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Protocol status: In development
We are still developing and optimizing this protocol

Created: Mar 19, 2023

AMR Detection by dPCR

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ABSTRACT

Overview

Microbes resistant to antimicrobial agents are a major worldwide health challenge. Rapid detection of antimicrobial resistant pathogens and surveillance efforts are critical. Information about **antimicrobial resistance (AMR) genes** is available in databases, and quantitative PCR (qPCR) has been proven suitable for detecting AMR genes (Abram et al. 2019; Galhano et al. 2021; Wu et al. 2022). In this research session, we will use the epMotion 5075 and a custom 3D-printed adapter to leverage the power of automation and the new QIAGEN QIAcuity Digital PCR system to detect and quantify a target antimicrobial resistance gene in metagenomic DNA samples from soils and compost. Take a virtual tour of the [QIAcuity](#) that we will be using for our research!

Digital PCR (dPCR) allows for absolute quantification of template DNA or RNA molecules based on Poisson statistics. But, what does that mean and how does dPCR even work?

Read about the fundamentals of dPCR in these articles and watch this short video about dPCR:

- Read [Digital PCR for beginners](#)
- Read [Fundamentals of Digital PCR](#)
- Watch [Principles of dPCR Explained](#)

During our second research session, we will set up the epMotion 5075 to dilute metagenomic DNA samples. We will set up dPCR by combining a commercial PCR master mix (includes: dNTPs, polymerase, buffers, Mg²⁺, water) with our research specific primers, probes, and diluted DNA template to carefully pipette into a QIAGEN [Nanoplate](#) with partitions for use in the QIAcuity. We will run dPCR on AMR genes from a mixed population of soil microbes to investigate whether soils used as our samples contain microbes with AMR genes.

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Funders Acknowledgement:

Biotechnology Program

GUIDELINES

Additional Resources:

[QIAcuity Probe PCR Kit Protocol](#)

MATERIALS

Materials

- QIAGEN PowerSoil Pro DNA extraction kit
- Invitrogen Qubit BR DNA kit and standards to quantify DNA
- QIAGEN QIAcuity 4X Probe Mix
- QIAGEN QIAcuity Probe Kit ultraclean water
- Forward and Reverse AMR primers diluted to 100 µM
 - 1. Forward: 5' TTCTTCAGCACCGCG 3'
 - 2. Reverse: 5' CGAATTAGAGCGGCAGTC 3'
- Probe
 - 1. /5'FAM/CATCGCAAAGCGCTCATCAGCACGATAAAGT/3'BHQ2/
- Teknova TE Buffer in 2 ml aliquots
- New England BioLabs EcoRI HF enzyme
- Test DNA at 1 ng/µl
- Multichannel pipettors: p10, p100
- Barrier tips
- QIAGEN Nanoplate 96-well, 8.5k partitions
- Eppendorf Twin Tec PCR plate

Equipment

- Eppendorf epMotion 5075 liquid handler
- Custom 3D-printed Eppendorf epMotion QIAGEN Nanoplate adapter
- Biosafety hood for dilutions and primer/probe preparation to avoid contamination
- QIAcuity Digital PCR (one plate, five plex) instrument

Metagenomic DNA Extraction

- 1 Obtain soil and compost samples from different environments. We have access to soil and compost samples from Christopher Hopkins from efforts to learn about the composting process of "swine lagoon" sludge and have dried and "tar-like" samples. Your instructors (Horton and Phillips) also prepared solid metagenomic DNA samples from around the campus for comparison.

