Product Name: Long ssDNA Preparation Kit (LsODN Preparation Kit)

Cat. #	Product	Size	
DS610	Long ssDNA Preparation Kit for 1.5 kb (LsODN Preparation Kit)		
	pLSODN-1	10 µg	(0.5 µg/µl)
	pLSODN-2D	10 µg	(0.5 µg/µl)
	Denaturing Gel-Loading Buffer	1 ml	(100 loadings)
	DynaMarker® Prestain Marker for RNA High	90 µl	(18 loadings)
DS620	Long ssDNA Preparation Kit for 3.0 kb (LsODN Preparation Kit)		
	pLSODN-3	10 µg	(0.5 µg/µl)
	pLSODN-4D	10 µg	(0.5 µg/µl)
	Denaturing Gel-Loading Buffer	1 ml	(100 loadings)
	DynaMarker® Prestain Marker for RNA High	90 µl	(18 loadings)

Storage Conditions: Plasmids and Denaturing Gel-Loading Buffer should be stored at -20℃. DynaMarker® Prestain Marker for RNA High should be stored at -80℃ directly from a dry ice shipping box.

Introduction: Single-stranded DNA (ssDNA) is widely used for molecular biology and biotechnology applications. Several methods were developed to generate ssDNA from double-stranded DNA (dsDNA), including asymmetric PCR, lambda exonuclease digestion, separation with denaturing-urea PAGE or separation using streptavidin-coated magnetic beads (ref. 1, 2 and 3).

The Long ssDNA Preparation Kits (LsODN Preparation Kits) give a simple and easy method for generation of a long ssDNA (pLSODN-1 and -2D for ssDNA fragments within 1,500 base and pLSODN-3 and -4D for ssDNA fragments within 3,000 base). A long ssDNA prepared by this method has defined sequence and length because it does not include inside mutation and terminal deletion caused by PCR, exonuclease side reaction, not high-fidelity reverse transcriptase reaction or not high-fidelity synthetic oligonucleotides.

The procedure of this method is almost the same as the method to obtain dsDNA fragments. The DNA of interest is cloned into a plasmid. The resulting plasmid harboring the DNA is digested with a pair of two nicking endonucleases or a combination of a nicking endonuclease and a restriction enzyme. The nicked plasmid is denatured by mixing with Denaturing Gel-Loading Buffer and then subjected to agarose gel electrophoresis. The band corresponding to a long ssDNA is excised and extracted with commercially available kits.

Additional Materials Needed:

To prepare a long ssDNA, the following materials are required.

- Nicking Endonuclease (Nt.BspQI, Nb.BsrDI, Nb.BtsI, Nb.BsmI or Nb.BbvCI)
- Agarose for gel electrophoresis (e.g. Seakem GTG™ Agarose)
- Ethidium bromide, SYBER® Green, crystal violet or any other DNA staining reagent
- DNA gel extraction kit (e.g. QIAquick® Gel Extraction Kit)

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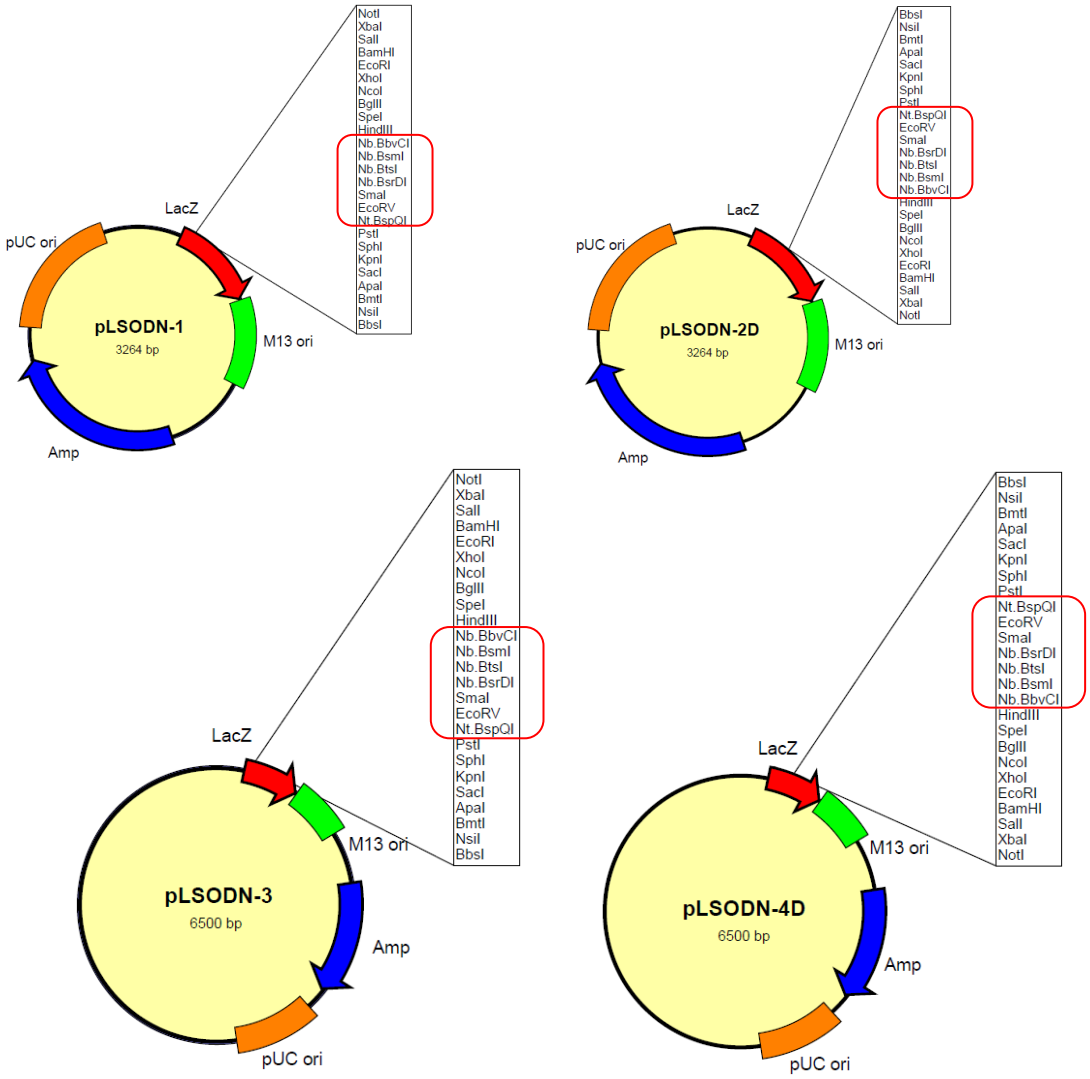
Kit Components

