### 4.6.2. ADSORPTION EXPERIMENTS

Adsorption phenomena in silicates are long known and widely exploited for the intentional adsorption of desired reagents, such as polar molecules in the silica columns of high-performance liquid-chromatography (HPLC) or the attachment of DNA-molecules to silicon substrates in hybridization chips. Nonetheless, the phenomena of undesired adsorption in silicon chips has not been deeply studied. Adsorption to silica surfaces is caused by the selective action of $SiO_x$-surface silanol (Si-OH) groups on polar molecules, and it is a result of the combination of ionic and hydrogen bonding effects [Tswett1906]. Therefore, DNA and Taq polymerase (which are both polar molecules) could be theoretically adsorbed in silicon oxide passivated or bare silicon (which displays always a parasitic $SiO_2$ layer) chips, posing a problem in the determination of the exact causes of PCR inefficiency described in PCR-chips.

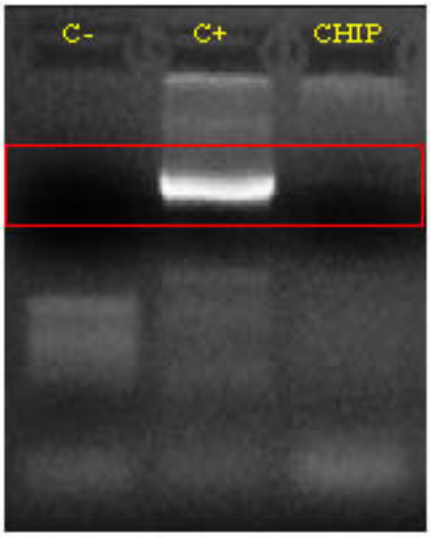


**Figure 101** - Typically negative Is200-fragment chip PCR results with a standard reaction mix and no adjuvants.

The most direct route to reach conclusive data was to perform several different PCR experiments in PCR-chips, varying the concentration of the different reagents (template DNA, primers, dNTPs and Taq polymerase), in order to determine which elements were being adsorbed and, thus, limited PCR efficiency in chips. Although literature reports show low, but still significant, efficiency when conducting standard PCR on silicon dioxide passivated chips ([Shoffner1996], [Taylor1997]), repeated PCR experiments with

the here-developed rhomboidal chips yielded consistent negative results (see Figure 101). Hence, after discarding possible effects of inaccurate temperature control (see p.170), it was determined that some other circumstance (like a greater surface-to-volume ratio, due to the use of 300 μm wafers, than those used by other researchers) was impeding standard PCR amplification (a fact already noted in results with serpentine-like chips, see p.140). Moreover, the multifaceted nature of PCR optimization (see p.69), in which the variation of any concentration might lead to unforeseen effects, made the direct approach less reliable. Hence, a roundabout and more secure route towards determining the nature of adsorption phenomena in silicon dioxide passivated chips was set.

**Initial experiments**

Since either DNA or polymerase molecules could be actively adsorbed at the walls of PCR-chips, an initial batch of experiments was carried out to determine whether DNA was being actively adsorbed. In a standard PCR mix, there are three kinds of DNA molecules: template DNA, primers and free oligonucleotides. Even though an excessively low concentration of any of them can result in diminished PCR product, it was assessed that, of the three, template DNA was the most critical when facing adsorption, since a lowered concentration of template at the initial steps of PCR would yield strongly lower efficiencies, while primers and dNTPs should act more indirectly via a net effect on specificity. Moreover, template DNA was a best-fitted candidate for the prevailing hypothesis of literature reports: that polymerase, and not DNA, was being adsorbed. Since template DNA is, by far, the longest of the three kinds of DNA molecules present in a PCR mix, and taking into account the kinetic nature of adsorption, which makes smaller molecules more prone to be adsorbed, a negative result (no adsorption) with template DNA would also discard adsorption of the other two DNA molecules.

