Product Name: Long ssDNA Preparation Kit

Cat. #	Product	Size	
DS615	**Long ssDNA Preparation Kit for 1.5 kb**		
Box 1 (-20℃)	**pLSODN-1**	**10 µg**	**(0.5 µg/µl)**
	pLSODN-2D	**10 µg**	**(0.5 µg/µl)**
	Denaturing Gel-Loading Buffer	**1 ml**	
	Nicked pLSODN-1 (1.5 kb Fragment)	**100 µl**	**(0.1µg/µl, 50 loadings)**
Box 2 (RT)	**Long ssDNA Gel Extraction Kit for 3kb (#DS640)**	**25 preps**	
DS625	**Long ssDNA Preparation Kit for 3.0 kb**		
Box 1 (-20℃)	**pLSODN-3**	**10 µg**	**(0.5 µg/µl)**
	pLSODN-4D	**10 µg**	**(0.5 µg/µl)**
	Denaturing Gel-Loading Buffer	**1 ml**	
	Nicked pLSODN-3 (3 kb Fragment)	**100 µl**	**(0.1µg/µl, 50 loadings)**
Box 2 (RT)	**Long ssDNA Gel Extraction Kit for 3kb (#DS640)**	**25 preps**	

Storage Conditions:

Box 1 should be stored at -20℃.
Box 2 should be stored at 15-25℃, stable for up to 24 months from the date of receipt.

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 give a simple and easy method for generation of a long ssDNA (pLSODN-1 and -2 for ssDNA fragments within 1,500 bases and pLSODN-3 and -4 for ssDNA fragments within 3,000 bases). 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, non-high-fidelity reverse transcriptase reaction or non-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 the Long ssDNA Gel Extraction Kit.

The Long ssDNA Gel Extraction Kit is highly optimized for long ssDNA purification. It gives high yield and high purity of a long ssDNA.

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
- Ethanol and isopropanol.
- Heat block for 50-70℃ incubation for gel extraction.

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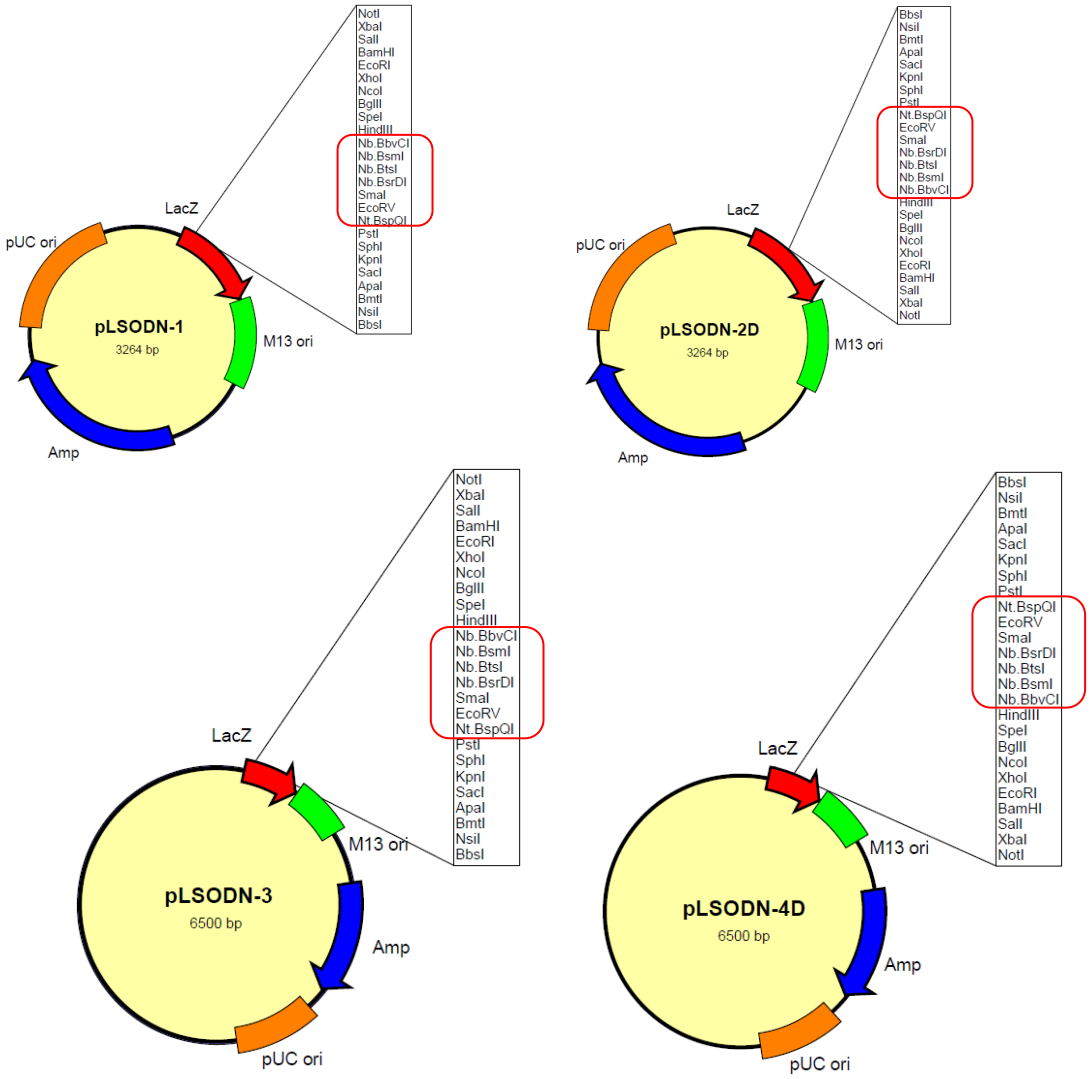
KIT COMPONENT

