BSA titration experiments

Methodology

To conduct BSA titration experiments, a 200 µg/µl master mix for *iroN* promoter fragment amplification was elaborated using the above-described conditions (see p.198) and distributed among eight eppendorf tubes. To avoid inequalities due to chip extraction problems, PCR experiments were carried out using only 20 µl of mix. Therefore, in this assay control tubes were loaded with only 20 µl of mix, while test tubes were loaded with 30 µl, enough to completely fill the chip, and thus yielding pre-BSA master mixes of 50 µl (20+30 µl) for each different BSA concentration. BSA was introduced in incremental 0.5, 1.0 and 5.0 µg/µl concentrations among the 50 µl pre-BSA mixes by depositing 1.25, 2.5 and 12.5 µl of 20 mg/ml BSA stock solution. After distribution of the BSA-containing mix among the final eppendorf tubes, 1 µl of 100 ng/µl template DNA was inserted into the tubes. The contents of test tubes were then inserted into astringently cleansed PCR-chips for 30 min at 4 ºC. Afterwards, they were extracted and 20 µl of each tube were deposited into new eppendorf tubes that, together with positive and negative control tubes, underwent PCR amplification in a conventional thermocycler using the PCR protocols described (Table 8, p.195). The experimental data for these assays is shown in Table 11. Regarding the PCR-chip assay, the chip mix was loaded with an intermediate BSA concentration (2.5 µg/µl, that is 6.25 µl in a 30 µl mix) and a doubled, to be on the safe side, Taq polymerase and DNA concentration (0.4 µl of Taq polymerase and 2 µl of template DNA).

Sample tube	Correct insertion?	Inserted volume	Correct extraction?	Extracted volume	Analysis time
X0	Yes	29 µl	Yes	>22 µl	29:00 min
X1	Yes	29 µl	Yes	>22 µl	29:00 min
X5	~Yes	26 µl	Yes	>22 µl	29:30 min

Table 11 - Experimental data for BSA titration experiments. Samples are labeled according to their BSA concentration: 0 for 0.5 µg/µl, 1 for 1.0 µg/µl and 5 for 5.0 µg/µl.

Results

BSA titration results (see Figure 106) confirmed the suspicion that higher BSA concentrations were required to efficiently reduce polymerase adsorption in long exposures to the PCR-chip walls. Results for 0.5 and

1 μg/μl BSA concentration yielded very poor results, indicating that the amount of BSA was not high enough to counteract, in a durable way, polymerase adsorption. On the other hand, results for 5 μg/μl BSA indicated that this concentration was enough to partly neutralize polymerase adsorption, but they also showed (through a 5 μg/μl BSA positive control) that such large amounts of BSA had some indirect efficiency-lowering effect on PCR and, thus, they suggested that higher (>2.5 μg/μl) BSA concentrations were not recommended for PCR.

