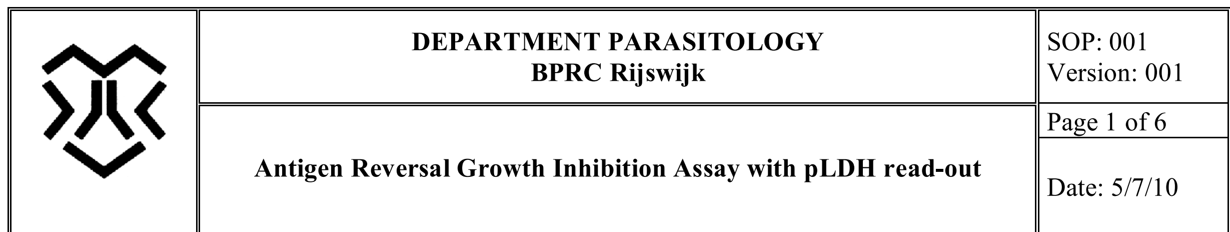

DEPARTMENT PARASITOLOGY
BPRC Rijswijk

SOP: 001
Version: 001

Antigen Reversal Growth Inhibition Assay with pLDH read-out

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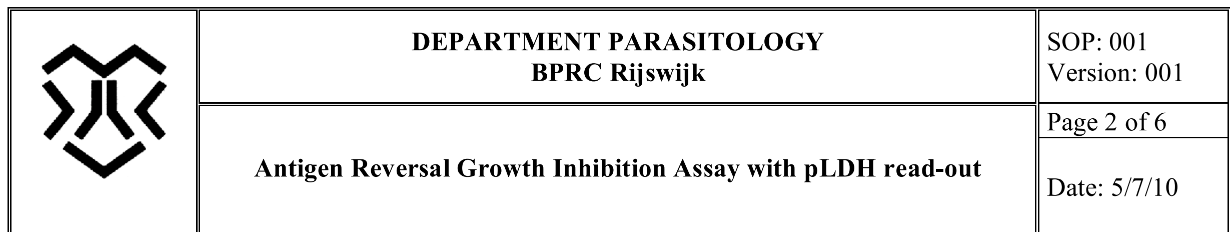


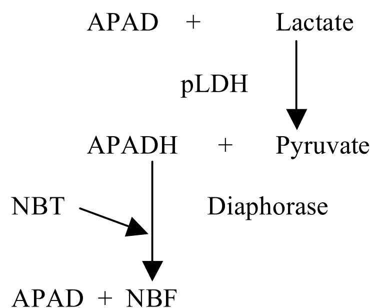
A.1. Aim

The aim of Antigen Reversal GIA is to study specificities responsible for growth inhibition in normal GIAs. This is achieved by culturing parasites in presences of different concentrations of antigens and a fix concentration of antibodies (48μl assay).
The read out system is detection of *p*LDH (*plasmodium* Lactate DeHydrogenase), this enzyme is produced by live parasites and well detectable when they are in late trophozoit and schizont phase.

A.2. Principle

3-Acetylpyridine Adenine Dinucleotide (APAD) and Lactate (present in substrate buffer) are converted by pLDH to APADH and Pyruvate. APADH reduces the chromogenic substrate Nitro Blue Tetrazolium (NBT) using the enzyme diaphorase. This results in the formation of Nitro Blue Formazan (NBF), a deep purple soluble stain that can be measured at a wavelength of 650 nm.

Schematic representation:



B. Samples

IgG's obtained from human trials or animal experiments, in which the individuals/animals were immunised against *P. falciparum*. Or sera from people naturally exposed to malaria.

C. Controls

Positive control: BG98 Rabbit standard at 6mg/mL

D.1. Equipment, instruments and materials

D.1.1. Equipement and instruments

1. 50 ml tubes: Greiner Bio-one #22761
2. 96-well flat-bottom half area culture plates with individual lids: Greiner Bio-one #675180
3. 96-well flat-bottom culture plates with individual lids: Greiner Bio-one #655180
4. Amicon Ultra Filter tubes: Amicon Ultra -15 #UFC903024
5. Centrifuges: for culturing and spinning down RBC's: Beckman Coulter Allegra X-22R Centrifuge
   for harvesting of GIA-plates: Beckman Spinchron R Centrifuge
6. Culture flasks: T25: Corning #430168
   T75: Corning #430720
   T175: Corning #731079
7. Eppendorf centrifuge: Eppendorf centrifuge 5424
8. Eppendorf tubes: Eppendorf Safe-lock 1.5 ml #0030 120.086
9. Filters: 0.45 μm: Whatman FP30/0,45 CA-S #10462100
   0.2 μm: Whatman FP30/0,2 CA-S #10462200
10. Flatbed shaker: Edmund Bühler TiMix CONTROL
11. Humidified box: plastic box with wet tissues or water at the bottom
12. Humidified incubator: Sanyo O_2/CO_2 incubator
13. Microscope: Zeiss Axioskop