Plasmids

These are plasmid maps of pLSODNs. Red boxes show the region containing nicking endonucleases.



Denaturing Gel-Loading Buffer

The Denaturing Gel-Loading Buffer is a gel-loading buffer to obtain a long ssDNA of interest using agarose gel electrophoresis. The Denaturing Gel-Loading Buffer enables denaturing of the nicked pLSODN plasmid harboring the DNA of interest. Mixture of the nicked plasmid and the Denaturing Gel-Loading Buffer can be subjected to agarose gel electrophoresis. During electrophoresis, DNAs derived from the nicked plasmid keep single-strand status. To use, three volumes of the Denaturing Gel-Loading Buffer are added to the nicked plasmid, then heated and chilled to load into wells of agarose gel.

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DynaMarker® Prestain Marker for RNA High

The DynaMarker® Prestain Marker for RNA High can be used for monitoring ssDNAs on agarose gel electrophoresis in real time, although it was developed as a visible single-strand RNA molecular weight marker in formaldehyde denaturing agarose gel electrophoresis. To use the DynaMarker® Prestain Marker for RNA High, just mix with one volume of the Denaturing Gel-Loading Buffer, then directly load into a well of agarose gel without heating in the case of conventional non-denaturing agarose gel electrophoresis.

Using the DynaMarker® Prestain Marker for RNA High, electrophoresis can be monitored in real time and stopped as soon as the sufficient resolution of ssDNA bands is obtained. After electrophoresis, the visible marker allows you to cut out the gel region including a long ssDNA band to prevent contamination and reannealing with other ssDNAs exuding out of the gel during the following staining step with dye.*5

The bands of the DynaMarker® Prestain Marker for RNA High migrate at approximately the same rate as linear ssDNAs of 9,487 base, 6,487 base, 4,751 base, 3,251 base, 3,000 base and 1,500 base derived from double nicked pLSODN plasmids harboring 1.5 kb or 3.0 kb DNA fragment, pLSODN-1 (1.5 kb Fragment) and pLSODN-3 (3 kb Fragment) *1, in 1.2% non-denaturing agarose gel with 1×TAE at 100 V constant voltage.*2, *3, *4

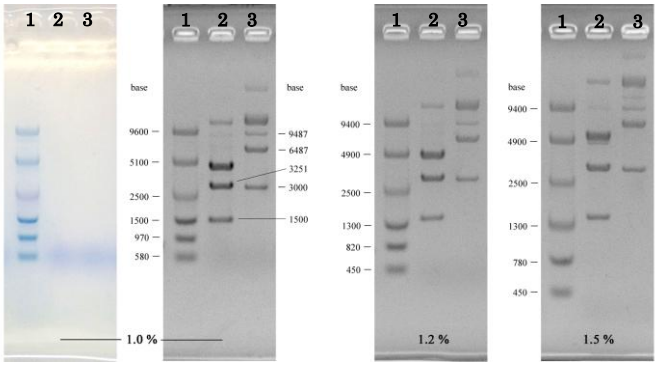

Figure 1. Electrophoresis profile of DynaMarker® Prestain Marker for RNA High, double nicked pLSODN-1 (1.5 Kb Fragment) and double nicked pLSODN-3 (3 Kb Fragment) on 1.0%, 1.2% and 1.5% non-denaturing agarose gel with 1 x TAE at 100 V constant voltage.

Lane 1: DynaMarker® Prestain Marker for RNA High
Lane 2: pLSODN-1 (1.5 Kb Fragment), double nicked and denatured
Lane 3: pLSODN-3 (3 Kb Fragment), double nicked and denatured

The left photo is the gel under white light. The others are the gels stained with ethidium bromide under the UV light.

*1 The pLSODN-1 (1.5 Kb Fragment) was constructed by inserting a 1.5 kb fragment of DNA between the Nt.BspQI and the Nb.BsrDI sites of pLSODN-1. The pLSODN-3 (3 Kb Fragment) was also constructed in a similar way. The double nicked plasmids were obtained by double digestion with Nt.BspQI and Nb.BsrDI. The double nicked pLSODN-1 (1.5 Kb Fragment) gives three linear ssDNAs (1,500 base, 3,251 base and 4,751 base) and one circular ssDNA (4,751 base). Similarly, the double nicked pLSODN-3 (3 Kb Fragment) gives three linear ssDNAs (3,000 base, 6,487 base and 9,487 base) and one circular ssDNA (9,487base).

*2 Two linear ssDNAs (4,751 base and 9,487 base) are derived from randomly broken circular ssDNAs. They originate in open circular form of plasmid DNA. The band of 4,751 base linear ssDNA often overlaps with the band of 4,751 base circular ssDNA on agarose gel electrophoresis.

*3 Each band of the DynaMarker® Prestain Marker for RNA High is calibrated against linear ssDNAs' bands derived from the double nicked plasmids above. Although bands of the linear ssDNAs derived from the double nicked plasmids may not exactly migrate according to their molecular weight due to the ssDNAs' complementarity, the usage of the relationship between molecular weights and apparent migration positions of the ssDNAs' bands derived from the double nicked plasmids is experimentally useful for identification and preparation of ssDNAs' bands derived from nicked pLSODN plasmids harboring a DNA of interest in agarose gel electrophoresis.

