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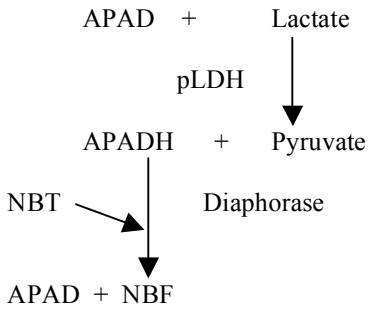
A.1. **Aim**

To quantify IgG's obtained from patients or animals in their ability to inhibit invasion or intraerythrocytic development of *P. falciparum* into erythrocytes. The read-out system is detection of *p*LDH (*plasmodium* Lactate DeHydrogenase).

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 Rb standard at 6mg/mL
Negative control: Negative rabbit, rhesus or human IgG fraction, purified similarly as the samples.

D.1. **Equipment, instruments and materials**

D.1.1. Equipement and instruments

1. 50 ml syringes: BD Plastipak #300866
2. 50 ml tubes: Greiner Bio-one #22761
3. 96-well flat-bottom half area culture plates with individual lids: Greiner Bio-one #675180
4. 96-well flat-bottom culture plates with individual lids: Greiner Bio-one #655180
5. Amicon Ultra Filter tubes: Amicon Ultra -15 #UFC903024
6. Centrifuges: for culturing and spinning down RBC's: Beckman Coulter Allegra X-22R Centrifuge
   for harvesting of GIA-plates: Beckman Spinchron R Centrifuge
   for IgG purification: Beckman Coulter Avanti J-E Centrifuge
7. Columns: Econopack columns Bio-Rad #7371511;
   Econo-column Flow Adaptor Bio-Rad #738-0016
8. Culture flasks: T25: Corning #430168
   T75: Corning #430720
   T175: Corning #731079
9. Eppendorf centrifuge: Eppendorf centrifuge 5424
10. Eppendorf tubes: Eppendorf Safe-lock 1.5 ml #0030 120.086

11. Filters: 0.45 µm: Whatman FP30/0,45 CA-S #10462100
    0.2 µm: Whatman FP30/0,2 CA-S #10462200
12. Flatbed shaker: Edmund Bühler TiMix CONTROL
13. Humidified box: plastic box with wet tissues or water at the bottom
14. Humidified incubator: Sanyo O_2/CO_2 incubator
15. Microscope: Zeiss Axioskop
16. Nanodrop: Nanodrop ND-1000 Spectrophotometer
17. Pipettes: 2 ml: ALP #PN2E1
    5 ml: ALP #PN5E25
    10 ml: ALP #PN10E25
    25 ml: ALP #PN25E1
18. Platereader: Bio-Rad Model 680 Microplate Reader
19. Pump: Watson Marlow 205U
20. Screw-cap tubes: Sarsteadt 2ml PP #72.694.006
21. Shaking incubator: innova44
22. Slides: Menzel-Gläzer 76x24 mm
23. Stative
24. Tips: 20 µl: CLP #BT20
    200 µl: CLP #BT200
    1000 µl: CLP #BT1000
25. 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. Bindingbuffer: Protein G IgG Binding Buffer: Pierce #21011
6. Cmed = Complete Culture Medium:
   RPMI1640 + 10% Human serum + 15 µg/ml Gentamycin
7. 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.
8. 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.
9. Elutionbuffer: Pierce #21009
10. Gentamycin: Gibco #15750-037; stock = 50 mg/ml
11. Giemsa buffer: Merck #1.09468.0100; $Na_2HPO_4*2H_2O$ KH_2PO_4
12. 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.
13. Heat-inactivated Human Serum A+: Bloedbank Leidsenhage, store at -20 °C.

