In vivo cloning of PCR products in *E.coli*

Jonathan D.Oliner, Kenneth W.Kinzler and Bert Vogelstein*

The Johns Hopkins Oncology Center, Baltimore, MD 21231, USA

Received July 12, 1993; Revised and Accepted September 30, 1993

ABSTRACT

This report describes an efficient method to clone PCR products exploiting endogenous *Escherichia coli* enzymatic activities. PCR products are engineered to contain terminal sequences identical to sequences at the two ends of a linearized vector. PCR products and vector DNA are then simply co-transfected into *E. coli* strain JC8679, obviating the requirement for enzymatic treatment of the PCR product or *in vitro* ligation. The high rate of homologous recombination in this strain results in efficient incorporation of the insert into the vector, a process we refer to as *in vivo* cloning (IVC).

INTRODUCTION

It is often desirable to clone PCR products into plasmid vectors. Unfortunately, such products have proven unusually resistant to standard cloning procedures. One recognized impediment to cloning is that PCR products contain non-templated residues at their 3' termini. *Thermus aquaticus* (Taq) DNA polymerase, like other eucaryotic and procaryotic DNA polymerases, has a propensity to add a single dATP to the 3' termini of blunt-ended DNA duplexes (1). Numerous cloning techniques have been developed to handle such products. One method entails polishing the ends of the DNA fragment with the Klenow fragment of DNA polymerase I so that it can be cloned by blunt-end ligation (2–5). Another procedure takes advantage of the non-templated dATP addition by ligating the PCR product to a vector containing 5' T-overhangs (6–8).

Alternatively, approaches have been developed that circumvent the complication of dATP addition. One such approach involves the design of PCR primers which contain restriction endonuclease recognition sequences (9). While this is the most commonly used method for cloning PCR products, it carries an additional limitation: PCR-derived DNA fragments are often resistant to digestion by restriction endonucleases (9–12). A further constraint inherent to this method is that restriction sites internal to the PCR product are also susceptible to cleavage. Another tactic for cloning PCR products involves the use of a different thermostable DNA polymerase in the amplification reaction that, unlike Taq, generates blunt-ended products due to an intrinsic 3' to 5' exonuclease activity (e.g. Vent polymerase [New England Biolabs]). A final class of methods have been developed which entail the selective degradation of linear duplex DNA from the 3' (13,14) or 5' (15,16) ends of both strands, leaving extensive

regions of 5' or 3' overhang, respectively, at the termini of PCR products and vectors. The vector and PCR product tails are designed to be complementary to one another; once annealed, the PCR product/vector circles are stable enough to forego *in vitro* ligation.

The above procedures are successful in many instances, but not universally. Moreover, most of these procedures require significant vector and PCR product preparation. We describe a method for cloning PCR products in *E. coli* which is highly efficient and requires minimal *in vitro* manipulation prior to transformation. This strategy is modelled on previous work showing that yeast transfected with a gapped plasmid and an appropriate linear template have the capacity to 'patch' the plasmid gap by homologous recombination with the template (17). This approach has since been used to clone PCR products in yeast (18). We reasoned that such an approach might be feasible in a procaryotic system, as *E. coli* also have the ability to repair DNA gaps (19). The advantages of using *E. coli* over yeast include greater transfection efficiency, more rapid growth rates, and higher plasmid yields.

Using this method, we have cloned PCR products ranging in size from 608 to 2132 bp with high efficiency. Cloning efficiency was found to be directly related to the extent of homology between the PCR product and vector. With as little as 22 and 27 base pairs (bp) of homology on the two ends, colonies containing appropriate recombination products could be obtained. However, if the homologous stretches on each end were increased, the number of colonies with insert-containing plasmids rose by over two orders of magnitude. Such large regions of homology can be easily engineered into PCR products by choosing appropriate primers.