*4 The bands' migration of the DynaMarker® Prestain Marker for RNA High may be affected by gel concentration (as shown in figure 1), voltage of electrophoresis, buffer composition, etc. Because the DynaMarker® Prestain Marker for RNA High was optimized for single-strand RNA, its migration is different from ssDNA even on formaldehyde denaturing agarose gel electrophoresis (ref. 4). It is necessary to check the relationship with ssDNA bands on small scale analytical agarose gel electrophoresis under the same conditions as a preparative one in advance.

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*5 The DynaMarker® Prestain Marker for RNA High and DynaMarker® DNA High D (DM122) are very convenient, but if you need a highly purified long ssDNA without any impurities in the following steps, we do not recommend their use in preparative electrophoresis, as with other DNA molecular weight markers.

Experimental Outline

The procedure to obtain a long ssDNA using this method is almost the same as that of a conventional method to obtain dsDNA fragment. First, the DNA fragment of interest is cloned into the multiple cloning site of pLSODN plasmid using at least one nicking endonucleases site. The resulting pLSODN plasmid harboring the DNA fragment is digested with a pair of bottom and top nicking endonucleases or a combination of a nicking endonuclease and a restriction enzyme. The nicked plasmid is denatured and then subjected to agarose gel electrophoresis. After electrophoresis, the gel is stained with DNA staining reagent. The band corresponding to a long ssDNA is excised and extracted.

The table below describes the major steps necessary to obtain a long ssDNA of interest

Step	Action	Page
1	Cloning of the DNA of your interest into pLSODN plasmid	4-5
2	Digestion of plasmid harboring the DNA and following desalting	6
3	Analytical agarose gel electrophoresis	7-8
4	Preparative agarose gel electrophoresis	9-11

PRODUCT USAGE

Cloning of the DNA of your interest into pLSODN plasmid

The sequences below are the multiple cloning sites of pLSODNs. To obtain a long ssDNA, it is necessary to clone the DNA fragment of interest between appropriate nuclease cleavage sites in the multiple cloning sites. Figure 2 shows a schematic representation of the possible choices of cloning sites to generate a long ssDNA. There are three recommended methods:

1. Two nicking endonuclease sites (a bottom-strand nicking endonuclease and a top-strand nicking endonuclease).
2. An Nt.BspQI site and a restriction enzyme site (a top-strand nicking endonuclease and a restriction enzyme generating 5' overhang).
3. An Nb type nicking endonuclease site and a restriction enzyme site (a bottom-strand nicking endonuclease and a restriction enzyme generating 3' overhang) or BbsI.

At least one nicking endonuclease site must be used for cloning to give three ssDNAs different in size: a long ssDNA (a), a linear single-stranded vector DNA (b) and a circular or linear single-stranded whole plasmid DNA (c, d). Cloning sites should be chosen with consideration of nicking endonuclease sites and restriction enzyme sites in the DNA fragment of interest and terminal sequences of the resulting long ssDNA. If there are no appropriate cloning sites in the DNA fragment of interest because of the existence of some nicking endonuclease sites or restriction enzyme sites in it, some of these sites in the DNA fragment may need to be changed by site-directed mutagenesis not to affect the function of a long ssDNA, for example silent mutation.

* If you want a highly purified ssDNA, the quality of the pLSODN plasmid harboring the DNA of interest is important. Amounts of host RNA, host genomic DNA, degradated plasmid DNA and open circular plasmid DNA should be minimized.

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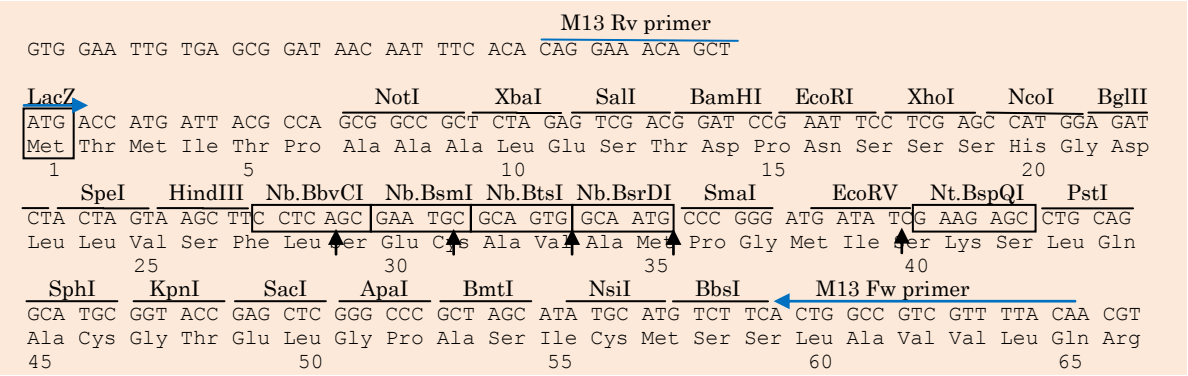
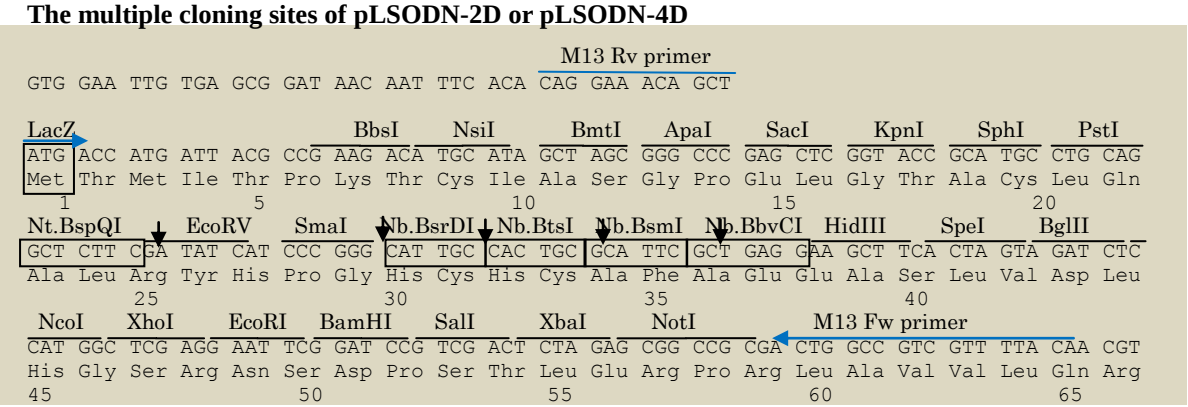
The multiple cloning sites of pLSODN-1 or pLSODN-3

