Supplementary Information

Comprehensive identification of diverse ribosomal RNA modifications by targeted Nanopore direct RNA sequencing and JACUSA2

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Supplementary Table 1: Oligonucleotides for IVT generation and Nanopore sequencing

18S		
	Primers for IVT generation	TAATACGACTCACTATAGTACCTGGTTGATCCTGCCAG (T7-18S fw) TAATGATCCTTCCGCAGGTTC (18S rv)
	ONT adapter	GAGGCGAGCGGTCAATTTTCCTAAGAGCAAGAAGAAGCCtaatgatcct (18S v1 oligo B) GAGGCGAGCGGTCAATTTTCCTAAGAGCAAGAAGAAGCCtaatgatcct tccgcaggtt (18S v2 oligo B)
universal		
	ONT adapter	/5PHOS/GGCTTCTTCTTGCTCTTAGGTAGTAGGTTC (oligo A)

Supplementary Table 2: Oligonucleotides for CRISPR-Cas9 cell line generation and validation

DIMT1L-Y131G		
	Donor DNA	TGAAAACAGATTTGCCATTCTTTGATACTTGTGTGGCAAATTTGCCTGGGCAGGTATGTCCTCACATTTTCAGGAACATCATACTAACTGTTCCTCTGAT (ssDL003)
	Guide RNA	ATGTGAGGACATACCTGATA (crDL049)
	Diagnostic PCR	GCTATGTTCACCACCTGAACTG (LD4372) GGTTGTTGCTTGTGAACTTGACC (LD4373)
WBSCR22-D82K		
	Donor DNA	CTGAGTGGAAGTTATCTGTCAGATGAAGGGCACTATTGGGTGGGACTGAAAATCAGCCCTGCCATGCTGGGTAAGTATGTCCTGTCTGGCACCAGGGTGG (ssDL001)
	Guide RNA	GCATGGCAGGGCTGATATCC (crDL026)
	Diagnostic PCR	GAACTCCTTTACCATGTCC (LD4067) GCAGGAATTAAAGACCCTC (LD4068)
SNORD13 KO		
	Guide RNAs	AGAATGGATGTATCGCATTA (crDL051) TAAATCGATCCTTGAAGTTC (crDL052)
	Diagnostic PCR	AATCACAGAATCTCAGTGGG (LD4467) AAACTAGGCCACCTGTTATC (LD4468)
	Northern blot probe	GCCCACGTCGTAACAAGGTTCAAGGGTGGC (LD2684)

Supplementary Table 3: Sequence of oligonucleotides used for detection of 18S rRNA modifications

Primer extension		
	m^6_2A m^6_2A and ac^4C detection on 18S rRNA helix 45	CGAGCGAGCGAACGAACGGGC (LD2141)
	Primer extension for m^7G detection on 18S rRNA	GTACAAAGGGCAGGGACTTAATC (LD2120)
Misincorporation Assay		
	18S_H45_rev (used as RT primer)	TAATGATCCTTCCGCAGGTTCACCTAC
	18S_H45_fwd (used for Sanger sequencing)	CGTCGCTACTACCGATTGGATGG

