Detection and characterization of oncogene mutations in preneoplastic and early neoplastic lesions.

Detection and Characterization of Oncogene Mutations in Preneoplastic and Early Neoplastic Lesions

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Running Title: *K*-ras *oncogene mutations in fresh and fixed tissue samples*

Abstract

While it has been more than 20 years since its discovery, the *ras* family of genes has not yet lost its impact on basic and clinical oncology. They remain central to the field of molecular oncology as tools for investigating carcinogenesis and oncogenic signaling, as powerful biomarkers for the identification of those who have or are at high risk of developing cancer, and as oncogene targets for the design and development of new chemotherapeutic drugs. Mutational activation of the K-*ras* proto-oncogene is an early event in the development and progression of colorectal, pancreatic and lung cancers that are the major causes of cancer death in the world. The presence of point mutational "hot spots" at sites necessary for the activation of this proto-oncogene has led to the development of a number of highly sensitive PCR-based methods that are feasible for the early detection of K-*ras* oncogene mutations in the clinical setting. In the light of these facts, mutation at the K-*ras* oncogene has the potential to serve as a useful biomarker in the early diagnosis and risk assessment of cancers with oncogenic ras signaling. This chapter describes a highly sensitive method for detecting mutant K-*ras*, enriched PCR, and its application to early detection of this oncogene in preneoplastic and early neoplastic lesions of the colon and rectum.

Key Words: oncogene, K-*ras*, enriched PCR, molecular diagnosis, risk assessment, biomarker, colorectal cancer, pre-neoplastic lesion, aberrant crypt foci

1. Introduction

A detailed scenario of molecular alterations, including epigenetic events, has been identified in the development and progression of human cancers. Multistep genetic alterations are known to affect oncogenes and tumor suppressor genes, including the genes for DNA mismatch and excision repair ***(1,2)***. Genetic testing for susceptibility has become part of the standard management of patients with well-defined and uncommon hereditary cancers, in which cancer predisposing mutations occur in the germ line ***(3)***. However, a molecular diagnostic approach to sporadic cancers, which comprise the majority of clinically documented malignant tumors, is still under development.

Of the first identified oncogenes, some of the best characterized are the *ras* family of genes (H-*ras*, N-*ras* and K-*ras*), the products of which regulate GTP-signal transduction. Common to all mutant forms of *ras* genes is a mutation pattern that results in constitutive activation of signaling cascades, including PI3K, PKB/AKT and MAPK, and the stress kinases ***(4)***. Activated *ras* genes efficiently override cellular growth control and attenuate its ability to initiate programmed cell death. Somatic mutational activation of *ras* genes is frequent in various human cancers ***(5,6)***. Activation of K-*ras* is an early event in the development of certain types of cancer, i.e., colorectal, pancreas and lung cancers, both in human beings and experimental animals. Accordingly, this mutation has the potential to serve as a useful biomarker, both in early diagnosis and in susceptibility assessment ***(7)***. In clinical samples subjected to molecular diagnosis and risk assessment, the ratio of neoplastic or preneoplastic to normal cells is extremely low, and varies in different target organs, and among individuals. The fact that K-*ras* mutations occur exclusively in three hot spots (codons 12,13 and 61) ***(5,6)*** has led to the development of various PCR-based methods that are much more sensitive and more feasible for the early detection of cancer ***(8,9)*** than methods developed for other genes (i.e., *p53*, *APC*), in which mutations are distributed throughout their entire sequences. Modified PCR protocols have been established to enhance the amplification of mutant, but not wild type, alleles in non-transformed tissues that appear normal. These modifications include combinations of PCR with restriction fragment length polymorphisms (RFLP) or single strand conformation polymorphisms (SSCP),

mutant-enriched PCR (EPCR), EPCR-SSCP, mutant allele-specific amplification (MASA), and the mutation-ligation assay ***(8,9)***.

The procedure of mutant-enriched PCR (EPCR), as outlined in **Fig. 1A**, consists of a mismatched primer-mediated two-step PCR amplification, intervened by digestion of the wild-type PCR product with a restriction enzyme ***(10)***. The upstream primer (K5') encodes a G to C substitution at the first position of codon 11, creating a product with a recognition site (CCTGG) specific to the DNA restriction enzymes *Bst* NI or *Mva* I that overlaps the first two nucleotides of the wild type codon 12. Since this restriction enzyme site is absent in the product amplified from the K-*ras* gene with a mutant codon 12, RFLP analysis of the PCR products can distinguish wild type and mutant genes (**Fig. 1B** and **1C**). Of particular importance is the strategic incorporation of a second *Bst* NI or *Mva* I site into the downstream primer (K3'), as an internal control for restriction enzyme activity and fidelity ***(11)***.

As shown in **Fig. 1A**, the first exon fragments of K-*ras* are PCR amplified with the set of upstream primer K5' and a new downstream primer, K3'wt that lacks an internal copy of the restriction enzyme site. The 157 base pair (bp) fragment amplified in this first step PCR is digested with *Bst* NI or *Mva* I, thereby cleaving the wild type products and rendering them inaccessible for subsequent amplification. The products of the intermediate digestion, enriched in full length mutant codon 12 sequences, are then amplified by the second step PCR with primers K5' and K3'. The resultant products are subjected to RFLP analysis by digestion with the same restriction enzyme and native polyacrylamide gel electrophoresis. The 157 bp product of mutant K-*ras* is cleaved only at the control site created by the primer K3', to give fragments of 143 bp and 14 bp, while the wild type product is cleaved at two sites mediated by the primers K5' and K3', generating fragments of 114 bp, 29 bp and 14 bp. Elimination of normal alleles from the amplification process has been found to enable detection of one mutant allele of *ras* among 1,000-10,000 normal alleles (**Fig. 2**) ***(10,12)***. Similarly, the two-step EPCR amplification of a K-*ras* gene with mutant codon 13 is possible using the upstream primer K5'-13, which encodes substitutions of A to C in the third nucleotide of codon 10, and G to C in the first position of codon 11 (**Fig. 1B**). When the wild type K-*ras* codon 13 is amplified with the downstream primer K3'wt, the primer K5'-13

creates a new recognition site (GCCNNNNNGGC) specific to the restriction enzyme *Bgl* I (**Fig. 1C**).

*1.1. Detection of K-*ras *Oncogene Mutations in Normal Appearing (Non-neoplastic) Tissues of Colorectal Cancer Patients*

Along with early diagnosis of tumors, one of the optimal ways to reduce mortality from, or prevent colorectal cancer, is to identify those who are at increased risk of developing sporadic cancer (by analogy with the known hereditary cancer syndromes ***[2,3]***). The precise localization of *ras* oncogene activating mutation allows for their detection even when they are present in a very small fraction of cells, as is often found in the early stages of tumor development. In fact, mutant *ras* has served as a classic paradigm of the ability to detect mutations in oncogenes in tissues that appear normal at histopathological examination ***(7)***. A good representative of this paradigm comes from our experiences in analyzing matched pairs of normal and tumor tissues from colorectal cancer patients, since the molecular foundation of the multistep carcinogenesis process in human colorectal cancer has been established in great detail **(*13*)**. Many independent studies have been directed toward sensitive detection of mutations in K-*ras* **(**reviewed in ***7,14*)**, which occur in more than 40% of colorectal cancers.

