SUPPLEMENTAL MATERIAL

Simulation of nanopore sequencing signal data with tunable parameters

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Generating the simulated NA12878 dataset

The simulated NA1278 dataset was generated using Squigulator, with the intention to emulate the real experimental dataset above. BCFtools consensus (v1.16) was used to incorporate high-confidence NA12878 variants (SNVs and indels) from Genome in a Bottle (v3.3.2) into the human reference genome sequence (hg38; FASTA format). To minimise computational resources for resulting benchmark experiments, we restricted this to Chr22. The commands used were as follows:

```
bcftools consensus --haplotype 1 -f chr22.fa giab_na12878.vcf > hap1.fa
bcftools consensus --haplotype 2 -f chr22.fa giab_na12878.vcf > hap2.fa
cat hap1.fa hap2.fa > na12878_chr22.fa
```

These commands generate two separate Chr22 reference sequences with variants incorporated from NA12878 haplotype 1 and haplotype 2, respectively (homozygous variants are incorporated into both references). We then used Squigulator to generate simulated nanopore signal data from this custom diploid reference. To match the data to the NA12878 experimental dataset, we used the *-x dna-r9-prom* pre-set parameter configuration. We adjusted the read-length mean, read-length standard deviation and sequencing depth so as to approximate the equivalent metrics measured from the experimental dataset. The command used was as follows:

```
squigulator na12878_chr22.fa -o reads.blow5 -n 135000 -r 10800 -x dna-r9-prom -t 8 -K 4096
```

Details of analysis workflow and evaluation with RTG

Signal data was basecalled with ONT's Guppy software (using the Buttery-eel wrapper for SLOW5 data access; Buttery-eel v0.0.1 on Guppy v6.0.6). Basecalled reads were aligned to the hg38 reference genome with no alternate contigs using minimap2 (v2.17). Alignment statistics were derived with SAMtools stats (v1.9). Reference:read identity scores were retrieved using PAFtools, which is a companion tool in the minimap2 repository:

```
samtools view reads.bam -h chr22 | paftools.js sam2paf -p - | awk '{print $10/$11}'
```

Variant calling was performed separately using Nanopolish (v0.14.0) and Clair3 (v0.1-r11; r941_prom_sup_g5014). Variant evaluation was performed using *rtg vcfeval* against the GIAB NA12878 high confidence truth-set (the same callset that was used during the simulation) with QUAL field as the --vcf-score-field. The commands used for basecalling, alignment, variant calling and evaluation were as follows:

```
buttery-eel -i reads.blow5 -o reads.fastq --guppy_bin ont-guppy-6.0.6/bin --port 5887 --config
dna_r9.4.1_450bps_${MODEL}_prom.cfg  -x cuda:all --chunk_size 1500 --max_queued_reads 1000 #
MODEL is fast or hac or sup

minimap2 -x map-ont -a -t32 --secondary=no hg38noAlt.fa reads.fastq  > reads.sam
$samtools sort -@32 reads.sam > reads.bam
$samtools index reads.bam

run_clair3.sh --threads=32 --include_all_ctgs --bam_fn=reads.bam --ref_fn=hg38noAlt.fa  --
platform=ont
--model_path=r941_prom_sup_g5014/ --output=out/ --sample_name=reads --enable_phasing --
longphase_for_phasing

nanopolish variants -o output.vcf -w ${1}  -r  reads.fastq -g hg38noAlt.fa -b reads.bam -p 2 -t 4
-q cpg --fix-homopolymers

 rtg RTG_MEM=32G vcfeval  -b highconf_PGandRTGphasetransfer.vcf.gz  -c merge_output.vcf.gz  -t
hg38noAlt.sdf -o compare_clair --region chr22:1-50818468  -e
highconf_nosomaticdel_noCENorHET7.bed  --vcf-score-field QUAL
```

Details of parameter exploration experiment

For the parameter exploration experiments presented in **Fig3** and **Supplemental FigS3**, we repeated the simulation and analysis workflows described above, each time varying the simulation parameters. We independently varied the dwell-time mean (--dwell-mean), dwell-time standard deviation (--dwell-std) and amplitude noise factor (--amp-noise), whilst holding the other parameters at the default value. Example commands are as follows:

```
squigulator na12878_chr22.fa -o reads.blow5 -n 135000 -r 10800 -t 8 -K4096  -x dna-r9-prom  --amp-noise <FACTOR> --dwell-mean <MEAN> --dwell-std <STD>
```

For each simulation, the analysis workflow and evaluation was described exactly as above.

