COMMUNICATION

ChemComm
www.rsc.org/chemcomm

An efficient method to perform milliliter-scale PCR utilizing highly controlled microwave thermocycling†

Kristina Orrling,[a] Peter Nilsson,[a] Mats Gullberg*[b] and Mats Larhed*[a]

[a] *Organic Pharmaceutical Chemistry, Department of Medicinal Chemistry, Uppsala Biomedical Centre, Uppsala University, P. O. Box 574, SE-751 23 Uppsala, Sweden*

[b] *The Beijer Laboratory, Department of Genetics and Pathology, Rudbeck Laboratory, SE-751 85 Uppsala, Sweden*

Received (in Cambridge, UK) 6th January 2004, Accepted 20th February 2004
First published as an Advance Article on the web 8th March 2004

This communication describes the development of a controlled microwave methodology for rapid milliliter-scale PCR.

Polymerase chain reaction (PCR) technology[1] is critical to almost every discipline involving molecular biology, including clinical diagnosis and proteomics.[2] In addition, nucleic acids have proved to be useful as both therapeutic agents[3–6] (aptamers) and in the field of organic synthesis, *e.g.* RNA molecules selected and amplified by combinatorial methods efficiently catalyze asymmetric Diels–Alder reactions.[7] A common theme throughout genomics, proteomics and drug discovery research is the need for speed. In response to this demand, several methods for achieving high-speed heating and cooling during PCR have been developed, including microwave heating.[8]

Surprisingly, methods to increase the scale of each individual amplification reaction are rare. With classic heating of a milliliter-scale sample the time required for heat transfer through the wall of the reaction tube and to obtain an even temperature in the whole sample is substantial. In practice, the slow distribution of heat together with the importance of short process times and reproducibility limits the volume for most reactions to 0.2 mL. At present, the generation of large quantities of DNA by PCR is restricted to parallel processing, followed by pooling of the separate reaction mixtures. This tedious procedure is a severe bottleneck when large amounts (mg) of long nucleic acids need to be produced or when large quantities of DNA are desired from scarce samples. Scaling up from the normal 0.1 mL reaction volume to a multi-milliliter scale would allow the amplification to be carried out at a higher sample dilution, thus yielding the possibility to remove the sample preparation step where a potential loss of scarce material may occur *e.g.* in forensic applications, without risking that inhibitors from the sample affect the PCR performance.

Another interesting application would be where the amount of PCR product is currently setting the limit. Recently a series of methods have been developed that perform multiplexed PCR using a common set of primers.[9–11] However, as PCR is a product inhibited reaction an obvious disadvantage when increasing the complexity of the amplification reaction is that the amount of each individual product decreases with an increasing complexity of the reaction down to a point where it is undetectable. The only option to avoid this using PCR is to run larger reactions. Thus, milliliter-scale PCR has the potential to increase the number of amplicons that can be simultaneously interrogated by a factor of more than a hundred. In the case of padlock, or molecular inversion probes, this would correlate to more than a million Single Nucleotide Polymorphism:s (SNP:s) typed in one reaction.[12]

High density microwave *in situ* heating is in many ways superior to traditional heating, avoiding large temperature gradients and hot walls of the reaction vessel. Compared to classic wall heating, the advantages with microwave heating increase in the interval 0.1 mL to 20 mL due to the instant and smooth heating of the full sample.[13] Further, important instrument innovations now allow for a highly reproducible computer control of pulsed microwave irradiation (2450 MHz), paving the way for controlled thermocycling. The available microwave instruments are not primarily designed for PCR, but for organic synthesis.[14,15] Despite application, milliliter-scale samples can now be rapidly heated to the desired temperature and also efficiently cooled by in-line air-jet cooling in a sequential fashion (Fig. 1).

Temperature can be measured with two different methods during microwave exposure, by an infrared (IR) pyrometer detecting the temperature of the surface layer, or by a fluoroptic (FO) probe inside the vessel, measuring the temperature at a specific *in situ* position. With the microwave instrument used, the software setting of the IR-pyrometer controls the temperature. In the cooling phase the jet-flow of air streams on the outside of the vessel and cools the glassware first (Fig. 1). Therefore, the IR-pyrometer supplied with the instrument showed an erroneously low minimum temperature.

With this background information in mind, we decided to perform a proof-of-principle investigation on the potential of microwave-assisted PCR in the milliliter-scale. The reaction tubes employed were made of microwave transparent boronic glass and were utilized together with a magnetic stirrer for increased temperature homogeneity of the reaction. The sequence to be amplified was a 53 bp fragment from human chromosome 13. All completed PCR reactions were analyzed using agarose gel-electrophoresis, capillary electrophoresis and real-time PCR.

