General outline for extraction of nucleic acids from samples with prokaryotes – version 3 April 2013:

1) For optimal extraction of nucleic acids (NA), consider which protocol may be appropriate for your particular case. If unsure, perform pre-studies using different types of nucleic acid extraction protocols. For general diversity studies, try to employ at least two different protocols.

2) Basic considerations prior to harvesting cells:
   a. Select appropriate harvest time for best yield (i.e. to retrieve a high NA concentration and, with respect to complex samples, as diverse as possible). Thus, where possible, harvest cells during exponential growth phase. If you are processing environmental samples where the growth stage of the cells is unknown, treat the samples as soon as possible, e.g. by freezing samples immediately on the field (e.g. by immersing in liquid nitrogen or by adding preservaties) or cooling the samples if they need to be transported to the lab until they can be further processed.
   b. Consider if the sample needs to be washed with e.g. an appropriate sterile buffer prior to NA extraction.
   c. If you plan to extract RNA, consider which kind of preservative such as RNAlater (Ambion) should be added to your sample, e.g. see review in Ritalahti et al., RNA extraction and cDNA analysis for quantitative assessment of biomarker transcripts in groundwater. In K. N. Timmis (ed.), Microbiology of Hydrocarbons, Oils, Lipids, and Derived Compounds. Springer, Berlin.

   d. Consider sample treatment prior to harvest, does the sample need to be:
      i. Excised from larger particles (like leaves, animals etc)?
      ii. Dissolved in a solution (if solid like rock or soil)?
      iii. Concentrated by e.g. centrifugation or filtration (e.g. groundwater)?
      iv. Diluted (if the cell concentration is extremely high)?
      v. Detached from debris, biofilm matrix?

      Consult literature on how to treat various types of samples!

3) Harvest samples (if solution, centrifuge down to a visible (if possible) pellet) and dissolve in an appropriate buffer in sterile tubes with screw caps (e.g. 2 ml or 15 ml tubes). Depending on size of sampling tube, different amounts of sample are required, from ~10 mg up to 5 gram or more. Normally, ~ 0,5 g solid sample for 2 ml tubes is sufficient. For water samples, different sampling may be required, depending on the cell concentration (e.g. 10 ml to 1 Liter, or even more) If the samples will not be processed for NA extraction shortly after, freeze samples (-20 or -80 ° C).

   Be sure to produce replicates of your samples and that other appropriate analyses of the samples have been made and documented (e.g. activity, environmental conditions, other microbiological analyses, etc).

4) Select homogenization mode in order to break down the cell wall package and nucleases:
    a. No treatment.
    b. Simple homogenziation (e.g. with a tissue grinder, mortar or stomaker).
    c. Sonication.
    d. Chemical (e.g. by a lysing buffer such as GuTC or CTAB).
    e. Enzymatic, chemical (by different types of enzymes and SDS).
    f. Physical stress – thermal schocks (multiple freeze-thaw cycling).
    g. Mechanical (by e.g. bead beating using different types of beads, French press etc).

5) Extraction by phenol-chloroform-isoamylalcohol, followed by Chisam (chloroform-isoamyl alcohol 24:1). Where appropriate, use phaselock gel. *Comment: Phenol denatures proteins and removes solutes, but it does so more completely in the presence chloroform. Chloroform also stabilizes the phenol/aqueous phase boundary and improves yield by reducing the amount of aqueous phase retained by phenol. Isoamyl alcohol is used to enhance the separation of organic and aqueous phases and to reduce foaming. (cited from http://onlinelibrary.wiley.com/doi/10.1002/0471142905.hga03cs00/pdf).*

6) Precipitation by Na-acetate and isopropanol.

7) Washing step with 70 % EtOH.

8) Vaccum dry and then dissolve precipitate of nucleic acids in an appropriate solution.

9) Measure with nanodrop (to determine concentration and purity) and where appropriate with gel electrophoresis (to explore the size of extracted nucleic acids). Where appropriate, remove DNA/RNA with enzymes.

