IFAT/T.b.rhodesiense (*)
Serodiagnosis of human African Trypanosomiasis (sleeping sickness) due to T.b.rhodesiense

I. PRINCIPLE

Infection with Trypanosoma brucei rhodesiense results in production of circulating antibodies against several surface antigens of the parasite. Such antibodies can be demonstrated in the blood, plasma or serum of the infected host by indirect immunofluorescence. The IFAT-antigens are freeze dried suspensions of purified, fixed bloodstream form trypanosomes expressing the predominant variable antigen types Etat 1.1 and Etat 1.18 of Trypanosoma brucei rhodesiense. In order to obtain maximal sensitivity, each sample is tested separately with Etat 1.1 and Etat 1.18.

(*) IFAT = Indirect Fluorescent Antibody Test

II. REAGENTS

1. IFAT ANTIGENS [2.5 ml / vial]
   - Freeze dried suspensions of purified, formaldehyde fixed trypanosomes (VAT's Etat 1.1 and Etat 1.18).
   - Preservative: sodium azide (0.1 %).
   - Storage: Refrigerator (+2°C / +8°C) or freezer (-20°C).

2. IFAT BUFFER [Phosphate buffered saline (pH 7.2)]
   Composition: - NaCl ............................................. 7.20 g
   - Na2HPO4.2H2O .......................... 1.86 g
   - KH2PO4 ----------------------------- 0.43 g
   - Na2S (sodium azide) ............... 0.20 g
   - Distilled water up to ............ 1000 ml
   - Use for reconstitution of the IFAT-antigen, the Positive and Negative controls, preparation of sample dilutions and washing of the test slides.
   - Storage: Refrigerator (+2°C / +8°C).

3. POSITIVE CONTROL [0.2 ml / vial]
   - Freeze dried undiluted human positive reference serum.
   - Storage: Refrigerator (+2°C / +8°C) or freezer (-20°C).

4. NEGATIVE CONTROL [0.2 ml / vial]
   - Freeze dried undiluted human negative reference serum.
   - Storage: Refrigerator (+2°C / +8°C) or freezer (-20°C).

5. EVANS BLUE [Stock solution 1:1000]
   Composition: 50 mg of Evans Blue + 50 ml of IFAT-buffer.
   - Storage: Refrigerator (+2°C / +8°C) – Maximum 4 weeks!
   - Use Multi-spot slides with 10 reaction areas (diameter 6 mm).
   - Wipe the slides with a tissue soaked in alcohol.
   - Using a micropipette, dispense 10 microliter of antigen suspension Etat 1.1 onto each reaction area and spread out using the pipette tip.
   - Dry the antigen preparations for 1 hour by leaving them on the bench or in the cold airstream of a fan.
   - Storage of antigen-coated slides: Wrap the slides in absorbent paper and pack them individually or in small series in hermetically sealed plastic bags containing Silica gel.

Note: In a negative test, this counter stain gives the trypanosomes a red fluorescence which contrasts with the green fluorescence of the positive test.

6. CONJUGATE
   - Use a monospecific anti-human IgG (λ-chain specific) fluorescein conjugated antisum to order to obtain maximal specificity.
   - Storage: Refrigerator (+2°C / +8°C).
   - Prepare the working dilution (** in 1 volume Evans Blue (1:1000) + 9 volumes IFAT-buffer.

(**) Prior to use, each batch of conjugate should be titrated in order to determine the optimal working dilution. This is done by testing serial twofold dilutions of the Positive and the Negative Controls (1:50, 1:100, 1:200, 1:400, 1:800) in combination with Serial twofold dilutions of the conjugate (e.g. 1:25, 1:50, 1:100, 1:200).

7. BUFFERED GLYCEROL
   Composition: 1 volume of Glycerol + 1 volume of IFAT-buffer.
   - Storage: refrigerator (+2°C / +8°C) – Maximum 2 weeks.

III. TEST SAMPLES

The test can be performed blood impregnated filter papers or on serum/plasma.

1. BLOOD SAMPLES ON FILTER PAPER
   - Blood obtained by finger prick is collected on filter paper Whatman N° 4.
   - The samples are dried in the shadow for 1 hour. Exposure to sunlight causes denaturation!
   - Filter papers are packed in small series in hermetically sealed plastic bags containing Silica gel and stored in the freezer at ≤ -20°C.

2. SERUM- or PLASMA SAMPLES
   - Serum or plasma is obtained by centrifugation of respectively coagulated or heparinized blood.
   - Small volumes of plasma can be obtained by finger prick and collection of blood in heparinized capillary tubes. The plasma is separated by centrifugation.
   - The samples are stored in a freezer at ≤ 20°C.

