Analytica Chimica Acta 1163 (2021) 338470

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Liquid biopsy genotyping by a simple lateral flow strip assay with visual detection

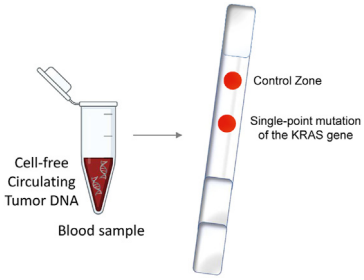
Panagiota Kalligosfyri [a], Sofia Nikou [b], Vasiliki Bravou [b], Despina P. Kalogianni [a, *]

[a] Department of Chemistry, University of Patras, 26504, Rio, Patras, Greece
[b] Department of Anatomy-Histology-Embryology, Medical School, University of Patras, 26504, Patras, Greece

HIGHLIGHTS

- A rapid gold nanoparticle-based lateral flow assay with visual detection was developed for genotyping.
- The DNA-based lateral flow assay was applied for the detection of cell-free/circulating tumor DNA in blood samples.
- Four alleles of the KRAS gene were detected by the proposed assay.
- The detection is accomplished through bare eye and is completed within 10 min.
- The proposed lateral flow assay is simple, rapid, cost-effective, sensitive, portable and universal.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 23 February 2021
Received in revised form
24 March 2021
Accepted 29 March 2021
Available online 12 April 2021

Keywords:
Dipstick
Gold nanoparticles
Biosensor
KRAS
Cell-free DNA
Circulating tumor DNA

ABSTRACT

Liquid biopsy, as a minimally invasive method that allows real-time monitoring of the tumor genome, represents a competing approach for cancer diagnosis, prognosis and therapy decision making. Liquid biopsy in cancer patients mainly includes analysis of circulating tumor cells (CTC) and cell-free circulating tumor DNA (ctDNA). ctDNA is the tumor-derived fraction of the cell-free DNA present in the blood. ctDNA is detected based on cancer-specific genomic aberrations (mainly mutations) and represents a challenging analyte due to high fragmentation and low concentration. Several methodologies have been developed for ctDNA analysis in cancer patients but many of these technologies are too time-intensive, complicated and expensive for implementation in diagnostic testing. Herein, we developed a novel lateral flow strip assay for mutational analysis of ctDNA in blood samples and visual detection that is based on gold nanoparticles as reporters. As a model, common single-point mutations of the KRAS gene, related to colorectal cancer (CRC), have been selected for method development. The proposed DNA biosensor has been successfully applied for the detection of three KRAS mutations (KRAS G12D/A/V), along with the wild-type KRAS gene in synthetic DNA targets, cancer cell lines and cfDNA from blood samples of healthy individuals and CRC patients. The main advantages of the proposed lateral flow assay are simplicity, rapid analysis time (~10 min) and visual detection without the requirement of special instrumentation. The assay is also cost-effective with high detectability, specificity and reproducibility and has the potential to be used as a portable and universal device. In conclusion, the proposed assay offers a rapid diagnostic strip test for visual genotyping, as an alternative approach for liquid biopsy applications.

* Corresponding author.
E-mail address: kalogian@upatras.gr (D.P. Kalogianni).

https://doi.org/10.1016/j.aca.2021.338470
0003-2670/

1. Introduction

Cancer diagnosis, prognosis and therapy decisions in the modern era of personalized medicine are largely based on cancer-specific genomic aberrations (tumor genotyping) [1]. In colorectal cancer (CRC) for example, KRAS mutations are among the most common aberrations in the disease related to worse prognosis, poor response to conventional chemotherapy and resistance to anti-EGFR therapy and detection of KRAS mutations guides patient's treatment [2–4]. Moreover, novel genomic biomarkers with diagnostic, predictive and prognostic significance are being discovered in an unprecedented rate. However, tumor heterogeneity and especially clonal evolution and selection that lead to disease progression and therapy resistance introduce several hurdles in clinical decision making based on tumor genotyping [1,5,6]. While tissue biopsy still remains the gold standard for initial cancer diagnosis and prognosis, in terms of disease progression and therapy monitoring, tissue biopsy shows limitations related to the invasive procedure required and the snapshot information obtained [1,5–7]. On the other hand, liquid biopsy analyzing circulating tumor cells (CTC) or cell-free circulating tumor DNA (ctDNA) in blood samples of cancer patients represents a rapid, minimally-invasive approach with important clinical applications in early cancer diagnosis, assessment of the risk of recurrence, detection of minimal residual disease, real-time monitoring of therapy response or resistance and therapy tailoring [7–10].

Cell-free DNA (cfDNA) refers to extracellular DNA molecules released by all types of cells in the circulation and body fluids and ctDNA is the tumor-derived fraction of cfDNA. As ctDNA represents a surrogate marker of the entire tumor genome, ctDNA analysis in blood samples is a very promising non-invasive biomarker allowing early cancer detection and monitoring of cancer-specific aberrations at all times during the course of the disease, bypassing the limitations of tumor biopsies [7–10]. In CRC for example quantification of cfDNA and detection of KRAS mutant ctDNA has been used as a diagnostic and prognostic tool and also as an approach to monitor resistance to anti-EGFR therapy [11,12].

Detection of ctDNA (and discrimination from normal cfDNA) is based on the presence of cancer-specific aberrations, usually gene mutations. The ctDNA usually represents only a small fraction (<1.0%) of total cfDNA in blood circulation and several factors affect the amount of ctDNA in body fluids such as tumor stage and burden, tumor proliferation rate and turnover, tumor type and therapeutic interventions that cause inflammation and necrosis [13–15]. ctDNA is released mainly from necrotic and apoptotic tumor cells. The average size of ctDNA varies between small fragments of 70–200 bp which are generated by cellular apoptosis and long fragments (200 bp–21 Kbp) generated by necrosis. These characteristics i.e high fragmentation and very low concentration especially in the context of early cancer detection, detection of minimal residual disease and cancer chemotherapy (the later increases cfDNA from non-tumor cells and dilutes ctDNA), render ctDNA a challenging analyte [16,17]. Analytical methods that are mainly used for ctDNA analysis include PCR-based techniques (such as digital PCR, real-time PCR and HRM (high-resolution melting) analysis, etc), next-generation sequencing, mass spectrometry, DNA microarrays and southern blotting. In general, analysis of ctDNA can be performed via targeted approaches that detect known gene mutations (e.x. KRAS in the context of anti-EGFR therapy in CRC) and un-targeted approaches (e.x. whole genome or exome sequencing) to screen the genome for new genomic aberrations [7–10]. Although both strengths and limitations of the above techniques exist, as previously reviewed, it should be emphasized that many of these techniques cannot overcome the analytical challenge of ctDNA due to their low detectability, sensitivity and specificity, expensive and complex instrumentation, high cost, tedious process, extensive analysis time and sample pretreatment. For example, technologies such as DNA sequencing (Sanger method) are not able to detect such low amount of ctDNA, and droplet or real-time PCR have limited multiplexity potential [18–22]. For these reasons, new, rapid, simpler, and cost-effective analytical methods with high sensitivity for liquid biopsy application have still to be developed. Biosensors represent promising analytical tools for liquid biopsy applications and several biosensors have been developed. However, there are limited data regarding application of biosensors in genotyping ctDNA from patients' blood samples [23–40].

In this work, we developed a rapid diagnostic strip test, a lateral flow assay, based on gold nanoparticles, that was applied for the first time for visual detection of KRAS mutations in ctDNA for liquid biopsy applications. The new strip test was successfully applied for the detection of four single nucleotide polymorphisms (SNPs) that correspond to the normal KRAS gene and three of the most significant/common mutations in KRAS gene related to CRC in synthetic DNA samples, cancer cell lines and ctDNA isolated from blood samples of CRC patients. The main advantages of the proposed diagnostic test are simplicity and cost-effectiveness, rapid analysis that is completed within 10 min, high detectability, sensitivity, specificity and reproducibility, universality and portability, while the detection is accomplished by bare eye and no expensive instrumentation or highly trained personnel are required.

