Fig. S1

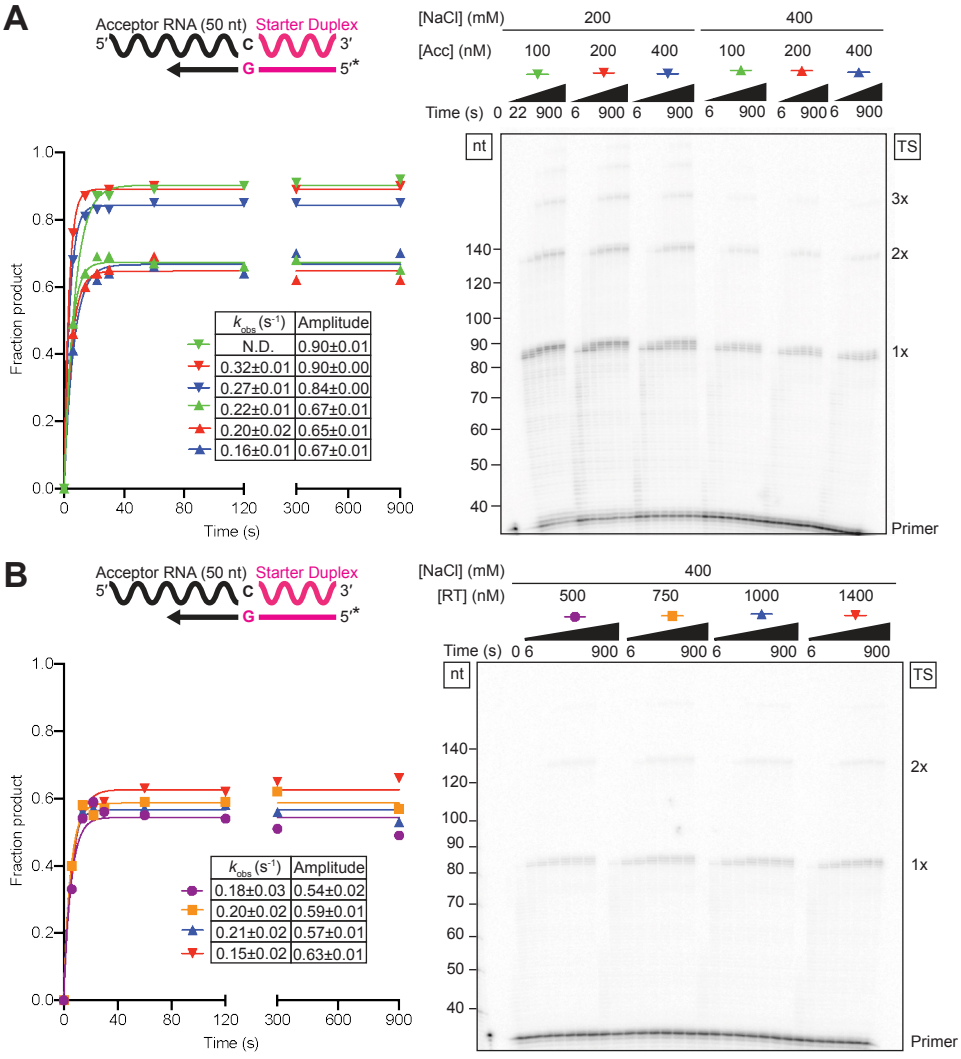

A
Acceptor RNA (50 nt)
Starter Duplex
5′
3′
C
G
5′*
[NaCl] (mM)
200
400
[Acc] (nM)
100
200
400
Time (s)
0 22 900 6 900 6 900 6 900 6 900 6 900
nt
TS
3x
2x
1x
Primer
Fraction product
Time (s)
k_{obs} (s^{-1}) | Amplitude
N.D. | 0.90±0.01
0.32±0.01 | 0.90±0.00
0.27±0.01 | 0.84±0.00
0.22±0.01 | 0.67±0.01
0.20±0.02 | 0.65±0.01
0.16±0.01 | 0.67±0.01
B
Acceptor RNA (50 nt)
Starter Duplex
[NaCl] (mM)
400
[RT] (nM)
500
750
1000
1400
Time (s)
0 6 900 6 900 6 900 6 900
nt
TS
2x
1x
Primer
Fraction product
Time (s)
k_{obs} (s^{-1}) | Amplitude
0.18±0.03 | 0.54±0.02
0.20±0.02 | 0.59±0.01
0.21±0.02 | 0.57±0.01
0.15±0.02 | 0.63±0.01

Fig. S2

[NaCl] (mM)	rate (nt/s)	Amplitude
100	~9	~0.79
200	~7	~0.79
300	~5	~0.79
400	~4	~0.81

