

Long ssDNA Preparation Kit for 10kb



Cat. #	Product	Size
DS635	Long ssDNA Preparation Kit for 10kb	
	pLSODN PCR Template	20 μ l
	Denaturing Gel-Loading Buffer (DS611, DS612)	1 ml
	λ /HindIII DNA for long ssDNA preparation kit (DS670)	200 μ l (25 ng/ μ l)
	Long ssDNA Gel Extraction kit for 10kb (DS650)	25 preps

Storage Conditions:

pLSODN PCR template should be stored at -80°C .

Denaturing Gel-Loading Buffer should be stored under -20°C .

λ /HindIII DNA for long ssDNA preparation kit should be stored at -80°C .

Long ssDNA Gel Extraction kit for 10kb should be stored at $15-25^{\circ}\text{C}$, stable for up to 2 years from the date of receipt.

Introduction:

3-10 kb long single-stranded DNA (ssDNA) can be prepared simply and easily with this kit. A long ssDNA prepared by this kit has defined sequence and length. 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.

In this method, at first the DNA fragment of interest is cloned into a plasmid. This plasmid is digested with a pair of two nicking endonucleases or combination of nicking endonuclease and restriction enzyme. The nicked plasmid is denatured by Denaturing Gel-Loading Buffer and subjected to agarose gel electrophoresis. The band of long ssDNA is excised and extracted with Long ssDNA Gel Extraction Kit for 10kb.

Additional Materials Needed:

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

- DNA polymerase for PCR suitable for long amplicons (PrimeSTAR GXL DNA Polymerase (Takara Bio Inc.) is recommended)
- DpnI
- DNA extraction kit from agarose gel (e.g. Monarch DNA Gel Extraction Kit (New England Biolabs Inc.))
- DNA seamless assembly kit (NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs Inc) is recommended).
- Competent cell which has high competency (e.g. DH5 α).
- Plasmid purification kit (e.g. QIAGEN plasmid kit (QIAGEN N.V.))
- Nicking Endonuclease (Nt.BspQI, Nb.BsrDI, Nb.BtsI, Nb.BsmI or Nb.BbvCI)
- Agarose for gel electrophoresis (Seakem GTG Agarose (Lonza Rockland, Inc.) is recommended)

The sequence of pLSODN PCR Template:

You can obtain the sequences of pLSODN PCR template from our website.

(http://www.biodynamics.co.jp/e/prd_ds610.htm)

You will find the access ID to open the files inside the package.

Kit Components

pLSODN PCR template

pLSODN PCR Template has pUC Ori and Ampicillin resistance gene.

The following Nicking Endonuclease sites and Restriction Enzyme sites are not present in pLSODN PCR Template, and these enzymes can be used to prepare ssDNA.

Nicking Endonucleases available for ssDNA preparation:

Nt.BspQI, Nb.BbvCI, Nb.BsrDI, Nb.BtsI, Nb.BsmI

Restriction Enzymes available for ssDNA preparation:

AatII, AbsI, AflIII, AgeI, ApaI, AscI, AvrII, BamHI, BbsI, BbvCI, BglII, BmrI, BmtI, BsaI, BsgI, BsiWI, BsmBI, BsmI, BspEI, BspQI, BsrDI, BsrGI, BssHII, BtgZI, BtsI, EagI, EcoRI, EcoRV, FseI, HindIII, KasI, KpnI, MauBI, MfeI, MluI, MreI, NcoI, NgoMIV, NotI, NsiI, PciI, PstI, PvuII, SacI, SalI, SbfI, SgrDI, SmaI, SpeI, SphI, TspMI, XbaI, XhoI

Denaturing Gel-Loading Buffer

The Denaturing Gel-Loading Buffer is a gel-loading buffer to obtain a long ssDNA using agarose gel electrophoresis. The Denaturing Gel-Loading Buffer denature the nicked plasmid harboring the DNA of interest. A mixture of the nicked plasmid and Denaturing Gel-Loading Buffer is 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.

λ /HindIII DNA for long ssDNA preparation kit

The approximate size of ssDNA can be estimated by λ / HindIII DNA for long ssDNA preparation kit. The mobility of ssDNA is more changeable depending on the sequence than dsDNA, therefore λ / HindIII DNA indicate the rough size.

To use, aliquot 20 μ l into new tube, heat and chill before loading into wells of agarose gel. See figure 3 for fragment size.

Long ssDNA Gel Extraction Kit for 10kb (# DM650)

Kit Components: 25 reactions

Components	Contents or Comments	Size
Crystal Violet Solution	Crystal Violet 4mg/ml (2,500 \times).	5 ml
Gel-Dissolving Buffer	It contains guanidine thiocyanate.	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

- If precipitated material has formed in the Gel-Dissolving Buffer, heat to dissolve at 37°C.

The Long ssDNA Gel Extraction Kit is a highly-specialized kit for long ssDNA (lssDNA) purification from agarose gel. The spin column, buffer formulations and purification protocol are optimized for high recovery yields and high purity of lssDNA. LssDNA purified with the kit is high-quality, with low mechanical degradation and without ultraviolet light-induced damage from the purification process. The purified lssDNA is suitable for molecular biology and biotechnology applications.

