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Enhanced Detection of DNA Sequences Using End-Point PCR Amplification and Online Gel Electrophoresis (GE)-ICP-MS: Determination of Gene Copy Number Variations

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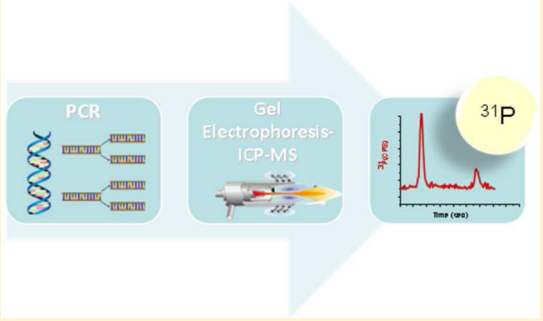
ABSTRACT: The design and evaluation of analytical methods that permit quantitative analysis of specific DNA sequences is exponentially increasing. For this purpose, highly sensitive methodologies usually based on labeling protocols with fluorescent dyes or nanoparticles are often explored. Here, the possibility of label-free signal amplification using end-point polymerase chain reaction (PCR) are exploited using on-column agarose gel electrophoresis as separation and inductively coupled plasma−mass spectrometry (ICP-MS) for the detection of phosphorus in amplified DNA sequences. The calibration of the separation system with a DNA ladder permits direct estimation of the size of the amplified gene fragment after PCR. With this knowledge, and considering the compound-independent quantification capabilities exhibited by ICP-MS for phosphorus (it is only dependent on the number of P atoms per molecule), the correlation of the P-peak area of the amplified gene fragment, with respect to the gene copy numbers (in the starting DNA), is then established. Such a relationship would permit the determination of copy number variations (CNVs) in genomic DNA using ICP-MS measurements. The method detection limit, in terms of the required amount of starting DNA, is ∼6 ng (or 1000 cells if 100% extraction efficiency is expected). The suitability of the proposed label-free amplification strategy is applied to CNVs monitoring in cells exposed to a chemical agent capable of deletion induction, such as cisplatin.



Changes in the copy number of genes contribute to the pathogenesis of various genetic disorders and cancer.[1] Copy number variations (CNVs) are imbalances that alter the diploid status of a locus, so that copy numbers increase (duplications) or decrease (deletions).[2] The screening of genome-wide CNVs in single cells is of special importance for a variety of applications in basic research and clinical diagnosis of diseases such as Alzheimer's disease, autism, schizophrenia, breast cancer, or obesity.[3,4] In addition, recent studies have associated genomic CNVs with chemoresistance (tumoral cells that are intrinsically resistant to chemotherapy or become resistant after an initial partial response).[5,6] Therefore, the development of suitable technologies for whole-genome amplification protocols (particularly in single cells) and appropriate platforms for evaluating the amplification products is highly demanded. The first generation of polymerase chain reaction (PCR) users performed end-point analysis by gel electrophoresis and added fluorogenic intercalating dyes such as ethidium bromide or SYBR Green for signal monitoring.[7] The introduction of real-time (or so-called quantitative) polymerase chain reaction (qPCR) spawned a second generation of assays that enabled quantitation by monitoring the progression of the amplification after each cycle using fluorescence probes.[8,9] In real-time PCR, quantitative information is obtained from the cycle threshold (CT), which is a point on the analogue fluorescence curve where the signal increases above the background.[10] External calibrators or normalization to endogenous controls are required to estimate the concentration of an unknown sample. When relative quantification is not sufficient and absolute numbers are required, it is necessary to sort to quantification standards (RNA, cDNA, plasmid DNA, genomic DNA) and the accuracy of the result is largely dependent on the accuracy of the standard that is used. Therefore, accurate estimation necessitates that the same reaction conditions are used for a standard and for the sample.[11,12] In general, qPCR assays are characterized by a wide dynamic range of quantification of 7−8 logarithmic decades, a high technical sensitivity (<5 copies), and a high precision (<2% standard deviation). These reasons make qPCR the golden standard for gene expression analysis[13] and also for CNV, although applied to a lower extent.[14]

