Protocol

Execution Phase -

For partners sending isolates
Two Weeks in the World

V. 1.1

4 February 2020 DTU Food

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

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

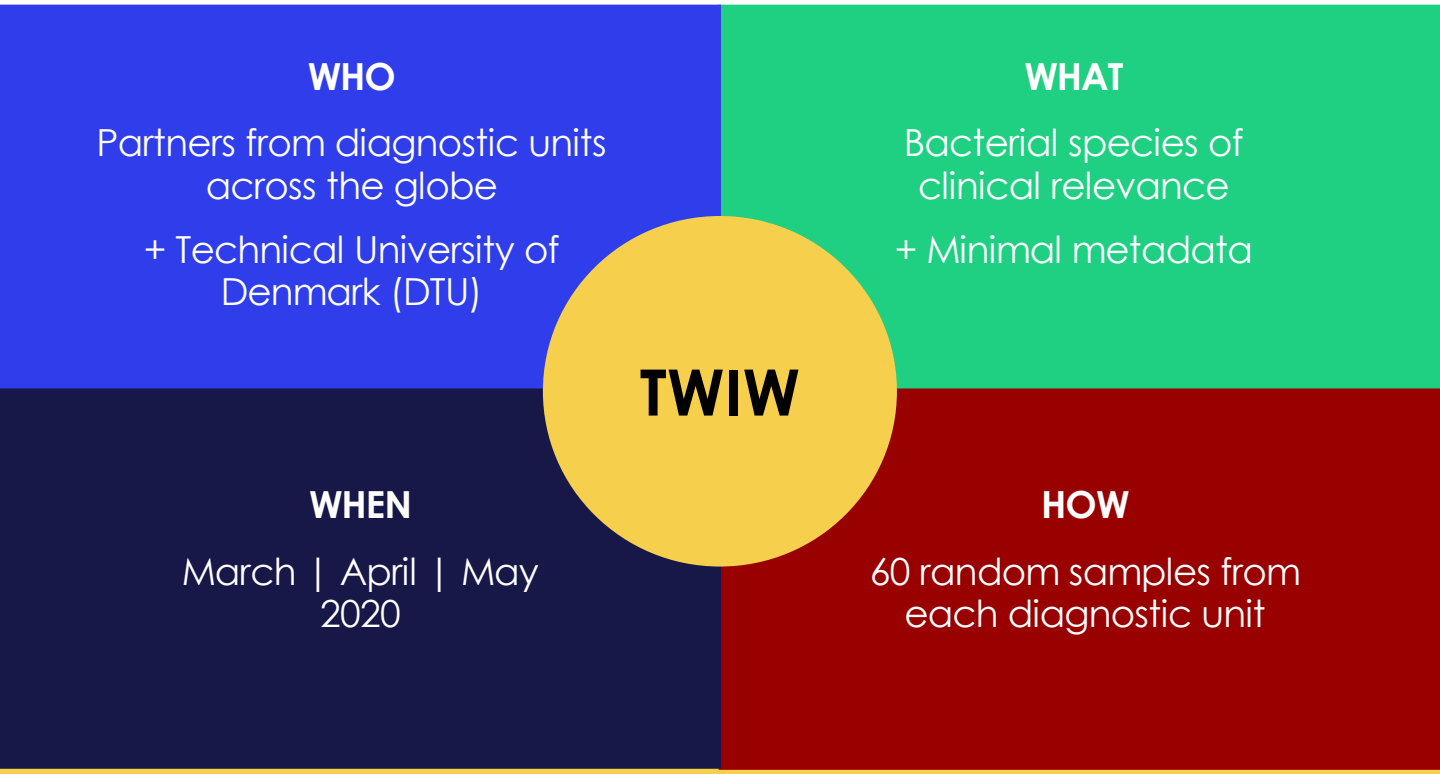
Table of contents

Summary

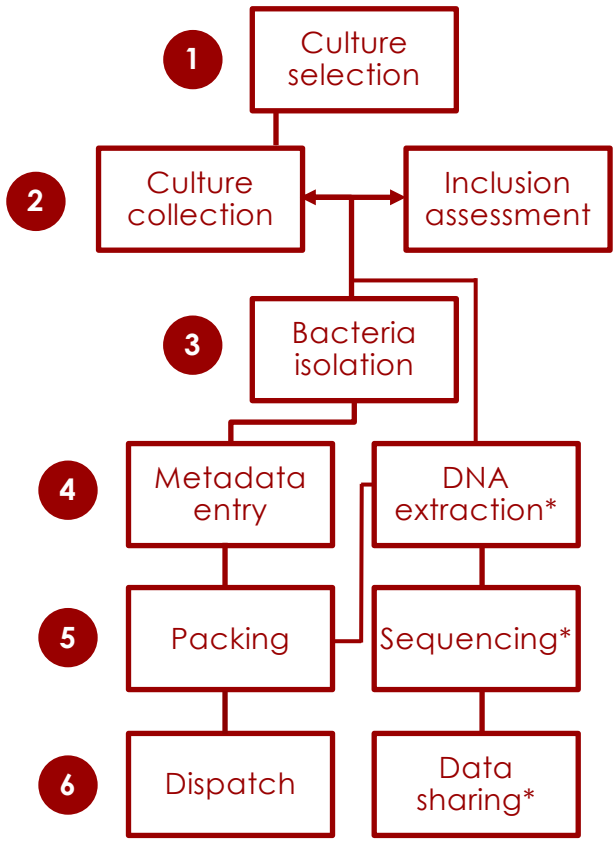
Two Weeks in the World (TWIW) is a global research collaboration to take a snap-shot in time of the prevalence of bacterial pathogens across the globe, as well as their antimicrobial resistance and virulence profiles.

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 isolate the bacteria (3). If you are performing DNA extraction, you do not have to first isolate the bacteria. Once your bacteria are isolated or your DNA is extracted, you can enter the minimal metadata online (4). Finally, you pack your isolates or DNA (5) and dispatch (6).

