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Valentina Casadio
Samanta Salvi *Editors*

Cell-free DNA as Diagnostic Markers

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Cell-free DNA as Diagnostic Markers

Methods and Protocols

Edited by

Valentina Casadio and Samanta Salvi

Biosciences Laboratory, IRCCS I.R.S.T, Meldola, Italy

Editors

Valentina Casadio
Biosciences Laboratory
IRCCS I.R.S.T
Meldola, Italy

Samanta Salvi
Biosciences Laboratory
IRCCS I.R.S.T
Meldola, Italy

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Preface

In the last decade, cell-free DNA (cfDNA) is one of the most studied issues in the oncologic field, counting for more than 15,000 published papers (PubMed search: “cell-free DNA and cancer”). Its role has been intensively pursued also in other important areas such as fetal prenatal diagnosis and physical activity monitoring.

Three important characteristics (noninvasiveness, relatively low cost, simplicity) render cfDNA an excellent diagnostic marker. The technological advancement allows to study cfDNA for an increasing number of alterations (in some cases the whole exome), even its very low amounts.

The great number of publications on this topic makes mandatory to straighten up the main important concepts and findings, from the methodological approaches to the clinical applications.

The present volume aims at describing some of the most important techniques used for studying cfDNA in the different samples (serum, plasma, urine). It will also provide information regarding the different alterations that could be found and studied for the different disease types (adult and pediatric cancer, prenatal diagnosis) and physiological condition as physical activity.

The target audience will be biologists, technicians, or clinicians that are interested to study and deepen the knowledge on cfDNA methods and potential applications.

Meldola, Italy

Valentina Casadio

Samanta Salvi

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Contributors

GIANNI BISOGNO • *Department of Woman's and Children's Health, Hematology and Oncology Unit, University of Padova, Padova, Italy*

VALENTINA CASADIO • *Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy*

FRANCESCA COLIZZI • *Immunopathology and Cancer Biomarkers, Department of Translational Research, Centro di Riferimento Oncologico di Aviano (CRO) IRCCS, Aviano, PN, Italy*

ALESSIA COVRE • *Center for Immuno-Oncology, Division of Medical Oncology and Immunotherapy, Department of Oncology, University Hospital of Siena, Siena, Italy*

PIETRO FICI • *Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy*

ELISABETTA FRATTA • *Immunopathology and Cancer Biomarkers, Department of Translational Research, Centro di Riferimento Oncologico di Aviano (CRO) IRCCS, Aviano, PN, Italy*

PETER B. GAHAN • *Fondazione Enrico Puccinelli, Perugia, Italy*

GIORGIA GURIOLI • *Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy*

HIROTAKA IWASE • *Department of Breast and Endocrine Surgery, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan*

PEIYONG JIANG • *Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong; Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong*

PASQUALINO MAIETTA LATESSA • *Department for Life Quality Studies, Alma Mater Studiorum—University of Bologna, Bologna, Italy*

FILIPPO MARTIGNANO • *Department of Medical Biotechnologies, University of Siena, Siena, Italy*

MENG NI • *Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong; Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong*

XIANLU LAURA PENG • *Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC, USA*

ROSSELLA RANUCCI • *Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy*

SARA RAVAIOLI • *Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy*

SAMANTA SALVI • *Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy*

HEIDI SCHWARZENBACH • *Department of Tumor Biology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany*

LUCA SIGALOTTI • *Istituto di Patologia Clinica, Azienda Sanitaria Universitaria Integrata, Udine, Italy*

TAKASHI TAKESHITA • *Department of Breast and Endocrine Surgery, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan*

ANDREA TAROZZI • *Department for Life Quality Studies, Alma Mater Studiorum—University of Bologna, Bologna, Italy*

MICHELA TEBALDI • *Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy*

LUCIA TOMBOLAN • *Department of Woman's and Children's Health, Hematology and Oncology Unit, University of Padova, Padova, Italy*

LEYDI NATALIA VITTORI • *Department for Life Quality Studies, Alma Mater Studiorum—University of Bologna, Bologna, Italy*

ANGELICA ZIN • *Institute of Pediatric Research (IRP), Fondazione Città della Speranza, Padova, Italy*

Part I

An Overview



Chapter 1

Cell-Free DNA: Applications in Different Diseases

Rossella Ranucci

Abstract

Since its discovery in human blood plasma about 70 years ago, circulating cell-free DNA (cfDNA) has become an attractive subject of research as noninvasive disease biomarker. The interest in clinical applications has gained an exponential increase, making it a popular and potential target in a wide range of research areas.

cfDNA can be found in different body fluids, both in healthy and not healthy subjects. The recent and rapid development of new molecular techniques is promoting the study and the identification of cfDNA, holding the key to minimally invasive diagnostics, improving disease monitoring, clinical decision, and patients' outcome.

cfDNA has already given a huge impact on prenatal medicine, and it could become, in the next future, the standard of care also in other fields, from oncology to transplant medicine and cardiovascular diseases.

Key words Cell-free DNA, Liquid biopsy, Cell-free fetal DNA, Circulating tumor DNA, Clinical application

1 Introduction

The existence of circulating cell-free DNA (cfDNA) was reported for the first time in 1948 by Mandel and Métais that demonstrated the presence of free nucleic acids in blood plasma of healthy subjects [1].

This cfDNA comprises double-stranded deoxynucleic acid fragments that are characterized by an approximate length lower than 200 bp, a low molecular weight and a low concentration [2, 3].

Such a free-floating DNA is present in the cell-free component of whole blood, such as plasma and serum, and in the other human body fluids [4–6]; it is not associated with cells [7], but it forms complexes with proteins or membrane-bound structures [8].

Even if its function and source are still unknown, there are different reports about the origin and the possible mechanisms of release of cfDNA.

The most common hypothesis advanced for circulating cfDNA in plasma of healthy subjects believes that cfDNA could derive primarily from apoptosis of normal hematopoietic lineage cells [9, 10] or of other nucleated cells [11] that, in physiological circumstances, are cleared by infiltrating phagocytes; in fact cfDNA levels in healthy subjects are relatively low [12, 13].

Several characteristics of cfDNA sustain this hypothesis: its rapid turnover [14] and its short half-life in the circulation [15] suggest a model of ongoing release from apoptotic cells with a consequent rapid degradation or filtration [13, 16–19].

Consequently, cfDNA is highly fragmented (about 160 bp) [6, 20]; this size could correspond to the length of DNA occupied by a nucleosome, such as the primary unit for spatial organization of DNA in the nucleus [16].

To date, apoptosis and necrosis are not the only mechanisms responsible for the presence of circulating cfDNA. It seems that lymphocytes and many other types of living cells can spontaneously release newly synthesized DNA [21–24]; this could indicate a role of cfDNA in the normal functioning of cells or its involvement in a kind of active intercommunication between cells via release of nucleic acids as an intercellular signaling pathway [25]. Recent evidences from different fields indicate an active trafficking of nucleic acids between cells through carrier mechanisms on the cell membrane [26], during development, epigenetic remodeling, tissue regeneration, and fine tuning of the adaptive immune system. In addition, this traffic mechanism may be involved in cancer development and immune surveillance.

cfDNA source and functions could differ between different physiological and pathological conditions. cfDNA is released into circulation mostly by pathological mechanisms: research on cfDNA started only few years later its discovery, when high levels of circulating cfDNA were reported firstly in patients with systemic lupus erythematosus [27] and then in patients with cancer [28, 29].

The detection of circulating cfDNA of tumoral origin, named as circulating tumor DNA (ctDNA), inspired researchers to look for other DNA molecules: in 1997 cfDNA of fetal origin in maternal plasma was discovered [30].

Elevate concentrations of cfDNA were reported in plasma or serum of patients with many disease states, such as stroke [31], trauma, myocardial infarction [32], and diverse types of cancer, suggesting the hypothesis that cfDNA could be in a feedback relationship with inflammation and its release seems to activate a proinflammatory cytokine response [33].

Even if plasma and serum cfDNA are the best characterized, cfDNA can also be found in other fluids, like urine [34]. Urine cell-free DNA (ucfDNA) seems to originate from epithelial necrotic or apoptotic cells that are released or in direct contact with the urinary tract but could also derive from cfDNA present in blood [35, 36]. In

fact, Botezatu et al. [37] demonstrated that kidney barrier is partially permeable particularly to fragmented DNA derived from the circulation.

ucfDNA seems to be a promising biomarker [38], mostly for genitourinary-derived cancers, but also for other non-uurological disease [39].

2 cfDNA Applications

2.1 cfDNA in Prenatal Diagnosis

Due to its association with several human disorders, cfDNA has assumed a relevant clinical importance in the recent years and its clinical utility as a noninvasive, accurate, sensitive and rapid biomarker is an area of active research.

One of the fields in which the application of circulating cfDNA has obtained the best success and is used extensively till now is prenatal genetic testing [30, 40, 41]. In 2011 noninvasive prenatal testing (NIPT) has become a reality in clinical practice [42].

The identification of fetal-derived nucleic acids in maternal plasma during pregnancy was reported for the first time in 1997 [30]. Maternal plasma cfDNA is made of a mixture of both maternal and fetal DNA, where fetal fraction contributes only in a little percentage of the total and it seems to increase with gestational age [41] and pregnancy progress [15, 43, 44].

The fetal-derived cfDNA can be detected from 4–5 weeks' gestation, and it is rapidly cleared from the maternal circulation after delivery, suggesting that it is pregnancy specific [15, 41].

The first clinical applications were greatly potential but confined to the identification of alleles present in the fetus and not in maternal genome, such as paternally inherited or de novo mutations; these approaches included fetal sex assessment [30] and detection of paternally inherited single-gene disorders inherited in autosomal dominant manner, such as achondroplasia [45] and myotonic dystrophy [46].

Conversely, the determination of autosomal recessive or maternally transmitted autosomal dominant disorders resulted more complicated, even if several studies have been successful for the determination of excluding paternal alleles in recessive conditions, such as cystic fibrosis or beta-thalassemia.

Thanks to the continuous advances in technology, the perspective has rapidly changed and applications of fetal cfDNA have brought too many advantages in prenatal diagnosis [47].

Currently, sex determination and RhD and fetal blood group system genotyping are used in clinical practice in several countries because of their reliability and high accuracy [48, 49].

In addition, cfDNA technology is used for prenatal diagnosis of fetal aneuploidies including trisomy [13, 16, 17], sex chromosome aneuploidies and specific microdeletions [50]. For the applications

mentioned above, fetal cfDNA results to have a noninvasive nature, broad applicability, and availability at early gestational age. Even if it is considered a powerful screening tool, cfDNA has a few limitations. In the detection of aneuploidies, it cannot be considered a karyotype substitute; indeed the current detection of aneuploidy is limited to the most common trisomies. Moreover, the use of cfDNA for single-gene disorders and autosomal recessive and x-linked diseases diagnosis is an evolving not yet routine technology [42].

Since a large set of chromosomal abnormalities is currently not accessible [47], several studies have evaluated a possible introduction of genome-wide profiling in clinical practice that seems to hold great promises for the detection of a larger range of chromosomal rearrangements, such as rare autosomal trisomies [50], and contributing in lowering the incidence of false-positive results. However, the real clinical utility of genome-wide screening is an open issue.

2.2 *cfDNA in Tumors*

Seventy years after its discovery, circulating cfDNA has become particularly intriguing in the oncologic area, and recently its plasma genotyping is transforming cancer care [51], even if further steps are necessary for effective applications in clinical practice.

It has been known for decades that cfDNA is present in high concentrations in blood of cancer patients, but patients with advanced stage tumors present higher levels than those in early stage [52, 53].

ctDNA represents only a small fraction (<1%) of the total cfDNA [54–56] and derives from primary tumors, circulating tumor cells (CTCs), micrometastasis, or metastases [5, 12, 57, 58].

Several reports have confirmed that ctDNA harbors specific tumor-related alterations, such as point mutations, copy number variations (CNVs), amplifications, and methylation changes [59–63], acting as a circulating picture of a specific disease [64] and serving as a sort of liquid biopsy, though it is possible to reconstruct the status of tumor genome [35].

The increasing interest on tumor DNA is just due to its potential use as a liquid biopsy that holds great promise in a wide range of clinical applications, from noninvasive early diagnosis to prognosis, detection of minimal residual disease, and prediction of response to cancer treatment [51, 65, 66].

Even if surgical biopsy continues to be the gold standard for diagnosis and choice of treatment, it presents some disadvantages: besides its invasive nature, it can give only a static and spatially limited picture of the disease at the moment of surgical procedure [67].

Conversely, in addition to its noninvasive, rapid and low-cost nature, ctDNA allows a real-time cancer longitudinal monitoring and has the ability to capture tumor heterogeneity [67–70]. In addition, in the last 5 years, strong concordance has been shown

between plasma- and tissue-based genomic assays encouraging the exploration of its potential clinical utility [51].

In early tumor detection, ctDNA has received great attention and seems to be a potential source of biomarkers for different cancer types; anyway, its very low amount that needs more sensitive and reproducible methods and its variability among patients limits its use in clinical practice. An optimal promising application seems to be the combination of ctDNA detection with a panel of conventional cancer markers used for screening programs [35, 51].

Several studies on ctDNA ability to detect minimal residue disease after surgery or treatment have been done in different types of cancer, suggesting its high prognostic and predictive power of recurrence.

Plasma genotyping of ctDNA can also be used to guide choice of treatment, monitor in a dynamic way the response to therapies, give further details of potential genetic causes of progression, and make light on resistance mechanisms [51, 71–77].

These applications seem to be easier to achieve and closer to be introduced into clinical practice.

In 2016, the first ctDNA tested for epidermal growth factor receptor (EGFR) mutations in NSCLC was approved by FDA, paving the way to clinical implementation of liquid biopsy. Roche's Cobas plasma EGFR mutation test v2 is aimed to guide treatment decisions in patients with NSCLC, at progression after the first-generation EGFR tyrosine kinase inhibitor (TKI) therapy, not able to undergo tissue re-biopsy, necessary for monitoring cancer evolution and treatment response. Assaying cfDNA for EGFR T790M mutation on plasma samples has become an optimal alternative and leads to equivalent outcomes than traditional procedures, saving many patients from invasive biopsy [78–80].

The Roche's Cobas® EGFR mutation test v2 consists in a real-time PCR test for the qualitative detection and identification of 42 mutations in exons 18, 19, 20, and 21 of the EGFR gene, including the T790M-resistant mutation. This test is indicated for a semiquantitative analysis and provides a feature called semiquantitative index that reflects EGFR mutation load, allowing to understand tumor progression and management of NSCLC patients. The test is also provided as an aid in selection of patients for therapy with an EGFR TKIs [80].

Nowadays, it is still the only FDA-approved plasma genotyping assay. In the next 5 years, ctDNA is going to become a tool of choice for dynamic monitoring of patients on treatment or under surveillance, but its detection is still a great challenge because the extremely low level of ctDNA compared to cfDNA needs an accurate quantification of mutant fragments fraction in a sample [12].

2.3 cfDNA in Other Diseases

The investigation of possible applications of cfDNA has interested other types of pathological status like stroke, autoimmune disorders, myocardial infarction, and allograft transplant rejection, but until now no real clinical applications are provided.

After the discovery of high levels of cfDNA in systemic lupus erythematosus (SLE) patients in the 1970s, studies in systemic autoimmune disorders have gone on, and data have shown that increased cfDNA concentration seems to be correlated with antibody titers and active lupus nephritis, but not with disease activity. Anyway, cfDNA testing for diagnostic and prognosis of SLE remains questionable [81].

Circulating cfDNA has been detected also in patients with stroke symptoms. Rainer et al. [31] demonstrated that in the first 24 h of acute stroke DNA can be found in plasma, suggesting that could be used as a potential useful, quick and non-invasive test for patients' monitoring and risk stratification. Other studies have confirmed it, but, even if it seems to provide prognostic information for assessment of stroke severity and outcome, currently it is not proposed as a stroke biomarker with real clinical application [82].

Similarly, cfDNA has been detected also in patients with myocardial infarction; its measurement may be useful in the diagnosis of the disease, especially in combination with the traditional markers, troponin and CK-mb (isoenzyme of creatine kinase), and in the determination of infarct size, since both apoptosis and necrosis determine the death of myocytes, which seems to contribute to the increase of this parameter [32].

In transplant medicine, the surveillance of organ health, detecting transplant rejection, is essential for long-term survival of organ transplant recipients. Snyder et al. [83] have demonstrated that donor-derived cfDNA exists in plasma of recipients as a genetic signature, and allograft rejection events can be correlated with abnormally high levels of itself. Since rejection is associated with apoptosis of transplant-derived certain cells, measuring their genetic signature in the cfDNA in recipient's plasma, it can be possible to monitor organ health over time.

Currently, there are several emerging applications of circulating cfDNA in the post-transplant monitoring of rejection, infection and immunosuppression, but no one has been introduced in clinical practice.

3 Conclusions

Thanks to continuous technical advances in the genomic landscape, the technical barrier for cfDNA applications has been overcome, and now cfDNA analysis is a feasible approach in some medicine fields.

Despite the growing interest in cfDNA analysis and independently from the different clinical fields, currently, a preanalytical and analytical consensus is not available due to different hurdles in translating cfDNA analysis to clinical practice.

The heterogeneity among the various protocols for sample manipulation and analysis of cfDNA is one of the major obstacles, and till now a standardization of operating procedures does not exist. The preanalytical parameters potentially affecting cfDNA concentration and fragmentation comprise steps from blood collection tube choice to plasma processing, extraction and purification modalities, interpretation of findings, and sample storage conditions [84].

The development of standard techniques and protocols would pave the way to an effective use of cfDNA in clinical practice not only in research areas.

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Chapter 2

Cell-Free DNA: An Overview of Sample Types and Isolation Procedures

Filippo Martignano

Abstract

The study of cell-free DNA (cfDNA) is often challenging due to genomic DNA contamination, low concentration, and high fragmentation. Therefore, it is important to optimize pre-analytical and analytical procedures in order to maximize the performance of cfDNA-based analyses.

In this chapter, we report the most common methods for the correct collection, centrifugation, storage, and DNA isolation from cell-free biological sources such as plasma, urines, cerebrospinal fluid, and pleural effusion fluid.

Key words Cell-free DNA, Plasma, Serum, Urines, Cerebrospinal fluid, Pleural effusion fluid, Collection, Storage, Centrifugation, Nucleic acid extraction

1 Introduction

Cell-free DNA (cfDNA) consists in extracellular DNA fragments which are shed into biological fluids by necrotic and apoptotic cells [1]. The typical biological source for the isolation of cfDNA is the bloodstream, but it is possible to obtain it also from other biological materials such as urines [2–4], cerebrospinal fluid (CSF) [5–7], and pleural effusion fluid (PEF) [8–11]. If not specified otherwise, in this chapter we will refer to blood-derived cfDNA.

In healthy individuals, cfDNA belongs mainly from myeloid and lymphoid apoptotic cells due to the physiological turnover of hematopoietic cells [12, 13], while, in cancer patients, a fraction of the total cfDNA, termed circulating tumor DNA (ctDNA), comes from neoplastic lesions [14].

ctDNA fraction can vary from 0.01% to 60% of total cfDNA [15–18], and its concentration depends on different tumor features such as tumor volume, stage, vascularization, proliferation rate, and cell death rate [15, 19].

ctDNA is very informative for the study of oncological pathologies as it harbors tumor-specific genetic alteration that reflects the genomic status of the malignant cell of origin [19–23]. Its study is often challenging due to genomic DNA contamination, low concentration, and high fragmentation. Therefore, it is important to optimize pre-analytical and analytical procedures in order to maximize the performance of cfDNA-based analyses [24].

The typical length of cfDNA fragments in the bloodstream is around 169 bp that corresponds to the length of a DNA fragment wrapped in a chromatosome, which is a nucleosome bound to a H1 histone [25]; because of its short length, cfDNA is often called low-molecular-weight DNA (LMW-DNA).

The first mandatory step for cfDNA analysis is the centrifugation of the sample; this step causes the precipitation of the cellular component (typically healthy cells, e.g., buffy-coat cells in blood) whose genomic DNA (gDNA) represents a contaminant for cfDNA analyses. The centrifugation of the sample allows the collection of a supernatant which is actually “cell-free” (e.g., plasma), reducing the contamination by genomic DNA (gDNA) belonging to healthy cells [26–29]. As non-tumoral gDNA fragments are typically longer than cfDNA fragments [29], gDNA is often called high-molecular-weight DNA (HMW-DNA) [26]. The optimal centrifugation condition (speed, temperature, and duration) may vary depending on the type of biological fluid; this topic will be addressed in detail in the next paragraphs and summarized in Table 1.

2 Blood-Derived cfDNA

2.1 Choice of the Matrix: Serum or Plasma?

Blood-based cfDNA analysis can be performed both on serum and on plasma.

Some studies compared cfDNA levels in paired plasma and serum samples showing higher cfDNA concentrations in serum [30–32]; however, serum is not the preferable biological source as it is prone to contamination by gDNA [30]. The contamination derives from the storage procedure, which involves a clotting step before centrifugation that may lead to hematopoietic cell contamination or cell lysis [30, 33, 34]. As plasma is recommended for cfDNA analysis, the following paragraphs will focus on pre-analytical procedures suggested for plasma-based analysis.

2.2 Blood Collection and Storage

In order to preserve the quality and the quantity of cfDNA, many types of preservative tubes for blood collection are available.

The most common and cost-effective are the standard EDTA collection tubes. They successfully preserve cfDNA for up to 6 h before the processing [26, 35]; this is attributable to the inactivation of DNase activity by the EDTA [36]; however they are not

Table 1

Summary of collection, storage, centrifugation, and isolation methods for cell-free DNA from different biological sources such as plasma, urines, cerebrospinal fluid, and pleural effusion fluid

Procedure	Method	Application notes	References
Collection and storage (blood)			
	EDTA tubes	Preserve cfDNA for up to 6 h, blood processing is preferable within 2 h after venipuncture Short-term storage is recommended at 2–8 °C	[26, 35, 39]
	CellSave tubes	Preserve cfDNA for up to 48–96 h	[35, 38]
	Streck's Cell-Free DNA BCT	Preserve cfDNA for up to 48–96 h (extendable to 7–14 days, even if not suggested) Short-term storage is recommended at room temperature	[35, 38–40]
Centrifugation (blood)			
	Single centrifugation (2000–3000 × g , 10 min)		[43–49]
	Double centrifugation (first: 1600–3000 × g , 10 min; second: 10,000–15,000 × g , 10 min)		[50–58]
	4 °C centrifugation		[50–52, 54]
	Room temperature centrifugation		[53, 55–58]
Centrifugation (urines)			
	Single centrifugation (15,000–16,000 × g , 10–30 min)		[53, 97]
	Double centrifugation (first: 600–1800 × g , 10 min; second: 10,000–16,000 × g , 10 min)		[2, 75]
Centrifugation (CSF)			
	Single centrifugation (1000 × g , 10 min)		[6, 7]
Centrifugation (PEF)			
	Single centrifugation (250 × g , 10 min)		[8–10]
Long-term storage			
	–80 °C	Preserves cfDNA quality up to 2 weeks Increase of cfDNA degradation level by 30% after 1 year of storage	[59, 62]

(continued)

Table 1
(continued)

Procedure Method	Application notes	References
Isolation methods (blood)		
FitAmp® Plasma/Serum DNA Isolation kit (Epigentek)	Column-based	[1, 26]
QIAamp circulating nucleic acid kit (Qiagen)	Column-based	[1, 26, 63, 66]
Plasma/Serum Circulating DNA Purification Mini Kit by (Norgen)	Column-based	[80]
Plasma/Serum Cell-Free Circulating DNA Purification Mini Kit (Norgen)	Column-based	[80]
NucleoSpin® Plasma XS Kit (Macherey-Nagel)	Column-based	[1, 26]
QIAasymplicity PAXgene Blood cfDNA Kit (Qiagen)	Magnetic bead-based	[1, 47]
EpiQuick Circulating Cell-Free DNA Isolation Kit (Epigentek)	Magnetic bead-based	[66]
Maxwell RSC cfDNA Plasma Kit (Promega)	Magnetic bead-based	[1, 66]
MagMAX Cell-Free DNA Isolation Kit (Thermo Fisher Scientific)	Magnetic bead-based	[1]
MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche)	Magnetic bead-based	[64, 65]
NEXTprep-Mag cfDNA Isolation Kit (Bioo Scientific)	Magnetic bead-based	[66]
PME free-circulating DNA Extraction Kit (Analytik Jena)	Polymer-mediated enrichment	[1, 63, 66]
QIAamp DNA Blood Mini Kit (Qiagen)	Column-based Not recommended for cfDNA isolation, but still frequently used	[45, 50, 55, 70-74]
Yuan et al. and Hufnagl et al. phenol-chloroform homemade protocols	Similar performances compared to commercial kits Long processing time (2–3 days) Inexpensive	[78, 79]
Isolation methods (urines)		
Urine DNA Isolation Kit (Norgen)		[96]
Extract-all Urine DNA (Zymo)		[2]
QIAamp MinElute Virus Spin Kit (Qiagen)	Not specific for ucfdNA	[75]

recommended for a longer storage time as it can affect cfDNA quality and increase the risk of contamination with gDNA due to hematological cell lysis [37, 38].

According to one study, the best performances are obtained if EDTA-stored samples are processed within 2 h after venipuncture; alternatively, they can be stored at 2–8 °C as the prolonged storage at higher temperatures (22–37 °C) can increase the contamination by genomic DNA [39].

To overcome EDTA tube limitations, CellSave tubes and Streck's Cell-Free DNA BCT have been developed. These tubes are able to preserve the cfDNA quality up to 48–96 h after the blood collection [35, 38]; other studies claim that Streck tubes perform well even after longer storage periods such as 7 [39] and 14 days [40]. Another important advantage on using Streck tubes is that they can be conserved at room temperature without affecting DNA quality [35, 39, 40]. Interestingly, while for EDTA tubes it is a good practice to store them at low temperatures (2–8 °C), a prolonged storage of Streck tubes at lower (<10 °C) or higher (40 °C) temperatures is not recommended [35, 41].

It is important also to be careful during an eventual transportation of the blood samples before processing, as physical stress may promote hemolysis leading to an increase of plasma DNA (presumably increasing contamination by gDNA) [27]. One study simulated the sample transportation by shaking the tubes and demonstrated that Streck tubes, compared to EDTA tubes, can limit the negative effects of physical stress on DNA quality [39].

2.3 Centrifugation

As stated before, the centrifugation step is necessary for the removal of the cellular component from blood and, consequently, for obtaining the cell-free plasma fraction. Hence, a correct centrifugation procedure is important to minimize the contamination by gDNA belonging to hematopoietic cells.

After whole blood centrifugation, plasma is separated and can be subsequently collected with a pipet [26]: in this step, it's important to be careful not to touch the buffy coat.

After this first step, it is possible to perform a second centrifugation of the collected plasma in order to remove residues of cells and cell debris that may have been not successfully removed during the first step [1]. Page et al. demonstrated that a double centrifugation reduces the amount of longer, typically undesired, cfDNA fragments (>300 bp) [42].

Single centrifugation is usually performed at speed ranging from 2000–3000 × g for 10 min [43–49], while the double-centrifugation procedure consists in a first centrifugation step at 1600–3000 × g for 10 min followed by a faster second centrifugation step at 10,000–14,000 × g for 10 min [50–58]. These centrifugation steps are usually performed at 4 °C [50–52, 54] or room temperature [53, 55–58].

2.4 Long-Term Storage

After centrifugation, plasma (or serum) can be stored for long periods at -80°C . -20°C storage is not recommended as it can increase cfDNA fragmentation [59, 60].

Of course, it is recommended to store samples at -80°C only after the centrifugation step, as freezing whole blood samples leads to cell lysis and consequently to contamination with gDNA [61]. Plasma cfDNA levels are not affected by 2 weeks of storage at -80°C [59], while a storage of 1 year can increase by 30% the degradation level of cfDNA [62].

2.5 Isolation Methods

Today, many extraction kits for the isolation of plasma cfDNA are available, and they are mainly divided in two categories: column-based kits such as FitAmp[®] Plasma/Serum DNA Isolation kit (FA) by Epigentek, QIAamp circulating nucleic acid kit (CNA) by Qiagen, Plasma/Serum Circulating DNA Purification Mini Kit (PSN) by Norgen, Plasma/Serum Cell-Free Circulating DNA Purification Mini Kit (PScfN) by Norgen, and NucleoSpin[®] Plasma XS Kit (Macherey-Nagel) (NS) and magnetic bead-based kits such as QIAasympnhy PAXgene Blood ccfDNA Kit (QS) by Qiagen, EpiQuick Circulating Cell-Free DNA Isolation Kit (EQ) by Epigentek, Maxwell RSC ccfDNA Plasma Kit (RSC) by Promega, MagMAX Cell-Free DNA Isolation Kit (MM) by Thermo Fisher Scientific, MagNA Pure Compact Nucleic Acid Isolation Kit I (MPC) by Roche, and NEXTprep-Mag cfDNA Isolation Kit (NPM) by Bioo Scientific [1, 26, 47, 63–66].

The column-based approach is based on spin columns with usually a silica matrix able to bind DNA fragments during the passage of a preprocessed plasma sample, followed by washing steps for the removal of contaminants performed with a vacuum pump or a minicentrifuge.

The bead-based approach is based on magnetic beads able to bind nucleic acids that can be recovered with the help of a magnet without the need of centrifugation reducing the risk of damaging the DNA due to shear forces [67].

An important advantage of bead-based protocols is that they are often automatable.

One less common approach is the polymer-mediated enrichment: PME free-circulating DNA Extraction Kit (PME) by Analytik Jena [66].

These methods are optimized for cfDNA isolation and thus are preferable than “standard” extraction protocols. However most of them have been recently developed; for this reason, generic or homemade extraction methods have been often reported in studies [46, 57, 68, 69], as QIAamp DNA Blood Mini Kit (DBM) by Qiagen which is still frequently used [45, 50, 55, 70–74].

It is pivotal to take into account the short size of cfDNA when choosing an isolation protocol; despite its widespread use,

DBM is not the best choice for cfDNA extraction, as it is intended for the isolation of HMW-DNA [75].

Most of these methods are designed to extract cfDNA from starting volumes of plasma <2 mL; notably, Qiagen products tend to be oriented to larger starting volumes up to 3 mL with CNA, 4.8 mL with QS, and 10 mL with QIAamp MinElute ccfDNA Kit which combines a preliminary bead-based enrichment of cfDNA followed by a spin-column extraction approach.

As stated before, pre-extraction processing of blood samples can strongly affect cfDNA quality and quantity; hence, it is difficult to compare the performance of the extraction methods in studies with different pre-analytic procedures. With this purpose, some recent direct comparisons have been made. Page et al. tested CNA, DBM, NS, and FA kits: CNA and DBM kits produced comparable results, tenfold higher than NS and FA kits. Notably, they found that DBM kit isolates higher amounts of HMW-DNA compared to CNA and NS [26]. These results were confirmed by Devonshire et al. who found the CNA kit to be the best performing, followed by the DBM, NS, and FA kits. They also performed a spike-in with fragments of different sizes (115, 461, and 1448 bp) and found the DBM kit to be more efficient in recovering long DNA fragments (>400 bp) which is not preferable for cfDNA analysis, while CNA and NS kits showed the same performance irrespective of fragment length [76]. Sorber et al. measured the yield of five extraction kits with a digital droplet PCR-based method finding that CNA and RSC performed similarly, with yields remarkably higher compared to PME, EQ, and two consecutive versions of NPM (NPMv1 and NPMv2). They were able to detect KRAS mutation in five out of five patients with cfDNA extracted with CNA and RSC, in three out of five patients with NPMv2, and in no patients using the other kits.

According to the authors, the higher yield of RSC and CNA is partly due to the lysis step included in these protocols, which allows the recovery also of DNA bound to proteins or encapsulated in microvesicles [66]. Pérez-Barrios et al. observed similar yields with CNA and RSC, while MPC yields were lower compared to RSC. However, they obtained a better enrichment of 150–200 bp fragments using MPC compared to the other two kits [77]. Notably, according to Mauger et al., two phenol-chloroform homemade protocols proposed by Yuan et al. [78] and Hufnagl et al. [79] have similar performances compared to commercial kits (i.e., CNA, NS, PSN, PScfN). The drawback of using these protocols is the remarkably longer processing time (2–3 days rather than 2–3 h), but they can still be a valid cost-effective alternative [80].

3 Other Fluids

Blood is the most common and the most largely investigated source for cfDNA analysis. However, for the study of malignancies that affect specific body districts (i.e., the brain, lungs, urinary apparatus), the study of cfDNA in urines, CSF, and PEF has a big potential.

Unfortunately, standard methods for the collection, handling, centrifugation, and cfDNA extraction from such fluids are currently unavailable.

Hence, in the following paragraphs, a summary of the few studies published is reported; anyway, further methodological investigations are strongly needed to define gold-standard practices.

Note that some studies regarding liquid biopsy don't perform a centrifugation step or, in some cases, analyze specifically the cellular component obtained from the pellet after a centrifugation step [81–85]. Consequently, these studies have not been included in the following paragraphs as they are not focused technically on cfDNA, even if they are able to detect tumor-derived DNA from the biological fluid.

3.1 Urines

Urinary cfDNA (ucfDNA) is composed of two classes of DNA: DNA derived from apoptotic or necrotic cells of the urinary tract [4, 86] and transrenal DNA which belongs to the bloodstream that passed through the glomerular filtration [87, 88]. The former is useful for the study of urological cancers [3, 4] and the latter for both urological and non-urological cancer detection and monitoring [89, 90].

Glomerular pore size (3–11.5 nm) impedes the passages of molecules bigger than 70KDa (including vesicles and large protein complexes [88, 91]) from the circulation to the urinary tract; indeed transrenal DNA is typically LMW-DNA [88, 92]. This is supported also by studies conducted on urines of pregnant women, which observed very short fragments (29–45 bp) of fetal DNA in urines [93]. Hence, for the analysis of urinary transrenal DNA, it is particularly important to choose an extraction method that can efficiently recover LMW-DNA [92].

A recent study showed that ucfDNA has a shorter half-life time than cfDNA in blood-derived samples, suggesting that urinary nucleases and contaminants play a role in DNA degradation [94]. Urine samples should therefore be processed as soon as possible after collection.

As for plasma processing, a centrifugation of whole urines is needed to separate the cell-free supernatant from cell debris. Cell debris is composed mainly of cells of the urogenital tract whose gDNA may contaminate ucfDNA and impair biomarker detection [95, 96].

Uries are normally centrifuged immediately after collection [2–4]; however some workgroups freeze-stored urines until

centrifugation [53, 96]; this is not recommended as the freeze-thaw cycle will lead to cell lysis [61], increasing the contamination with gDNA.

Some studies performed a double-centrifugation protocol, which involves a first centrifugation at $600\text{--}1800 \times g$ for 10 min followed by a second, faster centrifugation at $10,000\text{--}16,000 \times g$ for 10 min [2, 75].

Other studies performed only a single high-speed centrifugation at $15,000\text{--}16,000 \times g$ for 10–30 min [53, 97].

For ucfDNA extraction from urinary supernatant, urine-specific kits such as Urine DNA Isolation Kit by Norgen (UDI) and Extract-all Urine DNA (EAU) by Zymo have been used [2, 96].

Alternatively, some groups used cfDNA-specific kits (CNA, PME) [2, 96] or, more frequently, conventional extraction kits (DBM) [53, 75, 97, 98].

One study demonstrated that QIAamp MinElute Virus Spin Kit by Qiagen is preferable, rather than DBM, as it performs better in isolating shorter DNA fragments [75].

Starting from an equal amount of urines, Ghanjati et al. obtained almost the double amount of DNA with CAN, compared to UDI [96].

According to Xia et al., among EAU, UDI, and PME, PME is the more performing in terms of DNA yield; however, EAU produced higher-quality DNA extracts, more appropriate for next-generation sequencing approaches [2].

In order to obtain a satisfactory amount of DNA from urinary supernatant, it is suggested to use protocols that allow the use of large volumes of urine; typically, this is a common feature of the more recent urine-specific DNA extraction kits.

3.2 Cerebrospinal Fluid

CSF is a biological fluid that circulates throughout the central nervous system; this makes it a promising biological source for the study of ctDNA belonging to primary and metastatic brain tumors [99]. The presence of the blood-brain barriers limits drastically the passage of genetic material from the CSF to the circulation making blood-based analysis challenging, while increasing the concentration of ctDNA in CSF [1, 15]. In addition, one further advantage of CSF is the low cellular component ($0\text{--}5$ cells/ μL compared to $3500\text{--}10,500$ white blood cells/ μL in blood) which results in lower risk of contamination by gDNA [7, 15]; thanks to this, a second centrifugation is not crucial [7], while some studies analyzed CSF without even performing a centrifugation step [100, 101]. Anyway, at least a single centrifugation step is usually performed [5–7], and it is recommended as cfDNA is still a little fraction of total CSF-DNA, even with such a low cellularity [7]. Li et al. and Pan et al. perform a $1000 \times g$, 10 min centrifugation, followed with DNA extraction with CNA kit [6, 7].

3.3 Pleural Effusion Fluid

PEF is a biological fluid that derives from a frequent complication of lung cancer: malignant pleural effusions. It is often easy to collect in a noninvasive manner and can harbor tumor-specific mutations, so it has been proposed as a promising biological fluid for the monitoring of lung cancer [11]. As for CSF and urines, there are no standard procedures for PEF cfDNA analysis; based on the few studies published, a starting sample of 2–5 mL sample PEF is centrifuged at $250 \times g$ for 10 min at room temperature, and DNA is extracted from 0.8–2 mL of supernatant with different kits such as DBM, CNA, and QIAamp DNA Blood Midi Kit (Qia-
gen) [8–11, 102, 103].

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Part II

A Liquid Biopsy for Cancer Disease



Chapter 3

Circulating Cell-Free DNA and Cancer Therapy Monitoring: Methods and Potential

Peter B. Gahan

Abstract

The monitoring of therapy during the treatment of cancer patients is currently assessed by the levels of circulating tumor cells or by PET/CT scans. Neither approach has the sensitivity or specificity to be very sure of the efficacy of the treatment. Moreover, PET/CT scans can be both comparatively expensive and produce low levels of radiation for the patient. The advent of the possibility of using circulating DNA released from the tumor permits (1) a possible early marker of the presence of the cancer, (2) an indication of the success of the primary treatment, (3) an indication of the early presence of possible metastasis, (4) a marker of the success of secondary subsequent treatment, (5) determining which patients can benefit from a particular treatment, and (6) offering a prognosis. These aspects will be discussed concerning the application of circulating tumor DNA analysis to the monitoring of cancer patients undergoing therapy.

Key words cfDNA, ctDNA, Cancer therapy monitoring, Methodology, Current limitations, Prognosis

1 Introduction

It is important to monitor patients during and after chemotherapy/radiotherapy and surgery for cancers, to ensure the efficacy of the treatment and the continuing health prior to the possibility of detecting relapses when further treatment will also need monitoring. Currently, this is achieved by either frequent sampling for the presence of circulating tumor cells (CTCs) or through a sequence of computerized tomography/positron emission tomography (CT/PET) scans to reveal the presence of primary and secondary tumors. Both methods have their limitations.

In the case of CTCs, Economopoulos et al. [1] have evaluated that the presence of CTCs can be linked to poor clinical outcome in patients with metastatic breast cancer, lung cancer, colorectal cancer (CRC), and prostate cancer. Moreover, a longitudinal evaluation of CTCs in patients with early breast cancer can possibly show the

presence of chemo- and hormone therapy-resistant CTC subtypes that may be linked to an increased risk for disease relapse and disease-related death. They conclude that the molecular characterization of CTCs could be useful for monitoring and evaluating treatment efficacy in patients with various tumor types. More recently, a meta-analytical study by Yan et al. [2] also showed that CTC status can be employed to monitor treatment efficacy and guide subsequent therapies in breast cancer. However, Horn et al. [3] have stressed the uncertainty of the available methodology for monitoring CTCs [4, 5]. They suggest modifying the approach by discontinuing the use of the 5CTC static cutoff to stratify patients and, instead, to employ significant changes in CTC values as a grouping parameter for responders and nonresponders to chemotherapy among metastatic breast cancer (mBC) patients. They refer to such a change as “a significant difference between two serial measurements when taking the variability of the measuring method into account”—termed significant change value limits (SCV-limits).

In spite of these encouraging developments, Kowalik et al. [6] drew attention to the fact that, regardless of the technical improvements, during that past 150 years since the first identification of CTCs by Ashworth [7], few CTCs detection methods have been approved for routine clinical use. This would appear to reflect the remaining methodological problems linked to their cell biology, their heterogeneity, their varied metastatic potential, and the small number of CTCs released into the blood at the early disease stage [6].

During positron emission tomography (PET) scanning, the patient is injected with, usually, fluorodeoxyglucose which is accumulated by the tumor that is seen as a highly contrasted site. This is a rapid way to identify the presence of tumors although it is considered that the value of PET usage will never reach 100%. The sensitivity has been increased by combining PET with computer tomography (CT), but this does not change the fact that, in monitoring treatment, the sensitivity is not adequate to detect initial tumor formation. The use of CT also poses a problem in that the patient is exposed to low doses of radiation. Thus, with frequent scans during the monitoring of treatment, there will be an accumulative radiation effect leading, in some cases, to tumor induction. Hence, there are limitations to the routine use of PET/CT scans in the monitoring of cancer treatment due to the need for frequent scans so leading to possible radiation effects as well as high costs [8].

This leaves the use of circulating DNA in blood that, together with CTC analysis, forms the basis of the liquid biopsy approach to cancer studies for early diagnosis, prognosis, and treatment monitoring [9]. The circulating DNA in cancer patients will be of two forms: that released from healthy cells, cell-free DNA (cfDNA), and that from tumor cells (ctDNA). The ctDNA will be in lower amounts than the cfDNA. In cancer-free, healthy individuals, there will be only cfDNA.

2 Origins of cfDNA

When examining cfDNA and ctDNA, their divergent sources need to be considered. Thus, cfDNA from healthy individuals can be derived from leukocyte breakdown, bacteria, viruses, cell-surface DNA as neutrophil extracellular traps (NETs), eosinophil extracellular traps (EETs), transposons, retrotransposons, necrosis, apoptotic release of nucleosomes, and active release including exosomes and virtosomes [10]. Less than 3% will be due to xenogeneic sources [11].

In the case of ctDNA, the main sources are considered to be apoptosis [12, 13], necrosis [14], NETs [14], and active release mechanisms [15, 16]. It has been indicated that only 3% of this were found to be xenogeneic compared to 87% that were attributable to known database sequences.

2.1 cfDNA and ctDNA Content of Peripheral Blood

A tumor is comprised of “normal” cells, malignant cells, and tumor microenvironmental cells that release cfDNA/ctDNA. The ctDNA fraction will reflect the range of mutated DNA fragments found as a function of the particular mutation range present within each tumor type. Thus, while a particular mutant DNA will initially dominate the ctDNA fraction, after tumor treatment, this fraction can be replaced by new mutations that were not originally present in the ctDNA fraction but were present in very small numbers of mutant cells in the tumor [17]. In the case of the tumor presence, CTCs will also detach from the tumor in addition to the ctDNA fraction.

The amount of cfDNA in healthy individuals tends to be relatively low in amount 1.8–35 ng DNA/mL in serum [10] and 3.6–5.0 ng/mL in plasma [18]. However, there are higher levels of cfDNA (5–1500 ng/mL) found in cancer patients as compared to healthy individuals (1–5 ng/mL) [19, 20]. Working with serum from CRC patients, Schwarzenbach et al. [21] found a wide range of DNA concentrations (from 22 to 3922 ng/mL) compared to the cfDNA average concentration of healthy donors (5–16 ng/mL). The increased amounts of ctDNA may be accounted for tumor burden [22–24]. These will vary with the size of the tumor, only a low number of mutated ctDNA fragments being found on the initiation of the tumor and hence before being detectable by CT/PET scans. It appears that there is a point at which ctDNA will be identified, without detecting by CT/PET scan [25].

The differences reported in the literature for ctDNA concentration show variations between serum and plasma ctDNA levels. El Messaoudi and Thierry [26] examined these variations using SW620 xenografts and concluded that plasma levels gave the more reliable results, and they determined the ideal blood drawing, blood storage, and DNA extraction procedures to maximize the cfDNA and ctDNA levels per sample.

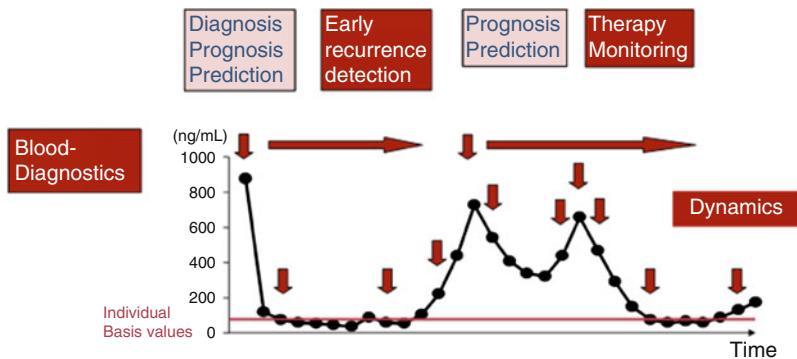


Fig. 1 During the course of cancer, circulating biomarkers can be exploited for (1) cancer detection and differential diagnosis, (2) estimation of prognosis, (3) prediction and monitoring of therapy response, and (4) early detection of therapy resistance and recurrent disease. Biomarker changes in relation to individual baseline values often indicate disease progress [27]

Thus, it is argued that the higher amounts of ctDNA are due to the increased release of ctDNA by the enlarging tumor and, hence, this may be used as a marker for the monitoring of treatment by determining the levels of ctDNA before and during treatment in order to establish if there is a reduction in the total amount of ctDNA circulating after treatment. Subsequent ctDNA levels could be used for monitoring the treatment response and to follow relapses by an increase in the ctDNA content. This can again be monitored during the second form of treatment and the patients' response observed. The detection of these ctDNA levels at different points in the cancer identification and treatment can be achieved with a sequence of liquid biopsies per patient (Fig. 1) [27].

2.2 Monitoring of cfDNA Levels in Peripheral Blood

The lower levels of cfDNA in healthy individuals are likely due to the presence of DNase I in the blood [28] as well as the uptake of cfDNA by the liver and elimination via the kidneys. Early studies on circulating DNA showed *Bacillus subtilis* DNA to be rapidly lost from the blood within 30 min [29]. Injected DNA in mammals was also rapidly removed from the circulatory system through DNase activity [30]. The liver was also identified as the site of calf thymus DNA removal [31] primarily by reticuloendothelial uptake.

Studies on nucleosomal material showed the liver to be the major point of their removal after intravenous injection in mice at doses lower than 11 µg with 71.0–84.7% being removed within the first 10 min. However, only $0.52 \pm 0.15\%$ was localized in the kidneys [32]. The timing of the removal of the nucleosomes by the liver equates well with that given for the removal of fetal DNA from the maternal blood stream by Lo et al. [33], the mean half-life for circulating fetal DNA being 16.3 min (range 4–30 min). In this case, removal by the liver was not determined

though plasma nucleases were found to account for only part of the clearance of the plasma fetal DNA.

ctDNA is at higher levels than cfDNA signals, and there is a greater release of DNA in the presence of the tumor, but much of the released DNA is in some way protected from DNase digestion and possibly from removal by the liver. This is most probably due to the DNA being protected from DNase activity through being associated with lipid and/or protein seen not just in the case of nucleosomal proteins but also with actively released DNA as is seen with the virtosomes from both normal cells and tissue and tumor cell lines [15, 34–37]. In contrast, naked DNA released from mitochondria (mcfDNA) is readily attacked by DNase due to its lack of histone protein protection. This can result in mcfDNA of <100 bp [38].

