Supplementary Information

Human RNA polymerase III termination favors decomposition over facilitated recycling

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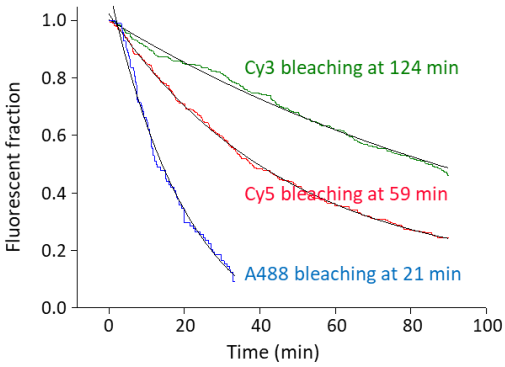
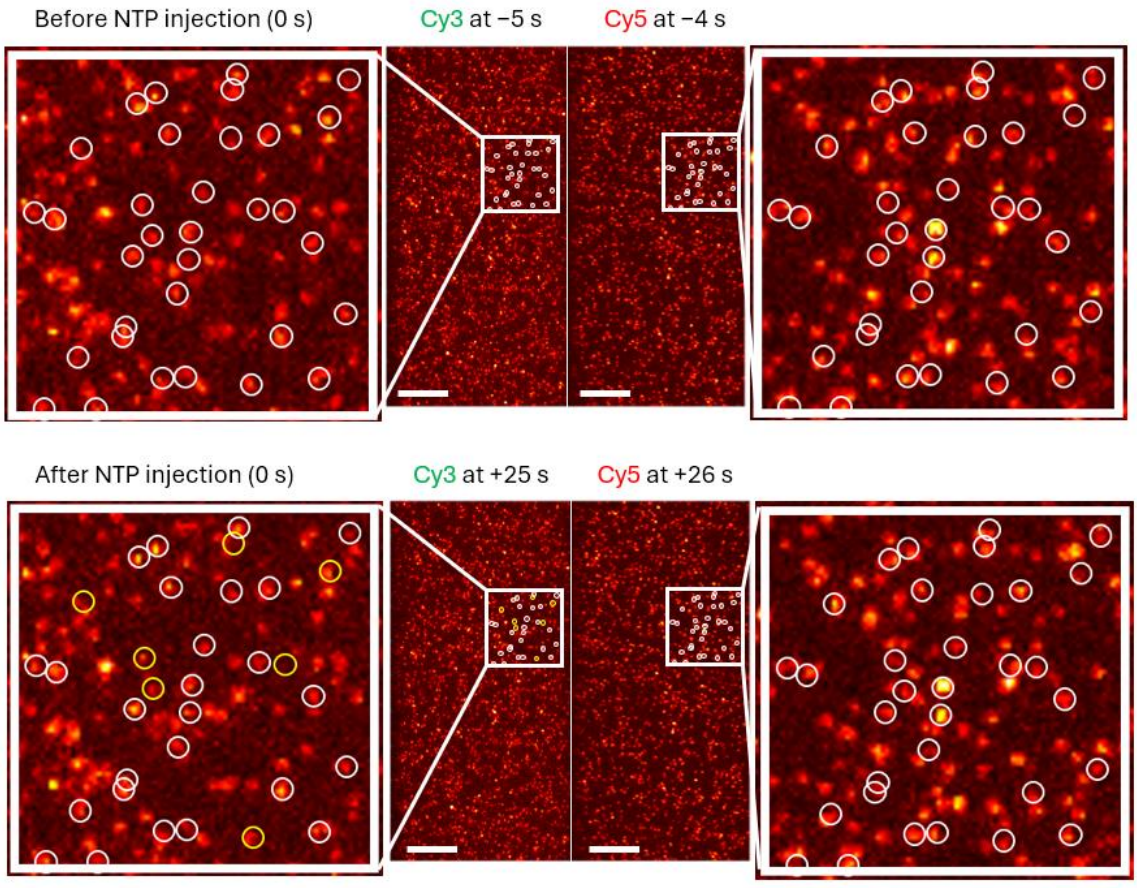
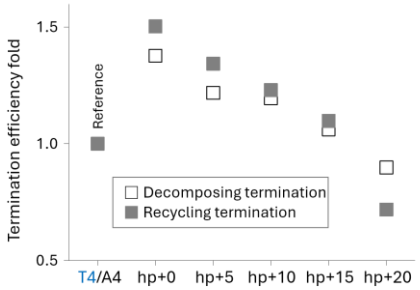
Supplementary Fig. 1 | Photobleaching kinetics of fluorophores. To determine the photobleaching kinetics of Cy3, Cy5, and Alexa Fluor 488 (A488), the fluorescence intensity decay of single-molecule spots was monitored under experimental conditions. Measurements were performed in imaging buffer without NTP injection to maintain stalled elongation complexes. This survival analysis reports the fraction of molecules remaining unbleached over the total experiment duration. Spots were initially identified based on colocalization and tracked independently. Photobleaching lifetimes were determined exclusively for spots exhibiting a one-step, irreversible decline in fluorescence intensity, ensuring rigorous single-molecule characterization. Spots displaying multi-step intensity decreases or evidence of overlapping fluorophores were excluded. Cy3 (green) and Cy5 (red) signals were monitored for 5,400 s (90 min), and A488 (blue) signals were monitored for 2,000 s (33 min). Single-exponential fitting of the survival curves yielded the following photobleaching lifetimes: 124 min for Cy3, 59 min for Cy5, and 21 min for A488.



