related to the use of short extension times (30 s) in our nested PCR assays, because longer extension times were related to unspecific PCR products. On average, we yielded 200 bp of intronic sequence at any intron of interest. These sequences are now available for routine genetic analysis in chromosome 7-linked forms of congenital long-QT syndrome. Taken together, MTV-PCR is highly efficient in determining the genomic structure of eukaryotic genes of interest despite the unknown position within the gene and despite often large sizes of the introns. In summary, this method has been proven to be exceedingly useful for determining unknown DNA sequences that are otherwise difficult to obtain.

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Rapid, Sensitive Detection of Mutant Alleles in Codon 12 of K-ras by REMS-PCR

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A wide variety of acquired and inherited diseases are associated with genetic abnormalities such as point mutations and deletions. Analysis of genetic material is likely to become routine in the clinic for assessing the risk of, or diagnosis of, disease; predicting a patient's prognosis or response to therapy; and/or for monitoring a patient's progress. Abnormalities within the *ras* oncogene family are frequently observed in human cancer, and these could provide a clinical marker of tumor cells. Point mutations at codon 12 within the *K-ras* gene are found in approximately 75% of pancreatic adenocarcinomas, 50% of colorectal adenocarcinomas and 40% of lung adenocarcinomas. The introduction of such genetic tests depends on the development of simple, rapid and inexpensive assays for genetic abnormalities.

Restriction endonuclease-mediated selective polymerase chain reaction (REMS-PCR) enriches for mutant sequences in a single reaction (3). The protocol requires the presence of a thermostable restriction endonuclease in the PCR mixture. During REMS-PCR wild-type (WT) products are cleaved, while mutant products are digestion resistant, and are thus selectively amplified.

This study describes a further development of REMS-PCR, which allows the detection of mutant products and the determination of the exact nucleotide substitution. In this method, two primers are used which simultaneously induce a restriction site that is present only in the WT allele for codon 12 of the *K-ras* gene and the restriction sites for multiple enzymes that are specific for all possible mutated sequences at this codon. Mutant products are produced when any mutation is present, and these products can be further analyzed, by post-PCR digestion, to identify the exact nucleotide substitutions in all cases. The 59-bp fragment produced by amplification with primers 5BKIT

Benchmarks

and 3AKIP contains an induced *Bst*NI site only if the first two bases of codon 12 are WT. Products from WT DNA are cleaved by *Bst*NI during the PCR, leaving only mutant DNA to amplify. Therefore, the presence of this “diagnostic PCR product” indicates the presence of mutant *K-ras* in the template. The 3' primer 3AKIP induces specific restriction sites in the products, which are cleaved with the restriction endonucleases *Bsa*JI, *Sty*I, *Avr*II, *Mn*II, *Ac*iI, *Rle*AI and *Bsu*36I following PCR (Table 1). The pattern of sensitivity and resistance of mutant products to cleavage with these enzymes depends upon the exact mutation present at codon 12.

DNA was isolated from the human cell lines SupT1 (CRL 1942; ATCC, Rockville, MD, USA), K562 (CCL 243; ATCC), Calu 1 (HTB 54; ATCC), A549 (CCL 185; ATCC) and SW480 (CCL 228; ATCC). SupT1 and K562 are both WT (GGT) at *K-ras* codon 12, Calu 1 is heterozygous mutant (TGT), A549 is homozygous mutant (AGT) and SW480 is homozygous mutant (GTT). A multiplex REMS-PCR system was used for amplification. The reactions contained 500 ng genomic DNA, each dNTP (dATP, dCTP, dTTP, dGTP) at 50 μ M (Amersham Pharmacia Biotech, Piscataway, NJ, USA), 40 U of *Bst*NI (New England Biolabs, Beverly, MA, USA) in 100 mM NaCl, 50 mM Tris-HCl (pH 8.3) and 4 mM MgCl₂ in a total volume of 50 μ L. The amounts, sequences and functions of the primers were as follow: 40 pmol of 5BKIT (TATAAACTTGTGGTAGTTGGACCT) and 3AKIP (GGATGAC TCATTAAGGCACTCTTGCCTACGCC) (produce products only if mutations at codon 12 are present), 2 pmol of 5K36 (CTAGAACAGTAGA-CACAAACCA) and 3K37 (GATTTG-CAGAAAACAGATC) (produce enzyme control products, a 130-bp sequence from exon 3 of *K-ras*, only if *Bst*NI activity fails during the PCR), 20 pmol of 5K38 (GTACACATGAAGC-CATCGTATA) and 3K39 (CCACTGTG-TACTAGTATGCCTTAAG) (produce PCR control products, a 215-bp sequence from exon 4b of *K-ras*, only when all reaction conditions are adequate for PCR). Six units of AmpliTaq[®] DNA Polymerase (PE Biosystems, Foster City, CA, USA) were

Table 1. Partial Sequences of Products Produced from all Alleles of *K-ras* Using Primers 5BKIT and 3AKIP

	Codon 11	Codon 12	Codon 13	Restriction Endonuclease(s)	Source of Enzyme
Sequence of WT Products	CCT	GGG	GGC	<i>Bst</i> NI	NEB
Sequence of Mutant Products	CCN	<u>NGG</u>		<i>Bsa</i> JI	NEB
	CCT	<u>IGG</u>		<i>Sty</i> I	BM
	CCT	<u>AGG</u>		<i>Avr</i> II/ <i>Sty</i> I	NEB/BM
	CCT	<u>C</u>		<i>Mn</i> II	NEB
		G <u>CG</u>	G	<i>Ac</i> iI	NEB
	T	G <u>T</u> G	GG	<i>Rle</i> AI	NA
	CCT	N <u>A</u> G	G	<i>Bsu</i> 36I	NEB
		G <u>A</u> G	G	<i>Mn</i> II	NEB

