Betaine (N, N, N-tri-methyl-glycine)

Betaine is supposed to work in a similar way to DMSO, equalizing the contribution of G+C and A+T base pairing to the stability of the DNA duplex and thus improving results in %G+C rich regions. The exact mechanism of betaine action is unknown, but is seems clear that betaine does not have an effect on primer annealing and that it just disrupts contorted DNA structures, helping polymerase advance at stuck ends; this later property has been demonstrated by the addition of betaine *after* polymerase had already become stuck.

The way in which betaine does breaks-up knots in DNA is not altogether clear either. It has been suggested that betaine disrupting effects come from its binding to A+T pairs in the major groove of DNA [Weissensteiner1996] or by increasing the hydration of G+C pairs in the minor groove [Mytelka1996]. In addition to its secondary structure disrupting effects, betaine is also a known osmo-protectant, meaning that it increases the resistance of polymerase to denaturation, and it can even help to overcome some contamination effects in low-quality templates [Weissensteiner1996]. Evidently, not everything about betaine is that sweet, but to date there is no direct evidence of PCR inhibition by betaine at common concentrations (~1 M) although its use is not recommended when not dealing with %G+C rich regions. Nuclear magnetic resonance studies have shown that betaine is a common additive in most PCR commercial optimization kits [Frackman1998].

Formamide (methanamide carbamaldehyde formimidic acid)

Formamide is also a long known adjuvant to PCR [Sarkar1990c] that, as DMSO, can dramatically improve the specificity of the reaction under certain circumstances (mainly %G+C regions). Formamide works in a similar way to DMSO, destabilizing primer annealing and increasing denaturation. Thus, it also presents many of its problems and is known to inhibit polymerase activity. Therefore, it should not be used above 5% (v/v) concentrations (typically between 1 and 5%). Since formamide also lowers the effective T_m of the primers, titration of the polymerase concentration and of the annealing temperatures is strongly recommended after the addition of formamide.

TMAC (tetra-methyl-ammonium chloride)

Closing the list of specificity enhancers that work by altering the effective primer annealing temperature is TMAC ([Hung1990], [Chevet1995]), which, to date, has not been shown to cause polymerase inhibition at working concentrations (10-100 μM). In addition, TMAC is reported to reduce RNA/DNA mismatches, a fact that is of considerable importance in special procedures, such as RT-PCR (see p.91).

Glycerol (1, 2, 3-propanetriol)

Glycerol, first described in 1989 [Boehm1989], is another relatively abundant adjuvant to PCR. It works by stabilizing the polymerase enzyme, helping it to withstand denaturation and thus improving the reaction yield. The ability to allow for higher denaturation temperatures (or longer times) makes glycerol suitable for improving yields in difficult (high %G+C content or unusually long) templates when used at 5-10% (v/v) concentrations ([Smith1990], [Pomp1991]).

Non-ionic detergents

Non-ionic detergents are known to stabilize polymerase [Bachmann1990] and are mainly used to overcome inhibition problems coming from extraction procedures, such as contamination from the ionic detergent SDS (Sodium-dodecyl-sulfate) [Gelfand1989] or polysaccharides [Adams1992]. Among non-ionic detergents, Tween-20, Triton X-100 and Nonidet P-40 are the most commonly used, generally at concentrations between 0.5 and 2% (v/v). Higher concentrations (above 10%) may result in PCR inhibition.

BSA (bovine serum albumin)

Bovine serum albumin is another quite often-cited facilitator of PCR. Its main hypothesized working basis is to act as a competitor with polymerase in inhibitor chelation [Kreader1996]. Thus, BSA sequesters many polymerase inhibitors (such as hemoglobin [Forbes1996] or melanin [Giambernardi1998]) and facilitates reactions that would not be functional without it. This fact has been readily exploited in amplification of aDNA (see p.92, [Pääbo1988]) and other difficult templates. Additionally, BSA is supposed to thicken the PCR mix, facilitating primer annealing, stabilizing both DNA and the polymerase enzyme and acting as an osmo-protectant of polymerase, and

has been shown not to inhibit polymerase activity in concentrations as high as 1 µg/µl [Henegariu1997]. Finally, BSA has also been suggested as a competitor to polymerase in adsorption-unspecific binding to surfaces [Rasmussen1994b], a theory that has been promptly adopted to improve PCR yields when dealing with large surface/volume ratio PCR vessels, as is the case of PCR conducted within capillaries (see p. 94) and PCR-chips (see p.96).

