Methodology

A 100 µl mix was elaborated in accordance with the previously described protocol (see Table 12), and distributed among 4 different eppendorfs: two 25 µl positive controls, one 20 µl negative control and a 30 µl chip tube. BSA (2.5 µg/µl) was added to one of the positive controls (C+B) and to the chip tube, and Taq polymerase concentration was doubled in the chip tube. Template DNA was added in equal amounts (1 µl of 70 ng/µl template) to both positive controls and the chip tube. Finally, PCR protocols were adapted for fast thermocycling in the chip, as shown in Table 13, and used also for positive control amplification in the conventional thermocycler.

Results

Results, shown in Figure 114, indicated that, as in the previous assays, 2.5 µg/µl BSA did not interfere with amplification. They also proved that chip PCR amplification was quite efficient using a lower amount of DNA (1 µl of DNA in a 30 µl mix) than positive controls (1 µl of DNA in 25 µl mixes) and that fast thermocycling (including fast cooling) operation was possible with the new set of template and primers. Also, the experiments showed that, with short hold times, fast transient times were capable of doubling the speed of PCR. Therefore, it was decided to validate these hypotheses with additional experiments, in which DNA concentration was further diminished and reaction times sped up.

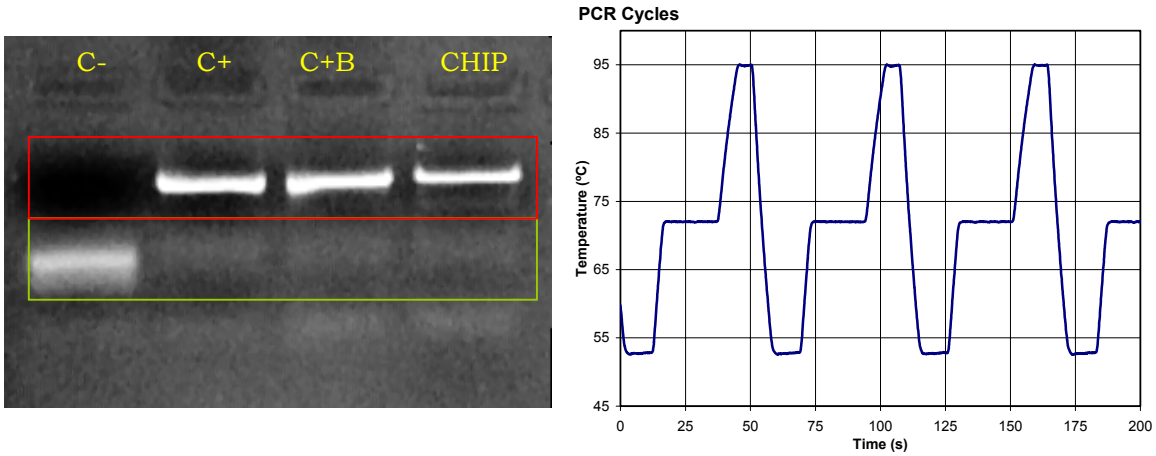


Figure 114 - Image of the PCR cycles and the 3% agarose gel (20 µl per well) for the initial validation of chip PCR amplification of the *hfq* gene (red box). Possible primer-trimer formation can be observed in the negative control lane and, more dimly, in the other lanes (green box). Total analysis time in conventional thermocycler: 1:37:42 h. Total analysis time in chip: 42:47 min.

Chip PCR optimization

In the following experiments, the effects of a diminishing DNA concentration were studied in PCR-chips, while different versions of PCR protocols were tried in order to determine the best-fitted protocol for chip PCR operation.

