Supporting information

Table S1. Sequences of oligonucleotides used in this work.

Name	**Oligo sequence** (5′-3′)
	Substrates for single-molecule FRET
$s_{26}3G4d_{17}$	AAGCAGTGGTATCAACGCAGAGAAAT(iCy3)GGGTTAGGGTTAGGGTTAGGGATGTATGACAAGGAAGG
$s_{26}4G4d_{17}$	AAGCAGTGGTATCAACGCAGAGAAAT(iCy3)GGGGTAGGGGTAGGGGTAGGGGATGTATGACAAGGAAGG
$s_{26}5G4d_{17}$	AAGCAGTGGTATCAACGCAGAGAAAT(iCy3)GGGGGTAGGGGGTAGGGGGTAGGGGGATGTATGACAAGGAAGG
$s_{26}1\text{-}1\text{-}1d_{17}$	AAGCAGTGGTATCAACGCAGAGAAAT(iCy3)GGGTGGGTGGGTGGGATGTATGACAAGGAAGG
$s_{26}1\text{-}9\text{-}1d_{17}$	AAGCAGTGGTATCAACGCAGAGAAAT(iCy3)GGGTGGGTGTAAGTGTGGGTGGGATGTATGACAAGGAAGG
$s_{26}1\text{-}2\text{-}1d_{17}$	AAGCAGTGGTATCAACGCAGAGAAAT(iCy3)GGGTGGGTAGGGTGGGATGTATGACAAGGAAGG
$s_{26}2\text{-}2\text{-}2_{17}$	AAGCAGTGGTATCAACGCAGAGAAAT(iCy3)GGGTAGGGTAGGGTAGGGATGTATGACAAGGAAGG
$s_{26}2\text{-}2\text{-}1d_{17}$	AAGCAGTGGTATCAACGCAGAGAAAT(iCy3)GGGTAGGGTAGGGTGGGATGTATGACAAGGAAGG
$s_{26}3\text{-}5\text{-}10d_{17}$	AAGCAGTGGTATCAACGCAGAGAAAT(iCy3)GGGTGTGGGTGTGTGGGTGTGGTGTGTGGGATGTATGACAAGGAAG G
$s_{26}3\text{-}10\text{-}10d_{17}$	AAGCAGTGGTATCAACGCAGAGAAAT(iCy3)GGGTGTGGGTGTGGTGTGTGGGTGTGGTGTGTGGGATGTATGACAA GGAAGG
$s_{26}10\text{-}8\text{-}10d_{17}$	AAGCAGTGGTATCAACGCAGAGAAAT(iCy3)GGGTGTGGTGTGTGGGTGTGGTGTGGGTGTGGTGTGTGGGATGTATG ACAAGGAAGG
$s_{26}32\text{-}3\text{-}3d_{17}$	AAGCAGTGGTATCAACGCAGAGAAAT(iCy3)GGGCTGGTGCTGAGCGTGTGCTTGTGCTTGTGCTTGGGCTTGGGCTT GGGATGTATGACAAGGAAGG
$s_{47}d_{17}$	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT(iCy3)TTTTTTTTTTTTTTTTTTTTTATGTATGACAAGGAAGG
Stem	Biotin-CCTTCCTTGTCAT(iCy5)ACAT
	Substrates for CD measurement
$s_{26}3G4d_{17}$	AAGCAGTGGTATCAACGCAGAGAAATGGGTTAGGGTTAGGGTTAGGGATGTATGACAAGGAAGG
$s_{26}4G4d_{17}$	AAGCAGTGGTATCAACGCAGAGAAATGGGGTAGGGGTAGGGGTAGGGGATGTATGACAAGGAAGG
$s_{26}5G4d_{17}$	AAGCAGTGGTATCAACGCAGAGAAATGGGGGTAGGGGGTAGGGGGTAGGGGGATGTATGACAAGGAAGG
$s_{26}1\text{-}1\text{-}1d_{17}$	AAGCAGTGGTATCAACGCAGAGAAATGGGTGGGTGGGTGGGATGTATGACAAGGAAGG
$s_{26}1\text{-}9\text{-}1d_{17}$	AAGCAGTGGTATCAACGCAGAGAAATGGGTGGGTGTAAGTGTGGGTGGGATGTATGACAAGGAAGG
$s_{26}1\text{-}2\text{-}1d_{17}$	AAGCAGTGGTATCAACGCAGAGAAATGGGTGGGTAGGGTGGGATGTATGACAAGGAAGG
$s_{26}2\text{-}2\text{-}2_{17}$	AAGCAGTGGTATCAACGCAGAGAAATGGGTAGGGTAGGGTAGGGATGTATGACAAGGAAGG
$s_{26}2\text{-}2\text{-}1d_{17}$	AAGCAGTGGTATCAACGCAGAGAAATGGGTAGGGTAGGGTGGGATGTATGACAAGGAAGG
$s_{26}3\text{-}5\text{-}10d_{17}$	AAGCAGTGGTATCAACGCAGAGAAATGGGTGTGGGTGTGTGGGTGTGGTGTGTGGGATGTATGACAAGGAAGG
$s_{26}5\text{-}10\text{-}3d_{17}$	AAGCAGTGGTATCAACGCAGAGAAATGGGTGTGTGGGTGTGGTGTGTGGGTGTGGGATGTATGACAAGGAAGG
$s_{26}3\text{-}10\text{-}10d_{17}$	AAGCAGTGGTATCAACGCAGAGAAATGGGTGTGGGTGTGGTGTGTGGGTGTGGTGTGTGGGATGTATGACAAGGAA GG
$s_{26}8\text{-}8\text{-}7d_{17}$	AAGCAGTGGTATCAACGCAGAGAAATGGGTTGGATATGGGTAATTGGAGGGTAACGGTGGGATGTATGACAAGGAA GG
$s_{26}10\text{-}8\text{-}10d_{17}$	AAGCAGTGGTATCAACGCAGAGAAATGGGTGTGGTGTGTGGGTGTGGTGTGGGTGTGGTGTGTGGGATGTATGACA AGGAAGG
$s_{26}10\text{-}8\text{-}15d_{17}$	AAGCAGTGGTATCAACGCAGAGAAATGGGTGTGGTGTGTGGGTGTGGTGTGGGTGTGGTGTGGTGTGTGGGATGTA TGACAAGGAAGG
$s_{26}12\text{-}3\text{-}18d_{17}$	AAGCAGTGGTATCAACGCAGAGAAATGGGTGTGGTGTGTGTGGGTGTGGGTGTGGTGTGGTGTGGTGTGGGATGTA TGACAAGGAAGG
$s_{26}32\text{-}3\text{-}3d_{17}$	AAGCAGTGGTATCAACGCAGAGAAATGGGCTGGTGCTGAGCGTGTGCTTGTGCTTGTGCTTGGGCTTGGGCTTGGG ATGTATGACAAGGAAGG
Stem	CCTTCCTTGTCATACAT
	Substrates for fluorescence melting
3G4	FAM-TGGGTTAGGGTTAGGGTTAGGGT-TAMRA
4G4	FAM-TGGGGTAGGGGTAGGGGTAGGGGT-TAMRA
5G4	FAM-TGGGGGTAGGGGGTAGGGGGTAGGGGGT-TAMRA
1-1-1	FAM-TGGGTGGGTGGGTGGGT-TAMRA
1-9-1	FAM-TGGGTGGGTGTAAGTATGGGTGGGT-TAMRA
1-2-1	FAM-TGGGTGGGTAGGGTGGGT-TAMRA
2-2-2	FAM-TGGGTAGGGTAGGGTAGGGT-TAMRA
2-2-1	FAM-TGGGTAGGGTAGGGTGGGT-TAMRA
3-5-10	FAM-TGGGTGTGGGTGTGTGGGTGTGGTGTGTGGGT-TAMRA
5-10-3	FAM-TGGGTGTGTGGGTGTGGTGTGTGGGTGTGGGT-TAMRA
3-10-10	FAM-TGGGTGTGGGTGTGGTGTGTGGGTGTGGTGTGTGGGT-TAMRA
3-8-12	FAM-TGGGTGTGGGTGTGGTGTGGGTGTGGTGTGTGTGGGT-TAMRA
10-8-10	FAM-TGGGTGTGGTGTGTGGGTGTGGTGTGGGTGTGGTGTGTGGGT-TAMRA
8-8-12	FAM-TGGGTAGTGTTAGGGTAGTGTGTGGGTGTGGTGTGTGTGGGT-TAMRA
10-8-15	FAM-TGGGTGTGGTGTGTGGGTGTGGTGTGGGTGTGGTGTGGTGTGTGGGT-TAMRA
10-12-11	FAM-TGGGTGTGGTGTGTGGGTGTGGTGTGTGTGGGTGTGTGTGTGTGGGT-TAMRA
8-3-27	FAM-TGGGTGTGGTGTGGGTGTGGGTGTGGTGTGGTGTGGTGTGTGGGT-TAMRA
19-3-16	FAM-TGGGATGATATGAACATGGACATGGGTATGGGTCATGATCAGTCCGAAGGGT-TAMRA
32-3-3	FAM-TGGGCTGGTGCTGAGCGTGTGCTTGTGCTTGTGCTTGGGCTTGGGCTTGGGT-TAMRA