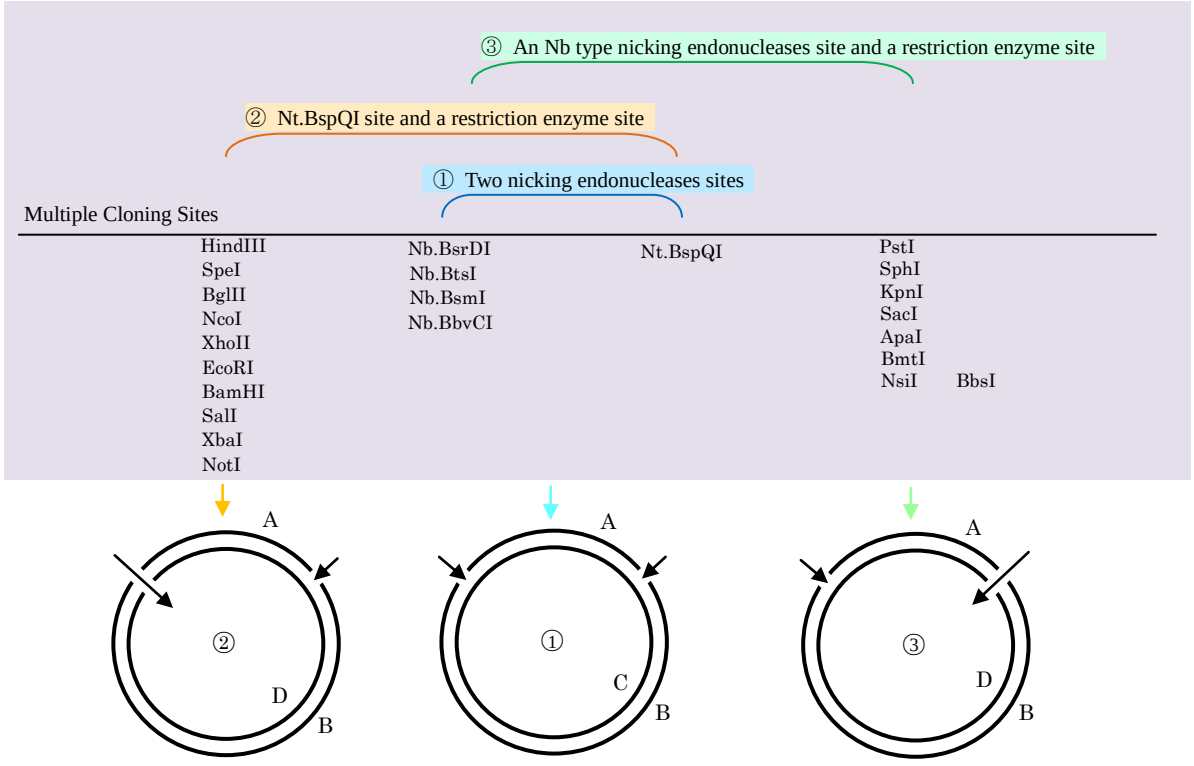


The multiple cloning sites of pLSODN-2D or pLSODN-4D



* Rectangular boxes show the nicking endonuclease sites. Downward and upward arrows show sense-strand nicking position and antisense-strand nicking position, respectively

Figure 2. A schematic representation of possible pairs of cloning sites to generate a long ssDNA



A: a long single-stranded DNA, B: a linear single-stranded vector DNA, C: a circular single-stranded whole plasmid DNA, D: a linear single-stranded whole plasmid DNA

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Digestion of plasmid harboring the DNA and following desalting

The plasmid harboring a DNA fragment of interest is digested with a pair of bottom and top nicking endonucleases or a combination of a nicking endonuclease and a restriction enzyme according to protocols of enzyme manufacturers. After digestion, the nicked plasmid solution must be desalted with ethanol precipitation, desalting spin column, etc. Desalting of nicked plasmid solution is very important for the following steps of analytical and preparative agarose gel electrophoresis of ssDNAs because excessive salt and Mg^{2+} stabilize the dsDNA and prevent complete denaturation of the nicked plasmids. One example of these procedures is shown below.

A 1.5 Kb DNA fragment of interest was cloned between the Nt.BspQI and the Nb.BsrDI sites of pLSODN-1, the resulting plasmids were designated as pLSODN-1(1.5 Kb Fragment). Similarly, pLSODN-2D (1.5 Kb Fragment), pLSODN-3 (3 Kb Fragment) and pLSODN-4D (3 Kb Fragment) were also constructed.

To generate a double nicked plasmid, pLSODN-1(1.5 Kb Fragment) was digested with Nt.BspQI and Nb.BsrDI as follows. The following 50 µl reaction mixture was set up.