Other adjuvants

The list of possible adjuvants to PCR is practically endless. The ones described above are the most common and well documented, but they are not alone, and there are many hints that the list may keep growing indefinitely. For example, *E. coli Ssb* (single-stranded binding) protein, involved in prokaryotes *lexA*-directed DNA repair mechanism, has been used to stabilize ssDNA and to facilitate annealing in difficult reactions ([Oshima1992], [Dabrowski1999]). Similarly, the bacteriophage T4 gene-32 protein, also involved in DNA replication, recombination and repair, has been found to enhance polymerase activity during PCR, improving yields of long products and reducing inhibition from fecal compounds ([Lyons1992], [Kreader1996]). PEG (polyethylene glycol) 6000, 7-Deaza dGTP, potassium glutamate or spermidine and other polyamines have also been suggested as PCR facilitators.

Additional strategies for optimization

Simpler than the fuzzy world of adjuvants, there is a whole set of modifications to the PCR that can circumvent many specificity and efficiency problems. These variations, based on very sensible principles inherent to the PCR, offer alternative methodologies to the analyst in cases where PCR becomes difficult to optimize.

Re-amplification

Obvious as it might seem, re-amplification is a sound strategy when dealing with short amounts of template DNA or with unusually long templates. In both cases, an inefficient initial amplification will produce some amplicate products, albeit the reaction can be quite unspecific. The trick is to extract and dilute this product and then re-amplify using the same set of initial primers. If there are unspecified products, one can still resort to gel

electrophoresis and retrieve the desired band for re-amplification (see *Materials and Methods*, p.320). This simple method is useful when dealing with degraded or excessively long DNA, since the amplicate that is re-amplified oversees both these problems.

Nested PCR

Nested PCR was early described as a specificity booster for PCR on difficult templates [Mullis1987]. Its basic modus operandi is quite straightforward: nested PCR is a two-step reaction in which a set of enclosed primers (two [outer] primers delimiting a region that includes the other [inner] primers' binding site) is used to minimize nonspecific products. The initial steps of the PCR are carried out with the outer primers, producing several nonspecific amplicates and the desired one. Then, the second set of inner primers is introduced and PCR starts amplifying the inner region of the desired amplicate. The probability that the inner set of primers displays annealing with previous non-specific amplicates is vanishingly small and hence the specificity of PCR is greatly enhanced. Of course, this improved efficiency does not come for free, since two sets of primers must be designed and synthesized, and the reaction must be optimized for two different conditions. In addition, the introduction of the second primer set during PCR may cause contamination. This last problem can be overcome by careful design of the primers, selecting sets of very different annealing temperatures and targeting the annealing of each set by simply switching temperature profiles. Notwithstanding its costs, the methodology of nested PCR is reliable and a good alternative when nonspecific products fail to disappear under conventional optimization pressures [Gibbs1990].

Touchdown method

First described in 1991 [Don1991], the touchdown method is a clever variation of the conventional PCR protocol. Since most unspecific product comes from the imperfect annealing of primers and this is mainly due to an excessively low annealing temperature, the touchdown method suggests to increase the T_a in order to favor more specific anneals. Unfortunately, this obvious strategy has a drawback, since PCR yield will be also reduced if the annealing temperature is excessively high. To circumvent this problem, and exploiting the exponential nature of PCR amplification, the touchdown method applies higher, stringent temperatures at the initial PCR cycles and progressively decreases them in order to get the best from both worlds.

Since PCR is exponential, a selective specificity in the initial cycles will give an evolutionary advantage to the desired specific priming pairs [Hecker1996]. And, since Dollo's Law [Farris1977] does not abide reversibility in evolution, the lowering of the annealing temperature in further steps will provide high yields of the desired, dominant amplicates.

Hot-start

Hot-start PCR has been the latest innovation in specificity-boosting techniques for amplification. As already stated, a primal factor in the production of nonspecific products is the mismatched anneal of primers to template due to low annealing temperatures. As seen above, this can be overcome by touchdown methodologies or the addition of adjuvants (like DMSO). However, primer mis-anneal at the first cycle of the PCR is inescapable, since the mix is put together at room temperature (and thus way below the primers melting temperature). The introduction of mis-anneals at the first cycle of PCR induces a great reduction in reaction specificity and efficiency, because non-desired amplified regions will tend to compete exponentially for resources with the desired amplicate. To overcome this problem, an initial long heating step is introduced at the beginning of the PCR (see p.79), but many mismatched anneals will have been already extended by the polymerase (which is partially active at room temperature and during the heat ramp) and, if long enough, these amplified regions will not be completely denatured in this initial stage.

