

The Isolation of High Quality Stool DNA and its Application to Quantitative Adenovirus Detection using TaqMan® Real-Time PCR

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INTRODUCTION

The use of stool as a biological sample for nucleic acid isolation and subsequent biomarker or viral nucleic acid detection has only recently been recognized. Despite the invasive nature and questionable sensitivity of colonoscopy, it is still used as the gold standard for colorectal carcinomas (CRC)¹. Stool DNA, on the other hand, has shown remarkable sensitivity rates considering its non-invasiveness, even detecting lesions that have been missed using colonoscopy¹.

Adenoviruses (AdV) were first discovered in the 1950s, and they naturally infect many species, including humans². AdV infections can be life threatening, and can be transmitted through haematopoietic stem cell transplantation (HSCT). Therefore, it has been recommended that transplant recipients receive repeated screening for AdV DNA as a routine procedure follow-up, using whole blood³. As blood collection requires a nurse, studies have assessed the utilization of stool for AdV detection, with great success. The use of cell culture for AdV visual CPE detection takes up to two weeks, and the cost of current stool AdV detection assays is a strong deterrent for healthcare industries to adopt routine stool DNA isolations as a screening tool for AdV infections¹.

Norgen's Stool Nucleic Acid Isolation Kit (Cat# 45600) is a fast, affordable method for the purification of high quality stool DNA, with DNA isolation to viral or oncogenic nucleic acid detection made possible in as little as 3 hours using qPCR.

In this study, we demonstrate the use of Norgen's Stool Nucleic Acid Isolation Kit for the purification and detection of AdV DNA using TaqMan® real-time PCR (Life Technologies, Grand Island, NY).

MATERIALS AND METHODS

Sample Collection and Stool Nucleic Acid Extraction

Stool samples were collected from three different individuals into sterile collection cups. Nucleic acid isolation took place in duplicate, using Norgen's Stool Nucleic Acid Isolation Kit, following the manufacturer's protocol. Briefly, 230mg of stool, suspended in stool suspension buffer, was added to a Norgen bead tube (Cat# 26550) along with 1mL of Lysis buffer. Samples were then vortexed for 3 minutes at 2500 RPM using the Vortex Genie® Pulse. Two microliters of RNase was added to each sample, and samples were incubated for 5 minutes at room temperature. Samples were then centrifuged for 3 minutes at 14 000rpm, and 700µl of supernatant was transferred to a new 2mL tube. At this point, 50µL of AdV stock (~ 5.0 x 10⁸ copy number) was spiked into each sample, and 100µl of Binding solution was added. Samples were then incubated on ice for 10 minutes. Samples were then centrifuged at 14 000rpm for 3 minutes, and supernatants were transferred to a new 1.5mL tube, with equal volume of 70% ethanol. Samples were then vortexed, and transferred to a spin column. Samples were then bound, washed and eluted according to the manufacturer's instruction. Samples were eluted in 75µl elution solution.

Nuclease Digestion, Agarose Gel Electrophoresis, and Nanospectrophotometry

Ten microliters of purified stool DNA was digested with 0.5µl of RNase A, and incubated at room temperature for 30 minutes to differentiate RNA and DNA on the gel. Similarly, 10µl of purified stool DNA was digested using 3µl of DNase I in 10µl of enzyme incubation buffer, and incubated at 37°C for 30 minutes. Digested and undigested samples were run on a 1X TAE 1.2% agarose gel, pre-stained with ethidium bromide, and viewed using the Alphamager 2200 (AlphaInnotech). Purified stool elutions were also quantified using the NanoVue™ Plus spectrophotometer (GE Healthcare).

Real-Time PCR

The TaqMan® assay consisted of Adenovirus serotype 5 (Ad5)- specific probes and the fluorescent label utilized was 6-FAM® (50-carboxyfluorescein) with internal ZEN® quencher and 3' Iowa Black® fluorescein quencher (IBFQ), that were synthesized in order to provide superior performance and reduce fluorescence background. The ZEN/Iowa Black® FQ is a double-quenched probe. The Ad5-specific primers and probe were designed by Norgen Biotek Corp. Two microliters of purified DNA was used in the reaction, along with Norgen's 2X PCR Mastermix (Cat# 28007). The PCR reactions were placed into an iCycler iQ® real-time thermocycler and were subjected to the following reaction parameters: initial template denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, followed by primer annealing and extension at 60°C for 30 seconds.

