Lambda FIX II Undigested Vector Kit

INSTRUCTION MANUAL

Catalog #248201

Revision A

For In Vitro Use Only

248201-12

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MATERIALS PROVIDED

Material provided	Quantity
Undigested Lambda FIX 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. On arrival, store the 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 FIX II Vector: –20°C
Bacterial Glycerol Stocks: –80°C

Revision A

INTRODUCTION

The Lambda FIX II vector is a replacement vector[1] used for cloning large fragments of genomic DNA (see Figures 1 and 2). The Lambda FIX 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 FIX II DNA are located on the stuffer fragment; therefore, the wild-type Lambda FIX II phage cannot grow on XL1-Blue MRA (P2). When the stuffer fragment is replaced by an insert, the recombinant Lambda FIX II vector 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 strain XL1-Blue MRA is also provided as a control strain and later for growth of the recombinant when the selection is no longer necessary. The unique arrangement of the polylinker for the Lambda FIX II vector permits the isolation of the insert and flanking T3 and T7 bacteriophage promoters as an intact cassette by digestion with *Not* I. 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.

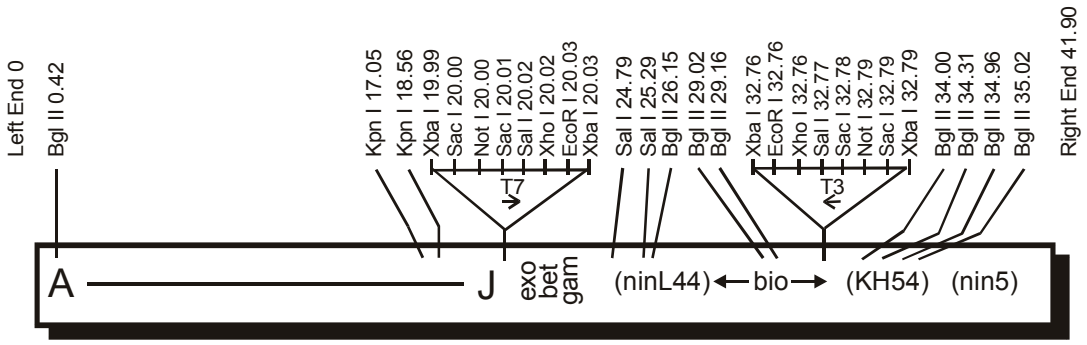

Figure 1 Map of the Lambda FIX II replacement vector.

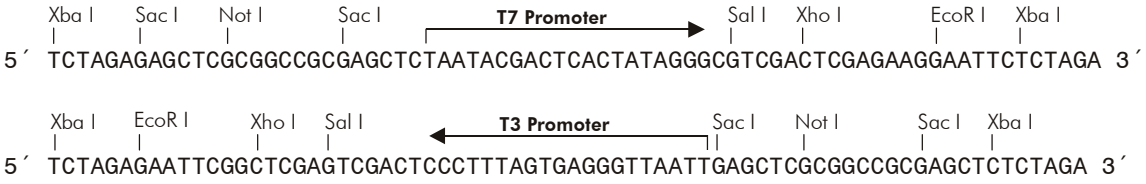

Figure 2 Multiple cloning site sequence of the Lambda FIX II replacement vector.

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 $MgSO_4$
VCS257 strain[b]	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.

[b] For use with Gigapack III packaging extract and wild-type control only. Supplied with Gigapack III packaging extract.

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. It is also best to 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§. 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.

§See *Preparation of Media and Reagents.*

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 (5 ml of glycerol + 5 ml of appropriate medium) to the bacterial culture from step 1. Mix well. 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 FIX II VECTOR

The undigested Lambda FIX 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.

We recommend performing a pilot digestion, covering a range of enzyme digestion incubation times. Package 0.4 µg from each time point and 0.4 µg of the undigested Lambda FIX II vector as a control. (Plate a dilution of the undigested Lambda FIX 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 FIX II vector.

1. Digest 2.5 µg of the Lambda FIX 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) from each time point and stop each reaction by adding 0.5 µl of 10× STE[§].

