

Review

The restriction site mutation assay: a review of the methodology development and the current status of the technique

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The restriction site mutation (RSM) assay has been employed in our laboratory, as a mutation detection system, since its first description in 1990. In principle the technique is capable of detecting mutations in ubiquitous restriction enzyme sites and is, therefore, readily applicable to any sequenced gene and/or organism. The RSM assay has been applied in our laboratory in various species, detecting rare mutations induced in mouse, rat, *Xenopus*, flatfish and human cells and tissues. This paper reviews the data accumulated by the RSM methodology in our hands and charts the developmental processes which have steadily improved the technique such that it is now applicable as a sensitive genotypic mutation detection system. This paper also includes PCR primer sequences and restriction enzymes employed in mutational analyses performed in the various species studied. We detail a variety of problems associated with the assay and the steps taken to solve them. The specific hurdles which have been overcome include the lack of quantitative data, the question of the contribution of DNA adducts to the induced mutation profile and the presence of false positives. Finally, the methods which have been developed to increase the sensitivity of the assay are also detailed. This paper describes our recommended RSM methodology, as it is routinely employed in our laboratory, which enables the analyses of mutations induced by chemical exposures and spontaneous endogenous processes. Our aim in presenting the developmental data on the RSM assay is to provide other researchers with sufficient information about the RSM methodology to facilitate its application in mutation analysis in other genes and organisms.

Introduction

The development of sensitive mutagenicity tests is essential if genotoxic agents are to be correctly identified and potential human exposures minimized, where possible. These mutation tests may also play a major role in the characterization of the specific mutational events responsible for carcinogenesis. The identification of specific mutational events accumulated in carcinogenesis may lead to a better understanding of the process and the identification of culprit mutagens, as well as allowing earlier diagnosis. The required mutation tests must be sensitive and readily applicable to the genes associated with tumour formation, whilst at the same time allowing rapid screening.

Currently, there are many methods available for studying DNA mutations, however, most involve phenotypic expression of the mutation and hence only a small number of selectable loci can be studied (Albertini *et al.*, 1990; Steingrimsdottir

et al., 1995). In addition, many of the current mutational tests have been developed in bacterial and yeast systems; the relevance of such data to mammalian exposures is not always obvious and, hence, does not allow accurate assignment of mammalian hazard and risk estimates. There are several genotypic techniques which are capable of analysing the DNA taken from mammalian tissues and cells for the presence of mutational events. These mutation tests can be employed in a scanning role to detect unknown mutations or in a diagnostic mode where well-characterized mutations can be screened for (Cotton, 1989). As genotypic tests have no prerequisite for living cells, they have the added advantage of being capable of detecting mutations in archival material (Cortopassi and Arnheim, 1992) and from biopsy samples (Sandy *et al.*, 1992). These genotypic mutation tests include direct sequencing, heteroduplex analysis and single-strand conformation analyses (Rossiter and Caskey, 1990; Axton and Hanson, 1998). These techniques, having low sensitivity, are better suited to the detection of diagnostic mutations and the presence of allele heterozygosity. Alternative genotypic techniques are needed to detect rare mutational events present in an excess of wild-type DNA. This situation is exemplified by a small number of pre-cancerous mutated cells surrounded by an excess of wild-type tissue. In 1990, such a genotypic mutation test, which we named the restriction site mutation (RSM) assay, was conceived (Parry *et al.*, 1990). In the RSM assay, DNA mutations in ubiquitous restriction enzyme sites which eliminate the ability of the restriction enzyme to recognize the specific DNA target sequence are detected (Figure 1). This methodology showed much promise and was developed in this and other laboratories as a mutation detection system. (Bridges *et al.*, 1990; Parry *et al.*, 1990; Palombo *et al.*, 1992; Pourzand and Cerutti, 1993).

The RSM assay allows the detection of mutations in restriction enzyme sites through selective PCR amplification of induced restriction enzyme-resistant sites and the concurrent destruction of wild-type sequences by enzymatic digestion. This molecular selection is essential in allowing the RSM assay to detect rare mutational events present in an excess of wild-type DNA sequences. Indeed, the detection sensitivity depends heavily upon, and is limited by, the efficient removal of these wild-type sequences (Parry *et al.*, 1990; Steingrimsdottir *et al.*, 1995; Parsons and Heflich, 1997). DNA sequencing of the resistant RSM product allows the identification of the DNA base changes involved.

The RSM assay is limited to analysing the four to six bases which make up a restriction enzyme recognition sequence, however, by studying multiple restriction sites, it is possible to increase the target size. In addition to its higher sensitivity, the RSM assay holds several advantages over other contemporary techniques. First, any gene can be analysed for mutations; silent mutations and phenotype altering mutations are equally detectable. Additionally, due to the RSM assay being tissue-independent, mutations can be sought in all tissue types,

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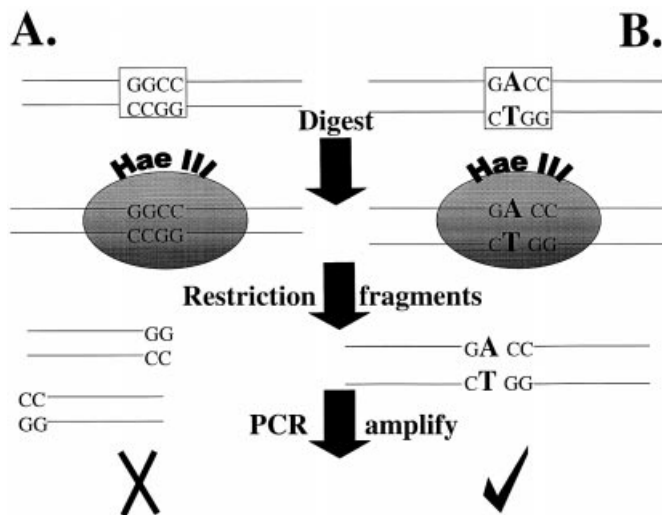


Fig. 1. Principles behind the RSM assay. (A) Wild-type DNA is recognized and cleaved by the restriction enzyme, preventing PCR amplification. (B) Mutant DNA is not recognized by the restriction enzyme, remaining undigested and providing a substrate for PCR amplification.

including germ cells, where currently there is a lack of mutagenicity data (Shelby, 1994). Furthermore, by using an endogenous target gene, the mutation pattern detected genuinely reflects the molecular events in the genome under study; this has been a criticism of transgenic techniques, where mutational data has to be extrapolated from the foreign DNA inserted into the genome of the organism under study.

