McCoy and Macpherson Lab
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Protocol 104_Preparation of bacterial LPS from *E. cloacae*

Method:

1. Grow 200ml overnight culture of E cloacae
2. Split in 4. Spin down and wash once in cold PBS
3. Resuspend in 10ml water at 65ºC
4. Add 10ml 90% phenol at 65ºC with vigorous stirring (water-bath in fume hood)
5. keep at 65ºC 15mins
6. Cool to 10ºC in ice bath
7. centrifuge at 3000 rpm (g?) for 45mins
8. Remove aqueous phase to fresh tube. Add back 10ml of 65ºC water and repeat

9. Combined aqueous extracts are dialyzed against ddH2O for 4 days
10. To the remaining organic phase add isopropyl alcohol to precipitate protein and some DNA still present as a control fraction. Spin and recover solids

11. Centrifuged at 3000 rpm (g?) to remove insoluble material
12. Lyophilized to a fluffy white powder yield approx 5% of starting dry bacterial mass which contains 40-50% RNA and 50-60% LPS

STEP 2! OR WE COULD USE hydrophobicity interaction chromatography HPLC... see Fischer 1990 Eur J Biochem. 194:655-661

1. Dissolve crude extract in 0.5M NaCl to 1%
2. Add 2% cetavlon (cetryltrimethlyammonium bromide) in 0.5M NaCl with stirring until proportion is 3:2 cetavlon: starting solution.
3. Gradually dilute the solution with water. RNA precipitates at 0.3M NaCl and can be collected separately
4. Remaining dilute solution is lyophilized, then dissolved in 0.5M NaCl
5. Solution is poured into a 10-fold volume of 100% ethanol to precipitate LPS
6. Centrifuge to collect solids and dissolve in water at 5mg/ml (approx)

STEP 3!

1. Resuspend LPS in 1ml ddH2O containing 0.2% TEA (sigma no T0886) – make fresh. Final conc of LPS 5mg/ml
2. Transfer 500µl to new eppendorf and keep remaining 500µl for analysis
3. Add 25µl of 10% sodium deoxycholate (Sigma no D5670) (final conc 0.5%)
4. Add 500µl rm temp water-saturated phenol
5. Vortex intermittently for 5mins, then allow to sit for 5mins at rm temp, then place on ice for 5 mins.
6. Spin at 4ºC for 2mins at 10000g
7. Transfer top (aqueous) layer to fresh tube (c425µl)
8. Add 425µl of 0.2% TEA plus 22µl 10% DOC to the remaining phenol phase and repeat mixing and centrifuging
9. Mix the two extracted aqueous phases, then split again into 2 tubes
10. To each, add 500µl of water-saturated phenol and repeat mixing and centrifuging
11. Mix the two aqueous phases then split again into 2 alqiuots of 400µl

12. To each, add 1100µl of 100% EtOH plus 15µl 3M sodium acetate pH 5.2
13. Vortex and incubate at -20øC for 1hr
14. Spin at 4ºC for 10mins at 10000g
15. Pour off ethanol supernatant and air-dry the LPS pellets
16. Dissolve in 0.2% TEA
17. Store at 4ºC

Analysis

Pour 15% Tris-glycine SDS-PAGE gel x 2
Pour agarose gel x 1

Run the 0.1 starting volume equivalents on SDS-PAGE x2 and agarose x1
Visualised agarose gel by ethidium bromide (RNA contamination)

Take one gel and stain with periodic silver acid stain (protein, carbohydrate, nucleic acid and lipid)
Transfer second gel to nitrocellulose and stain with oil red 0 (lipid only)

Adapted from:

Westphal and Jann, 1965. Methods in Carbohydrate Chemistry. 5:83

AND (Step 3)
C.L. Manthey, P.Y. Perera, B.E. Henricson, T.A. Hamilton, N. Qureshi, and S.N. Vogel. Endotoxin-induced early gene expression in C3H/HeJ (Lps^d) macrophages. *J. Immunol.* 153: 2653-2663 (1994).

C.L. Manthey and S.N. Vogel. Elimination of trace endotoxin protein from rough chemotype LPS. *J. Endotoxin Res.* 1: 84-91 (1994).

M. Hirschfeld, Y. Ma, J.H. Weis, S.N. Vogel, and J.J. Weis. Cutting Edge: Repurification of LPS eliminates signaling through both human and murine Toll-like receptor 2. *J. Immunol.* 165: 618-622 (2000).