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Award Number: W81XWH-04-1-0667

TITLE: Test for Sensitive Detection and Accurate Assignment of TP53 Variants in Tumor DNA

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REPORT DATE: September 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-09-2005	Final	1 Sep 04 - 31 Aug 05

4. TITLE AND SUBTITLE
Test for Sensitive Detection and Accurate Assignment of TP53 Variants in Tumor

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0667

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

SpectraGenetics LLC
Pittsburgh, PA 15213-2654

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Accurate, sensitive assessment of the mutational status of tumors enables genotype/phenotype studies and increases the understanding of tumor etiology, progression, recurrence and response to therapy. Preliminary Peptide Mass Signature Genotyping (PMSG) results using bacterial cultures indicated tremendous potential and proof of concept experiments demonstrated that the PMSG process could be converted to an in vitro test. Expansion of proof of concept protocols will provide a mutation detection process suitable for validation and clinical testing.

15. SUBJECT TERMS
No Subject Terms Provided

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	16	19b. TELEPHONE NUMBER *(include area code)*

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Introduction

New therapies for breast cancer and improved management of patients following tumor resections require a greater understanding of tumor progression and recurrence. Histology alone cannot define those subsets of tumors that develop and respond to treatment differently. Molecular characterization of tumors can provide signatures to categorize tumors. Tumors acquire many DNA changes but efforts to genotype breast cancer tissue DNA for mutations in critical oncogenes and tumor suppressor genes have met with limited success largely due to the limitations of existing DNA sequencing methods (Cooper et al. 2004). Tumors are very heterogeneous tissues due to contaminating stromal cells, adipose and vascular tissue and the microevolution that occurs within a tumor; therefore, mutant DNA is frequently only a minor component of a tumor DNA sample. A new technology called Peptide Mass Signature Genotyping (PMSG) has been shown to be more sensitive than dideoxy sequencing (Telmer et al. 2003) but in the current configuration it is difficult to implement. For PMSG, individual exons are PCR amplified, ligated into expression vectors and transformed into bacteria. The proteins expressed by the cultures are purified and analyzed using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). If a mutation is present in the sequence being analyzed a new peak will be observed in the mass spectrum. By expressing the exon sequence in multiple reading frames, one obtains a set of peptide masses, the *peptide mass signature*, that indicates the specific nature of the mutation.

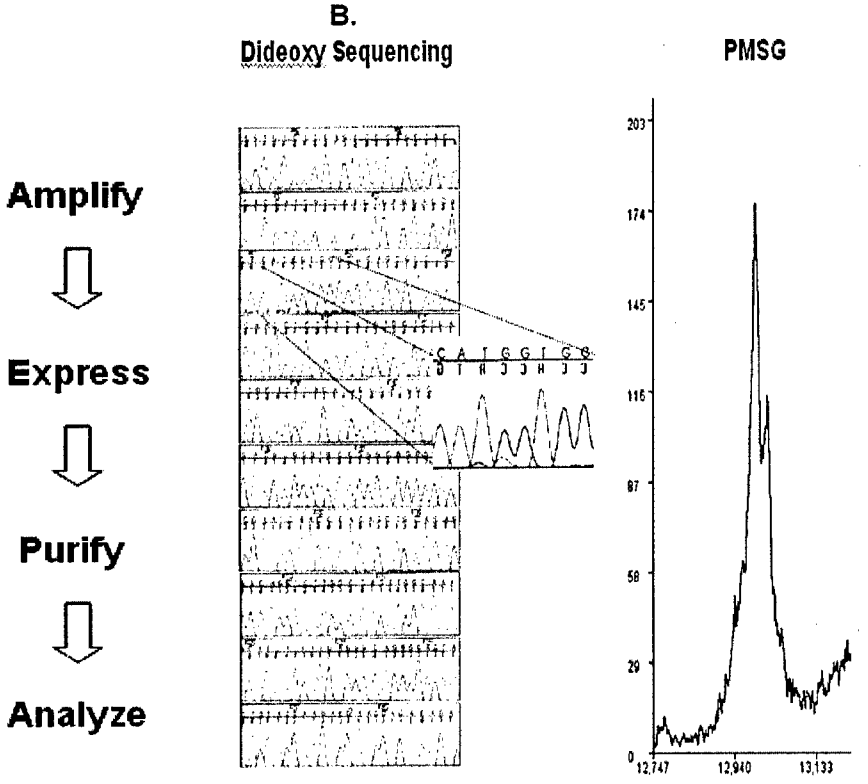

Figure 1A. An overview of the PMSG process. **B.** A comparison of the dideoxy sequencing chromatogram and the PMSG spectra of exon 8 of the *TP53* gene from a non-microdissected sample of a small cell carcinoma of the head and neck (T111) with an H179Y mutation.

