Journal of Visualized Experiments

Cell-free DNA integrity analysis in urine samples

--Manuscript Draft--

Manuscript Number:	JoVE55049R2
Full Title:	Cell-free DNA integrity analysis in urine samples
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	urine; cell-free DNA; integrity; Bladder; prostate; cancer
Manuscript Classifications:	3.12.758.409: Genital Neoplasms, Male; 3.12.758.409.750: Prostatic Neoplasms; 3.12.758.820: Urologic Neoplasms; 3.12.777.829: Urinary Bladder Diseases; 5.5.200.880: Molecular Diagnostic Techniques; 5.5.91: Biological Assay
Corresponding Author:	Casadio Valentina IRST Meldola Meldola, Forli Cesena (FC) ITALY
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	valentina.casadio@irst.emr.it
Corresponding Author's Institution:	IRST Meldola
Corresponding Author's Secondary Institution:	
First Author:	Casadio Valentina
First Author Secondary Information:	
Other Authors:	Samanta Salvi
	Filippo Martignano
	Roberta Gunelli
	Sara Ravaioli
	Daniele Calistri
Order of Authors Secondary Information:	
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 hypothesis 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 have frequently an increased DNA copy number in bladder and prostate cancer were chosen for the analysis, but the method is flexible and the 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.
Author Comments:	
Additional Information:	
Question	**Response**

If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	

TITLE:
Cell-Free DNA Integrity Analysis in Urine Samples

AUTHOR:
Valentina Casadio[1], Samanta Salvi[1], Filippo Martignano[1], Roberta Gunelli[2], Sara Ravaioli[1] and Daniele Calistri[1]

AFFILIATION:
[1]Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Via P. Maroncelli 40, Meldola 47014, Italy. [2]Department of Urology, Morgagni-Pierantoni Hospital, Forli, Italy.

CORRESPONDING AUTHOR:
Valentina Casadio valentina.casadio@irst.emr.it

KEYWORDS:
Urine; cell-free DNA; integrity; bladder; prostate; cancer

SHORT ABSTRACT:
A method for analyzing DNA integrity in the cell-free supernatant fraction of urine samples is proposed. The method is suitable for early detection of urological malignancies and has proven accurate for the early diagnosis of bladder cancer.

LONG 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 mL of urine needed to carry out the analysis.

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: The protocol consisted in isolating DNA from urine samples to perform a UCF DNA integrity analysis. A cell line was used to construct standards. DNA extraction, DNA quality and quantity control (spectrophotometer and real-time PCR for the control gene, *STOX1*), and real-time PCR for specific oncogenes were performed (Fig. 1).

1) Urine collection and processing

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

1.2) Maintain the urine at 4 °C for a maximum of 3 h and send it to the laboratory at the same temperature.

1.3) Mix each sample by inverting it twice immediately upon arrival in the laboratory and transfer into two 50-mL conical-bottomed polypropylene tubes.

1.4) Centrifuge the tubes at 850 x g for 10 min at 4 °C or at room temperature.

1.5) 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.

1.6) 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:

2.1) Thaw one aliquot of the urine supernatant at room temperature.

2.2) 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.

2.3) Add 100 µL of proteinase k directly to the sample.

2.4) Add 1 mL of AL buffer to the sample and mix well by pipetting.

2.5) Close the tubes and incubate the samples at 56 °C for 15 min.

2.6) 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).

2.7) Bring the samples back to room temperature and add 1 mL of absolute ethanol. Mix fully by pipetting.

2.8) 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.

2.9) 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).

2.10) Add 500 µL of buffer AW1 without wetting the rim of the column. Centrifuge it at 6,000 x *g* for 1 min.

2.11) Discard the tube containing the flow-through and replace it with a new, clean collection tube.

2.12) 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.

2.13) Discard the tube containing the flow-through and replace it with a new, clean collection tube.

2.14) Repeat step 2.12 to remove any residual washing buffer.

2.15) 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.

2.16) Centrifuge it at 8,000 x *g* for 1 min.

2.17) 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

3.1) 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.

3.2) 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.

3.3) 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

4.1) Thaw the primers (the concentration depends on the type of assay), green supermix, cell-line DNA standards, and UCF DNA diluted samples on ice.

4.2) 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.

4.3) 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.

