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Detection of heterozygous truncating mutations in the *BRCA1* and *APC* genes by using a rapid screening assay in yeast

CHIKASHI ISHIOKA*†, TAKAO SUZUKI*, MICHAEL FITZGERALD‡, MICHAEL KRAINER‡, HIDEKI SHIMODAIRA*, AKIRA SHIMADA*, TADASHI NOMIZU§, KURT J. ISSELBACHER‡, DANIEL HABER‡, AND RYUNOSUKE KANAMARU*

*Department of Clinical Oncology, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryo-machi, Aoba-ku, Sendai 980, Japan; ‡Massachusetts General Hospital Cancer Center, Building 149, 13th Street, Charlestown, MA 02129; and §Department of Surgery, Hoshi General Hospital, 2-1-16 Ohmachi, Koriyama 963, Japan

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ABSTRACT **The detection of inactivating mutations in tumor suppressor genes is critical to their characterization, as well as to the development of diagnostic testing. Most approaches for mutational screening of germ-line specimens are complicated by the fact that mutations are heterozygous and that missense mutations are difficult to interpret in the absence of information about protein function. We describe a novel method using *Saccharomyces cerevisiae* for detecting protein-truncating mutations in any gene of interest. The PCR-amplified coding sequence is inserted by homologous recombination into a yeast URA3 fusion protein, and transformants are assayed for growth in the absence of uracil. The high efficiency of homologous recombination in yeast ensures that both alleles are represented among transformants and achieves separation of alleles, which facilitates subsequent nucleotide sequencing of the mutated transcript. The specificity of translational initiation of the *URA3* gene leads to minimal enzymatic activity in transformants harboring an inserted stop codon, and hence to reliable distinction between specimens with wild-type alleles and those with a heterozygous truncating mutation. This yeast-based stop codon assay accurately detects heterozygous truncating mutations in the *BRCA1* gene in patients with early onset of breast cancer and in the *APC* gene in patients with familial adenomatous polyposis. This approach offers a rapid and reliable method for genetic diagnosis in individuals at high risk for germ-line mutations in cancer susceptibility genes.**

The identification of novel germ-line mutations in tumor suppressor genes presents a major difficulty in their initial characterization, as well as in the adaptation of reliable and effective approaches to clinical diagnostics. The large size of many of these genes and the fact that germ-line mutations are heterozygous have complicated analysis based exclusively on nucleotide sequencing. We have previously described a functional assay to detect germ-line mutations in *p53*, a gene that is affected primarily by missense mutations and whose function as a transcriptional activator can be tested in yeast (1). While the functional properties of other cancer susceptibility genes are not well understood, many of these are disrupted primarily by truncating mutations. Virtually all mutations in the colon cancer gene *APC* (2–5) and 80% of mutations in the breast cancer predisposition gene *BRCA1* (6) consist of nonsense or frameshift mutations, leading to the development of screening assays based on the *in vitro* production of truncated peptides, so-called PTT assays (7–11). These methods are reliable and effective, but require significant levels of technical expertise and interpretation of results. Here we describe a simple yeast-based method that makes use of homologous recombination in *Saccharomyces cerevisiae* to separate alleles and involves the production of a marker fusion protein to test for truncating mutations.

