

Bt-CoV HKU3 PCR/Sequencing Primers

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Abstract

This protocol details the primers and conditions used for forward and reverse PCR amplification and sequencing of Bt-CoV HKU3 genomes.

Introduction

This protocol details the steps, reagents, and conditions required to sequence Bt-CoV HKU3 genomes in the forward and reverse directions. The protocol begins with the RT-PCR step, assuming that BtCoV HKU3 RNA purified using a standard procedure (i.e., TRIzol extraction) has already been performed.

Reagents

Invitrogen SuperScript III kit

dNTPS (10 mM)

Random Hexamers

RNasin (if desired)

Thermo Phusion PCR enzyme

Primers (2 mM stock – see Tables 1 and 2)

Agarose

1X TAE Buffer

Ethidium Bromide

Equipment

70°C water bath

55°C water bath

Thermal cycler

Procedure

Reverse Transcription:

1. In a 1.5- μ L Eppendorf tube, add 1-4 μ L RNA and 1 μ L Random Hexamers.
2. Incubate at 70°C for 5 min.
3. Place reaction briefly on ice and assemble the reverse transcription reaction using the kit-supplied reagents (a master mix can be made if multiple reactions are being run): 4 μ L 5X First-Strand Buffer

2 μL DTT

1 μL SuperScript III reverse transcriptase

1 μL dNTPs

1 μL RNasin (if desired)

x μL H₂O to 20 μL

3. Incubate at 55°C for 45 min to 1 h.

4. Inactivate the reverse transcriptase at 70°C for 15 min. Place reaction on ice after inactivation.

5. Proceed with PCR setup.

PCR (with Phusion PCR kit):

1. Assemble PCR reactions to generate amplicons according to those detailed in Table 3 (for whole-genome sequencing) or with any combination of forward and reverse primers from Tables 1 and 2.

2. PCR reaction setup: 2 μL First-strand template

1 μL Forward Primer

1 μL Reverse Primer

5 μL 10X HF Buffer

1 μL dNTPs

0.5 μL Phusion polymerase

x μL H₂O to 50 μL

3. PCR reactions are run under standard PCR conditions: 98°C 5 min

35 cycles of:

98°C 15 sec

x°C for 30 sec*

72°C for ~45 sec/kb

72°C 10 min

8°C Hold

Annealing temperature is primer-dependent, but for most SARS-CoV primers in Tables 1 and 2, annealing temperatures 52-55°C will work.

Confirmation of PCR products and sequencing:

1. Run PCR products (5 µL/reaction) on a 0.8% agarose/1X TAE gel to verify PCR success.
2. Purify PCR products with PCR purification kit of choice (the Qiagen PCR Purification Kit works well).
3. PCR products can be diluted to 150-200 µL/reaction to ensure that enough product is present for assembling sequencing reactions.
4. Assemble sequencing reactions according to the primer/amplicon combinations outlined in Table 3.

Timing

Reverse transcription: 1-1.5 h

PCR: 2-4 h

Sequencing: facility-dependent

Anticipated Results

Primers have been designed to give clean, single-band PCR products. If multiple bands are detected, alternate annealing temperatures may be required. It may also be possible that alternate bands indicate multiple genome patterns at that locus.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

[Bt-CoV_HKU3_Sequencing_Primers.doc](#)

[Bt-CoV_HKU3_SRBD_Amplicon_Primer_Sets.doc](#)