AMERICAN SOCIETY FOR MICROBIOLOGY | mBio®

RESEARCH ARTICLE

Identification and Validation of *Loa loa* Microfilaria-Specific Biomarkers: a Rational Design Approach Using Proteomics and Novel Immunoassays

Papa M. Drame,[a] Zhaojing Meng,[b] Sasisekhar Bennuru,[a] Jesica A. Herrick,[a]* Timothy D. Veenstra,[b]* Thomas B. Nutman[a]

Helminth Immunology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA[a]; Protein Characterization Laboratory, Frederick National Laboratory for Cancer Research, Leidos, Inc., Frederick, Maryland, USA[b]

* Present address: Jesica A. Herrick, Division of Infectious Diseases, Immunology, and International Medicine, Department of Medicine, University of Illinois at Chicago, Chicago, Illinois, USA; Timothy D. Veenstra, Department of Applied Science, Maranatha Baptist University, Watertown, Wisconsin, USA.

ABSTRACT **Immunoassays are currently needed to quantify *Loa loa* microfilariae (mf). To address this need, we have conducted proteomic and bioinformatic analyses of proteins present in the urine of a *Loa* mf-infected patient and used this information to identify putative biomarkers produced by *L. loa* mf. In total, 70 of the 15,444 described putative *L. loa* proteins were identified. Of these 70, 18 were *L. loa* mf specific, and 2 of these 18 (LOAG_16297 and LOAG_17808) were biologically immunogenic. We developed novel reverse luciferase immunoprecipitation system (LIPS) immunoassays to quantify these 2 proteins in individual plasma samples. Levels of these 2 proteins in microfilaremic *L. loa*-infected patients were positively correlated to mf densities in the corresponding blood samples ($r = 0.71$ and $P < 0.0001$ for LOAG_16297 and $r = 0.61$ and $P = 0.0002$ for LOAG_17808). For LOAG_16297, the levels in plasma were significantly higher in *Loa*-infected (geometric mean [GM], 0.045 μg/ml) than in uninfected ($P < 0.0001$), *Wuchereria bancrofti*-infected ($P = 0.0005$), and *Onchocerca volvulus*-infected ($P < 0.0001$) individuals, whereas for LOAG_17808 protein, they were not significantly different between *Loa*-infected (GM, 0.123 μg/ml) and uninfected ($P = 0.06$) and *W. bancrofti*-infected ($P = 0.32$) individuals. Moreover, only LOAG_16297 showed clear discriminative ability between *L. loa* and the other potentially coendemic filariae. Indeed, the specificity of the LOAG_16297 reverse LIPS assay was 96% (with a sensitivity of 77%). Thus, LOAG_16297 is a very promising biomarker that will be exploited in a quantitative point-of-care immunoassay for determination of *L. loa* mf densities.**

IMPORTANCE ***Loa loa*, the causative agent of loiasis, is a parasitic nematode transmitted to humans by the tabanid *Chrysops* fly. Some individuals infected with *L. loa* microfilariae (mf) in high densities are known to experience post-ivermectin severe adverse events (SAEs [encephalopathy, coma, or death]). Thus, ivermectin-based mass drug administration (MDA) programs for onchocerciasis and for lymphatic filariasis control have been interrupted in parts of Africa where these filarial infections coexist with *L. loa*. To allow for implementation of MDA for onchocerciasis and lymphatic filariasis, tools that can accurately identify people at risk of developing post-ivermectin SAEs are needed. Our study, using host-based proteomics in combination with novel immunoassays, identified a single *Loa*-specific antigen (LOAG_16297) that can be used as a biomarker for the prediction of *L. loa* mf levels in the blood of infected patients. Therefore, the use of such biomarker could be important in the point-of-care assessment of *L. loa* mf densities.**

Received 7 December 2015 **Accepted** 14 January 2016 **Published** 16 February 2016

Citation Drame PM, Meng Z, Bennuru S, Herrick JA, Veenstra TD, Nutman TB. 2016. Identification and validation of *Loa loa* microfilaria-specific biomarkers: a rational design approach using proteomics and novel immunoassays. mBio 7(1):e02132-15. doi:10.1128/mBio.02132-15.

Editor Louis M. Weiss, Albert Einstein College of Medicine

Address correspondence to Thomas B. Nutman, tnutman@niaid.nih.gov.

This article is a direct contribution from a Fellow of the American Academy of Microbiology.

Loiasis, a tropical disease caused by the filarial parasite *Loa loa* (commonly known as the African eyeworm), affects approximately 13 million people in Central and West Africa, with the highest prevalences found in Angola, Cameroon, Gabon, Republic of the Congo, Central African Republic, Democratic Republic of the Congo, and Nigeria. Like most of the blood-borne filariae, the overwhelming majority of *L. loa* infections are clinically asymptomatic; moreover, *L. loa* has been viewed as a relatively unimportant infection (1, 2). However, *L. loa* has gained prominence in the past 20 years because of the serious adverse events (SAEs) associated with ivermectin distribution as part of mass drug administration (MDA) campaigns targeted toward elimination of onchocerciasis and lymphatic filariasis (LF) (3–5). These post-ivermectin SAEs, which include irreversible neurologic complications and deaths, have typically been observed in individuals with greater than 30,000 *L. loa* microfilariae (mf)/ml of blood (5). Consequently, ivermectin-based MDA programs have been delayed or paused in parts of Africa where *L. loa* is coendemic with either LF or onchocerciasis (6). The use of alternative "safer" treatment options (7–10) for onchocerciasis and LF has been proposed

TABLE 1 Details of *L. loa* mf-specific proteins identified in urine of patients[a]

Protein ID no.	Description	Peptide count	Mol mass (kDa)	FKPM	Homology to human		
					E value	% coverage	Homologue?
LOAG_00073	Heat shock protein 90	2	69.4	3,841.1	0E+00	99	Yes
LOAG_01395	WD repeats and SOF1 domain-containing protein	2	51.0	101.3	5E−174	97	Yes
LOAG_01611	Hypothetical protein	2	64.5	29.8	1E−85	74	Yes
LOAG_02628	Low-density lipoprotein receptor repeat class B-containing protein	2	196.0	33.7	4E−129	86	Yes
LOAG_03988	Hypothetical protein	2	54.0	152.3	2E−08	92	Yes
LOAG_04876	Peptidase M16 inactive domain-containing protein	2	47.4	40.7	2E−43	93	Yes
LOAG_05583	U4/U6 small nuclear ribonucleoprotein hPrp4	2	56.6	43.4	2E−157	99	Yes
LOAG_05701	14-3-3-like protein 2	2	28.3	2,392.0	1E−147	94	Yes
LOAG_05915	**Hypothetical protein**	2	**104.3**	**14.7**	**2E−02**	**33**	**No**
LOAG_06631	Troponin	2	171.1	8.8	1E−05	13	Yes
LOAG_09325	Hypothetical protein	2	33.0	68.3	1E−13	27	Yes
LOAG_10011	Hypothetical protein	2	13.6	3,257.3	7E−66	98	Yes
LOAG_16297	**Hypothetical protein**	2	**14.3**	**0.4**	**5E−04**	**67**	**No**
LOAG_17249	Pyruvate kinase	2	59.0	609.7	0E+00	99	Yes
LOAG_17808	**PWWP domain-containing protein**	2	**69.8**	**13.9**	**9E−04**	**5**	**No**
LOAG_18456	Cullin-associated NEDD8-dissociated protein 1	2	124.1	45.5	0E+00	98	Yes
LOAG_18552	**Hypothetical protein**	2	**106.5**	**2.1**	**1E−03**	**44**	**No**
LOAG_19057	Hypothetical protein	3	30.2	89.2	5E−128	77	Yes

