



# COMPARING METHODS FOR ISOLATION OF CIRCULATING-DNA (C-DNA). IMPLICATION OF C-DNA AS BIOMARKER IN THE CANCER

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**ABSTRACT: Background:** *In the last ten years, researchers actively studied cell-free nucleic acids present in plasma or serum with great expectations as potential biomarkers for cancer and other pathologic conditions. In the present manuscript the main findings related to the principal characteristics of C-DNA and some methodological considerations on sample collection and extraction as well as on some innovative assay methods have been summarized.*

**Materials and Methods:** *We compares different commercial kits for C-DNA isolation based on two criteria: A) procedure for capturing DNA by column impacted with patented Polymers so called Cartridge based-DNA isolation; and B) procedure based on a simple digestion of the lysate cell mixture digest by proteinase K and subsequent precipitation of DNA salt.*

*The measure of the best methods was based on the quantification of DNA integrity, amplifying the human Telomerase Reverse Transcriptase (hTERT) in a subset of C-DNA of healthy donors.*

**Results:** *The assays differs significantly as regard to the target genes (hTERT) but shares on the use of a common forward primer and reverse primers delimitating amplicons.*

**Conclusions:** *Recent reports on the importance of circulating nucleic acids in the intercellular exchange of genetic information between eukaryotic cells have been reviewed.*

**KEY WORDS:** *Cell-free DNA, plasmatic DNA, cancer circulating DNA, DNA biomarker*

## INTRODUCTION

All body fluids potentially containing circulating cell free Nucleic acids as lymphatic fluid, liquor, ascites, milk, urine, stool and bronchial-alveolar lavage).

Circulating-DNA (C-DNA) have been firstly identified in 1948<sup>1</sup>. Only 30 years later, in 1977, Leon et al<sup>2</sup> found C-DNA in plasma of patients affected by lung cancer.

The accounted hypotheses on the origin of C-DNA are supposed to derive from cells dead for necrosis or

apoptosis<sup>3</sup>, but some authors reported the possibility of an active release from cells<sup>4,5</sup>. The presence of DNA and RNA circulating freely in the blood stream of healthy subjects can be related to activated lymphocytes and to the lysis of other nucleated cells or to their active secretion<sup>6</sup>. In patients affected by neoplastic diseases it is supposed that normal and cancer cell can: i) detaches from the tumor mass and undergoes necrosis or apoptosis; and alternatively ii) they can actively release nucleic acids in the blood flow<sup>7,8</sup>.

The quantity of circulating DNA is generally very low in healthy subjects (less than 5 ng /ml of



plasma), while it increases (5 to 10 times) when considering subjects affected by a neoplastic disease<sup>9-12</sup> as well as in some physiologic conditions, such as pregnancy<sup>13,14</sup>. In physiologic conditions as well as in benign pathologies, C-DNA seems to derive almost entirely from apoptosis of circulating cells. C-DNA is double stranded and in the form of nucleoproteic complex. Studies on fetal DNA, reported an half life of 16.3 min for C-DNA<sup>15</sup> that can be reasonably extended to circulating DNA in general as confirmed by experiments conducted by injecting purified DNA into the blood stream of animals<sup>16</sup>. Moreover, the pattern of circulating DNA was found to be similar to that of apoptotic cells when analyzed by gel electrophoresis<sup>17,18</sup> and by sequencing reactions<sup>19,21</sup>. Nevertheless also high molecular weight DNA fragments have been retrieved and their presence was associated to the necrosis of tumor cells<sup>22</sup>. Results in this field are often in disagreement. Notwithstanding all the previous reports regarding the genetic analysis of C-DNA showed that high molecular weight fragments do not contain tumor specific mutations which on the contrary are frequent in small DNA circulating fragments, probably deriving from phagocitated necrotic cells<sup>23</sup>.

Differences in the nature of C-DNA may be an interesting pathological indicator, suggesting that in some pathologies, measurement of DNA integrity may provide a simple and inexpensive method for cancer detection<sup>3</sup>.

As regard to the biological significance of C-DNA, Garcia-Olmo and co-workers<sup>24</sup> suggested that metastases might develop as a result of transfection of susceptible cells in distant target organs with dominant oncogenes that circulate in the plasma and are derived from the primary tumor<sup>25</sup>.

