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TITLE: Quantification of Circulating Mtb Antigens for Rapid TB Diagnosis and Treatment Monitoring

PRINCIPAL INVESTIGATOR: Ye Hu, Ph.D.

CONTRACTING ORGANIZATION: TULANE UNIVERSITY
6823 SAINT CHARLES AVE
NEW ORLEANS LA 70118-5665

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Ye Hu, Ph.D.

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TULANE UNIVERSITY
6823 SAINT CHARLES AVE
NEW ORLEANS LA 70118-5665

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14. ABSTRACT

TB is considered to be among the most dangerous health threats to military men and women deployed to overseas battlefields and humanitarian missions. Time-consuming culture tests are still commonly used for TB diagnosis and to identify drug-resistant TB cases in much of the world. A rapid, easy-to-use and reliable diagnostic platform is therefore highly needed to allow rapid TB diagnosis and treatment of military forces in harsh environmental conditions, such as war zones. With the support of the CDMRP award, a translational research team, including the engineering lab at Tulane University, the clinician scientists at Baylor College of Medicine and NanoPin Technologies, Inc., aim to develop a nanoparticle-based approach to address the issues associated with detection of Mycobacterium tuberculosis (Mtb)-derived factors in human blood samples. This diagnostic platform can rapidly and sensitively detect and quantitate TB antigen levels directly from blood samples and provides quantitative results to allow prompt diagnosis and rapid evaluation of a patient's response to treatment. Ultimately, this proposal seeks to leverage our recent success in molecular diagnostics and biomedical nanotechnologies to produce a portable point-of-care diagnostic platform that has the potential to markedly improve TB treatment and control by improving the accuracy, speed and cost of TB diagnosis and treatment monitoring. If successful, our strategy should have a significant impact on populations in developing countries as well as military personnel deployed to areas where this disease is prevalent.

15. SUBJECT TERMS

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Year 2 ANNUAL REPORT FOR W8IXWH-19-1-0026

TITLE: Quantification of Circulating Mtb Antigens for Rapid TB Diagnosis and Treatment Monitoring

PI: Tony Hu PhD, Tulane University School of Medicine

1. INTRODUCTION:

With the support of the CDMRP award, a translational research team that includes scientists at Tulane University, clinician scientists at Baylor College of Medicine and researchers at NanoPin Technologies, Inc. will develop a nanoparticle-based approach to address issues associated with the detection of *Mycobacterium tuberculosis* (*Mtb*)-derived factors in human blood samples. This diagnostic platform will rapidly and sensitively detect and quantify TB antigen levels from blood samples to allow prompt disease diagnosis and evaluation of a patient's response to treatment.

In the second award year, we got the HRPO approved so the prospective collections have been initiated at all our study sites. At Tulalne research lab we also completed the method development for selecting the high-performance peptide marker and extract orthogonal chromatographic peak features. Thresholds of all features were integrated to aid in unambiguous identification of circulating antigen at a detection limit of 0.5 pM, or 50 attomoles in absolute amount. This method has shown a potential to translate to a general blood test for diagnosing various types of infectious diseases. In year 2, our industry partner (NanoPin Technologies) Inc. has made several modifications to its initial diagnostic protocol to optimize its workflow, increase its analytical sensitivity, and reduce costs, operator effort, variation, and potential sources of error. General workflow improvements include reducing the amount of serum/plasma required for analysis, the sample digestion time, and peptide resuspension conditions, and automation of the assay protocol. NanoPin has also successfully transitioned its assay from a research grade MS system to an instrument certified for use with clinical diagnostic applications, which also permitted assay workflow modifications that improved the performance time, reproducibility and limit of detection of the resulting assay. All these modification should enhance its feasibility for use as a clinical laboratory application. NanoPin is currently proceeding with analytical validation studies require to determine and document the performance of this refined assay.

2. KEYWORDS:

cGMP, scaled-up, assay kit, nanoparticles, monoclonal antibodies, sample digestion, mass spectrometry, protocols, tuberculosis diagnostics, technology transfer, quality management system

3. ACCOMPLISHMENTS:

- **What were the major goals of the project?**

Cohort Development: Obtain IRB approval for the retrospective cohorts and prospective sample collection (0-3 mon, **100% complete**).

Aim 1) Optimize the sensitivity and specificity of our existing blood-based prototype assay to meet or exceed minimum WHO performance criteria for screening and diagnosing TB.

Subtask 1.1: Organize the retrospective clinical samples (Month 0-24, **50% complete**)

1. Our Eswatini cohort (**Eswatini**) contains >800 subjects who were classified into one of four categories based on their diagnosis: active TB, LTBI, NTM, and close contacts of these three groups without evidence of mycobacterial disease.
2. The TB group (n=>350) spans the spectrum of TB disease severity. Individuals enrolled in this cohort were diagnosed at enrollment using microbial evidence from Xpert MTB/RIF and/or *Mtb* culture (69.9%) or clinically diagnosed (29.7%) using consensus guidelines.
3. This cohort also contains 290 asymptomatic household contacts of these TB cases, who were followed for 12-months after being offered preventative isoniazid therapy, these study participants were classified as close contacts without mycobacterial disease or as TB, LTBI, NTM cases based on their final diagnosis during this 12-month follow-up period.
4. We have tested ~250 retrospective samples from Eswantini cohort, and found out the ACD tube used in collecting plasma sample were not suitable for quantifying our antigen because the tube is specially designed for PBMC study and has minimal effect on preventing proteins from degradating. We have proactiavely contacted the resipository sample banks at IMPAACT and FIND to provide additional restrospective serum samples for us to complete the analytical validation of the assay.

Subtask 1.2: Evaluate Nanoparticle-MS assay diagnostic sensitivity improvements with additional *Mtb*-specific antigen peptides (Month 1-24, **100% complete**).

Subtask 1.3: Evaluate specificity Improvements by performing logistic analyses to evaluate the relative diagnostic specificity of individual peptide and protein combinations (1-12 months, **100% complete**)

Subtask 1.4: Evaluate the analytical performance of an established peptide biomarker panel. (5-30 months, **100% complete**).

Aim 2) Validate the performance of our optimized assay using blood samples previously collected from well-characterized TB and non-TB patients and determine if assay performance on a prototype mobile miniMS instrument is non-inferior to that observed on a standard MS instrument.

Subtask 1: Establish analysis and validation protocols for the Nanoparticle-MS system with clinical samples (7-36 months, **75% complete**)

Subtask 2: Optimize the direct analysis of nanoparticle-captured biomarkers (Month 13-18, **20% complete**)

Aim 3) Determine the clinical sensitivity and specificity of the final assay on a MiniMS-12 or bench-top MS using >3,000 blood samples prospectively collected from serial patients recruited from intended use settings in high (Swaziland), medium (Tanzania) and low (US) burden TB screening clinics.

Subtask 3.1: Submit documents for IRB approvals (Month 1-3, **100% complete**).

- The IRB for the prospective sample collection in Swaziland was approved and submitted to DOD on Sept. 4, 2019 (**Eswatini**).
- The IRBs for prospective sample collection in **Houston** and **Tanzania** (Site 2 and Site 4) were approved by the relevant institutions in both countries and submitted to DOD on February 17, 2020.

Subtask 3.2: Human sample collection (Month 4-35, **25% compete**, site preparation complete; with HRPO approval we have started the prospective collection):

- Enroll individuals being evaluated for TB once IRBs for the prospective sample collection is approved for **Houston, Tanzania and Eswatini** (recruitment will commence after receiving HRPO approval).
- Prospectively evaluate the sensitivity of the miniMS or bench-top MS instrument with serum from a cohort of individuals with microbiologically confirmed TB (**Tulane**).
- Prospectively evaluate the specificity of the miniMS or bench-top MS instrument with serum from asymptomatic controls without TB (**Tulane**).

Subtask 3.3: Perform clinical tests: sputum smear, culture and DST, HIV testing at Hosuton and Tanzania (Month 18-30, **on-going**, data collection > 90% to date).

Subtask 3.4: Determine the overall diagnostic sensitivity and specificity of Nanoparticle-MS for active TB cases (18-30 months, **not yet initiated**).

Subtask 3.5: Track *Mtb*-derived peptides in sera as a function of TB treatment for patients with documented therapies (25-36 months, **not yet initiated**).

Aim 4) Establish GMP product development processes to produce an optimized assay kit.

Subtask 4.1: Establish a cGMP fabrication process for nanoparticle by following internally established and externally validated Standard Operating Procedures (SOPs). The designated research staffs from Tulane University (Tulane) will work closely with Nanopin Technologies Inc. (Nanopin) to optimize the cGMP fabrication of nanoparticles, develop and verify the prototype and establish a quality control system (18-36 months, **40% complete**).

Subtask 4.2: Build a variety of product prototype configurations for the assay nanoparticle, using low-cost injection molding to study plate-to-silicon interfaces, including overmolding (20-30 months, **30% compete**).

Subtask 4.3: Conduct prototype verification (22-30 months, **15% compete**).

Subtask 4.4: Develop and implement a thorough ISO13485 quality management system (QMS) (24-30 months, **not yet initiated**).

Subtask 4.5: Identifying monoclonal antibodies for our assay targets (7-18 mon, **100% complete**).

Subtask 4.6: Evaluate the physical and functional stability of new antibody-conjugated nanoparticles by technical staff at Nanopin and Tulane during storage over a range of temperatures, both in solution and as a lyophilized powder (25-34 mon, **50% compete**).

- **What was accomplished under these goals?**

Progression for Aim 1.