An evident solution to this predicament is to add the polymerase *after* the initial denaturation step, in what has become known as the hot-start method. Early assays of the hot-start method involved the manual introduction of the polymerase across the oil evaporation barrier, after the PCR vessel had been placed on the thermocyclers and the first denaturing step had been carried out ([D'Aquila1991], [Ruano1992]). Unfortunately, as in the case of nested PCR, adding the polymerase in a non-clean environment may lead to contamination [Bassam1993]. To overcome contamination issues, several reagent providers have developed means to automatically heat-activate the polymerase enzyme after the denaturation step. AmpliTaq Gold (*Applied Biosystems*) polymerase is a modified version of the Taq polymerase that is chemically inactivated by a covalent bond at its active site [Birch1996]. Following a 10 min exposure to 95 ºC under a slightly lower pH than usual, AmpliTaq Gold becomes irreversibly activated and the reaction carries on with boosted specificity. TaqBead (*Promega*, [Smith1996])

uses a different approach, encapsulating the enzyme in a wax bead that releases the polymerase upon heating. Still, other methods rely on different principles, as tying up the enzyme with a specific antibody or an inert dye, to produce similar results.

1.4.4. PCR APPLICATIONS

As it has been pointed up (see p.63), one of the main advantages of PCR over rival amplification technologies is the huge number of applications with well-established protocols it covers. They are, in fact, too many to be even cited here. Nonetheless, some of them, due to their very special characteristics, their utility or their broad range of use have already become standards in the molecular genetics repertoire. The following is a brief summary of their basic ideas, use and importance.

Degenerate primer PCR

Contrary to the intuition of PCR optimization, the use of degenerate primers (primers that are not exact matches of the template DNA sequence) and lower-than-average annealing temperatures can be a useful methodology when part of the DNA sequence to be amplified is unknown [Compton1990]. This can be of great utility when trying to clone gene sequences from their partial protein sequence [Lee1988] or homologues of known genes in a novel organism [Mack1988]. Degenerate primer PCR has been carried to its extreme in fingerprinting analysis with RAPD (random amplified polymorphic DNA) PCR, in which amplicates are generated randomly with fixed primers, giving a unique set of products for each DNA sequence [Welsh1990].

Multiplex PCR

As its name implies, multiplex PCR is a method for carrying out multiple PCR at the same time. This can be useful in various circumstances, but particularly so when trying to identify sequence deletions in genes. A consistent approach to detect deletions is to perform a PCR with primers corresponding to the hypothetically deleted region. If the PCR generates a successful product, the deletion can be discarded (within an error margin). In case of large genes, like the 2 Mbp Duschenne muscular dystrophy (DMD) locus, many deletions can induce gene misbehavior and generate disease. A simple way to detect the presence or absence of various mutations in a single step is to perform a PCR with multiple primers

corresponding to each deletion site (multiplex PCR [Chamberlain1988]). Since each primer inherently sets its own melting temperature, primers must be carefully designed and temperatures wisely balanced to carry out multiplex PCR. Adjuvants can be also required to improve the yield of this technique [Henegariu1997].

RT-PCR

Reverse-transcriptase PCR, RT-PCR or RNA-PCR, is a method for amplifying RNA, instead of DNA. It works basically by first reverse-transcripting RNA into ssDNA and then routinely amplifying this DNA. Reverse-transcription can be accomplished with the addition of a specific enzyme (reverse-transcriptase) to the mix, or with the use of specific polymerases that do also display reverse-transcriptase functionality (see p.74). RT-PCR is of extraordinary significance in molecular microbiology, since it permits the evaluation of gene expression (transcribed copies of the gene in a cell or culture), rather than gene presence in the genome, thus allowing to evaluate gene functionality ([Veres1987], [Fuqua1990]).

