Recombination-Based Assay (RBA) for Screening Bacteriophage Lambda Libraries

BASIC PROTOCOL

The recombination-based assay represents a convenient way to screen a complex library constructed in bacteriophage λ for homology to a given sequence cloned into a specially designed plasmid. The technique serves to screen a bacteriophage library rapidly and efficiently with a sequence cloned into a plasmid; counterselection then yields the gene product of interest with its plasmid carrier deleted. Because 10^6 to 10^7 plaque-forming units (pfu) may be screened using several petri dishes, and the homology for crossing-over need only be >25 bp, the RBA represents an efficient way to screen complex λ libraries rapidly for homology to a given sequence.

In this procedure (outlined in Fig. 6.12.1), a λ library is screened using a specially designed R6K *supF* plasmid, pAD1 (Fig. 6.12.2), carrying the desired target sequence. Recombinants arising from cross-over events between the plasmid and a bacteriophage carrying a corresponding region of homology are selected by their ability to grow on strain DM21 (Fig. 6.12.3). Growth of λ on DM21 requires the presence of the *supF* allele encoded on the plasmid to suppress an amber mutation in the host strain that prevents λ propagation. Recovery of the original phage carrying the target sequence requires a reversal of the homologous recombination event. This reversal occurs spontaneously, and is detected by PCR amplification using primers that flank the cloning site in the λ vector (Fig. 6.12.4).

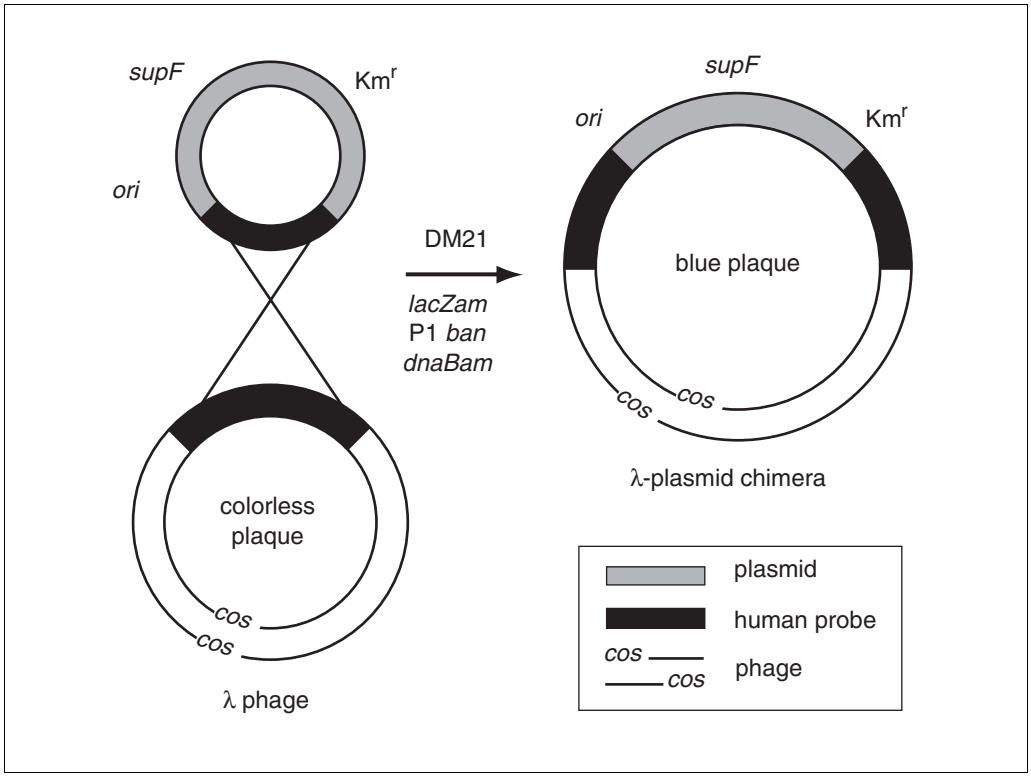

Figure 6.12.1 The recombination-based assay (RBA). Homology between sequences in a plasmid and a bacteriophage >25 bp long (Watt et al., 1985; Shen and Huang, 1986, 1989; King and Richardson, 1986) mediates a recombination event between the two vectors. As a result *supF* is integrated into the bacteriophage, allowing it to plate on the *dnaBam* host DM21 (see Table 6.12.1). The cointegrate yields a blue plaque in the presence of IPTG and Xgal on the *lacZam* host DM21, as *supF* suppresses the amber mutations in both the *dnaB* and *lacZ* genes. Different shadings indicate origins of DNA regions.