1) Plasmids

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



2) 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. A 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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3) Nicked pLSODN-1 (1.5 kb Fragment) or Nicked pLSODN-3 (3 kb Fragment) *1

The Nicked pLSODN-1 (1.5 kb Fragment) in DS615 or Nicked pLSODN-3 (3 kb Fragment) in DS625 can be used for molecular weight marker of long ssDNAs on analytical agarose gel electrophoresis.*2 Although the nicked plasmids give only three bands, they are useful to monitor experiments and to identify a long ssDNA of interest as the plasmid backbone is the same as the plasmids in the kit.*4

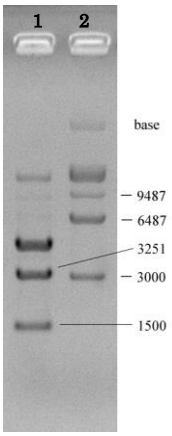

Figure 1. Electrophoresis profile of double-nicked pLSODN-1 (1.5 kb Fragment) and double-nicked pLSODN-3 (3 kb Fragment) *1, *2, *3

1.2% agarose gel in 1x TAE at 100 V.
Lane 1: pLSODN-1 (1.5 kb Fragment), double-nicked and denatured
Lane 2: pLSODN-3 (3 kb Fragment), double-nicked and denatured
The gel was stained with ethidium bromide under 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 bases, 3,251 bases and 4,751 bases) and one circular ssDNA (4,751 bases). Similarly, the double-nicked pLSODN-3 (3 kb Fragment) gives three linear ssDNAs (3,000 bases, 6,487 bases and 9,487 bases) and one circular ssDNA (9,487 bases).

*2 Both markers are supplied at a concentration of 0.1 µg/µl in 75% Denaturating Gel-Loading buffer. It is ready-to-use. To use, heat and chill before loading into wells of agarose gel.
Although ssDNAs give relatively weaker bands than dsDNAs on ethidium bromide staining, 150 ng – 3000 ng of nicked plasmid is enough for analytical agarose gel electrophoresis.

*3 Two linear ssDNAs (4,751 bases and 9,487 bases) are derived from randomly broken circular ssDNAs. They originate in an 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.

*4 The Nicked pLSODN-1 (1.5 kb Fragment) or Nicked pLSODN-3 (3 kb Fragment) is very convenient in analytical agarose gel electrophoresis. But if you need a highly purified long ssDNA without any impurities, we do not recommend their use in preparative electrophoresis.

4) Long ssDNA Gel Extraction Kit for 3kb (# DS640)

Kit Components: 25 preps

Components	Contents	Size
Crystal Violet Solution	Crystal Violet 4mg/ml (2,500 x).	5 ml
Gel-Dissolving Buffer	It contains guanidine thiocyanate as *chaotropic salts*.	45 ml
Wash Buffer 1	Tris-base buffer (Add 45 ml of 100 % ethanol to the Wash Buffer before use.)	8 ml
Wash Buffer 2	Tris-base buffer (Add 45 ml of 100 % ethanol to the Wash Buffer before use.)	11 ml
Elution Buffer	10 mM Tris-HCl buffer, pH 8.0.	5 ml
Spin Column		25 pieces
Collection Tube		25 pieces

• If precipitated material has formed in Gel-Dissolving Buffer, heat to dissolve at 37℃。

The Long ssDNA Gel Extraction Kit is a highly-specialized kit for long ssDNA purification from agarose gel. The spin column, buffer formulations and purification protocol are optimized for high recovery yields

and high purity of long ssDNA. Long ssDNA purified with the kit is high-quality, with low mechanical degradation and without ultraviolet light-induced damage from the purification process. The purified long ssDNA is suitable for molecular biology and biotechnology applications.

