Characterization of HIV proteins secreted in Extracellular Vesicles (EVs) during infection for use in at-home diagnostics.

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INTRODUCTION

Early diagnosis of HIV is key to obtaining access to treatment and reducing transmission. The OraQuick® HIV Self-Test is the only FDA-approved self test. It requires detection of antibodies and antigens and takes up to 90 days post-exposure to achieve 91.7% Sensitivity. Laboratory-based antigen/antibody tests detect the p24 capsid protein, a marker of early infection, and antibodies against the viral envelope protein. To reduce the time to diagnosis in an at-home testing format, we investigated extracellular particles (EPs) for HIV biomarkers. Using HIV+ cell lines and plasma from volunteers, HIV biomarkers were identified in tetraspanin containing extracellular vesicles (EVs), characterized for abundance and tetraspanin content, and developed for inclusion on a novel EV-capture and cargo detection platform in a lateral flow assay. This format would allow private, convenient, and low-cost screening for HIV from the home or mobile clinics that may reduce the test window period significantly.

METHODS

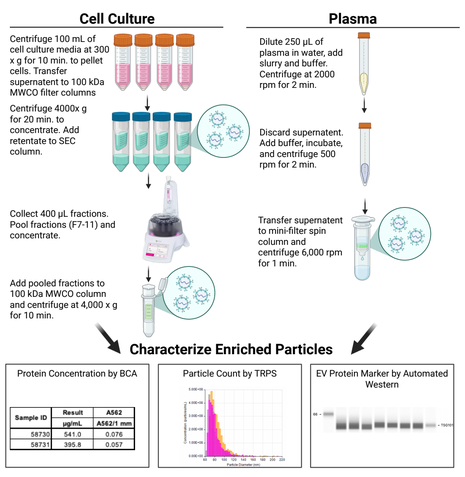
These experiments were performed following the MISEV2023 guidelines.

Cell culture. H9 (HTB-176) and U937 (CRL-1593.2) cells were cultured in RPMI 1640 supplemented with 10% FBS and Gentamicin. H9MN FI cells from Dr. H. Imamichi (NIAID). U1 (ARP-165) cells were obtained through the NIH HIV Reagent Program, contributed by Dr. Thomas Folks. Prior to enrichment cells were incubated in FBS-free DMEM in T-75 flasks until cellular confluence reached roughly 80%, approximately 48 hours.

Plasma. Plasma samples were remnants from the HVTN505 clinical trial (NCT00865566). Plasma selected from 10 HIV- volunteers from the placebo group. The 20 HIV+ samples were selected from volunteers 12 weeks after diagnosis. Additional "Normal" human plasma used as controls for Western blots or ELISA were acquired from Precision for Medicine (Frederick, MD) as a confirmed HIV negative sample. A seroconversion panel was acquired from SeraCare (Milford, MA). This panel was collected from a single person over 25 days in 2015 during the development of an HIV infection with reported values from FDA-approved HIV diagnostic tests.

EV enrichment. Cell culture medium was enriched using size exclusion chromotography (SEC) columns and a fraction collector following the manufacturer's instructions. After elution, fractions were pooled and concentrated using 100 kDa MWCO filter columns. For plasma, a resin-based method was used (Norgen Biotek). A total of 250 µL of plasma was enriched following the manufacturer's instructions resulting in approximately 200 µL of enriched EVs. See figure below for cell culture vs. plasma.

EV characterization. Protein concentrations were determined by BCA.Tunable resistive pulse sensing (TRPS) was performed using an Izon Exoid (Medford, MA)for size and particle concentration.



Workflow for EV enrichment and characterization from cell culture media (left) or plasma (right).

Transmission Electron Microscopy 5 µl of EV suspension in PBS added on bare metal electron microscopy for 1 min. The grid blotted dry with filter paper and 10 µL of distilled water added for 1 minute. The water droplet was removed by filter paper and 5 uL of Nano-W™ Methylamine Tungstate (Nanoprobes) was added, the grid was air dried for 5 minutes. Grids were then visualized using a transmission electron microscope (FEI CM120, FEI Company Hill).

