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Exploring the potential of rapid molecular techniques for Antimicrobial Resistance (AMR) and pathogens surveillance at Wastewater treatment plants

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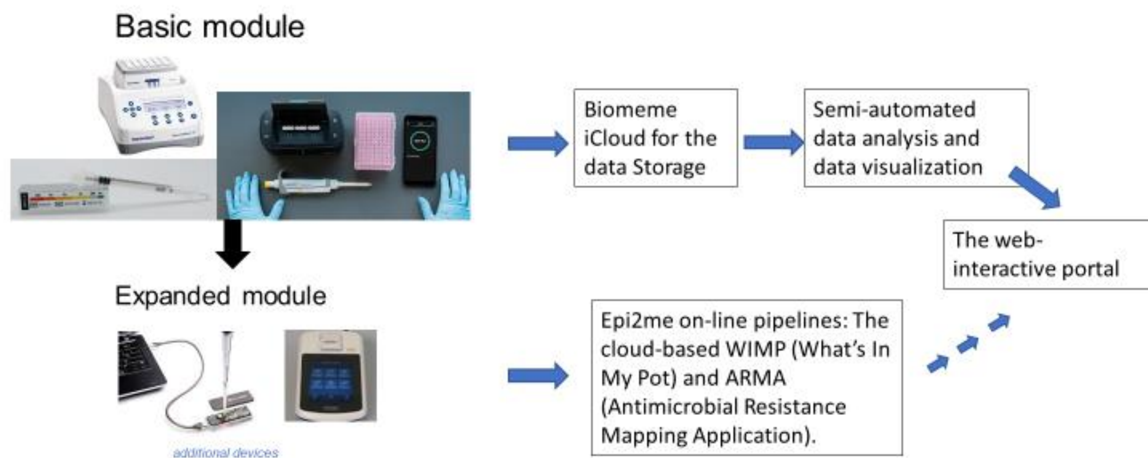
Brief introduction

Wastewater treatment plants (WWTPs) are not only a unique source of information on Antimicrobial Resistance (AMR) and pathogens, but also, they are gateways for AMR spreading and thus WWTPs constitute an engine for the escalation of global AMR issue. We propose a toolbox with portable solutions for implementation at the inlet and outlet of WWTPs, semi-autonomous data analysis and near-real time visualizations thus enabling early warning and detection of precursors of epidemic outbreaks as well as manner of quality control of wastewater treatment efficiency.

The draft protocol includes (1) a basic module for quantitative analysis of AMR proxies on the Franklin Biomeme instrument (2) as well as an extended module dedicated for metagenomic analysis on a Nanopore Oxford Sequencing MinION instrument.



Devices (on blue – required for the basic module, on black – required for the expanded module)

- Thermalmixer, Eppendorf ThermoMixer® C with 2,0 ml, thermoblock for 24×2,0 ml reaction vessels (Eppendorf)
- MinION Sequencing Device MIN-101B (Nanopore Oxford)
- Franklin™ Real-Time PCR Thermocycler (Biomeme)
- Qubit Fluorometer (Thermo Fisher Scientific)

Materials (on blue – required for the basic module, on black – required for the expanded module)

- Sterivex-GP Pressure Filter Unit, 0.22 µm pore size, polyethersulfone membrane, gamma irradiated, sterile with a bell SVGP010, Milipore
- 25 mm Cellulose Acetate Syringe Filters, sterile, pore size 0.2 µm, (VWR cat no. 514-1273)
- 3M guanidine thiocyanate lysis solution pH 8.9 (3M GuSCN, 50mM Tris, 15mM EDTA. 2% Sarkosyl and 0.2% SDS)