In-situ PCR

In-situ PCR is a technique for performing PCR directly on cell cultures. Cultures are placed on a glass slide and PCR reagents are poured directly onto it. Afterwards, the slide is hermetically sealed and PCR is carried out on the slide [Chiu1992]. This method is useful to evaluate the presence and distribution of specific DNA inside cells (for instance, bacterial or viral DNA inside eukaryote cells) or to visualize metabolic pathways in combination with RT-PCR [Embleton1992]. It is also far more sensitive than the corresponding direct-hybridization technique (FISH - fluorescence *in-situ* hybridization, [Gosden1994]). This innovative mapping method has been widely validated by conventional silver staining methods that can also reveal the location of bacterial cells within a host eukaryote [Tsongalis1994].

Long PCR

The amplification of long fragments is of fundamental interest in sequencing projects, since it can skip the longer conventional step of cloning the sample to be sequenced into a vector (like λ–phage or a plasmid) for culture in order to extract the necessary amount of DNA for sequencing. Therefore, amplification of fragments longer than 10 kb (the upper limit of the

conventional technique) has been a long sought goal in PCR and the major introducer of the use of adjuvants and modified PCR protocols. Long PCR involves the use of many optimization techniques, such as polymerase cocktails, touchdown methods, adjuvants (mainly DMSO, glycerol and BSA) or two-step techniques, and has already become a quite robust and commonly used methodology [Cheng1994].

Evolutionary PCR

The study of ancient DNA has given and continues to generate insightful approaches into evolutionary theory. The recovery and subsequent amplification of DNA from a 7000 year-old brain [Pääbo1988] has solidified controversial theories such as the "primordial Eve" mitochondria-mediated ancestry of human beings. As in the case of long PCR, aDNA amplification (together with forensic DNA analysis) requires specialized preparation (see p.72, [Goodyear1994] and optimization methods, and has already generated a whole subset of protocols for conducting PCR ([Pääbo1990], [Pusch1998]).

Mutagenic PCR

The conventional methods for altering the sequence of DNA fragments (chemical/UV mutagenesis or cloning of synthesized oligonucleotides) are limited by culture times and the selection of candidate clones for sequencing. In contrast, PCR can be applied for quick mutagenesis in two different ways. The simplest, is to make use of the own polymerase error rate (see p.74) to clone and sequence many PCR generated fragments. Nevertheless, this method can be unreliable due to the predominance of G→A transitions in polymerase mis-incoporation errors. Directed PCR mutagenesis ([Higuchi1988], [Weiner1995]) uses specific primers that flank and contain the desired mutation to introduce it into plasmid or genomic DNA and it is an invaluable technique for studying protein structure-function relationships, gene expression and vector modification.

SNP detection

By definition, single-nucleotide polymorphisms (SNPs) are common single-nucleotide mutations (present in more than 1% of the species population), and are probably the most common type of DNA sequence variation. In recent years, powerful links between SNPs at particular locii and heritable phenotype variations have been discovered, yielding a powerful tool for the

identification of diseases and other genetics-driven phenotypic traits (such as variation in drug metabolism) and thus conferring a strong importance to the fast and reliable detection and identification of SNPs. Even though SNPs can be readily detected with direct-hybridization techniques, PCR offers much more reliable methods [Tyagi1998].

The classical approach to PCR SNP-detection is a sort of modified sequencing reaction, in which only labeled terminator ddNTPs are used. The PCR primer is targeted to anneal just one base before the SNP and, thus, the incorporated terminator reveals the character of single-point mutation [Livak1995]. Evidently, highly specific assays like SNP-detection are prone to take an enormous advantage from enhanced specificity and, consequently, they are one of the target market niches for specificity-boosting PCR devices (such as capillary and chip PCR systems [Lohmann2000]).

1.5. PCR AND PCR-CHIPS

As it can be deduced from the previous section, the introduction of significant alterations into the PCR environment is a troublesome issue, since classical optimization rules of thumb may be rendered useless due to the new special circumstances in which PCR is carried out. And, as it will be seen in the course of this doctoral work, this is indeed a problem when working with PCR-chips, in which a novel environment consisting of micro-machined reservoirs and new materials substitutes the classical setting of thermocyclers and optimally silanized eppendorf tubes. Obviously, switching from a previous working system to a new setup in such a multifaceted reaction as PCR is only worth the trouble when huge advantages can be gained from the switching and, evidently, this is thought to be the case of PCR-chips and other predating approaches, which can boost PCR specificity, speed, versatility and efficiency due to, precisely, the special nature of the new substrates used.

