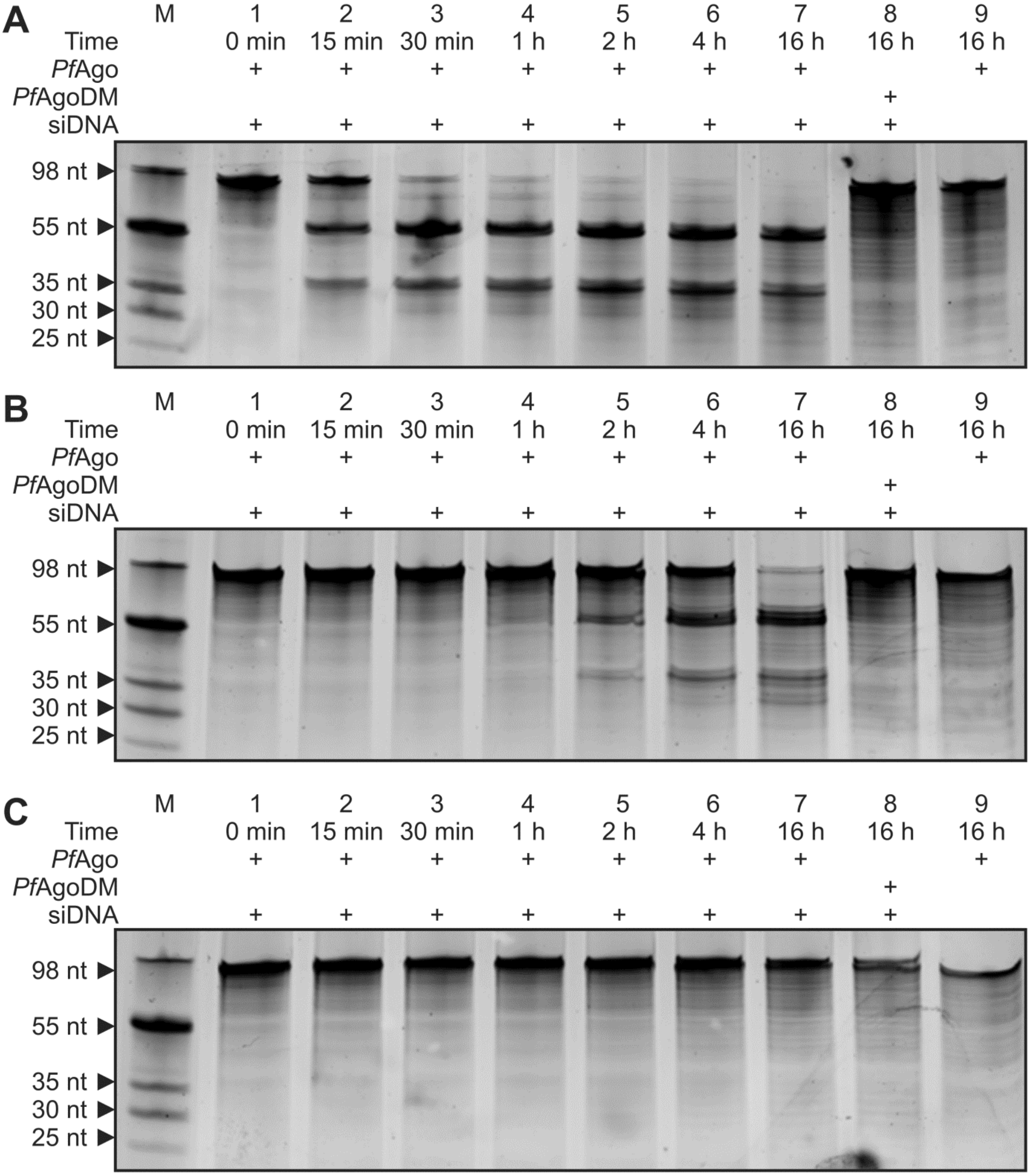
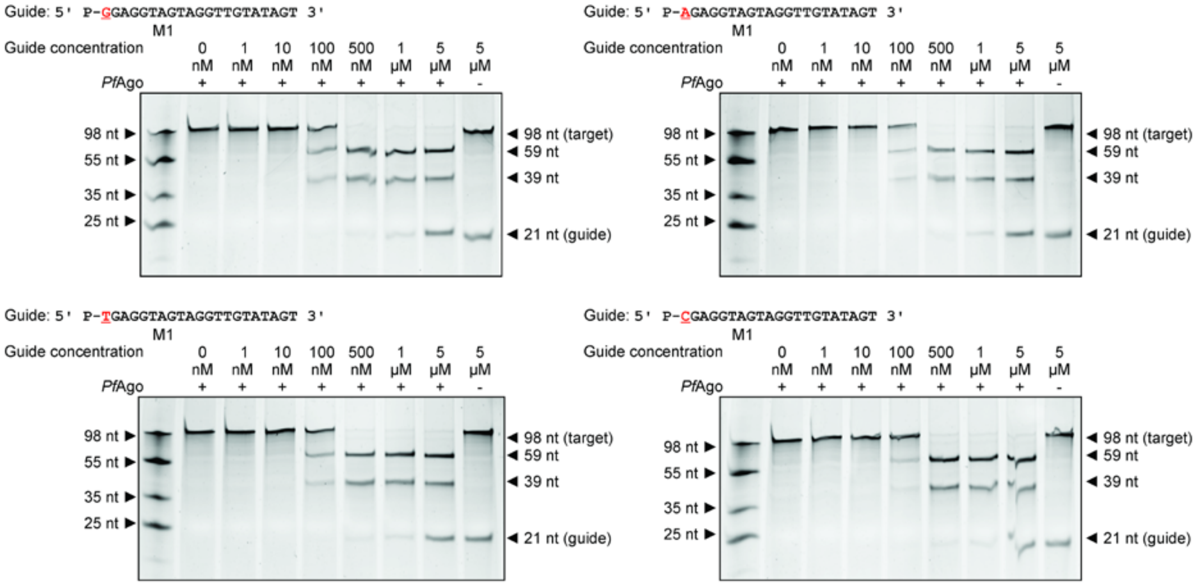
SUPPLEMENTARY DATA

