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Agglutination Assays of the *Plasmodium falciparum*-Infected Erythrocyte

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Abstract

The agglutination assay is used to determine the ability of antibodies to recognize parasite variant antigens on the surface of *Plasmodium falciparum*-infected erythrocytes. In this technique, infected erythrocytes are selectively labelled with a DNA-binding fluorescent dye and mixed with antibodies of interest to allow antibody–surface antigen binding. Recognition of surface antigens by the antibodies can result in the formation of agglutinates containing multiple parasite-infected erythrocytes. These can be viewed and quantified using a fluorescence microscope.

Keywords

Agglutination; Infected erythrocytes; *Plasmodium falciparum*; Variant surface antigens; PfEMP1; Malaria; Antibody; Naturally acquired immunity

1 Introduction

When *Plasmodium falciparum* parasites infect human erythrocytes, they insert parasite proteins into the infected erythrocyte surface. These proteins modify the immunological and cytoadhesive properties of the infected erythrocyte [1] and are targets of naturally acquired antibodies [2–5]. The major components of these proteins are variant surface antigens of the *P. falciparum* membrane protein 1 (PfEMP1) family, which undergo clonal antigenic variation and are extremely diverse [6, 7]. Thus, antibody recognition of antigens on the infected erythrocyte surface is usually variant specific, and antibodies that recognize one parasite isolate are often unable to recognize other isolates [8–11].

The agglutination assay was designed to test if antibodies in a plasma or serum sample of interest are able to recognize the surface antigens of erythrocytes infected by a particular parasite [8, 12, 13]. The assay is based on the fact that antibody recognition of similar or identical epitopes on multiple erythrocytes can lead to cross-linking of these cells to form agglutinates. The procedures for the agglutination assay are as follows: first, parasite-infected erythrocytes in a sample of interest are selectively stained with a DNA-binding fluorescent dye, which will not stain uninfected erythrocytes as they lack DNA. Next, the sample is rotated with serum or plasma (or any other antibody sample) of interest to allow antibody–surface antigen binding and agglutinate formation. Subsequently, the mixture is transferred onto a microscope slide, which is then examined under a fluorescence

microscope. Agglutinates appear as fluorescent clusters of cells indicating recognition of surface antigens by antibodies in the serum (Fig. 1).

The agglutination assay was used to demonstrate an association between antibodies against the infected erythrocyte surface and protection [3] and to estimate of the in vitro rate of antigenic switching in *P. falciparum* [14]. Despite the advent of high-throughput flow cytometry as a parallel tool to measure antibody recognition of infected erythrocytes [15], the agglutination assay is still useful. First, it is unclear whether agglutination and flow cytometry measure recognition of the same epitopes, as flow cytometry detects simple antibody-antigen binding but agglutination involves cross-linking of multiple cells, which may require higher affinity interactions or those that are directed towards parts of the surface antigens that are flexible enough to allow cross-linking. Second, analysis of identical samples using these two approaches has not revealed a clear correlation [16, 17]. Third, the agglutination assay can be used to determine whether infected erythrocytes from different parasite samples are simultaneously recognized by the same or different antibody subsets (see the mixed agglutination assay—Subheading 3.3). Finally, when using polyclonal antibody samples for agglutination, the size of agglutinates provides an idea of the proportion of infected erythrocytes within a sample being recognized by the same subset of antibodies. These findings would be difficult to discern through flow-based approaches.

Nevertheless, the agglutination assay has limitations that may render it unsuitable for certain objectives. First, it is not as high-throughput as flow-based techniques, as the examination of slides using a microscope can be time-consuming. Second, agglutination is at best semiquantitative, as it is difficult to determine whether infected erythrocytes that are not part of agglutinates do not join such structures due to lack of antibody recognition or because of kinetic/mechanical reasons. Also, it is difficult to count the percentage of cells agglutinated, especially when large agglutinates are formed. Finally, the agglutination assay is much less effective for samples with low parasitemia (<0.5 %), which may be an issue when dealing with clinical parasite samples [12].

In this chapter, we describe two variations of the agglutination assay, the individual agglutination assay and the mixed agglutination assay. The individual agglutination assay is used to determine if a serum of interest has antibodies that can recognize surface antigens on infected erythrocytes from a single parasite sample. This assay can be done using a wet slide preparation, which requires immediate slide examination, or a fixed slide preparation, which allows slides to be stored and examined at a later date [18, 19]. The mixed agglutination assay (wet slide preparation only) is used to determine if a serum has antibodies that can simultaneously recognize surface antigens from different parasite samples [8].

