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SQANTI-reads: a tool for the quality assessment of long read data in multi-sample lrRNA-seq experiments.

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ABSTRACT

SQANTI-reads leverages SQANTI3, a tool for the analysis of the quality of transcript models, to develop a quality control protocol for replicated long-read RNA-seq experiments. The number/distribution of reads, as well as the number/distribution of unique junction chains (transcript splicing patterns), in SQANTI3 structural categories are compiled. Multi-sample visualizations of QC metrics can also be separated by experimental design factors. We introduce new metrics for 1) the identification of potentially under-annotated genes and putative novel transcripts and 2) variation in junction donors and acceptors. All scripts are open source and customizable. Using two different datasets, one from *Drosophila* and one benchmark dataset from the LRGASP project, we demonstrate how low coverage does not automatically indicate low quality and how strong/weak splicing sites can be readily identified genome wide. SQANTI-reads is open source and available for download at GitHub.

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INTRODUCTION

Short-read RNA sequencing (srRNA-seq) is the most common and cost-effective approach for studying the transcriptome. However, in srRNA-seq, transcripts must be inferred computationally, which can lead to inaccuracies in transcript identification (Liu et al. 2016; Newman et al. 2018). Recent advances in single-molecule long-read sequencing technologies have opened new avenues for transcriptome analysis (reviewed in (Marx 2023; van Dijk et al. 2023)). In long-read RNA sequencing (lrRNA-seq), full-length transcripts can be observed as single sequencing reads, allowing for direct transcript detection without the need for an assembly step. However, like any technology, lrRNA-seq is not without errors, and factors such as mRNA degradation, library preparation failures, and sequencing errors can introduce biases into the data.

A database tracking bioinformatic tools for long-read sequencing (Amarasinghe et al. 2021) identifies numerous tools for the initial processing of lrRNA-seq data to assess the accuracy of base-calling and the length of the reads (e.g., pycoQC (Leger and Leonardi 2019), longQC (Fukasawa et al. 2020), nanoQC (De Coster et al. 2018)), critical first steps in evaluating lrRNA-seq read quality. Other tools, such as SQANTI3 (Pardo-Palacios et al. 2024a), TALON (Wyman et al. 2020), FLAME (Holmqvist et al. 2021), IsoSeq (https://isoseq.how/), and IsoTools (Lienhard et al. 2023), focus on evaluating consensus transcript models inferred from the data. However, most current tools for lrRNA-seq read quality control were developed during the early stages of these technologies and are generally limited in the number of evaluated features and/or samples. As long-read sequencing technologies rapidly evolve, improving both in quality and experimental scope, the need for comprehensive and comparative read quality assessment becomes increasingly critical.

The rapid decline in costs implies that the use of lrRNA-seq will continue to expand, with experimental designs involving multiple samples becoming more common (e.g. (Glinos et al.

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2022; Joglekar et al. 2024; Mahmoud et al. 2024; Patowary et al. 2024)). From a quality control perspective, this necessitates that datasets be homogeneous, without biases associated with experimental groups, and free of outliers. Moreover, the generated data must be sufficient to address the research questions that motivated the experiment. The increase in throughput now makes it possible to design experiments that include barcoding and multiplexing to balance library preparation and sequencing across experimental groups (Auer and Doerge 2010). This approach helps avoid confounding technical variation with the treatments of interest and facilitates the identification of failed technical replicates versus failed samples. Finally, technological advancements such as more accurate basecallers (https://github.com/nanoporetech/dorado) and novel library preparation methods (e.g. MAS-Iso-Seq (Al'Khafaji et al. 2024), CapTrap(Carbonell-Sala et al. 2024), and R2C2(Volden et al. 2018), Nano3P-seq (Begik et al. 2023) FLAM-seq(Legnini et al. 2019)) require tools that can easily evaluate how these improvements impact various aspects of data quality.

In this context, we present SQANTI-reads, an extension of SQANTI3 (Pardo-Palacios et al. 2024a), a tool originally designed for transcript model quality control, to jointly provide quality control metrics for long read data and to analyze multiple samples for consistency and bias. We demonstrate that SQANTI3's structural categories and other quality control metrics, repurposed in SQANTI-reads, are highly effective for assessing the consistency of a lrRNA-seq multi-sample experiment, identifying read quality control failures, and detecting outliers. Additionally, we have developed new metrics that provide insights into the potential utility and discovery power of the data, including variation at donor/acceptor sites and identification of potentially under-annotated genes and mis-annotated transcripts. SQANTI-reads offers an extensive array of summary output tables, is customizable to accommodate any experimental design, and is available as an open-source, freely accessible tool.

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METHODS

SQANTI-reads basics

SQANTI-reads is an adaptation of SQANTI3 designed to evaluate individual reads rather than transcript models. It allows for the comparison of multiple samples, providing quality control results across the entire experiment. Several new features have been introduced to address the specific needs of QC in multi-sample experiments, while some functionalities of SQANTI3 have been removed as they are not applicable to read-level processing. Table 1 highlights the major differences between SQANTI3 and SQANTI-reads, emphasizing the new features of SQANTI-reads, while Table 2 lists the names and descriptions of the output files.

