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Iridium(III) Luminescent Probe for Detection of the Malarial Protein Biomarker Histidine Rich Protein-II --Manuscript Draft--

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Abstract:	<p>This work outlines the synthesis of a non-emissive, cyclometalated Ir(III) complex, Ir(ppy)₂(H₂O)₂⁺ (Ir1), which elicits a rapid, long-lived phosphorescent signal when coordinated to a histidine-containing protein immobilized on the surface of a magnetic particle. Synthesis of Ir1, in high yields, is complete overnight and involves splitting of the parent cyclometalated Ir(III) chloro-bridged dimer into two equivalents of the solvated complex. To confirm specificity, several amino acids were probed for coordination activity when added to the synthesized probe, and only histidine elicited a signal response. Using BNT-II, a branched peptide mimic of the malarial biomarker Histidine Rich Protein II (pHHRP-II), the iridium probe was validated as a tool for HRP-II detection. Quenching effects were noted in the BNT-II/Ir1 titration when compared to L-Histidine/Ir1, but these were attributed to steric hindrance and triplet state quenching. Biolayer interferometry was used to determine real-time kinetics of interaction of Ir1 with BNT-II. Once the system was optimized, the limit of detection of rCHRP-II using the probe was found to be 12.8nM in solution. When this protein was immobilized on the surface of a 50µm magnetic agarose particle, the limit of detection was 14.5nM. The robust signal response of this inorganic probe, as well as its flexibility of use in solution or immobilized on a surface, can lend itself toward a variety of applications, from diagnostic use to imaging.</p>
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To whom it may concern,

We wish our manuscript, **“Iridium (III) Luminescent Probe for Detection of the Malarial Protein Biomarker Histidine Rich Protein-II ”** to be considered for publication in an upcoming issue of the *Journal of Visualized Experiments*.

While use of colorimetric and fluorescent probes has become a critical technique for rapid detection of biomarkers in well-equipped, first world laboratories, this need also extends to low-resource areas. Having a robust and sensitive reagent is one of the main necessities for low-resource diagnostics to succeed in the third-world disease management. In this submission, we demonstrate the synthesis and application of an emerging class of Ir(III) cyclometallated probe for detection of the malarial biomarker Histidine Rich Protein II (HRPII). $\text{Ir(ppy)}_2(\text{H}_2\text{O})_2^+$ (**Ir1**) is a unique probe in that it is non-emissive in the solvent state. Only upon binding the imidazole side chains of histidine does this probe emit a long-lasting blue green signal. We demonstrated low nanomolar limits of detection of **Ir1** toward HRPII when bound to the surface of a magnetic microparticle. This ELISA-based assay takes only 1.5-2 hours, compared to 5+ hours for a traditional ELISA. By integrating the probe with particle-based separation techniques, we envision coupling this detection method with readily available diagnostic designs.

This is a highly visual technique in that simply using a black light excites the probe. With the naked eye, the user is able to visualize a concentration dependent blue-green luminescent response of this probe with histidine rich peptides. Additionally, the probe can be imaged on the surface of magnetic particles when the biomarker is bound to the particle surface. Keersten Davis synthesized the probe and conducted the in-solution and on-bead titration experiments, while Anna Bitting assisted with these experiments. Christine Markwalter and Westley Bauer assisted with the biolayer interferometry experiments. The manuscript was written by Keersten Davis, with assistance from Anna Bitting.

I would like to suggest Dr. Dik-Lung Ma (edmondma@hkbu.edu.hk) of Hong Kong Baptist University, Dr. C. Bor Fuh (cbfuh@ncnu.edu.tw) of National Chi Nan University, Dr. Blake Peterson (brpeters@ku.edu) of the University of Kansas, Dr. A. Prasanna da Silva (a.desilva@qub.ac.uk) of Queen's University, Dr. Ola Soderberg (ola.soderberg@igp.uu.se) of Uppsala Universitet, and Dr. Ramon Martinez-Manez (rmaez@qim.upv.es) of the Universidad Politecnica de Valencia as people well suited to review and edit this material.

Sincerely,
David Wright

TITLE: Iridium(III) Luminescent Probe for Detection of the Malarial Protein Biomarker Histidine Rich Protein-II

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SHORT ABSTRACT:

Robust detection reagents are of increasing necessity for developing new malaria diagnostic tools. An iridium(III) probe was designed that emits long-lasting luminescent signal in the presence of a histidine-rich malarial protein biomarker. Detection of the protein either in solution or immobilized on a magnetic particle affords flexibility in application.

LONG ABSTRACT:

This work outlines the synthesis of a non-emissive, cyclometalated Ir(III) complex, $\text{Ir}(\text{ppy})_2(\text{H}_2\text{O})_2^+$ (**Ir1**), which elicits a rapid, long-lived phosphorescent signal when coordinated to a histidine-containing protein immobilized on the surface of a magnetic particle. Synthesis of **Ir1**, in high yields, is complete overnight and involves splitting of the parent cyclometalated Ir(III) chloro-bridged dimer into two equivalents of the solvated complex. To confirm specificity, several amino acids were probed for coordination activity when added to the synthesized probe, and only histidine elicited a signal response. Using BNT-II, a branched peptide mimic of the malarial biomarker Histidine Rich Protein II (*pf*HRP-II), the iridium probe was validated as a tool for HRP-II detection. Quenching effects were noted in the BNT-II/**Ir1** titration when compared to L-Histidine/**Ir1**, but these were attributed to steric hindrance and triplet state quenching. Biolayer interferometry was used to determine real-time kinetics of interaction of **Ir1** with BNT-II. Once the system was optimized, the limit of detection of rCHRP-II using the probe was found to be 12.8nM in solution. When this protein was immobilized on the surface of a 50 μm magnetic agarose particle, the limit of detection was 14.5nM. The robust signal response of this inorganic probe, as well as its flexibility of use in solution or immobilized on a surface, can lend itself toward a variety of applications, from diagnostic use to imaging.

