

Total microfluidic platform strategy for liquid biopsy

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Abstract. A liquid biopsy is a simple and non-invasive biopsy that examines a range of information about a tumor through a simple blood sample. Due to its non-invasive nature, liquid biopsy has many outstanding clinical benefits, including repetitive sampling and examination, representation of whole mutations, observation of minimal residual disease etc. However, liquid biopsy requires various processes such as sample preparation, amplification, and target detection. These processes can be integrated onto microfluidic platforms, which may provide a sample-to-answer system. The present review provides a brief overview of liquid biopsies, a detailed review of the technologies in each process, and prospective concluding remarks. Through this review, one can have a basic but cross-disciplinary understanding of liquid biopsy, as well as knowledge of new starting points for future research in each related area.

1. Introduction

A liquid biopsy, which is an alternative to surgical tissue biopsy, is a simple and non-invasive biopsy that examines a range of information about a tumor through a simple blood sample. Since the liquid biopsy yields outstanding advantages including non-invasive sampling, repetitive sampling and examinations, representations of whole mutation etc., it is rapidly propagated to microfluidic researchers as well as clinicians. In particular, the non-invasive nature of liquid biopsies, which requires only 5 ml of blood, is much easier and more tolerable than surgical biopsies. Tolerability and convenience of liquid biopsy can provide various benefits for patients and doctors. The biggest benefit lies in the potential of liquid biopsy to detect disease progression or treatment resistance before it triggers clinical symptoms or appears on imaging scans.

There have been debates on whether liquid biopsy, which works by detecting circulating tumor DNAs (ctDNAs), is as effective as traditional tissue biopsy. However, these debates are beginning to fade with recent comparison studies. For instance, Wyatt et al found a high correlation between driver mutations in plasma circulating tumor DNA (ctDNA) and metastatic tissue biopsies in castration-resistant prostate cancer [1]. This study demonstrates that, in the majority of patients, a ctDNA assay is sufficient to identify all driver DNA alterations present in matched metastatic tissue. This supports the development of DNA biomarkers to guide cancer patient management based on ctDNA alone.

Liquid biopsy, which is in an early stage of technical development, calls for innovative technology and the convergence of various disciplines. A promising technology is microfluidics, which has been brilliantly developed in the last few decades. A digital droplet polymerase chain reaction (ddPCR) yields the highest sensitivity to detect ctDNA in blood. However, the ddPCR cannot satisfy clinical

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needs, which require high-multiplexing of more than several hundreds of potential ctDNAs. Thus, the present protocol for liquid biopsy is based on NGS with additional ddPCR. In addition to detection technology, liquid biopsy requires plasma extraction from whole blood, DNA extraction from plasma, etc. As we review each theme below, we will recognize that there is sufficient room for microfluidic technology to contribute in the whole process of liquid biopsy.

Therefore, understanding the unmet needs of clinics, it is imperative to review each technology and methods. The first part of the review is about sample preparation for plasma extraction. The second is on DNA extraction from extracted plasma. The last part is about detection methods and techniques for liquid biopsy. After reviewing the updated technologies, comparisons will be given in specific measures such as throughput, purity, limit of detections, etc.

2. Blood cell separation: Plasma extraction

Blood plasma is the basic fluid of our body as well as an easily accessible source of circulating biomarkers for molecular diagnosis. Plasma is preferred over serum when taking blood samples, [2] although serum contains circulating DNA as well, [3] because the preparation of serum could cause unwanted release of nucleic acids due to cell lysis of non-cancerous cells [4]. Extraction of pure plasma is one of the most important steps for precise analysis of target biomarkers, especially in analyses using circulating nucleic acids that require compatibility with a gene amplification process such as the polymerase chain reaction (PCR) or isothermal amplification techniques [4]. It has been reported that impurities from blood cells, such as haemoglobin, [5] heme, [6] and lactoferrin, [5] could inhibit PCR by regulating the activity of the polymerase.

The most commonly used method to remove blood cells is centrifugation. It is currently the gold standard technique for plasma extraction with a high yield, high purity, and stable reproducibility. However, this method is hard to integrate with biosensors because it occupies considerable space, requires batch-processing, and is performed manually as a multi-step process, which can lead to human error. Furthermore, the transportation of a blood sample needs to be minimized because prompt centrifugation could reduce blood cell lysis and release of germline DNA, which otherwise would dilute the circulating target nucleic acids [2, 7–10].

2.1. General microfluidic approaches

In general, passive separation tends to be preferred over active separation because passive methods have less complex device design, can perform at relatively high flow rate, and are relatively easy to integrate with biosensors [11]. The passive methods are mainly utilizing biophysical effects of the blood cells in microscale channel flow and hydrodynamic forces to induce natural behaviors of red blood cells (RBCs) in the microfluidic channel, such as the cell-depletion layer, [12–17]. Fahraeus effect, [12, 18–21] the Zweifach-Fung bifurcation law, [22, 23] and deterministic lateral displacement (DLD), [24, 25] all three described next.

When blood flows through a channel, the RBCs tend to move towards the center so that blood flow is divided into a concentrated region in the central part and a depletion region near the wall [13]. It is called the cell-depletion layer. The Fahraeus effect refers to the decrease in hematocrit (concentration of blood cells) with decreasing capillary diameter. This effect is caused by the presence of a cell-depletion layer, which takes a larger fraction of a micro-sized than a large tube diameter. The effect of the cell-depletion layer is significant in capillaries with diameters less than 300–400 μm [19]. The Zweifach–Fung bifurcation effect means that the RBCs tend to prefer the large branch with high flow rate and is frequently observed at bifurcations of microchannels. When there is an asymmetric

bifurcation with different branch diameter, a lower density of RBCs and even pure plasma can be achieved in the small branch [26]. The DLD method determines the flow paths of particles, according to their sizes, through multiple microposts with controllable spacing. DLD is frequently used for cell separation as well as plasma extraction from whole blood [24, 25].

The Zweifach-Fung effect has been adopted in microfluidic devices for plasma extraction [27]. Additional geometric features, such as constriction and expansion of the channel and the appropriate repetition or curvature of the channel, can further improve plasma extraction [28–32]. The Agarwal group designed simple T [29], and Tau-microchannels [28, 30] and tested whole blood samples (Hct 7%–45%) at 0.3–0.5 ml/min [28]. A separation efficiency of around 80% from whole blood (Hct 45%) and near 100% from a diluted sample (Hct < 15%) was reported, while the yield was 3% using whole blood. In recent research, they not only developed a separation efficiency close to 100% at a wide range of Hct (7–62%) with a yield range from 1 to 6% but also conducted a quality check by measuring the glucose and human chorionic gonadotropin (hCG) [31]. Another case of improvement for plasma separation devices was provided by Marchalot et al. [33]. They connected two plasma separation units [34] in a multi-step set-up, obtaining a microfluidic system at an 18%–25% yield for 1:20 diluted blood. The quality of the plasma was investigated by contamination of other blood cells. Kerhoas et al. investigated circulating nucleic acids in a plasma extracted from a microfluidic device [32]. Although only the sample preparation for blood plasma extraction was done on a chip, this research involves the whole process (e.g., plasma separation, gene amplification, and detection), which a sample-to-answer system should cover.

Inertial microfluidics has been intensively studied and utilized as one of the main techniques for plasma separation [35]. Various applications, such as a constriction–expansion array, [36, 37] dean flow, [38, 39] and serpentine channels, [40] have been reported. Song et al. developed a device to extract plasma that can replace external pumping equipment with simple pipetting by the user [36]. The blood cells were autonomously focused along the walls in a series of expansions and the wall inclined to the vertical direction (85 degree) and then removed through outlets at the end of both sides. In recent work, a pattern of slant ridges was utilized to obtain high purity and high throughput with an additional air chamber to maintain equal pressure during the device operation [41, 42]. The inertial dean flow in the spiral channel can be a proper approach to extract plasma under dilute conditions, even though it could be suitable for a rare cell isolation technique, such as CTC (circulating tumor cells) [43–45]. Generally, however, samples applicable to extract plasma in inertial dean microfluidics tend to be restricted to highly diluted blood, i.e., less than 5%. A multiplexing device with tilted 16-spiral channels show a high throughput of 24 ml/min and 100% efficiency from 1:20 diluted blood [38]. In this study, the authors claimed a high yield close to 50%. Similarly, another recent study reported an efficiency of around 99% and yield of 95% with an optimized flow rate of 1.25 ml/min [39]. The yield was calculated by the difference in the fluorescent intensity from fluorescently labeled bovine serum albumin after passing through the Polydimethylsiloxane (PDMS) device, because the protein could be absorbed into the PDMS wall, which could cause loss of the target analyte. Even though the inertial dean flow shows a high plasma separation, the application of spiral dean flow in plasma separation requires further validation of clinical trials by quantifying the recovery rate for the circulating nucleic acids.

