

A Sensitive Method to Quantify Human Long DNA in Stool: Relevance to Colorectal Cancer Screening

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Abstract

Human long DNA in stool may reflect nonapoptotic exfoliation and has been used as a colorectal cancer (CRC) marker. Targeting human-specific *Alu* repeats represents a logical but untested approach. A real-time *Alu* PCR assay was developed for quantifying long human DNA in stool and evaluated in this study. The accuracy and reproducibility of this assay and the stability of long DNA during room temperature fecal storage were assessed using selected patient stools and stools added to human DNA. Thereafter, long DNA levels were determined in blinded fashion from 18 CRC patients and 20 colonoscopically normal controls. Reproducibility of real-time *Alu* PCR for quantifying fecal long DNA was high ($r^2 = 0.99$; $P < 0.01$). Long DNA levels in nonbuffered stools stored at

room temperature fell a median of 75% by 1 day and 81% by 3 days. An EDTA buffer preserved DNA integrity during such storage. Human long DNA was quantifiable in all stools but was significantly higher in stools from CRC patients than from normal controls ($P < 0.05$). At a specificity of 100%, the sensitivity of long DNA for CRC was 44%. Results indicate that real-time *Alu* PCR is a simple method to sensitively quantify long human DNA in stool. This study shows that not all CRCs are associated with increased fecal levels of long DNA. Long DNA degrades with fecal storage, and measures to stabilize this analyte must be considered for optimal use of this marker. (Cancer Epidemiol Biomarkers Prev 2006;15(6):1115–9)

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related death in the United States (1). Although CRC mortality is preventable if neoplasms can be detected at curable stage (2), only a minority of the population undergoes regular screening (3). Except for fecal occult blood testing, screening tools endorsed by the American Cancer Society are invasive and expensive (4).

The emergence of molecular stool testing provides a possible user-friendly alternative to conventional methods of CRC screening. A variety of DNA markers have been detected in the stools (5), including mutations of oncogenes (6) and tumor suppressor genes (7), microsatellite instability (8), and DNA methylation (9, 10). Owing to the continuous exfoliation of nonapoptotic neoplastic cells, long DNA occurs more abundantly in CRC stools than normal ones and serves as a candidate screening marker (11, 12). Colonocytes shed from normal epithelium undergo apoptosis, and their DNA is broken down by endonucleases into fragments shorter than 200 bp (12). However, there seems to be an escape from such apoptosis in exfoliated dysplastic cells, which results in long DNA sequences in stool that can be used for cancer detection (12).

Present methods for detecting long DNA use assay of multiple-specific target sequences on different genes (12, 13). Assay of *Alu* sequences represents a potentially simple approach to measure human long DNA in stool. *Alu* sequences embody the largest family of middle repetitive DNA sequences in the human genome. An estimated half million *Alu* copies are present per haploid human genome (14). Because *Alu* sequences are so abundantly distributed throughout the genome and specific to the genomes of

primates (14), an assay that amplifies DNA sequences longer than 200 bp within these 300-bp repeats should provide a genome-wide approach to quantify human long DNA in stool. *Alu*-based assays have been used to quantify human tumor xenograft burden in murine (15) or chicken embryo models (16) as well as integrated HIV-1 DNA in infected HeLa cells (17) but have not been applied to stool.

This study was designed to (a) validate a real-time *Alu* PCR assay for quantifying human long DNA in stool, (b) evaluate the stability of long DNA in stool stored at room temperature and the effectiveness of an EDTA buffer for stabilizing DNA integrity, and (c) explore the feasibility of fecal long DNA quantification for CRC screening.

Materials and Methods

The study was approved by the Mayo Clinic Institutional Review Board.

Stool DNA Extraction. Total DNA was extracted from stool samples with QIAamp DNA Stool Mini kit (Qiagen, Valencia, CA). Stool (2 g) was homogenized in 20 mL buffer ASL, and stool slurry (2 mL) was then used to extract total DNA following the instruction of the manufacturer. DNA was finally eluted in 100 μ L buffer AE.