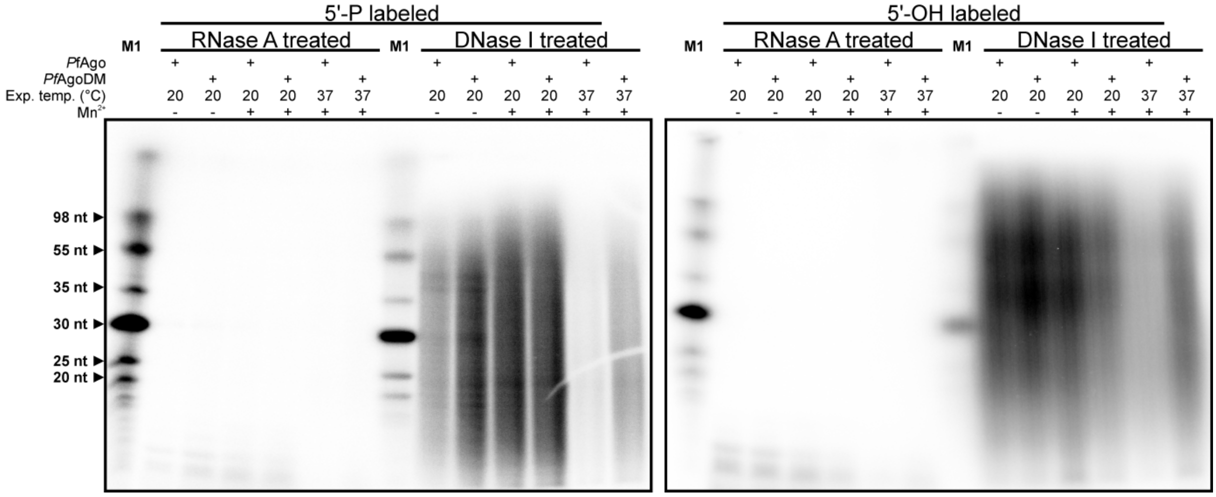


Supplementary Figure S1.
*Pf*Ago-siDNA complexes show multi-turnover activity. *Pf*Ago, siDNAs and target ssDNAs (Fig. 2C) were incubated in a 2.5:1:20 ratio (*Pf*Ago:siDNA:target) and incubated at 95 °C in buffer with (**A**) 250 mM NaCl, (**B**) 500 mM NaCl or (**C**) 1M NaCl. Nucleic acids are resolved on denaturing polyacrylamide gels. siDNA concentrations are below detection limits. M: ssDNA marker. nt: nucleotide.



