1997

Detection of the mutated K-Ras biomarker in colorectal carcinoma

Benjamin R. Doolittle
Yale University

DETECTION OF THE MUTATED K-RAS BIOMARKER IN COLORECTAL CARCINOMA

Benjamin R. Doolittle

March 1997

Detection of the Mutated K-Ras Biomarker in Colorectal Carcinoma

A Thesis Submitted to the Yale University School of Medicine
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Medicine

Benjamin R. Doolittle

March 1997

DETECTION OF THE MUTATED K-RAS BIOMARKER IN COLORECTAL CARCINOMA, Benjamin R. Doolittle, Janet Emanuel, José Costa, Department of Pathology, Yale University, School of Medicine, New Haven, CT.

This study describes an advantageous, effective protocol for detecting k-ras mutations in human stool as a prototype screen for colorectal carcinoma (CRC), the third most common malignancy in the United States. A reliable screening test that detects early lesions would contribute to a decrease in mortality. Currently, the only non-invasive screen for CRC is the heme-occult test which has a high false-positive rate. Previously, several investigators have identified genetic biomarkers for CRC in stool DNA. The k-ras oncogene, mutated in 46-50% of CRC tumors, serves as one molecular marker by which stool samples may be evaluated for early detection of adenocarcinomas.

DNA was isolated from stool samples by a new method we specifically designed for extracting high quality DNA using tetradecyltrimethylammonium oxalate (Catrimox-14). This protocol produces an optimal yield of high purity DNA, suitable for genotyping. Detection of the human gene in stool samples was enhanced by hybrid selection of the k-ras sequences, Polymerase Chain Reaction (PCR) and single-strand conformation polymorphism (SSCP analysis).

Tumor tissue and pre-operative stool samples for eight patients were k-ras genotyped and compared; stool samples from two asymptomatic, healthy patients were also evaluated in a double blind format. In seven of eight samples (87%), the genotype of the stool and colon tissue DNA was the same. Both healthy patients showed wild-type k-ras. This protocol shows promise for the development of an efficient and accurate screen for CRC.

Acknowledgments: For the guidance, friendship, trust and encouragement, I am indebted to Janet Emanuel who set me to soar in this project. For José Costa, I am grateful for the vision and enthusiasm he inspires. For Debbie Dillon, Veronica Dombi, and Lola Bautista, I am forever in their service. For Christine Damico, I am thankful for her preparation of the tumor DNA samples, and initial work on the gene enrichment procedure. Without their help, always offered in friendship, this work would not have been possible.

For Tanya, the joy of my life, I am ever so deeply thankful for her loving support.

Table of Contents

Tables and Figures

Detection of the Mutated K-Ras Biomarker in Colorectal Carcinoma
Yale Medical School Thesis

Benjamin R. Doolittle
Thesis Advisors: Janet Emanuel, José Costa

Introduction:

Colorectal carcinoma is the third most common human malignancy in the United States, accounting for 60,000 deaths each year. (1) Currently, the only non-invasive screen to detect colorectal cancer is the heme-occult test which has a high false-positive rate due to benign bleeding diseases such as hemorrhoids, diverticulitis, and interference by dietary peroxidases and catalases. Further, colorectal tumors less than 2 centimeters in diameter often do not bleed. (2) Among patients with malignancy, the sensitivity of the heme-occult test is 50-70%. (2) Currently, data are insufficient to indicate if screening for colorectal carcinoma with the heme-occult test decreases mortality. (2)

Colorectal carcinoma is curable in 90% of cases if the tumor is localized to the inner lining of the colon. Yet, five year survival drops to 10% if distant metastasis is present. Because the single most important prognostic indicator of colorectal carcinoma is the extent of

the tumor at the time of diagnosis, a reliable screening tool that detects early lesions shows promise to decrease mortality.(3)

Mutant oncogenes and tumor suppressor genes provide specific markers from which a screening test may be developed. (1) The disorganized, unregulated cell growth of malignant cancer is associated with a multi-step series of somatic cell mutations. Three to six genetic mutations are necessary before the regulatory pathways of cell growth are disrupted and dysplastic growth occurs.(4) Thus, a mutation acquired in one generation is passed to the cell's progeny which then accumulate new mutations, until the ability to regulate cell growth is disrupted. Most cancers occurs in the later years of life because this multi-step process may require decades.(4) Malignant cells in a tumor have the same mutations as the benign cells with the addition of at least one further mutation.(4) In colorectal carcinoma, these sequential mutations have been characterized.(4) The mutation of k-ras can cause an intermediate adenoma. A later "hit" which inactivates the DCC gene may cause a late adenoma. Invasive carcinoma is characterized by the loss of p53.(4)

46-50% of of colorectal adenomas and carcinomas show mutations of the k-ras oncogene.(2,6) The k-ras protein is located on the cytoplasmic side of the cell membrane and functions as a signal transducer which promotes cell growth. In the active state, k-ras can bind and cleave GTP. The k-ras protein is inactive when bound to GDP. A defective k-ras gene which loses GTP-ase activity becomes continuously activated, and promotes unregulated, proliferative cell growth. (7) Since k-ras is a signal transducer, only one copy of k-ras

need be inappropriately activated for unregulated cell growth to occur. (6)

Human stool provides a non-invasive specimen of the colonic epithelium and may be used as the material from which DNA can be extracted. Genetic markers can then be amplified, and analyzed for mutation. Since colonic renewal is estimated at 1% per hour, with an entire colonic turnover each 3-4 days, tumor cells are likely being shed into stool. (2) While stool provides a non-invasive specimen of the colonic carpet, it is a hostile environment for identifying intact genes of human origin. Stool contains an abundance of bacteria, undigested food particles, bile, mucous, as well as enzymes that can break down nucleic acids.(3)

Initial reports identifying genetic markers from stool DNA show promising results. Sidransky et al (1992) first reported that among nine patients with k-ras mutations identified in their colon tumor, eight (89%) were positive for the same k-ras mutations in their stool samples.(1) Smith-Ravin et al (1995) report that 50% of stool samples exhibit k-ras mutations identical to the mutation found in tumor cells.(2) Eguchi et al (1996) report that among 11 patients with a p53 mutation identified in their tumor sample, 7 (64%) show evidence of a p53 mutation in their stool sample.(8) Hasegawa (1994) was able to detect the identical k-ras mutation in DNA extracted from stool in 15 of 19 patients (79%) with colorectal tumors.(9)

More recent studies analyze colonic washings in conjunction with endoscopic evaluation to correlate observed endoscopic findings with the detectability and genotype of k-ras mutations. Tobi (1994)

surveyed 39 healthy patients who were undergoing endoscopy and was able to detect a k-ras mutation in 7 of 39 patients (18%). (10) Among the colonic washing of 230 patients, Villa was able to amplify the k-ras gene sequence in 103 patients (45%) and found only 45 k-ras mutations.(11)

While Sidransky's initial study demonstrated that existing technology could amplify the k-ras gene and identify the mutation in the stool, his study was not blinded. More recent, blinded studies show a lower yield. Tobi (1994) and Villa (1996) were able to amplify less than half of their samples for k-ras. A protocol which consistently recovers DNA from stool and reliably amplifies the genetic marker would greatly enhance the development of a screening test for colorectal carcinoma.

This study refines the methodology of genetic analysis in human feces and incorporates new technology to more accurately and efficiently extract and quantify DNA, amplify the k-ras gene, and analyze the gene product for mutation.