1.5.1. ORIGINS

The advantages of miniaturizing PCR were advanced by the pioneering work of *Carl T. Wittmer* [Wittmer1989]. Although distant from PCR-chips in its underlying technology, the capillary-PCR system devised by *Wittmer's* team demonstrated most, albeit not all, of the advantages of miniaturizing amplification that have been taken over by PCR-chips and, to date, it has

become a much more successful commercial product than the later (the Lightcycler, see [Wittwer1997a], [Wittwer1997b]).

The capillary air oven

The basic idea behind *Wittmer's* work was not so much to miniaturize the volumes of reagents in PCR, but to reduce heat-transfer rates in order to attain shorter transition times between the different temperature steps in PCR. To achieve this objective, he manufactured a custom closed-loop fan-driven oven (see Figure 43) that, in his own words, "resembled a re-circulating hair dryer" and was capable of providing fast transition times. The idea of a low-cost air-driven cycling oven had already been proposed. *Hoffman et al.* [Hoffman1988] had carried out PCR experiments adapting a gas chromatograph and a similar idea had been laid out by *Denton et al.* [Denton1990]. Nevertheless, the decisive insight in *Wittmer's* work was that, to obtain high heat-transfer rates, he replaced the conventional PCR plastic vessels with custom, 10 cm long, thin-walled glass capillaries, which he sealed by fusing the ends with an oxygen-propane torch.

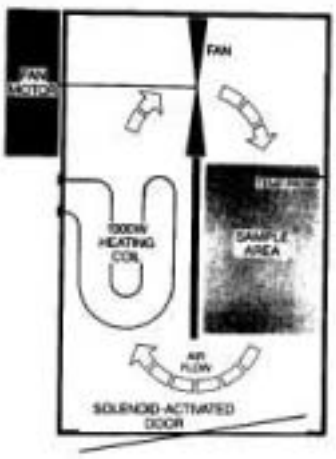

Figure 43 - Closed-loop hot-air driven oven. Source: [Wittmer1990].

Thus, the trick in *Wittmer's* design was to use a low heat-capacity medium (air), which could be warmed and cooled very rapidly, together with thin-walled capillaries that provided a larger surface/volume ratio and maximized heat-transfer between air medium and sample. To attain a homogeneous temperature distribution (±1 °C) across the oven, *Wittmer* introduced in his design an array of structural baffles to prevent laminar airflows and vortices that could cause local heat-concentration effects. With

this system, *Wittmer* was capable of carrying out successful amplifications in less than 15 min [Wittmer1990].

Quicker is better

In his seminal work on quick-PCR with capillary air-driven thermocyclers [Wittmer1990], *Wittmer* uncovered some very interesting facts on the nature of PCR. For one, he noticed that the time spent in temperature transition was usually wasted, even deleterious to PCR, and that it could only be functional when a slow transition between annealing and extension was required for a poorly annealing primer. He also proved that denaturation and annealing did in fact occur very quickly in DNA amplification (a fact that had been previously predicted by theoretical studies [Wetmur1968]), with good-yield amplifications occurring even when the denaturation and annealing times were reduced to spikes. He went on to assess [Wittmer1993] that "*limitations on the speed of DNA amplification arise not from kinetic requirements of the underlying reactions, but rather from the instrumentation usually used for temperature cycling*" and concluded that the limiting factor in quick-PCR was the extension time required by Taq polymerase.

Wittmer finished off his article with an insightful and detailed description of the optimal PCR vessels. These should have water-vapor tight closures, low thermal mass, good thermal conductivity, minimal internal condensation and small sample volumes, and should also display easy sample recovery without cross-contamination and no amplification inhibition. Shrewd as they were, his remarks on vessel construction were not the most valuable conclusions of his work. With rapid thermal cycling, *Wittmer* was able to demonstrate that both the specificity and the efficiency of PCR could be extraordinarily boosted in quick-PCR. The main reason for this improvement was the rapid cooling after denaturation and the use of short annealing times, which made annealing more stringent and favored the kinetic process (primer to template/product annealing) over the equilibrium process (product dimerization), a fact that has later been independently corroborated using conventional thermocyclers ([Mai1998], [Shaw1999]). Furthermore, short denaturation times also improved the half-life of the polymerase enzyme, providing better extensions at the critical final stages of PCR (as it had already been hypothesized using short denaturation times for short amplicates, [Yap1991]).

