Video Article

# Cell-Free DNA Integrity Analysis in Urine Samples

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## Abstract

Although the presence of circulating cell-free DNA in plasma or serum has been widely shown to be a suitable source of biomarkers for many types of cancer, few studies have focused on the potential use of urine cell-free (UCF) DNA. Starting from the hypotheses that normal apoptotic cells produce highly fragmented DNA and that cancer cells release longer DNA, the potential role of UCF DNA integrity was evaluated as an early diagnostic marker capable of distinguishing between patients with prostate or bladder cancer and healthy individuals.

A UCF DNA integrity analysis is proposed on the basis of four quantitative real-time PCRs of four sequences longer than 250 bp: *c-MYC*, *BCAS1*, *HER2*, and *AR*. Sequences that frequently have an increased DNA copy number in bladder and prostate cancers were chosen for the analysis, but the method is flexible, and these genes could be substituted with other genes of interest. The potential utility of UCF DNA as a source of biomarkers has already been demonstrated for urologic malignancies, thus paving the way for further studies on UCF DNA characterization. The UCF DNA integrity test has the advantage of being non-invasive, rapid, and easy to perform, with only a few milliliters of urine needed to carry out the analysis.

## Video Link

The video component of this article can be found at https://www.jove.com/video/55049/

## Introduction

Cell-free DNA can be detected in blood and urine due to cell death by apoptotic or necrotic mechanisms. Cell-free DNA in blood has been widely studied for diagnostic and prognostic purposes in various diseases, especially cancer[1]. However, less is known about the role of urinary cell-free (UCF) DNA. UCF DNA may originate from blood passing through the glomerular filtration system or from cells that come directly into contact with this body fluid [2] (*e.g.,* urothelial cells or prostatic cells). The use of UCF DNA as a source of biomarkers has mainly been investigated for the early diagnosis of renal, bladder, and prostate cancer due to the high percentage of UCF DNA coming directly from urinary tract cells[3,4].

Little is known about UCF DNA and the best methods for isolating and characterizing it. Given the hypothesis that tumor cells release longer DNA fragments than normal cells, the evaluation of cell-free DNA integrity has been studied in an attempt to elucidate the origin of DNA in the blood circulation[5]. Some studies have demonstrated that cell-free DNA integrity in blood represents a good diagnostic test for many types of cancer[6], and the same hypothesis has been proposed in relation to urine[7-9].

This paper describes a new method for UCF DNA integrity analysis with a potential application to bladder and prostate cancer detection. In particular, the integrity of UCF DNA fragments longer than 250 bp was tested in 4 regions known to have an increased DNA copy number in solid tumors, including prostate and bladder cancer: *c-MYC* (8q24.21), *HER2* (17q12.1), *BCAS1* (20q13.2), and *AR* (Xq12)[10-14]. Specific oncogenes, rather than random sequences, were chosen to increase the probability of finding them in the cell-free fraction of cancer patients. One of the main advantages of this method is that it is flexible and that other regions can also be selected on the basis of tumor type and characteristics.

## Protocol

The protocol follows the guidelines of the IRST Human Research Ethics Committee.

NOTE: In this protocol, we isolated DNA from urine samples to perform a UCF DNA integrity analysis. Lncap and MRC cell lines were used to construct standards. Techniques such as DNA extraction, DNA quantification (spectrophotometer and real-time PCR for the control gene, STOX1), and real-time PCR for specific oncogenes were performed (**Figure 1**).

## 1. Urine Collection and Processing

1. Obtain a clean-catch first-morning urine sample in a clean, dry, plastic cup. Collect at least 50 mL of the urine sample.

 

2. Maintain the urine at 4 °C for a maximum of 3 h and send it to the laboratory at the same temperature.
3. Mix each sample by inverting it twice immediately upon arrival in the laboratory and transfer into two 50-mL conical-bottomed polypropylene tubes.
4. Centrifuge the tubes at 850 x g for 10 min at 4 °C or at room temperature.
5. Carefully transfer 10 mL of the upper part of the urine supernatant into two 5-mL tubes, leaving at least 2 mL of the supernatant above the cell pellet.
   NOTE: Transferring the upper part of the supernatant reduces the risk of contamination by cells or cellular debris. There is no clear delineation between the upper and lower parts.
6. Discard the pellet and immediately freeze the supernatant at -80 °C until use.