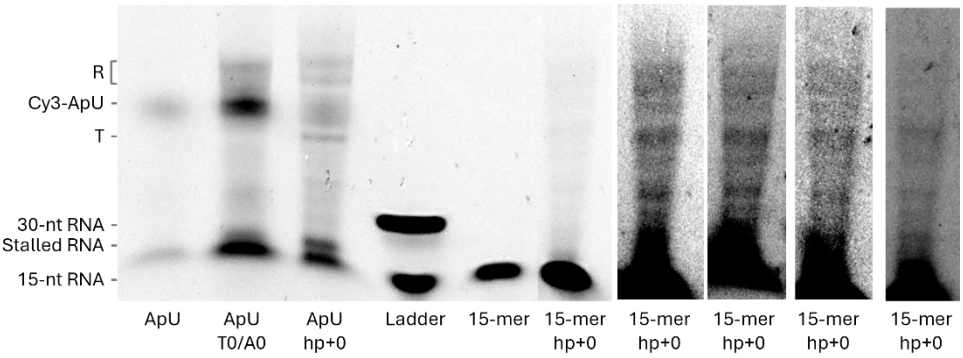
Supplementary Fig. 2 | Fluorescence images before and after NTP injection. Wide-field total internal reflection fluorescence (TIRF) microscopy images of the Cy3-labeled RNA and Cy5-labeled DNA channels are shown. The displayed images represent raw data without nonlinear processing. Channel identities (Cy3 and Cy5) are indicated in the panel labels. Magnified views (15 µm × 15 µm) of the boxed regions highlight individual single-molecule spots. White circles indicate colocalized signals in the Cy3 and Cy5 channels, while yellow circles mark Cy3 signals that decrease following NTP injection. For single-molecule analysis, active transcription events were defined strictly as those exhibiting a single-step Cy3 PIFE decline. All panels display the same field of view, with Cy3 and Cy5 channels recorded sequentially. Source data are provided as a Source Data file. Scale bar, 10 µm.



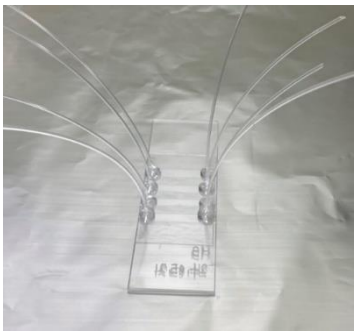
Supplementary Fig. 3 | RNA hairpin contributions to termination. The figure presents the fold changes in termination efficiencies for two distinct termination modes: decomposing (open squares) and recycling (filled squares) termination. The termination efficiencies are shown relative to the T4/A4 reference template. The data reveal that the hairpin-dependent enhancements in termination are comparable in magnitude across both the decomposing and recycling termination modes. This similarity indicates that the RNA hairpins exert a similar influence on both decomposing and recycling termination pathways.



Supplementary Fig. 4 | Bulk transcription with a 15-nt primer. This figure presents the results of a bulk transcription assay performed with a modified initiation component. A synthetic 15-nucleotide (5′-Cy3-AUCCAACCCAGACCG-3′) RNA primer (15-mer) at 200 nM was used, replacing the standard Cy3-ApU dinucleotide primer (ApU) used in other assays. The 15-nt primer was pre-annealed to the DNA template using the same procedure as the standard DNA annealing step described in the Methods section. All other reaction conditions were identical to those of the standard bulk assay. The band positions for expected runoff-sized RNAs (R) and terminated RNAs (T) are indicated to the left. Cy3-ApU migrates anomalously due to its hydrophobicity (see Fig. 7 legend) and is included as an intensity reference across gels. RNA size markers (ladder) are 15 and 30 nt.



