



Description

Stock and Custom cDNA Libraries

Novagen offers a unique collection of cDNA libraries featuring different developmental stages and tissues of *Drosophila* and mouse. All cDNA libraries are constructed in Novagen's advanced *lox* autosubcloning vectors by directional cloning strategies. Each library contains at least 1×10^6 primary recombinants and has undergone a single round of amplification. The cDNAs were size fractionated such that inserts are >500bp (oligo(dT) or >300bp (random primers) in length and are cloned directionally. The size range of cDNA inserts has been verified to be from 500-4000bp (oligo(dT) and 300-2000bp (random primers) by gel electrophoresis of DNA prepared from the amplified libraries. All libraries have been constructed without the use of carrier RNA, which eliminates the possibility of contamination with heterologous clones. Each Stock Library kit contains enough phage to perform 50-500 screenings.

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Components

- 100μl cDNA Library (high titer phage lysate)
- 0.2ml BM25.8 Glycerol Stock
- 0.2ml ER1647 Glycerol Stock
- 0.2ml BL21(DE3)pLysE Glycerol Stock

Available separately:

PhageFinder[™] Immunoscreening Kit for λSCREEN[™]-1/λEX*lox*[®] libraries 69260-3
mouse antibodies

PhageFinder Immunoscreening Kit for λSCREEN-1/λEX*lox* libraries 69261-3
rabbit antibodies

Growth of Host Cells

The appropriate host for immunoscreening λSCREEN-1 and λEX*lox* libraries is BL21(DE3)pLysE.

Inoculate a single colony of host cells into 50ml LB supplemented with 0.2% maltose, 10mM MgSO₄. Also add 34μg/ml chloramphenicol for BL21(DE3)pLysE. Incubate at 37°C overnight with shaking at 250rpm. Refrigerate cells until ready to use. Storage for 3 days at 4°C results in about a 25% loss of phage titer.

Note: The genotype of BL21(DE3)pLysE is: F⁻ *ompT hsdS_B(r_B⁻ m_B⁻) gal dcm* (DE3) pLysE (Cm^R). This host is a lysogen of λDE3, which contains the gene for T7 RNA polymerase under *lacUV5* control. λDE3 is of immunity group 21 so it does not interfere with the infection of λSCREEN-1 and λEX*lox*, which is *imm*⁴³⁴. λDE3 will not lyse the host cells because the inserted T7 RNA polymerase gene interrupts the *int* gene, whose product is required for excision from the host chromosome. (The *int* gene is deleted in λSCREEN-1 and λEX*lox* (deletion *b538*) so that it cannot serve as a helper to excise λDE3.) The host strain also contains the plasmid pLysE, which carries the gene for T7 lysozyme. T7 lysozyme is a natural inhibitor of T7 RNA polymerase and its presence is required to allow plaque formation by λSCREEN-1 and λEX*lox* phage. Without pLysE, even the small amount of T7 RNA polymerase that is produced under basal conditions is sufficient to inhibit lambda functions upon infection with a phage containing T7 transcription and translation signals.

Plating of Phage

1. For primary screening of libraries, make serial dilutions of the phage in SM (if necessary) and mix 2×10^4 pfu in 100μl SM with 200μl cells in a culture tube for every 82mm plate to be screened. Use up to 5×10^4 pfu and 600μl cells with 150mm plates. Incubate at 37°C for 30 min to allow the phage to adsorb to the host cells. Add 3ml top agarose (8ml for 150mm plates) warmed to 50°C and quickly pour on 2X YT plates and spread evenly over plate. Use slightly dry plates (2 days old) so that the agarose will tend to stick to the plates when filters are lifted.



2. Invert plates and incubate at 37°C until plaques are about 0.5-1mm in size (approximately 5-7 hours for λ SCREEN-1 and λ EXlox).
3. Overlay the plates with a dry nitrocellulose filter. To avoid trapping bubbles, slightly bend the filter in the center by holding it at the edges and allow the middle of the filter to make contact with the plate first. Allow the rest of the filter to be pulled down on the plate by capillary action. Always handle nitrocellulose filters with gloves. We do not recommend using IPTG-soaked filters when screening a λ SCREEN/ λ EXlox library for the first time, because some proteins appear to affect phage viability when expressed in very large amounts in infected cells. In these cases plaques may not form upon secondary plating. In addition, sufficient protein levels appear to accumulate to allow detection in the absence of induction under most conditions. If positives are difficult to detect using non-induction conditions, rescreen the same plates using IPTG-soaked filters.
4. Incubate plates and filters in an inverted position for an additional 3.5 hours at 37°C. Alternatively, put the plates and filters at 4°C overnight and incubate for 4 hours at 37°C in the morning. When the density of plaques is low (less than 1000/82mm plate), incubation may continue overnight at 37°C. A second filter may be overlaid after the first one has been removed and incubated for an additional 3 or more hours at 37°C if a duplicate is required.