The Long ssDNA Preparation Kit can be universally used for purification and clean-up of long ssDNA.

Features and Specifications:

- Optimized for long ssDNA.
- High recovery yield (typically 75-90%).
- High purity.
- Low mechanical degradation.
- No UV light-induced DNA damage.
- Direct monitoring of the migration of long ssDNA blue bands in the gel during electrophoresis.
- Excision of long ssDNA under ambient light.
- Guanidine thiocyanate is used as chaotropic salts. NaI is not used.*1
- ssDNA size for excision: 500 - 3,000 bases. *2
- Binding capacity on a Spin Column for ssDNA binding is up to 5µg.
- Elution volume: ≥ 15 µl.

*1 Residual NaI may be difficult to remove, and reduces the efficiency of downstream enzymatic reactions.

*2 200 bases of long ssDNA also can be extracted using the kit, but the recovery yield is not so high (about 40-45%).

Caution! Wear gloves and protective clothing while handling the Crystal Violet.

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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-strand 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.

PRODUCT USAGE

STEP 1: 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 ssDNA fragments: 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

```
                                                                M13 Rv primer
GTG GAA TTG TGA GCG GAT AAC AAT TTC ACA CAG GAA ACA GCT

LacZ                                    NotI      XbaI      SalI      BamHI     EcoRI     XhoI      NcoI      BglII
ATG ACC ATG ATT ACG CCA GCG GCC GCT CTA GAG TCG ACG GAT CCG AAT TCC TCG AGC CAT GGA GAT
Met Thr Met Ile Thr Pro  Ala Ala Ala Leu Glu Ser Thr Asp Pro Asn Ser Ser Ser His Gly Asp
  1               5                  10                  15                  20
     SpeI    HindIII  Nb.BbvCI  Nb.BsmI  Nb.BtsI  Nb.BsrDI  SmaI     EcoRV   Nt.BspQI  PstI
CTA CTA GTA AGC TTC CTC AGC GAA TGC GCA GTG GCA ATG CCC GGG ATG ATA TCG AAG AGC CTG CAG
Leu Leu Val Ser Phe Leu Ser Glu Cys Ala Val Ala Met Pro Gly Met Ile Ser Lys Ser Leu Gln
         25                  30                  35                  40
  SphI     KpnI     SacI     ApaI     BmtI     NsiI     BbsI     M13 Fw primer
GCA TGC GGT ACC GAG CTC GGG CCC GCT AGC ATA TGC ATG TCT TCA CTG GCC GTC GTT TTA CAA CGT
Ala Cys Gly Thr Glu Leu Gly Pro Ala Ser Ile Cys Met Ser Ser Leu Ala Val Val Leu Gln Arg
45                  50                  55                  60                  65
```

