because it eliminates the need for blunt ends. Furthermore, since there is no need for cleavage of polymerized linkers attached to the termini of cDNA, the double-stranded cDNA is never at risk from restriction endonuclease cleavage at internal sites.]

### Acknowledgments

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## [40] Cloning cDNA into λgt10 and λgt11

*By* JERRY JENDRISAK, RICHARD A. YOUNG, and JAMES DOUGLAS ENGEL

This chapter provides instructions for generating cDNA libraries with the λ bacteriophage vectors gt10 and gt11.[1-8] Briefly, double-stranded cDNA containing *Eco*RI cohesive termini (this volume [38]) is ligated into the unique *Eco*RI cloning site present in λgt10 or λgt11 DNA. Recombinant DNA is then packaged into viable phage particles which are plated on appropriate *Escherichia coli* hosts for amplification and screening. When only nucleic acid probes are available for library screening, λgt10 is the vector of choice. When antibody probes are available for screening, λgt11 is used since it is an expression vector (meaning that a fusion protein is formed between *E. coli* β-galactosidase and eukaryotic protein from the cDNA inserts). Advantages of λgt10 and gt11 over the use of plasmid vector cDNA cloning include (1) the high efficiency of introduc-

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[3] R. A. Young and R. W. Davis, *Science* **222,** 778 (1983).
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[5] R. A. Young, V. Mehra, D. Sweetser, T. Buchanan, J. Clark-Curtiss, R. W. Davis, and B. R. Bloom, *Nature (London)* **316,** 450 (1985).
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[7] N. R. Landau, T. P. St. John, I. L. Weissman, S. C. Wolf, A. E. Silverstone, and D. Baltimore, *Proc. Natl. Acad. Sci. U.S.A.* **81,** 5836 (1984).
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ing recombinant DNA into *E. coli* by *in vitro* packaging followed directly by infection, and (2) the efficiency of screening bacteriophage at high plaque densities using either nucleic acid or antibody probes.

The subject of constructing and screening cDNA libraries in λgt10 and λgt11 has been reviewed recently by Huynh, *et al.*[1] The reader is referred there for details on vector construction, genetic maps, and for a more detailed discussion on the rationale for using either λgt10 or λgt11 for a particular application.

It is the intent of this chapter to serve as a practical, step-by-step guide beginning with double-stranded cDNA in hand (replete with *Eco*RI cohesive termini) and ultimately ending up with a cDNA recombinant library in either λgt10 or λgt11. Many methods used in this process are the subjects of other more detailed chapters in this volume; the reader is referred to chapters on microbiological techniques [13], the purification of phage λ DNA [13], electrophoresis of nucleic acids [8], the use of restriction enzymes [11], ligation of DNA [10] (see Process Guide), and the preparation and use of bacteriophage λ DNA *in vitro* packaging extracts [17].

## Generating cDNA Libraries in λgt10

λgt10 is an insertion vector with a cloning capacity of up to 7 kb. The unique *Eco*RI cloning site is located in the λ repressor (*c*I) gene. Insertion of foreign DNA at this restriction site interrupts the *c*I coding sequence and causes the phenotype of the phage to change from $cI^+$ (wild type) to $cI^-$. Since $cI^-$ phage are unable to lysogenize the host, clear plaques are produced by recombinants. When plated on mutant bacteria which produce lysogeny (bacteriophage integration) at a high frequency, only recombinant $cI^-$ phage produce plaques. Nonrecombinants (e.g., λgt10 without an insert) are effectively suppressed from plaque formation. This phenomenon serves as the basis for the biological selection for recombinant phage during λgt10 library amplification.