INTRODUCTION:

Colorimetric and fluorescent labeling is an important method for the detection and tracking of biochemical molecules and processes.^{1,2} The most common fluorescent markers are low molecular weight organic dyes^{3,4}, but these molecules do not always have ideal optical properties. Fluorescent dyes are prone to photobleaching, often have small Stokes shifts, and may have overlapping excitation and emission spectra. Colorimetric labeling is often achieved by the use of enzymatic labels, which have an amplified signal useful in immunoassay quantification.^{5,6} These enzymes also have their drawbacks, including photosensitivity, reaction conditions, and short substrate shelf life. These properties tend to require immunoassays and protein labeling methods to be done under well-controlled conditions using costly reagents.

Emissive transition-metal complexes have been explored as an alternative labeling approach for biochemical detection. In particular, cyclometalated Ir(III) has been studied in the context of organic light emitting diodes (OLEDs)⁷⁻⁹ oxygen sensing¹⁰, catalysis¹¹, and protein/cell staining.¹²⁻¹⁴ High photostability and quantum efficiency make this class of probes a good candidate for biomolecule detection.^{15,16} It was previously found that cyclometalated Ir(III) complexes, of the form $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{solv})_2]^+$, irreversibly bind histidine and elicit a blue-green signal response.^{12,17} These complexes are non-emissive in the solvato state, but when histidine

displaces the solvent molecules and binds to the metal center, they release an intense phosphorescent signal after long-wave UV irradiation. This signal only occurs after ligand substitution, and is the result of a triplet state electron relaxing to the ground state through the activation of metal ligand charge transfer ($^3\text{MLCT}$) and ligand centered transfer (^3LC) pathways.^{8,15} These complexes can potentially be used as detection probes for histidine-rich proteins.

Histidine-rich proteins and their regulation levels are important in many diseases, including liver cirrhosis, cancer, and thrombotic disorders.¹⁸ *Plasmodium falciparum* Histidine Rich Protein II (*pfHRP-II*) in particular is a well-validated biomarker for malaria parasite infection. This protein is 67 kDa and contains 34% histidine, mostly within characteristic AHHAHHAAD repeating motifs.¹⁹ These histidine repeats can bind free metal ions¹⁹ and heme complexes²⁰ in host blood. *pfHRP-II* is commonly detected in low-resource settings using immunochromatographic rapid diagnostic tests (RDTs), but these tests are often inaccurate due to sample conditions, low biomarker concentration, poor manufacturing standards, and antibody degradation.²¹

Metal-based phosphorescent probes such as the cyclometalated Ir(III) complexes described above are attractive options for the detection of *pfHRP-II* due to their selective binding of histidine and their stable and efficient emission properties. In this paper, the use of $[\text{Ir}(\text{ppy})_2(\text{H}_2\text{O})_2]^+$ (**Ir1**) to detect BNT-II, a branched peptide mimic of *pfHRP-II* is explored.²² Kinetics of this interaction were monitored in real-time using biolayer interferometry techniques. The assay was also adapted to an on-bead ELISA-type format, where nanomolar limits of detection were achieved. This assay holds advantages over traditional ELISAs because it can be performed in under 2 hours with antibody-free reagents, instead of the 4-5 hours and biological reagents required with typical ELISAs.

PROTOCOL:

1. Synthesis of Iridium(III) Complex

1.1) Weigh out 53.6mg (50 μmol) of $[\text{Ir}(\text{ppy})_2\text{-Cl}]_2$ and dissolve in 5 mL of methylene chloride. Add this solution to a 50 mL Erlenmeyer flask equipped with a stir bar.

1.2) Weigh out 26.2 mg (100 μmol) of AgOTf and dissolve in 5 mL of methanol.

1.3) Add the dissolved AgOTf to the $[\text{Ir}(\text{ppy})_2\text{-Cl}]_2$ solution and stir for 1 hour. Note: A cream colored slurry should result.

1.4) After 1 hour, filter the slurry through silica gel into a 25x95mm dram vial and evaporate off remaining solvent using a rotary evaporator (5-10 minutes).

1.5) Once most of the solvent is removed, check that an oily yellow residue remains in the vial. To this residue, add 1mL of methanol to re-dissolve the product and lyophilize 1-2 days to yield the yellow solid product.

1.6) Characterize the $\text{Ir}(\text{ppy})_2(\text{H}_2\text{O})_2^+$ product (**Ir1**) by ^1H NMR (in CDCl_3), ESI and UV-Vis as

described previously.²³

2. Interactions of Ir1 with Various Amino Acids

2.1) Prepare a 2 mM solution of **Ir1** in methanol (MW 537.10 g/mol). Vortex to ensure complete dissolution of the solid.

2.2) Prepare 100 μ M solutions of the following amino acids and biomolecules in HEPES buffered saline (HBS, 100mM HEPES, 137mM NaCl, pH 7.4) Ala, Asp, Cys, His, Ile, Lys, Ser, Try, Val.

2.3) Pipette 100 μ L of each amino acid into a black 96-well plate. Add 100 μ L of HBS to the plate to serve as a blank.

2.4) Add 2.5 μ L of the 2 mM **Ir1** solution to each sample and shake the plate on a plate shaker for 10 minutes.

2.5) After 10 minutes, acquire the emission spectra of the samples using a 96-well plate reader (365ex/400-700em).

2.5)1. Place the plate in the instrument and open the plate reader software to set up a new experiment.

2.5)2. Set the new experiment to read a fluorescence scan with an excitation wavelength of 365 nm and a slit width of 9 nm with the optics at the top position.

2.5)3. Read the emission from 300-700 nm with a step size of 5 nm.

2.5)4. Export the data for data analysis.

2.6) Transfer the samples to a clear 96-well plate and image the emission using a UV Transilluminator.

3. Titration of Ir1 with BNT-II

3.1) Prepare a 1 mM stock solution of BNT-II (MW 8233.6 g/mol) in HBS as well as a 2 mM solution of **Ir1** in methanol.

