Protocol

Execution Phase -

For partners extracting DNA
Two Weeks in the World

V. 1.1

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Version	Date	Author	Edits	Contact
1.1	07.02.2020	Sidsel Nag	Major revision, format change, title change from "Execution Guidelines"	sidnag@food.dtu.dk
1.0	16.09.2019	Sidsel Nag		sidnag@food.dtu.dk

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Related documents	Availability
TWIW project portfolio v. 1.1	01.03.2020, via email and on the TWIW website (accessible from the same date)
TWIW project portfolio v. 1.0	Via previous email correspondance

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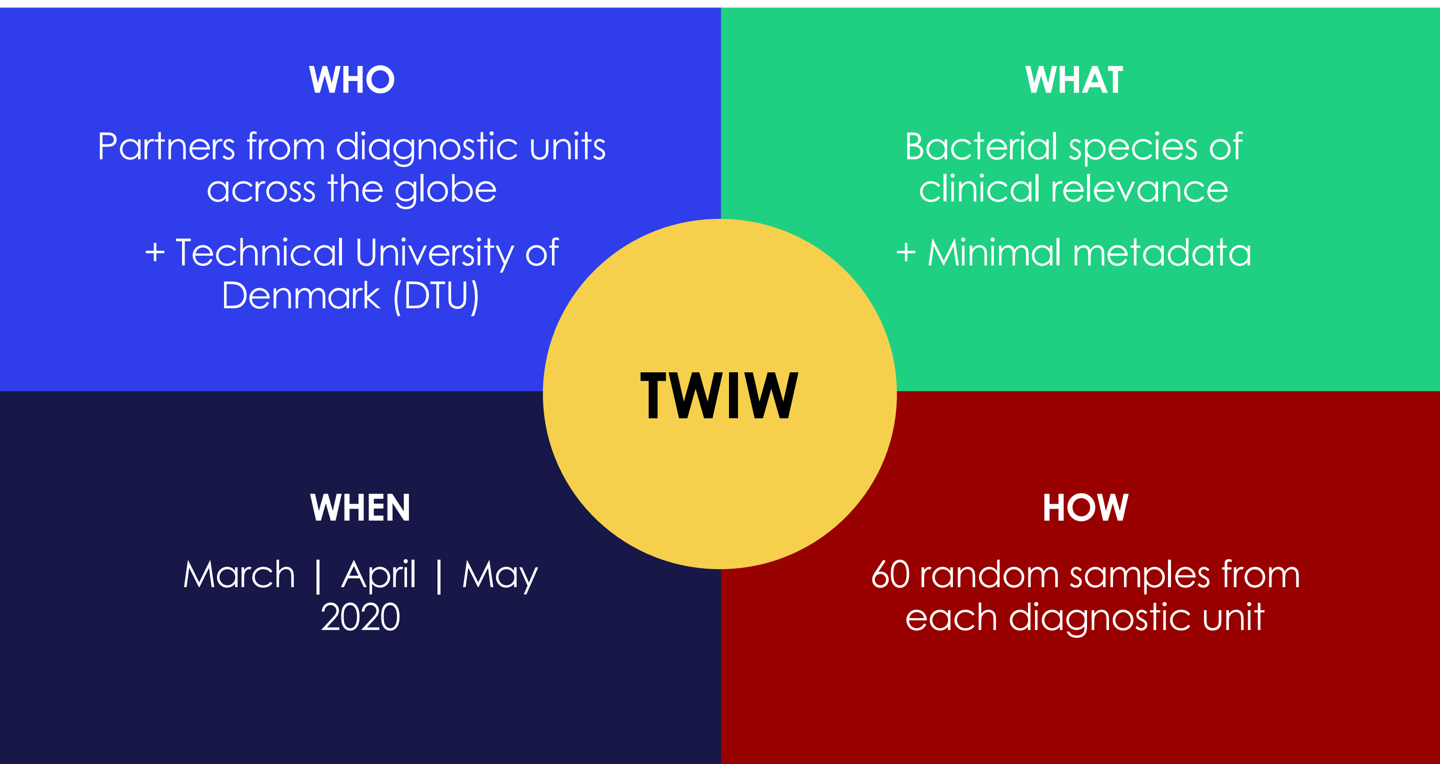
Summary

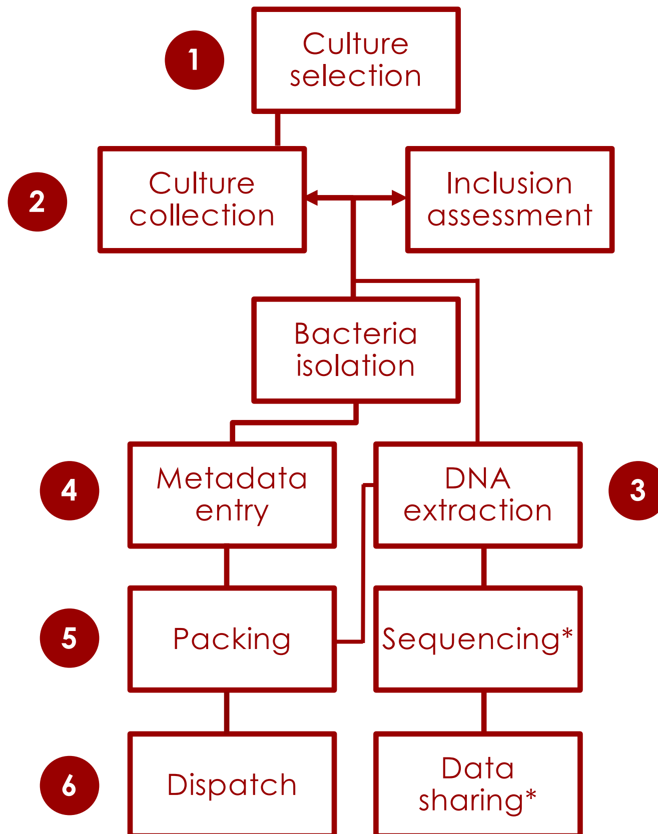
Two Weeks in the World (TWIW) is a global research collaboration to take a snap-shot in time of the prevalence of bacterial pathogens across the globe, as well as their antimicrobial resistance and virulence profiles.