The size of the tumor must also be considered since the smaller the tumor, the lower is likely to be the ctDNA concentration. The first point to consider is that the low levels of ctDNA will be more difficult to separate from the cfDNA released from healthy cells. The second point is that the ctDNA composition will also depend upon the treatment. Thus, the smaller the tumor size, the more likelihood of the presence of actively released DNA and apoptotic fragments. The larger tumor will develop hypoxic central tissues leading to necrosis and hence larger ctDNA fragments appearing in the blood. In addition, it is likely that there will be a reduction in actively secreted ctDNA from some of the tumor regions due to hypoxia. It was shown that the inhibition of virtosomal release from rat lymphocytes occurred when the inhibition of glycolysis and the electron transport pathway (ETP) was induced by 105 M Cu²⁺ that inhibits both phosphofructokinases and the ETP complex I [35, 39].

Nevertheless, small-sized fragments are increasingly found with increasing amounts of ctDNA in the presence of larger tumors [13, 40].

Nevertheless, an initial approach to the monitoring of tumor progression and the behavior of the tumor during treatment was developed based upon the concentration of nucleosomes in plasma. Given that most of the cells undergoing apoptosis are likely to be derived from the tumor, either through natural tumor cell death by apoptosis or apoptosis induced by the particular treatment provided, it was suggested that nucleosomes could form a useful marker for determining the success of cytotoxic chemo- and radiotherapy treatment in non-small cell lung cancer (NSCLC) [41]. Thus, in a prospective study of NSCLC patients treated by first-line chemotherapy, nonresponsive patients had higher initial nucleosome levels and higher maximum values together with a lower elimination value by the end of 1 week of treatment. In contrast, patients in remission had strongly decreasing nucleosome levels [42, 43]. Nucleosome levels indicating an early response to therapy were found also for colorectal [44], pancreatic [45], and

breast cancers [46]. However, the reliability of this approach was not clearly demonstrated, and a more sensitive approach was evidently needed.

2.3 cfDNA Fragment Size for ctDNA Analysis

Fragment size may also influence the procedure to be used in the monitoring of treatment, there being a wide size variation. Hence, ctDNA from necrotic cells has been shown to be comprised of 10,000 bp or larger [37], while apoptotic fragments have been demonstrated with sizes ranging from 180 to 1000 bp, subsequently being reduced to 180–200 bp fragments [47, 48]. Grunt et al. [49] have suggested the following size ranges, namely, healthy individuals <200 to >10,000 bp [50–53], ctDNA <150 bp [14, 51, 54], and mitochondria <100 bp [38, 40, 55].

Mouliere et al. [40] isolated fragments of 73, 145, and 300 bp released from tumors of increasing volume. It was found that only minor changes occurred in the quantities of 145 and 300 bp samples with never more than 10 ng/mL per tumor. However, there was a major increase of almost 70-fold for the 73 bp fragments to more than 70 ng/mL in the largest tumors.

qPCR quantification of the ctDNA depends on the amplified target length and is optimal for 60–100 bp fragments. Tumor-derived plasma ctDNA qPCR analysis from either xenografted mice or cancer patients exhibited a specific amount profile based on ctDNA size and a significantly higher ctDNA fragmentation. ctDNA isolated from the plasma of metastatic CRC patients ($n = 12$) yielded an almost fivefold higher mean ctDNA fragmentation than healthy individuals ($n = 16$) [40].

The small fragment size fraction associated with ctDNA was initially shown to contain mutated fragments [56]. This was confirmed by Jiang et al. [51] with massively parallel sequencing (MPS) when the size profile of plasma total DNA was characterized at single-base resolution for 225 samples. These included 90 hepatocellular carcinoma (HCC) patients and healthy individuals as well as individuals with hepatitis B virus infections and those either with or without liver cirrhosis as controls. A distribution of cfDNA fragment lengths was found to have a mode near 166 bp in all samples, while those fragments from the plasma DNA of patients with high fractions of ctDNA showed a greater fragmentation than in healthy individuals. The distribution of fragment size of sequences derived from chromosomal arm 8q, frequently amplified in HCC, showed a consistent trend toward small-sized ctDNA fragments.

Nevertheless, there is still some controversy that short DNA fragments primarily indicate the ctDNA fraction. Thus, although there was a significant enrichment of total short DNA fragments when using a library preparation protocol for the enrichment of short DNA fragments for a genome-wide analysis of plasma DNA samples from metastatic cancer patients [57], no enrichment of the tumor-derived fragments was seen. However, Heidary et al. [58]

and Heitzer [59] observed a correlation between high levels of ctDNA and larger fragments sizes, i.e., di- and tri-nucleosomal fragment sizes.

3 Methods

The use of the liquid biopsy approach gives the possibility to increase the frequency of blood sampling by a minimally invasive procedure versus the limited frequency of the invasive approach of tissue biopsy. Furthermore, the liquid biopsy provides the possibility of finding the full range of mutated DNA fragments; on the contrary, solid biopsy may only partially sample the full range of mutant DNA due to the nature of the imprecise procedure with 10–50% of biopsies failing to provide sufficient tissue [60]. Thus, it is possible to assay for the levels of total normal cfDNA, total ctDNA, as well as individual mutant ctDNA fragments. To achieve reproducible results, it is necessary to have strict protocols for handling the blood samples prior to analysis [26, 61].

A variety of methods are available for the separation and analysis of cfDNA and ctDNA [49]. At present, much of the literature relating to the analysis of ctDNA for monitoring treatment is restricted to massive parallel sequencing (MPS) and either digital PCR (dPCR) or real-time PCR (qPCR). qPCR has the advantage of high specificity with size measurement of chosen amplicons, while dPCR has improved sensitivity on detecting rare targets, offering absolute quantification, and having lower throughput than qPCR, but is more expensive [55].

Multiplexing can also be run using multiplex PCR which employs multiple primer sets within a single PCR mixture, thus producing amplicons of varying specific sizes to various DNA sequences. This results in the use of a single test run to target multiple sequences simultaneously. Ladas et al. [62] have proposed a method to enrich the KRAS mutation containing sequences using enzymatic digestion of the wild-type DNA.

In a cost comparison for KRAS 12 and 13 mutations, qPCR had the shortest turnaround time of 2 days and was the cheapest method [63]; these are important features for a test to be applied in the clinical application.

MPS permits millions of sequencing reactions to be performed in parallel. It has been developed to be used with both the total nuclear DNA content and cfDNA/ctDNA isolated from patients thus permitting multiplexing of various patient samples. Essentially, sequencing involves the use of clonally amplified DNA templates that are derived from a single DNA fragment. The resulting templates contain fluorescent markers that yield enhanced fluorescent signal during imaging. This means that the error rates for individual NGS reads are overestimated and there is a need to sequence given

regions many times to generate a consensus sequence. The assembly, alignment, and analysis of MPS data also need a sufficient number of overlapping reads. Nevertheless, this approach leads to cost-saving [64]. In practice, MPS has been applied to the analysis of ctDNA in a range of cancers and had most often employed either whole genome sequencing (WGS) or whole exome sequencing (WES) or epigenetic modifications [65]. A major problem in the application of WGS and WES is that they function adequately in the presence of high concentrations of ctDNA and hence when the tumor is likely to have significantly evolved, leaving targeted sequencing to be preferred in the presence of low amounts of ctDNA.

4 Approaches to Monitor the Cancer Clinical Evolution

4.1 Diagnostic Approach Prior to Primary Treatment

A first step in monitoring treatment can be considered to be the search for ctDNA in order to identify a patient with a tumor prior to its being detectable by, e.g., CT/PET scanning. A number of authors have demonstrated the increased sensitivity of ctDNA assessment over CT/PET scanning [14, 66]. Many earlier studies have shown the increase in the amount of ctDNA present when compared to the cfDNA levels in healthy individuals, but this is not an accurate approach for pretreatment diagnosis. Nevertheless, ctDNA levels correlate with tumor burden in, e.g., CRC for both animal experiments [14] and chemotherapy-naïve patients [67].

The search for the presence of mutant ctDNA released from the developing primary tumor has not yet been proven adequately to identify the first appearance of a tumor. Although the use of liquid biopsies was originally defined for DNA detection [9], such samples were used earlier for the monitoring purposes for blood protein markers [68, 69]. Given the fact that there is unlikely to be enough ctDNA especially in the earliest steps in the development of a cancer, a combined use of potential ctDNA and protein markers for the detection of tumors and their treatment offers an alternative approach. This has been essayed by Cohen et al. [70, 71]. One of the drawbacks of a liquid biopsy concerns the identification of the tissue origins of the ctDNA/protein markers [71]. However, based upon nucleosomal footprints, Snyder et al. [72] were able to begin to define the cell types from which the circulating DNA takes origin in pathological states including cancer.

The study of Cohen et al. [70] has been extended [71] to combine these two facets, i.e., using both ctDNA and protein markers to identify the presence of a tumor and to determine the tissue of origin with their CancerSEEK test using samples gathered from 1005 cancer patients at stages I–III versus those from 812 healthy individuals. ctDNA was determined using a multiplex PCR method resulting in ctDNA barcoding which, together with

eight identified proteins, was found to be important in identifying lung, ovarian, stomach, liver, pancreas, esophageal, colorectal, and breast cancers prior to treatment. The tests were positive in 70% of the cancer types with 99% specificity. This approach, combining both ctDNA and tumor-specific proteins, may provide a more reliable approach for early primary tumor detection prior to metastasis, thus permitting early treatment and a better opportunity to clear the tumor completely.

4.2 Treatment Monitoring

As observed in Fig. 1, the tumor may, or may not, have been identified early by ctDNA levels. Subsequent monitoring of the tumor system at the initial treatment provides a ctDNA level against which to compare a following series of ctDNA measurements. Clearly, the anticipated rapid decline of ctDNA following the treatment, e.g., surgery ± chemo-/radiotherapy, is a good marker of the initial treatment success. The subsequent low ctDNA levels can be considered as a reliable marker to continue successful treatment, these levels acting as baseline measurements against which to determine any further changes. At some stages there is likely to be the development of primary treatment resistance that will be observed by an increase in measurable ctDNA thus resulting in the need for an alternative treatment. This also permits the prediction of a prognosis. Subsequent monitoring for ctDNA will indicate the success—or not—of the secondary treatment [73].

4.3 Patient's Stratification for Treatment

The value of using ctDNA in the monitoring of cancer patients has been investigated in a range of studies [60, 73–76] and, in particular, for non-small cell lung cancer (NSCLC) [77], metastatic colorectal cancer (mCRC) [67], metastatic pancreatic ductal adenocarcinoma (mPDAC) prognosis [78], non-Hodgkin lymphoma [66], and metastatic breast cancer [79].

There is an important clinical requirement for the use of ctDNA, in particular to determine the mutation status of cancer patients with a view to their stratification. This can be achieved by the analysis of the nuclear DNA in tumor tissues taken from solid biopsy when there are inadequate levels of ctDNA or the ctDNA in a liquid biopsy as showed by Mohan et al. [80] for CRC genomes under anti-EGFR therapy. Shu et al. [75] examined a range of cancer types, and in one patient cohort, they compared the data from tissue samples and ctDNA, thus confirming the use of MPS-based profiling of ctDNA to reach a treatment decision. Thierry et al. [81] have established the validity of the use of ctDNA rather than nuclear DNA for CRC patients when in the latter case some KRAS fragments can be identified as wild type while the ctDNA data show them to be mutant KRAS fragments. The initiation of a number of treatments leads to the appearance of new mutated ctDNA fragments in the blood during primary treatment so resulting in resistance to that particular treatment. It

should be emphasized that the eventual ctDNA increase after commencing and during primary treatment is not due necessarily to an increase in the same mutant fragments as seen in the first series of measurements. The new mutant ctDNA fragments appear to be derived from the remaining tumor cell types that can move into the space left by the treatment of the original dominant mutant cells [75, 81–84]. The early identification of the appearance of new mutations in response to treatment and resulting in treatment inhibition was showed by Misale et al. [85]; they used *in vitro* systems concerning cetuximab-resistant variants of two CRC cellular models (DiFi, Lim1215) that are highly sensitive to epidermal growth factor receptor (EGFR) inhibition. Furthermore, they identified the acquisition of either G13D or G12R KRAS mutations by sequence analysis of the Lim1215 cetuximab-resistant variants. Similar results were obtained by Murtaza et al. [86] using exome sequencing of plasma ctDNA from patients with breast, ovarian, and lung cancers. Subsequently, Bettegowda et al. [87] studied 206 patients with metastatic colorectal cancers and showed an 87.2% sensitivity of ctDNA for detection of clinically relevant KRAS gene mutations with a specificity of 99.2%. Of 24 patients showing resistance to EGFR blockade and with a subsequent relapse after an initial response to therapy, 23 patients (96%) developed one or more mutations in genes involved in the mitogen-activated protein kinase pathway. The identification of the presence of mutant KRAS ctDNA fragments in the plasma of CRC patients should identify them as potential nonresponders to anti-EGFR treatment.

In the case of NSCLC, Singh et al. [88] have compared the use of both tissue DNA and ctDNA analysis. Although first-generation EGFR tyrosine kinase inhibitors (TKIs) yielded a good response by patients, the majority of them developed resistance usually within 1 year of therapy. The most common resistance mutation is T790M [83]. Continued ctDNA analysis could identify further driver EGFR mutations/resistance mutations, which could have important predictive and prognostic properties. Hence, the appearance of, e.g., T790M, could indicate a need to switch treatment to third-generation TKIs.

In a study regarding ESR1 mutation during therapy for metastatic breast cancer, Schiavon et al. [79] determined the presence of ESR1 mutations present in patient ctDNA samples. Their findings indicated that ESR1 mutations are detectable in the ctDNA fraction from women with estrogen receptor-positive breast cancer and that such mutations are acquired during metastatic disease therapy. Furthermore, using both dPCR and MPS, ESR1 mutations were rarely acquired during adjuvant aromatase inhibitors (AIs) treatment but were frequently found during metastatic AI therapy.

In the case of pancreatic ductal adenocarcinoma (PDAC), Perets et al. [78] were able to show in a preliminary study that,

using MPS, ctDNA contained KRAS mutations that could be used to monitor treatment response in PDAC patients. This could permit the avoidance of unnecessary chemotherapy in metastatic patients.

Thus, a general approach to the use of ctDNA for the determination of new mutant ctDNA fragments after the initiation of primary treatment may offer a general approach to determining the basis of treatment failure at an early stage, thus leading to an early modification of the available therapy. It is clear from the data so far obtained that ctDNA offers a viable alternative analytical approach to that of DNA derived from solid biopsy material which may not contain examples of all of the modified cells in the tumor and which may permit the misanalyses of some DNA.

4.4 Prognosis

A number of researchers have reported the successful exploitation of ctDNA to determine a prognosis. Early studies indicated that there was a high correlation between ctDNA and metastatic CRC (mCRC) patient survival; i.e., those with relatively low levels of ctDNA had a significantly longer lifetime than patients with higher levels [87]. Other studies indicated a similar association between ctDNA and survival for various cancers, e.g., advanced breast cancer [89], lung cancer [90, 91], prostate cancer [92], and pancreatic cancer [93]; Perets et al. [78] showed that mutant KRAS ctDNA fragments present in blood acted as a marker for poor prognosis in metastatic PDAC patients. A more in-depth study [24] on the overall survival (OS) of mCRC patients was based on the determination of the main KRAS exon2 and BRAF V600E mutations together with total cfDNA concentration, mutant cfDNA concentration, the proportion of mutant cfDNA, and cfDNA integrity index. Again, those patients with high levels of mutant ctDNA had significantly shorter OS with a high ctDNA level being shown to be an independent prognostic factor.

Thus, although total cfDNA and ctDNA levels cannot reliably indicate information concerning cancer patients, it is likely that ctDNA levels, and, more specifically, mutation levels, can be useful guides for OS for a number of different cancers.

5 Future Developments

It is clear that monitoring cancer patients using ctDNA has a basis on which can be built stronger and specific tests with good sensitivity and specificity to replace the current exploitation of biopsy material—the gold standard. This will result in a cheaper and faster turnaround time to obtain analytical results as well as permitting patient's progress through monitoring by a number of liquid biopsies as opposed to the very limited number of solid biopsies that could be reasonably taken from the patient.

One important aspect concerns the need for a methodology standardization. This includes drawing blood, preparation and storage of serum and plasma samples, DNA isolation method, and isolated DNA storage prior to analysis [26].

Given the need for frequent repeated liquid biopsies per patient and ctDNA analysis, the cheapest analytical approaches yielding rapid turnaround times will be important for the routine use of this approach. At present, PCR-based methods permit this with costs in the region of \$200 per test and turnaround times being as low as 2 days for the results. It is hoped that the costs and turnaround times using MPS methodology will be improved in the near future.

In the near future will be the analysis of liquid biopsies by PCR-based methods, and then it is reasonable to accept that each cancer treatment center will be able to sustain the technical costs. However, MPS brings additional analytical advantages in some cases, and this may be too expensive. Therefore, the question arises as to whether the equipment and experienced staff should be in a centralized facility available to a number of cancer centers. This question may be resolved by the recent development of a new version of a portable nucleic acid detection platform (SHERLOCK) that exploits CRISPR-Cas13 combined with Csm6 to detect single molecules of both RNA and DNA [94]. This approach has proved successful in preliminary studies with a range of sequences associated with ctDNA samples from a variety of cancer patients. It will be interesting to determine the application of this approach for all fractions of ctDNA in a full clinical situation. While the development of the use of liquid biopsies is still in its relatively developmental stages, new studies are essentially based upon adult cancers. It should not be forgotten that many major childhood cancer entities are exclusive to children [95, 96], and the extension of a ctDNA analytical approach to these cancers would provide an interesting extension of the liquid biopsy approach.

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Chapter 4

Cell-Free DNA in the Liquid Biopsy Context: Role and Differences Between ctDNA and CTC Marker in Cancer Management

Pietro Fici

Abstract

Liquid biopsy is a new diagnostic concept to investigate the molecular features of solid tumors by blood, saliva, urine, and any other body fluids which show a source of potential biomarkers. In cancer patients, it is a simple and less invasive mean, representing a sustainable alternative to interrogate all tumor cells longitudinally, quantifying and characterizing the biological materials (DNAs, RNAs, proteins) which originate from cancer tissues. Circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) analysis from a simple blood draw received enormous attention for the related clinical research results. A rich scientific literature demonstrates that liquid biopsy is a valid instrument to assess the tumor biomarkers in real time and profile the cancer genotype in diagnostic and prognostic field, as well to quantify minimal residual disease, during patient follow-up. This could be a breakthrough for a companion diagnostic and personalized medicine. Liquid biopsy needs further implementation in the methodological aspects as well as cost-based assessment. The number of new molecular diagnostic assays increases day by day, but the standards for their adoption and clinical validation are still to be achieved.

Key words Liquid biopsy, Circulating tumor DNA, Circulating tumor cells, ctDNA, CTC, Minimal residual disease, Methods

1 Introduction

A deep characterization of the molecular landscape of tumor tissue represents the main practice in cancer diagnosis and management. Understanding the genetic cause of disease in the primary tissue and profiling the known prognostic and predictive tumor markers represent an accurate guide to effective treatment, especially for a target therapy approach [1].

However, the molecular profile of tumor evolves dynamically over time and in a branched fashion, showing a high genetic and phenotypic heterogeneity, not only between different patients but even intra-tumor and between primary and metastatic lesion. It is a

result of a cancer cell response to endogenous and exogenous selective force, like for a “Darwin’s theory” [2]. Tumor heterogeneity is a result of two different but interconnected biological processes [3]: internal genetic instability that leads to fixed genetic changes and the “resilient” molecular divergence in gene expression. The first evolves gradually during the tumor progression by increasing the amount of clonal diversity, in particular in new metastases development. The second type could be influenced not only by a constitutive mechanism but also by environmental stress [4]. In clinical practice, both phenomena should be monitored in depth before and during the treatment to diagnose the cancer disease, monitor response to treatment, assess the emergence of drug resistance, and quantify minimal residual disease (MRD).

To date, surgical or biopsy tissue sample are the principal sources to develop a molecular diagnosis and profile of tumor tissue, and they are essential to define therapeutic strategy. However, some tumor types are inoperable (as metastatic disease), and biopsy could show some limits and biases. The risk of operator’s sampling bias, the difficulty to perform the biopsy due to the inaccessibility of the tumor site and metastatic lesion, especially in advanced cancer patients with multiple metastases, with the impossibility to obtain repeatedly sampling, makes the tissue biopsy an invasive and costly test with few and uncertain results. Also, the tumor heterogeneity and the ability of cancer genomes to evolve are not properly highlighted by tissue specimens.

Liquid biopsy is a new diagnostic concept to investigate the molecular landscape of solid tumors using blood, but also saliva, urine, cerebrospinal fluid, and other body fluids. In cancer patients, it is a simple and less invasive mean, representing a sustainable alternative to interrogate tumor cells longitudinally. The human blood contains a high quantity of materials originated from different tissues including cancer: circulating free DNA (cfDNA); circulating free RNA (cfRNA), especially miRNA, proteins, and extracellular vesicles (EVs); and circulating tumor cells (CTCs). Circulating marker analysis in the blood of patients with cancer has received enormous attention as it seems to be an optimal instrument for personalized medicine [1]. Thus, analyzing a single peripheral blood draw, it is possible to assess relevant tumor biomarkers in real time and to determine the cancer genomic profile with diagnostic, prognostic, and predictive purposes. This is helpful to define a patients’ stratification based on the presence of therapeutic targets or the emergence of resistance mechanisms or the MRD during follow-up [5].

CTC spread by tumor tissue and circulating tumor DNA (ctDNA) release from apoptotic or necrotic tumor cells contain genetic defects and molecular features identical to the tumor tissue and/or cells they originate from. Over the last years, liquid biopsy has begun to attract interest in the context of cancer molecular

diagnostics, for a routinely practice. Clinical applications of CTC and ctDNA in patients with cancer have paved new diagnostic avenues information obtained from both sources [6]. However, biological features and technologies make the liquid biopsy a still widespread practice. The application of CTC and ctDNA for the early detection of cancer faces serious challenges regarding specificity and sensitivity of the current assays. To date, the risk of false negative results for both biomarkers demonstrates/suggests the need to develop more sensitive cellular and molecular assays, which should allow researchers to thoroughly analyze blood for tumorspecific aberration [6]. Even if both these have been accused to be competing biomarkers, the scientific evidences suggested that the clinical and biological information could be different, complementary, and depends on the context of use [7, 8].

2 ctDNA

ctDNA is composed by DNA fragments released from primary tumors, CTC, micro or overt metastases into the bloodstream of cancer patients. The majority of such ctDNA is derived from apoptotic and necrotic tumor cells, and probably by other mechanisms that remain to be clarified. Fifty years ago the first description of the circulating free DNA dates reported the presence of high levels of DNA in the blood stream of patients with cancer compared to the health control groups [9], and the interest of the medical scientific world, in particular of oncologists, has been growing year by year.

Cell-free DNA is also loss by normal host cells died during the wild replacement of cells in tissue population. Apoptotic tissue and hematological cells which release their DNA in the circulation are the majority source of cfDNA in a body fluid, while the ctDNA generated also from the necrotic tissue that often characterizes tumor masses [10, 11]. Furthermore, phagocytosis of necrotic neoplastic cells by macrophages seems to have a role in the tumor DNA fragments release. Comparing the size distribution of cfDNA between healthy and cancer patients, data suggest apoptosis as the main source of both total cfDNA and ctDNA [12]. In a sample with high content of free DNA, ctDNA fraction could be reduced up to <1.0% of the total cfDNA, reducing the possibility to detect the tumor-specific fraction. It results in a low opportunity to detect the important molecular information, especially in cancer patients with tissue damage by surgery, chemotherapy, or radiotherapy. Thus, the absolute quantity of cfDNA (“the background”) determines the sensitivity of the assay to detect ctDNA, and quantification of the tumor-specific variant frequency (specificity of assay) depends both on the abundance of ctDNA molecules and on the total amount of cfDNA [13].

Due to the technological limitations in terms of sensitivity of the standard sequencing approaches (e.g., Sanger sequencing, pyrosequencing), the ctDNA analysis is limited only in patients with heavy tumor burden and high levels of ctDNA [12, 14]. A big breakthrough comes from technology improvements of the last 10 years in molecular assays currently available, facilitating the tumor genomes analysis by ctDNA in term of sensitivity.

This happened with the implementation of new targeted procedures such as the digital PCR (dPCR), the microfluidic system BEAMing digital PCR of single molecule on microparticles in water in oil emulsion, and the microfluidic droplet digital PCR (ddPCR), based on the same technology. These methodological advancements increased the sensitivity of the ctDNA assays, respectively, up to 0.01%, and between 0.05% and 0.001%, but for limited detection from 1 to 2 by dPCR up to 5–10 target sequences by ddPCR.

Nontarget procedure, as the conventional next-generation sequencing (NGS), shows a not deep sensitivity, around 2–5%, but this allows to detect a high number of molecular aberration as SNVs, insertion and deletion, simultaneously, through a massive parallel sequencing [15].

dPCR assays are quantitative and highly sensitive and are used extensively to quantify ctDNA levels. Nevertheless, the limited multiplexing capacity of these assays limits their use to investigate a small number of mutations and is often applied to analyze cancer hotspot mutation. To analyze a high number of loci, NGS by target sequencing, using PCR amplicons or hybrid capture probe, has been used with good results. In this field, using molecular barcoding or running multiple replicates to reduce the background error rate, ctDNA can be detected at allele fraction below 0.1% [16]. Sensitivity of these tests could be enhanced using multiplexed patient-specific panels in combination with targeted sequencing method, but reducing the amount of material for the single analysis by the sample split is possible to increase sampling error and may impair the overall performance of an assay for very low copy numbers of mutant DNA. Cancer-specific copy number variation of DNA, by amplification and deletion, can be analyzed by low-depth sequencing of the whole genome with a limited detection of a mutant allele fraction of 5% and 10%, which is not sufficient for an early detection [16]. On the contrary, a higher sensitivity may be achieved through targeted sequencing of single-nucleotide polymorphisms, which may detect copy number alterations as low as 0.5%.

The low fraction of ctDNA in blood samples in variable amounts of cfDNA remains a challenge. The specificity of ctDNA-based tests is also insufficient as demonstrated by cancer-associated driver mutations found out in more than 10% of non-cancer control groups of different studies, especially in persons older than 65 years, who never develop cancer during their lifetime [1].

Other most important factors impacting on the quality of ctDNA analysis are showed by the sampling time, the biological source used to obtain cfDNA, plasma or serum, the time intercourse from the collection to the cfDNA extraction, as well as the methods used. cfDNA clearance, which occurs via the spleen, liver, and kidney, is rapid with a half-life of few minutes, and it can be biased by biological process like circadian rhythms and inflammation or other pathological conditions and pharmacological treatments, but this aspect is poorly understood.

Both plasma and serum contain cfDNA, but plasma is preferable to serum for the analysis. Plasma samples showed lower background levels of wild-type DNA, proving to be a better source of ctDNA. Serum samples reveled high quantity of total cfDNA, up to more than 20-fold than in plasma, but this is probably due to contamination of DNA released from immune cell lyses during the clotting process.

The time lapses between the blood sampling and the cfDNA processing are strategic indeed, because here this increases the release of wild-type DNA from lysed hematological cells present in the sample tube [17].

Thus, to improve the cfDNA analysis with the aim to implement ctDNA in the current clinical practice, some standardization issues are needed.

To avoid pre-analytical variability, it is suggested to use specialized collecting tubes, such as Streck Cell-free DNA BCT, Roche CE-IVD Cell-free DNA Collection Tube, and Qiagen PAX-gene Blood cfDNA tubes, to stabilize blood samples at room temperature and prevent lysis of white blood cells, thus reducing the risk of cfDNA contamination from germinal DNA. Alternatively, it is possible to collect the blood by the ethylenediaminetetraacetic acid (EDTA) tubes, which are a less expensive but which need to perform the plasma separation from the blood sample immediately or within few hours (no much more than 6 h) after the draw, by centrifugation conditions to reach the maximum final ctDNA yield [6]. Afterward, an optimized ctDNA isolation protocol and defined quantification methods, using fluorescent dyes, spectrophotometry, or qPCR, are needed to obtain the maximum and certain final yield for the subsequent analysis.

Moreover, if important improvements are done in the last years with the development of much more sensitive methodologies for detecting ctDNA, digital genomic technologies that allow for enumeration of rare mutant variants in complex mixtures of DNA, monitoring multiple tumor-specific mutations in a single assay, are still not available [18]. Although, it's important to take into account that an analytical variability could be determined by the molecular analysis processes as: PCR errors, non-uniform genomic coverage of the target sequence and other technical errors. Indeed, at the moment of the analysis, it is important to consider the

inherent biological variability, both spatial and temporal tumor heterogeneity, which could be not totally represented by the blood sample collected in a specific moment [19].

As emerged by a joint review between the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP), there is not yet sufficient evidence of clinical validity and utility for the majority of ctDNA assays in advanced cancer [19]. Evidences show discordance between the results of ctDNA assays and genotyping tumor specimens and support tumor tissue genotyping to confirm undetected results from ctDNA tests. There is no evidence of clinical utility and little evidence of clinical validity of ctDNA assays for cancer screening of early-stage or residual disease detection. Given the rapid pace of research, reevaluation of the literature will shortly be required, along with the development of tools and guidance for clinical practice [19]. Despite uncertainties around pre-analytical considerations, analytical validity, and clinical validity and utility, except for clinical trials, the use of assays assessing genomic variants in ctDNA is increasing in the oncology clinical setting.

3 Circulating Tumor Cells (CTCs)

CTCs are cells shedding from solid primary tumor and/or metastatic lesions into the peripheral blood, directly or by lymphatic vessels. These cells can trek around in circulation throughout the human body as single cells or as aggregated tumor cells, which are defined as circulating tumor microemboli or “collective tumor cells migration” (CTM), which are associated with a higher metastatic potential [6]. CTCs are considered a major player in metastatic procedure resulting in the main keys to fully understand and counteract metastasis development.

CTCs were first described in 1869 by Thomas Ashworth who postulated that “cells identical with those of the cancer itself being seen in the blood may tend to throw some light upon the mode of origin of multiple tumors existing in the same person” [20]. Considering the theory proposed by Stephan Paget 20 years later, the “seed and soil,” explaining that “When a plant goes to seed, its seeds are carried in all directions; but they can only live and grow if they fall on congenial soil” [21], the CTC, as seeds for the subsequent initiation of distant site metastases, are responsible for the cancer-related deaths [3].

CTCs are multifaceted entities. They are cancer cells that actively or passively move from the primary tumor mass, following multiple potential patterns of dissemination to distant localization and, as a consequence, adaptation to different tissue microenvironments. These can reach the bloodstream by passive detached from tumor tissue, especially in highly vascularized solid tumor, or by the

epithelial mesenchymal transition (EMT) process which is considered the most CTC-producing mechanism. EMT in cancer cell with an epithelial origin induces a mesenchymal-like phenotype acquisition, becoming cancer cell with a motile and aggressive features, and it enhances their survivor ability in the bloodstream. Indeed, the same process induces a stemness-like features which helps cancer cell to acquire chemoresistance ability [22], and in CTC, it helps to survive to blood circulation stress, as to maintain their tumorigenic potential [2].

It is known that cancer tissues are not composed by a homogeneous cell population, but these show a multi-clonal collection of cells with distinct molecular signatures. Cancer heterogeneity results in a random distribution of genetically distinct tumor cell subpopulations in disease sites, known as spatial heterogeneity, and in temporal variations in the molecular makeup of cancer cells, which is named temporal heterogeneity [23]. This condition results in variable rates of proliferation, aggressiveness, and drug sensitivity, all of which affect a patient's diagnosis and prognosis. In addition, this heterogeneity is reflected in differential levels of sensitivity to treatment, determining one of the principal aspects of therapy resistance of a tumor.

CTCs are exceptionally rare and heterogeneous blood elements that include a mixture of subpopulations with dissimilar molecular, phenotypic, and functional features [24]. These could be considered a representation of the starting tumor tissue heterogeneity. Their characterization could be a help to dissect the complex clonal architecture of cancers, providing insights into tumor heterogeneity, thus a potentially highly accessible resource for molecular information of cancer disease in clinical assessments, with the opportunity to facilitate the development of more effective personalized therapies [2].

Despite the study on CTC isolation and analysis in clinical application began since 1930s, due to a long being in the technical limitation, only in the last decade the technical solutions developed allowed to investigate these rare cells in circulation, deeply. The main obstacles in CTC's isolation and characterization are due to their rarity, approximately $1 \text{ cell} \times 10^9 \text{ cells}$ (in metastatic cancer patients) or less in early cancer stage, their variability in different tumor types, and their heterogeneity consisting in different phenotypic and functional characteristics [25]. Indeed, the clinical relevance of CTC detection in cancer patients depends on the test method.

4 CTC Study Methods: Strengths and Weaknesses

Isolation and identification methods for CTC are closely related/connected to the biological and physical features of CTC, and their rarity makes it more difficult.

CellSearch system (Menarini Silicon Biosystems, Inc. 2018) is the only validated and cleared method by the US Food and Drug Administration for CTC detection and introduced in 2004 where the analytical accuracy, reproducibility, and linearity of the system were shown. The CellSearch system is designed for the enumeration of CTC from 7.5 mL of blood, by immunomagnetic enrichment-based method, using antibody directed on EpCAM expression, main marker antigen of epithelial cell, and a fluorescent labeling detection of cell. Cells positive to nuclear staining are identified as CTC for their epithelial origin by cytokeratins 8, 18, and/or 19 antibody and discriminate from blood cells for the negativity to the lymphocyte common antigen CD45. In this process, blood sample could be collect by the CellSave® blood draw tube, which were developed to process blood sample up to 96 h after blood draw, or by EDTA blood draw tube, if it needed to obtain viable cells for culture cell or extraction of RNA from the enriched cells [26].

Despite it is defined the gold standard CTC analytic platform, as it is based on epithelial markers enrichment and detection, this fails to detect all the potential CTC subpopulations, due to the connection with EMT and stemness. In addition, the low CTC presence in the peripheral blood leads to analyze a larger blood volume, or a larger number of blood samples, wishing to increase the chances for detecting CTC. A statistical study revealed that, to detect a frequency of 10 cells in a 10^6 cell population (at a 95% level of assurance), using flowcytometry, more than 15×10^6 cells need to be analyzed. Indeed, a study conducted on 7.5 mL sample showed a median of a single CTC (range from 0 to 4) in 7.5 mL of blood in 13% of analyzed samples, while analyzing 30 mL blood volume samples allowed a detection rate of two CTCs (range from 0 to 9) in 47% of the analyzed samples, demonstrating the high variability of CTC detection depending on sample volume [27].

Novel devices designed to enrich and detect CTC are more efficient compared to past technology where the detection rates of CTC might be 100-fold higher [28]. Nevertheless, CTCs are considered fragile cells, because they exist independently without the supporting tissue, and several studies have found CTC in apoptotic cascade. These aspects could further reduce the clearance of CTC in peripheral blood, and gentle isolation methods to preserve the integrity of CTC are required.

In the last decade, a number of methods and technical solutions based on different biological and/or physical properties have been developed to study CTC. CTC identification and enrichment methods can be classified into three main types. The first is positive selection strategy, which includes positive enrichment of CTC by identification of epithelial cancer-specific markers on membrane, cytoplasm, nucleus, or cell surface. The second is negative selection strategy, performed by a depletion of hematopoietic cell blood cells by any means, by target-specific cell

retention and discard. The third is label-free isolation strategy that is performed by exploiting intrinsic biophysical feature of CTC, as dimension and densitometry, including size-based, morphology-based, and/or other physical property-based isolation strategy. Moreover, a combination of two or more strategies mentioned above could be considered a fourth strategy. Even if the lack in technical standardization still limits a full translation of CTC in the clinical practice, thank to these alternative methods the real nature of CTC could be understood.

Almost all of these involve an enrichment phase, with detection and isolation of CTCs, followed by the identification and / or characterization test of the cells of interest. The main analysis approaches are shown in Table 1, reporting and integrating the previous summary table of Hsieh and Wu [29]. Many of the methods reported are able to efficiently analyze CTC, but each approach owns strengths and weaknesses for any single step. Following the enrichment step, CTC can be further analyzed and characterized, but it is important to specify that the intermediate recovery step is crucial to have a good molecular and functional analysis below. Recovery strategies, including cell and molecular biology techniques, depend on the material and method used for CTC enrichment step. The reduction of regional adhesive forces in cells, using shear forces, and enzyme digestion could be used for the active cell detachment from the functionalized support utilized in positive CTC selection. Using biomaterial and electrical engineering strategies, it is possible to employ polymers responsive to physical and electrophysical stimuli such as solvent composition, pH, temperature, ionic strength, light, mechanical force, and electric and magnetic fields. In this field, the aptamers, oligonucleotide, or peptide molecules able to bind to a specific target molecule have one of the best detachment properties, simplifying enrichment and detachment of intact CTC for good downstream analysis. However, the action of intact and live target cells still remains a challenge [28].

Downstream analyses consist of two main approaches for CTC detection, which are immunological assays using monoclonal antibodies and PCR-based assays (cytology and molecular biology approaches), and both exploit tissue- and/or tumor-specific antigen expression, comprising detection of specific somatic DNA variations and disease and/or phenotype-specific mRNA expression pattern, as well as morphology and functional characteristics (Table 1) [29, 30].

Table 1
Detection and molecular characterization CTC methods

Enrichment strategy	System	Enrichment and/or detection markers	Molecular analysis	CTC cell culture	Pros	Cons	Ref.
Positive selection	CellSearch	EpCAM, CKs, CD45, DAPI	DNA	No	FDA approved; reproducible; clinical relevance in metastatic breast, colorectal, and prostate cancer and early breast; semiautomated processing; capable of detecting smaller CTCs; standardize kits	EpCAM positivity dependent; expensive; cells losing EpCAM could not be detected; limited number of markers; identification by observation. Validation operator dependent	[33, 95]
CTC chip		EpCAM, CKs, CD45, DAPI	DNA and RNA	Yes	High detection rate, visual identification, viable cell	EpCAM positivity dependent; cells losing EpCAM could not be detected; require clinical trial validation	[96]
Ariol system		EpCAM, CKs, CD45, DAPI			High detection rate (versus CellSearch)	EpCAM positivity dependent	[97]
Laser scanning cytometer			DNA	No	Automated microscopic procedure, high detection rate	EpCAM positivity dependent, bias operator dependent	[98]
Adna test		EpCAM, MUC1, mucin-1, HER2, PSMA or EMT/Stem markers	Detection by RNA	No	High sensitivity; rapid processing; breast, lung, and prostate cancer	No morphology confirmation; EpCAM and MUC1 positivity dependent; operator dependent	[95]
GILUPI CellCollectors			DNA	No	High sensitivity	Invasive test by wired in the vein of patient; few blood volume screened	[99]

Negative selection	EPISBOT assay	CD45, CK19, mucin-1, cathepsin-D	DNA and RNA	Yes	Can detect viable CTCs by enzymatic activity	Lacks enough clinical trials for validation; operator and condition dependent [100]
PowerMag	CD45 depletion for four repeated times, EpCAM, Hoechst				Clinically validated in several cancer types; viable CTCs	Background noise; subjective judgment of CTCs; labor-intensive; limited markers
EasySep™	CD36 or CD56 depletion by immunomagnetic system	DNA and RNA	Yes	Several cancer types, viable CTCs,	Several cancer types, viable CTCs,	can be used for a sample
Direct Human CTC Enrichment/ RosetteSep™ CTC	CD45, EpCAM, CKs, CD133, CD44, or other custom solution	DNA and RNA (single cell also)		High sensitivity for multiple markers, high purity of isolation	High sensitivity for multiple markers, high purity of isolation	No clinical validity, background noise, subjective judgment of CTCs, operator-dependent [102]
Negative + flow cytometry, FACS	Size, CKs, Her2/neu, ALDH1, CD44, CD24	DNA and RNA		Rapid processing; multiplexed imaging and genetic analysis	Controls, cell aggregations, laser compensation, operator-dependent	[103]
Label-free (size)	ScreenCell® MB, Size CC, and Cyto	DNA and RNA (MB kit)	Yes (CC kit)	Fixed or live cell	Limited by size of CTCs variation	[30]
ISET	Size, CKs, EGFR, VE-cadherin, ki67	DNA and RNA	Yes	Rapid processing; nonantigen dependent; able to isolate CTC; cell illustrated by IHC staining; able to perform different molecular analysis	Few blood volume screened; operator dependent; different kits for different use	[104]

(continued)

Table 1
(continued)

Enrichment strategy	System	Enrichment and/or detection markers	Molecular analysis	CTC cell culture	Pros	Cons	Ref.
DEP force (DEPArray™)	Size, surface electricity; viability	DNA single cell	Rapid processing, can isolate single cell very precisely	Limited throughput; time-consuming; high cost	[106, 107]		
Tracheal carina-inspired bifurcated (TRAB) microfilter system	Size		High recovery rate; acceptable purity; viable isolation	Require clinical trial validation	[108]		
Label-free (gradient)	Ficoll + RT-PCR-qPCR	CK-19, HER2, h-MAM, CEA, maspin, GABA A, B726P and other	DNA and RNA	High sensitivity, customized	No morphology confirmation; not really captured CTCs	[30]	
	OncoQuick®	EpCAM, pan-CK, CK-8/18/19, h-MAM, or other customized	DNA and RNA	High sensitivity; quantification; customized	No morphology confirmation; not really captured CTCs. <i>Only enrichment</i>	[106, 107]	
Other	Magnetic activated cell sorting (MACS)	CD45 for neg selection, while EpCAM for positive enrichment	DNA	No	Negative or positive enrichment depending on the antibodies used	Limited to the targets chosen	[109]

5 Clinical Impact of Liquid Biopsy (CTC and/or ctDNA)

Liquid biopsy is a promising concept to comprehensively characterize a patient's disease and critical for the evaluation of cancer progression, in a minimally invasive way.

A tumor landscape characterization could be possible by both CTC and ctDNA analysis, which appeared as valid cancer biomarker sources from the results of different research studies. Many works have demonstrated the potential of liquid biopsy approaches to determine the genomic profile of cancer patients, as well as to monitor treatment response and quantify MRD, or assess the emergence of therapy resistance [12].

The existence and utility of CTC as a precondition of metastases formation and as a tool to understand the process have been recognized already in 2010, when a new classification of cancer stadiation, cM0(i+), was added to the newly released edition of the cancer staging system [31]. It was defined to include patients with detectable tumor cells in circulating blood or bone marrow but without other detectable metastases. A large amount of clinical studies on CTC enumeration indicates that the tumor cell loads in the peripheral blood of metastatic cancer patients correlate with a high disease aggressiveness, and it is an early prognostic marker of outcome as well in response to therapy.

Using the FDA-approved CellSearch system, CTCs were detected in the blood of patients with different carcinoma, including the colon, prostate, breast, ovary, and lung, but not in healthy subjects [32]. These findings as confirmed in a multicenter study by Cristofanilli et al. determined that a CTC enumeration ≥ 5 in 7.5 mL of blood, at baseline and follow-up in patients with metastatic breast cancer (MBC), is an independent predictor of progression-free survival (PFS) and overall survival (OS) in patients with metastatic breast cancer [33]. One or more CTC detection predicts early recurrence and decreases overall survival (OS) also in untreated patients with non-MBC compared to patients without detectable CTC, and independently to other clinical cancer markers [34]. Many other clinical trials performed using CellSearch system correlates the CTC presence with a poor outcome in other different metastatic and non-metastatic cancer disease [7, 12]. CTC count has been considered also a useful method to evaluate the response to therapeutic intervention and provides information for treatment selection [5, 6]. However, the CTC numbers are highly variable between different tumor types, as well intra-patient for different spread time, and it is subjected to CTC detection bias related to the methods used. Indeed, using different methods in the same patient cohort, it is possible to obtain nonconcordant results in terms of CTC count as reported by two different studies where Adna Test (Qiagen) and ISET

system (RareCells) were, respectively, compared with CellSearch system [35, 36].

Beyond enumeration, there is interest in genotypic and phenotypic heterogeneity characterization of CTC by cellular and molecular analysis, as showed in a study conducted on a group of metastatic colon cancer patients [37], where the concomitant presence of different cell phenotypic subgroups can dynamically change in CTC composition during cancer treatment and/or relapse, and correlating the mesenchymal CTC phenotype with disease progression and therapy resistance [37]. Another study showed the utility of CTC in downstream molecular analysis to understand their heterogeneity, which could be improved by the implementation of other tumor cell marker to increase the range of detectable cells, such as EMT-related markers, CSC-like markers, and other cancer-specific marker as HER-2 in breast and AR-v7 in prostate cancers [38, 39]. However, the major challenge for the CTC use in diagnostic field has been the low number of circulating tumor cell isolated from cancer patients, especially if we consider in any each subpopulation, and in the early stage of the disease. In this way, a solution was represented by the cellular and molecular assays at single-cell level, technological development of the last years which helps us to understand deeply the cancer events.

Recently, phenotypic and molecular analysis of single CTC from cancer patients with different solid tumors were helpful to demonstrate and confirm that the CTC molecular features could change during the course of the disease, acquiring a new marker expression in the metastatic setting [38], as well as presenting two different CTC subpopulations with alternative response to specific chemotherapies [38], or showing a potential mechanism of resistant therapy [40].

These results, together with a number of other evidences obtained comparing surgical biopsies of metastasis to the matched primary tumor, highlight the poor stability and dynamically change showed by the commonly biomarkers used in cancer management [6]. Also, monitoring patients longitudinally by CTC analysis may enable more early decisions in cancer treatment based on individual cases [6].

CTC studies have demonstrated to be helpful in patient stratification, evaluating the likelihood of a patient to respond to a specific therapy before the treatment administration, investigating the presence of drug target not yet identified in the tumor tissue, or as surrogate of tumor biopsy specimens when this is absent (i.e., after surgery). Studies developed by the analysis of CNV pattern of single-cell CTC samples from SCLC patients [41] identified patients to be chemosensitive or chemorefractory in 83% of tested sample, using a pretreatment blood specimens (in vitro), and the results have been confirmed by patient follow-up [41]. As well, the study of PD-L1 expression by CTC in luminal-A breast cancer (ER+, PgR+, HER-2

negative) [42] and in melanoma CTC [43], which were analyzed before to preceding with the immune checkpoint inhibitors treatment [43], demonstrating their role in patient stratification before and monitoring of response to immune-checkpoint blockade after the treatment.

In this way, CTC could be propagated ex vivo under hypoxic conditions using a combination of growth factors and low attachment plates for more than 6 months [44–46]. Thus, it is possible to test large series of single or combination treatment solutions, directly on CTC cell culture or in more physiological complex model as the xenograft [44, 47]. However, this still remains a limited possibility to the conditions in which it is possible to isolate and propagate CTC cell cultures and/or create xenograft models by CTC (CDX); also, the in vitro or in vivo models now available could not reflect the real condition and event which could happen during the course of the disease.

In terms of cancer diagnosis, CTC could be considered a big challenge. Indeed, it is really difficult to be sure about a false-negative result. However, focusing on patient with a high risk of developing cancer, a study conducted in patients with chronic obstructive pulmonary disease (COPD) analyzing 168 COPD patients and 77 health subjects reported that CTCs were detectable in subjects without clinically diagnosable lung cancer [48]. CTCs were detected in 5 of 168 patients. The annual surveillance of the CTC-positive COPD patients by computed tomography (CT) scan screening detected lung nodules 1–4 years after CTC detection, leading to prompt surgical resection and histopathological diagnosis of early stage lung cancer [48].

In counterpart, plasma-derived ctDNA is the most used type of blood-based biomarker. In clinical practice, ctDNA analysis has been used to monitor the response to therapy, to evaluate the therapy resistance development, and to detect MRD during and after the treatment [1]. Today, many works demonstrated the presence of high level of cfDNA in both plasma and serum samples of cancer patients harboring molecular and genomic alterations reported in primary tumor tissues: point mutations, rearrangements, amplifications, and even gene copy variations [18, 49].