2. Materials and methods

2.1. Materials and equipment

The synthetic oligonucleotides and primers were designed using the freeware application Oligo Analyzer by Teemu Kuulasma and obtained from Eurofins Genomics(Ebersberg, Germany) (Table 1). The NucleoSpin DNA Rapid Lysis kit for DNA extraction from cell lines and the Nucleo Spin Plasma XS kit for cfDNA extraction were purchased from Macherey-Nagel (Düren, Germany). Fetal bovine serum (FBS) and 0.25% trypsin/EDTA solution were from Gibco/Thermo Scientific (Waltham, MA, USA). The Mycoprobe Mycoplasma Detection Kit was from R&D Systems (Minneapolis, USA). Polymerase Chain Reactions (PCR) and primer extension (PEXT) reactions were performed in the PCR Thermal Cycler Dice TP60 (Takara Bio, Kusatsu, Japan) using the Kapa2G FAST PCR kit (Kapa Biosystems, Basel, Switzerland) and Vent (exo-) DNA polymerase kit (New England Biolabs, Ipswich, Massachusetts, USA), respectively. Mupid-one gel electrophoresis system and agarose Fast Gene were obtained from NIPPON Genetics (Duren, Germany). The ΦX174 DNA-HaeIII DNA marker was obtained from New England Biolabs (Ipswich, Massachusetts, USA) and ethidium bromide used for DNA staining from Research Organics (Cleveland, OH, USA). The 40 nm size gold nanoparticles (AuNPs) (7.7×10^7 particles/μL) were obtained from Nanobiosols (Liverpool, UK). Carboxylated microspheres Polybead (5.7×10^6 particles/μL), 2.00 μm in diameter, were from Polysciences Europe GMbH (Hirschberg, Germany). The coupling reagent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was from AppliChem (Maryland Heights, MO, USA). Bovine serum albumin (BSA) was purchased from Serva Electrophoresis GmbH (Duisburg, Germany) and EZ-Link Sulfo-NHS-LC-biotin (sulfosuccinimidyl-6-(biotin-amido) hexanoate) from Thermo Fisher (Waltham, Massachusetts, USA). The salt-free recombinant streptavidin (SA), from *Streptomyces avidinii* expressed in *E. coli* was used for AuNPs conjugates and was obtained from Roche (Basel, Switzerland). All the dNTPs

Table 1
Contains the KRAS gene PCR primers, the synthetic DNA targets used for optimization and the PEXT primers, at the 3′ ends of which the KRAS mutations are located.

Oligonucleotide	Sequence (5′ → 3′)
PCR Primers	
KRAS_F	GCCTGCTGAAAATGACTGAATA
KRAS_R	CAAGAGACAGGTTTCTCCATCA
Synthetic Targets	
tKRASNormal	CTGAATTAGCTGTATCGTCAAGGCACTCTTGCCTACGCCACCAGCTCCAACTACCACAAG
tKRASG12D	CTGAATTAGCTGTATCGTCAAGGCACTCTTGCCTACGCCATCAGCTCCAACTACCACAAG
tKRASV	CTGAATTAGCTGTATCGTCAAGGCACTCTTGCCTACGCCAACAGCTCCAACTACCACAAG
tKRASG12A	CTGAATTAGCTGTATCGTCAAGGCACTCTTGCCTACGCCAGCAGCTCCAACTACCACAAG
PEXT Primers	
KRAS_NORMAL	A_{24} CTTGTGGTAGTTGGAGCTGG
KRAS_G12D	A_{24} CTTGTGGTAGTTGGAGCTGA
KRAS_G12V	A_{24} CTTGTGGTAGTTGGAGCTGT
KRAS_G12A	A_{24} CTTGTGGTAGTTGGAGCTGC

(dATP, dGTP, dTTP) were purchased from Invitrogen (Carlsbad, California, USA) and biotin-dCTP from Jena Biosciences GmBh (Jena, Germany). Glycerol was from Carlo Erba (Barcelona, Spain). Sodium hydrogen carbonate was from Merck (Darmstadt, Germany) mono- and dihydrate sodium phosphate were purchased from Lachner (Neratovice, Czech Republic). 2-(N-morpholino)ethanesulfonic acid (MES), penicillin, streptomycin and all the other common reagents were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA).

For the construction of the strip, the nitrocellulose membrane Immunopure FP Whatman was purchased from GE Healthcare Life Sciences (Buckinghamshire, UK), the immersion pad, conjugate pad, and absorbent pad were obtained from Schleider & Schuell (Dassel, Germany).

The composition of 1 × Phosphate-buffered saline (PBS), pH 7.4 was 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 2 mM KH_2PO_4. The 4 × Saline-sodium citrate (SSC) buffer consisted of 600 mM NaCl and 60 mM $Na_3C_6H_5O_7$. 1 × TE buffer was constituted by 10 mM Tris-HCl pH 8.0 and 1 mM EDTA.

The centrifuge 5415 D was obtained from Eppendorf (Hamburg, Germany) and the Ultrasonic device was from Branson Ultrasonics (Danbury, CT, USA). The images of the strips were obtained with the scanner EPSON Perfection V500 PHOTO (Seiko Epson Corporation, Suwa, Japan). The NanoDrop 1000 spectrophotometer from Thermo Scientific (Waltham, MA, USA) was used for DNA and cfDNA determination.

2.2. Samples

2.2.1. Synthetic DNA samples

Synthetic single-stranded DNA targets that contained the SNP of interest of the KRAS gene were used for method development and optimization studies. The targets had a size of 60 bp and carried a poly(dA) tail at the 3' end to enable the detection with the lateral flow strip assay.

2.2.2. Cell lines

The human colorectal adenocarcinoma cell line Caco-2 (Caco2, human colorectal adenocarcinoma), with wild-type KRAS, was generated from American Type Culture Collection (ATCC, Manassas, VA) and was kindly provided by Prof. Kalofonos H Laboratory (Department of Medicine, University of Patras, Patras, Greece). The acute lymphocytic leukemia cell line CCRF-CEM with mutant KRAS [CCRF CEM, c.35G > A (p.G12D)] was generated from the ATCC and was kindly provided by Prof. Stathopoulos GT Laboratory (Department of Medicine, University of Patras, Patras, Greece). The human colon adenocarcinoma cancer cell line with mutant KRAS [LS174T, c.35G > A (p.G12D)] was purchased from the American Type Culture Collection (ATCC, USA). Cell lines were cultured in humidified incubator at 37 °C in 5% CO_2 and 95% air using full culture medium (DMEM, GIBCO-TM) supplemented with 10% FBS and 100 units of penicillin and streptomycin. All cell lines were cultured and passaged in T25 tissue culture flasks before subsequent harvesting using 0.25% trypsin/EDTA. Passage 3 to 4 cell lines were used for all experiments. For experiments, cells were grown to 80–90% confluency, while all cell lines were periodically tested for Mycoplasma contamination using Mycoprobe Mycoplasma Detection Kit.

2.2.3. Blood samples

Blood samples were collected from three healthy donors and three CRC patients with advanced disease and known G12D KRAS mutations, prior to or soon after initiation of first adjuvant chemotherapy in the Oncology Department, University Hospital of Patras Greece. Presence of KRAS mutations was confirmed in tissue samples by Next Generation Sequencing (Ion Gene Studio S5 Prime System, Thermo Fisher Scientific) using the Ion AmpliSeq NGS Panel (Thermo Fisher Scientific). Informed consent was available for all patients and the study has been approved by Institutional Ethics & Research Committee (protocol number 86436/14.10.2019).

2.3. DNA extraction from cell lines

After vigorous mixing by vortex, the cell pellet was washed with PBS and DNA extraction was performed using NucleoSpin DNA Rapid Lysis kit, according to manufacturer's instructions. DNA was eluted in 100 μL of elution buffer RLE followed by centrifugation for 1 min at 11000 g. The purity and concentration of DNA was determined using the NanoDrop 1000 spectrophotometer. DNA extracts were stored at −20 °C.

2.4. Cell-free DNA (cfDNA) extraction

Plasma was prepared from fresh human K3EDTA blood samples by centrifugation for 10 min at 2000 g. Plasma was carefully aspired from the cell pellet in order to avoid cell-pellet carryover. Plasma samples were then frozen and stored at −80 °C before use. Prior to DNA isolation, plasma samples were defrosted in room temperature, re-centrifuged for 3 min at 11000 g and the supernatant was further analyzed. cfDNA isolation from plasma was performed using the NucleoSpin Plasma XS kit according to the manufacturer's instructions, utilizing a column which directs the sample to a silica

membrane. DNA was finally eluted with 20 μL of elution buffer by centrifugation for 30 s at 11000 g (rapid protocol). The purity and concentration of DNA was again assessed using the NanoDrop 1000 spectrophotometer.

2.5. Biotinylation of bovine serum albumin (BSA)

For the biotinylation of BSA, 200 μL of 1 g L^{-1} BSA (0.2 mg) were mixed with 50 μL of freshly prepared 0.5 M $NaHCO_3$ (pH 9.1). Then, 1 mg of EZ-Link Sulfo-NHS-LC-biotin was added to the final reaction mixture. After a 1-h incubation in dark, the biotinylated BSA was ready to be used at a final concentration of 0.8 mg mL^{-1}.