In particular, this study uses Catrimox-14 (CM) (Iowa Biotechnology Corp., Iowa, USA) to extract DNA from human stool. Catrimox-14 is a cationic surfactant, tetra decyltrimethyl-ammonium oxalate, that can lyse cells, inhibit ribonucleases, and precipitate RNA and DNA in reverse micelles.(13) Catrimox-14 is shown to remove inhibitors of Taq polymerase and reverse transcriptase that are present in stool.(15) Macfarlane and Dahle (1993) have successfully extracted RNA from cells in culture and whole blood.(16,17) Uwatoko et al (1996) report that Catrimox-14 has been shown to extract viral DNA from fecal specimens of cat, chicken, cow, dog, and

gerbil.(14) The role of Catrimox-14 in extracting human DNA from stool is not documented and is evaluated in this study.

Isolation of the k-ras gene sequences from the stool DNA is facilitated using oligonucleotide hybridization and streptavidan-coated magnetic bead technology. Because DNA quantitation using optical absorption has been shown to vary widely compared to gel analysis techniques, DNA quantitation was acheived using both traditional optical absorption techniques and microfluorimetry with SYBR-Green dye.(12) Accurate quantitation of DNA by fluorescence determination allows efficient Polymerase Chain Reaction (PCR) and Single-Strand Conformational Polymorphism Analysis (SSCP).

Statement of Purpose:

This study shows an improved protocol to consistently and efficiently recover DNA from human stool, reliably amplify the genetic marker and analyze the marker for mutation. This study furthers the development of a mutation based screening test for colorectal carcinoma.

Table 1. Literature Summary of Stool Biomarkers in Colorectal Carcinoma

Study	Mutation	Technique	Sample	Findings mutations/tumor	mutations/stool sample
Eguchi (1995)	p53 exon 5-8	pcr(oligo spc probes)	25 pts w/ colorectal ca	11/25 w/ p53 mut in tumor	7/11 w/ p53 in stool (smallest 2.8x2. 3/5 w/ p53 mutation were heme neg
Hasegawa (1994)	k-ras codon 12,13	pcr (allele specific)	55 pts w/ adeno ca	19/55 w/ k-ras mut in tumor	15/19 w/ "pcr-able" DNA in feces 3/15 w/ k-ras in stool tumors 4x4 cm in sigmoid or rectum
Sidranksy (1992)	k-ras codon 12,13	pcr (phage DNA transfer)	9 pts w/ colorectal ca & k-ras mut. prox. & distal tumors		8/9 pts w/ mutation smallest 1.5x1.5x0.6 in sigmoid
Smith-Raven (1994)	k-ras codon 12	pcr(allele specific)	11 pts w/ colorectal ca prox & distal tumors	7/11 w/ k-ras mut in tumor	5/11 w/ mut in stool pellet 4/11 w/ mut in stool supernatant smallest tumor 3.5x4.0
Tobi (1994)	k-ras codon 12	pcr	39 pts for colonoscopy 7 w/ family hx, 5 post-op check, 13 IBD, 1 F.A.P., 6 normal		7/39 w/ k-ras mut in "colonic effluent 3/7 w/ +fh & nl scope 0/6 w/ nl, 0/13 w/ IBD 1/1 w/ FAP, 3/5 w/ post-op
Villa (1996)	k-ras exon 1	pcr (oligo spc probes)	230 pts for colonoscopy 6 rectal bleed, 31 post-op check 67 s/p excision, 19 IBD 68 diarrhea, 39 w/ family hx	5/5 w/ k-ras mut in tumor	103/230 w/ wt kras in stool 30/103 w/ k-ras mutant 4/5 w/ ca found on scope 11/67 s/p adenoma excisio smallest tumor 1.0x1.0 cm

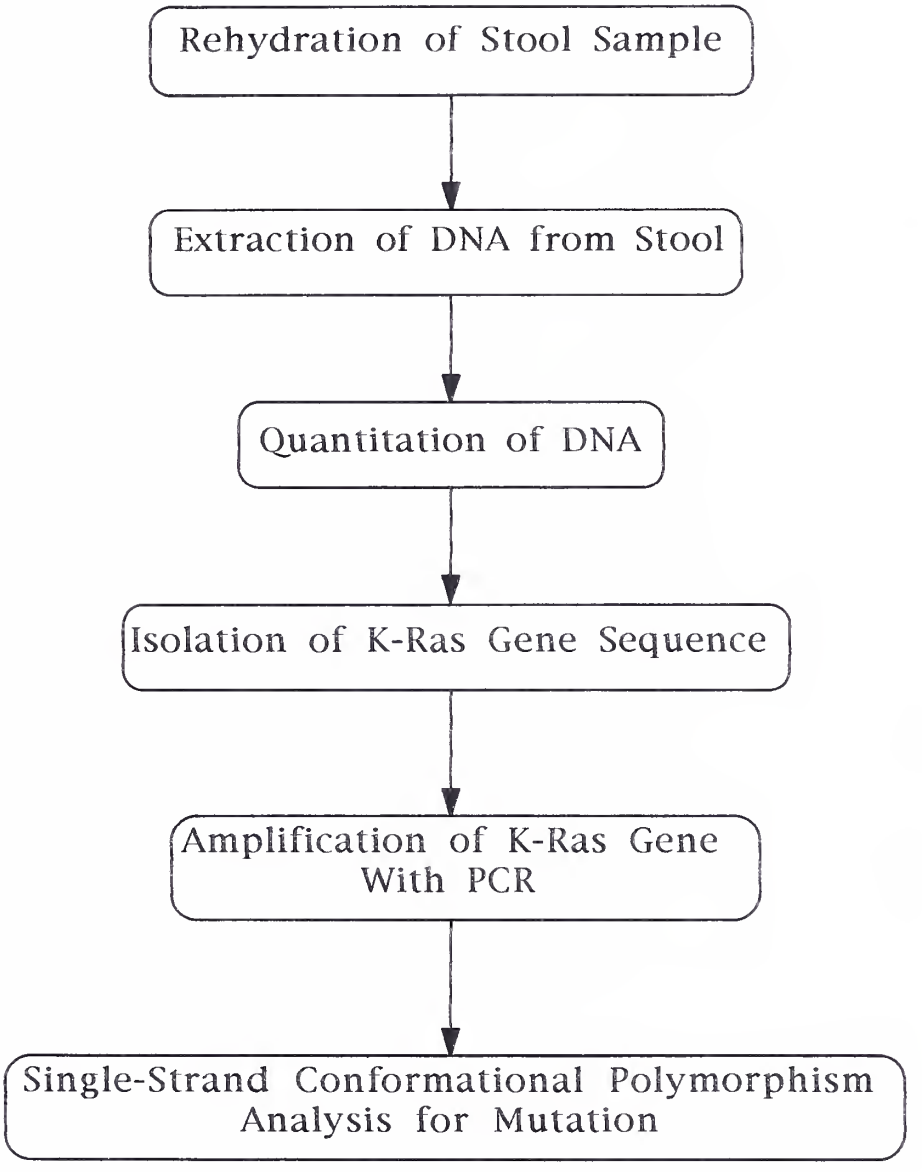
Materials and Methods:

Samples: Fresh stool samples from a healthy, asymptomatic subject were collected and held 1-3 days at 4 °C before processing. Samples weighed between .5-1.5 grams.

Stool and matched tissue samples were collected from patients with colorectal cancer and random patients with no personal or family history of colorectal cancer. The stool samples were archived by Dr. Robert Dubrow (Yale School of Epidemiology and Public Health). For archiving purposes, the stool samples had been lyophilized at -70 °C. Dry weight of samples were .12-.14 grams. From this bank of samples, 11 stool samples and 23 colon samples were processed in a double-blind fashion.

Samples of tumor and normal tissue for DNA extraction were microdissected from sections of paraffin embedded patient tissue.(19)

Table 2. Protocol for Detection of Mutated K-Ras Biomarkers of Colorectal Carcinoma



DNA Extraction Procedure:

Two different protocols were followed for DNA extraction from stool, a standard organic extraction protocol and the use of Catrimox-14, a cationic surfactant.