Measuring individual tumor mutations, ctDNA concentration in plasma has been shown to correlate with tumor size [50] and stage [18, 51]. In a study of 640 cancer patients with different types and stages of diseases, the concentration of ctDNA in plasma correlates with tumor size, suggesting a potential role as tumor stage assay [51].

These and other studies showed that patients with positive ctDNA analysis have poorer outcomes than those negative [52, 53]. The relationship between ctDNA levels and cancer stage suggests prognostic utility for ctDNA. In addition to ctDNA levels,

mutational patterns detectable in ctDNA can help to adopt a patient stratification into molecular subtypes with different prognosis [54].

With the aim to improve the patient outcome, an important application for the ctDNA could be the cancer diagnosis at an early stage, particularly before metastatic spread [16].

An important study demonstrated the concordance between molecular alteration detected in plasma ctDNA and tumor tissue [51, 55], as well as in early stage of the disease [56]. One of the first study conducted on 106 patients with mCRC, comparing ctDNA and traditional primary tissue analysis, proved that a quantitative PCR (qPCR) analysis of ctDNA was a valuable method for the detection of BRAF and KRAS mutations, as compared to traditional tissue biopsies [57]. KRAS mutation results were confirmed successively by Taly et al. by ddPCR, and different other studies also suggested a high rate of concordance between mutations observed in tumor biopsy and those identified in ctDNA [15, 58, 59]. Despite all these considerations, the detection of ctDNA is tricky for four main reasons: the discrimination of ctDNA from normal cfDNA; the commonly low amount of ctDNA in blood sample, especially at early stage, which needs a high sensitivity of the ctDNA assay; the low accuracy for mutant fragment quantification [18]; and the sample collection time which could be not representative of total tumor burden. Up to date, it appears possible in saliva and plasma from individuals in which mutations were detected up to 2 years prior to cancer diagnosis [60, 61], and other cancer types using different biological sources of ctDNA such as urine for bladder cancer [62] or stool for colorectal cancers [63], uterine lavage [64], or esophageal brushings [65] for gynecological or esophageal cancers, respectively. Also, in cancers with a viral etiology (e.g., nasopharyngeal carcinoma or cervical cancer), the discovery of an early stage of disease or premalignant lesion presence with a high risk for cancer is possible, thanks to the cancer-associated viral DNA detection [66, 67]. Unfortunately, a ctDNA analysis screening in asymptomatic populations is not still applicable, because the risks of overdiagnosis and false positives are too high [16].

ctDNA finds a good application also in MRD as demonstrated in prospective studies. Patients with different cancer diseases showed a positivity to ctDNA after adjuvant chemotherapy treatment, and it has been associated with a worse recurrence-free survival [12, 68]. In a study on patients with stage II colon cancer, ctDNA was detected postoperatively in 7.9% in patients without adjuvant chemotherapy, and among them, 79% had a recurrence [69]. ctDNA has also been shown to be relevant to predict recurrence after resection of locally advanced rectal cancer or liver metastases from CRC [69, 70], as well as in early breast cancer setting [56].

ctDNA detection may show the presence of few expressed mutated alleles, by minority clones, which could be missed by

standard-of-care as demonstrated by Bachet et al. in a multicenter study [58]. Thus, ctDNA shows a great potential role in molecular characterization of both intra-tumor heterogeneity [71, 72] and spatially separated disease foci [73–75].

On prediction and monitoring response to therapy, ctDNA analysis before the therapy and/or after represents a surrogate of tumor tissue biopsy to evaluate the response to therapy or the onset of resistance.

The variation of ctDNA concentration under treatment appears as a relevant early biomarker of personalized medicine in different cancer types. In a prospective study of 53 mCRC patients undergoing first-line chemotherapy, a ctDNA reduction among tenfold was registered between the first and second cycle of chemotherapy, correlating with the expected and with a long PFS trend [76].

Likewise, quantitative measurement of the known aberration in the plasma may be used to monitor patients under treatment, as a dynamic biomarker to monitor response to therapy. Considering that targeted drugs are only effective when certain mutations are present, Shu et al. identified a specific unique mutation spectrum in Chinese lung cancer patients which could be used to guide treatment decisions and monitor drug-resistant mutations [77]. In this study, certain mutations were identified avoiding unnecessary treatment, or in the recent PLACOL study by Garlan et al., designing a composite marker based on the “normalization” of the ctDNA concentration, they predict the efficacy of first- or second-line chemotherapy in CRC patients [78].

ctDNA analysis was proposed also to assess the development of therapy resistance, and it has begun to form the basis for treatment decision making. Different works demonstrated that ctDNA can monitor clonal evolution and identify resistance mechanisms to treatment [79]. In patients with NSCLC undergoing treatment with EGFR inhibitors, resistance conferring mutations emerged in plasma ahead of clinical progression [49, 80]. Similarly, results from a study on KRAS-mutated colorectal cancer revealed that under the selective pressure of target treatment, some patients developed secondary resistance to anti-EGFR antibody treatment, between 5 and 6 months following treatment [79]. They suggested that the emergence of KRAS mutations is a mediator of acquired resistance to EGFR blockade, and these mutations can be detected by liquid biopsy [79]. In another study, breast cancer patients resistant to endocrine therapy showed estrogen receptor 1 (ESR1) mutations in ctDNA which were not previously identified in the primary tumor [81, 82].

Siravegna et al. identified a subset of 8 among 100 patients (8%) with RAS mutations only detected in blood but not in the matched tumor tissue samples, interpreting the result as dynamic clonal com-

petition, a molecular evidence that rechallenge with EGFR inhibitors after a withdrawal period could be used for some patients [83].

In clinical practice, today, the determination of EGFR mutations in ctDNA is the first companion diagnostic test which has been approved by the regulatory agencies in Europe and in the USA. These interventional tests can now be used to guide anti-EGFR treatment in EGFR-mutated non-small cell lung cancer patients using blood when access to tissue is impaired [6].

Taking in consideration all the above studies, it is possible to understand that performing liquid biopsies, specifically by capturing CTCs and ctDNA in the plasma or serum of cancer patients, is a perfect strategy for clinical practice [8].

To investigate a liquid biopsy approach in advanced colorectal cancer (CRC), Kidess-Sigal and colleagues analyzed 15 patients with advanced CRC with liver metastasectomy, collecting 41 blood samples at 3 time points before and after surgery [84]. CTC analysis was conducted by Vortex Chip system for enrichment and downstream molecular analyses [85–87], to compare CTC's mutational status to that of primary and/or metastatic tumor tissue and ctDNA. KRAS, BRAF, and PIK3CA hotspot mutational profile (46 mutations in the 4 genes: KRAS, BRAF, PIK3CA, and EGFR) was done by SCODA (sequence-specific synchronous coefficient of drag alteration) mutation enrichment and detection technology [88], and the different patterns were compared to evaluate their concordance. Here, 80% of patient's blood samples were CTC positive, using a healthy baseline value as threshold (0.4 CTCs/mL), and they observed a relatively high concordance of 77.8% (7/9) between mutations found in CTCs compared to tissue biopsies, suggesting CTCs as noninvasive tools to monitor the mutational pattern [84]. CTC and ctDNA sample concordance was found for 23 samples with a 78.2% for KRAS, 73.9% for BRAF, and 91.3% for PIK3CA mutations, while in some samples CTCs exhibited a mutation undetected in relation to ctDNA, and vice versa [84]. However, the additional mutations detected either in CTCs or in ctDNA confirm the importance of both combined approaches.

In another precedent study, Madic et al. used the high prevalence of TP53 mutations in triple negative breast cancer (TNBC) to compare ctDNA and CTCs detection rates and evaluate their prognostic value in metastatic TNBC patients [89]. By different markers and different methodologies to compare ctDNA and CTCs detection rates, the presence of TP53 for ctDNA was verified by NGS, and the CellSearch™ for the enumeration of CTC. They showed that CTC numbers were correlated with OS of patients, while they reported an absence of prognostic impact of baseline ctDNA level. They thus suggested that mechanisms of ctDNA release in metastatic TNBC may be involved beyond tumor burden and that these biological features not dramatically affect patient outcome [89].

During the last year, other studies tested different solution of liquid biopsy combining ctDNA and CTC analysis to characterize the tumor landscape in different cancers, especially with the aim to improve patient stratification.

With the coming availability of third-generation EGFR TKI drugs, noninvasive approaches to T790M detection will become necessary for the patient management in non-small cell lung cancers (NSCLC) [90]. With this aim, Sundaresan and colleagues in a recent work used liquid biopsy to investigate 40 patients with EGFR-mutant tumors progressing on EGFR TKI therapy and to compare tumor biopsies with simultaneously collected CTC and ctDNA samples for a T790M genotype associated to tyrosine kinase inhibitor (TKI) resistance. They used a specific ^{HB}CTC-Chip system [37, 91] for EpCAM-positive CTC enrichment and a target sequencing by cobas[®] EGFR Mutation Test v2 (Roche Molecular Systems, Inc., Pleasanton, CA). T790M genotypes were effectively found in 30 (75%) tumor biopsies, 28 (70%) CTC samples, and 32 (80%) ctDNA samples. The T790M was detected in half part of total patients using each genotyping assay, with a 57–74% of concordance among them, while in 20–30% of cases, CTC- and ctDNA-based genotyping were unsuccessful [90]. In addition, the two assays together enabled genotyping in all patients with an available blood sample, identifying the T790M mutation in 35% of patients in whom the biopsy was negative or undetermined. These results showed that two complementary approaches (CTC and ctDNA) may suggest a more specific therapy solution, but this should be validated [90].

In a recent study conducted on 153 breast cancer patients (92 early and 61 metastatic breast cancer), the researchers analyzed the plasma-derived ctDNA and the DNA from EpCam-positive CTC isolated from peripheral blood draws. Comparing the SOX17, CST6, and BRMS1 promoter methylation status in the two liquid biomarkers, and in 75 FFPE primary tissue samples by real-time methylation-specific PCR assay, they investigated whether the “liquid phase” of the cancer reflects the status of the primary tumor tissue [92]. Researchers reported no concordance between the EpCAM-positive CTC fraction and paired primary tumors for the promoter methylation status of any of the genes studied. However, they evidenced an association between the EpCAM-positive CTC fraction and ctDNA for SOX17 promoter methylation in both early and metastatic breast cancer patients ($P = 0.001$ and $P = 0.046$, respectively), but not for the other genes studied. These findings suggest that it is difficult to characterize the heterogeneity of tumor burden and a massive parallel sequencing of single CTC sample could be more informative. In addition, SOX17 methylation status, associated with CK-19 expression, both predicted a worse overall survival, but did not reflect the status of primary tumors in breast cancer. These data confirmed the important

significant prognostic value of ctDNA and CTC analysis, especially if they were integrated with an evaluation of methylation status of related cancer genes as SOX17. Nevertheless, the authors highlighted that further studies are needed to improve liquid biopsy protocols and data handling [92].

Recently, a retrospective study of Rossi and colleagues provided a real-time assessment of metastatic breast cancer (MBC), evaluating the utility to combine CTC and ctDNA for the prognosis prediction in these patients. CTCs were enumerated by CellSearch system, and the ctDNA samples were analyzed for a panel, called Guardant360, of more than 50 cancer genes associated with solid tumors by next-generation sequencing (NGS) (Guardant Digital Sequencing). This assay has a single-molecule analytical sensitivity and a 99.9999% specificity. The results showed a median of 2 CTC (0–5612) and the detection of expected mutations in 84% of ctDNA samples (232 of 277). TP53 (52%), PIK3CA (40%), and ERBB2 (20%) were the most commonly mutated genes. The median follow-up of the patients for both CTC and ctDNA was 9 and 9.9 months, respectively. For CTCs and ctDNA, respectively, progression-free survival (PFS) was 4.2 and 5.2 months, and OS was 18.7 and 21.5 months. There was a statistically significant difference in PFS and OS for baseline $\text{CTC} < 5$ versus $\text{CTC} \geq 5$ with a $P = 0.003$ and $P = 0.012$, respectively, and a significant association was found between the number of alterations and the percentage of ctDNA in the baseline sample ($P < 0.0001$) [93]. By these results, the researchers assumed that patients with a greater burden of disease were at higher risk of disease progression and had a poor outcome, requiring attention for treatment selection based on both subtype-specific guidelines and molecularly driven treatments, when appropriate [93]. They also confirmed the independent prognostic value of CTCs in OS ($P = 0.006$), whereas ctDNA results an independent prognostic factor of PFS, suggesting a possible use of ctDNA in catching the progression and the relapses. Here, the study of ctDNA added to CTC in a comprehensive liquid biopsy analysis, demonstrated that a combined liquid biopsy is an effective prognostic tool [93].

6 Conclusion

In clinical practice, the best treatment solution should start as early as possible to limit the cancer to the local site, reducing the metastasization events and avoiding unnecessary toxicity. Currently established diagnostic markers, such as tumor size, clinical and pathological features, and metastatic status, are thought to be insufficient for a treatment selection, and the use of tissue biopsies as a reference standard is questionable in the era of personalized medicine. However, tissue biopsies, at the present, remain the

gold standards reference for liquid biopsy analyses, and this concept is even adopted by regulatory agencies to approve tests [1].

The early dissemination of ctDNA and/or CTC may be exploitable events for early detection of cancer progression. The intra-tumor heterogeneity of the lesion biopsied used as a reference can be derived from lesions that were not biopsied and may contain a divergent genomic composition. In patients without metastatic lesions detectable by current imaging modalities (stage M0), occult micrometastases may contribute to the pool of ctDNA or CTC and, therefore, lead to different genomic landscapes than the primary tumor used as Ref. 1.

The concept of liquid biopsy aims at simple, fast, and cost-efficient monitoring of disease status or response to treatment. It offers several advantages compared to “conventional” tissue biopsy: liquid biopsy is less burdensome than a tissue biopsy, because body fluids like blood, saliva, or urine are much easier to access [94].

CTCs and ctDNA currently appear to be complementary: ctDNA provides a cost-effective and highly sensitive tool for the detection of mutations, in particular in selection of cancer-associated hotspots; CTCs represent a valid tool to investigate drug sensitivity, tumor aggressiveness and heterogeneity, mutations at a genome-wide scale, and also RNA and protein expression. It is likely that liquid biopsy will evolve in a cancer-specific fashion, focusing more on CTCs or ctDNA depending on the cancer type (or subtype) and the nature of its main alterations and mechanisms of drug resistance [6]. In this way, the approval by EMA and FDA for mutation detection in plasma and the use of CTC enumeration with the purpose to introduce the new classification of cM0(i+) are the first signs of the importance for liquid biopsy in clinical practice.

The increasing interest for liquid biopsy approach has determined the emergence of an overabundance of assays. The implementation of liquid biopsy on a large scale in the clinical setting will require a better understanding of the full potential and limitations of this technology, which will be only possible to dissect with large patient cohorts in multiple cancer types and across multiple centers [6]. In this intention, the European consortium CANCER-ID encompasses 37 institutions and provides a new platform for international assay validations (www.cancer-id.eu).

However, the possibility to use standardized pre-analytical sample handling procedures in addition to robust and reproducible workflows for the molecular analysis of liquid biopsy samples is urgently needed [94]. Up to date, CTC- and ctDNA-based investigations have been in essence proof-of-concept reports [6], but the future studies in these fields will represent the foundation of liquid biopsies and companion diagnostic, to improve clinical practice.

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Part III

Methods in Cancer



Chapter 5

Cell-Free DNA Integrity: Applications

Sara Ravaioli

Abstract

Cell-free DNA integrity (cfDNAi) could be a valuable biomarker for solid tumors, to define prognosis and response to therapy. Several elements have been studied for cfDNAi, such as specific genes involved in cancer progression or repetitive DNA sequences as surrogate markers for the whole circulating DNA.

However, the lack of a standardized method for cfDNAi evaluation remains one of its main critical issues. Apoptotic index (AI) and integrity index (II) of cfDNA could be useful biomarkers to identify the patients likely to recur, progress, or relapse, which is an urgent need in translational research in oncology.

Here it is provided an adequate method of cfDNAi analysis: a cheap and reproducible tool, robust and performable in all laboratories using a real-time PCR instrument.

Key words cfDNA integrity, Biomarker, Real-time PCR, Integrity index, Apoptotic index

1 Introduction

DNA is released into the bloodstream by the apoptotic and necrotic cells of the primary tumor from an early phase of the disease, thus permitting the determination of its genetic and epigenetic characteristics for different purposes [1]. Apoptotic cells release DNA fragments of 180–200 base pairs, whereas necrotic cells release higher molecular weight DNA fragments [1–5]. It has been shown that DNA from normal apoptotic cells is highly fragmented, whereas DNA from necrotic cancer cells maintains its integrity [2–5].

Apoptotic index (AI) and integrity index (II) could be evaluated as biomarkers to identify the patients likely to recur, progress, or relapse, which is an urgent need in oncologic translational research. The latter is calculated as the ratio of the quantity of long/short fragments; the former is inversely calculated [2–5].

Studies have long suggested that cell-free DNA integrity (cfDNAi) could be used as a potential prognostic biomarker for solid tumors [3–6]. However, the lack of a standardized method for its evaluation remains one of its main critical issues. It is well known

that cell-free DNA (cfDNA) evaluation on serum/plasma/urine samples even stored for a long period of time is feasible and reproducible [2–6]. In serum of patients with colorectal cancer, cfDNAi was demonstrated to be significantly increased in patients with stage III/IV disease as compared to patients with stage I/II disease and healthy controls [7]. There are also conflicting reports, as Madhavan and colleagues, who determined that decreased cfDNAi (defined as the concentration ratio of 260/100 bp fragments of ALU and LINE by quantitative PCR) correlates with worse outcome [8]. The decreased integrity may be due to higher apoptotic rates, occurring in highly proliferative tumors [8]. Persistent high cfDNAi in patients with nasopharyngeal carcinoma after radiotherapy was associated with reduced disease-free survival [9]. These findings are in line with Maltoni et al., which demonstrated that early breast cancer patients had significantly higher median values of cfDNA quantity (ng) than healthy controls, who showed higher II and lower AI [2].

There is no unique and validated method for studying cfDNAi, and it could be analyzed in different types of tumors, with different diagnostic, prognostic, and predictive purposes [2, 7, 10]. Several approaches have been used to study cfDNAi, including measurement of noncoding DNA sequences like repetitive ALU sequences [8, 11] or LINE1 (long interspersed nucleotide elements) [8, 11], GAPDH-gene [12], β -globin-gene [13], and hTERT [9]. Regarding the methods, there is a consensus among the different studies that a quantitative PCR approach remains the standard method for testing cfDNAi.

Conversely, no standard upstream procedures are defined to obtain the cfDNA from biological fluids. Usually plasma samples are preferred as compared to serum, due to the lysis of cellular blood components that cause an increase in cfDNAi. It is worthy of note that delayed blood sample processing results in elevated cfDNA levels [13–15]. In addition to plasma and serum, cfDNAi could be evaluated also from urine samples, with significant results in terms of sensitivity and specificity for urological tumor diagnosis [4–6].

Here it is provided an adequate method of analysis of cfDNAi that represents a cheap and reproducible tool, robust and performable in all laboratories that have a real-time PCR equipment.

2 Materials

Prepare all solutions with nuclease-free water. Wear gloves to avoid contaminating the reaction mixture or reagents and follow all waste disposal regulations.

2.1 DNA Samples Preparation

1. Extract and purify DNA from at least 200–500 µL of serum/plasma/urine (different recommended volume of sample could be used according to the specific purification kit) by spin column-based nucleic acid purification kit according to the manufacturer's instructions. At the same time, extract and purify DNA from a human genomic control or a cell line using the same kit.
2. Quantify the cfDNA by fluorimetric method.

2.2 Real-Time PCR

1. Dilute the human genomic/cell line control DNA to create six 100 µL standards of different concentrations: 0.001, 0.01, 0.1, 0.5, 1.0, and 2.0 ng/µL. Store the standards at –20 °C until use.
2. Dilute the cfDNA to 1 ng/µL and store the diluted samples at –20 °C until ready for the integrity test.

Primer set mixes are prepared at the final concentration of 5 µM, mixing equal amounts of forward and reverse primer in 100 µL of nuclease-free water (*see Note 1*) for further recommendations for primer design.

Sequences >260 bp (“long fragments”) are analyzed in addition to shorter sequences (\leq 125 bp, “short fragments”) of the same genes. An example of primer sequences and amplicons length are shown in Table 1.

Table 1
Primer sequences and amplicons length

Locus	Gene	Primer sequence	Fragment size (bp)
<i>17q12</i>	HER2 long	Fw-CCAGGGTGTTCCTCAGTTG Rev-TCAGTATGGCCTCACCCCTTC	295
	HER2 short	Fw-CCAGGGTGTTCCTCAGTTG Rev-GGAGTTCCTGCAGAGGACAG	126
<i>8q24</i>	MYC long	Fw-TGGAGTAGGGACCGCATATC Rev-ACCCAACACCACGTCTAAC	264
	MYC short	Fw-GGCATTAAATTTCGGCTCA Rev-AAAAGCCAATGCCAACTT	128
<i>20q.13.2</i>	BCAS1 long	Fw-GGGTCAGAGCTTCCTGTGAG Rev-CGTTGTCTGAAACAGAGCA	266
	BCAS1 short	Fw-GGGTCAGAGCTTCCTGTGAG Rev-TATCATGCCTTGAGAACCA	129
<i>3q26.3</i>	PI3KCA long	Fw-CTC CACGAC CAT CATCAGGT Rev-CGAAGGTACAAAGTCGTCT	274
	PI3KCA short	Fw-CTCCACGACCATCATCATCAGGT Rev-TGGTTATAATGAGCCTCACGG	129

3 Methods

Arrange all reagents needed for the cfDNAi test in a freshly filled ice bucket, and let them thaw completely before setting up a reaction. Thaw the cfDNA samples, the standards, the appropriate PCR primers, and green supermix (i.e., IQ SYBR Green—Bio-Rad, Milan, Italy) on ice. Vortex and spin the tubes briefly, when completely thawed. Then, set up PCR tubes/plate as shown in Fig. 1a. Keep the reagents on ice throughout the experiment. All real-time PCR reactions should be performed in duplicate on 10 ng of each DNA sample.

3.1 Real-Time PCR

1. Place two 10 μ L aliquots of each sample and standard into wells.
2. Prepare two wells of 10 μ L nuclease-free water as negative controls.

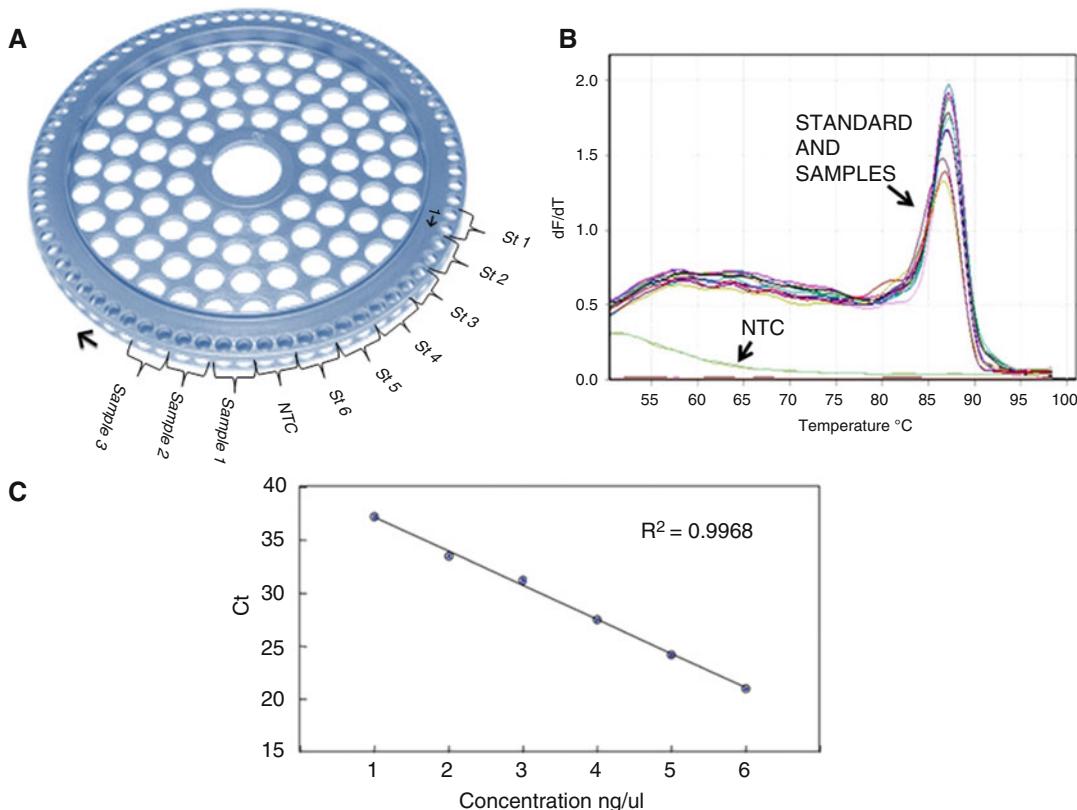


Fig. 1 (a) Example of a plate set up. Rotor-Disc 72 rotor (to be used with Rotor Gene 6000 detection system—Corbett Research, St. Neots, UK) set up with the six standards (St), no template control (NTC), and the samples in the subsequent positions. (b) Example of melting curve analysis of the six standards and samples and the NTC. X-axis shows the melting temperature (T_m); Y-axis shows the derivative of detected fluorescence. (c) Example of a standard curve. X-axis shows the DNA concentration in ng/ μ L; Y-axis shows the Ct

Table 2
PCR conditions for long and short fragments

Step	Temperature (°C)	Time	Number of cycles	
Initial denaturation	95	3 min	1	<i>Long fragments</i>
Denaturation	94	40 s	45	
Annealing	56	40 s		
Elongation	72	1 min		
Initial denaturation	95	90 s	1	<i>Short fragments</i>
Denaturation	95	15 s	40	
Annealing	54	45 s		

- For each filled well, prepare a mix of 1 µL of each primer, 12.5 µL of green supermix, and 6.5 µL of nuclease-free water. Prepare the PCR mix considering two extra samples.
- Transfer 15 µL of the mix into each filled well, pipetting slowly and placing the pipette tip at an angle (10°–45°) against the inside wall of the receiving well/tube. Once the plate/tubes are prepared, proceed immediately with the PCR, or you can store them at 4 °C in the dark for a maximum of 2 h [stopping point, optional].
- Perform the PCR with a real-time instrument according to the manufacturer's specifications. See Table 2 for PCR conditions for each primer set (*see Note 2*).

3.2 Analysis of Results

- Using the instrument software, perform melting analysis (Fig. 1b) on each sample to evaluate the PCR product specificity (*see Note 3*).
- Using the instrument software, check if any replicates have a cycling threshold (Ct) value difference of one or more. Discard those samples from the analysis and evaluate the mean Ct for the remaining samples (*see Note 4*).
- Various amounts of DNA from the standards (0.01, 0.1, 1, 5, 10, and 20 ng) are analyzed to draw a standard curve (Fig. 1c). Then, use the standard curve to determine a concentration in ng/µL for each sample by interpolation. The total cfDNA concentration is calculated for each gene (*see Note 5*).
- Calculate II as the ratio of quantity of long/short fragments. The cfDNA AI is 1/II.

4 Notes

1. These points should be considered for designing primers:
 - (a) Primer length should be 15–30 bases.
 - (b) Optimal GC content should range between 40 and 60%.
 - (c) The melting temperatures (T_m) of each primer should be similar for forward and reverse primer in order to easily select the most adequate annealing temperature. Optimal T_m for primers ranges between 52 and 58 °C, although the range can be expanded to 45–65 °C. The final T_m for both primers should differ by no more than 5 °C.
 - (d) The 3' end of primers should contain a G or C in order to clamp the primer and prevent “breathing” of ends, increasing priming efficiency. DNA “breathing” occurs when ends do not stay annealed but fray or split apart. The three hydrogen bonds in GC pairs help prevent breathing but also increase the T_m of the primers.
 - (e) Dinucleotide repeats (e.g., ATATATATAT) or single base runs (e.g., CCCCC) should be avoided as they can cause slipping along the primed segment of DNA and/or form hairpin loop structures. If unavoidable, due to nature of the DNA template, then only include repeats or single base runs with a maximum of four bases.

There are many online programs to help in designing primers, such as NCBI Primer design tool Primer3. In addition, it could be useful to run a blast on NCBI to check for the target specificity of the primers.

2. The PCR conditions reported in Table 2 are specific for the genes listed in Table 1. Adjust PCR conditions considering the melting temperature (T_m) of each primer set.
3. Carefully analyze the melting curve for each sample (Fig. 1c), to check the specificity of the amplified fragments. Discard a sample and retest it when the profile doesn't fit properly with the specific melting profile. Check the profile also for negative (no template) controls and repeat the whole plate if a specific melting profile is observed, since it could be due to a mix contamination.
4. The threshold for Ct determination should be set up as close as possible to the base of the exponential phase, when all reagents are still in excess, the low amount of product will not compete with the primers' annealing capabilities, making data more accurate. It is recommended to set the same Ct for the different PCR experiments.

5. Use the instrument software to calculate the R^2 value, to test the linearity of the standard curve (Fig. 1c). It should be as close as possible to 1, likely ≥ 0.99 .
6. It is recommended to perform real-time PCR experiments independently in duplicate on the same at least eight samples to test assay imprecision. Calculate coefficients of variation for long and short fragments of each gene to test the reproducibility, defined as the ratio of the standard deviation to the mean value of the replicates.

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Chapter 6

Copy Number Variation Analysis on Cell-Free Serum DNA

Heidi Schwarzenbach

Abstract

Genome diversity comprises single nucleotide polymorphisms, deletions, insertions, and duplications. These gains and losses of DNA segments leading to rearrangements of sequences are termed copy number variations (CNVs). CNVs may disrupt genes and/or alter gene dosage and, thereby, have an impact on both protein-coding and noncoding genes. Accordingly, they affect the activity of various signaling pathways and influence the cell phenotype. They are associated with risks for several severe diseases, in particular cancer. In the current chapter, I introduce a rapid profiling method to identify CNVs in circulating, cell-free DNA by multiplex ligation-dependent probe amplification (MLPA). MLPA represents an efficient method for the detection of CNVs among numerous genes on various chromosomal regions in serum.

Key words Copy number variation, Multiplex ligation-dependent probe amplification, Cell-free serum DNA, Screening, Cancer

1 Introduction

Genome variability among individuals includes abnormal number of copies of DNA sequence that has usually two copies per diploid genome. These copy number variations (CNVs) are usually caused by deletions, insertions, and duplications. The diverse variants can range in size from several dozens of bases to megabases. They have either no phenotypic effect, accounting for adaptive traits, or have an effect resulting in severe diseases. It has been estimated that up to 9.5% of the genome contributes to CNVs and approximately 100 genes can be completely deleted without producing apparent phenotypic consequences suggesting that these genes may be functionally redundant [1].

In 1936, it was shown for the first time that a phenotype can reflect CNVs. In this regard, Bridges et al. demonstrated that duplication of the Bar gene triggers that *Drosophila melanogaster* has a Bar eye phenotype [2]. Currently, CNVs are an important genetic source of pathogenic variants. Due to their particular locations that can be relative to or directly overlap genes or key

regulatory regions, CNVs have been linked with many complex neurological and developmental disorders, such as Down's syndrome (trisomy 21) [3]. In particular, they are a hallmark of different cancer types. A prominent example is HER2 gene amplification in breast cancer [4]. Growing evidence suggests that CNVs arise as a result of preferential selection that favors cancer development and progression. Moreover, it is assumed that the frequency and size of constitutional CNVs are also significantly increased in cancer-affected individuals. The functional impact of CNVs in cancer relies on the alteration of the activity of tumor-associated signaling pathways and anticancer drug sensitivity as well as toxicity in patients [5, 6]. Finally, CNV signatures are useful for determining the characteristics of origin of a primary tumor, an important issue for clinical follow-up in a broad cancer screening. Since they reflect the underlying molecular pathogenesis of a tumor, the identification of recurrent gains and deletions is crucial to improve our biological understanding of these malignancies. Besides, the detection of CNVs may be of important clinical relevance to assess diagnosis, prognosis, and targeted therapy of malignant as well as benign diseases. Thus, in clinical settings, detection of CNVs is essential for patient genotyping.

Numerous studies have demonstrated the ability to detect tumor-derived genetic aberrations in circulating cell-free DNA (cfDNA) [7–9]. However, cfDNA tests for cancer screening are impeded by a low fraction of tumor DNA present in cfDNA, which masks tumor-derived genetic alterations. Regular apoptotic cell death of lymphoid and myeloid cells as part of the hematopoietic homeostasis constitutes the majority of cfDNA. Depending on the cancer burden and type, the fraction of tumor DNA in human blood varies substantially from 0.1% to more than 60% of alleles [10]. As the amount of tumor DNA in blood is limited and prone to contamination by high molecular genomic DNA from leukocytes, sensitive and specific detection methods of tumor-derived genetic alterations are challenging. On the other hand, it is easier to detect CNVs, since each individual tumor CNV contributes to a much larger number of tumor DNA fragments to the overall pool of cfDNA in blood [7]. In the last years, CNV-based screening has dramatically improved sensitivity and demonstrated the technical feasibility of findings of CNVs in the pool of fragmented cfDNA. Most laboratories use fluorescence in situ hybridization (FISH), comparative genomic hybridization, and single and nucleotide genotyping, but high-throughput whole-exome and whole-genome sequencing are also increasingly used or offered as a diagnostic test for large-scale profiles of CNVs [11]. However, these methods are labor-intensive and/or use special equipment. Moreover, the throughput of these instruments may deliver some biases. In particular, the costs of DNA sequencing are so high that such screening tests cannot be performed routinely. Digital and real-time

quantitative PCR are currently very sensitive approaches to be used, but the detection of CNVs is limited on a few single genes in a single assay and cannot be scaled to high-throughput and multiplex testing [9].

To overcome these issues, my laboratory established a simple, efficient, and rapid assay starting from the extraction of cfDNA by the PME free-circulating DNA Extraction Kit (Analytik Jena, Germany) over the detection of CNVs in cfDNA by multiplex ligation-dependent probe amplification (MLPA, MRC Holland) to their statistical evaluation by a custom-developed data analysis software [12]. MLPA is a semiquantitative technique for determining the relative CNVs of multiple tumor suppressor genes and oncogenes in a single multiplex PCR-based reaction. Currently, MLPA allows analyzing CNVs in 43 chromosomal regions containing 37 genes (www.mlpa.com).

In the following paragraphs, the single steps of the detection of CNVs in serum cfDNA are described.

2 Materials

2.1 Preparation of Serum

Prepare serum from 6 mL whole blood by two centrifugation steps of $3000 \times g$ and $13,000 \times g$ for 10 min. Following centrifugation, the supernatant contains the serum (*see Note 1* and *2*).

2.2 Isolation of Leukocytes

1. Lysis buffer: 106.9 g sucrose (fc 0.3 M), 10 mL 1 M Tris-HCl pH 7.5 (fc 10 mM), 5 mL 1 M MgCl₂ (fc 5 mM), and 10 mL 100% Triton X100 (1%). Add aqua bidest up to a volume of 1 L. Mix and store the buffer at 4 °C. Filtrate the solution steriley (*see Note 3*).
2. Do the following steps on ice: incubate 5 mL EDTA blood mixed with 45 mL lysis buffer for 15 min. Centrifuge at $3000 \times g$ for 20 min. Remove the supernatant, and resuspend the pellet in 30 mL lysis buffer. After incubation for 15–30 min, centrifuge at $3000 \times g$ for 15 min. Resuspend the pellet in 1 mL buffer, and centrifuge at $16,000 \times g$ for 5 min. Store the pellet containing the leukocytes at –20 °C.

2.3 DNA Extraction

1. Extract cfDNA from 2 mL serum using the PME free-circulating DNA Extraction Kit (Analytik Jena) and according to the manufacturer's instructions (*see Note 4*). The SE/SB system of the kit should be used, since it delivers the best results. Solve the extracted cfDNA in a volume not higher than 30 µL and in nuclease-free water. Determine spectrophotometrically the quantity and quality of the extracted cfDNA.
2. Extract genomic DNA from leukocytes (reference) using QIAamp DNA Blood Mini Kit (Qiagen) and according to the manufacturer's instructions. Solve the extracted DNA in

200 µL nuclease-free water. Determine spectrophotometrically the quantity and quality of the extracted DNA.

2.4 MLPA Assay

Determine CNV using the SALSA MLPA probemix kit (MRC Holland) and according to the manufacturer's instructions. This kit contains a probe mix of 43 sequences of 37 genes to be analyzed and 22 reference genes [12]. The panel of the 37 genes is assembled for specific cancer types, such as urological or gynecological tumors (www.mlpa.com).

3 Methods

3.1 Verification of Hemolysis in Serum Samples

To avoid contamination of genomic DNA from hemolytic serum samples that mask genetic alterations, at first hemoglobin measurements should be carried out by spectrophotometry at wavelengths from 350 to 650 nm (see Note 5).

1. Prepare a dilution series of red blood cells lysed by lysis buffer (described above under Subheading 2.2), serving as a standard curve for the measurement of hemolysis in the serum samples. Mix 7 mL of whole blood red blood cells with lysis buffer in a ratio of 1:1, 1:3, 1:4, 1:6, 1:8, 1:10, 1:12, 1:14, 1:18, and 1:20.
2. Measure 50 µL of each sample and standard in duplicates on a microplate reader. The degree of hemolysis is determined based on the optical density (OD) at 414 nm (absorbance peak of free hemoglobin, called Soret band), with additional peaks at 541 and 576 nm. Samples are classified as being hemolyzed if the OD at 414 exceeds 0.25. The higher the absorbance in the samples is, the higher is the degree of hemolysis (Fig. 1).

3.2 Extraction of cfDNA from Serum and Leukocytes from Whole Blood

1. Prepare serum and leukocytes from whole blood by two centrifugation steps of $3000 \times g$ and $13,000 \times g$ for 10 min each. Following centrifugation, the supernatant contains the serum, and the intermediate white layer contains the leukocytes.
2. Extract cfDNA and genomic DNA from 2 mL serum and lysed leukocytes (reference), respectively, using the PME free-circulating DNA Extraction Kit (Analytik Jena, Germany). The SE/SB system of the kit should be used, since it delivers the best results. The extraction is to be carried out according to the protocol of the kit. However, at the end of the approach, the extracted DNA should be solved in a volume of not higher than 30 µL and in water.
3. Quantification and quality of the extracted cfDNA can be determined spectrophotometrically using, for example, the NanoDrop.

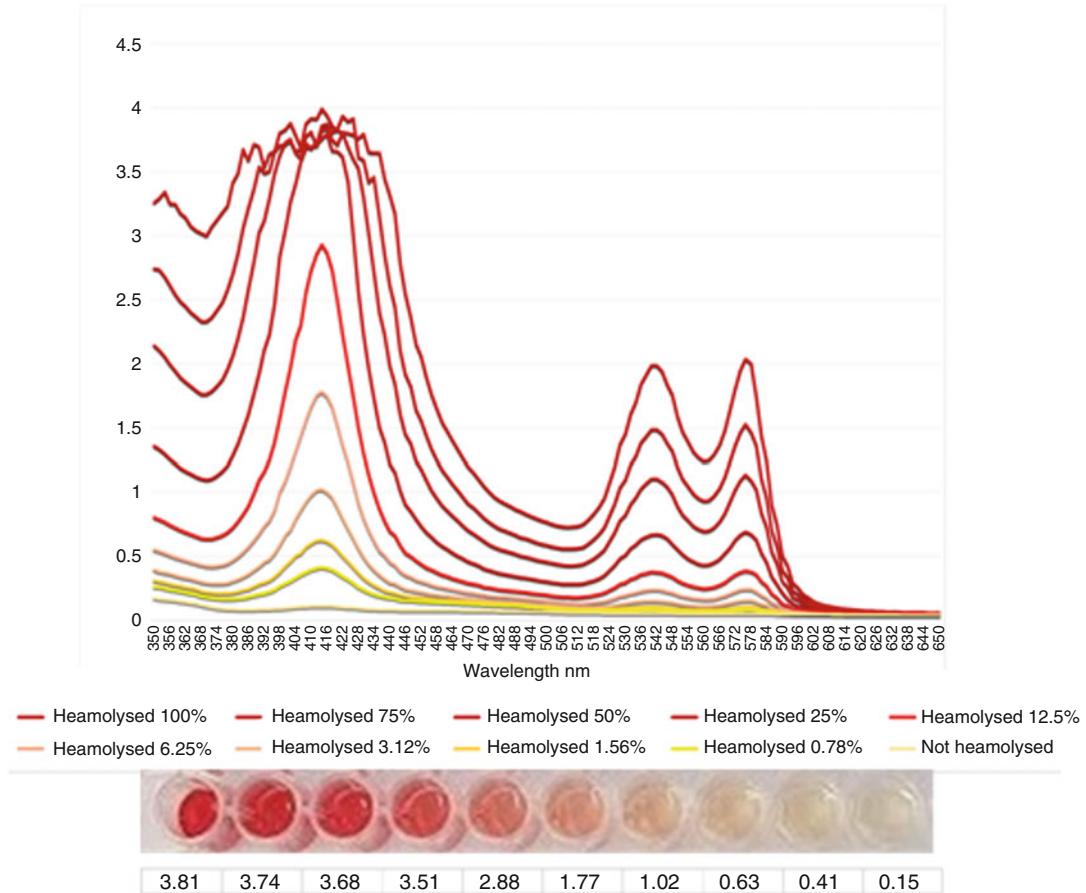


Fig. 1 Measurements of free hemoglobin. The chart shows the curves that indicate the percentage of hemolysis. A dilution series of lysed red blood cells in plasma is shown below the chart

3.3 MLPA Assay

Perform the assay according to the manufacturer's instructions of the MLPA kit (*see Notes 6 and 7*). The protocol includes the following steps:

1. DNA denaturation (day 1): In a thermocycler denature 5 µL (50 ng) cfDNA and 5 µL (100 ng) leukocyte (reference) DNA at 98 °C for 5 min. Cool down the samples to 25 °C.
2. Hybridization reaction (day 1): Mix hybridization master mix containing 1.5 µL MLPA buffer plus 1.5 µL probemix for each reaction. Add the 3 µL hybridization master mix to each denatured DNA sample. Incubate the reaction at 95 °C for 1 min and then at 60 °C for 16–20 h.
3. Ligation reaction (day 2): Prepare a Ligase-65 master mix. For each reaction, mix 25 µL aqua bidest, 3 µL Ligase buffer A, and 3 µL Ligase buffer B. Then, add 1 µL Ligase-65 enzyme. Continue thermocycler program of the reaction at 54 °C for 15 min by adding the 32 µL ligase master mix to each tube.

Mix by gently pipetting up and down. Inactivate the reaction at 98 °C for 5 min and pause it at 20 °C.

4. PCR reaction (day 2): During the subsequent PCR, all ligated probes are amplified simultaneously using the same PCR primer pair, of which one PCR primer is fluorescently labeled. Prepare polymerase master mix. For each reaction, mix 7.5 µL aqua bidest, 2 µL SALSA PCR primer mix, and 0.5 µL SALSA polymerase on ice. At room temperature, add the 10 µL polymerase master mix to each tube. Mix by pipetting gently up and down. Place the tubes in a thermocycler, and perform the thermocycler program: 35 cycles of 30 s at 95 °C, 30 s at 60 °C, and 60 s at 72 °C. End with 20-min incubation at 72 °C and stop it at 15 °C.
5. Fragment separation can be done by capillary electrophoresis on an automated DNA analyzer (e.g., Applied Biosystems), yielding a specific electropherogram (Fig. 2).

3.4 Data Normalization

Data normalization consisting of intra- and intersample normalization can be carried out by the custom-developed data analysis software created for MLPA (Coffalyser.Net analysis software, www.mlpa.com) (*see Note 8*).

1. Intrasample normalization: Within each sample, each probe peak is compared with the peaks of the reference probes. Thus, the fluorescence signal of each target-specific probe is divided by the signal of every single reference probe in the probe. The median of all these ratios of the probe is the normalization constant. The determined relative probe signals are then used for intersample normalization.
2. Intersample normalization: Final probe ratios are calculated by dividing the normalization constant of each probe of the sample by the average normalization constant of all reference (leukocyte) samples (Fig. 3). Reference probes located on various chromosomes detect sequences that are expected to have a normal copy number in all samples. Leukocyte DNA samples are therefore expected to have a normal copy number for both the reference and target probe.

4 Notes

1. Instead of plasma, serum should be used for cfDNA extraction. Although in our previous investigations [12], the extracted plasma cfDNA amounts were sufficient for the assay, they did not deliver reliable data. For the preparation of plasma heparin, acid citrate dextrose (ACD) or EDTA is commonly used, and traces of these anticoagulants may still be available in the extracted cfDNA, presumably affecting MLPA.

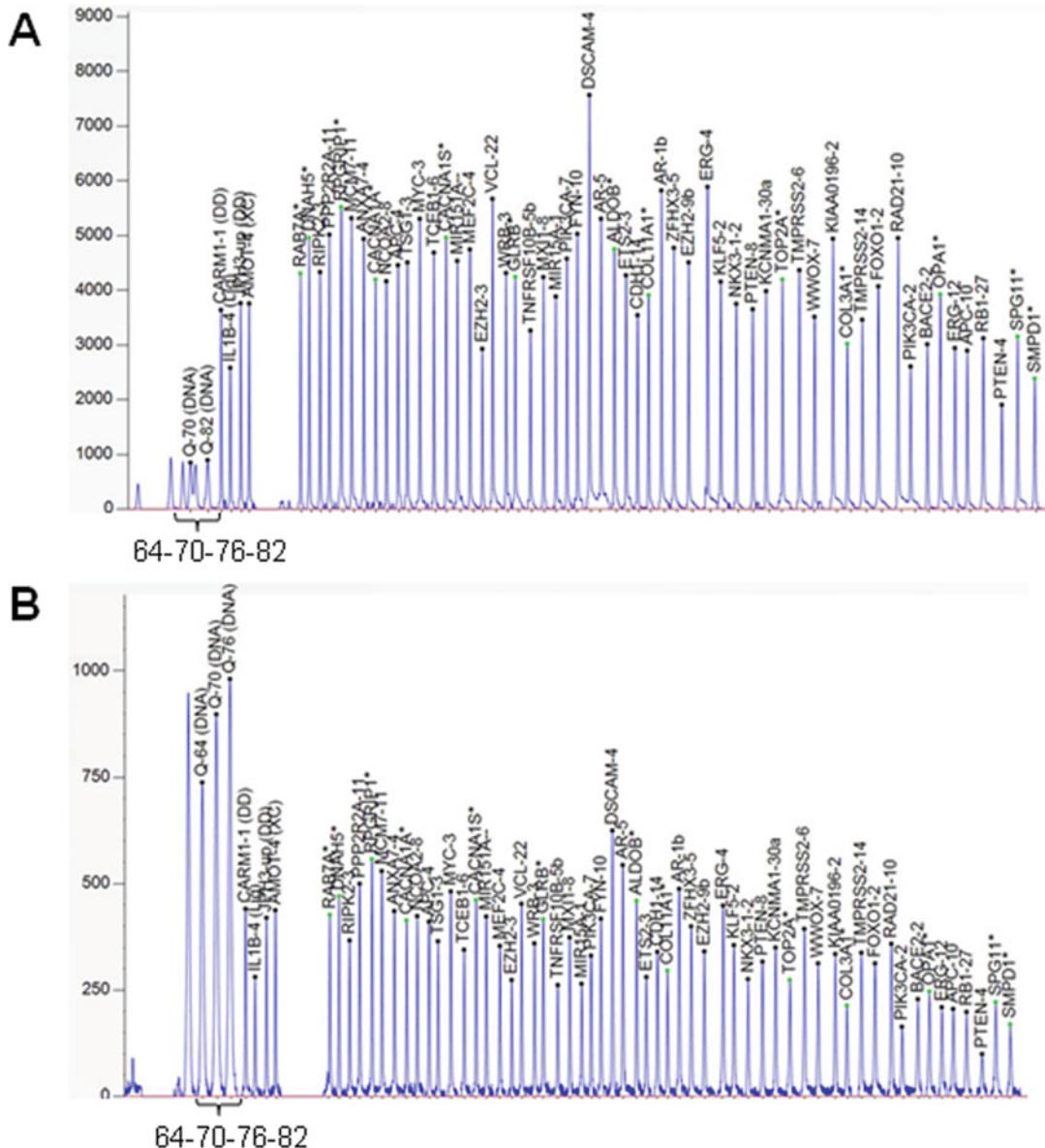


Fig. 2 Electropherograms derived from capillary electrophoreses. Each probe in an MLPA probemix has a unique amplicon length, typically ranging between 130 and 500 nucleotides (nt). The first four peaks (quantity fragments, at 64–70–76–82 nt) are complete fragments that do not need to hybridize to DNA or be ligated to be amplified during PCR. The more sample DNA is added, the lower they get. If these peaks are higher than the subsequent probe peaks, then the diagram is not evaluable. **(a)** Analyzable and **(b)** unanalyzable experiment

- Following withdrawal and prior to the first step of centrifugation, the whole blood should stay at room temperature for at least 30 min, so that thrombin can reach its full potential of coagulation. However, the serum should be prepared from whole blood within 3 h to avoid cell lysis.