2.6. Synthesis of poly(dT)-functionalized polystyrene microspheres

An aliquot of 12.8 μL from the stock solution of carboxylated polystyrene microspheres was added to 125 μL of MES buffer, pH 4.5. A 2-min centrifugation step at 15700 g was followed, and the supernatant was removed. The microspheres were resuspended in 40 μL MES buffer by vortex-mixing and sonication for 2 min. Then, 1 μL of 400 pmol μL^{-1} of NH_2-$dT_{(30)}$ oligonucleotide and 1.25 μL of 0.4 mg μL^{-1} of fresh EDC solution were added to the microspheres' solution. The reaction mixture was incubated for 30 min in the dark and with frequent stirring, while another aliquot of 1.25 μL of freshly prepared EDC solution was added at the end of the incubation step and the mixture was let for another 30-min period as above. Then, 2 μL of a Tween-20 solution (10% v/v) were added and the reaction mixture was centrifuged for 2 min at 15700 g. The supernatant was discarded, and the functionalized microspheres were washed twice with 100 μL of 1 × TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and 2 μL of a 10% Tween-20 solution. After each washing step, a 2-min centrifugation at 15700*g* was performed. Finally, the functionalized microspheres were resuspended in 100 μL of 1 × TE buffer pH 8.0.

2.7. Preparation of streptavidin-modified gold nanoparticles (SA-AuNPs)

A volume of 200 μL of 50 mg L^{-1} (particle concentration 7.7 × 10^{10} mL^{-1}) gold nanoparticles (10 μg) was used for the conjugation reaction. Firstly, the pH of the AuNPs solution was adjusted to 6.0 with 10 mM phosphate buffer (pH 6.8). Then, 1.5 μL of 1 g L^{-1} streptavidin (1.5 μg), diluted in H_2O, was added to AuNPs and a 2-h incubation step followed at room temperature and in the dark. The reaction mixture was regularly stirred during incubation, followed by addition of BSA diluted in H_2O at a final concentration of 10 g L^{-1} as blocking agent. The mixture was incubated for 30 min at room temperature. The SA-functionalized AuNPs were precipitated by centrifugation at 3300*g* for 20 min and finally reconstituted in 20 μL of proper storage buffer (1 × PBS, 1% BSA, 0.25% sucrose, 1% Tween-20, 0.05% NaN_3). The conjugate was finally stored at 4 °C for further use.

2.8. Construction of the lateral flow strip assay

The DNA-based strip had a size of 4 mm × 70 mm and is consisted of an immersion pad, a conjugate pad, a nitrocellulose membrane, an absorbent pad and under these parts a plastic adhesive surface to provide a stable support. All the pads and the membrane were placed on the plastic surface with overlapping ends, in order to ensure the appropriate capillary flow of the running buffer through the strip (Fig. 1). The construction of the control and the test zone on the membrane of the strip was performed as follows: a volume of 0.5 μL of 50 ng/μL of biotinylated BSA and a volume of 1 μL of the poly(dT)-functionalized microspheres were deposited onto the membrane of the strip to form a control and a test spot, respectively. The membrane was then let to dry at ambient temperature for 10–15 min before use.

2.9. Polymerase chain reaction (PCR)

PCR was performed in a gradient PCR Thermal Cycler at a final volume of 25 μL for the amplification of exon 12 of the KRAS gene. The reaction mixture contained 1 × KAPA 2G FAST Master Mix (supplemented with 1.5 mM $MgCl_2$), 0.6 μM of the upstream and downstream KRAS primers and about 100 ng of isolated DNA. The PCR parameters were as follows: an initial denaturation step at 95 °C for 3 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and a final extension step of the primers at 72 °C for 10 min. For the determination of the PCR products a 2% agarose gel electrophoresis with ethidium bromide staining were used. The quantification was carried out with densitometric analysis using the open-source ImageJ image processing and gel analyzer tool by National Institutes of Health (NIH) (Bethesda, Maryland, USA) and Laboratory for Optical and Computational Instrumentation, University of Wisconsin (Wisconsin, USA).

2.10. Primer extension reaction (PEXT)

The amplified DNA products were subjected to four separate primer extension (PEXT) reactions using four different PEXT primers specific to the normal gene and the three KRAS mutations. The reaction mixture, with a final volume of 20 μL, contained 1 × Thermopol buffer (20 mM Tris-HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100), 2.5 μM each of dATP, dTTP and dGTP, 2.5 μM of biotin-dCTP, 0.05 μM of the normal or mutants PEXT primers, 0.5 U of Vent(exo-) DNA polymerase and finally 200–400 fmol of the target DNA. The conditions of the PEXT reactions for the synthetic DNA targets were: a denaturation step at 95 °C for 3 min, and then 3 cycles of 95 °C for 15 s, 62 °C for 10 s and 72 °C for 15 s, while 400 fmol of each synthetic target was used in

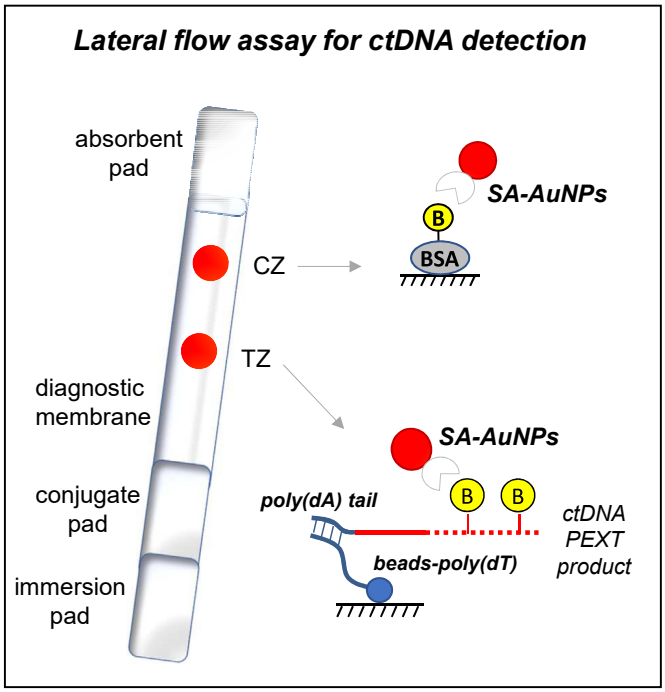


Fig. 1. Principle of the lateral flow assay. TZ, test zone; CZ, control zone; SA, streptavidin; AuNPs, gold nanoparticles; B, biotin; BSA, bovine serum albumin.

the reaction. The amount of 400 fmol of the DNA isolated from the cell line was also used in PEXT reactions with the following conditions: a denaturation step at 95 °C for 3 min, and then 10 cycles of 95 °C for 15 s, 58 °C for 10 s and 72 °C for 15 s. As for cell-free DNA isolated from the blood samples, 200 fmol were added to the reaction pool, while the PEXT parameters were the same as for the cell lines, but 15 cycles were required for a positive result. Finally, the PEXT products were denatured at 95 °C for 3 min and then incubated on ice for 2 min, just before their application onto the AuNPs-based strip test.

2.11. Gold nanoparticle-based lateral flow assay

For the performance of the lateral flow assay, 3 μL of streptavidin-functionalized gold nanoparticles (SA-AuNPs) and 5 μL of the denatured PEXT product were applied onto the conjugate pad of the strip. The strip was then immersed into 300 μL of the running buffer (4x SSC, 1% glycerol, 1% Tween, 1% BSA, 0.5% SDS). After 10 min, the strip was removed from the running buffer. The images of the strips were finally obtained using a conventional scanner.

3. Results

In the present work, we developed a gold nanoparticle-based DNA lateral flow assay to detect single KRAS mutations in cell-free DNA for non-invasive liquid biopsy applications. The proposed lateral flow assay was developed for the rapid visual detection of the normal allele and three major single-point mutations of the KRAS gene, that are located in exon 12: G12D (35G > A), G12V (35G > T), G12A (35G > C), in cell-free DNA from blood samples. The method was initially optimized using four synthetic DNA targets that contained the four analyzed SNPs (Table 1), as well as using DNA extracted from cancer cell lines carrying the normal and the mutant G12D KRAS allele. The strip test was finally applied for analysis of cell-free DNA in plasma from healthy individuals and CRC patients with known G12D KRAS mutations.

