TITLE: **Immunofluorescent Antibody Assay (IFA)**

SCOPE: IFA test using pre-prepared slides *of Plasmodium falciparum* culture (for preparation of slides from culture or from patient blood see alternative SOPs in same folder

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RECORD OF AMENDMENTS:

VERSION	SECTION	AMENDMENT	CURRENT VERSION
1.0			

SAFETY PRECAUTIONS

Plasmodium falciparum is a Category 3 pathogen (with some derogations). All culturing must be done in a Category 3 facility and the safety Code of Practice adhered to (see separate SOP for Category 3 guidelines).

1. Materials

1.1. PBS (PHOSPHATE BUFFERED SALINE PH 7.3

1.9mM Na_2HPO_4 (VWR International UK Ltd Cat No.301584L)
8.1mM $NaH2PO_4$ (VWR International UK Ltd Cat No.444433M)
150mMNaCl (VWR International UK Ltd Cat No. 27788.366)

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

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

1.3. ACETONE

1.4. FITC- (OR RHODAMINE-) CONJUGATED ANTI-MOUSE (ANTI-RABBIT, ANTI-HUMAN, ANTI-RAT ETC.. AS APPROPRIATE) IMMUNOGLOBULIN

(RECOMMENDED SUPPLIERS FOR SECONDARY ANTIBODIES ARE DAKO OR JACKSON IMMUNORESEARCH**)**

1.5. DAPI STAIN

4',6'-diamino-2-phenylindole, (Sigma Cat No. D8417)
Prepare at a concentration of 1:100,000 w/v in PBS

1.6. EVANS BLUE COUNTERSTAIN

0.1% (w/v) Evans blue in PBS (Fisher Scientific UK Ltd Cat no. 19555-0050)
(suitable only when using FITC-conjugates; do not use for rhodamine-conjugates).
N.B. DAPI stain 1.5 above and Evans Blue can be made up together in the same solution.

1.7. MULTISPOT IFA SLIDES

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

1.8. 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 1% (w/v) bovine serum albumin in phosphate buffered saline (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. (see SOP for IFA Slide preparation).

2.3 Using a P100 or P200 pipette and a **fresh** tip for each antibody, place 25µl of working dilutions of Abs on separate wells and incubate at room temperature for 30 minutes in a wet box to prevent evaporation. Note: Do not completely empty the tip as this may scatter microdroplets all around causing contamination of neighbouring wells. Normally each antibody concentration is tested in duplicate on the same slide. (see separate sheet for IFA recording template.

2.4 After 30 minutes remove the mAbs using a plastic pipette tip attached to a vacuum pump; wash the tip in distilled water or PBS between mAbs, taking all possible care not to cause cross-contamination between individual wells. Add 25µl PBS to each well and aspirate off. Slides now need to be washed 3x with PBS before the addition of the secondary antibody. This may be done as follows:

2.5 For a small number of slides, use a Pasteur pipette or similar to add one drop of PBS to each spot, aspirate off and repeat twice more. For a larger batch of slides, it is possible to wash slides 1x by quick dipping in PBS, and then 2x in fresh PBS for about 5 minutes per wash (this can be done in glass 'troughs' designed to hold standard histological slides).

2.6 Gently air-dry the slides until the plastic coating is completely dry (the wells may remain a little moist). For the drying process, a warm plate set at about 50°C or a stream of air from an air-conditioner (or hair-drier) may be used.

2.7 Place 15 µl of a working dilution of a commercial FITC- (or rhodamine-) conjugated anti-mouse (or appropriate secondary) immunoglobulin on each well (prior to initial use these secondary reagents need to be titrated for optimal dilution - usually 1 in 80-100 v/v). Incubate in a wet box at room temperature for 30 minutes.

2.8 Wash slides 2x by dipping in PBS for about 5 minutes / wash..

2.9 If staining of parasite DNA is desired, counter stain for 5 minutes in DAPI, 4',6'-diamino-2-phenylindole, Sigma (1 part in 100,000, (w/v) in PBS). Care should be taken as DAPI is carcinogenic. Alternatively add DAPI to counter stain (see 2.10 below).

2.10 Counter stain slides for 5 minutes in 0.1% (w/v) Evans blue in PBS (suitable only when using FITC-conjugates; do not use for rhodamine-conjugates).

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

2.12 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. During this time the DAPI stain may 'leak' slightly from the parasites but the fluorescence should remain intact and give sharp fluorescence.

3.0 EXAMPLES OF POSSIBLE IFA ARTEFACTS:

False positive reactions due to cross-contamination among different mAbs and wells.
An area of staining at one side of an otherwise negative well = contamination from the 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.
Uniform staining all over the well but usually of lower intensity than expected for a positive reaction of the mAb or, sometimes, of a wrong morphological pattern. Usually caused by contamination in the common 'buffer bath' during the washing steps for removal of mAbs.

False positive reactions due to transfer of flakes of fixed blood among different wells or even slides.
Irregularly sized patches of brightly stained parasites on an otherwise negative well. This can occur if slides had been made with blood suspensions more concentrated than 3-5% haematocrit.
Similar artefacts can be caused by microdroplets of other mAbs scattered over the slide due to careless pipetting.

Fluorescence of cells other than parasites.
Human lymphocytes can be stained with anti-mouse Ig due to cross-reactivity of most reagents with human Ig. Also, some types of bacteria and yeast react with Ig and can be stained if a culture or reagents become contaminated. All such artefacts can be recognised easily because of differences in size/shape of malaria parasites and the other cells.

Other false positives.
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', usually very bright, in the middle of wells; nature unknown - possibly traces of human Ig if blood 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 antibodies.
Inactivated (or overdiluted) monoclonals and/or second antibody. Working dilutions should be pre-tested for specificity on known parasite material. It is advisable to use in every test a positive standard; perhaps most convenient "standard" for this would be a batch of slides prepared with a mixture of several genetically different "strains" of *P.falciparum*.

False negatives due to parasite material.
If schizonts are absent or very immature, many typing mAbs will fail to react since most are specific for proteins expressed only after 36 hrs or later in the asexual cycle. Use the mAb 9.8 (anti-MSP1) to assess the quality and number of schizonts present in the tested preparation.

Parasites may not be accessible to antibody reagents due to poor acetone-fixation. This problem may arise as a result of repeated wetting/drying during preparation or storage of slides. Once prepared, **keep slides desiccated** at all times until used in the IFA tests. If the problem occurs, it may be overcome by prolonging acetone-fixation to 10 minutes.

References:

CONWAY D.J GREENWOOD B.M. & McBRIDE J.S. (1991) The epidemiology of multiple-clone Plasmodium falciparum infections in Gambian patients. Parasitology, 103: 1-6.

CONWAY D.J and McBRIDE J.S. (1991) Population genetics of Plasmodium falciparum within a malaria hyperendemic area. Parasitology, 103: 7-16.