Notes:

- a. Do not incubate λ SCREEN/ λ EXlox plates at 42°C because phage form plaques poorly at this temperature.
- b. It is important to use 2X YT media for λ SCREEN/ λ EXlox; other media such as LB are unsuitable for immunoscreening with these vectors.
- c. Overlay filters when plaques are 0.5-1mm diameter. Expressed fusion proteins diffuse rapidly into the top agarose and will produce weak, fuzzy signals if the plates are incubated too long at 37°C or are stored at 4°C prior to filter overlay. In addition, do not overlay filters over plaques that are too small (pinpoint in size) since these will contain only low amounts of expressed protein.
- d. If plaque formation takes longer than 7 hours, expression is usually poor. Plaque formation can be delayed by using top agarose at too high a concentration (> 0.8%; sometimes caused by pipetting from the bottom of an insufficiently mixed bottle) or by using plates which are thick (>20ml/82mm plate or >60ml/150mm plate).

Immunoscreening

1. To aid separation of the top agarose from the filters, refrigerate plates plus filters for 1 hour or longer. Mark filters in 3 asymmetric locations by stabbing an 18 gauge needle through filter and into the agar. Dip the needle into waterproof black ink prior to stabbing to make visible marks on filters and plates.
2. Carefully remove filters and rinse off any residual agarose in TBST. Number the filters and plates.
3. Block non-specific protein binding sites by incubating filters in TBST + 1% gelatin for 30 minutes. The 10% gelatin in the kit will liquefy upon warming to approximately 40°C. Use about 5ml per 82mm filter (15ml/150mm filter). Filters may be placed in Petri dishes (up to 5 per dish, given adequate agitation) for incubations and washes. Do the following incubations and washes at room temperature with gentle agitation.
4. Incubate filters with primary antibody diluted in TBST for 30-60 minutes. Use 7.5ml of diluted antibody per filter (15ml per 150mm filter), although less can be used when multiple filters are incubated together. The diluted antibody can be reused several times (add sodium azide to 0.02% and store at 4°C up to 1 week).
5. Remove antibody solution and wash filters three times with 15-20ml TBST for 10 minutes each.
6. Incubate filters with the appropriate dilution of secondary antibody alkaline



- phosphatase conjugate in TBST for 30-60 minutes. Use 7.5ml per 82mm filter.
7. Remove antibody solution and wash filters three times with 15-20ml TBST for 10 minutes each.
 8. After the last wash, place the filters on Whatman 3MM filter paper (protein side up) for several minutes to allow excess liquid to be absorbed. Do not allow the filters to dry completely; they should remain damp. Prepare 1X alkaline phosphatase buffer containing NBT and BCIP from the supplied solutions, adding 4 μ l of each substrate per ml of 1X buffer. Transfer the filters to Petri dishes and add enough color development solution to cover the filters (7.5ml/82mm filter is more than sufficient). Dark purple positive plaques usually appear within 5-10 minutes, although development can be allowed to proceed for 4 hours (expect the background to increase upon prolonged incubation). Since the fusion protein is synthesized in infected cells at the periphery of the plaque, positives often appear as "doughnuts" with clear centers. Stop the color development reaction by rinsing filters several times in water. Air-dry filters.
 9. Retest positive plaques by removing an agar plug corresponding to the positive signal on the filter with a Pasteur pipet and put into 1ml SM for 1 hour at room temperature or 4-16 hrs at 4°C. Replate at dilutions of 1:50 and 1:500. Repeat screening procedure and repurify positive plaques. Repeat immunoscreening procedure until all plaques on a plate produce a signal.

