Immunoprecipitation of Cell Surface Proteins from Gram-negative Bacteria

Carlos Eduardo Pouey da Cunha[1, 2, #], Jane Newcombe[1, #],

Odir Antonio Dellagostin[2] and Johnjoe McFadden[1, *]

[1]School of Biosciences and Medicine, Faculty of Health and Medical Sciences, University of Surrey, Guildford, Surrey GU2 7XH, UK; [2]Biotecnologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Pelotas, Rio Grande do Sul, Brasil

*For correspondence: j.mcfadden@surrey.ac.uk

#Contributed equally to this work

[Abstract] The meningococcus (*Neisseria meningitidis*) remains an important threat to human health worldwide. This Gram-negative bacterium causes elevated disabilities and mortality in infected individuals. Despite several available vaccines, currently there is no universal vaccine against all circulating meningococcal strains (Vogel *et al.*, 2013). Herein, we describe a new protocol that is capable of identifying only cell surface exposed proteins that play a role in immunity, providing this research field with a more straightforward approach to identify novel vaccine targets. Even though *N. meningitidis* is used as a model in the protocol herein described, this protocol can be used for any Gram-negative bacteria provided modifications and optimizations are carried out to adapt it to different bacterial and disease characteristics (*e.g.*, membrane fragility, growth methods, serum antibody levels, *etc.*).

Keywords: Gram-negative, Immunoproteome, Immunoprecipitation, Cell surface antigen, Outer membrane protein, Exposed antigen

[Background] Attempts to develop novel vaccines against *N. meningitidis* often rely on 2D SDS-PAGE (two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and Western blot followed by MS (mass spectrometry) (Wheeler *et al.*, 2007). However, such approach employs whole cell lysate, identifying a plethora of proteins that do not have vaccine potential (Mendum *et al.*, 2009). We therefore aimed at developing a method capable of identifying only cell surface exposed proteins that might play important role in immunity. Briefly, our protocol consists in growing the pathogen of interest, immunoprecipitating surface antigens with sera of immune individuals, and identifying immunoprecipitated proteins by liquid chromatography-tandem mass spectrometry. We were able to identify 23 meningococcal surface antigens using this new protocol, some of which are components of commercially available vaccines (Newcombe *et al.*, 2014). We also have adapted this protocol to other Gram-negative bacteria and have obtained promising results: we identified previously described surface-exposed proteins, many of which have already been tested as vaccine or diagnostic test candidates. These results show this is a robust technique that can be applied to a diverse range of Gram-negative bacteria and capable of yielding high-quality results that can be further exploited by a myriad of applications (*e.g.*, vaccines, diagnosis, *etc.*).

Materials and Reagents

1. Disposable Petri dishes (Cromwell Group, catalog number: STS3855002B)
2. L-shaped cell spreaders (Fisher Scientific, catalog number: 14-665-231)
3. Disposable inoculating loop (Sigma-Aldrich, catalog number: I8388)
4. 1.5 ml microcentrifuge tubes (Corning, Axygen®, catalog number: MCT-150-C)
5. Protein LoBind tube (Eppendorf, catalog number: 022431102)
6. 20 ml plastic universals (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 128BFS)
7. Plastic sealable bags (Fisher Scientific, catalog number: 10366984)
   Manufacturer: MINIGRIP, catalog number: BAJ-340-091N.
8. Pierce™ spin columns (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 69725)
9. Disposable sterile scalpel No. 10 (WMS, catalog number: W259)
10. 10 µl Filter tips (STARLAB INTERNATIONAL, TipOne®, catalog number: S1121-3810)
11. 20 µl Filter tips (STARLAB INTERNATIONAL, TipOne®, catalog number: S1120-1810)
12. 200 µl Filter tips (STARLAB INTERNATIONAL, TipOne®, catalog number: S1120-8810)
13. 1,000 µl Filter tips (STARLAB INTERNATIONAL, TipOne®, catalog number: S1122-1830)
14. Neisseria meningitidis (strains L9153 and MC58, Public Health England)
15. Phosphate-buffered saline (PBS) (Thermo Fisher Scientific, Gibco™, catalog number: 10010001)
16. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A9418)
17. Human serum (Sigma-Aldrich, catalog number: H6914)
18. Disease state serum (acquisition of this varies and depends on the pathogen being investigated)
19. Pierce™ Protein A/G UltraLink™ Resin (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 53132)
20. SeeBlue® protein marker (Thermo Fisher Scientific, Novex™, catalog number: LC5925)
21. Dithiothreitol (DTT) (Sigma-Aldrich, catalog number: 43815)
22. Iodoacetamide (Sigma-Aldrich, catalog number: I6125)
23. Invitrogen™ Novex™ NuPAGE™ 10x sample reducing agent (Thermo Fisher Scientific, Novex™, catalog number: NP0004)
24. Invitrogen™ Novex™ NuPAGE™ 4x LDS loading buffer (Thermo Fisher Scientific, Novex™, catalog number: NP0007)
25. Invitrogen™ Novex™ NuPAGE™ 12% Bis-Tris 1 mm–10 wells (Thermo Fisher Scientific, Invitrogen™, catalog number: NP0341BOX)
26. Invitrogen™ Novex™ NuPAGE™ MOPS running buffer 20x (Thermo Fisher Scientific, Novex™, catalog number: NP000102)
27. SimplyBlue™ Safe Stain (Thermo Fisher Scientific, Novex™, catalog number: LC6060)
28. Acetonitrile for HPLC-MS (Fisher Scientific, catalog number: 10616653)