Halving of template DNA

Methodology

A first experiment was carried out to determine the sensitivity of PCR-chips and the robustness of the quick, spike-like, denaturation approach that the use of chips, and their fast thermal distribution, could theoretically rend feasible. In this assay, a 100 µl mix was prepared according to preceding protocols (see Table 12, p.208) and distributed into 20 µl negative and positive control tubes and two 30 µl chip tubes. A standard 2.5 µg/µl BSA concentration was added to all tubes, including the negative control, and doubled polymerase concentration was used in the chip tubes. Finally, DNA was added as follows: 1 µl to CHIP1 tube, 0.5 µl to CHIP2 tube and 0.66 µl (the equivalent concentration to 1 µl in a 30 µl mix) to the 20 µl positive control tube. The PCR protocol was adapted for faster thermocycling, reducing denaturation time to a short spike (1 s) and slightly increasing the denaturation temperature (95.5 ºC). The complete protocol can be seen in Table 14.

Cycling protocol:

95.5 ºC - 2 min
95.5 ºC - 1 s \
53 ºC - 10 s x45
72 ºC - 19 s /
72 ºC - 1 min
4 ºC - ∞

Table 14 - PCR protocol for fast thermocycling with spike denaturation.

Results

The assay results shown in Figure 115 were very promising. Not only did the chip PCR outdo the positive control at equivalent template concentrations (CHIP1), but it also exhibited a good sensitivity at half the original template concentration (CHIP2). Moreover, spike denaturation was

validated, even though analysis times remained in the whereabouts of 45 min due to the increased number of cycles.

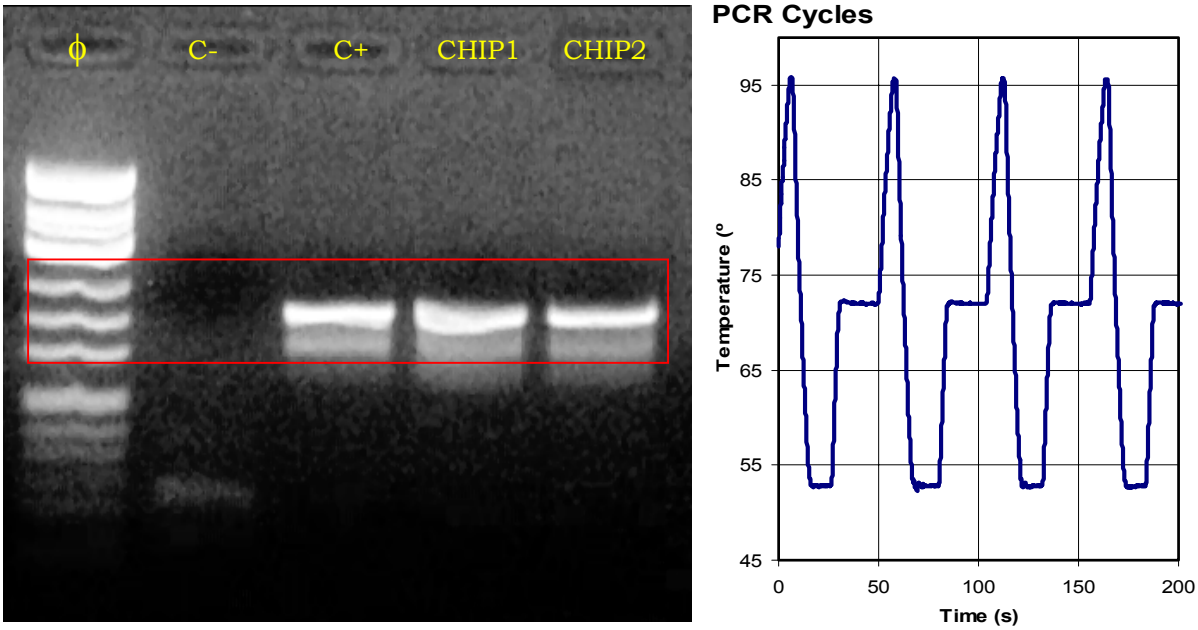


Figure 115 - PCR cycles and 3% agarose slab gel results (15 µl per well) for spike-denaturation PCR experiments. CHIP1 lane corresponds to the 1 µl template DNA mix and CHIP2 to the 0.5 µl template DNA mix. Total analysis time in conventional thermocycler: 1:31:23 h. Total analysis time in chip: 43:29 min.

