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QUANTITATIVE ANALYSIS OF K-RAS MUTATION IN URINE AS AN INDICATOR OF DISEASE STATUS IN PATIENTS WITH STAGE II OR HIGHER COLORECTAL CANCER

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By

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ABSTRACT

QUANTITATIVE ANALYSIS OF K-RAS MUTATION IN URINE AS AN INDICATOR OF DISEASE STATUS IN PATIENTS WITH STAGE II OR HIGHER COLORECTAL CANCER

By

Shital Darshan Parikh

Colorectal cancer (CRC) is the 3rd most commonly diagnosed cancer and the 4th most frequent cause of cancer deaths worldwide. Sequential mutation in various genes can lead to CRC. K-ras mutation is seen in about 50% of CRC patients and is acquired early and remains throughout the process of tumorigenesis. Therefore, detection of mutant K-ras in combination with various screening and surveillance tests may provide early diagnosis, which may enhance the survival rate as well as provide a new tool for determination of prognosis and identification of proper treatment in patients with mutated K-ras. The objective of this study was to develop a validated method to detect and quantitate mutant K-ras in biological specimen. A Restriction Enriched Polymerase Chain Reaction was developed to selectively amplify mutant K-ras, which was then qualitatively detected using gel electrophoresis and quantified using capillary electrophoresis (CE) method. CE method was developed and validated to selectively quantify mutant K-ras at a level as low as 0.05%.

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RATIONALE

Cancer is a genetic disease characterized by genomic instability. It has been estimated that cells have to acquire at least five to seven successive mutations to allow tumor growth, invasion, and metastasis (Fearon 1990; Boland 1999; Luebeck 2002). Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the fourth most frequent cause of cancer deaths worldwide. In CRC, various cancer susceptibility genes such as oncogenes, tumor suppressor genes and others are involved in tumor initiation and progression in a defined series of stages from normal mucosa to carcinoma (Fearon 1990; Vogelstein 1993). A large number of oncogenes like K-ras (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog), Myc [v-myc myelocytomatosis viral oncogene homolog (avian)], SRC [v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)], beta-catenin, BRAF (v-raf murine sarcoma viral oncogene homolog B1) have been identified to play a role in CRC, among all these K-ras is found to be most frequently mutated. In CRC, K-ras oncogene has been found mutated in 10-15% of screened adenomas <1 cm, in 30-60% of adenomas >1 cm, and in approximately 50% of adenocarcinomas (Bos J.L. 1989; Kressner U 1998; Fearon E.R. 1990; Boguski M.S. 1993). Current practice uses fecal occult blood test (FOBT), flexible sigmoidoscopy, double-contrast barium

enema, and colonoscopy (Kahi C.J. 2004). FOBT is a non-invasive test which detects the presence of hidden blood in the stool. Such blood may come from anywhere along the digestive tract. Hidden blood in stool is often the first, and in many cases the only warning sign that a person has colorectal disease, including CRC. The main advantages of this test are non-invasiveness and low cost. But the disadvantages include detection of blood in stool, but not its cause, false positive results are also common which may cause anxiety about cancer and lead to unnecessary further tests, and false negative results are also common and may miss disease in its early stages. Due to the non specificity in detecting CRC and chances of false positive and false negative results, it would be of interest to develop a test that is more specific and can be used with other techniques to identify CRC more reliably and in early stages. We therefore propose to detect K-ras mutation for screening using urine specimen. Urine is a sample that may be more reliably and safely obtained (in non-invasive manner) and in virtually unlimited amount. Also, molecular testing would be simpler as urine contains less amount of protein for DNA isolation.

INTRODUCTION

Cancer

One of the major characteristics of all higher eukaryotes is the defined lifespan of the organism. Normal cells appear to be mortal, with highly regulated growth and division, and have potential to become terminally differentiated (Trosko 1998; Hanahan 2000). Once the cells are terminally differentiated they can no longer proliferate. Unlike normal cells, cancer cells do not have growth control and they proliferate indefinitely if provided with adequate nutrients, which suggests that cancer cells may become immortal. Therefore, a cancer can be defined as an unregulated proliferation of cells.

Cancer has different types, depending on tissue and cell type from which they arise, (example: carcinomas, sarcomas, lymphomas, and leukemias) but all types of cancer share one common characteristic - uncontrolled growth that progresses towards limitless expansion. Cancers arising from epithelial cells are called carcinomas. The epithelial cells form the outer layer of tissues, surface of the skin, and line the intestines and lungs. About 90% of human cancers are carcinomas. This may be because most of the cell proliferation occurs in epithelia and the cells in epithelial tissues are most frequently exposed to various

forms of physical and chemical damage. Sarcomas are cancers of connective tissue or muscle cells. Leukemias are cancers of bone marrow cells resulting in excess production of leukocytes. Lymphomas are cancers that arise in the lymph nodes and tissues of the body's immune system.

Cancer is a major public health problem in the United States and other developed countries. Currently, one in four deaths in the United States is due to cancer. A total of 1,399,790 new cancer cases and 564,830 deaths from cancer, corresponding to more than 1,500 deaths per day, are expected in the United States in 2006 (Ref. Cancer statistics, 2006).

Molecular Genetics of Cancer

Cancer, in essence, is a genetic disease characterized by genomic instability. Since 1960, cytogenetic and molecular techniques have provided supporting evidence that tumors expand as a clone from a single altered cell, and that clinical 'progression' is the result of sequential somatic, genetic, or epigenetic changes followed by selection, generating increasingly aggressive subpopulations within the expanding clone (Nowell 1976; Nowell 2002). This prevailing model of tumorigenesis as

somatic evolution has been confirmed by numerous molecular studies and it has been estimated that cells have to acquire five to seven successive mutations to allow tumor growth, invasion, and metastasis (Fearon 1990; Boland 1999; Luebeck 2002). The role of cancer susceptibility genes in tumor initiation and progression is best illustrated by the identification of sequential mutations of APC (adenomatous polyposis coli), K-ras, Smad4 (SMAD, mothers against DPP homolog 4), and p53 (tumor protein p53) gene in a defined series of stages from normal mucosa to carcinoma in colorectal tumorigenesis (Fearon 1990; Vogelstein 1993).

Even though no two cancers are genotypically identical, they share some fundamental phenotypes. The loss or abnormal gains in functions of cancer susceptibility genes cause most cancers to acquire the same set of characteristics during their development. These characteristics are: (i) self-sufficiency in growth signals; (ii) insensitivity to antigrowth signals; (iii) evading apoptosis; (iv) limitless replicative potential; (v) sustained angiogenesis; and (vi) tissue invasion and metastasis (Hanahan 2000). Cancer susceptible genes can be classified into three classes: gatekeepers, caretakers, and landscapers.

➢ **Gatekeepers**

Gatekeepers regulate cell proliferation through oncogenes and tumor suppressor genes (Kinzler 1997; Michor 2004). The mutation in both oncogenes and tumor suppressor genes drive the neoplastic process by increasing tumor cell number through the stimulation of cell birth or the inhibition of cell death or cell cycle arrest (Hanahan 2000; Vogelstein 2004).

- **Oncogenes**

The oncogenes hypothesis of cancer was proposed based on the discovery that some endogenous viruses contained transforming elements, and the activation of these endogenous transforming elements could cause cancer (Huebner 1969). An oncogene is the mutant form of the proto-oncogene - a cellular gene involved in the control of cell growth and division. These proto-oncogenes can be classified into five broad classes based on their functional and biochemical properties: (a) secreted growth factors [e.g. SIS (simian sarcoma)], (b) cell surface receptors [e.g. ErbB (avian erythroblastosis virus)], (c) components of intracellular signal transduction systems [e.g. the RAS (rat sarcoma) family], (d) DNA-binding nuclear regulatory proteins, including transcription

factors [e.g. Myc, JUN (jun oncogene)], (e) components of the network of cyclins, cyclin-dependent kinases and kinases inhibitors that regulate process through the cell cycle [e.g. MDM2 (Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse))]. Oncogenes are frequently activated by gain of functional mutations or fusion with other genes or they are aberrantly expressed due to amplification, increased promoter activity, or protein stabilization (Munger 2002), and hence they play important roles in diverse signaling pathways that are involved in various stages of human cancers-tumors initiation, progression, angiogenesis and metastasis (Michor 2004). An activating somatic mutation in one allele is generally enough to confer a selective growth advantage on the cell. A large number of cellular oncogenes have been identified to play a role in colorectal cancer, such as K-ras, Myc, SRC, beta-catenin, BRAF, and others. It has been reported that approximately 50% of colorectal carcinomas have K-ras gene mutated (Bos J.L. 1989; Boguski M.S. 1993). The RAS family and association of K-ras with colorectal cancer are discussed in details in following sections.

- **Tumor Suppressor Genes**

Tumor suppressor genes function to suppress proliferation, promote apoptosis, and maintain integrity of the genome. Mutations in these genes impair this growth-suppressor mechanism resulting in uncontrolled growth. Examples are the genes - Rb1 (Retinoblastoma 1), p53, and p16 (cyclin dependent kinase inhibitor 2A). The tumor suppressor genes regulate diverse cell activities including cell cycle checkpoint response, detection and repair of DNA damage, protein ubiquitination and degradation, mitogenic signaling, cell specification, differentiation and migration, and tumor angiogenesis (Sherr 2004). The activation of tumor suppressor genes is usually achieved by deletion of one allele via a gross chromosomal event such as loss of heterozygosity (LOH) coupled with an intragenic mutation of the other allele. Most of the identified susceptible genes responsible for familial cancer syndromes are tumor suppressor genes. The inactivating somatic mutations in tumor suppressor genes are also found in sporadic counterpart of the familial cancer syndromes. (Gailani 1996; Ponder 2001; Voglestein 2004). For example, germline mutation of APC predisposes to familial adenomatous polyposis (FAP), but mutation in APC is also detected in more than 80% of sporadic colorectal cancers.

The tumor suppressor genes besides APC that are found involved in pathogenesis of colorectal cancer, including p53, DPC1/Smad4 (Deleted in pancreatic carcinoma at loci 1), STK11 (Serine/threonine kinase 11), PTEN (phosphatase and tensin homolog), DCC (deleted in colorectal carcinoma), Smad4, and others.

- **Caretakers**

Caretakers, or stability genes, function in maintaining the genomic integrity of the cell and regulate DNA repair mechanisms, chromosome segregation, and cell cycle checkpoints (Kinzler 1997). Defects in a caretaker gene does not promote tumor initiation, but leads to genetic instabilities that contribute to the accumulation of mutations in other genes, including gatekeeper genes that directly affect cell proliferation and survival, thus indirectly promoting tumorigenesis (Lengauer 1998; Rajagopalan 2003; Iwasa 2005). Since deoxyribonucleic acid (DNA) is vulnerable to many types of damage, DNA repair systems are shown to arise early and are highly conserved, in evoluation process (Hoeijmakers 2001). At least four main, partly overlapping DNA repair pathways operate in mammals: the nucleotide-excision repair (NER), base-excision repair (BER), and mismatch repair (MMR) systems

are responsible for repairing subtle mistakes made during normal DNA replication or induced by exposure to mutagens (Hoeijmakers 2001; Vogelstein 2004). The homologous recombination and non-homologous end joining (NHEJ) system is responsible for repairing DNA damage involving large proportions of chromosomes, such as double-strand DNA breaks (David 2000; Hoeijmakers 2001; Vogelstein 2004). Each DNA repair system is a complex biochemical process that requires participation of many genes, and still remains poorly understood.

The role of defective DNA repair systems causing tumorigenesis is shown by the existence of human cancer syndromes with germline mutations in caretaker genes. For example, inborn defects in NER cause syndromes of xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy (TTD) are all characterized by extreme sun sensitivity. Germline mutations in MMR genes, such as hMLH1 (human MutL homolog 1 gene), hMSH2 (human MutS homolog 2 gene), and hMSH6 (mutS homolog 6 (E. coli)), are known to predispose to hereditary nonpolyposis colorectal cancer (HNPCC) (Bandipalliam P 2004). As with tumor suppressor genes, both alleles of caretaker genes generally need to be inactivated for a pathological effect to result.

- **Landscaper**

Landscaper defects do not directly affect cellular growth, but generate an abnormal stromal environment that contributes to the neoplastic transformation of cells (Michor 2004; Iwasa 2005). It is now well accepted that cancer develops with malignant transformation of an epithelium occurring within the context of a dynamically evolving tissue stroma that is composed of multiple cell types surrounded by an extracellular matrix (Bhowmick 2004; Weaver 2004). There are many studies to show that cancers can arise as a consequence of inherited or acquired genetic mutations in stromal cells. It has been reported that distinct genetic alterations and chromosomal rearrangements were absent in the malignant carcinoma cells, but present in neighboring stromal cells instead (Moinfar 2000). Moreover, studies of the familial juvenile polyposis syndrome also indicate that it is actually the genetic alteration of the DPC/Smad4 gene in stromal cells that predispose colonic epithelial cells to carcinomas (Jacoby 1997; Howe 1998). The dysfunction or deregulation of landscaper genes can disrupt normal tissue homeostasis, reduce host immune surveillance and defense, induce angiogenesis and inflammation, and promote tumor growth and migration (Muller 2004).

Colorectal Cancer

Epidemiology

Colorectal cancer (CRC), a cancer of colon and rectum, is the third most commonly diagnosed cancer and the fourth most frequent cause of cancer deaths worldwide. The World Health Organization estimates more than 11 million new cases diagnosed yearly, with 7 million deaths. CRC is the second leading cause of cancer-related death in the United States; accounting for approximately 148,610 new cases with nearly 55,170 deaths in 2006 (Cancer statistics, 2006). This disease is more common in developed countries than developing countries. The lifetime incidence is 5% in developing countries, but the incidence and mortality are now decreasing (Strward 2003; Russo MW 2004; Jemal A 2004). The overall 5-year survival rate in the Unites States exceeds 60%, but is less than 40% in less developed countries (Strward 2003). This variability in disease outcome is proportional to access to specialists and availability of modern drug therapy (Strward 2003).