3.1. Principle of the method

Each DNA target obtained from the synthetic targets, the cell lines and the blood samples was amplified by PCR using KRAS-specific primers for the exon-12 region. The PCR products had a size of 171 bp and were quantified by agarose gel electrophoresis and ethidium bromide staining. Each PCR product was then subjected to four separate PEXT reactions using PEXT primers specific to the four SNPs examined: the normal allele and three single-point mutations of the KRAS gene. All PEXT primers had the same sequence with a difference in the base at the 3′ end, where the analyzed SNPs are located and were tagged with a poly-adenine tail at the 5′ end to enable the detection with the lateral flow assay. The DNA polymerase used in PEXT reaction lacks the 3′-5′ proofreading exonuclease activity, so that the primers are extended by the polymerase only if they are full-complementary to the target sequence. During the extension of the primers, biotin molecules were incorporated in the PEXT product, by using biotin-dCTP, for their detection by the SA-AuNPs conjugates (Fig. 1).

For the preparation of the diagnostic strip test, biotin molecules were immobilized on the nitrocellulose membrane using b-BSA conjugates. The deposition of the b-BSA on the membrane represented the control zone (CZ) of the strip, where the excess of SA-AuNPs is bound through biotin-streptavidin interactions. The CZ must be always visible as it ensures that the lateral flow assay works properly. For the test zone (TZ) of the strip, $NH_2dT_{(30)}$-beads were also pre-deposited on the membrane in order to capture the DNA sequences of interest. The PEXT products were denatured just before their application onto the strip. A 5-μL aliquot of the denatured products and 3 μL of the SA-AuNPs conjugates were deposited on the conjugate pad of the strip. The strip was then immersed into the running buffer for 10 min. The running buffer entrained the reagents upwards. The PEXT products were captured at the TZ of the biosensor by the immobilized poly(dT) sequences through hybridization to the poly(dA) tail of the PEXT primers. SA-AuNPs were also accumulated at the same zone through streptavidin-biotin interactions with the biotinylated PEXT products, forming a visual red spot. The appearance of a red spot at the TZ denoted the presence of the specific SNP examined, while the excess of the SA-AuNPs formed a second red spot at the CZ of the strip by binding to the immobilized b-BSA, ensuring the strip's proper function. The detection was accomplished by naked eye within 10 min.

3.2. Analysis of the synthetic DNA targets with the lateral flow assay

The method was initially developed and all conditions were optimized using synthetic DNA targets that contained the analyzed SNPs. The optimization studies aimed to signal improvement by eliminating any non-specific binding at the TZ of the biosensor. The parameters studied included the protocol for cell-free DNA isolation, the conditions of the coupling reaction of the protein streptavidin to gold nanoparticles, the reconstitution buffer of the SA-AuNPs conjugates, the amount of reagents and DNA target, the number of cycles and the annealing temperature of the PEXT reactions, as well as the composition of the running buffer of the lateral flow assay. Each synthetic DNA target was subjected to four different 3-cycle PEXT reactions using the PEXT primers for each SNP. The annealing temperature was set at 62 °C. Each PEXT product was then analyzed separately with the lateral flow assay. A red spot was formed at the TZ of the strip, thus a PEXT product was produced, only if the PEXT primer was fully complementary to the synthetic DNA target. The images of the strip for the normal gene, as well as for the three single-point mutations of the KRAS gene are shown in Fig. 2. We noticed that each PEXT product gave a positive result with the strip test, only with its corresponding PEXT primer, ensuring the specificity of the method.

3.3. Analysis of the DNA extracted from cell lines with the lateral flow assay

Optimization experiments were also performed using DNA extracted from cell lines carrying the normal (Caco2) or the G12D mutant (LS174T and CCRF-CEM) KRAS gene allele. Isolated DNA from each cell line underwent four separate PEXT reactions with the four different PEXT primers. High signal color intensity at the test zone of the strip was obtained by a 10-cycle PEXT reaction with an annealing temperature at 58 °C. Differences in the experimental conditions between synthetic DNA and DNA from cell lines are attributed to the fact that the synthetic targets are single-stranded small DNA sequences showing more efficient hybridization albeit with more intense non-specific interactions compared to the double-stranded genomic material isolated from the cell lines. As presented in Fig. 3, red spots were only formed at the TZ of the strip in case of the normal allele for the wild-type KRAS cell line (Caco2) and both the normal allele and the G12D KRAS mutation for the LS174T and CCRF-CEM cell lines as expected, highlighting the good specificity of the lateral flow assay.

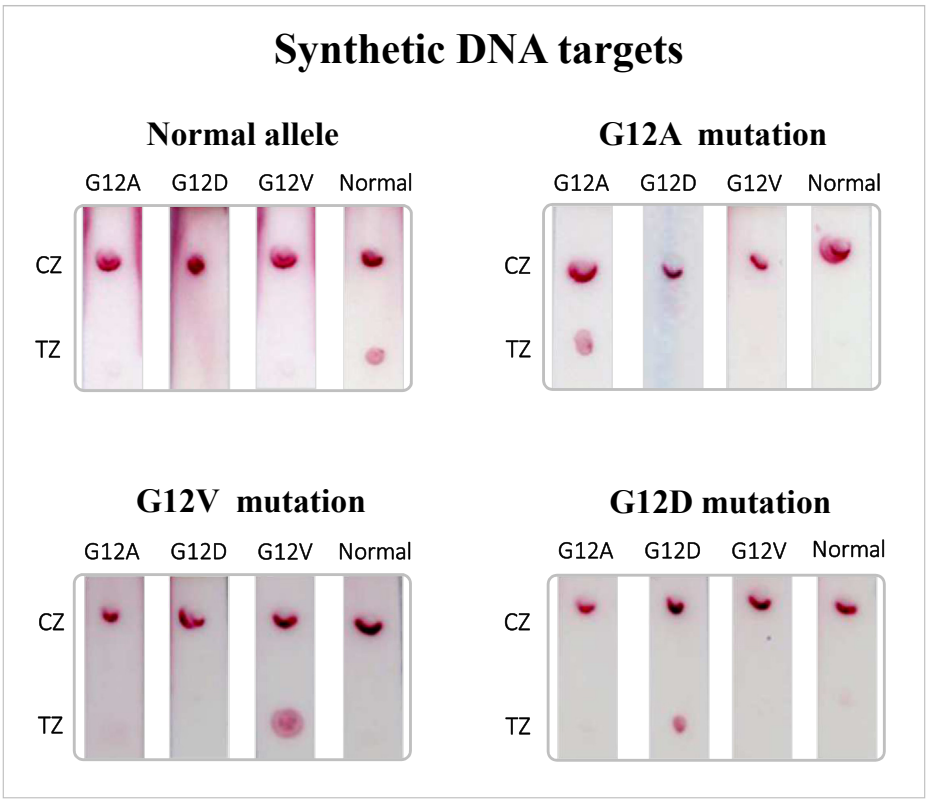


Fig. 2. Analysis of the synthetic DNA targets, that contain the SNP of interest, with the lateral flow assay. Each DNA target was subjected to four separate PEXT reactions using the four different PEXT primers specific to the normal allele of the KRAS gene and the G12A, G12D and G12V KRAS gene mutations. The PEXT products were then analyzed with four different strips that corresponded to the normal allele and the G12A, G12D and G12V KRAS gene mutations. TZ, test zone; CZ, control zone.

3.4. Analysis of cell-free/circulating tumor DNA in blood samples with the lateral flow assay

Following optimization studies, blood samples from three healthy individuals and three CRC patients that carried the G12D KRAS mutation were analyzed by the lateral flow strip assay. Cell-free DNA was firstly isolated from plasma and then subjected again to four PEXT reactions as described previously. An amount of 200 fmol of the isolated cfDNA was used for the PEXT reaction. As the mutated ctDNA is present in very low amounts in the blood circulation compared to the cfDNA carrying the normal KRAS allele, a number of 15 cycles of the PEXT reaction was required to get a positive outcome. The annealing temperature was also set at 58 °C. The PEXT products were then heat-denatured and applied onto the strip. Analysis of healthy donors' samples resulted in a red spot appearing at the TZ of the strip only for the normal allele. On the other hand, analysis of patients' samples resulted in a red spot that was formed at the TZ of both strips that corresponded to the normal allele and to the G12D KRAS mutation, as expected. Regarding patients' samples, it was also observed that the density of the color of the test spot for the G12D mutation was lower than that of the test spot for the normal allele consistent with the lower concentration of mutated ctDNA compared to non-tumor derived cfDNA in the circulation. Images of cfDNA analysis by the lateral flow assay are presented in Fig. 4. The results of the analysis are also presented in Table S4.

