

# A Realization of DNA Molecular Machine That Walks Autonomously by Using a Restriction Enzyme

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**Abstract.** In this paper, we propose an autonomous molecular walking machine using DNA. This molecular machine follows a track of DNA equipped with many single-strand DNA stators arranged in a certain pattern. The molecular machine achieves autonomous walk by using a restriction enzyme as source of power. With a proposed machine we can control its moving direction and we can easily extend walking patterns in two or three dimensions. Combination of multiple legs and ssDNA stators can control the walking pattern. We designed and performed a series of feasibility study with molecular biology experiments.

## 1 Introduction

Several molecular machines, which can walk along a DNA track, have been proposed [1-6]. Two of six molecular machines [1-2] need fuel DNA and cannot walk autonomously. The others have achieved autonomous walk by using DNA cleaving activity of a restriction enzyme or DNzyme. “Walking DNzyme”[3] and “free-running DNA motor”[4] can walk along only one dimensional track. “Spider molecules”[5] has multiple legs and can walk along patterns in two or three dimensions, but it can only walk to random direction. There are few molecular machines that can walk autonomously on two or three dimensions along the designed route. “Unidirectional DNA walker”[6] satisfies these features but it requires to design an appropriate ground pattern to program target patterns when we extend it to two or more dimension.

In this paper, we propose new molecular walking machine that can achieve these requirements by using a restriction enzyme and a track of DNA equipped with many single-strand DNA stators arranged in a certain pattern. In the following, we first propose the conceptual scheme and show feasibility experiments with a restriction enzyme.

## 2 A Molecular Walking Machine

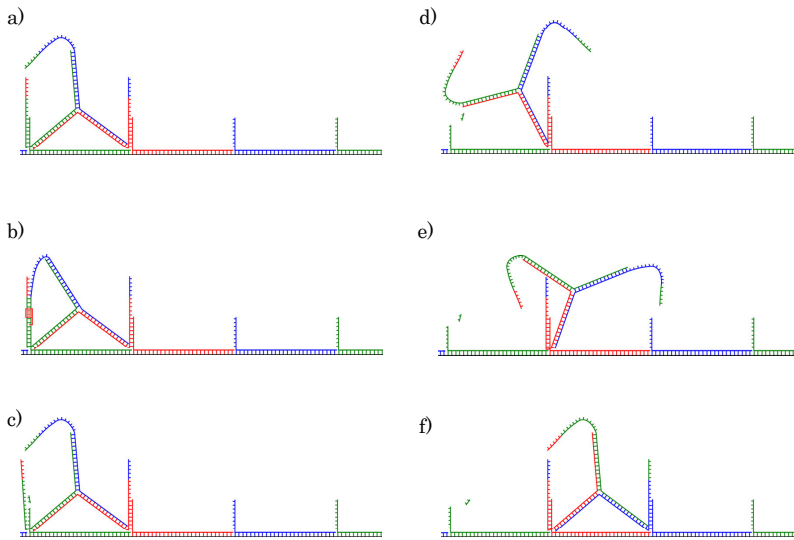
### 2.1 Mechanism How the Molecular Machine Walks

The molecular machine presented in this paper, walks a track of DNA embedded with many single-strand DNA stators. We must prepare a set of ssDNA stators and arrange

them in a certain pattern to form the track which the molecular machine follows. The molecular machine has more than three legs. Each of legs anneals one ssDNA stator, and ssDNA stators are equipped with enough distance so that one leg cannot anneal more than two ssDNA stators at once. We can design a route which the molecular machine walks, by patterning the ssDNA stators and legs.

If nothing has occurred, a molecular machine walks randomly like “spidar molecules”. We introduced the idea for the molecular machine to cleave the ssDNA stators in the correct order to walk along the route we designed.

Fig. 1 shows the reaction of three legs molecular walking machine. Fig. 1a shows a three legs molecular machine anneals the ssDNA stators. Green leg and red leg anneal green and red ssDNA stators. This is the basic state that the molecular machine binds the track of DNA. Blue leg cannot reach to blue ssDNA stators and is free-floating. The molecular machine must keep more than one legs free-floating to cleave ssDNA stators.