4.4) Aliquot 15 µL of the mix into each well. Do not pipette **or** spin the tubes.

4.5) 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] (Fig. 2).

5.1) Evaluate the replicates; sample replicates with a difference ≥1 Ct must be discarded and re-evaluated in a second experiment.

5.2) Calculate the median Ct for each sample and consider samples with a Ct value ≤36.

5.3) Evaluate the specificity of the PCR products **using** melting analysis.

5.4) 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 **(Fig. 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 AND TABLE LEGENDS:
Figure 1. Urine cell-free DNA integrity workflow and timeline. The workflow of the method is divided into different steps and times.

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.

Figure 3. UCF DNA integrity analysis workflow. A simple workflow for the UCF DNA integrity analysis is reported.

Table 1. Primer sequences and assay conditions.

Table 2. Real-time PCR reproducibility for each gene.

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.

ACKNOWLEDGEMENTS:

The authors thank Gráinne Tierney and Silvia Bellissimo for their editorial assistance.

DISCLOSURES:

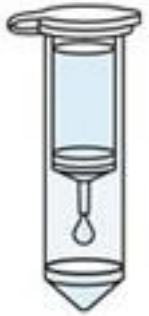
The authors declare no competing financial interests.

REFERENCES:

1. Francis, G. & Stein, S. Circulating Cell-Free Tumour DNA in the Management of Cancer. *Int.J.Mol.Sci.* **16** (6), 14122-14142, doi:10.3390/ijms160614122 [doi] (2015).
2. Bryzgunova, O.E. & Laktionov, P.P. Extracellular Nucleic Acids in Urine: Sources, Structure, Diagnostic Potential. *Acta Naturae.* **7** (3), 48-54 (2015).
3. Szarvas, T., *et al*. Deletion analysis of tumor and urinary DNA to detect bladder cancer: urine supernatant versus urine sediment. *Oncol.Rep.* **18** (2),405-409, DOI: 10.3892/or.18.2.405 [doi] (2007).
4. Togneri, F.S., *et al*. Genomic complexity of urothelial bladder cancer revealed in urinary cfDNA. *Eur.J.Hum.Genet.*, doi:10.1038/ejhg.2015.281 [doi] (2016).
5. Zonta, E., Nizard, P. & Taly, V. Assessment of DNA Integrity, Applications for Cancer Research. *Adv.Clin.Chem.* **70**, 197-246, doi:10.1016/bs.acc.2015.03.002 [doi] (2015).
6. Hao, T.B., *et al*. Circulating cell-free DNA in serum as a biomarker for diagnosis and prognostic prediction of colorectal cancer. *Br.J.Cancer.* **111** (8), 1482-1489, doi:10.1038/bjc.2014.470 [doi] (2014).
7. Salvi, S., *et al*. Urine Cell-Free DNA Integrity Analysis for Early Detection of Prostate Cancer Patients. *Dis.Markers.* **2015**, 574120, doi:10.1155/2015/574120 [doi] (2015).
8. Casadio, V., *et al*. Urine cell-free DNA integrity as a marker for early prostate cancer diagnosis: a pilot study. *Biomed.Res.Int.* **2013**, 270457, doi:10.1155/2013/270457 [doi] (2013).
9. Casadio, V., *et al*. Urine cell-free DNA integrity as a marker for early bladder cancer diagnosis: preliminary data. *Urol.Oncol.* **31** (8), 1744-1750, doi:10.1016/j.urolonc.2012.07.013 [doi] (2013).
10. Salvi, S., *et al*. Circulating cell-free AR and CYP17A1 copy number variations may associate with outcome of metastatic castration-resistant prostate cancer patients treated with abiraterone.*Br.J.Cancer.* **112** (10), 1717-1724, doi:10.1038/bjc.2015.128 [doi] (2015).
11. Ishkanian, A.S., *et al*. High-resolution array CGH identifies novel regions of genomic alteration in intermediate-risk prostate cancer. *Prostate.* **69** (10), 1091-1100, doi:10.1002/pros.20959 [doi] (2009).
12. Oxley, J.D., Winkler, M.H., Gillatt, D.A. & Peat, D.S. Her-2/neu oncogene amplification in clinically localised prostate cancer. *J.Clin.Pathol.* **55** (2), 118-120 (2002).
13. Nord, H., *et al*. Focal amplifications are associated with high grade and recurrences in stage Ta bladder carcinoma. *Int.J.Cancer.* **126** (6), 1390-1402, doi:10.1002/ijc.24954 [doi] (2010).
14. Tabach, Y., *et al*. Amplification of the 20q chromosomal arm occurs early in tumorigenic transformation and may initiate cancer. *PLoS One.* **6** (1), e14632, doi:10.1371/journal.pone.0014632 [doi] (2011).