MATERIALS AND METHODS

Vector preparation

The cloning vector used in all experiments was pBS (= pBluescript II SK⁺ [Stratagene]), prepared by digestion with HindIII and PstI, followed by phenol/chloroform extraction and ethanol precipitation. In some experiments, this vector was treated with calf intestine alkaline phosphatase (Boehringer Mannheim) according to manufacturer's specifications, or purified by a Spin-X column following agarose gel electrophoresis (20). A second vector, referred to as pBC (= pBC KS⁺) was also obtained from Stratagene.

* To whom correspondence should be addressed

PCR product preparation

All PCR reactions were performed using the Bind-Aid Amplification Reagent Kit (United States Biochemical) in 50-microliter reactions containing 5 units Amplitaq DNA polymerase (Perkin Elmer Cetus). PCR amplifications from plasmid templates contained 1 ng of plasmid and 350 ng of each PCR primer (95° 30 sec, 45° 1 min, 70° 1 min for 30 cycles). Genomic amplifications contained 100 ng of normal human lymphocyte DNA, 350 ng of UNIV1 and UNIV2, and 35 ng of APC1 and APC2 (95°×30 sec, 50°×1 min, 70°×1 min for 3 cycles; 95°×30 sec, 40°×1 min, 70°×1 min for 4 cycles; 95°×30 sec, 55°×1 min, 70°×1 min for 29 cycles). PCR primer sequences were as follows: UNIV1 (5' ATTAACCTC ACTAAAGGGA ACAAAGCTG GAGCTCCACC GCG-GTGGCGG CCGCTCTAGA ACTAGTGGAT CCCCCG-GGCT GCA 3' [83 nt]); UNIV2 (5' AATACGACTC ACT-ATAGGGC GAATTGGGTA CCGGGCCCCC CCTCGAG-GTC GACGGTATCG ATAAGCT 3' [67 nt]); APC1 (5' CCC-CGGGCTG CAGGATATTA AAGTCGTAAT TTTGT 3' [35 nt]); APC2 (5' CCGTATCGAT AAGTCTATGC ACTA-CGATGT AACT 3' [35 nt]); T3 (5' ATTAACCTCACT-AAAG 3'), T7 (5' AATACGACTCACTATAG 3'), SK (5' TCTAGAACTAGTGGATC 3'), and KS (5' CGAGGTCG-ACGGTATCG 3') primers were the same as those described in the Stratagene catalog. Sequences shared between UNIV1 and APC1 are shown in bold, and sequences shared between UNIV2 and APC2 are underlined. All PCR products were purified by one of the following four methods: 1) Phenol/chloroform extraction and ethanol precipitation; 2) phenol/chloroform extraction and isopropanol precipitation as described (21), resulting in the removal of DNA fragments smaller than 200 bp (including primer dimers and unincorporated dNTPs and primers); 3) agarose gel electrophoresis and recovery with a Spin-X column (20), followed by phenol/chloroform extraction and ethanol precipitation; or 4) agarose gel electrophoresis and recovery with a Prep-A-Gene matrix, according to the manufacturer's instructions (Bio-Rad; 22). Following all purifications, the PCR products were dissolved in LOTE (3 mM Tris (pH 7.5), 0.2 mM EDTA).

Bacterial transformation

E. coli strain JC8679 (ref. 23) (*recB21*, *recC22*, *sbca23*, *his-328*, *thr-1*, *ara-14*, *leuB6*, Δ (*gpt-proA*)62, *lacY1*, *tsx-33*, *glnV44*(AS), *galK2*, *rpsL31*, *kdgK51*, *xylA5*, *mtl-1*, *argE3*(Oc), *thi-1*, *Lam*⁻, *Rac*⁺, *Qsr*¹⁺) was transformed by electroporation using a Bio-Rad Gene Pulser set at 2.5 kV, 200 ohms, and 25 μ FD. The transformed cells were suspended in 600 μ l L-broth in a 2.2 ml eppendorf tube and incubated with shaking at 37° for 1 hour. Dilutions of each transformation were plated on ampicillin-containing (200 μ g/ml) L-agar. The colony counts given in tables represent the number of colonies which would result from plating the entire electroporation mix. Electroporation-competent bacterial cells were made as follows: JC8679 cells were grown in 500 ml S.O.B. medium to O.D.(550 nm) = 0.8 from a 1:500 dilution of overnight culture. Cells were harvested and washed twice in 500 ml ice-cold 10% glycerol. The cells were resuspended in the 10% glycerol (about 3 ml) remaining in the centrifuge bottle after the second wash, divided into 30- μ l aliquots, and stored at -80°C. For each electroporation, one 30- μ l aliquot of cells was thawed and added to a mix containing one microliter of vector DNA and one microliter of PCR product in LOTE.