Internal Cy3 (iCy3) or Cy5 (iCy5) in oligos was modified by the N-hydroxysuccinimide (NHS) ester form of Cy3 or Cy5 in thymine with a 12-atom linker.

Table S2. G4 motifs in *S. cerevisiae* which were named according to the loop sizes.

Name	**Oligo sequence** (5′-3′)		
30-nt		**35-nt**	
3-5-10	GGGTGTGGGTGTGTGGGTGTGGTGTGTGGG	5-10-8	GGGTGTGTGGGTGTGGTGTGTGGGTGTGGTGTGGG
10-3-5	GGGTGTGGTGTGTGGGTGTGGGTGTGTGGG	8-5-10	GGGTGTGGTGTGGGTGTGTGGGTGTGGTGTGTGGG
5-3-10	GGGTGTGTGGGTGTGGGTGTGGTGTGTGGG	8-12-3	GGGTGTGGTGTGGGTGTGGTGTGTGTGGGTGTGGG
5-10-3	GGGTGTGTGGGTGTGGTGTGTGGGTGTGGG	3-12-8	GGGTGTGGGTGTGGTGTGTGTGGGTGTGGTGTGGG
8-3-7	GGGTGTGGTGTGGGTGTGGGTGTGTGTGGG	3-8-12	GGGTGTGGGTGTGGTGTGGGTGTGGTGTGTGTGGG
7-3-8	GGGTGTGTGTGGGTGTGGGTGTGGTGTGGG	8-3-12	GGGTAGTGTGTGGGTGTGGGTGTGGTGTGTGTGGG
3-3-12	GGGTGTGGGTGTGGGTGTGGTGTGTGTGGG	12-3-8	GGGTGTGGTGTGTGTGGGTGTGGGTGTGGTGTGGG
6-7-5	GGGCCAGCAGGGATGTATTGGGCACAAGGG	8-8-7	GGGTTGGATATGGGTAATTGGAGGGTAACGGTGGG
5-5-8	GGGTGTGTGGGTGTGTGGGTGTGGTGTGGG	11-8-4	GGGCAAAGTTCTGCGGGCAACGTGTGGGCCAAGGG
		9-8-6	GGGTATTATAATGGGTTCTGTGAGGGCAAAGAGGG
		20-1-2	GGGATCCTCATTCTCGTGGTAGTGGGTGGGTTGGG
		3-10-10	GGGTGTGGGTGTGGTGTGTGGGTGTGGTGTGTGGG
		10-3-10	GGGTGTGGTGTGTGGGTGTGGGTGTGGTGTGTGGG
40-nt		15-5-3	GGGTGGATTTCGCCCGACGGGTGAATGGGTGTGGG
10-8-10	GGGTGTGGTGTGTGGGTGTGGTGTGGGTGTGGTGTGTGGG		
10-10-8	GGGTGTGGTGTGTGGGTGTGGTGTGTGGGTGTGGTGTGGG		
8-10-10	GGGTGTGGTGTGGGTGTGGTGTGTGGGTGTGGTGTGTGGG		
8-8-12	GGGTAGTGTTAGGGTAGTGTGTGGGTGTGGTGTGTGTGGG		
15-3-10	GGGTAGTGTGTGGTGTGTGGGTGTGGGTGTGGTGTGTGGG		
9-2-17	GGGCGAATCGGTGGGCCGGGACGGCTCTGACAGTTTCGGG		
13-13-2	GGGTTTTGCTATAATAGGGATGCCATAAGTGAGGGCAGGG		
17-10-1	GGGTTATGGAAGATTTTGATGGGTACAATACATGGGTGGG		
21-6-1	GGGCAGGAGTGTCATCTAACCAGAGGGTCGCCAGGGCGGG		
1-18-9	GGGAGGGTAATCTACTAAATTCTTAGGGTTATTGGTAGGG		
2-24-2	GGGTAGGGTCGAGGATCATTACGCTTTCTCGTGGGAAGGG		
8-15-5	GGGTGTGGTGTGGGTGTGGTGTGGTGTGTGGGTGTGTGGG		
45-nt			
10-8-15	GGGTGTGGTGTGTGGGTGTGGTGTGGGTGTGGTGTGGTGTGTGGG		
13-10-10	GGGTTGGATTAGAATAGGGTTAGAGTAGTGGGTGTGGTGTGTGGG		
10-12-11	GGGTGTGGTGTGTGGGTGTGGTGTGTGTGGGTGTGTGTGTGTGGG		
9-12-12	GGGTGATAAATTGGGTAGAATCCATATGGGCAAACAAGATTTGGG		
4-1-28	GGGAACTGGGTGGGCTTTCACAATGCTGGCCTTTATCGTCTTGGG		
1-8-24	GGGAGGGTTGGTAATGGGAAGATGGCTGATGTCGACGCCAAAGGG		
3-27-3	GGGTGTGGGTGTGGTGTGGTGTGGTGTGTGGTGTGTGGGTGTGGG		
25-2-6	GGGTCGTTGCATACGCATTTGTTCCAATGGGCTGGGTACTGAGGG		
12-3-18	GGGTGTGGTGTGTGTGGGTGTGGGTGTGGTGTGGTGTGGTGTGGG		
18-3-12	GGGAAATAAAACAAATTTTTAGGGCTAGGGCAATTGTTCCTTGGG		
23-8-2	GGGTAAGTTGAGAGACACGTTCATCAGGGTTAGAATAGGGTAGGG		
