[Table 3 and Geier, Trigg, and Merril (*4*)].

While λgt*WES* phage are at an obvious survival disadvantage because of their suppressor requirements, they have a further disadvantage in that fragment-containing phage lack the λ general recombination functions (*red*) required for normal phage growth (*5*). The yield of such *red*-deficient phage is one-half to one-tenth that of the λ wild type. Nevertheless, sufficient recombinant phage may be prepared from 1 to 2 ml of lysates to permit detailed enzymatic, hybridization, and electron microscopic analyses (*6*). The specific safety features of the λgt*WES* system are summarized in Table 4 (*2*). The presence of each genetic feature, including the amber mutations, the temperature-sensitive repressor gene, the *nin*5 deletion, and the absence of the specialized recombination genes, can be determined by simple genetic tests (*2, 3*).

In addition to other detailed tests required for EK2 certification (*7*), we have tested the possibility that a cloned fragment could form a persistent association with a bacterial host used in its propagation. The experiments described in Table 5 represent a typical preparative phage growth using the model gal^+ phage recombinant and the attenuated host strain *E. coli* K12 DP50/*sup*F (*8*). The gal^+ genes carried by the model recombinant represent a cloned DNA fragment. Any persistent association with the DP50/*sup*F host (in which the entire galactose operon is deleted) during lytic growth of the λ*WES* gal^+ phage will give rise to gal^+ colonies among the bacterial survivors in the lysate. As shown in Table 5, no gal^+-containing bacteria were detected among the bacteria surviving either at the time of lysis or 24 hours thereafter.

λgt*WES*·λC was certified as an EK2 vector by the NIH Advisory Committee on Recombinant DNA Research at its January 1976 meeting, but has been superceded by λgt*WES*·λB, which was certified in February 1977. The Advisory Committee has further required that the phage be grown preparatively in the attenuated host strain *E. coli* K12 DP50/*sup*F (*8*).

We have considerable experience in the use of λgt*WES* derivatives for cloning of partially purified DNA fragments derived from total mouse genomic DNA. We generally obtain about 4500 hybrid phage per microgram of Eco RI DNA fragment (*6*). This efficiency suggests that any unique sequence purified about 1000-fold could be identified by available screening techniques (*9*). We recently cloned an Eco RI fragment containing a segment of the mouse ribosomal RNA sequences (*6*). This fragment occurs about once per 5000 mouse Eco RI fragments and is approximately 200-fold reiterated with respect to unique sequences. McClements and Skalka (*10*) cloned a similar ribosomal fragment from genomic chicken DNA using the λgt*WES*·λC vector. While the requirements of EK2 biological containment clearly reduce the flexibility of the cloning technology, the λgt*WES* system should permit cloning of unique sequences of genomic DNA from higher organisms. Other attenuated strains of λ, such as those constructed by Blattner and his associates (*11*) and Donoghue and Sharp (*12*) should be useful in this respect as well.

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7 February 1977

Chemical Synthesis of Restriction Enzyme Recognition Sites Useful for Cloning

Abstract. *By a triester chemical synthesis method, three decameric DNA's have been made; these act as substrates for several restriction endonucleases, including Eco RI, Bam I, and Hind III. These homogeneous decamers form duplexes that can be efficiently blunt-end ligated to themselves or to other DNA molecules by the action of T4 DNA ligase and thus are useful tools for molecular cloning experiments.*

Restriction endonucleases recognize specific sequences in double-stranded DNA. Many of these endonucleases cleave their recognition sites to produce blunt ends, while others such as Eco RI, Bam I, and Hind III, leave cohesive or "sticky" ends (*1–3*). DNA fragments with these cohesive ends can be inserted easily into, and subsequently excised from, a suitable cloning vehicle such as the plasmid pMB9 (*4*), which has one site each for Eco RI, Bam I, and Hind III.

Unfortunately, cohesive ends are lacking in many DNA fragments of interest, such as randomly cleaved DNA, complementary DNA (cDNA), and many restriction endonuclease fragments. We have recently introduced a new general procedure suitable for cloning almost any DNA molecule (*5*). This method (Fig. 1) involves the addition by blunt-end ligation of short DNA segments to both ends of the DNA to be cloned. The added segments contain restriction endonuclease cleavage sites, and treatment with the corresponding endonuclease leaves cohesive ends on the subject DNA. This DNA then can be cloned by incorporating it into a plasmid or other vector that has been opened with the same restriction enzyme. The feasibility of this new method was demonstrated by adding an octadeoxyribonucleotide containing the Eco RI recognition sequence to a 21-base-pair duplex bearing the *lac* operator sequence. A clonable, excisable DNA fragment was obtained, and was shown to function as *lac* operator should, both in vitro and in vivo (*5*).