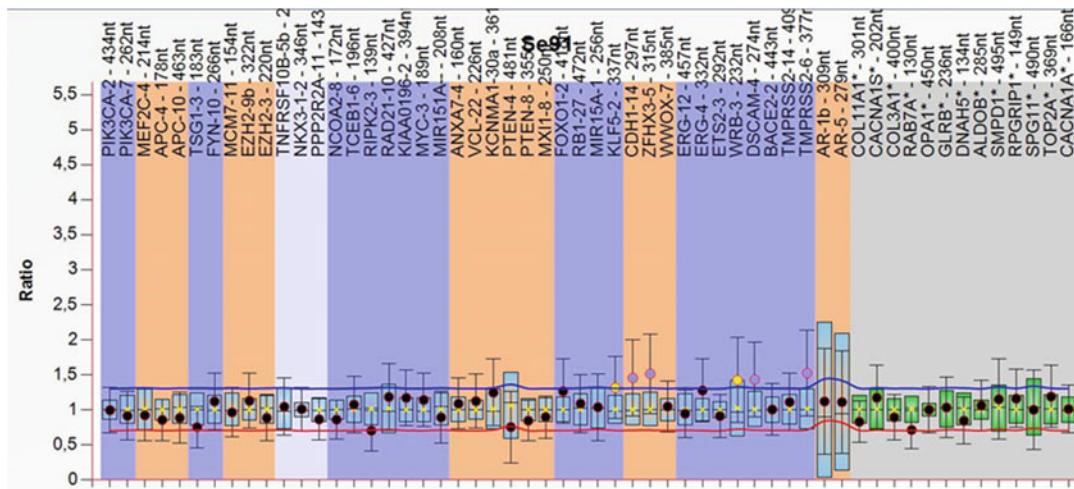


Fig. 3 A box plot showing data of an exemplary serum sample (as calculated by Coffalyser.Net software). The DNA probes are arranged by chromosomal locations. The target-specific probes have a blue (dark and light) and orange background (left side), whereas the reference probes have a gray background (right side). Above the chart, the genes with the exon on which they are located and the size in nt are indicated. Blue circles indicate a significant increase in CNV, whereas yellow circles are ambiguous. An increased copy number is clearly detected in the genes CDH1, ZFHx3, DSCAM, and TMPRSS2

- The lysis buffer for preparing leukocytes and red blood cells should be prepared usually before starting the experiments, because storing of the lysis buffer longer than 1 week at 4 °C may cause mold in the solution.
- The PME cfDNA Extraction Kit should be used for cfDNA extraction. My laboratory tested the combination of MLPA with several other commercial DNA extraction kits, but the combination with the PME cfDNA Extraction Kit has provided the best and most reliable data of CNVs in cfDNA. Possibly, the components of the other extraction kits may have an influence on the performance of MLPA by cfDNA.
- Hemoglobin measurements should be absolutely carried out, since hemolysis of serum samples is often not visually apparent.
- All buffers available in the MLPA kit should be vortexed before use, but not the enzyme solutions.
- At least 50 ng cfDNA extracted from serum should be used to get evaluable data. As shown in Fig. 2, the first four peaks of the electropherograms refer to the DNA amounts. The more sample DNA is added, the lower they get. If these peaks are higher than the subsequent probe peaks, then the diagram is not evaluable.
- The reliability of the CNV detection should also be examined by a repetitive analysis of serum cfDNA samples and by the fact that genomic leukocyte DNA should not harbor any CNVs.

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Chapter 7

Studying Copy Number Variations in Cell-Free DNA: The Example of AR in Prostate Cancer

Samanta Salvi and Valentina Casadio

Abstract

Serum and plasma cell-free DNA (cfDNA) has been shown as an informative noninvasive source of biomarkers for different diseases, including cancer. Starting from the hypothesis that the gain of androgen receptor (*AR*) gene is a frequent aberration in advanced prostate cancer patients, we analyzed it in cfDNA as a potential predictive biomarker of specific treatments. Here we report a general protocol that may be considered to analyze gene copy number variations in serum or plasma fluids.

Key words Androgen receptor, AR, Cell-free DNA, Copy number variation, Prostate cancer

1 Introduction

In the cancer field, cell-free DNA (cfDNA) analysis could be able to replace or give supplement information on tissue biopsies, resulting in a useful noninvasiveness source of biomarker. Since a long time, it has been widely demonstrated that the copy number variations (CNV) are associated with cancer risk and development [1].

Prostate cancer (PCa) is the most common cancer in men [2]. In PCa patients, androgen receptor (*AR*) gene CNV has been shown to be one of the principal mechanisms of hormone-sensitive to hormone-resistant transition [3]. For this reason, we propose to study *AR* gain in serum or plasma DNA of advanced PCa patients, aiming to identify a novel predictive biomarker for specific treatments. CNV analysis on cfDNA has several advantages: firstly the methods are generally noninvasive, fast, and simple, and secondly they could be performed starting from low volume of serum or plasma material [4–6].

Here we report a workflow to isolate DNA from serum or plasma and to detect the *AR* CNV by real-time quantitative PCR (qPCR) or digital PCR (dPCR) techniques.

Table 1
A summary of techniques which can be used for CNV analysis

Technique	Brief description	Resolution	Suitable for	Throughput	References
FISH	Cytogenetic technique that uses fluorescence probes complementary at only one specific DNA sequence	Deletions >100 kb or duplications <500 kb	Tissue, cells, and circulating tumor cells	Low (few targets)	[7]
aCGH	Hybridization with fluorescent probes of sample and reference DNA	Copy number detection resolution at 5–10 kb	Tissue, cells, whole blood DNA, and cfDNA	High (whole-genome or targeted analysis)	[8]
MLPA	Multiplex polymerase chain reaction-based method	Single exon	Tissue, cells, and whole blood DNA	Medium (up to 50 genes)	[9]
qPCR or dPCR	Real time based on the comparison of the target gene with a reference region	Up to 1 kb	Tissue, cells, whole blood DNA, and cfDNA	Low (few targets)	[4–6, 10]
NGS	Deep sequencing	<10 kb	Tissue, cells, whole blood DNA, and cfDNA	High (whole-genome or targeted sequencing)	[11]

FISH fluorescence in situ hybridization, *aCGH* array comparative genomic hybridization, *cfDNA* cell-free DNA, *MLPA* multiplex ligation-dependent probe amplification, *qPCR* quantitative PCR, *dPCR* digital PCR, *NGS* next-generation sequencing

The detection of CNV could be evaluated using various techniques (summarized in Table 1), mainly depending on the number of the targets of interest and the quality and quantity of biological material. Usually, short CNV are more difficult to detect than alterations involving longer sequences.

2 Materials

2.1 Serum or Plasma Collection

Two different types of tubes are necessary to collect serum or plasma: a tube without anticoagulant for serum and a tube with EDTA for plasma. Both tubes could be stored at 4 °C up to 24 h until the processing. After the centrifugation, serum or plasma could be transferred in cryovials to consent a long-term storage at -80 °C.

2.2 DNA Isolation and Quantification

DNA was isolated using QIAamp Mini kit (Qiagen), following the protocol for “DNA Purification from Blood or Body Fluids (Spin Protocol)” (see Note 1). Ethanol 100% is not included in the kit, and it is mandatory for activation of wash buffers 1 and 2 (included).

DNA extracted could be quantified by NanoDrop® ND-1000 (Thermo Fisher Scientific) (see Note 2).

2.3 Real-Time Quantitative PCR

CNV detection could be performed using TaqMan™ Universal PCR Master Mix and TaqMan™ real-time assays (Thermo Fisher Scientific), designed for the target of interest. We evaluated AR CNV using two different assays (*AR1*, assay ID (Hs04107225); *AR2*, assay ID (Hs04511283)) and two reference genes (*RNaseP*, TaqMan™ Copy Number Reference Assay, and *AGO1*, assay ID (Hs02320401), modified with VIC-labeled probe) (see Notes 3 and 4). We combined the assays (*AR1/AGO1* and *AR2/RNaseP*) to perform a duplex fluorescence detection for each DNA sample. We also analyzed a pooled cfDNA from healthy male donors, as a sample with normal copy number of *AR* gene (calibrator sample).

qPCR was performed using Applied Biosystems® 7500 instrument (Thermo Fisher Scientific) (see Note 3). CNV analysis was performed using CopyCaller™ Software.

2.4 Digital PCR

For dPCR approach we used the QuantStudio 3D MM2 (Thermo Fisher Scientific) and the same TaqMan™ real-time assay combination (*AR1/AGO1* and *AR2/RNaseP*). dPCR analysis was performed using QuantStudio 3D Digital PCR System (Thermo Fisher Scientific), and data were analyzed by QuantStudio 3D AnalysisSuite Cloud Software (Thermo Fisher Scientific).

3 Methods

3.1 Serum or Plasma Collection

1. Collect whole blood in a tube without anticoagulant for serum or with EDTA for plasma.
2. Maintain blood tubes at 4 °C until the processing up to 24 h.
3. Centrifuge the tubes for serum at $1000 \times g$ for 15 min or at $1800 \times g$ for 15 min for plasma tube.
4. Carefully transfer supernatant into 2 mL cryotubes (*see Note 5*).
5. Discard the collecting tube, and immediately freeze the supernatant at –80 °C until use.

3.2 DNA Isolation from Serum or Plasma

Isolation of DNA from serum or plasma should be performed using QIAamp Mini kit (Qiagen), following the protocol for “DNA Purification from Blood or Body Fluids (Spin Protocol).” We suggest to start from at least 0.5 mL of serum or plasma, adjusting the volume of each reagent. Moreover, we suggest to activate the wash buffers AW1 and AW2 before the isolation starts, adding the ethanol volume suggested in the protocol.

1. Thaw one aliquot of serum or plasma at room temperature.
2. Vortex and mix the sample, and transfer 0.5 mL of serum or plasma into a clear 1.5 mL tube.
3. Add 50 µL of Proteinase K.
4. Add 0.5 mL of AL buffer and mix well by pipetting (lysis step).
5. Perform an incubation at 56 °C for 15 min.
6. Bring to room temperature, and add 0.5 mL of absolute ethanol, and mix well by pipetting.
7. Add the mixture obtained to the specific column, and centrifuge at $6000 \times g$ for 1 min.
8. Discard the tube containing the flow through, and place the column on a new clean collection tube. Repeat Subheading 3.2, steps 7 and 8, one more time.
9. Add 500 µL of wash buffer AW1 and centrifuge at $6000 \times g$ for 1 min.
10. Discard the tube containing the flow through, and place the column on a new clean collection tube.
11. Add 500 µL of wash buffer AW2 and centrifuge at full speed for 3 min.
12. Discard the tube containing the flow through, and replace it with a new clean collection tube.
13. Centrifuge at full speed for 3 min to remove any residual washing buffer.

14. Place the column into a clean 1.5 mL tube, and add 150 µL of elution buffer AE (*see Note 6*).
15. After 7 min at room temperature, centrifuge at $8000 \times g$ for 1 min.
16. To ensure maximum recovery of DNA, pipet the eluted from Subheading 3.2, step 15, again into the same column, and centrifuge at full speed for 1 min.
17. Quantify the DNA by NanoDrop® ND-1000 (Thermo Fisher), using 2 µL of DNA on bench-top spectrometer.

3.3 Real-Time qPCR Approach

1. Equilibrate at room temperature the master mix stored at 4 °C.
2. 60 ng of plasma cfDNA samples and of the calibrator were splitted in three wells (20 ng for each well) to perform three technical replicates, required for the experiment.
3. Prepare a mix of 10 µL of Universal Master Mix (Life Technologies), 1 µL of target assay (*AR1 or AR2*), and 1 µL of reference gene assay (*RNaseP* or *AGO1*). Calculate three replicates of each patients and calibrator sample and one negative control (water).
4. Aliquot 12 µL of the mix into each well.
5. Add water to obtain 20 µL of final reaction volume.
6. Briefly spin down the plate using a microcentrifuge for plates.
7. Run the experiment using the following protocol: hold stage at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min.
8. Set the Ct threshold at 0.2 for each sample and each assay analyzed, and omit the replicate with high Ct difference (*see Note 7*).
9. Export the real-time PCR file in “.txt” extension.
10. Import the file “.txt” in CopyCaller™ Software.
11. Specify the copy number of the calibrator sample (e.g., one copy for *AR* gene).
12. Evaluate the results by the copy number bar plot or the table (Fig. 1).
13. Define *AR* gain when the CNV was greater than 1.5.

3.4 Digital PCR Approach

1. Equilibrate at room temperature the master mix stored at 4 °C.
2. Dilute cfDNA to obtain 2.5 ng/µL of final concentration.
3. Add 6.8 µL (17 ng) of cfDNA samples or calibrator to a new tube.
4. Prepare a mix of 0.725 µL of target assay (*AR1 or AR2*), 0.725 µL of reference gene assay (*RNaseP* or *AGO1*), and

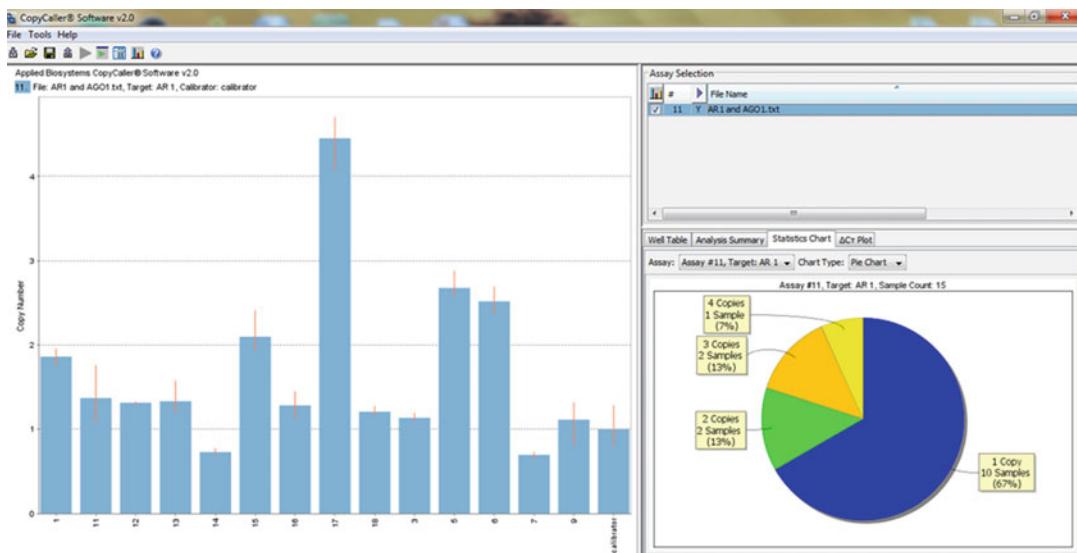


Fig. 1 qPCR *AR1* and *AGO1* copy number analysis using CopyCaller software

7.25 µL of QuantStudio 3D MM2 (Life Technologies) for each DNA sample including the calibrator and a negative control (water).

5. Aliquot 8.7 µL of mix in each tube containing the diluted DNA sample and pipetting well (*see Note 8*).
6. Aliquot 14.5 µL of the DNA mix sample onto a new loading blade in the loader (*see Note 9*) (Fig. 2).
7. Prepare a new chip and lid.
8. Press the loading button to distribute the sample volume on the chip.
9. Add approximately 20 drops of immersion fluid using a syringe/pipette to the chip, and move the loader arm to close the chip.
10. Press the loader arm for 20 s.
11. Dispense other immersion fluid with a syringe to remove air bubbles and to fully cover the chip area.
12. Remove the sticky label and close the chip.
13. Run the experiment using the following protocol: hold stage at 96 °C for 10 min, 45 cycles at 60 °C for 2 min and 98 °C for 30 s, and a final hold stage at 60 °C for 2 min.
14. After the protocol run, remove the chips, and clean it with laboratory wipe that has been sprayed with ethanol.
15. Insert a USB pen for data storage in the detector system, and proceed to read each chip.

3.4.1 Data Analysis and Interpretation

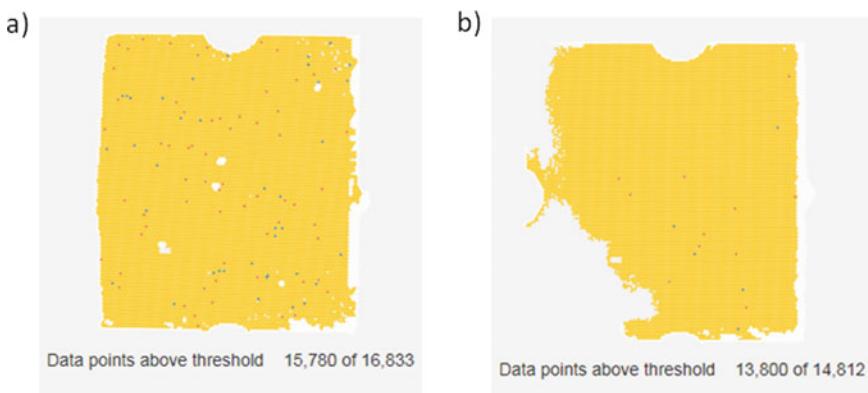


Fig. 2 dPCR chip distribution. On the left (**a**) the figure shows a well distribution of sample on the chip respect to a worst distribution (**b**)

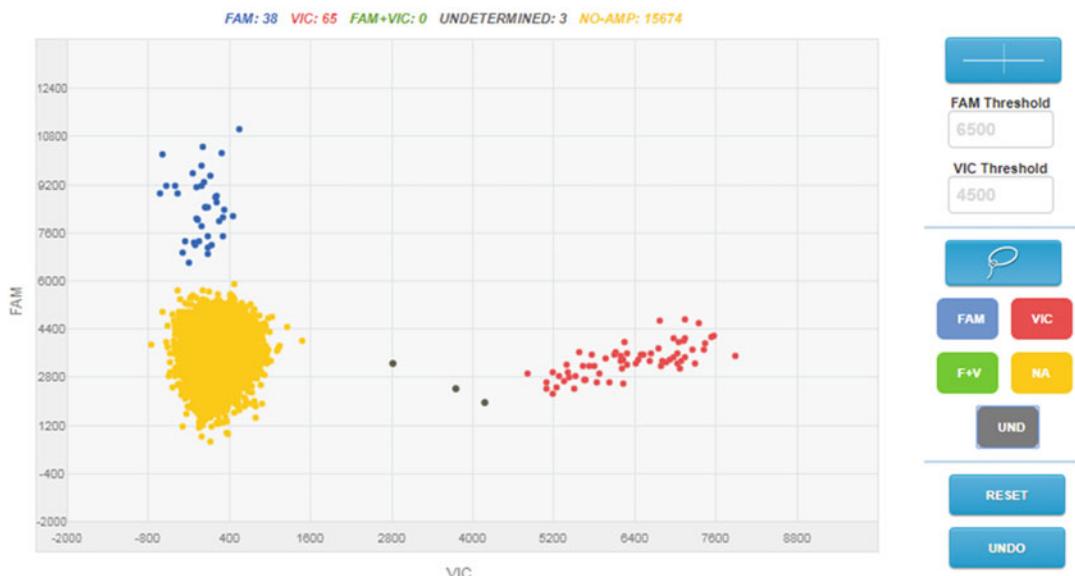


Fig. 3 dPCR plot for the duplex of *AR1* (blue dots) and *AGO1* (red dots) assays. We set a specific threshold of 6500 for *AR1* (FAM threshold) and 4500 for *AGO1* (VIC threshold). No amplified DNA is representative by yellow dots. Black dots are manually defined as “undetermined”

16. Create a project, and import the data files using the QuantStudio 3D AnalysisSuite Cloud Software.
17. Set the sample name of each chip.
18. Check in the review data section the quality of sample by the number of evaluable data points ($>13,000$).
19. Set the FAM threshold at 5000 for *AR2* assay and at 6500 for *AR1* assay (Figs. 3 and 4).

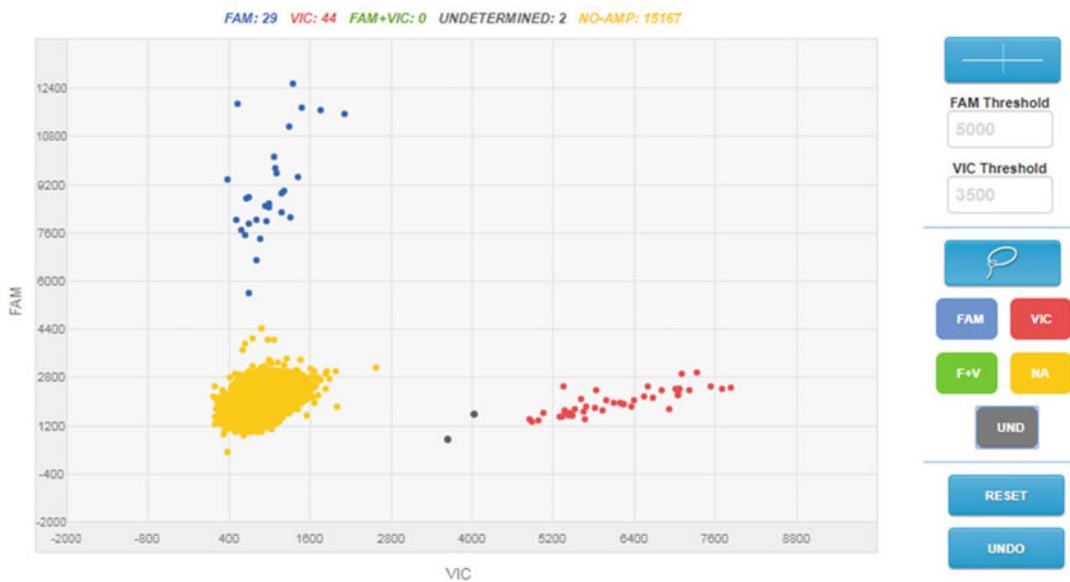


Fig. 4 dPCR plot for *AR2* (blue dots) and *RNaseP* (red dots) assays. Specific thresholds were set: 5000 for *AR2* (FAM) and 3500 for *RNaseP* (VIC). Yellow dots = no amplified DNA. Black dots = undetermined amplification products

20. Set the VIC threshold at 3500 for *RNaseP* assay and at 4500 for *AGO1* assay (Figs. 3 and 4).
21. Remove manually the false-positive points, and define it as “undetermined.”
22. To calculate the CNV, make a ratio between copies/ μL of the target and the reference genes for each sample. Then, perform a normalization on calibrator CNV result.
23. Define *AR* gain when the CNV was greater than 1.5.

4 Notes

1. You can also use other column-based commercial kits for the isolation of DNA from serum or plasma.
2. You can also use other types of quantification methods based on fluorometric approach, such as Qubit Fluorometer (Thermo Fisher Scientific). In this case, we suggest to quantify cfDNA using Qubit dsDNA HS (High Sensitivity) assay kit that is specific for low yield of DNA.
3. It is recommended to aliquot TaqMan™ real-time assays to avoid thawing cycles. We suggest to aliquot about 100 μL of each assay that are sufficient for 96-well plate.
4. It is recommended to test different assay combinations to define the most efficient.

5. You can also use other real-time instrument.
6. We increased the elution volume obtaining a better final DNA yield. You can also use sterile water for molecular biology, if downstream applications required it.
7. Generally, we exclude the replicate with >1 Ct difference thus decreasing the coefficient of variation.
8. It is very important to accurately pipet the dPCR mix with the DNA sample to prevent a well distribution of material in the chip.
9. It is very important to prevent the air bubbles formation at this step. We suggest to slowly pipet the DNA and mix into the loading blade.

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Chapter 8

dPCR Mutational Analyses in Cell-Free DNA: A Comparison with Tissues

Takashi Takeshita and Hirotaka Iwase

Abstract

Digital PCR (dPCR) enables the detection and characterization of fragmented DNA that is in low abundance in blood. Here, we describe the comparative analysis of mutations in tumor tissue DNA and plasma cell-free DNA (cfDNA) using a dPCR method. Tumor cells are captured by laser microdissection to obtain only cancerous cells from a small quantity of metastatic tissue samples and to exclude inflammatory and stromal cells. We extracted cfDNA from 500 µL of plasma, which is sufficient for target mutation analysis using dPCR. The dPCR assay for the detection of the specific region in the target gene consists of a pair of primers and two probes labeled with a fluorescent dye capable to recognize the presence or absence of a specific mutation. Using the dPCR method, we can identify only a few mutations in tissue that are present also in plasma.

Key words Tumor tissue DNA, Cell-free DNA, Digital PCR, Liquid biopsy, Dilution and cross-reactivity assay

1 Introduction

Tumor tissue is the gold standard specimen for tumor genotyping. Furthermore, over the past 10 years, “liquid biopsy” using cell-free DNA (cfDNA) has attracted attention as a potential tool to more accurately reflect tumor burden and tumor kinetics in breast cancer patients. This attention is due to the rapid development of genome analysis in recent years revealing that the half-life of fragmented DNA in blood is as short as approximately several minutes to 2 h and the value fluctuates depending on the increase in the tumor volume due to disease progression [1]. As an example of clinical utility, drug-resistant clones can be monitored through analysis of cfDNA in plasma obtained before and after treatment. Thus, cfDNA analysis appears to be extremely promising for elucidating the mechanism of drug resistance, choosing the next treatment and reducing the tumor volume with therapy and surgery [2–4].

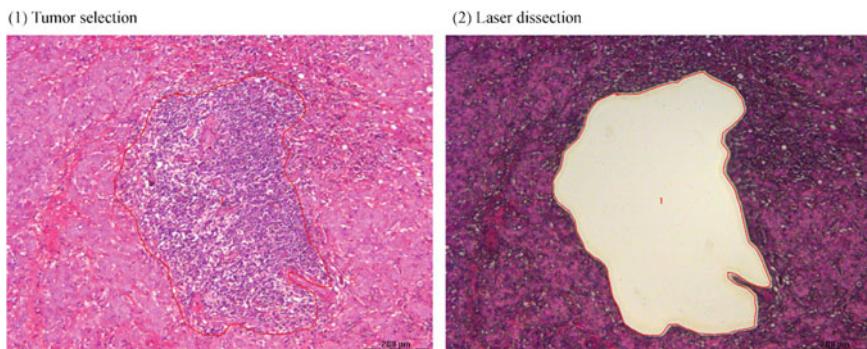


Fig. 1 The image of sample preparation by laser microdissection. Hematoxylin and eosin staining was carried out on 8- μm -thick sections. Tumor cells were captured by LMD using a Leica LMD 7000 (Leica Microsystems K.K.) to obtain only cancerous cells from a small quantity of metastatic tissue samples and to exclude inflammatory and stromal cells. Abbreviation: *LMD* laser microdissection

Digital PCR (dPCR), such as droplet dPCR (ddPCR) [5] and beads, emulsion, amplification, and magnetic PCR [6], is a method capable to detect one molecule of DNA using two probes designed for a specific mutation or a wild-type sequence in one single experiment. By counting the number of fluorescent dyes that show the presence or absence of a mutation, dPCR can detect about 0.01% of tumor-derived DNA [7].

The main drawback to the use of cfDNA as a surrogate for tissues is the high degree of variability in the concordance rate between the genetic alterations detectable in tumor tissues and those in the corresponding plasma. Here, we show a comparative analysis of mutations in tumor tissue DNA (ttDNA) and plasma cfDNA using the ddPCR method. Tumor cells are captured by laser microdissection (LMD) to obtain only cancerous cells from a small quantity of metastatic tissue samples and to exclude inflammatory and stromal cells (Fig. 1). We extracted the cfDNA from 500 μL of plasma. This amount is sufficient for target mutation analysis using ddPCR (Fig. 2). We adopted the Custom *TaqMan*[®] SNP Genotyping Assays to design primers and probes for the ddPCR assay. The annealing temperature should be adjusted using a temperature gradient assay (Fig. 3). After confirming the accuracy of detection probes by the dilution assay (Fig. 4) and the cross-reactivity assay (Fig. 5), we identified only a few mutations present in the tissue and plasma in this assay.

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to obtain a sensitivity of 18 M Ω cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature unless indicated otherwise. Please be sure to follow all waste disposal regulations when disposing of waste materials.

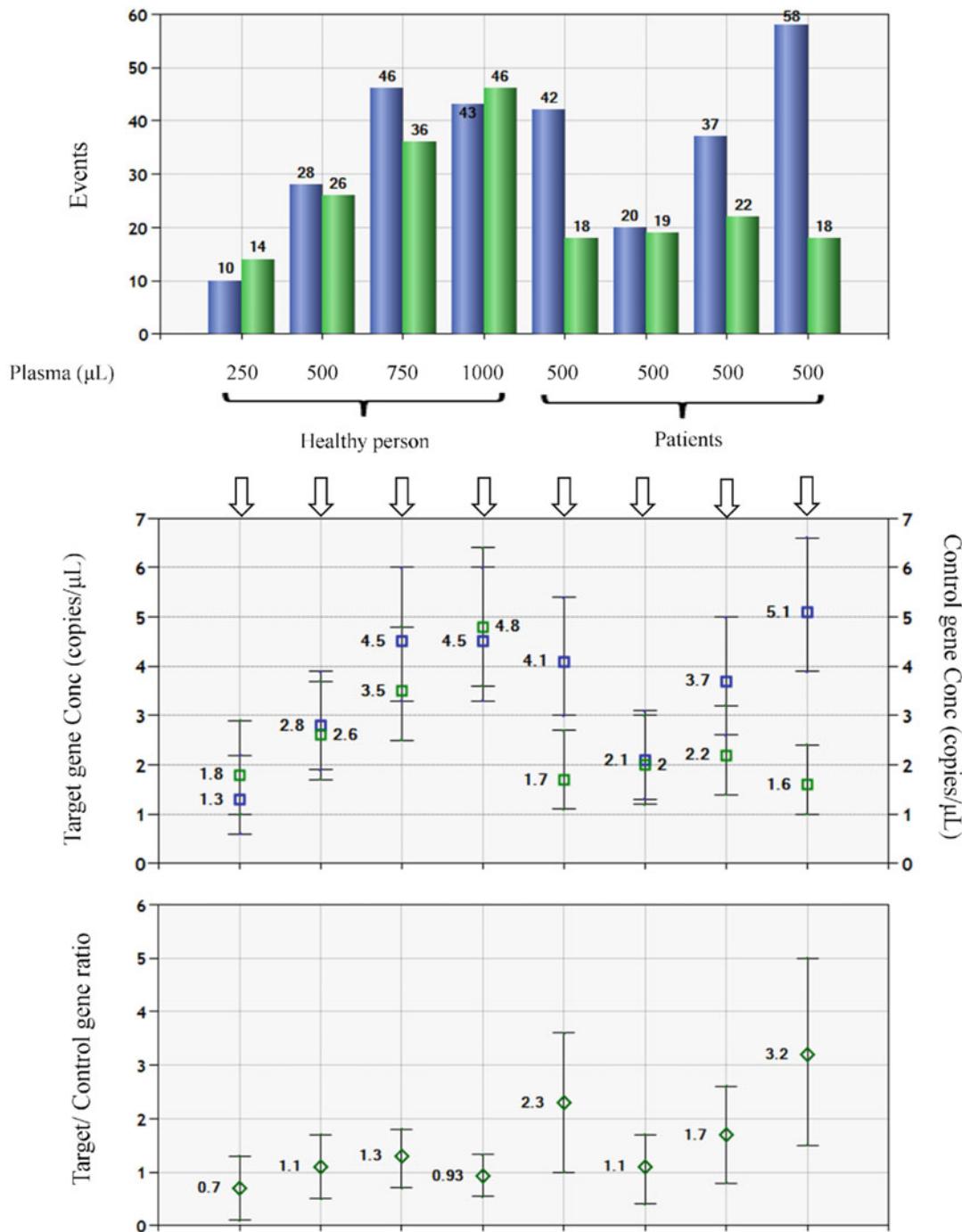


Fig. 2 The image of verifying the optimum adsorption capacity of the extraction column. We use cfDNA extracted from 500 μL of plasma for the ddPCR mutation analysis. The upper panel shows the absolute number of target genes and control genes in healthy people and patients. The middle panel shows the absolute copies/μL of the target gene and the control gene, and the lower panel shows the target/control gene ratio

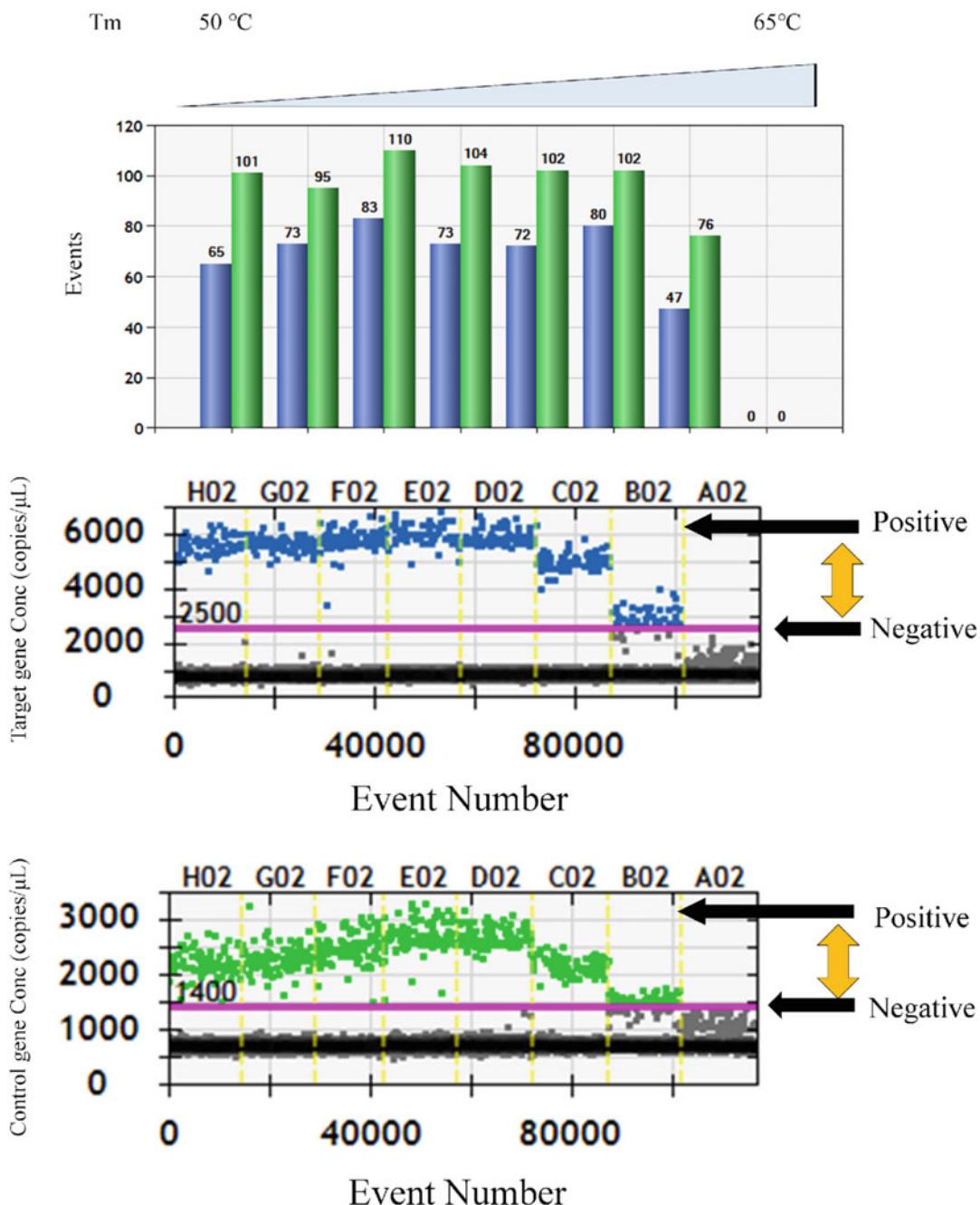


Fig. 3 The image of optimizing the annealing temperature using a temperature gradient assay that clearly separates positive and negative fluorescence intensities. The temperature at which the difference between the positive and negative amplitudes is maximum is optimal (double-headed arrow). The upper panel shows the absolute number of indicated synthetic mutation oligonucleotides and normal wild-type human DNA according to each Tm. The middle and lower panels show the absolute copies/ μ L of indicated synthetic mutation oligonucleotides and normal wild-type human DNA

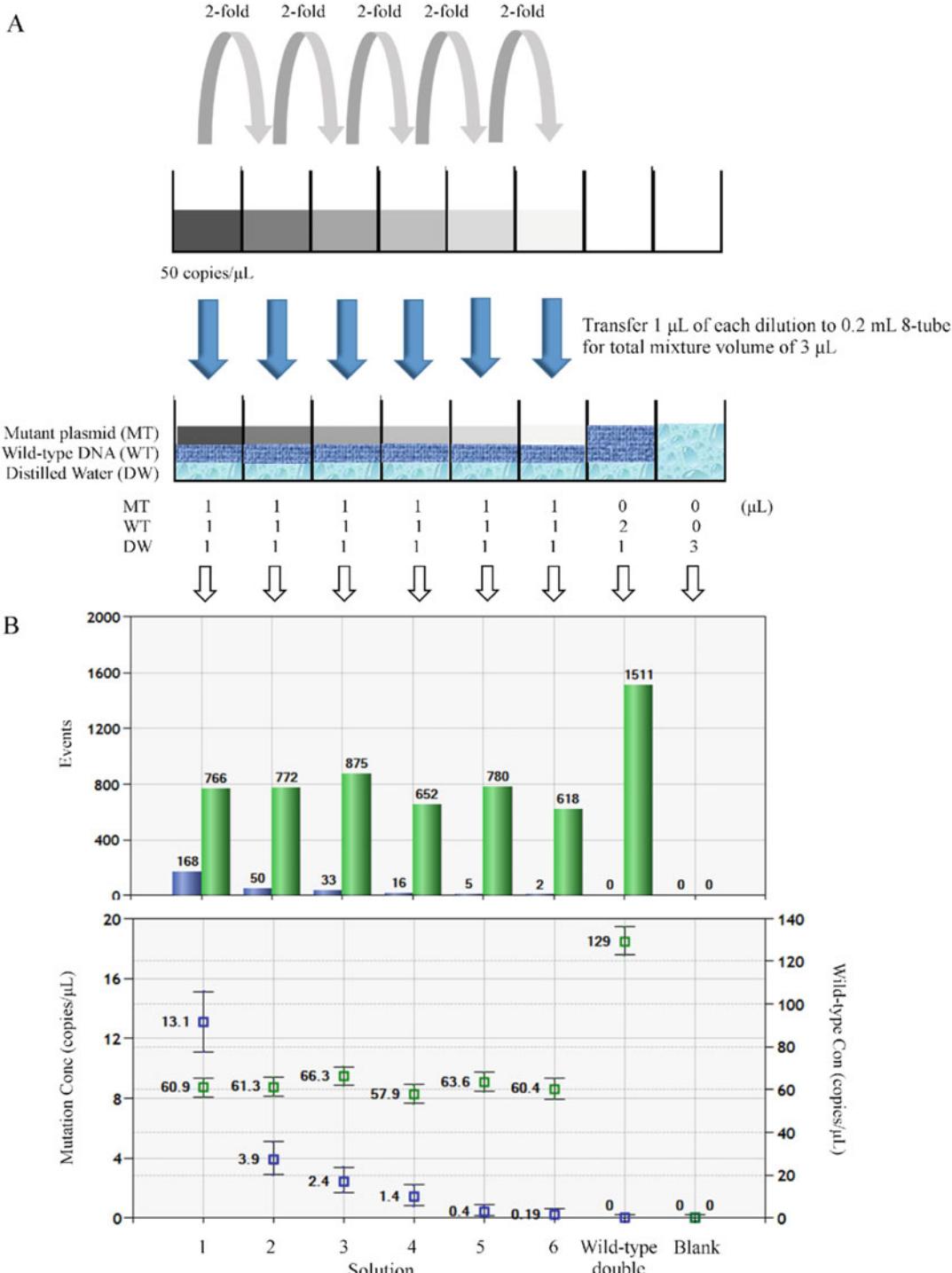


Fig. 4 (a, b) A comparative analysis of the dilution series of the indicated synthetic mutation oligonucleotides in a background of normal wild-type human DNA using the ddPCR assay. **(a)** The image of the dilution experiments that are prepared by twofold serial dilution of the synthetic mutation stock oligonucleotide in a background of normal wild-type human DNA. **(b)** The upper panel shows the absolute number of indicated synthetic mutation oligonucleotides and normal wild-type human DNA, and the lower panel shows the absolute copies/μL of the indicated synthetic mutation oligonucleotides and normal wild-type human DNA. Abbreviation: *ddPCR* droplet digital PCR

2.1 Extraction of Tumor Tissue DNA

1. All Prep DNA/RNA Mini kit (Qiagen).
2. Microtome.
3. Xylene.
4. Ethanol.
5. Hematoxylin and eosin.
6. Coated foil-covered slides processed with poly-L-lysine.
7. Leica LMD 7000 (Leica Microsystems K.K.).
8. PicoPure® DNA Extraction Kit (Life Technologies): Proteinase K and Reconstitution buffer.
9. Microcentrifuge (capable of $12,000 \times g$ with microfuge tube rotor).
10. Standard laboratory incubator set at 65 °C.
11. Heating block for 0.5 mL microcentrifuge tubes (95 °C).

2.2 Extraction of cfDNA

1. Ethylenediaminetetraacetic acid (EDTA) tubes.
2. Freezer set at -80 °C.
3. ISOSPIN Blood & Plasma DNA kit (Nippon Gene): Proteinase K, BE Buffer, spin column, collection tube, washing buffer, ethanol containing washing buffer, and elution buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 9.0).
4. Ethanol.
5. Microcentrifuge (capable of $12,000 \times g$ with microfuge tube rotor).
6. Standard laboratory incubator set at 56 °C.
7. Heating block for 0.5 mL microcentrifuge tubes (95 °C).

2.3 Quantification of cfDNA

1. The Agilent 2100 Bioanalyzer.
2. Chip Priming Station.
3. IKA Vortex Mixer.
4. Agilent DNA 1000 Kit.
5. Agilent RNA 6000 Pico Kit.
6. Microcentrifuge (capable of $\geq 13,000 \times g$ with microfuge tube rotor).
7. Standard laboratory incubator set at 70 °C.
8. Heating block for 0.5 mL microcentrifuge tubes (95 °C).
9. Ice in ice bucket.

2.4 Selecting the Target Sequence

1. Software: mfold.

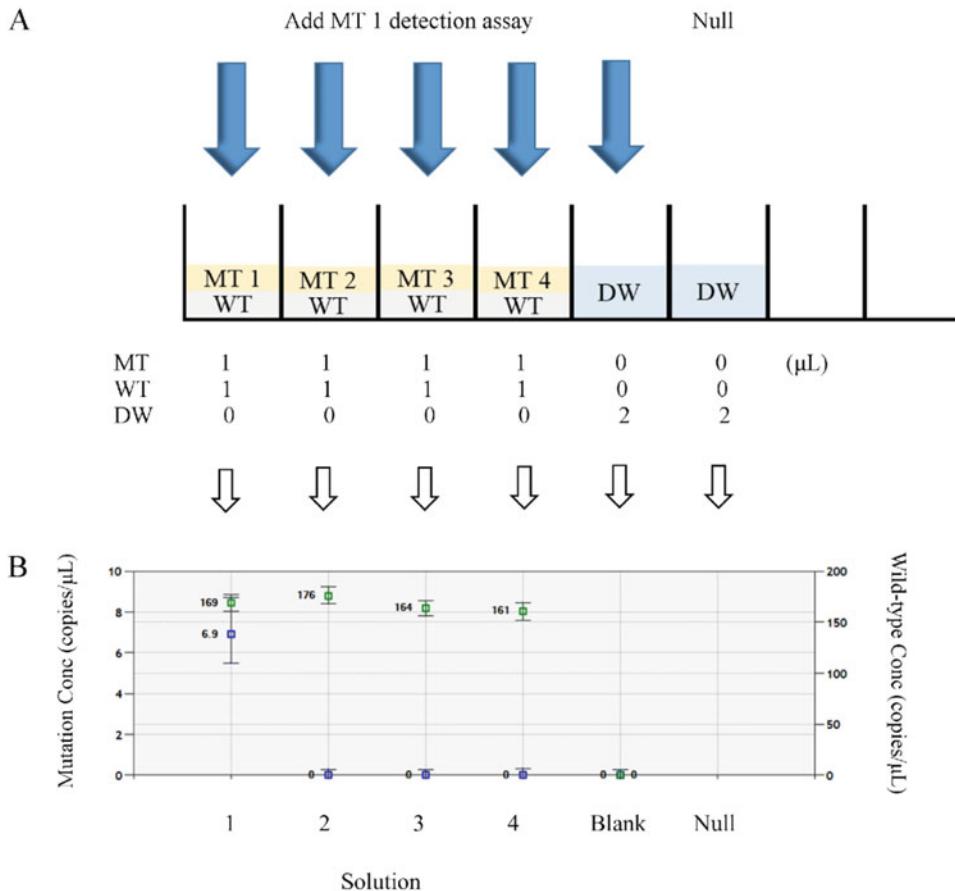


Fig. 5 (a, b) A comparative analysis of the cross-reactivity of the indicated synthetic mutation oligonucleotides in a background of normal wild-type human DNA using the ddPCR assay. **(a)** The image of the experiments for cross-reactivity between mutations that are prepared by each synthetic mutation stock oligonucleotide in a background of normal wild-type human DNA. **(b)** The absolute copies/ μL of the indicated synthetic mutation oligonucleotide and normal wild-type human DNA are shown (Fig. S3 in [13] is modified and used). Abbreviations: *ddPCR* droplet digital PCR, *MT* mutation, *WT* wild-type, *DW* distilled water

2.5 Designing Primers and Probes for a dPCR Assay

2.6 Droplet Digital PCR

1. Custom *TaqMan*® SNP Genotyping Assays.

2. The PCR mix.

3. Droplet generation oil.

4. Gasket.

5. 96-well plate.

6. Pierceable foil heat seal.

7. QX200 Droplet Digital PCR System (Bio-Rad Laboratories): droplet generator, PCR plate sealer, thermal cycler, and the droplet reader.
8. QuantaSoft software (Bio-Rad Laboratories).

2.7 Comparative Analysis of the Dilution Series and Cross-Reactivity

1. The synthetic target mutation stock oligonucleotide.
2. Normal wild-type human DNA.
3. 0.2 mL 8-tube strip.
4. Microcentrifuge (capable of $4000 \times g$ with 0.2 mL 8-tube strip rotor).
5. QX200 Droplet Digital PCR System (Bio-Rad Laboratories).
6. QuantaSoft software (Bio-Rad Laboratories).