20-10-3	GGGTGTGGTGTGTGTGTGTGTGGGTGTGGTGTGTGGGTGTGGG		
50-nt			
13-10-15	GGGCTGCCATCTACGTGGGTAAGAATTCTGGGTTTGAAATCATTCAAGGG		
8-22-8	GGGTAATAGCAGGGTAATGGTAGTGGAGTTGGATATGGGTAATTGGAGGG		
7-23-8	GGGTAATGGAGGGTAAGTTGAGAGACAGGTTGGCCAGGGTTAGATTAGGG		
7-11-20	GGGATTGGCAGGGAAGAACTTCGAGGGAGGTGGTCGGAGAAGAGAGTGGG		
8-26-4	GGGTAAATTACGGGTAGGACTACATATACAGAGAACTTCTGGGACCTGGG		
5-32-1	GGGATTTTGGGATTTCGAGTGGCGTCCAACGGAAAAGCCGGTCGGGAGGG		
32-3-3	GGGCTGGTGCTGAGCGTGTGCTTGTGCTTGTGCTTGGGCTTGGGCTTGGG		
8-3-27	GGGTGTGGTGTGGGTGTGGGTGTGGTGTGGTGTGGTGTGTGGTGTGTGGG		
2-13-23	GGGAAGGGATGCTAAGGTAGAGGGTGAACGTTACAGAAAAGCAGGCTGGG		
23-2-13	GGGTTGATCTATGGTGTCAGTTTTGAGGGATGGGTTACGCTAGCTTTGGG		
19-3-16	GGGATGATATGAACATGGACATGGGTATGGGTCATGATCAGTCCGAAGGG		
16-2-20	GGGTGGATAATTCATTTGAGGGAAGGGAAACAATTCCCAGTGTAATTGGG		
55-nt			
19-12-12	GGGTGTTGGCCAGTGCTACCGCGGGCGGCGCTGCACAGGGAGTAAGCAGAGCGGG		
19-8-16	GGGTAATAAAGTGTACGTTACTGGGTCTTTTACGGGATGGAGAAAGATGATCGGG		
15-15-13	GGGTACTGAAGGTGCAGAGGGTACTGGAGGTGCAGAGGGTGCAGAAGGCACTGGG		
15-8-20	GGGTGGACGAGGAGTGGAGGGAGTTATTAGGGAAAATATGAGTTCCATCCACGGG		
39-1-3	GGGATCGTTTCAATTTTATAATGAGGTAGTGTACAGAGAGGAGGGAGGGAGTGGG		
1-17-25	GGGAGGGTATGATTAGCAATACCAGGGTCCATTATGGAAAAATTTTCGTACTGGG		
17-25-1	GGGAAGGCGGATGGCTACTTGGGTTGAGCATTACTCTCTTTGTGAAAAGGGAGGG		
23-19-1	GGGTATTGAAATGTATGAAGACCAGTGGGAGAAGATTGCTGACCACGTGGGTGGG		
19-21-3	GGGAAGTGATCAGCAGAGTTTCGGGTACCCTAATCAAAGCTTGGTCGGGTTTGGG		
26-15-2	GGGTGTGAGGCATTTCTAGAACGGATTTTGGGTGCCGTTTGTCACGTGGGACGGG		
12-30-1	GGGCTGTCCCCAAGTGGGTGATTTTCCATTTGATCCTTGTATCAGCTTGGGAGGG		
20-20-3	GGGATGGTGTGTTATCCAGGAATGGGCGGCACACATTGCCATAAACGGGACCGGG		
60-nt			
17-16-15	GGGTAACGAGTGGAGAGGTAGGGTAATGGAGAGTAAGTTGGGAGACAGGTAAAATCAGGG		
17-19-12	GGGAAGGTGGCAGCCCTATTGGGAACGCGCTCCTTCCCTTTTGGGATATTGTCAGTAGGG		
11-25-12	GGGCAGTAAAAAGAGGGCAATACGATGTGAACGCCAATAGATGGGATGACCATTTTCGGG		
23-1-24	GGGAATCTCCGGATTTACTTTAAAGTGGGAGGGAGAAGTTCCTTTCTTCTGATTTGCGGG		
35-1-12	GGGTGGTCGTACCTGCTAGTTCCATTGGTGCGGTGTATGGGCGGGTACCCTCAAAAAGGG		
29-3-16	GGGACATCCGTCAGCGCTAGCTGAGGCCATCCGGGATAGGGACTGGATCAGATCTCTGGG		
13-32-3	GGGTAAATACTATCCTGGGACCATCCAAGGACAATATGATCAAACTGGTGAGGGTAAGGG		
27-18-3	GGGAATCATAGGCAATGTGTACAAACAAGAGGGTTTTAGAAGCTTGTTTAAGGGTTTGGG		
1-27-20	GGGTGGGTTTCGTCTTCTGTTCACCATGAATGACGGGCACAAGTGAAACATGGATCAGGG		
11-36-1	GGGCAGAATTAGACGGGCCCAGTACGGTAGAAAGAAACAAGGCTTTACGTTCTGGGCGGG		
7-40-1	GGGTAGGCGTGGGCAAATCTCCGATGTTCTCATAATTTTGAGGATGTTCTAACGGGAGGG		
4-43-1	GGGAGCCGGGACCACTACCGATAAGTGGCGGTAATATTGGATTTGGCGTCTCCGGGCGGG		

