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Quantification of DNA Targets by Droplet Digital PCR (ddPCR)

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Melissa Schussman¹

¹UW-Milwaukee



Melissa Schussman

UW-Milwaukee

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We use this protocol and it's working

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Abstract

Quantification of DNA targets by Droplet Digital PCR (RT-ddPCR)

Materials

PCR reactions performed on **BioRad QX200 Droplet Digital PCR System**

Additional Equipment and Materials needed:

ddPCR Supermix for Probes (No dUTP) (Bio-Rad, Catalog no. 1863024)

PCR Plate Heat Seal, foil, pierceable (Bio-Rad, Catalog no. 1814040)

ddPCR 96-Well Plates (Bio-Rad, Catalog no. 12001925)

DG8 Gaskets for QX200/QX100 Droplet Generator (Bio-Rad, Catalog no. 1863009)

DG8 Cartridges for QX200/QX100 Droplet Generator (Bio-Rad, Catalog no. 1864008)

ddPCR Droplet Reader Oil (Bio-Rad, Catalog no. 1863004)

Before start

1. Cleaning working space by wiping down using 70% ethanol and RNase away.
2. Always make sure the pipettes are calibrated.
3. Prepare an ice bucket. Take out reagents from -20°C freezer, including BioRad ddPCR Supermix for Probes (No dUTP), primers, probes and other reagents needed. Allow superman to warm to room temperature, and primers/probes to thaw on ice for half an hour.
4. Prepare a cold low temp PCR rack/ice box for reaction set up.
5. Take out TNA samples from -80°C or -20°C and thaw on ice before setting up reaction.

Reaction Setup

- 1 Once all components have thawed, mix thoroughly by vortexing each tube to ensure homogeneity, then centrifuge briefly to collect contents to the bottom of the tube.
- 2 Prepare samples at the desired concentrations before setting up the reaction mix.
 - For primers, spike 18 μL forward and 18 μL reverse 100 μM primers into 64 μL DNA/RNA free water.
 - For probes, spike 5 μL 100 μM probe into 95 μL DNA/RNA-free water.
- 3 Prepare the reaction matrix (for one well, beside sample DNA) according to the table below. Prepare Use a low-binding tube of appropriate volume to mix all the components according to the reaction numbers. Always include extra wells when setting up reaction to avoid potential volume shortage caused by pipetting.

	A	B	C
	Component	Volume per Reaction, μL	Final Concentration
	2x ddPCR Supermix for Probes (No dUTP)	11	1x
	Primer mix	1.1	900 nM
	Probe	1.1	250 nM
	DNA/RNA-free water	3.3	
	Total	16.5	

Table 1. Preparation of reaction mixture

Note: water volume can be replaced accordingly by other ingredients, such as another assay (e.g., duplex assay) or another NRA template (e.g., inhibition test).

- 4 Disperse 16.5 μL of reaction matrix into each well of a 96-well PCR plate. For runs with multiple columns, calculate (the matrix total volume/8) and evenly distribute the matrix into an 8-well PCR strip. Then use an appropriate multichannel pipette (e.g., 2–20 μL range) to add 16.5 μL of matrix into each well, column by column.

Note: Always prepare a total of $8 \times (N \text{ columns})$ wells for droplet generation, or use ddPCR Buffer Control for Probes (#1863052) to fill empty wells on the last cartridge.



- 5 Gently vortex at half speed to mix the DNA sample. Make sure no liquid is attached on the lid. Add 5.5 μL of sample nucleic acid into each well containing 16.5 μL of reaction matrix, making the total volume of each reaction 22 μL .
- 6 Seal the PCR plate. Centrifuge down gently at 1000 rpm for 30s. Take out the plate and vortex on a 96-well plate mixer at 1600 rpm for 30s. Centrifuge again at 1000 rpm for 30s to settle down the plate.

Droplet Generation

- 7 Prepare materials/reagents on the working bench top, e.g., cartridges, gaskets, ddPCR 96-well plate, droplet generation oil, foil cover. Label the cartridges with corresponding column numbers (e.g., 1 to 12 for a full plate).
- 8 Remove the sealing on the PCR plate. Place the cartridge in the cartridge holder. Align well the PCR plate and the cartridge on the bench.
Use an appropriate multichannel pipette (e.g., 2- 20 μL) for liquid transfer. Adjust the pipette at 20 μL and make sure the tips are well positioned. Gently mix the liquid by aspirating up to 2/3 of the tip height and then releasing to a lower level of the tip height, i.e., not to the end of the tip, to avoid creating bubbles. Repeat this mixing step 10 times. In the last movement, slowly aspirate to the full volume of 20 μL .
- 9 Transfer the 20 μL reaction matrix to the middle column of the cartridge. Position the tip end to the ridge in well (where the well wall connects to the bottom) at 15° angle. Avoid creating bubbles when releasing liquid from tips into the cartridge; this can be realized by only pressing the plunger to position 2 and not position 3 before pull the tips out from the cartridge. Make sure the cartridge wells are in the same direction as on the PCR plate.
- 10 Fill in 70 μL of Droplet Generation Oil into bottom wells of the cartridge and cover the cartridge with a red gasket. Loop outer holes of red gasket around hooks on left and right sides of cartridge holder. Place the gasket equipped cartridge into the droplet generator.
- 11 When droplet generation is done, take out the cartridge from the droplet generator and remove the red gasket. Using a Rainin multichannel with recommended Rainin tips, in a leaning position, count to 5 to aspirate all the liquid (i.e., 40 μL) from the droplets column, and press against side of wells of the corresponding column in the ddPCR 96-well plate (i.e., not the previous PCR plate), count to 5 to expel the droplets into the wells.
- 12 Turn on the PX1 PCR plate sealer and let heat to 180°C. Correctly place the plate support block, the ddPCR plate, foil cover (i.e., red line up) and the metal holder. Seal the plate at 180°C for 5s and remove the plate immediately from the sealer.