3.2) From the stock solution, prepare 1 mL of 100 μ M BNT-II in HBS. Vortex the microcentrifuge tube to ensure the sample is mixed.

3.3) Serially dilute the 100 μ M BNT-II by half in HBS to a final concentration of 1.56 μ M BNT-II.

3.4) Add 100 μL of each dilution, in triplicate, with HBS serving as a blank, to the wells of a 96 well plate. To each sample, add 2.5 μL of 2 mM **Ir1**.

3.5) Shake the plate for 10 minutes on a plate shaker.

3.6) After shaking, read the emission intensity at 510nm (excitation at 365nm) using a 96-well plate reader.

3.6)1. Place the plate in the instrument and open the plate reader software to set up a new experiment.

3.6)2. Set the new experiment to read a fluorescence endpoint with an excitation wavelength of 365nm and emission wavelength of 510nm. Set the slit width for both wavelengths to 9nm, with the optics at the top position.

3.6)3. Read the plate and export the data for analysis.

3.7) Transfer the samples to a clear 96-well plate and image the titration using a UV transilluminator.

4. Real-Time Kinetic Analysis of the Ir1/BNT-II System using Biolayer Interferometry

4.1) Add 200 μL of kinetic buffer (KB; 1x phosphate buffered saline with 0.2% Tween-20) to 8 wells in the first column of a black 96-well plate and insert the plate into the plastic sensor holder. Carefully transfer 8 Ni(II)NTA biosensors to the first column in the plastic holder such that the tips are suspended in the wells of buffer. Place the plastic holder in the left side of the interferometry instrument.

4.2) Prepare a 2 mL of a 0.5 μM solution of BNT-II in KB.

4.3) Prepare 500 μL of a 10 μM solution of **Ir1** in KB, and serially dilute by half down to 0.156 μM in KB.

4.4) To a new black 96 well plate, pipette 200 μL of KB to all 8 wells in column A and C.

4.5) In the same plate, pipette 200 μL of the 0.5 μM BNT-II solution to the wells in column B.

4.6) Pipette 200 μL of each dilution of **Ir1** into column D, starting with KB as the blank in well D1. Add the dilutions so that the wells increase in **Ir1** concentration down the column.

4.7) Place this plate in the instrument to the right of the plate containing the pre-wetting sensors.

4.8) In the biolayer interferometry software, set up a basic kinetic experiment by defining

the plate, assay, and sensor tips.

4.8)1. To define the plate, select a column on the screen, right click, and choose the appropriate definition for the wells. Select “Buffer” for columns A and C, “Load” for column B, and “Sample” for column D.

4.8)2. Define the assay in the next tab by adding the following steps (Click “Add”): Equilibration (Custom), 60 seconds; Loading, 120 seconds; Baseline, 60 seconds; Association, 120 seconds; and Dissociation, 300 seconds. Select the Equilibration step and double-click on column A. Repeat for Loading (column B), Baseline (column C), Association (column D), and Dissociation (column C). Select the Ni(II)NTA sensors.

4.8)3. Move to the next tab and confirm that the sensors are in column A on the plastic sensor holder.

4.8)4. Confirm the experiment is outlined correctly, insert a filename, ensure that “delay experiment” is checked, and press “Go.”

Note: Due to the automated nature of the instrument, once the kinetic experiment is outlined, the steps will occur as programmed.

4.9) Once the kinetic run is complete, process the data in the provided processing software.

4.9)1. Double-click on the folder with the filename of interest in the bottom portion of the panel on the left. Then, double-click the corresponding folder under “Kinetics” in the top portion of the panel. Move to the Processing tab.

4.9)2. Check the Subtraction box on the left to open the sensor selection screen. Right click on well A4 and change the well type to Reference Well.

4.9)3. Check the box next to Align Y axis, and ensure the selected step is Baseline. Specify the time range to be the last five seconds of the baseline (approximately 55 – 60 s).

4.9)4. Check the box for Inter-step Correction and select Align to Dissociation. Ensure that the Savitzky-Golay Filtering box is checked and press “Process Data!”

4.9)5. Move to the Analysis tab. Ensure that “Association and Dissociation” is the selection for steps to analyze and that the Model is 1:1. Select a Local, Full fit and press “Fit Curves!”

4.9)6. Highlight all curves in the table and right click to set all curves to one color. Next, select a Global (Full) fit, grouped by color and Rmax Unlinked by Sensor. Press “Fit Curves!” to obtain global kinetics data.

5. On-Bead Detection of BNT-II and rcHRP-II with Ir1

5.1) Prepare 1 mL each of a 1 μ M solution of BNT-II and rcHRP-II in HBS with Tween (HBST; HBS with 0.025% Tween 20).

5.2) Serially dilute each protein by half in HBST to a final concentration of 15.6 nM.

5.3) Pipette 100 μ L of each dilution, in triplicate, into a white 96-well plate, with the dilutions in order from lowest to highest concentration down a column.

5.4) Pipette 10 μ L of 50 μ m Ni(II)NTA magnetic agarose particles into each dilution well.

5.4)1. Vortex the microcentrifuge tube containing the magnetic particles after each pipetting to ensure the particles stay suspended.

5.5) Place the plate on a plate shaker for 15 minutes to incubate the samples with the particles.

5.6) After this incubation period, place the plate on a 96-well plate magnet and wait 30 seconds for the particles to pull out of solution.

5.7) Using a multichannel pipette, pull off the original sample and discard as waste. Remove the plate from the magnet.

5.8) Add 200 μ L of HBST to each well using the multichannel pipette. Pump the buffer up and down several times to wash the magnetic particles.

5.9) Place the plate back onto the magnet and wait 30 seconds for the particles to pull out of solution.

5.10) Using the multichannel pipette, pull off the 200 μ L of buffer and discard as waste. Remove the plate from the magnet.

5.11) Repeat steps 5.8 through 5.10 two times to complete three washings of the particles.

5.12) Add 100 μ L of HBST to each well containing magnetic particles followed by 2.5 μ L of 2 mM **Ir1**.