Supplementary Fig. 5 | Quartz slide chamber used in the assay. This figure shows the flow chamber used for the single-molecule experiments. The chamber was constructed by assembling a quartz slide and a glass coverslip, following the procedure detailed in the Methods section. The chamber includes wires (ports) that enable controled buffer exchange via a syringe pump during real-time imaging. This control is critical for resuming the transcription elongation (e.g., by injecting NTPs) and maintaining stable imaging conditions.



Supplementary Table 1 | Termination, arrest, and activity data. Each active transcription complex was classified into one of three mutually exclusive outcomes: decomposing termination, recycling termination, or readthrough, which together accounted for 100% of observed events. The recycling fraction of termination is defined as recycling termination / (decomposing termination + recycling termination). Transcription activity was estimated by the fraction of colocalized complexes exhibiting a one-step Cy3 PIFE decline, signifying successful elongation resumption. Values represent means ± standard deviations from three or more independent experiments. The total number of active complexes analyzed per template is provided in Supplementary Table 2.

Template	Decomposing termination (%)	Recycling termination (%)	Readthrough (%)	Recycling fraction of termination	Transcription activity (%)
5S rRNA	62±1.6	11±1.4	27±0.26	0.14±0.019	12±0.16
tRNA-Ala	65±2.9	8.4±2.0	26±2.8	0.11±0.026	13±4.0
U6 snRNA	66±4.9	7.5±1.9	27±3.0	0.10±0.030	16±2.1
RNase P RNA	44±6.4	6.2±2.0	50±8.4	0.12±0.021	16±1.9
7SL RNA	65±2.8	1.5±1.1	34±2.5	0.023±0.016	12±1.5
T0/A0	19±1.4	1.5±1.4	80±1.1	0.075±0.066	18±5.2
T1/A1	20±0.27	1.4±1.2	79±1.4	0.064±0.055	18±6.1
T2/A2	22±2.5	2.3±2.3	76±2.9	0.093±0.085	14±1.7
T3/A3	20±2.8	1.2±1.1	79±3.0	0.057±0.053	18±4.6
T4/A4	45±3.7	6.9±2.4	48±5.5	0.13±0.034	18±5.1
T5/A5	45±0.61	9.4±2.4	45±2.8	0.17±0.035	18±2.6
T6/A6	49±1.8	8.4±2.0	43±0.61	0.15±0.035	20±1.4
T7/A7	49±2.3	8.4±0.23	42±2.6	0.15±0.0028	20±2.8
T8/A8	55±3.8	9.9±4.0	35±4.8	0.15±0.058	20±5.7
T9/A9	54±1.5	9.9±2.5	36±2.6	0.15±0.035	18±1.7
T9/T9	47±5.5	9.4±2.8	44±3.9	0.17±0.056	17±4.7
A9/T9	29±0.87	4.8±2.0	66±2.8	0.14±0.046	17±3.7
A9/A9	30±1.0	1.4±1.3	68±1.4	0.044±0.042	19±2.7
hp+0	62±0.85	10±0.68	27±0.69	0.14±0.0091	22±2.3
hp+5	55±3.9	9.3±1.3	35±4.5	0.14±0.015	13±0.91
hp+10	54±5.2	8.5±2.2	37±3.7	0.14±0.041	12±0.87
hp+15	48±2.1	7.6±1.7	44±2.8	0.14±0.026	16±1.1
hp+20	41±5.2	5.0±2.3	54±7.0	0.11±0.041	13±4.0
Three-color assay					
T4/A4	45±4.6	7.3±1.3	47±3.7	0.14±0.030	15±1.5
With mutant RNAP3				Arrest (%)	
T0/A0	19±0.91	1.0±1.8	43±7.4	37±7.5	16±2.2
T4/A4	19±2.9	1.7±1.9	41±2.9	39±4.7	18±4.9
T8/A8	41±5.6	2.7±0.65	22±1.5	35±7.0	18±1.3

Supplementary Table 2 | Kinetics measurements and sample sizes. Termination times (transcript release) were defined as the interval between Cy3 PIFE decline (marking elongation resumption) and Cy3 signal loss (indicating RNA release). End-reaching times for readthrough were defined as the interval between Cy3 PIFE decline and Cy5 PIFE onset, indicating the arrival of readthrough RNAP3 at the Cy5-labeled downstream end of the DNA template. Values represent means ± standard deviations from three or more independent experiments.