Notes: Signal to noise ratios obtained with this procedure depend on several factors. First, the amount of antigen that is deposited on the filter varies with each fusion protein based on its expression level and stability in the host cells. (The λ SCREEN/ λ EX λ ox system tends to minimize this variability by producing a strongly expressed, stable gene 10 fusion protein.) Second, the quality (titer and affinity) of the screening antibody is of obvious importance. Generally, antibodies that produce good signals on Western blots are likely to produce good results in immunoscreening. Antibodies directed against carbohydrate determinants and some conformation-dependent antibodies are not suitable for immunoscreening. Third, crude antisera and ascites occasionally contain IgG components that bind to *E. coli* proteins that can produce high backgrounds on plaque lifts. The effect of these factors can be minimized by using the least amount of antibody that still gives a strong signal with a given amount of antigen (the amount of antigen in a plaque can range from about 30 to 800pg), and gives little reactivity with *E. coli* proteins. Various antibody dilutions can be easily tested by a dot blot assay using known amounts of antigen in parallel with similar amounts of *E. coli* extract (prepared by sonicating *E. coli* to break the cells and centrifuging to remove cell debris). In general, dilutions from 1:200 to 1:10,000 are appropriate for antisera, ascites fluids, or purified antibodies, whereas hybridoma tissue culture supernatants usually require dilutions from 1:10 to 1:100.

If non-specific background due to cross-reactivity with filter-bound *E. coli* proteins remains to be a problem, the antibody preparation can be preabsorbed with an *E. coli* lysate. For this purpose, Novagen offers a lysate of strain BL21 (Cat. # 69458-1). As a starting point, incubate 1mg/ml extract with antisera diluted 1:1000 for 30 minutes prior to incubating the antisera with filters. The amount of extract and the antibody dilution can be varied to optimize signal to noise ratios.

Preparation of plaque lifts for screening with nucleic acid probes

1. Determine the number of recombinants to be screened and prepare the required number of plates. Plan to plate about 3×10^4 phage per 82mm plate or 5×10^4 phage per 150mm plate. Plate the phage as described above, but use top agarose instead of top agar.
2. When plaques reach desired size (1-2mm diameter), cool the plates for 15-30 minutes at 4°C.
3. Holding a dry nitrocellulose membrane by the edges (wear gloves!), bend it slightly to produce a bow in the center. Carefully lay the membrane on top of the plate, center first, and let the sides drop on to the plate as the membrane wets. The objective is to avoid trapping air bubbles. Leave the membrane on



- the plate for one minute once it is completely wet.
4. Mark the filters in 3 asymmetric locations by stabbing an 18 gauge needle through filter and into the agar. Dip the needle into waterproof black ink prior to stabbing to make visible marks on filters and plates.
 5. Using a blunt forceps, carefully peel the membrane away from the top agarose surface. Float the membrane (DNA side up) in a shallow tray of denaturing solution (1.5M NaCl, 0.5M NaOH) for 60 seconds. Duplicate filters can be made from the same plate by the same procedure. For each replicate, the incubation time on the plate should be increased by 30 seconds.
 6. Transfer the membrane to another tray containing neutralizing solution (1.5M NaCl, 0.5M Tris-HCl, pH 8.0) for 60 seconds.
 7. Place the membrane, DNA side up, on filter paper and let dry.
 8. Put the membrane between sheets of filter paper, wrap in aluminum foil and bake in vacuum oven at 80°C for 15-30 min. Store membranes, wrapped in plastic, at 4°C.

Autosubcloning

After positive recombinant phage are isolated, it is often desirable to convert them to plasmid subclones. The λ SCREEN/ λ EXlox vectors feature the simple and reliable *loxP*-cre system for “automatic” subcloning *in vivo*. Complete plasmids are built in to the phage and are flanked by two 34bp *loxP* sites derived from bacteriophage P1. Plasmid subclones are generated simply by infection of hosts expressing the P1 cre recombinase, which recognizes the *loxP* sites and forms the plasmid by site-specific recombination. When plated in the presence of ampicillin (or, preferably, carbenicillin), colonies appear which are the result of plasmid excision. Inserts are faithfully and efficiently subcloned without the generation of rearrangements or deletions. λ SCREEN/ λ EXlox vectors have an additional advantage over other expression vectors because expression from the T7 promoter is virtually silent in the plasmid in the absence of a source of T7 RNA polymerase. Therefore, cDNAs coding for proteins toxic to *E. coli* will form stable plasmid subclones in the non-expression strain provided (BM25.8). This is in contrast to autosubcloning in vectors which employ other promoters, such as *lac*, to drive expression from cloned cDNAs because even basal expression levels can be too high to allow subcloning of cDNAs encoding toxic proteins as plasmids.