Thermal Cycling

- 13 Load the plate on to a PCR thermocycler. Our lab's assay conditions are shown as below. We use Eppendorf Mastercycler Pro and the ramp speed is set to 50% for RT-ddPCR.

	A	B	C	D
	Step	# of cycles	Temp, °C	Time
	Activation	1	95	10 min
	Denaturing	40	94	30 sec
	Annealing	40	53-60*	60 sec
	Deactivation	1	98	10 min
	Stabilization	1	4	30 min - infinite

Table 2. Thermal cycling conditions

*annealing temperature is dependent on target, corresponding temperatures are indicated in Table 3.

	A	B	C	D
	Assay	Sequence 5'-3'	Size, bp	Annealing temperature, °C
	Salmonella (invA gene)	F: TCGTCATTCCATTACCTACC	119	53
		R: AAACGTTGAAAACTGAGGA		
		P: FAM/TCTGGTTGA/ZEN/TTTCCTGATCGCA/IABkFQ		
	Shiga toxin1-producing bacteria (Stx1)	F: ACATTGTCTGGTGACAGTAGC	114	60
		R: CGACATTAAATCCAGATAAGAAGTAGT		
		P: FAM/ATCAGTCGT/ZEN/ACGGGGATGCAGATAAAT/IABkFQ		
	Shiga toxin 2-producing bacteria (Stx2)	F: ATGACAACGGACAGCAGTTAT	116	60
		R: CTGAACTCCATTAACGCCAGATA		
		P: HEX/ATGCAAATC/ZEN/AGTCGTCACTCAC		



A	B	C	D
	TGG/IABkFQ		
blaKPC	F: GGCCGCCGTGCAATAC	60	58
	R: GCCGCCCAACTCCTTCA		
	P: FAM/TGATAACGC/ZEN/CGCCGCCAATTT GT/IABkFQ		
blaOXA-24/40-like	F: GATGACCTTGACATAACCG	150	54
	R: CAGTCAACCAACCTACCTGTG		
	P: HEX/AGTAACACC/ZEN/CATTCCCCATCCA CTTTT/IABkFQ		
blaOXA-48-like	F: ACGGGCGAACCAAGCAT	59	54
	R: GCGATCAAGCTATTGGGAATTT		
	P: FAM/TTACCCGCA/ZEN/TCTACC/IABkFQ		
blaVIM	F: GTGAGTATCCGACAGTCARCGAAA	142	60
	R: TCACCATCACGGACAATGAGACCA		
	P: FAM/CYGATGGTG/ZEN/TTTGGTCGCATAT CGCAAC/IABkFQ		
blaIMP	F: GGCTTAATTCTCGATCTATCCC	113	53
	R: CTAGCCAATAGTTAACTCCGC		
	P: HEX/GACGGTAAG/ZEN/GTTCAAGCCACA AAT/IABkFQ		
blaNDM	F: CTGGGCGGTCTGGTCATCGGTC	127	60
	R: CTGGCAGCACACTTCCTATCTCG		
	P: HEX/AAGCGA+CT+GCCCCG+A+A+A+CC C/IABkFQ		
C. auris	F: ATCGAATCTTTGAACGCACATTGC	119	59
	R: GTGCAAGCTGTAATTTTGTGAATGC		

	A	B	C	D
		P: FAM/CCTGTTTGA/ZEN/GCGTGATGTCTTC TCACC/IABkFQ		
	<i>C. glabrata</i>	F: GCGCCCCTTGCCTCTC	120	60
		R: CCCAGGGCTATAACACTCTACACC		
		P: FAM/TGGGCTTGG/ZEN/GACTCTCGCAGC /IABkFQ		
	<i>C. albicans</i>	F: CTTGGTATTTTGCATGTTGCTCTC	128	60
		R: GTCAGAGGCTATAACACACAGCAG		
		P: HEX/TTTACCGGG/ZEN/CCAGCATCGGTT T/IABkFQ		

Table 3. Assay information: primers, probes, size, annealing temperature

Protocol references

Original Source of PCR assays:

Salmonella (invA gene): DOI:10.1128/jcm.38.9.3429-3435.2000

Shiga toxin1-producing bacteria (Stx1): DOI:10.1016/j.ijfoodmicro.2011.07.039

Shiga toxin 2-producing bacteria (Stx2): DOI:10.1016/j.ijfoodmicro.2011.07.039

blaKPC assay: <https://www.cdc.gov/hai/settings/lab/kpc-ndm1-lab-protocol.html>

blaOXA-24/40-like: Designed by the Wisconsin State Lab of Hygiene

blaOXA-48-like: DOI: [10.1099/jmm.0.045823-0](https://doi.org/10.1099/jmm.0.045823-0)

blaVIM: Designed by the Wisconsin State Lab of Hygiene

blaIMP: DOI: 10.4172/2155-9597.1000299

blaNDM: Designed by the Wisconsin State Lab of Hygiene

C. auris: Designed by the Wisconsin State Lab of Hygiene

C. glabrata: PMCID: PMC150045

C. albicans: PMCID: PMC150045