3.5. Reproducibility

The reproducibility of the developed method, regarding the PEXT reaction and the performance of the lateral flow assay, was finally assessed and expressed as coefficient of variation (%CV) values. For this study, DNA isolated from cell line Caco2 carrying the normal KRAS allele was selected. The DNA sample (400 fmol) was subjected to PEXT reaction for eight times using its corresponding PEXT primer that targets the normal allele. The PEXT products were then analyzed with eight different strips. Also, the PEXT product of a CRC patient that carried the G12D mutation was analyzed in triplicate with the lateral flow assay for the normal allele and the G12D mutation. The strips where then scanned, and the density of the red spots formed at the TZ of the sensor was determined using the open-source image processing software ImageJ. Finally, the % CVs calculated were 3.0% (n = 8) for the cell line and 3.3% (n = 3) for the normal allele and 4.9% (n = 3) for the G12D mutation of the cell-free DNA from the CRC patient, proving the very good reproducibility of the method and the lateral flow assay. The reproducibility results are presented in Fig. 5.

4. Discussion

Compared to the conventional methods, biosensors are excellent analytical tools for ctDNA screening and detection due to their high detectability/sensitivity and specificity in complex samples, rapid readout, cost-effectiveness, increased multiplicity, portability and ease of use. Due to their unique characteristics, biosensors have rapidly been emerged as promising tools in ctDNA analysis and liquid biopsy [41]. On the other hand, the use of nanomaterials in biosensing has received wide attention by scientists as a promising alternative to traditional techniques mainly due to their contribution to non-enzymatic signal enhancement [42]. However, current nanomaterial-based approaches for ctDNA sensing are still in their early stage, facing many drawbacks in terms of detectability and

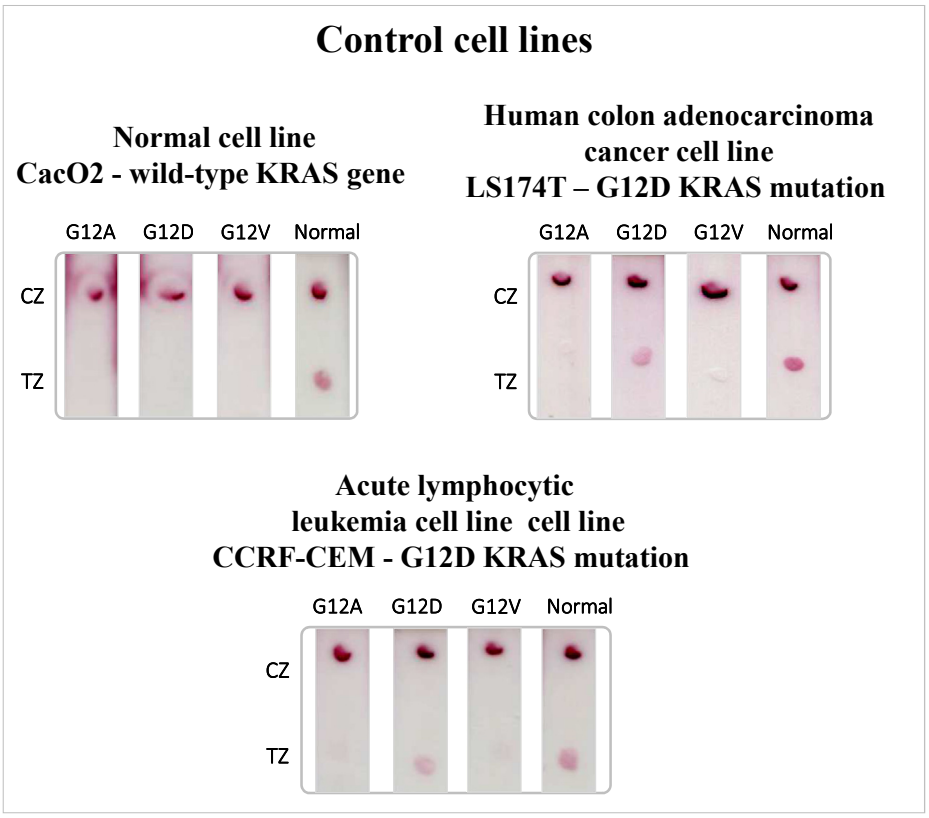


Fig. 3. Analysis of the control cell lines with the lateral flow assay for the wild-type (normal allele) of the KRAS gene (CacO2) and the cancer cell lines (LS174T and CCRF-CEM) that express both the normal allele and the G12D KRAS mutation. Each isolated DNA from the cell lines was subjected to four separate PEXT reactions and analyzed with four different strips that correspond to the normal allele and the G12A, G12D and G12V KRAS gene mutations. TZ, test zone; CZ, control zone.

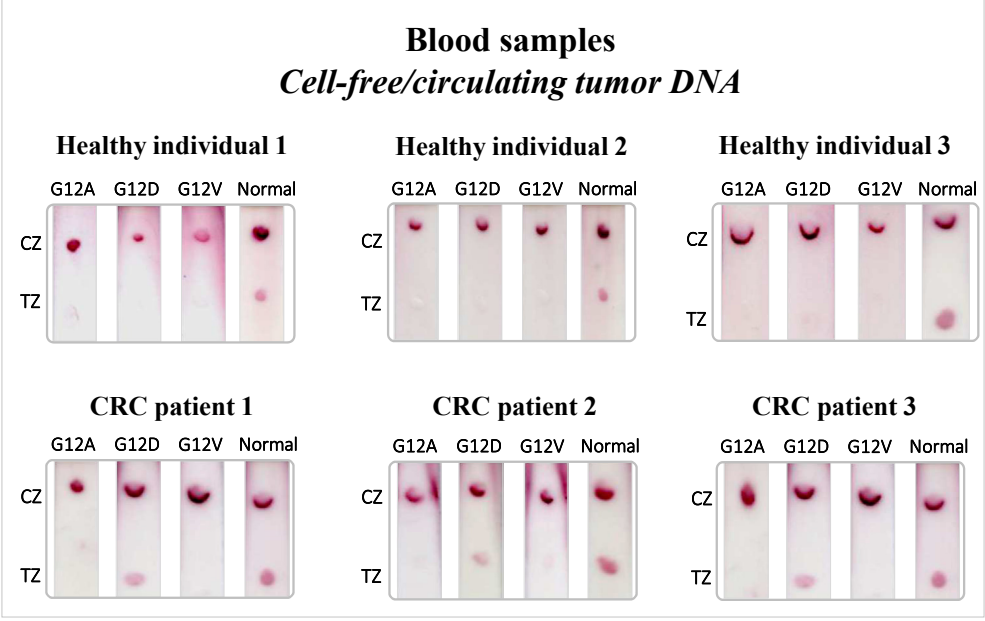


Fig. 4. Analysis of clinical blood samples and cell-free DNA of three healthy individuals and three CRC patients with known G12D KRAS mutation. Each isolated cell-free DNA from the blood samples was subjected to four separate PEXT reactions and analyzed with four different strips that correspond to the normal allele and the G12A, G12D and G12V KRAS gene mutations. TZ, test zone; CZ, control zone; CRC, colorectal cancer.

specificity. So, new nanotechnology-driven strategies have to be developed for clinical practices. Although many electrochemical, colorimetric, fluorescent, surface plasmon resonance (SPR) and surface-enhanced Raman spectroscopy (SERS) biosensors have been reported for ctDNA analysis, their evaluation is limited to spiking experiments using single-stranded synthetic

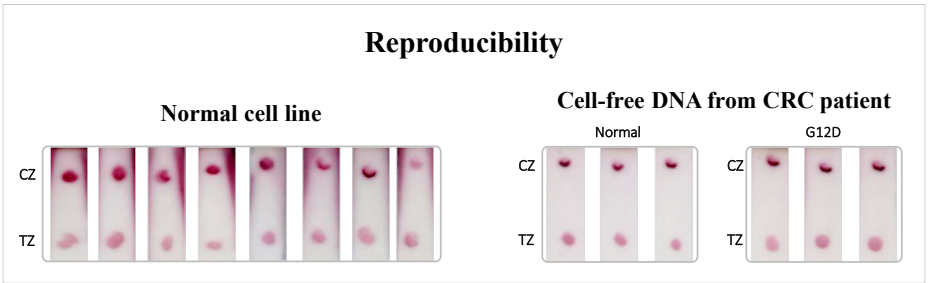


Fig. 5. Reproducibility study. The DNA of the wild-type KRAS gene cell line was subjected to PEXT reaction and analyzed with the lateral flow assay for 8 repeated times using the PEXT primer specific to the normal allele. Also, the cell-free DNA of a CRC patient was analyzed in triplicate with the lateral flow assay for the normal allele and the G12D mutation. TZ, test zone; CZ, control zone.