Our recent series of EPCR-based studies has clearly demonstrated activating K-*ras* mutations in apparently normal tissues taken from surgical specimens of patients who underwent operation for colorectal cancer ***(12,15-17)***. When one sample is taken from the adjoining non-neoplastic mucosa of surgical specimens, mutant K-*ras* is detected in 5 to 18% of patients ***(12,15)***. As shown in **Fig. 3**, when multiple (three to seven) samples are collected from each patient for EPCR analysis of K-*ras*, activating mutations are detected in 20% of the patients ***(16,17)***. The latter study also shows confined localization of the epithelial cells harboring this mutant gene in the non-neoplastic mucosa. Interestingly, sequencing analysis showed that the specific mutant K-*ras* allele found in mucosa that appeared to be normal was not always the same as that found in the tumors (**Fig. 3**). We have also demonstrated microsatellite DNA instability in normal appearing tissues adjacent to

the tumor ***(18)***, suggesting that colorectal cancer patients with mutant K-*ras* in apparently normal tissue may harbor genetically unstable mucosa that may predispose to development of second primary tumors. These findings suggest that the mutant K-*ras* identified in non-neoplastic mucosa may sometimes represent *de novo* mutations, and serve as a useful biomarker for identifying persons at higher risk of colorectal cancer. Presupposing either of these speculations in a pilot study, we could detect mutant K-*ras* by EPCR in colonic lavage fluids (effluents) in persons who are at high risk for development of colorectal cancer ***(19)***.

These surprising findings also raise concern as to the biological importance of *ras* mutations, and consequently the value of their early detection. It is clear today that *ras* mutation per se is not sufficient to yield a transformed phenotype, and that cooperation of mutant *ras* with other oncogenes and tumor suppressor genes must take place. For these reasons, it is important to analyze *ras* oncogene mutation in combination with the analysis of other markers known to be associated with tumor development ***(7,14,18,20)***.

*1.2. Detection of Mutant K-*ras *Oncogenes in Aberrant Crypt Foci of Human Colon*

Studies on very minute preneoplastic or early neoplastic lesions are essential for understanding the molecular details of the mechanism of colorectal carcinogenesis. Among these lesions are aberrant crypt foci (ACF), first identified in methylene blue-stained, whole mount preparations of colon mucosa from carcinogen-treated rodents. An aberrant crypt is two to three times larger than normal crypts in the same field, has a thickened epithelial layer, frequently has a slit-, asteroid- or oval-shaped lumen, has an increased pericryptal zone separating it from the surrounding normal crypts, and is microscopically elevated above the surrounding mucosa. Multiple aberrant crypts frequently appear together as a cluster, forming a single unit that is referred to as an ACF (**Fig. 4A**) ***(21)***. Multiple phenotypic alterations, i.e., decreased hexosaminidase activity, have been identified in ACF. These minute foci are also found in the surgical specimens of human colon cancer (**Fig. 4A**) **(*22*)**, and have recently been identified in patients with or without colorectal tumors by magnifying dye-endoscopy ***(23)***. Histological characteristics of ACF include findings of hyperplasia,

dysplasia, adenoma, and adenocarcinoma ***(24,25)***.

K-*ras* mutation was one of the first molecular alterations reported in human ACF ***(26)***. Practically, ACF are identified and dissected from the methylene blue-stained mucosal strips of formalin-fixed surgical specimens under a stereo microscope (**Fig. 4A and B**). Each focus is divided into two pieces, one for histopathological examination and the other for DNA extraction (**Fig. 4B**). By conventional PCR-RFLP and EPCR, K-*ras* mutations at codon 12 and 13 are detected in 46% and 12% of ACF, respectively (**Fig. 4C**) ***(22)***. Sequencing shows that, in ACF, GAT mutants are as frequent as GTT mutations in codon 12, while the latter type of mutation is predominant in adenocarcinomas. The high frequency of these oncogene mutations and subsequent demonstration of molecular alterations in these lesions ***(27)*** support the idea that ACF are genetically monoclonal in their evolution. Although ACF are very heterogeneous biologically and morphologically, and their fate has yet to be determined definitely, these minute foci represent one of the plausible candidates for prenoplastic colorectal epithelial foci mutated at various tumor related genes, including the K-*ras* locus.

2. Materials

Chemicals and reagents not specifically sourced may be purchased from local vendors or international suppliers (e.g., Sigma-Aldrich, St. Louis, MO).

2.1. Control DNA for Validation of EPCR

1. Sources of homozygous mutant DNA: human colon cancer cell lines SW480 (codon 12: GGT to GTT) and HCT116 (codon 13: GGC to GAC) (American Type Culture Collection, Rockville, MD).
2. RPMI cell culture medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY).
3. 10 mL pipettes and pipette aid.
4. 10 and 15 cm tissue culture dishes (Falcon, BD Biosciences, Franklin Lakes, NJ).
5. Cell culture incubator.
6. Trypsin-EDTA solution (Gibco).

7. Phosphate-buffered saline (PBS), pH 7.4: 137 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* Na_2HPO_4 (Gibco).
8. 50 mL polypropylene conical tubes (Blue Max, BD Biosciences).
9. Cell scraper (e.g., Cell Lifter, Costar, Cambridge, MA).
10. Table top centrifuge with swing rotor (e.g., KN-70, Kubota, Tokyo, Japan).
11. 1.5 mL polypropylene microcentrifuge tubes (Sarstedt AG, Nümbrecht, Germany).
12. Refrigerated microcentrifuge (e.g., Kubota 1920).
13. 1 mL micropipetter and appropriate tips.
14. Cell lysis buffer: 10 m*M* Tris-HCl, pH 7.5, 10 m*M* EDTA, 15 m*M* NaCl, 2% SDS. 10 mL of this buffer is sufficient for 25-50 cell samples, and can be stored at –20°C for over a year.
15. Proteinase K (Beckman, San Ramon, CA): small volume of stock solution (at 20 mg/mL) initially made by dissolving in deionized distilled water. This stock solution is then diluted in cell lysis buffer to a concentration of 100 μg/mL immediately prior to use. 2 mL of cell lysis buffer with proteinase K should be sufficient for at least 5 samples. Once the proteinase K has been dissolved or added, these solutions must be used or discarded, they cannot be stored.
16. 37°C water bath.
17. Phenol equilibrated with Tris-HCl pH 8.0 (Wako, Osaka, Japan).
18. Chloroform.
19. Ethanol: 99% (absolute), 95%, 70% solutions.
20. RNase A stock solution (10 mg/mL) (Roche Diagnostics, Mannheim, Germany) **(***see* **Note 1)**. Thaw one aliquot per experiment (can include multiple samples) and discard unused portion.
21. Human placenta DNA (Sigma).