A. Organic Extraction:

The stool was digested in 3 ml of proteinase K solution (250 mg/ml in 0.01 M Tris-HCl, pH 8.0, 0.1 M NaCl. 0.001 M Na EDTA, 0.5% SDS) overnight at 37 °C and extracted twice with equal volumes of phenol and twice with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1). Separation of aqueous and non-aqueous phases was facilitated by the presence of Phase-Lock-Gel in the tubes (5Prime-3Prime, Boulder, CO, USA).

Each sample was further purified with a potato flour extraction. 500 mg of potato flour was added to the aqueous sample, rotated for 1 hour, and centrifuged at 1,500 x g for 7 minutes. Potato flour further purifies DNA samples by binding to bile salts and other pigments. (20)

DNA was precipitated from the aqueous phase by addition of 2 volumes 100% ETOH and .1 volume 3 M NaOAc (f.c. 70% ETOH, .1M NaOAc). The samples were stored at -20 °C overnight to ensure adequate precipitation. After recentrifugation, the samples were suspended in 1-4 ml of TE (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and analyzed using spectrophotometry, microplate fluorimetry with SYBR-I Green dye, and agarose gel analysis. (12)

B. Catrimox Extraction (Table 3):

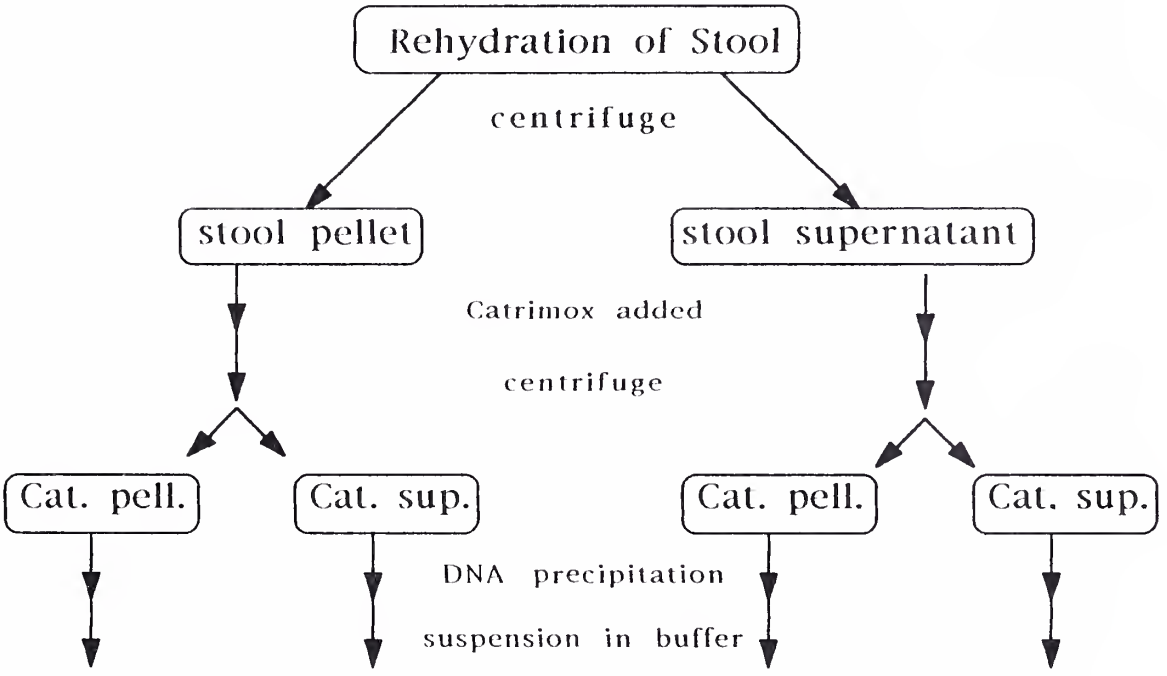
DNA from several lyophilized and fresh stool aliquots were extracted using Catrimox-14 (CM) (Iowa Biotechnology Corp., Iowa, USA).

.12-.15 mg of lyophilized stool samples were mixed with 2.5 ml TE (10 mM Tris, 1 M EDTA, pH 8.0) and agitated until a uniform sluice formed. The samples were centrifuged at 2,000 x g for 7 minutes. The supernatant and the stool pellet were processed separately. 3 ml of CM was added to both the supernatant and stool pellet. The samples were agitated on a shaker for 2-5 hours until a uniform sluice formed from the stool pellet. The samples were centrifuged at 1,500 x g for 7 minutes. The supernatant was removed from each of the CM pellets and processed separately.

To the CM pellets and CM supernatants, 2 volumes of ethanol were added to dissolve the DNA from the CM pellet. Precipitation followed with 1 volume of TE and .1 volume of 3 M NaOAc. The samples were stored at -20 ^{o}C overnight to ensure DNA precipitation.

Samples were then centrifuged for 10 minutes at 2,500 x g. The DNA pellets were washed twice in 70% ethanol, air dried, and resuspended in 1 ml of TE. (Figure 1, 2)

Table 3: Catrimox Extraction Protocol
(Cat.= Catrimox, pell.=pellet, sup.=supernatant)



DNA Quantitation:

DNA samples were quantified using both optical absorption (OD 260/280) and the CytoFluor II Multi-Well Fluorescence Reader (PerSeptive Biosystems, Inc., USA).(12) The CytoFluor measures emission of 520 nm light from fluorescent SYBR-Green I dye complexed with DNA. Samples were quantified by diluting 2 ul, 5 ul, and 10 ul aliquots of DNA sample into a total volume of 150 ul of TE with 1/2000 dilution of SYBR-Green I dye in a Dynatech Microfluor 96 Black "U" Bottom Plate (Dynatech, Virginia, USA). Each dilution was repeated in triplicate. Each well was scanned 10 times with a fluorescent excitation wavelength of 485+/-20 nm and measured the emission wavelength of light at 530 +/- 30 nm.

A standard curve using known DNA amounts of placental DNA was generated. The average of the triplicate values of each dilution was used to calculate total DNA per well and DNA per microliter of sample. Data were analyzed using Microsoft Excel spreadsheet (version 5.0).

DNA was also quantified using optical absorption for comparison. Each sample was diluted to 1/100 and 1/200. 50 ul of each diluted sample was placed in a microcuvette and absorption was measured at 260 and 280 nm using a Lambda-2 Spectrophotometer (Perkin-Elmer Corp., Connecticut, USA).

Enrichment of K-Ras DNA Sequence from Stool DNA:

75 µg of DNA extracted from the lyophilized stool samples was denatured at 98 °C for 8 minutes. The samples enriched for the k-ras gene sequence were the CM extracted DNA and the CM supernatants of the TE sluice (table 3). 1 picomole of biotinylated primers R33 and 13 bx, specific for k-ras, were added to the sample (R33 5'-GTATCAAAGAATGGTCCTG-3', 13bx, 5'-GTAGTTGGAGCTGGTGG-3').(19) Primers and DNA annealed at 55 °C for 1 hour.

25 ul of 2 mg/ml Streptavidin magnetic beads (SAMB) suspended in 50 mM Tris pH 8.4 was added to each sample. The mix was rotated at room temperature for 2 hours. Streptavidin magnetic beads (Dynal M-280 Streptavidin) are uniform superparamagnetic, polystyrene beads with streptavidin covalently attached to the bead surface.(18) Streptavidin is a protein with a high affinity for biotin and enables separation of DNA hybridized to biotinylated oligonucleotides.

