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Dielectrophoretic recovery of DNA from plasma for the identification of chronic lymphocytic leukemia point mutations

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Practice points

- We used a novel dielectrophoresis microarray chip capable of extracting circulating cell free DNA from the plasma of chronic lymphocytic leukemia (CLL) patients to verify the existence of specific cancer mutations in the *SF3B1*, *NOTCH1* and *TP53* genes.
- This new microarray chip enables dielectrophoresis to be performed in highly-conductive media and enables the rapid isolation of circulating cell free-DNA from plasma samples within 20 min. This is faster than traditional gold standard methods, such as Qiagen kits.
- The system allows for DNA to be extracted from small volumes of just 25 µl of plasma, which is a much smaller volume than what is recommended for the gold standard methods.
- The circulating cell free-DNA collected from the plasma of five CLL patients revealed identical mutations to those previously identified by extracting DNA from CLL cells from the same patients.
- To the best of our knowledge, this work is the first study using dielectrophoresis to collect DNA from plasma to detect specific cancer related mutations.
- The rapid isolation time of the dielectrophoresis process and its ability to operate with a small plasma volume makes it an excellent candidate for further development as a point-of-care diagnostic.

Aim: Circulating cell free (ccf) DNA contains information about mutations affecting chronic lymphocytic leukemia (CLL). The complexity of isolating DNA from plasma inhibits the development of point-of-care diagnostics. Here, we introduce an electrokinetic method that enables rapid recovery of DNA from plasma. **Materials & methods:** ccf-DNA was isolated from 25 µl of CLL plasma using dielectrophoresis. The DNA was used for PCR amplification, sequencing and analysis. **Results:** The ccf-DNA collected from plasma of 5 CLL patients revealed identical mutations to those previously identified by extracting DNA from CLL cells from the same patients. **Conclusion:** Rapid dielectrophoresis isolation of ccf-DNA directly from plasma provides sufficient amounts of DNA to use for identification of point mutations in genes associated with CLL progression.

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KEYWORDS

• biomarkers • chronic lymphocytic leukemia • circulating cell free DNA • dielectrophoresis • electrokinetic • genetics • point mutations

B-cell chronic lymphocytic leukemia (CLL) is one of the most common forms of leukemia in human adults [1,2]. In the USA alone, more than 14,000 new cases are diagnosed annually, with over 4500 deaths every year [1,2]. CLL is rarely curable and its clinical course is heterogeneous [1,3].

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Some patients are free of symptoms for many years, during which time treatment is typically not necessary. For others, the disease is relatively aggressive and requires therapy soon after diagnosis. Because standard therapies are associated with significant side effects and are not considered curative, current recommendations are to withhold treatment until the patient manifests disease-related complications or clear evidence of disease progression [2,4,5].

One of the main factors that influences the clinical course of CLL is the acquisition of mutations in genes that may expedite disease progression. Therefore, the early identification of these mutations is essential for CLL diagnostics in the clinical setting [3,4,6,7]. Mutations in *TP53*, *NOTCH1* and *SF3B1* genes can occur in approximately 5–20% of CLL patients at the time of diagnosis [4,8,9]. These genes have been shown individually to have significant correlations with poor prognosis and treatment resistance in several studies [8–10]. Although these mutations may be absent at initial diagnosis, there is a high probability of development of new high-risk genetic lesions over the course of the disease (>25% at 10 years) [11], and occurrence of these lesions can directly affect survival time [11,12]. These genetic lesions give us clues as to which divergent clinical prognosis is likely for a given CLL patient and can be used to guide the management of CLL [9,11,12]. Detection of these genetic lesions can be performed by extracting DNA from B-CLL cells, followed by targeted PCR, gel electrophoresis and DNA sequencing. However, mutations can also be detected in circulating cell-free (ccf) DNA isolated from CLL plasma or CLL blood [13–15].