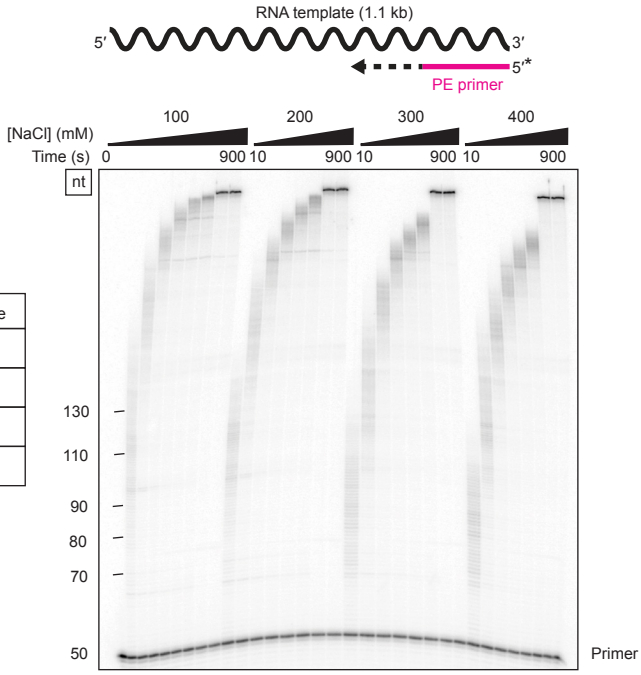


Fig. S3

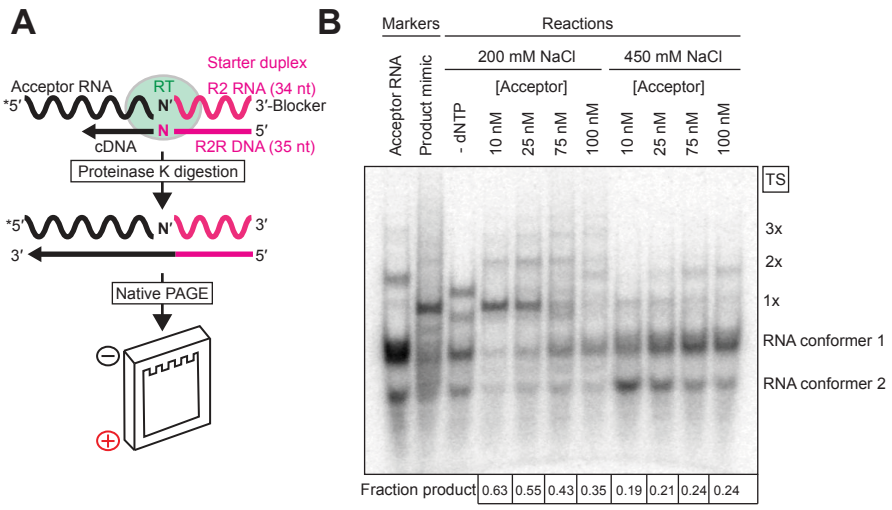
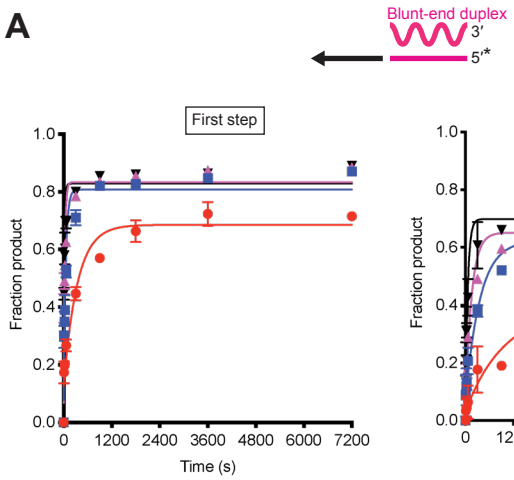
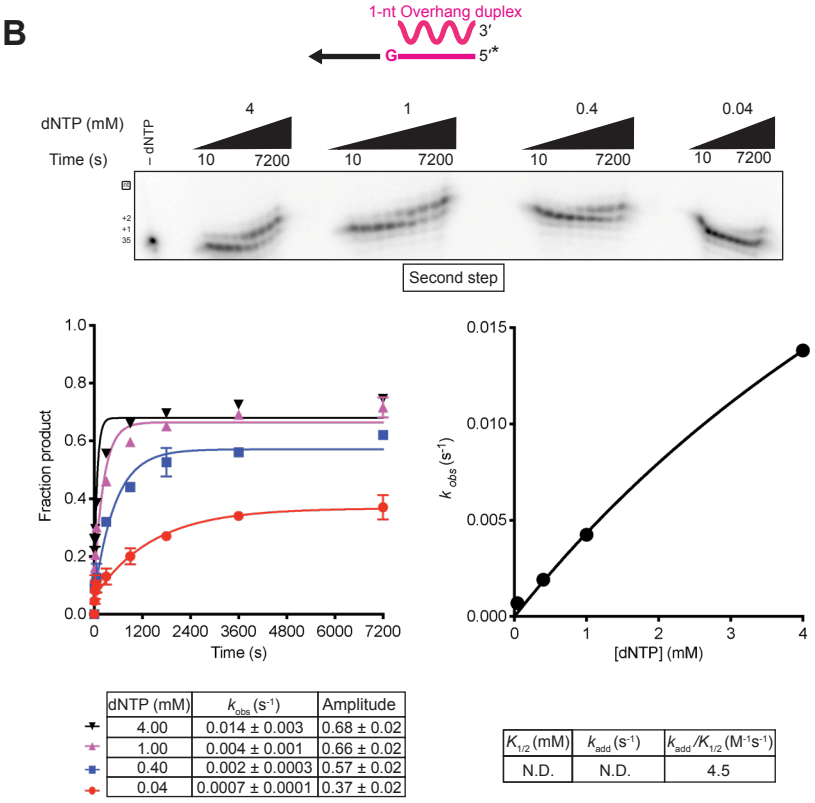

A
Acceptor RNA
*5′
RT
N′
Starter duplex
R2 RNA (34 nt)
3′-Blocker
N
5′
cDNA
R2R DNA (35 nt)
Proteinase K digestion
*5′
N′
3′
3′
5′
Native PAGE
B
Markers
Reactions
Acceptor RNA
Product mimic
200 mM NaCl
450 mM NaCl
[Acceptor]
[Acceptor]
- dNTP
10 nM
25 nM
75 nM
100 nM
10 nM
25 nM
75 nM
100 nM
TS
3x
2x
1x
RNA conformer 1
RNA conformer 2
Fraction product
0.63
0.55
0.43
0.35
0.19
0.21
0.24
0.24

Fig. S4



dNTP (mM)	k_{obs} (s^{-1})	Amplitude
4.00	0.035 ± 0.005	0.83 ± 0.02
1.00	0.052 ± 0.006	0.83 ± 0.02
0.40	0.019 ± 0.003	0.81 ± 0.02
0.04	0.003 ± 0.0006	0.69 ± 0.02

dNTP (mM)	k_{obs} (s^{-1})	Amplitude
4.00	0.017 ± 0.003	0.70 ± 0.02
1.00	0.006 ± 0.001	0.65 ± 0.02
0.40	0.003 ± 0.0005	0.62 ± 0.02
0.04	0.001 ± 0.0003	0.39 ± 0.03



dNTP (mM)	k_{obs} (s^{-1})	Amplitude
4.00	0.014 ± 0.003	0.68 ± 0.02
1.00	0.004 ± 0.001	0.66 ± 0.02
0.40	0.002 ± 0.0003	0.57 ± 0.02
0.04	0.0007 ± 0.0001	0.37 ± 0.02