Here, we propose an alternative strategy that combines the advantages of end-point PCR (high amplification rate) with a more precise and accurate method of quantification, based on the use of elemental mass spectrometry (in particular, inductively coupled plasma−mass spectrometry (ICP-MS))

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for phosphorus determination in combination with on-column agarose-gel electrophoresis. The column-type gel adopted the traditional slab gel preparation that has been used for proteins[15] and for the separation of DNA fragments.[16,17] The objective of the present work is to evaluate the suitability of GE-ICP-MS to detect gene copy variations and to address such variations quantitatively after PCR amplification of the sought gene. The "model" gene selected for our study corresponds to part of the promoter of *POLQ* gene, which encodes a DNA polymerase and helicase protein involved in defense against genomic instability and whose overexpression seems to correlate with poor outcomes for breast and colorectal cancer patients.[18]

EXPERIMENTAL SECTION

Instrumentation. The continuous elution gel electrophoretic system (Mini Prep Cell, including a high-voltage power supply PowerPac3000, BioRad Laboratories, Munich, Germany) is described in detail elsewhere.[16] The outlet of the GE system was connected to a concentric nebulizer with a flow rate of 0.7 mL min^{-1}. The gel housed in glass tubes (inner diameter (ID) of 2.2 mm) contained 2% (w/v) agarose SeaKem LE prepared in 50 mmol L^{-1} ammonium acetate buffer (pH 8.0), which served also as electrode and as elution buffer. The gel length was 30 mm. DNA separations were carried out at a voltage of 250 V. Sample volume injected on the top of the gel was 8 μL (4 μL of sample, 2 μL of 40% sucrose solution, and 2 μL of 5 μg mL^{-1} PO_4^{3-}).

The ELEMENT 2 inductively coupled plasma–sector field-mass spectrometer (ICP-SF-MS) from Thermo Fisher Scientific (Bremen, Germany) allowed the interference-free detection of ^{31}P in the medium resolution mode of 4,000. The PCR was a MWG Biotech, Inc., Primus 25 Thermal Cycler (Cole–Parmer, Madrid, Spain).

Materials and Methods. The 100 bp DNA ladder was obtained from Fisher Scientific (Madrid, Spain). The ammonium acetate buffer was prepared by dissolving the corresponding solid salt (Merck, Gemany) in 18 MΩ cm deionized water obtained from a Milli-Q system (Millipore, Bedford, MA, USA).

The A2780CIS cell line was purchased from the European collection of cell cultures (ECACC) through Sigma–Aldrich (St. Louis, MO, USA) and the GM04312 cell line was a gift from Dr. J. Surrallés (Autonomous University of Barcelona, Spain). The A2780CIS cell line was grown on a RPMI1640 medium (Invitrogen, Madrid, Spain) and GM04312 was grown on a DMEM medium (Labclinics, Barcelona, Spain); in both cases, the growing media was supplemented with 10% fetal bovine serum and Plasmocin (5 μG/mL). All cells were cultured at 37 °C with 5% CO_2. DNA was extracted with the Purelink Genomic DNA kit (from Invitrogen) and DNA concentrations were determined using a Nanodrop 2000c Spectrophotometer (Thermo Scientific, Wilmington, DE, USA)

The PCR used for this work was performed with primers PQ-F:5′-TTACAAAAGGGACAACACAGATGC-3′ and PQ-R:5′-CCAGTCTTCAAACTCAAACCTCC-3′ that anneal with the POLQ gene promoter and allow the amplification of a 623 bp amplicon. The 40 μL reaction volume contained 1X buffer, 20 μM dNTPs, 1.5 mM $MgCl_2$, 8 pmol of each primer, and 2.5 U of BioTaq DNA polymerase (Bioline, London, U.K.). The PCR stages can be described as follows: (1) 5 min at 95 °C; (2) 45 s at 95 °C, 45 s at 57 °C, and 2 min at 72 °C; and (3) 10 min at 72 °C. The temperature was held at 8 °C for at least 15 min at the end of the process. The PCR product (35 μL) was purified with the Ilustra GFX PCR DNA and gel band purification kit (GE Healthcare, Barcelona, Spain) and recovered in a final volume of 50 μL.