- M1 Sample Prep Cartridge Kit For DNA, DNA-HI (Biomeme # 3000536R)
- Go- Strips and caps (Biomeme #3000152)
- AMPure XP Reagent, 5 mL (Beckman Coulter # A6880)
- Absolute ethanol
- EquiPhi29 DNA Polymerase (ThermoScientific, A39390)
- Exo-resistant random primers (ThermoScientific, SO181)
- dNTPs Mix 10 mM
- Flow Cell (R9.4.1), FLO-MIN106D and Rapid PCR Barcoding Kit, SQK-RPB004, Nanopore Oxford (**or** the latest Nanopore Kit 14 chemistry Flow Cell R10.4.1- FLO-MIN114 and Rapid Barcoding Kit 24 V14 SQK-RBK114.24)
- 5 ml and 60 ml sterile syringes with Luer Lock
- DynaMag™-2 Magnet rack- Thermo Fisher Scientific or similar

Sampling at WWTP

Collect a composite sample (composed of sub-samples taken over a 24-hour period) at the inlet and outlet using an automatic water sampler (such as Endress+Hauser, Liquistation CSF48) and store (the samples) at 4°C for the period of collection. The sewage samples are collected usually in accordance with flow, so the sampling frequency is set per m3 based on the expected hydraulic load (e.g., every 1000 m3).

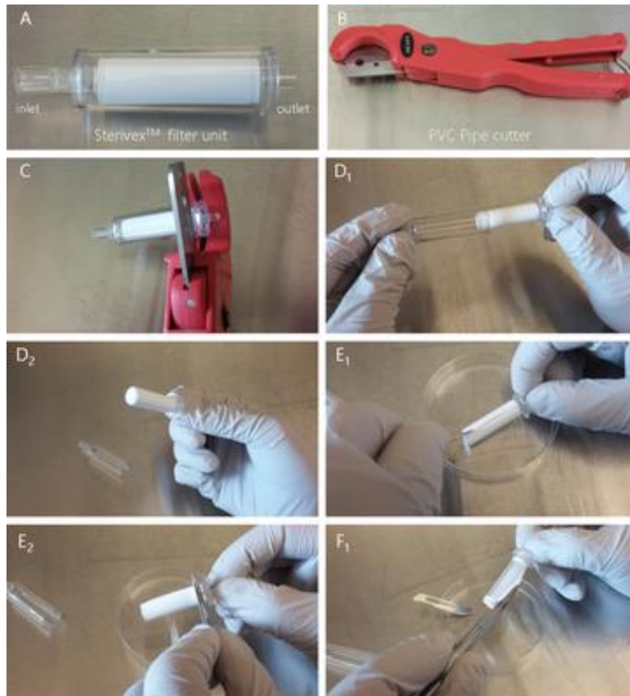
Pretreatment of wastewater samples (samples at inlet and outlet)

NB: Preferably filter 3 samples and continue the analysis in 3 replicates for outlet and inlet samples respectively

1. Use a large 60 ml Luer Lock Syringe and Sterivex filter (Figure 1.) to filter max volume, repeat filling the syringe several times if needed (aim at maximum volume, note the volume (at WWTP Mekjarvik it was possible to filtrate 240-250 ml sample at outlet and 30-35 ml at inlet)
2. Optionally store the filters at – 80 °C or – 20 °C (for shorter periods) otherwise perform DNA extraction immediately

Figure 1. Filtration of wastewater samples

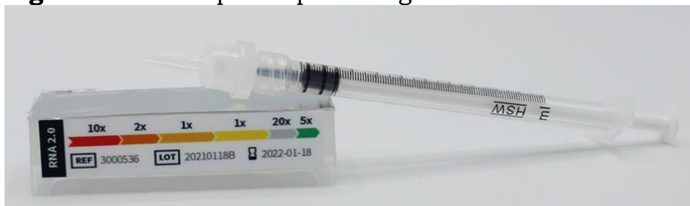
Figure2. Opening the Sterivex capsule, after Cruaud et al., 2017



