# *Shigella* IpaD and IpaB Surface Localizations

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**[Abstract]** *Shigella* uses a type III secretion system to invade host cell and to cause disease. Secretion control and insertion of a translocation pore into cell membrane are critical steps for pathogenesis and are tightly linked to the formation of the needle tip complex formed by the IpaB and IpaD proteins (Veenendaal *et al.*, 2007). Surface localizations of IpaD and IpaB were monitored by FACS analysis according to the localization protocol for *Pseudomonas aeruginosa* homolog PcrV (Lee *et al.*, 2010).

## Materials and Reagents

1. *Shigella* strains
2. Tryptic Soy Broth (TSB) (VWR International, catalog number: for Europe 1.00525.5000 and for U.S. EM1.00525.5007)
3. Agar (MP Biomedicals, catalog number: 0210026291)
4. Congo Red (VWR International, for Europe catalog number: 34140.184)
5. Anti-IpaD and -IpaB polyclonal antibodies (house made)
6. PBS (Fischer Scientific, catalog number: BP399-20)
7. Paraformaldehyde (PFA) (Sigma-Aldrich, catalog number: P6148)
8. Triton X-100 (VWR International, catalog number: 1.08603.1000)
9. Trizmabase (Sigma-Aldrich, catalog number: T1503)
10. Bovine serum albumin (BSA) (VWR International, catalog number: 422361V)
11. Anti-mouse secondary antibody CF647-conjugated (Sigma-Aldrich, catalog number: SAB4600351)
12. PBS + PFA 4% stock solution (see Recipes)
13. Congo Red agar plates (see Recipes)

## Equipment

1. 250 ml Erlenmeyer
2. Microtubes
3. CR agar plate

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4. Polystyrene tube for FACS analysis
5. Centrifuge Heating magnetic stirrer
6. 37 °C shaker
7. Rotator mixer
8. Flow cytometry with a four-colour FACS Calibur cytometer (Becton Dickinson and Company)

## Procedure

A. Preparation of bacterial samples

1. Launch overnight precultures in TSB (37 °C with shaking) from Congo Red (CR) positives colonies of *Shigella* on CR agar plates.
2. Dilute 1:100 precultures in fresh TSB (for volume see step A3) and incubate at 37 °C with shaking until an $OD_{600}$ ≈ 1.5 is reached. (Medium must be filtered or autoclaved with stirring to avoid glucose caramelization which interferes with type III secretion.)
3. Harvest 2 x $10^8$ bacteria per tested conditions at 2,000 *x g* for 4 min at room temperature (RT) ($OD_{600}$ of 1 correspond to approximately 5 x $10^8$ bacteria).
4. Wash twice with 500 µl of ice-cold PBS with 0.1% Triton X-100 (centrifugation conditions as in step A3). Be careful when you take out the liquid supernatant as the pellet can detach gradually all along the procedure.
5. Resuspend in 500 µl of ice-cold PBS with 0.1% Triton X-100 and add 500 µl of PBS + PFA (4%) to fix bacteria.
6. Mix by inversion and incubate 20 min at RT.
7. Add 50 µl Tris HCl 1 M (pH 7.5), mix by inversion and incubate 5 min at RT to quench the cross-linker.
8. Harvest bacteria at 10,000 *x g* for 2 min at RT (all further centrifugation steps are performed with these parameters).
9. Wash once with 1 ml PBS + 0.1% Triton X-100 and once with 1 ml PBS.

B. Immunological staining

1. Harvest bacteria and resuspend in 500 µl of the blocking solution (PBS + 4% BSA).
2. Incubate bacteria on rotator mixer 1 h at 4 °C.
3. Harvest bacteria and resuspend in 250 µl PBS + 4% BSA with mouse sera diluted 1:100. Sera were from Swiss mice immunized with GST-IpaD$^{131-332}$ (Schiavolin *et al.*, 2013) or His-IpgC + IpaB (Page *et al.*, 1999). Negative controls for IpaD and IpaB localizations are respectively an IpaD protein lacking its last residues which do not bind the needle tip (Espina *et al.*, 2006) and an *ipaD* KO mutant where IpaB is not retained at the tip

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(Veenendaal *et al.*, 2007). In both cases secreted IpaD or IpaB do not interfere with the assay.

4. Incubate overnight at 4 °C with agitation.
5. Add 750 µl PBS to bacteria and then wash twice with 1 ml PBS. All further steps are made in the dark to preserve fluorescent properties of the secondary antibody. Microtubes or rack are covered with aluminum foil.
6. Resuspend in 250 µl of PBS + 4% BSA with goat CF647-conjugated anti-mouse IgG antibody diluted 1:500.
7. Agitate 1 h or longer at 4 °C (incubation time may last overnight).
8. Add 750 µl PBS to bacteria and then wash twice with 1 ml PBS.
9. Resuspend bacterial pellet in 500 µl of PBS and dilute 1:10 in FACS polystyrene tube.
10. Analyze by flow cytometry with a four-colour FACS Calibur cytometer for instance (you will find examples of results in the supporting information of the third reference (Schiavolin *et al.,* 2013) available for free on *Mol. Microbiol*. website).
11. Parameters used (no compensation):

| detectors | voltage | mode |
|---|---|---|
| FSC | E02 | Log |
| SSC | 366 | Log |
| FL4 | 800 | Log |

## Recipes

1. PBS + PFA 4% stock solution (100 ml)
   Weigh out 4 g of paraformaldehyde in a 250 ml erlenmeyer
   Add 80 ml of double-distilled water
   Add 50 µl of 1 M NaOH
   Add a magnetic bar and close the erlenmeyer
   Heat at 70 °C until complete solubilization (the solution should become clear)
   Put on ice and allow the solution to cool down to RT
   Adjust volume to 90 ml with double-distilled water
   Add 10 ml of PBS 10x and mix
   Filter solution with a 0.2 µm 25 mm nylon syringe filter and aliquot in 15 ml falcon tube
   Freshly prepared PFA can be stored at -20 °C for further assays (thaw gently at RT)
2. Congo Red agar plates
   Prepare a 30 g/l TSB solution with bidistilled water and dissolve 15 g/l agar
   Autoclave the medium for 15 min at 120°C (if you autoclave your medium for 20 min, sugar will caramelize and your plates will be darker, and even darkest after incubation with bacteria at 37°C)

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Add the Congo Red at a final concentration of 250 µg/ml (stock solution is prepared in bidistilled water at a concentration of 10 mg/ml) when the bottle can be safely handled with a protective glove.

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