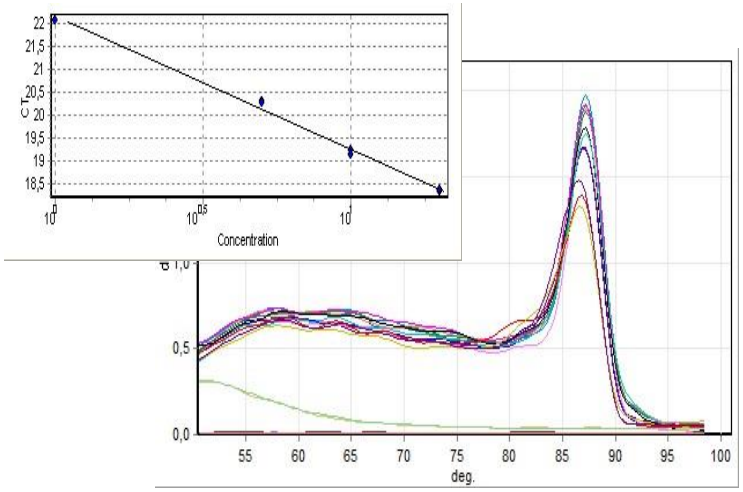
Fig. 1

1. **Urine sample collection and processing (1 hour)**

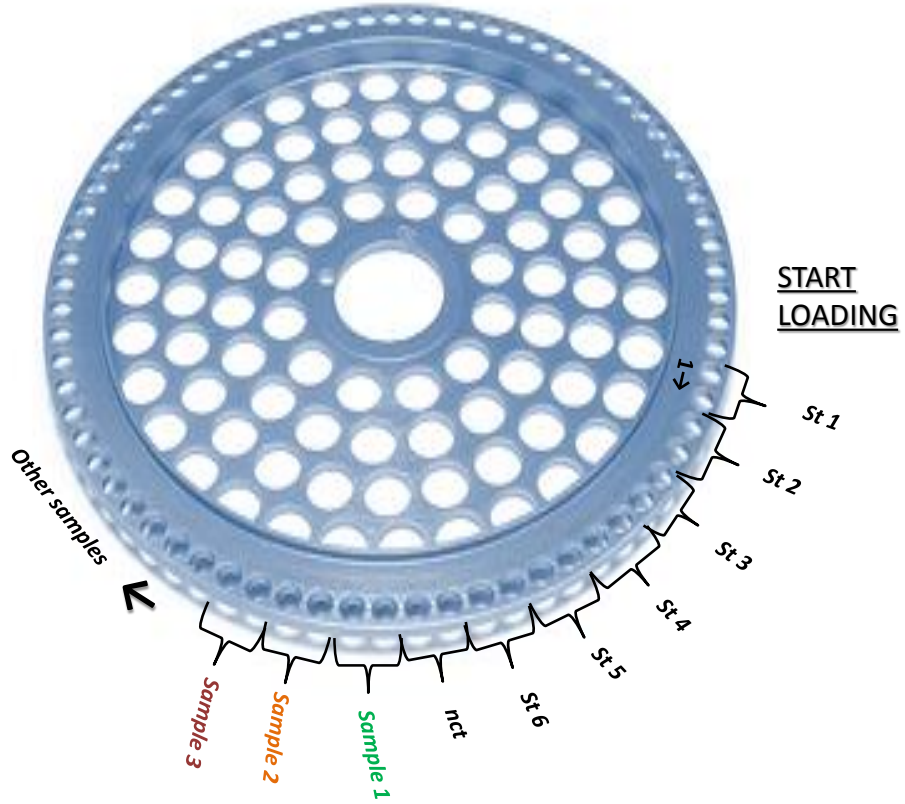


2. Cell free DNA isolation and quantification (3 hours)