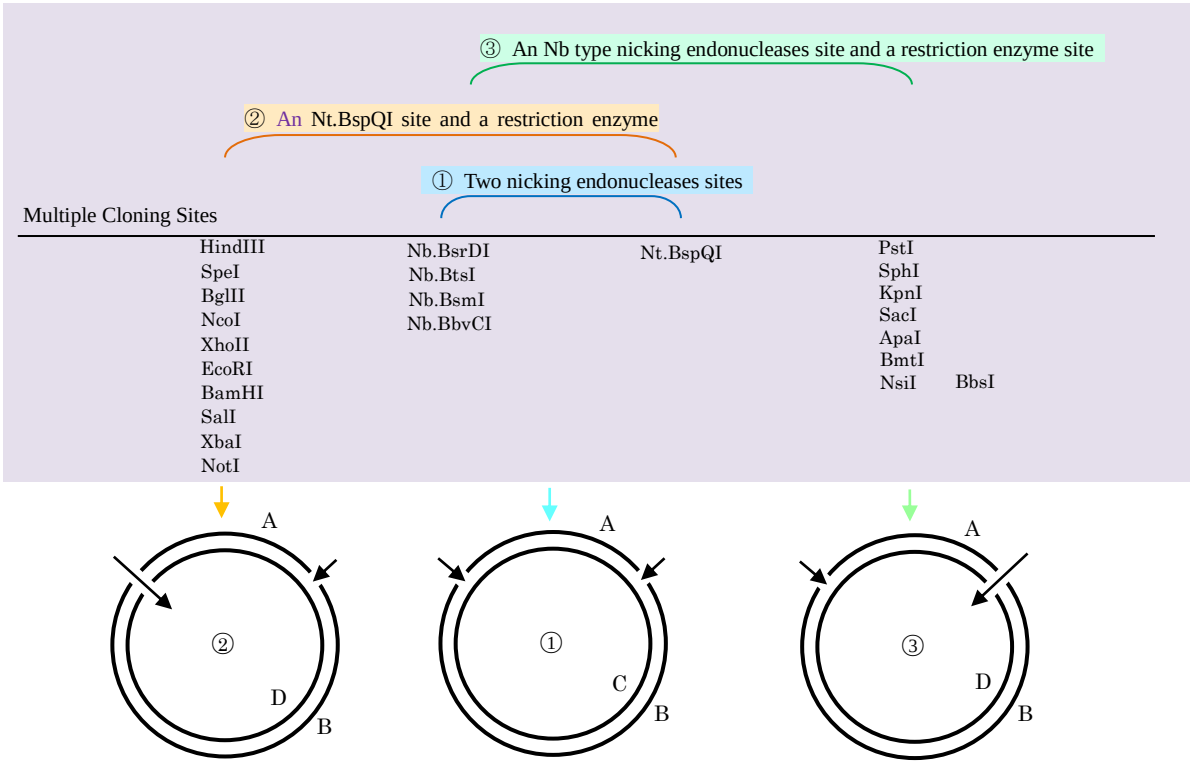
The multiple cloning sites of pLSODN-2D or pLSODN-4D

```
                                                                M13 Rv primer
GTG GAA TTG TGA GCG GAT AAC AAT TTC ACA CAG GAA ACA GCT

LacZ                                    BbsI     NsiI      BmtI     ApaI     SacI     KpnI     SphI     PstI
ATG ACC ATG ATT ACG CCG AAG ACA TGC ATA GCT AGC GGG CCC GAG CTC GGT ACC GCA TGC CTG CAG
Met Thr Met Ile Thr Pro Lys Thr Cys Ile Ala Ser Gly Pro Glu Leu Gly Thr Ala Cys Leu Gln
  1               5                  10                  15                  20
Nt.BspQI   EcoRV    SmaI   Nb.BsrDI  Nb.BtsI  Nb.BsmI  Nb.BbvCI  HidIII    SpeI     BglII
GCT CTT CGA TAT CAT CCC GGG CAT TGC CAC TGC GCA TTC GCT GAG GAA GCT TCA CTA GTA GAT CTC
Ala Leu Arg Tyr His Pro Gly His Cys His Cys Ala Phe Ala Glu Glu Ala Ser Leu Val Asp Leu
         25                  30                  35                  40
 NcoI     XhoI     EcoRI    BamHI    SalI     XbaI     NotI     M13 Fw primer
CAT GGC TCG AGG AAT TCG GAT CCG TCG ACT CTA GAG CGG CCG CGA CTG GCC GTC GTT TTA CAA CGT
His Gly Ser Arg Asn Ser Asp Pro Ser Thr Leu Glu Arg Pro Arg Leu Ala Val Val Leu Gln Arg
45                  50                  55                  60                  65
```

* 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



At least one nicking endonuclease site must be used.

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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STEP 2: Digestion of plasmid harboring the DNA

The plasmid harboring a DNA fragment of interest is digested with a pair of bottom and top-strand nicking endonucleases or a combination of a nicking endonuclease and a restriction enzyme according to protocols of enzyme manufacturers. 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

The reaction mixture was incubated at 50℃ for 60 min and then incubated at 60℃ for 60 min.

STEP 3: Desalting with ethanol precipitation

After digestion, the nicked plasmid solution must be desalted with ethanol precipitation. **Desalting of nicked plasmid solution is very important** for agarose gel electrophoresis of ssDNAs because residual salt and Mg^{2+} prevent complete denaturation of the nicked plasmids. Imcomplete denaturation of the nicked plasmid appears as a poor band of a long ssDNA on gel electrophoresis, resulting in low yield of the long ssDNA.

The double-nicked plasmid shoud be desalted with ethanol precipitation as follows.

