# A Rapid Direct Fluorescent Assay for Quantification of Circulating Cell-free DNA vs. Circulating Tumour DNA Analysis Review

# Introduction

Cancer, or malignant tumour, is a disease category that is defined by abnormal cell growth and their unusual spreading. In recent years, several advances were presented in investigating and understanding molecular and cellular mechanisms that cause tumor development, as well as necessary biomarkers that help to identify pre-cancerous and cancerous states. As one of the possible biomarkers could serve fragmented DNA that circulates in the cell free part of whole blood. This circulating cell-free DNA (cfDNA), found in blood plasma and serum is, therefore, being researched in many medical areas.

Even though cancer is in some cases deadly disease, in other cases it can be treatable. The success of a treatment depends highly on early detection and the possibility of monitoring treatment responses. Lately, circulating tumour DNA (ctDNA), derived from tumours, has been investigated as a potential biomarker for breast cancer (1–3), gastric cancer (4,5), non-small cell lung cancer (6–8), melanoma (9), and colorectal cancer (10,11). However, ctDNA investigation is being challenged for three main reasons, which include: low levels of ctDNA even in cancer patients; the difficulty of distinction between ctDNA and normal cfDNA; the mutation quantification accuracy of ctDNA (12). The added challenge can be the cost of ctDNA analysis and longer time that is needed for such diagnosis.

The step involved in ctDNA analysis is always ctDNA extraction before ctDNA can be analysed using various methods (genotyping, SNP detection, etc.). These methods differ in cost and time effectiveness. The widely used real-time or quantitative polymerase chain reaction (qPCR), which measures PCR amplification as it appears in real time, is considered as highly reliable, fast and high-throughput (13,14). However, in the case of cancer and the need of early diagnosis such method can be inaccessible in the required time or unaffordable. Therefore, there is a need of fast, cheap and accessible method that would serve as a prognostic tool.

A rapid direct fluorescent assay for quantification of cfDNA (15–17) seems as a potential diagnostic method, which can be used in various medical settings that do require specialised genetics laboratory. This fluorescent assay uses the fluorochrome SYBR Gold (18) without previous elaboration of tested samples, therefore the steps of DNA extraction and amplification are omitted. This review will highlight some of the advantages of the rapid direct fluorescent assay, and will compare it with widely used ctDNA analysis, with addressing the biology of cfDNA and ctDNA, and lastly will discuss possible future applications in early cancer detection.

# Biology of Cell-free DNA and Tumour

Cancer is a disease characterised not only by abnormal cell growth, but also by the increasing number of necrotic and apoptotic cells due to increased cellular turnover caused by tumour spread (19–21). Accordingly, patients with cancer show higher levels of cfDNA than healthy individuals (22–26). In healthy individuals infiltrating phagocytes relieve the physiological environment from apoptotic and necrotic cell residues, however, this mechanism is not sufficient during abnormal cancerous conditions, and cellular remains firstly accumulate and then enter the blood circulation.

The apoptotic character of release is more plausible than cell lysis due to the presence of a serum or plasma DNA ladder pattern resembling the one of apoptotic cells when run on electrophoresis (27). The length of cfDNA ladder pattern usually displays in multiplies of 180 base pairs, which is characteristic for processes where apoptosis in involved (20,28). Another recent study (29) confirmed that the tumour derived DNA fragments are shorter than fragments in healthy individuals. They also observed that the most noticeable peak was at 166bp, which suggest its apoptotic origin.

There can be found distinct characteristics across tumours (30), accordingly, the spread of cfDNA into plasma or serum depends not only on the apoptotic or necrotic processes, but also on the individual tumour characteristics that are determined by specific genetic mutations, and which contribute towards its proportions, location, and bloodstream connectivity. All these factors play important role in the quantity of cfDNA in plasma or serum, giving the possibility of using plasma or serum as an oncomarker.

In some cases tumours contribute to the higher levels of cfDNA in plasma or serum in cancer patients directly, however, some proportions of cfDNA are derived indirectly. Studies have implicated that ctDNA contribution towards overall cfDNA in plasma or serum varies considerably, these levels have a span of more than 90% (31,32). For example, Perrone et al. (33) has shown that the rate of KRAS mutations in cfDNA in plasma was detected in malignant lesions of adenocarcinomas (45%), however the rate of KRAS mutations in cfDNA in plasma was markedly lower (3%) in high-grade intraepithelial neoplasia in adenomas patients (benign tumour).

This indicates great differences in ctDNA in plasma and serum within distinct tumour characteristics that vary among cancer patients. Even though the rate of ctDNA in cfDNA diverge, cfDNA in plasma or serum increases in majority of cancer patients (11,17). This can be due to many factors, one can be the death of healthy cell because of tumour growth, which thereafter release DNA fragments into the bloodstream. Methods focusing on quantifying cfDNA are able to monitor these changes as well and are not limited to ctDNA. As discussed afterwards in this review, a rapid direct fluorescent assay for quantification of cfDNA overcomes this problem and stands as promising alternative to ctDNA analysis.

