D-JRP15-FED-AMR-WP2.1

Laboratory Operating Procedure (LOP)
Protocol for Extracellular DNA Separation and Extraction

Version 2
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1. Description

This protocol describes the procedure to separate **extracellular free DNA** (**eDNA**) from the **intracellular DNA** fraction, the precipitation of eDNA and its further purification, in the 11 matrices (compartments) included in the FED-AMR project. All DNA extractions rounds will include blanks and will be carried out in triplicate for metagenomics. In addition, **total DNA** will be extracted in paralel with the corresponding commercial kit at least in the soil samples. Extraction controls should be included to exclude contamination of the DNAs.
Do not vortex! Do not freeze the samples! (vortexing and freezing temperatures can break the cells and release intracellular **DNA**).

2. Material and Equipment

- Shaker
- Cooling centrifuge
- Microfuge
- Filter tips
- Falcon tubes, 50 mL
- Disposable 10 mL pipettes
- Sterile 1.5 mL microtubes
- Thermobloc
- Qubit®
- 0.1 M sodium phosphate buffer (see Annex)
- Glycogen, 20 mg/mL aqueous solution
- Absolute Ethanol
- Ethanol 70%
- Sodium acetate 3M pH=5.2, sterile
- TE Buffer 1x (see Annex)
- Acid washed Polyvinylpolypyrrolidone (PVPP) (see Annex)
- 0.22 µM and 0.45 µM filters (e.g: PES filter membranes are more suitable)
- Scale

3. Procedure

3.1Compartment 1: Pig feces

Note that DNA (eDNA and total DNA) will be extracted from the individual fecal samples and the composite sample as described in the FED-AMR fecal sampling protocol.

eDNA extraction (1x for qPCR and 3x for metagenomics):

- Weigh 2.5 g of **feces** in a 50 mL universal tube;
- Add 0.5 g of acid washed polyvinylpolypyrrolidone (PVPP);
- Add 7.5 mL of 0.1 M sodium phosphate buffer (NaP buffer);
- Shake 5 min at 150 rpm;
- Place the sample on ice for 3 min;
- Shake 5 min at 150 rpm;
- Centrifuge 10 min at 500 g, at 4°C;
- Collect the supernatant (contains the eDNA) and tranfer it to a sterile tube;
- Discard the sediment pellet (contains the iDNA);
- Centrifuge the supernatant 30 min at 10,000 g, at 4°C;
- Transfer the supernatant into a new sterile tube and discard the pellet. This is a **SAFE STOPPING POINT**. You can now freeze the supernatant at -20°C or continue with the precipitation.
- **OPTIONAL STEP:** Precipitate the eDNA from the supernatant as follows:
    - Add 1/10 volume of 3 M sodium acetate to the supernant;
    - Add glycogen 20 mg/mL to a final concentration of 0.05-1 µg/µL; Use up to 1 µL of glycogen per 20 µL of the solution;
    - Add 2.5 volumes of ethanol (preferably use ethanol, if not possible, use 1 volume of isopropanol) to the solution;
    - Mix gently but thoroughly;
    - Incubate the mixture at -20°C for up to 60 min, or at -70°C for 30 min; Longer incubation and lower temperature (up to 24h) provide better recovery of nucleic acids;
    - Centrifuge the mixture for 10-15 min at 10,000 rpm;
    - Discard the supernatant;
    - Rinse the pellet with 800 µl of cold 70% ethanol;
    - Air-dry the pellet. Avoid over-drying the pellet, as it then takes more time to dissolve;
    - Dissolve the eDNA pellet in 100 µL ultra-pure water and you will have now the eDNA ready for upstream applications. For high-thoughput analysis like metagenomics, a subsequent purification of the eDNA with Powersoil is needed. If this is your case dissolve the eDNA pellet

in 650 µL TE buffer or ultra-pure sterile water instead and continue with the protocol below.

- Extract the dissolved eDNA with the DNAeasy Powersoil Pro kit omitting steps 1-7;
- Approximately 100 µL will be eluted. Eluting with nuclease free-water instead of TE or other elution buffer is recommended.