DNA extraction and purification

1. Extract the filter from the capsule as follows. Open the Sterivex by cutting the bottom of the Sterivex casing with a flame sterilized PVC tube cutter (Figure 1). Remove the filter from the casing using flame sterilized forceps. Transfer the filter pieces into a 2 ml Eppendorf tube (note: it is better if the filters are fragmented)
2. Add 1,5 ml 3M guanidine thiocyanate lysis solution pH 8.9 (3M GuSCN, 50mM Tris, 15mM EDTA. 2% Sarkosyl and 0.2% SDS)
3. Incubate at 10 min 85 °C in ThermomixerC with 500 rpm speed.
4. Prepare a clean (sterile) syringe (with capacity 5 -10 ml) and syringe filter. Pass the lysate though the syringe filter to a Falcone tube (note: patience is required, do it slowly). Puncture 2 holes in the first section DNA Sample Prep Cartridge Kit DNA. **Transfer lysate to the first section of DNA Cartridge** (important: try avoiding the formation of foam!).
5. Continue with M1 Sample Prep Cartridge Kit DNA kit in 3 replicates according to the Biomeme instruction below except the stage- DNA elution (NB: discard **650 µl** elution buffer for the last section!).

Figure 3. M1 Sample Prep Cartridge Kit For DNA



Before beginning to puncture 2 holes in all remaining sections (except the section air day where 1 hole is needed). Do not transfer any liquid from one section of the sample prep cartridge to the next. This applies to each remaining step of the sample extraction protocol.

Secure the Sample Prep Column to the 1mL syringe and puncture the first red hole of the Sample Prep Cartridge, labeled “START”. Set aside the syringe.

Lysis & Binding (30 pumps) – red section

Place the syringe with the attached Sample Prep Column back into the red section of the Sample Prep Cartridge and transfer 1,5 ml lysate fluid all the way up the syringe and pump all the way back out. Repeat for a total of 20 pumps.

Push all fluid in the syringe into the red section of the Sample Prep Cartridge prior to beginning the next step.

Protein Wash (3 pumps)- red-orange section

Move the 1mL syringe with the attached Sample Prep Column into the red-orange section of the Sample Prep Cartridge (Biomeme Protein Wash - BPW) and pierce through the foil. Remember to pierce 2 holes per section of the cartridge to minimize liquid splatter, except during the Air-Dry step.

Draw the BPW fluid all the way into the syringe and pump all the way back out. Repeat twice ensuring that no buffer remains in the syringe before beginning the next step.

Salt Wash (2 pumps)- orange section

Move the 1mL syringe with the attached Sample Prep Column to the orange section of the Sample Prep Cartridge (Biomeme Wash Buffer - BWB) and pierce through the foil.

Draw the BWB fluid all the way into the syringe and pump all the way back out once ensuring that no buffer remains in the syringe before beginning the next step.

Drying Wash (2 pump)- yellow section

Move the 1mL syringe with the attached Sample Prep Column to the yellow section of the Sample Prep Cartridge (Biomeme Drying Wash - BDW) and pierce through the foil.

Draw the BDW fluid all the way into the syringe and pump all the way back out once ensuring that no buffer remains in the syringe before beginning the next step.

Air Dry (40 pumps)

Move the 1mL syringe with the attached Sample Prep Column to the blue section of the Sample Prep Cartridge and pierce through the foil to remove excess buffer.

Draw air up through a new larger (10-50 mL) syringe for air-drying and quickly pump back out. Repeat pumping vigorously 40 times until the Sample Prep Column appears dry and does not spray fluid droplets.

Elution

(Remember to discard **650 µl** elution buffer for this section, ***as there is little elution buffer left, you have to slightly tilt the DNA extraction cartridge to effectively carry out the elution*****)**.

Move the 1mL syringe with the attached Sample Prep Column to the green section of the Sample Prep Cartridge (Biomeme Elution Buffer - BEB) and pierce through the foil.

Elute by drawing the BEB fluid all the way up through the syringe and slowly pump back out for a total of 20 pumps.

Transfer the entire volume of the eluted DNA from the green section of the Sample Prep Cartridge into an Eppendorf tube.

Basic module - quantitative analysis on Biomeme Franklin qPCR instrument

The protocol below includes details for the analysis of marker genes such as korB and int1 indicating the overall level of ARGs burden (conjugative gene and class 1 integron-integrase gene respectively) and selected ARGs genes described in the literature as essential for monitoring at WWTPs (Keenum et al., 2022). The standard curve has been plotted in advance and slope-based formulas are included in the scripts to calculate gene copies of individual genes. The detailed information about the qPCR assays is included in the table 2.

