*Electronic Supplementary Information (ESI)*

# Multi-line Lateral Flow immunoassay for Detection and Subtyping of Breast Cancer Derived Small Extracellular Vesicles

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## Experimental Sections:

### Chemicals and reagents

All chemicals were purchased from Sigma-Aldrich, including Sodium tetraborate decahydrate ($Na_2B_4O_7{\cdot}10H_2O$; pH9), Boric acid ($H_3BO_3$; pH9), Bovine serum albumin (BSA), Tween-20, Sucrose ($C_{12}H_{22}O_{11}$), phosphate buffered solution (PBS), 5% skim milk, chloroauric acid ($HAuCl_4$), Sodium citrate ($Na_3C_6H_5O_7$) and Milli-Q water.

All antibodies were purchased from Abcam, except where noted. Antibodies used in the assay include HER2 (3B5, # ab16901), EpCAM (323/A3, # ab85987), CD9 (MEM-61, # ab2215), CD81 (M38, # ab79559), CD63 (MEM-259; # ab8219) and Goat Anti-Mouse IgG (# a16092; purchased from Thermofisher scientific).

### Lateral Flow assay components

All lateral flow assay components were purchased from MDI Advanced Microdevices (Haryana, India). The LFA is composed of a membrane (200CNPH-N-SS60-L2-P25;75x260 mm) and absorbent pad (AP045;27x260 mm).

### Plasma Samples and ethical approval

Ethics approval was obtained from the Macquarie University Human Research Ethics Committees (approval no. 52024923058548) and all studies followed the National Statement on Ethical Conduct in Human Research (2007) guidelines in accordance with the National Health and Medical Research Council Act 1992. Informed consent was obtained from human subjects. All blood plasma samples were provided by St Vincent Hospital Biobank from a collaboration with general surgeon A/Prof Simon Tsao and were stored at -20 °C for use. Each sample was assigned a unique code either starting with 'BB' to indicate Biobank or 'H' to indicate Healthy.

### Synthesis of 50 nm spherical gold nanoparticles (AuNPs)

AuNPs were synthesized using the seeded growth method. Briefly, chloroauric acid ($HAuCl_4$; 0.5 mL; 25 mM) was added to the boiling milli-Q water (48 mL) under stirring conditions (650 rpm) for 10 min. To synthesize the gold seed, sodium citrate (1% w/v; 1.5 mL; 34 mM) was then added rapidly and refluxed for a further 30 min. The reaction was then cooled to room temperature before being used as the gold seeds for the growth of 50 nm AuNPs. All glassware was cleaned using aqua regia for the following steps. Milli-Q water (45.5 mL) was boiled under stirring conditions (650 rpm) before adding Tris-buffer (2 mL; 100 mM) and incubating for 5 min. The as prepared AuNP seeds (2 mL) was then added with extra chloroauric acid ($HAuCl_4$; 0.5 mL; 25 mM) successively. The reaction was refluxed for 30 min under continuous stirring before allowing to cool and performing characterisation.

**Passive bioconjugation**

Borate buffer (100 mM; 30 µL) was added to the AuNPs to change the solution pH to be 9 for passive bioconjugation. EpCAM antibodies (0.5 mg/mL; 3.3 µL) were then added and incubated under shaking conditions for 30 min (600 rpm; room temperature). The AuNPs@ EpCAM was then centrifuged (5500 rpm; 8.5 min; room temperature) and resuspended in a storage buffer (10% sucrose, 0.5% BSA and 10 mM borate buffer).

**Cell line-derived sEV Isolation**

Breast cancer cell lines MCF7 and SKBR3 were used for the proof-of-concept experiments and optimisation. The cell lines were obtained from ATCC. The cell culture and isolation of the sEVs was conducted by a laboratory technician. Briefly, cell culture was performed using aspirated culture medium (RPMI+10% FBS and 1/100 unit of Penicillin/Streptomycin) combined with PBS and trypsin (warmed to 37°C). The cells were washed with PBS (5 mL) before being combined with trypsin (1 mL). The cells were then incubated at 37°C for 5 minutes for the cells to detach from the flask. The cells were then centrifuged (1200 rpm) for 5 minutes before resuspending in media (5 mL) 31.