### *Step 1. Plaque Purify λgt10 Phage and Prepare a Stock*

a. Prepare plating bacteria: grow a 50-ml overnight culture in LB of *E. coli* C600 (*hsdR*$^-$, *hsdM*$^+$, *supE44*, *lacYI*, *ton A21*) starting from a single colony. Sediment the cells (5000 *g* for 5 min) and resuspend them in 0.5 volume of 10 m*M* $MgSO_4$. Store the cells at 4°. The plating bacteria can be used for up to 1 week, but highest plating efficiencies are obtained when cells are 0–2 days old.

b. Streak λgt10 phage (λimm434, b527) from a phage stock or a plaque using a sterile wire loop onto the surface of an LB plate. Mix 200 μl of

C600 plating bacteria with 2.5 ml of top agar (at 47°) and pour on the streaked plate. Incubate the plate (inverted) for 8 hr to overnight.

c. Pick a well-isolated plaque (they should all be "turbid" in appearance) with a sterile glass capillary or Pasteur pipet (1–3 mm diameter). Suspend the plug containing the single plaque in 1 ml of λ diluent (10 m*M* Tris–HCl at pH 7.5, 10 m*M* $MgSO_4$) and add a single drop of chloroform (to kill any residual live bacteria); allow the phage to diffuse out of the agar plug for several hours at 4°. The titer is generally $10^6$ phage/ml (do not vortex, and allow the chloroform to settle before the next step).

d. Mix 100 μl of phage (0.1 of the plaque) with 100 μl of *E. coli* C600 plating bacteria and incubate at 37° for 20 min. Transfer to 50 ml of LB containing 10 m*M* $MgSO_4$. Incubate overnight at 37° with shaking (the culture should be completely lysed after 12 hr). Add 1 ml chloroform and continue shaking at 37° for 20 min. Sediment the lysis debris for 15 min at 5000 *g*. Collect the supernatant into a sterile screw-capped tube (preferably glass), and add 50 μl of chloroform. Titer the primary bacteriophage stock on *E. coli* C600 plating bacteria; the titer should be $>10^9$ pfu/ml. (See this volume [13] for a more detailed description of these techniques.)

*Step 2. Make a Large-Scale λgt10 Bacteriophage Preparation*

a. Mix 1 ml of C600 plating bacteria with $10^7$ λgt10 phage and incubate for 15 min at 37°. Transfer to 1 liter of LB containing 10 m*M* $MgSO_4$ in a 4-liter flask. Shake at 37° overnight. Add 5 ml of chloroform and shake for 15 min. Centrifuge 15 min at 5000 *g* for 5 min and collect the supernatant. Save a small sample for titer; it should be $>10^{10}$ pfu/ml.

b. Precipitate the phage by adding 54 g of NaCl and 63 g PEG 8000 to the supernatant and stir gently until dissolved.

c. Chill in an ice bath for 2–3 hr.

d. Sediment the phage by centrifugation at 5000 *g* for 20 min at 4°. Discard the supernatant.

e. Resuspend the pellet in 5 ml of λ diluent by scraping and stirring (not strong pipetting) and transfer to a 15-ml polypropylene centrifuge tube.

f. Extract with 5 ml chloroform using agitation (shake gently by hand). Centrifuge 5000 *g* for 15 min. Save the opalescent aqueous (phage-containing) upper phase.

*Step 3. Purify the λgt10 Phage*

Purify the phage by two sequential CsCl density gradient centrifugations.

a. Measure the volume of phage. Add 0.5 g CsCl/ml.

b. Make a step gradient in Beckman SW41 centrifuge tubes. One 11.4-ml nitrocellulose or cellulose acetate centrifuge tube will be enough for one preparation. Add the phage solution to the tube first (~5.75 ml), and then insert a long Pasteur pipet to the bottom of the tube. First add 2.5 ml of $\rho$ = 1.5 g/ml CsCl (in λ diluent) to the Pasteur pipet, and when that solution has completely underlayed the phage solution, add another 2.5 ml CsCl solution of $\rho$ = 1.7 g/ml (in λ diluent) to the pipet. Carefully withdraw the pipet, and add mineral oil to the rim of the tube.

c. Centrifuge at 30,000 rpm for 16 hr in an SW41 rotor or an equivalent *g* force × time in another rotor.

d. Remove the opalescent phage band (~1 ml) through the side of the tube using a syringe and a 20-gauge needle.

e. Add an equal volume of CsCl solution ($\rho$ = 1.5 g/ml in λ diluent) and centrifuge overnight at 30,000 rpm.

f. Collect the phage (as described above) in a volume of approximately 0.5 ml.