The first 2.5 mL PCR reaction was set up to mimic the conditions known to afford good results in a classic thermocycler: 95 °C denaturation for 15 s followed by 60 °C for 60 s in a combined annealing and extension step. The thermocycling was controlled *via* the IR-signal, but the *in situ* temperature was also determined with a FO-probe.

Real-time PCR analysis of the amount of amplified nucleic acid product after 33 cycles disclosed incomplete amplification. Since the IR-regulation of the microwave equipment was not originally optimized for this type of application, we suspected the poor result to be a consequence of temperature "over-shooting" at the denaturation phase and subsequent deactivation of the Taq polymerase.[16] This, together with the discrepancy obtained between the IR- and FO-measurements motivated a reduction of the programmed top temperature. By this maneuver the amplification effectiveness drastically improved. Thus, heating the 2.5 mL sample for 35 s with a target temperature of 88 °C,[17] cooling for 50 s and finally heating at 60 °C for 85 s resulted in a final

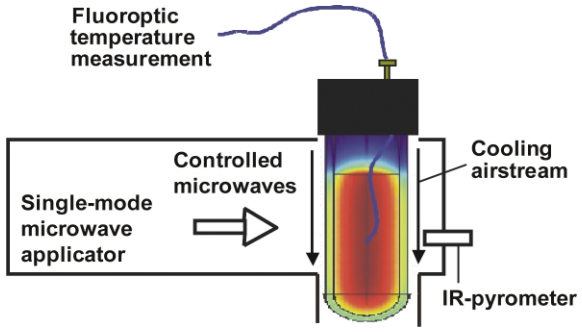

Fig. 1 Experimental set up for microwave heated PCR.

† Electronic Supplementary Information (ESI) available: experimental details. See http://www.rsc.org/suppdata/cc/b3/b317049g/

DOI: 10.1039/b317049g

concentration of PCR product between 10–30 nM, giving an amplification efficiency of 92–96% (analyzed using capillary electrophoresis) with 33 cycles performed in 1 h and 34 minutes (see ESI†). As illustrated in the inserted graph in Fig. 2, there was a difference of about 8 °C between the instrument IR-pyrometer and the FO-probe at the lowest measured temperatures. Regardless of the method for temperature measurement, the reproducibility between each individual cycle was impressive.

We also performed a microwave experiment running a 15 mL PCR reaction. The amplification mixture was rapidly heated to 88 °C for 50 s, jet-cooled for 95 s and held at 60 °C for 85 s. The overall process time was despite the large volume only 2 h and 7 min. Four of the 33 cycles are shown in Fig. 3.

Analysis of the reaction revealed a concentration of PCR product comparable to the concentration achieved with 2.5 mL reaction and a calculated amplification efficiency of close to 100% (see ESI). To our knowledge, it has not been possible to perform productive PCR or to achieve similar temperature profiles on this scale with conventional heating techniques. The importance of reproducible heating can not be understated, for while early pioneers in the field recognized the potential of microwave heating,[18] the situational specificity with the available domestic equipment completely inhibited the impact of the technique for small-scale PCR. Now, modern microwave cavities can deliver a very even field density without "hot-spots" enabling microwave heating for use in a wide range of biocatalytic areas.

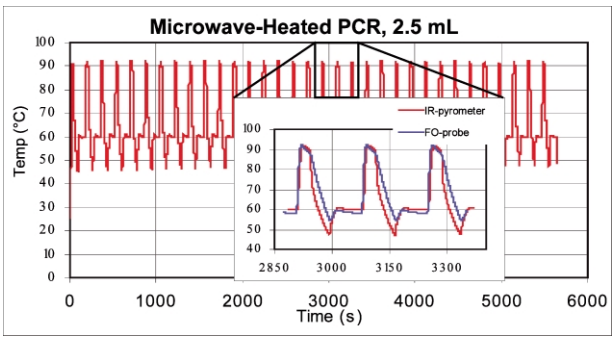

Fig. 2 Temperature curves for a microwave-heated 2.5 mL PCR experiment. In the inserted picture both the reading from the external IR-pyrometer (red) and from the internal FO-probe (blue) are presented.