ccf-DNA is becoming an important biomarker for early detection of cancers and residual disease and can also be used for therapy monitoring and cancer management [1,13,16–20]. The use of ccf-DNA isolated from plasma (liquid biopsy) for the detection of cancer mutations may become a better alternative to more invasive tissue biopsies [1,16,17]. Some plasma proteins inhibit PCR amplification and require that the genetic material be purified or isolated from plasma in order to perform PCR analysis to determine DNA sequence. Unfortunately, recovering the DNA from human plasma samples is a challenge that prohibits the use of ccf-DNA for point-of-care diagnostics. The conventional techniques for separation of ccf-DNA from plasma are highly complex and time consuming and usually require at least 1 ml of plasma. Traditional methods used to recover DNA from plasma involve several extraction steps including the introduction of special solvents [21,22]. This involves a large number of manipulations including multiple steps of pipetting and filtration that can shear DNA into smaller pieces. These manipulations increase the chance of human error and can also cause degradation in the quantity and quality of the DNA [18,21,22].

To reduce DNA degradation and simplify the recovery process, we present a new electrokinetic method that uses dielectrophoresis (DEP) to recover ccf-DNA from undiluted human plasma. Recently, an enabling technological advance in the design of the DEP microelectrode chips allows DEP to be performed in high conductance media, such as whole blood, plasma and serum [21–24]. This allows for the rapid isolation of nanoscale entities including high molecular weight DNA within 20 min.

Dielectrophoresis is the result of electric field gradients acting on differences in the dielectric properties between suspended particles and medium (such as ccf-DNA and plasma) to create a force [25]. When dielectric particles such as DNA are placed in an electrical field, they become polarized and create a dipole moment. This dipole moment is frequency dependent and its magnitude depends on the polarizability of the DNA relative to the plasma as described by the Clausius–Mossotti factor. A polarized particle in a nonuniform electric field will experience a net force along the electric field gradient. A particle with a higher polarizability than its suspending medium will experience a positive DEP force, which pushes the particle towards the region with higher electric field strength (high field), and the particle with lower polarizability compared with its medium will be driven to the area with lower electric field strength (low field) by the force of negative DEP [26]. Using an appropriately designed microelectrode device, dielectric particles can be concentrated in the areas of high or low electric field, offering a method to separate particles based on their respective dielectric properties. In this study, we used microelectrode arrays produced by Biological Dynamics (CA, USA) to trap and isolate ccf-DNA from CLL plasma samples using DEP. Sufficient amounts of ccf-DNA can be recovered and used to identify the characteristic point mutations in genes associated with CLL with this method.

Materials & methods

• Sample acquisition

Peripheral blood mononuclear cells (PBMCs) and plasma samples were collected from 12 patients who satisfied diagnostic and immunophenotypic criteria for common B-cell CLL [5]. These individuals have signed the written informed consent in compliance with the Declaration of Helsinki. PBMCs were isolated by density centrifugation using Ficoll-Hypaque 1077 (Sigma-Aldrich, MO, USA) and suspended in fetal calf serum containing 10% dimethylsulfoxide (Sigma-Aldrich) for storage in liquid nitrogen [27]. Each sample contained >90% CLL cells.

DNA was extracted from the viable frozen CLL PBMCs (QIAquick PCR purification kit, 70 Qiagen). Exons 14 and 15 of the *SF3B1* gene, exons 5–8 of the *TP53* gene, and exon 34 of the *NOTCH1* gene were amplified and sequenced. Parameters for the PCR amplifications were as previously described [28]. The products were purified with a QIAamp DNA minikit (Qiagen) and were confirmed by 2% agarose gel. Sanger sequences were compared with the corresponding germline RefSeq using the software Mutation Surveyor® v.4.0.6 (SoftGenetics, PA, USA).

Frozen plasma samples collected from the same 12 patients were stored in -80°C and used to extract ccf-DNAs.