3. Urine cell free DNA integrity – PCR (2 hours for control gene STOX1, 3 hours for each integrity gene)



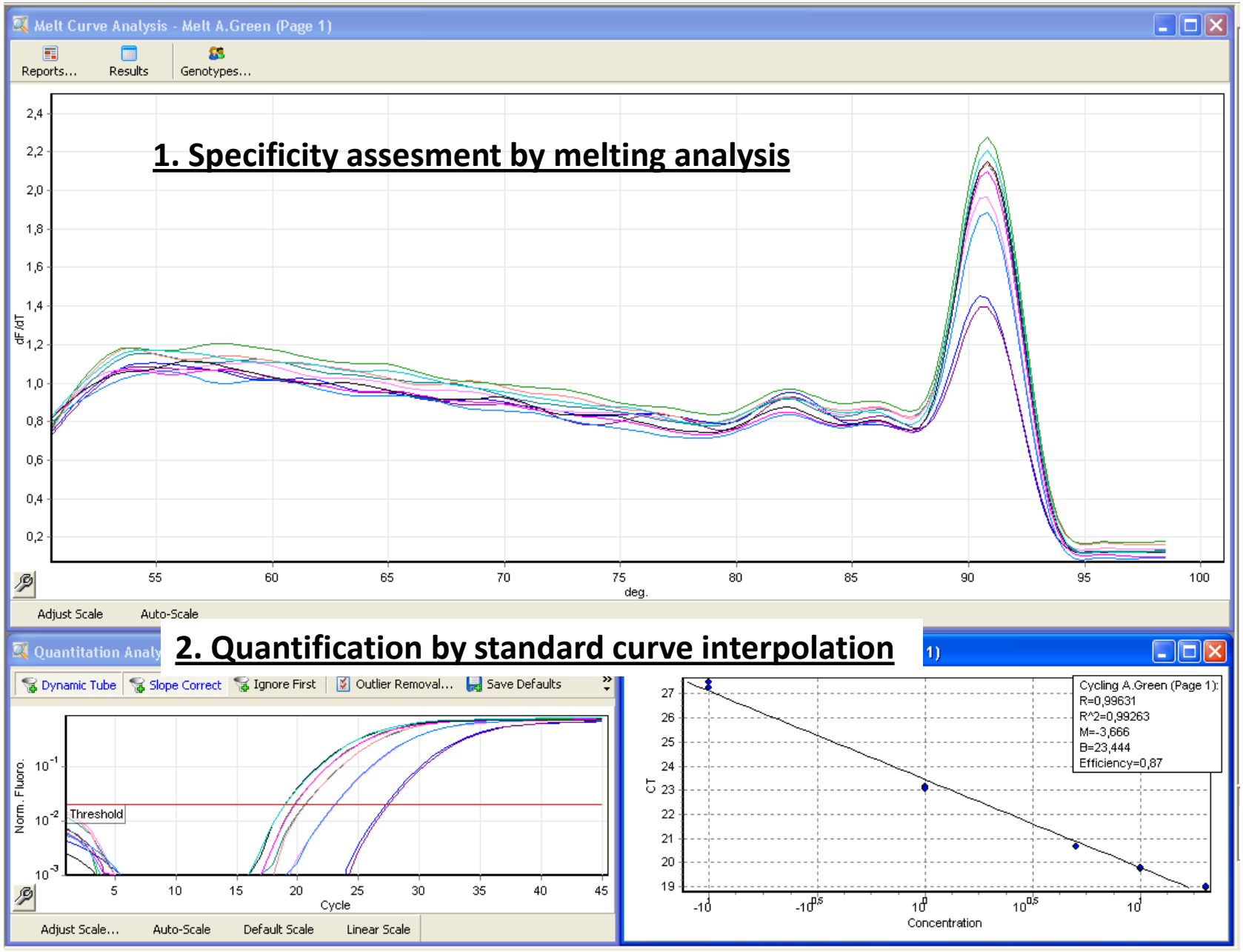
4. Urine cell free DNA integrity – analysis (1 hour)