2.2. Preparation of Tissue Samples and DNA Extraction

1. Non-neoplastic mucosa and tumor tissues taken from fresh surgical specimen of patients with colorectal cancer.

2. Aberrant crypt foci of colon dissected from formalin-fixed mucosal strips of surgical specimen.
3. Frozen storage container.
4. Liquid nitrogen.
5. Mortar and pestil.
6. Tissue lysis buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 10 m*M* NaCl, 0.1% SDS. 10 mL of this buffer is sufficient for 25-50 tissue samples, and can be stored at –20°C for over a year.
7. Proteinase K (Beckman): small volume of stock solution (at 20 mg/mL) initially made by dissolving in deionized distilled water. This stock solution is then diluted in tissue lysis buffer to a concentration of 100 μg/mL immediately prior to use. 2 mL of tissue lysis buffer with proteinase K should be sufficient for at least 5 samples. Once the proteinase K has been dissolved or added, these solutions must be used or discarded, they cannot be stored.
8. 45˚C water bath.
9. Phenol equilibrated with Tris-HCl pH 8.0 (Wako).
10. Chloroform.
11. Ethanol: 99% (absolute), 95%, 70% solutions.
12. Tris-EDTA solution (TE): 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA **(***see* **Note 2)**. This solution is also commercially available from a number of sources.
13. RNase A stock solution (10 mg/mL) (Roche) **(***see* **Note 1)**. Thaw one aliquot per experiment (can include multiple samples) and discard unused portion.
14. Glass slides used for conventional microscopic examination.
15. 0.2% methylene blue solution — will require 2 mL per ACF sample. This solution can be stored indefinitely at 4°C and can be reused (filter before returning to storage container).
16. Light microscope (e.g., Olympus model BX50, Tokyo, Japan).
17. Pasteur pipettes.
18. Ampoule cutter (or file).

19. DNA concentrater (e.g., Microcon model 100, Amicon, Beverly, MA).

2.3. Enriched PCR

1. Primers **(***see* **Note 3)**:

   K5': 5' ACTGAATATAAACTTGTGGTAGTTGGACCT 3'

   K3'wt: 5' TCAAAGAATGGTCCTGCACCAG 3'

   K3': 5' TCAAAGAATGGTCCTGGACCAG 3'

   K5'-13: 5' ACTGAATATAAACTTGTGGTAGTTGGCCCT 3'

2. Taq DNA polymerase (Applied Biosystems, Foster City, CA).
3. 10 X PCR buffer containing $MgCl_2$, comes with commercial purchase of Taq DNA polymerase (Applied Biosystems): 1 X buffer consists of 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$ and 0.01% gelatin.
4. dNTPs (Applied Biosystems).
5. AmpliWax PCR Gems 100 (Applied Biosystems).
6. Distilled and sterilized water in ampoules for medical use commercially available from pharmaceutical companies (e.g., Ohtsuka or Kobayashi Seiyaku, Tokyo, Japan).
7. Thin wall tubes for PCR: GeneAmp Thin-Walled Reaction Tubes with Flat Caps (Applied Biosystems), or Thin-Wall Tube with Flat Cap (MJ Research, Waltham, MA).
8. Thermal cycler: GeneAmp PCR System 9600 (Applied Biosystems), or DNA Engine Peltier Thermal Cycler PTC-200 (MJ Research).
9. Restriction enzymes:

   Mva I (Takara, Kyoto, Japan)

   Bst NI (New England Biolabs, Beverly, MA)

   Bgl I (Toyobo, Osaka, Japan)

10. 4 *M* NH_3COOCH_3.

2.4. Polyacrylamide Gel Electrophoresis (PAGE)

1. Acrylamide stock solution: 40% acrylamide/bis-acrylamide (ratio 29:1) dissolved in distilled water and filtered through Whatman chromatography paper (3MM Chr,

Whatman International, Maidstone, England) or a bottle top filter (Nalgene, Nalge Nunc International, Rochester, NY). Stored in a dark bottle at 4°C, this solution will be stable for more than a year, so can be prepared in bulk (500 mL). Recently, the same solution has become commercially available (Sigma). A 2.5 mL aliquot of this stock solution is used for preparation of 10 mL of 10% native acrylamide gel solution for a 1.0-mm-thick mini-gel.

2. 20 X TBE buffer: 1 *M* Tris base, 1 *M* boric acid, 0.02 *M* EDTA (EDTA-2Na-2H_2O). 500 to 1,000 mL of this solution can be prepared at a time, as it stable for more than a year at room temperature. Precipitate, when it appears after longer storage, can be re-dissolved with warming. An aliquot of 0.5 mL is used for preparation of a 1.0-mm-thick mini-gel (10 mL).
3. 10% ammonium peroxodisulfate (APS, Sigma). APS solid powder is stable when stored in the dark at room temperature. Practically, aliquots of 100 mg (0.1 g) of APS powder are stored in dark-colored, 1.5 mL microcentrifuge tubes and stored at 4°C. Prior to use, an aliquot of the powder is dissolved in 1.0 mL of distilled water to make 10% solution. It is important to inscribe the date of preparation on the tube, because this solution is stable only for one or two weeks at 4°C. Typically, 100 μL of APS solution is used for preparation of 10 mL acrylamide gel solution for a 1.0-mm-thick mini-gel.
4. N, N, N', N'-tetramethyl ethylene diamine (TMED, Wako, Osaka, Japan).
5. 6 X gel loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll (Type 400, Amersham Biosciences Corp., Piscataway, NJ) dissolved in water. Typically, 10 mL of this solution is prepared, which is stable at room temperature for over a year. Similar solutions are now available commercially.
6. Mini-slab gel apparatus (BioRad, Hercules, CA, or Hoeffer Pharmacia Biotech, San Francisco, CA) and power supply (Crosspower 500L, ATTO, Tokyo, Japan, or PowerPac 300, BioRad).
7. Ethidium bromide (stock solution, 10 mg/mL), or SYBR Green I nucleic acid stain (Molecular Probes, Eugene, Oregon). 10 to 100 mL of ethidium bromide stock

solution is prepared, depending on the frequency of electrophoresis, size of gels to be stained, etc., in each laboratory. The stock solution is stable in the dark at 4°C. Similar solutions are now available commercially. For staining mini-gels, a working solution is prepared by dissolving 10 μL of ethidium bromide stock solution in 100 mL of distilled water. A 1.0 mm-thick 10% acrylamide mini-gel can be stained in this solution in 5 min. This working solution can be used several times if recovered and stored in the dark.