**Western Blot**

Immunoblotting analysis of MCF7 derived sEVs. The protein lysates from sEVs were mixed with 4×Laemmli loading buffer (Bio-rad) and heated at 95 °C for 10 minutes. The protein ladder (Precision Plus Protein Dual Color Standards, Bio-Rad) and the samples with approximately 5 µg of protein MCF7 derived sEVs) were loaded onto the mini-protean TGX gel (Bio-rad). The gel was run under the conditions of 80 V to settle the sample into the gel and 130 V until the loading buffer reached the bottom of the gel. The protein ladder and samples were then transferred to the polyvinylidene fluoride (PVDF) membrane (Bio-rad) by using a Trans-blot machine (Bio-Rad) for 10 minutes. The membrane was later blocked by using 5% skim milk dissolved in PBS + 0.1% Tween 20 (PBST) for 1 hour. After three rounds of washing, the membrane was incubated with either a 1 : 1000 dilution primary antibody including anti-CD9 (#ab2215), anti-CD81 (#ab79559, Abcam), anti-CD63 (#ab271286, Abcam), anti-ALIX (#ab275377, Abcam), anti-TSG101 (#ab83), anti-calnexin (#ab112995) and anti-EpCAM (#ab216136, Abcam) at 4 °C, overnight and shaken at RT for 1 hour in the following day. Next, the unbound primary antibodies were washed five times with PBST. Then the nitrocellulose membrane was incubated with 1: 10000 dilution goat anti mouse IgG horseradish peroxidase (HRP) for 2 hours at RT. After the washing, the membrane was soaked in the HRP substrate for 1 minute and exposed to the SYNGENE Gbox for recording the images.

**Instruments**

For the characterisation of the gold nanoparticles (AuNPs), Jasco V-760 spectrometer (Jasco Corporation, Japan), Malvern Zetasizer Nano ZSP (Malvern Panalytical Ltd., United Kingdom) and JEOL JSM 7100F Field Emission Scanning Electron Microscope (JEOL Ltd., Japan; TEM) were utilised. For UV-Vis, samples were diluted by ½ with milli-Q water before being placed in a UV-Vis cuvette (Cat# Z637092, Sigma Aldrich) for scanning at a wavelength of 400-700 nm. For dynamic light scattering (DLS) measurements, Zetasizer was used to determine the size and surface charge of the AuNPs. Each sample was run three times with a minimum of 11 runs, using a UV-Vis cuvette was used with a 633 nm laser at 25°C to determine the size. The surface charge was determined with ¼ diluted in milli-Q water using folded capillary cells (DTS1070; Malvern Panalytical). TEM was used to image the AuNPs and the sEVs. The instrument was used at 100 kV voltage and 60,000x magnification. For characterisation by TEM, a copper grid was prepared by dropping 10 µL onto the grid, allowing to process for 2 min before drying with filter paper. This was repeated three times to ensure adequate concentration on the grid. To prepare the sEV samples for TEM, 5 µL of the sample was placed on the grid and left for 3 minutes (min) before removing excess liquid using a filter paper. Uranyl acetate (5

µL; 2 wt. %) was subsequently added on the grid and left for 3 mins before being removed with filter paper. The grids were left to dry overnight. The LFIAs were constructed using a dispenser (KinBio XYZ dispenser HM3035, China) and Lateral flow assay guillotine (Automatic Strip Cutter ZQ2002, China). The LFIAs were cut to 2.5 mm and each line was dispensed at 0.6 µL/cm and 80 mm/s for both test (1 mg/mL) and control lines (1 mg/mL). For characterisation of the sEV samples, NanoFCM flow cytometry (Tokyo; Japan) was utilised. Each sample was run three times diluted 1 in 10 in filtered 0.1 mM PBS. The samples were run using a 488 nm continuous wave (CW) laser at a power of 6/50 mW with 1.0 Kpa pressure.

**Data analysis**

Image J analysis was conducted on the scanned images of the assays to find the overall intensity of the bands indicating EpCAM expression. This was done through the 'gels' feature and the overall intensity was taken as the sums of the area under the curves for each test line. The summed intensity of three replicates was then used for statistical analysis. The data was tested for Gaussian distribution using the Shapiro-Wilk test. The non-parametric Kruskal-Wallis test and Dunn multiple comparison test was then used to determine if the means were significantly different using the obtained p-values.