Another technique, similar in principle to the RSM assay, has been developed, called restriction fragment length polymorphism/polymerase chain reaction (RFLP/PCR) (Zijlstra *et al.*, 1990; Felley Bosco *et al.*, 1991; Chiocca *et al.*, 1992; Sandy *et al.*, 1992). In RFLP/PCR, mutations in restriction sites rendering them resistant to digestion and allowing their amplification by PCR are similarly detected. The RFLP/PCR technique has been optimized as a very sensitive mutation detection system (Felley Bosco *et al.*, 1991). However, this sensitivity comes at a price; the methodology involved is complex (Steingrimsdottir *et al.*, 1995) and has only been described for two regions of human genomic DNA. The technique involves the isolation of the gene of interest by preparative restriction digestion, in order to enrich the DNA for the target gene. After exhaustive digestion with the test enzyme, the amplified mutants are cloned and identified by oligonucleotide probing. The RFLP/PCR technique has been employed to examine mutation induction in the *H-ras* gene (Sandy *et al.*, 1992) but mainly the *p53* gene (Aguilar *et al.*, 1993) of cultured human cells. Specifically, this technique has analysed *p53* hotspot mutations at codons 248–250 (Hollstein *et al.*, 1991), employing two restriction enzymes *Hae*III and *Msp*I. The detected mutations were induced by aflatoxin B1 (Aguilar *et al.*, 1993; Mace *et al.*, 1997), *N*-ethyl-*N*-nitrosourea (ENU) (Perwez Hussain *et al.*, 1994a), reactive oxygen species (Perwez Hussain *et al.*, 1994b), UV light (Amstad *et al.*, 1994), nitric oxide (Felley Bosco *et al.*, 1995), benzo[*a*]pyrene (B[a]P) (Cherpillod and Amstad, 1995) and radon particles (Perwez Hussain *et al.*, 1997).

Whilst sharing the same principles, the two techniques have separate applications. The RFLP/PCR technique continues to answer questions on mutation induction in the *p53* gene hotspot region of human cells exposed *in vitro* to mutagenic agents.

In contrast, the RSM assay, being more adaptable, is better suited to *in vitro* and *in vivo* mutational analysis in a wide range of organisms, genes and restriction sites (see below). The RSM assay offers mutational analysis without the labour intensive methodology of the RFLP/PCR protocol, allowing the screening of a large number of samples in a matter of days.

Summary of mutational data obtained by the RSM assay

The RSM assay has been developed in our laboratory with the implicit aim of producing a genotypic assay readily applicable to a wide range of genes and species, allowing rapid mutagen screening. Additional requirements for the RSM assay include the ability to detect rare mutations in non-tumour tissues plus the detection of a wide range of mutational events and the production of both qualitative and quantitative mutational data. Furthermore, we aimed to produce a mutation assay capable of detecting mutational events in endogenous genes producing mutation data free from artifacts.

We have tested the applicability of the assay by studying mutation induction in 14 different target gene regions of six different species. These targets, along with PCR primer sequences, restriction sites employed and other technical information are identified in Table I. The basic protocol employed in early experiments is shown as a flow chart in Figure 2. This cut–PCR–cut methodology is the simplest form of the assay and allows mutations to be screened rapidly. However, to achieve this simplicity, there is a trade-off with regard to sensitivity and, hence, this methodology also represents the least sensitive form of the assay. Nonetheless, this methodology has been successfully employed *in vivo* in our laboratory to detect ENU-induced mutations in mouse tissues (Myers and Parry, 1994), B[a]P-induced mutations in *Xenopus* tissues (Kennerley and Parry, 1994) and *N*-methyl-*N*-nitrosourea (MNU)-induced mutations in rat tissues (Suzen *et al.*, 1998).

The basic RSM methodology has been continually developed in our laboratory, in order to improve the sensitivity of the assay and overcome some of the initial technical difficulties. The specific alterations to the methodology are outlined in the following sections of this review. During these developments we have tried to keep to our original goal of producing a mutation test that is rapid, simple and may be applicable to any gene and/or organism.

Since its first description, the RSM assay has produced a number of obstacles for researchers employing this technique. It should be mentioned that the same problems also affect other genotypic mutation systems. The main problems that have been associated with the RSM methodology are: (i) a perceived lack of sensitivity; (ii) the lack of quantitative mutational data; (iii) the interference of DNA adducts with mutational events; (iv) the presence of false positives.

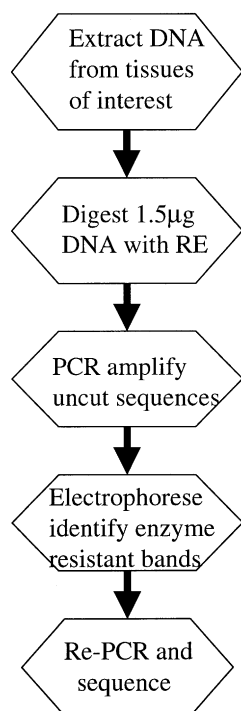
Overcoming these four hurdles has been a major focus of our investigations to date; the results of these developments are presented here. Table II contains recently published mutational data obtained by the RSM assay in various genes and species, indicating the power of the assay in mutation detection. These studies were able to detect mutations induced by exposure to test mutagenic agents and also produced data on the tissue specificity and mutational signature of the mutagens.

Sensitivity improvements in the RSM assay

The sensitivity of the RSM assay has been substantially improved since the initial mutation detection experiments.