Contributed by David M. Kurnit
Current Protocols in Molecular Biology (1994) 6.12.1-6.12.12

Materials

For recipes, see ***Reagents and Solutions*** *in this unit (or cross-referenced unit); for common stock solutions, see* ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

DNA fragment encoding sequence of interest
Plasmid pAD1 (Fig. 6.12.2; available from Dr. D. Kurnit)
recA$^+$ *E. coli* strain (Table 1.4.5 or commercial suppliers)
L broth (see recipe) with 50 μg/ml kanamycin (Table 1.4.1)
Bacteriophage λ library (*UNIT 5.8*)
Lambda top agar (see recipe)
Lambda plates (see recipe), some with 50 μg/ml kanamycin and some with 100 μg/ml streptomycin (Table 1.4.1)
Suspension medium (SM; see recipe)
Chloroform
E. coli DM21, DM75, DM392, and DM1061 (Fig. 6.12.3 and Table 6.12.1), saturated overnight cultures freshly grown in LB medium (*UNIT 1.1*) with 100 μg/ml streptomycin
100 mM IPTG (isopropyl thiogalactoside; Table 1.4.2)
2% Xgal in DMF (see recipe)

Additional reagents and equipment for subcloning DNA into plasmids (*UNIT 3.16*), culturing (*UNIT 1.1*) and transformation (*UNIT 1.8*) of bacteria, plating and titering λ phage (*UNIT 1.11*), β-galactosidase assay (*UNIT 1.4*), and PCR amplification (*UNIT 15.1*)

NOTE: All incubations are at 37°C unless otherwise specified.

Screen library and select recombinants

1. Clone the sequence of interest into a pAD1 plasmid and transform into *recA*$^+$ *E. coli* strain yielding a kanamycin-resistant *recA*$^+$ strain. Prepare a saturated overnight culture grown with aeration in L broth containing 50 μ/ml kanamycin.

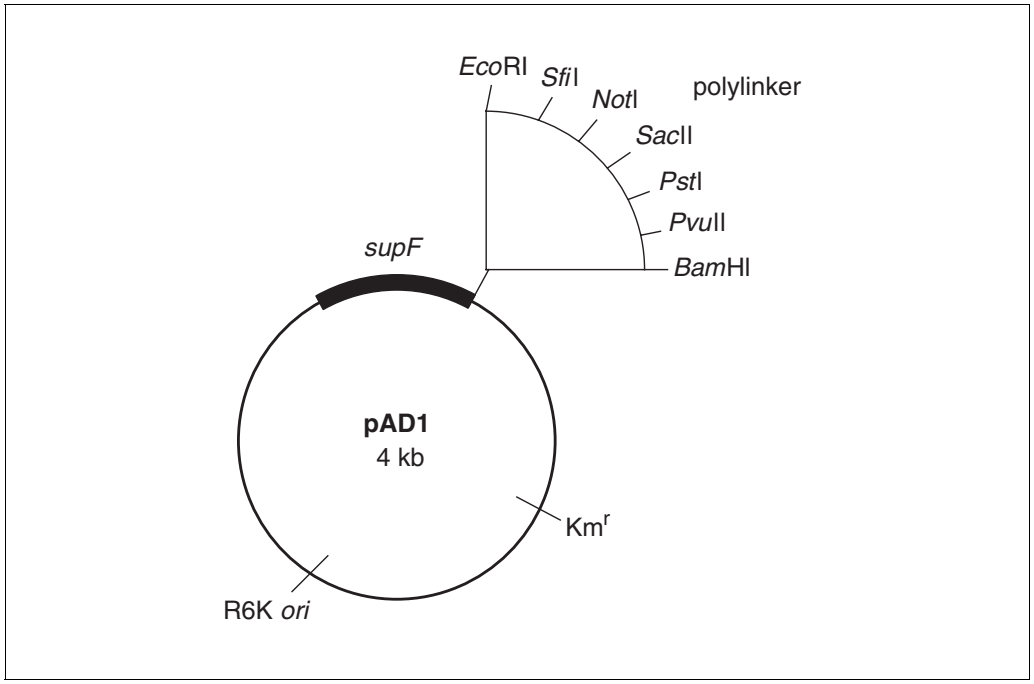

Figure 6.12.2 Structure of pAD1. This plasmid incorporates the R6K replicon, Km^r, *supF*, and a polylinker. It is not homologous to ColE1 plasmids.

2. Mix 3 ml lambda top agar, 200 µl of overnight culture, and 10^6 to 10^7 pfu of a bacteriophage λ library. Mix well and pour mixture onto a lambda/kanamycin plate. Incubate 7 hr to overnight until total lysis occurs.

   If more convenient, incubation overnight is perfectly acceptable, because there is no need to harvest the plates just as lysis occurs.

3. Add 3 ml SM and 0.5 ml chloroform to each plate. Swirl lightly. Incubate 2 hr to overnight at room temperature to allow the plates to elute.

   SM and chloroform are immiscible; swirling them together ensures that the SM is saturated with chloroform, killing any eluted bacteria and minimizing phage adsorption to bacterial debris. The easiest method is to rotate a stack of plates slowly by hand after adding the liquid. Care should be taken not to get chloroform on the petri dish cover, as this can cause fusion of the cover and the plate bottom. If fusion occurs, the cover can be pried from the bottom (e.g., with a screwdriver).

4. Using a nonsterile disposable transfer pipet, harvest the eluate from each plate into a 1.5-ml polypropylene microcentrifuge tube.

   Although the transfer pipets are polyethylene, they hold chloroform-saturated SM for too short a time-span to be damaged by the solvent.

   At this stage harvested eluates can be stored ≤1 week at 4°C before continuing the procedure.

5. Add 50 µl of eluate (5×10^8 to 1×10^9 pfu) to 200 µl DM21 culture. Add 3 ml lambda top agar and pour mixture onto a lambda/streptomycin plate. Incubate 7 hr to overnight until plaques form.