RESULTS AND DISCUSSION

Optimization of the Separation and Quantification. As previously described, the amplified fragment of the gene *POLQ* contains 623 bp; therefore, the first experiments were focused on optimizing the separation–detection conditions for the analysis of similar size DNA fragments with the GE-ICP-MS system. Figure 1 shows an electropherogram corresponding to the separation of 100 bp ladder with fragments ranging from 100 bp to 1000 bp after separation in a gel containing 2% agarose. The separated fragments were then taken into the ICP-MS for online P-monitoring using a 50 mM ammonium acetate buffer continuously introduced using a peristaltic pump. Figure 1 also shows the correlation of the observed retention times with the number of base pairs in the studied range (adjusted to a polynomial equation, r^2 = 0.999). Although this standard was not spiked with any inorganic phosphate, some traces of this compound were naturally present in the solution and showed a minor signal that came out at ~550 s (see Figure 1). This was verified by adding phosphate to the standard of the ladder (data not shown), which was further used as internal standard (IS)

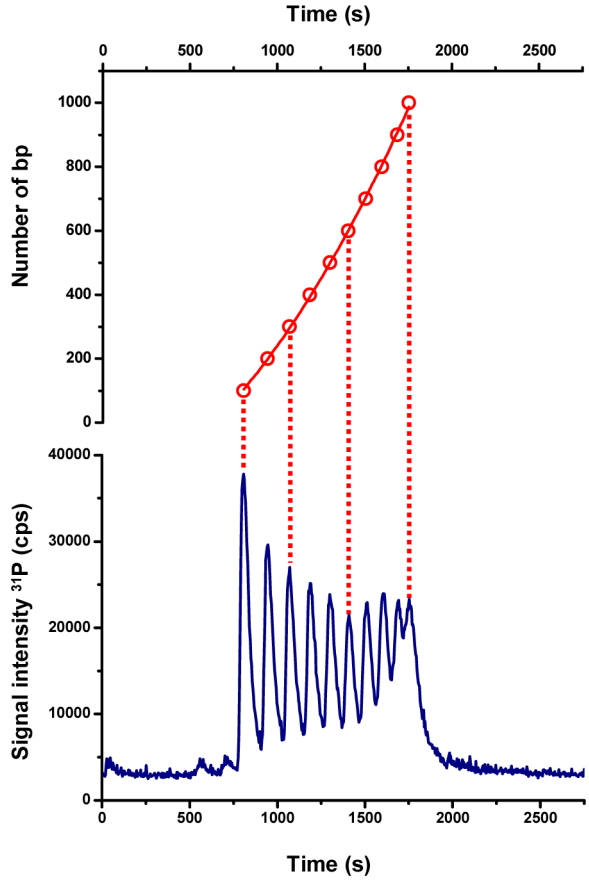

Figure 1. Separation of a 100 bp DNA ladder using on-column gel electrophoresis and ICP-MS detection of ^{31}P and plot of the retention times observed for the different sequences versus the number of base pairs. There is a polynomial relation ($y = -224.14 + 0.16x + 0.0003x^2$) with a correlation coefficient of r^2 = 0.9993.

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for correcting possible variations on the migration times and also for quantification purposes, as will be explained later.