Table 1. Comparison between SQANTI3 and SQANTI-reads

Feature	**SQANTI3**	**SQANTI-reads**
Sequences analyzed	transcript models	reads
Annotation with SQANTI3 categories	yes, for transcript models	yes, for reads and UJCs
Computation of SQANTI3 quality metrics	yes	yes
Samples processed	One	Multiple
Visualizations across samples	no	yes
PCA analysis between samples	no	yes
Summary of read counts (per gene, per UJC)	no	yes
Donor/acceptor variation metrics	no	yes
Identification of putative under annotated genes	no	yes
Identification of putative novel transcripts	no	yes
Machine learning validation of transcript models	yes	no
IsoAnnot annotation	yes	no

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Table 2. SQANTI-reads specific output files

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Output file	Description	Default output	Multiple Samples/ summary file
gene_counts.csv	Provides the number of reads in each structural category, per gene and per sample	Yes	Multiple samples
ujc_counts.csv	Provides a list of junction hashes in each sample and the number of reads in each sample associated with each junction string Flags the most expressed UJC per gene	Yes	Multiple samples
length_summary.csv	Provides the number and percentage of reads in length categories per sample	Yes	Multiple samples
cv.csv	Provides metrics on the coefficient of variance of reference junctions for each sample	Yes	Multiple samples
jxn_counts.csv	Provides the number of known canonical, novel canonical, known non-canonical, and novel non-canonical junctions in reads of each sample	- - all-tables	Multiple samples
cv_acc_counts.csv cv_don_counts.csv	Provides the number of detected annotated donors and acceptors in each junction variation category	- - all-tables	Multiple samples
FSM_counts.csv ISM_counts.csv NIC_NNC_counts.csv	Provides the number of reads in each subcategory for FSMs, ISMs, NICs, and NNCs	- - all-tables	Multiple samples
err_counts.csv	Provides the number and percentage of reads with evidence of intrapriming, RT-switching, and non-canonical junctions per samples	- - all-tables	Multiple samples

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pca_loadings.csv	Gives loadings of PC1 and PC2 for the PCA analysis	–pca-tables	Summary
pca_variance.csv	Gives the variance explained by each PC	–pca-tables	Summary
gene_classification.csv	For genes with coverage meeting a user-defined threshold, provides the annotation category of each gene	Yes	Summary
putative_underannotation.csv	Provides metrics on NIC and NNC UJCs and flags putative novel transcripts	Yes	Summary

The input files for SQANTI-reads include: 1) a GTF file of read alignments, 2) a reference genome FASTA file, 3) a GTF file of the reference transcript model annotation, and 4) a design file containing metadata for multiple samples. The first step of SQANTI-reads involves using the SQANTI3-QC module to generate SQANTI3-like classification and junction files, with the classification file containing one row for each mapped read. Reads are classified according to the SQANTI categories (Tardaguila et al. 2018) as full-splice match (FSM), incomplete-splice match (ISM), novel-in-catalog (NIC), novel-not-in-catalog (NNC), antisense, fusion, genic genomic, and intergenic. SQANTI3 subcategories are also included, based on 5' and 3' end positions relative to the annotated transcription start sites (TSS) and transcription termination sites (TTS) (Pardo-Palacios et al. 2024a). Additionally, the reverse transcriptase (RT) switching algorithm of SQANTI3-QC identifies reads with evidence of RT switching events, while reads with more than 60% adenines in the 20 bp downstream of the reported TTS at the genomic level are flagged as potential intrapriming events. The length of each read and the number of exons in each read are also recorded in the classification file.

Junction Metrics

The SQANTI-reads junction file follows the same format as the SQANTI3 junction file, with each row representing a junction in a read, including the start and end positions of the junction. The

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distance from the junction start and end to the nearest annotated junction start and end in the reference GTF is calculated. It's important to note that the nearest annotated start and end positions may not belong to the same annotated junction. SQANTI classifies junctions as known or novel, and as canonical or non-canonical, based on the dinucleotide pairs at the junction's start and end. By default, dinucleotide combinations of GT-AG, GC-AG, and AT-AC are considered canonical, while any other combinations are classified as non-canonical, although the user can specify additional canonical sites.

SQANTI-reads introduces new metrics to evaluate the relationship between the junctions in mapped reads and the annotated donors and acceptors. In the SQANTI3-QC junction file, the distance from each donor/acceptor in each read to the nearest annotated donor/acceptor is recorded. In SQANTI-reads, the mean absolute distance in nucleotides from the annotated donor/acceptor site, the standard deviation, and the coefficient of variation (CV = standard deviation/mean) are calculated and included in the cv.csv file. Each detected junction is classified as 1) Reference Match junction if the mean distance and the standard deviation to an annotated junction are both equal to 0; 2) CV = 0 Junction when the mean distance is greater than 0 and the standard deviation equals 0, and 3) CV > 0 Junction when the CV is greater than 0.

Unique Junction Chain and gene-level information

SQANTI-reads groups mapped reads based on their junctions, referred to as Unique Junction Chains (UJCs). Each UJC is labeled with a string that includes the chromosome and junction coordinates (Nanni et al. 2024). To enhance computational efficiency, UJC strings are encoded as an index in a hash table (JxnHash). The read count for each JxnHash is calculated and included in the ujc_counts.csv file. Additionally, the number of known canonical, known non-canonical, novel canonical, and novel non-canonical junctions within each UJC is annotated, along with the SQANTI structural category of the UJC. The number of reads within each structural category for each gene, as well as the total number of reads per gene, is stored in the summary file gene_counts.csv.

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Identifying genes that may be under-annotated and transcripts that may be mis-annotated

For expressed genes, a high proportion of reads from a UJC classified as NIC/NNC may indicate the presence of a potentially novel transcript. SQANTI-reads includes a customizable pipeline to identify genes with such potential under-annotation events. The procedure identifies NIC/NNC UJCs that meet a minimum number (R) and proportion (P) of reads, with default values set at 100 reads and 20%, respectively. To mitigate the risk that the NIC/NNC UJC is merely a degradation product, an additional condition is applied: the candidate UJC must include at least 80% of the gene's junctions (Q) (Figure 3A). The R, P, and Q thresholds are pipeline parameters that can be adjusted by the user. Furthermore, SQANTI-reads allows for the evaluation of under-annotated genes and novel transcripts within a specific subset of samples associated with a particular experimental factor (e.g., developmental stage or technology) using the --factor-level option.

