Enabling improved diagnostic assay sensitivity for Ebola virus and other pathogens

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ABSTRACT

Sample preparation and stabilization are critically important to downstream diagnostic assay sensitivity, as they directly impact the abundance and detection of analytes. Moreover, the Warfighter requires solutions for diagnostic sample processing that are simple, fast, and robust when operating in austere environments. Nanotraps® are hydrogel nanoparticles functionalized with chemical affinity baits that can capture low abundance proteins, peptides, metabolites, nucleic acids, small molecules, and whole virions, while also reducing high abundance interfering substances. Previously published studies have shown that Nanotraps can capture both virions and proteins from influenza virus, Rift Valley fever virus, HIV, and Venezuelan equine encephalitis virus (VEEV). Here, we applied Nanotrap® technology to Ebola virus (EBOV) diagnostic applications and found that Nanotrap® sample processing increased assay sensitivity for EBOV-specific nucleic acid tests and protein immunoassays in spiked human clinical matrices, and in matrices collected from nonhuman primate models of EBOV infection.

BACKGROUND

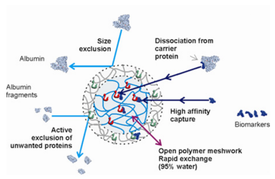
Challenges for diagnostic testing:

- Extremely low concentration of high value biomarkers present in body fluids
- Overwhelming presence of interfering substances
- Propensity for degradation of high value pathogen and host biomarkers in the absence of cold chain

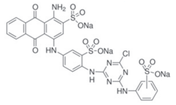
There is a pressing need for front-end sample processing approaches that can concentrate, enrich, and preserve diagnostic targets. Meeting this need will result in more sensitive and accurate diagnostics and may enable utilization of minimally invasive sample collection modalities.

Nanotrap® Technology:

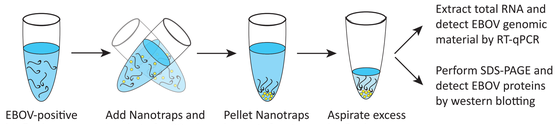
- Hydrogel microspheres consisting of crosslinked *N*-isopropylacrylamide (NIPAm) copolymers that are functionalized with a variety of chemical affinity baits
- Capture proteins, peptides, metabolites, nucleic acids, small molecules, and whole virions
- Compatible with complex biological matrices such as blood, serum, plasma, urine, saliva and nasopharyngeal fluids
- May be integrated into many existing diagnostic and analytical platform workflows, including nucleic acid amplification tests, immunoassays, immunochromatographic assays, protein microarrays, proteomics and mass spectrometry
- Recently published data indicate Nanotraps® can capture Rift Valley fever virus, human immunodeficiency virus, Venezuelan equine encephalitis virus, influenza A virus, adenovirus, and human coronavirus



Cibacron Blue Affinity Bait



Nanotrap® Sample Processing Workflow:



Potential Point-of-Care Integrated Nanotrap Sample Processing Workflows:

Oral fluid collection tubes with integrated Nanotrap® particles. The particles rapidly sequester, protect and concentrate.

Biospecimen collection swabs are placed into an extraction buffer containing Nanotrap® particles. The particles rapidly sequester, protect and concentrate.

Nanotrap®-functionalized urine cups equipped with magnetic particles enable the rapid collection, concentration, and processing of analytes from large sample volumes.

Nanotrap®-functionalized dried matrix collection substrates enable improving multi-analyte sample collection and storage.

Nanotrap®-functionalized tubes provide fractionation, concentration and preservation of target analytes present in blood samples for improved sample transport, analysis and storage.