8. Molecular marker DNA: Bluescript SKII (+) plasmid DNA (Stratagene, La Jolla, CA) digested with *Hpa* II (TaKaRa or Toyobo, Tokyo, Japan) **(***see* **Note 4)**.
9. UV transilluminator (UVP, Upland, CA).
10. Gel photography equipment (e.g., LightCapture Type AE-6960/C/FC, ATTO, Tokyo, Japan).

3. Methods

3.1. Preparation of Mutant DNA used for Validation of the Sensitivity of EPCR

1. Plate an aliquot of frozen stock of the SW480 and/or the HCT116 human colon adenocarcinoma cell line into a 10 cm tissue culture dish containing 10 mL RPMI supplemented with 10% FBS. When this culture is semi-confluent, it is used to initiate three 15 cm tissue culture dishes.
2. Grow the three cell cultures to semi-confluent to confluent status, then decant the medium and gently wash each dish three times with 10 mL ice cold PBS (introduce PBS with a pipette along the inner wall of the dish and swirl gently before decanting). Then harvest cells in the first dish by scraping in a further 10 mL ice cold PBS. Transfer this cell suspension into a second dish and harvest it by scraping; meanwhile wash the first dish with a further 10 mL ice-cold PBS. Transfer the cell suspension from the second dish to the third and harvest by scraping; transfer the wash solution from the first dish to the second and wash. Transfer the cells suspension from the third dish into a 50 ml polypropylene conical tube containing 10 mL ice cold PBS, and wash the third dish with the wash solution from the second dish. Finally, transfer the wash solution to the tube,

such that it contains harvested cells from all three dishes in about 30 mLs ice-cold PBS. Centrifuge this cell suspension at 3,000 to 5,000 rpm for 5 to 10 min to pellet cells.

3. After centrifugation, remove and discard the supernatant by gentle decantation. Wash the cell pellet three times with 40 mL of ice-cold PBS (resuspend by vortexing, pellet and decant wash as in **section *3.1.***2.). Resuspend the cell pellet in 1.0 mL of ice-cold PBS by gentle pipetting, and transfer the suspension into a 1.5 mL polypropylene microcentrifuge tube. Centrifuge the cell suspension at 10,000 rpm for 5 min at 4°C to pellet cells. After centrifugation, aspirate the supernatant through a 1 mL micropipette tip using a vacuum pump system and discard.
4. Resuspend the cell pellet in 100-200 µL (1 µL per initial mg of wet weight, can be estimated with experience) cell lysis buffer containing 100 µg/mL proteinase K by pipetting, and incubate for 1-2 h at 37°C in a water bath, with occasional tapping to keep the cells suspended in the lysis buffer.
5. Extract genomic DNA and purify by serial treatment with phenol and chloroform, then precipitate with 99 % ethanol, all according to standardized methodologies ***(28-30)***.
6. Serially wash the precipitated DNA with 1 mL of 95% and 70% ethanol (resuspension by gentle mixing or vortexing, followed by microcentrifugation), then dissolve in distilled water at a concentration of 1 µg/µL. Divide this DNA solution into conveniently sized aliquots (~50 µL) and store at –20°C.
7. To prepare mixtures with different ratios of mutant to wild-type control DNAs, 1 µg/µL of SW480- or HCT116-derived mutant DNA is diluted ten-fold with a 1 µg/µL solution of placenta-derived wild-type DNA. Use 0.5 or 1.0 µL of each mixture for validation of EPCR sensitivity.

3.2. DNA Extraction from Fresh Tissue Samples

1. Take 0.1 to 0.2 g (wet weight) samples of fresh tissue from normal mucosa and colon cancer immediately after removal of surgical specimens **(***see* **Note 5)**. Samples of this size range should correspond to volumes of 100-200 mL.
2. Snap-freeze samples **(***see* **Note 6)** and store at –80˚C until use.

3. To begin DNA extraction, selected frozen tissue samples are homogenized and powdered in a mortar containing liquid nitrogen **(***see* **Note 7)**.
4. Suspend powdered tissue in one or two volumes (assume 0.1 g weight weight = 100 μL) of tissue lysis buffer containing 100 μg/mL of proteinase K in a microcentrifuge tube, and incubate in a water bath at 45˚C for 2-5 h.
5. Extract genomic DNA and purify by serial treatment with phenol and chloroform, precipitating with ethanol, all according to standardized methodologies ***(28-30)***.
6. Wash precipitated DNA serially in 95% and 70% ethanol (resuspension by gentle mixing or vortexing, followed by microcentrifugation), then dissolve in 100 to 200 μL TE (exact volume is dependent on the amount of input tissue and the efficiency of the extraction), and treat with 25-50 ng/μL of RNase A at 37˚C for 30 min.
7. Repurify DNA by treatment with phenol and chloroform, and precipitate with ethanol ***(31)***.
8. Wash precipitated DNA again serially in 1 mL 95% and 70% ethanol, then dissolve in distilled and sterilized water or TE at a concentration of 1.0 μg/μL and store at –20˚C. Again, exact volume of water or TE is dependent on the amount and quality of the original tissue sample and the efficiency of the DNA extraction.

3.3. Extraction of DNA from Formalin-fixed ACF

1. Strip off the grossly normal mucosal layer from the formalin-fixed surgical specimen of colorectal cancer.
2. Cut mucosal strips into pieces the size of conventional glass slide used for light microscopic examination.
3. Stain each piece of mucosal strips in 2 mL of 0.2 % of methylene blue solution for a few minutes, and then wash briefly by immersion in distilled water and gentle shaking. This distilled water may be exchanged several times until no excess dye appears in the wash.
4. Place the stained mucosal strip on a glass slide and overlay it with distilled water, suitable for observation under the microscope at 40-fold magnification.

5. Under microscopic observation, excise ACF showing characteristic morphological findings (**Fig. 4A**) from mucosal strip using a Pasteur pipette, tip of which has been precut with an ampoule cutter.
6. Divide each isolated ACF into two pieces (**Fig. 4B**), one for histopathological examination and the other for DNA extraction after removal of as many normal crypts as possible, under microscopic observation.
7. Extract genomic DNA from each individual ACF by serial treatment with proteinase K (100 μg/mL) and RNase A (25-50 ng/μL) in 20 to 50 μL of tissue lysis buffer, depending on the size of the sample.
8. Purify extracted DNA and concentrate according to manufacturer‘s instructions. An aliquot of 0.5 to 1.0 μL of this DNA solution is used for polymerase chain reaction analysis.