Preparation of PCR mastermixes: LyoGreen™ 2.0 Master Mix and LyoDNA™ 2.0 + IPC Master Mix.

Resuspend lyophilized PCR matrix (LyoGreen™ 2.0 Master Mix or LyoDNA™ 2.0 + IPC Master Mix) in 325 µl in the low TE solution, this will constitute 4 X concentrated working solution.

One bottle LyoGreen™ 2.0 Master Mix will be sufficient for analysis of sewage at inlet and outlet for 3 replicates for a single time point. LyoDNA™ 2.0 + IPC Master Mix will last longer but as soon as resuspended prepare aliquots and freeze.

Preparation of qPCR reactions

Internal Positive Control run.

<table>
<tr><td>Prepare master mix for IPC run (190 µl in total)</td></tr>
<tr><td>47,5 µl - LyoDNA™ 2.0 + IPC Master Mix x 4</td></tr>
<tr><td>47,5 µl -DNA (to 6 wells) or dH2O (to 3 wells) (5 µl added separately)
<table>
<tr><td colspan="3">IPC</td><td colspan="3">Inlet</td><td colspan="3">outlet</td></tr>
<tr><td>H2O</td><td>H2O</td><td>H2O</td><td>Inlet sample 1</td><td>Inlet sample 2</td><td>Inlet sample 3</td><td>oulet sample 1</td><td>oulet sample 2</td><td>oulet sample 3</td></tr>
<tr><td>well1</td><td>well2</td><td>well3</td><td>well4</td><td>well5</td><td>well6</td><td>well7</td><td>well8</td><td>well9</td></tr>
</table></td></tr>
<tr><td>95 µl dH2O</td></tr>
</table>

Mix using a pipette and tap a tube 10 times on the bench. Add to each of 9 tubes (3 strips) 15 µl of prepared mix and add 5 µl H2O or DNA (as shown in the table above) . Close the strip with rubber caps (Void Filling Caps). Perform qPCR rection using the standard Biomeme LyoDNA program (Table 1).

Run of qPCR assays

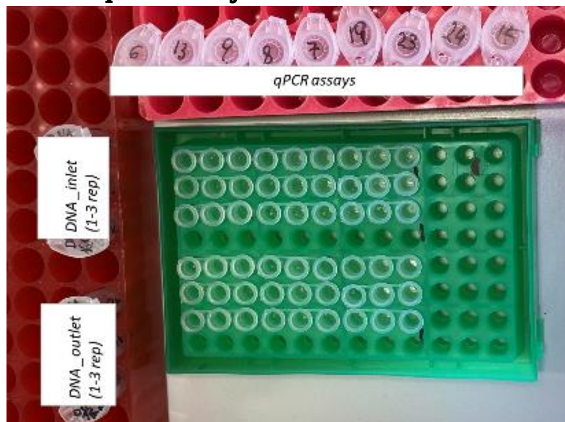


Figure 4. Preparation and setup of qPCR reactions for ARGs per one sampling point, and order of qPCR assays in a single run

Prepare master mix by adding to a single Eppendorf tube as shown below (this is reaction per 1 assay, you will need to prepare 9 master mixes per each time point) (130 µl total volume)
32,5 µl -PCR master mix (LyoGreen™ 2.0 Master Mix) x 4
32,5 µl -DNA (5 µl added separately)
6,5 µl working solution/primers mix (10 pmol/µl) to the final concertation of 500 nM
58,5 µl dH2O

Mix using a pipette and tap a tube 10 times on the bench. Add to each of 6 tubes (arranged vertically, as shown on the picture above, Figure 4) 15 µl of prepared mix. Add DNA to individual 9 tubes (3 strips, as shown on the picture above). Close the strip with rubber caps (Void Filling Caps). Perform qPCR rection using the standard Biomeme LyoDNA program (Table 1). The cycling program is saved on the mobile**.**

