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Background

Porcine reproductive and respiratory syndrome virus (PRRSV) is a rapidly evolving, highly pathogenic porcine arterivirus among the most common infectious agents of swine disease, affecting between 30% to 40% of the breeding herds in the US each year¹. Traditionally, PRRSV diagnosis involves amplifying (reverse-transcription PCR) and Sanger sequencing of several conserved open reading frames (ORFs), to detect and classify the virus for outbreak management². Herds are often coinfecting with both (pathogenic) wild-type viruses and (benign) modified live virus (MLV) vaccines, which are challenging to discriminate by Sanger sequencing³. However, long-read sequencing can rapidly generate full-length amplicon sequences from multiple strains in the same sample, allowing for greater discrimination, increased reliability, and improved turn-around time.

Concordance between Nanopore and Sanger

We evaluated the Oxford Nanopore platform for PRRSV-2 amplicon sequencing. ORF5, ORF6 and ORF7 were amplified from 15 PRRSV-2 RNA extracts collected from oral fluid, lung, serum and processing fluid, with Ct values of 15.0-27.5. Amplicons were sequenced on MinION flow cells using the native barcoding kit (NBD), and by Sanger sequencing (Fig. 1a).

Nanopore sequencing was found to have 100% concordance with Sanger sequencing for 9 ORF5 (n=10), 11 ORF6, and 10 ORF7 (n=12) generated consensus sequences (Fig. 1b). Four amplicons displayed concordance below 100% due to unresolved mixed infection reported as ambiguous bases in Sanger reference. In contrast, Nanopore could generate discrete, full-length amplicon sequences for each strain as a consensus of reads at the single-molecule level. In the case depicted in Fig. 1c), Nanopore sequencing revealed presence of three populations with distinguishable SNPs.

The protocol can be upscaled to simultaneously analyse up to 96 amplicons. We determined the minimal sequencing time required to reach depth of 1,000x for each of 94 amplicons to 2 h (Fig. 1d).

