

COMMUNICATION

Construction of optically controllable CRISPR-Cas9 system using a DNA origami nanostructure

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We demonstrated the photo-controlled Cas9-mediated dsDNA cleavage using a DNA origami structure. Cas9 was incorporated inside the nanoring to prevent the reaction. The release of the Cas9 by photoirradiation regenerated the activity for site-selective cleavage of dsDNA.

Artificial control of biomolecular functions is one of the key technologies for the regulation of specific biological system such as gene expression and signal transduction in living system. For this purpose, the integrated system to assemble biomolecules responsive to the signals should be created. DNA molecules are practically used for building up designed nanostructures and arranging various functional molecules in the nanoscale precision for expression of the higher-ordered functions.^{1–3} DNA nanotechnology using DNA origami has been growing rapidly because designed nanostructures can be constructed in a programmed fashion, and various applied research has been conducted.² By using these properties, it is possible to arrange biologically relevant molecules and substrates at desired positions and dynamically control the activity of biomolecules using 3D dynamic nanostructures.^{4–6} In addition, a number of researches have been conducted to introduce them into cells applicable for drug delivery systems and cellular control.⁷ DNA origami also has been widely used as a carrier for intracellular transport,^{7–9} regulation of cellular signals,⁴ and application to the living body.¹² Further, the optical control of the activity of biomolecules has been emerging due to its temporal and spatial control and permeability.^{6,13,14} Using DNA origami system, the activity of the enzyme caged in a nanospace should be silenced and the release of the enzyme could initiate the reaction.

We employed a DNA nanostructure and photoreaction to control the sequence-selective DNA cleavage using CRISPR-Cas9 system. The Cas9 protein with a single guide RNA (sgRNA) has evolved into a powerful tool for sequence-selective

genome editing.^{15–16} Cas9 is an endonuclease associated with the prokaryotic adaptive immune system CRISPR (clustered regularly interspaced palindromic repeats). The sgRNA directs a sequence-selective recognition of the target sequence which contains a protospacer adjacent motif (PAM), and Cas9 subsequently cleaves the target site of the dsDNA.¹⁶

In this study, we created a photoresponsive DNA nanodevice that can trap and release Cas9 in a DNA origami cavity. We introduced Cas9 into the cavity of the DNA origami nanoring using a photocleavable (PC) linker, and released the Cas9 by UV irradiation. We examined the photo-induced expression of the Cas9 activity for the substrate dsDNA.

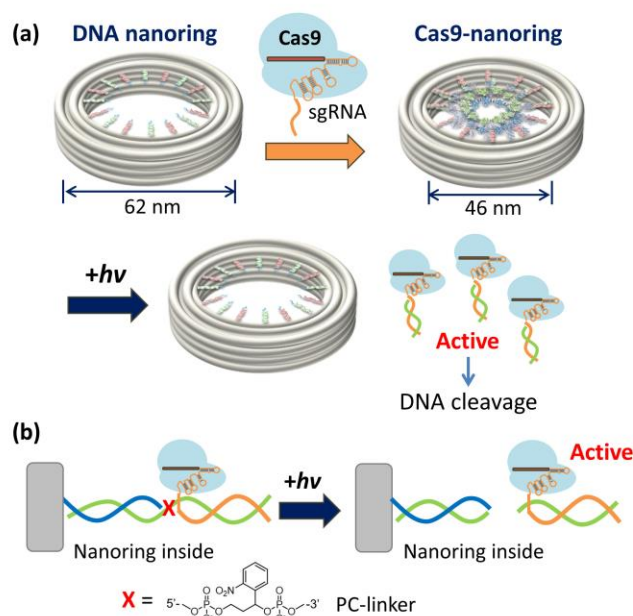


Figure 1. Schematic illustration of a DNA nanoring that releases target molecules in response to photoirradiation. (a) Anchoring strands were incorporated inside the nanoring to assemble Cas9 via hybridization. (b) Using a photocleavable (PC) linker, Cas9 incorporated inside the nanoring can be released to be activated for the sequence-specific DNA cleavage.

A ring-shaped DNA origami structure was employed in this research to incorporate multiple Cas9 (Figure 1 and S1).^{17–19}

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Biomolecules such as proteins can be incorporated inside and functional molecules such as fluorescent dyes and ligands can be introduced outside of the nanoring. The nanoring was assembled using p8046 single-stranded DNA (ssDNA) scaffold and staple strands by optimizing stepwise annealing. The assembled ring-shaped structure was observed by atomic force microscopy (AFM) (Figure 2a).

Next, Cas9 was incorporated into the nanoring. Cas9 can recognize and cleave dsDNA in a sequence-specific manner.¹⁶ Single guide RNA (sgRNA) was prepared to target specific dsDNA. An RNA linker was added to the 3'-end of sgRNA to hybridize with complementary DNA strand to attach to the nanoring via anchoring strands (Figure 1b). After the formation of Cas9/sgRNA complex, the complex was incubated with the nanoring having anchoring strands and a photocleavable (PC) linker containing complementary strand to attach Cas9 inside the nanoring via hybridization. From the agarose gel electrophoresis, we observed a band slower migrated band compare with that of the initial nanoring (Figure 2b, lane 1 and 2).

We next purified the Cas9-attached nanoring to remove excess Cas9 in the solution. In the AFM image, we observed the Cas9 attached inside the nanoring and also observed excess Cas9 on the mica surface (Figure 2c). We performed the affinity purification using magnetic beads (Figure S2). The Cas9 attached nanoring was captured to the beads via hybridization. After the washing, the product was recovered by strand displacement. From the AFM image after the purification, excess Cas9 was removed, and the Cas9 attached inside the nanorings were clearly observed (Figure 2d). The number of the attached Cas-9 was varied, and we found maximum six Cas9 were attached inside the nanoring. These results indicate that the attachment of Cas9 and its purification were successfully achieved.

