2.**Lohman, T.M. and K.P. Bjornson.** 1996. Mechanisms of helicase-catalyzed DNA unwiding. Annu. Rev. Biochem. *65*:169-214.
3.**Sambrook, J., E.F. Fritsch and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, NY.

Address correspondence to Jessica L. Epstein, Department of Molecular and Cell Biology, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138, USA. Internet: epstein4@husc.harvard.edu

Received 23 January 1996; accepted 4 June 1996.

Jessica L. Epstein, John W. Kozarich and David Bramhill
Merck Research Laboratories
Rahway, NJ, USA

Rapid Preparation of Affinity-Purified Lipopolysaccharide Samples for Electrophoretic Analysis

BioTechniques 22:230-236 (February 1997)

Lipopolysaccharides (LPS) are the major components of the outer membrane of gram-negative bacteria. These surface molecules are relevant both for the outer membrane stability and for the interaction of the bacteria with other organisms and with the environment (18). Extensive literature is available concerning LPS physiology in different symbiotic (parasitic) and pathogenic host-bacterial interaction systems (5,20). Physiological, biochemical and chemical approaches to study LPS functions commonly require LPS extraction and purification. In the literature, LPS have been prepared by a number of different cell disruption procedures. Protocols for LPS isolation include organic solvent extractions followed by exhaustive dialysis or evaporation steps, and their modifications (11,22).

An alternative procedure was described by Leive (17), which involves washing the bacteria with EDTA. In this procedure, incubation of cells with aqueous EDTA releases a significant proportion of LPS (ca. 30% to 50% in *E. coli* and *Salmonella*) without bacterial lysis, thus avoiding drastic protein, DNA and RNA contamination. All these protocols have been widely used for LPS chemical analysis (5,18), for the investigation of in vitro LPS biological activities (20) and also for analysis of LPS micro-heterogeneity using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (4). As a preliminary tool before chemical analysis, electrophoretic characterization of LPS can give valuable information concerning size distribution of different LPS molecular forms and general indications about structural changes in the LPS that produce modifications in the electrophoretic mobility (8). A quick method for LPS characterization by SDS-PAGE have been described by Hitchcock and Brown (12) using a whole SDS bacterial lysate. Although this method avoids tedious LPS preparations, it includes a strong protease treatment in the presence of SDS to clean samples after bacterial lysis. Technical aspects associated with different protocols for LPS isolation and its further SDS-PAGE analysis are summarized in Table 1.