Table I. PCR primers and restriction sites analysed by the RSM assay

Species	Gene	PCR primers (5'→3')	Length (bp)	T _{anneal}	Cycle no.	Restriction sites	Reference
Mouse	N-ras exon 1	atgactgagtacaaactggt ttatactagggtggtatctc	111	55°C	30–35	AluI (AGCT)	Parry <i>et al.</i> (1990)
<i>Xenopus</i>	α-globin exon 3	ttgggtgggtgttgctatgaa ctgctgctgtgttgctga	133	50°C	24	BsiLI (CCTGG)	Kennerley <i>et al.</i> (1994)
Mouse	α-globin exon 1	actgggtaggagcaagcaca tagcaaacatcctgggagaa	376	57°C	35	BamHI (GGATCC) PstI (CTGCAG)	Myers and Parry (1994)
	α-globin exon 2	tctcttctctctctccca gacaccctgatgcctctgct	344	58°C	35	BanI (GGpypuCC) MspI (CCGG)	
Rat	p53 exon 5	actcaattccctcaataag atgagcgttgctctgatgt	180	59°C	30	NcoI (AACTGG) DdeI (CTNAG)	Suzen <i>et al.</i> (1998)
	p53 exon 8	tgggaatcttctgggacggg ccccaggaggtgcaagaga	136	66°C	28	BsII (CCNGG)	
	H-ras exon 1	ggcaggagaccctgtaggag cccacctctggcaggtatgc	171	54°C	32	HindIII (AAGCTT) CfoI (GCGC)	
	H-ras exon 2	ctaccggaagcaggtggcatt atgccatgggcccagggcctag	261	52°C	28	CfoI (GCGC)	
Human	p53 exon 7	atgtgtaacagttcctgcatgg ctgacctggagcttccagtg	79	60°C	34	HaeIII (GGCC) MspI (CCGG)	Jenkins <i>et al.</i> (1998a)
Mouse	p53 exon 4	ccatcacctcactgcatggac	226	60°C	33	BanII (GAGCTC)	Jenkins <i>et al.</i> (1997, 1998a, 1999b)
	p53 exon 5	aggaagcccaggtggaag ctctctcccctcaataagc	146	55°C	31	NcoI (CCATGG) HaeIII (GGCC)	
	p53 intron 6	gtctcagacctccgtcatgtg ctttctctagcaaccgtttgc	305	62°C	33	NcoI (CCATGG) ApaI (GGGCCC)	
Flounder	p53 exon 8	ccctacctactacaggtgacc ctttgtgttgagcagcttaggg	311	58°C	34	TaqI (TCGA) MspI (CCGG)	Unpublished results
		gtgtgtggagcagagttactcg				RsaI (GTAC)	
<i>Escherichia coli</i> / mouse	lacZ	tgcggattgaaaatggctctg tgattcattccccagcgac	384	62°C	31	RsaI (GTAC) HaeIII (GGCC)	Unpublished results

**Fig. 2.** Outline of the basic RSM methodology.

These initial studies reported estimated mutation frequencies of 10^{-2} – 10^{-3} , indicating the initial sensitivity of the assay (Kennerley and Parry, 1994). The current sensitivity of the RSM assay has been improved by at least 1000-fold. There are two main reasons for the improvements in the sensitivity

of the RSM assay. First, due to the experience gained in our laboratory with the technique, we have been able to identify those restriction enzymes which are most suitable for RSM analyses, hence maximizing the digestion efficiency. We have also been able to couple this digestion to optimized PCR amplification conditions for mutation detection. The performance of multiple analyses has also improved the detection limit, especially where the mutations are present at low copy numbers. Second, we have been able to show that mutation induction in non-coding intron regions is substantially higher than in coding exon regions (Jenkins *et al.*, 1997, 1998a; Parry *et al.*, 1999). This observation presumably reflects the reduced selective pressure acting on non-coding gene regions compared with coding gene regions. Through the targeting of intron regions, we have been able to detect mutational events, including spontaneous mutations that have been undetectable in coding exon regions. These spontaneous mutations have been detected at mutation frequencies of $\sim 10^{-5}$ – 10^{-6} (Jenkins *et al.*, 1997, 1998a). These represent the first reports of spontaneous mutations detected by the RSM assay, attesting to the increases in sensitivity achieved.

The ultimate sensitivity of the RSM assay is determined by the amount of DNA that can be screened in each analysis. The maximum amount of DNA that supports optimal PCR amplification has been estimated to be 1–2 µg (McPherson *et al.*, 1994), representing $3\text{--}6 \times 10^5$ copies of the genome and, hence, the gene under analysis (Strickberger, 1976). Therefore, no more than 1–2 µg of DNA can be screened in each RSM analysis, limiting the sensitivity to 1.6×10^{-6} . The practical sensitivity of the RSM assay is ~ 10 -fold lower than the theoretical level, due to the fact that ~ 10 mutations are needed to produce an enzyme-resistant band (Jenkins, 1997;