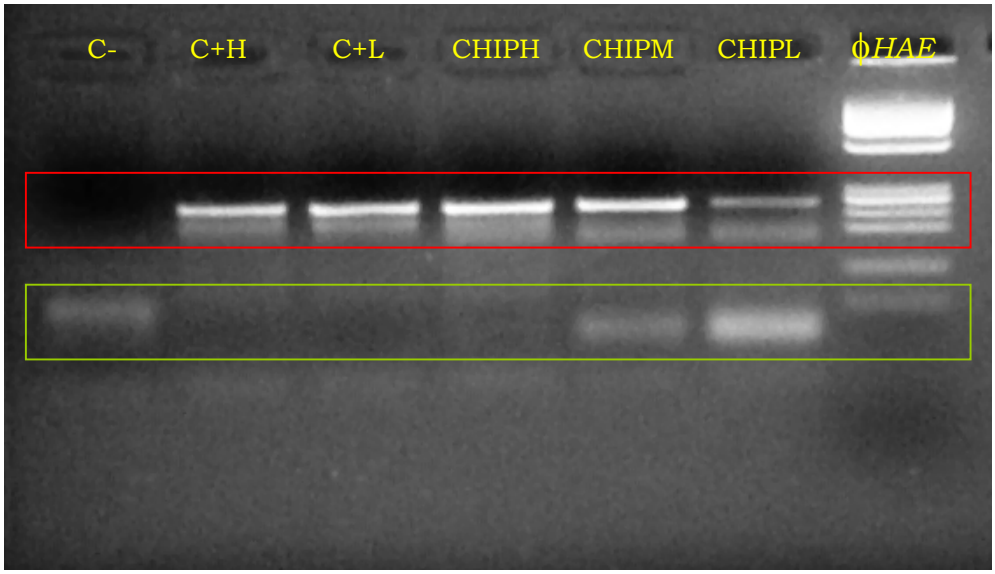
Sensitivity limit

Methodology

A further assay was done to evaluate the ultimate sensitivity of the chip and its correlation to standard thermocycling sensitivity. In this experiment, different template DNA concentrations were studied: 3.3%, 0.825% and 1.6% (v/v). A 150 µl mix was elaborated with a standard 2.5 µg/µl BSA concentration and distributed among three 20 µl tubes (two positive and one negative controls) and three 30 µl chip tubes, in which double polymerase concentration was maintained. Template DNA was then distributed as follows: 0.66 µl to C+H and 1 µl to CHIPH (3.3% v/v - high concentration), 0.165 µl to C+L and 0.25 µl to CHIPL (0.825% v/v - low concentration). Additionally, 0.5 µl (1.6% v/v) were added to a medium concentration chip tube (CHIPM). The PCR protocol used was the spike-denaturation one from the previous assay (see Table 14) and was applied both to chip and conventional thermocycler PCR amplifications.

Results

Gel analysis results (see Figure 116) indicated that chip sensitivity, under the present conditions, was somewhat lower than that of conventional thermocycler amplification. Nevertheless, it was again assessed that, over that sensitivity threshold, chip PCR yields were equal, or even superior, to conventional thermocycler ones, a remarkable fact when taking into account that analysis times had been halved. The occurrence of primer-trimer products was here validated too, since these appeared most in the low template-concentration and the negative control lanes, indicating that they were probably the product of unbound primers (green box).