Aim 1. Optimize the sensitivity and specificity of our existing blood-based prototype assay to meet or exceed minimum WHO performance criteria for screening and diagnosing TB.

The Mycobacterium tuberculosis (*Mtb*) virulence factor 10-kDa culture filtrate protein (CFP-10) represents a good candidate diagnostic biomarker for tuberculosis (TB), which can be difficult to diagnose by current methods in several patient populations at high risk for morbidity and mortality(1). CFP-10 is actively secreted by virulent *Mtb* strains, and transfer of its gene loci to attenuated *Mtb* strains has been shown to increase *Mtb* survival and virulence(2). Furthermore, its expression and secretion is likely regulated by *Mtb* bacteria at distinct stages of infection, allowing the bacteria to evade recognition by T cells specific for it(3). Our previous work indicate that CFP-10 shows a wide dynamic range in patient's blood(4). Its detection in blood samples can serve as direct evidence of an active *Mtb* infection and diagnose multiple TB manifestations(5). However, several non-tuberculous mycobacteria (NTM) express CFP-10 orthologs with significant sequence conservation(6), including two of the six NTM responsible for >80% of human mycobacterial respiratory isolates(7), limiting the utility of immunoassays for full-length CFP-10 protein. As shown in **Fig. 1a&b**, sequence differences in these conserved proteins can uniquely identify closely related pathogens and serve as the basis for more precise diagnostics than traditional protein immunoassays but these assays require tandem MS analysis to identify sequence variations specific for individual pathogens.

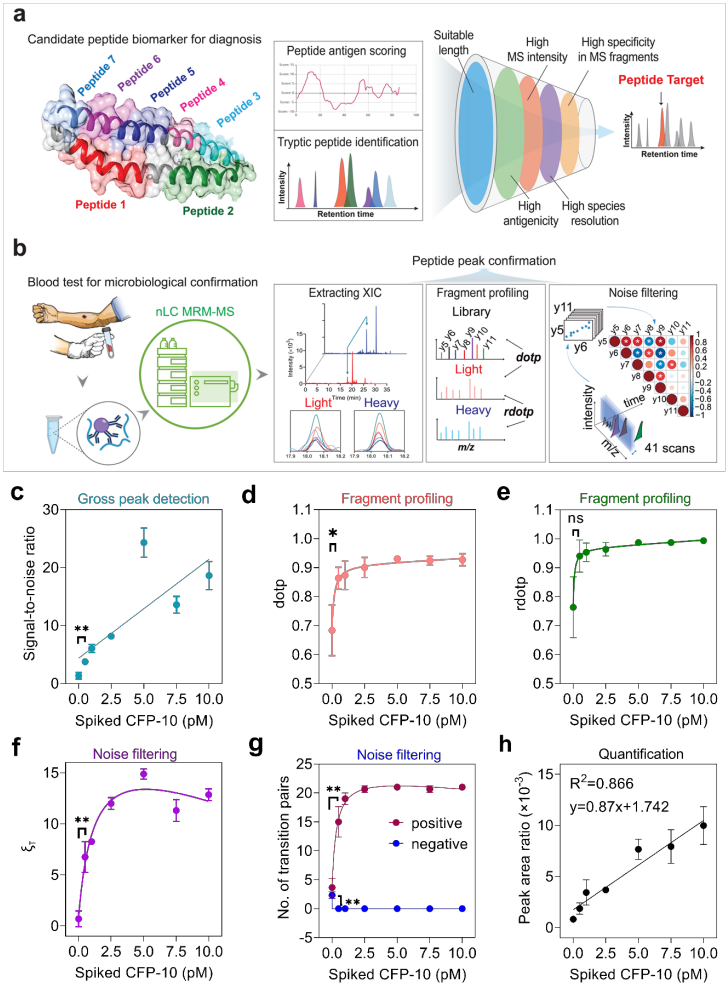

Figure 1. Development of MRM-MS assay for detecting scarce circulating antigen peptide biomarker with high pathogen species-specificity. **a** To offer diagnosis specificity, standard selection criteria for pathogen species-specific biomarker peptides are developed. Peptide target (red peak) is selected from all candidates of a protein (cartoon structure of CFP-10 antigen, PDB accession 3FAV) based on sequence specificity, antigenicity scores and MS signal intensities to identify peptides that can differentiate closely related pathogen species, induce high affinity antibodies enrichment, and yield unique MRM transition signal for detection and quantification. **b** A blood test for microbiological confirmation of pathogen infection by targeting the peptide marker. The peptide marker is enriched from serum or plasma digest together with its stable isotope labeled peptide standard (IS) and undergoes nanoflow LC MRM-MS analysis for its identification. The extracted ion chromatograms of IS peptide precursors (dark blue line) and endogenous peptide precursor (red line) in the whole run are aligned first to identify the appropriate retention time window (light blue arrows) of a total ion chromatographic peak, which is used to extract each MRM transition as deconvoluted peaks (colored lines). Standard selection criteria for precursor ion and MRM transition peaks, including SNR, dotp, rdotp and correlation coefficients are developed. MS data from IS-spiked negative control samples define thresholds for each MS feature for a true peak of light endogenous peptide and integrate them into a single threshold to distinguish the peptide target MS peak (red) from chemical or electronic noise (grey). **c** SNR, **d** dotp, **e** rdotp, **f** sum of significant correlation coefficients, **g** number of positive and negative correlated transition pairs, and **h** light-to-heavy peptide peak area ratio changes in relation to spiked CFP-10 concentrations in serum. Data are shown as mean ± SD (n=3) indicating significant (*p<0.05 and **p<0.01) and non-significant differences (n.s.) versus the blank (0 pM) sample as detected by unpaired parametric t-test.

1. Finlization of standard Curve for CFP-10pep assay

Seven y-ions (y5-y11) of CFP-10pep were carefully selected as MRM transitions due to their observed high MS intensities in trypsin digests of recombinant CFP-10 protein and reduced number of predicted interferences versus other detected CFP-10pep ions. Co-elution of these seven y-ions produced a characteristic chromatographic peak in the retention time (RT) window defined by a stable-isotope-labeled (heavy) IS identical to the CFP-10pep sequence (**Fig. 1c**). Correlation data was therefore employed to evaluate the morphological similarity of co-eluting target ions (See Extended Data Discussion for details). The CFP-10pep elution window was defined by its full width at base as 0.38 min or 41 MRM scans for endogenous peptide precursor ion from CFP-10 spike-in experiment results.

To distinguish true IP-MS CFP-10pep signal from random MS noise, we extracted feature information from MS peaks that eluted within the CFP-10pep target window and evaluated the SNR, dot product (dotp) and relative dot product (rdotp), and correlation matrix of these peaks and their transition ions (**Fig. 1b**). SNR was employed to evaluate the probability that signal detected within the target window represented actual signal rather than electronic noise, while

dotp and rdotp were used to evaluate how well the relative peak areas of transition ions detected within this window matched those produced by a reference library sample or the IS of the same sample, respectively. SNR, dotp and rdotp values are commonly employed to improve the accuracy of target peak identifications(8), but analyze all signal detected within a target window regardless of how well the elution profiles of each target ion align.

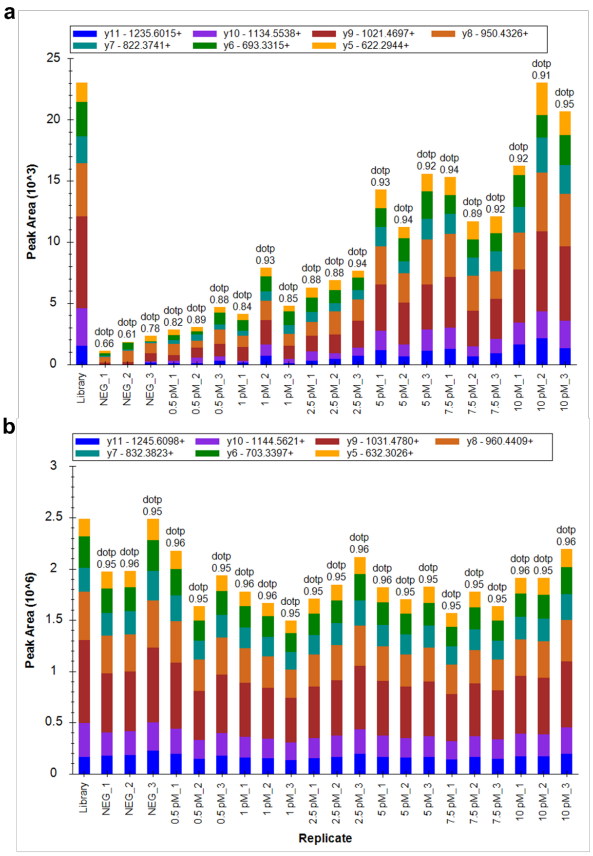

Figure 2. Peak area of seven selected transition ions XICs for (**a**) CFP-10pep and (**b**) its heavy-isotope-labeled IS peptide in affinity enriched samples from three replicates of trypsin-digested healthy human serum spiked with 12.5 nM of the IS peptide and 0-10 pM CFP-10. The fragment ion m/z is shown in the legend above. A dotp score measuring the similarity of relative ion abundances between library spectrum and query spectrum is indicated for each sample.