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

Features and Specifications of the Long ssDNA Gel Extraction Kit for 10kb (# DS650) :

- Optimized for long ssDNA.
- High recovery yield (Typically 55-75%).
- 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.*
- ssDNA size for excision: 3,000-20,000 bases.
- Binding capacity on a Spin Column for ssDNA binding is up to 10µg.
- Elution volume: $\geq 15 \mu\text{l}$.
 - * Residual NaI may be difficult to remove, and reduces the efficiency of downstream enzymatic reactions.

Caution!

Wear gloves and protecting clothing while handling the Crystal Violet.

Experimental Outline

Using this kit, long ssDNA can be obtained by almost the same procedure as the conventional method for dsDNA preparation.

At first, the DNA fragment of interest is cloned into the PCR product of “pLSODN PCR Template” by seamless cloning. At cloning, a nicking endonuclease site or restriction enzyme site is put between the primer and the insert (at least one nicking endonuclease site). This cloned plasmid is digested with a pair of nicking endonucleases or a combination of nicking endonuclease and restriction enzyme. The nicked plasmid is denatured and then subjected to agarose gel electrophoresis. The band of long ssDNA is excised and extracted.

The table below describes the procedure of long ssDNA preparation.

Outline	STEP	Action	Page
Design of Construct	1	Selection of Nicking Endonuclease and Restriction Enzyme	7
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PCR and purification of pLSODN PCR Template	3	PCR using pLSODN PCR template	10
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Cloning of insert into pLSODN plasmid	8	Preparation of insert	12
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Digestion of the plasmid with nicking endonuclease and extraction of long ssDNA	14	Digestion of plasmid with nicking endonuclease	13
	15	Desalting	13
	16	Analytical agarose gel electrophoresis	14
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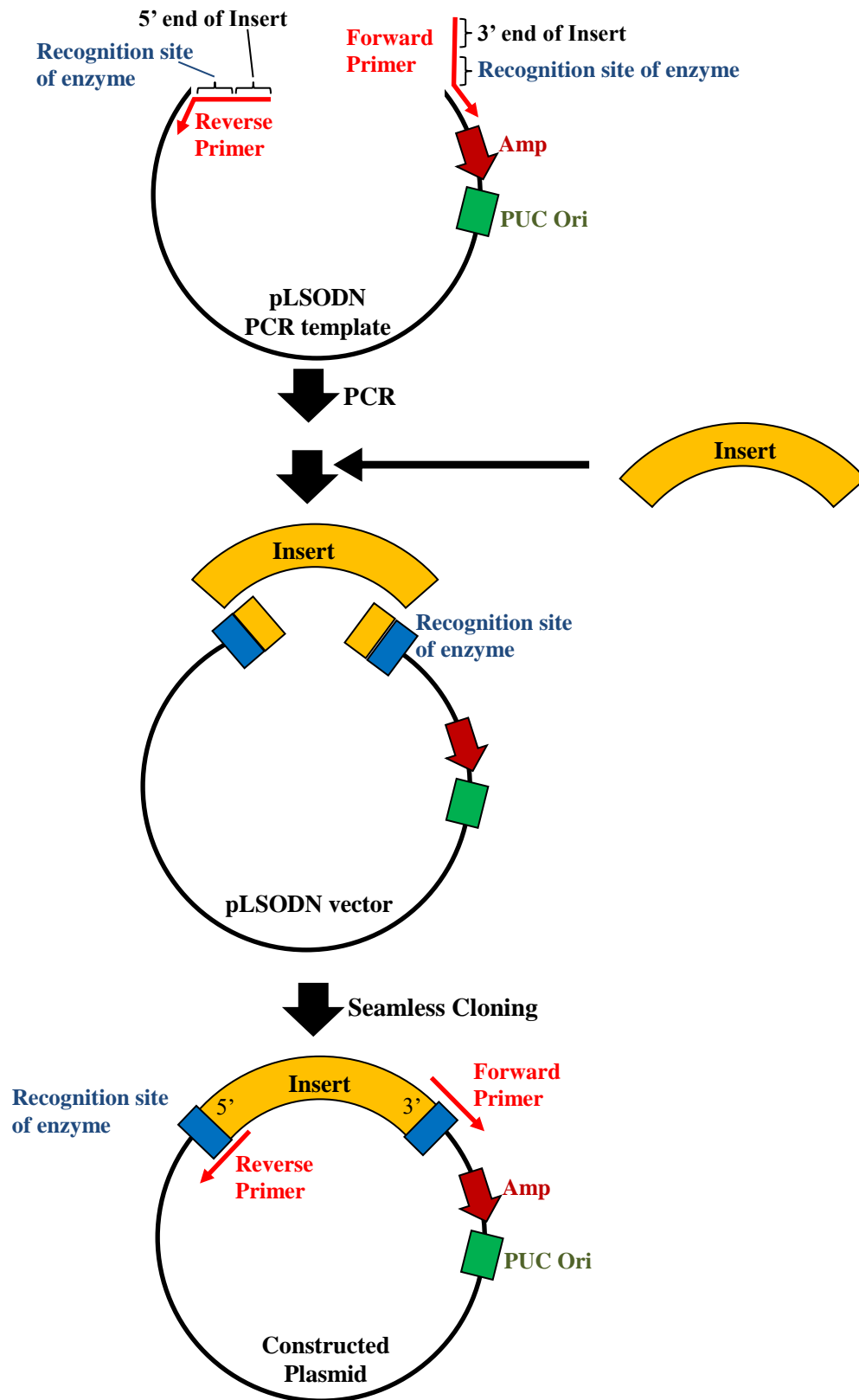
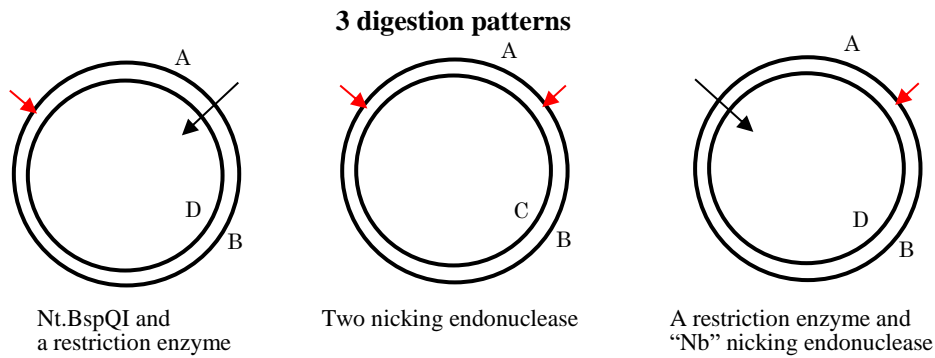
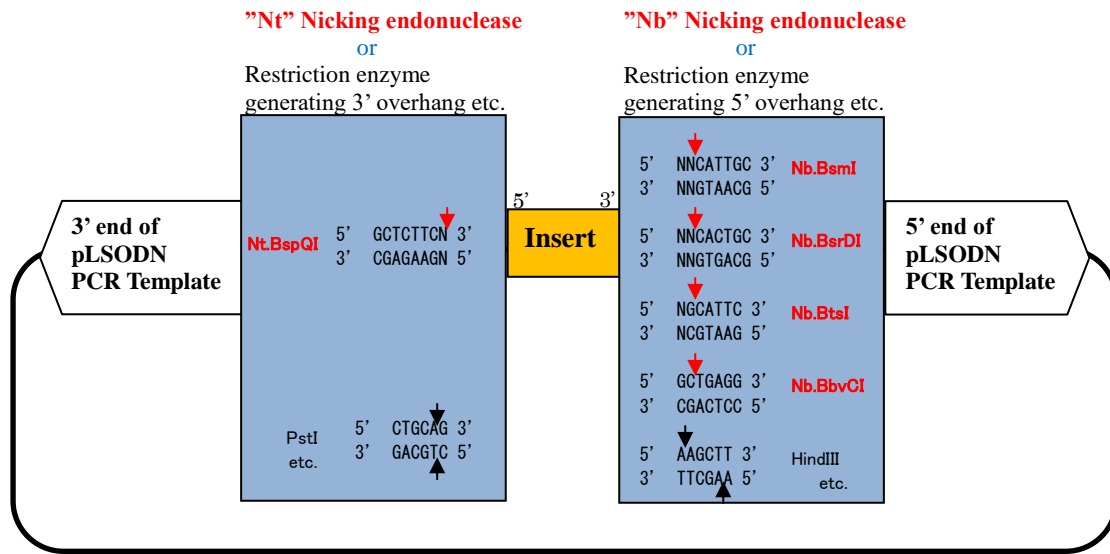