Details for DeepSimulator comparison

DeepSimulator generates simulated signal data via either of two approaches. The main mode is the 'context-dependent' moded which uses a Bi-LSTM trained model to generate realistic nanopore signal data. The alternative mode is the 'context-independent' mode, which utilises a k-mer model provided by ONT. The context-independent mode has some methodological similarity to Squigulator, in that it uses a k-mer pore model, then applies noise to emulate real data. The two algorithms differ significantly in the way they apply noise to the data:

1. Squigulator uses both the signal-level mean and signal-level standard deviation of the pore-model, where as DeepSimulator relies on the level mean only.

2. Squigulator uses the standard deviation of each k-mer in the pore-model to generate noise, so the noise is k-mer specific. DeepSimulator applies noise at two levels: at the signal level and event level. However, this noise is determined by a single standard deviation for each of the two levels of noise and is not *k*-mer specific. This is an important difference, as we observe that different k-mers have quite different noise characteristics.

3. DeepSimulator applies a low-pass signal to the signal, whereas Squigulator does not.

4. The dwell time distribution in Squigulator uses a normal distribution, whereas *DeepSimulator* uses a mixture alpha distribution.

DeepSimulator 1.5 main branch on Github (https://github.com/liyu95/DeepSimulator) has an install.sh script for building a conda environment and setting up various other tools required. This script does not work with conda v4+ and thus modifications were made to successfully install DeepSimulator. Similarly, the deepsimulatr.sh script for running the DeepSimulator pipeline needed modifications to work with conda v4+. Basecalling was excluded from the pipeline when running benchmarks.
To generate simulated libraries for comparison with Squigulator, the following commands were run:

```
## for context-independent mode:
deep_simulator.sh -i na12878_chr22_1.fa -o chr22_1_context_ind -n 67500 -l 10800 -c 16
deep_simulator.sh -i na12878_chr22_2.fa -o chr22_2_context_ind -n 67500 -l 10800 -c 16
## for context-dependent mode:
deep_simulator.sh -i na12878_chr22_1.fa -o na12878_chr22_1_context_dep -n 67500 -l 10800 -M 0
deep_simulator.sh -i na12878_chr22_2.fa -o na12878_chr22_2_context_dep -n 67500 -l 10800 -M 0
```

The modified deep_simulator.sh scripts can be found here: https://github.com/Psy-Fer/DeepSimulator_benchmark

Supplemental Table S1: Comparison of minimap2 alignment statistics for experimental vs Squigulator NA12878 datasets.
Basecalled data was generated using Guppy HAC model.

	NA12878 experimental	**NA12878 simulated**
sequences	135,083	134,999
reads mapped	134,001	134,987
reads unmapped	1,082	12
reads MQ0	661	153
total length	1,458,924,348	1,430,013,633
bases mapped (cigar)	1,491,898,469	1,430,001,381
mismatches	154,930,196	76,290,809
error rate	1.04E-01	5.34E-02
average length	10800	10592
maximum length	187345	87341
average quality	20.1	18
insertions (1-base)	11,508,645	10,218,115
deletions (1-base)	16,023,165	16,965,569