1. Add 1/10 volume of Sodium Acetate (3 M, pH 5.2) or NaCl (1M) to the nicked plasmid solution.*1, *2
2. Add 2.5 volumes ethanol or 0.7 volume isopropanol and mix well.
3. Incubate at room temperature or on ice for 10 min.
4. Spin at maximum speed for 10 min at 4℃- room temperature. Discard supernatant.
5. Spin again for 1 min. Discard residual supernatant completely with pipette tip.*3
6. Add 70% ethanol and vortex vigorously.*4
7. Spin for 5 min. Discard supernatant.
8. Spin again for 1min. Discard residual supernatant completely with pipette tip.
9. Repeat rinse with 70% ethanol (Step 8-12). *5
10. Dry the pellet.
11. Dissolve pellet in TE or dH_2O.*6

*1 Before ethnol precipitaton, denaturation of nicking enzyme or restriction enzyme with heat or phenol extraction might be needed. In the case of Nt.BspQI and Nb.BsrDI, denaturation was not needed.

*2 Before addition of salt, NaCl concentration in reaction buffer should be checked. (e.g. already 100 mM NaCl in 1x 3.1 NEBuffer).

*3 **Completely remove supernatant in order to desalt sufficiently.**

*4 The pellet might be swimming in the 70% EtOH but it is OK. Don't be afraid of the pellet dissolving, be afraid of any salt remaining.

*5 **Rinse twice with 70% ethanol in order to desalt sufficiently.**

*6 The final concentration of nicked plasmid might be 1-3 µg/µl. It may be easy to handle.

STEP 4: Analytical agarose gel electrophoresis

The nicked plasmid obtained in the above procedure is analyzed with agarose gel electrophoresis.

1. Prepare loading samples by mixing the nicked plasmid 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 with 1 x TAE. Run at 100 V constant voltage.*4, *5
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).

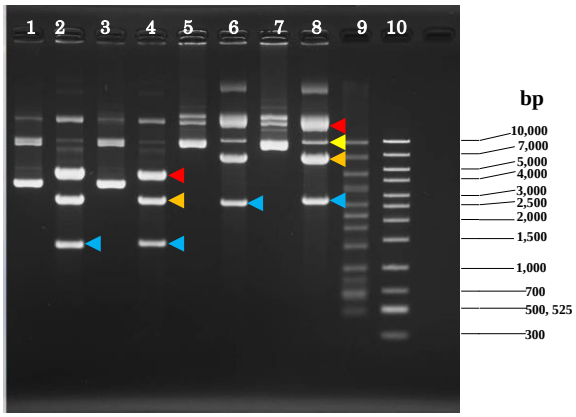

Figure 3. Analytical agarose gel electrophoresis of denatured and double-nicked plasmids.

1.2% agarose gel in 1x TAE.

Lane 1:	pLSODN-1 (1.5 kb Fragment)	112.5 ng	
Lane 2:	pLSODN-1 (1.5 kb Fragment), double-nicked and denatured	250 ng	
Lane 3:	pLSODN-2D (1.5 kb Fragment)	112.5 ng	
Lane 4:	pLSODN-2D (1.5 kb Fragment), double-nicked and denatured	250 ng	
Lane 5:	pLSODN-3 (3 kb Fragment)	112.5 ng	
Lane 6:	pLSODN-3 (3 kb Fragment), double-nicked and denatured	250 ng	
Lane 7:	pLSODN-4D (3 kb Fragment)	112.5 ng	
Lane 8:	pLSODN-4D (3 kb Fragment), double-nicked and denatured	250 ng	
Lane 9:	DynaMarker® DNA High D, denatured*6	5 μl	(loading volume 20 μl)
Lane10:	DynaMarker® DNA High D, non-denatured	5 μl	

The gel was stained with ethidium bromide under UV light. The 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 an open circular form of plasmid DNA.

*1 **Desalting of the nicked plasmid solution in advance is important.** 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, resulting in a weak band of long ssDNA of interest (see "STEP 3: Desalting with ethanol precipitation" at page 7).

*2 Although ssDNAs give relatively weaker bands than dsDNAs on ethidium bromide staining, 150 ng – 300 ng of nicked plasmid is enough for analytical agarose gel electrophoresis.

*3 After mixing with the Denaturing Gel-Loading Buffer, **the concentration of the nicked plasmid in the loading sample should be under 0.5 μg/μl.** If it exceeds 0.5 μg/μl, the band of a long ssDNA is weakened as a result of incomplete denaturation. It results in a low recovery yield of the long ssDNA. The acceptable maximum concentration of a nicked plasmid may vary depending on the melting temperature of the long ssDNA. It could be checked by carrying out analytical agarose gel electrophoresis by loading several concentrations of the nicked plasmid.

