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4-Gene Standard Uniplex PCR Protocol for Antibiotic Resistance Genes and Determinants *sul*1, *erm*(B), ctx-m$_{32}$, and *intI*1

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We use this protocol and it's working

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Abstract

A suite of four antibiotic resistance determinants were selected as a general screening tool for agricultural and environmental samples. It included antibiotic resistance genes from three commonly assayed drug classes (*sul*1, *erm*(b) and ctx-m) and one determinant often associated with human-impacted samples (the integrase gene *intI*1). This protocol describes the correct procedures on how to set-up and run the PCR assay and gel electrophoresis.

Image Attribution

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Guidelines

Scope

- Polymerase Chain Reaction (PCR) is a technique that enables researchers to rapidly and efficiently amplify (copy) a specific region of DNA (Deoxyribonucleic Acid) or RNA (Ribonucleic Acid) into millions of copies.
- A simple PCR reaction consists of target DNA, a set of synthetic oligonucleotide primers that flank the target DNA sequence, a thermostable DNA polymerase (usually Taq polymerase), and nucleotides.
- Thermal cycling exposes these contents to repeated cycles of heating and cooling that promotes DNA replication. There are three steps involved in the process:

1. denaturation
2. annealing
3. elongation

This assay is for the detection of *sul1*, *erm*(B), *ctx-m*-32, and *intI*1. In June 2020, the forward and reverse primers for *intI*1 were flip-flopped from the Hardwick orientation and are now based according to Dr. Lana Castleberry's recommendations and her use of the primers in the 4G multiplex assay. See 'References' section for references.

Responsibility

This SOP applies to all staff members and students. These individuals must be knowledgeable about the requirements set forth within this document. The lab manager or designee shall ensure that all staff and students know the proper techniques.

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Materials

Reagents and Materials

- JumpStart™ REDTaq® ReadyMix™ Reaction Mix **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P0982
- Forward and Reverse Primers – See **Table 2** (Step 4.3)
- PCR Water (HyClone SH30538.01 Sigma, St. Louis, MO)
- Positive Controls – gBlocks– See **Appendix** (Steps 31-34)
- Agarose **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A9539
- SYBR SAFE DNA stain **Invitrogen - Thermo Fisher Catalog #**S33102
- DirectLoad™ PCR 100 bp Low Ladder **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D3687
- 0.2 mL x 8 PCR reaction strips (T320-2N, Simport, Quebec, Canada)
- 2 mL sterile centrifuge tube (02.681.299 Fisher Scientific, Pittsburg, PA)
- Aerosol barrier no retention pipette tips (USA Scientific, Orlando, FL)
- 1X SB buffer (20X Sodium Borate (**SB**) Buffer - SB20-4, FasterBetter Media, Hunt Valley, MD)

Equipment

- PCR Prep Cabinet (3620804 Labconco, Kansas City, MO)
- Pipettors for various volumes from 1-1000µl (Gilson, Middleton, WI)
- Thermal cycler (T100, BioRad, Hercules, CA)
- Mini Centrifuge (C1301P, Labnet International, Big Flats, NY)
- 1 Liter or 500ml sterile Pyrex glass bottle
- Gel casting tray, gel combs, and gel boat
- Electrophoresis machine (PowerEase 300W, Life Technologies, Carlsbad, CA)
- Gel imaging apparatus (Gel Doc XR+, BioRad, Hercules, CA)
- Lighter

Safety warnings

Wear proper personal protective equipment (PPE) when working through PCR: nitrile gloves and lab coat.

When working with the hot agarose, wear thermal hot-pad gloves.

Procedure - PCR

15m

1 If frozen, thaw all reagents and samples and keep on an ice block or ice for stability. If kept in the refrigerator, keep there until ready for use, and keep on an ice block or ice for stability when in use.

2 Label tubes for the samples and controls.

3 Prepare the PCR prep area for setting up a PCR reaction to eliminate contamination – UV 0.2mL 8-strip PCR tubes, PCR water, and microcentrifuge tube for 00:15:00 .

15m

4 Combine the Master Mix components for the number of reactions (allow for 10% extra Master Mix) using the recipe from **Table 1 (** go to step #4.2 **)** with the correct primer sets **(Table 2 (** go to step #4.3 **))** for 20µL reactions.

4.1 Before adding to the Master Mix - mix the primers by aspirating and expelling with the pipette tip.

Note

***Beware, overmixing can degrade the DNA.**

4.2

	A	B	C	D
	Component	**Description**	**Volume/RXN (µl)**	**Final Concentration**
	Primer 1	Forward Primer (100µM)	0.4	2µM
	Primer 2	Reverse Primer (100µM)	0.4	2µM
	TAQ	JumpStart RedTaq ReadyMix Reaction Mix (Cat.#P0982)	10	1X

	A	B	C	D
	PCR Water	PCR Water (HyClone SH30538.01)	4.2	N/A
	Sample	Template (& positive control)	5.0	

Table 1. PCR Master Mix recipe. 20µL total reaction volume.