Total DNA extraction (1x for qPCR and 3x for metagenomics):

- Weight 0.25 g of feces and extract the total DNA with DNAeasy Powersoil Pro kit (eluate contains the **total DNA**);
- Approximately 100 µL will be eluted.
- Store the DNA aliquots at -20°C. For genomic analysis (WP2) DNA is transported at -80ºC.

- Compare the DNA concentrations of both DNA types using Qubit.

3.2 Compartment 2: Manure

- Weigh 2.5 g of **manure** in a 50 mL falcon tube.
- Add 0.5 g of acid washed polyvinylpolypyrrolidone (PVPP)
- Add 7.5 mL of 0.1 M sodium phosphate buffer (NaP buffer).
- Shake 5 min at 150 rpm
- Put on the sample on ice for 3 min
- Shake 5 min at 150 rpm
- Centrifuge 10 min at 500 g, at 4°C
- Collect the **supernatant (contains the eDNA)** and tranfer it to a sterile tube
- Discard the sediment pellet (contains the iDNA)
- Centrifuge the pooled supernatants 30 min at 10,000 g, at 4°C

- Transfer the supernatant into a new sterile tube and discard the pellet. This is a **SAFE STOPPING POINT**. You can now freeze the supernatant at -20°C or continue with the precipitation.
- **OPTIONAL STEP:** Precipitate the eDNA from the supernatant as follows:
    - Add 1/10 volume of 3 M sodium acetate to the supernant
    - Add glycogen 20 mg/mL to a final concentration of 0.05-1 µg/µL. Use up to 1 µL of glycogen per 20 µL of the solution.
    - Add 2.5 volumes of ethanol (or 1 volume of isopropanol) to the solution.
    - Mix gently but thoroughly.
    - Incubate the mixture at -20°C for up to 60 min, or at -70°C for 30 min. Longer incubation and lower temperature (up to 24h) provide better recovery of nucleic acids.
    - Centrifuge the mixture for 10-15 min at 10,000 rpm.
    - Discard the supernatant.
    - Rinse the pellet with cold 70% ethanol.
    - Air-dry the pellet. Avoid over-drying the pellet, as it then takes more time to dissolve.
    - Dissolve the eDNA pellet in 100 µL ultra-pure water and you will have now the eDNA ready for upstream applications. For high-thoughput analysis like metagenomics, a subsequent purification of the eDNA with Powersoil is needed. If this is your case dissolve the eDNA pellet in 650 µL TE buffer or ultra-pure sterile water instead and continue with the protocol below.

- Extract now all the disolved eDNA with the DNAeasy Powersoil Pro kit omitting steps 1-7.
- Approximately 100 µL will be eluted. Eluting with nuclease free-water instead of TE or other elution buffer is recommended.

- Now weight 0.25 g (solid) or 0.25 mL (liquid) manure and extract the total DNA with DNAeasy Powersoil Pro kit (the eluate contains the **total DNA**).

- Compare the DNA concentrations of both DNA types using Qubit.
- Store the DNA aliquots at -20°C. For genomic analysis (**WP2**) DNA is transported at -80ºC.

3.3 Compartment 3: Soil

- Weigh 2.5 g of **soil** in a 50 mL universal tube.
- Add 0.5 g of acid washed polyvinylpolypyrrolidone (PVPP)
- Add 7.5 mL of 0.1 M sodium phosphate buffer (NaP buffer).
- Shake 5 min at 150 rpm
- Place the sample on ice for 3 min
- Shake 5 min at 150 rpm
- Centrifuge 10 min at 500 g, at 4°C
- Collect the supernatant (contains the eDNA) and tranfer it to a sterile tube.

- Discard the sediment pellet (contains the iDNA)
- Centrifuge the pooled supernatants 30 min at 10,000 g, at 4°C
- Transfer the supernatant into a new sterile tube and discard the pellet. This is a **SAFE STOPPING POINT**. You can now freeze the supernatant at -20°C or continue with the precipitation.
- **OPTIONAL STEP:** Precipitate the eDNA from the supernatant as follows:
    - Add 1/10 volume of 3 M sodium acetate to the supernant
    - Add glycogen 20 mg/mL to a final concentration of 0.05-1 µg/µL. Use up to 1 µL of glycogen per 20 µL of the solution.
    - Add 2.5 volumes of ethanol (or 1 volume of isopropanol) to the solution.
    - Mix gently but thoroughly.
    - Incubate the mixture at -20°C for up to 60 min, or at -70°C for 30 min. Longer incubation and lower temperature (up to 24h) provide better recovery of nucleic acids.
    - Centrifuge the mixture for 10-15 min at 10,000 rpm.
    - Discard the supernatant.
    - Rinse the pellet with cold 70% ethanol.