3.4. Enriched PCR

1. Prepare the two reaction mixtures for the first step PCR; dividing them into two layers with AmpliWax according to the supplier’s instruction: the lower layer should contain 100 ng of the primer K5', 70 ng of primer K3'wt (not K3'), and 0.2 m*M* each of dNTP in a volume of 13.5 μL; the upper layer should contain 7.5 μL of 10 X PCR buffer with $MgCl_2$, 1.25 units of Taq DNA polymerase and 0.5 to 1.0 μg of genomic DNA in a volume of 61.5 μL **(***see* **Notes 8, 9 and 10)**.
2. Run the first-step amplification for 20 cycles of 1 min denaturation at 94˚C, 1 min annealing at 59˚C and 1 min extension at 72˚C, and followed by 10 min extension at 72˚C **(***see* **Note 11)**.
3. Intermediate digestion: digest 1 μL of the first step PCR products with 10 units of *Mva* I in a final volume of 10 μL at 37˚C for more than 2 h **(***see* **Note 12)**.
4. Prepare the reaction mixtures for the second step PCR in the presence of AmpliWax. The mixture should contain 140 ng of primer K5', 100 ng of K3' (not K3'wt), 0.2 m*M* each of dNTP, 1.25 units of Taq DNA polymerase, and 1 μL of *Mva* I-digested first step PCR product in a final volume of 75 μL (13.5 μL of lower layer + 61.5 μL of upper

layer) of 1 X PCR buffer **(***see* **Notes 8, 9 and 10)**.

5. Run the second step amplification for 30 cycles of 1 min denaturation at 94˚C, 1 min annealing at 59˚C and 1 min extension at 72˚C, followed by 10 min extension at 72˚C **(***see* **Note 10)**.

*3.5. Detection of Mutant K-*ras *by RFLP Analysis*

1. Digest 8 μL of the second step PCR product with 10 units of *Mva* I in a total volume of 10 μL containing 1 μL of 10 X enzyme buffer, at 37˚C for 1 h.
2. Add 2 μL of 6 X gel loading buffer to the digested PCR product solution.
3. Separate digested products on a 10% native polyacrylamide (29:1) mini-slab gel at 100 V for 1 h in the presence of 1 X TBE buffer (*see* **Note 13**) ***(32)***.
4. Stain the gel with 0.5 μg/mL ethidium bromide solution at room temperature for 5-10 min with gentle shaking.
5. Wash the stained gel with distilled water for 1 min with gentle shaking.
6. Detect and photograph the characteristic mutant signal (142 bp) under the UV transilluminator, and save the picture.

*3.6. Characterization of Mutant K-*ras *Detected by EPCR*

1. If a mutant band is detected in a sample, the second step PCR is repeated in duplicate.
2. Digest 8 μL of PCR product from each tube with *Mva* I and separate on a native polyacrylamide gel (*see* section ***3.5.***3.), to confirm reproducibility of enrichment of the mutant.
3. Digest the remaining PCR product (about 130 μL in total from two tubes) with 50 units of *Mva* I in 160 μL solution containing 1 X enzyme buffer, at 37˚C for 1 h.
4. Mix the digested PCR product with an equal amount of 4 *M* NH_3COOCH_3, and precipitate with ethanol at –80˚C for 15 to 30 min.
5. Dissolve the precipitated PCR product in 20 μL of TE, and separate on a 10% native polyacrylamide gel as described previously (section ***3.5.***3.).
6. Cut out a piece of gel (~5 X 1-2 mm, 1 mm thick) containing the mutant band from the

ethidium bromide-stained gel under the UV transilluminator.

7. Elute the mutant PCR product from the cut-out piece of gel in 50 μL of sterilized water in a 1.5 mL microcentrifuge tube by heating at 80˚C for 20 min.
8. Analyze this purified product by sequencing by one of the following methods **(***see* **Note 14)**:

   i) dideoxy chain-termination method using the Sequenase DNA Sequencing Kit (USB, Cleveland, OH)

   ii) dye terminator method using the Dye Primer Cycle Sequencing Kit (Applied Biosystems, Foster, CA) after cloning PCR product by blunt-end ligation

   iii) direct sequencing by the dye terminator method

4. Notes

1. Dissolve 20 mg of RNase A powder in 2 mL of 10 m*M* Tris-HCl (pH 7.5) buffer containing 15 m*M* NaCl. Heat the enzyme solution at 100˚C for 15 min, and then allow to cool down slowly to room temperature. Aliquot this stock solution (~50 μL) and store at –20˚C (should be usable for a year or longer).
2. TE is chemically stable but easily biologically contaminated. Whether small amounts are made fresh or a larger stock solution is kept on hand must be decided by the individual laboratory.
3. As a prerequisite, to maximize the sensitivity of EPCR, all synthesized primers must be gel purified.
4. Prepare the molecular weight marker DNA as follows: digest 10 μg of Bluescript SKII (+) plasmid DNA with 30 units of *Hpa* II in 30 μL of 1 X enzyme buffer at 37˚C for 1 h. Add 6 μL of 6 X gel loading buffer to this solution. A 1-2 μL aliquot of this marker solution is sufficient for a lane of a gel.
5. When collecting tissue samples, normal tissue should be excised prior to tumor tissue, using different forceps and scissors. Before storage, normal tissue is extensively washed with cold PBS to remove desquamated tumor cells.
6. “Snap” freezing simply implies freezing samples as fast as possible. We use a unique

method with equipment of our own design: two metal plates joined with hinges. This piece of equipment is chilled in liquid nitrogen prior to use. Then, each tissue sample is placed in a 8 X 5 cm plastic bag, sealed, frozen by compression between the two plates (this also causes the tissue to be flattened), and stored at –80°C.

7. Pour liquid nitrogen into a mortar just before homogenization of tissue sample to pre-chill both mortar and pestle.
8. In the original method ***(10)***, 10 ng each and 150 ng each of primers were used in the first and second step of PCR amplification, respectively. However, the optimal enrichment of mutant is obtained in the amounts of primers given in this protocol.
9. Concentration of each NTP should be kept at 0.2 m*M* to obtain optimal amplification.
10. AmpliWax is used to minimize nonspecific amplification.
11. To confirm the validity of the assay and to avoid contamination, placenta DNA (wild-type control), SW 480 cell–derived DNA (mutant control) and sterilized water instead of DNA are amplified in parallel with DNA samples in every PCR run.
12. When a mutant band is detected, the intermediate digestion mixture is stored at –20˚C for further use with the sequencing reactions.
13. Detection and separation of a mutant signal is more sensitive and feasible on a 10 to 15% polyacrylamide gel than on an agarose gel. The method for elution of the target DNA band from the former type of gel is also simpler and more feasible than from the latter type.
14. The exact protocols of the respective sequencing methods are not described here, due to a limitation of space. These protocols are available from the suppliers' instructions. The upstream primer K5' is used for sequencing in forward direction. The sequence of the downstream primer SK3' used for sequencing in the reverse direction is: 5' CTCTATTGTTGGATCATATTC 3' ***(12)***.