2 Materials

Prepare all solutions at room temperature using deionized water. Make all media and culture the infected erythrocytes under sterile conditions. However, reagents used only on the day of the agglutination assay do not need to be sterile. Store all solutions at 4 °C unless stated otherwise.

2.1 Media

1. Roswell Park Memorial Institute (RPMI-1640) incomplete medium (RPMI-I): 0.2 % w/v glucose, 2 mM L-glutamine, 25 µg/mL gentamicin, 37.5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid 0.05 mg/mL hypoxanthine (dissolved in 1 M NaOH) in RPMI 1640. Remove 33 mL from a new 500 mL bottle of RPMI 1640. Add 5 mL 20 % w/v glucose, 5 mL 200 mM (100×) L-glutamine, 1.25 mL 10 mg/mL gentamycin, 18.75 mL 1 M HEPES, and 3 mL 8.4 mg/mL hypoxanthine in 1 M NaOH to the bottle (*see* Note 1). Mix thoroughly by inversion.
2. RPMI complete medium (RPMI-C): RPMI-I with 10 % blood group AB serum from malaria-naïve human donors. Heat-inactivate serum at 56 °C for 30 min. Add one volume of serum to nine volumes of RPMI-I. Mix thoroughly by inversion.

2.2 Infected Erythrocytes

1. Use either acute parasites from patients or culture-adapted parasites:
 - (a) Acute parasites (*see* Note 2): Gently layer 5 mL of the patient's blood sample (or, if using erythrocytes after separation from plasma, up to 5 mL of a 1:1 ratio of erythrocytes and RPMI-I) onto 3 mL of Lymphoprep in a 15 mL tube and centrifuge at $440 \times g$ for 20 min (minimum acceleration and no brake) to remove peripheral blood mononuclear cells. Collect the erythrocyte pellet and rinse once with 10 mL of RPMI-I. Add a 1.5× pellet volume of RPMI-I (previously warmed to 37 °C) to resuspend the erythrocyte pellet. Next, mix an equal volume of Plasmion (Bellon, France) (previously warmed to 37 °C) and allow the tube to stand upright for 10 min at 37 °C. Discard the supernatant as it contains the granulocyte fraction. Rinse the erythrocyte pellet with 10 mL of RPMI-I and culture in RPMI-C to the mid- to late trophozoite stage in the same cycle (*see* Notes 3 and 4). Parasite cultures should be gassed with a sterile high-carbon dioxide gas mixture (92 % nitrogen, 5 % carbon dioxide, 3 % oxygen) and incubated at 37 °C.
 - (b) Culture-adapted parasites: culture infected erythrocytes to the late trophozoite/early schizont stage in RPMI-C (*see* Notes 3 and 4), adding washed blood group O erythrocytes to adjust parasitemia whenever necessary (*see* Note 5). Parasite cultures should be gassed with a sterile highcarbon dioxide gas mixture (92 % nitrogen, 5 % carbon dioxide, 3 % oxygen) and incubated at 37 °C.
2. Culture-adapted parasites can be bulked up at the trophozoite stage, and cryopreserved in liquid nitrogen [20], but if this is done, each aliquot should be used on the same day it is thawed. Thawed parasites that were stored as mature trophozoites cannot be put back into culture.

3. The final parasitemia used in the assay should ideally be between 1 and 5 % for optimal results (*see* Note 6). When comparing the recognition of multiple parasite isolates, the parasitemia of each isolate should be adjusted to a constant level (e.g., 1 %) by the addition of a known number of uninfected washed blood group O erythrocytes at the start of the assay.

2.3 Components for Agglutination Assay: Wet Slide Preparation

1. RPMI-I (*see* Note 7).
2. Infected erythrocytes.
3. 96-well round-bottom microtiter plates.
4. 200 µg/mL ethidium bromide in RPMI-I (*see* Note 7): Use 200 µg/mL final concentration for the mixed agglutination assay [14], but dilute in RPMI-I to 10 µg/mL final concentration for the individual agglutination assay (*see* Note 8). [Ethidium bromide excitation: 510 nm; emission: 595 nm.]
5. 1 mg/mL stock DAPI (4,6-diamidino-2-methylphenylindole in dimethyl sulphoxide) (*see* Note 8). [DAPI excitation: 350 nm; emission: 470 nm.]
6. Petroleum jelly. Store at room temperature.
7. 18 mm square coverslips.
8. Glass microscope slides.
9. Fluorescence microscope that has a channel for DAPI (excitation wavelength ~350 nm) and ethidium bromide (excitation wavelength ~510 nm) and that detects fluorescence emissions at 470–600 nm.
10. Sera containing antibodies of interest (*see* Note 9).
11. Plate rotator: plates can be attached to rotating wheels or tube rotators.