Real-time *Alu* PCR. The *Alu* sequence consists of conserved regions and variable regions. In the putative consensus *Alu* sequence, the conserved regions are the 25-bp span between nucleotide positions 23 and 47 and 16-bp span between nucleotide positions 245 and 260 (14). Although primers may be designed in any part of the *Alu* sequences for more effectively amplifying *Alu* sequences, the PCR primers should completely or partially (at least the 3'-regions of the primers) locate in the conserved regions. Primers specific for the human *Alu* sequences [sense (5'-ACGCTGTAATCC-CAGCACTT-3') and antisense (5'-TCGCCAGGCTG-GAGTGCA-3')] were used to amplify sequences ~245 bp inside *Alu* repeats (Fig. 1; ref. 16). Stool DNA was diluted 1:5 with 1 $\times$ Tris-EDTA buffer (pH 7.5) for PCR amplification. Tris-EDTA buffer-diluted stool DNA (1 μ L) was amplified in a total volume of 25 μ L containing 1 $\times$ iQ SYBR Green Supermix

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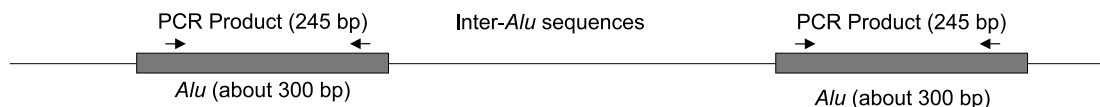


Figure 1. The design of the real-time *Alu* PCR. Primers with 3'-ends complementary to the conserved regions of consensus sequence were used to amplify products ~245 bp inside *Alu* repeats.

(Bio-Rad, Hercules, CA), 200 nmol/L each primer under the following conditions: 95°C for 3 minutes followed by 23 cycles of 95°C and 60°C for 30 seconds and 72°C for 40 seconds. Standard curve was created for each plate by amplifying 10-fold serially diluted human genomic DNA samples (Novagen, Madison, WI). Melting curve was made after each PCR to guarantee that only one product was amplified for all samples. Amplification was carried out in 96-well plates in an iCycler (Bio-Rad). Each plate consisted of stool DNA samples and multiple positive and negative controls. Each assay was done in duplicate.

Long DNA Stability Analysis. Five fresh stools from CRC patients were used to test the stability of human long DNA in stool stored at room temperature. Four aliquots (2 g each) from each of the five stools were stored at room temperature for 0, 1, 3, and 8 days. Total stool DNA was extracted from each aliquot with QIAamp DNA Stool Mini kit as described above. Human long DNA in total stool DNA sample was quantified with real-time *Alu* PCR as described above. Long DNA levels in stool aliquots extracted in days 1, 3, and 8 were divided by long DNA level in the stool aliquot extracted in day 0 for each stool sample to calculate the percentage of intact long DNA kept in the stool aliquots stored at room temperature for different durations. The median percentage of long DNA kept at each time point for five stools was then calculated.

Stabilizing Human DNA Integrity. Four fresh normal stools with added human genomic DNA were used to test the effectiveness of an EDTA-based buffer for stabilizing DNA integrity in stools. Human genomic DNA (1 µg) was spiked into two aliquots (4 g each) of each stool, and then aliquots of each stool were homogenized with 40 mL of two different buffers, including buffer with 100 mmol/L EDTA [0.5 mol/L Tris, 10 mmol/L NaCl, 100 mmol/L EDTA (pH 7); ref. 18] and buffer with 16 mmol/L EDTA [0.5 mol/L Tris, 10 mmol/L NaCl, 16 mmol/L EDTA (pH 7)]. Homogenized stool slurry was stored at room temperature, and 2 mL of it was used for stool DNA extraction at each of four different time points (day 0, 1, 3, and 8). Total stool DNA was extracted from each aliquot with QIAamp DNA Stool Mini kit with some modifications. Human DNA in total stool DNA sample was quantified with real-time *Alu* PCR. The median percentage of human DNA kept at each time point was calculated.