www.bio-protocol.org/e2250

Vol 7, Iss 09, May 05, 2017
DOI:10.21769/BioProtoc.2250

29. Ammonium bicarbonate (Sigma-Aldrich, catalog number: 09830)

30. Trypsin Gold, mass spectrometry grade (Promega, catalog number: V5280)

31. Columbia blood agar base (Oxoid, catalog number: CM0331)

32. Horse blood, defibrinated (Oxoid, catalog number: SR0050)

33. Tris base (Roche Diagnostics, catalog number: 10708976001)

34. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S9625)

35. EDTA (Sigma-Aldrich, catalog number: E6758)

36. Triton X-100 (Sigma-Aldrich, catalog number: X100)

37. Sodium deoxycholate (Sigma-Aldrich, catalog number: 30970)

38. Sodium dodecyl sulfate (SDS) (Sigma-Aldrich, catalog number: L3771)

39. Formic acid LC/MS (Fisher Scientific, catalog number: A117-50)

40. Water for HPLC-MS (Fisher Scientific, catalog number: 10777404)

41. Methanol (Sigma-Aldrich, catalog number: 179957-1L)

42. Acetic acid (Sigma-Aldrich, catalog number: A6283)

43. CAB medium (see Recipes)

44. PBS plus 1% BSA (see Recipes)

45. Solubilization buffer (see Recipes)

46. 1% SDS (see Recipes)

47. 1 M dithiothreitol stock solution (see Recipes)

48. 1 M Iodoacetamide (see Recipes)

49. 25 mM ammonium bicarbonate (see Recipes)

50. Acetonitrile (50% v/v) in 25 mM ammonium bicarbonate (see Recipes)

51. Acetonitrile (50% v/v) (see Recipes)

52. Solvent A for HPLC (see Recipes)

53. Solvent B for HPLC (see Recipes)

54. 0.1% formic acid/50% acetonitrile solution (see Recipes)

Equipment

1. Gilson Pipette Pipetman Classic P2 (Gilson, catalog number: F144801)

2. Gilson Pipette Pipetman Classic P20 (Gilson, catalog number: F123600)

3. Gilson Pipette Pipetman Classic P200 (Gilson, catalog number: F123601)

4. Gilson Pipette Pipetman Classic P1000 (Gilson, catalog number: F123602)

5. Eppendorf® Micro centrifuge 5415R (Eppendorf, model: 5415 R)

6. BOD low temperature refrigerated incubator (VWR, model: BOD Incubator 2005, catalog number: 35960-056)

7. CO_2 incubator (LEEC, model: Culture Safe Precision 190D)

8. Stuart Gyratory rocker (Cole-Parmer, Stuart, model: SSL3)

9. Vortex Mixer SA8 (Cole-Parmer, Stuart, model: SA8)

10. Ultrasonic bath (Fisher Scientific, model: Fisherbrand™ S-Series, catalog number: 10611983)

11. Ultimate 3000 nano HPLC system (Thermo Fisher Scientific, Thermo Scientific™, model: UltiMate™ 3000 RSL Cnano System)

12. Acclaim™ C-18 Pepmap™ column (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 164569)

13. QSTAR® XL hybrid LC/MS/MS system (Applied Biosystems, model: QSTAR® XL Hybrid LC/MS/MS System)

14. Eppendorf MixMate™ (Fisher Scientific, catalog number: 21-379-00)
   Manufacture: Eppendorf, catalog number: 022674200.