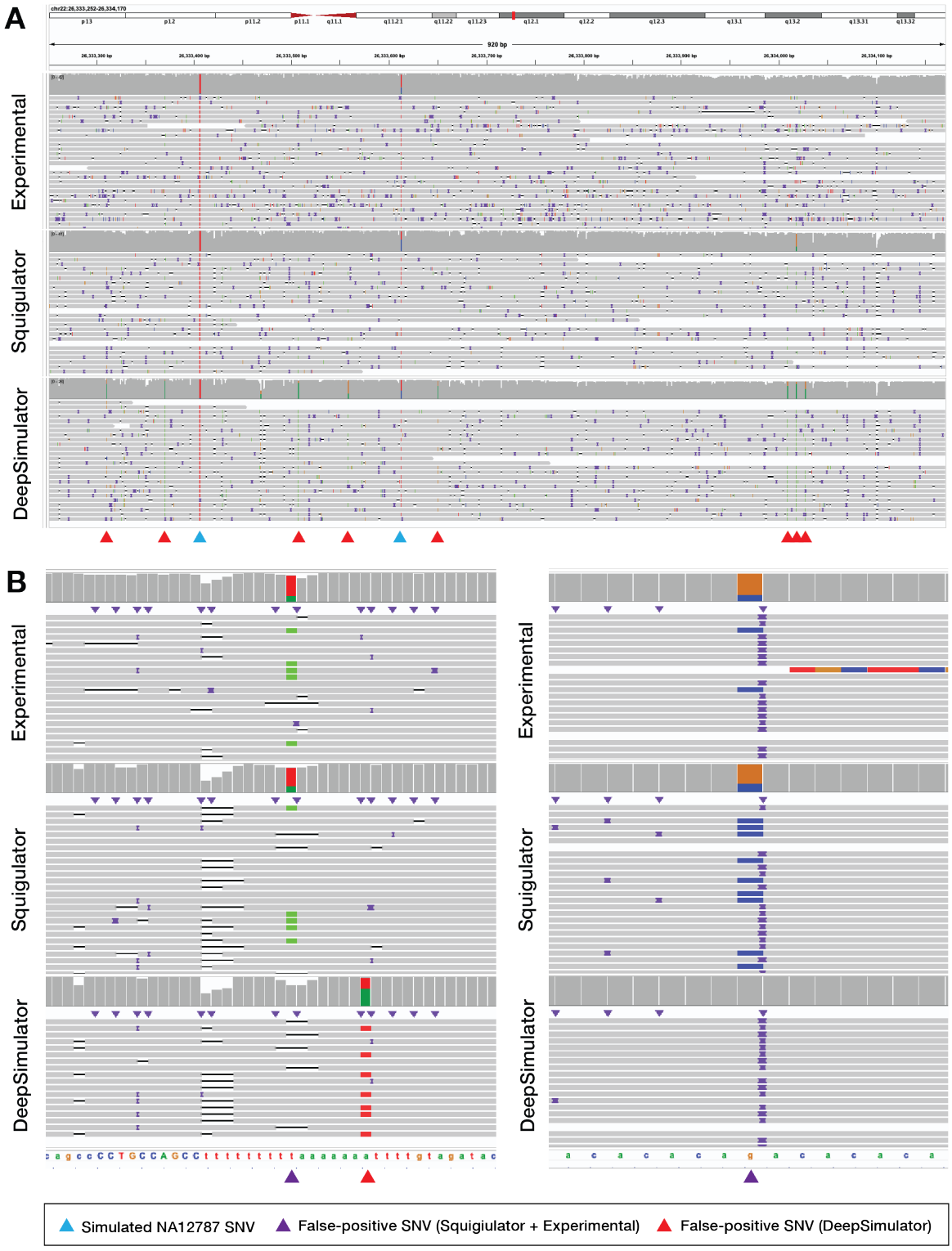
Supplemental Table S2: Comparison of Clair3 and Nanopolish SNV detection statistics for experimental vs Squigulator NA12878 datasets

Basecalled data was generated using Guppy SUP model.