Determination of AdV Copy Number Isolated

In order to determine the AdV copy number isolated from each spiked stool sample, a 10-fold dilution was performed using the 5.0×10^8 AdV stock. Using the TaqMan® assay described above, Ct values were generated from all dilutions, run in quadruple reactions. Using the calculator for determining the number of copies of a template provided by the URI Genomics & Sequencing Center⁴, a standard curve was generated. Sample Cts were then used as "X" in the equation of the line to determine the average copy number of AdV isolated.

RESULTS AND DISCUSSION

AdV detection in stool samples is a non-invasive, sensitive screening method to minimize the burden placed on patients who may become infected post-HSCT. As most methods of detection require either a great deal of time and/or money, Norgen Biotek Corp. has developed a method of AdV detection from stool by first isolating high quality nucleic acids from stool, followed by TaqMan® real-time PCR (Life Technologies, Grand Island, NY) using Ad-5 primers designed by Norgen Biotek. In this study, we demonstrated the detection of spiked Ad-5 from 250mg of stool isolated using Norgen's Stool Nucleic Acid Isolation Kit. Following the purification of nucleic acids from the stool samples, portions of the purified DNA were used for RNase and DNase digestions to determine the relative amounts of both nucleic acids in the sample (Figure 1). As we were looking to isolate DNA only, an RNase digestion

was also performed during the isolation process to minimize RNA contamination. The digested and undigested samples were run on a 1X TAE 1.2% agarose gel. Figure 1A shows the results from the RNase digestion, with the main differences between the digested and undigested samples being the amount of low MW nucleic acids found on the gel. The DNA was found to be sheared, which is common for high intensity homogenization methods such as bead tubes. Figure 1B shows the results for the DNase I digestions. This time, the nucleic acids in the digested samples were completely eliminated, demonstrating that the smear found on the gel is indeed DNA.

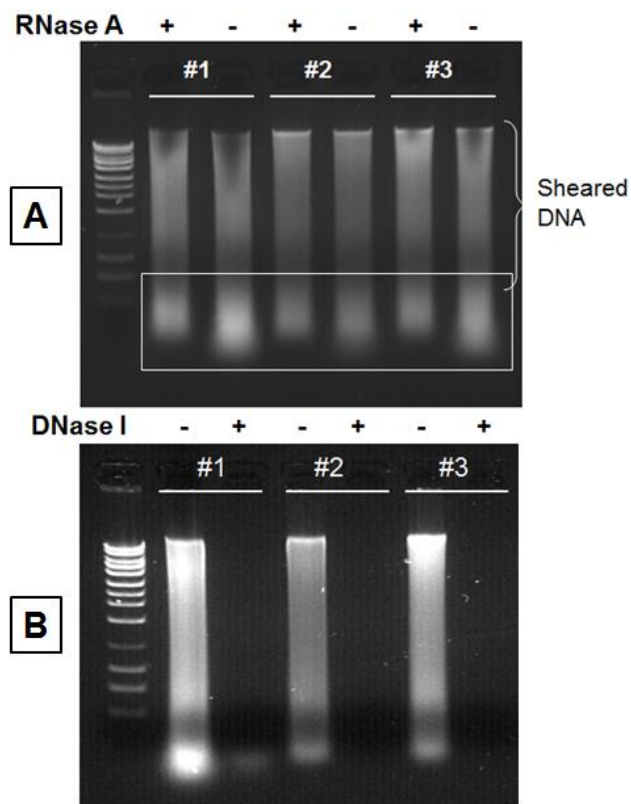


Figure 1. Nuclease digestions from stool nucleic acids isolated from 3 different donors, run on a 1X TAE 1.2% agarose gel. A) Digested and undigested samples from an RNase A digestion involving 10µl of nucleic acid elutions and 0.5µl of RNase A incubated at room temperature for 30 minutes. B) Digested and undigested samples from a DNase I digestion involving 10µl of elutions, 3µl of DNase I, and 10µl of enzyme incubation buffer, incubated at 37°C for 30 minutes (+ = digested, - = undigested).

