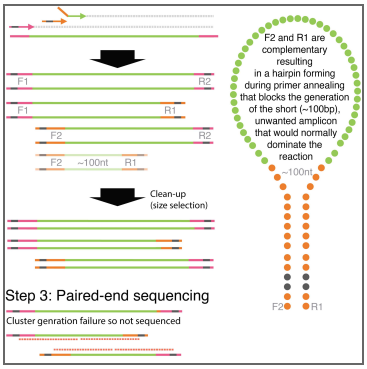

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Version created by Jason D Limberis

hissPCR: A simple, single-tube overlapping amplicon-targeted Illumina sequencing assay. V.1

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ABSTRACT

Targeted amplicon sequencing to identify pathogens, resistance-conferring mutations, and strain types is an important tool in diagnosing and treating infections. However, due to the short read limitations of Illumina sequencing, many applications require the splitting of limited clinical samples between two reactions. Here, we outline hairpin Illumina single-tube sequencing PCR (*hiss*PCR) which allows for the generation of overlapping amplicons containing Illumina indexes and adapters in a single tube, effectively extending the Illumina read length while maintaining reagent and sample input requirements.

ATTACHMENTS

hissPCR.png

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

Created: Mar 24, 2023

Last Modified: Sep 18, 2023

PROTOCOL integer ID: 79422

MATERIALS

Required

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

dNTPs **Contributed by users**

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

PowerUp SYBR Green Master Mix **Contributed by users Catalog #A25741**

A thermocycler and a qPCR machine

A magnetic rack

Primers

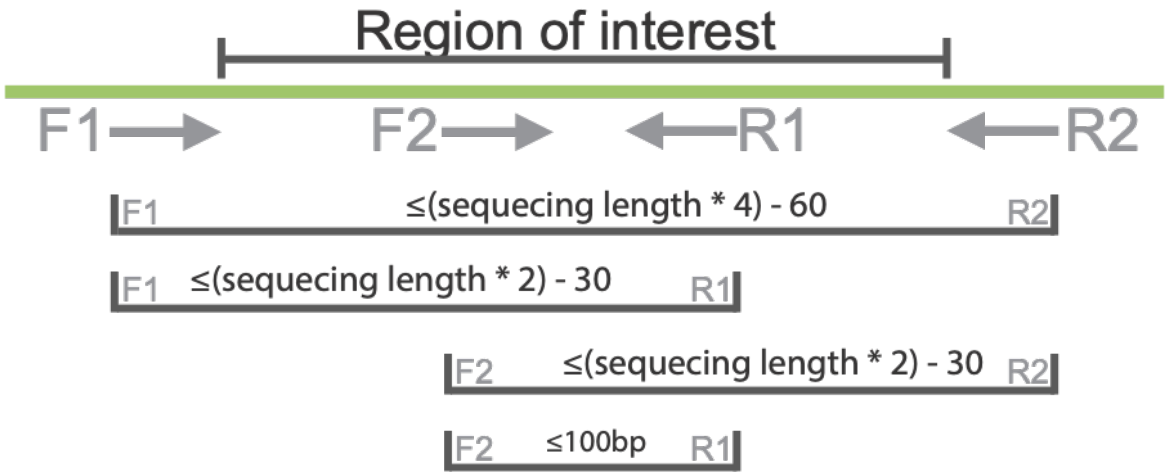
	A	B	C
	Primer Set	Direction	Sequence
	Rv0678	F1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTtcgatccgctgtggcttggc
	Rv0678	F2	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTgccgctcggatcacacacc
	Rv0678	R1	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTttgactcggttggcgggtcg
	Rv0678	R2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTcgacgagcgcctcgttgttg
	Illumina adapter primer	F	AATGATACGGCGACCACCGAGATCTACAC[i5]ACACTCTTTCCCTACACGACGCTCTTCCGATCT
	Illumina adapter primer	R	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC[i7]ATCTCGTATGCCGTCTTCTGCTTG
	Illumina_quant_Inner	F	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
	Illumina_quant_Inner	R	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
	Illumina_quant_Outer	F	CAAGCAGAAGACGGCATACGAGAT
	Illumina_quant_Outer	R	AATGATACGGCGACCACCGAGATC

*e.g., [i5] = A501 = TGAACCTT; [i7] = A701 = ATCACGAC

Lowercase bases are gene specific regions, change these if designing your own primer set

Primer design

1 Primers must be designed as shown in the figure below, if any amplicon is larger than the 2 x sequencing length, the inner portion of the amplicon will not be covered. We have developed a pipeline to assist in creating primers, which is available here: hisPCR primer design, and is used as follows.