**Fig. 1.** The structure and reaction of the molecular walking machine

In this stage, blue leg plays an important role to cleave green ssDNA stator. Fig. 1b shows the free-floating blue leg anneals green leg. In the later experiments, we selected a nicking enzyme “*N.A/w I*” to cleave the ssDNA stators. *N.A/w I* can bind and cleave green ssDNA stators when blue leg anneals green leg on the green ssDNA stator.

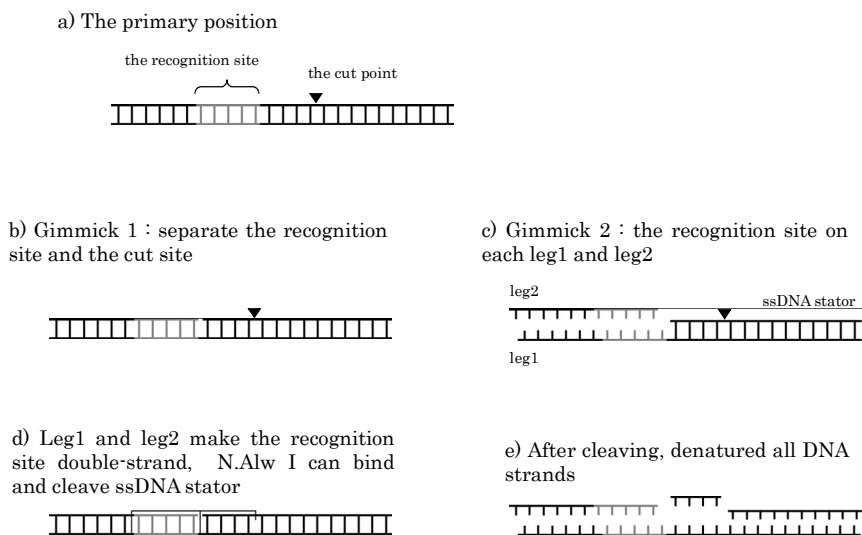
After cleaving green ssDNA stator, green leg cannot keep annealing there. And blue leg is denatured from green leg, because that double-strand is designed unstable (Fig. 1c). After denatured from green ssDNA stator, blue leg gets to reach blue ssDNA stator. The molecular machine moves ahead to anneal blue ssDNA stator (Fig. 1d, e).

Finally, blue leg anneals blue ssDNA stator. Red leg and blue leg anneal ssDNA stators (Fig. 1f). This state is the same as the state Fig. 1a. Green leg acts to cleave red ssDNA stator in the next step. Red leg will act to cleave blue ssDNA stator after

the next step. A molecular machine can cleaves ssDNA stators in the order of green, red, blue, green, and so on, to repeat these motions. This is the mechanism that the track of ssDNA stators is cleaved in the correct order.

## 2.2 Function of Cleaving ssDNA Stators

It is important for this molecular walking machine to cleave the ssDNA stators. A nicking enzyme like *N.Alw I* binds to double-strand DNA at the recognition site and cleaves only one strand at the cut point to introduce a nick. So the molecular machine can cleave only ssDNA stators.



**Fig. 2.** Function of cleaving DNA stators

*N.AlwI* has another characteristic that is the cut point isn't included in the recognition site (Fig. 2a). A molecular machine uses this characteristic to control the activity of *N.Alw I*. We designed the molecular machine with following two gimmicks for the control.

First gimmick is that the cut point and the recognition site of *N.Alw I* are separated into the different two DNA strands (Fig. 2b). All ssDNA stators have the cut point and all legs of the molecular machine have the recognition site. The way of control a restriction enzyme is similar to that of using at "Programmable and autonomous computing machine"[7].

"Programmable and autonomous computing machine" uses a restriction enzyme "*Fok I*". As the first choice, we considered using *Fok I* for the molecular machine. *Fok I* isn't the nicking enzyme, so *Fok I* cleaves the molecular machine's leg unless we protect that strand. We first examined to make the activity of *Fok I* the same as that of a nicking enzyme by using of phosphorothioate-modified DNA [8-9]. Since, we couldn't achieve expected activity, we determined to use *N.Alw I*. See the appendix for detail.