A critical advantage of ICP-MS as detector in this application was its ability to provide compound-independent quantification strategies, exploited also for DNA sequences after labeling with lanthanides[19] or nanoparticles.[20] Since ICP-MS analytical response is virtually compound-independent, the introduction of a P-containing inorganic standard (e.g., inorganic phosphate) allowed direct calculation of the phosphorus concentration in the corresponding PCR product (amplicon), and by knowing the P/amplicon stoichiometry (directly obtained from Figure 1) and the amplicon concentration. Thus, the proposed strategy provides simultaneously fragment size and quantitative data of the amplified fragment, in connection with PCR amplification. For example, Figure 2A shows the electropherogram obtained from human genomic DNA (32 ng) after amplification of the fragment of the *POLQ* gene (t_m = 1430 s). The DNA was extracted from cultured cells (A2780CIS cell line) and the amplification was performed using 30 cycles of PCR. The signal of the PO_4^{3-} standard added for quantification can be also observed in Figure 2A (t_m = 550 s), and, using this signal (and the corresponding phosphate concentration), the concentration of the amplicon was directly extracted from the electropherogram, accounting for 0.014 ± 0.004 μM (n = 5).

To establish a generic quantitative method for the quantification of PCR products, it is important to address the amplicon peak area, depending on the number of PCR cycles and the starting DNA concentration. These two parameters are inversely related, because lower DNA starting concentrations will require higher PCR cycles to obtain the same amount of the amplified product. However, there is a limit in the amplification rate (a "plateau" region in the PCR curve) given by the limitation in the concentration of the reaction components. To evaluate this effect, different starting DNA amounts (nanogram level) and PCR cycles were studied and the obtained results were plotted in Figure 2B, where the represented signal corresponds to the amplicon peak area divided by the area of the IS. According to these results, an increase in the peak area ratios was observed with increasing the starting DNA amounts for the number of assayed cycles (25, 30, and 35). When comparing among cycles, the highest amplification rates were observed for 30 cycles, in the range of the evaluated starting DNA amounts (16–320 ng). However, if lower starting DNA amounts were to be used (<16 ng), higher amplification rates could be obtained with higher number of cycles, as suggested by the results of Figure 2B.

Figure 2C shows that, at 30 PCR cycles, the amplicon peak area correlated linearly with the logarithm of the starting DNA amounts (correlation coefficient of r^2 = 0.99). This dynamic range for quantification can be converted into copy numbers of a gene in the starting DNA (taking into account that the size of the human haploid genome is 3.3 × 10^9 bp, which corresponds to ~3.3 pg DNA and, when starting from genomic DNA, this corresponds to one copy of any single copy gene). In our case, this spanned from 4800 (lowest concentration tested) to ~100 000 copies. Therefore, working under the established conditions (30 PCR cycles and starting DNA amounts within the assayed range), it is possible to obtain the starting copy numbers of the amplified fragment (in absolute and relative terms) with this calibration curve. Of course, this work represents only an innovative proof of concept, but a simultaneous measurement of different genes (multiplex experiments) could be done with the proposed setup.

