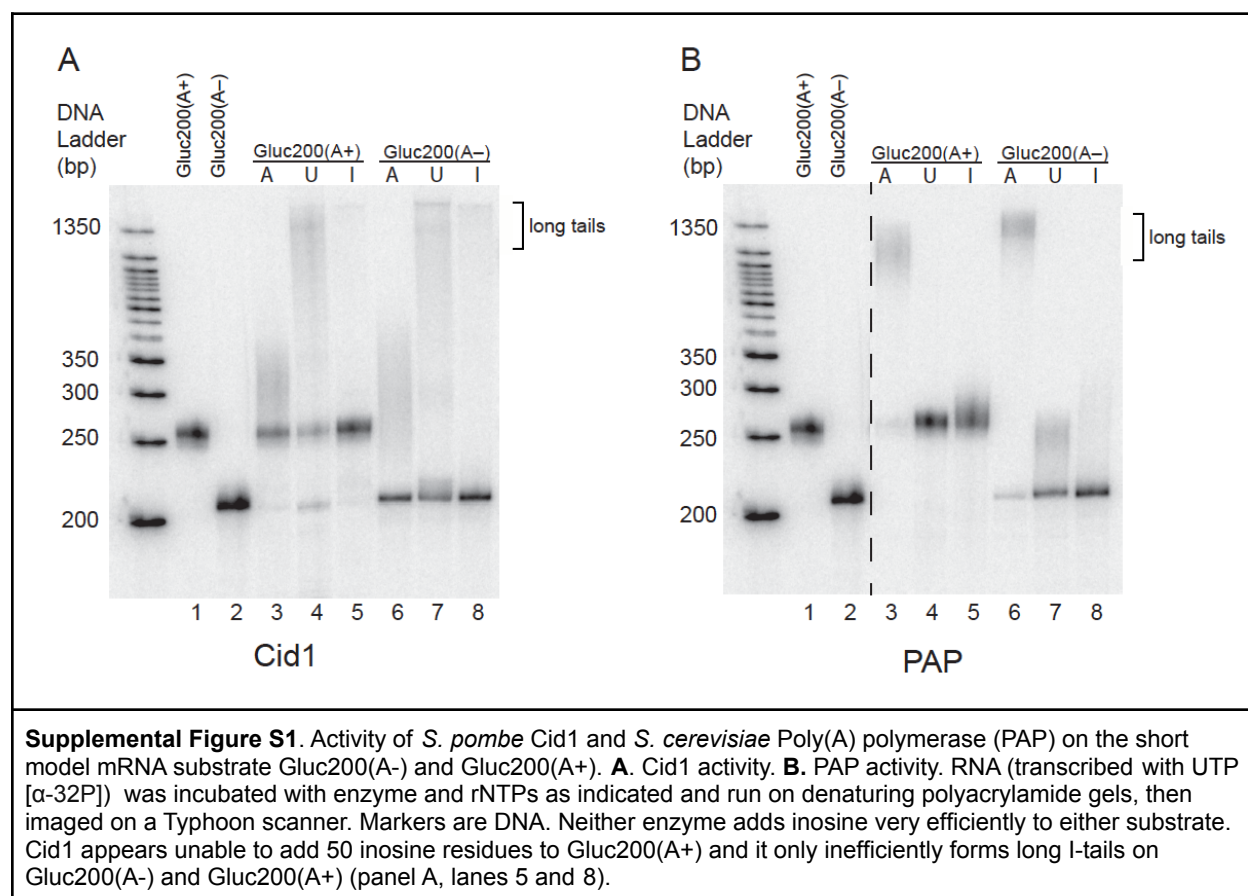


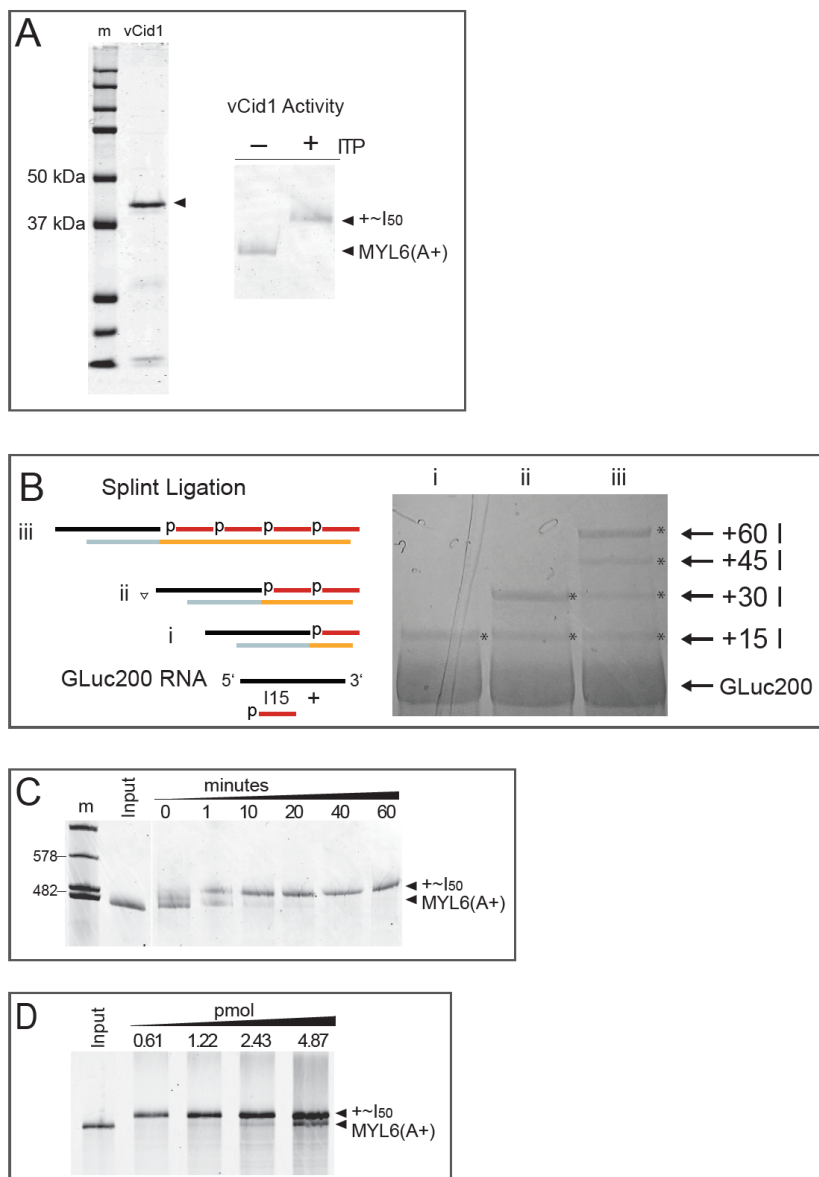
Supplemental Figures and Methods

Synthesis of modified nucleotide polymers by the poly(U) polymerase Cid1: Application to nanopore RNA sequencing.

