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easyDB – Circularization of rv0678 for genotypic bedaquiline resistance testing of Mycobacterium tuberculosis

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ABSTRACT

We designed primers with a tail sequence that forms a six-nucleotide hairpin at temperature <55^{o}C, but not ≥55^{o}C. These primers contain six phosphorothioate bonds starting at the complementary region to inhibit exonuclease T7 activity. The primers successfully amplified the target and, following incubation with a mixture of T7 exonuclease, DNA polymerase, and Taq DNA ligase, pseudo-circular double-stranded DNA formed.

ATTACHMENTS

222.png

OPEN ACCESS

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Protocol status: Working
We use this protocol and it's working

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70165

Keywords: circular, rolling circle amplification, RCA, sequencing

MATERIALS

Rv0678 amplification primers, you can tack the tail (GGCGTCTCAAAACGCCCGT*targetedPrimerS*eq) onto any primer set but remember to add the PTO modifications.

	A	B
	Forward primer	GGCGTCTCAAAACGCCCGT*T*T*T*C*T*GTTGGTGC TGATATTGC
	Reverse primer	GGCGTCTCAAAACGCCCGT*A*C*T*T*GCCTGTCGC TCTATCTTC

Q5 Hot Start High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #M0493L**

Agencourt AMPure XP **Beckman Coulter Catalog #A63880**

Optional

Exonuclease III (E.coli) - 5,000 units **New England Biolabs Catalog #M0206S**

Exonuclease VIII truncated **New England Biolabs Catalog #M0545S**

Reagents for buffers

beta-Nicotinamide adenine dinucleotide (NAD+) - 0.2 ml **New England Biolabs Catalog #B9007S**

100mM dNTPs

Polyethylene Glycol 8000 **Contributed by users**

Dithiothreitol

T7 Exonuclease - 5,000 units **New England Biolabs Catalog #M0263L**

Phusion polymerase

Taq DNA Ligase - 10,000 units **New England Biolabs Catalog #M0208L**

Prepare Buffers

1

	A	B
	ISO buffer (2.5X)	Volume (ul)

	A	B
	1M Tris-HCl pH 7.5	100
	200mM MgCl2	50
	100mM dGTP	2
	100mM dATP	2
	100mM dTTP	2
	100mM dCTP	2
	100mM DTT	100
	40% PEG 8000	90
	50 mM NAD	20

Aliquot 100µl and store at -20°C for up to two years

	A	B
	easyDB Master Mix	Volume (ul)
	2.5X ISO buffer	640
	T7 exonuclease (10 U/µl)	0.64
	2 U/µl Phusion polymerase	20
	40 U/µl Taq DNA ligase	160
	H20	379.36

Aliquot 10 µl and store at -20°C

Amplicon PCR

2

	A	B
	Component	Volume (ul)
	5X Reaction Buffer	10
	5X Q5 High GC Enhancer	10
	10 mM dNTPs	1

	A	B
	Forward primer	2.5
	Reverse primer	2.5
	DNA (5ng)	2
	Q5 High-Fidelity DNA Polymerase	1.5
	Nuclease-Free Water	20.5

	A	B	C	D
	Step	Temp (C)	Time (s)	Cycles
	Denaturation	98	30	1
	Denaturation	98	10	34
	Annealing	62	10	
	Extension	72	20	
	Extension	72	2	1

Cycle parameters

3 Add 40 µL of resuspended AMPure XP beads 10m 30s
Mix by pipetting 10x
Incubate 00:05:00 at Room temperature
Place on magnet
Wash 2x with 200 µL freshly-prepared [M] 70 % (v/v) ethanol
Air dry for 00:00:30, don't allow the beads to become cracked
Resuspend in 20 µL Tris-low EDTA
Mix by pipetting 10x
Incubate 00:05:00 at Room temperature
Place on the magnet, aspirate 20 µL of the eluant into a new 200ul tube

easyDB reaction

4 Thaw a 10ul aliquot of easyDB Master Mix on ice
Add 5ul (~150ng) DNA to the tube
Mix thoroughly by pipetting 10X
Incubate at 50°C for 60min *(will be reduced, probably to 10min)*

5 Add 20 µL of resuspended AMPure XP beads 10m 30s

Mix by pipetting 10x

Incubate 00:05:00 at Room temperature

Place on magnet

Wash 2x with 200 µL freshly-prepared [M] 70 % (v/v) ethanol

Air dry for 00:00:30, don't allow the beads to become cracked

Resuspend in 12 µL Tris-low EDTA

Mix by pipetting 10x

Incubate 00:05:00 at Room temperature

Place on the magnet, aspirate 12 µL of the eluant into a new 200ul tube

Exonuclease Treatment - optional 10m 30s

6 *Optional*

	A	B
	Component	Volume (ul)
	H20	7
	Cutsmart	2
	DNA	10
	Exonuclease VIII, truncated	0.5
	Exonuclease III	0.5

7 Add 20 µL of resuspended AMPure XP beads 10m 30s

Mix by pipetting 10x

Incubate 00:05:00 at Room temperature

Place on magnet

Wash 2x with 200 µL freshly-prepared [M] 70 % (v/v) ethanol

Air dry for 00:00:30, don't allow the beads to become cracked

Resuspend in 12 µL Tris-low EDTA

Mix by pipetting 10x

Incubate 00:05:00 at Room temperature

Place on the magnet, aspirate 20 µL of the eluant into a new 200ul tube