| FIEBRE Standard Operating Procedure | | | |
|---|---|---|---|
| **Title** | **Performing the Direct Agglutination Test (DAT) for Leishmania** | | |
| ***SOP Reference*** | | ***Version*** | ***Date of effect*** |
| | | 1.1.1 | |

**SOP Development**

| | **Name** | **Title** | **Signature** | **Date** |
|---|---|---|---|---|
| **Author** | Victoria Gould | LSHTM Laboratory Co-ordinator | Victoria Gould | 02/03/21 |
| **Reviewer** | Chrissy h Roberts | LSHTM FIEBRE laboratory expert |  | 02/03/21 |
| **Approver** | Heidi Hopkins | FIEBRE scientific program coordinator | Signed by email | 17/02/21 |

**Review Tracker**

| **Due date for next review** | **Reviewer name** | **Signature** | **Date reviewed** |
|---|---|---|---|
| | | | |
| | | | |
| | | | |
| | | | |

**Revision History**

| **Version No.** | **Effective date** | **Reason for change** |
|---|---|---|
| | | |
| | | |
| | | |
| | | |
| | | |

**SOP User Confirmation**

I acknowledge that I have read, understood and agree to follow this SOP

| # | Name (print) | Signature | Date |
|---|---|---|---|
| 1 | | | |
| 2 | | | |
| 3 | | | |
| 4 | | | |
| 5 | | | |
| 6 | | | |
| 7 | | | |
| 8 | | | |
| 9 | | | |
| 10 | | | |

1. **Title: Performance of the Direct Agglutination Test (DAT) for Leishmania**
2. **Purpose:** To describe the procedure for carrying out the DAT on serum samples from the FIEBRE study. This SOP describes procedures for selection, dilution and assaying of serum samples for agglutination with the DAT assay to determine positivity for Leishmaniasis infection.
3. **Responsible staff:** FIEBRE laboratory staff at LSHTM
4. **Background & Rationale:** The first primary objective of the FIEBRE study is to determine the treatable and/or preventable causes of fever in our study population. One of the potential causes in the study areas are Leishmania species. The DAT assay is well established and works on the principle that where antibodies are present agglutination with Leishmania antigen occurs providing a visible readout.
5. **Supplies and Materials**
    - Serum sample aliquot allocated for Leishmania testing
    - Freeze dried, Coomassie blue stained, Leishmania antigen
    - V bottom microtitre plates
    - Saline
    - Urea saline
    - Beta Mercaptoethanol
    - Reagent reservoirs
    - Pipettes (including Multichannel)
    - Suitable pipette tips

- Biohazard disposal system
- Lab surface disinfectant (e.g. Virkon)
- Sterile filtration units
- Heat block

**6. Procedures:**

***6.1*** Sterilisation of solutions

*6.1.1* Make saline to 1x solution (if required).

*6.1.2* Filter through a unit containing 0.2µM pore.

***6.2 Reconstitution of Leishmania antigen***

6.2.1. Reconstitute the freeze-dried antigen in 5mL of sterile saline and rotate (**DO NOT SHAKE**) to ensure a homogenous solution

***6.3 Selection of samples***

*6.3.1* Carry out initial testing on day 28 samples where there is sufficient sample to allocate for Leishmania testing. If positive, or where no sample is available, test the day 0 sample.

*6.3.2* Remove the test samples from storage at -80$^0$C and thaw prior to testing. Test the samples in the Cryptococcal antigen test, if required, then heat inactivate the sample at 56°C. Place samples in the fridge after use to facilitate the titration of any positive samples as soon as possible without any additional freeze thaw cycles.