CFP-10pep SNR, dotp and rdotp values decreased with CFP-10 concentration (**Fig. 1c-e**), with SNR and dotp, but not rdotp, values distinguishing CFP-10pep target peaks in serum spiked with and without 0.5 pM CFP-10. SNR values were more variable than dotp and rdotp (CVs as 43.6%, 12.8 % and 13.8%, respectively) in serum replicates not spiked with recombinant CFP-10 protein. More positively correlated transition ion pairs were detected in the CFP-10pep RT window of serum samples spiked with versus without 0.5 pM CFP-10 (median 15 vs. 4), and the sum of all significant correlation coefficients increased with CFP-10 concentration (**Fig. 1f**). Negatively correlated pairs were detected only in blank serum samples (**Fig. 1g**), and represented ~40% of the significant correlations in these samples. All correlation parameter values distinguished CFP-10pep target peaks in serum spiked with and without 0.5 pM CFP-10 (**Fig. 1f-g**).

CFP-10pep-to-IS peptide peak area ratios, used to absolutely quantify CFP-10pep abundance, were linear across a 0.5-10 pM range in trypsin digests of healthy human serum spiked with serial dilutions of recombinant CFP-10 and a constant amount of IS peptide (R^2=0.866, **Fig. 1h** and **Fig. 2**), and revealed CFP-10pep limit of background (LOB) and detection (LOD) estimates of 0.0011 and 0.0018, respectively (9). The CFP-10pep LOD value was found to be close to the mean peak area ratio detected in serum spiked with 0.5 pM CFP-10 (0.00186), implying the CFP-10pep LOD for this approach is approximately 0.5 pM.

However, four IP-MS target ions detected in CFP-10-free serum matched the CFP-10pep y-ions y5, y6, y8 and y9), but exhibited poor co-elution (**Fig. 3**), and thus produced only sporadic positive integrated signal peaks with SNR >2 in CFP-10-free serum replicates (e.g., two negatives and one positive peak with a SNR of 2.01). Signal detected in these control samples thus likely represents variable electrical or chemical noise, and our proposed chromatographic peak feature

cutoff algorithm aided in accurately distinguishing weak CFP-10pep peaks from random MS spectrum noise.

Development of a threshold of peak features

To increase the accuracy of CFP-10pep identification at concentrations near 0.5 pM in the presence of signal interference, we determined empirical thresholds for peak detection using SNR, dotp, rdotp, peak area ratio and two correlation matrix parameters: the sum of coefficients that were significantly different from zero and negative-to-total ratio of significant target ion correlations (**Fig. 4a**). Minimum SNR, dotp, and rdotp thresholds for peak acceptance were defined as the mean minus 3 times the standard deviation (SD) of CFP-10pep signal detected at the 0.5 pM assay LOD. Minimum and maximum thresholds for the sum and negative-to-total ratio of the significant fragment ion correlations were respectively set as the lower and upper 95% confidence interval of these values in the non-spiked standard curve sample.

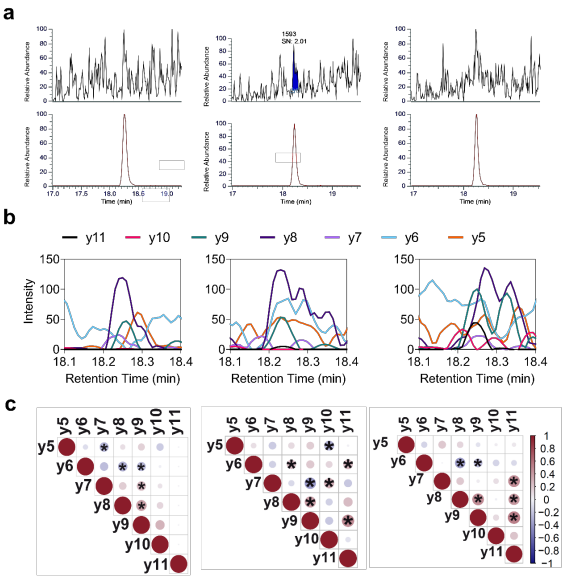

Figure 3. Peak recognition and differentiation of noise from signal by transition correlation analysis. **a** XICs for (upper panel) CFP-10pep and (lower panel) its heavy-isotope-labeled IS peptide in affinity enriched samples from three replicates of trypsin-digested healthy human serum spiked with 12.5 nM of the IS peptide. Recognized peaks (blue) are labeled with their signal-to-noise ratio (SN). **b** Targeted retention time window XICs for the seven selected transition ions in the blank serum samples in (**a**). **c** Kendall rank order correlation coefficient matrix for the seven transitions in blank serum samples. Correlation coefficients are color coded, and the circle size represented for their significance p value. Asterisks indicate the correlation coefficients that are significantly different from zero ($p<0.05$).

A proof-of-concept study using all five criteria to analyze MS results from negative control plasma samples obtained from 20 commercialized serum controls correctly identified 95% of these samples as deriving from individuals without TB (**Fig. 4b-c**), if peak area data was excluded from the analysis. Values for six samples passed the dotp and rdotp cutoffs, and two met the SNR threshold (**Fig. 4b**), while four and six met the cutoff criteria for the sum and negative-to-total ratio of significant correlations (**Fig. 4c**). However, all 20 samples met the peak area ratio LOD threshold, indicating that this parameter was not suitable

a

	1. Peak acceptance features			2. Noise filtering features			
	XIC peak parameters			Corr. transitions		Corr. intensity	
	dotp	rdotp	SNR	(+)	(-)	(-) corr. %	ξ_T
Mean	0.863	0.94	3.753	3.7	2.3	40.7	0.698
SD	0.038	0.056	0.528	1.5	0.6	19.6	0.776
Cutoff	***0.75***	***0.77***	***2.2***			***22***	***1.58***

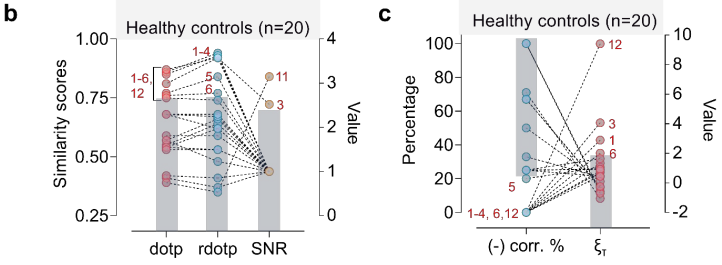

Figure4. Performance of peak feature criteria with clinical samples. a Cutoff threshold for peak acceptance features; dotp, rdotp and SNR thresholds employ Mean-3×SD values from the LOD concentration standard (0.5 pM), while those for noise filtering features; the percent of negative ion correlations and the sum of significant correlations (ξ_T) utilize the lower and upper limits of the 95% confidence interval detected in PBS-spiked healthy serum. **b-c** Distribution of (**b**) three peak acceptance criteria and (**c**) two noise filtering criteria in plasma from 20 healthy donors. Red numbers indicate specific samples that exceed the indicated thresholds. Blue shaded box indicates the cutoff for each feature.

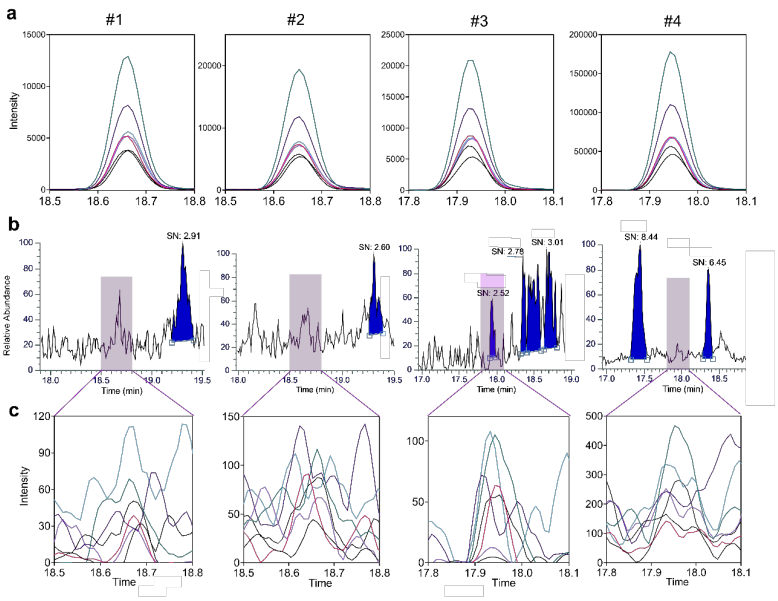

Figure 5. CFP-10pep assay results from four healthy donors with dotp and rdotp values above the selected cutoffs for these parameters (#1-4 in **Fig. 2a-b**). **a** EIC peaks corresponding to the integrated target transition ions (y5-y11) of the spiked-in stable-isotope-labeled IS peptide, where elution times for samples 1-2 and 3-4 reflect minor differences in column elution behavior after replacing one column with an identical column. **b** SNRs for non-specific integrated EIC peaks generated by serum-derived transition ions that co-elute with IS peptide transition ions, where blue shading indicates algorithm-recognized peaks with SNR values ≥2. **c** EIC peaks for non-specific signals detected within this target window that matched the seven targeted CFP-10pep fragment ions.

for accurate peak identification, and this parameter was therefore excluded from the peak identification algorithm for all further analyses. Single parameters for the integrated intensity, abundance similarity, and correlation of target fragment ions thus had poor specificity for peak identification, although the combination of dotp/rdotp data and SNR data excluded all but one non-specific peak in these non-TB samples. Elution profiles of the four samples with the highest dotp and rdotp values (#1-4) found they exhibited co-localization of spikes in the MS background noise, with only one (#3) revealing fragment ion peaks that aligned and were centered in the CFP-10pep elution window (**Fig. 5**). SNR and correlation criteria excluded three and two of the peaks detected in these samples, respectively, and all four integrated peaks had SNRs less than those of adjacent peaks. Only other sample with a peak that met both correlation criteria (#12, **Fig. 4c**), had rdtop and SNR values below their cutoff thresholds, and represented a spurious peak produced by the edge of a peak that eluted outside the CFP-10pep target window (**Fig. 6**).