Figure 1. The flow of plasmid construction

Diagram of insertion site



At least one nicking endonuclease site must be used

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

Figure 2. The possible pairs of recognition site to generate a long ssDNA

DESIGN OF CONSTRUCT

Selection of nicking endonuclease and restriction enzyme, and design of PCR primer

STEP 1: Selection of Nicking Endonuclease and Restriction Enzyme

ssDNA can be prepared from the constructed plasmid using “two nicking enzymes” or “nicking enzymes and restriction enzymes” that satisfy the following three conditions.

- (1) The enzymes are in the list of below "Nicking enzymes and restriction enzymes available for ssDNA excision".
- (2) There is no recognition site of the enzymes inside your insert.
- (3) The enzymes cleave the same strand of dsDNA.

If it is necessary to minimize extra bases at the end of ssDNA from the recognition site of enzymes, consider the design shown in Figure 2 and below.

Nicking endonucleases and restriction enzyme available for ssDNA preparation

The site suitable for adding to 5' end of insert

Nt.BspQI *1

AatII, ApaI, BbsI, BmrI, BmtI, BsaI, BsgI, BsmBI, BsmI, BspQI, BsrDI, BtgZI, BtgZI, BtsI, FseI, KpnI, NsiI, PstI, SacI, SbfI, SphI *2

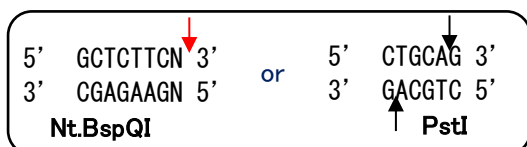
The site suitable for adding to 3' end of insert †

Nb.BsmI, Nb.BsrDI, Nb.BtsI, Nb.BbvCI *1

AbsI, AflII, AgeI, AscI, AvrII, BamHI, BbvCI, BglII, BsiWI, BspEI, BsrGI, BssHII, EagI, EcoRI, HindIII, KasI, MauBI, MfeI, MluI, MreI, NcoI, NgoMIV, NotI, PciI, SalI, SgrDI, SpeI, TspMI, XbaI, XhoI *2

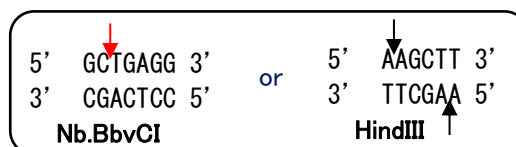
*1 Nicking endonucleases are shown in red.