- Air-dry the pellet. Avoid over-drying the pellet, as it then takes more time to dissolve.
- Dissolve the eDNA pellet in 100 µL ultra-pure water and you will have now the eDNA ready for upstream applications. For high-thoughput analysis like metagenomics, a subsequent purification of the eDNA with Powersoil is needed. If this is your case dissolve the eDNA pellet in 650 µL TE buffer or ultra-pure sterile water instead and continue with the protocol below.

- Extract now all the disolved eDNA with the DNAeasy Powersoil Pro kit omitting steps 1-7.
- Approximately 100 µL will be eluted. Eluting with nuclease free-water instead of TE or other elution buffer is recommended.

- Now weight 0.25 g of soil and extract the total DNA with DNAeasy Powersoil Pro kit (eluate contains the **total DNA**).

- Compare the DNA concentrations of both DNA types using Qubit.

- Store the DNA aliquots at -20°C. For genomic analysis (WP2) DNA is transported at -80ºC.

3.4Compartment 4: Crops

A) Extracellular matrix of grains and leaves

Before starting the laboratory work, first see the Protocol for Crop Sampling within **D-JRP15-FED-AMR-WP2.1**.

Carefully peel off the grains with two sterile tweezers – do not carve the grains, otherwise, exDNA will be mixed with intracellular DNA! Carefully separate the leaves from the plant too.

Aliquote:

- 2,5g of grains & 2,5g leaves for eDNA workflow

- For Duplicates: **2 x 0,25g** of grains and **2 x 0,25g** leaves for full DNA workflow

exDNA extraction

- Weight 2.5g of grains and 2.5g of leaves in two different 50ml universal tubes
- Wash the surface with 70% EtOH to sterilize the surface and avoid contamination with external microorganism. Afterwards rinse with nuclease free water alternately twice– be careful not to carve the grains and leaves!
- Let the samples dry and then make a small cut in the middle of each grain or leaf.
- Add 7.5 mL of 0.1 M sodium phosphate buffer (NaP buffer)
- Incubate for about 1h
- Shake 5 min at 150 rpm
- Centrifuge 10 min at 500 g, at 4°C
- Collect the supernatant (**contains the eDNA**) and tranfer it to a sterile tube

- Discard the pellet (**contains the iDNA**)
- Centrifuge the supernatants 30 min at 10,000 g, at 4°C
- Transfer the supernatant into a new sterile tube and discard the pellet. This is a **SAFE STOPPING POINT**. You can now freeze the supernatant at -20°C or continue.

- **OPTIONAL STEP:** Precipitate the eDNA from the supernatant as follows:
  - Add 1/10 volume of 3 M sodium acetate to the supernant
  - Add glycogen 20 mg/mL to a final concentration of 0.05-1 µg/µL. Use up to 1 µL of glycogen per 20 µL of the solution.
  - Add 2.5 volumes of ethanol (or 1 volume of isopropanol) to the solution.
  - Mix gently but thoroughly.
  - Incubate the mixture at -20°C for up to 60 min, or at -70°C for 30 min. Longer incubation and lower temperature (up to 24h) provide better recovery of nucleic acids.
  - Centrifuge the mixture for 10-15 min at 10,000 rpm.

- Discard the supernatant.
- Rinse the pellet with cold 70% ethanol.
- Air-dry the pellet. Avoid over-drying the pellet, as it then takes more time to dissolve.
- Dissolve the eDNA pellet in 100 µL ultra-pure water and you will have now the eDNA ready for upstream applications. For high-thoughput analysis like metagenomics, a subsequent purification of the eDNA with Powersoil is needed. If this is your case dissolve the eDNA pellet in 650 µL TE buffer or ultra-pure sterile water instead and continue with the protocol below.