OBJECTIVES

1. **To determine if Nanotraps® can concentrate EBOV virions and antigens from simulated human clinical matrices**
2. **To demonstrate improved sensitivity by RT-qPCR, NGS, and immunoassays**
3. **To verify Nanotrap® performance using samples collected from EBOV-infected nonhuman primates**

RESULTS

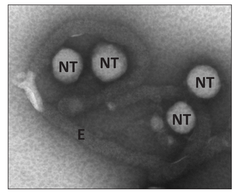


Figure 1. Physical interaction between EBOV and Nanotraps®. EBOV/Mayinga culture supernatant ($1.9x10^7$ PFU/ml) was combined with 100 µl of Nanotraps® and then incubated for 30 min at room temperature. Afterwards, Nanotraps® were pelleted by centrifugation, and then resuspended in 4% glutaraldehyde fixative buffer for approximately one week. Samples then were adsorbed to Formvar/carbon coated grids, and were negative stained with 1% phosphotungstic acid for 10 sec. Samples were evaluated on a JEOL 1011 transmission electron microscope at 80kV and digital images were acquired using an AMT camera system. NT, Nanotrap® particles; E, EBOV virion.

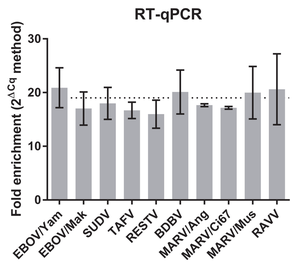


Figure 2. Enrichment of diverse filoviruses from buffer using Nanotraps®. Each of the viruses indicated was diluted to a concentration of $1x10^4$ PFU/ml in phosphate buffered saline (PBS) and then 1.9 ml of each was combined with 100 µl of prewashed Nanotrap® slurry while rotating the samples for 30 min. Nanotraps® were recovered by centrifugation, resuspended in 100 µl water and 300 µl Trizol LS, and then RNA was extracted using an EZ1 Advanced XL sample processor (Qiagen). The eluted RNA was analyzed on a Roche LightCycler 480 with real-time reverse-transcriptase PCR (RT-qPCR) assays targeting each virus. Fold enrichment relative to samples processed without Nanotraps® was determined using the $2^{\Delta Cq}$ method. A dotted line indicates the maximum theoretical enrichment (19-fold). Error bars indicated standard errors of the means. EBOV, Ebola virus; SUDV, Sudan virus; TAFV, Tai Forest virus; RESTV, Reston virus; BDBV, Bundibugyo virus; MARV, Marburg virus; RAVV, Ravn virus.

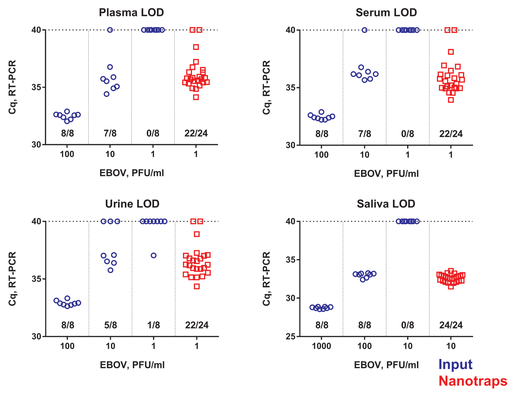


Figure 3. Improved EZ1 RT-qPCR assay sensitivity following Nanotrap® processing of EBOV-spiked human matrices. Pooled human plasma, serum, urine, and saliva from healthy donors were diluted with two equal volumes of PBS, and then spiked with EBOV/Kikwit to the indicated concentrations. Samples were then processed as described in Figure 2 with Nanotraps® (red squares) and then compared to unprocessed input dilutions (blue circles) using an RT-qPCR assay equivalent to the FDA EUA-approved Ebola EZ1 diagnostic assay currently being fielded by the US Army. Eight independent replicate samples were analyzed for each input dilution, and twenty-four independent samples were processed with Nanotraps. Samples with a Cq value less than 40 (dotted line) were considered positive. The number of positive samples/total samples tested is indicated for each condition.