***Methodology***

The methodology of the experiments was quite straightforward and focused on minimizing any other factors that might affect PCR: a standard PCR mix lacking Taq polymerase was prepared and inserted into PCR-chips for 5 to 20 minutes at 4 ºC. The ~200 bp IS200 fragment was used again as template DNA, but this time at lower concentrations (10-12 ng/μl) in order to easily betray any adsorption effects. Afterwards, the mix was extracted

and deposited into a standard eppendorf tube, into which Taq polymerase was added, and PCR was carried using conventional means and protocols (see p.187 and *Materials and Methods*, p.322). Polysilicon and silicon dioxide passivated PCR-chips were used in these initial experiments and, in order to reuse them, they were washed by rinsing with 40 ml of pressure driven milliQ water and $N_2$ flow between mix insertions. The kind of chip and the exposed time for each experiment are summarized in Table 5.

| **Assay #** | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| **Material** | **Exposed time (min)** | | | | |
| Silicon dioxide (S) | 5 | 5 | 10 | 10 | 20 |
| Polysilicon (P) | 5 | 10 | 15 | 15 | 25 |

**Table 5 -** Type of surface passivation and exposed time for each assay. Assays will be hereafter referred as S1 or P3, indicating the kind of material (S or P) and the assay number (1 to 5).

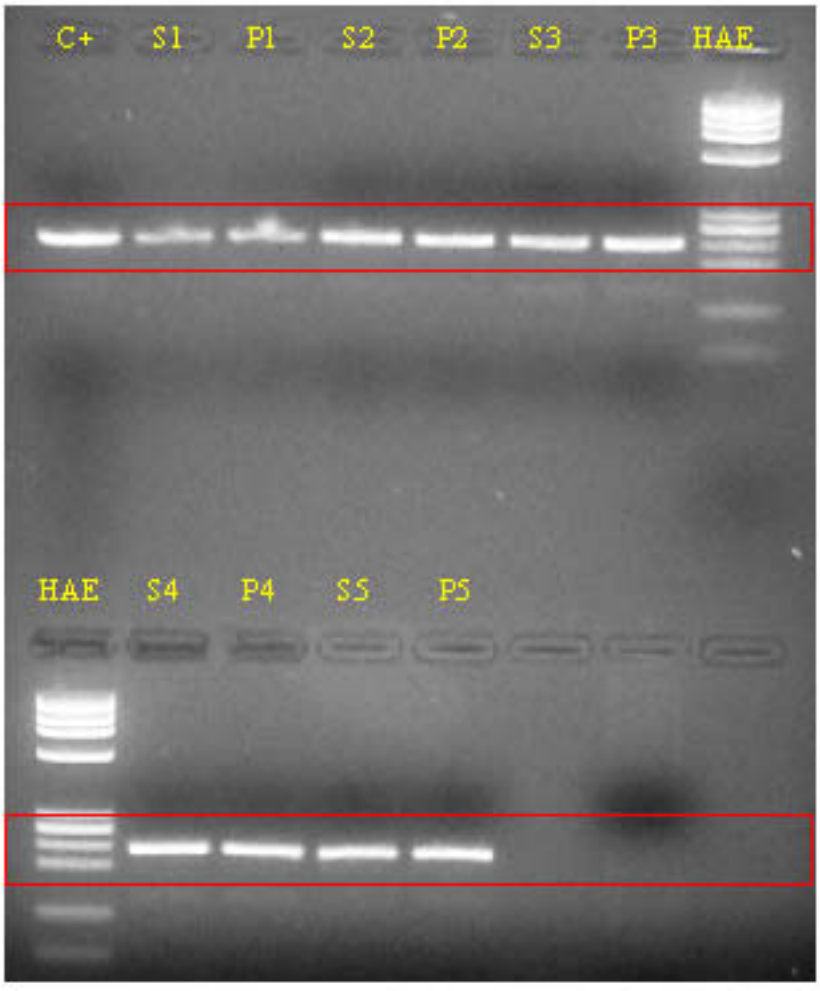


**Figure 102 -** Results for the initial adsorption experiments on a 3% agarose gel (20 µl per well). Assay 1 results (S1 and P1) were discarded due to inefficient extraction of reagents from the chip.

