Supplementary Material to:

HELIOS NAD-Seq: A Next-Generation Capture and Sequencing Protocol for NAD-Capped RNAs with Superior Targeting and Processing

Marvin Möhler[a‡], Tae Wha Seong[a‡], Frederik Weber[a], Héctor Gabriel Morales Filloy[a], Marleen Wölke[a], Elif Tosun[a], Edrea Mendonca[a], Andrijana Crenner[a], Yaqing Zhang[a], Frank Preugschat[b], Andres Jäschke[a]*

Content:

Supplementary Figures:

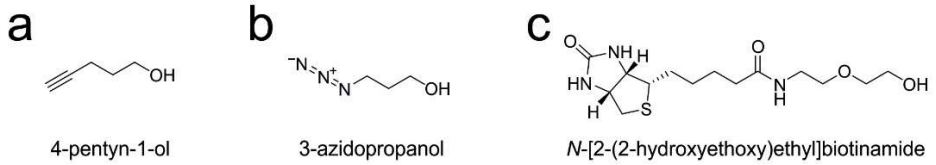

Figure S1: Chemical structures of currently applied substrates for ADPRC transglycosylation reactions of NAD-capped RNA. a) 4-pentyn-1-ol, applied in NAD captureSeq and NAD tagSeq. b) 3-azidopropanol, applied in NAD tagSeq II, SPAAC NAD-seq and NADcapPro. c) *N*-[2-(2-hydroxyethoxy)ethyl]biotinamide (HEEB), applied in ONE-seq and DO-seq.

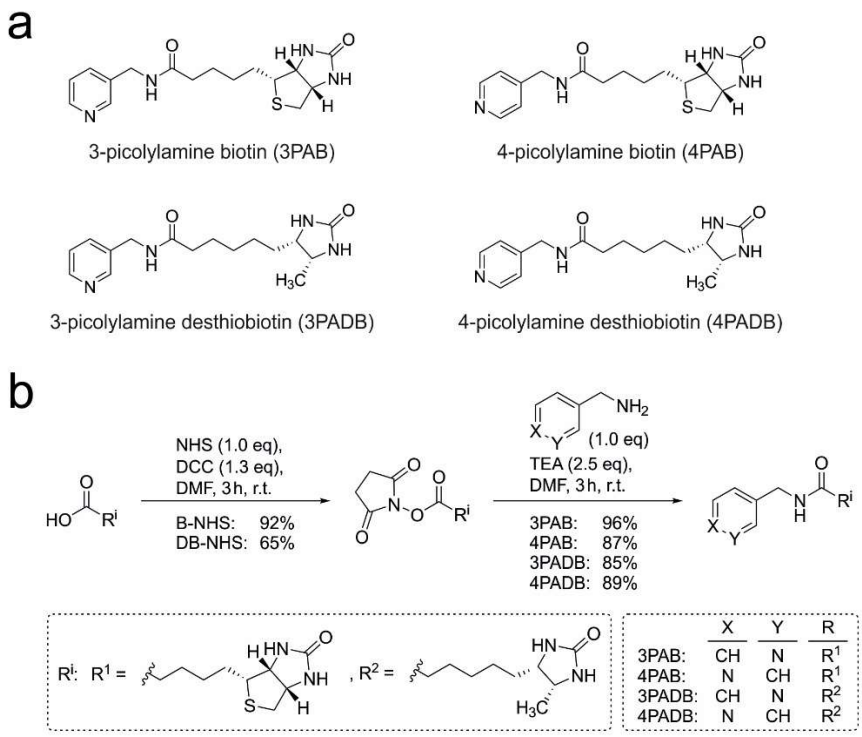

Figure S2: Chemical structures and synthesis of PAB conjugates as novel substrates for ADPRC transglycosylation reactions of NAD-capped RNA. a) Chemical structures of 3-picolylamine biotin (3PAB), 4-picolylamine biotin (4PAB), 3-picolylamine desthiobiotin (3PADB), and 4-picolylamine desthiobiotin (4PADB). b) Synthesis route for 3PAB, 4PAB, 3PADB, and 4PADB via NHS-ester activation of biotin (B-NHS) or desthiobiotin (DB-NHS) and amide coupling to 3- or 4-picolylamine.

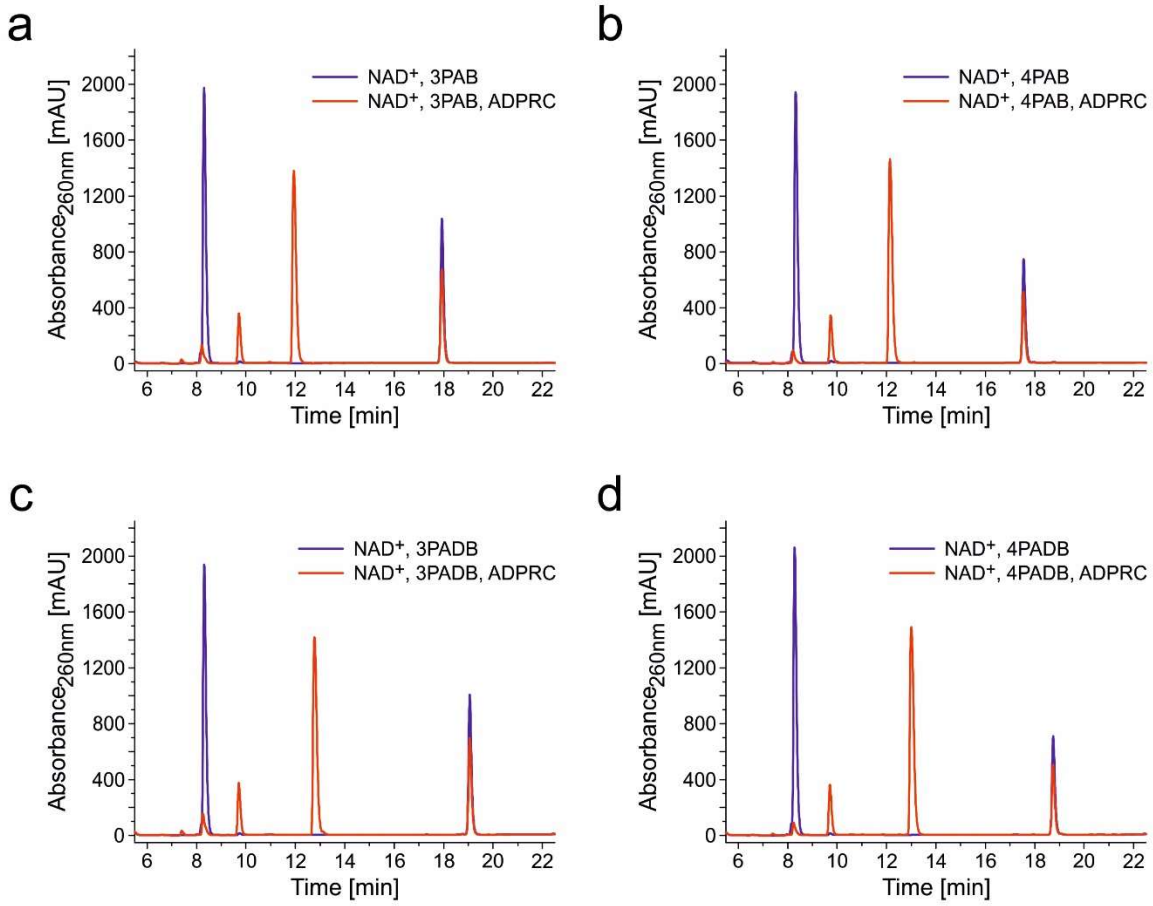

Figure S3: HPLC analysis after ADPRC transglycosylation reactions with NAD^+ and different PAB conjugates. Chromatograms are shown for a reaction mixture with (red curve) and without ADPRC enzyme (blue curve), with the NAD^+ peak at 8.5 min and the PAB or PADB conjugate peak between 17.5 and 19.0 min. The panels show reactions with a) 3PAB, b) 4PAB, c) 3PADB, and d) 4PADB. Close to quantitative conversion in the ADPRC-containing reactions (red curve) to the desired (desthio)biotinylated ADPR reaction products (peak between 12.0 and 13.0 min) and nicotinamide (peak at 10.0 min) was observed. Product identities were confirmed by HR-MS. ADPR-3PAB ($C_{31}H_{43}N_9O_{15}P_2S$): m/z (ESI, positive) found 876.2118 $[M+H]^+$, 898.1945 $[M+Na]^+$, and m/z (ESI, negative) found 874.2005 $[M-H]^-$. ADPR-4PAB ($C_{31}H_{43}N_9O_{15}P_2S$): m/z (ESI, negative) found 874.2010 $[M-H]^-$. ADPR-3PADB ($C_{31}H_{45}N_9O_{15}P_2$): m/z (ESI, positive) found 846.2557 $[M+H]^+$, 868.2376 $[M+Na]^+$, and m/z (ESI, negative) found 844.2445 $[M-H]^-$. ADPR-4PADB ($C_{31}H_{45}N_9O_{15}P_2$): m/z (ESI, positive) found 846.2548 $[M+H]^+$, 868.2364 $[M+Na]^+$, and m/z (ESI, negative) found 844.2445 $[M-H]^-$.

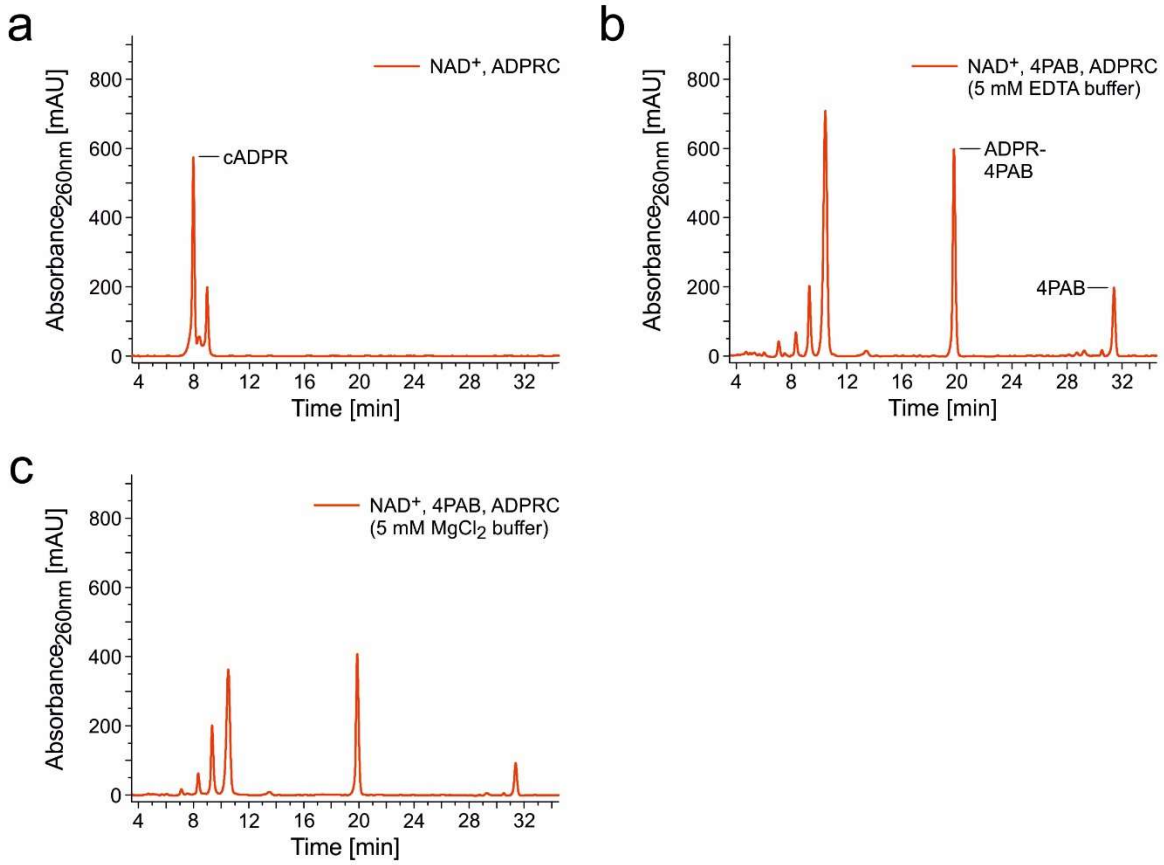

Figure S4: HPLC analysis after ADPRC transglycosylation reactions with NAD^+ under various reaction conditions, showing that the substitution reaction with PAB conjugates proceeds in the presence and absence of Mg^{2+}-cations. a) HPLC chromatogram for a reaction mixture with 5 mM $MgCl_2$ and without PAB conjugate. The main product cyclic ADP-ribose (cADPR) was eluted after 8 min. b) HPLC chromatogram for a reaction mixture with 5 mM $MgCl_2$ and 1.5 mM 4PAB. The main product ADPR-4PAB was eluted after 20 min. c) HPLC chromatogram for a reaction mixture with 5 mM EDTA and 1.5 mM 4PAB. The main product ADPR-4PAB was eluted after 20 min. Product identities were confirmed by HR-MS.

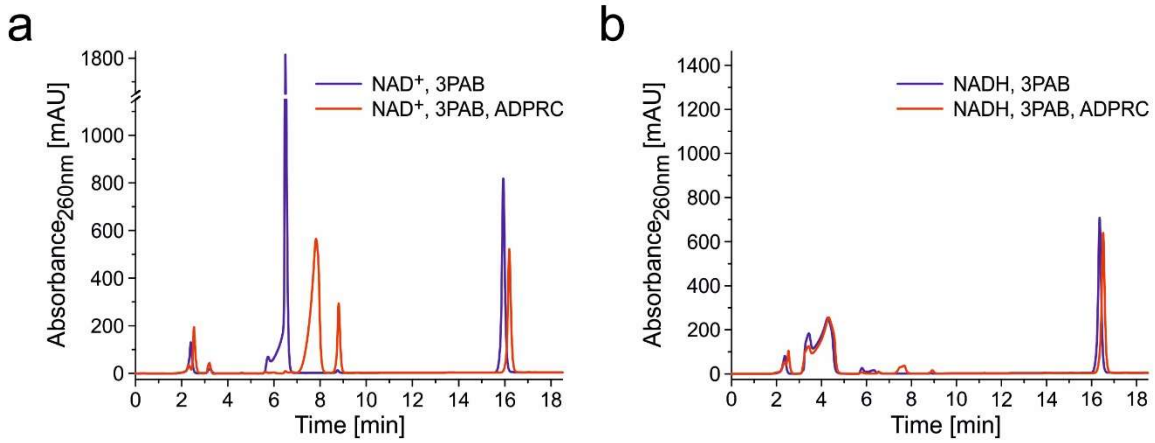

Figure S5: HPLC analysis after ADPRC transglycosylation reactions (30 min time-point) with 3PAB and NAD^+ or NADH. a) HPLC chromatogram for a reaction mixture with NAD^+ (peak at 5.5 – 6.5 min), which was accepted as a substrate to yield ADPR-3PAB (peak at 7.5 min). b) HPLC chromatogram for a reaction mixture with NADH (double peak at 3.0 – 4.0 min), which was not accepted as a substrate, but contained low amounts of the oxidation product NAD^+ (peak at 6.0 min), from which the corresponding amount of ADPR-3PAB (peak at 7.5 min) was formed. Product identities were confirmed by HR-MS.

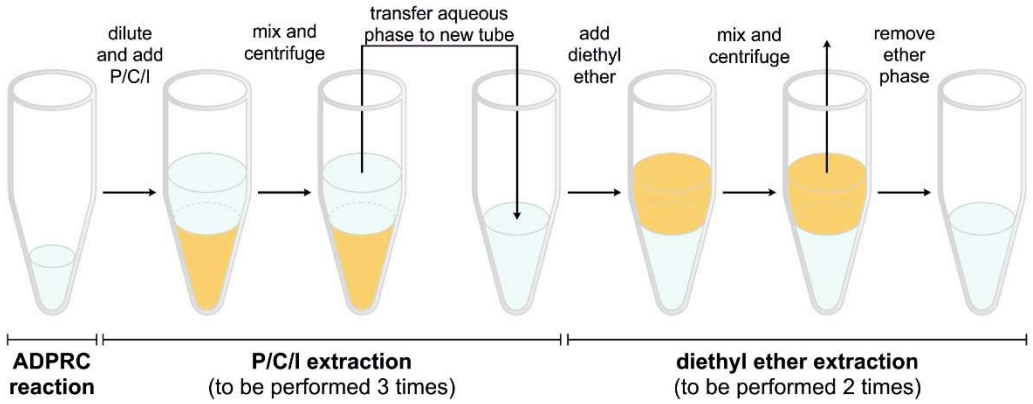

Figure S6: Quenching and extraction protocol after ADPRC transglycosylation reactions. The reactions are regularly performed with a volume <200 µL in 1.5 mL reaction vessels. For quenching, a mixture of 300 µL P/C/I and the corresponding amount of H_2O to reach an equal volume of aqueous phase is added, and thoroughly mixed. The aqueous phase is extracted three times with P/C/I to remove ADPRC protein (interphase) and unreacted PAB conjugate (organic phase), and two times with diethyl ether. In each case, the phases are thoroughly mixed and separated again by centrifugation, before the upper phase is transferred into a new 1.5 mL vessel (aqueous phase) or discarded (organic phase). For better understanding, the aqueous phase is depicted with a light blue color, whereas the organic phases are shown in a light orange color.

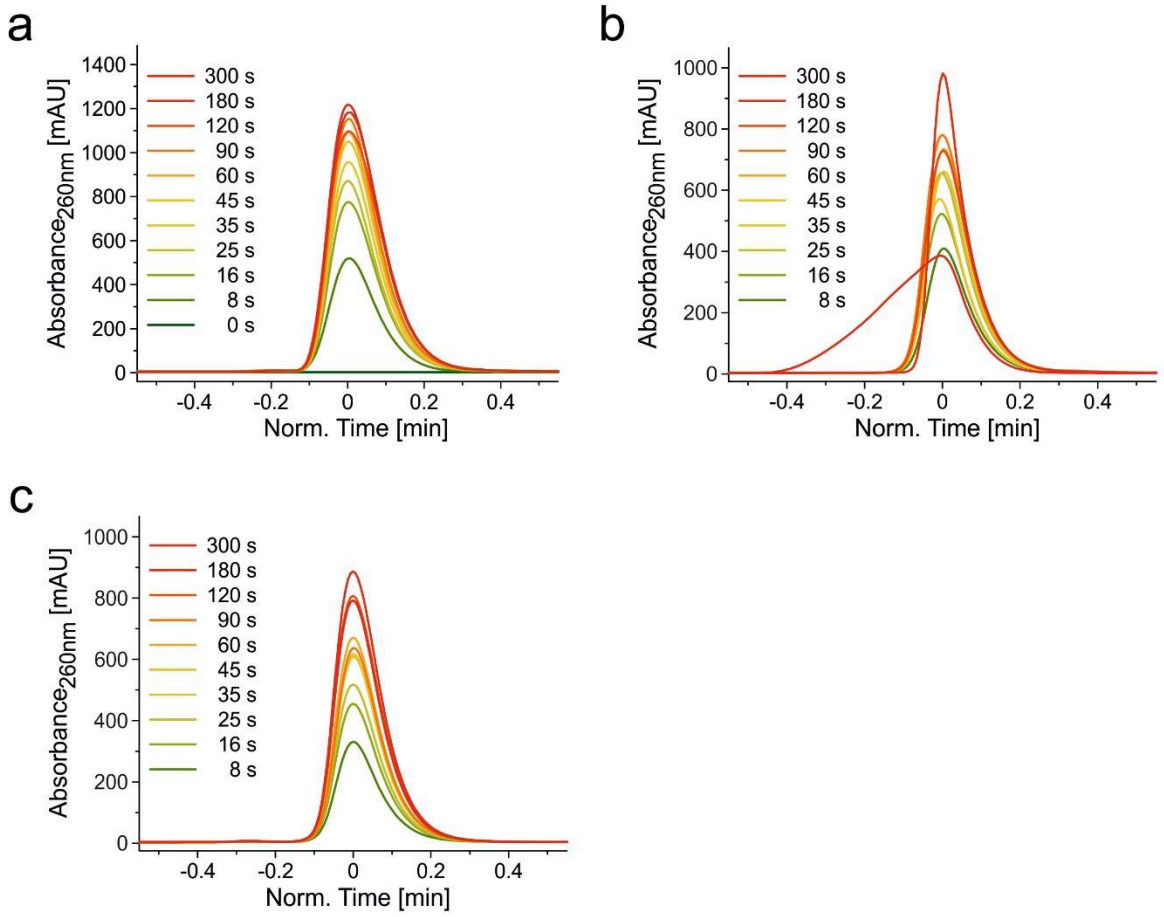

Figure S7: Kinetic analysis for ADPRC transglycosylation reactions with NAD^+ and PAB substrates following the preparation shown in Fig. 1f – HPLC analysis. HPLC chromatograms for a reaction mixture with 4PAB, 3PADB, or 4PADB showing an overlay of a) ADPR-4PAB, b) ADPR-3PADB, and c) ADPR-4PADB product peaks at different time-points of one reaction replicate, each.

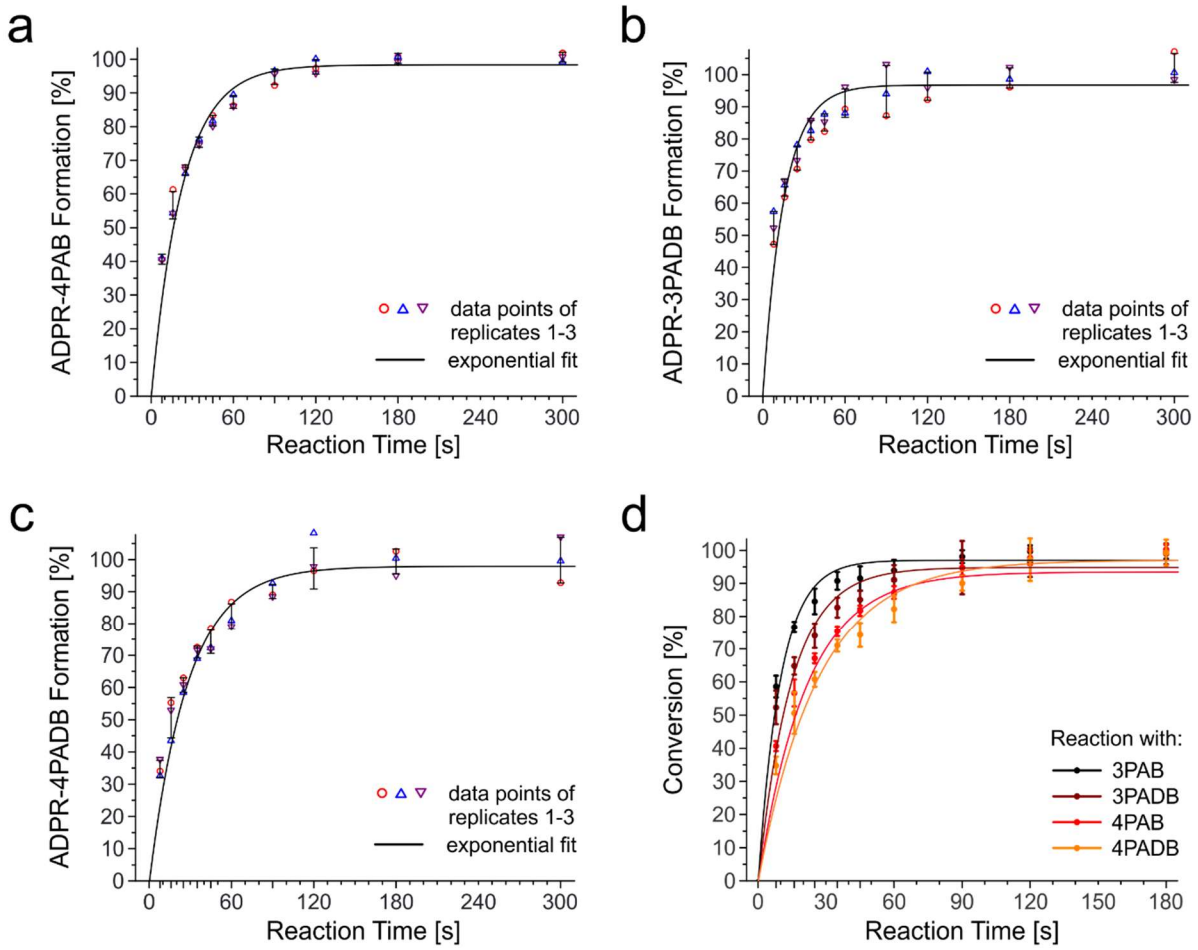

Figure S8: Results of the kinetic analysis for ADPRC transglycosylation reactions with NAD^+ and PAB substrates, with a) ADPR-4PAB, b) ADPR-3PADB, and c) ADPR-4PADB formation data for each time-point of the reaction replicates plotted against reaction time, and exponential fitting applied (x-axis: large ticks represent time-points 8 s, 16 s, 25 s, 35 s, 45 s, 60 s, 90 s, 120 s, 180 s, 300 s). d) Comparison of the results of the kinetic analysis after exponential fitting. The reactivity of tested PAB substrates decreases in the order of 3PAB > 3PADB > 4PAB > 4PADB. The k_{cat} / K_m values are 7.59×10^5 $M^{-1}s^{-1}$, 5.16×10^5 $M^{-1}s^{-1}$, 3.52×10^5 $M^{-1}s^{-1}$, 2.81×10^5 $M^{-1}s^{-1}$ respectively.

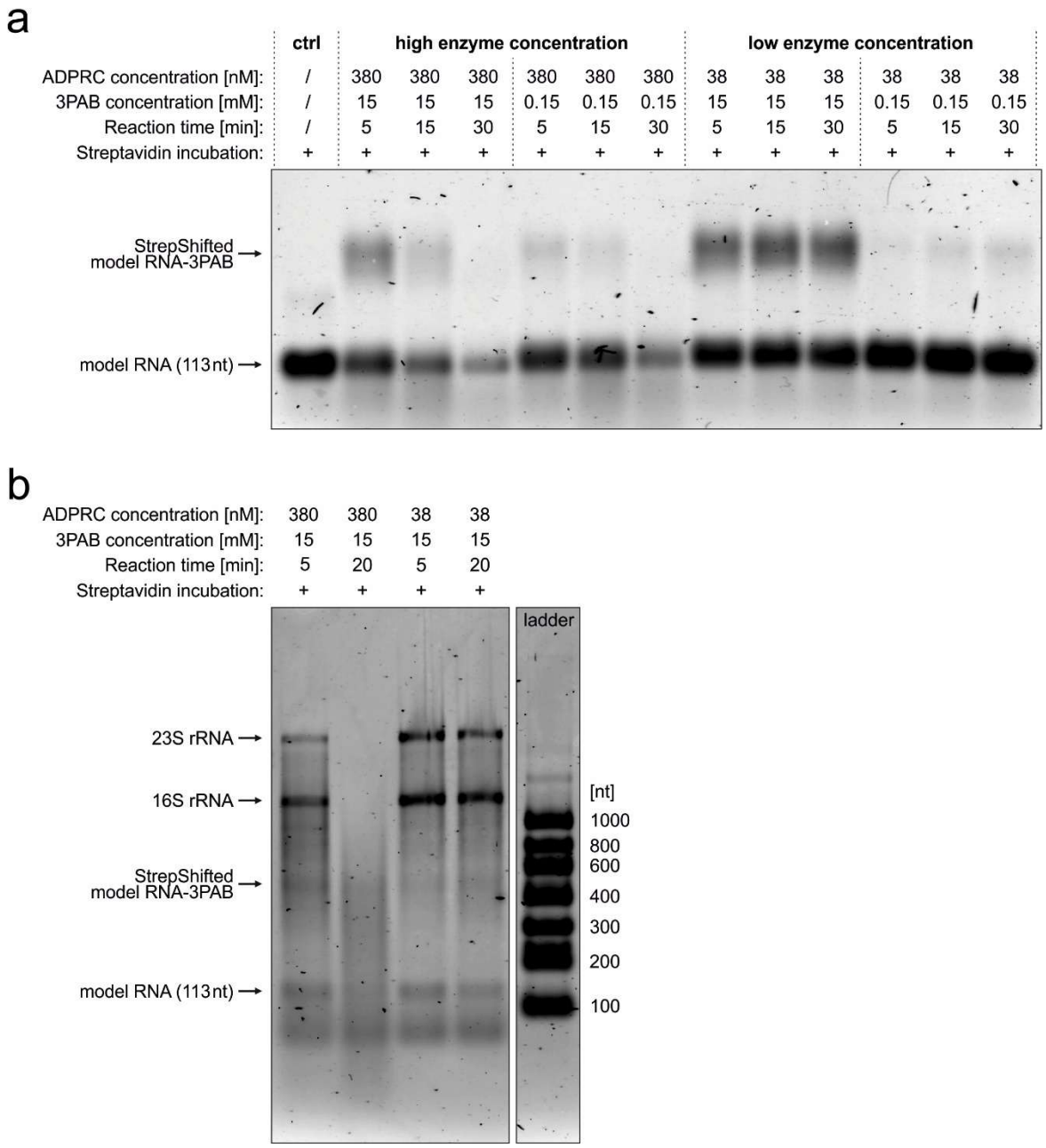

Figure S9: Optimization of the enzyme concentration in ADPRC transglycosylation reactions with 3PAB and RNA. a) StrepShift analysis on 2% agarose gel after ADPRC reactions starting from 5 µg (or 136 pmol) of a 113 nt model RNA (~1:1 NAD/pppRNA) with varied enzyme concentrations (380 nM, 38 nM), 3PAB concentrations (15 mM, 150 nM), and reaction times (5 min, 15 min, 30 min). b) StrepShift analysis on 2% agarose gel after ADPRC reactions starting from 0.5 µg of a 113 nt model RNA (~1:1 NAD/pppRNA) and 4.5 µg of *E. coli* total RNA with varied enzyme concentrations (380 nM, 38 nM) and reaction times (5 min, 20 min), and a 3PAB concentration of 15 mM. The conditions of choice with a fast conversion to the biotinylated RNA product and low degradation for all tested RNA mixtures and reaction times were found at an ADPRC concentration of 38 nM and a 3PAB concentration of 15 mM.