Risk Factors

The strongest risk factor for developing CRC is advancing age. The current statistics on CRC indicate that

incidence rates rise from 13.2 per 100,000 at age 40-44 to 338.7 per 100,000 at age 75-80 (Ref. SEER data 1975-2001). The overall lifetime risk of CRC is 1 in 17 for men and 1 in 18 for women (Cancer Statistics, 2005). Inflammatory bowel disease, a personal or family history of CRC or colorectal polyps, and certain hereditary syndromes are also associated with high risk of developing CRC. Other factors that also contribute to the risk for CRC are: lack of regular physical activity, low fruit and vegetable intake, a low-fiber and high fat diet, obesity, high alcohol consumption and use of tobacco. From a public health perspective, prevention and screening for early diagnosis of CRC is essential to help reduce deaths from CRC. In addition, patients with one diagnosed colon cancer are at risk to develop further colon cancers.

Table 1. Risk factors associated with colorectal cancer.

Sporadic Colorectal cancer

- Older age
- Cholecystectomy (Surgical removal of the gallbladder)
- Ureterocolic anastomosis
- Hormonal factors
  - o Nulliparity (never having carried a pregnancy),
  - o Late age at first pregnancy,
  - o Early menopause
- Environmental factors
  - o Diet rich in meat and fat, and poor in fiber, folate, and calcium
  - o Sedentary lifestyle
  - o Obesity
  - o Diabetes mellitus
  - o Smoking
  - o Previous irradiation
  - o Occupational hazards (e.g. asbestos exposure)
  - o High alcohol intake
- Personal history of sporadic tumors
  - o History of colorectal polyps
  - o History of colorectal cancer (risk is 1.5-3% for second such cancer in first 5 years)
  - o History of small bowel, endometrial, breast, or ovarian cancer
- Familial colorectal cancer

  First or second degree relatives with this cancer, criteria for hereditary colorectal cancer not fulfilled:
  - o One affected first-degree relative increases risk by two to three fold
  - o Two or more affected first-degree relatives increase risk by four to twenty five fold
  - o Index case less than 45 years increase risk by three to nine fold
  - o Familial history of colorectal adenoma increase risk by two fold

Table 1 (cont'd)

Colorectal cancer in inflammatory bowel disease
• Ulcerative colitis • Crohn's colitis
Hereditary colorectal cancers
• Polyposis-syndromes o familial adenomatous polyposis (FAP) o Gardner's syndrome o Turcot's syndrome o Attenulated adenomatous polyposis coli o Flat adenoma syndrome • Hereditary non-polyposis colorectal cancer (HNPCC) • Hamartomatous polyposis syndromes o Peutz-jeghers syndrome o Juvenile polyposis syndrome o Cowden syndrome
Source: Strward B.W. 2003

Clinical Background

Sporadic Colorectal Cancer

Although most cases of CRC are sporadic; genetic and environmental factors also play an important role (Table 1) (Strward B.W. 2003). The characteristic of sporadic CRC is the step-wise progression from normal colonic epithelium to malignant growth associated with sequential molecular abnormalities in each step (Kinzler 1996). Fearon et al. showed that colorectal tumors arise as a consequence of the

accumulation of activated oncogenes and inactivated tumor-suppressor genes (Fearon E.R. 1990). About 20% of all patients with this cancer are estimated to have some component of familial risk without fulfilling the strict criteria for hereditary colorectal cancer (Lynch H.T. 2003). Therefore, family history should always be taken into account when assessing a patient.

Colorectal Cancer in Inflammatory Bowel Disease

Colorectal cancer accounts for about a third of deaths related to ulcerative colitis, and risk depends on disease duration (2% of affected people by 10 years, 8% by 20 years, and 18% by 30 years), extent of inflammation, presence of primary sclerosing cholangitis, and backwash ileitis (Itzkowitz S.H. 2004; Krok K.L. 2004). Crohn's colitis is also associated with increased risk of colorectal cancer; the relative risk is similar to that for ulcerative colitis (Itzkowitz S.H. 2004).

Hereditary Colorectal Cancer

Roughly 5-10% of all CRCs develop in the setting of defined hereditary cancer syndromes. Hereditary nonpolyposis colorectal cancer (HNPCC, also called Lynch Syndrome) and familial adenomatous polyposis (FAP) are the

two main forms (Lynch H.T. 2003). Various hamartomatous polyposis syndromes are also associated with an increased risk of such cancer, such as Peutz-jeghers syndrome, juvenile polyposis syndrome, Cowden syndrome (Lynch H.T. 2003; Half E.E. 2004).

HNPCC is an autosomal dominantly inherited disorder caused by germline mutations of mismatch repair (MMR) genes. Tumors with HNPCC typically have a molecular characteristic called microsatellite instability. The microsatellite instability is defined as frequent mutations in microsatellites, which are short repeated DNA sequences (Grady W.M. 2003). The penetrance of colorectal cancer in HNPCC is 70-85%. Risk is also increased for tumors of the genitourinary system, stomach, biliary system, pancreas, small intestine, and CNS (Lynch H.T. 2003; Half E.E. 2004; Vasen H.F. 1999). On average, affected patients develop colorectal cancer by age 44, tumors tend to be right-sided, and have classical histological features (Bandipalliam P 2004).

FAP is an autosomal-dominant disease. In about 80% of affected individuals, a germline mutation can be identified in the adenomatous polyposis coli (APC) gene (Lynch H.T. 2003; Half E.E. 2004). A subset of people with FAP and attenuated FAP, a mild form of FAP which is characterized

by the occurrence of fewer colonic adenomas, has biallelic mutations of the mut Y homolog (MYH) gene (Sieber O.M. 2003; Venesio T 2004). Patients with attenuated FAP typically have mutations at the 5' (proximal to codon 1517) or the 3' end (distal to codon 1900) of the APC gene (Grady M 2003). FAP patients can develop more than 100 colorectal adenomas (50% of patients by age 15 years, 95% by age 35 years); if left untreated, colorectal cancer arises in almost all patients by age 40.

Pathogenesis of Colorectal Cancer

CRC develops in a series of genetic steps, corresponding with histological progression from normal colonic epithelium to adenomatous dysplasia through microinvasion, adenocarcinoma and, finally metastasis. Two major pathways are known to be involved in the tumorigenesis of CRC. The classical pathway (sometimes called chromosome instability syndrome), is responsible for about 85% of sporadic CRC and is the mechanism of carcinogenesis in patients with FAP, in which cancer cells are characterized by chromosomal instability with mutation of multiple oncogenes, including K-ras, (others oncogenes such as c-myc, c-neu, c-erb-2, c-src are also involved) and many tumor suppressor genes, such as APC, p53, DCC,

DPC4/Smad4, and nm32 (Chaung D.C. 2000; Calvert P.M. 2002).

Other major pathway that leads to colorectal cancer is via microsatellite instability. Microsatellite instability occurs in approximately 15% of all CRCs and can arise through two mechanisms (De La Chapella A 2003). In the first mechanism, germline mutations of mismatch repair genes (e.g. MLH1, MLH2, and MSH6) in hereditary nonpolyposis colorectal cancer (HNPCC) leads to deficient DNA-mismatch repair. Nucleotide mismatches occur when two strands of DNA replicate, but almost all such errors are quickly corrected by a molecular proofreading mechanism. Studies of HNPCC revealed mutation in mismatch repair genes, which encode proteins that repair nucleotide mismatches. Defective mismatch repair presumably facilitates malignant transformation by allowing the rapid accumulation of mutations that inactivate genes with key functions in the cell. The defective MMR genes fail to produce proteins that correct nucleotide mismatches during replication and thus promote mutations in other genes. In addition, genes carrying microsatellite in their coding sequences such as BAX and TGFBR2 are also involved in carcinogenesis via frame shift mutation.

In the second mechanism, epigenetic silencing of MLH1 occurs when methylation of the CpG sites in the promoter region of MLH1 silences its transcription and when both the alleles are affected, leads to a mismatch repair deficiency (Herman J.G. 1998). This second mechanism is not heritable and accounts for the majority of all sporadic CRCs that are positive for microsatellite instability (Veigl M.L. 1998; Cunningham J.M. 1998).

Additional pathways could exist, for example, the serrated pathway as well as distinct pathways for carcinogenesis of flat and depressed colorectal neoplasms and for carcinogenesis in inflammatory bowel disease (Krok K.L. 2004; Jass J.R. 2002; Soetikno R.M. 2003; Itzkowitz S.H. 2004). Epigenetic mechanisms such as change in DNA methylation, loss of imprinting, and histone acetylation, as well as modifier genes (cyclooxygenase-2 gene and peroxisome proliferators-activating receptor gene) also seem to be involved in the genesis of CRC (Lynch J.P. 2002; Kondo Y 2004; Rao M 2004; Sinicrope F.A. 2004; Harman F.S. 2004). Other genes, such as tyrosine phosphatases (Wang Z 2004), activin type2 receptor (Jung B 2004), phophatidylinositol 3-kinases (Samuels A 2004), and hCDC4 (F-box and WD repeat domain containing 7) (Rajagopalan 2004) might also contribute to colorectal carcinogenesis.

Screening and Prevention

Screening is effective in reducing mortality from CRC. Reducing the number of deaths from CRC depends on detecting and removing precancerous colorectal polyps, as well as on detecting and treating the cancer in its early stages. CRC can be prevented by removing precancerous polyps or growth, which can be present in the colon for years before invasive cancer develops (Winawer S.J. 1993). Screening procedures include fecal occult blood test, flexible sigmoidoscopy, double-contrast barium enema, and colonoscopy (Kahi C.J. 2004). One of these options should be offered to asymptomatic people aged 50 years or older (Winawer S.J. 2003). The ideal screening method is still controversial, with no test unequivocally better than another. The American Cancer Society (ACS) and U.S. Preventive Services Task Force (USPSTF) recommend yearly stool for occult blood tests. The colonoscopy has the advantage of allowing assessment of the entire colon with the possibility of simultaneous biopsy or polypectomy. For patients with personal or familial history of colorectal neoplasms, FAP, HNPCC or inflammatory bowel diseases, special guidelines exist, taking into consideration the higher risk (Winawer S.J. 2003).

Staging of CRC

If CRC is suspected, various combinations of tests, recommended by ACS and National Comprehensive Cancer Network (NCCN), such as medical history and physical exam, colonoscopy, blood count and blood chemistry, carcinoembryonic antigen (CEA) blood test, ultrasound (imaging test), computed tomography (CT or CAT scan), chest x-ray are performed. Tissue biopsy is performed to determine if the disease is really present. The most reliable prognostic factor identified to date in CRC is the staging of disease at the time that treatment is initiated. Staging of colorectal cancer should be done using the current TNM classification of the American Joint Committee on Cancer (AJCC) and International Union Against Cancer (UICC) (Ref: American Joint Committee on Cancer, 2002).

TNM (Tumor, Node, Metastasis) Classification

The current joint AJCC-UICC staging system for colorectal cancer is now the only classification system that should be used (Compton C.C. 2000). The TNM system (Table 2 and Table 3) classifies colorectal tumors on the basis of the invasiveness (not size) of the primary tumor (T stage), the number (not size or location) of local and regional lymph nodes containing metastatic cancer (N

stage), and the presence or absence of distant metastatic disease (M stage) (Ref: American Joint Committee on Cancer).

Table 2. TNM classification of CRC.

Stage	**Tumor (T)**	**Lymph Nodes (N)**	**Metastasis (M)**
0	Tis	N0	M0
I	T1	N0	M0
	T2	N0	M0
IIA	T3	N0	M0
IIB	T4	N0	M0
IIIA	T1 - T2	N1	M0
IIIB	T3 - T4	N1	M0
IIIC	Any T	N2	M0
IV	Any T	Any N	M1
* Abbreviations are in Table 3.			
Source: Greene F.L. 2002			

Table 3. American Joint Committee on Cancer- Union Internationale Contre le Cancer Tumor, Node, Metastasis Staging of CRC

Primary Tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	*In situ* adenocarcinoma (Tis): cancer confined to the glandular basement membrane or lamina propria
T1	tumors invade into but not through the submucosa
T2	tumors invade into but not through the muscularis propria
T3	tumors invade through the muscularis propria into the subserosa or into nonperitonealized pericolic or perirectal tissue
T4	tumors directly invade other named organs or structures (T4a)* or perforate the visceral peritoneum (T4b)
Regional Lymph Nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	denotes that all nodes examined are negative
N1	includes tumors with metastasis in one to three regional lymph nodes
N2	indicates metastasis in four or more regional lymph nodes
Distant Metastasis (M)	
MX	Distance metastasis cannot be assessed
M0	no evidence of distant metastases is present
M1	Identification of distant metastases
Source: Greene F.L. 2002	

Treatment and Surveillance

Once CRC has been detected, the main treatment for this disease is surgical excision. Up to 50% of CRC patients will develop a recurrence of tumors and 30-50%

will subsequently develop metastases (August D.A. 1984). As 80% of recurrences after resection of CRC occur within the first two years after surgery, it is important to undertake more intensive follow-up during this period (Cochrane J.P.S. 1980). Therefore, after resection and initial treatment, surveillance is necessary to address recurrence, treatment complications, and related medical issues. This provides a basis to optimally manage recurrent disease, monitor the development of new primary cancers, and precancerous polyps (Winawer S.J. 1993). The surveillance program for CRC patients usually includes periodic history taking and physical examinations; some combination of laboratory tests such as CEA tests, liver-function tests, complete blood counts, and fecal occult blood tests (FOBT); diagnostic imaging studies such as chest radiography, ultrasonography, CT, magnetic resonance imaging, and barium enema; and endoscopic procedures such as sigmoidoscopy and colonoscopy. There are potentially thousands of possible combinations of frequency and diversity of these surveillance tests (Kieveit J 1995). Different recommendations of follow-up protocols has been formulated but no consensus has been reached (Vernava A 1994; Kjeldsen B.J. 1997; Audisio R 1996; Schoemaker D 1998; Anthony T 2000; C Desch 1999; C Desch 2000). NCCN has outlined the

surveillance guidelines for patients with resected stage II and III CRC. Among patients who have undergone resection for localized CRC disease, the five-year survival rate is 90 percent; the rate decreases to 65 percent when metastasis to regional lymph nodes is present (Jemal A 2003). Depending on the location, CRC tumors commonly metastasize to the liver, local sites, abdomen, and lungs (Figueredo A 2003).

