Bioplatforms Australia Antibiotic Resistant Sepsis Pathogens Initiative
Highly purified Genomic DNA from *Streptococcus pyogenes* for PacBio sequencing (Amanda Walker)

- The protocol below works very well for both overnight and log phase cultures.
- The advantage of this method is that it should result in very long sequence reads in PacBio sequencing allowing the production of just one or two contigs for the genome and plasmids from any strain.
- It has also been used with success on *Salmonella* and other enteric pathogens. This protocol can be scaled down for smaller volumes.
- All chemicals should be obtained from Roche where possible. The quality of PacBio directly relates to quality of the gDNA.
- For Sanger PacBio, aim is to submit >5 ug of high quality (low sheared) gDNA.

1. Grow up overnight 10mls of culture. Any media is fine (I used THY). Shaking or non-shaking also does not significantly affect DNA integrity.
2. Spin down at 7, 000 x g for 10 min.
3. Re-suspend cell pellet in 2mls of 25% Sucrose in TE (25 grams Sucrose in TE consisting of 10mM Tris and 1mM EDTA pH8.0).
4. Add 50ul of 100mg/ml Lysozyme and 20 ul of 5000U/ml mutanolysin, leave at 370C for >1 hour.
5. Add 100ul of 20mg/ml Proteinase K (Roche product 03 115 828 001 - it is supplied as a 18mg/ml solution so use neat).
6. Add 30ul of 10mg/ml RNase A (Roche product 10 109 142 001 - boil before use then freeze).
7. Add 400ul of 0.5M EDTA pH8.0 (Na salt).
8. Add 250 ul of fresh 10% Sarkosyl NL30 (BDH/Fisher 442753R - comes as 30% solution). Now leave on ice for 1 hour or until lysis is almost complete (slightly turbid but viscous) or if possible complete (almost clear but still viscous).
9. Leave in 50°C water bath for 2 hours.
10. Make up to 6 mls with TE (10mM Tris, 1mM EDTA pH8.0) and mix gently to equilibrate.

The next stages involve Phenol-Chloroform extraction (Fluka-Sigma is fine for these products)

1. Mix DNA sample with 5ml **Phenol:Chloroform:Isoamyl Alcohol** (25:24:1) in a phase lock tube (PLG)
2. Centrifuge for 15 minutes at 4000 rpm to separate the phases.
3. Remove aqueous phase into a fresh PLG tube and repeat **step 1**. Centrifuge for 10 minutes at 4000 rpm to separate the phases once again.
4. Remove aqueous phase into a fresh PLG tube and **extract for a third time** with **Phenol:Chloroform:IAA (**25:24:1). **With experience** you could just do two Phenol:Chloroform:IAA extractions.
5. Centrifuge for 5 minutes at 4000 rpm and recover aqueous top layer.

6. Remove aqueous phase into a fresh PLG tube and extract once with equal volume of **Chloroform:IAA** (24:1).
7. Centrifuge for 5 minutes at 4000 rpm.
8. **Repeat Chloroform:IAA extraction once more**
9. Transfer final aqueous phase from both tubes to a 50ml Falcon tube and add **2.5 volumes of Ethanol to precipitate the DNA**. Incubate at -80°C for 15 minutes. It may be possible to see the DNA easily as 'cotton wool' like effect afterwards. If you do, scoop it out or spin it down at 4000 rpm for 10 minutes.
10. Wash pellet with 25mls of **70% Ethanol**.
11. Dry gently to remove traces of Ethanol but leave the pellet slightly moist and re-suspend in 350ul of 10mM Tris pH7.4 buffer. It should go into solution very quickly. To speed up this step, leave at 55°C for 1 hour to aid solubility and then store in cold-room (do not freeze so to maintain maximum DNA lengths without nicking!).
12. The DNA is now ready to use....

Eppendorf 1.5ml Phase-lock gels, light (VWR cat no 713-253) or
Eppendorf 15ml Phase-lock gels, light (VWR cat no 713-2537)