Table S3. Melting temperatures (°C) derived from fluorescence melting curves for oligonucleotides.

	3G4	**4G4**	**5G4**	**1-1-1**	**1-2-1**	**1-9-1**	**2-2-2**	**2-2-1**	**3-5-10**	**3-10-10**	**10-8-10**	**32-3-3**
50 mM NaCl	41.5	43.3	57.6	60.5	49.6	38.2	41.6	43.4	--[a]	--[a]	--[a]	--[a]
100 mM NaCl	47.5	50.7	63.1	66.2	55.9	41.6	43.8	47.3	--[a]	--[a]	--[a]	--[a]
50 mM KCl	54.3	76.2	>95	>95	>95	70.8	59.3	74.6	41.4	38.4	39.2	35.3
100 mM KCl	60.1	83.5	>95	>95	>95	76.8	65.8	79.7	50.6	46.8	46.8	40.6

[a] not determined.

Table S4. Apparent dissociation constant K_D (nM) determined by DNA binding assay with Pif1.

	s_{26}3G4 d_{17}	**s_{26}4G4 d_{17}**	**s_{26}5G4 d_{17}**	**s_{26}1-1-1 d_{17}**	**s_{26}1-2-1 d_{17}**	**s_{26}1-9-1 d_{17}**	**s_{26}2-2-2 d_{17}**	**s_{26}2-2-1 d_{17}**	**s_{26}3-5-10 d_{17}**	**s_{26}3-10-10 d_{17}**	**s_{26}10-8-10 d_{17}**	**s_{26}32-3-3 d_{17}**
50 mM NaCl	21.0	22.3	18.6	21.0	18.3	18.9	19.7	20.8	--[a]	--[a]	--[a]	--[a]
100 mM NaCl	17.2	18.1	14.3	18.6	13.6	15.0	16.7	14.2	--[a]	--[a]	--[a]	--[a]
50 mM KCl	21.1	16.6	16.7	19.5	21.2	19.8	15.1	17.5	14.9	16.6	16.0	16.1
100 mM KCl	17.8	15.8	12.7	13.9	19.8	18.1	18.8	14.9	17.4	18.8	16.0	19.2

[a] not determined.

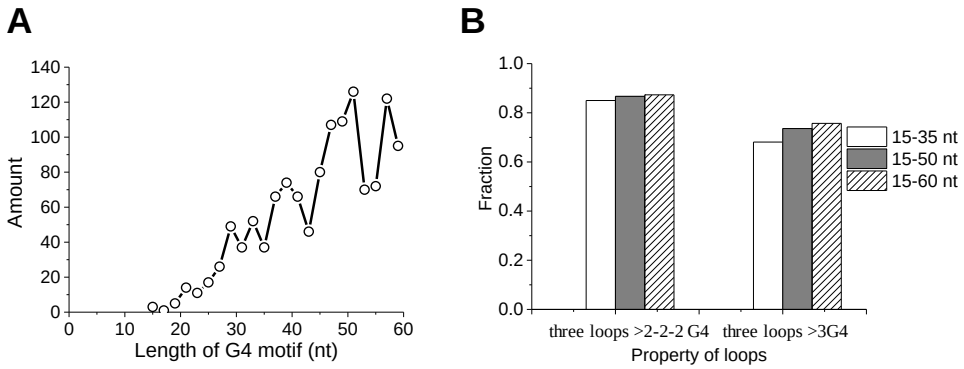

Figure S1. G4 motifs in *S. cerevisiae* genome according to the bioinformatics prediction by Hershman *et al*. (1). (A) Distribution of the amount of G4 motifs with different sequence lengths. Bin size was 2. (B) Fractions of G4 motifs with different properties of loops. The lower limit of sequence length for a G4 motif is 15-nt. The upper limit of sequence length was set as 35-, 50-, and 60-nt respectively.