[a] Proteins that do not share significant sequence homology to human proteins are highlighted in bold. FPKM represents the relative mRNA expression level obtained using transcriptome sequencing (RNA-seq) (22).

in regions of *L. loa* coendemicity, but none has been found to be practical and/or efficacious. The strategy that has gained the most traction in these settings has been termed "test and (not) treat" (TNT), whereby those at risk for post-ivermectin SAEs are identified and excluded from ivermectin-based MDA programs. Such a TNT strategy, however, requires a rapid and point-of-care (POC) test allowing for the quantification of *L. loa* mf loads.

Currently, the definitive identification and quantification of *L. loa* mf can be made either by the traditional microscopic methods using calibrated stained slide-based methods (11, 12) or by using quantitative PCR (qPCR) tests, the latter adding an additional level of sensitivity (13). However, both of these methods are time intensive, relatively expensive (qPCR), or impractical for rapid testing at the POC. Recently, a mobile phone-based video microscopy system (CellscopeLoa) has been developed as a POC tool that allows rapid and accurate counting of *L. loa* mf (14). However, such a device is not as precise as other methods when assessing low *L. loa* mf densities (<150 mf/ml of blood) because of sampling limitations, and manufacturing for widespread use is lacking. Therefore, a POC quantitative immunoassay for mf-derived antigens could provide a second-generation POC assessment tool.

During their life cycle, *L. loa* parasites have five distinct morphological stages in their human and invertebrate (*Chrysops*) hosts (15). The mf, released by adult female worms into the human circulation, produce large and presumably measurable amounts of proteins and glycoproteins (16–18), either through excretion or active secretion (so-called "ES products"). Studies with *Brugia malayi* indicate that relatively more ES products are produced by the mf by any of the other stages (adult and other larval stages) of the parasite *in vitro* (19). However, unlike *B. malayi*, for which the life cycle can be maintained in animal models, providing large numbers of parasites of all stages, the biology of *L. loa* mf (e.g., their proteome/secretome) has been difficult to explore because parasite material is limited as it must be obtained from infected human subjects.

We postulated that certain *L. loa* parasite antigens secreted or excreted into the human bloodstream might not be fully reabsorbed following filtering by the renal glomeruli and could thereby be concentrated in the urine of *Loa*-infected individuals. Studies have shown that urine is a sample source of high importance for biomarker discovery because it is easily available, can be collected noninvasively in large quantities (20, 21), and, from a protein point of view, is much less complex than human serum or plasma. In the present study, utilizing a nontargeted (shotgun) nanobore reversed-phase liquid chromatography tandem mass spectrometry (RPLC-MS/MS) proteomic approach, we attempted to identify *L. loa* mf proteins present in the urine of *Loa*-infected patients that could be used as the basis of quantitative immunoassays for the detection of *L. loa* mf-specific biomarkers in either plasma/serum or in urine.

RESULTS

Specificity of identified and selected proteins. Mass spectrometry analyses of urine samples from an *L. loa*-infected individual resulted in the identification of spectra matching those of 70 *L. loa* proteins, of which 18 proteins were detectable by at least 2 unique peptides and not present in normal uninfected urine (Table 1). All 18 proteins were identified to be *L. loa* mf proteins. Their corresponding mRNA expression (22) ranged from 2.07 to 3,841.10 fragments per kilobase per million (FPKM). Eight (44.4%) of the 18 *L. loa* urine-specific proteins were annotated as "hypothetical" proteins with unknown function (Table 1).

Further filtering the data for proteins with little or no sequence homology with human proteins shortlisted four *L. loa* proteins: LOAG_05915, LOAG_16297, LOAG_17808, and LOAG_18552 (Table 1). These four proteins were then assessed for having homologues in the other filariae sequenced to date—*B. malayi*, *Wuchereria bancrofti*, and *Onchocerca volvulus* (Table 2). As can be seen, LOAG_05915, LOAG_17808, and LOAG_18552 share significant sequence homology *W. bancrofti*, *B. malayi*, and *O. volvulus* proteins, while LOAG_16297, a small 14-kDa hypothetical

TABLE 2 Specificity of the four downselected *L. loa* mf proteins

Protein ID (accession no.)	Immunogenic peptide sequence	*B. malayi*		*W. bancrofti*		*O. volvulus*	
		% identity	E value	% identity	E value	% identity	E value
LOAG_05915 (EFO22569.1)	CMRDKYRDTENE CLDDEKEQYNKNL	76	0	65	0	No hit	
LOAG_18552 (EJD74082.1)	CEEKQNRNEKPANGD CEQEKLEKPKSKKPNP	No hit		87	0	42	0
LOAG_17808 (EJD74956.1)	CEGENKRDGKRRMDKSP CRPFDDERNSYDKNGN	91	0	91	0	No hit	
LOAG_16297 (EFO12236.1)	CVETRKYENRK CDSDTGNRNDESYKFKQ	No hit		No hit		No hit	

protein, showed no homology to proteins of any of these filarial parasites nor to any nematode for which genomic sequences are available.

Immunogenicities of the four selected *L. loa* proteins. The immunogenicities of the protein antigens were assessed using hyperimmune rabbit antisera in a standard luciferase immunoprecipitation system (LIPS) assay. As shown in Fig. 1, there was minimal reactivity with the respective prebleed sera and robust reactivity with the hyperimmune sera (and their purified IgG) from two of the four fusion proteins, LOAG_17808 and LOAG_16297. In addition, the LOAG_17808 fusion protein was also recognized by purified IgG antibodies raised against *L. loa* somatic mf antigen (Fig. 1).