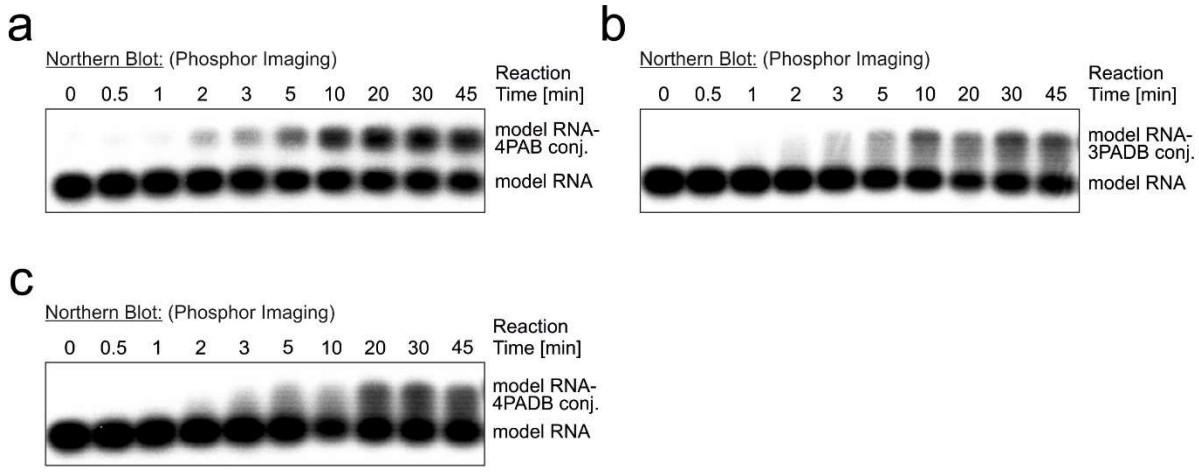

Figure S10: Kinetic analysis for ADPRC transglycosylation reactions (triplicates) with NAD-RNA (113 nt model RNA: 47.9% NAD-capped) and PAB substrates following the preparation shown in Fig. 1j – StrepShift analysis. Northern blots for a reaction mixture with 4PAB, 3PADB, or 4PADB showing the shifted a) 4PAB-modified, b) 3PADB-modified, and c) 4PADB-modified NAD-RNA bands at different time-points of one reaction replicate, each.

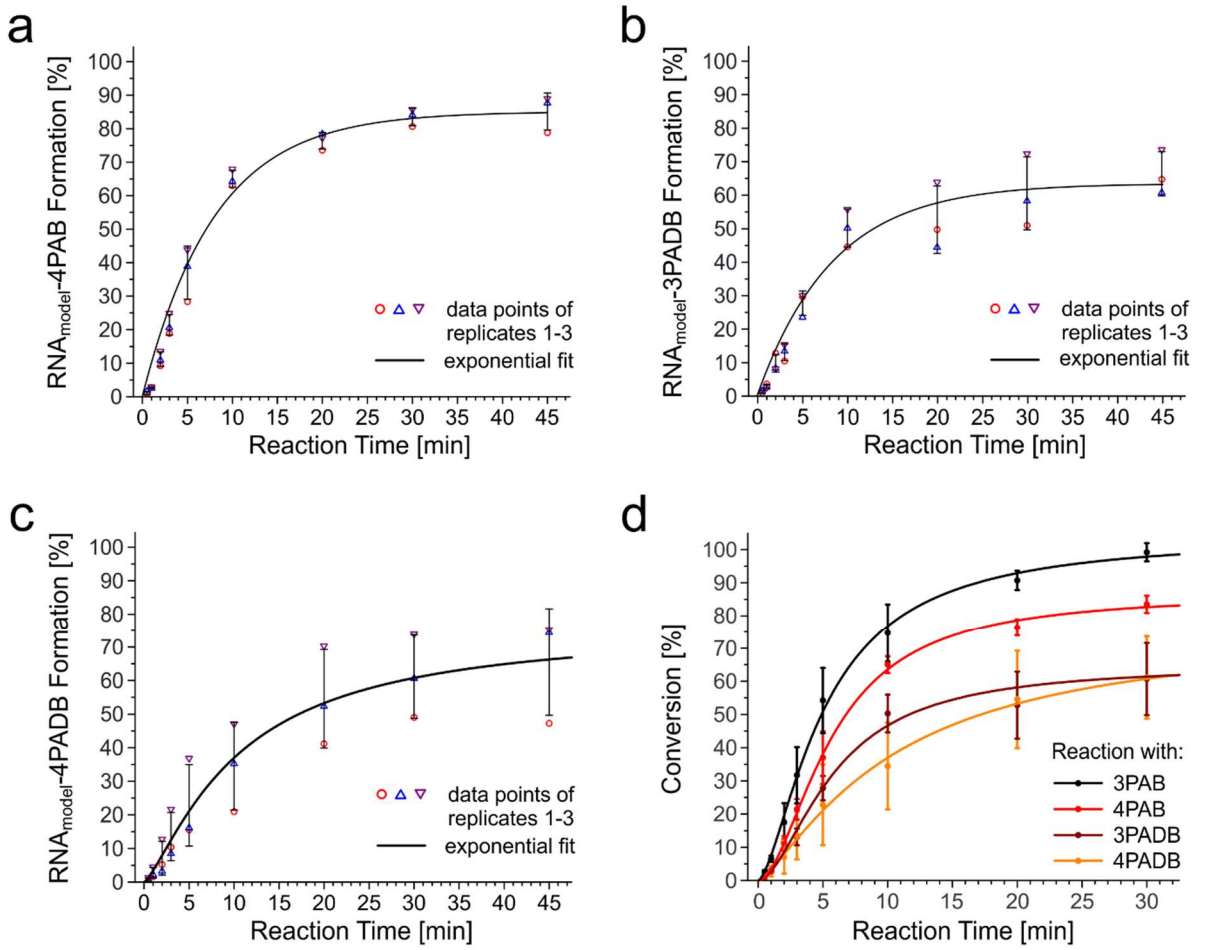

Figure S11: Results of the kinetic analysis for ADPRC transglycosylation reactions with NAD-RNA (113 nt model RNA: 47.9% NAD-capped) and PAB substrates, with product formation data for a) 4PAB-modified, b) 3PADB-modified, and c) 4PADB-modified RNAIII leader for each time-point of the reaction replicates plotted against reaction time, and exponential fitting applied. d) Comparison of the results of the kinetic analysis after exponential fitting. The reactivity of tested PAB substrates decreases in the order of 3PAB > 4PAB > 3PADB > 4PADB. The k_{cat} / K_m values are 6.17 ×10^5 $M^{-1}s^{-1}$, 5.47 ×10^5 $M^{-1}s^{-1}$, 5.25 ×10^5 $M^{-1}s^{-1}$, 3.43 ×10^5 $M^{-1}s^{-1}$ respectively.

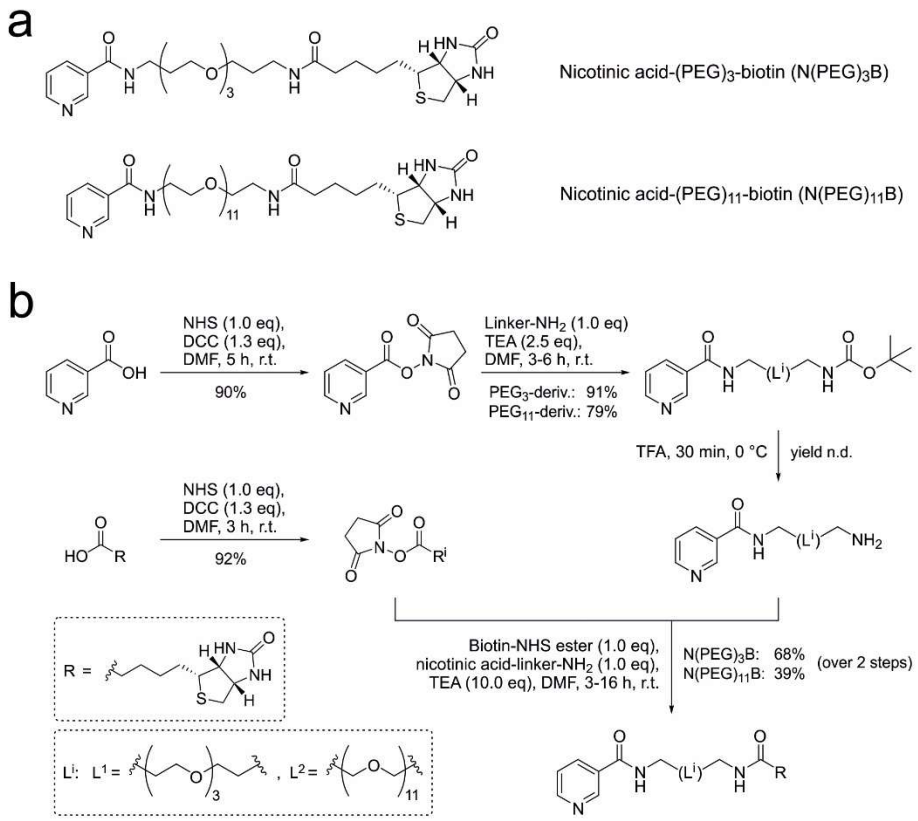

Figure S12: Chemical structures and synthesis of N(PEG)$_x$B conjugates as substrates for ADPRC transglycosylation based on 3PAB, but with extended polyethylene glycol (PEG) linkers. a) Chemical structures of nicotinic acid-(PEG)$_3$-biotin (N(PEG)$_3$B) and nicotinic acid-(PEG)$_{11}$-biotin (N(PEG)$_{11}$B). b) Synthesis route for N(PEG)$_3$B and N(PEG)$_{11}$B via NHS-ester activation of nicotinic acid (N-NHS) and biotin (B-NHS) and subsequent amide coupling to both ends of the initially Boc-protected linker molecules.

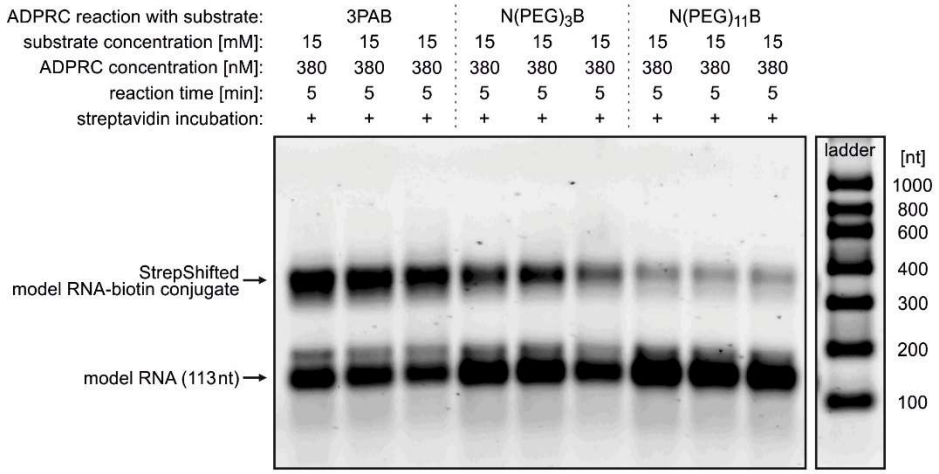

Figure S13: StrepShift analysis on 2% agarose gel after ADPRC reactions (triplicates) with nucleophilic substrates of varied linker length (3PAB, $N(PEG)_3B$, $N(PEG)_{11}B$) starting from 5 µg (or 136 pmol) of a 113 nt model RNA (~1:1 NAD/pppRNA) with depicted concentrations and reaction times shows a decrease in biotinylation efficiency with increasing linker length.

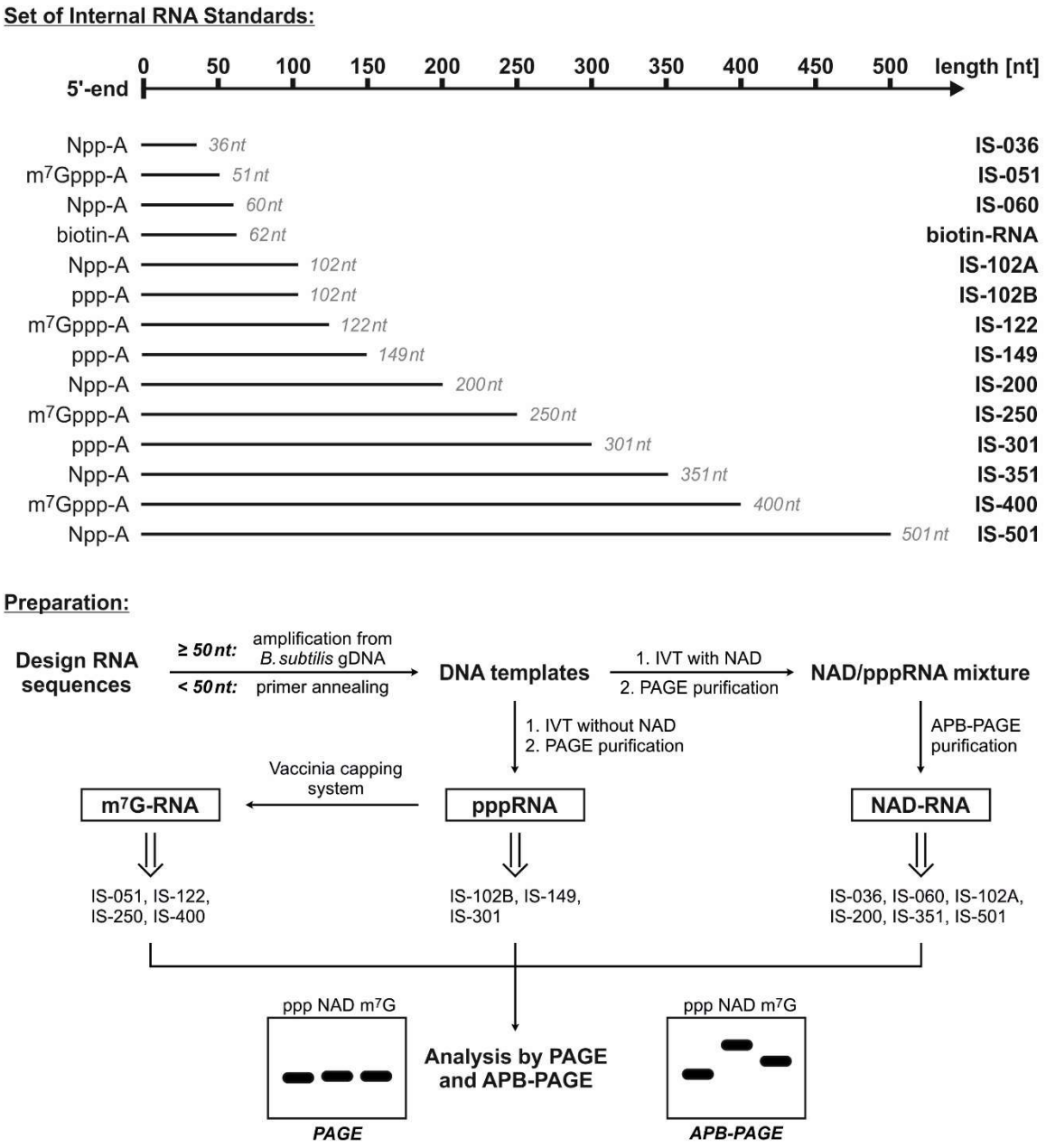

Figure S14: Set of internal RNA standards. The sequences were picked, amplified and *in vitro* transcribed from random regions of the *B. subtilis* genome and named with the identifier IS (internal standard) and the length of the RNA transcript by nucleotide count (e.g. IS-036 has a length of 36 nucleotides). Six of those transcripts were NAD-capped (Npp) and purified by APB-PAGE. Eight transcripts, which comprise of four m^7G-capped RNAs (m^7Gppp), three pppRNAs, and one biotin-RNA, serve as controls in the internal standard mix.

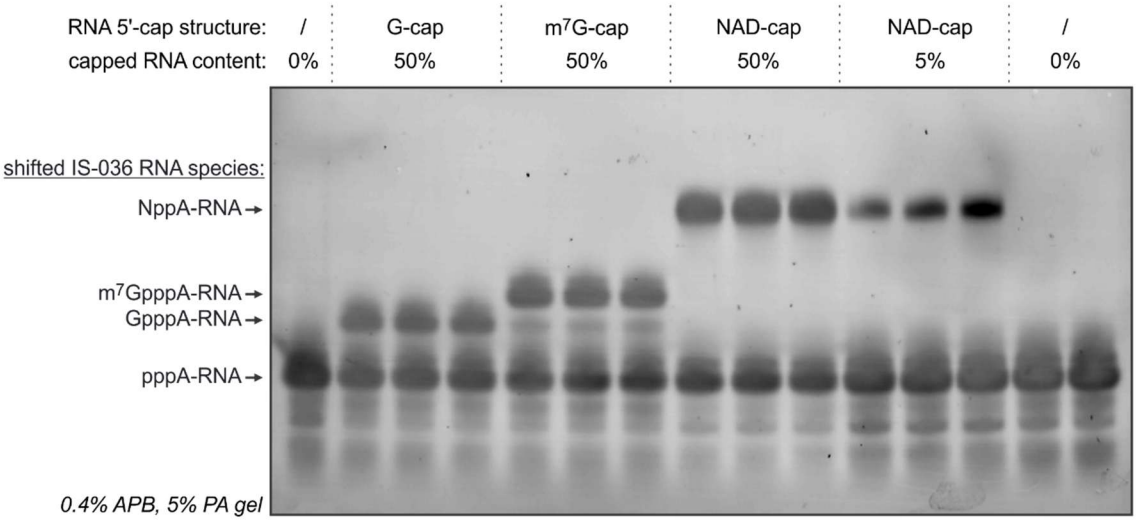

Figure S15: APB-PAGE analysis of IS-036 model RNAs with different 5'-end structures (NAD-cap: NppA, m^7G-cap: m^7GpppA, G-cap: GpppA). The various cap structures have different migration properties in the APB gel with the NAD cap structure leading to the slowest migration speed.

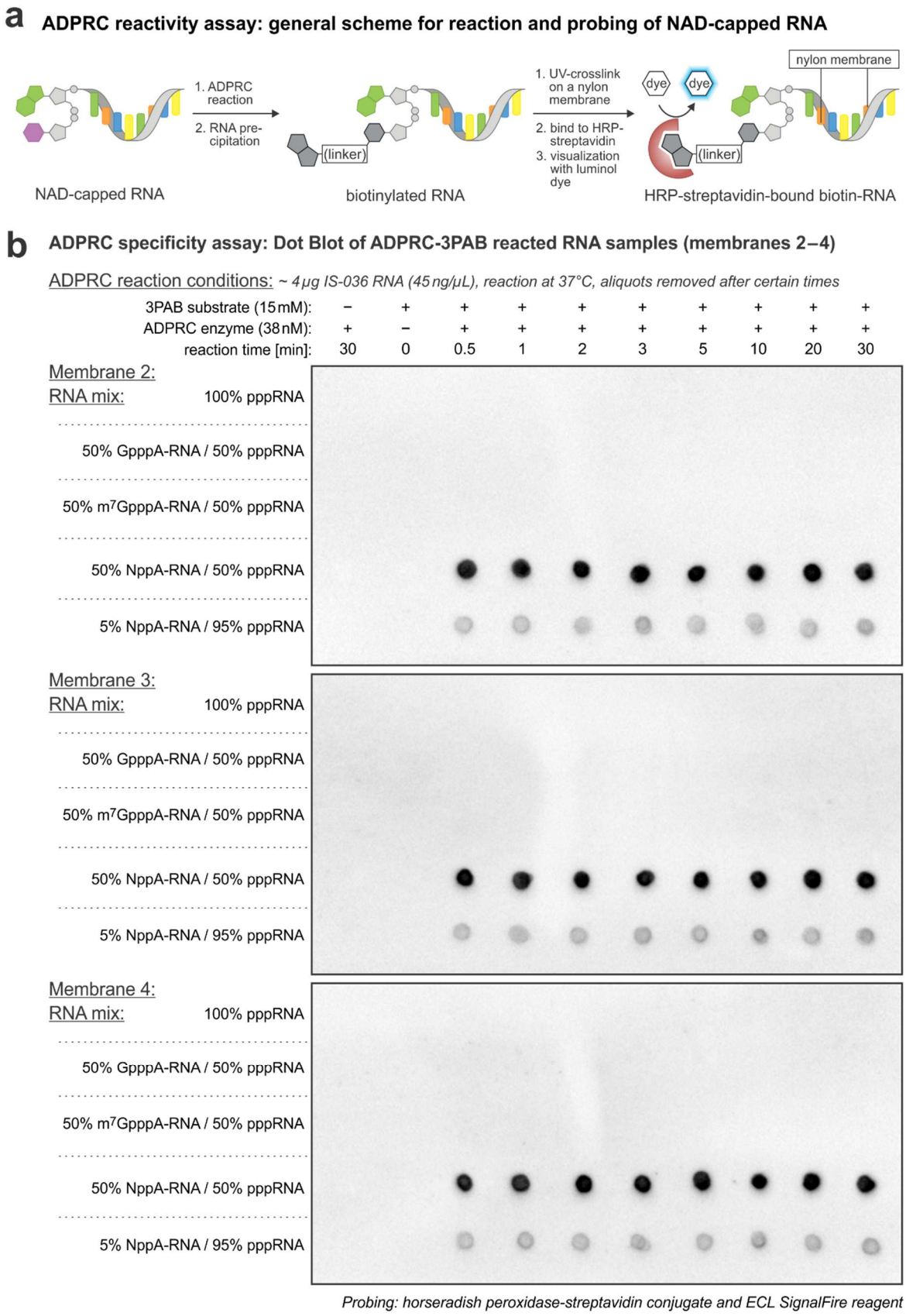

Figure S16: Specificity assay for the ADPRC transglycosylation reaction with 3PAB via dot blot (quadruplicates). a) Concept and workflow of the ADPRC specificity assay. b) 4 µg of IS-036 model RNA (45 ng/µL) were reacted in the presence of ADPRC (38 nM) and 3PAB (15

mM) at 37°C, while removing aliquots after certain reaction times, which were quenched and extracted with P/C/I and diethylether. 1 µL of the recovered RNAs (~50 ng/µL) were dotted onto a nylon membrane, UV-crosslinked, incubated with horseradish peroxidase-streptavidin conjugate, and probed with ECL SignalFire reagent before fluorescence readout.

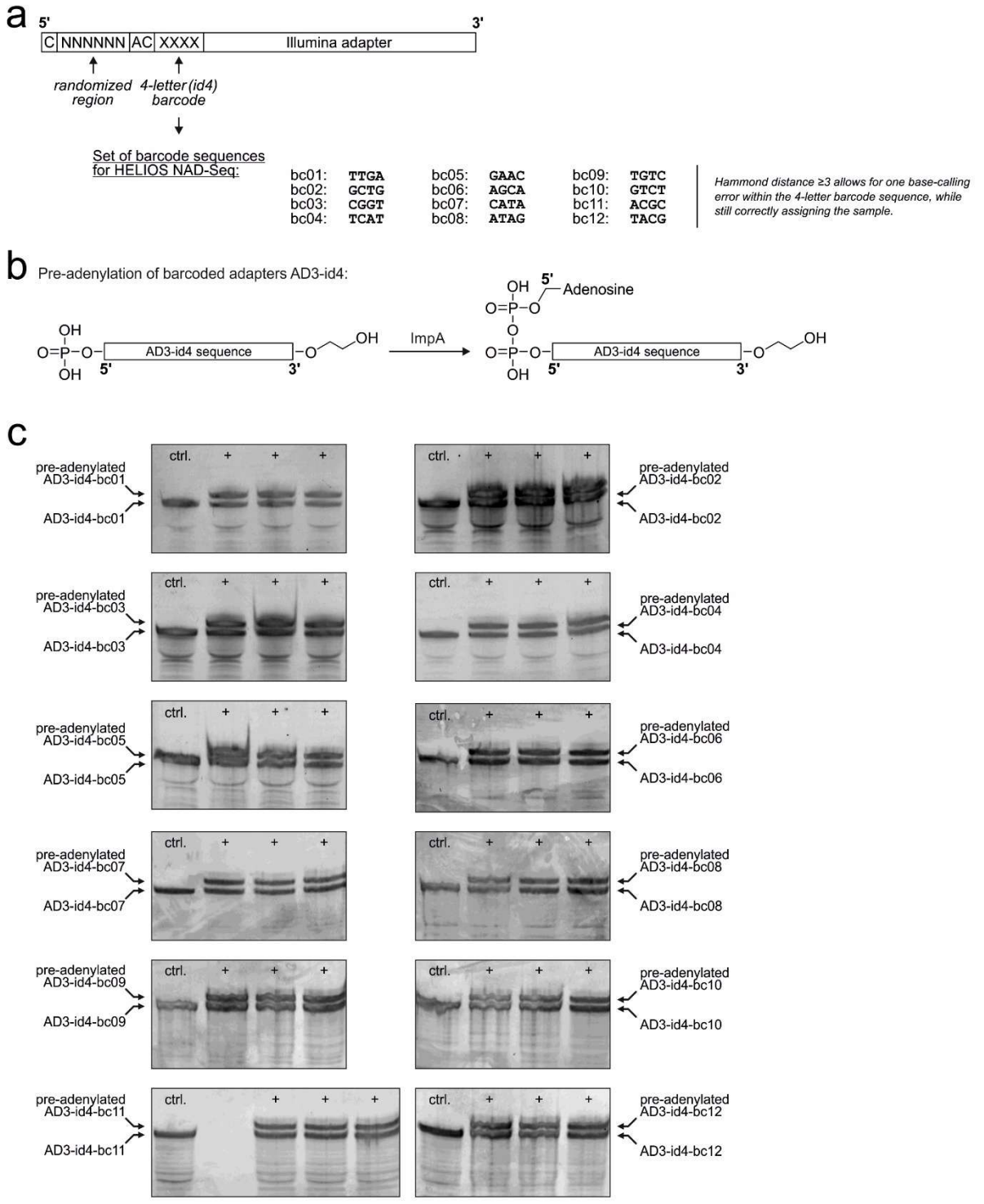

Figure S17: Design of 4-letter barcodes for 3'-ligation adapters (AD3-id4) applied in HELIOS NAD-Seq and pre-adenylation of AD3-id4 adapters. a) General design of AD3-id4 adapters and sequences for barcodes bc01 – bc12. The depicted set of bc01 – bc08 is chosen in HELIOS NAD-Seq for early multiplexing of a complete sample group (four biological replicates and four negative controls). b) Pre-adenylation reaction of adapters AD3-id4. c) Anaylsis of the pre-adenylation reaction of barcoded adapters AD3-id4 by 20% PAGE (SYBR Gold staining). Adenylation levels of around 50% are readily reached and suffice for application in HELIOS NAD-Seq.

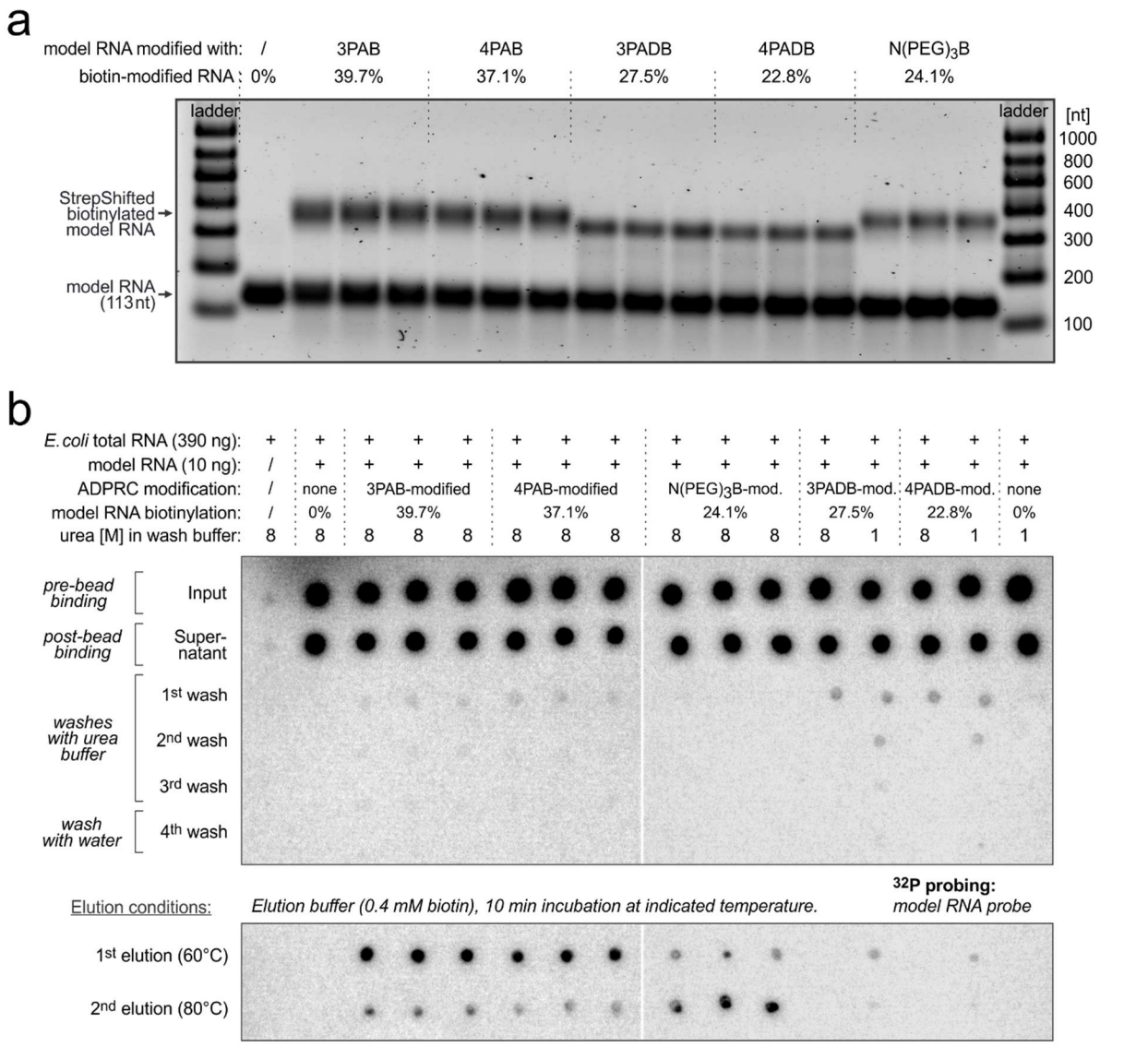

Figure S18: ADPRC transglycosylation reactions of a 113 nt model RNA and evaluation of binding interactions with streptavidin magnetic beads. a) StrepShift assay (triplicates) after shortened ADPRC transglycosylation reactions of model RNA (113 nt) with 3PAB, 4PAB, 3PADB, 4PADB, and $N(PEG)_3B$, which led to lower modification ratios in order to monitor specific and unspecific binding of the model RNA on streptavidin magnetic beads. b) Dot blot analysis after streptavidin magnetic bead purification and elution of 3PAB-, 4PAB-, $N(PEG)_3B$-, 3PADB-, and 4PADB-modified model RNAs (113 nt) in the presence of *E. coli* total RNA. Supernatant, wash and elution aliquots were blotted, UV-crosslinked and [^{32}P]-labeled with a complementary model RNA probe.