*4 The heating and chilling procedure of samples should be carried out just before loading.

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

*6 Commercially available DNA molecular weight markers sometimes could not give clear bands after mixing with Denaturing Gel-Loading Buffer. It may be caused by different migration of complementary strands due to their secondary structure or incomplete denaturation.

STEP 5: Preparative agarose gel electrophoresis

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

ⅰ) Preparation of 1.2% agarose gel containing Crystal Violet

An example of this is given for making 100 ml of a 1.2% agarose gel.

1. Add 1.2 g of agarose powder to 100 ml of 1×TAE buffer and mix them.
2. Dissolve agarose in the microwave.
3. Add 40µl of Crystal Violet Solution to the agarose (100 ml) and mix them by swirling.
4. Pour the agarose into a mold and set a comb.* Allow the gel to harden.

* It is recommended to make thick gel with deep wells so as not to cause the overflow of a loaded sample in a well, resulting in contamination.

ⅱ) Preparative agarose gel electrophoresis and excision

A simple way is shown in the following example.

1. Pre-chill 1xTAE running buffer containing Crystal Violet (add 40µl Crystal Violet /100ml 1xTAE) on ice or in a refrigerator. *1
2. Put the horizontal gel electrophoresis apparatus on ice in an ice bucket in a draft chamber. *2, *3, *4
3. Pour pre-chilled 1xTAE running buffer containing Crystal Violet into the electrophoresis apparatus. *5
4. Put the agarose gel into the electrophoresis apparatus.
5. Prepare loading samples by mixing the long ssDNAs sample (e.g. the nicked plasmid) and 3 volumes of the Denaturing Gel-Loading Buffer. *6, *7, *8
6. Heat the mixture at 70°C for 5 min, then chill on ice for 1 min. Subject the mixture to agarose gel electrophoresis. *9, *10
7. Run the gel at 100 V at a low temperature (less than 20℃). *2, *3, *4
8. Monitor the long ssDNA blue bands moving in the gel during electrophoresis (Figure 1). Stop the electrophoresis after the bands are sufficiently resolved. *11, *12
9. Excise the long ssDNA band of interest from the agarose gel with a scalpel. *13, *14

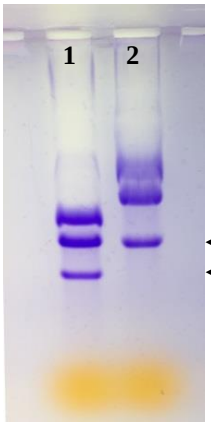

Figure 1. Agarose gel electrophoresis using the gel containing Crystal Violet.
1.2% agarose gel in 1x TAE, 5.5cm x 6.0 cm. The loading volume of each sample is 24 µl.
Lane 1: pLSODN-1 (1.5 kb Fragment), double-nicked *15 12 µg
Lane 2: pLSODN-3 (3 kb Fragment), double-nicked *15 12 µg

*1 1xTAE with Crystal Violet (add 40 µl Crystal Violet /100ml TAE) should be used as running buffer.

*2 For the safe use of Crystal Violet, electrophoresis with Crystal Violet should be carried out in a draft chamber. Don't do electrophoresis with Crystal Violet or ethidium bromide in a closed space (especially in a cold room). These dyes diffuse into the air with the water vapor or bubbles generated by electrolysis during electrophoresis.

*3 Electrophoresis at a low temperature is recommended (less than 20℃). It gives a better resolution and strong Crystal Violet staining. If the temperature of gel and running buffer exceeds 25℃, Crystal Violet staining of long ssDNA becomes weak. If so, the gel can be restained by gentle shaking in a dilute Crystal Violet solution (40 µl of Crystal Violet Solution / 100 ml of water) for 0.5 to 1 hour after electrophoresis.

*4 To keep the gel temperature low during electrophoresis in a draft chamber, we recommend placing the electrophoresis apparatus on ice in an ice bucket.

*5 At least, use a pre-chilled 1xTAE running buffer on ice to keep a low temperature.