To evaluate the reactivity of these proteins in humans, sera/plasma from *L. loa*-infected patients and uninfected control subjects were used and compared to the reactivity to *L. loa* SXP-1, a previously described *L. loa* antigen (23). As expected, healthy-control samples had very low signals, with median anti-LOAG_16297, anti-LOAG_17808, and anti-SXP-1 antibody titers of 236, 97, and 746 light units (LU), respectively (Fig. 2). For *L. loa*-infected patients, the median values were 6 times higher for LOAG_16297 (1,423 LU), 148 times higher for LOAG_17808 (14,317 LU), and 905 times higher for SXP-1 (674,990 LU) than the median titers of the uninfected healthy controls. The differences between *L. loa*-infected patients and uninfected controls

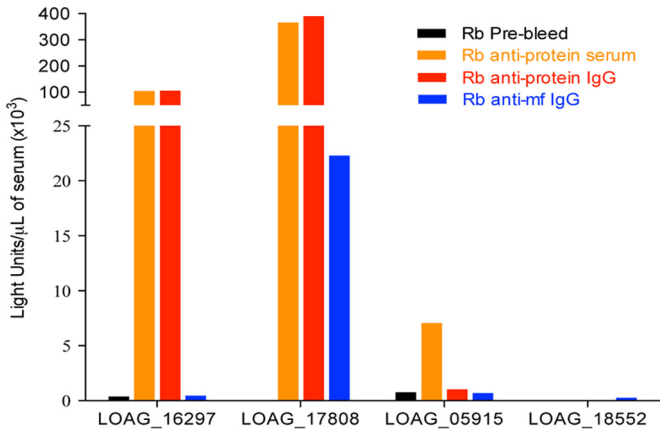

FIG 1 Reactivities of the four fusion proteins to their specific antibodies. Antisera (orange) raised against the two most immunogenic peptides of each protein, IgG purified from those antisera (red), and purified IgG anti-*L. loa* mf somatic antigen (blue) were used to test reactivities to the four fusion proteins. "Rb prebleed" antisera (black) are antisera collected prior to immunization. The protein names are indicated under the *x* axis.

were significant for all tested fusion proteins ($P < 0.0001$). In addition, a receiver operating characteristic (ROC) analysis shows that the two *L. loa* mf antigens were able to accurately distinguish *Loa*-infected from *Loa*-uninfected individuals: LOAG_16297 with 96.7% sensitivity and 100% specificity, using a threshold of 760 LU (Fig. 2A), and LOAG_17808 with 100% sensitivity and 96.7% specificity, using a threshold of 862 LU (Fig. 2B). In comparison, *L. loa* SXP-1 showed 100% sensitivity and 100% specificity using a threshold of 10,785 LU on the same set of 31 patients and 31 controls (Fig. 2C).

Competitive LIPS assay results. The ability to identify LOAG_16297 and LOAG_17808 in an antigen detection system was next tested using a heretofore-undescribed competitive LIPS assay (Fig. 3A) in *L. loa*-, *W. bancrofti*-, and *O. volvulus*-infected individuals and uninfected healthy controls. Using pooled human AB serum spiked with increasing concentrations of the appropriate antigen, we were able to generate standard curves that allowed us to relate the percentage of inhibition in the competitive LIPS assay to the antigen concentration present in the sera (Fig. 3B). We then used these standard curves to quantitate the levels of circulating protein in the serum of *Loa*-infected patients and in the control groups. For LOAG_16297 (Fig. 4A), the geometric mean level of detectible protein in serum/plasma was 17.88 ng/ml in *Loa*-infected subjects, whereas it was negligible in *W. bancrofti*- and *O. volvulus*-infected subjects and in uninfected subjects. Using a cutoff based on an ROC analysis (5 ng/ml), we can see that there were measurable antigen levels in 12/26 microfilaremic *Loa*-infected individuals compared to 0/5 amicrofilaremic *Loa*-infected, 0/31 uninfected ($P < 0.0001$), 0/15 *O. volvulus*-infected ($P = 0.004$), and 1/15 W. *W. bancrofti*-infected ($P = 0.03$) individuals. For LOAG_17808 (Fig. 4B), the geometric mean levels of protein were 36.68 ng/ml in *Loa*-infected, 21.04 ng/ml in *W. bancrofti*-infected, and 1.86 ng/ml in *O. volvulus*-infected individuals and 4.97 ng/ml in uninfected individuals. Again using ROC analysis (with an upper threshold of 39 ng/ml), there were detectible LOAG_17808 levels in 9/26 microfilaremic *Loa*-infected subjects for 0/5 in the amicrofilaremic *Loa*-infected group, 0/31 in the uninfected control group ($P = 0.002$), 0/15 in the *O. volvulus*-infected group ($P = 0.02$), and 4/15 in the *W. bancrofti*-infected group ($P = 0.9$).

LOAG_16297 and LOAG_17808 LIPS assay performance compared to microscopy. To further assess the performance of the LOAG_16297- and LOAG_17808-based LIPS antigen detection assays, optimized competitive LIPS assays were run using plasma samples from 26 *Loa* microfilaremic (*Loa* mf$^+$) subjects with a range of mf counts and from 25 healthy (uninfected) indi-

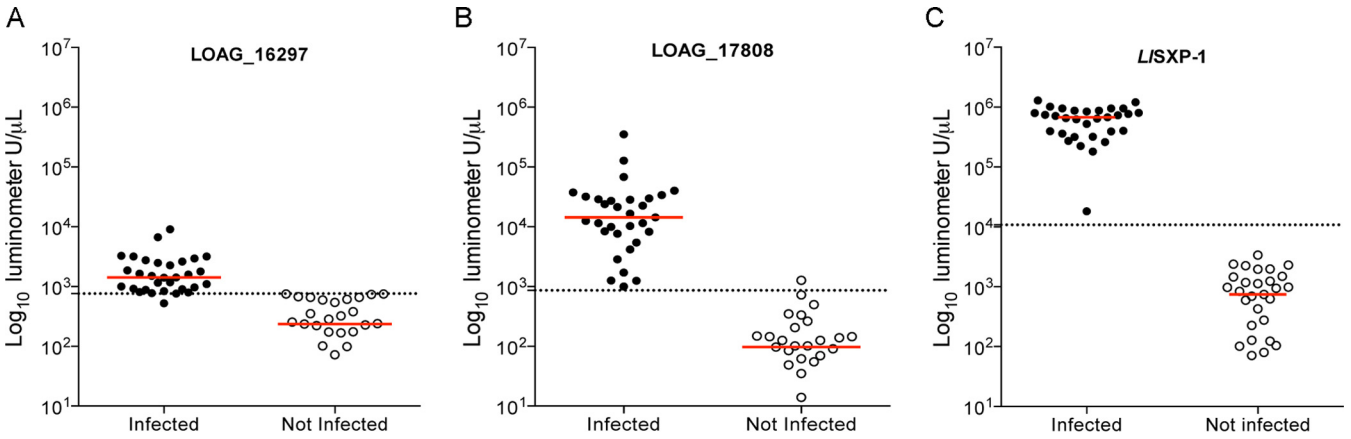

FIG 2 Immunogenicity of LOAG_16297, LOAG_17808, and SXP-1 in humans. The levels of IgG specific to LOAG_16297 (A), LOAG_17808 (B), and SXP-1 (C) were assessed by LIPS assay, and light units (LU) were compared between *Loa*-infected subjects and uninfected controls. The horizontal red solid line represents the median level for each group, and the horizontal black dotted line indicates the threshold of sensitivity/specificity of the assay determined by ROC analysis. Each individual is represented by a single dot, with closed circles used for the *Loa*-infected individuals and open circles for the uninfected individuals.

