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TITLE:

Optimized Griess Reaction for UV-Vis and Naked-Eye Determination of Anti-Malarial Primaquine

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KEYWORDS:

Antimalarial primaquine, 8-aminoquinoline, colorimetric detection, naked-eye detection, UV-vis spectrum, azo product

SUMMARY:

This protocol describes a novel colorimetric method for antimalarial primaquine (PMQ) detection in synthetic urines and human serums.

LONG ABSTRACT:

Primaquine (PMQ), an important anti-malarial drug, has been recommended by the World Health Organization (WHO) for the treatment of life-threatening infections caused by *P. vivax*

and *ovale*. However, PMQ has unwanted adverse effects that leads to acute hemolysis in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency. There is a need to develop simple and reliable methods for PMQ determination with the purpose of dosage monitoring. In early 2019, we have reported an UV-Vis and naked-eye based approach for PMQ colorimetric quantification. The detection was based on a Griess-like reaction between PMQ and anilines, which can generate colored azo products. The detection limit for direct measurement of PMQ in synthetic urine is in the nanomolar range. Moreover, this method has shown great potential for PMQ quantification from human serum samples at clinically relevant concentrations. In this protocol, we will describe the technical details regarding the syntheses and characterization of colored azo products, the reagent preparation, and the procedures for PMQ determination.

INTRODUCTION:

PMQ is one of the most important antimalarial drugs that work not only as a tissue schizontocide to prevent relapse but also as a gametocytocide to interrupt disease transmission[1-4]. Intravascular hemolysis is one of the concerning side effects of PMQ, which becomes extremely serious in those deficient in G6PD. It is known that the G6PD genetic disorder is distributed worldwide with a gene frequency between 3-30% in malaria endemic areas. The severity of PMQ weakness depends on the degree of G6PD deficiency as well as the dose and the duration of PMQ exposure[5,6]. To lower the risk, the WHO has recommended a single low dose (0.25 mg base/kg) of PMQ for malaria treatment. However, this is still challenged by the variations in patient drug sensitivity[5,7]. Dose monitoring is necessary to assess the pharmacokinetics after PMQ administration, which can effect dosage adjustment for a successful treatment with limited toxicity.

High-performance liquid chromatography (HPLC) is the most widely used technique for PMQ clinical determination. Endoh et al. reported a HPLC system with a UV detector for serum PMQ quantification using a C-18 polymer gel column[8]. In their system, serum proteins were first precipitated with acetonitrile, and then the PMQ in the supernatant was separated for HPLC. The calibration curve was linear over the concentration range from 0.01-1.0 μg/mL[8]. Another method based on a reverse-phase HPLC with UV detection at 254 nm has been reported for the quantification of PMQ and its major metabolites[9]. The calibration curve for PMQ was linear in the range between 0.025-100 μg/mL. An additional liquid-liquid extraction with mixed hexane and ethyl acetate as organic phase was used for PMQ separation with percentage recovery reached to 89%[9]. More recently, Miranda et al. developed an UPLC method with UV detection at 260 nm for PMQ analysis in tablet formulations with a detection limit at 3 μg/mL[10].

Though HPLC methods exhibit promising sensitivity in drug determination and the sensitivity can be further improved if the HPLC is equipped with a mass spectrometer, there are still some disadvantages. Direct drug measurements in biological fluids are usually inaccessible by HPLC, since many biomolecules can greatly influence the analysis. Additional extractions are required to remove endogenous molecules before HPLC analysis[11,12]. Moreover, PMQ detection by a HPLC-UV detector is typically performed at its maximum absorption wavelength (260 nm).; however, there are many endogenous molecules in biological fluids with a strong absorbance at

260 nm (e.g., amino acids, vitamins, nucleic acids and urochrome pigments), thus interfering with PMQ UV detection. There is a need to develop simple and cost-effective methods for PMQ determination with reasonable sensitivity and selectivity.

The Griess reaction was first presented in 1879 as a colorimetric test for nitrite detection[13-16]. Recently, this reaction has been extensively explored to detect not only nitrite but also other biologically relevant molecules[17-20]. We have previously reported the first systematic study of an unexpected Griess reaction with PMQ (**Figure 1**). In this system, PMQ is able to form colored azos when coupled with substituted anilines in the presence of nitrite ions under acidic conditions. We have further found that the color of azos varied from yellow to blue when increasing the electron donating effect of the substituent on anilines[21]. A UV-vis absorption based colorimetric method for PMQ quantification has been developed through the optimized reaction between 4-methoxyaniline and PMQ. This method has shown great potential for sensitive and selective detection of PMQ in bio-relevant fluids. Here, we aim to describe the detailed procedures for PMQ determination based on this colorimetric strategy.

PROTOCOL:

1. Synthesis of colored azos

1.1. In a 25 mL round bottom flask (RBF), dissolve aniline (0.1 mmol) and primaquine bisphosphate (45.5 mg, 0.1 mmol) into 10 mL of H_3PO_4 solution (5% v/v). Put the RBF on an ice bath, add a stir bar with the proper size into the solution, and put the RBF on a stir plate.

NOTE: For the synthesis of azo **3g** (**Figure 2**), use 0.2 mmol of primaquine bisphosphate.

1.2. Dissolve $NaNO_2$ (6.9 mg, 0.1 mmol) in 1 mL of cooled water and then add into the reaction mixture dropwise. Remove the ice bath, and keep the reaction mixture stirred at room temperature.

1.3. Monitor the reaction with a silica gel coated thin-layer chromatography (TLC) plate. Use a dichloromethane (DCM)/methanol (MeOH) mixture (vol/vol=5:1) as the eluent for TLC. The azo product exhibits colored spots on the TLC plate, which is easy to distinguish by naked eyes. Stop the reaction when the PMQ spots disappear on TLC.

1.4. Adjust the reaction mixture to pH >10 by NaOH (2 M) on an ice bath. Use a 50 mL separation funnel to extract the mixture 3 times with 20 mL of ethyl acetate for each, combine and concentrate the organic phase under vacuum using a rotary evaporator.

NOTE: Before extraction, adjust the pH value of reaction solutions over 10. This can maintain the primary amine as its non-ionized form, thus facilitating extraction.

1.5. Purify the residues by flash chromatography with reverse-phase silica gel under normal pressure, using MeOH/H_2O as the eluent. Dry the product solution through lyophilization to

give desired azo products.

NOTE: The same reaction can also be performed in diluted HCl solutions (0.2 M).

2. UV-Vis measurements and theoretical calculation

2.1. Dissolve pure azo (50 µM) in distilled water or in 5% H_3PO_4 solution (pH 1.1), respectively. Record UV-vis absorption spectra (250 nm-700 nm) on a spectrophotometer at room temperature (25 °C). Export the data as .xls/.xlsx files for further analysis.

2.2. Perform all theoretical calculations for PMQ itself and azo products using the Gaussian 16 program. Use time dependent density functional theory (TD-DFT) with a 6-31G basis set. Include solvent effects by polarizable continuum model (PCM) formalism using water.