Multisample processing

SQANTI-reads processes multiple samples to generate classification and junction files when a design file (Supplementary file 1) is provided to the sqanti-reads.py command. If individual samples have already been pre-processed with SQANTI3-QC, SQANTI-reads can be run in --fast mode, where the design file links the individual classification and junction files to sample IDs for the calculation of SQANTI-reads metrics, summaries, and a series of visualizations. If pre-processing has not been done, SQANTI-reads is run in --simple mode where SQANTI3 is run on each sample, followed by the calculation of SQANTI3 metrics and summaries. The output also includes a summary for each sample, reporting the mean, median, upper quartile, and lower quartile of mapped read length, as well as the number and proportion of reads that are shorter than 1 kb, between 1 and 2 kb, between 2 and 3 kb, and greater than 3 kb in length, all of which are included in the length_summary.csv file.

***Drosophila melanogaster* data**

A total of 24 samples corresponding to 2 developmental stages (0-1 hours and 3-8 hours post-hatching) and four genotypes (dmel 11037, 11255, 12272 and 12279) (3 samples per

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experimental condition) were sequenced using Oxford Nanopore Technology (ONT). One barcoded cDNA library was built per sample, the 12 samples from the 0-1 hour and the 12 samples from the 3-8 day stages were pooled and sequenced on a MinION. Data were evaluated with PycoQC (Leger and Leonardi 2019) which focuses on read length and base quality. All samples passed this basic QC, libraries were re-pooled and run on the PromethION. Detailed metadata for these samples are provided in Supplementary Table 1. Raw electrical data were processed by default basecallers on the machines during the run (Guppy). Samples were stored in .fast5 format and as .fastq files. The .fast5 files were converted to the Dorado compatible .pod5 format using pod5 (v 0.3.6) and then processed in Dorado (v 0.5.2) (https://github.com/nanoporetech/dorado) using options --recursive --device "cuda:0,1" --kit-name SQK-PCB109 --trim none. Reads were demultiplexed using the demux mode of Dorado (v 0.5.2) with options --no-classify --emit-fastq resulting in separate Dorado fastq files for each sample. The fastq files generated by Guppy/Dorado were both processed using pychopper (v 2.7.1), the oriented fastq files were aligned to *D. melanogaster* 6.50 and the resulting sam files were converted to gtf using samtools (v 1.10) (Li et al. 2009) and bedtools (v 2.29.2) (Quinlan and Hall 2010). The resulting gtf files (67 technical replicates from 24 samples), the *D. melanogaster* 6.50 fasta reference file (https://ftp.flybase.net/releases/FB2023_01/dmel_r6.50/ (Öztürk-Çolak et al. 2024)), and a design file (Supplementary File 2) were used as input to SQANTI-reads. The *Drosophila* dataset includes, therefore, two experimental conditions (time and genotype) and two technical conditions (sequencing platform and base caller) with the experimental samples multiplexed and evaluated with both technical conditions. The SQANTI-reads output for this dataset is provided in Supplementary File 3.

Human Cell line WTC11

We used publicly available lrRNA-seq data from the Long-read RNA-seq Genome Annotation Assessment Project (Pardo-Palacios et al. 2024b) to illustrate the utility of SQANTI-reads. Specifically, we used triplicate measurements of the transcriptome of the WTC11 human cell line that were profiled by cDNA PacBio Sequel II, cDNA Oxford Nanopore Minion, and direct

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RNA Oxford Nanopore Minion long reads methods. Data were downloaded from the ENCODE website (https://www.encodeproject.org/search/?type=Experiment&internal_tags=LRGASP). Accession numbers for these samples are provided in Supplementary Table 2. The fastq files were pre-processed by LRGASP researchers as described in (Pardo-Palacios et al. 2024b). We used the gtf files of read alignments, GENCODE's GRCh38.p13 reference genome gtf and fasta for release 38 (https://www.gencodegenes.org/human/release_38.html), and a design file (Supplementary file 4) to run SQANTI-reads on the WTC11 samples. The SQANTI-reads output is provided in Supplementary File 5.

RESULTS

SQANTI-reads can be used to evaluate technology for a common set of libraries

Our *Drosophila* experimental design serves as an excellent example to evaluate the technological aspects of long-read methods using SQANTI-reads. One of the notable advancements in Oxford Nanopore Technology (ONT) is the introduction of the new basecaller, Dorado (https://github.com/nanoporetech/dorado), which claimed to significantly improve base call accuracy. A robust long-read QC tool should be able to clearly identify improvements in technology. Therefore, we first compared the Guppy and Dorado basecallers using SQANTI-reads. As anticipated, Dorado resulted in more reads with assignable barcodes, a higher number of mapped reads, more reads aligning to annotated genes, and more reads aligning to annotated transcripts, without an increase in the proportion of reads with technical artifacts (Supplementary Figure 1, Supplementary file 2). This confirms that Dorado improves base-calling accuracy without introducing unwanted biases. Based on these SQANTI-reads QC results we chose to move forward with Dorado basecalled reads.

In the *Drosophila* experiment, libraries were barcoded, pooled, and multiplexed across different MinION and PromethION runs, with a re-pooling step between the two machines. We used SQANTI-reads to compare the quality of the MinION and the PromethION runs, and to evaluate the consistency of the PromethION technology across technical replicates. The first MinION run

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(TR1) had higher percentages of reads with NIC/NNC and with non-canonical junctions compared to the second and third technical MinION runs (TR2,TR3) and compared to the PromethION runs for the same libraries (Supplementary Figure 2). The other MinION runs (TR2, TR3) were similar in their quality metrics (described below) to the PromethION run of the same samples, and the technical replicates of the 3-8 day libraries on the PromethION were similar. These results indicate that the technology performs consistently across instruments and runs. Based on these SQANTI-reads QC results we aggregated data across technical replicates to further evaluate the quality of the lrRNA-seq experiment.