Second gimmick is that the two legs of the molecular machine make the recognition site double-strand (Fig. 2c). When only one leg anneals ssDNA stator, the recognition site is still single strand and *N.A/w* I cannot bind to cleave ssDNA stator. The other leg can anneal to make the recognition site double-stranded. Then *N.A/w* I can cleave ssDNA stator (Fig. 2d). To achieve this function, a leg has two recognition sites; one is for the binding ssDNA stators and another is for the neighborhood ssDNA stators. A combination of the two recognition site leads to the cleaving reaction of Fig. 1.

### 3 Experiment

#### 3.1 Activity of *N.A/w* I

We first designed an experiment to know the activity of *N.A/w* I. We confirmed the DNA cleaving activity for the case that the recognition site and the cut point are in the different DNA strands.

##### 3.1.1 Materials and Methos

For this experiment, we prepared four sets of DNA strands with different separation point, and one set of DNA strands without separate point for a control (Table. 1) (Fig. 3). We named each strands N-X-Y, where X denotes the number of the separate point and Y denotes the length of each strands. “N-none” means a strand of no separate point and “N-c” means a complement of N-none.

Each five sets of DNA strands were mixed at 0.5  $\mu$ M in hybridization buffer. NEBuffer2 from New England Biolabs was used as the hybridization buffer. 3 units of *N.A/w* I from New England Biolabs were added to each five sets 20  $\mu$ l solution. The five sets were incubated at 37°C by 24hours.

**Table 1.** DNA strands. The recognition site of *N.A/w* I is shown in bold type. The cut point is indicated by /.

Name	Separate Point	sequence(5'...3')
N-1-10	1	GATACAT <b>GGA</b>
N-2-11	2	GATACAT <b>GGAT</b>
N-3-12	3	GATACAT <b>GGATC</b>
N-4-13	4	GATACAT <b>GGATCA</b>
N-1-18	1	<b>TCACGG</b> /CTGAGACACTCT
N-2-16	2	<b>CACGG</b> /CTGAGACACTCT
N-3-17	3	<b>ACGG</b> /CTGAGACACTCT
N-4-15	4	<b>CGG</b> /CTGAGACACTCT
N-none	None	GATACAT <b>GGATC</b> ACGG/CTGAGACACTCT
N-c		AGAGTGTCTCAGCC <b>GTATCC</b> ATGTATC

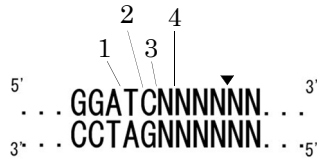


Fig. 3. Separate points

We ran the resulting solutions in 16% PAGE (non-denaturing gel and denaturing gel).

3.1.2 Results

Fig. 4 shows the results. We confirmed the activity of *N.A/w* I at the all sets. We found in the denaturing gel that *N.A/w* I at lane 8 cleaved the DNA strand “N-c”