The practical approach is based on random sampling of bacteria cultured for diagnostic purposes, transfer of minimal metadata, and whole-genome sequencing.

This protocol describes how to select samples for TWIW, how to isolate bacteria and how to send the isolates to Denmark for analysis. It also touches upon DNA extraction and sequencing, although these procedures are desribed in more detail in the respective protocols.



Work flow



The work flow corresponds to that of partner diagnostic units during the execution phase of the project. The work flow can vary by partner, some partners are sending isolates, others are extracting DNA in-house, while a few are also performing in-house sequencing. The numbering corresponds to the steps described in this document.

You will first determine which samples to collect for the study (1). You will then set aside the selected cultures as you process them in your diagnostic work (2). This is done in parallel with the inclusion assessment (2). Collected cultures are stored appropriately as the week passes, and once 60 cultures have been collected for inclusion in the study, you will perform the DNA extraction (3). It is also an option to perform the DNA extraction alongside the culture collection, as the week passes. Once your DNA is extracted, you can enter the minimal metadata online (4). Finally, you pack your DNA (5) and dispatch (6).

This protocol is for you specifically, because you are performing DNA extraction, and you therefore do not have to first isolate the bacteria (*). Isolation of bacteria with coal swabs is covered in the "Protocol Execution Phase – for partners sending isolates". Sequencing is not covered in these execution guidelines, but there are notes related to this step, containing our recommendations. The procedures are covered in detail in the protocols provided by library preparation kit and flow cell manufacturers. Data sharing from partners performing sequencing, will depend on the platforms used by these partners.

Step 1

Culture selection

Why is the selection method important?

It is important to avoid **"logistical bias"**.

Diagnostic units are often structured with specific schedules and logistical frameworks. These schedules and frameworks allow them to organize themselves and be more efficient in performing their diagnostic services. The efficiency gains are achieved by organizing themselves according to sample types and requirements, as well as patient groups and employee expertise. Because of the way diagnostic units typically organize themselves, it becomes highly likely that the logistics of the diagnostic unit will introduce a "logistical bias" into your selection of cultures, if not specifically avoided.

So how do we avoid logistical bias while retaining randomness?

We avoid logistical bias, by performing **"structured, prospective random sampling"**. This means collecting **every Nth culture** processed in your unit over an entire week.

In the following, you will see how to **calculate N** (this sounds more complicated than it is), how to store your collected cultures as well as what the inclusion criteria consist of.

Calculating N

N is a number corresponding to the interval of processed cultures between collecting another culture for the study.

Example: if your N = 10, you will collect every 10th culture your unit processes.

How to calculate N

Check how many bacterial samples your unit has processed during the week prior to your collection week. You will assume that you will process approximately the same number of samples during the collection week.

Example: if your unit processed 600 samples during the week prior to the collection week, your estimated total number of cultures for the collection week = 600.

N = your estimated total number of cultures / 60

Example: Your estimated total number of cultures for the collection week = 600. Therefore,

N = 600/60 = 10

Your collection interval is 10, so you will collect every 10th sample processed by your unit, during the collection week.

If your estimated total number of cultures < 60, collect all samples.

Step 2

Collection week

Culture collection

Since N is based on an estimate of how many samples you will be processing during the collection week, you may find that it will take you shorter or longer than a week to include 60 cultures.

- If you find you have collected 60 samples in less than a week, proceed to collect samples until the week is over. We have included extra coal swabs for you to use.
- If you find you have not collected 60 samples in a week, proceed to collect samples for longer than a week to reach 60.

Collected cultures should be kept refrigerated (at approximately 4°C), until you are ready to extract DNA.

Remember to assess whether the collected cultures should be included in the study according to the inclusion criteria, which includes acquisition of the minimal metadata belonging to the cultures.

Inclusion criteria

Cultured species is clinically relevant

Is the cultured bacteria thought to be the cause of infection in the patient diagnosed?

Culture without contaminants

Is the culture clean?

Minimal metadata is accessible

Minimal metadata consist of:

- date of sampling from the patient
- source of sample from the patient
- date of isolation from the culture
- identified species (if known)

You may have to go back to your collection process and include more samples in order to have 60 samples that fulfill the inclusion criteria.

Step 3.A

Preparing for DNA extraction

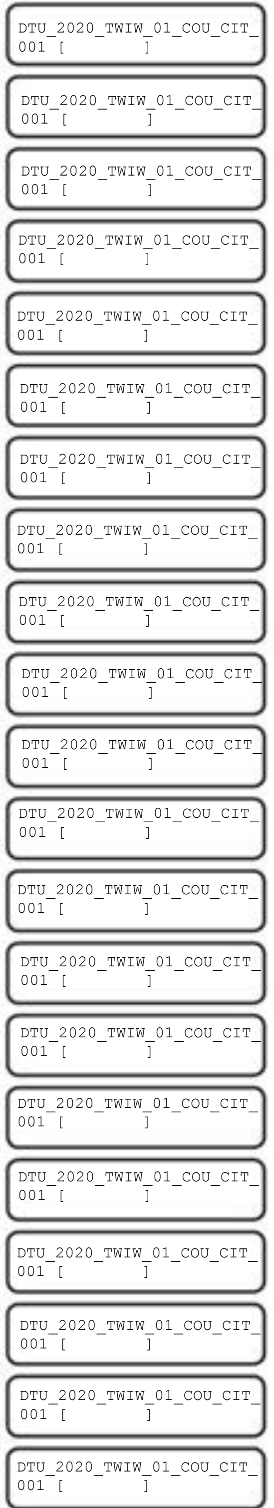
Labeling

If necessary, put a label on the culture plate. This may help you stay organized and help you to match metadata with the correct sample.