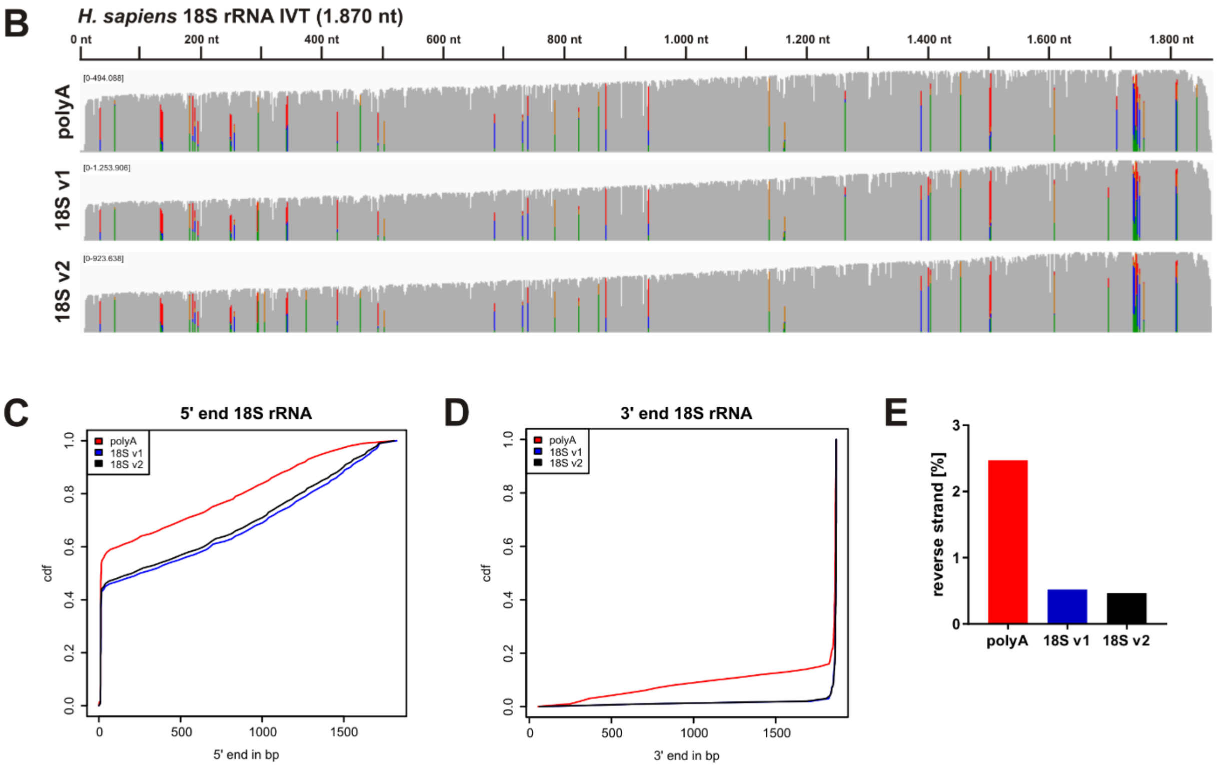
Supplementary Table 4: Run time of tools in rRNA benchmark

tool	threads	rep	time_1 [s]	time_2 [s]	time_3 [s]	mean [s]	sd
EpiNano DiffErr	1	1	70.66	66.17	67.18	68.01	1.92
JACUSA2 scores	1	1	**28.06**	**29.47**	**28.34**	28.62	0.61
JACUSA2 scores	1	123	49.22	**44.98**	45.12	46.44	1.97
EpiNano DiffErr	8	1	55.26	54.36	53.08	54.24	0.89
JACUSA2 scores	8	1	88.92	88.41	88.13	88.49	0.33
JACUSA2 scores	8	123	**48.72**	45.33	**44.15**	46.06	1.94
Eligos2	8	1	147.28	149.52	168.51	155.10	9.52
Nanocompore	8	1	1306.03	1275.48	1243.38	1274.96	25.58
Nanocompore	8	123	4308.22	4230.77	4173.13	4237.37	55.35
xPore	8	1	653.60	621.96	616.46	630.67	16.37
xPore	8	123	2191.97	2171.71	2177.88	2180.52	8.48