15. Eppendorf ThermoStat Plus interchangeable block heater (Eppendorf, model: ThermoStat Plus, catalog number: 022670204)

16. Eppendorf Vacufuge™ concentrator (Eppendorf, model: Vacufuge™ Concentrator, catalog number: 022820001)

17. Heraeus Tube roller Spiramix (Fisher Scientific, catalog number: 10600653)
   Manufacturer: Thermo Fisher Scientific, Thermo Scientific™, catalog number: DS507.

18. Fume hood

Software

1. Chromeleon v6.8 or later (Dionex, Thermo Fisher Scientific)
2. Analyst QS 1.1 (Applied Biosystems)
3. Mascot v2.2.04 or later (Matrix Science, London)

Procedure

A. Cell surface immunoprecipitation

1. Grow *N. meningitidis* from frozen stock: Obtain a small amount of frozen culture with a sterile 200 μl tip, inoculate Columbia agar base (CAB) plate and spread to give single colonies. Incubate overnight (~18 h) at 35 °C with 5% CO_2. Collect 20-30 colonies and spread over the entire surface of a CAB plate, using a sterile loop, and incubate for 4 h at 35 °C with 5% CO_2. Individual colonies will not be seen at this stage.

2. Recover bacteria by adding PBS to the plate and mixing the colonies into the PBS using a sterile loop. Remove the PBS/bacteria suspension using a P1000 micropipette (1 ml per plate, use half plate for each immunoprecipitation), centrifuge for 2 min at 13,000 *x g* at room temperature, Wash pellet three times with 1 volume PBS plus 1% BSA. For these and subsequent wash steps, pellet cells by centrifugation for 2 min at 13,000 *x g* at room temperature, remove supernatant, add fresh buffer and resuspend the pellet.
   Notes:

    a. If using liquid culture instead of plates, we suggest harvesting cells at mid-log growth phase and conditions that favor the expression of virulence factors. We suggest researchers do a growth curve with their organism of interest or have good knowledge of how the organism grows in liquid media to be able to properly identify growth phases.

    b. Centrifugation conditions may vary according to the bacterium in question. The presence of lysed cells will increase the selection on non-surface proteins.

    c. Make every effort to ensure that early centrifugation steps will not disrupt cells while removing secreted proteins or components of lysed, dead cells (see Note 2).

3. Suspend pellet from previous step in 1 ml of serum sample (control or test [items 17 and 18 of Materials and Reagents list, respectively]) and rotate for 90 min at 4 °C.

   Note: Proteins identified in the control serum samples should be disregarded in future analysis as they are likely to play no role in the immune response against the pathogen. Researchers should make sure the control sera they have is indeed negative against for the pathogen they are investigating.

4. Pellet the cells (13,000 x g, 5 min, RT) and wash twice with PBS plus 1% BSA.

5. Resuspend cells in 1 ml solubilization buffer and incubate for 60 min at 37 °C.

6. Centrifuge sample (13,000 x g, 60 min, RT), collect supernatant and incubate with protein A/G UltraLink beads (50 µl) for 18 h at 4 °C with agitation or rolling.

7. Centrifuge sample (2,500 x g, 2 min, RT), collect beads.

8. Wash beads five times (2,500 x g, 2 min) with 1 volume solubilization buffer.

9. Elute antibody:antigen complex with 1 volume SDS (1%) and boiling (10 min).

10. Add 4 volumes of water and vortex to elute proteins. Centrifuge (2,500 x g, 3 min, RT), and collect supernatant; the increase in volume allows for a more efficient removal of proteins during vortexing.

11. Concentrate sample back to 50 µl so SDS concentration is back to 1% (approximately 45 min at 45 °C using Speed-Vac). At this point, the samples can be mixed with loading buffer and run on an SDS PAGE gel. Proceed to step B1. If the samples are being prepared for mass spectrometry analysis, the proteins can be reduced and alkylated prior to separation. Reduce the eluted proteins by adding DTT to a final concentration of 10 mM and boil for 2 min (for 50 µl, add 0.5 µl of 1 M DTT stock solution).