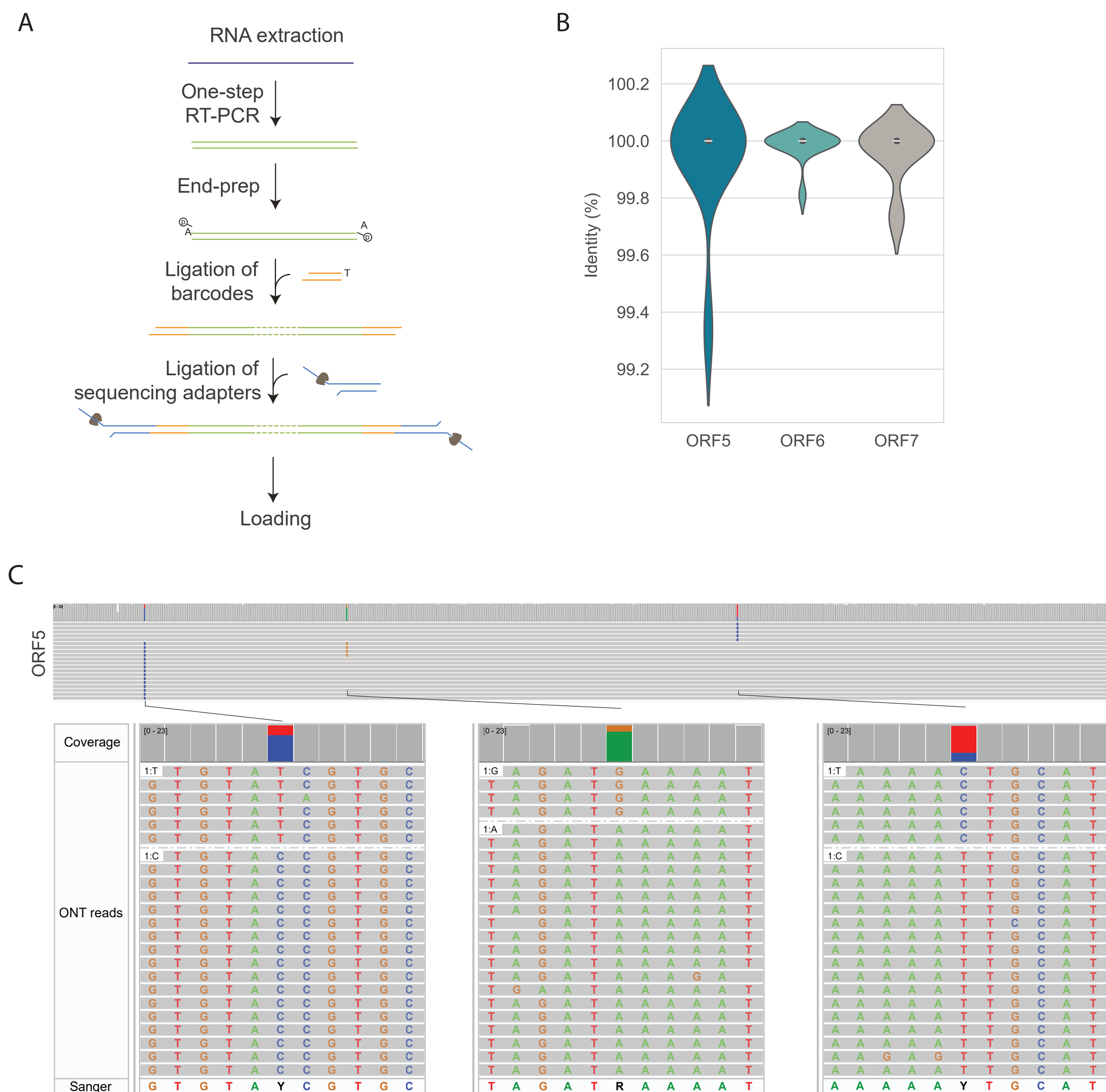


Fig. 1 a) Library preparation workflow for Nanopore sequencing. b) BLASTing identity between Sanger and Nanopore consensus sequences. c) Nanopore reads resolve ambiguous bases in Sanger consensus. d) Relation between time and coverage depth when sequencing 94 PRRSV amplicons (Ct up to 27.5).

Discrimination of mixed infections

To assess applicability of Nanopore sequencing for detection of mixed infections, a clinical sample containing MLV and wild type strains was subjected to amplification and sequencing. Generated sequencing reads spanned the full ORF5 region which allows to discriminate strains (Fig. 2a).

Furthermore, in vitro mixtures of amplicons were generated to simulate mixed infections at different frequencies, and sequencing correctly detected the presence of mixed amplicons at ratios as low as 100:1 (Fig. 2b).