Table II. Summary of recently published RSM mutational data

Species	Gene	Chemical	Tissue	Induced mutations/ no. analysed	Spontaneous mutations/no. analysed	Most prevalent mutation induced	Reference
Human	<i>p53</i> exon 7	4-NQO	Cells	100/690 (14%)	0/690 (0%)	GC→AT (100%)	Jenkins <i>et al.</i> (1998a)
Mouse	<i>p53</i> exon 4	DMH	Liver	4/24 (16%)	0/24 (0%)	GC→AT (53%)	Jenkins <i>et al.</i> (1997)
	<i>p53</i> exon 5	DMH	Liver	2/24 (8%)	0/24 (0%)	GC→TA (26%)	
	<i>p53</i> intron 6	DMH	Liver	9/24 (38%)	2/24 (8%)		
Rat	<i>p53</i> exons 5+8	MNU	Liver	4/32 (13%)	0/32 (0%)	GC→AT (57%)	Suzen <i>et al.</i> (1998)
	<i>p53</i> exons 5+8	MNU	Brain	4/32 (13%)	0/32 (0%)	AT→GC (43%)	
	<i>H-ras</i> exons 1+2	MNU	Pancreas	5/32 (16%)	0/32 (0%)		
	<i>H-ras</i> exons 1+2	MNU	Spleen	2/32 (6%)	0/32 (0%)		
Mouse	<i>p53</i> exon 4	ENU	Testes	2/54 (4%)	0/52 (0%)	GC→AT (50%)	Jenkins <i>et al.</i> (1998a)
	<i>p53</i> exon	5 ENU	Testes	1/44 (2%)	1/47 (2%)	GC→TA (28%)	
	<i>p53</i> intron 6	ENU	Testes	7/67 (10%)	2/60 (3%)		
Flounder	<i>p53</i> exon 8	B[a]P	Liver	16/225 (7%)	11/255 (4%)	GC→AT (82%)	Unpublished results
Mouse	<i>p53</i> intron 6	ENU	Spleen	19/65 (29%)	4/55 (7%)	GC→AT (54%)	Jenkins <i>et al.</i> (1999b)
	<i>p53</i> intron 6	ENU	Bone marrow	7/36 (19%)	1/45 (2%)	GC→TA (71%)	

Steingrimsdottir *et al.*, 1996). This has also been demonstrated by reconstruction experiments involving the recovery of the mutant internal standard (MIS) molecules (see below for quantification of mutation frequency). It has been shown that at least 10 copies of the MIS were needed to be visible on a gel after RSM analysis (Jenkins, 1997). Hence, the practical sensitivity of the RSM assay is 1.6×10^{-5} per analysis; this is consistent with other sensitivity estimations (Steingrimsdottir *et al.*, 1995). This sensitivity can be improved by performing multiple analyses, increasing the chance of rare mutations being detected. However, in order to increase the number of, for example, *p53* genes screened by the RSM assay in each analysis, an attempt was made to isolate the target *p53* gene region from the genomic DNA. As mentioned earlier, this has already been achieved by restriction fragment isolation of target genes from agarose gels with the RFLP/PCR methodology (Cerutti *et al.*, 1994). However, this preparative process was deemed too laborious and time consuming and had not been entertained until a more rapid isolation method was recently reported (Teng *et al.*, 1997), reminiscent of a method proposed by Bridges *et al.* (1990). Figure 3 shows the method employed by us in isolating the murine *p53* gene, however, the same principles are readily applicable to other target genes. The gene was isolated by restriction enzyme digestion with an enzyme possessing target sites flanking the region of interest (*TaqI* in this case). The isolated restriction fragment bearing the mouse *p53* gene was pulled out from the rest of the genomic DNA by use of a biotinylated DNA probe complementary to one end of one DNA strand (if both strands are needed, two such probes can be employed). The biotinylated probe is captured on streptavidin-coated magnetic beads and the isolated gene is separated from the rest of the genomic DNA. The isolated target strand is made double-stranded by primer extension using a high fidelity polymerase and is then available for analysis with the RSM assay. Due to the isolated gene representing only one millionth the size of the genome, it is theoretically possible to include a million times more copies of the isolated gene in the RSM assay than was possible when using 1–2 µg of genomic DNA, representing a theoretical sensitivity of 10^{-8} – 10^{-9} . We envisage that this gene separation step may prove essential in improving the sensitivity of the assay to a point where spontaneous coding region mutations may be detected. Apart from increasing the sensitivity, the gene isolation procedure will also aid in the removal of

excess wild-type sequences and hence improve the digestion efficiency, which has been the limiting step in the assay. The isolation procedure has been successfully demonstrated in our laboratory and is currently being incorporated into current RSM analyses.

The problem of obtaining quantitative mutation frequency data

The production of mutation frequency data is essential if comparisons are to be made between the effects of different chemical mutagens and the role of such mutagens in tissue-specific mutation induction. Therefore, the RSM assay needs to be able to estimate the level of mutational load as well as being able to qualitatively determine which mutations are present. Initial studies employing the RSM assay attempted to ascertain approximate mutation frequencies by an estimation of the copy number of the enzyme-resistant RSM products through a comparison with known amounts of DNA size standards (Kennerly and Parry, 1994). This did not account for the inherent variation in PCR amplification efficiency and was not satisfactory, hence an alternative strategy was required.

The lack of mutation frequency data was overcome by employing internal standard molecules and performing quantitative fluorescent PCR. Initial copy numbers of the mutant sequence in genomic DNA could be determined from the final amplified copy number of the mutant RSM product by comparing the amplification efficiency with that of the internal standard. The target and internal standard molecules were distinguished, after electrophoresis, by a small difference in size, facilitated by the deletion of the RSM restriction sites from the MIS molecules. In order to estimate the mutation frequency using an internal standard, the internal standard has to utilize the same PCR primers as the target and have as similar a DNA sequence as possible (Ferre, 1992; Siebert and Larrick, 1992; Cottrez *et al.*, 1994).

If the RSM target regions were short enough (<150 bases) the MIS molecules could be created with deleted restriction sites by use of a DNA synthesizer (Myers, 1994; Suzen, 1996). Longer targets required more elaborate protocols to generate MIS molecules with deleted restriction sites. For example, two megaprimers were synthesized with a 20–30 base 3' overlap allowing the generation of MIS molecules of 200 bases or so (Jenkins, 1997). However, when longer MIS molecules were