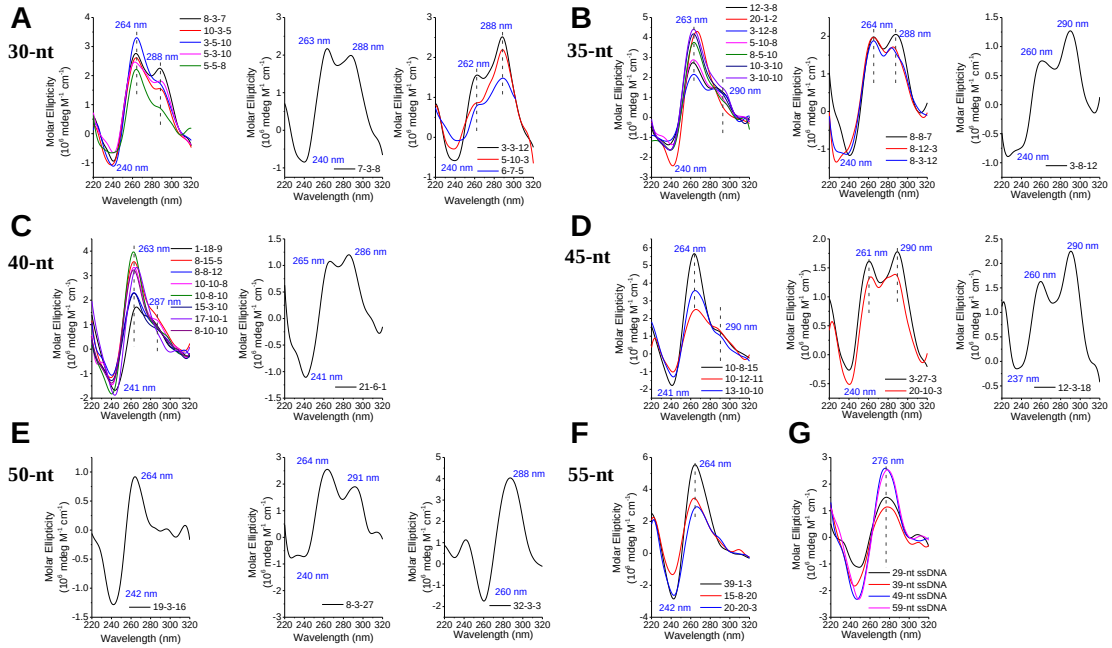

Figure S2. CD spectra of G4 structures in *S. cerevisiae* sequences in 150 mM KCl. The sequences were listed in Table S2. Majority of the folded structures are a mixture of parallel and antiparallel conformations, and they are categorized into three types including mainly parallel, a mixture with similar fractions and mainly antiparallel. (A) All nine 30-nt G4 motifs fold into G4 structures. (B) Eleven 35-nt G4 motifs out of fourteen randomly selected sequences fold into G4 structures. (C) Nine 40-nt G4 motifs out of twelve randomly selected sequences fold into G4 structures. (D) Six 45-nt G4 motifs out of twelve randomly selected sequences fold into G4 structures. (E) Three 50-nt G4 motifs out of twelve randomly selected sequences fold into G4 structures. (F) Three 55-nt G4 motifs out of twelve randomly selected sequences fold into G4 structures. (G) ssDNA without the G4 motif demonstrate the distinct CD spectra. The sequences for 29-, 39-, 49- and 59-nt ssDNA are GCGTGGCACCGGTAATAGGAAATAGGAGA$(T)_{0,10,20,30}$ respectively.

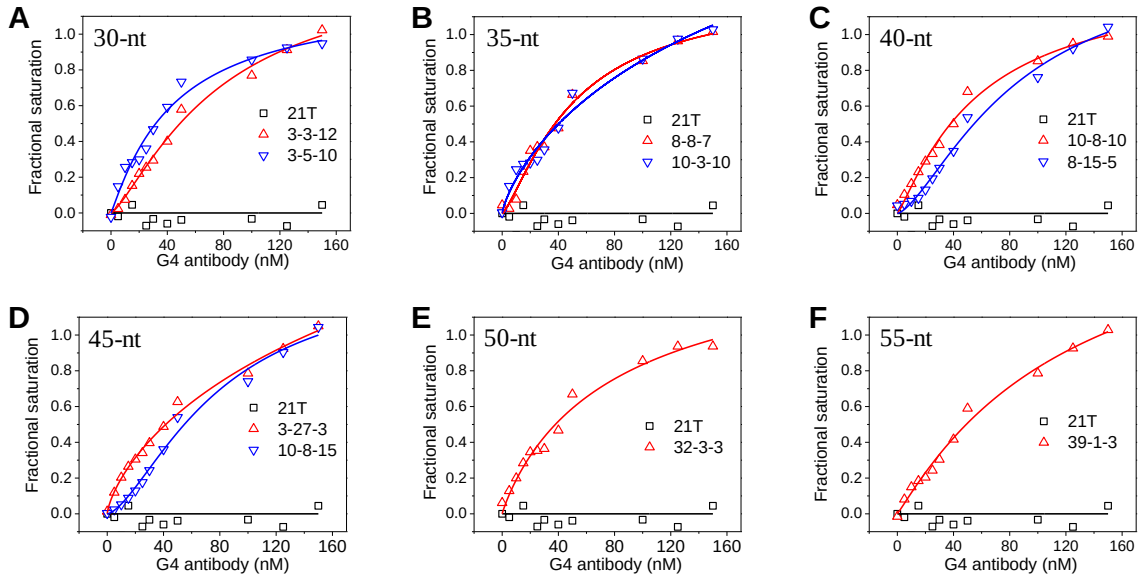

Figure S3. Detection of G4 structures by a G4 antibody. The antibody does not bind to ssDNA 21T with the sequence of TTTTTTTTTTTTTTTTTTTTT-FAM. By contrast, the antibody is able to bind to G4 structures with the sequence lengths of 30-nt (A), 35-nt (B), 40-nt (C), 45-nt (D), 50-nt (E), and 55-nt (F) from Table S2. The result for 21T has been reused in each panel to display the difference between G4 structures and ssDNA more clearly. Those sequences were all labelled with FAM at the 3′ end. The G4 structures were prepared in 150 mM KCl by incubating the solution at 95°C for 5 min, followed by slow cooling to room temperature in about 7 hours. The binding curve was fitted by Hill equation $y = [\text{antibody}]^n / (K_D^n + [\text{antibody}]^n)$, where y is binding fraction, n is Hill coefficient, and K_D is the apparent dissociation constant.

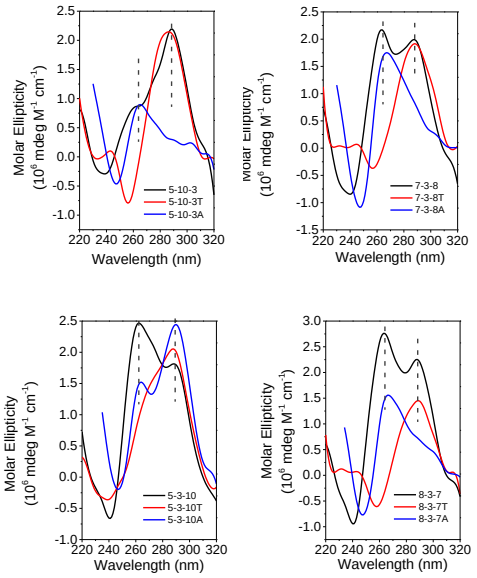

Figure S4. G4 motifs with the same loop lengths but different loop sequences fold into G4 structures with different conformations. The loops in 30-nt G4 motifs including 5-10-3, 7-3-8, 5-3-10 and 8-3-7 in Table S2 were all replaced by T or A. Therefore, they were named as 5-10-3T/5-10-3A, 7-3-8T/7-3-8A, 5-3-10T/5-3-10A and 8-3-7T/8-3-7A. The CD spectra were recorded in 150 mM KCl.