*DNA extraction and sequencing are not covered in these execution guidelines, but there are notes related to both steps, containing our recommendations for protocols. The procedures are covered in detail in the protocols provided by DNA extraction kit manufacturers, as well as library preparation kit and flow cell manufacturers. Data sharing from partners performing sequencing, will depend on the platforms used by these partners.

For a video guide of the bacterial isolation procedure, as well as packaging for dispatch, visit the TWIW Resources Youtube channel.

Step 1

Culture selection

Why is the selection method important?

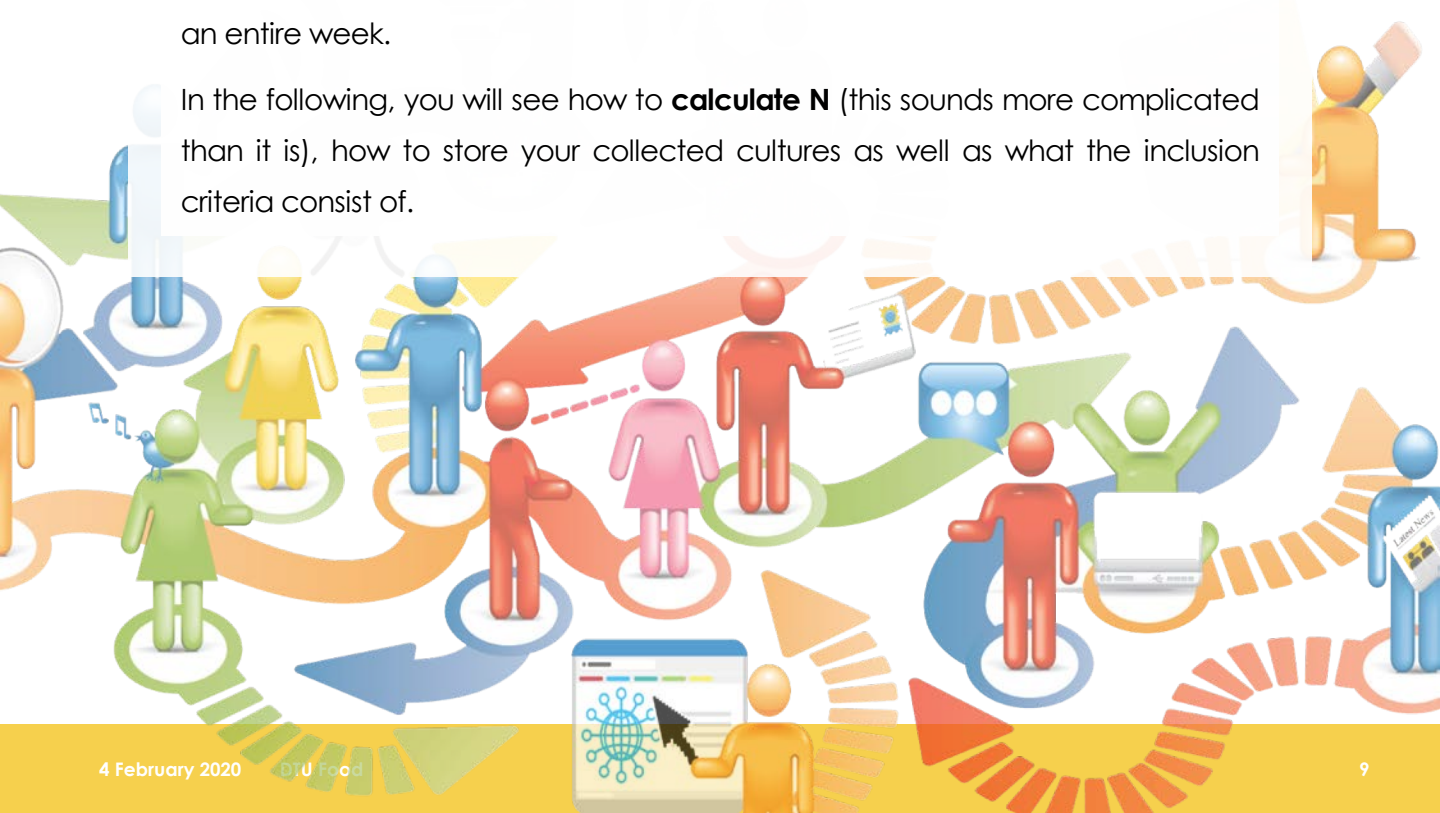
It is important to avoid **"logistical bias"**.