We report here the chemical synthesis by the improved triester method of Itakura *et al.* (*6*) of three new decameric "linker" molecules (see Fig. 1), which together contain seven different recognition sites cleavable by 23 different restriction endonucleases including Eco RI, Bam I, and Hind III (*2*). These linker molecules should add great flexibility to cloning methodology, and also provide substrates for physicochemical studies on restriction enzyme recognition, methylation, and cleavage mechanisms.

The synthetic scheme in Fig. 2 outlines the strategy used in the convergent synthesis of the Bam I decamer by the triester method, using 2,4,5-triisopro-

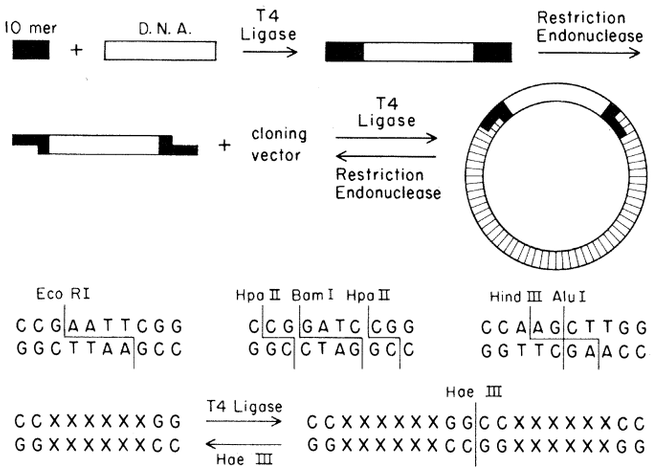


Fig. 1. (Top) A decameric linker (10 mer) bearing a restriction enzyme site is joined by T4 DNA ligase to both ends of the DNA to be cloned, and cohesive ends are then produced by treatment with restriction endonuclease. This "sticky-ended" DNA then can be incorporated into a vector that has been cut open with the same restriction endonuclease. (Bottom) The three chemically synthesized decameric linker molecules investigated in this study. The ends of the decamer sequences are designed so that, after blunt-end ligation with T4 DNA ligase, a site recognized by the Hae III restriction enzyme is produced.

pylbenzenesulfonyl tetrazolide (TPST) as a condensing reagent (*7*). The other two decamers were made similarly. Procedures and reaction conditions are given in the legend of Fig. 2. Two milligrams of fully deblocked Eco RI decamer were obtained, whereas for the Bam I and Hind III decamers the yields were 26 mg and 80 mg, respectively. The lower yield of synthesis of the Eco RI decamer may be attributable to the particular deblocking conditions used for the removal of the β-cyanoethyl group on the 3′ ends. These conditions subsequently were changed for the Hind III and Bam I decamer syntheses (see legend of Fig. 2). The pyridine-ethanol mixture provided a faster, more efficient removal of β-cyanoethyl protecting groups than tetrahydrofuran did. The use of pressure silica gel chromatography for separation of protected oligonucleotides greatly aids the triester synthesis. Chromatography under pressure leads to improved and faster separation (4 hours). It also produces 5 to 10 percent higher yields at each step, resulting in significant increases in yields of the final product. Moreover, use of NH_4OH rather than NaOH in organic solvents for removing protecting groups also increases the yield and purity. The three decamers travel as a single band on 20 percent polyacrylamide–7*M* urea gel electrophoresis, with the same R_F value as bromphenol blue (Fig. 3).

