Please cite this article as: Hong and Mohammed , (2017). Crude Preparation of Lipopolysaccharide from *Helicobacter pylori* for Silver Staining and Western Blot, *Bio-protocol* 7 (20): e2585. DOI: 10.21769/BioProtoc.2585.

bio-protocol www.bio-protocol.org/e2585 Vol 7, Iss 20, Oct 20, 2017
DOI:10.21769/BioProtoc.2585

Crude Preparation of Lipopolysaccharide from *Helicobacter pylori* for Silver Staining and Western Blot

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[Abstract] This protocol provides an easy and rapid method to prepare lipopolysaccharide from the gastric pathogen *Helicobacter pylori* for visualization on acrylamide gels by silver staining and for detecting the presence of Lewis antigens by Western blot. The silver staining is a four-step procedure, involving a 20 min-oxidation step, a 10 min-silver staining step, a 2-10 min color development step and finally a 1-min color termination step. Lipopolysaccharide from *H. pylori* wild-type and corresponding mutants analyzed by this method are described in a recent publication (Li *et al.*, 2017). This crude preparation of LPS for silver staining is also applicable in other Gram-negative bacteria.

Keywords: Lipopolysaccharide, Crude preparation, Silver staining, Western blot, *Helicobacter pylori*

[Background] Lipopolysaccharide (LPS) is a large and variable complex glycolipid that makes up the outer leaflet of the outer membranes of most Gram-negative bacteria. It is typically composed of three domains: a hydrophobic domain termed lipid A (or endotoxin), which is embedded in the outer membrane; a relatively conserved non-repeating core-oligosaccharide; and a variable O-antigen, which extends from the cell to the external environment. A unique feature of *H. pylori* lipopolysaccharide O-antigen is the presence of fucosylated oligosaccharide structures that mimic human Lewis antigens. Large-scale extraction of highly pure LPS from Gram-negative bacteria is labor-intensive and time-consuming. Here, in this protocol, we describe in detail the use of an easy and rapid crude preparation of LPS from the gastric pathogen *Helicobacter pylori* for visualization by silver staining and Lewis antigen expression by Western blot.

Materials and Reagents

1. Pipette tips
2. Inoculating loops (10 µl) (Copan Diagnostics, catalog number: 8177CS20H)
3. Aluminum foil
4. PVDF membrane (0.2 µm) (Merck, catalog number: ISEQ00010)
5. *H. pylori* cells
6. Columbia blood agar (CBA) plates (Autobio Diagnostics, catalog number: M0109-2)
7. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: 746398-500G)

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8. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: 746436-500G)
9. Sodium phosphate dibasic (Na_2HPO_4) (Sigma-Aldrich, catalog number: 795410-500G)
10. Potassium phosphate dibasic (K_2HPO_4) (Sigma-Aldrich, catalog number: 795496-500G)
11. NaOH (Sigma-Aldrich, catalog number: S5881-500G)
12. Proteinase K (Sigma-Aldrich, catalog number: P8044-1G)
13. Ethanol (Sigma-Aldrich, catalog number: 24102-5L-R)
14. SDS (Sigma-Aldrich, catalog number: L3771-1KG)
15. Glycerol (Sigma-Aldrich, catalog number: G9012-1L)
16. Tris base (Sigma-Aldrich, Roche Diagnostics, catalog number: 11814273001)
17. Glycine (Sigma-Aldrich, catalog number: G8898-1KG)
18. Bromphenol blue (AMRESCO, catalog number: 0449-50G)
19. β-Mercaptoethanol (AMRESCO, catalog number: 0482-100ML)
20. Periodic acid (Sigma-Aldrich, catalog number: P7875)
21. Acetic acid (BDH, catalog number: 100015N)
22. Ammonium persulfate (Sigma-Aldrich, catalog number: A3678)
23. Ammonium hydroxide (Sigma-Aldrich, catalog number: 320145)
24. Silver nitrate (Sigma-Aldrich, catalog number: 209139)
25. Citric acid (Sigma-Aldrich, catalog number: C7129)
26. Formalin (37% formaldehyde) (Sigma-Aldrich, catalog number: 252549)
27. Freshly-made 15% SDS-PAGE gels
28. 30% Acrylamide/Bis (Bio-Rad Laboratories, catalog number: 1610157)
29. TEMED (Bio-Rad Laboratories, catalog number: 1610801)
30. Methanol (Sigma-Aldrich, catalog number: 34860-4X4L-R)
31. Bovine serum albumin (BSA) (AMRESCO, catalog number: 0332-100G)
32. Tween-20 (Solarbio, catalog number: T8220-500 ml)
33. Mouse anti-Le^x (Santa Cruz Biotechnology, catalog number: sc-59471)
34. Mouse anti-Le^y (Santa Cruz Biotechnology, catalog number: sc-59472)
35. Mouse anti-Le^a (Santa Cruz Biotechnology, catalog number: sc-51512)
36. Mouse anti-Le^b (Santa Cruz Biotechnology, catalog number: sc-51513)
37. Secondary rabbit anti-mouse peroxidase-conjugated IgM antibody (Jackson ImmunoResearch Laboratories, catalog number: 315-035-049)
38. Chemiluminescent peroxidase substrate-1 (Sigma-Aldrich, catalog number: CPS1120)
39. PBS (pH 7.2) (10x) (see Recipes)
40. SDS-PAGE running buffer (10x, see Recipes)
41. 0.1 N NaOH solution (see Recipes)
42. LPS lysis buffer (see Recipes)
43. Oxidation solution (see Recipes)
44. Silver staining solution (see Recipes)
45. Color developer solution (see Recipes)