*Step 4. Purify λgt10 DNA*

a. Dialyze the CsCl from the phage suspension against λ diluent for several hours with two dialysis changes.

b. Add one-fourtieth volume of 0.5 *M* EDTA, pH 8.0 (EDTA chelates the $Mg^{2+}$ inside the phage head, and thus disrupts the compacted bacteriophage DNA) to the dialyzed phage solution and extract with phenol–chloroform 3–4 times. Phage DNA must be handled gently to avoid random shearing; do not vortex (see this volume [13]).

c. Extract with chloroform one additional time.

d. Dialyze versus DNA buffer (10 ml Tris–HCl at pH 7.9, 0.1 m*M* EDTA) for several hours to overnight with at least three buffer changes.

e. Determine the DNA concentration by absorbance measurement at 260 nm (this volume [6]). The yield should be about 1 mg.

*Step 5. Prepare λgt10 Phage Vector DNA for Cloning*

a. Starting with λgt10 DNA at a concentration of approximately 200 μg/ml, add 0.1 volume of 10× ligase buffer (0.5 *M* Tris–HCl at pH 7.8, 0.1 *M* $MgCl_2$, 0.2 *M* dithiothreitol). Incubate at 50° for 15 min to anneal the *cos* sites.

b. Cool to 16°, add 0.1 *M* ATP to 1 m*M* (final concentration) and T4 DNA ligase to a final concentration of 100 Weiss units per ml, and incubate for 2–3 hr at 16°.

c. Heat at 70° for 10 min (to inactivate the ligase).

d. Add 0.1 volume of 10× *Eco*RI digest buffer (1 *M* Tris–HCl at pH

7.5, 0.5 *M* NaCl, 50 m*M* $MgCl_2$) and *Eco*RI restriction enzyme to 200 units/ml. Incubate 1 hr at 37°.

e. Add an additional 200 units/ml *Eco*RI and incubate 1 additional hr at 37°.

f. Add DNase-free calf intestinal alkaline phosphatase to a final concentration of 5 glycine units/ml to the remainder and incubate at 37° for 30 min. (One glycine unit is that amount of alkaline phosphatase that will hydrolyze 1 μmol of *p*-nitrophenyl phosphate in 1 min at pH 10.4 at 37° in a reaction containing 0.1 *M* glycine, 1 m*M* $ZnCl_2$, 1 m*M* $MgCl_2$, and 6 m*M* *p*-nitrophenyl phosphate.)

g. Add one-thirtieth volume 0.5 *M* EDTA and gently extract DNA with phenol–chloroform three times (this volume [4] and [13]). Extract once with chloroform. Precipitate with 2 volumes of absolute ethanol.

h. Microcentrifuge for 5 min at 4°; aspirate the supernatant and then rinse the pellet once with 70% ethanol. Allow the rinsed pellet of DNA to air dry until the residual supernatant ethanol evaporates. Resuspend the damp pellet at a final concentration of approximately 500 μg/ml in DNA buffer.

i. Check the DNA concentration by absorbance measurement at 260 nm.

j. Check the quality of the vector DNA by self-ligating a small amount in the absence or presence of T4 polynucleotide kinase (PNK) (kinase is fully functional in ligase buffer). Package the DNA and titer the phage on *E. coli* C600 (see this volume [13,17]; also step 7a below). The *Eco*RI-digested and phosphatase-treated vector should produce less than $10^5$ pfu/μg. Self-ligation in the absence of PNK should not change this background number, but ligation in the presence of PNK (0.1 Weiss units) and 0.1 m*M* ATP should produce greater than $10^7$ pfu/μg. If the vector passes these quality control tests it is suitable for use in subsequent cloning steps. (Note: alkaline phosphatase-treated λgt10 vector DNA is commercially available, e.g., from Promega, which allows you to proceed directly to step 6.) If it does not pass these tests, start over from purified phage, step 4.