2.2.1. Use software (e.g., Chemdraw Office) to draw the structures and then save the structure as a Gaussian input file (.gif).

2.2.2. Open the gif file with Gauss View and click the button **Calculate**. Select **Gaussian Calculation Setup**, **Opt+Freq**, and **ground state-DFT-B3LYP-6-31G**; then click **Submit**. The geometry optimization will generate a .log file.

2.2.3. Following the procedure above, use Gauss View to open this log file. Click **Calculate-Gaussian Calculation Setup** and select **energy** and **TD-SCF-DFT-B3LYP-6-31G-Singlet only**. Then **Submit**. The energy calculation will generate another log file and a cube file.

2.2.4. Use Gauss View to open the log file from the energy calculation. Click **Results-UV/Vis** to see the predicted absorption.

2.2.5. Use Gauss View to open the cube file. Click **Result**s and select **surface and contours-surface actions** and **new surface** to see the orbits.

2.3. Compare the results from both experimental measurement and Gaussian calculation. Calculate the percent error between the calculated and measured values, according to the following equation.

$$\text{Error} = | (W_{max}\ \text{cal.} - W_{max}\text{exper.}) / W_{max}\text{exper.} | \times 100\%$$

where W_{max} cal. represents the maximum absorbance wavelength from theoretical calculation and W_{max} exper. represents the wavelength from experimental result.

3. PMQ determination

3.1. PMQ measurement using a 96-well plate (Figure 5)

3.1.1. Dissolve 4-methoxyaniline in 0.2 M HCl for a 200 mM aniline solution, R1. Dissolve sodium nitrite in distilled water to obtain a 5 mM solution, R2. Keep all the solutions in the

fridge at 4 °C before use.

3.1.2. Add 100 µL of R1 into a 96-well plate, and add 50 µL of PMQ containing sample into the plate to mix with R1. Then, add 50 µL of R2 into the plate. Mix the solutions by repeated pipetting.

3.1.3. Keep the plate at room temperature for 15 min, and then record the UV-vis absorbance at 504 nm. Repeat 3x for each test. The azo product is stable with room light exposure; it not necessary to keep the plate under dark.

3.1.4. Export the data as .xls/.xlsx files for further analysis.

3.2. **Calibration curve for direct PMQ measurement in a urine sample**

3.2.1. Prepare PMQ solutions using synthetic urine with PMQ concentrations at 0, 1, 2, 5, 10, 20, 50, 100, 200 µM, respectively.

3.2.2. Add 100 µL of R1 into a 96-well plate, and add 50 µL of PMQ urine solution to mix with R1. Then, add 50 µL of R2 to the above mixture. Mix the solutions by repeated pipetting. Keep the plate at room temperature for 15 min, and then record the UV-vis absorbance at 504 nm.

3.2.3. Generate a calibration curve based on the absorbance I_{504} and PMQ concentrations. Use the values from the wells without PMQ as a blank, and subtract the blank values from all tests before data processing.

3.2.4. Perform a linear fit to generate the linear equations as **Y = aX+b**, where **Y** is the absorbance intensity at 504 nm, **X** is the concentration of PMQ, **a** is the slope, and **b** is the y-intercept of the linear line.

3.3. **Calibration curve for direct PMQ measurement in a human serum sample**

3.3.1. Prepare PMQ solutions using human serum with PMQ concentrations at 0, 1, 2, 5, 10, 20, 50, 100, 200, µM respectively.

3.3.2. Add 100 µL of R1 into a 96-well plate and add 50 µL of PMQ serum solution to mix with R1. Add 50 µL of R2 to the above mixture and mix the solutions by repeated pipetting. Keep the plate at room temperature for 15 min and then record the UV-vis absorbance at 504 nm. Export the data as .xls/.xlsx file for further analysis.

3.3.3. Generate a calibration curve based on the absorbance I_{504} and PMQ concentrations. Use the values from the wells without PMQ as a blank, and subtract the blank values from all tests before data processing.

3.3.4. Perform a linear fit to generate the linear equations as **Y = aX+b**, where **Y** is the

absorbance intensity at 504 nm, **X** is the concentration of PMQ, **a** is the slope, and **b** is the y-intercept of the linear line.

3.4. **PMQ extraction from serum**

3.4.1. Add a certain amount of PMQ into human serum to simulate PMQ-containing serum. For PMQ extraction, add 6 mL of mixture of ethyl acetate/hexane (7:1 v/v) into 2 mL of PMQ-containing serum in a 15 mL centrifuge tube.

3.4.2. Add 100 µL of sodium hydroxide (2 M) solution to the extraction system. Violently shake the tube using a vortex mixer for 30 s. Collect the organic layer and concentrate it using a rotary evaporator under vacuum.

3.4.3. Redissolve the residue with 200 µL of distilled water and remove insoluble lipid components by filtration through a disk-shaped membrane with 220 nm pore size. Use the final solution for test.

3.5. **Determine PMQ from the serum with extraction**

3.5.1. Follow steps 3.2 or 3.3 to generate the calibration curve for PMQ in distilled water. Extract PMQ from PMQ-containing serums according to step 3.4.

3.5.2. Add 100 µL of R1 and 50 µL of PMQ solution into a 96-well plate. Add 50 µL of R2 to above mixture, and mix the solutions by repeated pipetting.

3.5.3. Keep the plate at room temperature for 15 min and record the UV-vis absorbance at 504 nm. Use the wells with R1 and R2 but without PMQ as controls. Export the data as .xls/.xlsx files for further analysis.

3.5.4. Subtract the control values from the absorbance values I_{504} for each test, and then use the result for concentration calculations according to the liner equation from the calibration curve.

NOTE: The limit of detection (LOD) for PMQ in all cases can be calculated according to a standard method[22]. Calculation was based on the calibration function: LOD = 3.3 × SD/*b*, where SD is the standard deviation of the blank and *b* is the slope of the regression line

REPRESENTATIVE RESULTS:

To optimize the reaction conditions (**Figure 2**), various anilines were used to couple with PMQ through the Griess reaction. We have achieved a series of azos with different colors. It has been found that anilines with an electron donating substituent can cause a red-shift in the UV-vis absorption spectrum. Theoretical calculations were carried out through time dependent density functional theory (TD-DFT). As presented in **Figure 2A**, the calculation result was in good agreement with optical measurements with average error of 3.1%. 4-methoxyaniline was then

used to conduct the PMQ detection reaction due to its good performance in reaction rate, product solubility, and stability[21]. Moreover, the azo product from 4-methoxyaniline is red in color, which is easy to distinguish with naked eyes. Therefore, this reaction offers potential for naked-eye PMQ detection (**Figure 3**).