Colony hybridization

Bacterial colonies containing recombinant plasmids were identified as follows. Colony lifts (Colony/Plaque Screen, Dupont NEN) were treated for 2 min with 2× SSC/5% SDS, microwaved at full power until dry (4–5 min), and probed with ³²P-labelled oligonucleotides as described (24). Oligonucleotides used as probes were APC #22 (5' CTCTCCAGAACGGCTTGA 3') and APC #6 (5' TCAGGCTGTGAGTGAATGA 3'). The APC #22 probe was used for hybridization to all colony lifts described in Tables 1–4, while APC #6 was used for hybridization to the colony lifts described in Table 5.

RESULTS

It has been previously shown that *E. coli* strain JC8679 had the capacity to repair a gapped plasmid by intramolecular homologous recombination (19). We wished to determine whether this strain could additionally perform *intermolecular* homologous recombination between a PCR product and a linearized plasmid,

Figure 1. General scheme for testing IVC. PCR products were amplified from a plasmid which shared a multiple cloning site (MCS) and flanking primer sequences with the cloning vector. Note, however, that the MCS was oppositely-oriented with respect to the T3 and T7 primer sequences in the two vectors. Thus, the T3 and T7 sequences would not be expected to contribute to the vector/PCR product homology, despite being common to the two DNA fragments. The plasmid used to generate PCR products (pBC, containing a 749 bp cDNA fragment of the APC gene) contained a different selectable marker (chloramphenicol resistance) than that within the pBS cloning plasmid (ampicillin resistance). Therefore, any bacteria transformed with contaminating pBC plasmid would be unable to grow on ampicillin-containing plates. Homologous recombination between the PCR product and vector can occur at the sequences demarcated by 'X's.

thereby cloning the PCR product. As a preliminary test of the feasibility of the *in vivo* cloning (IVC) approach (general scheme shown in Figure 1), PCR products were amplified from a pBC plasmid containing a 749 bp fragment of the APC gene (25–28) cDNA cloned into the EcoRI site. With the primers T3 and T7, the resultant product shared 88 bp of homology (50 bp on one end, 38 bp on the other) with the non-insert-containing pBS which had been linearized by digestion with HindIII and PstI (homology measured from the center of each restriction site to the divergence between the pBS and pBC sequences). With the primers SK and KS, the resultant PCR product contained 49 bp of homology to pBS (27 bp on one end, 22 bp on the other). Two other PCR products were also amplified, utilizing different combinations of

the above primers. The four PCR products were mixed with the pBS vector, electroporated into JC8679, and the resultant colonies screened for the presence of recombinant plasmids. To determine the fraction of colonies which contained the appropriate inserts, colony lifts from the bacterial plates were hybridized with a radioactively-labelled oligonucleotide (APC #22) homologous to an 18 bp segment of the APC cDNA sequence.