Procedure

1. Grow an overnight culture of the host strain BM25.8 in LB supplemented with 10mM MgSO₄, 0.2% maltose, 50μg/ml kanamycin and 34μg/ml chloramphenicol.
2. Mix 100μl of an appropriate dilution (see Step 6) of the phage with 100μl of host cells.
3. Incubate the host/phage mixture at 37°C for 30 min to allow the phage to adsorb to the host.
4. Spread 100μl of the host/phage mixture directly on the surface of a carbenicillin plate with a bent glass rod. Use LB agar plus 50μg/ml carbenicillin (available from Sigma Chemical Co.). Ampicillin can also be used, but tends to result in more satellite colonies.
5. Incubate the plate inverted at 37°C overnight.
6. Count the number of colonies. Expect an efficiency of 10 to 20%. For example, if the phage titer is 10⁴ pfu/ml, there should be 1-2 × 10³ drug-resistant cfu/ml.

BM25.8 genotype is: [F' *traD36 lac^llacZΔM15 proA⁺B^r*] *supE thi Δ(lac-proAB)* P1 Cm^R *hsdR* (r_{K12}⁻ m_{K12}⁺) (λ *imm*⁴³⁴Kan^R). Maintain the strain on minimal media such as M9 containing 1μg/ml thiamine, 34μg/ml chloramphenicol and 50μg/ml kanamycin. The strain is a double lysogen of P1 and λ *imm*⁴³⁴, each of which is maintained *via* antibiotic selection.

Note: colonies obtained in this manner contain plasmids which have been excised from the phage by site-specific recombination. The cre recombinase generates a mixture of plasmid multimers, and plasmids prepared directly from colonies in BM25.8 by



standard miniprep procedures are unsuitable for mapping by gel analysis or sequencing. Plasmids that are subcloned in BM25.8 should be isolated by a miniprep protocol and then transformed into another host for these purposes. Suitable hosts include NovaBlue, HB101, JM109, and DH5. pSCREEN/pEX/ox recombinants can be transformed into any pET expression strain for protein production (see the Novagen catalog for information about frozen competent cells of NovaBlue and the pET host strains).

Recipes

<u>2X YT (plates)</u> Per liter: 16g Bacto tryptone 10g Yeast extract 5g NaCl 15g agar Autoclave	<u>Top agarose</u> Per liter: 10g Bacto tryptone 5g Yeast extract 5g NaCl 6g agarose Autoclave	<u>SM</u> Per liter: 20ml 5M NaCl 2g MgSO ₄ ·7H ₂ O 50ml 1M Tris-HCl pH 7.5 100mg gelatin Autoclave
<u>10X TBST</u> Per liter: 100ml 1M Tris-HCl pH 8.0 88g NaCl 25ml 20% Tween 20 Add azide to 0.02% Store at room temp	<u>LB</u> Per liter: 10g Bacto tryptone 5g Yeast extract 10g NaCl pH to 7.5 with 1N NaOH Autoclave	<u>M9</u> Per liter: 6g Na ₂ HPO ₄ 3g KH ₂ PO ₄ 0.5g NaCl 1g NH ₄ Cl Autoclave After autoclaving, add: 10ml 0.01M CaCl ₂ 2ml 1M MgSO ₄ 10ml 20% glucose 0.5ml 1% thiamine
<u>20% Maltose</u> Autoclave	<u>1M MgSO₄·7H₂O</u> Autoclave	

10mM IPTG (Isopropyl β-D-Thiogalactopyranoside)
0.238g IPTG in 100ml H₂O. Filter sterilize and store at -20°C.

Nitrocellulose filters

(Optional; see protocol) Soak nitrocellulose filters in a solution of 10mM IPTG for 5 minutes. Air dry. Wrap in plastic and store at 4°C up to one week.

Troubleshooting

Possible problems/Causes	Controls/Solutions
Non-binding antibody: no positive plaques	Check antibody reactivity with antigen on Western blot. Determine proper dilutions of primary and secondary antibodies or other signal reagents.
No expression: no positive plaques	Be sure to use host BL21(DE3)pLysE or BL26(DE3)pLysE for immunoscreening. Be sure to use 2X YT media for plating.
Positive plaques inviable: secondary screens negative	Do not use IPTG induction for expression; can result in lack of phage production for certain clones.
No plaques or pinpoint plaques	Regrow fresh plating cells; check incubator temperature and top agarose concentration. Also, the source and lot of agarose can make a difference.

References

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