

# Evaluation of extracted circulating cell free dna concentration by standard nucleospin plasma xs (NS) kit protocol compared to its modified protocol

## Abstract

**Background:** One of the earliest and most common processes for cell-free DNA analysis is its extraction. Our study was to analyze cell-free plasma DNA of prostate cancer (PCA) and of benign prostatic hyperplasia (BPH) patients.

**Methods:** We extracted circulating cell-free DNA (ccfDNA) from 24 patients' plasma samples (14 PCA; 10 BPH) by NucleoSpin plasma XS (NS) Kit using the standard kit protocol alongside with a second protocol with some modifications. Subsequently, NanoDrop, PCR and Real-time-PCR were used to evaluate the concentrations of the obtained DNA, respectively.

**Results:** Concentrations of the extracted cell free DNA with our modified protocol was significantly higher compared to the same DNA samples which were extracted with the company protocol (p value<0.001).

**Conclusion:** We could increase the rate of cell free DNA recovery to a much significant amount which is beneficial for investigators who need to evaluate gene expression in blood rather than not accessible organs and tissues. We recommend the modified protocol for NucleoSpin plasma XS (NS) Kit while the original protocol could not result in appropriate recovery of blood cell free DNA.

**Keywords:** circulating cell-free dna, prostate cancer, modified protocol

Volume 4 Issue 4 - 2017

Maryam Khani,<sup>1</sup> Farkhondeh Pouresmaeili,<sup>2</sup>  
Reza Mirfakhraie<sup>1</sup>

<sup>1</sup>Department of Medical Genetics, Shahid Beheshti University of Medical Sciences, Iran

<sup>2</sup>Infertility and Reproductive Health Research Center (IRHRC) and Department of Medical Genetics, Shahid Beheshti University of Medical Sciences, Iran

**Correspondence:** Farkhondeh Pouresmaeili, Infertility and Reproductive Health Research Center (IRHRC) and Department of Medical Genetics, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran, Email pouresfar@gmail.com

**Received:** March 18, 2017 | **Published:** April 11, 2017

## Introduction

To exclude invasive procedures like organ and tissue biopsy to detect changes associated with the disease in clinical approaches and in amniocentesis for prenatal diagnosis of genetic conditions, replacement of non-invasive methods like the use of peripheral blood samples for its content of circulating cell free DNA from fetal source or originating from dead tumor cells, is recommended by previous investigators. Standardization and strengthening easier, more accessible, reliable, low-cost and non-invasive methods meet the needs of the global economy. So far, different kits with manual or semi-automated or fully-automated protocol are used for extracting cell-free-DNA such as QIAamp Circulating Nucleic Acid (Qia), PME free-circulating DNA Extraction Kit (PME), Maxwell RSC ccfDNA Plasma Kit (RSC), EpiQuick Circulating Cell-Free DNA Isolation Kit (EQ), NEXTprep-Mag cfDNA Isolation Kit (NpM<sub>V1/2</sub>), Quick-cfDNA™ Serum & Plasma Kit, NucleoSpin plasma XS (NS).

The QiAamp kit is often used for cell free DNA extract, but it is necessary to have a vacuum device to perform the operation.<sup>1</sup> Nucleospin (NS) is a much cheaper and easy to use kit which is commonly used by postgraduate students. So, to analyze two genes of prostate cancer for the purpose of our study, we used NS kit for extracting cell free DNA from plasma. The amount of circulating DNA in blood serum is very low and different in people based on their health condition. For example, concentrations in healthy subjects is 20-30 ng/ml and in men with metastatic prostate cancer is close to 40 ng/ml and almost 1200 ng/ml in patients with other types of

cancer.<sup>2</sup> However, when NanoDrop, PCR and Real-time-PCR were used to evaluate the concentrations of cell free DNA, we noticed that the extracts did not meet our expectation as was written in the kit manual. In order to avoid losing time and not to stop the project, we tried to change the kit protocol to achieve the desirable concentration. Thus, the purpose of the present case report was to provide a new protocol for NS kit which makes it more effective to carry out projects similar to ours.