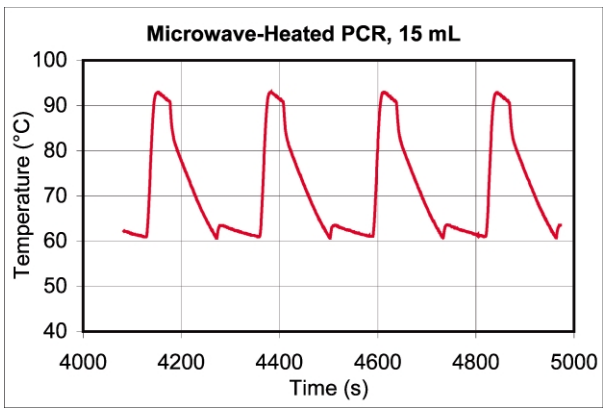

Fig. 3 Four IR-measured thermocycles representing the 15 mL microwave-heated PCR experiment.

Microwave-irradiation has been claimed to induce non-thermal irreversible denaturation of proteins, including destruction of enzymatic function.[19,20] However, the result from this study clearly proves that the Taq polymerase survives 33 microwave pulses and maintains the same efficiency regardless of the energy source. In conclusion, we believe this protocol represents an unprecedented example of an efficient milliliter-scale PCR process.

We thank the Swedish Foundation for Strategic Research, the Swedish Research Council, Personal Chemistry and Dr Kristofer Olofsson. MG has been supported by grants to Professor Ulf Landegren from the Wallenberg and Beijer Foundations and from the Swedish Research Council.

Notes and references

1 R. K. Saiki, S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich and N. Arnheim, *Science*, 1985, **230**, 1350.
2 S. Fredriksson, M. Gullberg, J. Jarvius, C. Olsson, K. Pietras, S. M. Gustafsdottir, A. Ostman and U. Landegren, *Nat. Biotechnol.*, 2002, **20**, 473.
3 L. C. Bock, L. C. Griffin, J. A. Latham, E. H. Vermaas and J. J. Toole, *Nature*, 1992, **355**, 564.
4 J. R. Lorsch and J. W. Szostak, *Acc. Chem. Res.*, 1996, **29**, 103.
5 D. J. King, D. A. Ventura, A. R. Brasier and D. G. Gorenstein, *Biochemistry*, 1998, **37**, 16489.
6 L. S. Green, D. Jellinek, R. Jenison, A. Ostman, C. H. Heldin and N. Janjic, *Biochemistry*, 1996, **35**, 14413.
7 F. Stuhlmann and A. Jaschke, *J. Am. Chem. Soc.*, 2002, **124**, 3238.
8 C. Fermer, P. Nilsson and M. Larhed, *Eur. J. Pharm. Sci.*, 2003, **18**, 129.
9 R. Lucito, M. Nakimura, J. A. West, Y. Han, K. Chin, K. Jensen, R. McCombie, J. W. Gray and M. Wigler, *Proc. Natl. Acad. Sci. USA*, 1998, **95**, 4487.
10 P. Hardenbol, J. Baner, M. Jain, M. Nilsson, E. A. Namsaraev, G. A. Karlin-Neumann, H. Fakhrai-Rad, M. Ronaghi, T. D. Willis, U. Landegren and R. W. Davis, *Nat. Biotechnol.*, 2003, **21**, 673.
11 G. C. Kennedy, H. Matsuzaki, S. Dong, W. M. Liu, J. Huang, G. Liu, X. Su, M. Cao, W. Chen, J. Zhang, W. Liu, G. Yang, X. Di, T. Ryder, Z. He, U. Surti, M. S. Phillips, M. T. Boyce-Jacino, S. P. Fodor and K. W. Jones, *Nat. Biotechnol.*, 2003, **21**, 1233.
12 Based on the authors own calculations.
13 P. Lidström, J. Tierney, B. Wathey and J. Westman, *Tetrahedron*, 2001, **57**, 9225.
14 M. Larhed and A. Hallberg, *Drug Discov. Today*, 2001, **6**, 406.
15 M. Larhed, C. Moberg and A. Hallberg, *Acc. Chem. Res.*, 2002, **35**, 717.
16 J. Dietrich, P. Schmitt, M. Zieger, B. Preve, J. L. Rolland, H. Chaabihi and Y. Gueguen, *FEMS Microbiol. Lett.*, 2002, **217**, 89.
17 The very first heating step was adjusted to 90 °C for 45 s because of the starting room temperature.
18 C. R. Strauss and R. W. Trainor, *Aust. J. Chem.*, 1995, **48**, 1665.
19 H. Bohr and J. Bohr, *Phys. Rev. E*, 2000, **61**, 4310.
20 M. Porcelli, G. Cacciapuoti, S. Fusco, R. Massa, G. dAmbrosio, C. Bertoldo, M. DeRosa and V. Zappia, *FEBS Lett.*, 1997, **402**, 102.