Western Analysis. Enriched EVs were separated on a SimpleWestern Abby using a 12-230 kDa Separation Module (Bio Techne). Primary antibodies were detected using anti-rabbit or anti-mouse detection modules. Area under the curve (AUC) band was determined using the Compass software (ver. 6.1.0).

Lateral flow strip testing. EVs were diluted to the desired concentration in 50 µL PBS and added to the sample pad of the lateral flow strip. The sample was chased by adding 125 µL of lysis buffer to the sample pad and incubating for 25 minutes prior to visualization.

Antibodies and antigens. Mouse anti-p24 (24-3, Santa Cruz), rabbit anti-Nef R0026, Intuitive Biosciences), and rabbit anti-TSG101 (JJ0900, Abcam) were used for Western analysis. Mouse anti-p24 and recombinant p24 for lateral flow development (Meridian Biosciences). Anti-CD9, anti-CD63, and anti-CD81 antibodies were used in lateral flow development (Abnova).

ONI Microscopy. EVs were processed using an ONI EV profiler2 kit using phosphatidylserine (PS) capture and fluorophore conjugated antibodies as noted in figures.

RESULTS

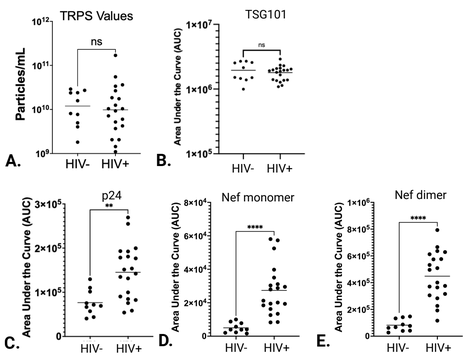
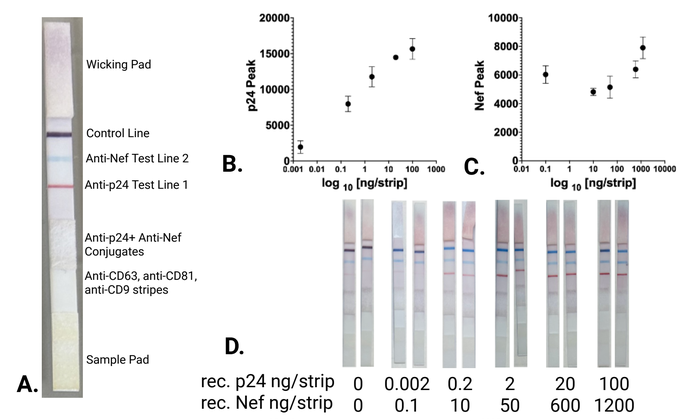


Figure 1. Detection of EV markers and HIV proteins in EVs enriched from plasma. (A) TRPS analysis demonstrating equivalent particle concentration between HIV- and HIV+ enriched EVs. **(B)** Western analysis demonstrating equivalent levels of TSG101 in 200 µg HIV- and HIV+ enriched EVs quantified by Area Under the Curve (AUC). **(C)** Western analysis showing the detection of p24 in EVs enriched from HIV+ individuals. **(D)** Western analysis showing the detection of Nef in EVs enriched from HIV+ individuals. Values for the AUC of the Nef monomer at approximately 33kDa shown. **(E)** Western analysis showing the detection of Nef in EVs enriched from HIV + individuals. Values for the AUC of the Nef dimer at approximately 36kDa shown. ** indicates a p-value <0.01, **** indicates a p-value<0.0001. "ns" indicates a p-value that is "not significant" or greater than 0.1.