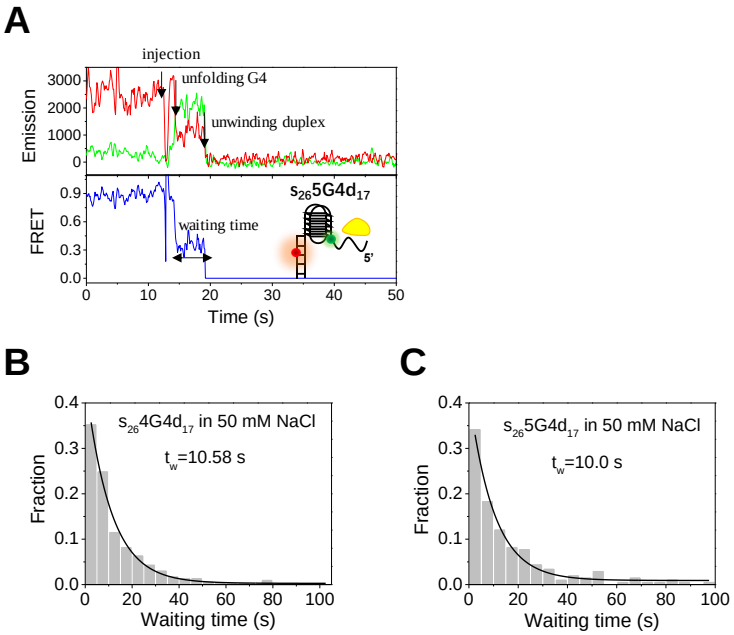

Figure S5. Pif1-catalysed downstream duplex DNA unwinding at 80 nM Pif1 and 2 mM ATP. (A) Representative fluorescence emission and FRET traces for Pif1-catalysed unwinding of $s_{26}5G4d_{17}$ in 50 mM NaCl. (B-C) Distributions of the waiting time for $s_{26}4G4d_{17}$ and $s_{26}5G4d_{17}$. The waiting time was measured manually from each trace, and more than 300 traces were included. Single-exponential decay was used to fit those histograms, generating a time constant t_w.

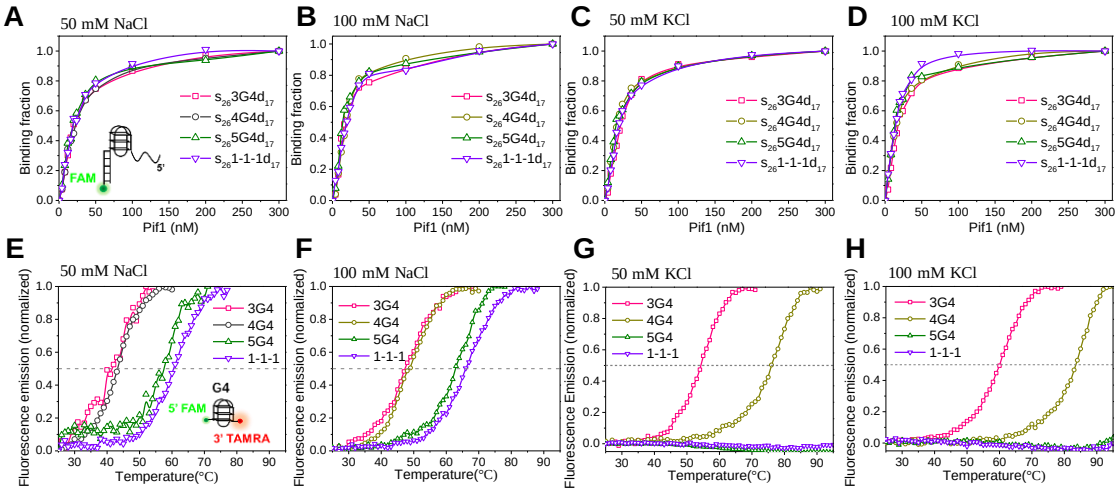

Figure S6. Determination of Pif1 binding affinities and thermal stabilities of different G4 structures. (A-D) Binding fractions of Pif1 towards different DNA substrates in 50-100 mM NaCl or KCl determined by fluorescence polarization assay. The binding curve was fitted by Hill equation $y = [Pif1]^n / (K_D^n+[Pif1]^n)$, where y is binding fraction, n is Hill coefficient, and K_D is the apparent dissociation constant. (E-H) Normalized melting curves from FRET melting assays for different G4 substrates. 5G4 and 1-1-1 are too stable in both 50 and 100 mM KCl, therefore no melting can be observed between 25-95°C, indicating their T_m values are above 95°C. In fact, the FAM emission itself will slightly decrease with the increase of temperature. Therefore, the net increase of FAM intensity induced by G4 melting will be more than that we detected, and the actual T_m value will be a little higher than that in Table S3.

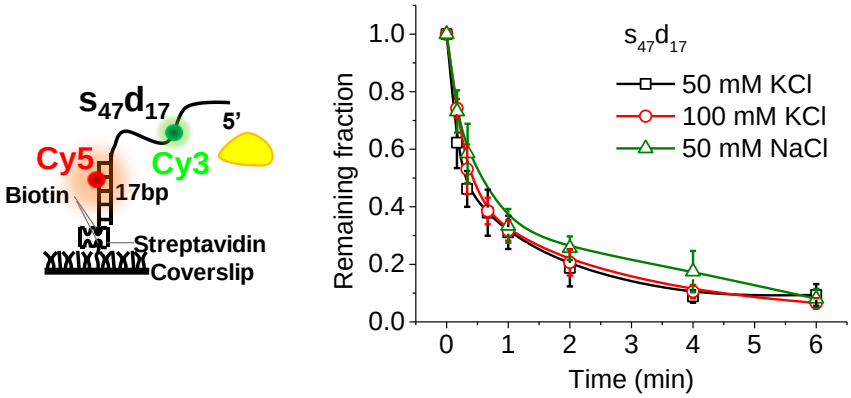

Figure S7. Pif1-catalysed unwinding of the partial duplex DNA $s_{47}d_{17}$ at 80 nM Pif1 and 2 mM ATP. Fractions of remaining DNA molecules on coverslip *versus* time in 50 mM NaCl, 50 mM KCl and 100 mM KCl. All lines are the simple connections of the individual data points by Origin 8.0. These results suggest that Pif1 unwinding activity is similar in all those buffer conditions.