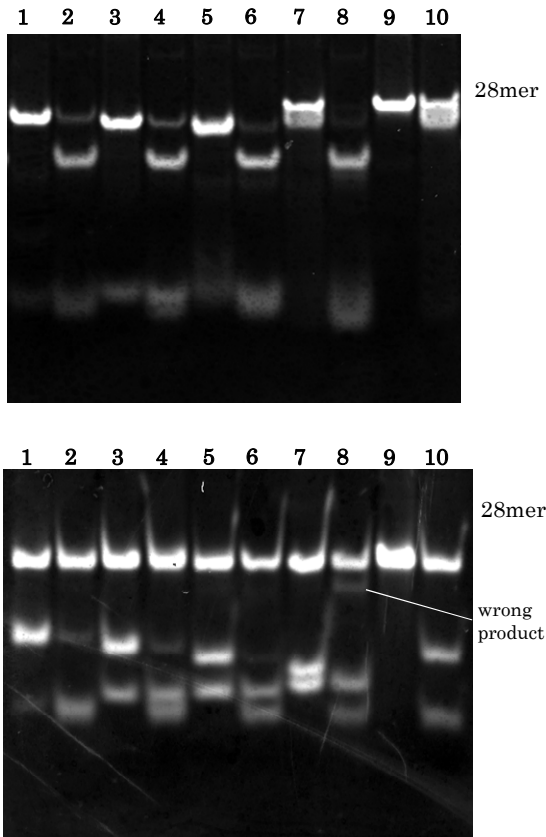


Fig. 4. Result of cleaving activity. 1)2) Separate point 1, 3)4) Separate point 2, 5)6) Separate point 3, 7)8) Separate point 4, 9)10) No separate, Odd number of lane : without *N.A/w* I , and even number of lane : with *N.A/w* I.

which didn't have the cut point. There were the set of DNA with separation point 4 in this solution. We supposed that double-strand DNA twists the recognition site and the cut point at the separation point, and *N.Alw* I cleaved wrong DNA strand. If we used separation point 4 at this molecular machine, *N.Alw* I would cleave not only ssDNA stators but also the legs of the molecular machine. Except for the result of lane 8, *N.Alw* I had the cleaving activity which this molecular machine needs.

We determined to use separation point 1 for later experiments about the molecular machine. The molecular machine needs to anneal the ssDNA stators and to be denatured after cleavage. We need a significant difference in melting temperature before and after cleaving to lead to this behavior. For this reason, the molecular machine should cleave ssDNA stator into as large fragments as possible.

### 3.2 Half Step of the Molecular Walking Machine

As the next stage, we confirmed the behavior of this molecular machine's legs. Each leg has two recognition sites which are for cleaving ssDNA stators and for cleaving the neighborhood ssDNA stators. The molecular machine cleaves ssDNA stators using two legs. We experimented to confirm that the two legs can make the recognition site double-stranded and cleave ssDNA stators.

#### 3.2.1 Materials and Methods

We prepared three DNA strands for two legs molecular machine and one DNA strand for ssDNA stator (Table. 2). In two legs, leg1 is a strand "N-L1", leg2 is a strand "N-L2". Strand "N-L3" is to be a leg3, when the molecular machine has third leg. "N-S1" is ssDNA stator, annealed and cleaved by leg1. Fig. 5 shows N-L1, N-L2 and N-L3 build two legs molecular machine and the molecular machine anneals to N-S1. Reaction condition is the same as the experiment in chapter 3.1.

**Table 2.** DNA strands. The recognition site of *N.Alw*I is shown in bold type. The cut point is indicated by /.

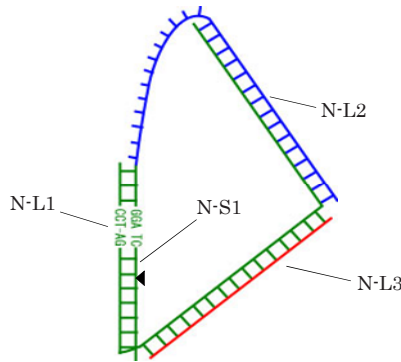
Name	Sequence(5'...3')
N-L1	TATAGATATCAAGTAGTCGATATGCTTCACAGTCTGATCTGAGGTGTGGAGACGTC ATGTGCATGCCAGTGTACGAT <b>CT</b> CCTGCAACG
N-L2	GTGAAGCATATCGACTACTTGATATCTATAGACCTAGTAGTGCCG <b>AT</b> CCAGATCT ACGTTGCAG <b>GA</b>
N-L3	ACATGACGTCTCCACACCTCAGATCAGACT
N-S1	CGTA/CACTGGCATGC