*Step 6. Prepare Recombinant λgt10 Phage DNA*

a. Mix double-stranded cDNA containing cohesive *Eco*RI termini with an equimolar amount of prepared λgt10 vector DNA. (For cDNA of average length of 1 kb, this is a 50:1 weight ratio of vector to insert.)

b. Add 0.1 volume of 10× ligase buffer, 0.01 volume of 0.1 *M* ATP, and T4 DNA ligase to a concentration of 100 Weiss units/ml. Incubate for 2 hr at 16°. (See this volume [10] and the Process Guide for protocols for ligation.)

*Step 7. In Vitro Package Recombinant λgt10 Phage DNA*

a. Package the ligated DNA using *in vitro* packaging extracts prepared by any of several methods listed in another chapter ([17]) or obtained commercially from a variety of suppliers. Use a wide-bore microcapillary pipet for transferring the viscous ligated DNA to the packaging extract. (Packaging efficiency should be greater than $10^8$ pfu/$\mu$g ligated λ DNA. Most extracts are capable of packaging from 1 to several micrograms of DNA.)

b. Dilute packaging extract to 0.5 ml with λ diluent and add a drop of chloroform and gently mix. This diluted packaging extract is termed "the library." Titer the library on *E. coli* C600hfl A150.

*Step 8. Plate the Packaging Mixture to Produce an Amplified λgt10 Library*

a. Mix aliquots of the packaging mixture containing $10^4$ to $2 \times 10^4$ bacteriophage with 200 $\mu$l of C600 *hfl* plating bacteria. Incubate at 37° for 20 min.

b. Mix 6.5 ml of melted top agar (46°) with each aliquot of infected bacteria and spread onto a freshly poured 150-mm LB plate.

c. Incubate at 37° for a maximum of 8 hr (until plaques are ~0.5 mm in diameter).

d. Overlay with 12 ml λ diluent and store plates at 4° overnight to allow the phage to desorb.

e. Remove the overlay solution containing the phage with a Pasteur pipet. Rinse the plate with 4 ml λ diluent and pool with initial overlay solution. Add chloroform to 5%. Allow the phage solution to equilibrate for 15 min at room temperature with occasional shaking.

f. Remove debris by centrifugation at 4000 *g* for 5 min at 4°.

g. Remove the supernatant to tight screw-capped glass tubes, vials, or bottles. Add chloroform to 0.3% and store aliquots at 4°. An amplified library stored in this manner should be stable for several years without significant loss of titer after the first month of storage.

(Note that the primary, unamplified library may be screened with nucleic acid probes directly during the initial plating for amplification. See chapters [44,45] on screening bacteriophage λ with hybridization probes.)

## Generating cDNA Libraries in λgt11

λgt11 is also an insertion vector with a cloning capacity of up to 7 kb. The single *Eco*RI site for cloning is located at the carboxy-terminal end of the β-galactosidase (*lacZ*) gene present in the vector molecule. Insertion

of foreign DNA (up to 7 kb) into this site causes the phenotype of the phage to change from *lacZ*$^+$ to *lacZ*$^-$. When plated on an appropriate host (which is deleted for *lacZ* function), parental λgt11 phage produce blue plaques in the presence of isopropyl-β-D-thiogalactopyramoside (IPTG; a synthetic inducer of the *lac* repressor) and X-Gal (a chromogenic substrate for β-galactosidase activity). Under the same conditions, recombinant λgt11 phage produce colorless plaques due to the interruption of the coding sequence in *lacZ* by inserted DNA, which generally produces an enzymatically inactive β-galactosidase fusion protein. Since there is no biological selection against the growth of parental λgt11 phage, background reduction relies solely on the use of alkaline phosphatase-treated vector molecules in the vector preparation step. Properly functional λgt11 vector should result in obtaining greater than 60% recombinant (colorless) plaques on X-Gal IPTG plates. As discussed by Huynh *et al.*, expression of foreign DNA as a β-galactosidase fusion under transcriptional control improves the probability of detecting foreign DNA gene products by antibody screening procedures (see this volume [51]).