*6 Denaturing Gel-Loading Buffer is not included in the kit. It can be purchased individually from BioDynamics Laboratory Inc. (see “Related Product “on page 4)

*7 **Desalting of the long ssDNAs sample (or the nicked plasmid) solution in advance is important** for agarose gel electrophoresis. If the long ssDNAs sample (or the nicked plasmid) solution contains excessive salt and Mg^{+2}, it adversely affects denaturation, resulting in giving poor bands and low recovery yields of the long ssDNA of interest. If your long ssDNA band is extremely weak, first you should consider the existence of residual salt in the long ssDNAs sample (or the nicked plasmid) solution. We recommend removing the salt in the long ssDNAs sample through ethanol precipitation. Importantly, wash with 70% ethanol twice, vortex vigorously during washing.

*8 To get a highly purified long ssDNA, the long ssDNA of interest should be loaded greater than 1µg/band. **At the same time, the concentration of the nicked plasmid in the loading sample should be under 0.5 µg/µl** after mixing with the Denaturing Gel-Loading Buffer.

*9 The heating and cooling procedure of the long ssDNAs sample (e.g. the nicked plasmid) mixture should be carried out just before loading into wells of the gel.

*10 Do not load a sample too much in a well because it may overflow and cause contamination. It is recommended to make deep wells with thick gel.

*11 **Long ssDNA bands moving in Crystal Violet-containing gel can be directly seen under ambient light in real time during electrophoresis.** The electrophoresis can be stopped as soon as the bands are sufficiently resolved.

*12 Because crystal violet (positively-charged molecule) migrates toward the cathode, the dye concentration of the running buffer and the gel on the anode side gradually decreases. Check the dye concentration and if needed, add dye into the running buffer on the anode side during electrophoresis (e.g. 25 µl of Crystal Violet Solution is added to 250 ml running buffer on the anode side every 30 min at 100V constant voltage).

*13 The long ssDNA band can be visible as a blue band and can be easily excised from the agarose gel on the bench.

*14 Minimize the size of the gel slice by removing extra agarose.

*15 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 (see the Data Sheet of the Long ssDNA Preparation Kit #615 & #625). 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 bases, 3,251 bases and 4,751 bases) and one circular ssDNA (4,751 bases). Similarly, the double-nicked pLSODN-3 (3 kb Fragment) gives three linear ssDNAs (3,000 bases, 6,487 bases and 9,487 bases) and one circular ssDNA (9,487 bases).

STEP 6: DNA extraction and purification

Before start:

- Add 45 ml of 100 % ethanol to Wash Buffer 1 and Wash Buffer 2 bottles, respectively.
- Isopropanol is required in the step.
- All centrifugation steps should be carried out at 16,000×g (around **13K rpm** in a conventional microcentrifuge) at room temperature (20℃- 25℃). Centrifugation at lower temperature might affect long ssDNA yield.
- This kit can also be used for long ssDNA clean-up from enzymatic reactions. For this purpose, add 3 volumes of Gel-Dissolving Buffer and 1 volume of isopropanol to the reaction, mix well, and proceed with step 5 of the protocol.

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1. Transfer the gel slice to a 1.5 ml microcentrifuge tube and weight the gel slice.
2. Add 3 volumes of Gel-Dissolving Buffer.
3. Incubate the tube at 50℃, vortexing periodically until the gel slice is completely dissolved for 10-15 min. *1
4. Add one gel volume of isopropanol to the dissolved gel and mix well.
5. Insert a Spin Column into a Collection Tube.
6. Load the sample to the Spin Column and centrifuge for 1 min. Discard the flow-through in the Collection Tube with a 1ml pipette tip. *2, *3, *4
7. Centrifuge the Spin Column again for 1 min. Remove the residual flow-through completely with a 10 µl or a 100 µl pipette tip. *5
8. Add 500 µl of Wash Buffer 1 to the Spin Column and centrifuge for 1 min. Discard the flow-through in the Collection Tube with a 1ml pipette tip (1st Wash). *3, *4
9. Repeat wash with Wash Buffer 1 (Step 8, 2nd Wash). *3, *4
10. Add 500 µl of Wash Buffer 1 to the Spin Column, close the cap tightly and vortex for 5 seconds to wash the whole inner wall of the Spin Column (3rd Wash).
11. Centrifuge for 1 min. Keep the flow-through in the Collection Tube and vortex for 5 seconds to wash both the outer wall of the Spin Column and the inner wall of the Collection Tube.
12. Centrifuge for 1 min. Discard the flow-through in the Collection Tube using a 1ml pipette tip. *3, *4
13. Add 500 µl of Wash Buffer 2 to the Spin Column and centrifuge for 1 min. Discard the flow-through in the Collection Tube with 1ml pipette tip (4th Wash). *3, *4
14. Centrifuge the Spin Column again for 1 min to remove residual Wash Buffer 2 completely.
15. Transfer the Spin Column into a new microcentrifuge tube.
16. Add 15-40 µl of Elution Buffer onto the Spin Column and incubate it at 70℃ for 5 min. *6
17. Directly Centrifuge the Spin Column for 1 min to elute long ssDNA. *7

