**Protocol 2: Ethanol preserved filters**

Sarah A. Tomke @ University of Kentucky

1/23/2020

Adopted from Spear et al. 2015

Day 1

1. Take out all samples that will be processed during this round of extraction from the -20℃ freezer.
2. One at a time, remove as much ethanol as possible from each tube using a **filter** tip. Using the pipette tip, position the filter paper as best you can so that it is not forming a seal with the side of the tube. Change pipette tips in between each tube.
3. Place all samples into a tube rack. Open the tops carefully as to avoid splatter between tubes.
4. Place the rack on a high shelf or inside a lab hood where it will not be accidently bumped and where the samples will not be exposed to dust.
5. Leave to air-dry overnight.

------------------------------------------------------------------------------------------

Day 2

1. Ensure that all filters have dried completely. If ethanol is still in any of the tubes it will need more time to dry.
2. Close the caps on all sample tubes.
3. Turn heat block to 55°C.

1. Add an extra tube with a clean, unused filter membrane to the set of samples as a negative DNA extraction control. This will ensure that the entire extraction process is monitored with a negative control.

1. Wipe down the work bench with a 10% bleach solution. Make sure bleach dries completely—bleach will destroy any DNA if exposed to filter samples.
2. Set up two beakers for rinsing forceps and scissors: DI water, 50% bleach. Change DI water occasionally throughout extraction procedure since bleach will accumulate in DI water and can degrade DNA.
3. Place **previously** **autoclaved** forceps and scissors onto a clean piece of bench paper/paper towel.
4. Place clean paper towel onto bench to process first sample. Switch paper towels between every sample during the extraction process to avoid any cross-contamination.
5. Wear new, clean gloves. Open the first sample tube. Take out the half-filter carefully with the tweezers and open it onto your work-bench paper towel. Store the second half in the -20℃ (short-term storage) or -80℃ (long-term storage) freezer as a backup.

*\*\*\*NOTE: Filter paper that has been air-dried after being stored in ethanol can be VERY static-y and can be difficult to work with. If this is the case, adding a few drops of* ***DI water*** *to the paper prior to removing it from the tube can help\*\*\**

1. Cut half-filter into about 15 pieces and put pieces back into original pop-top tube using tweezers. Avoid extremely small pieces since these are difficult to transfer to the Qiashredder spin column. Relabel tube if label was wiped off. Place in 4℃ refrigerator for the time being.

*\*\*\*NOTE: When processing filters from the same site/replicate, the same paper towel, scissors, and tweezers can be used\*\*\**

1. Place tweezers and scissors into bleach solution and let sit for at least 5 minutes before transferring to DI rinse water.
2. Wipe down bench with 10% bleach and replace with clean paper towel. Change gloves in between each sample or when possible contamination with other DNA or bleach is believed to have occurred.
3. Repeat steps 8-11 for all samples.
4. Add 250 ul ATL to each tube.

1. Add 20 ul ProK to each tube **individually** with a **filter** tip. Use the tip to push all the filter material down into the liquid at the bottom, even puncturing some holes in the filter paper to help squash it down. It should be fully immersed but seem to take up the whole amount of liquid. Squish it around a little.
2. *Gently* vortex each tube for a few seconds as you finish with it. Intense vortexing will cause the filter pieces to dislodge. If all the paper is not covered with solution, make sure all of it is at least covered by bubbles. If there really doesn’t seem to be enough liquid to cover the filter material, set is aside and top it off with ATL at the end.
3. Parafilm each tube to prevent it from popping open overnight.
4. Incubate at 55°C degrees in a heatblock overnight, vortexing a few more times while incubating.

------------------------------------------------------------------------------------------

Day 3

1. Wipe down the bench with a 10% bleach solution. Place clean paper towel on bench for transferring the samples to the Qiashredder columns, in case any filter paper falls out while being moved. Change paper towel in between each sample or if contamination of DNA or bleach occurs.
2. Set up two beakers for rinsing forceps: DI water, 50% bleach. Change DI water occasionally throughout extraction procedure since bleach will accumulate in DI water and can degrade exposed DNA.
3. Place bleached/rinsed forceps from Day 1 onto a clean piece of bench paper.
4. Remove samples from heatblock and turn up to 70°C. Put Buffer AE on heatblock to warm for later.

1. Gently vortex samples (do not vortex for too long or too intensely as the buffer will become very bubbly and difficult to pipette).
2. Spin down briefly at 8000 rpm for 1 minute.
3. Set out Qiashredder spin columns and **label the sides** of each tube with the sample information.

1. While working over clean paper towel, move the filter pieces to a Qiashredder spin column with sterilized tweezers. Then pipette the liquid using a **filter** tip. Be careful not to lose liquid while transferring the filter pieces. Spin the tube down if necessary and try to get all the liquid. Throw out the original pop-top tube.

*\*\*\*NOTE: When processing filters from the same site/replicate, the same paper towel, scissors, and tweezers can be used\*\*\**

1. Place tweezers into bleach solution and let sit for at least 5 minutes before transferring to DI rinse water.
2. Wipe down bench with 10% bleach and replace with clean paper towel. Change gloves in between each sample or when possible contamination with other DNA or bleach is believed to have occurred.
3. Centrifuge Qiashredder spin columns 5 minutes at 8000rpm.

1. Remove the Qiashredder columns (throw away) and add 200 ul Buffer AL to each sample. Use the caps from Qiashredder kit to close the tubes. Change gloves if potential contamination occurred.
2. Vortex and spin down at 8000 rpm for 1 minute.

1. Incubate at 70°C for 10 minutes.

1. Add 200 ul 100% ethanol (200 proof) to each tube, vortexing aggressively and **immediately** each time (with the caps on well!).
2. **Label the caps** of the spin columns from the DNeasy DNA extraction kit with the Site abbreviation and Replicate number (i.e. 1, 2, or 3). Do not label with liter or filter information. For example, label with SCC 3 (site, replicate), not SCC 3Aa (site, replicate, liter, filter).

1. At this stage the tubes containing the same samples will be combined into the same DNeasy Kit Qiamp Spin column (each tube will have ~660 mL of solution). Add ~500-600 uL of the mixture to a Qiamp Spin column using a **filter** tip. Do NOT overfill the Qiamp spin column or liquid will splatter out when the cap is closed. If liquid is very bubbly in spin column, suck out some bubbles to reduce risk of cross-contamination via splattering.
2. Centrifuge DNeasy Kit Qiamp spin column at 10,000 rpm for 2 minutes.
3. Pour the filtrate into a waste container.

1. Repeat steps 17-19 with the remaining liquid for each sample using the same DNeasy Kit Qiamp Spin Column.
2. Place the Qiamp spin column into a new collection tube. Pour the excess filtrate into the waste container, and discard the old collection tube.

1. Add 500ul AW1 to each sample. Centrifuge at 8000 rpm for 1 minute.

1. Place the Qiamp spin column into a new collection tube. Pour the filtrate into the waste container, and discard the old collection tube.

1. Add 500ul of AW2 and spin at 11,000 rpm for 3 minutes.

1. Label new, clean, screw-top tubes that will hold final DNA.
2. Take out Qiamp spin column carefully so no AW2 splashes on the filter. If this happens centrifuge the sample down again. Place the spin column in the new screw-top tube.

1. Elute the DNA with 100ul Buffer AE (preheated to 70°C). Incubate at room temperature for 5 minutes.
2. Spin at 8000 rpm for 1 minute.

1. Store in the refrigerator (short-term storage) or -20℃ freezer (long-term storage) until ready for PCR.