The samples were placed in the Dynal Magnetic Particle Concentrator (MPC) to concentrate the beads bound with the biotinylated DNA. The supernatant was removed. The magnetic beads were washed twice with 1 M STE (10 mM Tris, 1 mM EDTA, 1 M NaCl).

The DNA was eluted from the magnetic beads by adding 30 ul of ddH20 and heated to 70 °C for 10 minutes. After placing the sample in the MPC, the ddH20 was removed. 3 ul of 10x TE was added to the ddH20 for storage of DNA.

Polymerase Chain Reaction:

K-ras was amplified from both the original CM treated DNA and from the enriched samples of k-ras DNA. A 151 base pair product of exon 1 of k-ras was amplified from stool using the primers R53 (5'-GACTGAATATAAACTTGTGG-3') and R32 (5'-AATGGTCCTGCACCAGTAAT-3'). (19)

The DNA from the SAMB enrichment was amplified in two consecutive PCR reactions ("booster" PCR) in a 30 ul reaction containing .1 uM of each R32 and R53 primer, 100 uM of each dNTP, 0.16 ul of 5 U/ml Ampli*Taq* polymerase (Perkin-Elmer, Corp., Wilton, CT, USA) and .16 ul of Taq Start Antibody (TSA) in a buffer containing 40 mM KCl, 10 mM Tris HCl pH 8.4, 0.1% Triton X-100, 0.01% Gelatin, and 2.5 mM MgCl2.

3 ul of the 33 ul isolated enriched k-ras gene was amplified. After a 5 minute denaturing step at 96 °C, 5 cycles of stringent amplification was completed at 94 °C/30s, 59°C/45s, 73°C/45s, was followed by 20 cycles at 92 °C/30s, 56°C/45s, 73°C/45s.

From this PCR product, 3 ul was re-amplified in a 30 ul reaction subject to a denaturing step at 96 °C, followed by 40 cycles of amplification at 92 °C/30s, 56 °C/45s, 73 °C/45s in a Hybaid OmniGene (Sun Biosciences, Madison, CT, USA). All PCR reactions were run with positive and negative DNA controls.

Quantitation and size of DNA product was evaluated by comparison with a standard 100 base pair ladder, GelMarker (Research Genetics, Huntsville, AL, USA).

Single-Strand Conformational Polymorphism:

Single-Strand Conformational Polymorphism (SSCP) is a technique where mutations may be easily visualized by denaturing the DNA sample and running the sample through a high-voltage acrylamide gel. Each strand of denatured PCR product forms a unique conformation which can be compared to the known wild-type and mutant patterns. Genes with even a point mutation are shown to exhibit different conformations.

Conditions for SSCP that distinguish each k-ras mutation have been developed.(19) 20 ng of PCR product was denatured using 3 volumes of DDS (950 ul formamide, 2 ul 5.0 N NaOH, 25 ul 0.1% brom-phenol blue, and 25 ul 0.1% xylene cyanol). The mixture was heated to 96 ^{o}C for 7-10 minutes, quickly placed on ice and loaded immediately into a 15% acrylamide mini gel (37.5:1) polymerized with 0.075% ammonium persulfate and 0.05% TEMED. The gel was run for 80 minutes at 300 volts. 1.0 x TBE buffer was used for gel electrophoresis. The gel was quickly washed with TE pH7.4 and stained in the dark with SYBR-Green I stain, diluted 1:10,000 in TE pH 7.4 for 15 minutes. Imaging was accomplished using an alpha-Innotech IS1000 digital imager with an SG 3 filter (Figure 5, 6). Samples were compared with sequence identified mutant controls.

Results

Extraction and Quantitation:

Catrimox-14 extraction of DNA is an efficient and viable alternative to traditional phenol/chloroform extraction techniques. Figure 4 shows that the yield from comparative aliquots of stool are much greater with the use of Catrimox-14. The yield from the clinical stool samples was 664-1750 micrograms of DNA from .12-.15 mg of lyophilzed stool sample. Traditional extraction methods yielded 250-700 micrograms of DNA from .5 - .75 mg of fresh stool sample.(Figure 4) DNA extracted from the TE/Catrimox pellet was the most abundant compared to the stool pellet/Catrimox pellet and supernatant samples. (Table 3, Figure 2)

Table 4 compares the amount of DNA quantitated using the CytoFluor Fluorescence Emission reader and the optical absorption spectrophotometer for the samples visualized in Figure 1. Quantitation using the fluorescence analysis with SYBR dyes shows consistent readings among diluted samples and confirm the relative amounts of DNA visualized on agarose gel analysis. (Figure 1)

The optical absorption technique shows wide discrepancy in readings. (Table 4) For example, optical absorption of the CM pellet #2 showed a DNA concentration of 243 ng/ul with a standard deviation of 228 ng/ul. An aliquot of sample #2 extracted with the phenol/chloroform method showed a five-fold difference in DNA concentration between the 1/100 and 1/200 dilutions with an average of 1565 ng/ul and a standard deviation of 1973 ng/ul. This

inconsistency may be due, in part, to handling and contaminants which affect the absorption at the 260 nm and 280 nm wavelengths. Purity of the stool DNA samples were low. OD 260/280 ratios, a measurement of DNA purity, were between 0.88 - 2.06. (Table 4)

Comparison between the fluorimetric analysis and optical absorption was consistent in only stool sample #6 Catrimox-14 supernatant which showed an average DNA concentration 273 ng/ul and 278 ng/ul respectively. (Table 4) Inconsistencies between fluorimetic analysis and optical absoprtion varied by as much as 10-fold.

Table 4: DNA Quantitation By CytoFluor Plate Reader and Optical Density

Stool Sample	Cytofluor Plate Reader DNA ng/ul +/- std.dv.	Optical Density DNA ng/ul +/- std.dv.	OD 260/280
#2 cm pellet	617 +/- 33	243 +/- 228	1.26
#2 cm supernatant	12 +/- 1.7	113 +/- 3.6	2.06
#6 cm pellet	182 +/- 20	33 +/- 39	1.50
#6 cm supernatant	273 +/- 20	278 +/- 46	1.51
#2 phenol/choloform	135 +/- 18	1565 +/- 1973	1.07
#3 ""	685 +/- 91	390 +/- 0	1.21
#4 ""	2779*	205 +/- 63	0.88
#5 ""	616 +/- 183	438 +/- 591	0.95
#6 ""	359 +/- 9	35 +/- 7	0.90

legend: "cm" represents DNA quantified from a Catrimox-14. "phenol/chloroform" repesents DNA quantified from phenol and chloroform extraction. * sample #4 has one cytofluor reading.

Compare with quantitation by gel electrophoresis, Figure 1.

k-Ras Enrichment and Polymerase Chain Reaction:

Isolation of the k-ras gene sequence with the SAMB, and amplification by a PCR boost protocol was successful in 10 of 11 samples.

When the stool DNA samples were concentrated 5-fold, but not enriched for the k-ras gene sequence with the SAMB, no amplification of the k-ras gene sequence occured using the booster PCR protocol. (data not shown) In five samples of stool DNA enriched for the k-ras gene sequence with SAMB (A, B, C, D, J), the k-ras gene sequences was amplified in both the CM pellet and supernatant. In three samples (E, F, I), the k-ras gene was amplified from the CM pellet only. In two samples of stool DNA enriched for the k-ras gene sequence (G, H), k-ras was amplified only in the CM supernatant. (Table 5)

Single-Strand Conformational Polymorphism:

Among the 11 stool samples, 10 amplified k-ras gene sequence well enough to provide enough product (20ng) to be genotyped with SSCP. The detected mutations in stool samples were 5^{wt}, 2^{gat}, $2^{gat/gac}$, $1^{gat/agt}$, 1^{tgt}, and 1 unspecific mutation. (Figure 5, Table 5)

Among the colon tissue samples which amplified the k-ras gene sequence, 20 provided enough product to be genotyped using SSCP. The mutations detected in the colon samples were 6^{wt}, $8^{gat/agt}$, 4^{gtt}, $2^{gat/gac}$. (Figure 6, Table 5)

When the k-ras gene sequences from the stool samples and colon samples were compared, 2 wild-type stool samples (B, D) correllated with two wild-type colon samples. Sample D came from a patient with a 2.2cm invasive carcinoma that did not harbor a k-ras mutation. Sample J showed wild-type k-ras and came from an asymptomatic subject for which no colon tissue specimen was available. Sample I also came from an asymptomatic, healthy patient without family history for colorectal carcinoma. This patient's stool showed a wild-type mutation and a low-level GAT/GAC mutation in I pellet. No gastrointestinal work-up was available to confirm if the patient was disease free.