Samples originating from the same source (patient sample), can be indicated in the [] field on the labels by writing the number of the other sample(s) originating from the same source.

<u>Remember to use the labels for:</u>

- the DNA-containing Eppendorf tubes, once you have finished extracting your DNA
- The metadata sheets corresponding to the bacterial DNA in the tubes with the corresponding label



Material needed for extracting DNA

Cultured bacteria

Minimal metadata

Pen

Vortex

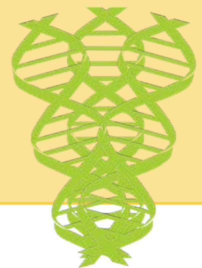
Pipettes and pipette tips

Provided by you

Microcentrifuge (positioned at room temperature)

Non-denatured ethanol, ≥ 95 %

Heating block or water bath, preferably with shaker option

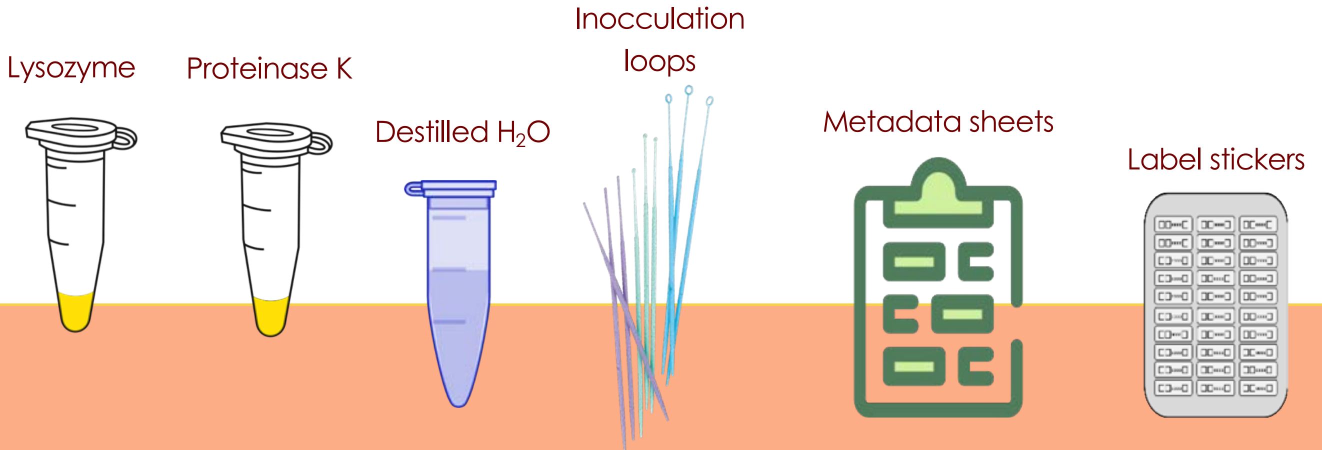


Equipment to measure DNA concentration

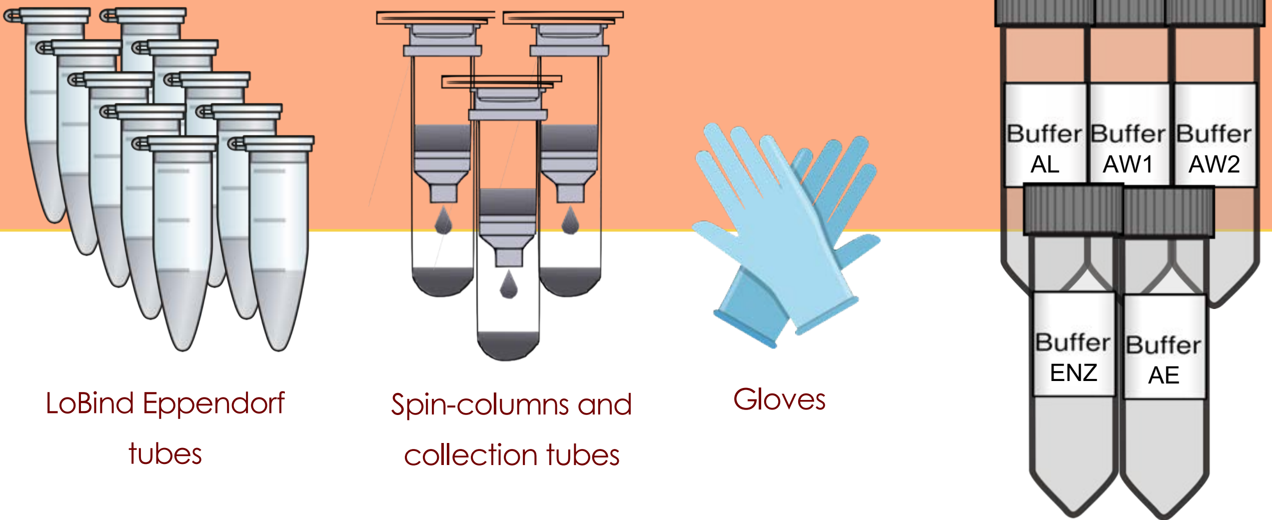
Why not denatured ethanol?