2.2. Centrifugal microfluidics

In the conventional method, the plasma can easily be separated by using a centrifuge because of the density difference between blood cells and plasma. Likewise, applying centrifugation in microfluidics is a simple and suitable approach to separate plasma from whole blood [47, 48]. The centrifugal force in microfluidics can provide an automatic, fast, and cost-effective system with many applications, such as a driving force to induce sample flow, flow control through valves, volume measuring, mixing, and

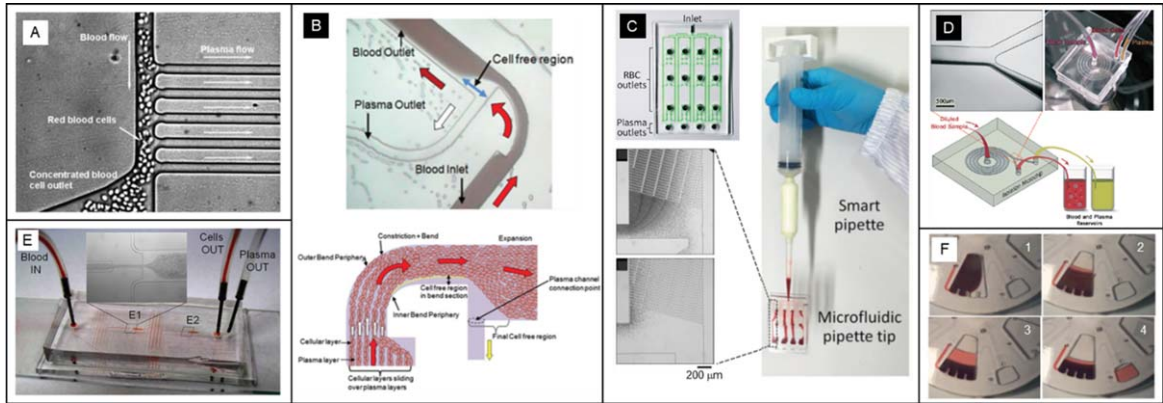


Fig. 1. (A) Image of plasma extraction using a bifurcating channel (adapted with permission, reference [27]). (B) Top: Image of a blood plasma separation device using multiple hydrodynamic effects and Bottom: plasma separation in a curved and expanded microchannel (adapted with permission, reference [28]). (C) Smart pipette device with 8-parallel channels (top), operating image of the smart pipette for high throughput plasma separation (right bottom). Expanded images show the migration of blood cells due to the slant array ridges (adapted with permission, reference [42]). (D) Top left and bottom: microscope image at the end of the spiral microchannel for plasma separation and Top right: picture of the fluidic device (adapted with permission, reference [38]). (E) Picture of a device composed of multi-step units for plasma separation utilizing the optimized cell free layer effect according to the channel design (adapted with permission, reference [33]). (F) CD-based plasma separation device that can handle large volume of blood sample. Pictures 1 to 4 indicate sample loading, primary sedimentation, total plasma separation, and plasma transferring into another chamber, respectively (adapted with permission, reference [46]).






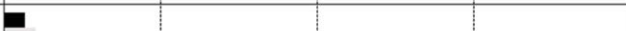

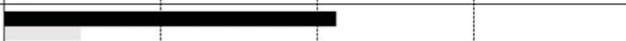

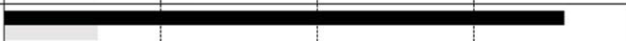

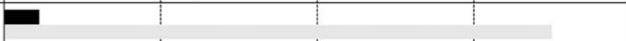

flow switching [47]. Furthermore, the centrifugal microfluidics have been studied with high potential to achieve a sample-to-answer system that can be integrated into a single disc [49–51].

It is important to note here that the Lab-on-a-Disk (LOD) based technique has a higher yield of plasma extraction than other separation techniques. For example, the extraction yield of hydrodynamic plasma separation from whole blood is generally low (around 1–10%). It means that most of the blood sample is consumed without being delivered to the detection unit. Centrifugal microfluidics can reduce the required volume in sample preparation to a level similar to centrifugation. In addition, as the dilution issue is not critical for LOD-based sample preparation, blood samples with high hematocrit can be loaded in the device.

Plasma separation from whole blood in centrifugal microfluidics has been researched by several research groups [46, 52–57]. Zhang et al. separated plasma from a diluted blood sample (12% HCT) with high purity (around 90%) in a simple design of the channel [56]. Plasma separation by flow-induced membrane deflection with pneumatic valve control was conducted with a high purity (<20 residual cells/ μL) as well as a high yield (around 80%) [53]. Haeberle et al. successfully extracted 2 μl of metered pure plasma from 5 μl of whole blood through sedimentation by a decanting mechanisms [53]. Zehnle et al. utilized capillary action to separate plasma from 40 μl of whole blood in a siphon device by pneumatic action [55, 57]. Considering its high purity and yield, centrifugal microfluidics could be a proper approach for the sample preparation technique of a sample-to-answer system. However, the technical barriers to this method are quite high. A high level of technological maturity is required to design appropriate valves and channels and to control the flow sensitively. To treat a clinical sample, a high throughput separation technique at the milliliter scale is required because the concentration of cfDNA is very low in plasma. These are necessary technological advancements because blood samples are typically restricted to a relatively small volume in Lab-on-a-CD devices, as complete packing should be ensured to prevent leakage during high speed rotation in a confined device.

Table 1

Plasma extraction techniques in microfluidics. Throughput (mL/min) indicates loading flow rate of blood sample in microfluidic device and it was calculated as sample volume/duration in centrifugal microfluidics. Yield was derived as volume fraction of extracted blood plasma (%). Throughput and yield were normalized by haematocrit of the sample on the basis of 45%. Normalized throughput and normalized yield are shown in black and gray bar graphs, respectively. Purity indicates blood cell removal percentage in extracted plasma

Separation technique	Group	Normalized Throughput (mL/min)				Purity (%)
		0	0.25	0.50	0.75	
Bifurcation law	Yang et al. ²⁶					100
Biophysical and geometrical effects	Prabhakar et al. ²⁸					80
	Tripathi et al. ²⁹					100
Constriction-expansion bifurcation law	Sollier et al. ³⁴					99
	Marchalot et al. ³³					100
	Kerhoas et al. ³²					99
Inertial Dean flow in spiral channels	Robinson et al. ³⁹					99
	Rafeie et al. ³⁸					100
Inertial effect in slant array	Kim and Choi ⁴¹					93
	Kim et al. ⁴²					100
Centrifugal microfluidics	Zhang et al. ⁵⁶					90
	Zehnle et al. ^{55,57}					100
	Amasia et al. ⁴⁶					100

Normalized Yield (%)

normalized Through put

normalized Yield

Large-volume plasma extraction was tried by Amasia et al [46]. They developed a device of centrifugal microfluidics to treat a large-volume undiluted blood sample. The device was designed as 1/4-inch-thick and with a CD radius. It successfully extracted plasma from 2 mL of whole blood with 99% purity and at a 77% yield. After finishing the cell sedimentation phase, the cell-free fluid was transferred to a collection chamber through a siphon valve that was controlled by the slow deceleration of the rotation. However, the quality of the bio-analyte in the plasma extracted through the microfluidic device was not verified.

2.3. Perspective on blood cell separation in sample-to-answer system

There is no doubt about the high potential of microfluidic plasma separation for integrating sensor systems. However, further considerations have been raised for clinical application of plasma extraction in microfluidics. Being the first sample preparation process in a sample-to-answer system, the purity and yield of plasma extraction are highly crucial in ctDNA analysis. Although the strategies for plasma separation have been improved, the dilution and high throughput should be significantly improved to take advantage of microfluidics in real clinical situations. The crucial issue is the purity of plasma extraction in liquid biopsy. Any inclusion of blood cells or lysed components would aggravate the subsequent process of liquid biopsy such as PCR or a sensing process. Considering these issues, current microfluidic technology for liquid biopsy of cancers are not satisfactory compared to the conventional centrifugation method.