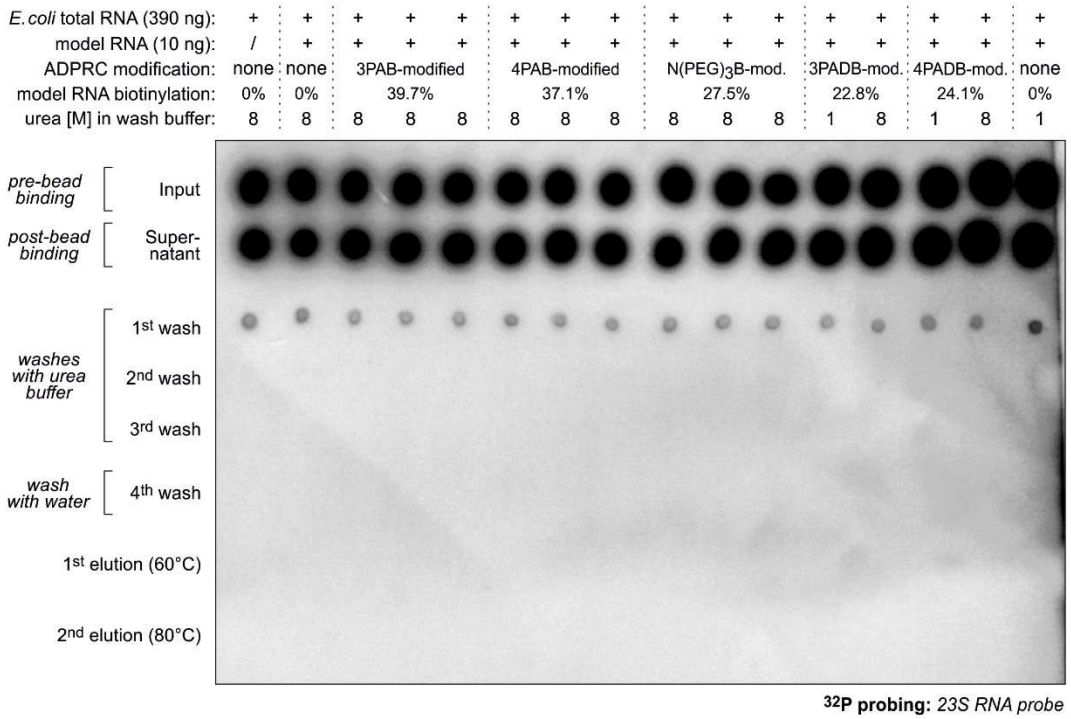

Figure S19: Dot blot analysis after streptavidin magnetic bead purification and elution of different ADPRC-modified model RNAs (113 nt) in the presence of *E. coli* total RNA. Supernatant, wash and elution aliquots were blotted, UV-crosslinked and [^{32}P]-labeled with a complementary 23S RNA probe.

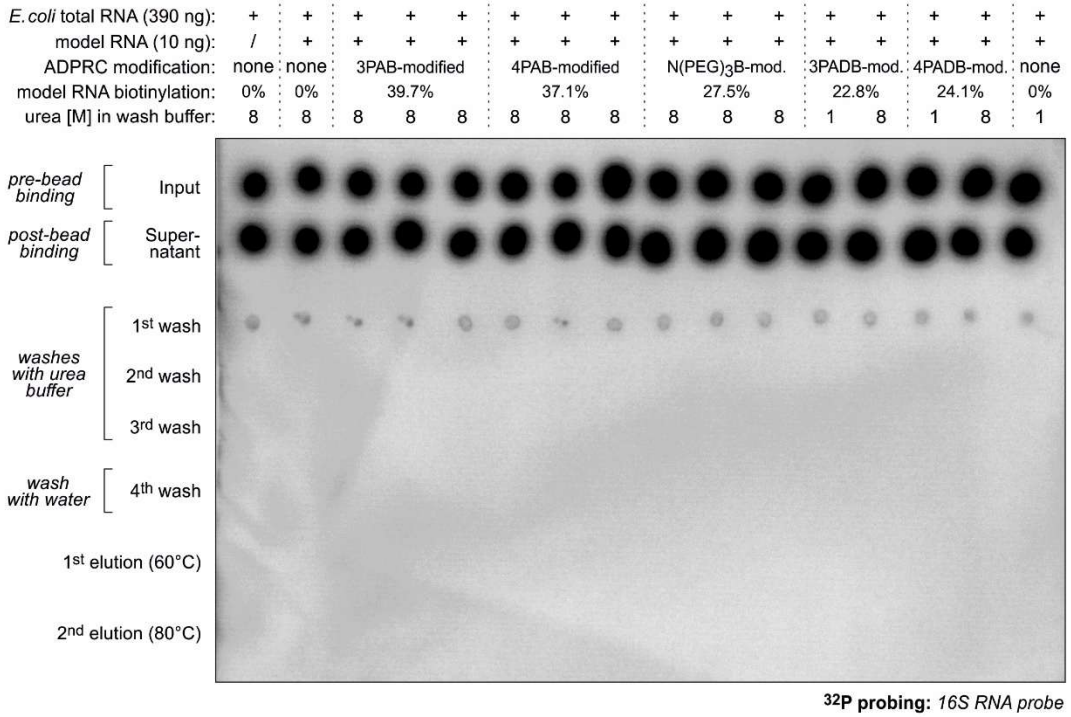

Figure S20: Dot blot analysis after streptavidin magnetic bead purification and elution of different ADPRC-modified model RNAs (113 nt) in the presence of *E. coli* total RNA. Supernatant, wash and elution aliquots were blotted, UV-crosslinked and [^{32}P]-labeled with a complementary 16S RNA probe.

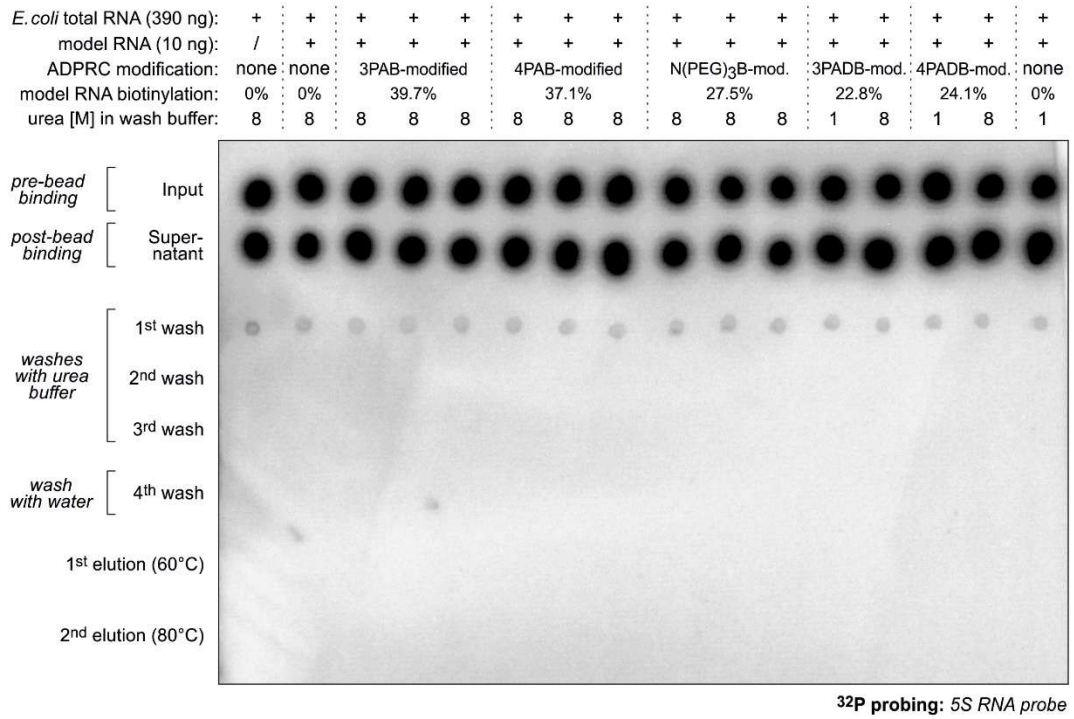

Figure S21: Dot blot analysis after streptavidin magnetic bead purification and elution of different ADPRC-modified model RNAs (113 nt) in the presence of *E. coli* total RNA. Supernatant, wash and elution aliquots were blotted, UV-crosslinked and [^{32}P]-labeled with a complementary 16S RNA probe.

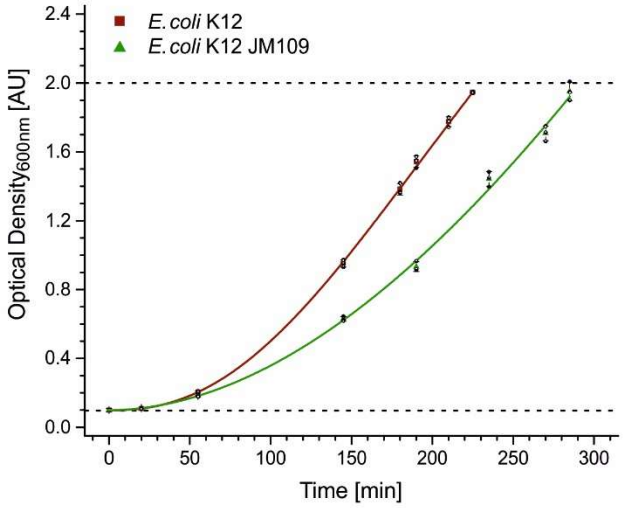

Figure S22: Growth curve of *E. coli* K12 JM109 (biological triplicates) and comparison with *E. coli* K12 strain. Cultures were harvested at the late exponential phase (last data point) at an OD_{600} of approximately 2.0. The three datasets per strain were averaged and sigmoidal fits applied for visualization purposes.

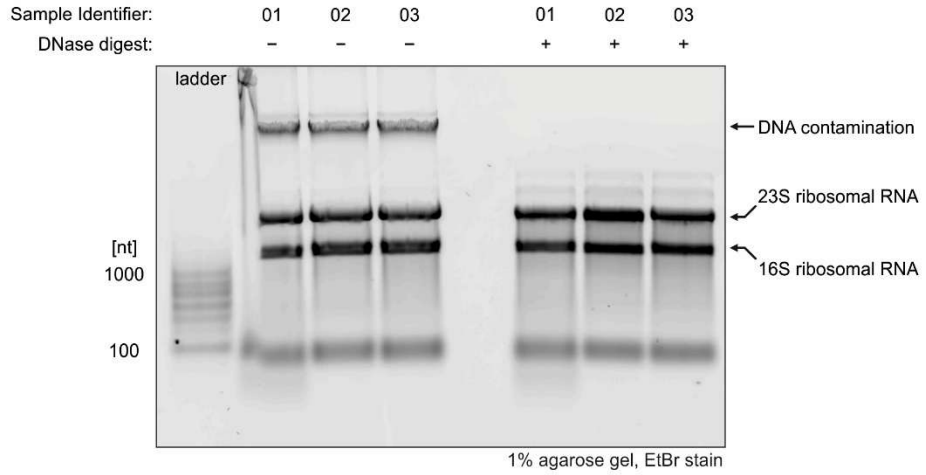

Figure S23: Agarose gel electrophoresis to analyze hot phenol-extracted total RNA samples (biological triplicates) from *E. coli* K12 JM109. After DNase-digest, the DNA contamination was successfully removed.

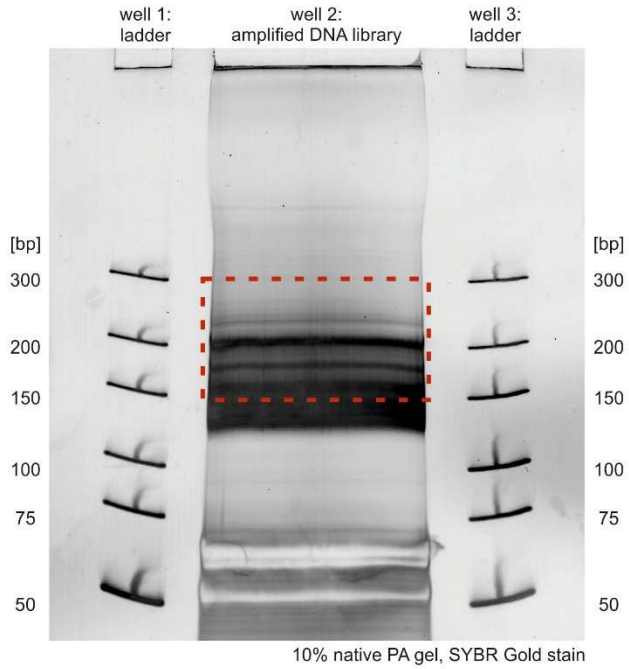

Figure S24: Native PAGE (10%) purification for HELIOS NAD-Seq with E. coli *K12 JM109* samples. Marked with a red-dashed box is the excision area for size selection (150–300 bp), making sure that the adapter/primer artifact visible at around 140 bp was removed before sequencing. DNA ladder: GeneRuler UltraLow Range DNA Ladder.

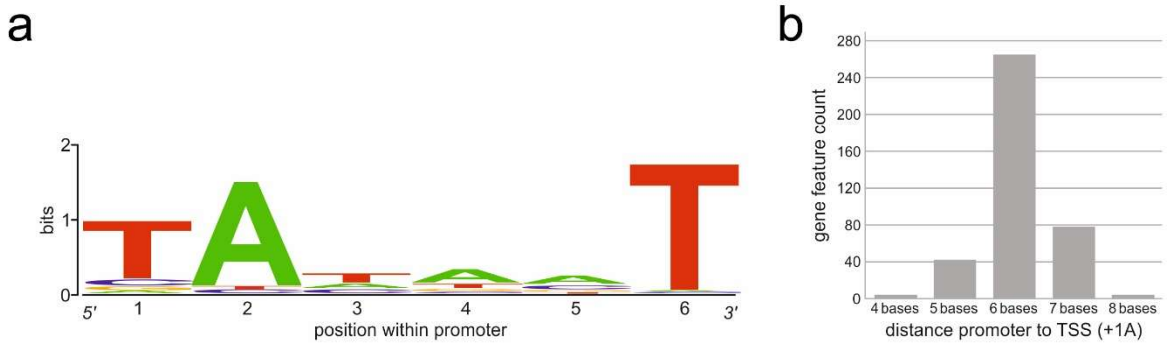

Figure S25: Results of HELIOS NAD-Seq with *E. coli* K12 JM109 samples – promoter for identified NAD-capped transcripts. a) −10 promoter motif for identified NAD-capped transcripts assigned to gene features from *E. coli* K12 JM109 Escherichia coli K-12 substr. MG1655 genome annotation (assembly ASM584v2, RefSeq accession GCF_000005845.2), and b) distance of the promoter motif to the TSS (+1A).

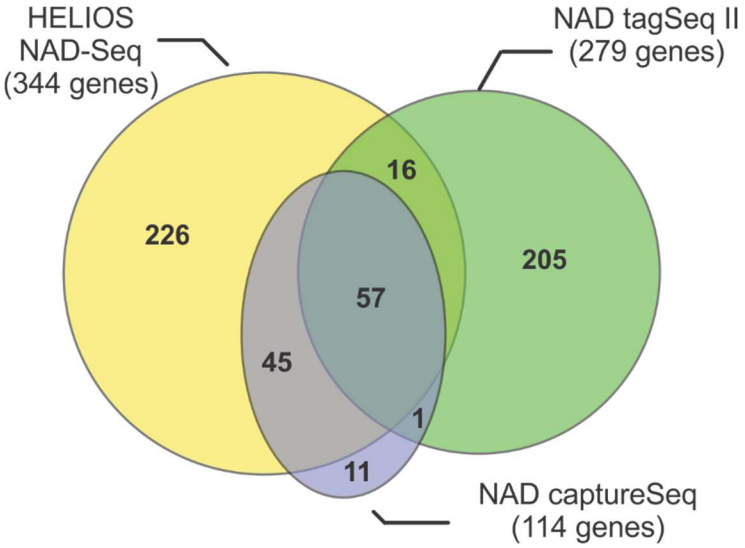

Figure S26. Comparison of overlapping gene features giving rise to NAD-capped RNAs between HELIOS NAD-Seq, NAD captureSeq (all three datasets), NAD tagSeq II

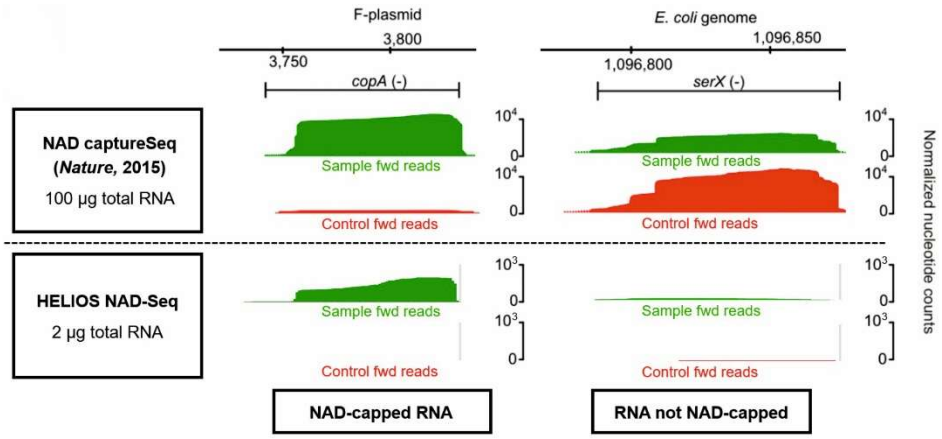

Figure S27: Example for read coverage of an NAD-capped RNA and a non-capped RNA from *E. coli* K12 JM109 and comparison between NAD captureSeq and HELIOS NAD-Seq. High enrichment for the NAD-RNA can be observed for both protocols, while only HELIOS NAD-Seq shows close to 0 reads in the control at roughly 6 million read sequencing depth for the copA transcript. Also, the non-capped transcript from the serX gene, which showed a major background signal in NAD captureSeq[1], is clearly identified with low sample and control read counts in HELIOS NAD-Seq.

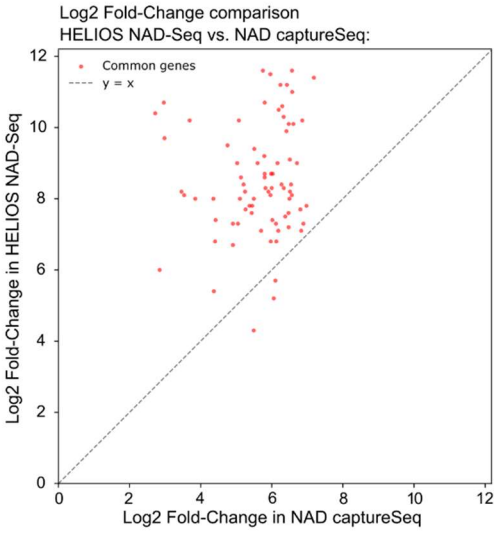

Figure S28: Comparison of $\log_2$ fold-changes between HELIOS NAD-Seq and NAD captureSeq with E.coli total RNA isolated at O.D ≈ 2.0. Except for 3 genes, HELIOS NAD-Seq generated higher $\log_2$ fold-changes.

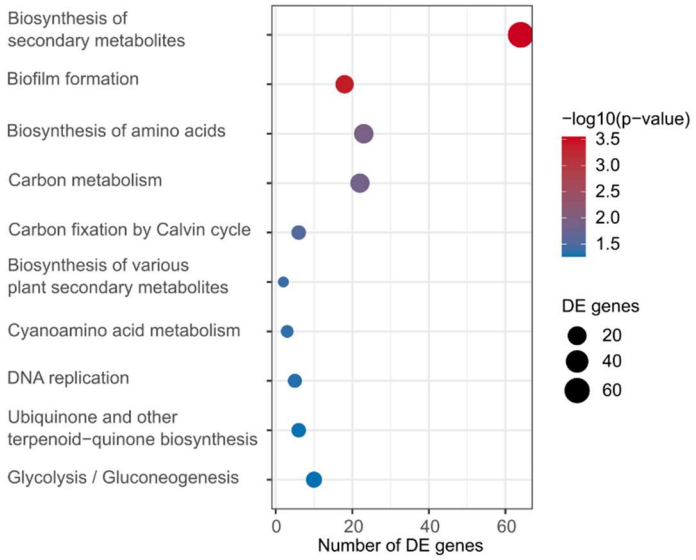

Figure S29: KEGG pathway enrichment analysis of NAD-capped transcripts at timepoint 7 (3 hours and 30 minutes after inoculation). Enriched pathways are shown with number of genes related to each pathway and the corresponding adjusted *p*-values.

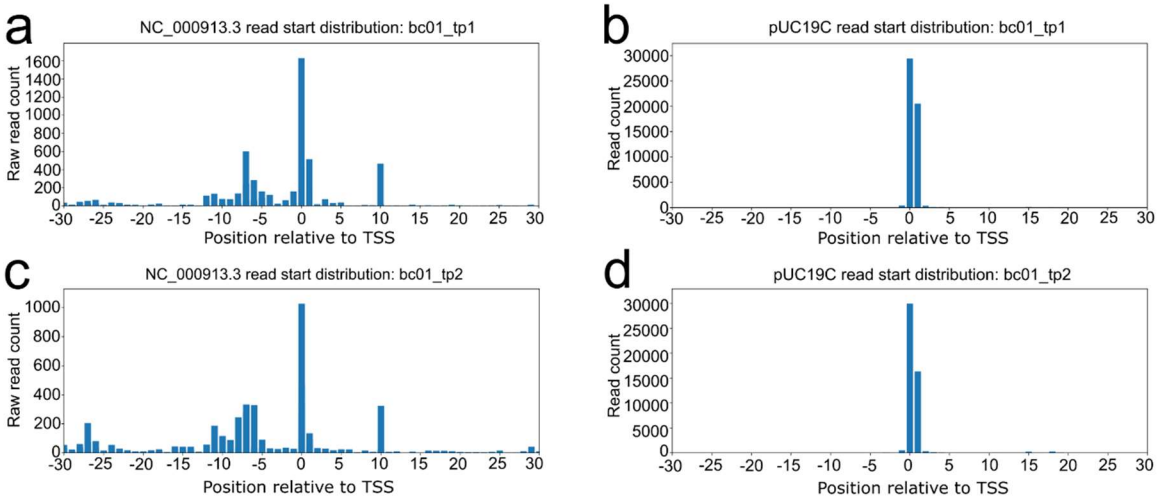

Figure S30: Transcript start site (TSS) mapping of NAD-capped RNAs to the *E. coli* K-12 JM109 genome (NC_000913.3) and the pUC19 plasmid. 4 representative bar plots from timepoints 1 and 2 for barcode 1 (positive sample) show enrichment at the +1 position (labelled as 0 position relative to TSS) from the -30 to +30 relative TSS position.

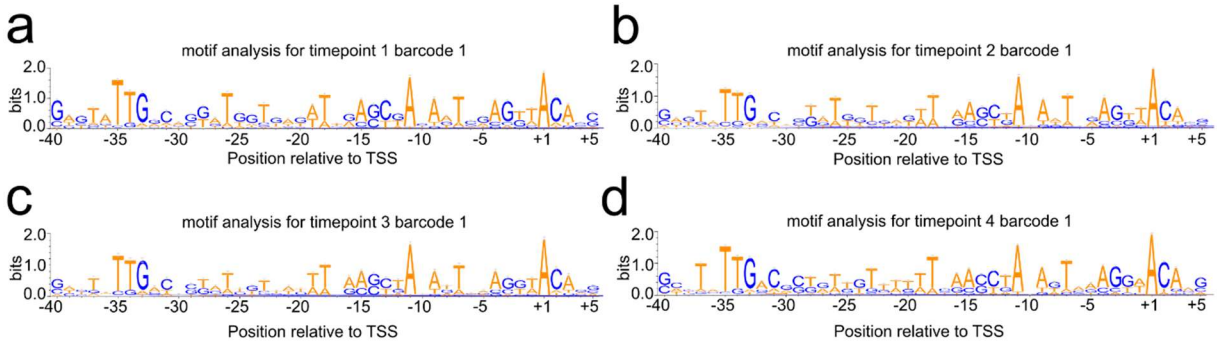

Figure S31: Motif analysis of upstream promoter regions of NAD-capped transcripts. The −10 promoter element (TANNNT) was detected across a majority of timepoints and positive samples that align to the *E. coli* K-12 JM109 genome (NC_000913.3). Four representative motif analysis plots from time points 1 to 4 for barcode 1 is shown.

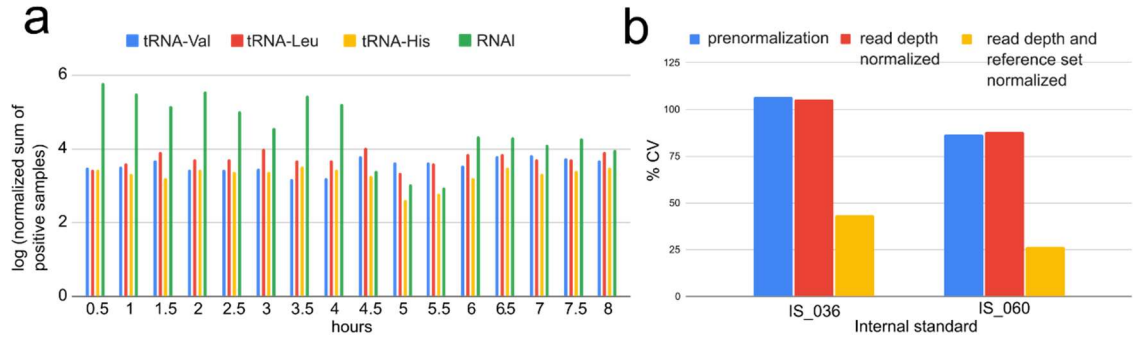

Figure S32: a) Stability of reference genes used for normalization. Expression profiles (normalized sum of all positive samples) of three representative tRNAs (tRNA-Val, tRNA-Leu, tRNA-His) are shown across 16 timepoints, demonstrating stable expression compared to the dynamic profile of RNA1. b) Effect of two-step normalization on internal standard variability. Coefficient of variation (%CV) values for IS036 and IS060 before and after normalization are shown, highlighting significant reduction in %CV after the second step.

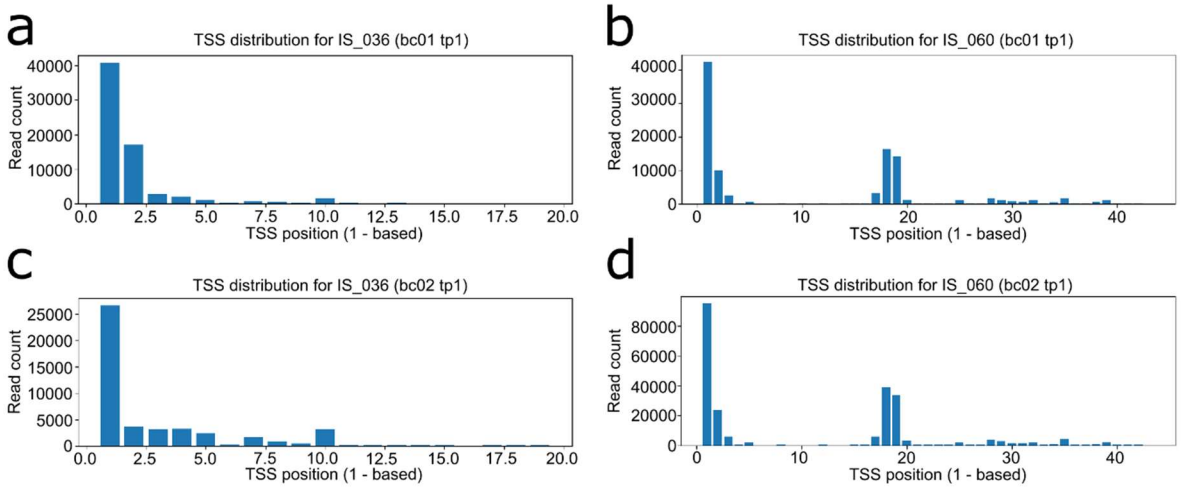

Figure S33: Internal standards IS036 and IS060 show consistent +1 TSS capture across all positive samples and timepoints. Representative TSS plots are shown for the two internal standards for time point 1 barcodes 1 and 2.

