

Real-time quantitative PCR array to study drug-induced changes of gene expression in tumor cell lines

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ABSTRACT

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Aim: Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is still the "gold standard" for quantitative analysis of mRNA and the study of differentially expressed genes. **Methods:** The authors describe a RT-qPCR array that exploits SYBR Green dye-based detection to perform reliable gene expression analysis on 41 genes involved in several pathways linked to DNA damage response, cell cycle progression, cellular senescence, and programmed cell death. To validate the RT-qPCR array, the authors investigated changes of the gene expression profile of HeLa cells treated with two well-characterized antiproliferative molecules such as cisplatin (CDDP) and sodium butyrate (NaBu). **Results:** The results showed a gene expression profile compatible with both biological and gene expression data already reported in literature. **Conclusion:** Importantly, the assay allowed the monitoring of additional and not reported gene regulations, indicating that this custom-made RT-qPCR array is a cheap, robust, and rapid tool for the study of drug-induced effects in human biological models.

INTRODUCTION

The study of gene expression profile of cancer cells has become an essential tool to understand the biological alterations involved in disease development, to individuate new potential markers, to predict clinical outcome, to create personalized pharmacological therapies for patients, and to investigate the molecular effects of drug exposure with the aim of improving

treatment efficacy.^[1]

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)-based methods has emerged as the "gold standard" method for a rapid and robust analysis of gene expression.^[2] Currently, many PCR arrays are commercially available for the study of gene expression modifications involved in hundreds of molecular pathways. However, based on our



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experience, many of these RT-qPCR arrays are characterized by technical limits that could compromise the success of a gene expression study. The first limit is represented by the absence of technical replicates that could monitor the inter-well reproducibility for each gene transcript. The amplification of each target in a single well does not allow one to identify possible incidental mistakes that can occur, for example, during preparation of the PCR reaction mix. Another critical aspect is amplification of genes expressed at very low levels (e.g. CDK inhibitors) that is often difficult and requires a highly efficient DNA polymerase. In fact, the different DNA polymerase master mixes commercially available are not always able to amplify very low represented mRNA species in a detectable way.

The rationale of the study was to design and test a RT-qPCR array able to analyze the behaviour of 41 human genes involved in cell cycle regulation, DNA damage response, apoptosis, and senescence induction. Our goal was to develop a qPCR array that could overcome the technical limits described above (e.g. low efficiency of amplification and technical replicates controls) in order to obtain an inexpensive and easy-to-use tool for the reliable monitoring of transcriptional modulations induced by exposure of human cells to drug treatments.

We applied this qPCR array to investigate the response of HeLa cells to two different anti-proliferative drugs such as cisplatin [cis-diamminedichloroplatinum(II)-CDDP] and sodium butyrate (NaBu). CDDP remains one of the principal chemotherapeutic agents used for cancer treatment. CDDP cytotoxicity is mediated by its ability to form DNA adducts, primarily intra-strand adducts, which activate a DNA-damage cellular response and subsequent programmed cell death.^[3,4] Cellular exposure to CDDP, for example, is known to cause up-regulation of cyclin dependent kinase inhibitors (e.g. p27 and p21) and down-regulation of anti-apoptotic protein Bcl2 in cancer models.^[5,6] NaBu is a widely known histone deacetylase inhibitor and is considered the prototype of an epigenetic modulator. NaBu exerts anticancer effects by inhibiting histone deacetylase enzymes, thus inducing increased histone acetylation levels, elaboration of chromatin structure, and consequent reactivation of aberrantly silenced genes.^[7] Previous studies showed that several molecular pathways are affected by NaBu treatment. In particular, cyclin dependent protein kinase (CDK) down-regulation,^[8] cell-cycle inhibitors up-regulation, together with modulation of several apoptosis-related genes were observed.^[9,10]

Finally, the described modulation of gene expression

induced in HeLa cells, when subjected to CDDP or NaBu exposure, was compared with the results obtained by our RT-qPCR array using the same biological model of study.

METHODS

Cell culture and treatments

HeLa cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). All cellular populations were cultured in Dulbecco's Modified Eagle Medium (DMEM, Cambrex, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% glutamine.

HeLa cells in exponential phase of growth were subjected to treatment with 10 μ mol/L CDDP (Sigma-Aldrich, St Louis, MO, USA) or 5 mmol/L NaBu for 24 h and 16 h, respectively. At the end of the treatment period, viability of cellular populations was analyzed by the trypan blue dye exclusion method.^[11]

Primers design

Primer pairs, purchased from Primm S.r.l. (Milan, Italy) and listed in [Table 1](#), were designed with Primer Express 2.0 Abi Prism software (PE Applied Biosystem, Foster City, CA, USA) as previously described,^[12] employing common design parameters [[Table 2](#)]. All amplicons primers, except for those amplifying an intronic region of GAPDH (control for possible genomic DNA contamination), were designed encompassing exon-exon boundaries to avoid genomic DNA amplification. The specificity of amplicons and primer pairs was checked *in silico* using BLAT (UCSC Genome Browser) and BLAST (National Center for Biotechnology Information) alignment tools.