2.4 Additional Components for Fixed Slide Preparation

1. Absolute methanol.
2. 5 µg/mL acridine orange in phosphate buffered saline (PBS) (*see* Note 8). [Acridine orange excitation: 500 nm; emission: 525 nm.]
3. Fluorescence microscope: the fluorescence channel that is used for ethidium bromide can also be used for acridine orange.

3 Methods

Perform all steps at room temperature unless stated otherwise. The agglutination assay does not have to be conducted under sterile conditions. All experiments should be carried out away from direct light to avoid excessive photobleaching of the fluorescent dyes. The procedure described herein is for a single experiment but can be adapted for multiple experiments as required.

3.1 Individual Agglutination Assay (Wet Slide Preparation)

1. Centrifuge the infected erythrocyte sample at $1,500 \times g$ for 1 min.
2. Transfer the required volume of erythrocyte pellet into a tube with 10 $\mu\text{g/mL}$ ethidium bromide in RPMI-I to give a 5 % hematocrit (pellet) (*see* Notes 7 and 10).
3. Resuspend the pellet thoroughly by pipetting up and down gently.
4. Add 10 μL of the infected erythrocyte suspension to a well of a 96-well round-bottom microtiter plate (*see* Note 11).
5. Add 2.5 μL of the serum of interest to the same well (*see* Note 11). A negative control must be included by adding 2.5 μL of serum from a malaria-naïve donor into a separate well containing 10 μL of the infected erythrocyte suspension (*see* Note 12). Similarly, a positive control should be carried out by adding 2.5 μL of a malaria hyper-immune serum (known to cause agglutination) into a separate well containing 10 μL of the suspension.
6. Cover the plate with its lid and gently mix the contents of the well by swirling the plate (*see* Note 13).
7. Wrap the plate with aluminum foil and place it onto a plate rotator. Rotate the plate at a $45\text{--}60^\circ$ angle at room temperature for 60 min (*see* Note 14).
8. Remove the plate from the rotator. Swirl the plate gently to resuspend the pellets and gently pipette the contents of each well onto a labelled microscope slide (*see* Note 15). If multiple slides are to be examined and blinding is necessary, the slides should be labelled in such a way as to enable blinding (*see* Note 16).
9. Gently place a coverslip with its edges previously lined with petroleum jelly onto the mixture (*see* Note 17).
10. Protect the microscope slide from light and transfer it to a dark fluorescence microscope room (*see* Note 18).
11. View the slide under bright field and then under fluorescence at $200\times$ or $400\times$. Use a channel that allows fluorescence excitation at $\sim 510\text{ nm}$ and fluorescence emission at $\sim 595\text{ nm}$.
12. Count the agglutinates, which will appear as fluorescent clusters of cells (Fig. 1) (*see* Note 19 for counting tips).
13. The method of counting should include both the number and maximum size of agglutinates (Table 1). We suggest a counting scheme that assigns scores of 0–4 to each sample as follows (adapted from ref. 10):

Size of agglutinates divided into four categories:

Category A: 5–20 infected erythrocytes
Category B: 21–50 infected erythrocytes
Category C: 51–100 infected erythrocytes

Category D: >100 infected erythrocytes

Final scores of 0–4 are awarded based on the number of agglutinates:

Score 0: <5 category A agglutinates

Score 1: 5–9 category A agglutinates

Score 2: 10 category A agglutinates or 1 category B agglutinate

Score 3: 5 category B agglutinates or 1 category C agglutinate

Score 4: 5 category C agglutinates or 1 category D agglutinate

3.2 Individual Agglutination Assay (Fixed Slide Preparation)

The wet slide preparation technique requires immediate slide examination to avoid rupture of infected erythrocytes. If agglutinates need to be counted on a different occasion (e.g., when a large number of slides is prepared), an alternative approach is to prepare fixed slides. This method involves post-agglutination staining and hence can only be used for the individual agglutination assay and not the mixed agglutination assay (*see* Note 20).