## 2. DNA Isolation from the Urine Supernatant and Cell Lines

NOTE: Isolate the DNA from a cell line (*e.g.,* Lncap for prostate cancer or MRC for bladder cancer) using a commercial kit and following the manufacturer's instructions. Isolation of DNA from the urine supernatant should be performed using the commercial protocol, modified as follows:

1. Thaw one aliquot of the urine supernatant at room temperature.
2. Vortex and mix the urine sample and transfer 1 mL of the urine supernatant into a clear 5-mL tube. Freeze the residual urine at -80 °C.
3. Add 100 µL of proteinase k directly to the sample.
4. Add 1 mL of AL buffer to the sample and mix well by pipetting.
5. Close the tubes and incubate the samples at 56 °C for 15 min.
6. During incubation, prepare one column for each sample and prepare the wash buffers, AW1 and AW2, as per the manufacturer's instructions (add the indicated amount of 100% ethanol).
7. Bring the samples back to room temperature and add 1 mL of absolute ethanol. Mix fully by pipetting.
8. Add 650 µL of the mixture obtained in step 2.7 to the column and centrifuge it at 6,000 x g for 1 min.
9. Discard the tube containing the flow-through and place the column on a new, clean collection tube. Repeat steps 2.8 and 2.9 until all of the sample mixture has been used (5 times).
10. Add 500 µL of buffer AW1 without wetting the rim of the column. Centrifuge it at 6,000 x g for 1 min.
11. Discard the tube containing the flow-through and replace it with a new, clean collection tube.
12. Add 500 µL of buffer AW2 without wetting the rim of the column. Centrifuge it at full speed (20,000 x g) for 3 min.
13. Discard the tube containing the flow-through and replace it with a new, clean collection tube.
14. Repeat step 2.12 to remove any residual washing buffer.
15. Place the column into a clean 1.5-mL tube. Add 50 µL of elution buffer AE and wait 7 min to ensure that the buffer wets the column.
16. Centrifuge it at 8,000 x g for 1 min.
17. Pipette the eluent from step 2.15 into the mini-column and centrifuge it at maximum speed for 1 min to ensure the maximum recovery of DNA.

## 3. DNA Quantification and Dilution

1. Using a spectrophotometer, perform the quantification of DNA from both the cell line and the urine supernatant samples. Use 2 µL of sample on a bench-top spectrometer, as per the manufacturer's instructions.
2. Dilute the UCF DNA samples to obtain a concentration of 1 ng/µL and store the DNA at -20 °C until the DNA integrity analysis.
   NOTE: If the DNA quantity is not sufficient to proceed with real-time PCR (at least 100 ng), perform a new DNA isolation process.
3. Dilute the DNA from cell line samples to obtain six standards with different concentrations: 0.001, 0.01, 0.1, 1, 0.5, and 2 ng/µL, each with a volume of 100 µL (st1, st2, st3, st4, st5, and st6). Store the cell line DNA standards at -20 °C until the DNA integrity analysis.

## 4. DNA Integrity Test — PCR

1. Thaw the primers (the concentration depends on the type of assay), green supermix, cell-line DNA standards, and UCF DNA diluted samples on ice.
2. Prepare strip tubes in the plate for the 72-well rotor disc. Aliquot 10 µL in duplicate for each standard and diluted sample and 10 µL of RNase-free water for the negative control.
3. Prepare a mix of 1 µL of each primer (the concentrations are indicated in **Table 1**), 12.5 µL of green supermix, and 6.5 µL of RNase-free water for each sample. When preparing the mix, use the following number of samples: 6 standards for 2 replicates, number of samples for 2 replicates, negative control for 2 replicates, and 2 extra samples.
4. Aliquot 15 µL of the mix into each well. Do not pipette or spin the tubes.
5. Start the protocol with the PCR conditions indicated in **Table 1**.
   NOTE: For the UCF DNA value, 29 DNA samples were processed for each real-time experiment using the 72-well rotor disc: 29 x 2 samples + 6 x 2 standards + negative control x 2 = 72 (UCF DNA value).
   NOTE: The protocol for the UCF DNA integrity analysis can also be performed using another PCR real-time instrument (when using another device, ROX dye may need to be added).