- Extract the eDNA using the **DNAeasy Powersoil Pro kit** omitting steps 1-7.
- Approximately 100 µl will be eluted. Eluting with nuclease free-water instead of TE or other elution buffer is recommended.

Total DNA extraction

- Weight 0.25g of grains/leaves, cut them and extract the total DNA with **DNAeasy Powersoil Pro kit** (eluate contains total DNA).
- Approximately 100 µL will be eluted.
- Store the DNA aliquots at -20°C. For genomic analysis (WP2) DNA is transported at -80ºC.

- Compare the DNA concentrations of both DNA types using Qubit.

B) Vase experiment (optional)

- For this experiment, you will not separate the leaves from the grains, but you will need to leave some stem.
- Cut the plant at stem level (approx. 50 cm height) into a 50 ml measuring glass.
- Add 1/3 of the volume of the measuring glass of nuclease free water.

- Seal the upper part oft he measuring glass PARAFILM to avoid water evaporation.
- Leave the plant standing for **two to three days** at room temperature.
- After the incubation time use a 0.45µm syringe-filter to filter the remaining water.
- This is a **SAFE STOPPING POINT**. You can now freeze the filtrate at -20°C or continue with the precipitation of the eDNA in the filtrate.
- **OPTIONAL STEP:** Precipitate the eDNA (contains the most part of the free fraction of the eDNA) as follows:
    - Add 1/10 volume of 3 M sodium acetate to the supernant
    - Add glycogen 20 mg/mL to a final concentration of 0.05-1 µg/µL. Use up to 1 µL of glycogen per 20 µL of the solution.
    - Add 2.5 volumes of ethanol (or 1 volume of isopropanol) to the solution.
    - Mix gently but thoroughly.
    - Incubate the mixture at -20°C for up to 60 min, or at -70°C for 30 min. Longer incubation and lower temperature (up to 24h) provide better recovery of nucleic acids.
    - Centrifuge the mixture for 10-15 min at 10,000 rpm.
    - Discard the supernatant.
    - Rinse the pellet with cold 70% ethanol.
    - Air-dry the pellet. Avoid over-drying the pellet, as it then takes more time to dissolve.
    - Dissolve the eDNA pellet in 100 µL ultra-pure water and you will have now the eDNA ready for upstream applications. For high-thoughput analysis like metagenomics, a subsequent purification of the eDNA with Powersoil is needed. If this is your case dissolve the eDNA pellet in 650 µL TE buffer or ultra-pure sterile water instead and continue with the protocol below.

- Extract now all the disolved eDNA with the DNAeasy Powerwater kit omitting steps 1-7.

- Approximately 100µl will be eluted. Eluting with nuclease free-water instead of TE or other elution buffer is recommended.
- Store the DNA aliquots at -20°C. For genomic analysis (WP2) DNA is transported at -80ºC.

Total DNA extraction (1x for qPCR and 3x for metagenomics):

- Directly take 2 x 1ml of the remaining water (unfiltered) and follow the **DNAeasy PowerWater Pro kit** protocol. → directly put 1ml in the powerbead tube (there is no filter). The eluate contains the total DNA!
- Approximately 100 µL will be eluted.
- Store the DNA aliquots at -20°C. For genomic analysis (WP2) DNA is transported at -80ºC.

- Compare the DNA concentrations of both DNA types using Qubit.

3.5Compartment 5: Field drainage

eDNA extraction (1x for qPCR and 3x for metagenomics):

- Filter two times up to 0.2 L of water through a 0.45 µM filter.
- This is a **SAFE STOPPING POINT**. You can freeze the filtrate at -20°C or continue with the precipitation of the eDNA in the filtrate.
- **OPTIONAL STEP:** Precipitate the eDNA (contains the most part of the free fraction of the eDNA) as follows:
    - Add 1/10 volume of 3 M sodium acetate to the supernant
    - Add glycogen 20 mg/mL to a final concentration of 0.05-1 µg/µL. Use up to 1 µL of glycogen per 20 µL of the solution.
    - Add 2.5 volumes of ethanol (or 1 volume of isopropanol) to the solution.
    - Mix gently but thoroughly.

