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# 🌐 Wet-lab identification of Leishmania donovani antigens detected by human IgG1

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Bruno Hinckel<sup>1</sup>, Tegwen Marlais<sup>2</sup>, Stephanie Airs<sup>2</sup>

<sup>1</sup>University of Antwerp, Coris BioConcept; <sup>2</sup>London School of Hygiene & Tropical Medicine, University of London



Bruno Hinckel

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**Protocol status:** Working

We use this protocol in our group and it is working. Small changes (wording) and details might follow. Protocol was already applied multiple times

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## Abstract

This protocol describes a wet-lab method to identify Leishmania proteins bands recognized by human IgG1. The cytosolic proteins obtained by cell lysis are initially separated by tricine gel electrophoresis using a long (14 cm) apparatus (SG-200, CBS Scientific, USA). Upon completion of the gel electrophoresis, the proteins are transferred onto a nitrocellulose (NC) membrane (10600046, GE Healthcare Life Sciences, UK) using a semi-dry electroblotting transfer system (1703940, Bio-Rad, UK). The NC membrane is sliced into several thin (4mm) strips and each of the strip is immunoassayed with individual sera from Indian patients with different clinical status of visceral leishmaniasis - VL (active VL, relapse, endemic healthy controls and non-endemic healthy controls) using an HRP-conjugated anti-human IgG1 secondary antibody (ab99774, Abcam, Cambridge, UK)

## Materials

Tris, tricine, SDS, HCl, acrylamide, bisacrylamide, ammonium persulphate, TEMED, dH<sub>2</sub>O, glycerol, Coomassie blue G-250, PBS, acetic acid, non-fat milk powder, Tween 20, mouse anti-human IgG1-HRP monoclonal antibody, SigmaFast DAB tablets

## Safety warnings

- ⚠ Acrylamide and bisacrylamide are highly neurotoxic. When handling these chemicals, wear gloves, use a pipetting aid and always work under the hood

## Protein separation by tricine SDS-PAGE

- 1 Prepare 10x solutions of cathode buffer (Tris 1M, Tricine 1M, SDS 1%, pH ~8,25) and anode buffer (Tris 1M, HCl 0.225M, pH 8.9)
- 2 Prepare an acrylamide-bisacrylamide (AB-3) stock solution by dissolving 48 g of acrylamide and 1.5g of bisacrylamide (each twice-crystallized; Serva) in 100 ml of water
- 3 Prepare a 3x gel buffer solution (Tris 3M, HCl 1M, SDS 0,3%, pH 8,45)
- 4 To prepare 20ml of a 10% resolving gel solution, mix 4ml of AB-3, 6.66ml of 3x gel buffer solution and 9.33ml of dH<sub>2</sub>O.
- 5 Add the polymerising agents: 100 µl of 10% amonium persulphate (APS) and 10 µl of TEMED
- 6 Pour the 10% resolving gel solution into the gel apparatus with a pipette, leaving a space of about the height of the comb that will be used + 1,5cm (where the 4% stacking gel will be later casted)
- 7 Store the gel overnight with a dH<sub>2</sub>O overlay at 4°C. Cover the top of the gel with a plastic film to minimise evaporation
- 8 On the following day, carefully pour off the dH<sub>2</sub>O on the top of the (now solidified resolving gel) and prepare about 4 ml of a 4% stacking gel solution by mixing 0.33ml of AB-3, 1ml of gel buffer 3x and 2,67ml of dH<sub>2</sub>O
- 9 Add the polymerising agents: 67.5 µl of 10% APS solutions and 6.75 µl of TEMED
- 10 Pour the 4% stacking gel solution into the gel apparatus with a pippete (onto the top of the resolving gel, now solidified)
- 11 Directly insert the comb (avoid the formation of air bubbles underneath the comb)
- 12 Store the gel at 4°C until complete solidification of the stacking gel

- 13 Carefully and slowly remove the comb in order to avoid damaging to the stacking gel
- 14 Carefully rinse the wells multiple times with dH<sub>2</sub>O.
- 15 Prepare the samples to be loaded into the wells by mixing 3 parts of *L. donovani* lysate with 1 part of non-reducing sample buffer (2% SDS (wt/vol), 30% glycerol (wt/vol), 0.05% Coomassie blue G-250 (Serva), 150 mM Tris/HCl (pH 7.0). The final *L. donovani* concentration should be 4 µg/mm lane. Depending on the initial concentration of the *L. donovani* lysate, use PBS to dilute it to a final total protein load of 4 µg/mm lane
- 16 Mount the gel electrophoresis apparatus and fill the cathode and anode tanks with 1x cathode and anode buffer, respectively
- 17 Load the wells with the samples and protein molecular weight marker (1610374, Bio-Rad, UK)
- 18 Run the gel at 30V constant for 55min, then 120V constant for a total of 5h

## Protein transfer

- 19 Upon gel completion, disassemble the gel apparatus and remove the stacking gel
- 20 Soak three layers of blotting paper (Whatman GB005) with blotting buffer (300 mM Tris, 100 mM acetic acid (pH 8.6). Do not flood the semi-dry system (1703940, Bio-Rad, UK)
- 21 Add the NC membrane (10600046, GE Healthcare Life Sciences, UK) on the top of the soaked blotting paper
- 22 Carefully add the gel on the top of the NC membrane. Ensure full contact between gel and NC membrane along the whole surface
- 23 Add four layers of blotting paper previously soaked with blotting buffer on the top of the gel. Add more blotting buffer if necessary. Do not flood the system
- 24 Squeeze out air bubbles by gently rolling any cylindrical object (e.g. a Falcon tube) against the top layer of blotting paper

- 25 Cover the transfer system with the lid
- 26 Run for 120 minutes at constant 60 mA
- 27 Upon completion of the transfer, air dry the membranes. Place heavy objects onto the four corners of the membrane, to avoid curling.

## Immunoassay

- 28 Upon completion of the protein transfer, block the membrane with blocking buffer (PBS + 3% w/v non-fat milk powder overnight at 4°C followed by three 5 minute washes in PBST (PBS + 0,05% Tween 20) and one wash of PBST + 0.02% w/v sodium azide
- 29 Tape the top of the membrane (for identification, see step below) and slice the blocked NC membrane into 4 mm strips using a paper trimmer (Cathedral Products, UK)
- 30 Number the strips sequentially (including the MWM). Store in airtight containers at 4°C until use.
- 31 Pre-wet the individual strips with PBS and place them in wells of an 8-well immunotray (octa-101, Pateof, Denmark)
- 32 Add enough serum samples diluted 1:400 in blocking buffer to cover the strips
- 33 Incubate at rocking for 1h at room temperature (RT)
- 34 Wash the strips with PBST six times for 10 minutes each
- 35 Add enough mouse anti-human IgG1-HRP (ab99774, Abcam, UK) diluted 1:1000 in blocking buffer to cover the strips
- 36 Incubate at rocking for 1h at RT
- 37 Wash the strips with PBST six times for 10 minutes each



- 38 To reveal the protein bands, dissolve 1 tablet pair of SigmaFast DAB (D0426, Sigma-Aldrich) in 15ml of dH<sub>2</sub>O immediately prior to use.
- 39 Add enough volume to cover the strips and incubate for 4 minutes in darkness
- 40 Wash thoroughly with water
- 41 Air-dry the strips with heavy objects on top and bottom to avoid curling.