MATERIALS AND METHODS

Plasmid Construction. The plasmid pCI-HA(URA3)-2 was constructed as follows: a fragment spanning nucleotides 423–1239 of the plasmid pRS316 (12) (GenBank U03442), which contains *URA3* coding sequence from codon 5 to the natural termination codon, was amplified by PCR using a set of primers containing a *Bam*HI site or a *Bgl*II site at the 5′ end. The *Bam*HI/*Bgl*II fragment was inserted in-frame into the *Bam*HI site of the plasmid pRS-PGK (13) to produce pCI-HA(URA3). This vector was digested with *Nsi*I and *Pst*I and religated to produce pCI-HA(URA3)-2 (see Fig. 1*a*). Three fragments spanning nucleotides 96–908 (BRCA1a), nucleotides 789–4214 (BRCA1b), and nucleotides 4089–5708 (BRCA1c) of the *BRCA1* cDNA (GenBank U14680), were amplified and inserted in-frame into the *Bam*HI site of the pCI-HA(URA3)-2 to produce pCI-BR1a, pCI-BR1b, and pCI-BR1c, respectively (see Fig. 1*b*). Four fragments spanning nucleotides 19–1977 (APCa), nucleotides 1978–5256 (APCb), nucleotides 1978–3570 (APCc), and nucleotides 3571–5256 (APCd) of the *APC* cDNA (GenBank M74088) were amplified and inserted in-frame into the *Bam*HI site of pCI-HA(URA3)-2 to produce pCI-APCa, pCI-APCb, pCI-APCc, and pCI-APCd, respectively (see Fig. 1*b*). All the vectors described above result in a uracil-independent (Ura^+) phenotype, following introduction into YPH499 strain. The gap vectors, pCI-BR1ag, pCI-BR1bg, pCI-BR1cg, pCI-APCag, pCI-APCbg, and pCI-APCcg (see Fig. 1*b*) are identical to pCI-BR1a, pCI-BR1b, pCI-BR1c, pCI-APCa, pCI-APCb, and pCI-APCc, respectively, except that the central portions of the inserted fragments, BRCA1a, BRCA1b, BRCA1c, APCa, APCb, and APCc, between nucleotides 183–827, nucleotides 888–4111, nucleotides 4215–5609 (GenBank U14680), nucleotides 109–1899, nucleotides 2054–5201, and nucleotides 2086–3489 (GenBank M74088), were replaced by the unique restriction sites *Bgl*II, *Stu*I/*Bam*HI/*Sma*I, *Bgl*II, *Bgl*II, *Nsi*I, and *Bgl*II, respectively. All the gap vectors except pCI-APCbg were produced by PCR using ExTaq (Takara Shuzo, Kyoto) and the original plasmids with full-length insertion as templates, followed by ligation using the unique restriction sites described above and transformation of *Escherichia coli* (DH5α). The pCI-APCbg was obtained by removing the central portion of APCb fragment of the pCI-APCb using two *Nsi*I sites.

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Abbreviations: HA, hemagglutinin; FAP, familial adenomatous polyposis; SC assay, stop codon assay.

†To whom reprint requests should be addressed. e-mail: chikashi@idac.tohoku.ac.jp.