Clinical Pilot Study. A completely independent set of fresh stools from 18 CRC patients and from 20 colonoscopically normal individuals were analyzed in blinded fashion. The

demographic and clinical characteristics of the CRC patients and controls are shown in Table 1. All stools were collected before colonoscopy or surgery. None of the CRC patients had undergone chemotherapy or radiotherapy before stool collection. Any previous instrumentation had occurred >2 weeks before stool collection. A plastic bucket device was used to collect whole stool. Stools in sealed buckets were immediately transported to our laboratory, and total DNA was extracted from all stools within 4 hours from defecation.

Statistical Analysis. For human long DNA levels obtained by real-time *Alu* PCR, the median for each group of stool samples was calculated, and Wilcoxon signed-rank test was used to compare the human long DNA levels of different stool groups. Spearman's rank correlation was used to calculate the correlation coefficient of the reproducibility. Statistical tests were done using SAS statistical software (SAS Institute, Inc., Cary, NC). All *P*s were two sided.

Results

Validating Real-time *Alu* PCR Assay. To determine the dynamic range of the real-time *Alu* PCR, human genomic

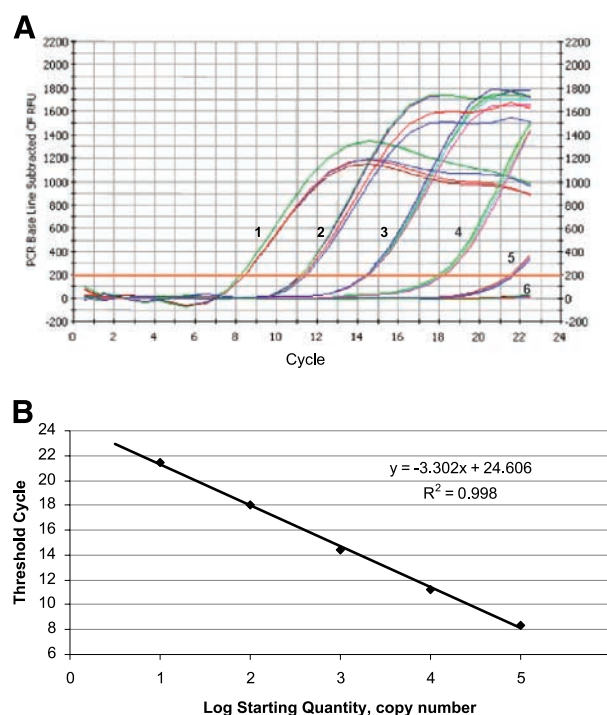


Figure 2. **A.** Human genomic DNA samples, which had been serially diluted by 10-fold (lines 1, 2.5 ng; lines 2, 250 pg; lines 3, 25 pg; lines 4, 2.5 pg; and lines 5, 250 fg), were amplified with real-time *Alu* PCR. Water control, 2.5 ng of each genomic DNA from pig, bovine, and chicken, and 2.5 ng *E. coli* genomic DNA were not amplified with real-time *Alu* PCR (lines 6). **B.** A standard curve was created with the log starting quantity and threshold cycle of the 10-fold serially diluted human genomic DNA samples.

Table 1. Demographic and clinical characteristics of subjects

	Cancer	Normal
Number	18	20
Sex (M/F)	12/6	11/9
Mean age (y)	62	71
Site (proximal/distal)	5/13	
Median size, cm (range)	3.5 (1.1-10.0)	
Stage (Dukes AB/CD)	8/9*	

*Duke stage information was not available for a patient who did not have surgery.