• DNA extraction using dielectrophoresis

AC electrokinetic microarray chips, from Biological Dynamics, were used for isolation of ccf-DNA from the plasma samples using the Biological Dynamics Generation 4 Elution System. These microarray devices contain 1000 platinum microelectrodes, which are fabricated on a silicon base and coated with a hydrogel (PolyHEMA) layer. Each microarray chip is contained within an acrylic microfluidic cartridge that allows the chip to hold approximately 25 µl of fluid. Each plasma sample was stained with 5000× diluted YOYO®-1 Iodide (Molecular Probes® – ThermoFisher Scientific, MA, USA). The 5000× diluted YOYO stain was prepared by first diluting a sample of the original purchased YOYO-1 stock solution 100× into MilliQ purified water. One microliter of this solution was then diluted into 49 µl of plasma and incubated for 2 min. A total of 25 µl from each stained sample was then placed into the microarray device. The AC electric field was applied to the microarray chip with a magnitude of 12 V peak-to-peak (V_{pp}) and a sinusoidal waveform of 15-KHz for about 10 min.

At these DEP parameters, the ccf-DNA is more polarized than the plasma and experiences a positive DEP force that causes it to concentrate around the edge of the circular microelectrodes in the DEP high field region. A top view of the microarray chip for each sample showing an image of fluorescent DNA collected on the electrode edges is shown in **Figure 1**. Maintaining the same voltage and frequency, each chip was then washed at 20 µl/min with 1× TE buffer (Sigma-Aldrich) for an additional 10 min. The buffer wash and the electric field were then turned off. The DNA collected at the array electrodes was allowed to diffuse into the TE buffer by Brownian motion for approximately 1 min. The wash buffer containing the collected ccf-DNA (~20 µl) was then eluted into a micro-centrifuge tube and stored at 4°C for further analysis.

• Quantification of collected DNA fluorescence intensity

A custom MATLAB script was used to quantify the fluorescence intensity of the isolated DNA on the edge of the electrodes. 3D graphs with the x and y axes representing the physical dimensions of the image and the z axis showing the fluorescence intensity were created for each sample **(Figure 1)**.

• DNA quantification of DEP recovered DNA

The ccf-DNA recovered by DEP from the plasma samples were separated into three groups according to the presence of specific mutations previously identified on DNA samples extracted from CLL cells: *SF3B1* mutated (samples 1–5), *NOTCH1* mutated (samples 6–9) and *TP53* mutated (samples 10–12). For DNA quantification, 2 µl of each eluted sample was combined with the Qubit® dsDNA HS Assay Kit (Invitrogen™) and was quantified by Qubit Fluorometric Quantitation (ThermoFisher Scientific).

• PCR, gel electrophoresis & Sanger sequencing

In order to verify the presence of the specific mutations in each sample, 5 µl of the eluted DNA, equivalent to approximately 5 µl of plasma sample, was amplified by PCR using Phusion High–Fidelity DNA polymerase (New England Biolabs) and 5 sets of forward and reverse primers to specifically amplify the above

mentioned exons in each gene (Table 1). The same PCR cycling conditions were used for all samples and are as follows. Initial denaturation was performed at 98°C for 5 min followed by 40 cycles of denaturation (98°C, 15 s), annealing (67°C, 15 s), and extension (72°C, 15 s). The