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 N^{th} 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 the last cultures are collected. This is due to the fact that bacteria survive much better on their growth media and at 4°C, than on the coal swabs. Once you have collected all 60 cultures, you are ready to isolate bacteria with the coal swabs that you have recieved from us.

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:

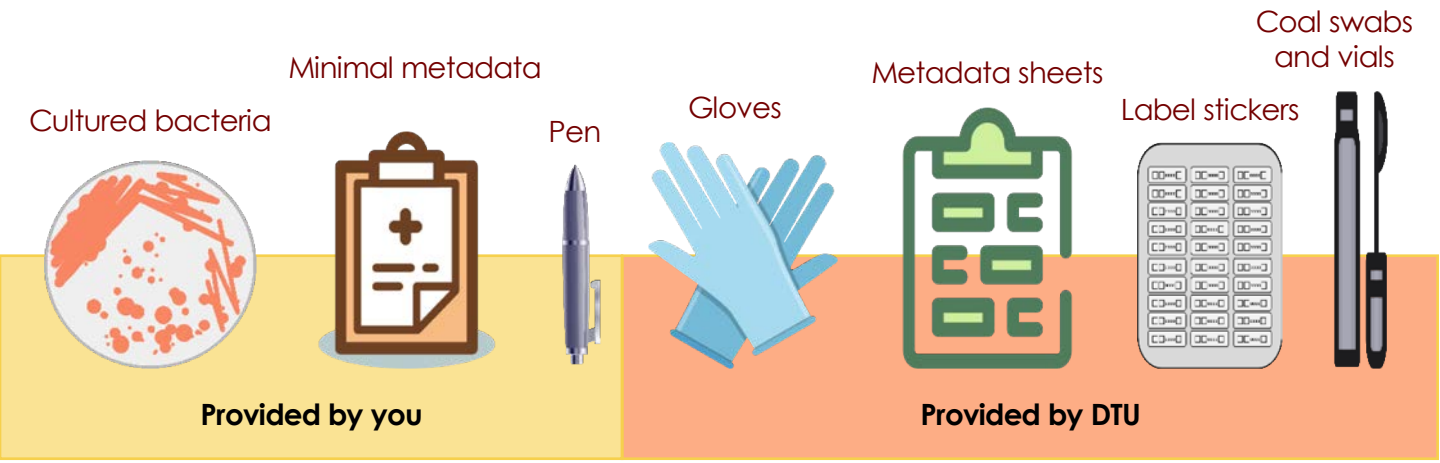
- date of sampling from the patient
- source of sample from the patient
- date of isolation from the culture
- identified species (if known)

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

Sampling bacterial isolates

Material needed for sampling bacteria



What are coal swabs and why do we use them for this purpose?