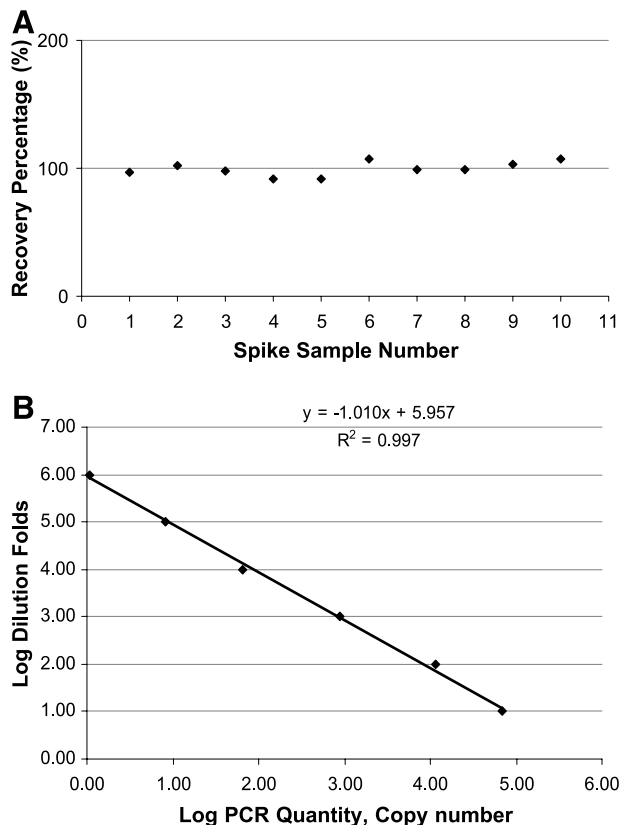


Figure 3. A. Human genomic DNA (25 pg) added into 10 different stool DNA samples was recovered by real-time *Alu* PCR. Recovery percentage (%) equals to human DNA amount recovered divided by human DNA amount added and then multiplied by 100. **B.** A stool DNA sample was 10-fold serially diluted, and human long DNA was then quantified using real-time *Alu* PCR. Linear recovery of human long DNA in serially diluted stool DNA samples.

DNA samples serially diluted over a 10-fold range (2.5 ng, 250 pg, 25 pg, 2.5 pg, 250 fg, and 25 fg) were amplified with the real-time *Alu* PCR. *Alu* sequences were linearly detected from 250 fg up to 2.5 ng human genomic DNA per PCR (Fig. 2A and B).

To confirm the human specificity of the real-time *Alu* PCR, genomic DNA samples from pig, bovine, and chicken, which are three nonhuman species typically consumed in the diet,

and *Escherichia coli*, a common bacterium in stool, were tested by this method. The *Alu*-based PCR assay was negative for all nonhuman mammalian DNA and *E. coli* DNA (Fig. 2A).

Because stool contains PCR inhibitors (19), quantification could be affected by PCR inhibitors. To check whether assay accuracy was affected by potential PCR inhibitors, 500 pg human genomic DNA (2 μ L) was added into 10 different stool DNA samples (38 μ L each), and mixed DNA (1 μ L), which contained 25 pg human genomic DNA, was then quantified with real-time *Alu* PCR. The mean recovery percentage of the added samples was 99.6% (range, 91.4–107.8%; Fig. 3A). For further confirming that PCR inhibitors did not affect the quantitative accuracy of the assay, one stool DNA sample from a CRC patient was 10-fold serially diluted and then quantified with real-time *Alu* PCR. Linear recovery of long DNA from these serially diluted stool DNA aliquots ($r^2 = 0.997$) confirmed the absence of interference by PCR inhibitors (Fig. 3B).

The reproducibility of the real-time *Alu* PCR was studied in frozen stool samples from eight CRC patients and eight normal individuals. Human long DNA in these stool DNA samples was quantified in duplicate. The human long DNA levels of duplicate runs correlated highly ($r^2 = 0.99$; $P < 0.01$; Fig. 4).

Instability of Human Long DNA in Stools Stored at Room Temperature. Compared with stools tested on day 0, median long DNA levels in stools stored at room temperature for 1, 3, and 8 days after defecation fell 75%, 81%, and 89%, respectively (Fig. 5A).