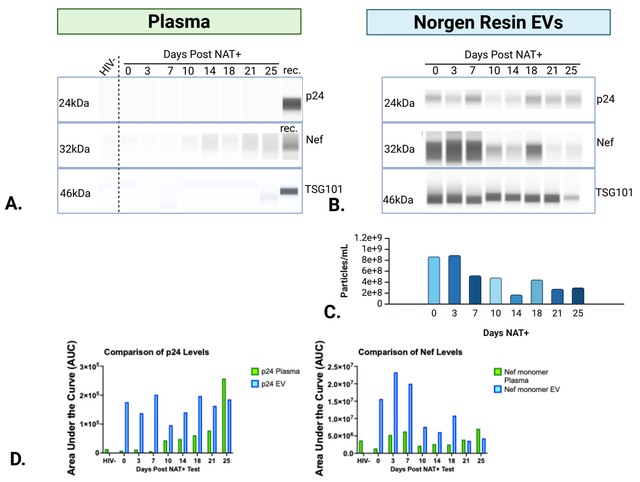


Figure 2. Detection of HIV proteins in plasma and enriched EVs from a person living with HIV over the course of infection. (A) Western analysis of 4 µg of plasma for p24, Nef, and TSG101. An HIV- "normal" human plasma sample is shown in the left lane for reference. Days after first nucleic acid test (NAT) positive result corresponding to that sample is shown above each lane. **(B)** Western analysis of approximately 4 µg of resin enriched EVs for p24, Nef, and TSG101. Molecular weights determined from ladder run on same cartridge. **(C)** Mean particles per mL measured in triplicate by TRPS for each sample. **(D)** Comparison of quantified area under the curve (AUC) values for p24 and Nef between plasma and EVs from the seroconversion panel., demonstrating higher signal in the EVs.