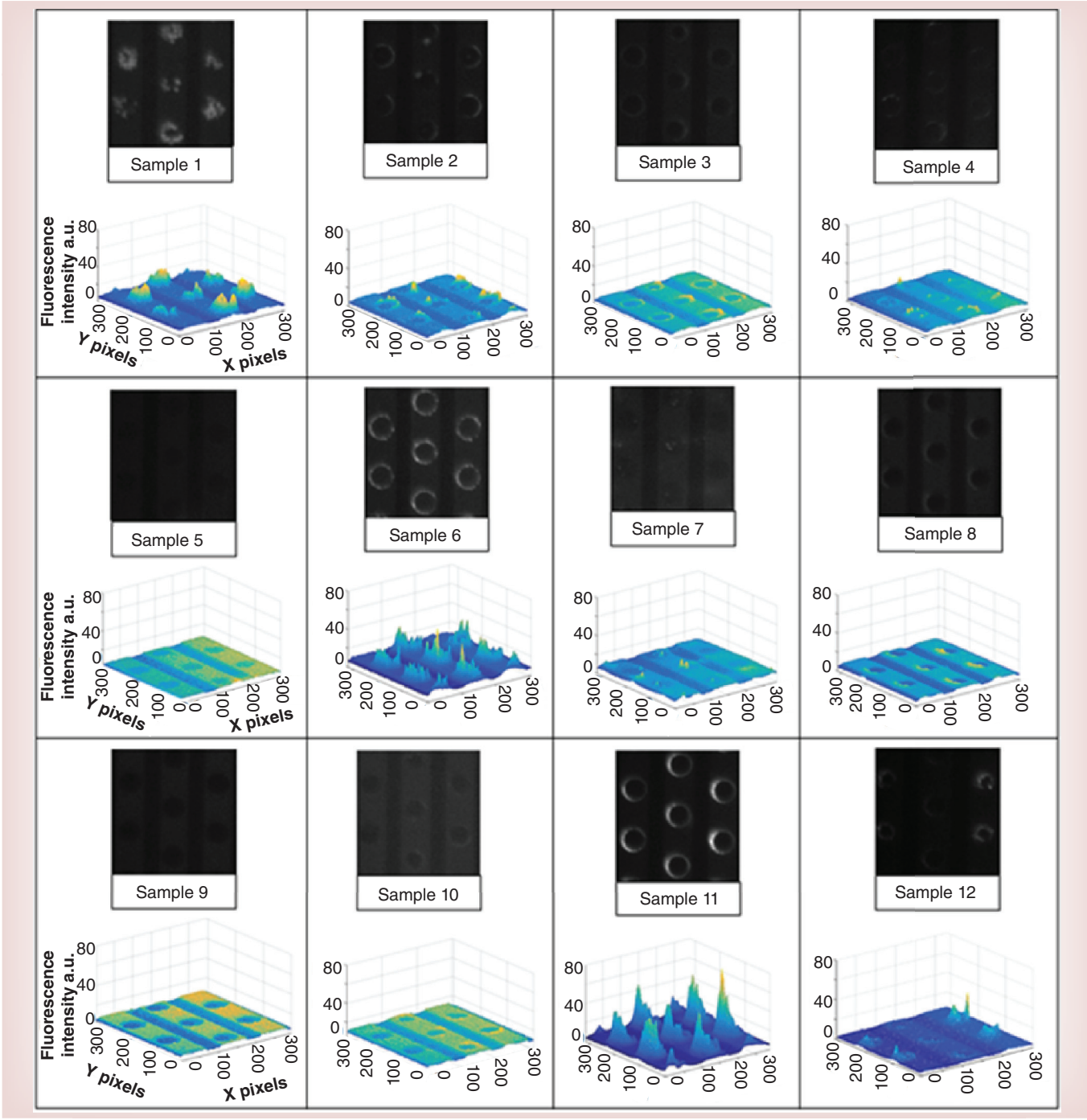

Figure 1. Fluorescence detection of circulating cell free-DNA collected by dielectrophoresis in 12 chronic lymphocytic leukemia plasma samples. Each sample is stained with YOYO-1 florescent dye to enable us to obtain these mono-color fluorescence images. These images were taken after 15 min of dielectrophoresis application and washing to remove the bulk plasma. The circulating cell free-DNA stained by fluorescent dye concentrated in the microarray dielectrophoresis high field region as seen in samples 1, 2, 6, 11 and 12. For each fluorescence image, a 3D fluorescence intensity plot has been created in order to quantify the fluorescence intensity of the isolated circulating cell free-DNA.

Table 1. List of the PCR primer sets used for each sample.

Sample number	Gene	Forward primer 5′->3′	Reverse primer 5′->3′
11	*TP53* exon 5–6	GACTTTCAACTCTGTCTCCTT	CCAGAGACCCCAGTTGCAA
10, 12	*TP53* exon 7–8	AAGGCGCACTGGCCTCAT	AAGTGAATCTGAGGCATAAC
6, 7, 8, 9	*Notch1* exon 34	GTGACCGCAGCCCAGTT	AATGCGGGCGATCTGGGACT
2, 5	*SF3B1* exon 14	TCTGTTTATGGAATTGATTATGGAA	GGGCAACATAGTAAGACCCTGT
1, 4	*SF3B1* exon 15	TTGGGGCATAGTTAAAACCTG	AAATCAAAAGGTAATTGGTGGA

PCR products then were analyzed using gel electrophoresis on a 2% agarose gel (E-Gel® EX, Invitrogen). The gels were analyzed under a UV transilluminator to determine if the amplicons were the correct size. The samples which showed a distinct band were identified and sequenced using Sanger sequencing (Eton Bioscience Inc.). The use of Sanger sequencing enabled us to do a side-by-side comparison between point mutations found in the DEP recovered DNA and the previously known Sanger sequencing data provided by the UCSD Moores Cancer Center. Purification of the PCR product was performed at the sequencing location.