2.8 Comparative Analysis of Mutations in Tissue and Plasma

3 Methods

3.1 Extraction of Tumor Tissue DNA

For primary breast cancer specimens, DNA from formalin-fixed paraffin-embedded (FFPE) tissue samples, which included more than three tissue cores, are extracted using the All Prep DNA/RNA Mini kit (Qiagen, Germantown) according to the manufacturer's instructions.

For metastatic breast cancer specimens, tumor cells are captured by LMD using a Leica LMD 7000 (Leica Microsystems K.K.), and DNA is extracted from the dissected tissues using the PicoPure® DNA Extraction Kit (Life Technologies) according to the manufacturer's instructions (*see* the description in ref. 8):

1. Carry out hematoxylin-eosin staining on 8- μ m thick FFPE tumor sections placed on coated foil-covered slides processed with poly-L-lysine.
2. Perform LMD to obtain only cancerous cells from a small quantity of metastatic tissue samples such that the samples do not include inflammatory and stromal cells (*see Note 1*) (Fig. 1).
3. Carefully scrape 1.5 μ g of the dissected tissue into a 1.5 mL microcentrifuge tube for DNA extraction.
4. Add 150 μ L of the Proteinase K/Reconstitution buffer mix to the tube.
5. Vortex the sample gently to mix it thoroughly.
6. Incubate the sample at 65 °C for 3 h (*see Note 2*).

7. After heat inactivation of Proteinase K at 95 °C for 10 min, directly use the solution, in which all DNA extracts are adjusted to a concentration of 20 ng/μL, as ctDNA for the mutational analysis.

3.2 Extraction of cfDNA

Blood collected in tubes coated with ethylenediaminetetraacetic acid (EDTA) is processed as soon as possible (within 2 h if possible) and is centrifuged at $1500 \times g$ for 10 min. The supernatant in the tube is plasma, which is stored frozen (-80°C is desirable) in a 1.5 mL microcentrifuge tube with a dropper until DNA extraction. cfDNA is extracted from 500 μL aliquots of plasma using the ISOSPIN Blood & Plasma DNA kit (Nippon Gene) according to the manufacturer's instructions:

1. Add 20 μL of Proteinase K to a 1.5 mL microcentrifuge tube to inactivate nucleases.
2. Return the plasma sample to room temperature (RT) and stir it by inversion. Add 250 μL of plasma to the microcentrifuge tube (see Note 3).
3. Add 250 μL of BE Buffer to the sample, and stir it for 15 s using a vortex mixer. Spin down lightly (see Note 4).
4. Incubate the sample at 56 °C for 10 min.
5. Add 250 μL of ethanol to the sample. Vortex it for 15 s, and spin down gently (see Note 5).
6. Repeat these procedures twice to treat 500 μL aliquots of plasma.
7. To adsorb DNA onto the membrane, add half of the whole mixture to the spin column, and centrifuge it at $12,000 \times g$ for 1 min at RT (see Note 6, Fig. 2).
8. Discard the filtrate along with the collection tube, and place the column in a new collection tube.
9. For washing, add 750 μL of washing buffer to the spin column, and centrifuge it at $12,000 \times g$ for 1 min at RT. Discard the filtrate along with the collection tube, and place the column in a new collection tube.
10. For washing, add 500 μL of ethanol containing washing buffer to the spin column, and centrifuge it at $12,000 \times g$ for 1 min at RT. Discard the filtrate along with the collection tube, and place the column on a new 1.5 mL microcentrifuge tube (see Note 7).
11. To elute the DNA, add 40 μL of elution buffer to the center of the membrane, and leave it at RT for 3 min. Centrifuge the sample at $12,000 \times g$ for 1 min at RT.
12. Collect the DNA solution in a 1.5 mL microcentrifuge tube.
13. Quantify all cfDNA extracts using the DNA 1000 kit and the RNA 6000 Pico kit (Agilent Technologies Inc.).

3.3 Quantification of cfDNA

A precise quantification of cfDNA may be necessary for the following two reasons. First, fragmented DNA in plasma contains double-strand DNA and single-strand DNA. Second, although dPCR can only measure the amount of the target site of fragmented cfDNA, the total DNA copy numbers can also be obtained from the dPCR results.

Therefore, we analyze the concentration and the quality of total alleles in the plasma using not only the DNA 1000 kit but also the RNA 6000 Pico kit (Agilent Technologies Inc.), which can identify the total amount of one allele that reacts with the detection probe, according to the manufacturer's instructions.

3.4 Selecting the Target Sequence

When choosing a region of the target for amplification, follow these guidelines:

1. For higher efficiency and discrimination from any primer dimers that may form, the number of bases in the target region should be 75–180 bp.
2. Avoid regions that may form secondary structures, such as hairpin loops, self-complementary structures, and primer dimers using programs such as “mfold.”
3. Avoid regions with more than four repeats of a single base.
4. Select a region with a GC content of 50–60%.

3.5 Designing Primers and Probes for a dPCR Assay

We adopt Custom *TaqMan*® SNP Genotyping Assays to design primers and probes for a dPCR assay. The dPCR assay for the detection of the specific region in the target gene consists of a pair of primers and two *TaqMan* minor groove binding probes.

Each primer is designed to anneal to the antisense strand of the target gene where functional mutations most frequently occur (*see Note 8*). One probe is designed to be specific to the wild-type sequence, and the other is designed to be specific to the target mutation (*see Note 9*). When the region is mutated, the mutant probe anneals to the template, hence giving rise to a 6-carboxyfluorescein signal. This phenomenon will not occur with the wild-type-specific probe. Conversely, when the region is not mutated, the mutant probe is unable to anneal to the template, but the wild-type-specific probe would be able to, hence giving rise to a secondary dye with either a HEX or VIC signal.

Aside from uniplex detection of the target mutation described above, multiplex detection of mutations also becomes important practically and clinically because it saves biological samples and simplifies the inspection (*see Note 10*).

3.6 Droplet Digital PCR

We performed the ddPCR assay using the QX200 Droplet Digital PCR System (Bio-Rad Laboratories), which makes thousands of droplets. Each droplet contains 0–1 copies of the target gene sequence that reacts with a pair of primers and 2 *TaqMan* probes.

For this assay, the wild-type and mutant signals represent the presence of wild-type and mutant molecules, respectively.

1. Fit an exclusive cartridge into the holder.
2. Load 20 μL of the PCR mix and 70 μL of droplet generation oil into an exclusive chamber (*see Notes 11 and 12*).
3. Install the gasket on the cartridge (*see Note 13*). Set the cartridge in the bay of the droplet generator. The droplet generator produces approximately 20,000 droplets per sample.
4. The amount to be drawn up is 40 μL . After transferring the droplets to a 96-well plate and heat sealing, place the plate into a thermal cycler for PCR and detection of reporter signals (*see Note 14*).
5. Apply the following cycling profile of the detection assay for the target mutation: incubation at 95 °C for 10 min followed by 40 cycles of 94 °C for 30 s for denaturation and optimum temperature for 1 min for annealing (*see Note 15*, Fig. 3).
6. Quantify the PCR data using QuantaSoft software (Bio-Rad Laboratories), and determine the cutoff according to the next section below (3.7).

3.7 Comparative Analysis of the Dilution Series and Cross-Reactivity

3.7.1 Dilution Assay (Fig. 4)

We confirmed that the ddPCR assay is able to detect each target mutant molecule in a background of normal wild-type human DNA with the lowest concentration and does not detect any false positives in the normal wild-type human DNA. Based on these experiments, the lower limit of detection of a true mutation must be estimated and presented.

1. Perform a twofold serial dilution five times of a solution adjusted to 50 copies/ μL of the synthetic target mutation stock oligonucleotide (*see Note 16*).
2. Add 1 μL of the solution to six tubes, and add 2 μL of distilled water to the “Blank” tube in the 0.2 mL 8-tube strip.
3. Add 1 μL of 20 ng of normal wild-type human DNA to all of the tubes except the “Blank” tube.
4. Add 1 μL of 20 ng of normal wild-type human DNA to the “Wild-type double” tube, and add 1 μL of distilled water to the other tubes to prepare a 3 μL sample per tube (*see Note 17*).
5. Carry out ddPCR using these solutions.

3.7.2 Cross-Reactivity Assay (Fig. 5)

1. Add 1 μL of the synthetic target mutation stock oligonucleotide and 1 μL of 20 ng of the normal wild-type human DNA to the required number of tubes, and add 2 μL of distilled water to two tubes in the 0.2 mL 8-tube strip.

2. Add a specified amount of target mutation detection assay to all of the tubes except the “Null” tube.
3. Carry out ddPCR using these solutions.

3.8 Comparative Analysis of Mutations in Tissue and Plasma

We compared the mutations in the tissues and plasma using the cutoff determination method described above (*see* the description in refs. 9, 10). We have shown the concordance 74.3% of mutations detected in tissues and plasma (*see* the description in ref. 9). With regard to tumor tissue, it may be difficult for the dPCR assay to determine the cutoff level since the quality of the tumor tissue is uneven in the state of preservation and degradation and DNA damage and cross-linking often cause nonspecific reactions. In this case, it may be necessary to determine the cutoff by adding a comparative examination of the specimens to the above method (*see* the description in ref. 8).

4 Notes

1. With regard to FFPE tissue samples, it may be difficult for the dPCR assay to determine the cutoff level since the quality of tumor tissue is uneven in the state of preservation and degradation and DNA damage and cross-linking often cause non-specific reactions. Therefore, as a negative control, it is desirable to collect normal breast tissue simultaneously with tumor tissue.
2. If incubation is insufficient, there may be differences in the amount of DNA extracted even in the same sample. The incubation time can be adjusted up to 24 h.
3. If stirring is insufficient, there may be differences in the amount of DNA extracted even in the same sample.
4. Stir the sample and BE Buffer to mix thoroughly. If stirring is insufficient, the extraction efficiency decreases.
5. Stir the mixture with ethanol to mix thoroughly. If stirring is insufficient, the extraction efficiency decreases.
6. There is a possibility that the column will be clogged if the number of additions of the mixture increases. Therefore, verifying the optimum adsorption capacity of the column is necessary (Fig. 2).
7. Carefully remove the column so that the filtrate does not adhere, attach it to a new tube, centrifuge it at $12,000 \times g$ for 1 min at RT with the spin column empty, and dry the membrane.
8. We designed primers for a chosen target sequence using the following guidelines:

- (a) The GC content of the designed primer is 50–60%.
 - (b) The Tm of the designed primer is between 50 and 65 °C.
 - (c) The forward and reverse primer sequences do not form any secondary structures, such as hairpin loops, self-complementary structures, and primer dimers.
 - (d) The G or C in the primer for the target sequence is not repeated for longer than three bases.
 - (e) The primer for the target sequence is confirmed to be specific and to not contain SNPs using tools such as the Basic Local Alignment Search Tool (BLAST).
9. We designed the probes using the following guidelines:
- (a) The probe does not have a G at its 5' end since it may quench the fluorescent signal.
 - (b) The probe contains more Cs than Gs.
10. We demonstrated the multiplex ddPCR assay using LBx® Probe ESR1 Multi (A082) as the detection probe for *ESR1* Y537S/Y537N and D538G; LBx® Probe PIK3CA Screen1 (A087) as the detection probe for *PIK3CA* E542K/V, E545V/G/A/Q/K, and Q546L/R/P/E/K; and LBx® Probe PIK3CA Screen2 (A088) as the detection probe for *PIK3CA* H1047L/R/Y and G1049R/S (Riken Genesis) (*see* the description in refs. 11, 12).
11. The PCR mix for 1 well is set up by mixing 3 µL of the cfDNA sample, 10 µL of 2× ddPCR Supermix (Bio-Rad Laboratories), and 5 µL of the 4× *Taq*Man Single-Nucleotide Polymorphism Genotyping Assay in a reaction volume of 20 µL, which was adjusted with sterile water.
12. All samples are compared with the mutant molecule as the positive control. A water-only (no template) control and normal wild-type human DNA (*Taq*Man Control Genomic DNA) are run in parallel for each ddPCR reaction as the negative control.
13. Maintain the gasket so it does not dry out. When the gasket is dry, the droplet generator does not work.
14. Start the PCR within 30 min after sealing.
15. Optimize the annealing temperature using a temperature gradient assay that clearly separates positive and negative fluorescence intensities. The temperature at which the difference between the positive and negative amplitudes is greatest is ideal (Fig. 3).
16. If the diluent amount is small, the plasmid does not sufficiently diffuse, and it does not dilute properly. Each diluent should be at a volume of at least 5 µL.
17. In every ddPCR plate, several wells of wild-type DNA (summing to >20 ng) should be analyzed to ensure that there are no false positives (or to determine the degree of false positives).

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Chapter 9

From cfDNA to Sequencing: Workflows and Potentials

Michela Tebaldi and Samanta Salvi

Abstract

Cell-free DNA (cfDNA) is acquiring increasingly importance in oncologic clinical practice, mostly due to its role in predicting the onset of therapy resistance by following the mutation status changes of patients. In this field, high-sensitivity methods like next-generation sequencing (NGS) could help to accurately detect somatic mutations at low frequency. Here, we report some advantages and limitations of NGS approaches for cfDNA mutation analyses with the aim of choosing the most suitable in terms of sensitivity, specificity, data output, costs, and time work.

Key words Cell-free DNA, Next-generation sequencing, Sequencing, Amplicon-based panel, Hybridization capture-based panel

1 Introduction

Recently the use of circulating cell-free DNA (cfDNA) as biomarkers has become common in clinical practice for early detection screening, prognosis, minimal residual disease, and monitoring tumor dynamics over time. In the era of personalized medicine, the cfDNA is very useful to acquire genetic tumor-related information, able to predict the treatment efficacy or the onset of resistance, thus avoiding an invasive biopsy.

Notably, in the lung cancer research, cfDNA is used as a diagnostic and prognostic biomarker analyzed by next-generation sequencing (NGS) to reveal mutations in EGFR and ALK genes to prescribe cancer-specific tyrosine kinase inhibitors (TKIs) such as erlotinib, gefitinib, or crizotinib [1, 2].

cfDNA may also be useful for monitoring tumor burden, a central aspect in the management of cancer patients that is typically assessed with imaging. Circulating biomarkers like prostate-specific antigen (PSA), cancer antigen (CA) 19-9, carcinoembryonic antigen, or CA-125 have played an increasing important role in assessing specific disease burden, especially in cases with poor imaging results. However, these circulating protein markers often lack

specificity leading to inaccurate results on tumor growth or progression. Nevertheless, cfDNA could be informative of patient's status in real time and could integrate the information given by imaging studies or protein biomarkers [3].

Another potential application of cfDNA analysis is the detection of minimal residual disease after surgery or therapy. In breast and colon cancer, the surgery resection can permit the localized remission of disease, but there is still an open question about the presence of minimal residual disease [4, 5]. By comparison between cfDNA profile before and after surgery, it may be possible to identify the minimal residual disease and its changes.

In all these applications, for many types of tumors, the availability of tumor tissue is a barrier but can be easily overcome by the use of cfDNA.

The cfDNA derived from tumor, known as circulating tumor DNA (ctDNA), is a small part of total cfDNA, and its characterization is very important to understand the nature of the tumor.

The low amount of ctDNA, which could represent <0.1% of the total amount of cfDNA, is a critical aspect that requires highly sensitive and accurate techniques for its detection [6]. NGS and digital PCR provide a deep sensitivity, but they differ in the number of targets that can be investigated at the same time [7]. Thus, each technique could be useful for specific biological queries and investigations.

For example, for tumor burden analysis and the monitoring of tumor dynamics over time, it is necessary to investigate a high number of genetic informations, thus making the NGS the most performant technique.

NGS has emerged in the past decade as an efficient method for sequencing DNA and obtaining huge informations. This technique allows massively parallel sequencing of millions of DNA templates and can generate millions of sequence reads at a tiny fraction of the cost when compared to conventional Sanger sequencing [8]. Furthermore NGS enables to analyze single nucleotide variants (SNVs), indels, copy number variations (CNVs), and fusions for multibiomarker analysis, with variant detection as low as 0.1% [9].

Digital PCR analysis can provide a higher level of sensitivity (up to about 0.01%) than NGS, but it is useful for only few molecular targets assessing an absolute quantification of mutated target compared to the total DNA. For these reasons, digital PCR is often used to confirm NGS results [10].

2 Plasma cfDNA Preparation

cfDNA is present in serum and plasma samples. The plasma can be collected using EDTA or specific tubes which stabilize nucleated blood cells; serum should be collected in tubes containing no

anticoagulant. Literature data showed that cfDNA from plasma seems to be more clean than serum cfDNA, due to the prevention of DNA derived from clotting process of whole blood cells [11].

Different extraction methods are useful and feasible for cfDNA. However, for downstream approach as NGS, it is necessary to obtain high-quality and high-quantity DNA using protocols which require few purification steps. For all these reasons, the standard method is a column-based extraction starting from at least 1 mL of plasma. Nevertheless, it is mandatory to take into consideration that raising the plasma volume, the ctDNA fraction should be enriched. The amount of cfDNA is variable because it is influenced by physiological and pathological status, but generally cancer patients present higher level of cfDNA than healthy individuals [12].

cfDNA is generally released in blood through apoptotic events, which give the characteristic size distribution profile in fragments of about 167 bp or multiples, thus as well as the nucleosomes finger-print generated by apoptotic cells [11]. However, the presence of large DNA fragments due to genomic contamination is frequent. For this reason, it should be important to check the DNA quality by Agilent Bioanalyzer or similar micro-capillary-based instruments. If long genomic DNA fragments are present, it is mandatory to verify that the selected NGS approach will be not affected by them.

3 NGS Approaches

NGS approaches can be essentially described as two categories: amplicon-based and hybridization capture-based. Both methods are successful to analyze the cfDNA; however, some specific critical issues must be considered to choose the right assay.

The amplicon-based method requires oligonucleotide primers designed to amplify the regions of interest by polymerase chain reaction (PCR). The most critical point of amplicon-based technique is that PCR may introduce errors resulting in a loss of sensitivity to detect true mutations in the original DNA fragments [13, 14] and limits the number of regions to be investigated as a result of pooling of primers.

However, this technology has some advantages: rapid preparation of libraries and the flexibility for a wide range of sample types and for the experimental design.

With regard to cfDNA analysis, this method can be useful because it could start from a little amount of DNA; despite this, the introduction of amplification-related errors leads to potentially losing the mutations at low frequency which is often the most important goal when studying somatic mutations.

The amplicon panel is the most widely used method when studying diseases where hotspot sites have already been identified

and the primary interest is to check their presence for patient monitoring.

The second method is the hybridization capture-based or enrichment strategy in which the nucleic acid strands, derived from the sample, are specifically hybridized to selected oligonucleotide fragments complementary to the regions of interest. This technique has several advantages such as (1) there is the possibility of analyzing up to hundreds of targets simultaneously; (2) the performance does not suffer of GC-rich regions; and (3) it is not necessary to use steps that involve numerous amplification cycles. This last characteristic is very relevant in the cfDNA analysis because it implies the possibility to detect low-frequency mutations. In addition, another advantage of the hybridization strategy in cfDNA investigation is the possibility of combining it with molecular barcodes. The molecular barcodes or molecular indexes can be defined with several names such as unique identifiers (UID), unique molecular identifiers (UMI), primer ID, duplex barcodes, etc. They are usually designed as a string of random nucleotides (such as NNNNNNNN), partially degenerate nucleotides (such as NNNRNYN), or defined nucleotides (when template molecules are limited). UID or UMI are introduced to target templates by ligation or through primers during PCR. These tags, combined with deep sequencing, are a great promise to limit PCR biases and sequencing errors, providing sequencing accuracy to the detection of rare and ultra-rare mutations [15, 16]. Another edge of the enrichment strategy is the possibility to use the out-of-target regions for the analysis of CNVs making it more efficient and reliable than the amplicon-based method.

The disadvantages of this type of library construction are the long time required to do the several purification steps and the amount of ng of DNA necessary for the hybridization step (approximately around 500–750 ng).

Several studies have demonstrated how the increasing of depth coverage of sequencing is an efficient way to improve the detection sensitivity of tumor-specific mutations especially with the use of UMI. The deep sequencing of large panel genes leads to the use of large platforms of sequencing to provide a high reading depth, increasing the costs. This is often not applicable in the clinical practice.

With regard to bioinformatic analysis, the hybridization capture-based is more tricky than amplicon-based approach. Specifically, the hybridization capture-based analysis requires several additional steps from the standard workflow (demultiplexing, alignment, and variant calling) such as duplication removal and the clustering analysis of UMI families after the alignment step. On the contrary, amplicon-based approach needs only the trimming of primers before the alignment. In Fig. 1, NGS approach workflows are summarized.

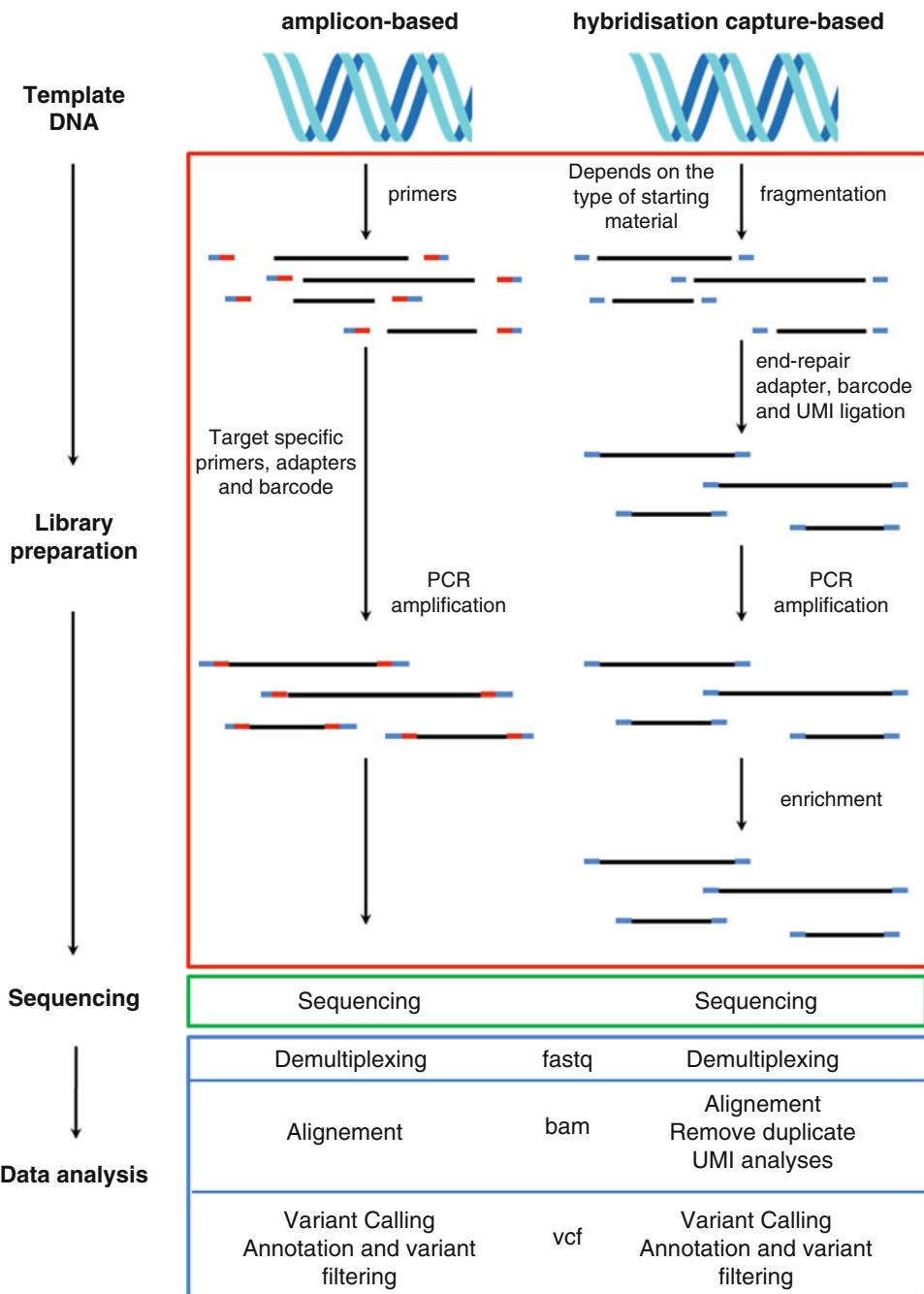


Fig. 1 Here we describe a general workflow from library preparation to bioinformatic analysis of amplicon- and hybridization capture-based approaches in NGS

A very important element to take into consideration to detect low-frequency mutation is the depth coverage of sequencing [17]. For each method described above, the identification of mutations in cfDNA requires ultra-deep sequencing as a read depth at least of $1000\times$ to ensure the assessment of mutation at a frequency of 0.1%.

4 Conclusions

Detection of rare mutations associated with treatment response, early detection, and the ability to recognize tumor-specific changes have made the cfDNA a promising biomarker in cancer research [18, 19].

cfDNA profiling of cancer patients offers several advantages for real-time monitoring. Indeed, cfDNA is a noninvasive, easy, and low-cost marker to identify the therapy resistance and the tumor heterogeneity.

However, tumor-derived DNA usually constitutes only a small percentage of total cfDNA, so the capability to detect rare genome aberrations is an essential requirement for cfDNA analysis.

For cfDNA analysis is necessary a huge deep sequencing, consequently is preferable to investigate few genes to avoid high costs and resulting feasible for clinical application.

To date, the sequencing technology is constantly evolving toward reduced costs making tumor burden and exome profile of cfDNA close to clinical practice. It will help to detect a wide and new range of aberrations important for managing metastasis and resistance to therapy, also in asymptomatic individuals.

The possibility to customize targeted gene panels for different and specific tumor types gives to NGS approach a good flexibility and the advantage to include also new discoveries.

We have described two different NGS approaches: amplicon-based and hybridization capture-based. On the first hand, amplicon-based approach permits to construct the libraries in short time, and it could be useful to investigate hotspot regions, but it affects by PCR biases that can lead to sequencing errors. On the second hand, hybridization capture-based approach has high performance for large target panel up to the whole exome analysis and can detect broad genomic arrangements, but at least 3 working days are needed.

In conclusion, the use of NGS approach for cfDNA analysis is already a great source for target therapy field. Moreover, it is still evolving toward the clinical practice resulting in a relevant and reliable technique in the next future.

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Part IV

Epigenetic Modifications



Chapter 10

Epigenetic Characterization of Cell-Free DNA

Giorgia Gurioli

Abstract

Cell-free DNA can be evaluated for the epigenetic component. Epigenetic alterations consist of changes in gene functions that do not involve changes in DNA sequence. The mainly studied epigenetic alteration is DNA methylation occurring at CpG islands in the promoter regions for which several literature data showed clinical relevance. This chapter is an overview of the epigenetic alterations detected in cell-free DNA.

Key words Cell-free DNA, Epigenetic alterations, DNA methylation, Plasma, Serum, Urine

1 Introduction

Cell-free DNA (cfDNA) is a double-stranded nucleic acid with lower molecular weight than genomic DNA that circulates in body fluids such as plasma/serum, urine, saliva, or cerebrospinal fluids. In recent years, cfDNA has been considered in diseases including thrombosis, sepsis, trauma, and cancer. cfDNA is extensively used as a biomarker in cancer because it requires early detection, it is suitable for circulating biomarkers, and the concentration of circulating plasma DNA in patients with solid tumors is higher than healthy individuals [1]. In patients with tumor, cfDNA mainly derives from normal cells, while a small proportion of fragments derives from tumor cells. Accordingly, tumor-specific genetic alterations detected in primary tumor may be found in plasma or serum of tumor cells, but plasma/serum DNA also show the same methylation pattern of primary tumor [2].

The cancer epigenome can be described as the global somatic alterations not related to DNA sequence, and data on cancerogenesis indicate that it is a multistep process, in which both genetic and epigenetic changes are involved. Epigenetic defects frequently target nonrandom, chromosomal, and subchromosomal domains or genes belonging to specific pathways [3–5]. Epigenetics can be considered as a link between the environment and the genome.

Indeed, lifestyle (e.g., smoking and alcohol consumption) and environmental factors (e.g., chemical compounds and pollution, diet, etc.) can modify the epigenome with negative effects on health, including cancer development. Epigenetic alterations seem to be the most promising biomarkers in cfDNA studies. They consist of not permanent alterations that include DNA methylation (promoter hypermethylation or hypomethylation, hypomethylation of DNA repetitive elements).

2 DNA Methylation

It has been demonstrated that individuals with different pathologies including metabolic diseases and cancer show higher levels of cell-free methylated DNA [6]. DNA methylation is considered the most researched epigenetic change, and it is often an early phenomenon in the process of carcinogenesis. For this reason it could be more suitable for early detection than mutations or other genetic alterations. Cancer cells are generally characterized by global demethylation and hypermethylation of the promoters of tumor suppressor genes [7].

DNA methylation consists of the binding of a methyl group to a cytosine (5mC) and is considered as the master epigenetic mechanism for the organization of chromatin remodeling [8]. It mainly occurs at CpG sites of the promoter regions and regulates gene expression: in the presence of methylated CpGs, gene transcription is generally silenced, whereas unmethylated CpGs are common in chromatin regions accessible to the transcriptional machinery. Methylation at non-CpG islands has an important role in the maintenance of genomic stability by preventing the instability of transposable elements [9]. In mammals, the enzymes responsible for 5mC formation are DNMT1, DNMT3A, and DNMT3B. DNMT1 recognizes hemimethylated DNA and is responsible for maintaining existing methylation patterns, while DNMT3A and DNMT3B insert methyl groups into unmethylated cytosines. Moreover, cytosine methylation intermediates have been identified: 5-hydroxymethyl cytosine is generated by the oxidation of 5mC by the TET family of enzymes (TET1, TET2, and TET3) and plays an important role in the regulation of gene expression by acting as an intermediate state of active demethylation and by modulating the binding of factors responsible for transcription [10].

CpG-island methylation testing has been proposed as a tool for cancer detection, prognosis, and detection of residual disease in both blood and other body specimens, showing that methylation status is of clinical relevance [11, 12]. Literature data showed that plasma DNA is more reliable than serum because it seems to be less affected by sample artifacts but, since the methylated component is a smaller subfraction of circulating plasma DNA, technologies with high sensitivity and specificity are necessary.

2.1 DNA Methylation in Plasma Sample

Studies evaluating epigenetic changes in plasma of patients with cancer have been performed in different tumor types and could be useful biomarkers of tumor burden and risk assessment [13, 14]. Tumor-specific methylation in circulating plasma DNA is a potential target for the development of noninvasive, blood-based assay for cancer diagnosis [15].

Aberrant methylation in circulating plasma DNA has been found in different cancer types.

SEPT9 gene promoter methylation has been largely investigated in colorectal cancer [16–18] and has been validated as highly sensitive and specific biomarker for the detection of colorectal cancer in plasma, obtaining approval as screening marker by the Food and Drug Administration [19, 20].

SEPT9 methylation is a good example of transferring a methylation blood-based biomarker from the bench to the clinic. Other potential biomarkers have been tested: *RASSF1A*, *APC*, and *E-cadherin* [21, 22]. *GSTP1* methylation in prostate cancer (PCa) is an early event in carcinogenesis, and it represents one of the most studied epigenetic markers with a diagnostic role [23–25]. *GSTP1* hypermethylation is specifically correlated with its underexpression in malignant gland tissue, and the progressive loss of *GSTP1* expression may correlate with the progressive transition from a benign phenotype to PCa [26]. It can also be used as a “liquid biopsy biomarker” detected in circulating cell-free DNA and urinary DNA [27]. Good concordance between *GSTP1* methylation in plasma DNA and matched paraffin-embedded tissue has been identified [28, 29].

CST6 was differentially methylated between breast cancer and healthy samples, and the methylation of its promoter was included in an eight-gene panel that reaches a sensitivity of 91.7% and a specificity of 90% for breast cancer detection [30]. However, larger studies with breast cancer subtype partition should be recommended. In lung cancer, the most frequently studied biomarker in plasma DNA is *CDKN2A* promoter methylation, but it seems to work better when it is part of a biomarker panel rather than a single gene [31–33]. Moreover, *SHOX2* [34] and *SEPT9* methylation [35] have been identified as potential biomarkers in lung cancer. The choice of correct control group in lung cancer studies is to be taken into account because it is a disease linked to smoking and smoking leads to gene methylation changes [36]. MethDet56 is a microarray test panel of 56 frequently methylated genes used to identify pancreatic and ovarian cancer biomarkers: decreased methylation was found in five promoter genes for pancreatic cancer vs healthy controls [37], whereas several genes were found to be informative for ovarian cancer discrimination [38, 39].

2.2 DNA Methylation in Serum Sample

Studies using serum samples are less numerous and sometimes controversial. For example, Ibanez de Caceres et al. identified *RASSF1A* and *BRCA* hypermethylation in serum DNA of ovarian cancer patients with 82% sensitivity and 100% specificity [40]. Bastian et al. found that patients with localized PCa could benefit from *GSTP1* methylation detection in serum samples [41]. Sunami et al. found that 13% of serum DNA deriving from PCa patients showed *GSTP1* methylated, and its methylation was correlated with higher stages of the disease [42]. However, Brait et al. found that, although the frequency of *GSTP1* methylation in primary prostate cancer is high, it is not a suitable methylation marker in serum because of low sensitivity [43].

On the other hand, global genomic hypomethylation is also a hallmark of cancer in humans. Indeed, hypomethylation of Alu elements has been investigated by Chen et al. in serum of patients with glioma, suggesting that it could be used in clinical practice for the diagnosis of glioma [44].

Detection of target-methylated sequences in serum or plasma can be indicative of aggressive phenotype and/or large volume of tumor, both of which correlate with poor prognosis [45]. Otherwise cfDNA methylation analysis could serve as a biomarker for tracking tumor response to therapy or to monitor treatment. Takahashi et al. showed that in patients with methylated circulating tumor DNA before neoadjuvant chemotherapy, methylation decreased after neoadjuvant chemotherapy in those with disease that responded to therapy compared to those who did not respond [46].

In circulating plasma DNA studies, control samples need to be accurately chosen: the methylation status of healthy tissue of origin could not be the best negative control, whereas leukocyte population is better because it represents the most likely source of circulating plasma DNA [47, 48] or plasma directly [15].

2.3 DNA Methylation in Urine Sample

Urine cfDNA represents an ideal biomarker for noninvasive detection. Indeed, methylation of urine cfDNA of both urological and non-urological cancer has been investigated [49, 50]. For example, *LINE-1* hypomethylation is considered a promising DNA methylation biomarker for diagnostic purposes in bladder cancer [51, 52]. *LINE-1* hypomethylation is also associated with stroke and ischemic heart disease [53]. Moreover, Payne et al. showed that *GSTP1* methylation detection in whole urine significantly discriminate PCa from biopsy-negative patients with higher sensitivity than detection in plasma [54]. Hoque et al. found a 48% concordance between *GSTP1* methylation in primary prostate cancer tissue and in urine [55].

In addition to that, the epigenetic alterations in cancer cells are closely related to dysfunctional enzymes of the machinery dedicated to the establishment and maintenance of DNA methylation/demethylation and histone code information [56]. About

700 epigenetic enzymes involved in histone modifications, chromatin remodeling, and DNA modifications have been identified [57]. Accordingly, mutations in gene coding for epigenetic modifiers result in epigenetic anomalies in terms of DNA methylation, histone modifications, and miRNA deregulation leading to global dysregulation of gene expression. Mutation of *IDH1* or *IDH2* in glioma represents an example: this genetic alteration influences chromatin remodeling, leading to hypermethylation at a large number of loci [58].

To sum up, the main challenge is to identify the most tumor-specific methylated gene since there is not a well-accepted tumor-related methylated gene until now.

3 Conclusions

This chapter summarizes the epigenetic alterations mainly found in cfDNA. DNA methylation is considered the most studied epigenetic mechanism, and it regulates gene expression. It is evaluated in plasma, serum, and urine samples for several genes with different approaches.

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Chapter 11

Quantitative Methylation-Specific PCR: A Simple Method for Studying Epigenetic Modifications of Cell-Free DNA

Luca Sigalotti, Alessia Covre, Francesca Colizzi, and Elisabetta Fratta

Abstract

Aberrant DNA methylation of cell-free circulating DNA (cfDNA) has recently gained attention for its use as biomarker in cancer diagnosis, prognosis, and prediction of therapeutic response. Quantification of cfDNA methylation levels requires methods with high sensitivity and specificity due to low amounts of cfDNA available in plasma, high degradation of cfDNA, and/or contamination with genomic DNA. To date, several approaches for measuring cfDNA methylation have been established, including quantitative methylation-specific PCR (qMSP), which represents a simple, fast, and cost-effective technique that can be easily implemented into clinical practice. In this chapter, we provide a detailed protocol for SYBR Green qMSP analysis which is currently used in our laboratory for cfDNA methylation detection. Useful information regarding successful qMSP primers design are also provided.

Key words Circulating cell-free DNA, DNA methylation, Bisulfite conversion, Quantitative methylation-specific PCR, Plasma

1 Introduction

In the last years, the discovery of cell-free circulating DNA (cfDNA) in body fluids has led to investigate its potential use as a biomarker for several diseases, including cancer. To date, cfDNA in plasma and serum is the best characterized, whereas little is known about the role of urinary cfDNA [1]. Although the molecular mechanism by which cfDNA is released into the circulation is still not fully understood, it is likely that a primary source of cfDNA may be the apoptosis and necrosis of normal and tumor cells [2, 3]. The cfDNA concentration is generally low in healthy individuals since defective cells are efficiently removed from the circulation by phagocytes [4, 5]. In cancer patients, increased amounts of cfDNA are released by necrotic and apoptotic tumor cells [6], but the levels can vary widely with a range from 0 to >1000 ng/mL with respect to healthy individuals with cfDNA concentrations ranging from

0 to 100 ng/mL [7, 8]. Since molecular alterations can be detected in cfDNA [3, 6], analysis of cfDNA isolated from plasma or serum represents a potential noninvasive approach for studying genetic and epigenetic changes in cancer patients.

Epigenetics studies heritable changes of gene expression that do not derive from alterations of the nucleotide sequence of DNA. In this respect, methylation of genomic DNA represents one of the most intensively studied epigenetic modifications of gene expression. DNA methylation occurs at the C5 position of cytosine in the context of CpG dinucleotides, and it is carried out by different DNA methyltransferases (DNMT) that show distinct substrate specificities. DNMT1, which preferentially methylates hemimethylated DNA, has been associated with the maintenance of DNA methylation patterns. On the other hand, DNMT3a and 3b do not show preference for hemimethylated DNA and are thus been implicated in the generation of new methylation patterns. Promoter DNA methylation inhibits gene expression either by directly blocking the binding of transcriptional activators or by binding methyl-CpG-binding proteins which are part of multiproteic complexes exhibiting transcriptional repressor activities. Tumor progression is characterized by a complex deregulation of the cellular DNA methylation homeostasis, resulting in genome-wide DNA hypomethylation along with promoter hypermethylation of tumor suppressor gene (TSG) (for review, see [9, 10]). In 2005, high levels of TSG promoter methylation were revealed in cfDNA of lung cancer patients [11], thus demonstrating that specific epigenetic alterations, detected in the genomic DNA in tumor cells, could also be found in patient serum/plasma. Within the last decade, aberrant methylation of several genes has been described in cfDNA of patients with different tumor types [1], even before clinical evidence of malignancy [12]. These studies have led to investigate the potential use of cfDNA methylation as a valuable noninvasive tool for early cancer detection [13–16]. More recently, the evaluation of methylation status of cfDNA has been also proposed as a noninvasive approach for treatment monitoring [17] and detection of tumor recurrence [18].

1.1 Technical Aspects Associated with cfDNA Methylation Analysis

Some characteristics render aberrant cfDNA methylation particularly attractive as clinically applicable biomarker [19], including the stability of CpG methylation modifications [20, 21], and the availability of different methylation profiles that may be either shared among several tumor types or be tumor-specific, as well as profiles potentially associated with prognosis or response to therapy [22–29]. However, to translate cfDNA methylation analysis into clinical practice, an optimization of the pre-analytical procedures is needed, since the quality and the specific amount of cfDNA remain the major limitations. Therefore, before examining the technologies applicable to cfDNA methylation analysis, we will briefly review

some critical pre-analytical aspects stemming from the intrinsic characteristics of cfDNA.

cfDNA is composed of very short fragments, usually in the range of 160–180 bp [30, 31]. This size distribution needs to be clearly taken into account when choosing the method for DNA purification and for designing the assays for methylation detection. In addition, since cfDNA can be derived from both normal and cancer cells, the fraction of tumor-specific DNA in cfDNA can be very low, especially in early-stage tumors in which a higher background of normal DNA could dramatically lower the sensitivity of detection for tumor-specific DNA alterations. If nothing can be done to alter the normal tumor proportion in cfDNA, all care must be taken to avoid any contamination of the cfDNA with genomic DNA accidentally released during blood sample manipulation prior to DNA extraction. Indeed, it is well documented that an improper processing of the blood sample may lead to an important lysis of leukocytes, which release their genomic DNA in the sample, significantly diminishing the possibility to detect cancer-specific DNA modifications [31, 32]. Accordingly, specific pre-analytical optimizations include (1) standard blood collection precautions used for preventing hemolysis; (2) choice of plasma over serum, since the clotting process can trigger a certain degree of white blood cell lysis; (3) processing the blood sample as soon as possible after venipuncture or using blood collection tubes containing cell-preserving agents; (4) and a double centrifugation to remove all possible contaminating cellular remnants [31–34].

1.2 Sodium Bisulfite cfDNA Modification

DNA methylation pattern is lost following in vitro DNA amplification. To retain methylation information, DNA is usually modified by sodium bisulfite treatment, which represents the most widely used method to discriminate methylated from unmethylated CpG residues. DNA bisulfite treatment is reproducible and can be coupled with a variety of technologies, including PCR-based assays [35, 36]. Sodium bisulfite treatment uses a chemical reaction that rapidly converts all unmethylated, but not methylated, cytosines to uracil [37]. Accordingly, incomplete bisulfite conversion can affect the accurate measurement of DNA methylation, especially when using PCR-based assays. In the original protocol, DNA is denatured using NaOH and subsequently incubated for 16 h in the presence of 3.1 M sodium bisulfite and 0.5 M hydroquinone [38]. Bisulfite treatment causes random DNA breaks, thus leading to the degradation of about 90% of the incubated DNA [39]. Given the often limited starting amount of cfDNA, and its high fragmentation [30, 31, 40], such extensive degradation can be problematic. In fact, if the number of available and/or intact cfDNA molecules is drastically reduced, the PCR reaction obviously fails. Hence, various attempts have been made in order to improve the recovery of bisulfite-treated cfDNA [41]. Optimization includes a reduced

incubation time in a highly concentrated bisulfite solution at higher temperatures and combined purification procedure after deamination step [41]. In the last years, several bisulfite conversion kits that allow for the modification of DNA from body fluids have become commercially available [42]. Therefore, it is strongly recommended to use one of these kits to ensure complete bisulfite DNA modification.

1.3 **Methylation-Specific PCR**

Although numerous bisulfite conversion-based techniques can be applied for detection of DNA methylation, the choice of the right method usually depends on the research question but also on what technology is available in the research center. These techniques can be mainly separated into those suitable for the large-scale analysis of methylated DNA and those that can be used for the study of the DNA methylation status of selected genes [43]. However, some of these methodological approaches cannot be applied to analyze cfDNA aberrant methylation, since their use is limited by the low content and the size of cfDNA fragments [44].

During the last decades, methylation-specific PCR (MSP) has been one of the methods most frequently used to evaluate cfDNA methylation status [3]. MSP is a two-tube approach, in which the bisulfite-treated DNA is separately amplified with two primer sets; the first recognizes and anneals only to methylated DNA sequences, whereas the second set amplifies unmethylated DNA region [45] (Fig. 1). Well-optimized MSP reactions can provide detection of a single methylated allele among one thousand unmethylated ones, thus representing a high-sensitivity method for diagnostic purposes [46]. However, the major disadvantage of MSP is that it can only be used for qualitative analysis.

Based on MSP, several real-time PCR adaptations have been developed. The quantitative MSP (qMSP) is highly specific and more sensitive than conventional PCR since it enables the detection of a methylated allele in a 10.000-fold excess of unmethylated alleles, thus representing a suitable method for cfDNA methylation analysis [47]. Furthermore, because of its simplicity and requirement of commonly used equipment, it is indicated for routine analyses. MethylLight, the first qMSP assay, combines MSP with a TaqMan probe that anneals only to a specific methylated or unmethylated sequence [47]. However, this technique is relatively expensive compared to other SYBR Green-based qMSP method, which eliminates the need for fluorescently labeled probes, thereby reducing the overall cost of the assay. Furthermore, SYBR Green dye-based qMSP allows melting curve analysis to confirm the specificity of the amplified product after PCR reaction. In fact, the software calculation of the melting temperature (T_m) differentiates the amplified product from primer dimers, which also results in the fluorescent signal with SYBR Green but usually melt at a lower temperature being shorter than the amplicon product. The T_m of a

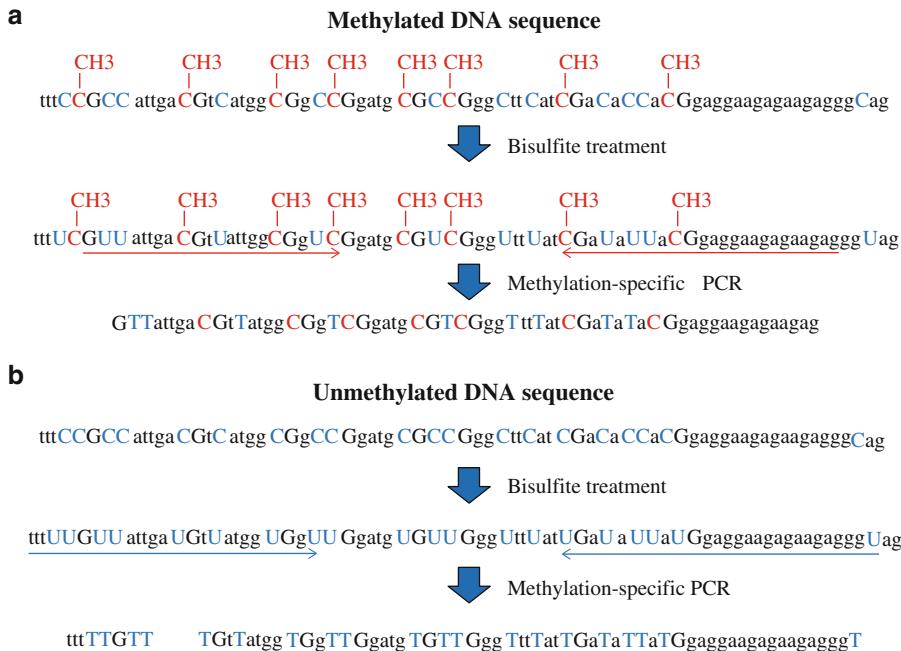


Fig. 1 Sequence modifications during bisulfite treatment and subsequent methylation-specific PCR (MSP) of a methylated (**a**) or unmethylated (**b**) sequence. Methylated cytosines are indicated in red, whereas unmethylated cytosines are in blue. DNA is treated with sodium bisulfite which converts unmethylated cytosines of the original DNA sequence to uracil (U), whereas methylated cytosine remains unchanged. Bisulfite-treated DNA is then amplified in two separate MSP reactions, each containing either a methylated (red arrows) or an unmethylated (blue arrows) specific primer set

DNA molecule depends not only on its size but also on its nucleotide composition. Therefore, PCR products resulting from a DNA sequence that was initially methylated should have a higher T_m than those derived from unmethylated templates.

Accordingly, the methodological section of the chapter specifically focuses on quantitative measurement of cfDNA methylation by SYBR Green dye-based qMSP assays.

