Wet lab protocol for the Global Sewage Surveillance Project

Pre-handling of sewage samples

Pre-handling of the samples includes spinning down procedures and portioning into fractions. Pre-handling can be performed independently from the next steps (i.e. the DNA extraction). The exact lab set-up is shown in the pictures below

Find the label with the correct sample name, and add a consecutive number in red to the label. Cover the label with a transparent PCR seal, and place the covered label inside the plastic bag with the sample.

Place the sample in the -80°C (Bordeaux)

Later:

Place the samples on ice. Write the number in red on the bottle and prepare 4 labels /number, using the label template.

Prepare for each sample:

2 x 50 ml tube – write sample number and 1 and 2 respectively.
1 x 15 ml tube – place a label – place tape around the label – add 5 ml of 50% glycerol.
2 x 250 ml bottles - place labels and tape around the label - write 1 and 2, respectively, on lid.
1 x 500 ml bottle - place a label and tape around the label
2 x 2 ml Eppendorph tubes – write sample number and 1 and 2 respectively.

Work in the LaminAir flow bench.

Workload, 6 wastewater samples per person per day (two persons a day)

Shake the samples and take a photograph.

Using a pipette, transfer 10 ml of wastewater to the 15 ml tube with the 5 ml glycerol. Place the tubes in -80°C freezer.

Pour 50 ml wastewater to each of the 50 ml tubes.

Only after the first pouring:

Mix the content and take a photography of the two tubes, one tube with the number visible and the other tube with the sample visible.

Centrifuge the tubes 10 minutes at 10.000xg at 5°C.

Decant the supernatant to the 250 ml bottle – no. 1 into bottle no. 1, and no. 2 into bottle no. 2.

Repeat 5 times in all. The last time you decant, leave some liquid (3-4 ml) then it is easier to transfer pellet to the 2 ml Eppendorph tube.

Transfer the pellet to the 2 ml tube using a disposable pipette.

Centrifuge 2 min at 8000xg at 4°C.

Discard the supernatant and repeat the pellet transference/centrifuging until there is no more pellet left in the 50 ml tubes.

All the waste water that are left is poured into the 500 ml bottle

Place the Eppendorph tubes in -80°C. Pellets from 1 and from 2 are collected in different boxes.

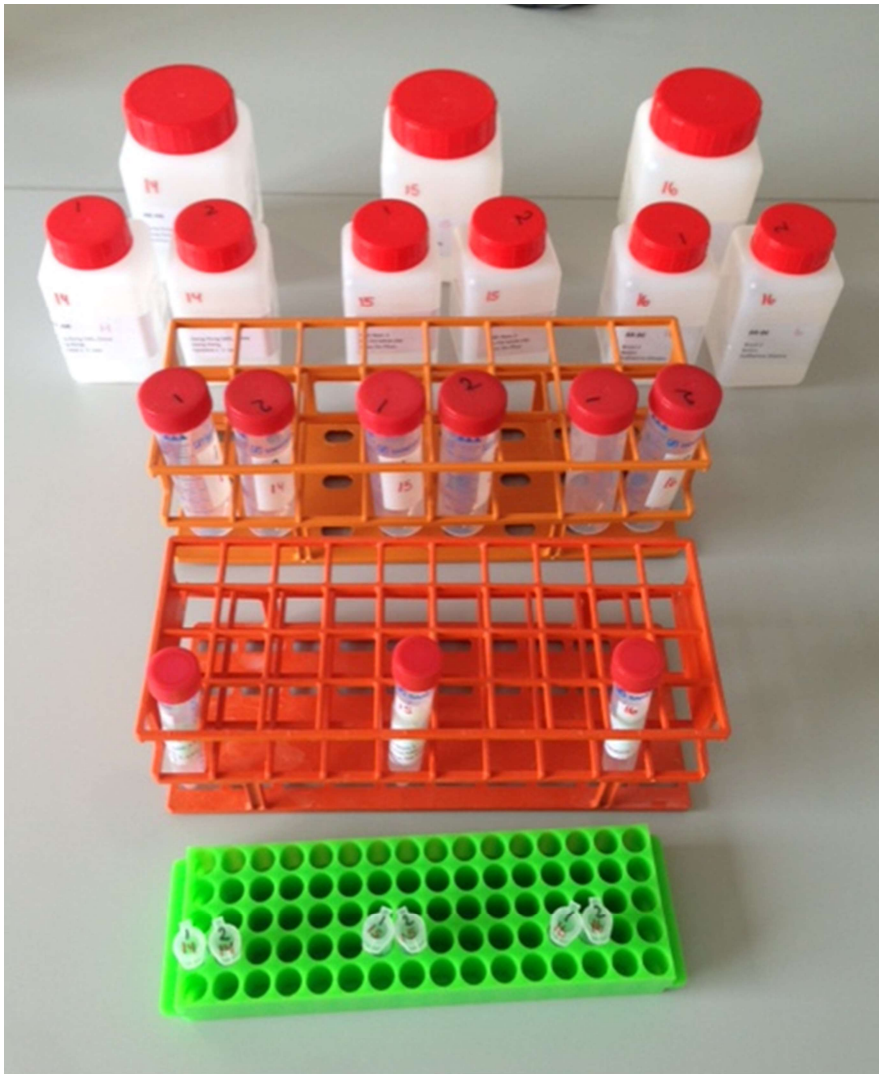
The 500 ml bottle and the 2 x 250 ml bottles are placed in plastic bags with a zip lock and placed in -80°C freezer.