Analyzing the results currently present in the literature, discordant results can be attributed to several factors, but the source of discrepancies can be the use of plasma or serum as the starting material. Several anticoagulants (EDTA, heparin etc.) have been used and, within the same biological fluids, the variability depend upon the variation in protocol for sample processing (i.e. sample collection and nucleic acid extraction procedure)<sup>26</sup>.

In particular, little is known about blood sample processing and especially about the delay in separating the plasma or serum, variation in centrifugation steps and duration and conditions of storage. On the contrary all the studies reported the extraction method used so that the results could be read in the light of the different procedure utilized. Last but not least, every original article implies a different technique for detecting the C-DNAs, some being quantitatively oriented, whereas others evaluate only the qualitative aspects.

Moreover, even the quantitative real time methods differs in the choice of the template gene and consequently in the assay characteristics.

Thus the mechanism and the nature of nucleic acids circulating in serum and plasma has not been clarified yet, notwithstanding many articles reported biomarkers validations among C-DNAs. Recently, comparison of C-DNAs with genomic DNA by next-generation sequencing<sup>21</sup> suggests that non specific DNA release is not the sole origin for C-DNAs and that apoptotic genomic DNA is the major but not the sole source of C-DNAs in apparently healthy individuals. The authors did not find a circulating DNA pool consisting purely of unspecific apoptotic or necrotic nuclear DNA, failing in evidencing classes of sequences differing from circulating and genomic DNA, apart for the detection of over-represented Alu sequences in serum DNA<sup>21</sup>.

The apparent higher content of DNA in serum relatively to plasma can be explained by the clotting process of blood cells that can cause nucleic acid release. This is the reason why plasma seems more convenient and adequate to the study of C-DNAs, since its analysis avoids the simultaneous testing of material originally associated to cells<sup>28</sup>.

## MATERIALS AND METHODS

### Sample collection

It was reported that both a delay in blood processing and storage temperature can influence the amount of DNA extracted from plasma<sup>27</sup>. Anticoagulants do not influence the quantity of the recovered DNA from plasma, but EDTA shows a stabilizing effect on blood during the time between sample drawn and processing, both at room temperature and at 4°C.

In order to get rid of contaminating DNA deriving from cells, both filtration<sup>29</sup> and repeated centrifugations at low and high speed were reported<sup>30,31</sup>, demonstrating that no release of circulating nucleic acid was induced from blood cells even at maximum centrifugation speed<sup>33</sup>. As regard to the stability of C-DNAs in the frozen samples, some authors showed that plasma can be conserved frozen for years (at least 2 for RNA and 6 for DNA)<sup>34,35</sup> at -70 or -20°C without affecting C-DNA concentration, while other authors reported a decay of 30% in DNA from stored plasma<sup>36</sup>.

Plasma sample collection procedure in our lab was adopted as following:

- a) Samples are collected in EDTA-containing tubes;
- b) The tube arrive within 1 hour from blood draw. They are submitted to a first centrifugation step at 1600 g, 4 °C for 10 minutes for Plasma recovering;

- c) A second centrifugation is performed at maximum speed, at 4°C, for 10 minutes. Pellets eventually formed in this step will be discarded. Plasma will be divided in one- extraction-volume aliquots (500  $\mu$ l) in 1,5mL tubes and stored at -80°C until extracted.

### **Circulating DNA extraction**

Extraction method is an important issue to be addressed in the field of C-DNA, for which there is no agreement in literature and several protocols have been reported<sup>37</sup>.

The isolation of C-DNA from plasma and serum represent a challenge, due to their small quantity and fragmented nature<sup>38</sup>.