Template	RNA release time (s) in decomposing termination	RNA release time (s) in recycling termination	End reaching time (s) in readthrough-runoff	Number of molecules, N
5S rRNA	120±20	49±32	27±1.5	115 = 33+41+41
tRNA-Ala	90±32	49±24	29±13	179 = 100+49+30
U6 snRNA	99±19	60±42	23±8.3	187 = 54+78+55
RNase P RNA	113±20	62±29	21±9.3	213 = 52+52+36+39+34
7SL RNA	106±23	14±2.5	28±11	207 = 57+62+50+38
T0/A0	79±6.7	52±49	22±2.8	234 = 70+112+52
T1/A1	85±36	58±57	14±4.7	137 = 46+50+41
T2/A2	119±45	41±14	23±6.1	132 = 44+46+42
T3/A3	138±16	103±50	17±3.4	226 = 61+68+97
T4/A4	67±9.5	43±26	34±8.0	123 = 34+31+58
T5/A5	71±10	30±6.1	31±6.4	172 = 67+50+55
T6/A6	91±11	35±3.3	33±6.3	143 = 50+44+49
T7/A7	71±14	44±7.5	30±6.3	201 = 59+93+49
T8/A8	90±7.6	34±11	33±7.8	146 = 46+45+55
T9/A9	98±30	57±11	34±6.1	123 = 33+42+48
T9/T9	120±18	100±9.8	23±6.0	237 = 109+87+41
A9/T9	125±18	77±15	8.0±4.8	261 = 140+64+57
A9/A9	110±16	27±2.8	15±5.2	179 = 75+64+40
hp+0	52±18	57±51	25±4.2	154 = 45+41+68
hp+5	58±15	43±10	20±11	128 = 47+37+44
hp+10	72±9.1	65±41	17±11	106 = 44+33+29
hp+15	78±21	39±7.0	13±3.1	128 = 56+35+37
hp+20	73±9.8	40±9.1	17±12	122 = 45+43+34
Three-color assay				
T4/A4	70±15	33±11	30±12	137 = 40+52+45
With mutant RNAP3				
T0/A0	251±36	127±41	112±51	110 = 42+35+33
T4/A4	243±52	127±10	118±21	156 = 71+53+32
T8/A8	209±37	113±20	110±40	176 = 49+90+37

Supplementary Table 3 | DNA oligomers for template construction. The non-template strand is denoted as NT, and the template strand as T.

