Detection of HIV Proteins in Extracellular Particles by Immunocapture Lateral Flow Method

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INTRODUCTION

Human Immunodeficiency Virus (HIV) is a major source of human morbidity and mortality around the world. HIV proteins have been shown to be packaged in Extracellular Vesicles (EVs) produced from virally infected cells. By capturing Extracellular Vesicles on a lateral flow strip we hope to enrich for HIV proteins, allowing for increased sensitivity of detection of those HIV proteins.

Vaccine Induced Seropositivity (VISP) is a factor that complicates HIV diagnostics as patients who have been vaccinated with clinical trial vaccines for HIV may present as HIV+ by antibody based diagnostics.

HIV capsid protein p24 is a protein detected early during HIV infection. It is a common protein target for current generation HIV Ag/Ab diagnostics.

The goal of this project is to use enriched EPs to detect viral proteins from patient samples on a lateral flow strip. With the long term goal to develop an HIV protein-centric diagnostic tool.

METHODS

These experiments were performed following the MISEV2018 guidelines.

Cell culture. Cells were maintained at 37°C in 5% CO_2. Complete media is RPMI supplemented with 10% FBS, 10 mM HEPES, 2 mM glutamine and 10 µg/mL gentamicin. EV free media (11-875-119, Fisher Scientific) used 10% EV-depleted FBS (EXO-FBS-250A-1, System Biosciences) in place of standard FBS.

EV purification. Cell culture and human plasma EVs were purified per the manufacturer's instructions. The sample was clarified by centrifugation followed by incubation on Norgen resin and collection by centrifugation.

EV characterization. EV protein concentrations were characterized by BCA according to the manufacturer's instructions. Nanoparticle Tracking Analysis was done using a Nanosight NS300 at the Wisconsin Center for NanoBioSystems.

Western Analysis. Abby Western analysis was using 12-230 kDa Separation Module (SM-W004, Bio Techne) and either anti-rabbit or anti-mouse detection modules (DM-001 or DM-002, Bio Techne).

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 pAb, HIV Reagent Network), rabbit anti-Tat was used for western analysis (HXB2, HIV Reagent Network) and rabbit anti-TSG101 (JJ0900, Abcam). Mouse **a**nti-p24 antibodies were used for lateral flow development (Meridian Biosciences). Recombinant p24 was used for western analysis and lateral flow development from Abcam. Anti-CD9, anti-CD63, and anti-CD81 antibodies were used in lateral flow development (Immunostep).

ONI Imaging. EVs were processed using an ONI EV profiler kit. The anti-CD81 antibody was removed and replaced with with an Alexa-fluor 647 conjugated anti-p24 antibody (Meridian, C01655M).

RESULTS

A.

EV Source	Mean size (nm)	Mode size (nm)	Total count (particles/mL)	[Protein] (µg/mL)
H9	168.45	102.55	8.77E+08	1088
H9MN FI	164.05	105.5	1.33E+09	1205

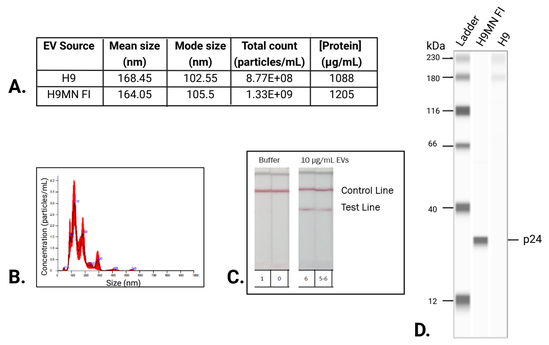


Figure 1. Characterization of EVs from cell lines. A. Mean size, mode size, particles/mL and protein concentration in EVs H9 and H9MN FI cell lines. B. NTA analysis of EVs purified from H9 cells. C. Detection of 10 µg EVs standard on EV capture zone using anti-CD9 gold. D. Western analyzer of 200 µg purified EVs probed for p24.