Assay no.	6	13	9	8	7	19	23	24	15
Target gene	ESBL-blaCTX-M-1	The class 1 integron-integrase (int1)	Sulfonamide Resistance Gene (sul1)	Tetracycline resistance	Vancomycin-resistance (Van)	secE/A. baumannii	ESBL-blaCMY	ESBL -blaSHV	Conjugative factor (korB)

	POSITIVE CONTROLs		LyoDNA™ 2.0 + IPC Master Mix	NAME of the run :DATE , type of samples for instance: 2023.06.16 IPC						
		IPC			inlet			outlet		
IPC	H2O	H2O	H2O	inlet sample 1	inlet sample 2	inlet sample 3	oulet sample 1	oulet sample 2	oulet sample 3	
	well1	well2	well3	well4	well5	well6	well7	well8	well9	
	INLET samples	LyoGreen™ 2.0 Master Mix								
	rep 1	inlet sample 1		NAME of the run :DATE , type of samples for instance: 2023.06.16 inlet1						
no. assay	6	13	9	8	7	19	23	24	15	individual run on Biomeme
	well1	well2	well3	well4	well5	well6	well7	well8	well9	
	rep 2	inlet sample 2		NAME of the run :DATE , type of samples for instance: 2023.06.16 inlet2						
no. assay	6	13	9	8	7	19	23	24	15	individual run on Biomeme
	rep3	optional								
	OUTLET samples									
	rep 1	outlet sample 1		NAME of the run :DATE , type of samples for instance: 2023.06.16 outlet1						
no. assay	6	13	9	8	7	19	23	24	15	individual run on Biomeme
	rep 2	outlet sample 2		NAME of the run :DATE , type of samples for instance: 2023.06.16 outlet2						
no. assay	6	13	9	8	7	19	23	24	15	individual run on Biomeme
	rep3	optional								
	INLET samples	Melting curve								
	rep 1	inlet sample 1		NAME of the run :DATE , type of samples for instance: 2023.06.16 inlet1M						
no. assay	6	13	9	8	7	19	23	24	15	individual run on Biomeme
	OUTLET samples									
	rep 1	outlet sample 1		NAME of the run :DATE , type of samples for instance: 2023.06.16 inlet1M						
no. assay	6	13	9	8	7	19	23	24	15	individual run on Biomeme

Names of runs (in the order of processing)

Program LyoDNA

Internal Positive Control – IPC-2023.08.08

Biological replicate no. 1 – influent sample -DATE-inlet1 (np. 2023.08.08-inlet1)

Biological replicate no. 1 – effluent sample -DATE-outlet1 (np. 2023.08.08-outlet1)

Biological replicate no. 2 – influent sample -DATE-inlet2 (np. 2023.08.08-inlet2)

Biological replicate no. 2 – effluent sample -DATE-outlet2 (np. 2023.08.08-outlet2)

Biological replicate no. 3 – influent sample -DATE-inlet3 (np. 2023.08.08-inlet3)

Biological replicate no. 3 – effluent sample -DATE-outlet3 (np. 2023.08.08-outlet3)

Biological replicate no. 3 – influent sample -DATE-inlet3 (np. 2023.08.08-inlet3)

Biological replicate no. 3 – effluent sample -DATE-outlet3 (np. 2023.08.08-outlet3)

Program melting (optional)

Biological replicate no. 3 – influent sample -DATE-inlet3melt (np. 2023.08.08-inlet3melt)

Biological replicate no. 3 – effluent sample -DATE-outlet3melt (np. 2023.08.08-outlet3melt)

Biomeme Go App.

