

Cyclic Nucleotide (cAMP and cGMP) Assays and Capture ELISA for Quantitative Analysis of *Plasmodium falciparum* Blood-stage Egress

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[Abstract] Upon rupture of *Plasmodium falciparum* (*P. falciparum*) schizonts *in vitro* (an event known as egress), merozoites are released into the culture medium. The merozoites invade fresh red blood cells, a process that involves shedding of a microneme protein called apical membrane antigen-1 (AMA1) from the merozoite surface. This shedding, which takes place even in the absence of invasion, is therefore a surrogate marker for the degree of egress taking place in a culture, and can be measured using a specific capture ELISA to quantify AMA1 levels in culture supernatants (Collins *et al.*, 2013). The assay uses a monoclonal antibody specific for AMA1 (called 4G2dc1) (Kocken *et al.*, 1998; Collins *et al.*, 2009) to capture and immobilize the protein from culture supernatants, then uses a specific rabbit polyclonal antiserum to detect the immobilized antigen. A phosphatase-conjugated goat anti-rabbit antibody is finally used to quantify the binding of the second antibody. Egress is absolutely dependent upon the activity of a parasite cGMP-dependent protein kinase, PKG, and so is influenced by levels of intracellular cGMP (Collins *et al.*, 2013). This is regulated by the interplay between guanylate cyclases and phosphodiesterases. The latter enzymes may also degrade cAMP, so it may also be informative to measure intracellular cAMP levels.

Materials and Reagents

1. *Plasmodium falciparum* schizonts
2. DetectX Direct cGMP or cAMP immunoassay kit (Arbor Assays, catalog number: K020-H1 or K019-H1)
3. Protein-free RPMI 1640 (Life Technologies, Invitrogen™)
4. Albumax
5. Zaprinast (Sigma-Aldrich, catalog number: Z0878)
Note: Make up as a 50 mM stock solution in DMSO and stored at -20 °C.
6. PKG inhibitor compound 1 {4-[2-(4-fluorophenyl)-5-(1-methylpiperidine-4-yl)-1H-pyrrol-3-yl] pyridine} (Merck KGaA)
Note: Make up as a 10 mM stock solution in DMSO and stored at -20 °C.
7. Anti-AMA1 monoclonal antibody (clone 4G2dc1) (Kocken *et al.*, 1998)
8. Complete RPMI 1640 culture medium (Blackman, 1994)
9. Tween 20
10. Rabbit polyclonal anti-AMA1 serum (Collins *et al.*, 2009)

11. Phosphatase-conjugated goat anti-rabbit IgG (whole molecule) (Sigma-Aldrich, catalog number: A3687)
12. Phosphatase substrate (Sigma-Aldrich, catalog number: S0942)
13. Phosphate-buffered saline (PBS)
14. Sodium azide [10% (w/v) stock solution in water]
15. Dry ice-ethanol mix for freezing
16. Gelatine-tween stock solution (blocking buffer) (see Recipes)
17. 0.1 M Sodium carbonate/bicarbonate (pH 9.6) (see Recipes)
18. Diethanolamine buffer (pH 9.8) (see Recipes)

Equipment

1. Centrifuge
2. 96-well Immulon plates (Nunc[®])
3. ELISA reader
4. Incubator

Procedure

A. Assay of cGMP levels in the malaria parasite

1. For measurement of intracellular cyclic nucleotide levels, Percoll-enriched schizonts were suspended at a 5% haematocrit in gassed, protein-free RPMI 1640 at 37 °C. Standard procedures were used to culture the parasites and enrich schizonts (Blackman, 1994; Yeoh *et al.*, 2007). The suspension was divided into aliquots each containing 2×10^8 schizonts (2×10^8 schizonts corresponds to ~20 μ l packed cells).
2. Duplicate zero time samples (each containing 2×10^8 schizonts) were pelleted by centrifugation (1 min, 13,000 \times g) and snap-frozen in a dry ice-ethanol mix.
3. Further duplicate samples were then taken at various time intervals following addition of 75 μ M zaprinast, zaprinast plus the 2.5 μ M PKG inhibitor compound 1, or vehicle only (DMSO), and similarly processed. Zaprinast is an inhibitor of parasite phosphodiesterase (which degrades cGMP and cAMP), and so results in an increase in cGMP levels, inducing egress.
4. All samples were stored at -70 °C until required for assay.
5. cGMP and cAMP levels in schizont extracts were measured using a DetectX Direct cGMP or cAMP immunoassay kit, precisely as directed by the manufacturer.

B. Capture ELISA for quantitative analysis of *P. falciparum* blood-stage egress

1. Coat 96-well Immulon plates with 100 μ l/well of a stock solution of purified monoclonal antibody 4G2dc1 made up at 2.5 μ g/ml in carbonate/bicarbonate buffer (pH 9.6).

2. Seal the plates and incubate overnight in a humidified atmosphere at 4 °C. This and all subsequent incubations are done whilst stationary.
3. Wash the plates once with PBS 0.5% (v/v) Tween 20 (PBS/T), then block by adding 250 µl/well of a 1: 4 dilution of gelatine-Tween stock solution, and incubating for 2 h at room temperature.
4. Prepare serial 2-fold dilutions of test culture supernatant samples in a separate 96-well plate. Dilute the samples into RPMI 1640 culture medium containing 0.5% Albumax. For negative control wells, use RPMI Albumax medium only. Add samples to the washed ELISA plates, then seal and incubate the plates for 1 h at room temperature.
5. Wash plates 3x in PBS/T.
6. Make up a solution of rabbit polyclonal anti-AMA1 serum diluted 1:1,000 in PBS/T. Add 100 µl/well, then seal and incubate the plates a further 1 h at room temperature.
7. Wash plates 3x with PBS.
Note: No Tween at this stage, as it interferes with the alkaline phosphatase activity in the next step of the assay.
8. Add 100 µl/well phosphatase-conjugated goat anti-rabbit IgG (whole molecule), diluted 1: 30,000 in PBS. Seal the plates, then incubate 1 h at room temperature.
9. Wash plates 5x with PBS (no tween) then add 50 µl/well substrate (1 tablet of phosphatase substrate, in 5 ml diethanolamine buffer). Incubate in the dark at 37 °C for at least 30 min. Determine OD₄₀₅ using an ELISA reader.
10. Use the data to produce titration curves of antigen levels in the various culture supernatants.

Recipes

1. Gelatine-tween stock solution (blocking buffer)
 - 5 g Gelatine
 - 2.5 ml Tween-20
 - 150 µl 10% (w/v) sodium azide
 - 500 ml PBS

Warm at 37 °C to dissolve the gelatine
 Use at 1: 4 dilution in PBS
 Stored stock at 4 °C
 May require warming before coaxing out of the bottle
2. 0.1 M Sodium carbonate/bicarbonate (pH 9.6)
 - Make up the two component buffers first
 - These are
 - 7.155 g Na₂CO₃·10H₂O made up to 250 ml water
 - 2.1 g NaHCO₃ made up to 250 ml water

Mix the above buffers to obtain a buffer with a pH of 9.6

As an approximate measure, this requires ~37 ml 0.1 M Na₂CO₃·10H₂O plus 73 ml 0.1 M NaHCO₃

3. Diethanolamine buffer (pH 9.8)

48.5 ml Diethanolamine

982 µl 1 M MgCl₂

2.5 ml 10% (w/v) sodium azide

Add 400 ml water then adjust pH to 9.8 with 1 M HCl before making up to a total of 500 ml with water

Stored in the dark at 4 °C

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