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Multiplex polymerase chain reaction in combination with gel electrophoresis-inductively coupled plasma mass spectrometry: A powerful tool for the determination of gene copy number variations and gene expression changes

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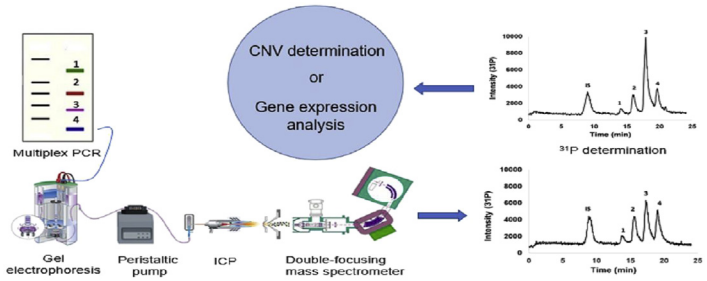
HIGHLIGHTS

- Conventional multiplex PCR was combined with GE-ICP-MS for CNV and gene expression analyses in cancer cells.
- Amplicons were separated, identified and label-free quantitate through ^{31}P monitoring.
- CNVs results obtained with this method were in accordance to literature data and to uniplex qPCR results.
- This method provided the same information on relative gene expression for three genes, as single reaction RT-qPCR.

GRAPHICAL ABSTRACT



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ABSTRACT

During the last few years multiplex real-time or quantitative polymerase chain reaction PCR (qPCR) has become the method of choice for multiplex gene expression changes and gene copy number variations (CNVs) analysis. However, such determinations require the use of different fluorescent labels for the different amplified sequences, which increases significantly the costs of the analysis and limits the applicability of the technique for simultaneous amplification of many targets of interest in a single reaction. In this regard, the use of the coupling between gel electrophoresis (GE) separation with inductively coupled plasma mass spectrometry (ICP-MS) detection allows the label-free determination of multiplex PCR-amplified sequences (amplicons) by monitoring the P present in the DNA backbone. The quantitative dimension is obtained since under optimal and controlled multiplex PCR conditions the peak areas of the separated amplicons are directly proportional to the amount of DNA template in the original sample. Moreover, the calibration of the GE-ICP-MS system with a DNA ladder permits direct estimation of the size (bp) of the PCR products. The suitability of the proposed multiplex strategy has been evaluated addressing two different situations: determination of CNVs and gene expression changes in human ovarian cancer cells. In the first case, the results obtained for the simultaneous quantitation of

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CNVs of four genes (*HER2*, *CCNE1*, *GSTM1*, *ACTB*) on DNA obtained from OVCAR-3 cells were in accordance with the literature data, and also with the results obtained by conventional simplex qPCR. In the second case, multiplex gene expression changes of *BAX*, *ERCC1* and *CTR1* genes, using *ACTB* as constitutive gene, on A2780cis respect to A2780 cells, resistant and sensitive to cisplatin, respectively, provided the same information as single reaction reverse transcription (RT)-qPCR.

1. Introduction

Determination of variations in DNA gene copy number under the form of deletions and duplications of more than one gene or gene fragment [1,2] has become increasingly important, since these copy number variations (CNVs) are extensively related to a great number of human diseases and syndromes [2–5], from infectious, autoimmune and neuropsychiatric disorders to cancer [6,7]. Therefore, reliable and efficient determination of CNVs will be essential to develop diagnostic, prognostic and therapeutics tools for CNVs-related diseases.

Similarly, gene expression analysis, which provides quantitative information about the population of messenger RNA (mRNA) transcripts in cells and tissues, is important for identifying genes involved in the development of diseases such as cancer [8,9]. Gene expression is crucial in all normal cellular events but it is also a necessary step to the pathogenic events that drive the development and progression of a disease, as well as governing response to therapy [10–12]. Thus, determination of gene expression in a multiplex way, with the aim of improving the diagnosis of a disease, predict therapeutic response, and determine disease prognosis is a current topic of active research [8,9].