Component	Volume
plasmid 100 µg	variable
10x 3.1 NEBuffer	5 µl
Nt.BspQI (10 unit/µl)	5 µl (50 unit)
Nb.BsrDI (10 unit/µl)	5 µl (50 unit)
dH_2O	variable
Total volume	50 µl

Reaction mixture was incubated at 50℃ for 60 min and then incubated at 60℃ for 60 min. The double nicked plasmid was desalted with ethanol precipitation. To the reaction solution, 125-µl ethanol was added. It was mixed well and spun at a maximum speed for 10 min. Supernatants was removed. Then, 500 µl of 70% ethanol was added to the pellet. It was vortexed well and spun at maximum speed for 5 min. Supernatant was removed. The pellet was dried and resuspended in 50-µl water. The resulting final concentration of nicked plasmid was 2 µg/µl.

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Analytical agarose gel electrophoresis

The nicked plasmid obtained in the above procedure is analyzed with conventional non-denaturing agarose gel electrophoresis.

1. Prepare loading samples by mixing the nicked plasmid and 3 volumes of the Denaturing Gel-Loading Buffer. *1, *2, *3, *4, *10
2. Heat the mixture at 70°C for 5 min, then chill on ice for 1 min. Subject the mixture to 1.2% agarose gel electrophoresis with 1 x TAE. Run at 100 V constant voltage. *5, *6, *7
3. After electrophoresis, immerse the gel in a solution containing 0.5 µg/ml of ethidium bromide for approximately 20 min. Then examine the gel on a UV illuminator (figure 3). *8

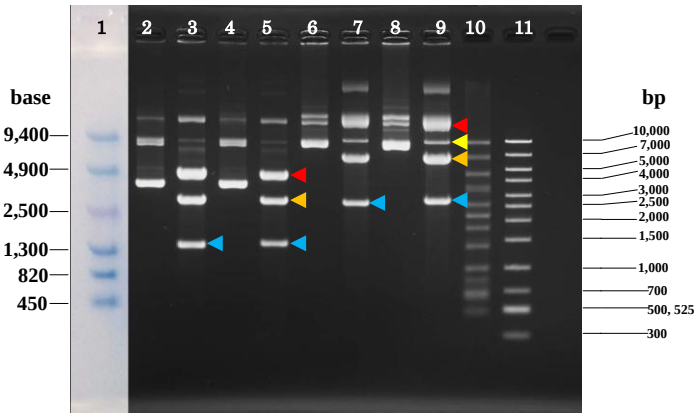

Figure 3. Analytical agarose gel electrophoresis of denatured and double nicked plasmids.
1.2% non-denaturing agarose gel in 1x TAE.

Lane 1:	DynaMarker® Prestain Marker for RNA High	5 µl	(loading volume 10 µl)
Lane 2:	pLSODN-1 (1.5Kb Fragment)	112.5 ng	
Lane 3:	pLSODN-1 (1.5Kb Fragment), double nicked and denatured	250 ng	
Lane 4:	pLSODN-2D (1.5Kb Fragment)	112.5 ng	
Lane 5:	pLSODN-2D (1.5Kb Fragment), double nicked and denatured	250 ng	
Lane 6:	pLSODN-3 (3 Kb Fragment)	112.5 ng	
Lane 7:	pLSODN-3 (3 Kb Fragment), double nicked and denatured	250 ng	
Lane 8:	pLSODN-4D (3 Kb Fragment)	112.5 ng	
Lane 9:	pLSODN-4D (3 Kb Fragment), double nicked and denatured	250 ng	
Lane 10:	DynaMarker® DNA High D, denatured *9	5 µl	(loading volume 20 µl)
Lane 11:	DynaMarker® DNA High D, non-denatured	5 µl	

Lane 1 is under white light. The other lanes are stained with ethidium bromide under the UV light. Blue, orange and red arrows show long ssDNAs, linear single-stranded vector DNAs and circular single-stranded whole plasmid DNAs, respectively. The yellow arrow shows randomly broken circular single-stranded whole plasmid DNAs. They originate in open circular form of plasmid DNA. Similar bands exist in double nicked pLSODN-1(1.5 Kb Fragment) and double nicked pLSODN-2D (1.5 Kb Fragment). However their bands overlap with bands of circular single-stranded whole plasmid DNAs.

*1 If the nicked plasmid solution contains excessive salt and Mg^{+2}, it cannot be completely denatured even by mixing with the Denaturing Gel-Loading Buffer. Desalting of the nicked plasmid solution in advance is required.

*2 Although ssDNAs give relatively weaker bands than dsDNAs do on ethidium bromide staining, 100 ng – 250 ng of nicked plasmid is enough for analytical agarose gel electrophoresis.

*3 After mixing with the Denaturing Gel-Loading Buffer, the total volume of the sample should exceed 4 µl.

*4 The DynaMarker® Prestain Marker for RNA High should be mixed with one volume of the Denaturing Gel-Loading Buffer before loading in the case of non-denaturing agarose gel electrophoresis.

*5 The composition of 1 x TAE buffer is 40 mM Tris, 20 mM acetic acid and 1 mM EDTA (ref. 6).

*6 The heating and chilling procedure of samples should be carried out just before loading. After heating and chilling, quickly load the samples into wells of the gel and promptly start electrophoresis.

The nicked plasmid DNAs denatured by the heating and chilling procedure are gradually reannealed even in the solution containing the Denaturing Gel-Loading Buffer. Furthermore, once the samples are loaded into the wells, diffusion and dilution of the Denaturing Gel-Loading Buffer in the sample solution could occur gradually.

*7 The DynaMarker® Prestain Marker for RNA High mixed with one volume of the Denaturing Gel-Loading Buffer

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does not need to be heated. It can be directly loaded into a well of the gel.

*8 In order to use the DynaMarker® Prestain Marker for RNA High in the next step, preparative gel electrophoresis, you should carry out the analytical gel electrophoresis under the same conditions as the preparative gel electrophoresis (for example the concentration of the nicked plasmid in loading sample, agarose gel concentration, buffer composition and voltage) and check the relative positions between the ssDNA bands derived from the nicked plasmid and bands of the DynaMarker® Prestain Marker for RNA High on the gel in advance. During preparative gel electrophoresis, the DynaMarker® Prestain Marker for RNA High is used as a visible ssDNA molecular indicator to monitor the electrophoresis.