Results & discussion

In this study, 12 plasma samples from CLL patients were analyzed. We categorized these samples into three different groups based on the presence of specific gene mutations. ccf-DNA from the plasma samples was successfully collected on the DEP microarray as shown in **Figure 1**. As seen in both the fluorescence image and its associated fluorescence intensity graph, samples 1, 2, 6, 8, 11 and 12 show larger amounts of ccf-DNA centered on the edge of the electrodes in the high DEP field region. For the other samples, it was concluded that there was insufficient DNA in the original plasma to be detected.

The concentration of DNA eluted from the DEP microarray chips was quantified using the Qubit Fluorometric Quantitation system and is shown in **Figure 2**. The concentration of DNA varies from sample to sample, which is expected as there is natural variability amongst individual patients and samples were collected from patients at different stages of the disease.

At this early stage of DEP chip development, there can be variability in chip electrode performance, which may also contribute to the observed variation in the amount of captured DNA collected on the electrodes. As seen in **Figure 2**, no usable DNA was recovered from sample 3 due to a technical malfunction. However, DNA at a concentration ranging from 37.3–472 ng/ml was recovered for each of the other samples.

Five microliter of each eluted sample was used for PCR amplification using the specific primer sets shown in **Table 1**.

Gel electrophoresis showed the correct sized band of PCR amplification products for seven of the CLL plasma samples (CLL-1, -2, -6, -8, -10, -11, -12). The intensities of these bands were seen to have variability between different samples; samples with higher levels of ccf-DNA collected from the DEP chip had a higher chance of successfully amplifying. CLL samples 1, 2, 6 and 11 had ≥100 ng/ml of ccf-DNA collected from the DEP chips and all successfully amplified. Only CLL samples 8, 10 and 12, which had ≥100 ng/ml of ccf-DNA, amplified. This trend can also be seen in the fluorescence images of each chip where chips showing higher levels of fluorescence also had a better chance of successfully amplifying, with the exception of CLL-8 and CLL-10. Several possible reasons for samples not amplifying include less ccf-DNA present in the original plasma samples, less DNA eluted from the microarray chips, and the need for further optimization of the PCR protocol for low concentrations of ccf-DNA. Future optimization of the design and manufacturing of the microarray devices may also lead to more DNA recovered from the chips.

After gel electrophoresis, the remainder of the successfully PCR amplified DNA were sequenced using Sanger sequencing. The results were compared with the sequencing results obtained from PCR amplification of DNA extracted from CLL cells. Five samples (CLL-1, -2, -8, -10 and -11) exhibited matching sequences. These results reveal that at least some of the ccf-DNA isolated by DEP was from the leukemia cell population. **Figure 3** shows the sequencing results for representative mutations in each gene. The sequencing results from the DNA and the ccf-DNA revealed a heterozygous missense mutation (E622D) in *SF3B1* gene in

Sample ID	DNA conc. ng/ml
CLL-1	472
CLL-2	267
CLL-3	N/A
CLL-4	62.7
CLL-5	43.3
CLL-6	118
CLL-7	46.7
CLL-8	77.3
CLL-9	61.3
CLL-10	64.2
CLL-11	355
CLL-12	37.3