2 Materials

2.1 Blood Sample Collection and Plasma Separation

1. 10 mL Vacutainer K₂EDTA Blood Collection Tubes (Becton Dickinson) or 10 mL Cell-Free DNA BCT tubes (Streck).
2. 15 mL polypropylene centrifuge tubes with conical bottom.
3. Low DNA binding polypropylene microcentrifuge tubes (1.5 mL).
4. Micropipettes (100–1000 µL) and polypropylene pipette tips.
5. Swinging-bucket rotor centrifuge.
6. Fixed-angle rotor centrifuge/microcentrifuge.

2.2 cfDNA Extraction

1. QIAamp Circulating Nucleic Acid (QIA) (Qiagen) (*see Note 1*).
2. QIAvac 24 Plus vacuum manifold (Qiagen).
3. QIAvac Connecting System (Qiagen).
4. Vacuum pump capable of producing a vacuum of 800–900 mbar (Qiagen).
5. Centrifuge tubes (50 mL).
6. Microcentrifuge tubes (1.5 mL).
7. Pipettes (2–20, 20–200, 100–1000 µL) and plastic pipette tips.
8. 100% ethanol.
9. 100% isopropanol.
10. Microcentrifuge.
11. Water bath or heating block capable of holding 50 mL centrifuge tubes at 60 °C.

2.3 Qubit Assay

1. Qubit dsDNA HS Assay Kit (0.2–100 ng) (Thermo Fisher Scientific).
2. Microcentrifuge tubes (0.5 mL).
3. Pipettes (2–20, 20–200, 100–1000 µL) and plastic pipette tips.
4. Vortexer.
5. Qubit fluorometer (Thermo Fisher Scientific).

2.4 Bisulfite Modification of cfDNA

1. EZ DNA Methylation-Gold Kit (Zymo Research) (*see Note 2*).
2. Microcentrifuge tubes (1.5, 0.2 mL).
3. Pipettes (2–20, 20–200, 100–1000 µL) and plastic pipette tips.
4. 96–100% ethanol.
5. Vortexer.
6. Thermal cycler.

2.5 M.SssI Treatment and Whole Genome Amplification (WGA) of Genomic DNA

1. M.SssI CpG methyltransferase [NEB, kit contains NEBuffer 2 and S-adenosyl methionine (SAM)].
2. REPLI-g Mini Kit (Qiagen).
3. Microcentrifuge tubes (0.2, 1.5 mL).
4. Pipettes (2–20, 20–200 µL) and plastic pipette tips.
5. Vortexer.
6. Thermal cycler.

2.6 Preparation of Methylated and Unmethylated Standards

1. Microcentrifuge tubes (0.2, 1.5 mL).
2. Pipettes (2–20, 20–200, 100–1000 µL) and plastic pipette tips.
3. Vortexer.

4. Thermal cycler.

5. MSP reagents:

- (a) Platinum Taq DNA Polymerase [Thermo Fisher Scientific, kit contains: 10× PCR buffer, 50 mM MgCl₂, and 5 U/μL Platinum Taq DNA polymerase].
- (b) 100 mM dNTPs set (Thermo Fisher Scientific). Before use, dilute each dNTP to 2.5 mM with nuclease-free water.
- (c) Primers specific for methylated (pM) and unmethylated (pU) templates. Upon arrival, dissolve primers using ultrapure water to 100 pmol/μL stock solution, and store at -20 °C.

6. Electrophoresis reagents:

- (a) Agarose.
- (b) Ethidium bromide.
- (c) 6× Orange G loading buffer (30% glycerol, 0.25% Orange G).
- (d) 100 bp DNA ladder, 500 μg/mL. Prior to use, dilute 40 μL DNA ladder into 160 μL of nuclease-free water. Add 40 μL of 6× Orange G loading buffer, and store at +4 °C.

7. Electrophoresis apparatus.

8. ChemiDoc Imaging Systems.

9. TOPO TA Cloning Kit for Subcloning with One Shot TOP10 Chemically Competent *E. coli* (Thermo Fisher Scientific).

10. Water bath, shaking and non-shaking incubator, and ice bucket with ice.

11. LB medium and agar plates (for preparation refer to TOPO TA Cloning Kit user manual).

12. Spreaders and tubes (5 mL).

13. NucleoSpin Plasmid kit for purification of plasmid DNA (Macherey Nagel).

14. 96–100% ethanol.

15. Microcentrifuge.

16. NanoDrop spectrophotometer (Thermo Fisher Scientific).

17. Sequencing reagents:

- (a) ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, kit also contains M13 forward and reverse primers for sequencing).
- (b) 5× Sequencing Buffer (Thermo Fisher Scientific).
- (c) Hi-DiT M Formamide (Thermo Fisher Scientific).
- (d) ABI PRISM 3100 Genetic Analyzer (Thermo Fisher Scientific).

2.7 qMSP Analysis

1. Microcentrifuge tubes (0.5, 1.5 mL).
2. Pipettes (2–20, 20–200, 100–1000 µL) and plastic pipette tips.
3. Optical 96-well microtiter plates (Thermo Fisher Scientific).
4. Optical adhesive films (Thermo Fisher Scientific).
5. SYBR Green PCR Master Mix 2× (Thermo Fisher Scientific).
6. Primers for qMSP (*see Subheading 3.5*).
7. Buffer for standard curve preparation: 1 mM Tris–HCl, pH 8.3, 10 ng/µL Hind III digested Lambda DNA.
8. Vortexer.
9. Real-time PCR instrument (ABI prism 7000 Sequence Detection System, Thermo Fisher Scientific).

3 Methods

3.1 Blood Draw and Processing for Plasma Preparation

Blood draw should follow the general good practice indications as per minimizing hemolysis, among which using of 21G or 22G needles, pre-warming the skin puncture site, not leaving the tourniquet on for longer than 1 min, filling the tubes fully to prevent excessive concentration of anticoagulant, and avoiding vigorous mixing of the sample.

The choice of the collection tube depends on whether it can be processed immediately or needs to be processed several time after venipuncture, possibly in a different place. In case of immediate processing, Vacutainer K₂EDTA tubes and Cell-Free DNA BCT tubes are equivalent, while in case of a delayed processing, or if centralized processing is needed, the choice of cfDNA BCT tubes (or similar) is needed as they preserve the integrity of nucleated blood cells for at least 72 h (14 days as per supplier instructions sheet). Specific indications for the processing of the cfDNA BCT are available at the suppliers.

Blood sample processing:

1. After blood draw, blood collection tubes must be gently inverted 10 times to allow full mixing of blood and anticoagulants/cell preservation agents.
2. Blood should be kept at room temperature until processing, which must start within 2 h (1 h ideally) from draw, when collected in Vacutainer K₂EDTA tubes, and within 72 h from draw, when collected in cfDNA BCT tubes. Whenever possible, standardizing shorter processing times is recommended (*see Note 3*).
3. Centrifuge the blood samples at 1600 × *g* for 10 min at 20 °C in a swinging-bucket rotor centrifuge to separate plasma from the formed element fraction.

4. Using a 1 mL micropipette, carefully transfer the upper plasma layer into a 15 mL conical bottom tube, taking care to not disturb the interphase (leave a few of plasma to be on the safe side). Usually, 4–5 mL of plasma is obtained from 10 mL blood.
5. Centrifuge the plasma at $16,000 \times g$ for 10 min at +4 °C in a fixed-angle rotor centrifuge. If a centrifuge suitable for allocating 15 mL tubes is not available, plasma can be aliquoted in 1.5 mL Eppendorf tubes and centrifuged following the same conditions using a microcentrifuge.
6. Using a 1 mL micropipette, recover the plasma without disturbing the pellet, and either proceed directly to DNA extraction or divide the samples in 1 mL aliquots in 1.5 mL Eppendorf low DNA binding tubes, and store at –80 °C until use.

3.2 Extraction of cfDNA from Plasma

cfDNA is extracted from plasma obtained in Subheading 3.1 using QIA and following the manufacturer's protocol (*see Note 4*). Below there are recommendations to optimize the protocol [48]:

1. Process up to 5 mL of plasma in order to isolate sufficient amounts of cfDNA.
2. Carrier RNA must be dissolved in Buffer AVE and then mixed with buffer ACL. Unused carrier RNA should be aliquoted and frozen at –20 °C.
3. Add an additional wash with Wash buffer 1 to ensure complete removal of the binding buffer excess and possible lysate contaminates [49].
4. Heat the AVE buffer to 40 °C to maximize the cfDNA recovery [49].
5. Elute twice the purified cfDNA in a low volume of buffer AVE (50 µL and then 30 µL) to obtain high concentrations [49].

3.3 Quantification of cfDNA

The quantification of cfDNA is performed using the Qubit dsDNA HS Assay Kit on a Qubit fluorometer (*see Note 5*), following the manufacturer's instructions. To make a measurement, 1 µL of sample is added to 199 µL of working solution. Note that air bubbles in the sample preparation can interfere with fluorescence detection. The capacity to determine accurate cfDNA concentration can be further evaluated using quantitative real-time PCR (qRT-PCR) of housekeeping genes [50] or repetitive DNA sequence elements [51] (*see Note 6*).

3.4 Bisulfite Modification of cfDNA

Bisulfite conversion of cfDNA from Subheading 3.2 is performed using the EZ DNA Methylation-Gold Kit, according to the manufacturer's instructions. For optimal conversion results, the points to be considered in respect to the kit handbook are as follows:

1. The amount of input cfDNA should be from 50 to 500 ng.
2. After the addition of 200 μ L of M-desulfonation buffer to the column, incubate the sample at room temperature for 20 min.
3. Use small (e.g., 20 μ L) volumes of M-Elution buffer to maintain high bisulfite-converted cfDNA concentrations.
4. Use immediately the bisulfite-converted cfDNA for qMSP amplification, or store at -20°C .

3.5 Primers Design for qMSP Analysis (See Note 7)

Although qMSP represents a simple technique that can be easily integrated into the routine analysis, the ability to accurately determine cfDNA methylation status largely depends on design of primers [52]. As reported above, qMSP requires two sets of primers specifically designed to anneal to the same bisulfite-converted CpG-containing region and to yield different PCR products. To this end, qMSP primer pairs must (1) be specific for bisulfite-converted DNA, in order to preclude the generation of false-positive results caused by incomplete bisulfite conversion (*see Note 8*) and (2) clearly discriminate between methylated and unmethylated DNA sequences following bisulfite treatment.

First of all, it is necessary to define the genomic region of interest and to analyze its CpG density, since this step could affect the results of the experiment. In fact, qMSP primers should be located within CpG-rich DNA sequences in order to distinguish native from bisulfite-treated DNA. Gene promoters are often targets of qMSP assay since approximately 60–70% of all human genes contain CpG-rich regions in their 5'-promoter region, called CpG islands, which are usually unmethylated in normal cells [53]. QMSP primers are generally designed within the region placed 1000 bp upstream and 500 bp downstream of the transcription start site (TSS). This solution frequently works in laboratory practice since most CpG islands are located around TSS, and their hypermethylation usually represses gene transcription [54]. However, some CpG islands containing transcription factor binding sites can be found upstream, downstream, or even distant from TSS [55]. Based on these considerations, literature data can be helpful in identifying DNA sequences where CpG island methylation may play a biological role.

Once the genomic region of interest has been defined, qMSP primer pairs can be designed using MethPrimer [56], a web-based program freely accessible that facilitates the study of DNA methylation by providing several tools and databases, including those for (1) predicting CpG islands within the candidate genomic region and (2) designing primers for qMSP assays. The process of qMSP primer design with MethPrimer is demonstrated with an example in Fig. 2.

(a)



MethPrimer - Input

MethPrimer allows you to design primers for most bisulfite conversion based PCR primers and to predict CpG islands on an input sequence. It also allows you to search for predesigned primers for human and mouse genes.

- Design primers on your input sequence:** Paste an ORIGINAL DNA [sequence](#). Input sequence doesn't need virtual bisulfite conversion (e.g. convert 'C' to 'T'). Try this [Sample sequence](#).
- Search for predesigned primers for human and mouse genes:** Type in the box below gene symbol, RefSeq ID, to search for pre-designed primers for protein coding genes, lncRNAs and miRNAs (e.g., ESR1, NM_000044, mir-1-1, PANDA).
- CpG island prediction:** You can also use the program to predict CpG islands in a sequence.

```

ACCCG CAGGCATTCTCAAGGGGTGCACTGTGCAAATGCTCACAGGTGACAGAAAC
GAGCATCTCTGCCATACCTTCAACAGCAGGGTGACGAGACGACCTCTGA
GTGAGGACTGAGGGTCAACGCCCGCCACCCACACCATAGAGGGACACAGAAAT
CCAGCTCAGCCCCCTTGTGCGCTTAAACGCAAGCAGTGTGTCACCCAGACACA
CCCCCTCCCCCAATGCGCTTCACTTCAAGGTCAAGACTGTGGTGTGAGGGAGC
AGAAAGCAATCTGCAAGAGATGGGTCAGGCTCAAGCTCAAGGACCTG
AGGGATGACCGAAAGCCGCCACCAACCCCACCTCCCGACCCACCAAGGATCTAC
AGCTCAAGGACCCGCCACCACTTAACTCTTGCCCCATCACATCTTATGCTTAC
TCCACCCCCATCCGGTCCATCACTTCAAGGATTTCAAGTTCACCCCTGCGCGAACCCAG
GGTAGTACCGTTGCCAGATGTGACGCACTGACTTGCGATTGGAGGTGACAGAACGGC
GAGATTCTGCCCTGAGCAACGAGCGACGGCTGACGCTGGCGGGAGCTGGCGGGCCAG
GCTCGGGTGAAGGGAGCAAGGTAAGCCTGAGGGAGGACTGAAGCGGGCCCTACCTCAGAC
AGAGGGCCTCAAATAATCCAGTGTGCTCTGCTGCGGGCTGGGCAACCCCGCAGGG
AAAGACTTCCAGGCTGGGGACAGAGCTTAATGTGGCCAGGGGGGGGTGTGAGAGGTCAGGG
GGGAACCTTGGGGACAGAGCTTAATGTGGCCAGGGGGGGGGGTGTGAGAGGTCAGGG
CCACAGCTGGCAGGAATCAAGGTCAAGGACCCCGAGAGGGAACTGAAGGGCAGCTTACCC
ACACCCCTGACCACTTCCGGCCCAACACCAACCCCGACCCCATCCCCATTCCCC

```

Pick Bisulfite sequencing PCR (BSP) primers.

Pick MSP primers.

Pick Methylight BSP primers.

Pick Methylight MSP primers.

Pick Nested BSP primers.

Pick Nested MSP primers.

Pick CORBRA primers.

Use CpG island prediction for primer selection?

Window

Shift

Obs/Exp

GC%

100

1

0.6

50

Design primers on the Minus strand?

Blat Genome:

None

Use Degeneracy for primer selection?

Submit

Reset

General Parameters for Primer Design

Sequence name (optional):

Output Number (optional):

5

Target Regions (optional):

"start, size", such as (560, 30)

Excluded Regions (optional):

"start, size", such as (160, 50 1100, 50)

Primer Tm:

Min: 57

Opt: 59

Max: 60

Primer Size:

Min: 20

Opt: 25

Max: 30

Primer GC%:

Min: 10

Opt: 40

Max: 80

Primer GC clamp:

0

Primer non-CpG 'C's:

4

Primer Poly_X:

5

Primer Poly_T:

8

Primer Tm diff:

5

Product Size:

Min: 80

Opt: 150

Max: 200

Parameters for MSP primers

3'CpG constraint:

1

CpG in primer:

1

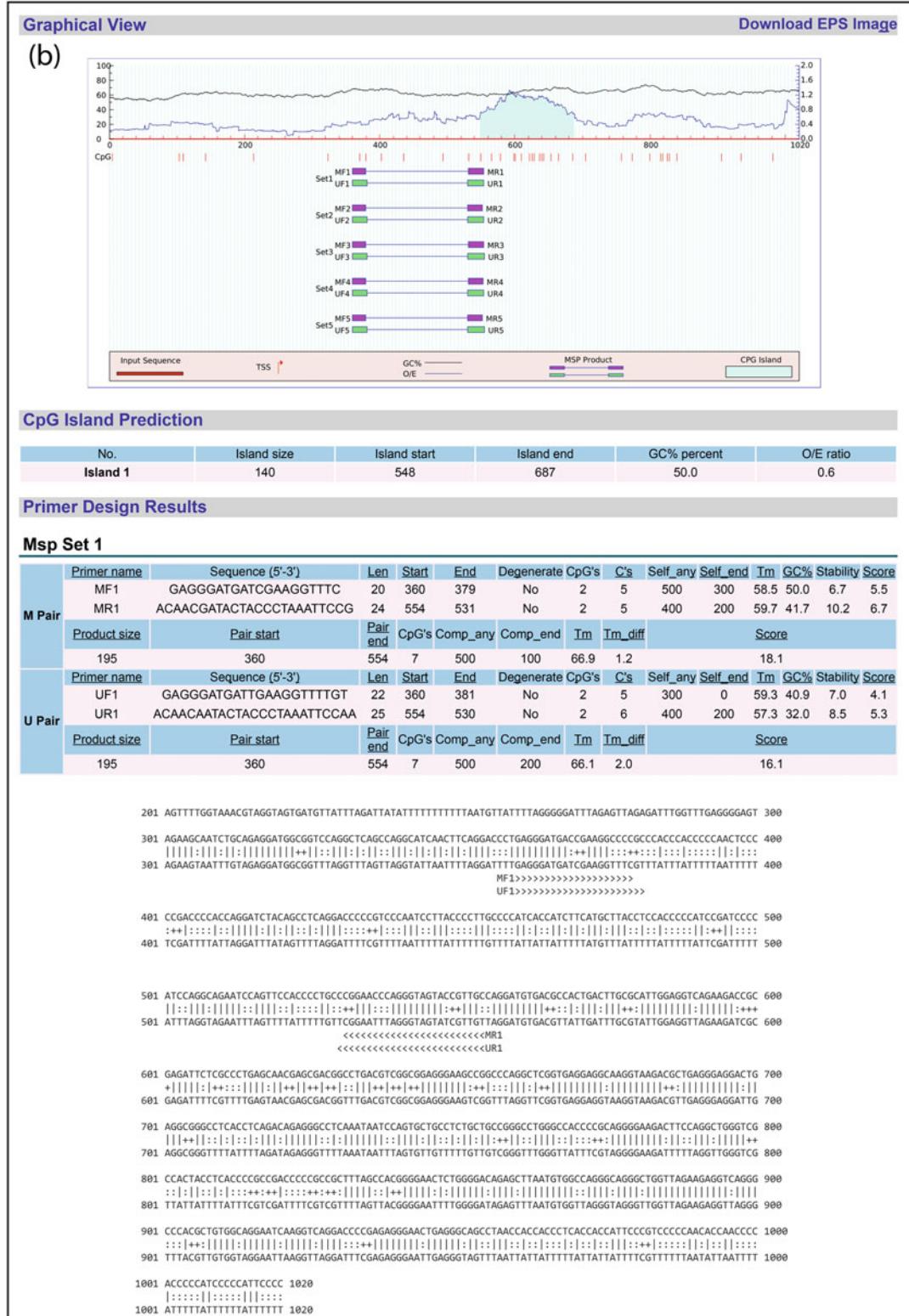
Max Tm difference:

3

Submit

Reset

Fig. 2 MethPrimer is a web-based program freely accessible at <http://www.urogene.org/methprimer2/>. (a) MethPrimer provides several tools and databases for studying DNA methylation, including those for designing primers for qMSP assays. Genomic DNA sequence is copied and pasted in the appropriate field of MethPrimer which offers several options regarding primer features. (b) MethPrimer accepts the native DNA sequence as input, transforms it into the bisulfite-modified one, and searches the sequence for potential CpG islands. Primers are usually picked around the predicted CpG islands. Results are given in the form of graphic presentation and text view showing sequence alignment and primers location. All "C"s followed by "G" that are potential candidate for DNA methylation are indicated with "+" along the sequence

**Fig. 2 (continued)**

Guidelines to consider when designing qMSP primer:

1. Go to NCBI gene bioinformatics website (<https://www.ncbi.nlm.nih.gov/gene/>) or similar websites to retrieve the genomic sequence of interest.
2. Open MethPrimer (<http://www.urogene.org/methprimer2/>), and select “Design PCR primers.”
3. In the window called “MethPrimer—Input,” copy and paste the candidate genomic region, select “Pick MSP primers,” and click submit.
4. To choose the adequate qMSP primers, you should select a T_m range of 57–60 °C (primer T_m) and a maximum T_m difference (max T_m difference) between pM and pU pairs of 2–3 °C.
5. In general, pU has a lower annealing temperature than pM. In order to have pM and pU pairs with a closer annealing temperature, we suggest to include few more bases at the 5' end of pU.
6. To prevent the amplification of unmodified or incompletely modified DNA, at least four scattered Cs that are not followed by G should be included in primer sequences (primer non-CpG “C”s).
7. To provide maximal discrimination between methylated and unmethylated bisulfite-treated DNA, qMSP primers should contain up to three CpG sites within their sequence (CpG Cs in primer), and one of these polymorphic C/T sites should preferably be located at the 3' end of the primer (3' CpG constraint).
8. To facilitate the application of qMSP to cfDNA, where amplification of large fragments is not possible, the product size should be from 80 to 150 bp. If the candidate genomic DNA region contains more than one CpG island, any of the CpG islands can be selected for primer design.

Here we report some additional tips to improve qMSP primers design:

1. Following bisulfite treatment the converted DNA strands are no longer complementary, so either strand can be selected for qMSP primers design. Consequently, the submission of the reverse complement sequence is recommended when the other fails to provide relevant qMSP primers.
2. Upstream or downstream sequences in addition to the genomic imported region may also help in optimizing qMSP primer design.
3. The incorporation of locked nucleic acids (LNAs) [57] at one or more CpG sites in primer sequences can improve the analytical specificity of qMSP by enhancing discrimination between methylated and unmethylated templates [58] (see Note 9).

MethPrimer provides up to nine possible pM and pU pairs (output number) together with the unconverted genomic DNA sequence (upper sequence) and the bisulfite-converted sequence (lower sequence) (Fig. 2). Before proceeding with qMSP analysis, specificity and sensitivity of pM and pU pairs must be checked using fully methylated and unmethylated DNA templates (*see Note 10*). Furthermore, we recommend to verify PCR bias, a condition that leads to preferential amplification of either methylated or unmethylated DNA (*see Subheading 3.8*) [59].

3.6 Preparation of Fully Methylated and Unmethylated DNA Controls

Fully methylated (mDNA) and unmethylated (uDNA) DNA can be easily prepared from any genomic DNA sample using M.SssI methyltransferases and WGA, respectively (*see Note 11*).

M.SssI methylase is an enzyme that globally methylates cytosines in the context of the CpG dinucleotide using SAM as a methyl donor. Fully mDNA control is prepared by incubating up to 4 µg of genomic DNA (1 µg/µL) in a 20 µL reaction containing 1 U of M. SssI methylase, 1× NEBuffer 2, and freshly prepared 160 µM SAM (*see Note 12*). The reaction is incubated for up to 4 h at 37 °C and then stopped by heating at 65 °C for 20 min. To repeat M.SssI treatment on the same genomic DNA should ensure complete conversion of cytosine to 5-methylcytosine.

Fully uDNA control is obtained through WGA by phi 29 DNA polymerase, which creates a copy of the genome with all methylated cytosines replaced by unmethylated cytosines. WGA is performed according to instructions provided with the REPLI-g. In brief, 10 ng of genomic DNA (4 ng/µL) is denatured for 3 min at room temperature with 2.5 µL of Buffer D1 and then neutralized by adding 10 µL of Buffer N1. To amplify the DNA template, denatured and neutralized sample is incubated overnight at 30 °C with 40 µL of Master Mix containing the phi 29 DNA polymerase (REPLI-g DNA polymerase). After amplification, the REPLI-g DNA polymerase is heat-inactivated at 65 °C for 10 min. To perform WGA on the same genomic DNA, twice is beneficial for obtaining completely uDNA.

Before proceeding with bisulfite conversion as described in Subheading 3.4, M.SssI-treated and amplified DNA are cleaned up by phenol/chloroform/isoamylalcohol extraction followed by ethanol precipitation or by using a commercial DNA purification kit (e.g., Genomic DNA Clean & Concentrator, Zymo Research) and normalized to a concentration of 10 ng/µL using Qubit fluorometer (*see Note 13*).

3.7 Preparation of Methylated and Unmethylated Standards

To quantify cfDNA methylation levels by qMSP, standards with known number of mDNA and uDNA molecules are required. This could be achieved by using serial dilutions of control genomic mDNA and uDNA with known concentration. Cloning the control genomic mDNA and uDNA into plasmid vectors is an alternative

method that provides (1) higher accuracy and (2) the availability of a large amount of standard DNA.

To generate plasmid standard curves, bisulfite-treated mDNA and uDNA are amplified by MSP in two separate reactions, as reported below.

1. For each amplification reaction, 2 µL of bisulfite-treated DNA is added to 48 µL of PCR mix containing:
 - (a) 5 µL of 10× PCR buffer.
 - (b) 3 µL of MgCl₂ 50 mM.
 - (c) 1 µL of each dNTP 2.5 mM.
 - (d) Each primer set diluted at the final working concentration (*see Note 14*).
 - (e) 0.25 µL of Platinum Taq DNA polymerase (5 U/µL) (*see Note 15*).
2. Mix gently, and spin briefly. Place the PCR reactions in a thermal cycler, and incubate at 95 °C for 5 min, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C.
3. Verify the MSP products by gel-based electrophoresis. The presence of a single and specific band is considered as a successful MSP amplification (*see Note 16*).
4. Clone both methylated and unmethylated MSP products using the TOPO TA Cloning Kit, following the manufacturer's instructions. By using this kit, MSP products can be incorporated into the pCR 2.1-TOPO vector and transformed into One Shot TOP10 competent cells.
5. Once transformed, plate bacteria on LB agar plates containing 100 µg/µL ampicillin (*see Note 17*). Plates are then inverted and incubated overnight at 37 °C.
6. The next morning pick five to ten white colonies from each plate and inoculate separately into 3 mL LB cultures with 100 µg/mL ampicillin. Grow the cultures on constant agitation overnight at 37 °C.
7. Extract plasmid DNA from each culture using the NucleoSpin Plasmid kit.
8. Verify the sequence of the cloned PCR fragments by Sanger sequencing, with the M13 forward primer and the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit, according to the manufacturer's protocol. Sanger sequence electrophoresis and data sampling are performed using an automated sequencer (e.g., ABI PRISM 3100 Genetic Analyzer). Plasmids containing verified methylated and unmethylated MSP inserts are referred to as methylated (Std_M) and unmethylated (Std_U) standards, respectively.

9. Quantify the concentration of Std_M and Std_U at the Nano-Drop spectrophotometer, and calculate the number of molecules per microliter ($\text{mol}/\mu\text{L}$) of both standards using the following formula:

$$\text{No. of mol}/\mu\text{L} = [\text{plasmid concentration } (\text{g}/\mu\text{L}) / (\text{vector length} + \text{insert length}) \times 660] \times N_A$$

where N_A = number of Avogadro (6.022×10^{23}).

10. Use buffer for standard curve preparation to dilute each plasmid, starting from a concentration of 1×10^{10} molecules per $2 \mu\text{L}$ ($5 \times 10^9 \text{ mol}/\mu\text{L}$) (see Note 18). Use $10 \mu\text{L}$ of this dilution plus $90 \mu\text{L}$ of buffer for standard curve preparation to make the second dilution, and repeat this process until you have serially diluted both plasmids to $5 \text{ mol}/\mu\text{L}$, as shown in Fig. 3. Below are some recommendations:
- Vortex each dilution before preparing the next one to ensure adequate suspension of the DNA.
 - Change pipette tip before every dilution to prevent carry-over of more concentrated DNA dilutions.
 - Make at least $100 \mu\text{L}$ of each standard, and store them at -80°C .

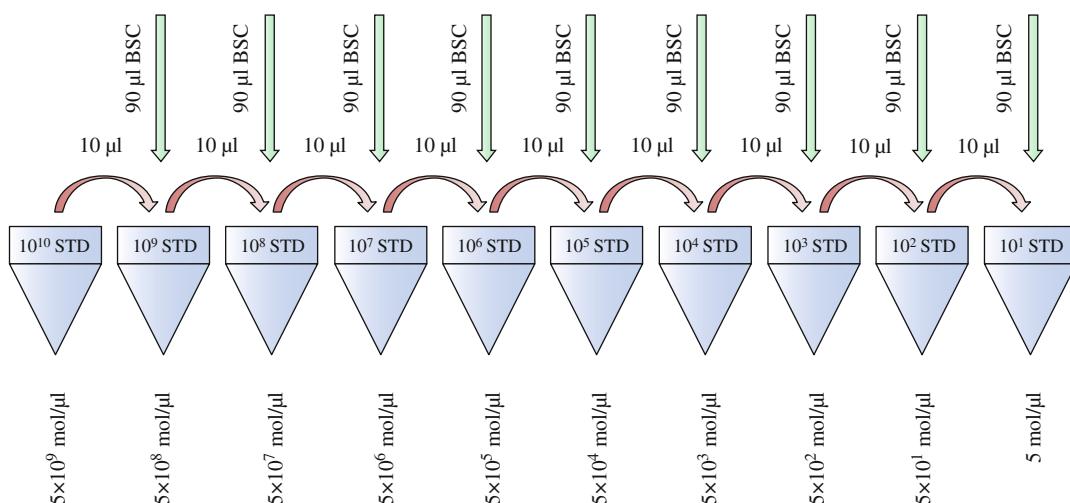


Fig. 3 Standard curves are made of tenfold serially diluted plasmids containing either completely methylated or fully unmethylated DNA insert and indicated as standards (STD). $90 \mu\text{L}$ of buffer for standard curve (BSC) preparation is dispensed into 0.2 mL tubes. $10 \mu\text{L}$ of STD diluted to 1×10^{10} molecules per $2 \mu\text{L}$ ($5 \times 10^9 \text{ mol}/\mu\text{L}$) is pipetted into the 10^9 standard tube. Subsequently, $10 \mu\text{L}$ of $5 \times 10^8 \text{ mol}/\mu\text{L}$ STD is transferred into the 10^8 standard tube. This process is repeated until each plasmid has been diluted to 10^1 STD. Each dilution must be vortexed and spun down before preparing the next one

Table 1
Mix setup for qMSP and qUSP reactions

Reagents	TriPLICATE AMOUNT VOLUME^{a,b}	
	qMSP mix	qUSP mix
SYBR Green Mix 2×	37.5 µL	37.5 µL
pM forward (7.5 pmol/mL) (see Note 14)	3 µL	
pM reverse (7.5 pmol/mL)	3 µL	
pU forward (7.5 pmol/mL) (see Note 14)		3 µL
pU reverse (7.5 pmol/mL)		3 µL
H ₂ O	To 69 µL	To 69 µL

^aReactions are carried out in triplicate

^bVolumes are referred to a single sample; for the final volume, each value is multiplied for the total number of samples and increased by 10% to allow for pipetting

3.8 Specificity, Sensitivity, and Quantitative Accuracy of qMSP Assay

Before proceeding with cfDNA methylation analysis, we recommend to verify that the new qMSP assay is suited to amplify and to detect methylated and unmethylated copies of the target DNA and displays the correct proportion of methylated and unmethylated DNA amounts.

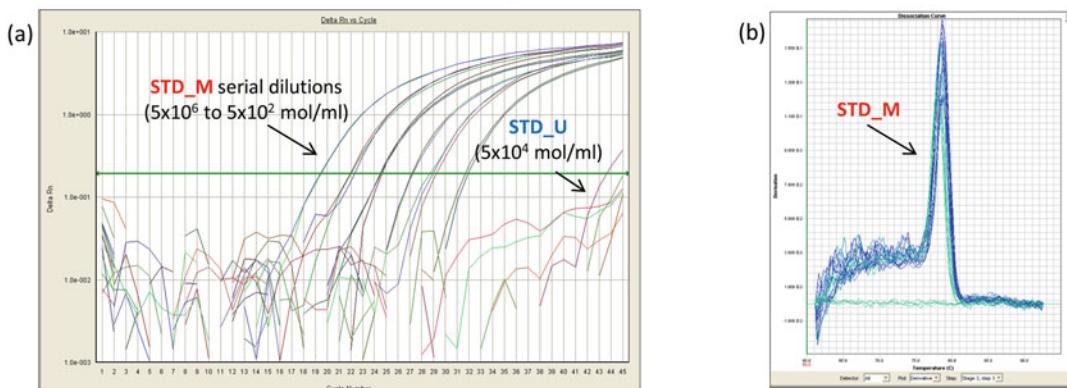
To test the specificity of qMSP primers in differentiating methylated versus unmethylated templates, serial dilutions of both Std_M and Std_U are amplified in two separate reactions using either pM (qMSP reaction) or pU (qUMP reaction).

1. Assemble qMSP and qUSP master mixes as described in Table 1.
2. Transfer 69 µL of qMSP reaction mix to 0.5 mL tubes, and add into each tube 6 µL of plasmid or water blank. Five serial dilutions of STD_M (5×10^6 to 5×10^2 mol/µL), one serial dilution of STD_U (5×10^4 mol/µL), and water blank are amplified in the qMSP reaction. Do the same for qUSP reaction.
3. Mix well by vortexing, and pipette 20 µL of the above mix into each well of a 96-well microplate.
4. Seal the microplate with an optical grade sealing tape.
5. Spin the microplate to collect the reactions at the bottom of the wells and to eliminate any air bubbles that may be present.
6. Run the qMSP using the following program: 95 °C for 10 min (activation of AmpliTaq Gold DNA polymerase present in the SYBR Green Mix), followed by 45 cycles at 95 °C for 15 s (denaturing template) and 1 min at 60 °C (primer annealing and template extension). Fluorescence data are collected during the annealing/extension step for determination of the cycle threshold.

7. To ensure amplification of desired products, perform the melting curve analysis which consists of one cycle at 95 °C for 15 s, 60 °C for 20 s, and 95 °C for 15 s with data collection throughout the linear increase of temperature from 60 to 95 °C.

The qMSP and qUSP reactions can be considered 100% specific if (1) Std_M and Std_U yield pure products only with pM and pU pairs, respectively, and (2) pM pair does not amplify Std_U in the qMSP assay and vice versa (Fig. 4).

qMSP reaction



qUSP reaction

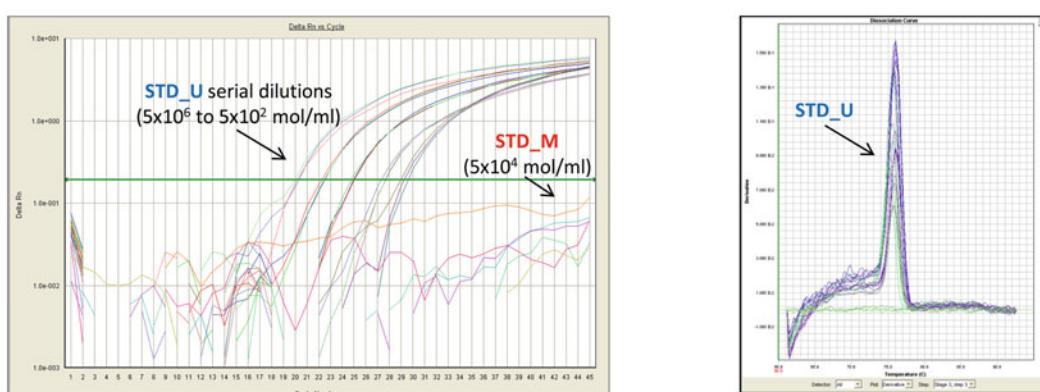


Fig. 4 Specificity and cross-reactivity of primers for methylated (pM) and unmethylated (pU) DNA templates. (a) Graphs showing PCR cycles (X-axis) plotted against the fluorescence intensity of accumulated amplified product in SYBR Green reaction mixture (Y-axis). Serially diluted methylated (STD_M) and unmethylated (STD_U) plasmids are used as a template in two separate reactions with either pM (qMSP reaction) or pU (qUSP reaction). Both qMSP and qUSP reactions are 100% specific since pM do not cross-react with pU and vice versa. (b) Specificity of pM and pU pairs is confirmed by melting curve analysis: serially diluted STD_M and STD_U yield pure products only with pM and pU pairs, respectively (melting temperatures, 78.5 °C for pM and 76 °C for pU). No extra peaks indicative of unspecific products or primer dimerization are present

Under ideal conditions qMSP sensitivity has been estimated to be 1 methylated copy within 10.000 copies of unmethylated genomic DNA [47]. Therefore, a minimum of 10.000 copies of DNA template in each PCR reaction is required to achieve optimal sensitivity. Based on these considerations, to assess the sensitivity of qMSP assay:

1. Serially dilute Std_M in Std_U solution. The amount of Std_U is kept constant (10^5 copies/well), and the amount of Std_M is decreased (e.g., 10^3 –0.1 copies/well).
2. Perform qMSP on these dilutions.
3. Do the same for qUSP reaction.

Both pM and pU pairs should detect dilutions up to 1:10.000.

Finally, the proportional PCR amplification of methylated and unmethylated templates can be checked with a control mDNA: uDNA 50:50 mix, as indicated below:

1. Prepare the following ratio of mDNA:uDNA: 100:0, 50:50, 0:100.
2. Proceed with bisulfite conversion as described in Subheading 3.4.
3. Amplify the bisulfite-treated mDNA:uDNA control mixes in qMSP and qUSP reactions.

Melting curve analysis should show an equal proportion of mDNA/uDNA 50:50 mix compared to the control amplification of 100% mDNA and uDNA, respectively (Fig. 5).

3.9 qMSP Assay

Bisulfite-converted cfDNA is quantified by preparing two parallel reaction mixes as described above (*see* Subheading 3.8). In addition to cfDNA samples, each run must include:

1. STD_M and STD_U (5×10^4 to 5 mol/ μ L).
2. mDNA and uDNA diluted to 5 ng/ μ L.
3. Bisulfite-untreated genomic DNA diluted to 5 ng/ μ L.
4. A water blank.

As suggested above, cfDNA, standards, and controls should be run in triplicates.

The copy number of methylated or unmethylated sequences for each cfDNA sample is established by extrapolation from the respective standard curves. To make the results easily usable in clinical practice, the methylation index (MI) is calculated as follows:

$$\text{MI} = [\text{methylated DNA copies}/(\text{methylated DNA copies} + \text{unmethylated DNA copies})]/100$$

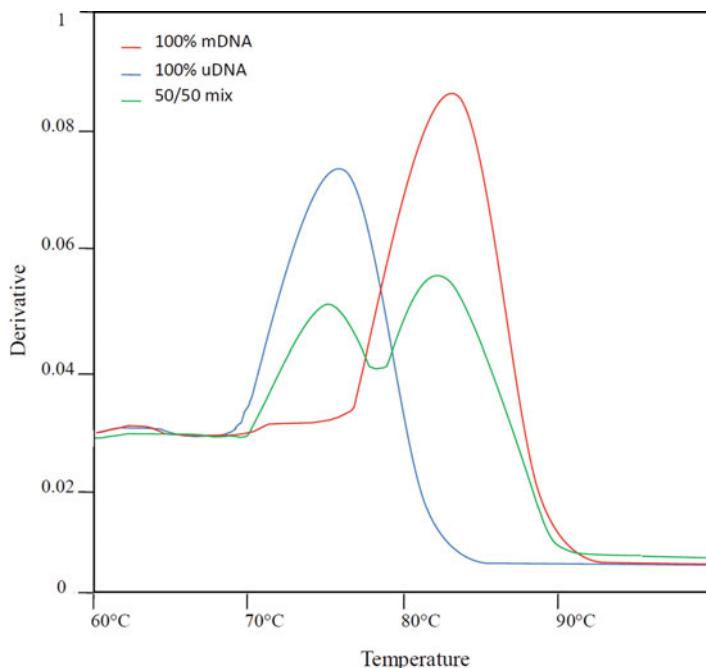


Fig. 5 Melting curve analysis reports an equal proportion of fully methylated (mDNA) and completely unmethylated (uDNA) 50:50 DNA mix (green line) when compared to the control amplification of 100% mDNA (red line) and 100% uDNA (blue line, U), which show higher and lower melting temperatures, respectively

The threshold for determining the threshold cycle for each well must be set within the logarithmic phase of the amplification plot. Note that increasing the threshold value gives a higher sensitivity, while the specificity decreases. On the other hand, a lower threshold value increases the specificity but reduces the sensitivity. A threshold level for the % of cfDNA methylation in cancer is often undetermined, and the cutoff value is evaluated and determined in each individual case. However, setting the threshold on the highest % of DNA methylation value detected in normal controls could give the best discrimination between normal and malignant sample.

4 Notes

- Upon arrival, QIAamp Mini columns can be stored at room temperature for up to 4 weeks and at 2–8 °C at least 1 year.
- The CT Conversion Reagent is provided as a solid mixture and must be dissolved by adding 900 µL of ultrapure water, 300 µL of M-Dilution Buffer, and 50 µL M-Dissolving Buffer to the tube. This is enough for 11 cfDNA samples. If the CT Conversion Reagent does not entirely dissolve, we recommend to centrifuge the solution and to recover the supernatant. To

ensure high yield and efficient bisulfite conversion, the CT Conversion Reagent solution should be used immediately following preparation. Otherwise, it can be stored overnight at room temperature, 1 week at 4 °C, or up to 1 month at –20 °C. Always keep the dissolved CT Conversion Reagent in the dark.

3. The effect of refrigeration on preventing genomic DNA release from white blood cells when collected in K₂EDTA tubes has not been fully investigated. While some reports suggest that it might be of benefit when long-term storage of the sample is required, refrigeration appears an unnecessary protocol complication when K₂EDTA tubes are processed in a timely manner [31, 33].
4. QIA is one of the most commonly used commercial kits for manual cfDNA isolation, and it has been reported to produce high cfDNA yields. For automated cfDNA isolation, other highly efficient kits are commercially available [60].
5. The Qubit fluorometer is more sensitive and specific for quantifying cfDNA than the NanoDrop spectrophotometer since it measures the fluorescence of a double-stranded DNA-specific dye. Therefore, contaminants such as RNA or nucleotides in the sample do not affect the quantification.
6. QRT-PCR of repetitive elements is also used to evaluate cfDNA integrity [61].
7. Primer sequences for several qMSP assays can also be found in the scientific literature. However, we suggest to design your own primer set since published primers (1) could not have been extensively validated for their specificity and sensitivity and (2) would not produce the same results under different experimental conditions (e.g., if they were not designed for analysis of cfDNA).
8. Bisulfite-untreated genomic DNA should be included in qMSP analysis. The presence of any products in unmodified genomic DNA template sample could indicate that (1) bisulfite treatment is incomplete and (2) primers are not specific for the bisulfite-treated DNA.
9. Note that the addition of LNA bases to qMSP primers could increase the predicted T_m by up to 8 °C per LNA monomer substitution.
10. In an ideal qMSP assay, pM pair would only amplify the mDNA control, whereas pU pair would be positive for only uDNA control. If unmethylated product is amplified in the reaction that uses pM pair and/or vice versa, we suggest additional optimization steps which might include (1) adjusting qMSP condition (e.g., increasing annealing temperature) and (2) designing new pM and pU pairs.

11. If only small quantities of methylated and unmethylated genomic DNA controls are required, we suggest to use commercial control DNA (e.g., EpiTect PCR Control DNA Set, Qiagen) in order to save effort and time.
12. A 50 μ L reaction volume is recommended for more diluted DNA samples (<1 μ g/ μ L).
13. To test the success of the M.SssI treatment and WGA, we recommend qMSP reactions using well-established pM and pU pairs. To maximize mDNA and uDNA quantification, LINE-1 repetitive elements can be used as qMSP target. Approximately 18% of the human genome consists of LINE-1 repetitive elements which are moderately CpG-rich, thus representing a surrogate marker of global DNA methylation (for LINE-1 qMSP primers, see [62]).
14. In our experience, a final primer concentration of 7.5 pmol/ μ L works well for most qMSP assays, but in some cases, improved efficiency reactions may be obtained by higher or lower working primer concentration. To optimize primer concentration, we suggest to prepare individual working dilutions of forward and reverse primers (e.g., from 3.75 to 12.5 pmol/ μ L) and to test their combinations leaving all the other qMSP conditions unmodified. Optimal final primer concentrations should (1) achieve the maximal amount of product necessary for quantification and (2) avoid unspecific bindings and primer dimerization.
15. The pCR2.1 TOPO vector is a TA cloning system and requires an extra adenosine added to the 3' end of both strands during the PCR reaction. Therefore, in case alternatives to Platinum Taq have to be used, choose an appropriate enzyme or enzyme combination that is able to add the required 3' deoxyadenosine overhangs.
16. If unspecific bands are also visible in the agarose gel (e.g., pM and/or pU dimers), commercially available kits such as Zymoclean Gel DNA Recovery Kit (Zymo Research) can help in purifying the target MSP product.
17. The pCR2.1 TOPO plasmid is a blue/white selection plasmid in which white colonies are considered positive for the insert. For blue/white screening of recombinants, we recommend to include IPTG and X-gal in the LB agar. It is possible to obtain false positives (white colonies with no MSP products), so an alternative method to confirm the presence of the insert in white colonies is suggested. For example, plasmid DNA obtained in Subheading 3.7 can be screened with restriction enzymes (e.g., EcoRI, NEB) to identify pCR2.1 TOPO vectors with inserted MSP products.

18. In qMSP reaction, we add 2 µL of standard per well. If a different volume of standard is used, calculations must be changed accordingly.

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Part V

Fetal and Pediatric Diseases



Chapter 12

Cell-Free DNA in Pediatric Rhabdomyosarcoma: Potential and Challenges

Lucia Tombolan, Angelica Zin, and Gianni Bisogno

Abstract

Rhabdomyosarcoma is an aggressive solid tumor that may disseminate hematogenously giving metastasis which represents the most important prognostic factor. Chances of an effective cure in childhood cancer rely on the capacity to make an early and accurate diagnosis, detect metastatic disease or relapse, and predict the response to treatment.

Liquid biopsy is a very promising blood test for cancer detection and noninvasive disease monitoring. This method has a great advantage to use blood and plasma, a more accessible biological material, quick and easy to obtain with minimal pain and risk for patients. In particular, circulating free DNA (cfDNA) represents a tumor biomarker detected in plasma that gives information on biology and genetic background of tumor.

Moreover, cfDNA mutation detection could be a reliable method to monitor the efficacy of treatment and to discover novel targets for a personalized treatment in pediatric solid tumor. Here, we describe an optimized protocol to cfDNA isolation from small amount of plasma, as well as a method to assess the quantity and quality of cfDNA. Finally, we propose ddPCR as a reliable method to detect mutations at low frequency in cfDNA obtained from pediatric rhabdomyosarcoma samples.

Key words Cell-free DNA, Rhabdomyosarcoma, ddPCR, Pediatric solid tumors, Mutation detection

1 Introduction

Rhabdomyosarcoma (RMS) is an aggressive tumor of mesenchymal origin that typically affects young children and represents up to 4% of all childhood malignancies with evidence of metastasis at diagnosis in 20–25% of cases. It can develop in almost any part of the body, but preferential sites are the head and neck, genitourinary organ, and extremities. RMS is characterized by a variable degree of myogenic differentiation, and two main subtypes have been identified: embryonal RMS (ERMS), which accounts for approximately 70% of all RMS, and aggressive alveolar RMS (ARMS) (20%) [1].