## Supplementary Results:

**Characterisation of AuNPs and MCF7 cell derived-EVs**

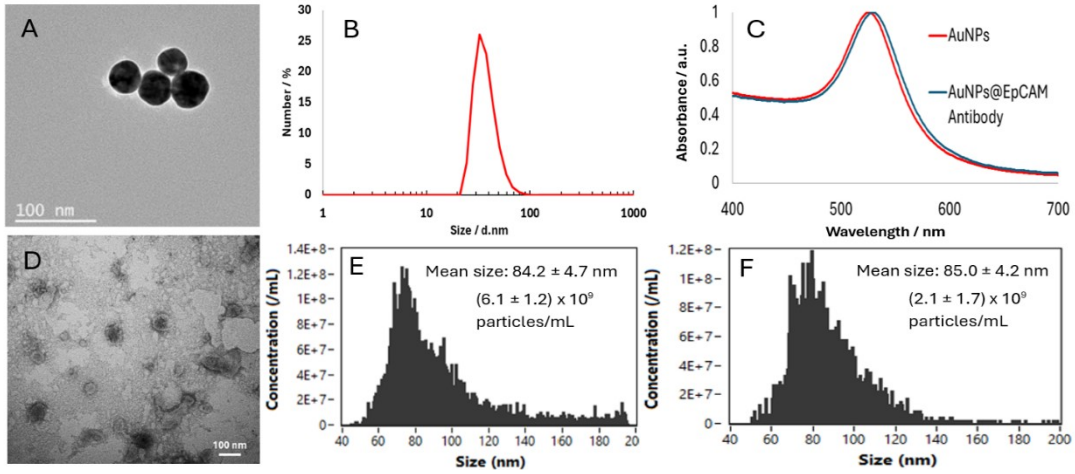


**Figure S1.** Characterisation of the AuNPs and MCF7 cell line-derived sEVs. **(A)** Transmission electron microscopy (TEM) image of AuNPs. **(B)** Size distribution of AuNPs using Dynamic light scattering (DLS). **(C)** Peak shift using ultra-violet visible spectroscopy (UV-Vis) demonstrating a red shift when AuNPs are conjugated with EpCAM antibodies. **(D)** TEM image of MCF7 cell-derived sEV. **(E)** Size and concentration of MCF7 cell-derived sEVs measured by Nano-Flow cytometry (NanoFCM). **(F)** Size and concentration of SKBR3 cell-derived sEVs by NanoFCM.

**Stability of sEVs in running buffer**

The running buffer contains harsh environment conditions for sEVs. To ensure they were sufficiently stable, sEVs incubated for two hours were run using NanoFCM to obtain the particle count. The particle counts were (5508 ± 206) particles in running buffer before incubation. After incubation with 1% Tween-20 for 2 hours, the particle counts were (4235 ± 327) particles. A decline (28%) in the particle counts indicates a minor loss of sEVs due to harsh buffer conditions. The particle count however does indicate a large portion of the sEVs are stable over the 2-hour incubation period. For running conditions with the LFIA, 1% Tween-20 buffer was found to be ideal. Thus, the sEVs were

confirmed to be sufficiently intact following a 2-hour incubation in the running buffer with 1% tween-20.

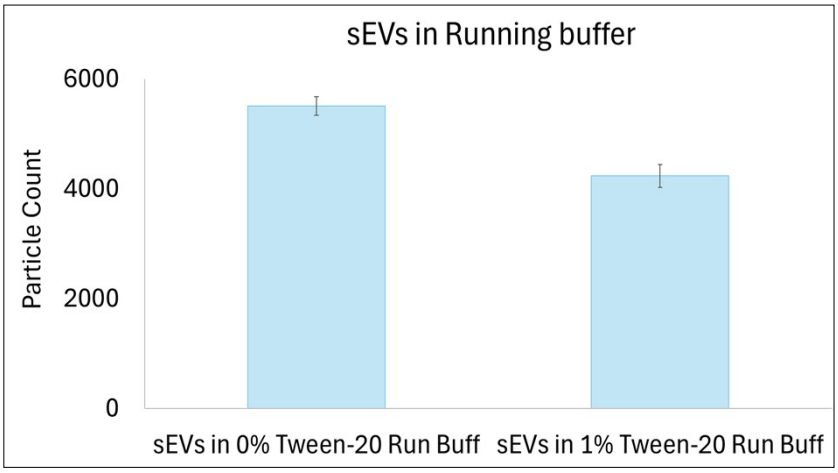


***Figure S2.*** Particle count obtained for sEVs in 0% and 1% tween-20 running buffer.

**Characterisation of tetraspanin expression in MCF7 cell- derived sEVs**

The tetraspanin (CD9, CD81 and CD63) expression for MCF7 cell line derived sEVs was obtained using NanoFCM. The unstained (***Figure S3A***) shows the background which is sufficiently low while the DiO staining (***Figure S3B***) indicates the entire lipid population in the sample. CD81 (***Figure S3C***) was found to be 0.9% while CD9 (***Figure S3D***) showed an expression of 6.2% and CD63 (***Figure S3E***) showed 2.2%.