2

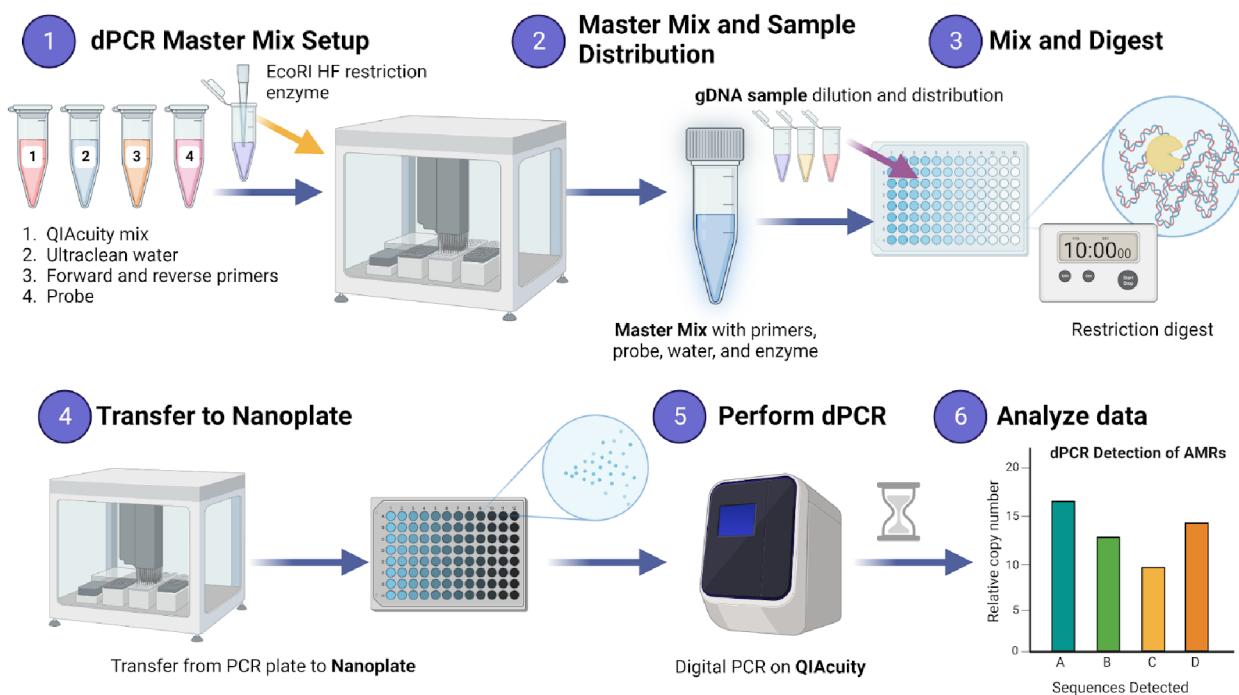


Figure 1. Schematic of procedure to use liquid handler to seed plates with samples and digital PCR master mix. The epMotion 5075 is used to set up a master mix for digital PCR by combining the QIAcuity mix, ultra clean water, forward and reverse primers/probe (**Table 1**) and EcoRI HF restriction enzyme according to **Tables 2**. In Step 2, the master mix is distributed to a 96-well PCR plate and genomic DNA is diluted and transferred to the PCR plate. A ten-minute incubation at 22 °C is started to allow the EcoRI enzyme to digest large pieces of DNA for mobility in the Nanoplate. In Step 4, the reactions are transferred to a QIAGEN Nanoplate placed on the custom Nanoplate adapter. In Step 5, the QIAcuity digital PCR is loaded with the sealed Nanoplate from Step 4. Thermocycling conditions (listed in **Table 3**) are followed before data is downloaded to analyze in Step 5. Created with BioRender.com