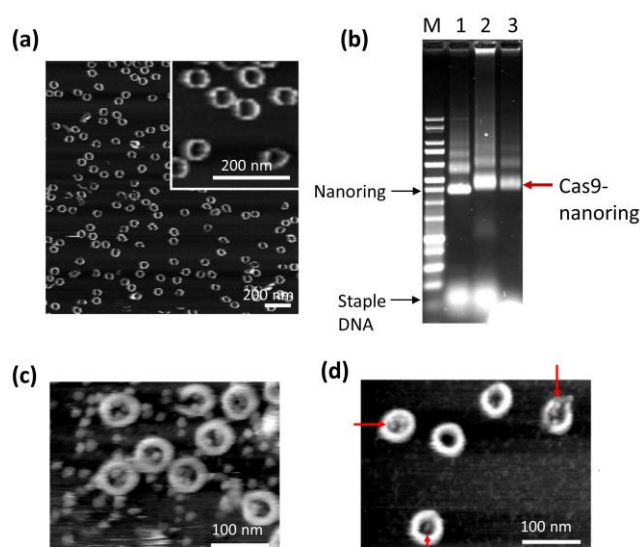


Figure 2. Construction of Cas-9 incorporated nanoring. (a) AFM image of DNA origami nanoring with anchoring strands to incorporate Cas9. (b) Incorporation of Cas9 into the nanoring via hybridization. Agarose gel electrophoresis image of Cas9 (lane1), Cas9-attached nanoring without purification (lane 2), and Cas9-nanoring after purification

(lane 3). (c) AFM image of reaction mixture of Cas9 and nanoring for attachment before purification. (d) AFM image of Cas9-nanoring after purification.

Finally, Cas9 was activated by releasing from the nanoring using photoirradiation. Cas9 incorporated in the nanoring was dissociated to check its activity. To dissociate Cas9, we used a PC-linker in the complementary strand which was used to introduce the enzyme (Figure 1b). Release of Cas9 by photoirradiation was confirmed by gel electrophoresis and AFM observation. UV-irradiation (350 nm, 25 °C, 10 min) caused the band shift of Cas9-nanoring to be similar migration position to the nanoring (Figure 3a, lane 3 and lane 4), indicating that Cas9 was dissociated from the nanoring. By comparing AFM images before and after photoirradiation, Cas9 inside the nanoring disappeared (Figure 3b).

We next examined the activity of released Cas9 after photoirradiation to the Cas9-nanoring. Specific site in the 850 bp DNA dsDNA encoding EGFP (enhanced green fluorescent protein) gene was used as a target substrate. Cas9 should recognize the target site and cleave it. By exposing UV light to a solution containing the Cas9-nanoring and the substrate dsDNA, the substrate should be cleaved into 650 bp and 200 bp fragments (Figure 3c). By using this system, the activity of released Cas9 was evaluated. Without photoirradiation, the cleaved band was not observed in the gel image (Figure 3c, lane 2), showing the activity of Cas9 was properly suppressed in the nanoring. After the photoirradiation, a band appeared at around 650 bp in 46% yield (Figure 3c, lane 3). The result indicated that the cleavage of the target dsDNA was induced by photoirradiation and subsequent release of Cas9. Therefore, it can be concluded that in this system, Cas9 was properly introduced into the nanoring, and did not deactivated during the purification, and Cas9 was released from the nanoring with photoirradiation, and the released Cas9 maintained the activity for selective cleavage of dsDNA.

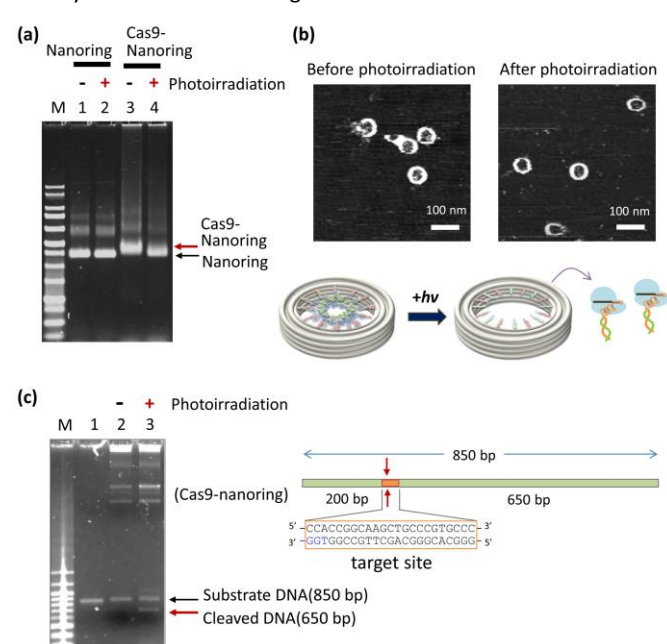


Figure 3. Photo-induced activation of Cas9 using dissociation from the nanoring. (a) Agarose gel electrophoresis image of the photoinduced band shift of Cas9-nanoring.

Lane 1 and 2, nanoring only; lane 3 and 4, Cas9-nanoring. In lane 2 and 4, UV-light was irradiated to nanoring and Cas9-nanoring, respectively. (b) AFM images of Cas9 release from the nanoring before and after photoirradiation. (c) Agarose gel electrophoresis image of the site-selective DNA cleavage. Lane 1, substrate dsDNA only; lane 2, substrate DNA with Cas9-nanoring without photoirradiation; lane 3, substrate DNA with Cas9-nanoring with photoirradiation. The right figure presents a target site and sequence in the substrate dsDNA.

Using the system described above, enzymes can be incorporated into the nanoring and purified, and subsequent release and activation can be controlled by photoirradiation. For its application, this system can control the release of any target molecules from DNA origami carrier and should work inside the cells. This work is part of an intensive research effort in different fields such as DNA origami and light control, and is expected to be used for a carrier for introduction of target molecules. The research is expected to contribute to the application of DNA nanotechnology in the cellular studies and therapeutics.

Conflicts of interest

There are no conflicts to declare.

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Construction of optically controllable CRISPR-Cas9 system using a DNA origami nanostructure

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Experimental Procedure

Materials. All the staple DNAs for the DNA nanoring were purchased from Eurofins Genomics (Tokyo, Japan). Single-stranded p8064 DNA was purchased from Tilibit nanosystems (Garching, Germany). DNA strand containing a cleavable linker was purchased from Japan Bio Services (Saitama, Japan). Cas9 and Streptavidin Magnetic Beads for purification were purchased from New England Biolabs (Ipswich, MA). The gel-filtration column and the Sephacryl S-300 and S-400 were purchased from BioRad Laboratories (Hercules, CA) and GE healthcare (Buckinghamshire, UK), respectively.

Preparation of DNA nanoring. A solution was prepared with 40 nM p8046 ssDNA (template strand), 4 equivalents of staple strands, 10 mM MgCl₂, 1 mM EDTA, and pH was adjusted with 20 mM Tris-HCl (pH 7.6) as a buffer. This solution was incubated at 65 °C for 15 min, then cooled stepwise by 1 °C every 60 min from 60 °C to 40 °C to assemble nanoring. Sequences of staple DNAs used here are listed in Table 1.