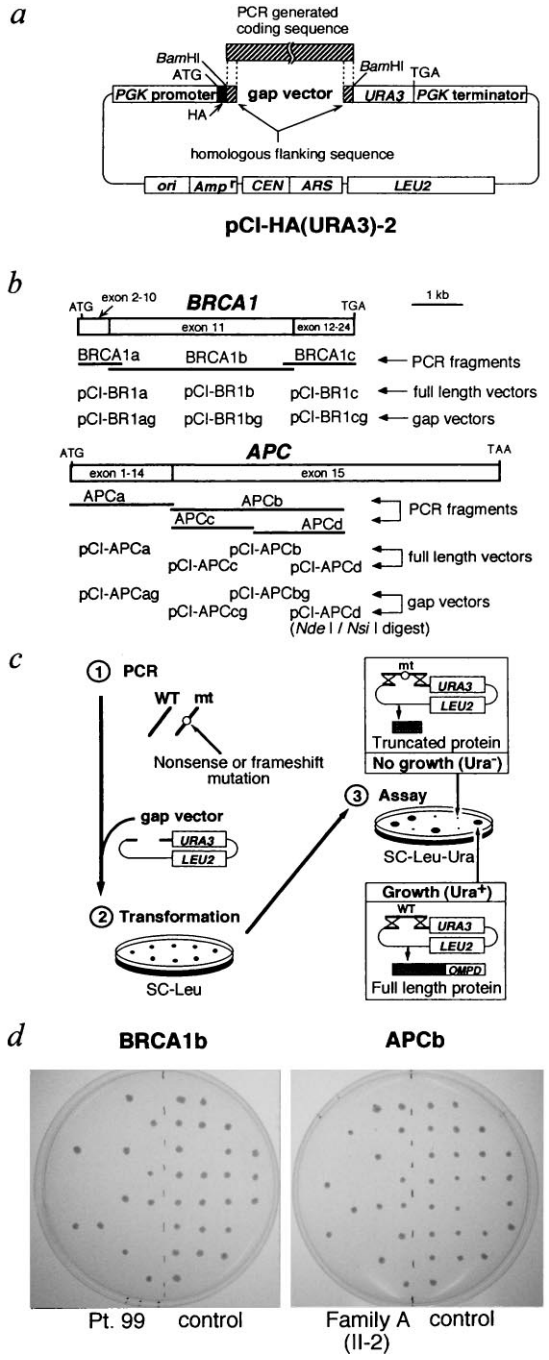

FIG. 1. Schematic representation of SC assay. (*a*) pCI-HA(URA3)-2 vector. In-frame insertion of a coding sequence of interest into the unique *Bam*HI site results in constitutive expression of a hemagglutinin (HA)-tagged URA3 fusion protein, driven from the *PGK* (3-phosphoglycerate kinase) promoter. In addition, the vector contains the *PGK* terminator downstream of the *URA3* fragment, the *LEU2* gene as a second selectable marker, and *CEN* and *ARS* for stable low-copy-number replication. The derived gap vector lacks the central portion of the inserted fragment, which can be replaced by a PCR-generated fragment inserted by homologous recombination, using the remaining flanking sequences. (*b*) Sequences of *BRCA1* and *APC* chosen for analysis. cDNA or genomic fragments denoted BRCA1a-c and APCa-d were inserted in-frame into the *Bam*HI site of pCI-HA(URA3)-2, producing plasmids pCI-BR1a, -b, and -c and pCI-APCa, -b, -c, and -d. Plasmids pCI-BR1ag, -bg, and -cg and pCI-APCag, -bg, and -cg are gap vectors. (*c*) Schematic representation of SC assay. Step 1, PCR amplification of cDNA or genomic fragment containing wild-type (WT) or truncated mutant (mt; nonsense or frameshift) is combined with the appropriate gap vector, which contains 100 bp flanking the PCR fragment to allow for efficient homologous recombination. Step 2, transformation of *leu2*- and *ura3*-deficient yeast with the PCR product and gap vector yields leucine prototrophic transformants that have undergone recircularization of the plasmid following homologous recombination. Step 3, selection of Leu^+ prototroph following replating in the absence of uracil distinguishes Ura^+ prototrophs (wild-type inserted sequence) from Ura^- auxotrophs (truncating mutation). OMPD, orotidine-5′-phosphate decarboxylase. (*d*) Representative SC assay for *BRCA1* (fragment BRCA1b) and *APC* (fragment APCb), showing growth of yeast transformants in the absence of uracil. In both cases, the left half-plate represents a specimen derived from a patient with a heterozygous truncating mutation, and the right half-plate is a control sample.

PCR. For *BRCA1* and *APC* analysis, genomic DNA and/or total cellular RNA was isolated from Epstein–Barr virus (EBV)-immortalized cell lines or peripheral blood mononuclear cells. cDNA was synthesized by using a First-Strand cDNA synthesis kit (Pharmacia). BRCA1a–c and APCa (see Fig. 1*b*) were amplified from cDNA. BRCAb and APCb–d were amplified from genomic DNA. Primers for amplification of *BRCA1* fragments were
5′-GAAAGTTCATTGGAACAGAAAGAA-3′ and
5′-ACCCTGATACTTTTCTGGATG-3′ for BRCA1a,
5′-CCCAGATCTGCTGCTTGTGAATTTTCTGAG-3′ and
5′-CCCAGATCTTAAGTTTGAATCCATGCTTTG-3′ for BRCA1b, and
5′-ATGAGGCATCAGTCTGAAAGC-3′ and
5′-GTAGTGGCTGTGGGGGATCT-3′ for BRCA1c.
Primers for amplification of *APC* fragments were
5′-ATGGCTGCAGCTTCATATGAT-3′ and
5′-CTGTGGTCCTCATTTGTAGC-3′ for APC1a,
5′-CAAATCCTAAGAGAGAACAAC-3′ and
5′-GTCCATTATCTTTTTCACACG-3′ for APCb,
5′-CAAATCCTAAGAGAGAACAA-3′ and
5′-GGCATATTTTAAACTATAATC-3′ for APCc, and
5′-ACAGATATTCCTTCATCACAG-3′ and
5′- GTCCATTATCTTTTTCACACG-3′ for APCd.
All PCR fragments were obtained by using ExTaq DNA polymerase (Takara Shuzo). The PCR parameters are available from the authors upon request.