The methods for use of λgt11 are very similar to those used for λgt10. The only major differences are that the parent phage are produced by lysogen induction rather than infection, and the use of special host strains for library amplification and screening which were designed to optimize insert protein expression and detection.

*Step 1. Colony Purify the λgt11 Lysogen BNN97*

a. Streak the λgt11 (λcI857, *lac5*, S100) lysogenic strain BNN97 for single colonies on an LB plate and incubate overnight at 32°.

b. Pick a colony and restreak it onto two LB plates. Incubate one plate at 32° and the other at 42° to be sure that this colony harbors a temperature-sensitive lysogenic phage (i.e., there should be very many fewer colonies of BNN97 at 42 than at 32°.

*Step 2. Produce λgt11 Phage*

a. Grow a 50-ml overnight culture of BNN97 at 32° with shaking.

b. Use 10 ml of this stock to inoculate 1 liter of prewarmed LB (pH 7.5) in a 4-liter flask. Grow at 32° with shaking until the $A_{600\ nm}$ is 0.6 (3–4 hr).

c. Shift the temperature rapidly to 42–43° and hold at that temperature with shaking for 15 min.

d. Grow at 38° for 3 more hr (if need be, adjust the pH to 7.5–8 with NaOH) with vigorous shaking.

e. Add 5 ml of chloroform and continue shaking at 38° for 10 min. Lysis should occur.

f. Sediment the cell debris at 7000 *g* for 10 min at 4°.

g. Proceed with steps 2b–f of the section on λgt10.

*Step 3. Purify the λgt11 Phage.* Proceed as described in step 3 of the section on λgt10.

*Step 4. Purify the λgt11 Phage DNA.* Proceed as discussed in step 4 of the section on λgt10.

*Step 5. Prepare λgt11 Phage DNA for Cloning.* Proceed as described in step 5 of the section on λgt10. Again, one has the option to obtain commercially available alkaline phosphatase-treated, *Eco*RI-digested λgt11 DNA and proceed from step 6.

*Step 6. Prepare λgt11 Recombinant Phage.* Proceed as described in step 6 of the section on λgt10.

*Step 7. In Vitro Package Recombinant λgt11 Phage.* Proceed as described in step 7 of the section on λgt10 (save 10 μl of the diluted packaging mixture for a later determination of the number of recombinants in the initial λgt11 library).

*Step 8. Plating to Amplify the Recombinant λgt11 Phage Library*

a. Prepare *E. coli* Y1090 ($R^+M^+$) and Y1088 ($R^-M^+$) for plating bacteria (first section, step 1), or *E. coli* Y1090 ($R^-M^+$). [If you have the latter strain (available from Promega Biotec, Madison, WI) you can plate and screen the phage library directly. In order to screen λgt11 recombinants using the original strains, you must amplify the library first on *E. coli* Y1088 (to methylate host specifically inserts containing a recognition site for *E. coli* K restriction endonuclease) and then screen, using antibodies, by plating the Y1088-amplified library on the original ($R^+M^+$) strain Y1090].

b. Mix aliquots of the packaging mixture containing 10,000–20,000 bacteriophage with 200 μl of host strain Y1090 ($R^-M^+$) or Y1088. Incubate at 37° for 15 min.

c. Mix the bacteriophage solution with 6.5 ml of top agarose (46°) and pour onto a fresh 150-mm LB plate.

d. Incubate at 37° for ≤8 hr.

e. i. If you used strain Y1090 ($R^-M^+$), you can either screen the plate directly using antibodies (this volume [51]) and prepare the amplified library (after isolation of the positive plaques), or prepare the amplified library directly (prior to screening) at this time (step ii, below).

ii. If you used strain Y1088, overlay the plates with 12 ml λ diluent and store in a level position at 4° overnight.

f. Repeat steps 8, e–g, in the section on λgt10 but using Y1090 ($R^+M^+$).