Preparation of sgRNA. Single-guide RNA (sgRNA) was prepared by in vitro transcription from a template dsDNA containing T7 promotor sequence. The template dsDNA was prepared by performing overlap extension PCR with an 84 nt and 56 nt template DNA strands (Table S1). The PCR amplification was performed using Go Taq polymerase (Promega) with a forward and reverse primer (Table S1) by following the manufactures protocol. The amplified product was purified using a QIAquick PCR purification Kit (Qiagen).

The in vitro transcription reaction was performed using 30 nM dsDNA template, 40 mM Tris-HCl (pH 8.0), 5 mM DTT, 8 mM MgCl₂, 2 mM spermidine, 2 mM NTP and 0.25 uM T7 RNA polymerase (Takara) at 37 °C for 2 h. The resulting sgRNA was purified by RNA purification kit (Qiagen) and the quality was confirmed by running the sgRNA on an 8% denaturing polyacrylamide gel. Sequence of template dsDNA and sgRNA are listed in Table S2.

Introduction of protein. For incorporation of Cas9/sgRNA into the nanoring (Figure S1), 1 μM Cas9 and 500 nM sgRNA were incubated in a buffer containing 20 mM HEPES, 100 mM NaCl, 5 mM

MgCl₂ and 0.1 mM EDTA at 25 °C for 10 min. A solution of 250 nM Cas9/sgRNA complex was added to a solution of 10 nM DNA nanorings with PC-linker-containing complementary strand in a buffer (10 mM HEPES, 50 mM NaCl, 5 mM MgCl₂, 0.01 mM EDTA), and then incubated at 37 °C for 1 h. Excess staple DNAs were removed by gel filtration using Sephacryl-S400.

Purification of nanoring. For purification of a Cas9-attached nanoring, a single-stranded DNA (ssDNA, 25 nt) was introduced to the nanoring as a capture strand (Figure S2). A 50 μM of biotinylated complementary strand (16 nt) to hybridize with above 25-nt capture ssDNA was added to a mixture of streptavidin-attached magnetic beads (4 mg/mL), and the mixture was kept at rt for 5 min, then the magnetic beads was washed to remove excess biotinylated strand. The Cas9-attached nanoring with 25-nt capture ssDNA was added to the magnetic beads mixture. The mixture was kept at 25 °C for 30 min, then the beads were trapped using a magnet stand, and the remaining supernatant was removed to remove excess Cas9. After three times washing, the Cas9-attached nanoring was separated from the beads using strand displacement with 50 μM complementary ssDNA (25 nt) by incubating at 25 °C for 30 min, and the purified Cas9-attached nanoring was recovered. The sequences of DNA strands used for purification are listed in Table S3.

Confirmation of the Cas-9 activity. For photoirradiation, the purified Cas9-attached nanoring (7 nM) was irradiated with UV light (350 nm) for 10 min using Xenon Light Source (ASAHI SPECTRA MAX-303, 300W). After irradiation, PCR-amplified EGFP-coding dsDNA (850 bp, 10 nM) was added to the solution of photoirradiated Cas9-nanoring, and the mixture was incubated at 37 °C for 60 min. The reaction solution was then heated at 65 °C for 10 min to dissociate Cas9 from the substrate dsDNA, and then the decomposed stapled DNAs were removed by Sephacryl-S300 gel filtration and the reaction substrate was recovered. The cleavage reaction was observed by agarose gel electrophoresis. Sequence of a target dsDNA (EGFP gene) is presented in Table S4

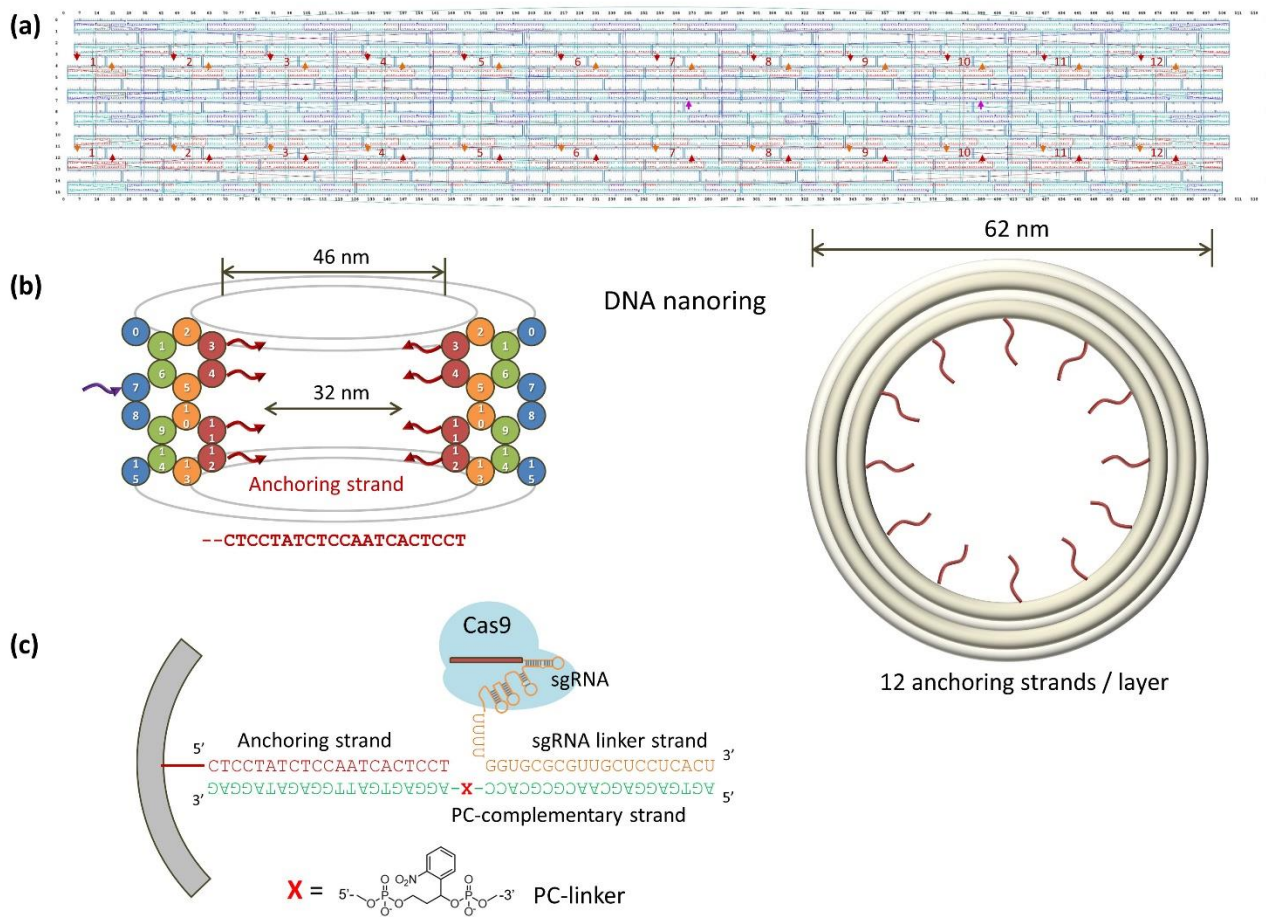


Figure S1. Schematic illustration of introduction of Cas9 into the nanoring. (a) caDNA design of DNA origami nanoring. (b) Illustration of the nanoring with anchoring strands inside the cavity. Twelve anchoring strands were incorporated for one layer, and total 48 strands were incorporated. (c) Incorporation of Cas9 inside the nanoring. Linker RNA strand was added to 3'-end of sgRNA. Cas9/sgRNA with linker strand was bound to the anchoring DNA strand inside the nanoring with the PC-linker-containing complementary strand.