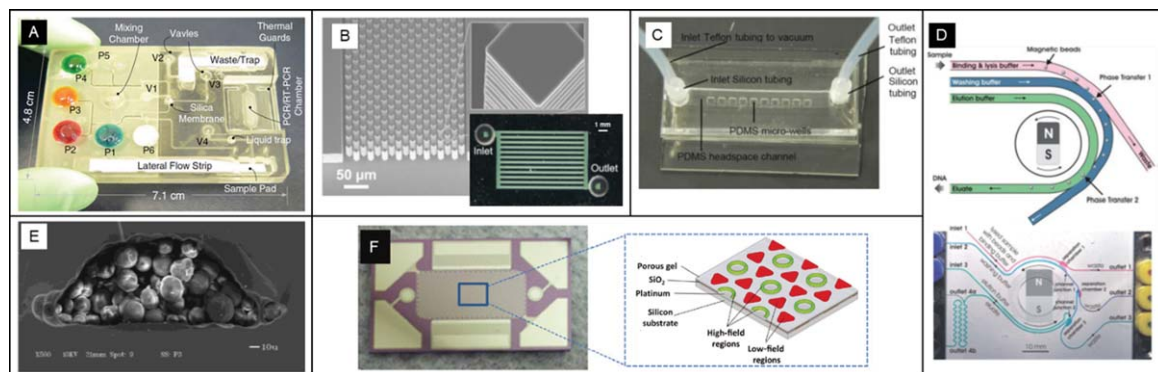


Fig. 2. (A) A polycarbonate microfluidic cassette capable of DNA and RNA extraction through a silica membrane to (RT)-PCR (adapted with permission, reference [60]). (B) Left: SEM image of micropillar structure in a flow channel and Right: microscope image of glass-silicon bonded device (adapted with permission, reference [61]). (C) Image of microfluidic nucleic acid purification chip using phenol-chloroform method (adapted with permission, reference [62]). (D) Schematics of flow description (top) and picture of a microfluidic device for a continuous nucleic acid extraction kit using paramagnetic beads (adapted with permission, reference [63]). (E) SEM image of cross section of a bead packing channel for microfluidic DNA purification (adapted with permission, reference [64]). (F) Image of electrokinetic microarray device with detailed description of materials and DEP positioning at a specific AC (alternating current) frequency (adapted with permission, reference [65]).

164 As we reviewed, the proposed microfluidic approaches are unsatisfactory for clinical application.
 165 However, these methods can be greatly improved by combining with either each other or adopting active
 166 separation technology. Although not mentioned in this section, active separation utilizing various fields
 167 (e.g., acoustic field, electrical field, magnetic field, and field flow fractionation), can be utilized for
 168 development of hybrid plasma separation with the passive methods [58, 59]. In fact, centrifugation-
 169 based microfluidics would be the most potent technique to extract pure plasma with high-throughput
 170 capacity, even though it requires further validation. Thus, one can start a liquid biopsy study with plasma
 171 rather than with whole blood because the plasma is extracted and stored in an easy and routinely manner
 172 in clinical environments until more advanced microfluidic technology is developed. After this section,
 173 the initial sample will be regarded as a plasma extracted by centrifugation.

174 3. Nucleic acids extraction

175 Because circulating DNA is known as a major biomarker for precision medicine, liquid biopsy
 176 techniques are rapidly being developed such as genome-wide sequencing, next-generation sequencing
 177 (NGS), and droplet digital polymerase chain reaction (ddPCR). Most of these efforts have been focused
 178 on detection methods because of their extremely low concentrate of ctDNAs. Although many studies
 179 have reported considerable results with excellent sensitivity and specificity of sensing, all results
 180 assumed that the target DNAs were successfully extracted and purified for their sample. Without ade-
 181 quate nucleic acid extraction and purification technology, liquid biopsy using ctDNA cannot be made.
 182 Thus, efficient extraction of target analytes would determine the clinical applicability in molecular
 183 diagnostics. The loss of biomarkers could cause serious analytical errors in accurate molecular diag-
 184 nosis and limit the reliability of the test. Furthermore, post-processing, such as gene amplifications or
 185 detection, could be inhibited according to the buffer condition of the final elute.

186 The microfluidic technique is specialized for a small amount of target sample and has high potential
 187 to be integrated with the sensor. The miniaturization and automation of the nucleic acid purification on
 188 a microfluidic chip has been broadly investigated by many researchers. Even though cancer-derived

189 cfDNA has its original characteristics, the fundamental principle of nucleic acid extraction can encom-
190 pass liquid biopsy application regardless of the type of nucleic acids. Therefore, we provide a brief
191 overview of the applicable techniques for cfDNA extraction in a microfluidic sample-to-answer system
192 as well as in conventional ones.

193 3.1. Conventional extraction of nucleic acid

194 The extraction of nucleic acids has been performed manually in the laboratory using either a spin
195 column or magnetic beads. The common feature of these methods is to utilize DNA separation by
196 silica adsorption, i.e., DNA molecules binding to silica surfaces in the presence of certain salts and
197 under certain pH conditions [66, 67]. While a sample consisting of mixtures of various proteins, DNAs,
198 phospholipids, etc., flows through the silica surface conduit, DNAs are adsorbed by the silica surface,
199 while other components, such as plasma proteins, flow away. The DNA-silica binding (adsorption)
200 involves a reduction of the negative charge of the silica surface, which leads to a decrease in the
201 electrostatic repulsion between the negatively charged DNA and the negatively charged silica. However,
202 because residual salts could inhibit the gene amplifying reaction of the enzymes [68], the salts as well
203 as non-specific protein contaminants should be removed in a washing step using ethanol. The residual
204 ethanol then needs to be removed because of the inhibition of DNA amplification [68, 69]. Then, the
205 captured nucleic acids are eluted in a low salt buffer.

206 Thus, the spin column and magnetic beads use a silica membrane and silica-coated beads,
207 respectively. However, these manual operations suffer from contamination and operator-dependence
208 problems, because the procedures have a batch-type work flow, such as changing tubes and buffers or
209 applying centrifugation under various conditions. To prevent cross contamination and improve the sta-
210 bility of the experiment, a few automated systems for extraction of nucleic acids are now commercially
211 available. However, these systems have a high initial installation cost and a large volume consumption
212 of buffers because the large device is using a liquid handler or because of the motion control of the
213 magnet.

214 3.2. Solid phase nucleic acid extraction in microfluidics

215 Solid phase extraction refers to a technique for selectively capturing nucleic acids on the surface
216 of a solid material under specified buffer conditions. Typically, such techniques have been studied
217 extensively utilizing membranes, micro-patterned devices, and beads.

218 3.2.1. Microstructure-based technique: Membrane and micro-patterning

219 Membranes can be considered microstructural materials because they are composed of bundles of
220 micro-sized capillaries. Silica membranes have been used widely in commercialized spin columns for
221 solid phase nucleic acid extraction. Integration of silica membranes in microfluidics is one of the simple
222 methods for DNA extraction in a fluidic device [60, 70, 71]. Similar to conventional DNA extraction,
223 high concentrations of salts are frequently adopted in microfluidics. Typical salts are chaotropic salts
224 such as guanidinium thiocyanate and sodium perchlorate [66, 67]. After binding the DNAs on the silica
225 surface, the residual salts as well as unwanted protein contaminants should be removed in a washing
226 step using ethanol. Furthermore, any residual ethanol should be removed because it inhibits DNA
227 amplification [68, 69]. Then, the captured nucleic acids are eluted in a low salt buffer. Many studies
228 introduced silica membrane-based microfluidic systems for nucleic acid extraction [60, 70, 71]. and
229 some of them are fully automated, self-contained, and disposable. A few studies introduced sample-
230 to-answer systems, fully integrated with gene amplification processes and target detection sensors
231 [60, 72].

Likewise, aluminum oxide membranes can be utilized instead of silica membranes with strong binding of nucleic acids at high salt concentrations [73, 74]. The major advantage of aluminum oxide membranes is that they do not inhibit PCR because of elimination of ethanol in the washing process, as aqueous NaCl [75] or KCL [73]. solutions can be utilized in the membrane. It should be noted that the nucleic acids stay immobilized on the alumina surface because of the tight bond, even in the presence of components of the PCR reaction. While it was reported that DNA can be eluted by adding bovine serum albumin and Taq polymerase to the PCR buffer, [74] the tight binding property allows the post-PCR reaction to be applied continuously to the membrane. In recent research, chitosan-modified filter paper was embodied in a microfluidic device and DNA was successfully extracted under pH control; binding at pH 5 and elution at pH > 8 [76]. Although these systems successfully purified and detected target nucleic acids with elaborately designed channels and valves to control each phase of the extraction process, they were primarily developed to detect virus and bacteria from whole blood [70, 76] or saliva [60, 71].

Similarly, the microstructural patterning to apply maximized surface area have been investigated. Microstructure patterning increases the flow disturbance and improves mixing, which can provide more opportunities for DNA to contact the binding region [77]. The fluidic devices consisting of microstructures were generally fabricated ON silicon or glass substrates, which patterned the micropillars [61, 78–81] or parallel channels [82]. Generally, the microstructure using a silica surface has the following advantages; it is reusable, no post-fabrication is required, and high flow rates are possible [83]. Especially, the compatibility for loading a high sample volume in the device is critical for most of clinical applications. West et al. showed that the micro-patterned channel can adapt a high flow rate, ranging from 5 to 500 $\mu\text{l}/\text{min}$, while the DNA elution at a high flow rate decreases with flow rate. The yield of DNA was 55% at 500 $\mu\text{l}/\text{min}$ compared to 5 $\mu\text{l}/\text{min}$. The key factor to determine the DNA extraction efficiency is the shape of the microstructural patterning of the channel. Min et al. investigated the DNA extraction efficiency according to various micro-patterning dimensions (pillar diameter, gap size, chamber volume, surface area, and surface to volume ratio) and they indicated that the DNA binding capacity was proportional to the surface to volume ratio [84]. Pyramidal pillars showed better DNA extraction efficiency than frustoconical pillars due to increased flow disturbance [77]. The limitations of micro patterning are: high cost, time-consuming, and complex fabrication process. However, because the semiconductor manufacturing industry has widened its application in the biomedical fields, micro-patterning could be a potent technique of liquid biopsy in clinics.

