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Detection of Plasmodium Sporozoites in Anopheles Mosquitoes Using an Enzyme-linked Immunosorbent Assay

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TITLE:

Detection of *Plasmodium* Sporozoites in *Anopheles* Mosquitoes Using an Enzyme-linked Immunosorbent Assay

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KEYWORDS:

Plasmodium falciparum, *Plasmodium vivax*, malaria, sporozoite, CSP, VK210, VK247, ELISA, Anopheles, mosquito

SUMMARY:

This protocol describes a sandwich enzyme-linked immunosorbent assay to detect salivary gland sporozoites in mosquitoes. Using easily available monoclonal antibodies, the method enables cost-effective, high-throughput detection of mosquitoes carrying *Plasmodium falciparum* or *Plasmodium vivax*. The method is suitable for malaria transmission research, including vector surveys.

ABSTRACT:

Plasmodium sporozoites are the infective stage of malaria parasites that infect humans. The sporozoites residing in the salivary glands of female *Anopheles* mosquitoes are transmitted to humans via mosquito bites during blood feeding. The presence of sporozoites in the mosquito salivary glands thus defines mosquito infectiousness. To determine whether an *Anopheles* mosquito carries *Plasmodium* sporozoites, the enzyme-linked immunosorbent assay (ELISA) method has been the standard tool to detect the *Plasmodium* circumsporozoite protein (CSP),

the major surface protein of the sporozoites. In this method, the head along with the thorax of each mosquito is separated from the abdomen, homogenized, and subjected to a sandwich ELISA to detect the presence of CSP specific to *Plasmodium falciparum* and each of the two subtypes, VK210 and VK247, of *Plasmodium vivax*. This method has been used to study malaria transmission, including the seasonal dynamics of mosquito infection and the species of the major malaria vectors in the study sites.

INTRODUCTION:

Plasmodium sporozoites are the infectious stage of the malaria parasites in the mosquitoes. The sporozoites are delivered to humans via mosquito bites. In the mosquito, the sporozoites first form inside the oocysts on the midgut wall. Once ready, they are released into the hemocoel and travel to the mosquito salivary glands. There, they mature and become ready for transmission to humans during blood feeding. In humans, the sporozoites are deposited in the dermis. Then, they enter the blood vessel and travel along the blood circulation to reach the liver to establish infection in the hepatocytes[1,2].

Three different methods have been used to determine sporozoite infection of the mosquito salivary glands. The first method is the dissection of the salivary glands followed by direct examination of sporozoites under a light microscope. This method is the gold standard to detect and quantify sporozoites in *Anopheles* mosquito salivary glands[3]. However, it requires a technician well trained in both dissection and microscopic examination. Moreover, it cannot be used to determine *Plasmodium* species and CSP subtyping (for *P. vivax*)[4,5]. The second method uses polymerase chain reaction (PCR) to detect *Plasmodium* DNA in the upper part of the mosquito body[6]. Given the specificity of PCR, both species and subtyping of the parasite are possible[7-10]. Although PCR is increasingly used, it requires relatively expensive equipment and well-trained staff. The last method, the ELISA to detect the *Plasmodium* specific circumsporozoite protein (CSP), has been the mainstay for three decades[11-13]. CSP is present in both oocyst sporozoites and salivary gland sporozoites[12,14]. Using specific antibodies, this method allows *Plasmodium* species identification and CSP subtyping of *P. vivax* sporozoites. The rationale for this assay is the requirement of a simple high-throughput assay to examine a large number of wild mosquitoes to understand malaria transmission (i.e., determine the sporozoite infection rate).

The ELISA method has two key advantages over microscopic examination. First, it allows researchers to keep mosquito samples until they are ready for sample processing. Second, the ELISA method can be used to differentiate *Plasmodium* species through species-specific monoclonal antibodies. In addition, ELISA can accommodate a larger number of mosquito specimens, permitting a much higher throughput[15]. Compared to PCR, which detects sporozoite DNA, the ELISA procedure takes more time but costs less[16]. The ELISA assay described here was developed to determine the mosquito infectivity and separately detect CSP of *P. falciparum* and each of the two CSP variants of *P. vivax,* VK210 and VK247. This ELISA method has been used in many studies to determine the seasonal dynamics of mosquito infection and identify the species of the major malaria vectors in the field[12,13,17,18]. To perform this assay, a standard laboratory equipped with an ELISA plate reader is sufficient.

The overall approach is summarized in **Figure 1**. In this sandwich ELISA, the primary (capture) monoclonal antibody (mAb) specific for each *Plasmodium* species/subtype is first used to coat the ELISA plate. Each plate is coated with a single capture mAb. The function of the mAb is to capture the corresponding CSP antigen in the mosquito homogenates. After antigen capture and plate washes, a second CSP-specific antibody labeled with peroxidase is used to detect the presence of CSP bound to the capture mAb. The chemical reaction catalyzed by peroxidase results in color development in wells positive for CSP.

PROTOCOL:

1. Preparation of reagents

NOTE: Refer to the **Table of Materials** for a list of equipment, reagents, and other consumables used in this protocol and to **Table 1** for a list of solutions and their composition.

1.1. Capture and peroxidase-conjugated mAbs

1.1.1. To reconstitute the mAb, resuspend the lyophilized mAb in a 1:1 mixture of distilled water:glycerol at 0.5 mg/mL. Make aliquots as needed to avoid repeated freeze-thawing, and store them at -20 °C.

1.2. Blocking buffer (BB)

1.2.1. Prepare the blocking buffer by dissolving 5 g of ELISA-grade casein in 100 mL of 0.1 N NaOH. Add 900 mL of phosphate-buffered saline (PBS) (see **Table 1**) to bring the final volume to 1.0 L.

1.2.2. Add 0.02 g of phenol red as an indicator and adjust the pH to 7.4 with HCl. Store BB at 4 °C for up to one week, or aliquot into 50 mL for long-term storage at -20 °C.

1.3. Positive controls

1.3.1. To reconstitute the positive controls, rehydrate the lyophilized proteins by adding 1,000 µL of BB. Make aliquots of the stock positive control solutions as needed, and store them at -20 °C.

1.3.2. For serial dilution, further dilute each positive control to the final working concentration in BB as follows: Pf, 2 pg/µL; Pv (VK210), 182 (pg/µL); Pv (VK247), 89 pg/µL.