5.13) Incubate the particles with **Ir1** on a plate shaker for 1 hour.

5.14) After 1 hour, read the emission intensity at 510nm (excitation at 365nm) using a 96-well plate reader.

5.14)1. Place the plate in the instrument and open the plate reader software to set up a new experiment.

5.14)2. Set the new experiment to read a fluorescence endpoint with an excitation wavelength of 365nm and emission wavelength of 510nm. Set the slit width for both wavelengths to 9nm, with the optics at the top position.

5.14)3. Read the plate and export the data for analysis.

5.15) Transfer the samples to a clear 96-well plate and image the titration using a UV Transilluminator.

REPRESENTATIVE RESULTS:

As shown in Figure 1, synthesis of the $[\text{Ir}(\text{ppy})_2(\text{H}_2\text{O})_2]^+$ (**Ir1**) solvento-complex involved splitting of the chloride bridge in the parent dimer by precipitation of the chloride in the form of insoluble AgCl and association of water molecules to the metal center. The formation of **Ir1** was confirmed by ^1H NMR and ESI. Additionally, UV-visible bands characteristic of metal ligand charge transfer and π - π^* transitions were assigned to the spectrum, further validating the formation of **Ir1**. This solvated complex exhibits no emissive character when excited at 365nm.

[Place Figure 1 here]

Once the complex was synthesized and characterized, the amino acid selectivity was analyzed, as previously described using a similar iridium analog.¹⁷ Figure 2A shows that only histidine elicited a signal response at 510nm, when excited at 365nm.

[Place Figure 2 here]

This “on-switch” of the phosphorescence of the iridium probe occurs because the singlet excited state ($^1\text{MLCT}/^1\text{LC}$) of the Ir(III) bioconjugate undergoes intersystem crossing to the triplet excited state ($^3\text{MLCT}$), when histidine is coordinated to the metal center. This probe was applied to BNT-II a peptide mimic of the malarial biomarker *Plasmodium falciparum* Histidine Rich Protein II (*pf*HRP-II). In a titration of BNT-II with **Ir1**, a concentration dependent signal response was observed (Figure 2B). Recombinant HRP-II (rcHRP-II) also exhibited a similar response in solution. The limits of detection of BNT-II and rcHRP-II in solution were 54.8nM and 12.8nM, respectively. Using biolayer interferometry techniques, the K_D of **Ir1** binding to BNT-II immobilized on a Ni(II)NTA surface was found to be 2.05 μM (Figure 3).

[Place Figure 3 here]

When transitioning from a solution-based assay to a magnetic particle platform, the added complexity of the particle in the system had to be taken into account. It was known from previous work, that these particles efficiently bind the malaria biomarker *pf*HRP-II.²⁴ Black fluorescent plates worked well for the solution assay describe above, but white plates were better suited for the on-particle detection platform, as seen in figure 4A. In a white plate, the

light is reflected back into the sample, thus allowing for better absorbance by the **Ir1** bound to the surface of the particle. In the on-particle assay, the limit of detection of rcHRP-II was determined to be 14.5nM (Figure 4B). The limit of detection for rcHRP-II in-solution and on-bead were statistically the same based on an unpaired t-test ($p=0.731$).

[Place Figure 4 here]

Figure Legends

Figure 1: Synthesis and Characterization of Ir1. Chloride bridge splitting reaction of Dichlorotetrakis(2-(2-pyridinyl)phenyl)diiridium(III) to create the aquo complex $\text{Ir}(\text{ppy})_2(\text{H}_2\text{O})_2^+$ (**Ir1**). Upon addition of L-His to the aquo complex in aqueous buffer, luminescent signal is switched on.

Figure 2: Amino Acid Selectivity and Titration of Ir1 with Histidine Rich Peptides. A) Interaction of 200 μM of various amino acids (Cys, Ser, Asp, Glu, Phe, Lys, Arg, Tyr, Trp, His) with 50 μM **Ir1** in HBS. Spectral scans were taken from 400-700 nm at an excitation wavelength of 365 nm in a black 96 well plate. Signal in relative fluorescence units (RFU) from all amino acids besides histidine (dashed black trace) was negligible. B) Nanomolar concentrations of BNT-II (■) and rcHRP-II (◆) were titrated with **Ir1** in HBS. The histidine rich peptides were incubated with the probe for 15 minutes in solution before reading the emission at 510 nm after excitation with 365 nm light. Limit of detection was calculated as the value of x when $y=3\sigma_{\text{blank}}$.

Figure 3: Real-time Kinetic Analysis of Ir1 with BNT-II. Biolayer interferometry for kinetic analysis of various concentrations of **Ir1** binding to BNT-II on the surface of a Ni(II)NTA glass sensor. After equilibrating the sensors in KB (Region A), the sensors are loaded with 0.5 μM BNT-II (Region B). Once the peptide is loaded on the sensors, a baseline is established (Region C) prior to measuring the association of **Ir1** to the BNT-II (Region D). After a period of association, the sensors are placed back into KB to measure dissociation (Region E). The whole kinetic analysis process takes less than 30 minutes.

Figure 4: On-Bead Detection of BNT-II and HRP-II. A) Difference between the signal detected from **Ir1** bound to BNT-II on the surface of 50 μm Ni(II)NTA magnetic agarose particles in a black 96-well plate (solid line) versus a white 96-well plate (dashed line). The particles were excited with 365 nm light, and emission was measured at 510 nm. B) Titration of rcHRP-II immobilized on the magnetic particles and detected using **Ir1**. Limit of detection was calculated as the value of x when $y=3\sigma_{\text{blank}}$.

Figure 5: Schematic Representation of the On-Bead Detection of rcHRP-II with Ir1. General scheme of HRP-II binding to the surface of Ni(II)NTA particles and labeled with **Ir1**. The particles are incubated with a histidine rich peptide for 15 minutes. After this incubation period, the particles are washed with HBST using a magnet, in order to pull the particles out of solution. Finally, the peptide bound particles are incubated with **Ir1** for 1 hour prior to reading the

emission at 510 nm after excitation with 365 nm light.