In this report, we describe an alternative, practical and rapid method for

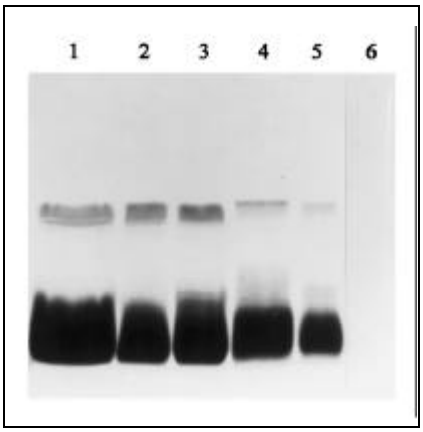

Figure 1. Comparison of SDS-PAGE pattern of *R. meliloti* LPS samples obtained by phenol-water extraction and by the ETP protocol. Slab gel electrophoresis was performed in an 80-mm × 50-mm × 1-mm 15% acrylamide gel as described by Laemmli (15) in a Mini-Protean® II unit (Bio-Rad). LPS preparations were mixed with sample buffer (1× final), heated 5 min in a boiling water bath and sonicated 15 min at high power before gel loading (see text). The gel was run at 25 mA until bromophenol blue reached the bottom of the gel, fixed and silver stained for LPS as described by Tsai and Frash (21). Lane 1: EDTA-TEA extract corresponding to LPS obtained from approximately 10 mg wet weight of *R. meliloti* 2011 cells. One mg wet weight of *R. meliloti* 2011 cells corresponds to approximately 5×10^8 colony-forming units. Lanes 2 and 3: ETP extracts corresponding to LPS obtained from approximately 10 mg wet weight of *R. meliloti* 2011 cells using Affi-Prep Polymyxin (Bio-Rad) and Detoxi-Gel® Endotoxin Removing Affinity Gel (Pierce, Rockford, IL, USA), respectively, during the LPS removal step of the protocol (see text). Lane 4: phenol-water extracted LPS from *R. meliloti* 2011 corresponding to approximately 1–2 mg wet weight of cells (positive control). Lane 5: phenol-water extracted LPS from *R. meliloti* 2011 (same as lane 4) followed by an LPS recovering using Affi-Prep Polymyxin. Lane 6: protein sample to assess the specificity of the LPS silver staining procedure (2 µg of molecular weight markers Dalton Mark VII-L™; Sigma Chemical, St. Louis, MO, USA). The upper bands of the LPS profiles correspond to complete LPS molecules (namely lipid A-core-O antigen). The lower (and broader) components correspond to lipid A-core LPS molecular forms.

Table 1. Summary of Relevant Characteristics of the Principal Methods Used to Prepare LPS Samples Suitable for SDS-PAGE Analysis

	Aqueous Phenol Extraction of LPS[22]	Ether-Phenol-Chloroform Extraction of LPS[11]	LPS Release by Whole Cell Lysis[12]	LPS Release by EDTA Treatment[17]	LPS Release by EDTA-TEA Treatment Coupled to Polymyxin B-LPS Removal (ETP)
Extractions with Organic Solvents/Phenol	yes	yes	no	no	no
Dialysis or Evaporation Steps	yes	yes	no	yes	no
Bacterial Lysis	yes	yes	yes	no	no
Protease Treatments	not necessary	not necessary	yes	no	no
Scale Type	analytical/ preparative	analytical/ preparative	analytical	analytical/ preparative	analytical

the preparation of affinity-purified LPS samples suitable for electrophoretic analysis. The procedure that yields samples with low protein and nucleic acid content includes a water-phase LPS extraction from cells using a combination of LPS releasing agents, followed by an in-batch affinity purification of LPS. The affinity purification uses the ability of the well-known antibiotic, polymyxin B, to bind LPS (19).

Briefly, cells are treated with a combination of EDTA as LPS-releasing agent (17) and triethylamine (TEA), a strong LPS dispersant (10). This combination has been previously used as running buffer to dissociate phenol-extracted LPS during gel permeation chromatography (6). The crude EDTA-TEA extract is mixed with a polymyxin B-bound resin, which efficiently removes LPS. Subsequently, LPS is released for SDS-PAGE analysis by boiling the resin in Laemmli's sample buffer (15). The step-by-step protocol is as follows: the bacteria were grown on agar plates and approximately 20 mg of wet cells were transferred to a 1.5-mL polypropylene microcentrifuge tube. Then 50 µL of 100 mM EDTA, titrated with TEA to reach pH 7.0, were added to the cells. The bacterial suspension was mixed with gentle agitation and incubated at room temperature (ca. 20°C) for 15 min. The bacterial suspension was centrifuged for 2 min at 10 000× *g,* and the supernatant was transferred to a new microcentrifuge tube. After the cell pellet was discarded, 30 µL of a polymyxin B resin suspension were added to the supernatant (Affi-Prep Polymyxin®; Bio-Rad, Hercules, CA, USA) supplied in 1.0 mM EDTA, 0.1 M HEPES, pH 7.5, 0.05% sodium azide. The mixture was incubated at room temperature for 15 min with gentle agitation and centrifuged for 2 min at 10 000× *g*. The supernatant was discarded. The resin pellet was washed with 100 µL of 100 mM KH_2PO_4 and 150 mM NaCl, pH 7.0, and centrifuged as before. The supernatant was discarded. Then 50 µL of Laemmli's sample buffer were added to the pellet and heated in a boiling water bath for 5 min. Before applying the sample to the polyacrylamide gel, the sample was sonicated in a water bath at high power for 15 min to disperse LPS. The sonication step is important to enhance dispersion of structurally different LPS forms in the sample. SDS-PAGE was performed using 15% gels and silver-stained as previously described by Tsai and Frash (21).