viduals (Table 3). Considering microcopy to be the "gold standard" for *L. loa* mf quantification, the LOAG_16297 antigen LIPS assay had a sensitivity of 76.9% (95% confidence interval [95% CI], 56.3 to 91.0%), a specificity of 96.0% (95% CI, 79.6 to 99.9%), a positive predictive value (PPV) of 95.2% (95% CI, 76.2 to 99.9%), and a negative predictive value (NPV) of 80% (95% CI, 61.4 to 92.2%). For the LOAG_17808 competitive LIPS assay, the sensitivity, specificity, PPV, and NPV were 80.7 (95% CI, 60.6 to 93.5%), 37.5 (95% CI, 18.8 to 59.4%), 58.3% (95% CI, 40.8 to 74.5%), and 64.3% (95% CI, 35.1 to 87.2%).

Correlation between the amount of LOAG_16297 antigen and the number of microfilariae. To evaluate if the levels of antigen circulating in the plasma of *Loa*-infected individuals with a range of mf counts were correlated with the density of mf, Spearman's rank correlation was performed between the plasma concentrations of the LOAG_16297 and LOAG_17808 proteins and the corresponding counts of *L. loa* mf as determined by microscopy (Fig. 5). As can be seen, there were significant positive correlations for LOAG_16297 ($r = 0.71$, $P < 0.0001$) (Fig. 5A) and for LOAG_17808 ($r = 0.61$, $P = 0.0002$) (Fig. 5B).

DISCUSSION

L. loa infection has recently gained prominence because of the SAEs occurring after ivermectin administration in some individuals harboring high *L. loa* mf densities (4, 5). Most of the currently available tools and methods (11, 13, 23, 24) that are being used to quantify *L. loa* mf are impractical for POC field-testing. Developing a quantitative immunoassay for *L. loa* mf that could be used for field screening to identify individuals at high risk of SAE would be of great benefit to MDA programs.

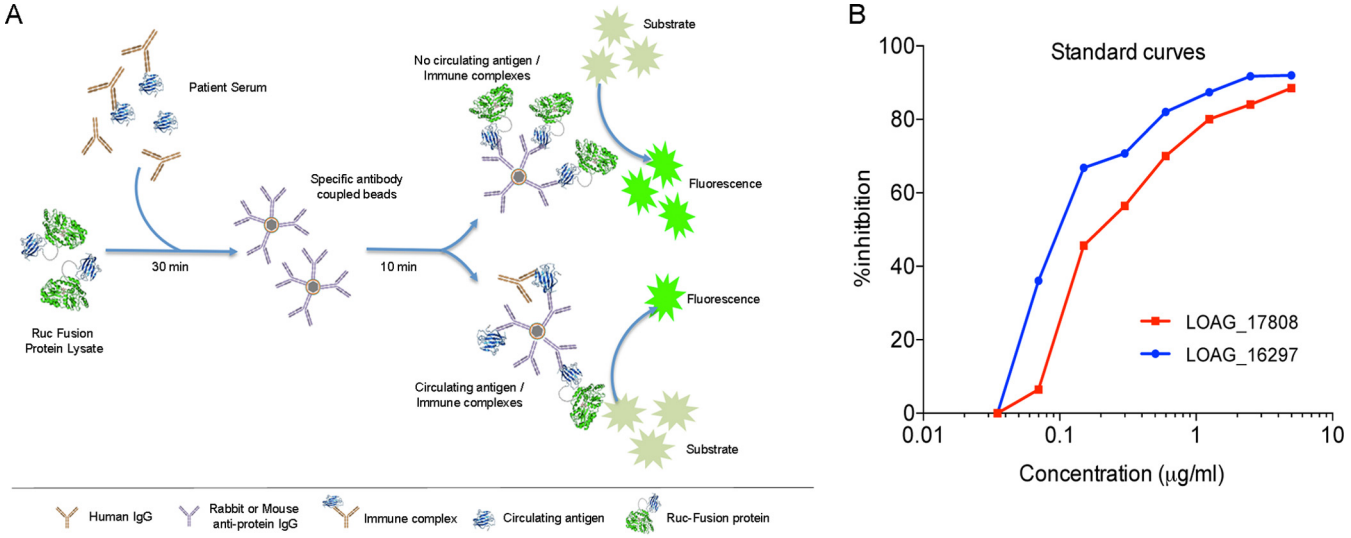

FIG 3 Principle of the antigen LIPS assay and relationship between the percentage of protein inhibition and amount of protein. (A) Schematic of the general steps involved in competitive LIPS antigen detection in which the *Renilla* luciferase (Ruc) fusion constructs of the antigen of interest are incubated with serum containing unfused antigen. These antigens are then immobilized on agarose beads containing antigen-specific IgG. After washing, the amount of specific antigen present is determined by the inhibition of the Ruc fusion construct by the unfused antigen after addition of luciferase substrate. Panel B shows the percentage of inhibition as a function of spiked recombinant protein in human AB serum for LOAG_16297 (blue) and LOAG_17808 (red).

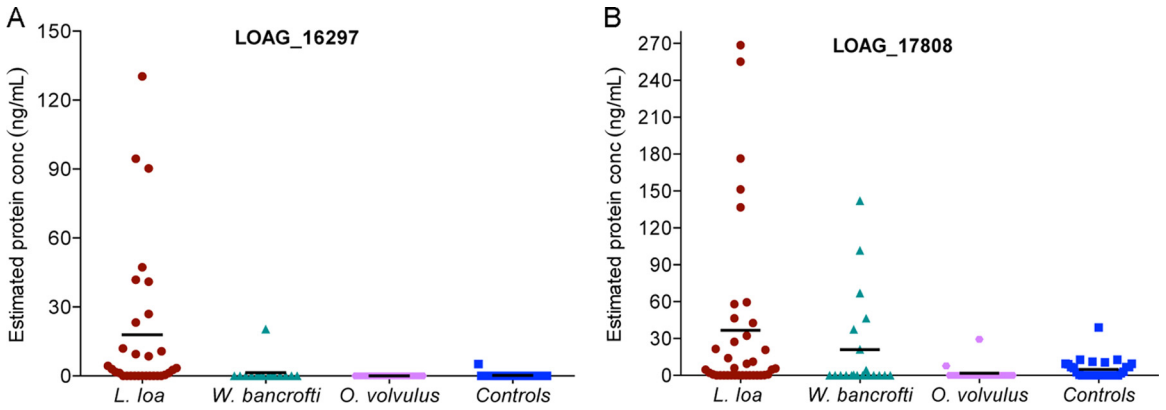

FIG 4 Detection of LOAG_16297 and LOAG_17808 in plasma samples by LIPS assay. The quantities of LOAG_16297 (A) and LOAG_17808 (B) were estimated for 31 *L. loa*-infected (red), 15 *W. bancrofti*-infected (green), and 15 *O. volvulus*-infected (purple) individuals and 25 uninfected (blue) individuals, extrapolating from standard curves as represented in Fig. 3B. The horizontal solid black line in each group indicates the geometric mean in nanograms per milliliter of protein, and each value is represented by an individual dot.