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Figure Legends

Figure 1. Schematic representation of the two-step procedure of EPCR amplification of mutant K-*ras* codon 12 sequences (A), the primers used for EPCR (B), and comparison of the sequences of wild-type K-*ras* exon 1, mismatched primers, and restriction sites of *Bst* NI/*Mva* I and *Bgl* I (C). In the first step of amplification (1st PCR), a set of primers K5' and K3'wt is used for amplification of 157 bp fragments including codon 12 sequences. The K5' contains a nucleotide substitution at the first position of codon 11, creating a *Bst* NI/*Mva* I restriction site (CCTGG, cross-hatched box) that overlaps the first two nucleotides of wild type codon 12. Intermediate digestion of the first step PCR product with *Bst* NI or *Mva* I leaves products enriched in mutant codon 12 sequences (closed box). An aliquot of the undigested products is subjected to the second step PCR (2nd PCR) with a set of primers K5' and K3' (that contains a control site specific to *Bst* NI/*Mva* I). RFLP analysis with *Bst* NI/*Mva* I distinguishes the mutant fragments of 143 bp from wild-type alleles of 114 bp, as shown in **Figure 2**.

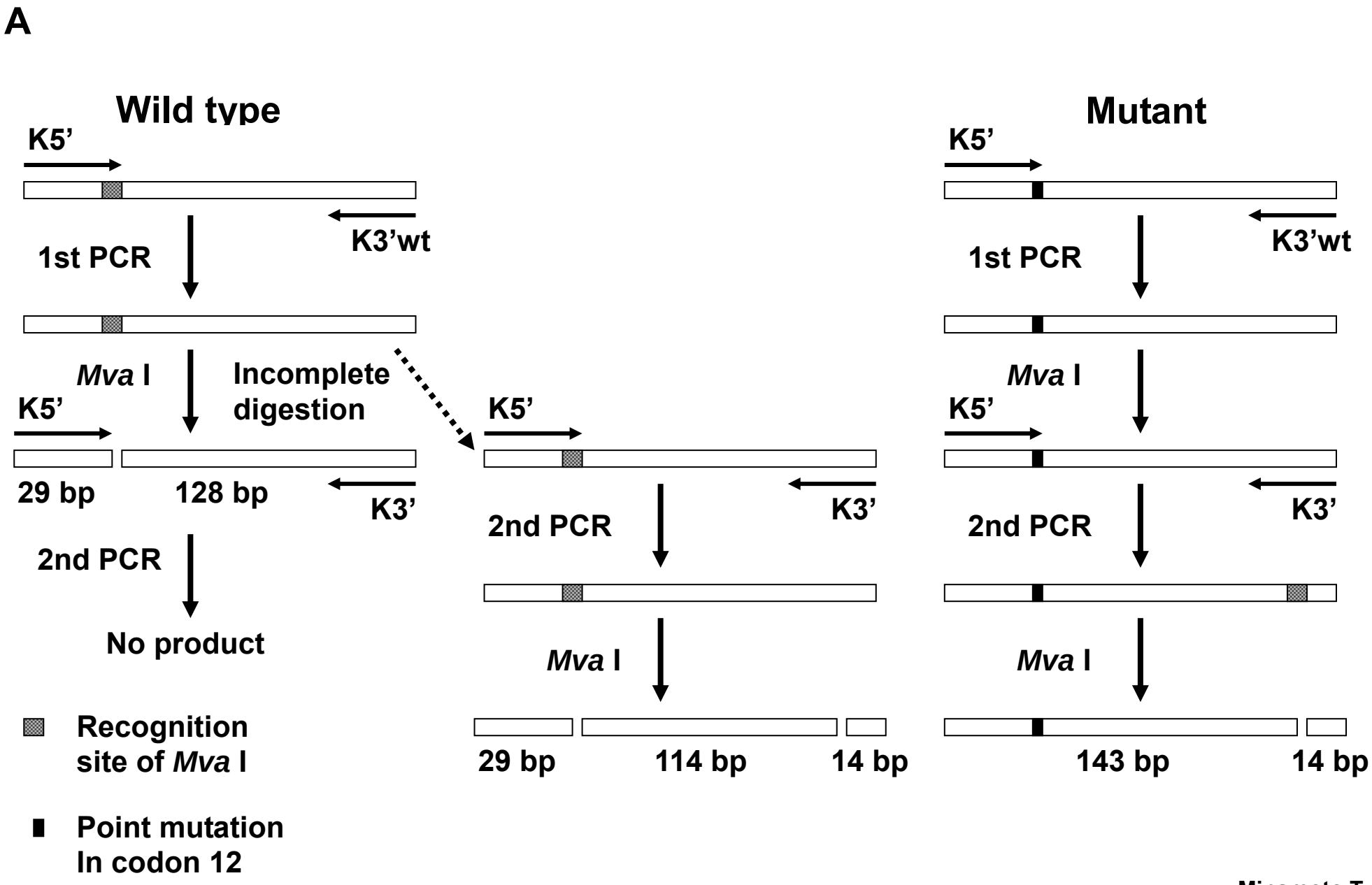
Figure 2. Sensitivity of EPCR for detecting mutant K-*ras*. The genomic DNA mixtures with different ratios of mutant DNA (derived from the SW480 colon cancer cell line) to wild-type (human placenta) DNA were amplified by the EPCR to determine the sensitivity for detecting mutant K-*ras* gene (143 bp fragment). Each PCR product was digested with *Mva* I, and subjected to electrophoresis on a 10 % native polyacrylamide mini-gel. Ratios of SW480 cell line-derived DNA to human placental DNA in templates for EPCR are shown on the respective sample lanes. An EPCR product from a negative control, containing no DNA, was loaded in the W (distilled water) lane. U, undigested EPCR product from SW480 DNA; M, molecular weight marker DNA (ϕX174 DNA digested with *Hae* III).

Figure 3. Representative detection patterns of mutant K-*ras* in the non-neoplastic mucosa (N) and tumors (T) of the colon and rectum, by conventional PCR-RFLP and EPCR. In each case, the sample numbers shown above the lanes of 10 % polyacrylamide gel electrophoresis are identical to those depicted in the figures of corresponding surgical specimen. (A) A

59-year old woman with rectal cancer. The conventional PCR-RFLP analysis shows mutant K-*ras* (143 bp) in the tumor. By the EPCR, a mutant with a base pair alteration identical to that of the tumor is detected in one (proximal to the tumor) of four non-neoplastic mucosal samples. (B) A 65-year-old woman with ascending colon cancer. The EPCR detects a mutant in a non-neoplastic sample taken from the cecum. The base pair alteration in this sample is different from that in the tumor. (C) A 75-year-old man with sigmoid colon cancer. The different types of mutants are identified in two of four non-neoplastic samples, by the EPCR. The tumor has no mutant allele.