RAS Family

A mammalian cell contains at least three distinct ras proto-oncogenes: v-Ha-ras Harvey rat sarcoma viral oncogene homolog (H-ras), K-ras, and neuroblastoma RAS viral (v-ras) oncogene homolog (N-ras) (Lowy D.R. 1993). H-ras and K-ras were first identified as viral (v-Ras) oncoproteins of Harvey and Kirsten murine sarcoma viruses, and were found to be capable of cellular transformation. The N-ras oncoprotein was identified in a neuroblastoma cell line. The human genomic DNA sequences spread over 6.6 kb (H-ras), 8 kb (N-ras), and 35 kb (K-ras) and are located in chromosomes 11p15.5, 1p13, 12p12.1 respectively (Ref. Website: Infobiogen). The K-ras gene is alternatively spliced into two isoforms: K-rasA and K-rasB (Pells S 1997). Other member of the Ras family genes are M-ras, R-

ras, Rap 1/2 and Ral that share at least 50% sequence identity.

H-, K-, and N-ras have similar structure and sequences, with four coding exons, and conserved splicing sites, even if the introns have various dimensions and sequences. The protein product of the ras gene is a closely related monomeric G protein of approximately 21 kD in size, containing 189 (H-ras, K-rasB, and N-ras) or 188 (K-rasA) amino acids. The Ras gene protein is able to bind and hydrolyze guanosine triphosphate (GTP). These Ras GTPases are essential mediators in signaling pathways that convey extracellular signals from surface receptors to the interior of the cell, functioning as molecular switches in processes governing cell proliferation, survival, and differentiation (Malumbres M 1998; Crespo P 2000). The three genes are ubiquitously expressed although mRNA analysis suggests different tissue expression levels (Leon J 1987). Variation in the expression pattern also exists throughout embryonic development (Leon J 1987) and during differentiation processes (Crespo P 2000). The H-ras is highly expressed in the skin and in the skeletal muscles; K-ras is mostly expressed in the colon and thymus, and N-ras in the male germinal tissue and the thymus suggesting that ras family members probably are expressed in a tissue

specific fashion (Lowy D.R. 1993). In support to this hypothesis, mutations in each one of these genes, frequently found in various types of tumors, are involved in the development of specific neoplasia, such as mutation of K-ras in lung, colorectal, and pancreas tumors, H-ras in bladder, kidney and thyroid tumors, and N-ras in melanoma, hepatocellular carcinoma, hematological malignancies, and neuroblastoma (Fujita J 1984; Visvanathan K.V. 1988; Bos J.L. 1989; Bos J.L. 1987; Hesketh R 1995).

Functional Roles of RAS Proteins in Cancer

Various literature reviews, on the RAS genes and functions, are proposed to contribute to genesis and progression of several tumor types (Bollag G 1991; Macaluso M 2002; McCormick F 1999; Bos J.L. 1987; Barbacid M 1987). RAS proteins in mammalian cells are located on the inner plasma membrane layer and may be activated [Guanine triphosphate (GTP)-bound status) or inactivated [Guanine diphosphate(GDP)-bound status] by extracellular physiological stimuli or signals (i.e. growth factors, cytokines, and adhesion signals) via specific cell surface receptors. Activation of RAS is obtained by means of several guanine exchange factors (GEFs) able to induce a RAS protein conformational change leading to GDP

dissociation. In physiological conditions, several GTPase activating proteins (GAPs) inactivate the RAS protein after short time by inducing GTP hydrolysis and setting the formation of the RAS/GDP inactive complex. Specific growth factors (i.e. epidermal growth factor and platelet derived growth factor) interacting with their receptor tyrosine kinases induce receptor autophosphorylation on specific tyrosine residues. Subsequently, adaptor proteins, like the Growth factor Receptor-Bound protein 2, interact with receptor phosphorylated tyrosines by their SH2 domains and bind to RAS/GEFs by the SH3 domain (Bollag G 1991; Macaluso M 2002; McCormick F 1999; Bos J.L. 1987; Barbacid M 1987). RAS activation by specific stimuli and specific GEFs promote different RAS functions via several transduction pathways including RAS/RAF, RAS/PI3-K and RAS/RAL.

K-ras Gene and CRC

The K-ras (Kirsten-rat sarcoma) gene, located on the short arm of chromosome 12, encodes a 21 kD protein ($p21^{ras}$) which is able to bind and hydrolyze GTP. The protein acts as a mediator in the transduction of extracellular signals to the cytoplasm and nucleus. Mutations in K-ras, most commonly at codons 12, 13, and 61 (Bos J.L. 1989), lead to the formation of constitutively active (GTP-bound)

proteins, which trigger the transduction of proliferative and/or differentiative signals even in the absence of extracellular stimuli (Boguski M.S. 1993). In human cancers, the most common mutations are found at codon 12. For example, 90% of human pancreatic cancers, 50% of colon cancers, and more than 30% of smoking-related lung cancers have a mutation at codon 12 of the K-ras gene (Bos J.L. 1989; Rodenhuis S 1992; Slebos R.J. 1991).

In CRC, the K-ras oncogenes has been found mutated in 10-15% of the screened adenomas <1 cm, in 30-60% of adenomas >1 cm, and also, in 50% of the adenocarcinomas (Bos J.L. 1989; Kressner U 1998; Fearon E.R. 1990). The most frequently mutated position in sporadic and familial adenomatous polyposis (FAP) - associated colorectal tumors is codon 12 (Bos J.L. 1989). Because these mutations are acquired early in tumor development (Vogelstein B 1988), and remain through out the process of tumorigenesis, detection of the mutated K-ras in DNA of patients may improve early detection in combination with other screening methods, allowing removal of even small polyps. Testing for mutated K-ras during follow-up after resection from colorectal cancer may provide new tools for detection of recurrence or second primary tumors. Recent evidence implies that tumors with mutated K-ras do not respond to

EGFR [epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)] inhibitors (Van Cutsem 2008; Bokemeyer C 2008; Amado R 2008) and thus may serve to identify appropriate treatments or to avoid ineffective treatments, and thus may increase survival.

Presence and Detection of DNA from Serum/Plasma

During apoptosis (programmed cell death), cellular components are dismantled and usually phagocytosed by the macrophages or neighboring cells. During the early stages of apoptosis, nuclear DNA is degraded in nucleosomes and their oligomers (Wyllie A.H. 1980). Taking into account that in human adults approximately 10^{11} cells die daily, the amount of released DNA should be approximately 0.6 gram/day (g/day) (Lichtenstein A.V. 2001). However, some chromatin (DNA) degradation products are further degraded into acid-soluble products and are reutilized within an organism, whereas others escape phagocytosis and appear in the blood-stream. Circulating DNA has been detected in serum and plasma of both healthy and diseased individuals (Anker P 2001). DNA levels in the blood-stream presumably reflect the amount of cell death occurring in the whole body and are increased during destructive pathological processes,

including cancer and during pregnancy (Chiu R.E. 2004). It has been shown that the serum contains cell-free circulating fragmented DNA in the size of nucleosomal DNA (Jahr S 2001). DNA with a cancer "signature" (mutation or hypermethylation) has been found in the plasma or serum of patients with small cell lung cancer (Chen X.Q. 1996), head and neck cancer (Nawroz H 1996), clear cell renal cancer (Goessl C 1998), pancreatic cancer (Mulcahy H.E. 1998), breast cancer (Chen X.Q. 1998), hepatocellular carcinoma (Wong IHN 1999), non-small cell lung cancer (Esteller M 1999), and colorectal carcinoma (Kopreski M 1997). It has also been demonstrated that the mutation found in tumor tissue is the same identical mutations found in plasma or serum of respective patients (Chex X.Q. 1996). The appearance of DNA in plasma from cells dying in a body was also shown by detection of fetus-specific sequences in the plasma of pregnant women (Lo Y.M. 1997; Bischoff F.Z. 1999).

Detection of DNA from Urine

Experiments on animals demonstrated that DNA fragments of several hundreds base pairs originating from apoptotic cells can cross the renal barrier and preserve their matrix functions in PCR (Polymerase Chain Reaction) (Botezatu I

2000). This conclusion was extrapolated to humans, as it was possible to identify sequences filtered from the blood-stream through the kidney barrier into urinary DNA. The possibilities that there are two type of DNA in urine: excreted DNA, originating from cells dying in various tissues of an organism and second, locally degraded DNA, originating from cells dying in the urinary tract itself. For example, sequences specific for masculine Y chromosome were detected in urine of women after blood transfusion from male donors and in pregnant women with male fetuses (Botezatu I.V. 2000). Earlier studies demonstrated the possibility of PCR analysis of urinary DNA for diagnosis and monitoring of tumor growth (Su Y.H. 2004). The advantages of detecting mutant sequences in urinary DNA over the analysis of plasma or serum DNA are obvious: it is noninvasive, DNA can be isolated from greater volumes of initial material and is easier to isolate due to low concentration of total proteins (1000-fold lower protein content).

OBJECTIVES

The main objective of this research was to develop and validate a method to identify and quantify mutant K-ras in biological specimen. The specific objectives of this research are:

- Isolation of DNA
  - Isolation of low molecular DNA from urine sample
  - Isolation of High molecular DNA from SW480 cell line and Peripheral Blood Leucocytes
- Adaptation of qualitative method for the detection of mutant K-ras sequences
- Identification of the lowest concentration of mutant K-ras that can be detected qualitatively using RE-PCR and gel electrophoresis by serially diluting mutant K-ras with wild-type K-ras
- Develop and validate a method to quantitate mutant K-ras using a Capillary Electrophoresis
  - Design an anti-sense fluorescent primer to quantify mutant K-ras using fluorescence

METHODS

All the materials and equipment mentioned in this section are listed in the appendix.

➢ Preparation of Samples

- **Positive Control**

Genomic DNA from SW480 cell line (American Type Culture Collection (ATCC)) possessing a mutation in codon 12 of the K-ras proto-oncogene was used as a positive control.

SW480 cell line cells were obtained in suspension. The suspension was thawed at 37°C in a water-bath (Precision Water Bath) for approximately 5 minutes. The suspension was then centrifuged for 5 minutes at 300 x gravity (g) in a 1.5 mL microcentrifuge tube and the supernatant was removed and discarded. The cell pellet was resuspended in PBS (Phosphate Buffered Saline), pH 7.4 to a final volume of 200 µL. The isolation was carried out using the method described below.

- **Negative Control**

Genomic DNA from Peripheral Blood Leucocytes (PBLs) was used as a negative control for K-ras codon 12.

The whole blood was collected in ACD (Acid Citrate Dextrose), yellow-top tube. The tube was centrifuged at 655 x g for 10 minutes, and 200 µL of buffy coat (leucocytes) was used for DNA isolation as described below.

- **Serial Dilution of Positive and Negative Controls to Identify Sensitivity of the Method to Detect K-ras**

The serial dilution assay was performed by diluting SW480 cell line (100% mutant) used as positive control with PBLs (100% wild-type) used as negative control. The dilution range for SW480 cell line was 100, 75, 50, 25, 10, 5, 1, 0.5, 0.25, 0.1, 0.05, 0.02, 0.01, and 0.002%. The RE-PCR was performed (discussed in the sections to follow) on all dilution samples, negative control (PBLs, 100% wild-type), and blank control of PCR. A 12% PAGE was performed (discussed in the sections to follow) after RE-PCR. The procedure is performed in triplicate.

- **Urine Sample**

  - **Urine Collection**

Twenty five to fifty milliliter (mL) of fresh urine was collected (the first void of the day was not used) in a sterile container and immediately mixed with appropriate

volume of 0.5 mole per Liter (M) EDTA (Ethylenediaminetetraacetic acid), pH 8.0 to achieve a final EDTA concentration of 10 millimole per Liter (mM). This concentration of EDTA is known to inhibit nuclease activity (Milde A 1999). Urine samples were stored in 15 mL conical polypropylene screw cap tubes in 10 ml aliquots at -70 °C (degree centigrade). The tubes were thawed and centrifuged (Sorvall TC6 tabletop centrifuge) at 200 x g for 5 minutes and placed on ice before isolation. The urine samples were obtained from a volunteer.

Ability of Urine Isolation Method to Detect Low Molecular Weight Mutant K-ras Sequences

To evaluate the ability of the method to detect K-ras in urine sample and to determine the sensitivity of the method following samples were prepared: Eight 10 mL aliquots of same urine sample, negative for mutant K-ras were thawed. A different amount (80 µL, 15 µL, 5 µL, 1 µL, 0.1 µL, 0.01 µL, and 0.001 µL) of SW480 cell line first round PCR (discussed in the sections to follow) products were added to the seven of the eight urine sample tubes. The tubes were mixed and centrifuged at 200 x g for 5 minutes and placed on ice.

➢ DNA Isolation of Samples

• Positive and Negative Controls

The QIAamp® DNA Mini Kit, designed to isolate high molecular weight DNA, was used to obtain genomic DNA from PBLs and SW480.

Twenty micro-liter of QIAGEN protease (provided with kit) and 200 µL buffer AL (a cell lysis buffer, provided with kit) was added to 200 µL of cells or buffy coat. The tube was vortexed (Vortexer, Type 16700 Mixture) for 15 seconds, centrifuged, and the mixture then was incubated at 56°C (Precision Water bath-micro) for 10 minutes. After incubation, the tube was centrifuged, and precipitation of DNA was achieved by adding 200 µL of ethanol and vortexing the tube for 15 seconds. Tube was then centrifuged and the mixture was transferred to the QIAamp spin column (in a 2 mL collection tube, provided with kit) and centrifuged at 9295 x g for 1 minute. The column was placed in a new clean 2 mL collection tube, 500 µL of wash buffer AW1 (provided with kit) was added to the column and centrifuged at 9295 x g for 1 minute. The column was placed in a new clean 2 mL collection tube, 500 µL of wash buffer AW2 (provided with kit) was added to the column and centrifuged at 9295 x g

for 3 minutes. The column was placed in a clean 1.5 mL UV-treated (90 seconds at 254 nm) (GS Gene Linker UV chamber) microcentrifuge tube and 200 µL of buffer AE (elution buffer, provided with kit) was added to the column and allowed to incubate for 5 min at room temperature. The DNA was collected by centrifugation at 9295 x g for 1 minute.

The GeneQuant RNA/DNA Calculator was used to measure the optical density at 260nm. Five micro-liter of DNA was diluted to 200 µL with sterile deionized distilled water. The protein contamination, purity, and 260nm/280nm ratio were also recorded. The concentration of double stranded, high molecular weight DNA was calculated using equation 1. The DNA samples from SW480 cell line and PBLs were stored at -20°C until further use.

