SAFETY PRECAUTIONS

Plasmodium falciparum is a Category 3 pathogen (with some derogations). All culturing must be done in a derogated Biosafety CL3 facility and the malaria safety Code of Practice adhered to.

1. MATERIALS

1.1. PBS (PHOSPHATE BUFFERED SALINE pH 7.3

1.1.1. 1.9mM Na_2HPO_4 (VWR International UK Ltd., Cat No.301584L)
1.1.2. 8.1mM NaH_2PO_4 (VWR International UK Ltd., Cat No.444433M)
1.1.3. 150mMNaCl (VWR International UK Ltd., Cat No. 27788.366)

1.2. PBS CONTAINING 1% BOVINE SERUM ALBUMIN (BSA) AND 0.01% SODIUM AZIDE

1.2.1. Bovine Serum Albumin (Sigma, Cat No. A4503)
1.2.2. Sodium Azide, NaN_3 (VWR International UK Ltd., Cat No 301112G)

1.3. ACETONE

1.3.1. (Analar grade or equivalent) (Fisher Scientific Ltd., Cat No.A/0600/17)

1.4. PRIMARY ANTIBODIES

Primary antibodies may be from a variety of sources, including monoclonal antibodies, polyclonal animal (mouse, rabbit, rat) serum or human serum. These may be used either in their whole serum or as purified Ig preparations. Suitable known positive and negative control samples should be included in each assay. Each sample should be tested in duplicate. Duplicate control wells of the IFA buffer alone should be used as a further negative control in each assay. Primary antibodies should be diluted in IFA Buffer and are normally titrated in doubling or tripling dilutions to achieve an end-point reading.

1.5. SECONDARY ANTIBODIES

1.5.1. FITC-Conjugated Anti-human IgG/Ig (or anti-rabbit, anti-mouse, anti-rat as appropriate)
1.5.2. anti-human IgG, Dako rabbit anti-human IgG (Cat. No. F0202)
1.5.3. anti-rabbit Ig, Dako swine anti-rabbit Ig (Cat. No. F0205)
1.5.4. anti-mouse Ig, Dako goat anti-mouse Ig (Cat.No. F0479)
(Recommended suppliers are Dako, Denmark or Jackson Immunoresearch Inc.)

Appropriate secondary antibodies are diluted to appropriate, pre-tested, concentrations in IFA buffer at a concentration determined for each batch and supplier. Optimal concentrations may vary slightly according to the microscope used, and should be tested with appropriate positive and negative controls for specificity and sensitivity.

1.6. DAPI STAIN

1.6.1. 4',6'-diamino-2-phenylindole, (Sigma Cat No. D8417)
1.6.2. Prepare at a concentration of 10mg L^{-1} in PBS

1.7. MULTISPOT IFA SLIDES

1.7.1. Hendley-Essex 12-SPOT or 10-SPOT multispot microscope slides.
PTFE & specialised coatings from C.A.Hendley (Essex) Ltd., Oakwood Hill Industrial Estate, Loughton, Essex, Fax: UK 0181 502 0430 or equivalent

1.8 COVERSLIPS
Menzel-Gläser 22x64mm Ref 6776321) or similar

1.9 MOUNTANT
Citifluor AF1 mountant containing ant-fade agent (Citifluor Ltd London. http://www.citifluor.co.uk/products.htm) or similar.

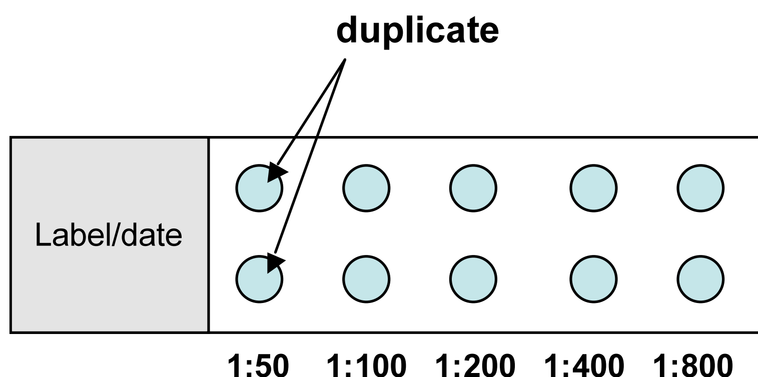
1.10. MICROSCOPE
50x or 100x objective, and incident light of 450-490 nm for FITC-fluorescence, 390-440 nm for DAPI or 515-560 nm for rhodamine.(Leitz Dialux 20 microscope or similar).

2. Methods

2.1 Prepare working dilutions of antibodies in IFA (PBS, pH 7.3) containing 0.01% sodium azide. It is convenient to prepare 5-10 ml of working dilutions at a time; the dilutions can be kept at 4°C for months until used up.

