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Ligation-mediated PCR amplification of specific fragments from a Class-II restriction endonuclease total digest

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ABSTRACT

A method is described which permits the ligation-mediated PCR amplification of specific fragments from a Class-II restriction endonuclease total digest. Feasibility was tested using *BclI* and phage λ DNA as a model enzyme and amplicon system, respectively. *BclI* is one of many widely used restriction enzymes which cleave at palindromic recognition sequences and leave 5′-protruding ends of defined sequence. Using a single pair of universal primers, a given fragment can be specifically amplified after joining the fragments to adaptors consisting of a duplex primer region and a 9-nucleotide protruding single-stranded 5′-end containing the sequence complementary to the cleaved restriction site and a 4-nucleotide 'indexing sequence.' The protruding strand anneals to a restriction fragment by displacing its corresponding strand in the same fragment-specific indexing sequence located juxtaposed to the restriction site. The adaptor is covalently linked to the restriction fragment by T4 DNA ligase, and amplification is carried out under conditions for long-distance PCR using the M13 forward and reverse primers. The technique discriminated robustly between mismatches and perfect matches for the 16 indexing sequences tested to allow individual λ *BclI* fragments to be amplified from their respective adaptor pairs. A strategy is proposed enabling a non-cloning approach to the accession, physical mapping and sequencing of genomic DNA. The method could also have application in high-throughput genetic mapping and fingerprinting and should expand the enzyme base for ligation-mediated indexing technology which has previously been limited to the Class-IIS and IP restriction endonucleases.

Recent advances in PCR have enabled amplification of DNA regions up to 40 kb in length, making use of the exonucleolytic proof-reading activity of certain thermostable DNA polymerases (1,2). Although there are now several kits available commercially that perform such 'long distance' PCR, reproducibly amplifying such long regions still requires extensive optimizations in most cases. Easily extending amplicon lengths beyond 40 kb does not appear to be imminent, and therefore it would be advantageous to develop an alternative strategy by which PCR can reach even further into unknown regions of a complex genome. To do this, we have been developing a method by which Class-II restriction fragments are individually amplified from a total digest using a single pair of universal primers. As a means to bypass conventional molecular cloning techniques, ligation-mediated PCR of specific restriction fragments was first described by Smith (3), and later by Unrau and Deugau (4) using a similar strategy which the latter authors called 'indexing'. These approaches used either the class IIS (5) or IP restriction enzymes (4), the former cleaving DNA asymmetrically at precise distances outside their recognition sequences, and the latter cutting symmetrically within their interrupted palindromic (IP) binding sites. Overhangs of different length (two to five bases) and polarity (3′ or 5′) are created which vary in base composition as determined by the locations of the enzymes' respective cleavage sites in a genome. The overhanging sequence at any one cut site serves as an annealing site for an 'indexer', a cohesive-end adaptor specific for that particular restriction site. Four-base overhangs, for example, yield 256 (4^4) possible indexers, and these authors demonstrated how pairs of indexers can be specifically ligated to the ends of restriction fragments which are then selectively PCR amplified from the total digest using primer sequences built into the adaptors. In this paper, we demonstrate the feasibility of using our method to expand this indexing capability to the widely used Class-II restriction endonucleases which cleave palindromic recognition sequences and leave overhanging sequences of invariant base composition.

Feasibility of the methodology was tested here using bacteriophage λ DNA as a model amplicon system and the 6-cutter, *Bcl*I, as a model Class-II restriction endonuclease. *Bcl*I cuts the λ genome eight times, producing nine fragments with the same 5′-overhang sequence. As shown in Figure 1, our *Bcl*I restriction site indexers are partially double-stranded oligodeoxynucleotide adaptors containing either the –21M13 forward or M13 reverse primer, 5 nucleotides (nt) complementary to the sequence produced by *Bcl*I cleavage, and 4 nt which reside immediately adjacent to the restriction recognition sequence in the target (λ) DNA molecule. The shorter strand of the adaptor contains the

