MATERIAL AVAILABLE IN THE LABORATORY

LABORATORY EQUIPMENT

- 1-2 microwaves to dissolve agarose solution
- 1 fridge freezer (working)
- 1 ice machine (working)
- eppendorf centrifuges
- 1 transilluminator (under the hood)
- 4 power supplies
- vortexes

COMMUNAL MATERIAL ON THE BENCH

- Electrophoresis grade agarose
- Balance
- Spatulas
- Paper sheets for agarose weighting
- Parafilm
- Scissors
- Gloves (S-M-L)
- Deionized water (1 tank each 2-3 groups)
- Tape
- Blue or black Magic marker (medium tip)

COMMUNAL MATERIAL UNDER THE HOOD

- 2-20µl micropipette
- tips for 2-20µl micropipette
- tank for liquid waste
- transilluminator
- 2 racks containing respectively phaselock tubes with prealiquoted Phenol:Chloroform mix and phaselock tubes with prealiquoted chloroform

PER GROUP ON THE BENCH

- 1 timer
- 1 icebox
- 250ml flask
- 100 ml cylinder
- 500ml cylinder
- 20ml pipette tips
- pipette
- a falkon type tube with 30 ml of TAE 50X

- a set of micropipettes (p1000, p200, p20)
- a box of filtered tips for p1000; p200; p20 micropipettes
- rack for eppendorf type tubes
- 4-5 sterile “DNA low-bind” eppendorf type tubes (2ml)
- blue or black magic marker (fine tip)
- a falkon type tube with 50 ml of ultrapure nuclease free water
- a complete set for agarose gel electrophoresis (box, gel holder and combs)
- 1 aliquot of lysis buffer
- 1 aliquot of isopropanol 100%
- 1 aliquot of ethanol 100%
- 1 aliquot of 3M Sodium Acetate pH 5.5
- 1 aliquot of Sarkosyl 30% stock solution
- 1 aliquot of 8M Lithium Cloride

PER GROUP IN THE FREEZER

- 1 tube containing sliced filter and cleaned quartz powder
- 1 tube for all of proteinase K in the fridge
- 1 aliquot of loading dye 2.5X (green)
- 1 aliquot of ladder (blue)

EXPERIMENTAL PROCEDURES

1ST DAY

Wear gloves and make sure that you will be wearing gloves during all the exercitation

CELL BREAKAGE

Filters were frozen in liquid nitrogen immediately after removal from the filtering apparatus. We sliced the filters using clean scissors, we added clean chilled quartz powder (SIGMA) and we prepared 13 aliquots of the filter powder mix. Aliquots were stored at -20°C until the moment of use. We cleaned quartz powder by washing twice in RNAse Zap, rinsing in sterile H_2O (DNAse and RNAse free, Sigma) and drying in the stove at 300°C (sterile cycle).

1. Collect one sample from the freezer. Keep it in ice. Label the tube with your group number or initials. Vortex the sample for at least 2 minutes at maximum speed. *This step allows the quartz powder to hit the bacteria trapped in the filter producing a mechanical damage of their cell wall.*

NUCLEIC ACIDS EXTRACTION

2. Add to the sample 665 µl of lysis buffer (0.1M Tris-HCl pH 8.5, 0.1 M EDTA pH 8.0, 0.2 M NaCl, 2.5%(w/v) SDS).
3. Immediately after the addition of lysis buffer add also 35 µl of Proteinase K (RNAse and DNAse free, Invitrogen). You will obtain a final concentration of 1mg/ml Proteinase K in lysis buffer (added fresh right at the moment of use).
4. Incubated on a rocking platform in gentle agitation for 15 minutes at room temperature. *This step allows the solubilization of cell membranes and the digestion of proteins, including endogenous DNAses and RNAses.*
5. After the 15 minutes incubation add 50 µl of Sarkosyl from a 30% stock solution. You will obtain a final concentration of 2% Sarkosyl in lysis buffer. Incubated on a rocking platform in gentle agitation for 30 minutes at room temperature. *This step will produce a further denaturation of proteins, nucleic acids and protein-nucleic acids complexes. In particular with this addition we will denature nucleosomes, ribosomes and proteinase K.*
6. Centrifuge the sample at maximum speed for 10 minutes. *This centrifuge eliminates quartz powder, filter slices and unbroken cells. In the supernatant you will recover all the solubilized cellular lysate.*
7. In the flow hood, you can find phaselock tubes containing 750µl of phenol:chloroform mix. Collect your supernatant and transfer it to one of the phaselock tubes containing phenol:chloroform mix. Close the lid tightly and mix by inverting the tube (do not vortex!). Label your phaselock tube.
8. Centrifuge the phaselock tube for 5 minutes at maximum speed at room temperature.
9. In the flow hood, you can also find phaselock tubes containing 750µl of chloroform. Collect the upper aqueous phase from your first extraction and transfer it to one of the

phaselock tubes containing chloroform. Close the lid tightly and mix by inverting the tube (do not vortex!). Label your phaselock tube.