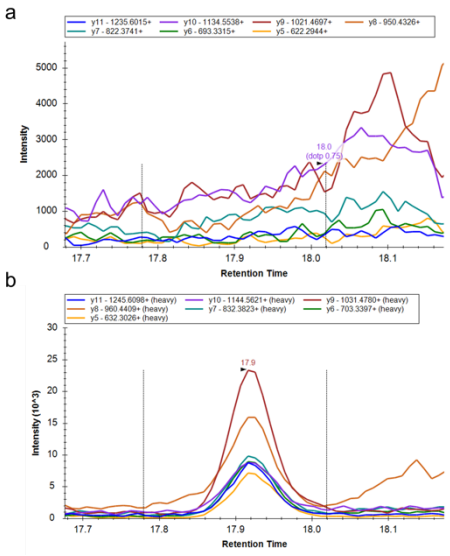

Figure 6. Example of highly correlated non-specific signal detected in a healthy donor serum sample (#12 in Fig. 4A-B, C12 in Table S2) depicting the EICs of (**a**) serum-derived signal and (**b**) the IS peptide relative to the target elution window (dashed lines), where it appears that rising signal from ions belong to peaks outside this window are responsible for the false-positive signal.

Candidate peaks coeluting with the IS peptide were first filtered by dotp/rdotp, SNR, and peak area criteria to identify similar and weak signal, then analyzed to determine if the rank-ordered correlations of their transition time series significantly differed from zero. The sum of significant correlation coefficients and negative-to-total ratio of all their significant correlations were used to identify random correlation. Correlation analysis are widely applied to MS dataset for peak quality control, one is cross-

correlation of ligh/heavy ion pairs, such as AuDIT. The other is cross-correlation of all transitions from endogenous peptide, such as mProphet. Here we compared Kendall's rank order correlation and cross-correlation for peak quality control. The aim was to find a better feature that is orthogonal to similarity scores dotp and rdotp and can aid in noise filtering.

Progression for Aim 2.

Aim 2) Validate the performance of our optimized assay, using blood samples previously collected from well-characterized TB and non-TB patients, and determine if assay performance on a prototype mobile miniMS instrument is non-inferior to that observed on a standard bench-top MS instrument.

CFP-10pep detection algorithm performance in pediatric cohorts

Pathogen-derived biomarkers may circulate at low levels, particularly during early infection, and biomarker-derived peptide detection may be masked by MS fragment ion overlaps with co-eluting peptides contaminants in immunoprecipitation (IP) samples. While we are still evaluating the archived specimens, we evaluated archived retrospective samples from a completed studies in Dominic Republic (sponsored by Micah Batchelor Award for Excellence in Research in Children; PI: Charles Mitchell) that were provided by our collaborators on other non-DOD funded projects.

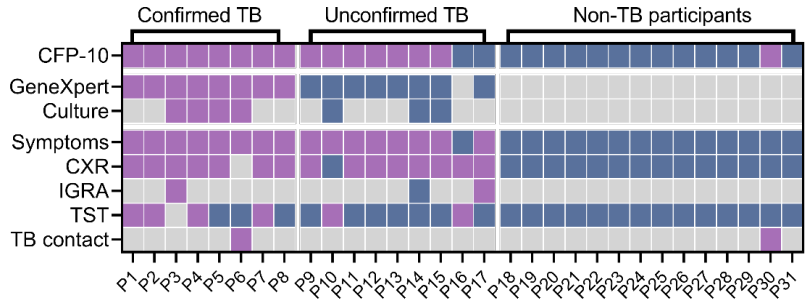

Fig 7. CFP-10pep diagnosis of TB in pediatric patient with *Mtb* infection. Plasma CFP-10 positivity and diagnostic information for patients from Dominican Republic retrospective cohort. Blue, purple and grey squares indicate negative, positive, and missing data respectively.

The proposed CFP-10pep assay correctly diagnosed microbiologically confirmed 8/8 TB cases, 6/8 unconfirmed TB cases by related symptoms and chest X-ray findings, and 13/14 non-TB cases in *Mtb* infected children (**Fig. 7**). The lone case in non-TB cases was a household contact of active TB patient. We next applied this approach to a diagnostically challenging pediatric cohort consisting of hospitalized HIV-infected children aged <12 years with suspected TB, most of whom exhibited evidence of severe immunosuppression. These children were classified into three categories using an algorithm proposed by an NIH expert panel: Confirmed TB (microbiologic evidence of TB); Unconfirmed TB (no microbiologic evidence but matched at least two other criteria: TB-associated symptoms, a TB-consistent chest radiograph, close TB exposure or immunologic evidence of Mycobacterium tuberculosis infection, or a positive TB treatment response); or Unlikely TB (negative for both Confirmed and Unconfirmed TB criteria).

Progression for Aim 3.

Aim 3) Determine the clinical sensitivity and specificity of the final assay on a portable miniMALDI or bench-top MS using >3,000 blood samples prospectively collected

from serial patients recruited from intended use settings in high (Eswatini), medium (Tanzania) and low (US) burden TB screening clinics.

- The IRB for the prospective sample collection in Eswatini has been approved and was submitted to DOD on Sept. 4, 2019 (**Eswatini**). The approval for the protocol was approved by DOD HRPO on May 14th, 2021. Since then, 50 participants have been enrolled into the prospective study. In Houston, 28 people have been enrolled into the prospective study since DoD HRPO approval in May. Due to the COVID-19 pandemic and associated safety protocols implemented by the hospital and by Baylor, research coordinator access to the hospital has been restricted thus far. We believe this has acted as a limiting factor to our ability to recruit participants, and that moving forward, as restrictions are lifted, this will not curb recruitment.
- The IRBs for prospective sample collection in Tanzania (**Baylor and Tanzania**) were submitted and approved by both the National Institute for Research (NIR) and the Mbeya Medical Research and Ethics Committee (MMREC), and submitted to DOD on February 17, 2020.
- Non-DOD sources of funding were used to collect and store plasma in the existing Eswatini cohort biorepository. These plasma samples were collected in ACD (acid citrate dextrose) tubes, processed within 4 hours of collection, frozen at -80C freezer, and then rapidly shipped on ice with no more than 2 freeze-thaws occurring before Nanoparticle-MS analysis that were conducted as part of Aim 1. Specimens collected in this fashion have thus far not been amenable to successful completion of Nanoparticle-MS assay. As a result, we have revised specimen collection, storage and shipment SOPs. The prospectively collected specimens are now being collected into standard red top tubes and shipped on dry ice.
- Specimen storage capacity has been optimized at all sites through the introduction of freezer works, including related training. The clinical database has been refined and improved during this year.
- Training on data collection and prospective enrollment took place over several months in Tanzania and Eswatini.
- All three sites (**Eswatini**, **Houston, and Tanzania**) stopped non-essential research in March 2020 due to the global COVID-19 pandemic and institutional requirements. However, by September 2020, clinical research was allowed to begin at both **Eswatini** and **Tanzania** following national guidelines for implementing clinical research and COVID-19 precautions. Following HRPO approval, prospective sample collection commenced in Eswatini , Tanzania, and Houston. Since July 2021, there has been significant political instability that has disrupted and slowed study recruitment in Eswatini.
- All team members have completed Good Clinical Practice or Good Laboratory Practice courses conducted *via* CITI training available through Baylor College of Medicine.

Profession for Aim 4.

Progression Details

Modifications to the assay protocol and its analysis system

NanoPin has made several modifications to its initial diagnostic protocol to optimize its workflow, increase its analytical sensitivity, and reduce costs, operator effort, variation, and potential sources of error. All these modification should enhance its feasibility for use as a clinical laboratory application. We have made the following assay modifications and adjustments to achieve this:

General workflow improvements:

Reduced serum/plasma volume requirement: We have reduced the amount of plasma or serum required for analysis from 200 µL/test to 100 µL/test without compromising assay sensitivity or specificity. This has several advantages, including reducing the per test reagent cost, simplifying liquid handling by eliminating the need to split samples during sample dilution, and reduces the assay demand in cases where there may be limited serum or plasma available for an array of diagnostic tests, including newborns and infants.

Reduced serum/plasma digestion time: We have also modified our sample digestion protocol that required a 12 hour digestion step at 37°C with sequencing grade trypsin to achieve near complete digestion of a serum or plasma sample. Our new digestion procedure employs a 2 hour digestion at ambient temperature (~22°C) with immobilized TPCK trypsin to achieve the same results. This modification markedly reduced the assay performance time to allow same-day sample-to-answer times, assay material costs, and potential for non-specific cleavage by residual contaminating protease activity in the sample or trypsin.

Refined peptide resuspension conditions: Our workflow optimization studies also allowed us to identify and correct a significant source of sample loss and assay variation encountered during the reconstitution of the affinity-enriched target peptide prior to its MS analysis. Specifically, this analysis found that resuspension of the lyophilized peptide samples captured by affinity enrichment in deionized water containing 10% acetonitrile and 0.1% formic acid markedly reduced non-specific binding of the target peptide with the sample vials to improve assay sensitivity and reproducibility, particularly for samples containing low levels of the target biomarker.