- Incubate the mixture at -20°C for up to 60 min, or at -70°C for 30 min. Longer incubation and lower temperature (up to 24h) provide better recovery of nucleic acids.
- Centrifuge the mixture for 10-15 min at 10,000 rpm.
- Discard the supernatant.
- Rinse the pellet with cold 70% ethanol.
- Air-dry the pellet. Avoid over-drying the pellet, as it then takes more time to dissolve.
- Dissolve the eDNA pellet in 100 µL ultra-pure water and you will have now the eDNA ready for upstream applications. For high-thoughput analysis like metagenomics, a subsequent purification of the eDNA with Powerwater is needed. If this is your case dissolve the eDNA pellet in 650 µL TE buffer or ultra-pure sterile water instead and continue with the protocol below.

- Extract now all the disolved eDNA with the DNAeasy Powerwater kit omitting steps 1-7.
- Approximately 100 µL will be eluted. Eluting with nuclease free-water instead of TE or other elution buffer is recommended.
- Measure DNA concentration using Qubit.
- Store the DNA aliquots at -20°C. For genomic analysis (WP2) DNA is transported at -80ºC.

3.6 Compartment 6: River water

- Filter two times up to 0.2 L of **water** through a 0.45 µM filter.
- This is a **SAFE STOPPING POINT**. You can now freeze the filtrate at -20°C or continue with the precipitation of the eDNA in the filtrate.
- **OPTIONAL STEP:** Precipitate the eDNA (contains the most part of the free fraction of the eDNA) as follows:
  - Add 1/10 volume of 3 M sodium acetate to the supernant
  - Add glycogen 20 mg/mL to a final concentration of 0.05-1 µg/µL. Use up to 1 µL of glycogen per 20 µL of the solution.

- Add 2.5 volumes of ethanol (or 1 volume of isopropanol) to the solution.
- Mix gently but thoroughly.
- Incubate the mixture at -20°C for up to 60 min, or at -70°C for 30 min. Longer incubation and lower temperature (up to 24h) provide better recovery of nucleic acids.
- Centrifuge the mixture for 10-15 min at 10,000 rpm.
- Discard the supernatant.
- Rinse the pellet with cold 70% ethanol.
- Air-dry the pellet. Avoid over-drying the pellet, as it then takes more time to dissolve.
- Dissolve the eDNA pellet in 100 µL ultra-pure water and you will have now the eDNA ready for upstream applications. For high-thoughput analysis like metagenomics, a subsequent purification of the eDNA with Powerwater is needed. If this is your case dissolve the eDNA pellet in 650 µL TE buffer or ultra-pure sterile water instead and continue with the protocol below.

- Extract now all the disolved eDNA with the DNAeasy Powerwater kit omitting steps 1-7.
- Approximately 100 µL will be eluted. Eluting with nuclease free-water instead of TE or other elution buffer is recommended.
- Measure DNA concentration using Qubit.
- Store the DNA aliquots at -20°C. For genomic analysis (WP2) DNA is transported at -80ºC.

3.7 Compartment 7: Groundwater/drinking water

- Filter two times up to 0.2 L of **water** through a 0.45 µM filter.
- This is a **SAFE STOPPING POINT**. You can now freeze the filtrate at -20°C or continue with the precipitation of the eDNA in the filtrate.
- **OPTIONAL STEP:** Precipitate the eDNA (contains the most part of the free fraction of the eDNA) as follows:
    - Add 1/10 volume of 3 M sodium acetate to the supernant

- Add glycogen 20 mg/mL to a final concentration of 0.05-1 µg/µL. Use up to 1 µL of glycogen per 20 µL of the solution.
- Add 2.5 volumes of ethanol (or 1 volume of isopropanol) to the solution.
- Mix gently but thoroughly.
- Incubate the mixture at -20°C for up to 60 min, or at -70°C for 30 min. Longer incubation and lower temperature (up to 24h) provide better recovery of nucleic acids.
- Centrifuge the mixture for 10-15 min at 10,000 rpm.
- Discard the supernatant.
- Rinse the pellet with cold 70% ethanol.
- Air-dry the pellet. Avoid over-drying the pellet, as it then takes more time to dissolve.
- Dissolve the eDNA pellet in 100 µL ultra-pure water and you will have now the eDNA ready for upstream applications. For high-thoughput analysis like metagenomics, a subsequent purification of the eDNA with Powerwater is needed. If this is your case dissolve the eDNA pellet in 650 µL TE buffer or ultra-pure sterile water instead and continue with the protocol below.