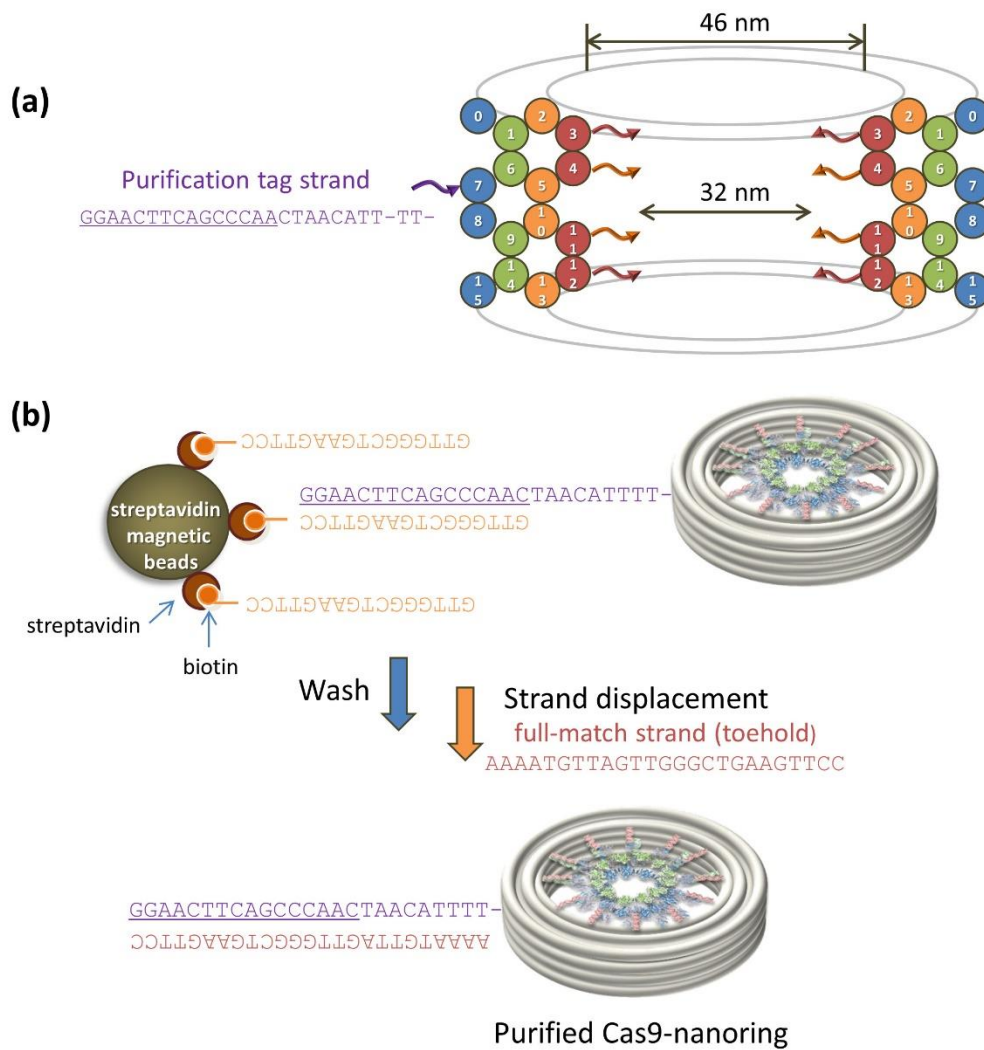


Figure S2. Schematic illustration of the purification. (a) Purification tag strands were incorporated to the outer side of the nanoring. (b) Biotinylated DNA strand was immobilized on the streptavidin magnetic beads. Purification-tag attached Cas9-nanoring was attached onto the magnetic beads to wash out excess Cas9, and then separated to recover the purified Cas9-nanoring using strand displacement.