*9 Although the bands' mobility of the DynaMarker® DNA High D (DM122, denatured by mixing with 3 volumes of the Denaturing Gel-Loading Buffer) is highly consistent with the mobility of linear ssDNAs derived from the double nicked plasmids on the non-denaturing agarose gel electrophoresis as shown in figure 3 (for example 9,487 base, 6,487 base, 4,751 base, 3,251 base, 3,000 base and 1,500 base), it should not be defined as an ssDNA molecular weight marker. Because each band of the DynaMarker® DNA High D is composed of complementary strands, it is possible that the bands do not keep complete single-strand status throughout electrophoresis.

The other commercially available DNA molecular weight markers mixed with the Denaturing Gel-Loading Buffer sometimes could not give clear bands. This may be caused by different migration of complementary strands due to their secondary structure or incomplete denaturation.

*10 The concentration of the nicked plasmid in the loading sample is important for preparative agarose gel electrophoresis in the next step. After mixing with the Denaturing Gel-Loading Buffer, the concentration of the nicked plasmid in the loading sample should not be over 0.5 μg/μl in the case of pLSODN-1 (1.5 Kb Fragment), pLSODN-2D (1.5 Kb Fragment), pLSODN-3 (3 Kb Fragment) or pLSODN-4D (3 Kb Fragment). If it exceeds 0.5 μg/μl, the yield of a long ssDNA decreases as a result of incomplete denaturation. Acceptable maximum concentration of a nicked plasmid may vary depending on the melting temperature of the long ssDNA. It should be checked by carrying out analytical agarose gel electrophoresis and loading various concentrations of the nicked plasmid in advance. If the acceptable maximum concentration of your nicked plasmid is too low, you should try using urea (4 M) agarose gel electrophoresis*11 and check it again.

*11 Urea (4 M) agarose gel electrophoresis is useful for stabilization of denaturing status of the nicked plasmid in the gel. In urea (4 M) agarose gel electrophoresis, 1.0% agarose gel containing 4 M urea and 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) and running buffer consisting of 4 M urea and 1x TAE buffer are used. Urea (4 M) agarose gel is made by mixing equal volumes of 1) 2% agarose gel containing 1x TAE melted by microwave and 2) 8 M urea and 1x TAE solution (not cold). After pouring the mixture into the gel mold, place it at low temperature, for example in a cold room, because it takes more time to solidify completely than non-denaturing agarose gel.

In the case of urea (4 M) agarose gel electrophoresis, the procedures are similar to those of non-denaturing agarose gel electrophoresis. Loading samples are prepared by mixing the nicked plasmid and 3 volumes of the Denaturing Gel-Loading Buffer. The mixture is heated at 70°C for 5 min, and then chilled on ice for 1 min. It is subject to urea (4 M) agarose gel electrophoresis at 100 V constant voltage. It would give a better resolution at low temperature, for example in a cold room. After electrophoresis, the gel is immersed in a solution containing 0.5 μg/ml of ethidium bromide for approximately 30 min. A sufficient amount of the solution containing DNA staining dye is needed to stain bands and dilute urea in the gel. It takes more time for staining than non-denaturing agarose gel. Then the gel is examined on a UV illuminator.

To use the DynaMarker® Prestain Marker for RNA High, it should be mixed with 3 volumes of the Denaturing Gel-Loading Buffer before loading because the density of the running buffer containing 4 M urea is higher than 1x TAE. It does not need to be heated. It can be directly loaded into a well of the gel.

The apparent molecular weights of the DynaMarker® Prestain Marker for RNA High in urea (4 M) agarose gel electrophoresis are different from those in non-denaturing agarose gel electrophoresis. They show 8,500 base, 4,400 base, 2,000 base, 1,200 base, 700 base and 350 base when they are calibrated against linear ssDNAs' bands derived from the double nicked plasmids, pLSODN-1 (1.5 Kb Fragment) or pLSODN-3 (3 Kb Fragment).

In order to use the DynaMarker® Prestain Marker for RNA High in preparative gel electrophoresis using urea (4 M) agarose gel, you should carry out the analytical gel electrophoresis under the same conditions as the preparative gel electrophoresis (for example the concentration of the nicked plasmid in the loading sample, agarose gel concentration, buffer composition and voltage) and check the relative positions between the ssDNA bands derived from the nicked plasmid and bands of the DynaMarker® Prestain Marker for RNA High on the gel in advance. During preparative gel electrophoresis, the DynaMarker® Prestain Marker for RNA High is used as a visible ssDNA molecular indicator to monitor the electrophoresis.

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Preparative agarose gel electrophoresis

The nicked plasmid is subject to preparative agarose gel electrophoresis. The procedure is similar to that of analytical gel electrophoresis.

1. Prepare loading samples by mixing the nicked plasmids and 3 volumes of the Denaturing Gel-Loading Buffer.*1, *2, *3
2. Heat the mixture at 70°C for 5 min, then chill on ice for 1 min. Subject the mixture to 1.2% agarose gel electrophoresis. Run at 100 V constant voltage.*4, *5, *6, *7, *12
3. After electrophoresis, the gel is immersed in a solution containing DNA staining reagent (figure 4).*8, *9, *10
4. After staining, a single-strand DNA band can be excised from the gel and extracted with a commercial gel extraction kit (e.g. QIAquick Gel Extraction Kit, not included in the kit) (figure 5).*11