From four fresh normal stools added to human DNA and mixed with low concentration EDTA (16 mmol/L), recoveries of human DNA after room temperature storage for 1, 3, and 8 days were 65%, 19%, and 3%, respectively, compared with day 0. However, for stool aliquots mixed in buffer with high EDTA concentration (100 mmol/L), median recoveries of added human DNA were preserved at 121%, 118%, and 100%, respectively (Fig. 5B).

Human Long DNA Levels in CRC Stools and Normal Controls. Human long DNA levels in 18 CRC and 20 normal fresh stools, which were collected immediately after defecation, were quantified by real-time *Alu* PCR in blinded fashion. Human long DNA was detected in all 38 stool samples but was significantly higher in CRC stools (median, 309 ng/g stool; range, 5–21,115) than in normal stools (median, 70 ng/g stool; range, 2–2,870; $P = 0.04$; Fig. 6). At a long DNA cutoff of 2,900 ng/g stool, sensitivity for CRC was 44% (8/18), and specificity was 100% (20/20). Median long DNA in five proximal CRC stools was 48 ng/g (range, 10–506 ng/g) and in 13 distal CRC stools was 4264 ng/g

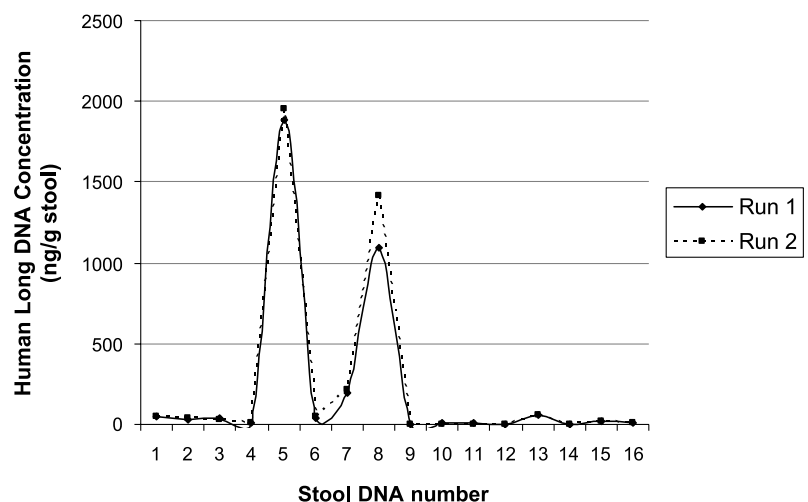


Figure 4. Stool DNA samples from eight CRC and eight normal stools were quantified with real-time *Alu* PCR twice. The human long DNA levels from these two runs showed good reproducibility ($r^2 = 0.99$).

(range, 5-21,115; $P = 0.09$). In this small series, tumor size did not significantly affect long DNA levels in stool.

Discussion

This report describes a new method to quantify human long DNA in stool using real-time PCR amplification of a 245-bp sequence within *Alu* repeats. The method is very sensitive with a dynamic range of 250 fg to 2.5 ng human genomic DNA, accurately detects human DNA added into stools, and yields highly reproducible results. Furthermore, this real-time *Alu* PCR method may have advantages of simplicity and speed compared with other approaches that describe use of multiple gene targets to assay long human DNA in stool (12, 13).

With this validated new method, we found that human long DNA was present in all stools tested, but levels were significantly higher in stools from CRC patients than from normal individuals. When human long DNA in stool was used as a marker at a 100% specificity cutoff, about half of CRC patients could be detected, which is consistent with the performance of long DNA as a marker in earlier reports (11-13). The abundance of human long DNA in stools from CRC patients likely reflects the nonapoptotic exfoliation that occurs with CRC described by others (11-13).