Yeast Transformation. The yeast strain used in this study was YPH499 (*MAT***a**, *ura3–52, lys2–801*^{amber}, *ade2–101*^{ocher}, *trp1*Δ*63, his3*Δ*200, leu2*Δ*1*) (Stratagene). Competent yeast cells were prepared by lithium acetate (LiOAc) treatment of the strain cultured in YPD liquid medium (1) and were stored at −80°C in the presence of 5% (vol/vol) dimethyl sulfoxide (DMSO) until use. Frozen competent yeast retain high transformation efficiency for at least 3 months. Gap repair assays were performed by cotransformation of unpurified PCR product (≈200 ng) and linearized gap vector (≈30 ng) by the LiOAc method (14) with minor modifications (1). To analyze the PCR fragments BRCA1a, BRCA1b, BRCA1c, APCa, APCb, APCc, and APCd, linearized gap vectors pCI-BR1ag (*Bgl*II digest), pCI-BR1bg (*Bam*HI/*Sma*I digest), pCI-BR1cg (*Bgl*II digest), pCI-APCag (*Bgl*II digest), pCI-APCbg (*Nsi*I digest), pCI-APCcg (*Bgl*II digest), and pCI-APCg (*Nde*I/*Nsi*I digest) were used, respectively. Transformants were selected on synthetic complete medium lacking leucine (SC-Leu); 25 transformants were then assayed for the Ura^+ phenotype by growth on synthetic complete medium lacking leucine and uracil (SC-Leu-Ura). If more than 85% of transformants are Ura^+, the sample is scored as homozygous wild-type, whereas if all transformants are Ura^-, the sample is scored as homozygous mutant. If 40–50% of colonies are Ura^+, the sample is scored as heterozygous for a truncation mutant.

Sequencing. Yeast DNA extraction from pooled Ura^- transformants was described previously (15). Template *APC* fragments were amplified as described above and were sequenced using a CircumVent Thermal Cycle Dideoxy DNA Sequencing

Kit (New England Biolabs). Appropriate *APC*-specific oligonucleotides were used as sequencing primers after end-labeling by [γ-^{33}P]dATP.

RESULTS AND DISCUSSION

Stop codon (SC) Assay. The SC assay benefits from two advantages of yeast systems. First, the ability to synthesize fusion proteins with orotidine-5′-phosphate decarboxylase (OMPD) encoded by the *URA3* gene and, second, the use of gap repair and homologous recombination to efficiently insert a PCR-generated sequence into this fusion construct and separate the products of different alleles. The outline of the assay is summarized in Fig. 1*c*. We constructed a centromeric yeast expression vector, pCI-HA(URA3)-2, with two selectable markers: *LEU2* and *URA3* (codons 5–267), which complement the genetic defects of the YPH499 strain, allowing growth in the absence of leucine and uracil. The *URA3* gene is driven by the strong *PGK* promoter, tagged at the N terminus by hemagglutinin (HA) and interrupted by a *Bam*HI site to allow introduction of exogenous DNA fragments. To test whether fusion of proteins to the N terminus of URA3 preserves the Ura$^+$ phenotype, 15 different coding sequences of 0.8–3.4 kb in size, derived from 7 different genes, were inserted in-frame. All insertions demonstrated the Ura$^+$ phenotype, confirming that this selectable marker is not disrupted by N-terminal fusion to a variety of protein domains (data not shown). The Ura$^+$ phenotype was dependent on use of the correct upstream promoter and translational initiation codon, as demonstrated by its loss following placement of the *URA3* fragment in the reverse orientation, by insertion of 4 bp into the *Bam*HI site, or by insertion of exogenous DNA fragments containing an out-of-frame insertion or in the reverse orientation. Synthesis of the expected full-length URA3 fusion protein from Ura$^+$ colonies was confirmed by immunoblotting analysis using anti-HA antibody. As predicted, insertion of out-of-frame sequences into pCI-HA(URA3)-2 led to the expression of HA-positive truncated fusion proteins in Ura$^-$ colonies (data not shown).