## 5. DNA Integrity Test — Data Analysis and Interpretation

NOTE: The UCF DNA value for each sample was obtained by a real-time instrument-detection system software using a standard curve construction for each individual PCR gene evaluation and using standard curve interpolation, as previously described[7-9] (**Figure 2**).

1. Evaluate the replicates; sample replicates with a difference ≥1 Ct must be discarded and re-evaluated in a second experiment.
2. Calculate the median Ct for each sample and consider samples with a Ct value ≤ 36.
3. Evaluate the specificity of the PCR products using melting analysis.

4. Evaluate the concentration of each amplicon by interpolation with the standard curve; obtain a concentration value (ng/µL) for each amplicon.

## Representative Results

The total free DNA concentration was quantifiable by spectrophotometry for all samples analyzed, showing a range of between 1.51 and 138 ng/µL. Five control samples were used for reproducibility of the data: two independent real-time experiments were performed for *c-MYC*, *HER2*, *BCAS1*, *AR*, and *STOX1*. The coefficients of variation (CV) were then calculated for each gene (**Table 2**), with a good degree of reproducibility between the two independent experiments (**Table 2**).

The 125-bp *STOX1* sequence was analyzed to exclude the presence of PCR inhibitors. If the samples showed an amplification of *STOX1*, the UCF DNA integrity test was performed. A lack of amplification meant that DNA quantity was not sufficient to perform the UCF DNA integrity test, indicating the need to repeat the analysis with a new urine collection. As there is little information available about the amplification or deletion of STOX1 in bladder and prostate cancer, this gene could be used as a control sequence for these tumor types. Finally, the UCF DNA integrity evaluation was performed by adding together the values obtained for the three oncogenes (**Figure 3**).

The use of the sum of *c-MYC*, *HER2*, and *BCAS1* genes has been proposed for bladder cancer detection[9], while *c-MYC*, *AR*, and *HER2* have been suggested for prostate cancer[7,8]. The best cut-off values identified for bladder and prostate cancer detection are 0.1 ng/µL and 0.04 ng/µL, respectively. Using these cut-off values, a sensitivity of 73% and a specificity of 84% were obtained in detecting bladder cancer versus symptomatic individuals, while 58% sensitivity and 44% specificity were observed in detecting prostate cancer versus patients with benign urogenital tract diseases[7-9]. In conclusion, the UCF DNA integrity test is flexible, so the genes used in the present study could be substituted with other long sequences of interest, depending on the disease.