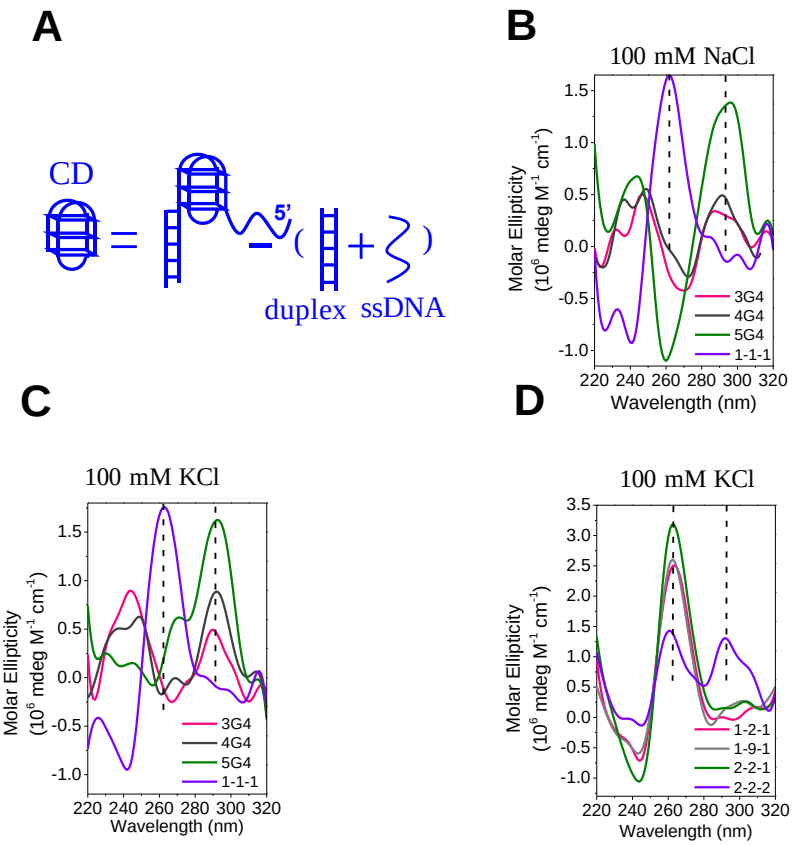

Figure S8. Folding conformation of different G4 structures with the existence of proximal duplex and ssDNA. (A) Schematic representation of the experiment design. CD spectrum of G4 with duplex and ssDNA overhangs were measured instead of the bare G4, as the presence of proximal DNA may influence of G4 folding significantly. CD spectrum of pure G4 was obtained by separating the contributions of the adjacent duplex and ssDNA. (B) CD spectra of different G4 structures in 100 mM NaCl. (C) CD spectra of different G4 structures in 100 mM KCl. (D) CD spectra of G4 structures with short loops in 100 mM KCl. 2-2-2 folds into both parallel and antiparallel forms, while all other G4 sequences adopt only parallel conformation.

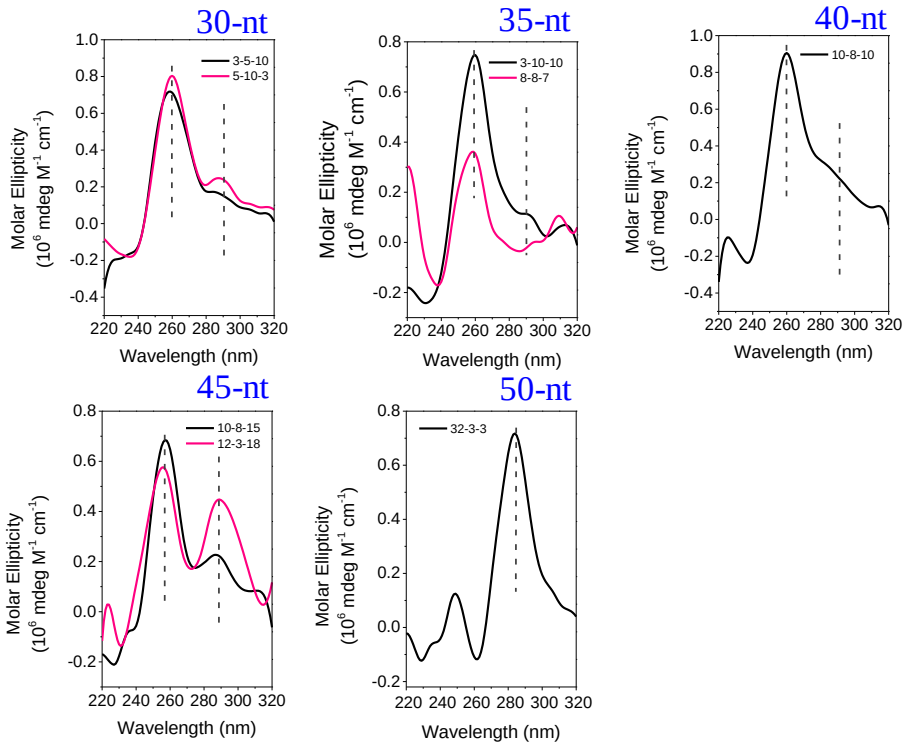

Figure S9. Formation of G4 structures with long DNA sequences from 30- to 50-nt in 100 mM KCl. Those G4 motifs have a 26-nt ssDNA at 5′ end and a 17-bp dsDNA at 3′ end.

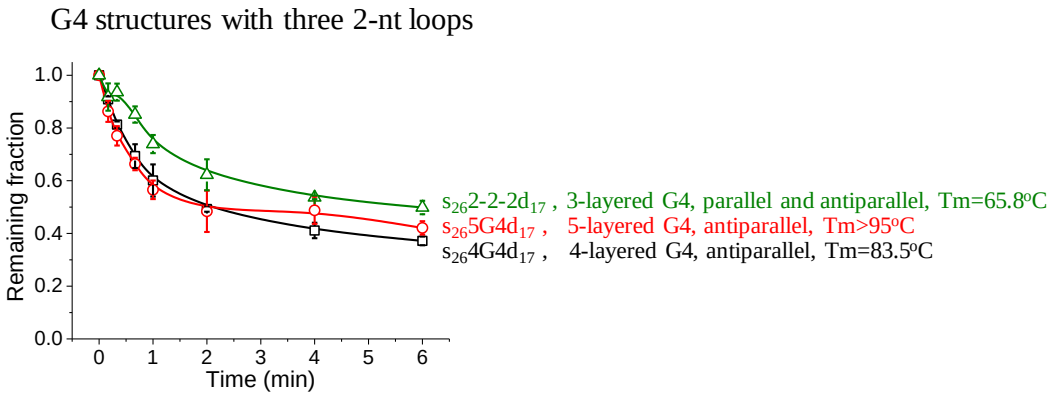

Figure S10. Fractions of the remaining DNA molecules on coverslip *versus* time for substrates with 3-, 4- and 5-layered G4s containing the same loops in 100 mM KCl. All lines are the simple connections of the individual data points by Origin 8.0.