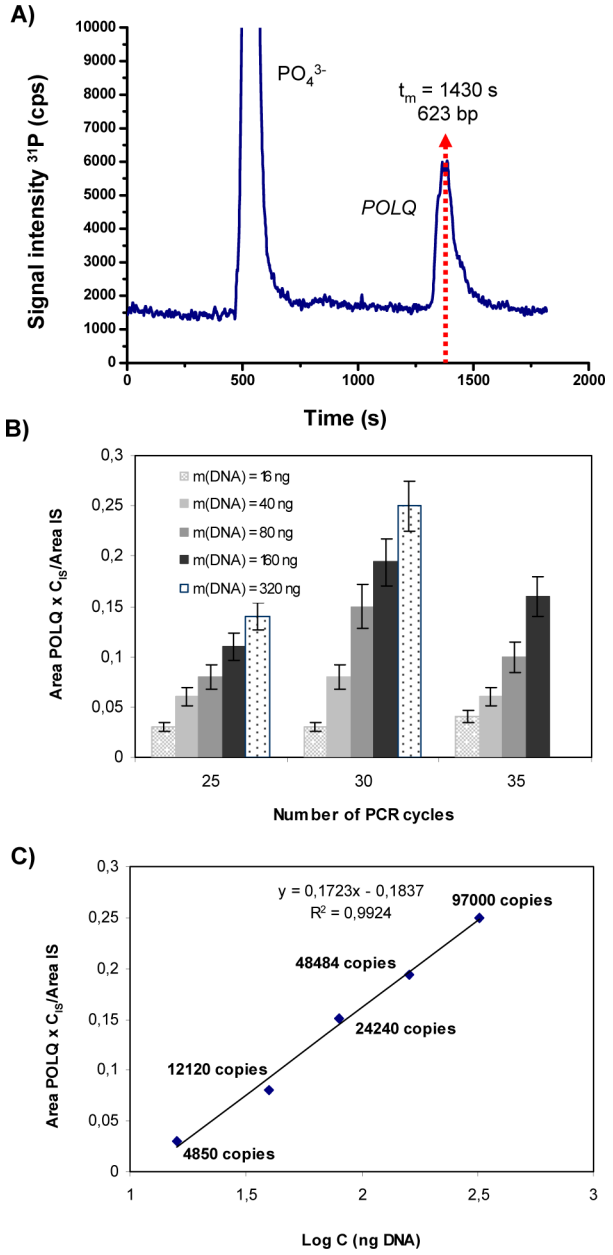

Figure 2. (A) Separation of amplified fragment of the POLQ gene (623 bp) in the on-column GE-ICP-MS system with the signal of the PO_4^{3-} added as an inorganic standard. (B) Peak areas ratio of the amplicon/phosphate for different DNA concentrations and PCR cycles. (C) Variation of the amplicon/phosphate peak areas ratio with the concentration of DNA in the template (and with the corresponding number of copies).

Application of the (PCR)-GE-ICP-MS to Gene Copy Number Determination. The application of this proof of concept in health and disease was checked using human DNA obtained from cultured cells treated with the chemotherapeutic drug cisplatin. This chemical induces DNA damage, mainly intrastrand cross-links that can block replication and may induce deletions.[21] Moreover, apparently CNV in specific genes is related to the poor outcome of the drug in ovarian cancer patients and might also be related to cisplatin resistance.[22] The cell line used in these experiments (GM04312) is deficient for the nucleotide excision repair mechanism (NER) and then sensitive toward cisplatin action, since NER is the main DNA

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repair system involved on the removal of cisplatin-induced DNA damage.[23,24] Therefore, this cell line was selected to conduct the experiments for the purpose of detecting possible CNV ascribed to the treatment with cisplatin. Thus, three replicates of GM04312 (one control and two exposed to 5 and 20 μM cisplatin, respectively) were studied. After DNA extraction, the same amount of genomic DNA was used for PCR amplification (30 cycles) in each case, and the amplicon was analyzed under the optimized conditions.

Figure 3 shows the electropherograms obtained for the control and the 20 μM cisplatin samples by GE-ICP-MS. The

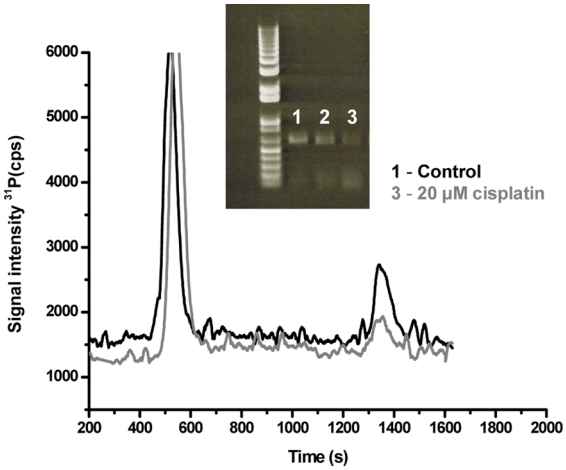

Figure 3. Separation of the amplified fragment of the POLQ gene (623 bp) after amplification with 30 cycles of PCR in the slab gel and in the on-column GE-ICP-MS system. The samples correspond to the cell line GM04312 of fibroblasts: line 1, control (black trace in the online system); line 2, 5 μM cisplatin (not shown in the online system); and line 3, 20 μM cisplatin (gray trace in the on-line system). At 550 s, in GE-ICP-MS, the signal corresponds to the inorganic phosphate used as an internal standard (IS).

