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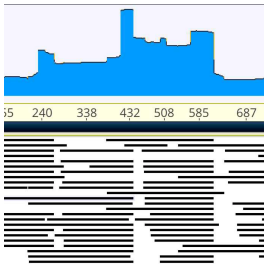
Whole genome sequencing of H5N1 from dairy products with tiled 250bp amplicons



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

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Keywords: HPAI, Influenza, H5N1, Amplicon, Sequencing, Dairy, Milk, Whole Genome, Illumina, Oxford Nanopore, Tiled Amplicon

Disclaimer

In our first batch of sequences we found that the amplicon defined by primers HPAI-PB1-250_7_RIGHT and LEFT mostly failed to amplify. This is the only amplicon we found to drop out completely and consistently. In the attached primer list, we have called this primer pair HPAI-PB1-250_7a and have included an additional HPAI-PB1-250_7b primer pair to supplement the coverage in that particular spot. These changes, while in the primer ordering sheets, are not reflected in the BED file.

We also advise that users analyze their sequence data carefully with a workflow that handles multi-segment viruses well. At the time of posting this protocol we are still determining what data analysis workflow works best on our end.

Abstract

Here we present a new short tiled amplicon scheme for the whole-genome sequencing of H5N1 from dairy product samples, and the methods we have used to successfully obtain whole-genome H5N1 sequences from dairy product samples using these primers. The majority of these primers were created using **primalscheme** with recent H5N1 sequences provided by the Andersen Lab (https://github.com/andersen-lab/avian-influenza/tree/master/alignments) used as a reference.[1-2] The primalscheme amplicons left coverage gaps at the very 5' and 3' ends of each gene segment, so we manually added additional primers to supplement these gaps informed by primers developed by the Moncla Lab (https://github.com/moncla-lab/h5-sequencing-protocol-dev/blob/main/RT_and_PCR_protocol.md).[3] The attached BED file contains the primer sequences aligned to gene segment sequences from A/Bovine/texas/24-029328-01/2024(H5N1).[4] We were inspired by the effectiveness of the QIAseq DIRECT SARS-CoV-2 250bp tiled amplicon scheme at amplifying difficult environmental samples for sequencing, and continue to use QIAseq Direct SARS-CoV-2 reagents and protocols in this workflow for that reason.[5] We plan to add our qPCR and sequencing results from commercial dairy samples to our github at https://github.com/dholab/dairy-hpai-monitoring.

Attachments

PDF ligation-sequencing-... 1.7MB

PDF HB-2880-003_HB_QIAse... 4.9MB

XLSX 2024.06.03_opoolsent... 15KB

FASTA DHO_CN_H5specific01_.. 0B

FASTA DHO_CN_H5specific01_.. 0B

XLSX 2024.06.03_named_opo.. 16KB

FASTA AVRLVS_fluMgene_prim.. 0B

FASTA AVRLVS_fluMgene_prob.. 0B

FASTA A_Bovine_texas_02932... 13KB

TXT AVRL_H5N1_250bpAmpWG... 8KB

Materials

*Not accounting for plastic wear and lab instrumentation.

Isolation, cleaning, and concentration:
- MagMax Wastewater Ultra Nucleic Acid Isolation Kit (#A52610), ThermoFisher Scientific
- TURBO DNAse (#AM2238), ThermoFisher Scientific
- RNA Clean and Concentrator 5 DNAse not included (#R1015 or R1016), Zymo Research

qPCR:
- H5N1 primers/probe (see **github** for fastas)
- qPCR master mix (see TaqMan, ThermoFisher Scientific)

Reverse transcription, PCR, and sequencing library prep:
- AVRL_H5N1_250bpAmpWGS primers from IDT (see protocol)
- QIAseq DIRECT SARS-CoV-2 Kit A (#333891), Qiagen
- QIAseq DIRECT SARS-CoV-2 Enhancer (#333884), Qiagen
- Qubit High Sensitivity dsDNA Kit
- For ONT sequencing only
  - Native Barcoding Kit 96 v14 (#SQK-NBD114.96), Oxford Nanopore Technologies
  - Supplemental New England Biosciences reagents for ONT Native Barcoding Kit 96

Sequencing
- Illumina Miseq
  - Miseq Reagent Kit V2, 2x150, Illumina
- Oxford Nanopore MK1C/MK1B or Gridion
  - R10.4.1 FLO-MIN114 Minion/Gridion flow cell, Oxford Nanopore Technologies

Before start

The protocol below describes how we have generated whole-genome H5N1 sequences from commercial dairy products using a 250bp tiled amplicon scheme. The methods described are not the only way to sequence H5N1 with this primer set. We invite others to use these primers however they think will work best. Here we document what has worked best for us.