Figure 4A shows the pH effect on the UV-vis absorption spectrum of the azo product 3d. I_{504} does not change when increasing pH from 1.0 to 6.0. I_{504} under pH 7.0 exhibits a slight decrease, while a basic pH (8.0 and 9.0) greatly affects the absorption. **Figure 4B** shows the pH effects of PMQ solutions on the Griess reaction. PMQ (50 μM) in PBS buffer with various pHs (4.0, 5.0 ,6.0, 7.0, 8.0, 9.0) were individually mixed with the testing reagent as described in section 3.1. I_{504} was then measured after 15 min at room temperature. As indicated, basic pHs (8.0, 9.0) of PMQ solutions potentially influence the reaction. **Figure 5** shows the general procedure to perform the Griess reaction for PMQ detection. As described in the protocol section, four steps are required to obtain the absorption data I_{504} for analysis. **Figure 6A** and **6B** show the calibration curves for direct detection of PMQ from urine and serum samples, respectively, without sample pretreatments. An excellent linear relationship (R^2 = 0.998) was found when PMQ in synthetic urine ranges from 0 to 200 μM. In term of the serum sample, a linear relationship was found at the concentration ranging from 10 to 200 μM.

Figure 7A shows the procedure to extract PMQ from serum. The residues were redissolved in distilled water after extraction and concentration, and then filtrated. To simulate a real PMQ-containing serum, PMQ was added into human serum with final concentrations at 0, 0.2, 0.5, 1.0, 2.0 μM. Using steps 3.4 and 3.5, the concentrations of PMQ in serums were found to be 0.02, 0.14, 0.44, 0.90 and 1.78 μM, respectively (**Figure 7C**). Based on the result, the percentage of PMQ recovery was found to be around 90% when PMQ was over 0.5 μM in serum, which was comparable to previous reports[9].

TABLES AND FIGURES LEGENDS:

Figure 1: **Schematic of the Griess reaction on PMQ.** (**A**) A classical Griess reaction for nitrite analysis. (**B**) The Griess reaction in the proposed PMQ detection method. This figure has been modified with permission from previous work[21].

Figure 2: Photophysical properties of synthetic azos. (**A**) UV-vis measurement and theoretical calculation of the maximum absorption of azos generated from different anilines. The numbers outside the brackets represent for the maximum absorbance measurement in distilled H_2O near neutral pH conditions; the numbers in the brackets refer to the measurement in 5% H_3PO_4 solution (pH ≈ 1.1). λ_{abs}/nm exper. represents the experiment data and λ_{abs}/calc. represents the theoretical calculation data. E_{exc} is the excitation energy (eV), and f is the oscillator strength. (**B**) Photo images of PMQ and the azo products with different substituents, 50 μM in 5% phosphoric acid solution. (**C**) UV-vis spectra of the synthetic products. The values were normalized to a range between 0 and 1. This figure has been modified with permission from previous work[21].

Figure 3: Colorimetric determination of PMQ. (**A**) Monitoring the absorbance changes at

maximum I_{504} in a time dependent way. The reaction was performed using 4-methoxyaniline, and PMQ was used at 100 μM; (**B**) Color changes of the reaction with different concentrations of PMQ: 400 μL of 4-methoxyaniline solution (200 mM in 0.2 M HCl) and 200 μL of sodium nitrite in water (5 mM), with 200 μL of PMQ solution of different concentrations (0, 1, 2, 5, 10, 20, 50, 100 μM).

Figure 4: pH effect on PMQ detection. (**A**) pH effects on the UV-vis absorbance of azo product **3d** (50 μM); (**B**) PMQ (50 μM) in PBS buffer with different pHs (4.0, 5.0, 6.0, 7.0, 8.0, 9.0) were used to perform the reaction as described in step 3.1. Fifteen min later, the absorbance at 504 nm was measured.

Figure 5: **PMQ determination through a Griess reaction on a 96-well plate based system**. R1 refers to 200 mM 4-methoxyaniline solution in 0.2 M HCl; R2 refers to 5 mM sodium nitrite in distilled water.

Figure 6: **Calibration curves for PMQ determination from (A) synthetic urine and (B) human serum samples**. The concentration of PMQ ranges from 0-200 μM.

Figure 7: PMQ determination from serum samples. (**A**) Schematic illustration of PMQ extraction from serum samples for the quantitative analysis. (**B**) The linear relationship found between I_{504} and PMQ concentration within the range from 0 to 100 μM. (**C**) PMQ in serum was quantify by the Griess reaction-based method in comparison with the exact amount added into the serum. This figure has been modified with permission from previous work[21].

Table 1. Theoretical calculation of Log D and the percentage of water distribution of PMQ and CPMQ.

DISCUSSION:

We described a colorimetric method for convenient PMQ quantification. It is potentially the most simple and cost-effective current method. More importantly, this method offers enables naked-eye based PMQ measurement without using any equipment.

The optimized Griess reaction for PMQ detection can generate a red color azo with a maximum absorption at 504 nm. The potential influence from UV-vis absorption of endogenous biomolecules is limited, thus making the method promising for direct measurement of PMQ in biological fluids. As indicated by the result, an excellent linear relationship (R^2 = 0.998) was found for urine PMQ detection over the concentration range of 0-200 μM (**Figure 6A**). The limit of detection (LOD) for PMQ was found to be 0.63 μM. This method has also shown great potentials for direct measurement of PMQ in human serum. An excellent linear relationship was found in the concentration ranging from 10 to 200 μM for serum PMQ detection (**Figure 6B**). We can further improve the sensitivity by pre-treating the serum sample through extraction and concentration. As **Figure 7** shows with a simple extraction process, this method can quantify serum PMQ at clinically relevant ranges. Based on the reaction mechanism, the main carboxyl metabolite of PMQ (CPMQ) can potentially form an azo product with similar UV-

Vis properties. However, the liquid-liquid extraction under basic pH conditions can potentially minimize the interference from CPMQ. **Table 1** shows the calculated log D and water distribution of both PMQ and CPMQ. As shown, at pH > 10, less than 6.33% of PMQ will be found in the water phase, whereas over 98.54% of CPMQ will be in the water phase. Therefore, theoretically, more than 93.7% of PMQ and less than 1.56% of CPMQ could be extracted out for test. It can be concluded that the interference from the main metabolite CPMQ is limited.

The procedure for PMQ detection is very easy to handle. Taking the 96-well plate-based system as an example, the entire procedure consists of four steps: 1) adding 100 µL of 4-methoxyaniline solution (200 mM in 0.2 M HCl) R1 into a 96-well plate; 2) adding 50 µL of PMQ concentration-unknown sample to mix with R1; 3) adding 50 µL of R2 (5 mM sodium nitrite solution) to perform the reaction at room temperature; and 4) recording the UV-vis absorption at 504 nm using a spectrometer. The concentration of PMQ from an unknown sample can be calculated based on the absorption intensity $\boldsymbol{I}_{504}$ and the linear equation from calibration curve. The entire procedure is performed at room temperature without the need of incubation. A dark environment is not necessary for the entire procedure, as the colored product is not sensitive to room light.