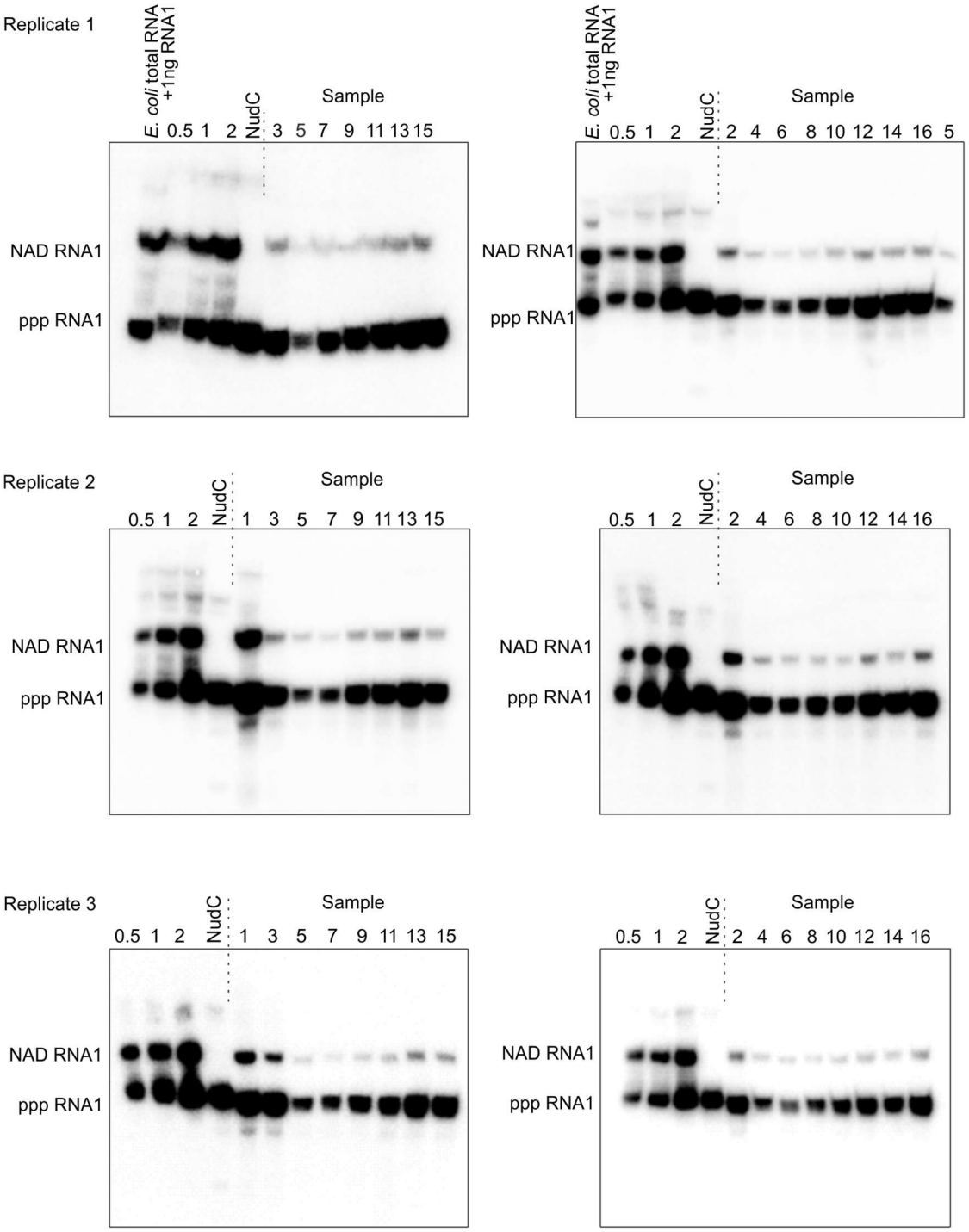

Figure S34: Northern blot detection of RNA1 abundance across 16 timepoints using APB-PAGE and 5'-end γ-[^{32}P]-ATP–labeled probes. ppp-RNA1 abundance is shown in the lower band and NAD-RNA1 abundance is shown in the upper band. (n=3)

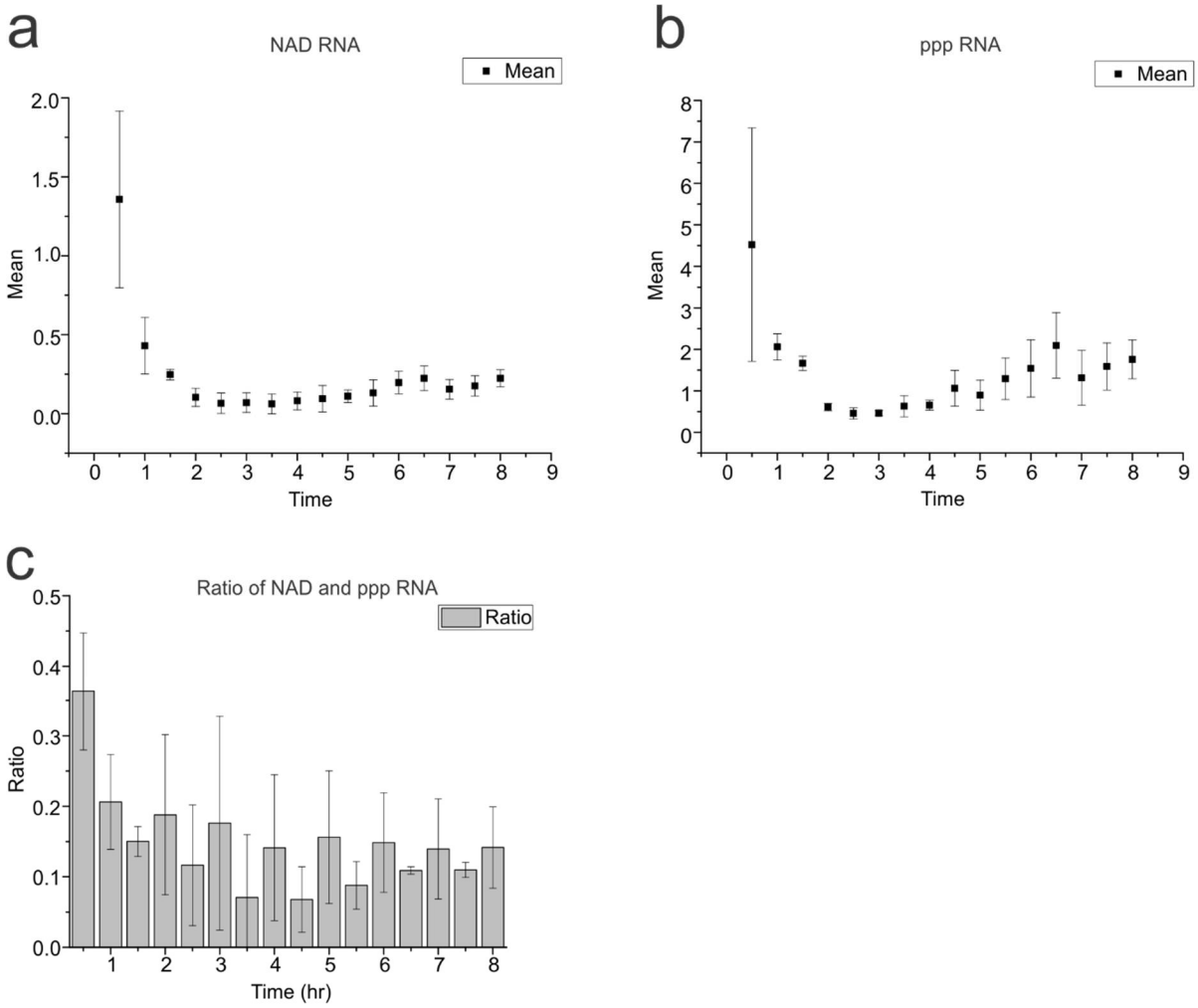

Figure S35: Quantification of changes in a) NAD-RNA1, b) ppp-RNA1 and c) capping ratio changes across the time points. Highest abundance of both non-capped and NAD-capped RNA1, with highest NAD capping ratios were found in time point 1 (30 minutes after inoculation).

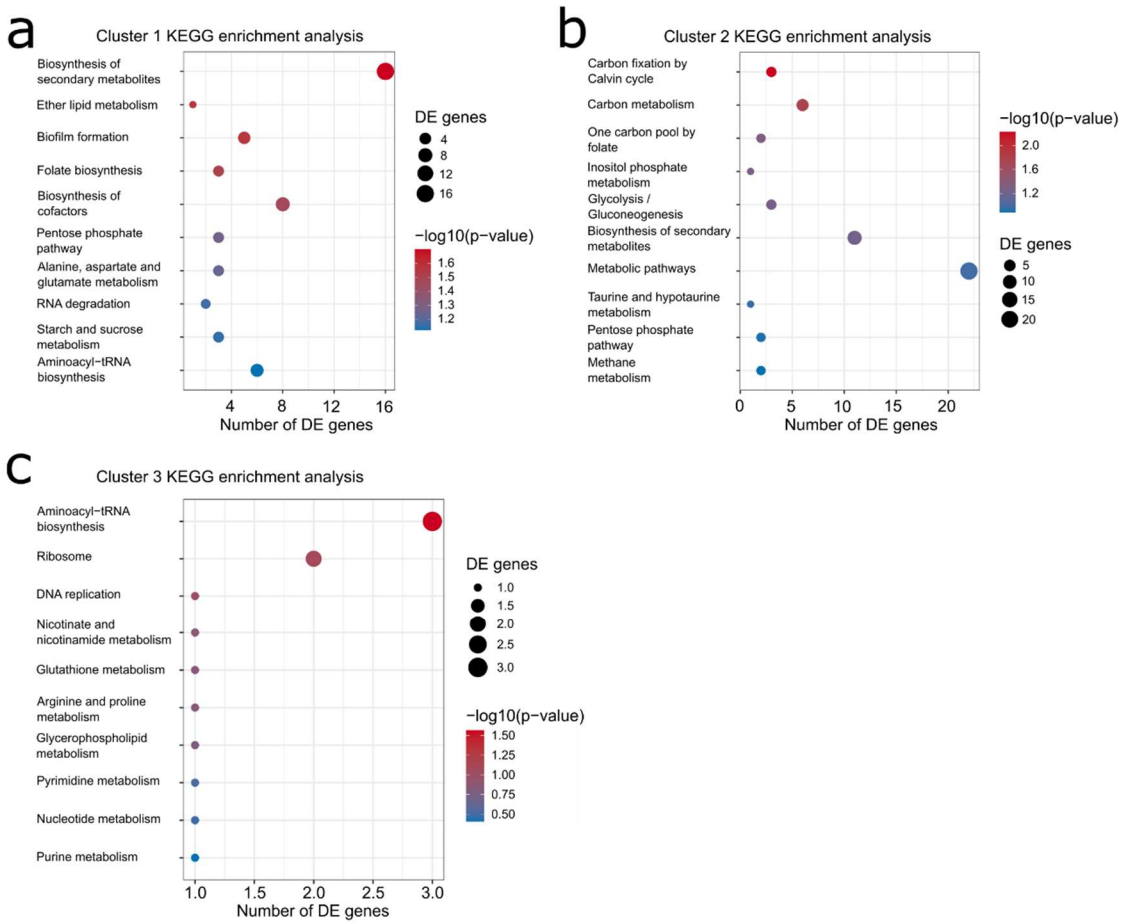

Figure S36: KEGG pathway enrichment analysis for genes in a) cluster 1 and b) cluster 2 of the NAD-capped RNA expression heatmap (Figure 3 e,f). Enriched pathways are shown with number of genes related to each pathway and the corresponding adjusted *p*-values.

Supplementary Tables:

Table S1: Results from the analysis of the sequencing reads aligning to internal RNA standards for the sequencing data set from HELIOS NAD-Seq with *E. coli* K12 JM109 samples. Enrichment factors and log_2-fold changes were calculated from the 5'-corrected raw read counts (Table S2) after normalization to the internal biotin-RNA standard.

internal standard	5'-end structure	RNA length	log_2-fold change
biotin-RNA	biotin	62	0
IS-102B	ppp	102	0
IS-149	ppp	149	0
IS-301	ppp	301	0
IS-051	m^7G	51	0
IS-122	m^7G	122	4.9
IS-250	m^7G	250	5.5
IS-400	m^7G	400	0
IS-036	NAD	36	12.0
IS-060	NAD	60	12.5
IS-102A	NAD	102	10.6
IS-200	NAD	200	13.5
IS-351	NAD	351	9.0
IS-501	NAD	501	12.4

Table S2: Raw sequencing read counts with 5'-end correction (5'-adenosine), which align to the internal RNA standards for the sequencing data set from HELIOS NAD-Seq with *E. coli* K12 JM109 samples. Positive samples were tagged with barcodes bc01–bc03, negative controls with barcodes bc04–bc06.

internal standard	**5'-end structure**	**read counts for each sample/control**					
		bc01	**bc02**	**bc03**	**bc04**	**bc05**	**bc06**
Raw sequencing counts		35265254	37681136	42392083	5942629	5589215	6076486
biotin-RNA	*biotin*	1309929	937470	1054815	764752	766684	806062
IS-102B	*ppp*	0	0	0	0	0	0
IS-149	*ppp*	0	0	0	0	0	0
IS-301	*ppp*	0	0	0	0	0	0
IS-051	m^7G	0	0	0	0	0	0
IS-122	m^7G	76	0	0	0	0	0
IS-250	m^7G	91	0	15	0	0	0
IS-400	m^7G	0	0	0	0	0	0
IS-036	*NAD*	707257	1528346	674869	55	126	380
IS-060	*NAD*	303981	336582	712091	11	133	26
IS-102A	*NAD*	36853	23308	32575	26	5	10
IS-200	*NAD*	10420	7015	7076	0	0	0
IS-351	*NAD*	552	259	332	0	0	0
IS-501	*NAD*	6129	1875	4148	0	0	0

Table S3: Results from the bioinformatic analysis of the sequencing data set from HELIOS NAD-Seq with *E. coli* K12 JM109 samples and the gene features the identified RNA transcripts align to. A very high probability of NAD-capping was assigned to those transcripts, as they fulfill a challenging set of restrictions (baseMean ≥50, Log2-fold change ≥3, adjusted p-value ≤0.01). The transcripts marked with an asterisk (*) are RNA species that were also identified in at least one of the NAD captureSeq data sets[1].

RNA transcript	Category	BaseMean	log_2-fold change	adjusted p-value
RNAI *	sRNA	173637	7.6	1.1E-19
QUAD, sibD *	sRNA	22656	10.6	2.7E-12
QUAD, sibE *	sRNA	11910	9.1	4.2E-16
sfsA *	protein-coding	9967	10.5	6.8E-13
sroB (chiX) *	sRNA	7329	9.0	2.8E-11
GadY *	sRNA	6017	11.4	5.7E-19
raiA *	protein-coding	4451	7.4	1.2E-09
QUAD, sibC *	sRNA	3889	11.6	1.1E-11
yfjI	protein-coding	2299	9.1	1.7E-16
ykgS	pseudogene	2294	9.9	3.2E-15
SraC_RyeA *	sRNA	2225	6.8	2.7E-04
rlmI *	protein-coding	1999	11.6	1.6E-06
bsmA *	protein-coding	1961	11.5	4.0E-05
GcvB *	sRNA	1571	11.2	5.7E-07
yaiO *	protein-coding	1558	11.2	1.4E-06
gatY *	protein-coding	1351	9.2	4.9E-14
hdeD *	protein-coding	1323	11.0	1.3E-06
glgX *	protein-coding	1102	10.7	4.1E-06
ompP *	protein-coding	1098	10.7	2.0E-06
epd *	protein-coding	947	9.5	7.9E-09
ymdB	protein-coding	887	10.4	3.5E-06
traG *	protein-coding	877	10.4	3.9E-06
csdA *	protein-coding	859	10.3	7.7E-06
bfr	protein-coding	837	10.3	5.7E-06
aspA *	protein-coding	787	10.2	2.2E-05
yuaQ	protein-coding	786	10.2	6.3E-06
ykgN *	pseudogene	770	10.2	7.5E-06
nuoG	protein-coding	770	5.6	9.6E-03
erfK	protein-coding	762	10.2	7.2E-06
tqsA *	protein-coding	754	10.2	1.2E-05
cdsA *	protein-coding	736	10.1	5.3E-06
yiaT	protein-coding	735	10.1	2.4E-05
efp	protein-coding	732	9.2	2.6E-07
repA2	protein-coding	709	10.1	1.2E-05
ydfJ *	pseudogene	701	8.2	5.0E-09
racR *	protein-coding	699	10.1	6.3E-06
talA	protein-coding	665	6.6	1.1E-06
ydjN *	protein-coding	639	7.4	5.3E-10

mnmA	protein-coding	623	9.9	7.7E-06
lrhA *	protein-coding	616	9.9	8.9E-05
yhcF	protein-coding	585	9.8	2.2E-05
ybaJ	protein-coding	574	9.8	2.4E-05
ribA	protein-coding	569	9.8	1.2E-05
traY *	protein-coding	549	9.7	1.1E-05
gltF	protein-coding	512	9.6	1.5E-05
nadR	protein-coding	466	9.5	5.2E-05
betT	protein-coding	453	9.4	1.0E-04
CopA *	sRNA	432	9.4	6.2E-05
yhbT	protein-coding	430	9.4	3.5E-05
poxA *	protein-coding	418	8.4	6.7E-07
ygcB	protein-coding	416	9.3	2.4E-05
ybgC	protein-coding	415	8.3	1.5E-06
rplT	protein-coding	395	9.2	3.6E-05
ycfH	protein-coding	394	8.3	1.7E-06
ycbJ *	protein-coding	383	8.2	2.5E-06
yahN	protein-coding	376	8.2	5.3E-07
osmB	protein-coding	372	9.1	8.3E-05
pqqL	protein-coding	366	8.2	1.5E-06
yifK	protein-coding	362	9.1	1.7E-04
kdsD *	protein-coding	361	7.3	5.0E-09
fimH	protein-coding	351	9.1	2.5E-04
aroG	protein-coding	346	7.0	7.7E-06
CsrC *	sRNA	339	8.1	2.3E-06
thrS *	protein-coding	336	9.0	1.7E-04
pyrG *	protein-coding	335	8.0	1.2E-06
allR	protein-coding	335	9.0	1.2E-04
yniB *	protein-coding	331	9.0	2.1E-04
ygdH *	protein-coding	329	9.0	5.7E-05
ubiC	protein-coding	322	8.9	1.8E-04
trmA	protein-coding	322	8.9	1.5E-04
glgS	protein-coding	315	8.9	5.6E-05
hycH	protein-coding	314	8.9	2.1E-04
yfeX	protein-coding	313	8.9	1.7E-04
tRNA-Met	tRNA	308	7.9	8.9E-05
yhjS	protein-coding	300	8.8	1.5E-04
lptC	protein-coding	296	8.8	2.7E-04
yoeG	pseudogene	292	8.8	2.1E-04
yagJ	pseudogene	281	8.7	1.5E-04
sfmD	protein-coding	278	8.7	1.5E-04
yghB *	protein-coding	276	8.7	1.5E-04
zapA *	protein-coding	274	8.7	2.4E-04
thrA *	protein-coding	269	8.7	2.0E-04
rfaY	protein-coding	268	8.7	7.7E-04
yrfF	protein-coding	264	8.6	1.5E-04

ytfB *	protein-coding	262	8.6	2.3E-04
yuaO	protein-coding	261	8.6	4.5E-04
tRNA-Glu	tRNA	254	3.5	6.9E-03
cbpA *	protein-coding	253	8.6	1.5E-04
uraA	protein-coding	249	8.6	6.4E-04
GlmZ_SraJ	sRNA	249	5.8	1.0E-03
rob *	protein-coding	237	6.7	3.6E-08
fieF	protein-coding	236	7.5	2.4E-05
bla *	protein-coding	231	5.2	6.6E-04
yfcO *	protein-coding	229	8.4	1.6E-04
trpH	protein-coding	227	8.4	2.2E-04
DsrA *	sRNA	223	8.4	1.6E-04
tRNA-Gly	tRNA	219	8.4	2.0E-04
slp	protein-coding	218	7.4	7.7E-06
fucP	protein-coding	210	3.7	3.7E-06
melR *	protein-coding	210	8.3	8.6E-04
exuR *	protein-coding	209	8.3	3.3E-04
yefM	protein-coding	206	8.3	2.4E-04
osmE *	protein-coding	206	8.3	1.8E-03
matA	protein-coding	205	8.3	2.3E-04
ymgD	protein-coding	204	8.3	2.7E-04
ydbA	pseudogene	202	7.3	6.9E-06
ycdT	protein-coding	200	8.2	5.4E-04
fxsA *	protein-coding	199	8.2	5.7E-04
yhhA *	protein-coding	196	7.2	2.2E-05
dicA *	protein-coding	196	8.2	4.4E-04
yfhB	protein-coding	195	8.2	5.8E-04
ypfM	protein-coding	193	8.2	4.5E-04
yjiT	pseudogene	192	8.2	3.2E-04
rplN	protein-coding	191	8.2	3.0E-04
tnaB	protein-coding	187	7.2	3.5E-05
yebW *	protein-coding	186	8.1	3.8E-04
mqsR	protein-coding	186	8.1	4.5E-04
mdaB	protein-coding	185	8.1	5.3E-04
dnaA	protein-coding	183	8.1	1.4E-03
dnaG	protein-coding	183	8.1	6.0E-04
kdsC	protein-coding	182	8.1	5.8E-04
glpA *	protein-coding	181	8.1	5.4E-04
flgG	protein-coding	178	8.1	3.3E-04
mgsA *	protein-coding	178	7.1	5.3E-05
amyA	protein-coding	176	8.1	5.2E-04
ypfJ *	protein-coding	174	8.0	9.5E-04
dcrB *	protein-coding	173	8.0	6.7E-04
ucpA *	protein-coding	173	8.0	5.4E-04
btuB	protein-coding	172	8.0	4.7E-04
insH4	protein-coding	172	8.0	5.3E-04

insH9	protein-coding	171	8.0	5.4E-04
setA	protein-coding	171	8.0	7.9E-04
yqgA	protein-coding	168	8.0	6.0E-04
emrK	protein-coding	165	8.0	5.5E-04
ascF	protein-coding	165	7.0	2.5E-05
ybaO *	protein-coding	164	8.0	5.5E-04
hdeA	protein-coding	162	7.9	2.7E-03
spy	protein-coding	160	7.9	1.1E-03
cysP	protein-coding	159	7.9	5.4E-04
yibA	protein-coding	157	7.9	6.1E-04
fabR	protein-coding	155	7.9	4.3E-04
yqeJ	protein-coding	153	4.0	7.1E-04
pifA	protein-coding	152	6.9	4.2E-05
mgrB *	protein-coding	150	7.8	9.5E-04
miaA	protein-coding	150	7.8	7.8E-04
insK *	protein-coding	150	7.8	7.5E-04
FinP	sRNA	150	7.8	2.4E-03
yeaI	protein-coding	149	7.8	5.2E-04
yfiR	protein-coding	149	7.8	1.4E-03
yjgD	protein-coding	149	6.8	4.2E-05
ykgH	protein-coding	147	7.8	9.2E-04
cutC	protein-coding	145	7.8	4.7E-04
yihI	protein-coding	143	7.8	9.2E-04
trmJ *	protein-coding	143	7.8	6.4E-04
setC	protein-coding	141	7.7	1.5E-03
cysB	protein-coding	139	7.7	2.2E-03
nagA *	protein-coding	139	7.7	1.5E-03
rlmE *	protein-coding	139	7.7	1.9E-03
yhdJ	protein-coding	139	7.7	8.1E-04
rraA	protein-coding	139	7.7	1.0E-03
tusB	protein-coding	138	7.7	2.0E-03
atoS	protein-coding	136	6.7	5.6E-05
cytR	protein-coding	136	7.7	6.7E-04
ygdR	protein-coding	136	7.7	3.4E-03
sfa	protein-coding	136	7.7	8.5E-04
tynA	protein-coding	135	7.7	1.9E-03
yhbX	protein-coding	135	7.7	5.7E-04
yfdY	protein-coding	134	7.7	8.1E-04
dapA *	protein-coding	132	7.6	8.9E-04
puuP	protein-coding	131	7.6	2.4E-03
hemD	protein-coding	127	7.6	8.1E-04
ydcJ	protein-coding	127	5.5	1.2E-03
yncN	protein-coding	125	7.6	2.2E-03
glcB	protein-coding	124	7.6	9.8E-04
ccdA	protein-coding	124	7.6	2.2E-03
holC	protein-coding	124	7.6	2.0E-03

ylaB	protein-coding	124	7.6	1.4E-03
yfhK	protein-coding	123	7.5	1.1E-03
yfiL	protein-coding	122	6.5	1.7E-04
rppH *	protein-coding	122	7.5	1.9E-03
traI	protein-coding	121	7.5	1.5E-03
rnc	protein-coding	121	5.7	1.0E-04
mdtE	protein-coding	117	7.5	1.0E-03
rihA	protein-coding	115	7.4	3.9E-03
betA	protein-coding	114	7.4	2.2E-03
alaS	protein-coding	114	7.4	1.1E-03
rffG	protein-coding	113	7.4	1.1E-03
udk	protein-coding	113	7.4	3.8E-03
yejF	protein-coding	112	7.4	8.3E-04
yabP	pseudogene	112	7.4	8.8E-04
gmhB	protein-coding	112	7.4	9.3E-04
holE	protein-coding	110	7.4	1.8E-03
yuaU	protein-coding	110	7.4	1.5E-03
rfbX	protein-coding	109	7.4	1.8E-03
cmk	protein-coding	108	7.4	1.9E-03
otsA	protein-coding	108	7.4	2.5E-03
skp	protein-coding	108	7.4	1.2E-03
ylbH	pseudogene	108	7.4	1.3E-03
pflB	protein-coding	107	7.3	1.7E-03
tRNA-Ser	tRNA	107	7.3	1.1E-03
yhiJ	protein-coding	106	4.0	1.4E-03
yciC	protein-coding	106	7.3	2.6E-03
nirB	protein-coding	105	7.3	1.7E-03
ubiD	protein-coding	105	7.3	1.0E-03
ytfN	protein-coding	103	7.3	1.1E-03
yaiS	protein-coding	103	7.3	2.9E-03
melB	protein-coding	103	7.3	3.5E-03
yfcB *	protein-coding	103	7.3	1.3E-03
csrA *	protein-coding	103	7.3	6.2E-03
cycA *	protein-coding	102	7.3	5.1E-03
ybdM	protein-coding	99	7.2	9.6E-03
ydfG	protein-coding	99	4.8	4.0E-05
SraB_PsrD	sRNA	99	7.2	1.1E-03
yfjN	protein-coding	99	7.2	1.5E-03
yqeA	protein-coding	99	7.2	5.8E-03
ybhQ	protein-coding	97	7.2	2.5E-03
lptF	protein-coding	97	7.2	2.0E-03
dusC	protein-coding	96	6.2	3.4E-03
kdsA	protein-coding	96	7.2	2.3E-03
yidE	protein-coding	96	7.2	2.3E-03
ppiA	protein-coding	94	6.2	1.8E-03
gcvR	protein-coding	94	7.2	1.7E-03

yiaJ	protein-coding	93	7.1	1.4E-03
yqiB *	protein-coding	93	7.1	2.7E-03
iraP	protein-coding	93	7.1	2.5E-03
ybdR	protein-coding	91	7.1	1.9E-03
yecH	protein-coding	91	7.1	1.3E-03
ais	protein-coding	91	7.1	8.0E-03
hisL *	protein-coding	90	7.1	1.4E-03
modE	protein-coding	89	7.1	2.2E-03
yjbQ	protein-coding	88	7.1	2.1E-03
yicO	protein-coding	88	7.1	1.7E-03
dcuC	protein-coding	88	7.1	2.0E-03
yjeH	protein-coding	87	7.0	8.0E-03
ycgX	protein-coding	86	7.0	5.2E-03
ycbB	protein-coding	86	7.0	2.0E-03
htrE	protein-coding	86	7.0	1.8E-03
mviN	protein-coding	85	7.0	2.0E-03
hupB	protein-coding	84	7.0	5.8E-03
metG	protein-coding	84	7.0	1.9E-03
yebG	protein-coding	84	7.0	2.9E-03
asnA	protein-coding	83	7.0	1.9E-03
metK	protein-coding	83	7.0	5.4E-03
sodB *	protein-coding	82	6.0	4.9E-04
yedK	protein-coding	82	7.0	6.3E-03
yifL	protein-coding	82	7.0	4.2E-03
ymdF	protein-coding	82	7.0	2.5E-03
lldD	protein-coding	81	7.0	2.8E-03
nepI	protein-coding	81	6.9	6.1E-03
csiE	protein-coding	81	6.9	2.5E-03
ydcS	protein-coding	80	6.9	2.1E-03
tRNA-Glu	tRNA	79	4.5	3.8E-04
emtA	protein-coding	79	6.9	5.4E-03
yedY	protein-coding	79	6.9	2.0E-03
yhbE	protein-coding	79	6.9	5.4E-03
ygaM	protein-coding	79	6.9	3.4E-03
sulA	protein-coding	79	6.9	3.6E-03
yehY	protein-coding	78	6.9	8.8E-03
paaX	protein-coding	78	6.9	4.7E-03
dcuA	protein-coding	77	5.9	6.5E-04
phoP	protein-coding	77	6.9	2.3E-03
yliF	protein-coding	77	6.9	2.5E-03
ybhT	protein-coding	76	6.9	2.4E-03
ycgN	protein-coding	76	6.9	3.3E-03
ascB	protein-coding	76	6.8	3.6E-03
potD	protein-coding	76	6.8	4.3E-03
rpmE2	protein-coding	76	6.8	9.8E-03
frvA	protein-coding	75	6.8	2.2E-03

tRNA-Gly	tRNA	75	6.8	2.3E-03
ycgE *	protein-coding	75	6.8	6.6E-03
yihF	protein-coding	74	5.8	5.5E-04
yjgM	protein-coding	74	6.8	5.8E-03
ydbK *	protein-coding	74	6.8	2.7E-03
arsB	protein-coding	73	6.8	2.1E-03
ypjB	protein-coding	72	6.8	3.0E-03
ydgI	protein-coding	72	6.8	7.9E-03
polA	protein-coding	72	6.8	5.3E-03
chpS	protein-coding	71	6.8	2.4E-03
yehB	protein-coding	71	5.7	6.4E-03
yafM	protein-coding	70	6.7	2.5E-03
pgpB	protein-coding	70	6.7	4.7E-03
tnaL	protein-coding	70	5.8	5.2E-04
rplP	protein-coding	70	6.7	5.7E-03
def	protein-coding	69	6.7	5.9E-03
hofD	protein-coding	69	6.7	2.3E-03
yjeI *	protein-coding	69	5.7	2.0E-03
gudD	protein-coding	68	6.7	4.9E-03
barA	protein-coding	68	6.7	3.6E-03
fliY	protein-coding	67	6.7	2.6E-03
gadX	protein-coding	67	6.7	5.5E-03
ydcH	protein-coding	67	6.7	3.1E-03
menA	protein-coding	65	6.6	5.1E-03
yjgN	protein-coding	65	6.6	8.5E-03
purA	protein-coding	65	6.6	5.2E-03
bglF	protein-coding	64	6.6	7.4E-03
ydeO	protein-coding	64	6.6	4.4E-03
ygaQ	pseudogene	63	4.7	1.0E-03
fnr	protein-coding	63	6.6	9.2E-03
STnc560_mgrR *	sRNA	63	4.3	2.7E-03
cadC	protein-coding	62	6.6	5.9E-03
yjiV	pseudogene	61	6.5	6.1E-03
hslJ	protein-coding	61	6.5	3.4E-03
cysK	protein-coding	61	6.5	9.0E-03
folA	protein-coding	61	6.5	3.4E-03
ytfI	protein-coding	60	6.5	9.8E-03
dcuB	protein-coding	60	6.5	5.5E-03
ydjM	protein-coding	60	6.5	3.1E-03
ychJ	protein-coding	60	6.5	4.6E-03
greB	protein-coding	60	6.5	3.4E-03
ycgG	protein-coding	60	6.5	3.7E-03
yhhZ	protein-coding	60	5.5	1.4E-03
glxK	protein-coding	60	6.5	7.8E-03
yedQ	protein-coding	60	6.5	5.9E-03
mnmG	protein-coding	59	6.5	8.0E-03

chiA	protein-coding	59	6.5	3.6E-03
intB	pseudogene	59	6.5	8.6E-03
ybeL	protein-coding	58	6.5	8.8E-03
kdpD	protein-coding	58	6.5	6.7E-03
yaeT	protein-coding	58	6.5	4.8E-03
yjiE	protein-coding	58	6.4	3.8E-03
yihW	protein-coding	56	6.4	5.3E-03
yhiD	protein-coding	56	6.4	6.8E-03
yfjW	protein-coding	56	6.4	4.3E-03
sopB	protein-coding	55	6.4	6.7E-03
infC	protein-coding	55	6.4	5.8E-03
helD *	protein-coding	55	5.4	5.3E-03
torT	protein-coding	54	6.4	8.0E-03
yneK	protein-coding	54	6.4	6.6E-03
yrhB	protein-coding	53	6.3	4.8E-03
yohK	protein-coding	53	5.4	3.6E-03
rstB	protein-coding	53	6.3	5.7E-03
alkA	protein-coding	53	6.3	4.9E-03
yicR	protein-coding	53	6.3	6.9E-03
truD	protein-coding	52	6.3	6.1E-03
repE	protein-coding	52	6.3	6.0E-03
rpsC	protein-coding	51	6.3	8.9E-03
tgt	protein-coding	51	6.3	6.9E-03
ygeN	pseudogene	51	6.3	7.7E-03
caiA	protein-coding	51	6.3	6.3E-03
hemC	protein-coding	51	6.3	7.9E-03
yjcE	protein-coding	50	6.3	5.2E-03
glpQ	protein-coding	50	6.3	8.5E-03
ydcK	protein-coding	50	6.3	5.2E-03
fsaB	protein-coding	50	6.2	8.6E-03

Table S4: Results of the manual analysis of read coverage plots for the HELIOS NAD-Seq data set for *E. coli* K12 JM109 samples. The table highlights all identified gene features with transcription start sites (TSS) near or before the 5'-end of RNA transcripts and the identified location of the TSS (assembly ASM584v2, RefSeq accession GCF_000005845.2) for NAD-capped transcripts. Gene features are ordered in descending order based on abundance (by read count). Gene features that were identified by manual analysis of read coverage plots appear at the end, as no baseMean was assigned to them.