The procedure (EDTA/TEA/polymyxin B LPS extraction, known as ETP) yields samples containing LPS in sufficient amounts to be characterized by SDS-PAGE (Figure 1). LPS profiles obtained either with crude EDTA/TEA extracts (lane 1) or with the complete ETP procedure (lanes 2 and 3) were both identical to the pattern resulting from the control phenol-extracted LPS (lanes 4 and 5). Samples obtained with the ETP protocol had a low protein content. The amount of protein in the crude LPS extract released after the treatment with EDTA/TEA was less than 40 µg/mL, as estimated by the method of Bradford (3). Protein silver

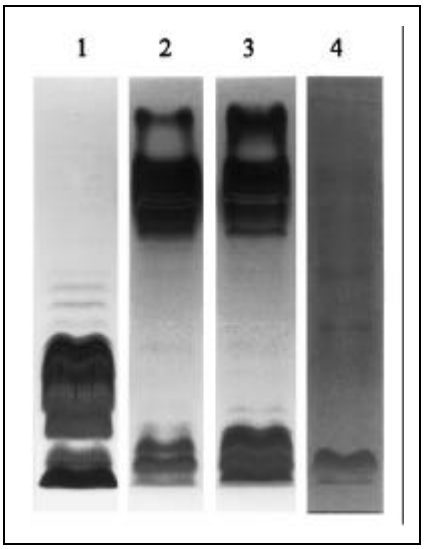

Figure 2. SDS-PAGE pattern of ETP-LPS samples obtained from different gram-negative bacteria. LPS preparation and slab gel electrophoresis were performed as described in the legend to Figure 1. In all cases, ETP extracts corresponded to LPS obtained from approximately 10 mg wet weight of bacterial cells. The well samples were as follows: lane 1, *E. coli* LPS; lane 2, *S. typhimurium* LPS; lane 3, *S. paratyphi* LPS; and lane 4, *V. cholerae* O139 Bengal AI1837 LPS.

staining (2) of LPS samples after SDS-PAGE revealed a small number of minor bands and a predominant protein of approximately 40-kDa molecular weight (not shown). None of these bands were detected when gels were specifically stained for LPS as described by Tsai and Frash (21) (Figure 1, lanes 1–3). If small amounts of negatively charged-lipid A-free polysaccharides were released from the bacteria during the EDTA/TEA extraction (i.e., some exopolysaccharides, EPS), they should not associate to polymyxin B. A crude extract of the succynoglycan EPS from *Rhizobium meliloti* 2011 (100 µg/mL) was incubated in the presence of polymyxin B as described in the protocol for LPS removal. After the polymyxin B treatment of the EPS solution, no change in the original concentration of hexoses was observed as estimated by the phenol-sulfuric method (9). No DNA/RNA was detected in crude EDTA/TEA LPS extracts when samples were run in 0.5 µg/mL ethidium bromide 1% agarose gels (detection limit ca. 10 ng of double-stranded DNA, 50–100 ng RNA). Crude EDTA-TEA LPS extracts can also be directly analyzed avoiding the use of the polymyxin B resin, although with markedly lower resolution (Figure 1, lane 1).