It should be noted that the time for the reaction solution to reach its saturated $\boldsymbol{I}_{504}$ is temperature dependent. As shown in **Figure 3**, at least 12 min was required at room temperature (25 °C). The reaction time would be longer if performing the reaction at temperatures below 25 °C. The basic pH condition of PMQ solutions can potentially affect the absorbance $\boldsymbol{I}_{504}$. To address this issue, adjust the pH of PMQ solution to be less than 7.0. Otherwise, a new calibration curve is needed for the solution with pH over 7.0. In addition, intrinsic nitrites in the tested samples can influence the detection. However, this may only occur when the concentration of intrinsic nitrites is extremely high since a high concentration of nitrite (5 mM) was used in a standard test.

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DISCLOSURES:
The authors have nothing to declare.

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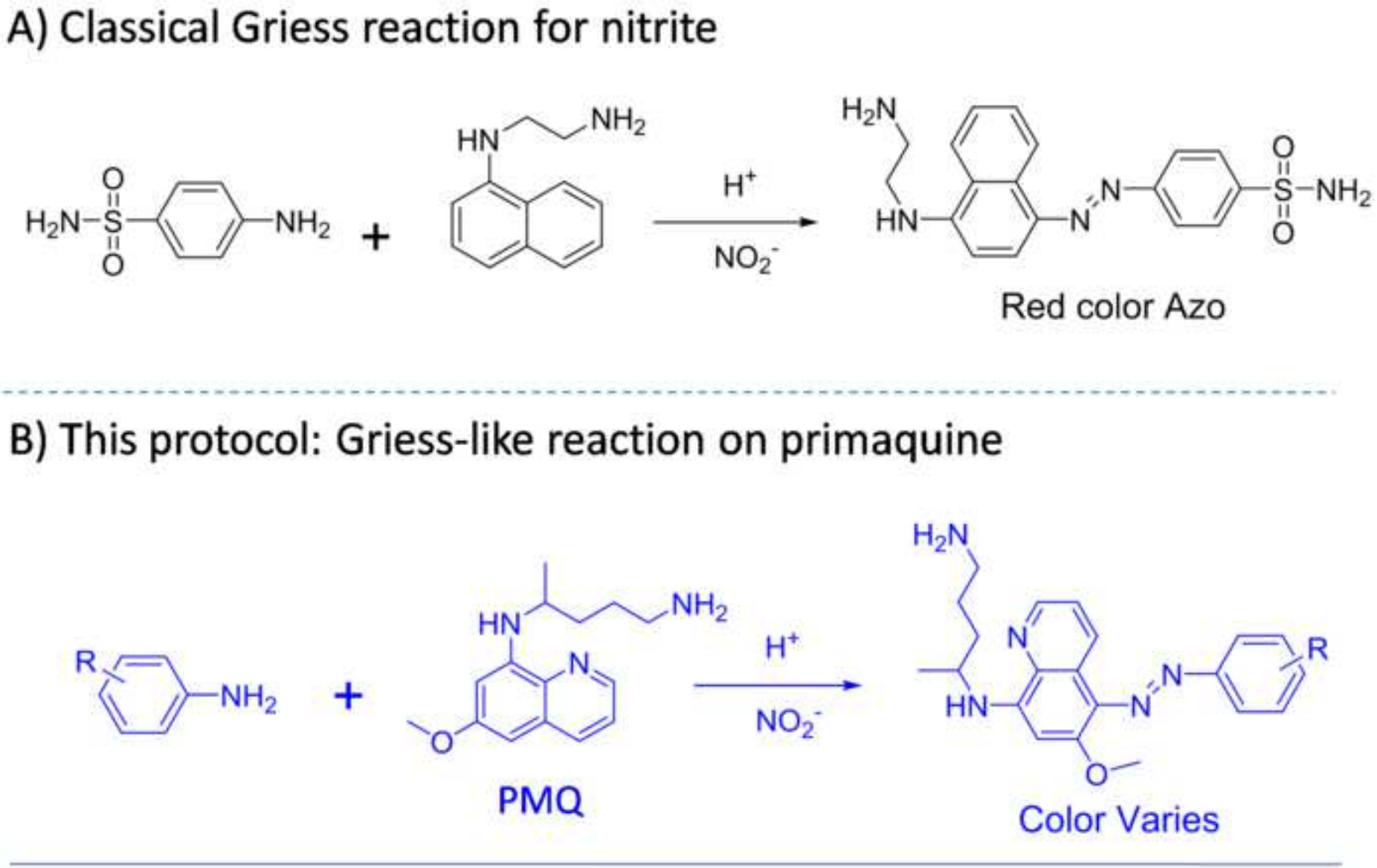
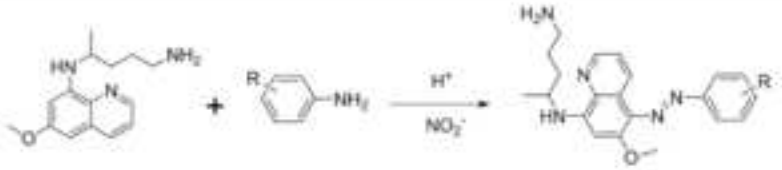
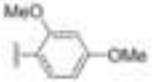
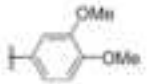


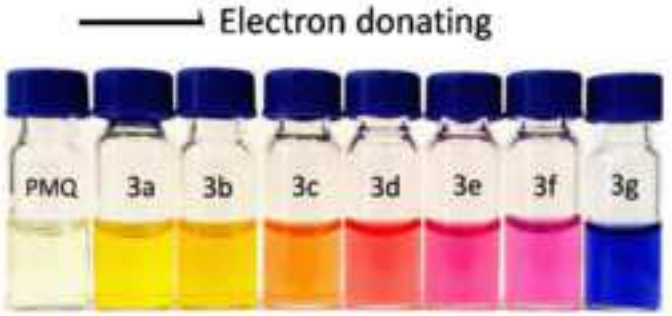
Figure 2

A



	Aniline	Azo	λ_{abs}/nm exper.	λ_{abs} calc.	E_{exc} (cv)	f
2a		**3a**	470(470)	472.92	2.62	1.13
2b		**3b**	476(481)	478.92	2.59	1.02
2c		**3c**	488(492)	480.42	2.58	0.44
2d		**3d**	500(504)	497.57	2.49	0.73
2e		**3e**	512(512)	491.83	2.52	0.54
2f		**3f**	526(518)	510.47	2.42	0.43
2g		**3g**	582(584)	639.58	1.94	1.78