oligonucleotide targets [23–40]. Until now, there are only few reports of novel biosensors that have been applied for genotyping of ctDNA isolated from body fluid samples. Specifically, Wang et al., 2020 developed a tetrahedral DNA nanostructure-decorated electrochemical biosensor for EGFR genotyping in ctDNA from plasma samples. ctDNA was amplified by amplification refractory mutation system (ARMS)-based linear-after-the-exponential-PCR to obtain asymmetric biotinylated PCR products that were then detected by the biosensor through avidin-HRP (horseradish peroxidase) conjugates. Two-single point mutations of the EGFR gene were detected by the proposed biosensor [43]. A novel electrochemical biosensor was also developed by Huang et al., 2020 for PIK3CA E545K mutation detection in ctDNA. This biosensor was based on hybridization chain reaction (HCR) on a gold electrode's surface for signal enhancement, while the HCR products were detected through a biotin-streptavidin sensing system using streptavidin-alkaline phosphatase [44]. Another electrochemical biosensor was grounded on urchin-like gold nanocrystal-multiple graphene aerogel and target DNA-induced recycling for ctDNA analysis allowing signal enhancement [45]. Das et al. (2019 and 2016) reported new electrochemical sensors that capture ctDNA through specific immobilized PNA probes onto the electrode's surface. The detection was based on the electroactive compounds $[Ru(NH_3)_6]^{3+}$ and $[Fe(CN)_6]^{3-}$ that were electrostatically bound to nucleic acids sequences, as well as DNA clutch probes and PNA clamps to enhance the hybridization efficiency and specificity. The biosensor offered high specificity and increased multiplexing potential [46,47]. A dual electrochemical biosensor was also developed for the detection of tumor-specific mutation and epigenetic methylation of ctDNA. The use of PNA probes conjugated to gold nanoparticles enabled the capture of two mutated ctDNAs of PIK3CA gene and led to increased electrical signal. Also, lead phosphate apoferritin conjugated to anti-5-mC recognized the methylation sites of ctDNA. The sandwich complex formed was finally detected by square-wave voltammetry and the lead ions released from apoferritin [48]. A dual enzyme-assisted electrochemical sensor was also constructed for signal amplification and ctDNA analysis. The sensor was based on target recycling through RNase HII, while terminal transferase was used to produce DNA dendritic nanostructures for signal enhancement. The electroactive compound methylene blue was finally used as reporter and the sensor offered very good detectability [49]. SERS has also been exploited for ctDNA analysis. An allele-specific PCR-based SERS assay was developed for multiplex detection of ctDNA using multicolor SERS nanotags (AuNPs). The PCR products were labelled with biotin at one end and captured through streptavidin-coated magnetic beads, while overhanging ends at the other end were used to allow binding to the complementary oligonucleotides coupled to the SERS tags. After magnetic separation, the complexes were analyzed with SERS spectrum by a portable Raman microscope [50,51]. In another SERS assay, positively charged gold/silver nanostars were exploited for the detection of allele-specific PCR-amplified ctDNA through electrostatic interactions with the negatively charged DNA backbones. The SERS measurements were also performed in the same portable Raman microscope [52]. Single-walled carbon nanotubes and copper nanoparticles were also used for the SERS-based detection of single-stranded ctDNA in blood samples. Target recycling was here also performed using RNase HII [53]. Finally, an SPR sensor was developed for the detection of 11 single nucleotide variants in circulating RAS mutated DNA in plasma samples from colorectal cancer patients. The wild-type and a mutated allele of the KRAS and NRAS genes were detected simultaneously in two parallel microfluidic channels of the sensor. The great advantage of this sensor lies in the fact that it avoids the ctDNA extraction and PCR amplification steps, while gold nanoparticles were used for signal enhancement [54]. Advantages, disadvantages and details for the analytical performance of the above biosensors and the proposed lateral flow strip assay are presented in Supplementary Table 1 (Table S1).

Compared to the above biosensors used for ctDNA analysis, lateral flow hybridization-based tests also represent good candidates for liquid biopsy applications in terms of rapidness and simplicity, along with high detectability and reproducibility [55,56]. Although many lateral flow assays have been developed for microRNA detection, there are no reports regarding their use in ctDNA analysis. A lateral flow assay that was based on oligonucleotides-functionalized gold nanoparticles and allele-specific PCR was previously reported for the detection of six KRAS mutations in genomic DNA isolated from cancer cell lines [57].

Herein, we have developed a new rapid lateral flow assay, exploiting gold nanoparticles as visual reporters, for KRAS mutation detection on ctDNA of CRC patients. Compared to the aforementioned reported biosensors for ctDNA analysis in clinical samples, as shown in Table S1 of the Supplementary Material, this lateral flow test is significantly superior in the terms of simplicity, visual detection, rapid analysis time, short time for biosensor's fabrication and limited steps for the analysis (as no washing and incubation steps are acquired) along with high specificity, the ease of use and the multiplexing and universality potential.

5. Conclusions

In conclusion, we developed a rapid gold nanoparticle-based lateral flow assay that was for the first time exploited to detect single KRAS mutations in cell-free DNA for non-invasive liquid biopsy applications. The strip test was applied for the detection of three major single-point mutations of the KRAS gene in cell-free circulating tumor DNA. The proposed lateral flow assay offered high detectability, as the very low amount of the ctDNA was easily detectable by the strip test, good specificity and good

reproducibility (%CVs ~ 3–5%). Detection of the KRAS mutations was accomplished by bare eye and completed within 10 min after the application onto the strip. The whole protocol (DNA isolation, PCR, PEXT and analysis with the strip test) had an analysis time of about 3–3.5 h. The rapidly obtained results of high sensitivity, specificity, reproducibility, simplicity, along with low cost, render this strip test a very promising minimally invasive tool for the detection of KRAS mutations in CRC patients with important clinical applications in early diagnosis, prognosis and treatment tailoring [11,12]. Moreover, considering that the allele discrimination reaction is accomplished prior to the application to the strip and the reaction's products are detected based on dA/dT hybridization, the proposed DNA lateral flow assay may be used as a universal targeted approach of ctDNA analysis, meaning that it can be applied for the detection of any SNP or single-point mutation of any gene of interest. The later advantage along with the potential to increase the number of mutations and genes analyzed strongly support the promising future use of this lateral flow assay in many liquid biopsy applications.

CRediT authorship contribution statement

Panagiota Kalligosfyri: Methodology, Validation, Formal analysis, Investigation, Visualization, Writing – original draft. **Sofia Nikou:** Methodology, Formal analysis, Investigation, Resources, Writing – original draft. **Vasiliki Bravou:** Conceptualization, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing. **Despina P. Kalogianni:** Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research is co-financed by Greece and the European Union (European Social Fund - ESF) through the Operational Programme « Human Resources Development, Education and Lifelong Learning 2014–2020» in the context of the project "Gold nanoparticle-based biosensor for rapid liquid biopsy applications" (MIS 5047147).

We also acknowledge Professor Kalofonos Haralabos and Karteri Sofia (Division of Oncology, Department of Medicine, University Hospital, University of Patras, Patras, Greece) for providing blood samples from CRC patients.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2021.338470.