$K_{1/2}$ (mM)	k_{add} (s^{-1})	$k_{add}/K_{1/2}$ ($M^{-1}s^{-1}$)
N.D.	N.D.	4.5

Fig. S5

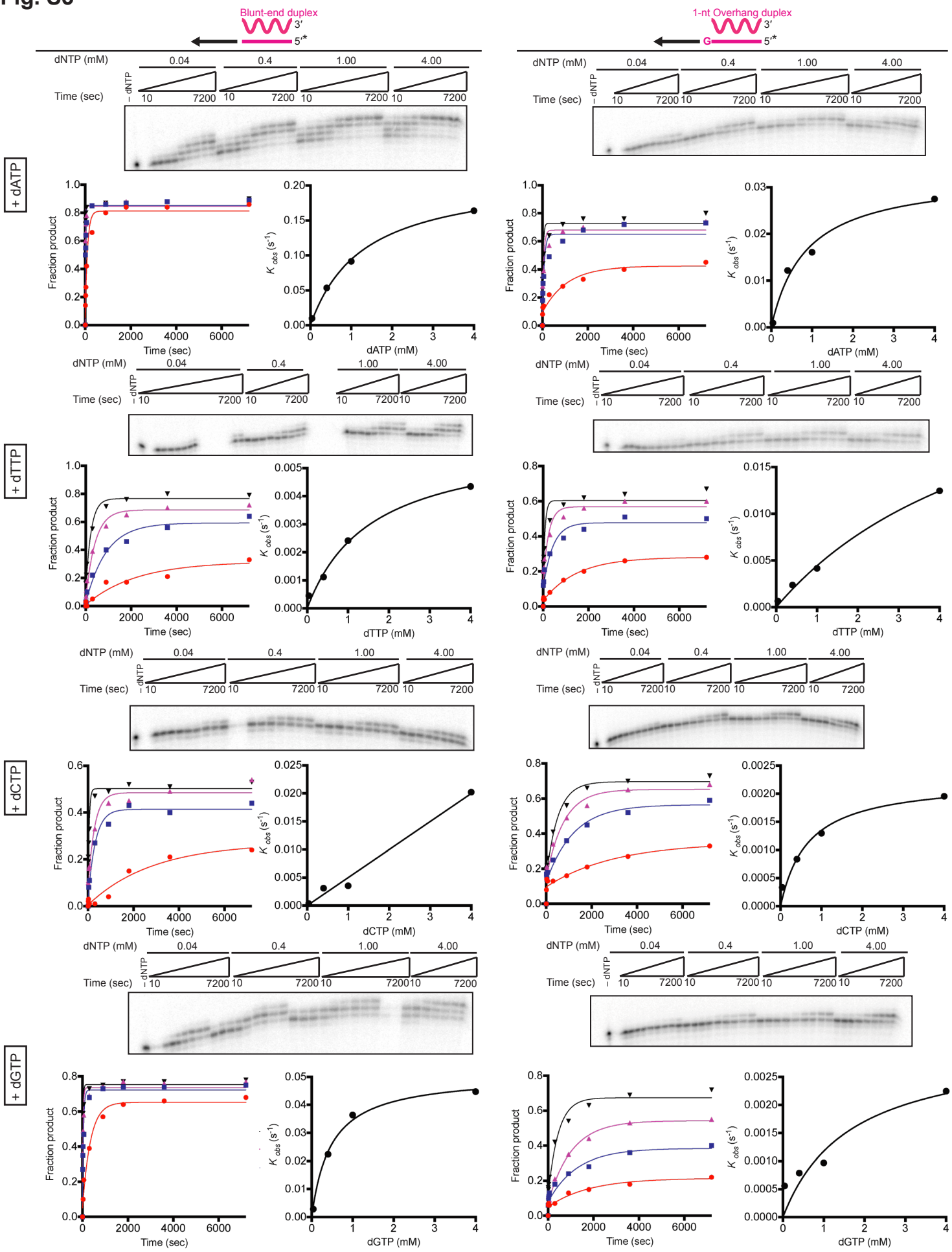

Blunt-end duplex
3′
5′*
1-nt Overhang duplex
3′
G
5′*
dNTP (mM)
0.04
0.4
1.00
4.00
Time (sec)
– dNTP
10
7200
+ dATP
+ dTTP
+ dCTP
+ dGTP
Fraction product
Time (sec)
K_{obs} (s^{-1})
dATP (mM)
dTTP (mM)
dCTP (mM)
dGTP (mM)