B



C

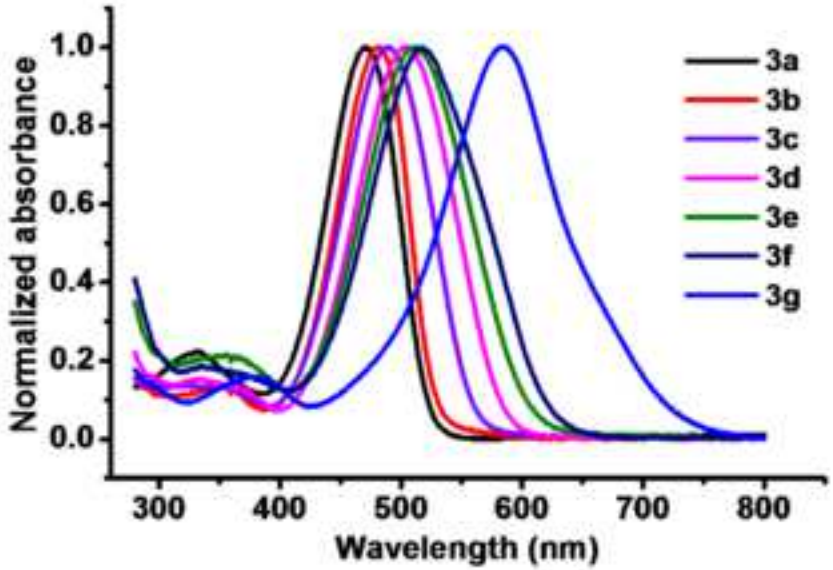


Figure 3

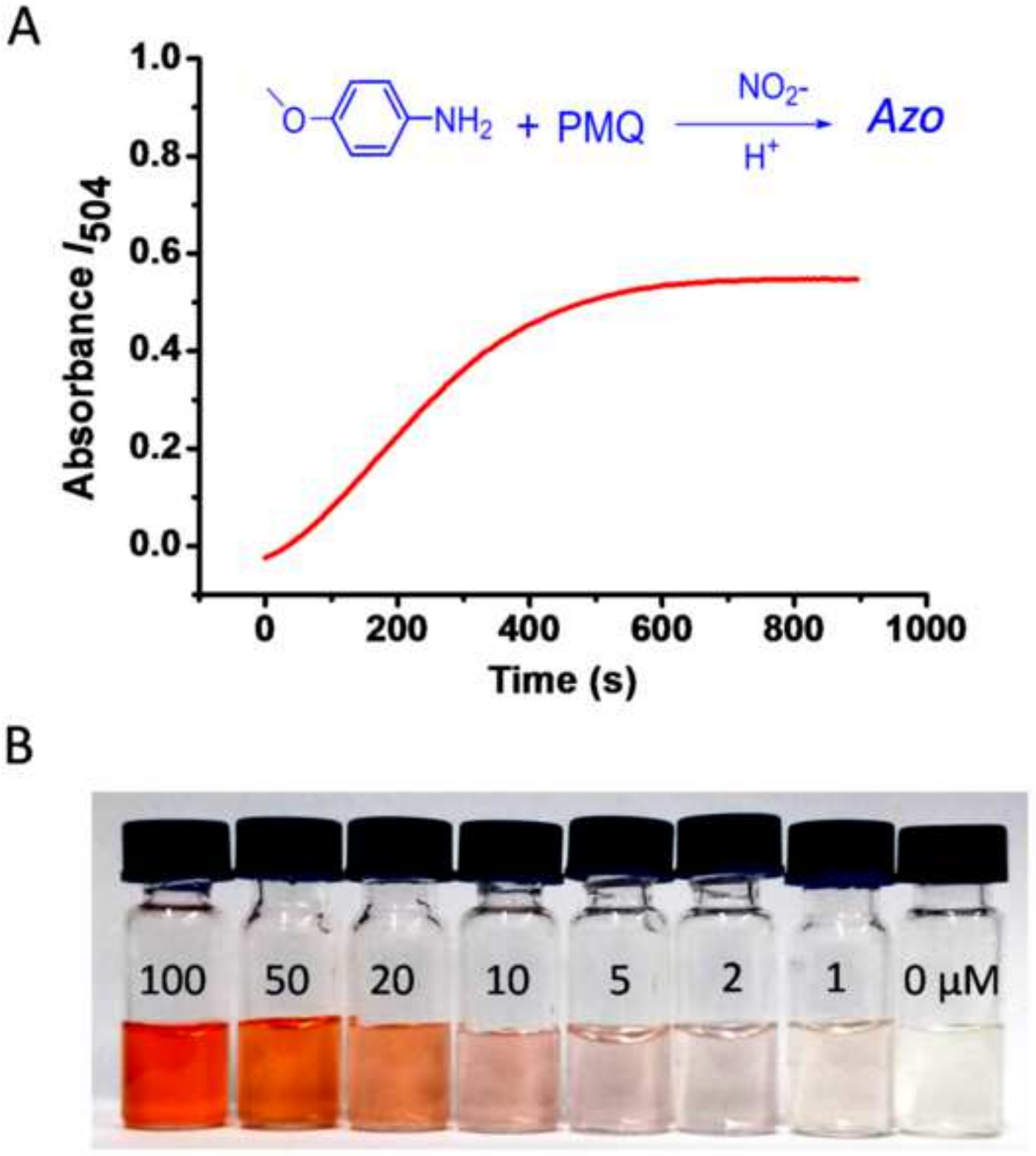


Figure 4

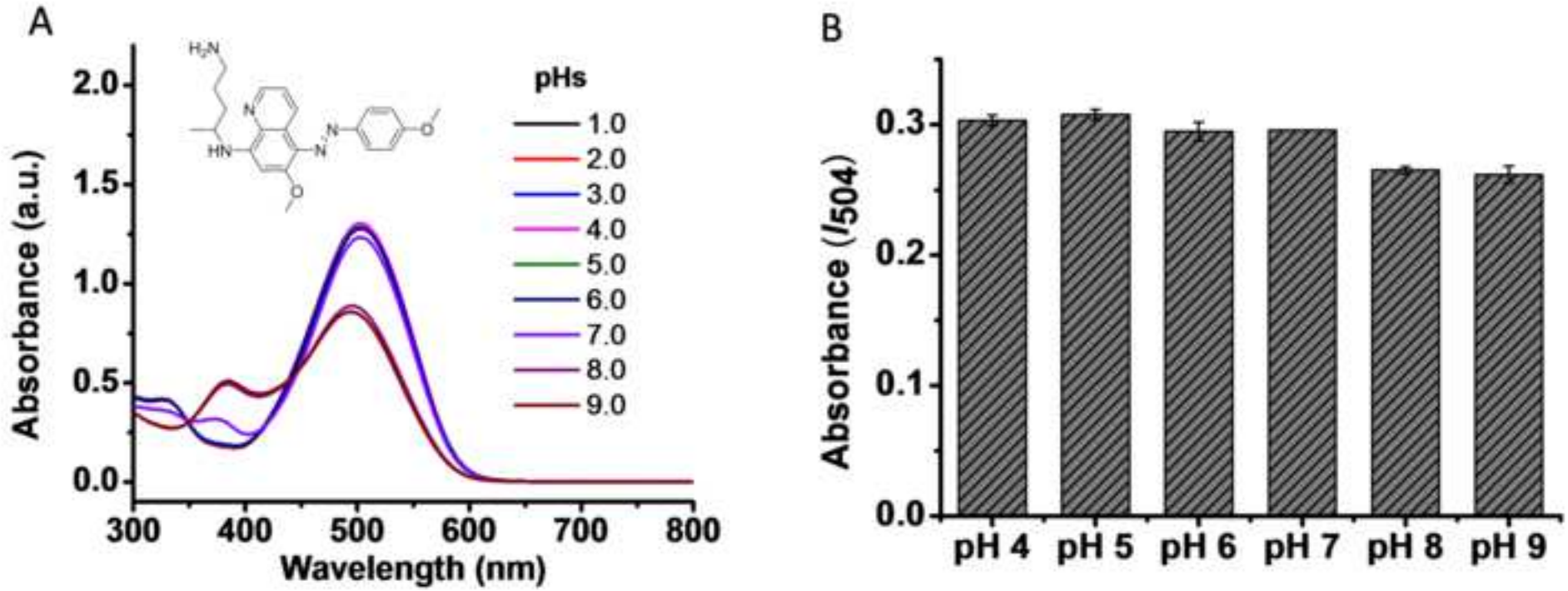


