

Work plan

For all these reasons, it was decided to set a work plan with specific objectives focused at discarding possible sources of inefficiency while setting up the necessary equipment for taking full advantage of PCR-chips features. The following is an outline of the proposed work plan for optimization of PCR amplification in PCR-chips:

- Optimization and standardization of the technological process for production of PCR-chips.
- Development of novel designs and associated devices that would allow efficient insertion/extraction of reagents whilst minimizing the possible effects on PCR kinetics posed by serpentine-like structures.
- Design and development of an external fast thermocycler.
- Assessment and evaluation of alternative capping (sealing) methodologies and independent comparison of yields with acrylic tape based capping.
- Evaluation and optimization of passivation layers and their effects on PCR amplification.
- Optimization of the PCR mix and evaluation of adjuvants.

The following sections cover the development and experimental validation of the aforementioned points. As it happened in previous sections, the following is a sequential account of a mainly parallel research effort. Therefore, jumps across sections or repetitions of some instances will be, not without regret, unavoidable.

4.2. TECHNOLOGICAL PROCESS OPTIMIZATION

The basic technological process depicted above for serpentine-like chips was already an optimized version of preceding work with PCR-chips and, thus, after some deliberation concerning design issues (see next section), it was decided to maintain the same basic processes, which had already proven to be convincingly reliable and reproducible, in further PCR-chip developments, introducing only minor changes to the general procedure. These changes concern mainly the fabrication of some polysilicon-passivated PCR-chips to functionally assess their compatibility with PCR and the switch to thinner (0.5 mm) glass wafers in order to facilitate the potential incorporation of optical detection methods and to minimize the

thermal mass of the devices. The details of these technological changes and their integration into the mainstream technological process, together with a detailed discussion of the later, can be found in *Materials and Methods*, p.279.

4.3. CHIP RE-DESIGN

As it has been previously stated, design issues related to insertion/extraction procedures or to effects on PCR kinetics have not been largely addressed in the literature. From previous experimentation (see p.131), it was known that rectangular reservoirs with centered or side located access ports yielded difficult and irreproducible insertions and extractions, and that placing silicon-etched access ports directly onto the PCR chamber membrane resulted in a high rate of membrane breakages. Conversely, experimentation with serpentine-like chips had positively demonstrated that efficient insertion and extraction of reagents were feasible in PCR-chips, but at the cost of unknown effects on the kinetics of PCR due to the excessive linearity of the PCR chamber.

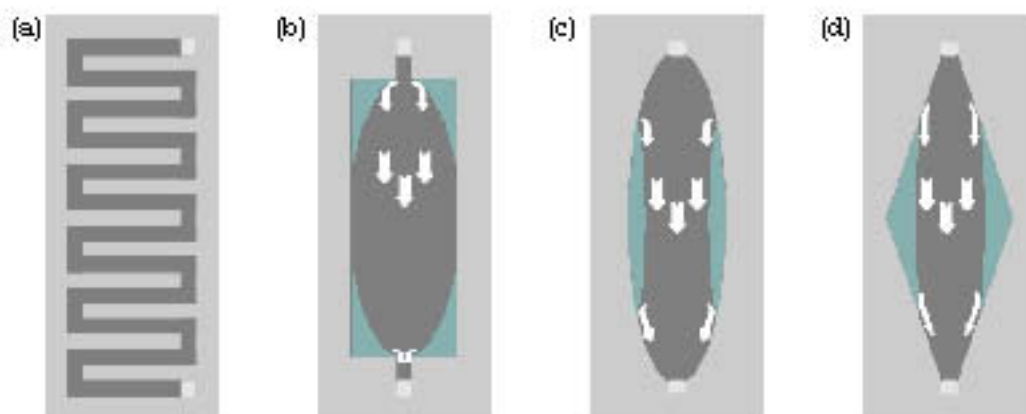


Figure 64 - Different widening strategies for the serpentine-like capillary structure (a) and their predicted reagent stacking and airflow problems: rectangular broadening (b), ellipsoid (c) and rhomboid (d).

