

ITMA-DAT/VL

Direct Agglutination Test for Visceral Leishmaniasis

with FREEZE DRIED ANTIGEN on SERUM

I. PRINCIPLE

Infection with *L.donovani* or *L.infantum*, agents of visceral leishmaniasis, results in production of circulating antibodies against surface antigens of the parasite. Even before the onset of clinical symptoms, antibodies can be demonstrated in the blood or serum of the infected host by direct agglutination.

The **DAT/VL antigen** is a freeze dried suspension of trypsin-treated, fixed and stained culture form promastigotes of *L.donovani*.

The **test** is performed in V-shaped multi-well micro-plates, by mixing 50 µl of antigen with 50 µl of diluted blood (filter paper eluate) or serum. The plate is incubated overnight at ambient temperature. In the presence of antibodies to leishmania, the DAT/VL antigen is agglutinated and forms a pale blue film over the bottom of the well. In the absence of antibodies, the cells settle on the bottom of the V-well as a small dark blue dot.

The **DAT/VL** is intended for use at all levels of health services, from first-line screening of rural communities at risk to local reference laboratories for epidemiological studies including antibody titration.

II REAGENTS

1. DAT-ANTIGEN (2.5 ml / vial)

- Freeze dried suspension of purified, trypsin-treated, fixed and stained promastigotes of *L. donovani* strain 1-S.
- Preservative: sodium azide (0.1%)
- Storage:** refrigerator (+2°C / +8°C) or freezer (-20°C).

2. DAT-BUFFER (≥30 ml/vial)

- Phosphate Buffered Saline (PBS – pH 7.2) supplemented with protein
- Use for reconstitution of DAT-antigen, positive and negative controls
- Use to prepare the diluent
- Preservative: sodium azide (0.1%)
- Storage:** refrigerator (+2°C / +8°C) or freezer (-20°C)!

3. POSITIVE CONTROL (0.6 ml/vial)

- Freeze dried serum diluted in PBS
- Preservative: sodium azide (0.1%)
- Storage:** refrigerator (+2°C / +8°C) or freezer (-20°C).

4. NEGATIVE CONTROL (0.6 ml/vial)

- Freeze dried serum diluted in PBS
- Preservative: sodium azide (0.1%)
- Storage:** refrigerator (+2°C / +8°C) or freezer (-20°C)

6. 2-MERCAPTO-ETHANOL (1.0 ml/vial) [TOXIC!!!]

- Commercial 2-ME (Merck Ref.805740)
- Use to prepare DAT/VL-Diluent
- Storage:** refrigerator (+2°C / + 8°C)

III. PREPARATIONS PRIOR TO TESTING

1. Reconstitution of the Antigen

- Using the 2.5ml-syringe, add 2.5 ml of buffer to a vial of freeze dried antigen.
- Immediately gently shake the vial for a few seconds so as to obtain a homogeneous suspension.
- The antigen is ready for use.

Notes: 1. Before each use, gently shake the vial for a few seconds.
2. Keep the antigen out of the sun and dust.

2. Reconstitution of the controls

- Using the 2.5ml-syringe, add 0.6 ml of buffer to the vial with the positive control and to the vial with the negative control.

3. Preparation of diluent (+ 2-ME)

- 2-ME is a volatile substance. Prepare diluent just before use and only prepare the volume needed.
- Prepare a 0.8vol% solution.
- F.i. Using the 1.0 ml-syringe, add ±0.08 ml of 2-ME to 10 ml of buffer.
- Use this solution to prepare the sample dilutions (IV.1, IV.2)

IV. DAT ON SERUM SAMPLES

1. Procedure for screening (dilutions 1:200 to 1:3200)

- Prepare the micro-plates according to the example below (End-dilutions are given between brackets)

	Age-control	1:100 (1:200)	1:200 (1:400)	1:400 (1:800)	1:800 (1:1,600)	1:1,600 (1:3,200)	Age-control	1:100 (1:200)	1:200 (1:400)	1:400 (1:800)	1:800 (1:1,600)	1:1,600 (1:3,200)
A	○	○	○	○	○	○	○	○	○	○	○	○
B	○	○	○	○	○	○	○	○	○	○	○	○
C	○	○	○	○	○	○	○	○	○	○	○	○
D	○	○	○	○	○	○	○	○	○	○	○	○
E	○	○	○	○	○	○	○	○	○	○	○	○
F	○	○	○	○	○	○	○	○	○	○	○	○
G	○	○	○	○	○	○	○	○	○	○	○	○
H	○	○	○	○	○	○	○	○	○	○	○	○

Notes:

- On plates containing control sera, **row A 1-6** is reserved for the **positive control** and **row B 1-6** for the **negative control**. In this case, **DO NOT FILL wells A2 and B2**
- When several plates are to be prepared, every fifth plate should include control sera
- Dispense 50 µl of **diluent** (with 2-ME!) into every well of the vertical rows 1,-,3,4,5,6 but **NOT in row 2**. and into every well of the vertical rows 7,-,9,10,11,12 but **NOT in row 8**
- Dispense 100 µl of **diluent** into every well of the vertical rows 2 and 8
- Controls:** Dispense 100 µl of the "**positive control**" in well **A2** and 100µl of the "**negative control**" in **B2**.
- Add 1µl per serum sample into every well of row 2 and row 8
- Sample dilutions:** Using a multi-channel pipette, transfer 50 µl from Row 2 to Row 3, mix 3 times, transfer 50 µl to Row 4, mix 3 times, continue until row 6, take off 50 µl and discard the tips. Repeat for rows 8 to 12
- Antigen:** Mix and re-suspend the DAT-antigen by gently shaking the vial. Using a dispenser or a multi-channel pipette, add 50 µl antigen to every well. Avoid touching the liquid in the wells!
- Seal** the plate with an adhesive plate sealer.
- Gently **shake** the plate by quickly "rubbing" it back and forth over the tabletop. Avoid moistening the underside of the plate sealer.
- Incubate** overnight at ambient temperature in a horizontal position and do not touch the plate.
- Read the results:** see section V