Coal swabs are transport swabs, usually used in a clinical setting to sample directly from the patient. They work equally well when used for cultured bacteria, and these ones contain charcoal in the agar. The charcoal makes the environment suitable for both aerobic and anaerobic species.

Store them at 3 – 25 °C. Never freeze.

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 isolation here.
- Wear gloves.
- Keep petri dishes closed and upside down when not interacting with them.
- Prepare the coal swabs a few at a time.
    - Put the coal-containing vials in a rack.
    - Do not open the lids.
    - Keep the swabs in the sterile packaging.

Isolating bacteria

1. Swab the cultured bacteria

- Open your petri dish.
- **Scrape** the cultured bacteria with a swab.

  NOTE: It is necessary with more than a single colony on the swab.
- Put the petri dish back down on its lid.

2. Place the swab into the coal-vial.

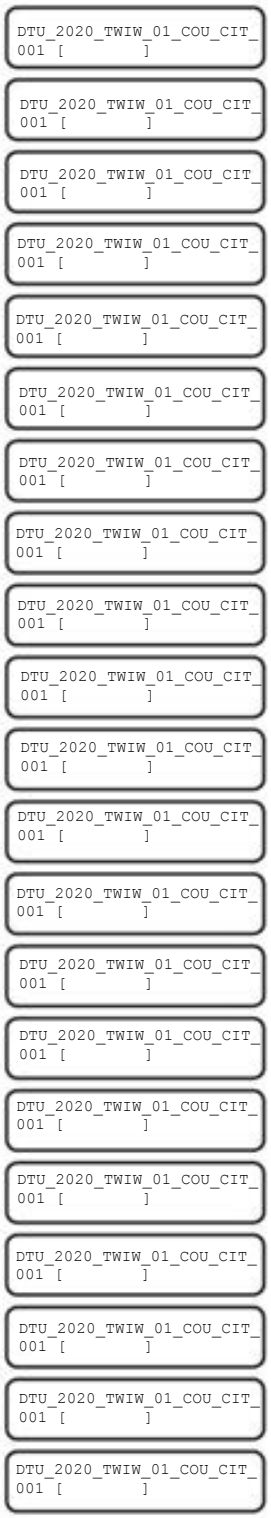
- Take one vial.
  - Remove the lid.
  - Insert the swab.
  - Close **tightly**.
- Close off with parafilm.

We have made a video guide of the bacterial isolation procedure for you to see on the TWIW Resources Youtube channel.

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>If you are collecting isolates:</u>

- Place a label sticker on the vial now containing a coal swab with isolated bacteria.
- Place a matching label sticker on the metadata sheets.
    - One sheet is for minimal metadata.
    - One sheet is for "nice to know" metadata.
- Fill in the metadata relevant for the isolate.
    - Minimal metadata is mandatory.

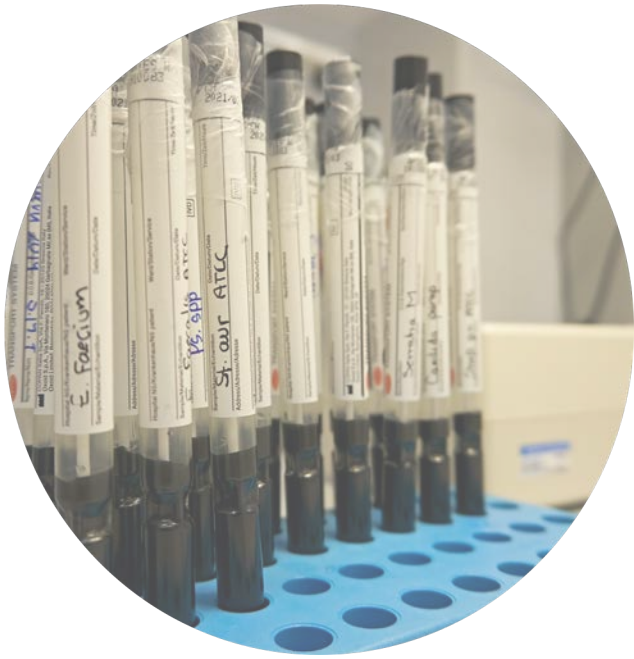
<u>If you are extracting DNA:</u>

- Label the DNA-containing Eppendorf tubes.
- Label metadata sheets as above.