Fig. S6

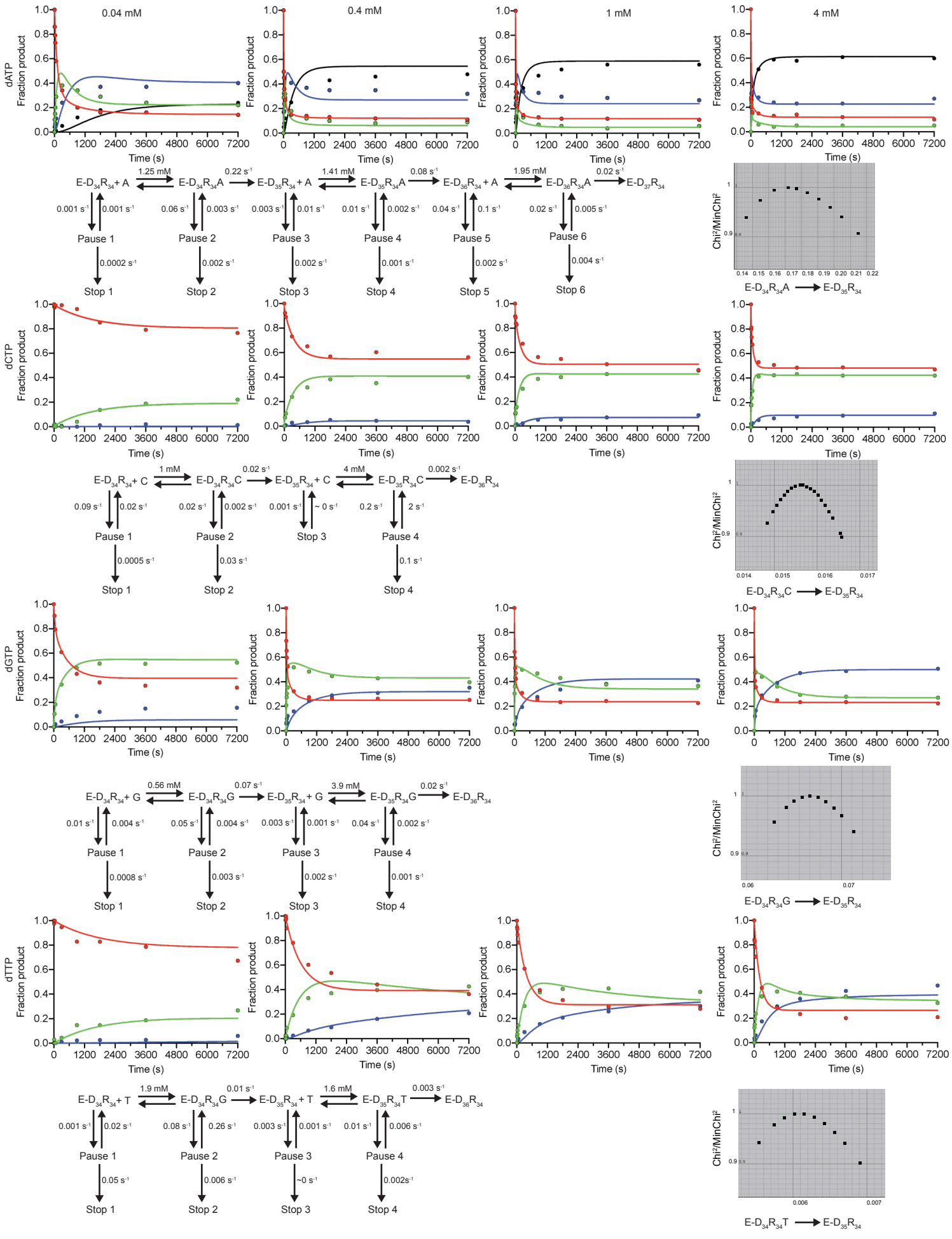
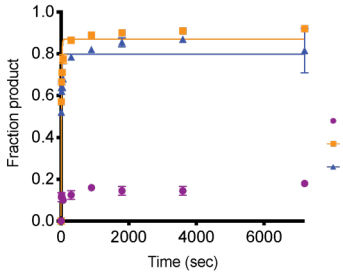

0.04 mM
0.4 mM
1 mM
4 mM
dATP
dCTP
dGTP
dTTP
Fraction product
Time (s)
E-D34R34+ A
E-D34R34A
E-D35R34+ A
E-D35R34A
E-D36R34+ A
E-D36R34A
E-D37R34
1.25 mM
0.22 s-1
1.41 mM
0.08 s-1
1.95 mM
0.02 s-1
Pause 1
Pause 2
Pause 3
Pause 4
Pause 5
Pause 6
Stop 1
Stop 2
Stop 3
Stop 4
Stop 5
Stop 6
Chi²/MinChi²
E-D34R34A → E-D35R34
E-D34R34+ C
E-D34R34C
E-D35R34+ C
E-D35R34C
E-D36R34
1 mM
0.02 s-1
4 mM
0.002 s-1
E-D34R34C → E-D35R34
E-D34R34+ G
E-D34R34G
E-D35R34+ G
E-D35R34G
E-D36R34
0.56 mM
0.07 s-1
3.9 mM
0.02 s-1
E-D34R34G → E-D35R34
E-D34R34+ T
E-D34R34T
E-D35R34+ T
E-D35R34T
E-D36R34
1.9 mM
0.01 s-1
1.6 mM
0.003 s-1
E-D34R34T → E-D35R34

Fig. S7



	k_{obs} (s^{-1})	Amplitude
ssDNA	N.D.	N.D.
RNA/DNA	0.07	0.87
DNA/DNA	0.07	0.80

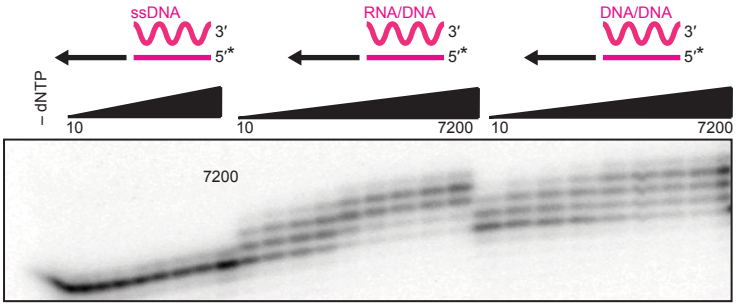
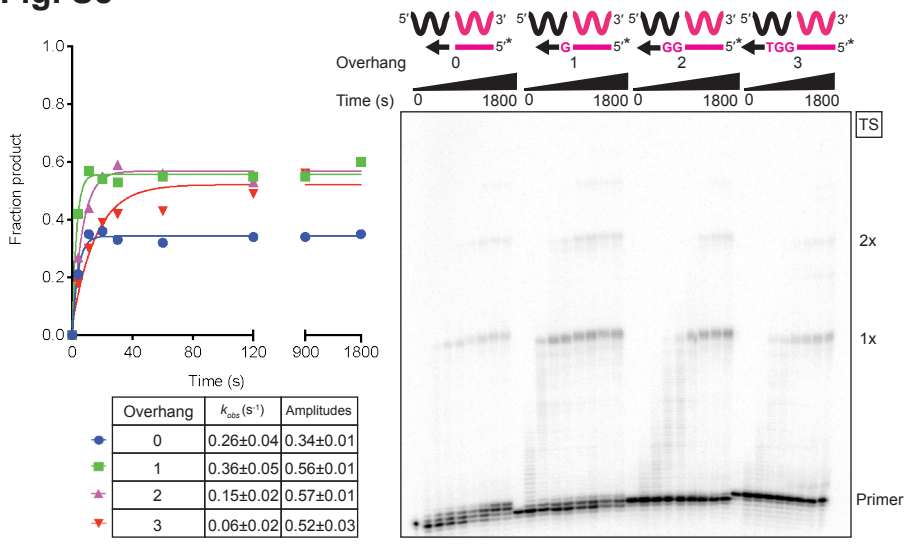