## Case presentation

In our study we needed to extract cell free DNA from plasma samples taken from patients with prostate cancer, benign prostatic hyperplasia and healthy men. According to NS kit protocol to eliminate contamination with DNA blood cells, we took the blood samples without cell lysis in EDTA tubes (6ml) and transported on ice from hospital to laboratory in 2 hours. Samples were centrifuged at 3000 rpm for 15 min at 4°C. The upper part, the plasma, was isolated from each blood sample and stored at -20°C until use. The cell free DNA was extracted according to the kit manual.<sup>3</sup> Unfortunately, NanoDrop, PCR and Real-time-PCR showed no visible materials of extracted cell free DNA in the Elute (Table1). Since it was not possible to change the kit for the study, either due to more financial burden or time consuming, we asked NS-company to help us in achieving the expected result. For this purpose, reduction in the elution volume in the kit manual steps 6 and 7, or increase the eluted volume in PCR reactions was necessary, as they recommended. The results were unsatisfactory. Thus we changed numerous steps of the protocol to see whether we

could get the right concentration. According to our instruction, we warmed the buffers before test to receive a better dilution. To provide a replacement for the vacuum condition of QiaGen, we waited for 10 to 15 minutes before each centrifugation, increased the time of spin, and reduced the Relative Centrifugal Force (RCF). The results of the new protocol are presented in Tables 1,2 and in Figures 1,2.

**Table 1** Comparison between standard protocols with modified protocol at a glance

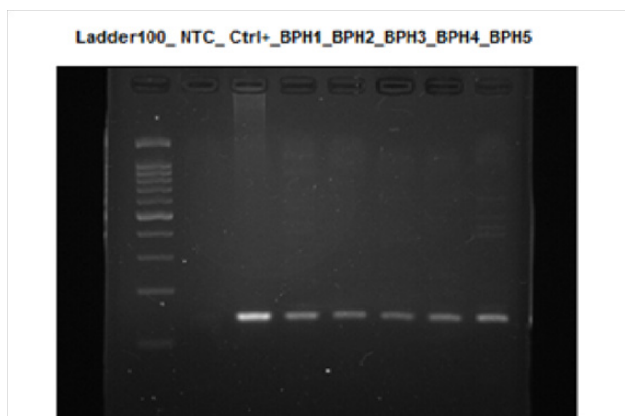
Our Protocol with Modifications	Original Protocol	Steps
-Warm buffers up to 40 °C 30min before use (water bath), then shake. -Warm EB up to 60 °C 10 min before use. -Molten plasma 8000xg for 3min -warm up 250 µl upper Clear Plasma to 40 °C for use	Use up to 240 µl plasma	1. Prepare sample
-Add 25 µl Proteinase K -Mix -Incubate at 40 °C for 20 min	-Add 20 µl Proteinase K -Mix -Incubate at 37 °C for 10 min	1a. Optional: Proteinase K treatment
Add 360 µl BB	Add 360 µl BB	2. Adjust binding conditions
-Invert tube 3X -Vortex 3s -Spin down briefly	-Invert tube 3X -Vortex 3s -Spin down briefly	3. Mix sample
-Wait 15 min -Load lysate -1500xg 1 min -7000xg 30s -Wait 10 min	-Load lysate -2000xg 30s -11000xg 5s	4. Bind DNA
-1 <sup>st</sup> Wash 500 µl WB -Wait 10 min -7000xg 1 min  -2 <sup>nd</sup> Wash 250 µl WB -Wait 10 min -7000xg 5 min	-1 <sup>st</sup> Wash 500 µl WB -11000xg 30s  -2 <sup>nd</sup> Wash 250 µl WB -11000xg 3 min	5. Wash and dry silica membrane
-25 µl EB -Wait 15 min -16000xg 1.5min	-20 µl EB -11000xg 30s	6. Elute DNA
-80 °C 12min -Vaporize all EB -Add 15-20 µl TEB or Aquapura	-90 °C 8min	7. Removal of residual ethanol

BB: Binding Buffer; WB: Wash Buffer; EB: Elution Buffer; TEB: Tris-EDTA Buffer

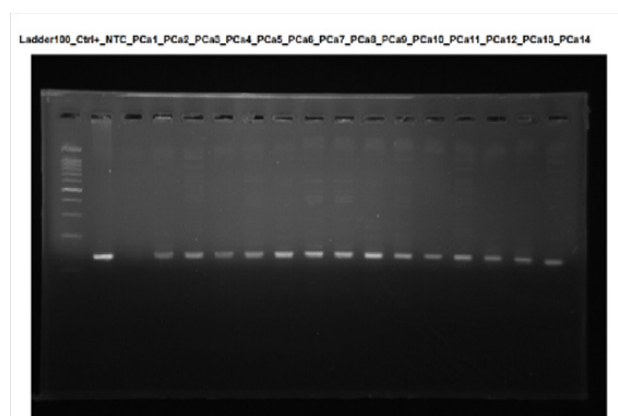
**Table 2** Results of concentration quality between standard protocol and modified protocol