RNA extraction

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and following supplier's instructions. Elution was performed in a solution of 1 volume of PBS and 5 volumes of RNeasy Lysis Buffer. For total RNA extraction RNase-free water and RNase-free supplies were used. Total RNA concentration was measured with Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) and RNA integrity evaluated through 1.3% agarose gel electrophoresis, as described.^[13]

Reverse transcription

One microgram of RNA from each sample was retrotranscribed (RT) using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and T Personal thermocycler (Biometra), according to

Table 1: List of genes amplified, relative primers, and main pathways

Gene	Forward primer	Reverse primer	Length	Pathways ^a
<i>ABL1</i>	5'-TACCCGGAATTGACCTGTC	5'-GGATTTTCAGCAAAGGAGGGC	151	Cell cycle
<i>ATM</i>	5'-GGTATAGAAAAGCACCAGTCCAGTATTG	5'-CGTGAACACCGGACAAGAGTTT	152	DNA damage
<i>ATP5B</i>	5'-GTCTTCACAGGTCATATGGGGA	5'-ATGGGTCCCACCATATAGAAGG	122	Housekeeping
<i>ATR</i>	5'-AGTAGCTTCCTTCGCTCCAAA	5'-ACTGACTCCGGCCACTCCAT	154	DNA damage
<i>BAX</i>	5'-CAAACCTGGTGCTCAAGGCC	5'-GGGCGTCCCAAAGTAGGAGA	151	Apoptosis
<i>BCL2</i>	5'-CTGGTGGACAACATCGCCCT	5'-TCTTCAGAGACAGCCAGGAGAAAT	151	Apoptosis
<i>BIRC5</i>	5'-CCGGTTGCGCTTTCCTTTC	5'-CGCACTTTCCTCGCAGTTTC	151	Apoptosis
<i>BRCA1</i>	5'-GCATGCTGAAACTTCTCAACCA	5'-GTGTCAAGCTGAAAAGCACAATGA	151	DNA damage
<i>BRCA2</i>	5'-AGACTGTACTTCAGGGCCGTACA	5'-GGCTGAGACAGGTGTGGAACA	151	DNA damage
<i>CCNA2</i>	5'-AGTAAACAGCCTGCGTTCACC	5'-GAGGGACCAATGGTTTTCTGG	151	Cell cycle
<i>CCNB1</i>	5'-ATGACATGGTGCCTTTCCTCC	5'-GCCAGGTGCTGCATAACTGG	151	Cell cycle
<i>CCNB2</i>	5'-GATAACGAAGATTGGGAGAACCC	5'-CCACTAGGATGGCACGCATG	151	Cell cycle
<i>CCND1</i>	5'-TGAAGGAGACCATCCCCCTG	5'-TGTTCAATGAAATCGTGCGG	151	Cell cycle
<i>CCNE1</i>	5'-AAATGGCCAAAATCGACAGG	5'-CGAGGCTTGCACGTTGAGTT	151	Cell cycle
<i>CDC2</i>	5'-ACAGGTCAAGTGGTAGCCATGA	5'-ACCTGGAATCCTGCATAAGCA	151	Cell cycle
<i>CDC16</i>	5'-ATGCTGAGGCCTTGATTACC	5'-TCTCGCCTAAGACCAAGGGC	151	Cell cycle
<i>CDC20</i>	5'-AGATGGACGACATTTGGCCA	5'-ATTGGACTGCCAGGGACACC	151	Cell cycle
<i>CDC34</i>	5'-GGATTCCGCGTGACACTGGT	5'-ACCGAAAGCTGGTGGAGAG	151	Cell cycle
<i>CDK2</i>	5'-TTCTCATCGGGTCTCTCACC	5'-TCGGTACCACAGGGTCACCA	151	Cell cycle
<i>CDK4</i>	5'-CTGTGCCACATCCGAACTG	5'-GCCTCTTAGAACTGGCGCA	151	Cell cycle
<i>CDK6</i>	5'-CCGAAGTCTTGCTCCAGTCC	5'-GGGAGTCCAATCACGTCCAA	151	Cell cycle
<i>CDK7</i>	5'-TCACATCTTCAGTGCAGCAGG	5'-TGGCAGCTGACATCCAGGT	151	Cell cycle
<i>CDK8</i>	5'-AGCGGGTCGAGGACCTGTTT	5'-CATGCCGACATAGAGATCCCAG	151	Cell cycle
<i>CDKN1A</i>	5'-TACCCTGTGCCTCGCTCAG	5'-GGCGGATTAGGGCTTCTCT	151	Cell cycle - senescence
<i>CDKN1B</i>	5'-AGACTGATCCGTCGGACAGC	5'-CACAGAACCGGCATTTGGG	152	Cell cycle - senescence
<i>CDKN2A</i>	5'-CAACGCACCGAATAGTTACGG	5'-CTGCCATCATCATGACCTG	54	Cell cycle - senescence
<i>CDKN2B</i>	5'-ATCCCAACGGAGTCAACCG	5'-CTGCCATCATCATGACCTG	58	Cell cycle - senescence
<i>CDKN3</i>	5'-TGAAGCCGCCAGTTCAATA	5'-CAACCTGGAAGAGCACATAAACC	151	Cell cycle
<i>CHEK1</i>	5'-GAGCGTTTGTGAACAAGATGTG	5'-GTTGGTCCCATGGCAATTCT	151	Cell cycle
<i>CHEK2</i>	5'-TCAGACAAGAGGCAGACACC	5'-ACAGCTCTCCCCCTCCATC	151	Cell cycle
<i>CUL3</i>	5'-GGTAAACACACAGCGGGT	5'-CTGGGTGCGATTACCTTGT	151	Cell cycle
<i>DNMT1</i>	5'-AGAACGCCTTTAAGCGCCG	5'-CCGTCCACTGCCACCAAAT	110	Cell proliferation
<i>E2F4</i>	5'-GCATCCAGTGAAGGGTGTG	5'-ACGTTCCGGATGCTCTGCT	151	Cell cycle
<i>GADD45A</i>	5'-GATGCCCTGGAGGAAGTGCT	5'-GAGCCACATCTCTGTCGTCGT	151	Cell cycle - senescence
<i>GAPDH</i>	5'-GCAAATTCATGGCACCGT	5'-TCGCCCCACTTGATTTTGG	106	Housekeeping
<i>KNTC1</i>	5'-ATAGTCAACCCAGAGTGGGCTGT	5'-TTTACGTTTTTCGTCCTGCG	151	Cell cycle
<i>MCM2</i>	5'-TGCCACTGTCCTAGCCA	5'-GATGGAAGGCAATGCTGG	151	Cell cycle
<i>MKI67</i>	5'-TGTGCTGTCTCAGCCCTACA	5'-TGAAATAGCGATGTGACATGTGCT	151	Cell proliferation
<i>PCNA</i>	5'-TTTGGTGCAGCTCACCCTG	5'-CGCGTTATCTTCGGCCCTTA	151	Cell proliferation
<i>RB1</i>	5'-GACCCAGAAGCCATTGAAATCT	5'-GGTGTGCTGGAAGGGTCC	151	Cell cycle
<i>RPA3</i>	5'-TTCGTAGGGAGGCTGGAAAA	5'-CCTTGGCGTTACTCTTCCAA	151	DNA damage
<i>RPLP0</i>	5'-TTCATTGTGGGAGCAGAC	5'-CAGCAGTTTCTCCAGAGC	156	Housekeeping
<i>TP53</i>	5'-GCGTGTGTTGTCCTGTCCTG	5'-TGGTTTCTCTTTGGCTGGG	151	Cell cycle - senescence
<i>UBA1</i>	5'-CCATAAACGCCTTCATTGGG	5'-TGGAGGCACTGTCTCTGTG	151	Cell cycle