14. Human RBC O^+, 12 heparin tubes of individual donors, after processing store at 4 °C.
15. 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.
15. plasmodium falciparum, e.g. FCR3 or 3D7 strain
16. 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.
17. NaAz: Sigma #S2002; NaN_3, M=65.01 Sodium azide
18. NBT: Sigma #N6639; $C_{40}H_{30}N_{10}O_6$*2Cl, M=817.64 Nitro Blue Tetrazolium chloride
19. PBS: Gibco #10010 Phosphate Buffered Saline pH 7.4
20. Protein G: Pierce #20399
21. RPMI 1640: Gibco #52400
    RPMI1640 + L-glutamine + 25 mM HEPES
22. TRIS: Merck #1.08382.2500; $H_2NC(CH_2OH)_3$, M=121.14
    Tris(hydroxymethyl)aminomethane
    To prepare 1 liter of 1M TRIS pH 9.0: Dissolve 121.14 g of TRIS in 900 ml of distilled water, adjust pH to 9.0, fill up to 1 liter with distilled water.

D.2. Procedure

D.2.1. Culture and synchronisation of parasites

1. * *Routine culturing:*
    The culture can routinely be maintained at 0.1 – 1% parasitaemia.
    The hematocrit will be kept at 5%.
    The culture must be refreshed 3 times a week.
1.1. Place Cmed at room temparature before culturing.
1.2. Take culture out of the shaking incubator.
1.3. Transfer ± 200 µl of culture-suspension to an eppendorf-tube.
1.4. Spin for 10 seconds at maximum speed (~20000*g*) in an Eppendorf centrifuge.
1.5. Remove supernatant carefully, leave about the same amount of supernatant as pelletvolume.
1.6. Resuspend pellet in the supernatant left.
1.7. Place a small drop onto an slide and use another slide under ± 45°-angle to make a thin smear.
1.8. Air-dry the slide.
1.9. Fix the slide in 100% methanol and airdry.
1.10. * Prepare a fresh Giemsa-stain.
1.11. Filter through a 0.45 µm filter and allow 0.5 ml to drain away before using the rest of the stain for the staining.
1.12. Stain for 5 minutes at room temparature.
1.13. Pour off stain solution and immediately rinse the slide in water.
1.14. * Dry the slide thoroughly before observation through a microscope.
1.15. Put a drop of oil on the slide and estimate the parasitaemia under the x100 objective.
1.16. Count at least 2000 RBC, in case they are evenly distributed throughout the slide, one quarter of one field can be representative for the RBC-count. To get the number of RBC, multiply the quarter-count of RBC by 4 and than by the number of fields counted.
1.17. Count the number of infected RBC for each field.
1.18. % parasitaemia = (total counted parasites / total RBC counted) x 100

1.19. * To determine the developmental stage of the parasites perform a differential count (rings, trophozoites and schizonts are counted individually).
1.20. Transfer all of the culture to a 15 or 50 ml tube.
1.21. Spin for 5 minutes at 900*g*.
1.22. Remove supernatant carefully
1.23. Take out the amount of pellet needed and place back into the culture flask containing new Cmed.
1.24. Add RBC 50% HT to a final hematocrit of 5%.
1.25. Gas the culture with 5% CO_2, 5% O_2, 90% N_2, using sterile filters, tips and pipettes.
1.26. Place the culture in the shaking incubator at 37°C.

2. *Synchronization of parasites with alanine:*
Alanine-treatment removes schizonts/trophozoites from the culture, leaving behind the rings and early trophozoites (time window of 0-16 hours).
Before you start the alanine-treatment you should do a differential count of your culture to make sure there are rings in your culture.
It is advised to repeat the alanine-treatment 2 to 3 times before using the culture for a GIA.

2.1. Pre-warm a solution of 0.3 M alanine, 10 mM Hepes pH 7.5 at 37°C.
2.2. Transfer the culture to a 50 ml tube.
2.3. Spin for 5 minutes at 900*g*.
2.4. Remove supernatant carefully.
2.5. Resuspend the pellet in 5x pellet volume of 0.3 M alanine, 10 mM Hepes pH 7.5.
2.6. Incubate for 20 to 30 minutes at 37°C.
2.7. Fill up the 50 ml tube with Cmed.
2.8. Spin for 5 minutes at 900*g*.
2.9. Remove supernatant carefully.
2.10. Resuspend pellet in 50 ml Cmed.
2.11. Spin for 5 minutes at 900*g*.
2.12. Remove supernatant carefully.
2.13. * Take out the amount of pellet needed and transfer to a new culture flask containing new Cmed.
2.14. Add RBC 50% HT so that the hematocrit will be 5%.
2.15. Gas the culture with 5% CO_2, 5% O_2, 90% N_2, using sterile filters, tips and pipettes.
2.16. Place the culture in the shaking incubator.