4.3.1. TOWARDS RHOMBOIDAL CHIPS

With the hindsight provided by this previous know-how, a re-design strategy was sought to minimize the capillary-like structure of serpentine-like chips while still maintaining efficient and reliable insertion/extraction yields. To confront this re-design process, two facts were taken into account. On the one hand, it had been widely observed that the apparition of air bubbles invariably led to reagent stacking in the chip. On the other

hand, albeit the occurrence of air bubbles could not be completely avoided, it was evident that their effects on extraction could be minimized if the establishment of an air path between access ports was thermodynamically unstable, that is, untenable against tensional forces, as in the case of serpentine-like chips, where the extreme length/wideness ratio of chambers trimmed nearly down to zero the probability of air channel occurrences. Hence, the obvious intuitive solution was to widen the PCR chamber without losing the benefits of its capillary shape. Different schemes (see Figure 64) can be thought of for this purpose.

On the one hand, the capillary can be simply widened (with a prolonged thin appendix to avoid placing the access holes onto a wide fragile membrane, see Figure 64b), but this leaves convection free zones at the capillary ends that may induce air path formation and that obviously lead to stuck ends for reagents. On the other hand, the capillary may be gradually widened, reaching a maximum wideness at mid-length, in the form of an ellipsoid or, more feasibly with wet chemical etching, a rhomboid (see Figure 64c/d). The problem with rhomboids (and to a lesser, but still persisting, degree with ellipsoids) is that the mid-length zones will induce stronger tensional forces in the liquid, promoting reagent accumulation and the formation of air paths once the reservoir has been partly drained. In ellipsoids, this problem is less acute, but it is nonetheless prone to happen. Moreover, within the developed framework, ellipsoids required switching back to RIE etching technologies, and that meant a too great technological redefinition process (incorporating metal masks and other factors, see p.122) that might curtail the benefits of the optimization processes based on the experience gained from serpentine-like PCR-chips. Hence, since wet chemical etching was the ready technological basis, an intermediate approach was devised to gain the most from both strategies (straight broadening and rhomboidal broadening) without incurring deeply into the problems both kinds of structures convey. This was the origin of the rhomboidal chips, depicted in Figure 65, that are discussed in the next section.

4.3.2. RHOMBOIDAL CHIPS

Principles

Intuitively, the inherent principles of the rhomboidal chip are plainly apparent. The rhomboidal structure takes advantage of the absence of dead

volumes at the ends of a true rhomboid, but prolongs the structure in a lineal way (creating a sort of trapezoidal rhomboid), and thus avoids fluid stacking at in the mid-length region. That was, at least, what theoretical intuitiveness hinted at. To demonstrate the functional feasibility of such a design, rhomboidal PCR-chips were fabricated following the technological process already described, and introducing some minor variations in design (length, overall wideness, etc.). After fabrication, the rhomboidal structures were thoroughly tested for injection and extraction procedures.