The protocol described here was used in our laboratory to extract LPS from different bacterial genera including *Escherichia*, *Salmonella*, *Vibrio* and *Rhizobium* species (Figure 2). The distribution of LPS forms for *Vibrio cholerae* O139 Bengal AI1837 and *R. meliloti* 2011 paralleled with the LPS profiles previously published for the same bacterial strains (13,16). The ETP method was also useful in extracting LPS from *E. coli* strains with either rough (R) or smooth (S) phenotype (not shown). In agreement with previous studies using *E. coli* and *Salmonella tiphymurium*, the release of LPS by EDTA (14) and the LPS removal by polymyxin B (19) were both operative irrespective of the length of the O-antigen. LPS with structurally different lipid A regions including *Rhizobium etli* (non-phosphorylated lipid A) (1), and *R. meliloti* (sulfated lipid A) (7) were also all efficiently extracted.

The ETP procedure was simple, fast and reliable for microscale preparation of LPS samples for SDS-PAGE analysis. The method that can be applied for preparation of up to 24 samples/h using a standard microcentrifuge yields samples with low protein content avoiding enzymatic treatments and the routine use of caustic chemicals and organic solvents.

REFERENCES

1. **Bhat, U.R., L.S. Forsberg and R.W. Carlson.** 1994. Structure of lipid A component of *Rhizobium leguminosarum* bv.*phaseoli* lipopolysaccharide. Unique non-phosphorylated lipid A containing 2-amino-2-deoxygluconate, galacturonate and glucosamine. J. Biol. Chem. *269*:14402-14410.
2. **Blum, H., H. Beier and H. Gross.** 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis *8*:93-99.
3. **Bradford, M.M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. *72*:248-254.
4. **Carlson, R.W.** 1984. Heterogeneity of *Rhizobium* lipopolysaccharides. J. Bacteriol. *158*: 1012-1017.
5. **Carlson, R.W., U.R. Bhat and B. Reuhs.** 1991. *Rhizobium* lipopolysaccharides: their structures and evidences for their importance in the nitrogen-fixing symbiotic infection of their host legumes, p. 39-50. *In* P.M. Gresshoff (Ed.), Current Topics in Plant Molecular Biology, Vol. I. Chapman and Hall, New York.
6. **Carlson, R.W., R. Sanders, C. Napoli and P. Albersheim.** 1978. Host-symbiont interaction III. Purification and partial characterization of *Rhizobium* lipopolysaccharides. Plant Physiol. *62*:912-917.
7. **Cedergreen, R.A., J. Lee, K.L. Ross and R.I. Hollingsworth.** 1995. Common links in the structure and cellular localization of *Rhizobium* chitolipooligosaccharides and general *Rhizobium* membrane phospholipid and glycolipid components. Biochem. *34*:4467-4477.
8. **Clover, R.H., J. Kieber and E.R. Signer.** 1989. Lipopolysaccharide mutants of *Rhizobium meliloti* are not defective in symbiosis. J. Bacteriol. *171*:3961-3967.
9. **Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. *28*:350.
10. **Galanos, C. and O. Luderitz.** 1975. Electrodialysis of lipopolysaccharides and their conversion to uniform salt forms. Eur. J. Biochem. *54*:603-610.
11. **Galanos, C., O. Luderitz and O. Westphal.** 1969. A new method for the extraction of R lipopolysaccharides. Eur. J. Biochem. *9*:245-249.
12. **Hitchcock, P.J. and T.M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. *154*:269-277.
13. **Hisatsune, K., S. Kondo, Y. Isshiki, T. Iguchi, Y. Kawanata and T. Shimada.** 1993. O-antigenic lipopolysaccharide of *Vibrio cholerae* O139 Bengal, a new epidemic strain for recent cholera in the Indian subcontinent. Biochem. Biophys. Res. Comm. *196*:1309-1315.
14. **Hukari, R., I. Helander and M. Vaara.** 1986. Chain length heterogeneity of lipopolysaccharides released from *Salmonella thyphimurium* by ethylenediaminetetraacetic acid or polycations. Eur. J. Biochem. *154*:673-676.
15. **Laemmli, U.K.** 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature *227*:680-685.
16. **Lagares, A., G. Caetano-Anollés, K. Niehaus, J. Lorenzen, H.D. Ljunggren, A. Pühler and G. Favelukes.** 1992. A *Rhizobium meliloti* lipopolysaccharide mutant altered in competitiveness for nodulation on alfalfa. J. Bacteriol. *174*:5941-5952.
17. **Leive, L.** 1965. Release of lipopolysaccharide by EDTA treatment of *E. coli*. Biochem. Biophys. Res. Commun. *21*:290-296.
18. **Lüderitz, O., M. Freudenberg, C. Galanos, V. Lehmann, E. Rietschel and D. Shaw.** 1982. Lipopolysaccharides of gram-negative bacteria. Current Topics in Membranes and Transport. Vol. 17. Academic Press, New York.
19. **Morrison, D.C. and D.M. Jacobs.** 1976. Binding of polimixin B to the lipid A portion of bacterial lipopolysaccharides. Mol. Immunol. *13*:813-818.
20. **Raetz, C.R.H.** 1993. Bacterial endotoxins: extraordinary lipids that activate eukariotic signal transduction. J. Bacteriol. *175*:5745-5753.
21. **Tsai, C.M. and C.E. Frash.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. *119*:115-119.
22. **Westphal, O. and K. Jann.** 1965. Bacterial lipopolysaccharides, p. 83-92. *In* R. Whistler (Ed.), Methods in Carbohydrate Chemistry. Vol. 5. Academic Press, New York.