1. The individual agglutination assay is performed up to **step 8** as described in Subheading 3.1, except that the cells are resuspended in RPMI-I instead of 10 µg/mL ethidium bromide at **step 2** (*see* Note 7).
2. Gently spread the cells on the microscope slide in a circle with a 1–1.5 cm diameter using a 20–200 µL plastic pipette tip.
3. Allow the cells to dry at room temperature for 30 min (a hairdryer can be used to expedite drying).
4. Add absolute methanol to fix the cells for 1 min, allow the methanol to evaporate, and store the slides at room temperature (or at –80 °C if they are to be kept for >1 month) until microscopy is to be conducted.
5. For slide examination, stain with 13 µL of 5 µg/mL acridine orange in PBS.
6. Carry out **steps 9–13** of the previous section (Subheading 3.1) to complete the assay. The same fluorescence channel that is used for ethidium bromide can generally be used for acridine orange, but note that the optimal fluorescence emission for acridine orange (when bound to DNA) is at ~525 nm.

3.3 Mixed Agglutination Assay

1. This assay can be performed on two different infected erythrocyte samples.
2. Determine the parasitemia of each sample using flow cytometry or by counting from blood smears (*see* Note 21).
3. Centrifuge each infected erythrocyte sample at $1,500 \times g$ for 1 min.
4. Transfer the required volume of pellet (*see* Note 22) into a new microcentrifuge tube containing a previously added 100× volume of dye (*see* Note 23).

Separately stain the first sample with 200 µg/mL ethidium bromide and the second with 5 µg/mL DAPI (*see* Note 24).

5. Wrap the tubes with aluminum foil and rotate at room temperature for 1 h to keep the pellets from settling (*see* Note 25).
6. Centrifuge the tubes at $1,500 \times g$ for 1 min and remove as much of the dye as possible using micropipettes. Rinse the stained erythrocytes with RPMI-I five times (*see* Note 7). Each rinse consists of resuspending the erythrocyte pellet in a large volume of medium (at least 100× the volume of pellet), centrifuging at $1,500 \times g$ for 1 min and removing as much medium as possible without disturbing the pellet (*see* Note 26).
7. Add a 50× pellet volume of RPMI-I and completely resuspend each pellet. Mix the two stained erythrocyte samples in a volume ratio to obtain equal parasitemia of both samples (*see* Note 27).
8. Centrifuge the parasite mixture at $1,500 \times g$ for 1 min and remove most of the supernatant, leaving exactly 10 µL of supernatant per 0.5 µL of pellet.
9. Resuspend the pellet thoroughly and pipette 10 µL of the suspension into a well of a 96-well round-bottom microtiter plate (*see* Note 11).
10. Add 2.5 µL of the serum of interest to the well (*see* Note 11).
11. Carry out **steps 6–10** of the individual agglutination assay (Subheading 3.1) to complete this assay.
12. View the slide under bright field and then under fluorescence at 200× or 400× to count agglutinates (Table 2) (*see* Note 28). The presence of dual-color mixed agglutinates (Fig. 2) would signify simultaneous antibody recognition of the two different parasites, whereas the presence of two different types of single-color agglutinates (Fig. 3) would indicate distinct antibody subsets that recognize each parasite individually with no cross-reactivity (*see* Note 29).

4 Notes

1. All ingredients for RPMI-I should be sterile-filtered if not commercially obtained as sterile reagents. All ingredients should be stored at 4 °C except for L-glutamine, which should be kept at –20 °C. The L-glutamine and hypoxanthine in NaOH should be made as single-use aliquots.
2. To avoid blood group-mediated agglutination, ensure that the patients' ABO blood group antigens will not be recognized by antibodies in the serum of interest. For instance, do not use serum from an individual with blood group A to agglutinate a parasite sample from an individual with blood group B.
3. Maximum agglutination generally occurs during the late trophozoite/early schizont stage [12].
4. Warm RPMI-C to 37 °C before using it for parasite culture.