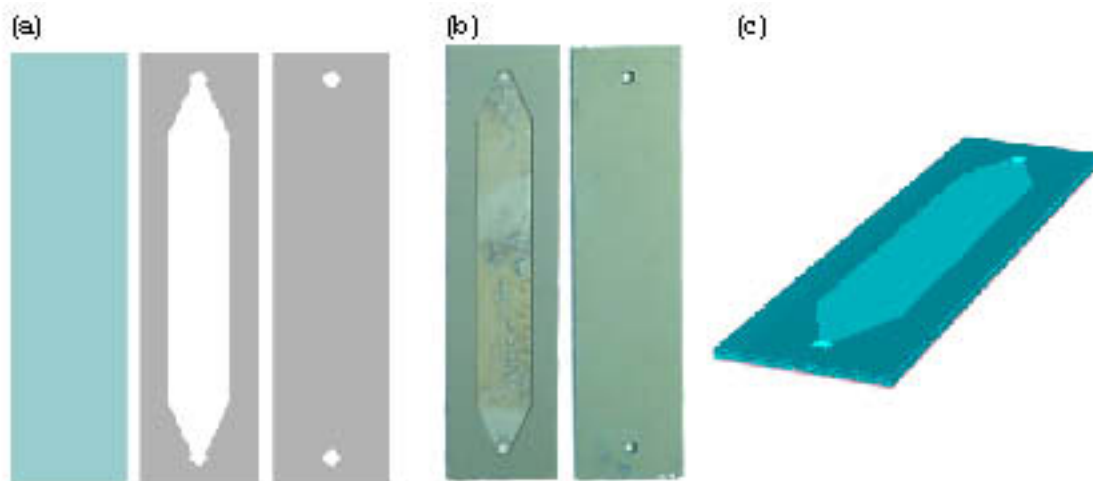


Figure 65 - (a) Schematic view of a rhomboidal chip (glass wafer and back and front-side views of the silicon wafer), (b) fabricated rhomboidal chip and (c) three-dimensional CAD view of the integrated system.

Insertion and extraction experiments

Initial experiments

Initial insertion and extraction trials were conducted using the serpentine-like PCR-chips protocol. Insertion was carried out by depositing a sample drop onto the access hole and making use of capillary effects within the chip to carry on with the insertion. Conversely, extraction was carried out by forcing a positive air pressure at one end with a syringe and suctioning the emerging droplet of sample at the other port, using a custom methacrilate device as the ones shown in Figure 56c, p.129.

The results of these initial experiments confirmed the intuitive hypothesis that rhomboidal chips should take profit from the combination of rhomboidal and straight broadening strategies, yielding non-capillary PCR-chips that would allow easy insertion and extraction. Nevertheless, results with rhomboidal chips were not as successful as those with serpentine-like

chips. On the one side, insertion yields ranged between 60-80% of chip capacity, meaning that capillary effects (as was to be expected) were not so powerful in rhomboidal chips and could not fully overcome the tensional force of surface-liquid interactions, suggesting that an active pumping mechanism should be devised for insertion. On the other side, extraction yields were also poorer (in the 60% of inserted sample range), due a less efficient pumping in non-completely filled reservoirs and to the frequent emergence of sample in sequential droplet spurts (occasioned by air discontinuities in the liquid sample), complicating external recovery with the micropipette.

Pipette-tip envelope-shape methacrilate devices

Insertion and extraction problems of this kind can be solved by simply switching to airtight connection methacrilate devices (Figure 56b, p.129) connected to 6 mm silicone tubes that can be coupled to pumping sources (either vacuum or airflow sources), but this setup requires the use of large amounts of reagents for insertion and poses the problem of reagent staking at tube walls during extraction. To overcome this kind of problems, new methacrilate devices were fabricated providing two access holes that matched the external shape of a pipette tip (see Figure 66). Methacrilate devices with conical holes were fabricated using design specifications from pipette tip suppliers (see *Materials and Methods*, p.299) and, although they did not match unerringly the exact shape of the pipette tip, they provided the necessary tightness for providing vacuum and positive pressures to the chip using the pipette.