*2 Besides these enzymes, EcoRV, PvuII and SmaI which generate blunt ends can also be used.



"Nt." Nicking endonuclease or restriction enzymes that leave 3' overhangs are suitable for attaching to the 5' side of insert (attach to reverse primer of pLSODN PCR template).

For example, at Nt.BspQI site, a single strand is cleaved after "N" indicated by the arrow. Therefore, extra bases do not remain in the ssDNA.



"Nb." Nicking endonuclease or restriction enzymes that leave 5' overhangs are suitable for attaching to the 3' side of insert (attach to forward primer of pLSODN PCR template).

If the terminal sequence of the insert is shared with the restriction enzyme site, the extra sequence will not be added to the end of the prepared ssDNA. For example, at Nb.BbvCI site, a single strand is cleaved after "GC" indicated by the arrow as shown below. Therefore, if the 3' end of insert is "GC", extra bases do not remain in the ssDNA.

Therefore, there are three recommended methods:

5' end of Insert	3' end of Insert
1) "Nt" nicking endonuclease	"Nb" nicking endonuclease
2) "Nt" nicking endonuclease	Restriction enzyme generating 5' overhang etc.
3) Restriction enzyme generating 3' overhang etc.	"Nb" nicking endonuclease

At least one nicking endonuclease site must be used to give three ssDNA fragments different in size. If there are no appropriate recognition sites in the DNA fragment of interest because of the existence of some nicking endonuclease or restriction enzyme sites in it, some of these sites in the DNA fragment may need to be changed by site-directed mutagenesis so as not to affect the function of a long ssDNA, for example silent mutation.

STEP 2: Design of PCR primer

. If the insert length is 3-6 kb, a 13 kb vector backbone is amplified. And if the Insert length is 6-10 kb, a 19 kb vector backbone is amplified by PCR Then the insert is cloned in vector backbone by seamless cloning method. (Figure 1, 2)

Insert	Vector backbone
3-6kb	13kb
6-10kb	19kb

■ Primer design when insert length is 3-6 kb (13 kb vector backbone is amplified by PCR)

Reverse primer for 13 kb vector backbone:

Reverse primer is **complementary strand of:**

[5'-GTTCCGGAGCACTAGGTAAGATGG

TTATGC-3'

(3' end of pLSODN PCR Template)]

+ [Nicking Endonuclease or Restriction Enzyme recognition site]

+ [5' end of insert (20 base)]

Forward primer for 13 kb vector backbone:

[3' end of insert (20 base)]

+ [Nicking Endonuclease or Restriction Enzyme recognition site]

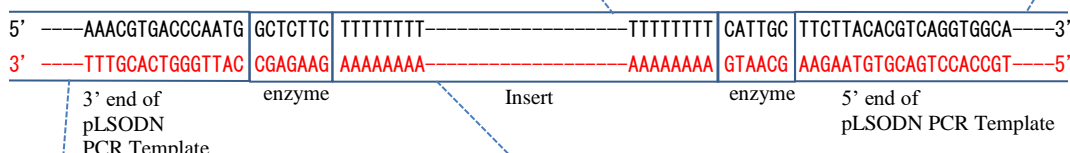
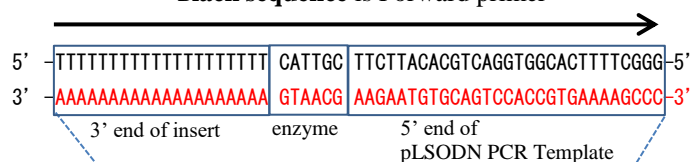
+ [5'-TTCTTACACGTCAGGTGGCACTTT

TCGGG-3'

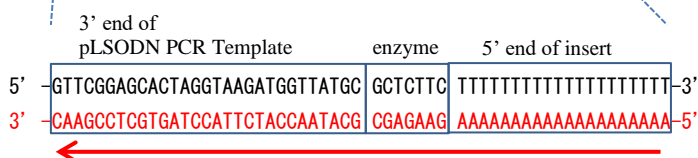
(5' end of pLSODN PCR Template)]

Example of Forward Primer

Black sequence is Forward primer



Example of Reverse prime



Red sequence is Reverse primer

**Primer design when insert length is 6-10 kb
(19 kb vector backbone is amplified by PCR)**

Reverse primer for 19 kb vector backbone

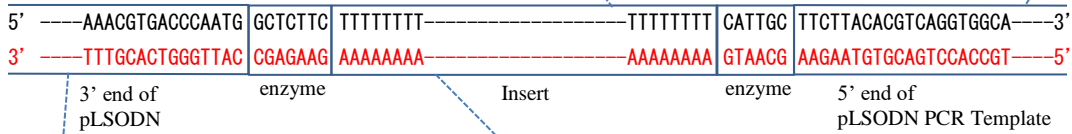
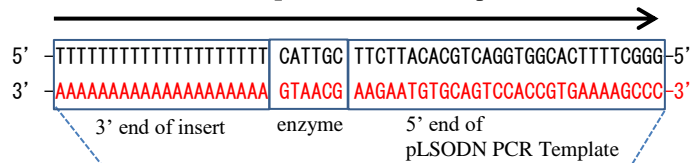
Reverse primer is **complementary strand of:**
 [5'-AATCGAATCCTCACAAACGTGA
 CCAATG-3'
 (3' end of pLSODN PCR Template)]
 + [Nicking Endonuclease or Restriction Enzyme
 recognition site]
 + [5' end of insert (20 base)]