gene feature	type of transcript	(pre-)5'-TSS	TSS location (NAD-capping)
RNAI	sRNA_antisense	known	−1
SibD	sRNA_antisense	known	+1
SibE	sRNA_antisense	known	+1
ChiX (SroB)	sRNA_regulatory	known	+1
GadY	sRNA_regulatory	known	+1
raiA	protein-coding RNA	alternative	−30
SibC	sRNA_antisense	known	+1
RyeA (SraC)	sRNA_antisense	known	−2
GcvB	sRNA_regulatory	known	+1
gatY	protein-coding RNA	known	−29 through −27
hdeD	protein-coding RNA	known	−35
ompP	protein-coding RNA	suspected	−31
pgk (lies in epd gene)	protein-coding RNA	known	−235
ymdB	protein-coding RNA	alternative	−23
csdE (lies in csdA gene)	protein-coding RNA	suspected	−59
bfr	protein-coding RNA	known	−23
aspA	protein-coding RNA	known	−104
yuaQ	protein-coding RNA	suspected	−113
insX (ykgN)	pseudogene	known	−14/−13
ldtA (erfK)	protein-coding RNA	known	−30
tqsA	protein-coding RNA	known	−23
repA2	protein-coding RNA	suspected	−33
ynfT (ydfJ)	protein-coding RNA	alternative	−176
racR	protein-coding RNA	known	+1
tomB (ybaJ)	protein-coding RNA	known	−87/−86
ribA	protein-coding RNA	known	−30

traY	protein-coding RNA	suspected	−30
nadR	protein-coding RNA	known	−14 / −12
CopA	sRNA_antisense	known	+2
ubiT (yhbT)	protein-coding RNA	known	−25
frdA (wrongly assigned to poxA)	protein-coding RNA	known	+96
rplT	protein-coding RNA	known	−14
ycbJ	protein-coding RNA	alternative	−25
osmB	protein-coding RNA	known	−43
kdsC (wrongly identified as kdsD)	protein-coding RNA	known	−555
kdsC (2nd)		known	−263
aroG	protein-coding RNA	alternative	−4
CsrC	sRNA_regulatory	known	+1
thrS	protein-coding RNA	known	+162
eno (wrongly assigned to pyrG)	protein-coding RNA	alternative	−497
yniB	protein-coding RNA	known	−28/−29
ygdH	protein-coding RNA	suspected	−37
ubiC	protein-coding RNA	alternative	−24
glgS	protein-coding RNA	alternative	−50
hycI	protein-coding RNA	suspected	−59
yfeX	protein-coding RNA	suspected	−10
bcsE (yhjS)	protein-coding RNA	known	−15
yoeG	pseudogene	suspected	−27
yghB	protein-coding RNA	known	−34
ssrS (wrongly assigned to zapA)	Non-coding RNA	known	−232
ytfB	protein-coding RNA	known	−102 (worse)
ytfB (2nd)		alternative	−25 (better)
cbpA	protein-coding RNA	known	−53
uraA	protein-coding RNA	alternative	−15
GlmZ (SraJ)	sRNA_regulatory	known	−1
rob	protein-coding RNA	known	−43
bla	protein-coding RNA	suspected	−35
DsrA	sRNA_regulatory	known	+2

tRNA-Gly_glyU	tRNA	suspected	−12
slp	protein-coding RNA	alternative	−14
melR	protein-coding RNA	known	−22
exuR	protein-coding RNA	alternative	−28
yefM	protein-coding RNA	known	−18
osmE	protein-coding RNA	known	−27
ymgD	protein-coding RNA	suspected	−10
dgcT (ycdT)	protein-coding RNA	known	−37 / −35
fxsA	protein-coding RNA	known	−61
yhhA	protein-coding RNA	known	−23
dicA	protein-coding RNA	suspected	−3
pgpC (yfhB)	protein-coding RNA	alternative	−42
ypfM	protein-coding RNA	alternative	−8 / −10
yebW	protein-coding RNA	known	−24
mqsR	protein-coding RNA	known	−18
ygiN (wrongly assigned to mdaB)	protein-coding RNA	known	−64
recF (wrongly assigned to dnaA)	protein-coding RNA	known	−410
recF (2nd)		alternative	−268
glpA	protein-coding RNA	alternative	−26
glpA (2nd)		known	−63
mgsA	protein-coding RNA	known	−26
ypfJ	protein-coding RNA	suspected	−15
dcrB	protein-coding RNA	known	−38
ucpA	protein-coding RNA	known	−41
btuB	protein-coding RNA	alternative	−37
yqgA	protein-coding RNA	known	−29
decR (ybaO)	protein-coding RNA	known	−26
cysP (or cysPUWAM)	protein-coding RNA	known	−30
mgrB	protein-coding RNA	known	−26
sokA (wrongly assigned to insK?)	pseudogene	suspected	+1252 (insK) or −145 (sokA)
FinP	sRNA_antisense	known	+3

yfiR (2nd)	protein-coding RNA	known	−51
rraB (yjgD)	protein-coding RNA	alternative	−28
MicL (wrongly assigned to cutC)	sRNA_regulatory	known	−227
trmJ	protein-coding RNA	suspected	−24
cysB	protein-coding RNA	alternative	−94
nagC (wrongly assigned to nagA)	protein-coding RNA	known	−271
rlmE	protein-coding RNA	known	−67
atoS	protein-coding RNA	suspected	−28
ygdR	protein-coding RNA	known	−21
gnsA (sfa)	protein-coding RNA	alternative	−72
dapA	protein-coding RNA	known	−24
ymjA (wrongly assigned to puuP)	protein-coding RNA	known	−157
hicA (yncN)	protein-coding RNA	known	−11
ccdA	protein-coding RNA	suspected	−32
pdeB (ylaB)	protein-coding RNA	suspected	−14
pdeB (ylaB) (2nd)		suspected	−25
rppH	protein-coding RNA	alternative	−28
hscC (wrongly assigned to rihA)	protein-coding RNA	known	−176
alaS	protein-coding RNA	known	−79
gmhB	protein-coding RNA	known	−27
rpsA (wrongly assigned to cmk)	protein-coding RNA	alternative	−304
tRNA-Ser (serU)	tRNA	known	−8
ubiD	protein-coding RNA	known	−16
ytfP (wrongly assigned to tamB (ytfN))	protein-coding RNA	suspected	−232
yaiS	protein-coding RNA	alternative	−16
aroC (wrongly assigned to prmB (yfcB))	protein-coding RNA	suspected	−154
csrA	protein-coding RNA	known	−43
csrA (2nd)		known	−127
cycA	protein-coding RNA	alternative	−52
ydfG	protein-coding RNA	known	−26

SraB (PsrD)	sRNA	known	+2
rnlB (wrongly assigned to rnlA (yfjN))	protein-coding RNA	known	−277
ybhQ	protein-coding RNA	known	−30
lptF	protein-coding RNA	known	−96
yohJ (wrongly assigned to dusC)	protein-coding RNA	known	−129
ysaA (wrongly assigned to yiaJ (plaR))	protein-coding RNA	suspected	−145
cpdA (wrongly assigned to yqiB)	protein-coding RNA	suspected	−132
yecH	protein-coding RNA	alternative	−10
hisL	protein-coding RNA	known	−33/−31
yjbQ	protein-coding RNA	known	−25
ldTD (ycbB)	protein-coding RNA	alternative	−12
ldTD (ycbB) (2^{nd})		known	−116
hupB	protein-coding RNA	known	−12
yebG	protein-coding RNA	alternative	−27
asnA	protein-coding RNA	known	−23
sodB	protein-coding RNA	known	−53
yedK	protein-coding RNA	suspected	−19
yifL	protein-coding RNA	known	−31
ymdF	protein-coding RNA	suspected	−19
csiE	protein-coding RNA	known	−24
ydcS	protein-coding RNA	known	−58
emtA	protein-coding RNA	alternative	−4
ygaM	protein-coding RNA	known	−25
yehX (wrongly assigned to yehY?)	protein-coding RNA	suspected	−175
paaX	protein-coding RNA	known	−29
phoP	protein-coding RNA	alternative	−25
acrZ (ybhT)	protein-coding RNA	known	−22
ycgN	protein-coding RNA	known	−18
ykgO (wrongly assigned to ykgM (rpmE2)?)	protein-coding RNA	suspected	−173

frvA	protein-coding RNA	suspected	−14
bluR (ycgE)	protein-coding RNA	known	−54
ydbK	protein-coding RNA	known	−61
polA	protein-coding RNA	known	−27
chpS	protein-coding RNA	known	−17
pgpB	protein-coding RNA	known	−21
def	protein-coding RNA	known	−40
blc (yjeI)	protein-coding RNA	known	−26
barA	protein-coding RNA	suspected	−297
tcyJ (fliY)	protein-coding RNA	known	−25
tcyJ (fliY) (2nd)		alternative	−83
gadX	protein-coding RNA	known	−31
rimL (wrongly assigned to ydcH)	protein-coding RNA	alternative	−109
purA	protein-coding RNA	known	−22
fnr	protein-coding RNA	known	−26
MgrR (STnc560)	sRNA_regulatory	known	+2
hslJ	protein-coding RNA	known	−34
folA	protein-coding RNA	alternative	−35
ydjM	protein-coding RNA	alternative	−24
ychJ	protein-coding RNA	known	−45
greB	protein-coding RNA	known	−175
pdeG (ycgG)	protein-coding RNA	known	−64
dgcQ (yedQ)	protein-coding RNA	suspected	−24
ybeL	protein-coding RNA	known	−25
rpmI (wrongly assigned to infC)	protein-coding RNA	known	−431
helD	protein-coding RNA	known	−28
umpG_surE (wrongly assigned to truD)	protein-coding RNA	alternative	−345
repE	protein-coding RNA	suspected	−28
yjcE	protein-coding RNA	alternative	−63
ydcK	protein-coding RNA	alternative	−22
gldA (wrongly assigned to fsaB)	protein-coding RNA	alternative	−62
gldA (wrongly assigned to fsaB)	protein-coding RNA	suspected	−45

ytgA (counted with lptF)	protein-coding RNA	known	−127
purU (wrongly assigned to ychJ)	protein-coding RNA	alternative	−117
yhgF (wrongly assigned to greB)	protein-coding RNA	alternative	−7
tcyP (ydjN)	protein-coding RNA	alternative	−61
rpmE	protein-coding RNA	known	+1
GlmY (SroF)	sRNA_regulatory	known	+1
IspZ (an sRNA)	sRNA_regulatory	known	+1
McaS	sRNA_regulatory	known	−39
acpP	protein-coding RNA	known	−42
glpD	protein-coding RNA	known	−32
ilvL	protein-coding RNA	known	−38
yfgG	protein-coding RNA	known	−100
clpP	protein-coding RNA	known	−71
glpF	protein-coding RNA	known	−29
rbsD	protein-coding RNA	known	−59
yafD	protein-coding RNA	known	−27
yafV	protein-coding RNA	known	−53
ychH	protein-coding RNA	suspected	−36
ynaJ	protein-coding RNA	suspected	−10
ynhF (cydH)	protein-coding RNA	known	−38
lacZ	protein-coding RNA	known	−168
ihfA (wrongly assigned to pheT)	protein-coding RNA	known	−42
thrL	protein-coding RNA	known	−61
rsuA	protein-coding RNA	alternative	−50
bamE (smpA)	protein-coding RNA	known	−50
wrbA	protein-coding RNA	known	+4
RNAII	primer RNA	known	−37
uspE	protein-coding RNA	known	−1

Table S5: Results from the analysis of the eight most abundant transcripts (by normalized read count) identified in the HELIOS NAD-Seq sequencing data set for *E. coli* K12 JM109, and comparison of log_2-fold change to the results from NAD captureSeq data sets 1 and 3 by *Cahová et al*[1].

Top 8 RNAs (abundance)	**HELIOS NAD-Seq**	**NAD captureSeq (data set 1 / 3)**
	log_2-fold change	**log_2-fold change**
RNAI	7.6	3.1 / 6.5
SibD	10.6	3.8 / 6.3
SibE	9.1	2.9 / 6.5
sfsA	10.5	5.3 / 6.2
ChiX	9.0	4.7 / 4.3
GadY	11.4	4.1 / 6.1
raiA	7.4	2.5 / 4.4
SibC	11.6	– / 5.7

Table S6: Comparison of identified transcripts from NAD captureSeq data sets 1–3 with the hits from the HELIOS NAD-Seq data set for *E. coli* K12 JM109. NAD captureSeq data sets 1 and 3 were derived from *E. coli* K12 JM109 samples, whereas in NAD captureSeq data set 2 the *E. coli* K12 strain was analyzed[1]. In total, 102 transcripts from NAD captureSeq were also identified as NAD-capped RNAs by HELIOS NAD-Seq (75 of 80 transcripts of data set 1, 42 of 44 transcripts of data set 2, and 64 of 70 transcripts from data set 3 of NAD captureSeq). 12 gene features (from which 11 were identified in only a single data set of NAD captureSeq) were not found enriched using HELIOS NAD-Seq.

RNA transcript	**NAD captureSeq data set 1 (n = 80)**	**NAD captureSeq data set 2 (n = 44)**	**NAD captureSeq data set 3 (n = 70)**	**also found with HELIOS NAD-Seq?**	**also found with NAD tagSeq?**
acpP	x	x	x	yes	yes
aroM			x	*no*	*no*
aspA	x	x	x	yes	yes
bla	x		x	yes	*no*
bsmA	x		x	yes	yes
cbpA	x			yes	*no*
cdsA (rseP?)	x		x	yes	yes
clpP	x			yes	yes
CopA	x		x	yes	*no*
csdA			x	yes	*no*
csrA	x	x		yes	yes
CsrC	x		x	yes	yes
cycA	x		x	yes	yes
dapA			x	yes	yes
dcrB	x			yes	yes
dicA		x		yes	*no*
DsrA	x		x	yes	*no*
epd (pgk)	x	x	x	yes	yes
eptB		x		*no*	*no*
exuR	x		x	*no*	yes
fxsA		x		yes	yes
GadY	x	x	x	yes	*no*
gatY	x	x	x	yes	*no*

GcvB	x	x	x	yes	yes
glcD	x			*no*	*no*
glgX (glgC?)	x		x	yes	yes
GlmY_tke1		x	x	yes	yes
glpA	x		x	yes	yes
glpD	x		x	yes	yes
glpF	x			yes	yes
hdeD	x	x	x	yes	*no*
helD	x			yes	*no*
hemY		x		*no*	*no*
hisL	x	x		yes	*no*
hspQ	x			yes	yes
ilvL	x	x	x	yes	*no*
insK	x			yes	*no*
IS061 (McaS)	x	x	x	yes	*no*
kdsD	x			yes	*no*
lacZ			x	yes	*no*
lrhA			x	yes	yes
melR			x	yes	yes
mgrB	x			yes	yes
mgsA		x	x	yes	yes
nagA (nagC)	x	x	x	yes	yes
nsrR	x			yes	yes
ompP	x			yes	*no*
osmE	x	x		yes	yes
pheT (ihfA)		x	x	yes	yes
polB	x			*no*	*no*
poxA	x		x	yes	*no*
pyrG (eno?)	x	x	x	yes	*no*
QUAD (SibC)		x	x	yes	yes
QUAD (SibD)	x	x	x	yes	yes
QUAD (SibE)	x	x	x	yes	yes

racR	x		x	yes	*no*
raiA	x	x	x	yes	yes
rbsD	x			yes	*no*
rlmE	x	x		yes	yes
rlmI			x	yes	*no*
RNAI	x		x	yes	*no*
RNAII	x		x	yes	*no*
rob	x			yes	yes
rppH	x		x	yes	yes
rsuA		x		yes	*no*
secY (rpmJ?)	x	x	x	yes	yes
sfsA (dksA?)	x	x	x	yes	yes
smpA		x		yes	*no*
sodB	x			yes	yes
SraC_RyeA	x		x	yes	yes
SroB	x	x	x	yes	*no*
STnc560 (MgrR)	x	x	x	yes	*no*
thrA (thrB?)	x		x	yes	*no*
thrL		x		yes	*no*
thrS	x		x	yes	yes
tqsA			x	yes	*no*
traG			x	yes	*no*
traY	x		x	yes	*no*
trmJ	x	x	x	yes	yes
ucpA	x		x	yes	yes
uspE	x	x	x	yes	yes
wrbA		x		yes	*no*
yafD	x			yes	yes
yafV	x			yes	yes
yaiO			x	yes	*no*
ybaO			x	yes	*no*
ycbJ	x		x	yes	yes

ycgE	x		x	yes	*no*
ychH	x			yes	yes
ydbK	x			yes	*no*
ydcW			x	*no*	*no*
ydfJ			x	yes	*no*
ydiS			x	*no*	*no*
ydjN			x	yes	yes
yebW		x		yes	*no*
yegR			x	*no*	*no*
yfcB (aroC?)	x	x	x	yes	yes
yfcO (yfcB?)	x	x		yes	yes
yfgG	x	x	x	yes	yes
ygdH	x			yes	yes
yghB	x	x		yes	yes
ygjR	x			*no*	*no*
yhhA			x	yes	*no*
yidF	x			*no*	*no*
yjel		x		yes	*no*
yjhB			x	*no*	*no*
ykgN	x			yes	*no*
ynaJ	x			yes	yes
ynhF	x			yes	*no*
yniB			x	yes	yes
ypfJ	x			yes	*no*
yqiB (cpdA?)	x		x	yes	yes
ytfB		x		yes	yes
zapA (ssrS)	x	x	x	yes	yes

Table S7: Overlap of sequencing hits from NAD captureSeq data sets 1–3 with the HELIOS NAD-Seq data set for *E. coli* K12 JM109. Individual transcripts found by both methods are summarized in Table S6. NAD captureSeq data sets 1 and 3 were derived from *E. coli* K12 JM109 samples, whereas in NAD captureSeq data set 2 the *E. coli* K12 strain was analyzed[1].

comparison of sequencing hits	**number of hits fulfilling the condition**	**number of hits <u>also</u> found with HELIOS NAD-Seq**	**number of hits <u>not</u> found with HELIOS NAD-Seq**
overlap of NAD captureSeq data sets 1, 2 and 3 (all)	23	23	0
overlap of NAD captureSeq data sets 1 and 3 (*E. coli* K12 JM109)	47	46	1
overlap of NAD captureSeq data sets 1 and 2	29	29	0
overlap of NAD captureSeq data sets 2 and 3	27	27	0
hits exclusive to NAD captureSeq data set 1	27	23	4
hits exclusive to NAD captureSeq data set 2	11	9	2
hits exclusive to NAD captureSeq data set 3	19	14	5
hits identified in NAD captureSeq data set 1, but not in 3	33	29	4
hits identified in NAD captureSeq data set 3, but not in 1	23	18	5

Table S8: NAD-capped transcripts detected in at least two of the timepoints, including gene name, functional category, maximum baseMean, maximum $\log_2$ fold change, and lowest adjusted *p*-value.

gene name	gene category	Num Time Points	Time Points	adjusted pvalue (best)	log2-fold change (max)	Base Mean (max)
rna1	ncRNA	15	tp1,tp2,tp3,tp4,tp5,tp6,tp7,tp8,tp9,tp10,tp11,tp12,tp14,tp15,tp16	1.90E-42	11.1	16631
hicA	protein_coding	13	tp1,tp3,tp4,tp6,tp7,tp8,tp9,tp10,tp11,tp12,tp14,tp15,tp16	6.20E-08	10.7	439
gadY	ncRNA	12	tp1,tp2,tp3,tp4,tp6,tp7,tp8,tp9,tp11,tp12,tp14,tp16	6.80E-08	10.1	738
rlmI	protein_coding	11	tp1,tp2,tp3,tp4,tp5,tp6,tp7,tp8,tp9,tp10,tp12	2.00E-11	10.6	477
yjeI	protein_coding	10	tp1,tp2,tp3,tp4,tp5,tp6,tp7,tp8,tp9,tp12	1.50E-10	10.9	553
cdsA	protein_coding	10	tp1,tp4,tp7,tp8,tp9,tp10,tp11,tp12,tp14,tp15	2.00E-05	10	227
sibD	ncRNA	9	tp1,tp2,tp3,tp4,tp5,tp6,tp7,tp8,tp12	1.30E-13	9.4	2742
chiX	ncRNA	9	tp1,tp2,tp3,tp4,tp5,tp6,tp7,tp8,tp12	1.40E-11	10.6	892
gcvB	ncRNA	9	tp1,tp2,tp3,tp4,tp5,tp6,tp7,tp8,tp12	5.50E-11	9.6	377
tomB	protein_coding	9	tp1,tp2,tp3,tp4,tp5,tp6,tp7,tp8,tp12	1.80E-09	10.9	439
sibE	ncRNA	8	tp1,tp2,tp4,tp5,tp6,tp7,tp8,tp12	2.50E-31	10.7	1210
sibC	ncRNA	8	tp1,tp3,tp4,tp5,tp6,tp7,tp8,tp12	2.40E-09	11.6	613
tpiA	protein_coding	8	tp1,tp3,tp4,tp5,tp6,tp7,tp8,tp12	8.00E-08	10.8	344
yfgG	protein_coding	8	tp1,tp3,tp4,tp5,tp6,tp7,tp8,tp12	9.70E-08	10.9	366
csrC	ncRNA	8	tp1,tp3,tp4,tp5,tp6,tp7,tp8,tp12	1.00E-05	7.7	338

ppnN	protein_coding	8	tp4,tp6,tp7,tp8,tp9,tp11,tp12,tp15	5.80E-05	9.6	510
glyW	tRNA	7	tp1,tp2,tp3,tp4,tp5,tp6,tp7	1.80E-12	9.1	626
secY	protein_coding	7	tp1,tp3,tp5,tp6,tp7,tp8,tp12	1.10E-09	9.7	268
nagA	protein_coding	7	tp1,tp3,tp4,tp6,tp7,tp8,tp12	2.80E-09	9.4	209
leuS	protein_coding	7	tp1,tp4,tp5,tp9,tp10,tp11,tp14	2.50E-06	9.8	197
yejF	protein_coding	7	tp1,tp3,tp4,tp5,tp6,tp8,tp12	4.00E-06	9.1	103
kdsD	protein_coding	7	tp1,tp4,tp5,tp6,tp7,tp8,tp12	4.50E-06	9.2	229
insH21	protein_coding	7	tp1,tp4,tp7,tp8,tp9,tp11,tp14	9.80E-06	8.7	159
insH4	protein_coding	7	tp4,tp7,tp9,tp10,tp11,tp14,tp15	1.80E-05	10	240
serV	tRNA	6	tp1,tp2,tp3,tp4,tp5,tp6	1.10E-21	8.1	1791
gatY	protein_coding	6	tp4,tp5,tp6,tp7,tp8,tp12	3.80E-13	11.4	879
thrU	tRNA	6	tp1,tp2,tp3,tp4,tp5,tp6	5.30E-09	4.9	536
pdhR	protein_coding	6	tp1,tp2,tp3,tp4,tp5,tp8	8.40E-09	6.2	1601
betT	protein_coding	6	tp1,tp4,tp6,tp7,tp8,tp9	1.30E-08	7.2	201
ileX	tRNA	6	tp1,tp2,tp4,tp5,tp6,tp7	4.80E-06	5.9	186
ackA	protein_coding	6	tp1,tp3,tp4,tp7,tp8,tp12	6.50E-06	8.9	92
miaA	protein_coding	6	tp3,tp4,tp7,tp8,tp9,tp12	2.50E-05	7.7	87
insH3	protein_coding	6	tp4,tp7,tp9,tp10,tp11,tp14	2.80E-05	8.1	240
ftsE	protein_coding	6	tp3,tp4,tp6,tp7,tp8,tp12	8.00E-05	8.3	62
rplN	protein_coding	6	tp6,tp7,tp8,tp9,tp11,tp12	5.00E-04	7.2	41

cutC	protein_coding	6	tp7,tp9,tp11,tp12,tp15, tp16	9.70E-04	10.2	248
yghB	protein_coding	5	tp4,tp6,tp7,tp8,tp12	6.20E-17	9.4	538
sfsA	protein_coding	5	tp6,tp7,tp8,tp12,tp14	2.70E-10	11.8	709
slyD	protein_coding	5	tp4,tp6,tp7,tp8,tp12	6.60E-10	9.8	274
zapG	protein_coding	5	tp4,tp6,tp7,tp8,tp12	7.00E-08	8.8	142
epd	protein_coding	5	tp4,tp6,tp7,tp8,tp12	7.80E-08	8.5	362
dnaG	protein_coding	5	tp1,tp4,tp6,tp7,tp9	8.40E-08	7	111
ymdB	protein_coding	5	tp4,tp6,tp7,tp8,tp12	1.00E-07	10.2	225
ssrA	ncRNA	5	tp1,tp2,tp3,tp4,tp7	1.80E-07	6	815
ldtE	protein_coding	5	tp6,tp7,tp8,tp9,tp12	2.20E-07	10.3	266
nrdH	protein_coding	5	tp1,tp2,tp3,tp4,tp7	1.00E-06	8.5	113
bcsE	protein_coding	5	tp4,tp6,tp7,tp8,tp12	1.20E-06	9.7	160
proK	tRNA	5	tp5,tp9,tp10,tp11,tp12	1.30E-06	5.2	686
insH1	protein_coding	5	tp7,tp9,tp10,tp11,tp14	1.10E-05	8.4	288
insX	pseudogene	5	tp1,tp4,tp7,tp8,tp12	1.20E-05	8.8	87
insH6	protein_coding	5	tp7,tp9,tp10,tp11,tp14	9.80E-05	9.9	214
frdA	protein_coding	4	tp6,tp7,tp8,tp12	4.10E-12	10.5	447
gcvT	protein_coding	4	tp6,tp7,tp8,tp12	1.30E-09	11.7	660
fruB	protein_coding	4	tp5,tp6,tp7,tp8	1.30E-08	11	395
bluR	protein_coding	4	tp4,tp7,tp8,tp12	2.30E-08	10.7	329

acpP	protein_coding	4	tp1,tp4,tp7,tp8	6.80E-08	8.7	133
tqsA	protein_coding	4	tp4,tp7,tp8,tp12	5.90E-07	9.6	151
aaeR	protein_coding	4	tp6,tp7,tp8,tp12	6.20E-07	10.1	211
ymcE	protein_coding	4	tp4,tp6,tp7,tp8	2.20E-06	9.4	125
dcrB	protein_coding	4	tp4,tp7,tp8,tp9	2.30E-06	9.6	147
yfjI	protein_coding	4	tp7,tp8,tp11,tp12	2.50E-06	9.2	110
pheT	protein_coding	4	tp4,tp7,tp8,tp12	3.00E-06	9.1	107
fimH	protein_coding	4	tp4,tp7,tp8,tp12	3.60E-06	9.3	120
yaiO	protein_coding	4	tp6,tp7,tp8,tp12	4.50E-06	9.8	169
ychJ	protein_coding	4	tp6,tp7,tp8,tp12	8.00E-06	9.1	107
rimJ	protein_coding	4	tp4,tp6,tp7,tp8	9.80E-06	9.1	107
yadM	protein_coding	4	tp3,tp7,tp8,tp12	1.70E-05	8.9	95
mepH	protein_coding	4	tp6,tp7,tp8,tp12	1.80E-05	8.5	67
ldtD	protein_coding	4	tp7,tp9,tp10,tp11	4.40E-05	8.2	47
yehA	protein_coding	4	tp10,tp11,tp14,tp15	4.50E-05	9.9	220
aspA	protein_coding	4	tp6,tp7,tp8,tp12	4.60E-05	7.9	463
ppiD	protein_coding	4	tp9,tp10,tp11,tp14	4.60E-05	9.4	152
adeQ	protein_coding	4	tp7,tp8,tp11,tp12	6.30E-05	8.4	67
tamB	protein_coding	4	tp3,tp4,tp7,tp12	8.10E-05	8.8	78
yheS	protein_coding	4	tp4,tp6,tp7,tp12	9.30E-05	7.9	45

ispZ	ncRNA	4	tp1,tp4,tp7,tp9	1.00E-04	8.3	62
ydhF	protein_coding	4	tp7,tp9,tp11,tp14	3.80E-04	9.2	125
casD	protein_coding	4	tp6,tp7,tp8,tp12	4.40E-04	8.6	71
cspD	protein_coding	4	tp4,tp6,tp8,tp12	1.10E-03	7.9	44
osmE	protein_coding	3	tp7,tp8,tp12	3.80E-13	11.2	732
mcaS	ncRNA	3	tp4,tp7,tp12	2.50E-10	7.4	309
cas3	protein_coding	3	tp4,tp7,tp8	3.20E-10	9.7	261
cnu	protein_coding	3	tp1,tp4,tp7	1.30E-08	7.6	97
ydbK	protein_coding	3	tp4,tp7,tp8	4.40E-08	10.6	296
rpsT	protein_coding	3	tp4,tp7,tp8	5.20E-08	10.5	285
ychH	protein_coding	3	tp7,tp8,tp12	6.80E-08	10.4	265
yajO	protein_coding	3	tp1,tp7,tp8	6.90E-08	9.9	306
trmA	protein_coding	3	tp7,tp8,tp12	8.40E-08	8.8	98
uspB	protein_coding	3	tp7,tp8,tp12	1.40E-07	7.9	62
insK	protein_coding	3	tp1,tp4,tp7	5.60E-07	9.8	161
waaU	protein_coding	3	tp4,tp7,tp8	7.50E-07	9.6	153
exbB	protein_coding	3	tp4,tp7,tp8	2.00E-06	9.6	145
pepD	protein_coding	3	tp7,tp9,tp11	2.20E-06	9.4	127
yaaJ	protein_coding	3	tp4,tp7,tp8	2.20E-06	9.2	116
yfcO	protein_coding	3	tp4,tp7,tp12	2.30E-06	9.4	127

nadR	protein_coding	3	tp7,tp8,tp12	4.40E-06	9	100
dsrA	ncRNA	3	tp1,tp4,tp7	4.50E-06	8.9	113
rraB	protein_coding	3	tp4,tp7,tp12	5.10E-06	9	98
fxsA	protein_coding	3	tp4,tp7,tp8	5.50E-06	6.7	77
mgrB	protein_coding	3	tp1,tp4,tp7	6.90E-06	8.8	83
valU	tRNA	3	tp2,tp4,tp5	7.40E-06	5.9	153
ycgZ	protein_coding	3	tp4,tp5,tp7	9.80E-06	8.7	78
raiA	protein_coding	3	tp6,tp7,tp8	1.00E-05	5.8	1091
betA	protein_coding	3	tp7,tp8,tp12	1.70E-05	8.5	71
efp	protein_coding	3	tp7,tp8,tp12	1.80E-05	8.6	72
citC	protein_coding	3	tp1,tp4,tp7	1.90E-05	8.7	81
kdsC	protein_coding	3	tp7,tp8,tp12	1.90E-05	8.6	73
nuoG	protein_coding	3	tp7,tp8,tp12	2.20E-05	8.4	67
ybdD	protein_coding	3	tp9,tp10,tp11	2.80E-05	8.2	32
cysZ	protein_coding	3	tp7,tp8,tp12	2.90E-05	8.6	76
purA	protein_coding	3	tp4,tp7,tp8	3.10E-05	8.2	58
betI	protein_coding	3	tp3,tp4,tp7	4.00E-05	8.3	62
cspA	protein_coding	3	tp3,tp4,tp7	5.00E-05	6.6	59
ypfJ	protein_coding	3	tp4,tp7,tp12	5.40E-05	8.3	62
fbaA	protein_coding	3	tp7,tp8,tp12	6.00E-05	8.4	63