Next, to determine the quality of DNA isolated from the three stool samples, 2µl of the purified DNA was analyzed using the NanoVue™ Plus spectrophotometer (GE Healthcare). The average A260:A280 and A260:A230 for all three samples (isolated in duplicate) is depicted in Figure 2. The purified DNA isolated from all three stool samples was found to be of the utmost quality, with all A260:A280 and A260:A230 ratios falling in the 1.8-2.2 range.

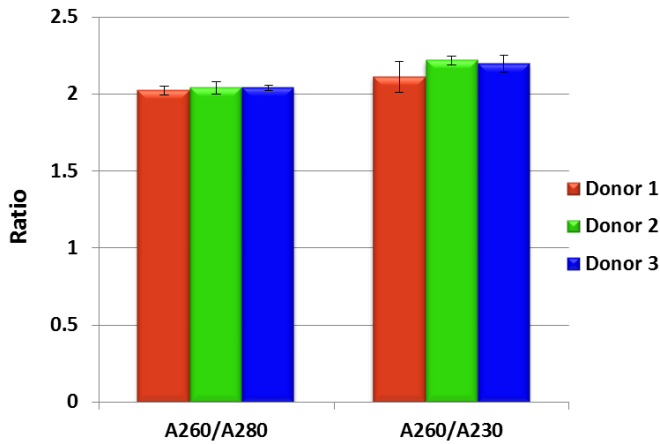


Figure 2. The average A260:A280 and A260:A230 ratio generated for all three purified stool DNA samples, isolated in duplicate, determined using the NanoVue™ Plus spectrophotometer.

Purified stool DNA was then used in a TaqMan® real-time PCR detection assay, using Ad-5 primers to detect the AdV spiked into each sample during the isolation procedure. The results can be found in **Figure 3**. Figure 3A is the amplification graph, and Figure 3B is the graphical representation of the average Ct value generated for all three samples, isolated in duplicate. All three samples were found to amplify around a Ct of 16, with small standard deviations generated between the two replicates. As each sample received the same amount of AdV spike-in, this indicates that the isolation procedure is consistent and reliable, isolating high quality DNA ready for sensitive downstream applications, such as qPCR.

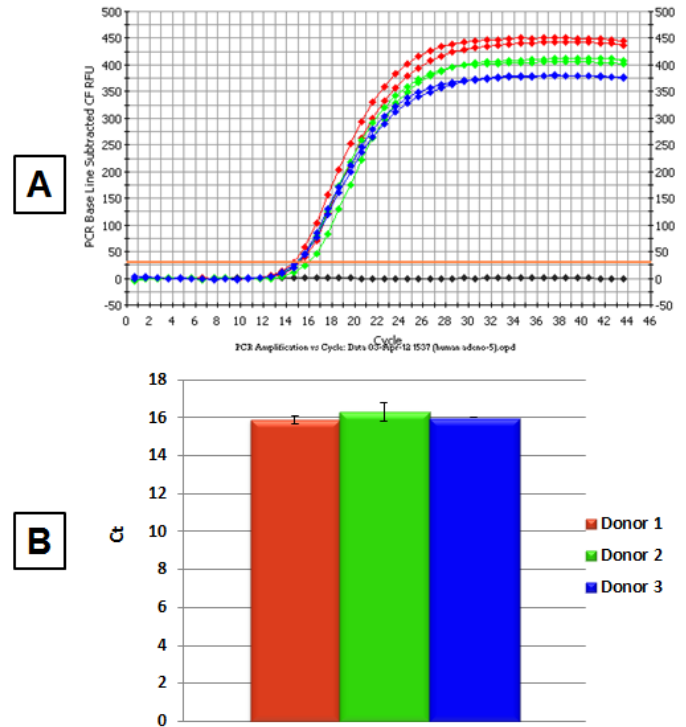


Figure 3. TaqMan® real-time PCR detection of AdV. Ad5-specific probes were used, designed by Norgen Biotek Corp., as well as 6-FAM®, with internal ZEN®, and 3' IBFQ. Two microliters of purified DNA was used in the reaction, along with Norgen's 2X PCR Mastermix. The reaction took place in the iCycler iQ® real-time detection system.

Finally, to determine the AdV copy number isolated from each spiked stool DNA sample, a standard curve was generated from a 10-fold dilution series of a 5.0×10^8 AdV stock (**Figure 4**). The curve consisted of the average Ct value of a quadruplicate TaqMan® qPCR reaction of each dilution, plotted against the plasmid copy number, determined using the calculator for determining the number of copies of a template, provided by the URI Genomics & Sequencing Center⁴.