12. Add iodoacetamide to a final concentration of 50 mM (for 50 µl, add 2.5 µl of 1 M IAA stock solution) and incubate for 30 min at room temperature in the dark.

B. 1D SDS-PAGE and sample digestion

1. Mix reduced, alkylated samples with loading buffer (item 23 of Materials and Reagents list) and boil for 5 min.

2. Separate proteins by SDS-PAGE (12%, 1 mm thick, 10 cm long).

3. Stain with SimplyBlue™ for 1 h and de-stain with ultra-pure water (according to manufacturer's instructions–see Figure 1).

Figure 1. Representative image of immunoprecipitated (IP) surface proteins of the meningococcus. Reproduced from Newcombe *et al.* (2014).

4. Cut gel lanes in as many slices as necessary based on sample protein concentration and place slices in low protein bind tubes.

   Note: A full gel lane can be cut into 20 slices, however, it may only be necessary to run a shortened gel and cutting it into 5 slices.

5. Wash gel slices with 50% acetonitrile in 25 mM ammonium bicarbonate twice with vortexing for 15 min at room temperature to remove stain–use enough volume to cover the gel slices.

6. Dehydrate gel slice by incubation with 100% acetonitrile (20 min, room temperature).

7. Dry gel pieces using a Speed-Vac to dryness (about 20 min).

8. Rehydrate in 25 mM ammonium bicarbonate containing modified trypsin (12.5 ng/µl–the final volume will depend on the size of the gel slice, 4 °C for 10 min). If required add more 25 mM ammonium bicarbonate to cover gel pieces, incubate overnight at 37 °C.

9. Remove digest solution, extract digested proteins by incubation with 30 µl 0.1% formic acid/50% acetonitrile for 20-30 min with vortexing followed by 5 min in a sonicating bath (40 kHz)–repeat once and collect supernatant each time.

10. Completely dry extracted digested proteins using Speed-Vac and resuspend in 20 µl water. Store at -20 °C.

C. Liquid chromatography-electrospary ionization-MS/MS (please see Notes)

   1. Separate peptides using Ultimate 3000 HPLC and C18 pepman (Dionex) column at 40 °C, 350 nl/min, gradient of 2-50% solvent B for 30 min, 90% solvent B for 5 min–controlled by Chromeleon software.

   2. Set QSTAR XL to 2.3 kV electrospray, curtain gas at 8 arbitrary units, and 1 sec survey from 400 to 1,200 *m/z* with charge states 2-4.

   3. Acquire MS data with Information Data Acquisition with Analyst QS 1.1 software.

   4. Select most intense product ions with *m/z* of between 65 and 1,200 amu for MS/MS.

D. Database analysis (please see Notes)

   1. Submit peak lists of MS/MS spectra to Mascot Server (v2.2.04; Matrix Science) and analyse using the MS/MS Ions search programme.

   2. Allow up to one missing trypsin cleavage with fixed modification of carbamidomethyl (C) and variable modification of oxidation (M).

3. Set peptide and MS/MS fragment tolerance to 1.2 and 0.6 Da respectively and select MH_2^{2+} and MH_3^{3+} as the precursor charge states.

4. Search identified proteins against the National Center for Biotechnology Information nonredundant (NCBInr) database with the taxonomy set *N. meningitidis* (taxonomy code 487). Analyse the data with the appropriate database for the organism being used.