Because the DNA strands synthesized are self-complementary, they form the duplexes shown in Fig. 1. These duplexes are relatively stable, having a T_m of about 48°C in 0.3*M* NaCl, 0.1 m*M* EDTA, 10 m*M* tris-HCl, *p*H 7.5, at a DNA concentration of 50 μg/ml. These duplexes are homogeneous and act as restriction enzyme substrates (Fig. 3). The Eco RI decamer is totally cleaved by its endonuclease to produce the labeled trimer as shown in Fig. 3, lanes 1 and 2. The Bam I decamer is also completely digested by its endonuclease to a labeled trimer and a second band running close to the [γ-^{32}P]ATP marker (lanes 3 to 6). Since this second band does not appear in the decamer without enzyme, it most likely can be ascribed to a nuclease contamination of the enzyme preparation. The Hind III decamer is cleaved to two identical five-base-pair pieces by Alu I, which recognizes adenine-guanine-cytosine-thymine (AGCT), but is not affected by Hsu I endonuclease, which recognizes AAGCTT (lanes 12 to 14). (Hsu I and Hind III recognize the same base sequence.) However, if the decamer is first polymerized with T4 DNA ligase under blunt-end joining conditions (legend of Fig. 3), then the polymer is cleaved by Hsu I endonuclease to pieces of approximately 30 base pairs in length, with no further degradation of these pieces (lanes 15 and 16). Thus it appears that Hsu I, unlike Eco RI, Bam I, or Alu I, requires a DNA strand of 30 base pairs or more as a substrate.

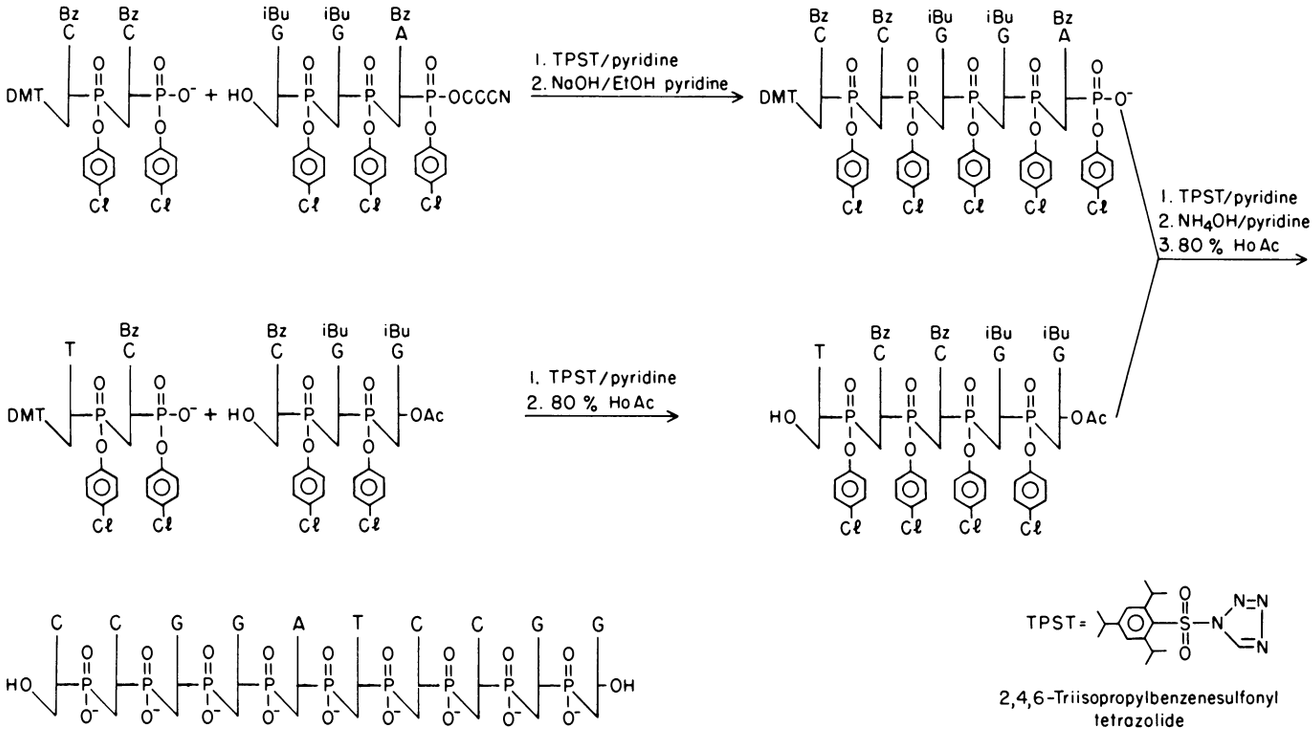
A comparable story is found with the Bam I decamer. It has two Hpa II sites per decamer (Fig. 1). In spite of this, Hpa II will not digest the decamer, apparently because the sites are too near the ends. However, if the decamers are polymerized with the T4 DNA ligase, then the polymer is quantitatively cleaved to smaller fragments by Hpa II endonuclease (lanes 8 to 10). The specific recognition by the Hpa II enzyme of the outer four base pairs of the Bam I decamer after polymerization is a confirmation of the sequence of the ends.