DISCUSSION:

As microparticle-based diagnostic tools come to the forefront of modern diagnostic technology, detection of target biomolecules directly on the surface of the particle is a major advantage. Traditional molecular detection methods, such as ELISA and PCR, are advantageous, in that they can achieve very low limits of detection.²⁵ However, these assays require extensive reagents and lengthy protocols. This assay was designed to mimic ELISA-type sandwich interactions without the time and reagent requirements (Figure 5). Additionally, the cost of the **Ir1** probe per sample was estimated to be around a penny, whereas the cost of antibodies for a typical ELISA is \$0.20-0.30 per sample.

[Place Figure 5 here]

Since the methods described above rely on a cyclometalated iridium probe for detection of the malarial biomarker, this probe would not be susceptible to failure modes common to other detection methods (i.e. fluorophore quenching and antibody/enzyme degradation). Histidine is unique in its metal binding properties. The outlined work takes advantage of this phenomenon by replacing the antibodies in a typical ELISA sandwich with metal containing complexes. Ni(II)NTA captures rCHRP-II on the surface of the particle and **Ir1** signals the presence of the protein. The most critical step in the assay is allowing the magnetic particles to mix with the sample and the probe. In a 96-well plate ELISA, the biomolecules and reagents reach equilibrium with the two-dimensional surface of the bottom of the well. Magnetic particles have the tendency to settle out of solution due to their dense iron-oxide core, which would reduce the available binding sites. Thus, the particles must be mixed during the assay time to ensure maximal binding.

When designing a reagent for disease diagnostics, one must keep in mind the form of patient sample as well as the physiological concentration of the biomarker. For malaria, the concentration of *pf*HRP-II in a patient's blood can vary from low picomolar to high nanomolar. While this assay is clinically relevant for higher levels of infection, the limit of detection needs to be improved in order to detect asymptomatic patients with low picomolar circulating *pf*HRP-II. In the methods outlined above, the "switch-on" signal generated by **Ir1** occurs in the presence of histidine. While the malarial biomarker is rich in histidine, other serum proteins, such as human serum albumin and histidine rich glycoprotein, would elicit a signal response with the probe. This would in turn lead to false positive diagnoses. This makes the capture of the protein on the surface of the particle a beneficial step, in that the protein of interest can be rapidly extracted from a patient sample. Additionally, designing a bifunctional probe, that couples a histidine rich peptide to a robust molecular recognition element (i.e. aptamer), could add another layer of specificity to the assay. The peptide would be loaded with iridium before coupling to the aptamer. In such a design, the stable **Ir1** probe can still be utilized while achieving target specificity with the aptamer. This would allow for application of the probe to detect native HRP-II in a complex matrix (i.e. plasma or whole blood) and reduce non-specific

binding effects. In order to further enhance the signal of rapid diagnostic tests, the assay could be incorporated into an electrochemiluminescent (ECL) system, where *pf*HRP-II can be detected on the RDTs as a electrochemical readout.²⁶ This next generation iridium probe would greatly enhance our on-bead ELISA assay for the detection of disease biomarkers.

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DISCLOSURES:

The authors have nothing to disclose.

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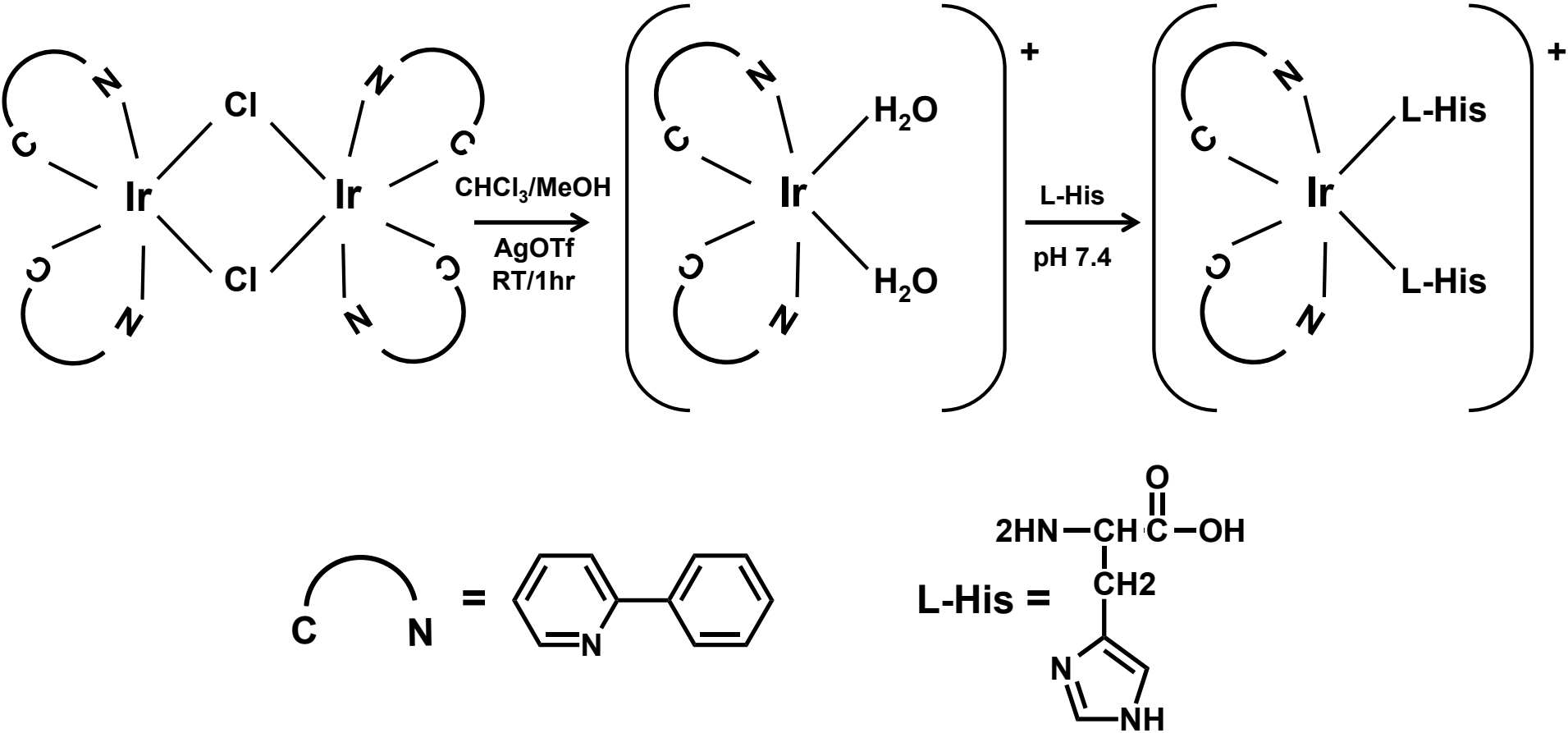