   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

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

3. Package 0.4 µg (4 µl) and plate according to the instructions outlined in *Packaging* section of this manual.

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

    a. Digest 5 µg of the Lambda FIX II vector in a final 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.

    d. Add an equal volume of 4 M NH_4OAc to the aqueous phase.

    e. Add 2.5 volumes of room temperature 100% (v/v) ethanol. Microcentrifuge the reaction for 15 minutes at room temperature at a maximum speed to pellet the DNA.

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

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

    h. 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 into the Lambda FIX II Vector

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 FIX II vector. 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 yellow pipet tip prior to pipetting.*

1.0 µl of the digested Lambda FIX II DNA (1 µg)
X µl of partially digested genomic DNA (use an equal molar ratio of insert:vector)
0.5 µl of 10× ligase buffer
0.5 µl of 10 mM rATP (pH 7.5)
2 U of T4 DNA ligase (Stratagene Catalog #600011)
Water up to a final volume of 5 µl

Incubate the ligation at 4°C overnight.

When ligating the insert, use a volume up to 2.5 µl. Use an equal molar ratio of the *Bam*H I- *Mbo* I-, or Sau 3A-digested insert with the Lambda FIX II vector. The Lambda FIX 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 5×10^5–1×10^7 recombinant plaques when using high-efficiency packaging extracts, such as Gigapack III Plus or Gigapack III Gold packaging extracts ([Catalog #200201 (Gold-4), #200202 (Gold-7), and #200203 (Gold-11)] Gigapak III Plus packaging extract [Catalog #200204 (Plus-4), #200205 (Plus-7), and #200206 (Plus-11)]).

Note *Protocols for preparing the partially digested DNA are outlined in Reference 2. It is recommended to size fractionate the DNA and/or to calf intestine alkaline phosphatase (CIAP)-treat the ends of the DNA to prevent 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.

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

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).

Both recombinant and nonrecombinant phage will grow on XL1-Blue MRA, but only recombinant phage will grow on XL1-Blue MRA (P2). Plaques should be visible after 8–12 hours of incubation at 37°C.

Note *Historically, the host strain LE392 has been used with this vector; however, E. coli restriction systems in this strain have a significant negative effect on the efficiency of DNA cloning and the ability to generate libraries representative of the gene population. The strains provided, XL1-Blue MRA and XL1-Blue MRA (P2), are McrA⁻, McrB⁻ and Mrr⁻; these modifications have been demonstrated to cause up to a 10-fold increase in the yield of recombinant phage containing methylated DNA. In addition, these strains have been further modified to enhance the stability of nonstandard DNA structures.*

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. 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 NZY 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. We recommend storing 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 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 membrane 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.

6. Blot briefly on a Whatman® 3MM paper.

7. Crosslink the DNA to the membranes 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.[2,3] Following these procedures, perform secondary and tertiary screenings also as outlined in the standard methodology texts. [2,3] After an isolate is obtained, refer to Sambrook *et al.*[2] for suggested phage miniprep and maxiprep procedures.

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

* Stratagene Catalog #400071 (1800) and #400075 (2400).

RAPID RESTRICTION MAPPING

The insertion sites of the Lambda FIX 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 FIX II vector has unique *Not* I sites flanking the RNA promoters, which permits the excision from the lambda vector of insert DNA with the T3 and T7 promoter sequences as an intact fragment.

TROUBLESHOOTING

Observations	Suggestions
Packaging efficiency is too low	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 use of ligase buffers containing PEG, which can inhibit packaging
	Ensure the concentration of DNA is sufficient. Ligate at DNA concentrations of 0.2 µg/µl or greater and package between 1 and 4 µl of the ligation reaction
	Never package >4 µl of the ligation reaction, which causes dilution of the proteins contained within the packaging extract
Neither a bacterial lawn nor plaques is observed on the plate	Be sure to spin down the chloroform completely prior to removing an aliquot for titering to avoid chloroform contamination during 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 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)

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

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

REFERENCES

1. Frischauf, A. M., Lehrach, H., Poustka, A. and Murray, N. (1983) *J Mol Biol* 170(4):827-42.
2. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
3. 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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