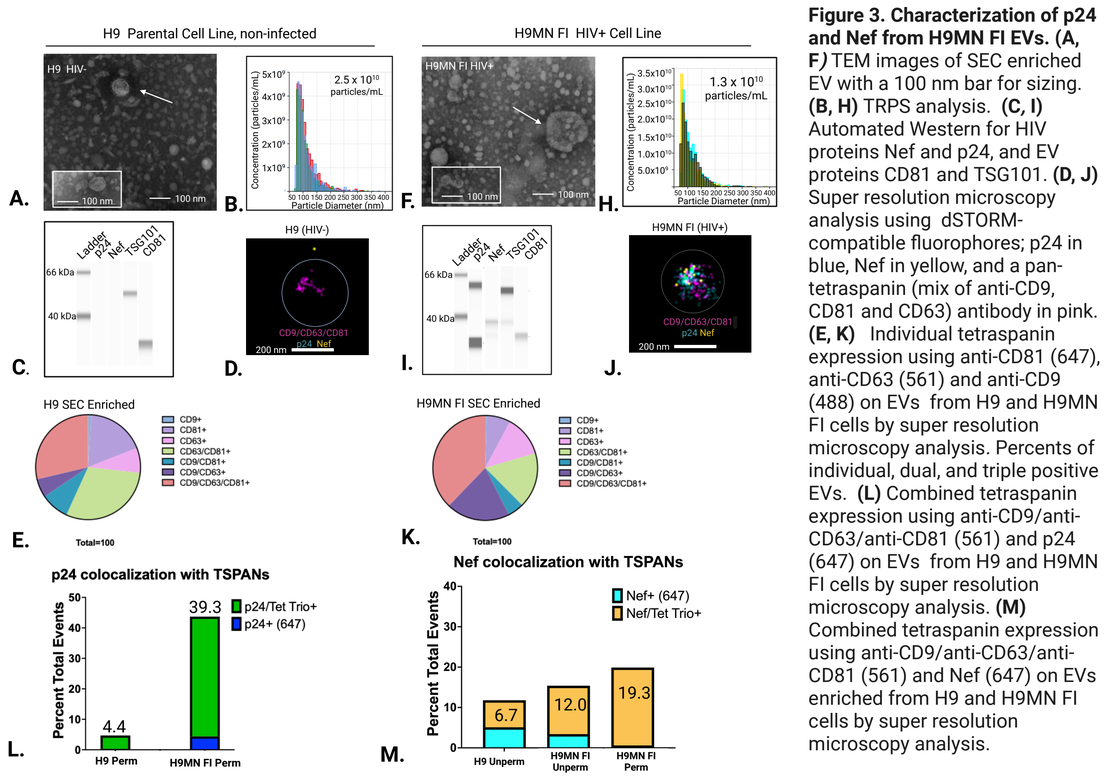


Figure 3. Characterization of p24 and Nef from H9MN FI EVs. (A, F) TEM images of SEC enriched EV with a 100 nm bar for sizing. **(B, H)** TRPS analysis. **(C, I)** Automated Western for HIV proteins Nef and p24, and EV proteins CD81 and TSG101. **(D, J)** Super resolution microscopy analysis using dSTORM-compatible fluorophores; p24 in blue, Nef in yellow, and a pan-tetraspanin (mix of anti-CD9, CD81 and CD63) antibody in pink. **(E, K)** Individual tetraspanin expression using anti-CD81 (647), anti-CD63 (561) and anti-CD9 (488) on EVs from H9 and H9MN FI cells by super resolution microscopy analysis. Percents of individual, dual, and triple positive EVs. **(L)** Combined tetraspanin expression using anti-CD9/anti-CD63/anti-CD81 (561) and p24 (647) on EVs from H9 and H9MN FI cells by super resolution microscopy analysis. **(M)** Combined tetraspanin expression using anti-CD9/anti-CD63/anti-CD81 (561) and Nef (647) on EVs enriched from H9 and H9MN FI cells by super resolution microscopy analysis.

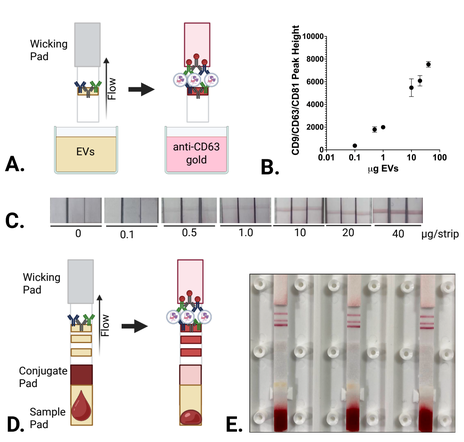


Figure 4. Purified EVs and EVs from whole blood can be detected in a lateral flow format. (A) Schematic of EV detection on lateral flow dip stick. Anti-tetraspanin antibodies striped onto nitrocellulose with a wicking pad placed into a well with diluted EVs. EVs flow up the strip and bind to the capture line. The strip is transferred to a well with an anti-CD63 gold conjugate to detect EVs. **(B)** Triplicate measurements of peak heights for each dilution of A375 EVs are graphed with standard deviations. **(C)** Images of triplicate strips of purified EVs detected with an ani-CD9 gold conjugate to determine the visual LOD of detection. **(D)** Schematic of 3 line mixed anti-tetraspanin dip stick test for EV capture zone. **(E)** Test strips run with 20 µL whole blood, chased with 80 µL buffer. Liquid flows up the strip, allowing release of the anti-CD9/CD63/CD81 gold conjugate to mix with EVs as they are captured as they flow past the 3 mixed anti-CD9/CD63/CD81capture lines.

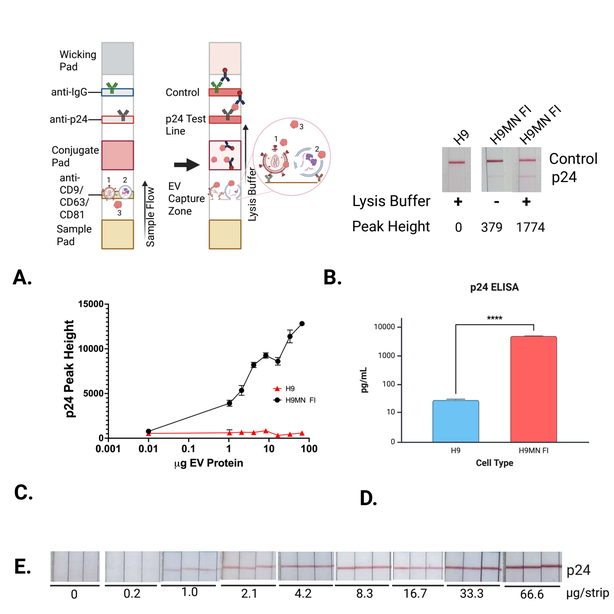


Figure 5. Detection of p24 from HIV-EVs by lateral flow. (A) two-stage lateral flow design that includes an EV capture zone and a test zone. **(B)** p24 detection from EVs with and without Lysis Buffer.**(C)** Triplicate measurements of p24 test line from enriched EVs from H9 (HIV-) or H9MN FI (HIV+) cells spiked into human plasma. **(D)** Mean p24 values from 3 µg of H9 or H9MN FI EVs measured by p24 sandwich ELISA after treatment with Lysis Buffer. **(E)** p24 test line from the triplicate lateral flow strips.