Figure 4. Detection of mutant K-*ras* in human colon ACF. (A) An ACF, identified in methylene blue-stained grossly normal mucosa, consists of a cluster of aberrant crypts showing thickened epithelial layer, slit-, asteroid- or oval-shaped lumen, increased pericryptal zone and microscopic elevation above the surrounding normal mucosa. (B) Under microscopic observation, an ACF is isolated and divided into two pieces, one for conventional histopathological examinations and the other for DNA extraction. (C) EPCR and RFLP analysis of K-*ras* mutations in codon 12 (upper panel) and codon 13 (lower panel) in ACF, carcinomas and microscopically normal mucosa sampled from the same patients with colorectal cancer. N1 and N2, normal colorectal mucosa; A1 to A7, ACF; T1 and T2, colorectal carcinomas (N1, A1 to A3 and T1 were taken from a 53-year-old woman with rectal cancer. N2, A4 to A7 and T2 were from a 63-year-old man with sigmoid colon cancer); W, wild-type DNA derived from human placenta; Mu, mutant DNA controls derived from a colon cancer cell line SW 480 (codon 12) and from an adenocarcinoma known to harbor a heterozygous mutation in codon 13; U, undigested PCR product (157 bp); N, a PCR product from a negative control reaction with no DNA template; M, molecular size marker (ϕX174 DNA digested with *Hae* III). In the former case, mutation was detected in two ACF and a carcinoma, and the sequence of one mutant (GAT) in ACF was different to that (GTT) in the carcinoma. In the latter case, K-*ras* mutations were detected in three of four ACF: two ACF showed mutation in codon 12 and one in codon 13, while the carcinoma harbored no mutation.

Figure 1



Minamoto T

Figure 1

B

Primers	Sequence
K5’	5’ ACTGAATATAAACTTGTGGTAGTTGGA<u>C</u>CT 3’
K3’wt	5’ TCAAAGAATGGTCCTGCACCAG 3’
K3’	5’ TCAAAGAATGGTCCTG<u>G</u>ACCAG 3’
K13-5’	5’ ACTGAATATAAACTTGTGGTAGTTGG<u>CC</u>CT 3’

C

Codon	• • •	10	11	12	13	• • •
K-*ras* Exon 1 (wild type)	• • •	GGA	GCT	GGT	GGC	• • •
Primer K5’	• • •	GGA	<u>C</u>CT			
Bst N1/*Mva* I Site			CCN	GG		
Primer K13-5’	• • •	GG<u>C</u>	<u>C</u>CT			
Bgl I Site		GC	CNN	NNN	GGC	