10) Aliquot and freeze (-20°C for shorter periods, -80°C for longer periods) or lyophilize.

General flexible protocol for coextraction of DNA and RNA (composed from Liu et al., 1997; AEM; Griffiths et al., AEM 2000), Urakawa et al., AEM 2010; MOBIO kits; http://primerdigital.com/dna.html; Qiagen DNA/RNA handbook, http://www.qiagen.com/default.aspx .

Note, for RNA extraction special precautions must be undertaken (all reagents must be treated with DEPC etc, check e.g. http://www.protocol-online.org/prot/Molecular_Biology/RNA/RNA_Extraction/Total_RNA_Isolation/index.html).

Solutions/material:

A. Select buffer type:

- *Extraction buffer 1 (CTAB):*
  Mix equal volumes (1:1) of:
  - 10% (wt/vol) CTAB (hexadecyltrimethylammonium bromide) in 0.7 M NaCl
  - 240 mM potassium phosphate buffer, pH 8.0 – can be replaced with 100 mM TrisHCl pH 8.
  - Autoclave (or sterile filter into a sterile bottle). Store at room temperature.

- *Extraction buffer 2 (GuTC) – often employed in RNA extractions):*
  - 1 M guanidine thiocyanate (can be varied between 0,5-2 M), 1% N-lauroylsarcosine (Na salt, Sarkosyl), 0.1 M MOPS pH 4.6. Autoclave all else except, GuTC – add this afterwards aseptically (dissolve GuTC in sterile bottle with sterile dH2O). Or, sterile filter all into a sterile bottle. Store at dark, room temperature.

    Safety note: Upon contact with acids, guanidine thiocyanate can produce a toxic gas (HCN), thus avoid contact with acids! Prepare guanidine thiocyanate containing buffers in a fume hood. Heating in a 60-65˚C water bath with shaking may speed up the dissolution.

Comment: A low amount of autoclaved 1-10 mM Na_3EDTA may be considered for buffers 1 and 2, but watch up that EDTA will not inhibit PCR in subsequent steps!

- Extraction buffer 3 (sucrose):
  - 100 mM Tris-HCl pH 8.
  - 100 mM EDTA
  - 0,75 M Sucrose

B. Select enzymes and incubation conditions:

- *Lysozyme (for general degradation of the cell wall of mainly gram negative organisms)*, prepare working solution around 1-10 mg/ml. For standard applications, prepare 50 mg/5 ml of Fluka product number 62970; 81 821 U/mg. Add between1,9 and 19 µl of this to your sample (corresponds to ~150-1500 U in your sample) but even more may be better, depending on your sample (needs to be checked up by yourself). Dissolve in sterile tube with **sterile 50 mM Tris/HCl pH 7** (tolerable pH range 3,5-7, pH optimum at 6,6; solubility ~ 10 mg/ml in water). Do not autoclave. Store stock solution +4 °C for a short period (some day(s)). For longer periods store at -20°C. Aliquot frozen solutions to avoid too frequent freezing-thawing cycles.

- *Achromopeptidase (for degradation of especially gram-positive organisms),* prepare working solution around 0,2 mg/ml. For standard applications, prepare 1 mg/5 ml of Sigma product number A3547; 4590 U/mg. Add between 1 to 44 µl of this solution to your sample (this corresponds to ~1-40 U in your sample, see comment below about recommendations on concentrations). Dissolve in sterile tube with **sterile 50 mM Tris/HCl pH 8** (pH optimum 8.5-9; solubility 10 mg/ml in water). Do not autoclave. Store stock solution +4 °C for a short period (some day(s)). For longer periods store at -20°C. Aliquot frozen solutions to avoid too frequent freezing-thawing cycles.