Among the 7 stool samples which showed mutations in their k-ras gene sequence, 5 were the same as those of their corresponding tumor. One stool sample showed a non-specific mutation pattern (E pellet) which might have reflected both the GTT and AGT mutation pattern detected in the colon samples. In the 7th sample (I), a mutation was detected in the stool for which there was no colon tissue sample to correlate.

The sensitivity of detecting the same genotype in the stool as in the colon sample is (7/8) 87%. To screen for *any* k-ras sequence mutation in the stool for which there is a mutation in the colon, the sensitivity is (6/6) 100%. The specificity of detecting a wild-type genotype is 100%. However, only two stool samples were tested for which a corresponding wild-type sample was obtained. From two asymptomatic patients (I, J) for which no colon samples were available, both showed wild-type k-ras with a low-level GAT/GAC mutation in I pellet.(Table 5)

Table 5: Genotype of Stool and Corresponding Tissue DNA

Stool Sample	Mutation	Colon Sample	Mutation	Comments
A pellet	GAT/wt	A site 1	wild-type	polyp
A supernatant	wild-type	A site 2	wild-type	polyp
		A site 3	GAT/AGT	2x2 rt transverse
B pellet	wild-type	B site 1	wild-type	2.2 cm invasive
B supernatant	wild-type	B site 2	wild-type	carcinoma
C pellet	GAT	C site 1	GAT	3.5x7.5x9 cm
C supernatant	TGT	C site 2	GAT	rectal carcinoma
D pellet	wild-type	D site 1	no amplification	1 x .2 cm margin
D supernatant	wild-type	D site 2	wild-type	sigmoid colon
E pellet	GTT/unspecified mutation	E site 1	GTT	1x.5x.5 cm
		E site 2	no amplification	sigmoid
E supernatant	no amplification	E site 3	AGT	appendix
		E site 4	GTT	sigmoid
		E site 5	GTT	polyp
F pellet	GAT/AGT	F site 1	GAT	mucosa
F supernatant	no amplification	F site 2	GTT	5x5x1cm sigmoid
G pellet	no amplification	G site 1	AGT	2.5x2.5x.6cm
G supernatant	GAC	G site 2	no amplification	sigmoid
		G site 3	AGT	
		G site 4	AGT	
H pellet	no amplification	H site 1	GAT/GAC	metastasis
H supernatnat	GAT/GAC	H site 2	wild-type	primary tumor
		H site 3	GAT/GAC	metastasis
I pellet	GAT/GAC/wt	asymptomatie		
I supernatant	no amplification	healthy patient	no sample	available
J pellet	wild-type	asymptomatic		
J supernatant	wild-type	healthy patient	no sample	available

note: Capital letters note the stool sample. Numbers note the colon sample. Thus, "A site 1" matches both the colon biopsy sample and the corresponding stool sample.

Discussion:

This study describes an accurate, efficient protocol for detecting genetic mutations in human stool. While the sample size is small, identifying the mutation in the stool had a high sensitivity and specificity for predicting the same genotype as in the tumor. This study suggests that a screening protocol for colon cancer is possible using efficient DNA extraction and isolation techniques and should be explored with a larger study sample incorporating a more representative population. The use of colonic washings of patients undergoing colonoscopy as in the studies of Tobi (1994) and Villa (1996) would provide a broad sampling of wild-type and mutants.

In particular, the use of Catrimox-14 has greatly reduced the time involved to extract DNA from human stool. Using the Catrimox-14 extraction protocol, the total time for the extraction of the stool samples was four hours. Extracting DNA from stool using traditional phenol/chloroform extraction methods required at least 3 days to allow for adequate digestion with proteinase K, the multiple phenol/chloroform/potato flour extractions, and overnight precipitation of DNA. The ease and yield of using Catrimox-14 to extract DNA from stool shows promise towards developing an inexpensive screening test.

DNA Quantitation Using Microfluorimetry and Spectrophotometry

Microfluorimetry with SYBR-Green dyes quicky and efficiently quantitates DNA extracted from stool. The amounts are consistent

with that seen on gel analysis. (Figure 1, Table 4) Impurities in the stool DNA do not affect the excitation and emission of the SYBR-Green dye in the Cytofluorimeter as they do in the optical density technique, since fluorescence of dye increases 1000-fold when bound to DNA and background fluorescence is very low.(12) Thus, SYBR dye fluoresence quantitation permits rapid evaluation of less pure samples. The lower limit of the microfluorimetry assay is 100 picograms.(12) This ability to measure a lower threshold of DNA is important when quantifying small amounts of DNA obtained from stool or other minimal patient samples.

The practical lower limit of spectrophotometry is 50-100ng of DNA in a 50-100 ul cuvette. (19) Purity of the stool DNA samples extracted with Catrimox-14 was poor with A260/280 ratios between 1.1-1.8. Given the impurities which remain in the DNA samples, absorption of light by other materials such as proteins distort the accurate quantitation of DNA.

The SYBR dye fluorescence detection is also an easier, more accuarate method than optical absorption for detecting small samples of DNA. The samples can be processed simultaneously, with only 1 ul of sample required. The optical absorption method requires that samples are processed sequentially and results between samples are shown to be inconsistent. (Table 4) Fluorescence analysis proves to be consistent at several dilutions. Also, several samples can be processed simultaneously since they are loaded on a plate with 96 wells.

Character of DNA Extracted from Stool with Catrimox-14

The k-ras gene sequence was amplified in both Catrimox-14 pellets and supernatants. This provides a clue to the behavior of Catrimox-14 as well as the nature of DNA being shed in stool. The DNA present in the supernatant was a very low amount detectable only by the CytoFluor Fluorescence reader (data now shown). Further, due to the low amount of DNA, only 25 µg was used in the isolation technique by the SAMB, while 75 µg of DNA was used from the Catrimox-14 pellet. Although only one-third the amount of DNA was used for the SAMB isolation, 7 of 10 CM supernatant samples were able to amplify their k-ras gene sequence. The "boost" PCR protocol was used since 9 % (3 ul of 33 ul) of the DNA eluted from the magnetic beads was used in the PCR reaction. An actual diagnostic assay could use all of the eluted DNA in a more concentrated solution. Since the entire colon sloughs cells into stool, then most human DNA in stool would be from non-tumor cells. Yet, this study shows that mutated biomarkers in stool which correlate with tumor tissue are present in detectable amounts. Tumor tissue may be shedding cells at a rate higher than normal tissue.

Human DNA present in stool may come from cells lysed by the Catrimox-14 or from necrosed cells sloughed from tumor. Much of the nucleic acids in stool derive from both bacterial RNA and DNA. Only 4-10% of DNA in stool is estimated to be human (unpublished data, C. Damico, J Emanuel). DNA present in the supernatant may be human DNA that was sloughed off from necrosed tumor cells rather from intact, normal cells. Since Catrimox-14 is a cationic surfactant that lyses cells, perhaps Catrmox has a preference for nucleic acids

within the cell rather than free DNA that is derived from necrosed tissue. DNA present in the CM supernatant may also be due to insufficient amount of Catrimox-14 used. The 3 ml of Catrimox used for the .12 - .15 mg lyophilized stool samples might not provide enough binding capacity on the reverse micelles to acquire the total amount of DNA in the stool samples.