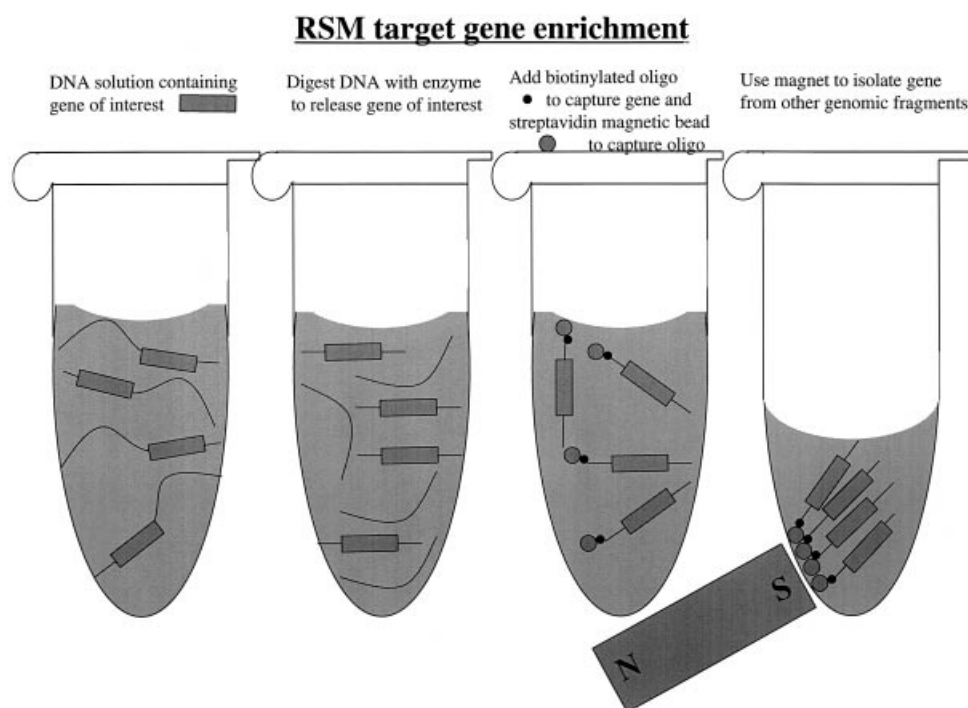


Fig. 3. Outline of the gene separation technique. In the case of the murine *p53* gene, the DNA is digested with *TaqI* to release a 1 kb fragment bearing the *p53* gene. The digested DNA is annealed to a biotinylated probe (B*AAAAACACAGAAAGGGAGGAAGAAGGAAAGG) and eluted out of the digested DNA by the use of streptavidin-coated magnetic beads (Dynal).

needed, we employed novel PCR protocols to generate MIS molecules. Specifically, we were able to demonstrate the ligation of two PCR products flanking the target restriction sites, producing a truncated PCR product devoid of the target restriction sites (Jenkins and Parry, 1997). After construction, these MIS molecules were cloned into plasmids, in order to overcome any preferential amplification associated with short DNA molecules. The plasmids were isolated using commercial kits and diluted to a copy number of ~ 1000 copies/ μ l. Approximately 50 copies of fresh plasmid were included in the PCR step of each RSM analysis, allowing comparative intensity calculations to determine the copy number of any mutations detected, as described previously (Jenkins *et al.*, 1997, 1998a). If <10 molecules of the MIS were added to the samples before PCR, no MIS band was evident. This indicates that, similarly, 10 mutated restriction sites are necessary to produce a resistant RSM product.

Alternative methods to assess the mutation frequencies were also sought due to the identification of some technical problems with the use of internal standards. The diluted plasmids were found to be unstable when stored at -20 or -70°C . Reproducible results were only achieved using freshly extracted plasmids. In addition, the construction of the internal standards was extremely complex and hence the RSM assay was not easily applied to new target regions.

Due to the temperature uniformity of modern PCR machines, it is not necessary to account for tube to tube variation by employing an internal standard. External standards can be employed (Ferre, 1992). These external standards take the form of a DNA dilution series amplified alongside the RSM products. In addition to being less complicated than internal standard strategies, the quantitation does not rely on a single data point, as with the internal standard methodology, but involves reading unknown copy numbers off a calibration curve of PCR intensity against copy number, thus improving

the accuracy of the data obtained. This quantitation method has been employed to assess the mutation frequencies in a recent RSM study (unpublished results) and is routinely used in our laboratory to assess mutation frequencies.

The problem of DNA adducts interfering in mutation detection

When studying DNA mutation induction at early time points post-exposure by PCR-based methods, it is not always possible to distinguish true mutations from *ex vivo* misreplications produced by the action of *Taq* polymerase when encountering DNA adducts. This is also potentially problematical for other genotypic tests, including transgenic systems, where bacterial polymerases process DNA adducts originating from the transgenic animals during the blue/white selection step in *Escherichia coli* (Sommer and Ketterling, 1994). The interference of DNA adducts with RSM mutational data was of concern to us in situations where early time points post-exposure were studied. However, after following several lines of investigation, it appears that the interference of DNA adducts may not be as significant as first envisaged. Persuasive evidence was provided by an experiment set up to analyse the contribution of DNA adducts to detected mutations. The fate of DNA adducts and DNA mutations were concurrently measured in human fibroblasts treated *in vitro* with the model mutagenic agent 4-nitroquinoline 1-oxide (4-NQO), by application of both the ^{32}P -post-labelling method and the RSM assay (Jenkins *et al.*, 1998a). Figure 4 demonstrates the results of these analyses. It can be seen that the DNA adducts were detectable, with a peak at 4 days, whereas two mutational peaks were found, a minor peak at day 4, coinciding with that of the adducts, and a major peak at day 46. The first mutational peak is presumably due to *ex vivo* misreplication of the DNA adducts, with the second peak due to genuine mutational

A Comparison of the persistence of 4-NQO induced DNA adducts and DNA mutations

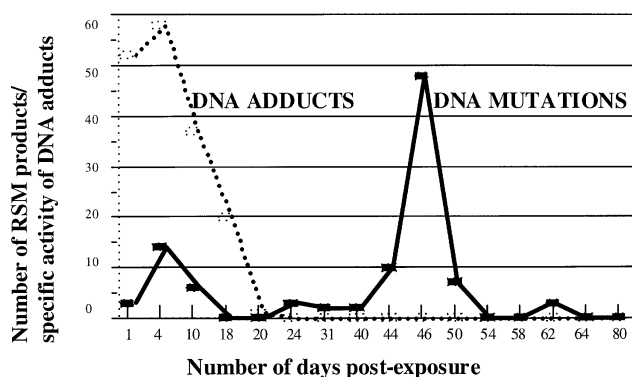


Fig. 4. Graphical representation of an experiment analysing the *in vitro* formation of DNA adducts and mutations in human cells by the application of ^{32}P -post-labelling and RSM analysis. Taken from Jenkins *et al.* (1998a).