As recent study<sup>39</sup> we compares different commercial kits based on two criteria:

**Cartridge based-DNA isolation:** this methods use a procedure for capturing DNA by column impacted with patented Polymers. Commercial kits used in this issue includes: QIAamp DNA Mini Blood Kit (Qiagen, Hilden, Germany) AgencourtGenfind Blood and Serum GenomicDNA IsolationKit (Agencourt Bioscience Corporation), QIAamp Virus Spin Kit (Qiagen), Invitrogen ChargeSwitch gDNA 1 mL Serum Kit (Invitrogen, Milan Italy), NucleoSpin Plasma XS Kit by Macherey-Nagel (Duren, Germany), NucliSpin Circulating DNA (Diachem, Naples, Italy) reaching the conclusion that the QIAamp Virus Spin kit gives the best yields from serum and NucliSpin Circulating DNA from plasma.

**Salt precipitation:** is a procedure based on a simple digestion of the lysate cell mixture digest by proteinase K and subsequent precipitation of DNA salt using propanol and potassium acetate. Commercial kits used in this issue includes: Wizard Genomic DNA purification Kit (Promega, Milan, Italy).

Other authors reported the superiority of the NucleoSpin Plasma XS Kit by Macherey-Nagel in terms of DNA yield, purity, and retrieval of small DNA fragments with respect to the QIAamp DNA Blood Mini Kit (QIAGEN), which is widely used by investigators in the field of C-DNA<sup>38</sup>.

In a report on circulating fetal DNA in maternal plasma<sup>40</sup> the NucliSens Magnetic Extraction system (BioMérieux) and the QIAamp DSP Virus Kit (Qiagen) are compared with QIAamp DNA Blood Mini Kit (QIAGEN). Both methods were assessed to improve total DNA yield. QIAamp DSP Virus Kit provided the best results for fetal versus total DNA, as it preferentially extracts small size DNA fragments.

In our experience the comparison between the two most widely used platforms (*Cartridge based-DNA isolation vs Salt precipitation*) confirmed this findings as explained in the section dedicated to the Results “DNA integrity index”.

### **DNA quantity**

Quantification of DNA was measured by detection of hTERT (Telomerase Reverse Transcriptase) gene, using instruction of manufacturer AMPLI Set Quant DNA Real time (Diachem, Naples Italy). The locus amplified span from 13059-13156 (geneBank AF128893), and amplicon size was 98bp.

### **Statistical Data**

Analysis of coefficient of variation (CV), calculated on the slope values from daily standard curves, were used to evaluate intra-assay and inter-assay reproducibility. The slope of standard curves has been used to determine the efficiency of target amplification using the equation  $E=10e^{-1/\text{slope}}$ . In theory this slope should not be lower than  $-3.3$  because this implies a PCR efficiency of more than 1 ( $> 100\%$ ) and indicates that more than twice as many amplicons are being made per PCR cycle.

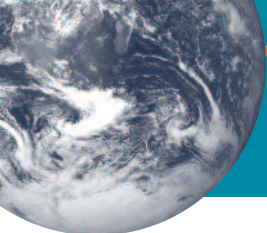
Analysis of variance (ANOVA) was calculated to study variability within the CV% data and expressed as Fisher’s exact test

## **RESULTS**

The need to correctly quantify C-DNA was evident when significant differences between cancer patients and the control population had been evidenced<sup>41,42</sup>. Total plasma DNA concentration is now considered as an unspecific biomarker, but easily identifiable in neoplastic patients as well as in some benign diseases. Initial methods ranged from diphenylamine staining<sup>43</sup>, Dipstick Kit<sup>44</sup>, counter immuno-electrophoresis<sup>45</sup> spectrophotometric measurement<sup>46</sup>, picogreen assay<sup>47</sup> and real time quantitative PCR<sup>48</sup>. Fleischhacker M. and co-workers reported in their extensive review<sup>37</sup>, as well as in the web site [www.cnaps.net](http://www.cnaps.net), a table summarizing the updated findings on different population of cancer patients. A general observation is related to the lack of correlation between plasma DNA concentration and tumor size, stage or location. An increased plasma concentration can be observed at an early stage of tumor development<sup>49</sup> as well as at the first trimester of pregnancy<sup>50</sup>.