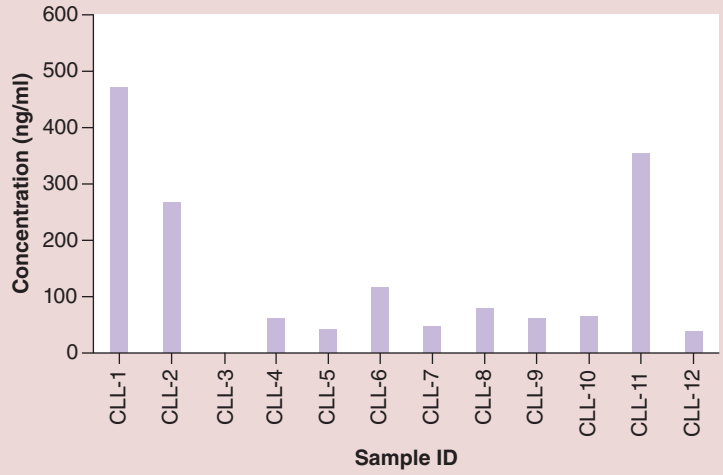

Figure 2. Quantification of DNA recovered from the dielectrophoresis microarray chips. 2 µl of each sample eluted from the chips was used to quantify the DNA. The amount of DNA recovered varied from sample to sample ranging from 37.3 to 472 ng/ml. No DNA was recovered from the chip containing sample CLL-3 due to a malfunction.
CLL: Chronic lymphocytic leukemia; conc.: Concentration.

the CLL-2 sample, a heterozygous deletion in *NOTCH1* gene in the CLL-8 sample, and a homozygous missense mutation (520A>T) in *TP53* gene in the CLL-11 sample.

Conclusion

Frozen CLL plasma samples from 12 patients were obtained from the UCSD Moores Cancer Center biorepository for this study. Specific mutations in these samples were identified after collecting CLL cells from patients, extracting DNA, and then amplifying and sequencing specific exons in the *SF3B1*, *NOTCH1* and *TP53* genes. In this study, we verified the existence of specific mutations in such genes using a novel microarray chip capable of performing dielectrophoresis in highly-conductive media. This electrokinetic technique enables rapid isolation of ccf-DNA from plasma samples within 20 min. This is faster than traditional gold standard methods, such as Qiagen kits [21]. These DEP chips can also recover this DNA from 25 µl of plasma, which is a much smaller volume than what is recommended for the Qiagen kits.

DEP was successfully applied to all CLL samples using only 25 µl of plasma. After a microfluidic washing step using 1× TE buffer to remove the bulk plasma, the concentrated ccf-DNA was recovered from each microarray chip. DNA was recovered in a sufficient amount to allow for PCR amplification and sequencing for five of the 12 CLL patient samples. The sequencing results matched those obtained from DNA extracted from CLL cells. The CLL-1 and CLL-2 samples showed mutations in exons 15 and 14 of the *SF3B1* gene, respectively, the CLL-8 sample showed a two-bp deletion in exon 34 of the *NOTCH1* gene, and the CLL-10 and CLL-11 samples showed missense mutations in exons 7 and 5 of the *TP53* gene, respectively. The PCR amplification was not successful for most samples where less than 100 ng/ml of ccf-DNA was recovered from the DEP chip. These samples may have come from patients who had an insufficient amount of ccf-DNA in circulation to reach this threshold for consistent detection. In general, the amount of DNA that can be recovered from plasma is much less than what can be recovered directly from isolated CLL cells. There is also variability in the performance of the chips at this early stage of development that could contribute to the observed overall variability.

To the best of our knowledge, this work is the first study using dielectrophoresis to collect DNA from plasma to detect specific cancer related mutations. The rapid isolation time of the