The results shown in Table 1 indicate that the IVC procedure succeeded, as all co-transformations led to the production of recombinant plasmids. Further, both the absolute and relative numbers of hybridizing colonies rose as the homology between the vector and PCR products increased. Colonies present on the vector-alone plate were probably due to the presence of residual

Table 1. Effect of length of homology on IVC

pBS Vector ^a (50 ng)	Residues shared between vector and PCR product ^b (bp)	Total colonies	Fraction Recombinant
–	88	0	0 %
+	88	2412	74 %
–	77	0	0 %
+	77	534	47 %
–	60	0	0 %
+	60	120	20 %
–	49	0	0 %
+	49	342	4 %
+	–	858	0 %

^agel purified by Spin-X method

^bPCR products purified by phenol/chloroform extraction and ethanol precipitation; 40 ng of PCR product used

Table 2. Effect of varying vector:insert ratios on cloning of inserts with 49 bp of homology

pBS Vector ^a (ng)	PCR Product ^b (ng)	Total colonies	Fraction Recombinant
40	100	162	11 %
40	2000	90	26 %
10	100	168	36 %
120	2000	294	49 %
40	0	678	0 %
0	100	0	0 %

^agel purified by Spin-X method

^bphenol/chloroform extracted and ethanol precipitated; PCR product and vector shared 49 bp homology

Table 3. Effect of PCR product purification method on IVC

pBS Vector ^a (50 ng)	Method of PCR product purification ^b	Total colonies	Fraction Recombinant
+	Ethanol ppt	5040	>90 %
+	Isopropanol ppt	2790	>90 %
+	Prep-A-Gene	366	61 %
+	Spin-X	732	58 %
+	No purification ^c	300	78 %
+	No PCR product	528	0 %
–	Ethanol ppt	0	0 %

^agel purified by Spin-X method

^bas described in Materials and Methods; 75 ng of PCR product used

^cone microliter of product directly out of the PCR reaction was mixed with one microliter of vector and used for electroporation

Table 4A. Effect of vector preparation method on IVC

PCR product (75 ng) ^a	Gel purification of pBS vector ^b	Phosphatase treatment of pBS vector ^c	Total colonies	Fraction Recombinant
—	—	—	4200	0 %
—	+	—	2616	0 %
—	—	+	1560	0 %
—	+	+	1272	0 %
+	—	—	33600	96 %
+	+	—	24300	94 %
+	—	+	21300	100 %
+	+	+	2544	77 %

^aphenol/chloroform extracted and ethanol precipitated; shared 88 bp of homology with vector^bgel purified by Spin-X method; 35 ng of vector used^cas described in Materials and Methods**Table 4B.** Effect of vector amount on cloning inserts with 150 bp of homology

pBS Vector ^a (ng)	PCR product ^a (50 ng)	Total colonies	Fraction Recombinant
35	—	990	0 %
35	+	21000	86 %
18	+	17400	95 %
8.8	+	5160	85 %
4.4	+	2250	92 %
0	+	0	0 %

^aphenol/chloroform extracted and ethanol precipitated; vector and PCR product shared 150 bp of homology**Table 5.** Cloning genomic PCR products by IVC

pBS Vector (35 ng)	Genomic APC PCR Product ^a (ng)	Total colonies	Fraction Recombinant
+	0	990	0 %
+	75	7500	84 %
+	38	6900	87 %
+	19	3120	65 %
+	9.4	1080	53 %
+	4.7	270	0 %
—	75	0	0 %

^aphenol/chloroform extracted and isopropanol precipitated; PCR product obtained with 350 ng of each universal primer and 35 ng of each APC-specific primer

uncut pBS vector, or cut pBS which had been intramolecularly repaired by the bacteria. As expected, no colonies were observed on the PCR product-alone plates.

To determine whether the hybridization-positive recombinant plasmids contained inserts of the correct size, plasmid DNA from 10 such colonies was digested with EcoRI to excise the inserts. All of the vector and insert fragments migrated with the predicted electrophoretic mobilities. The sites of recombination were then analyzed by sequencing 5 of these plasmids, and all were shown to contain the expected sequence, with recombination occurring exactly at the points of identity between PCR products and vector. Sequence data obtained from plasmids propagated in strains JC8679 and XL-1 Blue (Stratagene) were equivalent in quality (data not shown).