Denatured ethanol contains additives to make it poisonous or bad-tasting, to discourage consumption of the ethanol. Some of the additives used for these purposes can interfere with the down stream applications of the extracted DNA.

Material needed for extracting DNA



Provided by DTU



Why LoBind Eppendorf tubes?

When stored in standard tubes, a large portion of the extracted DNA may bind the inside surface of the tubes. LoBind tubes have a hydrophilic surface (non-silicone) which minimises surface binding of the DNA.

Preparing your workspace

Avoiding environmental contaminants

- Make sure you are working on a clean surface.
    - If a LAF-bench or other sterile environment is accessible, perform extraction here.
- Wear gloves.
- Keep petri dishes closed and upside down when not interacting with them.
- If using a water bath for temperature control, make sure to not submerge the tubes completely in the water bath. Avoid water from the water bath around the lid opening of your tubes.

Safety first

The material sent to you for DNA extraction is from the Qiagen DNeasy® Blood & Tissue Kit (250 reactions, cat. n. 69506). This protocol contains all steps and information necessary to perform the DNA extraction according to Qiagen's protocol.

If you wish to consult Qiagen's protocol, you can find it on the TWIW website, (under resources for participants): https://www.globalsurveillance.eu/projects/global-surveillance-of-antimicrobial-resistance/two-weeks-in-the-world

We have attached the pages relevant for extraction of Gram+ and Gram- bacteria, at the end of this protocol, as appendices for your convenience. You do not necessary need to consult these pages.

All MSDS documents for buffers and reagents can also be found ont he TWIW websites, under resources for participants (above link).

Buffers AL, AW1 and the **Proteinase K** contain hazardous ingredients, and Qiagen's safety information is printed and packed with the buffers, as well as included as an appendice to this protocol. Please note that Qiagen has not updated the health hazard codes in their safety information. Their MSDS documents are updated with the accurate codes.

Before you begin to extract DNA

We have included the following in your parcel:

• Enzymatic buffer	10 ml
• Lysozyme (powder)	10 mg[1]
• Destilled H_2O	app. 1.2 ml
• Proteinase K solution	1.9 ml
• Buffer AL	15 ml
• Buffer ATL	10 ml
• Buffer AW1(concentrate)	17,5ml[2]
• Buffer AW2 (concentrate)	12 ml[2]
• Buffer AE	10 ml
• Spin columns	70 pcs
• Collection Tubes (2 ml)	140 pcs
• Low bind eppendorf Tubes 1,5 ml	150 pcs
• Blue loops	100 pcs

[1] Add 1 ml destilled H_2O, to get a 10 mg/ml solution. Store frozen once dissolved.

[2] Add non-denatured ethanol (≥ 95%) before use. (Ethanol is provided by you)

Step 3.B

Gram-positive DNA extraction

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DNA extraction for Gram+ bacteria

Step 1. Bacterial lysis

- Set your heating block/water bath to 37°C
- Suspend ¾ blue loop bacteria in 180 µl Enzymatic Buffer in an Eppendorf tube
- Add 20 µl lysozyme (10mg/ml) to the suspension
- Incubate the suspension at 37°C for 60 min (until the suspension clears up)

- Remove your Eppendorf tube with the bacterial suspension from the heating block/water bath
- Set the heating block/water bath to 56°C
- Add 25 µl proteinase K to the bacterial suspension
- Add 200 µl Buffer AL to the suspension
- Vortex the suspension

- Incubate the suspension at 56°C 30 minutes
- Remove the suspension from the heating block/water bath
- Add 200 µl non-denatured ethanol (≥ 95%)
- Vortex the suspension

DNA extraction for Gram+ bacteria

Step 2. Cleaning the bacterial lysate

- Prepare a DNeasy spin-column and its 2 ml collection tube
- Pipet the bacterial suspension from your Eppendorf tube into the prepared DNeasy spin-column.
- Centrifuge the spin-column for 1 min. at 8000 rpm

! Remember to balance the centrifuge

- Discard the flow-through[1] and collection tube

- Place the spin-column in a new 2 ml collection tube
- Add 500 µl buffer AW1 to the spin-column.
- Centrifuge for 1 min. at 8000 rpm.
- Discard the flow-through and collection tube

- Place the spin-column in a new 2 ml collection tube
- Add 500 µl Buffer AW2 to the spin-column.
- Centrifuge for 3 min. at 14000 rpm.
- Discard the flow-through and collection tube

[1] rpm = revolutions per minute. You can convert rpm to g force/rcf (relative centrifugal force). The conversion depends on the size of the rotor in your centrifuge. This is a website where you can do the conversion: http://www.endmemo.com/bio/grpm.php

[2] Flow-through refers to the liquid which has passed through the spin-column into the collection tube during centrifugation

DNA extraction for Gram+ bacteria

Step 3. Elution of the DNA

- Place the spin-column in a 1,5 ml Lobind Eppendorf tube
- Add 100 µl Buffer AE to the spin-column
- Centrifuge for 2 min. at 8000 rpm.
- Discard the spin-column and close the Lobind Eppendorf tube

If the lid on the Lobind Eppendorf tube breaks off during centrifugation, it is ok to still use the broken lid.

Step 4. Checking your DNA

- Check the eluted DNA for a pellet in the bottom of the tube. If there is a visible pellet, transfer the supernatant to a new Lobind Eppendorf tube, discard the tube with the pellet.
- The DNA is now ready for measuring the concentration.
- Send at least 50 µl DNA per sample.

NOTE! If the DNA concentration < 6 ng/µl, send at least 80 µl.

- Store extracted DNA in the cardboard box provided by DTU, at 4°C (preferred) or at room temperature until dispatch. Do not freeze.

Qiagen has made a (general) visual protocol which can be found on YouTube.