^aMain molecular pathways in which genes are known to be involved

manufacturer's protocol. Briefly, the RT assay was conducted with 1 µL Oligo (dT) 500 mg/mL, 1 µL dNTPs mix 10 mmol/L, each, 1 µg of total RNA and sterile RNase-free water up to 13 µL final volume. The RT mix was heated to 65 °C for 5 min, enriched with 4 µL of first-strand buffer 5X and 2 µL of DTT 0.1 mol/L, and then incubated at 42 °C for 2 min with addition of 1 µL SuperScript II RT. Lastly, 20 µL of final solution

was incubated at 42 °C for 50 min and successively warmed up to 72 °C for 15 min.

Quantitative real-time PCR

Quantitative real-time PCRs (qPCR) were performed with Rotor-Gene 6000 (Corbett Life Sciences, Sydney, Australia) in 100-wells Gene Discs, using a final volume reaction of 15 µL containing 0.3 µmol/L of each

Table 2: Parameters employed for primers design

Primer Tm requirements	Min Tm (°C)	58
	Max Tm (°C)	62
	Optimal Tm (°C)	60
	Max Tm Difference (°C)	2
Primer GC content requirements	Min GC (%)	40
	Max GC (%)	60
	Min length (bp)	12
Primer length requirements	Max length (bp)	40
	Optimal length (bp)	20
	Min length (bp)	150
Amplicon requirements	Max length (bp)	250

Tm: melting temperature

forward and reverse primer, 1x SYBR Green mastermix (as indicated in Results section) and 0.2 µL of cDNA solution, as described.^[14] The following thermal profile was applied: 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 10 s, 64 °C for 30 s, and 72 °C for 15 s. Melting curve analysis was performed ramping from 60 °C to 90 °C and rising by 0.5 °C every 2 s.

Gene expression variations were evaluated in term of fold induction respect to the untreated cellular population (control) by both the $2^{-\Delta\Delta CT}$ method and 'Comparative Quantitation' tool of the Rotor Gene 6000 Series software 1.7. Expression stability values of the different housekeeping genes were calculated by Norm Finder software^[15] to choose the best reference gene for normalization. Filtering of results was carried out as follows: genes were considered differently expressed when their change was greater than ± 2.5 fold respect to the transcript levels of untreated sample, as already described.^[16] All experiments were conducted in triplicate.