- Extract now all the disolved eDNA with the DNAeasy Powerwater kit omitting steps 1-7.
- Approximately 100µl will be eluted. Eluting with nuclease free-water instead of TE or other elution buffer is recommended.
- Measure DNA concentration using Qubit.
- Store the DNA aliquots at -20°C. For genomic analysis (WP2) DNA is transported at -80ºC.

3.8 Compartment 8: Feed

- Weigh 2.5 g of **feed** in a 50ml universal tube.
- Add 0.5g of acid washed polyvinylpolypyrrolidone (PVPP)
- Add 7.5 mL of 0.1 M sodium phosphate buffer (NaP buffer).
- Shake 5 min at 150 rpm
- Place the sample on ice for 3 min

- Shake 5 min at 150 rpm
- Centrifuge 10 min at 500 g, at 4°C
- Collect the supernatant (contains the eDNA) and tranfer it to a sterile tube.
- Discard the sediment pellet (contains the iDNA)
- Centrifuge the pooled supernatants 30 min at 10,000 g, at 4°C
- Transfer the supernatant into a new sterile tube and discard the pellet. This is a **SAFE STOPPING POINT**. You can now freeze the supernatant at -20°C or continue with the precipitation.
- **OPTIONAL STEP:** Precipitate the eDNA from the supernatant as follows:
    - Add 1/10 volume of 3 M sodium acetate to the supernant
    - Add glycogen 20 mg/mL to a final concentration of 0.05-1 µg/µL. Use up to 1 µL of glycogen per 20 µL of the solution.
    - Add 2.5 volumes of ethanol (or 1 volume of isopropanol) to the solution.
    - Mix gently but thoroughly.
    - Incubate the mixture at -20°C for up to 60 min, or at -70°C for 30 min. Longer incubation and lower temperature (up to 24h) provide better recovery of nucleic acids.
    - Centrifuge the mixture for 10-15 min at 10,000 rpm.
    - Discard the supernatant.
    - Rinse the pellet with cold 70% ethanol.
    - Air-dry the pellet. Avoid over-drying the pellet, as it then takes more time to dissolve.
    - Dissolve the eDNA pellet in 100 µL ultra-pure water and you will have now the eDNA ready for upstream applications. For high-thoughput analysis like metagenomics, a subsequent purification of the eDNA with Powersoil is needed. If this is your case dissolve the eDNA pellet in 650 µL TE buffer or ultra-pure sterile water instead and continue with the protocol below.
- Extract now all the disolved eDNA with the DNAeasy Powersoil Pro kit omitting steps 1-7.
- Approximately 100µl will be eluted. Eluting with nuclease free-water instead of TE or other elution buffer is recommended.

- Now weight 0.25g of **feed** and extract the total DNA with DNAeasy Powersoil Pro kit (eluate contains the **total DNA**).

- Compare the DNA concentrations of both DNA types using Qubit.
- Store the DNA aliquots at -20°C. For genomic analysis (WP2) DNA is transported at -80ºC.

3.9 Compartment 9: Farmers

- Weigh 2.5 g of feces in a 50ml universal tube.
- Add 0.5g of acid washed polyvinylpolypyrrolidone (PVPP)
- Add 7.5 mL of 0.1 M sodium phosphate buffer (NaP buffer).
- Shake 5 min at 150 rpm
- Place the sample on ice for 3 min
- Shake 5 min at 150 rpm
- Centrifuge 10 min at 500 g, at 4°C

- Collect the supernatant (contains the eDNA) and tranfer it to a sterile tube.
- Discard the sediment pellet (contains the iDNA)
- Centrifuge the pooled supernatants 30 min at 10,000 g, at 4°C
- Transfer the supernatant into a new sterile tube and discard the pellet. This is a **SAFE STOPPING POINT**. You can now freeze the supernatant at -20°C or continue with the precipitation.
- **OPTIONAL STEP:** Precipitate the eDNA from the supernatant as follows:
    - Add 1/10 volume of 3 M sodium acetate to the supernant
    - Add glycogen 20 mg/mL to a final concentration of 0.05-1 µg/µL. Use up to 1 µL of glycogen per 20 µL of the solution.
    - Add 2.5 volumes of ethanol (or 1 volume of isopropanol) to the solution.
    - Mix gently but thoroughly.