### *Results*

Preliminary results (see Figure 102) showed that no apparent adsorption of template DNA was taking place in the chip, regardless of the time the PCR mix had been exposed to it and of the type of passivation material. Conclusions drawn by visual inspection of the electrophoresis slab gel were validated by taking a picture of the gel and analyzing it with specialized electrophoresis software (see *Materials and Methods*, p.326). The phiX174 DNA/Hae-III ladder 271 bp band was used as a concentration normalizer, shedding no visible deviations on DNA concentration and confirming visual inspection conclusions (see Table 6).

| Tube | C+ | S2 | P2 | S3 | P3 | S4 | P4 | S5 | P5 |
|---|---|---|---|---|---|---|---|---|---|
| Mass (ng) | 71.16 | 68.83 | 68.99 | 64.92 | 69.82 | 67.05 | 66.93 | 65.23 | 68.14 |

**Table 6 -** Estimated mass (in ng) for each of the analyzed bands after normalization with known mass (50.3 ng) of the 271 bp ladder band.

### Astringent cleansing experiments

Although the results from the initial experiments were highly convincing, the reuse of chips introduced a putative distortion effect. It was a feasible hypothesis that simple rinsing with de-ionized water would not have the necessary ionic strength to overcome DNA-wall ionic bonds. In such a case, repeated experiments would experience no observable adsorption due to the saturated nature of the chip walls with previously adsorbed DNA. This problem was aggravated in the initial experiments that had been carried out, since the first batch of assays (the one that could reveal an initial adsorption taking place) was not reliable due to extraction problems.

Therefore, a second run of DNA adsorption assays was carried out, introducing a severe astringent cleansing methodology that could, theoretically, render the chips anew to their original adsorption properties after each experiment.

### *Methodology*

Assays were carried out using much the same methods and materials as in the initial experiments (see p.191). However, big fragments of pBluescript II SK (pBSK for short) plasmid DNA were used instead of the aforementioned

IS200 fragment. pBSK was grown in *S. thymurium* culture, fragmented using an EcoRI digestion enzyme and a linear band of it (~2500 bp) recovered by gel electrophoresis (see *Materials and Methods*, p.325). Also, only $SiO_2$ chips were used in this second round of experiments, to diminish the variability of the experiments and thus draw more certain conclusions.

Washing of the chips between assays was carried out in two different manners: as in initial experiments (with de-ionized water) to provide a control measure, and with a sequential $HCl:H_2O_2$ / $NH_3:H_2O_2$ / ethanol rinsing (see *Materials and Methods*, p.317) for astringent washing.

| Chip | Assay | Correct injection? | Inserted volume | Correct extraction? | Extracted volume | Time in chip |
|---|---|---|---|---|---|---|
| 1 | 1 | Yes | ~30 µl | Yes | >26 µl | 10:00 min |
| 2 | 1 | Yes | ~30 µl | Yes | >26 µl | 11:49 min |
| 3 | 1 | Yes | ~30 µl | Yes | >26 µl | 14:15 min |
| 1 | 2 | Yes | ~30 µl | Yes | >26 µl | 20:00 min |
| 2 | 2 | Yes | ~30 µl | Yes | >26 µl | 21:00 min |
| 3 | 2 | Yes | ~30 µl | Yes | >26 µl | 23:00 min |
| 1 | 3 | Yes | ~30 µl | Yes | >26 µl | 10:00 min |
| 2 | 3 | Yes | ~30 µl | Yes | >26 µl | 11:10 min |
| 3 | 3 | Yes | ~30 µl | Yes | >26 µl | 12:45 min |

**Table 7 -** Experimental data for DNA adsorption experiments.

Three $SiO_2$-passivated chips were used for experimentation. In the first assay, these were previously non-used chips. They were then astringently washed before the second assay, and only mildly washed prior to the third assay. Table 7 summarizes the experimental data.

### *Results*

Results for this second round of experiments (see Figure 103) were conclusive. No significant (after normalized analysis with electrophoresis software) template DNA adsorption was observed, independently of the time and the type of washing procedure used, a fact that suggested that prior results for polysilicon chips were already valid.