NOTE: The exact of concentrations of the positive controls may vary from one lot to the next. Consult the product information sheet for the exact concentration needed. The positive control concentrations, starting from the working concentration above, are 2, 1, 0.5, 0.25, 0.13, 0.06 pg/µL for Pf; 182, 91, 46, 23, 11, 5.7 pg/µL for PV210; and 89, 45, 22, 11, 5.6, 2.8 pg/µL PV247.

1.4. Negative controls

NOTE: The ideal negative control is the head-thorax homogenate of female Anopheles mosquitoes prepared identically as the test samples. However, BB can also be used as a negative control.

1.4.1. With BB as the negative control, use the 2-fold absorbance threshold for reliable positive readouts.

2. Mosquito sample preparation

2.1. Separate the head and the thorax of each collected adult mosquito from the abdomen with a sterile razor blade. Place the head and thorax in a prelabeled 1.5 mL centrifuge grinding tube. Pool heads and thoraces of up to 10 mosquitoes if desired.

NOTE: For sample preparation, typically, the salivary gland from an infected mosquito will be dissected and subjected to CS-ELISA. However, the head and thorax of collected mosquitoes can also be used to perform CS-ELISA directly (without dissecting for salivary glands)[12,13,19].

2.2. Add 50 µL of Grinding Buffer (GB) into each tube and homogenize the sample with a clean pestle (washed with soap). Rinse the used pestle with 250 µL of GB into the tube containing the homogenized mosquito(es) to a final volume of ~300 µL.

2.3. Keep the sample in a freezer (-20 °C) until use or proceed immediately to perform ELISA.

3. Sporozoite ELISA

3.1. Fill out the sporozoite ELISA worksheet (see **Supplemental Material 1**). Prepare one ELISA plate for each CSP (Pf, Pv-210, or Pv-247).

3.2. Prepare the capture mAb working solution by dissolving the antibody in PBS: 4 µg/mL Pf; 2 µg/mL Pv-210; 2 µg/mL of Pv-247. Calculate the volumes required based on the addition of 5 mL per plate. Vortex the solution gently.

3.3. Pipette 50 µL of each working mAb solution from step 3.2 into each well of the ELISA plate. Cover the plate with a plastic lid and incubate for 30 min or overnight at room temperature.

3.4. Aspirate the well contents and tap the plate upside down on paper towels at least 5 times to remove all liquid.

NOTE: If an aspiration system (multichannel vacuum suction connected to clean tips) is not available, dump out the antibodies into the sink and then tap the plate on paper towels.

3.5. Add 200 µL of BB to fill all wells in the plate. Cover the plate with a plastic lid. Incubate the plate for 1 h at room temperature. Aspirate or dump out the well contents. Tap the plate upside down on paper towels 5 times to remove all liquid.

3.6. Load the mosquito homogenate and the control on the plate as follows.

3.6.1. Add 50 µL of the positive control to wells H1 and H2.

3.6.2. Add 50 µL of BB to wells in columns 1 and 2 from row C to G. Then, add 50 µL of the positive control into wells G1 and G2. Make a serial dilution of the positive control starting from G1 and G2 followed by F1 and F2 until C1 and C2.

NOTE: All positive control wells should contain 50 µL.

3.6.3. Add 50 µL of BB (negative control) to wells A1, A2, B1, and B2.

3.6.4. Add 50 µL of each homogenate sample to an Unknown (Unk) well.

3.6.5. Cover the plate and incubate for 2 h at room temperature.

3.7. After approximately 2 h, start preparing the substrates. For the ABTS substrate 2-component kit, mix substrate A and substrate B in a 1:1 ratio.

NOTE: A full 96-well plate requires 5 mL of substrate A and 5 mL of substrate B.

3.8. Prepare the working solutions of peroxidase-labeled mAbs for Pf, Pv-210, and Pv-247 by adding BB to the reconstituted conjugate mAb to obtain a working concentration of 1 µg/mL.

3.8.1. Calculate the required volumes based on the addition of 5 mL of working conjugate mAb solution per plate.

3.8.2. Test peroxidase activity by mixing 5 µL of the peroxidase-labeled mAb made in step 3.8 with 100 µL of the substrate made in step 3.7 in a separate 1.5 mL tube. Vortex gently.

NOTE: There should be a rapid color change from clear to green, indicating that the peroxidase enzyme and the substrates are working.

3.9. Aspirate or dump the well contents and tap the plate upside down on paper towels 5 times to remove all liquid.

3.10. Wash the wells twice with 200 µL of PBS-Tween, aspirate the well contents, and tap the plate 5 times each.

3.11. Add 50 µL of peroxidase-labeled mAb made in step 3.8 to each well. Cover the plate and incubate for 1 h at room temperature in the dark. Aspirate or dump the well contents and tap the plate upside down on a paper towel 5 times.

3.12. Wash the wells 3 times with 200 µL of PBS-Tween, aspirate the well contents, and tap the plate 5 times each.

3.13. Add 100 µL of the substrate solution prepared in step 3.7 to each well. Cover the plate and incubate for 30 min at room temperature in the dark.

3.14. After 30 min, read the absorbance at 405–414 nm using an ELISA plate reader.

NOTE: Follow the specific instructions for the ELISA plate reader used. For details on the instructions for the software used for this protocol, refer to **Supplemental Material S2**. There should be a noticeable color change from clear to green in the positive control wells.

4. Analysis

4.1. Detecting positive samples.

4.1.1. Label samples with absorbance values above the cut-off (twice the mean absorbance value of the negative samples) as positive.

4.2. Quantifying CSP

4.2.1. Estimate the CSP concentration in the sample using the standard curve constructed from the control dilution series as follows.

4.2.1.1. Create the standard curve by plotting the absorbance values (y-axis) of the serially diluted controls against their concentrations (x-axis).