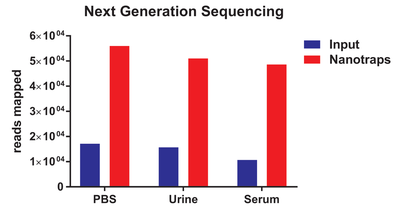


Figure 4. Increased EBOV-specific sequencing coverage following Nanotrap® processing of EBOV-spiked human matrices. EBOV/Kikwit was diluted to a concentration of $1x10^5$ PFU/ml in PBS, or in 1:3 diluted pooled human urine or serum, and then processed with Nanotraps® as described previously. EBOV-specific reads were identified by metagenomic next generation sequencing using an Illumina MiSeq isntrument. Total reads mapped in unprocessed and Nanotrap® processed samples are indicated.

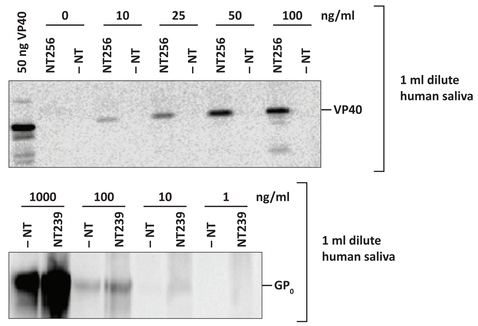


Figure 5. Improved immunoassay limit of detection for EBOV VP40 or GPΔTM in saliva. Recombinant EBOV VP40 and GPΔTM proteins were diluted to final concentrations of 1-1000 ng/ml into 1:3 diluted pooled human saliva in PBS, and then 1 ml of each of these samples was incubated with 100 µl NT256 or NT239 (cibacron blue core Nanotraps®) at room temperature for 30 minutes. Nanotraps® were pelleted by centrifugation, and then the pellets were resuspended in SDS-PAGE sample buffer. All samples were denatured for 10 min at 95 °C, separated by SDS-PAGE, and then analyzed by western blotting using EBOV VP40- or GP- specific polyclonal antisera. Control samples without Nanotraps® (-NT) were processed in parallel.

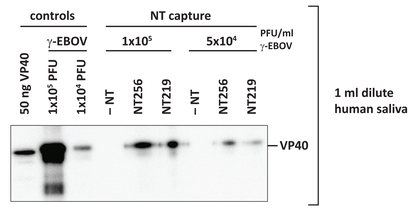


Figure 6. Nanotrap® particles enrich EBOV VP-40 from irradiated EBOV samples. Gamma-irradiated virus was spiked into 30% pooled human saliva in PBS to final concentrations of $1x10^5$ or $5x10^4$ PFU/ml. For each 1 ml of sample, 100 µl of NT256 or NT219 (cibacron blue core Nanotraps®) was added and then incubated for 30 min at room temperature. In parallel, samples were also processed without Nanotraps® (-NT). After Nanotrap® binding, samples were processed and analyzed as described in Figure 5.

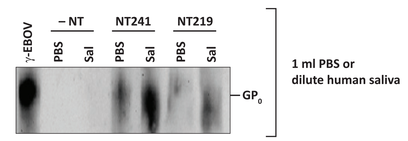
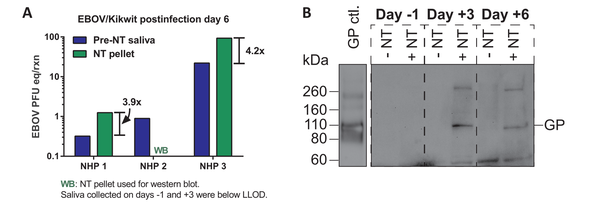


Figure 7. Nanotrap® particles enrich EBOV GP from irradiated EBOV samples. Gamma-irradiated EBOV cell culture supernatant was diluted in PBS or 10% saliva in PBS to a concentration of $5x10^5$ PFU/ml. For each 1 ml of sample, 100 µl of NT219 or NT241 (cibacron blue cores) was added and then incubated for 30 min at room temperature. In parallel, samples were also processed without Nanotraps (-NT). After Nanotrap® binding, samples were processed and analyzed as described in Figure 5. The positive control (γ-EBOV) was 10 µl of irradiated virus at $1x10^7$ PFU/ml. Sal = sample diluted in 10% saliva in PBS.