events. By comparing the peak heights of the DNA adducts and DNA mutations it appears that only a small number of the DNA adducts were converted into DNA mutations *ex vivo*. Hence the *ex vivo* misreplication of 4-NQO adducts was found to be inefficient and hence there was probably a low risk of DNA adducts interfering with genuine mutational events. This data supports other reports that DNA adducts do not contribute to mutational events as detected by RSM or RFLP/PCR (Steingrimsdottir *et al.*, 1996; Perwez Hussain *et al.*, 1994a). The reason for inefficient adduct misreplication by *Taq* polymerase is that a wide range of DNA adducts can potentially block PCR amplification, by inhibiting *Taq* polymerase extension (Moore and Strauss, 1979; McCarthy *et al.*, 1996). This effect has been exploited by many groups, including our own, in an attempt to detect the presence of DNA adducts by the accompanying reduction in PCR amplification (Jenkins *et al.*, 1998b). It has also been shown that thermostable polymerases containing inherent proof-reading capabilities are more effectively blocked by DNA adducts than *Taq* polymerase, without proof-reading capability (Strauss and Wang, 1990). The use of such proof-reading enzymes in the RSM assay may reduce *ex vivo* adduct misreplication even further, such that misreplication events become negligible. Recent RSM analyses, including those of mutagen-treated marine organisms, have employed proof-reading polymerases (unpublished results) in order to reduce the interference of DNA adducts with RSM mutation data.

The presence of false positives in RSM analyses

Perhaps the most serious drawback of the RSM assay to date has been the presence of false positives (Kortenkamp *et al.*, 1997). These false positives represent wild-types scored as mutants and, as such, introduce noise into the assay, preventing the detection of some genuine mutations (Steingrimsdottir *et al.*, 1996). These false positives tend to take the form of enzyme-resistant bands which upon sequencing reveal both wild-type and mutant restriction sites. In these samples, the wild-type component should have been completely removed by digestion. In our hands we have found that, on average, the wild-type component comprised 50–75% of the sequence of the restriction enzyme-resistant product (Jenkins, 1997). The presence of these false positives reduces the effectiveness of the assay to detect rare mutational events by masking their

presence with wild-type sequences. False positives, as well as interfering with the mutational data, has meant that sequencing reactions have to be performed on every positive RSM analysis for confirmation of the mutational event. There are a number of possible explanations for the persistence of wild-type sequences in the enzyme-resistant RSM products. These include poor enzyme digestion efficiency and heteroduplex formation.

The level of wild-type sequences present in resistant RSM products has been shown to vary with the particular restriction enzyme employed (Pourzand and Cerutti, 1993), presumably due to variations in digestion efficiency. It has been found that thermostable restriction enzymes such as *TaqI* cut extremely efficiently even during the PCR step and so yield the lowest level of false positives (Pourzand and Cerutti, 1993). Our results have also indicated that *TaqI* is the most effective enzyme for use in RSM analyses (unpublished results). In addition, we have found that four-base cutters, in general, are more efficient at digesting DNA than six-base cutters.

Two factors have been shown to improve the efficiency of digestion such that false positives are avoided. These factors are especially important for restriction enzymes that do not possess the high digestion efficiency of *TaqI*. The first factor involves the use of a proof-reading polymerase in the PCR step. Presumably, the higher fidelity of such enzymes removes the risk of polymerase-induced misincorporations (even though misincorporations by *Taq* polymerase have not been evident in our analyses; see Discussion). The second factor which aids in the removal of false positives is a mid-PCR digestion (Steingrimsdottir *et al.*, 1995). PCR products are digested after five cycles of PCR with the restriction enzyme under test, removing more wild-type sequences. This mid-PCR digestion step has been shown not to interfere with efficient amplification of resistant RSM products.

The second possible explanation for the presence of the false positives in the RSM assay is the formation of heteroduplex PCR products. This complication is linked to restriction enzyme inefficiency, as the heteroduplexes only form when wild-type DNA persists. Heteroduplexes form between mutant and wild-type PCR products during the final cycles of PCR amplification, due to their DNA sequences being identical except for the presence of a point mutation. These heteroduplexes are undigestible with the restriction enzyme and allow wild-type sequences to persist in the final resistant RSM product (Serth *et al.*, 1998). Another problem associated with heteroduplex formation involves the assignment of quantitative mutation frequency data, as heteroduplex formation has been shown to affect the quantitation of PCR products (Ferre, 1992; Iland and Todd, 1992). We believe that the formation of these heteroduplexes is a major source of false positives in analyses where the wild-type sequences are not digested efficiently.

We have been able to demonstrate that heteroduplexes can form *in vitro* when mixtures of DNA sequences differing by a single base are PCR amplified together. We have used an enzyme, T4 endonuclease VII (Pharmacia, Uppsala, Sweden), which is capable of cutting mismatched heteroduplexes (Youil *et al.*, 1996) to identify and to potentially eliminate heteroduplex formation. This enzyme has been shown to be capable of recognizing all mismatches (Golz *et al.*, 1998a) and nicking heteroduplexed DNA (Golz *et al.*, 1998b). Figure 5 demonstrates the production of heteroduplexes when mixtures of wild-type and mutant sequences are PCR amplified together. As can be seen, the mixtures show heteroduplex formation,

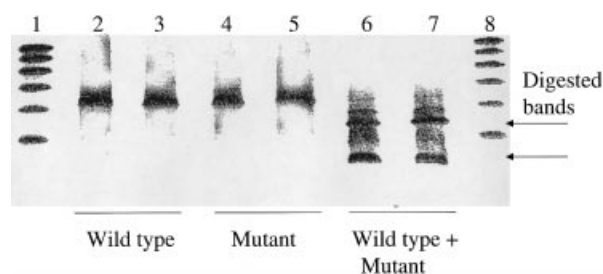


Fig. 5. Demonstration of the formation of heteroduplexes during PCR amplification. Lanes 2 and 3 contain PCR-amplified products from wild-type DNA from the *p53* gene of flounder; lanes 4 and 5 contain PCR-amplified mutated DNA from the same species differing by one base from the wild-type sequence (TCGA→TTGA). Lanes 6 and 7 contain a 1:1 mixture of mutant and wild-type DNA amplified by PCR. After PCR lanes 2–7 were digested with T4 endonuclease VII, which recognizes and cleaves mismatches. Only lanes 6 and 7 show digestion products indicating the formation of heteroduplexes between mutant and wild-type strands during PCR amplification.