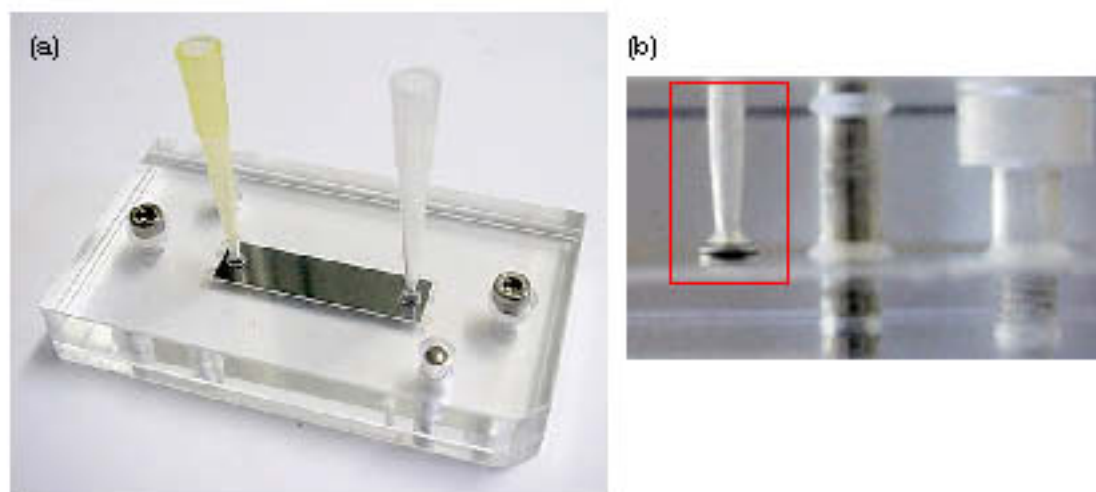


Figure 66 - Pipette-tip envelope-shape methacrilate device (a) and cross-section detail of the conical pipette-like structure (b; red box)

Results with the new methacrilate devices surpassed the best expectations. Using only the micropipette, insertions could be routinely carried out with 90-99% efficiency (there was always a 1-2% of dead air volume at the chip holes). Extraction yields with the micropipette also surpassed those previously obtained for serpentine-like chips. On average, an 80-85% of the inserted sample could be extracted from the chip by simply positioning the pipette tip into the envelope hole and vacuum suctioning. Due to the rhomboidal nature of the chip design, a small amount of sample invariably got stuck near the exit port (see Figure 67), but this 10-15% remnant could then be partly recovered by switching to a traditional pump-coupled methacrilate device and forcing liquid out with a N₂ flow, recovering, on average, 50% of the remaining fluid. Thus, the resulting global extraction procedure gave typical yields between 90-95% of the inserted sample, exceeding, by far, the best results reported to date in the literature ([Lin2000a], [Zhan2000]).



Figure 67 - Surface-stuck reagent in rhomboidal PCR-chips.

4.4. EXTERNAL FAST THERMOCYCLER

4.4.1. ALTERNATIVE EXPERIMENTAL SETUPS

Due to several reasons, the development of a fast external thermocycler to conduct assays on passive PCR-chips was considered a fundamental issue in the evaluation of these systems. On the one hand, it had already been assessed that temperature gradients between the thermocycler wells and block surface could be a source of problems when trying to characterize and optimize PCR and, although there existed simple solutions to this problem (like the development of an accurate surface temperature sensing system to calibrate temperatures at the thermal block surface or the acquisition of a FISH plate from the thermocycler manufacturer), they were clearly limited in scope and usefulness. For instance, the development and integration of an accurate surface temperature sensor would be already halfway towards the development of an external thermocycler, and it would also require constant recalibration to account for non-homogeneities in the mineral oil

layer that acts as the heat conductor between wells and surface. This does not hold true for a FISH (fluorescence in-situ hybridization) plate. These devices are exact replicas of the block surface that, when placed on top of the thermocycler block, cover the wells and distribute temperature homogeneously on the plate surface. FISH plates can be acquired (together with temperature compensation tables and expected heat-transfer times) from some thermocycler manufacturers, but they are a costly add-on. Moreover, both strategies implied extensive, nearly monopolistic use of a commercial thermocycler, an untenable perspective for prototype validation in view of the operating budget.

Most importantly, on the other hand (and notwithstanding whichever of the above mentioned solutions was chosen), the use of a conventional thermocycler meant that it was not possible to exploit the main predicted advantages of a fully integrated PCR-chip [Wittmer1990]: fast cycling and, through it, better specificity and efficiency on PCR amplification. These are advantages that a conventional thermocycler (due to its inherent thermal mass) cannot provide and, since the ultimate goal of this research was to produce working active PCR-chips, it was deemed essential to optimize reaction parameters in similar conditions with passive chips. Hence, the conclusion was that the development of a reliable and fast external thermocycler was a foremost prerequisite in experimentation with passive PCR-chips.