Thus, we have identified 18 proteins present only in *L. loa* mf-infected urine by using a high-throughput RPLC-MS/MS proteomic approach. We then developed antigen-based competitive LIPS assays for the 2 (LOAG_16297 and LOAG_17808) that were immunogenic and highly (and/or relatively) specific to *L. loa* mf. One of these, LOAG_16297 showed excellent diagnostic performance and has great promise for a potential field use as a POC diagnostic tool.

The presence of parasite proteins in urine should be a surrogate for their availability in the circulation and, therefore, should provide an accurate source for biomarker discovery useful for disease diagnosis (25). In our study, only 18 were found exclusively in the urine of the *Loa*-infected patient compared to urine from uninfected individuals, suggesting some promiscuity in this proteomic approach. This number of urine-identified proteins was relatively low considering the total number of putative *L. loa* proteins (15,444) (22). That is certainly due to the fact that there are many fewer proteins in urine than in the plasma (26), the majority of proteins found in the blood being reabsorbed through the renal glomeruli. We also cannot exclude the role played by variability among the analytes in the urine (27), factors related to the MS/MS instrument itself (28), or the fact that so many *Loa* proteins had similar human sequences. Therefore, it would be more advantageous to perform proteomics of urine samples from multiple *Loa*-infected individuals. Nevertheless, the data gleaned from one urine sample from an infected individual allowed us to identify potential biomarker candidates and then validate the most important ones.

Most of the identified *Loa*-specific urine proteins (14/18) have orthologues in humans. Among the four *Loa* mf proteins unrelated to human proteins, only two, LOAG_16297 and LOAG_17808, could be studied closely as they were immunogenic in rabbits. In addition, antibodies raised against *L. loa* crude somatic mf antigen recognized LOAG_17808 and LOAG_16297 (to a lesser degree), suggesting that they make up a significant fraction of the mf-specific antigen mix. Furthermore, antibodies to both LOAG_16297 and LOAG_17808 were present in *L. loa*-infected patients. Although not the main purpose of the present study, these antigens show promise in an antibody-based immunoassay with sensitivities and specificities similar to or close to what has been observed with *L. loa* SXP-1 (23, 29) antibody profiling for *L. loa* diagnosis.

In general, antibody-based assays often are unrelated to parasite burden, and a correlation between antibody level and parasite density is likely to be difficult (if not impossible). In addition, specific antibodies cannot distinguish between previous and new infections and often persist indefinitely after treatment or exposure (29). Antigen-based assays are then the sine qua non for diagnosis of infectious diseases. Thus, we developed methods for rapidly testing the validity of such assays using a single antibody specific for the protein in question and a mammalian-expressed recombinant protein that could be used without purification. Such an assay, termed reverse (or competitive) LIPS, relies on the ability of the antigen(s) in serum (or other biological samples) to inhibit a fixed concentration of the same protein that is luciferase fused (Fig. 3A). In addition to its simplicity, the competitive LIPS assay could identify *L. loa*-infected patients and quantify the *L. loa* mf level rapidly and with good accuracy. Only 45 to 60 min of total processing time (including preparation and wash times) per 94 plasma samples is needed compared to hours for enzyme-linked immunosorbent assays (ELISAs). Moreover, ELISAs for antigen

TABLE 3 Performance of *L. loa* mf-specific proteins on clinical samples using a LIPS competitive assay[a]

LIPS assay type	Status	No. of samples: mf$^+$	No. of samples: mf$^-$	% sensitivity (95% CI)	% specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
LOAG_16297	Positive	20	1	76.9 (56.3–91.0)	96.0 (79.6–99.9)	95.2 (76.2–99.9)	80.0 (61.4–92.3)
	Negative	6	24				
LOAG_17808	Positive	21	15	80.7 (60.6–93.5)	37.5 (18.8–59.4)	58.3 (40.8–74.5)	64.3 (35.1–87.2)
	Negative	5	9				

[a] PPV, positive predictive value; NPV, negative predictive value. The 95% confidence interval (95% CI) is indicated for each parameter.

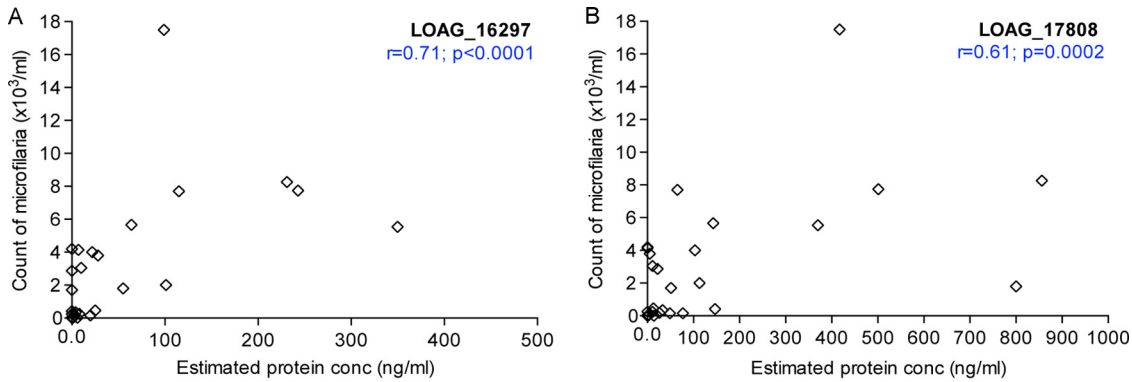

FIG 5 Correlation between the quantities of detected antigen (represented by detected protein in *L. loa* mf-infected individuals) and *L. loa* mf count.

often require at least two different antibodies (e.g., directed against different epitopes of the same protein), a requirement that often cannot be satisfied. The presumed increased sensitivity of the LIPS-based system is likely due to the assay performance in solution, our ability to detect many more conformational epitopes than a standard solid-phase ELISA (23), and the fact that highly purified recombinant antigen is not needed.

In the present study, we have shown that both circulating LOAG_16297 and LOAG_17808 antigens can be detected in some plasma of *Loa*-infected individuals. Interestingly and more in line with its potential utility as a POC method to identify those at risk for SAEs following ivermectin, there was a significant positive relationship between the amounts of LOAG_16297 and LOAG_17808 proteins detected in *Loa*-infected samples and the mf levels in blood of the same samples. Furthermore, the assay to detect LOAG_16297 described herein showed the best correlation to mf data and had the best specificity, PPV and NPV (Fig. 5 and Table 3). The lower specificity of the assay for LOAG_17808 is not surprising since the protein shares 91% sequence identity to a PWWP domain protein of *W. bancrofti* (and other filariae).