Screening for *BRCA1* Mutations. To test the SC assay in detecting truncating mutations in *BRCA1*, the coding sequence was divided into three overlapping fragments, which were amplified by reverse transcription (RT)-PCR and inserted in-frame into the *Bam*HI site of the pCI-HA(URA3)-2 vector. The resulting constructs, pCI-BR1a, -b, and -c, showed preservation of the Ura$^+$ phenotype following introduction into yeast. The plasmids were converted into the corresponding "gap vectors," pCI-BR1ag, pCI-BR1bg, and pCI-BR1cg, by removing most of the *BRCA1* insert, leaving 100 bp of flanking *BRCA1* sequence to allow for homologous recombination (see Fig. 1*b* and *Materials and Methods*). The three corresponding *BRCA1* fragments were then amplified by RT-PCR from peripheral blood mononuclear cells of patients with known *BRCA1* truncating mutations and controls. Cotransformation of unpurified PCR products and corresponding linearized gap vectors into yeast allowed homologous recombination and growth of leucine auxotrophs containing the recircularized plasmid. Twenty-five independent transformants were assayed for growth in the absence of uracil. Following gap repair with PCR products derived from control lymphocytes, 88–100% of transformants were Ura$^+$, demonstrating efficient homologous recombination of the *BRCA1* fragment and reconstitution of the URA3 fusion protein (Table 1). The small fraction of Ura$^-$ transformants is presumably due to infidelity of the ExTaq DNA polymerase and to recombination error, as described previously (1). In contrast, PCR products derived from the lymphocytes of patients with known heterozygous *BRCA1* mutations (11) led to 44–64% Ura$^+$ transformants. These included specimens from two patients (nos. 231 and 253) with a heterozygous 2-bp deletion at codon 23 (the so-called 185delAG mutation) (gap vector pCI-BR1ag), one patient (no. 99) with a 2-bp deletion at codon 327 (gap vector pCI-BR1bg), one patient (no. 364) with a nonsense mutation at codon 563 (gap vector pCI-BR1bg), and one patient (no. 250) with a 1-bp insertion at codon 1756 (the so-called 5382insC mutation) (gap vector pCI-BR1cg). Another 6 samples that only contained *BRCA1* polymorphisms (11) scored as wild type (Table 1). The distribution of Ura$^+$ and Ura$^-$ colonies derived from specimens with or without *BRCA1* truncating mutations was reproducible and nonoverlapping (Fig. 2), demonstrating the reliability of this assay for diagnostic purposes.

Screening for *APC* Mutations. To test the ability of the SC assay to detect unknown mutations, we chose the familial adenomatous polyposis (FAP) gene *APC*, which is inactivated exclusively ($\approx$93%) by truncating mutations located within the N-terminal 60% of the coding sequence (16). This portion of the *APC* cDNA was divided into two fragments, APCa and APCb, which contains the so-called mutation cluster region (MCR) (17), was further divided into two overlapping fragments, APCc and APCd. As expected, in-frame insertion of these fragments into the *Bam*HI site of pCI-HA(URA3)-2 preserved the Ura$^+$ phenotype. We then analyzed 24 individuals derived from six unrelated families with FAP (Table 2). Analysis of the mutation cluster region for 6 patients (individuals I-1 from families B, D, E, and F; individual I-7 from family C; individual II-2 from family A) who were clinically