hemC	protein_coding	3	tp4,tp7,tp12	6.20E-05	8.4	65
tyrU	tRNA	3	tp9,tp10,tp11	6.40E-05	5.3	89
metK	protein_coding	3	tp4,tp7,tp8	8.80E-05	7.9	45
exuR	protein_coding	3	tp4,tp7,tp8	9.30E-05	8.4	86
mlaA	protein_coding	3	tp7,tp9,tp12	9.30E-05	8.9	88
lpxD	protein_coding	3	tp1,tp4,tp7	1.50E-04	7.7	39
cvrA	protein_coding	3	tp4,tp7,tp12	1.60E-04	7.7	40
yfeO	protein_coding	3	tp7,tp8,tp12	1.60E-04	7	40
puuD	protein_coding	3	tp4,tp7,tp12	2.00E-04	8.1	52
tig	protein_coding	3	tp4,tp6,tp7	2.10E-04	7	36
glvC	protein_coding	3	tp4,tp7,tp12	2.60E-04	8	50
rsmE	protein_coding	3	tp4,tp6,tp7	2.70E-04	8	49
yraH	protein_coding	3	tp6,tp7,tp8	2.70E-04	7.9	46
rplT	protein_coding	3	tp7,tp8,tp12	2.80E-04	7.5	35
hemA	protein_coding	3	tp1,tp4,tp7	3.50E-04	7.2	60
tpx	protein_coding	3	tp7,tp8,tp12	6.00E-04	7.9	42
zntA	protein_coding	3	tp7,tp9,tp11	8.40E-04	7.3	30
ycbZ	protein_coding	3	tp4,tp7,tp8	1.30E-03	6.9	89
ptsG	protein_coding	2	tp4,tp7	6.80E-08	6.9	192
zapA	protein_coding	2	tp7,tp8	3.40E-07	9.8	173

hdeD	protein_coding	2	tp4,tp7	8.90E-07	9.6	148
ribA	protein_coding	2	tp4,tp7	1.20E-06	9.4	128
yagJ	protein_coding	2	tp7,tp8	1.20E-06	6.9	67
ycfH	protein_coding	2	tp7,tp8	2.50E-06	9.6	148
ydgA	protein_coding	2	tp7,tp8	2.70E-06	9.4	129
ybeL	protein_coding	2	tp7,tp12	2.80E-06	6.6	58
ybiI	protein_coding	2	tp4,tp7	3.00E-06	9.1	104
opgG	protein_coding	2	tp4,tp7	3.90E-06	9.1	100
yifL	protein_coding	2	tp4,tp7	6.00E-06	9.1	106
ybhA	protein_coding	2	tp4,tp7	6.50E-06	9	98
ispF	protein_coding	2	tp7,tp8	7.40E-06	7.6	61
fliZ	protein_coding	2	tp7,tp12	7.80E-06	7.2	47
thrL	protein_coding	2	tp4,tp7	8.00E-06	7.7	65
dnaK	protein_coding	2	tp4,tp7	1.00E-05	8.6	77
ubiC	protein_coding	2	tp4,tp7	1.30E-05	8.7	82
metZ	tRNA	2	tp4,tp5	1.40E-05	6.6	102
yhcD	protein_coding	2	tp4,tp7	1.40E-05	8.8	86
yieE	protein_coding	2	tp4,tp7	1.40E-05	8.5	70
ryfD	ncRNA	2	tp7,tp8	1.70E-05	9.1	103
talA	protein_coding	2	tp7,tp8	1.80E-05	6.1	35

moeA	protein_coding	2	tp4,tp7	2.50E-05	8.5	70
yagK	protein_coding	2	tp7,tp8	2.60E-05	8.5	69
ydbA	pseudogene	2	tp7,tp12	2.90E-05	8.4	64
moaA	protein_coding	2	tp4,tp7	3.00E-05	8.3	61
alaA	protein_coding	2	tp4,tp7	3.10E-05	8.4	64
epmA	protein_coding	2	tp7,tp12	3.10E-05	8.2	55
osmB	protein_coding	2	tp7,tp8	3.10E-05	6.1	171
wrbA	protein_coding	2	tp7,tp8	3.10E-05	8.5	69
ybdO	protein_coding	2	tp4,tp7	3.10E-05	8.5	70
mraZ	protein_coding	2	tp4,tp7	3.60E-05	5.5	78
ydfG	protein_coding	2	tp4,tp7	3.70E-05	8.3	61
sulA	protein_coding	2	tp7,tp12	3.80E-05	8.1	52
tusB	protein_coding	2	tp7,tp8	4.30E-05	7.4	55
bsmA	protein_coding	2	tp7,tp12	4.70E-05	9	96
dgcE	protein_coding	2	tp4,tp7	4.80E-05	8.1	52
rlmJ	protein_coding	2	tp4,tp7	4.80E-05	8.1	51
ybgC	protein_coding	2	tp7,tp12	5.10E-05	8.1	52
thrS	protein_coding	2	tp4,tp7	5.40E-05	8.6	73
rbsZ	ncRNA	2	tp3,tp4	5.60E-05	7.2	48
rpmE	protein_coding	2	tp7,tp8	5.80E-05	8	50

ybdM	protein_coding	2	tp4,tp7	6.30E-05	8.2	56
cyoA	protein_coding	2	tp4,tp7	7.10E-05	8	47
ysgD	protein_coding	2	tp3,tp4	7.10E-05	8.1	53
ung	protein_coding	2	tp4,tp7	8.60E-05	7.8	43
ybdR	protein_coding	2	tp7,tp8	8.80E-05	8.2	55
yabP	protein_coding	2	tp7,tp12	9.00E-05	7.8	44
yigG	protein_coding	2	tp4,tp7	9.00E-05	8.1	52
glgX	protein_coding	2	tp7,tp12	1.10E-04	7.9	47
bamE	protein_coding	2	tp4,tp7	1.30E-04	6.9	31
gltF	protein_coding	2	tp4,tp7	1.30E-04	7.7	39
ftsK	protein_coding	2	tp3,tp4	1.50E-04	7.6	37
hemY	protein_coding	2	tp4,tp7	1.50E-04	8	49
ubiE	protein_coding	2	tp4,tp7	1.50E-04	7.6	36
rluE	protein_coding	2	tp4,tp7	1.60E-04	7.6	38
ygjR	protein_coding	2	tp7,tp12	1.60E-04	7.7	41
ykfJ	pseudogene	2	tp4,tp7	1.60E-04	8.2	56
ygiD	protein_coding	2	tp7,tp8	1.70E-04	7.9	47
yqiB	protein_coding	2	tp4,tp7	1.70E-04	7.5	35
insJ	protein_coding	2	tp4,tp7	1.80E-04	7.6	36
atoS	protein_coding	2	tp4,tp7	2.00E-04	7.7	41

dadX	protein_coding	2	tp4,tp7	2.00E-04	7.4	33
hdeA	protein_coding	2	tp4,tp7	2.00E-04	7.8	42
uspE	protein_coding	2	tp8,tp12	2.00E-04	7.6	56
ftnB	protein_coding	2	tp7,tp12	2.10E-04	6.3	36
lgoT	protein_coding	2	tp4,tp7	2.10E-04	7.7	40
mscS	protein_coding	2	tp4,tp7	2.10E-04	7.9	45
yjhF	protein_coding	2	tp4,tp7	2.10E-04	7.6	36
asnA	protein_coding	2	tp4,tp7	2.50E-04	7.4	31
pfkA	protein_coding	2	tp4,tp7	2.50E-04	7.9	47
ompF	protein_coding	2	tp4,tp7	2.70E-04	7.4	31
ykgS	protein_coding	2	tp7,tp12	2.90E-04	7.3	30
amyA	protein_coding	2	tp4,tp7	3.00E-04	6.5	29
yaiT	pseudogene	2	tp4,tp7	3.00E-04	7.3	30
talB	protein_coding	2	tp4,tp7	3.20E-04	7.6	37
yccA	protein_coding	2	tp4,tp7	3.50E-04	7.2	27
zapB	protein_coding	2	tp4,tp7	3.50E-04	6	21
groS	protein_coding	2	tp5,tp7	3.70E-04	7.3	31
cmk	protein_coding	2	tp7,tp12	4.20E-04	7.3	30
fucP	protein_coding	2	tp7,tp12	4.20E-04	7.4	33
yoeG	pseudogene	2	tp4,tp7	4.20E-04	7.5	35

ygiF	protein_coding	2	tp4,tp7	4.40E-04	7.1	26
yjjV	protein_coding	2	tp7,tp12	4.40E-04	8.7	74
yfcH	protein_coding	2	tp7,tp12	4.50E-04	7.2	28
cycA	protein_coding	2	tp4,tp7	5.40E-04	6.8	54
nhaA	protein_coding	2	tp4,tp7	6.30E-04	7.4	33
sraG	ncRNA	2	tp4,tp7	7.20E-04	7.2	28
lysU	protein_coding	2	tp4,tp7	7.70E-04	7.2	28
uxuR	protein_coding	2	tp4,tp7	7.90E-04	7	25
uraA	protein_coding	2	tp4,tp7	8.30E-04	7	24
sodA	protein_coding	2	tp4,tp7	8.90E-04	6.9	47
wzzB	protein_coding	2	tp4,tp7	8.90E-04	6.9	22
amiB	protein_coding	2	tp4,tp7	9.20E-04	7.2	28
gspL	protein_coding	2	tp4,tp7	9.20E-04	6.1	17
glcD	protein_coding	2	tp7,tp12	9.40E-04	7.3	28
yadC	protein_coding	2	tp4,tp7	1.00E-03	6.9	23
yfiL	protein_coding	2	tp7,tp12	1.00E-03	7.9	43
pqqL	protein_coding	2	tp8,tp12	1.10E-03	6.7	31
hns	protein_coding	2	tp4,tp7	1.30E-03	6.8	21
trpR	protein_coding	2	tp4,tp7	1.40E-03	6.6	19
lptD	protein_coding	2	tp7,tp12	1.50E-03	6.8	21

ycgX	protein_coding	2	tp7,tp14	1.50E-03	9.4	150
yciC	protein_coding	2	tp7,tp12	1.60E-03	7.5	34
yhfA	protein_coding	2	tp4,tp7	1.70E-03	6.6	20
pldA	protein_coding	2	tp4,tp7	1.80E-03	6.6	19
yliM	protein_coding	2	tp4,tp7	1.80E-03	6.6	19
nirC	protein_coding	2	tp4,tp7	1.90E-03	6.3	19
parC	protein_coding	2	tp7,tp12	1.90E-03	6.8	21
srkA	protein_coding	2	tp4,tp7	2.00E-03	6.7	20
ytjE	protein_coding	2	tp4,tp7	2.00E-03	6.6	18
bfd	protein_coding	2	tp4,tp8	2.10E-03	6	23
rplP	protein_coding	2	tp9,tp12	2.10E-03	7.5	31
serU	tRNA	2	tp4,tp7	2.10E-03	6.6	18
envC	protein_coding	2	tp4,tp7	2.30E-03	6.5	17
accD	protein_coding	2	tp4,tp7	2.40E-03	6.6	19
lptB	protein_coding	2	tp7,tp8	2.40E-03	6.8	21
pgi	protein_coding	2	tp4,tp7	2.90E-03	6.3	15
yhhA	protein_coding	2	tp7,tp9	3.30E-03	6	32
yncE	protein_coding	2	tp4,tp7	3.30E-03	6.7	20
gmk	protein_coding	2	tp4,tp7	3.40E-03	6.3	17
ahpC	protein_coding	2	tp4,tp7	3.60E-03	6.4	16

ilvI	protein_coding	2	tp4,tp7	5.10E-03	6.5	18
fdhD	protein_coding	2	tp4,tp7	6.10E-03	6.2	14

Table S9: Gene membership of Clusters 1–3 from the dynamic NAD-capped RNA heatmap (Figure 3e), with functional categories, KEGG pathway annotations, and cluster assignment

Gene name	Gene biotype	KEGG pathway	Cluster
serV	tRNA	Aminoacyl-tRNA biosynthesis	1
yejF	protein_coding	ABC transporters	1
ssrA	ncRNA	Non-coding RNAs	1
yjeI	protein_coding	N/A	1
amyA	protein_coding	Starch and sucrose metabolism	1
purA	protein_coding	Nucleotide metabolism	1
tomB	protein_coding	Prokaryotic defense system	1
alaA	protein_coding	Alanine, aspartate and glutamate metabolism	1
rlmJ	protein_coding	Ribosome biogenesis	1
atoS	protein_coding	Protein kinases	1
metK	protein_coding	Cysteine and methionine metabolism	1
yccA	protein_coding	Transporters	1
rna1	ncRNA	N/A	1
moeA	protein_coding	Folate biosynthesis	1
yoeG	pseudogene	N/A	1
fliZ	protein_coding	Biofilm formation	1
yajO	protein_coding	N/A	1
asnA	protein_coding	Alanine, aspartate and glutamate metabolism	1
yjhF	protein_coding	Transporters	1
ygiD	protein_coding	N/A	1
groS	protein_coding	Chaperones and folding catalysts	1
wzzB	protein_coding	Lipopolysaccharide biosynthesis proteins	1
yfcH	protein_coding	N/A	1
sibE	ncRNA	Prokaryotic defense system	1
betI	protein_coding	Transcription factors	1
yncE	protein_coding	N/A	1
ybhA	protein_coding	N/A	1
uxuR	protein_coding	Transcription factors	1
ysgD	protein_coding	N/A	1
hns	protein_coding	Chromosome and associated proteins	1
ahpC	protein_coding	N/A	1
nirC	protein_coding	Transporters	1
zapG	protein_coding	N/A	1
thrL	protein_coding	N/A	1
dadX	protein_coding	D-Amino acid metabolism	1
dgcE	protein_coding	Biofilm formation	1
gmk	protein_coding	Purine metabolism	1
yieE	protein_coding	N/A	1
pgi	protein_coding	Glycolysis / Gluconeogenesis	1
zapB	protein_coding	Chromosome and associated proteins	1
citC	protein_coding	Two-component system	1

exuR	protein_coding	Transcription factors	1
gspL	protein_coding	Bacterial secretion system	1
yigG	protein_coding	N/A	1
rbsZ	ncRNA	N/A	1
moaA	protein_coding	Folate biosynthesis	1
yqiB	protein_coding	N/A	1
ybdO	protein_coding	N/A	1
thrS	protein_coding	Aminoacyl-tRNA biosynthesis	1
parC	protein_coding	DNA replication proteins	1
pldA	protein_coding	Glycerophospholipid metabolism	1
mgrB	protein_coding	N/A	1
kdsD	protein_coding	Biosynthesis of various nucleotide sugars	1
hemY	protein_coding	N/A	1
glyW	tRNA	Aminoacyl-tRNA biosynthesis	1
cyoA	protein_coding	Oxidative phosphorylation	1
amiB	protein_coding	Cationic antimicrobial peptide	1
dnaK	protein_coding	RNA degradation	1
cnu	protein_coding	N/A	1
sodA	protein_coding	Peroxisome	1
talB	protein_coding	Pentose phosphate pathway	1
yadC	protein_coding	N/A	1
cysZ	protein_coding	N/A	1
ribA	protein_coding	Riboflavin metabolism	1
ung	protein_coding	Base excision repair	1
ubiC	protein_coding	Ubiquinone and other terpenoid-quinone biosynthesis	1
sraG	ncRNA	N/A	1
ryfD	ncRNA	N/A	1
ytjE	protein_coding	N/A	1
bamE	protein_coding	Transporters	1
secY	protein_coding	Protein export	1
ydfG	protein_coding	Pyrimidine metabolism	1
ygiF	protein_coding	N/A	1
ubiE	protein_coding	Ubiquinone and other terpenoid-quinone biosynthesis	1
acpP	protein_coding	Fatty acid biosynthesis	1
nhaA	protein_coding	Transporters	1
thrU	tRNA	Aminoacyl-tRNA biosynthesis	1
ftsK	protein_coding	Chromosome and associated proteins	1
cvrA	protein_coding	Transporters	1
dsrA	ncRNA	Biofilm formation	1
ompF	protein_coding	Two-component system	1
rluE	protein_coding	Ribosome biogenesis	1
accD	protein_coding	Pyruvate metabolism	1
pfkA	protein_coding	Glycolysis / Gluconeogenesis	1
chiX	ncRNA	Messenger RNA biogenesis	1

nagA	protein_coding	Amino sugar and nucleotide sugar metabolism	1
rraB	protein_coding	Messenger RNA biogenesis	1
uraA	protein_coding	Transporters	1
yliM	protein_coding	N/A	1
insX	pseudogene	N/A	1
hemA	protein_coding	Porphyrin metabolism	1
envC	protein_coding	N/A	1
cycA	protein_coding	Transporters	1
yaaJ	protein_coding	N/A	1
insK	protein_coding	N/A	1
ileX	tRNA	Aminoacyl-tRNA biosynthesis	1
lpxD	protein_coding	Lipopolysaccharide biosynthesis	1
bfd	protein_coding	N/A	1
yfcO	protein_coding	N/A	1
gcvB	ncRNA	Biofilm formation	1
lptD	protein_coding	Transporters	1
lgoT	protein_coding	Transporters	1
yheS	protein_coding	Translation factors	1
yhfA	protein_coding	N/A	1
insJ	protein_coding	N/A	1
yifL	protein_coding	N/A	1
csrC	ncRNA	Biofilm formation	1
ydbK	protein_coding	Glycolysis / Gluconeogenesis	1
sibC	ncRNA	Prokaryotic defense system	1
cspA	protein_coding	N/A	1
sibD	ncRNA	Prokaryotic defense system	1
glvC	protein_coding	Starch and sucrose metabolism	1
rsmE	protein_coding	Ribosome biogenesis	1
tig	protein_coding	N/A	1
trpR	protein_coding	Transcription factors	1
nrdH	protein_coding	N/A	1
hdeA	protein_coding	Chaperones and folding catalysts	1
gltF	protein_coding	N/A	1
opgG	protein_coding	N/A	1
metZ	tRNA	Aminoacyl-tRNA biosynthesis	1
zapA	protein_coding	Chromosome and associated proteins	1
pdhR	protein_coding	Transcription factors	1
serU	tRNA	Aminoacyl-tRNA biosynthesis	2
pheT	protein_coding	Aminoacyl-tRNA biosynthesis	2
valU	tRNA	Aminoacyl-tRNA biosynthesis	2
fxsA	protein_coding	N/A	2
ykfJ	pseudogene	N/A	2
lysU	protein_coding	Aminoacyl-tRNA biosynthesis	2
ycfH	protein_coding	N/A	2
fbaA	protein_coding	Glycolysis / Gluconeogenesis	2
yagJ	protein_coding	N/A	2

mraZ	protein_coding	Transcription factors	2
tqsA	protein_coding	N/A	2
ybeL	protein_coding	N/A	2
fimH	protein_coding	Secretion system	2
cmk	protein_coding	Pyrimidine metabolism	2
frdA	protein_coding	Citrate cycle (TCA cycle)	2
ybiI	protein_coding	N/A	2
gatY	protein_coding	Galactose metabolism	2
fruB	protein_coding	Phosphotransferase system	2
yhcD	protein_coding	N/A	2
tamB	protein_coding	Transporters	2
cas3	protein_coding	Prokaryotic defense system	2
ilvI	protein_coding	Butanoate metabolism	2
yadM	protein_coding	N/A	2
ackA	protein_coding	Pyruvate metabolism	2
ybgC	protein_coding	N/A	2
ypfJ	protein_coding	N/A	2
rpsT	protein_coding	Ribosome	2
hemC	protein_coding	Porphyrin metabolism	2
rimJ	protein_coding	Ribosome biogenesis	2
casD	protein_coding	Prokaryotic defense system	2
epmA	protein_coding	Translation factors	2
osmB	protein_coding	N/A	2
tusB	protein_coding	Sulfur relay system	2
yciC	protein_coding	N/A	2
kdsC	protein_coding	Biosynthesis of various nucleotide sugars	2
mscS	protein_coding	Transporters	2
ydgA	protein_coding	N/A	2
tpx	protein_coding	N/A	2
tpiA	protein_coding	Glycolysis / Gluconeogenesis	2
exbB	protein_coding	Transporters	2
mepH	protein_coding	Peptidases and inhibitors	2
talA	protein_coding	Pentose phosphate pathway	2
slyD	protein_coding	Chaperones and folding catalysts	2
ychJ	protein_coding	N/A	2
uspB	protein_coding	Transporters	2
fdhD	protein_coding	N/A	2
yraH	protein_coding	Secretion system	2
bcsE	protein_coding	N/A	2
yaiT	pseudogene	N/A	2
rplT	protein_coding	Ribosome	2
ymcE	protein_coding	N/A	2
ptsG	protein_coding	Glycolysis / Gluconeogenesis	2
fucP	protein_coding	Transporters	2
aspA	protein_coding	Alanine, aspartate and glutamate metabolism	2

cspD	protein_coding	N/A	2
lptB	protein_coding	ABC transporters	2
sfsA	protein_coding	N/A	2
yfjI	protein_coding	N/A	2
uspE	protein_coding	N/A	2
rpmE	protein_coding	Ribosome	2
ybdR	protein_coding	N/A	2
wrbA	protein_coding	Ubiquinone and other terpenoid-quinone biosynthesis	2
glcD	protein_coding	Glyoxylate and dicarboxylate metabolism	2
ftnB	protein_coding	N/A	2
bluR	protein_coding	Transcription factors	2
ybdM	protein_coding	N/A	2
glgX	protein_coding	Starch and sucrose metabolism	2
yaiO	protein_coding	N/A	2
efp	protein_coding	Translation factors	2
raiA	protein_coding	Ribosome biogenesis	2
rlmI	protein_coding	Ribosome biogenesis	2
ydbA	pseudogene	N/A	2
nuoG	protein_coding	Oxidative phosphorylation	2
epd	protein_coding	Vitamin B6 metabolism	2
gcvT	protein_coding	Glyoxylate and dicarboxylate metabolism	2
osmE	protein_coding	N/A	2
yfgG	protein_coding	N/A	2
yghB	protein_coding	N/A	2
aaeR	protein_coding	Transcription factors	2
miaA	protein_coding	Prenyltransferases	2
yjjV	protein_coding	N/A	2
hdeD	protein_coding	N/A	2
ispF	protein_coding	Terpenoid backbone biosynthesis	2
srkA	protein_coding	N/A	2
ycgZ	protein_coding	Signaling proteins	2
betT	protein_coding	Transporters	2
betA	protein_coding	Glycine, serine and threonine metabolism	2
ftsE	protein_coding	ABC transporters	2
ychH	protein_coding	N/A	2
ymdB	protein_coding	N/A	2
pqqL	protein_coding	Peptidases and inhibitors	2
waaU	protein_coding	Lipopolysaccharide biosynthesis	2
ycbZ	protein_coding	Peptidases and inhibitors	2
mcaS	ncRNA	Biofilm formation	2
hicA	protein_coding	Prokaryotic defense system	3
pepD	protein_coding	Glutathione metabolism	3
proK	tRNA	Aminoacyl-tRNA biosynthesis	3
leuS	protein_coding	Aminoacyl-tRNA biosynthesis	3

ppnN	protein_coding	Purine metabolism	3
adeQ	protein_coding	Transporters	3
yabP	protein_coding	N/A	3
ycgX	protein_coding	N/A	3
yfeO	protein_coding	Transporters	3
cdsA	protein_coding	Glycerophospholipid metabolism	3
tyrU	tRNA	Aminoacyl-tRNA biosynthesis	3
ydhF	protein_coding	N/A	3
nadR	protein_coding	Nicotinate and nicotinamide metabolism	3
ldtD	protein_coding	Peptidases and inhibitors	3
sulA	protein_coding	Chromosome and associated proteins	3
yfiL	protein_coding	N/A	3
ykgS	protein_coding	N/A	3
insH1	protein_coding	N/A	3
yehA	protein_coding	N/A	3
mlaA	protein_coding	N/A	3
ygjR	protein_coding	N/A	3
trmA	protein_coding	Transfer RNA biogenesis	3
insH4	protein_coding	N/A	3
insH21	protein_coding	N/A	3
puuD	protein_coding	Arginine and proline metabolism	3
bsmA	protein_coding	N/A	3
cutC	protein_coding	N/A	3
ybdD	protein_coding	N/A	3
insH3	protein_coding	N/A	3
zntA	protein_coding	N/A	3
yagK	protein_coding	N/A	3
dcrB	protein_coding	N/A	3
yhhA	protein_coding	N/A	3
ppiD	protein_coding	Chaperones and folding catalysts	3
gadY	ncRNA	Messenger RNA biogenesis	3
rplP	protein_coding	Ribosome	3
rplN	protein_coding	Ribosome	3
insH6	protein_coding	N/A	3
ldtE	protein_coding	Peptidases and inhibitors	3
dnaG	protein_coding	DNA replication	3
ispZ	ncRNA	N/A	3

Table S10: Table of sequences for RNA oligonucleotides, DNA templates, primers, and DNA adapters

RNA oligonucleotides	sequence (5'–3')
113 nt model RNA	AGAUCACAGAGAUGUGAUGGAAAAUAGUUGAUGAGUUGUUUA AUUUUAAGAAUUUUUAUCUUAAUUAAGGAAGGAGUGAUUUCAA UGGCACAAGAUAUCAUUUCAACAAUCGG
113 nt model RNA probe (86mer)	AUUGAAAUCACUCCUUCCUUAAUUAAGAUAAAAAUUCUUAAAA UUAAACAACUCAUCAACUAUUUUCCAUCACAUCUCUGUGAUCU
E. coli 5S rRNA probe (60mer)	GGCGCUACGGCGUUUCACUUCUGAGUUCGGCAUGGGGUCAG GUGGGACCACCGCGCUACG
E. coli 16S rRNA probe (60mer)	GGCGUGGACUACCAGGGUAUCUAAUCCUGUUUGCUCCCCACG CUUUCGCACCUGAGCGUC
E. coli 23S rRNA probe (60mer)	GGCACCAUUUUGCCUAGUUCCUUCACCCGAGUUCUCUCAAGC GCCUUGGUAUUCUCUACC
biotin-RNA (internal standard)	AGGGAAGUGCUACCACAACUUUAGCCAUAAUGUCACUUCUGC CGCGGGCAUGCGGCCAGCCA
IS-036 (=36 nt model RNA)	ACUGACUUGAACGCGAUUCUUUAUCAGCGCGAUAAC
IS-051	ACGUACGCUCCGACACUUGGAGUACUCGGCGCUGUAAUCGG GCUGAUUGCC
IS-060	AUCGAAGAGCCAGUUGCAGCCGCCAUCGGAGCGGAUUUACCG GUUGAUGAGCCGGUUGCC
IS-102A	AUCAAGCAAUCCGCCGAUAAUCGCGACAUCUGUGCCGUGGCC CUUAUACGUUUCCGCAAACGAGCCGUAAAAAGAUACAAUGAU GCGCUCAGGCUCUCUGCC
IS-102B	ACAGUCUGGAUGUUUACGCCAACAUCGACGGUUACCUCACUG AUACGCUCAGCCAUAUUUUUGAAGAAGCAAUCGGAUUUGCGG GCUGUGAGCUGAUACGAC
IS-122	AGAUGACCUGCUCCCCAGUCUCUGGAUGUCUGCCGUUCAAUG CGAAUACAGAUCCGAUUUUCGCUAAAUCCAAGCUGUUCAUCU CAAUCGCGCAUUGCUUCGUAUAUAAAUCCAUUACUGCC
IS-149	ACUGCAGGAGCGAGGCGUCUCGCUGCCUGAAAGACUUCUUCU AUUGUCUCGCUCGACGUAAAUUCAGUAAUUUGCCAAUAAGCA ACAGGUGCAUCGAUACUGCCUGCGAUCGACUUCACUAACGUC GUCUUUCCGUUUCCAGGAGGUCC
IS-200	ACCUGGACCAGAAGUUUGAGCUCAUCAAAAUUUGCCCGCUUA UCCACUGGACAUGGGAUGACGUUUGGACGUACAUCCGCCUGC ACAAUUUGCCUUAUAACAAACUGCACGAUCAACAUUAUCCGAG CAUAGGCUGUGAAAUGUGUACACUGCCUUCCCCGGACCCGAA CGAUGAGAGGGCUGGAAGAUGGGCCGGACGG
IS-250	AUGAAUGCCGGGCGGCUGUUUCCGCGUCACGGCUUUGAAGC UGGCGGUCUCUAAUAACAGAAAGAGGCAGAAACGUCGCCCGG CCGAAGGAAUUCUGCUUUAAAUAUUGAAUCGCUUUGCGGGCA GAUUGUUCAUCGUCGGUCACGACGUGUUGAGCAGAAGCGCC GAGCGCUAUUUCAAUGGCCGUUUCAUACUUCUGUUCUGUAGA AAUCAGCUCAAGAACCGCUCCGCGAAUUCCGCCAAGGCGCUC