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46. Termination solution (see Recipes)
47. SDS-PAGE transfer buffer (10x, see Recipes)
48. TBS (10x, see Recipes)

Equipment

1. Pipettes (Eppendorf, catalog numbers: 4920000024, 4920000059, 4920000067, 4920000083)
2. Centrifuges (Eppendorf, catalog number: 5418 R)
3. Glass wares
4. Water bath
5. Rotary shaker
6. Cell electrophoresis tank (Bio-Rad Laboratories, catalog number: 1658001)
7. Electrophoresis power supply (Bio-Rad Laboratories, catalog number: 1645070)
8. Semi-dry electrophoretic transfer system (Bio-Rad Laboratories, catalog number: 1703940)
9. pH meter
10. Spectrophotometer (Shimadzu, model: UV-1601 UV-Visible)
11. Digital camera
12. Luminescent Image Analyzer (Fujifilm, model: LAS-3000)

Software

1. Image reader LAS 3000 V2.2

Procedure

A. LPS sample preparation
   1. Culture *H. pylori* strains on CBA plates, incubate at 37 °C for 24 h under microaerobic conditions.
   2. Use a loop (10 µl) to harvest *H. pylori* cells grown on CBA plates (24 h growth) into 1 ml of cold 1x PBS (see Recipes) to a turbidity of 3.0 at OD_{600}.
   3. Centrifuge the *H. pylori* cells at 5,000 *x g* for 5 min, discard supernatant.
   4. Resuspend the pellet in 100 µl LPS lysis buffer (see Recipes) and heat the sample at 100 °C for 10 min, and allow to cool at room temperature.
   5. Add 5 µl Proteinase K (20 mg/ml) to the cooled samples, and incubate in a 55 °C water bath overnight.
   6. Load 10 µl of the above-obtained LPS sample per well to the 15% SDS-PAGE gel and electrophorese with 1x SDS-PAGE running buffer (see Recipes).
   7. Once the gel electrophoresis is complete, remove the gel from the apparatus and trim away the stacking gel

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B. LPS silver staining

1. Rinse the gel 3 times with ddH_2O.
2. Transfer the gel to a separate ware with freshly-made oxidation solution (see Recipes), oxidize for 20 min on a rotary shaker (70 rpm/min) (all shaking in this protocol is done at room temperature).
3. Transfer the gel to a separate clean ware, rinse and wash with ddH_2O for 3 times, each time with 200 ml ddH_2O on a rotary shaker for 10 min.
4. Transfer the gel to a separate ware with freshly-made silver staining solution (see Recipes), stain for 10 min on a rotary shaker.
   Note: The silver staining solution must be freshly-made, which can be made during the final wash of step B4. During the staining process, the ware should be wrapped in aluminum foil, but make sure the foil is not in contact with the solution.
5. Transfer the gel to a separate clean ware, rinse and wash for 3 times, each time with 200 ml ddH_2O on a rotary shaker for 10 min.
6. Transfer the gel to a separate ware with freshly-made color developer solution (see Recipes), develop for 2-10 min on a rotary shaker.
   Note: The color developer solution must be freshly-made, which can be made during the final wash of step B6. The color will develop on the gel within to 2-10 min, the longer the developer solution remains in contact with the gel the darker the bands will become. Therefore, it is important to watch the gel develop. The operator must decide when the gel is sufficiently developed and transfer the gel to the termination solution (see Recipes).
7. Transfer the gels to a separate ware with freshly-made termination solution for 1 min. The gels are then rinsed and stored in ddH_2O.
8. Use a digital camera to take images of the silver-stained LPS gels.