Inverse RSM

GGGCCC Apa I
G to A mutational event
 GGACC C Ava II

- Step 1. Cut DNA with Apa I to remove wild type sequences
- Step 2. PCR amplify uncut molecules
- Step 3. Cut PCR product with Ava II
- Step 4. Electrophorese products and look for Ava II fragments

Fig. 6. Basic methodology behind inverse RSM. Mutations which transform one restriction site into another restriction site can be screened for by the use of the two enzymes before and after PCR amplification. The original restriction enzyme (*ApaI*) is used to digest the wild-type DNA; undigested DNA is amplified by PCR and the new enzyme (*AvaII*) is used to reveal any mutant sequences.

as detected by digestion with T4 endonuclease VII. The characterization of heteroduplex formation will hopefully lead to strategies to minimize their formation in RSM analyses.

Due to the problem of false positives in RSM analyses, we sought to modify the RSM assay such that mutations could be detectable by the creation of a new restriction site and by the appearance of new restriction fragments. Due to the fact that here we rely on the formation of new restriction sites and do not use the loss of digestion to detect mutations, false positives associated with enzyme digestion inefficiency are avoided. This is the opposite of RSM and hence it was named inverse RSM (Jenkins *et al.*, 1999a). Figure 6 demonstrates the basic principles behind the methodology. Analyses were scored as positive when one restriction site was successfully converted into another by mutation. The target region chosen to demonstrate this technique was the *ApaI* site of intron 6 of the mouse *p53* gene. This region was chosen because mutations were readily detectable in the *ApaI* site (Jenkins *et al.*, 1997, 1998a). In addition, a large percentage of these mutations transformed this *ApaI* site (GGGCCC) into an *AvaII* site (GGa/tCC). Hence, we could screen for mutations by removing the majority of wild-type DNA with an *ApaI* digest, amplifying the uncut molecules and screening for the specific mutations transforming the *ApaI* site into an *AvaII* site by digestion with *AvaII*. The presence of *AvaII* fragments indicates that mutations are

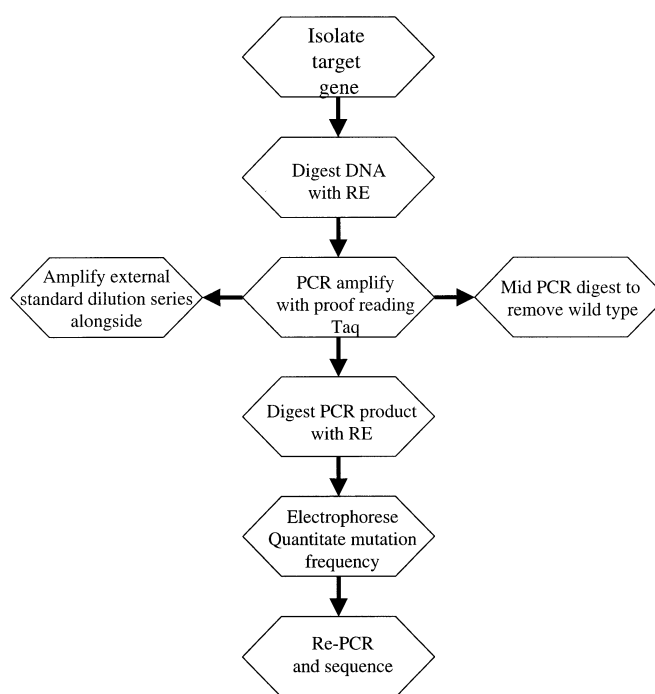


Fig. 7. Improved RSM methodology, including enrichment of the gene of interest, use of proof-reading polymerases and employment of an external standard for quantitative purposes.

present. In addition to removing the problem of false positives, this technique obviates the need for sequencing and hence analyses are rapid and may be amenable to high throughput screening.

Current RSM methodology

The current RSM methodology employed in our laboratory is shown in Figure 7. DNA is extracted from the tissues/cells of interest, yielding high quality, high molecular weight DNA. Enrichment of the target region is recommended, if appropriate restriction sites are available to isolate the target gene. RSM analysis is performed on aliquots of the DNA target using primers/restriction enzymes previously identified. The modifications to the original protocol shown in Figure 2 are as follows: proof-reading DNA polymerases are employed in the PCR; external standards are amplified alongside the samples in order to estimate mutation frequencies. In addition, all restriction enzymes apart from *TaqI* require a mid-PCR digest after five cycles of PCR, comprising the addition of 10 U of enzyme to the PCR reaction held at the appropriate incubation temperature for 30 min. If false positives interfere with the RSM analyses, a digestion step with the T4 endonuclease VII enzyme may be necessary to remove any heteroduplexes. Alternatively, inverse RSM analysis may provide mutational data without the interference of false positives, if the appropriate sequences are available in the target region.