- *Proteinase K / or Pronase (for removing proteinases)*, prepare working solution around 0,05-10 mg/ml. For standard applications, prepare 1 mg/1 ml of Pronase Roche product 10165921001; 7000 U/g. Add 54 µl of this to your sample (corresponds to ~375 U per sample). Dissolve in sterile tube with sterile **50 mM Tris/HCl pH 8.** Store at 2-8 °C for a short period (day(s)), at -20°C. for longer periods. Aliquot frozen solutions to avoid too frequent freezing-thawing cycles.
    - Proteinase K pH range 4-12,5 (optimum 8). Do not autoclave.
    - Pronase (e.g. Roche product number 10165921001; 7000 U/g). pH tolerance 6-9 (optimum 6-7,5); solubility 10-20 mg/ml in water. Do not autoclave.

Comment 1: Appropriate enzyme concentrations must be chosen with respect to the sample and its cell wall packages. Standard concentrations for efficient lysis normally are around several hundreds of U (end concentration up to e.g. 1500 U per sample).

Exception: Achromopeptidase which is used at concentrations generally around 1-40 U/µl. Note, higher concentrations may may shear the DNA and RNA!

Other enzymes like *labiase* may be more suitable for certain cases (e.g. Niwa et al 2005, J Microb Methods 61:251). Note that other enzymes may be more efficient than those listed here (e.g. lytostatin, mutanolysin and glyince can be useful for staphylococci).

Comment 2: Membrane lipids and nucleases can be removed via special buffers (e.g. CTAB, GuTC) and SDS (see paragraphs A and C).

C. Other reagents:

- 25 % SDS (place into an empty autoclaved bottle, add autoclaved dH2O, do not autoclave). To remove precipitates in SDS solution, simply heat it (add a magnetic bar and stir it).
- Phenol-chloroform-isoamyl alcohol (25:24:1) (pH 8.0). Ready made commercial product (e.g. from ROTH, Germany).
- Chisam (chloroform-isoamyl alcohol 24:1) – prepare yourself. Keep for maximum a few months in a tightly closed bottle (do not autoclave).
- 3 M Na-Acetat (pH 5,2), autoclave.
- Ice-cold isopropanol (do not autoclave).
- Ice-cold 70 % EtOH molecular grade (do not autoclave).
- For dissolution of nucleic acids:
    - Sterile (if DNA) or DEPC treated (if RNA) $H_2O_{bidest.}$
    - Or, sterile (where appropriate DEPC treated for RNA) 10 mM or 0.1 mM Tris-buffer + 1 mM EDTA buffer (for DNA, pH 8; for RNA, pH 7,5).
- GlycoblueTM (Ambion): The chained carbohydrate branches of glycogen help DNA to precipitate into a pellet, while the blue dye makes the pellet visible at the bottom of the microcentrifuge tube. Useful if the expected yield of NA concentration is low.
- If working with RNA: Treat all solutions and glassware with either RNAse (e.g. from Quiagen or MOBIO) or diethyl pyrocarbonate (DEPC, e.g. from SIGMA).
    - DEPC treatment:
        - Solutions: Add DEPC to a final concentration of 0,1 % and incubate overnight at 37° C. Autoclave.
        - Equipment: Soak items in a 0,1 % DEPC solution (dissolved in dH2O) overnight at 37° C. Autoclave.

Note DEPC is carcinogenic! A less toxic version is DMPC. For more info: http://openwetware.org/wiki/DEPC

D. Equipments:

- Sterile tubes and pipette tips (1,5, 2 ml, 15 ml; 1 ml and smaller).
- ≥ 2 Water baths (different temperatures).
- Spectrophotometer, nanodrop.
- Centrifuges for 2 ml and e.g. 15 ml tubes (or other sizes), where appropriate cool centrifuges (for 4° C).
- For difficult samples: Phaselock gel tubes (PLG) from 5Prime (for 1,5 or 15 ml tubes), https://de.vwr.com/app/catalog/Catalog?parent_class_id=4&parent_class_cd=116986 - note, prior to use, pellet phaselock gel tubes at > 12,000 x g for > 20-30 sec.
- For cell destruction: e.g. vortex with MOBIO adaptorors or similar, sonication bath, bead beater (including appropriate beads) etc.