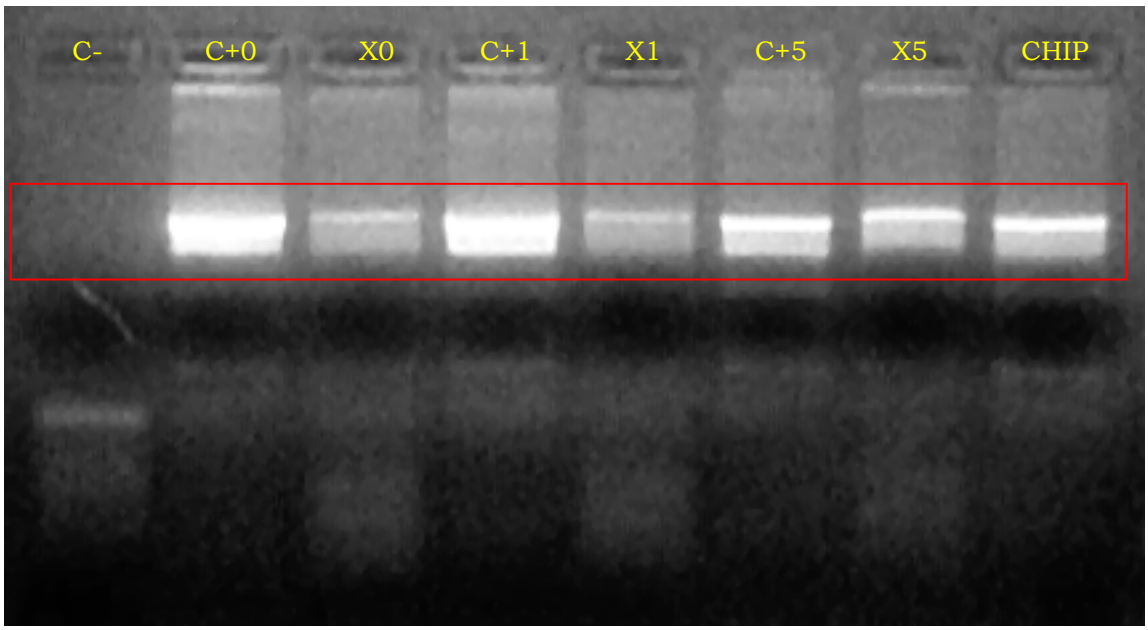

Figure 106 - Image of the 3% agarose gel (20 μl per well) for BSA titration experiments. C+ lanes correspond to positive controls and X lanes to test samples. The adjoining number labels the BSA concentration (0 - 0.5 μg/μl, 1 - 1.0 μg/μl, 5 - 5.0 μg/μl). Succesful PCR-chip amplification can be seen in the rightmost lane.

Nonetheless, the most exciting of Figure 106 results was the first success of PCR amplification in the PCR-chip, which finally confirmed the correct operation of the developed external fast thermocycler. Although Taq polymerase and template DNA had been doubled in the chip experiment, the moderate BSA concentration used (2.5 μg/μl) suggested that optimization of the chip results was possible. Hence, it was decided that further BSA titration experiments, as well as variations in PCR mix and protocols, would be conducted directly using PCR-chip amplification. These are the chip PCR optimization experiments related hereafter.

4.7. CHIP PCR OPTIMIZATION EXPERIMENTS

4.7.1. INITIAL OPTIMIZATION ASSAYS

After the initial success of PCR in PCR-chips (see Figure 106), a first batch of experiments was conducted to assess whether these results could be optimized both in terms of mix composition and PCR speed. Regarding mix composition, the main research line would be to further titrate BSA concentration and to see if successful PCR results could be achieved with normal amounts of Taq polymerase and template DNA (which had been doubled in previous experiments). On the other hand, the optimization of PCR speed was not such a straightforward matter. After initial chip PCR failures using low BSA concentrations (see Figure 105, p.199), two main conclusions had been drawn: either a higher BSA concentration was required (a fact that was later confirmed by the above-related BSA titration experiments) or denaturation-to-annealing ramps had to be soothed to allow efficient annealing.