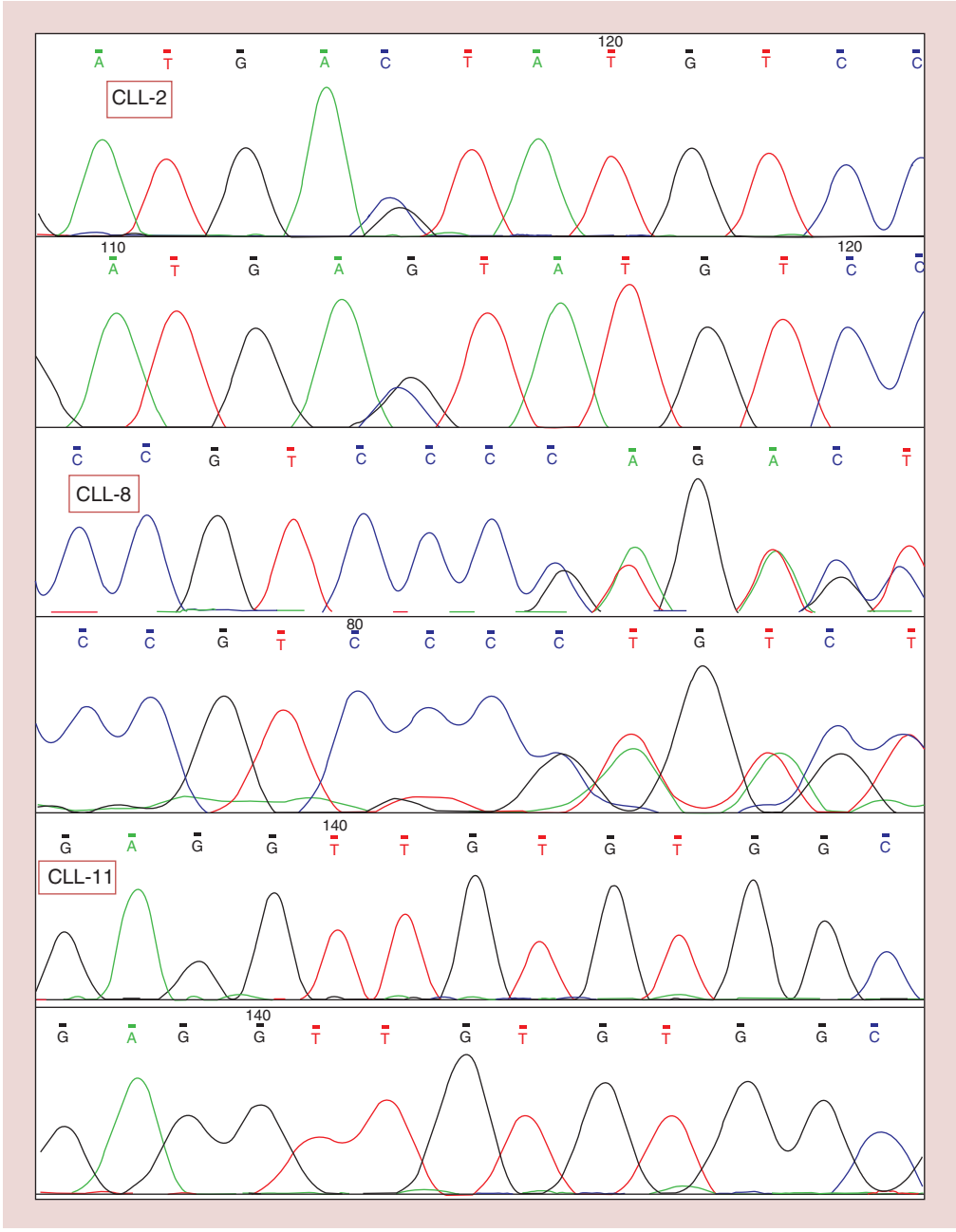

Figure 3. Sequencing results. For 3 samples (CLL-2, CLL-8 and CLL-11), the sequencing results obtained by sequencing DNA isolated from CLL cells are shown in the top panel and the sequencing results obtained from circulating cell free-DNA isolated by the dielectrophoresis technique are shown in the bottom panel. The sequencing results from the DNA and the circulating cell free-DNA in the CLL-2 sample show a heterozygous missense mutation (E622D) in the *SF3B1* gene, in the CLL-8 sample a heterozygous deletion in the *NOTCH1* gene, and in the CLL-11 sample a homozygous missense mutation (520A>T) in the *TP53* gene.
CLL: Chronic lymphocytic leukemia.

DEP process and its ability to operate with small plasma volumes makes it an excellent candidate for further development as a point-of-care diagnostic. Future work will continue to optimize this DEP technique and to make it available as a simple and rapid means to perform liquid biopsies for point-of-care cancer diagnostics and treatment monitoring.

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Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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