The results of *Wittmer* were independently validated by other groups [Swerdlow1993] and, in 1993, he was able to conduct reproducible studies of single-nucleotide polymorphisms (SNPs, single-base mutations) with PCR [Wittmer1993], a feat that could only be accomplished with extraordinary levels of PCR specificity (not allowing a primer to anneal when there is single-base mismatch). His basic idea led to the commercial development of the Lightcycler by *Idaho Technologies* [Wittwer1997b], which adds quantitative fluorescence detection of PCR to the rapid thermal-cycling scheme, and has also been developed in other ways, as in the constant capillary flow PCR [Nakano1994] (an scheme that has also been taken over in PCR-chips [Kopp1998]) or the use of indium-tin oxide covered capillaries, which, like PCR-chips, are capable of independent cycling [Friedman1998].

1.5.2. PCR-CHIPS

Although it may now seem plain obvious, the work of *Wittmer* did only indirectly hint at the use of chips for producing miniature PCR systems. It took the insight of someone used to micro-fabrication techniques and electronic circuitry, *Allen Northrup*, to put together the pioneering work of *Andreas Manz* [Manz1990] and *Carl T. Wittmer*, and deduce that silicon-based microsystems could be ideal candidates for the quick-PCR vessels that *Wittmer* had described. Although their last two properties (easy sample recovery and no inhibition) had yet to be demonstrated, silicon technology provided the perfect means for devising low thermal mass, good thermal conductivity, water-vapor tight vessels with minimal internal condensation, and *Northrup* decided it was worth the try.

Northrup's chip

Northrup's design [Northrup1993] is of special relevance to this doctoral work for various reasons. Firstly, *Northrup's* chip was the first approach to PCR-chips to be described in the literature (albeit tight in timing with *Findlay's* approach [Findlay1993]) and thus became a major milestone in the field. Secondly, and of most interest to this thesis, *Northrup's* chip was made on a silicon substrate (rather than plastic or glass, see [Findlay1993], [Anderson1998]), using standard micro-fabrication technologies and an insightful exploit of microsystems capabilities.

Northrup's chip (see Figure 44) was fabricated onto 0.5 mm silicon wafers that were chemically etched to provide 10x10 mm^2 reservoirs. These,

approximately, 50 µl pools were then sealed with a glass cover bound to silicon with silicone rubber. Polyethylene tubing was used to provide the system with inlets/outlets and the thermal cycling required for PCR was made available by a patterned polysilicon layer deposited by CVD (Chemical vapor deposition, see *Materials and Methods*, p.286) onto a heat-region defining silicon nitride (Si_3NO_4) membrane at the backside of the chip. The polysilicon resistance was electrically accessed through two aluminum contact pads for heating, and cooling was attained by passive air convection. Temperature was monitored using a chrome-aluminum thermocouple.

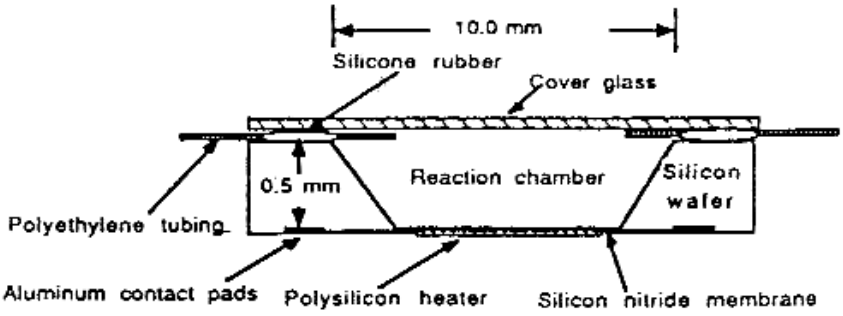

Figure 44 - Detailed cross-section of *Northrup's* chip. Source: [Northrup1993]

With this basic scheme, *Northrup*'s team was able to conduct PCR amplifications of a 142 bp region of the GAG gene of HIV (Human immunodeficiency virus). Starting with 10^8 initial copies, he was able to obtain successful amplification in twenty 1 min cycles (that is, 20 min), although the PCR yield was quite lower than the positive control run on a conventional thermocycler. The device displayed up to 15 °C/s heat/cooling rates with an accuracy of ±1.5 °C and a mean power consumption of 2.25W. These results prompted Northrup to conclude that PCR-chips were well fitted for the development of low-cost, portable, independent and efficient reaction-based micro-instruments and he soon moved on to form a spin-off company, *Cepheid*, to further develop his ideas.