4.2.1.2. Perform linear regression to determine the best fit using $y = A + Bx$, where A and B are free parameters.

4.2.1.3. Determine the CSP concentration for each positive sample by solving the equation for a given absorbance value.

REPRESENTATIVE RESULTS:

Representative ELISA results are shown in **Figure 2**. In this experiment, the *P. falciparum* ELISA detected sporozoite infection in well A7. The positive well could be visually detected by its faint green color (**Figure 2A**). The absorbance value of this well was above the cut-off threshold (twice the mean value of the four negative control wells) (**Figure 2B**). The distribution of the absorbance values of all 80 unknown wells is depicted in **Figure 2C**. CSP quantification of well A7 by the in-plate standard curve after background (negative control) subtraction suggests a CSP

concentration of 0.35 pg/μL (**Figure 2D**). The *P. vivax* assays for VK210 and VK247 were both negative for this sample set (data not shown), indicating that the sporozoite infection in A7 was mono-species *P. falciparum*.

FIGURE AND TABLE LEGENDS:

Figure 1: Overview of CSP sandwich ELISA. (**A**) Specific capture monoclonal antibody (capture mAb) is used to coat the surface of each well. CSP antigen in the mosquito homogenate binds to the mAb-coated wells. (**B**) HRP-labeled mAb is used to detect the captured Ag. Abbreviations: CSP = circumsporozoite protein; ELISA = enzyme-linked immunosorbent assay; Ag = antigen; mAb = monoclonal antibody; HRP = horseradish peroxidase; OD = optical density; BB = blocking buffer.

Figure 2: Representative results for *Plasmodium falciparum* CSP detection. (**A**) The image of the ELISA plate after 30 min incubation with ABTS. The red arrow and red circle represent the positive unknown well (A7). (**B**) The absorbance values read by the ELISA plate reader. The four upper left wells (A1, A2 and B1, B2) were the negative controls. Diluted positive controls were run in duplicates: C1/C2 (1:32), D1/D2 (1:16), E1/E2 (1:8), F1/F2 (1:4), and G1/G2 (1:2). The undiluted positive controls were H1/H2. (**C**) The absorbance distribution of the 80 unknown wells. The solid line represents the mean absorbance of the negative control wells. The dashed line represents the positivity threshold. (**D**) The standard curve constructed from the two-fold serial dilution of the positive control. The highest concentration of the positive control was 2 pg/μL. Linear regression of the data estimated the CSP concentration in A7 as 0.35 pg/μL. Abbreviations: CSP = circumsporozoite protein; ABTS = 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate); ELISA = enzyme-linked immunosorbent assay; Abs = absorbance.

Table 1: Recipes.

Supplemental Material S1: Sporozoite ELISA worksheet.

Supplemental Material S2: Guidelines for ELISA plate reader software.

DISCUSSION:

The CSP-ELISA provides a highly specific and cost-effective method to detect *Plasmodium* CSP. It allows discrimination between *P. falciparum* and *P. vivax* sporozoites as well as between the two subtypes, VK210 and VK247, of *P vivax*[11,13-15]. Certain critical points should be considered to obtain reliable and reproducible results. All solutions should be kept in the refrigerator for less than 1 week to prevent microbial growth. The mAb should be kept in diluent containing 50% glycerol and aliquoted as needed to prevent multiple freeze-thawing. The positive controls should be aliquoted for single use. The ELISA plate should be covered with the lid during the incubation period to prevent evaporation. Steps involving peroxidase-labeled mAb incubation should be carried out in the dark.

All steps involving solution change should be performed quickly to prevent drying out, which can lead to high background. The working substrate solution should be kept in the dark by wrapping

it with aluminum foil and added to the plate immediately after preparation. When working with frozen mosquito homogenates, the samples should be tested on the same day of thawing. The pH of the reaction should be maintained in the range of 7–7.4 as the reaction is inhibited at pH values outside this range. Washing should be done carefully to avoid false positives. The inclusion of the non-ionic detergent, Tween-20, in the washing solution can minimize signal from the background.

This protocol was modified from the protocol described by Wirtz et al.[19]. One difference is the lower number of negative controls to allow for the six-point standard curve. In addition, the protein standards are serially diluted in BB without the mosquito lysate. Therefore, their background composition differs from that of the test samples. These standards are used to provide consistent CSP quantification of the test samples across different plates. If more accurate quantification is needed, the standards can be prepared using the lysate of uninfected mosquitoes processed identically to the test samples but with a known amount of protein added. Lastly, as with most diagnostic assays, the CSP ELISA is not error-free[20]. All positive samples should be confirmed by repeating the assay with heated homogenate (100 °C, 10 min) or by *Plasmodium* species-specific PCR, using the remaining homogenate as the source of the DNA template[20].

When performed correctly, this CSP ELISA method can be highly reliable. It has been, and likely will continue to be, used in several studies of malaria transmission, with the goals to determine the seasonal dynamics of mosquito infection and identify the species of the major malaria vectors[12,13,17,18]. Compared to direct microscopic examination of sporozoites, this assay has a much greater throughput and is more suitable for research involving a large number of mosquitoes. Compared to the PCR detection of sporozoites, the ELISA procedure takes more time but costs less[16]. Overall, its simplicity, high throughput, and relatively low cost permit large-scale testing in a standard laboratory.

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DISCLOSURES:

The authors have no conflicts of interest to declare.