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sequence which is the same as that used for a PCR primer and which provides the 3′-OH group necessary for joining to the restriction fragment by DNA ligase. Two oligos, the 18mer primer strand and the 27mer 'invading' strand, are first annealed to form the adaptor. Then, the adaptor is hybridized and enzymatically ligated to the restriction fragment, resulting in a strand-displaced structure (Fig. 1). Five nucleotides are displaced from the double-stranded portion of the target molecule—one from the restriction site and the four identical to the indexing sequence of the adaptor's invading strand. To PCR amplify restriction fragments containing 5′-overhangs, extension of the displaced strand from its 3′-end by polymerase (dashed arrow in Fig. 1) is required in the first thermal cycle to regenerate template complementary to the PCR primers. (Note: Figure 1 shows the structure formed for a 5′-overhang. For 3′-protruding ends, the polarity of the strands are reversed in which case this fill-in step would be avoided.) We refer to the invading or displaced 4 nt region as the 'indexing sequence.' We considered that two isoenergetic displacement structures could form with equal probability: the one shown in Figure 1, and one whereby the indexing sequence of the adaptor is displaced by the restriction fragment. However, the latter structure is not favored for it results in a net loss of 5 nt available for annealing by the invading strand.

An adaptor is specific to one end of a restriction fragment based on its indexing sequence. An indexing sequence is unique to an end, where uniqueness is confined to the 256 possible 4mers which exist. Each end of a given restriction fragment is distinguished from the other by possessing a different indexing sequence, and all fragments are distinguishable by their respective indexing sequence pairs. In a preliminary study, we observed that a single mismatch at any position within an indexing sequence can provide the discrimination necessary to distinguish among different end-specific annealings/ligations (data not shown). Only mismatches located within 3 nt of the joining site are expected to affect ligase activity (6), and therefore mismatches located within the indexing sequence should exclusively exert their effect by duplex destabilization during hybridization. Also, strand displacement itself should provide a contribution to enhancing discrimination. That is, invading strands perfectly base-paired with their complementary sequences may displace more efficiently than those containing unstable base mismatches (7,8). We show here that individual λ *Bcl*I fragments can be amplified from the total digest by using pairs of known indexing sequence which are 'end-specific' for their respective fragments, or by using end-specific adaptors in conjunction with 'combinatorial' adaptors containing the complete mixture of 256 indexing sequences.

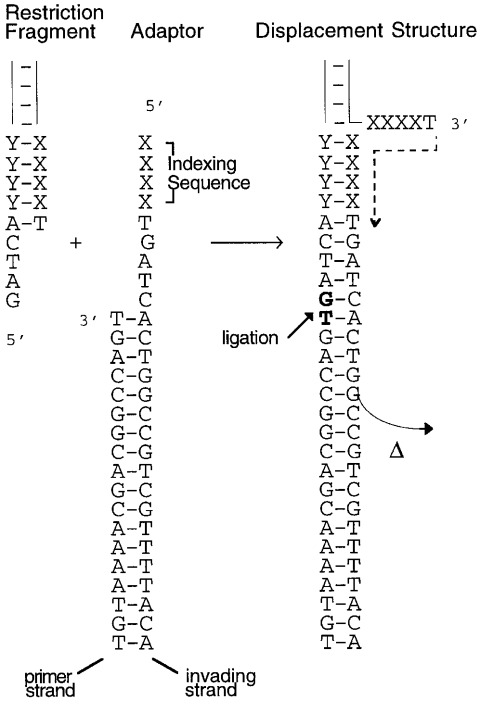

Figure 1. Displacement structure formed between an adaptor and the end of a *Bcl*I restriction fragment. The 9 nt single-stranded protruding end of the adaptor consists of the cleaved restriction site (*Bcl*I shown) and the indexing sequence (XXXX). Annealing to its complementary sequence in the restriction fragment displaces five 3′-end nt, shown as XXXXT. Highest specificity of adaptor-fragment annealing requires perfect X–Y matching within the indexing sequence. Ligase is used to covalently join the adaptor and the restriction fragment at the G–T nick (in bold). In the first cycle of the PCR, the invading strand is thermally dissociated (Δ), leaving the displaced 3′-end free to reanneal and prime extension by polymerase (---->) to regenerate template for binding to PCR primers. The –21M13-forward universal primer sequence is shown, and adaptors containing the M13revP for primer sequence are typically used at the other end of the restriction fragment (Fig. 2).