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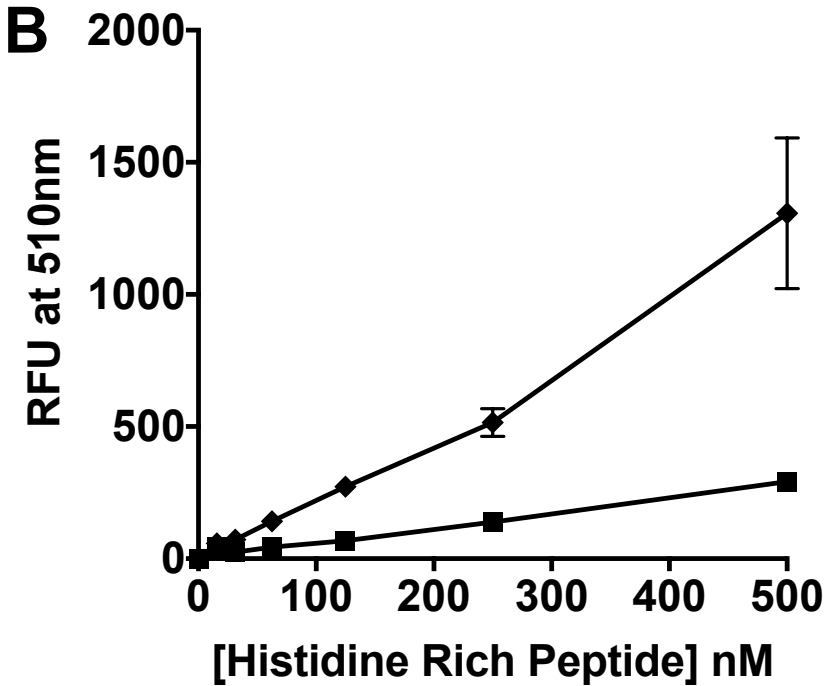
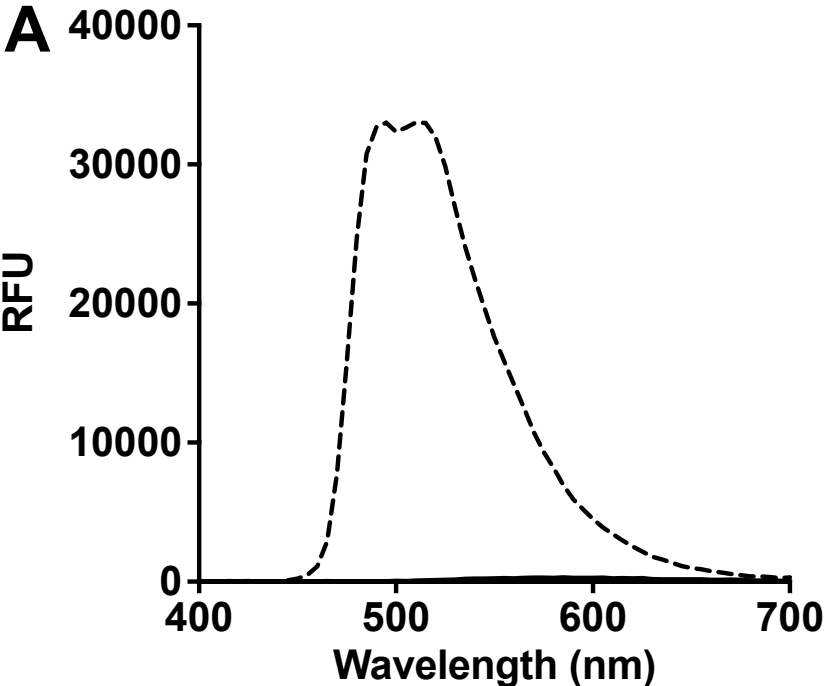