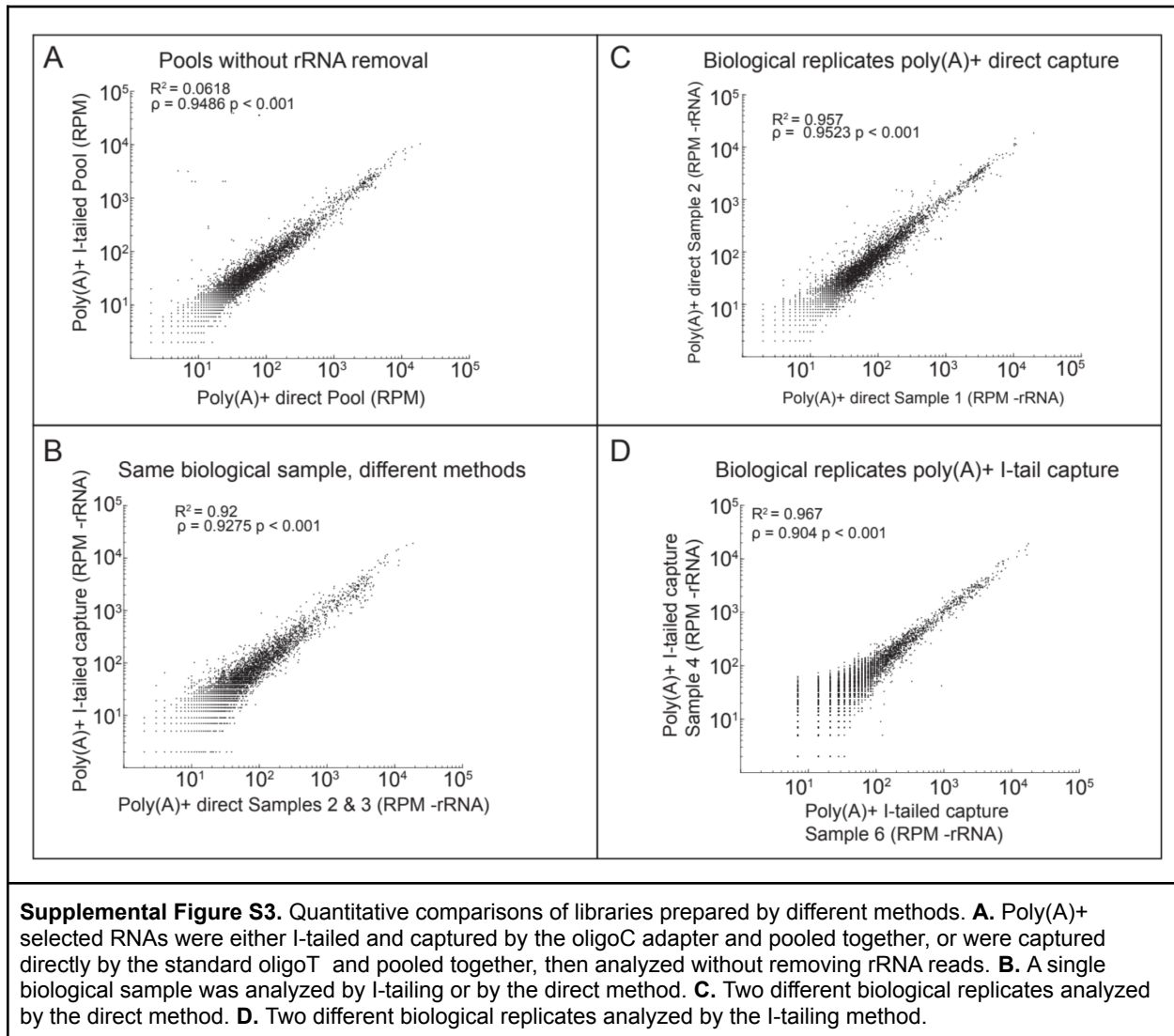
Jenny Vo, Logan Mulroney, Jen Quick-Cleveland, Miten Jain, Mark Akeson, Manuel Ares, Jr.

Supplemental Figures





Supplemental Figure S2. Methods of addition of inosine to RNA. **A.** Homemade purified Cid1 expressed from *E. coli* adds 50 I residues to a model poly(A)+ mRNA. Left, a protein gel showing purified N-terminal truncated Cid1 (vCid1). Arrow indicates expected protein product (theoretical MW ~ 44.9 kDa). Right, inosine adding activity of purified vCid1 on MYL6(A)+ substrate showing major +50 nt product (+~I₅₀). **B.** Splint ligation was used to add defined lengths of I₁₅ to the end of a model mRNA. A different splint oligo (i, ii, or iii, blue and gold) was used to align the model RNA (black) and a phosphorylated inosine 15-mer (red) for ligation by T4 DNA ligase. The products were separated on a denaturing gel and gel purified. **C.** Time course of I-tailing on a model poly(A)+ mRNA. Within 20 min, addition of 50 I residues to all the substrate molecules is complete under the standard conditions (see Methods). **D.** Capacity of the standard reaction. Increasing amounts of substrate were I-tailed in a standard reaction (see Methods). Reaction capacity is about 2 pmoles under these conditions.