END-SPECIFIC INDEXING SEQUENCE PAIRS

We made pairs of end-specific adaptors for all nine of the *Bcl*I fragments of the linear 48 502 bp λ genome. *Bcl*I was chosen because of the broad range of fragment sizes that the enzyme generates, as follows: 517, 560, 1576, 2684, 4459, 4623, 6330, 8844 and 18909 bp, the underlined ones being the terminal fragments. In Figure 2A, the fragments' displaced indexing sequences (in bold) are shown as part of the invading strand of their corresponding adaptors. As shown in Figure 3A and B, each of these fragments were individually amplified from the total *Bcl*I digest using a straightforward protocol: (i) digest λ DNA with *Bcl*I, (ii) use T4 DNA ligase to join 15 ng of the λ DNA with an excess (25 pmol) of end-specific adaptor pairs (left and right; see Fig. 2A), and (iii) PCR amplify 1.5 ng of the ligation product using 10 pmol of the universal primers. Incubation with T4 DNA ligase was only 20 min at 40°C, and only a single set of PCR parameters has been required to date. Also, no reactant removal or product purifications were required between each step making the overall procedure amenable to automation. The specificity obtained for the PCR products shown indicates that it is possible to achieve quite good discrimination among the adaptor pairs tested. Eight out of nine of the λ fragments were robustly amplified from the total *Bcl*I digest, including the 560 and 8844 bp terminal fragments, each amplified from an adaptor and a λ-specific 18mer primer (Fig. 2, legend). In the PCR reactions of Figure 3A and B, some non-targeted restriction fragments were weakly co-amplified along with the desired products (e.g. 0.56, 1.5 and 4.4 along with specific fragments 4.4, 4.6 and 0.560, respectively). Each case can be explained by homology in some indexing sequence positions and the potential for stable mismatch duplex formation in other positions (9). The source of the few non-specific products observed which do not co-migrate with the restriction fragments has not been determined. We found that treatment with the 3′→5′ exonuclease activity of Vent polymerase was important for increasing the yields of the PCR products

A. *END-SPECIFIC ADAPTORS*

LEFT *w/forward primer*

-21M13	5′	tgtaaaacgacggccagt
517L	3′	ACATTTTGCTGCCGGTCACTAGT**GGTC**
560L		ACATTTTGCTGCCGGTCACTAGT**GGTA**
1567L		ACATTTTGCTGCCGGTCACTAGT**GATA**
2684L		ACATTTTGCTGCCGGTCACTAGT**AGTC**
4459L		ACATTTTGCTGCCGGTCACTAGT**GGGC**
4623L		ACATTTTGCTGCCGGTCACTAGT**CAAG**
6330L		ACATTTTGCTGCCGGTCACTAGT**CAAA**
18909L		ACATTTTGCTGCCGGTCACTAGT**CGGC**

RIGHT *w/reverse primer*

M13RevP	5′	caggaaacagctatgacc
517R	3′	GTCCTTTGTCGATACTGGCTAGT**GAAG**
1576R		GTCCTTTGTCGATACTGGCTAGT**CAGT**
2684R		GTCCTTTGTCGATACTGGCTAGT**CGGA**
4459R		GTCCTTTGTCGATACTGGCTAGT**GGAG**
4623R		GTCCTTTGTCGATACTGGCTAGT**TCCT**
6330R		GTCCTTTGTCGATACTGGCTAGT**TGAC**
8848R		GTCCTTTGTCGATACTGGCTAGT**TTAG**
18909R		GTCCTTTGTCGATACTGGCTAGT**GGTG**

B. *COMBINATORIAL ADAPTORS w/forward or reverse primer*

Combo-FP	5′	tgtaaaacgacggccagt
	3′	ACATTTTGCTGCCGGTCACTAGT**NNNN**
Combo-RP	5′	caggaaacagctatgacc
	3′	GTCCTTTGTCGATACTGGCTAGT**NNNN**

Figure 2. Sequences of the (**A**) end-specific and (**B**) combinatorial adaptors used for amplification of λ *Bcl*I fragments (sizes in bp shown). The end-specific adaptors corresponding to the left (L) and right (R) ends of the fragments utilized the –21M13 (forward) and M13RevP (reverse) universal primer sequences (in lower case), respectively. The indexing sequences are shown in bold and the *Bcl*I site (after cutting) is underlined. The combinatorial adaptors are synthesized with a degenerate mixture of all 4 nt (NNNN) in the indexing sequence and contain the sequences for either the forward primer (combo-FP) or reverse primer (combo-RP). Adaptors were made by mixing 12.8 µM each of the primer and invading strands (Fig. 1) in 50 mM Tris–HCl, pH 7.8 at 85°C and annealing by slow cooling to room temperature. Note: when used in conjunction with an end-specific adaptor, combinatorial adaptors can be ligated to either end of a restriction fragment, resulting in four possible ligation choices: combo-FP(L); combo-FP(R); combo-RP(L); combo-RP(R). Amplification of the λ terminal fragments utilized the λ-specific 18mers CGTAACCTGTCGG-ATCAC and CGCGGGTTTTCGCTATTT for 560R and 8848L, respectively. All oligonucleotides were synthesized by the University of Wisconsin Biotechnology Center and purified on Sep-Pak C18 cartridges (Waters, Milford, MA).