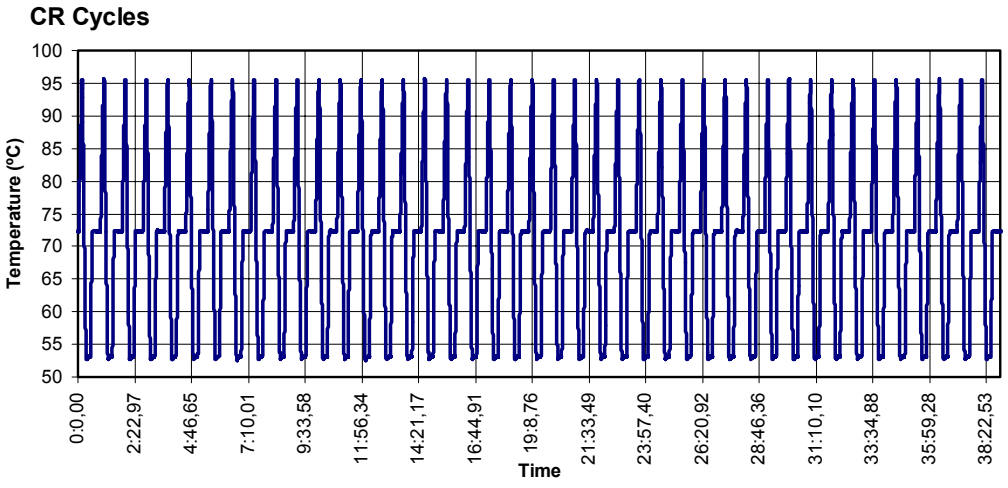


Figure 116 - Image of the 3% agarose slab gel results and the complete PCR cycles for CHIPM assay. There are 19 µl per well in all wells except in C+H (16 µl) due to a pipetting error. Total analysis time in conventional thermocycler: 1:30 h. Total analysis time in chip (CHIPM): 43:37 min.

5. ACTIVE PCR-CHIPS

5.1. INTRODUCTION

The integration of active heater/sensing elements in PCR-chips is a crucial step in the development of future fully automated chips that should be able to perform independent PCR reactions. Once thermal driving/sensing circuitry has been integrated into a PCR-chip, if the technological processes are still compatible with standard CMOS processes, there is no reason why signal conditioning, temperature control and even optical detection circuitry should not be included in such a device. This would yield a fully integrated chip capable of performing independent quantitative PCR assays in portable environments, with low power consumption and a high efficiency ratio, and, most importantly, such a device could be mass produced using standard silicon technology processes, rendering a disposable, one-use-only analysis system. Therefore, after the successful functional experimentation with passive PCR-chips described in the earlier section (see p.186 to 212), the integration of active heating/sensing circuitry was seen as one of the ultimate goals of the present research, in order to produce a working pre-industrial prototype that could be then extensively characterized and optimized with circuitry add-ons.

5.1.1. OBJECTIVES

With regard to this ultimate goal, a series of separate objectives were set to evaluate the feasibility and operational functionality of such devices. Basically, this set of partial objectives can be enumerated as follows:

- To determine the best electronic layer for integrating heating elements in current PCR-chips.
- To estimate the relevant design parameters concerning the chosen layer and present design restrictions and to create a set of prototypes for functional evaluation.
- To fabricate and functionally test different design alternatives in order to assess their efficiency both as heater and sensing elements.
- To integrate the best working design into the mainstream process for PCR-chip production.
- To fabricate and characterize active PCR-chips.

5.2. DESIGN RESTRICTIONS AND METHODOLOGY

5.2.1. HEATING/SENSING LAYER DETERMINATION

Principles

All conductor and semiconductor materials pose a certain resistance to the flow of current across them. The main part of this resistance work is dissipated in the form of heat, the amount of which follows Ohm's law and is determined by the thermal capacitance and the resistivity of the material. Thus, with relatively high current flows, conductor and semiconductor materials can be used to generate heat and/or other forms of electromagnetic radiation (as in the case of incandescent light bulbs). In addition, the measured resistance of a given material will vary depending on the temperature (approximately in a linear fashion over a limited temperature range), according to the material temperature coefficient of resistivity (TCR), and this provides the means for conducting temperature measures with conductor materials (such as the Pt100 resistor described earlier, see p.157).

Platinum vs. polysilicon

Conventional silicon processing technologies typically involve the deposition of a number of thin-film layers, with varying degrees of resistance and different thermal behaviors. Temperature sensing and heating is often attained through the deposition of platinum layers, a fact that has been exploited in PCR-chip development ([Poser1997], [Zhan2000]). Platinum offers a highly linear resistance deviation with temperature (TCR), which has been extensively typified in standard Pt100 resistors, and also provides a good thermal response at relative low voltages. However, the use of Pt layers imposes certain restrictions on technological processes, as the impossibility of undergoing further thermal oxidations (a fact already noted in the case of deep silicon etching, see p.122) or the need to use highly aggressive lift-off processes that pose incompatibilities and may damage other components of the system. Moreover, at the time this research was done, platinum deposition was not a standard fabrication process at CNM-IMB, a fact that posed a substantial restriction to its direct use as a standard material in PCR-chips.