### **DNA integrity**

DNA integrity is a parameter of interest for plasma CF DNA since it could be used to extrapolate the origin of circulating DNA. As stated in the introduction, the dimension of DNA fragments are related to the mechanism of release from the cells. This aspect can represent an useful parameter also



in the view to clarify this problem. Few papers compared DNA integrity index of cancer patients with those of healthy subjects generally leading to the common conclusion that circulating DNA from cancer patients poses higher integrity index than in the control population<sup>51-57</sup>. On the other hand, no difference was found between benign and malignant tumors<sup>58,59</sup>. The design of the assays differs significantly as regard to the target genes (ALU repeats, hTERT) but shares the common principle for assay design based on the use of a common forward primer and reverse primers delimitating amplicons of increasing dimension. The ratio between the quantity of the long versus the short amplicon represents the so called integrity index.

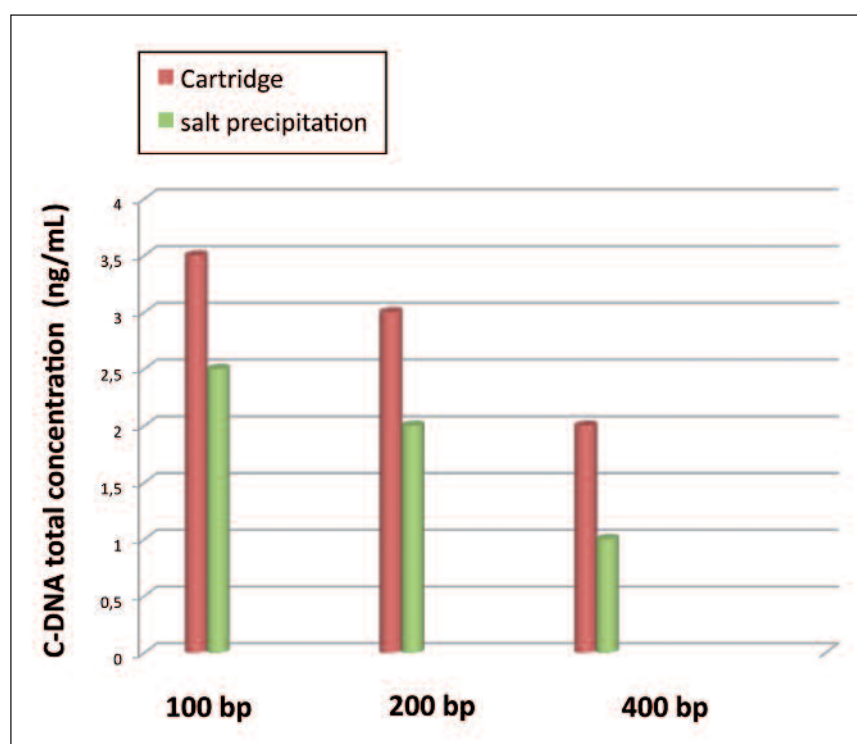
We built up an assay based on the hTERT gene detection by real time-PCR designing 4 different assays with comparable efficiency for the amplification of a 98 bp (for simplification cited as 100), 204 bp (cited 200), 306 bp and 416 bp (cited as 400) amplicons, respectively (unpublished data). This procedure allowed us to compare three integrity index (200/98, 300/98 and 400/98) to be used for the investigation of the circulating DNA integrity. Just to compare the extraction procedure we considered only healthy subjects whose plasma samples were submitted to the two different extraction procedures (*Cartridge based-DNA isolation vs Salt precipitation*). The results are reported in Figure 1, where it can be evidenced that plasma total DNA concentration varies on the basis of the

amplicon length. The two platforms differs in all 3 fragments size, clearly much more when fragment between 100 and 400 bp are considered (Figure 1).

## DISCUSSION

Most reports related to the diagnostic and prognostic applications of C-DNA are relative to neoplastic diseases. Nevertheless, the increase of circulating C-DNA is not a specific marker for malignant conditions. Plasma DNA levels are increased also in other physiological status and diseases, for example pregnancy and trauma. The detection in plasma/serum DNA of oncogene mutations, often encountered in a wide variety of cancer tissues, provides concrete evidence that circulating nucleic acids are released into the circulation by tumors<sup>41</sup>. Thus, methods for the detection of tumour-specific DNA variants have been developed.