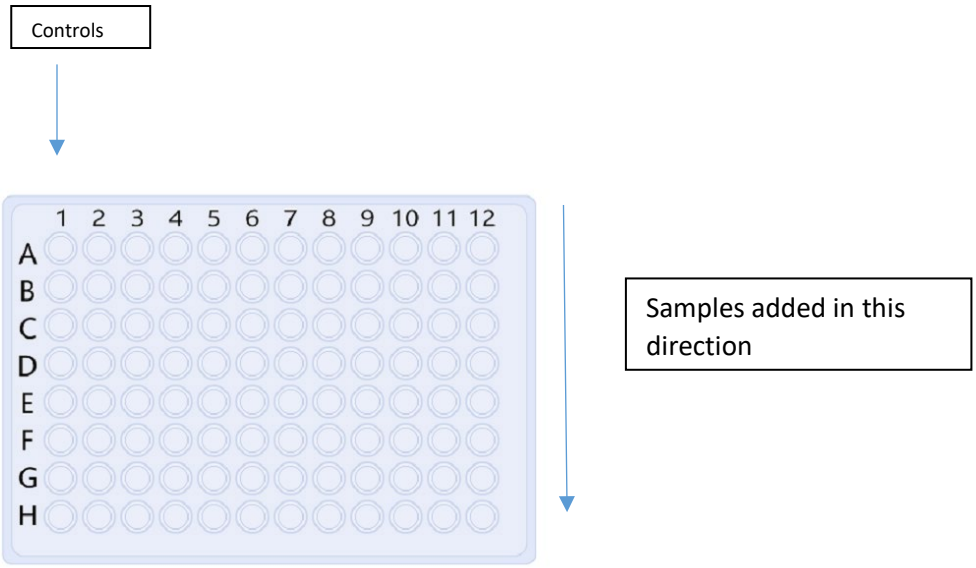
***6.4 Dilution of samples***

*6.4.1* Mix a suitable quantity of urea saline with Beta Mercaptoethanol (70 µL of 2-ME per 10 mL of saline) and pour into a reagent reservoir for use as sample diluent.

*6.4.2* Initially a single sample dilution will be screened. Carry out a Pre-dilution step by adding 318µL of diluent to all wells of a dilution plate using a multichannel pipette and suitable tips.

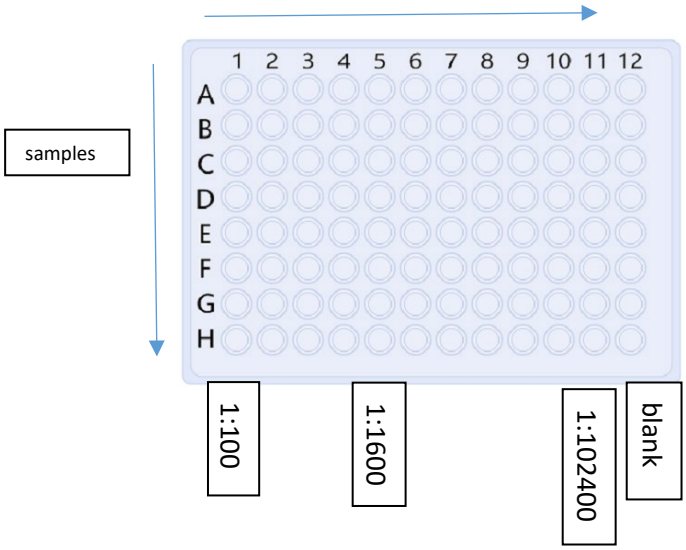
*6.4.3* Add 2 µl of sample to wells in columns 2-12 and mix. Add positive controls to each plate in appropriate wells in column 1, along with a negative control. Record the ID of samples added to each well in the test template (Appendix A). *See plate map.*

*6.4.4* Add 45µl of sample diluent to the test plate. Transfer 5 µl from the dilution plate to corresponding wells in the test plate and mix. This plate now contains the screening dilution of 1:1600.




*6.4.5* For positive samples at the screening dilution carry out a sample titration by adding 98µL of diluent into wells A1 to H1 and 50µL into the remaining wells. Add 2µl of sample to wells in column 1 along with controls.

*6.4.6* Remove 50µL from column 1 and serially dilute through columns 2-11. Discard the extra 50µl from column 11. Column 12 is left blank. *See plate map*



## *7 Addition of Antigen*

*7.1.1* Add 50µL of antigen to all wells. Start with the blank wells to minimise the risk of cross contamination.

*7.1.2* Incubate the plate for a minimum of 18 hours at room temperature, undisturbed, for any antibody/antigen reaction to occur

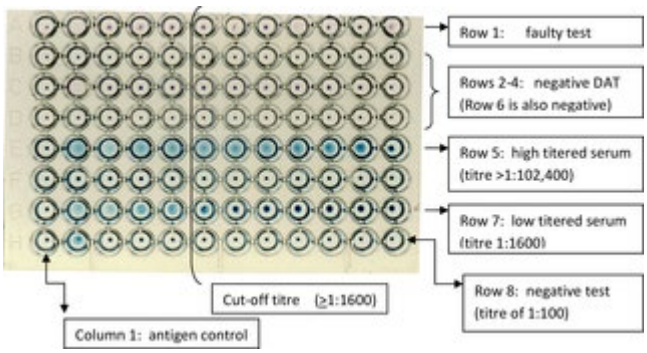
## *8* Reading of results

*8.1.1* After the incubation period examine the plate for signs of agglutination, where there will be a uniform blue colour across the well in comparison to a negative well where the unreacted promastigotes fall to produce a blue button in the point of the well.