Conclusions

The RSM assay has been employed in our laboratory for 10 years and in that time it has proved to be a valuable research tool, answering questions on the induction of mutations. Given its wide applicability, it has provided detailed data on mutation induction in various genes, tissues and organisms, as shown in Tables I and II. However, in order for the RSM assay to be

a useful genotoxicity test, several obstacles had to be overcome. These centred mainly on the assay sensitivity. We consider that these obstacles have now been largely removed, if not better characterized. Hence, we are confident about the employment of the RSM assay as a mutation detection system. As well as being employed as a sensitive somatic mutations test, the RSM assay may also be employed as a germline mutation test, providing data which is currently not available (Shelby, 1994). This has already been demonstrated in the case of ENU-induced germline mutations in mouse testes (Jenkins *et al.*, 1998a). The RSM assay also offers the ability to analyse tissue specificity, threshold effects and the influence of DNA repair, as well as answering questions on the long-term fate of mutational events and the constraining effects of selection on specific mutations (Holmquist and Gao, 1997).

In addition to identifying potential mutagenic agents, the RSM assay can provide useful data on mutational events involved in carcinogenesis. For example, the RSM assay can be used to screen for *p53* hotspot mutations, where they fall in suitable restriction enzyme sites. This has been demonstrated with codons 248–250, which are contained within the *HaeIII* and *MspI* restriction sites (Aguilar *et al.*, 1993; Perwez Hussain *et al.*, 1994a,b; Jenkins *et al.*, 1998a). Given that *p53* mutations are somewhat tumour specific (Hollstein *et al.*, 1991) and also to a large extent mutagen specific (Dennisenko *et al.*, 1996), genotypic mutation tests, like the RSM assay, can tie up the links between tumour mutations and defined mutagen exposure. Obviously, the RSM assay can only provide such data when the tumour mutations are present within restriction enzyme sites.

The sensitivity of the RSM assay currently allows the detection of mutations induced by both potent and weak mutagens and also the detection of spontaneous mutational events. This sensitivity may well be increased further by enrichment of target genes, as mentioned earlier. However, if greater sensitivity is required, another possible strategy is to target hypermutable cells and animals (Steingrimsdottir *et al.*, 1995). These may take the form of *p53*-deficient cells/animals or cells from repair-deficient (e.g. xeroderma pigmentosum) patients. Similar results can be obtained by targeting other hypermutable sequences, including those in mitochondrial DNA, which has been shown to accumulate 20-fold more mutations than genomic DNA (Khrapko *et al.*, 1997). The employment of multicopy targets (such as mitochondrial genes) in RSM analyses may obviate the need for target gene enrichment, as the higher copy number increases the sensitivity of detection substantially (Parsons and Heflich, 1997).

The current sensitivity of the RSM assay owes a lot to the discovery of the increased mutability of intron regions compared with exon regions. This difference in mutability between exons and introns has previously been shown through analysis of sequence divergence between closely related species, where mutations accumulated predominantly in introns (Turker *et al.*, 1993; Nickerson *et al.*, 1998). The increased accumulation of intron mutations is, we believe, a consequence of their neutrality, with regard to protein structure. Neutral mutations (ones inducing no amino acid change) are known to accumulate in the genome more frequently than non-neutral mutations (Krawczak and Cooper, 1996). Another possible explanation for the increased intron mutability is that there is differential DNA repair between exons and introns. However, it has been noted that mutations detected in both exons and introns preferentially accumulate on the non-transcribed strand (Jenkins *et al.*, 1997, 1998a; Suzen *et al.*, 1998); this strand bias is

indicative of efficient strand-specific DNA repair (Mellon *et al.*, 1987). Hence, if both exons and introns are subject to preferential strand-specific repair, this might indicate that exons and introns are generally repaired similarly. Therefore, the differences in mutability between exons and introns must, presumably, be due to some form of mutation selection.

Any molecular technique incorporating a PCR step has to assess the level of *Taq* polymerase error, which can markedly affect the results. However, we feel that at present the contribution of *Taq* error is negligible in the RSM assay, for the following reasons. First, we are employing high fidelity PCR regimes (McPherson *et al.*, 1994) and are currently using high fidelity polymerases. Second, we recognize that we require at least 10 mutations in order to obtain an amplified PCR product with the RSM assay. Therefore, there would have to be 10 identical errors induced in our target restriction site in order for them to be evident in the RSM assay. The likelihood of this is so low as to be ignored. In addition, we have noted that the mutations detected by RSM analyses contained a large proportion of transversion events, which are not caused by *Taq* polymerase error (Keohavong and Thilly, 1988; Chen *et al.*, 1992). This provides further evidence that mutations detected by RSM analysis are probably not induced by *Taq* polymerase errors.

In order to study the mutagenicity of specific mutagens the RSM assay can be tailored to match the mutagenic profile, by choosing restriction enzymes most likely to be mutated (Palombo *et al.*, 1992). Restriction enzymes cutting the highly mutable CpG sites have been shown to be more prone to mutation accumulation (Cooper and Krawczak, 1993). CpG sites are also known to be targets for certain types of DNA damage, including that produced by B[a]P and 2-acetylaminofluorene (Denissenko *et al.*, 1997; Chen *et al.*, 1998; Jenkins *et al.*, 1998a). Thermostable restriction enzymes offer the highest digestion efficiencies of all enzymes, as they continue to digest wild-type sequences even during the PCR step, hence, where possible, these should be chosen for RSM analysis. The restriction enzyme *TaqI* (TCGA) benefits from both of the above criteria, i.e. it is thermostable and cuts at a CpG dinucleotide. Additionally, CpG sites have been shown to be most mutable when in the sequence context TCGA (Krawczak *et al.*, 1998) and hence the *TaqI* restriction site represents the most mutable target RSM site and is the enzyme of choice for RSM analyses.

In summary, the RSM assay has been successfully developed as a mutation assay and, as demonstrated in this paper, has been applied to detect mutations in a variety of genes and organisms. The main problems associated with the RSM assay have been overcome, or at least better characterized. The RSM assay is now capable of answering questions on the mutability of DNA sequences which are contained within restriction enzyme sites. Obviously, due to the reliance of the RSM assay on such restriction enzyme sites, complementary techniques are required to study mutagenesis outside restriction sites. However, the RSM assay represents a powerful technique for mutation analysis, producing quantitative mutation data rapidly and allowing the screening of a large number of mutagen-treated samples.

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