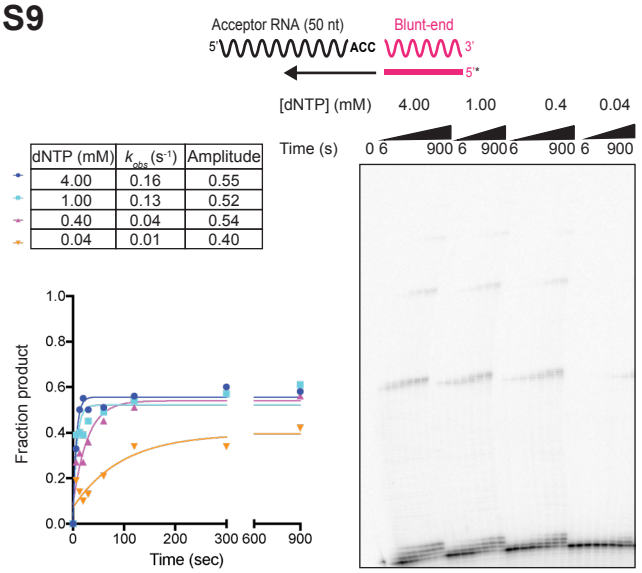
Fig. S8



Overhang	k_{obs} (s^{-1})	Amplitudes
0	0.26±0.04	0.34±0.01
1	0.36±0.05	0.56±0.01
2	0.15±0.02	0.57±0.01
3	0.06±0.02	0.52±0.03

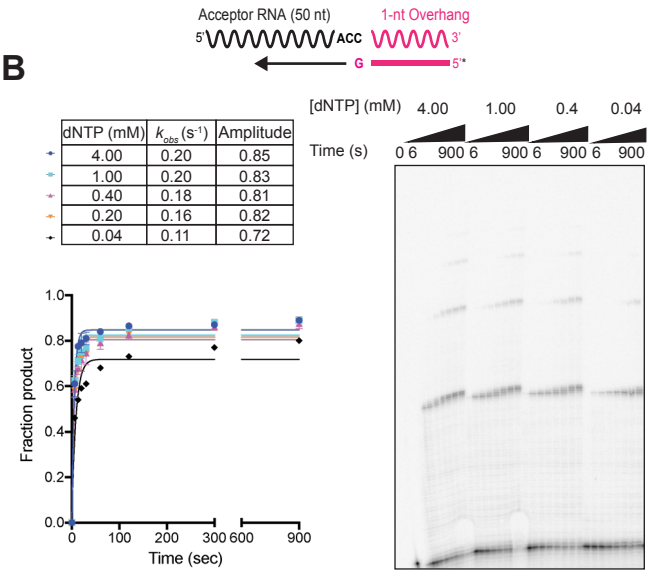
Fig. S9

A



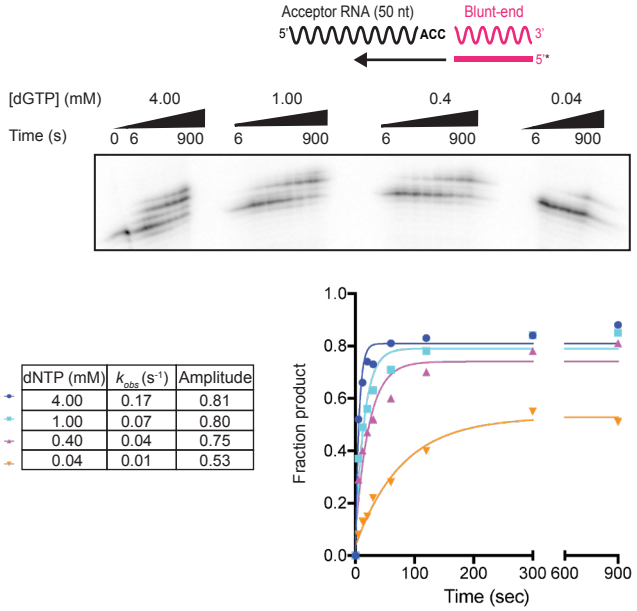
dNTP (mM)	k_{obs} (s^{-1})	Amplitude
4.00	0.16	0.55
1.00	0.13	0.52
0.40	0.04	0.54
0.04	0.01	0.40

B



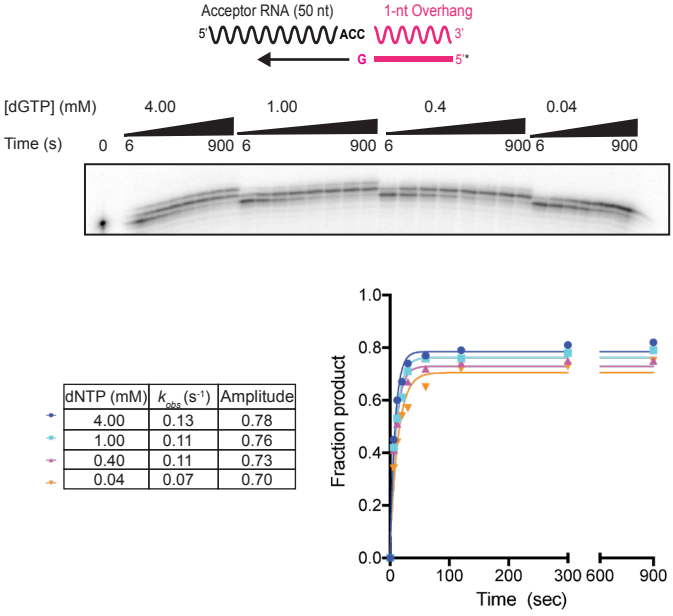
dNTP (mM)	k_{obs} (s^{-1})	Amplitude
4.00	0.20	0.85
1.00	0.20	0.83
0.40	0.18	0.81
0.20	0.16	0.82
0.04	0.11	0.72