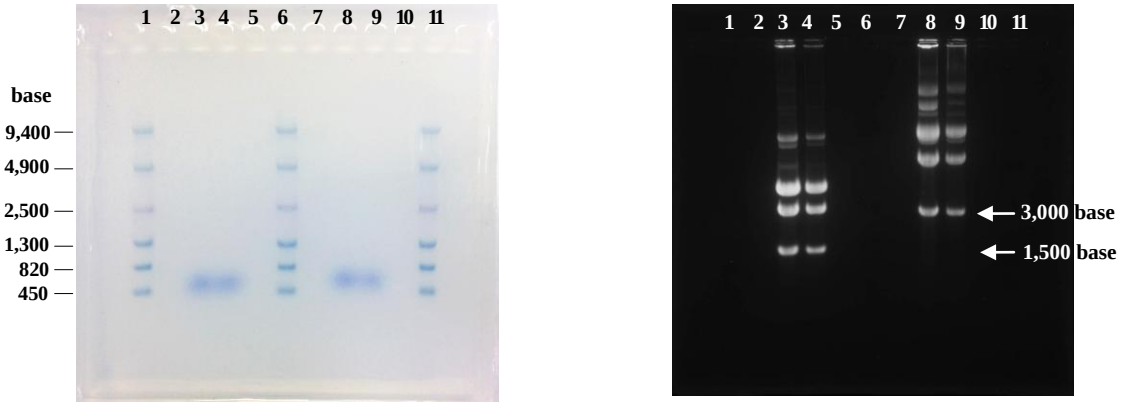

Figure 4. Preparative agarose gel electrophoresis.
1.2% non-denaturing agarose gel in 1x TAE, 12 cm x 12.5 cm. The left photo is the gel under white light. The right photo is the gel stained with ethidium bromide under the UV light.

Lane 1: DynaMarker® Prestain Marker for RNA High	10 µl	(loading volume 20 µl)
Lane 2:		
Lane 3: pLSODN-1 (1.5 Kb Fragment), double nicked	10 µg	(loading volume 20 µl)
Lane 4: pLSODN-1 (1.5 Kb Fragment), double nicked	5 µg	(loading volume 20 µl)
Lane 5:		
Lane 6: DynaMarker® Prestain Marker for RNA High	10 µl	(loading volume 20 µl)
Lane 7:		
Lane 8: pLSODN-3 (3 Kb Fragment), double nicked	10 µg	(loading volume 20 µl)
Lane 9: pLSODN-3 (3 Kb Fragment), double nicked	5 µg	(loading volume 20 µl)
Lane 10:		
Lane 11: DynaMarker® Prestain Marker for RNA High	10 µl	(loading volume 20 µl)

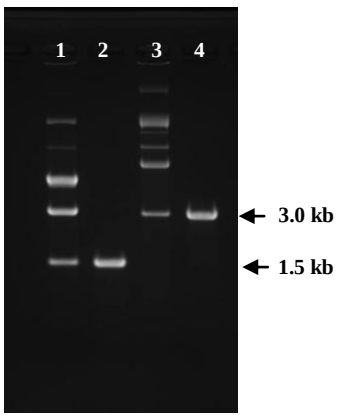

Figure 5. Agarose gel electrophoresis of purified long ssDNAs.
1.2% non-denaturing agarose gel in 1x TAE.

Lane 1: pLSODN-1 (1.5 Kb Fragment), double nicked and denatured	200 ng
Lane 2: a long ssDNA purified from pLSODN-1 (1.5Kb Fragment)	200 ng
Lane 3: pLSODN-3 (3 Kb Fragment), double nicked and denatured	200 ng
Lane 4: a long ssDNA purified from pLSODN-3 (3 Kb Fragment)	200 ng

*1 Desalting of the nicked plasmid solution in advance is also important for preparative agarose gel electrophoresis.

*2 Do not exceed acceptable maximum concentration of your nicked plasmid checked by analytical agarose gel

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electrophoresis.

*3 The DynaMarker® Prestain Marker for RNA High should be mixed with one volume of the Denaturing Gel-Loading Buffer before loading in the case of non-denaturing agarose gel electrophoresis.

*4 The heating and cooling procedure of the nicked plasmid should be carried out just before loading into wells of the gel. After loading, quickly start electrophoresis.

*5 Do not load a sample up to the top of a well because it may overflow and cause contamination.

*6 It can give a better resolution by running electrophoresis at low temperature, for example in a cold room.

*7 The DynaMarker® Prestain Marker for RNA High mixed with one volume of the Denaturing Gel-Loading Buffer does not need to be heated. It can be directly loaded into a well of the gel. You should make an empty lane between the marker and the sample to prevent contamination. During preparative gel electrophoresis, the DynaMarker® Prestain Marker for RNA High is used to monitor electrophoresis in real time. The positions of three ssDNAs can be easily elucidated from the DynaMarker® Prestain Marker for RNA High. If the distance between a long ssDNA and the other ssDNAs is sufficient, stop the electrophoresis.

*8 For example, a solution containing 0.5 µg/ml of ethidium bromide (for about 30 min) or a solution containing 1.6 µg/ml of crystal violet (for about 2–3 hr) are conventionally used for the gel staining.

Although crystal violet staining has a lower sensitivity and takes more time than ethidium bromide staining, ssDNA bands stained with crystal violet can be seen under white light. So, DNA damage by the crystal violet staining procedure must be lower than that of ethidium bromide staining, since UV light harmful to DNA is not used in the crystal violet staining procedure (ref. 5).

*9 Using the DynaMarker® Prestain Marker for RNA High, before staining you can cut out the agarose gel region including a long ssDNA with a razor blade referring to the DynaMarker® Prestain Marker for RNA High. This step may be useful for preventing a long ssDNA in the gel from contamination and rehybridization with a circular single-stranded whole plasmid DNA and then a linear single-stranded vector DNA exuding from the gel during the next staining step.

*10 You can use the 1.2% agarose gel and running buffer including 0.5 µg/ml of ethidium bromide or 1.6 µg/ml of crystal violet. In these cases, you can directly monitor the migration of ssDNA bands during electrophoresis by a handy UV lamp or with the naked eye in real time (as shown in figure 6). The electrophoresis can be stopped as soon as the bands are sufficiently resolved. A long ssDNA band may be excised and extracted just after electrophoresis. Although the sensitivity of crystal violet staining is lower than that of ethidium bromide staining, 100 ng of ssDNA band is sufficient for detection. The DNA bands moving in the gel including crystal violet can be seen with the naked eye without any additional apparatus and not be damaged by UV light harmful to DNA (ref. 5).