Forward primer for 19 kb vector backbone

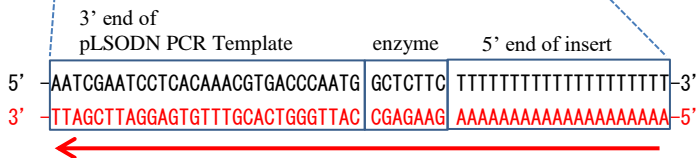
Forward primer is:
 [3' end of insert (20 base)]
 + [Nicking Endonuclease or Restriction Enzyme
 recognition site]
 + [5'-TTCTTACACGTCAGGTGGCAC
 TTTTCGGG-3'
 (5' end of pLSODN PCR Template)]

Example of Forward Primer

Black sequence is Forward primer



Example of Reverse primer



Red sequence is Reverse primer

PCR AND PURIFICATION OF pLSODN PCR TEMPLATE

The process is PCR, DpnI treatment, electrophoresis, isopropanol precipitation, electrophoresis, and gel extraction.

STEP 3: PCR using pLSODN PCR template

1.2 µl of pLSODN PCR Template is used for 300 µl of PCR reaction. Please use enzyme suitable for long amplicons such as PrimeSTAR GXL DNA Polymerase (Takara Bio Inc.). Inappropriate enzyme may not work well.

STEP 4: Treatment by DpnI

1. Add 60 units of DpnI to 300 µl of PCR product.
2. Incubate at 37°C for 1 hour.
3. Incubate at 80°C for 20 minutes.

STEP 5: Electrophoresis

Electrophorese at 100V for 40 minutes using 0.8 % agarose gel with 1×TAE to check the size of PCR product. *1, *2, *3

- *1 One µl of PCR product in STEP 3 is normally enough to recognize the band.
- *2 If too much DNA is loaded, DNA may remain in the wells or the bands may smear. Reducing the concentration of DNA may improve this problem.
- *3 The composition of 1×TAE buffer is 40 mM Tris, 20 mM acetic acid and 1 mM EDTA (ref. 2).

STEP 6: Isopropanol precipitation

1. Add 1/10 volume of 3M sodium acetate (pH 5.2), 1/50 volume of 5 mg/ml glycogen, 0.7 volume of isopropanol.
2. Mix and centrifuge at 20,000×g for 30 min at 4°C
3. Discard the supernatant.
4. Wash DNA pellet with 1 ml of 70% ethanol with vortexing.
5. Centrifuge at 20,000×g for 15 min.
6. Discard the supernatant.
7. Wash DNA pellet with 1 ml of 70% ethanol with vortexing
8. Centrifuge at 20,000×g for 15 min.
9. Discard the supernatant
10. Air-dry the pellet
11. Add 10µl of TE *1

- *1 For large size DNA, it may take some time to dissolve the precipitate. Please incubate for about 30 minutes at room temperature until precipitation dissolve completely. There may be a thin precipitate spreading on the wall of the tube.

STEP 7: Electrophoresis and gel extraction

1. Prepare 0.8% agarose gel by adding 40 µl of Crystal Violet Solution to 100 ml of melted agarose in 1×TAE. ^{*1}
2. Add 30 µl of Denaturing Gel-Loading Buffer to 10 µl of PCR product dissolved in TE (STEP 6). ^{*2}
3. Subject the mixture to 0.8% agarose gel electrophoresis in 1×TAE including Crystal Violet (add 40 µl Crystal Violet Solution / 100 ml TAE). Run at 100 V constant voltage for 40 minutes. ^{*3, *4}
4. Purify the DNA fragment from the excised gel using commercially available kit (e.g. Monarch DNA Gel Extraction Kit, New England Biolabs Inc.).

^{*1} **The DNA band in Crystal Violet-containing gel can be directly seen under ambient light.**

^{*2} If standard Loading Dye is added, large amounts of high molecular weight DNA may be left in the wells. In this step Denaturing Gel-Loading Buffer is necessary. Unlike separation of ssDNA (STEP 16, 17), the sample is not heated before electrophoresis.

^{*3} In the electrophoresis with Crystal Violet, the migration of DNA is influenced by the DNA amount in each band. Therefore molecular weight marker does not necessarily migrate to the expected position. However, DNA band in the gel including Crystal Violet can be directly seen in real time. So the band to excise can be identified by comparison with the result of analytical electrophoresis (STEP 5).

^{*4} Crystal Violet concentration in the running buffer and gel gradually decrease. Check the color of the gel and running buffer, and if needed, add Crystal violet into the running buffer on the anode side during electrophoresis.

CLONING OF INSERT INTO pLSODN PLASMID

STEP 8: Preparation of insert

Prepare the fragment of insert as appropriate.

STEP 9: Ligation and transformation

1. Ligate insert (STEP 8) and purified pLSODN PCR product (STEP 7) with commercially available seamless cloning kit (NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs Inc.) is recommended).
 2. Transformation it to appropriate Competent Cell. ^{*1}
- ^{*1} For large plasmids, transformation efficiency and ligation efficiency may be decreased, so competent cells with high competency is recommended.