Table S1. Staple DNA sequences for ring-shape DNA origami

Staple	sequence
ring 001 h0-1A	TTCACTACTTACCACTCTACC-GGGCGATGTTGGGGTCGAGGTAACGCTCATGTACCGCCAGCCTTGCT
ring 002 h0-2A	TTCACTACTTACCACTCTACC-TAAAGAACCCTTCTGACCTGCCTCATATTCAAACCACCAGTAACAT
ring 003 h0-3A	TTCACTACTTACCACTCTACC-CGAGATAGCAGAAATAAAGAACATATATTTTCAAAGAACAAATGCTG
ring 004 h0-4A	TTCACTACTTACCACTCTACC-GGTGGTCTTTAGTATCATATTTTAAATCATCCCGACTCTAAGAAC
ring 005 h0-5A	TTCACTACTTACCACTCTACC-AGCGGTCTTTATCCCAATCCGAGATTGAGGCCAAAGACAATAGAAA
ring 006 h0-6A	TTCACTACTTACCACTCTACC-CAACAGCTCATTAGCAAGGCCACTTCGGAACATAAACAGGGTCAGT
ring 007 h0-7A	TTCACTACTTACCACTCTACC-TGCGTATTATATAAGTATAGCGGTATATTGCAACAACCTTGATACC
ring 008 h0-8A	TTCACTACTTACCACTCTACC-TGCCAGCAAAGACTTTTTCACAATTATACCACCTTATGTTAATTTT
ring 009 h0-9A	TTCACTACTTACCACTCTACC-TAATTGCGAATGCAGATACATCAACTAAAGTTGTAGCTCCTTAGAGC
ring 010 h0-10A	TTCACTACTTACCACTCTACC-CATAAAGTTATATTTTCATTTCCAGATTGTAATAATCAGAGCATGTC
ring 011 h0-11A	TTCACTACTTACCACTCTACC-GAAATTGTCTGGCCTTCCTGGCCTTTCTCCCCAGTCCCATCGGCGA
ring 012 h0-12A	TTCACTACTTACCACTCTACC-GGTACCGAAAAATCCCGTAAAGCGTGCACCTCGATCCAGCAGCGGTGC
ring 013	CGTGAACCATCCTCACAGTTGAGGATCCCCG
ring 014	CCAACGTCAAAGGGCGATTCTGGACACACGACCAGTAATGCCG
ring 015	TGTTGTTCCAGTTTGGTCAAAATGTTGGAAGGGTTAGAAAAAG
ring 016	GCAAAATCCCTTATACATAATTTATAAATAAGAATAAAATTG
ring 017	TTTGCCCCAGCAGGCGTACAAAACTTTCCAGAGCCTAAGCGT
ring 018	CTTACCGCCTGGCCCAAATCACGACCATTGGAATTTAAAT
ring 019	GGGTGGTTTTTCTTTATAAGTGCAGGGTTTTGCTCAGTGGA
ring 020	TGAATCGCCAACGCGGCTACAAAAGCATCGGAACGAGCCGG
ring 021	CACTGCCCGCTTCCATTTAGGATAGTAGAAAGATTCATTGAG
ring 022	TGGGGTGCTAATGAAATGGTCTCTTGACCATTAGATAAACG
ring 023	TCACAATTCCACACAAACGCCATTGAGCTCATTTTTTAAGGGG
ring 024	TTCGTAATCATGGTCACGGTTGTTGCTCATTTGCCGCCATAGC
ring 025	TAAAGCATTACATTGGCAGGTCTGATACCTATTGCTT
ring 026	CGTAAGAGTTTGATTATACAATATATTATCATGACGC
ring 027	CGTAGATCGACCGTGTGATTAATGGAATTTCCGACAA
ring 028	TATACAATCTTACCAACGCACAATTTAGTTGAAGACG
ring 029	AAGAAACATTATCACCGTCAATTCATGAAGGTACTTGA
ring 030	ACGTCACCAAGAGAAGGATAGAGGCTATTCTCACCA
ring 031	AATAGGTCGTCACCTCAGGCCGCTCTGAGGTTAACG
ring 032	GAAGTTTCTAACGGAACAACCGTTAAAGGACGGATTGT
ring 033	CCAAAAGTTCTGCGAACGAAACAGTTGTCTGTTGTCA
ring 034	CGCGAGCTCGCATTAATTAATATTCAAATAGACGGG
ring 035	CAGCTTTAGCGGATCAAATAAGAGATGAAGGCCGGA
ring 036	GCCGCACTACCGGGGTTTGCCGTGTGCTGCGGTCT
ring 037	AGGGAGCCCCATCTAAATCGGAACCTCGTGCCTGTTCTTCAAAA
ring 038	TATTTTGAATTATACGTGGCAGCCAAACAGAAAATACCG
ring 039	GATGAATATACACTTTCAGGTTTAACTATTTGCAGCTTGAA
ring 040	GCCAACGCTCAAATTTTACCAGTAAC TAGAAAATTTAGGC
ring 041	CAAAAATGAAAAGATTTTTTGTTTATAAACAGCGGGAGAAT
ring 042	GCAGCACCGTACTCAATGAAACCATCCAGTAGCATTTTCATCG
ring 043	AGGTTTAGTAATGTATCACCGTACTCCGTCGAGACCTCAT
ring 044	TACGTAATGCCACCATTAAACGGGTGAGGCTTTACTCATCT
ring 045	AAGAGCAACACAAGAATTACGAGGCAATACCACAGCTTTTGC
ring 046	TTCTACTAATATTGAAAAGGTGGCAAATAACCTCAAAATTA