10. Centrifuge the phaselock tube for 5 minutes at maximum speed at room temperature. *These two passages step extract selectively the nucleic acids from the cellular lysate. The majority of the denatured proteins and lipids dissolve in the organic phase, while nucleic acids dissolve preferentially in water.*
11. Collect again the upper aqueous and transfer it to a new tube. Label the tube with a description of the content and with your group initials or number.
12. Add to you sample 0.6 volumes of isopropanol (example: 1 ml of sample -> add 600µl of isopropanol. Final volume 1.6 ml)
13. Store at -20°C until the next day. *This step will precipitate selectively the nucleic acids form the cellular lysate.*

2ND DAY

Wear gloves and make sure that you will be wearing gloves during all the exercitation

RECOVERY AND RESUSPENSION OF PURIFIED NUCLEIC ACIDS

1. Recover your sample from the freezer and centrifuge at maximum speed for 30 minutes, possibly at 4°C. *This step allows you to recover the nucleic acids precipitated in the pellet and to get rid of the majority of the cellular contaminants in the supernatant.*

- While the centrifuge is working we suggest you to have a look at the section of the protocol titled "AGAROSE GEL ELECTROPHORESIS" and see if you can anticipate part of the work -

2. Discard the supernatant and rinse the pellet by adding first 700µl of ethanol and then 300µl of sterile H2O (DNAse and RNAse free, Sigma). Mix well.
3. Centrifuge for 15 minutes at maximum speed at room temperature. This step helps eliminating residual salts from the pellet.
4. Carefully remove the supernatant and discard it.
5. Dry the pellet under the flow hood.
6. Once the pellet is dried rehydrate it in 20µL of H2O (DNAse and RNAse free, Sigma).
7. Collect 10 µl of your purified nucleic acid solution and transfer to a new tube. Label the tube.
8. Store one of the two aliquots in the freezer at -20°C.
9. Collect one aliquot of 2.5X loading dye (green) from the freezer and one aliquot of ladder (blue). Let them thaw at room temperature.
10. Add to the second aliquot of your sample a suitable amount of 2.5X loading dye solution (green). The sample now is ready for loading on agarose gel electrophoresis. Store on ice until you load.

AGAROSE GEL ELECTROPHORESIS

1. Prepare 1 L of 1X TAE using a suitable amount of 50X stock solution available on your bench and diluting in distilled water.
2. You will prepare 70ml of 1% agarose solution in TAE buffer. Calculate the amount of agarose needed to prepare such a solution, weight it and pour it into the 250ml flask available on your bench. Label the flask.
3. Measure 70ml of 1X TAE buffer and add it to the flask containing the agarose.
4. Seal your gel holder using tape.
5. Dissolve the agarose by heating the solution in the microwave. Once it is completely dissolved (watch carefully through) bring the hot solution under the hood.
6. Collect your gel holder (already sealed) and bring it also under the hood together with the combs. Place the combs on the holder.

7. Compute the amount of DNA colouring solution that has to be added to the gel: the stock solution available is 10.000X. Add the colouring solution to your gel solution using the pipet and the tips available under the hood. Carefully mix the solution
8. Pour a small amount of the gel solution onto the gel holder. Make sure that the apparatus is not leaking
9. Once you've checked that the holder is sealed properly pour the rest of your gel solution onto the holder and leave the gel under the hood to solidify.
10. Bring the flask with you and wash it thoroughly using soap and water. Rinse using deionized water.
11. Once you're done throw away your gloves into the chemical waste and wear a new pair of gloves
12. Briefly spin the tubes containing the sample to be loaded and the ladder s in the eppendorf centrifuge to collect the liquid at the bottom of the tube
13. Once your gel will be completely solidified (the gel will turn cloudy) collect it from the hood. Remove the tape and place it into the box.
14. Cover the gel with 1X TAE buffer.
15. Only after the gel will be soaked in buffer, delicately remove the combs
16. Change your gloves again (through away the used gloves into the chemical waste)
17. Load all your sample into the gel and write down carefully the position
18. Load 5µl of the ladder in one or two lanes of the gel
19. Make sure that the gel is completely immersed in TAE buffer, close the box and connect the cables to the power supply
20. Switch on the electricity and switch on the power supply
21. Set the supply to a constant voltage of 100V while leaving the Amperes free to float according to the resistance of the gel
22. When the yellow marker will have reached the end of the run switch of the power supply, unplug the cables and open the box lid
23. Collect the gel and bring it to the transilluminator under the hood to see the results

ISOLATION OF RNA AND DNA FORM THE TOTAL NUCLEIC ACIDS SOLUTION

11. If your gel electrophoresis showed nicely purified nucleic acids, then you're ready to separate high molecular-weight RNA from genomic DNA. Add lithium chloride to your sample from a 3X stock solution (8M LiCl). Mixed and place at 4°C overnight.

3RD DAY

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RECOVERY AND RESUSPENSION OF ISOLATED DNA AND RNA

1. Recover RNA by centrifugation at 12,000xg for 30 min at 4°C.
2. Collect the supernatant, which contains DNA and tRNA, and transfer to a new tube. Label the tube.
3. Rehydrate RNA-containing pellet in 15µL of H_2O (DNAse and RNAse free, Sigma).
4. Add to both the two solutions: 1/10 of the volume of 3M sodium acetate and 2 volumes of ethanol.
5. Place in the fridge at –20°C for 1 hour.
6. Collected nucleic acids by centrifuging at maximum speed for 30 min, if possible at 4°C.
7. Remove the supernatant and wash the pellet with 70% ethanol (again add 700µl of ethanol first and then 300µl of H_2O).
8. Centrifuge for 15 minutes at maximum speed at room temperature.
9. Dry the pellet under the hood and rehydrated in 10µL of H_2O (DNAse and RNAse free, Sigma).
10. *Both the two preparations will be assessed for quality and concentration using respectively Nanodrop and Qbit by your teachers. The results will be published on the blog.*
11. *All the successful purifications will be pooled and used for library preparation by the sequencing service.*