*Step 9. Determination of the Number of Recombinant λgt11 Bacteriophage in the Phage Library*

In order to estimate the number of recombinant bacteriophage which must be screened for isolation of the recombinants of interest, one must first determine the number of recombinants in the libraries. When using λgt10 as a vector, if the packaging efficiencies of the vector alone (when compared to the vector plus cDNA inserts) are roughly the levels as outlined in the section on λgt10, one may assume that 95–99% of the recovered bacteriophage (after amplification on an *hfl* strain) are indeed recombinant. Thus the λgt10 library can be titered directly on an *hfl* strain to determine the number of recombinant bacteriophage.

In order to determine the number of recombinant phage in λgt11 libraries, we make use of the property of the (wild-type) phage to catabolize a chromogenic β-galactosidase analog (X-Gal) from an uncolored precursor to a blue product. Since cDNA inserts are ligated into an *Eco*RI site within the β-galactosidase structural gene, inactivation of the β-galactosidase protein leads to production of a colorless (clear) plaque in recombinant bacteriophage and production of a blue plaque in nonrecombinants.

a. Prepare *E. coli* Y1090 ($R^-M^+$) for plating bacteria.

b. Mix 100-μl dilutions of the amplified library (this section, step 8) or of the original library (this section, step 7) with 100 μl of Y1090 strain plating bacteria. Incubate at 37° for 20 min.

c. Add 2.5 ml of melted LB top agar containing 20 μl of IPTG stock solution (20 mg/ml) and 50 μl of X-Gal stock solution (20 mg/ml in dimethylformamide.) Pour onto a fresh, dry LB plate.

d. Incubate (inverted) for 6–12 hr at 37°.

e. Score the plates for number of clear plaques versus total number of plaques (this equals the percentage of recombinants).

## Screening Considerations

Screening λgt libraries for any particular sequence of interest follows relatively straightforward statistical probabilities on the one hand; on the other hand, problems arising in attempting to clone a particular cDNA sometimes obey nonpredictable criteria. These are both addressed in the two following sections.

*Statistical Probabilities*

A typical vertebrate cell contains between $10^4$ and $3 \times 10^4$ different mRNA sequences.[9] As discussed previously,[10] in order to ensure cloning of any particular sequence statistically, one can calculate the strict probability of the number of unique clones which must be screened in order to ensure isolation of a particular sequence using the equation

$$N = \frac{\ln(1 - P)}{\ln(1 - n)}$$

where $N$ is the number of clones required; $P$, the probability of isolating the clone (usually set at a desired value of 99%); and $n$, the fractional proportion of the total mRNA population represented by a single mRNA species (the clone you want).

A priori estimation of the abundance of the mRNA species encoding the gene of interest is difficult without a direct (e.g., nucleic acid probe) assay. Most often, rough estimates have been made using immune precipitation of radiolabeled *in vitro* translation reactions. Estimations of the relative mRNA levels based on such data can be quite misleading, and vary according to the isotope(s) used in *in vitro* translation reactions, the integrity of the mRNA used, and the size of the primary translation product. While all of these factors detract from the ability to afford an accurate assessment of mRNA concentration of a particular species, without nucleic acid probes such estimates sometimes afford the only possible avenue for gaining any knowledge of mRNA abundance prior to cloning.