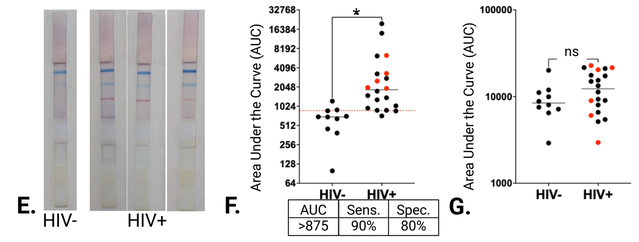


Figure 6. Plasma Samples Tested on Prototype Lateral Flow Tests. (A) Two-stage lateral flow prototype with an EV capture zone and a test zone that includes both the p24 and the Nef test line. **(B)** Limit of detection analysis of mixed recomb. p24+Nef in buffer. Measurement of the intensity of the p24 test line. **(C)** Measurement of the intensity of the Nef test line. **(D)** Representative images of the mixed recombinant proteins used to calculate results for B and C. **(E)** Representative images of HIV- and HIV+ plasma sample. **(F)** AUC of the p24 test line from 10 HIV- and 20 HIV+ plasma samples. The red dotted line represents the cut-off value determined by ROC analysis, at 90% Sensitivity and 80% Specificity. * represents statistical significance at a p-value less than 0.01. **(G)** Results for the Nef test line. Plasma samples tested were remnant samples collected during the HVTN505 vaccine trial (2010-2013). HIV+ specimen graphed in black were under ARV treatment. Those in red indicate no ARV treatment.

CONCLUSIONS

Previous work in our lab demonstrated immunocapture EVs by lateral flow, expanding on work done by Oliveira-Rodríguez et. al, 2011. Here, a two-stage lateral flow with an EV capture for concentration of EVs upstream of a standard antigen detection was designed for a highly sensitive antigen only HIV at-home test.

- **The HIV proteins p24 and Nef are found in EVs enriched from HIV+ plasma.** Figure 1.
- **In a seroconversion panel, p24 and Nef were detected in EVs earlier than in plasma.** Figure 2. By Western analysis, Nef and p24 were detected at earlier timepoints in enriched EVs at at higher signal intensities.
- **EVs that contain HIV proteins have all 3 tetraspanins.** Figure 3. EVs from H9MN FI cells were characterized to be used as controls for the LFA work.
- **EVs can be captured on a mixed tetraspanin capture line with an LOD <0.5 µg protein, and EVs can be captured from whole blood.** Figure 4.
- **p24 can be detected as cargo released from captured HIV-EVs on the two-stage lateral flow test with an LOD <1.0 µg of protein.** Figure 5.
- **A prototype EV-HIV LFA test can detect HIV+ plasma with 90% Sensitivity in a small-scale trial.** Figure 6. Despite high background making the Nef test line useless, the p24 signal alone resulted in a 90% Se and 80% Sp in the prototype testing.

Summary and Future Work. The EV-HIV immunocapture lateral flow assay can detect EV-associated p24 from both cell culture EVs and plasma from HIV+ volunteers. Key areas to improve the prototype tests will include:

- Optimization of anti-Nef monoclonals. Recombinant antibodies are currently under development.
- Optimization of the sample pad for use of whole blood as the input sample.
- Improving sensitivity with integration of the Nef test line.
- Maximizing capture in the EV Capture Zone from blood.
- Design of cartridge to accept maximum blood sample.
- Large scale testing with volunteers to determine usability, sensitivity, specificity with blood sample.

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