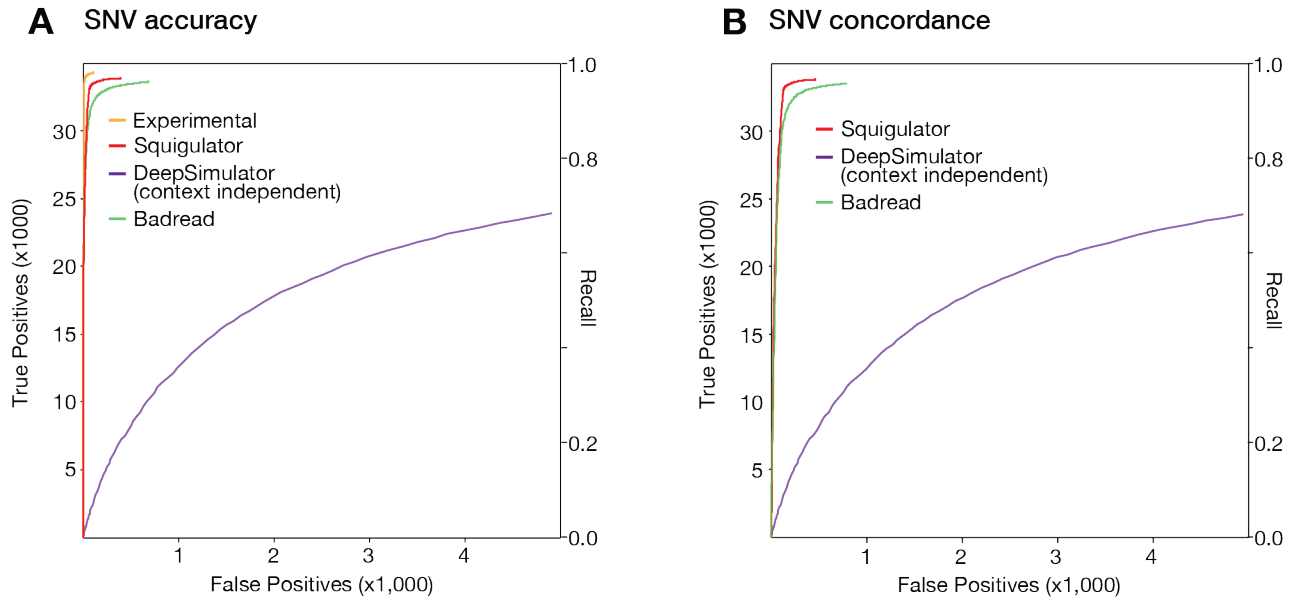
Data	Variant caller	Score threshold	True positives baseline	False positives	True positives	False negatives	precision	recall	f_score
Experimental data	**Clair3**	None	34302	118	34304	160	0.9966	0.9954	0.996
		2.15	34302	118	34304	160	0.9966	0.9954	0.996
	Nanopolish	None	32743	1532	32735	1719	0.9553	0.9501	0.9527
		20.9	32713	1479	32705	1749	0.9567	0.9492	0.953
Simulated	**Clair3**	None	33881	403	33883	581	0.9882	0.9831	0.9857
		6.48	33804	255	33807	658	0.9925	0.9809	0.9867
	Nanopolish	None	33418	317	33409	1044	0.9906	0.9697	0.98
		21.7	33418	316	33409	1044	0.9906	0.9697	0.9801

Supplemental Table S3: Comparison of Squigulator and DeepSimulator run-time and memory usage.
Run times and peak RAM usage were measured during simulations of NA12878 data from Chr22 at ~30X using 16 CPUs.

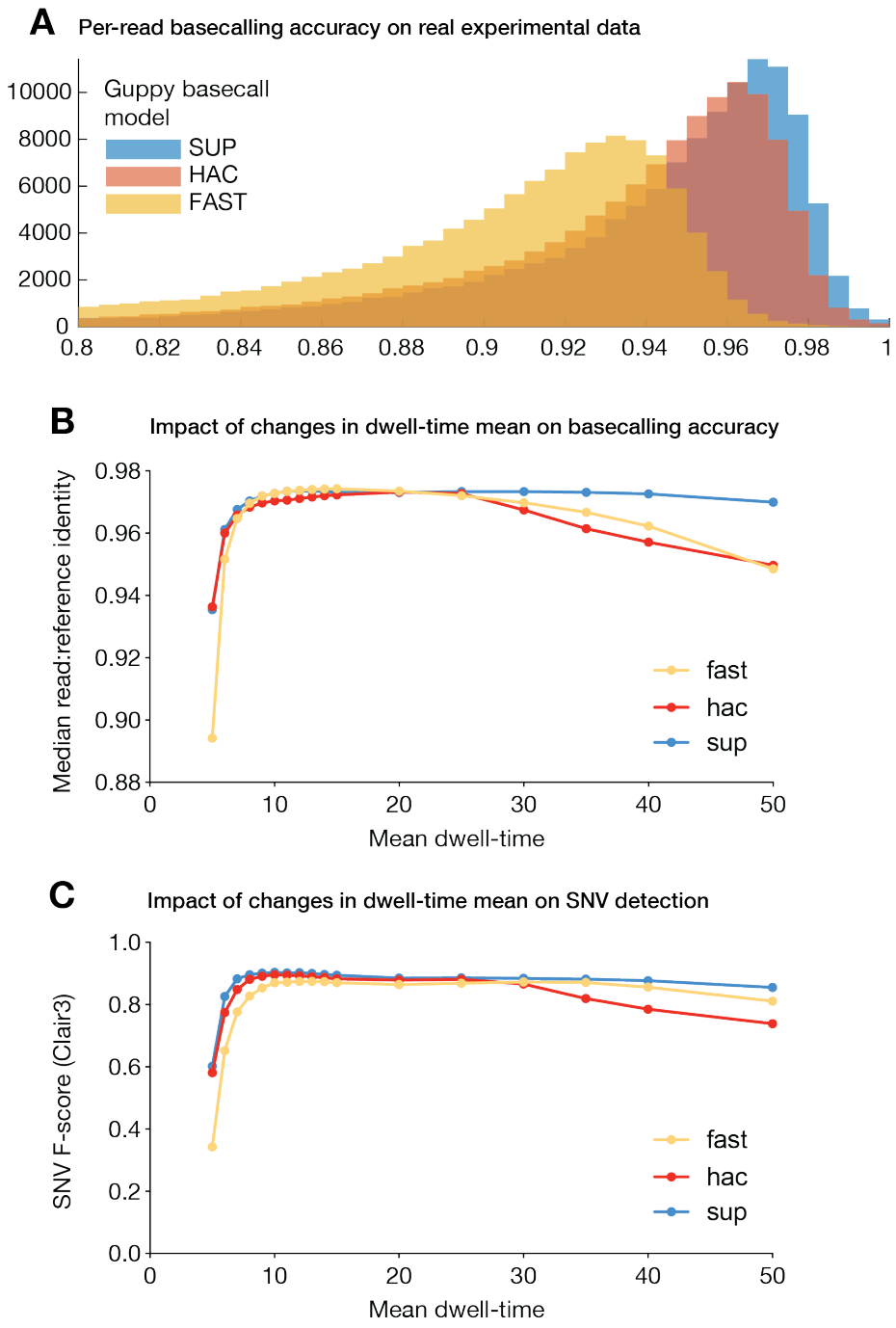
	Squigulator	**Deep Simulator (context independent)**	**Deep Simulator (context dependent)**
Execution time	156.194 seconds	3939.58 seconds	130.8 hours
Peak RAM usage	0.477 GB	2.117 GB	49.39 GB