5. Blood group O erythrocytes should generally be used for parasite culture (of culture-adapted parasites) to avoid blood group-mediated agglutination, unless the research question of interest requires the use of specific blood groups. If so, *see* Note 2.
6. Lower levels of parasitemia have been shown to result in the formation of smaller agglutinates [12]. The assay should be conducted with at least 1 % parasitemia (whenever possible) to give clear results.
7. PBS can be used as an alternative to RPMI-I to dilute ethidium bromide and to suspend and rinse infected erythrocyte pellets [12, 21].
8. Fluorescent dyes are light sensitive and should be stored at 4 °C in small tubes that are wrapped with aluminum foil to prevent photobleaching. Nevertheless, when in use during the assay, ethidium bromide, DAPI and acridine orange can be handled under dim lighting as they are relatively resistant to photobleaching. The working solutions should only be made in small amounts and used within a week. Stock solutions should be stored at –20 °C.
9. If plasma is to be used in agglutination assays this should be centrifuged at high speed (~16,000 × *g* for >10 min), followed by transfer of the supernatant to a new tube to remove residual platelets from the plasma. This should be done as platelets may mediate clumping of infected erythrocytes [22], which is a distinct phenomenon from antibody-mediated agglutination. Plasma or serum should also be heat-inactivated at 56 °C for 30 min to inactivate complement, which may also interfere with agglutination. Purified or monoclonal antibodies can also be used instead of sera if preferred.
10. It is preferable to make a suspension of infected erythrocytes and pipette equal volumes of the suspension into wells of a microtiter plate rather than transfer small volumes of pellet into each well, as the latter method will reduce the precision of the assay. A cell counter can be used to count and standardize the number of erythrocytes used in each assay.
11. To avoid bubble formation, do not push the pipette knob to the second stopping point during transfer of the fluorescent dye and serum into the microtiter plate well.
12. Match the negative control to the samples to be tested, i.e., use a plasma negative control for plasma samples and a serum negative control for serum samples. Examine an unblinded negative control first to determine the baseline level of non-specific agglutination, and include a blinded negative control with the samples to be examined.
13. One possible mixing method is to move the plate fairly rapidly using circular motions on a flat horizontal surface.
14. A longer rotation time generally results in the formation of larger agglutinates. An alternative to rotation is to secure the plate onto a shaker and shake gently [21].

15. Use pipette tips with larger apertures to prevent agglutinates from being disrupted upon ejection from the pipette tip. For instance, a 20–200 μL pipette tip is preferable to a 0.5–10 μL pipette tip. In our experience, agglutinates containing more than 10,000 cells can pass safely through the former with no observed disruption.
16. As the counting of agglutinates is only semiquantitative, if the experiment involves multiple slides with different expected results, it is essential to blind yourself from knowing the identity of each observed slide.
17. The petroleum jelly should be applied very thinly to the bottom four sides of the coverslip to prevent sample leakages and to provide space for the agglutinates (to avoid flattening them with the coverslip). This can be done by placing a very thin layer of petroleum jelly onto a flat surface, such as the bottom corner of a palm, and scraping that surface gently with each side of the coverslip.
18. Agglutinates should be scored immediately after slide preparation to minimize erythrocyte lysis. In our experience, leaving slides overnight at 4 °C for examination the following day sometimes results in some lysis. An alternative approach is to use a fixed slide preparation (Subheading 3.2).
19. General counting tips
 - (a) Ensure that clusters of parasites identified as agglutinates under fluorescence have clear erythrocyte membranes when observed under bright field, as parasites (surrounded by parasitophorous vacuole membranes) from lysed erythrocytes have a tendency to adhere and form clusters that look like agglutinates.
 - (b) Agglutinates tend to float and drift to the edges of the coverslip, and often cluster at one particular edge. Due to the bias in agglutinate distribution, it is essential to completely examine each slide rather than randomly select a specific number of fields to observe or count a predetermined number of cells.
 - (c) Due to their tendency to float, agglutinates are often slightly out of focus relative to unattached erythrocytes.
 - (d) An infected erythrocyte may contain more than one parasite but should only be counted as a single cell when determining agglutinate size. Hence, especially when determining the size of small agglutinates, constantly check bright field to ensure that agglutinate size is based on the number of infected erythrocytes and not the number of parasites.
20. An alternative to the fixed slide preparation method described here has been suggested in ref. [23].
21. To assess trophozoite parasitemia by flow cytometry, stain a small amount of pellet (~0.5 μL) with 100 μL of 10 $\mu\text{g/mL}$ ethidium bromide in 0.5 % bovine serum albumin (BSA) in PBS for 10 min at room temperature. Rinse twice with 200 μL of 0.5 % BSA in PBS and resuspend the pellet in 200 μL of 0.5 % BSA

in PBS. Set the flow cytometer to excite and detect fluorescence at the correct wavelengths for ethidium bromide (~510 nm and ~595 nm, respectively) and record the percentage of fluorescent cells. When assessing trophozoite parasitemia by a blood smear, count a total of 1,000 cells. It is essential to read multiple parts of the slide as trophozoites tend to have a heterologous distribution on glass slides.