Mutations present throughout the *TP53* gene in inherited and sporadic breast cancers are associated with recurrence and mortality (Bull et al. 2004) and with response to therapy (Geisler et al. 2003). Thus a test that scans the entire *TP53* coding sequence will be developed in this project. There is a vast literature examining the involvement of p53 in cancer but many of the studies utilize immunohistochemistry and do not directly determine whether the gene is mutant or not, nor do these studies characterize the codon 72 polymorphism that preliminary studies have associated with breast cancer risk (Huang et al. 2003).

The objective of this project was to develop a PMSG process for genotyping tumor DNA that requires fewer recombinant DNA steps and eliminates the need for transformation of *E. coli* and growth of cultures. The project will be encompassed by three specific aims: (1) Development of reliable protocols for the synthesis of DNA molecules consisting of several exons joined together by PCR overlap extension. (2) Development of robust protocols for adding an epitope tag and regulatory regions for coupled *in vitro* transcription/translation to produce peptide analytes. (3) Development of reproducible protocols for the purification, cleavage and MALDI-TOF MS of the peptides.

The DNA molecules will be designed to group exons for translation in the same alternate reading frames and will include formic acid cleavage sites between the exons in all of the frames to be expressed. The exons will be assembled using regions within the introns for overlap and extension using PCR. This step will reduce the number of peptides that need to be expressed and purified. Overlap extension will then be used to add the promoter, ribosome binding site, epitope tag and termination signals necessary for transcription and translation of the sequence in the coupled *in vitro E. coli* extracts supplemented with T7 RNA polymerase. Once the peptides are produced they will be purified from the complex reaction mix using the epitope tag for affinity capture. The peptides composed of several exons are too large for accurate measurement by MALDI-TOF MS; therefore protocols for formic acid cleavage at the engineered sites will be developed. MALDI-TOF MS techniques will be optimized for resolution and mass accuracy.

Development of a sensitive, accurate PMSG genotyping assay can provide high quality data that investigators need to characterize breast cancer initiation, progression and recurrence. This methodology can be applied to other genes involved in breast cancer providing a comprehensive analysis of the mutational status of a particular tumor. It is expect that in the future such data will be critical for physicians to effectively treat breast cancer patients.

Progress Report

This report details the progress made on each of the tasks (there are a total of 3) that were outlined in the Statement of Work. There needed to be some modifications to the plans involving overlap extension and formic acid digestion but the alternatives that were employed resulted in very promising progress towards achieving the objectives of the project.

Task 1. Development of reliable protocols for the synthesis of DNA molecules consisting of several exons joined together by PCR overlap extension. Months 1-4.

a. Analysis of the 10 *TP53* exon sequences to determine the alternate reading frames which are most informative.
b. Configuration of the exons to group together exons to be translated in the same alternate reading frames and optimal placement of formic acid cleavage sites.
c. Design of the primers to amplify the complete coding sequence and splice sites and then the intron sequence of the adjacent exon and incorporation of acid cleavage sites where required.
d. PCR amplification of the individual exons.
e. Overlap extension to join the exons and produce large DNA molecules to be used to synthesize peptides.
f. Confirm by sequencing.

The target gene for testing of this approach to generate template for PMSG analysis is the tumor suppressor gene, *TP53* (GeneID 7157; Locus tag HGNC11998; MIM 191170; Source Sequence AH002918, AH002919, U94788, X02469, Genbank NC_000017). The 19 kb gene contains 11 exons but the first exon and 28 bases of exon 2 are not translated. PMSG primers were designed to be within the intron sequence and in the correct reading frame. Mismatches were used to change a stop codon present in the primer sequences.

To create one long DNA molecule to serve as the template for in vitro translation it is necessary to join all of expressed exons. The plan was to perform PCR using primers that have 5' anchor sequences complimentary to the 3' end of other template sequences that are present in the PCR reaction mix. With this approach, one could create a PCR product that contains a set of contiguous segments that were not contiguous in the template. Further, one can engineer particular spacer sequences between the segments by including additional nucleotides between the 3' and 5' template-complimentary sequences in the primers. Using this plan, the amplifications would generate a minigene containing, all of the expressed *TP53* exon sequences, separated by spacers that encode sites for specific acid proteolysis of the minigene-encoded polypeptides (Figure 2).