- Incubate the mixture at -20°C for up to 60 min, or at -70°C for 30 min. Longer incubation and lower temperature (up to 24h) provide better recovery of nucleic acids.
- Centrifuge the mixture for 10-15 min at 10,000 rpm.
- Discard the supernatant.
- Rinse the pellet with cold 70% ethanol.
- Air-dry the pellet. Avoid over-drying the pellet, as it then takes more time to dissolve.
- Dissolve the eDNA pellet in 100 µL ultra-pure water and you will have now the eDNA ready for upstream applications. For high-thoughput analysis like metagenomics, a subsequent purification of the eDNA with Powersoil is needed. If this is your case dissolve the eDNA pellet in 650 µL TE buffer or ultra-pure sterile water instead and continue with the protocol below.

- Extract now all the disolved eDNA with the DNAeasy Powersoil Pro kit omitting steps 1-7.
- Approximately 100µl will be eluted. Eluting with nuclease free-water instead of TE or other elution buffer is recommended.

- Now weight 0.25g of feces and extract the total DNA with DNAeasy Powersoil Pro kit (eluate contains the **total DNA**).

- Compare the DNA concentrations of both DNA types using Qubit.
- Store the DNA aliquots at -20°C. For genomic analysis (WP2) DNA is transported at -80ºC.

3.10 Compartment 10: Wastewater

- Filter twice up to 0.2 L of water through a 0.2 µM filter (when the filter gets stuck, the filtration should be done through a 0.45 µM filter).

- This is a **SAFE STOPPING POINT**. You can now freeze the filtrate at -20°C or continue with the precipitation of the eDNA in the filtrate.
- **OPTIONAL STEP:** Precipitate the eDNA (contains the most part of the free fraction of the eDNA) as follows:
    - Add 1/10 volume of 3 M sodium acetate to the supernant
    - Add glycogen 20 mg/mL to a final concentration of 0.05-1 µg/µL. Use up to 1 µL of glycogen per 20 µL of the solution.
    - Add 2.5 volumes of ethanol (or 1 volume of isopropanol) to the solution.
    - Mix gently but thoroughly.
    - Incubate the mixture at -20°C for up to 60 min, or at -70°C for 30 min. Longer incubation and lower temperature (up to 24h) provide better recovery of nucleic acids.
    - Centrifuge the mixture for 10-15 min at 10,000 rpm.
    - Discard the supernatant.
    - Rinse the pellet with cold 70% ethanol.
    - Air-dry the pellet. Avoid over-drying the pellet, as it then takes more time to dissolve.
    - Dissolve the eDNA pellet in 100 µL ultra-pure water and you will have now the eDNA ready for upstream applications. For high-thoughput analysis like metagenomics, a subsequent purification of the eDNA with Powerwater is needed. If this is your case dissolve the eDNA pellet in 650 µL TE buffer or ultra-pure sterile water instead and continue with the protocol below.
- Extract now all the disolved eDNA with the DNAeasy Powerwater kit omitting steps 1-7.
- Approximately 100 µL will be eluted. Eluting with nuclease free-water instead of TE or other elution buffer is recommended.
- Measure DNA concentration using Qubit.
- Store the DNA aliquots at -20°C. For genomic analysis (WP2) DNA is transported at -80ºC.