Assay automation: We have also performed preliminary studies to evaluate the potential to automate the assay workflow since operator effort and operator-associated variability can limit the feasibility of clinical applications. Manual sample processing is the major source of variation in our assay, as the purified samples resulting from this process are loaded into the sample hander of the mass spec system for automated injection and analysis on the MS system. We found that the entire sample handling procedure up to the stage tip step could be performed on a relatively inexpensive liquid handling system that is already used in many clinical laboratories (Kingfisher Flex, ThermoFisher) without adversely affecting assay sensitivity, reproducibility, or performance time. Subsequent studies found that the stage tip procedure could be eliminated after the assay was transitioned to an analytical MS system, and thus operator involvement would be required only for sample loading to the Kingfisher liquid handling system and the MS autoloader.

System-specific improvements:

Adaptation to a clinical MS system: We have also achieved a major milestone by successfully transferring our assay from a nanoflow MS system to an analytical MS system (Vanquish Altis MD, ThermoScientific) certified for *in vivo* diagnostic analyses. This was a critical step in the

development of our proposed clinical application since nanoflow MS systems are not approved for use in clinical applications and require runtimes and workflows that would be prohibitive for most assays. This also yielded several additional benefits, as described below.

Stage tip elimination: The transition to the Vanquish Altis MD analytical MS system allowed us to remove the C18 spin-column stage tip employed for sample clean-up from the assay workflow. This elimination improved assay operation, cost, and sensitivity. The use of this stage tip is unavoidable when samples are analyzed using a nanoflow MS system, since the columns used in these systems are susceptible to irreversible and cumulative blockage by fine particulates. This can rapidly degrade column performance and result in the loss of the expensive column as a result of the high backpressure encounters during this progression. However, the column employed in the Vanquish Altis MD system does not require this sample clean-up step prior to analysis. We have shown that the omission of this stage tip does not alter the characteristic elution time of our target peptide or column backpressure during the elution gradient or reconditioning procedure, as validated by over 500 runs performed over several weeks. Samples evaluated without the stage tip produced sharp chromatograms with reproducible narrow gaussian curves without significant front- of back-tailing when analyzing affinity-enriched peptide samples that were captured from healthy human serum spiked with low concentration recombinant CFP-10 (**Fig. 8**).

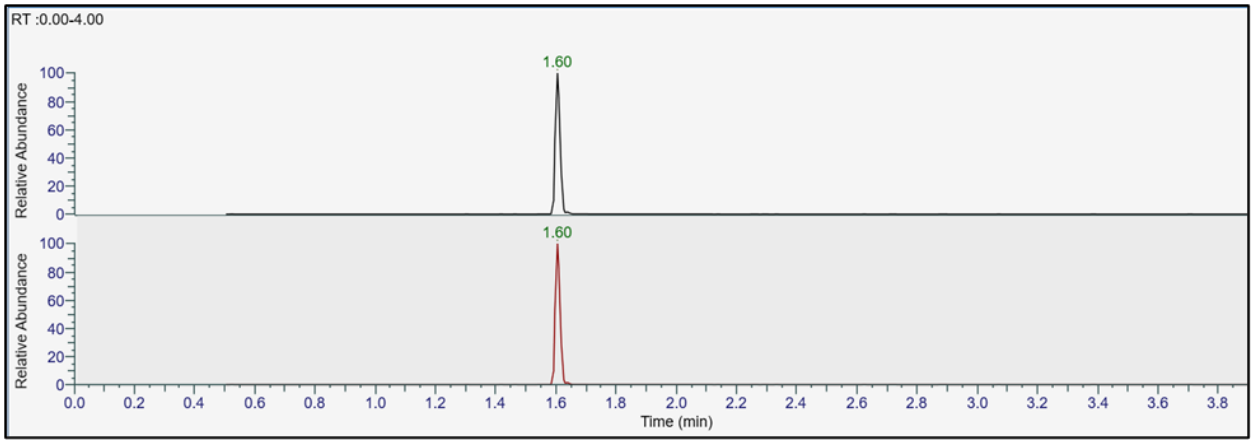

Figure 8. The MS peak retention time of the 1593 target peptide and its heavy isotope-labeled internal standard (IS) peptide (upper and lower panels, respectively) after affinity capture from serum spiked with 25 pM recombinant CFP-10 and 100 pm IS peptide.

Removing the stage-tip improved sample handling and eliminated a step that would have complicated assay automation, as discussed above. Its elimination also yielded a 25% reduction in the cost of the assay materials and decreased the lower limit of consistent biomarker detection (5 pm versus 10 pM) by reducing variable and non-specific sample losses associated with this step.

Stage-tip removal markedly enhance target signal detection during our assay refinement process, with results of two trials performed with healthy human serum spiked with low concentrations of recombinant CFP-10 (10 and 20 pM). In this analysis, omission of the stage tip purification step from one aliquot of each sample produced a 2- to 4-fold signal increase and permitted resolution of peptide signal produced these two concentrations (**Fig. 9**). These results indicated that non-

specific binding to the stage tip resulted in a substantial and variable amount of target peptide loss to decrease both assay sensitivity and reproducibility. Stage tip removal, in combination with our refined assay procedure, subsequently allow us to detect our target peptide with a high signal reproducibility when analyzing healthy human serum samples spiked with 5 pM recombinant CFP-10. Prior to this refinement, we experienced difficulty reliably detecting samples containing less than 10 pM CFP-10. This enhanced sensitivity and reproducibility should increase our ability to reliably detect individuals with early TB disease and monitor treatment responses and disease clearance.

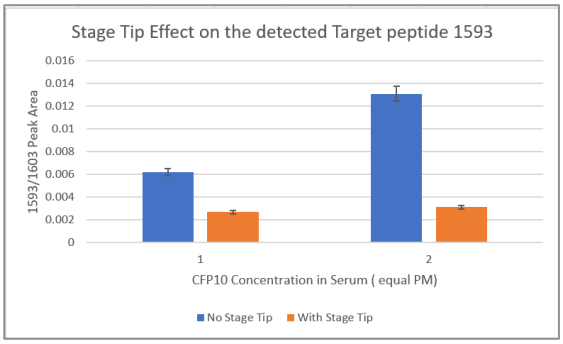

Figure 9. LC-MS signal detected for the 1593 target peptide in affinity enriched peptide fractions isolated from serum spiked with 10 pM (1) or 20 pM (2) recombinant CFP-10 protein, where samples were or were not passed through a stage tip prior to LC-MS

Increased LC loading volume: Switching from nanoflow LC-MS to an analytical LC-MS system also allowed us to increase the volume used to resuspend the lyophilized peptide samples that were captured by affinity-enrichment. In nanoflow analyses, the LC-MS liquid handler injected 6 µL of an 8 µL sample to minimize sample loss while using an acceptable loading time, while in the analytical flow analyses the system injected 20 µL of a 22 µL sample. This volume increase reduced inherent sample losses and variation associated with handling low sample volumes to improve injection consistency, and thus sample recovery, to improve assay sensitivity and reproducibility.

Reduced LC run-time: Shifting our assay from a nanoflow LC-MS to an analytical LC-MS system allow us to markedly reduce the time required for the target peptide elution gradient and column reconditioning protocol from 70 minutes to 7 minutes due to the much higher flow rates permitted by the latter system.

Vanquish MD LC gradient

Time (min)	Solvent A DI Water with 0.1% formic acid	Solvent B Acetonitrile with 0.1% formic acid
0	95	5
0.5	95	5
2.6	40	60
3	30	70
3.5	1	99
5.8	1	99
6.4	95	5
7	95	5

Optimized target ion signature: LC-MS optimization studies performed on the Vanquish MD consistently and specifically detected five of the seven initially selected biomarker product ions in positive samples and these five ions were therefore included in the SRM (selected reaction monitoring) scans for accurate target peak identification. The remaining two product ions were excluded for their tendency to appear in blank samples. Reducing the number of monitored

product ions employed for peak detection reduced noise in the SRM ion chromatograms to simplify peak identification.

SRM Scan Parameters

Compound	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)
Peptide 1593	797.12	822.4	27.16	117
Peptide 1593	797.12	950.5	26.29	117
Peptide 1593	797.12	1021.5	26.97	117
Peptide 1593	797.12	1134.5	27.16	117
Peptide 1593	797.12	1235.6	28.59	117
Peptide 1593 IS	802.45	832.4	27.16	117
Peptide 1593 IS	802.45	960.5	27.58	117
Peptide 1593 IS	802.45	1031.6	28.13	117
Peptide 1593 IS	802.45	1144.7	29.09	117
Peptide 1593 IS	802.45	1245.7	28.59	117

Our optimization studies determined that setting the cycle time to 0.4 sec and adjusting the Q1 and Q3 resolution to the full width at half maximum (FWHM) time divided by 1.2 significantly reduced noise and magnify the detected ions intensity in the SRM ion chromatograms to simplify peak identification. Notably, the detection and relative intensity pattern of these five target ions remained highly consistent even in healthy human serum samples spiked with our lowest CFP-10 concentration standard (5 pM) when compared to the matching ions from our isotope-labeled internal standard peptide of the same sequence (**Fig. 10** and **11**). This consistency permits accurate target identification at very low biomarker concentrations to aid in early disease diagnosis and treatment monitoring for disease clearance, when the ability to reliably detect low biomarker concentrations is critical.