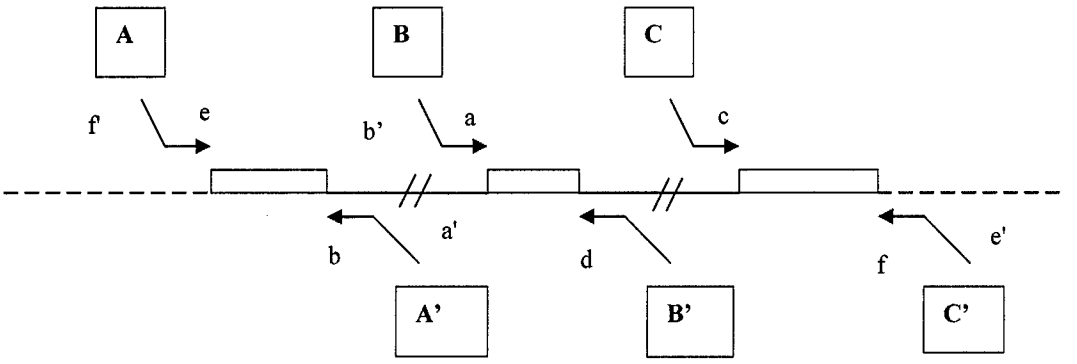

Figure 2. This diagram shows how the primers could be used to assemble a minigene. Exons would be PCR amplified using primer pairs A, A', and B, B' and C, C'. Then to omit the intron sequence the tails on the primers are designed complementary to the primer sequence of the previous and next primer. Primer B would consist of sequence b complementary to b' and primer A' which would be used to amplify the previous exon consists of sequence complementary to a, and thus omitting the intron sequence and bringing the exons adjacent to each other. In order to change the relative position of exons the tails match the exon that it is to be located next to. So primer A would contain intron A specific sequence, e, but the tail would complement sequence f and sequence complementary to e would constitute the tail of primer C'.

An essential feature of PMSG is the translation of the exons in multiple reading frames. In addition to the natural reading frame, most of the TP53 exons contain at least one other reading frame that is open (i.e., free of nonsense codons) across the entire sequence. The latter fact makes it possible to design a large number of minigene permutations in which the entire sequence is open in one reading frame and large segments, representing multiple exons, are open in additional reading frames (Table 1).

Table 1. This shows one permutation to include each exon in its natural reading frame and is configured to yield peptides covering the entire sequence in a second reading frame with three additional peptides.

Exon / *Frame*	**11**	**8**	**6**	**9**	**5**	**4.2**	**SD**	**2**	**7**	**10**	**3**	**4.1**
Natural	X	X	X	X	X	X	X	X	X	X	X	X
Forward+1	X	X	X	X	X							
Reverse								X	X	X	X	X
Reverse-1					X	X	X					

The plan was to begin by PCR amplifying the *TP53* exons from the genomic DNA sample with the primers listed in Table 2.

Table 2. Primer sequences to be used to synthesize the minigene. Lower case represents intron sequence and upper case, exon sequence. Parentheses indicate mismatches in the primers which are introduced to change a stop codon in a particular reading frame. The size in base pairs of the initial products are shown and the size of the complete minigene without the regulatory sequences is shown.

EXON	5' primer	reverse complement of 3' primer	Size (bp)
2	atg ctg gat ccc cac ttt t	AA CTg tg(a/c) gtg gat cca ttg gaa	150
3	aag cga aaa ttc cat gg	c tgg agg gct ggg gac	108
4.1	gct ctt ttc acc cat c(a/t)a cag TCC	CA CCA GCC CCC TCC	195
4.2	GCA CCA GCA GCT CC(t/g) ACA CC	GC ACG gtc (a/c)gt tgc cct gag ggg	168
5	tct gtc tcc ttc ctc ttc c	tg ggg ctg gag aga cga	234
6	ttg ctc tt(a/g) gGT CTG GCC CC	tgg ttt gca act ggg gtc	144
7	gtt atc tcc (t/g)ag GTT GGC TCT	C AGg tc(a/c) gga gcc act	135
8	ttc tct ttt cct atc c(t/g)g agt agT GG	ag caa gca gga caa gaa gcg gtg	186
9	ttc ctt gcc tct ttc c(t/g)a gCA CT	T CAG gta ct(t/c) agt ctt ggg	108
10	ata ctt act tct ccc cct cct c	GCT CAC TCC AGg tg(a/g) gtg acc	150
11	gtc tcc tac agC CAC C(t/g)G AAG TC	AC TCA GAC TG(a/c) cat tct cca ctt ctt gtt	111
SD(DP) (6His)	**c(GTGGTGGTGGTGGTGGTG)(GAT C CG)CATGGCTGTCCTCCTgg**		42
DP	**GAT CCG**		126
		Total	1857

The junction primers correspond to the reverse complement of the 3' primer of the preceding exon followed by the 5' primer sequence, with the coding for aspartate and proline, the formic acid cleavage site D-P (GAT CCG) between the two. For example, the 5' primer for exon 8 would be the exon 11 reverse complement, then the coding for DP, and exon 8, 5' primer **AC TCA GAC TG(c) cat tct cca ctt ctt gtt GAT CCG - ttc tct ttt cct atc c(g)g agt agT GG** and the reverse primer would follow the same pattern, exon 8 sequence-DP coding- exon 6 sequence.