   DM21 is selective (dnaBam lacZam) and resistant to streptomycin. DM75, DM1061, and DM392 (used in later steps) are also streptomycin-resistant, with growth and plating conditions identical to those for DM21.

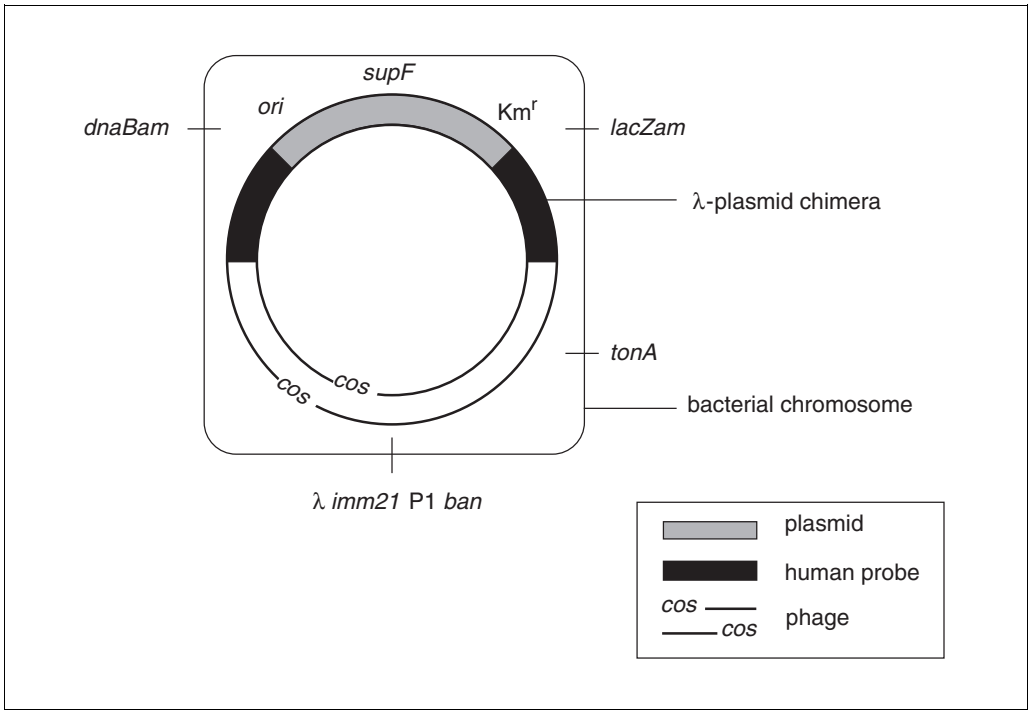

Figure 6.12.3 Bacterial strain DM21 (outer rectangle) containing λ plasmid chimera with *supF* integrated (inner circle). DM21 has the genotype $lacZ_{YA536}$*(am)*, *dnaB266(am)*, Sm^r, $hsdR^+$, $hsdM^+$, $tonA^-$ (λ *imm21 b*515 *b*519 *nin5 att*$^+$P1 *ban*), *supO lacZ(am) dnaB(am)*. The *dnaB* amber allele selects for λ phage that have *supF* integrated as shown. *SupF* also suppress the *lacZ* amber mutation, yielding blue plaques. Different shadings indicate origins of DNA regions.

Titer eluates on permissive strain

6. Add 10 µl of each eluate to be titered to 990 µl SM to obtain a 1/100 dilution. Prepare a 100-fold dilution series (to 10^{-8}) in SM.

   Several random eluates should be titered on the permissive (supF-bearing) strain DM392 to ensure that an appropriate number of phage have been added to the DM21 lawn.

7. Pour a lawn of DM392 (200 µl culture in 3 ml top agar) on a lambda/streptomycin plate. Drop 10-µl aliquots of each eluate dilution onto lawn. Dry 15 min in a forced-air hood (or for longer on bench or in incubator). Incubate 7 hr to overnight until total lysis occurs.

   This drop-titer procedure is the most convenient method of titering the eluates.

8. Count plaques in the lowest dilution that yields plaques. Convert the result to pfu/ml by multiplying it by the appropriate dilution factor and by a factor of 10^2.

   Titration ensures that sufficient phage have been added to the DM21 lawn. Should too many be added (more pfu than cells), the lawn will not materialize due to lysis from without. This phenomenon occurs because every cell that is infected with a bacteriophage will die, even though only cells infected by a phage carrying supF will yield a productive burst that then goes on to infect other cells. In rare cases of lysis from