#### 3.2.2 Results

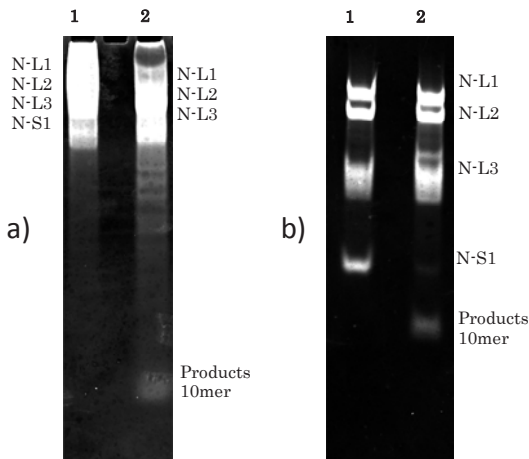
Fig. 6 shows the result that the two legs molecular machine achieved to cleave ssDNA stator. By analysis of denaturing gel electrophoresis, the band of 10 mer in lane 2 shows N-S1 was cleaved by *N.Alw* I. The band of 10 mer corresponds to the cleaving products of N-S1. From non-denaturing gel, we found the two legs molecular machine annealed N-S1 at lane1. And lane 2 shows that the two legs molecular

machine couldn't keep annealing after cleaving, because the band of the cleaving products appeared on the different position of a band which denotes the two legs molecular machine.

The result shows that the two legs cooperated to cleave N-S1 and the unconcerned parts for cleaving of the legs don't have a critical effect on cleaving. We think that the molecular machine presented in this paper, can cleave and be denatured from ssDNA stators. Thus we achieved the motion that the molecular machine lifts up its legs. The molecular machine can walk ahead a half step.



**Fig. 5.** Two Legs Molecular Machine



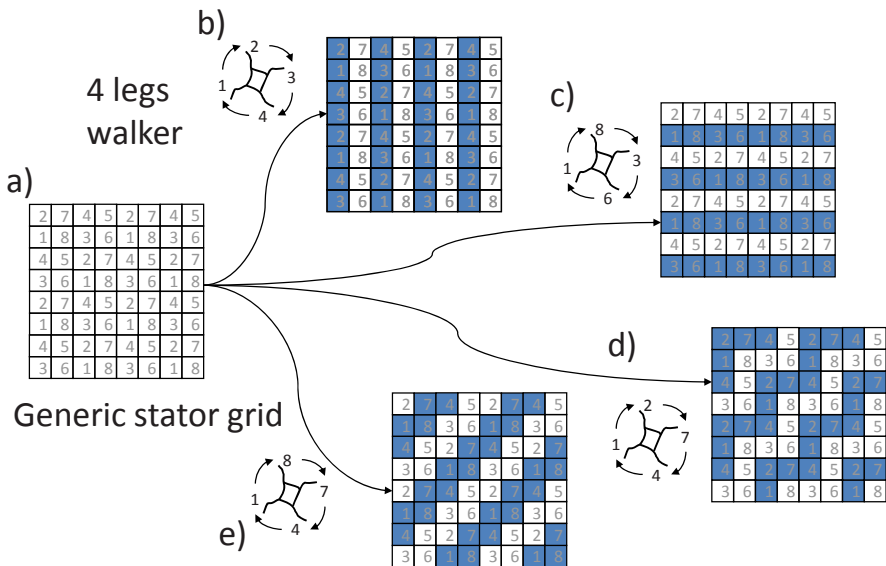
**Fig. 6.** Activity of two leg machine. a) non-denaturing gel, b) denaturing gel. 1) without *N.A/w* I, 2) with *N.A/w* I.

### 3 Discussion

There are several further issues to discuss. Firstly, we need the motion to drop off the lifted legs for other half step. The motion is decomposed into two moves. One is a

move to anneal the next ssDNA stator, the other is a move to anneal the leg which anneals the neighborhood ssDNA stator. To achieve other half step, we plan to examine the move to the leg annealing the neighborhood ssDNA stator at first. The move can be confirmed by an experiment using two legs machine and a track with two ssDNA stators. In this experiment, we should make the condition that leg1 and leg2 anneal each ssDNA stators. We are going to add a strand which should anneal leg1 and make the recognition site double-stranded, and then *N.A/w* I will be able to bind the recognition site and cleave the ssDNA stator. After cleaving, leg1 is going to move to anneal leg2 for cleaving the neighborhood stator. The move is similar to another move to the next ssDNA stator. If the two moves are realized, the molecular machine will achieve the motion to drop off the legs. To combine this motion and a motion to lift up the legs, the molecular machine can walk one step. If the molecular machine can repeat the one step, it should walk ahead.