RESULTS

RT-qPCR array design

The RT-qPCR array was developed to study modulation of transcript abundance of 41 human genes involved in regulation of key cellular pathways, such as cell cycle, DNA damage, cellular proliferation, apoptosis, and senescence [Table 1]. The RT-qPCR array was designed exploiting 100-well discs compatible with the Rotor-Gene 6000 instrument [Figure 1], but it could be easily adapted to standard 96-well plates. Most importantly, our array was designed to harbor several technical controls to statistically evaluate final results and to exclude possible experimental bias. Six wells were reserved for no template controls (NTC) to monitor possible contamination (amplifying 3 housekeeping genes *GAPDH*, *RPLP0*, and *ATP5B*-yellow disc section, Figure 1). In addition, one primer pair was designed to amplify part of an intronic region of the *GAPDH* gene in order to detect possible genomic DNA contaminations resulting from the RNA extraction

procedure (purple disc section, Figure 1). The same *GAPDH*-primer pair was used to amplify commercial genomic DNA as both positive PCR reaction control and internal standard control to compare the efficiency of amplifications performed at different times in different discs (orange disc section, Figure 1).

In order to better control the results, and also to extend the applicability of the assay to different experimental conditions, we designed the RT-qPCR array, including primer pairs able to amplify the 3 stable and housekeeping gene transcripts *GAPDH*, *RPLP0* and *ATP5B*, to be used as reference for gene expression normalization. All reactions were placed in the 100-well disc in duplicate (red disc section, Figure 1).

Primers were designed following parameters reported in Table 2 in order to optimize and make uniform all PCR reactions of the array. To achieve the best results, we chose primer pairs with the lowest penalty value given by the PrimerExpress 2.0 software.

PCR conditions optimization

In order to optimize the PCR experimental condition, we evaluated: (1) primer efficiency by analyzing the slope of the real-time amplification curves; (2) absence of primer-dimer amplification; (3) specificity of the product; (4) absence of unspecific products by both agarose gel electrophoresis (AGE), and analysis of the melting curve profiles generated after PCR amplification. First, we compared 4 different commercially available master mixes and selected the 1 that, in our conditions, gave the best results in terms

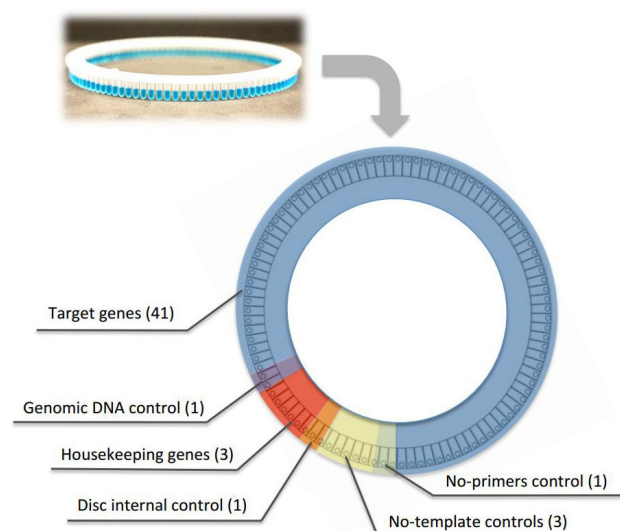


Figure 1: Schematic representation of the RT-qPCR array design. Distribution of the different primer pairs and relative experimental controls using the Rotor Gene 100-wells disc. RT-qPCR: reverse transcription-quantitative polymerase chain reaction

of product specificity and amplification efficiency (FastStart SYBR Green Master produced by Roche, Figure 2A-D). Then, we tested different thermal profiles by changing the annealing step temperature (from 56 °C to 66 °C, data not shown), identifying 64 °C as the best annealing temperature. We demonstrated that, at the selected qPCR condition, the slope of the amplification curves was comparable in all array samples [Figure 3A]. In addition, all the different primer pairs generated a unique PCR product as observed through both AGE separation [Figure 3B] and melting curves analysis (data not shown), highlighting the specificity of all the amplifications and the absence of primer-dimer products.

Modulation of the gene expression profile induced by cisplatin (CDDP)

Once optimized, the RT-qPCR gene array was used to study effects induced by CDDP on HeLa cells. To this end, cells were treated with sublethal doses of CDDP (10 $\mu\text{mol/L}$ for 24 h), obtaining a reduction of cell survival equal to 72.1%.

Total RNA was extracted from treated and untreated

HeLa cells and its integrity was evaluated by monitoring the 28S to 18S rRNA ratio through AGE (data not shown). After fluorometric quantitation, 1 μg of RNA was retro-transcribed and the resulting cDNA used for qPCR analysis.

Gene expression profile modulations were evaluated comparing Ct values between treated and non-treated cells, using the $2^{-\Delta\Delta\text{Ct}}$ method. HeLa cells treated with CDDP showed a clear increase of the abundance of the 2 cell cycle inhibitors CDKN1A (+4.93 fold) and CDKN2B (+7.24 fold), as well as of GADD45A (+23.1 fold, Figure 4A). In addition, other genes playing key functions in both DNA damage response and cell cycle regulation were found to be up-regulated, such as BRCA1 (+2.73 fold) and cyclin dependent kinases 1 and 2 (CDK1, +3.12 fold; CDK2, +2.74 fold), while a significant down-regulation of anti-apoptotic gene *BCL2* was observed (-3.63 fold, Figure 4A). Notably, all these regulations were confirmed applying the comparative quantitation method available on Rotor Gene 6000 Series software 1.7 (data not shown). Amplification efficiency was checked by monitoring the slope of