*8.1.2* Where titrations are being done the titre is the last dilution that shows a difference in agglutination compared to the blank or negative control.

*8.1.3* There is a cut off of 1:1600. Report titres of 1:100 to 1:800 as negative. Report titres of 1:1600 or above as positive.

*8.1.4* Example plate (NB antigen control is column 1 not 12 in this example)




## *9* Reporting of results

*9.1.1* Enter the results on to the DAT test template

*9.1.2* Report the results using the appropriate form (e.g on REDCap)

## *10* Appendix

**Leishmania DAT test template**

**Date** _____ **Set Up By** _____ **Ag Lot No.** _____

**Urea Saline Lot No.** _____ **Positive Control** _____ **Negative Control** _____

**Read By/Date** _____ **Check by/Date** _____

| Plate No | 1:100 | 1:200 | 1:400 | 1:800 | 1:1600 | 1:3200 | 1:6400 | 1:12800 | 1:2560 | 1:512000 | 1:102400 | 0 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Positive Control | | | | | | | | | | | | |
| Negative Control | | | | | | | | | | | | |
| Sample 1 | | | | | | | | | | | | |
| Sample 2 | | | | | | | | | | | | |
| Sample 3 | | | | | | | | | | | | |
| Sample 4 | | | | | | | | | | | | |
| Sample 5 | | | | | | | | | | | | |
| Sample 6 | | | | | | | | | | | | |

| Plate No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | +ve Ctrl | 1 | 9 | 17 | 25 | 33 | 41 | 49 | 57 | 65 | 73 | 81 |
| B | +ve Ctrl | 2 | 10 | 18 | 26 | 34 | 42 | 50 | 58 | 66 | 74 | 82 |
| C | Low +ve | 3 | 11 | 19 | 27 | 35 | 43 | 51 | 59 | 67 | 75 | 83 |
| D | Low +ve | 4 | 12 | 20 | 28 | 36 | 44 | 52 | 60 | 68 | 76 | 84 |
| E | -ve Ctrl | 5 | 13 | 21 | 29 | 37 | 45 | 53 | 61 | 69 | 77 | 85 |
| F | -ve Ctrl | 6 | 14 | 22 | 30 | 38 | 46 | 54 | 62 | 70 | 78 | 86 |
| G | blank | 7 | 15 | 23 | 31 | 39 | 47 | 55 | 63 | 71 | 79 | 87 |
| H | blank | 8 | 16 | 24 | 32 | 40 | 48 | 56 | 64 | 72 | 80 | 88 |

| Sample | ID | Result | Comment |
|---|---|---|---|
| 1 | | | |
| 2 | | | |
| 3 | | | |
| 4 | | | |
| 5 | | | |
| 6 | | | |
| 7 | | | |
| 8 | | | |
| 9 | | | |
| 10 | | | |
| 11 | | | |
| 12 | | | |
| 13 | | | |
| 14 | | | |
| 15 | | | |
| 16 | | | |
| 17 | | | |
| 18 | | | |
| 19 | | | |
| 20 | | | |
| 21 | | | |
| 22 | | | |
| 23 | | | |
| 24 | | | |
| 25 | | | |
| 26 | | | |
| 27 | | | |
| 28 | | | |
| 29 | | | |
| 30 | | | |
| 31 | | | |
| 32 | | | |
| 33 | | | |
| 34 | | | |
| 35 | | | |
| 36 | | | |
| 37 | | | |
| 38 | | | |
| 39 | | | |
| 40 | | | |
| 41 | | | |
| 42 | | | |

| | | | |
|---|---|---|---|
| 43 | | | |
| 44 | | | |
| 45 | | | |
| 46 | | | |
| 47 | | | |
| 48 | | | |
| 49 | | | |
| 50 | | | |
| 51 | | | |
| 52 | | | |
| 53 | | | |
| 54 | | | |
| 55 | | | |
| 56 | | | |
| 57 | | | |
| 58 | | | |
| 59 | | | |
| 60 | | | |
| 61 | | | |
| 62 | | | |
| 63 | | | |
| 64 | | | |
| 65 | | | |
| 66 | | | |
| 67 | | | |
| 68 | | | |
| 69 | | | |
| 70 | | | |
| 71 | | | |
| 72 | | | |
| 73 | | | |
| 74 | | | |
| 75 | | | |
| 76 | | | |
| 77 | | | |
| 78 | | | |
| 79 | | | |
| 80 | | | |
| 81 | | | |
| 82 | | | |
| 83 | | | |
| 84 | | | |
| 85 | | | |
| 86 | | | |
| 87 | | | |
| 88 | | | |