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

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dx.doi.org/10.17504/protocols.io.u8rezv6

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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)

TAGS

Western

I. donovani

Show tags

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Schägger H. Tricine-SDS-PAGE. Nat Protoc. 2006;1(1):16-22. doi: 10.1038/nprot.2006.4. PMID: 17406207.

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

MATERIALS TEXT

Tris, tricine, SDS, HCl, acrylamide, bisacrylamide, amonium persulphate, TEMED, dH2O, 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_2O.

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_2O overlay at 4^oC. Cover the top of the gel with a plastic film to minimise evaporation

8 On the following day, carefully pour off the dH_2O 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_2O

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^oC 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_20.

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^{o}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_2O 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.