ring 047	AGCGAGTAACACCATCAACATTAACAAAAATAGGTCACGT
ring 048	GAAGGGTAAAGCAAGGCGCCTTTAGGTACATCGCGTCGCTG
ring 049 h3-1C	TTCGATTTAGAGCTTGATGCGGCGGTGCCAGCACGAA-CTCCTATCTCCAATCACTCCT
ring 050 h3-2C	TGGCTATTAGTCTTTAAGCTCAATCATTCACCAGTCAA-CTCCTATCTCCAATCACTCCT
ring 051 h3-3C	ATAGTAACAGTACCTTTAATTCATCTTCTGAATAACA-CTCCTATCTCCAATCACTCCT
ring 052 h3-4C	TGAACAGTAGGGCTTAACCTAAATTAATAAGGCGTAA-CTCCTATCTCCAATCACTCCT
ring 053 h3-5C	GATAGCAGCCTTTACAGACCCAGCTTAACGAGCGTCGT-CTCCTATCTCCAATCACTCCT
ring 054 h3-6C	TCATCAGTAGCGACAGACGGAAATTCGACTTGAGTA-CTCCTATCTCCAATCACTCCT
ring 055 h3-7C	GGCCGCCACCCTCAGAAAAGTATTATAGGATTAGCGGG-CTCCTATCTCCAATCACTCCT
ring 056 h3-8C	AACTACGAAGGCACCAAAGTTAAAGCAGCGAAAGACAA-CTCCTATCTCCAATCACTCCT
ring 057 h3-9C	CCTATCATAACCCTCGTAAATCTAATTATTACAGGT-CTCCTATCTCCAATCACTCCT
ring 058 h3-10C	AAAGTAGTAGCATTAACTTCCATATGTAGATTTAGTAA-CTCCTATCTCCAATCACTCCT
ring 059 h3-11C	AACCCGTCGGATTCTCCTAAACGTTTTTGTAAATCTG-CTCCTATCTCCAATCACTCCT
ring 060 h3-12C	GTTTAAACGATGCTGATCAGGAAATAAATTTCTGAT-CTCCTATCTCCAATCACTCCT
ring 061	CGAAATGCGCGCCTGTCCGTGAGCCTCACCCAAATCGAAGAAAG
ring 062	AACGACAACTGCGCGAACTGAAATGACAGTATTACAAATCAA
ring 063	TACCAGAGCTACATCGGGAGAAATTAATTTACCTTGAGTGAA
ring 064	AGAGGTTGATTGAGAATCGCGTTCAGACAAAAGGTCCTAATT
ring 065	TAACTTTGCAGAGAATAACATAAGAGCTAATATCCGAACAAA
ring 066	GCATTCAAGATCAAGTTTGCCCTCATTCATAATACCAGAGC
ring 067	TTTACCCGACCGCCACCCTCCAGACAACTGAGTTTCTGTATG
ring 068	TTGACCAGGCCTAAAACGAACCTGCTACGGAGATAAAGAGGA
ring 069	AAAAGAAGATTACCAGACGAATCCCCACTGGATAGACTATTA
ring 070	AGCAAAACAATCCAATAAATCAACGCAAAACATTAGATTCAA
ring 071	TGGTGAATGGTGGGAACAAACAGCTTTGAGGGGATGGGAAGG
ring 072	GCAGCAGCTTGCCGTTCGGGGCAAGAGGACTTGTCATCAGCG
ring 073	AACAATATGAGAGAAAGGAAGGAGTTTTTGCCCACTA
ring 074	AACAAAGCTCATCGCCATTAAGATAGAACGTGGACT
ring 075	TCGCAAGAAGATTGCGCTGATTGTAAGGTTGAG
ring 076	AGCGAACCAGCGCCAACATGTAAAGCCTGCGAAATCG
ring 077	ACCAGCGGAAGCGCATTAGACCATATTACACGCTGG
ring 078	AGTGCCCGCTACTGTAGCGCGTCCATTACGATTGCC
ring 079 tag1	GGAACCTCAGCCCACTAACATTTT-GACAATGAGTCTCAGAGCCACCAGGGTTGGGGCGCCA
ring 080	GTGAATTAGATACTAAACGAGGACTTGCAATTA
ring 081	TATAATGCACAAATAGCGAGGTTCAACTTTGCGCT
ring 082 tag2	GGAACCTCAGCCCACTAACATTTT-CCCGGTTGTAGCAAAGAATTAGGTTTAGCGTAAAGCC
ring 083	ATGTTTAGTCGTAATGGGATAATTGCGGTTATCCGC
ring 084	CATCAGACTGGTTTTTTTCTGTCTACATAAAGCTCGAA
ring 085 h15-1B	GGTAATATCCACACCCGCGCGCATCAGTGAGCCAGAATCCTGAGA-TTCCTCTACCACCTACATCAC
ring 086 h15-2B	TATCATTCTGCAACAGTGCCCGGATTAGAGTATCTTTAGGAGCACT-TTCCTCTACCACCTACATCAC
ring 087 h15-3B	ATGCAATAGAAAGATGATGAACTAGCGATAGATTTTCCCTTAGAATC-TTCCTCTACCACCTACATCAC
ring 088 h15-4B	GCGAGGCGATTCTGTCCAGACTCATTAAACCTCTTCCCTTATCATT-TTCCTCTACCACCTACATCAC
ring 089 h15-5B	ATTCAATATAACCCACAAGAACAATTAAGACAACGGAATACCCAAAA-TTCCTCTACCACCTACATCAC
ring 090 h15-6B	GCCTTGAGACCGGAACAGAGGGAAATAAATAGACGATTGGCCTTGA-TTCCTCTACCACCTACATCAC
ring 091 h15-7B	GATAGTTGCAGTACAACTACGCGGAATTGCGAGTGAGAATAGAAA-TTCCTCTACCACCTACATCAC
ring 092 h15-8B	AACTTTAATCGCCTGATAAAACGACAAGAATGGCTGACCTTCATCA-TTCCTCTACCACCTACATCAC
ring 093 h15-9B	TTAATTGCATACTGCGGAATCTTATCGCGTTTAAAGAGGAAGCCCGAA-TTCCTCTACCACCTACATCAC
ring 094 h15-10B	AATCATATTGTAATACTTTTGGATGATAAATATCAATATGATATTC-TTCCTCTACCACCTACATCAC
ring 095 h15-11B	AACGTACAGTATCGGCCTCAACGCGATTAAGCTGGCGAAAGGGGGA-TTCCTCTACCACCTACATCAC

ring 096 h15-12B	CGGTGCCCCAGCGTGGTGCTGGCACTGTTGCGGGCGCGGTTGCGGTA-TTCCTCTACCACCTACATCAC
ring 097	ACTATCGGCCATTGCAAACCACCAGCAGAAGATAACGG
ring 098	AAAGTTTGAGAAGGAGCGAGTTACAAAATCGCGCAGAAA
ring 099	CTATATGTGCGAGAAAACCATTTTCGAGCCAGTAATATT
ring 100	CGGTATTTGCGGGAGGTGAACACCCTGAACAAAGTTTT
ring 101	TCACAATCAAAGGGCGATTTCGGTCATAGCCCCCTTAAC
ring 102	TTTTAACGGTTAATGCCCCGGGATAGCAAGCCCAATAGCC
ring 103	AAACAGCATCGCCACGCCCCAGCGATTATACCAAATT
ring 104	GAGATGGTCGATTTTAAGAAGTTTGGCCAGAGGGGGTAA
ring 105	GCGGATGGAACATGTTTTTAAAGCCTCAGAGCATAAAAC
ring 106	TAAAACTAAAAGCCCCATAGATGGGCGCATCGTAAGCC
ring 107	CGGAAACAGGAATTTGTGCTCCGGCCAGAGCACATCCTG
ring 108	CTGCAGCCGCAGTGTACGGAGCGGGCGCTAGGGCGCAG
ring 109	AATGCGCTGTAGCAATACTCACGCAACCGAG
ring 110	ACATCACCATTTTGGGAAAAAAGGGACAAAAACCGTCTATCA
ring 111	TGAGAGCTTTACAAACAATTTTAGAACAAATAG
ring 112	AATCCTTGATGATGATTATCATACCATAAACAAGAGTCCACTAT
ring 113	ACATCAAAATCATAGGTCTATAGTGGATTAA
ring 114	AACCTCCATCTTCTTTTCAAACCGGAATAATCAAAAGAATAGCC
ring 115	ACAATAAATCGTAGGAATCAGCCGTACCGCA
ring 116	GCAAGCACTATTTTTGAAGCCTGCCAGTAAAATCCTGTTTGAT
ring 117	GTTAAGCATAAAGGTGGCAAGTAGAATATTAC
ring 118	CAAAGACAAATATTTTCAACCGCCAGCATGAGAGAGTTGCAGCA
ring 119	CACCGGAGTTCCAGTAAGCAGTCTCATTTAA
ring 120	GATGATAGAAACATTGCCTATCAGGCGGTCACCAGTGAGACGGG
ring 121	CCTGTAGCCAAAAGGAGCCCTCCAAAATAAT
ring 122	TATCAGCCTTGCAAGTAACCGATAGCAACCGGGAGAGGCGGTT
ring 123	GTCGAAATCAGTGAATAAGGAACAAAATATTC
ring 124	AACACCATTGGGAACCTGGCTCGTTGAGAGTCGGGAAACCTGTCTG
ring 125	TAAATATACAGGTCAGGATCCGGAATTCGAG
ring 126	TTGCTCCGAAGTTTATATGCATTTTCGAGTGAGCTAACTCACAT
ring 127	AGAAGCCTATCAGGTCACTAGAGATGCCGGA
ring 128	CAACAATTTAAATAACAGGAAATAGGACATACGAGCCGGAAG
ring 129	GATCGCACGACGGCCAGTGCCACGACGGTAAC
ring 130	GGAGCCGATAGCTAGATAGAAGTTGGGTAGCTGTTTCCTGTGT
ring 131	GGTCAGCCATCCCTTACACCTTTGCCGGCTG
ring 132	TCGTTAACGGCCAGAACGGGGAAGCCGGGCGCGTGCAA
ring 133 h4-1C	TATGGTTGCTGTGCTACAGGCGAACGTGGCAAAATGGA-CTCCTATCTCCAATCACTCCT
ring 134 h4-2C	TCTAAAGCATCCCAGCAGCATAGCCCTAAAAGAATCCTGA-CTCCTATCTCCAATCACTCCT
ring 135 h4-3C	CATTTAACAATAAGAAAACAAAACAATAACGGTTTTTTGAA-CTCCTATCTCCAATCACTCCT
ring 136 h4-4C	TGCAGAACGCTCACAACATCATATTTAACAAATTTATCC-CTCCTATCTCCAATCACTCCT
ring 137 h4-5C	AAACAATGAAACCCAATAATAAAACAGGGAGGTAAAGGT-CTCCTATCTCCAATCACTCCT
ring 138 h4-6C	CGCCACCCTCACCACCGCCTCTTTAGCGTCAGATTGAGAC-CTCCTATCTCCAATCACTCCT
ring 139 h4-7C	TCATAGTTAGCTCATTTCCAAGAACCGCCACCCGTTTGCG-CTCCTATCTCCAATCACTCCT
ring 140 h4-8C	GTTACTTAGCCTTCCGCGAAGAGGCAAAAGATCTAAAACG-CTCCTATCTCCAATCACTCCT
ring 141 h4-9C	AATGCTTTAAACATCATTGACGATAAAAACAGGTGATTC-CTCCTATCTCCAATCACTCCT
ring 142 h4-10C	ATAAAAATTTGCTTTATTTATACAGGCAAGAGTTGTTA-CTCCTATCTCCAATCACTCCT
ring 143 h4-11C	GCACCGCTTCTACTCCAGCCGCGGATTGACGGCGCAGAA-CTCCTATCTCCAATCACTCCT
ring 144 h4-12C	CAACGGCAGCAAAAGCAACCCAAACGCGGTCTGTTACG-CTCCTATCTCCAATCACTCCT