SQANTI-reads metrics can be used to evaluate the global quality of the lrRNA-seq experiment

In a multi-sample lrRNAseq experiment, all samples should be of similar quality. SQANTI3 uses the FSM structural category to identify long-read sequences whose junctions are consistent with an annotated transcript model. However, for a lrRNA-seq experiment to accurately reflect the analyzed transcriptome, the reads should ideally also capture the distribution of transcript lengths of the expressed transcriptome. The distribution of transcript lengths depends on the species with *Drosophila* having overall less complex and shorter transcripts than human (Supplementary Figure 3). A dataset with reads substantially shorter than the targeted transcriptome but with still a high number of FSM indicates capture of short transcripts, while combining shorter than expected reads with a high proportion of ISM may indicate RNA degradation. We looked at these values for an initial assessment of the quality of the *Drosophila* experiment.

First, we compared the number of reads and length distributions for all samples. The difference in sequencing depth between the two developmental stages was evident (Figure 1A). For all samples, most reads were shorter than 1kb with less than 20% of them above the 1 kb threshold (Figure 1B, Supplementary Figure 4A). While between 53% and 67% of the reads across samples were classified as FSM, 20% to 38% were labeled as ISM, and NIC/NNC were under 10% of the reads (Figure 1C). For the reads greater than 1 kb, between 73% and 82% of the reads were FSM while 10% and 18% were ISM (Figure 1D).

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To further understand how read quality affects gene and transcript quantification, we examined these metrics aggregated by gene and UJC. We found that, despite the large sequencing depth differences between developmental stages, the number of detected genes was only slightly lower in the 0-1 h samples (Figure 1E). However, these genes were quantified with fewer reads (80% genes with < 50 reads) than the 38 d samples, which had between 30% and 50% of genes with more than 100 reads(Figure 1E). Interestingly, when evaluating UJC we found that, while the number of UJC mirrored the sequencing depth pattern (Figure 1F), with 3-8 d samples showing five times more UJC than 1 h samples, and a larger number of FSM and ISM UJC, there were many additional UJC detected by fewer than 10 reads, and usually by a single read (Figure 1F & 1G) and these UJC were most frequently NIC/NNC (Supplementary Figure 4B & 4C). Downstream analyses would therefore need to address whether this represents novel low-expressed transcripts or technology errors. In contrast, the percentage of FSM reads between the two time points differed by less than 1x in all replicates (Figure 1H). These results indicate that the higher sequencing depth of the 3-8 d samples does not change the number of detected genes or annotated transcripts (FSM). The higher read depth per gene/ UJC suggests that more genes and transcripts will be able to be quantitatively evaluated in the 3-8 day samples compared to the 0-1 hour samples.

In the *Drosophila* data, we noticed two samples (RIL 12279 rep 1 0-1 hour- orange arrow; RIL 11255 3-8 day rep 3 - teal arrow) that had the lowest percentage of FSM and highest percentage of ISM in the 0-1 hour and 3-8 day groups respectively (Figure 1C). To determine whether these two samples were of overall lower quality than the rest, we examined the SQANTI-reads metrics for these two samples. We found that RIL 12279 rep 1 had a lower proportion of FSM across all genes (Figure 1H) and a higher proportion of genes quantified with only one gene (Figure 1F), while RIL 11255 rep 3 had a similar gene (Figure 1H), UJC (Figure 1G) and % FSM in genes (Figure 1H) than other 3-8 day samples. We concluded that RIL 12279 rep 1 0-1 hour is a low-quality sample.

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Altogether, this example shows that SQANTI-reads metrics can be used to compare samples and experimental conditions in a multi-sample experiment, detect outliers, and suggest points of attention for downstream data processing.