# A Rapid Direct Fluorescent Assay for Quantification of cfDNA

As mentioned before, current methods measuring concentration of cfDNA in plasma or serum cannot be widely used due to expressivity and work-load because they require DNA extraction and amplification with further analysis. A rapid direct fluorescent assay as first tested by Goldstein et al. (17) used SYBR Gold stain for cfDNA quantification. SYBR Gold is a cyanine dye used in genetics as nucleic acids mark. To demonstrate its simplicity, diluted SYBR Gold (1:10000)[[1]](#footnote-1) is directly added to 96-well plates, where sera is already applied (10ul of serum, 40ul of diluted SYBR Gold). Goldstein et al. run the assay in fluorometer (Spectrafluor Plus, Tecan, Durham, NC, USA) at an excitation of blue light wavelength (485 nm), which SYBR Gold absorbs, at an emission of green light wavelength (535nm), which SYBR Gold emits.

Although presented fluorescent assay convinces by its easiness, the results presented by Goldstein et al. (17) and later by Czeigler et al. (34) points towards the need of refinement of the assay. Mainly, the standard deviations both in healthy controls and patients are too high. Goldstein et al. (17) reported that the mean cfDNA concentration in 47 healthy individuals (22 females, mean age 26.3 ± 4.7) was 471 ± 203 ng/mL, establishing the normal range between 65-877 ng/ml. On the other hand, Czeigler et al. (34) reported concentrations of cfDNA in 38 colorectal cancer patients (21 females, mean age 68), where the mean was 798 ± 409 ng/mL, with range between 165 and 2,590 ng/mL. Patients in the lower tier of the normal distribution or the range exhibit similar cfDNA concentrations as healthy individuals.

The differences are not necessarily limitations of the assay, as stated before, there considerable distinctions between tumours, which can affect the wide-spread range of cfDNA concentrations in healthy individuals and cancer patients. In the future research, it is also important to assign cfDNA concentration by age, gender day time, physical condition, and in patients by tumour type as they all might contribute towards miscellaneous cfDNA concentrations.

# The differences between ctDNA analysis and cfDNA analysis

There is a need to identify in which cases either ctDNA analysis or cfDNA analysis is sufficient, and to ask whether there are cases, in which both would be preferably performed. Both methods help to obtain different information about patient´s state. The more accurate information about type and stage of cancer can be obtained from ctDNA analysis. During ctDNA analysis the same genetic abnormalities are found as the ones in tumours themselves. These genetic defects include point mutations like EGFR (35–39) and KRAS (40–42), rearrangements like EML4-ALK (43–45), amplifications like HER2 (46–48) and MET (49–51), and also chromosomal changes like aneuploidy (19,52).

The fluorescent quantification method of cfDNA provides information related to overall patient´s health in specific time. During rapid direct fluorescent assay only the concentration of cfDNA is estimated. If the levels of cfDNA are above the mean of normal population, there is a higher chance that the individual suffers from cancer.

Even though both methods study changes in plasma or serum, which correlate with disease progression, they are specifically efficient in different stages of disease progression. The analysis of ctDNA can be done in healthy individuals not previously diagnosed with cancer, however such method is too expensive and work skills-demanding. DNA extraction, amplification and further analysis requires specifically designed laboratory, which makes this method in some regions inaccessible. On other hand, cfDNA quantification can be performed in any medical setting with basic equipment by using very simple protocol.

# Conclusion

As it has been stated, there is a necessity in distinguishing specific needs of a medical screening that are accommodated by these methods. The ideal would aim for cfDNA assay in every GP’s medical office that would screen each patient once or twice a year. If the normal level of cfDNA concentration in plasma abnormally rises, then ctDNA analysis would be performed. This is a case in which both methods could be efficiently used.

If cfDNA quantification is going to be used as a screening method, there have to be constant results in many assays applied to one sample. It also has to be corrected to limit quantifications of proteins and any other disruptive elements. There is also a need of an estimated line between normal levels for any age, gender, and physical condition and abnormal levels that may point towards a disease. Any other conditions that would cause differences in cfDNA levels should be tested and these include for example: smoking, alcohol drinking, diet, etc.

To conclude, cfDNA analysis by rapid fluorescent assay for its quantification is a promising future biomarker. It is cheap, fast, and does not require skilled handling. However, there is also a need for further testing and levels estimation. Analysis of ctDNA is also a promising assay, but it is more expensive and skills demanding. In the future, these two methods could work complementary and provide new screening methods for cancer.

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1. 1:1000 in dimethyl sulphoxide, Sigma-Aldrich, Rehovot, Israel, and then 1:8 in phosphate-buffered saline, Biological Industries, Beth Haemek, Israel. [↑](#footnote-ref-1)