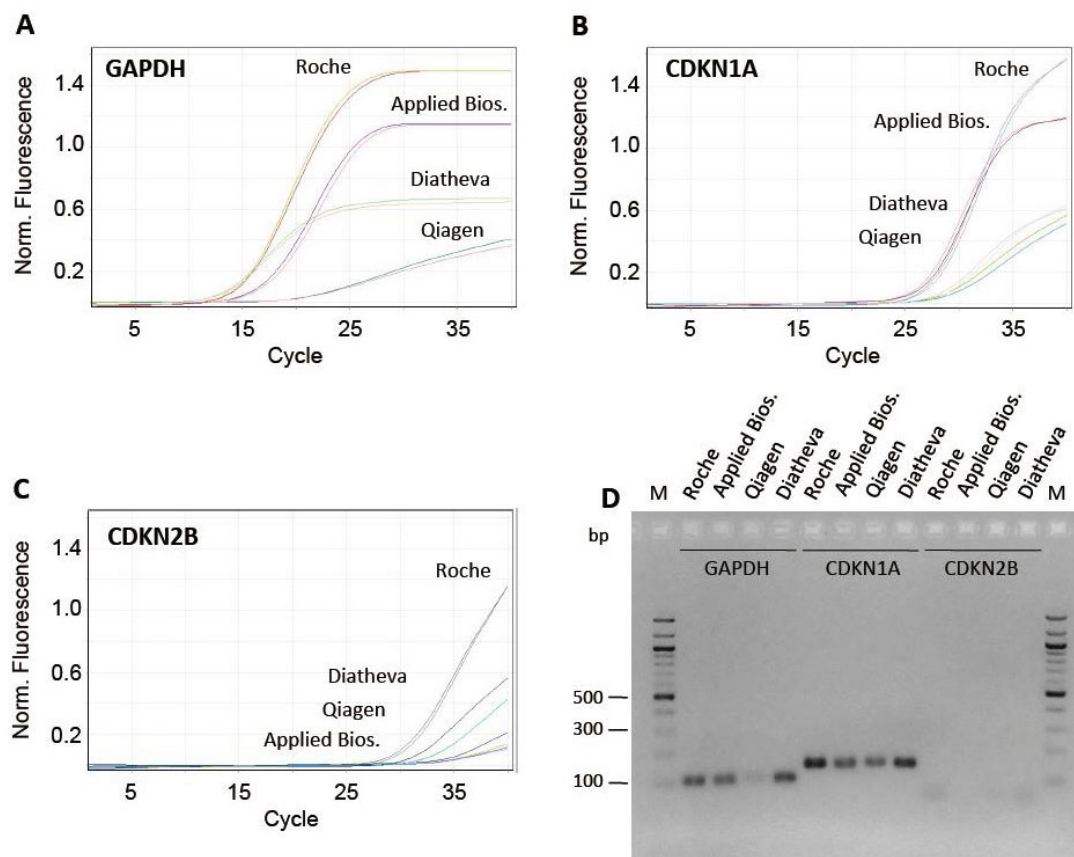


Figure 2: Test of different commercially available master mixes. (A-C) profiles relative to amplification of cDNA using primer pairs for GAPDH, CDKN1A and CDKN2B genes and the following commercially available SYBR-green master mixes: (1) FastStart SYBR Green Master (Roche); (2) SYBR Select Master Mix (Applied Biosystems); (3) RT2 SYBR Green FAST MasterMix (Qiagen); (4) 2X PCR Master Mix (Diatheva). (D) 2% agarose gel electrophoresis of GAPDH, CDKN1A and CDKN2B PCR products. PCR: polymerase chain reaction

amplification curves generated during real-time amplification [Figure 4B and C], while specificity was confirmed by analyzing the uniqueness of the PCR product by melting curve peaks analysis [Figure 4D and E] and AGE (data not shown).

Modulation of gene expression profile induced by sodium butyrate (NaBu)

Subsequently, the RT-qPCR array was used to investigate the effects induced by histone deacetylase inhibitor sodium butyrate (NaBu) on HeLa cells. Even in this case, cells were subjected to sublethal doses of NaBu (5 mmol/L for 16 h) to achieve a reduction of cell survival equal to 68.5%.