*hiss*PCR Primer design strategy

```
hisPCR_primer_designer.sh \
  --name "rpob_demo" \
  --seq_cycles 300 \
  --start 100 \
  --end 800 \
  --template
"ttgaccgatgaccccggttcaggcttcaccacagtgtggaacgcggtcgtctccgaacttaacggcg
accctaaggttgacgacggacccagcagtgatgctaatctcagcgctccgctgacccctcagcaaagg
gcttggctcaatctcgtccagccattgaccatcgtcgaggggtttgctctgttatccgtgccgagcag
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gacatcagatccaactcggggtccgcatcgctccgccggcgaccgacgaagccgacgacactaccgtg
ccgccttccgaaaatcctgctaccacatcgccagacaccacaaccgacaacgacgagattgatgacag
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attccgctaccgctggcgtaaccagccttaaccgtcgctacacctttgatacgttcgttatcggcgcc
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ctcaccgatctttcgttgcccaaaatcggccaagcgttcggccgtgatcacacaaccgtcatgtacgc
ccaacgcaagatcctgtccgagatggccgagcgccgtgaggtctttgatcacgtcaaagaactcacca
ctcgcatccgtcagcgctccaagcgctag"
```

Stage 1 PCR

2

	A	B
	COMPONENT	Volume (µl)
	5X Q5 Reaction Buffer	10
	5X Q5 High GC Buffer	10
	10 mM dNTPs	1
	Q5 High-Fidelity DNA Polymerase	0.5

	A	B
	10µM Forward primer 1	1
	10µM Reverse primer 2	1
	20 mg/ml BSA	5
	Template DNA (~1ng/ul)	5
	Nuclease-Free Water	18.5

The total volume is 50ul at this stage

	A	B	C	D
	Step	Temp (C)	Time (s)	Cycles
	Denaturation	98	120	1
	Denaturation	98	10	10
	Annealing	64	15	
	Extension	72	45	
	Extension	4	Forever	1

Cycle parameters

As soon as the sample hits 4 °C, proceed to step 2.

Stage 2 PCR

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

	A	B
	COMPONENT	Volume (µl)
	10µM Forward primer 2	0.15
	10µM Reverse primer 1	0.15
	10µM Forward Illumina adapter primer	0.35
	10µM Reverse Illumina adapter primer	0.35

The total volume is 51ul at this stage

	A	B	C	D
	Step	Temp (C)	Time (s)	Cycles
	Denaturation	98	10	1
	Denaturation	98	10	20
	Annealing	65	15	
	Extension	72	30	
	Extension	72	120	1

Cycle parameters

Bead cleanup

4 Add 40 µL of resuspended AMPure XP beads to each reaction in a PCR tube 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
Remove the tubes from the magnetic rack
Add 50 µL 10 mM Tris-HCl pH 8.0 with 50 mM NaCl
NOTE: The BSA in the reaction causes the beads to clump.
Flick the tubes to partially resuspend the beads
Mix by pipetting 10x
Incubate 00:05:00 at Room temperature
Place on the magnet, aspirate 50 µL of the eluant into a new tube
Run 10 µL on a 0.8% agarose gel

Optional: Determine the proportion of amplicons with both li... 1m 50s

5 *Library quantification can be done using a commercial kit such as the KAPA Library Quantification Kits or using the below custom protocol.*

Dilute the cleaned up amplicons 1:100.

Make the master mix below using Illumina_quant_Inner primer set and a second master mix for Illumina_quant_Outer primer set.

	A	B
	COMPONENT	Volume (µl)
	PowerUp SYBR Green Master Mix (2X)	5
	10µM Forward primer	0.5
	10µM Reverse primer	0.5
	Diluted amplicon	2
	Nuclease-Free Water	2

qPCR master mix

Aliquot 8 µL to each well and add in 2 µL of the diluted amplicon.

