**Responses to Peer Reviewer Comments:**

**Editorial comments:**

1) All of your previous revisions have been incorporated into the most recent version of the manuscript. In addition, Editor revised steps 1.5, 3.6.2 and 5.14.2 so that they are written in the imperative tense. On the JoVE submission site, you can find the updated manuscript under "file inventory" and download the microsoft word document. Please use this updated version for any future revisions.

2) Please add the sub-steps of 5.14 to the highlighting since the sub-steps describe how to perform step 5.14, which is highlighted.

**The sub-steps were included in the highlighted portion.**

3) In step 1.4: Do you mean 25X95 mm rather than 25X95 m? How long does it take to evaporate most of the solvent?

**This typo was fixed and the time to evaporation was added to the manuscript (lines 125-126).**

4) In step 2.3: Should the blank be PBS or HBS?

**This typo was fixed to be HBS (line 144).**

5) In step 4.2: Define KB. Possibly “kinetic buffer?”

**KB was defined as kinetic buffer in step 4.1 and it’s composition (line 199).**

6) Please ensure that references are numbered in the manuscript in chronological order. Currently, ref 9 appears after ref 5.

**These references were corrected so they are now in chronological order.**

7) Prior to peer review, the length of the Short Abstract is close to our 50 word limit. If, in response to peer review comments, changes are made to the Short Abstract, please ensure that the final length does not exceed 50 words.

**The short abstract remains under 50 words.**

8) When headings are included, the highlighted portion of your protocol is close to our 2.75 page highlighting limit. If, in response to peer review, additional details are added to the protocol, please adjust the highlighting to identify a total of 2.75 pages of protocol text (which includes sub-headings and spaces) that should be visualized to tell the most cohesive story of your protocol steps. The highlighting should include complete statements and not portions of sentences. See JoVE's instructions for authors for more clarification.

**The highlighted portion of the protocol still remains at 2.75 pages.**

9) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

**The manuscript was proofread for errors.**

10) Please disregard the comment below if all of your figures are original.

If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

**All of the images are original in this manuscript and not reprinted from the original publication.**

11) Please be consistent with formatting of journal titles in your references.

\* JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

**Reviewers' comments:**

**Reviewer #1:**

*Manuscript Summary:*

In this manuscript,the authors used an iridium(III) complex as new malaria diagnostic tool based on long-lasting luminescent signal of the complex in the presence of a histidine-rich malarial protein biomarker. Furthermore, the probe worked well either in solution or immobilized on a magnetic particle.However, some minor parts should be addressed by authors before publication.

*Major Concerns:*

N.A

*Minor Concerns:*

1) Please check and correct some format errors, such as "Iridium (III)" in title, "50µM" in line 66, "Ir1" in line 368;.

**These corrections were made in the document, both in the title and on line 66 and 368.**

2) Cyclometalated Ir(III) complexes have many other applications except applications mentioned by authors, such as protein staining, detection of metal ions and enzymes, drug etc. So some related papers could be cited, for example: 1) Angew. Chem. Int. Ed., 2012, 51, 9010; 2) Chem. Soc. Rev., 2013, 42, 3427; 3) Angew. Chem. Int. Ed., 2013, 52, 7666. 4) Acc. Chem. Res., 2014, DOI: 10.1021/ar500310z; 5) Chem. Sci., 2014, DOI: 10.1039/C4SC03094J;

**Several of these papers were cited in the article (line 85).**

*Additional Comments to Authors:*

As mentioned above, I recommend the publication of this manuscript in Journal of Visualized Experiments after minor revision.

**Reviewer #2:**

I think the paper is nice and well written and the results are important and it should be published. However, in the Introduction, authors refer to the detection of malaria using gold materials. There is a recent review dealing with the use of gold for detection and treatment of tropical diseases, namely malaria (Gold Bulletin, 2013, 46:65-79) that should also be cited.

**This bulletin was cited in the article (line 79).**

**Reviewer #3:**

*Manuscript Summary:*

depicts method using iridium to detect histidine rich proteins in malaria

*Major Concerns:*

As addressed in discussion the method is unproven in human whole blood or serum and may need extra layer of specificity

**More clarification was added to the discussion on how the method could be modified to be utilized in a more complex matrix (lines 439-441); however the described method was more a proof of concept before expanding on the probe with the molecular recognition element.**

*Minor Concerns:*

none

**Reviewer #4:**

*Manuscript Summary:*

The chemically synthesized cyclometalated Ir(III) complexes were introduced as detection probes to capture histidine-rich peptides. This manuscript gives a detailed description on the methods to synthesis of Iridium (III) Complex (Ir1) by splitting of the parent cyclometalated Ir(III) chloro-bridged dimer into two equivalents of the solvated complex. The end-product Ir1 was aimed to target repeated histidine residues in malarial biomarker PfHRP-II. A series of validation test has been performed to ensure the specific and functional of Ir1, including interating with various amino acids, BNT-II and recombinant HRP-II. Quenching effects were determined by comparing BNT-II/Ir1 titration with L-Histidine/Ir1 on 96-well magnetic plate. In addition to qualitative, the quantitative kinetic analysis of Ir1 with BNT-II was determined using Biolayer Interferometry platform. The results showed that Ir1 was able to detect histidine-based molecules. However, false positive diagnosis was found in Ir1 when it reacted with histidine-rich protein marker. The way to address it has also been presented in manuscript.

*Major Concerns:*

Nil

*Minor Concerns:*

1. In 1.4, 25×95 "mm" dram vial, not "m"

**This correction was made in step 1.4 (line 125).**

2. Though Ir1 probing assay sounds promising and time-saving, malaria RDTs only need 15 mins to detect an infected sample and are widely available elsewhere. Could you briefly explain whether the Ir1 can be used to replace antibody in classical RDT format?

**The Ir1 probe would not serve to replace the capture antibody on the strip, but it could potentially replace the antibody functionalized gold conjugate in the RDT. The probe could be incorporated into the RDT by using electrochemiluminescence as the readout, as mentioned on lines 441-443.**

3. Some of the current antibody-based ELISA are reported to possess high sensitivity with as low as < 200 p/µL parasitaemia detection limit. Can Ir1 probe achieve this cut-off level?

**As mentioned on line 428, this method cannot yet achieve this cut-off level. With the design of the molecular recognition element and potentially using electrochemiluminesence as a detection strategy, we hope to achieve these lower limits of detection in the future.**

4. Do you plan to use malaria native protein as a antigen model in further works?

**Once the bifunctional probe is designed and synthesized, we hope to incorporate the probe into a complex matrix and assay the probe again native protein. This is addressed on lines 439-441.**

5. Would be good if to write out the source of BNT-II, rc-HRP-II, and other relevant reagents.

**These reagents are listed in the materials spreadsheet to be supplied with the manuscript.**

6. Undoubtedly, the development in this study is excellent. I was wondering the Ir1 probe will also react with other irrelevant recombinant proteins with His-6X tag. Would this problem be possibly addressed by "bifunctional probe" as suggested in the discussion?

**In a complex matrix/biological application, there wouldn’t be as much of a worry about His-tagged proteins interfering but other histidine containing serum proteins (ie. human serum albumin). By designing the bifunctional probe, we hope to mediate this problem by specifically targeting *pf*HRP2 with an aptamer, as addressed in the discussion.**

*Additional Comments to Authors:*

Nil