- **Urine Sample**

Fragmented DNA that appears in urine due to apoptosis has low molecular weight than the high molecular weight genomic DNA from the positive and negative controls. Therefore, a different method was used to isolate fragmented DNA in urine than the isolation method used for positive and negative control mentioned in the following sections. DNA was isolated as described previously (Botezatu I 2000; Ying-Hsiu Su 2004) with some modifications. The

changes to the original method were obtained via personal communication with Samuil Umansky (Xenomics, Inc.). The isolation method is described below.

A 20 mL of 6 M GITC (Guanidine Isothiocyanate) was added to 10 mL of urine in a 50 mL conical polypropylene screw cap tube and mixed vigorously. One mL of resin (Wizard® Plus Minipreps DNA Purification System) was added and the tube was incubated for 4 hours at room temperature with gentle mixing. The resin was pelleted by centrifugation at 200 x g for 10 minutes. The supernatant was carefully aspirated and discarded. The resin pellet was resuspended in 1 mL of 4 M GITC, and transferred into a 1.5 mL microcentrifuge tube. The tube was centrifuged in a microcentrifuge (Sorvall Biofuge® Pico) at 9295 x g for 1 minute. The supernatant was removed and discarded. The pellet was resuspended in 1 mL of column wash buffer (Wizard® Plus Minipreps DNA Purification System) and suspension was transferred to a Promega Wizard minicolumn (Wizard® Plus Minipreps DNA Purification System). The DNA was separated from the suspension by applying vacuum. The column was washed with 2 mL of column wash buffer. To remove residual washing buffer from the column, the column-tube assembly was centrifuged in a microcentrifuge at 9295

x g for 3 minutes. The column was placed in a clean 1.5 mL UV-treated (90 seconds at 254 nanometer (nm)) microcentrifuge tube and 100 microliter (µL) of sterile deionized distilled water was added to the column and allowed to incubate for 5 min at room temperature. The DNA was collected by centrifugation at 9295 x g for 1 minute.

The GeneQuant RNA/DNA Calculator was used to measure the optical density at 260 nm. Fifty microliter of DNA was diluted to 200 µL with sterile deionized distilled water. The protein contamination, purity, and 260nm/280nm ratio were also recorded. The concentration of double stranded DNA (dsDNA) was calculated using formula mentioned in Figure 1. The DNA samples were stored at -20°C until further use.

Formula for Double stranded DNA

DNA µg/µL = [OD_{260}* X 50(µg/mL) X DF** X 1 mL)]÷ 1000 µL

*OD_{260} = Optical density at 260 nm
**DF = Dilution factor
An optical density at 260nm of 1.0 corresponds to 50µg of DNA per mL for dsDNA.

Figure 1. Formula for the Calculation of DNA Concentration.

➢ **Primer Preparation**

Two primer sets were used for the Polymerase Chain Reaction (PCR). The sense and antisense primers of primer set I and sense primer of primer set II were published sequences (Su YH 2004). The antisense primer in primer set II was developed specifically for this research project. The antisense primer in the primer set II was labeled with 6-FAM (6-carboxyl fluorescein) at 5′ end. Incorporation of 6-carboxyl fluorescein imparted the fluorescence property needed in the detection and quantification of K-ras using the capillary electrophoresis. Capillary electrophoresis was performed using ABI PRISM® 310 Genetic Analyzer. Primers were synthesized at the Macromolecular Structure Facility at Michigan State University. The primer sequences of both the primer sets are listed in Table 4. The primers were dried under vacuum in a SpeedVac SC100 system. Dried primers were reconstituted in one mL of sterile deionized distilled water and stored at -20°C.

Table 4. PCR Primers.

Primer Set I	
Sense*	GCTCTTCGTGGTGTGGTGTCCATATAAACTTGTGGTAGTTGGACCT
Antisense*	GCTCTTCGTGGTGTGGTGTCCCGTCCACAAAATGATTCTGA
Primer Set II	
Sense*	ACTGAATATAAACTTGTGGTAGTTGGACCT
Antisense*	**6-FAMCTGAAGTCCACAAAATGATTCTGAATTAGC

*The primer sequence is in 5′ to 3′ form.

** The antisense primer in the primer set II was labeled with 6-FAM (6-carboxyl fluorescein) at 5′ end.

➢ **Restriction-Enriched Polymerase Chain Reaction (RE-PCR)**

K-ras mutation was detected in a two-stage PCR assay using selective restriction enzyme digestion of an artificially created recognition site to distinguish wild-type and mutant K-ras sequences. Mutant K-ras sequences were enriched by RE-PCR.

- **First-stage PCR**

PCR amplification was completed in 0.2 mL thin walled reaction tube with a total volume of 50 µL. A master mix

containing all the PCR reagents except for the DNA template was prepared. The reaction mixture includes 1X GeneAmp PCR Buffer, 1.25 Units of AmpliTaq Gold DNA polymerase, 200 µM of each of the deoxyadenosine triphosphate (dATP), deoxycytosine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), deoxythymine triphosphate (dTTP), and 0.9 µL of each of the sense and antisense primers (primer set I, Table 1). For positive and negative controls, DNA templates for SW480 or PBLs at a concentration of 0.1 µg were added. For urine samples the amount of DNA present in 0.2 mL of urine was used as DNA template. Also, a blank was included in each PCR assay, which contained only sterile deionized distilled water instead of DNA template. In each case the final volume of 50 µL was adjusted with sterile deionized distilled water.

Hot start (Perkin Elmer) PCR method was used to produce higher yields of specific PCR amplified products. PCR amplification was performed with the GeneAmp® PCR System 9700 with an initial incubation at 95°C for 15 minutes. Followed by 15 cycles, each cycle included following steps; a) denaturation at 94°C for 48 seconds, b) annealing at 56°C for 90 seconds, and c) extension at 72°C for 2 minutes. A final extension, 72°C for 5 minutes, was done to ensure complete amplification of PCR products with

a 4°C temperature hold if the PCR product was to remain in the thermal cycler. These first 15 cycles of PCR amplified both wild-type and mutated DNA and introduced an artificial BstNI site to the 5' end of amplified product derived from wild-type K-ras sequences.

- **First-round of BstNI Digestion**

The restriction endonuclease enzyme, BstNI [source: Bacillus stearothermophilus N (D. Comb)], was used to digest artificially created recognition site in wild-type K-ras sequences.

After the first-stage PCR, the amplified products were digested with BstNI restriction endonuclease enzyme to eliminate amplified products derived from wild-type K-ras sequences. The mixture of 10 µL of first-stage PCR product, 10 Units of BstNI enzyme, and 1X NEBuffer 2 in a total volume of 20 µL (adjusted with sterile deionized distilled water), was incubated at 60°C for 3 hours.

- **Second-stage PCR**

PCR amplification was completed in 0.2 ml thin walled reaction tube with a total volume of 50 µL. A master mix containing all the PCR reagents except for the DNA template was prepared. The reaction mixture includes 1X GeneAmp PCR

Buffer, 1.25 Units of AmpliTaq Gold DNA polymerase, 200 µM of each of dATP, dCTP, dGTP, and dTTP, and 6 µL of sense and 7.5 µL of antisense primers (primer set II, Table 1). One micro-liter of the first-round of BstNI products was added as DNA template for second-stage PCR. The final volume of 50 µL was adjusted with sterile deionized distilled water.

Hot start PCR method was used and PCR amplification was performed with the GeneAmp® PCR System 9700 with an initial incubation at 95°C for 15 minutes. Followed by 35 cycles, each cycle included following steps; a) denaturation at 94°C for 48 seconds, b) annealing at 56°C for 90 seconds, and c) extension at 72°C for 2 minutes. A final extension, 72°C for 5 minutes, was done to ensure complete amplification of PCR products with a 4°C temperature hold if the PCR product was to remain in the thermal cycler.

- **Second-round of BstNI Digestion**

10 µL of second-stage PCR product, 1 X NEBuffer 2, 10 Units of BstNI enzyme, and 16 µL of sterile distilled water were mixed and incubated at 60°C water-bath for 5 hours.

➢ **Polyacrylamide Gel Electrophoresis (PAGE)**

The final BstNI digestion products were separated by electrophoresis using a Mini-PROTEAN® II Cell electrophoresis chamber and a Model 1000/500 Power Supply. A 12% polyacrylamide gel (19:1 acrylamide to bisacrylamide ratio, 1X TAE) with the dimensions of 75 mm x 85 mm x 0.75 mm was used. A 10 µL of final BstNI digested product was mixed with 2 µL of 5X nucleic acid sample loading buffer and loaded on the gel along with one lane of molecular weight marker which contained 50-500 bps DNA fragments. The samples were electrophoresed at 150 volts for 1 hour.

➢ **Detection of Bands of DNA Fragments (Qualitative Method)**

Ethidium bromide dye was used to stain DNA fragments. One drop of ethidium bromide (10 milligram per milliliter (mg/mL)) was diluted in at least 500 mL of water. The gel was placed into diluted ethidium bromide solution and incubated with gentle rotation (American Rotator V) at room temperature for about 10 minutes. The gel was washed thrice with water to remove excess stain on the gel. DNA bands were visualized using Chromato-Vue Transilluminator model TS-36 at the wavelength of 254 nm. The gels were photographed with type 667 black and white Polaroid® film

using a Fotodyne FCR-10 camera. Film was exposed to gel with UV light for one second at f=8 and allowed to develop for one minute.

➢ Quantitation of Mutant K-ras Sequences using Capillary Electrophoresis

DNA fragments after the second-round of BstNI enzyme digestion were used for K-ras quantitation. The quantification of mutant K-ras sequences was done by capillary electrophoresis using ABI PRISM® 310 Genetic Analyzer. The combination of the DS-30 dye set and virtual filter set D was used for analysis of all samples.

• Matrix File Preparation

The matrix file contains the information necessary for software to correct the spectral overlap of the dyes in the virtual filter set. The matrix file was generated from a separate matrix standards run, and contains information about how much of the collected light falling on a filter is due to the intended light emission and how much is contaminating light.

To generate matrix file, a matrix standard set (DS-30) was used which contains 6-FAM (6-carboxyfluoresein), HEX

(4, 7, 2', 4', 5', 7',-Hexachloro-6-carboxyfluorescein), ROX (6-Carboxy-X-rhodamine,), and NED (Applied Biosystems Proprietary) fluorescent dyes. Each fluorescent dye emits a continuous spectrum of light upon laser excitation. The matrix standards preparation and the electrophoresis on the ABI PRISM 310 Genetic Analyzer occurred as follows: 2 µL of each of the matrix dyes (6-FAM, HEX, NED, and ROX) was added to 24 µL of deionized formamide in MicroAmp 0.2 mL sample tubes. The mixture was mixed by vortexing and centrifuging for 5 seconds, denatured (DNA Thermal Cycler) at 95°C for 5 minutes and chilled on ice for 5 minutes. The matrix standards were injected in the capillary by electrokinetic injection for 5 seconds and electrophoresed at 15 kilo Volt (kV) for 24 minutes in Performance Optimized Polymer (POP-4™ polymer) with a run temperature of 60°C. The matrix standard run data were collected using the ABI PRISM® 310 data collection software v3.0.0. The data collection software generated the sample file of each matrix standard. The sample files of each matrix standard were used to create a matrix file using ABI PRISM® GeneScan® Analysis Software v3.7 as described in the user's manual (Ref. Applied Biosystems).

- **Preparation and Capillary Electrophoresis of K-ras DNA Fragment Samples**

The DNA fragments from the second-round of BstNI digestion were diluted appropriately with deionized distilled water. The appropriate amount of diluted sample and 0.5 µL of GeneScan™-350 ROX® internal lane size standard (vortexed and centrifuged for 5 seconds) were added to 12 µL of deionized formamide in MicroAmp 0.2 mL sample tube. The mixture was vortexed and centrifuged for 5 seconds, denatured at 95°C for 5 minutes and chilled on ice for 5 minutes. Samples were injected in a capillary by electrokinetic injection for 5 seconds and electrophoresed at 15 kilo Volt (kV) for 24 minutes in Performance Optimized Polymer (POP-4™ polymer) with a run temperature of 60°C. The data were collected using the ABI PRISM® 310 data collection software application v3.0.0. The sample file for appropriate sample was created by data collection software.

➢ **Data Analysis**

The electrophoresis results were analyzed using ABI PRISM® GeneScan® Analysis Software v3.7. The analysis parameters for data processing are described in Table 5.

The peaks were interpreted when greater than or equal to 50 relative fluorescence units (RFUs). The matrix file was applied to the samples before analyzing the data. The GS350-250 size standard parameter was used for size calling of DNA fragments. Size calling, peak height, and peak area of samples were determined.

Table 5. Analysis Parameters

Analysis Parameters	Values
Analysis range	2500 to 4000 Data points
Size Call range	35 to 150 base pairs
Data Processing :Smooth options	None
Size Calling method	Local Southern Method
Peak Detection: o Peak Amplitude Thresholds: B Y G R o Minimum Point Half Width o Polynominal Degree o Peak Window Size o Slope Threshold for Peak Start o Slope Threshold for Peak end	 50 50 50 50 2 Points 3 15 Points 0.0 0.0
Baselining: BaseLine Window Size	 51 Points

Quantitation of Mutant K-ras Sequences in Serial Dilution Assay

- **Intra-sample Variability**

The second BstNI digestion products of RE-PCR from the serial dilution assay (described earlier) were taken to perform the quantitative analysis of mutant K-ras sequences using capillary electrophoresis as described above. The procedure was performed using the same sample three times to determine the intra-sample variability.

- **Inter-sample Variability**

The second BstNI digestion products of RE-PCR from the serial dilution assay as described earlier were taken to perform the quantitative analysis of mutant K-ras sequences using capillary electrophoresis as described above. The procedure was performed in triplicate; three different sample sets were prepared to determine the inter-sample variability.

RESULTS

DNA Isolation from Positive and Negative Controls

The concentration of DNA isolated from SW480 cell line (positive control), PBLs (negative control), and urine is shown in Table 6. The optical density at 260nm and 280 nm measured using spectrophotometer and 260nm:280nm ratio was calculated.

Table 6. DNA Concentration in SW480 cell-line and PBLs

	OD at 260 nm	OD at 280 nm	Ratio 260 nm/280 nm	DNA concentration in µg/µL
SW480 cell-line	0.059	0.031	1.879	0.118
PBLs	0.160	0.085	1.892	0.320
Urine	0.038	0.021	1.815	0.0076

Detection of K-ras Sequences after RE-PCR and Gel Electrophoresis (Qualitative Method)

The SW480 cell line (positive control), PBLs (negative control), and normal urine negative for mutant K-ras were amplified by RE-PCR as described in materials and 10 µL was electrophoresed on a 12% PAGE as described previously.