image shows the same separation also in a slab gel, together with a DNA ladder, all treated with ethidium bromide. As can be directly observed in the slab gel, the control sample (line 1) showed significantly higher intensity than the 20 μM cisplatin (line 3). Quantitatively, the absolute number of copies of the control sample could be determined using the proposed setup and the calibration line of Figure 2C. The obtained results revealed a decrease in the number of copies of ~20%, with respect to the control for the 20 μM cisplatin-treated sample (12 332 ± 120 vs 10 180 ± 296, n = 3). The observed differences were not statistically significant in the case of 5 μM cisplatin. Thus, the proposed strategy enabled to address quantitatively that the treatment with cisplatin might have induced deletions (or at least DNA damages that prevent primer annealing) at the region of the analyzed fragment. To summarize, Table 1 shows the obtained analytical figures of merit of the proposed system, including the accuracy obtained (by comparing the DNA concentration obtained with the proposed system after PCR and this obtained by measuring it in the original sample using spectrophotometric detection). The limit of detection estimates the minimum amount of DNA necessary to detect the amplicon with the proposed system and under the optimized set of conditions (using 30 PCR cycles). This value is slightly higher than that observed in the case of qPCR for which DNA concentrations above 0.1 ng are required. However, the LDs can be further improved by

Table 1. Analytical Figures of Merit of the Proposed Methodology Based on On-Column Gel Electrophoresis−Inductively Coupled Plasma−Mass Spectroscopy (GE-ICP-MS)

figure of merit	GE-ICP-MS[a]
limit of detection, LD[b]	6 ng or 1000 cells
percent relative standard deviation, %RSD[c]	8%
resolution[d]	1.02
percent relative standard deviation, %RSD (mig. time)[e]	0.8%
accuracy[f]	>95%

[a]Includes the use of different homemade gels for every analysis. [b]Minimum amount of DNA needed for detection (using 30 PCR cycles) and considering 100% extraction efficiency. [c]Calculated for n = 3 (measured different days with different gels). [d]Between 500 bp and 600 bp. [e]Evaluated on the POLQ gene (623 bp). [f]Calculated initial DNA concentration by GE-ICP-MS and compared with NanoDrop results.

increasing the number of PCR cycles. Therefore, the proposed strategy seems to provide an interesting alternative for quantitative evaluation of CNV associated with different pathological conditions.

CONCLUSIONS

In conclusion, we have shown that end-point polymerase chain reaction (PCR) in combination with on-column gel electrophoresis−inductively coupled plasma−mass spectroscopy (GE-ICP-MS) offers outstanding features for the quantitative assessment of changes in the number of copies of a given gene. From a qualitative point of view, the proposed approach can serve to evaluate the correct performance of the amplification process by PCR, since direct information on the size of the amplicon can be attained (ICP-MS-driven molecular information) by calibrating the system with the adequate ladder (with clearly improved resolution, with respect to more-conventional slab gel electrophoresis).

In addition, because of the direct correlation obtained between the area of the amplicon and the number of copies of a given gene (depending on the DNA starting concentration), the methodology is fully quantitative to address CNVs in different cells as alternative to qPCR (but not limited by the size of the amplicon) and could be further exploited simultaneously for different genes (multiplex CNV determinations). Among the main advantages of the proposed strategy, first is the absence of any labeling procedure, since the method is based on direct quantification of the phosphorus present in the amplicon after PCR (thus, errors in quantification associated with insufficient labeling reagent, quenching, etc. are minimized). When compared with qPCR that exhibits optimum performance for amplicons <300 bp, the presented strategy can be applied to any amplicon length (e.g., here, a fragment of ~600 pb is shown). Furthermore, no melting curves are required to address the outcome of the PCR reaction (identification of the amplified product in qPCR). Thus, the methodology here proposed can be used to address simultaneously the correct functioning of the PCR and the concentration of the amplicon (by sorting to a well-characterized phosphate standard).

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Notes

The authors declare no competing financial interest.

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