Figure 5

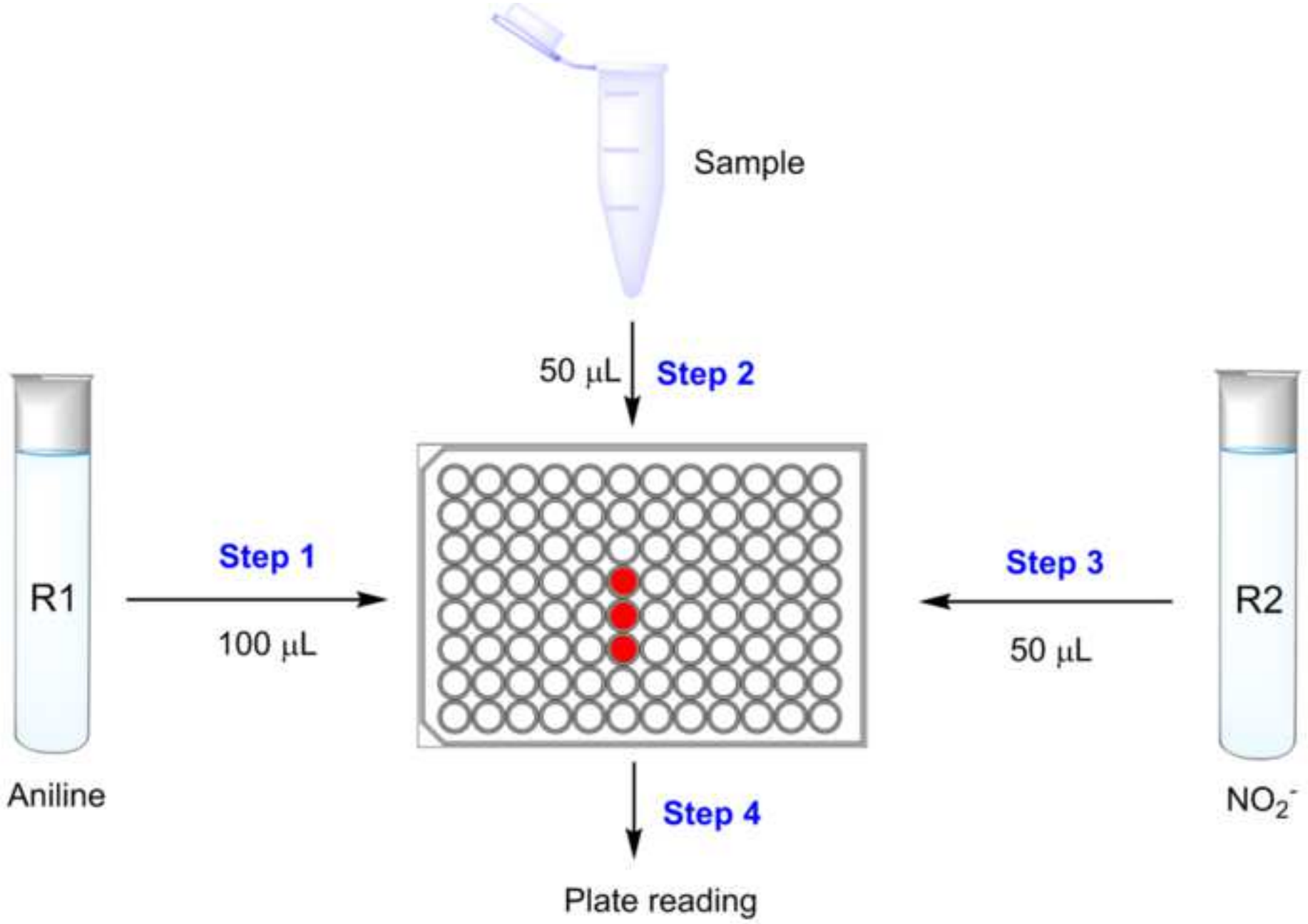
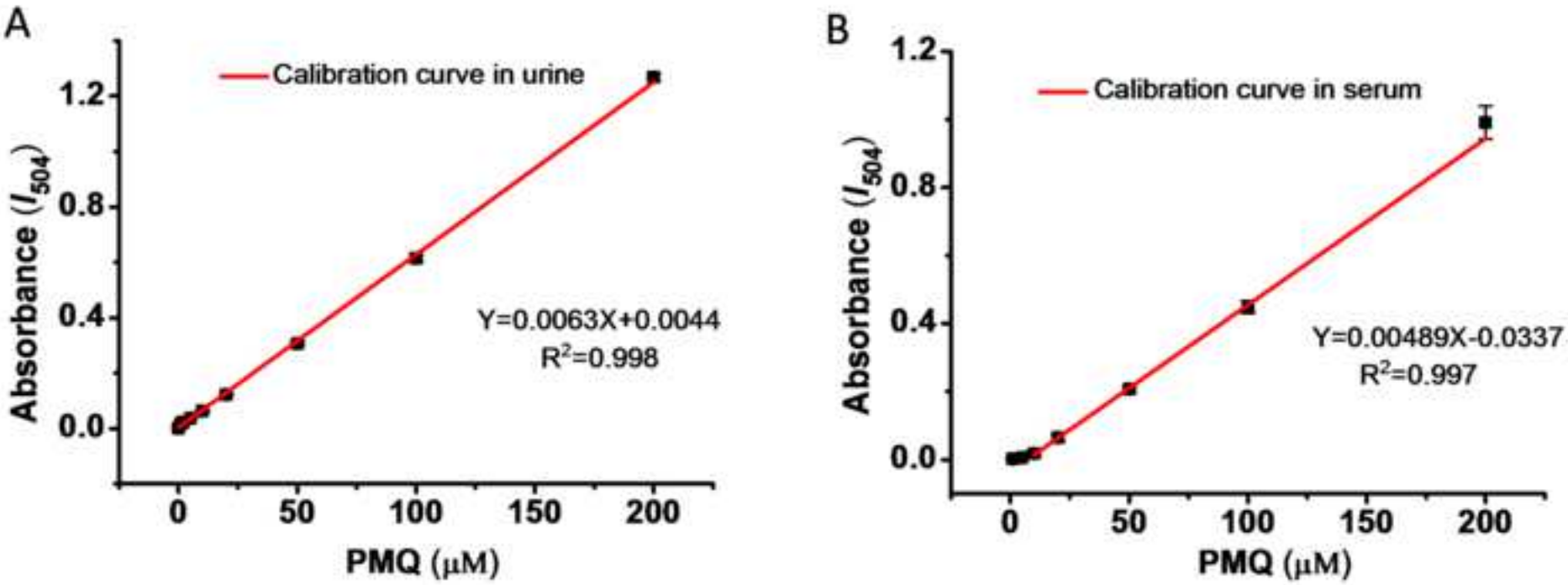
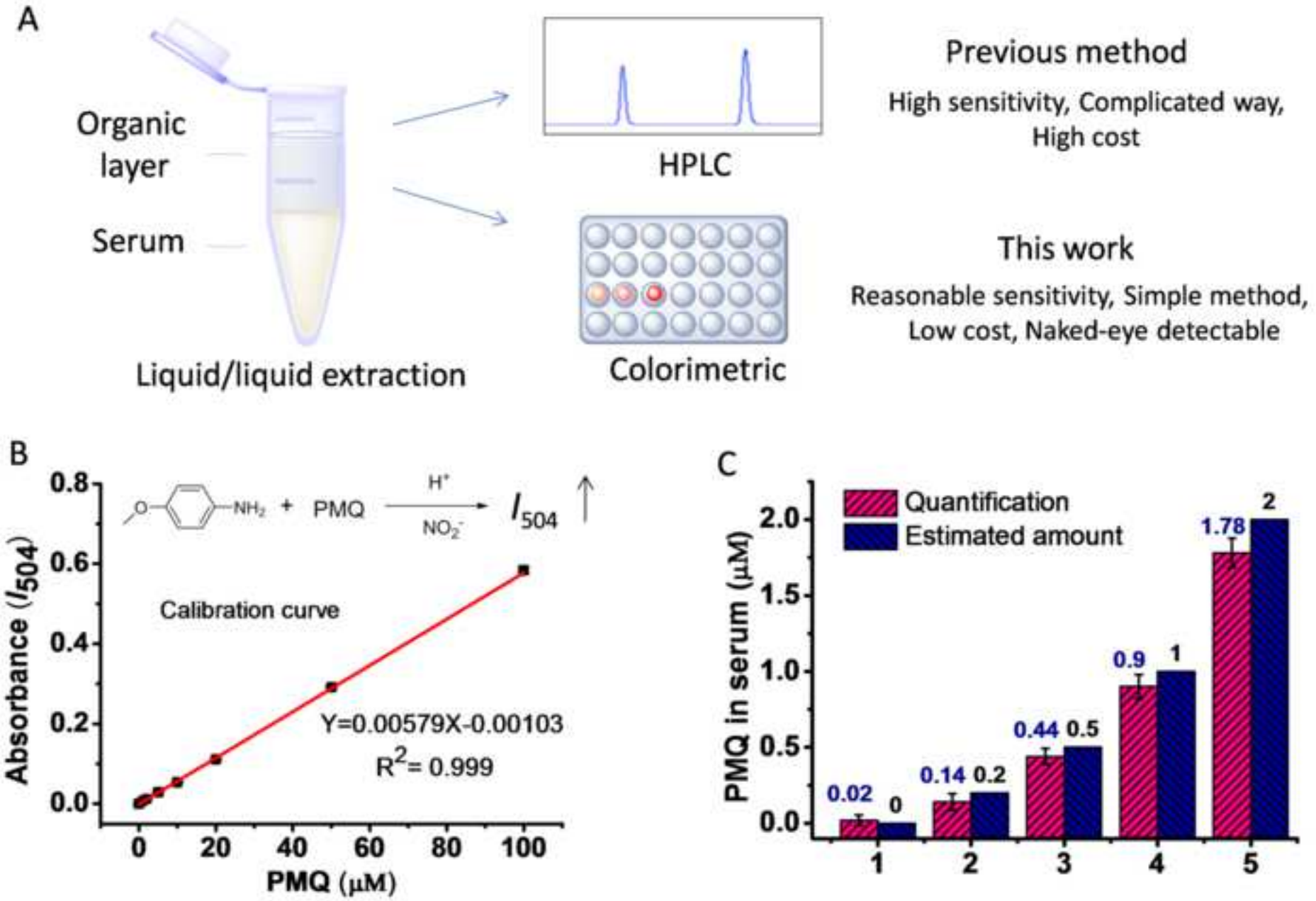