ring 145 h11-1C	CCTTGACGAGCACGTATAAAGGTTTTGGTGTGTTCAAC-CTCCTATCTCCAATCACTCCT
ring 146 h11-2C	AACCTTGCTGAACCTCACTGTCCATTCTTTGATTAGAA-CTCCTATCTCCAATCACTCCT
ring 147 h11-3C	TCTTCATTTGAATTACCTTGAGGATCGACAACTCGTA-CTCCTATCTCCAATCACTCCT
ring 148 h11-4C	TTGCCTGTTTATCAACAAAGAGTCAGAGAGACTACCAA-CTCCTATCTCCAATCACTCCT
ring 149 h11-5C	ATAGCAATAGCTATCTTAAACAAGCAATTACCGCGCCAG-CTCCTATCTCCAATCACTCCT
ring 150 h11-6C	TAGAACC GCCACCCTCATAGCAAACCATATAAAAGGC-CTCCTATCTCCAATCACTCCT
ring 151 h11-7C	GGCGTAACGATCTAAAGGAAAGCGCGTCATACATGGCC-CTCCTATCTCCAATCACTCCT
ring 152 h11-8C	AGGAACGAGGCGCAGACTTGAAAATTTTAATTGTATAT-CTCCTATCTCCAATCACTCCT
ring 153 h11-9C	TCCAGTTCAGAAAACGAATCAACGTCTTGCCCTGACA-CTCCTATCTCCAATCACTCCT
ring 154 h11-10C	AGTTAGAACCCTCATATGAACCAGATAGAGAGTACCGG-CTCCTATCTCCAATCACTCCT
ring 155 h11-11C	AGGTGCCGGAACCAAGGTATTTTTGGCCTGAGAGTCCG-CTCCTATCTCCAATCACTCCT
ring 156 h11-12C	TACCGTCGGTGGTGCCATTCCCAGTCAAGCTTTCAGC-CTCCTATCTCCAATCACTCCT
ring 157	TAAAAGATTTTATACTGAGTAGAAGAGTAATAATA
ring 158	ATAATACACTAATACGAACGTTATTATCATTATTA
16HB ring 159	GACGCTGAAAACATTAGGTTGGGTTACGCTTTTTT
16HB ring 160	CTCATCGAACGGGTAGATATAGAAGGCGCACATA
16HB ring 161	GCAGTATTGGCATGCGGAATAAGTTTTTGAAAACG
16HB ring 162	GCCAGAACACAAACAGTGTACTGGTAACGCCTTTT
16HB ring 163	TTTTTCAAACTAAATTTTCGAGGTGAACACCCGGTT
16HB ring 164	ATTACCCTAATCTTGAGTAGTAAATTAGCTCGAGA
16HB ring 165	CTTCAAATTCAAATGATAAGAGGTCACAAATTTAA
16HB ring 166	GAGGGTATTCTAGCATCGATGAACGGTTTCTGGAG
16HB ring 167	GCCAGGGCTGCAAGGGGAACGGATAACCATGAGGT
16HB ring 168	GGCCGAGCCGGGTCATCAGATGCCGGCACGCTGCGCAAACAGGA
16HB ring 169 h12-1C	GTAATGGGTAACGTGCTTTCCTCGTTAGAATCCCAATTAA-CTCCTATCTCCAATCACTCCT
16HB ring 170 h12-2C	CAGTTGTGTAATATCAAACCCTCAATCAATATGTGTATTAG-CTCCTATCTCCAATCACTCCT
16HB ring 171 h12-3C	TAACCCAAATTTTTTAAATGGAACAGTACATATAAATTTA-CTCCTATCTCCAATCACTCCT
16HB ring 172 h12-4C	TACGATGAGATAGATAAGTCTGAACAAGAAAGTTTTTAT-CTCCTATCTCCAATCACTCCT
16HB ring 173 h12-5C	GTTACAGAGACCGAAGCCCTTTTTAAGAAAAGCTAATACAT-CTCCTATCTCCAATCACTCCT
16HB ring 174 h12-6C	CGCCGACGTGAGCCACCACCCTCAGAGCCGCCTCTGAATT-CTCCTATCTCCAATCACTCCT
16HB ring 175 h12-7C	GGATTTTGTGTTGTGCTCTTTCAGACGTTAATAAAAAA-CTCCTATCTCCAATCACTCCT
16HB ring 176 h12-8C	CAGATACCGGTC AATCATAAGGGAACCGAACGGGCTGCTC-CTCCTATCTCCAATCACTCCT
16HB ring 177 h12-9C	TAGTCAGAAGAATGACCATAAATCAAAAATCAAAGCAAAC-CTCCTATCTCCAATCACTCCT
16HB ring 178 h12-10C	AAGGGACGCATTTTAAATGCAATGCCTGAGTAATCTACAA-CTCCTATCTCCAATCACTCCT
16HB ring 179 h12-11C	GCGATCGGCCAAAGCGCCATTGCGCATTGAGTGGTTGTAA-CTCCTATCTCCAATCACTCCT
16HB ring 180 h12-12C	GGGTCTGTTTCCACGCAACCAAGCTTACGGCTTGTCGTCA-CTCCTATCTCCAATCACTCCT
16HB ring 181	GGGAGCTGTAACCACCTGGCAAGTGTAGCGGTGTTAC
16HB ring 182	CAGTTGGACACCGCTTGAACAGAGGTGAGGCGACTCAA
16HB ring 183	ATATATGGAGCAAACCGGCGAATTATTCATTATTTTA
16HB ring 184	TATCCCATAAAGTATTAGAGAATATAAAGTACTATAA
16HB ring 185	AGATAGCAGAGAGATGGCAGAGGGTAATTGAGCTTATC
16HB ring 186	AACCACCCAAAATCTATTAGCGTTTGCCATCATTTTG
16HB ring 187	TGAATTTTCGTCACCGGAACCCATGTACCGTAATAAG
16HB ring 188	AACTTTGTTGTATCATCGCGCGAAACAAAGTATTTCTT
16HB ring 189	TTACCCTGCGTCCATGAATAGTAAATGTTTGGGCTT
16HB ring 190	TAGGTAAATGACCCGTGCTAAATCGGTTGTACTTTTT
16HB ring 191	CAACTGTCGACGACAGCCCGTGTCATCTGCCAGTAATCG
16HB ring 192	TGTCCAGAGAACGTCTCATAACGGAACGTGCCTCAC
16HB ring 193	AGTGAAAGGAATTGAGGAAGGTTATCTAAAACCTGGT