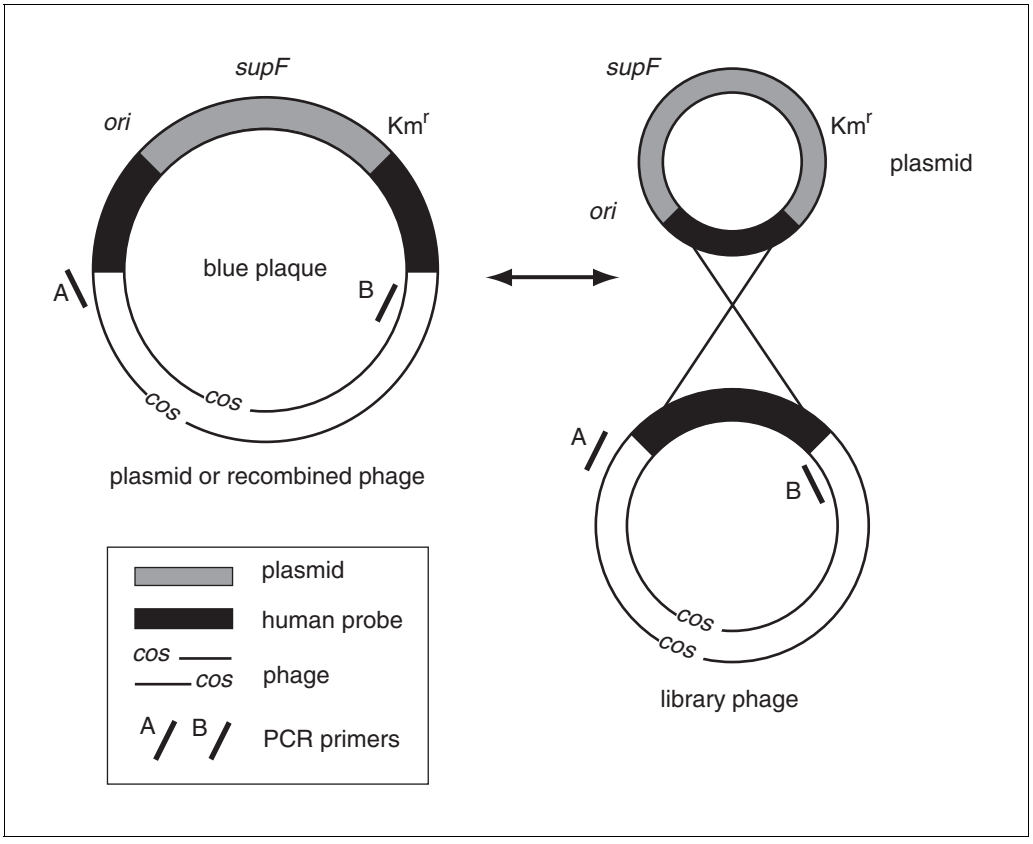

Figure 6.12.4 Counterselection. Reversal of the recombination event (which is an equilibrium event) occurs spontaneously. PCR using primers abutting the cloning site of the bacteriophage is employed preparatively to obtain the cDNA without the genomic sequence in pAD1 that was used to retrieve it. The cDNA insert + pAD1 + genomic insert is too large to be amplified by PCR; in contrast, the cDNA insert alone can be amplified. Because there is an equilibrium between the selected and the counterselected phage, the counterselected insert can be amplified directly from the selected blue plaque, which contains a mixture of the two phages. Different shadings indicate origins of DNA regions.

without, the plating should be repeated; either the eluate should be titered or less eluate used.

Plaques on DM21 are very small, because suppression of the dnaBam mutation (which is not fully efficient) is required for growth. This makes it difficult to confirm that supF is present via simultaneous suppression of the lacZam mutation by supF; therefore, phage must be transferred to another strain as described in the following steps.

Confirm phages have integrated supF

9. Elute plaques on DM21 (from step 5) into 100 µl SM. Mix:

   10 µl eluate
   200 µl DM75 culture
   3 ml lambda top agar
   10 µl 100 mM IPTG
   100 µl 2% Xgal in DMF.

   Plate on lambda/streptomycin plates. Incubate 7 hr to overnight until total lysis occurs.

   To mix water and DMF, the tubes of top agar must be inverted and righted several times, taking care not to create bubbles. It is best not to prepare more than several tubes at once, because cells do not tolerate the heating block for very long.

   Light blue plaques are the desired phage containing supF. A larger number of colorless plaques that have not integrated supF will also plate on this strain; these correspond to phage that were not adsorbed originally on DM21 and therefore remain viable. In addition, for a phage such as λgt11, in which interruption of an intact lacZ gene serves as evidence of successful cloning, blue color can result from an intact lacZ gene in the phage. To differentiate between the two, note that the desired supF suppression of the single-copy chromosomal lacZ locus results in a light blue color that extends only to the plaque margins, whereas the high-copy-number lacZ gene on λgt11 yields a dark blue halo that extends past the plaque margins.

10. Elute each plaque thought to contain an integrated *supF* (from step 9) into 100 µl SM. Pour lawns of DM75 and DM1061 (200 µl culture in 3 ml top agar/IPTG/Xgal, as in previous step) onto separate lambda/streptomycin plates. Drop 10-µl aliquots of each phage eluate onto a lawn of each strain. Incubate 7 hr to overnight until total lysis occurs.

    This serves to confirm that plaques result from phage with supF rather than lacZ. Phage with supF will be blue on DM75 (lacZam) but colorless on DM1061 (which contains a lacZ deletion), whereas phage carrying an intact lacZ gene will be blue on both strains.

Counterselect with PCR

11. Pour a lawn of 200 µl DM75 in 3 ml top agar onto lambda/streptomycin plate. Drop 10-µl aliquots of phage eluate onto lawn. Incubate 7 hr to overnight, until a single large plaque (“macroplaque”) appears.

12. PCR amplify the cloned product from the macroplaque using primers that abut the *Eco*RI cloning site of the λ phage vector used to construct the library.

    This reverses the selection process and accomplishes counterselection (see Fig. 6.12.4). Using the large macroplaque ensures that sufficient template is present.