3.2.2. Bead-based technique

Another approach to apply a large surface area in nucleic acid purification is the bead-based method. Bead-based nucleic acid extraction has been utilized as one of the basic concepts of a commercial kit to substitute for centrifugation. Some studies found that bead-based cfDNA extraction improved the extraction yield and the quality of the circulating nucleic acid [85–88]. Jorgez et al. explained the improvement by arguing that beads can maximize the contact between the surface matrix and DNA, resulting in better absorption of DNA on the silica surface [86].

Bead packing in a microchannel is a direct adaption of a macroscale bead-based extraction [64, 89–91]. Tian et al. showed that fast DNA purification (<10 min) was effectively miniaturized using packed silica particles in a capillary [91]. The binding capacity of silica beads for DNA as well as RNA, which was packed in a channel, was investigated [89]. In addition, bead packing using chitosan-coated silica particles showed enhanced nucleic acid extraction efficiency in both DNA and RNA due to the high affinity of the nucleic acids bound to the chitosan surface [90].

Because the bead can be easily collected by incorporating paramagnetic materials, the bead-based technology has successfully replaced centrifugation processes and eliminated the need for a syringe

Table 2
Nucleic acids extraction techniques in microfluidics

Category	Technique	Materials/Methods	Advantage/ Disadvantage	Integration difficulty
Solid phase extraction	Membrane	Silica membrane [60] Aluminium oxide membrane [74] Chitosan membrane [76]	Well developed in commercial product, Disposable Easily integrated into microfluidic devices Difficult to completely elute buffer inside the membrane Use of PCR inhibition buffer (silica membrane)	Low
	Micro-patterning	Micro-patterning [77, 84, 80]. Integration with gene amplification [77]	Handling high flow rate Large surface area, Reusable device Expensive and difficult to fabricate device Use of PCR inhibition buffer	High
	Bead packing	Silica bead packing [89, 91]	Inexpensive, Disposable Ease fabrication in a tube Low reproducibility of bead packing High flow resistance, Use of PCR inhibition buffer	Low
	Paramagnetic bead	Conventional [93] Unconventional [94] Phase-transfer magnetophoresis [63]	Well developed in commercial product Freely resuspended and collected, Large surface area Relatively expensive Restriction in channel design	Moderate
Liquid phase extraction	Electrophoretic isolation	Gel electrophoresis [95] DEP [65]	No PCR inhibition buffer used No flow driving parts required Additional device to generate electrical power required Difficult to be integrated with sensor	High
	Isolation using organic solvents	Phenol-chloroform [62]	High extraction yield Widely used method in manual extraction Use of toxic solvents Many handling steps	Moderate

279 pump to allow flow through a packing channel [92]. Nucleic acid extraction in a bead is still a restricted
280 solid phase extraction at the binding surface. However, the beads can be suspended homogeneously
281 several times using paramagnetic characteristics, so that nucleic acid binding could occur like in semi-
282 liquid phase extraction. Furthermore, freely mixed beads can maximize the binding capacity of the
283 bead surface and consequently reduce the use of the overall bead amount and total cost.

284 The current use of paramagnetic beads can be generally classified into conventional and uncon-
285 ventional techniques [83]. The conventional techniques are based on stationary beads with a flowing
286 buffer, whereas the unconventional techniques are based on a stationary buffer with moving beads.
287 The manual operation of the DNA extraction using magnetic beads is conducted in the conventional
288 manner, but commercialized automation machines (KingFisher instrument; Thermo Fisher Scientific,

289 and RCS instrument; Promega) prefer the unconventional technique by moving the magnetic handler
290 with a fixed buffer chamber. Both of the existing methods can be adapted into microfluidic systems
291 for cfDNA extraction (conventional [92, 93] and unconventional [94, 96] techniques), but it should be
292 noted that the paramagnetic bead-based device originally has fewer degrees of freedom in the geomet-
293 rical design to apply enough magnetic field, such as the thickness of the wall, [92] device material,
294 [92] and channel design to minimize loss of beads [83]. To control the motion of paramagnetic beads,
295 an additional electric or mechanical part is required. For example, Lien et al. used a built-in microcoil
296 array to generate a magnetic field by applying an electrical force [93] and Duarte et al. placed an
297 external magnet to hold the paramagnetic bead and motorized rotating magnet under the fluidic device
298 to mix beads and buffer due to bead agitation [92].

299 Solid phase DNA extraction including beads and membranes has a fundamental limitation in its
300 inconsistent work flow. However, microfluidics can significantly reduce the work load and integrate the
301 complex process into one continuous process. Karle et al. developed a phase-transfer magnetophoresis
302 device that continuously extracts DNAs in fluidics devices [63, 97]. They designed microchannels of
303 binding, washing, and elution buffers to flow around a magnet rotating at the center of the device.
304 Beads that captured DNAs were continuously transferred from channel to channel from the binding
305 buffer to the final elution buffer. Robust mixing was periodically employed in the rotating magnetic
306 field, changing it from strong to weak magnetic field. Magnetic beads were aggregated and forming
307 flagella-like chains in the strong magnetic field phase. Then, they were carried away by the flow in the
308 weak magnetic field phase. The authors claimed that the continuous on-chip DNA extraction improved
309 the extraction efficiency by on average 147% compared to batch-wise bead extraction test in a tube.
310 This technology has the potential to be directly integrated with continuously flow- through micro-PCR
311 and flow-type detector for sample-to-answer systems.

312 3.3. *Liquid phase nucleic acid extraction in microfluidics*

313 Liquid phase extraction is a method to purify nucleic acid without intermediate substances such
314 as solid material and target capturing probes. Solid phase extraction enhances the limitation of the
315 immobilized surface through resuspension of the paramagnetic beads. However, its extraction effi-
316 ciency depends on the ability of the solid material to capture nucleic acids. Liquid phase extraction
317 in microfluidic application can be categorized in two methods utilizing either an electrical field or an
318 organic solvent.

319 Gel electrophoresis, which is the basic approach of size-based nucleic acids separation based on the
320 electrophoretic mobility of negatively charged nucleic acids, has been investigated in microfluidic chips
321 [95, 98]. In microfluidic gel electrophoresis, the target nucleic acids, which migrated through the gel
322 region, was easily purified into a TBE buffer filled chamber without additional gel isolation processes
323 [98]. Another example of electrophoresis is microfluidic isotachopheresis (ITP), which consists of two
324 buffers (a leading and trailing electrolyte) to which are applied high electric field gradients, enabling
325 simultaneous concentration and separation of ionic analytes [99, 100]. Because the two buffers have
326 higher and lower electrophoretic mobilities than the target nucleic acids, the DNAs can be focused
327 in the interface of the heterogenous buffers. A direct application is to extract cancer-derived ctDNA
328 from human blood using dielectrophoresis (DEP), which isolates nucleic acids using the characteristic
329 movements of the analytes in an inhomogeneous electric field [65]. When applying an AC voltage,
330 the circulating DNAs from chronic lymphocytic leukemia patients were concentrated at a positive
331 DEP position, while other blood cells were located at a negative DEP position. After the blood cells
332 had been washed with a TE solution, the remaining 25 μ L of the TE solution was eluted when the
333 electrical field was turned off. The amount of ctDNA extracted in the electrokinetic microarray device
334 was comparable with the commercial QIAgen spin column kit. Furthermore, the IGHV sequencing

335 analysis of all 15 patients with chronic lymphocytic leukemia obtained by the microfluidic device and
336 QIAgen kit exactly matched that of leukemic B-cell DNA.

337 Another example of liquid phase extraction is by using an organic solvent. The most important
338 advantage of the nucleic acid extraction method is the high extraction efficiency. In general, phenol-
339 cholochrome isolation has been known as a high throughput method [101]. Although the phenol-
340 cholochrome method is time consuming, inconvenient, and relatively dangerous (it uses a toxic solvent),
341 the solid phase extraction is preferred, despite the high yield of the liquid phase extraction. However,
342 a high throughput extraction technology could be suited to detect extremely rare mutations in liquid
343 biopsy, because the loss of one target gene can cause diagnostic errors. Zhang et al. successfully
344 developed a microfluidic device for phenol-chloroform extraction [62]. They controlled the fluidic
345 solvent and nucleic acids extraction process with a vacuum pump and pinched valve. According to the
346 pH of the organic solvent mixture, RNA (pH = 8) or DNA (pH = 4.6) can be selectively purified in the
347 device. In addition, the recovery yield of nucleic acid was up to 10 times higher than for a conventional
348 column-based solid phase kit. For practical applications, the device should ensure safe handling of the
349 organic solvent and the used solvent should be properly disposed [83].