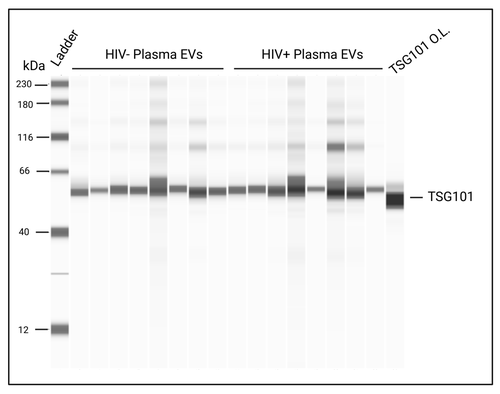


Figure 2. Western analysis of TSG101 levels in EVs enriched from HIV- and HIV+ plasma. An overexpression lysate was run as a positive control. TSG101 is expected to run at approximately 50 kDa.

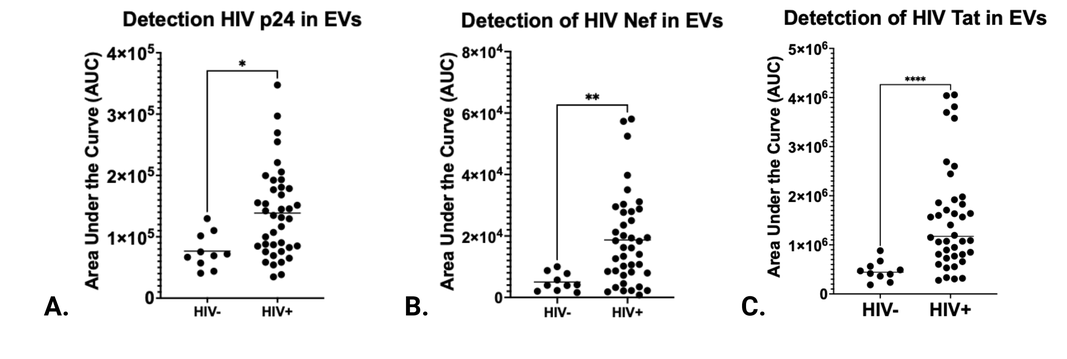


Figure 3. Comparison of HIV protein levels in enriched EVs. A. AUC for p24 from HIV- or HIV + enriched EVs compared by a student's t-test. B. AUC for Nef compared by student's t-test. C. AUC for Tat compared by student's t-test. A total of 10 HIV- samples and 40 HIV+ samples were tested. *p-val.<0.1, **p-val<0.01, ****p-val<0.0001

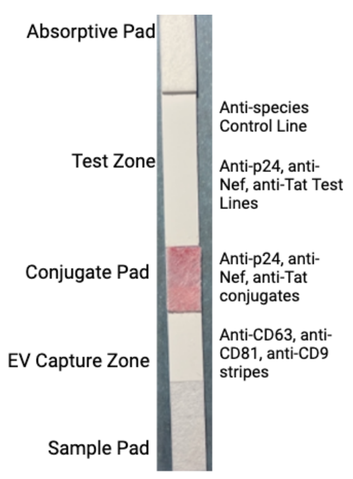


Figure 4. Construction of two-phase lateral flow strip. The EV Capture Zone is striped with anti-tetraspanin antibodies. The Test Zone is striped with antibodies to capture HIV proteins.

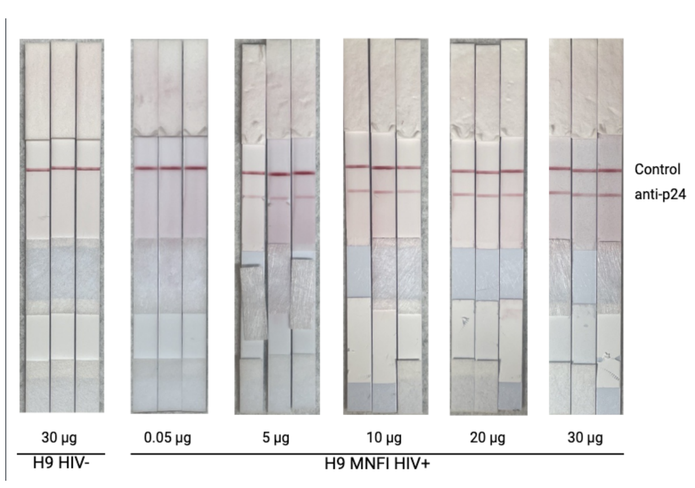
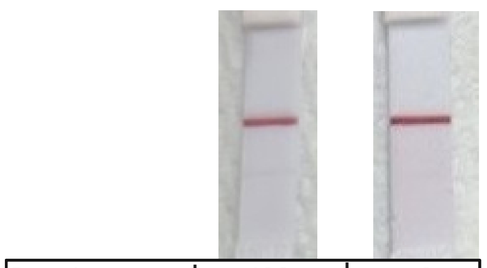


Figure 5. Demonstration of p24 detection in EVs from cell lines. Detection of p24 from 5 µg of purified EVs from H9 MNFI cells. The H9 MNFI cells were isolated from an individual on cART and produce viral proteins. The H9 cells are HIV- controls. EVs were purified from both cell lines and compared for detection of p24 on the two-phase lateral flow test. Purified EVs were added to the sample pad and chased with 50 µL of lysis buffer to release HIV cargo proteins. Unpublished data performed under NIH contract 75N93023C00007.