16HB ring 194	AATTGCTTCTGTAAATCGTCGCTATTAATTACTAATCA
16HB ring 195	CTGCATGTAGAAACCAATCAATAATCGGCTGAAAATAA
16HB ring 196	CCACAGAAGGAAACCGAGGAAACGCAATAATTCTAAGC
16HB ring 197	GACCAGCATTGACAGGAGGTTGAGGCAGGTCCCACCAG
16HB ring 198	TATTGCTAAACAACCTTTCAACAGTTTCAGCGGAGTAAA
16HB ring 199	GGAGAACGGTGTACAGACCAGGCGCATAGGCCCTGACC
16HB ring 200	AGAGAAGCAAAGCGGATTGCATCAAAAAGATTGGTCT
16HB ring 201	AGTGAGAAAGGCCGGAGACAGTCAAATCACCTAATGTG
16HB ring 202	AACCGGTGCGGGCCTCTTCGCTATTACGCCAGTCTGCG
16HB ring 203	TGATTGCAGGCGCTTTTCGCACTCAATCCGCCCCGGAGG
16HB ring 204	TGATTAAAGGGATTTTAGACAGGAACGGTACGGAGAGC
complementary strand A	GGTAGAGTGGTAAGTAGTGAA
complementary strand B	GTGATGTAGGTGGTAGAGGAA
complementary strand C	AGGAGTGATTGGAGATAGGAG

Table S2. Sequences of sgRNA and temple dsDNA.

	sequence
sgRNA + connector	GGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGA GUCGGUGCUUUUGGUGCGCGUUGCUCUCACU
T7 promoter +	TTCTAATACGACTCACTATA-
sgRNA template + connector top 84 nt	GGGCACGGGCAGCUUGCCGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTT
T7 promoter +	AGTGAGGAGCAACGCGCACC-AAAA-
sgRNA template + connector bottom 80 nt	GCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAAGTT
Forward primer for template	TTCTAATACGACTCACTATAGG
Reverse Primer for template	AGTGAGGAGCAACGCGCACCAA

Table S3. DNA sequences of biotinylated strand and fully complementary strand for purification.

Staple	sequence
biotinylated strand	GTTGGGCTGAAGTTCC [BIOTEG]
complementary strand	AAAATGTTAGTTGGGCTGAAGTTCC

Table S4. Substrate sequence (EGFP complementary strand, 714 bp)

GTACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCGGTCACGAACTCCAGCAGGACCATGTGATCGCGCTTCTCGTTGGGGTCTTTG
CTCAGGGCGGACTGGGTGCTCAGGTAGTGGTTGTCTGGGCAGCAGCACGGGGCCGTCGCCGATGGGGGTGTTCTGCTGGTAGTGGTCGG
CGAGCTGCACGCTGCCGTCTCGATGTTGTGGCGGATCTTGAAGTTCACCTTGATGCCGTTCTTCTGCTTGTCTGGCCATGATATAGAC
GTTGTGGCTGTTGTAGTTGTACTCCAGCTTGTGCCCCAGGATGTTGCCGTCCTCCTTGAAGTCGATGCCCTTCAGCTCGATGCGGTTTC
ACCAGGGTGTCTGCCCTCGAACTTCACCTCGGCGCGGGTCTTGTAGTTGCCGTCGTCCTTGAAGAAGATGGTGCGCTCCTGGACGTAGC
CTTCGGGCATGGCGGACTTGAAGAAGTCGTGCTGCTTCATGTGGTCGGGGTAGCGGCTGAAGCACTGCACGCCGTAGGTCAGGGTGGT
CACGAGGGTGGGCCAGGGCACGGGCAGCTTGCCGGTGGTGCAGATGAACTTCAGGGTCAGCTTGCCGTAGGTGGCATCGCCCTCGCCC
TCGCCGGACACGCTGAACTTGTGGCCGTTTACGTCGCCGTCCAGCTCGACCAGGATGGGCACCAACCCCGGTGAACAGCTCCTCGCCCT
TGCTCACCAT

The underline presents the target sequence.

Reference

P. Ketterer, A. N. Ananth, D. S. Laman Trip, A. Mishra, E. Bertosin, M. Ganji, J. van der Torre, P. Onck, H. Dietz and C. Dekker, *Nature Communications*, 2018, 9, 902.