Data analysis

Once a list of identified proteins is obtained it should be checked to ensure all proteins are surface exposed. This can be done using cellular localization prediction tools such as PSORTb v3.0 (Yu *et al.*, 2010), CELLO v2.5 (Yu *et al.*, 2004 and 2006) or Gneg-mPLoc (Shen and Chou, 2010). Searching for signal peptides or lipidation can also be useful. We recommend the following tools: LipoP 1.0 (Juncker, 2003), SignalP 4.1 (Petersen *et al.*, 2011), Signal-CF (Chou and Shen, 2007), and PrediSi (Hiller *et al.*, 2004). To further assess protein location prediction, researchers are encouraged to look for β-barrel proteins and dismiss proteins containing transmembrane α-helix (beware that this conformational 'rule' does not apply to lipoproteins, only outer membrane proteins). There are several techniques to assess experimentally the location of predicted proteins and they vary according to the bacterium being studied. Cell fractionation followed by Western blot, indirect immunofluorescence (unfixed samples), and cell surface proteolysis among other techniques may be suitable as well. To assess the localization of candidate proteins of the meningococcus, we produced serum against candidate proteins, probed intact bacteria with this serum, and added FITC-conjugated secondary antibodies and analysed single-cells by flow cytometry. The detection of fluorescence would confirm the surface exposure of a given candidate protein as demonstrated in Figure 2. Please refer to Newcombe *et al.*, 2014 for more details on this technique and other techniques we used.
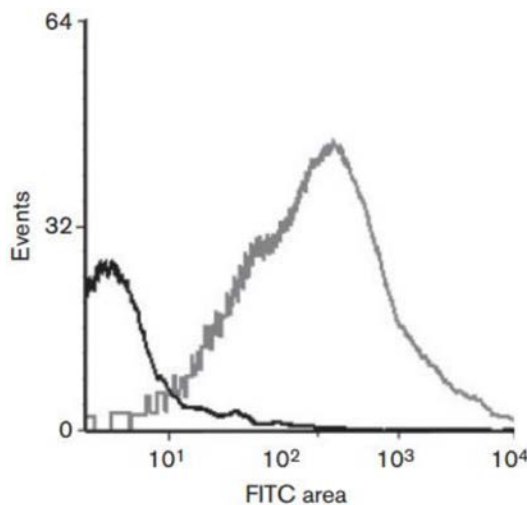

Figure 2. Representative data of flow cytometry experiment to assess whether a candidate protein is surface exposed or not. Cells probed with sera against surface exposed

proteins and FITC show a stronger signal (grey line) than the negative control (black line). Reproduced from Newcombe *et al.* (2014).

Notes

1. Serum samples should be selected based on immunity against the bacteria of interest, this can be healthy sera which has shown to be confer immunity or sera specifically raised against the target organism. The antibody titre against the antigen will determine the amount of serum required to immunoprecipitate sufficient antigenic proteins.

2. In addition to SDS-PAGE of immunoprecipitated proteins, we also recommend performing Western blots to confirm the presence of immunogenic proteins. The presence of other protein bands would indicate the extraction of non-specific proteins. It is essential to run controls including an antibody negative sample. It will be necessary to ensure the serum contains antibodies to the bacteria. The titre of the serum will affect the amount required to immunoprecipitate detectable levels of specific immunogenic proteins. The use of serum against non-surface proteins (*e.g.*, periplasmic proteins, inner membrane proteins, *etc.*) is also advised to ensure there are no contamination of proteins that are not surface exposed.

3. Step A7 of immunoprecipitation can be done using the spin columns or carefully pipetting off the supernatant.

4. Mass spectrometry parameters are specific to the instrumentation available to the researchers. We report here specifications that we used, but researchers should refer to their own equipment instructions to find out how to better adjust their equipment. There are also other ways to analyse MS files (Procedure D). Other software and methodologies are available. We demonstrate here what software we incorporated in our analysis. It is recommended that the immunoprecipitated sample be split and or multiple samples analysed to enhance the reliability of the data.

5. The preparation of peptides for the mass spectrometer analysis, the mass spectrometer data collection, and subsequent data analysis will depend on the facility performing the analysis. If this is in house or to be outsourced discuss the sample preparation before preparing the samples. All facilities have set procedures to ensure maximum output.

Recipes

1. CAB medium
   39 g Columbia agar base
   Distilled water to 1 L
   Autoclave (121 °C, 15 min)
   Cool to 50 °C and add 6% sterile defibrinated blood

2. PBS plus 1% BSA

    1 g BSA

    PBS to 100 ml final volume, keep refrigerated

3. Solubilization buffer

    3.0028 g Tris, 50 mM, pH 7.8

    4.38 g NaCl, 150 mM

    0.146 g EDTA (or dilute from 500 mM stock solution), 1 mM

    1% (v/v) Triton X-100

    1 g sodium deoxycholate, 0.2%

    0.5 g SDS (or dilute from 10% stock solution–weight in fume hood), 0.1%

    Distilled water (to a final volume of 500 ml)