    Because the recombination reaction is an equilibrium reaction, a small fraction of phage within a blue macroplaque represent colorless revertants that have excised the pAD1 plasmid and its insert. In contrast, the major product in the macroplaque carries the phage insert, the plasmid, and the insert. Because this is too large to be amplified efficiently by PCR, the technique preferentially yields the desired genic insert from the phage without the unwanted plasmid and its insert.

13. If desired, sequence the isolated genic clone (*UNITS 7.1-7.5*) and compare it to a database of known expressed sequences (*UNIT 7.7*) to obtain information about its possible significance, if available.

Repeatedly performing this protocol with different cDNA libraries allows determination of the timing of development and the tissue(s) in which the gene of interest is expressed. The latter can also be determined by using PCR primers specified by the sequence to see if amplification of different cDNA libraries occurs; given the sensitivity of this method, only cDNA library eluates, rather than DNA preparations, need be screened.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

Lambda plates

10 g tryptone
5 g NaCl
13 g agar
3 ml 1 M $MgCl_2$
H_2O to 1 liter

Sterilize by autoclaving. Allow to cool until comfortable to touch. Add antibiotics as needed, mix gently to avoid bubbles, and pour plates. Store up to several months at 4°C.

Lambda top agar

10 g tryptone
5 g NaCl
8 g agar
3 ml 1 M $MgCl_2$
H_2O to 1 liter

Sterilize by autoclaving. Maintain ≤1 month molten at 60°C.

L broth

10 g tryptone
5 g NaCl
5 g yeast extract
5 g $MgSO_4 \cdot 7H_2O$
1 g glucose
160 ml 12.5 M NaOH (to pH 7.2)
H_2O to 1 liter

Sterilize by autoclaving. Allow to cool until comfortable to touch. Add antibiotics as needed and mix. Store up to several months at 4°C.

Suspension medium (SM)

5.8 g NaCl
2 g $MgSO_4 \cdot 7H_2O$
50 ml 1 M Tris·Cl, pH 7.5 (*APPENDIX 2*)
5 ml 2% (w/v) gelatin
H_2O to 1 liter

Sterilize by autoclaving. Store up to several months at 4°C.

Gelatin is prepared by adding 2 g gelatin to 100 ml H_2O, then autoclaving to dissolve when needed.

Xgal, 2% (v/v) in DMF

Dissolve 2% Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; see Table 1.4.2) in dimethylformamide (DMF). Place in polypropylene tube (not polystyrene, which will be dissolved by DMF), wrapped in aluminum foil. Store indefinitely at −20°C (solution will not freeze).

COMMENTARY

Background Information

The recombination-based assay (RBA) permits screening of a complex library or group of libraries with a given probe using only two petri dishes. As a result, the RBA is unparalleled in its efficiency and speed. The crux of the RBA is the insertion of a DNA fragment into a plasmid containing *supF*, followed by screening of a complex λ library (10^6 to 10^7 recombinants) for homology to the fragment. If such homology exists, a recombination event ensues between the inserts in the plasmid and homologous phage at a frequency of 10^{-2} to 10^{-3} (see Fig. 6.12.1). As a result of this homology-mediated recombination event, the plasmid with *supF* is integrated into λ. Genetic selection for λ phage carrying the plasmid with *supF* results in the isolation of λ phage carrying an insert homologous to the insert in the plasmid. Given the high frequency of homologous recombination (10^{-2} to 10^{-3}), and the fact that 5×10^8 to 10^9 pfu can be plated onto a single petri dish, it is feasible to screen rapidly a λ library with a complexity of 10^6 to 10^7.

Bacterial host characteristics

This assay employs a bacterial strain, DM21 (see Figs. 6.12.1 and 6.12.3), that has been constructed to require the presence of *supF* in λ for phage propagation. As a result, sequences from a λ library that are homologous to a sequence cloned into the *supF*-bearing plasmid can be isolated on this strain. By screening a λ library carrying human genomic DNA sequences (Lawn et al., 1978), the copy number of a given sequence can be determined analytically. Plasmids carrying repetitive sequences rescue more phage clones from a human genomic library than do plasmids carrying nonrepetitive sequences (Neve and Kurnit, 1983). By screening a λ library corresponding to the genes encoded by a given tissue with single-copy sequences, the tissue and time in which a single-copy sequence is transcribed can be determined analytically. Selection for the desired *supF*-bearing phage is done using the *dnaB*/P1 *ban* balanced lethal system. In constructing the host, the *dnaB* unwinding protein that is normally essential for λ phage growth was replaced by the related, but not identical, P1 *ban* gene for *E. coli* growth. The resulting streptomycin-resistant *dnaBam* P1 *ban lacZam* host, DK21 (Kurnit and Seed, 1990), was then protected against a contaminating large (?T1) phage infection by a ?*tonA* mutation to yield the strain DM21 that is used in the protocol (the question mark notes characteristics that are likely but not definite). Analogously, strains LE392, LG75, and MC1061 have each been altered to carry a ?*tonA* mutation and resistance to streptomycin for use in the protocol, and have been renamed DM392, DM75, and DM1061, respectively (Table 6.12.1). DM21 selects for the plasmid-borne *supF* by requiring the suppression of an amber mutation in the *dnaB* gene to permit λ propagation. Furthermore, *supF* also suppresses the amber mutation in the *lacZ* gene of DM21, yielding a blue plaque upon addition of the chromogenic substrate Xgal in the presence of IPTG. This makes it possible to discard rare ($<10^{-9}$) mutant λ phages (probably P-gene mutants) that lack *supF* but can be successfully plated on the *dnaBam* P1 *ban* host DM21, because these contaminating phages will yield colorless plaques.