350 3.4. Perspective on nucleic acid extraction in sample-to-answer systems

351 Nucleic acids extraction in microfluidics has been successfully implemented through various tech-
352 niques. Although microfluidic studies aiming directly at circulating nucleic acid analysis have not
353 progressed much, most of the microfluidic techniques can be applied in the sample-to-answer systems
354 of liquid biopsy. In addition, because cell lysis process is not required for cfDNA isolation, the device
355 can be more simplified than genomic DNA extraction. Thus, microfluidic miniaturization can incorpo-
356 rate separate steps: DNA binding, washing, drying, and elution on a single chip using a small-volume
357 sample with a high extraction efficiency of the circulating DNA. However, the nucleic acid isolation
358 technique should be evaluated regarding whether it interferes with the gene amplification and target
359 sensing mechanisms, whether it can relate to a minimal driving device in a fluidic system, and whether
360 the DNAs especially short fragment of around 170 bp are sufficiently extracted to detect rare mutations.
361 In this review, the targeted capture of nucleic acids with specific oligonucleotides was not included,
362 considering universal sample preparation that would be broadly integrated with various sensor part,
363 because sequence-based hybridization in the DNA preparation process could restrict diversity of the
364 analytical application.

365 4. Nucleic acids detection

366 The final goal of liquid biopsy is biomarker detection for diagnosis of diseases. As discussed earlier,
367 typical biomarkers in liquid biopsy are CTCs, exosomes, proteins, and cfDNAs in the blood. Among
368 these, cfDNA is the most spotlighted biomarker. It contains important information for diagnosis, prog-
369 nosis, and monitoring of targeted therapies, because the cfDNAs contain critical genetic information
370 of the mutants in cancer diagnosis [102]. Because detecting gene mutations is a very sophisticated
371 analysis at the molecular level, the current method of analyzing the information of cfDNA in the clin-
372 ical field is split in two. One is to analyze the whole genomic information using either whole exome
373 sequencing (WES) or next-generation sequencing (NGS). The other is that the existence of a specific
374 target gene can be examined with an highly sensitive detection device such as digital PCR (dPCR)
375 [103]. The former can detect various gene mutations (ctDNA) from cfDNAs. However, because of
376 its sequencing techniques, this method suffers from low sensitivity, high cost, and a long turnaround
377 time. The latter is satisfied with its high sensitivity but is limited for multiplexing. Thus, the related

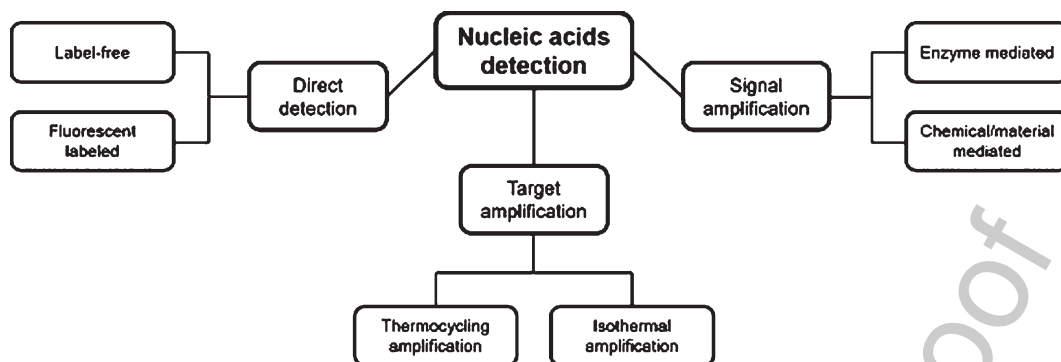


Fig. 3. The strategies of nucleic acids detection.

communities have a high demand to develop an innovative molecular diagnostic method with high sensitivity and multiplexing capability. Additionally, a cost-effective and fast test is frequently requested for clinical use.

A key issue in DNA detection methods is “how do we detect an extremely low concentration of ctDNAs sensitively and selectively?”. Various methods have been developed to detect low concentrations of target DNAs and these methods can be classified in three categories as follows: 1) Direct detection methods for target DNAs using high sensitivity sensors, 2) Pre-amplification and detection of target DNAs and methods, and 3) Signal amplification methods for amplifying the detection signals of the sensor. Each method combines various mechanisms, enzymes, signal amplifiers, and sensors to detect the target DNA. In this section, we provide a brief overview of the techniques for target DNA detection that can be applied to sample-to-answer systems.

4.1. Direct detection methods

Direct detection methods detect target DNA using highly sensitive sensors. The focus is on lowering the detection limit of the sensor. However, because the amount of cfDNA is very small, additional nucleic acid amplification steps are generally required prior to the detection step. Theoretically, if one copy of the target DNA is present, amplified DNA at the 2 pmol / 20 μ L (100 nM) level can be obtained by 40 cycles of PCR. The minimum sample volume used to analyze the amplified DNA may be greater than 20 μ L depending on the sensor used, so lower detection limits may be required considering sample dilution.

4.1.1. Label-free detection

Label-free methods detect target DNA without using any additional signal generation material. Therefore, it can be regarded as the simplest method to detect the target DNA present in the sample solution.

Electrochemical sensor-based label-free methods have been developed for direct detection of target DNA [104–107]. Wang et al. [106] applied thin-layer MoS₂ on the electrochemical sensor surface. MoS₂ is a 2D layer material that is spotlighted for its excellent optical, mechanical, and electrical properties. The single-stranded probe is absorbed by MoS₂ and immobilized on the surface of the DNA sensor. When the target DNA is hybridized with the probe DNA, the affinity is changed, and it is separated from the MoS₂ surface and a detection signal is generated. In this study, a very low detection limit of 19 fM was detected. A chip-based study using an electrochemical sensor has also been reported. Das et al. [104] detected circulating nucleic acid in serum using an electrochemical

409 clamp assay. Clamp DNAs, which bind only to the nucleic acids other than the target nucleic acid,
410 were introduced to allow selective detection. In this method, a chip-based electrochemical sensor
411 successfully detected mutations within 15 minutes with high sensitivity and specificity.

412 A label-free electrical characterization method for target DNA detection using a field-effect transistor
413 has been reported. Star et al. [108] described DNA detection using a chip with carbon nanotube network
414 field-effect transistors (NTNFETs). NTNFET is a random network of nanotubes, covering a relatively
415 large surface area between two metal electrodes. Notably, this study discriminated SNP. Because SNPs
416 are the most abundant variants of the human genome and are involved in a variety of diseases, the
417 need for sensitive and selective detection is important. This study was able to successfully discriminate
418 SNPs as well as detect target DNA with the help of NTNFET. A DNA detection chip based on an
419 organic thin-film transistor (OTFT) was also developed [109]. In this study, PNA was used as a probe
420 to detect low concentrations of DNA. PNAs have greater selectivity, affinity, and stability than DNA
421 due to charge-neutrality, which reduces the electrostatic repulsion between the two hybridized strands.
422 With the help of high sensitivity PNA and OTFT, DNA concentrations of 100 nM could be detected.

423 Plasmonic-based label-free detection studies have also been reported [110–116]. Plasmonic sensors
424 are very sensitive to changes in the evanescent field that occur when light enters a nano-sized metal.
425 The detection range of the evanescent field is very short, below a few hundred nanometers, making it
426 well suited for small molecule analysis. The method of analyzing the plasmonic phenomena occurring
427 on the surface of nano-sized metal particles is called nanoplasmonics. The nanoplasmonic biosensor is
428 applied to colorimetric analysis because the wavelength of the peak absorbance changes depending on
429 the refractive index of the material bound to the surface. Su et al. introduced a PNA probe to control the
430 aggregation and dispersion of gold nanoparticles (AuNP) [111]. In the well dispersed state of AuNPs,
431 the plasmonic phenomenon absorbs light of a specific wavelength band, resulting in a vivid plasmonic
432 color. On the contrary, when the AuNPs are aggregated, the plasmonic phenomenon is changed,
433 modifying the color band because the wavelength band of the absorbed light is changed. The target DNA
434 is identified by this color change. In this study, the target DNA was detected using a highly selective PNA
435 probe, and the surprising result of detecting single-base mismatches was also introduced. With the help
436 of the high sensitivity of nanoplasmonic biosensors, analyses yielding extremely low concentrations of
437 DNA have been introduced [117]. Nanoplasmonic phenomena are sensitive to changes in the refractive
438 index within a few tens of nanometers at the surface of a nanoparticle, so a very small amount of
439 target DNA can be detected. Truong et al. proposed a low detection limit concentration of 111 aM
440 as a result of detection using gold nanorods (AuNRs) [115]. In addition, Soares et al. showed that
441 a PCR-amplified target originated from clinical samples could be applied to the clinical field by
442 detection using nanoparticles [113]. This is a surface plasmon resonance (SPR) sensor that uses a
443 nanometer-thick metal film instead of nano-sized metal particles. The SPR sensor has a longer range
444 of evanescent fields than the nanoplasmonic sensor described above. Therefore, they are less sensitive
445 than nanoplasmonic sensors because they cannot focus on small areas. Carrascosa et al. conducted
446 an early detection of breast cancer using the SPR biosensor [112]. The detection limit condition
447 was optimized to discriminate successfully between deletion mutant DNA and wild-type DNA of
448 breast cancer, but the detection limit concentration was 50 nM. However, the SPR biosensors are still
449 highly sensitive sensors compared to other sensors and require further study because they are powerful
450 multiplexed detection platforms [114, 118].