One other sequence to be added will be located between exons 4.2 and 2 and will serve to provide a Shine Dalgarno sequence (SD in table 2) for an alternative reverse reading frame with the structure as follows:

(reading frame adjustment)-***SD*-spacer-INITIATION-***DP rev. compl*-**6HIS** with the sequence (cc)***AGGAGG*acagccATG** *CGG ATC* **CAC CAC CAC CAC CAC CAC** (g)

The sequence **c GT GGT GGT GGT GGT GGT *GAT CCG* GCA TGG CTG TCC TCC Tgg** could be added to the junction primer between the intron sequences in addition to the DP coding sequence (exon 2 contains 3 DP motifs in the natural reading frame). Also between exons 3 and 4.1 there will be no acid cleavage site because exon fragment 4.1 contains an endogenous formic acid cleavage site. DP coding sequences will also need to be incorporated in the alternative reading frames for downstream cleavage of the peptides for MALDI.

The minigene was designed so that it contains several multi-exon segments in alternative open reading frames. In particular, exons 11, 8, 6, 9, and 5 are open in an alternative forward frame (so the sequence cGAT CCG gg is added between intron sequences to code for the DP cleavage site; exons 4.1, 3, 10, 7 and 2 are open in a reverse reading frame (so GCC ATC is

added between those exons); and exons 5 and 4.2 are open in yet another frame (so cc GCC ATC g is added between them). The size of the complete product is 1857 bp. This molecule would then be ready for extension with three sets of regulatory oligos (see next section) to generate a complete minigene that will encode a set of peptides representing every part of the sequence in two different reading frames.

Having completed the design some pilot experiments were undertaken to test the feasibility of the PCR and overlap extension and the digestion with formic acid. Unfortunately the PCR/OLE experiments yielded many artifacts and formic acid was not a reliable cleavage agent. Therefore and alternative strategy was employed for generating template for the coupled transcription/translation reactions. Primers were redesigned to be placed in the introns and therefore include analysis of splice sites and the primers are shown in Table 3. These gene-specific PCR reactions all yielded amplicons as shown (Figure 3).

Table 3. PMSG primers for the *TP53* gene. The bases in parentheses ( ) designate a mismatch, T is changed to G in the primer so an A:G mismatch is present.

Primer Name	5' Primer Sequence	Primer Name	3' Primer Sequence
EI2tp53Sfi5	ctg gat ccc cac ttt tcc tc	**EI2tp53Sfi3**	ggc ctg ccc ttc caa t
EI3tp53Sfi5	ggg aag cga aaa ttc cat gg	**EI3tp53Sfi3**	gtc ccc agc cct cca g
EI4tp53Sfi5	act gct ctt ttc acc cat cta	**EX4.1tp53Sfi3c**	GGA GGG GGC TGG TG
EX4.2tp53Sfi5b	GCA CCA GCA GCT CC(G) ACA CC	**EI4tp53Sfi3**	ccc ctc agg gca act
EI5tp53Sfi5	aac tct gtc tcc ttc ctc ttc c	**EI5tp53Sfi3**	cag ccc cag ctg ctc
EI6tp53Sfi5	cct ctg att cct cac (g)ga ttg ctc	**EI6tp53Sfi3**	gac ccc agt tgc aaa cca g
EI7tp53Sfi5	tca tct tgg gcc tgt gtt at	**EI7tp53Sfi3**	tgc agg gtg gca agt gg
EI8tp53Sfi5	tgc ttc tct ttt cct atc ctg agt	**EI8tp53Sfi3**	ctc cac cgc ttc ttg tcc
EI9tp53Sfi5b	tca gat tca ctt tta tca cc	**EI9tp53Sfi3**	cac ttg a(g)a aga ggt ccc aag act ta
EI10tp53Sfi5	tat ata ctt act tct ccc cct cct	**EI10tp53Sfi3**	gga agg ggc (g)ga ggt cac tc
EI11tp53Sfi5	ctc cct gct tct gtc tcc tac	**EI11tp53Sfi3**	gtg ggg aac aag aag tgg aga at