WB: NT pellet used for western blot.
Saliva collected on days -1 and +3 were below LLOD.

Figure 8. Nanotrap® concentration improves assay sensitivity and diagnostic time-to-answer in EBOV-infected rhesus macaque saliva. Rhesus macaques were infected with 1000 PFU EBOV/Kikwit by the intramuscular route. Saliva was collected from anesthetized NHPs on days -1, +3, and +6 relative to EBOV challenge. One part clarified day +6 saliva (750 µl) was diluted with two parts PBS (1500 µl), and then incubated with 100 µl of Nanotrap® slurry for 30 min. Nanotraps® were recovered by centrifugation, and then prepared for **(A)** EBOV RT-qPCR assay or **(B)** EBOV GP western blot as described previously. The Nanotrap®-concentrated material (+ NT) was compared to dilute saliva prior to Nanotrap® addition (- NT). The fold enrichment by Nanotraps® for NHPs 1 and 3 are indicated for the RT-qPCR data.

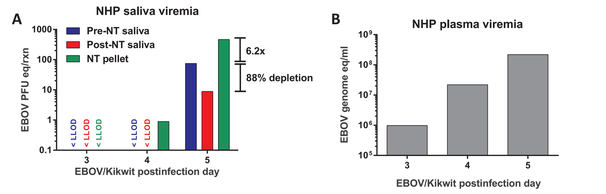


Figure 9. Nanotrap® concentration improves assay sensitivity diagnostic time-to-answer in EBOV-infected cynomolgus macaque saliva. A cynomolgus macaque was infected with 1000 PFU EBOV/Kikwit by the intramuscular route and then saliva and plasma were collected at various times postinfection. **(A)** One part clarified saliva (700 µl) was diluted with two parts PBS (1400 µl), and then incubated with 100 µl of prewashed Nanotrap® slurry for 30 min. Nanotraps® were recovered by centrifugation, and then prepared for the EBOV rRT-PCR assay as described previously. The NT222-concentrated material (NT pellet) was compared to dilute saliva prior to NT222 addition (Pre-NT saliva) and to unbound material after centrifugation (Post-NT saliva). EBOV RNA was detected using rRT-PCR and samples below the lower limit of detection (< LLOD) are noted. **(B)** For comparison, the plasma viremia for this NHP is shown over the same time period. Both panels utilized similar rRT-PCR assays, but different standard curves.

CONCLUSIONS

- Cibacron blue Nanotraps® directly interact with and bind a diverse panel of filoviruses
- The sensitivity of the EBOV EZ1-equivalent RT-qPCR assay was increased by ten-fold by use of Nanotrap® concentration
- Nanotrap® processing increased immunoassay sensitivity towards EBOV GP and VP40 by ten- to one hundred-fold over unprocessed samples
- NHP studies demonstrated the *in vivo* relevance of EBOV diagnostic targets in saliva, and Nanotrap® processing was able to shorten the diagnostic time-to-answer
- Proof-of-concept was achieved in about four months, demonstrating the robustness of the Nanotrap® sample processing approach and its potential application for rapid outbreak response

FUTURE DIRECTIONS

- Evaluate the potential for Nanotraps® to stabilize diagnostic analytes in clinical matrices at elevated temperatures and for extended periods of time
- Integrate Nanotrap® processing into additional filovirus diagnostic platforms such as NGS, MAGPIX, and lateral flow immunoassays to demonstrate improved sensitivity
- Continue verification of the Nanotrap® workflow using NHP infection models of biothreats
- Continue to expand the repertoire of viral and bacterial biothreats compatible with Nanotraps®

ACKNOWLEDGEMENTS & DISCLAIMERS

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- Research was conducted under an IACUC approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011.
- Opinions, interpretations, conclusions, and recommendations contained within this presentation are those of the authors and are not necessarily endorsed by the United States Army or George Mason University.