Supplementary Figure 1

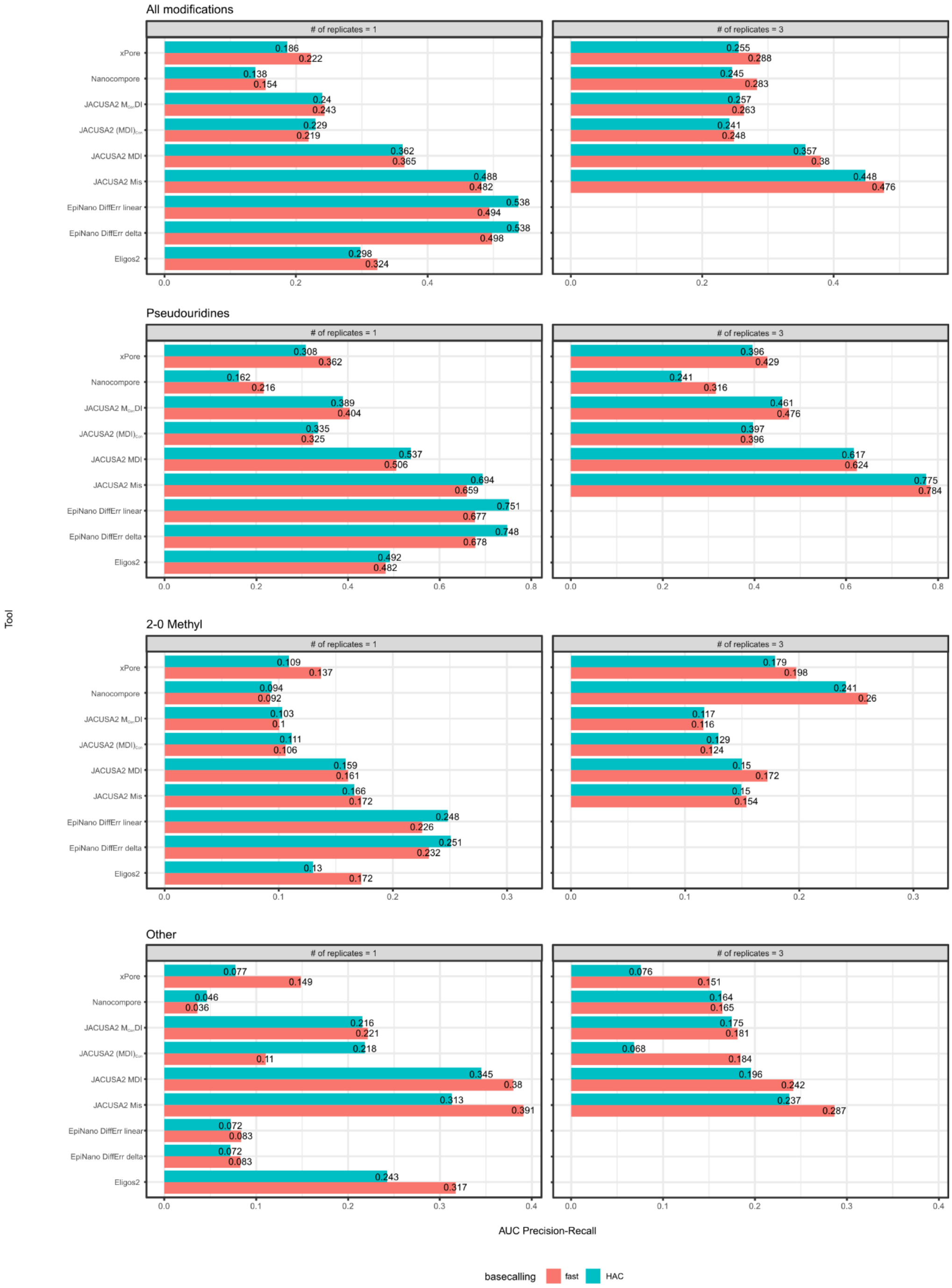
A

Sample	Available pores	K pass reads	Median read length (bp)	N50 – Pass (bp)	Median PHRED Score
18S polyA	920	662.5	1400	1760	9.046
18S v1	1642	1324.3	1480	1800	9.093
18S v2	1303	970.0	1550	1790	9.068



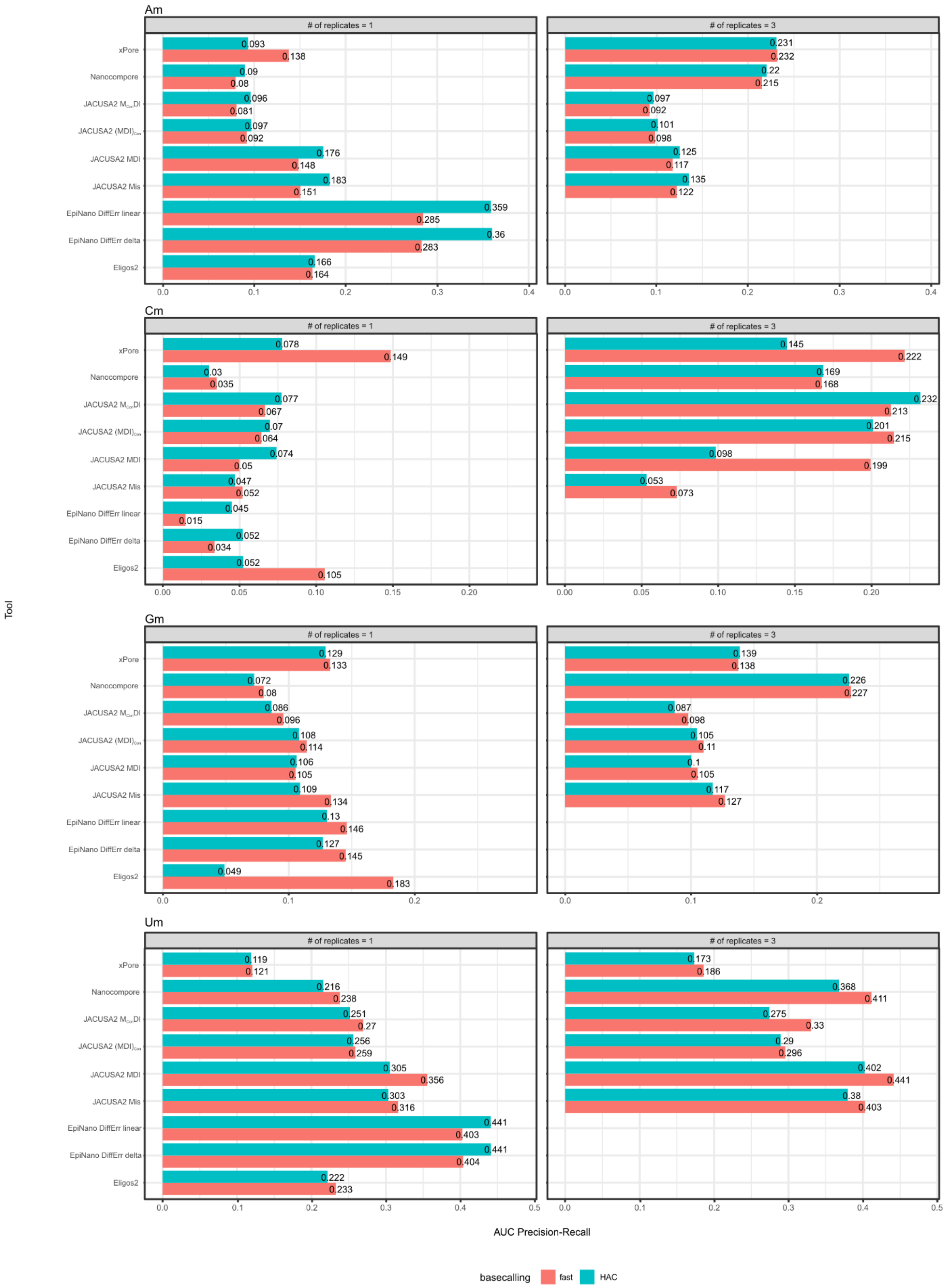
<span style="color:#00aeef">**Supplementary Figure 1**</span> A) Read statistics of Nanopore direct RNA-seq of unmodified 18S *in vitro* transcripts (IVT) with the standard oligo(dT) adapter (after polyadenylation of the IVT), 18S v1 (20-nts long) and 18S v2 (10-nts long) specific adapters on MinION R9.4.1 flow cells. B) Coverage of 18S IVT from IGV snapshots of the sequencing runs listed in A). Allele frequency threshold = 0.2. C) Cumulative distributive function (cdf) of the 5' read ends of the 18S rRNA IVT sequenced with either of the three adapters, as indicated. D) Cumulative distributive function (cdf) of the 3' read ends of the 18S rRNA IVT sequenced with either of the three adapters, as indicated. E) Proportion of reads mapping to the reverse strand for 18S IVT sequencing runs.