3.11 Compartment 11: Wild animals

- Weigh 2.5 g of pooled feces in a 50 mL universal tube.
- Add 0.5 g of acid washed polyvinylpolypyrrolidone (PVPP)
- Add 7.5 mL of 0.1 M sodium phosphate buffer (NaP buffer).
- Shake 5 min at 150 rpm
- Place the sample on ice for 3 min
- Shake 5 min at 150 rpm
- Centrifuge 10 min at 500 g, at 4°C
- Collect the supernatant (contains the eDNA) and tranfer it to a sterile tube.
- Discard the sediment pellet (contains the iDNA)
- Centrifuge the pooled supernatants 30 min at 10,000 g, at 4°C
- Transfer the supernatant into a new sterile tube and discard the pellet. This is a **SAFE STOPPING POINT**. You can now freeze the supernatant at -20°C or continue with the precipitation.
- **OPTIONAL STEP:** Precipitate the eDNA from the supernatant as follows:
    - Add 1/10 volume of 3 M sodium acetate to the supernant
    - Add glycogen 20 mg/mL to a final concentration of 0.05-1 µg/µL. Use up to 1 µL of glycogen per 20 µL of the solution.
    - Add 2.5 volumes of ethanol (or 1 volume of isopropanol) to the solution.
    - Mix gently but thoroughly.
    - Incubate the mixture at -20°C for up to 60 min, or at -70°C for 30 min. Longer incubation and lower temperature (up to 24h) provide better recovery of nucleic acids.
    - Centrifuge the mixture for 10-15 min at 10,000 rpm.
    - Discard the supernatant.
    - Rinse the pellet with cold 70% ethanol.
    - Air-dry the pellet. Avoid over-drying the pellet, as it then takes more time to dissolve.
    - Dissolve the eDNA pellet in 100 µL ultra-pure water and you will have now the eDNA ready for upstream applications. For high-thoughput analysis like metagenomics, a subsequent purification of the eDNA

with Powersoil is needed. If this is your case dissolve the eDNA pellet in 650 µL TE buffer or ultra-pure sterile water instead and continue with the protocol below.

- Extract now all the disolved eDNA with the DNAeasy Powersoil Pro kit omitting steps 1-7.
- Approximately 100µl will be eluted. Eluting with nuclease free-water instead of TE or other elution buffer is recommended.

- Now weight 0.25g of pooled feces and extract the total DNA with DNAeasy Powersoil Pro kit (eluate contains the **total DNA**).

- Compare the DNA concentrations of both DNA types using Qubit.
- Store the DNA aliquots at -20°C. For genomic analysis (WP2) DNA is transported at -80ºC.

4. Sample identifiers

See the corresponding sample identifiers in the document "*Sample identifiers FED-AMR*", also part of D-JRP17-FED-AMR-WP2.1.

5. References

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6. Annex

Considerations

Use Ultra-pure water when possible to prepare the buffers and other working solutions

0.1 M Sodium Phosphate Buffer:

1. Prepare 1 litre of Stock Solutions:
   - To prepare 1 litre NaH_2PO_4 0.2 M add 27.6 g NaH_2PO_4 per liter of destilled water.
   - To prepare 1 litre Na_2HPO_4 0.2 M add 28.4 g Na_2HPO_4 per liter of destilled water.
   - Autoclave both solutions
2. Prepare 500 mL of 0.1M Na_2HPO_4 pH8 (Working Solution)
   - Add 236.75 mL of Na_2HPO_4 0.2 M and 13.25 mL NaH_2PO_4 0.2 M up to 500 mL of destilled water
   - Adjust pH
   - Autoclave 15 min 121°C

TE Buffer 1X, pH=8:

To prepare 100 mL of TE Buffer 1X add in a Duran bottle:

- 1 mL of 1M Tris-Cl (pH 8.0): final concentration 10 mM
- 0.2 mL of 0.5M EDTA (pH 8.0): final concentration 1mM
- 98.8 mL destilled water
- Place the lid on the bottle and invert a few times to mix.
- Autoclave the solution on a liquid cycle (20 min at 15 psi).
- Store TE buffer at room temperature (+15°C – +25°C).

Acid washed Polyvinylpolypyrrolidone (PVPP):

1. Prepare 4 L of 3M HCL (slowly add 992.0 mL of 12.1N HCL to 3008 mL of distilled H2O in a 4L beaker);

2. Slowly add 300 g of PVPP with stirring, cover beaker, and stir overnight;
3. Filter suspension through Miracloth or several layers of cheesecloth (use a large Buchner funnel and a 4-L vacuum flask);
4. Resuspend the PVPP in 4 L of 20 mM potassium phosphate buffer (pH 7.4) and mix for 1 to 2h. Check the pH (desired pH is 7.0);
5. Repeat filtrations and washes in 20 mM potassium phosphate buffer until the PVPP suspension has a pH of 7.0;
6. Following the final filtration, spread the PVPP on lab paper and let air dry overnight.