Lambda DASH II Undigested Vector Kit

INSTRUCTION MANUAL

Catalog #247201

Revision A.01

For In Vitro Use Only

247201-12

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CONTENTS

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MATERIALS PROVIDED

Materials provided	Quantity
Undigested Lambda DASH II DNA[a]	10 µg
Host strains[b]	
XL1-Blue MRA strain	0.5-ml bacterial glycerol stock
XL1-Blue MRA (P2) strain	0.5-ml bacterial glycerol stock

[a] Shipped as a liquid at 1 µg/µl in 10 mM Tris-HCl and 1 mM EDTA (see *Preparation of Media and Reagents*). On arrival, store the Lambda DASH II vector at –20°C. After thawing, aliquot and store at –20°C. Do not pass through more than two freeze–thaw cycles. For short-term storage, store at 4°C for 1 month.

[b] For host strain shipping and storage conditions, please see *Preparing the Host Strains*.

STORAGE CONDITIONS

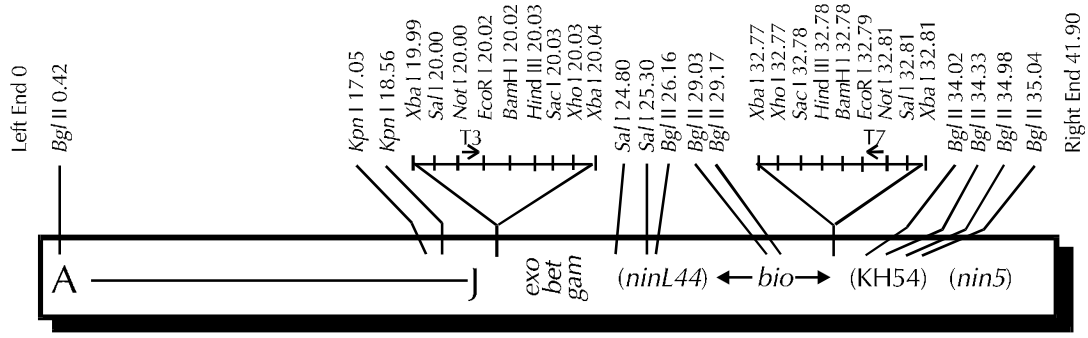
Lambda DASH II Vector: –20°C
Bacterial Glycerol Stock: –80°C

INTRODUCTION

Lambda DASH II is a replacement vector used for cloning large fragments of genomic DNA (see Figure 1). The Lambda DASH II system takes advantage of spi (sensitive to P2 inhibition) selection. Lambda phages containing active *red* and *gam* genes are unable to grow on host strains that contain P2 phage lysogens. Lambda phages without these genes are able to grow on strains lysogenic for P2 such as XL1-Blue MRA (P2), a P2 lysogen of XL1-Blue MRA. The *red* and *gam* genes in the Lambda DASH II DNA are located on the stuffer fragment; therefore, the wild-type Lambda DASH II phage cannot grow on XL1-Blue MRA (P2). When the stuffer fragment is replaced by an insert, the recombinant Lambda DASH II becomes red^-/gam^- and the phage is able to grow on the P2 lysogenic strain. Therefore, by plating the library on the XL1-Blue MRA (P2) strain, only recombinant phages are allowed to grow. The XL1-Blue MRA strain is also provided as a control strain and later for growth of the recombinant when the selection is no longer necessary. Target DNA cloned into the *Eco*R I through *Xba* I sites of the Lambda DASH II vector may be removed by digestion with *Not* I. The unique arrangement of the Lambda DASH II polylinker permits the isolation of the insert and flanking T3 and T7 bacteriophage promoters as an intact cassette by digestion with *Not* I. The T3 and T7 promoters flanking the insertion sites can be used to generate end-specific RNA probes for use in chromosomal walking and restriction mapping.