IS-301	AUGCAUCCAUAAACGAAUUGGCUGCCGCAUAAUCGGCUUGUC CAGCGUUUCCUAAACAUCCCGAAACAGAAGAGAAGAAAAUGAA AAAGUCCAAUGGGAAAUCCUUGCUGCAUUCGUCUACAUGUAA CAACCCGCUCACUUUUGGCUGCAACACUUCUUGAAACUCUUC AUUCGUUUUAUGAAUGAUGAAGCGAUCUUUACUGCUGCCUGC ACCGUGAAUAAUUCCAUUGAGAGUGCCAUAUCUUUCUUUGAU UUCCUCCAAAAGAUGACGAACCGCAUGCUGAUCACUUACAUC AGCCUC
IS-351	AGACCUCUUGUCCCGCCUGUAAUAAGCAGGACUUUGUCUGUU GAAAACGAUACUUGCUUAUGACUUAGAGCGGCUUCUGCUGGC UGCUCUGUCAAAUAUGCGCGAUAUCGCUGUCCGUUUCGAUAA CAGACUUCUGAUUCUGUGCUCUUGGCAUAAUAUUCAACUGCA AUCAGUCUGGCCAGCUCCUCAUGAGAGGCUUCGCAUUCCAUA UCUGCAUGUCUUGAAGUAAGAUGGCUGUACUCACUUUGAAGC AUGCGGUAUAAACCGGCCCGUGAAGCACCAGACAAAAGAACA CCCUCAUUUGCGUAGGCCUCCAGCCCCUUUGUUACACCCAAG ACCGUCAAAUGAUGC
IS-400	ACUGCCAGGUCUCCAAGCGCAACGAUCCCGACUAGAUUGUUU UGAUCUACAAUAGGAAGGCGGCGGAUUUGGUGCUGGGCCAU CAGCUGCGACGCGUCUUCCAGACUCAUAUUAGGGUUUCCUGA UACGAGUUCUGUUGACAUCACUUCUGAAACGGGUGUCUGGCC AUCACGGCCCUGAGCUGUAGUUCUUAAUGCAAUGUCACGGUC AGUCAGCAUUCCUUUUAAAACACCUUGCUCUACAACGGGGAU CGCCCCGACGUUAUGCUGUUUCAUUAGAGAAGCCGCUUCCUG AAUCGUUUGAUUCGGAGAAACGGUUGCCACCUGCGUUGUCAU UGUAUCUUUAACUGAACUCAUUGUCAGCACCCCUUUCAAUCA UUAGUAUGUUCAGACACGAGCCG
IS-501	ACAGGCUAACAUUUGCUGUAACCUCUGUCUCUACUUUUACCC GUUCUGUUCUUGCGACAAGCUGCAGGGCGCUGUCAAAGCAAG CUGCAUAACCUGCUGCAAACAGCUGCUCUGGAUUUGUCGCUU UUUCUAAUUUCUUGGCUCUCGGUGUCCCCGGCAUUGCGACAU CAAGCUCAAGAACGCGGUCUGAUGAAAUGACCUUUCCUUCUC UUCCUCCUACCGCUGAAACAGUUGCGGUAAAUAAUGGCUGAC UCAUUUUUCAUUCCCCCAAUAUAUUUUAUACAAUUAAAUUGUA CACAAUUAAAUUUGAAAUGUCAAUUUUUCGCGUAUAAAAAAGC UUAUCUCGUAAUGAGAUAAGCUUUUUCAUUGAACAUUACCCC GCUAUUCACGGAUUUGCCCAGUUCCUUUAAUGAUGUAUUUUG UAGAAGUCAGUGCAGGAAGCCCCAUCGGUCCUCUUGCAUGAA GCUUUUGCGUGCUGAUGCCGAUUUCGGCUCCGUAGCC
DNA templates	**sequence (5'–3') (with T7 promoter region underlined)**
113 nt model RNA	TAATACGACTCACTATTAGATCACAGAGATGTGATGGAAAATAG TTGATGAGTTGTTTAATTTTAAGAATTTTTATCTTAATTAAGGAAG GAGTGATTTCAATGGCACAAGATATCATTTCAACAATCGG
113 nt model RNA probe (86mer)	TAATACGACTCACTATTATTGAAATCACTCCTTCCTTAATTAAGA TAAAAATTCTTAAAATTAAACAACTCATCAACTATTTTCCATCACA TCTCTGTGATCT
E. coli 5S rRNA probe (60mer)	CAGTAATACGACTCACTATAGGCGCTACGGCGTTTCACTTCTGA GTTCGGCATGGGGTCAGGTGGGACCACCGCGCTACG
E. coli 16S rRNA probe (60mer)	CAGTAATACGACTCACTATAGGCGTGGACTACCAGGGTATCTAA TCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTC

E. coli 23S rRNA probe (60mer)	CAGTAATACGACTCACTATAGGCACCATTTTGCCTAGTTCCTTC ACCCGAGTTCTCTCAAGCGCCTTGGTATTCTCTACC
IS-036 (=36 nt model RNA)	CAGTAATACGACTCACTATTACTGACTTGAACGCGATTCTTTATC AGCGCGATAAC
IS-051	TAATACGACTCACTATTACGTACGCTCCGACACTTGGAGTACTC GGCGCTGTAATCGGGCTGATTGCC
IS-060	TAATACGACTCACTATTATCGAAGAGCCAGTTGCAGCCGCCATC GGAGCGGATTTACCGGTTGATGAGCCGGTTGCC
IS-102A	TAATACGACTCACTATTATCAAGCAATCCGCCGATAATCGCGAC ATCTGTGCCGTGGCCCTTATACGTTTCCGCAAACGAGCCGTAAA AAGATACAATGATGCGCTCAGGCTCTCTGCC
IS-102B	TAATACGACTCACTATTACAGTCTGGATGTTTACGCCAACATCG ACGGTTACCTCACTGATACGCTCAGCCATATTTTTGAAGAAGCA ATCGGATTTGCGGGCTGTGAGCTGATACGAC
IS-122	TAATACGACTCACTATTAGATGACCTGCTCCCCAGTCTCTGGAT GTCTGCCGTTCAATGCGAATACAGATCCGATTTTCGCTAAATCC AAGCTGTTCATCTCAATCGCGCATTGCTTCGTATATAAATCCATT ACTGCC
IS-149	TAATACGACTCACTATTACTGCAGGAGCGAGGCGTCTCGCTGC CTGAAAGACTTCTTCTATTGTCTCGCTCGACGTAAATTCAGTAAT TTGCCAATAAGCAACAGGTGCATCGATACTGCCTGCGATCGACT TCACTAACGTCGTCTTTCCGTTTCCAGGAGGTCC
IS-200	TAATACGACTCACTATTACCTGGACCAGAAGTTTGAGCTCATCA AAATTTGCCCGCTTATCCACTGGACATGGGATGACGTTTGGACG TACATCCGCCTGCACAATTTGCCTTATAACAAACTGCACGATCA ACATTATCCGAGCATAGGCTGTGAAATGTGTACACTGCCTTCCC CGGACCCGAACGATGAGAGGGCTGGAAGATGGGCCGGACGG
IS-250	TAATACGACTCACTATTATGAATGCCGGGCGGCTGTTTCCGCGT CACGGCTTTGAAGCTGGCGGTCTCTAATAACAGAAAGAGGCAG AAACGTCGCCCGGCCGAAGGAATTCTGCTTTAAATATTGAATCG CTTTGCGGGCAGATTGTTCATCGTCGGTCACGACGTGTTGAGC AGAAGCGCCGAGCGCTATTTCAATGGCCGTTTCATACTTCTGTT CTGTAGAAATCAGCTCAAGAACCGCTCCGCGAATTCCGCCAAG GCGCTC
IS-301	TAATACGACTCACTATTATGCATCCATAAACGAATTGGCTGCCG CATAATCGGCTTGTCCAGCGTTTCCTAAACATCCCGAAACAGAA GAGAAGAAAATGAAAAAGTCCAATGGGAAATCCTTGCTGCATTC GTCTACATGTAACAACCCGCTCACTTTTGGCTGCAACACTTCTT GAAACTCTTCATTCGTTTTATGAATGATGAAGCGATCTTTACTGC TGCCTGCACCGTGAATAATTCCATTGAGAGTGCCATATCTTTCT TTGATTTCCTCCAAAAGATGACGAACCGCATGCTGATCACTTAC ATCAGCCTC
IS-351	TAATACGACTCACTATTAGACCTCTTGTCCCGCCTGTAATAAGC AGGACTTTGTCTGTTGAAAACGATACTTGCTTATGACTTAGAGC GGCTTCTGCTGGCTGCTCTGTCAAATATGCGCGATATCGCTGTC CGTTTCGATAACAGACTTCTGATTCTGTGCTCTTGGCATAATATT CAACTGCAATCAGTCTGGCCAGCTCCTCATGAGAGGCTTCGCA TTCCATATCTGCATGTCTTGAAGTAAGATGGCTGTACTCACTTTG AAGCATGCGGTATAAACCGGCCCGTGAAGCACCAGACAAAAGA

	ACACCCTCATTTGCGTAGGCCTCCAGCCCCTTTGTTACACCCAA GACCGTCAAATGATGC
IS-400	TAATACGACTCACTATTACTGCCAGGTCTCCAAGCGCAACGATC CCGACTAGATTGTTTTGATCTACAATAGGAAGGCGGCGGATTTG GTGCTGGGCCATCAGCTGCGACGCGTCTTCCAGACTCATATTA GGGTTTCCTGATACGAGTTCTGTTGACATCACTTCTGAAACGGG TGTCTGGCCATCACGGCCCTGAGCTGTAGTTCTTAATGCAATGT CACGGTCAGTCAGCATTCCTTTTAAAACACCTTGCTCTACAACG GGGATCGCCCCGACGTTATGCTGTTTCATTAGAGAAGCCGCTT CCTGAATCGTTTGATTCGGAGAAACGGTTGCCACCTGCGTTGTC ATTGTATCTTTAACTGAACTCATTGTCAGCACCCCTTTCAATCAT TAGTATGTTCAGACACGAGCCG
IS-501	TAATACGACTCACTATTACAGGCTAACATTTGCTGTAACCTCTGT CTCTACTTTTACCCGTTCTGTTCTTGCGACAAGCTGCAGGGCGC TGTCAAAGCAAGCTGCATAACCTGCTGCAAACAGCTGCTCTGG ATTTGTCGCTTTTTCTAATTTCTTGGCTCTCGGTGTCCCCGGCAT TGCGACATCAAGCTCAAGAACGCGGTCTGATGAAATGACCTTTC CTTCTCTTCCTCCTACCGCTGAAACAGTTGCGGTAAATAATGGC TGACTCATTTTTCATTCCCCCAATATATTTTATACAATTAAATTGT ACACAATTAAATTTGAAATGTCAATTTTTCGCGTATAAAAAAGCT TATCTCGTAATGAGATAAGCTTTTTCATTGAACATTACCCCGCTA TTCACGGATTTGCCCAGTTCCTTTAATGATGTATTTTGTAGAAGT CAGTGCAGGAAGCCCCATCGGTCCTCTTGCATGAAGCTTTTGC GTGCTGATGCCGATTTCGGCTCCGTAGCC
primers	**sequence (5'–3') (with T7 promoter region underlined)**
RTp2, reverse transcription primer	CAGACGTGTGCTCTTCCGAT
113 nt model RNA_fwd	TAATACGACTCACTATTAGATCACAGAGATGTGATGGAAAATAG TTGATG
113 nt model RNA_middle strand	GGAAAATAGTTGATGAGTTGTTTAATTTTAAGAATTTTTATCTTAA TTAAGGAAGGAGTGATTTCAATGG
113 nt model RNA_rev	CCGATTGTTGAAATGATATCTTGTGCCATTGAAATCACTC
113 nt model RNA probe_fwd	TAATACGACTCACTATTATTGAAATCACTCCTTCCTTAATTAAGA TAAAAATTCTTAAAA
113 nt model RNA probe_rev	AGATCACAGAGATGTGATGGAAAATAGTTGATGAGTTGTTTAAT TTTAAGAATTTTTATC
E. coli 5S rRNA probe_fwd	CAGTAATACGACTCACTATAGGCGCTACGGCGTTTCACTTCTGA GTTCGG
E. coli 5S rRNA probe_rev	CGTAGCGCGGTGGTCCCACCTGACCCCATGCCGAACTCAGAAG TGAAAC
E. coli 16S rRNA probe_fwd	CAGTAATACGACTCACTATAGGCGTGGACTACCAGGGTATCTAA TCCTG
E. coli 16S rRNA probe_rev	GACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATA CCCTGG
E. coli 23S rRNA probe_fwd	CAGTAATACGACTCACTATAGGCACCATTTTGCCTAGTTCCTTC ACCCG

E. coli 23S rRNA probe_rev	GGTAGAGAATACCAAGGCGCTTGAGAGAACTCGGGTGAAGGAACTAGG
IS-036_fwd	CAGTAATACGACTCACTATTACTGACTTGAACGCGATTCTTTATCAGCGCGATAAC
IS-036_rev	GTTATCGCGCTGATAAAGAATCGCGTTCAAGTCAGTAATAGTGAGTCGTATTACTG
IS-051_fwd	TAATACGACTCACTATTACGTACGCTCCGACACTTGG
IS-051_rev	GGCAATCAGCCCGATTACAG
IS-060_fwd	TAATACGACTCACTATTATCGAAGAGCCAGTTGCAGC
IS-060_rev	GGCAACCGGCTCATCAACC
IS-102A_fwd	TAATACGACTCACTATTATCAAGCAATCCGCCG
IS-102A_rev	GGCAGAGAGCCTGAGCG
IS-102B_fwd	TAATACGACTCACTATTACAGTCTGGATGTTTACG
IS-102B_rev	GTCGTATCAGCTCACAG
IS-122_fwd	TAATACGACTCACTATTAGATGACCTGCTCCCCAG
IS-122_rev	CGTATATAAATCCATTACTGCCAAGC
IS-149_fwd	TAATACGACTCACTATTACTGCAGGAGCGAG
IS-149_rev	GGACCTCCTGGAAACG
IS-200_fwd	TAATACGACTCACTATTACCTGGACCAGAAGTTTGAGC
IS-200_rev	CCGTCCGGCCCATCTTCC
IS-250_fwd	TAATACGACTCACTATTATGAATGCCGGGCGGCTG
IS-250_rev	GAGCGCCTTGGCGGAATTCG
IS-301_fwd	TAATACGACTCACTATTATGCATCCATAAACGAATTGG
IS-301_rev	GAGGCTGATGTAAGTGATCAG
IS-351_fwd	TAATACGACTCACTATTAGACCTCTTGTCCCGCCTG
IS-351_rev	GCATCATTTGACGGTCTTGG
IS-400_fwd	TAATACGACTCACTATTACTGCCAGGTCTCCAAGC
IS-400_rev	CGGCTCGTGTCTGAACATAC
IS-501_fwd	TAATACGACTCACTATTACAGGCTAACATTTGCTG
IS-501_rev	GGCTACGGAGCCGAAATCGG
DNA adapters	**sequence (5'–3') (with 4-letter barcodes underlined)**
AD3-id4-bc01	/5Phos/CNNNNNNACTTGAAGATCGGAAGAGCACACGTCTG/3SpC3/
AD3-id4-bc02	/5Phos/CNNNNNNACGCTGAGATCGGAAGAGCACACGTCTG/3SpC3/
AD3-id4-bc03	/5Phos/CNNNNNNACCGGTAGATCGGAAGAGCACACGTCTG/3SpC3/

AD3-id4-bc04	/5Phos/CNNNNNNACTCATAGATCGGAAGAGCACACGTCTG/3SpC3/
AD3-id4-bc05	/5Phos/CNNNNNNACGAACAGATCGGAAGAGCACACGTCTG/3SpC3/
AD3-id4-bc06	/5Phos/CNNNNNNACAGCAAGATCGGAAGAGCACACGTCTG/3SpC3/
AD3-id4-bc07	/5Phos/CNNNNNNACCATAAGATCGGAAGAGCACACGTCTG/3SpC3/
AD3-id4-bc08	/5Phos/CNNNNNNACATAGAGATCGGAAGAGCACACGTCTG/3SpC3/
AD3-id4-bc09	/5Phos/CNNNNNNACTGTCAGATCGGAAGAGCACACGTCTG/3SpC3/
AD3-id4-bc10	/5Phos/CNNNNNNACGTCTAGATCGGAAGAGCACACGTCTG/3SpC3/
AD3-id4-bc11	/5Phos/CNNNNNNACACGCAGATCGGAAGAGCACACGTCTG/3SpC3/
AD3-id4-bc12	/5Phos/CNNNNNNACTACGAGATCGGAAGAGCACACGTCTG/3SpC3/
cDNA anchor (sense)	/5Phos/CAGATCGGAAGAGCGTCGTGT/3SpC3/
cDNA anchor (antisense)	ACACGACGCTCTTCCGATCTGGG

Supplementary Methods:

General methods. All water used for the work highlighted was ddH_2O grade, and additionally filtered through a MilliQ system (mQ). If not stated otherwise, chemicals, solvents, commercial buffers, nucleic acids, enzymes, *et cetera*, were used without further purification. Chemicals, stock solutions and commercial buffers were purchased from Acros Organics, Alfa Aesar, Applichem, Carl Roth, Grüssing, Honeywell Riedel-de Haën, Invitrogen, New England Biolabs, Rapidozym, Sigma-Aldrich (Merck), Thermo Fisher Scientific, VWR. Non-commercial buffers were mixed from its components and H_2O according to the composition and sterile-filtered using a 0.22 µm bottle-top filter. Moisture-sensitive chemical reactions were performed using anhydrous solvents and reagents, and under argon atmosphere using the Schlenk technique. Thin-layer chromatography (TLC) was performed using POLYGRAM SIL G UV254 polyester sheets as stationary phase, and product, reactant or reagent spots were visualized using a UV lamp. Oligonucleotide primers, (radioactive) nucleotides and cofactor derivatives were purchased from Hartmann Analytic, Integrated DNA Technologies, Rapidozym, Sigma-Aldrich (Merck), Thermo Fisher Scientific. Enzymes and enzyme mixes were purchased from Bio-Rad Laboratories, Invitrogen, New England Biolabs, Roche, Thermo Fisher Scientific. ADPRC enzyme was kindly provided by Dr. Yaqing Zhang (Jäschke Lab, IPMB, University of Heidelberg). Commercial kits for oligonucleotide preparation and analysis were purchased from Agilent Technologies, Cell Signaling Technology, Invitrogen, QIAGEN. Stocks containing oligonucleotides and enzymes were generally stored at −20 °C (or −80 °C for the aliqouts of mixed internal RNA standards). Reaction mixes were generally prepared in the following order: Water, reagents, reactants, oligonucleotides, enzymes. Lyophilization of products was performed from aqueous solution or water/acetonitrile mixtures after freezing in suitable containers using liquid nitrogen. Work with [^{32}P]-labeled, radioactive nucleotides and RNA species was performed behind protective Plexiglas screens and following the applicable safety regulations in a dedicated isotope lab. Nuclear magnetic resonance (NMR) spectroscopy was conducted with a Mercury plus 300 MHz or 500 MHz spectrometer. Analytes were dissolved in suitable deuterated solvents from Eurisotop. In general, recorded spectra involved ^{1}H- and ^{13}C-NMR experiments (^{13}C-NMR spectra with attached-proton test (APT)). Where applicable, ^{31}P-, COSY-, HSQC-, and HMBC-NMR spectra were recorded (data not shown). Chemical shifts δ were reported in the reference to the deuterated solvent and are generally given in the format »chemical shift [ppm] (multiplicity, coupling constant [Hz], atom count)«. The signal multiplicity was abbreviated as s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, and m = multiplet. The analysis of recorded spectra was performed using the MestReNova software. Mass spectrometry (MS) measurements were conducted on a Bruker micrOTOF QII system using electrospray ionization (ESI) in positive or negative mode. High-resolution mass spectrometry (HR-MS) was performed using ESI-L Low-Concentration Tuning Mix as a calibrant. UV-Vis spectroscopy for small molecules, nucleic acids, and peptides was performed with the Nanodrop One device using 1 µL of solution and the respective measurement program recommended by the manufacturer. OD_{600} measurements for bacterial cultures were performed on the BioPhotometer 6131 with suitable cuvettes using 1 mL of solution according to the manufacturer's instructions. Fluorescence spectroscopy measurements for DNA or RNA oligonucleotides were performed with a Qubit fluorometer 2.0 device using either the Qubit dsDNA Quantification Assay Kit (High Sensitivity) or the Qubit RNA High Sensitivity (HS) Assay Kit according to the manufacturer's instructions. High-performance liquid chromatography (HPLC) was generally performed with two setups: For analytical HPLC, either the Luna 5u C18(2) 100 Å (250 × 4.6 mm) or the VDSpher PUR 100 C18-SE 5u (250 × 4.6 mm) column was used as stationary phase. For semi-preparative HPLC, the Luna 5u C18(2) 100 Å (250 × 15 mm) column was used as stationary phase. The mobile phase typically either was a mixture of H_2O (mQ) and acetonitrile (ACN), or a buffer mixture consisting of buffer A (0.1 M triethylamine/acetic acid in water, pH 7.0) and buffer B (0.1 M triethylamine/acetic acid in

acetonitrile/water 4:1, pH 7.0). The flow-rate generally was set to 1 mL/min for analytical HPLC and 5 mL/min for semi-preparative HPLC. Chromatograms were typically recorded at 260 nm (absorption), and other suitable wavelengths. Analysis of the peak area was performed using the ChemStation software, all other analyses, base-line correction, and plotting were done using the OriginPro software. Automated electrophoresis for DNA libraries obtained after HELIOS NAD-Seq was performed on a 2100 Bioanalyzer device in combination with the Bioanalyzer High Sensitivity DNA Analysis Kit according to the manufacturer's instructions. Denaturing polyacrylamide (PA) gels were prepared using Rotiphorese Sequencing gel concentrate (25%), which was diluted with TBE buffer and a saturated urea concentrate. Native PA gels were prepared using Rotiphorese NF-Acrylamide/Bis-solution (30%), which was diluted with TBE buffer. Denaturing and native PA gel electrophoresis (PAGE) was performed in 1x TBE buffer at a constant power of 17 W (gel size 24x18 cm) or a constant voltage of 150 V (gel size 8.3x7.0 cm). 0.4% APB–5% PA gels were prepared using the required amount of solid acryloylaminophenyl boronic acid (APB) and Rotiphorese NF-Acrylamide/Bis-solution (30%), which was diluted with TAE buffer. APB-PA gel electrophoresis (APB-PAGE) was performed in 1x TAE buffer at a constant voltage between 150 V and 180 V (gel size 8.3x7.0 cm). Nucleic acids were stained with SYBR Gold and visualized on a Typhoon FLA 9500 device. Agarose gels were prepared using agarose (1–2% (w/v)) in 1x TBE buffer, and gel electrophoresis was performed in 1x TBE buffer at a constant voltage (120 V) for typically 25–40 min. Nucleic acids were stained with ethidium bromide (EtBr) and visualized on a Typhoon FLA 9500 device, or the gel was further used for application of the Northern blotting technique. Signal intensities from gel electrophoresis or Northern blot experiments were determined using the ImageQuant software.

PCR for DNA template generation. PCR amplification reactions for template generation (e.g. for IVT reactions) were generally performed using either Taq DNA polymerase or Q5 DNA polymerase, with a variable amount of template DNA (dsDNA, gDNA, plasmid, ssDNA with overlap to both forward and reverse primers). Taq DNA polymerase-based PCR amplifications for RNAI and RNAIII leader templates, as well as RNA probe templates, were performed in the presence of 0.2 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 2 µM of gene-specific forward and reverse primer, 1x GenTherm buffer, 3.4 mM $MgCl_2$, 2% DMSO, and 10 ng/µL Taq DNA polymerase. Denaturation was performed at 94 °C, annealing at typically 55–58 °C, and extension at 72 °C for 1 min, 1 min, and 30 s, respectively. Q5 DNA polymerase-based PCR amplifications for internal RNA standard templates were performed in the presence of 0.2 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 0.2 µM of gene-specific forward and reverse primer, 1x Q5 reaction buffer, and 0.02 U/µL Q5 high-fidelity DNA polymerase. Denaturation was performed at 98 °C, annealing at typically 61–68 °C, and extension at 72 °C for 10 s, 20 s, and 30 s, respectively. Generally, 35 amplification cycles were performed using a thermocycler device. The information from the manufacturer (Integrated DNA Technologies) as well as the NEB Tm Calculator web tool were consulted to determine the primer annealing temperature. The amplified DNA templates were purified using the QIAquick PCR Purification Kit according to the manufacturer's instructions, followed by elution with H_2O, concentration determination by UV-Vis spectroscopy using a Nanodrop device, and analysis by agarose gel electrophoresis.

***In vitro* transcription (IVT)**. *In vitro* transcriptions for pppRNAs were generally performed in the presence of varying amounts of DNA template, 2 mM ATP, 2 mM CTP, 2 mM GTP, 2 mM UTP, 40 mM Tris-HCl (pH 8.1), 1 mM spermidine, 22 mM MgCl2, 0.01% Triton-X-100, 10 mM DTT, 5% DMSO, and 0.1 µg/µL T7 RNA polymerase, and incubated at 37 °C for 3.5 h. By adding 4 mM NAD^+ and reducing the concentration of ATP to 1 mM, mixtures of NAD-capped RNAs and corresponding pppRNAs were obtained. After the full reaction time, 2 µL of DNase I (1 µg/µL stock) per 100 µL of reaction volume were added and the incubation continued for 30

min at 37 °C. RNA was typically purified by denaturing 10% PAGE, followed by excision of the band of interest, gel crushing, overnight elution (20 °C, 700 rpm shaking) in 0.3 M NaOAc (pH 5.5), removal of gel pieces using 50 mL filter falcon tubes, and recovery by isopropanol precipitation. After this, the concentration of the aqueous RNA stock was determined by UV-Vis spectroscopy using a Nanodrop device, and the stock solution was stored at −20 °C. To obtain pure NAD-capped RNA, additional purification by 0.4% APB–5% PA gel electrophoresis was performed, followed by RNA visualization after incubation with SYBR Gold using a Typhoon FLA 9500 device, excision of the band of interest, and elution following two main routes: For RNA longer than 125 nt, the gel band was crushed, followed by overnight elution (20 °C, 700 rpm shaking) in 0.3 M NaOAc (pH 5.5), removal of gel pieces using 50 mL filter falcon tubes, and recovery by isopropanol precipitation (see chapter 19.4). For shorter RNAs, the above described method was also mainly used, however, in some cases, an in-house method was applied. In said procedure for electroelution, the excised band was pushed into the wells of an agarose gel (containing two drops EtBr, see chapter 18.8), and while performing agarose gel electrophoresis, the travelling RNA band was followed with an AlphaImager device until it has run into a large well (about 3–5 cm after the regular wells in the direction of RNA travelling) filled with 1x TBE. Once the RNA has visibly entered the pool, the buffer inside the well was removed, brought to a final concentration of 0.3 M NaOAc (pH 5.5) using a 3 M stock, and the purified RNA was recovered by isopropanol precipitation.

***In vitro* transcription (IVT) for radioactively labeled RNA**. Radioactively labeled RNA probes were prepared accordingly to the procedures described above. The *in vitro* transcription reaction was performed in the additional presence of typically 1 µCi/µL α-[^{32}P]-CTP (optionally also α-[^{32}P]-ATP and α-[^{32}P]-UTP were used in addition to α-[^{32}P]-CTP, but the sum of radioactive nucleotide triphosphates was kept at 1 µCi/µL). RNA probes were typically purified by extractions with P/C/I (three times) and diethyl ether (twice), followed by removal of ether traces in an Eppendorf concentrator, and recovery by ethanol precipitation.

Vaccinia capping of pppRNA. For the m^{7}G-capping reaction of pppRNA, the NEB Vaccinia Capping System was used according to the manufacturer's instruction. First, a 2 mM *S*-adenosylmethionine (SAM) solution was freshly diluted from the contained 32 mM stock. Then, around 2.2 µg of pppRNA in a volume of 15 µL were incubated at 65 °C for 5 min, cooled down on ice, and mixed with 2 µL 10x capping buffer, 1 µL GTP stock, 1 µL SAM (2 mM), and 1 µL Vaccinia capping enzyme. The G-capping of pppRNA was performed following the same method, while excluding SAM from the reaction mixture. The total volume of 20 µL was incubated at 37 °C for 30 min, before the reaction was stopped by the addition of 20 µL 2x APB loading buffer. To obtain pure m^{7}G-capped RNA, purification by 0.4% APB–5% PA gel electrophoresis was performed, followed by RNA visualization after incubation with SYBR Gold, excision of the band of interest, and elution as described above for the purification of NAD-capped RNAs.

Recovery of RNA or DNA oligonucleotides by precipitation. Nucleic acid-containing, aqueous solutions were mixed with NaOAc (3 M, pH 5.5) to reach a resulting sodium acetate concentration of 0.3 M. For low amounts of nucleic acids (regularly < 1 µg), 1 µL of RNA-grade glycogen was added, followed by gentle shaking and incubation at room temperature for about 1 min. In a next step, either 1 volume of cold isopropanol (only for RNA) or 2.5 volumes of cold absolute ethanol (for both, RNA and DNA), both at −20 °C, were added to the nucleic acid-containing solution. The vessel was inverted between 5 and 10 times, and standardly stored at −20 °C for between 1 h and 3 days. The precipitated nucleic acids were centrifuged (~15000 g, 4 °C) for at least 60 min (DNA) or 90 min (RNA). The supernatant was removed and the formed pellet was washed twice with 70% aqueous ethanol solution (at −20 °C), each wash followed by centrifugation (~15000 g, ~10 min, 4 °C) and removal of the supernatant. Residual solvent was removed in an Eppendorf concentrator (~3 min), before the pellet was dissolved

in H_2O. The nucleic acid concentration of the recovered sample was determined by UV-Vis spectroscopy.

Pre-adenylation of 3'-adapters. For the pre-adenylation of different 3'-adapters, 5'-phosphorylated and 3'-blocked (C3-spacer) oligonucleotides were ordered from Integrated DNA Technologies. From the dried down oligonucleotides, stock solutions of 800 µM were prepared with H_2O (mQ), from which 100 µL were used per adenylation reaction. Those 100 µL solutions were either dried using an Eppendorf concentrator device or further used without solvent removal, and the oligonucleotides (80 nmol) were adenylated in a total volume of 200 µL in the presence of 100 mM ImpA and 50 mM $MgCl_2$ at 50 °C for 90 min. After this reaction time, another increment (200 µL) containing 100 mM ImpA and 50 mM $MgCl_2$ was added, and the incubation continued at 50 °C for 90 min. The reaction mixture was purified on Illustra NAP-5 columns according to the manufacturer's instructions and fractions of 2 drops (around 100 µL) taken, which were analyzed using a Nanodrop device. Fractions with ratios of A_{260}/A_{280} < 2.0 and A_{260}/A_{230} < 2.4 were combined as DNA adapter-containing fractions, and the adapter adenylation ratio was determined by 20% denaturing PAGE. Stocks with final concentration of 50 µM of pre-adenylated adapter (100 µM total oligonucleotide concentration) were prepared either by diluting or concentrating the combined solutions, and stored at −20 °C.

Cell cultivation and RNA extractions. Total RNA from *E. coli* cells was extracted using a hot phenol extraction. *E. coli* K12 JM109 cultures were grown in LB medium till OD ≈ 2.0, the solvent was removed by centrifugation and the cell pellets frozen at −80 °C. Then, 4 mL of an aqueous solution containing SDS (2% (w/v)) and EDTA (4 mM), which was previously heated to 95 °C, were added. After suspending by pipetting and mixing on a vortex mixer for 30 s, the suspension was incubated at 95 °C with shaking at 750 rpm for 3 min, with vortex mixing after every minute. This mixture was pipetted into 8 mL aqueous phenol, which was previously heated to 65 °C, in a 15 mL falcon. The suspension was mixed on a vortex mixer for 30 s, and incubated at 65 °C for 5 min, with vortex mixing after every minute. The mixture was centrifuged (15000 g, typically around 15 min, 20 °C) until phase separation, and the aqueous layer transferred into a new 15 mL falcon tube without disturbing the interphase. This was followed by extraction with equal volumes of P/C/I solution (four times) and diethyl ether (twice), each followed by centrifugation (10000 g, 5 min, 20 °C), and removal of ether traces in an Eppendorf concentrator. After this procedure, around 1 mL was left for every sample and the extracted total RNA was recovered by ethanol precipitation (overnight The concentration of the obtained nucleic acid samples was measured by UV-Vis spectroscopy, and, to remove DNA contamination, the total RNA sample was treated with 1x DNase I buffer and DNase I (0.5 U per 1 µg of RNA) at 37 °C for 45 min, followed by extractions with P/C/I (three times) and diethyl ether (twice), removal of ether traces in an Eppendorf concentrator, ethanol precipitation to recover total RNA, and analysis by 1% agarose gel electrophoresis.