Obtaining primer pools

1 Navigate to the **oPools primer pools product page** on IDT.

2 To order, upload the 2024.06.03_opoolsentrysample.xlsx 15KB file attached to this protocol. There should be 178 valid sequences. Order at a concentration of 50pmol/oligo.

3 Purchase AVRL_H5N1_250bpAmpWGS_Pool1 and AVRL_H5N1_250bpAmpWGS_Pool2. Together, the two pools should cost about $240. At 50 pmol/oligo, this order should contain enough primers to amplify 96 samples in separate 25ul reactions for each pool.

Isolating, cleaning, and concentrating viral RNA from milk

4 See our other protocol **RNA extraction from milk for HPAI surveillance**. When preparing samples for this sequencing protocol, we recommend following the optional clean and concentration steps in the RNA extraction protocol.

Quantifying H5N1 RNA concentration

5 We recommend using qPCR or dPCR to determine if your isolated samples have H5N1 RNA, and if so, to quantify how much. We have used both an influenza A primer/probe set AVRLVS_fluMgene_primers.fasta 0B AVRLVS_fluMgene_probe.fasta 0B and an H5 specific primer/probe set DHO_CN_H5specific01_primers.fasta 0B DHO_CN_H5specific01_probe.fasta 0B to test dairy samples. See our **dairy-hpai-monitoring** Github repository for more PCR primers and probe sequences we have found to work. Our qPCR results can be seen in the DETECTION_RESULTS.tsv, and primer/probe fastas can be found in the repository's assets folder. We will also post sequencing results in the same Github repository, so look there as well for examples of what sample concentrations can be successfully sequenced with our method.

Reverse transcription

6 Using reagents from the **QIAseq DIRECT SARS-CoV-2 Kit A** and **QIAseq DIRECT SARS-CoV-2 Enhancer**, follow the enhanced cDNA synthesis procedure starting on page 14 of the QIAseq DIRECT SARS-CoV-2 Handbook: HB-2880-003_HB_QIAseq_DIRECT_… 4.9MB [5].

6.1 Following the QIAseq DIRECT SARS-CoV-2 Handbook, for each sample assemble a cDNA synthesis reaction in strip cap tubes or 96-well plate **on ice** as follows (or add RNA to an aliquoted master mix of reagents 2-6).

	Reagent	Amount
	RNA from step 4	5ul
	RP primer	1ul
	Multimodal RT buffer, 5X	4ul
	RNAse inhibitor	1ul
	Nuclease-free water	8ul
	EZ reverse transcriptase	1ul

Total volume per reaction is 20ul.

6.2 Following the QIAseq Direct SARS-CoV-2 Handbook cDNA synthesis incubation protocol (page 15), use a thermal cycler to perform the cDNA synthesis incubation with the following conditions:

	Temperature	Time
	25°C	10 min
	42°C	50 min
	85°C	5 min
	4°C	Hold

Primer pool preparation

7 Add nuclease-free water or 1xTE buffer to lyophilized AVRL_H5N1_250bpAmpWGS_Pool1 and AVRL_H5N1_250bpAmpWGS_Pool2 to create 100uM stocks.

8 Dilute aliquots of 100uM stock 10x in nuclease free water to create 10uM working stock. You will need 4ul of each 10uM primer pool per sample, like in the **nCoV-2019 sequencing protocol v3 (LoCost) V.3**.[7]

PCR amplification

9 Using prepared 10uM H5N1 primer pools from above and reagents from the **QIAseq DIRECT SARS-CoV-2 Kit A** and **QIAseq DIRECT SARS-CoV-2 Enhancer**, follow the enhanced SARS-CoV-2 enrichment procedure starting on page 16 of the QIAseq DIRECT SARS-CoV-2 Handbook. **Use enrichment cycling protocol 5a.**

HB-2880-003_HB_QIAseq_DIRECT_… 4.9MB [5]