Mismatched bases introduced by primers are indicated in bold type; point mutations at codon 12 are underlined; N = T, A, C, G.
NEB, New England Biolabs; BM, Boehringer Mannheim (Indianapolis, IN, USA); NA, *Rle*AI is not commercially available.

mixed with TaqStart[™] Antibody (CLONTECH Laboratories, Palo Alto, CA, USA), to give a final molar ratio of *Taq* DNA Polymerase:TaqStart antibody of 1:5, and incubated for 15 min at room temperature. The reactions were placed in a GeneAmp[®] PCR System 2400 Thermal Cycler (PE Biosystems), denatured at 94°C for 3 min and then subjected to 30 cycles of 58°C for 1 min, followed by 92°C for 20 s.

A 15- μ L aliquot of each reaction was analyzed without subsequent manipulation by electrophoresis on a 5%

NuSieve[®] GTG[®] gel (FMC BioProducts, Rockland, ME, USA). Diagnostic products are only detected in samples containing mutants (Figure 1). The 59-bp diagnostic fragment was visible in reactions containing Calu 1, A549 and SW480 DNA, but was absent in those reactions containing SupT1 or K562 DNA. This confirms that Calu 1, A549 and SW480 all contain mutations, while SupT1 and K562 are WT at codon 12. A 215-bp PCR control fragment was present in all reactions, confirming that efficient PCR amplification

Figure 1. REMS-PCR and subsequent allele identification by post-PCR digestion. All PCRs, with the exception of lane 2, contained *Bst*NI in the PCR mixture. Lanes 6–8, 10–12 and 14–16 have been digested with a restriction enzyme following the PCR. Lanes 1 and 18 contain PhiX-174 DNA cut with *Hin*II, (Promega, Madison, WI, USA); lane 2, K562 product (no *Bst*NI control reaction); lane 3, K562 product; lane 4, Sup T1 product; lane 5, A549 product; lane 6, A549 product cut with *Sty*I; lane 7, A549 product cut with *Bsa*JI; lane 8, A549 product cut with *Avr*II; lane 9, Calu 1 product; lane 10, Calu 1 product cut with *Bsa*JI; lane 11, Calu 1 product cut with *Sty*I; lane 12, Calu 1 product cut with *Avr*II; lane 13, SW480 product; lane 14, SW480 product cut with *Bsa*JI; lane 15, SW480 product cut with *Ac*iI; lane 16, SW480 product cut with *Mn*II; lane 17, PCR with no DNA.

Benchmarks

Table 2. Identification of Mutant Alleles by REMS-PCR and Post-PCR Digestion

DNA Template	Products Generated from	Restriction Endonuclease	Result	Sequence at Codon 12 Positions 1 and 2 (N = A, C or T)
K562	5BK38/3K39 only	—	—	WT - GG
Sup T1	5BK38/3K39 only	—	—	WT - GG
A549	5BK38/3K39 and 5BKIT/3AKIP	<i>Bsa</i> JI	Cleaved	Mutant NG
		<i>Sty</i> I	Cleaved	AG or TG
		<i>Avr</i> II	Cleaved	AG
				Result: Mutant (AG)
Calu 1	5BK38/3K39 and 5BKIT/3AKIP	<i>Bsa</i> JI	Cleaved	Mutant NG
		<i>Sty</i> I	Cleaved	TG or AG
		<i>Avr</i> II	Resistant	not AG
				Result: Mutant TG
SW480	5BK38/3K39 and 5BKIT/3AKIP	<i>Bsa</i> JI	Resistant	Mutant Not NG (i.e., GN)
		<i>Ac</i> iI	Resistant	not GC
		<i>Mn</i> II	Resistant	not GA
				Result Mutant (GT)

had occurred in all reactions. The absence of a 130-bp fragment in reactions containing *Bst*NI confirms that the restriction enzyme was active and eliminates the possibility of false positives.

The diagnostic products generated from Calu 1, A549 or SW480 DNA were further analyzed by digestion with *Bsa*JI, *Sty*I, *Avr*II, *Mn*II and *Ac*iI. The restriction enzyme was incubated with 20 µL of PCR product, to which dithiothreitol (DTT) and MgCl₂ had been added to give final concentrations of 10 and 6.5 mM, respectively. Incubation was performed at the temperatures recommended by the manufacturers. The results of the analysis are shown in Figure 1 and are summarized in Table 2. This analysis by post-PCR digestion confirmed the known sequences of these cell lines at codon 12 of *K-ras*.

The technique described in this study allows detection of mutations at codon 12 of the *K-ras* oncogene. Subsequent analysis by restriction endonucleases confirms the presence of the mutation and identifies the specific base substitution. Specific base substitutions at codon 12 of the *K-ras* gene

have been associated with prognosis for various cancers. For example, a glycine (GGT) to valine (GTT) mutation is reported to be associated with a poorer prognosis for colorectal cancer (1), and mutation to aspartate (GAT) or valine (GTT) is associated with poor survival for lung adenocarcinomas (2). The method is suitable for use with other PCR systems including enriched PCR and induced restriction fragment-length polymorphism (RFLP)-PCR, and is a quick alternative to sequencing to determine the exact mutation present at codon 12 of *K-ras*.

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