451 4.1.2. Fluorescence labeled detection

452 While the label-free methods use a complex sensor system to sensitively detect target DNA, fluo-
453 rescence labeling has the advantage of detecting target DNA using a simple fluorescent reader.

454 A variety of fluorescence-based target DNA detection studies has been introduced [95, 119–123].
455 We will describe SNP detection using protein chip among fluorescence-based detection technologies

[121]. In this study, MutS protein was introduced to discriminate SNPs. The MutS protein, an important component of the DNA mismatch repair system, can specifically recognize and bind all possible single base mismatches as well as 1–4 base insertion or deletion loops. The mismatch binding activity of MutS was used to detect various types of DNA mutations. They utilized fluorescence-labeled DNA, which was synthesized as a wild-type sequence with no SNP. When the fluorescence-labeled DNA was injected, a heteroduplex was formed in which one base pair of DNA was not hybridized. The chip-immobilized MutS can then selectively bind single nucleotide mismatches of double-stranded DNA by introducing a sample mixture into the sensor. This study successfully discriminated various SNPs and presented detection limits of 90 pM.

A fluorescence paper based microchip study to detect two target DNAs simultaneously has been reported [120]. An origami paper chip called oPAD consisting of 3 layers coated with a fluorescent probe and quencher designed in each layer. Four analysis regions were provided on one chip, and the presence of two DNAs was multiplexed by an AND / OR logic gate method using a combination of a fluorescence probe and quencher in each region.

4.2. Target amplification methods

Target amplification methods amplify DNA so that a small amount of target DNA can be detected by the sensor with the signal generated from the amplified DNA. Because this approach involves the process of producing large amounts of amplified DNA products, a small amount of target DNA can be detected without investing excessive efforts to lower the detection limit of the sensor itself. Therefore, the target amplification methods are based on integrating the DNA amplification systems into the microfluidic devices or observing signals from amplified DNA products.

4.2.1. Thermocycling amplification-based detection

PCR is a thermal cycling technique that amplifies the target DNA sequence with millions of copies. The sample DNAs experience around 40 repeated temperature cycles, including denaturation, annealing, and extension steps. Theoretically, a single target DNA is amplified to two DNAs through each cycle.

Among the target amplification methods, the most suitable detection method for ctDNA detection is the digital DNA amplification method introduced in 1999 [124]. The biggest obstacle to the detection of ctDNA is that the amount of wild-type DNA is relatively high compared to the mutant DNA. Therefore, it is difficult to detect mutant DNA due to the presence of excess wild type coexisting in the PCR tube. However, digital PCR divides the PCR reaction into millions of small volumes to ensure that only one cfDNA is contained within the confinement. Each partition generates a fluorescent signal through PCR only if a target DNA exists in the partition. Thus, the number of mutant DNAs can be accurately determined by enumerating the fluorescence of each partition. The digital PCR technology is further developed as fully-commercialized products, such as BEAMing [125–127] and ddPCR [128]. Both products are based on water-oil emulsion droplet technology, even though the droplet generation method differs between them. In ddPCR, for instance, the PCR reagents are divided into droplets by a microfluidic technique, PCR thermal cycling is performed, and fluorescence information is analyzed using flow cytometry. The techniques for each step in ddPCR and its applications in target detection have been developed in various microfluidic chip based studies, e.g., droplet generation for ddPCR applications, [129, 130] microfluidic ddPCR, [131, 132] and digital droplet loop-mediated isothermal amplification (LAMP) [133].

A DNA detection technique based on the target amplification method was implemented as a lab-on-a-chip. PCR-based gene amplification and real-time detection techniques were developed using recirculating PCR technology and localized surface plasmon resonance (LSPR) sensors [134]. The

501 unidirectional flow is implemented as a microchip imbedded piezoelectric pump, and the sample
502 recirculation system was implemented in a closed-loop microchannel. Three heaters are attached to
503 the thermal cycling zone of the closed-loop microchannel and the target DNA present in the PCR
504 reagent is recycled and amplified. Part of the closed-loop microchannel is equipped with an LSPR
505 sensor, which monitors the hybridization of the PCR product in real time. The sensor, which is divided
506 into two probe regions, detects the amplification results of two kinds of DNA in real time. The LSPR-
507 based recirculation PCR system successfully detected the target DNA at 5 fg / mL within 15 min. This
508 technology can be applied as a sample-to-answer system when integrated with the sample preparation
509 process.

510 A study of a sample-to-answer system already implemented on a lab-on-a-chip has been reported
511 [135]. When diluted whole blood is injected into the microfluidic chip, a cell lysis process is performed.
512 The cell debris and PCR inhibitor are then removed by cross-flow filtration. Finally, the prepared
513 sample is mixed with the PCR reagent and injected into the PCR chamber. When the prepared sample
514 is injected, it is divided into two, mixed with PCR reagents, including different primers and probe sets,
515 and injected into the PCR chambers separated from each other by an oil plug, thereby implementing
516 multiple detection. The entire process could be completed within one hour. It should be noted that this
517 study proposes an example of the ultimate goal of the sample-to-answer system.

518 4.2.2. Isothermal amplification-based detection

519 An isothermal amplification method was also developed to amplify DNA at a constant temperature
520 without the 3-step thermal cycling of the PCR. Because PCR-based DNA amplification methods involve
521 different temperature setting for denaturation, primer annealing, and polymerase extension, there are
522 difficulties to place a thermal cycling block or three independent heating blocks to build an integrated
523 system. On the contrary, as isothermal amplification works at constant temperature and even at room
524 temperature, an advantage is that it is easily applied in a simple device set up.

525 Isothermal amplification has been developed using various combinations of enzymes and nucleic acid
526 probes. Furthermore, many studies utilizing isothermal target amplification have been implemented in
527 chip-based applications, e.g., rolling circle amplification (RCA), [136–138] recombinase polymerase
528 amplification (RPA), [139] LAMP. [140, 141] helicase-dependent amplification (HDA), [142–144]
529 and nucleic acid sequence-based amplification (NASBA) [145–147].

530 A microfluidic chip technology based RCA has been introduced for target DNA detection [136].
531 When a DNA amplification reaction occurs by the target DNA, a specially designed template is used to
532 increase the viscosity of the amplified RCA product to generate DNA hydrogel. A micro channel filled
533 with micro beads was used to widen the surface area where the reaction takes place and to reduce the
534 area blocked by the DNA hydrogel. To check the channel blockage, the inlet was filled by a colored dye,
535 and negative pressure was applied using a disposable syringe to examine the presence of target DNA
536 by the naked eye through the dye flow. The detection limit of this detection technique is 0.1 pM, and
537 the detection time is at least 15 minutes. It also enables multiple detection through 5 independent micro
538 channels. The technology is aimed at virus detection but can also be used for general gene detection.

539 Chip-based DNA detection technology using isothermal RPA has also been introduced [139]. RPA
540 amplifies DNA through the action of recombinase, single-stranded DNA binding protein (SSB), and
541 polymerase. Recombinase binds the primer to anneal dsDNA and SSB binds so that unbound ssDNA
542 does not become dsDNA again. The target DNA is replicated by primer extension with polymerase
543 binding. In this study, the sticky end was designed to be present in the RPA product using a special
544 primer. One side of the sticky end binds to the sensor surface and the other side binds to the reporter to
545 generate a detection signal. In this study, detection limits using fluorescence signals and paper-based
chips are 6 fM and 10 fM, respectively.

4.3. Signal amplification methods

The signal amplification method is an approach to increase the detection signal of sensors when the target DNA is present. For instance, target DNA would act as a catalyst to amplify the detection signal. The advantage of the method is to reduce the risk of error signal generation during DNA amplification. In fact, when the DNAs are amplified, an error DNA could be produced due to incorrect binding between DNAs. If such an error DNA is generated early in the amplification process, the error DNA products will continue to be amplified and lead to false detection results. However, in signal amplification methods, only the detection signal is amplified without amplifying the target DNA, reducing the risk for false signals.