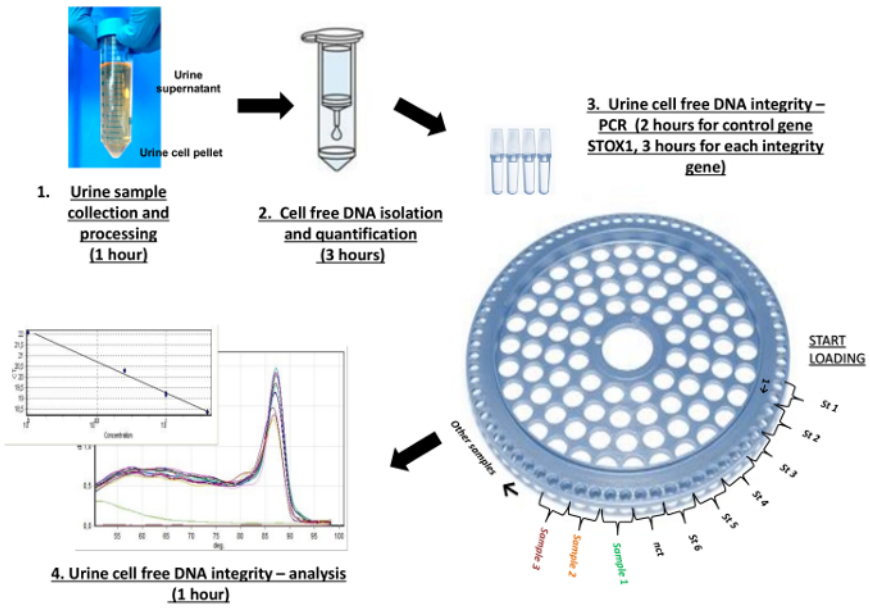


**Figure 1. Urine Cell-free DNA Integrity Workflow and Timeline.** The workflow of the method is divided into different steps and times. Please click here to view a larger version of this figure.

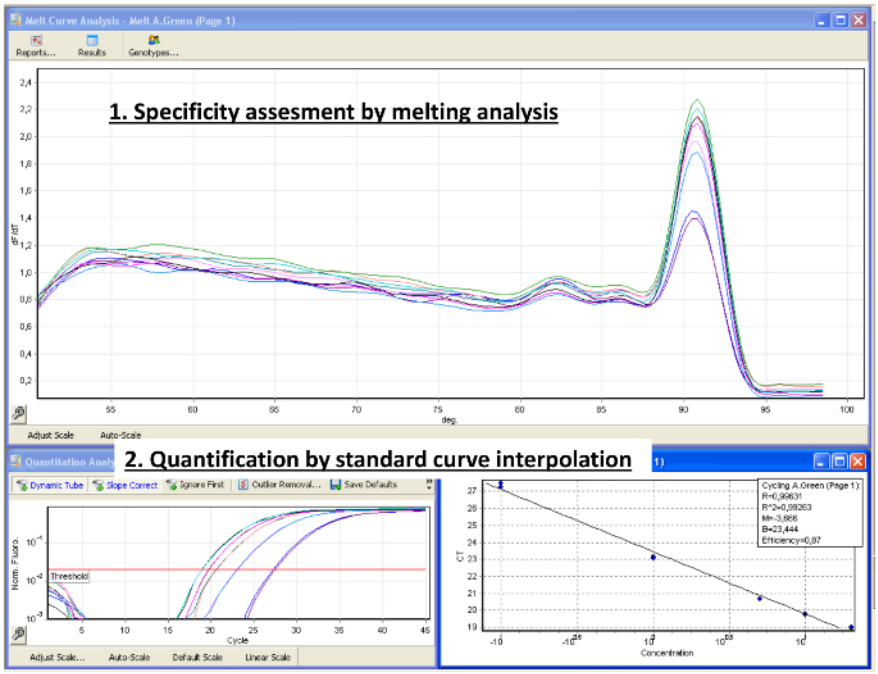


**Figure 2. Report for the *c-MYC* Amplicon Analysis.** An example of the melting analysis, amplification plot, and standard curve are reported for the *c-MYC* evaluation. Please click here to view a larger version of this figure.