In the case of CNVs, monitoring of such variations has been traditionally conducted by well-known and widely applied techniques [13,14] such as fluorescent *in situ* hybridization (FISH) and comparative genomic hybridization with DNA microarrays (array-CGH) [15,16], which is also widely used for gene expression determination. FISH analysis for targeted DNA regions is a difficult technique to implement as a multiplex assay, while array-CGH allows for simultaneous screening of many genes across the entire genome with high resolution [17–19] and even ultra-high resolution [20]. However, the lack of standardized reference samples and the large incongruities in the results from the different commercial array-CGH platforms highlight the need for standardizing array data collection, quality assessment and experimental validation with orthogonal techniques [18,19]. Nowadays, next generation sequencing (NGS) have greatly improved the resolution and accuracy of genome-wide CNVs analysis using different NGS-based tools [17,21,22]. However, the enormous variability in performance of these tools [21,23], as well as the massive amount of data generated constitute a great challenge for result interpretations [24].

Besides the above mentioned techniques, real-time or quantitative polymerase chain reaction (qPCR) is now the state-of-art technology for quantifying targeted genomic regions for both CNVs [25] and gene expression (in this case following a reverse transcription step to convert the mRNA to complementary DNA, cDNA) [25,26]. This is due to the low consumables costs, the high sensitivity and the open format (independent of a single supplier) of the qPCR technique.

With qPCR, the PCR product accumulation is measured in real-time resulting in a sigmoidal amplification curve. Several fluorescent detection chemistries are available to measure PCR product accumulation, including hydrolysis probes, molecular beacons, dual hybridization probes and double stranded DNA specific binding dyes. In this regard, for the amplification of multiple DNA sequences simultaneously, different dyes or fluorescent probes are required, what increases significantly the costs of the analysis and limits the applicability of the technique for simultaneous amplification of many targets of interest in a single reaction. In addition, the use of some of these dyes such as SYBR Green I exhibit a large number of disadvantages [27] such as extensive optimization requirements, inhibition of PCR in a concentration-dependent manner, effects on DNA melting temperature and preferential binding to certain DNA sequences. This last drawback prevents the analysis of multiple DNA sequences. To overcome this problem, alternative PCR-based approaches with a higher multiplexing capability, such as multiplex ligation-dependent probe amplification (MLPA) can be used for targeted screening of CNVs [28–30]. The MLPA technique allows the simultaneous measure of relative CNVs of up to 40 different DNA sequences [28], but due the time-consuming nature of the probe-generation process the vast majority of MLPA applications are restricted to genes for which it is possible to use commercially available kits [29–31].

In this work, we have tried to illustrate the combination of conventional multiplex PCR with an alternative strategy for the simultaneous quantification of the amplified DNA sequences, based on the use of gel electrophoresis (GE) with on-line elemental detection by inductively coupled plasma mass spectrometry (ICP-MS). The ICP-MS detector has unrivaled advantages in quantification compared to molecular MS detectors, such as Electrospray ionization (ESI)-MS and Matrix Assisted Laser Desorption/Ionization (MALDI)-MS. First, in ICP-MS the ionization is virtually compound independent and second, it is easier to interface with electrophoretic-based separation techniques (capillary electrophoresis or GE). The ICP-MS technique produces the complete atomization and ionization of different DNA sequences (fragments), and the obtained signal is proportional to the number of P-atoms in the sequence and, therefore, to the amount of the corresponding amplified fragment in the original sample. Using different initial DNA amounts a calibration curve can be established and then the amount of amplified fragment measured can be correlated with the initial amount of DNA used for the PCR reaction. The on-line coupling of ICP-MS to separation techniques that permit the isolation of DNA fragments as function of their size, such as column GE, allows the simultaneous identification of the amplified gene fragment size and its quantification, as previously demonstrated in the case of a single gene as proof of concept [32]. The work presented here shows the extension of the referenced work to relevant multiplex PCR amplification of genes used to detect CNVs and gene expression changes by means of ICP-MS detection of the P present in the amplified fragments. The results of multiplex CNVs determination were validated by analyzing genomic DNA from the ovarian carcinoma cell line OVCAR-3 which possesses substantial copy-number changes. These results were also confirmed by conventional simplex qPCR. In the case of multiplex gene expression analysis the results were compared to those obtained by single reaction reverse transcription (RT)-qPCR.

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