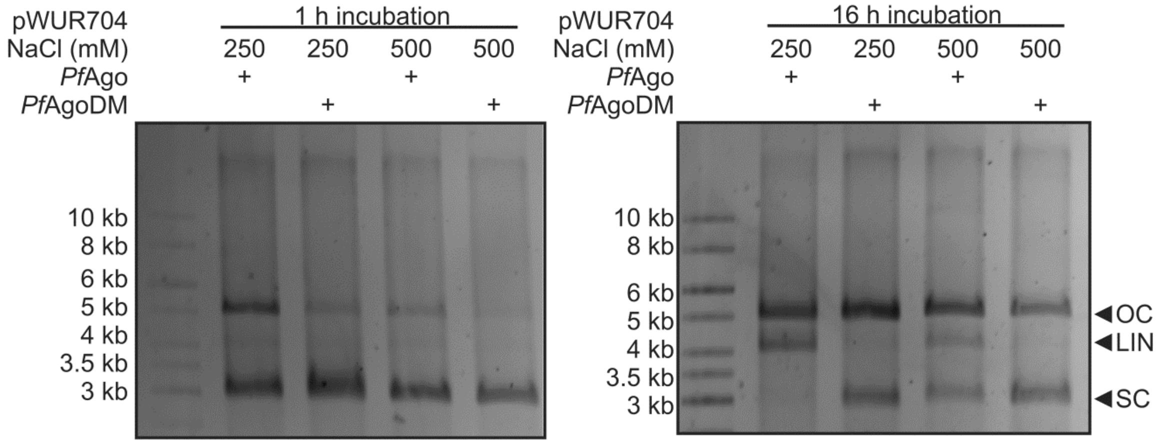
Supplementary Figure S2.

Effect of the variation of the 5’-end deoxynucleoside of the siDNA on *Pf*Ago cleavage efficiency. Cleavage of 98 nucleotide ssDNA targets by *Pf*Ago loaded with complementary siDNAs containing a different 5’-end deoxynucleoside, as shown in red above each gel. The concentrations of each siDNA were varied (indicated on top of the gels). Products of the reactions were resolved on denaturing polyacrylamide gels. M1: ssDNA marker. Samples without *Pf*Ago (indicated with ‘-‘) contain *Pf*AgoDM as control.



Supplementary Figure S3.

Nucleic acids co-purifying with *Pf*Ago and *Pf*AgoDM. Co-purified nucleic acids are 5’ phosphorylated in a T4 PNK exchange reaction (left panel; 5’-P groups, and to a lesser extend 5’-OH groups are labeled) or in a T4 PNK forward reaction (right panel; 5’-OH groups, and to a lesser extend 5’-P groups are labeled) using [γ-^{32}p] ATP and resolved on 20% denaturing polyacrylamide gels. Nucleic acids were not treated, RNase A treated or DNase I treated. M1: ssDNA marker, labeled in a T4 PNK forward reaction. Exp. temp: Expression temperature. Mn^{2+}: indicates whether the protein was purified in absence (-) or presence (+) of Mn^{2+}.



Supplementary Figure S4.

Guide-free *Pf*Ago activity on pWUR704. pWUR704 was incubated with *Pf*Ago and *Pf*AgoDM for 1 h or 16 h at 75°C at different final NaCl concentrations, and resolved on 0.75% agarose gels. Left lane: 1 kb generuler (Thermo Scientific). OC: open circular. LIN: linear. SC: Supercoiled.

Table S1: Strains

Strain	Abbreviations	Description	Source, reference
***Pyrococcus furiosus* JFW02**	Pfu, wild type	*Pyrococcus furiosus* JFW02	(17)
Pyrococcus furiosus* Δ*ago	PfuΔ*ago*, knockout	*Pyrococcus furiosus ago* gene (PF0537) knockout	This chapter
***Pyrococcus furiosus ago* overexpression strain**	Pfu-*ago*-O/E, overexpression strain	*Pyrococcus furiosus Pf*Ago overexpressing strain	This chapter
***Escherichia coli* KRX**	*E. coli* KRX	*E. coli* expression strain	Promega