- **First-stage PCR and First- round of BstNI Digestion**

The results of gel electrophoresis following first-stage PCR and first-round of BstNI digestion are shown in Figure 2.

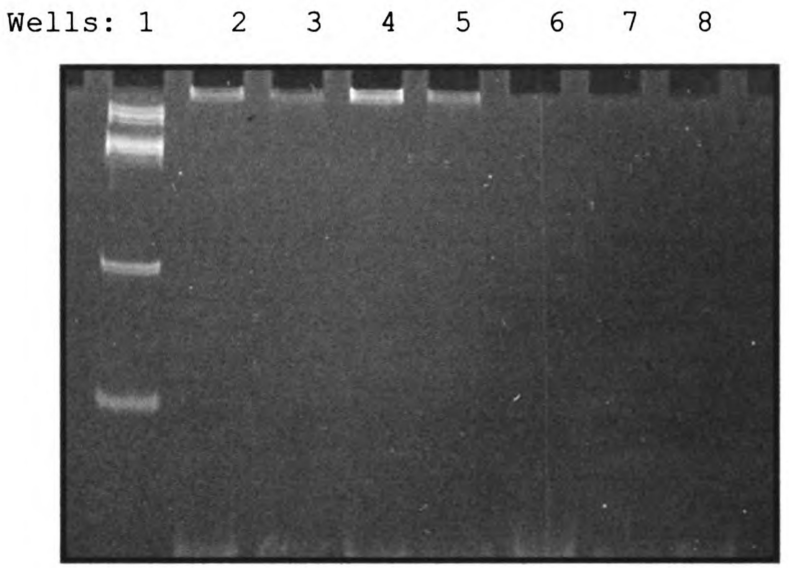


Figure 2. A gel photograph after first-stage PCR and first-round of BstNI digestion.
Well 1 - molecular ruler (50-500 bps)
Wells 2, 4, and 6 - First PCR products of SW480, PBLs, and normal urine negative for mutant K-ras sequences, respectively (125 bps)
Wells 3, 5, and 7 - SW480, PBLs, and normal urine negative for mutant K-ras, respectively, after first-stage PCR followed by first round of BstNI digestion
Well 8 - negative control for PCR (water, no DNA)

The first-stage PCR amplifies both mutant and wild-type K-ras sequences; the amplified fragment size was 125 bps. The BstNI digestion cut the amplified wild-type K-ras sequences in 85 and 40 bps fragments.

- **Second-stage PCR and Second-round of BstNI Digestion**

The results of gel electrophoresis following second-stage PCR and second-round of BstNI digestion are shown in Figure 3.

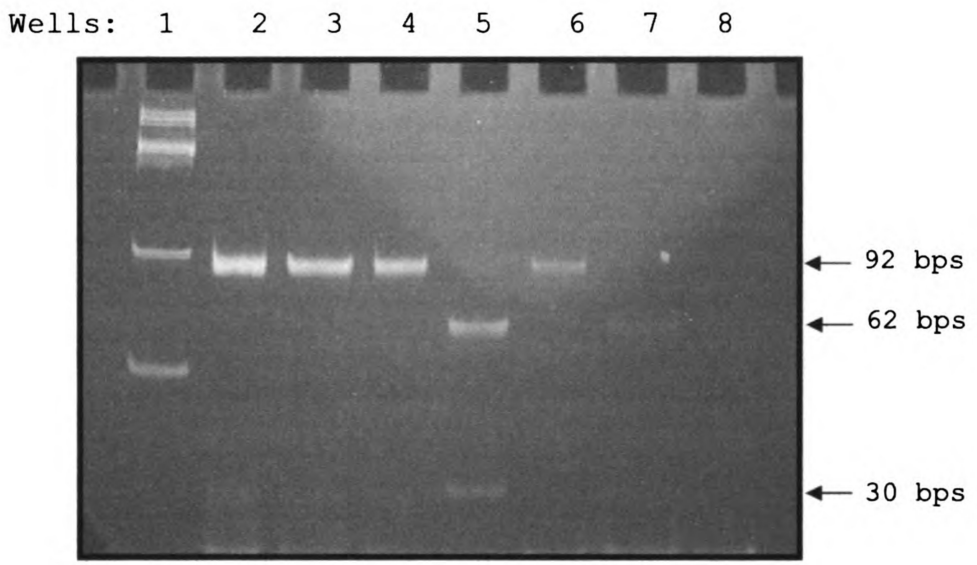


Figure 3. A photograph of gel electrophoresis after second-stage PCR and second-round of BstNI digestion.
Well 1 - molecular ruler (50-500 bps)
Wells 2, 4, and 6 - second PCR products of SW480, PBLs, and normal urine negative for mutant K-ras sequences, respectively (92 bps)
Wells 3, 5, and 7 - K-ras fragments after second BstNI digestion of SW480 (92 bps), PBLs (62 and 30 bps), and normal urine negative for mutant K-ras (62 and 30 bps), respectively
Well 8 - negative control for PCR (water, no DNA)

The fragment sizes of amplified products after second-stage PCR were 92 bps. The second-round of BstNI digestion cut the amplified wild-type K-ras sequences into 62 and 30 bps fragments.

Ability of Urine Isolation Method to Detect Low Molecular Weight Mutant K-ras Sequences

Gel electrophoresis data for the isolation of 125 bps fragments of SW480 cell line first-stage PCR by urine isolation method and RE-PCR is shown in Figure 4.

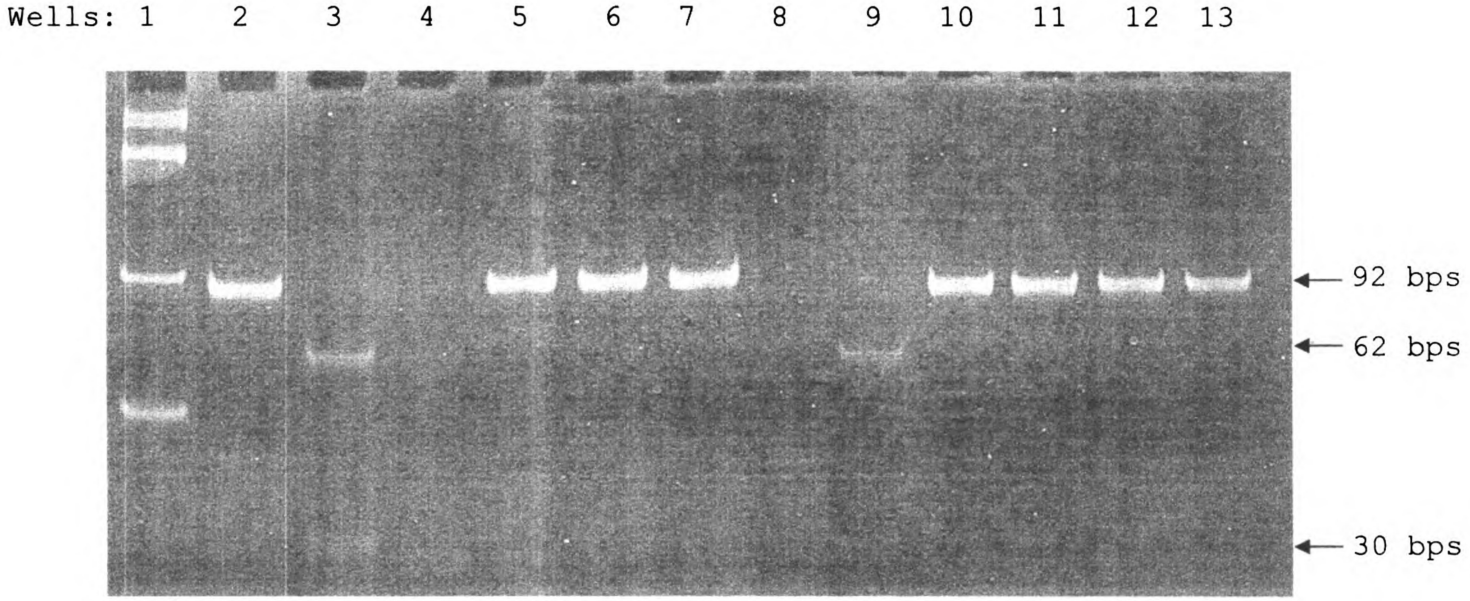


Figure 4. The photograph of gel electrophoresis after RE-PCR.
Well 1 molecular ruler (50-500 bps)
Well 2 SW480 cell line
Wells 3 and 9 PBLs
Well 4 normal urine negative for mutant K-ras
Wells 5, 6, 7, 10, 11, 12, and 13 are DNA isolated from normal urine mixed with 80 µL, 15 µL, 5 µL, 1 µL, 0.1 µL, 0.01 µL, and 0.001 µL of SW480 cell line first-stage PCR products which are 125 bps fragments respectively.
Well 8 - negative control for PCR (water no DNA)

Serial Dilution Assay

- **Qualitative Analysis of Mutant K-ras Sequences**

The serial dilution assay was performed to determine the sensitivity of RE-PCR method by diluting SW480 cell line (positive control) with PBLs (negative control) to obtain different mutant K-ras concentration as described previously. The gel photograph was obtained after 12% PAGE followed by RE-PCR as showed in Figure 5.

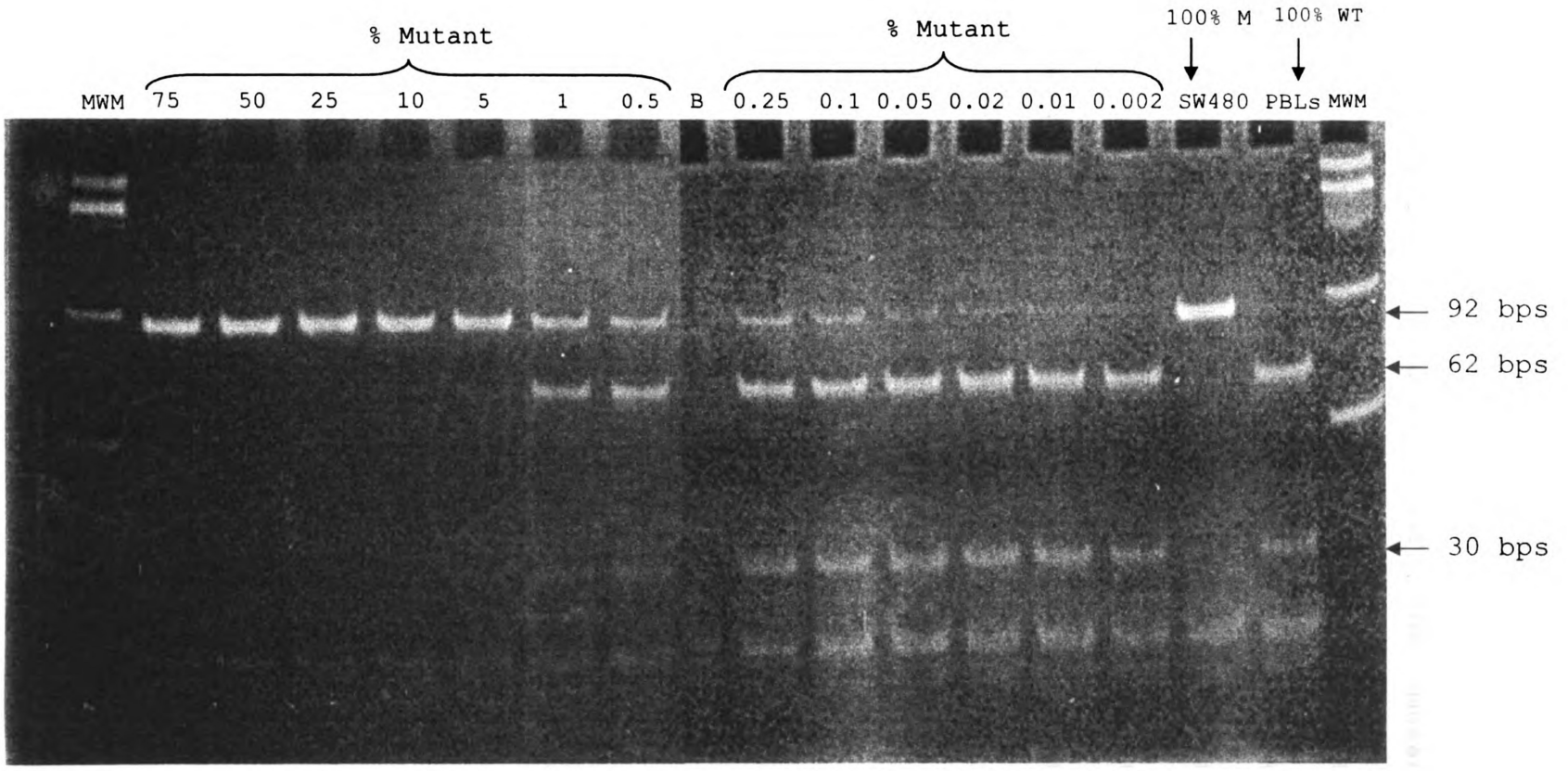


Figure 5. A gel photograph of serial dilution assay after RE-PCR and 12% PAGE.
MWM - molecular weight marker (50-500 bps)
B - Negative control of PCR (water no DNA)
SW480 referred to 100% mutant for K-ras codon 12 sequences
PBLs referred to 100% wild-type for K-ras codon 12 sequences

- **Quantitative Analysis of Mutant K-ras Sequences in Serial Dilution Assay**

Quantitative analysis of the mutant K-ras was performed using ABI PRISM® GeneScan Analysis software. The capillary electrophoresis followed by RE-PCR was performed as described earlier in serial dilution assay. Figures 6-8 show the fragment analysis by ABI PRISM® GeneScan Analysis software v 3.7. Y-axis indicated fluorescence intensity, while the X-axis indicated base pair size.