As a specific example, suppose we wish to clone a "rare" mRNA (10 copies/cell) from chicken red blood cells.[11] Rare mRNA sequences represent about 10% of the total polysomal message of an immature chick RBC (in which there exist only ~100 different mRNA species). Therefore the actual fractional representation of any mRNA of this class in the total mRNA of a red cell is ~1 in $10^3$ molecules. Therefore if $n = 10^{-3}$, to ensure cloning of this sequence to a 99% statistical probability, we need theoretically to construct and screen a library of only 5000 members to ensure that this sequence is adequately represented (Table I).

A more common example might be encountered in examination of the population of mRNAs in a transformed human fibroblast.[12] Williams has

[9] J. G. Williams, *in* "Genetic Engineering" (R. Williamson, ed.), Vol. 1, p. 1. Academic Press, New York, 1981.

[10] L. Clarke and J. Carbon, *Cell* (Cambridge, Mass.) **9,** 91 (1976).

[11] L. Lasky, N. D. Nozick, and A. J. Tobin, *Dev. Biol.* **67,** 23 (1978).

TABLE I
THEORETICAL CONSIDERATIONS FOR CLONING cDNA SEQUENCES

| A, Cell type | B, Abundance class | C, Percentage representation | D, Number of mRNAs | E, mRNA copies/cell | F, $n$ (fractional representation of each mRNA) | G, $N$ (total number of clones required) |
|---|---|---|---|---|---|---|
| Chick RBC | Abundant | 90 | 3 | 1,500 | 0.30 | 13 |
| | Rare | 10 | 100 | 10 | $10^{-3}$ | $4.6 \times 10^3$ |
| Fibroblast | Abundant | 20 | 30 | 3,500 | $6.7 \times 10^{-3}$ | $6.8 \times 10^2$ |
| | Moderately abundant | 50 | 1,000 | 200 | $5 \times 10^{-4}$ | $9.2 \times 10^3$ |
| | Rare | 30 | 11,000 | 10 | $2.7 \times 10^{-5}$ | $1.7 \times 10^5$ |
| Hypothetical | Abundant | 15 | 10 | 10,000 | $1.5 \times 10^{-2}$ | $3.1 \times 10^2$ |
| | Moderately abundant | 40 | 1,000 | 100 | $4 \times 10^{-4}$ | $1.2 \times 10^4$ |
| | Rare | 40 | 10,000 | 10 | $4 \times 10^{-5}$ | $1.2 \times 10^5$ |
| | Very rare | 5 | 10,000 | 1 | $5 \times 10^{-6}$ | $9.2 \times 10^5$ |

determined the number of clones necessary to ensure successful cloning of a single mRNA in the "rare" abundance class of message in this cell type.[9] These values are also shown in Table I. Note that simply because there are many more total mRNA species in fibroblasts than in red cells, in order to clone a message present at 10 copies per cell in fibroblasts one would need a library some 35-fold greater in total to isolate the same 10 mRNA/cell species as is present in red blood cells!

Finally, we have included the probability of cloning an mRNA represented once per cell in a hypothetical cell type which contains 21,000 different mRNA species, all at relatively different levels, an example which might well represent a "typical" mammalian cell. As shown in this hypothetical case, one would need to have an initial library of $10^6$ recombinants to ensure statistically that a very rare mRNA was contained within that library.

The numbers shown in Table I are the theoretical considerations for cloning of a particular cDNA sequence. Thus, those considerations would be straightforwardly applied to cloning of a cDNA into λgt10 and screening with (e.g.) oligonucleotide probes. When cloning into λgt11, however, one usually assumes faithful expression of a cDNA fusion of β-galactosidase and a eukaryotic mRNA copy to be only one-sixth the simple cloning

[12] J. G. Williams, M. M. Lloyd, and J. Devine, *Cell (Cambridge, Mass.)* **17**, 903 (1979).

efficiency deduced from Table I, since only one-third of the cDNA clones will be in the proper reading frame to produce a fusion gene with the correct eukaryotic amino acid sequence, and only half of the cDNA clones will be in correct reading orientation when cloning cDNA containing *Eco*RI linkers at either end into an *Eco*RI site.