C



dNTP (mM)	k_{obs} (s^{-1})	Amplitude
4.00	0.17	0.81
1.00	0.07	0.80
0.40	0.04	0.75
0.04	0.01	0.53

D



dNTP (mM)	k_{obs} (s^{-1})	Amplitude
4.00	0.13	0.78
1.00	0.11	0.76
0.40	0.11	0.73
0.04	0.07	0.70

Fig. S10

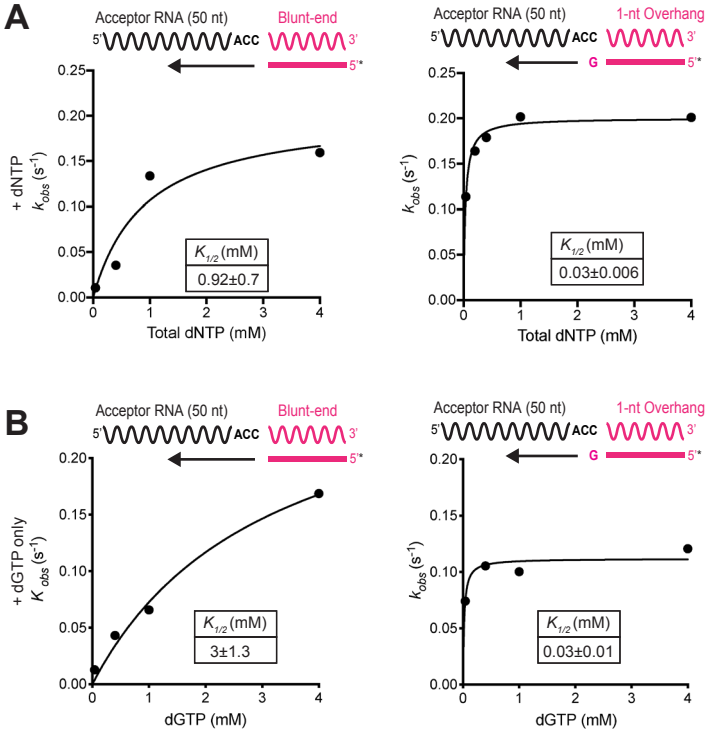

A
Acceptor RNA (50 nt)
Blunt-end
5'
ACC
3'
5'*
+ dNTP
k_{obs} (s^{-1})
$K_{1/2}$ (mM)
0.92±0.7
Total dNTP (mM)
Acceptor RNA (50 nt)
1-nt Overhang
5'
ACC
G
3'
5'*
k_{obs} (s^{-1})
$K_{1/2}$ (mM)
0.03±0.006
Total dNTP (mM)
B
Acceptor RNA (50 nt)
Blunt-end
5'
ACC
3'
5'*
+ dGTP only
K_{obs} (s^{-1})
$K_{1/2}$ (mM)
3±1.3
dGTP (mM)
Acceptor RNA (50 nt)
1-nt Overhang
5'
ACC
G
3'
5'*
k_{obs} (s^{-1})
$K_{1/2}$ (mM)
0.03±0.01
dGTP (mM)

Table S1

Type	Oligonucleotide name	Oligonucleotide Sequence
Acceptors nucleic acids	50 nt Acceptor A (RNA)	CGCCGGACCGUGCACCAUCUGGAGUUAUAGAGAUGAGUCUCACAUAGACA
	50 nt Acceptor U (RNA)	CGCCGGACCGUGCACCAUCUGGAGUUAUAGAGAUGAGUCUCACAUAGACU
	50 nt Acceptor G (RNA)	CGCCGGACCGUGCACCAUCUGGAGUUAUAGAGAUGAGUCUCACAUAGACG
	50 nt Acceptor C (RNA)	CGCCGGACCGUGCACCAUCUGGAGUUAUAGAGAUGAGUCUCACAUAGACC
	50 nt Acceptor C (DNA)	CGCCGGACCGTGCACCATCTGGAGTTATAGAGATGAGTCTCACATAGACC
	21 nt Acceptor OH (RNA)	GCCGCUUCAGAGAGAAAUCGC
	21 nt Acceptor P (RNA)	GCCGCUUCAGAGAGAAAUCGC3'P
	21 nt Acceptor 2Meo (RNA)	GCCGCUUCAGAGAGAAAUCG2'OMe
	21 nt Acceptor OH (DNA)	GCCGCTTCAGAGAGAAATCGC
	21 nt Acceptor OH (DNA)	GCCGCTTCAGAGAGAAATCG3'ddC
Components of starter duplexes	R2R A (DNA)	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTA
	R2R T (DNA)	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTT
	R2R G (DNA)	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTG
	R2R C (DNA)	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTC
	R2R GG (DNA)	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGG
	R2R GGT (DNA)	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGT
	R2R Blunt (DNA)	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
	Native PAGE Product Marker	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT- GGTCTATGTGAGACTCATCTCTATAACTCCAGATGGTGCACGGTCCGGCG
	Primer extension assay primer	TCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTC
Sequencing primers	R2 (RNA)	AGAUCGGAAGAGCACACGUCUGAACUCCAGUCAC
	R1R (DNA)	GATCGTCGGACTGTAGAACTCTGAACGTGTAG
	Multiplex primer	AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGACGATC
	Illumina barcode PCR primer	CAAGCAGAAGACGGCATACGAGAT**BARCODE**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