Figure 6





Name of Material/Equipment	Company	Catalog Number	Comments/Description
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2,4-Dimethoxyaniline	Heowns	10154207	
3,4-Dimethoxyaniline	Bidepharm	BD21914	
4-Methylaniline	Adamas-beta	P1414526	
4-Nitroaniline	Macklin	C10191447	
96-wells,Flat Botton	Labserv	310109008	
Gaussian@16 software	Gaussian, Inc		Version:x86-64 SSE4_2-enabled/Linux
Hydrochloric acid	GCRF	20180902	
Marvin sketch (software)	CHEMAXON		free edition: 15.6.29
Phosphoric acid	Macklin	C10112815	
Primaquine bisiphosphate	3A Chemicals	CEBK200054	
Sodium nitrite	Alfa Aesar	5006K18R	
Sulfonamides	TCI(shanghai)	GCPLO-BP	
Varioskan LUX Plate reader	Thermo Fisher		Supplied with SkanIt Software 4.1

FANG LIU, ASSOCIATE PROFESSOR
INSTITUTE OF TROPICAL MEDICINE,
GUANGZHOU UNIVERSITY OF CHINESE MEDICINE

Aug. 8, 2019

Dear Editor Dr. Bing Wu,

Thank you very much for your message regarding our submitted manuscript (**JoVE60136-r1**). We are very grateful for editor and referees' constructive comments and appreciate their detailed and valuable suggestions for the revision of the manuscript r1. We have addressed all the referees' comments point-by-point in the response below and in the appropriate sections of the manuscript. Changes can be found in the manuscript r1 with modification traces.

A new figure was added as figure 4, a new table was added as table 1. The table of materials has been renewed. Since table 1 is not able to upload as a figure, please kindly find it in supplemental files. We look forward to hearing from you regarding the status of the submission.

Sincerely,

Fang Liu
Associate Professor
Guangzhou University of Chinese Medicine

Editorial comments:

The manuscript has been modified and the updated manuscript, 60136_R1.docx, is attached and located in your Editorial Manager account. Please use the updated version to make your revisions.

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

Answer: We greatly thank editor for nice comments and suggestions. We have rechecked the spelling and grammar.

2. For steps that are done using software, a step-wise description of software usage must be included in the step. Please mention what button is clicked on in the software, or which menu items need to be selected to perform the step.

