Haake Lab

Author: Marija Pinne

Immuno-fluorescence assay of leptospiral surface-exposed proteins

This is an efficient method to assess surface-exposure of leptospiral proteins. The method is specifically designed to avoid disruption of the fragile outer membrane of leptospiral cells. This technique requires employment of several negative controls to assess the integrity of the outer membrane and specificity of antibody reaction.

Method:

1.) Fixation of *Leptospira* to glass slides

1.1) Grow *Leptospira* in EMJH medium, supplemented with 1% rabbit serum at 30° C until they reach mid- to late-log phase (density of 5 x 10^7 to 5 x 10^8 cells/ml).
1.2) Harvest the culture by centrifugation at ~2000 x g for 7 min at room temperature.
1.3) Remove the supernatant and gently resuspend the pellet in phosphate buffered saline (PBS)-5 mM $MgCl_2$, pH7.2 to a final concentration of 5 x 10^8 cells/ml.
1.4) Add 1 ml of cell suspension to each well of two-well chamber glass slides and incubate at 30° C for 80 min to allow cells to adhere.
1.5) Carefully remove the liquid containing unbound cells by aspiration.
1.6) Fix remaining intact bacteria to glass slides by adding 1 ml/well of 2% paraformaldehyde in PBS-5 mM $MgCl_2$. Incubate for 40 min at 30° C. These slides will be for assessment of surface-exposed proteins.
1.7) For control slides assessing sub-surface proteins, permeabilize the outer membrane by fixing with 1 ml/well of 100% ice-cold methanol. Incubate at -20° C for 20 min. Methanol acts in several ways: it permeabilizes the outer membrane, denaturates the proteins, and fixes cells to glass slides.

2.) Labeling with specific antibodies

2.1) Block non-specific binding by adding 1 ml/well of blocking buffer (Difco *Leptospira* Enrichment EMJH) Incubate at 30° C for 90 min.
2.2) Dilute the specific antibody (immune rabbit sera or mouse monoclonal antibodies) and pre-immune rabbit sera or mouse ascetic fluid containing no antibody (when utilized as negative controls) in blocking buffer. Dilutions for each antibody have to be empirically estimated depending on antibody titer, antigen-antibody reactivity and abundance of protein in the cell, the usual range is 1:50 to 1:600.
2.3) Remove the blocking buffer by aspiration and add 1 ml/well of diluted primary antibodies.
2.4) Incubate at 30° C for 1h.
2.5) Remove the liquid by aspiration and wash the wells three times with PBS (1 ml/well).

3.) Visualization of leptospires

3.1) Add 1 ml/well of Alexa Fluor 488-labeled secondary antibodies (either goat anti-rabbit IgG or goat anti-mouse IgG) diluted 1:2000 and fluorescent nucleic acid stain, 4'6-diamidino-2-phenyl-indole dihydrochloride (DAPI) diluted to a final concentration of

0.25 μg/ml in blocking buffer. This step ensures detection of antibody binding and the presence of spirochetes, respectively.

3.2) Incubate the slides at 30° C for 45 min.

3.3) Remove the liquid by aspiration and wash the wells twice with PBS and once with distilled water (1 ml/well).

3.4) Remove the chambers from the glass slides and air-dry for ~10 min.

3.5) Add ProLong Gold anti-fade mounting medium (2 x 20 μl per slide) and a 24 x 50 mm cover slip (Fisherfinest Premium Cover Glass).

3.6) Incubate overnight at room temperature in the dark. This step is necessary to cure (harden) the mounting medium.

3.7) Seal the cover slip with nail polish.

3.8) Visualize the staining by fluorescence microscopy using a cyan/blue detection filter for DAPI and a green detection filter for Alexa Fluor 488. Make sure you evaluate the entire chamber area for each sample before making conclusions.

3.9) Record the data by imaging a representative field for each sample. When imaging Alexa Fluor 488 fluorescence, use the same exposure time for all samples. Using a consistent exposure time will allow more accurate comparison of results with test antigens versus controls.

References:

1. Pinne, M. and Haake, D. A. "A comprehensive approach to identification of surface-exposed, outer membrane-spanning proteins of *Leptospira interrogans*," PLoS ONE 4:e6071 (2009). PMID: 19562037.

2. Pinne, M. and Haake, D. A. "Immunofluorescence assay of leptospiral surface-exposed proteins" J Vis Exp 53:2805 (2011). PMID: 21750491.