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Step 3.C

Gram-negative DNA extraction

DNA extraction for Gram- bacteria

Step 1. Bacterial lysis

- Set your heating block/water bath to 56°C
- Suspend ¾ blue loop bacteria in 180 µl Buffer ATL in an Eppendorf tube
- Add 25 µl proteinase K to the bacterial suspension
- Vortex the suspension
- Incubate the suspension at 56°C 30 minutes while shaking

If you do not have a shaker for eppendorf tubes to submerge in your water bath, or a heating block with a shaking function, you can manually invert the tubes 5 times every 10 minutes.

- Add 200 µl Buffer AL to the suspension
- Vortex the suspension
- Incubate the suspension at 56°C 30 minutes

- Remove the suspension from the heating block/water bath
- Add 200 µl non-denatured ethanol (≥ 95%)
- Vortex the suspension

DNA extraction for Gram- bacteria

Step 2. Cleaning the bacterial lysate

- Prepare a DNeasy spin-column and its 2 ml collection tube
- Pipet the bacterial suspension from your Eppendorf tube into the prepared DNeasy spin-column.
- Centrifuge the spin-column for 1 min. at 8000 rpm

! Remember to balance the centrifuge

- Discard the flow-through[1] and collection tube

- Place the spin-column in a new 2 ml collection tube
- Add 500 µl buffer AW1 to the spin-column.
- Centrifuge for 1 min. at 8000 rpm.
- Discard the flow-through and collection tube

- Place the spin-column in a new 2 ml collection tube
- Add 500 µl Buffer AW2 to the spin-column.
- Centrifuge for 3 min. at 14000 rpm.
- Discard the flow-through and collection tube

[1] rpm = revolutions per minute. You can convert rpm to g force/rcf (relative centrifugal force). The conversion depends on the size of the rotor in your centrifuge. This is a website where you can do the conversion: http://www.endmemo.com/bio/grpm.php

[2] Flow-through refers to the liquid which has passed through the spin-column into the collection tube during centrifugation

DNA extraction for Gram- bacteria

Step 3. Elution of the DNA

- Place the spin-column in a 1,5 ml Lobind Eppendorf tube
- Add 100 µl Buffer AE to the spin-column
- Centrifuge for 2 min. at 8000 rpm.
- Discard the spin-column and close the Lobind Eppendorf tube

If the lid on the Lobind Eppendorf tube breaks off during centrifugation, it is ok to still use the broken lid.

Step 4. Checking your DNA

- Check the eluted DNA for a pellet in the bottom of the tube. If there is a visible pellet, transfer the supernatant to a new Lobind Eppendorf tube, discard the tube with the pellet.
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- Send at least 50 µl DNA per sample.

NOTE! If the DNA concentration < 6 ng/µl, send at least 80 µl.

- Store extracted DNA in the cardboard box provided by DTU, at 4°C (preferred) or at room temperature until dispatch. Do not freeze.

Qiagen has made a (general) visual protocol which can be found on YouTube.

Step 4

Metadata entry

TWIW sample log

Enter the metadata via the TWIW Sample Log (Survey Monkey link).

You will be asked to fill in:

- Your contact information
- Name and location of the diagnostic unit
  - The types of samples that your unit processes in general
  - The diagnostic methods used
    - Whether your unit is able to culture anaerobic bacteria and other fastidious species
      - The metadata belonging to your samples
      - If you have extracted DNA: concentrations and elution volumes, as well as protocols used
        - If you have performed sequencing: quality assessments as well as protocols used

If you are performing WGS

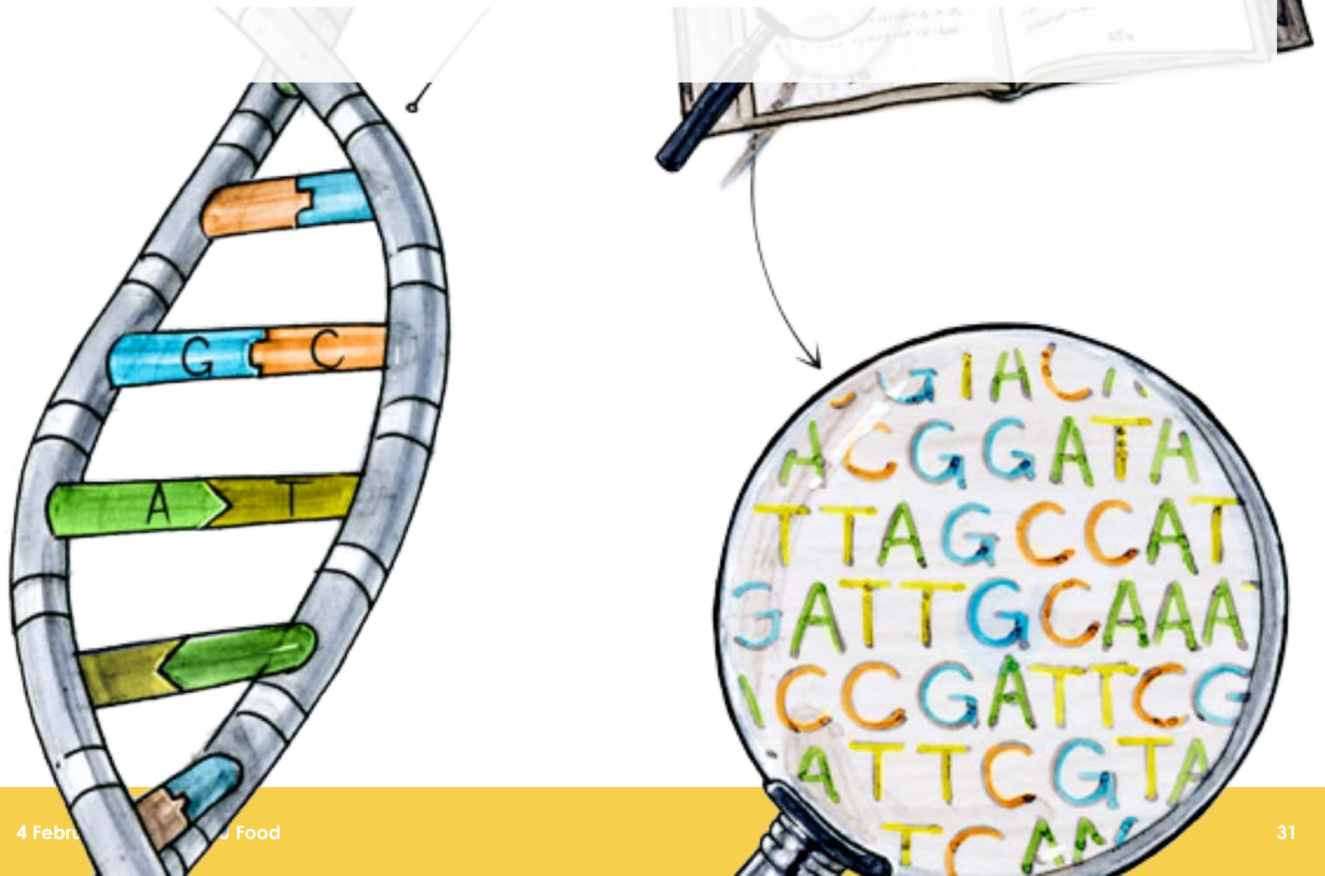
In order for data to be comparable across samples, it is important to streamline the protocols used as much as possible. If you are sequencing your samples, we recommend that you perform paired-end sequencing on one of Illumina's platforms:

Miseq (2x 250 bp)

Nextseq (2 x 150 bp)

Hiseq (2 x 150 bp)

At DTU, we use the Nextera XT kit for sequencing, and we sequence 30-40 isolates on a Miseq V3 flow cell, or 98 isolates on a Nextseq medium output flowcell.



Step 5

Packaging

Packing your DNA

DNA is not hazardous and does not need to meet any specific standards for shipping. Nevertheless, we wish to take good care of it in order to protect its quality.

- Seal the LoBind Eppendorf tubes containing your DNA with parafilm.
- Find the cardboard box that contained your buffers when you received the parcel, as well as the absorbing material that the buffers were in. Place the absorbing material in the bottom of the box.
- Insert the 81-compartment cardboard spacers into the box.
- Place the LoBind Eppendorf tubes containing your DNA in the 81-compartment cardboard box.
- Place the rubber bands around the cardboard box.
- Put the cardboard box into the Biosafety bag (which has stickers covering the UN3373/Biosafety text because this is not a hazardous parcel) and close it shut with the zip-lock. Take out as much air as possible.
- Put the biosafety bag and the metadata sheets into the protected envelope.
- Close the envelope.

Step 6

Dispatch

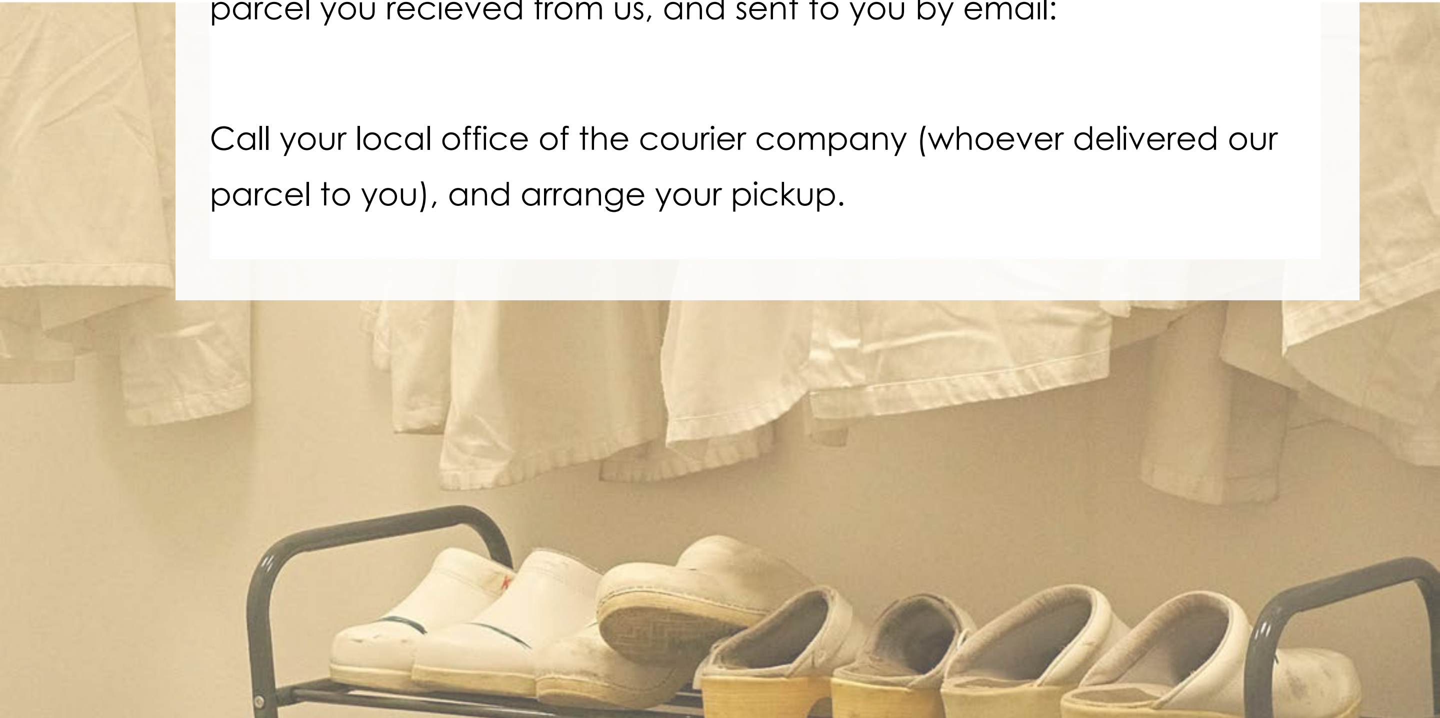
Ready to send!