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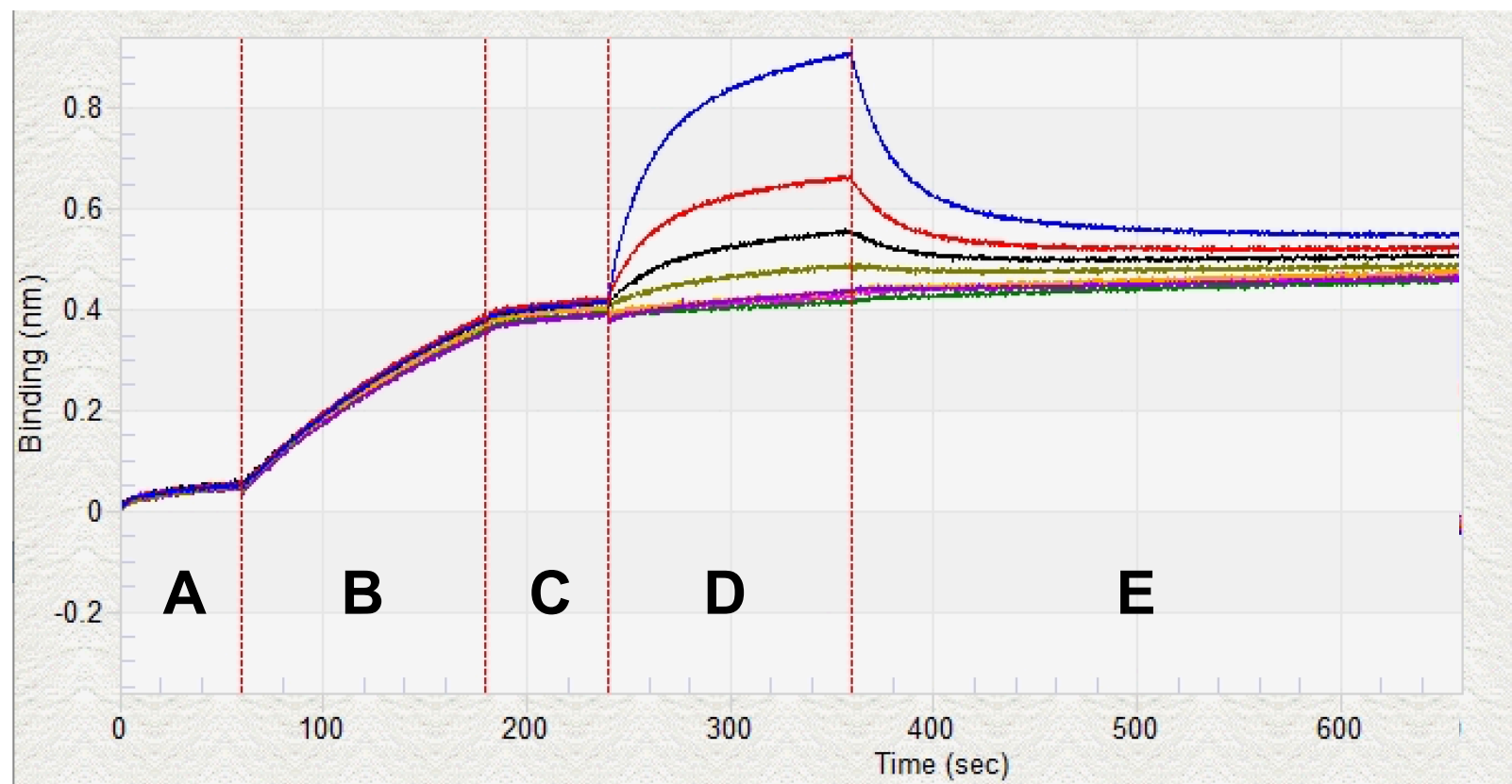