Supplemental Table S1: Summary of sequencing runs

Library name	RNA treatment	Capture Method	# reads	# pass reads
Poly(A) Control #1	Poly(A) #1	ONT standard	1,507,013	1,408,009
Poly(A) Control #2	Poly(A) #2	ONT standard	4,544,817	4,154,482
Poly(A) Control #3	Poly(A) #2	ONT standard	2,976,161	2,755,245
Poly(A) Poly(I) #1	Poly(A) #1	Poly(I)	496,472	435,203
Poly(A) Poly(I) #2	Poly(A) #1	Poly(I)	206,170	170,135
Poly(A) Poly(I) #3	Poly(A) #1	Poly(I)	347,347	302,453
Poly(A) Poly(I) #4	Poly(A) #3	Poly(I)	769,807	666,455
Poly(A) Poly(I) #5	Poly(A) #3	Poly(I)	34,427	26,291
Poly(A) Poly(I) #6	Poly(A) #3	Poly(I)	346,754	297,307
Poly(A) Poly(I) #7	Poly(A) #4	Poly(I)	367,679	319,259
Total RNA	Total RNA (none)	Poly(I)	128,919	57,276
RiboMinus	RiboMinus	Poly(I)	280,590	79,587
RNAPII-associated	biotinylated-RNAPII streptavidin pull down	Poly(I)	320,969	258,896

Supplemental Table S2. Supplemental_TableS2_Mapped_Reads.xlsx

Supplemental Table S3. Supplemental_Table3_Median_TailLengths.xlsx

Supplemental Methods

Sequence of T7-MYL6 transcription template

This insert sequence (capital letters) is cloned in the SmaI site in the polylinker (lower case) of pUC13. The T7 promoter sequence is italicized. The recognition sequences of BsmI (GCATTC) and BbsI (GTCTTC) are underlined. The slash indicates the position of the 5' end of the bottom strand of the cut DNA and is the last nucleotide transcribed by T7 RNA polymerase initiating at the T7

promoter, indicating the 3' nucleotide of the MYL6(A)– transcript (BsmI cut template) and the MYL6(A)+ transcript (BbsI cut template).

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gatccccTAATACGACTCACTATAGGGAGACAGTGGCCAAGAACAAGGACCAGGGCACCTATGAG
GATTATGTCTGAAGGACTTCGGGTGTTTGACAAGGAAGGAAATGGCACCGTCATGGGTGCTGAA
ATCCGGCATGTTCTTGTCACTGGGTGAGAAGATGACAGAGGAAGAAGTAGAGATGCTGGTG
GCAGGGCATGAGGACAGCAATGGTTGTATCAACTATGAAGCGTTTGTGAGGCATATCCTGTCGG
GGTGACGGGCCCATGGGGCGGAGCTCGTCCGCATGGTGCTGAATGGCTGAGGACCTTCCCA
GTCTCCCCAGAGTCCGTGCCTTTCCTGTGTGAATTTGTATCTAGCCTAAAGTTTCCCTAGGCT
TTCTTGTCTCAGCAACTTTCCTCATCTTGTCTCTTGGATGATGTTTGCCGTC/AGCATTACCAA
ATAAATTGCTCTCTGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA/CTGTCTTC
gggcgagctcgaattc
```

vCID1 expression in *E. coli*:

Plasmid 10H-tev-vCID was made by standard techniques using synthetic DNA. It carries a beta-lactamase gene and a colE1 origin of DNA replication, and expression is driven by a T7 promoter. The predicted protein is 391 amino acids long with a theoretical molecular weight of 44.9 kilodaltons and the following sequence:

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MGHHHHHHHHHHSSGAENLYFQSPNSHKEFTKFCYEVYNEIKISDKEFKEKRAALDTLRLCLKRISP
DAELVAFGSLESGLALKNSDMDLCVLMDSRVQSDTIALQFYEEIAEGFEGKFLQRARIPPIIKLTS
DKNGFGASFQCDIGFNNRLAIHNTLLLSSYTKLDARLKPMVLLVKHWAKRKQINSPYFGTLSSYGYV
LMVLYYLIHVIKPPVFPNLLLSPLKQEKIVDGFVDGFDKLEDIPPSQNYSSLGSLHGFRRFYAYK
FEPRKVVTFRRPDGYLTKQEKGWTSATEHTGSADQIIKDRYILAIEDPFEISHNVGRTVSSSGLYR
IRGEFMAASRLLNSRSYPIPYDSLFEETPIPPRRQKKTDEQSNKKLLNETDGDNSE*
```