9.1 Following the QIAseq DIRECT SARS-CoV-2 Handbook (replacing SARS-CoV-2 primer pools with H5N1 primer pools), for each 10uM H5N1 primer pool (AVRL_H5N1_250bpAmpWGS_Pool1 and AVRL_H5N1_250bpAmpWGS_Pool2) set up the following reaction for each sample in strip caps or a 96-well plate **on ice**:

Reagent	Amount	Final Concentration
cDNA from step 6	8ul	
10 uM primer pool	4ul	1.6 uM
UPCR buffer, 5X	5ul	1x
QN taq polymerase	1ul	
Nuclease-free water	7ul	

Total volume per reaction is 25ul. When pool 1 and pool 2 are combined for each sample, you will have 50ul per sample.

9.2 Following the QIAseq Direct SARS-CoV-2 Handbook enrichment protocol 5a (page 17), use a thermal cycler to perform the PCR using the following conditions:

Step	Time	Temperature (°C)	Number of cycles
Heat activation	2 min	98	1
Denaturation	20 s	98	4
Annealing/extension	5 min	63	
Denaturation	20 s	98	29
Annealing/extension	3 min	63	
Hold	∞	4	Hold

Sequencing Library Prep

10 Below are instructions on how to sequence using either Illumina (Miseq V2 2x150) or Oxford Nanopore (Native Barcoding 96 V14) sequencing chemistries.

11 Pool the AVRL_H5N1_250bpAmpWGS_Pool1 and AVRL_H5N1_250bpAmpWGS_Pool2 25ul PCR products for each sample (for 50ul total per sample), mix, and determine sample concentration using a Qubit Fluorometer.

STEP CASE

Sequencing with Oxford Nanopore 3 steps

12 Prepare for Oxford Nanopore sequencing following the **Native Barcoding 96 V14** protocol ligation-sequencing-amplicons-native… 1.7MB .[8] Add 200fmol or 11.5ul of sample into the End Prep step. We have not had problems loading more than 200fmol.

13 Load end-prepped, barcoded, and adapted sample library onto an R.10.4.1 flow cell and sequence on an ONT MK1C/MK1B or Gridion instrument. Load 50fmol or 12ul of prepared library. We have not had any problems loading more than 50fmol.

Protocol references

1) Quick J et al. Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples. Nat Protoc. 2017 Jun;12(6):1261-1276.

2) andersen-lab/avian-influenza [Internet]. Andersen Laboratory @ Scripps Research; 2024 [cited 2024 Jun 4]. Available from: https://github.com/andersen-lab/avian-influenza

3) moncla-lab/h5-sequencing-protocol-dev [Internet]. Moncla Laboratory @ University of Pennsylvania; [cited 2024 Jun 4]. Available from: https://github.com/moncla-lab/h5-sequencing-protocol-dev/blob/main/RT_and_PCR_protocol.md

4) Burrough ER, Magstadt DR, Petersen B, Timmermans SJ, Gauger PC, Zhang J, Siepker C, Mainenti M, Li G, Thompson AC, Gorden PJ, Plummer PJ, Main R. Highly Pathogenic Avian Influenza A(H5N1) Clade 2.3.4.4b Virus Infection in Domestic Dairy Cattle and Cats, United States, 2024. Emerg Infect Dis. 2024 Apr 29;30(7). doi: 10.3201/eid3007.240508. Epub ahead of print. PMID: 38683888.

5) QIAseq DIRECT SARS-CoV-2 Kits [Internet]. [cited 2024 Jun 4]. Available from: https://www.qiagen.com/us/products/next-generation-sequencing/rna-sequencing/qiaseq-direct-sars-cov-2-kits

6) Moncla LH, Ross TM, Dinis JM, Weinfurter JT, Mortimer TD, Schultz-Darken N, et al. A Novel Nonhuman Primate Model for Influenza Transmission. PLoS One. 2013 Nov 14;8(11):e78750.

7) Quick J. nCoV-2019 sequencing protocol v3 (LoCost). 2020 Aug 25 [cited 2024 Jun 4]; Available from: https://www.protocols.io/view/ncov-2019-sequencing-protocol-v3-locost-bh42j8ye

8) Native Barcoding Kit 96 V14 [Internet]. [cited 2024 Jun 4]. Available from: https://store.nanoporetech.com/us/native-barcoding-kit-96-v14.html

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