4.3 .

	A	B	C	D	E	F	G
	Gene	**Type**	**Sequence 5' to 3'**	**TM (°C)**	**TC Conditions**	**Amplicon Size (bp)**	**Sequence Reference**
	sul1	Forward	GACGAGATTGTGCGGTTCTT	64	1 cycle at 94°C for 2 min; 35 cycles at 94°C for 30s, 64°C for 30 s, 72°C for 2 min; 1 cycle at 72°C for 5 min.	185	Szczepanowski et al., 2009
		Reverse	GAGACCAATAGCGGAAGCC				
	***erm*(B)**	Forward	GATACCGTTTACGAAATTGG	58	1 cycle at 94°C for 2 min, 35 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 2 min; 1 cycle at 72°C for 5 min.	364	Chen et al, 2007
		Reverse	GAATCGAGACTTGAGTGTGC				
	ctx-m-32	Forward	CGTCACGCTGTTGTTAGGAA	63	1 cycle at 94°C for 2 min; 35 cycles at 94°C for 30 s, 63°C for 30 s, 72°C for 2 min; 1 cycle at 72°C for 5 min.	156	Szczepanowski et al., 2009
		Revers	CGCTCATCAGCACGATAA				

	A	B	C	D	E	F	G
		e	AG				
	intI1	Forward	ACATGCGTGTAAATCATCGTCG	60	1 cycle at 94°C for 2 min; 35 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 2 min; 1 cycle at 72°C for 5 min	473	Hardwick et al., 2008
		Reverse	CTGGATTTCGATCACGGCACG				Castleberry 2018

Table 2. Primer sequences, melting temperature, and thermal cycling conditions.

5 Vortex the completed Master Mix for at least 00:00:20 . 20s

6

Note

Check the pipette's volume as you go along, making sure it is exactly at the correct volume

7 Carefully pipette 5 µL of PCR Water to the non-template control (NTC) tube.

8 Add 15 µL of Master Mix to each labeled, 0.2mL PCR tubes.

9 Next, gently vortex to mix, then pipette 5 µL of sample (5 µL of working stock; or 1 µL straight DNA + 4 µL PCR water) into its same labeled tube.

Note

Use a new pipette tip for each sample.

9.1 To make a working stock dilution, see **SOP: IDT Primer and gBlock Hydration and Aliquots**.

10 Add 5 µL of positive control (mix first by aspirating and expelling with the pipette tip) to its same labeled tube.

11 Centrifuge the strip tubes so that the entire volume is at the bottom.

> Note
>
> **Make sure the centrifuge is balanced with both sides containing tubes.**

12 Load the tubes into the Thermocycler, keeping them in order.

13 Find the programmed gene conditions listed under '4G --' and double check that they match the conditions listed in **Table 2 [** go to step #4.3 **]**.

14 Run PCR

- When cycle is complete:

14.1 **Procedure - Gel Electrophoresis**, Steps go to step #15 - go to step #30.3 **OR**

14.2 Place the tubes in a labeled rack at -20 °C until ready to run a gel.

14.3 **Turn off the Thermocycler.**

Procedure – Gel Electrophoresis

3h 11m 30s

15 Using a spoon, measure out 2.6 g of powdered agarose into a weigh boat.

16 Measure 260 mL of 1X **SB** buffer into a graduated cylinder.

17 Add the powdered agarose and 1X **SB** into 500mL bottle and swirl to mix.

18 Microwave the **SB** and agarose mixture for 00:01:00 with bottle lid LOOSE. 1m

19 Remove bottle from microwave (**using hot pad gloves**), tighten the lid and gently swirl.

Safety information

For Steps 19 - 27 - Use thermal hot pad gloves to protect from burns.

20 LOOSEN the lid and put in microwave for another minute.

21 Check mixture visually, if flecks are present repeat go to step #19 , loosen lid and microwave again for 00:00:30 . 30s

22 Add 26 µL of SYBR safe to molten gel.

23 Swirl gently with a loose lid to distribute SYBR.

24 Place bottle in 55 °C water bath for 00:10:00 to temper. 10m

25 While the mixture is tempering, set up gel cast by putting two plastic stoppers into the ends of the cast, with the orange rubber side of the stopper facing out.

26 Put two (or three) 36-well combs into the first and fourth notches of the gel cast (gel cast is right side up if first notch is close to the top of the cast)

27 After the gel has tempered, slowly pour it (**using hot pad gloves**) into the gel cast.

28 If there are any noticeable bubbles, take a lighter and hold it over the bubble, which should make it pop.

29 Cover gel loosely with foil and allow it to set (up to 01:00:00) before use. 1h

Note

****SB gels will be flimsier, so be careful when removing the combs or transporting the gel****

30 **Electrophoresis Conditions**

30.1 10 µL of PCR amplicon will go into each well flanked by 5 µL of ladder.

