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Nanopore Direct Sequencing of RNA-DNA Hybrid Molecules

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Nanopore direct RNA sequencing (DRS) profiles full-length RNAs and detects base modifications, yet comparative studies are hampered by run-to-run variability and the lack of robust multiplexing. We present a hybrid basecalling strategy that decodes DNA segments and RNA reads within the same molecules prepared by incorporating DNA barcodes during DRS library construction. Unlike methods restricted to predefined barcode sets, our approach performs de novo DNA decoding of arbitrary sequences and lengths, enabling scalable pooling. We built training datasets from reporter DNAs covering hundreds of k-mers and from RNA–DNA hybrid libraries generated from human total RNA, and trained a new model to account for the distinct nanopore signal properties of DNA versus RNA. In pooled DRS runs, the method accurately demultiplexed samples across independent runs and reproduced DNA calls with high concordance, reducing batch effects and sequencing cost. Beyond demultiplexing, simultaneous DNA–RNA decoding allows single-molecule analysis of endogenous RNA–DNA hybrids across the transcriptome, creating opportunities for studies of chromatin-associated RNAs and RNA modification landscapes. This work expands DRS toward robust comparative designs and single-molecule interrogation of RNA–DNA interactions.