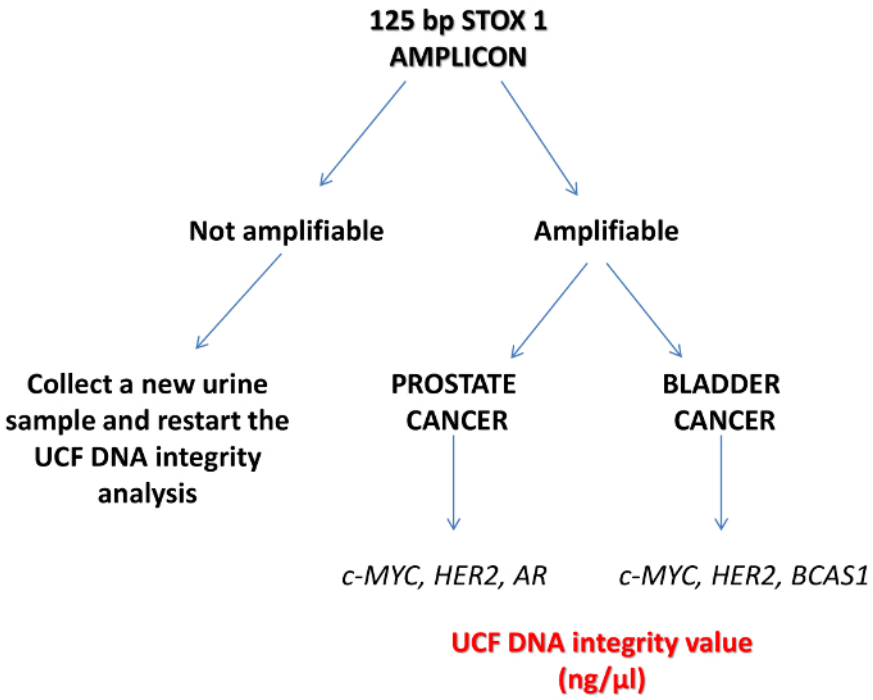


**Figure 3. UCF DNA Integrity Analysis Workflow.** A simple workflow for the UCF DNA integrity analysis is reported. Please click here to view a larger version of this figure.

| Gene | Reference sequence | Primer forward | Primer reverse | Amplicon length (bp) | Real time protocol |
|---|---|---|---|---|---|
| ***MYC (c-MYC)*** (V-Myc Avian Myelocytomatosis Viral Oncogene Homolog , locus 8q24.21) | NG_007161.1 | TGGAGTAGGG ACCGCATATC | CCCAACACCA CGTCCTAAC | **264** | 95 °C for 3 minutes followed by 45 cycles at 94 °C for 40 seconds, 56 °C for 40 seconds and 72 °C for 1 minute. |
| ***ERBB2 (HER2)*** (Erb-B2 Receptor Tyrosine Kinase 2, locus 17q12.1) | NG_007503.1 | CCAGGGTGTT CCTCAGTTGT | TCAGTATGGC CTCACCCTTC | 295 | |
| ***BCAS1*** (Breast Carcinoma Amplified Sequence 1, locus 20q13.2) | NC_000020 | GGGTCAGAGC TTCCTGTGAG | CGTTGTCCTG AAACAGAGCA | 266 | |
| ***AR*** (Androgen Receptor, locus Xq12***)*** | NG_009014.2 | AGCCCAGGTT CTCTCCTGAT | TGGCTAGTC CTCAGCTT | 265 | |
| ***STOX1*** (Storkhead Box1, locus 10q21.3) | NG_012975.1 | GAAAACAGG GCAGCAAGAAG | CAGACAGCAT GGAGGTGAGA | 125 | 95 °C for 90 seconds followed by 45 cycles at 94 °C for 40 seconds and 54 °C for 1 minute. |

**Table 1. Primer Sequences and Assay Conditions.**

| Sample | HER2 | | CV (%) | BCAS1 | | CV (%) | c-MYC | | CV (%) | AR | | CV (%) | STOX1 | | CV (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Replicate 1* | Replicate 2* | | Replicate 1* | Replicate 2* | | Replicate 1* | Replicate 2* | | Replicate 1* | Replicate 2* | | Replicate 1* | Replicate 2* | |
| **1** | **0.0** | **0.0** | **3.5** | **0.1** | **0.1** | **3.4** | **0.1** | **0.1** | **14.0** | **0.6** | **0.5** | **29.1** | **0.6** | **0.8** | 4.5 |
| **2** | 2.6 | 2.7 | 2.3 | 0.0 | 0.0 | 0.0 | 0.9 | 1.5 | 28.6 | 0.1 | 0.1 | 22.0 | 2.6 | 3.0 | 6.1 |
| **3** | 0.4 | 0.2 | 17.0 | 0.1 | 0.1 | 5.9 | 2.6 | 3.2 | 11.6 | 0.0 | 0.0 | 0.0 | 0.7 | 1.2 | 23.1 |
| **4** | 0.0 | 0.0 | NE | 1.1 | 1.0 | 3.0 | NE | 0.0 | NE | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | NE |
| **5** | 2.5 | 3.1 | 9.8 | 0.1 | 0.1 | 14.0 | NE | 0.0 | NE | 0.1 | 0.0 | 27.1 | 0.0 | 0.0 | NE |