Table 6.12.1 Bacterial Strains Used

Strain	Genotype[a]	Reference	Comment
DM21	*lacZ*$_{YA536}$(*am*), *dnaB266*(*am*), Sm^r, *hsdR*$^+$, *hsdM*$^+$, ?*tonA*$^-$ (λ *imm21 b*515 *b*519 *nin5 att*$^+$ P1 *ban*)	Kurnit and Seed, 1990	*sup0 lacZam dnaBam*
DM75	*lacZ*$_{YA536}$(*am*), Sm^r, *hsdR*$^+$, *hsdM*$^+$, ?*tonA*$^-$	Guarente et al., 1980	*sup0 lacZam*; strain used by the author and collaborators is Sm^r, although the published genotype does not state this
DM392	*hsdR*514 (*hsdR*$^-$, *hsdM*$^+$), *supE*44, *supF*58, ?*lacY*1, *galK*2, *galT*22, *metB*1, *trpR*55, Sm^r, ?*tonA*$^-$	L. Enquist (unpub. observ.)	*sup*$^+$; made Sm^r by the author and collaborators
DM1061	*araD*139, Δ(*ara, leu*)7697, Δ*lacX*74, *galU*$^-$, *galK*$^-$, Sm^r, *hsdR*$^-$, *hsdM*$^+$, *mcrA*$^-$, *mcrB*$^-$, ?*tonA*$^-$	Casadaban and Cohen, 1980	*sup0*

[a]A question mark denotes characteristics that are likely, but not definite.

Counterselection

Regeneration of the phage as it existed before the recombination-based retrieval event requires deletion of the *supF*-bearing plasmid and its insert by reversal of the original homologous recombination event. Reversal of the selection event by PCR counterselection (Saiki et al., 1985; *UNIT 15.1*) allows preparative isolation of the transcribed sequence free of the sequence originally used to screen for it (Fig. 6.12.4). Fortunately, the frequency of this excision event is high in the presence of the λ *red* or *rap* genes: on the order of 10^{-2} to 10^{-3} per generation where perfect homology exists between the genomic insert in the plasmid and the cDNA insert in the phage. Following selection on DM21 and amplification on DM75, counterselection is employed to delete the screening DNA sequence, leaving only the DNA sequence obtained by selection. This counterselection is achieved by PCR using primers that abut the *Eco*RI cloning site in phage vectors (a specific set of primers is used for each phage vector; see Fig. 6.12.4). Each blue plaque contains both the selected sequence and a small proportion (~0.1%) of the counterselected sequence, which exists in equilibrium with the selected sequence (see Figs. 6.12.1 and 6.12.4). The selected sequence, which contains the R6K-derived *supF*-bearing pAD1 plasmid and its sequence as well as the (at least partially) homologous sequence in λ, is too large to be amplified efficiently by PCR, whereas the shorter counterselected cDNA sequence can be amplified. Thus, PCR counterselection yields the desired cDNA sequence free of pAD1 and of the genomic sequence originally used to retrieve the cDNA sequence.

Another useful aspect of counterselection (Hanzlik et al., 1993) is that it distinguishes legitimate from illegitimate recombinants (Kurnit and Seed, 1990): legitimate recombinants will reverse the recombination reaction at a high frequency (10^{-2}), whereas illegitimate recombinants will reverse the recombination reaction at a much lower frequency (10^{-9}; Ikeda et al., 1982; Marvo et al., 1983). On an analytical basis, a rough indication of which category a recombinant belongs to is provided by the number of plaques arising during selection: the existence of multiple plaques (indicating multiple events) rules out rare nonhomologous events as the cause of recombination. This differential in reversal frequency allows recombination mediated by full homology to be distinguished from that mediated by partial or no homology, as the latter reverses at a much lower frequency due to mismatching or absence of matching (Watt et al., 1985; King and Richardson, 1986; Shen and Huang, 1986; 1989). When counterselection is performed, this large difference results in the isolation of PCR product in the case of recombination mediated by legitimate homology versus no product in the case of poorly-matched or illegitimate recombination. Thus, in addition to yield-

ing a desired clone, reversal of the recombination reaction (counterselection) allows one to distinguish legitimate from the rarer and undesired illegitimate recombinants.

Avoiding plasmid–phage library homology

For recombination-based screening, there can be no homology between the screening plasmid and the library to be screened lest this homology yield false positives. As a result, the plasmid used must have no homology to sequences present in a recombinant library to be screened. To permit recombination-based screening of common cDNA libraries that contain ColE1 sequences, *supF* has been inserted into a R6K plasmid origin of replication that is not homologous to ColE1. The ColE1 origin is present in a number of common plasmid vectors, including pBR– and pUC– derivatives and the author's π *supF* vectors (Bolivar et al., 1977; Seed, 1983; Yanisch-Perron et al., 1985; Lutz et al., 1987; Kurnit and Seed, 1990; Stewart et al., 1991). The R6K replicon chosen in this case lacks homology with ColE1 and therefore with λ libraries carrying ColE1 sequences (Poustka et al., 1984; Stewart et al., 1991).