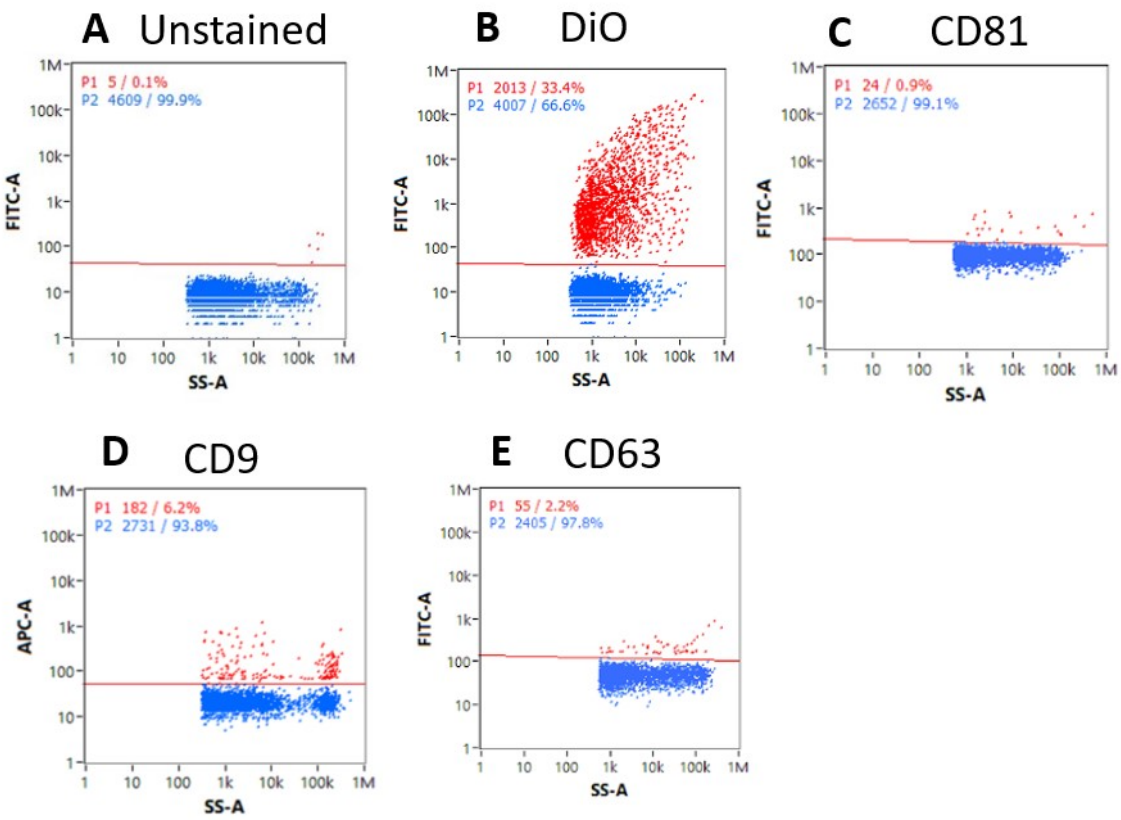

***Figure S3.*** Expression of tetraspanins on MCF7 cell-derived sEVs characterized by NanoFCM. (**A**) Unstained sEVs as control to show the background signal, (**B**) DiO staining of sEVs to show the entire population of lipid particles captured; The expression level of CD81 (**C**), CD9 (**D**) and CD63 (**E**) on MCF7-derived sEVs.

**Western Blot Validation of MCF7 Cell line-derived sEVs**

The biomarker expression for MCF7 cell line-derived sEVs were also validated by Western blot, as demonstrated in ***Figure S4***. The clear band of Alix (inner protein of sEVs) and the absence of mitochondria protein Calnexin indicated the presence of sEVs in the sample. The presence of sEVs surface proteins including CD9, CD81, and CD63, as well as cancer biomarker EpCAM corresponds the results obtained by NanoFCM which indicate the presence of each of the target biomarkers used in the LFIA.