General procedure (select between the various options for modifications, make notes which of these were chosen for your experiment):

Suggested time planning: Start early in the morning and complete work late afternoon; or start on day 1 in the afternoon, and complete work on day 2.

Day 1

1) Before you start: turn on water baths/ovens. Allow them to reach appropriate temperatures. Check out all reagents and other equipments that will be needed.

2) Prepare sample for sterile screw cap tubes. You can select between **2 ml up to 15 ml.** Before you start, calculate the total amount of volume needed for all steps and decide then which tubes you should use (advice: 2 ml tubes are more practical than 15 ml tubes – in the end you must scale down to 2 ml tubes because that is the only size that fits into the lab´s vacuum centrifuge). If you use 15 ml tubes, check that the 15 ml tube centrifuge is available (book it!). If you have multiple samples, see to that they have equal volumes/weights.

- If your sample is solid (e.g. pellet), dissolve in an appropriate buffer, e.g. CTAB or GuTC (see page 2). If you are using 2 ml tubes, add 500 µl buffer, otherwise scale up the volume accordingly so that the sample is dissolved in the buffer, however consider that there will be enough space available for subsequent reagents so that the tube will not be overfilled, and the centrifuge or bead beater will not be overloaded.

- If you plan to beadbeat, add appropriate lysing matrix and beads (see e.g. MP Biomedicals or from MOBIO).

- If your sample contains Gram-positive bacteria or unknown composition, treat it with e.g.:

  a) Lysozyme (see page 4), incubate 0,5-1 h between 30-90° C (optimum 55° C). Where appropriate, include also (or add afterwards) achromopeptidase (but note that achromopeptidase is usually incubated at 37° C, up to 2 h.

  b) Proteinase K / or Pronase (see page 4). If Proteinase K: Incubate ≥30 min between 37-60 ° C (do not exceed 65° C). If Pronase: Incubate 0,5 -1 h between 40-60° C.

  c) And/or other appropriate enzymes, see page 4.

  d) SDS. Dilute stock solution of 25 % SDS solution so that the final concentration in the sample is ~ 2 %. Incubate at 65º C for 0,5-2 h.

4) Add 1 volume (with respect to the total volume of your sample) phenol-chloroform-isoamyl alcohol (25:24:1, pH 8.0). For example if you are using 2 ml tubes with 500 µl sample, add 500 µl phenol-chloroform-isoamyl alcohol.

Choose between vortexing (for this, use the MOBIO vortex adaptors for multiple tubes, vortex at highest speed for at least several minutes) or beadbeating:

a) lysis for e.g. 30 s at machine speed 5,5 m/s (Bead beater), one or more cycles, at RT or cooled (only possible for 15 ml tubes). Advice: tighten your tubes and the lid before you beadbeat. Check that the machine does not get overloaded.

b) centrifugate 5 min at 4°C (15000 rpm for 2 ml tubes, or 9000 rpm for 15 ml tubes).

c) consider if you need to use PLG tubes after the phenol-chloroform-isoamyl alcohol (step 4), or the chisam extraction (step 7). Prepare tubes as described above (page 2).

d) transfer upper, aqueous phase (nucleic acids!) into a new sterile screw cap tube. Where appropriate, pipette into a phase lock gel tube (PLG), treated as described above. Note, PLG tubes can also be used after the chisam treatment (advisable if e.g. the tube after step 4 is too full). Estimate the total volume of your aqueous phase with a pipette.

5) Add 1 Volume chisam (chloroform-isoamyl alcohol 24:1) to your one volume sample.

6) Centrifugate 5 min at 4°C at 15000 rpm (or 9000 rpm if 15 ml tubes are used). Always note how you place the tubes in the centrifuge with a marker (place the sign where the pellet is expected to be).