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Figure 1

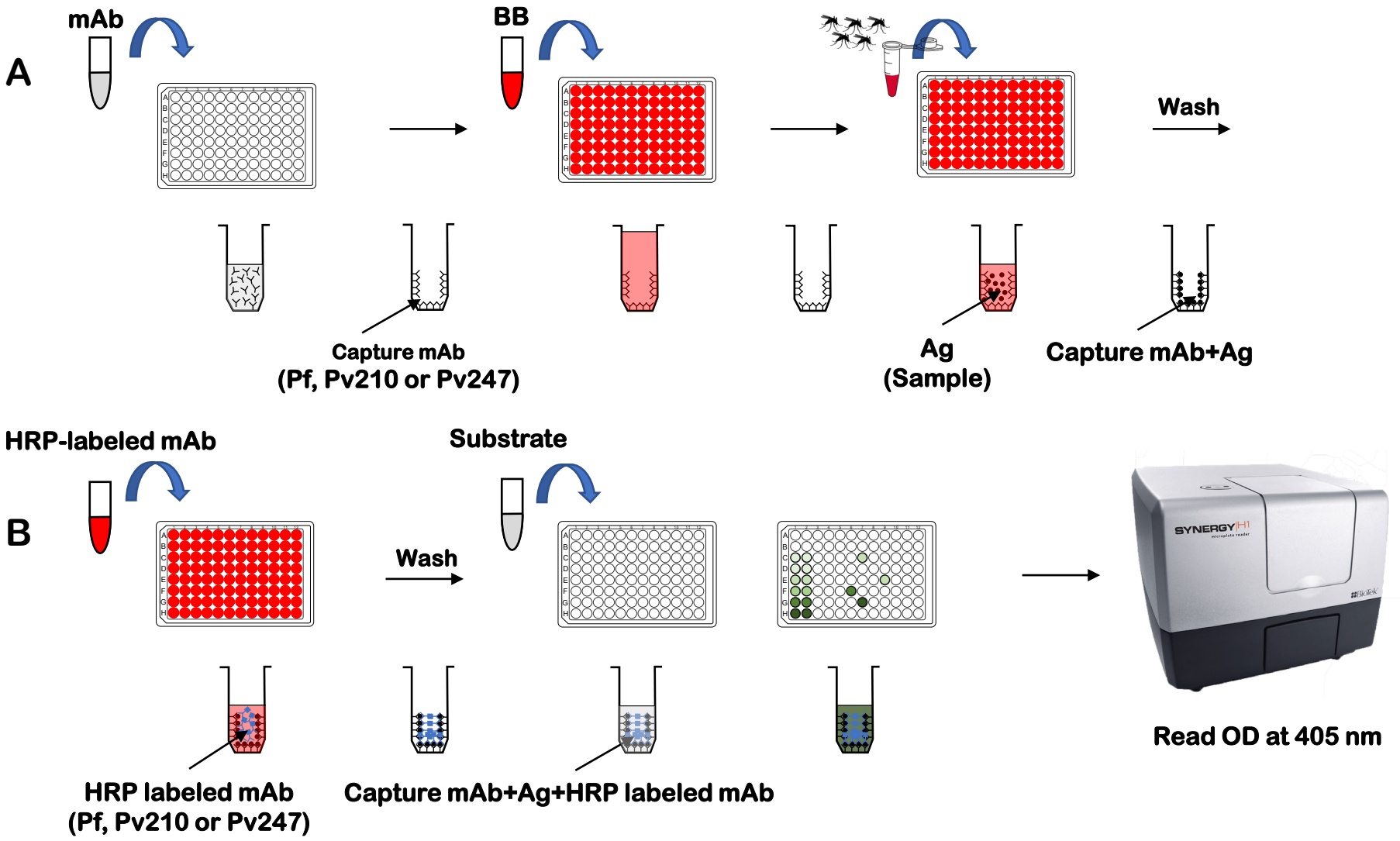
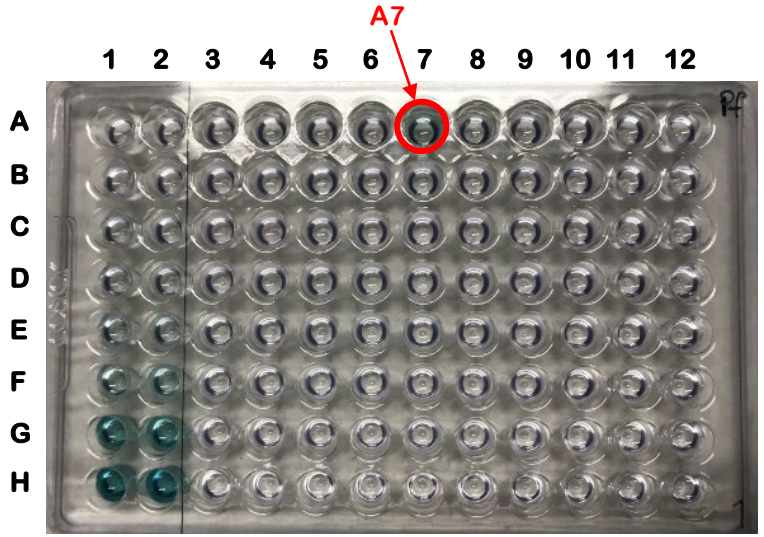


Figure 2.

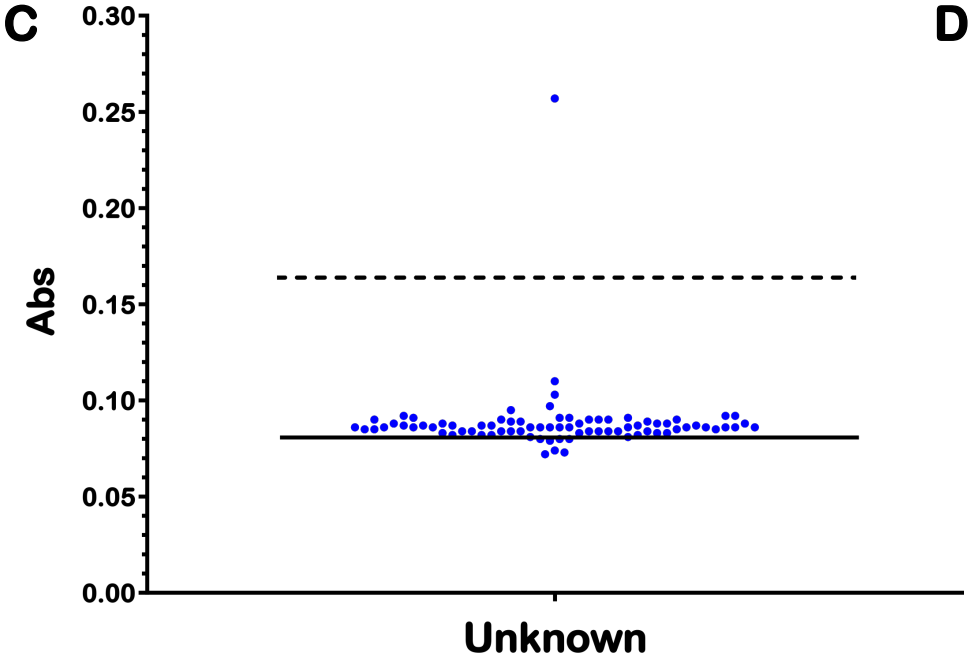
A



B

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.083	0.082	0.082	0.083	0.095	0.081	0.257	0.085	0.079	0.081	0.103	0.084
B	0.085	0.081	0.080	0.072	0.082	0.080	0.083	0.084	0.086	0.086	0.085	0.086
C	0.112	0.111	0.074	0.073	0.083	0.090	0.085	0.088	0.091	0.086	0.090	0.086
D	0.143	0.154	0.080	0.087	0.086	0.097	0.082	0.090	0.092	0.087	0.086	0.083
E	0.204	0.229	0.091	0.087	0.089	0.084	0.091	0.090	0.084	0.086	0.084	0.088
F	0.283	0.291	0.089	0.087	0.092	0.090	0.089	0.088	0.086	0.090	0.087	0.088
G	0.644	0.611	0.084	0.086	0.092	0.085	0.091	0.082	0.086	0.084	0.087	0.087
H	1.161	1.097	0.084	0.086	0.084	0.086	0.086	0.110	0.086	0.084	0.088	0.088