Each sample has to be weighed. Note the weight in the Excel sheet.

Purify DNA using QIAamp Fast DNA Stool Mini Kit, Cat. no. 51604, Qiagen. Remember to include a negative control.

Measure DNA concentration and dilute (note the concentration in the Excel sheet)

Set-up of bottles and tubes for spinning wastewater – for three samples:



Per sample:

1 x 500 ml Bottle
2 x 250 ml Bottle
Write resp. 1 and 2 on lid.

2 x 50 ml tubes
Write resp. 1 and 2 on the lid, and on the side of the tube.

1 x 15 ml tube,
add 5ml 50% glycerol

2 x 2 ml Epp. Rør
Write resp. 1 and 2 on the lid

Place the current label on 500, 2 x 250 and 15 ml tubes.
Cover the label with transparent tape wrapped all the way around the bottle.
Using red ink, write the current sample number on the bottles/tubes (in case the label fall off)
Write the current sample number on the 50 and 2 ml tube.

DNA extraction of sewage samples

DNA extraction is based on bead beating and performed according to a modified protocol of QIAamp Fast DNA Stool Mini Kit, Cat. no. 51604, Qiagen, based on the Handbook 1081060_HB_LS_QIAamp_FastDNAStool_0314_WW_Web.pdf.

Products: Qiagen Fast + InhibitEX Buffer (FIB). A number of bead beating tubes can be used without significant differences, we are usually using MoBio UltraClean Fecal DNA Kit Bead Tubes.

DNA quantity is assessed by use of fluorometric methods to measure the DNA concentrations. The required DNA quantity is dependent on the library kit specifications. We use the Qubit dsDNA HS assay kit Cat no. Q32854 (Invitrogen, Carlsbad, CA, USA) for quantification.

Prepare before starting:
Put ethanol (96–100%) in freezer
Preheat/turn-on the 70°C & 95°C water-bath or heatingblock
Get Styrofoam box with ice.
Place TissueLyzer ''Blocks'' in -20°C freezer (Can be done the day before. But do not store blocks in the freezer for days)

Continue with a pellet from the protocol ' Pre-preparation of sewage sample''

1. Weigh the tube of centrifugated waste water pellet. (Should be around 500 µl or 0.5 g)

2. Add the sample to a bead-beating tube.

3. Add 1 ml InhibitEX Buffer to the sample.

4. Vortex continuously until the sample is thoroughly homogenized. (10 sec.)

5. Treat sample in a TissueLyser at 30 Hz for 3 x 30 s, with cooling on ice between each treatment.

6. Heat at 95°C for 7 min.

7. Vortex for 15 s

8. Centrifuge sample at full speed for 2 min to pellet stool particles.
IMPORTANT: Do not transfer any solid material. If particles are still visible in the supernatant, centrifuge the sample again.

DO STEP 9-13 IN DOUBLE

9. Pipet 30 µl proteinase K into a new 1.5 ml microcentrifuge tube (not provided).

10. Pipet (400-)500 µl supernatant from step 8 into the 1.5 ml microcentrifuge tube containing proteinase K.

11. Add 400 µl Buffer AL and vortex for 15 s.

Note: Do not add proteinase K directly to Buffer AL. It is essential that the sample and Buffer AL are thoroughly mixed to form a homogeneous solution.

12. Incubate at 70 °C for 10 min.
Centrifuge briefly to remove drops from the inside of the tube lid (optional).

13. Add 400 µl of ethanol (96–100%) to the lysate, and mix by vortexing.
Centrifuge briefly to remove drops from the inside of the tube lid (optional).

14. Carefully apply 600 µl lysate from step 13 to the QIAamp spin column. Close the cap and centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2 ml collection tube (not supplied), and discard the tube containing the filtrate.
Close each spin column in order to avoid aerosol formation during centrifugation. If the lysate has not completely passed through the column after centrifugation, centrifuge again until the QIAamp spin column is empty.

15. Apply the remaining lysate from step 13 to the spin column, close the cap and centrifuge at full speed for 1 min. Place the spin column in a new 2ml collection tube, and discard the tube containing the filtrate.

16. Carefully open the QIAamp spin column and add 500 µl Buffer AW1. Centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2 ml collection tube, and discard the collection tube containing the filtrate.

17. Carefully open the QIAamp spin column and add 500 µl Buffer AW2. Centrifuge at full speed for 3 min. Discard the collection tube containing the filtrate.
Note: Residual Buffer AW2 in the eluate may cause problems in downstream applications. Some centrifuge rotors may vibrate upon deceleration, causing the flow-through containing Buffer AW2 to come in contact with the QIAamp spin column. Removing the QIAamp spin column and collection tube from the rotor may also cause flow-through to come into contact with the QIAamp spin column.

18. Place the QIAamp spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 3 min.
This step helps to eliminate the chance of possible Buffer AW2 carryover.

19. Transfer the QIAamp spin column into a new, labeled 1.5 ml Axygen (low-DNA bind) microcentrifuge tube (not provided) and pipet 50 µl Buffer ATE directly onto the QIAamp membrane. Incubate for 3 min at room temperature, then centrifuge at full speed for 1 min to elute DNA.

20. Pipet another 50 µl Buffer ATE directly onto the same QIAamp membrane. Incubate for 2 min at room temperature, then centrifuge at full speed for 1 min to elute DNA. Total volume ~100 µl.

21. The extracted DNA can be stored at -20° C until further use.

This lab protocol is written by and updated by the current DTU lab staff corresponding to the lab procedures in December 2019 .