3

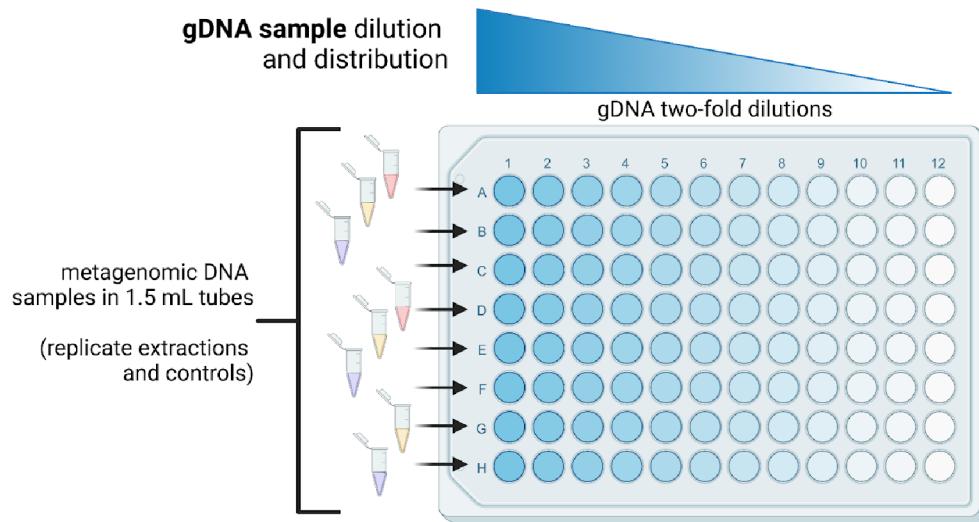


Figure 2. Dilution of metagenomic DNA samples from replicate extractions. Metagenomic DNA from replicate DNA extractions is diluted before using as a template for digital PCR. One microliter for each of eight metagenomic DNA samples in 1.5 ml tubes is transferred to the first column of a 96-well. The epMotion liquid handler is used to transfer ultra clean water to the remaining wells and perform two-fold dilutions from columns 2 through 12. One microliter of each well is then transferred to the PCR plate with the digital PCR master mix. Image created with BioRender.com.

As a group, we will use a common script and the liquid handler to prepare sets of plates for analyses. Each bay will set up one plate.

4 Weigh 250 mg into QIAGEN PowerSoil Pro bead beating tubes.

5 Extract DNA following the QIAGEN PowerSoil Pro protocol in triplicate.

6 Elute in 50 µL 5 of CD6 Elution buffer.

- 7 Quantify DNA by NanoDrop and use the Qubit BR DNA kit.
- 8 Run 1 μ L of sample on Agilent TapeStation using the Genomic DNA tape and reagents.

Note

The first six steps have been performed by your instructors in preparation for our lab session. Metagenomic DNA preparation will allow us to focus on the setup and automation of a plate full of genomic samples.

Digital PCR Setup

- 9 Obtain [M] 0.8 micromolar (μ M) Forward and [M] 0.8 micromolar (μ M) Reverse Primer for the blaCTX-M-1 antimicrobial resistance gene.
- 10 Obtain probe [M] 0.4 micromolar (μ M) and dilute it with 1 μ L of TE Buffer.
- 11 Transfer 1 μ L of diluted probe into tubes via pipette in a biosafety hood.
- 12 Transfer 400 μ L of buffer solution with 40 μ L of both the reverse and forward primers, and 20 μ L of the probe to make the primer probe mix

- 13** The primer probe mix, $\text{3 } \mu\text{L}$ of the master mix, and $\text{0.15 } \mu\text{L}$ of the restriction EcoRI enzyme per reaction were dispensed into the PCR plate. Then, the extracted compost DNA was added to each well that contained the overall reaction mix ($\text{1 } \mu\text{L}$ per reaction)
- 14** Transfer mixtures from the PCR plate to the QIAGEN Nanoplate (96 wells, 8.5k partitions) using the epMotion 5075.
- 15** Seal the Nanoplate properly with the provided seal and use roller to ensure proper seal.
- 16** Transfer the plate to the QIAcuity instrument, remove the white plate tray, and load into the digital PCR using a template for a full plate with a FAM-based probe.

Reagent Concentrations and Calculations

- 17** **Table 1.** Concentrations of stocks and working solutions for primer mix and probes.

A	B	C	D
Component	Initial Concentration	Final Concentration	Volume for 500 μl (in TE)
Forward Primer	100 μM	8 μM	40 μl
Reverse Primer	100 μM	8 μM	40 μl
Probe	100 μM	4 μM	20 μl
TE Buffer	-	-	400 μl

- 18** **Table 2.** List of reagents used for digital PCR on the QIAGEN QIAcuity instrument.