Figure

Click here to download Figure Fig. 2 Casadio.pptx

Fig. 2



Figure

Click here to download Figure Fig. 3 Casadio.pptx

Fig. 3

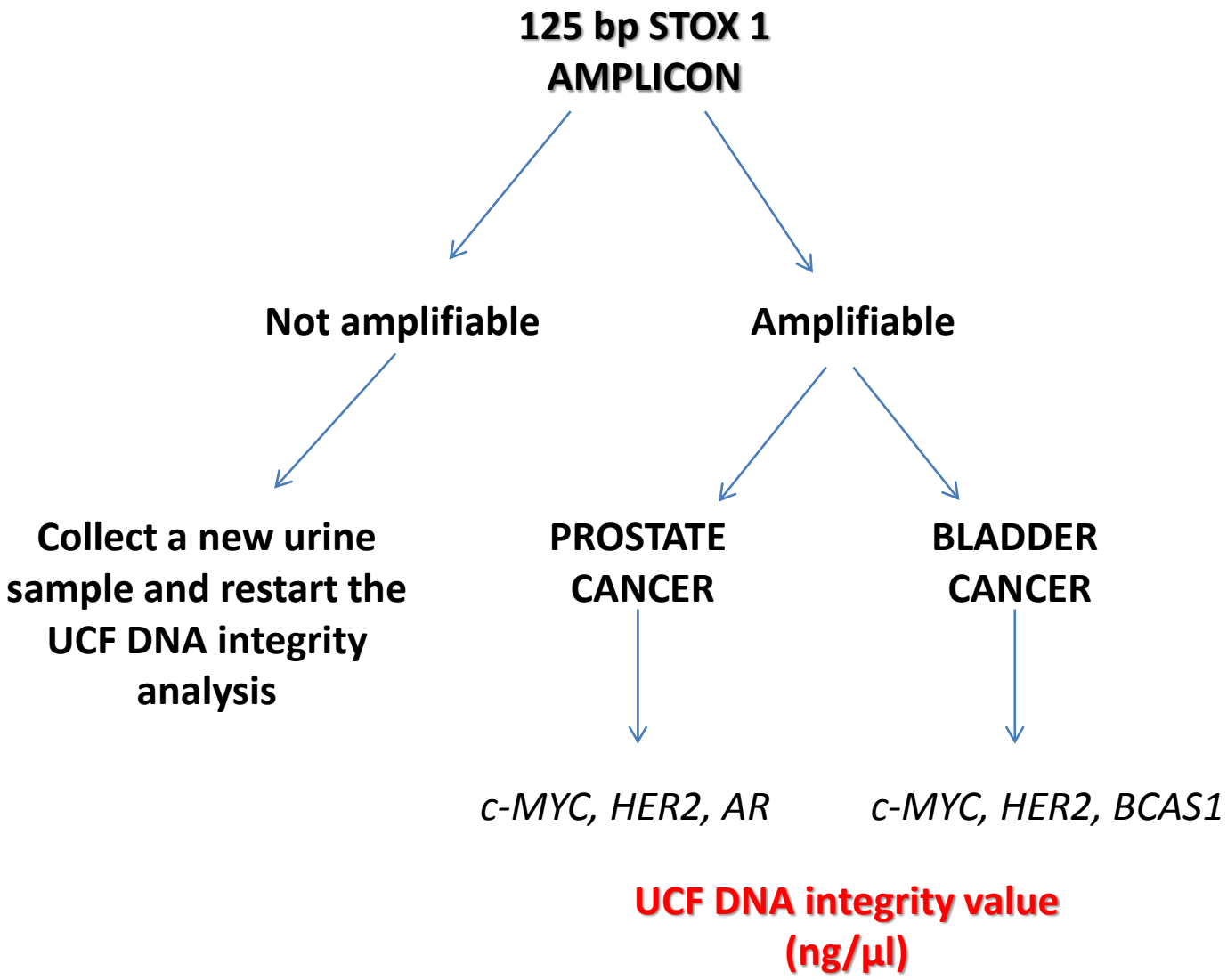


Table 1. Primer sequences and assay conditions

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	TGGAGTAGGGACCGCATATC	CCCAACACCACGTCCTAAC	**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	CCAGGGTGTTCCTCAGTTGT	TCAGTATGGCCTCACCCTTC	295	
BCAS1 (Breast Carcinoma Amplified Sequence 1, locus 20q13.2)	NC_000020	GGGTCAGAGCTTCCTGTGAG	CGTTGTCCTGAAACAGAGCA	266	
AR (Androgen Receptor, locus Xq12***)***	NG_009014.2	AGCCCAGGTTCTCTCCTGAT	TGGCTAGTCCTCAGCTT	265	
STOX1 (Storkhead Box1, locus 10q21.3)	NG_012975.1	GAAAACAGGGCAGCAAGAAG	CAGACAGCATGGAGGTGAGA	125	95°C for 90 seconds followed by 45 cycles at 94°C for 40 seconds and 54°C for 1 minute