4.4.2. HEAT SOURCE

A basic requirement in any thermocycler that plans to deliver temperature cycles above room temperature is the presence of a heat source. In the case of fast thermocyclers for PCR-chips, heat sources must comply with some evident constraints. Since one of the main advantages of the use of chips is their low thermal mass, heat sources should not add excessive thermal mass to the system, so that fast temperature transitions can be achieved. Additionally, this low thermal mass implies that power drain should be greatly reduced in such systems or that, at least, exceedingly quicker reactions ought to be carried out with the power requirements of a conventional thermocycler. Although there exist some commercial alternatives, like the use of small resistor mats, to date the main heat sources used in passive PCR-chips research have been Peltier cells ([Findlay1993], [Wilding1994], [Shoffner1996], [Taylor1997], [Wilding1998] and [Lin2000a]).

Peltier cells (see Figure 68, *Materials and Methods*, p.305) are interlaced arrays of PN junctions that can pump heat bi-directionally, depending on the sense of the electrical current that flows across them. This thermodynamic property, a reverse of the Seebeck effect used in thermocouples, makes Peltier cells a very appealing device for implementing fast chip thermocyclers, since, although they have a relatively large thermal mass (compared to that of the chip), they compensate for this drawback with bi-directional heat pumping. Unfortunately, the added thermal mass impedes the creation of a low-power thermocycler with Peltier cells. Nevertheless, Peltier cells can provide the necessary fast cycling and, judging from their almost unanimous use in the literature and setting aside power consumption issues, they were considered the best available option in heat sources for the implementation of a fast chip thermocycler.

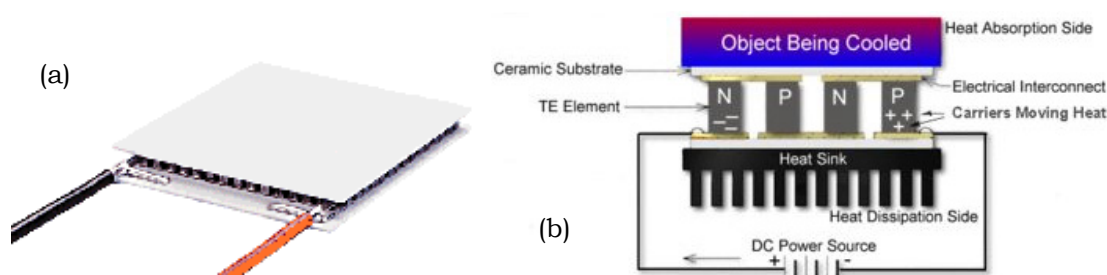


Figure 68 - A Peltier cell (a) and its cross-section schematic (b). Source: *Ferrotec America, Inc.*

4.4.3. TEMPERATURE CONTROL SETUP

Having decided which heat source to use, the remaining issues towards the development of a fast chip thermocycler were the choosing of the adequate Peltier cells, the elucidation of the main setup for the system and the development of all the ensuing aspects of the thermocycler.

Custom vs. commercial controllers

As it will be seen, setting up a system for accurate (± 0.5 °C) temperature control of PCR-chips is not a straightforward matter. Since there are some commercially available temperature controllers for Peltier cells, a first decision had to be reached between acquiring an already assembled temperature controller and assembling a custom one. Although the acquisition of a commercial temperature controller could have sped up the

setup of the system and the ulterior research, there were some inherent drawbacks in commercial systems that posed substantial problems on the development of the present research. Firstly, most commercial systems are aimed at controlling large mass reservoirs (larger than the Peltier cell) and were clearly not suited for control of a PCR-chip, since parameters could not be readily adapted to the new environment. Secondly, the vast majority of these devices are also closed systems, without programmable connection to an external controller. In the creation of a fast external (and obviously automated) thermocycler this was an important issue, since complex PCR cycles (not just the main stage thermal cycling, but also preheating and final extension steps) had to be programmed. External connection in commercial temperature controllers (usually an RS232 connection) could only be found in top-level controllers and, even in most of them, the connection was not a programmable one, but served only output data for monitoring and display. Even in the case of controllers that allow parameters to be externally programmed, this programming usually amounts only to setup temperatures and, in some cases hysteresis, but control parameters (like PID values) cannot be independently programmed (or not at all), a fact that, as it will be seen, can be of great importance when trying to optimize PCR fast-cycling. As in previous cases, the advantages and drawbacks of acquiring a top-level (and thus costly) temperature controller were carefully balanced against budget restrictions, and this option was finally discarded after consideration of a remaining issue: since the ultimate goal of this research was to produce active PCR-chips (and in further stages, active chips with integrated temperature control circuitry), the effort of developing a custom control system for Peltier cells was seen as a valuable know-how in the unavoidable subsequent elaboration of a custom temperature control for PCR-chips, for which commercial alternatives were not at hand.