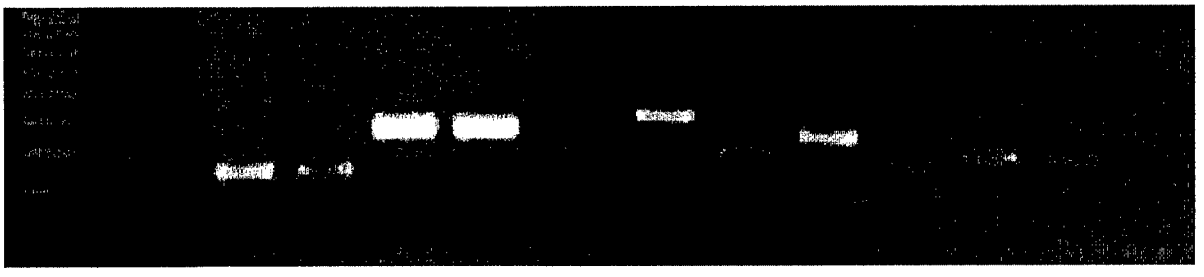

Figure 3. These gels show the PCR products representing the exon-specific amplicons, 14 lanes showing the molecular weight markers, exons 2, 3 forward, 3 reverse, 4.1 forward, 4.1 reverse, 4.2, 5, 6, 8, 9, 10 forward, 10 reverse, and 11.

The successful amplification of each of the exons from genomic DNA in this format achieved the broad goal of having the expressed exons and splice consensus sequences of *TP53* represented as PCR products for PMSG analysis.

Task 2. Development of robust protocols for adding an epitope tag and regulatory regions for coupled in vitro transcription/translation to produce the peptides for analysis. Months 5-8.

a. Design of 6His epitope tag and regulatory sequences to be added for transcription and translation.
b. PCR and overlap extension to produce template for the coupled in vitro transcription/translation reactions.
c. Confirm by sequencing.
d. Produce peptides using coupled in vitro transcription/translation reactions.
e. Confirm production of peptides using Ni-chelate agarose beads for IMAC purification followed by MALDI-TOF.

The exon specific PCR products were to be used in secondary overlap extension and amplification reactions described and diagrammed below (Figure 4). In order to achieve these reactions the 5' ends of each primer consisted of an extension that contained sequences for overlap extension (CGC AAG ACC AAA CAG AGA ACC and GCT CGA GAC TCC TTG TGC TGC). The orientation of reading frame is determined by forward and reverse pairs of primers for each exon. Reading frame is determined by the regulatory elements added.

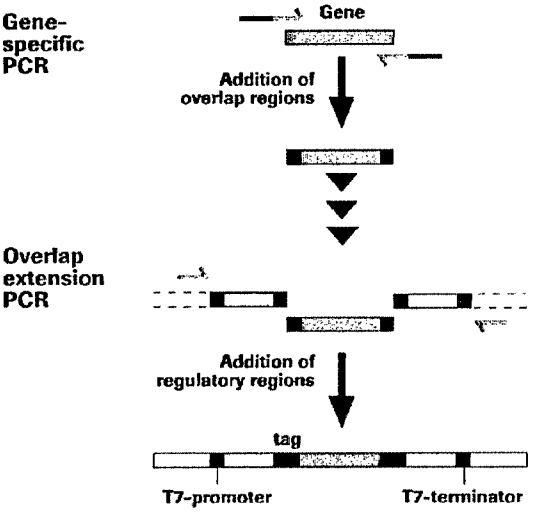

Figure 4. This figure diagrams the PCR followed by overlap extension to add the regulatory and tag sequences.

The design of the epitope tags and regulatory sequences was modeled after expression vectors that are routinely used in the lab for ligating exons into for expression in bacterial cells. This plasmid was used as the template for producing the regulatory sequences that need to be added to the exon specific PCR products using the overlap sequences. Sequence to code for 8XHis and the biotinylation signal sequence, GLNDIFEAQKIEWHE, was added after the ATG start codon. At the 3' end a CCACA repeat sequence, coding for PHHTT, the universal epitope, and stop codons in 3 reading frames were added. The general structure of the templates for in vitro expression are shown (Figure 5).

Figure 5. Diagram to show the structure of the sequences designed for overlap extension PCR. SD is the Shine Dalgarno sequence for ribosome binding in prokaryotic systems, RF shows the region where the reading frame is adjusted and UE is the universal epitope sequence.

Three sets of primers are used to generate the templates for in vitro reactions. Exon 3 is used as an example below (Figure 6).

A.