List of Non-Commercial Buffers. ADPRC buffer with EDTA (5x): Na-HEPES (250 mM, pH 7.0), EDTA (25 mM). ADPRC buffer with $MgCl_2$ (5x): Na-HEPES (250 mM, pH 7.0), $MgCl_2$ (25 mM). ADPRC storage buffer (1x): 0.5x ADPRC buffer with $MgCl_2$, glycerol (50% (v/v)) Agarose gel electrophoresis loading dye (6x): Glycerol (30% (v/v)), bromophenol blue (0.1% (w/v)), xylene cyanol (0.1% (w/v)). APB-PAGE loading buffer (2x): 2x TAE buffer, urea (8 M), optionally with bromophenol blue and xylene cyanol dye. Buffer A (for HPLC): 0.1 M triethylamine/acetic acid in water, pH 7.0. Buffer B (for HPLC): 0.1 M triethylamine/acetic acid in acetonitrile/water 4:1, pH 7.0. High-Salt Wash buffer (1x)(50): Tris-HCl (20 mM, pH 7.4), NaCl (1 M). Immobilization Buffer (2x): Na-HEPES (20 mM, pH 7.2), NaCl (2 M), EDTA (10 mM). IVT buffer (10x): Tris-HCl (400 mM, pH 8.1), spermidine (10 mM), $MgCl_2$ (220 mM), Triton-X-100 (0.1%). Low-Salt Wash buffer (1x)(50): Tris-HCl (20 mM, pH 7.4), NaCl (0.1 M). NaOAc stock (for nucleic acid precipitation or gel elution): NaOAc (3 M, pH 5.5). Native PAGE loading buffer (2x): 2x TBE buffer, glycerol (50% (v/v)), optionally with bromophenol blue and xylene cyanol dye. Northern Blot Wash Solution 1: SSC buffer (2x), 0.1% SDS. Northern Blot Wash Solution 2: SSC buffer (0.25x), 0.1% SDS. PAGE loading buffer (2x): 2x TBE buffer,

formamide (80% (v/v)), optionally with bromophenol blue and xylene cyanol dye. Streptavidin Wash buffer (1x) – high urea: Tris-HCl (50 mM, pH 7.4), urea (8 M). Streptavidin Wash Buffer (1x) – low urea: Tris-HCl (50 mM, pH 7.4), urea (1 M). TAE buffer (50x): Tris-acetate (2 M, pH 8.0), EDTA (50 mM). TBE buffer (10x): Tris-borate (1 M, pH 8.3), EDTA (20 mM). TBS buffer (10x): Tris-HCl (100 mM, pH 7.4), NaCl (1.5 M). TBS-T buffer (1x): 1x TBS buffer, Tween 20 (0.5% (v/v)).

Chemical synthesis:

Biotin-NHS ester. Anhydrous DMF (25.0 mL) was heated to 65 °C in a Schlenk flask under argon atmosphere. Biotin (750.0 mg, 3.07 mmol) was dissolved in the hot DMF and, after full dissolution, *N*-hydroxysuccinimide (NHS) (361.2 mg, 3.14 mmol) was added. The suspension was again stirred until complete dissolution of NHS, cooled back to room temperature and, after the addition of dicyclohexylcarbodiimide (DCC) (827.1 mg, 4.01 mmol), stirred for another 3 h. The formed urea precipitate was removed by vacuum filtration and the filtrate was concentrated under reduced pressure. The residual solid was washed several times with diethyl ether, before drying it again under reduced pressure. The crude biotin-NHS ester (965.3 mg, 92.1%) was obtained as a colorless solid and used without further purification in follow-up reactions. ^{1}H NMR (300.0 MHz, DMSO-d6): δ [ppm] = 6.42 (s, 1H), 6.36 (s, 1H), 4.35-4.26 (m, 1H), 4.18-4.11 (m, 1H), 3.15-3.06 (m, 1H), 2.90-2.82 (m, 1H), 2.81 (s, 4H), 2.67 (t, J = 7.4 Hz, 2H), 2.58 (d, J = 12.4 Hz, 1H), 1.77-1.38 (m, 6H).

3-Picolylamine-biotin conjugate (3PAB). Biotin-NHS ester (400.0 mg, 1.17 mmol) was dissolved in DMF (30.0 mL). Triethylamine (408.3 µL, 2.93 mmol) was added and the solution incubated at room temperature for 5 min before addition of 3-picolylamine (120.0 µL, 1.18 mmol). The reaction mixture was stirred for over 3 h and product formation was monitored by NP-TLC (R_f = 0.15, SiO_2, DCM/MeOH 9:1). The solvent was evaporated under reduced pressure, the residual solid re-dissolved in MeOH and mixed with Celite® 545. The methanol was removed and the crude product, adsorbed on Celite® 545, was purified by silica column chromatography (DCM/MeOH 9:1). The solvent was evaporated under reduced pressure, which yielded the product as an off-white solid (378.5 mg, 96.6%). ^{1}H NMR (499.9 MHz, DMSO-d6): δ [ppm] = 8.46 (dd, J = 2.4, 0.9 Hz, 1H), 8.44 (dd, J = 4.8, 1.7 Hz, 1H), 8.37 (t, J = 6.0 Hz, 1H), 7.63 (ddd, J = 7.8, 2.4, 1.7 Hz, 1H), 7.34 (ddd, J = 7.8, 4.8, 0.9 Hz, 1H), 6.42 (s, 1H), 6.36 (s, 1H), 4.30 (ddt, J = 7.8, 5.2, 1.1 Hz, 1H), 4.27 (d, J = 5.9 Hz, 2H), 4.12 (ddd, J = 7.7, 4.4, 2.0 Hz, 1H), 3.09 (ddd, J = 8.6, 6.1, 4.4 Hz, 1H), 2.82 (dd, J = 12.4, 5.2 Hz, 1H), 2.58 (d, J = 12.4 Hz, 1H), 2.14 (t, J = 7.5 Hz, 2H), 1.66-1.25 (m, 6H). 13C NMR (125.7 MHz, DMSO-d6): δ [ppm] = 172.2 (1C), 162.7 (1C), 148.7 (1C), 148.0 (1C), 135.2 (1C), 134.9 (1C), 123.4 (1C), 61.0 (1C), 59.2 (1C), 55.4 (1C), 39.8 (1C), 39.7 (1C), 35.1 (1C), 28.2 (1C), 28.0 (1C), 25.2 (1C). HR-MS (ESI, positive): m/z calculated for $C_{16}H_{22}N_4O_2S$, 335.1536 $[M+H]^+$, 357.1356 $[M+Na]^+$; found, 335.1520, 357.1344.

4-Picolylamine-biotin conjugate (4PAB). Biotin-NHS ester (400.0 mg, 1.17 mmol) was dissolved in DMF (30.0 mL). Triethylamine (408.3 µL, 2.93 mmol) was added and the solution incubated at room temperature for 5 min before addition of 4-picolylamine (120.0 µL, 1.18 mmol). The reaction mixture was stirred for over 3 h and product formation was monitored by NP-TLC (R_f = 0.15, SiO_2, DCM/MeOH 9:1). The solvent was evaporated under reduced pressure, the residual solid re-dissolved in MeOH and mixed with Celite® 545. The methanol was removed and the crude product, adsorbed on Celite® 545, was purified by silica column chromatography (DCM/MeOH 9:1). The solvent was evaporated under reduced pressure, which yielded the product as an off-white solid (342.6 mg, 87.4%). ^{1}H NMR (499.9 MHz, DMSO-d6): δ [ppm] = 8.48 (dd, J = 4.4, 1.8 Hz, 2H), 8.42 (t, J = 6.1 Hz, 1H), 7.22 (dd, J = 4.4, 1.9 Hz, 2H), 6.43 (s, 1H), 6.36 (s, 1H), 4.33-4.29 (m, 1H), 4.28 (d, J = 6.1 Hz, 2H), 4.15-4.11 (m, 1H), 3.14-3.05 (m, 1H), 2.83 (dd, J = 12.5, 5.1 Hz, 1H), 2.58 (d, J = 12.5 Hz, 1H), 2.18 (t,

J = 7.4 Hz, 2H), 1.68-1.27 (m, 6H). ^{13}C NMR (125.7 MHz, DMSO-d6): δ [ppm] = 172.4 (1C), 162.7 (1C), 149.5 (2C), 148.7 (1C), 122.0 (2C), 61.0 (1C), 59.2 (1C), 55.4 (1C), 41.0 (1C), 39.8 (1C), 35.1 (1C), 28.2 (1C), 28.0 (1C), 25.2 (1C). HR-MS (ESI, positive): m/z calculated for $C_{16}H_{22}N_4O_2S$, 335.1536 $[M+H]^+$, 357.1356 $[M+Na]^+$; found, 335.1541, 357.1359.

Desthiobiotin-NHS ester. Anhydrous DMF (20.0 mL) was heated to 65 °C in a Schlenk flask under argon atmosphere. Desthiobiotin (500.0 mg, 2.33 mmol) was dissolved in the hot DMF and, after full dissolution, *N*-hydroxysuccinimide (NHS) (215.7 mg, 1.87 mmol) was added. The suspension was again stirred until complete dissolution of NHS, cooled back to room temperature and, after the addition of dicyclohexylcarbodiimide (DCC) (482.4 mg, 2.34 mmol), stirred for another 3 h. The formed urea precipitate was removed by vacuum filtration and the filtrate was concentrated under reduced pressure. The residual solid was washed several times with diethyl ether, before drying it again under reduced pressure. The crude desthiobiotin-NHS ester (567.1 mg (only 80% activated according to the ^{1}H NMR spectrum), 65.8%) was obtained as a colorless solid and used without further purification in follow-up reactions. ^{1}H NMR (300.0 MHz, DMSO-d6): δ [ppm] = 6.30 (s, 1H), 6.11 (s, 1H), 3.67-3.55 (m, 1H), 3.53-3.43 (m, 1H), 2.81 (s, 4H), 2.66 (t, J = 7.2 Hz, 2H), 1.62 (quin, J = 7.2 Hz, 2H), 1.42-1.15 (m, 6H), 0.96 (d, J = 6.4 Hz, 3H).

3-Picolylamine-desthiobiotin conjugate (3PADB). Desthiobiotin-NHS ester (255.0 mg, 0.82 mmol) was dissolved in DMF (22.0 mL). Triethylamine (285.0 µL, 2.04 mmol) was added and the solution incubated at room temperature for 5 min before addition of 3-picolylamine (85.0 µL, 0.83 mmol). The reaction mixture was stirred for over 3 h and product formation was monitored by NP-TLC (R_f = 0.19, SiO_2, DCM/MeOH 9:1). The solvent was evaporated under reduced pressure, the residual solid re-dissolved in MeOH and mixed with Celite® 545. The methanol was removed and the crude product, adsorbed on Celite® 545, was purified by silica column chromatography (DCM/MeOH 9:1). The solvent was evaporated under reduced pressure, which yielded the product as a clear, slightly yellow, highly viscous oil (170.4 mg, 85.4%). ^{1}H NMR (499.9 MHz, DMSO-d6): δ [ppm] = 8.46 (dd, J = 2.4, 0.8 Hz, 1H), 8.44 (dd, J = 4.8, 1.7 Hz, 1H), 8.35 (t, J = 6.1 Hz, 1H), 7.63 (ddd, J = 7.8, 2.3, 1.7 Hz, 1H), 7.34 (ddd, J = 7.8, 4.8, 0.8 Hz, 1H), 6.29 (s, 1H), 4.27 (d, J = 6.0 Hz, 2H), 4.27 (d, J = 6.0 Hz, 2H), 3.60 (dq, J = 7.2, 6.5 Hz, 1H), 3.49-3.43 (m, 1H), 2.13 (t, J = 7.4 Hz, 2H), 1.51 (quin, J = 7.4 Hz, 2H), 1.38-1.13 (m, 6H), 0.95 (d, J = 6.5 Hz, 3H). ^{13}C NMR (125.7 MHz, DMSO-d6): δ [ppm] = 172.3 (1C), 162.8 (1C), 148.7 (1C), 148.0 (1C), 135.2 (1C), 135.0 (1C), 123.4 (1C), 54.9 (1C), 50.2 (1C), 39.7 (1C), 35.2 (1C), 29.5 (1C), 28.7 (1C), 25.5 (1C), 25.1 (1C), 15.5 (1C). HR-MS (ESI, positive): m/z calculated for $C_{16}H_{24}N_4O_2$, 305.1972 $[M+H]^+$, 327.1791 $[M+Na]^+$; found, 305.1970, 327.1794.

4-Picolylamine-desthiobiotin conjugate (4PADB). Desthiobiotin-NHS ester (270.0 mg, 0.87 mmol) was dissolved in DMF (22.0 mL). Triethylamine (300.0 µL, 2.15 mmol) was added and the solution incubated at room temperature for 5 min before addition of 4-picolylamine (90.0 µL, 0.89 mmol). The reaction mixture was stirred for over 3 h and product formation was monitored by NP-TLC (R_f = 0.19, SiO_2, DCM/MeOH 9:1). The solvent was evaporated under reduced pressure, the residual solid re-dissolved in MeOH and mixed with Celite® 545. The methanol was removed and the crude product, adsorbed on Celite® 545, was purified by silica column chromatography (DCM/MeOH 9:1). The solvent was evaporated under reduced pressure, which yielded the product as a clear, slightly yellow, highly viscous oil (189.1 mg, 89.5%). ^{1}H NMR (499.9 MHz, DMSO-d6): δ [ppm] = 8.48 (dd, J = 4.4, 1.8 Hz, 2H), 8.40 (t, J = 6.2 Hz, 1H), 7.22 (dd, J = 4.4, 1.8 Hz, 2H), 6.30 (s, 1H), 6.12 (s, 1H), 4.27 (d, J = 6.2 Hz, 2H), 3.60 (dq, J = 7.1, 6.5 Hz, 1H), 3.50-3.45 (m, 1H), 2.16 (t, J = 7.4 Hz, 2H), 1.53 (quin, J = 7.4 Hz, 2H), 1.40-1.15 (m, 6H), 0.96 (d, J = 6.5 Hz, 3H). ^{13}C NMR (125.7 MHz, DMSO-d6): δ [ppm] = 172.4 (1C), 162.8 (1C), 149.4 (2C), 148.7 (1C), 122.0 (2C), 54.9 (1C), 50.2 (1C), 41.0 (1C),

35.2 (1C), 29.5 (1C), 28.7 (1C), 25.5 (1C), 25.1 (1C), 15.5 (1C). HR-MS (ESI, positive): m/z calculated for $C_{16}H_{24}N_4O_2$, 305.1972 $[M+H]^+$, 327.1791 $[M+Na]^+$; found, 305.1965, 327.1788.

Nicotinic acid-NHS ester. Anhydrous DMF (30.0 mL) in a Schlenk flask was put under argon atmosphere. Nicotinic acid (498.5 mg, 4.05 mmol) was dissolved in the DMF and, after full dissolution, *N*-hydroxysuccinimide (NHS) (475.2 mg, 4.13 mmol) was added. The suspension was again stirred until complete dissolution of NHS and, after the addition of dicyclohexylcarbodiimide (DCC) (1085.2 mg, 5.26 mmol), stirred for another 5 h. The formed urea precipitate was removed by vacuum filtration and the filtrate was concentrated under reduced pressure. The residual solid was washed several times with diethyl ether, before drying it again under reduced pressure. The crude nicotinic acid-NHS ester (809.4 mg, 90.8%) was obtained as a colorless solid and used without further purification in follow-up reactions. ^{1}H NMR (300.0 MHz, $CDCl_3$): δ [ppm] = 9.32 (dd, J = 2.3, 0.9 Hz, 1H), 8.88 (dd, J = 4.9, 1.7 Hz, 1H), 8.38 (dt, J = 8.0, 2.0 Hz, 1H), 7.47 (ddd, J = 8.0, 4.9, 1.0 Hz, 1H), 2.92 (s, 4H). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ [ppm] = 169.0 (2C), 160.9 (1C), 155.3 (1C), 151.6 (1C), 137.9 (1C), 123.8 (1C), 121.8 (1C), 25.8 (2C).

***tert*-Butyl (1-oxo-1-(pyridin-3-yl)-6,9,12-trioxa-2-azapentadecan-15-yl)carbamate (nicotinamide-(PEG)$_3$-NH-Boc)**. Nicotinic acid-NHS ester (369.5 mg, 1.68 mmol) was dissolved in anhydrous DMF (25.0 mL) in a Schlenk flask under argon atmosphere. Triethylamine (560.0 µL, 4.02 mmol) was added and the solution incubated at room temperature for 2 min before addition of *N*-Boc-4,7,10-trioxa-1,13-tridecanediamine (517.5 mg, 1.62 mmol). The reaction mixture was stirred for 3 h. The solvent was evaporated under reduced pressure, the residual solid re-dissolved in MeOH and mixed with Celite® 545. The methanol was removed and the crude product, adsorbed on Celite® 545, was purified by silica column chromatography (DCM/MeOH 19:1). The solvent was evaporated under reduced pressure, which yielded the product as a dark yellowish, highly viscous oil (629.9 mg, 91.7%). ^{1}H NMR (300.0 MHz, CD_3OD): δ [ppm] = 8.99 (dd, J = 2.3, 1.0 Hz, 1H), 8.69 (dd, J = 4.9, 1.6 Hz, 1H), 8.28 (ddd, J = 8.0, 2.3, 1.7 Hz, 1H), 7.56 (ddd, J = 8.1, 4.9, 1.0 Hz, 1H), 3.70-3.46 (m, 14H), 3.53 (t, J = 5.6 Hz, 2H), 3.17-3.08 (m, 2H), 1.92 (quin, J = 6.8 Hz, 2H), 1.72 (quin, J = 6.5 Hz, 2H), 1.44 (s, 9H). ^{13}C NMR (75.4 MHz, CD_3OD): δ [ppm] = 174.9 (1C), 167.6 (1C), 152.6 (1C), 149.1 (1C), 137.0 (1C), 132.1 (1C), 125.1 (1C), 71.5 (1C), 71.5 (1C), 71.3 (1C), 71.2 (1C), 70.1 (1C), 69.9 (1C), 38.8 (2C), 30.9 (1C), 30.3 (1C), 28.8 (3C), 26.3 (1C).

***N*-(3-(2-(2-(3-Aminopropoxy)ethoxy)ethoxy)propyl)nicotinamide (nicotinamide-(PEG)$_3$-NH$_2$)**. Trifluoroacetic acid (TFA) (2.5 mL) was added to nicotinamide-(PEG)$_3$-NH-Boc (625.0 mg, 1.47 mmol) and the mixture reacted at 0 °C for 30 min. TFA was removed under reduced pressure using a rotary evaporator placed in a fume hood. Small volumes of MeOH were added to the residue and removed under reduced pressure, repeatedly. Successful deprotection was confirmed by NMR spectroscopy, which showed a complete disappearance of the CH_3-group signal from the Boc-protecting group (in CD_3OD: δ [ppm] = 1.44). The crude product was obtained as a yellowish oil and used without further purification in the follow-up reaction to obtain N(PEG)$_3$B. ^{1}H NMR (300.0 MHz, CD_3OD): δ [ppm] = 9.13 (dd, J = 2.2, 0.9 Hz, 1H), 8.85 (dd, J = 5.4, 1.5 Hz, 1H), 8.63 (ddd, J = 8.1, 2.2, 1.5 Hz, 1H), 7.89 (ddd, J = 8.1, 5.3, 0.8 Hz, 1H), 3.70-3.56 (m, 12H), 3.56-3.49 (m, 2H), 3.11 (m, 2H), 1.98-1.87 (m, 4H).

Nicotinamide-(PEG)$_3$-biotin conjugate (N(PEG)$_3$B). Biotin-NHS ester (511.2 mg, 1.50 mmol) and triethylamine (2.05 mL, 14.71 mmol) were dissolved in anhydrous DMF (12.5 mL) and incubated for 5 min at room temperature, before adding to a solution of crude, Boc-deprotected nicotinamide-(PEG)$_3$-NH$_2$ (1.47 mmol) in anhydrous DMF (12.5 mL). The reaction mixture was stirred for 3 h. The solvent was evaporated under reduced pressure, the residual solid re-dissolved in MeOH and mixed with Celite® 545. The methanol was removed and the crude product, adsorbed on Celite® 545, was purified by silica column chromatography (DCM/MeOH

19:1). The solvent was evaporated under reduced pressure, which yielded the product as a slightly yellow, highly viscous oil (552.0 mg, 68.1%). ^{1}H NMR (300.0 MHz, DMSO-d6): δ [ppm] = 8.98 (dd, J = 2.3, 0.8 Hz, 1H), 8.69 (dd, J = 4.8, 1.7 Hz, 1H), 8.64 (t, J = 5.7 Hz, 1H), 8.17 (dt, J = 7.9, 2.0 Hz, 1H), 7.74 (t, J = 5.7 Hz, 1H), 7.49 (ddd, J = 8.0, 4.8, 1.0 Hz, 1H), 6.41 (s, 1H), 6.35 (s, 1H), 4.30 (dd, J = 7.8, 5.1 Hz, 1H), 4.16-4.05 (m, 1H), 3.58-3.42 (m, 10H), 3.41-3.30 (m, 4H), 3.14-3.02 (m, 3H), 2.81 (dd, J = 12.4, 5.1 Hz, 1H), 2.57 (d, J = 12.4 Hz, 1H), 2.04 (t, J = 7.4 Hz, 2H), 1.76 (quin, J = 6.4 Hz, 2H), 1.60 (quin, J = 6.6 Hz, 2H), 1.55-1.21 (m, 6H). ^{13}C NMR (75.4 MHz, DMSO-d6): δ [ppm] = 171.9 (1C), 164.7 (1C), 162.7 (1C) 151.7 (1C), 148.3 (1C), 134.9 (1C), 130.0 (1C), 123.4 (1C), 61.0 (1C), 59.2 (1C), 55.4 (1C), 69.8 (1C), 69.8 (1C), 69.6 (1C), 69.5 (1C), 68.2 (1C), 68.1 (1C), 39.8 (1C), 36.7 (1C), 35.7 (1C), 35.2 (1C), 29.4 (1C), 29.3 (1C), 28.2 (1C), 28.1 (1C), 25.3 (1C). HR-MS (ESI, positive): m/z calculated for $C_{26}H_{41}N_5O_6S$, 552.2850 $[M+H]^+$, 574.2670 $[M+Na]^+$; found, 552.2857, 574.2481.

***tert*-Butyl (1-oxo-1-(pyridin-3-yl)-5,8,11,14,17,20,23,26,29,32,35-undecaoxa-2-azaheptatriacontan-37-yl)carbamate (nicotinamide-(PEG)$_{11}$-NH-Boc)**. Nicotinic acid-NHS ester (180.5 mg, 0.82 mmol) was dissolved in anhydrous DMF (20.0 mL) in a Schlenk flask under argon atmosphere. Triethylamine (270.0 µL, 1.94 mmol) was added and the solution incubated at room temperature for 2 min before addition of *O*-(2-aminoethyl)-*O*'-2-(Boc-amino)ethyldecaethylene glycol (500.0 mg, 0.78 mmol). The reaction mixture was stirred for 6 h. The solvent was evaporated under reduced pressure, the residual solid re-dissolved in MeOH and mixed with Celite® 545. The methanol was removed and the crude product, adsorbed on Celite® 545, was purified by silica column chromatography (DCM/MeOH 19:1). The solvent was evaporated under reduced pressure, which yielded the product as a pale yellow oil (461.2 mg, 79.3%). ^{1}H NMR (300.0 MHz, CD_3OD): δ [ppm] = 9.01 (dd, J = 2.3, 1.0 Hz, 1H), 8.71 (dd, J = 4.9, 1.7 Hz, 1H), 8.28 (ddd, J = 8.0, 2.3, 1.6 Hz, 1H), 7.57 (ddd, J = 8.0, 4.9, 1.0 Hz, 1H), 3.72-3.60 (m, 44H), 3.53 (t, J = 5.6 Hz, 2H), 3.23 (td, J = 5.6, 1.6 Hz, 2H), 1.46 (s, 9H). ^{13}C NMR (75.4 MHz, CD_3OD): δ [ppm] = 173.4 (1C), 166.4 (1C), 151.2 (1C), 147.8 (1C), 135.7 (1C), 130.6 (1C), 123.7 (1C), 78.6 (1C), 70.5-69.5 (PEG), 39.9 (PEG), 39.7 (PEG), 27.4 (3C) 24.9 (1C).

***N*-(35-amino-3,6,9,12,15,18,21,24,27,30,33-undecaoxapentatriacontyl)nicotinamide (nicotinamide-(PEG)$_{11}$-NH$_2$)**. Trifluoroacetic acid (TFA) (2.5 mL) was added to nicotinamide-(PEG)$_{11}$-NH-Boc (450.2 mg, 0.60 mmol) and the mixture reacted at 0 °C for 30 min. TFA was removed under reduced pressure using a rotary evaporator placed in a fume hood. Small volumes of MeOH were added to the residue and removed under reduced pressure, repeatedly. Successful deprotection was confirmed by NMR spectroscopy, which showed a complete disappearance of the CH_3-group signal from the Boc-protecting group (in CD_3OD: δ [ppm] = 1.44). The crude product was obtained as a colorless solid and used without further purification in the follow-up reaction to obtain N(PEG)$_{11}$B. ^{1}H NMR (300.0 MHz, CD_3OD): δ [ppm] = 9.14 (dd, J = 2.2, 0.9 Hz, 1H), 8.86 (dd, J = 5.5, 1.5 Hz, 1H), 8.69 (dt, J = 8.1, 1.8 Hz, 1H), 7.93 (ddd, J = 8.1, 5.5, 0.9 Hz, 1H), 3.76-3.70 (m, 2H), 3.70-3.55 (m, 44H), 3.17-3.10 (m, 2H).

Nicotinamide-(PEG)$_{11}$-biotin conjugate (N(PEG)$_{11}$B). Biotin-NHS ester (205.2 mg, 0.60 mmol) and triethylamine (0.84 mL, 6.03 mmol) were dissolved in anhydrous DMF (10.0 mL) and incubated for 5 min at room temperature, before adding to a solution of crude, Boc-deprotected nicotinamide-(PEG)$_{11}$-NH2 (0.60 mmol) in anhydrous DMF (10.0 mL). The reaction mixture was stirred for 16 h. The solvent was evaporated under reduced pressure, the residual solid re-dissolved in MeOH and mixed with Celite® 545. The methanol was removed and the crude product, adsorbed on Celite® 545, was purified by silica column chromatography (DCM/MeOH 9:1) and then again by a second silica column chromatography (DCM/MeOH 19:1). The solvent was evaporated under reduced pressure, which yielded the product as a faint yellow, highly viscous oil (207.3 mg, 39.4%). ^{1}H NMR (300.0 MHz, DMSO-d6): δ [ppm] =

9.00 (dd, J = 2.3, 0.8 Hz, 1H), 8.77 (t, J = 5.6 Hz, 1H), 8.69 (dd, J = 4.8, 1.7 Hz, 1H), 8.19 (dt, J = 7.9, 2.0 Hz, 1H), 7.84 (t, J = 5.7 Hz, 1H), 7.50 (ddd, J = 8.0, 4.8, 0.9 Hz, 1H), 6.41 (s, 1H), 6.35 (s, 1H), 4.30 (dd, J = 7.8, 5.1 Hz, 1H), 4.15-4.10 (m, 1H), 3.58-3.43 (m, 44H), 3.41-3.36 (m, 2H), 3.20-3.15 (m, 2H), 3.12-3.06 (m, 1H), 2.81 (dd, J = 12.4, 5.1 Hz, 1H), 2.57 (d, J = 12.3 Hz, 1H), 2.06 (t, J = 7.3 Hz, 2H), 1.68-1.25 (m, 6 H). ^{13}C NMR (75.4 MHz, DMSO-d6): δ [ppm] = 172.1 (1C), 164.8 (1C), 162.7 (1C) 151.8 (1C), 148.4 (1C), 134.9 (1C), 129.8 (1C), 123.4 (1C), 61.0 (1C), 59.2 (1C), 55.4 (1C), 69.8-69.5 (multiple C), 69.2 (1C), 68.8 (1C), 45.4 (1C), 38.4 (1C), 35.1 (1C), 28.2 (1C), 28.0 (1C), 25.3 (1C). HR-MS (ESI, positive): m/z calculated for $C_{40}H_{69}N_5O_{14}S$, 552.2850 $[M+H]^+$, 574.2670 $[M+Na]^+$; found, 552.2857, 574.2481.

((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl (1*H*-imidazol-1-yl)phosphonate (ImpA). The synthesis of ImpA was adapted from a literature protocol(58). Adenosine 5'-monophosphate monohydrate (5'-AMP) (421.4 mg, 1.15 mmol) was suspended in anhydrous DMF (3 mL) and added dropwise to a stirred solution of imidazole (519.9 mg, 7.64 mmol), 2,2'-dithiopyridine (613.0 mg, 2.78 mmol), triphenylphosphine (721.1 mg, 2.75 mmol) and triethylamine (460 µL, 3.30 mmol) in anhydrous DMF (9 mL). After approximately 10 min of reaction time, the suspension turned clear. The resulting solution was further stirred at room temperature for 3 h and poured into a cooled solution (0 °C) of sodium perchlorate (143.8 mg, 1.17 mmol) in anhydrous acetone (100 mL). The product was precipitated at 0 °C for 1 h and collected by centrifugation (4000 g, 5 min, 15 °C). The pellet was washed with acetone (3 x 10 mL) and diethyl ether (10 mL). Residual solvent was evaporated and the solid product further lyophilized, which yielded ImpA as sodium salt as a colorless solid (466.2 mg, 96.4%). The substance was stored in an argon-flushed Schlenk container at −20 °C for extended periods of time. ^{1}H NMR (499.9 MHz, DMSO-d6): δ [ppm] = 8.39 (s, 1H), 8.13 (s, 1H), 7.69 (dd, J = 1.2 Hz, 1H), 7.31 (s, 2H), 7.11 (dd, J = 1.2 Hz, 1H), 6.87 (dd, J = 1.2 Hz), 5.88 (d, J = 6.3 Hz, 1H), 4.58 (dd, J = 5.4 Hz, 1H), 4.04 (dd, J = 4.2, 3.3 Hz, 1H), 3.94 (dt, J = 7.2, 3.6 Hz, 1H), 3.81–3.69 (m, 2H). ^{13}C NMR (75.5 MHz, DMSO-d6): δ [ppm] = 155.9 (1C), 152.7 (1C), 149.7 (1C), 135.2 (1C), 128.2 (d, 1C), 121.6 (1C), 118.9 (1C), 86.8 (1C), 83.7 (d, 1C), 73.6 (1C), 70.9 (1C), 64.9 (1C). ^{31}P NMR (202.3 MHz, DMSO-d6): δ [ppm] = −9.78 (s, 1P). HR-MS (ESI, positive): m/z calculated for $C_{13}H_{16}N_7NaO_6P$, 420.0792 $[M+H]^+$, 442.0611 $[M+Na]^+$; found, 420.0787, 442.0603. MS (ESI, negative): m/z calculated for $C_{13}H_{16}N_7NaO_6P$, 396.0827 $[M-Na]^-$; found, 396.1.