Table S2

Duplex	dNTP	dNTP (mM)	k_{obs} (s^{-1})	Product amplitude	$K_{1/2}$ (mM)	k_{add} (s^{-1})	$k_{add}/K_{1/2}$ ($M^{-1}s^{-1}$)
Blunt	dATP	4.00	0.16 ± 0.03	0.85 ± 0.02	1.3 ± 0.14	0.22 ± 0.01	170
		1.00	0.09 ± 0.02	0.85 ± 0.02			
		0.40	0.05 ± 0.01	0.85 ± 0.03			
		0.04	0.01 ± 0.002	0.81 ± 0.03			
	dTTP	4.00	0.004 ± 0.001	0.76 ± 0.03	1.6 ± 0.4	0.006 ± 0.0006	4
		1.00	0.002 ± 0.0004	0.69 ± 0.02			
		0.40	0.001 ± 0.0002	0.59 ± 0.03			
		0.04	0.0004 ± 0.0002	0.32 ± 0.04			
	dCTP	4.00	0.02 ± 0.005	0.50 ± 0.02	N.D.	N.D.	9
		1.00	0.004 ± 0.001	0.49 ± 0.02			
		0.40	0.003 ± 0.0007	0.41 ± 0.02			
		0.04	0.0004 ± 0.0001	0.27 ± 0.04			
	dGTP	4.00	0.04 ± 0.006	0.75 ± 0.02	0.5 ± 0.08	0.05 ± 0.003	100
		1.00	0.04 ± 0.007	0.74 ± 0.03			
		0.40	0.02 ± 0.004	0.72 ± 0.03			
		0.04	0.003 ± 0.0006	0.65 ± 0.02			
Overhang	dATP	4.00	0.03 ± 0.007	0.73 ± 0.03	0.9 ± 0.25	0.03 ± 0.003	33
		1.00	0.02 ± 0.005	0.68 ± 0.04			
		0.40	0.01 ± 0.005	0.65 ± 0.04			
		0.04	0.001 ± 0.0004	0.42 ± 0.04			
	dTTP	4.00	0.012 ± 0.005	0.60 ± 0.03	6.0 ± 2.4	0.03 ± 0.008	5
		1.00	0.004 ± 0.002	0.57 ± 0.03			
		0.40	0.002 ± 0.0009	0.48 ± 0.03			
		0.04	0.0007 ± 0.0001	0.28 ± 0.01			
	dCTP	4.00	0.002 ± 0.0007	0.70 ± 0.04	0.7 ± 0.23	0.002 ± 0.0003	3
		1.00	0.001 ± 0.0005	0.65 ± 0.05			
		0.40	0.0008 ± 0.0003	0.57 ± 0.05			
		0.04	0.0003 ± 0.0002	0.35 ± 0.08			
	dGTP	4.00	0.002 ± 0.0006	0.67 ± 0.04	1.6 ± 1.5	0.003 ± 0.001	2
		1.00	0.001 ± 0.0002	0.54 ± 0.03			
		0.40	0.0007 ± 0.0003	0.38 ± 0.04			
		0.04	0.0006 ± 0.0002	0.21 ± 0.03			

FIGURE S1. **Higher concentrations of GsI-IIC RT or acceptor RNA do not increase amplitudes in template-switching reactions at high salt concentration.** *A,* Template-switching reactions with 500 nM GsI-IIC RT, 50 nM ^{32}P-labeled (*) starter duplex and varying concentrations of 50-nt acceptor RNA in reaction media containing 200 or 400 mM NaCl. *B,* Template-switching reactions with 100 nM of 50-nt acceptor RNA, 50 nM ^{32}P-labeled starter duplex, and varying concentrations of GsI-IIC RT in reaction medium containing 400 mM NaCl. Template-switching reactions were done and analyzed as in Fig. 1.

FIGURE S2. **High salt concentrations have relatively little effect on primer extension by GsI-IIC RT.** Primer extension reactions were done with 50 nM template-primer substrate (a 1.1-kb *in vitro* transcribed RNA with a 50-nt ^{32}P-labeled (*) DNA primer (PE primer; Table S1) annealed at its 3' end) and 500 nM GsI-IIC RT in reaction media containing varying NaCl concentrations. The reaction was stopped after times ranging from 10 to 900 s, and the products were analyzed by denaturing PAGE and quantified from a phosphorimager scan of the dried gel. cDNA synthesis rates were approximated by the mean progression of nucleotide bands. The fraction product was measured by quantifying products larger than the primer and remained largely unchanged throughout the reaction. Extension rates and the end point fraction product are shown to the left.

FIGURE S3. **Template-switching reactions with varying concentrations of a 5'-labeled acceptor RNA analyzed by nondenaturing PAGE.** Template-switching reactions with varying concentrations of a 5' ^{32}P-labeled (*) 50-nt acceptor RNA with a 3'-C residue and 50 nM of starter duplex with a 1-nt 3'-G overhang were done for 15 min at 60 ºC in reaction medium containing 200 or 450 mM NaCl. After treatment with proteinase K to remove GsI-IIC RT, the products were analyzed by electrophoresis in a nondenaturing 8% polyacrylamide gel. The acceptor RNA (denatured by heating to 82 °C in 1x TE and placed on ice), and a product mimic (labeled acceptor RNA and R2 RNA annealed to a complementary 84-nt oligonucleotide corresponding to the sequence of a full-length cDNA) were loaded in parallel lanes. The gel was run at 4 °C at a constant power of 3 W to maintain the duplex structure and then dried and scanned with a phosphorimager. The labels to the right of the gel indicate the products resulting from the initial template switch (1x) and subsequent end-to-end template switches from the 5' end of one acceptor to the 3' end of another (2x, 3x, *etc*.). Putative product bands were summed and divided by the product and substrate bands to obtain a measure of the fraction product formed, which is indicated under each lane.