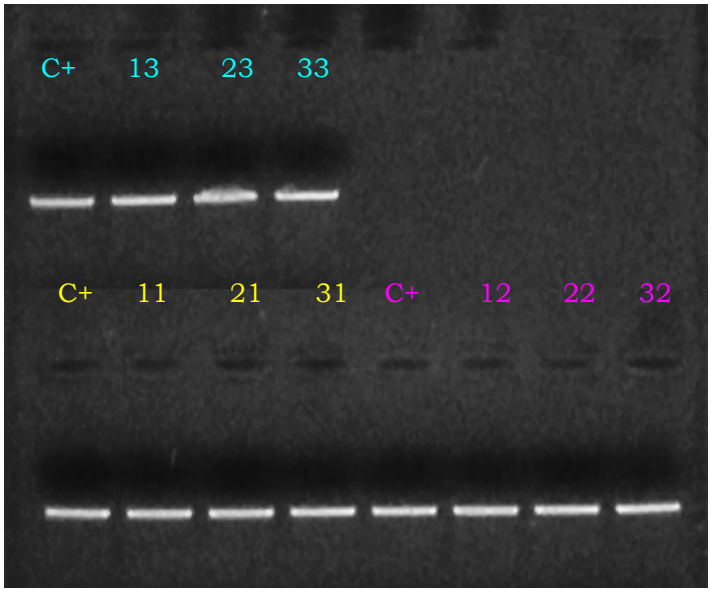


**Figure 103** - Image of the 0.7% agarose gel (20 µl per well) for the second round of DNA adsorption experiments. The yellow lane corresponds to assay #1 (unused chips), while purple and light-blue lanes correspond to assays #2 (astringent washing) and #3 (mild washing).

## Polymerase adsorption experiments

Having independently discarded template DNA (and thus other kinds of DNA, see p.191) adsorption, it was decided to assess the possibility of Taq polymerase adsorption onto the chip walls. With the knowledge of the non-adsorption of DNA, Taq polymerase adsorption was easy to check, since, as in the case of template DNA, diminished amounts of initial Taq polymerase should, in principle, have a strong and visible effect on PCR efficiency.

| Quantity | Reagent |
|---|---|
| 17 µl | milliQ $H_2O$ |
| 2.5 µl | 10x $MgCl_2$ Buffer |
| 2.5 µl | 10 nM dNTPs |
| 1.25 µl | 10 µM sense primer |
| 1.25 µl | 10 µM antisense primer |
| 0.2 µl | 3.5 U/µl Expand™ High Fidelity System (*Boehringer Mannheim Corp.*) |
| 1 µl | 100 ng/µl sample DNA |

**Cycling protocol:**

95 °C - 2 min
95 °C - 1 min \
61 °C - 1 min x30
72 °C - 2 min /
72 °C - 7 min
4 °C - ∞

**Table 8** - PCR protocols for Taq polymerase adsorption experiments using the *iroN* promoter amplicate.

### *Methodology*

The methodology followed in Taq polymerase adsorption experiments was very similar to that developed in DNA adsorption experiments (see p.191). The same three chips were used and previously washed by astringent rinsing (see *Materials and Methods*, p.317), while the amplicate used in this case was the band-recovered promoter region of *S. typhimurium* iron gene *iroN*, previously cloned into a pGEM®-T vector (see *Materials and Methods*, p.319). A 150 µl master mix, this time already including Taq polymerase, was elaborated in standard conditions and distributed among five 30 µl eppendorf tubes. Two tubes correspond to positive and negative controls, while the contents of the other three were extracted and inserted into each of the chips, left for a certain amount of time (1-4 min) at 4 ºC, and then extracted again and put into new eppendorf tubes. To normalize conditions regarding extraction problems, only 20 µl from all the final five eppendorfs were used in the subsequent PCR amplification, again using standard protocols. PCR and experimentation protocols are summarized in Table 8 and Table 9.

| Sample tube | Correct injection? | Inserted volume | Correct extraction? | Extracted volume | Time in chip |
|---|---|---|---|---|---|
| X1 | Yes | 30 µl | Yes | >22 µl | 2:00 min |
| X2 | ~Yes | 26 µl | Yes | >22 µl | 1:00 min |
| X3 | ~Yes | 28 µl | Yes | >22 µl | 4:00 min |

**Table 9 -** Experimental data for Taq polymerase adsorption experiments.

### *Results*

Taq polymerase adsorption assays were fairly conclusive. As it can be seen in Figure 104, the efficiency of the subsequent PCR amplification decreased proportionally to the time the prepared mix had stayed in the chip, meaning that, at the present concentration, Taq polymerase was not only being adsorbed to the chip walls, but it did not even achieve a significant concentration to attain the dynamic adsorption equilibrium that was to be expected in a kinetic phenomenon like that of adsorption. In other words, all (or, to put it more exactly, the significant fraction of Taq polymerase that is necessary for PCR amplification to occur) was being adsorbed at the chip walls.