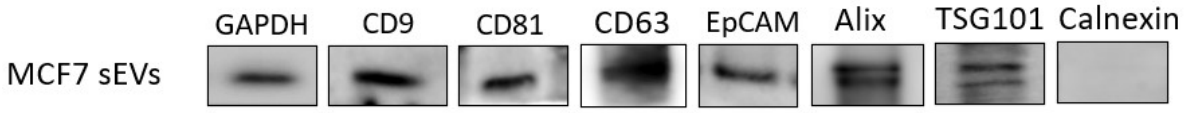


***Figure S4.*** Western Blot of GAPDH, CD9, CD81, EpCAM, Alix, CD63 and Calnexin from MCF7 cell line-derived sEVs.

**Characterisation of patient plasma sEVs by NanoFCM**

Two healthy (H0330 and H9243) and two breast cancer patients (BB0573 and BB0695) were characterised by NanoFCM to show the population distribution and concentration of each sample (***Figure S5***).

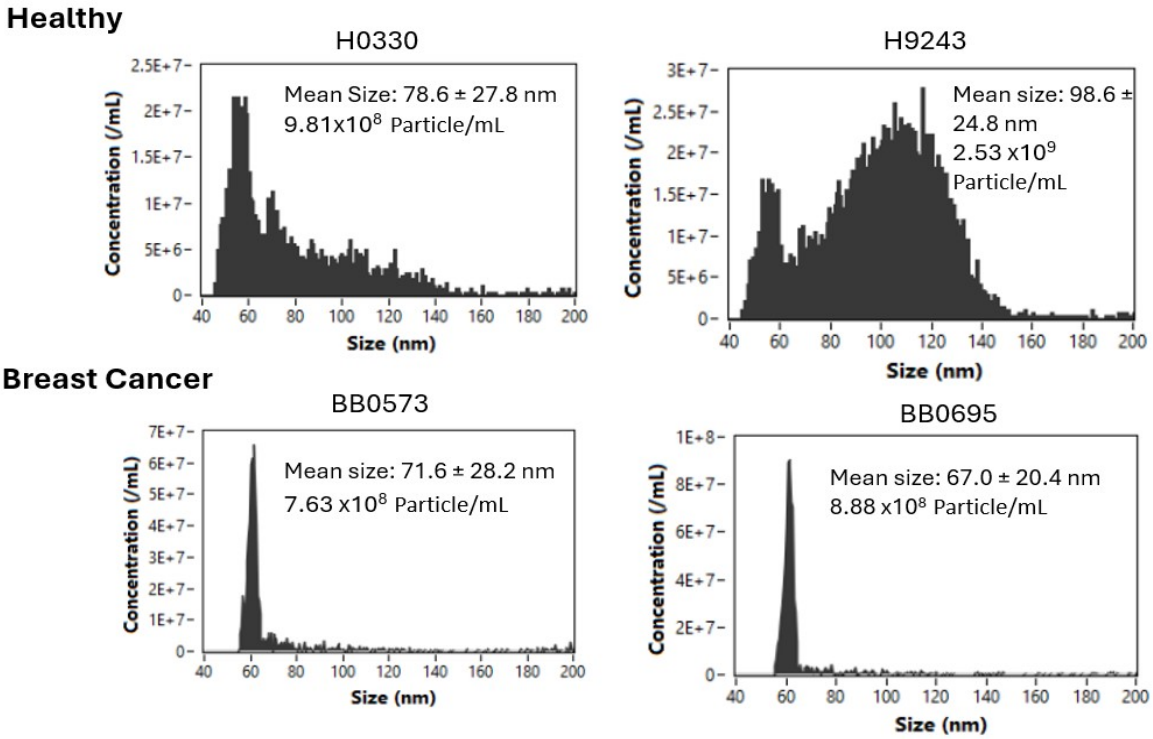


***Figure S5.*** Size distributions (by NanoFCM) for four sEV samples isolated from plasma including breast cancer patients (BB0573, BB0695) and healthy (H0330, H9243).

**Patient plasma samples**

Patient plasma samples were run directly with the LFIA. As a proof-of-concept demonstration, five breast cancer and five healthy donor samples were tested. The breast cancer patients ranged from stage 0 to stage IV as indicated in ***Figure S6***. One healthy patient showed faint false positive bands and was excluded as an outlier. The high background colour in these human plasma samples is attributed to the 'sticky' nature of plasma, hence it was found to cause excess nanoparticles to become stuck in the membrane. However, the bands intensity is clear, indicating either low or high amount of cancer sEVs detected. In the earlier stages (0, I and II), the low band intensity is attributed to the low number of cancer sEVs while in the later stages (III and IV), the bands are darker, indicating a high amount of cancer sEVs. The breast cancer patients were found to show more visible positive lines at advanced stages as compared to the faintly visible lines at early stages. In future studies, we will further optimize the assay to enhance the contrast of these lines.