D.2.2. Uninfected Red Blood Cell preparation

1. *Isolation of RBC from whole blood:*
1.1. first pool blood of all donors into a culture flask then divide the blood over 2 or 4 50ml tubes. These tubes can be spin down at 900*g*, for 10 minutes without brake.
1.2. Remove the serum and as much of the buffycoat (white cell layer) as possible.
1.3. Resuspend in a equal volume or excess of S.A.G.M. solution and wash the cells for 5 minutes at 900*g* without brake. Remove the S.A.G.M solution and any remaining visible white cell from the surface of the red blood cells.
1.4. Repeat the wash as stated in 1.3. twice more.
1.5. After the final wash, measure the red blood cell volume and resuspend in an equal volume of S.A.G.M. and divide the 50%HT cell suspension over 8-12 sterile 15ml tubes.
1.6. Store at 4 °C.

2. *Preparation of uninfected RBC for use in GIA:*
2.1. Use the same batch of RBC used for the culturing of parasites in the last few days.
2.2. Make a RBC suspension of 4% hematocrit in 2xCmed.

D.2.3. Preparation of the test samples

Use Protein G columns.

1. * Dilute 1 part serumsample in 2 parts bindingbuffer.
2. Leave o/n at 4°C.
3. Spin serum samples 10 minutes 2400*g*, and remove fat (white ring) on top.
4. Transfer supernatant in a clean tube and use for IgG-purification.
5. Wash columns with 2x columnvolume of bindingbuffer; pumpspeed = 10 rpm (~1.4 ml/min).
6. * Apply samples on columns; pumpspeed = 10 rpm (~1.4 ml/min), collect the flowthrough in 50 ml tubes labeled with the sample-name and "flowthrough".
7. Wash columns with 2x columnvolume of bindingbuffer; pumpspeed = 10 rpm (~1.4 ml/min),
8. * Wash columns with 35-40x columnvolume of PBS; pumpspeed = 20 rpm (~2.8 ml/min), Collect the flowthrough (total of 100 ml including the flowthrough of step 6 and 7), the rest of the flowthrough is waiste.
9. * Take a flowthrough sample to measure on the nanodrop (blank = PBS, sample has to be below 0.1 mg/ml, otherwise continue washing with PBS until sample is below 0.1 mg/ml).
10. Prepare 50 ml tubes with 3 ml of 1M TRIS (pH 9.0) (1 tube per sample), labeled with samplename.
11. Wash columns with elutionbuffer; pumpspeed = 10 rpm (~1.4 ml/min), collect the flowthrough in the prepared 50 ml tubes until a total volume of 30 ml.
12. * Take the last drop of flowthrough to measure on the nanodrop (blank = PBS, sample has to be below 0.1 mg/ml, otherwise continue washing with elutionbuffer until sample is below 0.1 mg/ml).
13. * Measure your collected flowthrough on the nanodrop for an indication of the IgG-yield.
14. Wash columns with 2x columnvolume of PBS; pumpspeed = 20 rpm (~2.8 ml/min).
15. Wash columns with 1x columnvolume of NaAz; pumpspeed = 20 rpm (~2.8 ml/min).
16. Pour 10 ml 70% EtOH on the Amicon Ultra Filter tubes and leave them for 5 minutes.
17. Pour off the EtOH and let the filters dry.
18. Pour 15 ml plain RPMI on the filters (to clean out the EtOH).
19. Spin for 10 minutes at 1900*g*.
20. Filter the eluted IgG's through a 0.2 µm-filter using a syringe.
21. Pour 15 ml of the eluted IgG's on the Amicon Ultra Filter Tubes.
22. * Spin for 30 minutes at 1900*g* (longer if there is more than 2 ml of fluid left above the filter).
23. * Collect the flowthrough in a new 50 ml tube labeled waiste1.
24. Pour the remaining 15 ml of the eluted IgG's on the Amicon Ultra Filter Tubes.
25. Spin for 30 minutes at 1900*g* (longer if there is more than 2 ml of fluid left above the filter).
26. Collect the flowthrough in the waiste1-tube.
27. Wash the tube used the eluted IgG's with 5 ml of plain RPMI and pour over to the Amicon Ultra Filter Tubes.
28. Fill the Amicon Ultra Filter Tubes up to 15 ml with plain RPMI.
29. Spin for 30 minutes at 1900*g* (longer if there is more than 2 ml of fluid left above the filter).
30. Collect the flowthrough in the waiste1-tube.
31. Fill the Amicon Ultra Filter Tubes up to 15 ml with plain RPMI.
32. Spin for 30 minutes at 1900*g* (longer if there is more than 2 ml of fluid left above the filter).
33. Collect the flowthrough in the waiste2-tube.
34. Fill the Amicon Ultra Filter Tubes up to 15 ml with RPMI.
35. Spin for 30 minutes at 1900*g* (longer if there is more than 2 ml of fluid left above the filter).
36. Collect the flowthrough in the waiste2-tube.
37. Transfer the concentrated IgG to a 1,8 ml screw-cap tube.
38. * Measure a 1 in 10 diluted (in RPMI) sample on the nanodrop.
39. * Dilute the IgG's to the wanted concentration, the concentration is 2x the end-concentration wanted in GIA.
40. Store at -20°C until use in GIA.