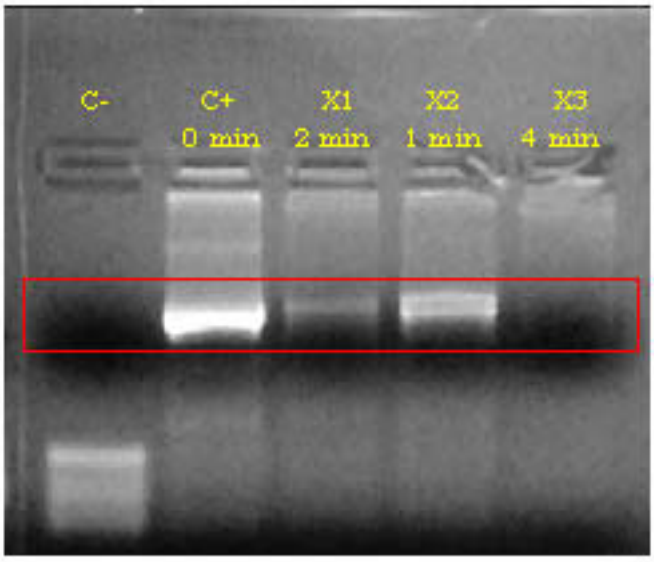


**Figure 104** - Image of the 3% agarose gel (20 µl per well) for Taq adsorption experiments. PCR product concentration can be seen to diminish in proportion to the time spent in the chips by the PCR mix.

### 4.6.3. ADSORPTION OPTIMIZATION EXPERIMENTS

In view of the clear-cut results for Taq polymerase adsorption experiments, it was decided to test different methodologies for overcoming the problem of polymerase adsorption on chip walls. The obvious solution in such a case is to increase Taq polymerase concentration until it saturates the medium and supersedes the dynamic equilibrium of adsorption processes. Nevertheless, this simple option is somewhat restricted in PCR applications. On the one hand, Taq polymerase is, by large, the most expensive reagent used in PCR and increasing its concentration in a fair amount is a costly and undesired trend. On the other hand, the exact nature of the adsorption of Taq polymerase onto chip walls has not been studied and it is not well understood. Therefore, it is plausible to suppose that polymerase adsorption is not produced by the active site of the enzyme. This would still imply a large decrease in efficiency, because bound polymerase, even with its active site freed, would strongly affect on the kinetics of PCR, almost inhibiting it. Nonetheless, if the active site of bound polymerase remains exposed, this has a direct influence on $Mg^{++}$ concentration, since bound polymerase still acts as a chelator of $Mg^{++}$ ions. Hence, even disregarding its costs, the increase of Taq polymerase concentration might not be such a straightforward methodology as it could seem, because an optimization by titration of $Mg^{++}$ ions and, as a consequence (see p.78), almost all the other

components of the PCR mix would be required. Another suggested method for decreasing Taq polymerase adsorption on chip walls is the use of bovine serum albumin (BSA, see p.86). In theory, BSA, which has otherwise been described as a standard chemical deterrent of polymerase inhibition and enhancer of PCR in various reports ([Kreader1996], [Forbes1996], [Giambernardi1998]), should act in chips by competing with polymerase for adsorption at the active sites of chip walls ([Rasmussen1994b], [Taylor1997], [Murakami2000]). To test this hypothesis and to evaluate its feasibility and the viability of the straight polymerase-increase method, the following series of tests were conducted.