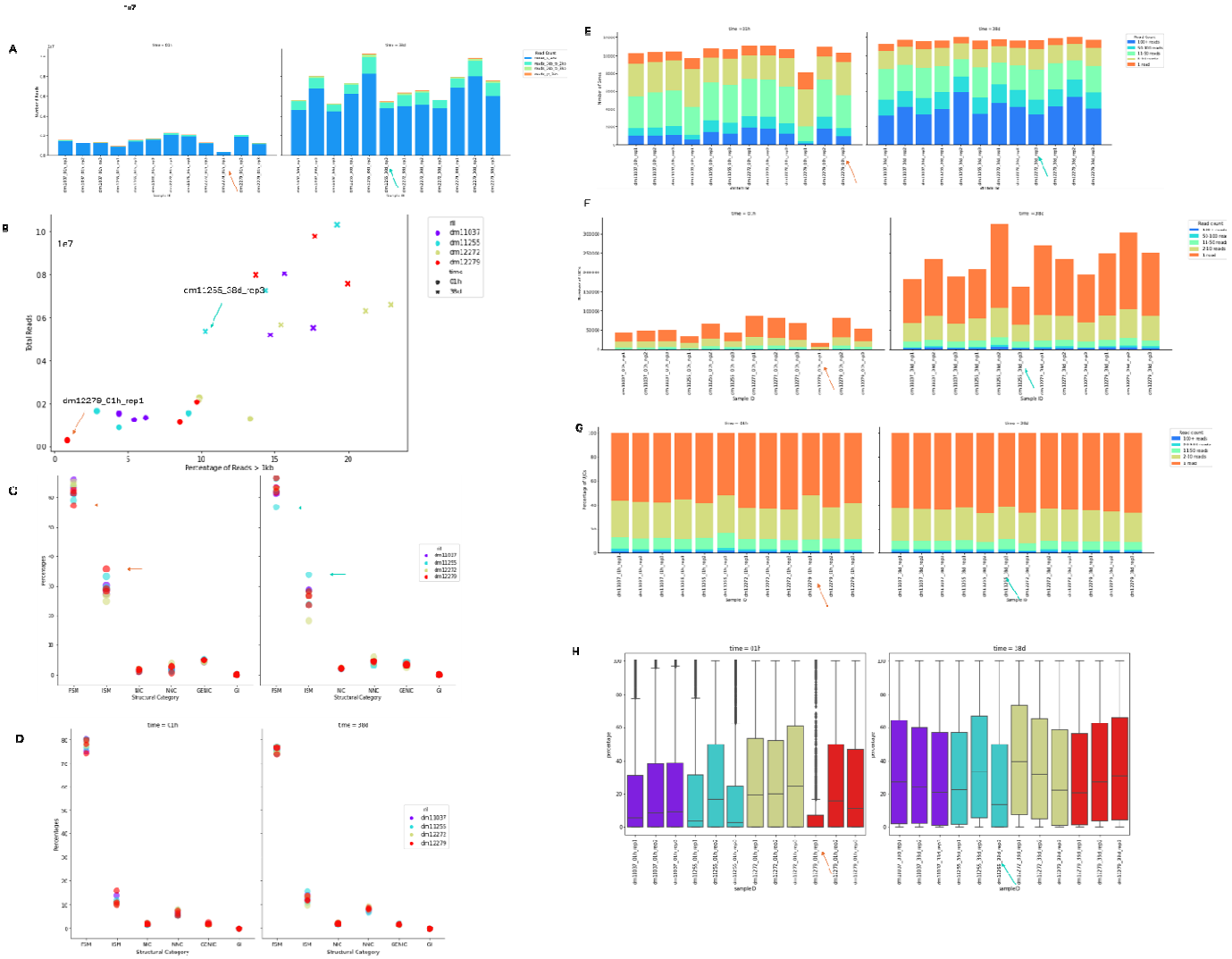

Figure 1: SQANTI-reads analysis of *Drosophila* samples. A) Number of mapped reads by experimental group labeled with read length. B) Percentage of mapped reads >1kb vs percentage of reads that are FSM. Dots represent early stage (0-1 hours after enclosure) and crosses indicate adult stage (3 to 8 days old). The four genotypes are indicated with four different colors. C) Percentage of reads mapping to genes in each SQANTI3 structural category D) Percentage of reads mapping to genes in each SQANTI3-QC structural category for reads >1kb E) Number of genes detected with breakdown by the number of reads mapped to each gene. F) Number of UJCs detected with breakdown by the number of reads associated with each UJC. G) Proportion of UJCs detected with breakdown by the number of reads associated with each UJC. H) Distribution of the percentage of FSM reads by gene across samples.

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SQANTI-reads metrics can be used to identify systematic differences among samples

The previous example demonstrated that SQANTI-reads metrics are effective in assessing dataset consistency. However, SQANTI-reads evaluates over 35 quality metrics, making it challenging to determine which features contribute to potential differences among samples. We include Principal Component Analysis (PCA) analysis to identify which metrics are the most relevant for quality variability when there are differences among samples or between groups. The percentage of reads and UJCs in each structural category, percentage of artifact reads (RT-switching, non-canonical junctions and intrapriming), percentage of junctions in each category, as well as length metrics, are included in the PCA.

We applied SQANTI-reads PCA analysis of quality features to investigate differences in read quality among various long-read sequencing methods used in the LRGASP challenge (Pardo-Palacios et al. 2024b), focusing on the WTC11 dataset. The analysis revealed that WTC11 samples clustered based on the long-read technology applied (Figure 2A). Specifically, PC1, which explains 56% of the variance, distinguished cDNA ONT samples from those generated by the other two technologies, while PC2, accounting for 35% of the variance, highlighted differences between dRNA ONT and cDNA PacBio. To further explore these differences, we examined the loadings for each principal component. Quality features with the highest positive loadings in PC1 included the number of reads, the percentage of reads and the proportion of UJCs in the NNC category, while features with high negative loadings included Intergenic and Genic Genomic reads. Several junction-related variables also exhibited high absolute loadings on PC1 (Figure 2B). SQANTI-reads plots confirmed these structural category differences between cDNA ONT samples and other library preparations. cDNA ONT had both the highest proportion of NNC reads and UJCs, (Figure 2C and 2D) and also had the lowest proportion of intergenic reads (Figure 2C). Other differences in sequencing throughput and junction characteristics were also confirmed (Supplementary Figure 5).

Upon examining the feature loadings for PC2, we found that variables with high contributions included several metrics related to read length (Figure 2B). Consequently, we evaluated the

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SQANTI-reads "Lengths of All Mapped Reads" plot for this experiment. Indeed, we observed that the cDNA PacBio method produced a significantly higher proportion of reads between 1-2 kb, 2-3 kb, and greater than 3 kb, as suggested by their negative loadings, compared to the dDNA ONT method, which predominantly generated reads shorter than 1 kb (Figure 2E). Similarly, percentage of reads assigned as ISM with high positive values was higher in dRNA ONT samples (Figure 2 C).

In conclusion, the SQANTI-reads PCA analysis is an effective tool for uncovering significant read quality differences between long-read sequencing methods and for revealing unexpected technology biases.