22. When calculating the amount of pellet required for the mixed agglutination assay, keep in mind that the final amount required for each test is 0.5 μ L of the pellet mixture. For example, if three mixed agglutination tests are being performed, a final pellet mixture of $0.5 \times 3 = 1.5 \mu$ L is required. Assuming equal parasitemia, initially staining 1 μ L of each pellet will lead to a final pellet mixture volume of 2 μ L, which is sufficient for the assay.
23. We have tried staining with different dye volumes and have found that staining the parasite-infected erythrocytes with a dye volume 100 $\times$ that of the erythrocyte pellet leads to increased breadth and intensity of staining compared to staining with a 20 $\times$ volume or a 50 $\times$ volume.
24. The high concentration of ethidium bromide (200 μ g/mL) is helpful when using a ~350 nm excitation wavelength to observe both dyes in a single channel, but its concentration can be reduced to 10 μ g/mL if it is observed separately from DAPI using ~510 nm excitation (see Note 28). Besides ethidium bromide and DAPI, acridine orange has been used by several groups to stain infected erythrocytes in agglutination assays [16, 18, 24]. However, in our hands, it stains infected erythrocytes multiple colors in a spectrum from green to orange. Hence, although acridine orange is suitable for the individual agglutination assay, it may be unsuitable for the mixed agglutination assay, which already involves two dyes of different colors.
25. Staining for a longer amount of time (e.g., 1 h) appears to increase breadth and intensity of staining compared to staining for a short time (e.g., 10 min). An alternative is to stain at 37 $^{\circ}$ C for 30 min.
26. Use a micropipette to remove the stain or rinse solution from each microcentrifuge tube. Initial liquid removal can be done using a larger micropipette, e.g., P1000, but removal of the final volumes of liquid should be done using a smaller micropipette, e.g., P10 or P200, to avoid accidental removal or disruption of the pellet.
27. Mixing liquid suspensions of each parasite rather than mixing pellets gives a more balanced mixture of the different parasites, which is critical for the mixed agglutination assay. The amounts mixed should be adjusted based on the initial parasitemia of each sample to give equal final parasitemias, e.g., if parasite A has double the parasitemia of parasite B, mix parasite A and B in a 1:2 ratio. To ensure accurate mixing, a cell counter can be used to determine the number of erythrocytes in each sample.

28. Ethidium bromide has a broad fluorescence excitation spectrum and can be excited at the same wavelength used to excite DAPI, i.e., ~350 nm (although this requires a higher concentration of ethidium bromide). Hence, both dyes can be viewed in a single channel if excitation is carried out at ~350 nm and a long-pass emission filter is used to allow capture of wavelengths from ~470 nm upwards. Alternatively, a microscope that can excite at different wavelengths consecutively (i.e., 350 nm and 510 nm) and obtain fluorescence images at different channels for image overlay can also be used.
29. If both mixed agglutinates and single-color agglutinates are seen, it is possible to estimate the degree of antigenic similarity between the two parasite samples based on the proportion of mixed agglutinates. Please refer to ref. [14] for details of the calculations required.

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Fig. 1.
An agglutinate stained with ethidium bromide in the individual agglutination assay.
Agglutination was conducted on a culture-adapted parasite isolate using plasma from an adult from a malaria-endemic area

Fig. 2.

An image of a mixed agglutinate captured using a single fluorescence channel. A culture-adapted parasite isolate was divided into two portions that were separately stained with DAPI (*blue*) or ethidium bromide (*orange*). Agglutination was conducted using plasma from an adult from a malaria-endemic area

Fig. 3.

An image of single-color agglutinates in a mixed agglutination assay captured using a single fluorescence channel. Two distinct parasite isolates were either stained with DAPI (*blue*) or ethidium bromide (*orange*). Agglutination was conducted using plasma from an adult from a malaria-endemic area

Table 1
Example of counting table for the individual agglutination assay

Sample	Agglutinate size			
	5–20	21–50	51–100	>100
1				
2				

Table 2
Example of counting table for the mixed agglutination assay

	Agglutinate size			
	5–20	21–50	51–100	>100
Orange (Ethidium bromide)				
Blue (DAPI)				
Orange + Blue				