Answer: We have made it much clear in manuscript r2.

3. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol steps (including headings and spacing) in yellow 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.

Answer: have done it in the manuscript r2, see yellow colored words.

4. Figure 1: Please add a title for the whole figure in figure legend. Please bold the title.
5. Figure 2: Please add a title for the whole figure in figure legend. Please bold the title.
6. Figure 3: Please add a title for the whole figure in figure legend. Please bold the title.

Answer: We have added titles to figure 1, 2, 3.

Reviewer #2:
Manuscript Summary:
Thank you for the responses. Overall the presentation is interesting and visually appealing.

Major Concerns:
The specificity of the assay with regard to other antimalarials is important but not surprising. My concern is the specificity with regard to PQ vs its metabolites. The consideration of the pH dependence of carboxyPQ extraction is important, and needs to be highlighted and included in the manuscript.
Bear in mind that the carboxyPQ concentrations will be 10-100 x the PQ concentrations in plasma.
But the major concern is the inadequate correlation of PQ plasma levels to biological responses. I still do not see how this can be used to "guide therapy". Is there any precedent for needing to know PQ plasma levels to make a decision in G6PD deficient therapy? I dont know of it.

Answer: Many thanks to reviewer 2 for the constructive comments. As suggested, we have added the calculation result as **table 1** in the manuscript r2, relative discussion has been added into the discussion part, line 347-354.

We have not included the contents "guide therapy" in both manuscript r1 and r2. Sometimes, dose monitoring is really necessary, which has often been done by HPLC method currently. Our method is really an alternative for PMQ quantification to assess to pharmacokinetics.

Reviewer #3:

Manuscript Summary:

This JOVE mss translates to video form a published method for determination of primaquine in serum. The method involves a nitrite coupling of primaquine and various anilines to form colored azo dyes.

Major Concerns:

1). The published procedure seems very straightforward, so it's not clear what benefit the video will confer. To address this concern, the author could describe potential sticking points of the assay in the script.

Answer: in the manuscript r2, new data regarding pH effects and the concerns about selectivity has been added. The relative concerns have been discussed in the manuscript r2. Please check Figure 4 and table 1, and the description.

2). I was surprised that there is no buffer or acid component added to control pH, which is important for the initial step in nitrite couplings (NO2- --> HONO). In biological samples, the pH may vary enough to cause trouble in clinical determinations. It would be a good idea to show that clinically relevant pH variations (5.5-8.5 seems like a reasonable range) don't mess up the assay.

Answer: Thanks for suggestion. Actually buffering effect has been considered in our study. For example, for urine PMQ quantification, the standard curve has been done by using PMQ in commercial synthetic urine. For direct PMQ measurement in serum, PMQ in human serums were used to make the standard calibration curve. But for selectivity study and spectrum measurement, PMQ in pure water was used. To strengthen this part, we have done some relevant studies.

1X PBS buffer with different pH values have been prepared for study of pH effect. Azo product **3d** was dissolved into PBS buffer with series of pH values. As shown from Figure 4, there no obvious change on UV-vis absorption when increasing pH from 1 to 7.0, whereas, basic pHs (8.0 and 9.0) can affect the absorption. In a further study, PMQ (50 μM) was prepared in these PBS buffer with increasing pHs. As shown in figure 4B, only negligible changes found when pH varied between 4.0-7.0. Basic pH medium can affect the intensity at 504 nm, but not much. The detection is still applicable for direct PMQ measurement in weak basic mediums. In case, a new calibration curve is needed for the PMQ solutions with pH over 7.0.

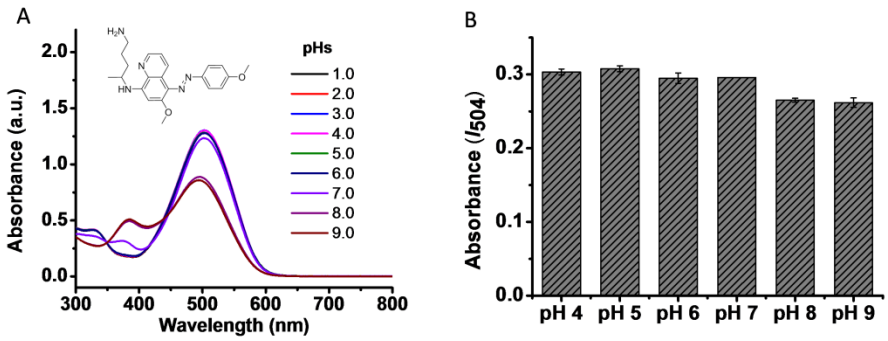


Figure 4: pH effect on PMQ detection. A) pH effects on the UV-vis absorbance of azo product 3d (50 μM); B) PMQ (50 μM) in PBS buffer with different pHs (4.0, 5.0, 6.0, 7.0, 8.0, 9.0) were used to perform the reaction as described in section 3.1, 15 min later, the absorbance at 504 nm was measured.

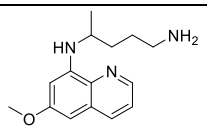
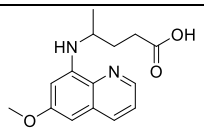
Minor Concerns:
Minor editing for spelling/grammar, only a few problems seen.

Answer: the entire manuscript has been rechecked.

Reviewer #4:
Minor Concerns:
It would have been better if you have some experiments for matrix effect.

Answer: matrix effect has been addressed, please find the answer to reviewer 3, and the figure 4 in manuscript r2.

Table 1. Theoretical calculation of Log D and the percentage of water distribution of PMQ and CPMQ.

	 PMQ		 CPMQ	
pH	**Log D**	**% in water**	**Log D**	**% in water**
0	-3.96	99.99	-0.18	60.22
1	-3.40	99.96	0.34	31.37
2	-3.20	99.94	0.52	23.20
3	-2.81	99.85	0.88	11.65
4	-2.19	99.36	1.30	4.773
5	-1.90	98.76	0.92	10.73
6	-1.80	98.44	0.03	48.27
7	-1.42	96.34	-0.91	89.04
8	-0.60	79.92	-1.60	97.55
9	0.36	30.39	-1.80	98.44
10	1.17	6.333	-1.83	98.54
11	1.52	2.932	-1.84	98.58
12	1.58	2.563	-1.84	98.58
13	1.58	2.563	-1.84	98.58
14	1.58	2.563	-1.84	98.58

Note: The pH dependent octanol/water partition coefficient, Log D, has been extensively used to evaluate the solubility of compounds under different pH conditions. To do calculation, open the structure files by Marvin sketch 15.6.29 (a free edition), click “calculation”, select “partitioning”, select “log D”, then on the parameter panel select log P method “ChemAxon”, then press “ok”. CPMQ stands for carboxyl primaquine.

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Author(s):	Yalan Wu, Shengjun Wu, Xin-an Huang, Qingping Zeng, Tao Deng, Fang Liu

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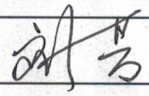
Signature:	
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