To produce protein, *E. coli* BL21(DE3) pLysS cells are transformed with the plasmid and transformants are selected on LB agar plates with 100 µg/ml ampicillin at 37°C overnight. A single colony is used to inoculate 50mL of LB supplemented with 100 µg/ml ampicillin, shaken at 300rpm overnight at 37°C and diluted the next day into 1L with LB supplemented with 25 µg/ml ampicillin. After growth OD600 = 0.6 at 37°C and 300rpm, cells are induced to express vCID by adding IPTG to 1mM and shaking at 300rpm for 16-18 hours at 18°C. Cells are harvested by centrifugation at 5000rpm for 10 minutes, and cell pellets are resuspended in 10mL of 50mM Tris-HCl, 1mM EDTA

pH 8.0 and centrifuged again at 4°C at 5000rpm, for 30 minutes. Supernatant is decanted, and washed pellets are stored at -80°C.

vCID1 Purification

Frozen cell pellets are resuspended in 14mL of a high salt-phosphate buffer (HSP buffer, 50mM sodium phosphate pH 8, 300mM NaCl, 100mM KCl, 1mM DTT, 10% glycerol) with 10 mM imidazole, with addition of lysozyme at 1 mg/mL and PMSF to 0.5mM, and then incubated on ice for 5 minutes. Cells are lysed with glass beads by vortexing for 3 minutes in 15 second intervals (12 x 15 sec) with incubation on ice for 15 seconds between vortexing. Lysates are clarified by centrifugation at 14000 x g for 1 hour at 4°C. The resulting supernatant is incubated for 30 minutes with 2.5mL of cobalt-chelate resin (pre-equilibrated in 10 mM imidazole HSP buffer) with gentle shaking at 4°C, and then is poured into a column. The column is washed 2x with 12mL of 20mM imidazole HSP buffer, and then 1x with 5mL of 50mM imidazole HSP buffer. Eluates are collected using 100mM, 150mM, 200mM, 250mM, 400mM, and 500mM imidazole buffers at 5mL each in succession. Each eluted sample is concentrated in a protein concentrator tube (Millipore) until the total volume is <500uL. The samples are resuspended in 10mL storage buffer (10mM Tris-Cl pH 7.5, 100mM NaCl, 1mM DTT, 0.1mM EDTA, 50% glycerol v/v), and concentrated again until the sample volume was under 500uL.

I15 Splint ligation for GLuc200I30 and GLuc200A44I30 library preparation

To prepare GLuc200I30 and GLuc200A44I30 samples, 15pmol of GLuc200 RNA with 30pmol of the appropriate bottom splint adapter (C25TTTTTTTTTTT (IDT) for GLuc200A44 or C25CCT AAG AGC AAG AAG AAG (IDT) for GLuc200), 1.4 nmols of a synthetic 5'p-15mer inosine homopolymer (Stanford PAN facility) in 10mM Tris-Cl pH 8.0, 1mM EDTA, and 50mM NaCl in 6ul reaction volume was heated to 55°C and slow cooled to 16°C in 25 minutes. Each reaction had 1ul 10X T4 ligation reaction buffer (NEB B0202S) and 2,000 units T4 DNA ligase (NEB M0202T) were added to each

reaction and brought to 10ul volume with water then incubated at 16°C overnight. 2X RNA loading dye (2X NEB N0362) was added to each sample and denatured at 95°C for 5 minutes before loading into a 10% acrylamide gel and run for 3.5 hours at 28 watts. The gel excision was performed by post-staining with 1X SYBR Gold in TBE and visualized on a UV transilluminator while cutting with a razorblade. The samples were eluted from the gel slice in 850µl of 1x TAE buffer using a D-tube™ Dialyzer Midi (Millipore Sigma 71507) for at least 90 minutes at 130 volts. The electro-eluted samples were precipitated with 85µl 0.3M NaOAc and 850µl isopropanol at -20°C overnight. The next day the samples were centrifuged at 4000 x g at 4°C for 30 minutes and the pellets were washed with 70% ethanol twice with subsequent centrifugations at 16000 x g for 15 minutes. The pellets were air dried for 15 minutes and resuspended in 10µl water with yields between 25-100ng. The libraries for each ligation product were prepared following ONT's Direct-RNA Nanopore sequencing library preparation with ~50ng of RNA using the polydC adapter. The optional reverse transcription step was skipped.