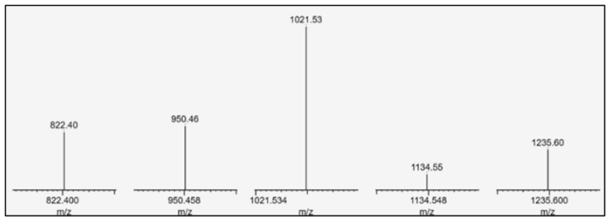

Figure 10. LC-MS product ions of the 1593 target peptide in affinity enriched peptide fractions isolated from serum spiked with 5 pM recombinant CFP-10 and detected by a SRM scan on the Vanquish Altis MD LC-MS system.

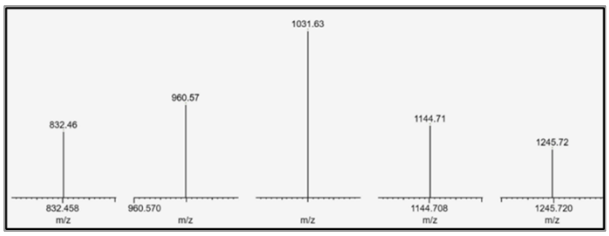

Figure 11. LC-MS product ions of the heavy-isotope-labeled 1693 internal standard (IS) peptide isolated from serum spiked with 100 pM of this IS pepdie and detected by a SRM scan on the Vanquish Altis MD LC-MS system.

Monoclonal antibody selection

NanoPin attempted to identify additional antibodies that could be used to identify other *Mtb*-specific peptides of candidate biomarker proteins to evaluate their relative analytical sensitivity and their ability to accurately identify serum spiked with very low concentrations of their target proteins. We successfully generated monoclonal antibodies to CFP-10 and ESAT-6 and

polyclonal antibodies to Ag85B, our three proposed targets. However, we chose to focus on CFP-10-specific peptide antibodies early in this process due to the poor performance observed when attempting to use the ESAT-6 and Ag85B peptide antibodies with clinical serum samples. The best ESAT-6-specific peptide monoclonal antibodies we evaluated detected positive signal in only about 25% the evaluated clinical samples and thus had no diagnostic benefit. Our polyclonal Ag85B-specific peptide antibodies also demonstrated poor performance to detect Ag85B in either spiked serum or TB patient samples. The reason(s) for the poor performance of antibodies to these two targets was not clear, but it is possible that their poor performance resulted from masking effects due to interaction with abundant environmental targets. Specifically, ESAT-6 has been reported to insert into cell membranes to exert its biological effects and Ag85B is known to interact with fibronectin. Both functions could serve to deplete these biomarker from serum to reduce their detection. We therefore focused on developing and validating the performance of high affinity rabbit monoclonal antibodies to *Mtb*-specific target peptides. We chose to generate and screen rabbit monoclonal antibodies for these studies since has been reported that the mean binding affinity of rabbit monoclonal antibodies tends to be higher than that of mouse monoclonal antibodies.

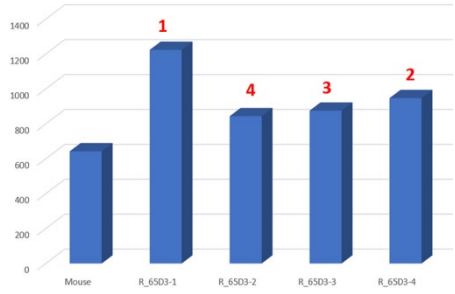

Figure 12. LC-MS signal detected for the 1593 target peptide in affinity enriched peptide fractions isolated from serum spiked with 25 pM recombinant protein using the indicated antibodies for peptide capture.

Biacore surface plasmon resonance (SPR) analysis of the target-specific affinity of our best CFP-10 peptide antibody candidate (65D3-1) determined that this antibody had an equilibrium dissociation constant (K_D) of 7.55×10^{-10}. We attempted to perform affinity maturation on this antibody to further improve its performance. The three best antibodies generated by targeted mutation of this antibody (65D3-2, -3, and -4), however, had slightly lower K_D values (**Table 1**) indicating decreased binding affinity. A second monoclonal antibody generated to the target sequence (67G8-1) revealed slightly better binding affinity than 65D3-1, but affinity maturation of this target also failed to further improve its binding affinity (data not shown).

Given this LC-MS data and the Biacore affinity data, further analyses focused on the rabbit 65D3-1 and 67G8-1 antibodies.

Analytical validation studies:

Nanopin has begun analytical validation studies required to establish and document the performance of our best antibody candidates. However, these ongoing validation studies were disrupted by large scale antibody delays and other supply issues associated with the pandemic and are not yet complete. Studies to determine assay linearity, precision, limit of detection (LOD) and lower limit of the measurement interval (LLMI), and matrix effects are now underway and have initial results. Stability testing studies will begin once these studies are complete.

Linearity: Serum from healthy subjects was spiked with low (0, 2, 5, 10 and 15 pM), medium (20, 25, and 30 pM) and high (35, 40 and 45 pM) concentrations of recombinant CFP10 protein and

analyzed in triplicate using both antibody candidates (65D3-1 and 67G8-1) to evaluate assay linearity across equidistant biomarker levels (**Fig. 13**). This analysis, which measured CFP-10 signal as the ratio of target to IS peptide signal versus the input protein concentration found that both antibodies produced similar responses, yielding linear curves with the same slope.

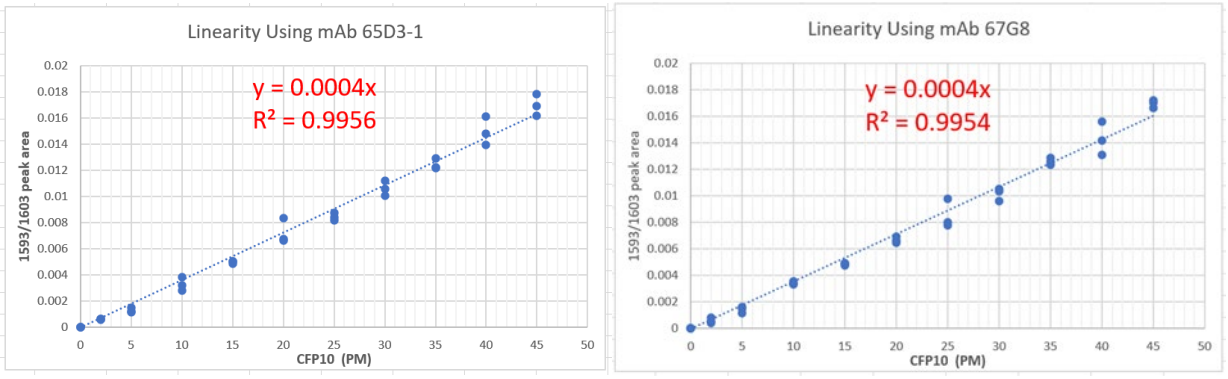

Figure 13. Linearity of the target-to-IS peptide signal ratio in samples spiked with the indicated concentrations of recombinant CFP-10 protein and analyzed by LC-MS assays using 65D3-1 (left) or 67G8 (right) for affinity enrichment.

Further, estimated of the limit of detection of this assay that were calculated using the formula LOD = limit of the blank (LOB) + 1.645 × the SD of the low concentration (SD_{Low}) sample . Using this formula and LODs of 2 to 10 pM standards, the 65D3-1 and 67G8-1 antibodies and the yielded mean LODs of 1.2 pM and 1.1 pM, respectively with estimates ranging from 0.2 – 2.6 pM and 0.9 – 1.4 depending upon the SD_{Low} sample chosen. This assay was able to reliable differentiate the three lowest standards from the blank sample and from each other. Achieving such high linearity and sensitivity is essential for the ability of the assay to evaluate patients with differing biomarker levels at various stages of their disease and treatment response.

Precision: The ability to obtain reproducible results is critical for clinical applications. We have initiated an analytical validation study to document the measurement precision of our assay. Within-run and within-laboratory precision are being evaluated by repeated analysis of three sets of four serum samples that are spiked with PBS (zero control) or equidistant concentrations of recombinant CFP-10 representing low (5 pM), medium (25 pM), and high (45 pM) biomarker concentrations. All samples are analyzed twice a day for twenty days to determine the mean, SD, and coefficients of variation for these samples when analyzed in by the two assays that use either the 65D3-1 or 67G8-1 antibody. This analysis is still ongoing, but our preliminary results from four analyses indicate that the mean percent coefficients of the 65D3-1 and 67G8-1 assays range from 8.6% to 15.5% and from 4.0% to 8.0%, respectively.

Matrix effects: Sample interference effects on target detection can significantly affect the reliability of assay results. We have therefore initiated a matrix effect evaluation study to determine the impact of several common clinical sample interferents on assay signal. This study will evaluate the effect of different concentrations of these interferents on standard curve values obtained when their replicate samples are spiked with low, moderate, and high levels. Preliminary results from this study indicate that 65D3-1 and 67G8-1 assays measuring the lowest concentration sample tested (5 pM) reveal moderate to strong target peak inhibition when spiked

with RBC hemolysate (1 – 10 mg/mL), triglyceride-rich lipoprotein (1.5 – 9 mg/mL), or abundant protein (1 – 10 mg/mL). Signal recovery ranged from 44 – 82% and 49 – 89% for the 65D3-1 and 67G8-1 assays, respectively, in this preliminary analysis, indicating that high levels of these factors would degrade accurate diagnosis of patients with low biomarker concentrations and inhibit accurate quantification at all concentrations.