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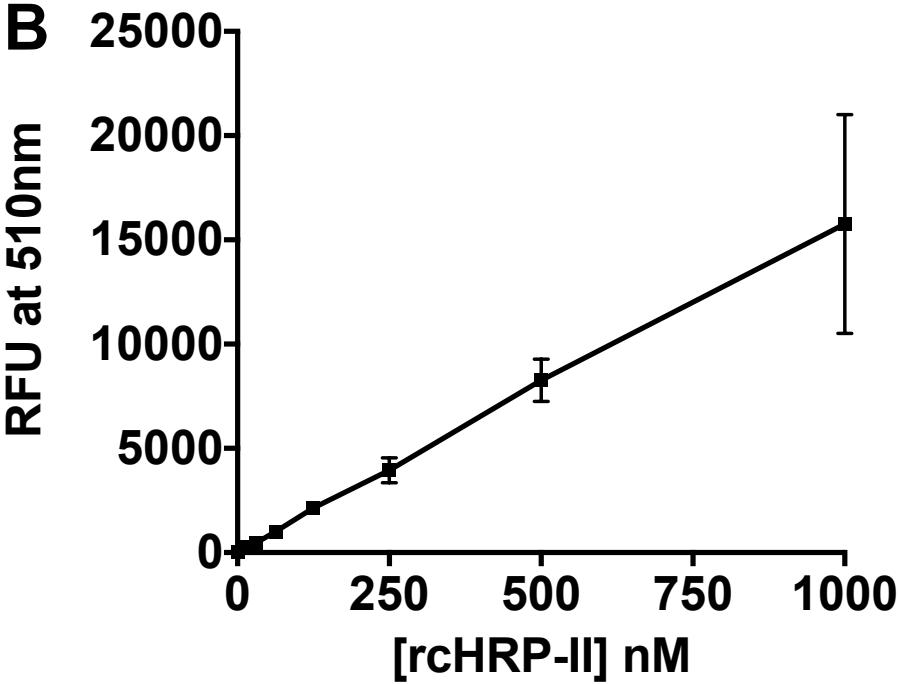
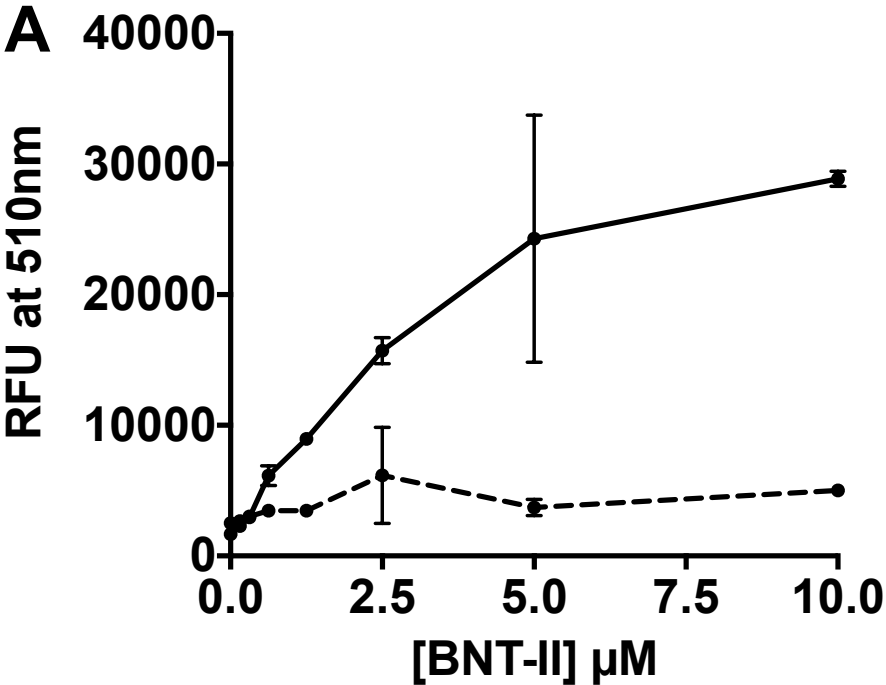
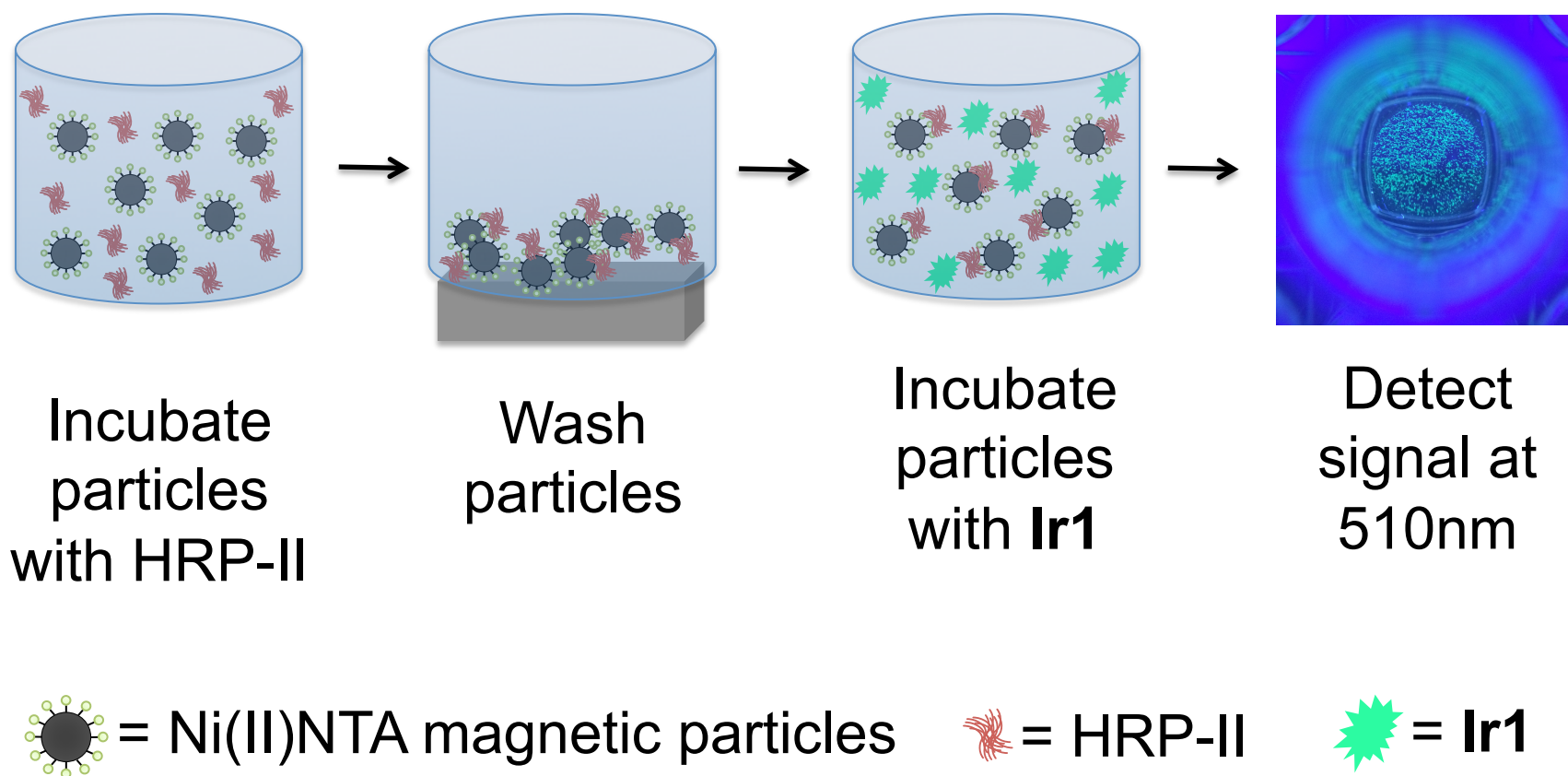


Figure 5
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Name
Dicholortetrakis(2-(2-pyridinyl)phenyl)diiridium(III)
silver trifluoromethanesulfonate
Silica gel
L-Amino acids
HEPES
BNT-II peptide
recombinant HRP II
Costar black polypropylene 96-well plate
Costar white polypropylene 96-well plate
Grenier black polypropylene 96-well plate
Ni(II)NTA magnetic agarose particles
Ni(II)NTA biolayer interferometry sensors
Octet Red96 Biolayer Interferometry Instrumet
Biotek Synergy H4 Microplate Reader
Fisher Biotech Transilluminator

Vendor	Catalog Number
Sigma-Aldrich	658383
Sigma-Aldrich	176435
Sigma-Aldrich	6858
Fisher Scientific	varies
Sigma-Aldrich	H3375
Synthesized in house	N/A
Immunology Consultants Laboratory inc.	AGPF-55
Fisher Scientific	07-200-567
Fisher Scientific	07-200-589
VWR	89089-582
Qiagen	36111
ForteBio	18-5101
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Biotek	
Fisher Scientific	FBDLT-88



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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.

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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, "50 μ M" 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 interacting 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 electrochemiluminescence 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 *p*/HRP2 with an aptamer, as addressed in the discussion.

Additional Comments to Authors:

Nil