This protocol is for DNA extraction and ONT library preparation for *rapid diagnostics* of:

1- Bacterial single isolates.

2- Metagenomic sample (e.g., faeces).

3- parasites, e.g., plasmodium.

Each case uses kits mentioned in each section, so please read through before starting to gather the needed kit.
Also please inspect that you have all the additional reagents required for each kit and protocol.

DNA extractions:

1- Rapid diagnostic of single bacterial isolate:

Sample preparation: Bacterial isolates can be transported at room temperature if they are on cultures for app. 10 days for DNA sequencing.
if you are starting from solid culture (plates or tubes), please make sure it is a general nutrient medium or selective medium to start from (i.e., LB, blood agar, MacConkey), and NOT a transport media (e.g., Cary Blair), such transport media are made for culturing but make DNA extraction very problematic. If the case is the latter, please culture the bacteria on LB before start extracting. From a plate, please collect 2 full white loop (app. 1 µl), and resuspend them in 200 µl PBS. Add the suspension to ZR BashingBead™ Lysis Tube from Zymo Quick-DNA Fungal/Bacterial kit, then continue from step 1 of the protocol named (quick-dna_fungal-bacterial_kit).

If you are starting from broth medium, same as above please make sure it is not a transport liquid medium. Centrifuge 2 ml of the broth culture (24 hours old) at 10 000 x g for 10 minutes, discard the supernatant and keep the bacterial pellet. Resuspend the bacterial pellet in 200 µl PBS. Add the suspension to ZR BashingBead™ Lysis Tube from Zymo Quick-DNA Fungal/Bacterial kit, then continue from step 1 of the protocol named (quick-dna_fungal-bacterial_kit).

2- Rapid diagnostic of faecal samples:

Sample preparation: faecal samples for DNA extraction are to be transported cooled down (on ice or frozen gel packs) until a freezer is available (24-48 hours chilled down are acceptable) without any buffer added, natural faeces. If RNAlater is available, please submerge the faeces in RNAlater, then the samples can be transported at room temperature. Faeces in RNAlater can be frozen when freezer is available.

Faecal samples without any buffer, follow directly protocol named (quick-dna_fecalsoil_microbe_kit). If faeces were submerged in RNAlater, weight out the starting material of the solid faeces (avoid liquid), if the faecal sample was diarrhoea, centrifuge the tubes at 10 000 x g for 10 minutes, discard the supernatant and start the protocol named (quick-dna_fecalsoil_microbe_kit).

3- Rapid diagnostic of plasmodium samples:

Sample preparation: from blood, follow the following protocol with Qiagen Blood and Tissue kit:

- Pipet 20 µl Proteinase K into a 1.5 ml or 2 ml Eppendorf. Add 50–100 µl anticoagulated blood. Adjust the volume to 220 µl with PBS.

- Add 200 µl Buffer AL (without added ethanol). Mix immediately and thoroughly by vortexing, and incubate at 56°C for 20 min.

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

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

- Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl Buffer AW1 (remember to add 100% EtOH to AW1 if new bottle), and centrifuge for 1 min at ≥6000 x *g* (8000 rpm). Discard flow-through and collection tube.

- Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl Buffer AW2 (remember to add 100% EtOH to AW2 if new bottle), and centrifuge for 3 min at 20,000 x *g* (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.

Make certain that the column is dry, if not, centrifuge same condition for an extra minute.

- Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml Eppendorf, and pipet 75 µl Buffer AE heated at 56°C directly onto the DNeasy membrane. Incubate at room temperature for 1min, and then centrifuge for 1 min at ≥ 6000 x *g* (8000 rpm) to elute.

- For maximum DNA yield, repeat elution once as described previous step.

ONT Library preparation and sequencing:

All three cases (single bacterial isolates, faeces and plasmodium) use the same library prep. Protocol. Please follow library prep. Protocol named (Ligation sequencing gDNA - Native Barcoding Kit 24 V14 (SQK-NBD114.24)) Or (Ligation sequencing gDNA - Native Barcoding Kit 96 V14 (SQK-NBD114.96)). Depending on how many samples you want to multiplex together on one flowcell.