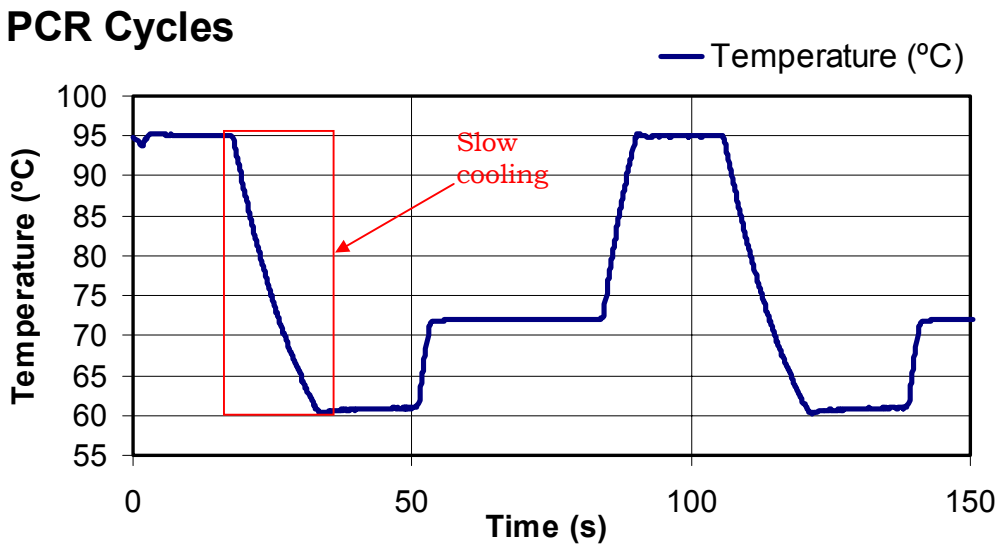

Figure 107 - Conventional thermocycler slow-cooling effect emulation to avoid ineffective annealing.

As it has been previously stated (see p.80), annealing temperatures are generally estimated from formulae that introduce information on the %G+C content of primers. Therefore, these temperatures are always rough estimates of the real annealing temperature, which has to be titrated in initial PCR experiments. However, due to the nature of conventional thermocyclers, which typically display low cooling ramps, even empirical

titration might not yield the real annealing temperature. In the case of the *iroN* promoter annealing temperature here used, which had been experimentally titrated to 61 ºC, it was quite possible that annealing was most efficient at 62 or even 63 ºC, a fact allowed by the slow cooling rate of the conventional thermocycler in which it annealing temperature had been titrated. Clearly, if this was so, fast cooling with the prototype external thermocycler could strongly affect PCR by driving down annealing rates. Consequently, fast cooling was switched off to emulate conventional thermocycler cooling in the successful chip PCR experiment described above (see Figure 107), and it still had to be assessed whether previous unsuccessful results stemmed from too low BSA concentration or too fast cooling (or both).

PCR validation in fast thermocycler

To check whether fast cooling rates were also an efficiency-lowering factor in chip PCR analyses, the preceding successful chip PCR experiment was first repeated with an equal BSA concentration and fast cooling, leaving all the other PCR parameters (mix composition and PCR protocols, see Table 8, p.195) unchanged.

