primediagnostics

Protocol

Indirect immuno fluorescence (IIF) protocol

Our reagents are optimized for use in IIF using object glasses with 4 mm diameter wells (Nutacon 10-342) and operating with a working volume of 5 µl per well.
Due to differences in working conditions and circumstances, the working dilution of the specific antibody can vary between 250 and 5000 times. The appropriate working dilution should be determined on-site after receipt of the antibody.
The incubations are performed in a tightly closed humid box. For washing: gently cover the glasses completely with washing solution, leave for 5 min and remove the washing solution gently by tilting of the glass. Repeat this 3 – 5 times. Gently cover the glasses completely with demineralized water, leave for 1 min and remove the water by tilting the glass. Dry the glass on a heating plate at 50ºC.

Buffers, chemicals & equipment:

- PBS 0.01 M: 8.18 gr NaCl, 0.15 gr KCl, 0.27 gr KH_2PO_4, 1.42 gr Na_2HPO_4 x 2 H_2O, pH 7.4
  Add demineralized water to 1000 ml total volume
- Washing buffer: 0.001 M PBS (dilute PBS 10 times)
- Specific antibody: Polyclonal IgG. Working dilution is dependent on local circumstances and must be determined before usage with sample material. Working dilution may range from 250 to 5000 times.
- GAR-FITC: Goat anti rabbit FITC (sigma F0382)
- Mounting-buffer: Vectashield mounting fluid (Brunschwig H1000)
- Mounting-Kpi: 3.2 gr Na_2HPO_4 x 12 H_2O, 0.15 gr NaH_2PO_4 x 2 H_2O, pH 7.6
  Add demineralized water to 100 ml total volume and add 50 ml glycerol
- Technical alcohol 96%
- Heating plate 50ºC
- Incubation chamber 27ºC
- Fluorescence microscope

Preparations:

The optimal working dilution of the specific antibody is dependent on local circumstances. We strongly recommend to perform a titre test before starting an experiment with sample material:

1. Prepare a dilution series of a positive control, preferable a pure culture of the target bacteria: 10^7, 10^6 and 10^5 cells/ml.
2. Prepare the IF-glasses as mentioned in step 1 of the protocol below.
3. Spot the bacterial cells according to the scheme below and follow step 2 of the protocol below.
4. Prepare a dilution series of the specific antibody in PBS: 500, 1000, 2000, 3000 and 5000 times. If you have more wells you may extend the dilution series.
5. Spot the antibody on the IF glasses according to the scheme below.
6. Follow steps 4 to 10 of the protocol below.
7. The optimal working dilution shows a fluorescence score 3-4 in all the bacterial concentrations[1].

Bacterial cells/ml	250	500	1000	2000	3000	5000
10^5	O	O	O	O	O	O
10^6	O	O	O	O	O	O
10^7	O	O	O	O	O	O

Dilution antibody

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Protocol:

1. Clean the object glasses extensively with alcohol before use.
2. Spot the samples and controls on the glasses; allow air-drying on the heating plate and fixing the cells by flaming or 96% alcohol.
3. Dilute the IgG to the optimal working dilution in PBS and spot the appropriate amount per well.
4. Incubate the glasses during 30 minutes at 27ºC.
5. Wash the glasses and allow air-drying on the heating plate.
6. Dilute the GAR-FITC 200 times in PBS and spot the appropriate amount per well.
7. Repeat steps 4 and 5.
8. Spread the vectashield or mounting-Kpi in droplets over the glasses and cover this with a fitting standard object glass.
9. Examine the wells with 490 nm (blue light) and look for green fluorescent cells with the same morphology as the positive control. Use a magnification of 100 times.
10. Scoring of the results:
    - 0 = no fluorescent cells visible
    - 1 = weakly fluorescent cells visible
    - 2 = moderately fluorescent cells visible
    - 3 = clearly fluorescent cells visible
    - 4 = bright fluorescent cells visible

Remarks:

1. When determining the optimal working dilution for the conjugate, please keep the following in mind that the fluorescence will be dimmed or disappear completely if:
    - The concentration of bacterial cells is too high.
    - The concentration of the antibody is too high.
    - The concentration of the antibody is too low.
    - The sample material contains auto-fluorescent particles.
    - The sample material contains an overflow of non-target bacterial cells.
    - The concentration of GAR-FITC is too low.
    - The samples were not fixed sufficiently to the glass.

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