To evaluate whether the efficiency of IVC could be improved when only small stretches of homology were used, we altered the ratios and absolute amounts of vector and PCR product in

the transformations (Table 2). While the fraction of colonies containing recombinants could be significantly boosted by such alterations, the total number of such colonies remained low despite the use of large amounts of PCR product.

We next sought to determine whether the efficiency of IVC would be affected by the method of PCR product purification. Aliquots of the PCR product were purified by four different methods (as described in Materials and Methods), and equivalent amounts of each were co-electroporated with the vector. The results in Table 3 indicate that the two simplest methods (precipitation with ethanol or isopropanol) were the most efficient. Cloning was possible (at lower efficiency) even without any purification of the PCR product (Table 3).

Since the method of PCR product purification appeared to affect the cloning efficiency, we wondered whether different procedures for preparing the vector might have similar effects. Specifically, we wished to determine the effects of gel purification and

Figure 2. Strategy for amplification of genomic sequences. Two sets of overlapping primers were used in a single PCR reaction to amplify an APC genomic sequence. Primer sequences shown on a diagonal with respect to the APC template were homologous to the pBS MCS. APC1 and UNIV1 shared 12 bp of sequence; APC2 and UNIV2 shared 15 bp of sequence (see Materials and Methods).

phosphatase treatment on the pBS vector. As might be predicted, the results shown in Table 4A suggest that both treatments decreased the background level of colony formation in the absence of PCR product. However, in the presence of PCR product, gel purification and phosphatase treatment of the vector had no beneficial effect on cloning efficiency. Optimal cloning efficiencies were found with 18 to 35 ng of vector, prepared simply by phenol/chloroform extraction and ethanol precipitation (Table 4B).

This data indicated that some of the factors affecting IVC efficiency were, 1) extent of homology between vector and PCR product, 2) the relative amounts of each, and 3) the methods used to prepare them. In order to maximize cloning efficiency, the homology between vector and insert was further increased. An 899 bp APC PCR product was synthesized which shared 150 bp of sequence homology with the vector (83 and 67 bp on the two ends). The increase in total homology resulted in a 20-fold elevation in cloning efficiency compared with an insert containing 88 bp of homology (data not shown). In a similar experiment, a 2132 bp PCR fragment of the human MDM2 gene (29), amplified from a plasmid and containing the same 150 bp of homology, cloned with about one-third the efficiency of the 899 bp APC product (data not shown).

We next attempted to amplify and clone a PCR product derived from a genomic DNA template. Two sets of overlapping primers were synthesized, as shown in Figure 2. Primers APC1 and APC2 contained at their 3' ends, 23 and 20 bp, respectively, of genomic sequences flanking APC exon 9. The 5' ends of the primers contained 12 and 15 bp, respectively, identical to sequences in the pBS multiple cloning site (MCS). The universal set of primers, UNIV1 and UNIV2, contained the MCS homology sequences, extending 83 and 67 bp, respectively, to the T3 and T7 sites. When the four primers were used in a single PCR reaction, a 608 bp product was synthesized which contained 150 bp of homology to the vector. The highest amounts of PCR products were observed when the universal primers were used at 350 ng per reaction and the APC-specific primers were used at 35 ng per reaction. Use of 175 ng of the APC primers resulted in less full-length PCR product and about one-eighth as many colonies (only 15 % of which harbored recombinant plasmids). When only 3.5 ng of the APC primers were used, no PCR product was observed (data not shown). As shown in Table 5, use of as little as 9.4 ng of the best PCR product resulted in

Figure 3. Optimized protocol for cloning genomic PCR products.

efficient cloning. Primer dimers did not interfere with cloning, as they were eliminated in the isopropanol precipitation step. This strategy has also been used successfully to amplify and clone a genomic DNA fragment containing exons 5 to 9 of the p53 gene (data not shown). A schematic of one method for cloning genomic PCR products is shown in Figure 3.