Storage

The bacteria on the coal swabs prefer to be stored refrigerated (approximately 4°C) until dispatch, if possible. You can, however, store them at 3-25 °C. They can also be shipped at these temperatures.

Please notify us by email if you have to store the coal swabs with bacteria for longer than a week prior to dispatch. We may ask you to send them in multiple batches, depending on the circumstances.



If you are extracting DNA

If you are extracting DNA in-house, please use one of the two following protocols:

1) Invitrogen (Life Technologies)' Easy-DNA kit

This is the protocol used at DTU, and it is part of our recommended protocol for extracting DNA and performing whole-genome sequencing, which can be found on the EU Reference laboratory site.

This protocol requires chloroform and you may therefore prefer an alterntive. In this case we recommend:

2) Qiagen's DNEasy Blood and Tissue kit.

Please note the kits require the user to provide Lysozyme (Qiagen) or Zymolase (Invitrogen) for pretreatment of Gram+ bacteria. In addition, for Staphylococci specifically, we recommend pretreatment with Lysostaphin.

Furthermore, we ask that you:

- Use LoBind Eppendorf tubes, we will provide these to the extent possible.
- Make sure to check your DNA concentrations.
- Send 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.

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 isolates

Isolates must be shipped according to UN3373 standards, as "Biological samples, Category B". We have sent you everything you need to pack the isolates according to these standards.

- Pack the coal swabs in the absorbing material lined with pockets. You can fit 2-3 coal swabs into each pocket, and therefore fit 10 coal swabs in each piece of absorbing material.

- Pile the filled pockets inside the Biosafety bag 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 Bioshipper box.

- Close down the front flap of the box, then the side flaps, and lastly, the lid.

- Seal the box with the Air Seal sticker.

If shipping with FEDEX, place the Bioshipper box into the FEDEX protective bag. Close.

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.

- Put your LoBind Eppendorf tubes containing your DNA into 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!

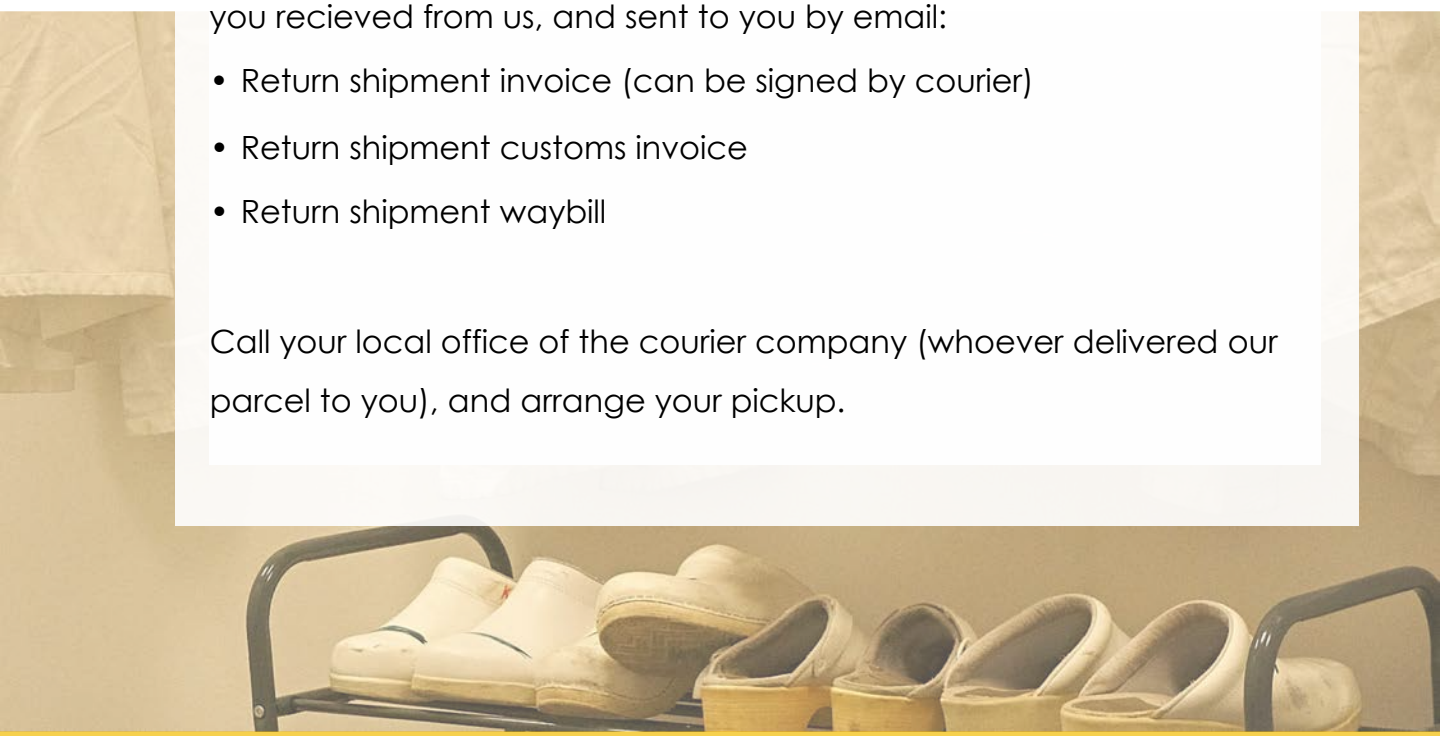
Once you have entered your metadata online in the TWIW sample log, and have packed your isolates or DNA, you are ready to contact the courier to pick it up.

