Dec 11, 2024 Version 1

spotPCR: A Rapid and Efficient Approach for Indexing Individual Template Molecules using Unique Molecular Identifiers V.1

DOI
https://dx.doi.org/10.17504/protocols.io.261ged66wv47/v1

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Protocol Citation: Jason D Limberis 2024. spotPCR: A Rapid and Efficient Approach for Indexing Individual Template Molecules using Unique Molecular Identifiers. **protocols.io** https://dx.doi.org/10.17504/protocols.io.261ged66wv47/v1

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

Created: June 16, 2023

Last Modified: December 11, 2024

Protocol Integer ID: 83554

Keywords: specific primer limited unique molecular identifier tagging pcr, using unique molecular identifier, incorporation of unique molecular identifier, unique molecular identifier, specificity of mutation detection, efficient approach for indexing individual template molecule, mutation detection, indexing individual template molecule, umi tagging process, polymerase, frequency mutation, sequencing, polymerase chain reaction, including drug resistance identification, deep sequencing, spotpcr, pcr, drug resistance identification, pcr amplification, primer, infectious disease research

Funders Acknowledgements:
John Metcalfe
Grant ID: R01AI177637

Abstract

Low-frequency mutations provide valuable insights in various fields, including drug resistance identification, cancer and infectious disease research. One promising strategy to enhance the sensitivity and specificity of mutation detection is the incorporation of unique molecular identifiers (UMIs) during polymerase chain reaction (PCR) amplification and before deep sequencing. However, conventional methods for UMI incorporation often necessitate multiple labor-intensive steps. spotPCR (Specific Primer Limited Unique Molecular Identifier Tagging PCR) overcomes these challenges, streamlining the UMI tagging process.

Materials

Required

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

Agencourt AmPure XP beads **Catalog #**A63880

dNTPs

A thermocycler and a qPCR machine

A magnetic rack

Optional

NEBNext Library Quant Kit for Illumina - 500 rxns **New England Biolabs Catalog #**E7630L

	A	B	C
	Primer Set	Direction	Sequence
	pncA	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCGCGGCGTCATGGACCCTAT
	pncA	R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNNNNNNNTTTCGAAGCCGCTGTACGCTCC
	gyrA	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCACCCGCAACGCCAAGGAT
	gyrA	R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNNNNNNNTTATTGCCTGGCGAGCCGAAGT
	Illumina adapter primer	F	CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG
	Illumina adapter primer	R	AATGATACGGCGACCACCGAGATCTACAC[i5]TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG

You can use any compatable Illumina adapters such as the IDT for Illumina DNA/RNA UD Indexes.

Stage 1 PCR

1

	A	B
	COMPONENT	Volume (µl)
	5X Q5 Reaction Buffer	5
	5X Q5 High GC Buffer	5
	10 mM dNTPs	0.5
	Q5 High-Fidelity DNA Polymerase	0.25
	1µM Forward primer 1	1
	1µM Forward primer 2	1
	0.0625µM Reverse primer 1*	1
	0.625µM Reverse primer 2*	1
	Template DNA	2
	Nuclease-Free Water	6.25

*We recommend doing a dilution series starting at 500fM to asses new primersets.

	A	B	C	D
	Step	Temp (C)	Time (s)	Cycles
	Denaturation	98	120	1
	Denaturation	98	10	3
	Annealing	62	45	
	Extension	72	120	
	Extension	4	Forever	1

Cycle parameters. Three cycles are performed to ensure there are amplicon copies with Illumina tags on both ends.

Stage 2 PCR

2 Add the below into the reaction while at 4 °C and proceed to the second PCR cycling

	A	B
	COMPONENT	Volume (µl)
	Illumina adapter primer F (10uM)	1
	Illumina adapter primer R (10uM)	1

	A	B	C	D
	Step	Temp (C)	Time (s)	Cycles
	Denaturation	98	120	1
	Denaturation	98	5	34
	Annealing	65	15	
	Extension	72	20	
	Extension	4	Forever	1

Cycle parameters

Bead cleanup 21m

3 Add 25 µL (1X) of resuspended AMPure XP Beads to the sample
Mix by pipetting 10x

4 Incubate 00:02:00 at Room temperature 2m

5 Place on the magnet, allow the beads to aggregate, and remove and discard the supernatant

6 Add 200 µL [M] 70 % (v/v) ethanol and incubate (still on the magnet) for 00:00:30 30s

6.1 Remove the supernatant

6.2 Repeat go to step #6 for a total of 2 washes

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

8 Immediately after the bead pellet becomes opaque, remove the tube from magnetic rack and resuspend in 20 µL of **Low EDTA Tris Buffer**. Ensure all beads are in solution.

9 Incubate at room temperature for 00:05:00

10 Place on magnetic rack, wait for the solution to become clear ~ 00:02:00 , and transfer the eluted DNA to a new well-labeled tube

Optional: NEB Illumina Quantification

11 Thaw the NEBNext Library Quant Master Mix and NEBNext Library Quant Primer Mix. Ensure mixing of NEBNext Library Quant Primer Mix by vortexing. Place reagents on ice.

12 Thaw the NEBNext Library Quant DNA Standards, tubes 1–6.
Mix by pulse vortexing on a low setting. Briefly spin to collect material from the sides of the tubes. Place on ice.

13 Thaw the NEBNext Library Quant Dilution Buffer (10X). Mix well by vortexing.
Centrifuge briefly to collect material from the sides of the tube.
Place on ice.

14 Add 100 µL NEBNext Library Quant Primer Mix to the tube of NEBNext Library Quant Master Mix (1.5 mL). Mix by vortexing. Write the date on the master mix tube to indicate that primer mix has been added.

15 Dilute the NEBNext Library Quant Dilution Buffer (10X) 1:10 with nuclease-free water. Mix by vortexing.
Prepare sufficient buffer for quantitating the desired number of libraries, allowing 1.2 mL for each library.

16 Prepare a 1:1,000 dilution of each library sample in NEBNext Library Quant Dilution Buffer (1X)

17 Aliquot 16 µL NEBNext Library Quant Master Mix (with primers) to each well

18 Add-in 4 µL of sample or standard per well

19

	A	B	C	D
	Step	Temp (C)	Time (s)	Cycles
	Denaturation	95	60	1
	Denaturation	95	15	35
	Annealing	63	45	

Cycle parameters

A denaturation/melt curve can be included if desired, but is optional.

	A	B
	Sample	Conc. (pM)
	DNA Standard 1	100
	DNA Standard 2	10
	DNA Standard 3	1
	DNA Standard 4	0.1
	DNA Standard 5	0.01

	A	B
	DNA Standard 6	0.001

20 Adjusted Conc. = Calculated Conc. × 399 / library size (bp)

Analysis

21 **Link to script (click here)**

```
bash spotPCR_process.sh \
  -R1 "Read1.fastq" \
  -R2 "Read2.fastq" \
  -Ref_name "Reference.fasta" \
  -sample_name "SampleName" \
  -threads "Threads" \
  -umi "UMI_Barcode_Pattern"
```

The output will allow you to determine if there was sufficient UNI tagging of the amplicons or if a lower initial primer concentration is needed.