obtained with rTth polymerase (2). We have not yet succeeded in specifically amplifying the 18.9 kb *Bcl*I fragment from its end-specific adaptors. This will likely require the use of longer primers (1,2) and/or more specifically defined reaction conditions or thermal cycling parameters.

COMBINATORIAL INDEXING

A powerful application of the method is the amplification of genomic regions which are longer than amplicons typically accessed by a pair of unique primers. This could be accomplished by generating an ordered set of amplified restriction fragments. For example, we are interested in directly accessing and sequencing regions of the human genome which are resistant to molecular cloning in bacteria. DNA located between two 'sequence-tagged sites' (10) could be isolated using one of a

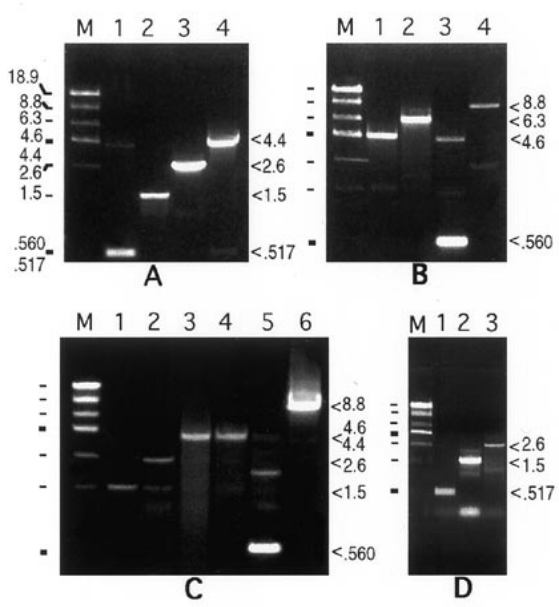

Figure 3. Amplification of specfic λ fragments from a *Bcl*I total digest. For (A), (B) and (C), 5 µg of N^6-methlyadenine-free λ DNA [New England Biolabs (NEB), Beverly, MA] was digested at 37°C for 2 h with 20 U of *Bcl*I in a volume of 100 µl using the manufacturer's (NEB) buffer. Three µl of a 10-fold dilution (15 ng λ DNA) was transferred to 50 µl ligation reactions containing 800 U T4 DNA ligase (NEB), manufacturer's 1× buffer and left/right adaptor pairs for a given restriction fragment (Fig. 2). Ligation reactions were pre-incubated for 5 min at 40°C, again for 20 min at 40°C after addition of enzyme and then stopped by heat at 65°C for 15 min. Five µl (1.5 ng λ DNA) of the ligation reactions were transferred to 100 µl PCR reactions for which the conditions used are described below. All PCR reactions contained 10 pmol of primer oligonucleotides. M = λ*Bcl*I total digest; fragment sizes are indicated on the left side of (A). The specific PCR amplified restriction fragments are indicated by (<) on the right side of each panel. All PCR reactions were performed with the XL-PCR kit from Perkin Elmer (Applied Biosystems Division, Foster City, CA), using 2 µl (4 U) of rTth DNA polymerase. PCR utilized 1.1 mM magnesium acetate (note: an additional 1 mM $MgCl_2$ brought in with ligase reaction), except for the reaction of lane C3 which utilized 1.65 mM $Mg(OAc)_2$, to obtain robust and specific amplification. All specific products shown could also be obtained using 0.55 mM $Mg(OAc)_2$ (data not shown). PCR was performed in the PTC-200 DNA Engine (MJ Research, Watertown, MA) using the following thermal cycling profile: 95°C for 1 min, 30 s followed by 30 cycles of: 94°C, 40 s; 55°C, 40 s; 72°C, 5 min. Twenty µl were loaded and electrophoresed on 0.8% agarose gels containing 0.5 µg/ml ethidium bromide. (**A** and **B**) Amplification of λ *Bcl*I fragments from end-specific adaptors: ligations were performed using 25 pmol of single-end adaptor pairs. (A) Lanes 1–4: amplification of the 0.517, 1.5, 2.6 and 4.4 kb fragments, respectively, using the corresponding left/right adaptor pairs and universal primers of Figure 2. (B) Lanes 1 and 2: amplification of the 4.6 and 6.3 kb fragments, respectively, using the corresponding left/right adaptor pairs and universal primers of Figure 2; lanes 3 and 4: amplifications of the 0.560 and 8.8 kb terminal fragments