Secondly, remark that these anneal—cut—denature—anneal cycle is similar to “free running DNA motor” [5] if the walker has only two legs. They have already achieved multiple cycles under isothermal environment which means autonomous behavior. So we believe we can also achieve these cycles autonomously. The difference is using more than three legs leads to control the moving direction. We are now considering more about the combination of phosphorothioate-modified DNA and restriction enzymes. The results shown in appendix indicate the half of phosphorothioate-modified DNA is cut by *Fok* I. There are two alternative oxygen sites to be phosphorothioated in DNA backbone. We guess *Fok* I was only blocked by the specific phosphorothioation site. The recognition site and the cut site of *Fok* I have longer distance than any known nicking enzymes. We expect this provides good amount of freedom for sequence design for walkers with more than three legs.



**Fig. 7.** Programming Two Dimensional Patterns with Four Legs Walker



Finally, we can program the behavior of our walking machine to make two or three dimensional patterns when we achieve complete walking steps on multiple legs. Fig.7 shows a portion of such “programmable patterns”. Consider a general purpose stator grid and four legs walker. Fig.7a shows an example of a general purpose stator grid with eight kinds of sequences arranged with some regularity. If we program four legs walker with execution order “1-2-3-4” to move “go up straightly”, then a solution of walkers make a vertical stripe on the stator grid by cutting stators in the same order “1-2-3-4” as shown in Fig.7b. We can easily program other patterns like horizontal stripe, checker board, and so on, only by changing four legs layout of the walker even on only one general purpose stators grid. Moreover, we need only one kind of restriction enzyme even for any number of legs. This feature can reduce experimental complexity on tuning reaction condition for many kinds of restriction enzymes working in the same efficiency.

## 4 Conclusion

We proposed an autonomous walking machine that can follow a track with many ssDNA stators along the certain route on two or three dimensions by cleaving ssDNA stators. From a series of feasibility studies, we confirmed the molecular machine can cleave and be denatured from ssDNA stators. So it is able to walk ahead a half step. Although our experiments are still in quite preliminary level, but we believe it is an alternative to construct programmable autonomous walking machines.

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## Appendix: *Fok I* as a Nicking Enzyme

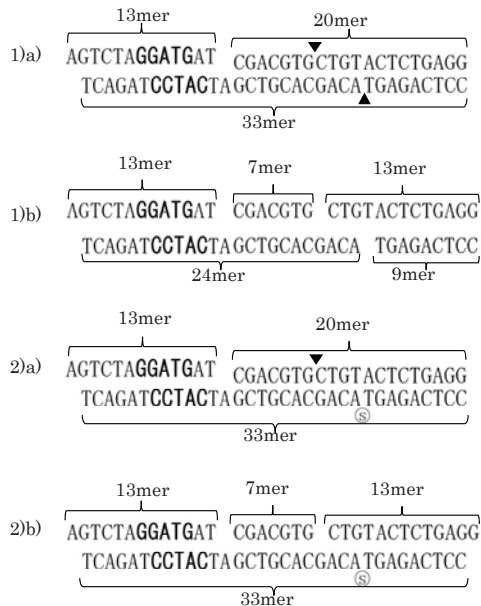
We examined to achieve a function which is the same as a nicking enzyme by using *Fok I* and phosphorothioate-modified DNA. *Fok I* cleaves both of double-strand, this cleaving activity isn't suitable for the molecular walking machine. We expected to block the cleaving activity at the cut point of one strand by phosphorothioate modified DNA (Fig. 7). Remark that for our walking machine, only legs need phosphorothioate modification.