### *Nonstatistical Considerations*

By far the most common problem encountered in isolating clones from cDNA libraries is the overall quality of the initial double-stranded cDNA inserted into prokaryotic λ or plasmid vectors. If, in comparison of an ethidium bromide-stained denaturing gel of the mRNA which you are using when run side by side with the cDNA you have prepared, the two are not at least comparable in average molecular weight, it is unlikely that any cDNA cloning method recommended will be successful. Several previous chapters in this volume deal with minimizing such initial technical problems in cDNA size.

Outside of this most common problem, several other considerations might be applicable in particular cases when screening λgt11 libraries with monoclonal antibodies and monospecific antisera. In general, if both are available, clean monospecific antisera are preferable to using monoclonal antibodies for λgt11 screening. This is because an antiserum may react with several epitopes in the fusion polypeptides, whereas (by nature) the monoclonals can only recognize a single epitope. One should then expect to find a particular clone more easily with a good antiserum if the epitopes are spread from the amino to the carboxy termini. Furthermore, one might also expect greater signal intensities by recognition of multiple epitopes in one cloned sequence.

In general, if a monoclonal recognizes an antigen on Western blots, it can be successfully used in λgt11 library screening, since a reasonable rule of thumb is that because since the antibody recognizes an SDS-denatured form of the protein on Western blots, the antigen is likely to be a simple (primary structural) epitope. The same does not hold true for antigens reactive with monoclonal antibodies after fixation for immunofluorescence. Since, in theory, antigens are "fixed" in place by aldehyde, acetone, or methanol treatment of cells, in many cases such complex epitopes (including appropriate secondary folds or tertiary interactions of the protein of interest with other cellular components) may only infrequently be present in λgt11 fusion polypeptides.

Of course, the monoclonal specificity may include a posttranslational modification of the protein (such as glycosylation or phosphorylation) as part of the epitope, and this "Western rule" may not always hold true.

However, such problems can usually be eliminated by testing immune precipitates of *in vitro* translation products from the mRNA to be used in initially forming the library.

Another theoretical problem which is a potential pitfall in the immunological approach in screening λgt11 libraries is the secondary structure of the mRNA substrate. If a strong secondary structure is encountered, the initial cDNA may be invariably prematurely truncated at a single nucleotide flanking the 3′ end (in mRNA sense) of a strong secondary structure, and therefore always leading to a β-galactosidase fusion product truncated at that point. Such a "strong stop" secondary structure has been observed in c-*abl* mRNA, and was overcome (as recommended in this volume [33]) by reverse transcription in the presence of methylmercury hydroxide (O. Witte, personal communication).

## [41] Full-Length cDNA Clones: Vector-Primed cDNA Synthesis

*By* PRESCOTT L. DEININGER

This chapter is concerned with methods which are of particular use for the formation of cDNA libraries containing high proportions of long or full-length inserts, specifically the use of vector-primed cDNA synthesis. Many methods exist which aid in the preparation of long cDNAs and do not require the use of vector priming. However, most of these methods can be, and are, incorporated into the vector-primed cDNA protocols.

### Scheme for Vector-Primed cDNA Cloning

The preparation of the vector–primer and linker molecules needed for these cDNA cloning procedures is illustrated in Fig. 1 and is essentially that of Okayama and Berg.[1] There are a number of variations on these protocols and, as discussed below, several procedures now do away with the preparation of the linker molecule altogether. The vector can be any number of plasmids that have the proper arrangement of restriction sites, and choice of vectors will be discussed in more detail later. It is helpful if the restriction enzyme cleavage site at Z (Fig. 1A) generates a 3′ overhanging end (e.g., *Kpn*I or *Pst*I) which will facilitate the tailing reactions,

[1] H. Okayama and P. Berg, *Mol. Cell. Biol.* **2**, 161 (1982).