2. Procedure for full-scale titration (1:200 to 1:204800)

- Prepare the micro-plates according to the example below. (End-dilutions are given between brackets)


	Ag-control	1:100 (1:200)	1:200 (1:400)	1:400 (1:800)	1:800 (1:1.600)	1:1.600 (1:3.200)	1:3.200 (1:6.400)	1:6.400 (1:12.800)	1:12.800 (1:25.600)	1:25.600 (1:51.200)	1:51.200 (1:102.400)	1:102.400 (1:204.800)
_____	A	○	○	○	○	○	○	○	○	○	○	○
_____	B	○	○	○	○	○	○	○	○	○	○	○
_____	C	○	○	○	○	○	○	○	○	○	○	○
_____	D	○	○	○	○	○	○	○	○	○	○	○
_____	E	○	○	○	○	○	○	○	○	○	○	○
_____	F	○	○	○	○	○	○	○	○	○	○	○
_____	G	○	○	○	○	○	○	○	○	○	○	○
_____	H	○	○	○	○	○	○	○	○	○	○	○

Notes:

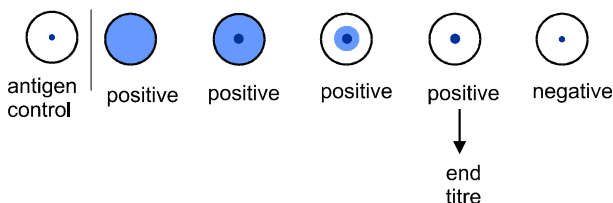
- On plates containing control sera, **row A** is reserved for the **positive control** and **row B** for the **negative control**. In this case, **DO NOT FILL wells A2 and B2**
- When several plates are to be prepared, every fifth plate should include control sera.
- Dispense 50 µl of **diluent** (with 2-ME !) into every well of the vertical rows 1, and 3 to 12 **but NOT in row 2**
- Dispense 100 µl of **diluent** (with 2-ME) into every well of the vertical row 2
- Controls:** Dispense 100 µl of the "positive control" in well **A2** and 100 µl of the "negative control" in **B2**.
- Add 1 µl per serum sample into every well of row 2
- Sample dilutions:** Using a multi-channel pipette transfer 50 µl from Row 2 to Row 3, mix 3 times, transfer 50 µl to Row 4, continue until row 12, take 50 µl off and discard the tips.
- Antigen:** Mix and re-suspend the antigen by gently shaking the vial. Using a dispenser or a multi-channel pipette, add 50 µl antigen into every well. Avoid touching the liquid in the wells!
- Seal** the plate with an adhesive plate sealer.
- Gently **shake** the plate by quickly "rubbing" it back and forth over the tabletop. Avoid moistening the underside of the plate sealer.
- Incubate** overnight at ambient temperature in a horizontal position and do not touch the plate.
- Read the results:** see section V

V. READING and INTERPRETATION

The DAT/VL test is best read by placing the plate onto a white background. Two independent readers should read the test.

NEGATIVE :  dark blue dot, of size **identical** to the size of the antigen control dot.

POSITIVE : from film to dot > antigen control dot



Note: Recommended Cut-off = 1.3200 (1:6400)
Borderline = 1:400 (1:800) till 1:1600 (1:3200)
 A difference in end-titre of one dilution between two separate titrations of the same sample is inherent to the test.

VI. STABILITY, STORAGE and EXPIRY DATE

1. Stability

- The freeze dried reagents (antigen, positive and negative controls) and the buffer remain stable for at least 6 months when stored in a refrigerator between +2°C and +8°C. At higher temperatures, i.e. + 45°C, the freeze dried reagents retain their activity for 1 week.
- After reconstitution, the reagents can be used during 1 week when stored between +2°C and +8°C, or up to 8 hours at 37°C.

Notes:

- These values are only an indication on the stability of the reagents but are by no means recommendations for prolonged storage!
- Do NOT freeze the reconstituted antigen suspension

2. Recommendations for storage and shipment

- Freeze dried reagents (antigen, controls) : in the refrigerator (+2°C to +8°C) or in the freezer (-20°C).
- DAT-Buffer in the refrigerator (+2°C to +8°C) or in the freezer (-20°C).
- During transport, storage and handling: avoid exposure to heat and direct sunlight.
- It is recommended to dispatch the reagents from a central storage centre to the field under refrigerated conditions (cold chain).

3. Shelf life / Expiry date (remain to be determined!)

When stored under the prescribed storage conditions, all the reagents will retain their activity until the expiry date mentioned on the "Reagent" boxes and on the packing list.

VII. PRESENTATION

1. KIT REAGENTS

Contents: - 10 vials DAT-Antigen
 - 1 vial Positive Control
 - 1 vial Negative Control
 - 2 vials DAT-Buffer
 - 1 instructions leaflet
 - 1 vial DAT-Diluent
 - 1 vial 2ME-solution

2. KIT ACCESSORIES

Contents: - 5 micro-plates + plate sealers
 - 2 syringes: (2.5 ml and 1.0 ml)

3. OTHER REQUIREMENTS (not included in the kit)

- Materials for collection of blood and preparation/storage of serum samples
- Dispensing and pipetting devices [single channel or multi-channel pipettes with adjustable volumes]