Peltier cell analysis & selection

Peltier cells come in a wide variety of shapes, operating ranges and prices (see *Materials and Methods*, p.307). It must be noted here that, due to their unique cooling properties, Peltier cells are most commonly used and optimized for cooling (rather than heating, for which cheaper alternatives do exist) purposes. Hence, finding an optimal Peltier cell for the purposes of this work was not simple. Most Peltier cells do not cover (cannot differentially pump) the wide range of temperatures (in the whereabouts of 70 °C) required for PCR operation at room temperature, and those that do

so are usually at their operational limit, yielding poor transitional times at the extremes (for instance, when trying to reach 95 °C, see p.169). To overcome these problems, there are commercially available multistage (superimposed) Peltier cells, but they are built in a pyramidal shape (see *Materials and Methods*, p.305), with the active surface being much smaller than the PCR reservoirs used in this work. Alternatively, fully custom-made Peltier cells can be ordered from the manufacturer, but this is an expensive option and it was decided to initially stick to conventional Peltier cells for initial development and ultimately order a custom cell once effective temperature control had been demonstrated and characterized. Eventually, this last step was to be skipped, by simply changing the working ambient temperature in order to fit the available Peltier cells operating range (see p.172).

Different standard Peltier cells were bought from several manufacturers and, after characterization (see *Materials and Methods*, p.308), two types of cells were selected for further work. The chosen cells, provided by *Melcor*, were a 33.4W, 30x30 mm³, CP1.0-127-05L and a 68.8W, 40x40x3.3 mm³ CP1.4-127-045L single-stage Peltier cell. Although near their operational limits, both cells could deliver the necessary temperature cycles and, moreover, they closely fitted the design parameters of rhomboidal chips, since the 30x30 mm² cell neatly covered the reservoir region, whilst the 40x40 mm² cell could hold the whole chip on its surface, providing alternative capping strategies (see Figure 69 and p.181).

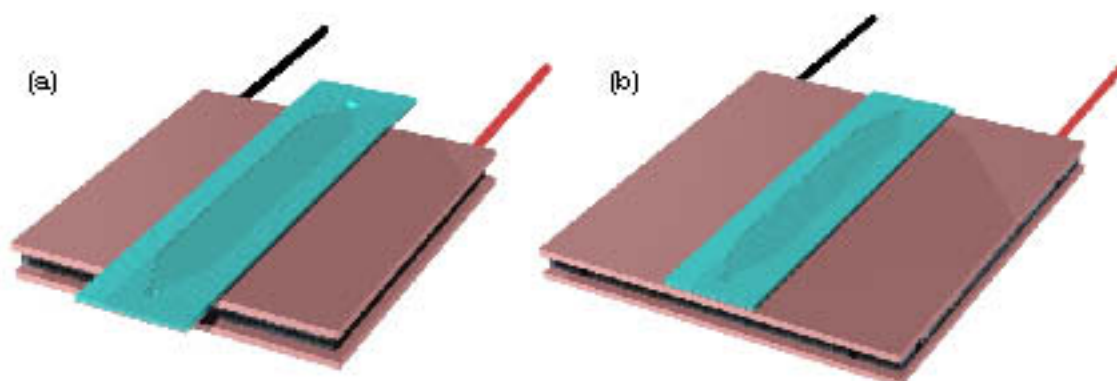


Figure 69 - Alternative 30x30 mm² (a) and 40x40 mm² (b) Peltier cells and their different distribution in relation to PCR-chips, which provides different capping strategies (see p.181)