The main limitation to the molecular diagnosis of solid tumors is the frequent need for invasive procedures to obtain adequate testing materials<sup>60</sup>. So if from a theoretical point of view, C-DNAs are well accepted as non-invasive markers readily available in any stage of the disease or at any time in the follow up period, their low concentration in plasma limits their use and represents a way of selection for the methods to be use for detection and quantization. During the last 10 years there was a growing



**Figure 1.** Cell-free DNA concentration in plasma from healthy donors (n=50). On the basis of the amplicon size used by the real-time PCR methods, it was possible to calculate the amount of cell-free DNA (ng/ml of plasma) of DNA fragments of dimension comprised in the following ranges: i) 1-98 bp (cited as 100); ii) 98-204 bp (cited as 200) and iii) 204-416 (cited as 500). Measurement of DNA concentration were performed following two different type of extraction procedure: Cartridge (light columns) and salt precipitation (Dark columns).

interest in C-DNA as demonstrated by the increasing number of publications. The analytical requirements of the methods used to study circulating DNA are so demanding for a strict specificity and high sensitivity that the ideal method is still far to be found. Obviously many attempts are ongoing in designing new methods able to overcome the sensitivity limitation of conventional PCR and sequencing methods, but the number of publications already available in the field is large so that representative paper will be chosen for discussion<sup>61</sup>. The mutation analysis to be performed on DNA extracted from plasma was related to the detection of variants in the K-ras oncogene. In fact, it was chosen as a tumor specific marker since it shows a very high mutation frequency. K-Ras mutations are precocious events in neoplastic development starting from codons 12, 13 and 61 and detectable with high specificity and sensitivity in patients affected by colorectal cancer<sup>62,63</sup>. It was reported an 83% correspondence between the results found in plasma with those obtained from tissues<sup>33</sup> thus bringing to the definition of plasma as a surrogate sample for tumour tissue. Moreover, some patients showed mutated DNA in the compartment also in the absence of an evident neoplastic disease and this could represent a risk factor for tumor development<sup>64</sup>. K-ras DNA in plasma was studied also for the diagnosis of other cancers. The results did not show any significant relationship with the development of non-small cell lung cancer (NSCLC), failing to confirm K-Ras as a marker of tumor presence in this tumor type<sup>65</sup>. On the contrary, K-Ras mutations were detected in patients with pancreatic cancer 5-14 months before tumor diagnosis and were absent in patients with benign pancreas pathologies and in healthy subjects<sup>66</sup>.

As a signature of tumour-derived DNA, mutations in oncogenes and tumor suppressor genes can be used as tumor biomarkers. Besides K-Ras mutation, the detection of common p53 mutations in plasma of smokers without cancer but not in the plasma of non-smokers indicated that the presence of p53 mutations in plasma DNA could reflect the exposure to carcinogens and, hence, the chance of developing lung cancer<sup>67</sup>. Other indicators of the presence of tumor-deriving DNA have been adopted such as microsatellite alterations<sup>68,70</sup>, viral DNA, hypermethylation of tumour suppressor gene<sup>71</sup>.

## CONCLUSIONS

We are optimistic that circulating nucleic acid analysis would become an important tool for the clinical management of cancer patients in the near future and that it could be used successfully for prenatal diagnosis even if, despite a great interest

in this field and ample possibilities, results are contradictory and confusing<sup>73</sup>.

Notwithstanding the lack of a precise knowledge on the origin and function of C-DNAs, many researchers are actively working on the discovery of sensitive and specific markers in plasma/serum based on the detection of DNA variants or anomalous RNA expression able to evidence the presence of pathological conditions, especially in neoplastic diseases and pregnancy. The involvement in studies related to C-DNAs derives from the conviction they represent a non invasive tool for disease screening and for the early detection of pathological conditions. It has to be taken in mind that, as often happens, new analytical principles, innovative methods with extraordinary sensitivity and specificity features, preanalytical suitable procedures and standardized protocols for sample collection and analysis are needed. A deeper understanding of the biology of C-DNAs can derive firstly from an advance in the methodological and technological support to the study of these molecules and secondarily to find medical expertise to interpret genomic results<sup>74</sup>.

## CONFLICT OF INTERESTS:

The Authors declare that they have no conflict of interests.

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