ColE1 sequences are present in a variety of desirable cDNA libraries that have been constructed to date. By necessity, λgt11 is propagated on a strain that contains *lac* sequences on the ColE1 replicon plasmid pMC9 to ensure repression of sequences downstream from the *lac* promoter in the λ phage. pMC9 thereby shares *lac* homology with λgt11, fostering recombination between the two mediated by that shared homology. As a result, phage incorporate pMC9 at a low, but for these purposes appreciable, frequency of ~10^{-4}. Once internalized, the pMC9 integrated in the phage can recombine with ColE1-derived *supF*-bearing plasmids because they share homology at the ColE1 *ori*. This shared homology prevents the use of ColE1-derived plasmids for background-free recombination-based screening of λgt11 libraries. Although theoretically λgt10 libraries do not suffer this problem, many λgt10 libraries do contain ColE1-derived sequences (Jankowski et al., 1990), indicating that these libraries have been passaged in strains intended for λgt11 or that accidental contamination with ColE1 sequences has occurred. The vector pYAC4 contains pBR322 (ColE1) sequences, which can be used to clone end fragments. This prevents background-free screening by recombination with a probe cloned in a ColE1 origin vector of λ libraries made from pYAC4 recombinants. Furthermore, the ColE1 replicon in phasmid vectors (i.e., recombinants arising from phage and plasmids) such as λZAP (Short et al., 1988) and CharonBS prevents recombination-based screening of libraries constructed in these vectors with inserts cloned in ColE1-based *supF*-bearing plasmids due to the shared ColE1 homology. To avoid these difficulties, we constructed a plasmid, pAD1 (Stewart et al., 1991; Fig. 6.12.2), based on the R6K replicon that is not homologous with ColE1 plasmids (Poustka et al., 1984). The sequences cloned in this plasmid may be used to screen all of the above λ libraries regardless of the presence of ColE1 sequences.

Other plasmid characteristics

Construction of the 4-kb plasmid pAD1 (Stewart et al., 1991) entailed cloning the R6K γ *ori*, *supF*, a kanamycin-resistance gene, and a polylinker containing sites for *Bam*HI, *Eco*RI, *Mlu*I, *Not*I, *Pst*I, *Pvu*II, *Sal*I, and *Sfi*I (Fig. 6.12.2). To confirm that the final vector, pAD1, indeed lacks homology to ColE1 sequences, it has been demonstrated by recombination (Seed, 1983; Kurnit and Seed, 1990) that this plasmid does not recombine with ColE1 DNA sequences. This lack of homology is consistent with the known sequence of R6K and with the finding that R6K-based cosmids do not recombine with ColE1-based plasmids (Poustka et al., 1984).

Recombination genes in host strain and phage

For recombination to function at a useful level of 10^{-2} to 10^{-3} if there is perfect homology, there must be genes promoting recombination in both the bacterial host and the bacteriophage. Thus, the bacterial host must be *recA*$^+$ and the bacteriophage must be either *red*$^+$ or *rap*$^+$. In the case where the bacteriophage is neither *red*$^+$ nor *rap*$^+$, the *rap* gene can be supplied in *trans* from the pACYC-derived plasmid, pOM-PRAP2, constructed by Kurnit and Seed (1990). Most λ vectors are *rap*$^-$, because *rap* lies within the *nin5* region deleted in most λ vectors.

Uses of the RBA

The RBA has special utility for two purposes:

1. Isolating single-copy sequences. A fragment whose copy number in the genome is to be elucidated is cloned into a plasmid with *supF*. Because cloning sequences into *supF*-bearing plasmids is also required for step (2) below, this procedure satisfies both require-

ments simultaneously. Sequence repetitiveness is assayed by the frequency with which a given insert in a *supF*-bearing plasmid mediates recombination between the plasmid and a recombinant bacteriophage library constructed from large random human genomic fragments (Neve et al., 1983; Neve and Kurnit, 1983). This author uses the library of Lawn et al. (1978) for this purpose, because it was constructed fortuitously (and unbeknownst at the time) in a Charon 4A λ vector that contains the ϕ80 *rap* gene (Kurnit and Seed, 1990). Although this older human genomic library is incomplete for single-copy sequences, this does not pose a problem because it is used merely to assay sequence repetitiveness, for which it is adequate. Subsequent human genomic libraries have been constructed in red$^-$ rap$^-$ vectors, which cannot be screened without the awkward placement of a *rap* gene in *trans* (Kurnit and Seed, 1990). The amount of recombination correlates with the degree of repetitiveness in the genome, with the understandable and benign caveat that more highly repetitive sequences, which manifest significant mismatching, show some depression of the recombination frequency (Neve and Kurnit, 1983). This depression does not interfere with the ability of the assay to sort out the desired single-copy sequences; it merely results in the finding that *Alu* sequences (Rubin et al., 1980), which are actually reiterated 10^6 times in the genome, behave in the RBA as if they are repeated only 10^3 to 10^4 times (Neve et al., 1983; Neve and Kurnit, 1983). The salient point is that the methodology allows rapid analysis and isolation of sequences of a given copy number in the genome: "single" (1 to 10 copies), low-order-repeated (10 to 100 copies), and more highly repeated (>100 copies; Neve and Kurnit, 1983).