Supplemental FigS1. Comparison of Squigulator and DeepSimulator to real experimental ONT data. Genome browser view shows basecalled reads (Guppy SUP model) aligned to the human reference genome (hg38). For each view, the top track shows real experimental data from ONT sequencing of NA12878 genomic DNA (R9.4.1 PromethION flow cells). The middle track shows simulated NA12878 data from Squigulator with *-x dna-r9-prom* pre-set configuration. The bottom track shows simulated NA12878 data from DeepSimulator running in context-independent mode. Blue triangle markers show the location of NA12878 SNVs that were incorporated into the simulation, and are correctly detected by Clair3. Red triangle markers show the presence of reproducible errors in basecalled reads from DeepSimulator, which are erroneously detected as SNVs by Clair3. Purple triangle markers show the presence of reproducible errors in basecalled reads from Squigulator and experimental data, which are erroneously detected as SNVs by Clair3. (**A**) Shows examples of false-positive SNVs in DeepSimulator data, which are not apparent in real experimental data, nor Squigulator data. (**B**) shows examples of false-positive SNVs that are apparent in real experimental data and Squigulator data, but not present DeepSimulator data.



Supplemental FigS2. Comparison of SNV detection between Squigulator, DeepSimulator and Badread. (**A**) ROC curves evaluate accuracy of SNV detection with Clair3 with real experimental NA12878 dataset (orange) vs simulated data from Squigulator (red), DeepSimulator (purple) or Badread (green). (**B**) ROC curves evaluate concordance of SNVs detected with real experimental NA12878 dataset vs simulated data from Squigulator (red), DeepSimulator (purple) or Badread (green). Left vertical axes in ROC curves show absolute numbers of detected SNVs and right vertical axes show fraction of true-positives detected (i.e. recall or sensitivity).



Supplemental FigS3. Parameter exploration regarding Guppy basecalling sequencing accuracy. (**A**) Guppy basecalling accuracy, as measured by read:reference identity score distributions, on real experimental NA12878 data with Guppy's FAST, HAC or SUP models. (**B**) Guppy basecalling accuracy, as measured by read:reference identity score medians, for repeated experiments in which the mean dwell time (--dwell-mean) is varied, while other parameters are held at default. Experiment was repeated with FAST, HAC and SUP basecalling models. Default value --dwell-mean=9 (for R9.4.1 flow cell). (**C**) Accuracy of SNV detection, as measured by F-score, by Clair3 on the same datasets and basecalling models as above (colours are matched).