T7up5/OverlapA3

TCCGGCGTAGAGGATCGAGA...xxxxx... ATC GAA TGG CAC GAG () AAT *CGC AAG ACC AAA CAG AGA ACC*
aggccgcatctcctagctca...xxxxx... TAG CTT ACC GTG CTC () TTA **GCG TTC TGG TTT GTC TCT TGG**

EI3tp53Overlap5/EI3tp53Overlap3

CGCAAGACCAAACAGAGAACCgggaagcgaaaattccatgg ...333...CTGGAGGGCTGGGGAC*GCAGCACAAGGAGTCTCGAGC*
*GCGTTCTGGTTTGTCTCTTGG*ccttcgcttttaaggtacc...333... GACCTCCCGACCCCTG**CGTCGTGTTCCTCAGAGCTCG**

GCAGCACAAGGAGTCTCGAGC...yyyyy...ggattggcgaatgggacg
CGTCGTGTTCCTCAGAGCTCG...yyyyy...**CCTAACCGCTTACCCTGC**

OverlapUE5/T7down3

B.

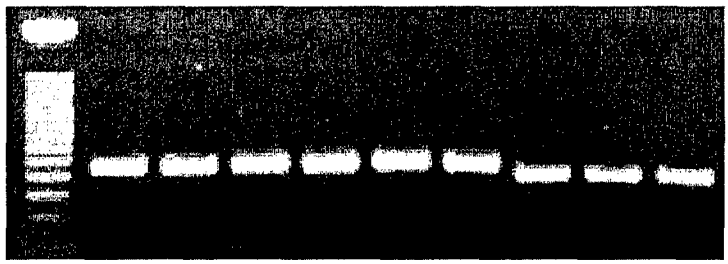

Figure 6. Details of the PCR reactions used to produce the template for the in vitro transcription/translation reactions. **A.** The 5' regulatory and 3' regulatory sequences are produced separately in addition to the exon specific product. Large amounts of the regulatory sequences are produced using plasmid DNA as template and the primers T7up5 and OverlapA3 for the 5' T7 promoter region and OverlapUE5/T7down3 for the 3' T7 termination region. In the next reaction the three pieces are combined so that the overlap sequences shown in italics anneal and extend. T7up5 and T7down3 primers are also included to provide primers for the amplification of the full length product. To produce reverse reading frames the exon-specific PCR is carried out using primers with the overlap sequences reversed. **B.** Agarose gel showing the regulatory regions produced by PCR. Lane 1, 50 bp molecular weight marker, lanes 2 to 7 show the T7 promoter fragment in 6 reading frames and lanes 8 to 10 show the T7 termination fragment.

By optimizing the second PCR reaction conditions and primer concentrations the exons were produced in the natural reading frame and some in alternate reading frames (Figure 7).

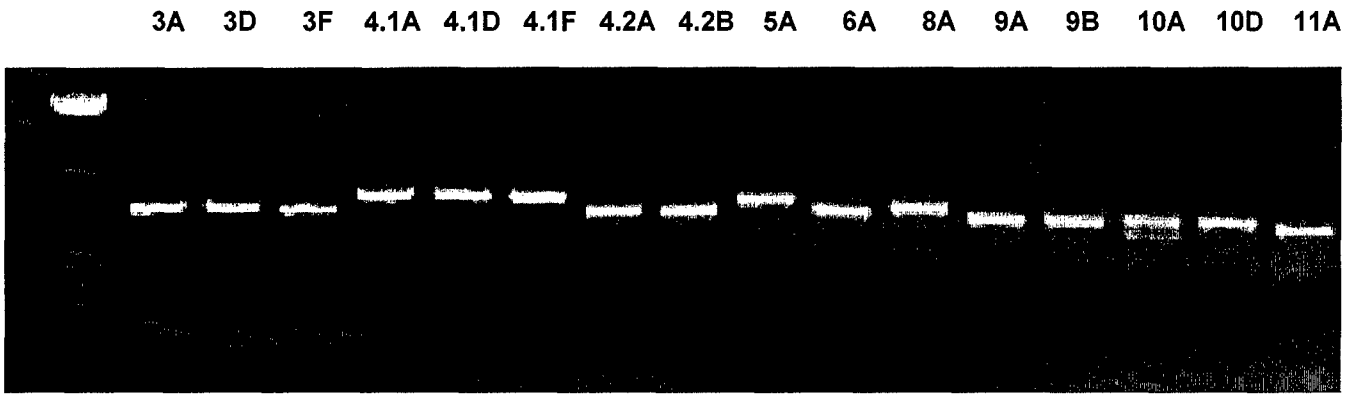

Figure 7. These gels show the overlap extension PCR products containing all of the sequences shown above. It is these DNAs that were used as template in the *in vitro* coupled transcription/translation reactions. The nomenclature is such that A is the natural reading frame, B is forward and D and F are reverse frames.