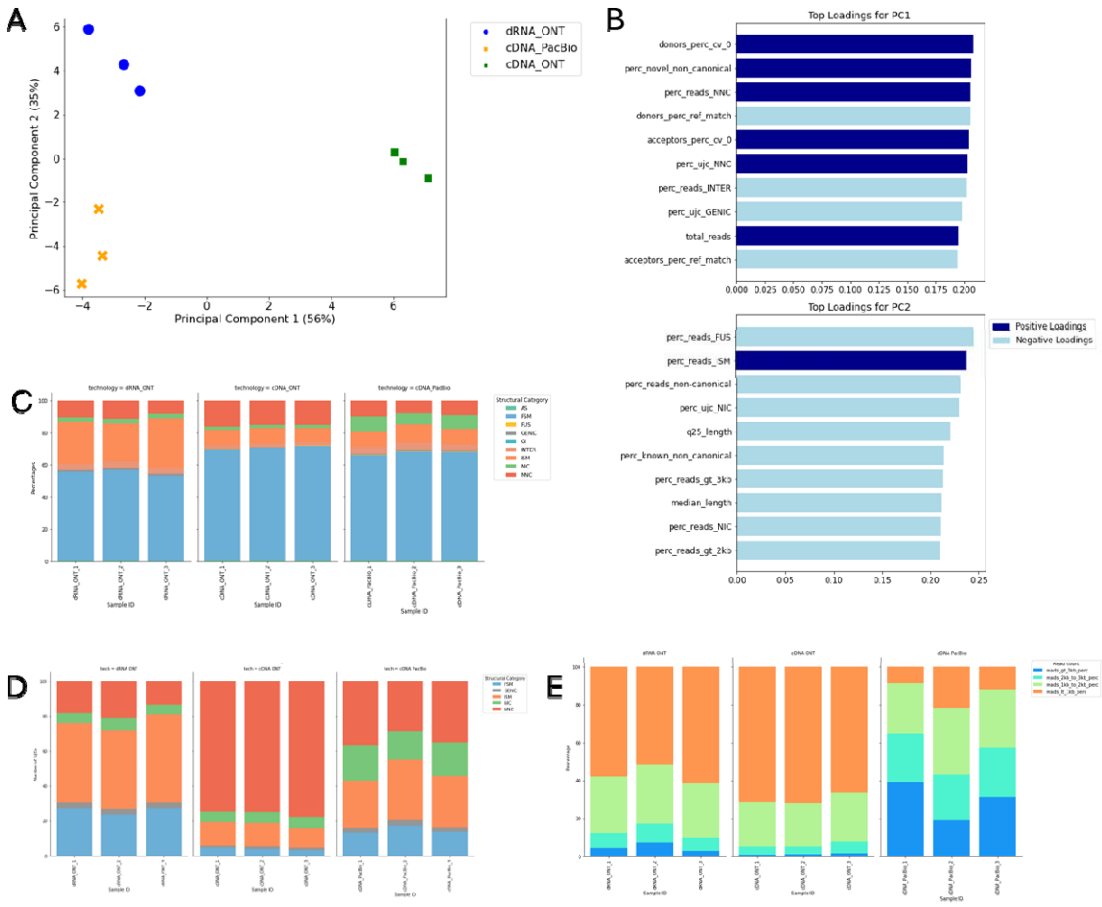

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Figure 2: SQANTI-reads PCA analysis of LRGASP WTC11 samples A) PCA using SQANTI-reads quality features. B) Top 10 Loadings for PC1 and PC2. C) Distribution of reads in SQANTI structural categories. D) Distribution of UJCs in structural categories E) Distribution of read lengths for all mapped reads.

SQANTI-reads identifies potentially under annotated genes.

Long-read data often reveal a large number of sequences that cannot be exactly matched to existing annotations. In many cases, these UJC belong to annotated genes and are identified by only a few reads, as illustrated by the SQANTI-reads analysis in Figures 1 and 2. This suggests they could be either low-expressed transcripts or technological artifacts. However, in some instances, a high proportion of reads in a gene may correspond to the same UJC, indicating the possibility of a novel previously unannotated transcript that warrants closer examination. SQANTI-reads includes a customizable decision tree to identify such cases (see Methods, Figure 3A). We applied this approach to the WTC11 PacBio data and identified 8556 well annotated genes, 88% of which have an annotated transcript with high coverage (>20% of total gene coverage) (Figure 3B). We also identified 101 underannotated genes, 54% of which have a candidate unannotated transcript. The annotation category for all genes evaluated (default: R > 100) are provided in the gene_classification.csv file. We also found 424 putative novel transcripts (Figure 3C). Of these, 316 were NIC and 108 were NNC transcripts. The SQANTI-reads output for putative novel transcripts flagged based on their length and read count are output in the putative_underannotation.csv file (Table 2).

For genes with at least one putative novel transcript (R=100, P=20, Q=80) and an annotated transcript (FSM) we selected the FSM with the highest proportion of reads. We then compared the structure of the putative novel transcripts to the most expressed annotated transcript using TranD (Nanni et al. 2024). For genes with an annotated transcript that is relatively highly expressed (>20% of the reads for that gene), 103 putative candidate transcripts differed from the annotated transcript by donor/acceptor variation, suggesting a possible alternative splice site. In addition, 10 putative candidate transcripts had an extra exon; 15 a skipped exon and 9 with both missing and skipped exons relative to the most expressed annotated UJC (Figure 3D).

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For the genes where the annotated transcript represented less than 20% of the reads in that gene, the putative novel transcript differed from the annotated transcript by an alternative exon in 147 cases (33 extra exons, 86 skipped exons, and 43 with both an extra and skipped exon) (Figure 3E). Details and scripts for this analysis are provided in the Supplementary Methods.

This analysis demonstrates that SQANTI-reads is effective in classifying genes as well annotated or underannotated and flagging putative novel transcript annotations that contain realistic alternative exonic patterns.