There are currently no commercial products available using LIPS-based tests. In addition, the development of a LIPS assay for a POC use may be difficult (if not impossible) mainly because of the high cost of the LIPS platform. Nevertheless, the use of LIPS has allowed us to validate a single identified antigen (LOAG_16297) as being quantitative and specific. With a potential candidate biomarker in hand, we hypothesize that generation of monoclonal antibodies for use in a cheaper standard antigen capture or lateral-flow immunoassay can be configured for POC testing. The use of monoclonal antibodies will increase the affinity of the searched antigens to their specific antibodies and likely improve the performance of the assay.

In summary, we have used an untargeted protein profiling approach (RPLC-MS/MS) to discover 18 new putative biomarkers of *L. loa* mf infection in the urine of a microfilaremic patient with *L. loa*. Among them, one immunogenic and highly *L. loa* mf-specific protein, LOAG_16297, can be detected in plasma/serum in a competitive LIPS assay format, with the amounts being detected correlating well with the quantity of *L. loa* mf found in the peripheral blood. Therefore, the use of LOAG_16297 as a biomarker could be important in a POC assessment tool to be used as the basis of an effective TNT strategy.

MATERIALS AND METHODS

Study population and samples. Samples were collected from subjects as part of registered protocols approved by the Institutional Review Boards of the National Institute of Allergy and Infectious Diseases for the filaria-infected patients (NCT00001345) and for healthy donors (NCT00090662). Written informed consent was obtained from all subjects.

Urine samples from one microfilaremic (17,000 mf/ml) *L. loa*-infected patient assessed at the NIH Clinical Center and one normal North American donor (who had never traveled outside the United States) were used for the profiling of specific *L. loa* mf proteins by RPLC-MS/MS.

Plasma samples used to validate the utility of potential biomarkers were from *L. loa*-infected individuals ($n = 31$ [26 microfilaremic and 5 amicrofilaremic]). Samples used as controls included those from subjects with *W. bancrofti* infection (mf^+; $n = 15$) from India and the Cook Islands (both nonendemic for *L. loa*), subjects with *O. volvulus* infection (mf^+; $n = 15$) from Ecuador (nonendemic for *L. loa*), and those from North America who had no history of exposure to filariae or other helminths and who had never traveled outside North America ($n = 31$). The parasitological diagnosis of all infections was made based on the demonstration of mf in the blood (for *W. bancrofti* and *L. loa*) or in the skin (for *O. volvulus*) using standard techniques (11, 30) or by finding adult parasites in the tissues (e.g., the eye for *L. loa*).

Sample preparation prior to mass spectrometric analysis. Urine samples were processed according to a workflow adapted from Nagaraj et al. (31). Briefly, urine samples were centrifuged for 15 min at 4°C and the supernatant was concentrated using a spin filter with a molecular mass cutoff of 3 kDa. Proteins were precipitated by acetone precipitation and subsequently treated in 10 mM Tris-HCl at 95°C for 5 min. The samples were then reduced, alkylated, and double digested with Lys-C in combination with trypsin overnight at 37°C. Tryptic peptides were further desalted, lyophilized, reconstituted in 25% acetonitrile with 0.1% formic acid, and further fractionated using strong cation exchange (SCX) chromatography. The SCX fractions of the urine samples were pooled into 32 fractions, lyophilized, and reconstituted in 0.1% trifluoroacetic acid (TFA) to be analyzed by liquid chromatography-mass spectrometry (LC-MS).

Nanobore RPLC-MS/MS. Nanobore reversed-phase liquid chromatography-tandem mass spectrometry (RPLC-MS/MS) was performed using an Agilent 1200 nanoflow LC system coupled online with an LTQ Orbitrap Velos mass spectrometer. The RPLC column (75-μm inside diameter [i.d.] by 10 cm) was slurry packed in-house with 5-μm, 300-Å pore-size C_{18} stationary phase into fused silica capillaries with a flame-pulled tip. The mass spectrometer was operated in a data-dependent mode in which each full MS scan was followed by twenty MS/MS scans, wherein the 20 most abundant molecular ions were dynamically selected for

collision-induced dissociation (CID) using a normalized collision energy of 35%.

Protein identification and quantification. The RPLC-MS/MS data were searched using SEQUEST through Bioworks interface against an *L. loa* database downloaded from the Broad Institute (version 2.2). Dynamic modifications of methionine oxidation as well as fixed modification of carbamidomethyl cysteine were also included in the database search. Only tryptic peptides with up to two missed cleavage sites meeting the following specific SEQUEST scoring criterion were considered legitimate identifications: delta correlation (ΔC_n) of ≥0.1 and charge-state-dependent cross-correlation (X_{corr}) of ≥1.9 for $[M+H]^{1+}$, ≥2.2 for $[M + 2H]^{2+}$, and ≥3.5 for $[M + 3H]^{3+}$.

Transcriptomics data. mRNA expression levels (putative proteins) of the mf state of *L. loa* were obtained using RNAseq as part of the *L. loa* genome project previously described (22).

Protein/peptide selection for immunoassays. *L. loa* mf proteins identified only in the infected urine (absent in the uninfected urine) were downselected for immunoassays based on comparison of sequence homologies against human proteins and those of *L. loa* and other related filarial species (*B. malayi*, *O. volvulus*, and *W. bancrofti*) or any other relevant nematode for which genome is available.

Proteins that showed no or little homology to non-*Loa* sequences were selected for identification of immunogenic peptides using Protean (Lasergene Suite). Among these, we chose the 2 peptides that were potentially the most immunogenic and *Loa* specific (i.e., with no significant hit to human or other filarial nematodes) per protein. These peptides were synthesized by the NIAID Peptide Facility as unconjugated free peptides and conjugated to keyhole limpet hemocyanin (KLH), the latter used to produce specific polyclonal antibodies in rabbits.

Generation of rabbit polyclonal antibodies. KLH-conjugated peptides were used to raise polyclonal antisera in rabbits using standard protocols as previously described (32). In addition, polyclonal antisera were raised against a somatic extract of *L. loa* mf using the same standardized protocols. After assessment of the reactivity of each of the antisera to its appropriate free peptide by enzyme-linked immunosorbent assay (ELISA), the IgG was purified from the sera using protein A/G (Pierce, Rockford, IL) columns. These purified IgG antibodies were used as capture antibodies in the luciferase immunoprecipitation system (LIPS) assay for antigen detection (see below).