STEP 10: Colony PCR

The following sequence can be used as colony PCR primer. However, in some case, PCR may not work well depending on the sequence of insert.

For 19 kb vector backbone

19 kb Forward Primer: TTTGTATGGCAAGACATCATGGGCGTTAAC

19 kb Reverse Primer: TGTTGAATACTCATACTCTTCCTTTTCAA

This primer combination gives “insert + about 350 bp”.

For 13 kb vector backbone

13 kb Forward Primer: AACAGGTAAGACTACCCGTTGGGAGAAAGA

13 kb Forward Primer: TTCGATGTAACCCACTCGGGCACCCAAC TG

This primer combination gives “insert + about 500 bp”.

STEP 11: Plasmid purification

1. Culture the selected clone using LB medium (100 µg/ml Ampicillin), SOB medium (100 µg/ml Ampicillin) etc. ^{*1}
 2. Purify the plasmid with a purification kit (e.g. QIAGEN Plasmid Kit (QIAGEN N.V.)).
- ^{*1} The plasmid of STEP 11 is large (16-29 kb). Mutation tends to be easily introduced into large plasmids, so multiple subculture should be avoided as much as possible.

STEP 12: Confirmation of insert size by restriction enzyme

There are restriction enzymes (double strand break) whose recognition site is the same as nicking endonucleases. Their name are the “name of the nicking endonuclease” minus “Nt.” or “Nb.” (e.g. Nicking Endonuclease: “Nt.BspQI”, Restriction Enzyme: “BspQI”). Using these restriction enzymes, the insert length can be checked.

STEP 13: Sequencing of insert

The sequence of insert should be confirmed before preparing the ssDNA.

DIGESTION OF THE PLASMID WITH NICKING ENDONUCLEASE AND EXTRACTION OF LONG ssDNA

The plasmid harboring your DNA fragment of insert (STEP 13) is digested with two nicking endonucleases or combination with nicking endonuclease and restriction enzyme. 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 ssDNA because remaining salt and Mg^{2+} stabilize the dsDNA and prevent complete denaturation of the nicked plasmids. One example of these procedures is shown below.

STEP 14: Digestion of plasmid with nicking endonuclease

The plasmid harboring your DNA fragment of interest (STEP 13) is digested with two nicking endonucleases or combination with a nicking endonuclease and restriction enzyme. ^{*1, *2}

^{*1} From 100 μ g of plasmid, about 2-6 μ g of ssDNA is often obtained.

^{*2} Reaction condition (Buffer, amount of enzyme and reaction time etc.) are in accordance with manufacturer's instructions.

(Example)

plasmid (100 μ g)	variable
10x 3.1 NEBuffer	10 μ l
Nb.BbvCI (10 unit/ μ l)	5 μ l
Nt.BspQI (10 unit/ μ l)	5 μ l
dH ₂ O	variable
Total volume	100 μ l

Incubate at 37°C for 3 hr.

STEP 15: Desalting

1. Add 1/10 volume of 3M sodium acetate (pH 5.2), 1/50 volume of 5 mg/ml glycogen, 2.5 volumes of ethanol.
2. Mix and centrifuge at 20,000 \times g for 30 min at 4°C
3. Discard the supernatant.
4. Wash the DNA pellet with 1 ml of 70% ethanol with vortexing.
5. Centrifuge at 20,000 \times g for 15 min.
6. Discard the supernatant.
7. Wash the DNA pellet with 1 ml of 70% ethanol with vortexing ^{*1, *2}
8. Centrifuge at 20,000 \times g for 15 min.
9. Discard the supernatant
10. Air-dry the pellet
11. Add 100 μ l of TE (The resulting final concentration of nicked plasmid was 1 μ g/ μ l). ^{*3}

^{*1} **Wash twice with 70% ethanol in order to desalt sufficiently.**

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

^{*3} For large size DNA, it may take some time to dissolve the precipitate. Please incubate for about 30 minutes at room temperature until precipitation dissolve completely. There may be a thin precipitate spreading on the wall of the tube.

STEP 16: Analytical agarose gel electrophoresis

The generation of ssDNA from the nicked plasmid (STEP 15) is analyzed with agarose gel electrophoresis.

The agarose concentration is determined as follows according to the insert length.

Length of Insert	Concentration of Agarose
3-6 kb	1.0%
6-10 kb	0.8%

1. Prepare loading samples by mixing the nicked plasmid (STEP 15) (300 ng) and 3 volumes of the Denaturing Gel-Loading Buffer. ^{*1, *2}
2. Heat the mixture at 70°C for 5 min. ^{*3}
3. Chill on ice for 1 min.
4. Subject the mixture to agarose gel electrophoresis with 1×TAE. Run at 100 V. ^{*4, *5, *6}
5. Stain the gel with 0.5 µg/ml of ethidium bromide for 20 min.
6. Examine the gel on a UV illuminator (figure 3).

^{*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 is required.

^{*2} After mixing with the Denaturing Gel-Loading Buffer, the concentration of the nicked plasmid in the loading sample should be under 0.25 µg/µl.