Results				Samples
Real Time PCR	PCR	Nano Drop (Concentration)		
No pick (5-6 µl)*	No band(5-6 µl)*	7-13 ng/µl	Standard protocol	PCa I- 14
CT: 20-30 (2µl)*	Band (2µl)*	60-110 ng/µl	Modify protocol	
No pick (5-6 µl *	No band (5-6 µl)*	7-10 ng/µl	Standard protocol	BPHI - 10
CT:25-30 (2µl)*	Band (2µl)* (Figure 1)	45-95 ng/µl	Modify protocol	

PCa: Prostate Cancer; BPH: Benign Prostatic Hyperplasia; CT: Cycle Threshold; \*: Used Elution Volume in PCR reaction (20µl total volume)



**Figure 1** Quality demonstration of the extracted samples on 2% agarose gel by modified protocol. BPH: benign prostatic hyperplasia, NTC: Negative control, Ctrl+: lymphocyte DNA, BPH1-5: extracted cell free DNA from 5 samples.



**Figure 2** Quality demonstration of the extracted samples on 2% agarose gel by modified protocol. PCA: prostate cancer, NTC: Negative control, Ctrl+: lymphocyte DNA, PCA1-14: extracted cell free DNA from 14 samples.

## Results

The first data is related to the concentrations of the standard protocol and the modified protocol using Kolmogorov-Smirnov normality test. The test indicated that the data were normally distributed. To compare

the average concentration of both standard and modified protocols, we used Paired-T-test (Table 3). The test results showed that the mean concentration of the modified protocol was significantly higher than the standard protocol (70.7, P value<0.001).

**Table 3** Results of the extraction protocol comparison by Paired-T-test

P-Value	Mean Difference ±SD	SD	Mean of Concentration	Number of Samples	
<0.001	70.7±17.71	3.07	9.71	24	Standard protocol
		19.93	80.42	24	Modified protocol

SD: Standard Deviation

## Discussion

The NS kit is often used for extracting cell-free-DNA from EDTA exposed plasma serum, and bronchial lavage in a range of 50-1000bp. However in our study it did not work with the proposed protocol in the user's manual. Therefore, we made several changes in the entire protocol through which we could get the satisfactory results. Previous researches have shown that the cell free DNA amounts in plasma depends on each individual and his/her health condition such as the kind of disease, cancer or pregnancy. The content of DNA in prostate cancer patients' plasma is less than other cancers. So, a qualified kit should contain fresh and effective reagents besides a well written protocol to provide high amounts and pure expected materials from the samples. However since the kit was effective when the protocol was changed, the only thing that needs to be changed in this kit is the guide.

We could increase the original concentration without decreasing the volume. This article specifically describes a possible method of increasing the rate of cell free DNA recovery as applicable to NucleoSpin plasma XS (NS) Kit. The modified protocol may prove a value to other researchers facing similar cfdDNA recovery yield problems although this is a single center experience report and its applicability needs to be replicated. We recommend the

investigators to follow our protocol for NS kits instead of the original kit to experience the advantageous results of our protocol. There are questions arising from our achievements for future studies. Can these modifications be performed on buffers of other extraction kits where the same separation steps and filtration are applied? The impact of these changes can be examined for cell free DNA studies when using blood samples and other extraction kits.

## Acknowledgment

We present our sincere thanks to Dr Saeed Zarein-Dolab (English Language Department, SBMU) for reading the manuscript and Dr Znoollah Azargashb (Social Medicine Department, SBMU) for his precise statistical advice.

## References

1. [http://www.calpoly.edu/~bio/ubl/protocols\\_files/qiaalu.pdf](http://www.calpoly.edu/~bio/ubl/protocols_files/qiaalu.pdf)
2. Rouprêt M, Hupertan V, Catto JW, et al. Promoter hypermethylation in circulating blood cells identifies prostate cancer progression. *Int J Cancer*. 2008;122(4):952–956 .
3. [http://www.mn-net.com/portals/8/attachments/redakteure\\_bio/protocols/genomic%20dna/um\\_circdnaplasm\\_nxsx.pdf](http://www.mn-net.com/portals/8/attachments/redakteure_bio/protocols/genomic%20dna/um_circdnaplasm_nxsx.pdf)