Once you have entered your metadata online in the TWIW sample log, and have packed your isolates or DNA, you are ready to contact the courier to pick it up.

We have pre-arranged for the shipment through our account with the courier service. We have placed a transparent envelope on your return envelope with the return shipment label and a copy of the return shipment customs invoice.

The documents you may need were placed into a plastic sheet in the parcel you recieved from us, and sent to you by email:

Call your local office of the courier company (whoever delivered our parcel to you), and arrange your pickup.



Appendices

- Appendix I: Qiagen DNeasy® Blood & Tissue saftey information
- Appendix II: Qiagen DNeasy Blood & Tissue handbook p. 28-30 and 44-46

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/ts/msds.asp where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

CAUTION: DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

Buffer AL and Buffer AW1 contain guanidine hydrochloride, which can form highly reactive compounds when combined with bleach. If liquid containing this buffer is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

The following risk and safety phrases apply to components of DNeasy Blood & Tissue Kits and DNeasy 96 Blood & Tissue Kits.

Buffer AL and Buffer AW1 (concentrate)

Contains guanidine hydrochloride: harmful, irritant. Risk and safety phrases:* R22-36/38, S13-26-36-46

Proteinase K

Contains proteinase K: sensitizer, irritant. Risk and safety phrases:* R36/37/38-42/43, S23-24-26-36/37

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

* R22: Harmful if swallowed; R36/37/38: Irritating to eyes, respiratory system and skin; R36/38: Irritating to eyes and skin; R42/43: May cause sensitization by inhalation and skin contact; S13: Keep away from food, drink, and animal feedingstuffs; S23: Do not breathe spray; S24: Avoid contact with skin; S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; S36: Wear suitable protective clothing; S36/37: Wear suitable protective clothing and gloves; S46: If swallowed, seek medical advice immediately, and show container or label.

Protocol: Purification of Total DNA from Animal Tissues (Spin-Column Protocol)

This protocol is designed for purification of total DNA from animal tissues, including rodent tails.

Important points before starting

- If using the DNeasy Blood & Tissue Kit for the first time, read "Important Notes" (page 15).
- For fixed tissues, refer to the pretreatment protocols "Pretreatment for Paraffin-Embedded Tissue", page 41, and "Pretreatment for Formalin-Fixed Tissue", page 43.
- All centrifugation steps are carried out at room temperature (15–25°C) in a microcentrifuge.
- Vortexing should be performed by pulse-vortexing for 5–10 s.
- Optional: RNase A may be used to digest RNA during the procedure. RNase A is not provided in the DNeasy Blood & Tissue Kit (see "Copurification of RNA", page 19).

Things to do before starting

- Buffer ATL and Buffer AL may form precipitates upon storage. If necessary, warm to 56°C until the precipitates have fully dissolved.
- Buffer AW1 and Buffer AW2 are supplied as concentrates. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- Preheat a thermomixer, shaking water bath, or rocking platform to 56°C for use in step 2.
- If using frozen tissue, equilibrate the sample to room temperature. Avoid repeated thawing and freezing of samples since this will lead to reduced DNA size.

Procedure

1. **Cut up to 25 mg tissue (up to 10 mg spleen) into small pieces, and place in a 1.5 ml microcentrifuge tube. For rodent tails, place one (rat) or two (mouse) 0.4–0.6 cm lengths of tail into a 1.5 ml microcentrifuge tube. Add 180 µl Buffer ATL. Earmark the animal appropriately.**

   Ensure that the correct amount of starting material is used (see "Starting amounts of samples", page 15). For tissues such as spleen with a very high number of cells for a given mass of tissue, no more than 10 mg starting material should be used.

We strongly recommend to cut the tissue into small pieces to enable more efficient lysis. If desired, lysis time can be reduced by grinding the sample in liquid nitrogen* before addition of Buffer ATL and proteinase K. Alternatively, tissue samples can be effectively disrupted before proteinase K digestion using a rotor–stator homogenizer, such as the QIAGEN TissueRuptor, or a bead mill, such as the QIAGEN TissueLyser (see page 56 for ordering information). A supplementary protocol for simultaneous disruption of up to 48 tissue samples using the TissueLyser can be obtained by contacting QIAGEN Technical Services (see back cover).

For rodent tails, a maximum of 1.2 cm (mouse) or 0.6 cm (rat) tail should be used. When purifying DNA from the tail of an adult mouse or rat, it is recommended to use only 0.4–0.6 cm.

2. **Add 20 µl proteinase K. Mix thoroughly by vortexing, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a thermomixer, shaking water bath, or on a rocking platform.**

   Lysis time varies depending on the type of tissue processed. Lysis is usually complete in 1–3 h or, for rodent tails, 6–8 h. If it is more convenient, samples can be lysed overnight; this will not affect them adversely.

   After incubation the lysate may appear viscous, but should not be gelatinous as it may clog the DNeasy Mini spin column. If the lysate appears very gelatinous, see the "Troubleshooting Guide", page 47, for recommendations.