In the case of electrophoresis using gel which includes DNA binding dye such as ethidium bromide or crystal violet, the migration of ssDNAs is influenced by many factors such as local and temporal fluctuations of dye concentration, the DNA amount in each band, the number and position of bands, etc. It is not predictable from any DNA molecular weight marker. We do not recommend the use of DNA molecular weight markers, such as the DynaMarker® Prestain Marker for RNA High, because they are not appropriate in this case. Actually, each ssDNA band in the gel including dye can be directly seen in real time during preparative agarose gel electrophoresis and easily identified from the result of previous analytical agarose gel electrophoresis (figure 6).

Because positively charged dyes such as ethidium bromide or crystal violet migrate toward the cathode, dye concentration of the running buffer and the gel on the anode side gradually decrease. Check the dye concentration and if needed, add dye into the running buffer on the anode side during electrophoresis.

It would give a better resolution and staining by running electrophoresis at low temperature, for example in a cold room.

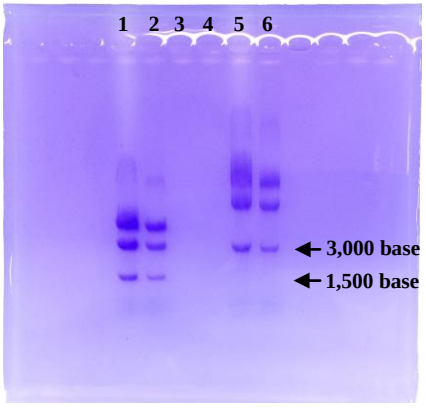

Figure 6. Preparative agarose gel electrophoresis using the gel including crystal violet.

1.2% non-denaturing agarose gel in 1x TAE, 12 cm x 12.5 cm. The loading volume of every sample is 20 µl.

Lane 1: pLSODN-1 (1.5 Kb Fragment), double nicked and denatured 10 µg
Lane 2: pLSODN-1 (1.5 Kb Fragment), double nicked and denatured 5 µg
Lane 3:
Lane 4:
Lane 5: pLSODN-3 (3 Kb Fragment), double nicked and denatured 10 µg
Lane 6: pLSODN-3 (3 Kb Fragment), double nicked and denatured 5 µg

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*11 The total yield of ssDNA recovery from a 40 μg of a nicked plasmid pLSODN-1(1.5 Kb Fragment) or pLSODN-3 (3 Kb Fragment) was about 30% using one column of QIAquick® Gel Extraction Kit.

*12 You can use urea (4 M) agarose gel for preparative agarose gel electrophoresis. In urea (4 M) agarose gel electrophoresis, 1.0% agarose gel containing 4 M urea and 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) and running buffer consisting of 4 M urea and 1x TAE buffer are used. Urea (4 M) agarose gel is made by mixing equal volumes of 1) 2% agarose gel containing 1x TAE melted by microwave and 2) 8 M urea and 1x TAE solution (not cold). After pouring the mixture into the gel mold, place it at low temperature, for example in a cold room, because it takes more time to solidify completely than non-denaturing agarose gel.

In the case of urea (4 M) agarose gel electrophoresis, the procedures are similar to those of analytical gel electrophoresis using urea (4 M) agarose gel and preparative agarose gel electrophoresis using non-denaturing agarose gel. Loading samples are prepared by mixing the nicked plasmid and 3 volumes of the Denaturing Gel-Loading Buffer. The mixture is heated at 70°C for 5 min, and then chilled on ice for 1 min. It is subject to urea (4 M) agarose gel electrophoresis at 100 V constant voltage. It would give a better resolution at low temperature, for example in a cold room. After electrophoresis, the gel is stained with a DNA staining reagent, for example a solution containing 0.5 μg/ml of ethidium bromide (for about 45 min) or a solution containing 1.6 μg/ml of crystal violet (for about 4-6hr). A sufficient amount of the solution containing DNA staining dye is needed to stain bands and dilute urea in the gel. It takes more time for staining than non-denaturing agarose gel.

The DynaMarker® Prestain Marker for RNA High should be mixed with 3 volumes of the Denaturing Gel-Loading Buffer before loading because the density of the running buffer containing 4 M urea is higher than 1x TAE. It does not need to be heated. It can be directly loaded into a well of the gel. You should make an empty lane between the marker and the sample to prevent contamination. The marker is used to monitor electrophoresis in real time. Using the DynaMarker® Prestain Marker for RNA High, before staining you can cut out the agarose gel region including a long ssDNA with a razor blade referring to the DynaMarker® Prestain Marker for RNA High. This step may be useful for preventing a long ssDNA in the gel from contamination and rehybridization with a circular single-stranded whole plasmid DNA and then a linear single-stranded vector DNA exuding from the gel during the next staining step.

After staining, a single-strand DNA band can be excised from the gel and extracted with a commercial gel extraction kit (e.g. QIAquick Gel Extraction Kit, not included in the kit) as the procedure for non-denaturing agarose gel. The total yield of ssDNA recovery from a 40 μg of a nicked plasmid pLSODN-1(1.5 Kb Fragment) or pLSODN-3 (3 Kb Fragment) was almost the same as non-denaturing agarose gel, about 30%, using one column of QIAquick® Gel Extraction Kit.

The usage of urea (4 M) agarose gel containing crystal violet is not suitable because DNA cannot be stained with crystal violet in the urea (4 M) agarose gel.

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Related Products:

DS611	Denaturing Gel-Loading Buffer	1 ml x 5	(500 loadings)
DS612	Denaturing Gel-Loading Buffer	1 ml x 2	(200 loadings)
DM260	DynaMarker® Prestain Marker for RNA High	180 µl	
DM122	DynaMarker® DNA High D	1 ml	(100 loadings)

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