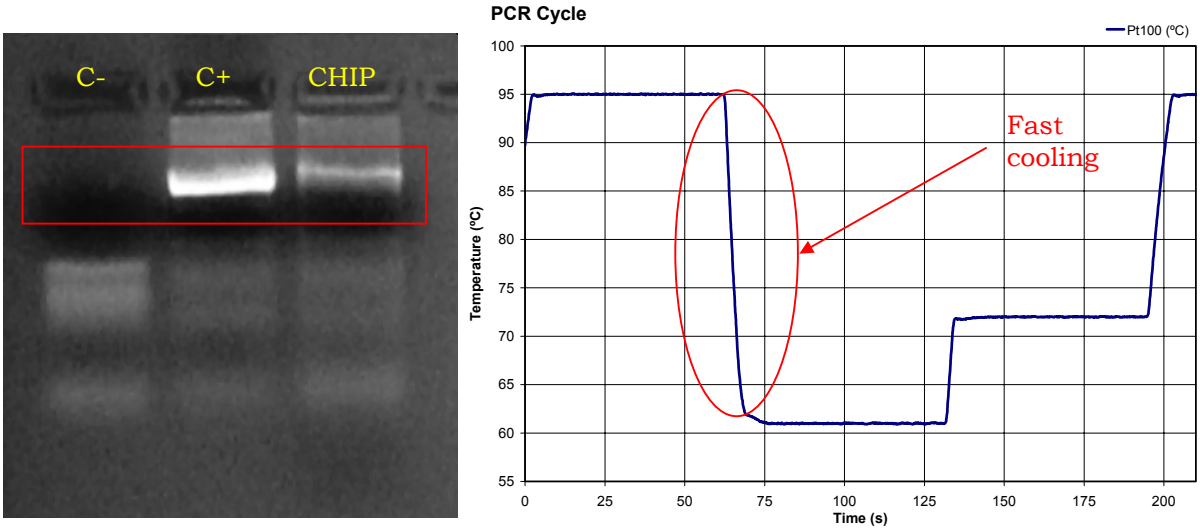

Figure 108 - Image of the 3% agarose gel (15 µl per well) results and the PCR-cycles used in the fast-cooling validation experiment. All tubes contain the same BSA concentration: 2.5 µg/µl. Chip contains doubled template DNA and polymerase concentrations. Total chip PCR analysis time: 1:50:08 h.

Results, shown in Figure 108, indicated that fast cooling was clearly undercutting PCR yields. To confirm this line of reasoning, the same experiment was repeated again, but this time with slow-cooling rates (see Figure 109).

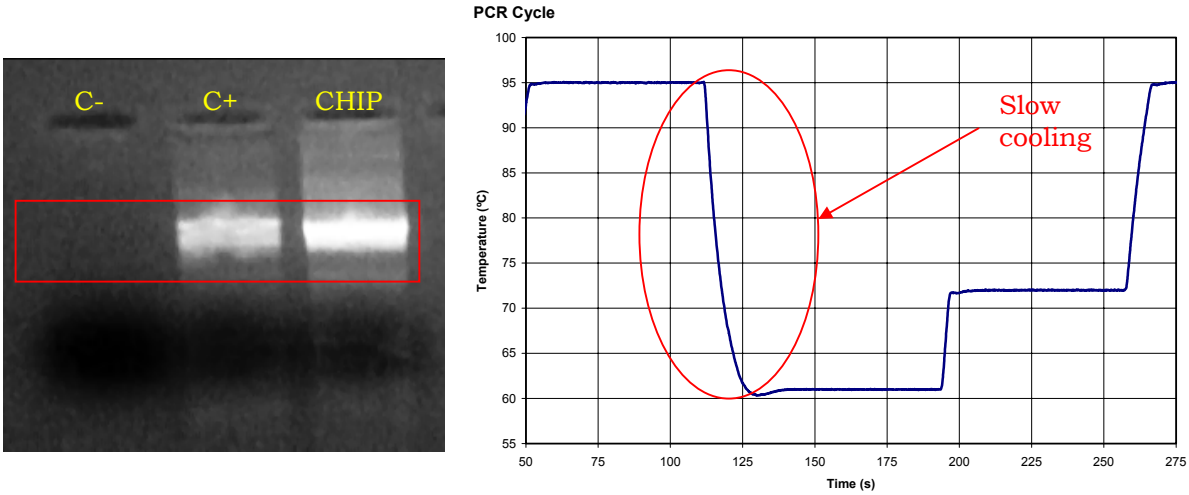

Figure 109 - Image of the 3% agarose gel (20 µl per well) results and the PCR-cycles used in the slow-cooling validation experiment. All tubes contain the same BSA concentration: 2.5 µg/µl. Chip contains doubled template DNA and polymerase concentrations. Total chip PCR analysis time: 1:54:32 h.

The above results confirmed the hypothesis of inaccurate titration of the annealing temperature and, therefore, it was decided to experimentally determine if higher annealing temperatures were, indeed, feasible.

Annealing temperature titration

Titration of the annealing temperature was conducted by directly repeating the previous experiments with increased annealing temperatures and fast cooling. Additionally, some BSA titration experiments were conducted in parallel, mainly to deduce if a higher (5 µg/µl) concentration was better than the already standard 2.5 µg/µl concentration; in these cases, the standard 61 °C annealing temperature was maintained. Also, a quick-PCR experiment (with 10 s denaturation and annealing times and a 45 s extension time) was carried out, with a 62 °C annealing temperature, to assess the feasibility of fast thermocycling with the present system. The results for all these experiments are shown in Figure 110 and discussed below.