7) Transfer upper, aqueous phase (contains the nucleic acids!) into a new sterile screw cap tube – if you used larger tubes, move now to smaller tubes (2 ml, 1,5 ml – screw cap tubes are now no longer needed).

If PLG tubes are used, the separation of the two phases should be distinct. Estimate the total volume of your aqueous phase with a pipette. If you did not yet use PLG tubes (treated as described above), you can do this now - if needed. If you had problems with phase separation or fear that your aqueous phase got contaminated, you can repeat the chisam extraction (go back to step 5).

8) Alternative option for DNA extractions (now or later at the end after the nanodrop measurement): Add 1 µL RNase (DNase-free) and incubate for 30 min at 37°C.

9) Choose one of the following steps for precipitation:

+ 0.1 Vol. 3M Na-Acetat (pH 5,2). + 0.6 Vol. icecold isopropanol. Free option: + 3 µl of GlycoBlue™. → turn tubes several times. → precipitate nucleic acids for at least 2 h (or overnight) at room temperature, or +4 °C, or –20°C.	or	+ 2 Vol. 30% (wt/vol) polyethylene glycol 6000 in 1.6 M NaCl. → precipitate nucleic acids for at least 2 h (or overnight) at room temperature, or +4 °C, or –20°C.

Day 2 (or if you started early in the morning of day 1, late afternoon on day 1):

10) Centrifugate 10 min at 4°C (15000 rpm). Mark the expected location of the pellet on the tubes before centrifugation, so that in those cases where the pellet is not visible, you will still know how to pipette carefully to minimize loss of the pellet.

11) Wash pelleted nucleic acids in ice cold 70% (vol/vol) ethanol by centrifugation. Remove carefully the supernatant as much as possible (if you don't see a pellet, then remove the supernatant carefully, avoiding coming close to the place where the pellet is expected to be and avoid removing all the supernatant. Repeat up to 3 times (especially if you could not remove the supernatant completely). Mark the expected location of the pellet on the tubes before centrifugation, so that in those cases where the pellet is not visible, you will still know how to pipette carefully to minimize loss of the pellet.

12) Vacuum dry nucleic acids (between 15 min or longer, depending on the amount of volume of EtOH left in the tube).

13) Resuspend pellet in a suitable volume of RNase free MQ or buffer. Allow appropritate time for dissolution (~ 15 min. or longer). Shake carefully (do not vortex vigorously! You may shear the genomic DNA!).

Guideline for suitable reagents for resuspension of DNA:

- Sterile RNAse free, sterile filtered neutral buffer, e.g. Tris-HCl or Tris-EDTA. Concentration e.g. 0,1-50 mM). If you use EDTA, be aware that the EDTA will not have a negative impact on subsequent PCR reactions.

- RNase free, sterile filtered MQ (however note, MQ may not dissolve and protect the nucleic acids so well due to low pH).

Guideline for suitable resuspension volumes:

- If the DNA concentration is low (i.e. the pellet after precipitation with NaAc and isopropanol) was not or hardly visible) add between 10-50 µl (general recommendation is 30 µl).

- If the DNA concentration is high (i.e. the pellet after precipitation with NaAc and isopropanol is thick) add > 50 µl up to x times 100 µl.

14) Measure concentration and purity (by spectrophotometer or nanoDrop).

Instructions for nanoDrop:

i) Select appropriate programm (e.g. DNA-50 for DNA).
ii) Wash the measuring spot with sterile water (add 1 µl droplet, do not scratch the surface). Wipe off with clean tissue (avoid touching the part that you use for cleaning).
iii) Calibrate the nanoDrop by adding 1 µl of the solution that you used to resuspend your DNA. Place the lid on the measuring spot. Click "calibrate". Check the result file on the computer screen. If you want, you can check that the calibration worked by clicking measure.
iv) Wipe off your calibration solution.
v) Add 1 µl of your DNA. Place the lid on the measuring spot. If needed, type the name of your sample into the data field. Click measure. Check the result file on the computer screen. Record the following data:
   - DNA concentration in ng/µl;
   - Ratio 260/230;
   - Ratio 260/230;
   - Absorbance value (e.g. 260 nm for DNA).
   - Check the graph.