In addition to lysing cells and forming reverse micelles with nucleic acids, Catrimox-14 is shown to remove inhibitors to PCR.(15) The removal of inhibitors may facilitate amplification of even the slightest amount of DNA. Prior studies used Catrimox-14 to isolate viral DNA and RNA from stool with success.(16, 17) Our protocol demonstrates excellent yield of DNA from human stool from which isolation for the k-ras gene sequence is feasible.

Further Evaluation is Necessary to Develop Screening Test

Further experiments on the role of Catrimox-14 in extracting DNA from stool remain to be explored before a screening test can be incorporated into the regimen of patient care. First, the stability of the stool-Catrimox-14 sample must be tested over time. If a patient sample is collected in an out-patient setting, the DNA must remain intact during the transportation to the lab and the lag time before processing. The DNA extracted from the lyophilized clinical samples yielded ample DNA. Would the DNA remain stable if it were not frozen? Would the most efficacious protocol call for the stool to be placed in the Catrimox-14 in the out-patient setting? Does the detergent quality of the Catrimox-14 degrade the DNA over time? To

apply this technology as a screening test, such practical concerns must be resolved.

Second, the use of hybrid enrichment probes should be further explored to incorporate multiple biomarkers for colorectal carcinoma. This study shows that oligonucleotide probes are an effective method for isolating k-ras as a biomarker in stool DNA. Since a large amount of DNA in stool is bacterial, and the number of mutated genes is small, efficient enrichment of the DNA sample for the specific biomarker is essential to enable PCR amplification. Further, incorporating other biomarkers would increase the catchment of the screening test. K-ras is mutated in only 46-50% of tumors. Thus, between 50-54% of tumors will not be detected since they will have wild-type k-ras. Among 230 patients, Villa (1996) amplified the k-ras sequence in only 103 patients' colonic washings and found only 45 mutations. In this study, sample B had a wild-type k-ras but the colon sample showed a 2.2 cm invasive carcinoma.

The tumor suppressor gene p53 is mutated in 60-86% of tumors. Isolating the p53 exons known to be commonly mutated (exons 5-8), in addition to the k-ras gene and other biomarkers, would augment the ability to identify early lesions. The use of hybrid enrichment for multiple biomarkers would broaden the scope of the screening test. Given the low and variable levels of human DNA in stool, enrichment for the biomarker gene sequence is essential for amplification by PCR. Our success with enrichment of the k-ras gene sequence with hybrid probes suggest that enrichment for multiple biomarkers is feasible.

Third, important to the screening test is the size of the tumor and its location in the colon. The smallest tumor detected in this study was a 1.5 x .5 cm tumor in the sigmoid colon. The smallest tumor in the literature cited for this paper is a .5 x .6 cm tumor in the rectum by Hasegawa (9) . The most proximal tumor detected in this study was 2.5 x 3.0 cm in the ascending colon. The most proximal tumor detected in the literature was in the cecum by both Sidransky (6 x 4 cm) and Smith-Ravin (7 x 7 cm).(1, 2) Since this screening test detects the DNA from shed cells, the tumor must be large enought to shed sufficient DNA to be detected. Given that tumors which have only invaded the mucosa have a 90% recovery, the tumor must be detected in its earliest stage for this screening test to be effective.(3) The tumor must be large enough to be detected, yet small enough to provide a good prognostic indicator for survival. The lower-limit of tumor size and its position in the colon remains to be evaluated.

Potential Role of Screening Test in Patient Care

The role of a screening test for colorectal carcinoma may be easily incorporated into the regimen of patient care in the same way that Papanicalou smears and mammography are incorporated into patient care. The expense and invasiveness of endoscopy are impediments towards its use. Further, endoscopy is not readily available in all parts of the country. Screening for biomarkers is not designed to replace the endoscopic work-up. Rather, screening for mutated biomarkers complements the role of physical exam and the more definitive endoscopic study. Screening for mutated biomarkers

helps raise the suspicion of disease in asymptomatic patients and provides a clue towards further work-up.

In an asymptomatic patient with a family history of colorectal carcinoma, for example, the genotype of biomarkers could be followed over the course of several years, beginning at an age prior to endoscopic work-up. Multiple wild-type biomarkers, such as both k-ras and p53, suggest that the patient is disease free. Discovering a subsequent mutated biomarker in the asymptomatic patient could allow intervention prior to the spread of invasive disease.

This study demonstrates that the technology exists for developing an efficient, viable screen for the third most common malignancy in the United States. This study outlines a previously unpublished protocol to efficiently extract DNA from human stool with a high yield. Further, this study incorporates new technology to accurately purify the DNA sample for a biomarker that is capable of PCR amplification. The genotype discovered in the stool with SSCP correlates with the genotype of the colon samples in 7 of 8 (87%) cases with 100% senstivity as a general screen for any k-ras mutation. These data are encouraging and strongly suggest that a screening test for colorectal carcinoma is accurate and feasible. Using this technology, a larger and more representative study population would evaluate the role biomarkers play in providing a powerful tool towards detection of colorectal carcinoma.

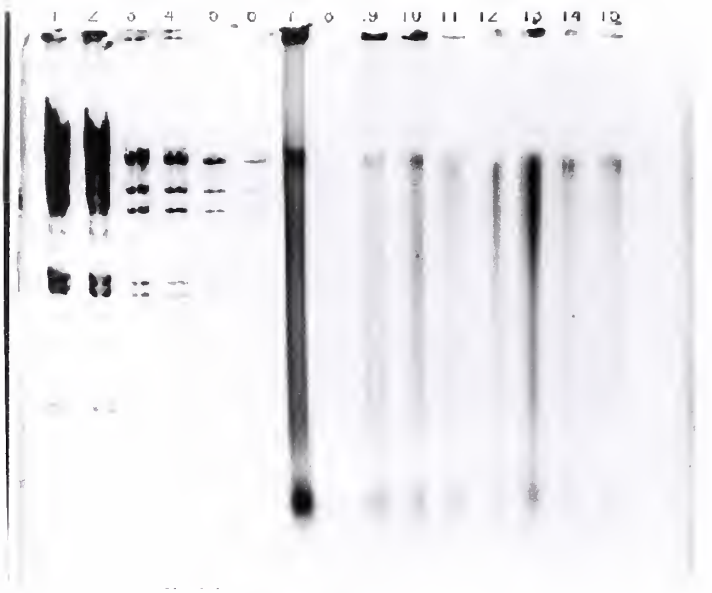


Figure 1. Comparison of DNA yield from stool aliquots. Lanes 7-10 are stool extracted with Catrimox-14. Lanes 11-16 are stool samples extracted with the traditional phenol/chloroform/potato flour method. The Catrimox-14 extracted samples are from an earlier protocol. Compare amounts of DNA by gel analysis with amounts measured with Cytofluor Plate Reader and Optical Density (Table 4).