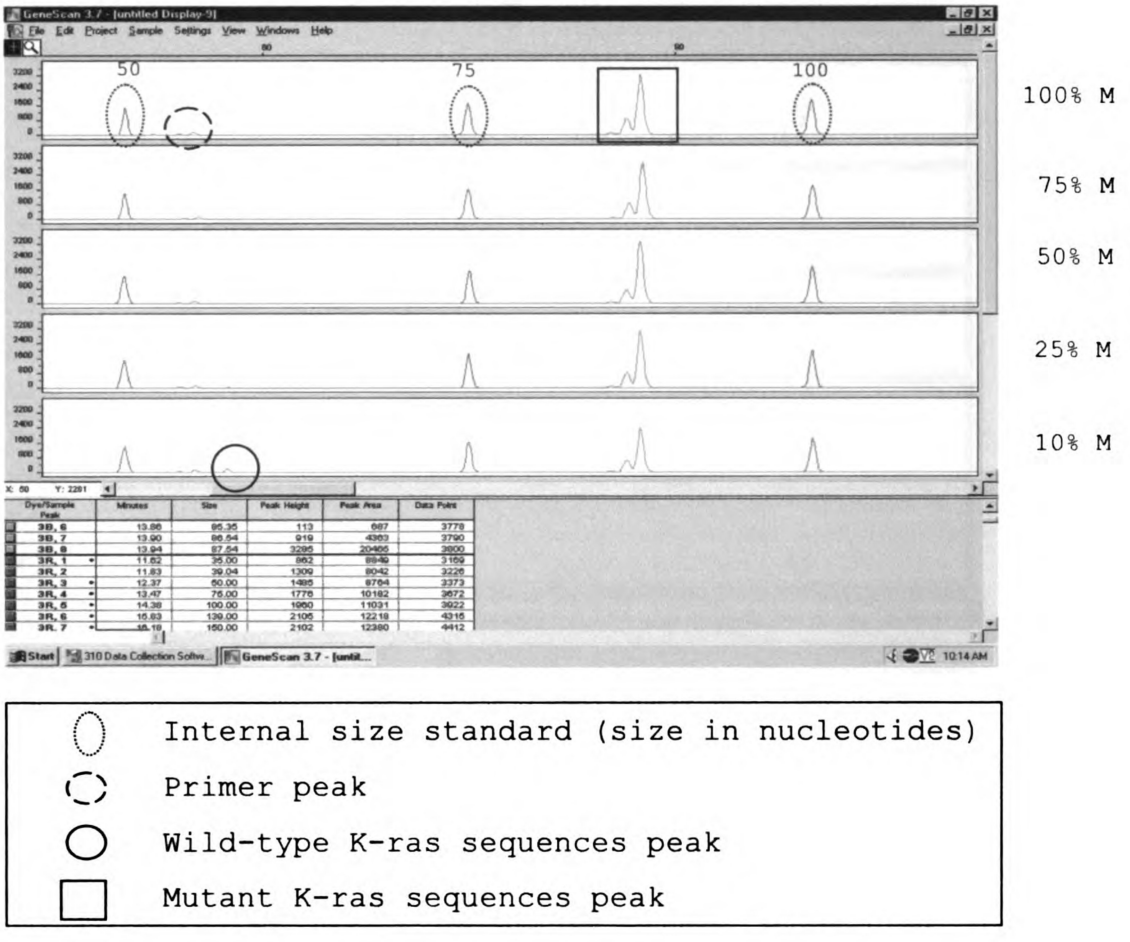


Figure 6. A fragment analysis picture of 100%, 75%, 50%, 25%, and 10% mutant K-ras sequences. All the peaks in different serial dilution samples were aligned by fragment size.

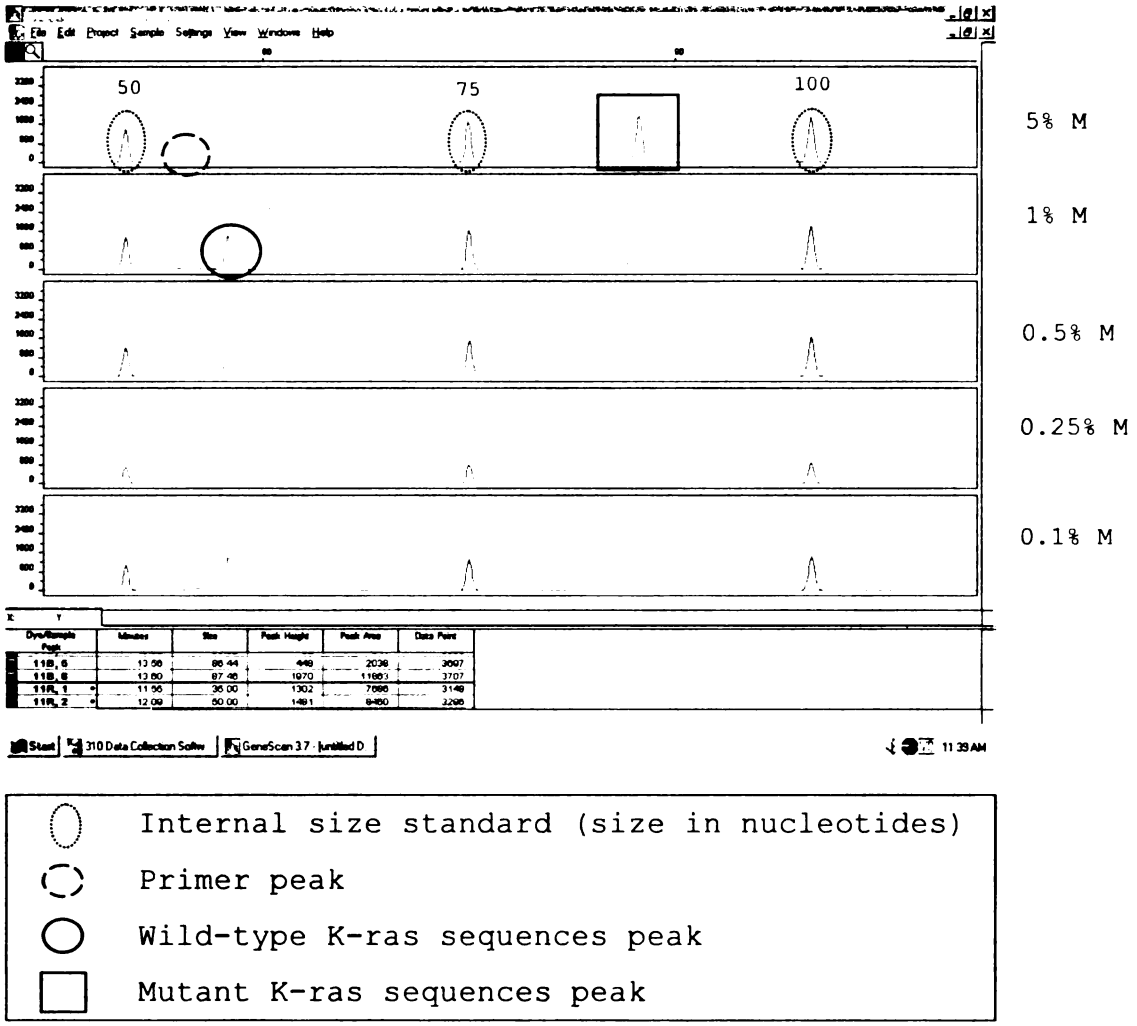


Figure 7. A fragment analysis picture of 5%, 1%, 0.5%, 0.25%, and 0.1% mutant K-ras sequences. All the peaks in different serial dilution samples were aligned by fragment size.

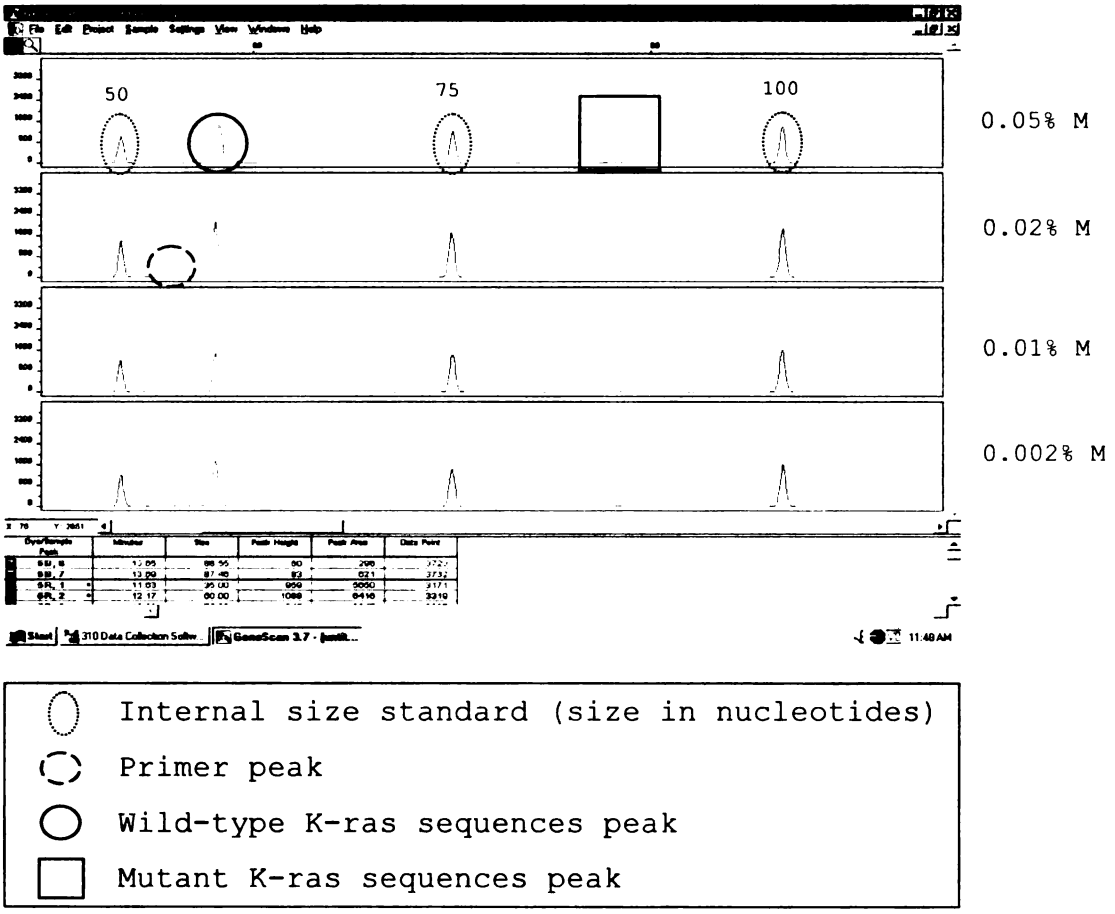


Figure 8. A fragment analysis picture of 0.05%, 0.02%, 0.01%, and 0.002% mutant K-ras sequences. All the peaks in different serial dilution samples were aligned by fragment size.

- **Intra-sample Variability**

The capillary electrophoresis followed by RE-PCR was performed as described earlier in serial dilution assay. To determine the intra-sample variability of analytical method, each % mutant sample was analyzed in triplicate and peak area was obtained as shown in Table 7. Figure 9 shows the data range that provided the best linear relationship

between log % mutant vs. log mean area. Data on log % mutant and log mean area are shown in Table 8.

Table 7. Intra-sample variation.

% Mutant	Area 1	Area 2	Area 3	Mean Area	SD	CV(%)
100	25677	25515	23167	24786	1405	5.669
75	25415	25667	24712	25265	495	1.959
50	24811	23961	25885	24886	964	3.874
25	23898	23603	21818	23106	1125	4.869
10	18414	18914	15143	17490	2048	11.710
5	12471	14591	11696	12919	1499	11.603
1	6902	6807	6129	6613	422	6.381
0.5	4596	4670	4376	4547	153	3.365
0.25	4110	4179	3111	3800	598	15.737
0.1	1924	1731	1701	1785	121	6.779
0.05	974	999	989	987	13	1.317
0.02	492	478	452	474	20	4.219
0.01	460	397	497	451	51	11.308
0.002	na	na	417	417	-	-

Table 8. Intra-sample variation. Calculation of log % mutant and log mean area of data shown in Table 7.

Log % Mutant	Log Area 1	Log Area 2	Log Area 3	Log Mean Area	SD
2.000	4.410	4.407	4.365	4.394	0.025
1.875	4.405	4.409	4.393	4.402	0.009
1.699	4.395	4.380	4.413	4.396	0.017
1.398	4.378	4.373	4.339	4.363	0.021
1.000	4.265	4.277	4.180	4.241	0.053
0.699	4.096	4.164	4.068	4.109	0.049
0.000	3.839	3.833	3.787	3.820	0.028
-0.301	3.662	3.669	3.641	3.658	0.015
-0.602	3.614	3.621	3.493	3.576	0.072
-1.000	3.284	3.238	3.231	3.251	0.029
-1.301	2.989	3.000	2.995	2.994	0.006
-1.699	2.692	2.679	2.655	2.676	0.019
-2.000	2.663	2.599	2.696	2.653	0.050
-2.699	na	na	2.620	2.620	-

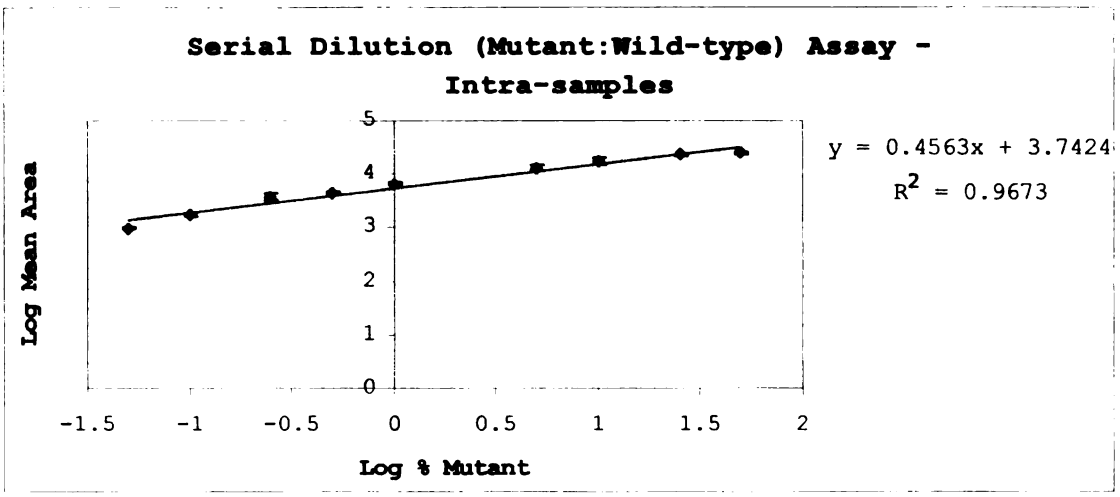


Figure 9. A plot of log % mutant vs. log mean area (Intra-sample variation).

