**Modified Protocol for Qiagen’s DNeasy 96 Blood & Tissue Kit**

**for use in McKenzie Lab**

This protocol largely follows the Qiagen DNeasy 96 Blood or Cells Protocol with modifications made for extracting from low biomass swab samples of amphibian skin.

**Lysozyme solution to lyse gram-positive bacteria: (from protocols):**

* 20mM Tris-Cl, pH 8
* 2 mM sodium EDTA
* 1.2% Triton X-100
* Immediately before use, add lysozyme to 20mg/mL

**Prepare stock solutions:**

* Autoclave 50mL of deionized water.
* Make 125mL of 0.2M EDTA by mixing 9.30595g of EDTA into distilled water , adding NaOH pellets until pH=8 (186.119g/L). Filter-sterilize.
* 1M Tris-Cl purchased at pH 8. Already sterile.
* Make 10mL of 10% Triton X-100 by weighing 1.07g (1mL volume) and diluting in 9mL sterile deioinized water. Mix thoroughly.

**Working solution (per reaction; multiply by number of samples + 1 “ghost”):**

* 3.6uL 1M Tris-Cl, pH8 (final concentration: 20mM)
* 1.8uL 0.2M EDTA (final concentration: 2mM sodium EDTA)
* 21.6uL 10% Triton X (final concentration: 1.2% Triton X-100)
* 153uL sterile distilled water

Right before use, add 0.0036g (3.6mg) of lysozyme and mix thoroughly to get 20mg/mL

Example calculation for 21 rxn of 180uL

* 75.6uL of 1M Tris-Cl, pH8
* 37.8uL of 0.5M sodium EDTA (372.238g/mol)
* 453.6uL of 10% Triton X-100 (1.07 g/cm³ density)
* 3213 of sterile distilled water
* 0.0756g of lysozyme

**Important points and things to do before starting**

* Do NOT add ethanol to Buffer AL
* If necessary, redissolve any precipitates in Buffer AL by warming to 56°C.
* Before using for the first time, add the appropriate amount of ethanol (96-100%) to Buffers AW1 and AW2 to obtain a working solution. Mark bottles as having ethanol added.
* Mix Buffer AW1 before use by inverting several times.
* Preheat bead bath and single heat block to 37°C for use in step 2; then 56°C for step 4.

\*\* Pre-procedure with lysozyme incubation \*\*

1. Resuspend bacteria in 180uL enzymatic lysis buffer
2. Incubate 30min at 37C (Rebollar et al 2016, ISME); after change heating black back to 56C
3. Add 25uL Proteinase K and 200uL buffer AL (without ethanol); mix by vortexing
4. Incubate 56C for 30 minutes
5. Add 200uL ethanol (96-100%) to sample, and mix thoroughly by vortexing. Ensure that sample is homogenous, and make sure to apply ALL sample (including precipitate) to spin column.

**Procedure**

1. ~~Cut swab samples into blue collection microtube 96 well rack using flame sterilized scissors. Add 200 μl of PBS (see page 14 of DNeasy Blood & Tissue Handbook 07/2006) or PCR water to each well. Add 20 μl proteinase K to each well. Save clear cover of plates for use in step 3.~~
2. ~~Add 200 μl Buffer AL (without added ethanol) to each sample.~~
3. ~~Seal the collection microtubes using the caps provided. Place clear cover over each rack of collection microtubes and shake the racks vigorously up and down for 15 sec. To collect any solution from the caps, centrifuge the plate. Allow the centrifuge to reach 3000 rpm and stop.~~

~~Do not prolong this step. Be sure to shake the plates well to obtain a homogeneous lysate. Save clear covers for use in step 6.~~

1. ~~Incubate plates at 56°C for 20 min in preheated dry bead bath. Place preheated heat block on top of caps during incubation. Mix occasionally to disperse the sample.~~

*~~Centrifuge??~~*

1. ~~Carefully remove the caps and add 200 μl ethanol (96-100%) to each well.~~
2. ~~Seal the collection microtubes using new caps. Place the clear cover over each rack and shake the racks vigorously up and down for 15 sec. Centrifuge briefly (allow centrifuge to reach 3000 rpm and then stop).~~

~~The lysate and ethanol should be mixed immediately and thoroughly to yield a homogeneous solution.~~

1. ~~Place DNeasy 96 plate on top of S-Block and label.~~
2. Remove and discard the caps from the collection microtubes. Carefully transfer the lysis mixture (maximum 900 μl) of each sample from step 6 to corresponding well of the DNeasy 96 plate.
3. Seal DNeasy 96 plate with an AirPore Tape Sheet (provided). Centrifuge for 4 min at 6000 rpm.

After centrifugation, check that all of the lysate has passed through the membrane in each well. If lysate remains in any wells, centrifuge for another 4 min.

1. Remove the tape. Carefully add 500 μl Buffer AW1 to each sample. Be sure ethanol has been added to Buffer AW1 before use.
2. Seal plate with a new AirPore Tape Sheet. Centrifuge for 2 min at 6000 rpm.
3. Remove the tape. Carefully add 500 μl Buffer AW2 to each sample. Be sure ethanol has been added to Buffer AW2 before use.
4. Seal plate with a new AirPore Tape Sheet. Centrifuge for 15 min at 6000 rpm.
5. Place DNeasy 96 plate in correct orientation on a labeled rack of Elution Microtubes RS. Remove the tape and let sit for 5 min to ensure evaporation of any remaining ethanol.
6. To elute the DNA, add 50 μl Buffer AE to each well and seal the plate with a new AirPore Tape Sheet. Incubate for 1 min at room temperature. Centrifuge for 4 min at 6000 rpm.
7. Seal Elution Microtube RS and store at -20°C.