Supplementary Figure 2



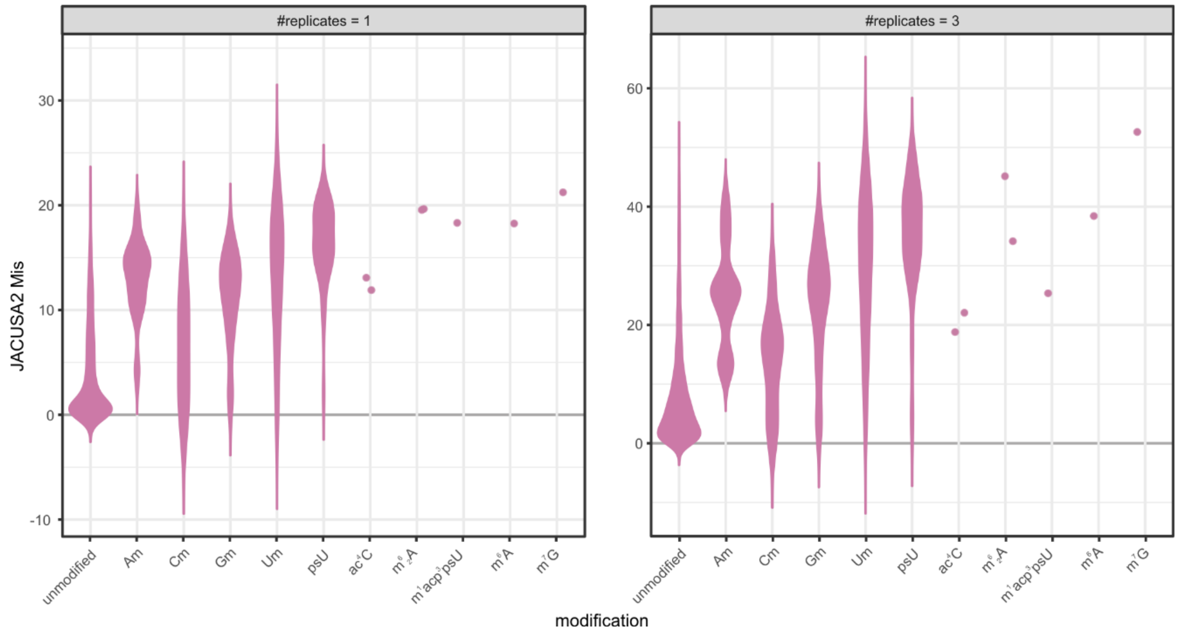
Supplementary Figure 2 Benchmark of tools designed for detection of RNA modifications on human 18S rRNA derived from HCT116 cells compared to an 18S IVT. All data sets were downsampled to 5000 reads per sample and basecalled using either the fast (red) or high accuracy (HAC, blue) basecalling mode. Left panels: analysis of a single replicate, right panels: analysis of three replicates. For EpiNano and Eligos2 a replicate analysis is not possible. Panel 1: AUC of a precision-recall analysis for all modifications. Panel 2: AUC of a precision-recall analysis for pseudouridine sites. Panel 3: AUC of a precision-recall analysis for 2’-O-methylation of ribose (Nm) sites. Panel 4: AUC of a precision-recall analysis for all other modifications found on the 18S rRNA (ac^{4}C, m^{1}acp^{3}psU, m^{6}A, m$^6_2$A and m^{7}G).