In the late 1970s, cooperative national and international study groups started to adopt a systematic multidisciplinary approach

including multidrug chemotherapy coordinated with surgery and radiotherapy. As a consequence, the chance of survival has progressively increased up to 80% in patients with localized tumors. Treatment modalities and intensity are tailored according to a series of prognostic factors with the most important being represented by the presence of metastasis at diagnosis (20–25% of patients). In this case, prognosis is dismal, with less than 30% of children surviving 3 years after diagnosis. Other prognostic factors include tumor size and site, histological subtype, nodal involvement, and patient age. In the last years, many efforts have been done to identify biological features that can integrate, or even substitute, clinical factors to predict tumor aggressiveness and the risk of relapse. The prognosis of patients with RMS after relapse is below 20% [2, 3]. More powerful methods to assess the potential of distant dissemination or relapse are, thus, warranted to address children at risk to more effective treatments during first-line therapy and improve their survival. Current tumor diagnosis and biological characterization depend on the availability of tumor biopsies. Recent studies have found that tumor cells detached from primary site can release their content in the bloodstream [4]. Therefore, possible tumor biomarkers, such as circulating tumor DNA (cfDNA), may be studied in blood and plasma (liquid biopsy) that represent a much more accessible biological material, easy to obtain with minimal risk and pain for patients.

Tumor DNA released by the tumor cells into the blood harbors the same alterations of the original tumor clones; therefore the detection of specific genetic aberrations in peripheral blood may provide a specific biomarker of disease [5] and may be of clinical relevance for the monitoring of minimal residual disease. Mutation detection in cfDNA also allows to monitor the efficacy of therapies, to understand potential resistance mechanisms, and, in perspective, to propose novel targeted therapies [6, 7]. Recently, cfDNA was studied in a cohort of pediatric neuroblastoma patients and characterized for genomic copy number alterations. This study demonstrated the feasibility to analyze cfDNA in pediatric solid tumors. Moreover, the comparison of copy number profile of cfDNA with respect to that of primary tumors highlighted as cfDNA may reflect genetic alteration of more aggressive cell clones [8]. Therefore, liquid biopsies, and in particular the analysis of cfDNA, could be transferred to another pediatric and aggressive solid tumor like RMS. The greatest technical challenge is the isolation of very low amounts of cfDNA from blood with a high variability between samples. This point is more difficult in pediatric patients where the quantity of peripheral blood that can be collected depends also on age and weight of the children. Ideally the time points of blood collection should be integrated into the clinical protocols in order to allow comparison of laboratory and clinical data (i.e., radiological tumor response).

In this study we optimized a protocol to isolate cfDNA from small amounts of plasma of patients with rhabdomyosarcoma. cfDNA isolated can be quantify with fluorimetric methods or by real-time PCR to obtain the total amount of cfDNA that may be correlated with clinical features. In addition, we evaluated the quality of cfDNA using Agilent Bioanalyzer to check the absence of gDNA contamination. cfDNA obtained can be used for downstream application such as next-generation sequencing or digital PCR to identify somatic mutations or other genetic aberrations. In particular, in this work, we describe the use of droplet digital PCR (ddPCR) to detect mutations at very low frequency and in small amount of cfDNA.

2 Materials

2.1 Equipment and Supplies

1. 10, 20, 100 µL micropipettes.
2. 10, 20, 100 µL micropipette filter tips.
3. 1.5 mL sterile microcentrifuge tubes.
4. 15 mL centrifuge tubes.
5. Centrifuge and microcentrifuge.
6. Magnetic stand OR QIAvac 24 plus vacuum manifold and vacuum pump.
7. Water bath and heating block.
8. Qubit fluorimeter or similar.
9. Real-time PCR instrument (Thermo Fisher or similar).
10. Agilent Bioanalyzer instrument.
11. Thermal cycler with 96-deep well reaction.
12. Droplets generator (QX200 Bio-Rad or similar).
13. Droplets reader (QX200 Bio-Rad or similar).

2.2 Plasma Isolation

Vacutainer EDTA tubes.

2.3 Extraction of Cell-Free DNA

1. QIAamp Circulating Nucleic Acid kit (Qiagen) or QIAamp MinElute ccfDNA kit (Qiagen) for cell-free DNA extraction.
2. Ethanol (96–100%).
3. Isopropanol (100%).
4. Water DNase-free.

2.4 Quantification of Cell-Free DNA by Fluorimetric Assay

1. Qubit High-Sensitivity DNA assay (Thermo Fisher).
2. Qubit assay tubes 0.5 mL.

2.5 Quantification of Cell-Free DNA by qRT-PCR

1. qRT-PCR master mix.
2. POLR2D TaqMan assay.
3. PCR plates.

2.6 Cell-Free DNA Quality Evaluation

2.7 Digital Droplet PCR (ddPCR)

1. High-sensitivity DNA kit (Agilent Technologies).

1. ddPCR supermix for probes, no dUTP (Bio-Rad Laboratories).
2. ddPCR mutation detection assay (Bio-Rad Laboratories).
3. PCR plates.

3 Methods

3.1 Plasma Isolation from Peripheral Blood

Peripheral blood samples preferably in EDTA (3/5 mL) should be processed within 24 h from drawing (*see Note 1*). For other sample collection considerations, refer to El Messaoudi et al. [9].

1. Centrifuge blood samples for 10 min at $890 \times g$ at room temperature setting.
2. Carefully aspirate plasma supernatant. About 1.5/2.5 mL plasma can be obtained from 3/5 mL of peripheral blood.
3. Aspirated plasma is transferred into fresh 15 mL centrifuge tubes.
4. Centrifuge plasma samples for 10 min at $16,000 \times g$ at room temperature setting (*see Note 2*).
5. Carefully remove supernatant and transfer to 1.5 mL tube.
6. For longer storage, keep plasma frozen at -80°C . Before using the plasma for circulating nucleic acid extraction, thaw plasma tubes at room temperature.

3.2 Isolation of Cell-Free DNA

Starting from at least 500 μL of plasma is recommended. Typically for pediatric samples, the amount of starting plasma is 1 mL or less.

cfDNA is extracted according to the manufacturer's instructions (QIAamp MinElute ccfDNA, Qiagen).

1. Mix plasma with magnetic bead suspension, proteinase K, and bead-binding buffer, and then incubate for 10 min at room temperature while shaking (low speed) (*see Note 3*).
2. Place the tube containing bead solution into a magnetic rack. Let stand for at least 1 min until the solution is clear. Discard supernatant.
3. Remove the tube from the magnetic rack, and add 200 μL bead elution buffer to the bead pellet. Pipette up and down to

resuspend beads. Transfer the mixture (including beads) into an elution tube.

4. Incubate for 5 min on a shaker at room temperature at 300/400 rpm.
5. Place the elution tube containing the bead solution into a magnetic rack. Let stand for at least 1 min or until the solution is clear.
6. Transfer the supernatant into a new elution tube. Add 300 μ L buffer ACB, and vortex to mix. Briefly centrifuge the tube.
7. Pipette the mixture from step 6 into a QIAamp MinElute column, and centrifuge for 1 min at $6000 \times g$.
8. Place the QIAamp MinElute column into a clean 2 mL collection tube.
9. Add 500 μ L of wash buffer to the QIAamp MinElute column, and centrifuge for 1 min at $6000 \times g$. Place the column into a clean 2 mL collection tube, and discard the flow-through.
10. Centrifuge at full speed ($20,000 \times g$; 14,000 rpm) for 3 min.
11. Place the column into a clean 1.5 mL elution tube. Open the lid, and incubate the assembly in a shaker (set to 300/400 rpm) for microcentrifuge tubes at 56 °C for 3 min to dry the membrane completely or incubate in a heat block at 56 °C for 8 min.
12. Carefully pipette 40 μ L of ultraclean water into the center of the membrane (*see Notes 4 and 5*). Incubate at room temperature for 1 min.
13. Centrifuge at full speed ($20,000 \times g$; 14,000 rpm) for 1 min to elute the nucleic acids.
14. Store cfDNA at -20 or -80 °C until use.

3.3 Cell-Free DNA Quantification

3.3.1 Fluorimetric Methods

Yields of cfDNA isolated from plasma are normally very low and therefore is difficult to determine the concentration using spectrophotometer. The use of high-sensitivity fluorimetric methods is preferable. Use the Qubit High-Sensitivity DNA assay (Thermo Fisher) or similar following the manual's instructions. Briefly the protocol is the following:

1. Prepare the Qubit working solution for all samples and the standards (included in the kit) diluting Qubit reagent 1:200 in Qubit buffer.
2. Prepare assay tubes (thin-wall 0.5 mL PCR tubes) mixing Qubit working solution with DNA samples to reach a final volume of 200 μ L. 2 μ L of cfDNA can be used for an optimal quantification.
3. Vortex all tubes for 2–3 s.
4. Incubate all tubes for 2 min at room temperature. Then, read the samples with dedicated instrument. The instrument

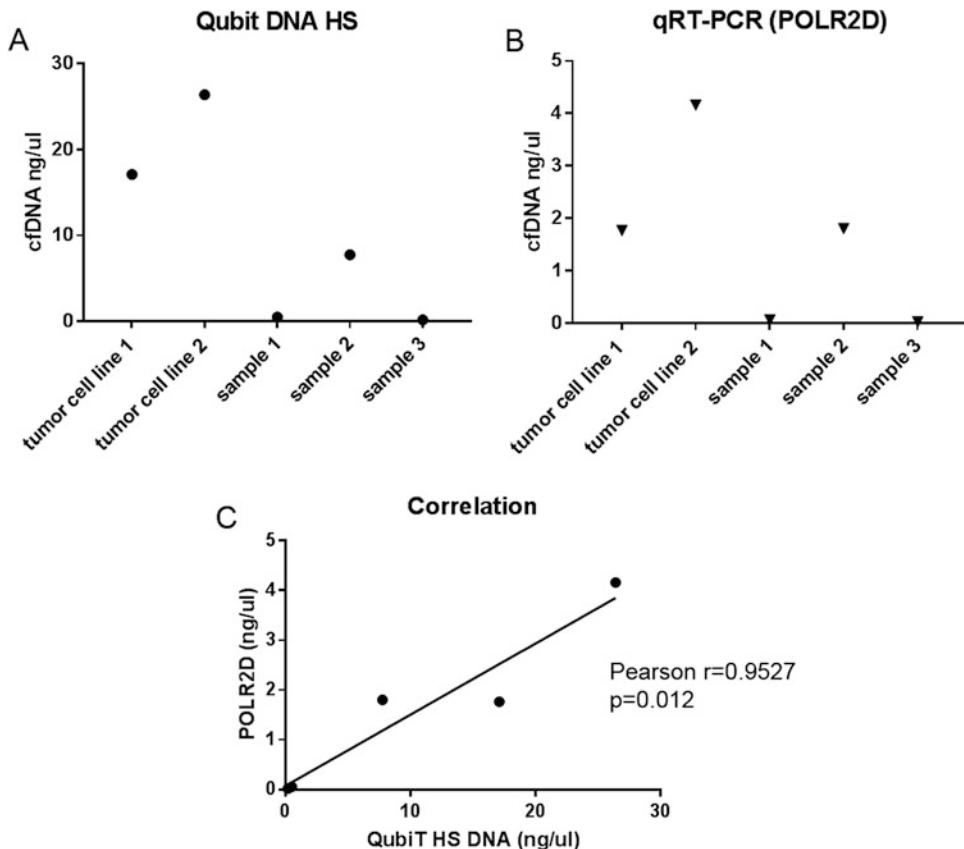


Fig. 1 Quantification of cfDNA. cfDNA can be quantified by fluorimetric assay (**a**) and qRT-PCR method (**b**). The correlation of two methods is optimal (**c**)

measures the DNA quantity in the assay tube and displays also the quantification of DNA in the stock solution based on volume of sample loaded (i.e., 2 μ L). The unit of measure more adequate can be set in the instrument. An example of fluorimetric quantification is reported in Fig. 1a.

3.3.2 Real-Time PCR Methods

Plasma ctDNA can be quantified using a TaqMan-based quantitative real-time PCR (qRT-PCR) assay for a target gene (i.e., *POLR2D*).

A standard curve is necessary to determine the starting target quantity.

Each sample or dilution could be tested in triplicate.

1. Prepare a standard curve using DNA of a cell line as starting material. Then, prepare fivefold serial dilutions of DNA in sterile water.
2. Prepare a mix of reaction using Master Mix for Probes (no UNG), primers at final concentration of 900 nM, and probe at final concentration of 200 nM. cfDNA must be used

without any dilution. Each sample is tested in triplicate, and the mean values of results are calculated.

3. Run the PCR reaction in qRT-PCR instrument using absolute quantification/standard curve template. The cycle used is the following (*see Note 6*):
 - 10-min initial activation at 95 °C.
 - 95 °C for 15 s and 60° for 1 min (40 cycles).
4. Analyze the data. The quantification using a target gene (POLR2D) will be relative to a standard curve. An example of quantification by qRT-PCR is shown in Fig. 1b. A comparison between fluorimetric assay and qRT-PCR to quantify cfDNA was reported in Fig. 1c.

3.4 Evaluation of Cell-Free DNA Quality

The size distribution of circulating DNA purified can be checked by analysis on an Agilent Bioanalyzer or similar device. Typically, cfDNA fragments corresponding to the size of the DNA wrapped around the nucleosomes (approx. 145/180 bp or multiples of these). This step is crucial to check the contamination of cfDNA with genomic DNA (gDNA) of blood cells that can occur during plasma preparation. Microfluidic electrophoresis using the Agilent 2100 Bioanalyzer and high-sensitivity DNA chips (Agilent Technologies) allows to assess DNA fragment length for a size range between 50 and 7000 base pairs (bp) based on manufacturer's recommended protocol. Briefly the protocol is the following:

1. Prepare gel-mix dye mixing high-sensitivity DNA dye with gel matrix. Vortex and spin at 2240 × φ for 10 min.
2. Put a new high-sensitivity DNA chip on chip priming station, and load gel-dye mix as indicated in the manual.
3. Pipette marker in all sample wells. Then, load ladder and 1 μ L of cfDNA per samples.
4. Run chip and analyze the data output. The peak corresponding to 160/180 bp can be undetectable if the amount of cfDNA is very low. A good cfDNA is reported in Fig. 2a, while a cfDNA with a marked contamination of gDNA was reported in Fig. 2b. In the last case, the quantification of cfDNA is not reliable and cannot be used to evaluate minimal residual disease.

3.5 Mutation Detection in Cell-Free DNA by Droplet Digital PCR Assay

A potential use of cfDNA is to detect known tumor mutations with high sensitivity. Specific ddPCR assay can be designed for many types of mutations (single-nucleotide variant—SNV—or small insertions/deletions, InDel) according to manufacturer's instruction (*see Note 7*).

1. Calculate the limit of detection (LOD) of each mutation detection assay.
The LOD is defined as the lowest mutant concentration that can be reliably distinguished from wild-type (mutation negative) control. This step can be achieved using mutation-

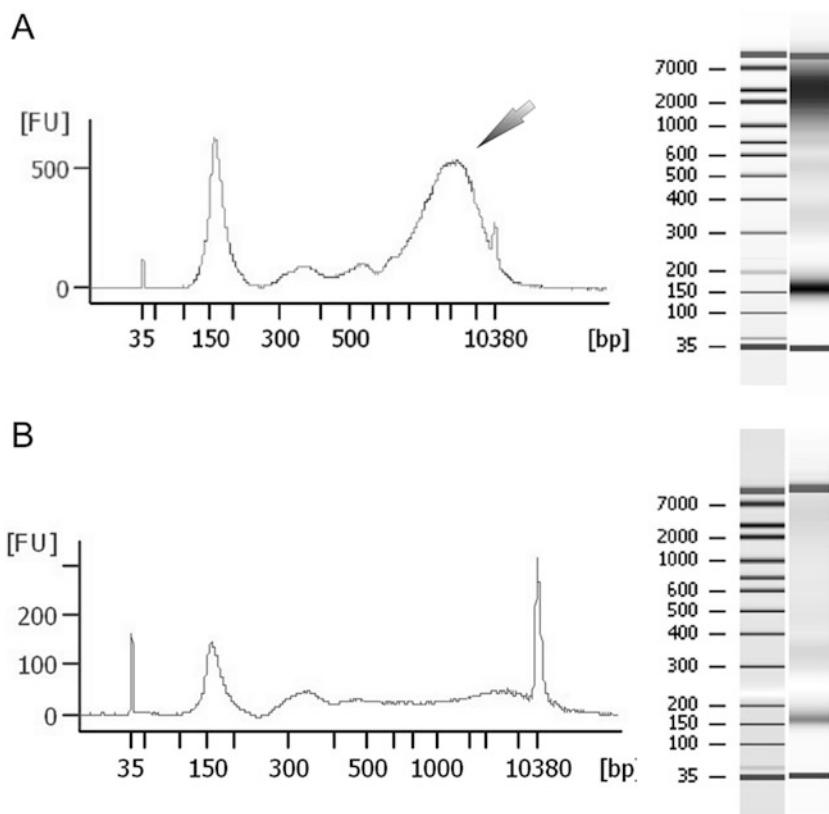


Fig. 2 Quality check of cfDNA assessed by Agilent Bioanalyzer. In (a) is shown an electropherogram with an evident contamination of gDNA (black arrow). Conversely, in (b) is reported a cfDNA with optimal quality. The peaks of tumor circulating DNA correspond to the size of 145/180 bp or multiple of these

positive control. Good option for positive control is (1) DNA from cell lines that show the specific mutation or (2) synthetic double-stranded sequence-verified blocks of DNA (gblock, IDT, or operon).

Then, prepare at least fivefold dilution series of DNA of mutation-positive control (*see Note 8*).

2. Prepare the mix of reaction using Master Mix for Probes (no UNG) and primers/probes (*see Notes 9, 10, and 11*). Samples are placed into a QX200 Droplet Generator to partition each sample into 20,000 nanoliter-sized droplets. Carry out the ddPCR in a PCR thermal cycler according to manufacturer's instruction. For cfDNA evaluation, at least 4–6 μ L of cfDNA should be loaded as template for each well. Each sample could be tested at least in triplicate.
3. Read plate with QX200 reader, and analyze data with dedicated software analysis available with the instrument (*see Note 12*). An example of ddPCR result is shown in Fig. 3.

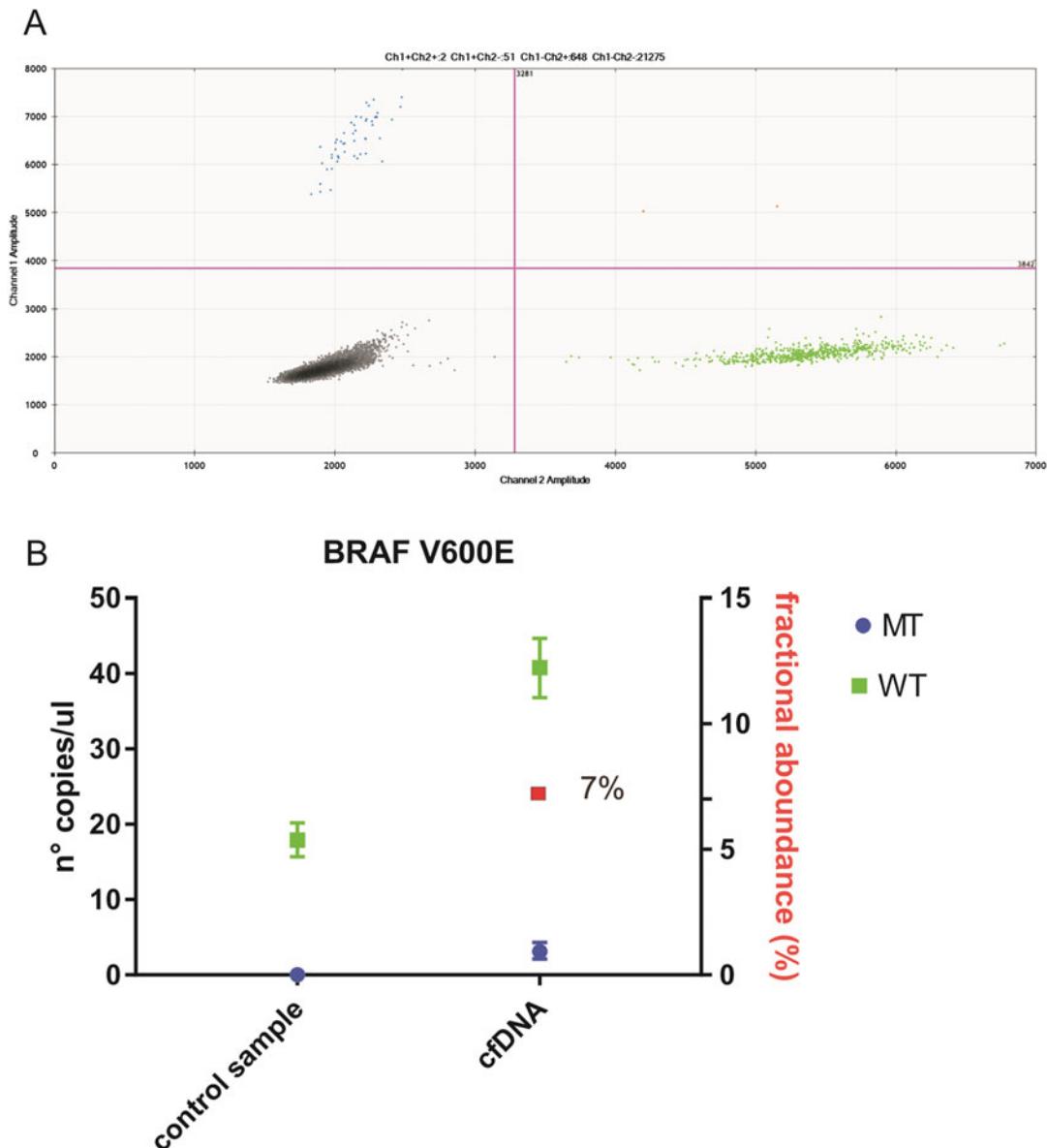


Fig. 3 Mutation detection on cfDNA by ddPCR. (a) Two-dimensional scatter plot demonstrating the four clusters obtained with a mutant and a wild-type allele. In blue, droplets contain only mutant template; in green, droplets contain only wild-type template; in red, droplets contain both template; and in gray, droplets contain no template. In (b) a ddPCR result for BRAF V600E mutation detection in cfDNA of a RMS patient. As shown in control sample (white blood cells), there are only wild-type copies (green) while in cfDNA occur both wild-type (green) and mutated (blue) molecules. In red (Y right axis) is indicated the percentage of mutation (n° mutated copies/ n° tot copies) in cfDNA sample

4 Notes

1. The use of EDTA or Streck tubes is mandatory to avoid contamination of gDNA, especially if peripheral blood is processed many hours after collection.
2. In order to isolate circulating cell-free nucleic acids from blood samples, two centrifugation steps should be performed in order to remove cellular debris and to reduce the amount of genomic DNA and RNA in the sample.
3. Adding DNA carrier to sample must be avoided particularly when isolated DNA will be used for next-generation sequencing or ddPCR.
4. Elution of cfDNA in ultrapure water is mandatory for NGS or ddPCR downstream applications.
5. The optimal elution volume when starting from 500 µL to 1 mL of plasma is 40–60 µL.
6. The thermal cycle depends on master mix and instrument used.
7. Newly designed ddPCR mutation detection assay must be run across a thermal gradient (53–60 °C) to determine the annealing/extension temperature that optimizes separation between positive and negative droplets.
8. When gblock is used for LOD detection, in each well will be loaded also DNA from a wild-type control to obtain a mix of wild-type and mutant sequences.
9. Prepare master mixes for ddPCR in a template-free environment.
10. Negative sample control wells (no DNA) must be run in each plate, and the observed positive droplets should be zero.
11. Mix of reaction of 22 µL per sample rather than 20 µL allows to avoid bubbles, to pipette errors, and to improve the total amount of droplets generated.
12. It is a good practice, in ddPCR, to require at least three single positive droplets in order to call a sample positive.

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Chapter 13

Bioinformatics Pipeline for Accurate Quantification of Fetal DNA Fraction in Maternal Plasma

Meng Ni, Xianlu Laura Peng, and Peiyong Jiang

Abstract

The discovery of circulating cell-free fetal DNA has profoundly transformed the landscape of noninvasive prenatal testing (NIPT) and rapidly found its way into global clinical applications. The fractional concentration of cell-free fetal DNA in plasma DNA of a pregnant woman is an important parameter for understanding and interpreting analytical results of NIPT. Thus, the accurate quantification of fetal DNA fraction is indispensable when NIPT is involved. In this protocol, we describe the bioinformatics workflow to calculate fetal DNA fraction using two programs developed by our group, which provide accurate estimation.

Key words Fetal DNA fraction, Maternal plasma DNA, Noninvasive prenatal testing, Bioinformatics pipeline

1 Introduction

The discovery of circulating cell-free fetal DNA in 1997 [1] has fundamentally revolutionized noninvasive prenatal testing (NIPT) in the modern obstetric clinics, for example, catalyzing a number of new approaches including fetal RhD blood group genotyping [2], chromosomal aneuploidy detection [3, 4], fetal sex determination [5, 6], and diagnosis of monogenic disorders [7–9]. In particular, NIPT for common chromosomal aneuploidies has rapidly found its way into global clinical practice [10, 11]. In these tests, the fetal DNA fraction is a key parameter for accurately interpreting the testing results. On one hand, the accurate quantification of fetal DNA fraction can allow us to identify testing samples with low fetal DNA fractions, reducing the false negatives [12, 13]. On the other hand, the parameter of fetal DNA fraction is used for deducing the theoretical thresholds for classifying whether the monogenic disorders would be inherited by an unborn fetus when using the relative haplotype dosage analysis [7]. Considering noninvasive

prenatal diagnosis of β -thalassemia as an example, the combinations of SNPs of maternal genome would form two haplotypes which would be present in maternal plasma. If one maternal haplotype carrying the mutation in HBB (hemoglobin subunit beta) gene causing the β -thalassemia is transmitted to an unborn fetus in a pregnant woman, the plasma DNA molecules derived from such a maternal haplotype harboring the disease-causing gene would be overrepresented in comparison with that of untransmitted haplotype. The degree of overrepresentation of the transmitted haplotype in maternal plasma is proportional to the fetal DNA fraction. Therefore, the fetal DNA fraction can be clinically used for determining the maternal haplotype inheritance. The higher accuracy of fetal DNA fraction quantification, the higher performance for the noninvasive prenatal diagnosis of monogenic disorders would be achieved, for example, detecting congenital adrenal hyperplasia, hemophilia A/B, Duchenne muscular dystrophy, Ellis-van Creveld syndrome, etc. [7]. To achieve an accurate quantification of the fetal DNA fraction, we previously developed two approaches, namely, FetalQuant [14] and FetalQuant^{SD} [15], by taking advantage of the targeted and nontargeted sequencing of maternal plasma DNA. In this methodology chapter, we elaborate on the bioinformatics steps utilizing FetalQuant and FetalQuant^{SD} to calculate the fetal DNA fraction from maternal DNA sequencing data.

2 Materials

A computing machine with 64-bit GNU/Linux operating system with at least 10 GB memory is required.

2.1 Datasets

1. Reference sequence of the human genome (Hg38 <http://genome.ucsc.edu>).
2. Two datasets of the sequencing reads for maternal plasma DNA of pregnant women.

2.2 Softwares

1. FetalQuant (<https://sourceforge.net/projects/fetalquant/>).
2. FetalQuant^{SD} (<http://www.cuhk.edu.hk/med/cpy/Research/FetalQuantSD/>).
3. SOAP (version 2.21) (<http://soap.genomics.org.cn/soapaligner.html>).
4. Trimmomatic (version 0.38) (<http://www.usadellab.org/cms/?page=trimmomatic>).

3 Methods

We use two datasets from FetalQuant and FetalQuant^{SD} to demonstrate fetal DNA fraction calculation.

3.1 Data Preparation

Download reference genome and build reference index for alignment in the processing directory.

1. \$ mkdir working_dir
2. \$ cd working_dir
3. \$ wget -c <http://hgdownload.cse.ucsc.edu/goldenPath/hg38/bigZips/hg38.fa.gz>
4. \$ gzip -d hg38.fa.gz
5. \$ 2bwt-builder hg38.fa

The output files from this step are hg38.fa and soap index files for human reference genome.

Then download raw sequencing reads from example datasets, trim adapters, and remove duplication.

6. \$ wget -c <http://www.cuhk.edu.hk/med/cpy/Research/FetalQuantSD/download/fetalquantsd.tar.zip>
 7. \$ tar zxf fetalquantsd.tar.zip
 8. \$ java -jar trimmomatic-0.35.jar PE -phred33 fetalQuantSD/example/test_seq_1.fq.gz fetalQuantSD/example/test_seq_2.fq.gz clean_1.fq.gz clean_2.fq.gz ILLUMINACLIP:TruSeq3-PE.fa:2:30:10
 9. \$ gzip -d clean_1.fq.gz; gzip -d clean_2.fq.gz
- The output files from this step are clean_1.fq and clean_2.fq.

3.2 Alignment and Result Formatting

\$ soap -a clean_1.fq -b clean_2.fq -D hg38.fa.index -2 SE.soap -r 0 -m 0 -x 600 -o align.soap

The output of this step is the alignment result: align.soap

3.3 Fetal DNA Fraction Calculation Using FetalQuant^{SD}

1. \$ R --slave --args example/training_dataset.dat < bin/linear-Regression.R
2. \$ sh bin/FetalQuantSD.sh example/test_iScan.bed align.soap test
3. \$ perl bin/calc_fetal_frac.pl test.Bfrac linear_parameters.dat

Using the previous output align.soap and test_iScan.bed in the example folder of FetalQuant^{SD}, the estimated fetal DNA fraction was found to be 5.12. The actual fetal DNA fraction for this sample is 5.47.

3.4 Fetal DNA Fraction Calculation Using FetalQuant

\$ FetalQuant 33 0.001 1 1 align.pileup > FetalQuant.txt 2> likelihood.log

Using the example file align.pileup in the FetalQuant folder, the above command will generate two result files of FetalQuant.txt,

and likelihood.log. FetalQuant.txt contains the modeling parameters used for deducing the fractional fetal DNA concentration and the final result of the deduced fetal DNA fraction. Likelihood.log file contains the detailed information of the likelihoods for each iteration step of the expectation-maximization (EM) algorithm.

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Conflict of Interest: P.J. is a consultant to GRAIL. P.J. has filed patents/patent applications regarding cell-free DNA molecules. The remaining authors declare no conflict of interest.

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Part VI

Physical Activity



Chapter 14

Circulating Cell-Free DNA in Physical Activities

Leydi Natalia Vittori, Andrea Tarozzi, and Pasqualino Maietta Latessa

Abstract

The interest about circulating cell-free DNA (cfDNA) concentration increased from several years because of its correlation with various conditions like osteoarthritis, cancers, stroke, and sepsis; recently it has become an important marker for overtraining syndrome or performance diagnostics.

Several studies have demonstrated that cfDNA increases in vigorous and exhausting exercise but also endurance exercise. Acute effect of exercise on cfDNA concentration seems to be correlated to stress factor, while chronic effect is associated with necrosis and apoptosis.

The intensity and duration seem to have effects on the variation of cfDNA concentration that is strongly correlated with other metabolic markers like acid lactate and creatine kinase, recognized as markers of muscle damage. Variation of cfDNA value could be used to predict overtraining syndrome.

Key words Physical activities, Acute exercise, Chronic exercise, Intensity cell-free plasma DNA, Inflammation, Overtraining

1 Introduction

Practicing regular physical exercise (PE) is widely recognized as a protection against obesity, insulin resistance, and atherosclerosis [1, 2]. Several studies showed that benefits depend on the intensity or amount of work performed during physical exercise [3, 4].

Physical exercise induces organism changes that may vary between damage, improved function, and even no changes, depending on workload adjustment to each individual level of fitness. However, if we consider PE as a discrete therapeutic modality, we also must consider that exercise mode, intensity, and duration must be on careful evaluation of risk and benefits. Certain level of physical activities may induce an increase in the production of inflammation reactive oxygen species (ROS) [5]. The increase in oxidative stress state induced by PE may vary with the characteristics of the exercise, such as intensity, time, and frequency [5].

It is widely accepted that oxidative stress results in molecular damage, which may be accumulated with age, probably causing

progressive physiological attrition increasing the susceptibility to disease and risk of death [6]. Oxidative stress, however, is able to stimulate several cell signaling pathways; thus, the cells can adapt, determining a better resistance to stress. Studies regarding regular physical exercise in the oxidant/antioxidant state have found an upregulation of antioxidative pathways; this occurs because of the expression of transcriptional factors for many antioxidant enzymes [7, 8].

Several studies have investigated the level of cell-free DNA (cfDNA) under pathological conditions [9, 10, 23, 24], elucidating its potential as a biomarker for diagnostics and disease progression, and in the last years, cfDNA is becoming interesting in physiological exercise.

Exhausting and vigorous exercise as half marathon [11] and ultramarathon [12], resistance exercise [13], continuous treadmill running [14], interval and incremental treadmill running [15, 16], and incremental rowing exercise [17] increase the level of inflammation cells and cfDNA. In most studies cfDNA levels returned to baseline levels within 2 h of recovery [14, 22], and only after an ultramarathon cfDNA levels remained elevated for up to 24 h [12].

Recently, Hallert et al. [18] demonstrated the increase of cfDNA during aerobic running below the steady state of lactate; these data highlight that cfDNA has an enormous potential as a biomarker for the exercise load in the aerobic and anaerobic status.

Chronic exercise reduces ROS production, increases antioxidant capacity, and improves mitochondria efficiency, therefore decreasing oxidative stress and damage. For this reason, we can expect a lower nuclear DNA damage, because it can prevent cell mutations associated to several diseases. Physical fitness has already been inversely related with DNA damage and mitochondria ROS production, revealing a positive effect on mitochondria function [19]. Considering these aspects, cfDNA can be a biomarker both for the exercise and for the chronic disease-related conditions.

Several studies demonstrated that comparing creatine kinase (CK) and cfDNA levels, the latter increased faster than CK after exercise because raising of cfDNA levels occurs within minutes.

2 Different Physical Activities on cfDNA Concentrations

2.1 Acute Effects

PE induces effects similar to immune-response patterns caused by acute infection and trauma [21]. The acute exercise induces multiple stressors for the body, by changing the ability to maintain homeostasis in response to thermal, oxidative, metabolic, hormonal, and mechanical stimuli [20].

The alterations of the acute immune system include the mobilization and activation of leukocyte subgroups, the generation of

ROS, the release of acute phase proteins and stress, platelet activation, and changes in thrombotic and fibrinolytic pathways.

Rapid increased of cfDNA and gradual increases in bleeding inflammatory markers were recorded in post-trauma patients [10]; the same value was recorded by Fatouros and coworkers [22] in response to exhaustive treadmill exercise where immediate and pronounced cfDNA peak preceded a blood inflammatory marker response.

Fatouros and coworkers investigated the time-course changes of cfDNA during 24 h after an acute bout exhaustive exercise in heavy exercise (experimental and control trial). The data showed that cfDNA raised immediately after exercise and remained elevated for 30 min and normalized thereafter; indeed hs-CRP (C-reactive protein levels) increased but did not vary in 24 h after exercise. CK increased immediately post exercise and during 24 h later. The acute exercise induced inflammation and increased the cfDNA, especially immediately post exercise.

The discovery of inflammation markers highlighted that acute bout exhaustive exercise induced inflammatory events: hs-CRP, CK, and UA high values found immediately post exercise reflected a muscle damage. These aspects influence also the cfDNA release.

The authors suggest that cfDNA could be correlated with hypoxia induced by exercise and radical production through leukocyte exudative burst observed during the post-exercise recovery period [22]. The peak reached immediately after exercise is typical in short protocols with respect to endurance exercises [12, 13, 22].

Atamaniuk and coworker tested the effect of cfDNA during and after half marathon race [11] and in long ultramarathon [12]. In the first study, cfDNA increased immediately and returned to the baseline after 2 h. In the second, cfDNA reached maximal value after ultramarathon performance and decreased after many hours, but the concentrations remain higher than baseline. cfDNA value returned to baseline after 24 h.

Atamaniuk and Fatouros studies' highlighted that cfDNA concentrations reach the maximum value immediately post the performance and, until 24 h after exercise, it still remains higher than pre-exercise.

Beiter et al. investigated the cfDNA and cell-free mitochondrial DNA (cf-mDNA) before and immediately after a 10-km cross-country run and 30 min after exhaustive short-term treadmill exercise [15]. The data did not report any correlation between cfDNA value and sex, age, and body max index post exercise in cross-country or treadmill runners. Kinetics of cfDNA is correlated with rapid lactate acid increasing during exhaustive treadmill exercise. To fully exploit the diagnostic potential of cf-mDNA, an improvement insight into the cellular and molecular mechanisms is necessary underlying the release and clearance of cf-mDNA.

Beiter and coworkers showed that strenuous exercise can serve as a suitable surrogate model to study cf-mDNA biology in vivo.

In agreement with Fatouros et al., Beiter demonstrated that gradual increases in blood inflammatory markers in response to exhaustive treadmill exercise are preceded by an immediate and pronounced cf-mDNA. cfDNA rapidly increasing during exercise was demonstrated with a similar kinetics also in patients after trauma but in contrast not accompanied by an increase in cf-mDNA.

Many studies showed cfDNA, lactate, and HMGB1 kinetics during exercise.

Beiter and coworkers in a subsequent study [15] highlighted that exhaustive exercise was not accompanied by an increase in cf-mDNA concentration; comparing data obtained by Beiter and Fatouros, the discrepancies can be attributed either to different sample-processing protocols [25] or to alternative mechanisms of cfDNA release.

A recent study evidenced an explanatory model for the rapid-release kinetics of cfDNA within minutes of activation [25, 26]. In autoimmune disorders, such as systemic lupus erythematosus [27], the impaired clearance of cfDNA is especially implicated in the disease development, and the athletes who experienced chronic fatigue syndrome displayed a higher seroprevalence of antinuclear antibodies [27].

Noteworthy, a recent study showed that cfDNA could depend from compliant cool-down behavior [17]. Tested ten rowing athletes undergone a stepwise incremental exercise until exhaustion; cfDNA analysis revealed a significant increase in the concentration till the end of exercise; post-exercise excessive cfDNA is gradually removed from circulations. Some of the athletes, who ceased the exercise after 7 min, had an increasing cfDNA concentration. DNase activity was inversely correlated with the kinetics of cfDNA. For the first time, indeed, this study revealed that significantly elevated levels of cfDNA in healthy trained subjects are efficiently reduced by adaptation of endogenously expressed DNase activity to regain homeostasis [17].

Nowadays, only some studies invested the acute effect of heavy weight lifting on cfDNA levels.

Firstly Atamaniuk [13] analyzed the acute effect of strength training in healthy male professional weight lifters on cfDNA value. Blood analyses revealed an increase on cfDNA blood value immediately after session training compared to baseline and returning base value after 2 h of recovery (Table 1). For other collected markers such as hypoxanthine and xanthine, a high correlation with cfDNA after training was found. These data revealed an association between ROS production and biological process. The authors concluded that high cfDNA concentrations might represent an index of muscle tissue damage, cellular necrosis, and apoptosis following an acute exercise-induced (muscle) injury [13].

In the second study, Thug and coworkers compared the effects of three strength training groups: conservation training (CT),

Table 1
cfDNA and acute exercise

Type of exercise	Subjects	Testing	Blood sampling	Results	Reference (n)
Acute endurance exercise	25 (12M/13F) recreational half marathon runners	Endurance	Before, immediately after the race, and 2 h after: UA, myoglobin, cfDNA	cfDNA: ↑ cfDNA higher immediately ↓ cfDNA 2 h after ↑ UA after immediately ↑ UA 2 h after ↑ Myoglobin after immediately ↑ Myoglobin 2 h after	Atamanuik et al. (2004) [11]
Acute endurance competition 6 h	14 (9M; 5F) recreational ultramarathon runners	Endurance	Before, immediately after the race, 2 h after, and 24 hours after: mRNA, Borg scale	cfDNA: ↑ cfDNA higher value after immediately ↓ cfDNA 2 h after ↓ cfDNA 24 h after return baseline value	Atamanuik et al. (2008) [13]
Acute stepwise endurance lab test	11M moderately trained athletes divided in control and experimental trial	45 min running to 70/75% VO _{2max} , 90%VO _{2max} until exhaustive	Before, immediately, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 8 h, 10 h after, and 24 h after: cfDNA, hs-CRP, CK, uric acid	cfDNA: ↑ cfDNA after immediately, 30 min ↓ cfDNA 1 h after ↑ hs-CRP, CK, uric acid 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 8 h, 10 h after ↑ hs-CRP, CK, uric acid peak 24 h after	Fatouros et al. (2010) [22]

(continued)

Table 1
(continued)

Type of exercise	Subjects	Testing	Blood sampling	Results	Reference (n)
Short-term treadmill running	9 well-trained endurance athlete	Inclination constant 1%, velocity increased by 2 km/h every 3 min until	cfDNA, cf-mDNA, HMGB	↑ cfDNA peak immediately ↓ cfDNA 30 min ↑ cf-mDNA peak immediately ↓ cf-mDNA 30 min ↑ HMGB1 peak immediately ↓ HMGB1 30 min	Beiter et al. (2011) [15]
Acute stepwise endurance exercise	53 (34M; 19F) recreational runners	10 * 1000 m distance with several minutes of rest between each	Before, immediately: cfDNA	↑ cfDNA peak immediately	Beiter et al. (2011) [15]
High-intensity exercise until volitional exhaustive	10 M highly trained rowers	Start to 200w, increased amount to 5w/4 min	Immediately post, 7 and 30 min post cfDNA (ng/mL), AR (DNase activity reduction)	↑ cfDNA during test ↑ cfDNA peak immediately ↓ cfDNA 7 min post exercise ↓ cfDNA 30 min post ↓ DNase peak immediately ↓ DNase 7 min post exercise ↑ DNase 30 min post	Velders et al. (2014) [17]
Acute resistance exercise	12M elite weight lifters	6 sets of 6 lifting exercises, 1–5 reps, 90–95% of 1 RM, rest between sets 4–5 min, rest between exercise 8–10 min	Baseline, immediately post, 2 h post: cfDNA, HX, and XA	↑ cfDNA immediately post ↑ cfDNA 2 h post ↑ HX immediately post ↑ HX 2 h post ↑ XA immediately post ↑ XA 2 h post	Atamanuk et al. (2010) [13]

Acute resistance exercise	16 (6CT,5 HT, 5DT)	2 sets of 8 exercises, 5 repetitions for about 60 min	cfDNA, CK	↑ cfDNA all groups each exercise ↑ cfDNA HT compare to CT cfDNA	Tug et al. (2017) [28]
		CT 60% IRM			
		HT 90% IRM			
		DT 60% IRM			

Notes: cfDNA cell-free DNA, IRM one-repetition maximum, CK creatine kinase, CRP c-reactive protein, mRNA messenger RNA, DNase deoxyribonuclease, HMGBl high mobility group box-1 protein, XA xantine, HT high intensity low repetition training, CT conservation training, UA uric acid

high-intensity low-repetition training (HT), and differential training (DT) with cfDNA concentration [20]. EDTA plasma samples were collected before every training session and on the first and last training day after every set of exercises. HT group showed higher cfDNA values during a single unit of strength training than the conservation training group. In a multifactorial analysis of variance across all types of training (CT, HT, and DT) and at both monitoring points in time (1st and 12th day), there was a significant increase of cfDNA after the 12th exercise compared to baseline. The authors did not find any significant difference of cfDNA concentrations compared to baseline levels, for each group.

Comparing the two studies mentioned above, we can conclude that in the first study, cfDNA reached the highest number than second. One reason could be the different intensity load of the training; in fact in the study by Tug et al., five subjects followed a 90% RM training.

2.2 Chronic Effects

The first researchers who observed the chronic effect of physical exercise in cfDNA concentration were Fatouros and coworkers in 2006. They investigated the effect of progressively resistance exercise on cfDNA in 12 weeks.

cfDNA concentration was analyzed at baseline and at 96 h after the last training session of each period [14]. The data, which were collected at baseline, after each training session (T1, T2, T3, and T4) showed that cfDNA increased during the training phase (T1, T2, T3) and decreased at 96 h after last training session reaching the value similar to the baseline (Table 2).

Fatouros and coworkers aimed to verify if the continuous excessive resistance training could lead to infection and immunologic marker response and create an overtraining effect. They demonstrated that biomarkers related to muscle damage and acute phase (e.g., CRP) were higher at the end of the training sessions, indicating an inflammatory condition [19]. The athletic performance could be impaired by muscular overtraining which increases susceptibility to infections and induces muscle tissue injury as well as psychological, immunological, and biochemical dysfunctions [28]. For these reasons the authors concluded that cfDNA could be used as a marker of overtraining if concentration returned to the baseline value within days after chronic progressive training load without fatigue syndrome.

Recently Tug et al. tested the effect on three different strength exercises (CT, HT, and DT) on cfDNA plasma value. As described above, the authors did not find any difference on cfDNA concentration in each group and also other markers, just like CK didn't show any alteration in the different types of training session (Table 2). The authors concluded that cfDNA value could be attributed to a local inflammation due to the lesion of the sarcomere [29] and indirect formation of oxygen free radicals [5, 30]. In particular, within

Table 2
cfDNA and chronic exercise

Type of exercise	Subjects	Testing	Blood sampling	Results	Reference (n)
Chronic resistance exercise: 12 weeks (t1, t2, t3, t4)	12M elite weight lifters	8 resistance multi-joint exercises selected to stress the entire musculature: t1 and t4 (2 training days per week, 2 sets per exercise, 10–12 reps per set at 70%RM; t2 high-volume training, 4 training days per week, 4 sets per exercise, 6–10 reps per set at 75–85% of 1RM; t3 6 training days per week, 6 sets per exercise, and 1–6 reps per set at 85%–100%RM	cfDNA, CRP, CK, UA	↑ cfCDNA t1 ↑ cfCDNA t2 ↑ cfCDNA t3 ↓ cfCDNA t4 ↑ CRP t2 ↑ CRP t4 ↑ CK t3 ↑ UA t2 ↑ UAT3	Fatouros et al. (2006) [14]
Acute resistance exercise 4 weeks	16 (6CT, 5HT, 5DT)	2 sets of 8 exercises, 5 repetitions for about 60 min CT 60% 1RM HT 90% 1RM DT 60% 1RM	cfDNA, CK, Hb, MCH, UA	↑ cfDNA day 2 ↑ cfDNA day 5 ↑ cfDNA day 8 ↑ cfDNA day 12 ↑ cfDNA day 2 compare to day 5 ↑ Ck day 5 ↑ Hb day 5 ↑ MCH day 5 ↑ UA day 5	Tug et al. (2017) [28]
Resistance exercise training with and without supplementation 6 months	117 older (Ct, RT, RTS)	Resistance training	cfDNA, TL, ULB	↓ cfDNA after 6 month RTS =TL all group =ULB all group	Ntassis- stathopoulos et al. (2013) [32]

Notes: CK creatine kinase, CRP c-reactive protein, Hb hemoglobin, MCH corpuscular hemoglobin, UA uric acid, TL telomere length, ULB unconjugated bilirubin

hours, neutrophils migrate into the injured skeletal muscle and remain there up to 24 h. Together with macrophages they degrade the damaged muscle tissue resulting in the formation of ROS [31]. In this study for the first time, Tug demonstrated that mostly fragments of polynucleosomal cfDNA in nature are caused by late increased level of cfDNA 24 h later the last force training session.

This lower level of degradation of the cfDNA may look like a release of less degraded DNA because of cellular damage. On study day 2, higher levels of cfDNA have been observed, with a highly significant correlation of polynucleosomal with all nucleosomal cfDNA concentrations. Hence, levels of polynucleosomal DNA and levels of total nucleosomal DNA are similar; this can imply that increase of cfDNA determined by a previous training session mostly involves a growth of polynucleosomal DNA. Therefore, they did not expect that the increased of cfDNA levels could be due to overtraining-induced inflammation.

Telomeres, the sequences of nucleotides at the end of the chromosomes that protect their integrity, are shortened with each cell division, so telomere length correlates with biological age [33]. Exercise has epigenetic effects on the telomere length, which help to prevent its deregulation by protecting it from transcription errors caused by transcription of noncoding RNA, which occur during cell division. In a randomized controlled intervention, Tosekova et al. investigated the relationship between cfDNA, serum unconjugated bilirubin (UCb), and telomere length (TL) in a predominantly female elderly population after 6-month resistance exercise training with or without supplementation [33]. The cfDNA was the only molecular markers, among TL and UCb, which decreased significantly after the period of training and dietary intervention.