C



D

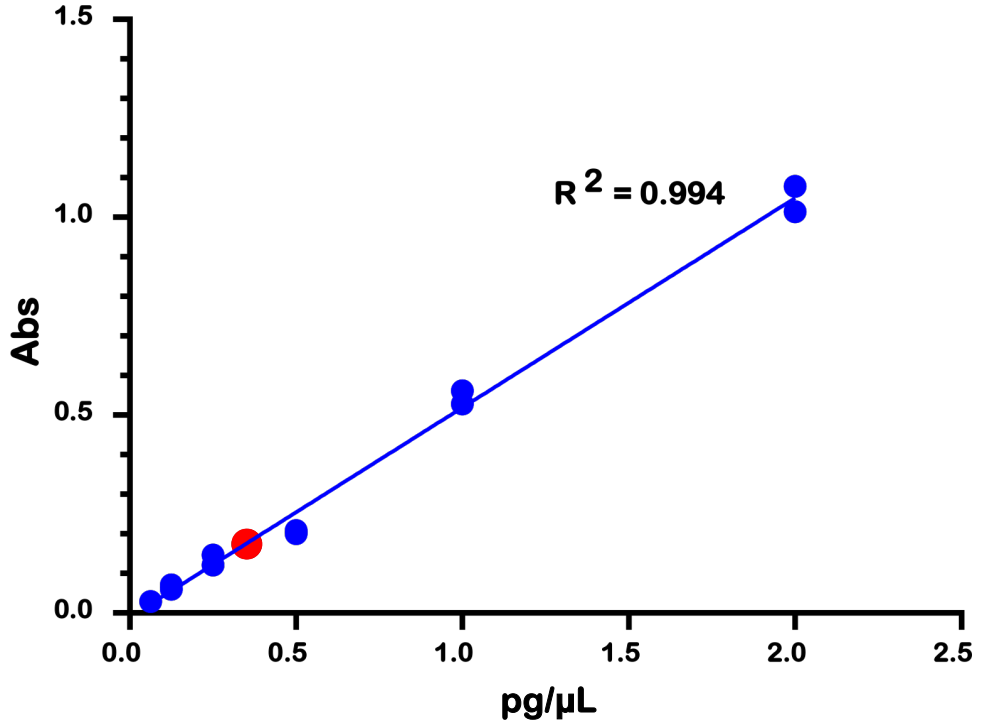


Table 1

Solution name	Materials	Preparation method	Shelf life and storage condition
ABTS Substrate (2-component)	The ABTS Peroxidase Substrate solution A and peroxidase Substrate Solution B	1. Mix equal volumes of ABTS Peroxidase Substrate Solution A and Peroxidase Substrate Solution B.	Stable for a minimum of 1 year at 2 - 8 °C
		2. Warm to room temperature before use.	
Blocking buffer (BB)	Casein 5 g; NaOH, 0.1 N 100 mL; 1x PBS, 10 mM, pH 7.4, 900 mL; and Phenol red 0.02 g	1. Bring 0.1 N NaOH to a boil in a flask with stir bar maxing on low.	1 week at 4 °C or may be frozen
		2. Slowly add the casein (Sigma) until dissolved in 0.1 N NaOH.	
		3. Allow solution to cool to room temperature.	
		4. Slowly add the PBS.	
		5. Adjust the pH to 7.4 with 1 N HCl.	
		6. Add the phenol red.	
		7. Store at 4 °C for up to 1 week or aliquot into 50 mL conical tubes for long-term storage at -20 °C.	
Grinding buffer	Igepal CA-630 125 µL and BB 25 mL	1. Combine 25 mL of BB and 125 mL of Igepal CA-630. This will be sufficient for approximately one plate.	1 week at 4 °C or may be frozen
		2. Mix well, using a vortex to dissolve the Igepal CA630 in the BB.	
		3. Store at 4 °C for up to one week or aliquot into 50 mL conical tubes for long-term storage at -20 °C.	
Monoclonal antibodies (mAb) capture and conjugate	Pf, Pv210 and Pv247 capture mAb and Peroxidase mAb	1. Monoclonal antibodies (mAb) capture and conjugate received from CDC will be lyophilized.	Stable for a minimum of 1 year at 2 - 8 °C or store at 20 °C
		2. The label will list the amount of glycerol:water to be added. Glycerol:water is a 1:1 mixture of distilled water and glycerol to get 0.5 mg/mL mAb capture stock.	
		3. Glycerol water allows for storage at -20 °C with freeze-thawing.	
		4. This step only needs to be performed when a new vial of capture antibody conjugate needs to be reconstituted.	
PBS-Tween (PBS-T) Wash solution.	1x PBS pH 7.4 and 0.05% Tween20	1. Add 0.5 mL of Tween20 to 1 L of 1x PBS.	2 weeks at 4 °C
		2. Mix well and store at 4 °C.	
10x Phosphate-buffered saline (PBS) pH 7.4	NaCl 80.0 g, KH_2PO_4 2.0 g, Na_2HPO_4 11.5 g, and KCl 2.0 g	1. Combine NaCl, KH_2PO_4, Na_2HPO_4, and KCl.	1 year at 4 °C
		2. Add to 1 L distilled water (dH_2O), mix, and adjust pH to 7.2-7.4.	
1x Phosphate-buffered saline (PBS) 10 mM pH 7.4	10x Phosphate-buffered saline (PBS) pH 7.4	1. Dilute 10x Phosphate-buffered saline (PBS), pH 7.4, to 1x Phosphate-buffered saline (PBS), pH 7.4, with distilled water (dH_2O).	1 year at 4 °C
		2. Mix well and adjust pH to 7.2-7.4.	
Positive controls	Pf, Pv210 and Pv247 positive control	1. Positive controls are lyophilized in-house.	Store at 20 °C
		2. The label will list the amount of blocking buffer (BB) to be added to get positive control stock.	
		3. Stock vials can be stored for future dilutions and aliquoted into smaller volumes to minimize freeze-thaw cycles.	
		The final concentration positive control solution of each species for standard serial dilution as Pf, Pv210, and Pv247 positive control are 2,000, 182,000, and 8,900 (pg/mL), respectively.	