Supplementary Figure 3



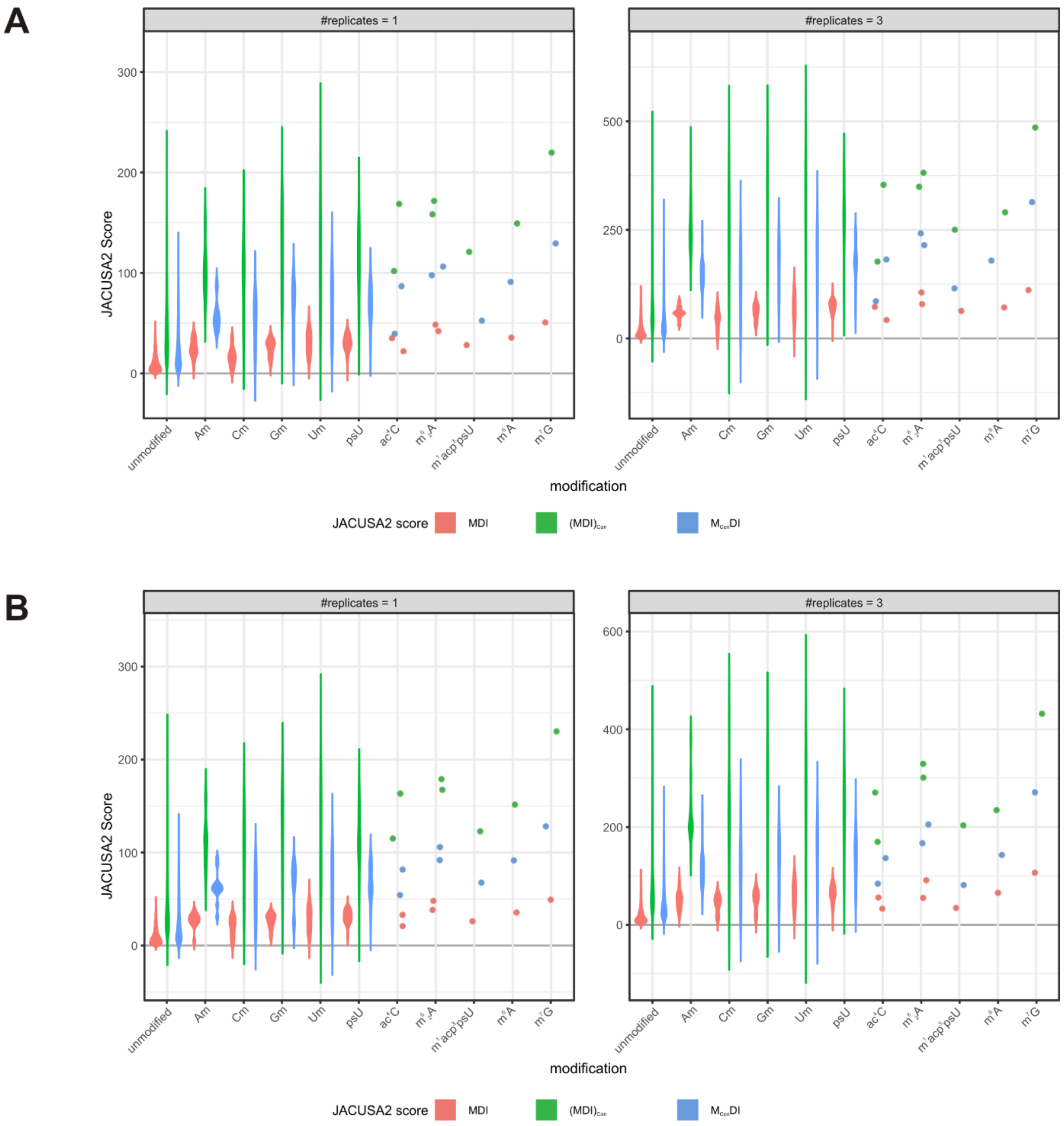
Supplementary Figure 3 Stratification of 2’-O-ribose methylation sites. Panel 1: AUC of a precision-recall analysis for Am modifications. Panel 2: AUC of a precision-recall analysis for Cm modifications. Panel 3: AUC of a precision-recall analysis for Gm modifications. Panel 4: AUC of a precision-recall analysis for Um modifications.