14. Nanodrop: Nanodrop ND-1000 Spectrophotometer
15. Pipettes: 2 ml: ALP #PN2E1
    5 ml: ALP #PN5E25
    10 ml: ALP #PN10E25
    25 ml: ALP #PN25E1
16. Platereader: Bio-Rad Model 680 Microplate Reader
17. Screw-cap tubes: Sarsteadt 2ml PP #72.694.006
18. Shaking incubator: innova44
19. Slides: Menzel-Gläzer 76x24 mm
20. Tips: 20 µl: CLP #BT20
    200 µl: CLP #BT200
    1000 µl: CLP #BT1000
21. Waterbath: Jubalo

D.1.2. Materials

1. 0.3 M alanine, 10 mM HEPES pH 7.5
   L-alanine: Sigma #A7469; $C_3H_7NO_2$, M=89.09
   HEPES: Gibco #11344-025; M=238.39
   To prepare 250 ml 0.3 M alanine 10 mM HEPES: dissolve 6.68 g alanine and 595.75 mg HEPES in 200 ml distilled water, adjust to pH 7.5, fill up to 250 ml with distilled water. Filter through a filtertop using vaccuum. Store at 4 °C.
2. 100% Methanol: Merck #1.06009.2500; CH_3OH, M=32.04
3. 2xCmed = 2x Complete Culture Medium:
   RPMI1640 + 20% Human serum + 30 µg/ml Gentamycin
4. APAD: Sigma #A5251; $C_{22}H_{28}N_6O_{14}P_2$, M=662.44
   3-Acetylpyridine Adenine Dinucleotide
   To prepare 10 ml stock solution of 10 mg/ml: dissolve 100 mg of APAD in 10 ml distilled water. Make 50 µl aliquots and store at -20 °C.
5. Cmed = Complete Culture Medium:
   RPMI1640 + 10% Human serum + 15 µg/ml Gentamycin
6. Diaphorase from *Clostridium kluyveri*: Sigma #D5540
   To prepare 30 ml stock solution of 50 units/ml: dissolve 1.500 units Diaphorase in 30 ml distilled water. Make 200 µl aliquots and store at -20 °C.
7. EDTA: Merck #1.00944.1000; $C_{10}H_{16}N_2O_8$, M=292.25
   Ethylenediamine tetraacetic acid
   To prepare an 8 mM EDTA solution: Dissolve 116.9 mg in 40 ml RPMI1640, adjust to pH 7.5, fill up to 50 ml with RPMI1640 and sterilise through a 0.45 µm filter, store at 4 °C.
8. Gentamycin: Gibco #15750-037; stock = 50 mg/ml
9. Giemsa buffer: Merck #1.09468.0100; $Na_2HPO_4*2H_2O$ KH_2PO_4
10. Giemsa stain: Sigma #GS500
    To prepare fresh Giemsa stain: dilute 1 part Giemsa stain in 4 parts Giemsa buffer, filter through a 0.45 µm filter, use immediately.
11. Heat-inactivated Human Serum A+: Bloedbank Leidsenhage, store at -20 °C.
12. Human RBC O^+, 12 heparin tubes of individual donors, after processing store at 4 °C.
13. S.A.G.M solution
    Saline: Sodium Chloride Sigma #S3014
    Adenine: Sigma #A2786
    Glucose: Sigma #16325
    D-Mannitol: Sigma #M9546
    To prepare 500 ml solution, dissolve 4.4 gr Sodium Chloride, 4.5 gr Glucose, 0.08 gr Adenine, 2.62 gr Mannitol in 500 ml distilled water and sterilise through a 0.2µm filter.
14. plasmodium falciparum, e.g. FCR3 or 3D7 strain