C. Western blot for the detection of Lewis antigen expression

Proceed from step 7 of Procedure A.

1. Transfer LPS from the gel to PVDF membrane with 1x SDS-PAGE transfer buffer (see Recipes).
2. Once the transfer is complete, remove the membrane from the apparatus.
3. Rinse the membrane 3 times with ddH_2O.
4. Block the membrane with TBST (see Recipes) containing 3% BSA, on a rotary shaker for 2 h or overnight at 4 °C.
5. Pour off the blocking solution.
6. Probe with primary antibodies: mouse anti-$Le^{x/y}$ or anti-$Le^{a/b}$ (1:1,500) (Santa Cruz) in TBST containing 1% BSA, on a rotary shaker for 2 h or overnight at 4 °C.
7. Pour off the primary antibody solution.
8. Rinse and wash with TBST for 3 times, each time on a rotary shaker for 10 min.

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9. Probe with secondary rabbit anti-mouse peroxidase-conjugated IgM antibody (1:10,000) (Jackson ImmunoResearch Laboratories) in TBST, on a rotary shaker for 1 h.
10. Pour off the secondary antibody solution.
11. Rinse and wash with TBST for 3 times, each time on a rotary shaker for 10 min.
12. Place the membrane on a flat sheet of plastic film.
13. Add approximately 5 ml of the chemiluminescent peroxidase substrate-1 working solution (Sigma-Aldrich) on the membrane.
14. Cover the membrane with a second flat sheet of plastic film.
15. Detect chemiluminescence using a LAS-3000 Intelligent DarkBox (Fujifilm) (software Image reader LAS 3000 V2.2).

Data analysis

Figure 1 below illustrates LPS prepared from *H. pylori* wild-type strains 26695, P1, B128 and J99 analyzed by silver staining and Western blot using anti-Lewis X (Le^x) and anti-Lewis Y (Le^y).

1. Silver staining: the fast migration bands stained black represent molecules comprising lipid A and core-oligosaccharide (Lipid A-core); the slower migration bands represent O-antigen. It is obvious that LPS preparations from the four *H. pylori* strains show a different staining pattern, indicating the strains carrying different LPS structures (Figure 1A).
2. Western blot: LPS from *H. pylori* strains 26695, P1 and J99 were detected expressing both Le^x and Le^y antigens (Figure 1B, Lanes 1, 2 and 4), whereas LPS prepared from strain B128 was not detected expressing Le^x/^{y} (Figure 1B, Lane 3).