Lane	Sample
1	Hind III marker - 500 ng
2	" - 250 ng
3	" - 100 ng
4	" - 50 ng
5	" - 20 ng
6	" - 10 ng
7	#2 Catrimox-14 pellet DNA extraction
8	#2 Supernatant from Catrimox-14 pellet
9	#6 Catrimox-14 pellet DNA extraction
10	#6 Supernatant from Catrimox-14 pellet
11	#2 Phenol/Chloroform/Potato Flour Extracted
12	#3 "
13	#4 "
14	#5 "
15	#6 "

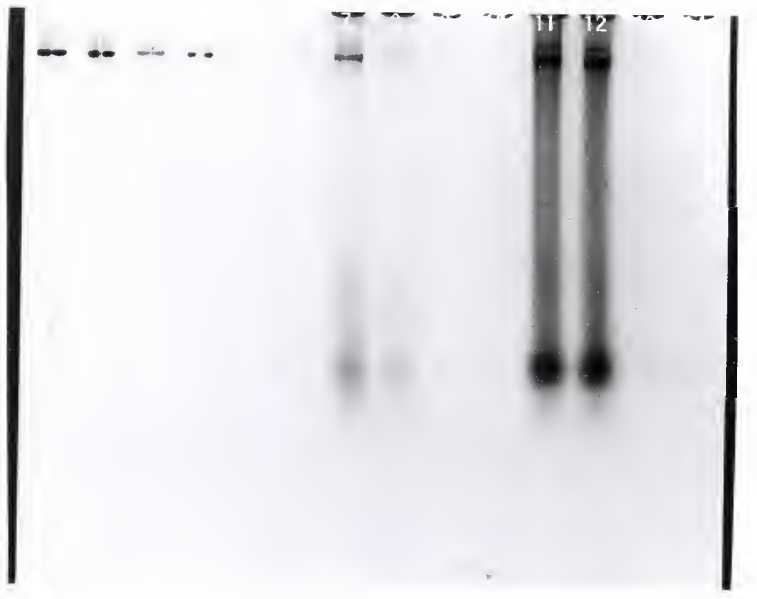


Figure 2. Catrimox-14 extraction of DNA from two aliquots of stool showing DNA yield from the stool/Catrimox-14 pellet, the stool/Catrimox-14 supernatant, the TE/Catrimox-14 pellet, and the TE/Catrimox-14 supernatant. Sample is from healthy male.

Lane	Sample
1	EcoRI digested placenta DNA - 300 ng
2	" - 200 ng
3	" - 100 ng
4	" - 50 ng
5	" - 20 ng
6	" - 10 ng
7	Stool/Catrimox-14 pellet #1
8	Stool/Catrimox-14 pellet #2
9	Stool/Catrimox-14 supernatant #1
10	Stool/Catrimox-14 supernatant #2
11	TE/Catrimox-14 pellet #1
12	TE/Catrimox-14 pellet #2
13	TE/Catrimox-14 supernatant #1
14	TE/Catrimox-14 supernatant #2

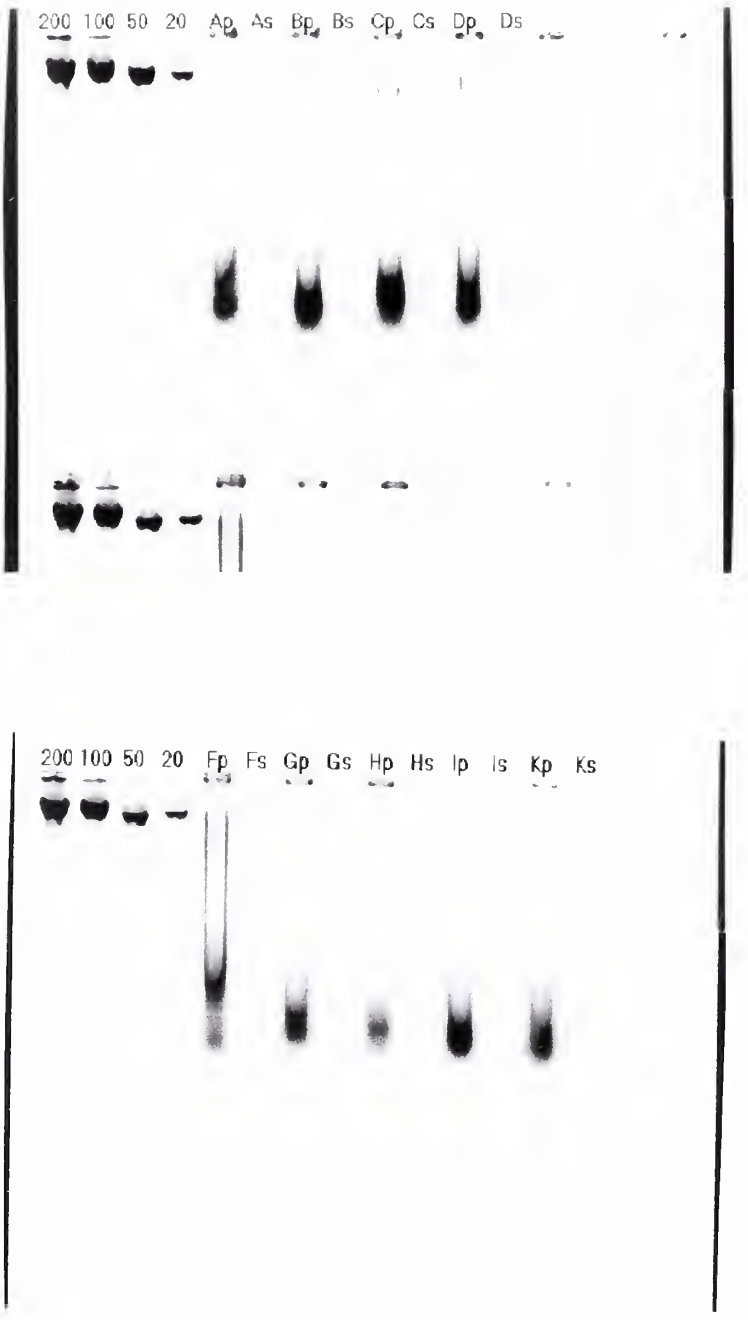


Figure 3. Catrimox-14 DNA Extraction of Clinical Stool Samples. The marker lanes of placental DNA are labelled in nanograms. The stool samples are labelled with "p" = Catrimox-14 pellet and "s" = Catrimox-14 supernatant.

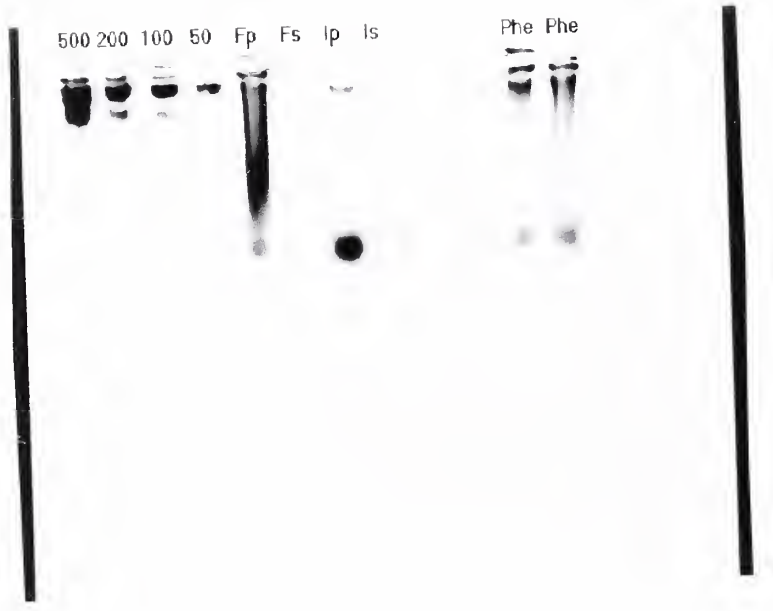


Figure 4. Comparison of DNA extracted with Catrimox-14 and Organic protocols. Lambda Hind III marker is labelled in nanograms. Samples 31 and 71 are clinical stool samples extracted with Catrimox-14. "phe" is phenol/chloroform extracted stool DNA.