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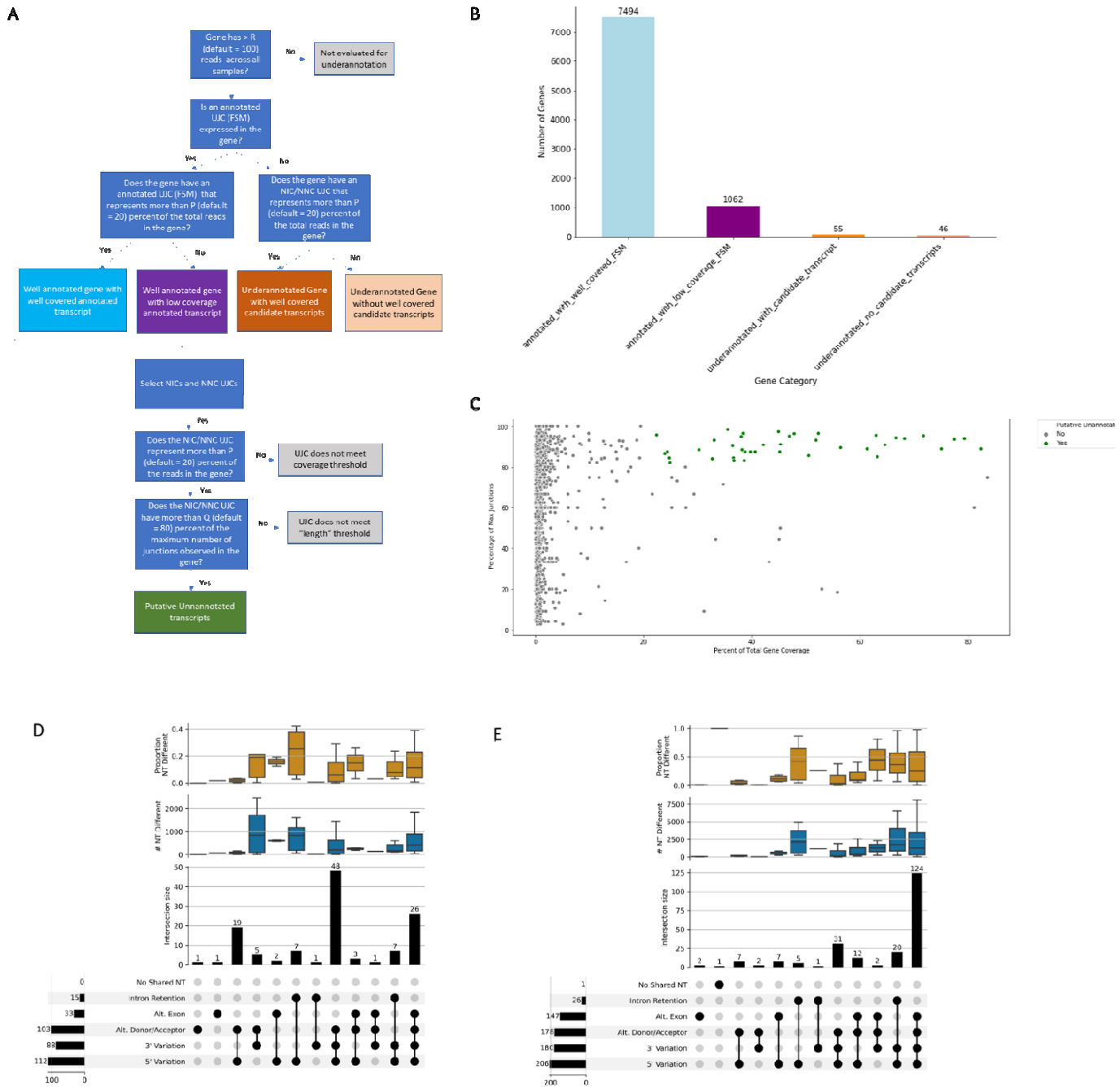

Figure 3: Evaluation of WTC11 PacBio samples for under-annotated genes. A) Decision tree for classifying genes as well annotated or under-annotated and classifying transcripts as putative novel candidate transcripts. B) Number of well-annotated and under-annotated genes according to the decision tree in A. C) The coverage (percent of total reads) vs length (percentage of maximum junctions) for all UJCs in underannotated genes with well covered candidate transcripts. UJCs that meet the thresholds for putative novel transcripts are coloured in green. D&E) Comparison of putative candidate novel transcripts with the most expressed

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annotated transcript in that gene for well-annotated genes with well-covered FSMs (D) and with an FSM detected but without < 20% of the total reads in the gene(E).