- **Inter-sample Variability**

Three completely different sets of samples were prepared and analyzed to determine the repeatability of the entire process. The data from this triplicate experiment are shown in Table 9. Figure 10 shows the data range that provided the best linear relationship between log % mutant vs. log mean area. Data on log % mutant and log mean area are shown in Table 10.

Table 9. Inter-sample variation.

% Mutant	Area 1	Area 2	Area 3	Mean Area	SD	CV(%)
100	25677	26186	22591	24818	1945	7.837
75	25415	26389	26338	26047	548	2.104
50	24811	24155	21052	23339	2008	8.604
25	23898	20563	20544	21668	1931	8.912
10	18414	16587	15342	16781	1545	9.207
5	12471	14242	14525	13746	1113	8.097
1	6902	6253	6222	6459	384	5.945
0.5	4596	4557	4342	4498	137	3.046
0.25	4110	4130	3343	3861	449	11.629
0.1	1924	1850	1670	1815	131	7.218
0.05	974	917	909	933	35	3.751
0.02	492	494	409	465	49	10.538
0.01	460	397	492	450	48	10.667
0.002	414	na	na	414	-	-

Table 10. Inter-sample variation. Calculation of log of % mutant and log mean area on data showed in Table 9.

Log % Mutant	Log Area 1	Log Area 2	Log Area 3	Log Mean Area	SD
2.000	4.410	4.418	4.354	4.394	0.035
1.875	4.405	4.421	4.421	4.416	0.009
1.699	4.395	4.383	4.323	4.367	0.038
1.398	4.378	4.313	4.313	4.335	0.038
1.000	4.265	4.220	4.186	4.224	0.040
0.699	4.096	4.154	4.162	4.137	0.036
0.000	3.839	3.796	3.794	3.810	0.025
-0.301	3.662	3.659	3.638	3.653	0.013
-0.602	3.614	3.616	3.524	3.585	0.052
-1.000	3.284	3.267	3.223	3.258	0.032
-1.301	2.989	2.962	2.959	2.970	0.016
-1.699	2.692	2.694	2.612	2.666	0.047
-2.000	2.663	2.599	2.692	2.651	0.048
-2.699	2.617	Na	na	2.617	-

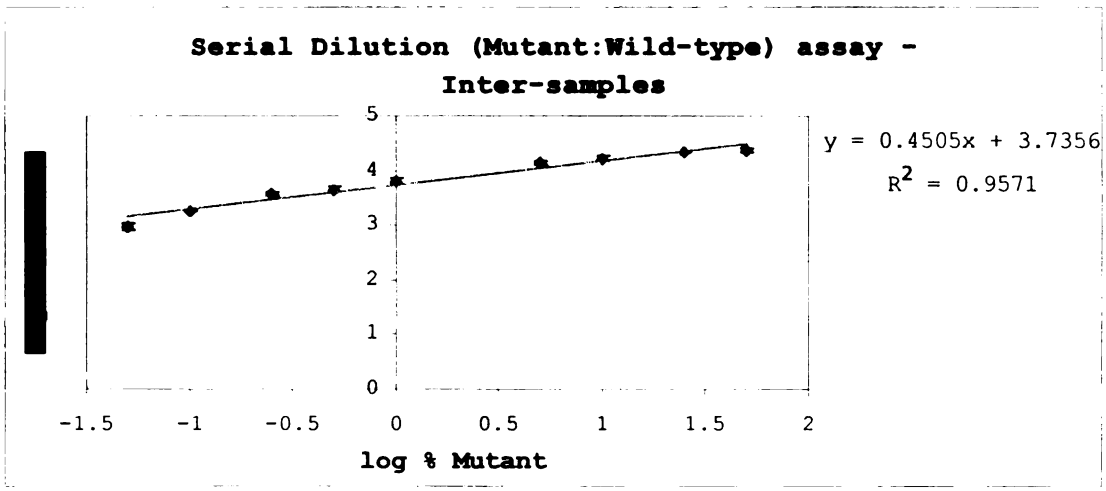


Figure 10. A plot of log % mutant vs. log mean area (Inter-sample variation).

DISCUSSION

DNA Isolation from Positive and Negative Controls

The concentration of DNA isolated from SW480 cell line (positive control), PBLs (negative control), and in Urine are presented in Table 1. The ratio of optical density measured at 260nm and 280nm is greater than 1.8 in all cases, indicating good purity of DNA in the isolated samples.

Detection of K-ras Sequences after RE-PCR and Gel Electrophoresis (Qualitative Method)

A previously described RE-PCR method was used as a starting method (Su Y.H. 2004) and modified in order to achieve the goals of this study.

- **First-stage PCR and First-round of BstNI Digestion**

First-stage PCR would amplify both mutant and wild-type K-ras sequences to 125 bps as shown in Figure 11 Sense primer creates an artificial BstNI digestion site at codon 11 and 12 in the wild-type K-ras; since mutant K-ras differs from wild-type at codon 12 the sense primer does not create the artificial BstNI digestion site on the mutant K-ras.

BstNI restriction enzyme would cut the wild-type K-ras at the BstNI digestion site into 80 and 45 bps fragments. Since, no BstNI digestion site was created in the mutant K-ras, the fragment remains unchanged at 125 bps. However, as observed from Figure 1 no bands were seen for positive control (SW480), negative control (PBLs), normal urine negative for mutant K-ras either after the first stage PCR or after the first BstNI digestion, this may have resulted from the very low amount of DNA in the samples.

- **Second-stage PCR and Second-round of BstNI Digestion**

The objective of the study was to qualitatively and quantitatively identify the mutant K-ras. The RE-PCR followed by gel electrophoresis method was developed by Su YH to detect K-ras qualitatively. In order to quantitatively detect both mutant and wild type K-ras sequences using capillary electrophoresis the K-ras sequences had to be labeled with fluorescence. In order to separate wild-type and mutant K-ras on capillary electrophoresis wild-type and mutant K-ras had to be cleaved differently during the second PCR and BstNI. An antisense primer, as shown in Table 1 (Antisense Primer Set II) was specifically developed for this purpose. The antisense primer in the primer set II was labeled with 6-

FAM (6-carboxyl fluorescein) at 5′ end. Incorporation of 6-carboxyl fluorescein imparted the fluorescence property needed in the detection and quantification of K-ras using the capillary electrophoresis. During second stage PCR; sense primer attaches to the K-ras sequences so that it introduces BstNI digestion site in the wild-type K-ras, while in mutant K-ras the base sequence is different because of the mutation, therefore, the BstNI digestion site is not created.

The gel picture (Figure 2) of SW480, PBL, and normal urine negative for mutant K-ras shows the fragment sizes of 92 bps. This would have resulted from the amplification of mutant or wild-type K-ras as explained by Figure 11. The gel picture of PBLs and normal urine following the second-round of BstNI digestion shows the presence of bands of 62 and 30 bps and no band of 92 bps. While the band for SW480 appear at 92 bps. This could be explained on the basis that sense primer used during second PCR would create an artificial BstNI digestion site at codon 11 and 12 in the wild-type K-ras; since mutant K-ras differs from wild-type at codon 12 the sense primer would not create the artificial BstNI digestion site on the mutant K-ras. During the second BstNI digestion, the BstNI restriction enzyme would cut the wild-type K-ras into 62 and 30 bps fragments.

Since, no BstNI digestion site is created; the mutant K-ras fragment remains unchanged at 92 bps.

The results show that the RE-PCR method followed by gel electrophoresis was able to specifically detect presence of mutant K-ras in the DNA samples.

Ability of Urine Isolation Method to Detect Low Molecular Weight Mutant K-ras Sequences

Gel picture in Figure 3 shows presence of bands at 62 and 30 bps for PBL and normal urine negative for mutant K-ras. This could be explained as described earlier (under RE-PCR section) and presented in Figure 11. Samples of various amounts of low molecular weight 125 bps SW480 after first PCR mixed with normal urine shows the presence of band at 92 bps. This could be explained as described earlier (under RE-PCR section) and presented in Figure 11. This data indicates that the urine isolation method was able to isolate low molecular weight DNA fragments.

Serial Dilution Assay

- **Qualitative Analysis of Mutant K-ras Sequences**

As observed in Fig. 4 the density of the band for fragment 92 bps correlates qualitatively with the concentration of SW480 in the samples. With the decrease in the density of band for fragment 92 bps as the concentration of SW480 decreases in the sample, as expected, there is a corresponding increase in the density of the bands for fragments 62 and 30 bps obtained by second BstNI digestion of 92 bps band of the wild type K-ras. From the gel picture it is clear that even at the concentration as low as 0.02% of SW480 band at 92 bps is visible. This clearly shows that the qualitative method used to detect K-ras is very sensitive.

- **Quantitative Analysis of Mutant K-ras Sequences in Serial Dilution Assay**

Quantitative Analysis

A typical electrpherogram is shown in figure 6. The fragments were sized by comparing to GS350-250 internal size standard. The expected sizes for the mutant K-ras sequences and wild-type K-ras sequences were 92 bps and 62

bps, respectively. The observed sizes were within 5 bps of expected sizes. This difference may be due to the conformation of the DNA or mobility variation among dyes. A previous research study had reported a similar shift of 5 bps during the quantitative analysis of the FAM-labeled fragment for factor V Leiden by capillary electrophoresis using ABI Prism 310 (Benson J 1999). Another study had reported a shift in 2 bps while quantifying Apolipoprotein using capillary electrophoresis using ABI Prism 310 (Sell S.M. 1997).

- **Intra-sample Variability**

Data of intra-sample variability as seen in Table 7 indicates that the analytical method was able to quantify the mutant K-ras consistently as indicated by low standard deviation even in the samples containing mutant K-ras as low as 0.05%. The coefficient of variation (CV) was found to be less than 15%.

Figure 9 shows plot of log % mutant vs. log mean area. A high value of coefficient of determination (R^2) 0.9673, close to 1, indicates a good correlation between log % mutant and log mean area in the range of 50 to 0.05% mutant K-ras.

- **Inter-sample Variability**

As seen from the data in Table 9, results obtained from all the three sample set is very consistent (low values of standard deviation) indicating that the sample preparation method is reproducible even for the dilute samples containing mutant K-ras as low as 0.05%. The coefficient of variation (CV) was found to be less than 15%.

CONCLUSIONS

The QIAamp® DNA Mini Kit was used to obtain genomic DNA from PBLs (negative control) and SW480 (positive control). Method to isolate DNA from urine sample was adopted from previous studies and modified to achieve the objectives of these studies.

Two stage Restriction Enriched Polymerase Chain Reaction followed by gel electrophoresis was used to detect mutant K-ras sequences without any interference from the wild-type K-ras sequences. This method was used as the qualitative method for the detection of mutant K-ras. First-stage PCR amplified both mutant and wild-type K-ras. First-round of BstNI digestion was carried out after first stage RE-PCR with primer set I in order to cut the amplified wild-type K-ras sequences to 85 and 40 bps. However, due to the lower DNA concentration; bands for mutant and wild-type K-ras were not observed in gel electrophoresis following first-stage RE-PCR and first-round of BstNI digestion. Second-stage PCR amplified both mutant and wild-type K-ras. Second-round of BstNI digestion was carried out after second-stage PCR with primer set II. The second-round of BstNI digestion cut the amplified wild-type K-ras sequences in 62 and 30 bps as observed in gel electrophoresis, while the mutant K-ras band was observed at 92 bps in SW480. Qualitative method was able to detect

presence of mutant K-ras in 0.02% SW480 sample, indicating the method is very sensitive.

Capillary electrophoresis was used as a quantitative method for analysis of mutant K-ras; quantification was performed using ABI PRISM® GeneScan Analysis software. Capillary electrophoresis was able to quantify the mutant K-ras consistently even in the dilute samples containing mutant K-ras as low as 0.05%. A good correlation between log % mutant and log mean area was obtained in the range of 50 to 0.05% mutant.

The whole process of DNA isolation followed by RE-PCR followed by quantitative analysis of mutant K-ras using capillary electrophoresis was found to be reproducible as indicated by the low correlation of variation (CV) (<15%) values between the three sets of samples prepared and analyzed at a different time. Also, the intra-sample variability was found to be low as indicated by low CV (<15%).

A qualitative and a quantitative method was developed to detect the presence of mutant K-ras in urine sample which could potentially be used in combination with other screening methods for the early detection of colorectal cancer in patients, which may improve the survival rate in future. Testing for mutated K-ras during follow-up after

resection from colorectal cancer may provide new tools for detection of recurrence or second primary tumors and thus may serve to identify appropriate treatments or to avoid ineffective treatments, and thus may increase survival.

APPENDIX

Materials

1. Positive Control, Genomic DNA from SW480 cell line (American Type Culture Collection (ATCC)) possessing a mutation in codon 12 of the K-ras proto-oncogene was used as a positive control.

2. Negative Control, Genomic DNA from Peripheral Blood Leucocytes (PBLs) was used as a negative control for K-ras codon 12.

3. Urine Specimen

4. EDTA(ethylenedinitrilo) tetraacetic acid), Disodium Salt, Dihydrate, Crystal, product # 8993-01, Mallinckrodt Baker , Inc., Phillipsburg, NJ.

5. Fifteen mL Conical Polypropylene Screw Cap Tube, part # 430052, Corning Inc., Corning, NY.

6. Guanidine Isothiocyanate, catalog # 15535-016, Invitrogen Corporation, Carlsbad, CA.

7. Fifty mL Conical Polypropylene Screw Cap Tubes, part # 430290, Corning, Inc., Corning, NY.

8. Wizard® Plus Minipreps DNA Purification Systems, catalog # A7100, Promega Corporation, Madison, WI.

9. 1.5 mL microcentrifuge tube (Eppendrof tubes), catalog # 2236411-1, Brinkmann Instruments Ltd., Canada.

10. SW480 cell line, ATCC® # CCL-228, American Type Culture Collection (ATCC), Manassas, VA.

11. Sodium Chloride, Crystals, product # 3624-05, Mallinckrodt Baker Inc., Phillipsburg, NJ.

12. Potassium Chloride, Crystals, lot # 707202, Columbus Chemical Industries, Inc., Columbus, WI.

13. Sodium Phosphate Monobasic (NaH2PO4), Anhydrous, lot # 126H04925, Sigma-Aldrich, St. Louis, MO.

14. Potassium Phosphate Monobasic (KH2PO4), Batch # 114K0240, Sigma-Aldrich, St. Louis, MO.

15. QIAamp® DNA Mini Kit, catalog # 51304, QIAGEN Inc., Valencia, CA.

16. Ethyl alcohol 200 proof (Ethanol), catalog # 111USP200, Pharmaco Products, Brookfield, CT.

17. 0.2 ml thin wall PCR tubes with attached cap, catalog # 501-PCR, Dot Scientific Incorporated, Burton, MI.