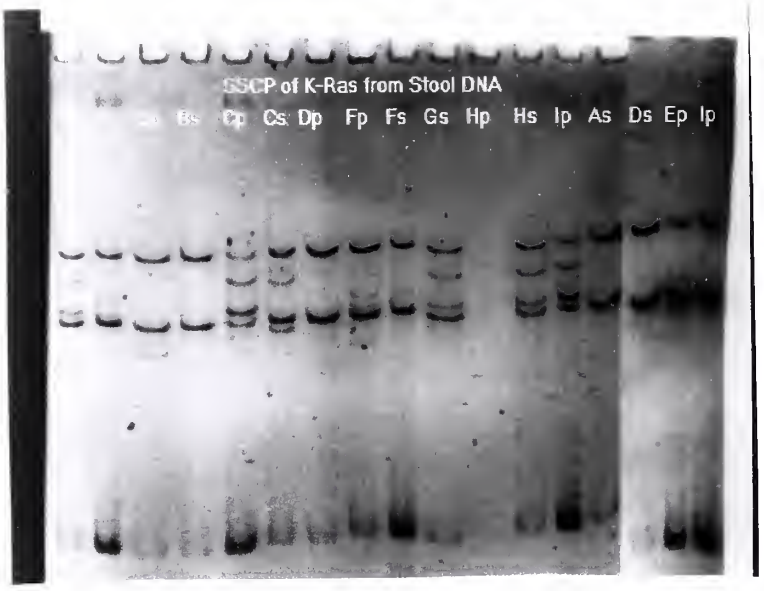


Figure 5. Single-Strand Conformational Polymorphism of K-ras Gene Sequence of Stool DNA. Each lane is marked with the corresponding stool sample. The "p" is the Catrimox-14 Pellet. The "s" is the Catrimox-14 supernatant.

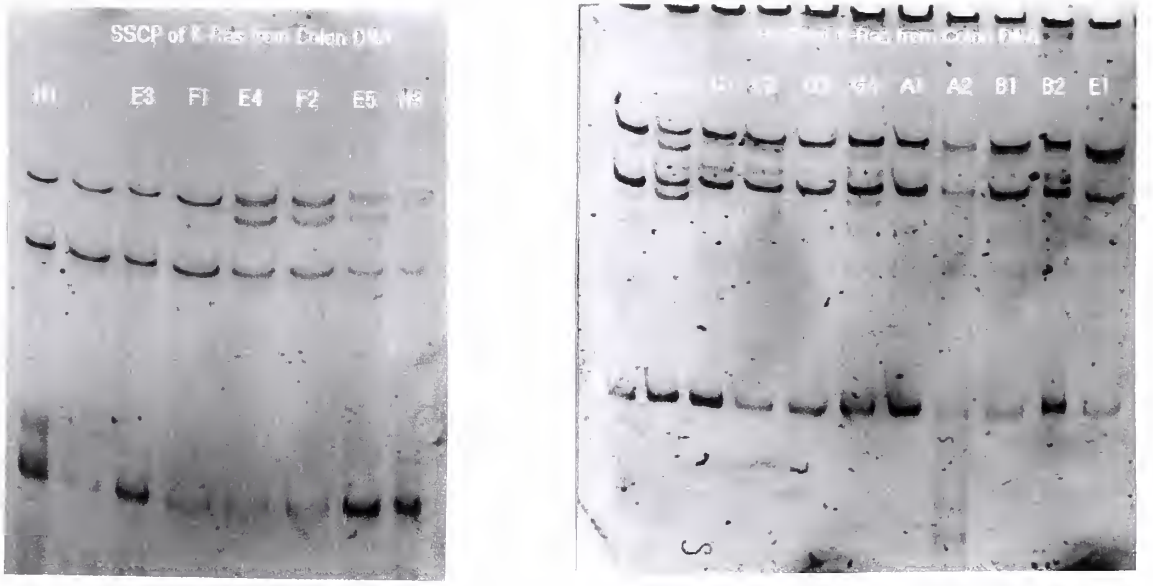


Figure 6. Single-Strand Conformational Polymorphism of K-ras Gene Sequence of colon DNA samples. Each lane is marked with the corresponding colon sample. "H1" corresponds to colon sample H - site 1.

Literature Cited:

1. Sidransky D et al, Identification of ras oncogene mutation in the stool of patients with curable colorectal tumors, Science 1992; 256:102-104.

2. Smith-Raven J et al, Detecion of c-ki-ras mutation in fecal samples from sporadic colorectal cancer patients, Gut 1995; 36:81-86.

3 Marx J.,Test Could Yield Improved Colon Cancer Detection, Science 3 April 1992; 256:32.

4. Fearon ER and Vogelstein B, A Genetic Model for Colorectal Tumorigenesis, Cell 1990; 61:759-67..

5. Carson DA, Lois A, Cancer Progress and p53, The Lancet, Oct 14th, 1995:1009-1011.

6. Morrin M et al, Mutations of k-ras and p53 Genes in Colorectal Cancer and Their Prognostic Significance, Gut 1994:1627-1631.

7. Feng LA, The Many Roads That Lead to Ras, Science 1993; 260:767-8.

8. Eguchi S et al, Mutations of the p53 gene in the stool of patients with resectable colorectal cancer, Cancer (supp) April 1996; 77(8):1707-1710.

9. Hasegawa Y et al, Detection of k-ras mutation in DNAs isolated from feces of patients with colorectal tumors by mutant allele-specific amplification (MASA), Oncogene 1995; 10:1441-1445.

10. Tobi M et al, Detection of k-ras mutation in colonic effluent samples from patients without evidence of colorectal carcinoma, J Natl Cancer Inst 1994;86:1007-1010.

11. Villa E et al, Identification of subjects at risk for colorectal carcinoma through a test based on k-ras determination in the stool, Gastroenterology 1996; 110:1346-1353.

12. Ahn SJ, Costa J, Emanuel JR, PicoGreen Quantitation of DNA: Effective Evaluation of Samples Pre- or Post-PCR, Nucleic Acids Research 1996; 24 (13): 2623-2625.

13. Catrimox-14-14 Surfactant Solution, Users Manual, Iowa Biotechnology Corp.

14. Uwatoko K et al, Rapid and Efficient Method to Eliminate Substance Inhibitory to the PCR from Animal Fecal Samples, Veterinary Microbiology 1996; 52:73-79.

15. Kobayashi and Ohshima, Rapid Detection of Viral Genes - Second Report, Bio View 1995; 14:15-16. (In Japanese)

16. Macfarlane DE, Dahle CE, Use of a Novel Cationic Surfactant in Isolation of RNA, Biomedical Products 1993; 18:60-62.

17. Macfarlane DE, Dahle CE, Isolating RNA from Whole Blood - the Dawn of RNA Based Diagnosis?, Nature 362:186-188.

18. -------, Biomagnetic Techniques in Molecular Biology, Technical Handbook, 2nd ed., Dynal A.S, Oslo, Norway.

19. Emanuel J.R. et al, Highly Sensitive Nonradioactive Single-strand Conformational Polymorphism Detection of Ki-ras Mutations, Diag. Mol. Path., 1996; 5:260-264..

20. Nollau P, Moser C, Cagener C, Isolation of DNA from stool and bodily fluids by PCR amplification, Biotechniques May 1996; 20(5): 784-788.

HARVEY CUSHING / JOHN HAY WHITNEY
MEDICAL LIBRARY

MANUSCRIPT THESES

Unpublished theses submitted for the Master's and Doctor's degrees and deposited in the Medical Library are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but passages must not be copied without permission of the authors, and without proper credit being given in subsequent written or published work.

This thesis by has been used by the following persons, whose signatures attest their acceptance of the above restrictions.

_____

_____

NAME AND ADDRESS DATE