15. LDH-buffer: Sigma #L7022; $C_3H_5NaO_3$, M=112.06
    Sodium L-lactate
    To prepare 500 ml of the buffer, mix 50 ml of 1M TRIS-HCl (pH 8.0) and 450 ml distilled water. Add 2.8 g Sodium L-Lactate and 1.25 ml Triton X-100. Mix on a magnetic stirrer at room temparature for at least 30 minutes. Make 50 ml aliquots and store at -20 °C.
16. NaAz: Sigma #S2002; NaN_3, M=65.01 Sodium azide
17. NBT: Sigma #N6639; $C_{40}H_{30}N_{10}O_6$*2Cl, M=817.64 Nitro Blue Tetrazolium chloride
18. PBS: Gibco #10010 Phosphate Buffered Saline pH 7.4
19. Protein G: Pierce #20399
20. RPMI 1640: Gibco #52400
    RPMI1640 + L-glutamine + 25 mM HEPES

E.1. <u>Assay preparation</u>

<u>E.1.1. Preparation of uninfected RBCs</u>

1. Use the same batch of RBC used for the culturing of parasites in the last few days.
2. Make a RBC suspension of 4% hematocrit in 2xCmed.

<u>E.1.2. Preparation of competitor Antigen</u>

1. Dilute competitor Ag to a start concentration of 200µg/mL (end concentration in the plate will start at 50µg/mL). 2-fold serial dilution will be prepared in the assay plates.
2. Serial dilution: 50µg/mL - 25µg/mL – 12,5µg/mL – 6,25µg/mL.

<u>E.1.3. Preparation of Antibody at fixed concentration</u>

Dilute Ab to the concentration yielding 70 - 80% inhibition in a normal GIA. This fixed concentration will be used to different concentrations competitor Ag.

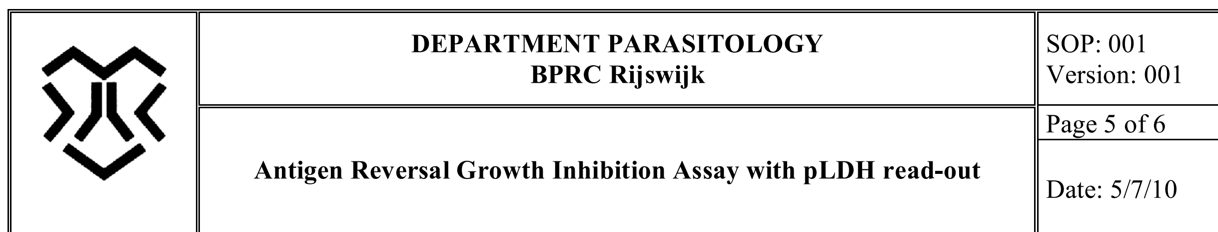
<u>E.1.4. Preparation of GIA parasite suspension</u>

1. Count the parasitaemia in a smear making sure all parasites are in the late-schizont stage.
2. Transfer the parasite-culture to a 50 ml tube.
3. Spin for 5 minutes at 900*g*.
4. Remove supernatant.
5. Dilute enough of the parasite-pellet in 2xCmed for 0.2-0.3% parasitaemia.
6. Add RBC 50% HT to get a 4%HT suspension.

E.2. <u>Procedure</u>

<u>E.2.1. Ag reversal GIA set-up</u>

1. Use 96-well flat-bottom half-area culture plates with individual lids.
2. Label all plates with the parasite-line used, date, initials and plate-number (numbers on both lid and plate).
3. Pipette 12µL plain RPMI in light grey area for serial dilution of competitor antigen.
4. Pipette 36µL competitor Ag in triplicate in 2nd wells of sample assay rows (dark grey area – row B).
5. Take 12µL competitor Ag out row B and pipette in wells above (row A). This will be the assay wells with just competitor Ag (and no Ab).
6. Again take 12µL competitor Ag out of row B and serially dilute from B to C, from C to D and from D to E, throw away the remaining 12µL.
   Row F will be assay wells with just Ab and no Ag.
7. Add 12µL Ab at fixed concentration yielding 70 - 80% inhibition in triplicate to 15 wells/sample (row B to F).
8. Pipette 24µL positive IgG to positive control wells.
9. Pipette 24µL plain RPMI to schizont control wells, RBC control wells and to the monitor wells.
10. Pipette 24µL 8mM EDTA to the EDTA-control wells.
11. Pre-incubate plates for 1hr at 37°C, so that Abs and Ags can interact.