D.2.4. Preparation of GIA parasite suspension

1. Count the parasitaemia in a smear (see D.2.2.1.3-D.2.2.1.19), 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.

D.2.5. GIA set-up

The assay could be performed with an end volume of 30 µl or 50 µl. A 30 µl assay is could be used when you do not have enough IgG sample, or just to save some for future assays.

1. Use 96-well flat-bottom half-area culture plates with individual lids.
2. * Pipette 15 - 25 µl IgG's in triplicates (30 - 50 µl if you make a serial dilution of the IgG's, than also pipette 15 - 25 µl of plain RPMI in the other wells for serial dilution).
3. * Pipette 15 - 25 µl positive IgG.
4. * Pipette 15 - 25 µl negative IgG.
5. * Pipette 15 - 25 µl plain RPMI for the schizont controls wells, RBC-control wells and to the monitor wells.
6. * Pipette 15 - 25 µl 8 mM EDTA for the EDTA-control.
7. * Add 15 - 25µl parasite-suspension to all wells, except RBC-control wells.
8. * Add 15 - 25µl RBC-suspension to the RBC-control wells.
9. 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.
10. Put the boxes with the plates in a humidified incubator at 37 °C, containing 5% CO_2, 5% O_2, 90% N_2.
11. Incubate for 40 – 44 hours.
12. 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 parasitaemia: 0.3 ± 0.1%

D.2.6. 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.

D.2.7. *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.

D.3. Plate-layout

Two examples of plate-layouts, with final IgG-concentrations.

Human samples

	1	2	3	4	5	6	7	8	9	10	11	12
A	EDTA	S1 10 mg/mL	S2 10 mg/mL	S3 10 mg/mL	S4 10 mg/mL	S5 10 mg/mL	S6 10 mg/mL	S7 10 mg/mL	S8 10 mg/mL	S9 10 mg/mL	S10 10 mg/mL	RBC
B												
C												
D												
E		5 mg/mL	5 mg/mL	5 mg/mL	5 mg/mL	5 mg/mL	5 mg/mL	5 mg/mL	5 mg/mL	5 mg/mL	5 mg/mL	
F												
G		monitor			-ve		-ve		+ve		SZ	
H												

Rabbit samples

	1	2	3	4	5	6	7	8	9	10	11	12
A	EDTA	S1 6 mg/mL	3 mg/mL	1,5 mg/mL	0,75 mg/mL	S3 6 mg/mL	3 mg/mL	1,5 mg/mL	0,75 mg/mL	S5 6 mg/mL	3 mg/mL	1,5 mg/mL	0,75 mg/mL	monitor
B														
C														
D														
E		S2 6 mg/mL	3 mg/mL	1,5 mg/mL	0,75 mg/mL	S4 6 mg/mL	3 mg/mL	1,5 mg/mL	0,75 mg/mL	-ve	-ve	+ve	SZ	RBC
F														
G														
H														

D.4. Remarks to the procedure

Ad. D.2.1.1 Culturing 3 times a week: Preferably on Monday morning, Wednesday mid-day, Friday afternoon.

