	97467	827	0	32765				459.5	0	77	52953	17677						
460.5	4115	0	0	1372				460.5	89650	710	0	30120				460.5	0	100	50018	16706						
461.5	4454	0	0	1485				461.5	81333	607	0	27314				461.5	0	129	47028	15719						
462.5	4831	0	0	1610				462.5	72775	518	0	24431				462.5	0	166	44013	14726						
463.5	5250	0	0	1750				463.5	64219	441	0	21553				463.5	0	213	41003	13738						
464.5	5714	0	0	1905				464.5	55884	375	0	18753				464.5	0	273	38024	12766						
465.5	6228	0	0	2076				465.5	47956	317	0	16091				465.5	0	349	35103	11817						
466.5	6795	0	0	2265				466.5	40580	268	0	13616				466.5	0	446	32260	10902						
467.5	7421	0	0	2474				467.5	33858	225	0	11361				467.5	0	567	29515	10027						
468.5	8108	0	0	2703				468.5	27855	189	0	9348				468.5	0	718	26885	9201						
469.5	8861	0	0	2954				469.5	22595	159	0	7584				469.5	0	906	24381	8429						
470.5	9684	0	0	3228				470.5	18070	132	0	6068				470.5	0	1139	22015	7718						
471.5	10581	0	0	3527				471.5	14249	110	0	4786				471.5	0	1425	19793	7073						
472.5	11555	0	0	3852				472.5	11077	92	0	3723				472.5	0	1776	17720	6499						
473.5	12608	0	0	4203				473.5	8490	76	0	2855				473.5	0	2202	15797	6000						
474.5	13742	0	0	4581				474.5	6416	63	0	2160				474.5	0	2716	14025	5580						
475.5	14959	0	0	4986				475.5	4780	52	0	1611				475.5	0	3332	12401	5244						
476.5	16259	0	0	5420				476.5	3511	43	0	1184				476.5	0	4067	10921	4996						
477.5	17639	0	0	5880				477.5	2542	35	0	859				477.5	0	4935	9580	4838						

478.5	19098	0	0	6366				478.5	1815	29	0	615				478.5	0	5954	8370	4775		
479.5	20629	0	0	6876				479.5	1278	23	0	434				479.5	0	7141	7285	4809		
480.5	22228	0	0	7409				480.5	887	19	0	302				480.5	0	8513	6317	4943		
481.5	23884	0	0	7961				481.5	607	15	0	207				481.5	0	10085	5458	5181		
482.5	25588	0	0	8529				482.5	409	13	0	141				482.5	0	11872	4698	5523		
483.5	27326	0	0	9109				483.5	272	10	0	94				483.5	0	13887	4029	5972		
484.5	29083	0	0	9694				484.5	179	8	0	62				484.5	0	16137	3444	6527		
485.5	30842	0	0	10281				485.5	115	7	0	41				485.5	0	18628	2934	7187		
486.5	32583	0	0	10861				486.5	74	5	0	26				486.5	0	21362	2491	7951		
487.5	34285	0	0	11428				487.5	46	4	0	17				487.5	0	24332	2108	8813		
488.5	35925	0	0	11975				488.5	29	3	0	11				488.5	0	27528	1778	9769		
489.5	37481	0	0	12494				489.5	18	3	0	7				489.5	0	30933	1495	10809		
490.5	38927	0	0	12976				490.5	11	2	0	4				490.5	0	34523	1254	11926		
491.5	40239	0	0	13413				491.5	6	2	0	3				491.5	0	38267	1048	13105		
492.5	41393	0	0	13798				492.5	4	1	0	2				492.5	0	42129	874	14334		
493.5	42367	0	0	14122				493.5	2	1	0	1				493.5	0	46066	726	15597		
494.5	43140	0	0	14380				494.5	1	0	0	0				494.5	0	50029	602	16877		
495.5	43694	0	0	14565				495.5	0	0	0	0				495.5	0	53967	498	18155		
496.5	44015	0	0	14672				496.5	0	0	0	0				496.5	0	57825	411	19412		
497.5	44090	0	0	14697				497.5	0	0	0	0				497.5	0	61547	338	20628		
498.5	43914	0	0	14638				498.5	0	0	0	0				498.5	0	65077	277	21785		
499.5	43483	0	0	14494				499.5	0	0	0	0				499.5	0	68360	227	22862		
500.5	42801	0	0	14267				500.5	0	0	0	0				500.5	0	71346	186	23844		

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