Supplementary Figure 4



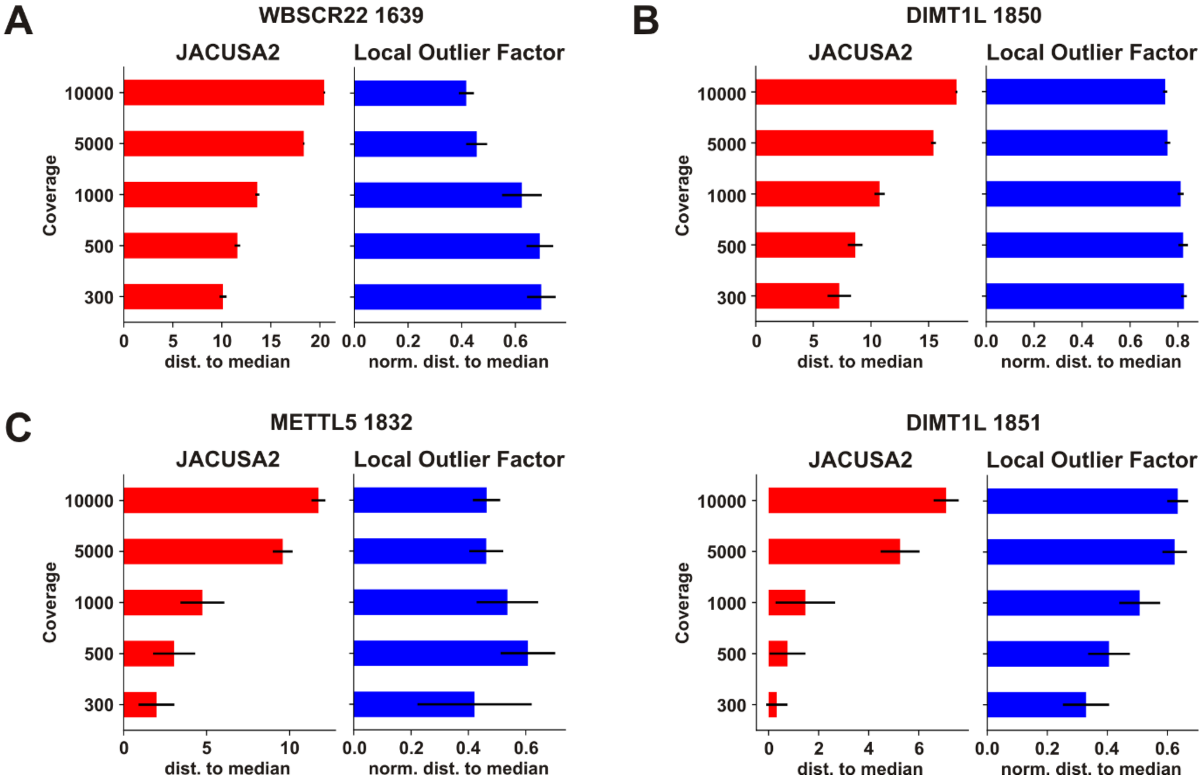
Supplementary Figure 4 Violin plot summarizing the JACUSA2 call-2 analysis of the 18S rRNA from HCT116 WT cells and 18S IVT. Shown is the JACUSA Mis score for all modification types on HAC basecalled data as indicated. Left panel: analysis of a single replicate, right panel: analysis of three replicates.