DISCUSSION

We have described a strategy for cloning PCR products in *E. coli* which involves minimal effort. Because this strategy requires the presence of sequence homology between vector and PCR product, one of the two DNA substrates must be appropriately engineered to match the other. As a universal approach to IVC, we have designed two sets of overlapping primers which match a specific vector (Figure 2). The inner primers can be devised to amplify any desired DNA sequence, while the outer 'universal' primers simply extend the PCR product/vector homology without regard to the target sequence amplified by the inner primers. To make an inner set of primers compatible with any given set of universal primers requires the addition of only 12 bp of 5' sequence, an amount of sequence only slightly greater than that required to append a restriction site. By mixing the inner and outer sets of primers at appropriate molar ratios, the final PCR product can be amplified in a single reaction. In some situations, however, it may be desirable to use a two-stage PCR protocol, employing the specific primers in the first stage and the universal primers in the second stage.

The success of IVC in *E. coli* appears, at least in part, to depend on the strain used. Kobayashi and Takahashi (19) have shown that strain JC8679, but not DH5 (30), was capable of performing intramolecular gap repair. This is probably the result of three mutations present in JC8679: *sbcA23* activates the RecE pathway of homologous recombination, while *recB21* and *recC22* inactivate the RecBCD enzyme, a double-stranded exonuclease. While this manuscript was under review, Bubeck *et al.* (31) published a technique similar to IVC. They reported successful cloning by homologous recombination using a number of common *E. coli* strains, including DH5 α . However, they were unable to clone when electroporation was used for transformation; instead, they relied on CaCl₂ transformation. We have replicated their experiments using DH5 α and confirmed the unsuitability of this strain for IVC following electroporation (data not shown). Following CaCl₂ transformation, we too were able to demonstrate successful cloning in DH5 α , but at an efficiency 25-fold lower than that obtained using electroporated JC8679

cells. Thus, JC8679 cells seem to be preferable for this type of PCR cloning.

There is no theoretical reason that IVC could not be used to clone DNA derived from sources other than PCR. For example, insert DNA could easily be shuttled from one vector to another without the requirement of convenient, compatible or unique restriction sites, provided that the plasmids share appropriate sequences. Additionally, two or more overlapping DNA fragments could be co-cloned into the same vector, thus facilitating, for example, the production of full-length cDNA expression constructs from multiple partial-length clones. It is likely that additional applications of this method will emerge.

ACKNOWLEDGEMENTS

We thank A.J. Clark for providing strain JC8679 and K.J. Smith for providing the APC cDNA-containing plasmid. This work was supported by the Clayton Fund and NIH grants CA35494 and CA41183. B.V. is an ACS Research Professor.