using the 560R and and 8848L primers, respectively (Fig. 2, legend), in conjunction with the corresponding left and right adaptors of Figure 2. (**C**) Amplification of λ *Bcl*I fragments using combinatorial adaptors in conjunction with end-specific adaptors: lanes 1–4: amplification of the 1.5, 2.6, 4.4 and 4.6 kb fragments, respectively, using 25 pmol of the corresponding right adaptors of Figure 2 in conjunction with the combinatorial adaptor (combo-FP; see Fig. 2); lanes 5 and 6: amplification of the 0.560 and 8.8 kb terminal fragments, respectively, using the 560R and 8848L primers in conjunction with the combo-FP and combo-RP adaptors, respectively. The amount of combinatorial adaptors ligated varied as follows: 0.5, 0.25, 25, 25, 0.0025 and 0.0025 pmol for the 1.5, 2.6, 4.4, 4.6, 0.560 and 8.8 kb fragments, respectively. (**D**) Amplification of λ *Bcl*I fragments from *E.coli* lysogen DNA: λ DNA was replaced with *E.coli* DNA (λc1857Sam7dam$^-$ lysogen; obtained from NEB), starting with 18 µg for digestion with *Bcl*I. Subsequent dilutions and reactions were performed the same as for λ DNA as described above. Lanes 1–3 show the PCR products obtained after ligation with 25 pmol of single-end adaptor pairs for the 0.517, 1.5 and 2.6 kb *Bcl*I fragments of the λ prophage DNA.

variety of techniques for site-specific excision (11–13), followed by digestion with several different 6-cutter restriction enzymes. However, all 65 536 (256 × 256) pairwise combinations of indexing sequences would need to be PCR amplifed for each enzyme in order to recover all possible restriction fragments from the total digests of the excised DNA. Utilizing such large arrays would be a formidable task even if automated. Instead, all indexing sequence pairs could be accessed in an array reduced to only 256 ligation (and PCR) reactions, each containing a single end-specific adaptor but sharing a 'combinatorial' adaptor. Besides the universal primer site(s) and a given restriction site sequence, the combinatorial adaptor contains the complete 256 degenerate mixture of indexing sequences made in one oligodeoxynucleotide synthesis (NNNN; see Fig. 2B). A simple calculation for 6-cutters predicts that 256 individual, sequence-ready restriction fragments could be amplified for a target DNA up to 0.5 Mb in size. [DNA of 1 Mb complexity digested with a 6-cutter enzyme, which cleaves a random sequence on average every 4096 bp, will produce on average 244 fragments. Dividing this by 256 indexers yields about one amplified fragment per end-specific adaptor/combinatorial adaptor pair used. An indexing sequence would be present twice in the full library (array) of adaptors, one contributed by the end-specific adaptor and one by the combinatorial adaptor. A fragment would be amplified twice but at different locations in the array, and therefore a 0.5 Mb target DNA segment would be accomodated bidirectionally for the isolation of individually amplified restriction fragments.] The use of several different enzyme-arrays would permit the fragments to be ordered by performing gel electrophoretic analysis of each product cross-digested with each enzyme used, followed by the generation of a restriction map.