Table 1. SC essay for *BRCA1* in women with early-onset breast cancer

		SC assay, % Ura$^+$ colonies					
	BRCA1 fragment:	1a	1b		1c	Mutation	
	Codons:	1–263	224–1365		1324–1863		
Patient	PCR template:	cDNA	cDNA	gDNA	cDNA	Sequence	Location
43		92	ND	88	92	WT	
79		88	92	ND	92	WT	
84		92	91	92	96	WT	
99		96	**44**	**48**	88	2-bp deletion (frameshift)	Codon 327 (exon 11)
103		88	ND	88	96	WT	
118		88	94	94	88	WT	
231		**44**	ND	88	92	2-bp deletion (frameshift)	Codon 23 (exon 2)
250		96	ND	88	**64**	C insertion (frameshift)	Codon 1756 (exon 20)
253		**48**	ND	88	100	2-bp deletion (frameshift)	Codon 23 (exon 2)
364		96	ND	**44**	92	CGA to TGA (nonsense)	Codon 563 (exon 11)

All patients are women with breast cancer before the age 30 and have been characterized for *BRCA1* mutations previously (11). Location of *BRCA1* fragment was indicated in Fig. 1*b*. A boldface number indicates existence of truncating mutation in examined DNA fragment by SC assay. gDNA, genomic DNA; ND, not determined; WT, wild-type in the entire coding sequence. The mutations detected in patients 231 and 253 are known as 185delAG. The mutation detected in patient 250 is known as 5382insC. These mutations are prevalent in the Ashkenazi Jewish population.

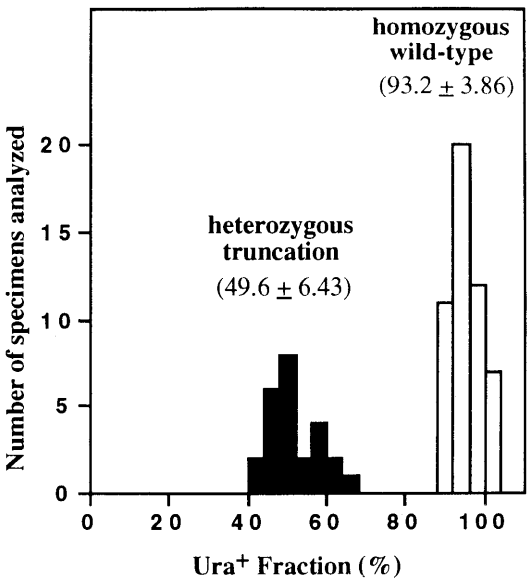

FIG. 2. Distribution of Ura$^+$ colonies derived from specimens with wild-type *BRCA1* and *APC* or containing a heterozygous truncating mutation. Results of the SC assay are shown for 75 specimens for which presence (filled bar) or absence (open bar) of a truncating mutation was confirmed by nucleotide sequencing. Values in parenthesis indicate mean ± SD.

diagnosed as affected, using gap vector pCI-APCbg, yielded yeast transformants, half of which retained the Ura$^+$ phenotype (mean 49%, range 38–60%), consistent with the presence of a heterozygous truncating mutation (Table 2). To identify the precise location of each mutation, gap repair assays were performed using the internal gap vectors pCI-APCcg and pCI-APCd (*Nde*I/*Nsi*I digest): individual II-2 from family A and individual I-1 from family B scored positive for mutations within fragment APCc, but not APCd, whereas the converse was true for individual I-7 from family C and individuals I-1 from families D-F. This analysis was extended to the remaining 18 members of these families, identifying 5 individuals as having heterozygous truncating mutations within the same fragment as the proband (Table 2). Six independent Ura$^-$ colonies were pooled and subjected to direct nucleotide sequencing. The separation of *APC* alleles resulting from the gap repair assay made it possible to specifically analyze the mutant allele, avoiding the difficulties inherent in sequencing heterozygous mutations. All specimens scored as positive by SC assay were found to have truncating *APC* mutations: a 4-bp deletion at codon 929 (3765del4) in family A, a 1-bp insertion at codon 938 (2831insT) in family B, a 2-bp deletion at codon 1249 (3765del2) in family C, a 5-bp deletion at codon 1309 (3945del5) in both families D and family E, and a 1-bp deletion at codon 1322 (3983delA) in family F (Table 2). Analysis of other members from each family demonstrated a complete concordance between the results of the SC assay and direct sequencing analysis (Table 2).