4.3.1. Chemical/material mediated signal amplification

Chemical/material mediated signal amplification methods have been introduced to analyze various bio-analytes, such as cells, proteins, antigens, and nucleic acids. In particular, the application of this method of signal amplification is essential for ctDNA detection. Because the size of ctDNA is 100 nm or less and the molecular weight is significantly small, the detection signal generated from ctDNA itself could be insufficient. Therefore, a variety of signal amplification methods have been developed through nanomaterials and chemicals that have characteristics suitable for sensor platforms.

The most popular sensor for chemical/material mediated signal amplification is an electrochemical sensor, which analyzes electrochemical interactions such as oxidation-reduction reactions. Because large detection signals cannot be obtained with only DNA bound to the sensor surface, various electrochemical signal amplification techniques have been introduced. The simplest signal amplification method is that one probe linker captures multiple target DNAs [148, 149]. A supersandwich assay was introduced, a signal probe DNA capable of binding to the target DNA captured on the probe linker immobilized on the sensor chip surface [149]. Because the signal probe DNA has a sticky end that can bind to the target DNA again, the signal probe DNA and the target DNA alternately and continuously combine on the target DNA bound to one probe linker to amplify the signal. The detection limit achieved in this study is 100 pM. However, because the principle of signal amplification occurs when the target DNA is continuously captured in the sensor, there is a limitation that the detection signal cannot be amplified when the amount of target DNA is extremely small.

A study to amplify the electrochemical signals was reported by Yao et al. [150]. Two materials were used for signal amplification of electrochemiluminescent (ECL) sensors. First, nano-gold was immobilized on the surface of the sensor chip to increase the surface area of the sensor, allowing more probe DNA to bind to the sensor surface. Secondly, the detection signal was amplified using ferrocene-labeled target DNA. The probe DNA used in this study exists as a stem-loop structure. One end of the stem-loops is fixed to the sensor surface and the other is modulated by ruthenium. Ruthenium generates an oxidation/reduction reaction on the surface of the ECL sensor. When the target DNA binds to the stem-loop probe, the loop structure changes to a linear structure, and the ruthenium moves away from the sensor surface and the signal decreases. Thus, a decrease in the signal means that target DNA was detected. When the target DNA binds to the probe, ferrocene is present near the ruthenium of the stem-loop, and the ferrocene quenches the signal of ruthenium. Therefore, it is possible to amplify the signal reduction effect due to binding of the target DNA. With the introduction of signal amplification technology, this study succeeded in detecting 0.1 pM. Although the signal amplification effect by ferrocene labeling is excellent, a limitation is that it takes overnight to label ferrocene in the target DNA.

A paper analytical device (PAD)-based microchip technology using a stem-loop structure has been reported [151]. In this study, methylene blue was labeled as a stem-loop probe instead of ruthenium as described above, but the basic principles of signal generation are similar. The advantages is that you

592 can analyze target DNA with a very simple operation by using a very small (2.3 cm wide and 4.0 cm
593 long) PAD sensor.

594 DNA detection using a microarray is the most appropriate method for diagnosing cancer that monitors
595 several mutations simultaneously. Spotted image results can be obtained using a single signal enhancer
596 after introducing the sample on the sensor surface spotted with different kinds of probes. Signal
597 amplification is essential for sensitive DNA microarray analysis. Taton et al. used an AuNP-labeled
598 probe instead of a fluorescence-labeled probe, which is commonly used in commercial applications
599 [152]. AuNP probes can be captured in a sandwich form on the array when the probe is immobilized
600 on the array surface and the target DNA binds. To amplify the signal, silver ions are reduced by
601 hydroquinone to silver metal at the surfaces of the AuNPs. This amplification allows us to provide a
602 clearer microarray image, thus achieving a detection limit of 50 fM. In addition, this study discriminated
603 single-base mismatched oligonucleotides by applying optimal detection temperatures.

604 Another silver enhancement-based technique has been reported to further increase the detection
605 signal. In the technique described above, one target DNA could capture only one AuNP labeled probe.
606 In this technique, the target DNA combined with the bar-code DNA carrier AuNP is collected with a
607 magnetic microparticle so that one target DNA can generate a large number of bar-code DNAs [153].
608 The amplified bar-code DNA binds to the microarray and the AuNP probe, and a larger number of
609 AuNP probes can be immobilized on the array surface. The signal amplification process based on
610 silver enhancement technology was then used to achieve a detection limit of 500 aM. On-chip based
611 microarrays have also been reported [154]. Nanophotonic structures, such as photonic crystals, can
612 provide advantages in sensitive detection by amplifying optical signals. In this study, a photonic crystal
613 array was fabricated using probe-linker immobilized 40 nm polystyrene particles. A photonic crystal
614 amplified the fluorescence probe signal and succeeded in detecting SNP with a detection limit of 50 aM.

615 An AuNP catalytic growth technique for DNA detection signal amplification using an SPR sensor
616 suitable for nano-size DNA analysis has been reported [155]. Catalytic growth of AuNP was employed
617 as a detection signal amplification method for various sensors. However, the SPR sensor is difficult to
618 apply because the surface of the sensor chip is also a thin gold film. In this study, a SiO₂ layer was
619 coated on the sensor chip surface so that catalytic growth did not occur on the sensor chip surface. A
620 probe immobilized on the surface of the sensor and a probe immobilized on AuNP were bridged and
621 amplified by catalytic growth. As a result, a detection limit of 4.8 pM was achieved. A DNA detection
622 method using SPR imaging (SPRi) has been reported for multiple detection, which is an advantage of
623 the SPR sensor. In this study, the detection signal was amplified using Exonuclease III [118]. When
624 the 5' end of the probe linker is immobilized on the surface of the sensor chip and the target DNA
625 binds to the probe linker, the exposed 3' end of the probe linker is digested by Exonuclease III to the
626 end of the dsDNA. Target DNA is released and bound to another probe linker and the same process is
627 repeated. Three different probe linkers were spotted on the SPRi sensor chip and it was confirmed that
628 multiple DNA sequences were detected simultaneously on one chip with a detection limit of 10 pM.

629 4.3.2. Fluorescence labeled detection

630 The key to enzyme-mediated signal amplification is to introduce enzymes and designing signal
631 probes to allow a single target DNA to act as a catalyst. While the chemical/material mediated signal
632 amplification methods mentioned above amplify only the signal generated from a single target DNA,
633 the single DNA reacts with multiple signal probes with the help of enzymes to generate detection
634 signals in enzyme mediated signal amplification methods. This method is called "target recycling"
635 because one DNA is repeatedly involved in the reaction. The major advantage of target recycling is
636 that it can greatly improve sensitivity because it can generate multiple detection signals even when low
637 concentration target DNA is present. In addition, it can be applied to develop various detection assays
638 using various signal probes suitable for each sensor systems.

639 Enzyme mediated signal amplification methods for electrochemical sensors have been reported. Wan
640 et al. amplified the detection signal by introducing terminal deoxynucleotidyl transferase (TdT), which
641 adds nucleotides to the 3' end of the target DNA immobilized on the surface of the sensor [156]. The 3'
642 end of the probe DNA is immobilized on the sensor surface; then, there is no externally exposed 3' end
643 until the target DNA is present. When the target DNA binds to the probe DNA, TdT recognizes the 3' of
644 the target DNA and adds nucleotides. Among the nucleotides, biotinylated dATP is included, so biotin
645 is present in the extended single-stranded DNA (ssDNA). Avidin-modified horseradish peroxidase
646 (Av-HRP) binds to biotin and Av-HRP generates an electrochemical signal. TdT amplifies the signal
647 with a mechanism that allows multiple Av-TdT to bind to each 3' end of the target DNA. Through this
648 mechanism, sensitive target DNA detection was achieved with a detection limit of 1 pM.

649 Another signal amplification method using exonuclease III has been reported by Xuan et al. [157].
650 An electrochemical molecular beacon (eMB) labeled methylene blue at the 3' end was used for signal
651 amplification. eMB is hampered by the negative charge of DNA to approach the negatively charged
652 sensor surface. When the eMB probe hybridizes with the target DNA, a probe-target duplex is formed
653 with a blunt end at the 3' end of eMB. The 3' end of the target DNA exists as an overhang. Because
654 exonuclease III prefers the blunt or recessed 3' end, the exonuclease III selectively digests the eMB
655 probe from the 3' terminus, releasing the labeled methylene blue. The released methylene blue can
656 be freely diffused towards the negatively charged sensor surface due to its low negative charge and
657 small size and generates an electrochemical signal. Notably, the target DNA can react with another
658 eMB because exonuclease III selectively digests eMB and target DNA does not. Hence, because one
659 target DNA continuously generates free methylene blue, the signal is amplified quantitatively. Target
660 recycling resulted in an excellent detection limit of 20 pM.