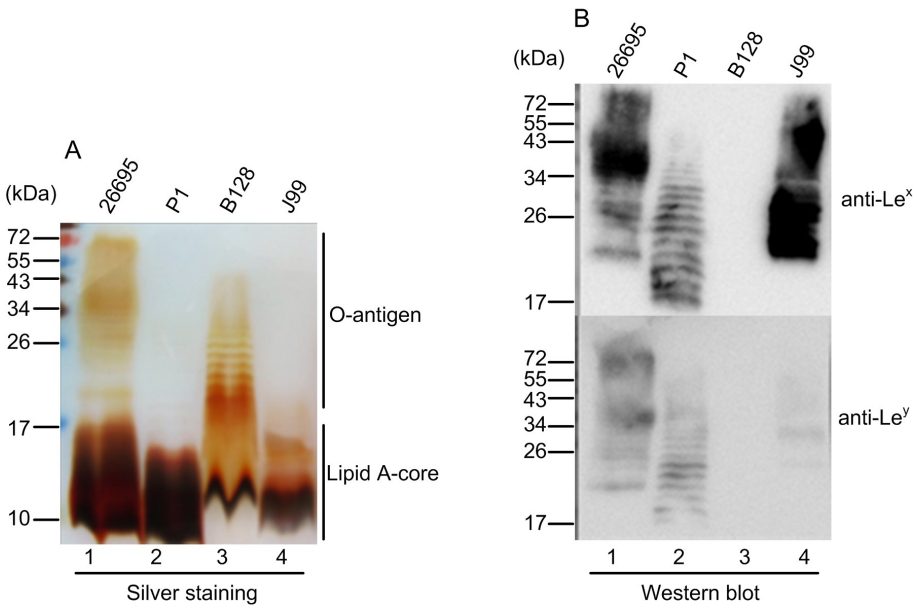

Figure 1. Crude preparation of LPS from *H. pylori* strains for silver staining and Western blot

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Recipes

Note: ddH_2O is used for preparing all the solutions unless otherwise indicated.

1. PBS (pH 7.2) (10x) (1 L)
   80 g NaCl
   2 g KCl
   14.4 g Na_2HPO_4
   2.4 g KH_2PO_4
   Add 900 ml H_2O to dissolve all the reagents and adjust the pH to 7.2, then add H_2O to make the total volume up to 1 L. The buffer is sterilized by autoclaving and stored at room temperature. The 1x working solution is prepared by mixing 100 ml 10x PBS buffer with 900 ml H_2O
2. 0.1 N NaOH (1 L)
   4 g NaOH
   The total volume is made up to 1 L with H_2O. The buffer was sterilized by autoclaving and stored at room temperature
3. LPS lysis buffer (20 ml)
   2 ml 20% SDS
   800 µl β-mercaptoethanol
   200 µl bromophenol blue
   2 ml glycerol
   15 ml 1 M Tris-HCl (pH 6.8)
4. Oxidation solution (100 ml)
   0.7 g periodic acid
   40 ml EtOH
   5 ml acetic acid
   55 ml H_2O
5. Silver staining solution (150 ml)
   28 ml 0.1 N NaOH
   2 ml ammonium hydroxide (29.4%)
   115 ml H_2O
   5 ml 20% silver nitrate (w/v)
   Note: This solution is mixed in the following order by adding 28 ml 0.1 N NaOH, 2 ml concentrated ammonium hydroxide (29.4%), and 115 ml H_2O in a dedicated ware, then transfer the ware on the rotary shaker (50 rpm/min). Finally, add the 5 ml 20% silver nitrate dropwise to the solution, waiting for the brown precipitate to dissolve before adding more of silver nitrate solution. If the brown precipitate fails to dissolve, discard the solution and remake the solution by adding the 20% silver nitrate more slowly.

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6. Color developer solution (200 ml)
   Dissolve 1 g citric acid into 20 ml H_2O, resulting into 5% citric acid:
   200 µl 5% citric acid
   100 µl 37% formaldehyde
   200 ml H_2O
7. Termination solution (100 ml)
   10 ml acetic acid
   90 ml ddH_2O
8. SDS-PAGE running buffer (10x) (1 L)
   30.3 g Tris base
   144 g glycine
   10 g SDS
   The total volume is made up to 1 L with H_2O. The buffer was sterilized by autoclaving and stored at room temperature. The 1x working solution is prepared by mixing 100 ml 10x running buffer with 900 ml H_2O
9. SDS-PAGE transfer buffer (10x) (1 L)
   30.3 g Tris base
   144 g glycine
   The total volume is made up to 1 L with H_2O. The buffer was sterilized by autoclaving and stored at room temperature. The 1x working solution is prepared by mixing 100 ml 10x transfer buffer with 700 ml H_2O and 200 ml methanol
10. TBS (10x) (1 L)
    80 g NaCl
    2 g KCl
    30 g Tris base
    Add 900 ml H_2O to dissolve all the reagents and adjust the pH to be 7.4, then add H_2O to make the total volume up to 1 L. The stocking buffer is sterilized by autoclaving and stored at room temperature. TBST is 1x TBS containing 0.1% Tween-20 (v/v)

Acknowledgments

This protocol described here and in Li *et al.* (2017) was adapted from protocols described in Apicella *et al.* (2008) and in Tsai *et al.* (1982). This work was supported by a grant from West China Hospital, Sichuan University entitled ‘1.3.5 project for disciplines of excellence, West China Hospital, Sichuan University’ (ZY2016201).

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