References

[1] F. Meric-Bernstam, G.B. Mills, Overcoming implementation challenges of personalized cancer therapy, Nat. Rev. Clin. Oncol. 9 (2012) 542–548, https://doi.org/10.1038/nrclinonc.2012.127.
[2] E. Dekker, P. Tanis, J. Vleugels, P.I. Kas, M. Wallace, Colorectal cancer, Lancet 394 (2018) 1467–1480, https://doi.org/10.1016/S0140-6736(19)32319-0.
[3] C. Tan, X. Du, KRAS mutation testing in metastatic colorectal cancer World J Gastroenterol, World J. Gastroenterol. 18 (2012) 5171–5180. https://dx.doi.org/10.3748/wjg.v18.i37.5171.
[4] E. Van Cutsem, A. Cervantes, R. Adam, A. Sobrero, J.H. Van Krieken, D. Aderka, E. Aranda Aguilar, A. Bardelli, A. Benson, G. Bodoky, F. Ciardiello, A. D'Hoore, E. Diaz-Rubio, J.Y. Douillard, M. Ducreux, A. Falcone, A. Grothey, T. Gruenberger, et al., ESMO consensus guidelines for the management of patients with metastatic colorectal cancer, Ann. Oncol. 27 (2016) 1386–1422, https://doi.org/10.1093/annonc/mdw235.
[5] M. Gerlinger, A.J. Rowan, S. Horswell, J. Larkin, D. Endesfelder, E. Gronroos, P. Martinez, N. Matthews, A. Stewart, P. Tarpey, I. Varela, B. Phillimore, et al., Intratumor heterogeneity and branched evolution revealed by multiregion sequencing, N. Engl. J. Med. 366 (2012) 883–892, https://doi.org/10.1056/NEJMoa1113205.
[6] M. Kleppe, R.L. Levine, Tumor heterogeneity confounds and illuminates: assessing and illuminates: assessing the implications, Nat. Med. 20 (2014) 342–344, https://doi.org/10.1038/nm.3522.
[7] E. Heitzer, P. Ulz, J.B. Geigl, Circulating tumor DNA as a liquid biopsy for cancer, Clin. Chem. 61 (2015) 112–123, https://doi.org/10.1373/clinchem.2014.222679.
[8] E. Heitzer, I.S. Haque, C.E.S. Roberts, M.R. Speicher, Current and future perspectives of liquid biopsies in genomics-driven oncology, Nat. Rev. Genet. 20 (2019) 71–88, https://doi.org/10.1038/s41576-018-0071-5.
[9] C. Alix-Panabières, K. Pantel, Clinical applications of circulating tumor cells and circulating tumor DNA as liquid biopsy, Canc. Discov. 6 (2016) 479–491, https://doi.org/10.1158/2159-8290.
[10] L. Keller, Y. Belloum, H. Wikman, K. Pantel, Clinical relevance of blood-based ctDNA analysis: mutation detection and beyond, Br. J. Canc. (2020) 1–14, https://doi.org/10.1038/s41416-020-01047-5.
[11] V. Vymetalkova, K. Cervena, L. Bartu, P. Vodicka, Circulating cell-free DNA and colorectal cancer: a systematic review, Int. J. Mol. Sci. 19 (2018) 3356, https://doi.org/10.3390/ijms19113356.
[12] H. Osumi, E. Shinozaki, K. Yamaguchi, H. Zembutsu, Clinical utility of circulating tumor DNA for colorectal cancer, Canc. Sci. 110 (2019) 1148–1155. https://dx.doi.org/10.1111/cas.13972.
[13] V. Kloten, N. Rüchel, N.O. Brüchle, J. Gasthaus, N. Freudenmacher, F. Steib, J. Mijnes, J. Eschenbruch, M. Binnebösel, R. Knüchel, E. Dahl, Liquid biopsy in colon cancer: comparison of different circulating DNA extraction systems following absolute quantification of KRAS mutations using Intplex allele-specific PCR, Oncotarget 8 (2017) 86253–86263. https://dx.doi.org/10.18632/oncotarget.21134.
[14] I.A. Cree, L. Uttley, H.B. Woods, H. Kikuchi, A. Reiman, S. Harnan, B.L. Whiteman, S.T. Philips, M. Messenger, A. Cox, D. Teare, O. Sheils, J. Shaw, The evidence base for circulating tumour DNA blood-based biomarkers for the early detection of cancer: a systematic mapping review, BMC Canc. 17 (2017) 697–714, https://doi.org/10.1186/s12885-017-3693-7.
[15] L. Giannopoulou, S. Kasimir-Bauer, E.S. Lianidou, Liquid biopsy in ovarian cancer: recent advances on circulating tumor cells and circulating tumor DNA, Clin. Chem. Lab. Med. 58 (2017) 186–197, https://doi.org/10.1515/cclm-2017-0019.
[16] Y. Chang, B. Tolani, X. Nie, X. Zhi, M. Hu, B. He, Review of the clinical applications and technological advances of circulating tumor DNA in cancer monitoring, Clin. Risk. Manag. 13 (2017) 1363–1374. https://dx.doi.org/10.2147/TCRM.S141991.
[17] A. Lopez, K. Harada, D.M. Kaya, X. Dong, S. Song, J.A. Ajani, Liquid biopsies in gastrointestinal malignancies: when is the big day? Expert Rev. Anticancer Ther. 18 (2018) 19–38, https://doi.org/10.1080/14737140.2018.1403320.
[18] X. Han, J. Wang, Y. Sun, Circulating tumor DNA as biomarkers for cancer detection, Genomics Proteomics Bioinf. 15 (2017) 59–72, https://doi.org/10.1016/j.gpb.2016.12.004.
[19] J. Wang, A. Wuethrich, A.A.I. Sina, R.E. Lane, L.L. Lin, Y. Wang, J. Cebon, A. Behren, M. Trau, Tracking extracellular vesicle phenotypic changes enables treatment monitoring in melanoma, Sci. Adv. 6 (2020), https://doi.org/10.1126/sciadv.aax3223 eaax3223.
[20] J. Wang, K.M. Koo, Y. Wang, M. Trau, Engineering state-of-the-art plasmonic nanomaterials for SERS-based clinical liquid biopsy applications, Adv. Sci. 6 (2019) 1900730, https://doi.org/10.1002/advs.201900730.
[21] J. Das, S.O. Kelley, High-performance nucleic acid sensors for liquid biopsy applications, Angew. Chem. Int. Ed. 59 (2020) 2554–2564, https://doi.org/10.1002/anie.201905005.
[22] X. Chen, L. Wang, J. Lou, Nanotechnology strategies for the analysis of circulating tumor DNA: a review, Med. Sci. Mon. Int. Med. J. Exp. Clin. Res. 26 (2020) 1–9, https://doi.org/10.12659/MSM.921040.
[23] M. Chen, D. Wu, S. Tu, C. Yang, D.J. Chen, Y. Xu, CRISPR/Cas9 cleavage triggered ESDR for circulating tumor DNA detection based on a 3D graphene/AuPtPd nanoflower biosensor, Biosens. Bioelectron. 173 (2021) 112821, https://doi.org/10.1016/j.bios.2020.112821.
[24] Q. Zhou, J. Zheng, Z. Qing, M. Zheng, J. Yang, S. Yang, L. Ying, R. Yang, Detection of circulating tumor DNA in human blood via DNA-mediated Surface-Enhanced Raman Spectroscopy of single-walled carbon nanotubes, Anal. Chem. 88 (2016) 4759–4765, https://doi.org/10.1021/acs.analchem.6b00108.
[25] L. Zhou, C. Wang, Q. Liu, Z. Teng, Z. Dai Wang, Quantification of cyclic DNA polymerization with lanthanide coordination nanomaterials for liquid biopsy, Chem. Sci. 11 (2020) 3745–3751, https://doi.org/10.1039/c9sc06408g.
[26] X. Chen, Y. Tang, R. Yan, P. Miao, Ultrasensitive detection of ctDNA by target-mediated in situ growth of DNA three-way junction on the electrode, ChemElectroChem 7 (2020) 64–68, https://doi.org/10.1002/celc.201901657.
[27] C. Huang, S. Hu, X. Zhang, H. Cui, L. Wu, N. Yang, W. Zhou, P.K. Chu, X.F. Yu, Sensitive and selective ctDNA detection based on functionalized black phosphorus nanosheets, Biosens. Bioelectron. 165 (2020) 112384, https://doi.org/