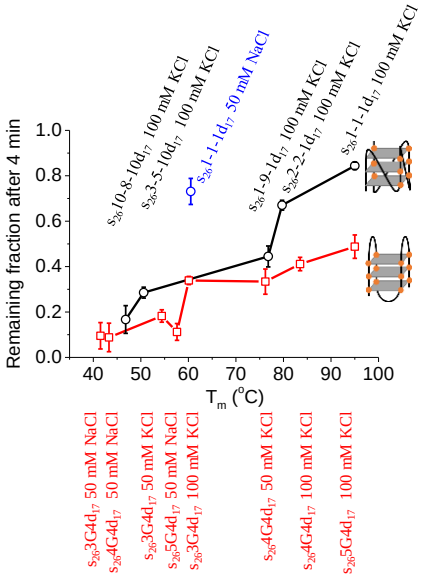

Figure S11. The response of Pif1 towards the stability change of antiparallel G4s and parallel G4s. All data points were obtained from Figure 3-6 in the main text. Fractions of the remaining DNA molecules with upstream antiparallel G4s are in red color. Fractions of the remaining DNA molecules with upstream parallel G4s are in black or blue color.

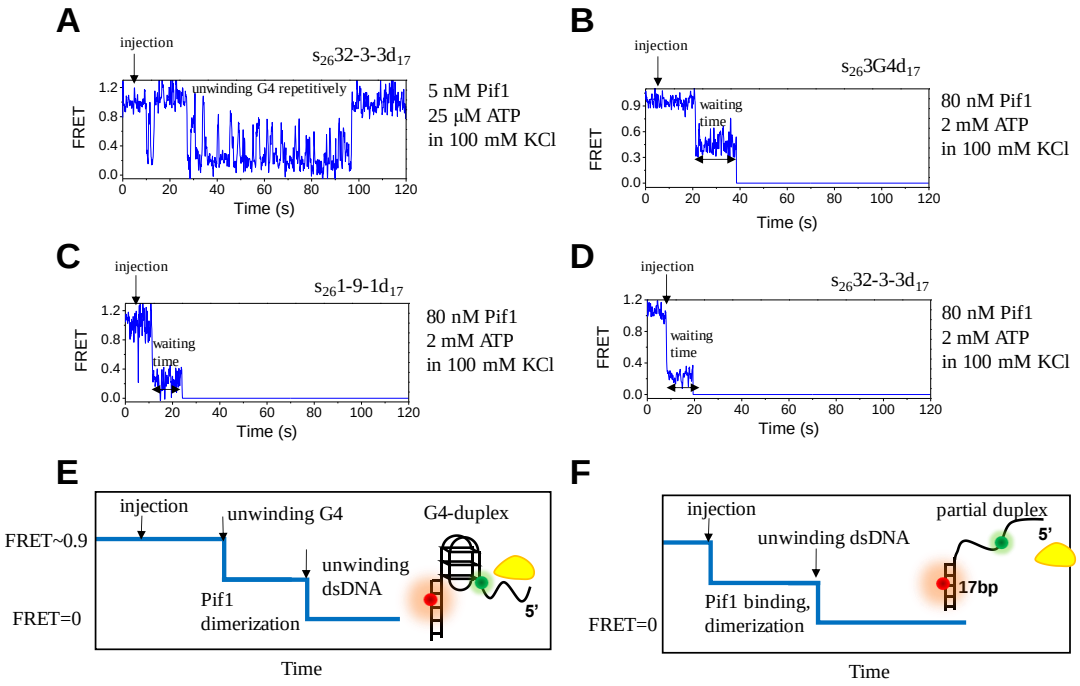

Figure S12. Pif1-catalysed unfolding of different G4 structures. (A) At low protein concentration, Pif1 unfolds the G4 structure with long loops repetitively. (B-D) Representative FRET traces for the unwinding of $s_{26}3G4d_{17}$, $s_{26}1\text{-}9\text{-}1d_{17}$, and $s_{26}32\text{-}3\text{-}3d_{17}$ at 80 nM Pif1 and 2 mM ATP in 100 mM KCl. After protein addition, FRET signal decreases, then keeps at a constant value until duplex unwinding. (E) Schematic representation of the FRET trace to show the unwinding a G4-duplex. Pif1 unfolds the G4 structure at some time later or immediately after Pif1 addition; afterwards, Pif1 undergoes dimerization at the ss/dsDNA junction until the duplex is separated. (F) For a partial duplex DNA without a G4 structure, Pif1 binds to the ssDNA rapidly, and then undergoes dimerization until the duplex is separated (2). The G-rich sequence from the unfolded G4 structure can accelerate the dimerization of Pif1, compared with the poly-T sequence in the partial duplex (2). However, if Pif1 takes a long time to unfold the G4 structure in the beginning (for example, 3G4 in 100 mM KCl), this G4 may exist as an impediment for the further unwinding of the downstream duplex.

Reference:

(1) Hershman, S. G., Chen, Q., Lee, J. Y., Kozak, M. L., Yue, P., Wang, L. S., and Johnson, F. B. (2008) Genomic distribution and functional analyses of potential G-quadruplex-forming sequences in Saccharomyces cerevisiae. *Nucleic acids research* **36**, 144-156

(2) Zhang, B., Wu, W. Q., Liu, N. N., Duan, X. L., Li, M., Dou, S. X., Hou, X. M., and Xi, X. G. (2016) G-quadruplex and G-rich sequence stimulate Pif1p-catalyzed downstream duplex DNA unwinding through reducing waiting time at ss/dsDNA junction. *Nucleic acids research* **44**, 8385-8394