All three of these decameric linker molecules have CC at the 5′ end and GG at the 3′ end. Hence, blunt-end ligation produces a new Hae III restriction endonuclease site (GGCC). Polymers of the three linkers are cleaved back to decamers by Hae III enzyme (lane 19 and data not shown). Recognition of the central portion of each decamer by its proper endonuclease, and recognition of the outer two base pairs by Hae III, confirm the sequences as synthesized. The complete cleavage of the molecules as judged by the appearance of the gels allows one to assess the overall purity in terms of length and sequence homology as 90 percent or better.

For cloning DNA fragments, three methods for joining to the vector are in common use: (i) restriction enzyme "sticky ends" (*8*); (ii) tailing, either with polydeoxyadenylate [poly(dA)] or polydeoxythymidylate [poly(dT)] (*9*) or, more recently, with polydeoxyguanylate and polydeoxycytidylate [(*11*); and A. Otsuka, personal communication]; and (iii) blunt-end ligation by T4 ligase (*12*). This latter method is based on the ability of T4 ligase to rather efficiently join even or blunt-ended DNA duplexes under the appropriate conditions (*13*). All of these methods are useful, but none is completely general.

The three "linkers" described here and the procedure outlined in Fig. 1 should allow almost any DNA fragment to be cloned in an existing vector and also aid in the construction of new cloning vectors. Eco RI, Bam I, or Hind III cohesive ends can be added at will to any DNA bearing 5′-phosphate/3′-hydroxyl flush ends. If not already blunt-ended, most DNA's can be made so, either by repair with DNA polymerase [see (*12*)] or treatment with S1 nuclease (*14*).

Suitable plasmid vectors are known for insertions at all three restriction sites: Eco RI, Bam I, and Hind III. The plasmid pMB9, mentioned earlier, has been described (*4*). The plasmid RSF2124 has an Eco RI site within its colicin gene (*10*), and therefore one can screen for insertion by looking for disruption of colicin production after recombination. The plasmids pBR316 and pBR313 have both ampicillin and tetracycline resistance genes, with a Bam I and Hind III site in the latter