6

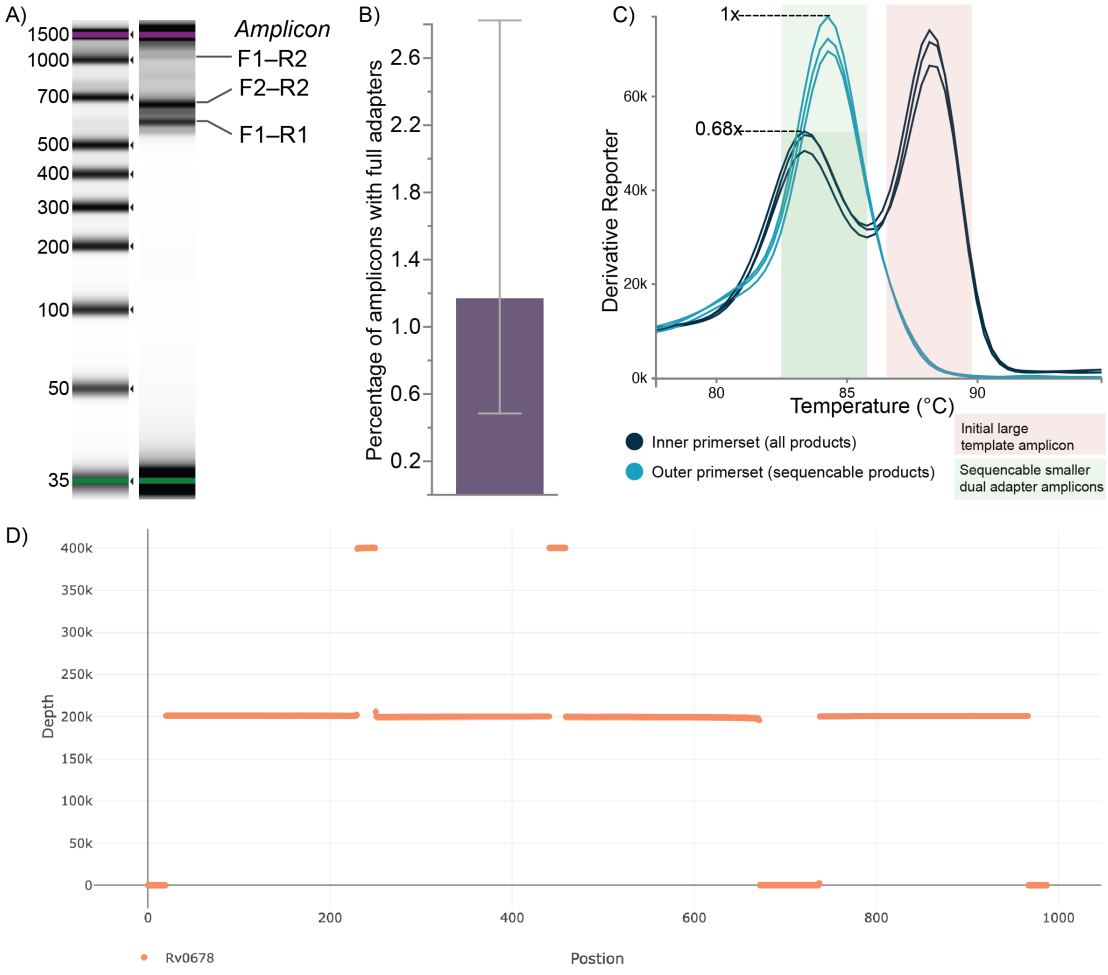
	A	B	C	D	E
	Step	Temp (C)	Time (s)	Cycles	Ramp Rate (C/s)
	UDG activation	50	120	1	2.73
	Denaturation	95	120	1	2.73
	Denaturation	95	1		2.73
	Amplification	60	30		2.11
	Capture	60	0	–	
	Melt Curve	95	1	1	2.73
		60	20	1	2.11
		95	–	1	0.15
		Capture	–	1	–

Cycle parameters for QuantStudio 3

7 The ratio of the Illumina _quant_Inner primer set to the Illumina _quant_Outer primer set will give the proportion of the DNA in the tube that contain both Illumina adapters and is sequencable (use this for pooling). While the melt curve will provide information on the proportion of each amplicon in the sample.

Expected Result

8



A) hissPCR generated amplicons using two forward and two reverse overlapping primers in a single tube. Amplicons contain either no Illumina adapter, one Illumina adapter, or two Illumina adapters per amplicon. B) The relative proportion of amplicons in the sample containing both Illumina adapters. C) Melt curves showing the expected amplicons for each primer set and the ratio of amplicons containing both Illumina adapters. D) An output from the *hiss*PCR analysis pipeline showing the expected read coverage over the target area using 250bp Illumina sequencing. (see step 10 in protocol)

Depth and Pooling Calculations

9 The amount of amplicon to be pooled and loaded can be calculated using the Illumina Sequencing Coverage Calculator by selecting DNA input then custom content and depends on numerous factors, including the amplicon size and number, and the kit and device

used. An example output is shown below.

	A	B
	Application or product:	Custom Content
	Genome or region size (Mbases)	0.001
	Read length	600
	On target (%)	95
	Coverage (x)	50000
	Duplicates (%)	0
	Instrument	MiSeq
	Run type	v3 Reagents
	Clusters	25,000,000 per flow cell
	Output per unit (flow cell or lane)	15,000,000,000 per flow cell
	Exceeds maximum read length?	Does not exceed maximum (2x300)
	Number of units per sample (flow cell or lane)	0.004 flow cells
	Samples per unit (flow cell or lane)	285/flow cell Most Illumina library prep kits enable up to 96 indexes; Nextera XT up to 384 indexes.
	Comments	Upgraded software; MCS v2.3 or later; MiSeq Reagent Kit v3 (150/600)
	Product	MiSeq Reagent Kit v3
	Link	http://www.illumina.com/products/miseq-reagent-kit-v3.html

Illumina coverage estimator output.

Data Analysis

10 The sequence data can be performed using most amplicon processing pipelines; however, the primer sequences must be trimmed from the reads to eliminate their effect on mutation identification. We have developed a pipeline to process data which is available at hissPCR analysis with further details, below is its basic usage command.

```
bash hissPCR.sh \
--R1 "test_data/read_R1_001.fastq.gz" \
--R2 "test_data/read_R2_001.fastq.gz" \
--ref "refs/BDQ_duplex.fasta" \
--primers "refs/primers.bed" \
```