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

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

- Return shipment invoice (can be signed by courier)
- Return shipment customs invoice
- Return shipment waybill

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



Appendices

- Appendix I: Oxoid Transport Swab – Amies agar with charcoal

Appendices attached if you are extracting DNA

- Appendix II: Genepi recommended pretreatment of Gram+ bacteria prior to DNA extraction
- Appendix III: EASY DNA (Invitrogen) extraction protocol (relevant chapters only)
- Appendix IV: DNEasy (Qiagen) extraction protocol (relevant chapters only)

Transport Swabs

Sector: Clinical

OXOID TRANSPORT SWABS
Code: TS

Effective transportation of throat, vaginal, wound and skin swabs conforming to highest medical device classification - Class IIa surgically invasive transient use.

AGAR GEL TRANSPORT SWABS
All swabs have soft rayon tips, which are inert and non-toxic to micro-organisms.
The addition of charcoal in the medium is considered to neutralise bacterial toxins and other inhibitory substances and has been shown to increase the recovery of *Neisseria gonorrhoeae.*

Effective
Swab tip submerged deep in 5ml agar gel - maximum protection for improved sample viability
Flushed with nitrogen to maintain optimum Eh potential of medium
Improved formulation contains scavengers to eliminate dissolved oxygen, superoxide and free radicals
Effective for aerobes and anaerobes

"Venturi" Hour-Glass Tube Design
Prevents disintegration of gel column during transport
Eliminates undesirable air pockets, bubbles and breaks in gel which may be harmful to fastidious bacteria
Centralises swab

MINI TIP TRANSPORT SWABS
Suitable for male urethral swab sampling. The narrow dimension of the swab shaft and tip provides a more practical device for some paediatric sampling

LIQUID MEDIA TRANSPORT SWABS
A soft polyurethane foam sponge, soaked in liquid transport medium, acts as a moisture reservoir. As the swab is inserted into the tube, the soft sponge yields and compresses down. Liquid medium is drawn immediately into the swab tip by capillary action. Preferred if performing rapid, direct testing, as agar gel can sometimes interfere with results.

Effective
Polyurethane sponge does not have the toxic effects that some other sponges can have on the sample
Liquid transport medium drawn into swab by capillary action- no dry samples
Extra large sponge reservoir, holds ample moisture and stays firmly anchored at the bottom of the tube

Quality Control
To ensure maximum product performance, Oxoid Transport Swabs are routinely tested using a wide range of organisms:
Neisseria gonorrhoeae ATCC® 43069
Bacteroides fragilis ATCC® 25285
Haemophilus influenzae ATCC® 10211
Streptococcus pyogenes ATCC® 19615
Campylobacter jejuni ATCC® 33291
Shigella flexneri ATCC® 12022
Yersinia enterocolitica ATCC® 9610

Traceability
Lot number, expiry date and product description clearly printed on the outside of every swab pack and on every individual tube.