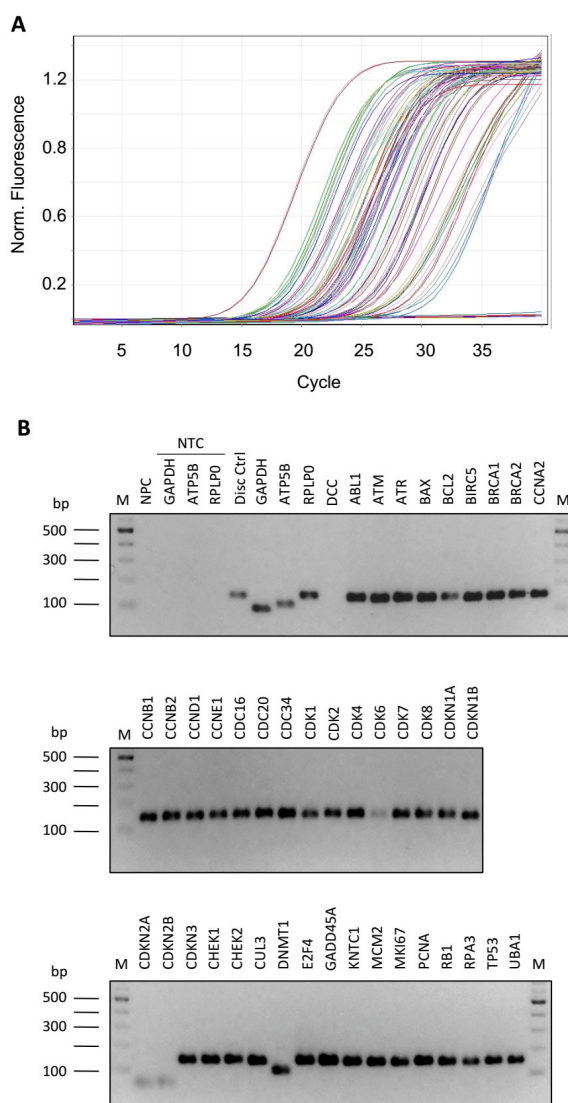


Figure 3: Optimization of PCR conditions. Amplification efficiency and specificity of all gene transcripts of RT-qPCR array. (A) Amplification plot showing profiles slope of different PCR reactions; (B) 2% agarose gel electrophoresis of all array amplicons. PCR: polymerase chain reaction; RT-qPCR: reverse transcription-quantitative polymerase chain reaction; M: 100 bp DNA ladder; NPC: no-primers control; NTC: no-template controls

Total RNA was extracted from treated and untreated HeLa cells and its integrity evaluated by monitoring 28S to 18S rRNA ratio through AGE (data not shown). After fluorometric quantitation, 1 μ g of RNA was retro-transcribed and the resulting cDNA used for qPCR analysis.

As for CCDP treatments, gene expression profile modulations were evaluated comparing Ct values between treated and not treated cells, using the $2^{-\Delta\Delta Ct}$ method. NaBu-treated HeLa cells showed down-regulation of cyclins A2 (CCNA2, -2.86 fold), B1 (CCNB1, -2.78 fold), and D1 (CCND1, -9.09 fold), as well as of cyclin-dependent kinase CDK6 (-5.00 fold, Figure 5A). A significant reduction of transcript abundance was monitored also for marker of proliferation MKI67 (-4.76 fold). Moreover, the array showed up-regulation of genes encoding for cyclin-dependent kinase inhibitors CDKN1A (+2.82 fold), CDKN1B (+3.65 fold), and CDKN2B (+3.88 fold), and of GADD45A (+2.72 fold, Figure 5A). Again, all regulations emerged from these analyses were confirmed applying the comparative quantitation method mentioned above (data not shown).

Analysis of the amplification curves showed that the efficiency of the PCR reaction was similar between the different primer pairs and samples [Figure 5B and C], while the peaks of the melting curves demonstrated the specificity of the PCR product [Figure 5D and E].

DISCUSSION

Here we report the design and validation of a RT-qPCR array that allows the reliable study of gene expression modulations occurring in biological models exposed to drug treatments or to any other different culture condition. In order to better control the results, and also to extend the applicability of the assay to different experimental requirements, we designed a RT-qPCR array to include primers able to amplify 3 different housekeeping genes *GAPDH*, *RPLP0*, and *ATP5B*. The presence of these 3 control genes is extremely important mainly for two reasons: (1) it is unlikely that there exists a “universal” housekeeping gene whose expression can be considered as reference for RNA normalization in any biological model (or treatment) under investigation; (2) despite the control of both the amount and integrity of RNA extracted from different samples, the RT-qPCR could be impaired also by additional experimental variables (e.g. the limit of spectrophotometric analysis, not homogeneous RNA purity).

Furthermore, to monitor possible DNA contamination

from the RNA extraction procedure, we also designed a primer pair that amplified an intronic region of the housekeeping gene *GAPDH*. Importantly, since each sample of cDNA would be amplified in a separated 100-well disc, we also added an additional control to verify the “inter-disc” variability by using, as template, a known amount of human genomic DNA and a primer pair that amplified genomic *GAPDH*.

To further increase the reliability of the assay, amplification of each target gene was performed in duplicate. Most commercially available PCR arrays contain more target genes but lacks replicates (e.g. 84 genes using the RT² Profiler™ PCR arrays by Qiagen, 92 genes using TaqMan Array Plates by

Thermo Fisher Scientific, 88 genes using real Time PCR Assay Panels by BioRad), increasing the risk of not identifying possible technical errors.