^{*3} After heating and chilling, quickly load the samples into wells of the gel and promptly start electrophoresis. The denatured DNA is gradually reannealed even in Denaturing Gel-Loading Buffer. Furthermore, once the samples are loaded into the wells, dilution of the Denaturing Gel-Loading Buffer in the sample solution could occur gradually.

^{*4} The approximate size of ssDNA can be estimated by “λ / HindIII DNA for long ssDNA preparation kit” (electrophoresis after incubating at 70 °C for 5 minutes and on ice for 1 minute). The mobility of ssDNA is more changeable depending on the sequence than dsDNA, therefore this λ / HindIII DNA indicates rough size.

^{*5} 23130 bases and 6557 bases of the “λ / HindIII DNA for long ssDNA preparation kit” may appear as double-band.

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

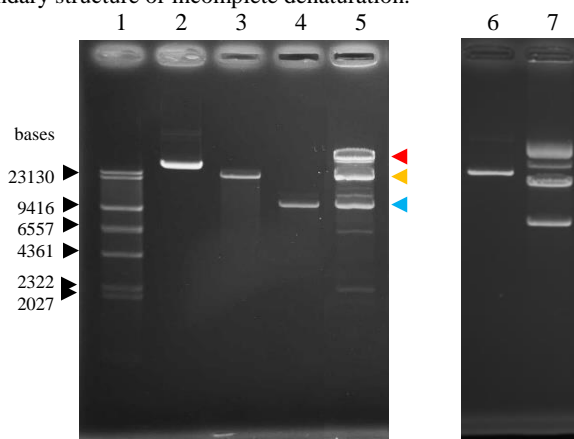


Figure 3. Analytical agarose gel electrophoresis of the nicked plasmid

Lane 1: λ/HindIII DNA for long ssDNA preparation kit (aliquoted 20 µl into new tube, heat and chill before loading)

Lane 2: Uncut plasmid

Lane 3: Purified pLSODN PCR product (STEP 7)

Lane 4: Insert prepared for cloning (STEP 8)

Lane 5: Nicked plasmid (digested by nicking endonuclease and restriction enzyme)

Lane 6: Uncut plasmid

Lane 7: Nicked plasmid (digested by two nicking endonucleases)

Samples of Lane 2-7 were added to 3 volumes of Denaturing Gel-Loading Buffer and incubated at 70 °C for 5 minutes and on ice for 1 minute before electrophoresis.

The blue, orange and red arrows show your interest 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).

STEP 17: Preparative agarose gel electrophoresis

The nicked plasmid is subject to agarose gel electrophoresis for ssDNA extraction.

The agarose concentration is determined as follows according to the insert length.

Length of Insert	Concentration of Agarose
3-6 kb	1.0%
6-10 kb	0.8%

1. Cool the 1×TAE to be used for electrophoresis on ice or in a refrigerator.
2. Prepare the agarose gel by adding 40 µl of Crystal Violet Solution to 100 ml of melted agarose in 1×TAE. ^{*1}
3. Prepare loading samples by mixing the nicked plasmids (STEP 15) and 3 volumes of the Denaturing Gel-Loading Buffer. ^{*2, *3}
4. Heat the mixture at 70°C for 5 min. ^{*4}
5. Chill on ice for 1 min.
6. Put the electrophoresis apparatus on ice in an ice bucket in a draft chamber. Subject the mixture to agarose gel electrophoresis in pre-chilled 1×TAE with crystal violet (add 40 µl Crystal Violet Solution / 100 ml TAE). ^{*5, *6, *7, *8}
7. Run at 100 V, low temperature (less than 20°C)
8. Monitor the ssDNA blue bands moving in the gel during electrophoresis. Stop the electrophoresis after the bands are sufficiently resolved (Figure 4). ^{*9, *10, *11}
9. Excise the ssDNA band from the gel. ^{*12}

^{*1} **ssDNA band in crystal violet containing gel can be directly seen under ambient light.**

^{*2} **Desalting of the nicked plasmid solution is important.**

^{*3} To get a highly purified long ssDNA, the long ssDNA of interest should be loaded greater than 0.5 µg/band. **At the same time, the concentration of the nicked plasmid in the loading sample should be under 0.25 µg/µl after mixing with the Denaturing Gel-Loading Buffer.**

^{*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 too much in a well because it may overflow and cause contamination. It is recommended to make deep wells with thick gel.

^{*6} For the safe use of Crystal Violet, electrophoresis with Crystal Violet should be carried out in a draft chamber. Do not 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.

^{*7} Electrophoresis at a low temperature is recommended (less than 20°C). It gives a better resolution and strong Crystal Violet staining. If the temperature of gel and running buffer exceeds 25°C, Crystal Violet staining of 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.

^{*8} To keep the gel temperature low during electrophoresis in a draft chamber, we recommend placing the electrophoresis apparatus on ice in an ice bucket. At least, use a pre-chilled 1×TAE running buffer on ice to keep a low temperature.

^{*9} 100 ng of ssDNA band is sufficient for detection with Crystal violet staining.

^{*10} In the electrophoresis with Crystal violet, the migration of ssDNA is influenced by DNA amount in each band. Therefore molecular weight marker is not necessarily migrated to the expected position. However, ssDNA band in the gel including crystal violet can be directly seen in real time. So the band to excise can be identified by compared with the result of analytical electrophoresis (STEP 16) (Figure 3, 4).