Fusion proteins and COS-1 cell transfection. Fusion proteins were made for each of the *in silico* selected proteins by cloning the full-length gene expressing the protein of interest into a FLAG-epitope-tagged mammalian *Renilla reniformis* luciferase (Ruc)-containing expression vector, pREN2 (33). Extracts (lysates) containing the light-emitting Ruc-antigen fusions were prepared from 100-mm^2 dishes of 48-h-transfected COS-1 cells as previously described (33, 34) and frozen until use for LIPS.

LIPS-based antibody and antigen detection systems. For evaluation of antibody titers, a standard LIPS antibody-based assay was used (23, 34, 35). Briefly, 100 μl of the assay master mix (20 mM Tris [pH 7.5], 150 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100), 1 μl of undiluted plasma/serum, and 2×10^6 light units (LU) of the Ruc-antigen fusion protein were added to each well of a 96-well polypropylene plate. This plate was then incubated for 10 min at room temperature. Next, 7 μl of a 30% suspension of Ultralink protein A/G beads (Pierce, Rockford, IL) in phosphate-buffered saline (PBS) was added to the bottom of a 96-well high-throughput-screening filter plate (Millipore, Bedford, MA). The 100-μl antigen-antibody reaction mixture from each microtiter well of the 96-well polypropylene plate was then transferred to the well of the filter plate, which was further incubated for 10 to 15 min at room temperature. The filter plate containing the mixture was then applied to a vacuum manifold. The retained protein A/G beads were washed with the assay master mix and with PBS (pH 7.4), the plate was blotted, and the LU were measured in a Berthold LB 960 Centro microplate luminometer, using a coelenterazine substrate mixture (Promega, Madison, WI).

For quantification of antigens, the original LIPS antibody-testing format was modified for use in a competitive LIPS assay (Fig. 3A). Having first coupled the purified antigen-specific IgG to Ultralink beads (Pierce, Rockford, IL), 5 μl of a 50% suspension (in PBS) of these beads (specific IgG-Ultralink beads) was added to the bottom of a 96-well filter plate. Glycine-treated plasma/serum (36) diluted 1/5 was added to the beads for 30 min at room temperature. Then, an optimized number of specific LU of Ruc-antigen fusions was added in each well and the mixture was incubated for 10 min at room temperature. Specific IgG-Ultralink beads were washed with the assay master mix and then with PBS. The plate was blotted, and LU were measured with a Berthold LB 960 Centro microplate luminometer. The percentage of inhibition was calculated for each sample, and the quantity of specific protein in each sample was estimated by using a standard curve designed using known concentrations of each protein in 1/5 diluted human AB serum (Fig. 3B).

All samples were run in duplicate. All LU data presented were corrected for background by subtracting the LU values of beads incubated with Ruc-antigens but no serum.

Statistical analysis. Figures and statistical analyses, including specificity and sensitivity calculations (ROC analysis) and correlations (Spearman's rank), were performed using Prism 6.0 (GraphPad Software, Inc., San Diego, CA). Fischer's exact test was used to compare the percentages of positivity between groups, and the nonparametric Mann-Whitney test was used to estimate differences in amounts of antigen between two groups. All differences were considered significant at the $P < 0.05$ level.

ACKNOWLEDGMENTS

We thank all the volunteers who participated in this study.

FUNDING INFORMATION

This study was funded by the Division of Intramural Research (DIR) of the National Institute of Allergy and Infectious Diseases. The funder had no role in study design, data collection and interpretation, or decision to submit the work for publication.

REFERENCES

1. **Hotez PJ, Molyneux DH, Fenwick A, Kumaresan J, Sachs SE, Sachs JD, Savioli L.** 2007. Control of neglected tropical diseases. N Engl J Med **357:**1018–1027. http://dx.doi.org/10.1056/NEJMra064142.
2. **Metzger WG, Mordmüller B.** 2014. Loa loa—does it deserve to be neglected? Lancet Infect Dis **14:**353–357. http://dx.doi.org/10.1016/S1473-3099(13)70263-9.
3. **Boussinesq M, Gardon J, Gardon-Wendel N, Kamgno J, Ngoumou P, Chippaux JP.** 1998. Three probable cases of Loa loa encephalopathy following ivermectin treatment for onchocerciasis. Am J Trop Med Hyg **58:**461–469.
4. **Chippaux JP, Boussinesq M, Gardon J, Gardon-Wendel N, Ernould JC.** 1996. Severe adverse reaction risks during mass treatment with ivermectin in loiasis-endemic areas. Parasitol Today **12:**448–450. http://dx.doi.org/10.1016/0169-4758(96)40006-0.
5. **Gardon J, Gardon-Wendel N, Demanga-Ngangue, Kamgno J, Chippaux JP, Boussinesq M.** 1997. Serious reactions after mass treatment of onchocerciasis with ivermectin in an area endemic for Loa loa infection. Lancet **350:**18–22. http://dx.doi.org/10.1016/S0140-6736(96)11094-1.
6. **Zouré HG, Wanji S, Noma M, Amazigo UV, Diggle PJ, Tekle AH, Remme JH.** 2011. The geographic distribution of Loa loa in Africa: results of large-scale implementation of the rapid assessment procedure for loiasis (RAPLOA). PLoS Negl Trop Dis **5:**e1210. http://dx.doi.org/10.1371/journal.pntd.0001210.
7. **Tabi TE, Befidi-Mengue R, Nutman TB, Horton J, Folefack A, Pensia E, Fualem R, Fogako J, Gwanmesia P, Quakyi I, Leke R.** 2004. Human loiasis in a Cameroonian village: a double-blind, placebo-controlled, crossover clinical trial of a three-day albendazole regimen. Am J Trop Med Hyg **71:**211–215.
8. **Kamgno J, Boussinesq M.** 2002. Effect of a single dose (600 mg) of albendazole on Loa loa microfilaraemia. Parasite **9:**59–63. http://dx.doi.org/10.1051/parasite/200209159.
9. **Boussinesq M.** 2012. Loiasis: new epidemiologic insights and proposed treatment strategy. J Travel Med **19:**140–143. http://dx.doi.org/10.1111/j.1708-8305.2012.00605.x.