- **What opportunities for training and professional development has the project provided?**

  Nothing to Report.

- **How were the results disseminated to communities of interest?**

  Nothing to Report.

- **What do you plan to do during the next reporting period to accomplish the goals?**

  In the Year 3, the research lab at Tulane aims to complete the development of a multi-factor blood-based TB assay and diagnostic algorithm that meets or exceeds the minimum WHO performance criteria for TB screening and diagnosis.

  During reporting year 3, the clinical team at Baylor College of Medicine plans to continue enrollment at all three sites, depending on pandemic conditions at each site. Enrollment began in September and October 2020 at both the Mbabane and Mbeya sites. Sample collection began following HRPO approval in Houston.

  We will determine the theoretical maximum sensitivity and specificity of our nanoparticle-MS assay under ideal laboratory conditions with blood samples from well-characterized prospective samples from the Baylor Eswantini, Houston and Tanzania sites, with the goal of achieving 90% sensitivity and specificity.

  We will start evaluating the analytical parameters of the integrated nanopartiple-miniMS assay, including linearity, precision, LOD and LLMI, accuracy, and matrix effects with the goal of achieving similar performance to that established for the current assays.

  NanoPin's Year 3 scope of work focuses on the physical and functional stability of the new antibody-conjugated particle platform during storage over a range of temperatures, in solution and as a lyophilized powder. This stability validation under a variety of standard clinical conditions is critical for the commercial acceptance of the TB assay for clinical use.

  NanoPin will test stability at several standard conditions encountered during normal handling and use, including standard laboratory temperature (25^{o}C) and common storage kit conditions

(4°C and -20°C). Conjugated nanoparticles will be examined for their stability in solution under these conditions and for their ability to be lyophilized and reconstituted without loss of function. This testing will process will also include an analysis of shipping and transportation of the assay kit components.

NanoPin will also evaluate the performance of the second-generation nanoparticle platform with the selected monoclonal antibody from Aim 4.2 to ensure that they demonstrate reliable results that meet or exceed those of the first generation Nanoparticles. This analysis will be performed with serum from healthy donors that will be spiked with recombinant CFP10 and matching heavy-isotope-labeled internal standard peptide, trypsin digested, and then immunoprecipitated with the second-generation particles to capture target and internal standard peptides for quantitative analysis. The new platform will be evaluated for performance for the sensitive and specific detection and quantitation of the analyzed target biomarkers.

4. **IMPACT:**

   - **What was the impact on the development of the principal discipline(s) of the project?**

     Nothing to report.

   - **What was the impact on other disciplines?**

     Nothing to report.

   - **What was the impact on technology transfer?**

     Nothing to report.

   - **What was the impact on society beyond science and technology?**

   The technology developed in this proposal is a necessary advancement in infectious disease diagnostics. Improved methods for tuberculosis (TB) detection in all patient groups will reduce the global burden of TB on countries and economies throughout the world. TB kills over 1.5 million persons with approximately 10 million new active TB cases annually.

5. **CHANGES/PROBLEMS:**

   - **Changes in approach and reasons for change**

     Nothing to report.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

  For our Tanzania site, the only "change" was delay of study launch due to COVID-19 and initial clinical research halting/restrictions in early/mid 2020. Dr. Jason Bacha (Site PI) has been working closely with the local organizations to resume project planning and site preparation. The PI has also explored the capacity of other nearby sites to provide additional study entry points. These pro-active explorations were completed to ensure that DOD recruitment targets will be met in light of declining TB case notification well-recognized globally due to COVID-19.

  Based on our preliminary results, we have determined that specimens stored in ACD tubes are inadequate for our prototype blood based assay. Hence, prospectively collected specimens will be collected into standard red top tubes once HRPO approval is received.

  The Tulane lab has experienced delayed delivery and installation of miniMALDI since April, 2021. Now the instrument has been shipped from Japan and arrived in the US in Sepember. Per the company, it will be installed in the first week of November, 2021. We are aiming to complete all sub-aims proposed in Aim 3 by September 2022.

- **Changes that had a significant impact on expenditures**

  Nothing to Report

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

  Nothing to Report

- **Significant changes in use or care of human subjects**

  Nothing to Report

- **Significant changes in use or care of vertebrate animals.**

  Nothing to Report

- **Significant changes in use of biohazards and/or select agents**

  Nothing to Report

6. **PRODUCTS:**

   - **Publications, conference papers, and presentations**

     - **Journal publications.**

Shu, Q., Rajagopal, M., Fan, J., Zhan, L., Kong, X., He, Y. Rotcheewaphan, S., Lyon, C. J., Sha, W., Zelazny, A.M., **Hu, T.** IP-MS Analysis of ESX-5 and ESX-1 Substrates Enables Mycobacterial Species Identification. *View.* **2021**, under revision.
Acknowledgement of federal support? Yes

Mao, L., Lacourse, S., Kim, S., Ning, B., Bao, D., Fan, J., Sun, Z., Nackman, S., Mitchelle, C., **Hu, T.** Evaluation of a blood-based antigen test for tuberculosis in HIV-exposed children younger than 5 years. *BMC Medicine*. **2021**, published.
Acknowledgement of federal support? Yes

He, Y., Lyon, C. J., Nguyen D. T., Liu, C., Sha, W. Graviss, E., **Hu, T.** Serum-based diagnosis of pediatric tuberculosis by assay of *Mycobacterium tuberculosis* factors: a retrospective cohort study. *J. Clin. Micro*. **2021**, published.
Acknowledgement of federal support? Yes

Shu, Q., Kenny, T., Fan, J., Lyon, C., Cazares, L., **Hu. T. *** Species-specific Quantification of Circulating Ebolavirus Burden using VP40-derived Peptide Variants. *PLOS Pathogens*. **2021.** Accepted.
Acknowledgement of federal support? Yes

- **Books or other non-periodical, one-time publications.**

  None.

- **Other publications, conference papers, and presentations.**

1. Detection of Infectious Disease at Species Resolution with Pathogen-derived Peptidomes. AACC Annual Meeting 2021. Sept. 26-30, **2021**. Atlanta, GA.
2. Keynote Talk. 2021 Research Experience Symposium. Tulane University Department of Chemical Engineering. Aug. 6, **2021**. New Orleans, LA.
3. Written in Blood. Rice BioE colloquium, April 20, **2021**. Rice University, Houston, TX.
4. Nanotechnology-Enabled Biomarker Discovery for Personalized Diagnosis. Kate Gleason College of Engineering, Rochester Institute of Technology. February 25, **2021**.
5. Detection of Infectious Disease at Species Resolution with Pathogen-derived Peptidomes. Louisiana State University Health Science Center. February 9, **2021**.
6. Spinning Biological Trash into Diagnostic Gold. Division of Pathology and Laboratory Medicine's Ground Rounds at the University of Texas MD Anderson Cancer Center. February 6, **2021.**
7. Evaluation of a blood-based antigen test for tuberculosis in HIV-exposed children younger than 5 years. *The 50th Union Conference -TB Science 2020 Virtual conference*. Oct. 20-24, **2020**.

8. Blood-based antigen test for rapid TB diagnosis. *CTVD 6th Annual Virtual Meeting*. June 16-17, **2020**.

a. **Website(s) or other Internet site(s)**

None.

b. **Technologies or techniques**

Several advantages were realized in the Aim 4.1 efforts.
1. The trypsin-based enzyme digestion method was translated to a commercially viable process in terms of both cost and time. The previous method was clinically non-viable due to cost (>$20 per test for the trypsin component itself) and time (overnight digestion required). The new method utilizes an agarose resin, TPCK treated immobilized trypsin that enables 1-3 hour digestion times with a >10x cost reduction (<$2 per test for the new trypsin component).
2. Commercial protocols for the entire assay procedure from sample preparation to LC-MS analysis were developed. A validated bill of materials (BOM) for the tuberculosis assay was confirmed and supply chain with commercial terms & conditions was established.
3. These advances were integrated in the NanoPin quality management system (QMS). The QMS and NanoPin's Quality Control incorporated these advances from the determination of user needs to design inputs and outputs, reagent validation and testing, supply chain management, and product labeling.

These advances were shared with the members of this grant and especially with the research group of Professor Tony Hu at Tulane University School of Medicine. Professor Hu's research group directly benefits from the validation of the assay reagents, extreme cost savings for the trypsin digestion step, and the time savings from the refined commercial protocols. The LC-MS maintenance, QC protocols, and information on other application changes has been shared with the entire team.

c. **Inventions, patent applications, and/or licenses**

Nothing to report.

d. **Other Products**

i. data or databases;

1. The dataset "mycobacterial culture filtrate proteins identified by LC-MS/MS" has been successfully submitted to ProteomeXchange via the PRIDE database.
Project Name: mycobacterial culture filtrate proteins identified by LC-MS/MS
Project accession: PXD019069

Project DOI: 10.6019/PXD019069

2. The dataset "Classification of Mycobacterium down to subspecies and strain level by Proteomic Analysis of Clinical MGIT Sample" has been successfully submitted to ProteomeXchange via the PRIDE database.
Project Name: Classification of Mycobacterium down to subspecies and strain level by Proteomic Analysis of Clinical MGIT Sample
Project accession: PXD022102
Project DOI: 10.6019/PXD022102

ii. biospecimen collections;

As described in Aim 3, a prospective pediatric cohort has been under development specially for the award.

iii. audio or video products;

None

1. *software;*

None

2. *models;*

None

ii. educational aids or curricula;

None

iii. instruments or equipment;

None

iv. research material (e.g., Germplasm; cell lines, DNA probes, animal models);

None.

v. clinical interventions;

None.

vi. new business creation; and

vii.other.