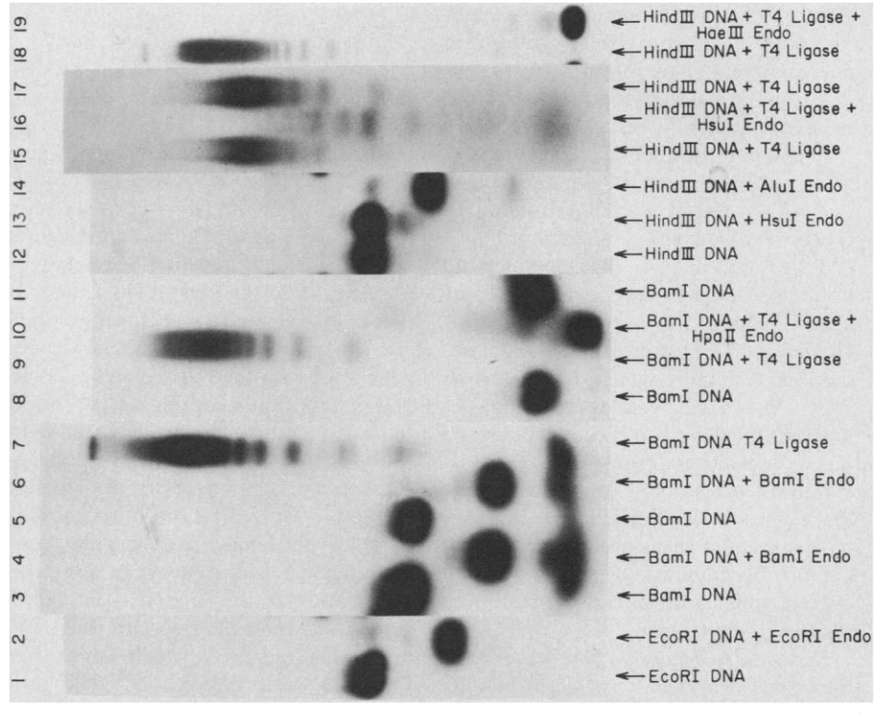


Fig. 2 (above). The convergent triester synthesis scheme used in preparing the Bam I decamer is shown. The other two decamers were made similarly. Most procedures, chemicals, and solvents have been described (*6, 7*); only new improvements are given here. Purification of the protected polynucleotide fragments was aided by the use of medium-pressure silica gel chromatography. Chromatronix glass columns with Fluid Metric RPG-50 solvent pumps were used to purify the intermediate and decameric products. The 2-inch columns were run at 25 pounds per square inch, 1-inch columns at 100 pounds per square inch, and ½-inch columns were run at 175 pounds per square inch in chloroform, with 5 to 10 percent methanol, depending on the polarity of the molecule being eluted. Removal of the β-cyanoethyl phosphate protecting groups at intermediate stages of the synthesis was carried out in a solution of 0.1*M* NaOH and tetrahydrofuran for preparation of Eco RI decamers, and in a solution of 0.05 *M* NaOH and a mixture of pyridine and ethanol (1 : 1) for the Bam I and Hind III decamers. All reactions were monitored on thin-layer silica gel plates (*6*). Final deblocking of the decameric products was done in a 1 : 2 mixture of pyridine and 30 percent NH_4OH at room temperature for 2 days, or at 70°C for 3 hours, followed by treatment with 80 percent acetic acid for 15 minutes. The deblocked products then were run on cellulose thin-layer chromatography plates in a mixture of 100 ml H_2O and 60 ml of isobutyric acid for 16 hours. The plates were dried, and the slowest moving band was recovered from the cellulose in 3 percent NH_4OH. Evaporation of solvent gave a white residue, which was dissolved in 70 m*M* phosphate buffer at *p*H 7, heated to 80°C, and then allowed to cool slowly at 4°C. The DNA duplex was then chromatographed on a Sephadex G-75 column (1 by 100 cm) at 4°C, eluted with 0.1*M* ammonium bicarbonate buffer, *p*H 7.5. Abbreviations: DMT, 4,4-dimethoxytrytyl protecting group for the 5′ end; CCCN, β-cyanoethyl protecting group for the 3′ phosphate; Ac, acetate protecting group for the hydroxyl end. For details about TPST, see (*7*). Fig. 3 (right). Accumulated electrophoretograms showing restriction enzyme reactions and blunt-end ligation. An explanation of each lane is in the text. The DNA's were phosphorylated and ^{32}P-labeled with T4 polynucleotide kinase (*5*), then treated as indicated. Conditions for T4 DNA ligase (blunt-end ligation) were 66 m*M* tris, *p*H 7.6, 6.6 m*M* $MgCl_2$, 10 m*M* dithiothreitol, 0.4 m*M* adenosine triphosphate (ATP), 1 μ*M* [5′-^{32}P]DNA, approximately 3.0 units of T4 ligase, 27°C, 16 hours, 15-μl volume. Conditions for Eco RI were 100 m*M* tris, *p*H 7.5, 5 m*M* $MgCl_2$, 100 m*M* NaCl, 0.02 percent Triton X100, 1 μg of [5′-^{32}P]DNA, 20°C, 5 hours, 10-μl volume. Conditions for Bam I were 100 m*M* tris, *p*H 7.5, 5 m*M* $MgCl_2$, 1 μg of [5′-^{32}P]DNA, 27°C, 5 hours, 10-μl volume. Conditions for Hsu I, Hpa II, and Hae III were T4 DNA ligase buffer, 1 μ*M* T4 ligated [5′-^{32}P]DNA, 37°C, 4 hours. Alu I: 6.0 m*M* tris, *p*H 8.0, 6.0 m*M* $MgCl_2$, 6.0 m*M* β-mercaptoethanol, 1 μg of [5′-^{32}P]DNA, 27°C, 5 hours, 10-μl volume. The enzymes Eco RI, Bam I, Hsu I, Alu I, Hae III, T4 DNA ligase, and T4 polynucleotide kinase were the gifts of Drs. H. Heyneker and P. J. Greene of the University of California, San Francisco. Hpa II was purchased from New England Bio-Labs. Gel electrophoresis was conducted in 20 percent acrylamide with 1.5 percent bis, 7*M* urea, at 15 volt/cm for various times in 0.05*M* tris-borate, *p*H 8.3.