Response to reviewers

Reviewer #1:

Manuscript Summary:

The authors describe the methodology for the detection of Plasmodium sporozoites in Anopheles mosquitoes using an enzyme-linked immunosorbent assay (ELISA). The theory behind malaria transmission and the applications of ELISAs are presented in the introduction section along with explanations of the functions of each of the reagents used in the protocol. The schematic summary of the assay given in Figure 1 is presented well with appropriate explanations provided in the figure legend. A well-detailed equipment and reagent list is provided with the relevant working concentrations of each reagent and where to source equipment and reagents. Each step of the protocol is written clearly and with appropriate detail and/ or explanation. There was no mention of adding phenol red when preparing the blocking buffer. Robert Wirtz, in his 2004 ELISA directions, notes to add 0.01g phenol red or 100ul of phenol red stock solution (1g/ 10ml water)/L PBS. The inclusion of phenol red provides a visual confirmation of correct pH levels of the buffer. Appropriate controls are suggested for both the positive and negative, with an explanation of the suggested alternative negative control given. The flow of the protocol is logical and easy to follow. The inclusion of the ELISA template worksheet is particularly useful and allows for visual confirmation of the plate-loading. The representative results include an image of the plate, a table of the optical density values for each sample, the absorbance distribution of the samples, and the standard curve constructed from the two-fold dilution of the positive control. Including these results is useful to the reader and provides a good reference for expected outcomes of the assay. Following this protocol would lead to the described outcome, however the addition of a follow-up repeat assay of CSP positive samples would reduce the risk of false positives. It is suggested that all unknown mosquito samples that present with CSP in the first assay be followed up with a repeat ELISA following a boiling step of the homogenate at 100℃ for 5 minutes. The authors did mention very briefly in the discussion that positive confirmation by PCR may be performed when the mosquito infection rate is very low, but this could be expanded on to include repeat ELISAs. Some useful tips are provided in the discussion section that may help readers with the assay, however some additional critical steps would contribute to this and are listed below. The references cited are relevant to this protocol and are presented appropriately in the reference list.

I wish to congratulate the authors on a very thorough and well-presented protocol.

Major Concerns:

None to report

Response: We thank the reviewer for the positive response and the highly constructive inputs. We very much appreciate your time.

Minor Concerns:

* Throughout manuscript: few typographical errors.

Response: We have carefully checked the manuscript and corrected all typographical errors we could find.

* Line 107, Blocking buffer step: 200uL of phenol red stock solution (1 gm/10mL water) is usually added as an indicator of correct pH after BB pH has been adjusted. See Wirtz 2004 Method, "Plasmodium falciparum and P. vivax sporozoite ELISA directions" for reference.

Response: We have now included phenol red to the protocol as follows.

Line 134-135: “0.02 g of phenol red is added as an indicator of correct pH.”

* Line 119: The catalogue number of the substrate must be included. If you specifically want the 2-component this must be included, but the 2 component is now less commonly used than the 1 component.

Response: The catalogue numbers of reagents, supplies and equipment have been included in a separate file name “JoVE_Table of Materials_revision.xls”

* Line 130, list of consumables: 1.5ml reaction tubes could be added to the list of consumables but not entirely necessary

Response: We have added 1.5 ml reaction tubes to the list of consumables (line 154).

* Line 135: If you are going to pool specimens the controls must be pooled equivalently, because increased protein itself will increase the OD. Therefore, you cannot have a control with one mosquito and a sample with 10 mosquitoes.

Response: The positive control in this particular protocol is the lyophilized protein serially diluted in the Blocking Buffer (BB) without mosquito lysate. This choice of standard samples was chosen for practical reasons. Although imperfect, these standards can be used as the internal positive controls and for comparing samples across different plates. For the most accurate CSP quantification, we agree that the standards should be prepared using the protein in the lysate of

the same number of uninfected mosquitoes. To emphasize this point, we have added the following text to the discussion section.

Line 336-341: “In addition, the protein standards are serially diluted in BB without the mosquito lysate. Therefore, their background composition differs from that of the test samples. These standards are used in our laboratory to provide consistent CSP quantification of the test samples across different plates. If more accurate quantification is needed, the standards should be prepared using the lysate of uninfected mosquitoes processed identically to the test samples, but with a known amount of protein added.”

* Line 136: Please define what is meant by "clean pestle"? Soap washed? Autoclaved? This choice has downstream effects, as more care needs to be taken if PCR is subsequently done on the sample.

Response: We have now defined the “clean pestle” as a detergent washed pestle on line 170-171: “Add 50 μL of Grinding Buffer (GB) into each tube and homogenize the sample with a clean pestle (soap washed).”

* Line 150, ELISA template: why were four negative controls chosen instead of the suggested seven control by Wirtz? Was it just to allow adequate well-space on the plate for the positive control serial dilution?

Response: The difference from the original protocol by Wirtz is due to the following reasons.

1. As the reviewer thought, the key reason is to make room for the standard curve.
2. We have used four negative controls extensively and are confident that they are sufficient. We rarely detect false positives in test samples.

* Line 202, Analysis: There is no mention of repeating CSP positive samples to confirm positivity. It is suggested that all unknown mosquito samples that present with CSP in the first assay be followed up with a repeat ELISA following a boiling step of the homogenate at 100℃ for 5 minutes.

Response: We have now added more discussion about confirming CSP positive samples.

