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

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

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Abstract

The ligation of dumbbell (hairpin) oligos to linear dsDNA produces pseudo-circular DNA. Including deoxyUridine in the PCR primer sequences causes Q5 and other high-fidelity polymerases to arrest elongation. This results in overhangs that were successfully ligated to a complementary hairpin structure. The deoxyUridine reduced the PCR product by approximately two-thirds, but this was ameliorated by increasing the Q5 DNA polymerase concentration three-fold.

Attachments

PNG

222.png
441KB

Materials

Rv0678 amplification primers (you can use any primer set here, and multiplex them, these are not yet optimized, the random sequence is for blunt-end cloning to detect *chimeras*)

	A	B
	Forward primer	/5Phos/GUCTATTTTCTGTTGGTGCTGATATTGC
	Reverse primer	/5Phos/GUCTATACTTGCCTGTCGCTCTATCTTC

	A	B
	uDumBeI	/5Phos/ATAGACCGAGACAGTAGAAGACCATGAACAAGCAGCACACGATAAACTAGACACCCTACTGTCTCG

Preferably PAGE purified

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

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

T4 DNA Ligase - 20,000 units **New England Biolabs Catalog #**M0202S

Optinal

Exonuclease I (E.coli) - 15,000 units **New England Biolabs Catalog #**M0293L

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

Troubleshooting

Amplicon PCR

1

	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

PCR using primer set

	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

Adapter ligation

2 Prepare the dumbell (hairpin) by incubating at 80 °C followed by cooling to room temperature over 00:30:00 (this only needs to be done once) 30m

3

	A	B
	Component	**Volume (ul)**
	T4 DNA Ligase Buffer (10X)	2
	PCR product (upto 1ug), as low as 50ng, probably much lower possible)	10
	dumbell adapter	3
	Ligase (add last, don't vortex)	1
	H20	4

Incubate as below, with the lid temperature set to 40 °C

	A	B
	Temp	**Minutes**
	22	30
	15	120
	4	120
	65	5

4 Incubate at Room temperature for 00:05:00 8m

Place on amagnetic rack

Aspirate supernatant

Add 200 µL [M] 70 % (v/v) ethanol

Wait for 00:00:30

Aspirate and discard the supernatant

Add 200 µL [M] 70 % (v/v) ethanol

Wait for 00:00:30

Aspirate and discard the supernatant

Resuspend beads in 20 µL of H20

Incubate for 00:02:00

Transfer to a clean PCR tube

Exonuclease treatment – optional

1h

5

1h

	A	B
	Component	**Volume (ul)**
	NEBuffer 4 (10x)	1
	Exonuclease VIII (truncated)	1
	DNA	18

Incubate at 37 °C for 00:30:00

Stop reaction by adding EDTA to at least 11 mM.

Heat Inactivation 70 °C C for 00:30:00