2. Determining tissue- and time-specific transcriptional activity of single-copy fragments and isolating genes. Gene libraries containing $>10^6$ independent recombinants are constructed: each corresponds to the totality of genes made in a given tissue at a given time in development. Screening a pool of 10^6 recombinants from a cDNA library requires only two petri dishes. The phage are first plated on a bacterial lawn carrying the sequence to be tested cloned in a *supF*-bearing plasmid. Following confluent lysis, 5×10^8 to 5×10^9 pfu are eluted and plated on DM21 to select for phage that have integrated *supF*. If no phage plaques are observed on DM21, this indicates that the sequence is not transcribed in the tissue at the developmental stage present when the cDNA libraries were made. If plaques are observed on DM21, this indicates that the sequence is transcribed at that stage. The transcribed sequence is isolated free of the genomic sequence initially used to screen for it by reversing the recombination event (Fig. 6.12.4). In all the libraries used to date—λgt10 (Huynh et al., 1985), λgt11 (Young and Davis, 1983), and Sumo 15A (Kurachi et al., 1989)—the desired sequence is liberated as an *Eco*RI fragment that can be subcloned. As well as liberating the sequence, the reversal also makes it possible to discard rare nonhomologous (or imperfect) recombination events, which are identified by the fact that they reverse at the same low 10^{-9} frequency that they occur (for nonhomologous events) and at an intermediate frequency (for partially homologous events). In contrast, homologous recombination events, which can occur in a forward direction at a similar 10^{-8} frequency (assuming a worst case where a sequence is present only once per genome equivalent in a phage library of 10^6 recombinants, which is multiplied by a 10^{-2} chance of recombining if there is homology), reverse at a much higher 10^{-2} frequency. Thus, reversal of the recombination reaction will yield the cDNA free of the genomic sequence and will simultaneously allow rarer nonhomologous or partially homologous exchange events to be identified and discarded.

The RBA can be employed to determine the tissue and time of transcription of candidate genes discovered by other technologies as well as to obtain the gene of interest (in the form of the larger gene sequence that is transcribed). The technique is useful either alone or in combination with other methods for defining single-copy transcribed sequences. If DNA sequencing (as part of the genome initiative) or techniques to define transcribed sequences are used to identify genes, the RBA is still useful for determining the tissue and developmental timing of transcription, as well as for isolating a larger gene of interest. Technologies for defining transcribed sequences include exon trapping/amplification (Nisson and Watkins, 1994; Duyk et al., 1990; Buckler et al., 1991), use of somatic cell hybrids (Liu et al., 1989), and the use of hybridization-based schemes (Hochgeschwender and Brennan, 1994; Hochgeschwender et al., 1989; Kao and Yu, 1991), including hybrid selection (Lovett, 1994; Lovett et al., 1991; Parimoo et al., 1991). The RBA will proceed cooperatively, rather than competitively, with these other methods be-

cause it efficiently accomplishes two necessary tasks: identifying the timing and tissue of gene transcription and isolating a large transcribed sequence.

Critical Parameters

Plaque size is a major issue in this assay because plaques on the *dnaB am* strain DM21 are so small. Fresh λ plates should be used to maximize plaque size, because plaques will be smaller on older (drier) plates; likewise, it is important to plate cells on lambda plates, because plaques will be smaller on richer (e.g., LB) plates.

It is essential that there be no homology between the screening plasmid and sequences in the λ libraries (see Background Information). Therefore, screening should be performed solely with R6K *supF* plasmids, not with ColE1 *supF* plasmids.

Although titering all eluates would be too time-consuming, a few eluates should be titered to ensure that lysis and elution are occurring as expected. This is especially important because a lysed plate may vary from clear to grainy, rendering it difficult to determine visually whether complete lysis has occurred. Eluates should be saved until the DM21 plates have been scored as a precaution in case too many phage have been added, resulting in lysis from without. If this happens, the eluate may be titered or a lesser amount plated on DM21.

Anticipated Results

The abundance of sequences in screened λ libraries should be reflected in the number of phage that plate on DM21. Assuming a recombination rate of 1/500 (the exact number that will depend on the extent of homology), a sequence abundance of $1/10^6$ should yield one plaque on DM21 per 5×10^8 phage plated. A higher abundance should yield a correspondingly greater number of plaques on DM21. If mismatching occurs in an interspersed "salt-and-pepper" manner (as for *Alu* sequences), recombination will be depressed (e.g., ~1000-fold for *Alu* sequences; Neve et al., 1983).

Time Considerations

The major advantage of the RBA is its rapidity: selection can be completed in four days using the following schedule. Day 1, grow bacterial cultures; day 2, add λ library and perform lysis; day 3, elute and plate on DM21; and day 4, identify plaques on DM21.

Counterselection takes an additional four days. One day is necessary for elution of plaques from DM21 that are plated on DM75 with IPTG and Xgal in top agar. A second day is required for elution of putative light blue plaques and confirmatory macroplaque plating on DM75 and DM1061 with IPTG and Xgal. PCR counterselection of macroplaques that are blue on DM75 and colorless on DM1061 takes one day and a final day is necessary to isolate the counterselected PCR band from the gel.

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