The results of Figure 3C indicate that specific fragments can be amplified from the total digest when adding both end-specific and the combinatorial adaptors in the same ligation reaction. When the end-specific and combinatorial adaptor DNA concentrations are equimolar, the concentration of a single indexing sequence within the combinatorial mixture is reduced by a factor of 1/256 relative to that of the end-specific adaptor. Thus, the end-specific adaptor is ligated to its targeted site at much higher efficiency than the combinatorial adaptor's specific indexing species targeted to the other end of the fragment. Subsequently, during the early cycles of PCR specific fragment amplification is driven predominantly by the primer annealing to the end-specific adaptor. The ability to co-ligate the end-specific and combinatorial adaptors renders the overall procedure amenable to automation by avoiding intervention of purification steps which remove ligation reactants or intermediate products prior to PCR. The highest specificities obtained to date when using the combinatorial adaptors are shown by the fragment amplifications of Figure 3C, and these were obtained over a range of end-specific:combinatorial adaptor DNA concentration ratios which varied from to 1:1 to 100:1 depending on the targeted fragment (Fig. 3, legend). Furthermore, an additional 100-fold dilution of the combinatorial adaptor yielded the most specific λ terminal fragment amplifications. We speculate that such fluctuations in relative adaptor DNA concentrations result from semispecific competition for the annealing step by indexing sequences in the combinatorial mixture which can form mismatched hybrids of varying stability. In contrast, numerous nonspecific products were typically observed when we replaced the combinatorial adaptor with one having just a 4 nt *Bcl*I cohesive end (data not shown).

TECHNICAL CONSIDERATIONS

Ideally, it would be desirable to establish a common set of annealing/ligation conditions which permit perfect discrimination among all possible indexing sequences. Toward this goal, we addressed *Escherichia coli* DNA using a λ lysogen (dam$^-$) as target. This more complex genome (4.7 Mb) has 1604 *Bcl*I sites (F. Blattner, personal communication) or 200 times as many sites as λ DNA. The results of Figure 3D indicate that despite this increase in target indexing sequence complexity, λ *Bcl*I fragments could still be specifically amplified. Although these results are encouraging, the full library of adaptors has yet to be tested. Eliminating nonspecificities and the above-described need for asymmetric adaptor ratios awaits further studies on the sequence and positional contributions of mismatches, particularly those arising as a result of stably mismatched duplex situations (9). Toward neutralizing effects due to indexing sequence differences, universal base analogues like 3-nitropyrrole (14) could be incorporated into the adaptors for enhancing discrimination (6,15). For choosing restriction mapping enzymes and designing their respective adaptors, it would be advantageous to minimize base compositional differences contributed by restriction site recognition sequences. For this purpose, there are ~50 class II 6-cutters (3′ and 5′, including isoschizomers) which generate 4 nt overhangs composed of all four bases (*Bcl*I is one such enzyme) but differ only in the one outermost base remaining after cutting, thus creating a small and almost inconsequential contribution to the T_m for adaptor-fragment annealing. Experiments are in progress to test these other enzymes as well as those which cleave palindromic sequences in opposite polarity, utilize a variety of base compositions and leave either blunt ends or variations in lengths of protruding ends. We are also exploring the use of 2–3 bp indexing sequence lengths in order to reduce array size to 16–64 for certain applications such as differential display of cDNAs (16–18).

For most applications of the described method (see below), we considered it advantageous to have the capability for direct bi-directional sequencing of the amplified fragments. Thus, for this study we chose to amplify the fragments with two different priming sequences which could be used for both PCR and the initiation of DNA sequencing by primer walking. However, amplifying with a unique priming sequence built into all adaptors moderately simplifies the procedure.

APPLICATIONS

Besides long distance gap-filling, the described method could be utilized to access uncharacterized regions of DNA which flank those of known sequence for applications including the end-sequencing of low-yielding cloned inserts, and the determination of genomic sequences which reside adjacent to gene exons or pathogenic integrons. As importantly, the method could be utilized as (i) an alternative to primer-directed indexing approaches for DNA fingerprinting such as AFLP (19), (ii) a means to generate 'sequence-ready' markers representing restriction fragment length polymorphisms (RFLP) that have been enriched by RDA (20) or RFLP-subtraction (21), and (iii) the characterization of differentially expressed mRNAs (16–18). Indexing has already been proposed (16) as a means to obtain more coding regions, allow lower redundancy and detect rare messages more efficiently than differential display techniques based on arbitrarily primed

PCR (17,18). The use of enzyme-specific adaptor libraries should provide an automatable '*in vitro* cloning' approach for the physical mapping and sequencing of whole microbial genomes as well as biochemically excised (11–13) or microdissected (22) sections of complex genomes, thereby avoiding replication of inserts in prokaryotic vectors and the concomitant risk of encountering chimerism, deletions and rearrangements. In contrast, chemical genetic strategies based on 'vectorette PCR' (23,24) require sequential walking steps coupled with *de novo* primer syntheses.

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