12. Add 24µl parasite-suspension to all wells, except RBC-control wells.
13. Add 24µl RBC-suspension to the RBC-control wells.
14. Put the plates in a humidified box (wet tissues or water at the bottom of the box), and place the lid on the box, but don't close it completely.
15. Put the boxes with the plates in a humidified incubator at 37°C, containing 5%CO_2, 5%O_2, 90%N_2.
16. Incubate for 40 – 44hrs.
17. Prepare a smear using contents of 3 monitor-wells for one or two plates at T=0 and after ~24hrs and stain with Giemsa to check parasite-stage and parasitemia.

Final hematocrite: 2%
Final parasitemia: 0.3 ± 0.1%

E.2.2. Template with final Ag-Ab-concentrations

	1	2	3	4	5	6	7	8	9	10	11	12
A		Ag 50µg/mL - Ab			Ag 50µg/mL – Ab			Ag 50µg/mL - Ab			Ag 50µg/mL - Ab	
B		Ag 50µg/mL + Ab			Ag 50µg/mL + Ab			Ag 50µg/mL + Ab			Ag 50µg/mL + Ab	
C		Ag 25µg/mL + Ab			Ag 25µg/mL + Ab			Ag 25µg/mL + Ab			Ag 25µg/mL + Ab	
D	S1	Ag 12,5µg/mL + Ab		S2	Ag 12,5µg/mL + Ab		S3	Ag 12,5µg/mL + Ab		S4	Ag 12,5µg/mL + Ab	
E		Ag 6,25µg/mL + Ab			Ag 6,25µg/mL + Ab			Ag 6,25µg/mL + Ab			Ag 6,25µg/mL + Ab	
F		only Ab			only Ab			only Ab			only Ab	
G	EDTA		+ve		MONITOR				Schizont		RBC	
H												

E.3. Harvesting and pLDH

E.3.1 GIA harvest

1. Prepare a smear using contents of 3 monitor-wells of each plate at T=~44hrs and stain with Giemsa to check parasite-stage and parasitemia. Harvest at schizont-stage.
2. * Fill new 96-well flat-bottom culture plates with 200 µl/well of cold PBS (one plate for each assay plate).
3. Mix the contents of the wells in the assay plate thoroughly using a multichannel and transfer the contents to the PBS-plate (in the same plate format).
4. Centrifuge plates for 10min at 1300xg at 4°C without brake.
5. Remove 190 µl of supernatant without disturbing the pellet.
6. Freeze the plates overnight at -20 °C (or until ready for *p*LDH-assay) to lyse erythrocytes.

E.3.2. *p*LDH assay

NOTE: NBT is light sensitive, so avoid direct light and keep solutions and plates with substrate in dark (cover with aluminium foil)

1. Thaw the plates and LDH-buffer and warm up to room temperature (10 ml LDH-buffer/plate).
2. Dissolve NBT in LDH-buffer at a concentration of 2mg/10ml. Mix gently and keep substrate in dark.
3. Add 50 µl APAD stock (10 mg/ml) to every 10 ml substrate.
4. Add 200 µl Diaphorase stock (50 units/ml) to every 10 ml substrate.
5. Use substrate immediately.
6. * Add 100 µl substrate per well of the harvested plates. One plate every minute.
7. Cover with aluminium-foil and place on a flatbed shaker at 400 rpm at room temparature.
8. Incubate for 30 minutes.
9. * Measure OD after 30 minutes at waveleangth 655 nm (A655). One plate every minute.

E.3.3. Remarks to the harvesting and pLDH

Ad.3.1.2. Put the cold PBS in the fridge the day before harvesting. Plate can be filled with PBS the day before, but than place the plates in the fridge to keep them cold. The cold PBS stops the parasites in their cycle.

Ad.3.2.6. Add substrate to 1 plate per minute. Wrap the plate with aluminium-foil and put on the flatbed shaker. This to ensure all plates are incubated exactly the same time.

Ad.3.2.9. Measure 1 plate per minute. This again to ensure all plates are incubated exactly the same time.

F. Results

OD-values measured by the platereader should be exported to a csv-file.
% inhibitions can be calculated using the following formula:

$$\% \text{ inhibition} = 100\% - \frac{(\text{A655 IgG sample} - \text{A655 RBC control})}{(\text{A655 Sz control} - \text{A655 RBC control})} \times 100\%$$