Revision A.01

LAMBDA DASH II VECTOR MAP



Lambda DASH II Multiple Cloning Site Regions

[sequences shown: 19.99–20.04kb (top sequence) and 32.77–32.81kb (bottom sequence)]

Xba I Sal I Not I **T3 Promoter** EcoR I BamH I Hind III Sac I Xho I Xba I

```
TCTAGAGAGCTGTCGACGCGGCCGCGGAATTAACCCTCACTAAAGGGAACGAATTCGGATCCAAGCTTGAGCTCCTCGAGAAATTCTCTAGA
```

Xba I Xho I Sac I Hind III BamH I EcoR I **T7 Promoter** Not I Sal I Xba I

```
TCTAGAGAATTTCTCGAGGAGCTCAAGCTTGGATCCGAATTCTTCGCCCTATAGTGAGTCGTATTACGCGGCCGCGTCGACAGCTCTCTAGA
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FIGURE 1 Linear map and multiple cloning site sequences for the Lambda DASH II replacement vector.

PREPARING THE HOST STRAINS

Host Strain Genotypes

Host strain	Genotype
XL1-Blue MRA strain	*Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 gyrA96 relA1 lac*
XL1-Blue MRA (P2) strain	XL1-Blue MRA (P2 lysogen)

Growing and Maintaining the Host Strains

The bacterial host strains are shipped as bacterial glycerol stocks. For the appropriate media, please refer to the following table:

Host strain	Agar plates for bacterial streak	Medium for bacterial glycerol stock	Medium for bacterial cultures for titering phage (final concentration)
XL1-Blue MRA strain[a]	LB	LB	LB with 0.2% (w/v) maltose–10 mM $MgSO_4$
XL1-Blue MRA (P2) strain[a]	LB	LB	LB with 0.2% (w/v) maltose–10 mM $MgSO_4$

[a] The XL1-Blue MRA and XL1-Blue MRA (P2) host strains are modified to enhance the stability of clones containing methylated DNA; in addition, these strains enhance the stability of nonstandard DNA structures.

On arrival, prepare the following from the bacterial glycerol stock using the appropriate media as indicated in the previous table:

Note *The host strains may thaw during shipment. The vials should be stored immediately at –20° or –80°C, but most strains remain viable longer if stored at –80°C. Avoid repeated thawing of the host strains in order to maintain extended viability.*

1. Revive the stored cells by scraping off splinters of solid ice with a sterile wire loop.
2. Streak the splinters onto an LB agar plate (see *Preparation of Media and Reagents*).
3. Incubate the plate overnight at 37°C.
4. Seal the plate with Parafilm® laboratory film and store the plate at 4°C for up to 1 week.
5. Restreak the cells onto a fresh plate every week.

Preparing a –80°C Bacterial Glycerol Stock

1. In a sterile 50-ml conical tube, inoculate 10 ml of appropriate liquid medium with one colony from the plate. Grow the cells to late log phase.

2. Add 4.5 ml of a sterile glycerol–liquid medium solution (prepared by mixing 5 ml of glycerol + 5 ml of appropriate medium) to the bacterial culture from step 1. Mix well.

3. Aliquot into sterile centrifuge tubes (1 ml/tube).

This preparation may be stored at –20°C for 1–2 years or at –80°C for more than 2 years.

PREPARING THE LAMBDA DASH II VECTOR

The undigested Lambda DASH II vector is shipped in 10 mM Tris-HCl, pH 7.0, and 0.1 mM EDTA and can be stored up to 1 month at 4°C. For long-term storage, aliquot and freeze at –20°C. Do not put the samples through multiple freeze–thaw cycles.

Note *The cos ends do not need to be ligated prior to digestion unless the vector will be filled in with Klenow.*

Digestion

The lambda DNA should be digested for the minimum time and with the minimal amount of enzyme required in order to obtain a complete digestion. Overdigestion with the restriction enzyme will lower ligation efficiency, while underdigestion will result in increased background.

Perform a pilot digestion series that covers a range of digestion incubation times. Package 0.4 µg from each time point and 0.4 µg of the undigested Lambda DASH II vector as a control. (Plate a dilution of the undigested Lambda DASH II vector packaging reaction in order to count a manageable number of plaques.) Choose the time point that gives <0.1% of the plating efficiency of the undigested Lambda DASH II vector.

1. Digest 2.5 µg of the Lambda DASH II vector with 2–5 U of enzyme/µg of DNA in a final volume of 25 µl.

2. Remove 0.5 µg (5 µl) for each time point and stop the reaction by adding 0.5 µl of 10× STE buffer (see *Preparation of Media and Reagents*) to the aliquot.

   Suggested time points are as follows:

   1 hour, 45 minutes