We experimented to confirm the effect of phosphorothioate-modified DNA. We prepared five DNA strands for the experiment (Table. 3). The five DNA strands compose the following four sets;

- 1) unmodified and separated the recognition site and the cut point
- 2) phosphorothioate-modified and separated the recognition site and the cut point
- 3) unmodified and unseparated
- 4) phosphorothioate-modified and unseparated

Each four sets of DNA strands were mixed at 0.3  $\mu\text{M}$  in hybridization buffer. NEBuffer4 from New England Biolabs was used as the hybridization buffer. 4 units of *Fok I* from New England Biolabs were added to each five sets 20  $\mu\text{l}$  solution. The four sets were incubated at 37°C by 2hours.

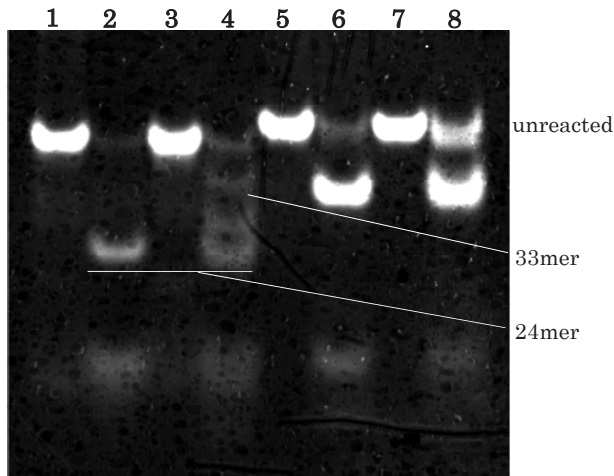
We ran the resulting solutions in 16% PAGE (non-denaturing gel).



**Fig. 8.** Phosphorothioate-modified point. 1) Unmodified (F-13, F-20, F-33), a) before cleaving b) after cleaving. 2) Phosphorothioate-modified (F-13, F-20, F-33s), a) before cleaving b) after cleaving.

**Table 3.** DNA strands. The recognition site of FokI is shown in bold type. The cut point is indicated by /, and s indicates the position of phosphorothioate-modified.

Name	Sequence(5'...3')
F-13	AGTCTAGGATGAT
F-20	CGACGTG/CTGTA <b>CT</b> CTGAGG
F-13+20	AGTCTAGGAT <b>GA</b> T <b>CG</b> ACGTG/CTGTA <b>CT</b> CTGAGG
F-33	CCTCAGAGT/ACAGCACGT <b>CG</b> AT <b>CA</b> TCCTAGACT
F-33s	CCTCAGAGT <b>s</b> ACAGCACGT <b>CG</b> AT <b>CA</b> TCCTAGACT



**Fig. 9.** Result of experiment of phosphorothioate-modified. 1)2) Unmodified and separated (F-13, F-20, F-33). 3)4) Phosphorothioate-modified and separated (F-13, F-20, F-33s). 5)6) Unmodified and not separated (F-13+20, F-33). 7)8) Phosphorothioate-modified and not separated (F-13+20, F-33s). Odd number of lane : without *Fok* I, Even number of lane : with *Fok* I.

For this result, we found phosphorothioate-modified DNA could block the cleaving activity a little (Fig. 8). There were phosphorothioate-modified DNA with separate point in lane 4, and no-modified DNA with separate point in lane 2. To compare the two lanes, we confirmed that phosphorothioate-modified DNA was cleaved. The length 24 mer band is the products of cleaving F-33 at the cut point, and there is the same band in lane 4. So this result shows phosphorothioate-modified DNA was cleaved.

In this regard, however there is the length 33 mer band in lane 4. The band shows a few F-33s remained not to be cleaved. And there is the band which shows unreacted

DNA strand. This band isn't observed in lane 2. Unmodified DNA couldn't block the cleaving activity.

The effort to block the cleaving activity by phosphorothioate-modified DNA has failed. But it was observed phosphorothioate-modified DNA had the weak inhibition of the activity. We expect that using phosphorodithioate-modified DNA leads to stronger inhibition of the activity. The molecular machine may be able to get the cleaving function which it needs by phosphorodithioate-modified DNA.