Oligomer	Sequence in the 5' to 3' direction
5S rRNA_NT	CCAACCCAGACCGCCAGGGACTGGAGACCGCCTGGGAATACCGGGTGCTGTA GGCTTTTTCTTTGGCTTTTTGCTGTTTCTTTCCTTTTCTTCCAGACG–[Cy5]
5S rRNA _T	CGTCTGGAAGAAAAGGAAAGAAACAGCAAAAAGCCAAAGAAAAAGCCTACA GCACCCGGTATTCCCAGGCGGTCTCCAGTCCCTGGCGGTCTGGGTTGGATAAA CAGTCAGAACAA-[Biotin]
tRNA-Ala_NT	CCAACCCAGACCGCCAGGGACTCCTGGGTTCGATCCCCAGTACCTCCAGGCCG TGTTTTCTTCCCCGGGACACCAGTAAGAAGCGGTCCATGATATTCC–[Cy5]
tRNA-Ala_T	GGAATATCATGGACCGCTTCTTACTGGTGTCCCGGGGAAGAAAACACGGCCTG GAGGTACTGGGGATCGAACCCAGGAGTCCCTGGCGGTCTGGGTTGGATAAAC AGTCAGAACAA-[Biotin]
U6 snRNA_NT	CCAACCCAGACCGCCAGGGACTAGGATGACACGCAAATTCGTGAAGCGTTCC ATATTTTTACATCAGGTTGTTTTTCTGTTTTTACATCAGGTTGTTTT–[Cy5]
U6 snRNA_T	AAAACAACCTGATGTAAAAACAGAAAAACAACCTGATGTAAAAATATGGAAC GCTTCACGAATTTGCGTGTCATCCTAGTCCCTGGCGGTCTGGGTTGGATAAAC AGTCAGAACAA-[Biotin]
RNase P RNA_NT	CCAACCCAGACCGCCAGGGACTCATAACCCAATTCAGACTACTCTCCTCCGCC CATTTTTGGAAAAAAAAAAAAAAAAAAAAACAAAACGAAACCGGG–[Cy5]
RNase P RNA_T	CCCGGTTTCGTTTTGTTTTTTTTTTTTTTTTTTTTCCAAAAATGGGCGGAGGA GAGTAGTCTGAATTGGGTTATGAGTCCCTGGCGGTCTGGGTTGGATAAACAGT CAGAACAA-[Biotin]
7SL RNA_NT	CCAACCCAGACCGCCAGGGACTTCCAGCCTGGGCAACATAGCGAGACCCCGT CTCTTTTGAACAATAAATACGTTAATTTTGGACTCTCAAGAGATGAA–[Cy5]
7SL RNA_T	TTCATCTCTTGAGAGTCCAAAATTAACGTATTTATTGTTCAAAAGAGACGGGG TCTCGCTATGTTGCCCAGGCTGGAAGTCCCTGGCGGTCTGGGTTGGATAAACA GTCAGAACAA-[Biotin]
T0/A0_NT	CCAACCCAGACCGCCAGGGACTACCGGGTGCTGTAGGCATGTCTCCATTCACT TCCCACGTGTGCAAGCACGTTACCC–[Cy5]
T0/A0_T	GGGTAACGTGCTTGCACACGTGGGAAGTGAATGGAGACATGCCTACAGCACC CGGTAGTCCCTGGCGGTCTGGGTTGGATAAACAGTCAGAACAA-[Biotin]
T1/A1_NT	CCAACCCAGACCGCCAGGGACTACCGGGTGCTGTAGGCTATGTCTCCATTCAC TTCCCACGTGTGCAAGCACGTTACCC–[Cy5]
T1/A1_T	GGGTAACGTGCTTGCACACGTGGGAAGTGAATGGAGACATAGCCTACAGCAC CCGGTAGTCCCTGGCGGTCTGGGTTGGATAAACAGTCAGAACAA-[Biotin]
T2/A2_NT	CCAACCCAGACCGCCAGGGACTACCGGGTGCTGTAGGCTTATGTCTCCATTCA CTTCCCACGTGTGCAAGCACGTTACCC–[Cy5]
T2/A2_T	GGGTAACGTGCTTGCACACGTGGGAAGTGAATGGAGACATAAGCCTACAGCA CCCGGTAGTCCCTGGCGGTCTGGGTTGGATAAACAGTCAGAACAA-[Biotin]
T3/A3_NT	CCAACCCAGACCGCCAGGGACTACCGGGTGCTGTAGGCTTTATGTCTCCATTC ACTTCCCACGTGTGCAAGCACGTTACCC–[Cy5]
T3/A3_T	GGGTAACGTGCTTGCACACGTGGGAAGTGAATGGAGACATAAAGCCTACAGC ACCCGGTAGTCCCTGGCGGTCTGGGTTGGATAAACAGTCAGAACAA-[Biotin]
T4/A4_NT	CCAACCCAGACCGCCAGGGACTACCGGGTGCTGTAGGCTTTTATGTCTCCATT CACTTCCCACGTGTGCAAGCACGTTACCC–[Cy5]
T4/A4_T	GGGTAACGTGCTTGCACACGTGGGAAGTGAATGGAGACATAAAAGCCTACAG CACCCGGTAGTCCCTGGCGGTCTGGGTTGGATAAACAGTCAGAACAA-[Biotin]
T5/A5_NT	CCAACCCAGACCGCCAGGGACTACCGGGTGCTGTAGGCTTTTTATGTCTCCAT