   All data can be saved in a excel table or as graphs (however, be aware that data may get lost on the computer so it is advisable to record data manually).

   Note, take Lamberts law into consideration, trust only values with Absorbance 260 nm (for DNA, if other compound like RNA, check appropriate wavelengths such as 230 nm etc) up to 1. If higher, dilute appropriately. Advice, dilute the DNA in a separate tube by taking e.g. 1 µl of your stock DNA and dilute then with the solution that you dissolved your DNA in (buffer or sterile water).

Instructions for spectrophotometer (note, this demands often more DNA solution which is a disadvantage if you have low amounts of DNA):

i) Select appropriate programm/wavelength (e.g. DNA and 260 nm).
ii) Pipette a diluted aliquot of your DNA solution into a clean cuvette. Note, the DNA solution may get contaminated so consider if you should dispose or reuse the aliquot you used for the measurement.
iii) Record the absorbance value.
iv) Calculate the concentrations, based on formulas below. File your data as described above for the nanoDrop.

Calculation of concentration with spectrophotometer or nanodrop:

Double stranded (ds) DNS: 1 $OD_{260nm} \cong 50$ µg/ml

Single stranded (ss) DNS: 1 $OD_{260nm} \cong 20$ µg /ml

Purity evaluation with spectrophotometer or nanodrop:

$E_{260}/E_{280} \geq 1{,}8$ (<1.8 high protein content)

$E_{260}/E_{230} \geq 2{,}2$ (<2.2 high RNA content)

15) Alternative option for DNA extractions Removal of nucleases (if needed (check e.g. after your nanodrop measurements):

DNA preparation	**RNA preparation (check Griffiths paper)**
→ incubate aliquot (25 µl) with RNase A (Sigma) at a final concentration of 100 µg/ml for 10 min at 37°C.	→ incubate aliquot (25 µl) with 3 U RQ1 RNase-free DNase (Promega Corp.) according to manufacturer's instructions ⇒ 3 µl RNase-free DNase (1 U/µl, Promega) + 3 µl 10x RQ1 DNase buffer → incubate at least for 30-60 min at 37°C + 3 µl RQ1 DNase stop solution → stop enzymatic reaction: 10 min at 65°C + 1 µl RNasin → store at **–80°C**

16) Aliquot extracted nucleic acids (depending on concentration, e.g. á 10 µl or more). Allow at least 15 min for dissolution, or longer.

For shorter periods, store at +4 °C, for longer periods store at -20 (though better at -80 for longer periods) °C. NA can also be lyophilized via a vacuum centrifuge.

Note: Avoid too frequent freezing-thawing of your nucleic acids (maximum 3 times for quantitative diversity analyses). Use a marker to make a stroke for each thawing session to keep track of how many times the samples got frozen and thawn up.

17) Record properly your samples in a properly labeled storage box and data in e.g. a excel table with the following data:

Label your tubes both on the lid (use an appropriate label) and on the tube itself – protect text with adhesive tape!!! Use a simple code for the lid (like a number 1-96 etc) and add some additional short information on the tube itself (to add more unique data to your tube to avoid confusion with other similarlyl labelled tubes).

Data for your table (excel, word or similar):

- Your name and project
- Which box is used for this and where it is located.
- Code of the tube on the lid, like a number on a label on the lid of the tube.
- Short name of your sample as it is labeled on the tube itself.
- Full description of you sample like:
  - Full name,
  - Volume and concentration (original and how much has been removed),
  - Origin,
  - Sampling date,
  - How the sample was processed, with a link to your lab book),
  - Analytical data (e.g. if DNA, concentration, quality – you may also make a link to your lab book etc).