(R. Rodriguez, F. Bolivar, H. Goodman, H. Boyer, and M. Betlach, personal communication). Hence, incorporation of DNA into a Bam I site can be monitored by looking for ampicillin resistance and tetracycline sensitivity in these two plasmid strains.

The linkers reported here have been used to clone eukaryotic DNA and complementary DNA (cDNA). As described (*15*), the Eco I decamer has been ligated to repetitive DNA from sea urchin sperm, and the resultant cohesive-ended fragments were cloned in RSF2124 plasmids. These clones will provide a source of repetitive DNA in quantity, helping to determine new features of the eukaryotic genome. The Bam I decamer has been added to cDNA's and cloned to the tetracycline gene of the pB313 plasmid (J. Shine and H. Goodman, personal communication). The decamers have also been shown to be useful for constructing new cloning vehicles by converting one restriction site to another, for example, Hind III to Eco RI (H. Heyneker, personal communication), and Eco RI to Bam I (A. Riggs, unpublished data). Therefore, the chemically synthesized decameric linkers reported here are generally applicable tools for molecular cloning experiments.

The three decameric linkers themselves were constructed with CC . . . . . GG at the ends for stability (T_m = 48°C), resulting in efficient blunt-end ligation, and so that polymerized decamers could be cloned and cut apart again by Hae III restriction enzyme. Cloning would provide a means of obtaining a steady supply of more linkers. However, "polylinker" clones have not yet been obtained, perhaps because of in vivo instabilities. Therefore, triester chemical synthesis may prove easier than cloning for moderate scale production of DNA molecules as small as decamers. At present, 25-mg amounts of greater than 90 percent pure material can be made in a relatively short time.

Note added in proof: The linker synthesis has also been pursued in parallel with this work by Bahl *et al.* (*16*).

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4 February 1977

Screening λgt Recombinant Clones by Hybridization to Single Plaques in situ

Abstract. *A rapid, direct method for screening single plaques of λgt recombinant phage is described. The method allows at least 10^6 clones to be screened per day and simplifies physical containment of recombinants.*

Recombinant DNA technology has made it possible, in principle, to isolate large quantities of DNA corresponding to any single gene. This isolation can be accomplished in any of three ways: (i) purification of the gene or its transcript before insertion into a vector for cloning (*1*); (ii) genetic selection of the desired gene after "shotgun" (*2*) cloning the genome of the donor organism (*3, 4*); or (iii) the use of complementary RNA or DNA probes to screen clones produced through a shotgun experiment. The first method is limited by the practical difficulty of physically purifying the complete nucleic acid sequence of any gene. Application of the second is limited to genes for which appropriate mutations are available in hosts which can express foreign genetic information. The third method is limited only by the availability of complementary nucleic acid probes and has been the most widely applied.

Screening methods have been developed for both recombinant plasmid (*5*) and bacteriophage (*6, 7*) cloning systems. The most rapid of these, the methods of Grunstein and Kramer, are based on a technology developed by Olivera and Bonhoeffer (*8*) for growing and lysing cells on membrane filters. The speed and simplicity of these methods is limited by the need to pick and spot plaques or colonies from plates onto filters for further growth. We have found that a single plaque of a λgt recombinant phage contains enough phage DNA for detectable hybridization to complementary labeled nucleic acid, and that this DNA can be fixed to a nitrocellulose filter by making direct contact between the plaque and the filter. These findings have allowed us to develop a method for screening recombinant phage which has significant advantages over previous methods: (i) physical containment of recombinant molecules (and organisms carrying them) is made simpler because this method requires fewer manipulations of viable phage, requires the growth and handling of much smaller quantities of recombinant DNA-containing phage and cells, and produces no contaminated materials other than the petri dishes on which the plaques are grown. (ii) At least 2×10^4 plaques can be screened per hour (*9*), which compares with about 100 per hour by the fastest previous method (*7*). (iii) Because up to 2×10^4 plaques can be screened on a single petri dish and nitrocellulose filter, the method requires smaller amounts of materials, including radioactively labeled probe. (iv) Because screening can be done directly from the transfection plate, all the independently constructed phages containing similar or identical inserts can be isolated and individually