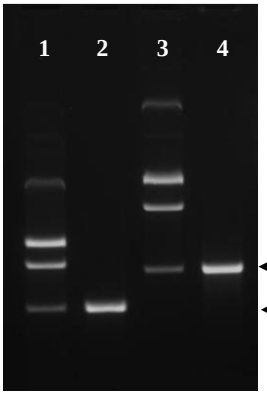

Figure 2. Agarose gel electrophoresis of purified long ssDNAs.
1.2% agarose gel in 1x TAE. 5.5cm x 6.0 cm.
Lane 1: pLSODN-1 (1.5 kb Fragment), double-nicked and denatured 800 ng
Lane 2: a long ssDNA purified from pLSODN-1 (1.5 kb Fragment) 400 ng
Lane 3: pLSODN-3 (3 kb Fragment), double-nicked and denatured 800 ng
Lane 4: a long ssDNA purified from pLSODN-3 (3 kb Fragment) 400 ng

*1 Dissolve the agarose completely. If not, it might decrease the recovery yield of long ssDNA and cause contamination of agar or buffers.

*2 If the volume of dissolved sample exceeds 500 µl, the loading of the sample onto the column should be performed in multiple rounds not to exceed 500 µl.

*3 Don't flip the Collection Tube to discard the flow-through. It contaminates the edge and upper inner wall of the Collection Tube. Use a 1 ml pipette tip to discard the flow-through.

*4 Be careful to ensure the column tip does not contact column flow-through. If possible, we recommend using a new Collection Tube at several washing steps to avoid contamination. However, spare collection tubes are not included in the kit. They can be purchased from many companies (e.g. Corning Axygen #MCT-200-NC).

*5 Don't wash the Spin Column with Gel-Dissolving Buffer only.

*6 Typical elution volumes are 15 µl-40 µl. Water can be used to elute the long ssDNA. The average elution volume is 38 µl from 40 µl Elution Buffer volume, and 13 µl from 15 µl.

*7 The total yield of ssDNA recovery from a 6 µg of a nicked plasmid pLSODN-1(1.5 kb Fragment) or pLSODN-3 (3 kb Fragment) was about 1.7 µg (100% recovery is 2 µg) using one column of the Long ssDNA Gel Extraction Kit for 3 kb with 40 µl Elution Buffer.

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Troubleshooting Guide, FAQ

TROUBLE	POSSIBLE CAUSE	SOLUTION
The ssDNA band does not clearly separate during electrophoresis.	Salt remaining	Desalting of the nicked plasmid solution is very important. ssDNA bands may not separate clearly if salt remains. Wash thoroughly with 70% ethanol in ethanol precipitation. Specifically, wash with 70% ethanol twice and vortex vigorously during washing.
Low ssDNA yield		
Is it possible to prepare ssDNA smaller than 1.5 kb using Long ssDNA preparation kit for 3.0kb (Cat. No. DS625)?	In principle, it is also possible to prepare ssDNA smaller than 1.5 kb with this kit, Long ssDNA Preparation Kit for 3kb (Cat. No. DS625). However, "Long ssDNA Preparation Kit for 1.5kb (Cat. No. DS615)" is more suitable for the preparation of ssDNA smaller than 1.5 kb. We recommend you to use #DS615. In order to obtain the same amount of ssDNA with #DS625 as compared with #DS615 a larger amount of plasmid is needed. Also, as the concentration of plasmid to apply to electrophoresis gel is limited (about 0.5 μg/μl), the ssDNA concentration in the band also decreases. This may affect the purification degree and yield.	

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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)
DS635	Long ssDNA Preparation Kit for 10 kb		
DS650	Long ssDNA Gel Extraction Kit for 10 kb		
DM122	DynaMarker® DNA High D	1 ml	(100 loadings)
DS230	JetGiga Competent Cell (DH5α)	100 μl x 10	

Purchaser Notification

The product shall be used by the purchaser for internal research purpose only. The products and plasmids that are modified or harboring a DNA may not be transferred to third parties, resold, modified for resale, or used to manufacture commercial products or to provide a service to third parties without prior written approval of Biodynamics laboratory Inc.

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