In separate experiments the expression vector was modified to replace the *lac* operator sequence. These constructs are being tested. The sequence inserted is shown below.

Bgl II **XbaI**
A/GATCTCGATCCCGCGAAAT[redacted]GGAGACCACAACGGTTTCCC**T/CTAGA**
TCTAG/AGCTAGGGCGCTTTAATTATGCTGAGTGATATCCCTCTGGTGTTGCCAAAGGG**AGATC/T**

Figure 8. The oligos shown above were inserted into the expression vector to insert the sequence in red in place of the lac operator sequence.

The production of the amplicons for use as template in coupled in vitro transcription/translation reactions successfully accomplished the goal of Task 2.

Task 3. Development of reproducible protocols for the purification, cleavage and MALDI-TOF MS of the peptides. Months 9-12.

a. Empirical optimization of purification protocols for high throughput microcolumns.
b. Empirical optimization of formic acid cleavage conditions.
c. Confirm presence of all expected peptides using MALDI-TOF mass spectrometry.
d. Empirical optimization of MALDI-TOF matrix and operating parameters.
e. High signal to noise for accurate mass determination and sensitive detection of new mutant peptides.
f. Validation of protocols using known mutations and large numbers of replications to assure reproducibility.

Experiments were conducted to select the extract to use for PMSG that gave consistently high yields of peptides of the expected mass with few proteolytic fragments. A comparison of commercial systems from Qiagen, Novagen, Invitrogen, Promega, Roche and Ambion revealed Qiagen's EasyXpress system to be the most reliable for providing the highest yields of peptides purified using IMAC agarose beads (Figure 9).

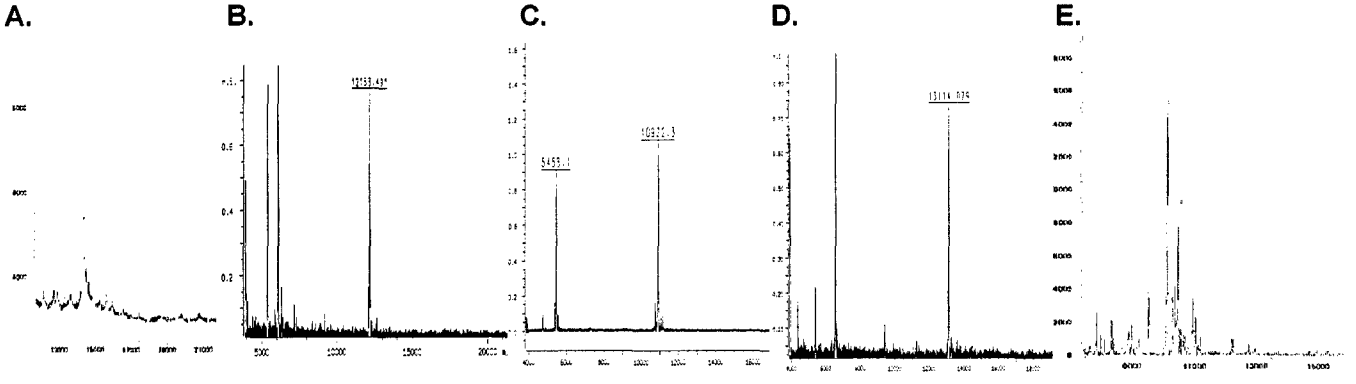

Figure 9. Spectra of peptides representing exons **A.** 5, **B.** 6, **C.** 7, **D.** 8, **E.** 9 of the *TP53* gene produced in coupled *in vitro* transcription/translation reactions.

Experiments were then conducted to improve the purity of the peptides of interest. Efforts to improve the purification using a single epitope proved insufficient and therefore the biotinylation signal sequence was employed. The addition of biotin ligase (Avidity) and biotin into the in vitro extracts results in biotin being covalently attached to the lysine in the signal sequence. This provides two independent methods to purify the peptides of interest. Protocols were empirically determined where the IMAC was first or second, different types of streptavidin matrices were tested, different binding and elution conditions were tested until a protocol was optimized and standardized.

The protocol utilizes IMAC capture with Strep Mutein agarose beads (Roche) followed by MagBeads (Qiagen's Ni-NTA magnetic agarose beads). The tandem affinity purification improved the purity of the peptides. Some examples are shown in Figure 10.