REFERENCES

- Clark, J.M. (1988) *Nucleic Acids Research* **16**, 9677–9686.
- Williams, J.F. (1989) *Amplifications* **3**, 19.
- Hemsley, A., Arnheim, N., Toney, M.D., Cortopassi, G., and Galas, D.J. (1989) *Nucl. Acids Res.* **17**, 6545–6551.
- Scharf, S.J. (1990) In Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J. (Eds.), *PCR protocols*. Academic Press, New York, pp. 84–91.
- Liu, Z.-G. and Schwartz, L.M. (1992) *BioTechniques* **12**, 28–30.
- Holton, T.A. and Graham, M.W. (1991) *Nucl. Acids Res.* **19**, 1156.
- Marchuk, D., Drumm, M., Saulino, A., and Collins, F.S. (1991) *Nucl. Acids Res.* **19**, 1154.
- Kovalic, D., Kwak, J.-H., and Weisblum, B. (1991) *Nucl. Acids Res.* **19**, 4560.
- Scharf, S.J., Horn, G.T., and Erlich, H.A. (1986) *Science* **233**, 1076–1078.
- Crouse, J. and Amores, D. (1986) *Focus* **8**, 9.
- Kaufman, D.L. and Evans, G.A. (1990) *BioTechniques* **9**, 304–306.
- Jung, V., Pestka, S.B., and Pestka, S. (1990) *Nucleic Acids Res.* **18**, 6156.
- Aslanidis, C. and deJong, P.J. (1990) *Nucleic Acids Research* **18**, 6069–6074.
- Haun, R.S., Serventi, I.M., and Moss, J. (1992) *BioTechniques* **13**, 515–518.
- Nisson, P.E., Rashtchian, A., and Watkins, P.C. (1991) *PCR Methods and Applications* **1**, 120–123.
- Rashtchian, A., Buchman, G.W., Schuster, D.M., and Berninger, M.S. (1992) *Analytical Biochemistry* **206**, 91–97.
- Guthrie, C. and Fink, G. (1991) *Methods in Enzymology* **194**.
- Scharer, E. and Iggo, R. (1992) *Nucleic Acids Research* **20**, 1539–1545.
- Kobayashi, I. and Takahashi, N. (1988) *Genetics* **119**, 751–757.
- Vogelstein, B. (1987) *Anal. Biochem.* **160**, 115–118.
- Kinzler, K.W. and Vogelstein, B. (1989) *Nucleic Acids Research* **17**, 3645–3653.
- Vogelstein, B. and Gillespie, D. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 615–619.
- Gillen, J.R., Willis, D.K., and Clark, A.J. (1974) In: *Mechanisms in Genetic Recombination* (R.F. Grell, ed.). Plenum, New York, pp. 123–135.
- Sidransky, D., von Eschenbach, A., Tsai, Y.C., Jones, P., Summerhayes, I., Marshall, F., Paul, M., Green, P., Hamilton, S.R., Frost, P., and Vogelstein, B. (1991) *Science* **252**, 706–709.
- Kinzler, K.W., Nilbert, M.C., Su, L.-K., Vogelstein, B., Bryan, T.M., Levy, D.B., Smith, K.J., Preisinger, A.C., Hedge, P., McKechnie, D., Finnear, R., Markham, A., Groffen, J., Boguski, M.S., Altschul, S.F., Horii, A., Ando, H., Miyoshi, Y., Miki, Y., Nishisho, I., and Nakamura, Y. (1991) *Science* **253**, 661–665.
- Joslyn, G., Carlson, M., Thliveris, A., Albertsen, H., Gelbert, L., Samowitz, W., Groden, J., Stevens, J., Spirio, L., Robertson, M., Sargeant, L., Krapcho, K., Wolff, E., Burt, R., Hughes, J.P., Warrington, J., McPherson, J., Wasmuth, J., LePaslier, D., Abderrahim, H., Cohen, D., Leppert, M., and White, R. (1991) *Cell* **66**, 601–613.
- Nishisho, I., Nakamura, Y., Miyoshi, Y., Miki, Y., Ando, H., Horii, A., Koyama, K., Utsunomiya, J., Baba, S., Hedge, P., Markham, A. Krush,

- A.J., Petersen, G., Hamilton, S.R., Nilbert, M.C., Levy, D.B., Bryan, T.M., Preisinger, A.C., Smith, K.J., Su, L.-K., Kinzler, K.W., and Vogelstein, B. (1991) *Science* **253**, 665–669.
- Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., Robertson, M., Sargeant, L., Krapcho, K., Wolff, E., Burt, R., Hughes, J.P., Warrington, J., McPherson, J., Wasmuth, J., LePaslier, D., Abderrahim, H., Cohen, D., Leppert, M., and White, R. (1991) *Cell* **66**, 589–600.
- Oliner, J.D., Kinzler, K.W., Meltzer, P.S., George, D.L., and Vogelstein, B. (1992) *Nature* **358**, 80–83.
- Hanahan, D. (1985) In: *DNA Cloning*, Vol. I, Glover, D.M. (ed.), IRL Press, Oxford.
- Bubeck, P., Winkler, M., and Bautsch, W. (1993) *Nucleic Acids Research* **21**, 3601–3602.