   Optional: If RNA-free genomic DNA is required, add 4 µl RNase A (100 mg/ml), mix by vortexing, and incubate for 2 min at room temperature before continuing with step 3.

   Transcriptionally active tissues such as liver and kidney contain high levels of RNA, which will copurify with genomic DNA. For tissues that contain low levels of RNA, such as rodent tails, or if residual RNA is not a concern, RNase A digestion is not necessary.

3. **Vortex for 15 s. Add 200 µl Buffer AL to the sample, and mix thoroughly by vortexing. Then add 200 µl ethanol (96–100%), and mix again thoroughly by vortexing.**

   It is essential that the sample, Buffer AL, and ethanol are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. Buffer AL and ethanol can be premixed and added together in one step to save time when processing multiple samples.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

A white precipitate may form on addition of Buffer AL and ethanol. This precipitate does not interfere with the DNeasy procedure. Some tissue types (e.g., spleen, lung) may form a gelatinous lysate after addition of Buffer AL and ethanol. In this case, vigorously shaking or vortexing the preparation is recommended.

4. **Pipet the mixture from step 3 (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at ≥6000 x *g* (8000 rpm) for 1 min. Discard flow-through and collection tube.***

5. **Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 min at ≥6000 x *g* (8000 rpm). Discard flow-through and collection tube.***

6. **Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x *g* (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.**

   It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution.

   Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000 x g (14,000 rpm).

7. **Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (not provided), and pipet 200 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥6000 x *g* (8000 rpm) to elute.**

   Elution with 100 µl (instead of 200 µl) increases the final DNA concentration in the eluate, but also decreases the overall DNA yield (see Figure 2, page 21).

8. **Recommended: For maximum DNA yield, repeat elution once as described in step 7.**

   This step leads to increased overall DNA yield.

   A new microcentrifuge tube can be used for the second elution step to prevent dilution of the first eluate. Alternatively, to combine the eluates, the microcentrifuge tube from step 7 can be reused for the second elution step.

   Note: Do not elute more than 200 µl into a 1.5 ml microcentrifuge tube because the DNeasy Mini spin column will come into contact with the eluate.

* Flow-through contains Buffer AL or Buffer AW1 and is therefore not compatible with bleach. See page 8 for safety information.

Protocol: Pretreatment for Gram-Negative Bacteria

This protocol is designed for purification of total DNA from Gram-negative bacteria, such as *E. coli*. The protocol describes the preliminary harvesting of bacteria before DNA purification.

Important points before starting

- See “Quantification of starting material”, page 17, for details of how to collect and store samples, and how to determine the number of cells in a bacterial culture.
- This pretreatment protocol has not been thoroughly tested and optimized for high-throughput DNA purification using the DNeasy 96 Blood & Tissue Kit. As a general guideline, we recommend to decrease the amount of starting material when using this protocol with the DNeasy 96 Blood & Tissue Kit.

Procedure

1. **Harvest cells (maximum 2 x 10^9 cells) in a microcentrifuge tube by centrifuging for 10 min at 5000 x *g* (7500 rpm). Discard supernatant.**
2. **Resuspend pellet in 180 µl Buffer ATL.**
3. **Continue with step 2 of the protocol “Purification of Total DNA from Animal Tissues (Spin-Column Protocol)”, page 29.**

Protocol: Pretreatment for Gram-Positive Bacteria

This protocol is designed for purification of total DNA from Gram-positive bacteria, such as *Corynebacterium* spp. and *B. subtilis*. The protocol describes the preliminary harvesting of bacteria and incubation with lysozyme to lyse their cell walls before DNA purification.

Important points before starting

- See "Quantification of starting material", page 17, for details of how to collect and store samples, and how to determine the number of cells in a bacterial culture.
- Ensure that ethanol has not been added to Buffer AL (see "Buffer AL", page 18). Buffer AL can be purchased separately (see page 56 for ordering information).
- This pretreatment protocol has not been thoroughly tested and optimized for high-throughput DNA purification using the DNeasy 96 Blood & Tissue Kit. As a general guideline, we recommend to decrease the amount of starting material when using this protocol with the DNeasy 96 Blood & Tissue Kit.

Things to do before starting

- Prepare enzymatic lysis buffer as described in "Equipment and Reagents to Be Supplied by User", page 14.
- Preheat a heating block or water bath to 37°C for use in step 3.

Procedure

1. **Harvest cells (maximum 2 x 10^9 cells) in a microcentrifuge tube by centrifuging for 10 min at 5000 x g (7500 rpm). Discard supernatant.**
2. **Resuspend bacterial pellet in 180 µl enzymatic lysis buffer.**
3. **Incubate for at least 30 min at 37°C.**

   After incubation, heat the heating block or water bath to 56°C if it is to be used for the incubation in step 5.
4. **Add 25 µl proteinase K and 200 µl Buffer AL (without ethanol). Mix by vortexing.**

   Note: Do not add proteinase K directly to Buffer AL.

   Ensure that ethanol has not been added to Buffer AL (see "Buffer AL", page 18). Buffer AL can be purchased separately (see page 56 for ordering information).
5. **Incubate at 56°C for 30 min.**

   Optional: If required, incubate at 95°C for 15 min to inactivate pathogens. Note that incubation at 95°C can lead to some DNA degradation.

6. **Add 200 µl ethanol (96–100%) to the sample, and mix thoroughly by vortexing.**

   It is important that the sample and the ethanol are mixed thoroughly to yield a homogeneous solution.

   A white precipitate may form on addition of ethanol. It is essential to apply all of the precipitate to the DNeasy Mini spin column. This precipitate does not interfere with the DNeasy procedure.

7. **Continue with step 4 of the protocol "Purification of Total DNA from Animal Tissues (Spin-Column Protocol)", page 30.**