10.1016/j.bios.2020.112384.
[28] D. Li, Y. Xu, L. Fan, B. Shen, X. Ding, R. Yuan, X. Li, W. Chen, Target-driven rolling walker based electrochemical biosensor for ultrasensitive detection of circulating tumor DNA using doxorubicin@tetrahedron-Au tags, Biosens. Bioelectron. 148 (2020) 111826, https://doi.org/10.1016/j.bios.2019.111826.
[29] G. Liu, X. Ma, Y. Tang, P. Miao, Ratiometric fluorescence method for ctDNA analysis based on the construction of a DNA four-way junction, Analyst 145 (2020) 1174–1178, https://doi.org/10.1039/d0an00044b.
[30] M. Rahman, D. Cui, S. Zhou, A. Zhang, D. Chen, A graphene oxide coated gold nanostar based sensing platform for ultrasensitive electrochemical detection of circulating tumor DNA, Anal. Methods. 12 (2020) 440–447, https://doi.org/10.1039/c9ay01620a.
[31] Z.O. Uygun, L. Yeniay, F. Gïrgïn Sağın, CRISPR-dCas9 powered impedimetric biosensor for label-free detection of circulating tumor DNAs, Anal. Chim. Acta 1121 (2020) 35–41, https://doi.org/10.1016/j.aca.2020.04.009.
[32] X. Mao, S. Pan, D. Zhou, X. He, Y. Zhang, Fabrication of DNAzyme-functionalized hydrogel and its application for visible detection of circulating tumor DNA, Sensor. Actuator. B Chem. 285 (2019) 385–390, https://doi.org/10.1016/j.snb.2019.01.076.
[33] A. Tadimety, Y. Zhang, K.M. Kready, T.J. Palinski, G.J. Tsongalis, J.X.J. Zhang, Design of peptide nucleic acid probes on plasmonic gold nanorods for detection of circulating tumor DNA point mutations, Biosens. Bioelectron. 130 (2019) 236–244, https://doi.org/10.1016/j.bios.2019.01.045.
[34] J. Zhang, Y. Dong, W. Zhu, D. Xie, Y. Zhao, D. Yang, M. Li, Ultrasensitive detection of circulating tumor DNA of lung cancer via an enzymatically amplified SERS-based frequency shift assay, ACS Appl. Mater. Interfaces 11 (2019) 18145–18152, https://doi.org/10.1021/acsami.9b02953.
[35] X. Zhai, J. Li, Y. Cao, X. Zhu, Y. Tang, G. Chen, K. Han, A nanoflow cytometric strategy for sensitive ctDNA detection via magnetic separation and DNA self-assembly, Anal. Bioanal. Chem. 411 (2019) 6039–6047, https://doi.org/10.1007/s00216-019-01985-x.
[36] W. Zhang, Z. Dai, X. Liu, J. Yang, High-performance electrochemical sensing of circulating tumor DNA in peripheral blood based on poly-xanthurenic acid functionalized MoS2 nanosheets, Biosens. Bioelectron. 105 (2018) 116–120, https://doi.org/10.1016/j.bios.2018.01.038.
[37] T. Tian, H. Liu, L. Li, J. Yu, S. Ge, X. Song, M. Yan, Paper-based biosensor for noninvasive detection of epidermal growth factor receptor mutations in non-small cell lung cancer patients, Sensor. Actuator. B Chem. 251 (2017) 440–445, https://doi.org/10.1016/j.snb.2017.05.082.
[38] R. Li, L. Zou, Y. Luo, M. Zhang, L. Ling, Ultrasensitive colorimetric detection of circulating tumor DNA using hybridization chain reaction and the pivot of triplex DNA, Sci. Rep. 7 (2017) 1–10, https://doi.org/10.1038/srep44212.
[39] Y. Chu, B. Cai, Y. Ma, M. Zhao, Z. Ye, J. Huang, Highly sensitive electrochemical detection of circulating tumor DNA based on thin-layer MoS2/graphene composites, RSC Adv. 6 (2016) 22673–22678, https://doi.org/10.1039/C5RA27625J.
[40] A.H. Nguyen, S.J. Sim, Nanoplasmonic biosensor: detection and amplification of dual bio-signatures of circulating tumor DNA, Biosens. Bioelectron. 67 (2015) 443–449, https://doi.org/10.1016/j.bios.2014.09.003.
[41] X. Li, M. Ye, W. Zhang, D. Tan, N. Jaffrezic-Renault, X. Yang, Z. Guo, Liquid biopsy of circulating tumor DNA and biosensor applications, Biosens. Bioelectron. 126 (2019) 596–607, https://doi.org/10.1016/j.bios.2018.11.037.
[42] D. Zhu, W. Liu, W. Cao, J. Chao, S. Su, L. Wang, C. Fan, Multiple amplified electrochemical detection of microRNA-21 using hierarchical flower-like gold nanostructures combined with gold-enriched hybridization chain reaction, Electroanalysis 30 (2018) 1349–1356, https://doi.org/10.1002/elan.201700696.
[43] X. Wang, J. Wu, W. Mao, X. He, L. Ruan, J. Zhu, P. Shu, Z. Zhang, B. Jiang, X. Zhang, A tetrahedral DNA nanostructure-decorated electrochemical platform for simple and ultrasensitive EGFR genotyping of plasma ctDNA, Analyst 145 (2020) 4671–4679, https://doi.org/10.1039/d0an00591f.
[44] Y.F. Huang, M.L. Tao, S.H. Luo, Y. Zhang, B. Situ, X.Y. Ye, P.W. Chen, X.J. Jiang, Q. Wang, L. Zheng, A novel nest hybridization chain reaction based electrochemical assay for sensitive detection of circulating tumor DNA, Anal. Chim. Acta 1107 (2020) 40–47, https://doi.org/10.1016/j.aca.2020.02.006.
[45] P. Yuanfeng, L. Ruiyi, S. Xiulan, W. Guangli, L. Zaijun, Highly sensitive electrochemical detection of circulating tumor DNA in human blood based on urchin-like gold nanocrystal-multiple graphene aerogel and target DNA-induced recycling double amplification strategy, Anal. Chim. Acta 1121 (2020) 17–25, https://doi.org/10.1016/j.aca.2020.04.077.
[46] J. Das, I. Ivanov, T.S. Safaei, E.H. Sargent, S.O. Kelley, Combinatorial probes for high-throughput electrochemical analysis of circulating nucleic acids in clinical samples, Angew. Chem. Int. Ed. 57 (2018) 3711–3716, https://doi.org/10.1002/anie.201800455.
[47] J. Das, I. Ivanov, E.H. Sargent, S.O. Kelley, DNA clutch probes for circulating tumor DNA analysis, J. Am. Chem. Soc. 138 (2016) 11009–11016, https://doi.org/10.1021/jacs.6b05679.
[48] C. Cai, Z. Guo, Y. Cao, W. Zhang, Y. Chen, A dual biomarker detection platform for quantitating circulating tumor DNA (ctDNA), Nanotheranostics 2 (2018) 12–20, https://doi.org/10.7150/ntno.22419.
[49] H.F. Wang, R.N. Ma, F. Sun, L.P. Jia, W. Zhang, L. Shang, Q.W. Xue, W.L. Jia, H.S. Wang, A versatile label-free electrochemical biosensor for circulating tumor DNA based on dual enzyme assisted multiple amplification strategy, Biosens. Bioelectron. 122 (2018) 224–230, https://doi.org/10.1016/j.bios.2018.09.028.
[50] N. Lyu, V.K. Rajendran, R.J. Diefenbach, K. Charles, S.J. Clarke, A. Engel, H. Rizos, M.P. Molloy, Y. Wang, Multiplex detection of ctDNA mutations in plasma of colorectal cancer patients by PCR/SERS assay, Nanotheranostics 4 (2020) 224–232, https://doi.org/10.7150/ntno.48905.
[51] E.J.H. Wee, Y. Wang, S.C.H. Tsao, M. Trau, Simple, sensitive and accurate multiplex detection of clinically important melanoma DNA mutations in circulating tumour DNA with SERS nanotags, Theranostics 6 (2016) 1506–1513, https://doi.org/10.7150/thno.15871.
[52] Y. Liu, N. Lyu, V.K. Rajendran, J. Piper, A. Rodger, Y. Wang, Sensitive and direct DNA mutation detection by Surface-Enhanced Raman Spectroscopy using rational designed and tunable plasmonic nanostructures, Anal. Chem. 92 (2020) 5708–5716, https://doi.org/10.1021/acs.analchem.9b04183.
[53] Q. Zhou, J. Zheng, Z. Qing, M. Zheng, J. Yang, S. Yang, L. Ying, R. Yang, Detection of circulating tumor DNA in human blood via DNA-mediated Surface-Enhanced Raman Spectroscopy of single-walled carbon nanotubes, Anal. Chem. 88 (2016) 4759–4765, https://doi.org/10.1021/acs.analchem.6b00108.
[54] R. D'Agata, N. Bellassai, M. Allegretti, A. Rozzi, S. Korom, A. Manicardi, E. Melucci, E. Pescarmona, R. Corradini, P. Giacomini, G. Spoto, Direct plasmonic detection of circulating RAS mutated DNA in colorectal cancer patients, Biosens. Bioelectron. 170 (2020) 112648, https://doi.org/10.1016/j.bios.2020.112648.
[55] W. Zheng, L. Yao, J. Teng, C. Yan, P. Qin, G. Liu, W. Chen, Lateral flow test for visual detection of multiple microRNAs, Sensor. Actuator. B Chem. 264 (2018) 320–326, https://doi.org/10.1016/j.snb.2018.02.159.
[56] M. Magiati, A. Sevastou, D.P. Kalogianni, A fluorometric lateral flow assay for visual detection of nucleic acids using a digital camera readout, Microchim. Acta 185 (2018) 2856–2859, https://doi.org/10.1007/s00604-018-2856-9.
[57] C. Wang, X. Chen, Y. Wu, H. Li, Y. Wang, X. Pan, T. Tang, Z. Liu, X. Li, Lateral flow strip for visual detection of K-RAS mutations based on allele-specific PCR, Biotechnol. Lett. 38 (2016) 1709–1714, https://doi.org/10.1007/s10529-016-2161-9.