IV. PREPARATION OF REAGENTS and TEST SAMPLES

1. Reconstitution of the IFAT antigens
   - Add 2.5 ml of IFAT-buffer to a vial of Etat 1.1-antigen and 2.5 ml of IFAT-buffer to a vial of Etat 1.18-antigen.
   - Resuspend the organisms by shaking the vial or by repeated aspiration into a Pasteur pipette.

2. Coating of the slides [Etat 1.1 and Etat 1.18 separately]
   - Use Multi-spot slides with 10 reaction areas (diameter 6 mm).
   - Dry the antigen preparations for 1 hour by leaving them on the bench or in the cold airstream of a fan.
   - Storage of antigen-coated slides: Wrap the slides in absorbent paper and pack them individually or in small series in hermetically sealed plastic bags containing Silica gel.
   - Store the bags in a freezer at ≤ -20°C until use.
   - In order to avoid condensation on the antigen spots, the frozen bags should be warmed up to ambient temperature before opening them.
   - Once unfrozen, the preparations should be used on the same day. They should not be frozen again!

3. Reconstitution of the controls
   - Add 0.2 ml of IFAT-Buffer to a vial of the Positive and the Negative Control and mix.

4. Preparation of test samples

4.1 Blood samples on filter paper
   In order to avoid condensation on the samples, the frozen bags should be warmed up to ambient temperature before opening them.

4.1.1 Qualitative test (Screening)
   - Disks of dried blood with a diameter of 6 mm are punched out of the filter papers.
   - Each disk is placed in a plastic tube or ‘multi-well’ tray and 0.1 ml of IFAT-Buffer is added.
   - Blood proteins are eluted for 1 hour at ambient temperature.
   - The tubes or plates are shaken from time to time.

4.1.2 Quantitative test (Titration)
   - Prepare serial twofold dilutions (1:2, 1:4, 1:8, 1:16 and 1:32) of the eluate(s) in IFAT-Buffer.
4.2 Serum or Plasma samples
Frozen samples are thawed and well-mixed before use.

4.2.1 Qualitative test (Screening)
- Prepare a 1:50 dilution by pipetting 10 microliters of serum/plasma into a tube or a well of a 'multi-well' tray containing 0.5 ml of IFAT-buffer.
- Mix the contents of the tube or shake the tray.

4.2.2 Quantitative test (Titration)
- Prepare serial twofold dilutions (1:50, 1:100, 1:200, 1:400 and 1:800) of the serum/plasma in IFAT-Buffer.

V. EXECUTION of THE TEST
Each sample is tested separately with Etat 1.1 and with Etat 1.18. All manipulations are done at ambient temperature. The different reagents are successively applied, shaken off after incubation and eliminated by washing.

- Rehydrate the antigen preparations for 15 minutes by dropping 50 microliters of IFAT-buffer onto each reaction area.
- Shake off the buffer and dry the slide(s) in-between the reaction areas with absorbent paper.
- Put 50 microliters of the test sample dilution(s) onto each of the reaction areas and let react for 30 minutes.
- Shake off the samples.
- Wash the slide(s) in 2 successive baths of IFAT-buffer [5 minutes each].
- Shake off the buffer and dry the slide(s) in-between the reaction areas with absorbent paper.
- Put 50 microliters of conjugate solution onto each of the reaction areas and let react for 30 minutes.
- Shake off the conjugate.
- Wash the slide(s) in 2 successive baths of IFAT-buffer [5 minutes each].
- Shake off the buffer and dry the slide(s) in-between the reaction areas with absorbent paper.
- Put a few drops of buffered glycerol on the slide(s) and cover the reaction areas with a cover slip.
- Remove excess liquid from below the cover slip with absorbent paper.
- Put the slide(s) on an underlay of absorbent paper until reading.
- The preparations can be stored in the refrigerator (2 days) or in the freezer (2 weeks).

VI. READING
- Prior to reading, the slide(s) are warmed up to ambient temperature to remove condensation.
- The preparations are read under the fluorescence microscope (magnitude 10 x 40).
- In a positive reaction, green fluorescence is observed at the entire periphery of the cell (= variable surface antigen coat). Fluorescence of the flagellum, the flagellar pocket or the kinetoplast should be considered aspecific.
- Depending on the intensity of the fluorescence results are scored as follows:

++  = STRONGLY POSITIVE (brilliant green fluorescence)
+   = POSITIVE (green fluorescence)
±  = WEAKLY POSITIVE (faint green fluorescence)
−  = NEGATIVE (red – absence of green fluorescence)

VII. INTERPRETATION
A positive reaction obtained with either Etat 1.1 and/or Etat 1.18 should be interpreted as significant.

Sensitivity
It is assumed that the test becomes positive within the first month of infection. After chemotherapy it may remain positive for several months (residual antibodies). At a sample dilution of 1:50, over 95% of untreated sleeping sickness patients show a positive result.