Ad. D.2.1.1.10. Giemsa-stock is 5x concentrated. A fresh Giemsa-stain is made by diluting 1 part Giemsa-stock in 4 parts buffer pH 7.2.

Ad. D.2.1.1.14. Use a tissue from the paper-dispenser. Press the tissue onto the slide without whiping the slide. Repeat this. The slide should be dry.

Ad. D.2.1.1.19. Determination of the developmental stage of the parasite-culture could be done during the counting of the parasites (D.2.2.1.16).

Ad. D.2.1.2.13. Use a new culture-flask after alanine-treatment because in the old culture-flask there are still some parasites left that are non-synchronised.

Ad. D.2.3.1. E.g. 10 ml of serum + 20 ml bindingbuffer.

Ad. D.2.3.6. First 100 ml of flowthrough is collected/sample in case the purification didn't work. Than it might be that all the IgG's are in this part of the flowthrough.

Ad. D.2.3.8. E.g. column-volume is 5 ml, than wash with at least 175 ml of PBS.

Ad. D.2.3.9. Nanodrop: Open ND-1000 V3.2.1-software. Clean the pedestal with aqua dest. Put on pedestal the blank (= PBS). Choose Protein A280. OK. Under sample-type choose "BSA". Reblank (without putting new blank on pedestal). Clean pedestal. Put on sample, name sample in sample-field. Measure. Clean pedestal etc. At end of measuring samples: Show report. Print and save window.

Ad. D.2.3.12. Nanodrop: Open ND-1000 V3.2.1-software. Clean the pedestal with aqua dest. Put on pedestal the blank (= PBS). Choose Protein A280. OK. Under sample-type choose "IgG". Reblank (without putting new blank on pedestal). Clean pedestal. Put on

sample, name sample in sample-field. Measure. Clean pedestal etc. At end of measuring samples: Show report. Print and save window.

Ad. D.2.3.13. Same way as Ad. D.2.4.12.

Ad. D.2.3.22. If there is a relative high concentration of IgG in your eluted IgG-solution, it could be that the filter needs more time to let the fluid through. Than most of the times an extra 10 minute spin could be sufficient. It could also be that the IgG concentration is very high. Than it's better to transfer part of it to a new Amicon Ultra Filter Tube and start over.

Ad. D.2.3.23. The flowthrough from the Amicon Ultra Filter tubes is collected in case the filters leak. When they leak, they well also pass the IgG's. By collecting the flowthrough, you can always try again by filtering the flowthrough again on a new Amicon Ultra Filter tube.

Ad. D.2.3.38. The concentration is now much higher than in the original eluted IgG solution, so dilute you sample 1 in 10 (in RPMI) for measurement on the nanodrop. Use of the nanodrop: see Ad. D.2.4.12.

Ad. D.2.3.39. When you need to dilute your sample 2x, it's better not to do it in 1 step. Most of the times you will dilute your sample too far, and you need to concentrate it again. So first dilute it 1.5x.
Concentrations wanted (with the error allowed):
Human IgG: 20 mg/ml (18 – 22 mg/ml)
Rhesus IgG: 12 mg/ml (10.8 – 13.2 mg/ml)
Rabbit IgG: 12 mg/ml (10.8 – 13.2 mg/ml)

Ad. D.2.5.2-8. See example plate-layouts in D.3.

Ad. D.2.6.2. 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. D.2.7.6. 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. D.2.7.9. Measure 1 plate per minute. This again to ensure all plates are incubated exactly the same time.

E. Results

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

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