## Counteracting polymerase adsorption

### *Methodology*

A first test was carried out to test which of the aforementioned options (Taq increase and BSA addition) worked better in the particular case of PCR chips. The three chips previously used for adsorption assays were reused here, after undergoing astringent washing (see *Materials and Methods*, p.317), and the general methodology was also similar to that of previous adsorption assays (see p.191). A 200 µl master mix for iron promoter amplification was elaborated using the protocols described above (see Table 8) and distributed among eight 25 µl eppendorf tubes. One was used as a negative control and another for a PCR-chip amplification trial, while the other 6 corresponded to positive control and test tubes for the following conditions: normal mix (N), BSA containing mix (B), increased polymerase mix (T). Following literature reports ([Wittmer1990], [Taylor1997], [Murakami2000]), which located the ideal BSA concentration in the 0.5-1.0 µg/µl range, it was decided to use an initial 0.5 µg/µl concentration, meaning that 0.625 µl of 20 mg/ml BSA stock were added to C+B and XB tubes. Regarding Taq polymerase concentration, it was arbitrarily increased 5-fold (from 0.2 to 1 µl), understanding that, even if viable, higher Taq concentrations would be markedly unaffordable. After elaboration of the master mix, the required amounts of BSA and extra polymerase were added to the tubes, together with 1 µl of 100 ng/µl template DNA, and the contents of the test tubes were inserted for 5 min at 4 ºC in the pre-washed PCR-chips (see Table 10). After extraction, 20 µl of each tube were transferred to new tubes and *iroN* promoter amplification was carried out following the protocol described in the earlier section (see Table 8, p.195).

Concerning PCR-chip amplification, it was conducted with standard Taq polymerase concentration (0.2 µl) and 0.5 µg/µl BSA concentration.

| Sample tube | Correct insertion? | Inserted volume | Correct extraction? | Extracted volume | Analysis time |
|---|---|---|---|---|---|
| XN | ~Yes | 27 µl | Yes | >23 µl | 5:00 min |
| XB | Yes | 29 µl | Yes | >23 µl | 5:00 min |
| XT | ~Yes | 26 µl | Yes | >23 µl | 5:00 min |

**Table 10 -** Experimental data for Taq polymerase adsorption contravening experiments.

***Results***

The results displayed in Figure 105 showed that the addition of BSA was a better method for contravening Taq polymerase adsorption than simply increasing polymerase concentration, although PCR-chip amplification results remained negative. Moreover, the positive control tubes revealed that BSA did not have any negative effects on PCR at 0.5 µg/µl concentrations.

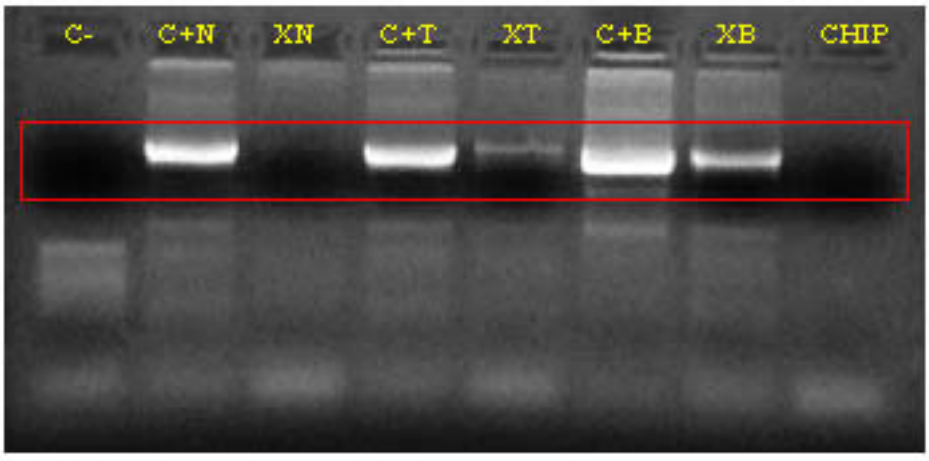


**Figure 105 -** Image of the 3% agarose gel (20 µl per well) for contravening adsorption experiments. X lanes correspond to chip-inserted samples, while C+ lanes are positive controls. N stands for normal PCR mix, while T stands for increased polymerase concentration and B for BSA addition, both in chip and positive control lanes.

In view of the results shown above, it was decided to discard polymerase increase in favor of BSA addition and, therefore, BSA titration was undertaken both to assess its optimal concentration and to observe the Taq polymerase adsorption contravening effects of BSA under longer exposition to PCR-chips.