1.5.3. ADVANTAGES OF PCR-CHIPS

Northrup's dreams were not seemingly unfounded. Although *Wittmer's* line of attack onto PCR had been much successful and already promised important commercial revenues, there were some points in which PCR-chips showed manifest superiority over their capillary counterparts. The

main one was the use of silicon as a substrate and micro-fabrication techniques as the manufacturing process. On the one hand, silicon is an excellent thermal conductor and, at chip sizes, it presents a very low thermal mass, allowing rapid heat transfer to and from the PCR sample and acting as a natural dissipating heat-exchanger due the augmented surface/volume ratio. On the other hand, silicon production technologies open up the way for a myriad of functional capabilities that can be implemented at the microscopic scale of PCR, and almost at the same cost of producing bare PCR-chips. For instance, independent cycle control in each chip with the on-chip integration of the heat-cycling machinery had been already demonstrated by Northrup (albeit he did not make use of it to control an array of chips). As a result, once integration of active elements had been proved, chip designers were free to speculate with the integration of further improvements and capabilities. For example, integrated temperature sensors and control circuitry could also be incorporated onto the chip and, theoretically, it could even be remotely programmed using an integrated telemetry coil. More interesting to the analyst, and without resorting to connection with other micro-analytical systems (such as electrophoresis chips), electrical or optical sensors could also be integrated into the device, providing the means for ultra-sensitive, in-situ detection of the PCR product and the development of extremely sensitive quantitative PCR. In essence, the use of silicon and its well-established production technologies theoretically enabled the development of a full range of PCR devices and it also foresaw low production costs in the event of mass-production.

1.5.4. EVOLUTION OF PCR-CHIPS

Albeit its promising overture, the symphony of PCR-chips has not reared a straight movement and, even though recent integration trends suggest a change in expectations (see p.58), its final closure as a commercial niche is still dim, nearly ten years after the inception of the first PCR-chips. As it will be seen, this failure to become a marketable product does not come from an inherent technical unfeasibility to carry out PCR in chips, but mainly from the inherited problems of PCR-chips parent field, μ-TAS, already acknowledged in a previous section (see p.49), which can be ascribed to two basic points: detection procedures and interface with the macroscopic world.

Sealing mechanisms

Close after the seminal work of *Northrup* came the nearly parallel development of plastic PCR-chips by *Findlay et al.* In their approach, which aimed at a complete μ-TAS for PCR analysis, *Findlay* used two sheets of heat-sealable plastic (polyethylene) to create a network of channels and reservoirs capable of conducting PCR and subsequent hybridization and detection of PCR products [Findlay1993]. Findlay's system also introduced novel analytical approaches, like the use of a modified touch-down/two-step (see p.88, p.80) technique and hot-start methodology (see p.89). Although their system was far from the production line (heaters and other mechanical actuators were manually glued to the system), the use of heat-sealable plastic introduced for the first time the concept of hermetic sealing of the devices, previously disregarded by *Northrup*, which was to become a major issue in microsystems for such a volatile reaction as PCR.

Anodic and thermal bonding

The next step in sealing techniques came shortly thereafter. *Wilding et al.* [Wilding1994] introduced the concept of silicon-glass field-assisted (or anodic) bonding (see *Materials and Methods*, p.293), first described by *Wallis et al.* in 1969 [Wallis1969] and commonly used in pressure and gas sensors within the microsystems field. Although anodic bonding places certain restrictions on the surface materials it can work with (see p.117, *Materials and Methods*, p.293), it produces strongly bonded devices that display efficient, water-vapor tight, sealing and has been extensively used in further approaches to PCR-chips (see [Shoffner1996], [Cheng1996a], [Cheng1996b], [Wilding1998], [Taylor1997], [Lin2000a] and [Lin2000b]).

In an alternative approach to hermetic sealing, μ-TAS pioneer *Andreas Manz* used a similar idea, glass-to-glass thermal bonding, to demonstrate continuous-flow PCR-chips (see p.103) in 1998 [Kopp1998], delving in his previous experience in the field of electrophoresis chips and the work of Nakano [Nakano1994] in capillary-PCR. This technique has been pursued further by other teams ([Waters1998a], [Waters1998b], [Lagally2001]) with remarkable success, although the use of a glass substrate does not accommodate so easily the integration of electronic components.