   2 hours, 0 minutes
   2 hours, 15 minutes
   2 hours, 30 minutes
   2 hours, 45 minutes

3. Package 0.4 µg (4 µl) and plate according to the protocol in the instruction manual for Gigapack packaging extract.

4. After determining the ideal time point, perform the steps outlined below:

   a. Digest 5 µg of the Lambda DASH II vector in a volume of 50 µl.

   b. Extract once with phenol–chloroform [1:1 (v/v)] and invert to mix (do not vortex). Repeat the extraction with chloroform only.

   c. Add an equal volume of 4 M ammonium acetate to the aqueous phase.

   d. Add 2.5 volumes of room temperature 100% ethanol. Microcentrifuge the reaction for 15 minutes at room temperature at maximum speed to pellet the DNA.

   e. Wash the DNA pellet with 70% (v/v) ethanol.

   f. Gently resuspend the DNA pellet in 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA.

   g. Store the resuspension at 4°C for up to one month or at –20°C for long-term storage. Do not repeatedly freeze-thaw the vector DNA.

Preparing and Ligating the Test Insert

When preparing the vector, it may be useful to prepare a test insert to confirm the quality of the lambda vector. The test insert is digested with the same restriction enzyme(s) used to prepare the Lambda DASH II vector arms. Prior to ligation, digest, phenol–chloroform extract, chloroform extract, ethanol precipitate, and quantitate the test insert.

Note *In all ligations, the final glycerol content should be less than 5% (v/v).* ***Do not exceed 5% (v/v) glycerol.*** *Due to the high molecular weight of the lambda vector, the contents may be very viscous. It is important to microcentrifuge the contents of the lambda vector tube briefly at 11,000 × g, then gently mix the solution by stirring with a pipet tip prior to pipetting.*

Prepare a ligation reaction mixture containing the following components:

1.0 µl of the digested Lambda DASH II DNA (1 µg)
X µl of partially digested genomic DNA (use an equimolar ratio of insert:vector)
0.5 µl of 10× ligase buffer (see *Preparation of Media and Reagents*)
0.5 µl of 10 mM rATP (pH 7.5)
2 U of T4 DNA ligase
Water to a final volume of 5 µl

Incubate the ligation at 4°C overnight.

When ligating the insert, use a volume of insert DNA up to 2.5 µl. Use an equimolar ratio of the *Sau*3A-partial, *Bam*H I, or *Mbo* I-digested insert with the Lambda DASH II vector. The Lambda DASH II vector can accommodate inserts ranging from 9 to 23 kb. If ligating a 20,000-bp insert to the vector, use 0.4 µg of insert for every 1 µg of vector. If the insert used is free from contaminants and contains a high percentage of ligatable ends, expect about 1×10^6–1.5×10^7 recombinant plaques when using high-efficiency packaging extracts, such as Gigapack III Plus or Gigapack III Gold packaging extracts.

Note *Protocols for preparing the partially digested DNA are outlined in Reference 1. It is recommended to size fractionate the DNA and/or to dephosphorylate the DNA ends using calf intestine alkaline phosphatase (CIAP) to prevent ligation of multiple inserts.*

TITERING PROCEDURE

1. Streak the bacterial glycerol stock onto the appropriate agar plates (see the table in *Preparing the Host Strains*). Incubate the plates overnight at 37°C.

2. Inoculate an appropriate medium, supplemented with 10 mM $MgSO_4$ and 0.2% (w/v) maltose, with a single colony.

3. Grow at 37°C, shaking for 4–6 hours (do not grow past an OD_{600} of 1.0). Alternatively, grow overnight at 30°C, shaking at 200 rpm.

   Note *The lower temperature keeps the bacteria from overgrowing, thus reducing the number of nonviable cells. Phage can adhere to nonviable cells resulting in a decreased titer.*

4. Pellet the bacteria at 500 × *g* for 10 minutes.

5. Gently resuspend the cells in half the original volume with sterile 10 mM $MgSO_4$.

6. Dilute the cells to an OD_{600} of 0.5 with sterile 10 mM $MgSO_4$.

   Note *The bacteria should be used immediately following dilution.*

7. Prepare dilutions of the final packaged reaction in SM buffer.[§] Add 1 µl of the final packaged reaction to 200 µl of host cells diluted in 10 mM $MgSO_4$ to an OD_{600} of 0.5. If desired, also add 1 µl of a 1:10 dilution of the packaged reaction in SM buffer to 200 µl of host cells.