In two recent multicenter studies (20, 21), human long DNA in stool was less informative than in earlier reports. This discrepancy seems to be due to degradation by bacterial DNAases during prolonged preassay fecal storage that occurred with mailed-in samples in these studies. Experimental observations in the present study and by others (18) corroborate the instability of human long DNA during fecal storage. Such degradation can be prevented by mixing stools with buffers containing a DNAase inhibitor like EDTA (18) as was shown in the present study. If human long DNA is to be

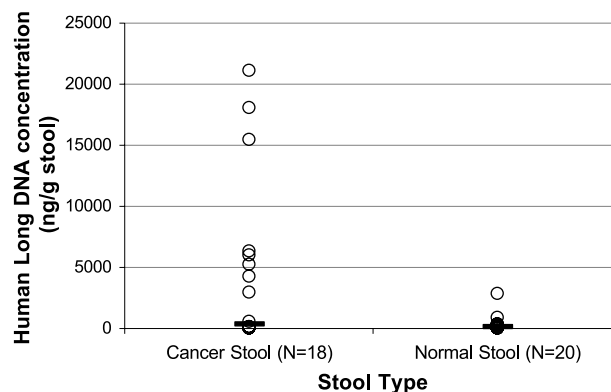


Figure 6. Human long DNA levels of stools in the blinded pilot clinical study. O, stool sample. Solid horizontal bar, median of human long DNA concentration within a group of subjects.

used clinically as a fecal marker, then attention must be given to incorporating a DNAase inhibitor as part of specimen collection and processing.

Human long DNA is not specific for CRC. Preliminary reports suggest that human long DNA in stool may detect cancers in the upper gastrointestinal track as well (22). Inflammatory bowel disease has also been shown to be associated with elevated levels of human long DNA in stools (23). In contrast to normal epithelial cells, which undergo apoptosis (anoikis) when shed from their basement membrane attachment (24), inflammatory cells are anchorage independent and logically contribute to long DNA in stools. The discriminant value of human long DNA measured by this method would need to be verified in a larger and more representative sample if it were to be considered for screening or other clinical applications.

Real-time *Alu* PCR is a simple, rapid, and inexpensive method for quantifying human long DNA in stools. This method may have useful applications for research observations and clinical testing.

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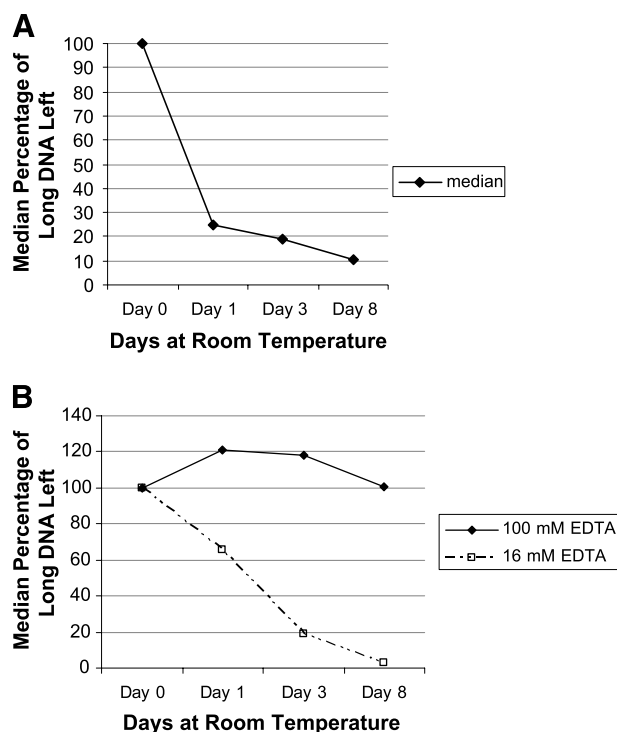


Figure 5. A. Long DNA levels of CRC stools stored at room temperature for 1, 3, and 8 days after defecation fell median percentages of 75%, 81%, and 89%, respectively. B. Buffers with 100 mmol/L EDTA and 16 mmol/L EDTA showed different effects on preserving human DNA added in stools stored at room temperature.

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