4. 1% SDS

    1 g SDS

    100 ml LC/MS water

    Weight in fume hood

5. 1 M dithiothreitol stock solution

    1.542 g DTT

    LC/MS water to 10 ml, keep at -20 °C

    Weight in fume hood

6. 1 M Iodoacetamide stock solution

    1.84 g IAA

    Distilled LC/MS water to 10 ml

7. 25 mM ammonium bicarbonate

    0.998 g ammonium bicarbonate

    LC/MS water to 500 ml

8. Acetonitrile (50% v/v) in 25 mM ammonium bicarbonate

    50 ml acetonitrile

    50 ml ammonium bicarbonate (see above)

9. Acetonitrile (50% v/v)

    50 ml acetonitrile

    50 ml LC/MS water

10. Solvent A for HPLC

    2 ml acetonitrile

    0.1 ml formic acid

    97.9 ml LC/MS water

11. Solvent B for HPLC

    90 ml acetonitrile

    0.1 ml formic acid

    9.9 ml LC/MS water

12. 0.1% formic acid/50% acetonitrile solution

0.1 ml formic acid

50 ml acetonitrile

49.9 ml LC/MS water

Acknowledgments

References

1. Chou, K. C. and Shen, H. B. (2007). Signal-CF: a subsite-coupled and window-fusing approach for predicting signal peptides. *Biochem Biophys Res Commun* 357(3): 633-640.

2. Hiller, K., Grote, A., Scheer, M., Munch, R. and Jahn, D. (2004). PrediSi: prediction of signal peptides and their cleavage positions. *Nucleic Acids Res* 32(Web Server issue): W375-379.

3. Juncker, A. S., Willenbrock, H., Von Heijne, G., Brunak, S., Nielsen, H. and Krogh, A. (2003). Prediction of lipoprotein signal peptides in Gram-negative bacteria. *Protein Sci* 12(8): 1652-1662.

4. Mendum, T. A., Newcombe, J., McNeilly, C. L. and McFadden, J. (2009). Towards the immunoproteome of *Neisseria meningitidis*. *PLoS One* 4(6): e5940.

5. Newcombe, J., Mendum, T. A., Ren, C. P. and McFadden, J. (2014). Identification of the immunoproteome of the meningococcus by cell surface immunoprecipitation and MS. *Microbiology* 160(Pt 2): 429-438.

6. Petersen, T. N., Brunak, S., von Heijne, G. and Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat Methods* 8(10): 785-786.

7. Shen, H. B. and Chou, K. C. (2010). Gneg-mPLoc: a top-down strategy to enhance the quality of predicting subcellular localization of Gram-negative bacterial proteins. *J Theor Biol* 264(2): 326-333.

8. Vogel, U., Taha, M. K., Vazquez, J. A., Findlow, J., Claus, H., Stefanelli, P., Caugant, D. A., Kriz, P., Abad, R., Bambini, S., Carannante, A., Deghmane, A. E., Fazio, C., Frosch, M., Frosi, G., Gilchrist, S., Giuliani, M. M., Hong, E., Ledroit, M., Lovaglio, P. G., Lucidarme, J., Musilek, M., Muzzi, A., Oksnes, J., Rigat, F., Orlandi, L., Stella, M., Thompson, D., Pizza, M., Rappuoli, R., Serruto, D., Comanducci, M., Boccadifuoco, G., Donnelly, J. J., Medini, D. and Borrow, R. (2013). Predicted strain coverage of a meningococcal multicomponent vaccine (4CMenB) in Europe: a qualitative and quantitative assessment. *Lancet Infect Dis* 13(5): 416-425.

9. Wheeler, J. X., Vipond, C. and Feavers, I. M. (2007). Exploring the proteome of meningococcal outer membrane vesicle vaccines. *Proteomics Clin Appl* 1(9): 1198-1210.

![bio-protocol logo]

10. Yu, C. S., Chen, Y. C., Lu, C. H. and Hwang, J. K. (2006). Prediction of protein subcellular localization. *Proteins* 64(3): 643-651.

11. Yu, C. S., Lin, C. J. and Hwang, J. K. (2004). Predicting subcellular localization of proteins for Gram-negative bacteria by support vector machines based on n-peptide compositions. *Protein Sci* 13(5): 1402-1406.

12. Yu, N. Y., Wagner, J. R., Laird, M. R., Melli, G., Rey, S., Lo, R., Dao, P., Sahinalp, S. C., Ester, M., Foster, L. J. and Brinkman, F. S. (2010). PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. *Bioinformatics* 26(13): 1608-1615.