8. Incubate the phage and the bacteria at 37°C for 15 minutes to allow the phage to attach to the cells.

9. Add 3 ml of NZY top agar[§] (48°C) and plate immediately on prewarmed NZY agar plates.[§]

10. Count the plaques and determine the titer in plaque-forming units per milliliter (pfu/ml).

[§] See *Preparation of Media and Reagents*.

AMPLIFYING THE LIBRARY

It is usually desirable to amplify libraries prepared in lambda vectors to make a large, stable quantity of a high-titer stock of the library. However, more than one round of amplification is not recommended, since slower growing clones may be significantly underrepresented.

Day 1

1. Prepare the host strains as outlined in *Preparing the Host Strains*.

Day 2

2. Dilute the cells to an OD_{600} of 0.5 in 10 mM $MgSO_4$. Use 600 µl of cells at an OD_{600} of 0.5/150-mm plate.

3. Combine aliquots of the packaged mixture or library suspension containing ~5×10^4 pfu of bacteriophage with 600 µl of host cells at an OD_{600} of 0.5 in 14-ml BD Falcon polypropylene round-bottom tubes (BD Biosciences Catalog #352059). To amplify 1×10^6 plaques, use a total of 20 aliquots (each aliquot contains 5×10^4 plaques/150-mm plate).

   Note *Do not add more than 300 µl of phage/600 µl of cells.*

4. Incubate the tubes containing the phage and host cells for 15 minutes at 37°C.

5. Mix 6.5 ml of NZY top agar, melted and cooled to ~48°C, with each aliquot of infected bacteria and spread evenly onto a freshly poured 150-mm bottom agar plate.

6. Incubate the plates at 37°C for 6–8 hours. Do not allow the plaques to get larger than 1–2 mm. On completion, the plaques should be touching.

7. Overlay the plates with ~8–10 ml of SM buffer. Store the plates at 4°C overnight (with *gentle* rocking if possible). This allows the phage to diffuse into the SM buffer.

Day 3

8. Recover the bacteriophage suspension from each plate and pool it into a sterile polypropylene container. Rinse the plates with an additional 2 ml of SM buffer and pool. Add chloroform to a 5% (v/v) final concentration. Mix well and incubate for 15 minutes at room temperature.

9. Remove the cell debris by centrifugation for 10 minutes at 500 × *g*.

10. Recover the supernatant and transfer it to a sterile polypropylene container. If the supernatant appears cloudy or has a high amount of cell debris, repeat steps 8 and 9. If the supernatant is clear, add chloroform to a 0.3% (v/v) final concentration and store at 4°C. Store aliquots of the amplified library in 7% (v/v) DMSO at –80°C.

11. Check the titer of the amplified library using host cells and serial dilutions of the library. (Assume ~10^9–10^{11} pfu/ml.)

PERFORMING PLAQUE LIFTS

1. Titer the library to determine the concentration (prepare fresh host cells to use in titering and in screening).

2. Plate on large 150-mm agar plates (≥2-day-old) to 50,000 pfu/plate with 600 µl of host cells at an OD_{600} of 0.5/plate and 6.5 ml of NZY top agar/plate. (Use 20 plates to screen 1×10^6.)

3. Incubate the plates at 37°C for ~8 hours.

4. Chill the plates for 2 hours at 4°C to prevent the top agar from sticking to the nitrocellulose membrane.

   Note *Use forceps and wear gloves for the following steps.*

5. Transfer the plaques onto a nitrocellulose membrane for 2 minutes. Use a needle to prick through the agar for orientation. (If desired, waterproof ink in a syringe needle may be used.)

   If making duplicate nitrocellulose membranes, allow the second membrane to transfer for ~4 minutes.

   Note *Pyrex® dishes are convenient for the following steps. All solutions should be at room temperature.*

   a. Denature the nitrocellulose-bound DNA after lifting by submerging the membrane in a 1.5 M NaCl and 0.5 M NaOH denaturation solution for 2 minutes.

   Note *If using charged nylon, wash with gloved fingertips to remove the excess top agar.*

   b. Neutralize the nitrocellulose membrane for 5 minutes by submerging the membrane in a 1.5 M NaCl and 0.5 M Tris-HCl (pH 8.0) neutralization solution.

   c. Rinse the nitrocellulose membrane for no more than 30 seconds by submerging the membrane in a 0.2 M Tris-HCl (pH 7.5) and 2× SSC buffer solution (see *Preparation of Media and Reagents*).

6. Blot the nitrocellulose membrane briefly on Whatman® 3MM paper.