661 Fluorescence based signal amplified detection methods have been realized through enzyme medi-
662 ated reactions. Target recycling-based signal amplification methods using exonuclease III have been
663 introduced. When duplex probe DNA and target DNA are combined, only the single strand connection
664 DNA of the duplex probe DNA is digested by exonuclease III [158]. The other single strand DNA
665 forms a quadruplex structure and the target DNA reacts with the other duplex probe DNA. N-methyl
666 mesoporphyrin IX (NMM) was introduced for fluorescence signal detection. It is weakly fluorescent
667 by itself but shows dramatic fluorescence enhancement upon binding to quadruplex DNA. Therefore,
668 the combination of quadruplex DNA and NMM generated during the repeated reaction can detect fluo-
669 rescence signals and achieve a detection limit of 36 pM. Target recycling technology using a molecular
670 beacon (MB) has been reported [159]. In a traditional molecular beacon strategy, the fluorescence
671 signal generation principle is that the distance between the fluorescent and the quencher is increased
672 when the target DNA binds to the fluorescence quenched MB. In this study, when target DNA binds to
673 MB, MB is digested by exonuclease III, and then the target DNA binds to another MB and the repeated
674 digestion continues. As a result, a remarkable detection limit of 20 aM was achieved.

675 Interesting detection methods for simultaneously amplifying the target and signal have been reported.
676 The target recycling technique of the MB-based signal amplification described above allows one target
677 DNA to react with multiple MBs. On the contrary, the two technologies to be introduced later allow
678 not only the fluorescence signal of MB but also target DNA to be amplified.

679 Theoretically, if the target DNA and signal are amplified at the same time, the detection sensitivity
680 can be amplified enormously. The method reported by Connolly et al. generates a "DNA trigger" by
681 introducing NbBvCl endonuclease and large fragment DNA polymerase [160]. The DNA trigger is a
682 duplicate of a portion of the target DNA. When the target DNA binds to MB to form a linear structure,
683 the primer binds to the unwinded molecular beacon. The large fragment DNA polymerase extends the
684 primer and the target DNA separates from the MB and binds to the other MB. The NbBvCl endonuclease
685 recognizes and nicks the nick site contained in the dsDNA duplex. By the nicking process, one strand
686 of DNA is divided into two, the shorter DNA of the two is again extended by the DNA polymerase,

687 and the other long DNA comes off and binds to the other MB. Long DNA is called a DNA trigger
688 because it can trigger additional reactions.

689 Target and signal amplification using RCA has been reported [161]. When the target DNA and linear
690 padlock probe bind, the padlock probe is circularized by ligase. The primer binds to the circularized
691 padlock probe, and is extended by DNA polymerase, producing a long ssDNA composed of the
692 complementary sequence of the padlock probe with a repeated complementary sequence for MB. The
693 MBs bind to the extended ssDNA, and the nicking enzyme recognizes and nicks the site included in
694 the MB sequence. The detection limits reported for each target and signal amplification method were
695 100 and 85 fM, respectively. Although the detection limit is reported to be 1000 times higher than the
696 20 aM reported in the target recycling technology using a molecular beacon (MB), a lower limit of
697 detection may be achieved by optimizing the enzyme and buffer conditions.

698 Nanoplasmonic-based sensitive colorimetric detection technology with target recycling has been
699 introduced [162–166]. When a linker DNA with a complementary sequence is exactly matched with
700 the target DNA, the nicking enzyme cleaves the nick site of the linker DNA. Two fragments of the
701 cleaved linker DNA can bind to each of the two types of AuNPs immobilized with the linker sequence
702 complementary ssDNA. If the target DNA is not present, the linker DNA does not cleave, so the linker
703 DNA binds the two AuNPs together. When AuNPs are combined, the absorption wavelength of the
704 light is changed due to the plasmonic phenomenon, and a color change is observed. Through this
705 method, a sensitive SNP detection of 500 aM was achieved.

706 4.4. Perspective on nucleic acid detection in a sample-to-answer system

707 The ctDNA analysis method for cancer diagnosis should satisfy three requirements. First, the ctDNA
708 detection method should have a high sensitivity because the amount of ctDNA present in the blood
709 is very small. Second, the ctDNA detection method must discriminate between wild-type DNA and
710 mutant DNA. The types of DNA mutations observed in cancer patients include insertion mutations that
711 add specific nucleotide sequences, deletion mutations that delete nucleotides, and point mutations that
712 change the sequence information of single nucleotides. Point mutations occur most frequently among
713 these mutations. Therefore, ctDNA detection should focus on SNP detection. Third, the essential
714 requirement for ctDNA detection is multiplexed detection. In cancer patients, various gene mutations
715 occur, and each mutation provides important information for targeted therapy or prognosis. Therefore,
716 the study of ctDNA detection for cancer diagnosis should satisfy all three of these requirements.

717 Techniques for detecting small amounts of DNA have been successfully implemented by vari-
718 ous enzymes, [148, 156–161, 167–169] signal amplifying materials, [149–154, 170, 171]. DNA
719 amplification methods, [124–139, 142–147, 172–179] sensors, [95, 104–116, 119–123, 180–187]
720 and appropriate combinations thereof. Not all sensor platforms have been integrated into a microflu-
721 idic chip, but further studies may incorporate them into microfluidic sample-to-answer system. Many
722 hybridization-based DNA detection methods use ssDNA as target DNA. However, the cfDNA present
723 in the blood is dsDNA, and the DNA amplified by PCR is also dsDNA [188, 189]. Therefore, a method
724 for detecting dsDNA should be considered. In addition, DNA detection should not only be focused
725 on lowering the detection limit but also be performed considering studies related to SNP detection.
726 Finally, the development of a sample-to-answer system will be in a not-so-distant future if we focus
727 on multi-detection sensor platforms and detection methods using detection assays.

728 5. Conclusion and perspectives

729 To achieve a sample-to-answer system for liquid biopsies, we have thoroughly reviewed the whole
730 process consisting of three themes: 1) sample preparation steps for blood cell separation, 2) and nucleic

731 acid extraction steps to help precise nucleic acid diagnosis, and 3) nucleic acid detection steps that
732 sensitively and selectively detect small amounts of target DNA. Many researchers tend to confine their
733 interests on the last theme because it is considered to be the major issue of liquid biopsy. However,
734 successful liquid biopsy cannot be achieved if the technologies of any of the above three elements
735 are not developed. For example, if extracted plasma is contaminated with lysed cells, lots of genomic
736 DNAs would be mixed with cfDNAs, making detection of ctDNA extremely difficult. Furthermore,
737 no matter how sensitive the sensor technology is, it cannot detect the target DNA successfully if target
738 DNAs are not successfully extracted from plasma.

739 A review of the all the technologies used in liquid biopsy, with microfluidics technology in mind,
740 has revealed that there are still many technological limits and challenges to overcome. First, in order
741 to extract blood plasma from microfluidic devices in real clinical situations, throughput should be
742 highly improved without sample dilution. Although current microfluidic technology is unsatisfac-
743 tory, combinations of proper techniques can improve extraction efficiency. Therefore, since plasma
744 is extracted and stored easily and routinely in a clinical environment, plasma that has been extracted
745 from centrifugation can be used in liquid biopsy until advanced microfluidic technology is further
746 developed.

747 Second, nucleic acids extraction in microfluidics has been successfully implemented through various
748 techniques. The key advantages of microfluidic nucleic acid extraction are its small sample volume and
749 high extraction efficiency by incorporating separate steps onto a single chip. Microfluidic nucleic acid
750 isolation techniques should be satisfied in whether it is compatible with gene amplification and target
751 sensing mechanisms, suitable to be implemented with minimal driving devices in a fluidic system, and
752 whether short fragment DNAs around 170 bp are sufficiently extracted.

753 Lastly, techniques for sensitive detection of small amounts of DNA have been developed through a
754 variety of enzymes, signal amplification materials, DNA amplification methods, sensors, and appro-
755 priate combinations thereof. Although the developed sensor platform has not been integrated into a
756 microfluidic chip yet, it can be integrated into microfluidic chips through future studies. Many studies
757 have suggested methods for detecting ssDNA, but since ctDNA or amplified DNA is actually dsDNA,
758 development of dsDNA detection methods should be considered. In addition, DNA detection should
759 not only focus on lowering the detection limits, but should be done considering studies related to
760 differentiating between wild-type DNA and mutant DNA.

761 However, even though we have listed various technical limits and challenges to overcome, the
762 development of a sample-to-answer system for liquid biopsy will not be a distant future. Microflu-
763 idic technology will be the main initiative to break through the present anticipated challenges. Again,
764 successful liquid biopsy can only be achieved if each element technology is developed together. Fur-
765 thermore, researchers should keep in mind clinical needs during development processes for practical
766 application.

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771 Conflict of interest

There are no conflicts of interest to declare.

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