Table S2: Plasmids

Plasmid	Description	Restriction sites used	Primers	Source, reference
pHSG298	*Pf*Ago overexpression cassette genome insertion vector		PF1223_1224_1-8 PfAgo_fow PfAgo_rev	This study
pYS3	*E. coli*/*P. furiosus* shuttle vector, Amp^R, pyrF under control of gdh promoter	BamHI	Pgdh_pyrF_fow Pgdh_pyrF_rev	(20)
pJFW18	*E. coli*/*P. furiosus* shuttle vector, Apr^R, pyrF under control of gdh promoter		-	(19)
pCDF-1b	Expression vector		-	Novagen
pWUR790	Synthetic codon optimized *P. furiosus ago* with N-terminal *strep(II)-tag* in pCDF-1b vector, expression vector for *Pf*Ago.	NcoI, AvrII	-	GenScript USA Inc.
pWUR791	pWUR790, *ago* active site residue substituted (D558A)		BG5480 BG5481	This study
pWUR792	pWUR790, *ago* active site residue substituted (E592A)		BG5482 BG5483	This study
pWUR793	pWUR790, *ago* active site residue substituted (E596A)		BG5484 BG5485	This study
pWUR794	pWUR790, *ago* active site residue substituted (D628A)		BG5486 BG5487	This study
pWUR795	pWUR790, *ago* active site residue substituted (H745A)		BG5488 BG5489	This study
pWUR796	pWUR791, two *ago* active site residue substituted (D558A,D628A)		BG5486 BG5487	This study
pWUR704	Target plasmid		-	(11)