Line 341-344: “Lastly, as with most diagnostic assays, the CSP ELISA is not error-free[1]. All positive samples should be confirmed by repeating the assay with heated homogenate (100 ℃, 10 minutes) or by *Plasmodium* species-specific PCR, using the remaining homogenate as the source of the DNA template [1].”

* Line 240, practices to follow to obtain reliable results should also include: 8) the reaction is inhibited if the pH of the reaction varies from the neutral pH of 7.0-7.4, 9) poor washing techniques can lead to false positives, 10) omission of the non-ionic detergent Tween-20 from the washing solutions will lead to high background.

Response: We appreciate the suggestions. All these suggested practices have been added to Discussion part on Line 330-333.

Reviewer #2:

Manuscript Summary:

In this protocol the authors describe a standard method used for the detection of Plasmodium spp. sporozoites from Anopheles mosquitoes. This method is very well established and is used all over the world. A video of this will likely be used widely. The detailed methods are described and steps outlined to allow for step-by-step instruction for readers to follow along with the method and the authors did a great job laying out the method clearly.

Response: We are thankful for the positive response and would like to thank the reviewer for the constructive comments.

Minor Concerns:

This method is nearly identical to the method provided by the US CDC in the Malaria Research and Reference Reagent Resource Center (MR4) manual https://www.beiresources.org/Anopheles Program/TrainingMethods.aspx; however, the authors do not include any reference to this document. While it is possible that one or more of the authors here were involved in the original development of the protocol in the 80s and 90s, the protocol submitted to JoVE is very similar and includes the same supply recommendations as the MR4 protocol, which I believe merits at least a citation to the MR4 manual (which is openly available to all and used as a primary resource in widespread malaria vector surveillance trainings).

Response: We have now cited this manual in the protocol.

Line 335: “This protocol described here was modified from Wirtz et al., 2016[2].”

Response to JOVE

Changes to be made by the Author(s):

We have highlighted in green for text we have made a change as suggested in the manuscript

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: We have thoroughly reviewed the manuscript and made several corrections as suggested.

2. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "The present protocol describes. …". Here the word limit is exceeding.

Response: The summary has been revised to 43 words

3. Please provide a 150- 300-word abstract. The current abstract is 119 words.

Response: We have expanded the abstracted to 152 words.

4. Please provide references for the following lines: 47-58

Response: The references have been added.

5. Please revise the Introduction to include all of the following:

a) A clear statement of the overall goal of this method.

Response: We have now stated the overall goal of the method on Line 79-81: "The ELISA assay described here was developed to determine the mosquito infectivity and separately detect CSP of *P. falciparum* and each of the two CSP variants of *P. vivax,* VK210 and VK247".

b) The rationale behind the development and/or use of this technique.

Response: The rationale of this technique is now stated on Line 70-72 : "The rational for this assay is the requirement of a high throughput assay to examine a large number of wild mosquitoes to understand malaria transmission (i.e., determine the sporozoite infection rate)."

c) The advantages over alternative techniques with applicable references to previous studies.

Response: We have now added the advantages of ELISA over alternative techniques on Line 74-79: "The ELISA method has two key advantages over microscopic examination. Firstly, it

allows researchers to keep mosquito samples until they are ready for sample processing. Secondly, The ELISA method can be used to differentiate *Plasmodium* species through the use of species-specific monoclonal antibodies. In addition, ELISA can accommodate a larger number of mosquito specimens, permitting a much higher throughput[3]. Compared to PCR, which detects sporozoite DNA, the ELISA procedure takes more time but costs less[4]."

d) A description of the context of the technique in the wider body of literature.

Response: We have mentioned about examples of use for this ELISA method on Line 79-81 : "This ELISA method has been used in many studies to determine the seasonal dynamics of mosquito infection and identify the species of the major malaria vectors in field[5-8]."

e) Information to help readers to determine whether the method is appropriate for their application

Response: We believe that information about the advantages of this assay compared to alternative methods and its application in the literature have helped the readers somewhat. We also added an additional sentence to state the laboratory requirement for the ELISA method.
Line 83-84: "To perform this assay, a standard laboratory equipped with an ELISA plate reader is sufficient."

6. Please consider providing reaction set-ups and solution compositions as Tables in separate .xls or .xlsx files uploaded to your Editorial Manager account. These tables can then be referenced in the protocol text.

Response: We now provide a table for solution compositions and reaction set-ups in separate file name "Reaction set-ups and solution compositions_JOVE".

7. Please include the ELISA worksheet template as a Supplementary File and reference in the protocol text.
Response: We now provide the ELISA worksheet template as a separate file name "ELISA WORKSHEET template_Jove".

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All commercial products should be sufficiently referenced in the Table of Materials.
For example: ABTS® 2-Component Microwell Peroxidase (KPL), casein (Sigma C7078), ELISA plate reader (Synergy H1), Biotek instrument, Inc, Grinder Pestle, Axygen scientific, PVS (Corning Life Science), etc.
Response: We have removed all commercial language from the manuscript.

9. Please use SI unit denotation for all units throughout the manuscript: L, mL, µL, cm, kg, etc. Hours, minute, and seconds can be written as h, min, s, respectively.
Response: All units have been changed to the SI format.

10. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.
Response: The numbering of this manuscript has revised following the JOVE instructions.

11. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.
Response: We have made revisions accordingly.

12. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
Response: The protocol has been revised as suggested.

13. Please add more details to your protocol steps.
Line 168/189: How was the aspiration performed? With a pipette? Please clarify.
Response: Aspiration was performed by multichannel vacuum suction connected to clean tips. The description has been added to line 199.

Line 172: What was the starting and ending serial dilution concentration of positive control? Please specify.

Response: We have added the information on line 119-121, “Note: The positive control concentrations, starting from the working concentration above, are 2, 1, 0.5, 0.25, 0.13, 0.06 pg/µL for Pf; 182, 91, 46, 23, 11, 5.7 pg/µL for PV210; and 89, 45, 22, 11, 5.6, 2.8 pg/µL PV247.”

Line 187: What does the color change to (For example clear to yellow)? Please mention.
Response: The color change is from clear to green. The color change is now mentioned on line 262-263: “II) There should be a noticeable color change from clear to green in the positive control wells.”

Line 200: Please include all the button clicks, command lines, etc. in the software as well as the instrument. Please ensure that the button clicks are bolded throughout.
Response: We now provide the software instruction in separate file name “Guideline for GEN5 program use for ELISA plate reading”.