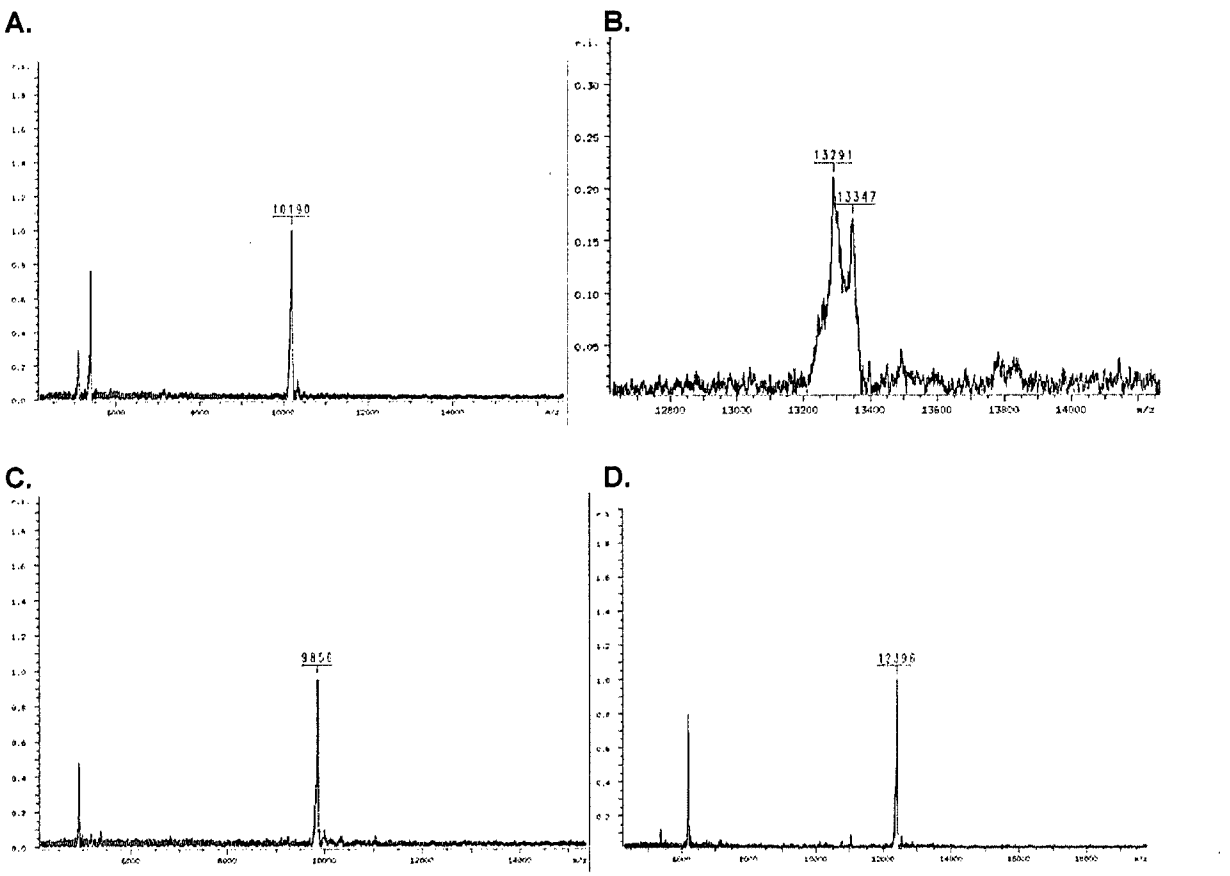

Figure 10. MALDI-TOF spectra acquired using linear PCR products directly in the coupled transcription translation reactions to express recombinant peptides in alternate reading frames and purification of the peptides via biotin and 8 His prior to spotting. **A.** Exon 3 reverse reading frame, **B.** first half of Exon 4 in the natural reading frame showing the R and P variants at codon 72, **C.** Exon 10 natural reading frame, **D.** Exon 10 reverse reading frame.

These results accomplish the overall Task 3 goals.

Key Research Accomplishments

The major accomplishments of this project show that PMSG can be transferred from a cumbersome assay that requires recombinant DNA steps followed by transformation and growth bacterial cultures to a very simple in vitro assay. The key elements for achieving this include optimized PCR protocols for amplification of each of the expressed exons of the *TP53* gene, production of linear template from each of the exons in the natural reading frame and alternative reading frames, addition of the regulatory elements required for in vitro coupled transcription/translation reactions and optimized double affinity purification protocols.

Reportable Outcomes

The outcome of this project is a simple, in vitro PMSG process that can sensitively and accurately determine the mutational status of a tumor.

Conclusions

PMSG is a very promising technology that could become the method of choice for analyzing breast cancer DNA samples for mutations. Accurately assessing panels of genes will uncover genotype/phenotype correlations that improve our understanding of tumor etiology, progression, recurrence and response to therapy. This understanding can be applied to develop better diagnostics and treatments that ultimately decrease the pain and suffering caused by cancer.

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