Table S3: Oligonucleotides

Experiment	Primers	Sequence (5’-3’)	Description, restriction sites
Pfu-*ago*-O/E strain	PF1223_1224_1	CCAGTGCCAAGCTTGCATGCACCGGTGCGGCCGCACGCGTTTAAACGGTTCTCAAGCGTATTTTTGG	PF1223 (FW)
	PF1223_1224_2	CTCAGCTCACTCCATTTTCAATCATCCATCCACTGAGAATATTGAAG	PF1233 (RV)
	Pgdh_PyrF_F	GATTGAAAATGGAGTGAGCTGAG	trpAB (FW)
	TrpAB_Csg_4	TAATTCGCCTTTTGCCGATAGTCGATTGGCTGAGCTCATG	trpAB (RV)
	Ptk_csg_fow	TATCGGCAAAAGGCGAATTATG	csg promoter (FW)
	Ptk_csg_rev	GAGGAAGCGGAGGTTCCA	csg promoter (RV)
	PF1223_1224_7	TGGAACCTCCGCTTCCTCTTCTTCTCTACTAGATCCGTTATC	PF1224 (FW)
	PF1223_1224_8	TATGACATGATTACGAATTCTACGTATCCGGATTAATTAATTTAAATGGTTAGATAAGAATCCGCTGAA	PF1224 (RV)
	PfAgo_fow	CAACCCAAGGAGGTGTTGTCATATGAAAGCGAAAGTTGTTATTAATCT	*ago* (FW)
	PfAgo_rev	AAAGAGGAGAAGAGAGGGGGGATCCTCAAACAAAATACAAAAATCCCTCA	*ago* (FW)
PfuΔ*ago*	PfAgo_del_1	TCTCTTCTGGCCGTTGATCT	Upstream region ago (FW)
	PfAgo_del_2	CTCAGCTCACTCCATTTTCAATCCATTAACTTTTTCTATTTAAACAATTTCTAACCT	Upstream region ago (RV)
	PfAgo_del_5	GGTGAAAGAATGGAGCTCAAGATAATTGAGGAATTTAGTTCTAGTTCTCAGG	Upstream region ago (FW)
	PfAgo_del_6	CATTAACTTTTTCTATTTAAACAATTTCTAACCT	Upstream region ago (RV)
	PfAgo_del_7	AGGTTAGAAATTGTTTAAATAGAAAAAGTTAATGATTATCACCAAGTGATTACAATTAATATCA	Downstream region ago (FW)
	PfAgo_del_8	TGTTCATCAACAGGGAGGAA	Downstream region ago (RV)
	Pgdh_PyrF_F	GATTGAAAATGGAGTGAGCTGAG	Pgdh pyrF cassette (FW)
	Pgdh_PyrF_R	TTATCTTGAGCTCCATTCTTTCACC	Pgdh pyrF cassette (RV)
pYS3	Pgdh_pyrF_fow	AGCTTCTCTGCAGGATATCTGGATCCGATTGAAAATGGAGTGAGCTG	Pgdh pyrF cassette (FW)
	Pgdh_pyrF_rev	GCCGAAGCTAGCGAATTCGTGGATCCGTCGATTGGCTGAGCTCATG	Pgdh pyrF cassette (RV)
Site directed mutagenesis of *ago* gene	BG5480	GATTATATCATTGGCATTGCTGTGGCACCGATGAAACG	*P. furiosus ago* D558A (FW)
	BG5481	CGTTTCATCGGTGCCACAGCAATGCCAATGATATAATC	*P. furiosus ago* D558A (RV)
	BG5482	CCGATTAAAATCGGTGCACAGCGTGGTGAAAGCG	*P. furiosus ago* E592A (FW)
	BG5483	CGCTTTCACCACGCTGTGCACCGATTTTAATCGG	*P. furiosus ago* E592A (RV)
	BG5484	GGTGAACAGCGTGGTGCAAGCGTTGATATGAACG	*P. furiosus ago* E596A (FW)
	BG5485	CGTTCATATCAACGCTTGCACCACGCTGTTCACC	*P. furiosus ago* E596A (RV)
	BG5486	CCTGCTGCTGCGTGCTGGTCGCATTACCAATAATG	*P. furiosus ago* D628A (FW)
	BG5487	CATTATTGGTAATGCGACCAGCACGCAGCAGCAGG	*P. furiosus ago* D628A (RV)
	BG5488	CCTGCACCGGTTCATTATGCAGCTAAATTTGCCAATGCCATTCG	*P. furiosus ago* H745A (FW)
	BG5489	CGAATGGCATTGGCAAATTTAGCTGCATAATGAACCGGTGCAGG	*P. furiosus ago* H745A (RV)
siDNA and siRNA sequences	BG3466	P-TGAGGTAGTAGGTTGTATAGT	21 nt DNA guide (FW), based on let-7 miRNA
	BG4017	P-TTATACAACCTACTACCTCGT	DNA guide (RV), based on reverse complement of let-7 miRNA
	BG4500	P-AGAGGTAGTAGGTTGTATAGT	DNA guide (FW), based on let-7 miRNA, 5’-end deoxyadenosine
	BG4501	P-GGAGGTAGTAGGTTGTATAGT	DNA guide (FW), based on let-7 miRNA, 5’-end deoxyguanosine
	BG4502	P-CGAGGTAGTAGGTTGTATAGT	DNA guide (FW), based on let-7 miRNA, 5’-end deoxycytidine
	BG4503	P-TGAGGTAGTAGGTTGTATAGT	DNA guide (FW), based on let-7 miRNA, 5’-end deoxythymidine
	BG4508	P-UGAGGUAGUAGGUUGUAUAGU	RNA guide (FW), based on let-7 miRNA
	BG5599	P-TGAGGTAG	8 nt DNA guide based on BG3466
	BG5600	P-TGAGGTAGT	9 nt DNA guide based on BG3466
	BG5601	P-TGAGGTAGTA	10 nt DNA guide based on BG3466
	BG5602	P-TGAGGTAGTAG	11 nt DNA guide based on BG3466
	BG5641	P-TGAGGTAGTAGG	12 nt DNA guide based on BG3466
	BG5713	P-TGAGGTAGTAGGT	13 nt DNA guide based on BG3466
	BG5714	P-TGAGGTAGTAGGTT	14 nt DNA guide based on BG3466
	BG5713	P-TGAGGTAGTAGGTTG	15 nt DNA guide based on BG3466
	BG5640	P-TGAGGTAGTAGGTTGT	16 nt DNA guide based on BG3466
	BG5603	P-TGAGGTAGTAGGTTGTATAGTATATTAAATT	31 nt DNA guide based on BG3466
Target sequences	BG4263	TCGACTTTATATTTAAATAATTTAATATACTATACAACCTACTACCTCGTATAAATTTTTAAATAAATATTGCATTCAAGCTTTTAATTTAATTAAAT	98 nt RV ssDNA target
	BG3678	AAACGACGGCCAGTGCCAAGCTTACTATACAACCTACTACCTCAT	45 nt RV ssDNA target
	BG4427	AAACGACGGCCAGUGCCAAGCUUACUAUACAACCUACUACCUCAU	45 nt RV ssRNA target