Line 203: If the analysis part needs to be filmed, please include all associated details of how absorbance was measured, the formula used for quantitation, plotting the standard curve, etc. Please remember that our scripts are directly derived from the protocol text. Please include all actions associated with each step.
Response: We have added these details to the Analysis section.
Line 270-280:

“**3.2 Quantifying CSP.** The CSP concentration in the sample can be estimated using the standard curve constructed from the control dilution series as follows:

3.2.1 Create the standard curve by plotting the absorbance values (y-axis) of the serially diluted controls against their concentrations (x-axis).

3.2.2 Perform linear regression to determine the best fit using y = A + Bx where A and B are free parameters.

3.2.3 Determine the CSP concentration for each positive sample by solving the equation at a given absorbance value.”

14. Please include a single line space between each step, substep, and note in the protocol section. Please highlight up to 3 pages of the Protocol (including headings and spacing) that **identifies the essential steps of the protocol for the video**, i.e., the steps that should be

visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Response: We have now added a single line space in each step as suggested. The essential steps of the protocol for making video are highlighted.

15. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

Response: The critical steps of this assay have been added.

Line 317-333: "To obtain reliable and reproducible results, the following practice should be followed.

1. All solutions should be kept in the refrigerator for less than 1 week to prevent the microbial growth.
2. The mAb should be kept in diluent containing 50% glycerol and aliquot as needed to prevent multiple freeze-thawing.
3. The positive controls should be aliquot for a single use.
4. The ELISA plate should be covered with lid during the incubation period to prevent evaporation. Steps involving peroxidase-labeled mAb incubation should be carried out the dark.
5. All steps involving solution change should be performed quickly to prevent dry out which can lead to high background.
6. The working substrate solution should be kept in the dark by wrapping with aluminum foil and applied to the plate immediately after preparing.
7. When working with frozen mosquito homogenates, the samples should be tested on the same day of thawing.
8. The reaction is inhibited if the pH of the reaction is outside the range of 7.0 - 7.4.
9. Poor washing techniques can lead to false positives.
10. Omission of the non-ionic detergent Tween-20 from the washing solution can lead to a high background."

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

Response: The following paragraph has been added to address these two points:

Line 335-344: “This protocol was modified from Wirtz et al., 2016[2]. One difference is the lower number of negative controls to allow for the six-point standard curve. In addition, the protein standards are serially diluted in BB without the mosquito lysate. Therefore, their background composition differs from that of the test samples. These standards are used in our laboratory to provide consistent CSP quantification of the test samples across different plates. If more accurate quantification is needed, the standards can be prepared using the homogenate of lab reared mosquitoes processed identically to the test samples, but with a known amount of protein added. Lastly, as with most diagnostic assays, the CSP ELISA is not error-free[1]. All positive samples should be confirmed by repeating the assay with heated homogenate (100 ℃, 10 minutes) or by *Plasmodium* species-specific PCR, using the remaining homogenate as the source of the DNA template[1].”

d) The significance with respect to existing methods
e) Any future applications of the technique

Response: The last paragraph of the discussion section has been revised to address these two points.

Line 346-353: “When properly performed, this CSP ELISA method can be highly reliable. It has been, and likely will continue to be, used in several studies of malaria transmission, with the goals to determine the seasonal dynamics of mosquito infection and to identify the species of the major malaria vectors[5-8]. Compared to direct microscopic examination of sporozoites, this assay has much greater throughput and is more suitable to research involving a large number of mosquitoes. Compared to the PCR detection of sporozoites, the ELISA procedure takes more time but costs less[4]. Overall, its simplicity, high throughput and relatively low-cost permit large-scale testing in a standard laboratory.

16. Please submit each figure individually as a vector image file to ensure high resolution throughout production.

Response: Each figure is now in the high resolution SVG format.

17. Figure 2D: In x-axis description, please write the unit as “pg/uL” is instead of “pg/ul”.

Response: The figure has been corrected as suggested.

18. Please revise the table of material to include all the essential supplies, reagents, and

equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

Response: The table of material has been revised as suggested and provided as separate file name “JoVE_Table of Materials_revision.xls”

19. Please do not abbreviate journal Titles in the References.

Response: We use output style as JOVE in Endnote. Please advise or point us to the proper endnote style file.

Reference

1 Durnez, L. *et al.* False positive circumsporozoite protein ELISA: a challenge for the estimation of the entomological inoculation rate of malaria and for vector incrimination. *Malar J.* **10** 195, (2011).

2 Wirtz, R. A., Avery, M., Benedict, M. & Sutcliffe, A. *Methods in Anopheles Research*. 333-343 (2016).

3 Rosenberg, R. *et al.* Circumsporozoite protein heterogeneity in the human malaria parasite Plasmodium vivax. *Science.* **245** (4921), 973-976, (1989).

4 Marie, A. *et al.* Evaluation of a real-time quantitative PCR to measure the wild Plasmodium falciparum infectivity rate in salivary glands of Anopheles gambiae. *Malar J.* **12** 224, (2013).

5 Wirtz, R. A., Burkot, T. R., Graves, P. M. & Andre, R. G. Field evaluation of enzyme-linked immunosorbent assays for Plasmodium falciparum and Plasmodium vivax sporozoites in mosquitoes (Diptera: Culicidae) from Papua New Guinea. *J Med Entomol.* **24** (4), 433-437, (1987).

6 Wirtz, R. A., Sattabongkot, J., Hall, T., Burkot, T. R. & Rosenberg, R. Development and evaluation of an enzyme-linked immunosorbent assay for Plasmodium vivax-VK247 sporozoites. *J Med Entomol.* **29** (5), 854-857, (1992).

7 Arevalo-Herrera, M. *et al.* Immunoreactivity of Sera From Low to Moderate Malaria-Endemic Areas Against Plasmodium vivax rPvs48/45 Proteins Produced in Escherichia coli and Chinese Hamster Ovary Systems. *Front Immunol.* **12** 634738, (2021).

8 Balkew, M. *et al.* An update on the distribution, bionomics, and insecticide susceptibility of Anopheles stephensi in Ethiopia, 2018-2020. *Malar J.* **20** (1), 263, (2021).

Fig1 svg file

Fig2 svg file

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