We thank G. Morris (Division of Infectious Diseases, Department of Medicine, University of Maryland, Baltimore, MD, USA) for providing the V. cholerae *O139 Bengal AI1837 strain, M.T. Painceira for providing the* E. coli *strain, H. Lopardo for providing the* S. typhimurium *strain and M.C. Magariños for providing the* Salmonella paratyphi *strain (the last three from Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina). We are greatly indebted to Catalina B. López for her excellent technical assistance. C.V. and D.F.H. were supported by the Universidad Nacional de Quilmes and Universidad Nacional de La Plata, respectively. A.L. is a member of the Research Career of CONICET (Argentina). This work was supported by grants from CONICET and CICBA (both from Argentina). Address*

correspondence to Antonio Lagares, Instituto de Bioquímica y Biología Molecular, Facultad de Ciencias Exactas, Universidad Nacional de La Plata. 47 y 115. La Plata (1900). Buenos Aires, Argentina.

Received 22 January 1996; accepted 8 July 1996.

Claudio Valverde[1], Daniela F. Hozbor and Antonio Lagares
Universidad Nacional de La Plata
Buenos Aires
[1]Universidad Nacional de Quilmes
Buenos Aires, Argentina

Cerium Oxide Removes Background Fluorescent Contaminants, "Green Smears", From Glass Plates Used for Automated Fluorescence-Based DNA Sequencing

BioTechniques 22:236-238 (February 1997)

The predominant automated DNA sequencers found in laboratories today use a laser-optics fluorescent dye detection system. In this format, fluorescent dye-labeled DNA fragments are excited by a laser as they electrophorese past a fixed point. The emitted fluorescence is scanned and collected by either a photomultipler tube (PMT) or a charge-coupling device camera (CCD) in the case of the Models 373A and 377 instruments (PE Applied Biosystems, Foster City, CA, USA). One of the problems associated with automated fluorescence-based DNA sequencing is the appearance of background fluorescence, which can obscure and interfere with signal collection. There appears to be two types of such backgrounds, referred to commonly as a "green smear". One such smear is mobile and migrates with electrophoresis. The second appears fixed and may be either transient or permanent. This second class may be generated by factors such as the general accumulation and deposits of chemicals used in acrylamide gel formation or during plate preparation. In addition, other mechanical problems, impacting gel pouring, may occur from a buildup of residues associated with repeated cleaning of glass plates such as carbonates and silicates from tap water, detergents or organic chemicals. Also, we have learned from experience that glass plates may even come from the supplier with preexisting fluorescent backgound contaminants. These contaminants have proven resistant to conventional cleaning methods. The cleanliness and clarity of the glass plates, especially at the signal collection point, is of utmost importance. We have solved this problem by polishing the glass plates with cerium oxide (CeO).