None.

9. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

a. What individuals have worked on the project

Name:	Tony Y. Hu
Project Role	PI
Researcher Identifier (e.g. ORCID ID):	0000-0001-7255-5409
Nearest person month worked:	3
Contribution to Project:	Dr. Hu has been providing scientific direction, overall project management and coordination, and financial management for the team. He has been also serving as the information conduit between the DOD and the scientific team, while ensuring that the shared resources and infrastructure at all participating institutions are leveraged to their fullest. Dr. Hu evaluates program activities such as discussions of project milestones and data presentation and dissemination.
Funding Support:	W8IXWH1910926, R01AI113725, R01HD090927

Name:	Christopher Lyon
Project Role:	Tulane Site co-investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6
Contribution to Project:	Dr. Lyon has been participating the analysis of clinical and preclinical biomarkers of multiple different human disease conditions, which he will

	employ to supervise the scientific rigor of all aspects of assay design, authentication, methodology and reproducibility.
Funding Support:	W8IXWH1910926, R21EB026347

Name:	Qingbo Shu,
Project Role:	Tulane site Postdoctoral Associate
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	12
Contribution to Project:	Dr. Shu has been focusing on developing mass spectrometry based detection method for *Mtb*-specific antigens in mycobacterium infected patient blood samples.
Funding Support:	W8IXWH1910926

Name:	Shu Wang
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	12
Contribution to Project:	Dr. Wang has been assisting Dr. Hu to process sample and nanoparticle operation. She focuses on the metallization of nanoparticles and surface modification for isolating, detecting and validating TB biomarker signatures from patient blood samples.
Funding Support:	W8IXWH1910926

Name:	Bo Ning
Project Role:	Tulane site faculty
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6
Contribution to Project:	Dr. Ning has been working on sample processing, data collection and analysis under the supervision of Both Dr. Hu and Dr. Lyon.
Funding Support:	W8IXWH1910926

Name:	Andrew DiNardo
Project Role:	Houston site co-investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Dr. DiNardo has overseen work for the project as per the previously described scope of work. He has coordinated previously collected specimen contributing to the existing biorepository and coordinated with Drs Hu and Tombler the initial evaluation of the study assay as well as clinical metadata for the study. He has been overseeing enrollment in Houston with the Houston study coordinator, Ms Spieler.
Funding Support:	W8IXWH1910926

Name:	Zoe Spieler
Project Role:	Houston Study coordinator

Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6
Contribution to Project:	Ms. Spieler has maintained regulatory documents, liased with the Baylor IRB to ensure compliance, and has been actively recruiting participants through Ben Taub Hospital, Houston, TX. She has organized the Houston-specific RedCap database for clinical epidemiologic data capture, and has been utilizing the database for data entry.
Funding Support:	W8IXWH1910926

Name:	Jason Bacha
Project Role:	Mbeya, Tanzania site co--investigator
Researcher Identifier (e.g. ORCID ID):	ORCID ID: 0000-0002-1093-5227
Nearest person month worked:	1
Contribution to Project:	Dr. Bacha has acted as the study site PI for Mbeya, Tanzania. He has assisted on pre-study preparation for Tanzania, orientation/training of Tanzanian team, recruitment strategies for Tanzania, procurement of study supplies for Tanzania, obtaining IRBs and ethical clearances for the study in Tanzania, regularly study team meetings participation, and assistance with preparation for data/specimen collection and data entry for Tanzania study participants.
Funding Support:	W8IXWH1910926

Name:	Lwijisyo Minga
Project Role:	Tanzania study nurse

Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	9
Contribution to Project:	Mrs. Minga is a TB study nurse dedicated to implementing the TB study. She has been involved in all aspects of study preparation, and pre-study orientation/training. She has been trained to be responsible for participant enrolment, sample collection, participant data collection/entry, participant follow-up and communications, and assisting with all clinical implementation of the study in Mbeya, Tanzania.
Funding Support:	W8IXWH1910926

Name:	Alexander Kay, MD
Project Role:	Site co-investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Dr. Kay has acted as the study site PI for Eswatini. He has assisted on pre-study preparation for Eswatini, orientation/training of Swati team, recruitment strategies for Eswatini, procurement of study supplies for Eswatini, obtaining IRBs and ethical clearances for the study in Eswatini, regularly study team meetings participation, and assistance with preparation for data/specimen collection and data entry for Eswatini study participants.
Funding Support:	W8IXWH1910926

Name:	Jose Euberto Mendez Reyes
Project Role:	Biostatistician
Researcher Identifier (e.g. ORCID ID):	N/A

Nearest person month worked:	1
Contribution to Project:	Supported database and analysis plan development
Funding Support:	W8IXWH1910926

Name:	Anna Mandalakas
Project Role:	Senior TB Expert
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Dr. Mandalakas has supervised and supported the development of all aspects of study implementation including the development of data collection tools within REDCap, standard operating procedures, and training among other activities. She has led weekly Baylor team meeting to ensure seamless communication across all study sites and smooth integration of DOD study activities within existing infrastructure. She has also led communications with stake holders to ensure robust support for planned study activities.
Funding Support:	W8IXWH1910926

Name:	Santiago Longlax
Project Role:	Research Technician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	5
Contribution to Project:	Dr. Carrero Longlax has been essential for participant recruitment in Houston, particularly recruitment and longitudinal follow-up of Spanish-speaking participants. He regularly processes samples, and is the Houston-based study team member that can perform phlebotomy for outpatient participants if and when necessary. He has also been organizing and utilizing Freezerworks

	to manage samples, and coordinates sample shipping and receiving between sites.
Funding Support:	W8IXWH1910926

Name:	Godwin Mtetwa
Project Role:	Research Nurse
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	4
Contribution to Project:	Coordinates the clinical research team in Eswatini and has led preparation for initiation of clinical cohort recruitment across 4 project sites.
Funding Support:	W8IXWH1910926

Name:	Qiniso Dlamini
Project Role:	Laboratory Technician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	7
Contribution to Project:	Mr. Dlamini has supported development of lab tools and introduction of freezer works to ensure complete specimens capture in accordance with study SOPs once prospective recruitment commences
Funding Support:	W8IXWH1910926

Name:	Nkulungwane Mthethwa
Project Role:	Data Manager
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2
Contribution to Project:	Has participated in the develop of data capture tools and RedCap for the project
Funding Support:	W8IXWH1910926

Name:	Gcebile Dlamini
Project Role:	Screening Officer
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	12
Contribution to Project:	Ms. Dlamini has supported site preparation for participant recruitment at one of three Eswatini sites
Funding Support:	W8IXWH1910926

Name:	Zanele Mashwama
Project Role:	Screening Officer
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	12
Contribution to Project:	Ms. Mashwama has supported site preparation for participant recruitment at one of three Eswatini sites
Funding Support:	W8IXWH1910926
Name:	Mduduzi Mbatha

Project Role:	Driver
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	12
Contribution to Project:	Specimen transport
Funding Support:	W8IXWH1910926

Name:	Thomas Tombler
Project Role:	Nanopin site co-investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3
Contribution to Project:	Dr. Tombler has overseen the project for the sub-award scope of work. He coordinated all the personnel involved in the project and ensured all objectives are being met. He has been responsible for supervision, document management, procurement, and development of supply contracts and licensing requirements for key components.
Funding Support:	W8IXWH1910926

Name:	Wael Abdelgaliel, Ph.D.
Project Role:	Site Co-Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	4

Contribution to Project:	Dr. Abdelgaliel ran the experiments for the particle standardization, trypsin digestion reagent validation, and limit of detection testing. He supported QMS integration and implementation. He has been responsible for leading sample preparation, nanoparticle testing, and running the mass spectrometry equipment.
Funding Support:	W8IXWH1910926

Name:	Rahul Rao, P.E.
Project Role:	Medical Device Engineer
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Mr. Rao has provided engineering support in setting up the initial quality system which meets requirements of ISO 13485 and 21 CFR 820. He assisted in the design control framework and review process of user needs and design inputs initially and supported the full development, review, and verification of the Quality Management System (QMS).
Funding Support:	W8IXWH1910926

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

  Nothing to Report.

b. **What other organizations were involved as partners?**

Nothing to Report.

10. **SPECIAL REPORTING REQUIREMENTS**
    a. **COLLABORATIVE AWARDS:** Not applicable.
    b. **QUAD CHARTS:** Attached.
11. **APPENDICES:** N/A

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4. C. Liu *et al.*, Quantification of circulating Mycobacterium tuberculosis antigen peptides allows rapid diagnosis of active disease and treatment monitoring. *Proc Natl Acad Sci U S A* **114**, 3969-3974 (2017).
5. T. T. Feng *et al.*, Novel monoclonal antibodies to ESAT-6 and CFP-10 antigens for ELISA-based diagnosis of pleural tuberculosis. *Int J Tuberc Lung Dis* **15**, 804-810 (2011).
6. S. M. Arend *et al.*, Tuberculin skin testing and in vitro T cell responses to ESAT-6 and culture filtrate protein 10 after infection with Mycobacterium marinum or M. kansasii. *J Infect Dis* **186**, 1797-1807 (2002).
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8. U. H. Toprak *et al.*, Conserved peptide fragmentation as a benchmarking tool for mass spectrometers and a discriminating feature for targeted proteomics. *Mol Cell Proteomics* **13**, 2056-2071 (2014).
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