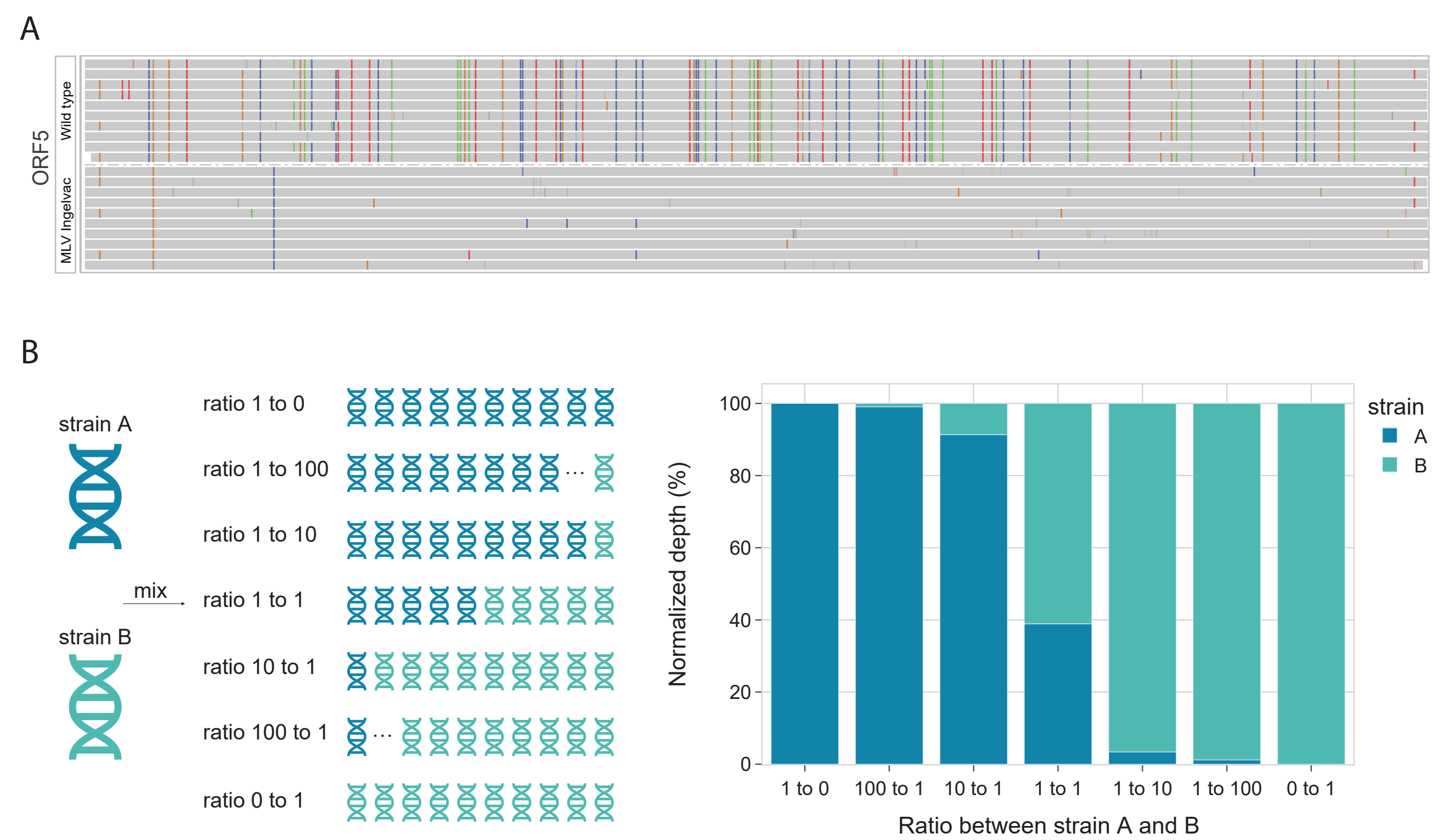


Fig. 2 a) Alignment of sequencing reads from RNA sample containing wild type PRRSV strain (top) and MLV Ingelvac (bottom) against PRRSV-2 strain SD95-21. b) Sensitivity of Nanopore sequencing to detect mixed strains.

Level of detection

We sought to determine detection threshold of PRRSV-2 amplicons by Nanopore sequencing. Serial dilution of selected RNA sample was used for quantitative PCR and reverse-transcription PCR followed by sequencing. Comparison of Ct value and number of reads detected for each dilution confirmed detection of ORF5 amplicons at Ct values as high as 30.6, and ORF6/ORF7 up to a Ct of 27.4.