Figure 2

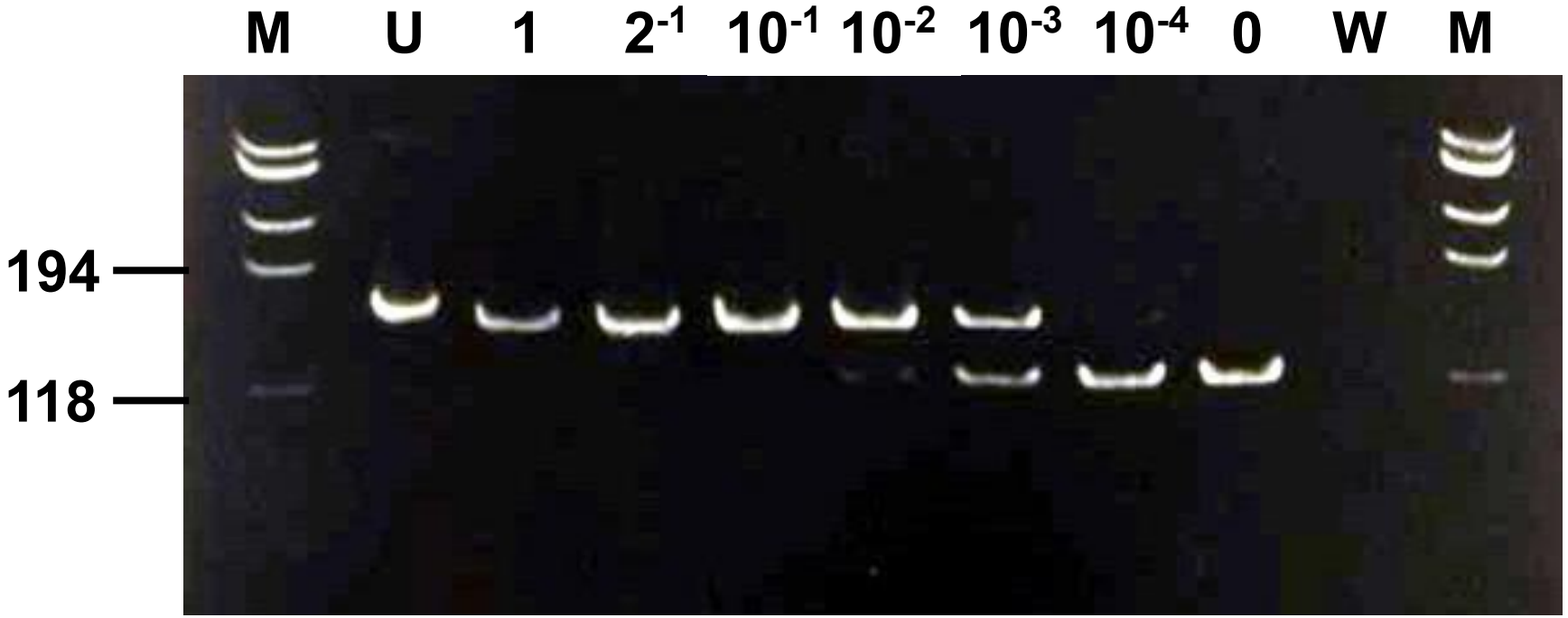


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Figure 3

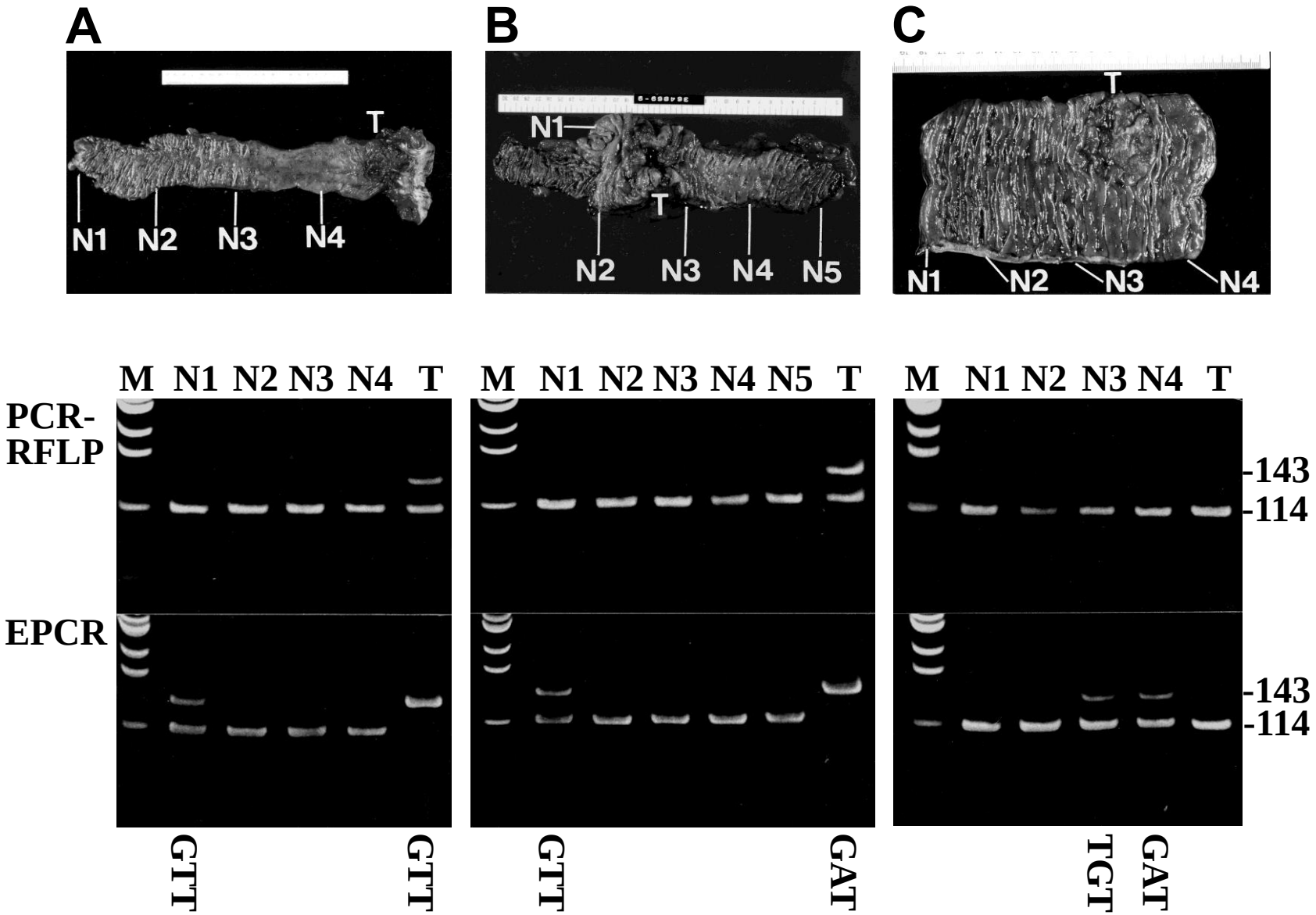

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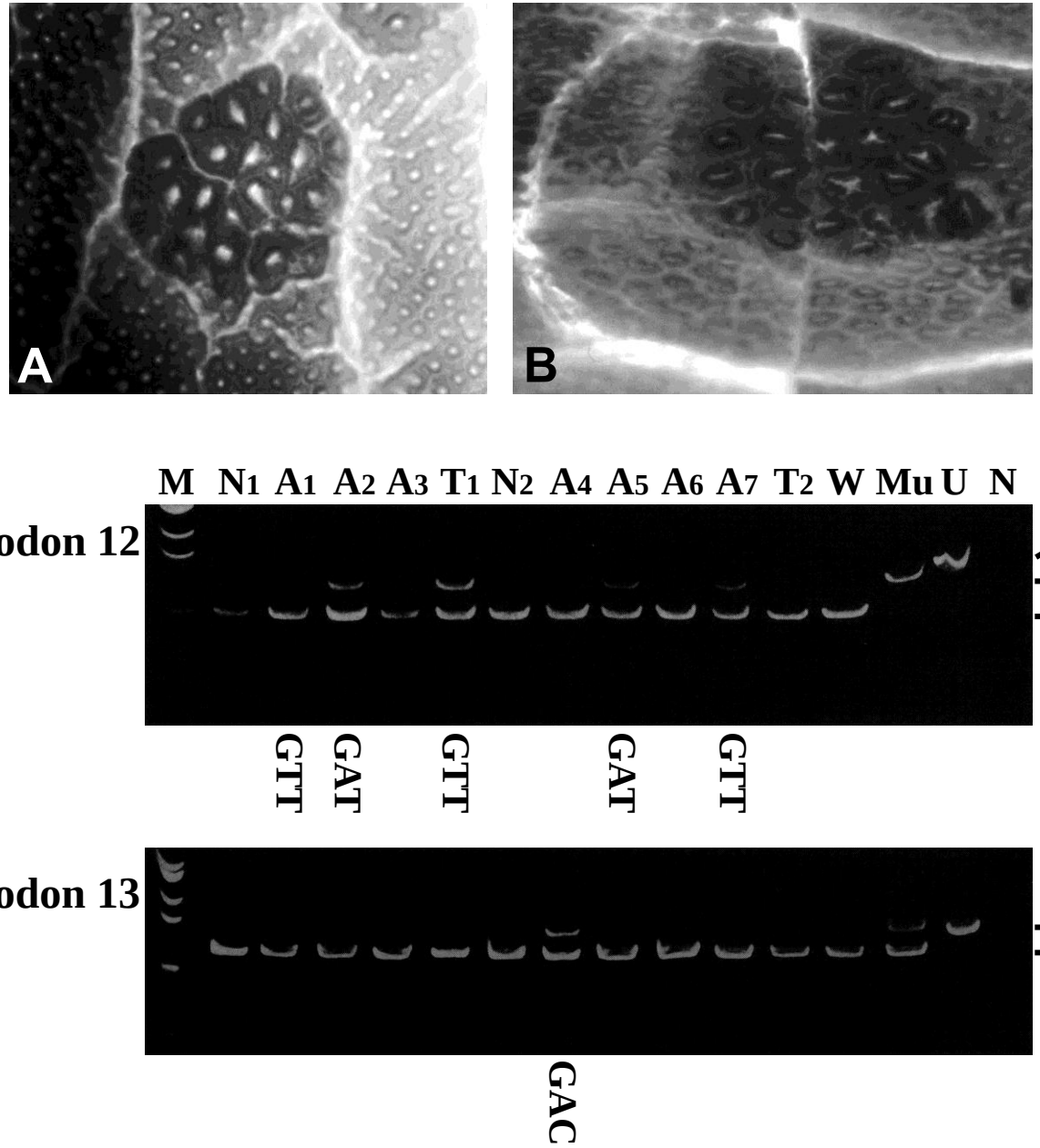
Figure 4



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