Table 1. The cycling program- LyoDNA

Protocol	LyoDNA			
Cycles	45			
	Denature	Cycling Denature	Anneal	Extension
Temp (Celsius)	95	95	60	0
Time (s)	60	1	20	0

Table 2. Q-PCR assays details

Assay no.	Target gene	Primers	Sequence (5`- 3`)	Size of amplicon (bp)	References
6	ESBL-blaCTX-M-1	blaCTX_Col_F blaCTX_Col_R	ACCAACGATATCGCGGTGAT ACATCGCGACGGCTTTCT	101	Colomer-Lluch et al., 2011
13	The class 1 integron-integrase (int1)	int1_Luo_F int1_Luo_R	GGCTTCGTGATGCCTGCTT CATTCCTGGCCGTGGTTCT	146	Luo et al., 2010
9	Sulfonamide Resistance Gene (sul1)	sul1_Pei_F sul1_Pei_R	CGCACCGGAAACATCGCTGCAC TGAAGTTCCGCCGCAAGGCTCG	163	Pei et al., 2006
8	Tetracycline resistance (tetA)	tetA_Bor_F tetA_Bor_R	TCAATTTCCTGACGGGCTG GAAGCGAGCGGGTTGAGAG	91	Borjesson et al., 2009
7	Vancomycin-resistance (Van)	Van_KlaF Van_KlaR	TCTGCAATAGAGATAGCCGC GGAGTAGCTATCCCAGCATT	376	Klare et al., 1995
19	secE/A. baumannii	SecEFP_F SecERP_R	GTTGTGGCTTTAGGTTTATTATAC AAGTTACTCGACGCAATTCG	94	Clifford et al., 2012
23	ESBL-blaCMY	CMY_fwd. CMY_rev.	GGCAAACAGTGGCAGGGTAT AATGCGGCTTTATCCCTAACG		Roschanski et al., 2014
24	ESBL -blaSHV	SHV_fwd SHV_rev	TCCCATGATGAGCACCTTTAAA TCCTGCTGGCGATAGTGGAT		Roschanski et al., 2014
15	Conjugative factor (korB)	F1kor F2kor R1kor R2kor R3kor	TCATCGACAACGACTACAACG TCGTGGATAACGACTACAACG TTCTTCTTGCCCTTCGCCAG TTYTTCYTGCCCTTGGCCAG TTCTTGACTCCCTTCGCCAG	107	Jechalke et al., 2013

qPCR Biomeme runs and storage of data.

Before start: Make sure that you are connected to WIFI (this is essential for the transfer of data from the Biomeme instrument and data storage transfer to Biomeme Cloud http://cloud.biomeme.com)
The Biomeme Go App is used to operate the Biomeme Franklin instrument.
Login to the app and the Biomeme Cloud:
Login:
Password:

Properly label each run in the Biomeme Go App; always select 9 samples.

Make sure that the data are saved on the Biomeme Cloud before the next Biomeme PCR run.

- To start a Biomeme run you will need to scan a code that can be found on a bag with go-strips
- In the Go App Always select the option for 9 samples
- You do not need to worry about your smartphone screen turning off or going to sleep. The experiment will continue to run. If the app freezes or crashes, the experiment will also continue to run, and your data can be found in the Incomplete Runs section of the app once you have reloaded the Biomeme Go app and reconnected to the thermocycler. For more information on recovering and reattaching test data, please see help.biomeme.com
- To operate the Go App, see also the Biomeme document “Biomeme Go Mobile App v1.1” in the Teams folder

Extended module - metagenomic analysis

The need to enrich the metagenome will depend on the amount of DNA extracted, for the older version of the Nanopore chemistry, 400 ng (in max volume 7,5 µl) is required, the latest Nanopore chemistry requires 50 ng (in max volume 7,5 µl).

Metagenomic DNA enrichment (WGA reaction)- isothermal amplification

1. Denature DNA in a 5 µL mix containing 1X final concentration of EquiPhi29 Reaction Buffer; 100 µM of exo-resistant random primers (#SO181); pg to ng DNA; and nuclease-free water. Heat at 95 °C in Thermomixer C for 3 min then immediately place on ice for another 3-5 min.
2. Amplify metagenomic (WGA reaction) or DNA in reaction mix containing:
3. Note: The enzyme requires active reducing reagent. 10X Reaction
4. Buffer for EquiPhi29 does not contain DTT. Fresh DTT should be added to the reaction mix separately. Thaw 100 mM DTT at room temperature just prior to use, add and refreeze immediately.
5. Prepare the master mix as it is shown in the table below.
6. Incubate at 45 °C for 1 to 3 hours. (Note: Reaction is saturated after 3 hours of incubation; longer incubation may result in non-specific)
7. DNA amplification. Inactivate polymerase at 65 °C for 10 min.
8. Check concertation of DNA on qubit