2.2 Remove antigen slides from -20°C freezer and fix immediately in acetone for 3-5 minutes. Do not allow frozen slides to absorb moisture from the air (see SOP for IFA Slide preparation). Label each slide on the frosted surface with a HB pencil. Use a number or code to indicate the antibodies being tested. Use the IFA scoring sheets and your lab notebook to keep records of all testing, including dates, parasite isolate used and dilutions of secondary antibodies used (see IFA scoring sheet for details)

2.3 Using a calibrated P100 or P200 pipette and a **fresh** tip for each antibody, place 25µl of working dilutions of the primary antibody on separate wells and incubate at room temperature for 30 minutes in a humidity chamber to prevent evaporation. Note: Do not completely empty the tip of liquid, as this may scatter microdroplets all around causing contamination of neighbouring wells. Each antibody concentration is tested in duplicate on the same slide, as illustrated below:



2.4 After 30 minutes incubation, remove the primary antibody by aspiration, using a plastic pipette tip attached to a low pressure vacuum pump with liquid trap or similar system; wash the aspiration tip in distilled water or PBS between wells, taking all possible care not to cause cross-contamination between individual wells. If using a dilution series of primary antibodies, start aspiration of wells at the highest dilution, working across the slide to the lowest dilution (i.e. finishing with the highest antibody concentration). Then add 25µl PBS to each well and aspirate off as before. Slides now need to be washed a further 3 times, with 5-minute incubations, in baths of PBS before the addition of the secondary antibody. These washes should be done in borosilicate glass staining jars or 'troughs' designed to hold standard histological slides (e.g Wheaton staining jars, VWR International UK Ltd., Cat No. 27788.366)

2.5 Gently air-dry the slides until the plastic coating around each well is completely dry (the wells can remain a little moist, but the coating must be dry). For the drying process, a warm plate set at about 50°C or a stream of air from an air-conditioner (or hair-drier on a cool setting) may be used.

2.6 Place 15 µl of a working dilution of the secondary antibody on each well (prior to initial use these secondary reagents need to be titrated to determine optimal dilution - usually this is 1:50-100 v/v, depending on the antibody batch). Incubate in a humidity chamber at room temperature for 30 minutes.

2.7 Wash slides 3 times by in PBS for 5 minutes per wash and dry as before.

2.8 Counter stain for 5 minutes in DAPI, 4',6'-diamino-2-phenylindole, Sigma (10mg L^{-1} in PBS). Care should be taken as DAPI is carcinogenic.

2.9 Rinse slides in PBS, drain and mount under coverslip in 2-3 drops of Citifluor (City University, London) or 50% (v/v) glycerol in PBS.

2.10 Read reactions using 50x or 100x objective, and incident light of 450-490 nm for FITC-fluorescence, 390-440 nm for DAPI or 515-560 nm for rhodamine. Mounted slides can be kept for re-examination at 4 °C for up to two weeks, if Citifluor mounting agent with anti-fade reagent is used. During this time the DAPI stain may 'leak' slightly from the parasites but the antigen-specific FITC fluorescence should remain intact and give sharp fluorescence patterns.

3 GUIDELINES FOR READING SLIDES

3.1 The set of samples supplied should be tested a minimum of twice, preferably 3 times, and each assay should be read by 2 microscopists, independently. If any of the results are anomalous they should be repeated until uniformity is obtained.

3.2 Control positive and negative reagent reactions should be read first in each assay and all the results in the assay compared to this standard.

3.3 Positivity of slides should be scored using the following grading system. The end point is defined as the highest dilution scoring a definite positive (+).

+++ very strongly positive fluorescence specific for parasitized red blood cells – this may be schizont-specific (e.g. anti-AMA-1 antibodies), or reactive with all stages, such as anti-MSP-1_{19} antibodies

++ brightly positive fluorescence specific for parasitized red blood cells

+ definite positive fluorescence specific for parasitized red blood cells – distinct from background fluorescence observed on non-parasitised erythrocytes

+/- equivocal fluorescence - most parasites showing little or no fluorescence at all, or not distinct from background fluorescence observed on non-parasitised erythrocytes

- negative, no parasite specific fluorescence (i.e. the same pattern as seen with the negative control). Occasionally the position and outline of the parasites may still be observed, as parasitized cells have different morphology to uninfected erythrocytes, but there is definitely no fluorescence

Any problems or queries? Please email David.Cavanagh@ed.ac.uk or Alison.Creasey@ed.ac.uk

4 EXAMPLES OF POSSIBLE IFA ARTEFACTS:

- False positive reactions due to cross-contamination among different Abs and wells. Do not splash primary antibody solutions during piptting – training in good pipetting technique is essential.
- An area of staining at one side of an otherwise negative well = contamination from a neighbouring well. This occurs most frequently within a slide from a well in the top row onto the well below. For this reason the common practice in routine screening is to place duplicate samples in top and bottom rows as illustrated in the protocol.
- Uniform staining all over the well but usually of lower intensity than expected for a positive reaction of the primary antibody or, a wrong morphological pattern. Usually caused by contamination in the common 'buffer bath' during the washing steps for removal of primary antibody. May also be due to non-specific reactivity or too low dilution of secondary antibodies.
- Precipitates of FITC-conjugates, particularly near the perimeter of the reagent drops where drying out may occur during incubations; make sure that the incubations are done in well-humidified boxes.
- Amorphous 'junk' or debris, usually very bright, non-cellular shapes in the middle of wells; nature unknown – possibly traces of human Ig if the blood was not well washed before preparation of the slides.
- Patches of the coating plastic may be present on some wells - these appear as yellow rather than green fluorescence.

- False negatives due to inactive antibodies. Inactivated (or overdiluted) primary antibodies and/or secondary antibodies. Working dilutions should be pre-tested for specificity on known parasite material.