Feb 28, 2023

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

DOI
dx.doi.org/10.17504/protocols.io.3byl4j24rlo5/v1

Jason D Limberis[1]

[1]University of California, San Francisco

Jason D Limberis
University of California, San Francisco

DOI: https://dx.doi.org/10.17504/protocols.io.3byl4j24rlo5/v1

Protocol Citation: Jason D Limberis 2023. easyDB – Circularization of rv0678 for genotypic bedaquiline resistance testing of Mycobacterium tuberculosis. **protocols.io https://dx.doi.org/10.17504/protocols.io.3byl4j24rlo5/v1**

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

Created: September 17, 2022

Last Modified: February 28, 2023

Protocol Integer ID: 70165

Keywords: circular, rolling circle amplification, RCA, sequencing , genotypic bedaquiline resistance testing of mycobacterium tuberculosis, genotypic bedaquiline resistance testing of mycobacterium, mycobacterium tuberculosis, genotypic bedaquiline resistance testing, mycobacterium, nucleotide hairpin at temperature, dna polymerase, nucleotide hairpin, stranded dna, circularization of rv0678, dna, taq dna ligase, easydb

Funders Acknowledgements:
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
Grant ID: 1R01AI153213-01A1

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

PNG

222.png

441KB

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*GCCTGTCGCT CTATCTTC

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

Dithiothreitol

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

Phusion polymerase

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

Troubleshooting

Prepare Buffers

1

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

	A	B
	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