Prior to using the array in an experimental model, we tested 4 commercially available SYBR Green master mixes, focusing attention on 3 genes that can recapitulate 3 different levels of gene expression: high levels (*GAPDH*), medium/low levels (*CDKN1A*), and very low levels (*CDKN2B*). In fact, it is known that expression level of some genes involved in the DNA-damage response (e.g. genes that are involved in cell cycle regulation, such as CDK-inhibitors) are at the limit of the detection and thus require a highly efficient DNA-polymerase. Therefore, we optimized our RT-

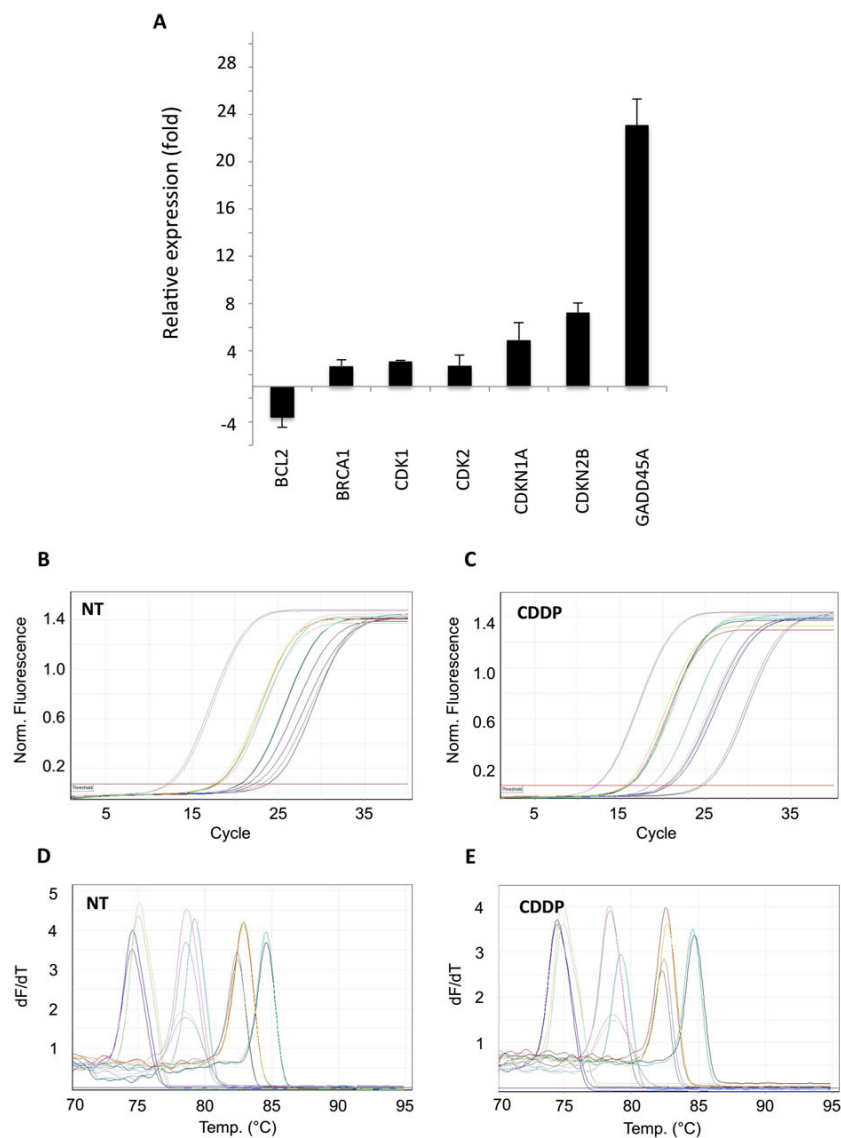


Figure 4: Application of PCR array to cisplatin (CDDP)-treated HeLa cells. Difference in transcriptional activity of cisplatin-treated HeLa cells, compared to non-treated cells, evaluated by $2^{-\Delta\Delta CT}$ method. Data reported as mean \pm standard deviation of 3 independent experiments. (A) Relative expression of differentially expressed genes; (B-E) examples of qPCR amplification plots and melting curves of genes found differentially expressed. PCR: polymerase chain reaction

qPCR array using SYBR-green master mix that showed the best efficiency in our conditions. This allowed us to monitor transcript levels of low-expressed genes (e.g. *CDKN2B*) [Figure 2B-D].

Then, the RT-qPCR array was validated using HeLa cells subjected to treatment with two different drugs, CDDP and NaBu, known to induce important modulations of the gene expression profile of this cellular model.

In particular, CDDP treatment was able to up-regulate growth arrest and DNA damage response genes *GADD45A* and *BRCA1*, as well as cell cycle inhibitors genes *CDKN1A* and *CDKN2B*. These transcriptional

elaborations have been widely documented in HeLa cells treated by CDDP.^[5,6,17,18] In addition, a significant down-regulation of anti-apoptotic gene *BCL2* also confirmed previously reported data.^[5,19] All these modulations were largely expected since CDDP is known to exert its activity by targeting DNA of cells and forming covalent adducts that lead to activation of the cellular DNA damage response.^[4] In fact, once damaged, cells respond by inhibiting its progression through the cell cycle and, in case of extensive damage, by activating the apoptotic cell death program.