CONCLUDING REMARKS

The mutational analysis that we describe here is comparable in its efficacy to previously described protein truncation (PTT) techniques that involve PCR amplification of gene fragments, followed by *in vitro* transcription-translation and resolution of encoded peptides by SDS/PAGE. However, the yeast-based SC assay provides a number of important technical advantages compared with these *in vitro* gel-based assays. Among these are (*i*) the ability to analyze larger (≈3.5-kb) DNA fragments, which reduces the number of PCRs required to scan an entire gene for truncating mutations; (*ii*) the ability to detect mutations that arise adjacent to the PCR primers, which minimizes

Table 2. SC assay for *APC* mutations in six FAP families

			SC assay, % Ura$^+$ colonies				
		APC fragment:	b	c	d	Mutation	
		Codons:	654–1748	654–1184	1185–1748		
Family	Individual	PCR template:	gDNA	gDNA	gDNA	Sequence	Location
A	II-1		100	92	ND	WT	
	II-2		**38**	**48**	92	4-bp deletion (frameshift)	Codon 929–930 (Exon 15)
	II-3		**56**	**48**	ND	4-bp deletion (frameshift)	Codon 929–930 (Exon 15)
B	I-1		**50**	**44**	100	T insertion (frameshift)	Codon 938 (Exon 15)
	II-1		96	92	ND	WT	
	II-2		88	100	ND	WT	
C	I-1		ND	ND	100	WT	
	I-2		ND	ND	100	WT	
	I-3		ND	ND	96	WT	
	I-7		**40**	92	**56**	2-bp deletion (frameshift)	Codon 1249–1250 (Exon 15)
	I-8		ND	ND	92	WT	
	II-1		ND	ND	**40**	2-bp deletion (frameshift)	Codon 1249–1250 (Exon 15)
	II-2		ND	ND	**48**	2-bp deletion (frameshift)	Codon 1249–1250 (Exon 15)
	II-3		ND	ND	**52**	2-bp deletion (frameshift)	Codon 1249–1250 (Exon 15)
D	I-1		**60**	92	**56**	5-bp deletion (frameshift)	Codon 1309–1311 (Exon 15)
	II-1		ND	ND	92	WT	
	II-2		ND	ND	96	WT	
	II-3		ND	ND	88	WT	
E	I-1		**44**	96	**48**	5-bp deletion (frameshift)	Codon 1309–1311 (Exon 15)
	II-1		ND	ND	92	WT	
	II-2		ND	ND	**48**	5-bp deletion (frameshift)	Codon 1309–1311 (Exon 15)
	II-3		ND	ND	92	WT	
F	I-1		**57**	92	**48**	A deletion (frameshift)	Codon 1322 (Exon 15)
	II-1		ND	ND	92	WT	

All individuals in the six FAP families are Japanese. Location of *APC* fragment is indicated in Fig. 1*b*. A boldface number indicates existence of truncating mutation in examined DNA fragment by SC assay. gDNA, genomic DNA; ND, not determined; WT, wild-type in the examined coding sequence.

the overlap required between fragments; (*iii*) the separation of alleles, which greatly simplifies confirmation of heterozygous mutations by nucleotide sequencing; and (*iv*) avoidance of the requirement for radioisotopes and for protein gel-electrophoresis. From a technical standpoint, the SC assay requires few manipulations, and the time required to perform the assay (4 days) reflects that required to clearly visualize yeast colonies after sequential plating on leucine- and uracil-deficient plates. The use of a mutational screening test based on the detection of truncating mutations also has important advantages in the adaptation of such approaches to clinical diagnostics. Protein truncating mutations constitute the majority of inactivating mutations for a number of important cancer predisposition genes, including *BRCA1*, *BRCA2*, *APC*, mismatch repair genes, and potentially *ATM*. Furthermore, the difficulty in interpreting missense mutations precludes their use in most clinical diagnostics. The SC assay thus provides a rapid and reliable test that can be readily adapted to detect heterozygous truncating mutations in cancer predisposition genes and other genes implicated in human disease.