10. **Klion AD, Horton J, Nutman TB**. 1999. Albendazole therapy for loiasis refractory to diethylcarbamazine treatment. Clin Infect Dis **29:**680–682. http://dx.doi.org/10.1086/598654.
11. **Moody AH, Chiodini PL**. 2000. Methods for the detection of blood parasites. Clin Lab Haematol **22:**189–201. http://dx.doi.org/10.1046/j.1365-2257.2000.00318.x.
12. **Molyneux DH**. 2009. Filaria control and elimination: diagnostic, monitoring and surveillance needs. Trans R Soc Trop Med Hyg **103:**338–341. http://dx.doi.org/10.1016/j.trstmh.2008.12.016.
13. **Fink DL, Kamgno J, Nutman TB**. 2011. Rapid molecular assays for specific detection and quantitation of Loa loa microfilaremia. PLoS Negl Trop Dis **5:**e1299. http://dx.doi.org/10.1371/journal.pntd.0001299.
14. **D'Ambrosio MV, Bakalar M, Bennuru S, Reber C, Skandarajah A, Nilsson L, Switz N, Kamgno J, Pion S, Boussinesq M, Nutman TB, Fletcher DA**. 2015. Point-of-care quantification of blood-borne filarial parasites with a mobile phone microscope. Sci Transl Med **7:**286re284. http://dx.doi.org/10.1126/scitranslmed.aaa3480.
15. **Boussinesq M**. 2006. Loiasis. Ann Trop Med Parasitol **100:**715–731. http://dx.doi.org/10.1179/136485906X112194.
16. **Sakwe AM, Evehe MS, Titanji VP**. 1997. In vitro production and characterization of excretory/secretory products of Onchocerca volvulus. Parasite **4:**351–358. http://dx.doi.org/10.1051/parasite/1997044351.
17. **Weinkopff T, Lammie P**. 2011. Lack of evidence for the direct activation of endothelial cells by adult female and microfilarial excretory-secretory products. PLoS One **6:**e22282. http://dx.doi.org/10.1371/journal.pone.0022282.
18. **Moreno Y, Geary TG**. 2008. Stage- and gender-specific proteomic analysis of Brugia malayi excretory-secretory products. PLoS Negl Trop Dis **2:**e326. http://dx.doi.org/10.1371/journal.pntd.0000326.
19. **Bennuru S, Semnani R, Meng Z, Ribeiro JM, Veenstra TD, Nutman TB**. 2009. Brugia malayi excreted/secreted proteins at the host/parasite interface: stage- and gender-specific proteomic profiling. PLoS Negl Trop Dis **3:**e410. http://dx.doi.org/10.1371/journal.pntd.0000410.
20. **Biarc J, Simon R, Fonbonne C, Léonard JF, Gautier JC, Pasquier O, Lemoine J, Salvador A**. 2015. Absolute quantification of podocalyxin, a potential biomarker of glomerular injury in human urine, by liquid chromatography-mass spectrometry. J Chromatogr A **1397:**81–85. http://dx.doi.org/10.1016/j.chroma.2015.04.003.
21. **Wood SL, Knowles MA, Thompson D, Selby PJ, Banks RE**. 2013. Proteomic studies of urinary biomarkers for prostate, bladder and kidney cancers. Nat Rev Urol **10:**206–218. http://dx.doi.org/10.1038/nrurol.2013.24.
22. **Desjardins CA, Cerqueira GC, Goldberg JM, Dunning Hotopp JC, Haas BJ, Zucker J, Ribeiro JM, Saif S, Levin JZ, Fan L, Zeng Q, Russ C, Wortman JR, Fink DL, Birren BW, Nutman TB**. 2013. Genomics of Loa loa, a Wolbachia-free filarial parasite of humans. Nat Genet **45:**495–500. http://dx.doi.org/10.1038/ng.2585.
23. **Burbelo PD, Ramanathan R, Klion AD, Iadarola MJ, Nutman TB**. 2008. Rapid, novel, specific, high-throughput assay for diagnosis of Loa loa infection. J Clin Microbiol **46:**2298–2304. http://dx.doi.org/10.1128/JCM.00490-08.
24. **Drame PM, Fink DL, Kamgno J, Herrick JA, Nutman TB**. 2014. Loop-mediated isothermal amplification for rapid and semiquantitative detection of Loa loa infection. J Clin Microbiol **52:**2071–2077. http://dx.doi.org/10.1128/JCM.00525-14.
25. **Fliser D, Novak J, Thongboonkerd V, Argilés A, Jankowski V, Girolami MA, Jankowski J, Mischak H**. 2007. Advances in urinary proteome analysis and biomarker discovery. J Am Soc Nephrol **18:**1057–1071. http://dx.doi.org/10.1681/ASN.2006090956.
26. **Rifai N, Gillette MA, Carr SA**. 2006. Protein biomarker discovery and validation: the long and uncertain path to clinical utility. Nat Biotechnol **24:**971–983. http://dx.doi.org/10.1038/nbt1235.
27. **Lutz U, Bittner N, Lutz RW, Lutz WK**. 2008. Metabolite profiling in human urine by LC-MS/MS: method optimization and application for glucuronides from dextromethorphan metabolism. J Chromatogr B Analyt Technol Biomed Life Sci **871:**349–356. http://dx.doi.org/10.1016/j.jchromb.2008.04.018.
28. **Parker CE, Borchers CH**. 2014. Mass spectrometry based biomarker discovery, verification, and validation—quality assurance and control of protein biomarker assays. Mol Oncol **8:**840–858. http://dx.doi.org/10.1016/j.molonc.2014.03.006.
29. **Klion AD, Vijaykumar A, Oei T, Martin B, Nutman TB**. 2003. Serum immunoglobulin G4 antibodies to the recombinant antigen, Ll-SXP-1, are highly specific for Loa loa infection. J Infect Dis **187:**128–133. http://dx.doi.org/10.1086/345873.
30. **Dickerson JW, Eberhard ML, Lammie PJ**. 1990. A technique for microfilarial detection in preserved blood using Nuclepore filters. J Parasitol **76:**829–833. http://dx.doi.org/10.2307/3282801.
31. **Nagaraj N, Mann M**. 2011. Quantitative analysis of the intra- and inter-individual variability of the normal urinary proteome. J Proteome Res **10:**637–645. http://dx.doi.org/10.1021/pr100835s.
32. **Suárez-Pantaleón C, Huet AC, Kavanagh O, Lei H, Dervilly-Pinel G, Le Bizec B, Situ C, Delahaut P**. 2013. Production of polyclonal antibodies directed to recombinant methionyl bovine somatotropin. Anal Chim Acta **761:**186–193. http://dx.doi.org/10.1016/j.aca.2012.11.041.
33. **Burbelo PD, Goldman R, Mattson TL**. 2005. A simplified immunoprecipitation method for quantitatively measuring antibody responses in clinical sera samples by using mammalian-produced Renilla luciferase-antigen fusion proteins. BMC Biotechnol **5:**22. http://dx.doi.org/10.1186/1472-6750-5-22.
34. **Burbelo PD, Ching KH, Klimavicz CM, Iadarola MJ**. 2009. Antibody profiling by luciferase immunoprecipitation systems (LIPS). J Vis Exp **32:**1459. http://dx.doi.org/10.3791/1549.
35. **Burbelo PD, Lebovitz EE, Notkins AL**. 2015. Luciferase immunoprecipitation systems for measuring antibodies in autoimmune and infectious diseases. Transl Res **165:**325–335. http://dx.doi.org/10.1016/j.trsl.2014.08.006.
36. **Henrard DR, Wu S, Phillips J, Wiesner D, Phair J**. 1995. Detection of p24 antigen with and without immune complex dissociation for longitudinal monitoring of human immunodeficiency virus type 1 infection. J Clin Microbiol **33:**72–75.