Supplementary Figure 5



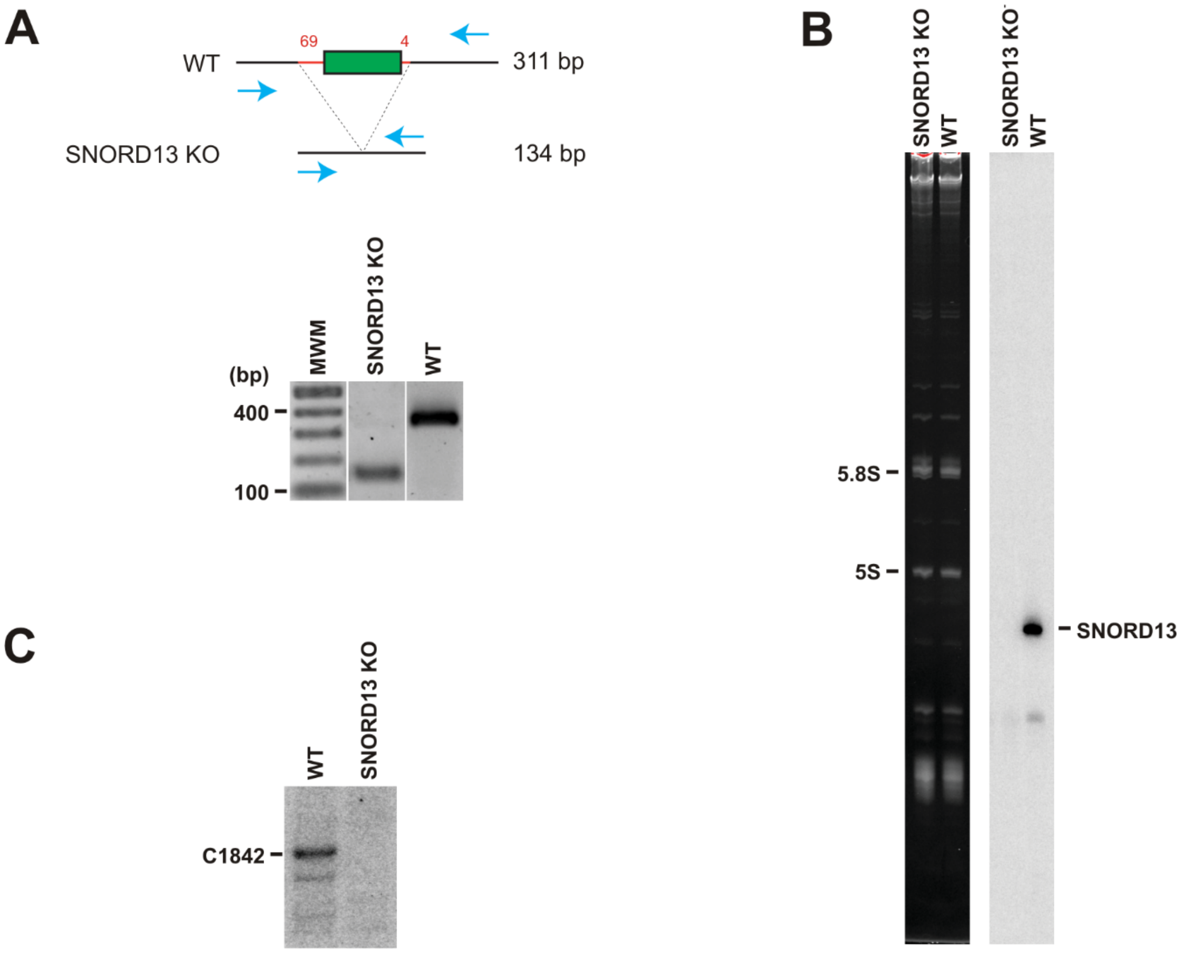
Supplementary Figure 5 A) Violin plot summarizing the JACUSA2 call-2 analysis of the 18S rRNA from HCT116 WT cells and 18S IVT. Shown are the JACUSA MDI, $(\mathrm{MDI})_{\mathrm{Con}}$ and $\mathrm{M_{Con}DI}$ scores for all modification types on fast basecalled data as indicated. Left panel: analysis of a single replicate, right panel: analysis of three replicates. B) Violin plots as in A on HAC basecalled data.

Supplementary Figure 6



Supplementary Figure 6 Detection of rRNA modifications with different read numbers. Indicated read numbers were sampled from MinION sequencing data and analyzed by JACUSA2 in pairwise comparisons. A) Downsampling analysis of WBSCR22-catalyzed m^7G_{1639}. B) Downsampling analysis of METTL5-catalyzed m^6A_{1832}. C) Downsampling analysis of DIMT1L catalyzed $m^6_2A_{1850}$. D) Downsampling analysis of DIMT1L catalyzed $m^6_2A_{1851}$. Left panels: Distance of the JACUSA2 Mis score for the respective target site to the median JACUSA2 score for the WT versus KO/MUT comparison. Right panels: normalized distance of the LOF score for the target site to the median LOF score. Shown are the mean and the standard deviation from down sampling employing different seeds (n = 15).