Densitometry score (p24)	430	0
EV source	HIV+ Plasma	HIV- Plasma

Figure 6. Detection of p24 from 20 µg of purified plasma EVs from HIV- or HIV+ samples. Tested using an EV capture lateral flow assay. EVs are captured by anti-tetraspanin antibodies. Lysed to release proteins and detected by anti-p24 Intense Red Conjugate.

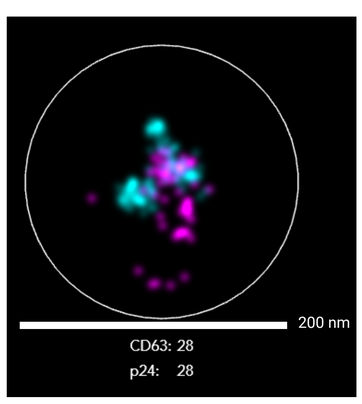


Figure 7. Colocalization of p24 (magenta) and CD63 (teal) in H9 MNFI purified EVs. EVs fixed, then stained and imaged on an ONI Nanoimager.

DISCUSSION

Previous work in our lab has demonstrated the ability to immunocapture EVs in a lateral flow based method (Fig 1C), expanding on work done by Oliveira-Rodríguez et. al, 2011. Building on this idea, we designed an EV capture zone for a two-phase lateral flow strip that could detect EV cargo by lysis of captured EVs and detection of released cargo downstream in the test lines.

The HIV protein p24 is found in EVs purified from H9 MNFI cells and not in H9 cells.

EVs were purified from H9 MNFI and the control parental line H9T and characterized by NTA for size and number, BCA for protein concentration and automated Western for presence of p24. As shown in Figure 1, the protein concentration, EV size, and number were comparable between the two cell types. The H9MN FI EVs show the presence of p24 by Western, while the H9T cell EVs do not. Because cell lines expressing viral proteins are easier to use than human samples for scale-up of EVs, H9 MNFI EVs were used for development of the EV-HIV prototype strips.

Several HIV proteins are found in EVs. Detection of HIV proteins was performed using an Abby automated western analysis sytem.. Equivalent EV material was used for western analysis as shown by TSG101 level in Figure 2. In Figure 3, p24, Nef, and Tat were detected at higher levels in EVs purified from the plasma of HIV-infected subjects over normal plasma. Due to lack of availability of antibody pairs for Nef and Tat, EV-HIV prototype strips were progressed with the well-characterized antibody pair for p24 only.

Lateral flow strips were developed with an EV capture zone and a HIV test zone as shown in Figure 4. These prototypes were used to perform screening of purified EVs from H9MN FI cells. **We demonstrated that the EV-HIV immunocapture lateral flow assay can detect EV-associated p24.** Figure 5 shows strip results after testing increasing amounts of EVs enriched from the H9MN FI cells. **EV-associated p24 can be detected in as little as 5 µg of purified H9 MNFI EVs. When testing purified EVs or plasma from HIV+ individuals, p24 is detected on the EV-HIV prototype strips (Fig.6).**

To further examine the location of the HIV proteins associated with EVs, super resolution microscopy was performed. While this work is ongoing, preliminary data in Figure 7 shows colocalization of p24 and CD63 in vesicles smaller than 200 nm.

CONCLUSIONS

- EVs can be captured on the anti-tetraspanin capture lines by lateral flow.
- HIV proteins p24, Nef, and Tat are detectable in EVs from HIV+ plasma.
- HIV-1 p24 is detected in lysed H9 MNFI EVs with little background in negative cell line EVs.

Next steps:

- Add Nef and Tat test lines to EV-HIV lateral flow strips.
- Test EVs purified from large samples set of HIV- and HIV+ plasma samples.

ACKNOWLEDGEMENTS

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