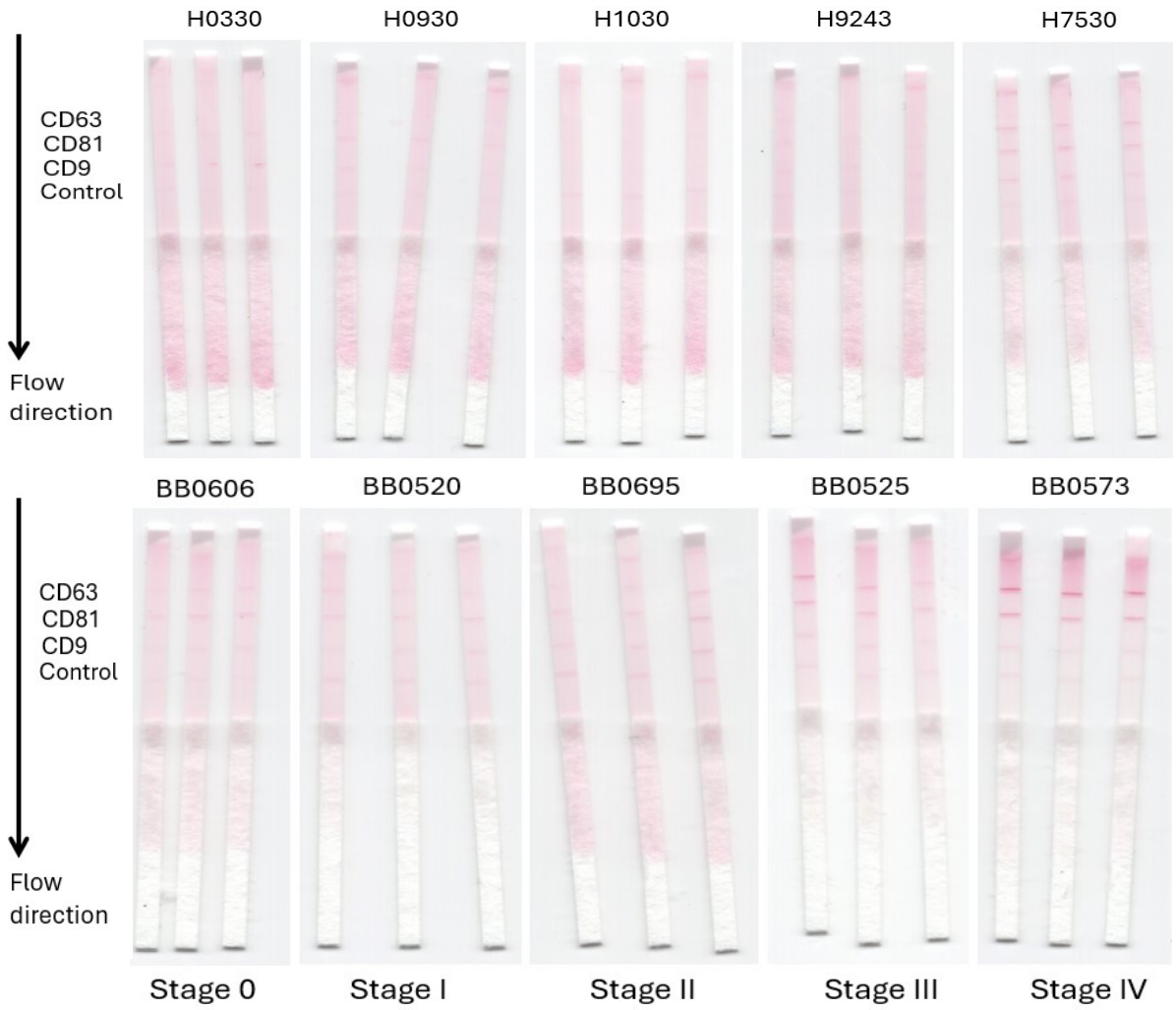


***Figure S6.*** Healthy (H0330; H0930; H1030; H9243; H7530) and breast cancer patient samples (BB0606; BB0520; BB0695; BB0525; BB0573) from stages (0-IV) samples run using plasma directly.