

Milli-Q® Biocel System

Optimized Ultrapure Water Quality for PCR

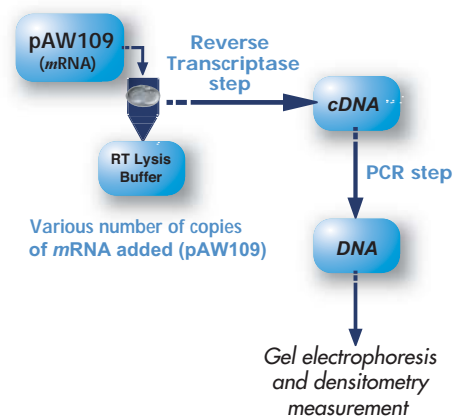
Introduction

Polymerase chain reaction (PCR) has become a well-known and widely-used technique for amplifying DNA molecules from a few DNA strands. Related techniques, such as reverse-transcriptase-PCR (RT-PCR) and quantitative real-time PCR, have emerged and developed as useful tools for gene expression studies and diagnostic assays and in fields such as pharmacogenomics and toxicogenomics.

While many of the parameters considered in PCR protocols have already been extensively studied and documented (e.g. ionic strength, pH, temperature, Mg concentration, primer length and structure and enzyme structures), the importance of the quality of water used to prepare buffer solutions throughout the experiment had still to be investigated.

The RT-PCR experiment can be formally divided into two steps (Figure 1): the retro-transcription of the *mRNA* strand to generate a *cDNA* strand, and the amplification (PCR) of this newly formed *cDNA* to obtain thousands of copies of double-stranded DNA molecules. The PCR reaction is a series of cycles during which the temperature is raised and lowered for defined periods of time. During each PCR cycle the number of DNA molecules is doubled.

Figure 1: RT-PCR sequence



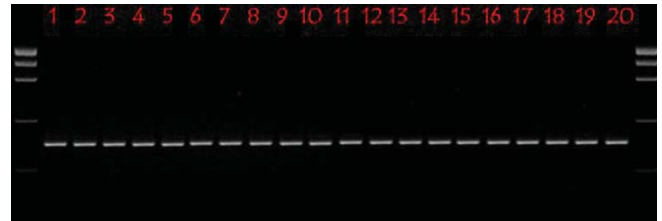
Results and Discussion

Known numbers of copies of *mRNA* (pAW109) were added to cell lysates, and the amounts of DNA obtained at the end of the RT-PCR were monitored by gel electrophoresis and quantified by densitometry measurement. Because the pAW109 *mRNA* has a known sequence of 305 bases, the theoretical quantities of DNA molecules expected at the end of the RT-PCR process could be anticipated and calculated if the experiment was quantitative at all steps (Figure 2). A theoretical curve can be built, corresponding to the maximum obtainable quantities of DNA when all the *mRNA* molecules are retro-transcribed and when every cycle of the PCR doubles the number of DNA copies.

Water, used to run the RT-PCR experiments, was purified via a combination of technologies. Reverse osmosis, a filtration method which removes the bulk of contaminants, was used as a pretreatment step, and water was further purified using the Milli-Q Biocel water purification system. This system combines high-grade mixed-bed ion-exchange resins, activated carbon and ultrafiltration. The quality of ultrapure water delivered at the point-of-use has the following characteristics:

- Resistivity: 18.2 M Ω ·cm @ 25 °C
- Total Organic Carbon: < 10 ppb
- RNase concentration = 0.003 ng/mL (below detection limits) - No RNase activity detected (using a fluorometric assay)

Figure 3: DNA bands obtained via RT-PCR



All three parameters are important for the success of the RT-PCR process. Low ionic concentration (water resistivity = 18.2 M Ω ·cm @ 25 °C) avoids interference with the enzymes present from ions such as Cd or Fe and prevents changes in the buffer concentrations of critical ions such as Mg and K. Organic molecules can also interact with enzyme active sites as well as with primers, DNA and RNA due to van der Waals forces and electrostatic bonds. Lowering the total organic carbon content to a few μ g/L (ppb) allows enzymes to be fully active and free nucleic acid molecules to be continuously available. Ribonucleases, that would obviously be an issue in the RT step, are removed via ultrafiltration.

These results highlight two very important points regarding:

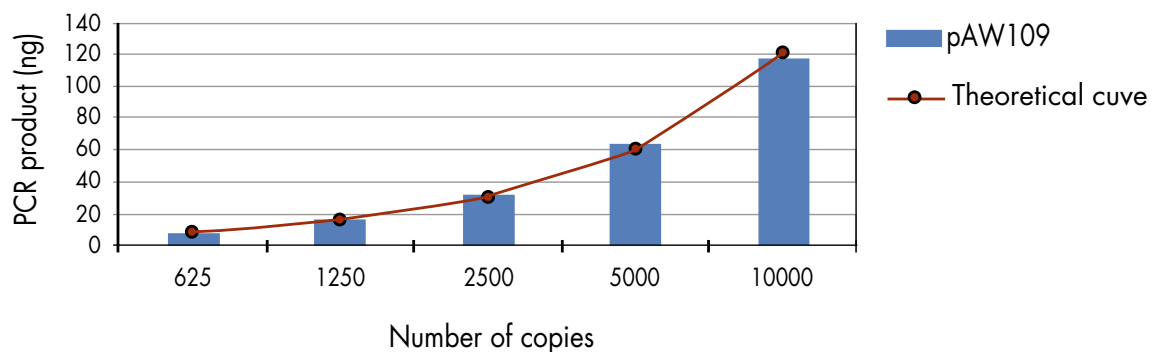
- *the ability to do quantitative RT-PCR assays.*

Data obtained with the RT-PCR process using Milli-Q Biocel water show that the amount of DNA generated exactly follows the theoretical curve (Figure 2).