The use of cerium oxide in the preparation of glass plates for manual radioactive DNA sequencing has been described previously by Millard and de Couet (1). Their main concern was the elimination of glass plate surface nonhomogeneity and nucleation sites due to chemical deposits and microscopic scratches. These problems, which also include "grease spots", are a consequence in the fluorescence-based sequencing format. In particular, the use of ultrathin gels (0.2-mm gels) for the Model 377 requires absolutely clean plates for gel pouring. As described, CeO is an inert nontoxic compound with mildly abrasive qualities that make it ideal for polishing glass and other optical surfaces. It is available in different particle sizes to accommodate a wide range of applications from polishing computer chips to automobile windshields. For example, CeO with an average particle size of 3.4 μm is used for polishing flat optical systems such as mirrors, TV/CRT faceplates and ophthalmic lenses (J. Ritter, personal communication, Universal Photonics, Hicksville, NY, USA). Because of the success in cleaning glass plates for manual radioactive DNA sequencing, we were interested in whether the green smear problem could also be solved with CeO. In contrast to its usage with manual radioactive sequencing systems, which do not rely on the optical clarity of a section of the plates, we had two concerns in applying this technique to fluorescence-based sequencing systems. Our first concern was that the

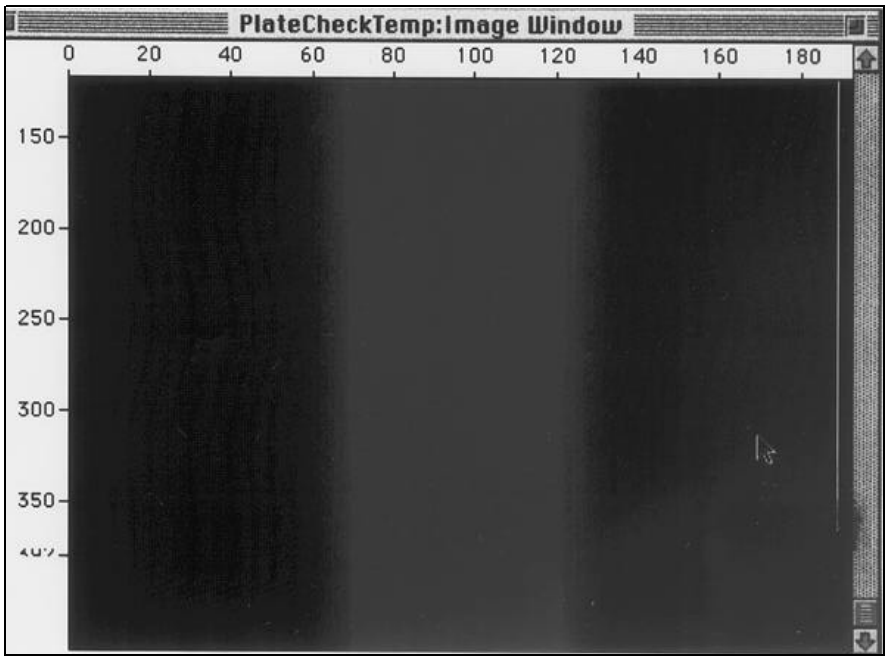

Figure 1. Plate check of an acrylamide "gel sandwich" with both glass plates. Before CeO treatment, the profile of a sequencing gel in the Model 377 DNA sequencer shows a background "green smear". The x-axis is a measurement of the "channels" (or 194 data points collected by sensors along the sequencing plate from side to side by the CCD-camera) and the y-axis represents arbitrary "ABI fluorescence units".