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1. Ishioka, C., Frebourg, T., Yan, Y.-X., Vidal, M., Friend, S. H., Schmidt, S. & Iggo, R. (1993) *Nat. Genet.* **5,** 124–129.
2. Kinzler, K. W., Nilbert, M. C., Su, L. K., Vogelstein, B., Bryan, T. M., *et al.* (1991) *Science* **253,** 661–665.
3. Nishisho, I., Nakamura, Y., Miyoshi, Y., Miki, Y., Ando, H., *et al.* (1991) *Science* **253,** 665–669.
4. Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., *et al.* (1991) *Cell* **66,** 589–600.
5. Joslyn, G., Carlson, M., Thliveris, A., Albertsen, H., Gelbert, L., *et al.* (1991) *Cell* **66,** 601–613.
6. Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P. A., Harshmann, K., *et al.* (1994) *Science* **266,** 66–71.
7. Roest, P. A. M., Roberts, R. G., Sugino, S., van Ommen, G. J. B. & Den Dunnen, J. T. (1993) *Hum. Mol. Genet.* **2,** 1719–1721.
8. Powell, S. M., Peterson, G., Krush, A. J., Booker, S., Jen, J., Giardiello, F. M., Hamilton, S. R., Vogelstein, B. & Kinzler, K. W. (1993) *N. Engl. J. Med.* **329,** 1982–1987.
9. van der Luijt, R., Meera Khan, P., Vasen, H., van Leeuwen, C., Tops, C., Roest, P., den Dunnen, J. & Fodde, R. (1994) *Genomics* **20,** 1–4.
10. Hogervorst, F. B., Cornelis, R. S., Bout, M., van Vliet, M., Oosterwijk, J. C., Olmer, R., Bakker, B., Klijn, J. G., Vasen, H. F., Meijers-Heijboer, H., Menko, F. H., Cornelisse, C. J., den Dunnen, J. T., Devilee, P. & van Ommen, G.-J. B. (1995) *Nat. Genet.* **10,** 208–212.
11. FitzGerald, M. G., MacDonald, D. J., Krainer, M., Hoover, I., O'Neil, E., Unsal, H., Silva, A. S., Finkelstein, D. M., Beer, R. P., Englert, C., Sgroi, D. C., Smith, B. L., Younger, J. W., Garber, J. E., Duda, R. B., Mayzel, K. A., Isselbacher, K. J., Friend, S. H. & Haber, D. A. (1996) *N. Engl. J. Med.* **334,** 143–149.
12. Sikorski, R. S. & Hieter, P. A. (1989) *Genetics* **122,** 19–27.
13. Ishioka, C., Ballester, R., Engelstein, M., Vidal, M., Kassel, J., The, I., Bernards, A., Gusella, J. F. & Friend, S. H. (1995) *Oncogene* **10,** 841–847.
14. Ito, H., Fukuda, K., Nurata, K. & Kimura, A. (1983) *J. Bacteriol.* **153,** 163–168.
15. Ishioka, C., Englert, C., Winge, P., Yan, Y.-X., Engelstein, M. & Friend, S. H. (1995) *Oncogene* **10,** 1485–1492.
16. Nakamura, Y. (1996) in *New Strategies for Treatment of Hereditary Colorectal Cancer*, ed. Baba, S. (Churchill Livingstone, New York), pp. 93–98.
17. Miyoshi, Y., Ando, H., Nagase, H., Nishisho, I., Horii, A., Miki, Y., Mori, T., Utsunomiya, J., Baba, S., Petersen, G., Hamilton, S. R., Kinzler, K. W., Vogelstein, B. & Nakamura, Y. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 4452–4456.