30.2 Run at 120 volts (V) for 02:00:00 in 1x SB buffer. 2h

Note

Make sure **SB gel** goes into a gel boat with **SB buffer**.

30.3 Remove processed gel and analyze using a gel imaging system.

Appendix – Positive Control (gBlock) Sequences

31

Forward, Reverse

*Note: 'Reverse' denotes the 'Reverse Complement'

gBlock 3 – *sul*1 (344 bp) - for 4G assay and primer set P2-sul1

Escherichia coli R46 sul1 gene for sulfonamide-resistant dihydropteroate synthase Sul1, complete CDS NCBI Reference Sequence: NG_048081.1

CCTTCGACCCGAAGACGCGCTCGACGAGATTGTGCGGTTCTTCGAGGCGCGGGTTTCCGCCTTGCGACGGAGCGG
GGTCGCTGCCGACCGGCTCATCCTCGATCCGGGGATGGGATTTTTCTTGAGCCCCGCACCGGAAACATCGCTGCAC
GTGCTGTCGAACCTTCAAAAGCTGAAGTCGGCGTTGGGGCTTCCGCTATTGGTCTCGGTGTCGCGGAAATCCTTCT
TGGGCGCCACCGTTGGCCTTCCTGTAAAGGATCTGGGTCCAGCGAGCCTTGCGGCGGAACTTCACGCGATCGGCA
ATGGCGCTGACTACGTCCGCACCCACGCGCCTGGAGATCTGC

32 **gBlock 17 – *erm*B (424 bp)** – for 4G assay and primer set P3-ermB

ATAATAAAACAATTGAATTTAAAAGAAACCGATACCGTTTACGAAATTGGAACAGGTAAAGGGCATTTAACGACGAAACTGGC
TAAAATAAGTAAACAGGTAACGTCTATTGAATTAGACAGTCATCTATTCAACTTATCGTCAGAAAAATTAAAACTGAATACTCG
TGTCACTTTAATTCACCAAGATATTCTACAGTTTCAATTCCCTAACAAACAGAGGTATAAAATTGTTGGGAATATTCCTTACAAT
TTAAGCACACAAATTATTAAAAAAGTGGTTTTTGAAAGCCGTGCGTCTGACATCTATCTGACTGTTGAAGAAGGATTCTACAAG
CGTACCTTGGATATTCACCGAACACTAGGGTTGCTCTTGCACACTCAAGTCTCGATTCAGCAATTGCTTAAGCTGCCAGCGGAA
TGCT

33 **gBlock 18 – ctxm-32 (249 bp)** – for 4G assay and primer set P4–ctxm32

GCGTCAGTTCACGCTGATGGCGACGGCAACCGTCACGCTGTTGTTAGGAAGTGTGCCGCTGTATGCGCAAACGGC
GGACGTACAGCAAAAACTTGCCGAATTAGAGCGGCAGTCGGGAGGAAGACTGGGTGTGGCATTGATTAACACAG
CAGATAATTCGCAAATACTTTATCGTGCTGATGAGCGCTTTGCGATGTGCAGCACCAGTAAAGTGATGGCCGTGGC
CGCGGTGCTGAAGAAAAGTGAAAG

34 **gBlock 19 – *intI*1 (535 bp)** - for 4G assay and primer set P5-intI1

GTGAGCGCACTCCGGCACCGCCAACTTTCAGCACATGCGTGTAAATCATCGTCGTAGAGACGTCGGAATGGCCGA
GCAGATCCTGCACGGTTCGAATGTCGTAACCGCTGCGGAGCAAGGCCGTCGCGAACGAGTGGCGGAGGGTGTGC
GGTGTGGCGGGCTTCGTGATGCCTGCTTGTTCTACGGCACGTTTGAAGGCGCGCTGAAAGGTCTGGTCATACATG
TGATGGCGACGCACGACACCGCTCCGTGGATCGGTCGAATGCGTGTGCTGCGCAAAAACCCAGAACCACGGCCA
GGAATGCCCGGCGCGCGGATACTTCCGCTCAAGGGCGTCGGGAAGCGCAACGCCGCTGCGGCCCTCGGCCTGGT
CCTTCAGCCACCATGCCCGTGCACGCGACAGCTGCTCGCGCAGGCTGGGTGCCAAGCTCTCGGGTAACATCAAGG
CCCGATCCTTGGAGCCCTTGCCCTCCCGCACGATGATCGTGCCGTGATCGAAATCCAGATCCTTGACCCGCAGTTG
CAAACCCTCACT

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