FIGURE S4**. Non-templated nucleotide addition activity to blunt-end and 1-nt 3' overhang starter duplexes using mixed dNTPs.** *A,* Reactions included 500 nM GsI-II RT and 50 nM of a blunt-end starter duplex with 5'-^{32}P-labeled (*) DNA primer in a solution containing 200 mM NaCl and varying dNTP concentrations (0.04, 0.4, 1, and 4 mM, where 4 mM is an equimolar mix of 1 mM dATP, dCTP, dGTP, and dTTP). Aliquots were quenched at times from 10 s to 7,200 s, and the products were analyzed by electrophoresis in a denaturing polyacrylamide gel, which was dried and scanned with a phosphorimager (gels and calculated kinetic parameters shown in Fig. 4). *B,* Reactions included 500 nM GsI-II RT and 50 nM of a 1-nt G overhang starter duplex and 200 mM NaCl. Reaction aliquots were stopped at times from 10 to 7,200 s, and the products were analyzed as in A. The observed rates for the first NTA from this template were used to calculate the kinetic parameters shown below the plot.

FIGURE S5. **Non-templated nucleotide addition reactions to blunt-end and 1-nt 3' overhang starter duplexes using individual dNTPs.** *A,* Assays of non-templated nucleotide addition to the ^{32}P-labeled blunt-end RNA/DNA duplex (left) and 1-nt 3'-overhang RNA/DNA duplex (right) using 0.04, 0.4, 1, and 4 mM dATP, dTTP, dCTP and dGTP individually. Reactions included 500 nM GsI-IIC RT and 50 nM of starter duplex in reaction medium containing 200 mM NaCl and were stopped at times ranging from 10 to 7,200 s. The reaction products were analyzed as in Fig. S4. The values obtained from these experiments were used for global fitting analysis in Fig. S6 and for the summary of second-order rate constants in Fig. 5.

FIGURE S6. **Global fitting of consecutive non-templated nucleotide addition using individual dNTPs.** Data and resulting models are shown for NTA of individual dNTPs. For dATP, three NTA steps were observed, and for the other dNTPs only two steps were observed. The plots show global data fitting of consecutive dNTP addition at the indicated concentrations of each dNTP. Each color represents a unique species in the reaction pathway: red, blunt-end starter duplex; green, product after first NTA; blue, product after second NTA; black, product after third NTA (if detected). For details on the global fitting process, see Experimental Procedures. A 1D fitspace plot is shown for each dNTP for the first reaction of nucleotide addition. These plots show the normalized chi^2 values for a range of values for the rate constant, with a chi^2 threshold of 0.9.

Figure S7. **Non-templated addition reactions of various starter molecule conformations.** Assays of NTA to 5'-^{32}P-labeled (*) single-stranded (ss) DNA (34 nt R2R DNA; left), a 34-bp RNA/DNA duplex (R2 RNA/R2R DNA; center), and a 34-bp DNA/DNA duplex (R2 DNA/R2R DNA; right). Reactions were done with 500 nM GsI-IIC RT and 50 nM substrate in reaction medium containing 200 mM NaCl and 4 mM dNTPs (an equimolar mix of 1 mM dATP, dCTP, dGTP, and dTTP) with time points ranging from 10 to 7,200 s. The reaction products were analyzed as in Fig. S4.

FIGURE S8. **Template switching to DNA acceptors is favored by a single nucleotide 3' overhang**. Template-switching reactions from starter duplexes with a blunt end (0 overhang) or 1, 2, or 3-nt 3' overhangs were done with 500 mM GsI-IIC RT, 100 nM 50-nt acceptor DNA, and 50 nM starter duplex with a ^{32}P-labeled (*) DNA primer in reaction medium containing 200 mM NaCl at 60 °C. Reactions were stopped after times ranging from 5 to 1,800 s, and the products were analyzed by denaturing PAGE and quantified from phosphorimager scans of the dried gel. The plots to the left of the gel show the data fit by a single exponential function to calculate the k_{obs} and amplitude for each time course, and the values are summarized in the table below the plots together with the standard error of the fit. The gel is labeled as in Fig. 1.

FIGURE S9. **Template-switching reactions from blunt-end and 1-nt 3'-overhang starter duplexes with varying concentrations of dNTPs.** *A* and *B*, Template-switching reactions with 50 nM blunt-end (A) or 1-nt overhang (B) starter duplexes with a ^{32}P-labeled (*) DNA primer and 100 nM 50-nt acceptor RNA were done with 500 nM GsI-IIC RNA RT and 0.04, 0.4, 1 or 4 mM dNTPs (where 4 mM dNTPs = 1 mM each of dATP, dCTP, dGTP, and dTTP). The reactions were stopped after times ranging from 6 to 900 s, and the products were analyzed by denaturing PAGE and quantified from a phosphorimager scan of the dried gel. The data were fit by a single exponential function to obtain the observed rates and amplitudes, which are summarized in the associated tables. *C* and *D*, The same template-switching reactions as in A and B but adding 0.04, 0.4, 1, or 4 mM dGTP, the nucleotide complementary to the last 2 nucleotides (CC) of the acceptor RNA, so that the template-switching products for the (C) blunt-end duplex is 2 nt longer than the DNA primer, and that for the (D) 1-nt overhang duplex is only 1 nt longer than the DNA primer.

FIGURE S10**. Template switching from a blunt-end duplex has a higher $K_{1/2}$ value for dNTPs than does template switching from a 1-nt 3' overhang duplex.** *A and* B, the observed rates for template switching at varying (A) dNTP or (B) dGTP concentrations from Fig. S9 were plotted against dNTP concentration and fit by a hyperbolic function to obtain $K_{1/2}$.

TABLE S1. **Sequences of oligonucleotides used in this study.** The oligonucleotides are divided into three sections indicated in the left-hand column: acceptor nucleic acids, components of starter duplexes, and sequencing primers.

TABLE S2. **Kinetic parameters for non-templated nucleotide addition of individual nucleotides.** The kinetic parameters of non-templated nucleotide addition with individual nucleotides, with the data shown in Figure S5 and second order rate constants in Fig. 5A.

TABLE S3. **RNA-Seq analysis of template-switching junctions.**