Table

Table 2. Real time PCR reproducibility for each gene

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

***results reported as ng/µl**

CV, coefficient of variation; NE, not evaluable

Table. 3 Summary of the results obtained for the early diagnosis of prostate and bladder cancer

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]

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
QIAamp DNA Mini Kit	Qiagen	51304	
iQ SYBR Green Supermix, 100 x 50 µl rxns, 2.5 ml (2 x 1.25 ml)	Biorad	1708880	
IDT custom DNA oligos	IDT		HPLC purification, 100nMole DNA oligo
NanoDrop 1000 Spectrophotometer	Thermo Scientific		Other spectrophotometric methods could also be used to quantify DNA
Rotor-Gene 6000	Corbett		Another Real Time PCR instrument could also be used
microcentrifuge			
one centrifuge for 50 ml tubes			
incubator			
-80°C freezer			
-20°C freezer			
10 ul pipette			
20 ul pipette			
200 ul pipette			
1000 ul pipette			
pipette tips (10;20;200;1000)			
1,5 ml tubes			
50ml tubes			
15 ml tubes			
Rotor-Disc 72 Rotor	Corbett	9018899	
Strip Tubes and Caps, 0.1 ml (250)	Qiagen	981103	
Collection Tubes (2 ml)	Qiagen	19201	
Buffer AL (264 ml)	Qiagen	19075	

Proteinase K (10ml)	Qiagen	19133

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: CELL - FREE DNA INTEGRITY ANALYSIS IN URINE SAMPLES

Author(s): VALENTINA CASADIO, SAYANTA SAU, FILIPPO MARTIGNANI, ROBERTA GUNELLI, SARA RAVAIOLI, DANIELE CALISTRI

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: ☒ Standard Access ☐ Open Access

Item 2 (check one box):

☒ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove
JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	VALENTINA CASADIO
Department:	BIOSCIENCES LABORATORY
Institution:	IRCCS IRST
Article Title:	CELL-FREE DNA INTEGRITY ANALYSIS IN URINE SAMPLES
Signature:	
Date:	27/5/2016

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

…08.2016

Jaydev Upponi, PhD
Science Editor, *JoVE*

Dear Dr. Upponi,

Re: JoVE55049R1
Cell-free DNA integrity analysis in urine samples by Valentina Casadio et al.

We are submitting the above manuscript, fully revised in accordance with the reviewers' suggestions, for further evaluation. As requested, we enclose a point-by-point reply to all the comments made by the reviewers and have marked all the changes made to the text in bold.
We look forward to hearing from you.
Yours sincerely,

Valentina Casadio, PhD
Corresponding Author

Editorial comments:

•Formatting:

-Reference citations – Some superscript numbers are bold and in italics – please correct this.

Reply: We have corrected reference citations throughout the text.

-Ethics statement should not be formatted as a note, just a line without a bullet number. Please also use 12 pt font size.

Reply: We have corrected the ethics statement (line 76).

-1.4 – ", better, at 4°C" – if there is a preferred option, that should come first, and the second should be an alternative. If there's a reason one is preferred over the other, that can appear in a note below the step.

Reply: We have corrected the text as suggested (line 88).

-Please include spaces between numbers and units.

Reply: We have checked and included spaces between all numbers and units.

-Please format table citations as "Table 1", etc. and figure citations as "Fig. 1" etc.

Reply: This has been done.

-Please use the American standard of punctuation, with periods instead of commas as decimal points (see Table 2).

Reply: This has been done.

-Line 265 – “Togneri et al” requires a superscript reference citation.

Reply: This has been done, now line 274.

•Grammar:

-Please copyedit the manuscript for numerous grammatical errors, some of which are indicated below. Such editing is required prior to acceptance and should be performed by a native English speaker.