Interestingly, we found that CDDP also induces up-regulation of cyclin dependent kinases 1 and 2 (CDK1 and CDK2). This result, which is to our knowledge the

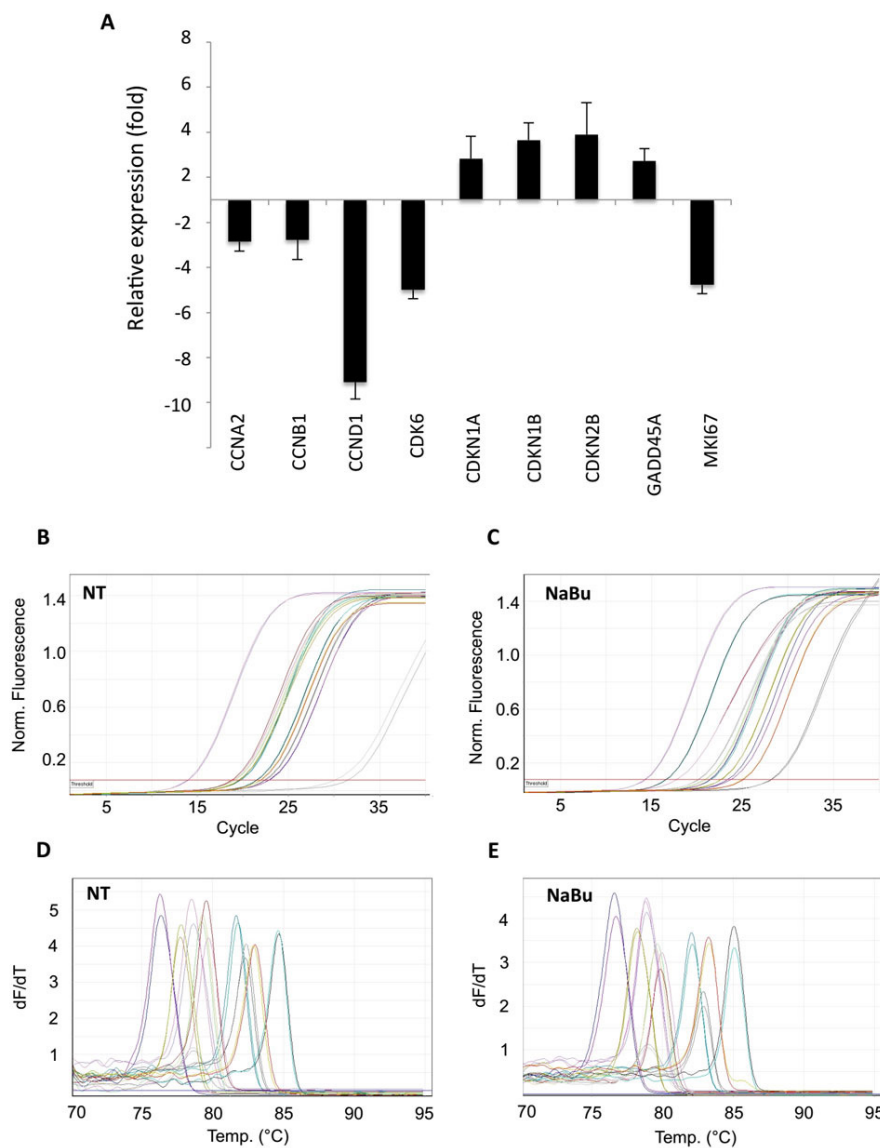


Figure 5: Application of the PCR array to sodium butyrate (NaBu)-treated HeLa cells. Difference in transcriptional activity of sodium butyrate-treated HeLa cells, compared to non-treated cells, evaluated by $2^{-\Delta\Delta CT}$ method. Data reported as mean \pm standard deviation of 3 independent experiments. (A) Relative expression of differentially expressed genes; (B-E) examples of qPCR amplification plots and melting curves of differentially expressed genes. PCR: polymerase chain reaction

first observation made in HeLa cells, could be put in relation with the known role of these CDKs in apoptosis, in addition to their known functions in regulation of cell cycle progression.^[20] This hypothesis is supported by evidence that CDK2 silencing by shRNA decreases cisplatin-induced apoptosis and PARP cleavage in embryonal carcinoma models^[21] and opens the door to possible future insights.

Regarding the effects of NaBu, our data confirm the down-regulation of cyclins and CDK6 and the up-regulation of three different CDK inhibitors,^[9,10] which reflect the long-known ability of this histone deacetylase inhibitor to regulate the progression of cells through the cell cycle.

Even in this case, the RT-qPCR array allowed us to provide new information about transcriptional activity of the genes under investigation. In fact, NaBu also induced in HeLa down-regulation of the gene encoding for the anti-proliferative antigen MKI67, and up-regulation of GADD45A, a typical sensor of stress involved in growth arrest and in the DNA damage response. These new observations, although already described in other cancer models (MKI67 in prostate cancer^[22,23] and GADD45A in colon carcinoma^[24]), have never been reported in HeLa cells subjected to NaBu treatment.

In summary, we have designed, optimized, and biologically validated an RT-qPCR array that can be exploited to robustly analyze expression of genes involved in cell cycle regulation, DNA damage response, apoptosis, and senescence induction.

This array represents a reliable, inexpensive, and rapid tool that could be exploited to provide an overview of molecular pathways activated by drug treatment in human cancer cells. Moreover, we believe that detailed description of the procedure will allow application of the RT-qPCR array in different experimental conditions and could contribute to understand the molecular mechanisms of action of new drugs in the context of pharmacologic studies.

Authors' contributions

Study conception and design: M. Fanelli, S. Amatori
Primers design and PCR conditions optimization: S. Amatori

Validation of the RT-qPCR array: G. Persico

Manuscript writing and editing: M. Fanelli, S. Amatori

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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