7. Crosslink the DNA to the membrane using the autocrosslink setting on the Stratalinker UV crosslinker (120,000 µJ of UV energy) for ~30 seconds. Alternatively, oven-bake at 80°C for ~1.5–2 hours.

8. Store the stock agar plates of the transfers at 4°C to use after screening.

HYBRIDIZING AND SCREENING

Following the preparation of the membranes for hybridization, perform prehybridization, probe preparation, hybridization, and washes for either oligonucleotide probes or double-stranded probes and then expose the membranes to film as outlined in standard methodology texts.[1, 2] Following these procedures, perform secondary and tertiary screens also as outlined in the standard methodology texts.[1, 2] After an isolate is obtained, refer to Sambrook *et al.*[1] for suggested phage miniprep and maxiprep procedures.

RAPID RESTRICTION MAPPING

The insertion sites of the Lambda DASH II vector are flanked by T3 and T7 promoters, which permit the generation of end-specific hybridization probes. End-specific probes can be made once a recombinant clone containing an insert is isolated. In addition, the Lambda DASH II vector has unique *Not* I sites flanking the RNA promoters, which permits the excision from the lambda vector of insert DNA plus the T3 and T7 promoter sequences as an intact fragment.

TROUBLESHOOTING

Observation	Suggestion(s)
Packaging efficiency is too low	Ensure that the packaging extracts are properly stored. Gigapack III packaging extract is very sensitive to slight variations in temperature; therefore, store the packaging extracts at the bottom of a –80°C freezer and avoid transferring tubes from one freezer to another. **Do not allow the packaging extracts to thaw.**
	Avoid the use of ligase buffers containing PEG, which can inhibit packaging.
	The DNA concentration in the packaging extract may be too low. Ligate at DNA concentrations of 0.2 µg/µl or greater and package between 1 and 4 µl of the ligation reaction.
	Packaging extract protein concentration may be too low. Never package >4 µl of the ligation reaction to prevent dilution of the proteins contained within the packaging extract.
During titering, neither a bacterial lawn nor plaques is observed on the plate	Chloroform, added after packaging to prevent bacterial contamination, may be present while titering. Be sure to spin down the chloroform completely prior to removing an aliquot of the viral stock for titering.

PREPARATION OF MEDIA AND REAGENTS

Note *All media must be autoclaved before use.*

LB Agar (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar
Add deionized H_2O to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclave
Pour into petri dishes (~25 ml/100-mm plate)

LB Broth (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
Add deionized H_2O to a final volume of 1 liter
Adjust to pH 7.0 with 5 N NaOH
Autoclave

NZY Broth (per Liter)

5 g of NaCl
2 g of $MgSO_4 \cdot 7H_2O$
5 g of yeast extract
10 g of NZ amine (casein hydrolysate)
Add deionized H_2O to a final volume of 1 liter
Adjust the pH to 7.5 with NaOH
Autoclave

NZY Top Agar (per Liter)

Prepare 1 liter of NZY broth
Add 0.7% (w/v) agarose
Autoclave

NZY Agar (per Liter)

5 g of NaCl
2 g of $MgSO_4 \cdot 7H_2O$
5 g of yeast extract
10 g of NZ amine (casein hydrolysate)
15 g of agar
Add deionized H_2O to a final volume of 1 liter
Adjust the pH to 7.5 with NaOH
Autoclave
Pour into petri dishes (~80 ml/150-mm plate)

SM Buffer (per Liter)

5.8 g of NaCl
2.0 g of $MgSO_4 \cdot 7H_2O$
50.0 ml of 1 M Tris-HCl (pH 7.5)
5.0 ml of 2% (w/v) gelatin
Add deionized H_2O to a final volume of 1 liter
Autoclave

20× SSC Buffer (per Liter)

175.3 g of NaCl
88.2 g of sodium citrate
800.0 ml of deionized H_2O
Adjust to pH 7.0 with a few drops of 10 N NaOH
Add deionized H_2O to a final volume of 1 liter

TE Buffer

10 mM Tris-HCl (pH 7.5)
1 mM EDTA

10× Ligase Buffer

500 mM Tris-HCl (pH 7.5)
70 mM $MgCl_2$
10 mM dithiothreitol (DTT)

Note *rATP is added separately in the ligation reaction*

10× STE Buffer

1 M NaCl
200 mM Tris-HCl (pH 7.5)
100 mM EDTA

REFERENCES

1. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
2. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G. et al. (1987). Current Protocols in Molecular Biology. John Wiley and Sons, New York.

ENDNOTES

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