18. GeneAmp PCR Buffer 10X, part # N808-0240, Applied Biosystems, Foster city, CA.

19. AmpliTaq® Gold DNA polymerase, part # N808-0240, Applied Biosystems, Foster city, CA.

20. dATP, dCTP, dGTP, and dTTP, part # N8080007, Applied Biosystems, Foster city, CA.

21. Primer set I and II (Table 1), Macromolecular Structure facility, Michigan State University, East Lansing, MI.

22. BstNI Endonuclease Enzyme (source: Bacillus stearothermophilus N (D. Comb)), catalog # R0168S, New England Biolabs Inc., Beverly, MA.

23. NEBuffer 2, 10X, catalog # R0168S, New England Biolabs Inc., Beverly, MA.

24. Acrylamide, lot # 14328820, Boehringer Mannheim corp., Indianapolis, IN.

25. BIS (N, N'-methylene-bis-acrylamide), catalog # 161-0201, Bio-Rad Laboratories, Hercules, CA.

26. Trizma® Base, catalog # T1503-500G, Sigma-Aldrich, St. Louis, MO.

27. Glacial acetic acid, lot # 39076, EM Industries Inc., Gibbstown, NJ.

28. N, N, N', N'-Tetramethylethylenediamine (TEMED), lot # 14336120, Boehringer Mannheim Corporation, Indianapolis, IN.

29. Ammonium Persulphate, Crystal, lot # C49331, Mallinckrodt Baker, Inc., Phillipsburg, NJ.

30. 5X Nucleic Acid Sample Loading Buffer, catalog # 161-0767, Bio-Rad, Richmond, CA. This loading dye was received along with EZ load 100 bp Molecular Ruler, catalog # 170-8352.

31. 50-500 bp Molecular Weight Marker, Ref # 74605-250, PEL-FREEZ, Dynal Biotech, Brown Deer, WI.

32. Ethidium Bromide Solution (10mg/mL), catalog # E1510, Sigma-Aldrich, St. Louis, MO.

33. Polaroid® Type 667 Black and White Instant Pack Film, Polaroid Incorporation, Cambridge, MA.

34. Matrix Standard Sets, Applied Biosystems, Foster city, CA.
    a) Fluorescent Amidite Matrix Standards Kit (contains one tube each of 6-FAM-, HEX-, TED-, TAMRA-, and ROX-labeled DNA), part # 401546.
    b) NED Matrix Standard, part # 402996.

35. Formamide, catalog # 11814320001, Roche Diagnostics, Indianapolis, IA.

36. MicroAmp 0.2 mL sample tube, part # N801-0531, Applied Biosystems, Foster city, CA.

37. Performance Optimized Polymer (POP-4™ polymer), part # 402838, Applied Biosystems, Foster city, CA.

38. 10X Genetic Analyzer Buffer with EDTA, part # 402824, Applied Biosystems, Foster city, CA.

39. GeneScan™-350 ROX® Internal Lane Size Standard, part # 401735, Applied Biosystems, Foster city, CA.

Instruments

1. GS Gene Linker UV chamber, serial # B05BR0581, Bio-Rad, Richmond, CA.

2. Precision Water bath-micro, serial # 9503-107, Precision Scientific Inc., Winchester, VA.

3. GeneQuant RNA/DNA Calculator, Serial # 80-2111-98, Biochrom Ltd, Cambridge Science Park, Cambridge, England.

4. Sorvall TC6 tabletop centrifuge, Serial # 9204183, Sorvall, Thermo Electron Corporation, Asheville, NC.

5. Lab Rotator, Model # 2314, Barnstead International, Dubuque, IA.

6. Sorvall Biofuge® Pico, Serial # 40245700, Sorvall, Thermo Electron Corporation, Asheville, NC.

7. Precision Water Bath, Model # 181, Precision Scientific Inc., Winchester, VA.

8. Vortexer, Type 16700 Mixture, Model # M16715, Barnstead Thermodyne, Dubuque, IA.

9. SpeedVac SC100 System, serial # SC100-OK47405-1A, Savant Instruments, Farmingdale, NY.

10. GeneAmp® PCR System 9700, serial # A965-2050316, Applied Biosystems, Foster city, CA.

11. Precision Shaking Water Bath, serial # 26AT-5, GCA Corporation, Precision Scientific Group, Chicago, IL.

12. Mini-PROTEAN® II Cell electrophoresis chamber, serial # 125BR 36702, and a Model 1000/500 Power Supply, Bio-RAD, Richmond, CA.

13. American Rotator V, serial # 030467, American Dade, American Hospital Supply Corporation, Miami, FL.

14. Chromato-Vue Transilluminator, Model # TS-36, UVP Incorporated, San Gabriel, CA.

15. Fotodyne FCR-10 camera, Fotodyne Incorporated, Hartland, WI.

16. ABI PRISM® 310 Genetic Analyzer, Serial # 310-95080145, Applied Biosystems, Foster city, CA.

17. DNA Thermal Cycler, serial # P3254, Perkin Elmer, Norwalk, CT.

Mutant and Wild-type K-ras Sequences during Restriction Enriched Polymerase Chain Reaction

- *Wild-type K-ras exon 1 DNA Sequence*

```
5' ATGACTGAAT ATAAACTTGT GGTAGTTGGA GCTGGTGGCG TAGGCAAGAG
   |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
3' TACTGACTTA TATTTGAACA CCATCAACCT CGACCACCGC ATCCGTTCTC

TGCCTTGACG ATACAGCTAA TTCAGAATCA TTTTGTGGAC 3'
|||||||||| |||||||||| |||||||||| ||||||||||
ACGGAACTGC TATGTCGATT AAGTCTTAGT AAAACACCTG 5'
```

- *Mutant K-ras Sequence*

Mutant K-ras sequence differs from the wild-type K-ras at the codon 12 (GGT, highlighted in the sequence above) and could have either of following sequence:

- GAT
- GTT
- GCT
- AGT
- TGT
- CGT

➢ **First-stage PCR**

Following Primers were used during the first-stage PCR:

- *Sense Primer Set I*

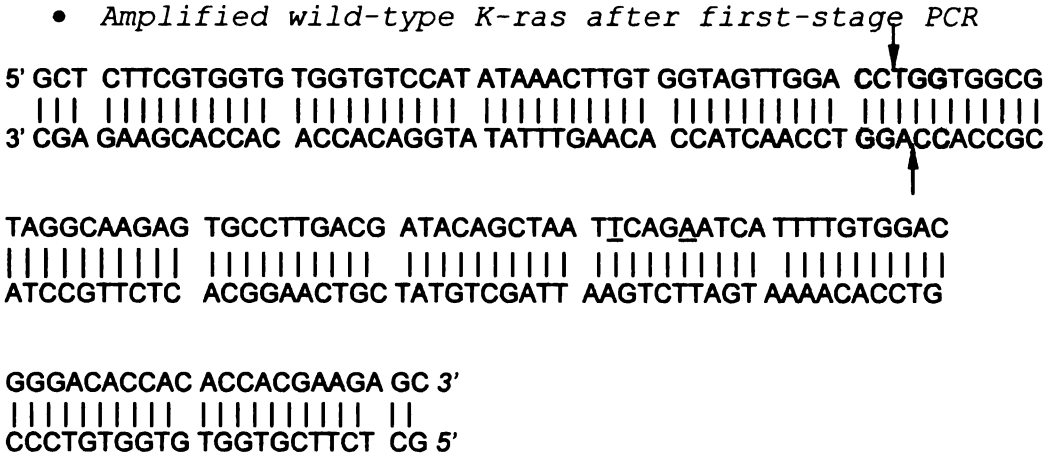
5′ GCTCTTCGTGGTGTGGTGTCCATATAAACTTGTGGTAGTTGGACCT 3′

- *Antisense Primer Set I*

5′ GCTCTTCGTGGTGTGGTGTCCCGTCCACAAAATGATTCTGA 3′

Mutant and Wild-type K-ras amplifies to 125 bps during the first stage PCR. Sense primer creates an artificial BstNI digestion site (by encoding G → C substitution at the first position of codon 11) at codon 11 and 12 in the wild-type K-ras (highlighted in the sequence below); since mutant K-ras differs from wild-type at codon 12 the sense primer does not create the artificial BstNI digestion site on the mutant K-ras.

- *Amplified wild-type K-ras after first-stage PCR*



- **First-round of BstNI Digestion**

BstNI restriction enzyme cuts the wild-type K-ras at the BstNI digestion site created by sense primer used in the first stage PCR into 80 and 45 bps fragments (shown

below). Since, no BstNI digestion site was created in the mutant K-ras, the fragment remains unchanged at 125 bps.

- *Wild-type K-ras 45 bps fragment following first-round of BstNI digestion*

```
5' GCT CTTCGTGGTG TGGTGTCCAT ATAAACTTGT GGTAGTTGGA CC
  ||| |||||||||| |||||||||| |||||||||| |||||||||| ||
3' CGA GAAGCACCAC ACCACAGGTA TATTTGAACA CCATCAACCT GGA
```

- *Wild-type K-ras 80 bps fragment following first-round of BstNI digestion*

```
TGGTGGCG TAGGCAAGAG TGCCTTGACG ATACAGCTAA TTCAGAATCA TTTTGTGGAC
||||||||  |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
CCACCGC  ATCCGTTCTC ACGGAACTGC TATGTCGATT  AAGTCTTAGT AAAACACCTG

GGGACACCAC ACCACGAAGA GC 3'
|||||||||| |||||||||  ||
CCCTGTGGTG TGGTGCTTCT  CG 5'
```

- *Mutant K-ras 125 bps fragments*

```
5' GCT CTTCGTGGTG TGGTGTCCAT ATAAACTTGT GGTAGTTGGA CCTGGTGGCG
  ||| |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
3' CGA GAAGCACCAC ACCACAGGTA TATTTGAACA CCATCAACCT GGACCACCGC

TAGGCAAGAG TGCCTTGACG ATACAGCTAA TTCAGAATCA TTTTGTGGAC
|||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
ATCCGTTCTC  ACGGAACTGC TATGTCGATT  AAGTCTTAGT AAAACACCTG

GGGACACCAC ACCACGAAGA GC 3'
|||||||||| |||||||||  ||
CCCTGTGGTG TGGTGCTTCT  CG 5'
```

➢ **Second-stage PCR**

In the second-stage PCR following sense and antisense primers were used. The antisense primer was labeled with 6-

FAM for the quantitative detection of the K-ras using capillary electrophoresis.

- *Sense Primer Set II*

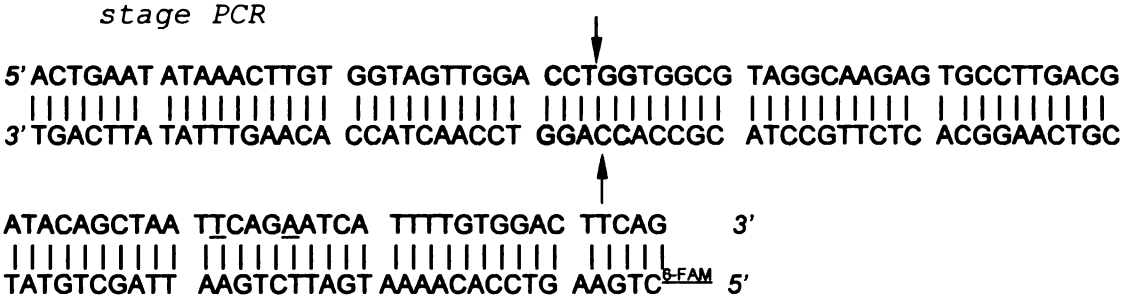
5' ACT GAA TAT AAA CTT GTG GTA GTT GGA CCT 3'

- *Antisense Primer Set II*

5' 6-FAM-CTG AAG TCC ACA AAA TGA TTC TGA ATT AGC 3'

Mutant and Wild-type K-ras amplifies to 92 bps during the second stage PCR. The 92 bps sequence of wild-type K-ras is shown below. Sense primer creates an artificial BstNI digestion site at codon 11 and 12 in the wild-type K-ras (highlighted in the sequence below); since mutant K-ras differs from wild-type at codon 12 the sense primer does not create the artificial BstNI digestion site on the mutant K-ras.

- *Wild-type K-ras Fragment size 92 bps after second-stage PCR*



➢ **Second-round of BstNI Digestion**

BstNI restriction enzyme cuts the wild-type K-ras at the BstNI digestion site created by sense primer used in the second stage PCR into 62 and 30 bps fragments (shown below). Since, no BstNI digestion site was created; the mutant K-ras fragment remains unchanged at 92 bps.

- *Wild-type K-ras 30 bps fragment following second-round of BstNI digestion*

```
5' ACTGAAT ATAAACTTGT GGTAGTTGGA CC  3'
   ||||||| |||||||||| |||||||||| ||
3' TGACTTA TATTTGAACA CCATCAACCT GGA 5'
```

- *Wild-type K-ras 62 bps fragment following second-round of BstNI digestion*

```
5' TGGTGGCG TAGGCAAGAG TGCCTTGACG ATACAGCTAA TTCAGAATCA TTTTGTGGAC
   |||||||| |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
3'  CCACCGC ATCCGTTCTC ACGGAACTGC TATGTCGATT AAGTCTTAGT AAAACACCTG

TTCAG     3'
|||||
AAGTC6-FAM 5'
```

- *Mutant K-ras 92 bps following second-round of BstNI digestion*

```
5' ACTGAAT ATAAACTTGT GGTAGTTGGA CCTGGTGGCG TAGGCAAGAG TGCCTTGACG
   ||||||| |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
3' TGACTTA TATTTGAACA CCATCAACCT GGACCACCGC ATCCGTTCTC ACGGAACTGC

ATACAGCTAA TTCAGAATCA TTTTGTGGAC TTCAG     3'
|||||||||| |||||||||| |||||||||| |||||
TATGTCGATT AAGTCTTAGT AAAACACCTG AAGTC6-FAM 5'
```

Figure 11. Mutant and Wild-type K-ras Sequences during Restriction-Enriched Polymerase Chain Reaction

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