-Line 21 – “it proved to be most accurate” – please rephrase in a more objective tone

-Line 32 – “Real Time PCRs on four sequences”

-Line 37 – “this open the way”

-Line 39 – “only few millililiters”

-Line 54 – “it.The”

-Line 66 – “One of the main goal of this method is that it is flexible”

-Section 2 heading – “and cell line”

-2.2, 2.7 – Please correct the run-on sentence.

-2.9 – “all of the sample mixture are used”

-3.1 – Should be “spectrophotometer”.

-3.2 – “diluition”

-Please do not use the personal pronoun “he”.

-Line 213 – “The best cut-off” – this implies there are other cutoff values.

-Line 223 – “reported divided”

-Line 237 – “The first advantage is the possibility of studying urine as a source of biomarkers is non-invasive method and the protocol is simple” – please re-write and clarify

Reply: The errors and queries have been addressed and rectified.

•Additional detail is required:

-1.3 – How are samples mixed?

Reply: They are mixed by inverting the sample twice. We have added this information to the text (line 85).

-1.5 – What constitutes the upper part? Can an approximate volume be given? Is there a clear delineation between the upper and lower parts?

Reply: The upper part to be transferred is constituted by a volume of 10 ml (two 5 ml vials obtained). There is no clear delineation between the upper and lower parts and we thus suggest leaving at least two ml above the cell pellet. This information has been added to the text (lines 90-91).

-Section 5 – Please provide a citation containing the details of the analysis.

Reply: We have added three citations (nos. 7-9) in which the analysis is described in detail.

•Branding: Please remove trademark symbols from the materials table.

Reply: Trademark symbols have been removed.

•Results:

-Please provide a graph or table to support the claims in the last paragraph of the results section, in addition to a citation if this has been previously published.

Reply: We have added a new table (Table3) to summarize the results obtained in the three different papers published by our group (the 3 references were already present in the original version of the paper (nos. 7-9).

-Table 2 – If two replicates are being evaluated, please label the columns with replicate 1 and replicate 2.

Reply: This has been done.

•Discussion: Please discuss the critical steps of the protocol as well as any troubleshooting/modifications that can be performed.

Reply: In our opinion we have already discussed the critical steps of the protocol in the Discussion. Does the editor need some specific clarifications?

•If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as “Re-print with permission from (reference#)” or “Modified from..” etc. And please send a copy of the re-print permission for JoVE’s record keeping purposes.

•JoVE reference format requires that the DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

Reply: We were not able to find the DOIs for 2 references (nos. 2 and 12).

•IMPORTANT: Please copy-edit the entire manuscript for any grammatical errors you may find. The text should be in American-English only. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol and the manuscript. Please thoroughly review the language and grammar prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

Reply: The paper has been thoroughly reviewed for its English language content by a native English speaker.

Reviewer #1:

Major Concerns:

-At the beginning of the protocol, clarify the starting material and the main steps of the experiment. For example you could list: urine samples (aim: UCF DNA integrity analysis), cell line (standards), DNA extraction, DNA Quality and quantity control (spectrophotometer and Real-time PCR for a control gene-STOX1), Real-time PCR for specific oncogenes.

From this experimental plan I guess you need to perform two Real-time PCR (one for the control gene and one for oncogenes).

Reply: As requested, we have added a brief summary of the protocol at the beginning of the section (page 2 lines 71-74).

Why did you say three Real-time PCR (page 5, line 242)? Please, clarify.

Reply: This was, in fact, an error on our part. We have substituted "three" with "two" (page 6, line 254).

-In Representative results section: specify at the beginning that you performed an experiment starting from 5 samples (how many bladder or prostate cancer samples?)

Reply: The experiment on 5 control samples was only made to test assay reproducibility. We have clarified this concept in the revised version of the manuscript (page 5, lines 208-210).

Clarify Table 2: did you performed tho Real-time PCR wiyh the same 5 samples, so did you replicate the experiment?

Reply: Yes, we replicated the experiment on the same 5 samples. We have emphasized this concept in the text (page 5, lines 208-210)

In Table 2 what the values of the sencond and third columns are? Ct values obtained from two indipendent experiments?

Reply: The values of the second and third columns are expressed as ng/µl and they were obtained in two independent experiments. We have added this information to Table 2.

-In Discussion section: page 6 lines 259-262

Specify the data separately for prostate and bladder cancer and cite the literature.