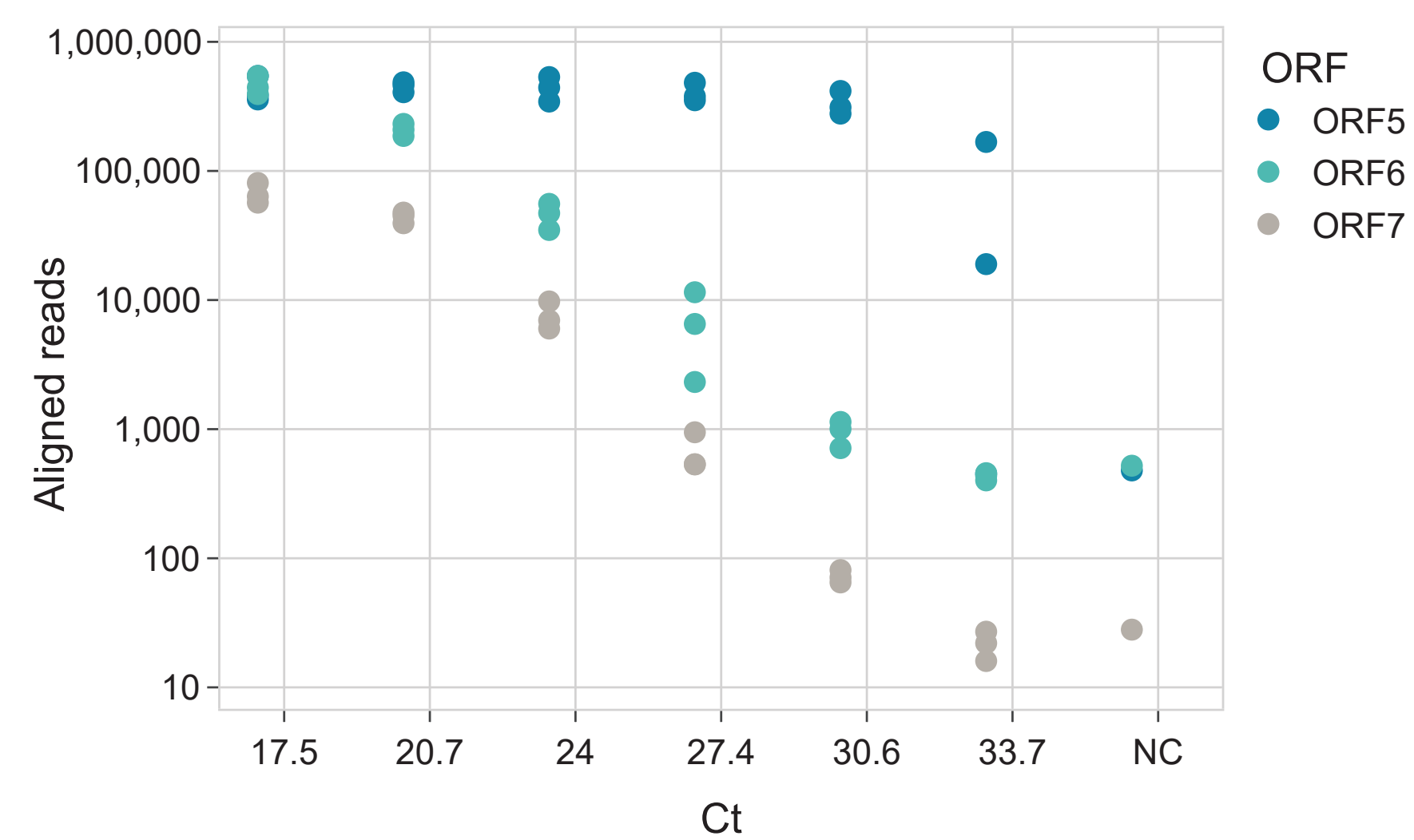


Fig. 3 Level of detection of PRRSV ORF5, ORF6 and ORF7 amplicons by Nanopore sequencing (n=3).

Conclusions

These results suggest that nanopore sequencing can be used as a drop-in replacement for Sanger sequencing of PRRSV-2 amplicons and has the potential to more accurately discriminate between amplicons generated from mixed infections or vaccine strains.

References

- ¹Tousignant et al "Temporal and spatial dynamics of porcine reproductive and respiratory syndrome virus infection in the United States" American journal of veterinary research 76 76.1 (2015): 70-76.
- ²Caserta et al. "Rapid genotyping of porcine reproductive and respiratory syndrome virus (PRRSV) using MinION nanopore sequencing." Plos one 18.5 (2023): e0282767.
- ³Kim et al. "Different biological characteristics of wild-type porcine reproductive and respiratory syndrome viruses and vaccine viruses and identification of the corresponding genetic determinants" Journal of clinical microbiology 46.5 (2008): 1758-1768.