Component	per 1 reaction 20 µL (final concetration)
10X Reaction Buffer for EquiPhi29	1.5 µL (1X)
DTT (100 mM)	0.2 µL (1 mM)
dNTP Mix 10 mM each	2 µL (1 mM each)
Denatured DNA mix 5 µL	5 µL
EquiPhi29 DNA Polymerase (10 U/ µL)	1 µL (10 U)
Nuclease-free water up to 20 µL	

Note: For one sampling point from WWTP two reactions with EquiPhi29 should be prepared for the inlet (pooled 3 replicates of extracted DNA) and the outlet samples (pooled 3 replicates of extracted DNA).

Metagenomic DNA purification using Agencourt AMPure XP

1. Transfer 20 µl metagenomic amplified DNA (rection mix from the step above) to a new tube
2. Resuspend the AMPure XP beads by vortexing.
3. Add an equal volume of resuspended AMPure XPbeads, and mix by flicking the tube.
4. Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. (or perform this step mixing by hand)
5. Prepare 500 µl of fresh 70% ethanol in Nuclease-free water.
6. Spin down the sample and pellet on a magnet.
7. Keep the tube on the magnet, and pipette off the supernatant.

8. Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet.
9. Remove the ethanol using a pipette and discard.
10. Repeat the previous step. Spin down and place the tube back on the magnet. Pipette off any residual 70% ethanol.
11. Briefly allow to dry. Remove the tube from the magnetic rack and resuspend pellet in 10 µl of 10 mM Tris-HCl pH 7.5-8.0 with50 mM NaCl.
12. Incubate for 2 minutes at RT. Pellet the beads on a magnet until the eluate is clear and colorless, for at least 1 minute.
13. Remove and retain 20 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.
14. Transfer the eluate to a new tube, measure concentration of DNA on Qubit Fluorometer and/or Nanodrop – 400 ng (in max volume 7,5 µl) is required for older Nanopore chemistry and 50 ng (in max volume 7,5 µl) for the latest chemistry.

This procedure ensures good quality and high molecular weight gDNA.

Metagenomic sequencing using Rapid gDNA barcoding (SQK-RBK004) or Rapid Barcoding Kit 24 V14 (SQK-RBK114.24)

Register on the Nanopore Community https://community.nanoporetech.com and then download the detailed protocol depending on the version of the sequencing kit and flow cell and follow the responding protocol.

MinKNOW™ software have to installed on your PC (check the min. PC specifications required on the Nanopore Oxford website, https://nanoporetech.com/sites/default/files/s3/2016-10/Computer%20requirements%20v4.2%20Sep2016.pdf)

MinKNOW™ is the software that controls Oxford Nanopore sequencing devices, i.e. MinION, performs several core tasks, including data acquisition, real-time analysis, feedback, basecalling, data streaming, controlling the device, and ensuring that the platform chemistry is performing correctly for sequencing. MinKNOW™ has integrated Guppy software, MinKNOW™ can perform basecalling and barcode demultiplexing during sequencing and through post-run analysis.

Analysis of metagenomic sequencing data

Epi2Me – The cloudbased WIMP (What's In My Pot) and ARMA (Antimicrobial Resistance Mapping Application).

The WIMP workflow aligns MinION reads using minimap2 (Li 2018) against the Centrifuge database (Kim et al. 2016) and ARMA aligns reads using LAST (Kiełbasa et al. 2011) against the Comprehensive Antibiotic Resistance Database (CARD; McArthur et al. 2013).

Epi2Me Labs – allows on more customized analysis.

Optionally to sort out plasmid- and chromosome derived read sequences use PlasFlow (version 1.0; Krawczyk et al. 2018).

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