Reply: In the sentences in question, we were only referring to our study on prostate cancer. The text has been modified to make this clear (page 6, lines 261-267).

-page 6, Line 272: I don't think that at this point of the research you can infer to use UCF DNA alone

Reply: We agree with the referee and have modified the sentence in question (page 6 line 280).

Moreover:

-Page 2, line 79: I suggest to refer to one sample, if not you can get confused. Mix samples...each sample or mix all the samples together (this hypothesis does not make sense, but to avoid misunderstanding, it is better if you speak about one sample. You can declare at the beginning of the protocol that you can perform the analysis starting from several samples

Reply: We agree with the referee and have modified the text accordingly (page 2, line 85).

-In Line 150 (Page 4) you say RT-PCR (Real time or Reverse trinscriptase? Please declare the abbreviation. Obviously it is a Real time PCR starting from DNA, performed to verify gene copy number, but if you are used to perform RT-Real time PCR for gene expression analysis, you can get confused for a while.

Reply: We have substituted "RT" with "Real Time".

In regard of this point, it should be better explained the expression "regions known to be frequently amplified in solid tumors" (Abstract and page 2, lines 62-63): is it a DNA amplification? Please clarify or at least cite literature such as Schwab, "Oncogene amplification in solid tumors"; 1999, where there is a good definition: "The term gene amplification refers to the selective increase of the gene copy number and is better designated as DNA amplification. It should not be confused with elevated gene expression". In this way, since the beginning of the article, it would be clear that you refer to Real-time PCR from DNA, to verify gene copy numbers.

Reply: We were, in fact, referring to DNA amplification. We have substituted the sentences in question with "regions known to have an increased DNA copy number" in order to avoid confusion with gene expression (page 2, lines63-64).

-Page 4 , line 178 and Table of Materials:

you say that the analysis can be performed using another PCR real-time instrument. Can you say the same for DNA extraction kit or Real-time PCR kit? Did you perform experiments with other kit and chose these ones?

Reply: We only tested the DNA extraction kit and Real Time instrument mentioned in the Table of Materials . However, we imagine that other kits or instruments would achieve the same results.

-Table 1: indicate Reference sequence for each gene (e.g NG_007503.1 for HER2) on which you have designed the primers, the complete gene name and use the official gene symbol (*e.g.* ERBB2, not HER2)

Reply: We have revised Table 1, adding the complete gene name and official gene symbol (first column) and the reference sequence (second column).

Minor Concerns:

-Both in the abstract and in the text declare the abbreviation of urine cell-free DNA (UCF DNA) the first time you use this expression and than always use the abbreviation.

Reply: This has been done.

-Page 2, line 62 and 67: genomic regions

Reply: This has been done.

-Page 5, lines 237-238-239 Check English

Reply: This has been done.

-Check formatting of the bibliography

Reply: This has been done.

-Check formatting of Table of Materials

Reply: This has been done.

-Check double spaces in the text

Reply: This has been done.

Reviewer #2:

Major Concerns:

-The authors have previously described this procedure in three other research articles, as they correctly reported in the paper (PMID 23141783, PMID23509700, PMID26412928) focusing on prostate cancer samples and bladder (PMID23141783).

-They enlarge the cohort to 314 urine samples, composed by prostate and bladder cancer patients, healthy and symptomatic individuals with benign disease of urogenital tract. Therefore, it is not clear how many samples are new and how many came from previous published results (cited before and in the references), probably a table (or supplementary table) could help the reader.

Reply: We agree with the referee that this was not clear. All the results came from our previously published papers. We have added a new table (Table 3) in which we provide details of the studies.

-STOX1 amplification or not, was suggested as control,the authors wrote "STOX1 is a gene that is not amplified or deleted in prostate and bladder cancer…". If they are sure they could use a reference, or I suggest to modify the sentence in "STOX1 is a gene that is rarely amplified or deleted in prostate and bladder cancer…" or "Amplification or deletion of STOX1 gene has not yet been reported in literature"

Reply: We have substituted the sentence in question with "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" (page 5 lines 217-218).

Minor Concerns:

Typographical errors:

-The abstract 13th line lack of a "T".

Reply: This has been corrected.

-Line 266 (page 6) please insert citation followed Togneri et al.

Reply: This has been done (ref. no. 4).