2.3 Intensity, Duration, and Metabolic Rate Hallmarks of cfDNA Levels

Intensity level in physical exercise is determined by percentage of maximal oxygen consumption ($\text{VO}_{2\text{max}}$) or 1RM which is the maximum weight that can be lifted for one complete repletion of the movement. High-intensity endurance gets about $>85\%$ $\text{VO}_{2\text{max}}$, and lower intensity is an exercise that gets about 60% $\text{VO}_{2\text{max}}$.

The effect of exercise doesn't depend only to the intensity but also to the mode, the type of contraction (concentric vs. eccentric vs. isometric), and the degree of complexity of exercise (muscle sloops vs. isolated muscles) [34].

Several studies showed a rapid increase in cfDNA in response to single bout of the exercise of different type and load. A study by Stawsky et al. tested the effect of repeated bout exhaustive exercise showing that plasma cfDNA raised 14 times after treadmill run and 15 times after similar treadmill run till exhaustion [16, 19]. Moreover, 10 km relay run induced the increase of 7.6-fold in cfDNA levels, while half marathon and ultramarathon caused the 18-fold difference between pre- and post-exercise cfDNA levels. The

different results could depend on by different protocols, exercise load, and study subjects [35].

Andreatta et al. observed that cfDNA value increased immediately after acute resistance exercise (80% of 1RM) at leg-press resistance exercise [36]. They tested muscular performance by both squat jump (SJ) and countermovement jump (CJ) exercises which are an effective measurement tool of the muscle explosive force of the lower limb.

After 24 h the value of cfDNA of 80% RM decreased and returned to the baseline value. Correlation between cfDNA measured immediately after resistance exercise (RE) and jump performance showed a moderate negative correlation between cfDNA immediately post exercise of SJ and CJ and 24 and 48 h after exercise session. There was a trend toward other significant relationships between cfDNA and SJ or CJ. Measuring cfDNA immediately after RE may indicate exercise-induced muscle damage at least in the early phase of the injury without need to wait for later markers, such as plasma CK and lactate dehydrogenase at 24 or 48 h. The data highlighted that cfDNA depends on the intensity of exercise (80RM vs. 40RM); thus cfDNA may be a better marker than lactate in short recovery to value exercise intensity. The authors concluded that cfDNA could be used as a marker to value the muscle performance until 48 h of exercise.

The concentration variation of cfDNA immediately after RE indicates that exercise induces muscle damage at least in the early phase of the injury.

Similar results were obtained by Haller during a controlled test-retest moderate-level aerobic run. The data showed a high concentration of cfDNA [18] steadily, while lactate did not increase during last 30 min of the 2 runs. cfDNA concentration was high for all subjects but higher for male participants. This difference between female and male could be ascribable to the different exercise intensity and not to the lactate level.

Lactic acid is always associated with the fatigue that appears during a very intense workout or long enough to create a buildup in the muscles and bloodstream. In the gym and in sport centers, it is very well known and mostly feared by athletes: its accumulation can in fact interrupt a workout, ruin a race, and become a risk factor for injuries. Several studies have shown a highly correlation between lactic acid and cfDNA.

cfDNA and lactate concentration followed a congruent curve during incremental treadmill running in Beiter et al. study [26]. In contrast, other studies showed that lactate levels value did not correlate between cfDNA and intensity of exercise. In particular, 15 mmol/L was observed in weight lifting intervention, and this depends on mechanical forces, and shear stress substantially affects the muscle tissue. Endurance exercise with respect to resistance exercise recruits larger muscle masses; for this reason, the local

oxidase stress is higher in weight lifting. cfDNA concentrations after resistance exercise increased to only 1/8 of those measured after a half marathon [11, 13]. Hudson et al. [38] expect that oxidative stress caused by resistance training is rather associated with exercise intensity (i.e., the energy consumption per minute) than with metabolism. Thus, the lower increase of cfDNA after weight lifting could be due to the duration of rests between sets (4–5 min) and exercises (8–10 min) [13].

Subsequently, lactate levels, the average energy expenditure [37, 39, 40], and the rate of anaerobic energy supply [39] decrease with possible attenuating effect on the cfDNA concentrations.

2.4 Overtraining Effects

To optimize increase by strength muscle, athletes and amateur subjects must maintain a balance between high-intensity training and recovery.

Overtraining syndrome is the condition in which this balance is altered. Typical signals are decreased performance, weight, appetite, and increased cardiac [41, 42]. Overtraining effect could result in high-intensity and high-value training [43], and it could interest individuals and team sport.

It's important to make a distinction between fatigue and overtraining syndrome; the first normally resolves in 24/48 h post exercise, and it's important to improve the performance; in the second the subject can't reach habitual performance also after several weeks of recovery.

Biomarkers for oxidative stress have been shown to be associated with overtraining; the data suggest a strong relationship with dose/response.

In this field, the studies try to find a special indicator value and method to evaluate overtraining: one is cfDNA level in blood.

The secretion of inflammatory markers and cytokines increased in overtraining syndrome; as a result, overtraining could induce tissue inflammation and affect T-lymphocyte differentiation, leading to a dysregulation of the Th1/Th2 (type 1 and type 2) balance and the attenuation of cellular immunity. Nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase is responsible for the neutrophil apoptosis and lymphocyte DNA damage seen in overtraining [44].

Margonis et al. observed that overtraining period led to higher levels of thiobarbituric acid reactive substances (TBARS) (56%), catalase (96%), and oxidized glutathione (GSSG) (25%) and to lower levels of glutathione (GSH) (31%) and GSH/GSSG (56%) in 12 participants after 3 weeks [45]. Furthermore, another study on seven subjects who were severely overtrained concluded that increased oxidative stress plays an important role in the pathophysiology of overtraining syndrome [46]. Pereira et al. showed that the current overtraining protocol based on downhill running sessions are linked to high concentrations of cytokines in serum, skeletal

muscles, and the liver [47]. Therefore, they concluded that the performance decrease induced by nonfunctional overreaching (NFOR) is associated with muscle damage and inflammation because muscle damage and inflammation are related to blood and skeletal muscle oxidative stress [48].

Recently Guo et al. [49] remarkable raises of plasma levels of cfDNA in overtraining rats which were associated with oxidative stress like T (testosterone), Cort (cortisterone), and CK (creatin kinase). This data suggests that plasma cfDNA might be a new molecular marker of overtraining.

Fatouros and coworkers [14] demonstrated that value of cfDNA could be used to monitor and quantify the overtraining; in fact, the data showed that chronic excessive resistance exercise induced an overtraining effect. This aspect was highlighted by a decrease performance level during T3 phase in which it was a lack of recovery and T4 during decreased training volume. Tissue damage and inflammation were confirmed by increased plasma DNA and CRP concentrations.

3 Conclusions

Recently the cfDNA in the plasma is one of the most fields of interest in medical research, and several studies showed its important role in some chronic disease and physiological conditions and also in training.

Knowledge about the effect of acute and chronic exercise are insufficient to explain the kinetics of cfDNA level concentration which seems to correlate with intensity, mode, and, more importantly, the individual response to exercise. Further studies could help to use this value like a parameter to design an optimal training load for a better performance in athletes or to monitor the training.

cfDNA analysis could be also a useful tool to monitor the athletes either the aim to early detection of overtraining syndrome. Moreover, cfDNA analysis is noninvasive and requires limited time and personnel.

Further research studies are needed on cfDNA kinetics to define its specific correlation with inflammatory process that is important during and after different types of performance.

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Part VII

Urinary Cell Free DNA



Chapter 15

Urinary Cell-Free DNA: Potential and Applications

Samanta Salvi and Valentina Casadio

Abstract

Urine could be a convenient source of biomarkers for different diseases and clinical applications, mostly for cancer diagnosis, prognosis, treatment monitoring, and prenatal diagnosis. The ultra-noninvasive sampling and the possibility to analyze large volume are the main undisputed advantages of urine-based protocols. Recent and comprehensive studies showed that urinary cell-free DNA (ucfDNA) is informative to identify the genomic signature of patients, resulting in a huge tool to track the tumor evolution and for personalized medicine in urological and non-urological cancer.

In this chapter, we reported the main published evidences on ucfDNA, with the aim at discussing its promising and translatable role in clinical practices.

Key words Urinary cell-free DNA, Urine, Cancer diagnosis, Cancer monitoring, Fetal urinary cell-free DNA

1 Introduction

Cell-free DNA (cfDNA) in body fluids represents an important source of biomarkers for a number of physiological conditions (e.g., physical activity monitoring) and different diseases, especially for cancer. The most studied is circulating cell-free DNA in the blood, already considered as a milestone in the liquid biopsy contest and personalized medicine thanks to the next-generation sequencing (NGS) technologies and the research advancements. Recently, urine has earning an emerging role as a convenient source of cfDNA-related biomarkers. As urine collection is even less invasive than blood collection, urinary cell-free DNA (ucfDNA) analysis guarantees patients compliance and large volumes of sample at different times can be collected from the same patients without health hazards.

Most of the studies regarding cfDNA in urine and blood are related to cancer disease. The heterogeneous genomic profile of cancer can be analyzed through the analysis of cfDNA, resulting in a noninvasive snapshot in time [1].

Analysis of cfDNA in blood or urine could be useful for early diagnosis, patient follow-up, and treatment selection when cancer tissues are not available for such evaluations [1, 2].

ucfDNA comes from the dying cells derived from the urogenital tract and exfoliated in urine or from the circulation. Only low molecular weight (MW) DNA from the blood can pass through the glomerular filtration [3] and be excreted into the urine cell-free component [4, 5]. Circulating DNA, however, is normally present as “nucleosome” (size 167bp), that is, a complex protein that could not pass through the glomerular barrier, due to its size exceeding the pore dimension.

Many papers have been published on bladder or other urological cancers that are directly in contact with urine samples [6, 7], but recently very encouraging results have been obtained also for non-urological cancer diseases, especially for lung cancer. The study of EGFR mutations in ucfDNA for lung cancer monitoring is one of the most intensively pursued objectives in the last years [8].

ucfDNA has been proposed since 20 years ago also for prenatal diagnosis (fetal DNA found in urine), but its role is still controversial. More recently, ucfDNA has been proposed as a source of biomarkers for the characterization of bacterial and viral urinary tract infections, with preliminary encouraging results [9].

This chapter aims at giving an overview of ucfDNA features, techniques, and clinical applications, giving some hypotheses for its potential use in the next future (Fig. 1).

2 Urinary Cell-Free DNA Features

ucfDNA could be divided into three categories, depending on its origin: urinary tract cells DNA, transrenal DNA [10], and nonhuman DNA coming from viruses and bacteria affecting the urogenital tract [9, 11]. Urinary tract cells DNA could be divided into high-MW DNA and low-MW DNA. High-MW DNA comes from necrotic cells exfoliated in urine (epithelial cells from the bladder, prostate, and kidney but also lymphocytes); low-MW DNA comes from apoptotic cells of the urogenital tract. This DNA can be used for urological cancer diagnosis and monitoring. Some papers demonstrated that, in the case of bladder cancer that is directly in contact with urine fluid, necrosis is a predominant source of cfDNA [6, 12]. This is an important issue, because long DNA fragments are easier to be analyzed by conventional molecular techniques with respect to short, degraded DNA.

Transrenal DNA is DNA coming from the circulation and passed through the glomerular filtration. It has a low MW [13], given that 3 nm and rare 11–11.5 nm glomerular pores limit the transport of >11.5 nm diameter molecules. Nucleosomes, exosomes, apoptotic bodies, and large protein complexes are thus

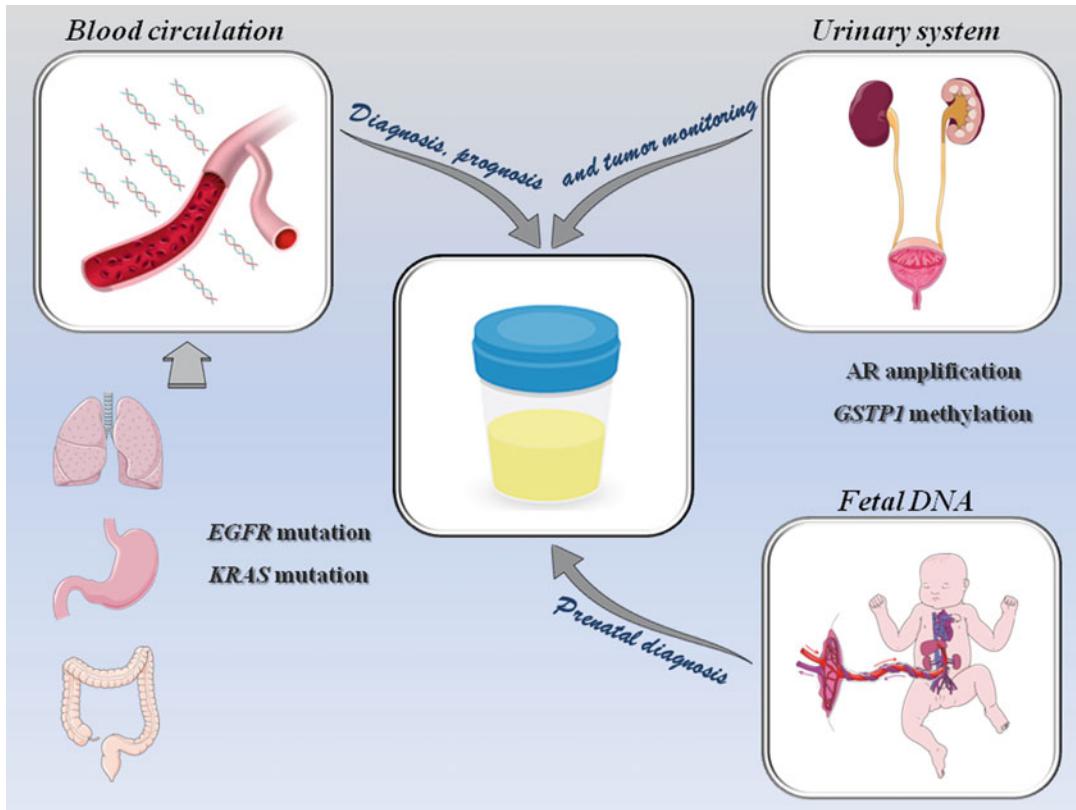


Fig. 1 The figure represents an overview of the main ucfDNA markers reporting some examples: EGFR, KRAS, and fetal DNA coming from blood circulation passed through the glomerular filtration; AR and GSTP1 coming from urinary tract cells

unable to pass from plasma to urine, with the exception of pathological condition [4].

Specific cancer sequences in ucfDNA can be detected from pancreatic, colon, and lung cancer patients, demonstrating that circulating cfDNA can pass through the glomerular filtration and appear in urine [3, 14]. Other studies on fetal DNA in pregnant women demonstrated that DNA from fetus appears in urine in very short sequences (between 29 and 45 bp long) [15].

3 Methods

3.1 Urine Collection and DNA Isolation

Urine sample is generally collected in sterile containers at large volume (up to 40 mL) during the first hours of the morning. To obtain urinary supernatant containing ucfDNA, one or more centrifugations at high speed ($10,000\text{--}16,000 \times g$, 10 min) are necessary [16]. ucfDNA has a shorter half-life time than cfDNA in serum or plasma, probably due to urine contaminants [17]. DNA degradation in urine specimens is also affected by the action of

hydrolyzing enzymes, such as DNase I and DNase II. For all these reasons, urine samples should be centrifuged and stored at -80°C or at room temperature, using a stabilization buffer, as soon as possible after their collection.

For DNA isolation, several commercial kits are available. In brief, they mainly perform a column-based purification or a DNA capture using beads. Most of published papers have used kits specific for isolating high-MW DNA (from 200 bp to 50 kb) such as QIAamp DNA Mini Kit or QIAamp DNA Blood Mini Kit (Qiagen) [16, 18]. Nevertheless, it is interesting to isolate also small DNA fragments which could derive from blood circulation, offering a more accurate information.

When choosing an isolation kit, an important feature to be considered is that it should start from a flexible urinary supernatant volume (from few up to 40 mL), in order to plan a heterogeneous case collection and to obtain as much as possible DNA yield.

To the best of our knowledge, the most performant commercial kits for ucfDNA isolation are Urine DNA Isolation Kit (Nor-gen) [19], QIAamp Circulating Nucleic Acid Kit (Qiagen) [20], and Quick-DNA Urine Kit (Zymo Research) [21]. All these approaches permit to start from different urine volumes.

Once isolated, ucfDNA can be quantified using different approaches. In a relevant paper of Zancan et al. [18], ucfDNA was measured using GeneQuant pro, Nanodrop, Qubit (Quant-iT DNA high-sensitivity assay), and real-time PCR with ABI Prism 7700 Sequence Detection System. The authors suggested that spectrophotometric methods are extremely affected by contaminants leading to an overestimation; the main reason is that a spectrophotometric approach quantifies both double-strand (ds) DNA and single-strand (ss) DNA fragments, whereas fluorimetric methods are specific for dsDNA. The advantage of PCR-based method is that it provides the quantification of amplifiable DNA, but it is more expensive than other approaches described above.

Finally, the quality of ucfDNA can be analyzed by microcapillary-based electrophoresis such as Bioanalyzer instrument (Agilent) which provides a profile of DNA fragment size.

3.2 DNA Evaluation Methods

Different techniques can be feasible and useful for detecting DNA aberrations such as copy-number variations, mutations, microsatellite instability (MSI), and DNA integrity.

In the last years, some limitations due to a low quantity and quality of ucfDNA have been overcome by more accurate and sensitive approaches as NGS and digital PCR (dPCR). The sensitivity and the possibility to simultaneously analyze several targets make NGS the most promising technique not only for few gene panels but also for whole-genome sequencing [21]. On the other hand, dPCR offers the highest analytic sensitivity (up to 0.01% for mutant allele fraction) and an absolute quantification of mutated

target compared to the total DNA; it is often used as confirmatory method for NGS.

Nevertheless, more classical and common techniques, such as mutation analysis by real-time PCR [22], MSI [23], and DNA integrity [6], are feasible and reliable when some cautions are considered to test and verify the biological and technical reproducibility and DNA quality.

4 Clinical Applications

4.1 Cancer Diagnosis

In diagnostic field, it is mandatory to prevent false-positive or false-negative results; thus a great accuracy in terms of sensitivity and specificity is the goal for a reliable test. Oncologic diagnosis is mainly based on circulating biomarkers such as prostate-specific antigen (PSA), cancer antigen (CA) 19-9, carcinoembryonic antigen, or CA-125, but currently the contribution of circulating nucleic acid, in particular DNA, isn't still considered in clinical routine.

Nevertheless, several studies highlighted the potential role of ucfDNA as a diagnostic marker, mainly for bladder [7] and prostate [24] cancers but also for non-urological tumors [22].

In a study of Togneri et al. [7], it is notably that ucfDNA seems to be more informative than circulating cfDNA in bladder cancer. Using OncoScan assay, the authors identified genomic alterations such as copy-number variations, loss of heterozygosity, and several somatic mutations detected at a sensitivity of 90% in ucfDNA compared to 61% in the cellular DNA from urine.

In addition, promising results were found by Casadio et al., studying ucfDNA integrity [6] at three DNA fragments longer than 250 bp of three genes frequently amplified in bladder cancer: *c-Myc* (8q24.21), *HER2* (17q12.1), and *BCAS1* (20q13.2). The authors obtained 79% sensitivity and 84% and 83% specificity for healthy individuals and patients with urological symptoms, respectively.

Regarding prostate cancer diagnosis, the most studied alteration involved *GSTP1* gene which is frequently hypermethylated in tissue and can be detected also in serum/plasma and urine. A recent study aiming to compare the *GSTP1* methylation in tissue, plasma, and urine of cancer patients to healthy and symptomatic individuals showed that its epigenetic status was confirmed in all the analyzed biological materials [24].

Non-urological cancer has been mainly investigated for epigenetic and mutation alterations detected in ucfDNA. An interesting example is the detection of *KRAS* mutations in urine/serum/plasma of colorectal cancer patients. In this study conducted by Su et al. [22], the authors conferred a more informative role of ucfDNA with respect to cfDNA in the blood, resulting in a detection of *KRAS* mutations of 95% vs. 35–40% of serum or plasma, respectively.

4.2 Cancer Monitoring

Recently, in the oncology field, there is much excitement about the possibility to monitoring the tumor progression and evolution in order to define tumor burden, mechanisms of therapy resistance, and minimal residual disease. The inability to investigate the primary or metastatic tumor tissue and their heterogeneities are leading over this year to focus on noninvasiveness and more feasible ways to typify the genetic of tumor.

EGFR mutation is the most studied alteration in ucfdNA for monitoring treatment resistance. An interesting example is a study conducted on 160 non-small lung cancer (NSCLC) patients at different stages where plasma and urine cfDNA were evaluated during 2 months [8]. Using droplet digital PCR, *EGFR* mutations were serially tested for monitoring the genetic profile in plasma and urine compared to tissue. This study showed that the genetic profile was concordant between tissue, plasma, and urine and correlated with different treatments. In NSCLC, *EGFR* mutation status was also analyzed for monitoring therapies such as EGFR tyrosine kinase inhibitors (TKI) [25] and osimertinib [14], showing its high detection rate in urine.

Urinary *EGFR* mutation was also detected in 120 gastric cancer patients and 100 healthy donors [26]. The serial monitoring of this mutation during the course of EGFR TKI therapy highlighted a concordance rate of *EGFR* mutation status between tissue and ucfdNA of 92% at baseline and 99% at different time points in gastric cancer patients. All these results suggested that ucfdNA can be used as a monitoring marker also in non-urological cancer.

Other interesting findings, regarding *KRAS* mutations, were showed by Fujii et al. [27]. The authors detected by NGS approach *KRAS* G12/*KRAS* G13 mutations in ucfdNA of patients with advanced cancer. In 71 patients, the concordance between tissue and ucfdNA was found at 73%, with a sensitivity of 63% and specificity of 96%. However, when a highest volume of 90–110 mL of urine was analyzed, the concordance increased at 89%, obtaining a sensitivity at 80% and specificity at 100% [27].

Some data were found also for urological cancer. In particular, in a case series of advanced prostate cancer patients, whole-genome sequencing was used for detecting copy-number variations (CNVs) in ucfdNA [21]. ucfdNA was analyzed in hormone-sensitive prostate cancer patients who received androgen deprivation therapy and in castrate-resistant prostate cancer patients who received docetaxel chemotherapy. The authors found significant changes on CNVs in 34 genomic loci during the course of the therapies.

4.3 Prenatal Diagnosis

Fetal DNA could be found as circulating cell-free DNA in maternal blood representing an important diagnostic tool for noninvasive prenatal diagnosis of a number of diseases caused by genetic disorders [28]. It has a rapid turnover (about 16 min) [29], and a part of it could pass the kidney barrier through the glomerular filtration;

Botezatu et al. [3] demonstrated that the kidney barrier is permeable to short DNA fragments (including fetal circulating cfDNA). This DNA is defined as “transrenal DNA”: it could be found in maternal urine and analyzed by molecular standard technologies. The main critical issue is that fetal DNA can be detected in maternal urine with a low specificity. Later, Koide and coworkers [30] compared fetal cell-free DNA in plasma and urine samples and found that fetal DNA in maternal plasma was often fragmented to <100 bp long, while fetal DNA in urine was even further fragmented and might be studied using small target size by conventional PCR.

Ten years ago, Majer and coworkers [31] starting from 1 mL of urine samples, using fluorescent PCR and quantitative real-time PCR, demonstrated that about 33% of urine samples were positive for fetal DNA presence. However they concluded that, due to the low sensitivity, ucfDNA didn't seem to be useful for prenatal testing.

Later, a study conducted by Tsui et al. [15] using massively parallel paired-end sequencing showed that transrenal fetal DNA in maternal urine was highly degraded: fragments were between 29 and 45 bp in length. They also found a sensitivity of about 71% in detecting fetal DNA in maternal urine.

The role of ucfDNA in prenatal diagnosis of genetic disease still remains controversial; the low concentration in urine samples and the high degradation due to the fragmentation make it less relevant with respect to plasma cell-free DNA, and few studies have evaluated its role.

Probably prenatal diagnosis will take advantages from the new molecular methodologies such as next-generation sequencing or digital PCR technologies to reach high-sensitivity levels.

5 Future Directions

Urine could be a convenient source of DNA in different clinical situations, given its main advantage to be an ultra-noninvasive sample. Nowadays, the concept of personalized medicine guides toward the need of individual's genomic signature not only at the time of the diagnosis but also during the tumor evolution using repeated sampling and studying the dynamic changes and the efficacy of treatments. As described above, ucfDNA is informative for diagnosis and prognosis and for the monitoring of urological and non-urological tumors. Given the possibility to analyze large volume of material in different time points, without any health complications for the patients, the onset of clonal evolution can be tracked through the detection of rare variants.

Recently, some papers have been focused on the ucfDNA analysis impact on non-urological tumors as colorectal, NSCLC, gastric, and pancreas. However, the low number of studies on ucfDNA

has not yet defined its application as a marker of cancer in the real clinical practice. Similarly, few papers are published regarding prenatal diagnosis, and the role of ucfDNA in this field still remains controversial.

In the near future, thanks to high-throughput approaches such as NGS, more data will be produced to give a focused and sharp picture of disease at several time points. More robust, sensitive, and reproducible techniques can help to evaluate biological materials containing contaminants, degraded and with different fragmentation profiles such as ucfDNA. In this way, more studies on ucfDNA can clarify its supplementary or exclusive informative role compared to cfDNA for different diseases.

In conclusion, we believe that ucfDNA is the most promising source of markers with a real impact on the clinical practice in different fields such as cancer and prenatal diagnosis, in the next future.

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Chapter 16

Urinary Cell-Free DNA: Isolation, Quantification, and Quality Assessment

Valentina Casadio and Samanta Salvi

Abstract

Urine cell-free DNA is an important source of diagnostic markers for different diseases (e.g., cancer and prenatal diagnosis). It is important to achieve a simple and fast protocol to maximize the recovery of DNA from urine supernatant and to assess its quality. Here we describe a simple approach from urine collection to DNA quality assessment for downstream analyses.

Key words Urine, Cell-free DNA, Isolation, Quantification, Quality, Protocol

1 Introduction

Circulating cell-free DNA has been widely studied in a variety of physiological and pathological conditions (e.g., cancer, physical activity, and prenatal diagnosis), and a number of robust and reproducible methodological approaches to isolate and analyze it have been described since many years ago [1, 2].

Less literature data are available about DNA coming from the cell-free component of urine; however, a particular emphasis has been placed on urine cell-free DNA (ucfDNA) in the last years.

ucfDNA takes its origin directly from dying cells exfoliated either in urine (bladder and prostatic cells) or from the circulation. ucfDNA could be a good source of biomarkers especially for urological cancers [3–6] but also for non-urological ones [7, 8].

It represents a good diagnostic tool also for prenatal diagnosis as fetal DNA could be found in the cell-free component of maternal urine [9, 10]. As urine collection is even less invasive than blood collection, patient compliance is guaranteed, large volumes are available, and urine can be collected routinely to monitor a disease evolution or a physiological process.

It is therefore an important goal to achieve cheap, fast, and simple procedures to obtain high-quality ucfDNA and to analyze

its alterations. Urine collection and preservation are crucial points to reach this aim. Nucleic acids in urine are more sensitive to degradation with respect to blood samples, due to urine composition (DNA nucleases, bacteria, variable pH, etc.); for these reasons some preservation buffers are commercially available (e.g., urine conditioning buffer (Zymo Research), urine preserve (Streck)) [11]. They are mainly used for whole urine, but they can be successfully added also to the cell-free component (urine supernatant obtained after whole urine centrifugation) for its maintenance. The choice of the DNA isolation protocol is also important to reach a sufficient and high-quality DNA yield. The suitability of the protocol depends on the urine volume to be processed. For volume larger than 3 mL, the Quick-DNA Urine Kit (Zymo Research) seems to be the best choice, whereas for 1 mL of urine or less, also other methods can be used (e.g., DNA Mini kit by Qiagen) [12]. As ucDNA has a low concentration, 1 mL of urine might be not sufficient to proceed with downstream analyses (e.g., gene copy number analysis and gene mutations). However, larger urine volumes are often available, thus to overcome this issue.

In this chapter, we describe an efficient method for DNA isolation, quantification, and quality assessment starting from 40 or less mL of urine sample (Fig. 1). DNA is extracted using Quick-DNA Urine Kit (Zymo Research) and quantified with a highly sensitive fluorometric approach (Qubit dsDNA High Sensitivity Assay Kit). The DNA quality in terms of length distribution is evaluated with a micro-capillary-based electrophoretic approach (Bioanalyzer, Agilent Technologies).

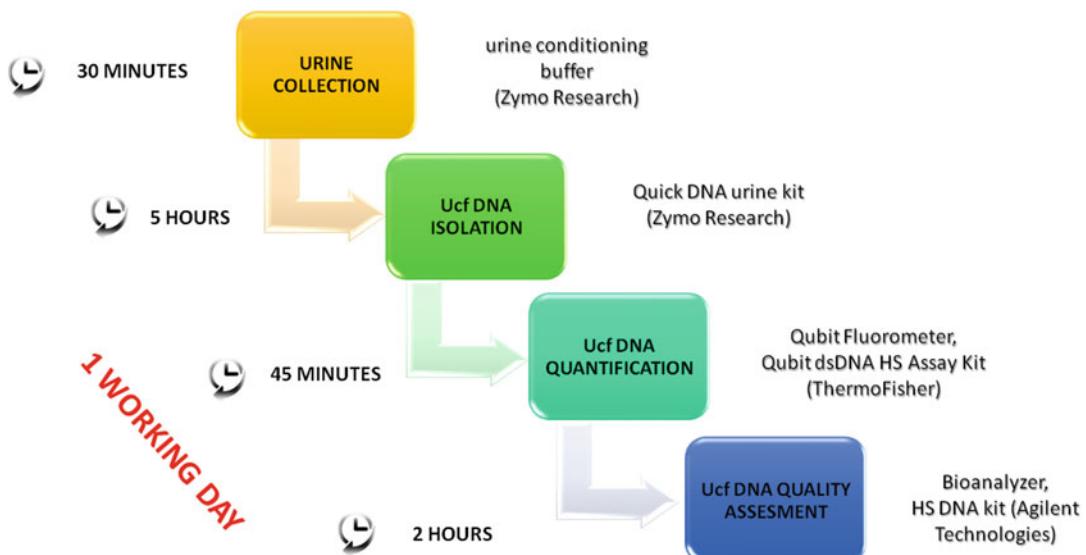


Fig. 1 The whole protocol is summarized: details regarding the duration of each single procedure and the reagents/instruments are given

2 Materials

2.1 DNA Isolation

For DNA isolation, we use Quick-DNA Urine Kit (Zymo Research). Ethanol and β -mercaptoethanol are not included in the kit. We suggest using a β -mercaptoethanol for molecular biology.

1. Genomic lysis buffer (Zymo Research): under a fume hood, add β -mercaptoethanol to a final dilution of 0.5% (V/V). To prepare 50 mL of genomic lysis buffer, add 250 μ L of β -mercaptoethanol.
2. DNA wash buffer: add 48 mL of 100% ethanol to the 12 mL urine DNA wash buffer.

2.2 DNA Quantification

To quantify urinary DNA, a fluorometric method is recommended (*see Note 1*). In this protocol, we use Qubit fluorometer (Thermo Fisher) and the Qubit dsDNA HS (High Sensitivity) Assay Kit.

HS reagent must be stored at room temperature and protected from light. HS buffer must be stored at room temperature. The two standards must be stored at 4 °C and warmed at room temperature when used.

2.3 DNA Quality Assessment

To assess DNA quality, we use the Bioanalyzer instrument (Agilent Technologies) and the High Sensitivity DNA Assay Kit (Agilent Technologies). Bioanalyzer instrument must be cleaned as suggested by the user's manual to avoid any contamination (e.g., by RNA when evaluating DNA); it is recommended to use only water for molecular biology to clean the instrument.

Chips must be stored at room temperature. All reagents supplied must be stored at 4 °C.

Prepare the gel-dye mix as follows:

1. Allow the HS DNA dye concentrate and the HS DNA gel matrix to equilibrate at room temperature for at least 30 min.
2. Vortex the HS DNA dye concentrate for 10 s and spin down. Make sure that dimethyl sulfoxide (DMSO) is completely thawed.
3. Pipette 15 μ L of the HS DNA concentrate into a HS DNA gel matrix vial.
4. Cap the tube and vortex until you visualize the correct mixing of gel and dye.
5. Transfer the gel-dye mix to the top of a spin filter.
6. Spin for 10 min at 2240 $\times g$. Discard the filter, label the tube with the date of preparation, and protect it from light (this mix is stable for 6 weeks at 4 °C).

3 Methods

3.1 Urine Collection

1. Obtain clean-catch first or second morning urine sample (*see Note 2*) in a clean dry plastic cup. Collect at least 50 mL of urine sample.
2. Maintain urine at 4 °C for a maximum of 3 h, and send to the laboratory at the same temperature.
3. Mix each sample by inverting it twice immediately upon arrival in the laboratory, and transfer into two 50 mL conical bottom polypropylene tubes.
4. Centrifuge tubes at $850 \times g$ for 10 min at room temperature (*see Note 3*).
5. Carefully transfer 40 mL of the upper part of the urine supernatant into a new, clean 50 mL conical bottom polypropylene tube, leaving at least 2 mL of the supernatant above the cell pellet (*see Note 4*).
6. Discard the pellet.
7. Add 70 µL of urine conditioning buffer (Zymo Research) for every 1 mL of urine supernatant collected.
8. Close the tube and mix the urine supernatant mixture by vortexing for at least 30 s to well homogenize sample.
9. Store urine samples at room temperature for a maximum of 1 month before proceeding with DNA isolation (*see Notes 5 and 6*).

3.2 DNA Isolation

1. Prepare clearing beads mixture: vortex for at least 1 min clearing beads mixture before use, and spin rapidly with a microcentrifuge, just to remove beads from the cap (*see Note 7*).
2. Vortex samples for at least 30 s.
3. Add 10 µL of clearing beads if processing ≤ 14 mL of urine supernatant or 20 µL of clearing beads if processing ≥ 14 mL.
4. Vortex the mixture obtained for at least 30 s.
5. Centrifuge at $3000 \times g$ for 15 min at room temperature. In this phase DNA precipitates with beads.
6. While samples are in centrifuge, prepare the genomic lysis buffer (*see Note 8*).
7. Without disturbing the pellet, slowly, pipette out the supernatant leaving behind about 400 µL of the pellet.
8. Add an equal volume (400 µL) of urine pellet digestion buffer to the pellet (*see Note 9*).
9. Add 5% (V/V) of proteinase K (40 µL) to the sample.

10. Resuspend the pellet mixture by vortexing. To completely resuspend the pellet, it is necessary to vortex for at least 1 min.
11. Incubate the pellet mixture at 55 °C for 30 min.
12. Transfer samples in a fume hood, and add 1 volume (840 µL) of genomic lysis buffer.
13. Mix samples well by vortexing.
14. Transfer 760 µL samples into a Zymo-Spin IC-S columns in a collection tube. Centrifuge at $\geq 16,000 \times g$ for 1 min (*see Note 10*).
15. Discard the tube containing the flow through, and place the column in a clean tube.
16. Repeat **steps 1** and **15** twice, till all the sample mixture is passed through the column.
17. Add 200 µL of urine DNA prep buffer to the spin column. Centrifuge at $\geq 16,000 \times g$ for 1 min, and discard the flow through.
18. Warm DNA elution buffer at 65 °C.
19. Add 700 µL urine DNA wash buffer to the column. Centrifuge at $\geq 16,000 \times g$ for 1 min, and discard the flow through.
20. Repeat **step 19** with 200 µL urine DNA wash buffer.
21. Transfer the spin column to a DNase/RNase-free microcentrifuge tube. Add 50 µL DNA elution buffer direct on the column matrix, and let stand for 5 min at room temperature.
22. Centrifuge at full speed for 1 min.
23. Reload the eluted DNA, and centrifuge for 1 min at full speed to maximize DNA recovery.
24. Store DNA samples at –20 °C.

3.3 DNA Quantification Using Qubit Fluorometer

1. Move all standard at room temperature about 20 min before starting quantification.
2. Thaw samples and place them at room temperature too.
3. Gently vortex and spin samples and HS reagent.
4. Prepare the working solution: add 199 µL of HS buffer and 1 µL of HS reagent for each sample (consider two standards plus the number of samples to be quantified plus 1 extra sample) (*see Note 11*).
5. Vortex and spin the working solution.
6. Set up the required number of 0.5 mL Qubit assay tubes (*see Note 12*) for samples and two for the standards.
7. Add 190 and 198 µL of working solution to the Qubit assay tubes, for standards and samples, respectively.

8. Add 10 μL of each standard and 2 μL of each sample (*see Note 13*). Pipette up and down samples and standard into the working solution.
9. Vortex standards and samples for 2–3 s without making bubbles to completely mix DNA and working solution.
10. Incubate samples at room temperature for 2 min, and proceed with reading samples.
11. Read standard and samples following the appropriate procedure for your instrument (Qubit 2.0 or 3.0).
12. In brief, perform the calibration with the two standards, proceed with reading samples, and annotate the results obtained.
13. Read the samples twice, without performing a new calibration with standards.
14. Result will be the mean of the two readings. To obtain the final concentration (ng/mL) of your samples, you must remember to multiply your result $\times 100$ (dilution factor) (*see Note 14*).

3.4 DNA Quality Assessment

1. Setting up the assay equipment and Bioanalyzer as suggested by user manual (*see Note 15*).
2. Replace the syringe if using a new reagent kit (*see Note 16*).
3. Set up the Bioanalyzer: open the lid and make sure that the electrode cartridge is inserted in the instrument. If not, open the latch and insert the electrode cartridge.
4. Set up the vortex mixer by adjusting the speed knob to 2400 rpm.
5. Start the 2100 software by following the user's manual.
6. Prepare the gel-dye mix or use an already prepared gel-dye mix stored at 4 °C.
7. Allow the gel-dye mix or the HS DNA dye concentrate and the HS DNA gel matrix to equilibrate at room temperature for at least 30 min.
8. Take a new HS DNA chip and place it on the priming station.
9. Pipette 9 μL of the gel-dye mix at the bottom of the well marked and dispense the gel-dye mix (*see Note 17*).
10. Set the timer to 60 s, make sure that plunger is positioned at 1 mL, and close the chip priming station.
11. Press the plunger of the syringe down until it is held by the clip.
12. Wait for 60 s and then release the plunger. The plunger moves back at least to 0.3 mL mark (*see Note 18*).
13. Wait for 5 s and slowly pull back the plunger to the 1 mL position.

14. Open the chip priming station, and pipette 9 μ L of the gel-dye mix in each of the wells marked.
15. Pipette 5 μ L of the HS DNA marker in the well marked with the ladder symbol and into each of the 11 sample wells.
16. Pipette 1 μ L of the DNA ladder in the well marked with the ladder symbol.
17. Pipette 1 μ L of sample in each of the 11 remaining wells or 1 μ L of marker in the unused wells.
18. Vortex for 60 s in the IKA vortex.
19. Run the chip following the user's instructions.
20. After the run is finished, immediately remove the chip from the instrument, and proceed with the cleaning as suggested by user's manual.

3.5 Bioanalyzer Results Interpretation

1. Verify that the ladder electropherogram is correct accordingly to the user's manual; if yes, proceed with sample analysis.
2. The software gives a sample report in which you can visualize an electropherogram trace, total concentration, and a table with all the peaks for each length and the relative concentrations (*see Note 19*).
3. For urinary samples, you can visualize three different electropherogram profiles. We have called them A, B, and C, and they have different characteristics in terms of DNA lengths distribution. We can describe them as follows:

Profile A: A peak or a number of different peaks that fall between 500 bp and the upper marker and a peak between 100 and 200 bp (Fig. 2).

Profile B: A peak or a number of different peaks that fall between 500 bp and the upper marker; a peak between 100 and 200 bp is absent or inferior to 1/3 of that at 500 bp and the upper marker (Fig. 3).

Profile C: The electropherogram is flat, only the upper and lower marker as visible (Fig. 4) (*see Note 20*).

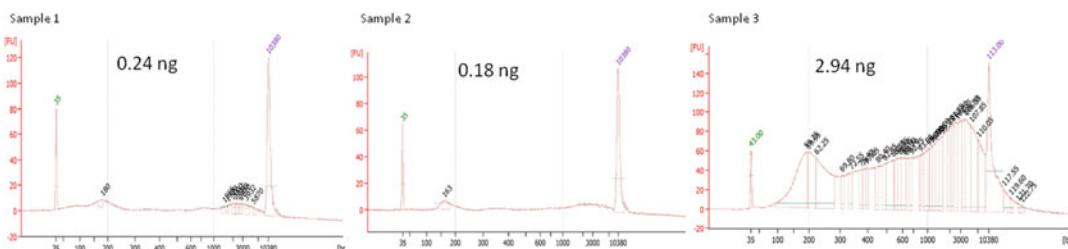


Fig. 2 Electropherograms representing three ucfDNA samples with a typical Profile "A"

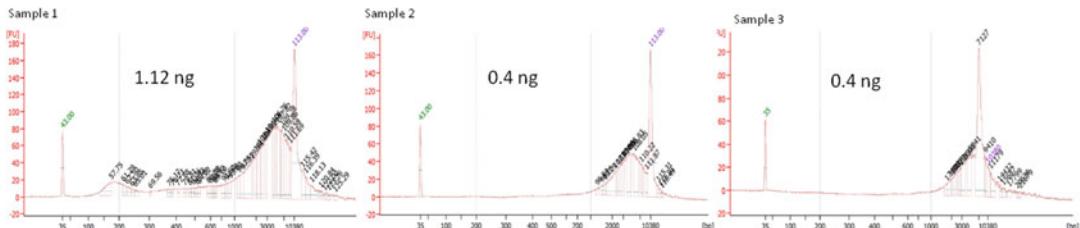


Fig. 3 Electropherograms representing three ucfDNA samples with a typical Profile “B”

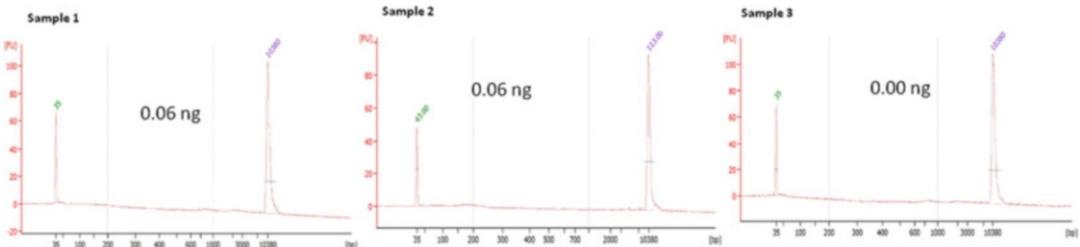


Fig. 4 Electropherograms representing three ucfDNA samples with a typical Profile “C”

4 Notes

1. For DNA quantification, we use a Qubit fluorometer (Thermo Fisher) and the Qubit dsDNA HS Assay Kit. The use of a different fluorometric approach will give similar results. For a precise quantification, a spectrophotometric approach is not recommended.
2. You can collect first morning urine or even second, third, etc. Take into account that if you choose to collect first morning urine, this sample contains a higher number of cells and cellular debris coming from the urological tract and exfoliated in urine during the night. Therefore, you will recover a higher DNA yield. This can help you if you are studying a urological cancer (you will probably find more DNA coming from cancer cells). On the other side, if you are approaching at other pathologies and you are searching for DNA coming from the circulation, the high amount of DNA derived from urological cells will be confounding and will probably reduce your test sensitivity.
3. When proceeding with the centrifugation of the whole urine sample, you can repeat the centrifugation twice (some protocols suggest it); however we found that it would not change the

yield and quality and a unique centrifugation at 850 g is sufficient to well separate cells and cellular debris.

4. When transferring the upper part of the supernatant, after the centrifugation, be careful to leave at least 2 mL over the pellet, thus to avoid contamination by cells or cellular debris.
5. We have verified that there is no difference in DNA yield and quality between different time storing (considering a maximum of 1 month at room temperature). You can proceed with the DNA isolation procedure when you have collected a number of samples (12 is the best), thus reducing time consumed.
6. We have verified that no difference in DNA yield and quality exists between samples stored at -80°C without urine conditioning buffer and those stored at room temperature with urine conditioning buffer. Therefore, the room temperature storage seems to be the best way, avoiding the use of a large space in -80°C freezer. However, if you plan to store samples for more than 1 month, it is better to keep them at -80°C (without adding the urine conditioning buffer). You will then add the buffer immediately before the isolation procedure, after thawing and vortexing samples.
7. It is important to carefully mix clearing beads by vortexing before use. As you will notice, the beads tend to separate from the water phase very quickly; we suggest to proceed with clearing beads addiction to samples immediately after vortexing and spin.
8. β -Mercaptoethanol is a toxic chemical. A fume hood is necessary to handle this solvent, and it is recommended to discard separately any pipette tips that touch β -mercaptopethanol and always any protective gloves used while handling this chemical directly after use. All buffer and samples containing β -mercaptopethanol must be opened under a fume hood.
9. When adding urine digestion buffer, do not touch the pellet, and do not pipette for mixing sample. This expedient is suggested to maximize DNA recovery.
10. When you're proceeding with the steps involving the columns, don't worry about the beads that are naturally retained in the columns. They will not interfere with DNA isolation, quantity, and quality. Simply add all buffer and samples on the column matrix.
11. When preparing the working solution, pipette up and down many times to be sure that HS reagent completely dissolves in the buffer.
12. Use only 0.5 mL tubes that are suitable for Qubit fluorometer. If using other 0.5 mL tubes, you can get a wrong quantification.

13. When using Qubit fluorometer with HS Assay, you might add from 1 to 20 μL of each sample. We have verified that 2 μL is the best volume: quantification is reproducible (low pipetting error), and the majority of samples are evaluable without using a lot of precious DNA sample. If you find many samples with a too low concentration, you must increase the loading volume.
14. Remember that Qubit fluorometer gives you a good DNA quantification, but it is not able to give you information regarding DNA quality assessment and sizing.
15. Components of HS DNA kit contain DMSO that must be treated as a potential mutagen. It is important to wear gloves and to follow good laboratory practices to protect the operators.
16. For setting up the chip priming station when you replace syringe, be careful to place the baseplate in the correct position and to adjust the level of the syringe clip for HS DNA assay.
17. When pipetting the gel-dye mix, be sure to insert the tip of the pipette to the bottom of the chip well when dispensing. This prevents the formation of air bubbles that can affect the subsequent analysis.
18. This step is particularly important for the analysis. If the plunger does not move back to 0.3 mL, probably you have pressed the plunger of the syringe with some hesitation. The first time you perform the experiment, you should train yourself with an old chip. This is an important operator-dependent step.
19. Bioanalyzer can give also an information regarding total DNA concentration. We have verified that Qubit and Bioanalyzer give concordant results. Given the high cost of Bioanalyzer, we suggest to use it only if you need a quality assessment. If a quality assessment is not necessary, it is better to use a fluorometer.
20. The different electropherogram profiles give you important information regarding what type of DNA you have isolated. Profile A: DNA coming from necrotic cells exfoliated by the urological tract (e.g., bladder and prostatic cells) and DNA coming from apoptotic cells derived from urological tract but also DNA coming from the circulation and passed through the glomerular filtration. Considering the first peak at about 162 bp, you can notice that the area covered by the peak frequently falls between 70–80 and 200 bp, so that it includes a short DNA fragments coming from the circulation. Profile B: genomic, long DNA component is prevalent and derived from urological tract cells. Profile C: DNA concentration is too low. You can proceed with concentrating your sample using a speed vac evaporator, and repeat the quality assessment analysis or proceed with a new urine sample and repeating the DNA isolation.

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