In contrast to platinum, polysilicon is a standard material in classical CMOS technological processes. Even though native polysilicon is a very poor conductor [Erill2000b], it can be, and in fact is, routinely doped with boron impurities to produce layers of increasing and tightly controlled conductivity, and it also presents a quite linear TCR above freezing temperatures. Moreover, albeit at other temperature ranges (800-1200 ºC), polysilicon had already been evaluated for heat driving and sensing elements in gas sensors at CNM-IMB with successful results [Götz1996] and had also been previously reported as the heating/sensing element of PCR-chip devices [Northrup1993]. Therefore, even though the use of polysilicon as a temperature driver hinted at possibly larger power consumptions and though its use as a sensor was not as well documented as for Pt resistances, it was considered that polysilicon still presented important advantages over platinum for direct development of active PCR-chips at CNM-IMB, and it was arbitrarily determined to develop these systems using polysilicon as the main active layer, introducing a substantial technological restriction into design parameters.

5.2.2. PASSIVE CHIP DESIGN RESTRICTIONS

The other main design restrictions on active PCR-chips stemmed from the work previously done in passive PCR-chips. Having acknowledged more than once the multifaceted nature of PCR and its susceptibility to changing environments, it was decided to stick to the present passive PCR-chip designs in the initial stages of active PCR-chip research, in order to keep a tight rein on the volume of changes introduced in the device. Therefore, 40x10x0.3 mm^3 chip dimensions were maintained, together with the rhomboidal structure of the PCR reservoir. This meant that the dimensions of the rectangular section of the reservoir would be 5x24x0.2 mm^3, which was the main area polysilicon heaters had to cover.

System restrictions

A last restriction onto the design of active heating elements was the already developed circuitry for power driving the Peltier cell. Since part of the research on heater elements was carried out simultaneously to PCR optimization research with Peltier driven passive PCR-chips, the use of the same power driver for both systems was considered a convenient option, and this set a further restriction on active heaters: mainly, the maximum

power they could dissipate, which was limited by the power driver output of 80W, with a peak maximum voltage of 10 V.

5.2.3. METHODOLOGY

Once the heating layer and the underlying design restrictions had been set into place, it was decided to split work into separate steps to assess the main problems of heating/sensing circuitry integration. Instead of directly moving onto the integration of such elements in complete PCR-chip systems, it was decided to first develop a heater/sensor only batch of devices, in order to separately evaluate their operational characteristics as sensors and heaters, and then get into the integration of already prototyped heater/sensor elements into the mainstream PCR-chip process. Prior to the development of the first heater/sensor only chips, a series of simulations and theoretical approximations were undertaken to obtain an a priori knowledge of the polysilicon layer behavior and its most suitable designs for PCR-chip operation. It must be stressed here that the main purpose of polysilicon layer integration was to provide active heating elements to the PCR-chips, and that its future use as a sensing element was regarded as a welcome but secondary goal. Therefore, simulations and initial experimentation with polysilicon layers were oriented at the achievement of working heater elements, assessing that, from their passive-mode study, the necessary insight for constructing polysilicon sensor elements could be also derived.

5.3. DESIGN SIMULATION

Conventional heater designs for heater and sensing elements using platinum layers often rely on serpentine-like structures to cover the heated/sensed region in the most homogenous possible way. However, the big (centimeter order) dimensions of PCR-chips and the high-resistance (in contrast to platinum) of doped polysilicon layers suggested that such an approach could not be feasibly taken in these devices. Therefore, a set of simulations and theoretical approximations was carried out to assess which were the appropriate dimensions for a polysilicon resistor that would efficiently and homogeneously heat the PCR reservoir.