A	B	C	D
Component and	Volume per Reaction	Final Concentration	Volume for 100 reactions

A Concentration	B	C in μM	D in 96-well plate
4X QIAcuity Probe PCR Master Mix	3 μl	1X	300 μl Master Mix
10X Primer Probe Mix (FAM channel)	1.2 μl	0.8 μM forward	120 μl Primer Probe Mix
Restriction EcoRI Enzyme 0.25 U/μl EcoRI-HF®, NEB®	0.15μl	0.8 μM reverse	15 μl NEB EcoRI-HF
RNase-free Water	6.65μl	primer	665 μl water
Template DNA	1.0 μl	0.4 μM probe	100 μl of DNA
Total Reaction Volume	12.0 μl	0.25 U/μl	1,200 μl

19 **Table 3.** Thermocycling Conditions.

A	B	C	D
Step	Time	Temperature (°C)	Number of Cycles
PCR initial heat activation	2 min	95°C	1 cycle (to activate the enzyme)
Denaturation	15 seconds	95°C	40 cycles
Combined annealing/extension	30 seconds	60°C	40 cycles

Source: [QIAGEN QIAcuity Probe Mix](#)

20

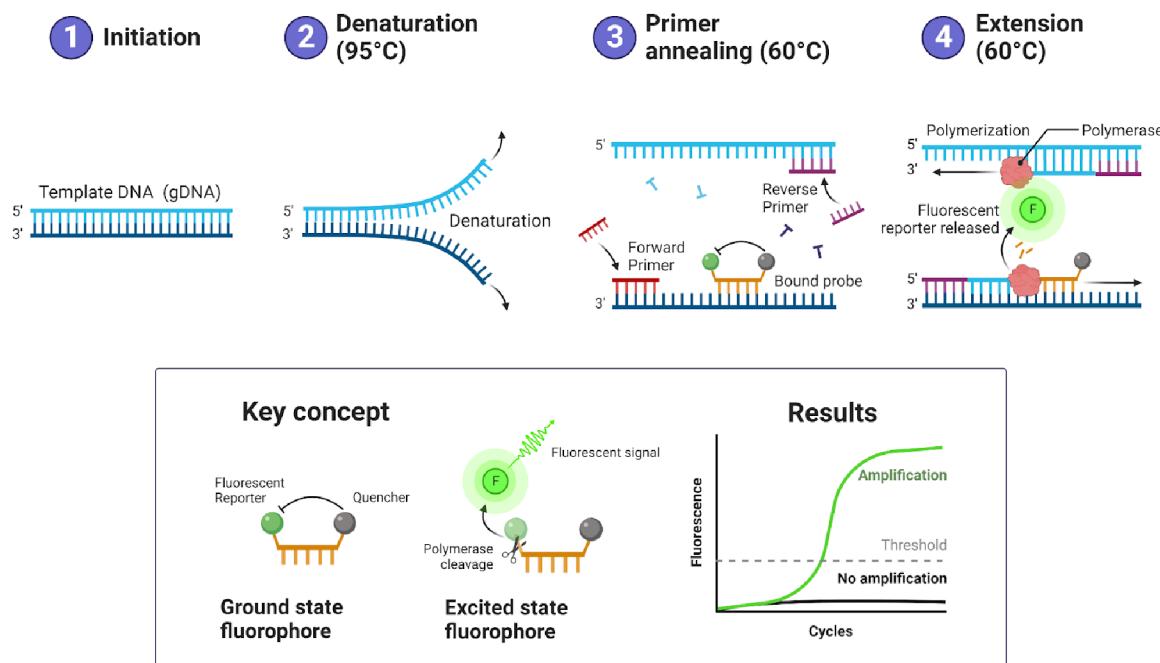


Figure 3. Schematic of the thermocycling steps with a probe with fluorophore and quencher.

Metagenomic template DNA (step 1) is denatured (step 2) to initiate the thermocycling procedure. Primer annealing (step 3) and extension (step 4) occur at the same temperature. Fluorescence is detected after the fluorescent reporter is released from the bound probe. Diagram created with BioRender.com

21

Note

Expected Results

We expect to detect AMR sequences in most samples and be able to detect a decreasing number of positive partitions as samples are diluted (left to right) across the plate.