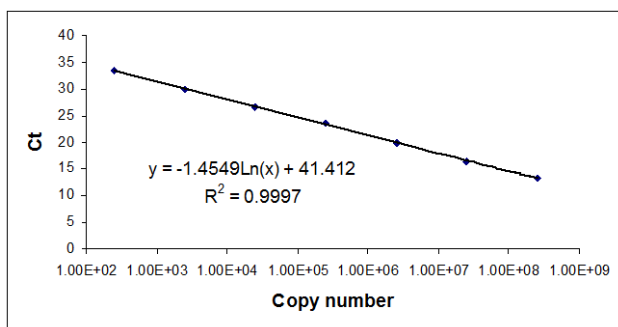


Figure 4. The standard curve generated from the amplification of a 10-fold serial dilution of the adenovirus 5 plasmid, starting from 5 ng down to 5 ag, in quadruple reactions. The amplification plot shows excellent linearity ($R^2 = 0.9997$) across the 7 orders of magnitude. Virus copy number was calculated using the calculator for determining the number of copies of a template, provided by the URI Genomics & Sequencing Center⁴.

Using the standard curve in Figure 4, the copy number isolated from each stool sample was determined. The average yields and copy numbers isolated from each stool sample is summarized in **Table 1**. Each stool sample contained a large amount of DNA, originating from human and bacterial cells, as well as from the spiked Ad-5 plasmid. Norgen's Stool Nucleic Acid Isolation Kit was able to isolate high yields of DNA from each sample, as well as a consistent amount of AdV spike-in.

Table 1. The average DNA yield and average copy number of AdV isolated from three stool samples, in duplicate. All three samples were found to isolate high DNA yields, translating to high copy numbers of AdV (determined using qPCR).

Donor	Average Yield (μg)	Average Copy Number
1	40.14 (± 8.83)	4.30×10^7
2	35.76 (± 7.56)	3.13×10^7
3	52.99 (± 17.55)	3.85×10^7

CONCLUSIONS

From the data presented in this report, the following can be concluded:

- 1) Convenience.** Stool nucleic acid isolation is a non-invasive method of detecting biomarkers of cancer, as well as viruses and bacteria. As shown in this study, Norgen's Stool Nucleic Acid Isolation Kit is an affordable option for the rapid isolation and detection of AdV from stool.
- 2) High quality DNA.** Norgen's Stool Nucleic Acid Isolation Kit allows for rapid DNA (or RNA) extraction from stool samples, producing high quality DNA ready for any downstream application, including qPCR. Quality measurements used in this study consisted of A260:A280 and A260:A230 ratios, which were all found to be in the 1.8-2.2 range, and qPCR Ct values, which are dependent on sample purity. Norgen's stool DNA samples were found to be quite pure, resulting in Ct values of ~ 16 .
- 3) Sensitivity.** Norgen's Stool Nucleic Acid Isolation Kit was found to consistently isolate both high yields and quality of DNA, which could then be used to detect AdV using the TaqMan[®] real-time PCR detection system, from as little as 1×10^2 plasmid copy number.

REFERENCES

1. Lucas AL, Hwang C, and Frucht H. 2012. Strengthening the case for stool DNA tests as first-line colorectal cancer screening: Are we there yet? Clin. Gastroenterol. H. 10(3): 214-215.
2. Roy S, Vandenberghe LH, Kryazhimskiy S, et al. 2009. Isolation and characterization of adenoviruses persistently shed from the gastrointestinal tract of non-human primates. PLoS Pathog. 5(7): e1000503.
3. Ahlquist DA, Taylor WR, Mahoney DW, et al. 2012. The stool DNA test is more accurate than the plasma septin 9 test in detecting colorectal neoplasia. Clin. Gastroenterol. H. 10: 272-277.
4. Staroscik A. 2004. Calculator for determining the number of copies of a template. URI Genomics & Sequencing Center. Accessed online April 19, 2012 from: <http://www.uri.edu/research/gsc/resources/cndna.html>

Related Products	Product #
Stool Nucleic Acid Isolation Kit	45600
Stool DNA Isolation Kit	27600
Ad5 Type C End-Point PCR Kit	EP42600
Bead Tubes	26533