Supplementary Figure 7



Supplementary Figure 7 Generation of the HCT116 SNORD13 KO cell line. A) The gene encoding the snoRNA U13 (SNORD13) was removed from both alleles of HCT116 cells by use of two CRISPR-Cas9 RNP complexes, one on each side of the gene. The entire sequence encoding SNORD13 was removed with an additional 69 nts upstream and 4 nts downstream. The deletion was diagnosed by differential PCR, and by DNA sequencing of the targeted genomic region (not shown). B) The loss of SNORD13 was demonstrated by northern blotting (probe: LD2684). Left panel, ethidium-bromide staining used to control loading. C) Loss of ac^4C_{1842} on helix 45 of the 18S rRNA was assessed by primer extension following $NaBH_4$ treatment, as described with oligonucleotide LD2141.

Supplementary text: rRNA Benchmark

Data and Processing

FAST5 files for HCT116 WT1-3 and IVT1-3 runs have been base called with Guppy 5.0.11 with fast and high accuracy mode. Subsequently, FASTQ files have been mapped with minimap2 (v2.22) to the 18S and 28S reference sequences.

Mapped BAM files have been filtered with “samtools -q 10 -F 2323” and only reads mapping to 18S rRNA have been retained for further evaluation.

To account for read coverage differences between runs, mapped BAM files have been converted with samtools to FASTQ file format and 5000 reads have been sampled with seqtk (v1.3-r106) by executing: “seqtk sample all_reads.fastq 5000 > 5000reads.fastq”. Finally, reads have been mapped as mentioned above.

Tools

The following tools have been considered for comparison:

Eligos2

Eligos v2.1.0 has been retrieved from the repository https://gitlab.com/piroonj/eligos2 and a docker container has been built and used with singularity as described in the documentation.

We used “eligos2 pair_diff_mod” to call modification sites:

```
eligos2 pair_diff_mod \
-tbam $WT_BAM -cbam $KO_BAM \
-reg $BED -ref $REF -t $THREADS -o results \
--max_depth 2000000 --min_depty 5 –esb 0 –oddR 1 –-pval 1
```

As suggested, when the odds-ratio is < 1, the adjusted p-value has been set to 1. A score has been created from the adjusted p-value by utilizing -log10 values.

EpiNano DiffErr

Epinano v1.2.1 has been installed from the repository https://github.com/novoalab/EpiNano. Epinano DiffErr does not support replicates but offers two modes (linear and delta) to identify RNA modification between a wild type and a control condition.

We followed the procedure documented in the examples in the repository to preprocess BAM files when using the sum of errors feature.

Site weres detected with:

```
 Rscript --vanilla $EPINANO/Epinano_DiffErr.R \
--coverage 5 \
--wt_sample $WT_EPINANO --ko_sample $KO_EPINANO\
--out_prefix $OUT_PREFIX
--feature sum_err
```

DiffErr results have been transformed to scores (1 - p-value) where greater values indicate the presence of a modification.

Nanocompore

nanocompore v1.0.4 has been installed in a conda environment.

Data preprocessing has been carried out following the instructions in https://nanocompore.rna.rocks/data_preparation.

The following command was used to identify sites:

```
nanocompore sampcomp \
--file_list1 $WT_COLLAPSE --file_list2 $KO_COLLAPSE \
--label1 wt --label2 ko \
--fasta $REF \
--outpath $OUTPUT_DIR/nanocompore \
--min_coverage 5 \
--allow_warnings \
--nthreads $THREADS
```

The p-value from statistical test results has been converted to a score by -log(p).

JACUSA2

JACUSA2 v2.0.1 has been downloaded from the repository https://github.com/dieterich-lab/JACUSA2.

The following command was used to identify sites:

```
jacusa2 call-2 \
-p $THREADS \
-q 0 -c 5 \
-D -I \
-r $RESULT \
$WT_BAMS $KO_BAMS
```

A custom R script was used to parse JACUSA2 results and create auxiliary scores: Mis+Del+Ins, MisContext+Del+Ins, and Mis+Del+Ins_Context.

The test score was extracted from the results and no transformations were carried out.

xPore

xpore v2.1 has been installed in a conda environment.

A customized $CONFIG with min reads = 5 and max reads 2000000 was used and the following command was executed to identify modifications:

```
xpore diffmod --config $CONFIG --n_processes $THREADS
```

The p-value from statistical test results has been converted to a score by -log(p).

Evaluation

All results from the tested tools have been converted to a common BED file format representation. Predictions have been merged and compared with a custom R script against known 18S rRNA modifications. The area under the precision recall curve has been used to compare the performance of different tools.

Running time comparison

Running times were captured using the features of snakemake to account for variance in running time, each tool has been run 3 times. Running time has been measured of all necessary preprocessing and actual identification steps. EpiNano and JACUSA2 scores were run on 1 and 8 threads, for all other tools only 8 threads were feasible.