**Table 2. Real-time PCR Reproducibility for Each Gene.**
*results reported as ng/µL
CV, coefficient of variation; NE, not evaluable

| Genes for UCF DNA integrity | Disease | Cancer Patients (n) | Controls (n) | Cut off (ng/µL) | Sensitivity rate (95% CI) | Specificity rate (95% CI) | Reference |
|---|---|---|---|---|---|---|---|
| *MYC HER2 BCAS1* | Bladder cancer | 52 | 46 symptomatic individuals 32 healthy individuals | 0.1 | 0.73 (0.61–0.85) | 0.83 (0.72–0.94) | Casadio V *et al.* 2013 [9] |
| *MYC HER2 BCAS1* | Prostate cancer | 29 | 25 healthy individuals | 0.04 | 0.79 (.62–0.90) | 0.84 (0.65–0.94) | Casadio V *et al.* 2013[8] |
| *c-MYC AR BCAS1* | Prostate cancer | 67 | 64 patients with benign diseases of the urogenital tract | 0.04 | 0.58 (0.46–0.73) | 0.44 (0.30–0.58) | Salvi S *et al.* 2015[7] |

**Table 3. Summary of the Results Obtained for the Early Diagnosis of Prostate and Bladder Cancers.**

## Discussion

UCF DNA integrity analysis is a new, non-invasive method for assessing DNA integrity in urine. It was recently proposed for the early diagnosis of bladder[9] and prostate cancers[7,8]. A number of advantages and disadvantages of the UCF DNA integrity test are discussed here, together with future prospects.

The main advantage of the approach is that it offers an inexpensive, non-invasive method and a simple protocol to study urine as a potential source of biomarkers, requiring only a basic knowledge of molecular biology techniques. The test is quick to perform, and the results, available

after 2 work days (**Fig. 1**), can easily be interpreted without the aid of a physician. Consisting of only DNA isolation processes and 2 real-time PCRs, the approach also has a good cost-benefit ratio. In terms of accuracy, UCF DNA integrity has high sensitivity (73%) and specificity (84%) in detecting bladder cancer in symptomatic patients[9]. Finally, the method is flexible, and the proposed genes can easily be substituted with other genes of interest, as long as they are longer than 250 bp.

The test also has a number of limitations. First, the DNA spectrophotometric quantification method is often imprecise and could be replaced with other, more accurate, fluorometric approaches (*e.g.,* qubit or picogreen). The DNA quality is also rather poor, as demonstrated by the frequently-low 260/280 and 260/230 ratios. Furthermore, in one of our studies, very low specificity (44%)was observed in prostate cancer patients versus patients with benign diseases of the urogenital tract[7], which was probably a result of benign inflammatory necrotic cells releasing more intact DNA into the circulation. This is a critical issue, because both prostate cancer patients and individuals with benign diseases may have an inflammatory component in their urinary cells. Thus, within the context of early diagnosis of prostate cancer, the results from a UCF DNA integrity analysis could be misleading.

UCF DNA integrity was evaluated in 314 urine samples from patients with prostate or bladder cancer, healthy and symptomatic individuals, and patients with benign diseases of the urogenital tract. A prospective study on a larger case series is needed to better define the role of this approach as an early diagnostic marker for urogenital tract cancers.

Although little has been published on the subject of UCF DNA as a source of biomarkers for cancer, interest in this area is increasing. Recently, Togneri *et al.*[4] published an interesting paper in which cell-free DNA extracted from the urine supernatant of bladder cancer patients showed a higher tumor genome burden than that of cellular DNA isolated from the urine pellet, suggesting that the study of the cell-free fraction of DNA in urine could be useful to characterize urological cancers.

In our experience, the UCF DNA integrity test did not prove to be a good early diagnostic test for prostate cancer. Conversely, it showed potential as a marker for the early detection of bladder cancer when used in combination with conventional urine cytology. A confirmatory study on a larger prospective case series is currently being planned.

## Disclosures

The authors declare no competing financial interests.

## Acknowledgements

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