^{*11} Crystal violet concentration in running buffer and gel gradually decrease. Check the color of gel and running buffer, and if needed, add Crystal violet into the running buffer on the anode side during electrophoresis.

^{*12} Minimize the size of the gel slice by removing extra agarose.

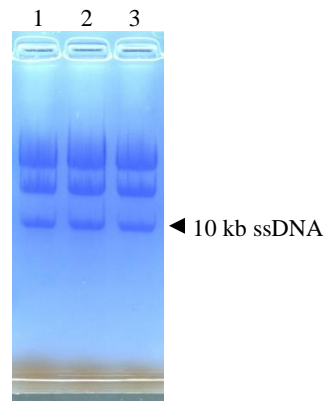


Figure 4. Preparative agarose gel electrophoresis.

Lane 1, 2, 3: digested plasmid

The photo was taken under white light.

STEP 18: Extraction of long ssDNA

Before start:

- Add 45 ml of 100 % ethanol to 8 ml of Wash Buffer 1 and 11 ml of Wash Buffer 2.
- Isopropanol is required.
- All centrifugation steps should be carried out at 16,000×g (around 13,000 rpm in a conventional microcentrifuge) at room temperature (20°C- 25°C). Centrifugation at lower temperature might affect the long ssDNA yield.

1. Transfer the gel slice to a 1.5 ml microcentrifuge tube and weight the gel slice (STEP 17).
2. Add 3 volumes of Gel Dissolving Buffer.
3. Incubate the tube at 50°C, 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 Buffer1 to the Spin Column and centrifuge for 1 min. Discard the flow-through in the Collection Tube with a 1ml pipette tip (1st Wash).
9. Repeat wash with Wash Buffer 1 (Step10, 2nd Wash).
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.
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 a 1ml pipette tip (4th Wash).
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°C for 5 min. ^{*6}
17. Immediately, centrifuge the Spin Column for 1 min to elute long ssDNA. ^{*7}

^{*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 μ l, the loading of the sample onto the column should be performed in multiple rounds so as not to exceed 500 μ l.
- *3 **Do not 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 Do not wash the Spin Column with Gel-Dissolving Buffer only or its dilution.
- *6 Typical elution volumes are 15 μ l -40 μ l. Water can be used to elute the long ssDNA. The average elution volume is 36.5 μ l from 40 μ l Elution Buffer volume, and 12 μ l from 15 μ l.
- *7 Generally, samples eluted from silica based spin columns contain a small amount of silica matrix. If necessary, it could be easily removed by spin down (e.g. 20,000 \times g for 10 min) or filtration using filter spin column (e.g. Merck Ultrafree-MC GV 0.22 μ m).

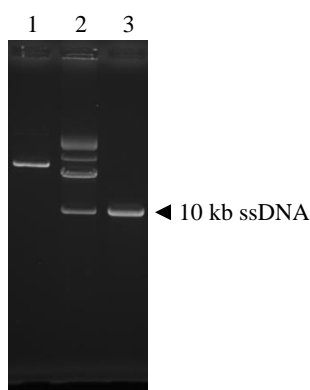


Figure 5. Agarose gel electrophoresis of purified long ssDNA

Lane 1: Uncut plasmid
Lane 2: Digested plasmid
Lane 3: Purified long ssDNA (10 kb fragment)

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 is remaining. Wash thoroughly with 70% ethanol in ethanol precipitation. Specifically, washing with 70% ethanol twice, vortex at washing.
Low ssDNA yield	Salt remaining	Desalting of the nicked plasmid solution is very important. ssDNA bands may not separate clearly if salt is remaining. Wash thoroughly with 70% ethanol in ethanol precipitation. Specifically, washing with 70% ethanol twice, vortex at washing.
	Low yield of nicked plasmid in ethanol precipitation	As in the above protocol, adding glycogen during ethanol precipitation may increase DNA recovery.
Is it possible to prepare ssDNA smaller than 3 kb using this kit?	<p>In principle, it is also possible to prepare ssDNA smaller than 3 kb with this kit, Long ssDNA Preparation Kit for 10kb (Cat. No. DS635).</p> <p>However, "Long ssDNA Preparation Kit for 1.5kb (Cat. No. DS615)" and "Long ssDNA preparation kit for 3.0kb (Cat. No. DS625)" are more suitable for the preparation of ssDNA smaller than 3 kb. We recommend you to use #DS615 or #DS625.</p> <p>In order to obtain the same amount of ssDNA with #DS635 as compared with #DS615 or #DS625, a larger amount of plasmid is needed. Also, as the concentration of plasmid to apply to electrophoresis gel is limited (about 0.25 µg/µl), the ssDNA concentration in the band also decreases, which may affect the purification degree and yield.</p>	

References

1. Yoshimi K, Kunihiro Y, Kaneko T, Nagahora H, Voigt B, Mashimo T. (2016) ssODN-mediated knock-in with CRISPR-Cas for large genomic regions in zygotes. *Nat Commun.* **20**;7:10431.
2. Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Related Products:

DS611	Denaturing Gel-Loading Buffer	1 ml x 5
DS612	Denaturing Gel-Loading Buffer	1 ml x 2
DS650	Long ssDNA Gel Extraction Kit for 10kb	
DS670	λ/HindIII DNA for long ssDNA preparation kit	
DS615	Long ssDNA Preparation Kit for 1.5 kb	
DS625	Long ssDNA Preparation Kit for 3.0kb	

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