	TCACTTCCCACGTGTGCAAGCACGTTACCC–[Cy5]
T5/A5_T	GGGTAACGTGCTTGCACACGTGGGAAGTGAATGGAGACATAAAAAGCCTACA GCACCCGGTAGTCCCTGGCGGTCTGGGTTGGATAAACAGTCAGAACAA-[Biotin]
T6/A6_NT	CCAACCCAGACCGCCAGGGACTACCGGGTGCTGTAGGCTTTTTTATGTCTCCA TTCACTTCCCACGTGTGCAAGCACGTTACCC–[Cy5]
T6/A6_T	GGGTAACGTGCTTGCACACGTGGGAAGTGAATGGAGACATAAAAAAGCCTAC AGCACCCGGTAGTCCCTGGCGGTCTGGGTTGGATAAACAGTCAGAACAA-[Biotin]
T7/A7_NT	CCAACCCAGACCGCCAGGGACTACCGGGTGCTGTAGGCTTTTTTTATGTCTCC ATTCACTTCCCACGTGTGCAAGCACGTTACCC–[Cy5]
T7/A7_T	GGGTAACGTGCTTGCACACGTGGGAAGTGAATGGAGACATAAAAAAAGCCTA CAGCACCCGGTAGTCCCTGGCGGTCTGGGTTGGATAAACAGTCAGAACAA-[Biotin]
T8/A8_NT	CCAACCCAGACCGCCAGGGACTACCGGGTGCTGTAGGCTTTTTTTTATGTCTCC ATTCACTTCCCACGTGTGCAAGCACGTTACCC–[Cy5]
T8/A8_T	GGGTAACGTGCTTGCACACGTGGGAAGTGAATGGAGACATAAAAAAAAGCCT ACAGCACCCGGTAGTCCCTGGCGGTCTGGGTTGGATAAACAGTCAGAACAA-[Biotin]
T9/A9_NT	CCAACCCAGACCGCCAGGGACTACCGGGTGCTGTAGGCTTTTTTTTTATGTCTC CATTCACTTCCCACGTGTGCAAGCACGTTACCC–[Cy5]
T9/A9_T	GGGTAACGTGCTTGCACACGTGGGAAGTGAATGGAGACATAAAAAAAAAGCC TACAGCACCCGGTAGTCCCTGGCGGTCTGGGTTGGATAAACAGTCAGAACAA-[Biotin]
T9/T9_NT	CCAACCCAGACCGCCAGGGACTACCGGGTGCTGTAGGCTTTTTTTTTATGTCTC CATTCACTTCCCACGTGTGCAAGCACGTTACCC–[Cy5]
T9/T9_T	GGGTAACGTGCTTGCACACGTGGGAAGTGAATGGAGACATTTTTTTTTTGCCT ACAGCACCCGGTAGTCCCTGGCGGTCTGGGTTGGATAAACAGTCAGAACAA-[Biotin]
A9/T9_NT	CCAACCCAGACCGCCAGGGACTACCGGGTGCTGTAGGCAAAAAAAAAATGTC TCCATTCACTTCCCACGTGTGCAAGCACGTTACCC–[Cy5]
A9/T9_T	GGGTAACGTGCTTGCACACGTGGGAAGTGAATGGAGACATTTTTTTTTTGCCT ACAGCACCCGGTAGTCCCTGGCGGTCTGGGTTGGATAAACAGTCAGAACAA-[Biotin]
A9/A9_NT	CCAACCCAGACCGCCAGGGACTACCGGGTGCTGTAGGCAAAAAAAAAATGTC TCCATTCACTTCCCACGTGTGCAAGCACGTTACCC–[Cy5]
A9/A9_T	GGGTAACGTGCTTGCACACGTGGGAAGTGAATGGAGACATAAAAAAAAAGCC TACAGCACCCGGTAGTCCCTGGCGGTCTGGGTTGGATAAACAGTCAGAACAA-[Biotin]
hp+0_NT	CCAACCCAGACCGCCAGGGACTGGTTGCGGCTTTCTGCTGCAATCTTTTATGTC TCCATTCACTTCCCACGTGTGCAAGCACGTTACCC–[Cy5]
hp+0_T	GGGTAACGTGCTTGCACACGTGGGAAGTGAATGGAGACATAAAAGATTGCAG CAGAAAGCCGCAACCAGTCCCTGGCGGTCTGGGTTGGATAAACAGTCAGAAC AA-[Biotin]
hp+5_NT	CCAACCCAGACCGCCAGGGACTGGTTGCGGCTTTCTGCTGCAATCCACATTTTT ATGTCTCCATTCACTTCCCACGTGTGCAAGCACGTTACCC–[Cy5]
hp+5_T	GGGTAACGTGCTTGCACACGTGGGAAGTGAATGGAGACATAAAAATGTGGAT TGCAGCAGAAAGCCGCAACCAGTCCCTGGCGGTCTGGGTTGGATAAACAGTC AGAACAA-[Biotin]
hp+10_NT	CCAACCCAGACCGCCAGGGACTGGTTGCGGCTTTCTGCTGCAATCCACATCAC

	ATTTTTATGTCTCCATTCACTTCCCACGTGTGCAAGCACGTTACCC–[Cy5]
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