- *the reproducibility of the assay.*

DNA bands obtained via the RT-PCR process have very similar intensities on the gel electrophoresis. Additionally the standard deviation in densitometry measurements is very small (Figure 3).

Figure 2: Quantitative RT-PCR: correlation between theoretical and experimental data





Conclusion

Quantitative and reproducible PCR results are achievable using high quality water (low conductivity and low TOC levels), that has been further purified by ultrafiltration, designed to efficiently remove RNases.

Material and Methods

Water purification system

Tap water (Pavia, Italy), purified using a RiOs™ reverse osmosis system (Millipore Corp, Billerica, MA), was stored in a PE reservoir designed to minimize the risks of water degradation by material extractables or airborne environmental contaminants, such as bacteria, particulates, CO₂ or solvents. Final purification was performed using a Milli-Q Biocel ultrapure water system (Millipore).

Fluorometric detection

The fluorometric RNaseAlert® test kit (Ambion Inc.) was used to test for RNases in ultrapure water. RNase Ambion® standard at 10 ng/mL was diluted using Ambion RNase-free water to obtain calibration curves. An SFM25 Kontron® Instrument fluorometer was used, with excitation set at 490 nm and emission at 520 nm, as indicated in the protocol.

RT-PCR methodology

Reagents and oligonucleotides for RT-PCR were purchased from Applera (Monza, Italy). The thermal cycler used was a GeneAmp® PCR System 9700 (Applera, Monza, Italy). To build an amplification curve, 625, 1250, 2500, 5000 or 10000 copies of pAW109 RNA were added to samples 1 to 5, respectively. Retro-transcription of RNA was performed in 20 µL of the following reaction mixture (final concentration): 3 µL sample, 1x PCR buffer (MgCl₂-free), 5 mM MgCl₂, 4 mM each dNTP, 2.5 µM oligo d(T)₁₆, 20 UE RNase inhibitor, 50 UE MuLV reverse transcriptase. Amplification was done via a two-step PCR process, with the first PCR reaction mixture prepared as follows (final concentration): 5 µL RT product, 1x PCR buffer (containing 1.5 mM MgCl₂), 200 µM each dNTP, 250 nM primer specific for pAW109 cDNA, 1.25 UE AmpliTaq™ polymerase. One microliter of the first PCR product was then used to individually re-amplify each single gene sequence, using 25 µL of the same reaction mixture of the first PCR with internal primers (nested PCR). Negative and positive controls were always run in each experiment.

Gel electrophoresis

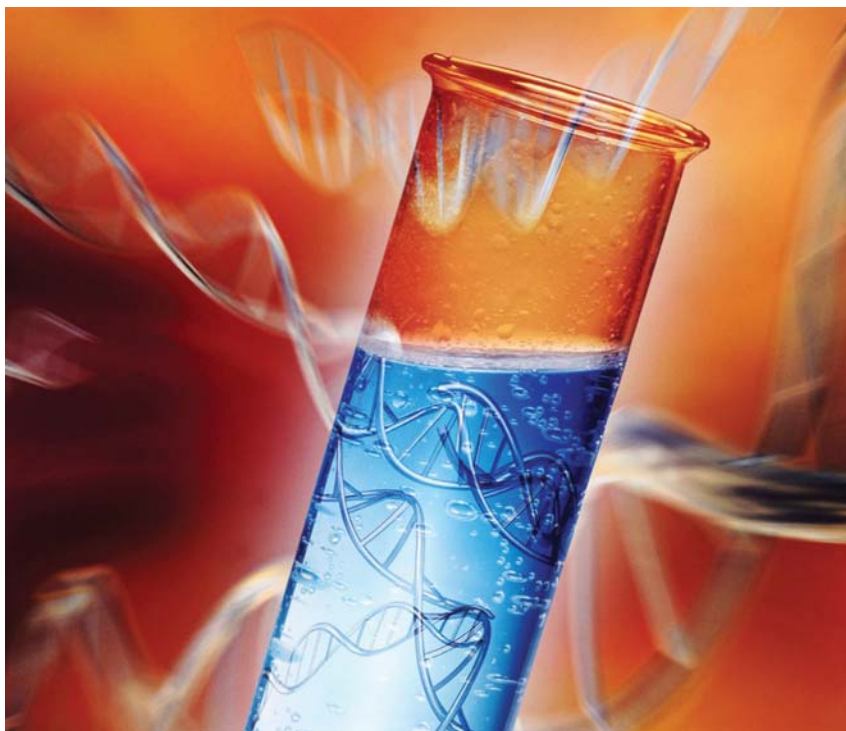
Ten microliters of each second PCR product were added to 4 µL loading buffer and run on a 2.5 % agarose gel in TBE 0.5 x at 6V/cm for 85 min. Low DNA Mass™ Ladder (Invitrogen, San Giuliano Milanese, Italy) was used as molecular weight marker and as a mass ruler. Bands were stained with ethidium bromide and visualized on a Bio-Rad Gel Doc™ instrument. The densitometric analysis was performed using Bio-Rad Quantity One® software. Statistical analysis was carried out using the ANOVA test (F test).

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Acknowledgments

Experiments were performed by Dr. L. Gentile and Dr. M. Zuccotti at the University of Pavia, Italy.

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