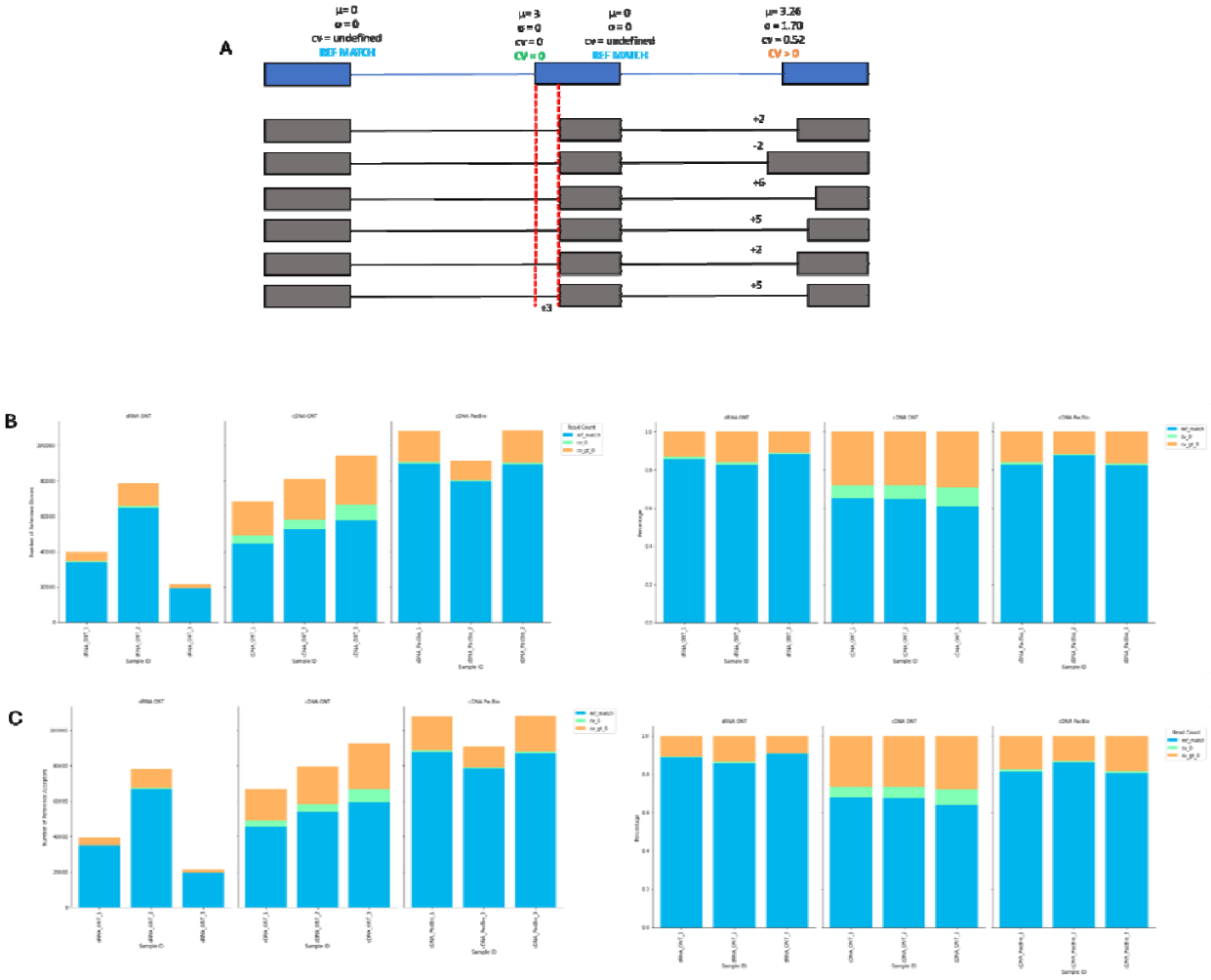

Figure 4. Variation in donors/acceptors. Metrics are only calculated for annotated donor/acceptors with a minimum threshold of reads (10 by default). A) Metrics for the classification of donor/acceptor variation. When all reads align to the annotated donor/acceptor this is classified as a reference match (Ref Match). When all reads align to the same donor/acceptor location, but this is not the annotated position this is classified as CV = 0. When reads align in multiple positions in proximity to an annotated donor/acceptor this is classified as CV > 0. B) Classification of the number (left) and proportion (right) of detected acceptors faceted by technology. C) Classification of the number (left) and proportion (right) of detected donors faceted by technology.

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SQANTI-reads metrics for donors/acceptors identify noisy splicing and potentially novel splice-sites

SQANTI-reads calculates the mean, standard deviation and coefficient of variation (CV) for all expressed annotated donors/acceptors (Figure 4A). A value CV > 0 indicates variability in the donor/acceptor, with higher CV values indicating more variability. Variability around a splice-junction may be due to weak splicing (Wang and Marín 2006) or to technology errors and mapping accuracy, for example due to junction ambiguity (Li 2018). We evaluated these metrics on the WTC11 dataset for reference junctions with at least 10 reads. We found similar patterns in the variability (CV > 0) in donors and acceptors (Figure 4B). All three technologies identify donors and acceptors with variability around the splice site (CV > 0). Donors/acceptors with CV > 0 consistently across the three technologies, are highly suspicious of 'noisy' splicing or a weak splice site and may be worth follow up (Supplementary Figure 6A and 6B). The SQANTI-reads output file cv.csv identifies the donors/acceptors with CV > 0 making it straightforward to follow up on particular locations with tools such as Integrative Genomics Viewer (IGV) (Robinson et al. 2011).

A reference match indicates that the splice signal is strong (Wang and Marín 2006; Dent et al. 2021). Results for cDNA PacBio and dRNA-seq were similar, with both showing a higher number and proportion of reference match donors/acceptors compared to cDNA ONT (Figure 4B and C) despite these technologies detecting similar numbers of FSM UJCs (Figure 2D). We compared the FSM UJCs identified by the three technologies (Supplementary Figure 7A). Most of the FSM UJCs are detected by all three technologies (n = 19690), with a similar number detected by cDNA PacBio only (n = 8110) and cDNA ONT (n = 9360) only. We hypothesized that the difference in the number of reference match donors/acceptors was potentially due to longer transcripts with more junctions being detected in cDNA PacBio compared to shorter transcripts with fewer junctions in dRNA ONT and cDNA ONT. For the FSMs detected only in one technology we plotted the distribution of the number of junctions and confirmed that the cDNA

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PacBio FSM transcripts had a larger number of junctions compared to dRNA ONT and cDNA ONT (Supplementary Figure 8). This is not surprising given that cDNA PacBio had longer reads than both ONT technologies (Figure 2E).

Donor/acceptor sites may differ from annotated sites due to the presence of novel donors/acceptors. The category CV = 0 identifies donor/acceptors with no variability, that differ from the annotated donor/acceptor. We evaluated the donors and acceptors with CV = 0 in all the technologies (Supplementary Figure 6E and 6F). Of the 5562 donors and 4474 acceptors with CV = 0 across all technologies, there were 47 donors and 35 acceptors with CV = 0 detected in all three technologies. These had a median distance from the annotated donor or acceptor of 4 nucleotides and 5 nucleotides respectively (Supplementary figure 9). These indicate potential robust detection of alternative splice sites.

DISCUSSION/CONCLUSION

SQANTI-reads compares multiple samples using summaries and visualizations of SQANTI3 categories and sub-categories that enable the researcher to evaluate the experiment for consistency, and identify any systematic differences between sample groups. The meta-data in the design file determines the sample groupings, and once the initial classification and junction files are created, SQANTI-reads can be invoked multiple times to compare different aspects of the experimental design.

In Oxford Nanopore Technology (ONT) the existence of multiple platforms at different price points for different numbers of pores (Flongle, MinION, GridION, PromethION) but with the same library protocols means that in a large experiment, samples can be initially evaluated at low cost on one of the lower throughput platforms (Flongle MinION) and if samples are of sufficient quality, then can be run on higher throughput platforms (GridION, PromethION).

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Sample multiplexing and running on multiple 'lanes' is also good experimental design practice (Auer and Doerge 2010).

SQANTI-reads introduces new metrics for identifying variation in donors/acceptors; under annotated genes; and putative novel transcripts for further evaluation. As the examples presented demonstrate, SQANTI-reads is flexible and can be customized to examine the impact of different aspects of the sample meta-data on the metrics for donor/acceptor variation and novel transcript identification. The output of SQANTI-reads can be easily mined for additional insights and can be used to direct attention and resources to interesting and novel features of lrRNAseq experiments. We expect SQANTI-reads to become an essential tool for the QC of multi-sample lrRNA-seq datasets.

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Competing interest statement

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